"Dedico todo o meu trabalho as pessoas mais importantes da minha vida, que amo muito: meu pai (Fernando), minha mãe (Auriene), minha avó (Helena), meu irmão (Philipe) e minha irmã (Fernanda)"

AGRADECIMENTOS

Agradeço em primeiro lugar a Deus, por ter colocado pessoas especiais na minha vida. Aos meus orientadores, Prof. José Teixeira da Universidade do Minho e Prof. Luiz Carvalho Jr. da Universidade Federal de Pernambuco. A estas duas pessoas, um muito obrigado é pouco para retribuir a oportunidade e apoio que os mesmos deram-me durante este período de doutoramento.

Ao Prof. Eduardo Beltrão, pela amizade e apoio sempre que preciso, mesmo que de longe, meu muito obrigado.

Ao Doutor Victor Balcão e Prof. Mª Graça Carneiro-da-Cunha, pelo incentivo e contribuição nos trabalhos.

Aos colegas de doutoramento Duarte Torres e Rafael Costa, pela amizade e colaboração em alguns trabalhos.

Ao Prof. José Oliveira da Universidade do Porto, pela disponibilidade e apoio dados no Instituto de Física dos Materiais.

Ao Doutor Fernando Dourado, na ajuda da análise dos dados de DSC e TGA, e a Engª Madalena Vieira, por todo apoio técnico.

A Todos os meus colegas de trabalho do Departamento de Engenharia Biológica, nomeadamente: Susana Moreira, Catarina Gonçalves, Vera Carvalho, Fábia Andrade, Reinaldo Ramos, Sílvia Ferreira, Marisa Cunha, Carla Oliveira, Joana Carvalho, Renata Pértile, João Machado, pela paciência, apoio e encorajamento.

A Gyrlene, pela atenção e carinho, e a todos os amigos e familiares que de alguma forma, contribuíram para a execução desta tese.

Ao Program Alban de bolsas de estudo de alto nível destinado à América Latina, pelo apoio financeiro (Scholarship no. *E05D057787BR*).

SUMÁRIO

Esta tese de Doutoramento é o resultado de um projecto de colaboração entre a Universidade Federal de Pernambuco, Brasil e a Universidade do Minho, Portugal, financiado pelo Programa Alban de bolsas de estudo de alto nível destinado à América Latina.

Lactases ou β-galactosidases (E.C. 3.2.1.23) são enzimas com um elevado interesse industrial quer na conversão da lactose em galactose e glicose quer na síntese de oligossacarídeos. O seu uso tem sido recomendado para hidrolisar a lactose no leite consumido por aqueles indivíduos com intolerância à lactose, na manufactura de sorvete e na produção de galactooliossacarídeos (GOS), compostos que se inserem nos denominados alimentos funcionais (pré-bióticos).

A imobilização desta biomolécula em suportes de baixo custo, mediante procedimentos de fácil execução, pode potenciar a eficiência catalítica do derivado enzimático por causa da melhor estabilidade ao pH e temperatura, proporcionando deste modo, menores custos operacionais e aumentando suas aplicações biotecnológicas em indústrias de alimentos.

O principal objectivo do trabalho desenvolvido foi avaliar a utilização de β-galactosidase imobilizada em diferentes matrizes insolúveis em água tanto na hidrólise da lactose como na síntese de GOS.

No decorrer do trabalho, foram usados quatro suportes magnéticos: (1) polisiloxano-Álcool Polivinílico magnético – mPOS-PVA; (2) magnetita revestida com polianilina – MAG-PANI; (3) polisiloxano revestido com polianilina – POS-PANI e (4) Dacron magnetizado. β-Galactosidase de duas origens diferentes (*Kluyveromyces lactis* e *Aspergillus oryzae*) foram usadas.

Todas as matrizes investigadas foram adequadas para a imobilização de β-galactosidase e para a produção de GOS usando lactose como substrato. A capacidade de formação de GOS pela enzima não foi afectada pela imobilização nos diferentes suportes magnéticos, não se tendo observado diferenças na cinética reaccional de sintese entre a enzima livre e a enzima imobilizada nos diferentes suportes. A caracterização

físico-quimica dos suportes contendo a enzima imobilizada permitiu confirmar a ausência de limitações difusionais à transferência de massa. Verificou-se também que a produção de GOS, a diferentes temperaturas 30 – 60ºC, foi praticamente inalterada, tanto para a enzima livre quanto para a imobilizada.

Comparando a eficiência de imobilização dos diferentes suportes, verificou-se que a maior retenção de actividade enzimática de hidrólise e de síntese foi obtida com a enzima imobilizada em Dacron magnetizado.

Foi também desenvolvido um modelo matemático que descreve adequadamente as reacções de hidrólise da lactose e sintese de GOS e efectuada a caracterização química, física e estrutural dos suportes MAG-PANI e POS-PANI.

ABSTRACT

This PhD thesis is the result of a collaboration project between the Universidade Federal de Pernambuco, Brazil, and the Universidade do Minho, Portugal, financed by the Programme Alßan, a high level scholarship programme specifically addressed to Latin America.

Lactases or β-galactosidases (E.C. 3.2.1.23) are enzymes with an increasing industrial importance for lactose hydrolysis and for oligosaccharides synthesis. Its use has been recommended for the hydrolysis of lactose in milk consumed by lactose intolerant individuals, for ice-cream manufacture and in the production of galactooligosaccharides (GOS), compounds known as functional foods (prebiotics).

The immobilization of β-galactosidase onto low-cost matrices by ease procedures can enhance the catalysis efficiency of the enzymatic derivatives because of the greater stability to pH and temperature, providing thus lower operational costs and increasing their applications in food industry biotechnology.

The main objectives of the developed work were to evaluate the immobilization of β-galactosidase onto different water insoluble matrices both for lactose hydrolysis and GOS synthesis.

Four magnetic matrices were used for enzyme immobilization: (1) polyvinyl alcohol polysiloxane-magnetic - mPOS-PVA; (2) magnetite coated with polyaniline - MAG-PANI; (3) polysiloxane coated with polyaniline - POS-PANI and (4) ferromagnetic Dacron. β-Galactosidase from two different origins (*Kluyveromyces lactis* and *Aspergillus oryzae*) were used.

All the investigated matrices were suitable for the β-galactosidase immobilization and the GOS production as well, using lactose as substrate. The ability of GOS formation by the enzyme was not affected by the immobilization on the different magnetic matrices and no differences on the kinetics of GOS synthesis were observed between immobilized and free enzymes. The physic-chemical characterization of the supports with the immobilized enzymes allowed the conclusion that diffusional mass transfer limitations were not a concern. It was observed that GOS production in

the temperature range of 30 - 60 º C was unchanged for both free and for immobilized enzyme.

In what concerns β-galactosidase immobilization efficiency of the different supports, it was conclude that magnetized Dacron allowed for the highest activity retention both for hydrolysis and synthesis.

Furthermore, a mathematical model describing lactose hydrolysis and GOS synthesis was developed and a detailed chemical, physical and structural characterization of the MAG-PANI and POS-PANI supports was done.

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ABBREVIATIONS

BCA – Bicinchoninic acid

BSA – Bovine serum albumin

DSC – Scanning calorimeter

EA – Elemental analyzer

FTIR - Fourier transform infrared spectroscopy

GOS – Galactooligosaccharides

GRAS – Generally recognized as safe

HPLC – High performance liquid chromatography

JCPDS-ICDD – Joint Committee on Powder Diffraction Standards - International Centre for Diffraction Data

MAG-PANI – Magnetite coated with polyaniline

mPOS-PVA – Polysiloxane-Alcohol Polyvinyl magnetic

MS – mass spectroscopy

NMR - Nuclear magnetic resonance

NODs – Non-Digestible oligosaccharides

ONP – Ortho- nitrophenol

ONPG – Ortho-nitrophenyl-b-D-galactopyranoside

PANI – Polyaniline

PET or DACRON – Polyethylene terephthalate

POS-PANI – Polysiloxane coated with polyaniline

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – Scanning electron microscopy

TD – Transgalactosylated disaccharides

TEOS – tetraethylorthosilicate

TGA – Thermogravimetric analyzer

VSM – Vibrating sample magnetometer

XRD – X-ray diffraction

LIST OF SYMBOLS

K_m^{app} – apparent Michaelis-Menten constant;

% – per cent;

cm^{-1} – per centimeter(s);

E_a – activation energies;

g – gram(s);

h – hour(s);

J – Joule;

Kj/mol – Kilojoules per mole;

mg/mL – miligram per mililiter

min. – minute(s);

mL – mililiter(s);

mM – milimolar;

ºC – centigrade;

ºK – Kelvin;

rpm - revolutions per minute;

v/v – volume per volume;

Vm – maximum velocity;

w/v – weight per volume;

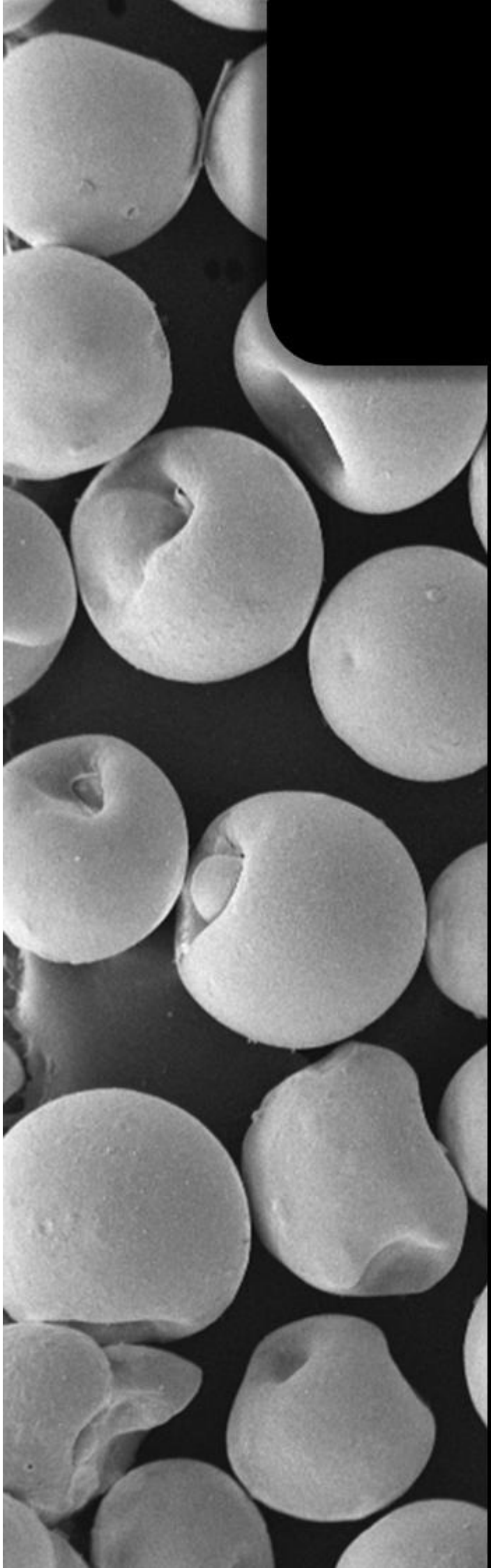
β – beta;

μg – microgram;

μm – micrometer(s);

μ – micro;

μM – micromolar.



Introduction

CHAPTER 1

The first chapter provides an introduction to the bio-molecule (β-galactosidase) used in the experiments, including the reactions in which the β-galactosidase are involved. We are going to introduce the different immobilization approaches and provide a short overview about the main advantages and disadvantages from these processes. The application of the β-galactosidase was also referred in this chapter, focusing in particular the milk lactose hydrolyze, the enzymatic whey hydrolysis and the oligossacharides synthesis. The functional foods focusing the prebiotics and the magnetic supports was also given.

"Todo homem determinado tem os seus sonhos concretizados"
Autor desconhecido

1.1. β-GALACTOSIDASE

β-Galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2.1.23) catalyzes the hydrolysis of β-D-galactosides and β-L-arabinosides (Whitaker, 1972). Even though the term "lactase" is obsolete, many authors continue to use this nomenclature in preference to the trivial name β-galactosidase (probably because of tradition and the ease in writing "lactase"). The enzyme is also capable of catalyzing synthesis of certain oligosaccharides via the galactosyl transfer reaction. The β-D-galactosyl transfer occurs preferentially at the primary alcohol of D-glucose with the formation of various di- and oligosaccharides (Kulp, 1975). Traditionally, much of the work has been on the *Escherichia coli* enzyme. However, this enzyme is widely distributed in nature and can be found in plants (especially almonds, peaches, apricots, and apples) animal organs, yeast, bacteria, and fungi. β-Galactosidase from different sources varies considerably in many of its properties although the specificity of the enzyme remains essentially the same. Several of these β-galactosidases have been purified, sequenced and extensively characterized. A few have been cloned and expressed in other hosts; however, the products require extensive testing before use. Consequently, the enzymes available commercially are derived from safe sources, principally, the yeasts *Kluyveromyces fragilis, Kluyveromyces lactis* and *Candida pseudotropicalis*, the fungi *Aspergillus niger* and *Aspergillus oryzae*, and a *Bacillus* species closely related to *Bacillus stearothermophilus* (Mahoney, 1997). The most widely used microbial sources are *Kluyveromyces* sp. and *Aspergillus* sp. The enzyme most studied is from *Escherichia coli* encoded by the *lacZ* gene, which serves as a model for understanding the catalytic mechanism of β-galactosidase action, but it is not considered suitable for use in foods owing to toxicity problems associated with the host coliform (Mahoney, 1997). Hence, the β-galactosidase from *E. coli* is generally not preferred for use in food industry (Joshi *et al*., 1989; Stred'ansky *et al*., 1993; Mahoney, 2003). *E. coli* β-galactosidase has a molecular weight of 464 kDa and is made up of four identical subunits, each containing 1023 amino acid residues and a binding site for magnesium, which is an activator. Amino acid sequences have been established for β-galactosidases from several other bacteria and comparison with the enzyme from *E. coli* shows extensive homologies and highly conserved regions (Mahoney, 2003). In contrast, relatively little is known about the enzyme structure from eukaryotes, although the enzyme from *Kluyveromyces lactis*

does show extended sequence homologies with the *E. coli* enzyme, suggesting a close evolutionary relationship.

Various methods are available for determining β-galactosidase activity. The activity can be determined readily by lactose as a substrate and determining the resulting glucose or galactose. Because of transfer reactions, it may be advisable to determine both appearance of glucose and disappearance of lactose (Kulp, 1975). Alternatively, o-nitrophenyl-β-D-galactopyranoside, abbreviated as ONPG, can be used as the substrate, and progress of the reaction can be followed by estimating the chromogen o-nitrophenol. In fact, ONPG is one of the best procedures available. Highest reaction velocities are obtained with this chromogen. The active site of the enzyme has one thiol and one imidazole group, and the reaction corresponds to a S_N2-like displacement mechanism (Shukla, 1975). Furthermore, a covalent intermediate involving a C-N bond was suggested. The number of active sites in the enzyme was temperature dependent.

The use of β-D-galactosidase has been suggested for the production of lactose hydrolyzed milk and whey hydrolysis to obtain glucose and galactose. As per the CAZy (carbohydrate active enzymes) database, this enzyme has been classified under the glycoside hydrolase 2 (GH 2) family of carbohydrate active enzymes. This enzyme has several applications in the food, dairy and fermentation industries (Mahoney, 1997). Technologically, lactose easily crystallizes, which sets the limits to certain processes in the dairy industry. Cheese manufactured from hydrolyzed milk ripens more quickly than that made from normal milk. Treatment of milk and milk products with lactase to reduce their lactose content seems to be an appropriate method to increase their potential uses and to deal with the problems of lactose insolubility and lack of sweetness. Furthermore, this treatment could make milk, a most suitable food, available to a large number of adults and children that are intolerant to lactose. Hydrolysis of lactose present in whey, converts whey into very useful sweet syrup, which can be used in the dairy, confectionery, baking and soft drinks industries. Thus, additionally it can help to solve problems related to the use of by-products from cheese manufacturing industries, avoiding serious pollution problems caused by their disposal. Therefore, lactose hydrolysis provides several advantages: nutritional, because a significant fraction of the world population suffers from lactase deficiency, technological, because

glucose and galactose are sweeter and more soluble than lactose, and environmental, associated with whey disposal. Furthermore, glucose and galactose are more readily fermented than lactose (Shukla, 1975; Gekas and Lopez-Leiva, 1985; Kumar *et al*., 1992).

1.1.1. β-Galactosidase reactions

For the mechanism of reaction by β-galactosidase the involvement of a minimum of three steps has been suggested, the last of which allows for hydrolysis or transferase activity (Wallenfels and Malhotra, 1961), viz:

Enzyme + Lactose → Enzyme-Lactose (1)

Enzyme-Lactose → Galactosyl-Enzyme + Glucose (2)

Galactosyl-Enzyme + Acceptor → Galactosyl-Acceptor + Enzyme (3)

Where the acceptor is water, free galactose is formed by hydrolysis (Figure 1.1). Where the acceptor is a sugar, the result is galactosyl-oligosaccharide formation (Figure 1.2).

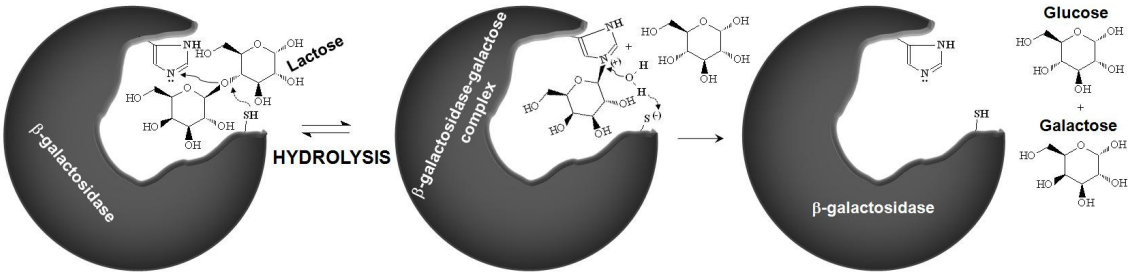


Figure 1.1. Mechanism of lactose hydrolysis by β-galactosidase (adapted from Shukla, 1975).

The effect of pH on the activity and the inhibition of enzyme thiol groups suggested that the active site of neutral pH enzymes contains a thiol group acting as a general base and an imidazole group acting as a nucleophile to facilitate splitting of the glycosidic bond (Wallenfels and Malhrotra, 1961).

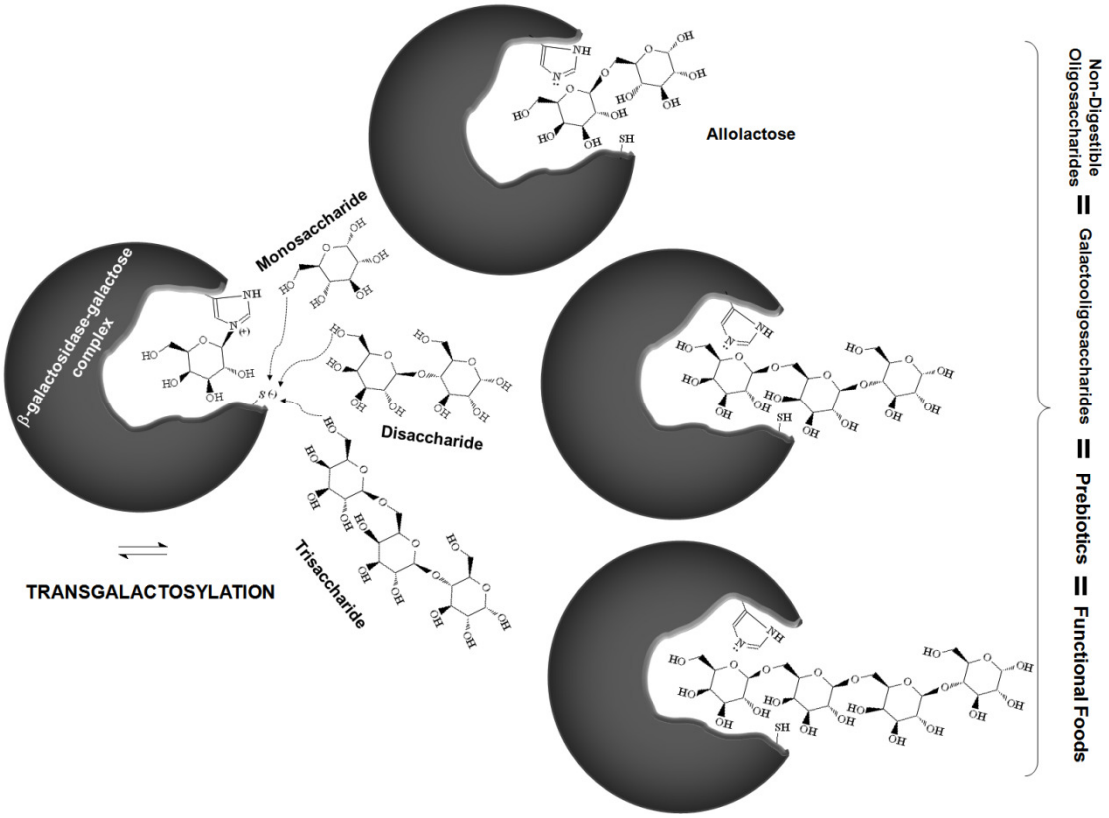


Figure 1.2. Mechanism of transgalactosylation reaction by β-galactosilase (adapted from Shukla, 1975).

The general reaction mechanism can then be depicted as shown in Figure 1.3. The mechanism indicates that β-galactosidase will transfer galactose to nucleophilic acceptors containing a hydroxyl group. Transfer to water produces galactose; transfer to another sugar produces di-, tri- and higher galactosyl-saccharides, collectively termed oligosaccharides. These in turn become substrates for the enzyme and are slowly hydrolyzed. In this scheme galactosyl transfer is the general reaction and hydrolysis can be regarded as a special instance of galactosyl transfer to water.

It was generally observed that the hydrolysis and transgalactosylation reactions occurred simultaneously. What dominates the product profile of the reaction is largely dependent on lactose concentration.

The products of lactose hydrolysis may inhibit β-galactosidase action. Galactose is often a competitive inhibitor but glucose is usually ineffective, except at higher concentrations when it is usually non-competitive. Complete hydrolysis is therefore very difficult to achieve unless high concentrations of the enzyme are used.

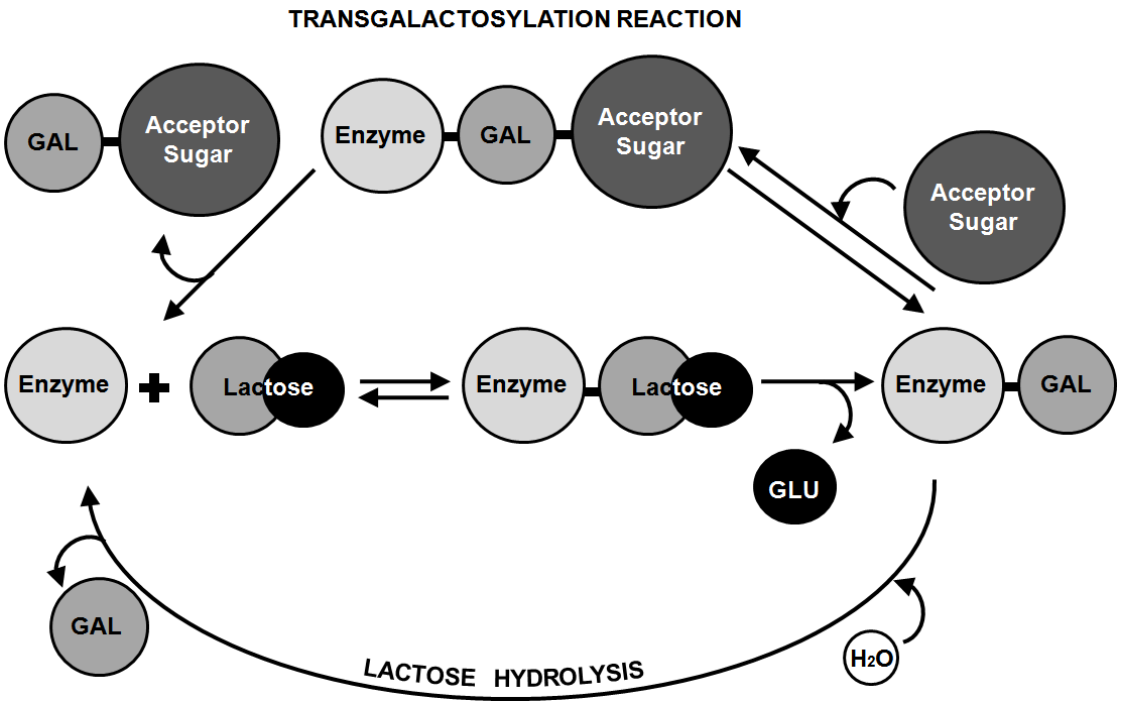


Figure 1.3. A proposed reaction mechanism for the action of β-galactosidase on lactose (adapted from Mahoney, 1998).

Yeast enzymes are generally inhibited by galactose (competitively) and glucose (non-competitively). *Aspergillus niger* enzyme is strongly inhibited by galactose; however, enzyme from *A. oryzae* is less subject to galactose inhibition (Mahoney, 2003).

1.2. IMMOBILIZATION OF β-GALACTOSIDASE

The immobilized enzyme is defined as "the enzyme physically confined or localized in a certain defined region of space with retention of its catalytic activity, which can be used repeatedly and continuously" (Chibata 1978). β-Galactosidase is one of the most studied enzymes as far as immobilization is concerned. Although many studies described the effective immobilization of β-galactosidase isolated from recombinant *Escherichia coli*, its application in food industry is limited, because this microorganism is not generally recognized as safe (GRAS) (Ladero *et al*. 2001; Di Serio *et al*. 2003). Currently, GRAS status is valid for *A. niger*, *A. oryzae*, *K. lactis*, *K. fragilis,* which are the main producers of β-galactosidase used in food industry. The choice of suitable enzymatic preparation depends on its properties and the purpose of its application. Yeast β-galactosidases are habitually used for the hydrolysis of lactose in

milk and sweet whey, whereas fungal β-galactosidases are more suited for acidic whey hydrolysis. Compared to yeast enzymes, fungal β-galactosidases are more thermostable, but they are more sensitive to product inhibition, mainly by galactose (Boon *et al*. 2000). Immobilization of β-galactosidases can dramatically affect enzyme's properties; *e.g.* pH and temperature stability, kinetic parameters, etc. (Rossi *et al*. 1999; Sun *et al*. 1999; Ladero *et al*. 2000). If an adequate technique is applied, immobilization can improve properties of β-galactosidases such as stability of the enzyme at high or low pH and temperatures. Therefore, in term of prevention of microbial growth in the reactor, these forms of enzymes are more useful for lactose hydrolysis processes (Zhou and Chen 2001b; Tanriseven and Dogan 2002). Moreover, the immobilization technology shows a promising role in reducing the product inhibition, what permits to reach higher conversion of lactose hydrolysis (Jurado *et al*. 2002; Pessela *et al*. 2003).

1.2.1. Techniques and matrices for immobilization of β-galactosidase

β-Galactosidases from different source were immobilized by several methods to a variety of matrices (see Table 1.1), including entrapment, cross-linking, adsorption, covalent binding or the combination of these methods. Since each method has its own advantages and drawbacks, the selection of suitable immobilization method (Figure 1.4) depends on the enzyme (different properties of various β-galactosidases, such as molecule weight, protein chain length, and position of the active site), matrix, reaction conditions, reactor, etc. (Tanaka and Kawamoto 1999).

Table 1.1. β-galactosidase from different source immobilized by several methods to a variety of matrices.

IMMOBILIZATION METHODS	MATRICES	SOURCE OF ENZYME	REFERENCE
Covalent coupling	mPOS-PVA	*K. lactis*	Neri *et al* ,2008
	oxides supports	*K. marxianus*	Di Derio *et al* ,2003
	chitosan	*A. oryzae*	Gaur *et al* ,2006
Entrapment	alginate-carrageenan	*K. fragilis*	Mammarella and Rubiolo,2005
	liposomes	*E. coli*	Rodriguez and Delgadillo,2006
	polyvinyl alcohol	*A. oryzae*	Rossi *et al* ,1999
Physical adsorption	ceramic monolith	*A. Niger*	Papayannakos and Markas,1993
	IMAC	*Tremus sp.*	Pessela *et al* ,2007a
	polyvinylcholoride and silica	*B. circulans*	Bakken *et al* ,1992
Cross-linker	gelatin with glutaraldehyde	*E. coli*	Pessela et al,2007b
	polyacrylamide gel	*Chichen bean*	Sun *et al* ,1999
	cellulose-gelatin	*K. lactis*	Numanoglu and Sungur,2004

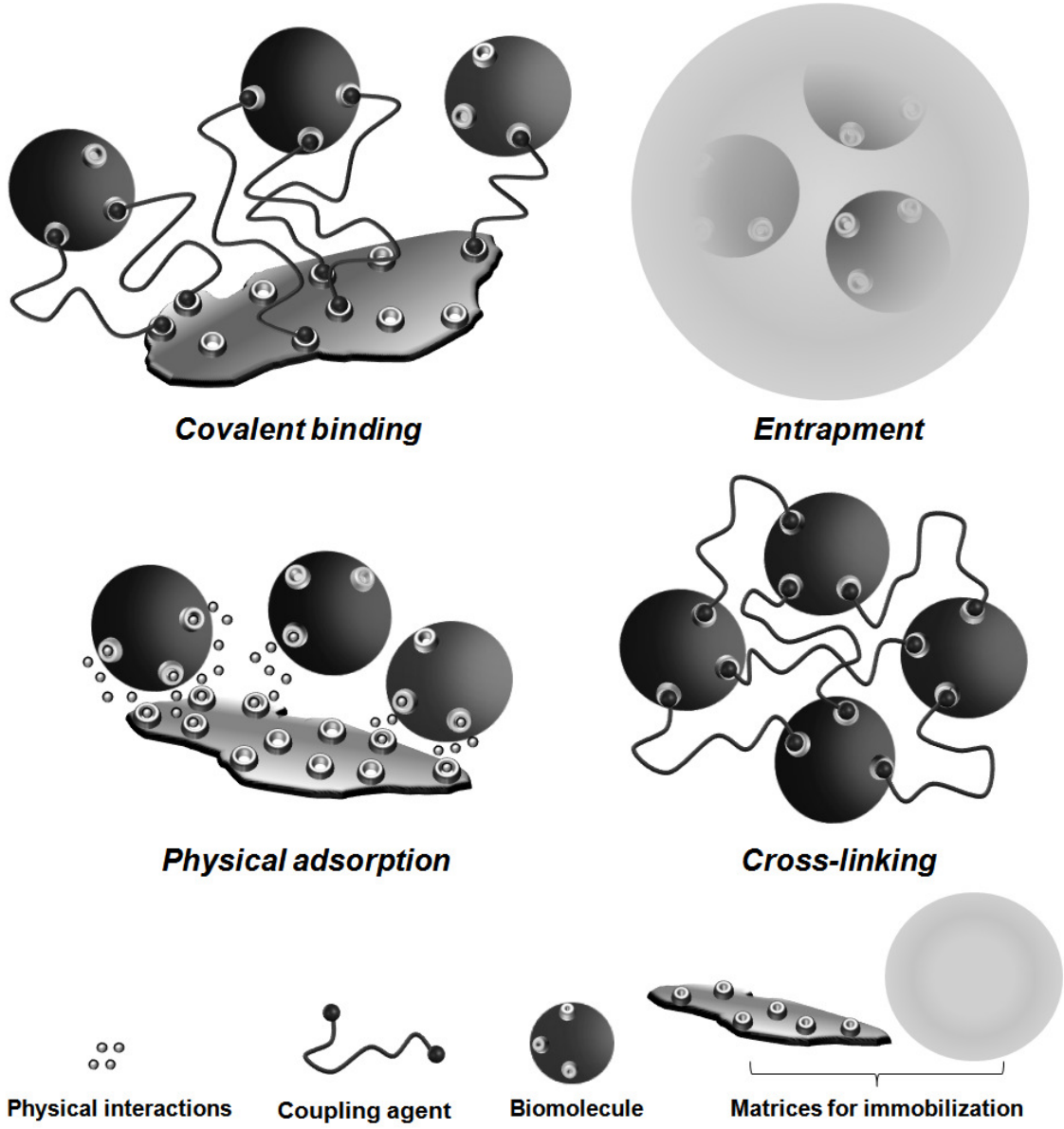
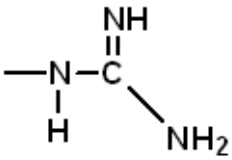
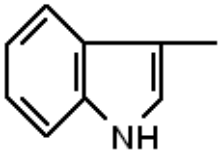
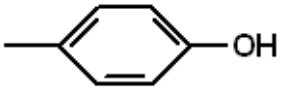


Figure 1.4. Methods of enzyme immobilization (adapted from Bickerstaff, 1997).

1.2.1.1. Covalent binding. This method is mostly used for β-galactosidases immobilization. Enzymes are covalently linked to the support through the functional groups in the enzymes that are not essential for the catalytic activity. Table 1.2 summarizes the residues of aminoacids that have functional groups in the side chain suitable for linking to a support (Cabral and Kennedy, 1991).

Table 1.2. Reactive residues of protein (Cabral and Kennedy, 1991).

REACTIVE RESIDUES OF PROTEIN	DESCRIPTION
—NH_2	t-Amino of L-Lysine (L-Lys) and N Terminus amino groups
—COOH	Carboxyl of L-Aspartame (L-Asp) and Glutamate (L-Glu)
—S—S—	Disulfide of L-Cystine
	Disulfide of L-Cystine
—CH_2OH	Hydroxyl of L-Serine (L-Ser) and L-Threonine (L-Thr)
	Imidazole of L-Histidine (L-His)
	Indole of L-Tryptophan (L-Trp)
	Phenolic of L-Tyrosine (L-Tyr)
H_3C—S—	Thioether of L-Methionine (L-Met)
—SH	Thiol of L-Cysteine (L-Cys)

Comparing to other techniques this method has the following advantages: enzymes does not leak or detach from the carrier and the biocatalyst can easily interact with the substrate, since being on the surface of the carrier. On the other hand, the major disadvantages are high costs and low activity yield owing to exposure of the biocatalyst to toxic reagents or severe reaction conditions (Tanaka and Kawamoto 1999). There were several matrices used for β-galactosidase immobilization. Oxide materials such as alumina, silica, silicate alumina were used for covalent binding of β-galactosidase from *K. marxianus* and applied in lactose hydrolysis processes. In spite of the derivatives to present good stability, the immobilization yields were less than 5% (Di Serio *et al*., 2003). β-Galactosidase from *K. fragilis* was covalently linked to silanized porous glass

beads via amino groups, using glutaraldehyde. The coupling efficiency was very high so that more than 90% of the enzyme was active and 87.5% of the protein was bound to the support. (Szczodrak, 2000). Graphite was also used for immobilization of β-galactosidase (from *K. lactis*). The immobilization on the graphite slabs was achieved by means of the Schiff 's base reaction between the active groups of graphite and the enzyme molecules to form covalent linkage using glutaraldehyde as a cross-linking reagent. The immobilization increased the enzyme thermal stability and shifts the optimal pH to a more alkaline value (7.7) compared to the free enzyme (6.6) (Zhou and Chen 2001a). Yeast β-galactosidase was covalently linked, via glutardaldehyde, to chemically modified corn grits. Although the immobilization material showed great characteristics; *e.g*. inexpensive, highly stable, good mechanical properties, the immobilization yield was very low (8%) (Siso *et al*. 1994). Among different fibrous matrices tested (non-woven polyester fabric, cotton wool, terry cloth, rayon non-woven cloth etc.), β-galactosidase (from *A. oryzae*) covalently bounded to cotton cloth activated with tosyl chloride showed the highest immobilized enzyme activity with coupling efficiency of 85% and enzyme activity yield of 55%. Thermal stability of the enzyme was increased by 25-fold upon immobilization and the immobilized enzyme had a half-life of 50 days at 50°C and more than one year at 40°C (Albayrak and Yang 2002c). Giacomini *et al*. (1998) compared the properties of immobilized *K. lactis* β-galactosidase using two different coupling carriers: glutaraldehyde-agarose gel and thiosulfinate-agarose gel. Glutaraldehyde-agarose exhibited lower yield after immobilization (36–40%) than thiosulfinate-agarose (60–85%), but better thermal properties.

1.2.1.2. Entrapment. The entrapment method is based on the localization of an enzyme within the lattice of a polymer matrix or membrane. Entrapment has been evolved and extensively used mostly for the immobilization of cells, but not for enzymes. The major limitation of this technique for enzymes immobilization is the possible leakage during repeated use due to the small molecular size compared to the cells. Next disadvantages of the method are diffusion limitations. Entrapment method is classified into five major types: lattice, microcapsule, liposome, membrane, and reverse micelle (Tanaka and Kawamoto 1999). For β-galactosidase immobilization, the lattice method is the most widely used. The enzyme is entrapped in the matrix of the various synthetic or natural polymers. Alginate, a naturally occurring polysaccharide that forms gels by ionotropic

gelation, is the most popular one. Mammarella and Rubiolo (2005) entrapped *K. fragilis* β-galactosidase in alginate-carrageenan gels to form beads. The presence of κ-carrageenan had favorable influence on the enzymatic reaction, because this gel is formed with K^+ ions, which increased the enzyme activity. Alginate as an immobilization matrix was also used in combination with gelatin to immobilized *A. oryzae* β-galactosidase in fibers. The immobilized enzyme showed good operational stability (35 days without decrease of activity). Furthermore, at 70°C the immobilized enzyme retained 27% of its initial activity (free enzyme lost all activity) and at pH 9 immobilized enzyme had 25% of initial activity (free enzyme retained just 11.5%) (Tanriseven and Dogan 2002). From synthetic polymers used for β-galactosidase entrapment, polyvinylalcohol gel was shown as very attractive because of its mild conditions of preparation, stability, biocompatibility, structural strength and diffusive properties (Rossi *et al*. 1999). Fungal β-galactosidase entrapped in polyvinyl alcohol cryogel beads was more thermostable than free enzyme, retaining 70% of activity after 24 h at 50°C and 5% activity at 60°C (Batsalova *et al*. 1987). The microcapsule type involves an entrapment to a semipermeable polymer. The preparation of enzyme micro capsules requires extremely wellcontrolled conditions. Taqieddin and Amiji (2004) developed a new encapsulation method in which the alginate-chitosan core-shell microcapsules were formed to immobilize β-galactosidase. The enzyme was localized and protected in the inner biocompatible alginate core while the outer chitosan shell established the transport properties. Microcapsules with liquid core were produced with 60% loading efficiency when using Ca^{2+} ions for crosslinking alginate, while using Ba^{2+} ions, microcapsules with solid core were produced and 100% loading efficiency was obtained. The liposome type employs entrapment within an amphipathic liquid-surfactant membrane prepared from lipid (usually phospholipids) (Kirby & Gregoriadis 1984). For the hydrolysis of milk lactose β-galactosidase – containing lipid vesicle (liposome) is added to milk and is disrupted into the stomach by the presence of bile salts, allowing *in situ* degradation of the lactose (Kim *et al*. 1999). The entrapment in liposome is increasingly recognized as a method of protecting biocatalysts from proteases inactivation. β-Galactosidase immobilized in lipossomes prepared by the dehydration-rehydration method showed high resistance to proteolysis, retaining about 93% and 75% of its initial activity after 6 h and 24 h of exposure to protease. Free enzyme retained only 7% of its activity after 24 h of exposure to protease. Moreover,

liposomal enzyme offers a noticeable increase in thermal protection. At 55°C, β-galactosidase- containing lipid vesicle retained 86% of its activity, compared to only 65% of the free enzyme activity at the same temperature (Rodriguez-Nogales and Delgadillo 2006). In the reversed micelle type, β-galactosidase is entrapped within the reversed micelles, which are formed by mixing a surfactant with an organic solvent, for example aerosol OT/isooctane reverse micelles (Chen and Ou-Yang 2004). And in the membrane type, the enzyme is separated from the reaction solution by an ultrafiltration membrane, a microfiltration membrane, or a hollow fiber (Chockchaisawasdee *et al*. 2005).

1.2.1.3 Physical adsorption. Physical adsorption is the simplest and the oldest method of immobilizing enzymes onto carriers. Immobilization by adsorption is based on the physical interactions between the biocatalyst and the carrier, such as hydrogen bonding, hydrophobic interactions, van der Waals force, and their combinations. Despite its simplicity, this immobilization method is significantly limited by the tendency of enzyme to desorb from the support and sensitivity to environmental conditions, such as temperature and ions concentration (Tanaka and Kawamoto 1999). The immobilized β-galactosidase particles (size 1 - 2 mm) prepared by physical adsorption of the enzyme on the porous ceramic support and intermolecular cross-linking with glutaraldehyde reached binding efficiency 80% and good operational stability. After 135 days in continuous operation, no activity loss was detected (Papayannakos and Markas 1993). Commercially available β-galactosidases were adsorbed on phenol formaldehyde resins of the Duolite type and applied in galactoside synthesis. The immobilization yields varied from 23% for *K. fragilis* β-galactosidase adsorbed on Duolite A-7 to 54% for *A. oryzae* β-galactosidase adsorbed on Duolite S-761 (Woudenberg-van Oosterom *et al*. 1998). Numbers of other supports for adsorption of β-galactosidase were tested; *e.g*. adsorption on celite (Gaur *et al*. 2006), on zeolite pellets (Poletto *et al*., 2005), on hydrophobic cotton cloth (Sharma and Yamazaki 1984), on chromosorb W (Bodalo *et al*. 1991), etc.

1.2.1.4 Cross-linking. The cross-linking method utilizes a bi- or multifunctional compounds, which serve as the reagent for intermolecular cross-linking of the biocatalyst (Tanaka and Kawamoto 1999). In case of β-galactosidase immobilization,

crosslinking is often used in combination with other immobilization method, mainly with adsorption and entrapment.

1.2.2. Drawbacks of immobilization

The immobilization process is associated with some disadvantages, such as drop of enzyme activity after immobilization, leakage and desorption of the biocatalyst from the matrix. The drop of activity after immobilization of β-galactosidase ranges from 0.01% to 90% depending on the immobilization method and the source of the enzyme (Table 1.2). Despite of this, the repeated use of the derivative can compensate this low activity retention. For example, although the yield of immobilized β-galactosidase by adsorption onto bone powder was high (83%), the immobilized enzyme lost its activity continuously and only 24% of the initial activity remained after four batch reactions (Carpio *et al.* 2000). On the other hand, β-galactosidase immobilized in fibers composed of alginate and gelatin cross-linked with glutaraldehyde retained only 56% of its activity, but the immobilized enzyme was active for 35 days without any decrease in its activity (Tanriseven & Dogan 2002). Szczodrak (2000) reported more than 90% of initial enzyme activity after covalent binding of the enzyme on porous silanized glass without any notable decrease in enzyme activity during 5 repeated batch conversions. Similar result of good operational stability of immobilized enzyme was obtained with β-galactosidase immobilized in/on poly(2-hydroxyethyl methacrylate) (pHEMA) membranes by two different methods: adsorption on Cibacron F3GA derivatised pHEMA membranes (pHEMA-CB), and entrapment in the bulk of the pHEMA membranes. After 15 repeated batch conversions the retained activity of the adsorbed and the entrapped enzymes was 80% and 95%, respectively (Baran *et al*. 1997). The problems of desorption of β-galactosidase from immobilization matrix and the leakage of the entrapped enzyme due to a small molecular weight compared to porous of gel in matrices can be overcome by cross-linking using bifunctional or multifunctional reagents. Cross-linking can be realized by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix using the functional groups of cross-linking reagents (Tanaka and Kawamoto 1999). Several studies have described various cross linking reagents used for improvement of β-galactosidase stability in immobilized state (Table 1.3). These reagents form covalent

bonds using their reactive functional groups, such as carbonyl groups of glutaraldehyde (Szczodrak 2000), imidoester groups of dimethyladipimidate (Khare and Gupta 1988), carbodiimide group of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Dominguez *et al*. 1988) etc. Among these, glutaraldehyde, which interacts with the amino groups through a base reaction, has been the most extensively used in the view of its GRAS status, low cost, high efficiency, and stability (Nakajima *et al*. 1993).

Table 1.3. Types of coupling agent and functional groups of original matrix used on β-galactosidase immobilization.

FUNCTIONAL GROUPS OF ORIGINAL MATRIX	ACTIVATING REAGENT	REFERENCE
Hydroxyl, Amino — CHITOSAN	tris(hydroxymethyl)phosphine	Cheng *et al*, 2006
Hydroxyl — COTTON CLOTH	Tosyl chloride	Albayrak & Yang, 2002a
Amino — SILANIZED GLASS	Glutaraldehyde	Szczodrak, 2000
Hydroxyl — SEPHAROSE	Cyanogen bromide	Pessela *et al*, 2007
Hydroxyl — COTTON CLOTH	Poly(ethyleneimine)	Albayrak & Yang, 2002b

For example, in β-galactosidase immobilization in fibers composed of alginate and gelatin, glutaraldehyde cross-links the enzyme and gelatin forming an insoluble structure and also stabilizes the alginate gel, helping in the prevent of the enzyme leakage (Tanriseven and Dogan 2002). Beneficial effect of glutaraldehyde as a cross-linker was also shown in immobilization of β-galactosidase from *A. oryzae* by

entrapment in cobalt alginate beads. Relative activity of the entrapped enzyme without crosslinking was 83%. After the first usage the relative activity dropped to 67.5% as a result of leakage. Relative activity of the entrapped enzyme crosslinked by glutaraldehyde was unchanged (83%) and stable even after the eighth use. However, the leakage of cobalt into the reaction mixture during lactose hydrolysis limits the use of this method in the food industry (Ates and Mehmetoglu 1997). Rogalski *et al*. (1994) compared the effect of two cross-linking reagents: glutaraldehyde and bis-oxirane on the pH and thermal stability of immobilized β-galactosidase on controlled porous glass (CPG). In the case of glutaraldehyde cross-linking, relatively high thermostability was observed (retaining 70% of the initial activity at 70°C). The enzyme immobilized on CPG and cross-linked with oxirane lost 50% of the initial activity at 70°C, but these immobilizates showed a very high stability at pH 8 (more than 80% of relative activity), compared to 20% of relative activity of glutaraldehyde cross-linked enzyme at the same pH value.

Transglutaminase has been used to stabilize immobilizates formed with β-galactosidase and acidic-processed gelatins of different qualities. N epsilon-(γ-L-glutamyl)-L-lysine bonds formed by transaminase (aminotransferase) were used for transformation of gelatin into an insoluble protein. Consequently, the enzyme was entrapped in the gelatin matrices with a yield of 8–46% (Fuchsbauer *et al*. 1996). The other problem associated with the immobilized enzyme system is microbial contamination. Therefore, using immobilized enzyme in the large-scale continuous processing of milk necessitates the introduction of intermittent sanitation steps, which includes the use of regular sanitation with basic detergent and a dilute protease solution (Novalin *et al*. 2005). Recently, two types of β-galactosidases: thermostable and cold-active enzymes have increasing interest in industrial lactose hydrolysis process to avoid microbial contamination. The thermostable β-galactosidases have the ability to retain their activity at high temperatures for prolonged period and could be used in the industrial processing of dairy products simultaneously with heat treatment to sterilize the product (Wolosowska and Synowiecki 2004). The cold- active β-galactosidases provide treatment of milk and dairy foods below 5°C so that the taste and nutritional values remain unchanged (Fernandes *et al*., 2002).

1.2.3. Scale-Up issues

Two types of processes are available for industrial scale hydrolysis of lactose. In the simplest process, β-D-galactosidase is added directly to whole milk. After lactose hydrolysis is completed at a desired level, the enzyme can be deactivated by heat treatment to avoid further reaction. Since the expensive enzyme cannot be reused, the resulting operation cost is high. To overcome this problem, another process is the hydrolysis of skim milk with immobilized β-D-galactosidase. After the desired lactose hydrolysis is achieved, cream is added to the hydrolyzed milk to adjust its fat content. Because of the different advantages of immobilized systems over soluble β-D-galactosidase, immobilized enzyme systems have been intensively investigated for possible industrial application. Although numerous hydrolysis systems have been investigated, only a few of them have been scaled up with success and even fewer have been applied at an industrial or semi-industrial level. The first company to commercially hydrolyze lactose in milk by immobilized lactase was Centrale del Latte of Milan, Italy, utilizing the SNAM Progetti technology. The process makes use of a neutral lactase from *Saccharomyces* (*Kluyveromyces*) *lactis* entrapped in cellulose triacetate fibres. Sumitomo Chemical, Japan, has developed an immobilization β-D-galactosidase preparation of fungal origin on the rugged surface of an amphoteric ion-exchange resin of phenol formaldehyde polymer and this technology was used by Drouin Cooperative Butter Factory for producing market milk and hydrolyzed whey (Honda *et al*., 1993). A rotary column reactor has been developed by Snow Brand's factory that could be used both as a stirred tank reactor and a packed bed reactor, since this reactor has the functions of both types of reactor (Honda *et al*., 1991). The reaction rate was greatly affected by the packing density of immobilized β-D-galactosidase in the rotary column. This reactor can also overcome the problem of channeling or severe pressure drop. The long-term testing of lactose hydrolysis of skim milk in a horizontal rotary column reactor was carried out and 70–80% lactose hydrolysis was obtained. Hydrolysis of lactose in the rotary column reactor and washing of immobilized β-D-galactosidase were carried out for 36 cycles, which indicated that the horizontal rotary column reactor was well suited for hydrolyzing lactose in milk with fibrous immobilized β-D-galactosidase. From the pilot plant experimentations, a commercial plant was set up in Snow Brand's factory (Honda *et al*., 1993). Although the immobilized β-D-

galactosidase was washed with phosphate buffer solution and pasteurized with Tego-51, the standard plate count of lactose-hydrolyzed milk increased sharply. Pasteurization of immobilized β-galactosidase using glycerol or propylene glycol was effective without inactivation of β-D-galactosidase. Thus, immobilized β-D-galactosidase technology can be successfully applied in the hydrolysis of lactose and it can overcome the problems associated with costs of soluble enzyme. However, major problems associated with the immobilized enzyme system are microbial contamination, protein adherence and channeling. Therefore, for long-term operations, using immobilized, periodic washing and pasteurization are indispensable during operation (Hirohara *et al*., 1981; Honda and Takafuji, 1987; Honda *et al*., 1987). The protein adhering to the enzyme can be easily dissolved using high- and low-pH solutions, because the immobilized enzyme has high durability over a wide range of pH. The immobilized enzyme can be pasteurized with benzalkonium chloride (quaternary ammonium salt) after removing the proteins. The use of acetic acid solution as a cleaning and pasteurizing agent instead of lactic acid can also be effective. The problem of channeling observed in the packed column system can be overcome by changing the flow direction of feed during operation (Honda *et al*., 1993; Honda *et al*., 1991; Honda and Takafuji, 1987). Thus, immobilization enzyme systems can certainly find greater a role in the future in the hydrolysis of milk and whey.

1.3. APPLICATION OF β-GALACTOSIDASE

β-Galactosidases can be used in a number of ways to hydrolyze lactose in milk, whey and whey permeate. The choice of process technology depends on the nature of the substrate, the characteristics of the enzyme, economics of production andmarketing of the product. The primary characteristic, which determines the choice and application of a given enzyme, is the operational pH range. Acid-pH enzymes from fungi are suitable for processing of acid whey and whey permeate and the neutral-pH enzymes from yeasts and bacteria are suitable for processing milk and sweet whey. The soluble enzyme can be used for batch processes and the immobilized form can be used either in batch-wise or in continuous operation (Panesar *et al*., 2006).

1.3.1. Hydrolysis of milk lactose

Lactose-hydrolyzed milk has been used for the preparation of flavoured milk, cheese and yoghurt. The hydrolysis of lactose in milk for food processing also prevents lactose crystallization in frozen and condensed milk products. Moreover, the use of hydrolyzed milk in yoghurt and cheese manufacture accelerates the acidification process, because lactose hydrolysis is normally the rate-limiting step of the process, which reduces the set time of yoghurt and accelerates the development of structure and flavor in cheese (Shukla, 1975).

The quality of ice milk and ice-cream was significantly improved by addition of lactozyme (β-galactosidase). It prevented the crystallization of lactose by breaking into glucose and galactose and reduced sandiness. Its addition helped to replace up to 75% of milk solid non-fat with demineralized whey powder, giving the final mix an improved creaminess, making this an acceptable product for lactose intolerants (Stevenson *et al*., 1983). β-D-Galactosidase has also been used to improve the production and ageing of Cheddar cheese by preliminary hydrolysis of lactose to glucose and galactose (Gooda *et al*., 1983). It resulted in an increase in the ratio of soluble nitrogen/total nitrogen and non-protein nitrogen in Cheddar cheese. By this method, cheese production became faster and cheaper with improved organoleptic properties. Permeable *Kluyveromyces fragilis* cells have shown about 90% lactose hydrolysis in pasteurized skim milk within 1 h of treatment at a cell concentration of 1–2%, while the digitonin-permeable yeast cells performed complete hydrolysis of the lactose present in milk within 2 h (Joshi *et al*., 1989; Joshi *et al*., 1987). The immobilized *E. coli* enzyme resulted in 60% hydrolysis of lactose at 55 ºC in 6 h, whereas, 45% hydrolysis was observed with native enzyme under similar conditions (Khare and Gupta, 1990). The addition of Mg^{2+} and Mn^{2+} enhanced the hydrolysis of 2-nitrophenyl β-D-galactopyranoside and lactose (Kim *et al*., 1997). High degrees of conversion (85–90%) of lactose in saline solution, whey, whey permeate and skimmed milk was obtained with immobilized β-D-galactosidase from *K. lactis* in batch-wise or in packed beds (Ovsejevi *et al*., 1998). The immobilized β-D-galactosidase from *K. fragilis* can be applied to the production of frozen dairy products to avoid lactose crystallization and to enhance the digestibility and flavor of such products (Ladero *et*

al., 2000). The commercial enzyme preparation of β-galactosidase known as Lactozym was immobilized on cellulose beads and resulted in 60% hydrolysis of milk lactose within 5 h (Roy and Gupta, 2003).

1.3.2. Hydrolysis of whey lactose

High concentration of lactose in whey is a major environmental problem since its disposal in local water streams increases the biological oxygen demand many times. The hydrolysis of whey using β-D-galactosidase is another important application of enzyme technology in the food industry. Concentrated hydrolyzed whey or whey permeates can be used as a sweetener in products such as canned fruit syrups and soft drinks (Shukla, 1975). Whole cells of *Kluyveromyces bulgaricus* showed 80% hydrolysis in lactose solution and whey, whereas 87–100% hydrolysis of lactose syrup from ultrafiltered sweet whey has been obtained using *S. lactis* β-galactosidase (Decleire *et al*., 1985; Chiu and Kosikowski, 1985). The hydrolyzed lactose syrup was effectively decolourized using activated carbon. Digitonin-permeabilized yeast cells hydrolyzed 70–80% of lactose content in whey within 2 h.7 β-D-Galactosidase immobilized on hydrogels resulted in 70–75% lactose hydrolysis in whey within 7 h (Kozhucharova *et al*., 1990). Recycling packed bed reactors have been successfully used for lactose hydrolysis. Lactose hydrolysis of 50% has been obtained using immobilized β-Dgalactosidase from *S. fragilis* in a recycling packed bed reactor (Siso *et al*., 1994). The system can be used up to five times before any significant drop in activity. *K. fragilis* β-D-galactosidase immobilized on silanized porous glass modified by glutaraldehyde binding resulted in lactose saccharification of 86–90% in whey permeate both in a batch process and recycling packed-bed bioreactor (Szczodrak, 2000). However, Lactozym ⇔ immobilized on cellulose beads hydrolyzed >90% whey lactose in 5 h. Moreover, the immobilized enzyme can be reused three times without any change in performance of the fluidized bed reactor (Roy and Gupta, 2003).

1.3.3. Synthesis of oligosaccharides

β-D-Galactosidase has been a popular enzyme for its hydrolytic action on lactose. The transferase activity by which the enzyme produces and hydrolyzes a series of oligosaccharides has also attracted the attention of researchers because oligosaccharides have a beneficial effect on the growth of desirable intestinal microflora. Moreover, the transferase reaction can be used to attach galactose to other chemicals and consequently have potential application in the production of food ingredients, pharmaceuticals and other biological active compounds. Different cultures having high transgalactosylation activity have been identified (Rabiu *et al*., 2001; Cho *et al*., 2003). The amount and nature of oligosaccharides formed depend upon several factors including the enzyme source, concentration and nature of the substrate and reaction conditions (Prenosil *et al*., 1987; Zarate and Lopez-Leiva, 1990; Mahoney, 1998). The yield of oligosaccharides can be increased by using higher substrate concentrations and/or decreasing the water content (Monsan *et al*., 1989). Earlier, presence of oligosaccharides in low-lactose milk has prompted nutritional concerns; however, recent studies suggested that oligosaccharides may have beneficial effects on human intestinal health as 'bifidus factors', promoting growth of desirable intestinal microflora (Mahoney, 1998). Oligosaccharides are increasingly being recognized as useful dietary tools for the modulation of colonic microflora toward a healthy balance. This usually involves selectively increasing the levels of gut bifidobacteria and lactobacilli at the expense of less desirable organisms such as *Escherichia coli, Clostridia* and proteolytic bacteroides (Fuller and Gibson, 1998). Novel oligosaccharide mixtures were synthesized using β-D-galactosidases from probiotic bacteria (Rabiu *et al*., 2001). These bacteria were used in glycosyl transfer reactions to synthesize oligosaccharides from lactose with oligosaccharide yields of 24.7–47.6%. β-DGalactosidase isolated from *Bifidobacterium bifidum* was effective in transgalactosylation reactions for synthesis of oligosaccharides even at low lactose concentrations (Jorgensen *et al*., 2001). The temperature, concentration of substrate and enzyme origin play an important role in the enzymatic synthesis of oligosaccharides (Boon *et al*., 2000). However, the influence of the initial lactose concentration can be much larger (Albayrak and Yang, 2002a; Chockchaisawasdee *et al*., 2005). In general, more and larger galactooligosaccharides (GOS) can be produced with higher initial

lactose concentrations. The higher temperatures can be beneficial in higher oligosaccharide yields. The higher yield at higher temperatures is an additional advantage when operating at high initial lactose concentrations and consequently elevated temperatures. β-D-Galactosidase from *B. circulans* produces the largest-sized oligosaccharides; however, the enzyme from *Kluyveromyces* sp. produces mainly trisaccharides. The molar ratio of water to surfactant *(ω_0)* of reverse micelles can strongly affect the transgalactosylation reaction of the enzyme synthesis of GOS from lactose in AOT/iso-octane reverse micelles (Chen *et al*., 2001). The packed bed reactor and a plug-flow reactor have been successfully used for continuous production of GOS from lactose using immobilized β-Dgalactosidase (Makkar and Sharma, 1983; Shin *et al*., 1998). The selectivity for GOS synthesis can be increased several-fold undermicrowave irradiation, using immobilized β-glucosidase and with added co-solvents such as hexanol (Maugard *et al*., 2003). Recently, *Bifidobacterium bifidum* NCIMB 41171 showed good conversion rate to GOS with high lactose concentration (Tzortzis *et al*., 2005).

1.4. FUNCTIONAL FOOD

Functional foods are defined as products that are demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either improved state of health and well-being and/or reduction of risk of disease (Diplock *et al*., 1999).

The original concept of functional foods has been originated in Japan from its development of a special seal to denote Foods for Specified Health Use (FOSHU) (The FOSHU system, 1991).

In Europe the European Commission's concerted action on Functional Food Science in Europe (FUFOSE), actively involving large number of the most prominent European experts in nutrition and related sciences were engaged by the International Life Science Institute (ILSI) (Diplock *et al*., 1999).

Although not yet completely agreed on by the scientific community, the concept of functional foods refers to: "foods similar in appearance to conventional foods that are

consumed as part of a normal diet and have demonstrated physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions" (Clydesdale, 1997).

Examples may include conventional foods; fortified, enriched or enhanced foods; and dietary supplements. These substances provide essential nutrients often beyond quantities necessary for normal maintenance, growth, and development, and/or other biologically active components that impart health benefits or desirable physiological effects.

The concept of functional foods includes foods or food ingredients that exert a beneficial effect on host health and/or reduce the risk of chronic disease beyond basic nutritional functions (Huggett and Schliter, 1996).

Recently, the functional food research has moved progressively towards the development of dietary supplementation, introducing the concept of probiotics and prebiotics, which may affect gut microbial composition and activities (Ziemer and Gibson, 1998).

1.4.1. Probiotic

Probiotic foods are defined as those that contain a single or mixed culture of microorganisms that affect beneficially the consumer's health by improving their intestinal microbial balance (Fuller, 1989). There is significant scientific evidence, based mainly on *in vitro* studies and on clinical trials using animals, suggesting the potentially beneficial effects of probiotic microorganisms. These include: metabolism of lactose, control of gastrointestinal infections, suppression of cancer, reduction of serum cholesterol, and immune stimulation (Gilliland, 1990; Salminen *et al*., 1998; Fooks *et al*., 1999). The necessity for epidemiological studies on healthy human populations to support the specific health promoting claims of a probiotic strain is generally highlighted (Sanders, 1998; Shortt, 1999; Saarela *et al*., 2000). Common microorganisms used in probiotic preparations are predominantly Lactobacillus species, such as *Lactobacillus acidophilus, L. casei, L. reuteri, L. rhamnosus, L. johnsonii*, and *L. plantarum* and *Bifidobacterium* species, such as *Bifidobacterium longum, B. breve, B.*

lactis (Shortt, 1999). The incorporation of probiotic strains in traditional food products has been established in the dairy industry, leading to the production of novel types of fermented milks and cheeses (Gomes and Malcata, 1999).

1.4.2. Prebiotic

A prebiotic is a food ingredient that is not hydrolyzed by the human digestive enzymes in the upper gastrointestinal tract and beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health (Gibson and Roberfroid, 1995).

For a dietary substrate to be classed as a prebiotic, at least three criteria are required: (1) the substrate must not be hydrolyzed or absorbed in the stomach or small intestine, (2) it must be selective for beneficial commensal bacteria in the colon such as the bifidobacteria, (3) fermentation of the substrate should induce beneficial luminal/systemic effects within the host (Cummings and Macfarlane, 1991).

Oligosaccharides are sugars consisting of between approximately two and 20 saccharide units, i.e. they are short-chain polysaccharides. Apart from those, which occur naturally in fruits and vegetables, and are extractable, others can be commercially produced through the hydrolysis of polysaccharides (*e.g*. dietary fibres, starch) or through enzymatic generation. The following oligomers have been suggested as having prebiotic potential: lactulose; fructo-oligosaccharides; galacto-oligosaccharides; soybean oligosaccharides; lactosucrose; isomalto-oligosaccharides; gluco-oligosaccharides; xylo-oligosaccharides; palatinose-oligosaccharides (Gibson, 2000) and some of them are lactose derivatives (see Figure 1.5)

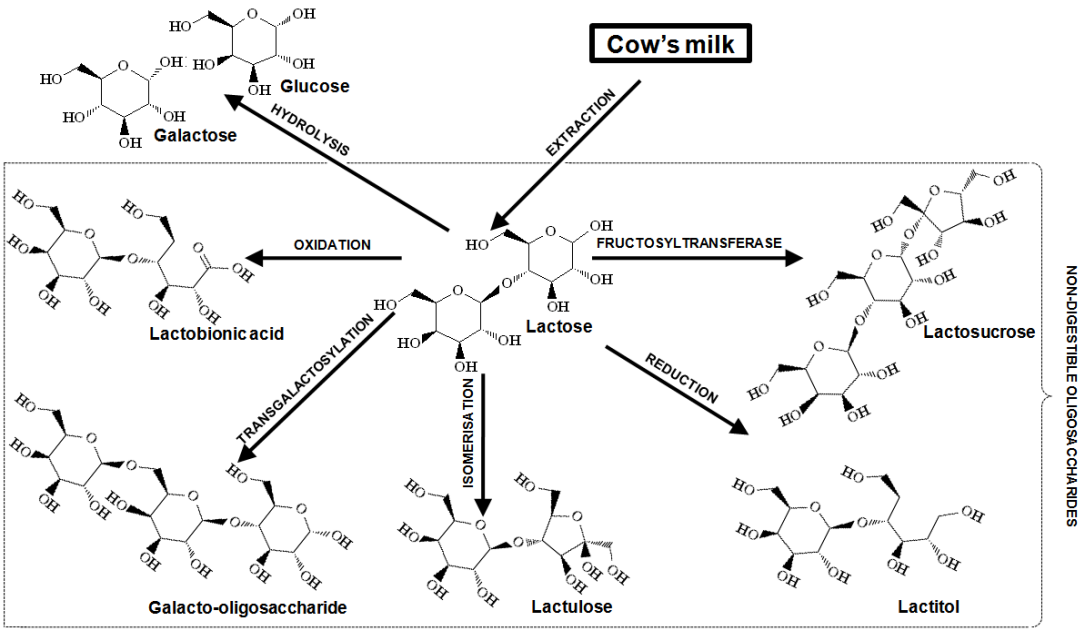


Figure 1.5. Prebiotic lactose derivatives.

1.4.2.1. Galacto-oligosaccharides

Galacto-oligosaccharide (GOS) derived from lactose in milk, have received less attention than the other prebiotics, although they are considered to have bifidogenic effects in humans. GOS is a collective term for a group of semi-synthetic non-digestible carbohydrates made using β-galactosidases as catalysts.

GOS contain zero to one glucose units and one to six galactose units bound to each other by different glycosidic bonds (β1–2, β 1–3, β 1–4, β 1–6). During production of GOS, mixtures of GOS of different chain length are formed (Alander *et al*., 2001; Ito *et al*., 1993; Tannock *et al*., 2004). During the last years, galacto-oligosaccharides were reported to be beneficial for human health (see Table 1.4), and they are now recognised as prebiotics (Gibson and Fuller, 2000). GOS, Non-Digestible oligosaccharides (NODs), are not hydrolyzed or absorbed in the upper intestinal tract, they pass on to the colon where they are then fermented selectively by beneficial intestinal bacteria, which implicate a balanced and advantageous microbiota (Boehm and Stahl, 2007). GOS are the products of transgalactosylation reactions catalysed by β-galactosidases when using lactose or other structurally related galactosides as the substrate. In aqueous systems transgalactosylation has to compete with hydrolysis, and therefore GOS mixtures

always contain considerable amounts of unreacted lactose and monosaccharides (Mahoney, 1998).

Table 1.4. Effects of galacto-oligosaccharides on human health.

GOS	Effects	Reference
Ingestion on intestinal microflora	Bifidogenic activity	Bouhnik *et al.*, 1997
	Metabolism in the colon	Kikuchi *et al*., 1996
Physiological	Improvement of defecation	Deguchi *et al* ., 1997
	Elimination of ammonia	Tamai *et al*., 1992
	Colon cancer prevention	van Dokkum *et al*., 1999
	Stimulation of mineral adsorption	Chonan and Watanuki, 1995
	Cholesterol and lipid metabolism	Hayashi, 1989

GOS are now widely used as a foodstuff beneficial to human health. The main drawback of GOS synthesis by these enzymes is that the reaction equilibrium is shifted to favor hydrolysis over synthesis in aqueous systems, which leads to a low yield in GOS production (Chen *e tal*, 2001). Galacto-oligosaccharides are present in human milk is believed to influence the establishment of a bifidus microflora in the gastro-intestinal tract of newly born, breast-fed infants (Matsumoto *et al*., 1993). Gyorgy (1973) showed that the galacto-oligosaccharide fraction from human milk (referred to as bifidus factor) enhanced the growth of bifidobacteria in the intestine not only of breast-fed infants but also of infants fed with cow's milk supplemented with this fraction.

The most abundantly supplied and utilized group of NDOs as food ingredients are Galacto-oligosaccharides (GOS) and Fructo-oligosaccharides (FOS) which are generally produced by enzymatic transglycosylation because of adequate supply of the raw materials and the high efficiency of the reaction (Sako *et al*, 1999). The stability of GOS is better than that of FOS (Voragen, 1998).

1.4.2.1.1. Production

GOS are produced from lactose by the action of β-galactosidases which have transgalactosylation activity. The linkage between the galactose units, the efficiency of transgalactosylation, and the components in the final products depend on the enzymes and the conditions used in the reaction. Glycoside bonds between two galactose units

are mainly β1-4 bonds (4'-GOS) when β-galactosidases derived from *Bacillus circulans* (Moza!ar *et al*., 1984) or *Cryptococcus laurentii* (Ozawa, Ohtsuka and Uchida, 1989) are used, and β1-6 bonds (6'-GOS) when enzymes derived from *A. oryzae* or *Streptococcus thermophilus* (Matsumoto, 1990) are used.

GOS synthesis has been studied by free (Reuter *et al.,* 1999) and immobilized enzymes (Albayrak and Yang, 2002a; Chen *et al*., 2003), using whole cells (Tzortzis *et al*., 2005), and by fermentation (Li *et al*., 2008).

In standardized large scale productions using the β-galactosidase derived from *B. circulans*, more than 55% of the lactose is converted to GOS (Ishikawa *et al*., 1995). Although tri- to hexa-saccharides with 2-5 galactose units are the main products of the reaction, transgalactosylated disaccharides (TD) consisting of galactose and glucose with different β-glycoside bonds from lactose and two galactose units are also produced. TD are considered to be NDOs, since they have similar physiological characteristics to longer GOS. Fig. 2 shows the industrial production process of GOS. A highly concentrated solution of lactose which is usually purified from cow's milk whey is used as a substrate solution in this reaction. The main products are trisaccharides, namely 4'- or 6'-galactosyllactose, and longer oligosaccharides consisting of 4 or more monosaccharide units. Substantial amounts of TD are also produced in these reactions (Matsumoto *et al*., 1990; Ishikawa *et al*., 1995).

1.5. MAGNETIC SUPPORTS

The immobilization of enzymes onto insoluble supports has been a topic of active research in enzyme technology and is essential for their application to industrial processes. A large number of enzymes were successfully immobilized with very high activity yields on appropriate supports. These immobilized products were intended for use in the construction of artificial organs, biosensors, or bioreactors. Immobilization is advantageous because (1) it extends the stability of the enzyme by protecting the active material from deactivation; (2) it enables repeated use; (3) it provides significant reduction in the operation costs; and (4) it facilitates easy separation and speeds up recovery of the enzyme. The availability of a large number of support materials and

methods of immobilization leave virtually no enzyme without a feasible route of immobilization. It is, thus, important that the choice of support materials and immobilization method over the free enzyme should be well justified (Arıca, *et al.*, 1995; Peterson and Kennedy, 1997; Uhlich, *et al*., 1996). Magnetic fields have been utilized in the support systems for the study of different enzyme immobilized (see Table 1.5).

Modified magnetic materials are nowadays wellknown and have benn investigated intensively due to their potencial applications in many areas, such as biology, medicine and the environment. Modified magnetic materials are composed of an iron oxide core coated with organic or inorganic molecules, which forms a chemical bond with the core surface. The iron oxide core is obtained as a fine powder containing nanometer-sized particles and presents superparamagnetic behavior.

Table 1.5. Enzymes immobilized onto different magnetic supports.

Biomolecule	Support	Reference
β-galactosidase	mPOS-PVA	Neri *et al*., 2008
Chitosanase	amylose-coated magnetic nanoparticles	Kuroiwa *et al*., 2008
Arg6-esterase	gold-coated magnetic nanoparticles	Jeong *et al*., 2006
Glucose oxidase	magnetic nanoparticles	Betancor *et al*., 2005
Invertase	magnetic polyvinylalcohol microspheres	Akgöl *et al*., 2001
Glucoamylase	poly(styrese) particles	Bahar and Çelebi, 1998
Trypsin	magnetic poly(GMA-MMA)-g-MAA beads	Bayramoglu *et al*., 2008
Lipase	magnetized Dacron	Pimentel *et al*., 2007

Use of magnetic particles in bioprocesses has many advantages. They can be easily separated from reaction medium and stabilized in a fluidized-bed reactor by applying a magnetic field. The use of magnetic particles so reduces capital and operational costs (Pieters and Bardeletti, 1992). The interest in using magnetic particles as supports for immobilization of enzymes as biocatalysts is constantly increasing nowadays. β-galactosidase immobilized on magnetic supports (1) can be immediately separated from the reaction mixture and the hydrolysis can be terminated easily before the yield of target higher oligosaccharides becomes lower due to further degradation; (2) the reaction can be stopped without any chemicals or heating; (3) enzyme catalysts can be recovered rapidly and reused repeatedly.

Based on the theoretical fundamentals above discussed this contribution aimed to covalently immobilize β-galactosidase onto four different low-cost magnetic supports and to apply the water insoluble derivatives on milk hydrolysis and oligosaccharides synthesis. Furthermore, to investigate their physical-chemistry and kinetics comparing to the native and free enzyme.

1.6. REFERENCES

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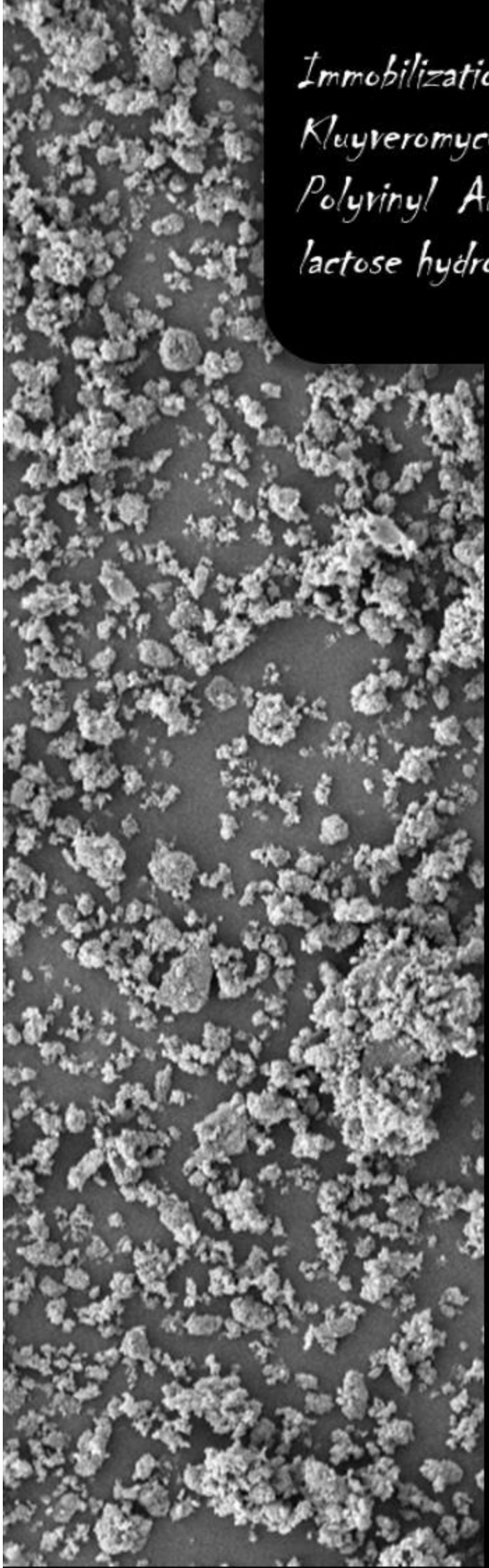

Immobilization of β-galactosidase from Kluyveromyces lactis onto a Polysiloxane-Polyvinyl Alcohol (mPOS-PVA) composite for lactose hydrolysis

CHAPTER 2

β-galactosidase from *Kluyveromyces lactis* was covalently immobilized onto a mPOS-PVA, using glutaraldehyde as activating agent and its properties were evaluated. The enzymatic water insoluble derivative displayed the same optimum pH (6.5) and optimum temperature (50°C) of the soluble enzyme. The apparent and activation energy for both soluble and immobilized enzyme derivative were found to be not significantly different. The mPOS-PVA β-galactosidase preparation presented a higher operational and thermal stability than the soluble enzyme. This immobilized β-galactosidase also was effective in hydrolyzing lactose from milk. Hence, one can conclude that mPOS-PVA is an attractive and efficient support for β-galactosidase immobilization.

Catalysis Communications | 2008 | *Published*

2.1. INTRODUCTION

In the last few years, immobilized enzymes have been extensively studied for its use in food applications (Bayramoglu *et al*., 2004). β-galactosidase (EC 3.2.1.23), commonly known as lactase, has received a particular interest as it can be used to produce an isomolecular mixture of glucose and galactose (Gekas and Lopez-Leiva, 1985). The major biotechnological application of this enzyme lies in the production of low-lactose milk (and its derived dairy products) for consumption by lactose-intolerant persons (Kretchmer, 1972). Additionally, galactooligosaccharides can also be formed by transgalactosylation.

Lactase can be found in microorganisms as well as in plant and animal tissues. However, the use of both native and soluble enzyme is limited by economic considerations (Gaur *et al*., 2006, Axelsson and Zachi, 1990). Such drawback may be overcome by using more stable and reusable immobilized enzyme biocatalysts that can be easily removed from the reaction medium, thus lowering the costs involved (Zhou and Chen, 2001a, Pessela *et al*., 2007a). Immobilization of β-galactosidase from *Kluyveromyces lactis* has been performed by covalently binding it on thiopropyl-agarose (Ovsejevi *et al*., 2004) and cellulose-gelatin (Numanoglu and Sungur, 2004) supports. Immobilized enzyme derivatives have also been reported for the lactase from *Kluyveromyces fragilis* (Roy and Gupta, 2003), *Thermus* sp. (Pessela *et al*., 2007b, Ladero *et al*., 2006), *Escherichia coli* (Bayramoglu *et al*., 2007) and *Aspergillus oryzae* (Gaur *et al*., 2006, Tanriseven and Dogan, 2002). The use of magnetic particles as support for enzyme immobilization presents the following advantages: higher specific surface area obtained for the binding of a larger amount of enzyme, on a mass basis, lower mass transfer resistance and less fouling, and selective separation of immobilized enzyme particles from a reaction mixture by simply applying a magnetic field around the reactor (Halling and Dunnill, 1980). In this research effort we describe a simple and inexpensive procedure to synthesize a magnetic enzyme derivative of β-galactosidase from *K. lactis*. Firstly, beads of a semi-interpenetrated network of polyvinyl alcohol (PVA) and polysiloxane (POS) is produced by sol-gel technique. Secondly, these beads (POS-PVA) are converted to powder and magnetized by co-precipitating Fe^{2+} and Fe^{3+} to form a composite with magnetite particles of Fe_3O_4 (mPOS-PVA). Finally, the

mPOS-PVA is activated with glutaraldehyde which acts as a chemical arm to link the enzyme molecules to the magnetic particles. This procedure has been previously and successfully used to immobilize antigens and lipase (Barros *et al*., 2002, Coêlho *et al*., 2002, Bruno *et al*., 2004, Bruno *et al*., 2005).

Some properties of the β-galactosidase immobilized on the mPOS-PVA acting on a synthetic substrate (*o*-nitrophenyl-β-D-galactopyranoside) have been investigated and compared to those established for the native enzyme. Finally, the action of the immobilized lactase derivative was investigated on the hydrolysis of whole milk lactose.

2.2. MATERIALS

β-Galactosidase from *K. lactis* was obtained from Novozymes (Bagsvaerd, Denmark). Polyvinyl alcohol, tetraethylorthosilicate, $FeCl_3$ and $MgCl_2$ were purchased from Fluka (Steinheim, Germany); $FeCl_2$ and *o*-nitrophenol (ONP) from Riedel-de Haën (Steinheim, Germany); glutaraldehyde and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) from Sigma-Aldrich (Steinheim, Germany) and bovine serum albumin from Pierce (Rockford, United States). Low fat milk containing 4.72 % (w/v) of lactose, pH 7.15, was acquired from the Pingo Doce (Minho, Portugal) supermarket. All other chemicals were of analytical grade or better.

2.3. EXPERIMENTAL PROCEDURES

2.3.1. POS-PVA synthesis and magnetization

POS-PVA beads were synthesized according to the procedure described by Barros *et al.* (2002). Briefly: 6 ml of 2% (w/v) polyvinyl alcohol, 5 ml of ethanol and 5 ml of tetraethylorthosilicate (TEOS) were mixed in a beaker. After heating up to 100 ºC, under magnetic stirring, 100 µl of concentrated HCl were added and the mixture incubated for an extra 50 min. Subsequently, the solution was distributed into the wells

of ELISA microplates (200 µl per well) and allowed to solidify for ca. 48 h at 25 °C. The resulting beads were smashed using a mortar and pestle; the powder (2 g) suspended in deionized water (100 mL) and 10 mL of a solution containing 0.6 M $FeCl_2$ and 1.1 M $FeCl_3$ (1:1) were added dropwise under magnetic stirring. Afterwards, the pH and temperature were adjusted, under overhead stirring, to 11.0 (using 33%, w/v, NH_4OH) and 100 °C, respectively, followed by incubation for 30 min. The resulting magnetized particles were thoroughly washed with deionized water until pH 7.0 was reached (Carneiro-Leão *e tal*., 1991) and collecting the magnetic particles by using a magnetic field (Ciba Corning; 6,000 Oe). The washed magnetic POS-PVA particles were dried at 50 °C overnight and finally sieved (<100µm).

2.3.2. β-galactosidase immobilization

For activation of the support, 10 mg of magnetized particles were incubated in 2.5 % (v/v) glutaraldehyde (100 µL) in 900µL of 0.1 M H_2SO_4 under orbital stirring (20 rpm) for 2 h at 25 °C. Following this incubation period, the particles were washed five times with 20 mM citrate-phosphate buffer, pH 6.5, containing 4 mM $MgCl_2$. The glutaraldehyde-activated POS-PVA magnetized particles (10 mg) were incubated with 1 mL β-galactosidase solution at different concentrations in the same buffer, for 18 h at 4 °C and 20 rpm. The enzymatic derivative thus produced was collected and washed 5 times with the aforementioned citrate-phosphate buffer. The immobilized enzyme was kept in the buffer at 4 °C until use and both the supernatant and washes were used for protein determination.

2.3.3. β-Galactosidase activity and protein determinations

The β-galactosidase activity assays were carried out using the artificial substrate ONPG (20 mM) prepared in citrate-phosphate buffer enriched with $MgCl_2$ since the hydrolysis is increased in the presence of the Mg^{2+} (Kim *et al*., 1997). The product released in the assay, ONP, was determined via absorbance readings at 410 nm. One β-galactosidase unit (U) was defined as the amount of enzyme which liberated one µmol of ONP per min per mg of protein at 25 °C. The protein concentration was determined

according to the method described by Smith *et al* (1985) using bovine serum albumin as standard. The amount of immobilized protein was calculated by the difference between the amount of protein offered to the support for immobilization and that found in the supernatant and the washing buffers.

2.3.4. Determination of kinetic parameters

The apparent Michaelis-Menten constant (K_m^{app}) and the maximum velocity (V_m) were determined, using Lineweaver-Burke plot, for both soluble and immobilized enzyme derivative by assaying activity at different concentrations of ONPG (0.625-40 mM).

The activation energies (E_a) for both soluble and immobilized enzyme derivative were calculated according to the Arrhenius law (Equation 1) by measuring the activities at different temperatures below the optimal temperature:

$$\log (\text{Activity}) = \log A - E_a / 2{,}303.RT \qquad \text{Equation (1)}$$

where E_a is the activation energy of the reaction, A the Arrhenius pre-exponential factor (or collision frequency), T the absolute temperature and R the universal gas constant. The values of A and E_a were obtained via nonlinear regression of the linearized Arrhenius equation to the experimental data.

The catalytic efficiency of the immobilized enzyme was calculated as follows:

$$\text{Catalytic efficiency} = \frac{\left(V_m / K_m^{app}\right)_{Immobilized}}{\left(V_m / K_m^{app}\right)_{Soluble}} \times 100 \qquad \text{Equation (2)}$$

2.3.5. Effects of pH and temperature on the catalytic activity

The effects of both pH and temperature on the activity of soluble and immobilized enzyme counterparts were investigated in the ranges 5.0-8.0 using 20 mM

citrate-phosphate buffer containing 4 mM $MgCl_2$, and 30-70 °C, respectively, using the aforementioned activity determination procedures.

2.3.6. Thermal stability characterization and reuse of the enzymatic derivative

Thermal stability of soluble and immobilized enzyme derivative was studied by incubating both enzymatic forms at 35 °C for 24 h. Appropriate aliquots of soluble and immobilized enzyme derivative were withdrawn at different time intervals and the activities determined after 30 min at 25 °C (temperature equilibration) using the protocol described above. The retention of activity of the immobilized enzyme derivative was evaluated by incubating the same immobilized preparation with ONPG for 20 times (between each successive use, the immobilized enzyme derivative was washed five times with 20 mM citrate-phosphate buffer, pH 6.5, containing 4 mM $MgCl_2$). The ONP production was evaluated spectrophotometrically as described above.

2.3.7. Lactose hydrolysis by the mPOS-PVA β-galactosidase derivative

Ten eppendorfs each containing 1 mL of low fat milk were incubated with the mPOS-PVA β-galactosidase (10 mg) at 25 °C under orbital stirring (20 rpm). At predetermined time intervals, one eppendorf was withdrawn and the support immediately separated from the reaction medium by applying a magnetic field. Then, the reaction medium was heated at 100 °C for 10 min and filtered with a 0.2 mm syringe filter (to eliminate eventual magnetic particle not separated by the magnetic field). Following this, 100 μL aliquots were withdrawn and assayed for lactose, galactose and glucose content by HPLC (Jasco AS-2057 Plus), employing a MetaCarb 87P 300 mm x 7.8 mm column (Varian) at 80 °C, a refractive index detector (Jasco RI-2031 Plus) and a mobile phase Milli-Q water at a flow rate of 0.4 mL min^{-1} (Jasco PU-2080 Plus).

2.3.8. Analysis of the stabilization of the quaternary structure of the enzyme

To check the stabilization of the quaternary structure of the protein, the enzyme derivative was sequentially washed with a series of buffer solutions (three washouts

were produced) and then with a series of urea 6 M solutions (three washouts were equally produced). This treatment releases from the support any enzyme molecules and/or subunits that were not covalently bound to it, while it is unable to break the enzyme-support covalent attachment; all the bonds established between the enzyme and the support are indeed very stable secondary amino bonds, and stand still at 6 M urea. In this way, any molecule that was not covalently attached to the support is released into the medium. Then, SDS-PAGE analysis of the supernatant together with all the washouts was performed, and the gel was stained with Coomassie Blue (when quantification of the enzyme molecules released into the supernatant was sought) and analyzed by densitometry.

2.4. RESULTS AND DISCUSSION

2.4.1. mPOS-PVA characterization

β-galactosidase immobilization has been carried out on several supports, *e.g.*, silica-alumina, thiopropyl-agarose, cellulose-gelatin, magnetic poly glycidylmethacrylate-methylmethacrylate (GMA-MMA) beads, glyoxyl and amino (MANAE) agarose and polyethyleneimine (PEI), alginate-gelatin fibers, celite and cellulose beads. These immobilization procedures involved physical adsorption, covalent coupling, cross-linking and entrapment. Figure 2.1A schematically displays the chemistry used in the support synthesis and immobilization procedure entertained in the present research effort, whereas Figures 2.1B and 2.1C display the scanning electron microscopy (SEM) analysis of non-magnetized and magnetized POS-PVA particles. The synthesized semi-interpenetrated network (first step: bead formation) combines the porous properties of glass (polysiloxane) with the vicinal hydroxyl groups of the organic polymer (polyvinyl alcohol). Afterwards, the beads are smashed to increase the immobilization surface (second step) and the particles are co-precipitated with Fe^{2+} and Fe^{3+} (third step) to provide them with magnetic properties in order to facilitate support recovery from the reaction medium. Finally, glutaraldehyde is attached to the polyvinyl alcohol vicinal hydroxyl groups under acid catalysis (Araujo *et al.*, 1997), and allowing the β-galactosidase immobilization by acting as a chemical spacer-arm. The linkage of

the enzyme to the polyvinyl alcohol-glutaraldehyde is schematically represented in Figure 2.1A as a Schiff's base, but glutaraldehyde may react in several ways (Migneault *et al*., 2004).

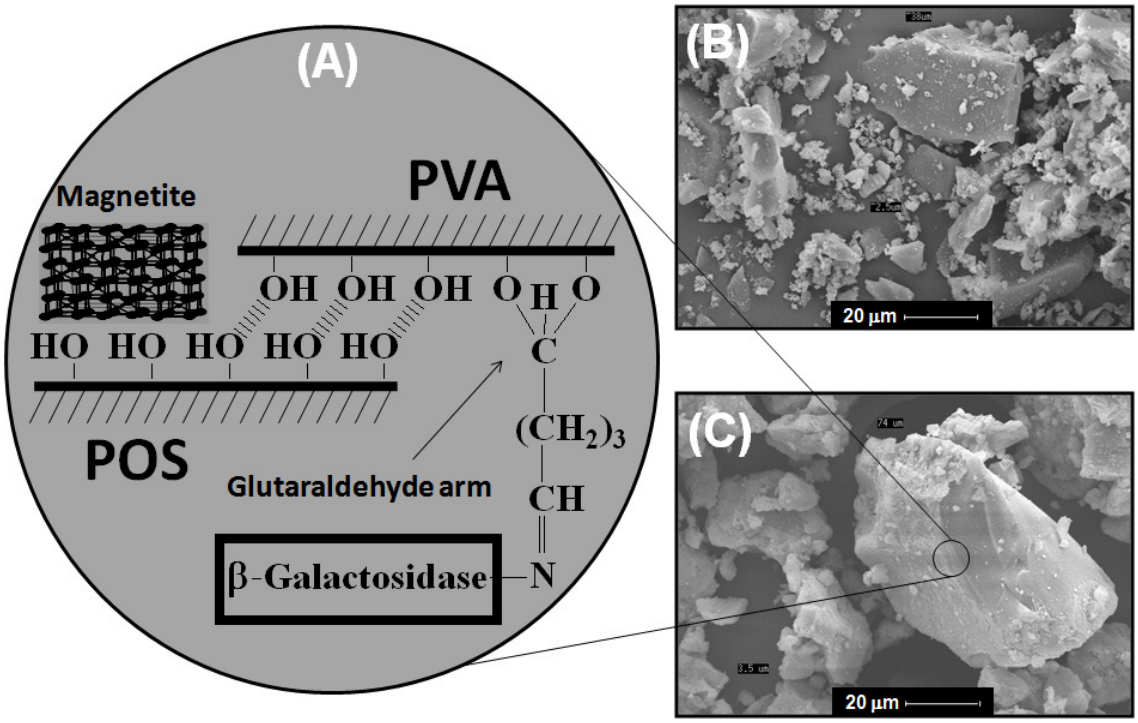

Figure 2.1. Schematic representation of the chemistry involved in the magnetic POS-PVA synthesis and β-galactosidase immobilization (A), scanning electronic microscopy particles of non magnetized (B) and magnetized (C) smashed beads of the semi-interpenetrated network of polysiloxane (POS) and polyvinyl alcohol (PVA).

The SEM images indicate rhombohedra particles and a slight increase in the size of the magnetized particles and a reduction on its sharpness that may be a consequence of the alteration of the electrical field following particle magnetization. The relationship between the amount of β-galactosidase offered to the mPOS-PVA particles and the retained protein, enzyme activity and the specific activity were studied. The fixed protein linearly increased with the amount of offered protein. A similar behaviour was observed for the amount of immobilized enzyme suggesting, as confirmed experimentally, that the retained specific activity remained constant for this range of offered enzyme concentrations. Based on the maintenance of the enzyme specific activity for different amounts of immobilized enzyme, it is possible to consider that mass transfer limitations are not a major concern. For the following experiments, an amount of offered enzyme of 0.19 mg/ml was used.

2.4.2. Kinetic parameters

The Km and K_m^{app} for the native and immobilized enzyme, respectively, were estimated as 2.525 ± 0.184 mM and 2.296 ± 0.208 mM. This difference did not show to be statistically significant according to a *t-Student* test analysis (F = 1.200; $p > 0.05$). This result suggests that the micro-events (partitioning, diffusional or mass transfer, conformational and steric effects) involving immobilized enzyme-substrate interaction and its microenvironment did not markedly disturb enzyme action. Higher K_m^{app} values after β-galactosidase immobilization compared to those calculated for the soluble enzymes have been reported by other authors with increases from 1.2-fold (Tanriseven and Dogan, 2002) up to 5.4-fold (Zhou and Chen, 2001a,). However, β-galactosidase from *K. lactis* immobilized on cellulose-gelatin showed a smaller value of K_m^{app} (11.8 mM) than that estimated for the soluble enzyme (13.3 mM) (Numanoglu and Sungur, 2004). The fact that the immobilization of the enzyme on the present support does not alter the value of K_m^{app} may be considered an important advantage when compared with most of the existing supports. Additionally, its magnetic characteristic provides easier recovery by a magnetic field. However, the catalytic efficiency of the immobilized enzyme was 12% compared to that found for the native enzyme. This reduced catalytic activity can be attributed to several factors, such as protein conformational changes induced by the support, steric hindrances and diffusional effects. These factors may operate simultaneously or separately, alternating the microenvironment around the bound enzyme (Bayramoglu *et al*., 2007).

The value of activation energy for the immobilized enzyme showed a marginal increase from 25.5 ± 8.7 for the soluble enzyme to 32.6 ± 5.8 KJ mol^{-1}. However, this difference was not significantly different according to a *t-Student* test analysis (F=2.240, p>0.05). Tu *et al* (1999) reported an increase of the activation energy value of the immobilized β-galactosidase from *Cicer arietinum* (gram chicken bean) on resin D202 from 41.6 KJ mol^{-1} (soluble enzyme) to 71.0 KJ mol^{-1}. On the other hand, El-Masry *et al* (2001) registered a decrease of the Aspergillus oryzae β-galactosidase activation energy (36.8 KJ mol^{-1}) after being immobilized on nylon membranes (25.1 KJ mol^{-1}).

2.4.3. Effects of pH and temperature on the catalytic activity

The optima pH and temperature values for both soluble and immobilized enzyme activities were found to be 6.5 (Figure 2.2A) and 50 ºC (Figure 2.2B), respectively. The immobilized enzyme derivative was lightly more stable at higher pH: the soluble and the immobilized enzyme retained 15 % and 25 %, respectively, of its initial activity at pH 8.0. This optimum pH is inside the range reported in the literature (6.0-7.0). Nevertheless, optima pH values of 4.5 (Tanriseven and Dogan, 2002) and 7.7 (Zhou and Chen, 2001b) have been cited for the *A. oryzae* β-galactosidase immobilized on fibers of alginate-gelatin and *K. lactis* β-galactosidase on graphite, respectively.

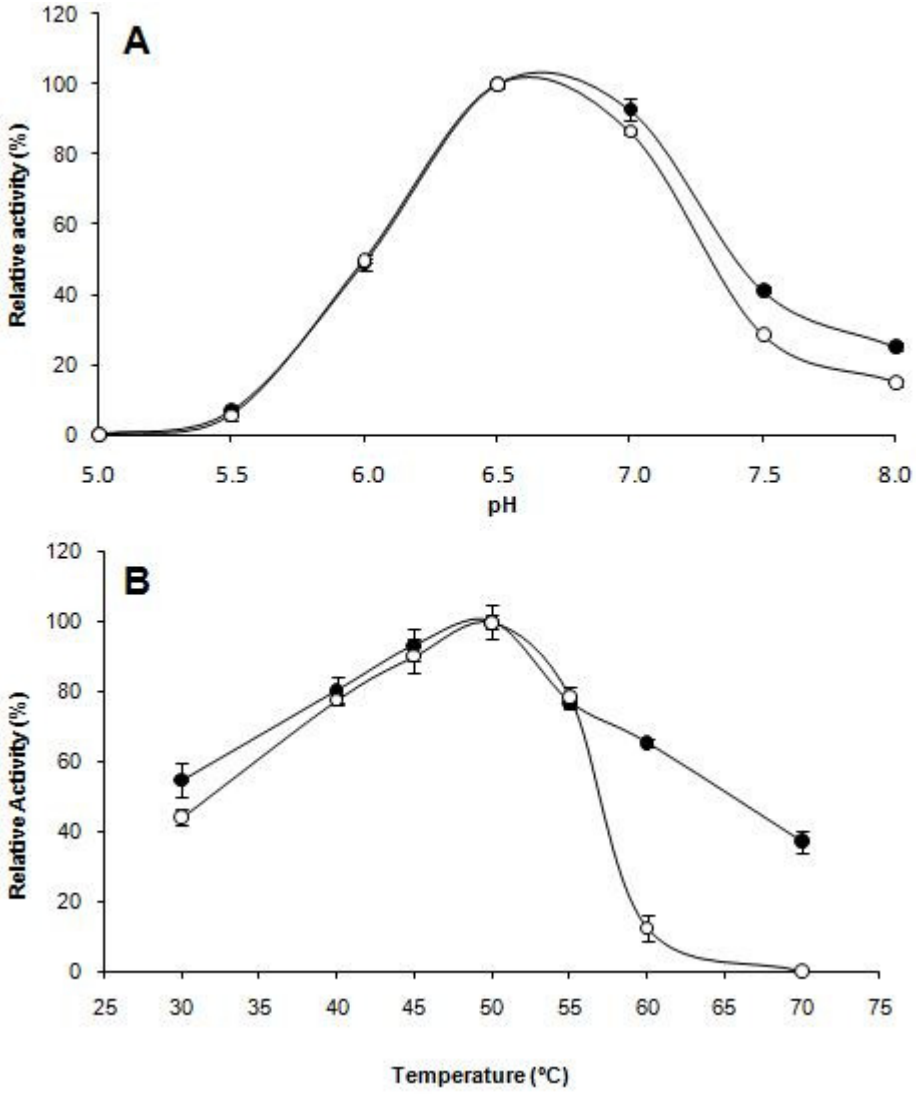

Figure 2.2. Effect of the pH (A) and the temperature (B) on the activity of the soluble (○) and the immobilized β-galactosidase (●) (N (number of replicates) = 3).

The optimum temperature value for the immobilized derivative is equal to those reported in the literature for other immobilized β-galactosidase obtained from *A. oryzae* (Tanriseven and Dogan, 2002), *K. lactis* (Zhou and Chen, 2001b) and *K. fragilis* (Roy and Gupta, 2003), and slightly lower (55 ^{o}C) than that found for the enzyme from *Bacillus* sp (Cheng *et al*., 2006). However, different optimum temperature values have been reported for the *C. arietinum* β-galactosidase immobilized on modified resin D202 (Tu *et al*., 1999) and the *E. coli* enzyme on silanized porous glass modified (Bayramoglu *et al*., 2007), namely, 60^{o} C and 35^{o}C, respectively. It is worthwhile to notice that at 70 ºC the soluble enzyme lost all activity but the immobilized enzyme retained 37 % of its initial activity. This thermal stability will be better demonstrated below.

2.4.4. Measurement of thermal stability and reutilization of the enzymatic derivative

The higher thermal stability of the β-galactosidase immobilized on mPOS-PVA compared to the soluble enzyme is depicted in Figure 2.3A. The soluble enzyme lost of all activity after 10 h of incubation at 35^{o}C whereas the immobilized one retained 47 % of its initial activity at the end of one day of incubation. The increased stability observed in the immobilized enzyme should be attributed to a reduction in the protein 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 and Amiji, 2004).

The immobilized β-galactosidase on mPOS-PVA was successively reutilized for 20 cycles at 25 ºC and at the end the enzymatic derivative retained approximately half of its initial activity (Figure 2.3B). This decrease may be caused by the mass loss of the enzymatic derivative during the washing procedure. This performance of the mPOS-PVA β-galactosidase derivative would be an additional advantage besides that from its easy of separation from the reaction medium by a magnetic field. Bruno *et al* (2005) also used this support for the immobilization of *Mucor miehei* lipase and reported that

after seven reutilization cycles the immobilized enzyme retained only 11.1 % of the initial activity.

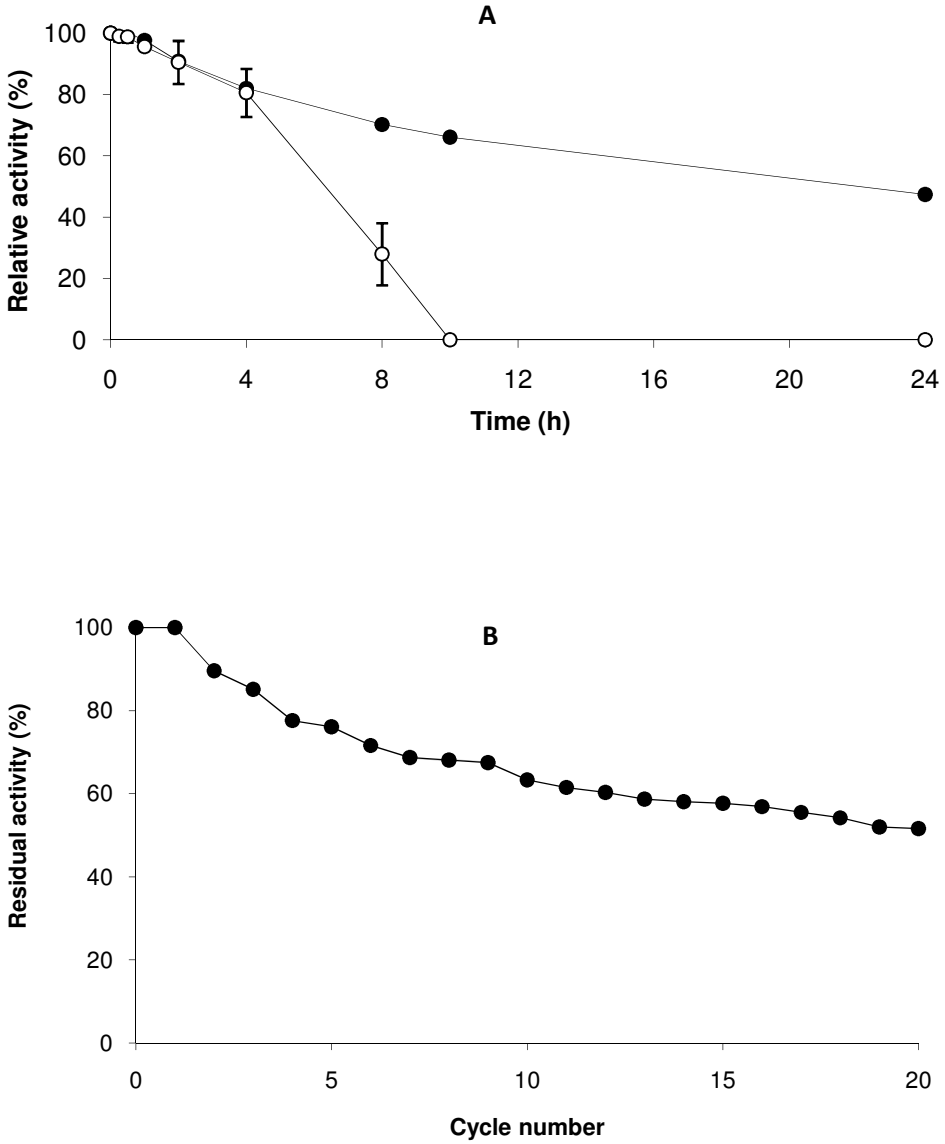

Figure 2.3. Thermal stability of the soluble (○) and immobilized (●) β-galactosidase at 35 ºC, using ONPG as substrate Aliquots were withdrawn at the indicated time interval cooled to 25 ºC and assayed for residual activity. The initial activity at 25 ºC was taken as 100 % activity (A) (N = 3). Effect of the number of reutilization cycles on the activity of β-galactosidase immobilized on mPOS-PVA (B) (N = 1).

2.4.5. Lactose hydrolysis by the mPOS-PVA β-galactosidase derivative

Figure 2.4 shows the action of the mPOS-PVA β-galactosidase on the lactose of low fat milk under batch reactor and at 25 ^{o}C. Almost all lactose (*c.a* 90%) was hydrolyzed in galactose and glucose after 120 min of the immobilized enzyme action. It

must be worthwhile to draw attention to the galactooligosaccharides formation. Similar results have been described by Giacomini *et al*. (2001) and Roy and Gupta (2003) using *K lactis* β-galactosidase immobilized on CPC-derivative and *K. fragilis* immobilized on cellulose beads acting on whey lactose, respectively. Zhou and Chen (2001a) working with *K. lactis* β-galactosidase immobilized onto graphite surface also reported galactooligosaccharides formation.

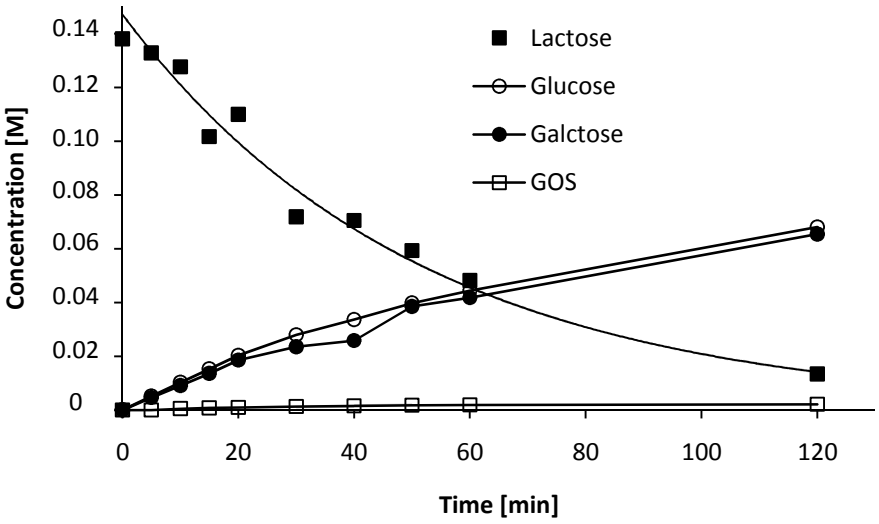

Figure 2.4. Time course of the defatted milk lactose(■) hydrolysis catalyzed by the β-galactosidase immobilized on mPOS-PVA and galactose(●), glucose(○) and galactooligosaccharides(□) release (N = 1).

2.4.6. Study of the structural stabilization of the quaternary structure of the enzyme via multisubunit immobilization

Figure 2.5 depicts the SDS-PAGE analysis of the supernatant, obtained after immobilizing the enzyme onto a mPOS-PVA, and of all enzyme derivative washouts produced (using both buffer and highly denaturating urea solution). From a simple inspection of the Coomassie-stained electrophoretogram produced, it is clear that the enzyme was indeed (irreversibly) covalently bound to the support, exerting a dramatic effect upon the structural stabilization of the enzyme. This can explain the high degree of retention of activity of the enzyme after the immobilization timeframe (see Figure 2.3B), and can be correlated with a high degree of rigidification of the enzyme molecules following immobilization onto a mPOS-PVA perhaps due to the lack of

geometrical constraints (*e.g*. if the dimeric enzyme is a planar one, it will be virtually impossible not to get the two subunits interacting with a plane surface).

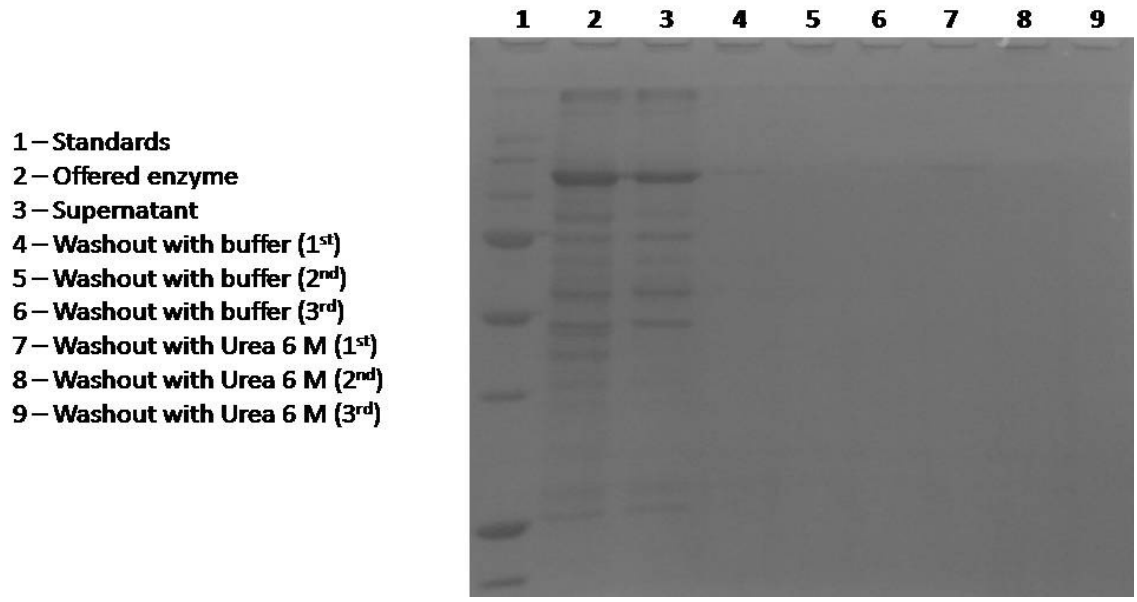

Figure 2.5. Coomassie-stained electrophoretogram of offered soluble β-galactosidase and corresponding washouts following the enzyme immobilization protocol (lane 1: low molecular weight markers; lane 2: soluble enzyme offered to the support; lane 3: supernatant solution after immobilization timeframe; lanes 4-6: washout of the support with buffer; lanes 7-9: washout of the support with urea 6 M).

2.5. CONCLUSIONS

Magnetic Polysiloxane-polyvinyl alcohol (mPOS-PVA) proved to be an attractive and efficient support for β-galactosidase immobilization due to the following arguments: the simplicity of the matrix synthesis and immobilization protocol; the easy removal of the reaction medium by simply applying a magnetic field on the reactor and the capability to catalyze the milk lactose hydrolysis into galactose and glucose and yielding galactooligosaccharides as well. The water insoluble synthesized enzymatic derivative acting on *o*-nitrophenyl-β-D-galactopyranoside displayed the same optima pH (6.5) and temperature (50 ºC) of the native enzyme and similar apparent Michaelis-Menten constant (*c.a.* 7mM on ONPG) and energy of activation (*c.a.* 30 KJ mol^{-1}) values. Furthermore, it retained about half of its initial activity after being reused 20-times at 25ºC or being incubated once at 35ºC for 24 h, whereas the soluble enzyme counterpart lost all its activity when used under these conditions.

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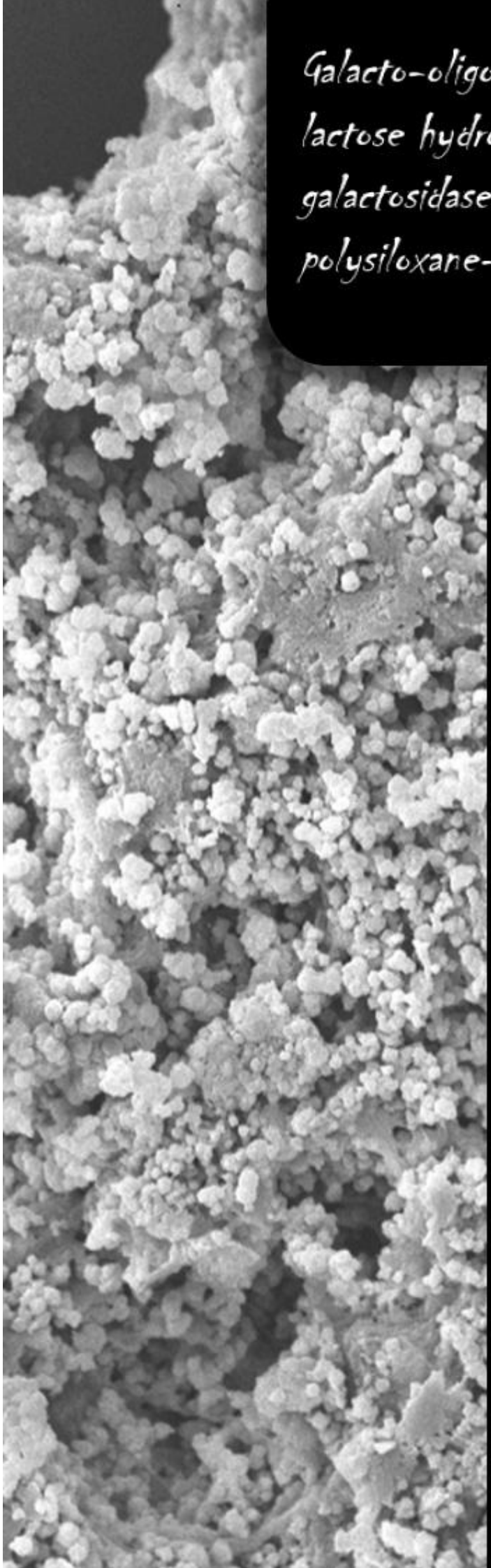

Galacto-oligosaccharides production during lactose hydrolysis by free Aspergillus oryzae β-galactosidase and immobilized on magnetic polysiloxane-polyvinyl alcohol

CHAPTER 3

The synthesis of galacto-oligosaccharides (GOS) by the action of *Aspergillus oryzae* β-galactosidase free and immobilized on magnetic polysiloxane-polyvinyl alcohol (mPOS-PVA) was studied. The initial lactose concentration in the reaction media affected the total amount and type of GOS and their time course production was described a "bell-shaped" curve as a result of the balance between transgalactosylation and hydrolysis. A maximum GOS concentration of 26% (w/v) of total sugars was achieved at near 55% lactose conversion from 50%, w/v lactose solution at pH 4.5 and 40°C. Trisaccharides accounted for more than 81% of the total GOS produced. GOS formation was not considerably affected by pH and temperature. The concentrations of glucose and galactose encountered near maximum GOS concentration greatly inhibited the reactions and reduced GOS yield. GOS formation was not affected by enzyme immobilization in the mPOS-PVA matrix, indicating the absence of difusional limitations in the enzyme carrier. Furthermore, this water insoluble magnetic derivative was reutilized 10-times and retained about 84% of the initial activity. A kinetic model for the interpretation of lactose hydrolysis and oligosaccharides synthesis for the free and immobilized β-galactosidase was used. Parameters were determined by a Systems Biology toolbox based on experimental data obtained at various initial lactose concentrations (10 - 50%; w/v). Data obtained showed that the model provides a good description of the process kinetics.

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3.1. INTRODUCTION

Galacto-oligosaccharides (GOS) are non-digestible food ingredients that beneficially affect the host by selectively stimulating the proliferation of bifidobacteria and lactobacilli in the intestine, which are considered to be beneficial to human health and can be synthesized from lactose when this sugar acts as the acceptor and transgalactosylation is catalyzed by β-galactosidase. However, if this acceptor is a water molecule galactose is released through a hydrolysis reaction (Sako *et al*., 1999).

In aqueous systems transgalactosylation has to compete with hydrolysis, and therefore GOS mixtures always contain considerable amounts of remaining lactose and monosaccharides (Mahoney, 1998). Recently, β-Galactosidase from *Aspergillus oryzae* was immobilized by three different techniques and used in oligosaccharides synthesis (Gaur *et al*., 2006).

Magnetic fields have been used in support systems for the study of enzyme immobilization (Pimentel *et al*., 2007; Kuroiwa *et al*., 2008; Bayramoglu *et al*., 2008). The use of magnetic particles for enzyme immobilization in bioprocesses has many advantages, as they can be used repeatedly and easily separated from reaction medium by a magnetic field (Bruno *et al*., 2005) reducing capital and operation costs (Pieters and Bardeletti, 1992).

Michaelis-Menten kinetic parameters can be determined using graphic approaches or linear regression methods (Yang and Okos, 1989; Ladero, 2001). Other methods use integrated rate equations by nonlinear regression to fit the model at different concentrations of substrate (Carrara and Rubiolo, 1996). Most of the models have neglected the synthesis of oligosaccharides, however when using higher lactose concentrations the synthesis reaction are necessary to be included in the model. In order to give a good description of the experimental data a reaction mechanism that includes both hydrolysis and synthesis was used. To better describe the process, a kinetic model was adapted from the work of Boon *et al.* (1999).

Although β-galactosidase is the most employed enzyme for the industrial production of GOS, kinetics studies considering the hydrolysis of lactose and oligosaccharides synthesis by these enzymes are limited (Bruins *et al*., 2003) and most of the works in the literature consider only the hydrolysis reaction (Nakkharat and

Haltrich, 2006; Alsan and Tanriseven, 2007). The aim of this work was to study lactose hydrolysis and oligosaccharides synthesis by β-galactosidase from *A. oryzae* free and covalently immobilized on magnetic polysiloxane-polyvinyl alcohol (mPOS-PVA) particles at various initial lactose concentrations, different temperatures and pH. In addition, the kinetic parameters for various initial lactose concentrations (10 - 50%; w/v) were determined and compared for the free and immobilized enzyme by using experimental data.

3.2. MATERIALS AND METHODS

3.2.1. POS-PVA synthesis and magnetization

POS-PVA beads were synthesized according to the procedure described by Barros *et al.* (2002). Briefly: 6 ml of 2% (w/v) polyvinyl alcohol, 5 ml of ethanol and 5 ml of tetraethylorthosilicate (TEOS) were mixed in a beaker. After heating up to 100ºC, under magnetic stirring, 100 µl of concentrated HCl were added and the mixture incubated for an extra 50 min. Subsequently, the solution was distributed into the wells of ELISA microplates (200 µl per well) and allowed to solidify for ca. 48 h at 25°C. The resulting beads were smashed using a mortar and pestle; the powder (2g) suspended in deionized water (100 mL) and 10 mL of a solution containing 0.6 M $FeCl_2$ and 1.1 M $FeCl_3$ (1:1) were added dropwise under magnetic stirring. Afterwards, the pH and temperature were adjusted, under overhead stirring, to 11.0 (using 33%, w/v, NH_4OH) and 100°C, respectively, followed by incubation for 30 min. The resulting magnetized particles were thoroughly washed with deionized water until pH 7.0 was reached (Carneiro-Leão *et al*., 1991) and the magnetic particles collected by using a magnetic field (Ciba Corning; 6,000 Oe). The washed magnetic POS-PVA particles were dried at 50°C overnight and finally sieved (<100µm).

3.2.2. β-galactosidase immobilization

For activation of the support, 10 mg of magnetized particles were incubated in 2.5% (v/v) glutaraldehyde (100 µL) in 900µL of 0.1 M H_2SO_4 under orbital stirring (20

rpm) for 2 h at 25°C. Following this incubation period, the particles were washed five times with 20 mM citrate-phosphate buffer, pH 4.5. The glutaraldehyde-activated POS-PVA magnetized particles (10 mg) were incubated with 1 mL β-galactosidase solution at different concentrations in the same buffer, for 18 h at 4°C and 20 rpm. The enzymatic derivative thus produced was collected and washed 5 times with the aforementioned citrate-phosphate buffer. The immobilized enzyme was kept in the buffer at 4 °C until use and both the supernatant and washes were used for protein determination. The protein concentration was determined according to the method described by Smith *et al* (1985) using bovine serum albumin as standard. The amount of immobilized protein was calculated by the difference between the amount of protein offered to the support for immobilization and that found in the supernatant and the washing buffers.

3.2.3. Galactooligosaccharides production

Production of GOSs from lactose was studied with free and immobilized enzyme on mPOS-PVA in different conditions. The reaction kinetics was studied at six different initial lactose concentrations (5, 10, 20, 30, 40, and 50%, w/v), five different pH values (3.5, 4.0, 4.5, 5.0, and 5.5), and four different temperatures (30, 40, 50, and 60ºC). The lactose solution was prepared by dissolving lactose in 20 mM citrate-phosphate buffer solution. Samples were taken at appropriate time intervals and analyzed for sugar content by high performance liquid chromatography (HPLC). The effects of galactose and glucose on GOSs production were also studied by adding galactose (3, 7, and 10%, w/v) and/or glucose (3, 7, and 10%, w/v) in the lactose solution.

3.2.4. Sugar analysis

GOSs, lactose, glucose and galactose concentration were determined using HPLC (Jasco AS-2057 Plus), with a MetaCarb 67H at 60 °C column and refractive index detector (Jasco RI-2031 Plus); used mobile phase was a 0,001N H_2SO_4 solution at a flow rate of 0.5 mL min^{-1} . As the concentration (w/v) of these sugars is proportional to their peak areas, normalized sugar concentrations as weight percentages of total sugars or initial lactose were determined from peak areas and are reported in this work. It should be noted that the accuracy of this approximation was verified by checking the

material balance. It is possible that the lactose peak shown in the chromatogram might also contain other disaccharides, although it has been reported that the extent of disaccharide formation is limited with this enzyme as compared with other lactases (Albayrak and Yang, 2002; Dombo *et al*., 1997).

3.2.5. Determination of kinetic parameters and data handling

The presented kinetic model for the trisaccharides synthesis and lactose hydrolysis mechanisms includes glucose and galactose competitive inhibition and was mathematically modelled following four ordinary differential equations:

The lactose hydrolysis (*L*) is given by,

$$\frac{dL}{dt}=\frac{n_{1L}[L][H_2O]+n_{2L}[Tri][H_2O]+n_{3L}[L]^2}{d_1} \quad \text{where,} \quad n_{1L}=\frac{-k_1k_2}{k_4};n_{2L}=k_2;n_{3L}=\frac{-2k_1k_3}{k_4} \qquad \text{Equation 1}$$

the glucose (*G*) formation by,

$$\frac{dG}{dt}=\frac{n_{1G}[L]^2+n_{2G}[L][H_2O]}{d_1} \quad \text{where,} \quad n_{1G}=\frac{k_1k_3}{k_4};n_{2G}=\frac{k_1k_2}{k_4} \qquad \text{Equation 2}$$

and the galactose (*Gal*) by,

$$\frac{dGal}{dt}=\frac{n_{1Gal}[H_2O][L]+n_{2Gal}[Tri][H_2O]}{d_1} \quad \text{where,} \quad n_{1Gal}=\frac{k_1k_2}{k_4};n_{2Gal}=k_2 \qquad \text{Equation 3}$$

Finally, the trisaccharides production (*Tri*) is described by the equation 4

$$\frac{dTri}{dt}=\frac{n_{1Tri}[Tri][H_2O]+n_{2Tri}[L]^2}{d_1} \quad \text{with,} \quad n_{1Tri}=-k_2;n_{2Tri}=\frac{k_1k_3}{k_4} \qquad \text{Equation 4}$$

and $$d_1=\frac{k_2}{k_4}[H_2O]+\left(\frac{k_3}{k_4}+\frac{k_1}{k_4}\right)[L]+[Tri]+\frac{k_2k_5}{k_4k_6}[G][H_2O]+\frac{k_3k_7}{k_4k_8}[Gal][L]+\frac{k_2k_7}{k_4k_8}[Gal][H_2O]+\frac{k_3k_5}{k_4k_6}[G][L]$$

Where k_1, k_2, k_3, k_4, k_5, k_6, k_7 and k_8 are reaction rate constants. The kinetic model was adapted from the work of Boon *et al.* (1999) and the rate expressions were derived with the King-Altman method (King and Altman, 1956; BioKin, Lda).

The model described (Equations 1 to 4) consists of a set of four differential equations thus representing four dependent state variables (lactose, glucose, galactose and trisaccharides concentration) and up to eight parameters (k_1, k_2, k_3, k_4, k_5, k_6, k_7 and k_8). In this model water or lactose can react with the galactosyl-enzyme complex and

the synthesis of the oligosaccharides is assumed to be reversible. The model used only lactose as substrate. Lactose inhibition, formation of allolactose (Huber *et al*., 1976), mutarotation of galactose (Bakken *et al*., 1992), formation of tetrasaccharides, difusional limitations and enzyme inactivation with time were not considered in the model. Also, eventual temperature and pH effects on the lactose hydrolysis and oligosaccharides synthesis were not included.

Initial values for the state variables were taken from the experimental data and their concentrations were analyzed off-line. The estimation of the model parameters was performed by the simulated annealing algorithm of the Systems Biology toolbox for *Matlab* (Mathworks) software (Schmidt and Jirstrand, 2006) using the results of the independent batch experiments, with the sets of experimental data from different initial lactose concentration (10%-50%; w/v) as substrate. All experimental data points were fitted simultaneously for each concentration. According to the model above mentioned the eight rate constants were as follows: k_1 is the overall reaction rate constant; $k_{2,}$ describes the reaction of water with the galactosyl-enzyme complex; k_3 is a measure of oligosaccharides synthesis; k_4 describes the hydrolysis of the formed oligosaccharides; and k_5/k_6 and k_7/k_8 account for glucose and galactose inhibition, respectively. The eight parameters were grouped into five parameters (k_1, k_4, k_3/k_4, k_5/k_6 and k_7/k_8) that were estimated and k_2, which was assumed constant. The rate equations were applied in the program, taking into account product galactose inhibition with (k_7, $k_8 \neq 0$) and without (k_5, $k_6 = 0$) glucose inhibition. The ordinary differential equations from the model were solved by numerical integration with an ODE23s method which is also offered in the same software.

3.3. RESULTS AND DISCUSSION

3.3.1. Effects of lactose concentration on GOS production by free and immobilized enzyme

The most significant factor on GOS formation is the initial lactose concentration (Boon *et al*., 2000). A typical GOS, glucose and galactose time course production is presented in Figure 3.1 for 50%, w/v intial lactose concentration, for free and

immobilized *A. oryzae* β–galactosidase. From this, it can be observed that: 1) the mass amount of consumed lactose equals the mass amount of GOS plus the monosaccharides mass amount, 2) different conversion rates were observed for free and immobilized enzyme experiments; this is a consequence of the different amounts of enzymes being used (an enzyme concentration of 0.149 mg/mL was used in the free form, while for the immobilized form, enzyme concentration was 0.383 mg/mL).

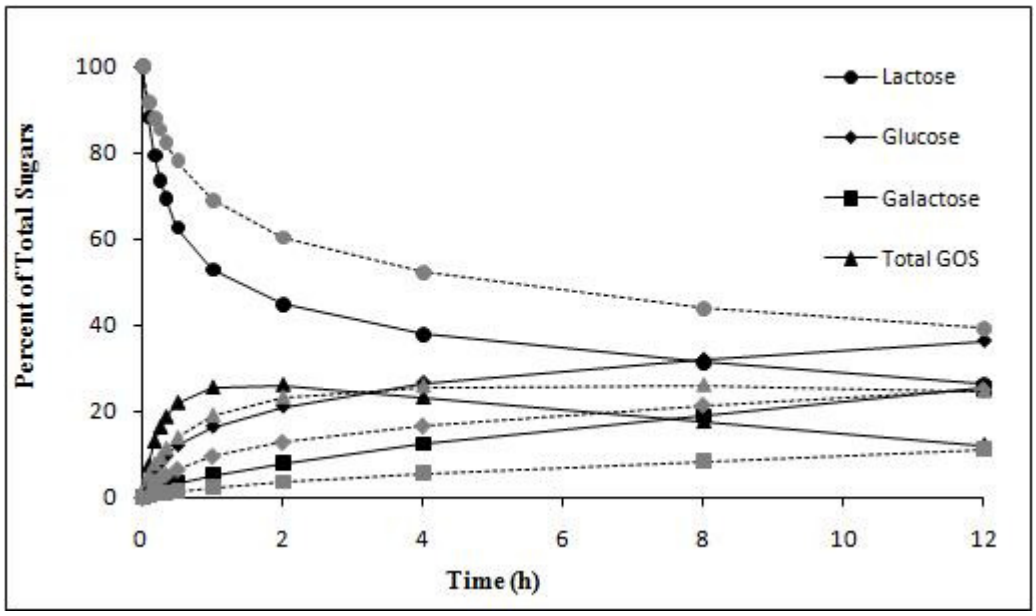

Figure 3.1. Lactose (50%, w/v) hydrolysis by free (grey symbol) and immobilized (black symbol) *A. oryzae* β-galactosidase on mPOS-PVA at pH 4.5 and 40° C. Total GOS, galactose and glucose production were estimated by HPLC.

Similar experiments were performed for different initial lactose concentrations (5 to 40%, w/v) and the tri- and the tetra-saccharides produced (expressed as percent of total sugars) were plotted against the percent of hydrolyzed lactose (Fig. 3.2).

Figure 3.2 shows that GOS (tri- and tetra-saccharides) production increased with increasing lactose concentration. However, for each lactose concentration the GOS production decreased after a certain degree of lactose conversion. This can be attributed to a preferential hydrolysis (formation of glucose and galactose) rather than GOS synthesis. Comparing Figures 3.2(A and D) and 3.2(B and E) one can notice that the trisaccharide production is higher (about 21% of total sugars for 50%, w/v, of lactose) than the tetrasacharide (about 7% of total sugars for 40%, w/v, lactose). Furthermore, there was no marked difference between the free and immbilized enzyme performances.

As the initial lactose concentration increased from 5% to 50%, the maximum GOS content in the product increased from 11.2% (at 35% conversion) to 26.1% (at 56% conversion) for the free enzyme and from 10.4% (at 30% conversion) to 26.0% (at 55% conversion) for the immobilized enzyme.

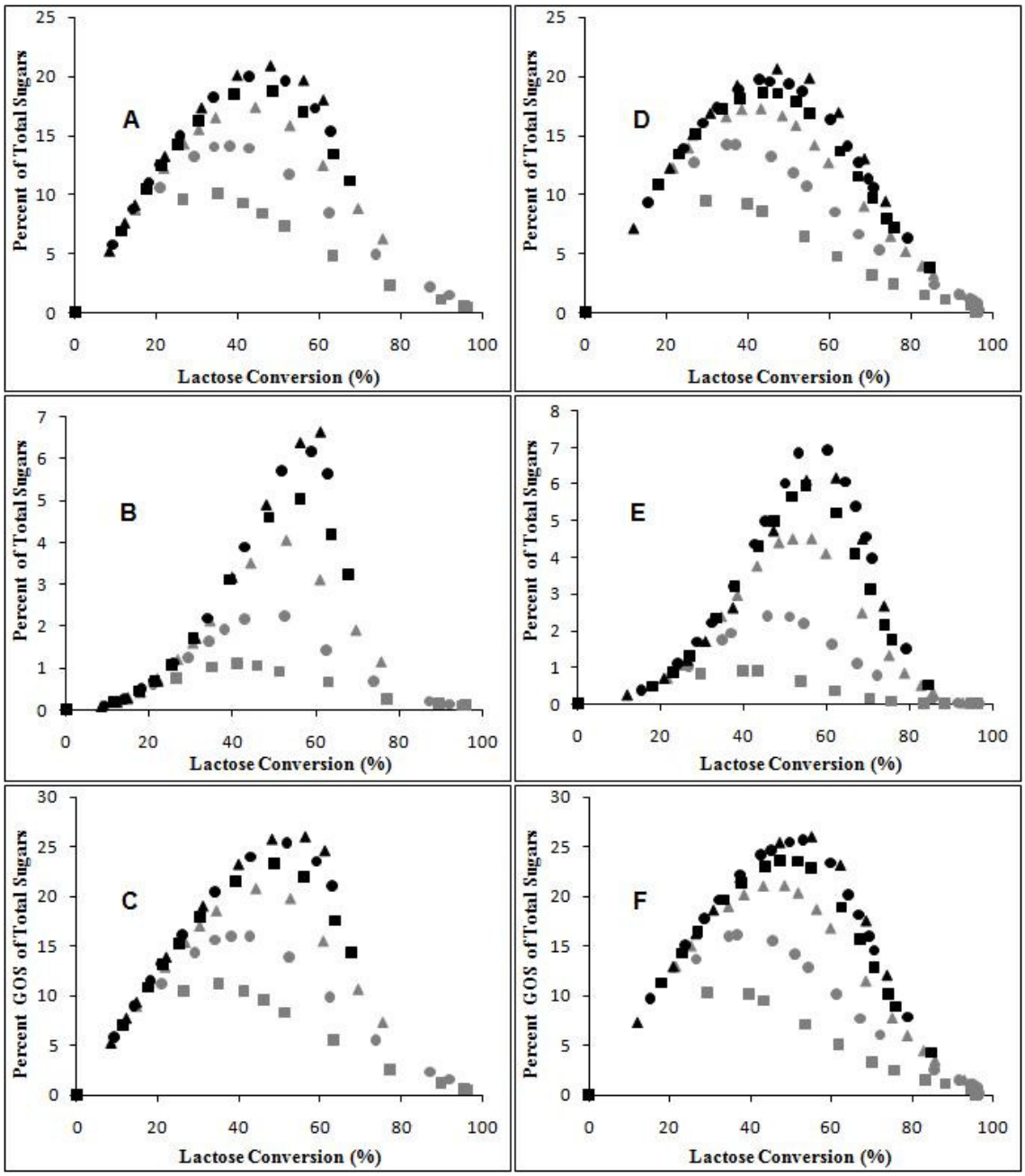

Figure 3.2. Trissacharide (A and D), tetrassacharide (B and E) and total GOS (C and F) formation by free (A, B and C) and immobilized (D, E and F) *A. oryzae* β-galactosidase on mPOS-PVA at pH 4.5 and 40º C catalysing the hydrolysis of diferent initial lactose concentrations 5% (■), 10% (●), 20% (▲), 30% (■), 40% (●), 50% (▲), w/v.

The maximum amount of tri-, tetra-saccharides and total GOS obtained for the immobilized enzyme were 103.4 g L^{-1}, 30.8 g L^{-1} and 129.9 g L^{-1} for a lactose

conversion of about 47%, 62%, and 55%, respectively, in 500 g L^{-1} of lactose. For the free enzyme, the corresponding values of 104.5 g L^{-1}, 33.2 g L^{-1} and 130.3 g L^{-1} were obtained for about 48%, 61% and 56% lactose conversion, respectively in 500 g L^{-1} of lactose. As expected, considering the small size of the particles, these results confirm that enzyme immobilization on mPOS-PVA does not impose any mass transfer limitations on GOS formation from lactose. Similar results were obtained with β-galactosidase from *A. oryzae* immobilized on cotton cloth (Albayrak and Yang, 2002). Higher operational and thermal stability, making possible its reuse, has been demonstrated to be one of the advantages of this system for enzyme immobilization (Neri *et al*, 2008). An immobilized β-galactosidase on mPOS-PVA preparation acting on 20% (w/v) lactose was successively reutilized for 10 cycles at 25 ºC and at the end the enzymatic derivative retained approximately 84% of its initial activity (data not shown), confirming the advantages of the mPOS-PVA β-galactosidase derivative. Similar results were previously reported (Neri *et al.*, 2008) using this support for the immobilization of *Kluyveromyces lactis* β-galactosidase acting on a synthetic substrate (ONPG; ortho-nitrophenyl-β-D-galactopyranoside), as after 20 reutilization cycles the immobilized enzyme retained about 50 % of the initial activity. Apart from this, the easy separation of the mPOS-PVA preparation from the reaction medium by the action of a magnetic field must be pointed out.

GOS yields during lactose hydrolysis catalyzed by the free and the immobilized β-galactosidase on mPOS-PVA are shown in Figure 3.3. The maximum GOS yield increased with the initial lactose concentration for both enzyme preparations The maximal values ranged from 39.5% (5%, w/v, lactose) to 64.1% (higher lactose concentrations) for the free enzyme and from 35.2% (5%, w/v, lactose) to 62.6% (higher lactose concentrations) for the immobilized one. It was observed that the hydrolysis and transgalactosylation reactions occurred simultaneously and that the product profile of the reaction is largely 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 (Iwasaki *et al*., 1996). The insert of Figure 3.3 presents these same data under a different way, cleary demonstrating that an increase in lactose concentration leads to an increase in GOS production. However, in terms of GOS yield, an increase was observed

up to 300 g L^{-1} of initial lactose concentarion, while for higher lactose concentrations the GOS yield was almost constant at 23.5–26% for both free and immobilized enzyme. Park *et al* (2007) observed the same behavior for high lactose concentration with a thermostable β-galactosidase from *Sulfolobus solfataricus*.

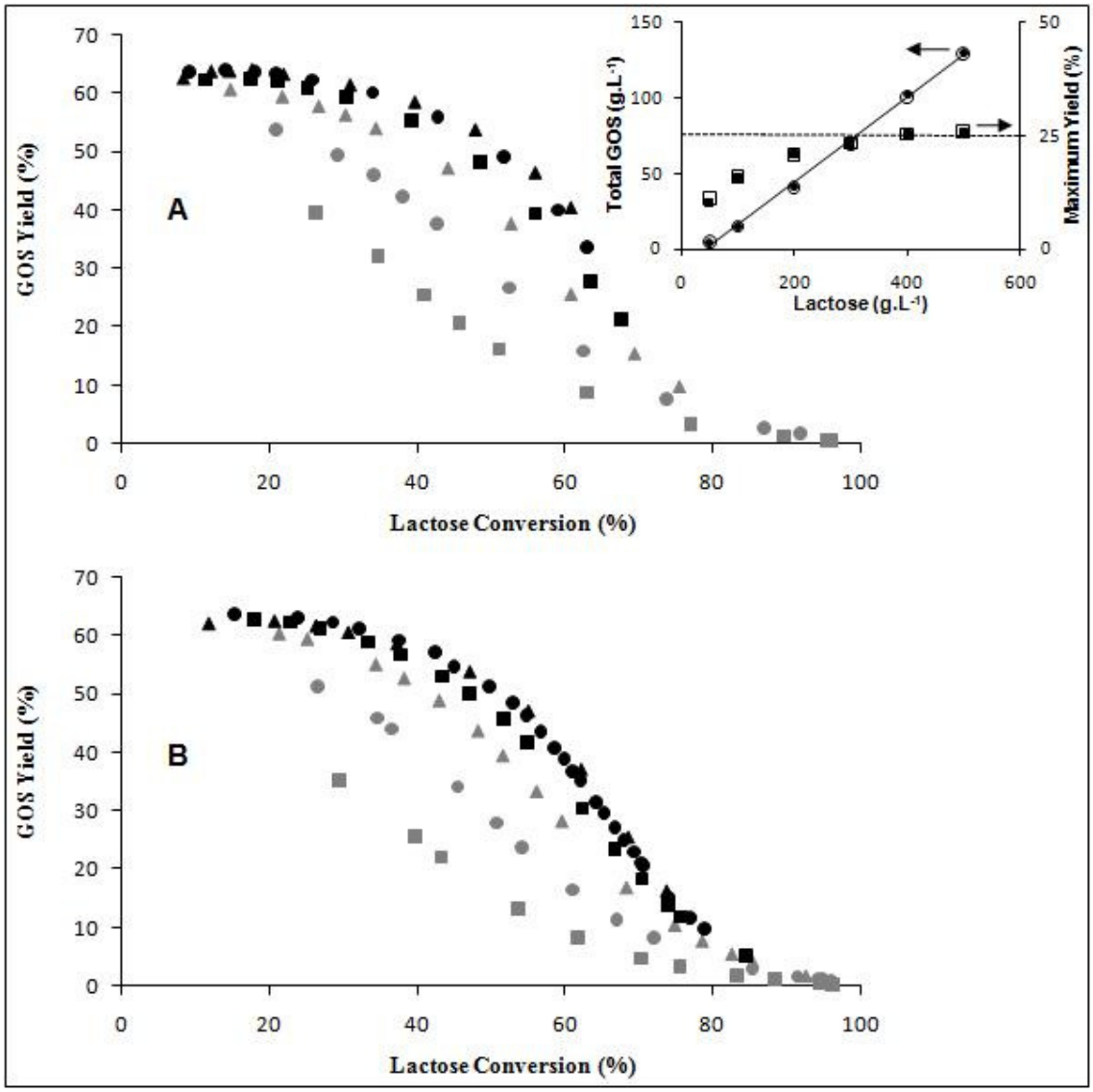

Figure 3.3. GOS Yield *versus* percent of lactose conversion by free (A) and immobilized (B) *Aspergillus oryzae* β-galactosidase. The insert presents the same relationship expressed in terms of total GOS (g/L) and maximum GOS yield by free (open symbols) and immobilized (closed symbols). The initial concentrations of lactose were 5% (■), 10% (●), 20% (▲), 30% (■), 40% (●), 50% (▲), w/v.

It is possible that some disaccharides such as allolactose and galactobiose are formed by the enzymatic transfer of galactose to glucose and galactose, respectively. The absence of this side reactions, was confirmed by checking the molar balances on glucose and galactose over the course of the reaction, assuming that all GOS only

contained one unit of glucose with galactose as the remaining sugar. It is also possible that different trisaccharides were also formed, but were not detected by HPLC.

3.3.2. Effects of galactose and glucose

Galactose is well known as a competitive inhibitor to the lactose hydrolysis reaction (Bakken *et al*., 1991; Portaccio *et al*., 1998; Shukla and Chaplin, 1993). From a thermodynamic point of view, a high galactose (product) concentration could favor the reversed hydrolysis reaction and cause an equilibrium shift towards condensation, thereby increasing GOS yield. However, the effects of monosaccharides on GOS formation rate and product yield have not been well characterized. Thus, the possible effect of monosaccharides on GOS formation was investigated by adding galactose and/or glucose into the initial lactose solution, both for free and immobilized enzyme (Fig. 3.4).

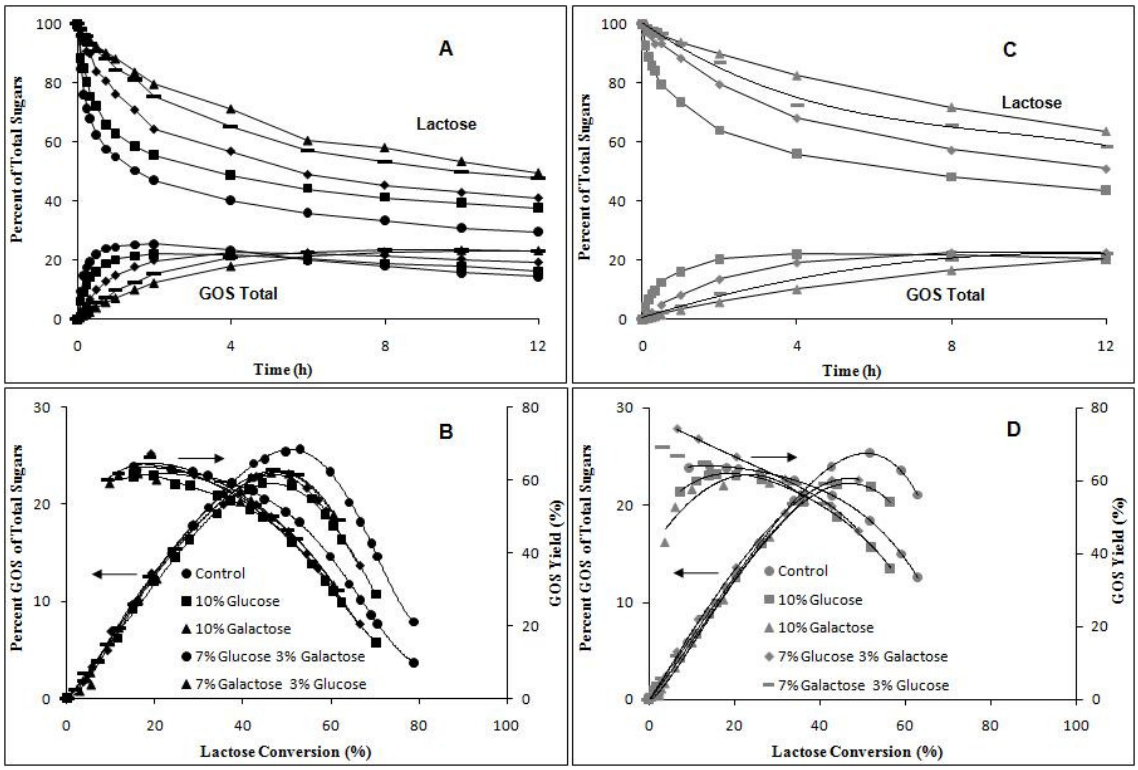

Figure 3.4. Lactose (40%, w/v) hydrolysis in the absence and the presence of galactose and/or glucose by free and immobilized *A. oryzae* β-galactosidase on mPOS-PVA at pH 4.5 and 40° C. The time course of the consumed lactose and total produced GOS is shown in A (immobilize enzyme) and C (free enzyme) as well as the produced GOS expressed as percent of total sugars and yield *versus* lactose conversion for the immobilized (B) and free (D) enzyme .

As expected, the reaction rate markedly decreased in the presence of galactose for both immobilized enzyme (Fig. 3.4A) and free enzyme (Fig. 3.4B). It is also shown that the addition of glucose either alone or mixed with galactose, simultaneously decreased lactose hydrolysis and GOS formation, indicating a different mechanism of action of glucose on the reaction kinetics Glucose did not act as an inhibitor to *A. oryzae* β-galactosidase and acted as a non-competitive inhibitor for some bacterial (Shin and Yang, 1998) and yeast enzymes (Cavaille and Combes, 1995). Overall, it is clear that the monosaccharides negatively affected the GOS formation from lactose by β-galactosidase. Hansson *et al*. (2001) reported that GOS formation by β-galactosidase action was more of a kinetically controlled transgalactosylation reaction rather than reversed hydrolysis.

Reversed hydrolysis or condensation may occur at high concentrations of monosaccharides upon prolonged incubations. Thus, it can be concluded that free glucose and galactose are not recommended for GOS formation and should be removed from the reaction mixture in order to increase GOS yield and formation rate.

3.3.3. Effects of temperature and pH

Temperature and pH normally have a pronounced effect on the enzyme reaction rates but showed to have a minimal effect, if any, on GOS production (Iwasaki *et al*., 1996; Monsan and Paul, 1995; Albayrak and Yang, 2002). As shown in Figure 3.5, GOS production was almost unchanged despite temperatures varying from 30^{o} C to 60^{o} C (Figure 3.5A and 3.5B) and pH from 3.5 to 5.5 (Figure 3.5C and 3.5D) for the conversion of 40% (w/v) lactose, for both the free and immobilized enzyme preparations. Albayrak and Yang (2002) reported similar results for β-galactosidase from *Aspergillus oryzae* immobilized on cotton cloth.

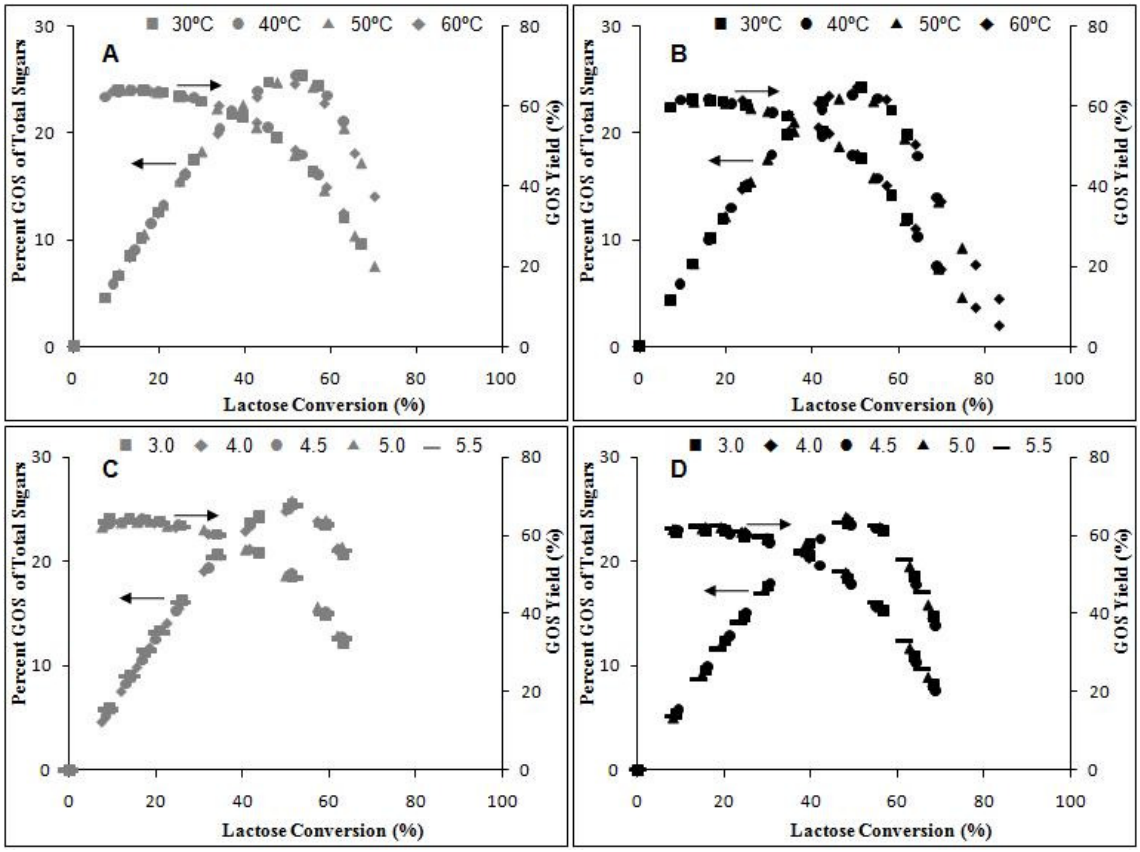

Figure 3.5. Effects of temperature (A and B) and pH (C and D) on the GOS production from the hydrolysis of 40% (w/v) lactose catalyzed by the free (grey symbol) and immobilized (black symbol) *A .oryzae* β-galactosidase on m-POS-PVA.

3.3.4. Kinetic analysis of free and immobilized enzyme

To verify the precondition that the model parameters are independent of the initial lactose concentrations, the 95% confidence interval of the slope of the linear dependence of the kinetic parameters on initial lactose concentration was estimated (data not shown) as, to determine one parameter set for various initial lactose concentrations by taking average parameters values, the estimated parameters have to be independent of the initial lactose concentration. Dependency of the parameters on the initial lactose concentration was observed for the free enzyme and $\log(k_3/k_4)$ for the immobilized enzyme. However, the averaged parameter values for the free and immobilized enzyme were used. Results presented in Figure 3.4 show that single, galactose addition was found to have the more pronounced inhibition effect on β-galactosidase activity. These results have also been found in a previous study (Friend and Shahani, 1982). In addition, enzyme inhibition by glucose plus galactose has also

been observed, but only the galactose inhibition effect was included in the considered models in this study (k_5, $k_6 = 0$ and k_7, $k_8 \neq 0$).

Figure 3.6 show a comparison of the experimental and calculated concentrations profiles with the averaged parameters (examples for 20 and 30% initial lactose concentrations) obtained for free and immobilized enzyme respectively. The kinetic model was validated by comparing the set of experimental data from the β-galactosidase to data obtained by model simulation with different initial lactose concentrations ranging from 10 to 50% (w/v). To determine the effect of the immobilization process on the kinetic parameters, in other words, on lactose hydrolysis and trisaccharides synthesis, experiments were carried out at various initial lactose concentrations. The good fitting quality of the model to the free enzyme and especially to the immobilized enzyme is remarkable for all initial lactose concentrations studied. The model accurately describes the time course lactose conversion and glucose formation with time, but only provides a reasonable fit for the trisaccharides production and galactose as shown in Figure 3.6. The production of trisaccharides and galactose is slightly overestimated.

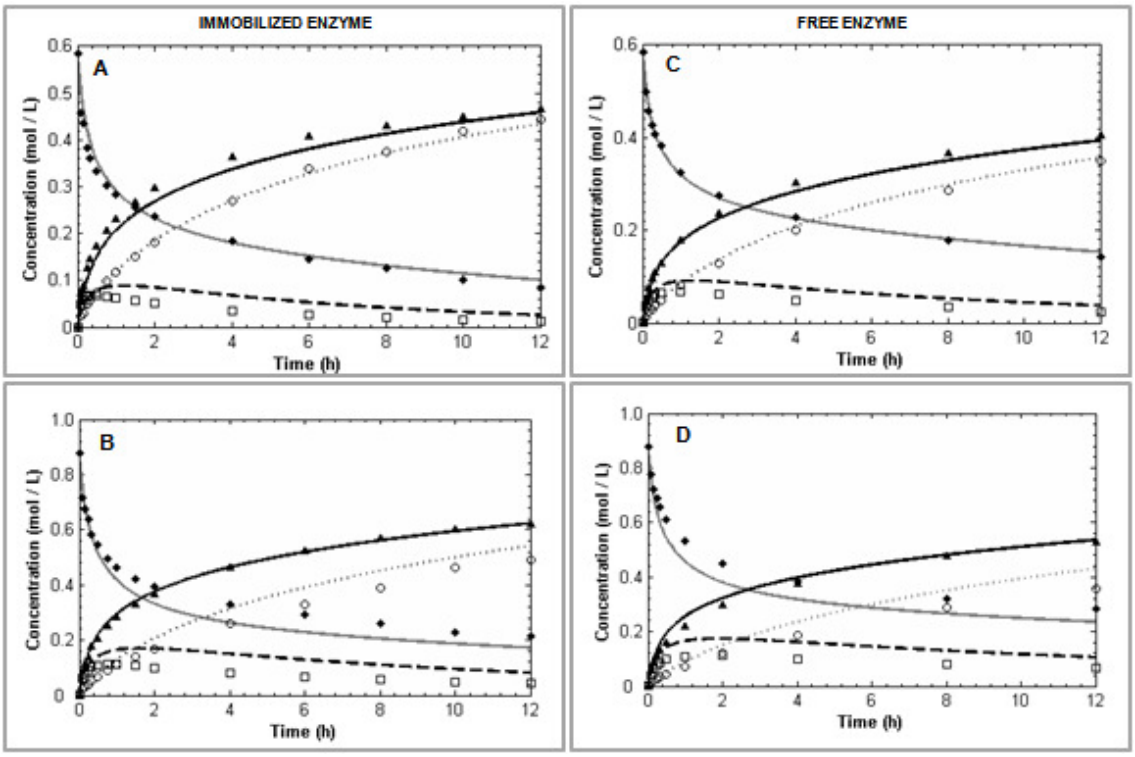

Figure 3.6. Comparison between experimental data (symbols) and simulated data (descending gray solid line: lactose; ascending black solid line: glucose; depicted line; galactose and dashed line: trisaccharides) of (♦) lactose, (▲) glucose, (○) galactose and (□) trisaccharides concentration by free and immobilized β-galactosidase from *A. oryzae* at pH 4.5, 40ºC for 20% (A = free and C = immobilized) and 30% (B = free and D = immobilized) of initial lactose concentrations. Lines represent the simulation using the averaged kinetic parameters of all fittings.

The parameter k_3, that regulates the oligosaccharides synthesis and k_4, which regulates the hydrolysis of the formed oligosaccharides are assumed to be key parameters in the overall process.

Table 3.1. Estimated average values and confidence intervals of parameters for the free and immobilized β-galactosidase, obtained by fitting the experimental data for *A. oryzae* at different initial lactose concentration (10 to 50%).

Parameter (units)	Immobilized	Free
k_1 (h^{-1})	1.41 ± 0.75	1.16 ± 0.92
k_3/k_4, Log(k_3/k_4) (-)	37.15, 1.57 ± 0.26	39.08, 1.59 ± 0.049
k_4 (h^{-1})	4.78 ± 0.35	10.50 ± 5.84
k_7/k_8, Log(k_7/k_8) (-)	32.36, 1.51 ± 0.12	51.29, 1.71 ± 0.19

Not surprisingly, as can be observed in Table 3.1, the ratio between k_3 and k_4 is of the same order of magnitude for both free and immobilized enzyme, indicating that the relative extent of the synthesis and hydrolysis reactions is not affected by the immobilization procedure. This is in accordance with the obtained experimental data.

3.4. CONCLUSIONS

Magnetic polysiloxane-polyvinyl alcohol proved to be an adequate support for *Aspergillus oryzae β*-galactosidase immobilization and its use on GOS production using lactose as substrate.

The initial lactose concentration in the reaction media affected the total amounts of produced galacto-oligosaccharides and their time course production was described as a "bell-shaped" curve as a result of the balance between transgalactosylation and hydrolysis. The galacto-oligosaccharides formation ability of the enzyme was not affected by its immobilization onto magnetic polysiloxane-polyvinyl alcohol, a support that can be easily recovered by applying a magnetic field and that made possible to retain 84% of the enzyme initial activity after 10 utilization cycles. The addition of galactose either alone or mixed to glucose decreased the lactose hydrolysis and galacto-oligosaccharides formation. The galacto-oligosaccharides production was almost

unchanged despite temperatures varying from 30° C to 60° C and pH from 3.5 to 5.5 both for the free and immobilized enzyme preparations. A model that adequately describes oligosaccharides production by the enzyme (free and immobilized) including galactose inhibition was successful used. Analysis of the model parameters shows that the more relevant parameters are those involved in the galactose-enzyme complex formation and those involved in the trisaccharides synthesis and hydrolysis. The model describes the experimental data well in the range of the initial lactose concentration used.

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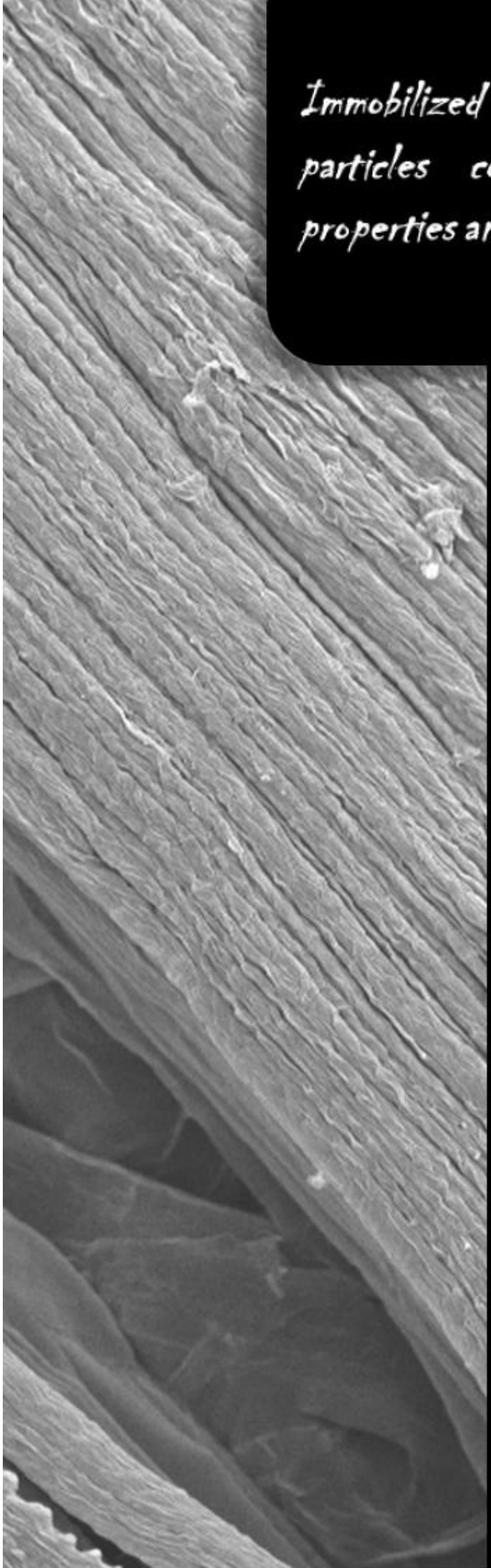

Immobilized b-galactosidase onto magnetic particles coated with polyaniline: catalytic properties and characterization

CHAPTER 4

Magnetite particles (<100µm) obtained by coprecipitation of Fe^{2+} and Fe^{3+} and coated with polyaniline (MAG-PANI) were used to immobilize *Aspergillus oryzae* b-galactosidase via glutaraldehyde. This magnetic enzymatic derivative was capable to act on lactose and to produce tri and tetragalactosides (transgalactosylation) and the catalytic properties were similar to the soluble enzyme. The tri and tetra-galactosides production by both soluble and immobilized enzyme was not affected by temperature in the range 30°C to 60°C. The MAG-PANI was characterized by X-ray diffraction, Fourier transform infrared spectroscopy, elemental analyzer, scanning electronic microscope, differential scanning calorimeter, thermogravimetric analyzer, vibrating sample magnetometer and thermomagnetization. These analysis showed rhombohedra particles presenting good magnetic response, evidences for the PANI coating and protein immobilization and magnetite as the predominant component. This magnetic β-galactosidase derivative presents the following advantages: simple synthesis using low cost reagents, catalytic properties similar to the soluble enzyme and easy removal from the reaction mixture by a magnetic field and reuse.

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4.1. INTRODUCTION

β-Galactosidase (EC:3.2.1.23) has been a popular enzyme for its hydrolytic action on lactose. The transferase activity has also attracted the attention of researchers because the produced oligosaccharides have a beneficial effect on the growth of desirable intestinal microflora. Moreover, the transferase reaction can be used to attach galactose to other chemicals and consequently have potential application in the production of food ingredients, pharmaceuticals and other biological active compounds (Panesar *et al*., 2006)**.** Magnetic fields have been utilized in support systems for the study of different immobilized enzymes (Kuroiwa *et al*., 2008, Pimentel *et al*., 2007, Bayramoğlu and Arica, 2008). The advantages of small magnetic particles for enzyme immobilization in bioprocesses are: improvement of the mass-transfer properties of immobilized enzymes suspended in a viscous solution, in which the promotion of mass transfer by agitation is difficult (Kuroiwa *et al* ., 2008); reuse and easy separated from reaction medium by applying a magnetic field (Bruno *et al*., 2005) and reduction of the capital and operation costs (Pieters and Bardeletti, 1992).

A magnetic immobilized β-galactosidase has been prepared using magnetite a functionalized by treatment with polyethyleneimine and crosslinked with glutaraldehyde as the magnetic material (Dekker, 1989). The aminated magnetic poly(glycidyl methacrylate-co-methylmethacrylate) beads were used for the covalent immobilization of β-galactosidase from *Escherichia coli* via glutaric dialdehyde activation (Bayramoglu *et al*., 2007). β-Galactosidase from *Kluyveromyces lactis* was covalently immobilized onto a polysiloxane–polyvinyl alcohol magnetic (mPOS–PVA) composite, using glutaraldehyde as activating agent (Neri *et al*., 2008).

A tremendous amount of research has been carried out in the field of conducting polymers since 1977 as has been recognized by the 2000 Nobel Prize in Chemistry awarded to Alan J. Heeger, Alan G. MacDiarmid and Hideki Shirakawa. Polyaniline (PANI) is also unique among conducting polymers as it has a very simple acid/base doping/dedoping chemistry (Huang, 2006). The organic metal PANI is one of the most widely used conducting polymers and can be used neat or as blends and in compounds with commodity polymers, such as polyethylene, polypropylene, polystyrene, soft PVC, poly-(methylmetacrylate), phenol-formaldehyde resins, melamineformaldehyde resins,

epoxies and thermoplastic elastomers. PANI has been used as a coating of different materials for immobilization of biomolecules (Oliveira *et al*., 2008; Seo *et al*., 2007; Coêlho *et al*., 2001).

In the present work magnetite (MAG) was coated with PANI, activated with glutaraldehyde and *Aspergillus oryzae* β-galactosidase was covalently immobilized for galactooligosaccharides production. The catalytic properties of the enzymatic derivative were investigated. Furthermore, MAG-PANI was characterized by Fourier transform infrared spectroscopy (FTIR), elemental analysis, X-ray diffraction (XRD), thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), vibrating sample magnetometer (VSD), scanning electron microscopy (SEM) and thermomagnetization.

4.2. EXPERIMENTAL

4.2.1. Magnetite synthesis and polyaniline coating

MAG support was prepared by the coprecipitation method, by adding NH_4OH into a mixed solution containing 0.6 M $FeCl_2$ and 1.1 M $FeCl_3$ (1:1) until obtaining pH 11.0, for 30 min at 100 ºC. MAG particles were thoroughly washed with deionized water until pH 7.0, dried at 105 °C overnight and finally sieved (<100µm). The MAG particles were treated with 0.1 M $KMnO_4$ solution at 50 ºC overnight, washed with distilled water and immersed into 0.5 M aniline solution prepared in 1.0 M HNO_3. Polymerization was allowed to occur for 2 h and after that the MAG-PANI were successively washed with distilled water, 0.1 M citric acid and distilled water.

4.2.2. β-Galactosidase immobilization

MAG-PANI particles (100 mg) were treated with 2.5 % w/v glutaraldehyde (1 mL) and 20 mM citrate-phosphate buffer, pH 4.5 (9 mL) for 2 h under stirring at 25ºC. Activated MAG-PANI were washed with distilled water 10 times and incubated overnight with 10 mL of β-galactosidase (4 mg solid/mL) from *Aspergillus oryzae* (SIGMA, Japan).

4.2.3. GOS production

GOSs formation kinetics with immobilized enzyme on MAG-PANI was studied and compared with free enzyme. The lactose solution was prepared by dissolving lactose in citrate-phosphate buffer solution pH 4.5. Samples were taken at appropriate time intervals and analyzed for sugar content by high performance liquid chromatography (HPLC). The reaction kinetics was studied at six different initial lactose concentrations (50, 100, 200, 300, 400 and 500 g/L) and four different temperatures (30, 40, 50, and 60ºC).

4.3.4. HPLC analysis

Determination of the concentration of all sugars present in the assay solution (GOSs, lactose, glucose, and galactose) was done by HPLC. An HPLC (Jasco AS-2057 Plus), employing a MetaCarb 87H at 60 °C, a refractive index detector (Jasco RI-2031 Plus) and a mobile phase 0,001N H_2SO_4 at a flow rate of 0.5 mL min^{-1} (Jasco PU-2080 Plus) was used. The concentration (w/v) of these sugars (*e.g.*, lactose, glucose, galactose, and oligosaccharides including tri-, and tetra-saccharides) is proportional to their peak areas. Thus, normalized sugar concentrations as weight percentages of total sugars or initial lactose were determined from peak areas and are reported in this work. It should be noted that the accuracy of this approximation was verified by checking the sugars mass balance.

4.2.5. Support characterization

The X-ray diffraction patterns of the samples were collected on a Philips-PW1710 diffractometer with Cu Kα radiation (λ = 1.54056 Å). Elemental analysis of dried samples was determined by a LECO Elemental Analyser CHNS 932 and Unicam 929 AA spectrophotometer (USA) at 1000ºC and sulphamethazine with standard. For SEM, the samples were mounted on stubs, coated with gold, examined in a Leica Cambridge S360 scanning electronic microscope and SEM micrographs were taken. Infrared spectra were recorded on an ABB FTLA2000 spectrometer in the range of 500-4000

cm^{-1} using KBr pellets. Thermal analyses TGA and DSC of MAG and MAG-PANI were done using a TGA-50 and a DSC-50 both from Shimadzu (Izasa, S.A., Portugal). Acquisition of the results was done by TA-50WS software (version 1.14). Samples were weighed (10 mg; the exact mass was recorded) into aluminium pans (Izasa, S.A., Portugal) and heated over the temperature range 25–500 ºC, at a scanning rate of 5 ºC/min, under 20 mL/min nitrogen flow. The magnetic properties of the sample MAG-PANI were investigated by measuring its hysteresis loop at room temperature using an Oxford instrument Vibrating Sample Magnetometer and the thermomagnetization from room temperature to 1000^{o}K using a Cahn 2000 Curie-Faraday balance.

4.3. RESULTS AND DISCUSSION

4.3.1. Effects of lactose concentration and temperatura on GOS production by free and immobilized enzime

A typical tri- and tetra-saccharides time course production is presented in Figure 4.1A during 50%, w/v, lactose conversion by the action of free and immobilized *A. oryzae* β-galactosidase. Two main observations can be made: 1) the mass of consumed lactose is stoichiometrically equal to the mass of GOS plus the mass of monosaccharides (glucose and galactose) formed and 2) the difference between the free and the immobilized enzyme results are caused by the different amounts of used enzyme in the experiments (an enzyme concentration of 0.149 mg/mL was used in the free form, while for the immobilized form, enzyme concentration was 0.204 mg/mL). Being so, a different approach was used to compare the results for the free and immobilized enzymes for the different experimental conditions considered. Figure 4.1B shows that the GOS (tri- and tetra-saccharides) production kinetics at 50 % of initial lactose concentration is closely related to lactose conversion and independent of the amount of used enzyme.

Identical experiments were performed for the other lactose concentrations (5 to 40%, w/v) and the total mass of produced GOS (expressed as percent of total sugars) plotted against the percent of hydrolyzed lactose (Fig. 4.1C).

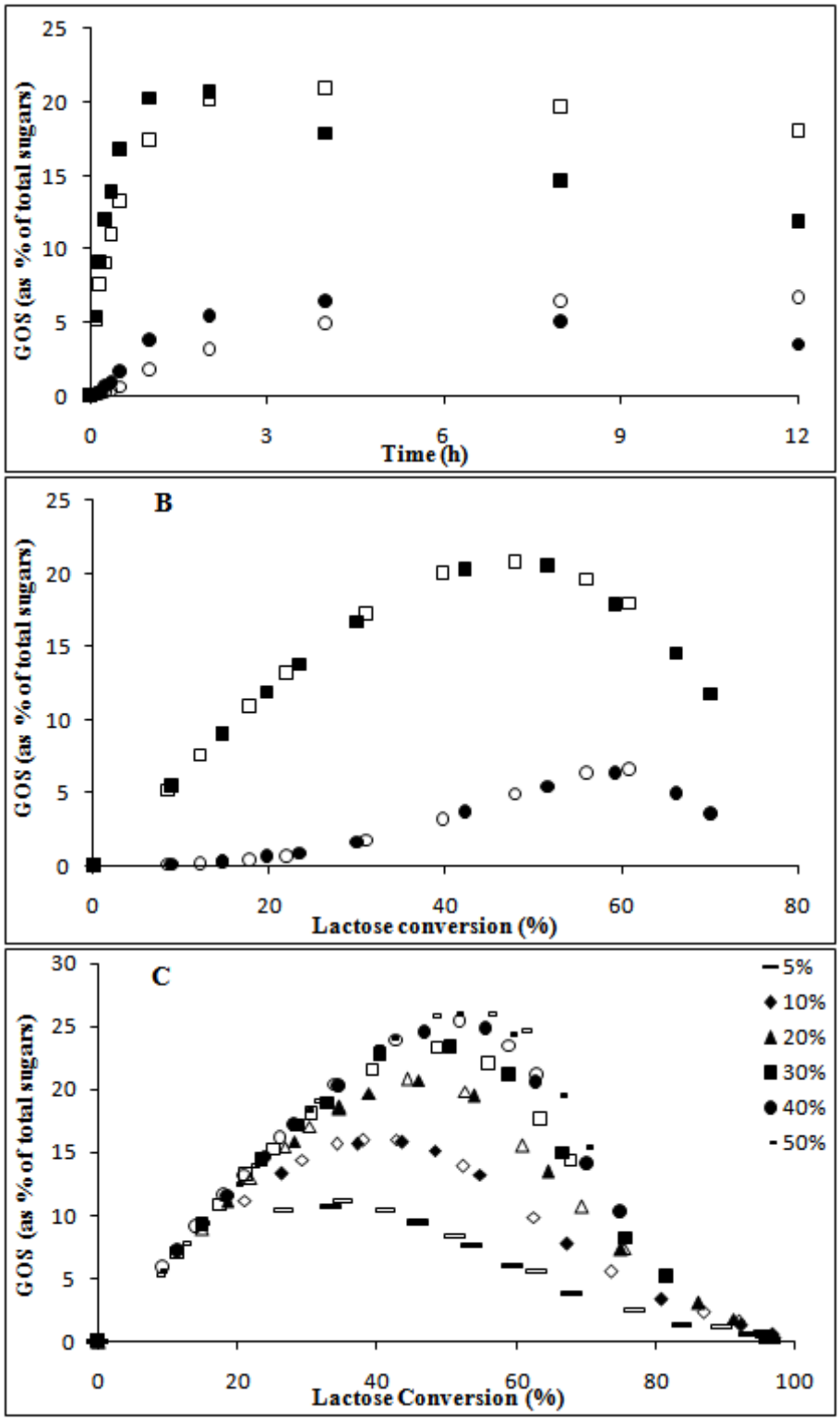

Figure 4.1. GOS (tri- and tetra-saccharides) formation at 50% (w/v) initial lactose concentration at pH 4.5 and 40° C represented as a function of time (A) and related to lactose conversion (B). GOS total production for different initial lactose concentrations (C) Results for free and immobilized enzyme are represent by open and closed symbols, respectively.

This result shows that GOS production increased with increasing lactose concentration. However, for each lactose concentration the GOS production decreased after a certain degree of lactose conversion. This can be attributed to a preferential hydrolysis reaction (formation of glucose and galactose) instead of GOS synthesis. In Figure 4.1B, it can be noticed that the trisaccharide production is higher (about 21% of total sugars for 50%, w/v, of lactose) than the tetrasacharide (about 6% of total sugars for 50%, w/v, lactose). Furthermore, there was no marked difference between the free and immobilized enzyme performances. As the initial lactose concentration increased from 5% to 50%, the maximum GOS content in the product increased from 11.2% (at 35% conversion) to 26.1% (at 56% conversion) for the free enzyme and from 10.8% (at 33% conversion) to 26.0% (at 52% conversion) for the immobilized enzyme. The maximum amount of tri-, tetra-saccharides and total GOS obtained for the immobilized enzyme were 103.2 g L^{-1}, 31.9 g L^{-1} and 130.2 g L^{-1} for a lactose conversion of about 52%, 59%, and 52%, respectively, in 500 g L^{-1} of lactose. For the free enzyme, 104.5 g L^{-1}, 33.2 g L^{-1} and 130.3 g L^{-1} were obtained for about 48%, 61% and 56% lactose conversion, respectively in 500 g L^{-1} of lactose. These results suggest that enzyme immobilization on MAG-PANI does not impose any limitation or changes on GOS formation from lactose. Similar results were obtained with β-galactosidase from *A. oryzae* immobilized on cotton cloth (Albayrak and Yang, 2002). Thus, the GOS formation ability of the enzyme was not affected by the immobilization of the enzyme onto MAG-PANI.

An immobilized β-galactosidase on MAG-PANI preparation acting on 20% (w/v) lactose was successively reutilized for 10 cycles at 25 ºC and at the end the enzymatic derivative retained approximately 85% of its initial activity (data not shown). This performance of the MAG-PANI β-galactosidase derivative would be an additional advantage besides that from its easy of separation from the reaction medium by a magnetic field.

GOS yields during lactose hydrolysis catalyzed by the free and the immobilized β-galactosidase on MAG-PANI are showed in Figure 4.2. The maximum GOS yield increased with the initial lactose concentration by the catalysis of both enzyme preparations This maximal values ranged from 39.5% (5%, w/v, lactose) to 64.1% (higher lactose concentrations) for the free enzyme and from 32.2% (5%, w/v, lactose)

to 63.5% (higher lactose concentrations) for the immobilized one. It was generally observed that the hydrolysis and transgalactosylation reactions occurred simultaneously.

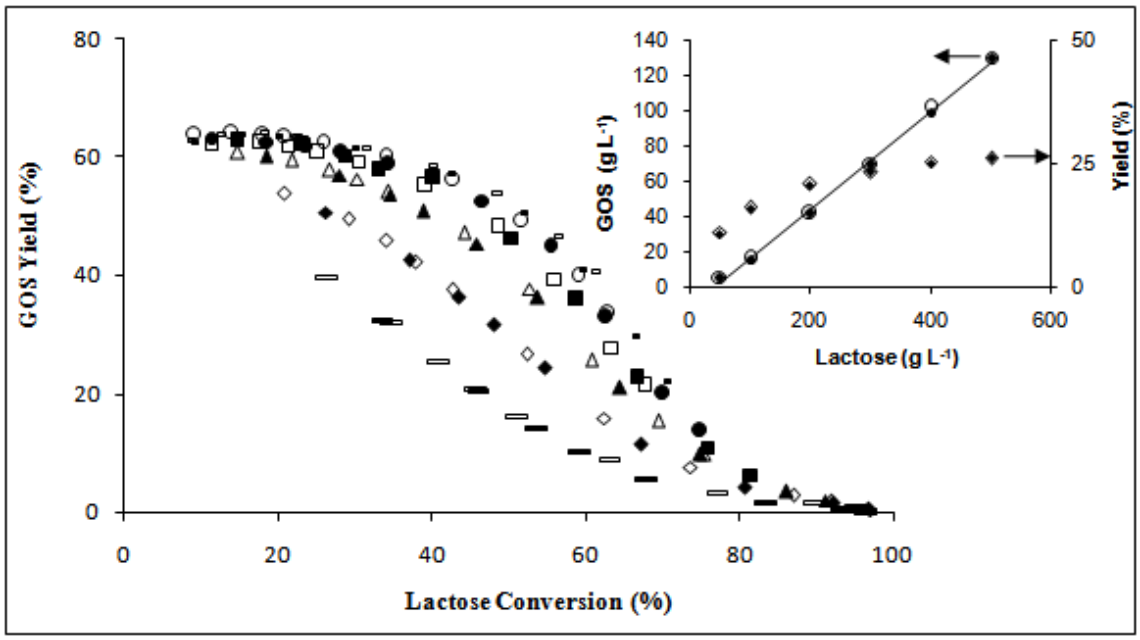

Figure 4.2. GOS yield *versus* percent of lactose conversion by free (open symbols) and immobilized (closed symbols) *Aspergillus oryzae* β-galactosidase. The insert presents the same relationship expressed in terms of total GOS (g/L) and maximum GOS yield. The initial concentrations of lactose were 5% (▬); 10% (◆); 20% (▲); 30% (■); 40% (●) and 50% (▪), w/v.

What dominates the product profile of the reaction is largely 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 (Iwasaki *et al*., 1996). The insert of Figure 4.2 presents these same data under other perspective, namely, increases in lactose concentration led to increases in GOS production. However, in terms of GOS yield, it was observed an increase until 30% of lactose and for higher the yield was almost constant at 23.3–26% for both free and immobilized enzyme. Park *et al* (2008) observed the same behavior for high lactose concentration with a thermostable β-galactosidase from *Sulfolobus solfataricus.*

Temperature normally affects the reaction rates but these factors showed minimal effects, if any, on GOS production (Albayrak and Yang, 2002, Monsan and Paul, 1995). As shown in Figure 4.3 the GOS production was almost unchanged despite temperatures varying from 30^{o} C to 60^{o} C for the conversion of 40% (w/v) lactose and

in all lactose percent of conversion for both the free and immobilized enzyme preparations. Albayrak and Yang (2002) reported similar results for β-galactosidase from *Aspergillus oryzae* immobilized on Cotton Cloth. It is well known that enzymes do not affect equilibrium constant of the reactions.

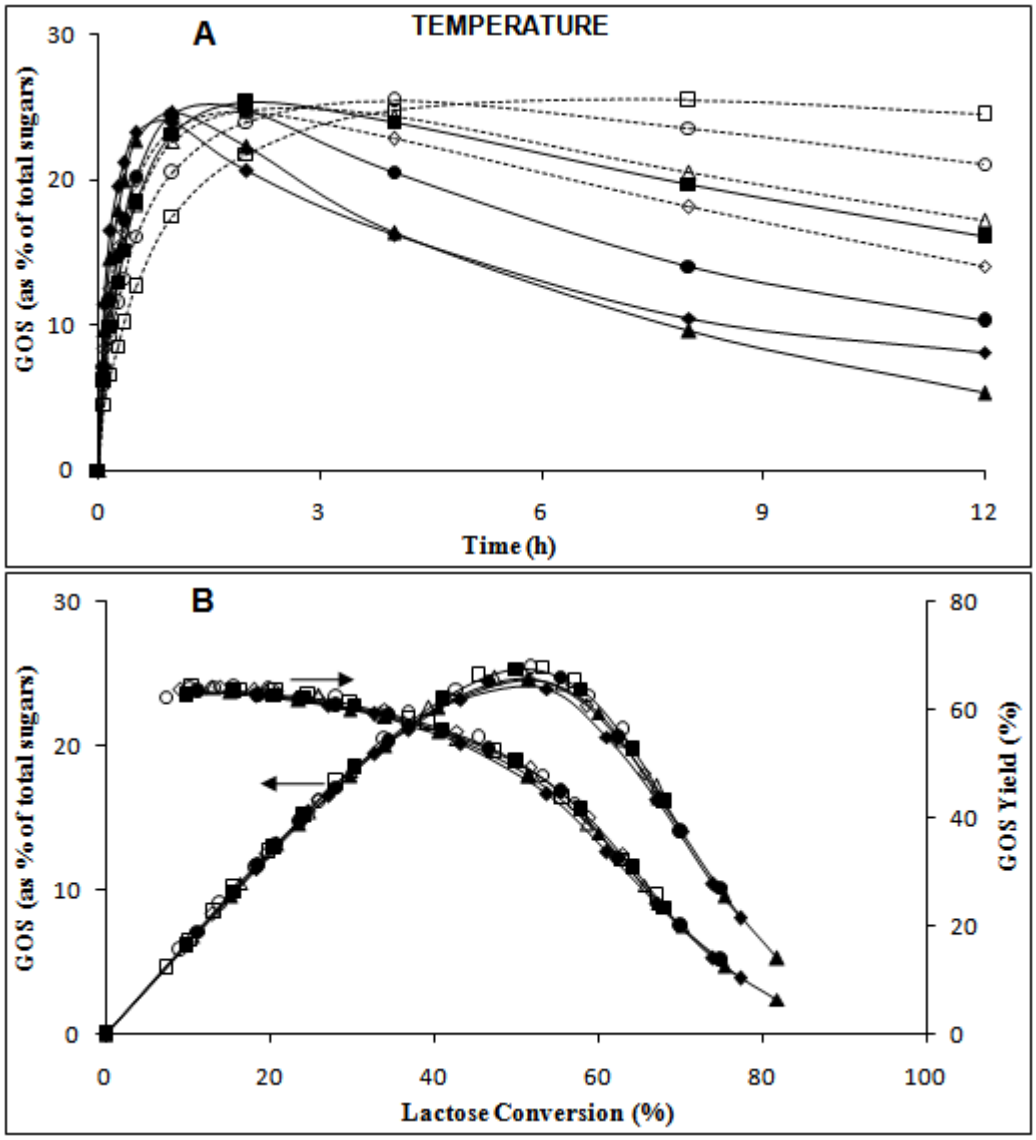

Figure 4.3. Effect of temperature on the GOS production during the hydrolysis of 40% (w/v) lactose catalyzed by the free (open symbols) and immobilized (closed symbols) *A. oryzae* β-galactosidase on m-POS-PVA. A: time course of GOS production; B: GOSS production *versus* lactose consumption. The temperatures were 30 ºC (■); 40 ºC (●); 50 ºC (▲); 60 ºC (◆).

4.3.2. Support characterization

Figure 4.4 shows the results of X-ray diffraction analysis for the MAG-PANI support. Patterns of iron oxides of the Joint Committee on Powder Diffraction Standards - International Centre for Diffraction Data (JCPDS-ICDD) database were included for

comparison. Both magnetite and maghemite have a spinel structure. Their lines are close and it is difficult to distinguish them from one another by X-ray diffraction pattern.

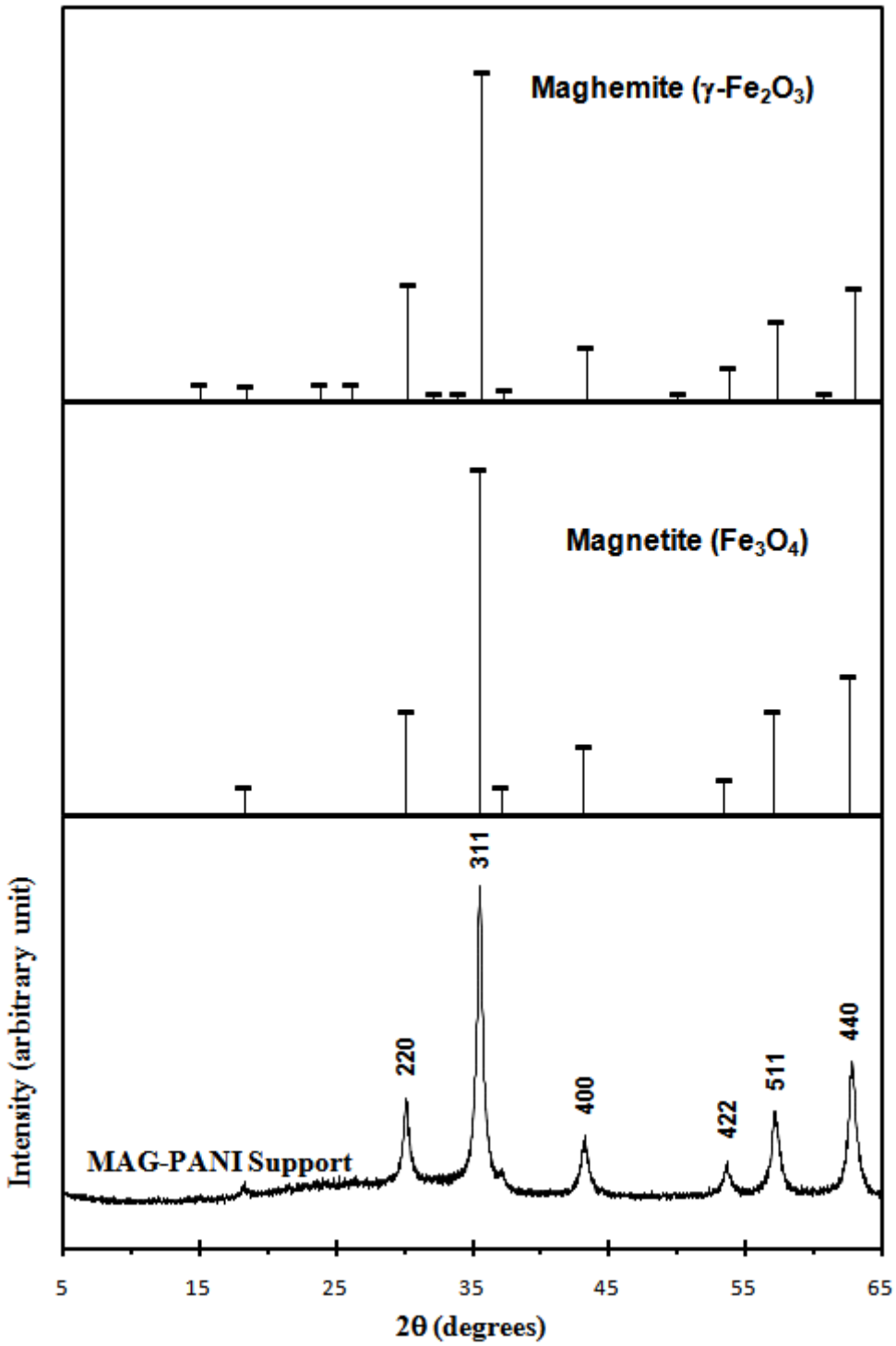

Figure 4.4. X-ray diffraction patterns of MAG-PANI and patterns of oxides from the JCPDS-ICDD database.

The X-ray diffraction data expressed in Å compared with those for magnetite according to the JCPDS database (bracketes) at the crystalline planes (*h/k*) 220; 311; 400; 422; 511 and 440 are, respectively, 2.962 (2.967); 2.516 (2.532); 2.090 (2.099); 1.705 (1.715); 1.609 (1.616) and 1.478 (1.485). From this result one can deduce that magnetite constituted the dominant phase in the MAG-PANI, although trace amounts of maghemite may probably have been formed as contaminants during the synthesis process.

Figure 4.5 shows the scanning electron microscopy (SEM) analysis of MAG and MAG-PANI particles. MAG particles were obtained of the co-precipitated with Fe^{2+} and Fe^{3+} and MAG-PANI particles were obtained with PANI coating on MAG. The SEM images indicate rhombohedra particles, the coating with PANI does not alter the MAG structure and MAG-PANI presents a similar format to MAG.

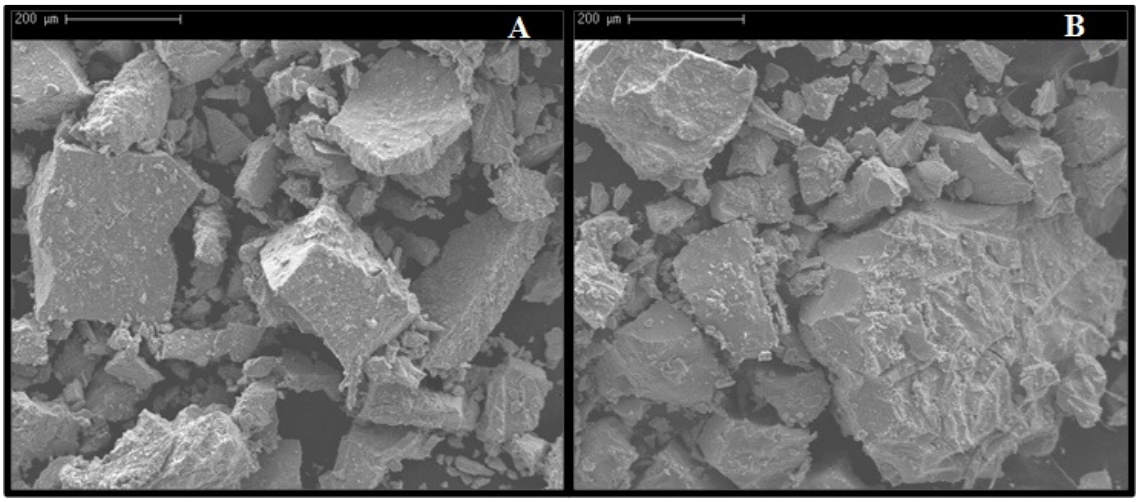

Figure 4.5. Ultrastructural image of MAG (A) and MAG-PANI (B).

Elemental analysis was done to determine the composition of each fraction: 1) MAG; 2) MAG-PANI and 3) MAG-PANI activated with glutaraldehyde plus protein immobilized (Table 4.1). This result shows that MAG alone did not have any analyzed element (N, C, H and S), whereas they are as expected present in the MAG-PANI composite, except sulfur which appears in the composite activated with glutaraldehyde plus immobilized protein.

Table 4.1. Elemental analysis to determine the composition of each fraction of magnetic particles (MAG free, MAG with PANI and MAG with PANI activated with glutaraldehyde plus β-galactosidase immobilized)

Fraction of magnetic particles	Element (%)			
	Nitrogen	Carbon	Hydrogen	Sulphur
MAG	0	0	0	0
MAG + PANI	0.951	5.657	0.378	0
MAG + PANI + b-galactosidase	0.834	5.397	0.415	0.316

Infrared spectra of MAG and MAG-PANI are shown in Figure 4.6 depicting considerable different vibrations. The characteristic peaks of MAG-PANI occur at 1588, 1504, 1384, 1306, 1145 and 828 cm^{-1}. Jiang *et al*. (2008) obtained similar results to PANI/magnetic ferrite nanocomposites. This result seems to be a consistent evidence of PANI coating on MAG.

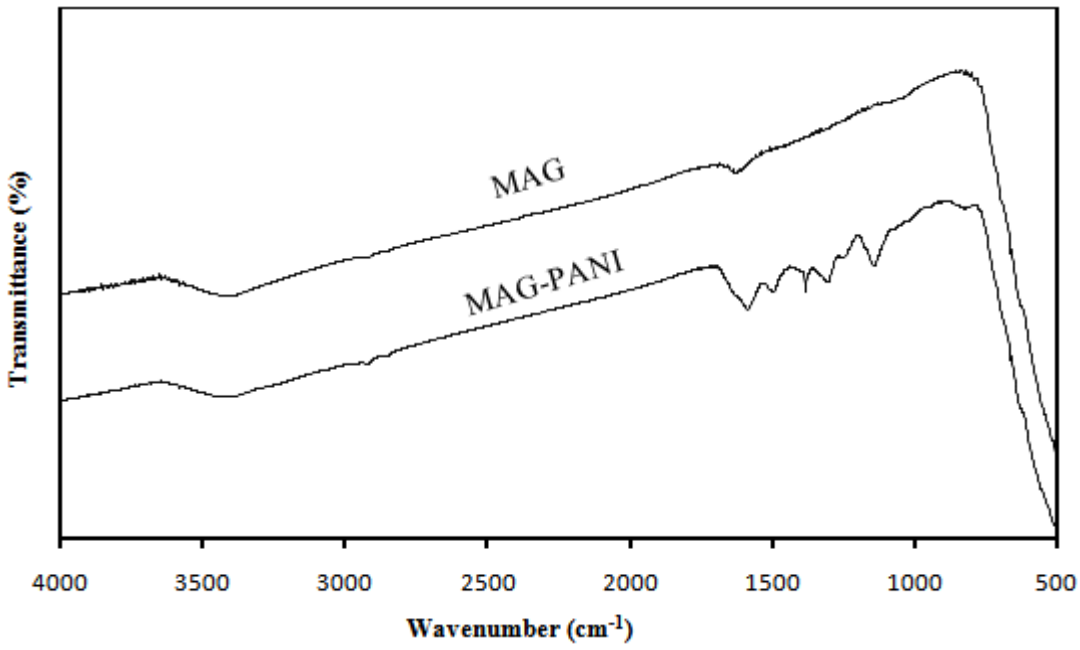

Figure 4.6. FTIR spectra of MAG and MAG-PANI.

The MAG-PANI presented a good magnetic response, being easily attracted by a magnetic separator as is shown in Figure 4.7. In order to study the MAG-PANI magnetic behaviour magnetization measurements were performed by analyzing its hysteresis loop at room temperature.

Figure 4.7. MAG-PANI being attracted by a magnetic separator.

As can be observed in Figure 4.8 the magnetization of the MAG-PANI exhibited a clear hysteretic behaviour. The saturation magnetization was measured to be approximately 60 emu/g (see Figure 8), this value being in fair well agreement with the value found for magnetite (Harrison and Putnis, 1995).

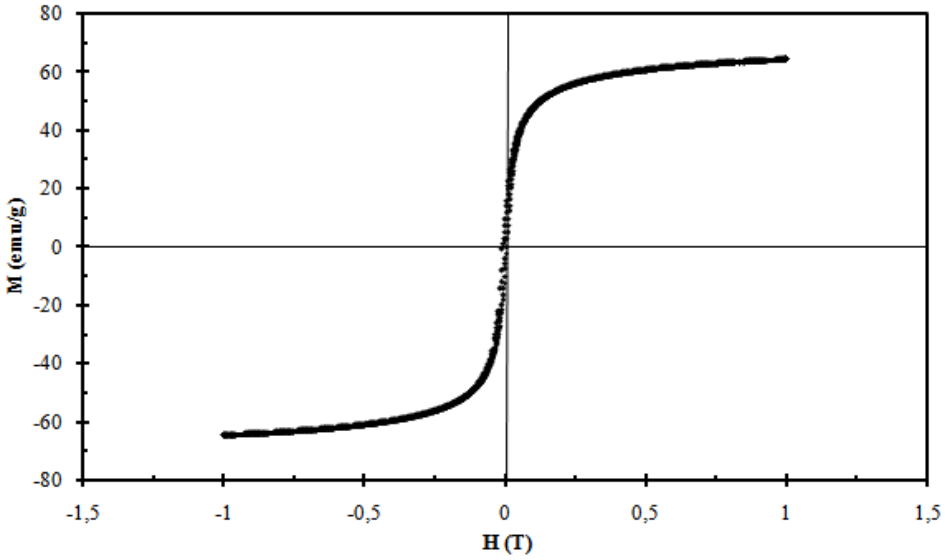

Figure 4.8. Hysteretic loop of the MAG-PANI at room temperature.

Another important parameter analysed was the curve of the magnetic moment against temperature (Figure 4.9) that shows the MAG-PANI sample has two phases possibly magnetite (dominant) and maghemite. The transformation of magnetite to maghemite can occur in the range 650-750^{o}K depending of the specific environment conditions and size of the particles (see Fig. 4.9A) (Lauer *et al*., 2001). In addition, an irreversible transformation to hematite due to oxidation occurs above 830^{o}K near the transition temperature Tc = 850^{o}K as can be seen in Figure 4.9B (Lauer *et al*., 2001; Janot and Guerard, 2002; Costa *et al*., 1998).

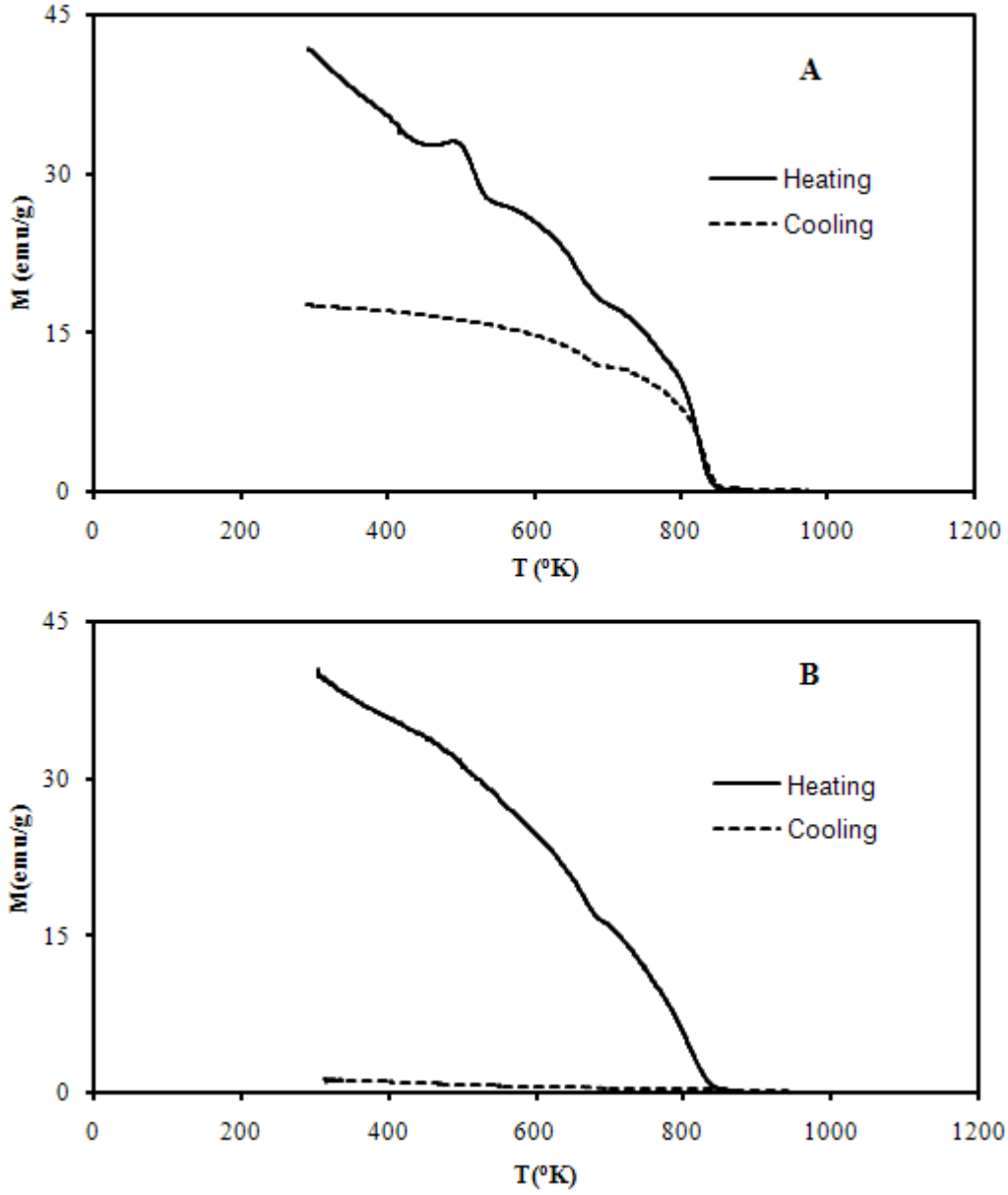

Figure 4.9. Thermomagnetization of MAG-PANI from room temperature to 1000°K.

Figure 4.10 shows the results from the thermal analysis relative to MAG and MAG-PANI. The TGA curves exhibit three steps of mass loss; these are more pronounced for MAG-PANI (Fig. 4.10 B). The two endothermic peaks (Fig. 4.10 A) up to 170 ºC correspond to the initial removal of moisture (Fig 4.10 B) and can possibly correspond to free and bound water. Above this temperature, exothermic peaks may be associated to the thermal degradation of PANI (Fryczkowski *et al*., 2006, Basavaraja *et al*., 2008). This is most visible within the range of 200 to 400 ºC for the DSC curve and within 150 to 330 ºC for the TGA curve (Fig. 4.10A and 4.10B, MAG-PANI). In this work a nitrogen flux was used during the calorimetric assays. The conversion of

magnetite to hematite (Fig. 4.10 A), detectable at 225 ºC (Lauer *et al*., 2003), can therefore be excluded as oxygen is required to complete the reaction.

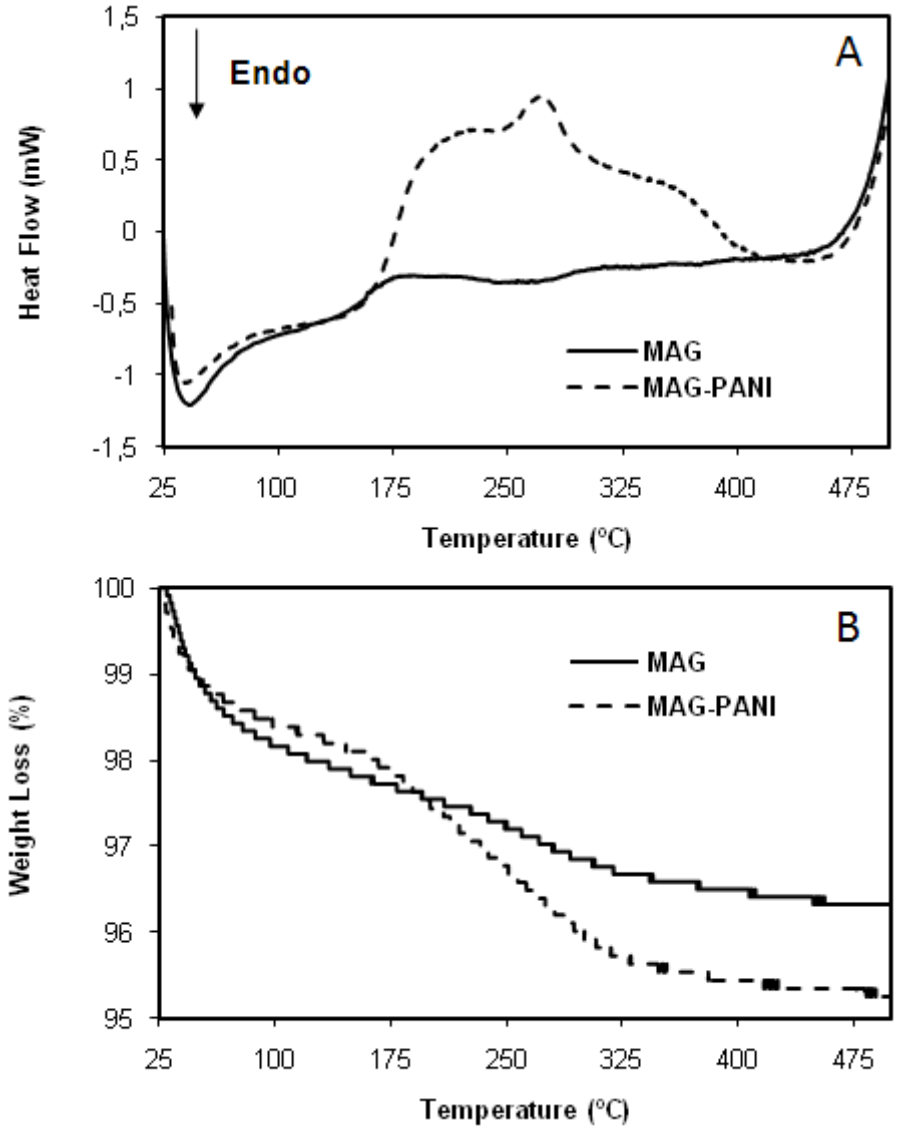

Figure 4.10. DSC heat flow (A) and thermogravimetry curves (B) for MAG and MAG-PANI.

4.4. CONCLUSIONS

According to the results above presented a catalytically active magnetic derivative of *A. orizae* β-galactosidase was synthesized. Firstly, magnetic particles were obtained by coprecipitation of Fe^{2+} and Fe^{3+} and coated with PANI afterwards. This magnetic preparation was capable to convert lactose into galactose and glucose and to produce tri and tetra-galactosides (transgalactosylation) as well. This performance did not differ

from that for the native and soluble enzyme. The tri and tetra-galactosides production by both soluble and immobilized enzyme was not affected by temperature from 30° C to 60° C. The analysis of this β-galactosidase-magnetic composite revealed: (1) rhombohedra particles showing good magnetic response; (2) the elemental analysis and infrared spectra provided evidences for the PANI coating and the protein immobilization; (3) x-ray diffraction, magnetization measurements at 25°C, curve of the magnetic moment against temperature and thermal analysis were in agreement with magnetite composition predominantly. Furthermore, this magnetic enzymatic derivative presented the following advantages: simple synthesis using low cost reagents, similar catalytic properties of the soluble enzyme and easy removal from the reaction mixture by a magnetic field and reuse.

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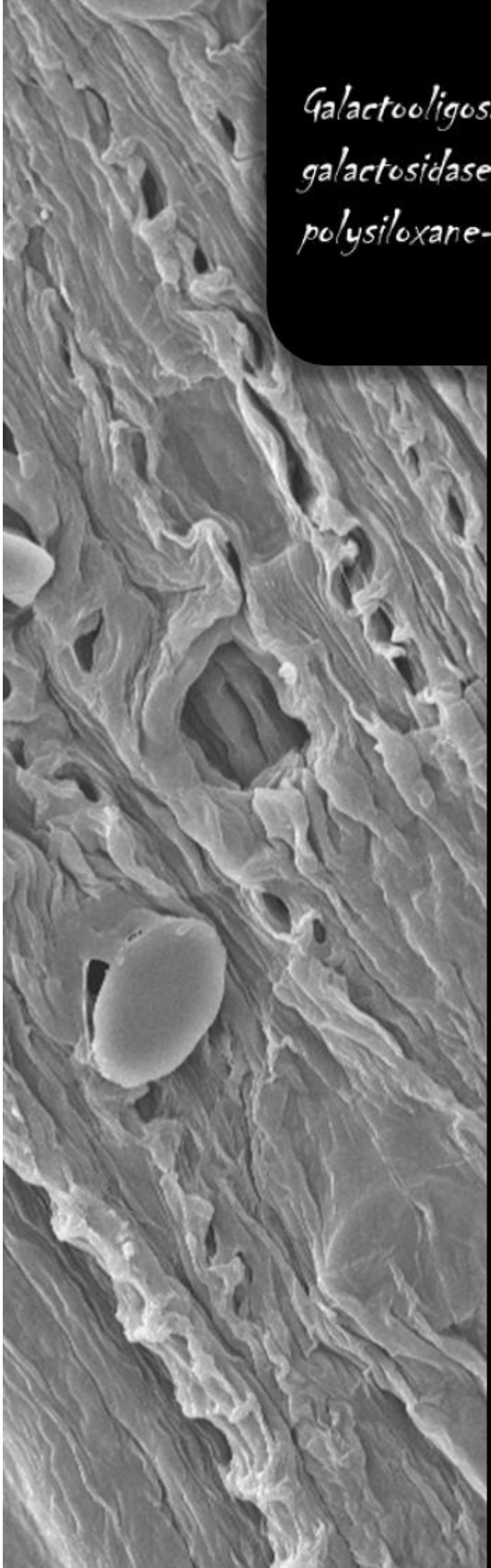

Galactooligosaccharides production by β-galactosidase immobilized onto magnetic polysiloxane-polyaniline particles

CHAPTER 5

Magnetized polysiloxane coated with polyaniline (mPOS-PANI) was used as a support for β-galactosidase immobilization via glutaraldehyde. The galactooligosaccharides (GOS) production by this derivative was investigated under different initial lactose concentrations (5 to 50%) and temperatures (30 to 60°C). The initial lactose concentration in the reaction media affected the total amounts of produced GOS and their time course production was described as a "bell-shaped" curve as a result of the balance between transgalactosylation and hydrolysis. No significative difference was observed for the free and immobilized enzymes. The reaction rates for lactose hydrolysis and GOS formation increased with increasing temperature from 30 to 60°C, but GOS production at all lactose conversion levels was almost unchanged with changing temperature. The mPOS-PANI matrix was also characterized by scanning electronic microscopy (SEM), X-ray diffraction (XRD), vibrating sample magnetometry (VSM), thermomagnetization, differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA).

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5.1. INTRODUCTION

Galactooligosaccharides (GOS) are produced by the β-galactosidase catalytic transfer of one or more D-galactosyl units into the D-galactose moiety of lactose (Mahoney, 1998). The properties of the final product depend on the source of the enzyme and conditions used in the reactions (Boon *et al*., 2000). GOS are nondigestible oligosaccharides acting as growth-promoting substrate for bifidobacteria in the human intestine (Ohtsuka *et al*., 1989, Kunz and Rudloff, 1993).

β-Galactosidase is present in a variety of sources, including plants, animals and micro-organisms (Prenosil *et al*., 1987). They are versatile biocatalysts used for lactose hydrolysis (Bakken *et al*., 1992) facilitating milk digestibility and improving the fuctional properties of dairy products and for GOS formation. GOS synthesis has been studied by free (Reuter *et al*., 1999) and immobilized enzymes (Albayrak and Yang, 2002; Chen *et al*., 2003), using whole cells (Tzortzis *et al*., 2005) and by fermentation (Li *et al*., 2008).

Immobilization is an important step in commercial and fundamental enzymology allowing the repetitive and economic utilization of enzymes (Oliveira *et al*., 2008). Compared with free enzyme in solution, enzyme immobilized on a solid support provides many advantages, including enzyme reusability, continuous operation, controlled product formation, and simplified and efficient processing (Albayrak and Yang, 2002). Recently, β-Galactosidase from *Kluyveromyces lactis* has been covalently immobilized onto a polysiloxane–polyvinyl alcohol magnetic (mPOS–PVA) composite, using glutaraldehyde as activating agent (Neri *et al*., 2008).

Polyaniline (PANI), a conducting polymer, has been used for immobilization of antigen from *Yersinia pestis* (Coêlho *et al*., 2001) and different enzymes, such as, lipase (Lee *et al*., 2008), horseradish peroxidase (Oliveira *et al*., 2008), glucose oxidase (Leite *et al*., 1994, Parente *et al*., 1992), xanthine oxidase (Nadruz *et al*., 1996).

In this study, magnetic particles of a composite of magnetite and polysiloxane (POS) coated with PANI (mPOS-PANI) were synthesized and characterized. β-Galactosidase was then covalent immobilized via glutaraldehyde on mPOS-PANI and used for lactose hydrolysis and GOS production.

5.2. EXPERIMENTAL

5.2.1. Synthesis, magnetization and polyaniline coating of support

POS particles were synthesized as follows: 5 mL of tetraethylorthosilicate (Fluka, Germany) and ethanol (Riedel-de Haën, Germany) were mixed in a beaker. After raising the temperature to 70 ºC, under stirring, 100 µL of concentrated HCl was added and incubated for 50 min. The solution was distributed into ELISA microplates (100 mL/well) and allowed to solidify for about 72 h at 25 ºC. The resulting beads were smashed using a mortar and pestle; the powder (2 g) was suspended in de-ionized water (100 mL) and 10 mL of a solution containing 0.6 M $FeCl_2$ and 1.1 M $FeCl_3$ (1:1) were added drop wise under magnetic stirring, pH was adjusted to 11.0 (using 33%, w/v, NH_4OH) and incubated for 30 min at 100 ºC. The resulting magnetized particles were thoroughly washed with de-ionized water until pH 7.0, were dried at 105 °C overnight and finally sieved (<100µm). The mPOS particles were treated with 0.1 M $KMnO_4$ solution at 50 ºC overnight, washed with distilled water and immersed into 0.5 M aniline solution prepared in 1.0 M HNO_3. Polymerization was allowed to occur for 2 h and after that the mPOS-PANI were successively washed with distilled water, 0.1 M citric acid and distilled water.

5.2.2. β-Galactosidase immobilization

mPOS-PANI particles (100 mg) were incubated with 2.5 % w/v glutaraldehyde (1 mL) and 20 mM citrate-phosphate buffer, pH 4.5 (9 mL) for 2 h under stirring at 25 ºC. Activated mPOS-PANI were successively washed with distilled water and incubated overnight with 10 mL of β-galactosidase (4 mg solid/mL) from *Aspergillus oryzae* (SIGMA, Japan). The amounts of free and immobilized enzyme in the experiments were 0.149 mg/mL and 0.341 mg support/mL, respectively.

5.2.3. GOS production

GOSs formation kinetics with free and immobilized enzyme on mPOS-PANI was studied using lactose as substrate that was prepared by dissolving lactose in citrate-phosphate buffer solution. Samples were taken at appropriate time intervals and analyzed for sugar content by high performance liquid chromatography (HPLC). The reaction kinetics was studied at six different initial lactose concentrations (50, 100, 200, 300, 400 and 500 g/L) and four different temperatures (30, 40, 50, and 60ºC).

5.2.4. HPLC analysis

An HPLC (Jasco AS-2057 Plus), employing a MetaCarb 67H at 60 °C, a refractive index detector (Jasco RI-2031 Plus) and a mobile phase 0,001N H_2SO_4 at a flow rate of 0.5 mL min^{-1} (Jasco PU-2080 Plus) was used for the determination of concentration of all sugars present in the assay solution (GOSs, lactose, glucose, and galactose). The concentration (w/v) of these sugars (lactose, glucose, galactose, and oligosaccharides including tri-, and tetra-sacacharides) are proportional to their peak areas. Thus, normalized sugar concentrations as weight percentages of total sugars or initial lactose were determined from peak areas and are reported in this work. It should be noted that the accuracy of this approximation was verified by checking the material balance.

5.2.5. Support characterization

The samples of uncoated magnetic particles and coated with PANI were mounted on stubs, coated with gold, and examined in a Leica Cambridge S360 scanning electronic microscope (SEM) and micrographs were taken. The X-ray diffraction patterns of the samples were collected on a Philips-PW1710 diffractometer with Cu Kα radiation (λ = 1.54056 Å). Thermal analyses (thermogravimetrical analysis (TGA) and differential scanning calorimetry (DSC)) of mPOS and mPOS-PANI were done using a TGA-50 and a DSC-50 both from Shimadzu (Izasa, S.A., Portugal). Acquisition of the results was done by TA-50WS software (version 1.14). Samples were weighed (10 mg; the exact mass was recorded) into aluminium pans (Izasa, S.A., Portugal) and heated

over the temperature range 25–500 ºC, at a scanning rate of 5 ºC/min, under 20 mL/min nitrogen flow. The magnetic properties of sample mPOS-PANI were investigated by measuring its hysteresis loop at room temperature using an Oxford instrument Vibrating Sample Magnetometer (VSM) and the thermomagnetization from room temperature to 1000 ºK using a Cahn 2000 Curie-Faraday balance.

5.3. RESULTS AND DISCUSSION

5.3.1. Effects of lactose concentration and temperature on GOS production by free and immobilized enzime

The initial lactose concentration is by far the most significant factor affecting GOS formation (Boon *et al*., 2000). This is caused by the reduced water activity of the reactive solutions as substrate concentration increases (Cruz *et al*., 1999). Higher substrate concentrations and consequently reduced water availability make the transfer reactions of galactose to water molecules less likely to occur leading to a greater degree of polymerisation of oligosaccharides being formed (Hansson and Adlercreutz, 2001). Figure 5.1A shows a typical tri- and tetra-saccharides time course production for 50%, w/v initial lactose concentration by the action of free and immobilized *A. oryzae* β–galactosidase. More detailed information on the catalytic behavior of free and immobilized enzymes can be obtained from the plots in Figures 1B and 1C. Oligosaccharide concentration increased initially to a maximum and subsequently decreased when trans-galactosylation activity became less pronounced than the hydrolytic activity (Boon *et al*., 2000). Figure 5.1B shows that the GOS (tri- and tetra-saccharides) production kinetics at 50% w/v initial lactose concentration is closely related to lactose conversion. Identical experiments were performed for the other lactose initial concentrations (5 to 40%, w/v) and similar results were obtained as can be seen in Figure 5.1C representing the total mass of GOS (expressed as percent of total sugars) against the percent of hydrolyzed lactose.

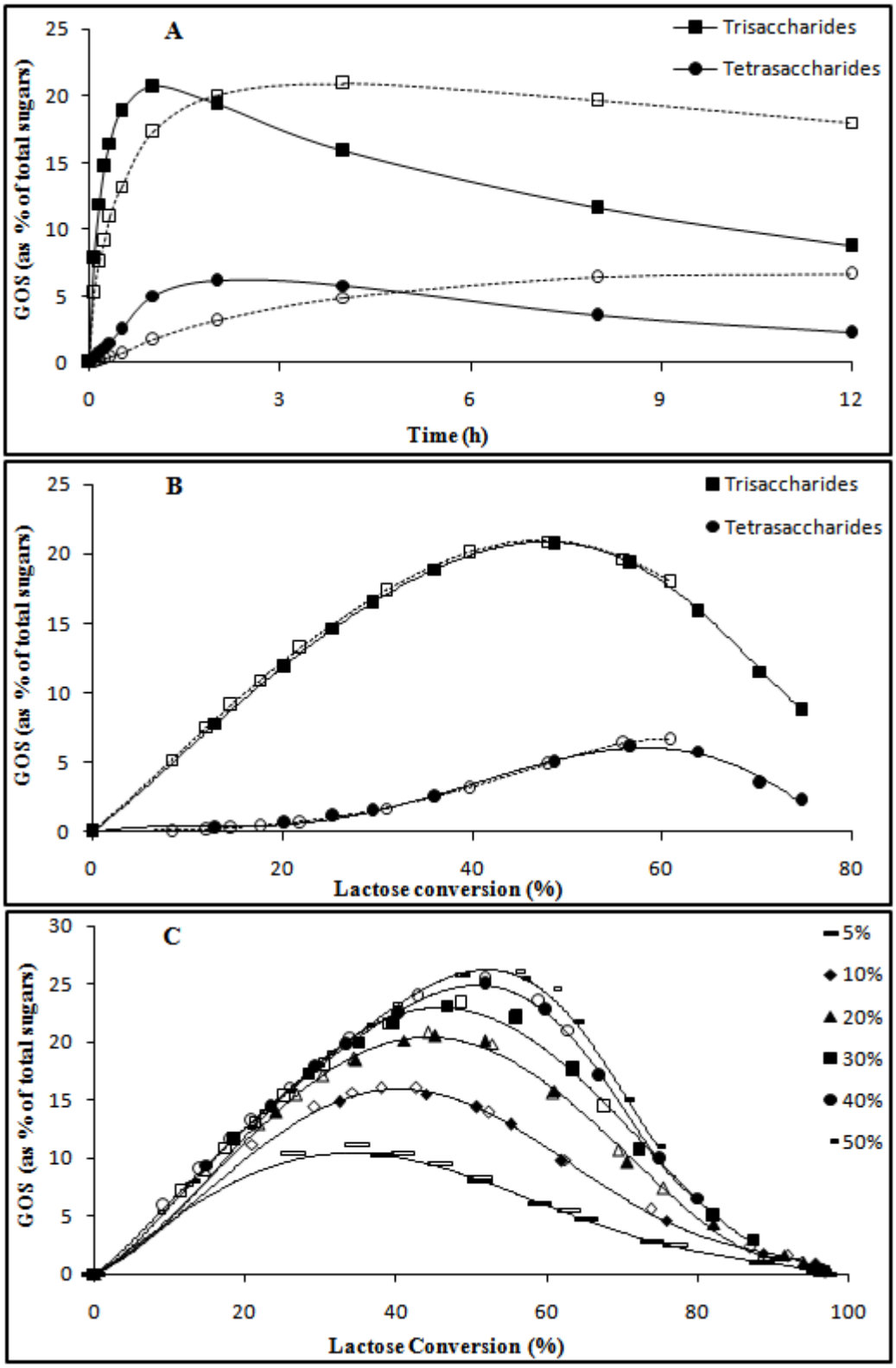

Figure 5.1. GOS (tri- and tetra-saccharides) production by free (open symbols) and immobilized (closed symbols) *A. oryzae* β-galactosidase onto mPOS-PANI at pH 4.5 and 40° C. Production using 50% (w/v) lactose in terms of time course (A); related to percent of lactose conversion (B) and using other lactose concentrations (C).

Figure 5.1C shows that GOS production increased with increasing lactose concentration. In Figure 1B, it can noticed that the trisaccharide (80.5% of GOS total) production is higher, about 20.7% of total sugars for 50%, w/v of lactose, than the tetrasacharide (24.2% of GOS total), about 6.2% of total sugars for 50% w/v of lactose.

No significative difference was observed for the free and immobilized enzymes. As the initial lactose concentration increased from 5% to 50%, the maximum GOS content increased from 11.2% (at 35% conversion) to 26.1% (at 56% conversion) for the free enzyme and from 10.3% (at 38% conversion) to 25.7% (at 49% conversion) for the immobilized enzyme. The maximum amount of tri-, tetra-saccharides and total GOS obtained for the immobilized enzyme were 103.5 g L^{-1}, 30.9 g L^{-1} and 128.6 g L^{-1} for a lactose conversion of about 49%, 57%, and 49%, respectively, in 500 g L^{-1} of lactose. For the free enzyme, 104.5 g L^{-1}, 33.2 g L^{-1} and 130.3 g L^{-1} were obtained for about 48%, 61% and 56% lactose conversion, respectively in 500 g L^{-1} of lactose. These results suggest that enzyme immobilization on mPOS-PANI does not impose any limitation or changes on GOS formation from lactose. Thus, the GOS formation ability of the enzyme was not affected by the immobilization of the enzyme onto mPOS-PANI.

β-galactosidase immobilized on mPOS-PANI acting on 20% (w/v) lactose was successively reutilized for 10 cycles at 25 ºC and the obtained activity was 87% of the initial one (data not shown). This performance of the mPOS-PANI β-galactosidase derivative is an additional advantage besides that from its easy of separation from the reaction medium by a magnetic field. It is worthwhile to draw attention that the free and immobilized *A. oryzae* β–galacotsidase were also capable to hydrolyze lactose into glucose and galactose but these results are not presented because the aim of this contirbution was to investigate the transgalactosylation action of the enzyme.

GOS yields during lactose conversion catalyzed by the free and the immobilized β-galactosidase on mPOS-PVA are shown in Figure 5.2. The maximum GOS yield increased with the initial lactose concentration by the catalysis of both enzyme preparations. The maximal values ranged from 39.5% (5%, w/v, lactose) to 64.1% (higher lactose concentrations) for the free enzyme and from 26.9% (5%, w/v, lactose) to 62.1% (higher lactose concentrations) for the immobilized one. It was generally observed that the hydrolysis and transgalactosylation reactions occurred simultaneously

and that the hydrolysis reaction dominates at low lactose concentration while GOS formation dominates at high lactose concentrations.

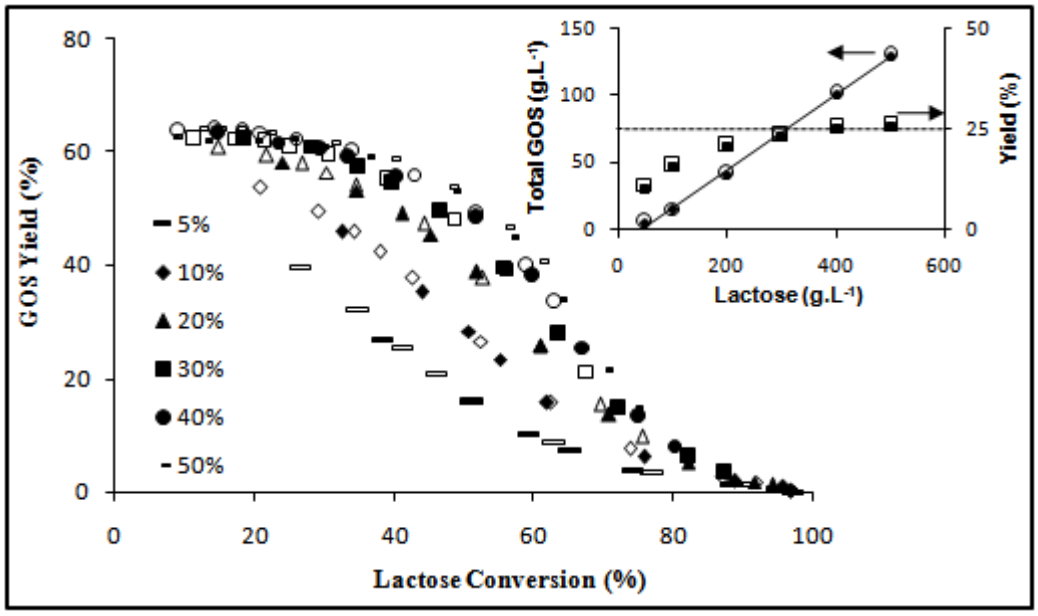

Figure 5.2. GOS yield *versus* percent of lactose conversion by free (open symbols) and immobilized (closed symbols) *A. oryzae* β-galactosidase onto mPOS-PANI at pH 4.0 and 40° C. The insert presents the same relationship expressed in terms of total GOS (g/L) and maximum GOS yield. The initial concentrations of lactose were 5% (—); 10% (◆); 20% (▲); 30% (■); 40% (●) and 50% (▪), w/v.

β-Galactosyl groups should have a higher probability of attaching to lactose than water at increasing lactose concentrations (Iwasaki *et al*., 1996). The insert of Figure 5.2 presents these same data under other perspective clearly showing that increases in lactose concentration lead to increases in GOS production. However, in terms of GOS yield, no increase was observed for lactose concentrations higher than 300 g L^{-1} , the yield value remaing constant at 23.3–26% for both free and immobilized enzyme. Park *et al* (2008) observed the same behavior for a high lactose concentration with a thermostable β-galactosidase from *Sulfolobus solfataricus*.

Figure 5.3 shows the effect of temperature on GOS production rate and yield during lactose hydrolysis catalyzed by free and immobilized enzyme preparations at pH 4.5 and 40% (w/v) initial lactose concentration.

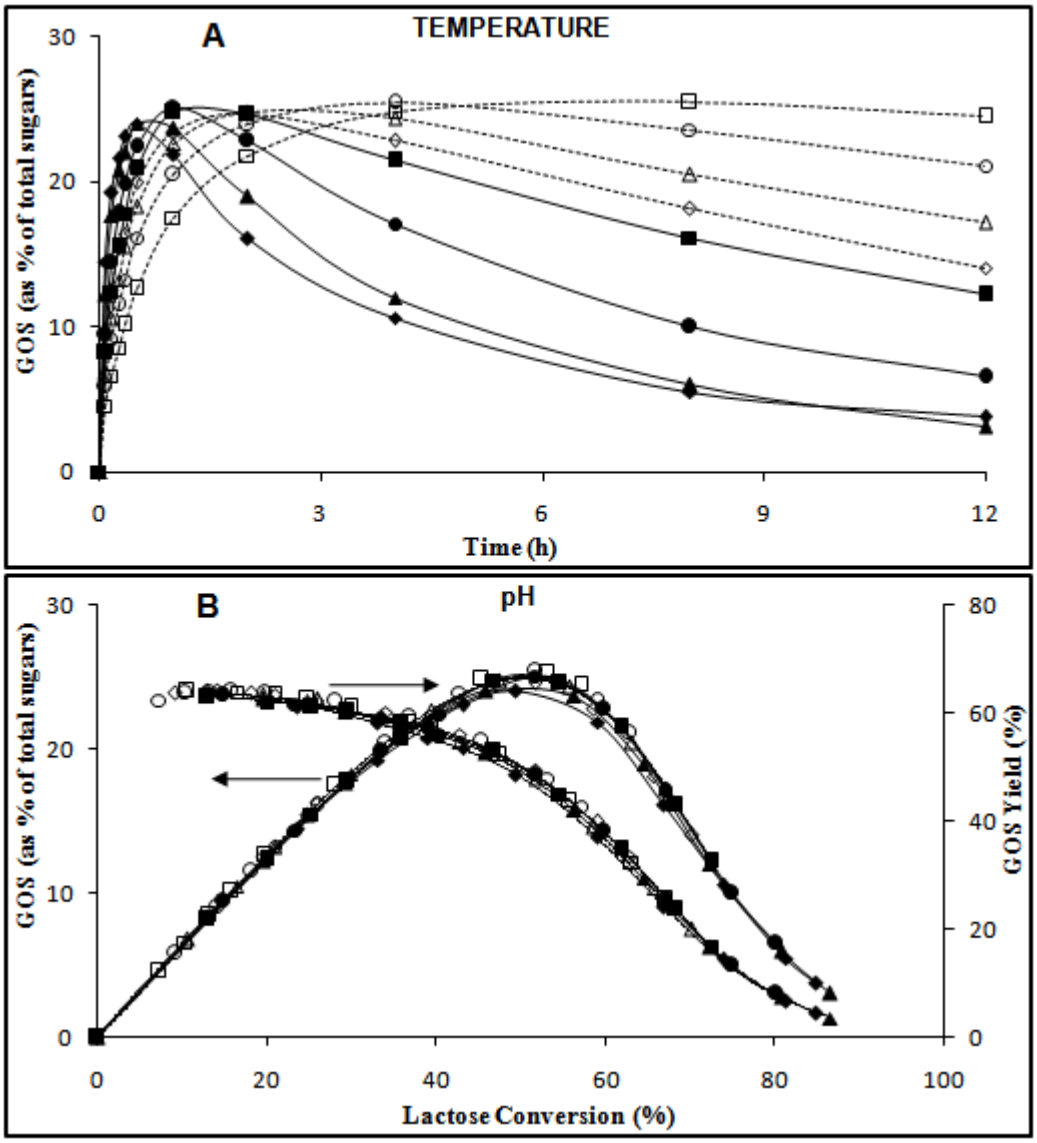

Figure 5.3. Effect of temperature on the GOS production during the hydrolysis of 40% (w/v) lactose catalyzed by the free (open symbols) and immobilized (closed symbols) *A. oryzae* β-galactosidase onto m-POS-PANI. A: time course of GOS production; B: GOS production *versus* lactose consumption. The temperatures were 30 ºC (■); 40 ºC (●); 50 ºC (▲); 60 ºC (◆).

The reaction rates for lactose hydrolysis and GOS formation increased with increasing temperature from 30 to 60 ºC, but GOS production at all lactose conversion levels was almost unchanged with changing temperature. This was consistent with results reported by Albayrak and Yang (2002) that reported similar results with *Aspergillus oryzae* β-galactosidase immobilized on Cotton Cloth.

5.3.2. Characterization

SEM analysis of the mPOS and mPOS-PANI revealed different sized 15-100 μm rhombohedra particles and no difference in the structure after coating with PANI was observed (Fig. 5.4).

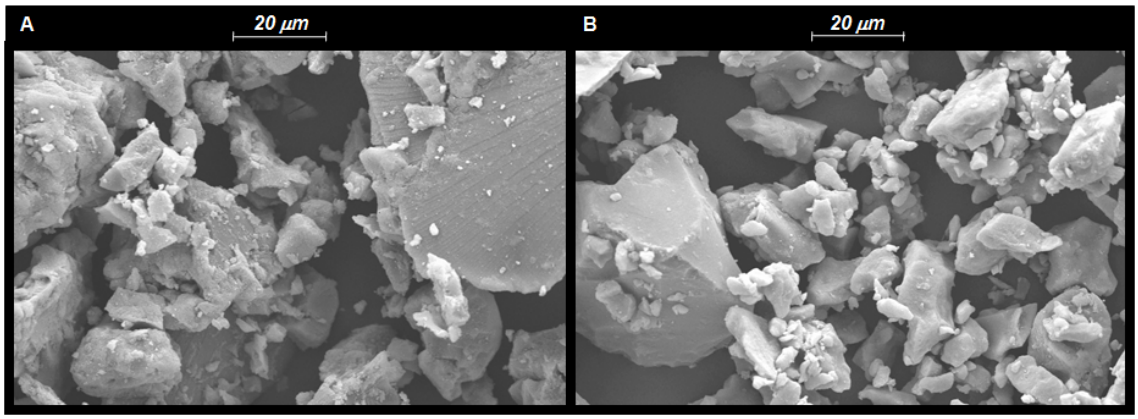

Figure 5.4. SEM image of the mPOS (A) and mPOS-PANI (B).

Figure 5.5 shows the results of X-ray diffraction analysis of uncoated magnetic composite of polysiloxane (mPOS) and coated with polyaniline (mPOS-PANI). Patterns of iron oxides of the Joint Committee on Powder Diffraction-International Centre for Diffraction Data database (JCPDS-ICDD) were included for comparison. Both magnetic composites presented broad peaks that can be ascribed to the spinel structure. Magnetite and maghemite are two iron oxides that crystallize in the spinel structure. They presented similar spectrum and it is difficult to distinguish one from another by X-ray diffraction pattern based on these features.

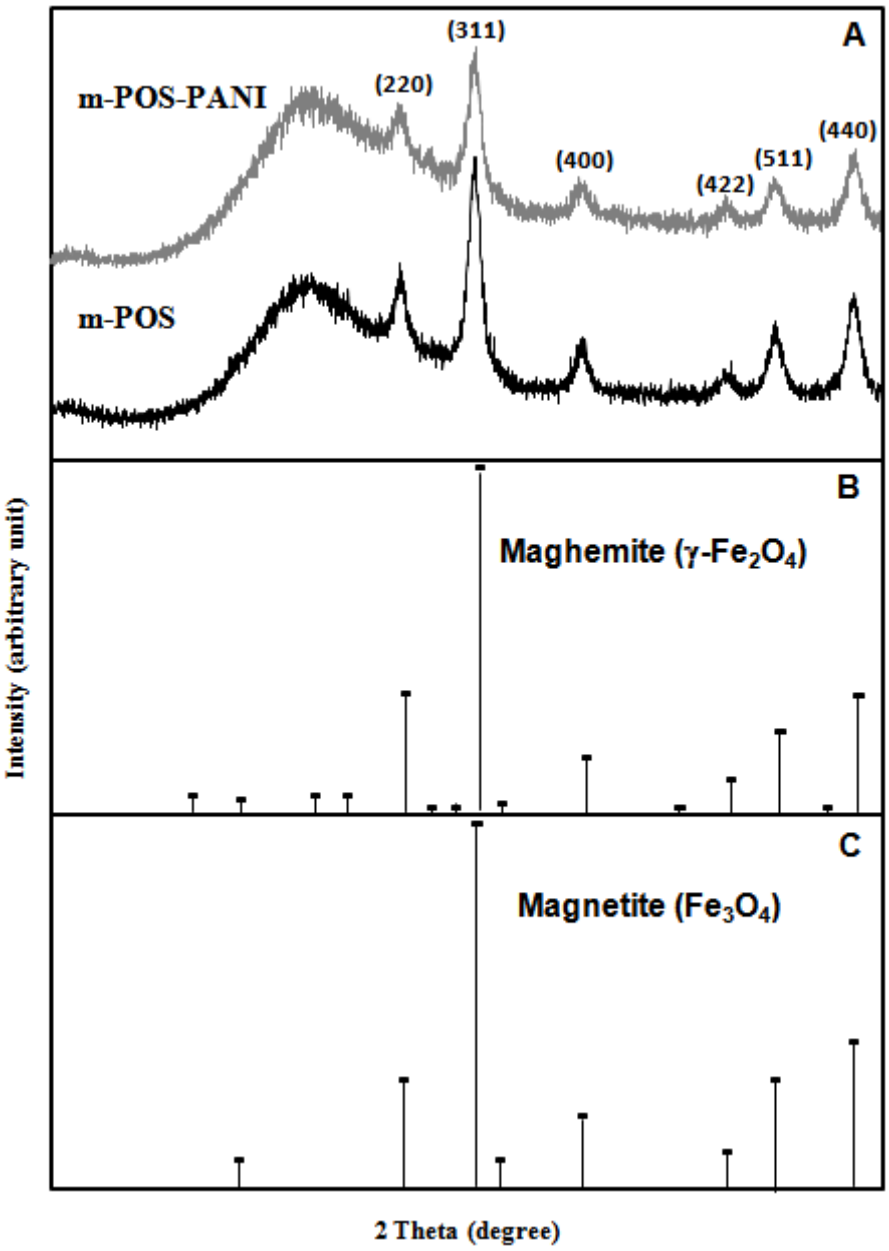

Figure 5.5. XRD spectra of the mPOS and mPOS-PANI support (A) and patterns of iron oxides maghemite (B) and magnetite (C) according to the JCPDS database.

Magnetization measurements were performed and as can be observed in Figure 5.6 the saturation magnetization was measured to be approximately 15 emu/g, lower than 60-70 emu/g reported for the small particles of magnetite (Chin *et al*., 2007, Lui *et al*., 2007). The presence of cations vacancy in maghemite can be responsible for the decrease in the saturation of magnetization compared to magnetite (Mendoza *et al*., 2005).

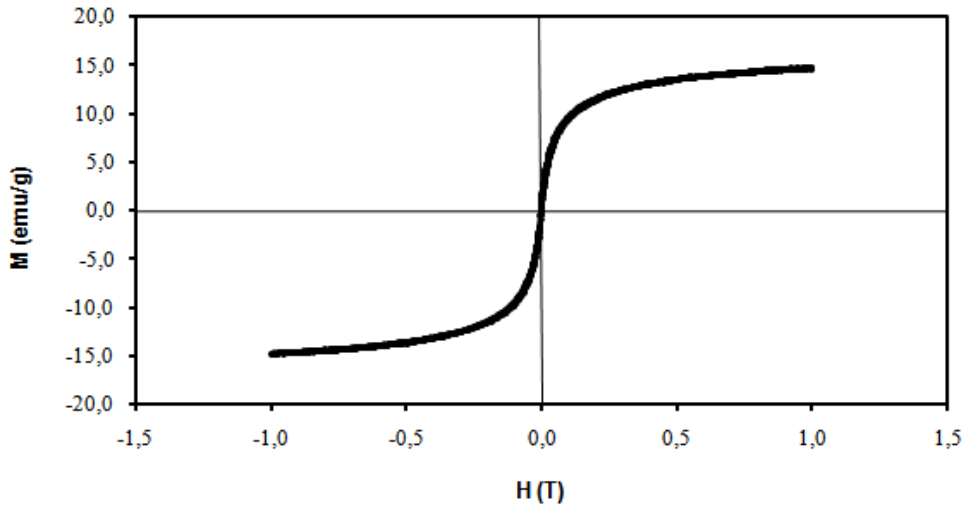

Figure 6. Hysteretic loop of the mPOS-PANI at 295 ºK.

The curves of the magnetic moment of the mPOS-PANI samples against temperature show a tendency to linear temperature dependence from 295 ºK until near the transition temperature Tc = 860 ºK (Figure 5.7).

This was observed for 2 distinct applied fields H = 0.51T and 0.071T (see Figures 5.7A and 5.7B). It should be noted that the behaviour of the temperature dependences, as well as the Curie temperature remains unchanged after heating-cooling cycles. As follows from literature, maghemite has a ferromagnetic structure with ferromagnetic ordering of ions in octahedral and tetrahedral positions. The sublattices are coupled through antiferromagnetic exchange (Janot and Guerard, 2002; Costa *et al*., 1998; Restrepo *et al*., 2006).

The model accounting for the change in the spin of iron ions in tetrahedral positions offers a satisfactory explanation for the linear dependence of the magnetization against temperature (Barinov and Aplesnin, 2006) as observed in our data.

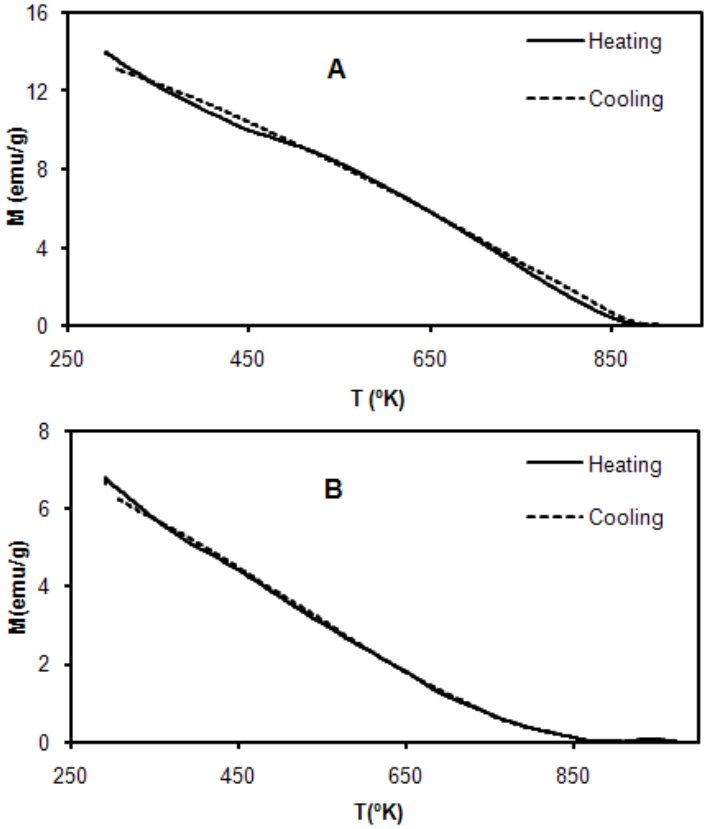

Figure 5.7. Thermomagnetization of mPOS-PANI from 295 °K to 1000 °K for H = 0.51T (A) and 0.071T (B).

Thermoanalytical techniques, such as DSC and TGA, have been used to characterize polymers and POS has been receiving an increasing attention, due to its thermal resistance and endurance at high temperatures (Zhixiong *et al*., 2006). Figure 5.8 shows the results from the thermal analysis relative to mPOS and mPOS-PANI. The DSC curves (Fig. 5.8A) show a clear endothermal peak, up to 175 ºC, associated mostly to the loss of residual moisture. This is also evidenced by the greater weight loss in Figure 5.8B. The remaining weight loss maybe mostly associated with the removal of water trapped within the porous structure of mPOS and also to the thermal degradation of PANI (starting near 325 ºC). A similar degradation was reported by Fryczkowski *et al*. (2006) and Basavaraja *et al*. (2008) for PANI complexes, though at lower temperatures. The degradation of PANI in mPOS-PANI does not, however, entail significant enthalpic changes (Fig 5.8A).

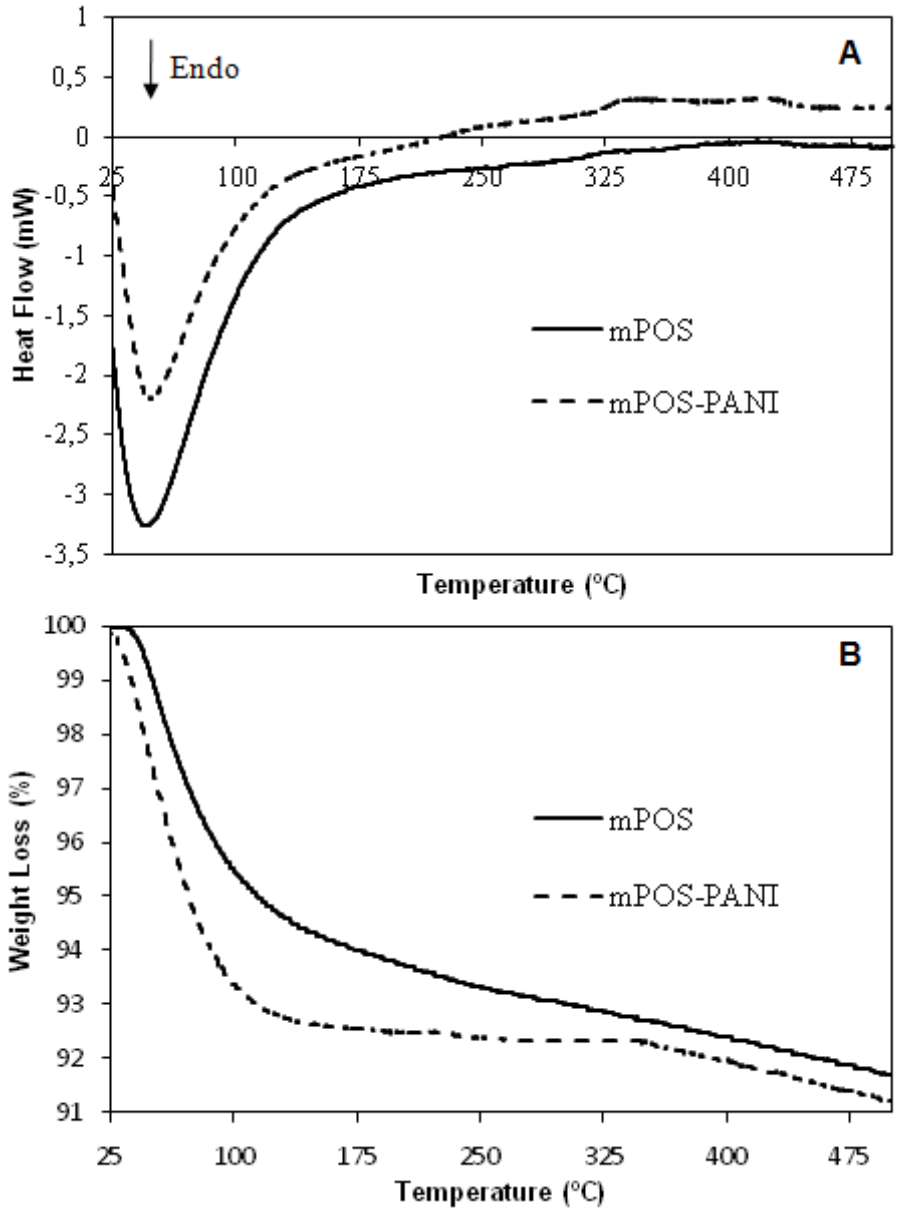

Figure 8. DSC heat flow (A) and thermogravimetry curves (B) for mPOS and mPOS-PANI.

5.4. CONCLUSIONS

From the above displayed results one can conclude that immobilized β-galactosidase onto mPOS-PANI derivative is capable to hydrolyze lactose and to produce GOS as the free enzyme. The initial lactose concentration in the reaction media affected the total amounts of produced GOS and their time course production was described as a "bell-shaped" curve as a result of the balance between

transgalactosylation and hydrolysis. This water insoluble enzymatic derivative can be reused and collected by a magnetic field easily. The reaction rates for lactose hydrolysis and GOS formation increased with increasing temperature from 30 to 60 ºC, but GOS production at all lactose conversion levels was almost unchanged with changing temperature. The magnetic particles have rhombohedra shape sizing 15-100 μm (SEM analysis) and are mostly maghemite (Magnetization measurements and X-ray diffraction analysis).

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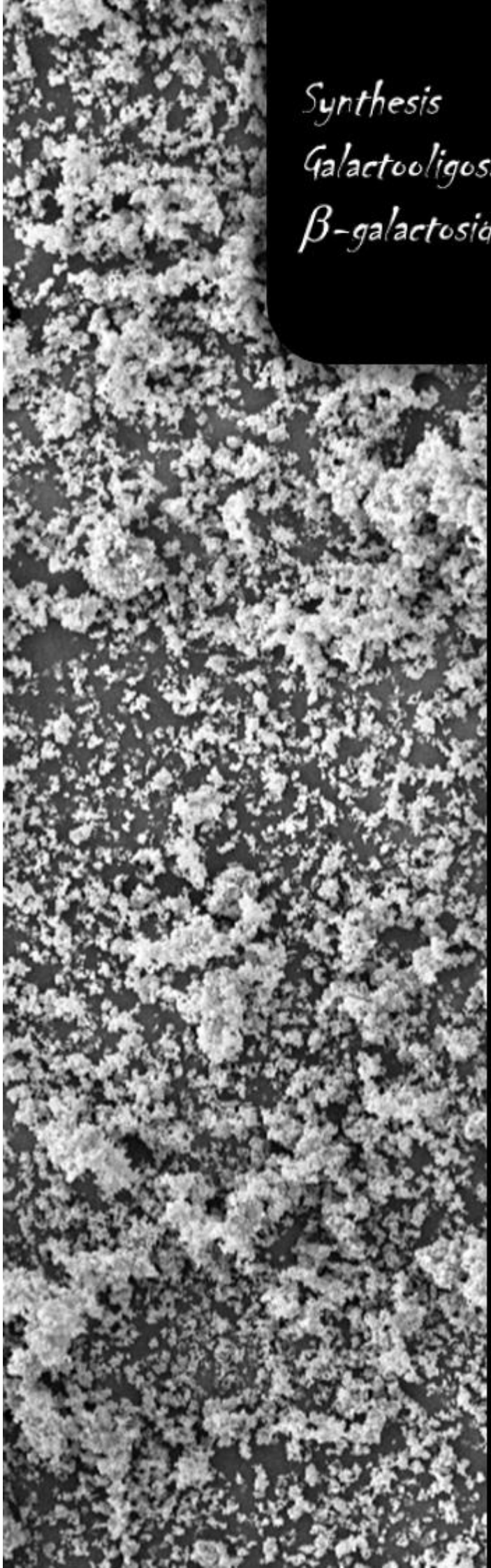

Synthesis and Characterization of Galactooligosaccharides Produced by Immobilized β-galactosidase onto Magnetized Dacron

CHAPTER 6

Dacron was hydrazinolyzed and the formed powder (hydrazide-Dacron) was coprecipitated with Fe^{+2} and Fe^{+3} ions under high temperature and alkaline pH. Then *Aspergillus oryzae* β-galactosidase was covalently attached, via glutaraldehyde, to this hydrazide-Dacron-magnetite composite. This magnetic immobilized β-galactosidase preparation was capable to hydrolyze lactose and also to produce galactooligosaccharides (GOS), which increased with increasing initial lactose concentration. Furthermore, the GOS production of GOS was almost unchanged despite temperatures varying from 30° C to 60° C. The immobilized enzyme retained 90% of its initial activity after 10 successive reuses. The NMR spectroscopy and mass spectrometry analysis of the HPLC fractions identified besides glucose and galactose the following GOS: β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-Glc*p*; β-D-Gal*p*-(1→4,6)-Glc*p*; and β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)Glc*p*. This magnetic β-galactosidase preparation besides the above properties is easily removed from the reaction mixture by a magnetic field. Furthermore, the low-cost of the reagents and the simplicity of the methodology used in the matrix synthesis are additional advantages.

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6.1. INTRODUCTION

Oligosaccharides are sugars consisting between two and approximately 20 saccharide units, i.e. they are short-chain polysaccharides. They can naturally occur in fruits and vegetables, and are extractable, others can be commercially produced through the hydrolysis of polysaccharides (*e.g*. dietary fibers, starch) or through enzymatic generation. The following oligomers have been suggested as having prebiotic potential (Gibson, 2000) and some of them are lactose derivatives: Lactulose; Fructo-oligosaccharides; Galacto-oligosaccharides; Soybean oligosaccharides; Lactosucrose; Isomalto-oligosaccharides; Gluco-oligosaccharides; Xylo-oligosaccharides; Palatinose-oligosaccharides.

Galactooligosaccharides (GOS) derived from lactose in milk have received less attention than the other prebiotics, although they are considered to have bifidogenic effects in humans. GOS is a collective term for a group of semi-synthetic non-digestible carbohydrates made using β-galactosidase as catalysts. GOS contain zero to one glucose units and one to six galactose units bound to each other by different glycosidic bonds (β1–2, β1–3, β1–4, β1–6). During production of GOS mixtures of oligosaccharides of different chain length are formed (Alander *et al*., 2001; Ito *et al*., 1993; Tannock *et al*., 2004).

β-Galactosidase is applied either in the lactose hydrolysis or galactooligosaccharides (GOS) production and has been immobilized on different magnetic supports, including polysiloxane-polyvinyl alcohol magnetic particles (Neri *et al*., 2008), magnetic poly(glycidylmethacrylate and methylmethacrylate) (Bayramoglu *et al*., 2007) and thermo-sensitive magnetic hydrogel microspheres (Kondo and Fukuda, 1997). Production of GOS by other immobilized β-galactosidase rather than magnetic supports has been considered in several studies (Shin *et al*., 1998; Albayrak and Yang, 2002; Li *et al*., 2008), but the GOS identification is relatively less investigated. The main products are trisaccharides, namely 4'- or 6'-galactosyllactose, and longer oligosaccharides consisting of 4 or more monosaccharide units. Substantial amounts of transgalactosylated disaccharides are also produced in these reactions (Ito *et al*., 1990; Ishikawa *et al*., 1995).

In this work, β-galactosidase was covalently immobilized onto magnetic Dacron particles and used to produce GOS from lactose. Also, the formed GOS were identified.

Dacron, chemically known as polyethyleneterephthalate, and also abbreviated as PET, is one of the polyester most worldly used. It has been already used for enzyme immobilization (Cooney *et al*., 1975; KO and Hersh, 1976; Carvalho *et al*., 1986; 1987; Carneiro-Leão *et al*., 1991; Amaral at al., 2006; Pimentel *et al*., 2007; Caramori and Fernandes, 2004; 2008). Many magnetic materials have been developed for enzyme immobilization because the use of them can also reduce operation costs (Pieters and Bardeletti, 1992).

6.2. EXPERIMENTAL

6.2.1. Hydrazinolysis of Dacron and Magnetization

Films of Dacron (4 g) were cut into strips and incubated in 50 mL of methanol containing 12.5 mL of hydrazine hydrate at 50 ºC for 72 h with stirring. Afterwards the hydrazide-Dacron (powder) was washed and filtered under vacuum twice with water. Then the hydrazide-Dacron (2 g) was stirred in deionized water (100 mL) and an aqueous solution (10 mL) containing 1.1 M $FeCl_3$ and 0.6 M $FeCl_2$ (1:1) was added dropwise. Under vigorous stirring, the mixture was adjusted to pH 11 by addition of (28% v/v) ammonium hydroxide solution and then incubated at 100 ºC for 30 minutes. The material obtained (magnetic hydrazide-Dacron) was filtered under vacuum and washed exhaustively with distilled water until pH 7.0, dried at 105 ºC overnight and finally sieved (<100μm).

6.2.2. β-Galactosidase immobilization

Magnetic hydrazide-Dacron particles (100 mg) were treated with 2.5 % w/v glutaraldehyde (1 mL) and 20 mM citrate-phosphate buffer, pH 4.5 (9 mL) for 2 h under stirring at 25 ºC. Activated magnetic hydrazide-Dacron particles were washed with distilled water 10 times and incubated overnight with 10 mL of β-galactosidase (4 mg/mL) from Aspergillus oryzae (SIGMA, Japan). Finally, the magnetic-β-galactosidase-Dacron preparation was 10 times washed with 20 mM citrate-phosphate buffer, pH 4.5 and kept in this buffer at 4^{o} C until use.

6.2.3. GOS production

The kinetics of GOSs formation by the immobilized enzyme on magnetized Dacron was studied as follows: lactose solution at increasing concentration (5, 10, 20, 30, 40 and 50%) prepared in 20 mM citrate-phosphate buffer solution, pH 4.5, was added (10 mL) to the immobilized preparation (100 mg) and samples were taken at appropriate time intervals and analyzed for sugar content by high performance liquid chromatography (HPLC). The reaction kinetics was also studied at four different temperatures (30, 40, 50, and 60ºC).

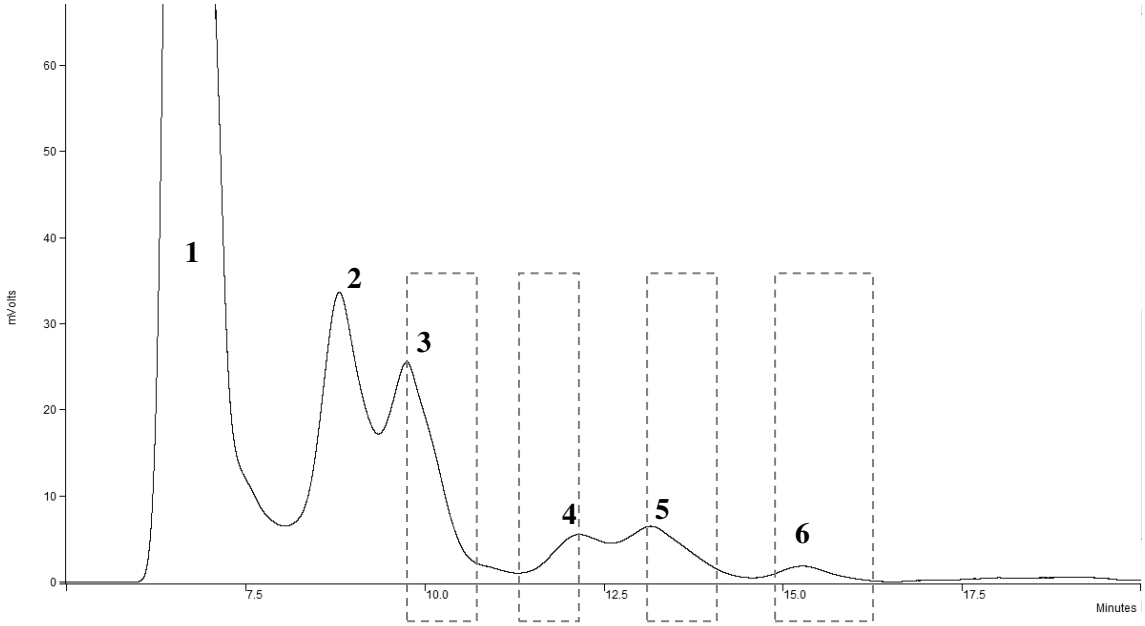
6.2.4. HPLC analysis

Determination of the concentration of all sugars present in the assay solution (GOSs, lactose, glucose, and galactose) was done by HPLC. An HPLC (Jasco AS-2057 Plus), employing a MetaCarb 87H at 60 °C, a refractive index detector (Jasco RI-2031 Plus) and a mobile phase 0.001N H_2SO_4 at a flow rate of 0.5 mL min^{-1} (Jasco PU-2080 Plus) was used. The concentration (w/v) of these sugars (*e.g.*, lactose, glucose, galactose, and oligosaccharides including tri-, and tetra-saccharides) is proportional to their peak areas. Thus, normalized sugar concentrations as weight percentages of total sugars or initial lactose were determined from peak areas and are reported in this work. It should be noted that the accuracy of this approximation was verified by checking the sugars mass balance.

6.2.5. GOS fractionation

The fractionation of different trisaccharides and tetrasaccharides was achieved using the methodology described by Dias *et al*. (2009). The liquid chromatograph above described was equipped with a Prevail Carbohydrate ES column (5 um, 250 x 4.6 mm, Alltech). A mixture of acetonitrile and 0.04% ammonium hydroxide in water (70/30 v/v) was eluted with a flow rate of 0.9 ml/min. 20 µl of the reaction solution (200 g/L lactose, pH 4.5, 40 ºC, after 29 hours) were injected. The RI signal was recorded and four fractions (3, 4, 5 and 6) were collected manually accordingly to the following scheme. The collected fractions were analysed by mass spectrometry and, additionally,

fractions 4, 5 and 6 were analyzed by NMR spectroscopy in order to determine their detailed structure.



Scheme 6.1- Typical HPLC chromatogram of the reaction solution. fraction 1: glucose + galactose; fraction 2: lactose; fraction 3: disaccharide (not lactose); fractions 4, 5 and 6: oligosaccharides. Dashed squares delimit the collection time of each compound.

6.2.6. Analysis of GOS by mass spectrometry

Positive-ion ESI-MS and ESI-MS/MS were carried out on LXQ ion-trap mass spectrometer (Thermo Finningan, San Jose, CA). For ESI analysis, oligosaccharides fractions were dissolved in methanol/water/formic acid (50:50:0.1, v/v/v). Samples were introduced at a flow rate of 5μL/min and the voltage applied was 5.5 kV. Nitrogen was used as the nebulizing and the drying gas. The heated capillary was kept at 350ºC. In each experiment, the ion transmission parameters were optimized automatically in order to improve the detector of the analyte of interest. Full scan mass spectra ranging from m/z 100 to 1500 were acquired in the positive mode. In the MS^n (n= 2, 3) experiments, collision energy varying between 20 and 25 of normalized collision energy.

6.2.7. Analysis of GOS by NMR

^{1}H and ^{13}C NMR spectra were recorded in D_2O on a Bruker Avance 500 spectrometer operating at 500.13 and 125.77 MHz, respectively; the chemical shifts are expressed in δ (ppm) values relative to sodium trimethylsilyl-2,2,3,3-d_4-propionate (TSS) as external reference. 2D COSYPR (homonuclear shift correlation with presaturation during relaxation delay) spectrum was recorded with 200 transients over 256 increments (zero-filled to 1K) and 1K data points with spectral widths of 2000 Hz. The repetition time was 1.5 s. The data were processed in the absolute value mode. The phase sensitive ^{1}H-detected (^{1}H,^{13}C) gHSQC (heteronuclear single quantum coherence, using gradient pulses for selection) spectrum was recorded with 200 transients over 256 increments (zero-filled to 1K) and 1K data points with spectral widths of 2000 Hz in F_2 and 7500 Hz in F_1. The repetition time was 2.0 s. A cosine multiplication was applied in both dimensions. The delays were adjusted according to a coupling constant $^{1}J(CH)$ of 149 Hz. The gHMBC (heteronuclear multiple quantum coherence, using gradient pulses for selection) spectrum was recorded with 200 transients over 256 increments (zero-filled to 1K) and 1K data points with spectral widths of 2000 Hz in F_2 and 7500 Hz in F_1. The repetition time was 2.0 s. A sine multiplication was applied in both dimensions. The low-pass J-filter of the experiment was adjusted for an average coupling constant $^{1}J(CH)$ of 149 Hz and the long-range delay utilized to excite the heteronuclear multiple quantum coherence was optimized for 7 Hz. α-Lactose (Sigma) was used as a reference compound for the structural elucidation of GOS. According to the interpretation of its ^{1}H, ^{13}C NMR, HSQC, COSY and HMBC spectra and further comparison to literature data (Bock *et al*., 1984), the ^{1}H and ^{13}C NMR chemical shifts of its monomeric units were assigned as follow: T-β-Galp (δ (ppm); C-1= 105.7; H-1= 4.31 (J=7.8 Hz); C-2= 73.8; H-2= 3.41; C-3= 75.4; H-3= 3.53; C-4= 71.4; H-4= 3.79; C-5= 78.2; H-5= 3.59; C-6= 63.9; H-6= 3.69); 4-β-Glcp (δ (ppm); C-1= 98.6; H-1= 4.53 (J=8.0 Hz); C-2= 76.7; H-2= 3.15; C-3= 77.2; H-3= 3.51; C-4= 81.1; H-4= 3.51; C-5= 76.7; H-5= 3.50; C-6= 62.9; H-6= 3.81 and 3.69); 4-α-Glcp (δ (ppm); C-1= 94.7; H-1= 5.08 (J=3.8 Hz); C-2= 74.0; H-2= 3.45; C-3= 74.3; H-3= 3.69; C-4= 81.2; H-4= 3.51; C-5= 73.0; H-5= 3.81; C-6= 62.8; H-6= 3.72).

6.3. RESULTS AND DISCUSSION

6.3.1. Magnetic β-galactosidase-Dacron particles

The magnetic support synthesis was carried out in two steps: 1 - the hydrazide-Dacron production based on the hydrazinolysis of the Dacron (Figure 6.1) and 2 - coprecipitation of this modified polymer with Fe^{+2} and Fe^{+3} ions under high temperature and alkaline pH yielding a hydrazide-Dacron-magnetite composite. The molecular weight of Dacron is reduced under this hydrazinolysis so that the film is converted to powder. Then the Dacron-hydrazide powder was magnetized in order to make its recovery easier by using a magnetic field. This procedure is very simple and has a very low cost due to the non-expensive polymer and employed reagents. Finally, β-galactosidase was then covalently attached to the hydrazide-Dacron-magnetite through the classical glutaraldehyde (Migneault *et al*., 2004).

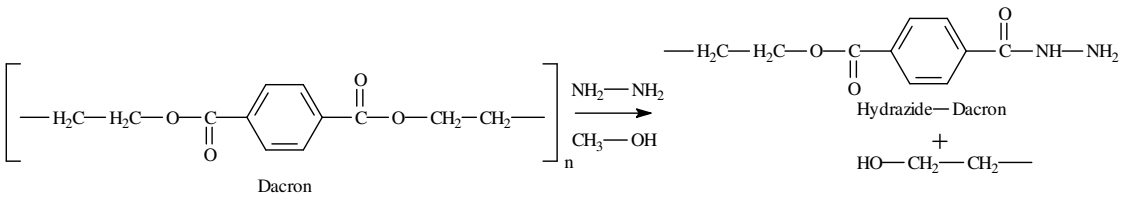


Figure 6.1. Dacron hydrazinolysis.

6.3.2. GOS formation

GOS are the products of transgalactosylation reactions catalyzed by β-galactosidase when using lactose or other structurally related galactosides as the substrate. Both transgalactosylation [Equation 3] and hydrolysis [Equation 2] reactions catalyzed by the enzyme acting on lactose are displayed below:

Enzyme + Lactose → Galactosyl-Enzyme + Glucose [Equation 1]

Galactosyl-Enzyme + H_2O → Enzyme + Galactose [Equation 2]

Galactosyl-Enzyme + Lactose ↔Enzyme + GOS3 [Equation 3]

In aqueous systems transgalactosylation has to compete with hydrolysis, and therefore GOS mixtures always contain considerable amounts of unreacted lactose and monosaccharides (Mahoney, 1998). Moreover, the linkage between the galactose units, the efficiency of transgalactosylation, and the components in the final products depend on the enzymes and the conditions used in the reaction. Glycoside bonds between two galactose units are mainly α,1-4 bonds (4'-GOS) when β-galactosidases derived from *Bacillus circulans* (Mozaffar *et al*., 1984) or *Cryptococcus laurentii* (Ozawa *et al*., 1989) are used, and α,1-6 bonds (6'-GOS) when enzymes derived from *A. oryzae* or *Streptococcus thermophilus* (Ito, 1990) are used. Only two types of GOSs, tri- and tetrasaccharides, were noted for β-galactosidases from Aspergillus oryzae.

Concentrations of the former and latter were 20.2% and 5.9% (w/v), respectively. Many authors demonstrated that trisaccharides were the main GOSs synthesized by β-galactosidase from different origin (Hsu. Thes *et al*., 2007, Li *et al*., 2008, Albayrak and Yang, 2002).

It was generally observed that the hydrolysis and transgalactosylation reactions occurred simultaneously. What dominates the product profile of the reaction is largely dependent on lactose concentration. When transfer to water produces galactose and to another sugar produces di-, tri- and higher galactosyl-saccharides, collectively termed oligosaccharides (Mahoney, 1998).

Figure 6.2 shows a time course of GOS production and lactose hydrolysis catalyzed by the immobilized Aspergillus oryzae β-galactosidase at pH 4.5 and 40 ºC using an initial lactose concentration of 50%. Initially, a rapid reduction in lactose concentration was accompanied by a high rate of GOS formation. The maximal amount of GOSs 26.2% (w/w) of total sugars in the reaction mixture was reached after 8 h of incubation. Meanwhile, the content of glucose in the reaction mixture was found to be much higher than that of galactose, indicating the involvement of galactose in GOS formation. These observations were similar to those reported for β-galactosidase from other microorganisms, where GOSs eventually decreased as result of hydrolysis (Park *et al*., 2008; Reuter *et al*., 1999; Cho *et al*., 2003). This demonstrated that transgalactosylation dominated early in the reaction, producing GOSs in a high yield, while hydrolytic activity of β-galactosidase takes over as the reaction further proceeds.

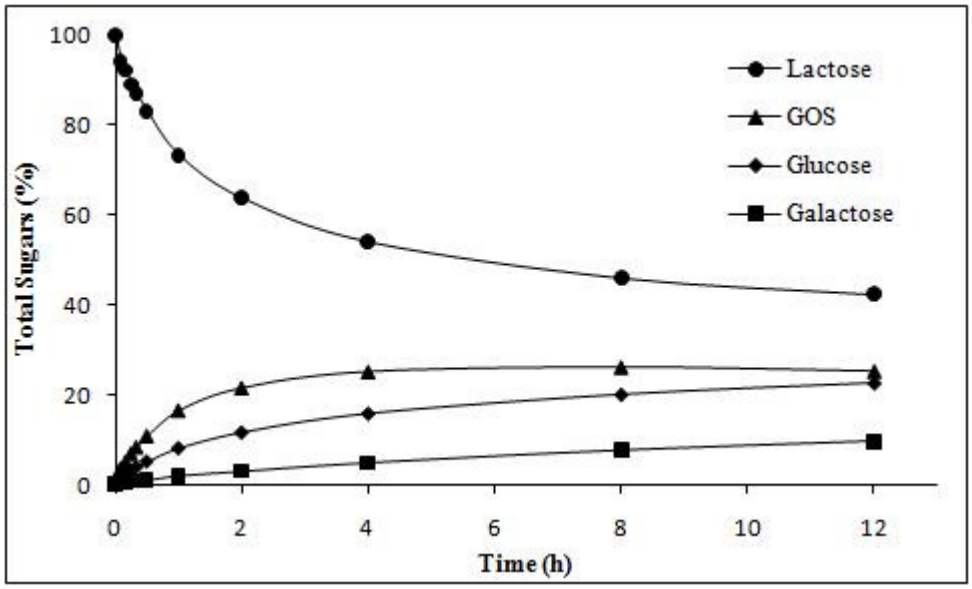


Figure 6.2. Time course of lactose hydrolysis and GOS formation catalyzed by immobilized Aspergillus oryzae β-galactosidase on magnetized DACRON. The reaction was performed at 40 ºC and pH 4.5 with an initial lactose concentration of 50%.

Further looking into the formation and degradation of GOSs during lactose conversion Figure 6.3 instead to show the time course (Figure 6.2) presents the GOS, glucose and galactose production as the lactose is hydrolyzed.

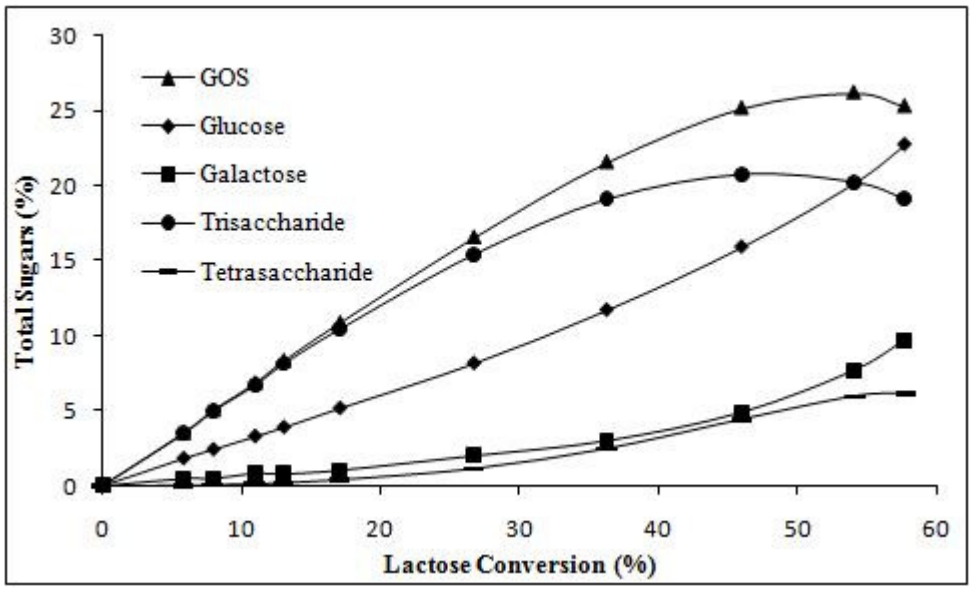


Figure 6.3. Formation and degradation of GOSs during lactose conversion by immobilized Aspergillus oryzae β-galactosidase on magnetized Dacron. The reaction was performed at 40 ºC and pH 4.5 and initial lactose concentration of 50%.

One can notice that before the maximum lactose conversion was reached at 54.1% there was an increase in the concentrations of GOSs, glucose, and galactose. On the other hand, the amounts of GOSs decreased and formation of monosaccharide (glucose

and galactose) increased as lactose conversion further proceeded. Furthermore, immobilized Aspergillus oryzae β-galactosidase showed a maximum GOS yield of 26.2% (w/w) at the lactose conversion of 54.1%. Similar result was reported for the Aspergillus oryzae β-galactosidase immobilized on cotton cloth that exhibited a maximum GOS yield of 26.6% (w/w) at the 50% conversion of lactose (Albayrak and Yang, 2002).

6.3.3. Effects of Lactose Concentrations

The initial lactose concentration in the reaction mixture has been reported by many investigators as the most significant factor affecting GOS formation (Li *et al*., 2008; Boon *et al*., 2000; Albayrak and Yang, 2002; Hsu *et al*., 2007). Figure 6.4 shows the carbohydrate yields of GOSs, glucose, and galactose in the reaction mixture after 8h of catalysis of the reaction by the immobilized β-galactosidase from Aspergillus oryzae.

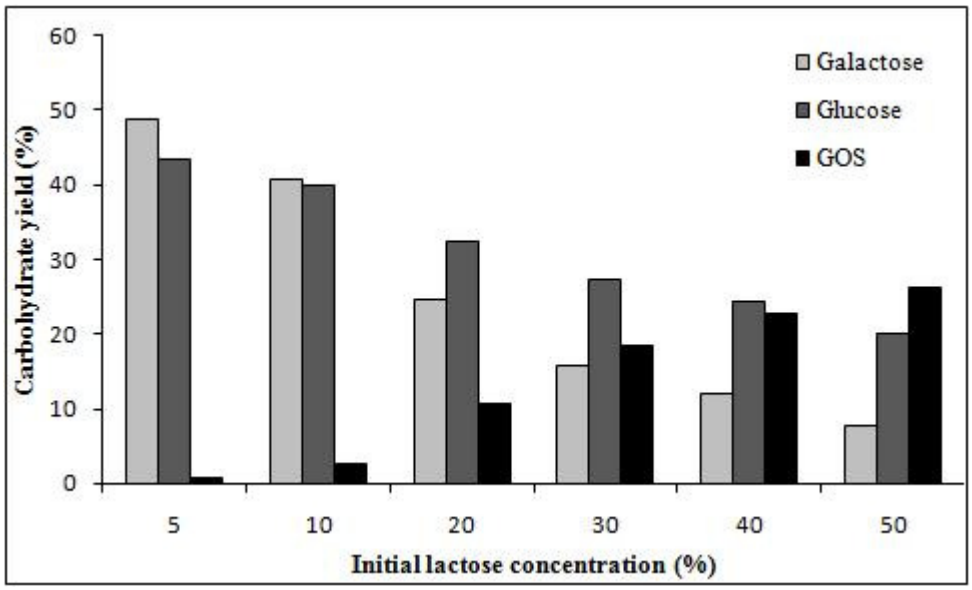


Figure 6.4. Effect of the initial lactose concentration on the GOS production catalyzed by immobilized Aspergillus oryzae β-galactosidase on magnetized DACRON. The reaction was performed at 40 ºC and pH 4.5 for 8 h.

It was found that the production of GOSs increased with increasing initial lactose concentration from 5% to 50%. The maximum GOS content in the product increased from 10.1% (at 34% conversion) to 26.2% (at 54% conversion). The maximum GOS yield from lactose reacted also increased with the initial lactose concentration, from 43% to 64%. As the increased of initial lactose concentration from 5% to 50%, the trisaccharides content doubled (9.4% to 20.7%), while tetrasaccharides increased by

6.9-fold (from 0.9% to 6.2%). It was also noted that the hydrolysis reaction dominates in reaction solutions containing a lactose concentration in the range 5% to 40%, while GOS formation dominated in reaction mixtures having a higher lactose concentration 50% (see Figure 6.4).

6.3.4. Effect of Temperature

Production of GOS by the immobilized enzyme preparation was almost unchanged despite temperatures varying from 30° C to 60° C for the initial concentration of 40% (w/v) lactose, as shown in Figure 6.5. Albayrak and Yang (2002) reported similar results for β-galactosidase from Aspergillus oryzae immobilized on cotton cloth. It is well known that enzymes do not affect equilibrium constant of the reactions.

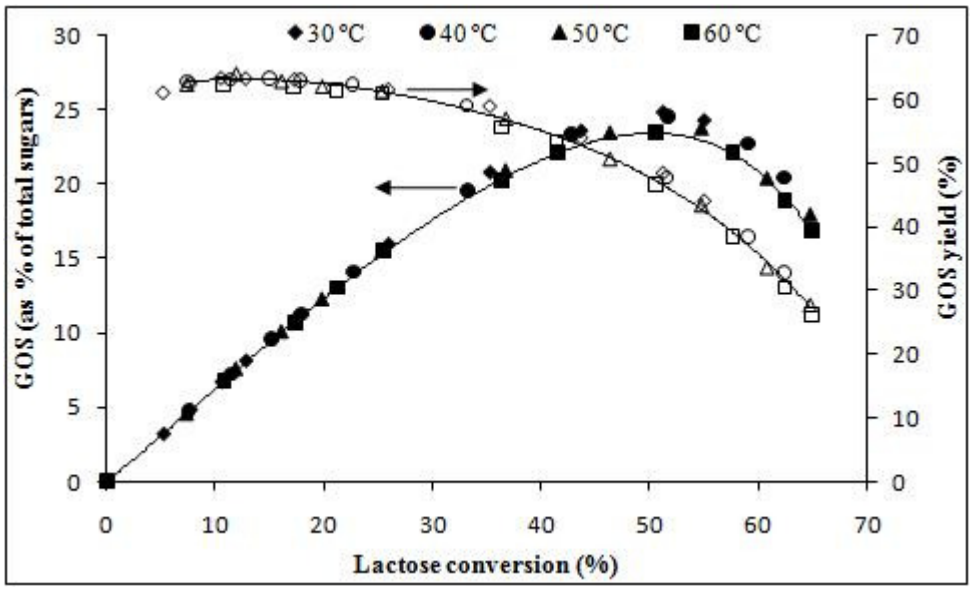


Figure 6.5. Effect of temperature on the GOS production from the hydrolysis of 40% (w/v) lactose catalyzed by immobilized Aspergillus oryzae β-galactosidase on magnetized DACRON. The production is expressed in terms of % total sugar (closed symbols) and yield (opened symbols).

6.3.5. Reuse of magnetized DACRON on lactose hydrolysis

One of the most important advantages of enzyme immobilization on magnetic support it is its easy removal and reutilization (Neri *et al*, 2008). The reuse of immobilized β-galactosidase from Aspergillus oryzae onto magnetized Dacron was

assayed successively up to 10 cycles at 25ºC and lactose (20% w/v) was used as substrate (Figure 6.6). The immobilized enzyme retained 90% of its initial activity after 10 uses as shown in Figure 6.6.

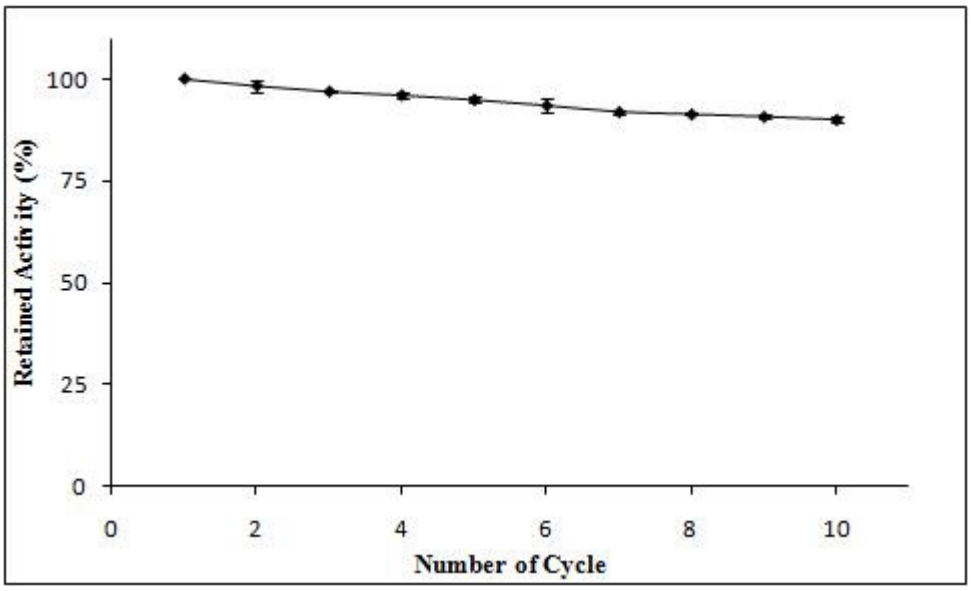


Figure 6.6. The reuse of the immobilized β-galactosidase from Aspergillus oryzae onto magnetized Dacron.

6.3.6. GOS characterization

The ESI-MS spectra obtained for the fractions 3, 4, 5 and 6 showed ions correspondent to the $[M+Na]^+$ ions of the oligosaccharides that eluted in each chromatographic fraction, allowing to identify the molecular weight of each oligosaccharide. The presence of only one ion also allowed confirming that in each peak only one oligosaccharide eluted. In fraction 3, the ESI-MS spectrum showed an ion at m/z 365, correspondent to a hexose disaccharide, while for the fractions 4 and 5 the ESI-MS spectra showed an ion at m/z 527, thus a trimer of hexoses eluted in these peaks and in the case of fraction 6, an ion at m/z 689 was identified permitting the identification of a tetramer of hexose.

ESI-MS/MS experiments were conducted for each ion identified, in order to confirm their composition in monosaccharide units. The results obtained are shown in Figure 6.7. The ion at m/z 365 ($[Hex_2+Na]^+$) of the disaccharide that eluted in fraction 3 fragmented by the loss of an hexose residue (-162 Da), yielding an ion at m/z 203, this latter corresponding to an $[Hex+Na]^+$ ion. The presence of the product ions at m/z 305, 275 and 245 result from the cross ring fragmentation of two glucose units linked by a 1-

6 type linkage, as previously described (Zaia J, 2004). This information is in agreement with the results inferred by the elution pattern, that suggest a different structure between the lactose (eluted in fraction 2), which has two hexose units linked by a 1-,4 linkage, from that of the disaccharide eluting in fraction 3. This latter, according with the MS/MS results, have two hexose units linked by a 1-6 linkage. The ESI-MS/MS spectra of the oligosaccharides with degree of polymerization 3 (those eluted in fractions 4 and 5 showed major product ions formed by loss of one and two hexose unitsof the $[M+Na]^+$ at m/z 527,with formation of the ions at m/z 365 and 203, respectively. This fragmentation pattern allowed concluding that the compounds eluted in these fractions are trisaccharides composed by three hexose units. Also, the fragmentation of the $[M+Na]^+$ of the oligosaccharide that eluted in the fraction 6, (m/z 689) fragmented by the loss of one, two and three hexose units, with formation of a $[Hex+Na]^+$ ion (m/z 203), thusconfirming that this compound is a tretamer of hexose units.

The MS/MS spectra of the trisaccharides present in fractions 4 and 5 were slighly distinct. In this sense, the major fragment ions obtained for the trimmer eluted in fraction 4 (at m/z 467 and m/z 407) were formed by the loss of 60Da ($-C_2H_4O_2$) and 120 Da ($-C_4H_8O_4$) , suggesting the presence of a 1-4 linkage , most probably at the reducing end. The sequential fragmentation (MS^3) of the product ion at m/z 365 ($[Hex_2+Na]^+$) showed product ions due to cross ring cleavages by losses of 60, 90 and 120 Da, indicating that these two monomers are linked by a 1-6 linkage. Together, the MS/MS results from the compound in fraction 4 suggested that this must have a 1-4 and a 1-6 linkage.

Concerning the MS/MS spectrum of the trisaccharide from fraction 5, this showed a small ion at m/z 437 along with abundant ions at m/z 467 and 407. However, the low abundance of the ion at m/z 437 is not enough to justify the presence of a 1-6 linkage in the reducing end. Still, the presence of a 1-6 linkage in this trisaccharide was confirmed by the MS^3 spectra of the product ion at m/z 365 (data not shown). Thus, although the correct assignment of the type of linkages present in the trisaccharide eluted in fraction 5 was not possible by MS/MS, this tecnhique indicated that the two trisaccharides have distinct structures. Moreover, the same approach for the compound eluted in fraction 6 allowed to suggest that this tretamer has a 1-4 (in the reducing end) and two 1-6 linkages.

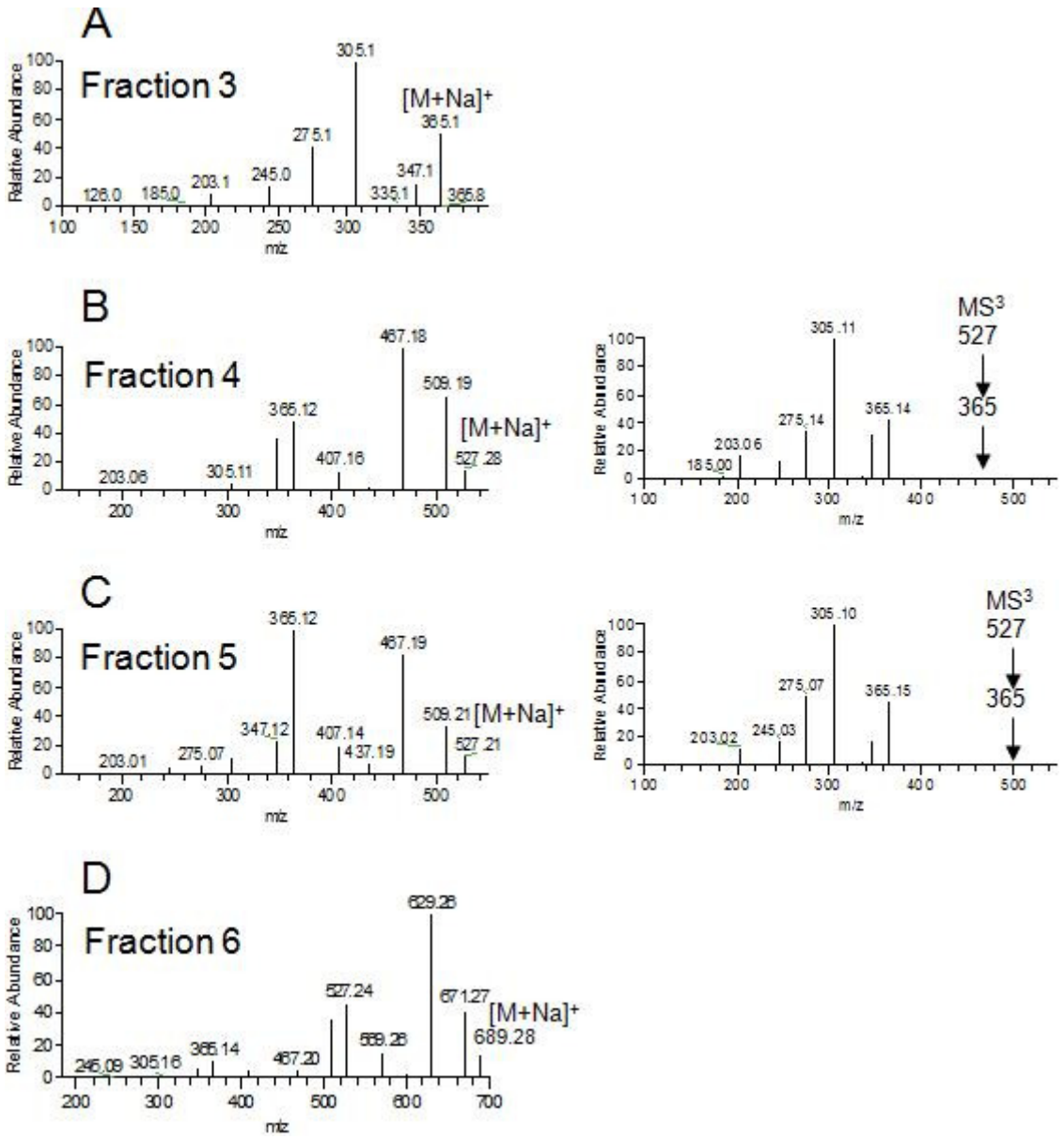


Figure 6.7. ESI-MSn spectra of the oligosaccharide that eluted respectively in (A) fraction 3 (m/z 365), (B) fraction 4 (m/z 527), (C) fraction 5 (m/z 527) and (D) fraction 6 (m/z 689).

NMR analysis of fractions 4, 5 and 6 confirmed the results from the MS analysis and also allowed to determine the detailed structure of the eluted compounds. For each oligosaccharide, the ^{1}C and ^{13}C chemical shifts were assigned by the interpretation of their ^{1}H NMR, ^{13}C NMR, HSQC, COSY and HMBC spectra (data not shown) and further comparison to those of lactose. According to that, compound eluted in fraction 4 showed characteristic ^{1}H and ^{13}C chemical shifts for T-β-Galp ($\delta_{H\text{-}1=}$ 4.47 ppm, J= 8.0, $\delta_{C\text{-}1}$= 106.0 ppm), 4-β-Glcp ($\delta_{H\text{-}1=}$ 4.67 ppm, J= 8.0 Hz, $\delta_{C\text{-}1}$= 98.6 ppm) and 4-α-Glcp monomers ($\delta_{H\text{-}1}$= 5.23 ppm, J= 3.7 Hz, $\delta_{C\text{-}1}$= 94.7 ppm) (Table 1). Also, the remaining

^{13}C resonances in its ^{13}C NMR spectrum at 106.2, 73.9, 75.3, 71.3, 76.9 and 71.9 ppm, as also their corresponding ^{1}H resonances in the ^{1}H NMR spectrum [4.4 (J= 7.9 and 1.6 Hz), 3.56, 3.68, 3.98, 3.95 and 4.08 ppm], indicated the presence of an internal 6-β-Galp-(1→ residue. The coupling constant values of H-1 allowed us to identify the β-configuration of the two galactosyl residues (J≈ 8.0 Hz) and the presence of the two anomers of glucose ($J_{H-1\beta}$= 8.0 Hz, $J_{H-1\alpha}$= 3.7 Hz). Thus, these results allowed concluding that the compound in fraction 4 is a β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-Glc*p*.

As shown in Table 6.1, the C-6 and H-6 resonances from the Glcp sugar units of fraction 5 (70.4 ppm and 4.29/4.22 ppm for the α and β anomers) were deshielded compared to those of lactose (62.8/62.9 ppm and 3.72/ 3.81 and 3.69 ppm), indicating that Glc was disubstituted at O-4 and O-6 (β-D-Gal*p*-(1→4,6)-Glc*p*). In accordance with that, resonances coherent with two T-β-Gal sugar units (δ_{H-1}= 4.51 ppm, J= 7.8 Hz; δ_{H-1}= 4.46 ppm, J= 7.8 and 5.4 Hz) were identified in this compound.

NMR data from fraction 6 indicated the presence of three β- galactosyl residues in which, two of those were linked to the O-6 of a β-Galp 6 monomer and the other was linked trough O-4 to a Glc residue. Due to the close environment of the two internal β-galactosyl residues of the tetrasacharide, most of their chemical shifts were not possible to discriminate. Still, the resonance data for H-1 and C-1 of these two β- galactosyl residues was clearly distinguishable (δ_{H-1}= 4.46 ppm, J= 7.8 Hz; δ_{C-1}= 106.2 ppm for the 6-β-Galp-(1→ sugar unit linked to the O-6 of a β-Galp and δ_{H-1}= 4.47 ppm, J= 7.7 Hz; δ_{C-1}= 106.3 ppm for the 6-β-Galp-(1→ sugar unit linked O-4 to the Glc residue).

In conclusion, the NMR analysis of this fraction indicated that the eluted compound was a β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)Glc*p*.

Table 6.1. ^{1}H and ^{13}C nuclear magnetic resonance data for fractions 4, 5 and 6 in D_2O.

GOS residues		Chemical shifts[a] 1	2	3	4	5	6
Fraction 4							
T-β-Gal*p*[b]	^{1}H	4.47 (J= 8.0 Hz)	3.56	3.66	3.95	3.72	3.78
	^{13}C	106.0	73.7	75.5	71.5	78.0	63.9
6-β-Gal*p*-(1→[c]	^{1}H	4.49 (J= 7.9 and 1.6 Hz)	3.56	3.68	3.98	3.95	4.08
	^{13}C	106.2	73.9	75.3	71.3	76.9	71.9
4-β-Glc*p*	^{1}H	4.67 (J= 8.0 Hz)	3.31	3.66	3.68	3.63	3.97
	^{13}C	98.6	76.6	77.4	82.1	77.6	63.0
4-α-Glc*p*	^{1}H	5.23 (J= 3.7 Hz)	3.60	3.83	3.68	3.97	3.89
	^{13}C	94.7	73.9	74.5	82.3	72.8	62.9
Fraction 5							
T-β-Gal*p*[d]	^{1}H	4.46 (J= 7.8 and 5.4 Hz)	3.56	3.66	3.93	3.72	3.79
	^{13}C	106.0	73.5	75.4	71.6	78.0	63.9
T-β-Gal*p*[c]	^{1}H	4.51 (J= 7.8 Hz)	3.56	3.66	3.93	3.72	3.79
	^{13}C	105.7	73.8	75.6	71.6	78.1	63.9
4,6-β-Glc*p*	^{1}H	4.70 (J= 5.7 Hz)	3.32	3.68	3.80	3.96	4.22
	^{13}C	98.8	76.5	76.6	80.8	77.2	70.4
4,6-α-Glc*p*	^{1}H	5.23 (J= 3.7 Hz)	3.60	3.84	3.77	3.91	4.29
	^{13}C	94.8	74.0	74.2	80.8	73.7	70.4
Fraction 6							
T-β-Gal*p*[b]	^{1}H	4.51 (J= 7.7 Hz)	3.56	3.66	3.97	3.71	3.78
	^{13}C	106.0	73.6	75.7	71.5	78.0	63.9
6-β-Gal*p*-(1→[b,*]	^{1}H	4.46	3.56	3.67	3.97	3.93	4.06
6-β-Gal*p*-(1→[c,*]	^{1}H	4.47 (J= 7.8 Hz)/ (J= 7.7 Hz)	3.56	3.67	3.92	3.91	4.06
	^{13}C	106.2	73.7	75.3	71.3	76.8	72.0
	^{13}C	106.3	73.7	75.4	71.5	76.6	72.1
4-β-Glc*p*	^{1}H	4.68 (J= 8.0 Hz)	3.31	3.66	3.68	3.63	3.96
	^{13}C	98.6	76.6	77.4	82.0	77.6	63.1
4-α-Glc*p*	^{1}H	5.23 (J= 3.7 Hz)	3.61	3.85	3.68	3.97	3.89
	^{13}C	94.7	73.9	74.5	82.3	72.9	62.9

[a] Assignments done in accordance to the data in the ^{1}H NMR, ^{13}C NMR, HSQC, COSY and HMBC spectra; [b] Residue linked to the O-6 of Galp; [c] Residue linked to the O-4 of Glcp; [d] Residue linked to the O-6 of Glcp; * Most of the chemical shifts of these two sugar residues were not possible to discriminate.

6.4. CONCLUSIONS

Based on the reported results one can conclude that an attractive magnetic and immobilized preparation of β-galactosidase can be synthesized by using inexpensive reagents and a simple methodology. For instance, the matrix is the widely employed polyester Dacron. Then *Aspergillus oryzae* β-galactosidase was covalently attached, via glutaraldehyde, to this hydrazide-Dacron-magnetite composite. This magnetic β-

galactosidase preparation showed to be capable to hydrolyze lactose and also to produce galactooligosaccharides. The galactooligosaccharides production showed to increase whereas the initial lactose concentration augmented and did not almost change despite temperatures varied in the range 30° C - 60° C. The immobilized enzyme retained 90% of its initial activity after 10 successive reuses. The NMR spectroscopy and mass spectrometry analysis of the HPLC fractions identified besides glucose and galactose the following galactooligosaccharides: β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-Glc*p*; β-D-Gal*p*-(1→4,6)-Glc*p*; and β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)Glc*p*.

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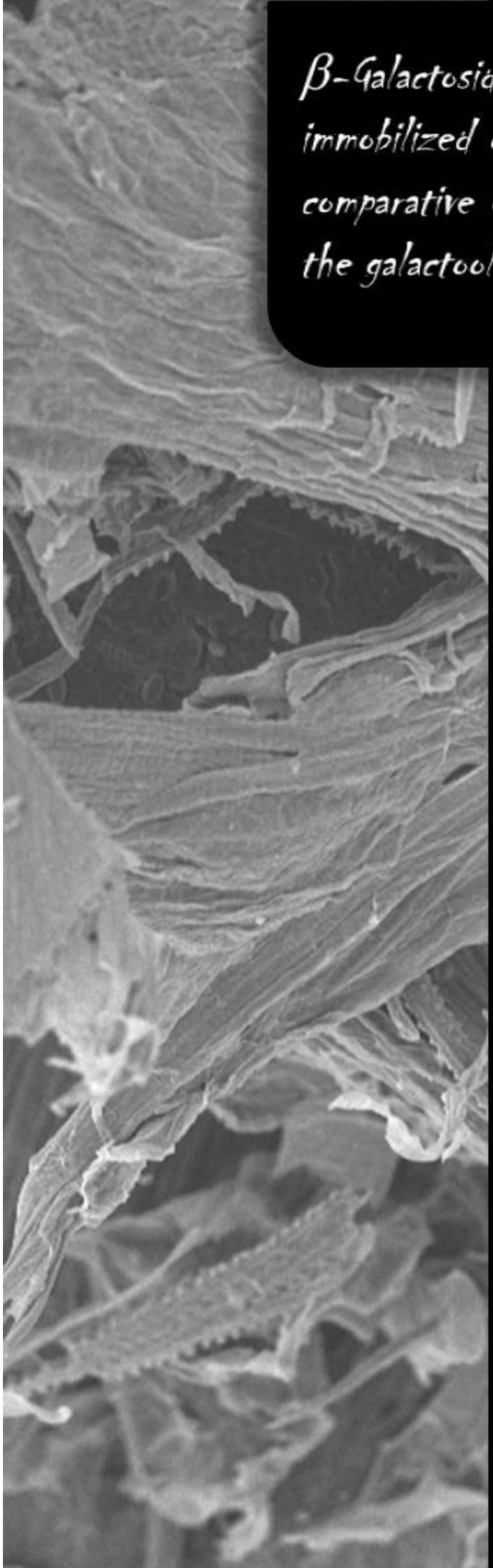

β-Galactosidase from Aspergillus oryzae immobilized onto different magnetic supports: A comparative experimental and modelling study of the galactooligosaccharides production

CHAPTER 7

β-Galactosidase from *Aspergillus oryzae* is an enzyme with a wide industrial application, mostly in the hydrolysis of lactose and, more recently, in the synthesis of oligosaccharides. Several advantages are associated with the application of immobilized enzymes on magnetic supports. In this work, β-galactosidase was covalently immobilised onto a Polysiloxane-Polyvinyl Alcohol Magnetic Composite (mPOS-PVA), Magnetic Polysiloxane with Polyaniline (mPOS-PANI), Magnetized Dacron (DACRON) and Magnetite with Polyaniline (MAG-PANI) using glutaraldehyde as activating agent being the synthesis of GOS evaluated and compared at different temperatures (30, 40, 50 and 60°C) and various initial lactose concentration (50, 100, 200, 300, 400 and 500 g/L). The kinetic parameters obtained by fitting the experimental data were compared in order to determine the effect of the immobilization process with different supports on the synthesis of oligosaccharides. These results clearly demonstrate that all supports may be used for β-galactosidase immobilization as, besides improving the enzyme hydrolytic and GOS synthesis properties, its separation from the obtained reaction products is easy to accomplish.

To be submitted

7.1. INTRODUCTION

During the lactose hydrolysis catalyzed by β-galactosidase GOS are produced by transgalactosylation activity and the production increases by increasing the initial concentrations of lactose in the reaction mixture (Albayrak and Yang, 2002). GOS are nondigestible oligosaccharides, which are prebiotics acting as growth-promoting substrates for bifidobacteria in the human intestine. Enzymes have been utilized in a large number of practical applications, particularly in biomedical and biotechnological fields, through immobilization on a variety of supports (Bayramoglu *et al*., 2008). The applications of immobilized enzymes may be challenged by difficulties that arise in enzyme recovery and recycling, which are the most important processes that can decrease the overall cost of the enzyme immobilization process (Lee *et al*., 2007). The use of magnetic micro-beads as supports for enzyme immobilization is mainly based on the magnetic feature of the solid-phase that enables to achieve a rapid separation in a magnetic field, as well as decrease of operation cost. In addition, the nano-scaled immobilized enzyme can greatly alleviate the transfer barrier, and thus improve the catalytic efficiency (Hong *et al*., 2008). Immobilization of enzyme or biomolecules to the magnetic microbeads is usually accomplished through reactive groups existing on their surface. Nowadays, these functional composites cold be produced in many ways, i.e. emulsion polymerization (Csetneki *et al*., 2004), in situ formation (Li *et al*., 2006) and sol-gel (Luo *et al*., 2006), but usually involve the coating of magnetically susceptible particles with synthetic polymers or biomacromolecules (Hong *et al*., 2008). Actually, the use of magnetic particles in enzyme technology is constantly increasing. Permanent magnetization could cause the particles to aggregate even if the supports are removed from the magnetic field (Neri *et al*., 2008, Bayramoglu *et al*., 2008). In this study, β-galactosidase from *Aspergillus oryzae* was covalently immobilized onto different magnetic supports (mPOS-PANI, DACRON, mPOS-PVA and MAG-PANI) for GOS production. The effects of the temperature, initial lactose concentration and reutilization were investigated. In addition, the kinetic parameters were determined and compared between the four supports using experimental data (10-50% initial lactose concentration).

7.2. MATERIALS AND METHODS

Supports synthesis: mPOS-PVA – beads were synthesized according to the procedure described by Barros *et al.* (2002) and the magnetized particles were obtained by Carneiro-Leão *et al*. (1991); MAG-PANI – magnetite (MAG) was obtained by co-precipitation (Carneiro-Leão *et al*., 1991) and after coated with polyaniline (PANI) as was done by Oliveira *et al*. (2008); mPOS-PANI – POS was prepared polymerizing the tetraethylorthosilicate and after was coated with polyaniline as described in Oliveira *et al*. (2008); DACRON – was converted to magnetic DACRON according to Pinheiro *et al*. (1999).

The immobilization of β-galactosidase was done using glutaraldehyde as activating agent. The supports activated (100 mg) were incubated with 10 mL β-galactosidase solution (4 mg solid/mL) in 20 mM citrate-phosphate buffer, pH 4.5, for 18 h at 4 °C and 20 rpm. Production of GOSs from lactose was studied with immobilized enzyme on various supports in different conditions.

The reaction kinetics was studied at six different initial lactose concentrations (5, 10, 20, 30, 40, and 50 %, w/v and four different temperatures (30, 40, 50, and 60ºC). GOSs, lactose, glucose and galactose concentration were determined using HPLC. As the concentration (w/v) of these sugars is proportional to their peak areas, normalized sugar concentrations as weight percentages of total sugars or initial lactose were determined from peak areas and are reported in this work.

Trisaccharides synthesis and lactose hydrolysis mechanisms including glucose and galactose competitive inhibition was mathematically modelled following four ordinary differential equations:

The lactose hydrolysis (*L*) is given by,

$$\frac{dL}{dt} = \frac{n_{1L}[L][H_2O] + n_{2L}[Tri][H_2O] + n_{3L}[L]^2}{d_1} \quad \text{where,} \quad n_{1L} = \frac{-k_1k_2}{k_4}; n_{2L} = k_2; n_{3L} = \frac{-2k_1k_3}{k_4} \qquad \text{Equation 1}$$

the glucose (*G*) formation by,

$$\frac{dG}{dt} = \frac{n_{1G}[L]^2 + n_{2G}[L][H_2O]}{d_1} \quad \text{where,} \quad n_{1G} = \frac{k_1k_3}{k_4}; n_{2G} = \frac{k_1k_2}{k_4} \qquad \text{Equation 2}$$

and the galactose (*Gal*) by,

$$\frac{dGal}{dt} = \frac{n_{1Gal}[H_2O][L] + n_{2Gal}[Tri][H_2O]}{d_1} \quad \text{where,} \quad n_{1Gal} = \frac{k_1k_2}{k_4}; n_{2Gal} = k_2 \qquad \text{Equation 3}$$

Finally, the trisaccharides production (*Tri*) is described by the equation 4

$$\frac{dTri}{dt} = \frac{n_{1Tri}[Tri][H_2O] + n_{2Tri}[L]^2}{d_1} \qquad \text{with,} \quad n_{1Tri} = -k_2; n_{2Tri} = \frac{k_1 k_3}{k_4} \qquad \text{Equation 4}$$

and $$d_1 = \frac{k_2}{k_4}[H_2O] + \left(\frac{k_3}{k_4} + \frac{k_1}{k_4}\right)[L] + [Tri] + \frac{k_2 k_5}{k_4 k_6}[G][H_2O] + \frac{k_3 k_7}{k_4 k_8}[Gal][L] + \frac{k_2 k_7}{k_4 k_8}[Gal][H_2O] + \frac{k_3 k_5}{k_4 k_6}[G][L]$$

The kinetic model was adapted from the work of Boon *et al.* (1999) and the rate expressions were derived with the King-Altman method (King and Altman, 1956; BioKin, Lda). The model used only lactose as substrate and temperature effect was not included. The parameters were grouped into five parameters (k_1,is the overall reaction rate constant; k_4, describes the reaction of water with the galactosyl-enzyme complex; k_3/k_4, ratio between rate constants oligosaccharides synthesis and hydrolysis of the formed oligosaccharides; k_5/k_6 and k_7/k_8 ,account for glucose and galactose inhibition, respectively) that were estimated and one fixed parameter (k_2, describes the reaction of water with the galactosyl-enzyme complex), which was assumed constant. The estimation of the model parameters was performed by the simulated annealing algorithm of the Systems Biology toolbox for *Matlab* (Mathworks) software (Schmidt and Jirstrand, 2006) using the results of the independent batch experiments, with the sets of experimental data from different initial lactose concentration (10%-50%; w/v) as substrate. All experimental data points were fitted simultaneously for each concentration and support.

7.3. RESULTS AND DISCURSION

7.3.1. Effects of lactose concentration on GOS production by free and immobilized enzyme on different magnetic supports

The percent of total GOS plotted against the percent of lactose conversion at 5% and 50% of initial lactose concentration, 40 ºC and pH 4.5 by different supports and free enzym, is shown in Figure 7.1A. Similar experiments were performed for different initial lactose concentrations (10 to 40%, w/v) and the tri- and the tetra-saccharides were quantified (data not show). GOS (tri- and tetra-saccharides) production increased with

increasing lactose concentration. However, for each lactose concentration the GOS production decreased after a certain degree of lactose conversion. This can be attributed to a preferential hydrolysis (formation of glucose and galactose) rather than GOS synthesis. Furthermore, there was no marked difference between the free and immbilized enzyme performances. As the initial lactose concentration increased from 5% to 50%, the maximum GOS content in the product increased from 11.2% (at 35% conversion) to 26.1% (at 56% conversion) for the free enzyme, from 10.3% (at 38% conversion) to 25.7% (at 49% conversion) for the immobilized enzyme on mPOS-PANI, from 10.1% (at 34% conversion) to 26.2% (at 54% conversion) for the immobilized enzyme on DACRON, from 10.4% (at 30% conversion) to 26.0% (at 55% conversion) for the immobilized enzyme on mPOS-PVA, and from 10.8% (at 33% conversion) to 26.0% (at 52% conversion) for the immobilized enzyme on MAG-PANI. The maximum amount of tri-, tetra-saccharides and total GOS obtained for the free enzyme were 104.5 g L^{-1}, 33.2 g L^{-1} and 130.3 g L^{-1} for a lactose conversion of about 48%, 61% and 56%, respectively, in 500 g L^{-1} of lactose. For the immobilized enzyme, the corresponding values of 103.7 g L^{-1} on DACRON, 31.9 g L^{-1} on MAG-PANI and 131.0 g L^{-1} on DACRON were obtained for about 46%, 59%, and 54% lactose conversion, respectively in 500 g L^{-1} of lactose. These results suggest that enzyme immobilization on different supports does not impose any limitation or changes on GOS formation from lactose. Similar results were obtained with β-galactosidase from *A. oryzae* immobilized on cotton cloth (Albayrak and Yang, 2002). Thus, the GOS formation ability of the enzyme was not affected by the immobilization of the enzyme onto different supports, what is a clear demontration of the absence of mass transfer limitations using mPOS-PANI, DACRON, mPOS-PVA and MAG-PANI as supports.

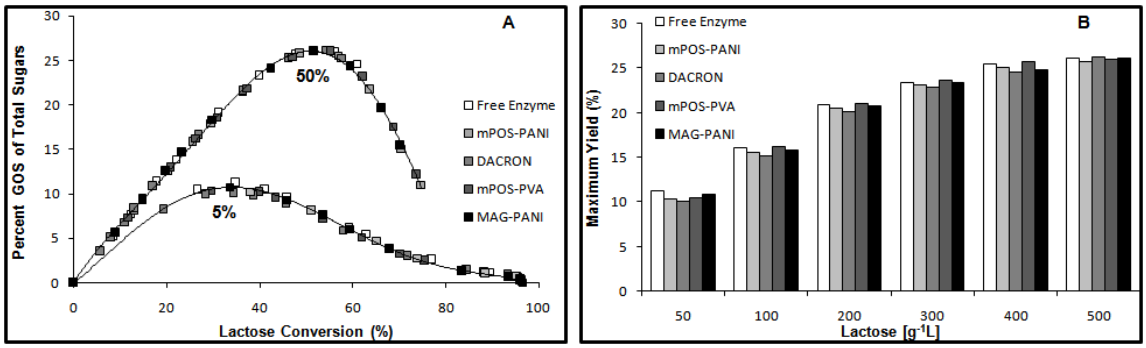

Figure 7.1. Total GOS (A) formation by free and immobilized (closed symbols) *A. oryzae* β-galactosidase on different supports at pH 4.5 and 40° C catalysing the hydrolysis of initial lactose concentrations (5 and 50%, w/v). GOS Yield (B) *versus* lactose concentration by free and immobilized *Aspergillus oryzae* β-galactosidase on different suports.

GOS yields during lactose hydrolysis catalyzed by the free and the immobilized β-galactosidase on different supports are shown in Figure 7.1B. The maximum GOS yield increased with the initial lactose concentration for both enzyme preparations. It was observed that the hydrolysis and transgalactosylation reactions occurred simultaneously and that the product profile of the reaction is largely 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 (Iwasaki *et al*., 1996).An increase was observed up to 300 g L^{-1} of initial lactose concentarion, while for higher lactose concentrations the GOS yield was almost constant at 22.8–26.2% for free and immobilized enzyme on different suports. Park *et al* (2008) observed the same behavior for high lactose concentration with a thermostable β-galactosidase from *Sulfolobus solfataricus*. It is possible that some disaccharides such as allolactose and galactobiose are formed by the enzymatic transfer of galactose to glucose and galactose, respectively. The absence of this side reactions, was confirmed by checking the molar balances on glucose and galactose over the course of the reaction, assuming that all GOS only contained one unit of glucose with galactose as the remaining sugar. It is also possible that different trisaccharides were also formed, but were not detected by HPLC.

Higher operational and thermal stability, making possible its reuse, has been demonstrated to be one of the advantages of this system for enzyme immobilization (Neri *et al*, 2008). An immobilized β-galactosidase on different supports preparation acting on 20% (w/v) lactose was successively reutilized for 10 cycles at 25 ºC and at the end the enzymatic derivative retained approximately 87% for mPOS-PANI, 90% for DACRON, 84% for mPOS-PVA, and 85% for MAG-PANI of its initial activity (see Figure 7.2), confirming the advantages of the magnetic supports β-galactosidase derivative.

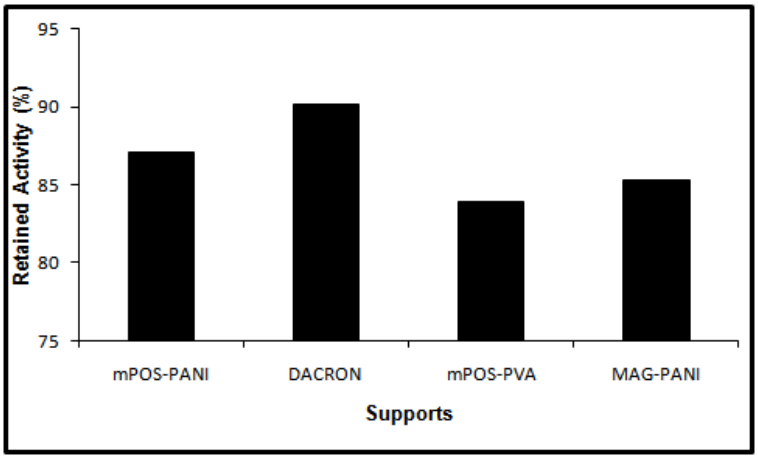

Figure 7.2. Effect on the activity of β-galactosidase immobilized on different supports after 10 reutilization.

7.3.2. Effects of temperature

Temperature normally has a pronounced effect on the enzyme reaction rates but showed to have a minimal effect, if any, on GOS production (Iwasaki *et al*., 1996; Monsan and Paul, 1995; Albayrak and Yang, 2002). As shown in Figure 7.3A and 7.3B, GOS production was almost unchanged despite temperatures varying from 30° C to 60° C (Figure 7.3B) for the conversion of 40% (w/v) lactose, for both the free and immobilized enzyme preparations. Albayrak and Yang (2002) reported similar results for β-galactosidase from *Aspergillus oryzae* immobilized on cotton cloth. It is well known that enzymes do not affect equilibrium constant of the reactions.

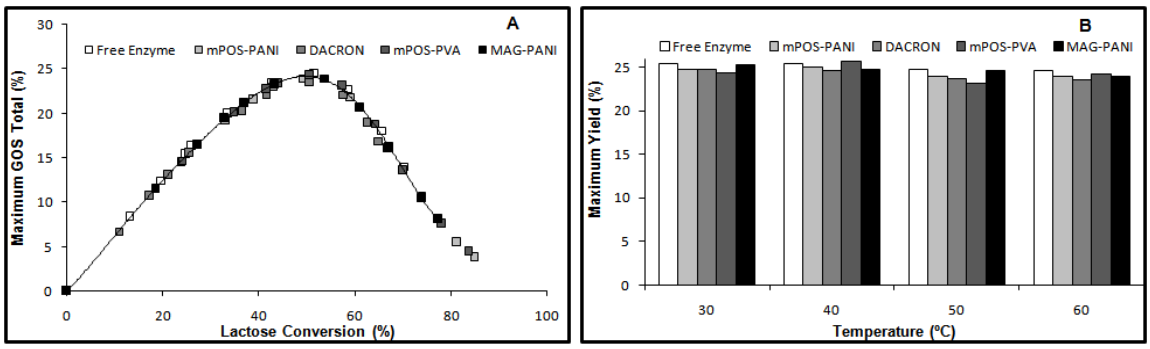

Figure 7.3. Effects of temperature on the GOS production from the hydrolysis of 40% (w/v) lactose catalyzed by the free and immobilized *A .oryzae* β-galactosidase on different supports at 60 ºC (A). GOS Yield (B) on different supports at various temperature.

7.3.3. Kinetic analysis β-galactosidase free and immobilized on different supports

To verify the precondition that the model parameters are independent of the initial lactose concentrations and in this way to use the parameters by taking the average values, the 95% confidence interval of the slope of the linear dependence of the kinetic parameters on initial lactose concentration was estimated (data not shown). For all the simulations runs the average parameters from each initial lactose concentration were used (Table 7.1).

To determine the effect of the different supports on the kinetic parameters, experiments were carried out at various initial lactose concentrations ranging from 10-50% (w/v). Figure 7.4 shows a comparison of the experimental and calculated concentrations profiles with the averaged parameters obtained for the supports at 20% initial lactose concentration. As can be seen, the good quality of the model is remarkable. The model describes well the time course of lactose conversion, glucose and galactose formation and overpredicts slightly the trisaccharides concentration for all the runs.

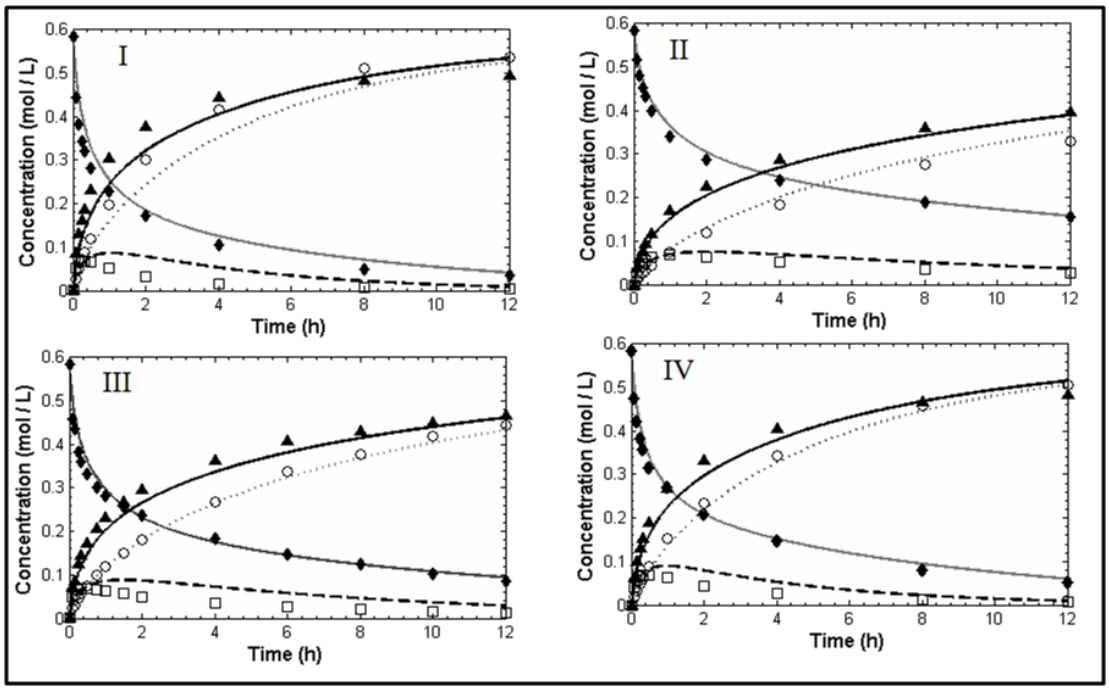

Figure 7.4. Comparison between experimental (symbols) and simulated data (descending gray solid line: lactose; ascending black solid line: glucose; depicted line; galactose and dashed line: trisaccharide) of (♦) lactose, (▲) glucose, (○) galactose and (□) trisaccharides concentration by immobilized β-galactosidase on Support I (mPOS-PANI), II (DACRON), III (mPOS-PVA) and IV (MAG-PANI) at pH 4.5, 40ºC for 20% initial lactose concentration. Lines represent the simulation using the fitted parameters.

The parameter k_3, that regulates the oligosaccharides synthesis and k_4, which regulates the hydrolysis of the formed oligosaccharides are assumed to be key parameters in the overall process. Not surpringly, as can be observed in Table I, the ratio between k_3 and k_4 is of the same order of magnitude for free and immobilized enzyme on different supports, indicating that the relative extent of the synthesis and hydrolysis reactions is not affected by the immobilization procedure. This is in accordance with the obtained experimental data (see Figure 7.1).

Table 7.1. Estimated average values and 95% confidence intervals of parameters for the immobilized at different supports and free β-galactosidase obtained by fitting the experimental data for *A. oryzae* at different initial lactose concentration, pH 4.5 and 40ºC.

Parameter (units)	mPOS-PANI	DACRON	mPOS-PVA	MAG-PANI	Free
k_1 (h^{-1})	1.93 ± 0.93	1.09 ± 0.78	1.87 ± 0.70	1.59 ± 0.76	3.06 ± 2.90
$Log(k_3/k_4)$ (-)	1.50 ± 0.043	1.52 ± 0.029	1.49 ± 0.013	1.50 ± 0.017	1.55 ± 0.22
k_4 (h^{-1})	3.69 ± 2.82	4.83 ± 1.613	4.59 ± 1.37	6.15 ± 2.50	3.45 ± 5.06
$Log(k_5/k_6)$ (-)	1.023 ± 0.22	1.12 ± 0.24	1.015 ± 0.17	1.012 ± 0.46	1.50 ± 0.33
$Log(k_7/k_8)$ (-)	0.75 ± 0.35	1.480 ± 0.13	1.45 ± 0.026	0.85 ± 0.41	1.51 ± 0.21

7.4. CONCLUSIONS

All supports proved to be an adequate support for *Aspergillus oryzae* β-galactosidase immobilization and its use on GOS production using lactose as substrate. The galacto-oligosaccharides formation ability of the enzyme was not affected by its immobilization onto the magnetic supports that can be easily recovered by applying a magnetic field. The galacto-oligosaccharides production was almost unchanged despite temperatures varying from 30^{o} C to 60^{o} C for the free and immobilized enzyme preparations on different supports. The model describes the experimental data well in the range of the initial lactose concentration used. Such kinetic model may be used bio-process engineering studies to increase knowledge of catalytic studies and the most appropriate support for immobilization process can be selected.

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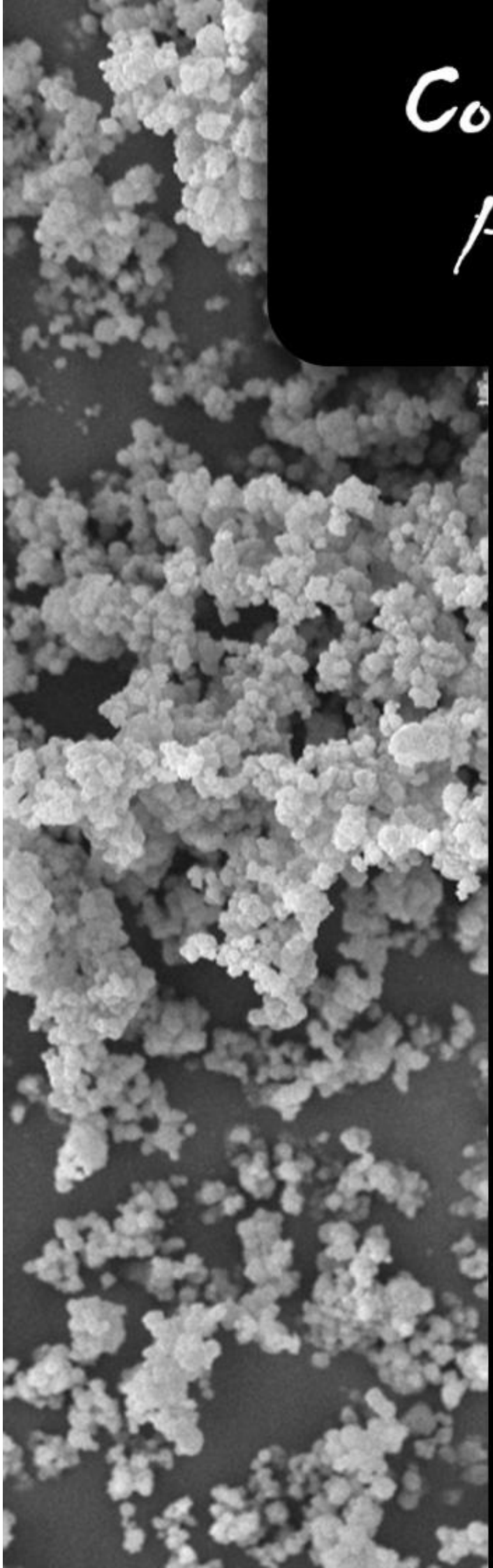

Conclusions and Perspectives

CHAPTER 8

In this last chapter, the main conclusions from this thesis are presented. More details about them can be found at the end of each individual chapter. Based on these results some suggestions are also presented for future works.

O fruto do trabalho é o sucesso e ele é doce.
David Neri

8.1. CONCLUSIONS

The main objective of this PhD thesis was a comparative study of lactose hydrolysis and GOS synthesis by free β-galactosidase and immobilized β-galactosidase derivatives synthesized onto four different magnetic matrices: (1) polyvinyl alcohol polysiloxane-magnetic - mPOS-PVA; (2) magnetite coated with polyaniline - MAG-PANI; (3) polysiloxane coated with polyaniline - POS-PANI and (4) ferromagnetic Dacron. This study sought to characterize the GOS synthesis reaction kinetics and to define the optimal GOS production conditions. To achieve these goals, many issues have been studied and various strategies have been implemented successfully.

The main conclusions achieved from this work are displayed below:

- β-galactosidase from two different origins (*Kluyveromyces lactis* and *Aspergillus oryzae*) was successively immobilized onto polyvinyl alcohol polysiloxane-magnetic.
- All four magnetic tested matrices proved to be attractive, efficient and inexpensive for β-galactosidase immobilization due to the following arguments: the simplicity of the matrices synthesis and immobilization protocol; the easy removal from the reaction medium by simply applying a magnetic field on the reactor; to be reused and the capability to catalyze the hydrolysis lactose into glucose and galactose and yielding galactooligosaccharides as well.
- The galacto-oligosaccharides formation ability of the enzyme was not affected by the immobilization onto the four tested magnetic matrices.
- The production of galacto-oligosaccharides by the different immobilized enzyme preparations did not change inside the investigated temperature range ($30^{o}C$–$60^{o}C$).
- It was possible to obtain an isomer of lactose, two, tri and tetrasaccharides; however, trisaccharides were produced in larger quantities.
- The addition of galactose either alone or mixed to the decreased glucose and lactose hydrolysis and galactooligosaccharides formation.

- The proposed mathematical model fitted the experimental data in the range of used initial lactose concentrations and may be used as the basis for choose the most appropriated immobilization protocol and matrix.

- The ability of conversion of lactose into glucose and galactose and the production of galacto-oligosaccharides by the immobilized enzyme in the different magnetic tested matrices do not differ from the free enzyme.

- The four investigated magnetic enzymatic derivatives retained activities higher than 84% of that initially estimated after 10 reuses using lactose as substrate.

- The sugars formed by the enzyme immobilized onto magnetic Dacron were characterized by Mass spectrometry and Nuclear Magnetic Resonance Spectroscopy.

- The properties of some magnetic matrices were characterized by X-ray diffraction, vibrating sample magnetometer and thermomagnetization.

- The elemental analysis and infrared spectrum of magnetite coated with polyaniline (MAG-PANI) and polysiloxane coated with Polyaniline (POS-PANI) proved that their surfaces were coated with polyaniline.

- It was possible to visualize the size and morphology of the matrices through the technique of Scanning Electronic Microscopy.

8.2. PERSPECTIVES

The results in this PhD thesis provide some interesting perspectives on the use of magnetic matrices in the biomolecules immobilization rather β-galactosidase. The recent study on the use of them confers many aspects to be explored, finding solutions to reduce operating costs and thereby increasing the options for application of enzyme technology in the food industries.

Some suggestions for future work are presented below:

- To use the magnetic β-galactosidase derivatives either in the lactose hydrolysis or in the galacto-oligosaccharides production on a continual basis using a reactor adapted with a magnetic field;
- To immobilize β-galactosidase from other sources looking for a more stable and more efficient catalyst in order to obtain a higher degree of hydrolysis and synthesis of GOS;
- To compare the efficiency of the investigated magnetic matrices with other magnetic matrices or without magnetic properties;
- To Increase the surface area of immobilization by using nanoparticles of the magnetic matrices;
- To attempt the immobilization of dead yeast cells containing β-galactosidase on the membrane surface onto the magnetic matrices, in order to reduce the time and cost of enzyme obtaining and purification and
- To test the immobilization of other enzymes on them and compare with those already described in the literature.