# Synthesis of *galacto-oligosaccharides* with *β-galactosidases*

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# Contents

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# Chapter 1

Introduction

1

## Functions of oligosaccharides

Many oligosaccharides, among which galacto-oligosaccharides (GOS), are regarded as prebiotics (Playne and Crittenden 2009). Prebiotics are food ingredients that can be selectively fermented by the intestinal microbiota and change the composition and/or activity of the gastro-intestinal microbiota to the benefit of the host's well-being and health (ISAPP 2012; Roberfroid et al. 2010). Prebiotic oligosaccharides are not digested in the upper part of the gastro-intestinal tract and reach the colon intact. Here, they stimulate the growth of beneficial bacteria, like bifidobacteria and lactobacilli, and inhibit the growth of harmful bacteria (Crittenden and Playne 1996; Macfarlane et al. 2008). Prebiotic effects include improved intestinal health, reduction in serum cholesterol, improved liver function, and anticarcinogenic effects (Blaut 2002; Crittenden and Playne 1996; Macfarlane et al. 2008; Mahoney 1998).

In human milk, relatively high concentrations of lactose-derived oligosaccharides are naturally present (the so-called human milk oligosaccharides (HMO)). These HMO seem to play an important role in the development of the intestinal microbiota and the immune system of infants. They have a protective role against infections and possibly allergy development (Macfarlane et al. 2008; Obihara et al. 2005; Playne and Crittenden 2009). Although GOS are not identical to HMO, they have structural and functional similarities (Crittenden and Playne 1996; Macfarlane et al. 2008) that make them a suitable ingredient to mimic HMO in infant nutrition.

## Market for GOS

GOS are currently used in a wide range of products, such as infant formulas, dairy products, sauces, soups, breakfast cereals, beverages, snack bars, ice creams, bakery products, animal feeds, and sugar replacements (Crittenden and Playne 1996; Macfarlane et al. 2008). GOS have various properties that make them suitable as ingredient in various commercial products. GOS preparations are usually transparent and have a high solubility. They have a low caloric value, but have about one third of the sweetness of sucrose. GOS have a low potential for the development of dental caries. Besides, they are not sensitive to high temperatures in acidic environments. These properties make GOS even suitable for acidic beverages and dairy products (Macfarlane et al. 2008).

Only few data is reported on the amount of GOS that is produced. In Japan the total production of oligosaccharides (including fructo-oligosaccharides and soybean oligosaccharides) reached 18,000 tons already in 1990 (Mahoney 1998). In 1995, the global production of GOS was estimated to be 15,000 tons (Crittenden and Playne

1996). Currently, the production of lactose-derived oligosaccharides (GOS, lactulose, and lactosucrose) is estimated to be 20,000 – 32,000 tons (Gänzle 2012). These numbers on production are difficult to compare since different categories of oligosaccharides were reported, however, they do indicate that the production of GOS has increased strongly over the last two decades.

## GOS production

Lactose, present in whey, used to be a by-product from cheese making (Macfarlane et al. 2008). Nowadays, lactose is a valuable component, as raw material for GOS. GOS are usually produced in a batch process with free β-galactosidase at high initial lactose concentrations and at high temperatures. An advantage of synthesis at high temperatures is the improved solubility of the substrates which makes higher substrate concentrations possible (Bruins et al. 2001) and results in a larger yield (Monsan et al. 1989). However, the thermal inactivation of the enzyme is faster as well (Bruins et al. 2003).

Typically, the enzymatic synthesis of GOS over time exhibits a maximum level (depending on the conditions) before hydrolysis dominates and smaller oligosaccharides, disaccharides and monosaccharides are produced. The actual amount of oligosaccharides at any time depends on the relative rates of synthesis and hydrolysis (Mahoney 1998). The final reaction mixture typically consists of galacto-oligosaccharides, lactose, glucose and a small amount of galactose. The presence of lactose in the final product is a disadvantage for consumers that suffer from lactose intolerance, while the presence of galactose and glucose inhibit the enzyme: galactose and glucose are often found to be inhibitors for β-galactosidases, dependent on their concentrations and the origin of the enzyme (Bakken et al. 1992; Boon et al. 1999; Greenberg and Mahoney 1982; Kim et al. 2004; Prenosil et al. 1987).

After the maximum level of GOS is obtained, the enzyme is inactivated and removed by filtration from the GOS mixture. In the further downstream processing, evaporation or drying might take place to obtain a concentrated or dry product (FDA 2007; Playne and Crittenden 2009).

## Molecular structure of GOS

The molecular structures of GOS may vary. GOS are oligosaccharides that generally consist of one glucose molecule and one to nine galactose molecules. Some definitions of GOS regard disaccharides consisting of glucose and galactose that are not digested by the human body as GOS, while other definitions of GOS restrict themselves to

oligosaccharides with a degree of polymerization (DP) of three and higher. (Barreteau et al. 2006; Crittenden and Playne 1996; Mahoney 1998; Playne and Crittenden 2009) GOS are in this thesis defined as oligosaccharides with a degree of polymerization (DP) of three and higher.

Besides variations in the degree of polymerization, GOS vary in regio-chemistry and in composition (Gosling et al. 2010). Between the galactose and glucose at the reducing end, β(1,2), β(1,3), β(1,4) and β(1,6) linkages have been identified and branched glucose residues occur. The other galactose residues are usually attached via (1,4) and (1,6) linkages. The enzymes and conditions used during GOS production determine the glycosidic linkages in the final product (Asp et al. 1980; Onishi et al. 1995; Toba et al. 1985; Yanahira et al. 1995).

## Enzymatic synthesis of GOS

GOS are usually produced from lactose via enzymatic synthesis with β-galactosidases (Barreteau et al. 2006; Mahoney 1998; Playne and Crittenden 2009; Prenosil et al. 1987). β-Galactosidases are systematically called β-D-galactoside galactohydrolases and are classified in the class of glycoside hydrolases, EC.3.2.1.23 (CAZY 2012). They catalyze the hydrolysis of the terminal non-reducing β-D-galactose residues in β-D-galactosides via a retaining mechanism. The β-galactosidases are divided over four glycoside hydrolase families: GH 1, 2, 35, and 42.

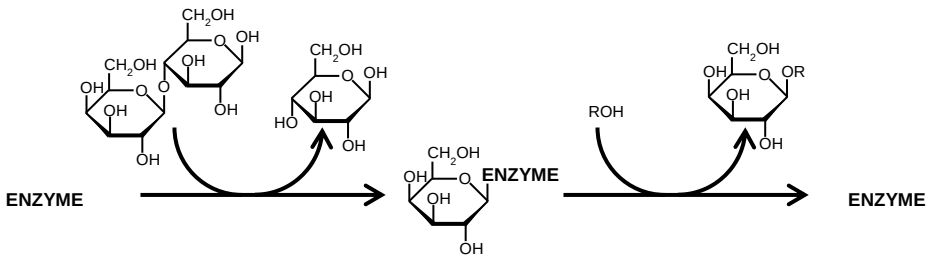


**Figure 1.** Reaction mechanism of the conversion of lactose with β-galactosidases.

Figure 1 shows the conversion of lactose into GOS with β-galactosidases. The first step is docking of the lactose molecule into the active site of the enzyme (Gosling et al. 2010). The enzyme catalyzes the hydrolysis of the β(1-4) linkage of lactose (galactosyl β(1-4) glucose) (Park and Oh 2010). Glucose is released and a covalent bond is formed between the galactosyl moiety and the enzyme (Gosling et al. 2010): an enzyme-galactose complex is formed. In the second step, the galactosyl moiety is transferred to the hydroxyl group of an acceptor molecule (Gosling et al. 2010). If this acceptor

molecule is water, hydrolysis takes place and galactose is released. If the acceptor molecule is another carbohydrate molecule present in the solution, transgalactosylation takes place and oligosaccharides are formed. This implies that a trisaccharide is formed if lactose acts as an acceptor molecule, and that a tetrasaccharide is formed if the trisaccharide acts as an acceptor molecule. In this way, GOS up to approximately a DP of ten can be synthesized.(Crittenden and Playne 1996; Prenosil et al. 1987) The formed products in turn can be used as substrate for the enzyme or can be hydrolyzed again (Mahoney 1998). The various linkages can be formed due to the transfer of galactose to free glucose, or due to internal rearrangement of galactose from the 4' position to the 6' position (e.g. in case of allolactose) of the glucose molecule, without first releasing the glucose from the active site (Huber et al. 1976).

The amounts and types of oligosaccharides are dependent on several factors, such as the initial lactose concentration, temperature, time of harvesting, and enzyme source (Gosling et al. 2010; Mahoney 1998; Prenosil et al. 1987). It is well known that β-galactosidases from different sources have different selectivities for water and other acceptor molecules (Gosling et al. 2010; Prenosil et al. 1987). The β-galactosidases from *Bacillus circulans* are known to have a higher productivity of GOS and to produce oligosaccharides with a higher degree of polymerization compared to β-galactosidases from *Aspergillus oryzae* and *Kluyveromyces lactis* (Boon et al. 2000; Urrutia et al. 2013). The differences in GOS yields and GOS structures are most likely a result of structural and/or mechanistic differences among β-galactosidases from different sources (Gosling et al. 2010). Typical GOS yields are in between 30-40% (w/w) of the initial lactose content (Gosling et al. 2010; Mozaffar et al. 1986; Onishi and Tanaka 1995; Otieno 2010; Palai et al. 2012; Splechtna et al. 2006), but incidentally yields of over 50% have been reported (Park et al. 2008). Although there are many studies on β-galactosidases and their GOS production, it is still a challenge to sufficiently understand the structure and activity of β-galactosidases to increase the efficiency of transgalactosylation (Gosling et al. 2010) due to many variations in the reaction conditions in these studies: the limiting factors in very concentrated media (as used in industrial practice) are very different from those under more dilute conditions (as often used in the scientific literature).

The time of GOS harvesting strongly determines the GOS yield and the composition of the GOS mixture, because GOS are simultaneously synthesized and hydrolyzed by β-galactosidases (Gosling et al. 2010). The maximum concentration of GOS is determined by the ratio between the rate of GOS synthesis and the rate of GOS hydrolysis. For example, Yanahira et al. (1995) reported that the yield of 4-galactosyllactose decreased from 95 to 35% in between 1 and 24 hours of reaction with β-galactosidase from

*B.circulans*, whereas other trisaccharides increased significantly within the same time range.

The initial lactose concentration may affect both rates: it increases the availability of acceptor molecules, which may increase the rate of GOS synthesis; and it decreases the availability of water molecules, which may decrease the rate of both GOS hydrolysis and lactose hydrolysis (Monsan et al. 1989). A higher reaction temperature increases the solubility of lactose, which enables the use of a higher lactose concentration. However, also independently of the increased lactose concentration, a higher temperature may enhance the GOS yield.

In general, the degree of polymerization and the type of linkages in GOS have been found to be controlled by the source of enzyme used and its specific mechanism (Gosling et al. 2010). Linkages between monosaccharides in the mixtures are formed and hydrolyzed at different rates that are specific for the enzyme source.

## β-Galactosidases from *Bacillus circulans*

Previous to the work described in this thesis, the β-galactosidase preparations from *Bacillus circulans* Biolacta N5 were known to consist of multiple β-galactosidase isoforms (Mozaffar et al. 1984; Vetere and Paoletti 1998).

Mozaffar et al. (1984) described the purification of two β-galactosidases. They identified two β-galactosidases that differed in their ratio between hydrolysis and transgalactosylation activity; β-galactosidase-2 showed substantially higher GOS yields than β-galactosidase-1. More than a decade later, Vetere and Paoletti (1998) identified three β-galactosidases in the enzyme preparation from *Bacillus circulans* with molecular weights of 212, 145, and 86 kDa. Each β-galactosidase was found to have a different set of $K_m$ and $v_{max}$ values, and temperature and pH optima and stable ranges. Vetere and Paoletti (1998) did not study the oligosaccharide production of the three isoforms. Recently, Song et al. (2011) identified four different β-galactosidases with molecular weights of 189, 154, 135, and 92 kDa, which were defined as β-gal-A, β-gal-B, β-gal-C, and β-gal-D, respectively. The largest β-galactosidase, β-gal-A, is thought to be truncated by protease activity into the smaller β-galactosidase isoforms, namely β-gal-B, β-gal-C, and β-gal-D. Although the isoforms have the same precursor, the isoforms diverge in their activity (Song et al. 2011). At low initial lactose concentrations, β-gal-A mainly hydrolyses lactose and produces only a small amount of trisaccharides, whereas β-gal-B, β-gal-C, and β-gal-D produce high amounts of tri- and tetrasaccharides (Song et al. 2011). Gosling et al. (2009) reported higher GOS yields and lower galactose formation at rather low lactose concentrations after heat treatment of the total enzyme

preparation, which was presumed to be caused by selective inactivation of the isoform responsible for hydrolysis. These findings suggests that β-gal-B, β-gal-C, and β-gal-D have a higher potential for GOS synthesis, and/or a lower potential for GOS hydrolysis, than β-gal-A and that removal/inactivation of the latter isoform might lead to a higher GOS yield. However, no data on GOS synthesis at high initial lactose concentrations, at which industrial GOS production typically takes place, are available.

## Concentrated systems

Many industrial processes, like the production of GOS, use highly concentrated solutions, because higher yields can be obtained. Besides, concentrated systems are much more sustainable than regular systems, since much less water has to be removed in the final ingredient preparation.

Biochemical reactions, like enzymatic conversions, are usually studied in diluted systems, whereas these reactions naturally occur in the living cell where the total concentration of components is much higher. Biochemical reactions are affected by the total concentration of intracellular components. This is called molecular crowding (Ellis 2001; Minton 2001). The dissolved molecules occupy a certain volume which is physically unavailable for other molecules. The smallest distance between the center of two molecules is equal to the sum of their radii (Minton 2001; van Boekel 2009). The volume that cannot be occupied by (the center of) other molecules is called the excluded volume (Chebotareva et al. 2004; Minton 2001; Zhou et al. 2008; Zimmerman and Minton 1993). Molecular crowding can have an effect on reaction rates, equilibria, enzyme activity and stability (Chebotareva et al. 2004; Elcock ; Minton 2001; Schnell and Turner 2004; Zhou et al. 2008; Zimmerman and Minton 1993). Crowding in general enhances the stability of folded proteins. This is important for the stability of enzymes: the denaturation temperature is higher under crowded conditions (Chebotareva et al. 2004; Schnell and Turner 2004; van Boekel 2009; Zhou et al. 2008). As a result, enzymes are stable for a longer period of time or they can be used at higher reaction temperatures. At higher reaction temperatures the enzyme may be more active, which results in a higher yield. In addition, association of macromolecules is favored under crowded conditions (Chebotareva et al. 2004; Schnell and Turner 2004; van Boekel 2009; Zhou et al. 2008; Zimmerman and Minton 1993): the association of macromolecules results in a reduction in volume and this is thermodynamically favorable in a crowded environment. If the volume of the enzyme-substrate complex is smaller than the total volume of the (dissociated) enzyme and substrate, molecular crowding will enhance the formation of the enzyme-substrate complex. This results in a

lower Michaelis-Menten constant $K_m$ (Schnell and Turner 2004; van Boekel 2009; Zhou et al. 2008), while $v_{max}$ will be enhanced in a crowded environment if the formation of the activated enzyme-substrate complex goes along with a volume decrease (van Boekel 2009). On the other hand, if the activated enzyme-substrate complex has a larger volume, $v_{max}$ will decrease (van Boekel 2009). Besides these crowding effects on the stability and activity of the enzyme, there is also the effect of crowding on diffusion, which is relevant for the enzyme-substrate encounter rate: the more crowded, the slower diffusion becomes (Chebotareva et al. 2004; Minton 2001; van Boekel 2009; Zhou et al. 2008; Zimmerman and Minton 1993).

In addition to molecular crowding, other interactions, like side reactions and inhibition, may play a role in concentrated systems. It is important that the reactions are studied at those conditions that are relevant to their use. In addition, we may expect that the enzymes have evolved to be optimally active under natural conditions (such as molecular crowding). These aspects should therefore be included in a study towards the enzymatic synthesis of GOS with β-galactosidase from *Bacillus circulans*.

## Enzyme immobilization

While in current industrial practice the enzyme is used free in solution during GOS production, many enzymatic reactions are carried out with enzymes that are immobilized into the system. One of the prime advantages of enzyme immobilization is that the reaction mixture can be easily separated from the enzyme without inactivating the enzyme. Thus, the enzyme can be re-used with fresh reaction mixture, leading to a more effective use of the enzyme.

There are three fundamental ways to immobilize an enzyme. The first one is by using a separation method, like ultrafiltration, by which the enzyme is retained in the system (Nakkharat et al. 2006; Pakizeh and Namvar-Mahboub 2011). In this way, one can construct a system in which the enzyme is immobilized into the reactor, while still being in solution, allowing for a continuous process. A disadvantage of such a system is the fact that the membrane will become fouled over time, and that most of the enzyme will accumulate in the gel layer on top of the membrane. This will hinder the reaction seriously.

A second way of immobilization is by trapping the enzyme in a matrix that still allows the enzyme to be active (Panesar et al. 2006; Sheldon 2007). An advantage of this method is that the enzyme is not chemically modified, nor adsorbed on a surface which may influence its configuration. However, a disadvantage is that the reactants have to diffuse through the matrix towards the enzyme, and the products need to diffuse from the

enzyme. This method is therefore not suited for reactions that are influenced by mass transfer limitation, such as with the synthesis of GOS from lactose.

The most often applied way of immobilizing enzymes is by pinning the enzyme on a solid surface. This can either be done by physical adsorption on a surface that has affinity to the enzyme, or by creation of a covalent bond between the enzyme and the surface. Physical adsorption, such as hydrophobic interactions, has the advantage that it is simple to carry out and it has little influence on the conformation of the enzyme (Panesar et al. 2010). On the other hand, the physical interaction with the surface is very unspecific, which often leads either to desorption of the enzyme during the process, or to denaturation of the enzyme, due to unfolding onto the surface, in case the interaction is too strong (Sheldon 2007).

With covalent binding, a reactive surface is brought into contact with a solution of the enzyme, and a reaction takes place. This technique has the advantage that no leaching of enzyme from the surface occurs (Sheldon 2007). However, the technique should be optimized so as not to change the conformational flexibility of the enzyme and to lose enzyme activity (Panesar et al. 2006). An added advantage is that aspecific adsorption of other components can be minimized. While many surfaces are suited for immobilization, typically porous beads are used. These beads allow for a packed bed of beads to be used as reactor, which is an efficient type of reactor for reactions that take some time.

In this thesis, the last method of immobilization is explored, since the other approaches are not feasible for the current reaction system. The work on immobilization in this thesis is not meant to be an exhaustive study into immobilization, as the emphasis of the thesis is on the reaction system itself; however it does show the potential of using immobilized enzymes for this type of reaction system that is conventionally done with enzymes free in solution.

## Aim of this thesis

The aim of this thesis is to better understand the mechanism of GOS production with Biolacta N5, and to learn why this enzyme preparation performs so much better in GOS production than β-galactosidases from other sources. Since Biolacta N5 consists of four β-galactosidase isoforms, we aim for more knowledge on the activities of the individual isoforms present in Biolacta N5. Focus is laid on a better understanding of β-galactosidases in highly concentrated systems. The obtained insight could ultimately be used to improve the GOS production from lactose with Biolacta N5 by constructing a process that results in a higher oligosaccharide yield and a lower lactose content. In

addition, an alternative process form such as the use of immobilized enzyme might help to get a higher enzymatic productivity by reusing the enzyme.

## Outline of this thesis

This thesis deals with the synthesis of GOS using mainly the β-galactosidase preparation from *Bacillus circulans*, Biolacta N5. Chapter 2 describes the characterization of the four β-galactosidase isoforms present in Biolacta N5 by considering kinetic and thermodynamic aspects. A method to purify the isoforms from Biolacta N5 is described and the four isoforms were used to convert lactose. Special attention is paid to the carbohydrate production at high initial lactose concentrations and to the thermodynamic properties of the conversion of lactose and some of its products (allolactose and 4-galactosyllactose) by using isothermal titration calorimetry.

Chapter 3 describes the effect of inert crowders and carbohydrate reactants on the behavior of Biolacta N5 during *o*NPG conversion over broad concentration ranges. Besides, the addition of reactants was studied for β-galactosidases from *A.oryzae* and *K.lactis* for better mechanistic understanding of the activity of various β-galactosidases and for identification of the reason why Biolacta N5 acts better in GOS production.

In chapter 4, the effect of the reactants on the four β-galactosidase isoforms of Biolacta N5 was investigated and a model was designed to describe the influence of the reactants on the *o*NPG converting activity and to predict the transgalactosylation and hydrolysis by the four isoforms under various conditions.

In chapter 5 of this thesis, the stability of Biolacta N5 was studied at various temperatures, in buffer and in systems with initially 5.0 and 30% (w/w) lactose. The enzyme activity was measured with the oNPG activity assay that was corrected for the presence of carbohydrates in the GOS mixture.

Chapter 6 compares the productivities of two GOS production processes to investigate whether a packed bed reactor (PBR) with immobilized enzyme can be an alternative for GOS production in a batch reactor with free enzyme.

The work of this thesis is further discussed in chapter 7. Based on the results, an outlook on various options for process optimization is given. Changes in the enzyme composition and in the substrate composition are considered. In addition, the sustainability of GOS production in a batch reactor with free enzyme and in a PBR with immobilized enzyme is discussed based on an exergy analysis.

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# Chapter 2

# Characterization of β-galactosidase isoforms from *Bacillus circulans* and their contribution to GOS production

2


## Abstract

A β-galactosidase preparation from *Bacillus circulans* consists of four isoforms called β-gal-A, β-gal-B, β-gal-C, and β-gal-D. These isoforms differ in lactose hydrolysis and galacto-oligosaccharide (GOS) synthesis at low substrate concentrations. For this reason, using a selection of the isoforms may be relevant for GOS production, which is typically done at high substrate concentrations. At initial lactose concentrations in between 0.44 and 0.68% (w/w), β-Gal-A showed the least oligosaccharide formation, followed by β-gal-B and β-gal-C; most oligosaccharides were formed by β-gal-D. The differences in behavior were confirmed by studying the thermodynamics of lactose conversion with isothermal titration calorimetry since especially β-gal-A showed a different profile than the other isoforms. Also during the conversion of allolactose and 4-galactosyllactose at 0.44 and 0.61% (w/w), respectively, β-gal-A and β-gal-D showed clear differences.

In contrast to above findings, the selectivity of the isoforms did hardly differ at an initial lactose concentration of 30% (w/w), except for a slightly higher production of galactose with β-gal-A. These differences were hypothesized to be related to the different accessibility of the active sites of the isoforms for different-sized reactants. The initial GOS formation rates of the isoforms indicate that β-gal-A and β-gal-B are the best isoforms for GOS production at high lactose concentrations.



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## Introduction

A β-galactosidase preparation from *Bacillus circulans*, Biolacta N5, which is known to produce high galacto-oligosaccharide (GOS) levels from lactose (Boon et al. 2000; Nakanishi et al. 1983) consists of several β-galactosidase isoforms and a protease (Mozaffar et al. 1984; Song et al. 2011; Vetere and Paoletti 1998). The latest study, by Song et al. (2011), identified four different β-galactosidases, which were defined as β-gal-A, β-gal-B, β-gal-C, and β-gal-D. These isoforms vary slightly in structure as well as in activity. β-Gal-B, β-gal-C, and β-gal-D all share the same precursor, namely β-gal-A. The isoforms are thought to be truncated trough protease activities into the shorter amino acid sequences that are still active (Song et al. 2011). Their molecular weights are 189, 154, 135, and 92 kDa, respectively (Song et al. 2011).

With the decreasing molecular weight of the enzymes, the activity of the enzyme changes. At an initial lactose concentration of 5%, β-gal-A has mainly hydrolytic activity and produces only a small amount of trisaccharides, whereas β-gal-B, β-gal-C, and β-gal-D produce large amounts of tri- and tetrasaccharides (Song et al. 2011). This suggests that the latter three β-galactosidases have a higher potential for GOS synthesis than the former one.

To further characterize the isoforms, we used isothermal titration calorimetry (ITC) in this study. ITC is a highly advanced and sensitive technique (Haq 2011; Todd and Gomez 2001) and only small amounts of reactants are required. Besides determining the binding constants, ITC is used in simple enzyme reactions to determine the kinetic constants and the apparent molar enthalpy of the reactions (Haq 2011; Todd and Gomez 2001). ITC can be useful in measuring enzyme kinetics because it directly measures the heat change in the system, which is proportional to the reaction rate (Haq 2011; Todd and Gomez 2001). In many cases, ITC offers benefits over spectrophotometric assays for determining enzyme kinetics. In spectrophotometric assays, an alternative substrate is often required since the substrate or product should absorb in the UV/VIS spectrum. In ITC, the natural substrate (or substrate of interest) can be used as such, and therefore, ITC gives more practically relevant results (Haq 2011; Todd and Gomez 2001).

However, interpretation of the data is complex if more than one reaction takes place, as is the case in lactose conversion with β-galactosidases. β-Galactosidases catalyze two types of reaction: the hydrolysis of lactose into glucose and galactose, and the synthesis of GOS from lactose, which in itself is a combination of many reactions. The reactions yield a complex mixture of carbohydrates with differences in the degree of polymerization, types of linkages, and monomer composition (Gosling et al. 2010) depending on the reaction conditions (concentration of substrate, temperature and enzyme type and source) (Boon et al. 2000). Lactose hydrolysis is known to be an

endothermic reaction with an enthalpy of approximately 0.50 and 0.58 kJ·mol$^{-1}$ at 30 and 40ºC, respectively (Goldberg and Tewari 1989). The enthalpy of GOS synthesis from lactose is not known to us. More information on the thermodynamic behavior of the β-galactosidase isoforms of *Bacillus circulans* could give us more insight in the kinetics of the enzymes.



Industrial-scale GOS production is usually performed at high initial lactose concentrations. The complete Biolacta N5 preparation is known to have a higher transgalactosylation activity and a lower hydrolysis activity at higher initial lactose concentrations (Boon et al. 2000; Huber et al. 1976). It is likely that not all isoform behave the same, and that some of them will show more transgalactosylation. It is therefore relevant to know more about the lactose conversion with the separate isoforms at high initial substrate concentration to investigate whether the productivity of the industrial GOS production can be increased when only a selection of the isoforms is used.

The aim of this research is therefore to characterize the β-galactosidase isoforms of *Bacillus circulans* in a kinetic and thermodynamic way, and to investigate their contribution in GOS production at high initial lactose concentrations.

First, a method to purify the four β-galactosidases from Biolacta N5 is described. Then, the four isoforms are used to convert lactose and their carbohydrate production at low and high initial lactose concentrations is analyzed in time. Besides, the thermodynamic behavior of lactose conversion and the conversion of some of its products, with the isoforms is studied with ITC.

## Materials and methods

### *Materials*

Lactose monohydrate (Lactochem), Vivinal-GOS, the DP3 fraction from Vivinal-GOS, and a β-galactosidase from *Bacillus circulans* called Biolacta N5 (Daiwa Kasei K. K., Japan) were supplied by FrieslandCampina (Beilen, The Netherlands). The activity of the solid enzyme preparation is 5550 LU/g (1 LU is defined as 1 µmol of lactose that is hydrolyzed per minute).

β-Galactosidase from *A.oryzae* (≥8U/mg solids), D(+)-galactose, D(+)-glucose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, glycine, hydrochloric acid, sulfuric acid, sodium hydroxide, o-nitrophenyl β-D-galactopyranoside (oNPG), o-nitrophenol (oNP), sodium dodecyl sulphate (SDS), bromophenol blue, 2-

mercaptoethanol, and bovine γ-globulin (BGG) were purchased from Sigma-Aldrich (Steinheim, Germany).

Glycerol and tris(hydroxymethyl)aminomethane were obtained from Acros Organics (Geel, Belgium). Protein assay dye reagent concentrate, Biosafe coomassie staining and SDS-PAGE high range standards were obtained from Bio-Rad (Veenendaal, The Netherlands).



McIlvaine's buffer was prepared by combining 0.1 M citric acid and 0.2 M disodium hydrogen phosphate in the correct ratio to reach a pH of 6.0. The final concentrations are 37 mM citric acid and 126 mM disodium hydrogen phosphate in McIlvaine's buffer of pH 6.0. Acetate buffer of pH 4.8 was prepared by adding together 200 mL of 138 mM sodium acetate solution and 200 mL of 108 mM acetic acid solution filled up to 1.0 L with MilliQ water. The final concentrations are 28 mM sodium acetate and 21 mM acetic acid in acetate buffer of pH 4.8. Sodium carbonate, citric acid monohydrate, disodium hydrogen phosphate, sodium acetate trihydrate, and acetic acid were purchased from Merck (Darmstadt, Germany).

Allolactose (6-O-b-D-galactopyranosyl-D-glucopyranose) and 4-galactosyllactose were obtained from Carbosynth Limited (Berkshire, UK). The Gel Filtration High Molecular Weight Calibration Kit was obtained from GE Healthcare (Buckinghamshire, UK). This kit contains ovalbumin (43 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa), thyroglobulin (669 kDa), and blue dextran (2,000 kDa).

### *Protein content determination*

The total protein content of Biolacta N5 was determined based on the method of Bradford as described in the Bio-Rad Protein Assay protocol with bovine γ-globulin (BGG) as a standard. Biolacta N5 was found to have a protein content of 19 ± 3% (BGG equivalents, 95% confidence interval based on nine measurements). The actual enzyme concentration is not known and for this reason the assumption is made that the total enzyme concentration is equal to the total protein concentration in all calculations.

### *Gel filtration Biolacta N5*

A gel filtration method was performed to fractionate the different isoforms. A solution of 50 $g \cdot L^{-1}$ Biolacta N5 was prepared in McIlvaine's buffer of pH 6.0. The solution was filtered with a 0.45 μm filter and 2 mL of the filtered solution was loaded onto a Superdex 200 prep grade column (GE Healthcare, Buckinghamshire, UK) at an Äkta Purifier with an UV detector at 280 nm (GE Healthcare, Buckinghamshire, UK).

McIlvaine's buffer of pH 6.0 was used as the running buffer and the applied flow rate was 1.25 $mL \cdot min^{-1}$.

Several fractions were collected and the fractions that together made up one protein peak were pooled in tubes. These tubes were stored at -20ºC until further usage. The subsequent pools were called pool 1 to 9. Pool 1 was the pool with the highest molecular weight and pool 9 was the pool with the lowest molecular weight. Later in this research, pool 2, 3, 4, and 5 are called β-gal-A, β-gal-B, β-gal-C, and β-gal-D, respectively.



The main molecular weight of the proteins in the pools was determined based on the standards from the high molecular weight gel filtration kit that was treated according to the accompanying protocol before the standards were loaded onto the column. The molecular weight of each pool was defined to be the molecular weight of the top of each peak.

The protein content of one pool was calculated based on the relative area of the peak compared to the total peak area in the chromatogram.

### *Concentration of pools*

The pools were concentrated in Stirred Amicon Ultrafiltration Cells (Millipore Corporation, Billerica, MA, United States). The membranes used had a cut-off of 5 kDa (for pool 1 to 6) and 1 kDa (for pool 7 and 8). A nitrogen flow was applied to obtain a pressure of 5 bars. The pools were concentrated until the protein concentration in the retentate was approximately 0.4 $g \cdot L^{-1}$.

### *Activity measurements*

The β-galactosidase activity was measured by using the *o*NPG activity assay adapted from Nakanishi et al. (1983). An Eppendorf tube with 790 µL of 0.25% (w/w) *o*NPG in McIlvaine's buffer of pH 6.0 was preheated in an Eppendorf Thermomixer at 40ºC and 600 rpm for 10 minutes. Subsequently, 210 µL of sample was added and these mixtures were incubated for another 10 minutes at 40ºC and 600 rpm. A volume of 1.0 mL of 10% (w/w) $Na_2CO_3$ solution was added to stop the reaction and, afterwards, the absorbance of *o*NP was measured at 420 nm. The *o*NP concentration was determined using the law of Lambert-Beer ($\varepsilon = 4576\ M^{-1} \cdot cm^{-1}$). The *o*NP formation was found to be linear during the first 10 minutes of the reaction. This initial rate of *o*NP formation was expressed in $mmol \cdot min^{-1} \cdot g\ protein^{-1}$. Measurements were performed in duplicate and the average enzyme activity was used.

### *SDS-PAGE*

SDS-PAGE was performed using a 7.5% Mini-Protean TGX Stain-Free precast SDS-PAGE gel (Bio-rad, Veenendaal). Biolacta N5 was dissolved in MilliQ water to a concentration of 1.2 g protein·$L^{-1}$. Pool 2, 3, and 4 were diluted with MilliQ water to a concentration of 0.1 g protein·$L^{-1}$. All enzyme solutions were diluted two times with SDS reducing buffer. The SDS reducing buffer and electrode running buffer were prepared as described in the Bio-Rad Mini-Protean 3 Cell instruction manual. Prestained SDS-PAGE high range standards were used with molecular weights of 202, 116, 98, and 47 kDa. After the SDS-PAGE run, the gel rinsed with MilliQ water and stained with Biosafe Coomassie Staining.

### *Lactose conversion at low initial lactose concentration*

The lactose conversion was measured with each pool that showed β-galactosidase activity. The pools were incubated for 20 minutes (similar to pre-equilibration time in ITC) in an Eppendorf thermomixer at 40ºC and 600 rpm. Subsequently, 150 mM lactose in McIlvaine's buffer of pH 6.0 was added. The volumetric ratio of enzyme solution and substrate solution of 9:1 was equal to their volumetric ratio in the ITC. Samples of 100 µL were taken from the reaction mixture every three minutes and transferred to a pre-weighted Eppendorf tube with 50 µL of 5% (w/w) sulfuric acid solution to inactivate the enzyme. Subsequently, the samples were stored at -20ºC until further preparation.

### *Isothermal titration calorimetry*

The conversion of several substrates was studied in a Microcal ITC-200 (GE Healthcare, Buckinghamshire, UK). Table 1 shows an overview of the performed ITC experiments.

Lactose was dissolved at 100 mM in acetate buffer of pH 4.8 or at 150 mM in McIlvaine's buffer of pH 6.0 depending on which enzyme was used. The allolactose and 4-galactosyllactose turned out to have a purity of only 89 and 81%, respectively. Therefore, the concentrations of allolactose and 4-galactosyllactose in the stock solutions were only 127 and 119 mM, respectively.

**Table 1.** Overview of conditions used in ITC

| Substrate | Enzyme | T [ºC] | [S]* [mM] |
|---|---|---|---|
| Lactose | β-galactosidase from *A.oryzae* | 30 vs. 40 | 100 |
| Lactose | Pool2 (β-gal-A) | 40 | 150 |
| Lactose | Pool3 (β-gal-B) | 40 | 150 |
| Lactose | Pool4 (β-gal-C) | 40 | 150 |
| Lactose | Pool5 (β-gal-D) | 40 | 150 |
| Allolactose | Pool2 (β-gal-A) | 40 | 127 |
| Allolactose | Pool5 (β-gal-D) | 40 | 127 |
| 4-galactosyllactose | Pool2 (β-gal-A) | 40 | 119 |
| 4-galactosyllactose | Pool5 (β-gal-D) | 40 | 119 |

* [S] *represents the substrate concentration in the syringe. The initial substrate concentration in the cell is approximately ten times smaller.*

A stock solution of 10 $g \cdot L^{-1}$ β-galactosidase from *A.oryzae* in acetate buffer of pH 4.8 was prepared. The pools were dialyzed against McIlvaine's buffer of pH 6.0 with Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific, Rockford, USA) with a cut off of 3.5 kDa and a capacity of 3-12 mL according to the accompanying protocol. The protein concentrations were adjusted for any volume change during the dialysis. The pools were stored at -20ºC until further usage.

### *Isothermal calorimetric (ITC) procedure*

The cells were heated up to the required temperature. All substrate, enzyme, and buffer solutions were degassed prior to the ITC experiments. Enzyme solution and Milli-Q water were transferred to the ITC sample cell (which is approximately 270 µL) and the reference cell, respectively. The syringe was filled with substrate solution. In blank measurements, buffer solution was used instead of the enzyme solution or the substrate solution. Once the sample cell was filled, we waited ten minutes before the experiment was started, to allow the solution in the cell to equilibrate and stabilize.

The experimental settings that were constant in each run were as follows, unless otherwise stated: total number of injections = 1; reference power = 12.25 µcal/s; initial delay = 60 s; stirring speed = 600 rpm; injection volume = 30 µL; injection duration = 60 s; filter period = 2 s. After starting the experiment, an equilibration of the system took place which took another ten minutes. Then, an initial delay of 60 s was recorded before

the solution from the syringe was injected into the sample cell and the reaction started. The thermal power supply was recorded every 2 s during each run.

After each ITC run, the solution in the sample cell (which is approximately 300 µL) was directly transferred to a pre-weighted Eppendorf tube with 100 µL of 5% (w/w) sulfuric acid solution to inactivate the enzyme. Subsequently, the samples were stored at -20ºC until further preparation.



*Lactose conversion at high initial lactose concentration*

The lactose conversion at an initial concentration of 30% (w/w) was followed in time by using a temperature controlled batch reactor with an anchor stirrer at 150 rpm. Lactose was dissolved in McIlvaine's buffer of pH 6.0 at 60ºC. The reaction was performed at 40ºC. The enzyme was added once the temperature was constant. The initial reaction volume was 25 mL. Samples of 200 µL were taken at regular time intervals and transferred to a pre-weighted Eppendorf tube with 100 µL of 5% (w/w) sulfuric acid solution to inactivate the enzyme. Subsequently, the samples were stored at -20ºC until further preparation.

*Sample handling for determination of the carbohydrate composition*

Before HPLC analysis, the enzyme was removed from the samples by filtering them at 14,000 x g at 18ºC for 30 minutes using pretreated Amicon® ultra-0.5 centrifugal filter devices (Millipore Corporation, Billerica, MA, United States) with a cut-off of 10 kDa in a Beckman Coulter Allegra X-22R centrifuge. The pretreatment of the filters consisted of two centrifugation steps: first, 500 µL of Milli-Q water was centrifuged at 14,000 x g at 18ºC for 15 minutes; and second, the filters were placed up-side-down in the tube and centrifuged at 14,000 x g at 18ºC for 5 minutes. After filtration, the samples were neutralized with 5% (w/w) sodium hydroxide. The pH was checked with pH paper.

*Measurements of the carbohydrate composition*

The filtered samples were analyzed with HPLC using a Rezex RSO oligosaccharide column (Phenomenex, Amstelveen, the Netherlands) at 80ºC. The column was eluted with Milli-Q water at a flow rate of 0.3 mL/min. The eluent was monitored with a refractive index detector.

The standards that were used for calibration of the column were lactose, glucose, galactose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose.

Galacto-oligosaccharides up to a degree of polymerization of 7, including allolactose and 4-galactosyllactose, were assumed to have the same response as the glucose-oligomers with equal degree of polymerization. This was confirmed with mass balances: the molar concentrations of monosaccharide residues in the varying carbohydrates were added up and compared with the initial concentration of monosaccharide residues in the substrate.



### *Measurement of the composition of the disaccharides*

The samples were diluted till approximately 10 ppm and analyzed with HPAEC using a CarboPac PA1: 250 x 4 mm anion-exchange column (Dionex, Sunnyvale, CA, USA) at 30ºC. The column was eluted with gradients of sodium hydroxide and sodium acetate at a flow rate of 1.0 mL/min. The eluent was monitored with a pulsed amperometric detector (PAD). The standards that were used for calibration of the column were lactose and lactulose. Other disaccharides were quantified by assuming that their response factor was equal to the response factor of lactose.

### *Data handling ITC*

The obtained data of the power supply in time was corrected with the reference power. The power supplies of the duplicates at each time point were averaged. The average power supply of the injections of substrate into buffer solution and the average power supply of the injections of buffer into enzyme solution (the blanks) were subtracted from the average power supply of injections of substrate into enzyme solution to correct for effects such as dilution effects (see (Velázquez-Campoy et al. 2001)).

If only one type of reaction takes place, the enthalpy of this reaction can be determined with this procedure. The total amount of heat generated was determined by integrating the area up to 3660 s between the calorimetric response and the baseline (Velázquez-Campoy et al. 2001). This area was used to determine the reaction enthalpy $\Delta H$ with equation 1:

$$\Delta H = \frac{Area_{3660}}{n} \tag{1}$$

where $n$ is the amount of moles of substrate that is converted which was determined from the HPLC analysis.

## Results and discussion

The isoforms of the β-galactosidase preparation Biolacta N5 were purified by fractionation before they were characterized. The isoforms were characterized based on *o*NPG converting activity, purity, and molecular weight. Besides, the lactose conversion at high and low initial substrate concentrations and the conversion of allolactose and 4-galactosyllactose with the isoforms were studied.



### *Fractionation of Biolacta N5*

Biolacta N5 was fractionated with gel filtration based on the molecular weight of the proteins. The gel filtration pattern that was obtained is shown in Figure 1.

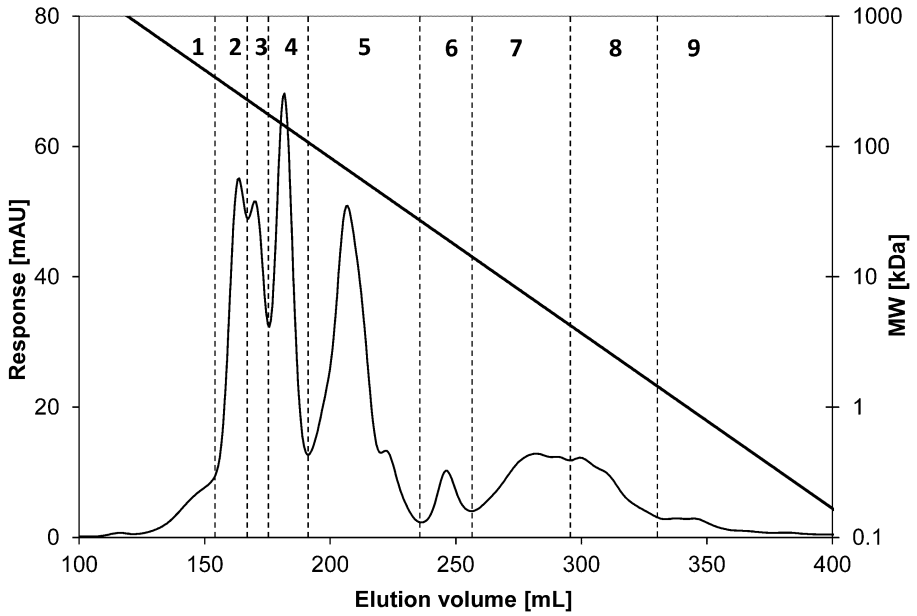


**Figure 1.** The gel filtration pattern of β-galactosidase isoforms from Biolacta N5 together with the molecular weight (line in bold). The volumes in between the dotted lines were pooled. The numbers 1 to 9 correspond to the name of the pool.

The pools could not be baseline-separated which might indicate that they are not completely pure. Additionally, some of the peaks were not symmetric. This might imply that they consist of more than one protein. The purity of the pools was later determined with SDS-PAGE.

### *Enzyme activity of the pools with oNPG*

After the pools were concentrated to a protein concentration of approximately 0.4 $g \cdot L^{-1}$, the *o*NPG converting activity was determined in each pool. Pool 1 to 5 showed *o*NPG converting activity, whereas pool 6 to 9 did not show any *o*NPG converting activity. The β-galactosidase isoforms appeared to be present in pool 1 to 5, and only pool 1 to 5 were characterized further. The measured enzyme activity of these pools on *o*NPG is shown in Table 2.

**Table 2.** Characteristics of pool 1 to 5: the mean molecular weight determined with gel filtration, the molecular weight determined with SDS-PAGE, *o*NPG converting activity (at 6.6 mM oNPG initially, 40ºC, and pH 6.0), and β-gal isoforms present in pool as identified by Song et al. (2011).

| | Mean molecular weight gel filtration [kDa] | Main molecular weight SDS-PAGE [kDa] | Molecular weight MALDI/TOF MS from Song et al. (2011) [kDa] | *o*NPG converting activity [$mmol \cdot min^{-1} \cdot g\ protein^{-1}$] | Corresponding β-gal from Song et al. (2011) |
|---|---|---|---|---|---|
| Pool 1 | 675-352 | - | - | 4.8 | (β-gal-A, B, C) |
| Pool 2 | 250 | 206 | 189 | 15 | β-gal-A |
| Pool 3 | 207 | 181 | 154 | 12 | β-gal-B |
| Pool 4 | 143 | 164 | 135 | 6.3 | β-gal-C |
| Pool 5 | 66 | 112 | 92 | 5.0 | β-gal-D |

### *Purity and molecular weight isoforms*

Figure 2 shows the result of SDS-PAGE of the complete Biolacta N5 preparation and its active pools. The molecular weights of the proteins are given in Table 2. Song et al. (2011) previously reported on the purification of the four β-galactosidases of Biolacta N5: β-gal-A, β-gal-B, β-gal-C, and β-gal-D. Based on the molecular weights and band patterns on SDS-PAGE, we postulate that pool 1 contains low amounts of β-gal-A, β-gal-B, and β-gal-C; pool 2 consisted of β-gal-A; pool 3 consists of β-gal-B; pool 4 consists of β-gal-C; and pool 5 contains β-gal-D among a few other (not active) proteins. Since pool 1 seems to be a mixture of similar amounts of three isoforms; we did not characterize pool 1 any further. β-Gal-A, β-gal-B, and β-gal-C were very well purified, whereas pool 5 was a mixture of several proteins of which we expect the largest band to be β-gal-D. Since all isoforms are present in different pools, we continue with pool 2, 3,

4, and 5 to further study the four isoforms. From here on, we will refer to pool 2, 3, 4, and 5 as β-gal-A, β-gal-B, β-gal-C, and β-gal-D.

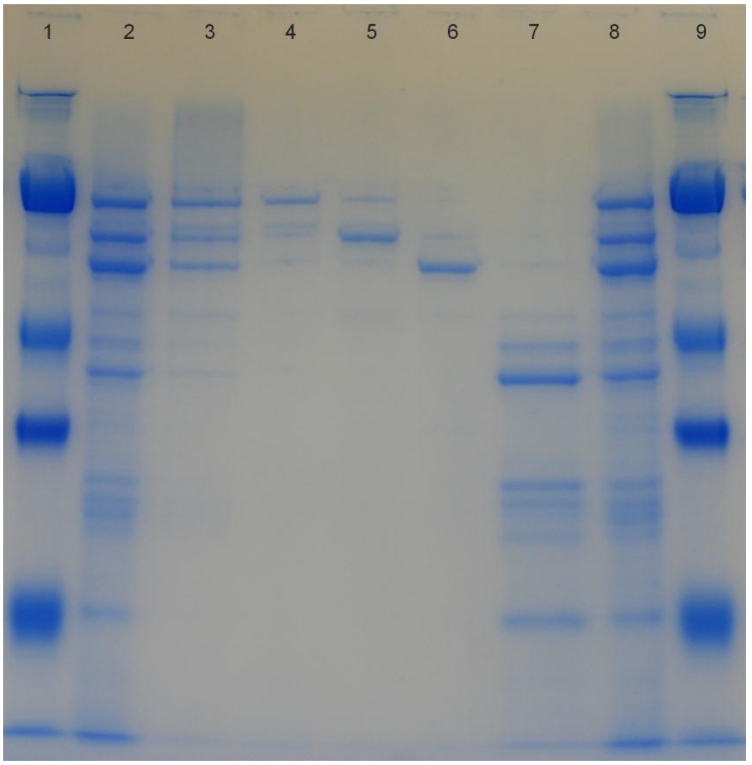


**Figure 2.** SDS-PAGE on a 7.5% gel of isoforms of Biolacta N5 and the crude enzyme preparation. Lane 1. marker (202, 117, 98, 47 kDa); 2. crude Biolacta N5; 3. pool 1; 4. pool 2 (β-gal-A); 5. pool 3 (β-gal-B); 6. pool 4 (β-gal-C); 7. pool 5 (β-gal-D); 8. crude Biolacta N5; 9. marker.

### *Lactose conversion at low initial lactose concentrations*

Lactose conversion was carried out with the various β-galactosidase isoforms of Biolacta N5 at an initial lactose concentration in between 0.44 and 0.68% (w/w). The results are shown in Figure 3.

During lactose conversion with β-gal-A at an initial lactose concentration of 0.44% (w/w), galactose and glucose were produced to approximately the same level. This means that mainly hydrolysis of lactose took place. The highest level of GOS with a DP of 3, which was only 3.5% (w/w) on dry matter basis, was produced when 30% of the lactose was converted. At a higher lactose conversion level, the concentration of trisaccharides decreased again due to hydrolysis. No larger GOS (DP>3) were detected during lactose conversion at an initial lactose concentration of 0.44% (w/w).

During lactose conversion with β-gal-B at an initial lactose concentration of 0.48% (w/w), GOS with a DP of 3 and 4 were formed up to 9.0 and 0.89% (w/w) on dry matter basis, respectively. The GOS concentration decreased when lactose was converted for more than 40%. Because of the formation of GOS, the glucose concentration is higher than the galactose concentration. When lactose conversion was completed, only galactose and glucose were present at similar levels.

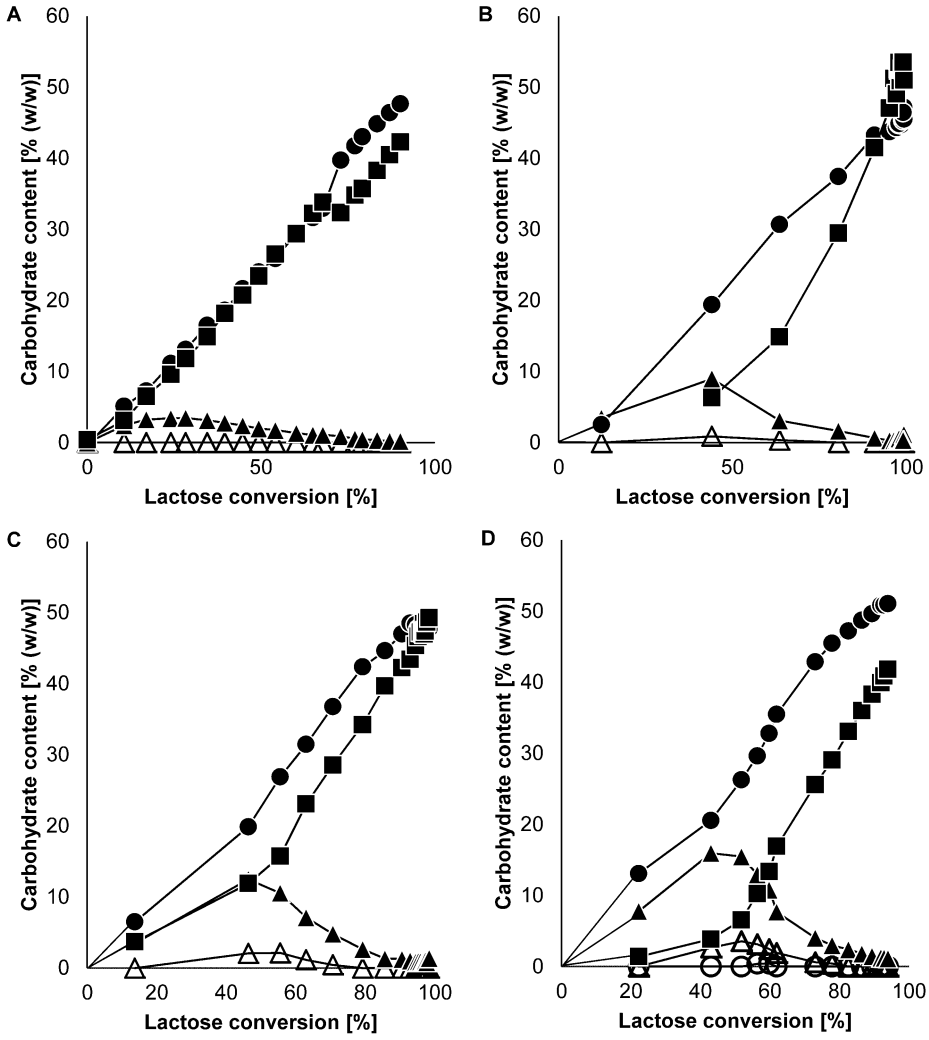


**Figure 3.** Production of carbohydrates as a function of the lactose conversion with (A) 0.04 g·L$^{-1}$ β-gal-A, (B) 0.4 g·L$^{-1}$ β-gal-B, (C) 0.4 g·L$^{-1}$ β-gal-C, and (D) 0.4 g·L$^{-1}$ β-gal-D at an initial lactose concentration of 0.44, 0.48, 0.48, and 0.68% (w/w) (13, 14, 14, and 20 mM), respectively, 40ºC, and pH 6.0. Symbols: ■ galactose; ● glucose; ▲ trisaccharides; Δ tetrasaccharides; ○ pentasaccharides. (Lines for guidance.)

Lactose conversion with β-gal-C shows the same profile as lactose conversion with β-gal-B, except for slightly higher levels of GOS formation and the formation of GOS with larger DP. The maximum concentrations of GOS with a DP of 3, 4, and 5 were 13, 2.1, and 0.18% (w/w) on dry matter basis, respectively.

β-Gal-D produced even higher concentrations of GOS with a DP of 3, 4, and 5 up to levels of 16, 3.6, and 0.41% (w/w) on dry matter basis, respectively. The lactose was not completely converted within the used reaction time and therefore, no equal levels of galactose and glucose were obtained. The slower reaction can be caused by the presence of inactive proteins in pool 5.



### *Isothermal titration calorimetry*

Isothermal titration calorimetry (ITC) was used to obtain more insight in the thermodynamics of substrate conversion with the isoforms and to investigate how these isoforms differ. Since various reactions take place simultaneously during lactose conversion, the system is complex.

A simpler reaction is lactose conversion with β-galactosidase from *A.oryzae*, because only glucose and galactose were detected which means that in effect only lactose hydrolysis took place. Since (effectively) only one reaction took place, the apparent molar enthalpy of lactose hydrolysis could be determined, which was found to be endothermic. However, repeated measurements were not exactly the same, and therefore, the average areas of measurements were used to calculate the apparent molar enthalpy which was found to be 0.65 and 0.38 $kJ \cdot mol^{-1}$ at 30 and 40ºC, respectively. These values correspond well with the study of Goldberg and Tewari (1989). The corresponding thermograms can be found in Figure A1 and Figure A2 in the Appendix.

Figure 4 shows the thermal power required to maintain a constant temperature after addition of lactose into the sample cell with β-gal-A, β-gal-B, β-gal-C, and β-gal-D together with the corresponding carbohydrate profiles in time to be able to link the thermodynamic data to the formation or degradation of reactants.

At the end of the ITC experiment, samples were taken from the ITC sample cell and their carbohydrate composition was measured. The results are presented in Table 3 and Table 4. They are in agreement with the carbohydrate compositions at one hour of conversion in Figure 4.

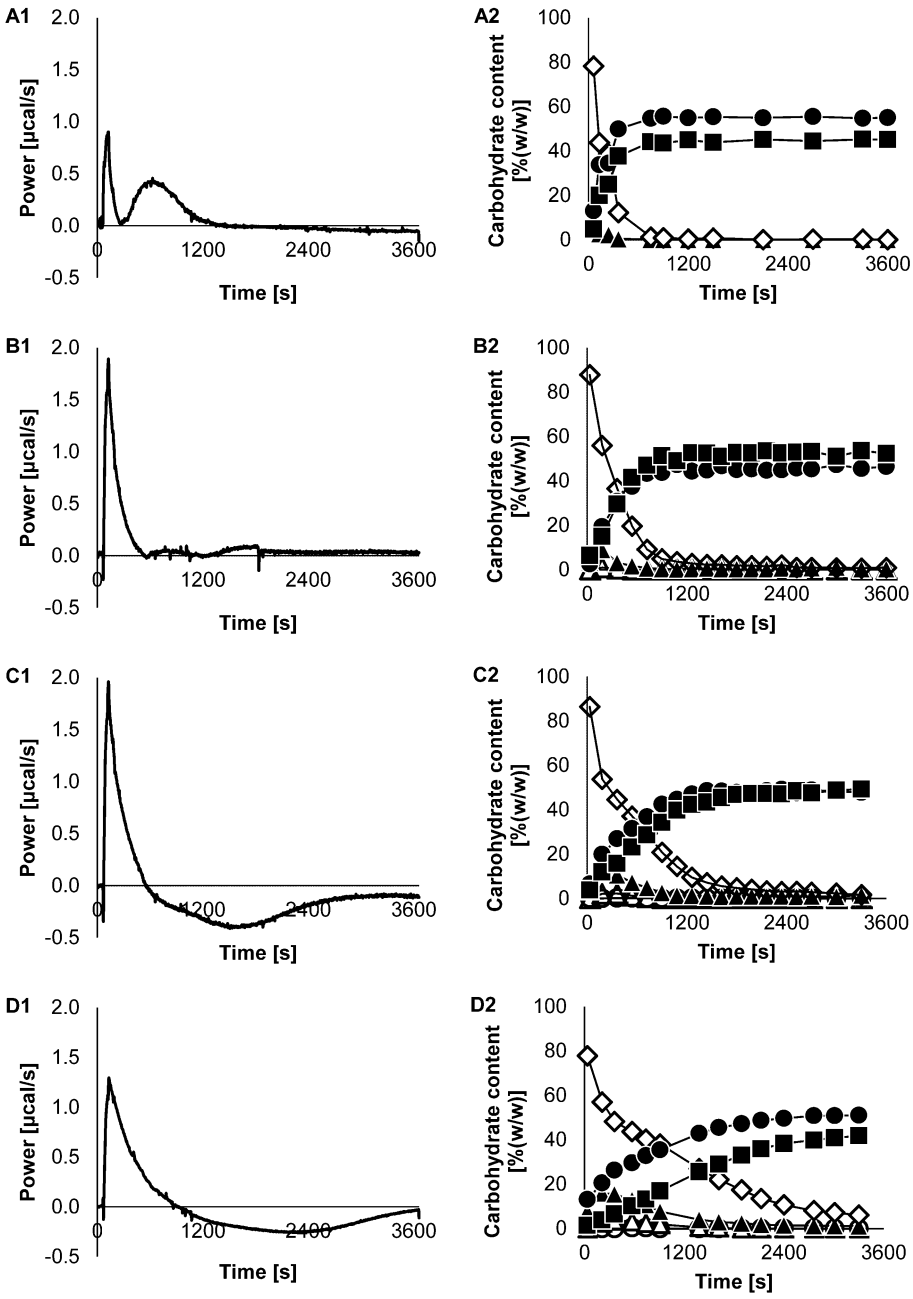


**Figure 4.** The thermal power supply of the ITC in time for an injection of 30 µL of 150 mM lactose into the sample cell with 0.4 $g \cdot L^{-1}$ (A1) β-gal-A, (B1) β-gal-B, (C1) β-gal-C, (D1) β-gal-D at 40ºC and pH 6.0 together with the corresponding carbohydrate profiles during lactose conversion at an initial concentration of 17, 14, 14, and 20 mM lactose with (A2) β-gal-A, (B2) β-gal-B, (C2) β-gal-C, (D2) β-gal-D, respectively. Symbols: ■ galactose; ● glucose; ◇ disaccharides; ▲ trisaccharides; Δ tetrasaccharides; ○ pentasaccharides. (Lines for guidance.) The raw data of ITC measurements and blanks can be found in Figure A3 in the Appendix.

**Table 3.** Carbohydrate composition of lactose conversion with pools in ITC after one hour of reaction (corresponding to Figure 4) measured with HPLC as a percentage of the dry matter content.

| | DP3 | DP2 | glucose | galactose |
|---|---|---|---|---|
| | % | % | % | % |
| β-Gal-A | 0.43 | 0.60 | 47 | 51 |
| β-Gal-B | 0.57 | 2.5 | 46 | 50 |
| β-Gal-C | 1.5 | 7.5 | 45 | 46 |
| β-Gal-D | 2.0 | 13 | 42 | 43 |

**Table 4.** Disaccharide composition (measured with HPAEC) after one hour of lactose conversion with pools in ITC corresponding with Table 3). Disaccharides are presented as a percentage of the total disaccharide content.

| | Galβ1-6gal | Allolactose | Lactose | Lactulose | Galβ1-4gal | Galβ1-3glu |
|---|---|---|---|---|---|---|
| | % of DP2 | % of DP2 | % of DP2 | % of DP2 | % of DP2 | % of DP2 |
| β-Gal-A | - | 3.1 | 67 | 28 | - | 1.3 |
| β-Gal-D | 1.7 | 24 | 44 | 11 | 2.7 | 17 |

The ITC profiles of lactose conversion with β-gal-A, β-gal-B, β-gal-C, and β-gal-D can be explained if we assume lactose hydrolysis to be endothermic (as was also measured with the lactose hydrolysis with β-galactosidase from *A.oryzae*), the synthesis of galacto-oligosaccharides to be endothermic, and the hydrolysis of galacto-oligosaccharides to be exothermic.

Lactose conversion with β-gal-A shows overall a positive area (Figure 4A1), which means that overall an endothermic reaction took place. This was expected based on the carbohydrate profile of β-gal-A (Figure 4A2) which shows net only lactose hydrolysis. The sharp endothermic peak at the beginning of lactose conversion with β-gal-A can be caused by fast oligosaccharide formation together with slower lactose hydrolysis (galactose was released from the beginning) since both reactions are endothermic. Then, the power supply decreased which is due to the oligosaccharide hydrolysis (exothermic) and lactose hydrolysis (endothermic). The power signals due to both reactions cancel each other to some degree and, therefore, the power signal approached zero. After this decrease in power supply, another peak in the power supply was recorded. In this time frame, in effect only lactose hydrolysis was measured (Figure 4A2), which is an endothermic reaction. The power supply became approximately zero after 1200 s, because all substrate was converted.

Lactose conversion with β-gal-B (Figure 4B) shows a sharp increase in power supply for the conversion of lactose with β-gal-B. Very soon, the reaction rate decreased again and returned to approximately zero. Overall an endothermic reaction took place. This is

in agreement with the final carbohydrate composition after one hour, which shows that mainly lactose hydrolysis took place.

Lactose conversion with β-gal-C (Figure 4C) shows initially a sharp increase in power supply because of the fast formation of oligosaccharides. Shortly after this, the power supply decreased and became negative, probably due to the hydrolysis of the oligosaccharides that were formed which is dominant over the simultaneous hydrolysis of the lactose. At approximately 1800 s, the power supply increased again and approached zero, since all reaction rates decreased and reached zero finally.

When the lactose was injected into a β-gal-D solution, a large endothermic peak was seen again (Figure 4D). During this time interval, oligosaccharide synthesis as well as lactose hydrolysis took place according to Figure 4D2. After this, the power supply decreased again and became negative: the dominant reaction now became the hydrolysis of the oligosaccharides, which is exothermic. This transition from overall endothermic towards overall exothermic corresponds well with the transition from mainly oligosaccharide synthesis to mainly oligosaccharide hydrolysis. At the end of the reaction, the power supply approached zero again: which corresponds with Figure 4D where the rate of formation or degradation of all components decreased. Although we have to keep in mind that the system consists of a complex combination of reactions, we can conclude from combining ITC with the carbohydrate analysis that lactose hydrolysis and oligosaccharide synthesis are endothermic, whereas oligosaccharide hydrolysis is exothermic.

The ITC measurements described above show that large variations exist among the conversions of lactose with the different isoforms. To get more insight in the behavior of the isoforms on some of the reactants, ITC experiments were performed with β-gal-A and β-gal-D (since they show the strongest variations), and allolactose and 4-galactosyllactose as substrates since allolactose and 4-galactosyllactose are products after lactose conversion.

### *Allolactose conversion*

Figure 5 shows a slightly increased power supply for both the conversion of allolactose with β-gal-A and β-gal-D. The reactions that took place were overall endothermic. HPLC analysis showed that within one hour only 13 and 21% of allolactose was converted with β-gal-A and β-gal-D, respectively (Table 5). Glucose and galactose were formed predominantly; only a small trace of carbohydrates with a DP of 3 was detected after one hour of reaction with both β-galactosidase isoforms. This means that both β-gal-A and β-gal-D catalyze at least two types of reactions with this reactant:

the hydrolysis of the allolactose as well as synthesis of oligosaccharides, which overall resulted in the enthalpy represented by the area under the lines in the graph. The hydrolysis of allolactose is a highly endothermic reaction since the increased power supply was only caused by a very small conversion of allolactose. Although allolactose has the same stoichiometric composition as lactose, it is a poor substrate for β-gal-A and β-gal-D in contrast to lactose. The explanation for this is the different linkage between the glucose and the galactose residue in allolactose and lactose. Allolactose contains a 1,6-linkage whereas lactose contains a 1,4-linkage. From these ITC experiments, we can conclude that β-gal-A and β-gal-D are well capable of hydrolyzing 1,4-linkages, but they are much less effective in hydrolyzing 1,6-linkages. Since β-gal-D converted slightly more allolactose than β-gal-A, β-gal-D seems to be less selective.

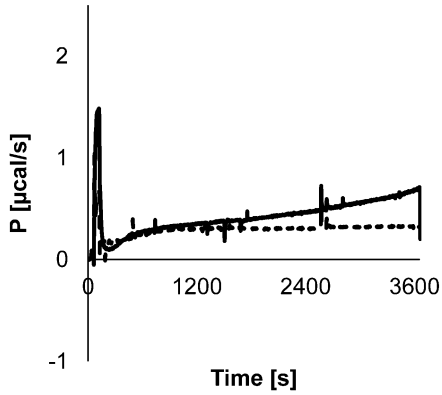


**Figure 5.** The thermal power supply of the ITC in time for injections of 30 µL of 127 mM allolactose into the sample cell with 0.4 $g \cdot L^{-1}$ β-gal-A (black line) and β-gal-D (dashed line) at 40ºC and pH 6.0. The concentration of allolactose during the reaction is 13 mM. The raw data of measurements and blanks can be found in Figure A4 in the Appendix.

**Table 5.** Carbohydrate composition of ITC samples after one hour of reaction (corresponding to Figure 5 and Figure 6) as a percentage of the dry matter content.

| | DP3 | DP2 | Glucose | Galactose |
|---|---|---|---|---|
| | % | % | % | % |
| Allolactose + β-gal-A | 0.1 | 87 | 3.9 | 9.4 |
| Allolactose + β-gal-D | 1.9 | 79 | 7.2 | 12 |
| 4-Galactosyllactose + β-gal-A | 5.5 | 0.0 | 28 | 66 |
| 4-Galactosyllactose + β-gal-D | 8.7 | 17 | 24 | 51 |

### *4-Galactosyllactose conversion*

A negative peak in power supply, indicating an exothermic reaction, was found for the conversion of 4-galactosyllactose with β-gal-A and β-gal-D (Figure 6). Right after injection of 4-galactosyllactose into β-gal-A, the net conversion was thus strongly exothermic, but the power supply returned to the original value after about 1200 s: the total conversion became less exothermic; after much more time, the signal indicated an endothermic reaction. After injection of 4-galactosyllactose into β-gal-D, the net conversion is slightly exothermic and the power supply increased slightly from approximately 1000 s onwards. The power supply does not completely return to zero within one hour, because the reaction still continues.

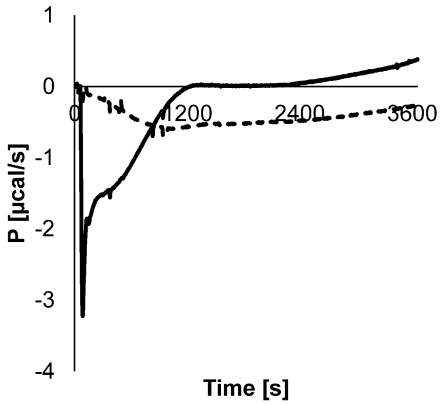


**Figure 6.** The thermal power supply of the ITC in time for injections of 30 µL of 119 mM 4-galactosyllactose into the sample cell with 0.4 $g \cdot L^{-1}$ β-gal-A (solid line) and β-gal-D (dashed line) at 40ºC and pH 6.0. The concentration of 4-galactosyllactose during the reaction in 12 mM. The raw data of measurements and blanks can be found in Figure A5 in the Appendix.

The total conversion of 4-galactosyllactose with β-gal-A and β-gal-D within one hour was 94 and 91%, respectively (Table 5). The final products of 4-galactosyllactose conversion with β-gal-A were only glucose and galactose. The conversion of oligosaccharides with a DP of 3 (Figure 7A) showed that the concentration of trisaccharides initially decreased very fast and leveled off afterwards. At the same time, the concentration of disaccharides increased very fast, which means that hydrolysis of oligosaccharides took place, and then decreased rapidly again. Also the concentration of glucose and galactose, which indicates hydrolysis of disaccharides increased quickly. Most likely, β-gal-A catalyzed two main reactions: the hydrolysis of the 1,4-linkage between the two galactose molecules of 4-galactosyllactose (exothermic), and the hydrolysis of the 1,4-linkage between the glucose and the galactose molecule next to it

(endothermic). Since the synthesis of larger oligosaccharides was minor, this reaction could not be observed in the ITC thermogram.

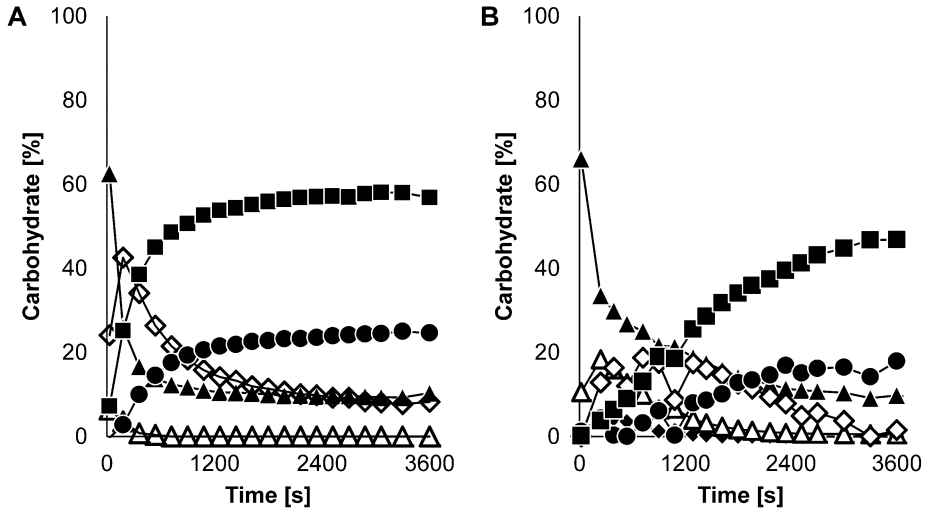


**Figure 7.** Carbohydrate profile during conversion of oligosaccharides with a DP of 3 (FrieslandCampina) at an initial concentration of (A) 17 mM with 0.4 g·L$^{-1}$ β-gal-A and (B) 21 mM with 0.4 g·L$^{-1}$ β-gal-D at 40ºC and pH 6.0. Symbols: ♦ pentasaccharides; Δ tetrasaccharides; ▲ trisaccharides; ◇ disaccharides; ● glucose; and ■ galactose. (Lines for guidance.)

The initial decrease in power supply was much stronger for β-gal-A than for β-gal-D. After one hour of 4-galactosyllactose conversion with β-gal-D, carbohydrates with a DP of 2 were detected after one hour of reaction, besides glucose and galactose. The carbohydrate composition analysis in time (Figure 7B) shows that the amount of oligosaccharides with a DP of 3 initially decreased slower than with β-gal-A, and that carbohydrates with a DP of 1 - 5 were formed. Oligosaccharide synthesis (endothermic) and breakdown (exothermic) took place at the same time, together with some lactose hydrolysis, since galactose was released. The breakdown of oligosaccharides dominated during the entire reaction since overall the reactions are exothermic. Around 1000 s, the exothermic net formation of disaccharides (breakdown of trisaccharides), changed to net hydrolysis of disaccharides, which is endothermic. This transition from net formation of disaccharides to net degradation of disaccharides explains the very small power signal of ITC around 1000 s. Besides this, also the decrease in the hydrolysis rate of the trisaccharides might explain the overall power signal of the ITC to become smaller, indicating less exothermic overall conversion.

β-Gal-D is well capable of hydrolysing 4-galactosyllactose into smaller carbohydrates, but its catalysis of disaccharide hydrolysis is less than the catalysis with β-gal-A.



### *Lactose conversion at high initial lactose concentrations*

The results at low substrate concentrations, as discussed above, prove that the β-galactosidase isoforms of Biolacta N5 differ in GOS production at low initial substrate concentrations. However, industrial GOS production takes place at a high initial lactose concentration. Therefore, we also investigated the use of the separate isoforms at an initial lactose concentration of 30% (w/w). Figure 8 shows the production of galactose, glucose, trisaccharides, tetrasaccharides, and pentasaccharides as a function of the lactose conversion for an initial lactose concentration of 30% (w/w) at 40ºC and pH 6.0 with β-gal-A, β-gal-B, β-gal-C, and β-gal-D. In contrast to the situation when using low lactose concentrations, a similar lactose conversion at high initial lactose concentrations resulted in a similar partition of carbohydrates for each of the pools. Only β-gal-A showed a small deviation. When only 45% of the lactose was converted with β-gal-A, the total mixture consisted out of 2.0% galactose, 12% glucose, 25% trisaccharides, 5.1% tetrasaccharides, and 0.4% pentasaccharides. When approximately 50% of the lactose was converted with β-gal-B, β-gal-C, and β-gal-D, the total mixture consisted out of 0.1% galactose, 14% glucose, 26% trisaccharides, 9.0% tetrasaccharides, and 0.8% pentasaccharides. This indicates that the β-galactosidase isoforms catalyze the conversions in much the same way at these high substrate concentrations.

The time necessary to synthesize equal levels of carbohydrates did vary, however. Table 6 shows the initial rates of lactose conversion, GOS formation, glucose formation, and galactose formation.

**Table 6.** Initial rates of lactose conversion, GOS formation, glucose formation, and galactose formation at an initial lactose concentration of 30% (w/w), 40ºC, and pH 6.0.

| | $V_{0,lactose}$ ($x10^3$ mol·min$^{-1}$ ·mol protein$^{-1}$) | $V_{0,GOS}^{*}$ ($x10^3$ mol·min$^{-1}$ ·mol protein$^{-1}$) | $V_{0,glucose}$ ($x10^3$ mol·min$^{-1}$ ·mol protein$^{-1}$) | $V_{0,galactose}$ ($x10^3$ mol·min$^{-1}$ ·mol protein$^{-1}$) |
|---|---|---|---|---|
| β-Gal-A | 14 | 6.6 | 7.0 | 0.58 |
| β-Gal-B | 11 | 6.2 | 5.8 | 0 |
| β-Gal-C | 6.7 | 3.8 | 3.6 | 0 |
| β-Gal-D | 5.9 | 3.1 | 3.1 | 0 |

**GOS is here defined as all carbohydrates with a DP of 3 and more.*

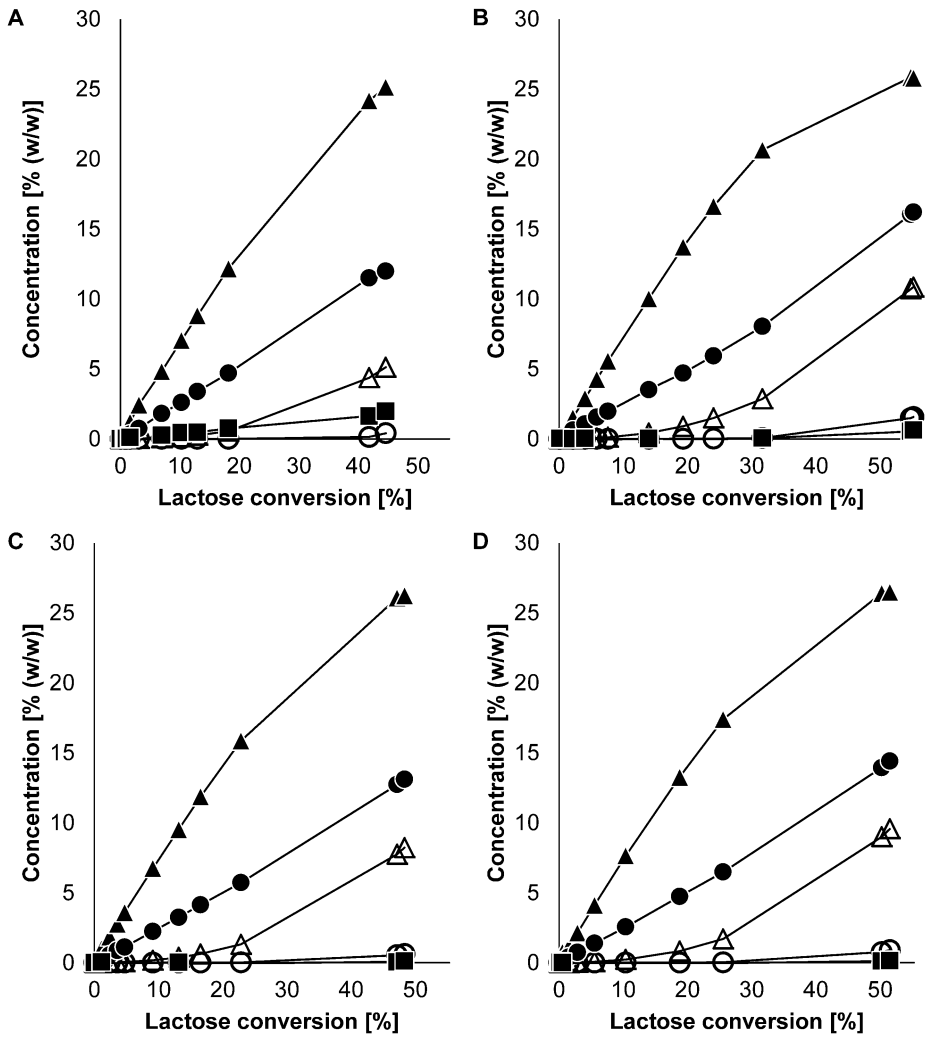


**Figure 8.** Production of carbohydrates as a function of the lactose conversion with (A) 8.3 mg·L$^{-1}$ β-gal-A, (B) 17 mg·L$^{-1}$ β-gal-B, (C) 17 mg·L$^{-1}$ β-gal-C, and (D) 16 mg·L$^{-1}$ β-gal-D at an initial lactose concentration of 30% (w/w) (1000 mM), 40ºC, and pH 6.0. Symbols: ○ pentasaccharides; Δ tetrasaccharides; ▲ trisaccharides; ● glucose; and ■ galactose. (Lines for guidance.)

Lactose was converted much faster with β-gal-A, shortly followed by β-gal-B, than with β-gal-C and β-gal-D. The total rate of GOS production and the rate of glucose formation were highest for β-gal-A, than β-gal-B, β-gal-C, and lowest for β-gal-D. The rate of galactose formation is only significant for β-gal-A.

Although β-gal-A was the isoform that performed the poorest in catalyzing GOS formation at low initial lactose concentrations, it showed the highest GOS formation rate at high initial lactose concentrations. Remarkably, the initial GOS formation rate

decreased when the isoforms decrease in the molecular weight. This result possibly suggests that the small isoform β-gal-D is not necessarily the best enzyme for GOS synthesis. In contrast, β-gal-A and β-gal-B might be the best isoforms for the GOS synthesis.

An explanation for this concentration dependent behavior might be the size of the enzymes. Since β-gal-A is the largest enzyme among the four isoforms, the active site of the enzyme might be more hidden in the folded enzyme than the active site of the other enzymes. The diffusion of carbohydrates into the active site of β-gal-A may then become more limiting than the rate of reaction. Since water molecules are much smaller than carbohydrate molecules and many more water molecules are available than carbohydrate molecules at low initial lactose concentrations, water molecules can more easily access the active site of the enzyme and hydrolysis takes place. Since the active site of the other isoforms may well be better accessible, the diffusion of carbohydrate molecules into the active site is much faster and does not limit the formation of oligosaccharides. At high initial lactose concentrations, with an excess of carbohydrate molecules available, this diffusion limitation does most likely not play a role.



## Conclusions

Fractionation of the enzyme preparation Biolacta N5 into four different enzyme isoforms resulted in four active β-galactosidase isoforms, in agreement with the work of Song et al. (2011). We observed differences between the isoforms in lactose conversion at low initial lactose concentrations. Especially β-gal-A yields less galacto-oligosaccharides and more hydrolysis than β-gal-B, β-gal-C, or β-gal-D. The differences among the β-galactosidase isoforms were confirmed with ITC analysis. In addition, β-gal-A showed a much faster catalysis of the hydrolysis of 4-galactosyllactose than β-gal-D. β-Gal-A and β-gal-D were only slightly capable of catalyzing allolactose conversion.

Although differences among the β-galactosidase isoforms were observed at low initial lactose concentrations, the β-galactosidase isoforms did not show major differences in performance at high initial lactose concentrations. The differences in GOS formation rate indicate that β-gal-A and β-gal-B are the best isoforms for GOS production. Differences in reaction rates were hypothesized to be related to the accessibility of the active site for different-sized reactants. At high lactose concentrations the GOS yield might be improved by using a Biolacta preparation which contains mainly β-gal-A and β-gal-B.

## Acknowledgements

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# Appendix

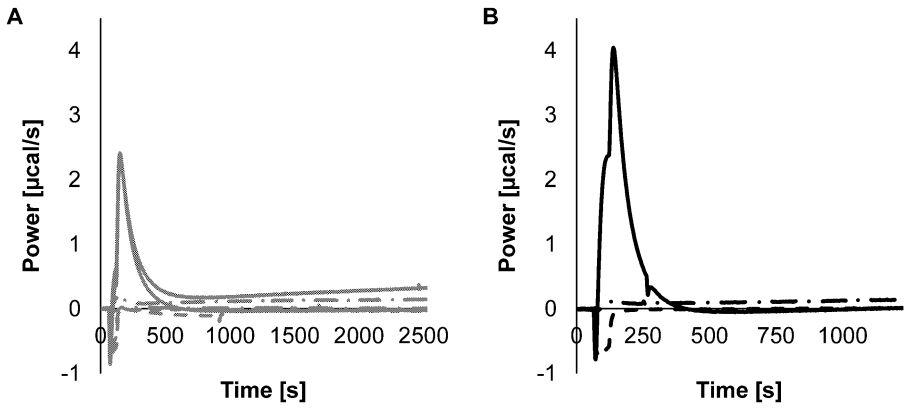


**Figure A1.** Raw data of the thermal power supply of the ITC in time for an injection of 30 µL of 100 mM lactose into the sample cell with 10 g·L$^{-1}$ β-galactosidase from *A.oryzae* at (A) 30 and (B) 40ºC and pH 4.8. The reference power was 5 µcal/s. Lines: ——— lactose into enzyme solution; – – lactose into buffer solution; — · — buffer into enzyme solution.

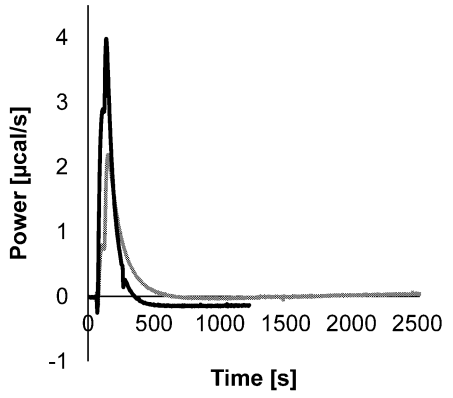


**Figure A2.** The thermal power supply of the ITC in time for an injection of 30 µL of 100 mM lactose into the sample cell with 10 g·L$^{-1}$ β-galactosidase from *A.oryzae* at 30 (grey line) and 40 (black line)ºC and pH 4.8 corrected for blanks.

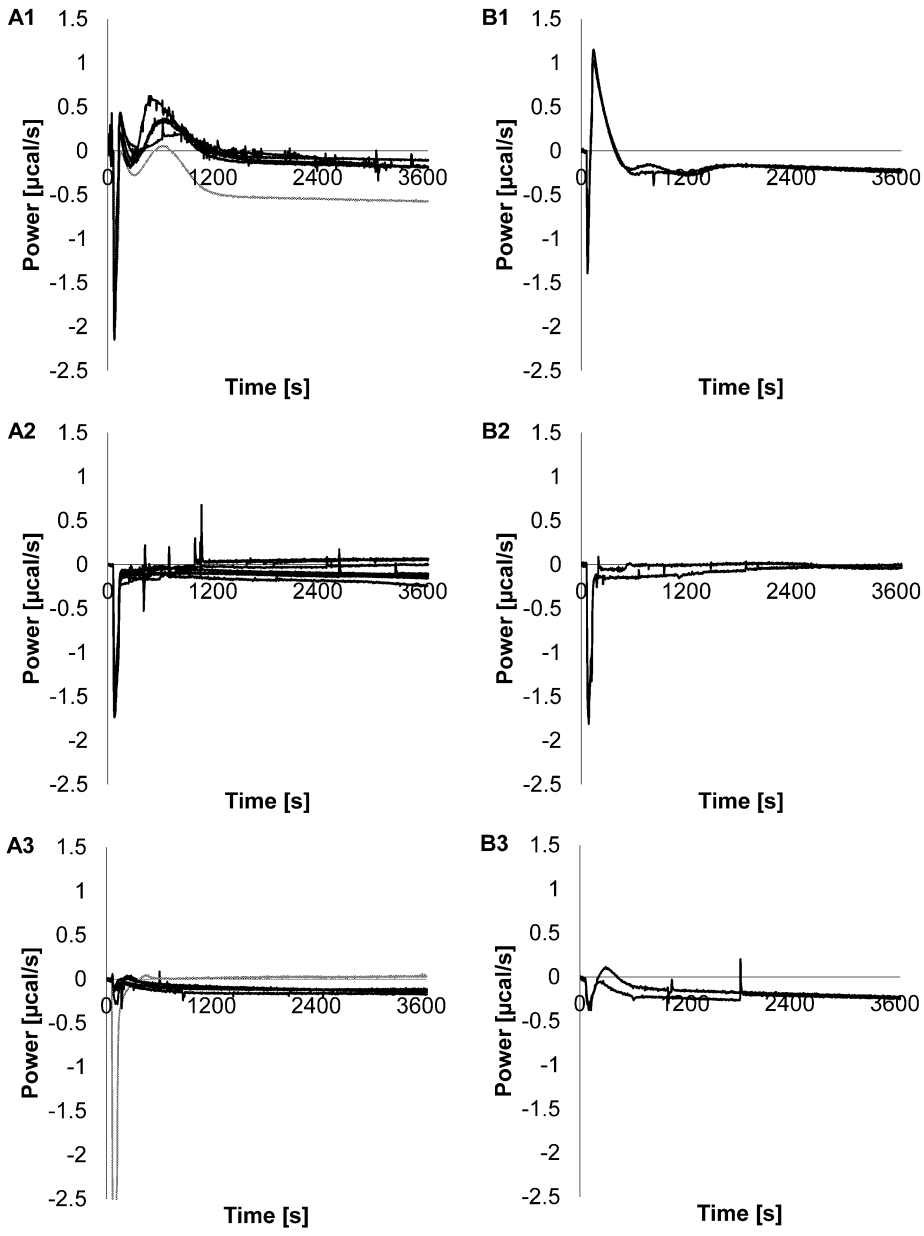


**Figure A3.** Raw data of the thermal power supply of the ITC in time for an injection of 30 µL of 150 mM lactose into the sample cell with 0.4 $g \cdot L^{-1}$ (A) β-gal-A, (B) β-gal-B, (C) β-gal-C, and (D) β-gal-D at 40ºC and pH 6.0. Figures x1: lactose into enzyme solution; figures x2: lactose into buffer solution; figures x3: buffer into enzyme solution. Grey lines were considered as outliers and were not taken into account.

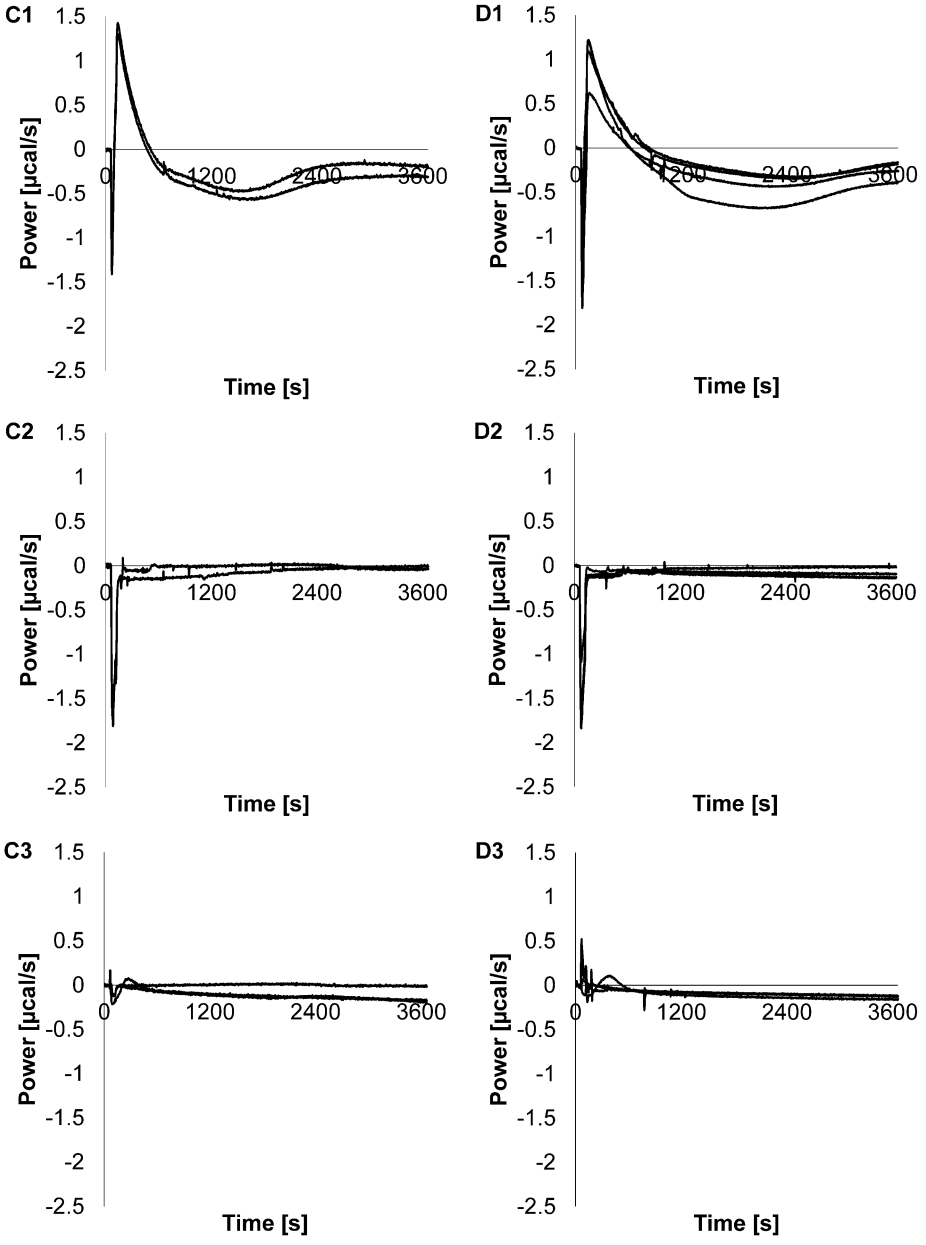


**Figure A3 continued.**

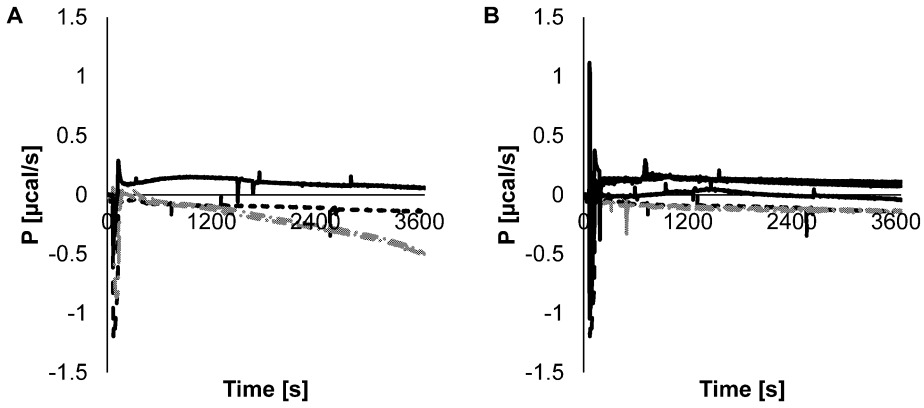


**Figure A4.** Raw data of the thermal power supply of the ITC in time for an injection of 30 µL of 127 mM allolactose into the sample cell with 0.4 g·L$^{-1}$ (A) β-gal-A, and (B) β-gal-D at 40ºC and pH 6.0. The concentration of allolactose during the reaction is 13 mM. Lines: ——— allolactose into enzyme solution; – – allolactose into buffer solution; — · — buffer into enzyme solution.

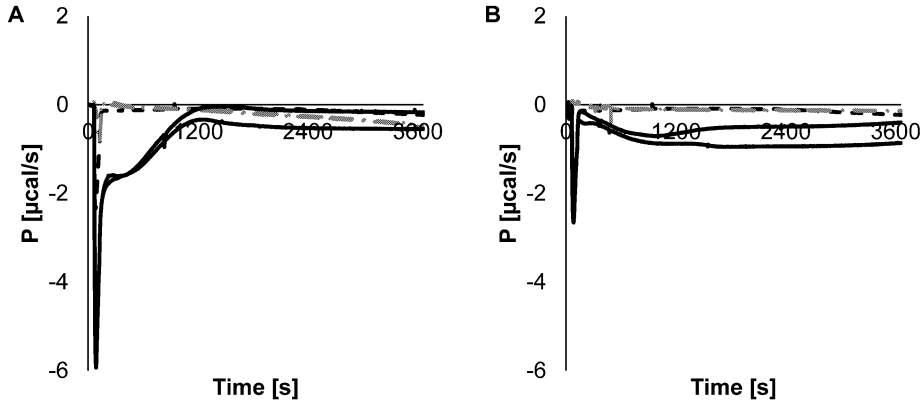


**Figure A5.** Raw data of the thermal power supply of the ITC in time for an injection of 30 µL of 119 mM 4-galactosyllactose into the sample cell with 0.4 g·L$^{-1}$ (A) β-gal-A, and (B) β-gal-D at 40ºC and pH 6.0. The concentration of 4-galactosyllactose during the reaction is 12 mM. Lines: ——— 4-galactosyllactose into enzyme solution; – – 4-galactosyllactose into buffer solution; — · — buffer into enzyme solution.

# Chapter 3

## Effects of carbohydrates on the *o*NPG converting activity of β-galactosidases




### Abstract

The effects of high concentrations of carbohydrates on the *o*NPG converting activity of β-galactosidase from *Bacillus circulans* are studied to get a better understanding on the enzyme behavior in concentrated and complicated systems in which enzymatic synthesis of galacto-oligosaccharides is usually performed. The components that were tested were glucose, galactose, lactose, sucrose, trehalose, raffinose, Vivinal-GOS, dextran-6,000, dextran-70,000, and sarcosine.

Small carbohydrates act as acceptor in the reaction. This speeds up the limiting step, which is binding of the galactose residue with the acceptor, and release of the product. Simultaneously, both inert and reacting additives seem to cause some molecular crowding, which results in a higher enzyme affinity for the substrate. The effect of molecular crowding on the enzyme activity is small compared to the effect of carbohydrates acting in the reactions as acceptors.

The effects of reactants on β-galactosidases from *B.circulans, A.oryzae* and *K.lactis* are compared.


*This chapter has been submitted as: Warmerdam A, Wang J, Boom RM, Janssen AEM. Effects of carbohydrates on the oNPG converting activity of β-galactosidases.*

## Introduction

Galacto-oligosaccharides (GOS) are widely recognized as prebiotics because they are indigestible in the human intestine and have a positive effect on the microflora in the colon (Barreteau et al. 2006; Macfarlane et al. 2008). They are commonly added to infant nutrition formula and to yoghurts and drinks (Macfarlane et al. 2008; Playne and Crittenden 2009).

GOS are typically produced from lactose via enzymatic synthesis with β-galactosidases (Barreteau et al. 2006; Mahoney 1998; Playne and Crittenden 2009; Prenosil et al. 1987). A β-galactosidase preparation from *Bacillus circulans*, called Biolacta N5, is known for its high transgalactosylation activity compared to other β-galactosidases (Boon et al. 2000; Nakanishi et al. 1983). It produces relatively large amounts of oligosaccharides with a higher degree of polymerization compared to β-galactosidases from *Aspergillus oryzae* and *Kluyveromyces lactis* for example (Boon et al. 2000; Urrutia et al. 2013). High GOS yields are obtained especially at high initial lactose concentrations (Boon et al. 2000; Huber et al. 1976).

Reaction rates, equilibria, and mechanisms of biochemical reactions are usually investigated in diluted systems. In order to better understand lactose conversion in concentrated systems, where large amounts and many different types of reactants and products are present, it is essential to study the effect of these carbohydrates on GOS synthesis. Each of these reactants and products may have its influence on GOS production: they might be converted into other products or might inhibit the reaction (Prenosil et al. 1987). These reactants and products influence the reaction, especially at high concentrations, and thus co-determine the yield of GOS.

Another phenomenon that might play a role in concentrated systems is molecular crowding (Ellis 2001; Minton 2001). The high concentration of molecules physically excludes some of the volume for other molecules. The excluded volume is the volume in a system that is occupied by molecules and cannot be occupied by the center of other molecules. This means that the effective concentration of components can be much larger than the actual concentration. Molecular crowding can affect the reaction rate, the reaction equilibrium, the enzyme activity, and the stability, and can cause diffusion limitation. (Kim and Yethiraj 2009; Zhou et al. 2008; Zimmerman and Minton 1993) Molecular crowding has been mainly studied for a better understanding of biochemical reactions in a cellular environment, but it is also relevant for enzymatic reactions under concentrated conditions.

The aim of this study is to investigate the effect of inert and reacting carbohydrates on the behavior of Biolacta N5 in concentrated systems. The effects of broad concentration ranges of several inert components (sarcosine and dextrans) and of

reacting carbohydrates on the behavior of β-galactosidase from *Bacillus circulans* during *o*NPG conversion were investigated.

The addition of reactants to the *o*NPG assay was investigated for β-galactosidases from both *A.oryzae* and *K.lactis*, to obtain a better mechanistic understanding of the activity of various β-galactosidases and to investigate the reason of the high GOS yields when using Biolacta N5.

## Materials and methods

### *Materials*



Lactose monohydrate (Lactochem), Vivinal-GOS, and a β-galactosidase preparation from *Bacillus circulans* called Biolacta N5 (Daiwa Kasei K. K., Japan) were gifts from FrieslandCampina (Beilen, The Netherlands). Biolacta N5 was previously found to have a total protein content of 19 ± 3% (Warmerdam et al. 2013). In all calculations, the total enzyme concentration was assumed to be equal to the total protein concentration, because the actual enzyme concentration is not known.

β-Galactosidase from *Aspergillus oryzae* (Lactase L017P) was a gift from Biocatalysts (Nantgarw, UK). β-Galactosidase from *Kluyveromyces lactis* (Lactozyme) was purchased from Sigma-Aldrich (Steinheim, Germany). The enzyme concentration was calculated based on the total mass of solid and liquid taken from the preparation for *A.oryzae* and *K.lactis*, respectively.

D(+)-Galactose, D(+)-glucose, sucrose, D(+)-trehalose dihydrate, D(+)-raffinose pentahydrate, sarcosine (*N*-Methylglycine), dextran-6,000 and 70,000 (from *Leuconostoc spp.*), maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, sulfuric acid, sodium hydroxide, *o*-nitrophenyl β-D-galactopyranoside (*o*NPG), and *o*-nitrophenol (*o*NP) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium carbonate, citric acid monohydrate, and disodium hydrogen phosphate were purchased from Merck (Darmstadt, Germany).

McIlvaine's buffer was prepared by adding together 0.1 M citric acid and 0.2 M disodium hydrogen phosphate in the right ratio to achieve a pH of 6.0 or 8.0.

### *Effect of carbohydrates on activity*

The enzyme activity measurements were adapted from Nakanishi et al. (1983). A stock solution of *o*NPG in buffer with a concentration of 0.25% (w/w) was prepared.

Besides, solutions of galactose, glucose, lactose, sucrose, trehalose, raffinose, sarcosine, dextran-6,000, and dextran-70,000 in buffer with varying concentrations were prepared. Stock solutions of Biolacta N5 were prepared in McIlvaine's buffer of pH 6.0. The stock solution of β-galactosidase from *A.oryzae* was prepared by adding 37 mg solid enzyme preparation into 50 mL of McIlvaine's buffer of pH 6.0 and the stock solution of β-galactosidase from *K.lactis* was prepared by adding 0.52 g liquid enzyme preparation into 4.6 g of McIlvaine's buffer of pH 8.0.

An Eppendorf tube with a mixture of 790 µL of 0.25% (w/w) *o*NPG solution and 189 µL of carbohydrate solution was preheated in an Eppendorf Thermomixer at 40ºC and 600 rpm for 10 minutes. Subsequently, 21 µL of enzyme solution was added and the mixtures were incubated for another 10 minutes at 40ºC and 600 rpm.



A volume of 1.0 mL of 10% (w/w) Na2CO3 solution was added to stop the reaction; afterwards, the absorbance of *o*NP was measured at 420 nm. The *o*NP concentration was determined using the law of Lambert-Beer ($\varepsilon$ = 4576 $M^{-1} \cdot cm^{-1}$). The carbohydrates did not affect the extinction coefficient. The *o*NP formation was found to be linear during the first 10 minutes of the reaction. This initial rate of *o*NP formation was expressed in $mmol \cdot min^{-1} \cdot g\ protein^{-1}$ unless otherwise stated.

### *Kinetics of oNPG conversion in the presence of carbohydrates*

In order to determine the $K_m$ and $v_{max}$ for *o*NPG conversion with Biolacta N5 in the absence and presence of several carbohydrates, *o*NPG solutions with concentrations varying from 3.4 to 67 mM and a Biolacta N5 solution of approximately 200 $mg \cdot L^{-1}$ were prepared. Besides, solutions were prepared with varying concentrations glucose, sucrose, trehalose, raffinose, and dextran-6,000 in buffer. During the activity assay, 790 µL of *o*NPG solution, 189 µL of carbohydrate solution and 21 µL of enzyme solution were incubated together for 0, 3, 5, and 10 minutes.

A volume of 1.0 mL of 10% (w/w) $Na_2CO_3$ solution was added to stop the reaction and, afterwards, the absorbance of *o*NP was measured at 420 nm. The initial rate of *o*NP formation was determined for each combination of initial *o*NPG concentration and carbohydrate concentration. The kinetic parameters $K_m$ and $v_{max}$ were determined with simple Michaelis-Menten kinetics:

$$v_1 = \frac{v_{max} \cdot [S_1]}{K_m + [S_1]} \quad (1)$$

where $v_1$ is the initial reaction rate of *o*-nitrophenol (*o*NP) formation in $mmol \cdot min^{-1} \cdot g\ protein^{-1}$, $v_{max}$ is the maximum reaction rate of *o*NP formation in $mmol \cdot min^{-1} \cdot g\ protein^{-1}$,

$[S_1]$ is the initial concentration of *o*NPG during the assay in mol·L$^{-1}$, and $K_m$ is the Michaelis-Menten parameter for *o*NPG in mol·L$^{-1}$.

### *Water activity of carbohydrate solutions*

The water activities of solutions of sucrose, trehalose, dextran-6,000, and dextran-70,000 in buffer at varying concentrations were measured with an Aqualab Water Activity Meter (Decagon Devices, Inc., Pullman, WA, United States) at 40ºC.

### *Solubility of oNPG in carbohydrate solutions*



The solubility of *o*NPG in several carbohydrate solutions was determined at 23 or 40ºC. Solutions of sucrose, trehalose, and raffinose with concentrations varying between 4.2 and 33 g·L$^{-1}$ were prepared. Subsequently, either 0.02 or 0.03 g of *o*NPG, for the solubility test at 23 or 40ºC, respectively, was dissolved in 1.0 mL of the carbohydrate solutions at a temperature of 65ºC. Afterwards, the temperature was cooled down to 23 or 40ºC and was constant during the night. In the meanwhile, crystallization occurred.

The obtained suspensions were centrifuged in a Beckman Coulter Allegra X-22R centrifuge for 15 minutes at 15,500 rpm and 23 or 40ºC to remove the crystals from the supernatant. The supernatant was transferred to cuvettes and the absorbance was measured with a spectrophotometer at 320 nm. The maximum concentration of *o*NPG that was soluble in the solutions was determined using the law of Lambert-Beer. The extinction coefficient was determined to be 2232 M$^{-1}$·cm$^{-1}$.

### *Sample preparation for analysis of the product composition*

Volumes of 790 µL of 0.25% (w/w) *o*NPG solution, 189 µL of 105 g·L$^{-1}$ galactose or 108 g·L$^{-1}$ glucose or 465 g·L$^{-1}$ trehalose solution, and 21 µL of Biolacta N5 (200 mg solids·L$^{-1}$) were incubated together at 40ºC. After 0, 10, 60, and 120 minutes of incubation, samples of 200 µL were taken and added to 100 µL of 5% (w/w) sulphuric acid to inactivate the enzyme. The same procedure was also carried out with reference samples in which one or more of the components were replaced with buffer solution.

Before the HPLC analysis, the enzyme was removed from the samples by filtering the samples at 14,000 x g and 18ºC for 30 minutes using pretreated Amicon® ultra-0.5 centrifugal filter devices (Millipore Corporation, Billerica, MA, United States) with a cut-off value of 10 kDa in a Beckman Coulter Allegra X-22R centrifuge. The pre-treatment of the filters consisted of two centrifugation steps: first, 500 µL of Milli-Q

water was centrifuged at 14,000 x g at 18ºC for 15 minutes; and second, the filters were placed up-side-down in the tube and centrifuged at 14,000 x g at 18ºC for 5 minutes. After filtration, the samples were neutralized with 5% (w/w) sodium hydroxide.

*Measurement of the carbohydrate composition*

The filtered samples were analysed with HPLC using a Rezex RSO oligosaccharide column (Phenomenex, Amstelveen, the Netherlands) at 80ºC. The column was eluted with Milli-Q water at a flow rate of 0.3 mL/min. The eluent was monitored with a refractive index detector.



The standards that were used for calibration of the column were lactose, glucose, galactose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose. Galacto-oligosaccharides up to a degree of polymerization of 7 were assumed to have the same response as the glucose-oligomers with equal degree of polymerization. This was confirmed with mass balances.

## Results and discussion

Figure 1 shows the *o*NPG converting activity of Biolacta N5 in the presence of several carbohydrates at varying concentrations. The activity was not greatly affected by the presence of sarcosine and dextran 70,000, over all tested concentrations. The activity in the presence of dextran-6,000 was slightly higher than in absence of dextran-6,000, but was 60% lower at a dextran-6,000 concentration of 80 $g \cdot L^{-1}$.

In the presence of sucrose, trehalose, and raffinose, the enzyme activity was much higher than without any additives: the activities increased up to 350%. This activity increase is similar to the activity increase of α-amylase by addition of a.o. sucrose and trehalose described by Yadav (2013). In the presence of lactose and Vivinal-GOS, the *o*NPG converting activity decreased with increasing lactose or Vivinal-GOS concentration. This may be due to the competition between *o*NPG and lactose or the oligosaccharides present in Vivinal-GOS, because both are substrates for the enzyme.

The products galactose and glucose have a strong positive effect on the *o*NPG converting activity: the activities increased up to 300% and 200%, respectively. Remarkable is the high activity at low galactose concentrations, and in the presence of glucose. Galactose and glucose are usually recognized to be inhibitors for β-galactosidases (Bakken et al. 1992; Boon et al. 1999; Chockchaisawasdee et al. 2005; Neri et al. 2009), but galactose and glucose here strongly promoted the conversion of *o*NPG into *o*NP. At galactose concentrations above 30 $g \cdot L^{-1}$, the activity decreased again

with increasing galactose concentration, which might be a sign of dominance of inhibition.

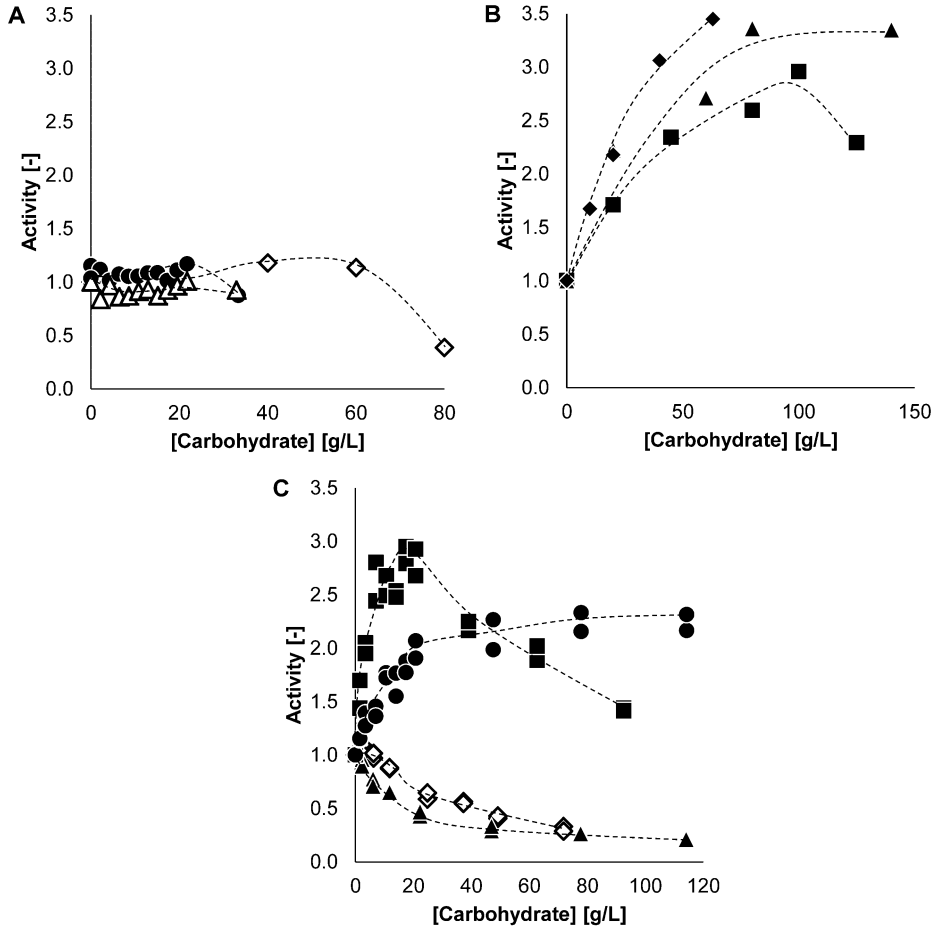


**Figure 1.** Initial oNPG converting activity of Biolacta N5 as a function of the carbohydrate concentration at 40ºC and pH 6.0 in the presence of (A) inert carbohydrates: Δ sarcosine[2], ◇ dextran-6,000[1], and ● dextran-70,000[2]; (B) small carbohydrates[1]: ■ sucrose, ▲ trehalose, and ♦ raffinose; (C) reactants[3]: ■ galactose, ● glucose, ◇ lactose, ▲ Vivinal-GOS. The activity is relative to the activity without added carbohydrates. (Lines for guidance.)

*[1] enzyme concentration during assay = 0.8 mg protein·L$^{-1}$; [2] enzyme concentration during assay = 1.6 mg protein·L$^{-1}$; [3] enzyme concentration during assay = 2.8 mg protein·L$^{-1}$.*

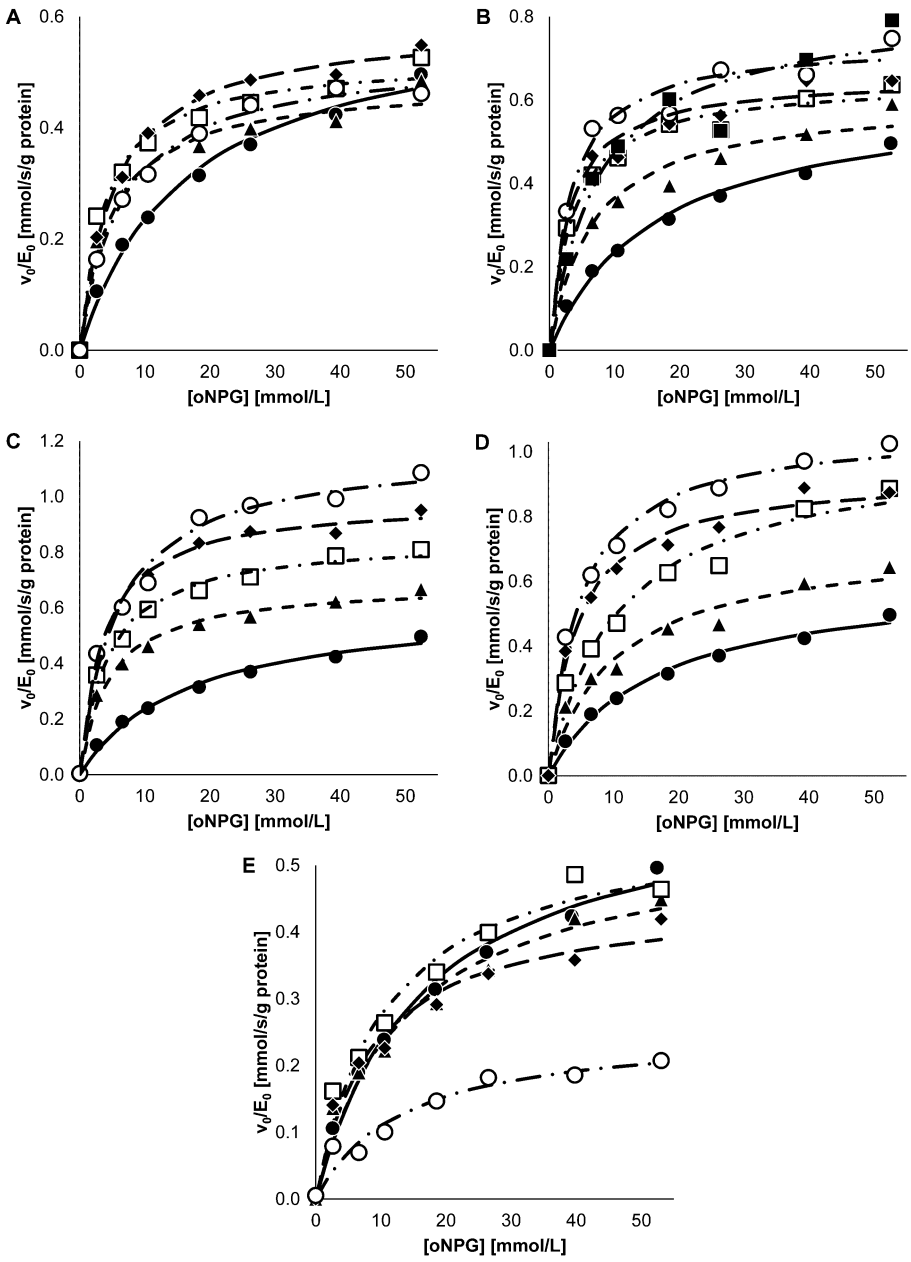


**Figure 2.** The initial rate of *o*-nitrophenol formation as a function of the initial *o*NPG concentration at 40ºC and pH 6.0 with an enzyme concentration of 0.8 mg protein·$L^{-1}$ (during the assay) in the presence of A. ●, —— 0; ▲,– – – 20; □,– · – · 40; ♦,— — 80; ○,— · – 131 g·$L^{-1}$ glucose; B. ●,—— 0; ▲,– – – 20; □, – · – · 45; ♦,— — 80; ○,— · – 100; ■,— · · 125 g·$L^{-1}$ sucrose; C. ●,—— 0; ▲,– – – 20; □,– · – · 60; ♦, — — 80; ○,— · – 140 g·$L^{-1}$ trehalose; D. ●,—— 0; ▲,– – – 10; □,– · – · 20; ♦,— — 40; ○,— · – 63 g·$L^{-1}$ raffinose; and E. ●,—— 0; ▲,– – – 20; □,– · – · 40; ♦,— — 60; ○,— · – 80 g·$L^{-1}$ dextran-6,000. The symbols are the measured data, the (dashed) lines represent the Michaelis-Menten model.

## *Kinetics of oNPG conversion in the presence of carbohydrates*

The initial rates of *o*NP formation in the presence of glucose, sucrose, trehalose, raffinose, and dextran-6,000 are shown in Figure 2. The data were fitted to the Michaelis Menten model to obtain the kinetic parameters $K_m$ and $v_{max}$, which are shown in Figure 3 and Figure 4, respectively.

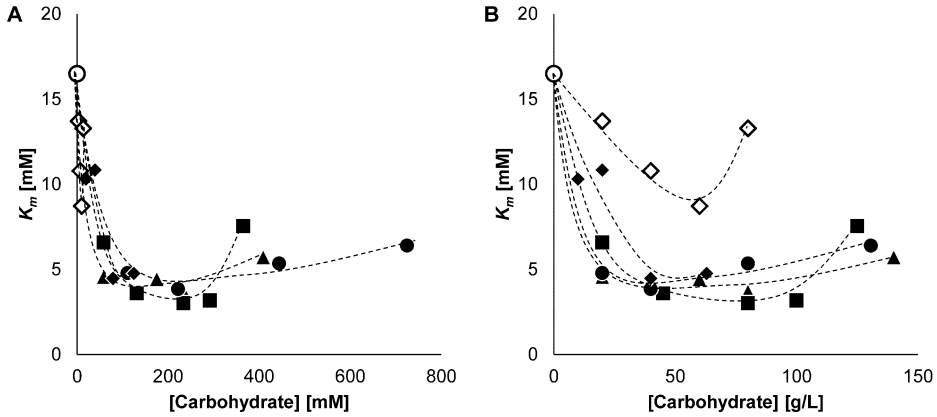


**Figure 3.** The Michaelis-Menten constant $K_m$ of β-galactosidase from *Bacillus circulans* for *o*NPG plotted as a function of the carbohydrate concentration, in (A) mM and (B) g·L$^{-1}$, in the presence of ○ no added carbohydrate; ● glucose, ■ sucrose, ▲ trehalose, and ♦ raffinose, and ◇ dextran-6,000 at 40ºC and pH 6.0. (Lines for guidance.)

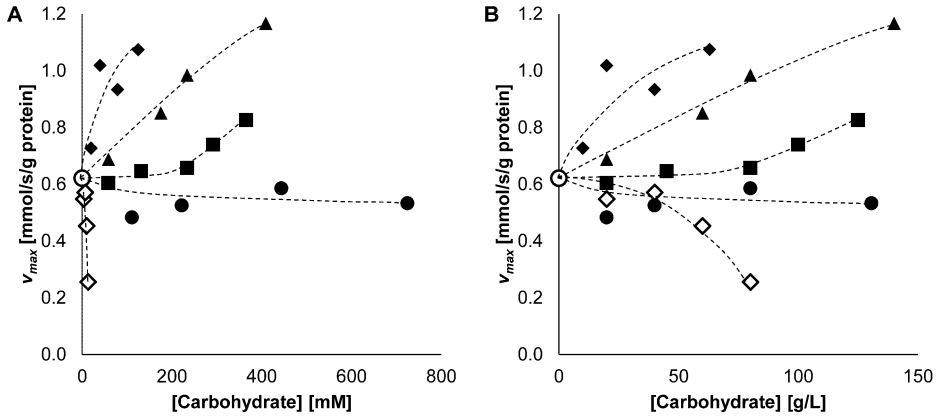


**Figure 4.** The maximum reaction rate of *o*NP formation $v_{max}$ with β-galactosidase from *Bacillus circulans* plotted as a function of the carbohydrate concentration, in (A) mM and (B) g·L$^{-1}$, in the presence of ○ no added carbohydrate; ● glucose, ■ sucrose, ▲ trehalose, and ♦ raffinose, and ◇ dextran-6,000 at 40ºC and pH 6.0. (Lines for guidance.)

Figure 3 shows that the Michaelis-Menten constant $K_m$ decreased with the addition of carbohydrates. On a molar concentration scale, this decrease was more or less independent of the type of carbohydrate added. However, on a mass concentration scale, dextran affected the $K_m$ less than smaller carbohydrates, due to its larger molecular weight. The smaller carbohydrates affected the $K_m$ all in a similar way.

Below carbohydrate concentrations of approximately 300 mM, the $K_m$ decreased with increasing carbohydrate concentration, which implies that the affinity of the enzyme for the substrate *o*NPG increased with the addition of these carbohydrates. At higher carbohydrate concentrations, the $K_m$ increased again slightly with further increase of the carbohydrate concentration.



Figure 4 shows more complex effects on $v_{max}$ for each of the tested carbohydrates (both on a molar concentration scale and on a mass concentration scale). $v_{max}$ did not change significantly with the glucose concentration, but in the presence of sucrose, trehalose, and raffinose, the $v_{max}$ increased strongly with increasing carbohydrate concentration, with the largest effect for raffinose, and the smallest for sucrose. Dextran-6,000 had a negative effect on $v_{max}$: it decreased with increasing dextran-6,000 concentration.

## *Characteristics of reaction medium*

To exclude effects by the medium from causing changes in enzyme activity and enzyme kinetics with the addition of carbohydrates, the water activity and the solubility of *o*NPG in the carbohydrate solutions were measured.

### *Water activity*

Figure 5 shows the water activities of solutions of sucrose, trehalose, dextran-6,000, and dextran-70,000 in buffer at 40ºC.

The fact that the water activity is not one at carbohydrate concentration zero, is due to the presence of the buffer. The water activities of each of the carbohydrate solutions were found to only show a very slight decrease from approximately 0.994 to 0.986 over a concentration range up to 130 $g \cdot L^{-1}$. This small decrease cannot explain the large changes in enzyme activity and enzyme kinetics. Gosling et al. (2011) found earlier that oligosaccharide levels were not affected by even larger changes in water activity.

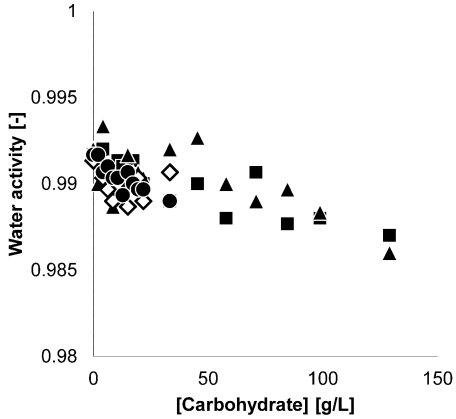


**Figure 5.** Water activity of solutions of ■ sucrose, ▲ trehalose, ◇ dextran-6,000, and ● dextran-70,000 in buffer at 40ºC.



*Solubility of oNPG in carbohydrate solutions*

The addition of carbohydrates could lead to a shift in the distribution of *o*NPG molecules between being free in solution and being bound to the enzyme, since the associated state will have a lower overall volume than the dissociated state. That would mean that with the addition of carbohydrates, the *o*NPG molecule prefers to leave the crowded environment and prefers to bind to the enzyme, which could result in a decrease in $K_m$ and an increase in the enzyme activity. If this is the case, the solubility of *o*NPG is expected to decrease with addition of carbohydrates when no enzyme is present. Figure 6 shows the solubility of *o*NPG in several carbohydrate concentrations at 23 and 40ºC.

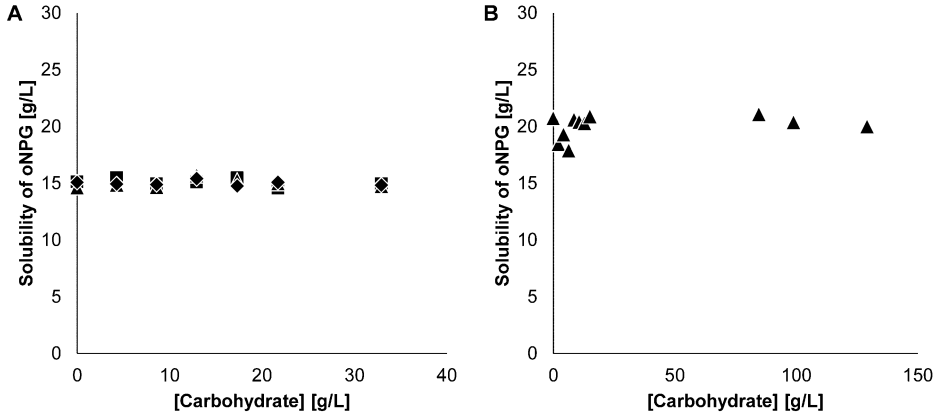


**Figure 6.** Solubility of *o*NPG in carbohydrate solutions at (A) 23ºC and (B) 40ºC. Symbols: ■ sucrose, ▲ trehalose, and ♦ raffinose.

The solubility of *o*NPG in buffer was determined to be 15 ± 1 and 21 ± 0 $g \cdot L^{-1}$ at 23 and 40ºC, respectively. The addition of sucrose, trehalose, and raffinose in the tested concentration ranges did not change the solubility, which means that the changes in enzyme activity and enzyme kinetics were not caused by a change in the solubility of *o*NPG.

Since the water activity and the solubility of *o*NPG do not change with increasing carbohydrate concentration, the characteristics of the reaction medium can be excluded as cause of the changes in enzyme activity and enzyme kinetics.



### *Characteristics of the enzyme*

Since the characteristics of the reaction medium do not change with the addition of the carbohydrates, we also investigated the effect of the carbohydrates on the enzyme itself.

In general, *o*NPG conversion with β-galactosidases takes place in two steps. First, an enzyme-galactose complex is formed and *o*NP is released, and second, the galactose is released since water is used as the acceptor molecule: hydrolysis takes place. However, in the presence of carbohydrates, transglycosylation might take place. The reaction mechanism that we propose is shown in Figure 7.

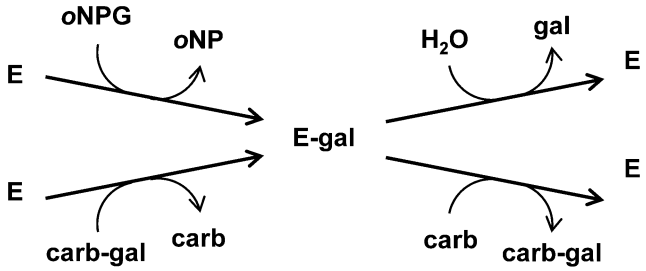


**Figure 7.** A schematic representation of the reaction mechanism of β-galactosidase from *Bacillus circulans*. Abbreviations: E, enzyme; *o*NPG, *o*-nitrophenyl β-D-galactopyranoside; *o*NP, o-nitrophenol; E-gal, enzyme-galactose complex; gal, galactose; carb, carbohydrate; carb-gal, oligosaccharide that consist out of one galactose unit attached to another carbohydrate.

Instead of water, the carbohydrates may also be used as an acceptor molecule, and form a product together with the galactose unit that is released from the enzyme-galactose complex. A similar phenomenon was observed by Gosling et al. (2011) during lactose conversion in the presence of sucrose. In this case, the product should have a degree of polymerization that is larger than the carbohydrate added. To test this

hypothesis, we measured the product composition during *o*NPG conversion with Biolacta N5 in presence of galactose, glucose, and trehalose. The results are shown in Figure 8.

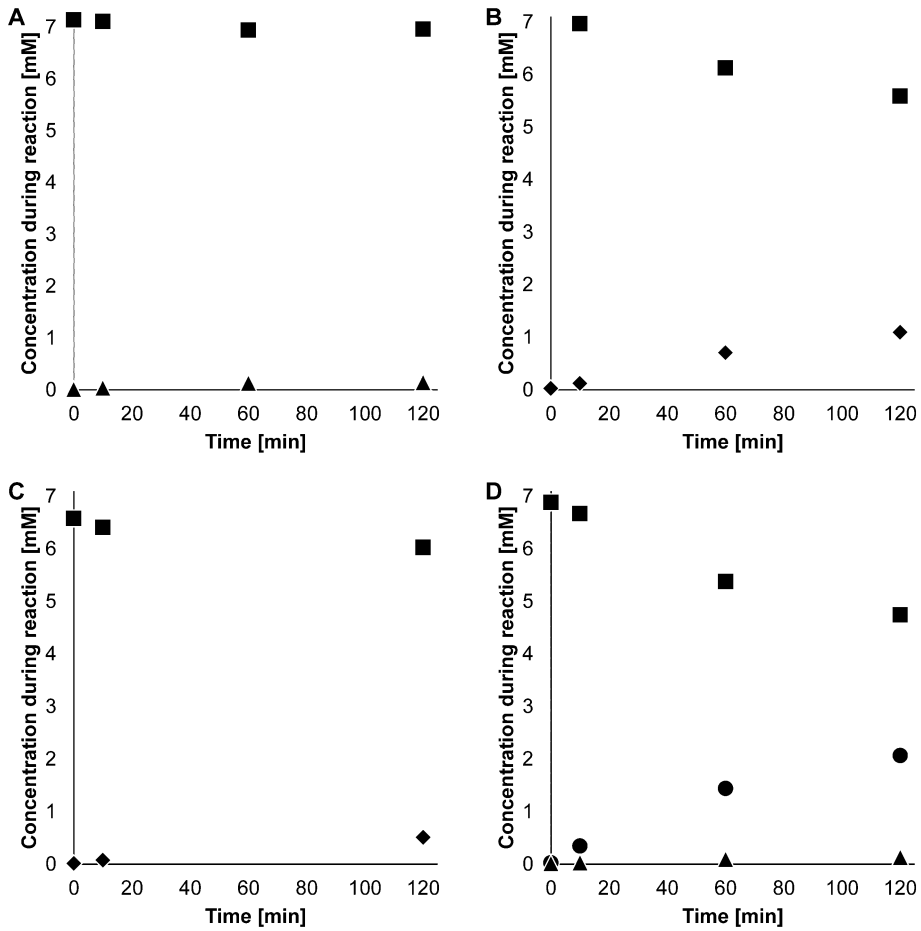


**Figure 8.** *o*NPG and carbohydrate profiles during *o*NPG conversion at 2.0 g·L$^{-1}$ with 0.8-0.9 mg·L$^{-1}$ Biolacta N5 at 40ºC and pH 6.0 in presence of (A) no added carbohydrates, (B) 20 g·L$^{-1}$ galactose, (C) 20 g·L$^{-1}$ glucose, and (D) 88 g·L$^{-1}$ trehalose. Symbols: ■ *o*NPG; ▲ galactose; ♦ disaccharides other than lactose; and ● trisaccharides. (The reaction was followed for two hours (whereas the activity assay is 10 minutes) to find larger differences in break down or formation of the components.)

During the conversion of *o*NPG without any added carbohydrates, a slight decrease in *o*NPG concentration and a slight increase in galactose concentration were detected. When galactose, glucose, or trehalose was added, much more *o*NPG was converted which is in agreement with the increases in activity (Figure 1). Besides, disaccharides were formed with addition of galactose and glucose, whereas trisaccharides were formed

in presence of trehalose. This confirms our hypothesis that the added carbohydrates participate in the reaction, and by removing galactose from the active center, frees up the enzyme for faster *o*NPG conversion.

Some carbohydrates that do not contain a galactose moiety can only act as acceptor and thus will speed up the overall reaction by freeing up the active center; other sugars (such as galactose itself) may also act as donor, and in this case both competitive inhibition and the acceptor-acceleration takes place. In this case, one may expect the optimum as was observed in Figure1C.



### *Changes in $K_m$ and $v_{max}$ explained*

The increase in affinity caused by the addition of carbohydrates may be explained by molecular crowding. A similar $K_m$ decrease was reported before by Jiang and Guo (2007) for addition of dextrans to isochorismate synthase. Yadav (2013) described an activity increase of α-amylase by addition of a.o. sucrose and trehalose which was due to molecular crowding. The association of the enzyme with *o*NPG probably results in a volume reduction which is thermodynamically favorable in a crowded environment (Ellis 2001; van Boekel 2009). Besides, if this enzyme is active as a folded protein, crowding will stabilize the folded proteins (Ellis 2001; van Boekel 2009).

The smaller carbohydrates have a stronger effect on a mass concentration scale on the $K_m$ than dextran, which is inert. This may be explained by dextran having a smaller total excluded volume per gram than the small carbohydrates, although the total volume of the carbohydrates is equal in each case. Figure 9 shows the calculated total excluded volume for various concentrations of added carbohydrates.

The calculations were carried out assuming the carbohydrate and the substrate to be spheres with radii $R_c$ and $R_s$, with $R = 8.26 \cdot 10^{-1} \cdot MW^{1/3}$ nm ($MW$ in kD), similar to the approach by Batra et al. (2009) for dextran. The total excluded volume is equal to the number of carbohydrate molecules multiplied by the excluded volume of each carbohydrate molecule with radius $R_c$+$R_s$, since the center of the substrate cannot enter there. This results in a larger total excluded volume when a certain mass of small carbohydrates is present compared to when the same mass of large carbohydrates is present (Minton 2001; Zhou et al. 2008). Therefore, the effect of molecular crowding in case of small carbohydrates on the $K_m$ is larger than in case of dextran. Besides, the interactions of the small carbohydrates might also play a role in the reaction.

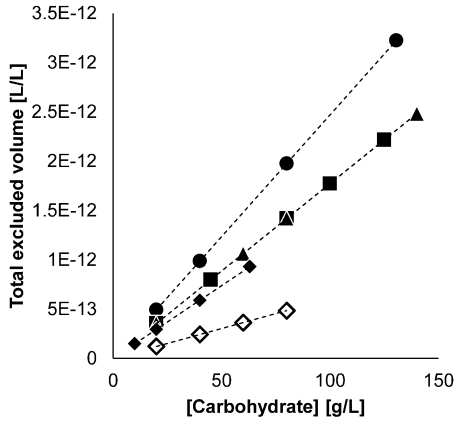


**Figure 9.** Calculated total excluded volume for substrate molecules as a function of the carbohydrate concentration. The total excluded volume is the amount of carbohydrate molecules multiplied with the excluded volume of each carbohydrate molecule. Carbohydrate and substrates are assumed to be spherical molecules with radii $R_c$ and $R_s$, with $R = 8.26 \cdot 10^{-1} \cdot MW^{1/3}$ nm ($MW$ in kD). The excluded volume of each carbohydrate molecule has a radius of $R_c$+$R_s$. Symbols: ● glucose, ■ sucrose, ▲ trehalose, and ♦ raffinose, and ◇ dextran-6,000.



The slight increase of $K_m$ at higher carbohydrate concentrations might be a result of diffusion limitation, because diffusion is slower in crowded systems, and thus the frequency of the encounters between enzyme and substrate is reduced (Ellis 2001; van Boekel 2009).

The maximum rate of *o*NP formation $v_{max}$ was higher in the presence of sucrose, trehalose, or raffinose than with dextran mainly because these carbohydrates are acceptor in the reaction. They cause a quick release of the enzyme (from the enzyme-galactose complex) and the enzyme is free in solution again, so that another *o*NPG molecule can form a complex with the enzyme. Dextran is inert and cannot be used as an acceptor molecule.

Since an increase in $v_{max}$ was found with increasing carbohydrate concentration, the attachment of an acceptor molecule (either water or a carbohydrate molecule) is supposed to be the rate limiting step. The reaction is accelerated when the carbohydrate concentration is increased. This implies that water is a very poor acceptor molecule as the molar water content is still very high compared to the carbohydrate concentration, and water molecules are very mobile. The affinity of the enzyme-galactose complex for the specific carbohydrate molecules therefore has to be much higher than the affinity for a water molecule.

The addition of glucose does not change $v_{max}$. According to Figure 8, the lowest conversion of *o*NPG was found with glucose among the tested carbohydrates. This can

be explained by glucose acting as an uncompetitive inhibitor (Boon et al. 1999; Deschavanne et al. 1978). If glucose inhibits the reaction at the same time that it may act as an acceptor, these two effects may cancel out each other. Next to glucose, sucrose is also an uncompetitive inhibitor (Deschavanne et al. 1978). However, the inhibition constant of sucrose is higher than that of glucose (Deschavanne et al. 1978), which means that the affinity for sucrose (as an inhibitor) is lower than for glucose. The inhibiting effect can explain the $v_{max}$ caused by glucose being lower than that of sucrose. Raffinose and trehalose do not have any inhibiting effect.

The presence of dextran decreased $v_{max}$ at high concentrations, which may be due to diffusion limitation.

Overall, the effect of carbohydrates acting in the reaction as acceptors, represented by a change in $v_{max}$, is large compared to the effect of molecular crowding on the enzyme activity, represented by a decrease in $K_m$.



### *Mechanistic understanding of β-galactosidase activity*

For a better mechanistic understanding of the activity of β-galactosidases, the *o*NPG converting activity of β-galactosidases of *A.oryzae* and *K.lactis* was measured in the presence of small carbohydrates. The results are shown in Figure 10.

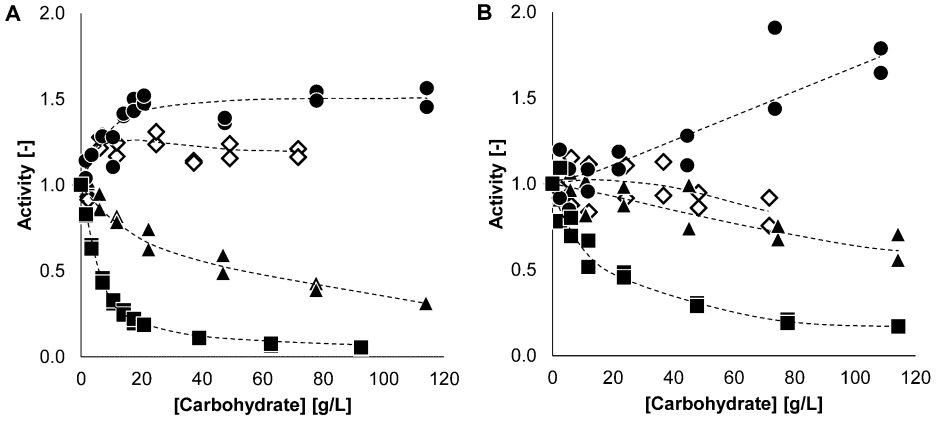


**Figure 10.** Initial *o*NPG converting activity of β-galactosidase from (A) *Aspergillus oryzae* at 15 mg solids·$L^{-1}$, 40ºC and pH 6.0, and (B) *Kluveromyces lactis* at 144 mg enzyme preparation·$L^{-1}$, 20ºC and pH 8.0, as a function of the carbohydrate concentration in presence of ◇ lactose, ▲ Vivinal-GOS, ■ galactose, and ● glucose. (Lines for guidance.)

Figure 10 shows, together with Figure 1C, that the reactants (lactose, oligosaccharides, galactose, and glucose) affect the three enzymes in very different

ways. In production systems with β-galactosidase of *B.circulans* with a high lactose concentration, where lactose is converted into GOS, the competition between lactose and Vivinal-GOS for association with the enzyme is advantageous: the enzyme has a high affinity for these components and will form products with a higher degree of polymerization. The latter was previously observed by Boon et al. (2000). The decrease in activity in the presence of galactose does not hinder the reaction, since hydrolysis is limited in concentrated solutions, and thus the galactose concentration remains low.

The *o*NPG converting activity of β-galactosidase of *A.oryzae* (Figure 10A) is (slightly) higher in presence of lactose and glucose, but lower in the presence of Vivinal-GOS and galactose, with the lowest activity in presence of galactose. The higher activity in the presence of lactose implies that lactose is used as an acceptor molecule instead of a donor molecule: the affinity of the enzyme for *o*NPG is much higher than for lactose, which will not be able to displace *o*NPG. We expect glucose to act as an acceptor as well. Galactose inhibits the enzyme already at low concentrations on, which is in agreement with the conclusion by Boon et al. (2000) that galactose is an inhibitor.

The *o*NPG converting activity of β-galactosidase of *K.lactis* (Figure 10B) showed similar trends. Vivinal-GOS reduced the activity. Lactose does not affect the activity much, but it probably acts simultaneously as an acceptor and as a donor. As with β-galactosidase from *A.oryzae*, glucose will be an acceptor; galactose once more acts as inhibitor (Boon et al. 2000). Since this enzyme shows much more hydrolysis activity, product inhibition by galactose would probably hinder the production of GOS even at high lactose concentrations.

Previously it was shown that Biolacta N5 was a productive enzyme preparation in terms of oligosaccharide yield compared to other β-galactosidases (Boon et al. 2000; Nakanishi et al. 1983). This can now be explained by the much higher affinity for lactose, and the suitability of glucose and galactose as acceptor molecules. Besides, galactose is not inhibiting the enzyme that strong. These mechanistic differences are the reason that the enzyme from *B.circulans* shows much higher production rates of GOS than the β-galactosidases of *A.oryzae* and *K.lactis* under comparable conditions.

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# Chapter 4

## Kinetic characterization of β-galactosidases


## Abstract

Many β-galactosidases show large differences in galacto-oligosaccharide production and lactose hydrolysis. In this study, a kinetic model is developed in which the effect of lactose, glucose, galactose, and oligosaccharides on the *o*NPG converting activity of various β-galactosidases is quantified. *o*NPG as competing substrate to lactose yields more information than only the conversion of lactose itself.

The reaction rate with lactose or oligosaccharides as substrate relative to that with water as acceptor is much higher for the β-galactosidase of *B.circulans* than the β-galactosidases of *A.oryzae* and *K.lactis*. Besides, the β-galactosidase of *B.circulans* has a high reaction rate with galactose as acceptor, in contrast to those of *A.oryzae* and *K.lactis*. The latter two are strongly inhibited by galactose. These differences explain why β-galactosidase of *B.circulans* gives higher yields in GOS production than other β-galactosidases. Many of the reaction rate constants for the β-galactosidase isoforms of *B.circulans* increase with increasing molecular weight of the isoform. This indicates that the largest isoform β-gal-A is most active in GOS production. However, its hydrolysis rate is also much higher, which results in a faster hydrolysis of oligosaccharides as well.




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## Introduction

The β-galactosidases of *Bacillus circulans* have already been a subject of investigation for several decades because of their high transgalactosylation activity compared to β-galactosidases from other origins (Boon et al. 2000; Gosling et al. 2011; Mozaffar et al. 1984; Palai et al. 2012; Rodriguez-Colinas et al. 2012; Song et al. 2011; Vetere and Paoletti 1998). High transgalactosylation activity is essential for the production of galacto-oligosaccharides (GOS). GOS have several health benefits and are recognized as prebiotics. They are added to infant nutrition and to yoghurts and drinks (Macfarlane et al. 2008; Mahoney 1998; Playne and Crittenden 2009).

The β-galactosidases of *Bacillus circulans* produce longer oligosaccharides and a higher yield compared to β-galactosidases from *Aspergillus oryzae* and *Kluyveromyces lactis* (Boon et al. 2000; Urrutia et al. 2013). However, it is not well understood why these differences arise among various sources. In addition, differences arise among the β-galactosidases from *Bacillus circulans*. The isoforms in the preparation called Biolacta N5 are called β-gal-A, β-gal-B, β-gal-C, and β-gal-D and have molecular weights of 189, 154, 135, and 92 kDa, respectively. The largest isoform is thought to be truncated by proteolysis into the smaller isoforms. Although they all have β-gal-A as precursor, the isoforms diverge in their activity (Song et al. 2011). β-Gal-A mainly hydrolyzes lactose and produces only a small amount of trisaccharides, whereas β-gal-B, β-gal-C, and β-gal-D produce high amounts of tri- and tetrasaccharides (Song et al. 2011). In a previous study (Warmerdam et al. 2013), we have purified Biolacta N5 into four active fractions which correspond with β-gal-A, β-gal-B, β-gal-C, and β-gal-D. Although the isoforms differed all in the ratios of transgalactosylation versus hydrolysis at low substrate concentrations, the selectivity hardly differed at high substrate concentrations, which are commonly used for GOS production. At high substrate concentrations, β-gal-A and β-gal-B showed the highest GOS formation rates. The differences are most likely due to the accessibility of the isoforms' active sites.

Besides, the reactants (lactose, galactose, glucose, oligosaccharides) were found to favorably influence the *o*NPG converting activity of Biolacta N5 (Warmerdam et al. Submitted for publication). Lactose and oligosaccharides were found to compete strongly with the artificial substrate *o*NPG for the active site of the enzyme. On the other hand, galactose and glucose were found to increase the *o*NPG converting activity of Biolacta N5 strongly because these carbohydrates act as acceptor molecules. (Warmerdam et al. Submitted for publication). While this has been done for complete enzyme cocktails such as Biolacta N5, it is not yet clear how individual galactosidases behave: since the isoforms show differences in GOS synthesis and hydrolysis, they are also expected to act different on the addition of reactants in the *o*NPG assay.

The aim of this study is therefore to obtain more insight in the specific reaction mechanism of individual β-galactosidases, through the development of a kinetic model on the influence of the reactants on the *o*NPG converting activity, which will quantify the differences among various β-galactosidases. The β-galactosidases that will be studied are preparations from *B.circulans, A.oryzae* and *K.lactis*. Besides, the effect of the reactants on the separate β-galactosidase isoforms from *B.circulans* will be studied.

## Theory

Figure 1 shows the reaction mechanism that we here propose to understand the effect of the reactants on the *o*NPG converting activity of β-galactosidases in presence of other molecules: lactose, Vivinal-GOS (oligosaccharides), glucose and galactose.

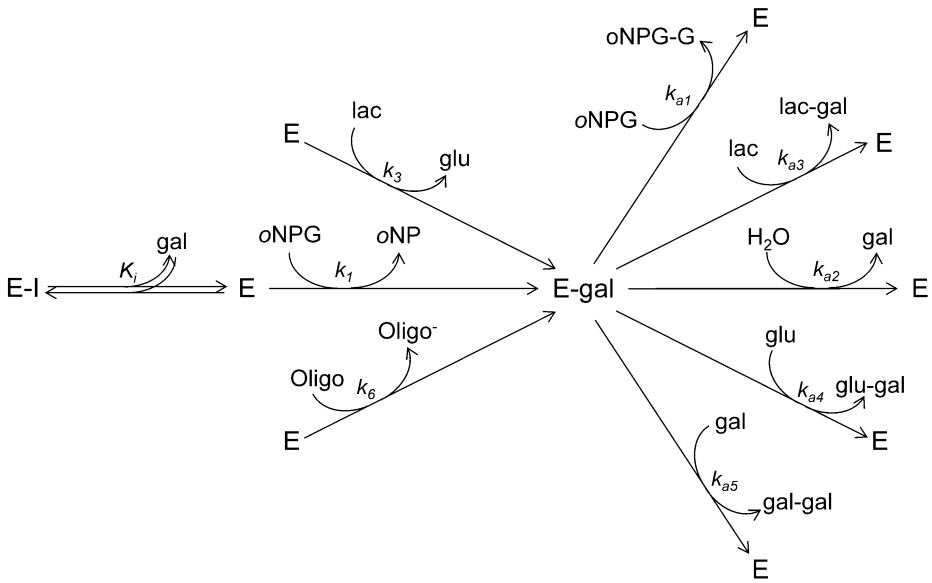


**Figure 1.** Proposed reaction mechanism of the conversion of *o*NPG in presence of various reactants. Abbreviations: E = enzyme; E-gal = enzyme-galactose complex; E-I; inhibiting enzyme-galactose complex; gal = galactose; gal-gal = disaccharide consisting of two galactose molecules; glu = glucose; glu-gal = disaccharide consisting of glucose and galactose; lac = lactose; lac-gal = trisaccharide consisting of lactose and galactose; Oligo = oligosaccharide; Oligo$^-$ = carbohydrate with one monosaccharide molecule less then precursor; *o*NPG-G = molecule consisting of *o*NPG and an extra galactose.

*o*NPG is hydrolyzed with β-galactosidases into *o*NP and galactose (gal) with formation of an intermediate enzyme-galactose complex (E-gal). Water is the acceptor

molecule. (Borralho et al. 2002; Ladero et al. 2001; Prenosil et al. 1987) These reactions are represented in Figure 1 with reaction rate constants $k_1$ and $k_{a2}$, respectively.

Lactose (lac) can be substrate ($k_3$) as well as acceptor ($k_{a3}$) in the reaction with β-galactosidases (Boon et al. 1999; Gosling et al. 2011; Huber et al. 1976; Prenosil et al. 1987). As substrate, glucose is released and an intermediate enzyme-galactose complex is formed. As acceptor, the galactose residue of the complex is attached to the lactose molecule and a trisaccharide (lac-gal) is formed. Next to lactose, many other molecules can act as acceptor molecule for the enzyme-galactose complex. In this model, glucose ($k_{a4}$) (Huber et al. 1976; Warmerdam et al. Submitted for publication), galactose ($k_{a5}$) (Vera et al. 2011; Warmerdam et al. Submitted for publication), and *o*NPG ($k_{a1}$) (Sauerbrei and Thiem 1992) are taken into account. Galactose can also act as an inhibitor for β-galactosidases (Bakken et al. 1992; Chockchaisawasdee et al. 2005; Greenberg and Mahoney 1982; Guven et al. 2011; Kim et al. 2004; Neri et al. 2009; Prenosil et al. 1987; Vera et al. 2011). The formation of an inhibiting enzyme-galactose complex (E-I) is described with inhibition constant $K_i$. Besides lactose and *o*NPG, oligosaccharides can be a substrate, which is described with reaction rate constant $k_6$.

Since we utilize initial rate measurements in this study, only reactants that are present prior to addition of the enzyme are taken into account in the model, i.e. formation of larger oligosaccharides is not considered.

The reaction scheme in Figure 1 can be used to derive the mole balances for the initial rate of *o*NP formation:

$$v_{0,oNP} = \frac{d[oNP]}{dt} = k_1 \cdot [oNPG] \cdot [E] \tag{1}$$

$$E_0 = [E] + [E\text{-}gal] + [E\text{-}I] \tag{2}$$

$$\begin{aligned} \frac{d[E\text{-}gal]}{dt} = {} & k_1 \cdot [oNPG] \cdot [E] - k_{a1} \cdot [oNPG] \cdot [E\text{-}gal] - k_{a2} \cdot [H2O] \\ & \cdot [E\text{-}gal] + k_3 \cdot [lac] \cdot [E] - k_{a3} \cdot [lac] \cdot [E\text{-}gal] - k_{a4} \\ & \cdot [glu] \cdot [E\text{-}gal] - k_{a5} \cdot [gal] \cdot [E\text{-}gal] + k_6 \cdot [oligo] \cdot [E] \end{aligned} \tag{3}$$

$$\frac{d[E\text{-}I]}{dt} = k_i \cdot [gal] \cdot [E] - k_{-i} \cdot [E\text{-}I] \tag{4}$$

$$K_i = \frac{k_{-i}}{k_i} \tag{5}$$

Assuming pseudo steady state conditions for the unknown concentrations of E, E-gal, and E-I, equation 6 may be derived:

$$\frac{v_{0,oNP}}{E_0} = \frac{k_1 \cdot [oNPG]}{1 + \frac{k_1 \cdot [oNPG] + k_3 \cdot [lac] + k_6 \cdot [oligo]}{k_{a1} \cdot [oNPG] + k_{a2} \cdot [H2O] + k_{a3} \cdot [lac] + k_{a4} \cdot [glu] + k_{a5} \cdot [gal]} + \frac{[gal]}{K_i}} \quad (6)$$

where $v_{0,oNP}$ is the initial rate of *o*NP formation in mM *o*NP·s$^{-1}$, $E_0$ is the initial enzyme concentration in g protein·L$^{-1}$ or in mmol protein·L$^{-1}$ with the reaction rate constants $k_1$, $k_{a1}$, $k_{a2}$, $k_3$, $k_{a3}$, $k_{a4}$, $k_{a5}$, and $k_6$ in mmol *o*NP·L·(mmol X·g protein·s)$^{-1}$ or in mmol *o*NP·L·(mmol X·mmol protein·s)$^{-1}$, respectively, with X being the corresponding reactant, and inhibition constant $K_i$ is in mM.



## Materials and methods

### *Materials*

Lactose monohydrate (Lactochem), Vivinal-GOS, and a β-galactosidase from *Bacillus circulans* called Biolacta N5 (Daiwa Kasei K. K., Japan) were gifts from FrieslandCampina (Beilen, The Netherlands). β-Galactosidase from *Aspergillus oryzae* (Lactase L017P) was a gift from Biocatalysts (Nantgarw, UK). β-Galactosidase from *Kluyveromyces lactis* (Lactozyme) was purchased from Sigma-Aldrich (Steinheim, Germany).

Sulfuric acid, sodium hydroxide, *o*-nitrophenyl β-D-galactopyranoside (*o*NPG), *o*-nitrophenol (*o*NP), and bovine γ-globulin (BGG) were purchased from Sigma-Aldrich (Steinheim, Germany). Protein assay dye reagent concentrate were obtained from Bio-Rad (Veenendaal, The Netherlands). 4-Nitrophenyl-β-D-glucopyranoside (*p*NPG), and 4-nitrophenyl 3-O-(α-D-glucopyranosyl)-α-D-glucopyranoside (*p*NPGG) were purchased from Carbosynth (Berkshire, UK).

McIlvaine's buffer was prepared by adding together 0.1 M citric acid and 0.2 M disodium hydrogen phosphate in the right ratio to achieve a pH of 6.0 or 8.0. Sodium carbonate, citric acid monohydrate, and disodium hydrogen phosphate were purchased from Merck (Darmstadt, Germany).

### *Protein content determination*

The total protein content of the β-galactosidase preparations from *A.oryzae* and *K.lactis* were determined based on the method of Bradford as described in the Bio-Rad Protein Assay protocol with bovine γ-globulin (BGG) as a standard. The protein content of the β-galactosidase preparations from *A.oryzae* and *K.lactis* was found to be 3.7 ± 0.2% and 3.2 ± 0.2% (BGG equivalents, 95% confidence interval), respectively. Biolacta N5 was earlier found to have a protein content of 19 ± 3 % (Warmerdam et al. 2013). The actual enzyme concentration is not known and for this reason the assumption is made that the total enzyme concentration is equal to the total protein concentration in all calculations.

### *Fractionation of Biolacta N5*



Fractionation of Biolacta N5 and characterization was performed as described by Warmerdam et al. (2013). The fractions used in this work were pool 2, 3, 4, and 5, which are called β-gal-A, β-gal-B, β-gal-C, and β-gal-D, respectively.

### *Enzyme activity measurements*

The enzyme activity measurements were adapted from Nakanishi et al. (1983). Stock solutions of *o*NPG in buffer with a concentration varying between 0.050 and 2.0% (w/w) were prepared and applied to the enzyme activity assay. The latter substrate concentration was limited by the solubility of *o*NPG which was determined to be 69 mM. Stock solutions of Biolacta N5 and the β-galactosidase preparation of *A.oryzae* were prepared in McIlvaine's buffer of pH 6.0. The stock solution of the preparation of *K.lactis* was prepared in McIlvaine's buffer of pH 8.0.

An Eppendorf tube with 790 µL of various concentrations *o*NPG-in-buffer and 189 µL reactant or buffer solution was preheated in an Eppendorf Thermomixer at 40ºC and 600 rpm for 10 minutes. When reactants were present, an *o*NPG stock solution of 0.25% (w/w) was used. Subsequently, 21 µL of enzyme solution was added and these mixtures were incubated for another 10 minutes at 40ºC and 600 rpm. A volume of 1.0 mL of 10% (w/w) $Na_2CO_3$ solution was added to stop the reaction and, afterwards, the absorbance of *o*NP was measured at 420 nm. The *o*NP concentration was determined using the law of Lambert-Beer of which the extinction coefficient was determined to be 4576 $M^{-1} \cdot cm^{-1}$. The *o*NP formation was found to be linear during the first 10 minutes of

the reaction. This initial rate of *o*NP formation was expressed in mmol·$s^{-1}$·g $protein^{-1}$ or in mmol·$s^{-1}$·mmol $protein^{-1}$.

## *Fitting procedure*

Equation 6 was fitted to the data after all terms in the equation were divided by $k_1$ to prevent correlation between parameters. This implies that the fitting parameters are $1/k_1$, $k_3/k_1$, $k_6/k_1$, $K_i \cdot k_1$, $k_{a1}$, $k_{a2}$, $k_{a3}$, $k_{a4}$ and $k_{a5}$. All fitting procedures were performed in MS Excel 2010. Parameters were determined by minimizing the sum of squared residuals with Solver. Besides, the 95 % confidence intervals of the parameters and correlation coefficients were determined with Solveraid. The corrected Akaike information criterion ($AIC_c$) (van Boekel 2009) was used to find out whether an extra parameter was allowed in the model. The model with the lowest $AIC_c$ was assumed to be the most suitable one.

### *Effect of oligosaccharides*

Vivinal-GOS was used in the activity assay as a mixture. The effect of only oligosaccharides on the activity assay was quantified. The concentrations of lactose, galactose, glucose and total oligosaccharides are 19 dm%, 1 dm%, 21 dm%, and 59 dm% in Vivinal-GOS, respectively. To develop mole balances, the oligosaccharides in the mixture were assumed to be mainly trisaccharides and have a molecular weight of 504.4 g/mol.



## *Sample preparation for analysis of oNPG conversion products*

Volumes of 790 µL of 2.0% (w/w) *o*NPG solution, 189 µL of McIlvaine's buffer, and 21 µL of the β-galactosidase isoforms of Biolacta N5 (80 mg protein·$L^{-1}$) were incubated together at 40ºC. After two hours of incubation (whereas the activity assay is 10 minutes, to find larger differences in break down or formation of the components), samples of 200 µL were taken and added to 100 µL of 5% (w/w) sulphuric acid to inactivate the enzyme.

Before the HPLC analysis, the enzyme was removed from the samples by filtering the samples at 14,000 x g and 18ºC for 30 minutes using pretreated Amicon® ultra-0.5 centrifugal filter devices (Millipore Corporation, Billerica, MA, United States) with a cut-off of 10 kDa in a Beckman Coulter Allegra X-22R centrifuge. The pretreatment of the filters consisted of two centrifugation steps: first, 500 µL of Milli-Q water was centrifuged at 14,000 x g at 18ºC for 15 minutes; and second, the filters were placed up-

side-down in the tube and centrifuged at 14,000 x g at 18ºC for 5 minutes. After filtration, the samples were neutralized with 5% (w/w) sodium hydroxide.

### *Determination of presence of oNPGG*

The filtered samples were analyzed with HPLC using a Rezex RSO oligosaccharide column (Phenomenex, Amstelveen, the Netherlands) at 80ºC. The column was eluted with Milli-Q water at a flow rate of 0.3 mL/min. The eluent was monitored with a refractive index detector.

The standards that were used for detection of *o*NPG and *o*NPGG were *o*NPG, *p*NPG, and *p*NPGG. Their elution times were 85.6, 110.1, and 93.1 minutes, respectively (see Figure A1 in the Appendix). *o*NPGG was not available as a standard. Therefore, the elution time of *o*NPGG ($t_{oNPGG}$) was estimated with equation 7:

$$t_{oNPGG} = \frac{t_{pNPGG} \cdot t_{oNPG}}{t_{pNPG}} \quad (7)$$

where $t_{pNPG}$, $t_{oNPG}$, and $t_{pNPGG}$ are the elution times of *p*NPG, *o*NPG and *p*NPGG, respectively. The elution time of *o*NPGG was estimated to be 72.4 minutes.

4

## Results and discussion

### *oNPG kinetics of β-galactosidases*

Figure 2 and Figure 3 show the initial *o*NP formation rate as a function of the initial *o*NPG concentration for each of the tested β-galactosidase preparations and for each of the isoforms from *B.circulans*.

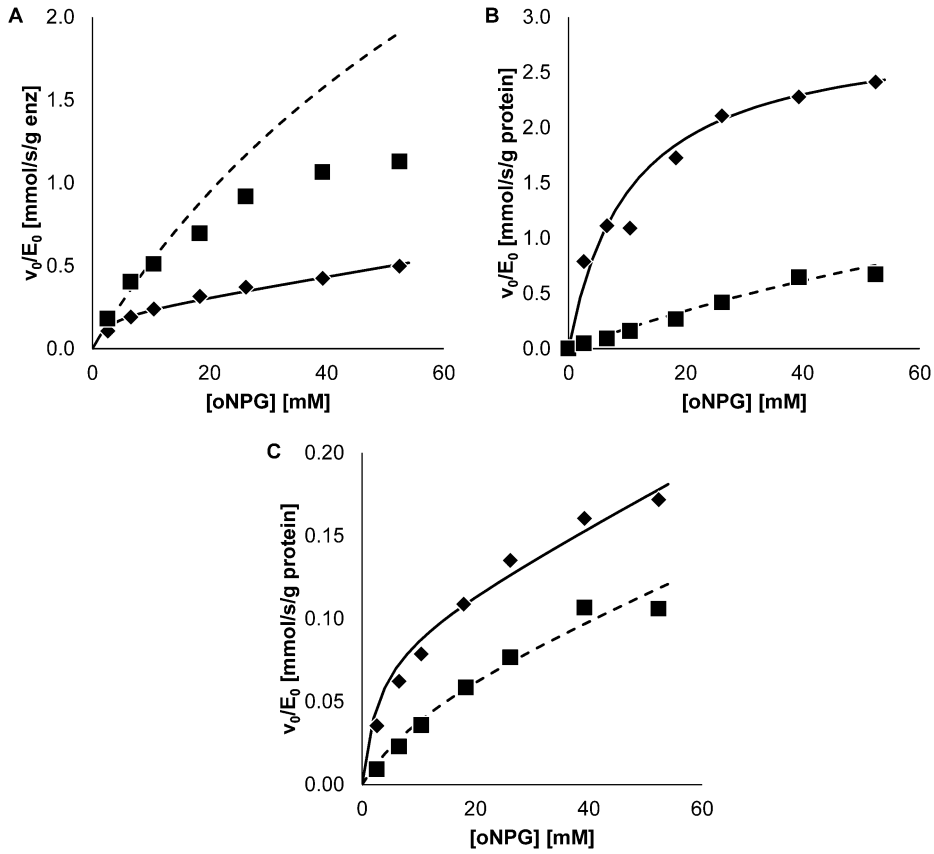


**Figure 2.** Initial rate of *o*NP formation as a function of the initial *o*NPG concentration with (A) Biolacta N5 complete (1.6 mg protein·$L^{-1}$) at 40ºC and pH 6.0; (B) β-galactosidase from *A.oryzae* (0.57 mg protein·$L^{-1}$) at 40ºC and pH 6.0; (C). β-galactosidase from *K.lactis* (4.6 mg protein·$L^{-1}$) at 20ºC and pH 8.0. Symbols: ♦ *o*NPG kinetics without added galactose; ■ *o*NPG kinetics with 35 g·$L^{-1}$ galactose added. Symbols represent measured data, lines represent modeled data.

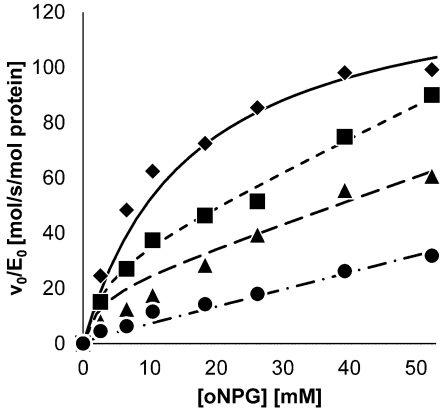


**Figure 3.** Initial rate of *o*NP formation as a function of the initial *o*NPG concentration at 40ºC and pH 6.0 with 1.7 mg protein·$L^{-1}$ ♦,—— β-gal-A; ■,- - - β-gal-B; ▲,— — β-gal-C; and ●,— · - β-gal-D. Symbols represent measured data, lines represent modeled data.



If only a simple cleavage of *o*NPG into *o*NP and galactose occurs, which can be described with Michaelis-Menten kinetics, the increase in the initial rate would level off with increasing *o*NPG concentration until the maximum reaction rate $v_{max}$ is reached. The β-galactosidase from *A.oryzae* shows more or less this behavior, but those from *B.circulans* and *K.lactis* do not. The initial rate with β-gal-A leveled off at high *o*NPG concentrations, but none of the other isoforms showed the maximum reaction rate within the measured *o*NPG concentration range. *o*NPG might act as acceptor (Sauerbrei and Thiem 1992). This was verified by using HPLC analysis. A peak with an elution time of 65.5 minutes was found in samples of *o*NPG conversion of two hours with β-gal-A, β-gal-C, β-gal-B, and β-gal-D (see Figure A2 in the Appendix). This elution time is rather close to the estimated elution time of *o*NPGG (72.4 minutes). Due to some small structural differences among the used standards, we believe that this peak with an elution time of 65.5 minutes is corresponding with the formation of *o*NPGG. The formation of *o*NPGG by β-gal-B, β-gal-C and β-gal-D was much higher than for β-gal-A.

The *o*NPG kinetics of the β-galactosidases was also measured in presence of 35 g·$L^{-1}$ galactose (Figure 2). The initial rate of *o*NP formation of the β-galactosidase from *B.circulans* was found to be much higher in presence of galactose than without galactose, whereas that of the β-galactosidases from *A.oryzae* and *K.lactis* was much lower. This indicates that β-galactosidase from *B.circulans* uses galactose as acceptor molecule, while the other two enzymes are inhibited by galactose.

*Effect of the reactants on the enzyme activity*

The measured initial rates at varying *o*NPG concentrations (Figure 2) were combined with the measured initial rates at varying concentrations of other reactants, which were published previously by Warmerdam et al. (Submitted for publication) (Figure 1). The fits with the model are shown in Figure 2 and Figure 4.

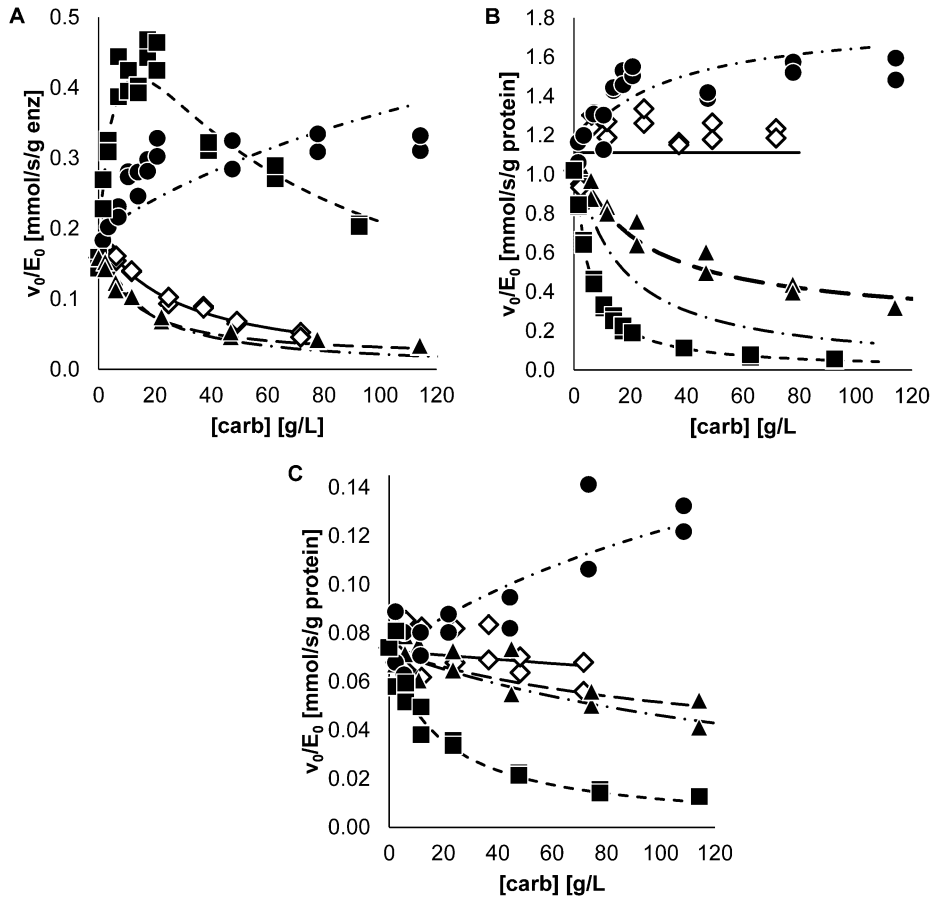


**Figure 4.** Initial rate of *o*NP formation as a function of the initial concentration of added carbohydrate at an initial *o*NPG concentration of 6.7 mM with (A) Biolacta N5 complete (2.8 mg protein·$L^{-1}$) at 40ºC and pH 6.0; (B) β-galactosidase from *A.oryzae* (0.57 mg protein·$L^{-1}$) at 40ºC and pH 6.0; (C) β-galactosidase from *K.lactis* (4.6 mg protein·$L^{-1}$) at 20ºC and pH 8.0. Symbols and lines: ◇,——— lactose; ▲,— — · Vivinal-GOS; ●, - - · · glucose; ■,- - - - galactose, and — · — oligosaccharides. Symbols represent measured data, (dashed) lines represent modeled data. Measured data from Warmerdam et al. (Submitted for publication).

*Elimination of parameters*

The reactions that are not taken into account for each β-galactosidase are shown in Figure 5.

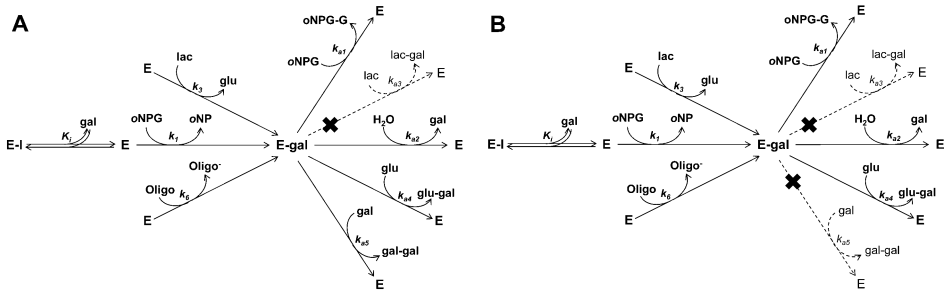


**Figure 5.** Reaction mechanism with parameters modeled for (A) Biolacta N5 complete, and (B) β-galactosidases from *A.oryzae* and *K.lactis*. Crossed reaction routes (dotted lines) are not taken into account for the specific β-galactosidase.



In the reaction schemes of each of the β-galactosidases, the reaction of using lactose as acceptor molecule ($k_{a3}$) is not taken into account. Since lactose can be used as substrate as well as acceptor, fitting both $k_3$ and $k_{a3}$ necessarily results in a high correlation between the two parameters. Since lactose mainly decreased the *o*NPG converting activity of the β-galactosidases, only $k_3$ (and not $k_{a3}$) was taken into account in the model (Figure 5).

All the models for the β-galactosidase preparations include the inhibition constant for galactose $K_i$. The models for the β-galactosidase preparations from *A.oryzae* and *K.lactis* do not include the reaction rate constant for galactose as acceptor ($k_{a5}$) (Figure 5B), but the model for the complete Biolacta N5 preparation does include $k_{a5}$ (Figure 5A). Including $k_{a5}$ in the models for the β-galactosidase preparations from *A.oryzae* and *K.lactis* resulted in a higher Akaike criterion. Therefore, the model without $k_{a5}$ for the β-galactosidase preparations from *A.oryzae* and *K.lactis* was assumed to be the more likely one.

It has to be noted that the model for β-galactosidase from *K.lactis* resulted in some correlation between $1/k_1$ and $k_{a2}$ and between $1/k_1$ and $k_{a4}$. The models for the other enzymes did not show correlation between parameters.

*Estimation of parameters*

Figure 6 and Table 1 show the parameters that were obtained by fitting equation 6 with the reaction rate constants shown in Figure 5 to all measured initial rates at various

*o*NPG concentrations and various reactant concentrations of one isoform. The fits are shown in Figure 2 and Figure 4.

The models describe the measured initial rates at various initial *o*NPG concentrations and with various concentrations of reactants well, except for the addition of galactose at high *o*NPG concentrations with the β-galactosidase from *B.circulans*. In the conversion of *o*NPG by β-galactosidases from *B.circulans* and *K.lactis* (Figure 2), *o*NPG was used as substrate as well as acceptor, whereas it was mostly used as substrate in the *o*NPG conversion by β-galactosidase from *A.oryzae*, which is evident in a very small $k_{a1}$. The reaction rate constant for *o*NPG as substrate for the β-galactosidase from *A.oryzae* is approximately three and fourteen times as high as for the β-galactosidases from *B.circulans* and *K.lactis*, respectively, which indicates the β-galactosidase from *A.oryzae* has a high affinity for *o*NPG. However, the reaction rate constant for water as acceptor ($k_{a2}$) for the β-galactosidase from *A.oryzae* is thirteen and thirty times higher than for the β-galactosidase from *B.circulans* and *K.lactis*, respectively, which means that hydrolysis is much faster with the β-galactosidase from *A.oryzae*. Although the β-galactosidase from *B.circulans* does not have the highest $k_1$ and $k_{a2}$, its ratio of $k_1/k_{a2}$ is approximately five times higher than that of the β-galactosidase from *A.oryzae*. The *o*NP formation rate with β-galactosidase from *B.circulans* and *K.lactis* is increased at higher *o*NPG concentrations by the use of *o*NPG as acceptor. The use of *o*NPG as acceptor at higher *o*NPG concentrations indicates that water, present in excess amounts, in the reaction is the rate limiting step of the reaction. The β-galactosidase from *A.oryzae* does not use *o*NPG as acceptor, because the use of water is more favorable.

In conclusion, the rate of using *o*NPG as substrate relative to the hydrolysis rate is higher for the β-galactosidase from *B.circulans* than for the other two enzymes, and moreover, use of *o*NPG as acceptor is much more favorable.

The reaction rate constants for lactose as substrate ($k_3$) for the β-galactosidases from *A.oryzae* and *K.lactis* are lower than that for the β-galactosidase of *B.circulans*. This means that the β-galactosidase from *A.oryzae* and *K.lactis* are less capable of using lactose as substrate than the β-galactosidase of *B.circulans*.

The reaction rate constants for glucose as acceptor ($k_{a4}$) for β-galactosidase from *B.circulans* and *K.lactis* are much lower than that for β-galactosidase from *A.oryzae*. That implies that the β-galactosidase from *A.oryzae* is better capable of using glucose as acceptor than the β-galactosidase from *B.circulans* and *K.lactis*. However, the fit of the model to the measured data with β-galactosidase from *B.circulans* and added glucose is rather poor. This could be due to inhibition by glucose (Boon et al. 1999). Taking into account glucose inhibition for the β-galactosidase from *B.circulans* would increase $k_{a4}$.

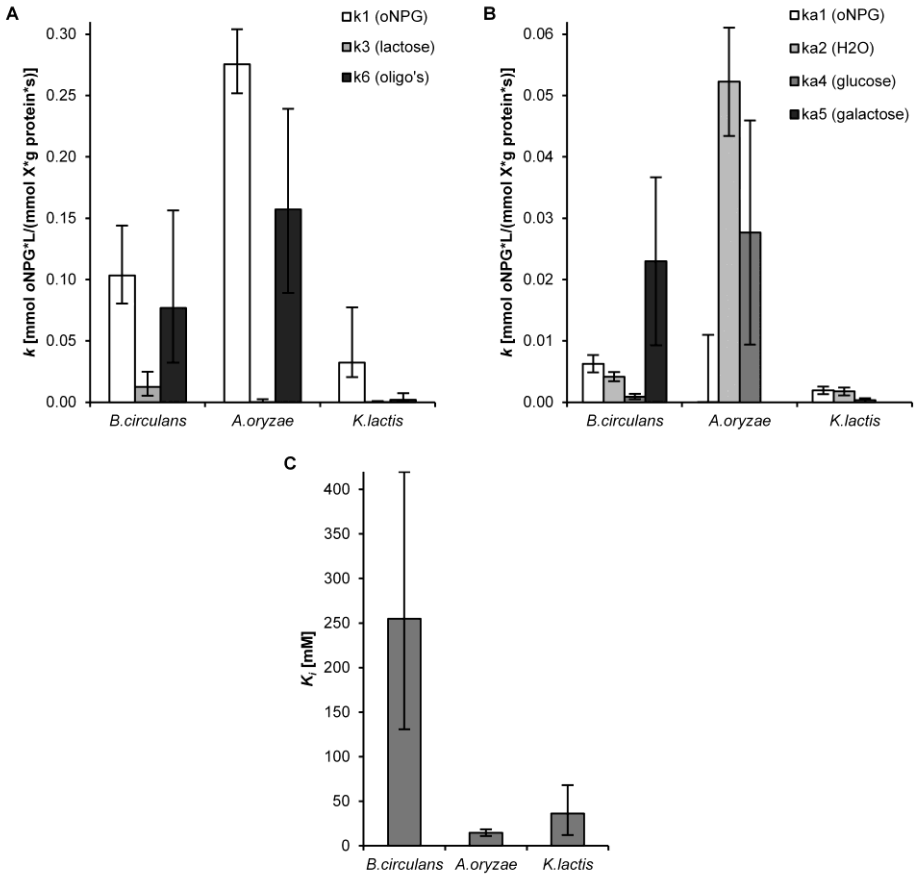


**Figure 6.** The parameters including their 95% confidence interval that were obtained by fitting equation 6 to the experimental data of the β-galactosidase preparations from *B.circulans*, *A.oryzae*, and *K.lactis*. (A) Reaction rate constants for substrates; (B) reaction rate constants for acceptors; and (C) inhibition constants for galactose.

**Table 1.** The parameters for the β-galactosidases from *B.circulans, A.oryzae, and K.lactis* that were obtained by fitting equation 6 to the experimental data. The 95% confidence intervals are shown in Figure 6.

| | *B.circulans* | *A.oryzae* | *K.lactis* |
|---|---|---|---|
| $k_1$ (mmol *o*NP·L·(mmol *o*NPG·g protein·s)$^{-1}$) | 0.10 | 0.28 | 0.032 |
| $k_3$ (mmol *o*NP·L·(mmol lactose·g protein·s)$^{-1}$) | 0.012 | 0.00000028 | 0.00014 |
| $k_6$ (mmol *o*NP·L·(mmol oligo·g protein·s)$^{-1}$) | 0.077 | 0.16 | 0.0019 |
| $k_{a1}$ (mmol *o*NP·L·(mmol *o*NPG·g protein·s)$^{-1}$) | 0.0063 | 0.0000010 | 0.0019 |
| $k_{a2}$ (mmol *o*NP·L·(mmol $H_2O$·g protein·s)$^{-1}$) | 0.0042 | 0.052 | 0.0018 |
| $k_{a4}$ (mmol *o*NP·L·(mmol glucose·g protein·s)$^{-1}$) | 0.00092 | 0.028 | 0.00032 |
| $k_{a5}$ (mmol *o*NP·L·(mmol galactose·g protein·s)$^{-1}$) | 0.023 | | |
| $K_i$ (mM) | 255 | 15 | 36 |
| | | | |
| $k_1/k_{a2}$ | 25 | 5.3 | 18 |
| $k_3/k_{a2}$ | 3.0 | 0.0000053 | 0.080 |
| $k_6/k_{a2}$ | 18 | 3.0 | 1.1 |
| $k_{a1}/k_{a2}$ | 1.5 | 0.000019 | 1.1 |
| $k_{a4}/k_{a2}$ | 0.22 | 0.53 | 0.18 |
| $k_{a5}/k_{a2}$ | 5.5 | | |

The reaction rate constant for galactose as acceptor ($k_{a5}$) could be determined only for the β-galactosidase from *B.circulans* due to very strong inhibition by galactose for the other two enzymes. The strong inhibition is especially important for the β-galactosidase from *A.oryzae*, because this enzyme has a high hydrolysis rate ($k_{a2}$), i.e. rapid galactose production. This would mean that certainly product inhibition by galactose will occur in a later stage of the reaction.

The addition of galactose at various *o*NPG concentrations with the β-galactosidase from *B.circulans* (Figure 2A) could not be fitted with the parameters that were determined based on all other measured data. This indicates that galactose at high *o*NPG concentrations causes an effect that is not included in the model. This effect can possibly be ascribed to the presence of multiple β-galactosidase isoforms in the preparation since they use galactose in a different way. This is described in the next section.

The *o*NP formation rate decreased with addition of Vivinal-GOS for β-galactosidases, however, for all enzymes to a different extend. We took into account that Vivinal-GOS is a mixture of 59% oligosaccharides, 21% glucose, 19% lactose, and 1% galactose. The reaction rate constant for just the oligosaccharides as substrate ($k_6$) for the β-galactosidase from *A.oryzae* is much higher than that for the β-galactosidase from *B.circulans* and *K.lactis*. This means that the β-galactosidase from *A.oryzae* is better capable of using oligosaccharides as substrates than the other two β-galactosidases. However, the ratio of $k_6/k_{a2}$ for β-galactosidase from *A.oryzae* is six times lower than that of β-galactosidase from *B.circulans*, which indicates that the hydrolysis of oligosaccharides can be much faster with β-galactosidase from *A.oryzae* than with β-galactosidase from *B.circulans*.

Overall, the high ratios of the reaction rate constants of lactose or oligosaccharide as substrate over the hydrolysis rate with β-galactosidase from *B.circulans*, and its high reaction rate constant of galactose as acceptor are very advantageous for GOS production.

### *Effect of reactants on enzyme activity of the isoforms*

Since Biolacta N5 is known to consist of multiple β-galactosidase isoforms, Biolacta N5 was further characterized by adding reactants to the activity assay with each of the isoforms and investigating how these reactants affected the *o*NPG converting activity of each isoform. Figure 7 shows the effect of lactose, galactose, glucose, and Vivinal-GOS

on the *o*NPG conversion with the various isoforms. The reactants affected most of the isoforms in a similar way as the complete Biolacta N5, however, all to a different extent.

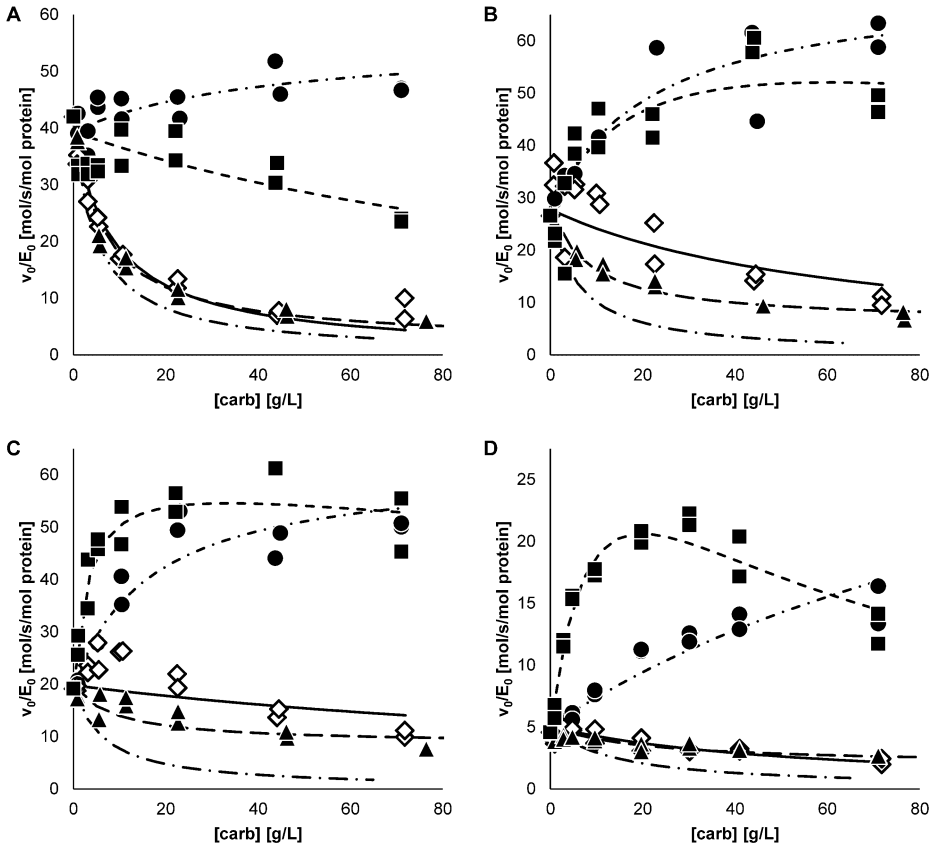


**Figure 7.** Initial rate of *o*NP formation as a function of the initial concentration of added carbohydrate at an initial *o*NPG concentration of 6.7 mM, 40ºC, and pH 6.0 with 1.7 mg protein·$L^{-1}$ (A) β-gal-A, (B) β-gal-B, (C) β-gal-C, and (D) β-gal-D. Symbols and lines: ◇,——— lactose; ▲,— — Vivinal-GOS; ●,- · - · glucose; ■, - - - galactose, and — · - oligosaccharides. Symbols represent measured data, (dashed) lines represent modeled data.

The presence of lactose in the activity assay caused a lower *o*NPG converting activity of β-gal-A. This was caused by substrate competition, since both *o*NPG and lactose are substrates for the enzyme.

The *o*NPG converting activity of β-gal-B, β-gal-C, and β-gal-D increased slightly with low lactose concentrations. However, the activity of these isoforms decreased at high lactose concentrations. This shift in behavior at different concentrations was most likely caused by the fact that lactose can act both as donor and as acceptor (Boon et al.

1999; Gosling et al. 2011): at low lactose concentrations, the main action is to act as donor, while at higher concentrations, the acceptor function becomes important as well.

The *o*NPG converting activity of each of the isoforms was higher in presence of glucose, because glucose is an acceptor (Kim et al. 2004; Warmerdam et al. Submitted for publication). The *o*NPG converting activity of β-gal-B, β-gal-C, and β-gal-D was higher in presence of galactose, because galactose is used as acceptor in the reaction (Warmerdam et al. Submitted for publication). In contrast to these isoforms, the *o*NPG converting activity of β-gal-A slightly decreased in presence of high concentrations of galactose. The decrease in activity indicates that galactose inhibits β-gal-A. Already years ago, inhibition by galactose has been reported by Mozaffar et al. (1984) when lactose was used as the substrate. They found that galactose inhibits a specific isoform called β-galactosidase-1 which might be similar to our β-gal-A. These different effects of galactose on the various isoforms most likely cause the bad fit of the addition of galactose to the complete Biolacta N5 preparation at high *o*NPG concentrations (Figure 2A).



The addition of Vivinal-GOS resulted in a lower *o*NPG converting activity of each of the isoforms. However, it is hard to say based on these measurements how the oligosaccharides affect the isoforms, since Vivinal-GOS is a mixture of oligosaccharides, glucose, lactose, and galactose. These effects are quantified separately with the model.

*Elimination of parameters*

Equation 6 with the reaction rate constants shown in Figure 8 was fitted to all the measured initial rates of *o*NP formation at various *o*NPG concentrations and various reactant concentrations of one isoform at once. The fits are shown in Figure 3 and Figure 7.

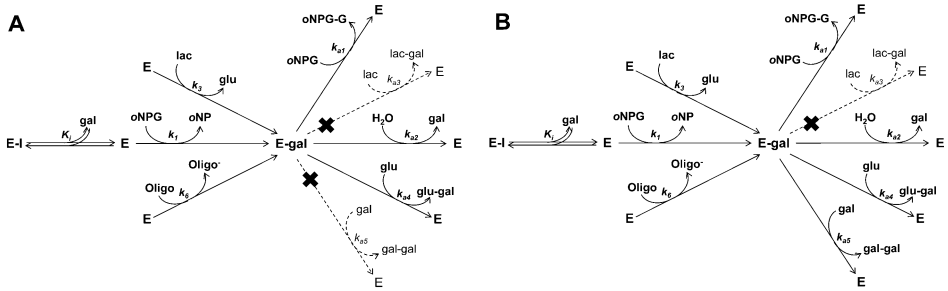


**Figure 8.** Reaction mechanism with parameters modeled for (A) β-gal-A, and (B) β-gal-B, β-gal-C, and β-gal-D. Crossed reaction routes (dotted lines) are not taken into account for the specific isoforms.

In the reaction schemes of each of the β-galactosidase isoforms (Figure 8), the reaction of using lactose as acceptor ($k_{a3}$) is not taken into account, similar as was described for the β-galactosidase preparations.

All the models for the isoforms include the inhibition constant for galactose $K_i$, whereas the reaction rate constant for galactose as acceptor ($k_{a5}$) was included for β-gal-B, β-gal-C and β-gal-D, but not for β-gal-A (Figure 8).

*Estimation of parameters*

Figure 9 and Table 2 show the parameters that were obtained by fitting equation 6 with the reaction rate constants shown in Figure 8 to all measured initial rates at various *o*NPG concentrations and various reactant concentrations of one isoform. The fits are shown in Figure 3 and Figure 7.

The models describe the measured initial rates as a function of the concentration of *o*NPG and other reactants well. Many of the rate constants decrease along with the molecular size of the isoform.

In the conversion of *o*NPG by β-gal-A, *o*NPG was only used as substrate and not as acceptor, which was evident in a $k_{a1}$ of zero, whereas it was also an acceptor for β-gal-B, β-gal-C, and β-gal-D (Figure 3). The reaction rate constants for *o*NPG as substrate $k_1$ hardly differed among the four isoforms: though the one for β-gal-B was slightly lower than for β-gal-D.

The reaction rate constant for water as acceptor ($k_{a2}$) was highest for β-gal-A, followed by β-gal-B, β-gal-C, and was lowest for β-gal-D. This means that the hydrolysis rate decreases with decreasing isoform size. The *o*NP formation rate with β-gal-B, β-gal-C, and β-gal-D is increased at higher *o*NPG concentrations by the use of *o*NPG as acceptor. Thus, the use of water, which is present in excess amounts, in the conversion with β-gal-B, β-gal-C, and β-gal-D is the rate limiting step of the reaction. The reaction rates for *o*NPG as acceptor ($k_{a1}$) decrease with decreasing isoform size when not taking into account β-gal-A.

$k_{a1}$ for β-gal-A was zero and this isoform does not seem to be capable of using *o*NPG as acceptor. Since $k_{a2}$ of β-gal-A is much higher, its hydrolysis step is not as rate limiting as for the other isoforms. The use of water by β-gal-A is more favorable than the use of *o*NPG as acceptor.

The reaction rate constant for lactose as substrate ($k_3$) for β-gal-A is much higher than that for the other isoforms. Therefore lactose strongly competes with *o*NPG for the active site. The reaction rate constant for lactose of the other isoforms is much lower: lactose will be less able to displace *o*NPG from their active site. Apparently, the high molecular weight of β-gal-A is needed for a high affinity of the active center for lactose.

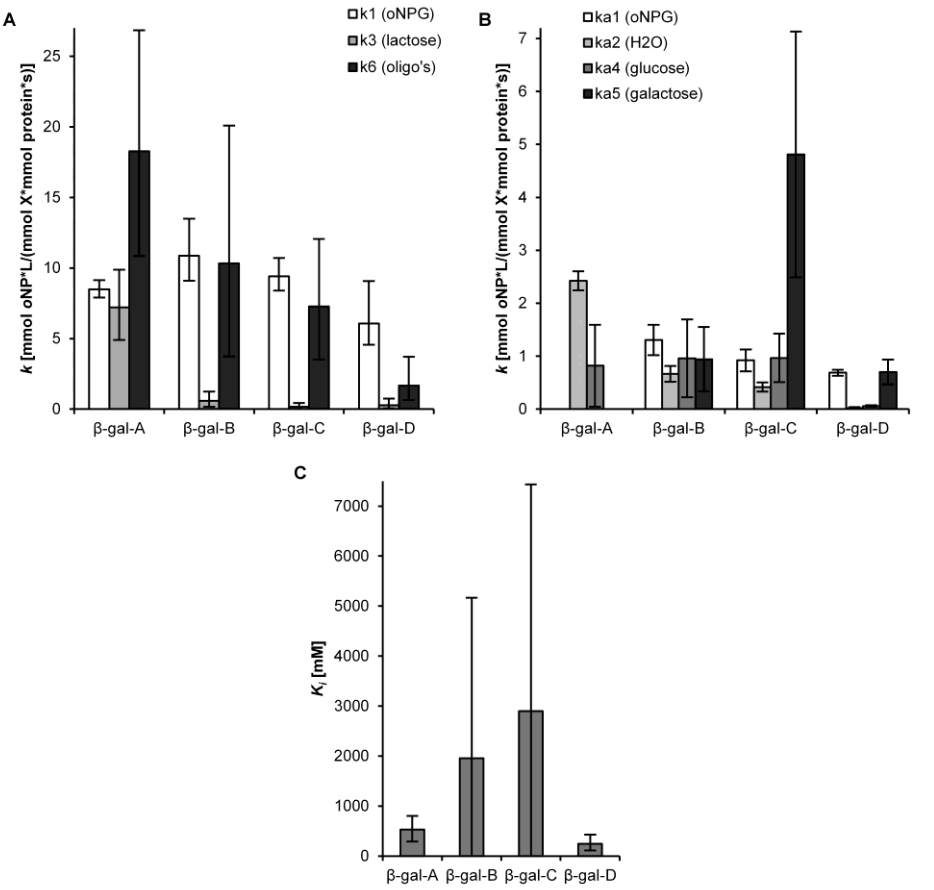


**Figure 9.** The parameters including their 95% confidence interval that were obtained by fitting equation 6 to the experimental data of the β-galactosidase isoforms from *B.circulans*. (A) Reaction rate constants for substrates; (B) reaction rate constants for acceptors; and (C) inhibition constants for galactose.

**Table 2.** The parameters for the β-galactosidase isoforms from *B.circulans* that were obtained by fitting equation 6 to the experimental data shown in Figure 3 and Figure 7. The 95% confidence intervals are shown in Figure 9.

| | β-gal-A | β-gal-B | β-gal-C | β-gal-D |
|---|---|---|---|---|
| $k_1$ (mmol *o*NP·L·(mmol *o*NPG·g protein·s)$^{-1}$) | 8.5 | 11 | 9.4 | 6.1 |
| $k_3$ (mmol *o*NP·L·(mmol lactose·g protein·s)$^{-1}$) | 7.2 | 0.60 | 0.18 | 0.29 |
| $k_6$ (mmol *o*NP·L·(mmol oligos·g protein·s)$^{-1}$) | 18 | 10 | 7.3 | 1.7 |
| $k_{a1}$ (mmol *o*NP·L·(mmol *o*NPG·g protein·s)$^{-1}$) | 0.0 | 1.3 | 0.92 | 0.69 |
| $k_{a2}$ (mmol *o*NP·L·(mmol $H_2O$·g protein·s)$^{-1}$) | 2.4 | 0.67 | 0.42 | 0.023 |
| $k_{a4}$ (mmol *o*NP·L·(mmol glucose·g protein·s)$^{-1}$) | 0.82 | 0.96 | 0.97 | 0.059 |
| $k_{a5}$ (mmol *o*NP·L·(mmol galactose·g protein·s)$^{-1}$) | | 0.94 | 4.8 | 0.70 |
| $K_i$ (mM) | 530 | 1952 | 2901 | 246 |
| | | | | |
| $k_1/k_{a2}$ | 3.5 | 16 | 23 | 261 |
| $k_3/k_{a2}$ | 3.0 | 0.90 | 0.43 | 12 |
| $k_6/k_{a2}$ | 7.5 | 16 | 17 | 72 |
| $k_{a1}/k_{a2}$ | 0 | 2.0 | 2.2 | 30 |
| $k_{a4}/k_{a2}$ | 0.34 | 1.4 | 2.3 | 2.5 |
| $k_{a5}/k_{a2}$ | | 1.4 | 12 | 30 |

The model does not include the use of lactose as acceptor molecule. Therefore, $k_3$ for especially β-gal-B and β-gal-C became a compromise between $k_3$ and $k_{a3}$. The *o*NPG conversion with β-gal-B and β-gal-C actually increased by adding lactose at low concentrations. *o*NPG acts mainly as the donor and lactose acts as the acceptor for the enzyme-galactose complex which results in the formation of a trisaccharide and the release of the free enzyme. On the other hand, at higher lactose concentrations, lactose molecules compete with *o*NPG as the donor molecule. Because of this competition, the *o*NPG converting activity of β-gal-B and β-gal-C is lower at high lactose concentrations. β-Gal-A, which possesses a much lower transgalactosylation activity, did not show this shift in behavior. β-Gal-A is able to use lactose much easier as donor than as acceptor.

The rate constant for glucose as acceptor ($k_{a4}$) did not seem to differ that much among the isoforms, although β-gal-D's rate constant for glucose was significantly lower than those of β-gal-B and β-gal-C. This means that β-gal-B and β-gal-C are better capable of using glucose as acceptor than β-gal-D. The $k_{a4}$ for β-gal-A is not significantly different from the other isoforms because of its large 95% confidence interval.



The rate constant for galactose as acceptor ($k_{a5}$) did not show a relation with the size of the isoforms. The $k_{a5}$ for β-gal-C was much higher than those of β-gal-B and β-gal-D. This would mean that β-gal-C is much better capable of using galactose as acceptor than β-gal-B and β-gal-D. An explanation for this was not found.

β-Gal-A and β-gal-D showed inhibition by galactose, although this was much weaker than for the β-galactosidases of *A.oryzae* and *K.lactis*, evident in a higher $K_i$. The other two isoforms do show inhibition by galactose, but their $K_i$ has a huge 95% confidence interval. Since β-gal-B and β-gal-C use galactose as acceptor, which increases the activity, inhibition might be hidden.

The *o*NP formation rate did decrease with addition of Vivinal-GOS for each of the isoforms. Taking into account that Vivinal-GOS is a mixture of 59% oligosaccharides, 21% glucose, 19% lactose, and 1% galactose, the effect of oligosaccharides on the activity of the isoforms was found. The reaction rate constant for oligosaccharides as substrate ($k_6$) seems to decrease with decreasing isoform size. However, the difference in $k_6$ among β-gal-A, β-gal-B, and β-gal-C was not significant. The oligosaccharides strongly compete with *o*NPG for the active site of β-gal-A, β-gal-B, and β-gal-C and seem to have a very high affinity for the active site. This affinity is much higher than the affinity of lactose, which makes it more likely that the oligosaccharides will be reduced

with one degree of polymerization than lactose when they are present in the same mixture.

*What do these parameters imply for GOS production?*

The main differences among the reaction rate constants of the β-galactosidases that are relevant for GOS production can be found in $k_{a2}$, $k_3$, $k_6$, $k_{a5}$, and $K_i$. The low hydrolysis rate $k_{a2}$ for β-galactosidases from *B.circulans* and *K.lactis* is very favorable for their GOS production. On their turn, the decrease in hydrolysis rate with decreasing size of the isoforms is very favorable for GOS production by the smaller isoforms of *B.circulans*. Less hydrolysis means that the smaller isoforms do not break the formed oligosaccharides down that fast.

On the other hand, the high $k_3$ (and $k_6$) for the β-galactosidase from *B.circulans* and its isoform β-gal-A indicate a very fast formation of an enzyme-galactose complex. The fast formation of the enzyme-galactose complex means that its galactose moiety is sooner available for donation to another acceptor molecule. This is especially advantageous at high substrate concentrations, where many acceptor molecules other than water are available.

The low $K_i$ for the β-galactosidases from *A.oryzae* and *K.lactis* is very disadvantageous for their GOS production. Especially for the β-galactosidases from *A.oryzae*, since this enzyme has a high hydrolysis rate, i.e. high galactose formation rate, which will result in product inhibition. The use of galactose as acceptor molecule by the β-galactosidase from *B.circulans* does make this enzyme even more favorable for GOS production than the other β-galactosidases, because this will lead to the formation of oligosaccharides.



## Conclusions

Initial rate reactions models were created for β-galactosidases from three different sources: *B.circulans, A.oryzae,* and *K.lactis.* In addition, the enzyme cocktail from *B.circulans* was fractionated and four different isoforms were analysed similarly. The use of *o*NPG next to other substrates enabled us to find differences in the mechanisms of the enzymes.

The β-galactosidase of *B.circulans* performs better in GOS production than those of *A.oryzae* and *K.lactis*, because its reaction rate for lactose and oligosaccharides is much higher than its hydrolysis rate (use of water as acceptor). The use of galactose as acceptor with β-galactosidase from *B.circulans* is advantageous for GOS production. While it is a strong inhibitor with the other enzymes, it is not with *B.circulans*.

Many of the reaction rate constants of the β-galactosidase isoforms from *B.circulans* for various substrates and acceptors increased with increasing molecular weight. This indicates that β-gal-A is the most active isoform: it shows the fastest formation of enzyme-galactose complexes ($k_3$); but it also has the highest hydrolysis rate.

## Acknowledgements

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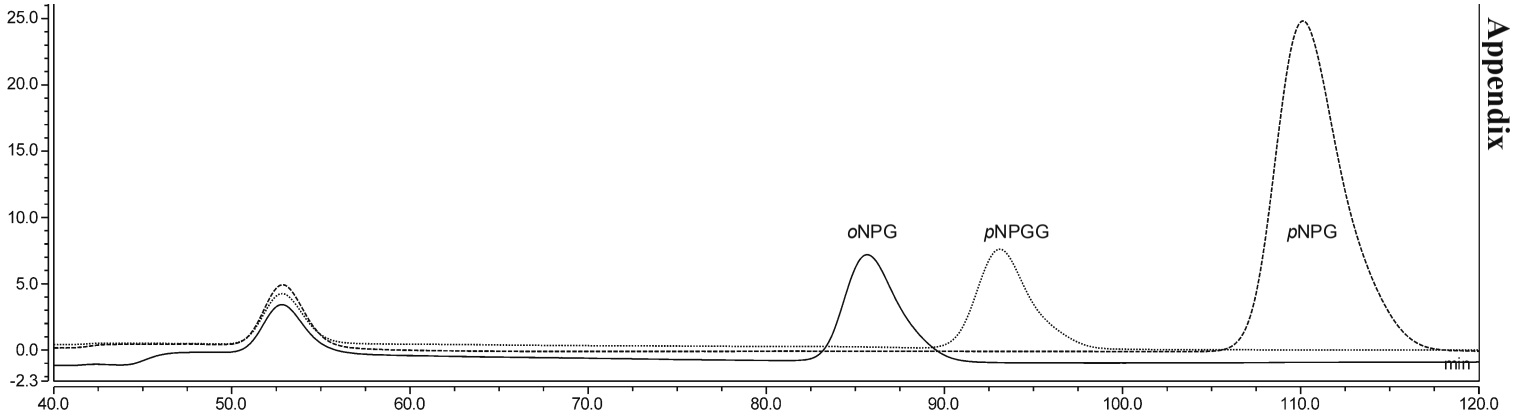


**Figure A1.** Chromatogram of the elution of *o*NPG, *p*NPGG, and *p*NPG.

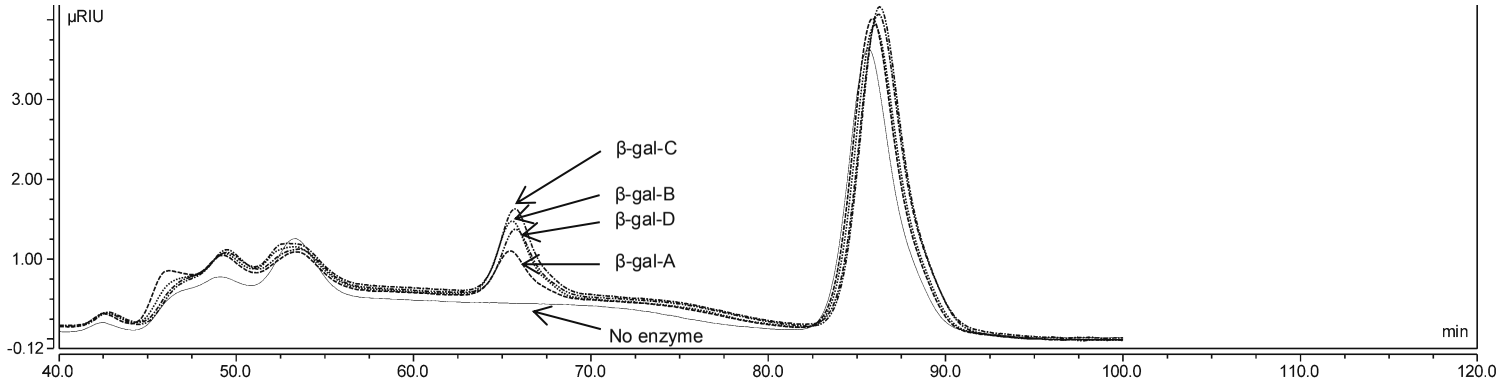


**Figure A2.** Chromatogram of the conversion of *o*NPG after 2 hours of incubation without enzyme and with β-gal-A, β-gal-B, β-gal-C, and β-gal-D.

# Chapter 5

## β-Galactosidase stability at high substrate concentrations


## Abstract

Enzymatic synthesis of galacto-oligosaccharides is usually performed at high initial substrate concentrations since higher yields are obtained. We report here on the stability of β-galactosidase from *Bacillus circulans* at 25, 40, and 60ºC in buffer, and in systems with initially 5.0 and 30% (w/w) lactose. In buffer, the remaining activity after 24 h was 70 and 16% at 25 and 40ºC, respectively, whereas the enzyme was completely inactivated after two hours at 60ºC. In systems with 5.0 and 30% (w/w) lactose, a mechanistic model was used to correct the *o*NPG converting activity for the presence of lactose, glucose, galactose, and oligosaccharides in the activity assay. Without correction, the stability at 5.0% (w/w) lactose was overestimated, while the stability at 30% (w/w) lactose was underestimated. The inactivation constant $k_d$ was found to strongly increase with temperature in buffer, whereas after correction only a slight increase in $k_d$ was found with temperature at high substrate concentrations. The enzyme stability/half-life time was found to increase strongly with the initial substrate concentrations, because of a much lower inactivation energy $E_a$ at high initial substrate concentrations.




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## Introduction

Using highly concentrated conditions are of interest for enzymatic production processes, since energy, water, and material costs can be saved. However, the activity and stability of enzymes is often investigated in aqueous systems, which may lead to irrelevant data. The enzyme activity of β-galactosidases, which is used in the production of galacto-oligosaccharides (GOS), in highly concentrated systems was studied before (Warmerdam et al. Submitted for publication-a) and was found to be strongly influenced by the concentration of reactants and products. The high concentration of reactants and products may not only lead to more reactions taking place, but it will also lead to molecular crowding, which can have large effects on enzyme activity (Ellis 2001; Minton 2001).

Besides the enzyme activity, their stability can as well be strongly affected by molecular crowding (Ellis 2001; Minton 2001). In 1985, Arakawa and Timasheff (1985) have already described the stabilization of the protein structure of lysozyme in the presence of osmolytes. De Cordt et al. (1994) described the influence of high concentrations of polyalcohols and carbohydrates on the enzyme stability by substrate binding or preferential hydration. They observed various situations in which the presence of inert crowding agents increases the thermo-stability of proteins (Perham et al. 2007; Stagg et al. 2007; Zhou et al. 2008). Recently, Yadav (2013) described that the presence of sucrose and trehalose strongly increased the half-life time of α-amylase.



GOS are usually produced with β-galactosidase at high temperatures and at high substrate concentrations in industry. An advantage of reactions at high temperatures is the improved solubility of the substrates which makes higher substrate concentrations possible (Bruins et al. 2001). However, the inactivation of the enzyme is faster as well (Bruins et al. 2003).

The stability of β-galactosidase from *Bacillus circulans* was investigated before in systems with low lactose concentration or in absence of lactose. Mozaffar et al. (1984), Vetere and Paoletti (1998), and Song et al. (2011a) studied the stability of several isoforms of β-galactosidase from *Bacillus circulans* in aqueous systems. They found that the enzyme preparation was (partly) stable up to 50ºC, whereas the highest activities of the isoforms that give high oligosaccharide yields were found at 74 and 60ºC. This would result in a compromise between working at optimal conditions and stable conditions. The stability of free enzyme in systems with high lactose concentrations, which are usually used in production systems, has to our knowledge never been investigated before.

When using high initial substrate concentrations, it is important to investigate the effect of reactants on the activity assay. Baks et al. (2006) found that starch and its

hydrolysis products may have large effects on the Ceralpha activity assay. This assay is comparable to the activity assay used for β-galactosidases with *o*NPG as an artificial substrate. Lactose and (some of) its conversion products are substrate for β-galactosidase as well as *o*NPG: they act as acceptor molecule for the enzyme-galactose complex, and they act as inhibitors and competitors (Borralho et al. 2002; Warmerdam et al. Submitted for publication-a; Warmerdam et al. Submitted for publication-b). Besides, galactose and glucose are usually found to be inhibitors for β-galactosidases (Greenberg and Mahoney 1982; Macfarlane et al. 2008; Prenosil et al. 1987; Warmerdam et al. Submitted for publication-a; Warmerdam et al. Submitted for publication-b). Because of the interactions of these carbohydrates, it is important to correct the activity measurements for their presence.

The aim of this study is therefore to investigate the stability of β-galactosidase from *Bacillus circulans* at various temperatures in buffer, and systems with initially 5.0 and 30% (w/w) lactose. The activity measurements are corrected for the effect of the carbohydrates present in GOS mixtures on the activity assay.

## Materials and methods

### *Materials*



Lactose monohydrate (Lactochem), Vivinal-GOS and a β-galactosidase from *Bacillus circulans* called Biolacta N5 (Daiwa Kasei K. K., Japan) were gifts from FrieslandCampina (Beilen, The Netherlands). Biolacta N5 was previously found to have a total protein content of 19 ± 3% (Warmerdam et al. 2013). In all calculations, the total enzyme concentration was assumed to be equal to the total protein concentration, because the actual enzyme concentration is not known. Sulphuric acid, sodium hydroxide, *o*-nitrophenyl β-D-galactopyranoside (*o*NPG), *o*-nitrophenol (*o*NP), D(+)-glucose, D(+)-galactose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium carbonate, citric acid monohydrate, and disodium hydrogen phosphate were purchased from Merck (Darmstadt, Germany).

McIlvaine's buffer was prepared by adding together 0.1 M citric acid and 0.2 M disodium hydrogen phosphate in the right ratio to achieve a pH of 6.0.

### *Lactose conversion*

The stability of Biolacta N5 was investigated in a 0, 5.0, and 30% (w/w) lactose-in-buffer solution in a temperature controlled batch reactor with an anchor stirrer at 150 rpm. The lactose was dissolved at approximately 60ºC prior to cooling the solution to the desired temperature. The initial reaction volume was 25 mL. Temperatures were kept at 25, 40, or 60ºC. A volume of 1.0 mL of 2.0 $g \cdot L^{-1}$ Biolacta N5 was added once the temperature was constant. Samples were taken at 30 s, 5, 10, 15, 30, 60, 120, 240, 360 minutes and 22, and 24 hours for determination of the carbohydrate composition (100 µL sample) and for determination of the enzyme activity (210 µL sample). The final reaction volume was 21 mL.

### *Sample handling for determination of the carbohydrate composition*

The sample (100 µL) taken from the reactor for determination of the carbohydrate composition was directly added into an Eppendorf tube with 50 µL of 5% (w/w) $H_2SO_4$ to inactivate the enzyme. Subsequently, the samples were stored at -20ºC until further preparation.

Before HPLC analysis, the enzyme was removed from the samples by filtering the samples at 14,000 x g at 18ºC for 30 minutes using pretreated Amicon® ultra-0.5 centrifugal filter devices (Millipore Corporation, Billerica, MA, United States) with a cut-off of 10 kDa in a Beckman Coulter Allegra X-22R centrifuge. The pretreatment of the filters consisted out of two centrifugation steps: first, 500 µL of Milli-Q water was centrifuged at 14,000 x g at 18ºC for 15 minutes; and second, the filters were placed up-side-down in the tube and centrifuged at 14,000 x g at 18ºC for 5 minutes. After filtration, the samples were neutralized with 5% (w/w) sodium hydroxide.

### *Measurement of the carbohydrate composition*

The filtered samples were analysed with HPLC using a Rezex RSO oligosaccharide column (Phenomenex, Amstelveen, the Netherlands) at 80ºC. The column was eluted with Milli-Q water at a flow rate of 0.3 mL/min. The eluent was monitored with a refractive index detector.

The standards that were used for calibration of the column were lactose, glucose, galactose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose. Galacto-oligosaccharides up to a degree of polymerization of 7 were assumed to have

the same response as the glucose-oligomers with an equal degree of polymerization. This was confirmed with mass balances.

### *Enzyme activity measurements*

The enzyme activity measurements, adapted from Nakanishi et al. (1983), were performed immediately after the sample was taken from the reactor. An Eppendorf tube with 790 µL of 0.25% (w/w) *o*NPG-in-buffer was preheated in an Eppendorf Thermomixer at 40ºC and 600 rpm for 10 minutes. Subsequently, 210 µL of sample was added and these mixtures were incubated for another 10 minutes at 40ºC and 600 rpm. A volume of 1.0 mL of 10% (w/w) $Na_2CO_3$ solution was added to stop the reaction and, afterwards, the absorbance of *o*NP was measured at 420 nm. The *o*NP concentration was determined using the law of Lambert-Beer of which the extinction coefficient was determined to be 4576 $M^{-1}\cdot cm^{-1}$. The *o*NP formation was found to be linear during the first 10 minutes of the reaction. This initial rate of *o*NP formation was expressed in $mmol\cdot min^{-1}\cdot g\ protein^{-1}$. Measurements were performed in duplicate and the average enzyme activity was used.

### *Modeling the effect of carbohydrates on the activity assay*

The effect of carbohydrates on the activity assay can be described with a mechanistic model; we refer to previous work for the mechanistic description of the model (Warmerdam et al. Submitted for publication-b) (equation 1). This model accounts for the use of *o*NPG as substrate ($k_1$) as well as acceptor ($k_{a1}$), the use of water as acceptor ($k_{a2}$), the use of lactose (lac) as substrate ($k_3$) as well as acceptor ($k_{a3}$), the use of glucose (glu) as acceptor ($k_{a4}$), the use of galactose (gal) as acceptor ($k_{a5}$) as well as inhibitor ($K_i$), and the use of oligosaccharides (oligo) as substrate ($k_6$) as follows:

$$\frac{v_{0,oNP}}{E_0} = \frac{k_1[oNPG]}{1 + \frac{k_1[oNPG] + k_3[lac] + k_6[oligo]}{k_{a1}[oNPG] + k_{a2}[H2O] + k_{a3}[lac] + k_{a4}[glu] + k_{a5}[gal]} + \frac{[gal]}{K_i}} \quad (1)$$

where $v_{0,oNP}$ is the initial rate of *o*NP formation in mM $oNP\cdot s^{-1}$, $E_0$ is the initial enzyme concentration in g $protein\cdot L^{-1}$ or in mmol $protein\cdot L^{-1}$ with the reaction rate constants $k_1$, $k_{a1}$, $k_{a2}$, $k_3$, $k_{a3}$, $k_{a4}$, $k_{a5}$, and $k_6$ in mmol $oNP\cdot L\cdot(mmol\ X\cdot g\ protein\cdot s)^{-1}$ or in mmol

*o*NP·L·(mmol X·mmol protein·s)$^{-1}$, respectively, with X being the corresponding reactant. The inhibition constant $K_i$ is in mM.

The respective parameters for Biolacta N5 were determined in previous work (Warmerdam et al. Submitted for publication-b) and are shown in Table 1.

**Table 1.** Parameters for Biolacta N5 in the conversion of *o*NPG, lactose, glucose, galactose, and oligosaccharides.

| | |
|---|---|
| $k_1$ (mmol *o*NP·L·(mmol *o*NPG·g protein·s)$^{-1}$) | 0.10 |
| $k_3$ (mmol *o*NP·L·(mmol lactose·g protein·s)$^{-1}$) | 0.012 |
| $k_6$ (mmol *o*NP·L·(mmol oligos·g protein·s)$^{-1}$) | 0.077 |
| $k_{a1}$ (mmol *o*NP·L·(mmol *o*NPG·g protein·s)$^{-1}$) | 0.0063 |
| $k_{a2}$ (mmol *o*NP·L·(mmol $H_2O$·g protein·s)$^{-1}$) | 0.0042 |
| $k_{a4}$ (mmol *o*NP·L·(mmol glucose·g protein·s)$^{-1}$) | 0.00092 |
| $k_{a5}$ (mmol *o*NP·L·(mmol galactose·g protein·s)$^{-1}$) | 0.023 |
| $K_i$ (mM) | 255 |

To investigate the effect of the present reactants compared to when no reactants are added in the activity assay, we normalized this initial rate with the initial rate that would have been obtained without addition of carbohydrates, which is given by equation 2:

$$\frac{v_{0,oNP}}{v_{0,oNP}^{[C]=0}} = \frac{1 + \frac{k_1[oNPG]}{k_{a1}[oNPG] + k_{a2}[H2O]}}{1 + \frac{k_1[oNPG] + k_3[lac] + k_6[oligo]}{k_{a1}[oNPG] + k_{a2}[H2O] + k_{a3}[lac] + k_{a4}[glu] + k_{a5}[gal]} + \frac{[gal]}{K_i}} \quad (2)$$

where $v_{0,oNP}^{[C]=0}$ is the initial rate of *o*NP formation without addition of carbohydrates *C*.

### *Activity measurements corrected for the presence of carbohydrates*

The activity measurements were corrected for the effect of lactose, glucose, galactose, and oligosaccharides on the activity assay with equation 3:

$$A_{corrected} = \frac{A_{measured}}{\frac{v_{0,oNP}}{v_{0,oNP}^{[C]=0}}} \quad (3)$$

where $A_{measured}$ and $A_{corrected}$ are the enzyme activity calculated directly from the absorbance measurements (see "Enzyme activity measurements"), and the enzyme activity corrected for the presence of lactose, glucose, galactose, and oligosaccharides, respectively.

For each sample made with Vivinal-GOS, the concentration of lactose, glucose, galactose, and total oligosaccharide was calculated. The concentrations of lactose, galactose, glucose and total oligosaccharides are 19 dm%, 1 dm%, 21 dm%, and 59 dm% in Vivinal-GOS. Oligosaccharides were assumed to be mainly trisaccharides with a molecular weight of 504 g/mol.

### *Determination of enzyme stability*

Enzyme inactivation during the running time of the experiment was modelled with a first order inactivation model with:

$$k_t = k_0 \cdot e^{-k_d \cdot t} \quad (4)$$

where $k_0$ and $k_t$ are the reaction rates at time zero and time $t$ in h, $k_d$ is the enzyme inactivation constant in $h^{-1}$, and $t$ is the running time at which the sample was taken in hours. The enzyme inactivation constant $k_d$ and the reaction rate at time zero $k_0$ were determined by linearization of equation 4.

The inactivation energy $E_a$ can be determined with the Arrhenius relation, equation 5:

$$k_d = k_\infty \cdot e^{-\frac{E_a}{R \cdot T}} \quad (5)$$

where $k_d$ and $k_\infty$ (the Arrhenius constant) are in $s^{-1}$, $R$ is the gas constant in $J \cdot mol^{-1} \cdot K^{-1}$, and $T$ is the temperature in K.

The half-life time of the enzyme $t_{½}$ can be determined with equation 6:

$$t_{1/2} = \frac{\ln(2)}{k_d} \quad (6)$$

## Results and discussion

### *Effect of temperature and initial lactose concentration*

Figure 1 shows the specific enzyme activity of Biolacta N5 during 24 hours of incubation in buffer (A), 5.0% (w/w) lactose (B), and 30% (w/w) lactose (C) at 25, 40, and 60ºC.

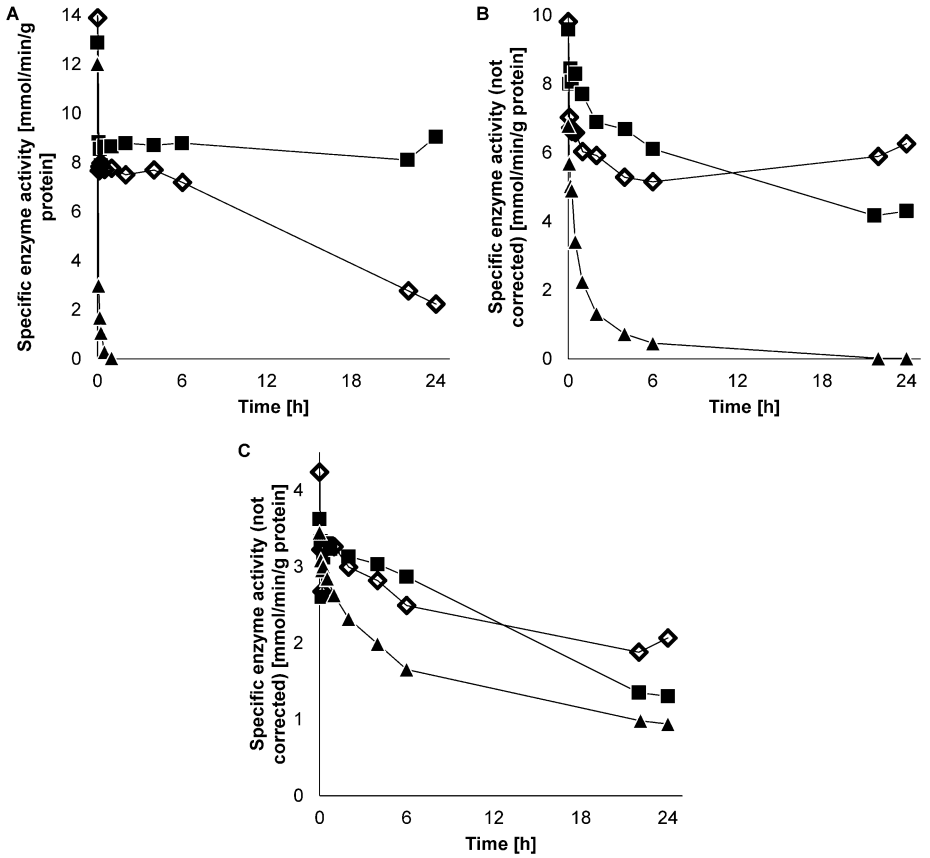


**Figure 1** Stability of Biolacta N5 in (A) buffer, (B) 5.0% (w/w) lactose, (C) 30% (w/w) lactose at ■ 25, ◇ 40, and ▲ 60ºC and pH 6.0 with an enzyme concentration of 16 mg·L$^{-1}$. The enzyme activity is measured in the *o*NPG activity assay. (Lines for guidance.)

The initial activity in buffer was approximately 13 mmol·min$^{-1}$·g enzyme$^{-1}$, while the initial activities were approximately 10 and 4 mmol·min$^{-1}$·g enzyme$^{-1}$ in 5.0 and 30% (w/w) lactose, respectively. The reduction in the initial activity with an increasing

lactose concentration is caused by the competition of lactose (that is present in the samples) with *o*NPG in the activity assay, as will be discussed later.

In buffer, the enzyme was stable at 25ºC, but lost 84% of its activity at 40ºC, and was completely inactivated after two hours at 60ºC. This complete inactivation in buffer at 60ºC was expected: Mozaffar et al. (1984), Vetere and Paoletti (1998), and Song et al. (2011b) described that its isoforms are stable up to at most 50ºC for one hour. The stability improves considerably in the presence of lactose. In a 5.0% (w/w) lactose solution, 45, 64, and 0% of the enzyme activity was left after 24 hours at 25, 40, and 60ºC, respectively. In a 30% (w/w) lactose solution, 36, 49, and 27% of the enzyme activity was left after 24 hours at 25, 40, and 60ºC, respectively.

The measured activity in Figure 1B and C after 24 hours of reaction at 25ºC was lower than at 40ºC. One would expect a better stability at a lower temperature. The unexpected stability values are the result of the presence of reactants during the activity assay. These reactants interfere with the activity measurements similarly as was described by Baks et al. (Baks et al. 2006) (Warmerdam et al. Submitted for publication-b). Therefore, the carbohydrate composition in the samples was determined and the effect of these reactants on the activity assay was determined with equation 2.

### *Carbohydrate profiles*



Figure 2 and Figure 3 show the concentrations of disaccharide (A), GOS (B), glucose (C) and galactose (D) as a percentage of the total carbohydrate content during lactose conversion with Biolacta N5 at 25, 40 and 60ºC and pH 6.0 for an initial lactose concentration of 5.0% (w/w) as well as 30% (w/w).

The carbohydrate content changed in time, and varied considerably between the initially different lactose concentrations. At an initial lactose concentration of 5.0% (w/w), the carbohydrate concentrations hardly changed anymore after 6 hours of reaction at 60ºC, whereas the GOS content decreased and the glucose and galactose content increased after 6 hours of reaction at 25 and 40ºC. The galactose production (indicating hydrolysis) was substantial at an initial lactose concentration of 5.0% (w/w) because of a high availability of water molecules, whereas no significant amounts of galactose were observed at an initial lactose concentration of 30% (w/w).

At an initial lactose concentration of 30% (w/w), GOS synthesis continued at all temperatures, including 60ºC, until at least 22 hours of reaction. Moreover, the reaction temperature had a strong effect on the carbohydrate composition. At both initial concentrations, higher amounts of oligosaccharides were present at 60ºC than at the lower temperatures. At an initial concentration of 30 w/w%, also the glucose content is

higher at 60ºC than at the lower temperatures, while at an initial concentration of 5.0 w/w% more glucose and galactose were present at 40ºC than at 25 and 60ºC after 24 hours of reaction. These differences are due to a higher initial activity and a lower stability at the higher temperatures.

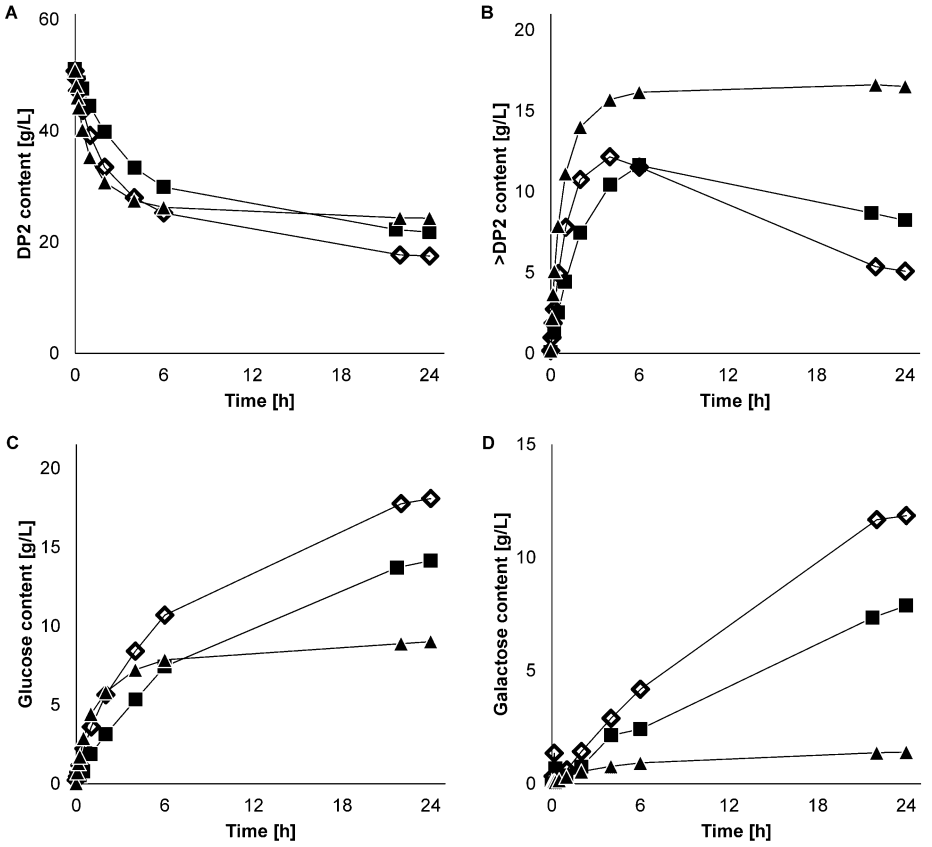


**Figure 2.** Carbohydrate profiles at an initial lactose concentration of 5.0% (w/w) at ■ 25, ◇ 40, and ▲ 60ºC and pH 6.0 with an enzyme concentration of 16 $mg \cdot L^{-1}$. A. Disaccharide conversion; B. GOS (all oligosaccharides larger than DP2) production; C. Glucose production; D. Galactose production. Figure corresponds with Figure 1B.

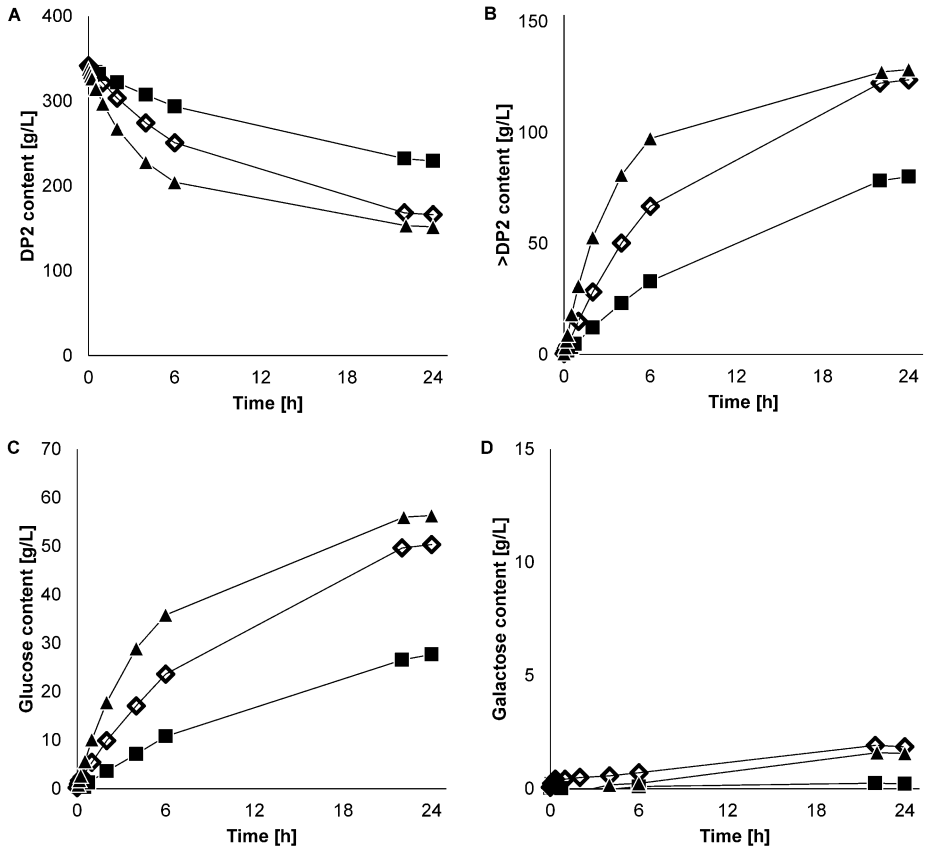


**Figure 3.** Carbohydrate profiles at an initial lactose concentration of 30% (w/w) at ■ 25, ◇ 40, and ▲ 60ºC and pH 6.0 with an enzyme concentration of 16 $mg \cdot L^{-1}$. A. Disaccharide conversion; B. GOS (all oligosaccharides larger than DP2) production; C. Glucose production; D. Galactose production. Figure corresponds with Figure 1C.

## *Correction for the presence of carbohydrates in stability experiments*

The enzyme activity that was determined at initial lactose concentrations of 5.0 and 30% (w/w) was evaluated once more. The influence of lactose, galactose, glucose, and oligosaccharides on the activity assay was taken into account using equation 2 and 3, because the carbohydrate content differed considerably during lactose conversion at various conditions (Figure 2 and Figure 3) and the carbohydrates have a strong effect on the activity assay (Warmerdam et al. Submitted for publication-a; Warmerdam et al. Submitted for publication-b). The corrected enzyme activities are shown in Figure 4.

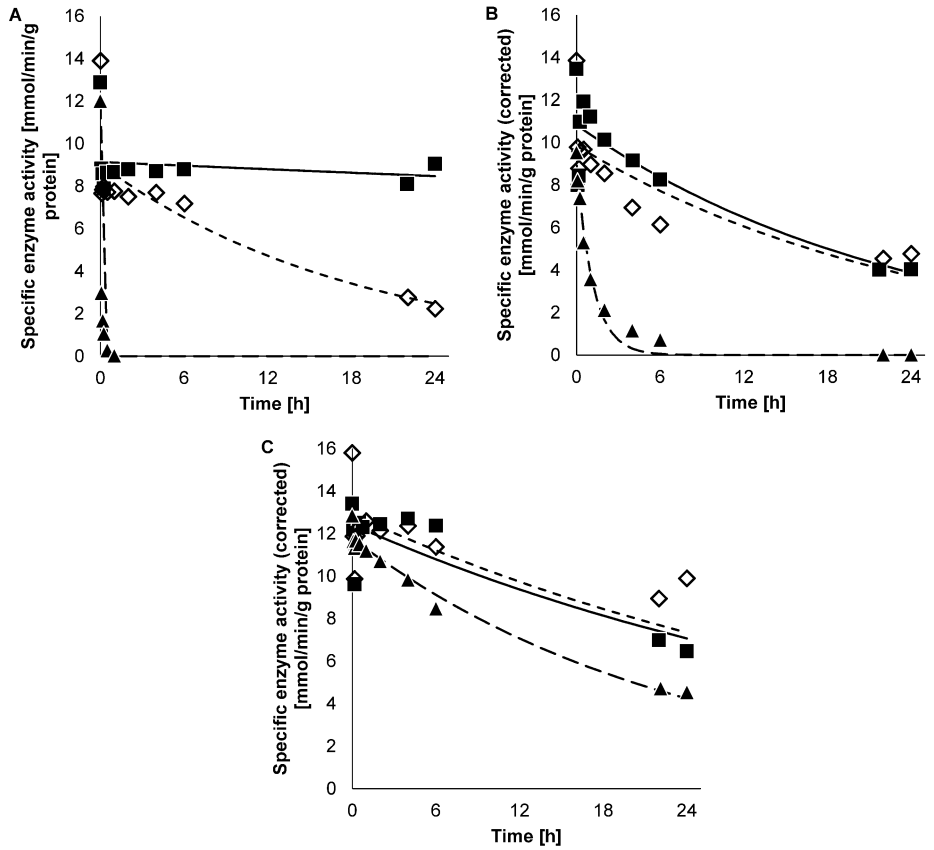


**Figure 4.** Stability of Biolacta N5 in (A) buffer, (B) 5.0% (w/w), and (C) 30% (w/w) lactose at ■, —— 25; ◇, – – – 40; and ▲, — — 60ºC and pH 6.0 with an enzyme concentration of 16 mg·L$^{-1}$, corrected for the influence of lactose, galactose, glucose, and oligosaccharides. Symbols represent measured data, (dashed) lines represent modeled data.

After correction for the presence of carbohydrates in the assay, the activities at time zero are more or less similar (Table 2) in contrast to when no correction is applied. Whereas the activity at 40ºC in a 5.0% (w/w) lactose solution seemed to increase (Figure 1), it decreased after correction for the presence of carbohydrates and the corrected enzyme activity decreased with increasing temperature up to six hours of reaction. The enzyme activity seems to be similar at 25 and 40ºC at 24 hours of reaction. The decrease of enzyme stability at 60ºC is in agreement with the breakdown of lactose and formation of glucose (Figure 2). The initial rate of lactose breakdown and glucose formation is higher with increasing temperature. However, after approximately one hour the rate of lactose breakdown and glucose formation clearly decreased due to the lower enzyme stability. The lower enzyme stability at 60ºC is evident in a higher $k_d$ (Table 3).

In a 30% (w/w) lactose solution, the enzyme activity was very stable for up to six and four hours of reaction at 25 and 40ºC, respectively. At 60ºC, the enzyme activity decreased almost linearly up to six hours. Also in this case, the decrease in enzyme stability at 60ºC is in agreement with the carbohydrate profiles, since the hydrolysis of lactose and formation of glucose level off in time (Figure 3). Afterwards, the enzyme activity decreased at all temperatures. At 24 hours of reaction, the activity at 25 and 40ºC was 48 and 63% of the initial activity, and at 60ºC, 35% of the initial activity remained. The lower enzyme stability at 60ºC is evident in a higher $k_d$ (Table 3).

**Table 2.** The initial *o*NP formation rate $k_0$ of Biolacta N5 in mmol·min$^{-1}$·g protein$^{-1}$ at various initial lactose concentrations and temperatures, together with its 95% confidence interval.

| [lactose]► ▼T | 0% (w/w) | 5.0% (w/w) | 30% (w/w) |
|---|---|---|---|
| 25ºC | 9.1 ± 1.0 | 11 ± 1 | 12 ± 1 |
| 40ºC | 9.0 ± 1.4 | 9.9 ± 1.2 | 13 ± 1 |
| 60ºC | 12 ± 1 | 8.9 ± 0.6 | 12 ± 0 |

**Table 3.** The inactivation constant $k_d$ of Biolacta N5 in h$^{-1}$ at various initial lactose concentrations and temperatures, together with its 95% confidence interval.

| [lactose]► ▼T | 0% (w/w) | 5.0% (w/w) | 30% (w/w) |
|---|---|---|---|
| 25ºC | 0.0032 ± 0.0112 | 0.043 ± 0.023 | 0.024 ± 0.011 |
| 40ºC | 0.054 ± 0.040 | 0.041 ± 0.025 | 0.024 ± 0.015 |
| 60ºC | 15 ± 3 | 0.85 ± 0.19 | 0.043 ± 0.006 |

**Table 4.** The half-life time $t_{½}$ of Biolacta N5 in hours at various initial lactose concentrations and temperatures.

| [lactose]► ▼T | 0% (w/w) | 5.0% (w/w) | 30% (w/w) |
|---|---|---|---|
| 25ºC | 220 | 16 | 29 |
| 40ºC | 13 | 17 | 29 |
| 60ºC | 0.048 | 0.82 | 16 |

The half-life time, and thus stability, at 60ºC in 30% (w/w) lactose is very high compared to that in buffer or in diluted lactose systems as the enzyme was completely inactivated in those systems. The half-life time of the enzyme (Table 4) (strongly) increased with increasing substrate concentration at 40 and 60ºC.

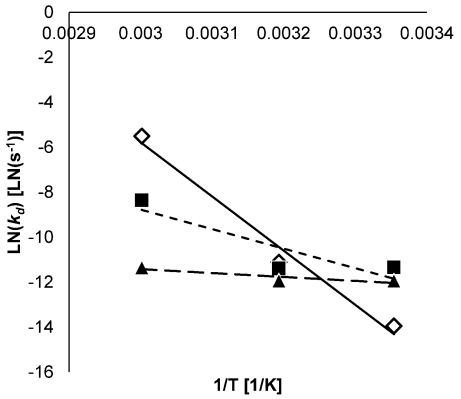


**Figure 5.** Linearized Arrhenius plot of ln($k_d$) as a function of 1/T. Symbols: ◇, —— 0% (w/w) lactose; ■, – – – 5.0% (w/w) lactose, and ▲, — — 30% (w/w) lactose.

**Table 5.** Inactivation energy $E_a$ for various lactose concentrations

| [lactose] [% (w/w)] | $E_a$ [kJ·mol$^{-1}$] |
|---|---|
| 0 | 200 |
| 5.0 | 72 |
| 30 | 14 |



Figure 5 shows the linearized Arrhenius plot of ln($k_d$) as a function of 1/T. The inactivation of the enzyme in buffer is strongly dependent on the temperature, whereas the inactivation in a system with 30% (w/w) lactose initially is hardly dependent on the temperature. The inactivation energy $E_a$, shown in Table 5, decreased with increasing substrate concentration. This is similar to what was found by De Cordt et al. (1994). The higher stability of the enzyme is might be caused by molecular crowding or by complexation with the substrate or with a remaining galactose moiety.

A higher thermostability at high substrate concentrations is very favorable in the production of GOS by β-galactosidases from *B.circulans*. At high substrate concentrations, the reaction temperature can be higher than the enzyme's stable ranges that were reported before in aqueous solutions, and it can be equal/closer to their optimal temperatures (Mozaffar et al. 1984; Song et al. 2011a; Vetere and Paoletti 1998), which will result in a higher enzyme activity.

## Conclusions

β-Galactosidase from *Bacillus circulans* was found to be quite stable against temperature at high substrate concentrations, when the enzyme activity measurements were corrected for the presence of various reactants.

This correction was of importance for both the reaction at 5.0 and 30% (w/w) lactose. Not correcting the enzyme activity at 5.0% (w/w) lactose resulted in an overestimation of the actual stability, whereas not correcting the enzyme activity at 30% (w/w) lactose resulted in an underestimation of the actual stability of β-galactosidase from *Bacillus circulans*. A high initial substrate concentration has a large positive effect on the enzyme stability.

The improved stability in more concentrated systems is very interesting for production conditions. The utilization of more concentrated systems for enzymatic conversions is economically more interesting in order to avoid the unnecessary use of water, to save energy as a smaller volume needs to be heated, and to save on capital expenditures as less equipment is necessary.

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# Chapter 6

## Galacto-oligosaccharide production with immobilized β-galactosidase in a packed-bed reactor vs. free β-galactosidase in a batch reactor


## Abstract

We report here that the usage of immobilized enzyme in a continuous packed bed reactor (PBR) can be a good alternative for GOS production that traditionally uses free enzyme in a batch reactor. The carbohydrate composition of the product of the PBR with immobilized enzyme was comparable to that of the batch reactor with free enzyme. The stability of the immobilized enzyme at a lactose concentration of 38% (w/v) and at 50ºC was very high: the half-life time of the immobilized enzyme was approximately 90 days. The enzymatic productivity of GOS production using immobilized enzyme in a PBR can be more than six times higher than that of GOS production with free enzyme in a batch reactor. Besides, when aiming for an equal volumetric productivity to the batch process in designing a PBR, the volume of the PBR can be much smaller than that of the batch reactor, depending on the enzyme dosage and the run time of one batch.




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## Introduction

The advantages of immobilized enzyme instead of free enzymes have been shown by existing large-scale processes such as the production of high fructose syrup (HFS) and penicillins such as 6-APA (Brodelius 1978; Linko et al. 1983). Due to the reuse of the immobilized enzymes, the cost of enzyme and carrier can be reduced to a few cent per kg finished product (Katchalski-Katzir and Kraemer 2000).

Usually, enzymes are immobilized onto insoluble polymeric beads, simplifying the recovery of the enzyme. Besides the ease of recovery, the immobilization often provides an increase in their stability (Boller et al. 2002; Campello et al. 2012; Cao 2005; Chen et al. 2009; Hernaiz and Crout 2000; Liu et al. 2012; Nakkharat and Haltrich 2007; Nguyen et al. 2005; Zhou and Chen 2001). The enhanced stability often comes at the expense of the activity of the enzyme (Sheldon 2007). Nevertheless, the ability to reuse the enzyme often compensates for the loss in activity.

The current industrial process for the production of galacto-oligosaccharides (GOS) uses free β-galactosidase (FDA 2007; FDA 2009; FDA 2010) in a batch reactor. GOS are produced on tonnage scale (Crittenden and Playne 1996; Gänzle 2012; Mahoney 1998) and are applied in infant nutrition because of their prebiotic benefits for health and similarity to human milk oligosaccharides (HMO) (Macfarlane et al. 2008; Mahoney 1998; Playne and Crittenden 2009).

GOS are synthesized from lactose via enzymatic synthesis with β-galactosidases (Barreteau et al. 2006; Mahoney 1998; Playne and Crittenden 2009; Prenosil et al. 1987). Besides forming GOS through transgalactosylation, β-galactosidases simultaneously hydrolyze lactose into galactose and glucose. Many factors such as the initial lactose concentration, the temperature, and the enzyme source, affect the ratio between hydrolysis and transgalactosylation (Boon et al. 2000).



Since in the GOS production process the enzyme is often still active after the reaction, it may be attractive to reuse the enzyme, which can be realized by immobilization of the enzyme. The aim of this study is therefore to investigate the feasibility of GOS production using immobilized beta-galactosidase in a continuous flow packed bed reactor (PBR) and to compare the performance of such system with the traditional batch system with free enzyme, in terms of enzymatic and volumetric productivity.

A β-galactosidase from *Bacillus circulans*, covalently immobilized on Eupergit C 250L was selected as our model immobilized enzyme. In order to evaluate the robustness

of this system, the activity and stability of the immobilized β-galactosidase were studied. Besides, the final product composition is also compared to the product composition obtained with the free enzyme. Finally, the productivities of the packed-bed reactor with immobilized enzyme and the batch system with free enzyme are compared.

## Materials and methods

### *Materials*

Lactose monohydrate (Lactochem), and Biolacta N5, a β-galactosidase from *Bacillus circulans*, (Amano Enzyme Inc., Nagoya, Japan) were gifts from FrieslandCampina (Beilen, The Netherlands). The enzyme preparation was previously found to have a protein content of 19 ± 3% (Warmerdam et al. Accepted for publication). In all calculations, the total enzyme concentration is assumed to be equal to the total protein concentration, because the actual enzyme concentration is not known.

Eupergit C 250L was a kind gift from Evonik (Darmstadt, Germany). D(+)-Galactose, D(+)-glucose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, sulfuric acid, sodium hydroxide, *o*-nitrophenyl β-D-galactopyranoside (*o*NPG), *o*-nitrophenol (*o*NP), and ethanolamine were purchased from Sigma-Aldrich (Steinheim, Germany).

McIlvaine's buffer was prepared by adding together 0.1 M citric acid and 0.2 M disodium hydrogen phosphate in the correct ratio to reach a pH of 6.0. Sodium carbonate, citric acid monohydrate, disodium hydrogen phosphate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, and sodium chloride were purchased from Merck (Darmstadt, Germany).



### *Immobilization of Biolacta N5*

Eupergit C 250L is used as a carrier for immobilization of Biolacta N5. Eupergit C 250L is well-known carrier consisting of porous acrylic beads with oxirane functionality (Boller et al. 2002; Sheldon 2007). Covalent immobilization proceeds through reaction of thiol and amino groups of the enzyme with the epoxide groups of the carrier.

Eupergit C 250L beads were washed with excess MilliQ water and dried over a glass filter by vacuum suction. The last washing step was performed with 0.2 M potassium phosphate buffer of pH 7.5. The resulting carrier, after drying over a glass filter with vacuum suction, was defined as wet Eupergit and had a mass of approximately four

times its initial dry mass. This is corresponding to the work of Bortone et al. (2012). The increase in mass is caused by the uptake of water by the carrier beads. An enzyme solution of 20 g Biolacta N5·$L^{-1}$ was prepared by dissolving Biolacta N5 in 1 M potassium phosphate buffer of pH 8.5. The carrier beads were added to the enzyme solution in a ratio of 1.0 g Biolacta N5 per 10 g wet Eupergit and incubated at room temperature in an IKA HS 250 horizontal shaker under gentle shaking for 24 hours. After washing with MilliQ water and 0.5 M NaCl, the beads were incubated in 20% (v/v) ethanolamine in 0.1M potassium phosphate buffer of pH 7.5 to block the remaining epoxide groups and providing a more hydrophilic surface of the carrier beads. After rinsing with excess MilliQ water again, the immobilized enzyme was stored in McIlvaine's buffer of pH 6.0 in the refrigerator. The amount of immobilized enzyme is defined as wet Eupergit with immobilized enzyme.

*Enzyme activity measurements*

The *o*NPG converting activity of the free enzyme, the supernatant after immobilization, the washing liquid, and the immobilized enzyme were measured. These measurements were performed as previously described (Warmerdam et al. Accepted for publication). An Eppendorf tube with 979 µL of 0.20% (w/w) *o*NPG-in-McIlvaine's buffer of pH 6.0 was preheated in an Eppendorf Thermomixer at 40ºC and 600 rpm for 10 minutes. Subsequently, 21 µL of sample was added and these mixtures were incubated for another 10 minutes at 40ºC and 600 rpm. A volume of 1.0 mL of 10% (w/w) $Na_2CO_3$ solution was added to stop the reaction and, afterwards, the absorbance of *o*NP was measured at 420 nm. The *o*NP concentration was determined using the law of Lambert-Beer of which the extinction coefficient was determined to be 4576 $M^{-1}$·$cm^{-1}$. The *o*NP formation was found to be linear during the first 10 minutes of the reaction. This initial rate of *o*NP formation was expressed in mmol·$min^{-1}$·g $protein^{-1}$. Measurements were performed in duplicate and the average enzyme activity was used.

The assay was slightly adapted to measure the enzyme activity of the immobilized enzyme, although the oNPG concentration in the assay was remained at 0.20% (w/w). A weighted amount of approximately 5 mg of wet Eupergit with immobilized enzyme was suspended in 1.21 mL McIlvaine's buffer and added to 790 µL preheated 0.50% (w/w) *o*NPG-in-buffer. After an incubation time of 10 minutes at 40ºC and 900 rpm, the *o*NPG-immobilized enzyme solution was transferred to a tube containing 2.0 mL $Na_2CO_3$ solution. Before measuring the absorbance, the solution was filtered with a 0.2 µm Minisart filter.

*Experimental setup PBR*

Lactose conversion in a PBR with immobilized Biolacta N5 was followed in time at various ingoing lactose concentrations. Lactose was dissolved in McIlvaine's buffer of pH 6.0 at 60ºC. The experiments at 5.0, 12, and 15% (w/v) were performed with a thermostatted XK16/20 column (GE Healthcare, Buckinghamshire, UK). The experiments at 25 and 38% (w/v) were performed with a Tricorn 5/20 column (GE Healthcare, Buckinghamshire, UK). The latter experiments were performed in an oven to control the temperature. Each reaction was performed at 50ºC. The packing of the PBR was 0.86 g immobilized enzyme per mL (0.22 g dry support per mL) of column volume with a loading of 20 mg protein per g immobilized enzyme. The lactose solution was pumped through the column at various flow rates. The initial flow rate corresponded to a residence time $\tau$ of 12 minutes, which was calculated with equation 1:

$$\tau = \frac{V_r \cdot (1 - \varepsilon)}{\phi} \tag{1}$$

where $V_r$ is the volume of the PBR in mL, $\varepsilon$ is the porosity of the bed in between the beads, and $\phi$ is the flow rate through the system in mL·min$^{-1}$. The porosity of the bed was assumed to be 0.60 equal to that used by Spieß et al. (1999).

After each variation in the flow rate, the flow rate with a residence time of 12 minutes was repeated (Figure 1). Carbohydrate conversions at this particular residence time were used to calculate enzyme inactivation.

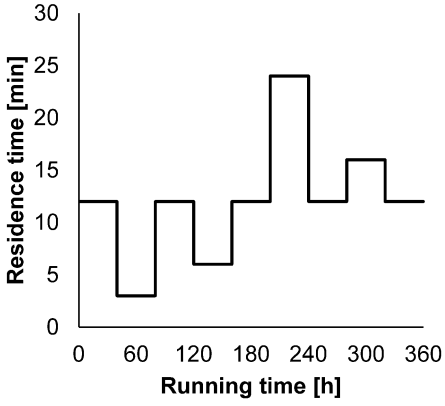


**Figure 1.** Example of the change in residence time during one PBR run

Table 1 shows the conditions of the various PBR experiments. Samples were taken at regular time intervals and stored at -20ºC until further preparation.

**Table 1.** Conditions of each PBR experiment

| $[lac]_{in}$ | Column | $D_{column}$ | $V_{column}$ | $m_{Biolacta\ N5}$* |
|---|---|---|---|---|
| % (w/v) | | cm | mL | g protein |
| 5 | XK16/20 | 1.6 | 7.0 | 0.12 |
| 12 | XK16/20 | 1.6 | 11 | 0.19 |
| 15 | XK16/20 | 1.6 | 7.8 | 0.13 |
| 25 | Tricorn 5/20 | 0.5 | 0.55 | 0.0092 |
| 38 | Tricorn 5/20 | 0.5 | 0.55 | 0.0092 |

** The density of column packing was 0.86 g immobilized enzyme per mL column volume. The loading of Biolacta N5 on the carrier was 20 mg protein per g immobilized enzyme.*

### *Experimental setup batch reactor*

Lactose conversion at various initial lactose concentrations was monitored in time in a temperature controlled batch reactor equipped with an anchor stirrer. The stirring speed was 150 rpm. The lactose was dissolved at approximately 60ºC prior to cooling the solution to the desired reaction temperature. The enzyme was added once the temperature was constant. The initial reaction volume was 25 mL. Samples were taken at regular time intervals and transferred to a pre-weighted Eppendorf tube with 5% (w/w) sulfuric acid solution to inactivate the enzyme. Subsequently, the samples were stored at -20ºC until further preparation.



### *Sample handling for determination of the carbohydrate composition*

The samples from the batch experiments were pretreated to remove the enzyme. Before HPLC analysis, the enzyme was removed from the samples by filtering the samples at 14,000 x g at 18ºC for 30 minutes using pretreated Amicon® ultra-0.5 centrifugal filter devices (Millipore Corporation, Billerica, MA, United States) with a cut-off of 10 kDa in a Beckman Coulter Allegra X-22R centrifuge. The pretreatment of the filters consisted out of two centrifugation steps: first, 500 µL of Milli-Q water was centrifuged at 14,000 x g at 18ºC for 15 minutes; and second, the filters were placed up-side-down in the tube and centrifuged at 14,000 x g at 18ºC for 5 minutes. After filtration, the samples were neutralized with 5% (w/w) sodium hydroxide.

### *Measurements of the carbohydrate composition*

The samples were analyzed with HPLC using a Rezex RSO oligosaccharide column (Phenomenex, Amstelveen, the Netherlands) at 80ºC. The column was eluted with Milli-Q water at a flow rate of 0.3 mL/min. The eluent was monitored with a refractive index detector.

The standards that were used for calibration of the column were lactose, glucose, galactose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose. Galacto-oligosaccharides up to a degree of polymerization of 7 were assumed to have the same response as the glucose-oligomers with equal degree of polymerization. This was confirmed with mass balances.

### *Measurement of the composition of the disaccharides*

The samples were diluted till approximately 10 ppm and analyzed with HPAEC using a CarboPac PA1: 250 x 4 mm anion-exchange column (Dionex, Sunnyvale, CA, USA) at 30ºC. The column was eluted with gradients of sodium hydroxide and sodium acetate at a flow rate of 1.0 mL/min. The eluent was monitored with a pulsed amperometric detector (PAD). The standards that were used for calibration of the column were lactose and lactulose. Other disaccharides were quantified by assuming that their response factor was equal to the response factor of lactose.

### *Determination of the enzyme inactivation*

The glucose formation rate $k_t^{glu}$, galactose formation rate $k_t^{gal}$, and disaccharide conversion rate $k_t^{DP2}$ were determined with equation 2, 3, and 4, respectively:

$$k_t^{glu} = \frac{c_{out}^{glu} - c_{in}^{glu}}{\tau} \quad (2)$$

$$k_t^{gal} = \frac{c_{out}^{gal} - c_{in}^{gal}}{\tau} \quad (3)$$

$$k_t^{DP2} = \frac{c_{in}^{DP2} - c_{out}^{DP2}}{\tau} \quad (4)$$

where $c_{in}^{x}$ and $c_{out}^{x}$ are the concentrations of component x (glucose, galactose, or disaccharides) in the ingoing and outgoing product stream in mM, and $\tau$ is the residence time in minutes. The decrease in reaction rate of each component was modeled with first order inactivation kinetics according to equation 5:

$$k_t^x = k_0^x \cdot e^{-k_d^x \cdot t} \quad (5)$$

where $k_0^x$ is the reaction rate at time zero, $k_d^x$ is the enzyme inactivation constant, and *t* is the running time at which the sample was taken in hours. The enzyme inactivation constant $k_d^x$ and the reaction rate at time zero $k_0^x$ were determined by linearization of equation 5. Only data points at a residence time of 12 minutes were used to determine the enzyme inactivation. The running time at which the reaction rate $k_t^x$ is half of the initial reaction rate $k_0^x$ is defined as the half-life time.

### *Correction of carbohydrate composition for inactivation*

To compare the glucose, galactose, and disaccharide content at the various residence times, the concentrations of glucose, galactose and disaccharides that were measured at the various sampling times were corrected for any enzyme inactivation. To determine $c_{out}^{x}$, corrected for the inactivation that took place at a certain running time, equation 2, 3, and 4 were used with $k_0^x$ (that was found for a residence time of 12 minutes) instead of $k_t^x$. The concentration of GOS $C_{out}^{GOS}$ was calculated with equation 6:

$$C_{out}^{GOS} = C_{in}^{DP2} - C_{out}^{DP2} - C_{out}^{glu} - C_{out}^{gal} \quad (6)$$

where $C_{in}^{DP2}$ is the ingoing lactose concentration, $C_{out}^{glu}$ is the glucose concentration, and $C_{out}^{gal}$ is the galactose concentration, all in g·L$^{-1}$.

### *Correction of residence time for inactivation*

To calculate the productivity at each ingoing lactose concentration, the residence time which resulted in the highest GOS concentration was determined from the plots of carbohydrate concentration versus residence time (Figure 5). Because the outgoing carbohydrate composition should remain constant, the residence time was adapted to the

enzyme inactivation. The adaptation of the residence time was based on glucose formation and was determined by rewriting equations 1 and 5 to equation 7:

$$\tau_t = \frac{\tau_0}{e^{-k_d^{glu} \cdot t}} \tag{7}$$

where $\tau_t$ and $\tau_0$ are the residence times at running time t and zero, respectively, in minutes.

### *Productivities*

The enzymatic productivity $Q_{enz}$ is defined as the amount of GOS produced per g of protein during one run. $Q_{enz}$ was calculated with equation 8:

$$Q_{enz} = \frac{m_{GOS}}{m_{BN5}} \tag{8}$$

where $m_{GOS}$ is the total mass of GOS with a DP larger than two in kg; and $m_{BN5}$ is the mass of Biolacta N5 protein in g. The length of one run in the PBR was assumed to be equal to the half-life time of the enzyme $t_{½}$, which is defined as the running time where the enzyme activity is half of the initial enzyme activity, in h.

We have chosen to express the productivity of the two systems per g protein and not per unit of enzyme activity. Expressing per g protein takes into account the activity loss during immobilization. Besides, expressing the productivity per unit of enzyme activity is difficult, since activity assays described in literature often differ in the applied conditions. Therefore, the activity units derived from different assays are not comparable. Using g GOS·g protein$^{-1}$ avoids this problem.

The volumetric productivity of the PBR and the batch reactor, averaged over the running time of the reaction, can be determined with equation 9:

$$Q_V = \frac{V_{GOS}}{t} \tag{9}$$

where $V_{GOS}$ is the total volume of product produced during one run in m$^3$ and $t$ is the running time in hours. The outgoing product composition has a constant GOS content during the entire run, since the flow rate is adapted to the enzyme inactivation, allowing the expression of $V_{GOS}$ in m$^3$.

## Results and discussion

### *Immobilization*

The *o*NPG converting activity of free Biolacta N5 was 9.7 mmol·min$^{-1}$·g protein$^{-1}$. The total enzyme activity loss measured in the supernatant after immobilization and the washing liquids was measured to be 1.4% of the initial enzyme activity. This means that 99% of the total amount of enzyme is immobilized on the Eupergit C 250L, which equals 20 mg protein per gram immobilized enzyme. The measured activity of the immobilized enzyme was 3.0 mmol·min$^{-1}$·g protein$^{-1}$ (or 59 µmol·min$^{-1}$·g immobilized enzyme$^{-1}$), which was 31% of the activity that would be obtained if all enzyme activity would be retained during immobilization. Higher immobilization yields have been described before by Hernaiz and Crout (2000) and Torres et al. (2012) for this enzyme and the same, or similar, carrier. The lower yield is possibly due to suboptimal enzyme to carrier ratio, pH value and the use of a different blocking agent.

### *Experiments with immobilized Biolacta N5 in a packed bed reactor*

PBR experiments were carried out with immobilized Biolacta N5 at a temperature of 50ºC. The lactose concentration in the feed was varied between 5 and 38% (w/v). The running time of each experiment was approximately 300 h, except for the experiment at an ingoing lactose concentration of 38% (w/v), because crystallization of lactose occurred in the PBR after approximately 170 h. The flow rate and residence time were varied during the running time.

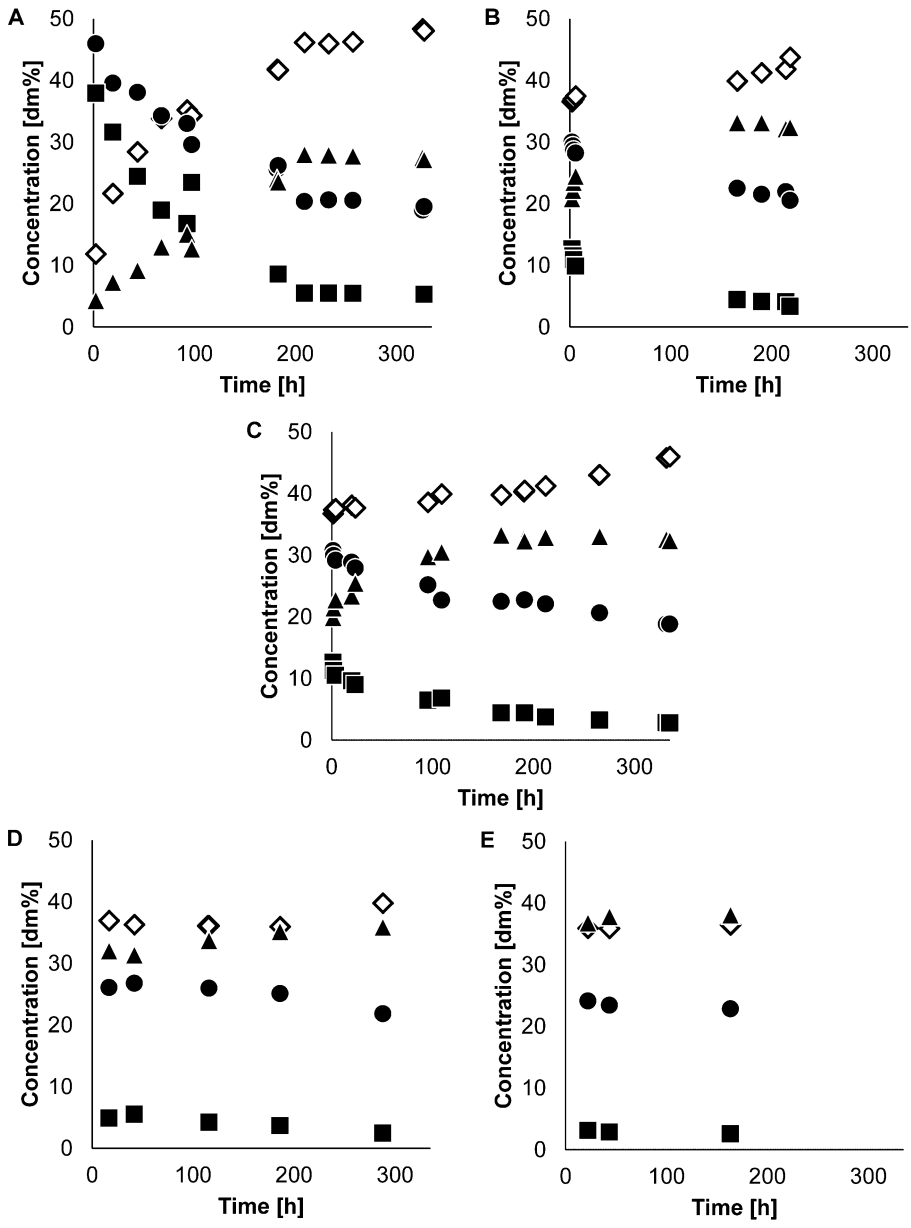


**Figure 2.** Carbohydrate concentrations in the outgoing product stream of the PBR after conversion of lactose with immobilized Biolacta N5 at 50ºC, pH 6.0, a residence time of 12 min, and a substrate concentration of (A) 5.0% (w/v); (B) 12% (w/v); (C) 15% (w/v); (D) 25% (w/v); and (E) 38% (w/v). Symbols: ■ galactose, ● glucose, ◇ disaccharides, and ▲ total GOS.

Figure 2 shows the carbohydrate content of the outgoing product stream of the PBR with immobilized Biolacta N5 at various ingoing lactose concentrations at 50ºC, and a

residence time of 12 minutes. Especially at low substrate concentrations, an increase in the disaccharide and GOS content was observed together with a decrease in the galactose and glucose content. This increase of disaccharides and GOS content became less pronounced with increasing substrate concentration. Also, the decrease in monosaccharaide content reduced with increasing substrate concentration. The ratio between the different products changed in time. This indicates that the rate of oligosaccharide synthesis increased and/or, more likely, the rate of oligosaccharide hydrolysis decreased during the running time of the PBR.

The changes in carbohydrate composition were due to enzyme inactivation. To determine the enzyme stability, the rate of glucose formation, galactose formation, and disaccharide conversion were determined with equation 2, 3, and 4, respectively. The results are shown in Figure 3.

Particularly at low substrate concentrations, the disaccharide conversion and glucose and galactose formation decreased, due to enzyme inactivation. Lower inactivation, and thus a higher stability, was observed at higher substrate concentrations.

The enzyme inactivation followed an exponential decrease at a substrate concentration of 5.0% (w/v) which can be described with first order inactivation kinetics. At higher substrate concentrations, the enzyme inactivation seems to be linear. The (nearly) linear decrease in enzyme activity was found because the measurements were only carried out in the initial regime of enzyme inactivation. Figure 4, Table 2 and Table 3 show the reaction rate constant $k_0$ and the inactivation constant $k_d$ at the various substrate concentrations, which were determined with equation 5.

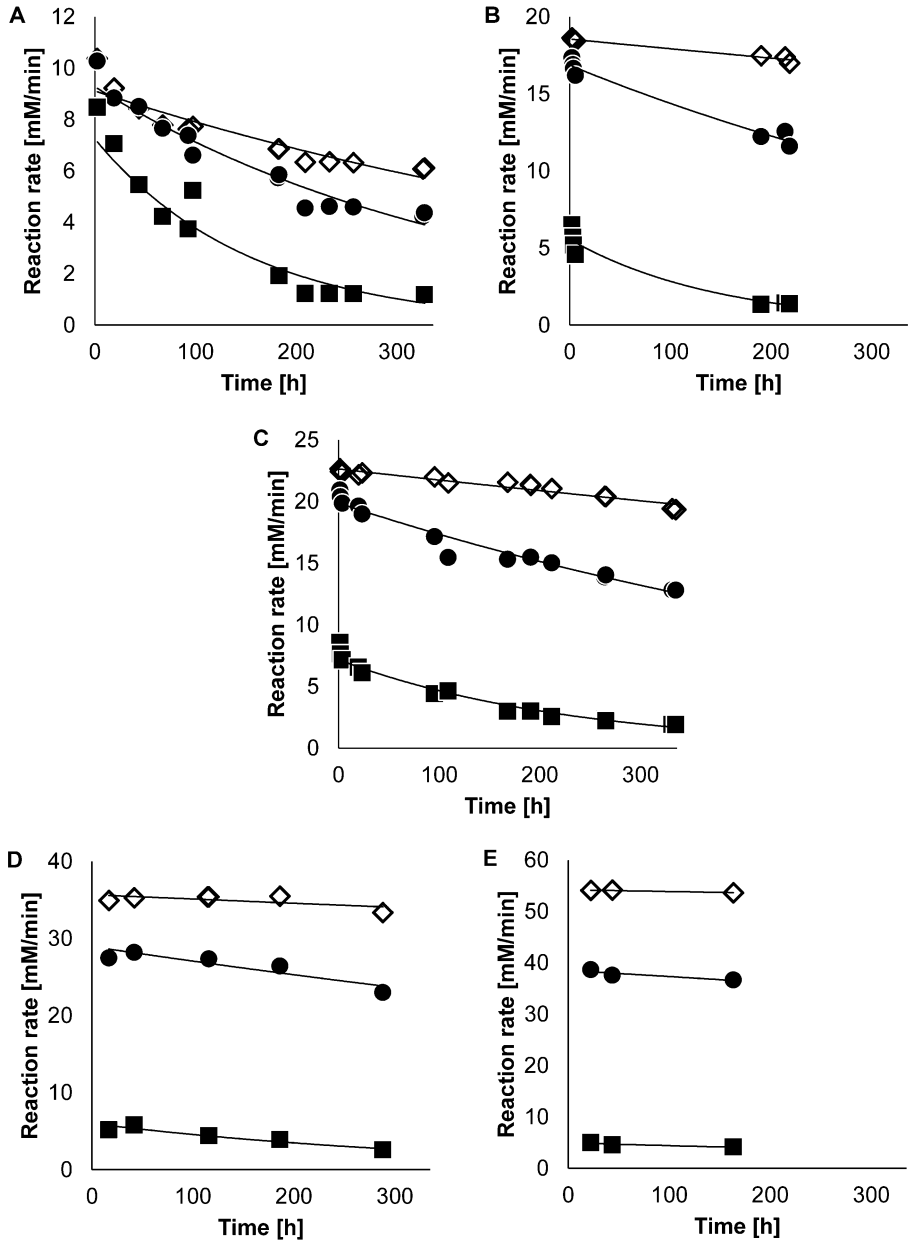


**Figure 3.** The rate of glucose formation (●), galactose formation (■), and disaccharide conversion (◇) of immobilized Biolacta N5 in time at 50ºC, pH 6.0, a residence time of 12 min, and an ingoing lactose concentration of (A) 5.0% (w/v), (B) 12% (w/v), (C) 15% (w/v), (D) 25% (w/v), and (E) 38% (w/v). The symbols represent the measured data, the lines represent the inactivation curves fitted with equation 2, 3, and 4.

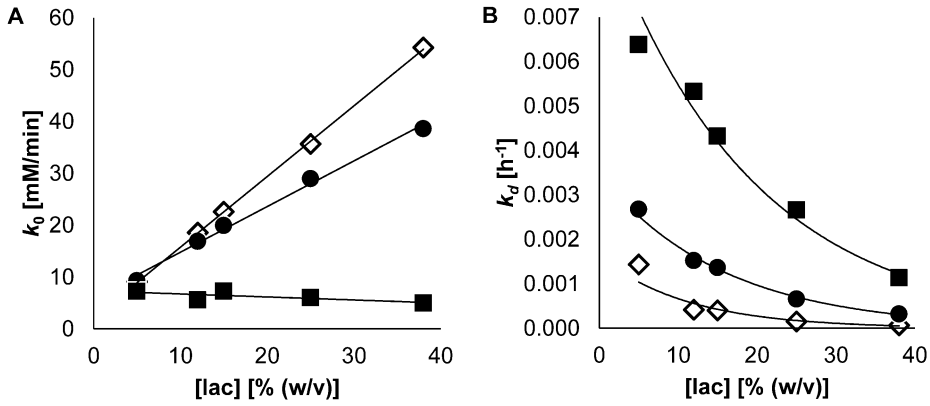


**Figure 4.** Inactivation parameters $k_0$ and $k_d$ based on ● glucose formation, ■ galactose formation, and ◇ disaccharide conversion at various initial lactose concentrations, 50ºC, and pH 6.0.

**Table 2.** The initial reaction rate for glucose formation, galactose formation, and disaccharide conversion at various initial lactose concentrations, 50ºC, and pH 6.0.

| $[lac]_{in}$ [% (w/v)] | 5 | 12 | 15 | 25 | 38 |
|---|---|---|---|---|---|
| $k_0^{glu}$ [mM$^{-1}$·min$^{-1}$] | 9.3 | 17 | 20 | 29 | 39 |
| $k_0^{gal}$ [mM$^{-1}$·min$^{-1}$] | 7.2 | 5.6 | 7.3 | 6.0 | 5.0 |
| $k_0^{DP2}$ [mM$^{-1}$·min$^{-1}$] | 9.1 | 19 | 23 | 36 | 54 |

**Table 3.** The inactivation constant based on glucose formation, galactose formation, and decrease in disaccharide content at various initial lactose concentrations, 50ºC, and pH 6.0.

| $[lac]_{in}$ [% (w/v)] | 5 | 12 | 15 | 25 | 38 |
|---|---|---|---|---|---|
| $k_d^{glu}$ [h$^{-1}$] | $2.7\cdot10^{-3}$ | $1.6\cdot10^{-3}$ | $1.4\cdot10^{-3}$ | $6.7\cdot10^{-4}$ | $3.2\cdot10^{-4}$ |
| $k_d^{gal}$ [h$^{-1}$] | $6.4\cdot10^{-3}$ | $6.7\cdot10^{-3}$ | $4.3\cdot10^{-3}$ | $2.7\cdot10^{-3}$ | $1.1\cdot10^{-3}$ |
| $k_d^{DP2}$ [h$^{-1}$] | $1.4\cdot10^{-3}$ | $3.4\cdot10^{-4}$ | $4.0\cdot10^{-4}$ | $1.6\cdot10^{-4}$ | $6.4\cdot10^{-5}$ |

The initial reaction rates $k_0^{glu}$ and $k_0^{DP2}$ increased linearly with increasing the initial lactose concentration, whereas the reaction rate $k_0^{gal}$ is more or less independent of the initial lactose concentration. The inactivation constant $k_d$ for each component decreased strongly. Consequently, the half-life time, i.e. the stability of the enzyme, increased substantially with increasing substrate concentration.

The real enzyme inactivation is best approximated based on glucose formation, as this combined both synthesis and hydrolysis. The inactivation constant based on glucose formation $k_d^{glu}$ at an ingoing lactose concentration of 15% (w/v) was equal to the inactivation constant based on lactose conversion $k_d^{DP2}$ ($1.4\cdot10^{-3}$ h$^{-1}$, determined with

HPAEC). The inactivation constant $k_d^{DP2}$ based on the decrease in disaccharide concentration gives an underestimated value, since the disaccharide concentration includes, besides lactose, all other disaccharides that are formed during the reaction. The inactivation constant based on galactose $k_d^{gal}$ only provides information on the decrease in hydrolysis rate and not on the oligosaccharide synthesis.

### *Residence times PBR and batch process compared*

During one PBR experiment with a specific lactose concentration, the residence time (flow rate) was varied (see Figure 1). To compare the different residence times, the reaction rates of the immobilized enzyme and the carbohydrate concentrations were corrected for the enzyme inactivation with equation 2, 3, and 4 using the parameters shown in Table 3. The GOS content was subsequently determined with equation 6.

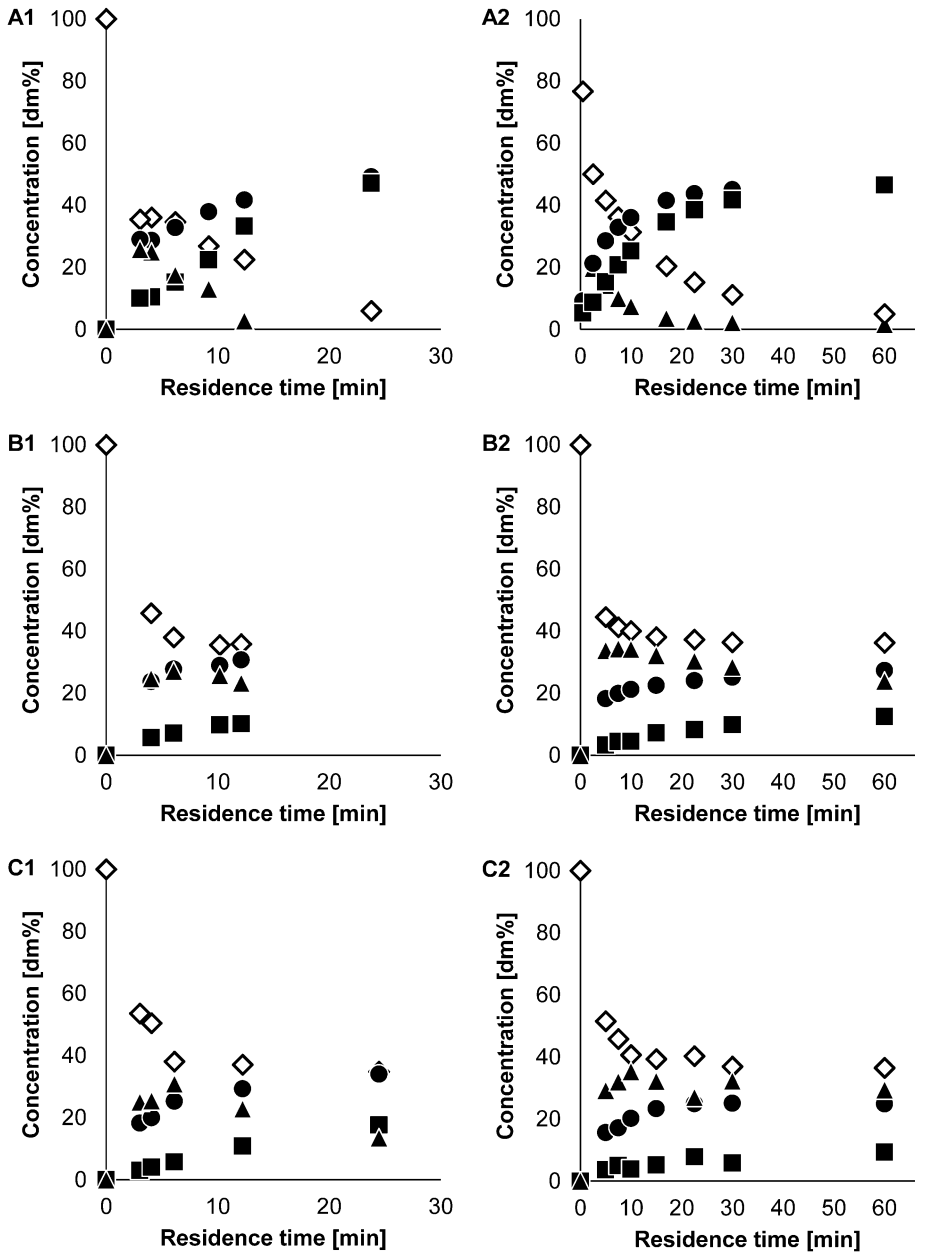


**Figure 5.** Carbohydrate profile as a function of residence time after lactose conversion with immobilized Biolacta N5 in a PBR (left figures) and free Biolacta N5 (at 1.7 g protein·$L^{-1}$) in a batch reactor (right figures) at 50ºC, pH 6.0, and an ingoing lactose concentration of (A) 5.0% (w/v), (B) 12% (w/v), (C) 15% (w/v), (D) 25% (w/v), and (E) 38% (w/v). Symbols: ■ galactose, ● glucose, ◇ disaccharides, and ▲ total GOS. (Note: the initial amount of enzyme in the PBR had an activity of 50 µmol·$min^{-1}$·$mL^{-1}$ vs. 16 µmol·$min^{-1}$·$mL^{-1}$ in the batch reactor.)

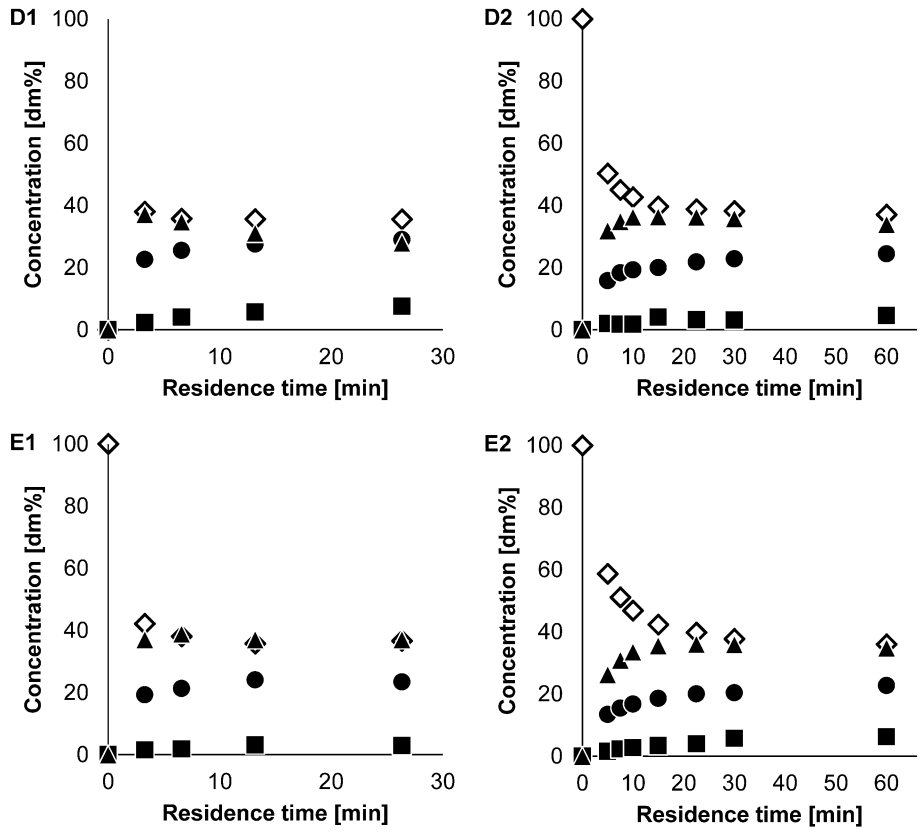


**Figure 5 continued.**

At short residence times in the PBR, the disaccharide content decrease rapidly. The glucose, galactose, and GOS content increased towards a certain level with increasing residence time. Although at short residence times, some GOS was formed at a low ingoing lactose concentration of 5% (w/v), the lactose was generally almost completely hydrolyzed into glucose and galactose at longer residence times. At intermediate ingoing lactose concentrations (12, 15, and 25%), the conversion into GOS increased with increasing residence time at short residence times, while at longer residence times the GOS levels decreased. At a very high ingoing lactose concentration of 38% (w/v), the carbohydrate composition did not change with the residence time.

Overall, the carbohydrate profiles of the outgoing PBR stream at various residence times show a similar profile as in the batch reactor, taking into account that the initial amount of enzyme in the PBR had an activity that was approximately three times higher than that in the batch reactor. Besides the difference in enzyme activity, some other small differences were observed. At a substrate concentration of 5% (w/v), the ratio

between galactose and glucose at intermediate residence times was lower in the PBR with immobilized enzyme than in the batch reactor with free enzyme, while GOS formation was higher. At substrate concentrations of 15% (w/v) and 25% (w/v), more galactose was formed in the PBR while slightly less GOS was formed than in the batch reactor at their maximum.

The latter differences are most likely due to diffusion limitation in the PBR with immobilized enzyme, which is a known drawback of Eupergit C 250L and similar carriers (Sheldon 2007). Lactose is depleted near the enzyme, due to either synthesis or hydrolysis. Therefore, it is less available for further synthesis, than water, that is always present in abundance. Thus hydrolysis is stronger than without diffusion limitations, and more galactose is released. This would explain the higher level of galactose and lower level of total GOS in the PBR at a substrate concentration of 15 and 25% (w/v) (Figure 5: C1 vs. C2 and D1 vs. D2). Additionally, the reduced accessibility of the enzyme's active site for GOS molecules could result in a lower rate of hydrolysis of GOS and slower galactose release as is the case for a substrate concentration of 5.0% (w/v) in the PBR (Figure 5: A1 vs. A2).

### *Productivities compared*

To calculate the maximum achievable productivity in the PBR with immobilized enzyme, the initial residence time $\tau_0$ was determined as the residence time that gave the highest GOS yield in Figure 5 (see Table 4). At 5% (w/v) lactose, the highest GOS yield was obtained at a very short residence time of 3.0 minutes, whereas at higher lactose concentrations the GOS production seems to be mostly independent of the residence time. Therefore, similarly short residence times for lactose concentrations of 12% (w/v) and higher were chosen to calculate the enzymatic productivity.



Due to enzyme inactivation, the composition of the outgoing product changes during the running time (Figure 2). When working under industrial conditions, a constant GOS concentration in the outlet is desired. A way to overcome the decrease in GOS production is to adapt (i.e., reduce) the flow rate and thus the residence time to the enzyme inactivation during the running time. The residence times at running time $t$, $\tau_t$, can be calculated with equation 7, based on the inactivation constant for glucose formation.

If we assume the running time of the PBR with immobilized enzyme to be equal to one half-life time of the immobilized enzyme, the enzymatic productivities (calculated with equation 8) for each of the substrate concentrations would be as indicated in Table 4.

**Table 4.** The optimum parameters during the PBR experiments with immobilized enzyme together with the maximum achievable enzymatic productivity. The productivity is based on a constant outgoing carbohydrate composition, i.e. on adapted residence time during the running time. The running time of the PBR is assumed to be equal to one half-life time of the immobilized enzyme.

| $[lac]_{in}$ | $t_{½}$ | $\tau_0$ | $[GOS]_{out}$ | $Q_{enz}$ |
|---|---|---|---|---|
| % (w/v) | h | min | dm% | kg GOS·g protein$^{-1}$ |
| 5 | 263 | 3.0 | 26 | 1.1 |
| 12 | 463 | 6.1 | 27 | 2.4 |
| 15 | 502 | 6.1 | 31 | 3.9 |
| 25 | 1028 | 6.6 | 35 | 14 |
| 38 | 2151 | 6.6 | 39 | 50 |

Although the calculated half-life time is an extrapolated value (the carbohydrate composition up till that time was not measured), Table 4 shows that the enzymatic productivity increased strongly with increasing the substrate concentration. This can be explained by higher GOS content together with a much longer enzyme half-life time. The productivity cannot be further increased by increasing the substrate concentration, since a substrate concentration of 38% (w/v) is a supersaturated solution. When raising the substrate concentration above 38% (w/v), crystallization will give flow blocking in the PBR. Using a higher temperature might be possible, however, β-galactosidases of Biolacta N5 are only stable up to 50ºC in aqueous solutions (Song et al. 2011; Vetere and Paoletti 1998).

The enzymatic productivity of the immobilized Biolacta N5 in the PBR can be compared with that of the free enzyme in the batch reactor. Table 5 shows the carbohydrate composition of lactose conversion with free Biolacta N5 at a substrate concentration of 34% (w/v) and a temperature of 40ºC for 24 hours.



**Table 5.** Carbohydrate composition after 24 hours of lactose conversion with free Biolacta N5 (at 0.016 g protein·L$^{-1}$) in a batch reactor at 40ºC, pH 6.0, and an initial lactose concentration of 34% (w/v).

| [GOS] | [Disaccharides] | [Glucose] | [Galactose] |
|---|---|---|---|
| dm% | dm% | dm% | dm% |
| 35 | 49 | 15 | 0.54 |

The disaccharide content decreased, while the total GOS and glucose concentration increased in time. The total GOS content was 35 dm% at 24 hours of reaction. The galactose content increased up to only 0.54 dm%. Table 6 shows the enzymatic

productivities of free β-galactosidase from *Bacillus circulans* together with productivities of the same enzyme from different enzyme preparations determined from Rodriguez-Colinas et al. (2012) and Palai et al. (2012). The running time of one batch reaction was determined as the time to reach the highest GOS content. GOS was considered as carbohydrates with a DP of three and higher. Therefore, the GOS content of Rodriguez-Colinas et al. (2012) was corrected for the incorporated amount of disaccharides. Since the protein content of Biolacta FN5 is not reported in literature, it is assumed to be equal to the protein content of Biolacta N5.

Although the substrate concentration in our experiment with free enzyme is slightly lower than in the experiments of Rodriguez-Colinas et al. (2012) and Palai et al. (2012), the enzymatic productivity of our experiment is higher than theirs. This may be due to a difference in activity of the enzyme preparation or differences in enzyme dosage and, related, in the running time at which the highest GOS content was found. Possibly the productivity of our experiment with free enzyme can be increased slightly further by raising the reaction temperature to 50ºC, and when the initial substrate concentration is raised to the maximum solubility at that temperature. 50ºC is the temperature up to which the β-galactosidases of Biolacta N5 are stable (Song et al. 2011; Vetere and Paoletti 1998), and more active (Vetere and Paoletti 1998) since an increase in temperature increases the conversion rate.

The enzymatic productivity of 50 kg GOS·g protein$^{-1}$ that we found for the immobilized Biolacta N5 (Table 4) is considerably higher than 7.6 kg GOS·g protein$^{-1}$ determined for the free enzyme (Table 6). Although the calculated half-life time is an extrapolated value, this implies that the amount of enzyme can be substantially reduced for the production of the same amount of GOS if immobilized Biolacta N5 is used instead of free Biolacta N5.



The volumetric productivities can be compared in a fair way if equal reactor volumes are considered or if equal production volumes are considered. If we consider the batch reaction with a final GOS content of 36 dm% from Figure 5E2 in a virtual reactor with a volume of 1 m$^3$, the volumetric productivity (calculated with equation 9) of this reaction would be 4.0 m$^3$·h$^{-1}$. A hypothetical PBR with the same productivity averaged over its whole running time should have a volume of 1.5 m$^3$, which is larger than that of the batch reactor.

**Table 6.** Enzymatic productivities of batch reactions with free β-galactosidase from *Bacillus circulans*.

| [lac] | Enzyme | [E] | $t_{batch}$ | [GOS][c] | $Q_{enz}$ | Reference |
|---|---|---|---|---|---|---|
| % (w/v) | preparation | g protein·L$^{-1}$ | h | dm% | kg GOS·g protein$^{-1}$ | - |
| 34 | Biolacta N5 | 0.016 | 24 | 35 | 7.6 | Table 5, this paper |
| 38 | Biolacta N5 | 1.7 | 0.25 | 36 | 0.084 | Figure 5E2, this paper |
| 40 | Biolactase | 0.082[a] | 50[b] | 33 | 1.6 | Rodriguez-Colinas et al. (2012) |
| 40 | Biolactase | 0.82[a] | 2[b] | 45 | 0.22 | Rodriguez-Colinas et al. (2012) |
| 53 | Biolacta FN5 | 0.30[d] | 8 | 39 | 0.68 | Palai et al. (2012) |

[a] *1.5 U/mL and 15 U/mL were recalculated to enzyme concentrations in g protein·L$^{-1}$ with an activity of 18.3 U/mg protein (Rodriguez-Colinas et al. 2012)*

[b] *time in batch reaction after which the GOS content did not increase significantly anymore.*

[c] *only GOS with a DP of three and higher are taken into account*

[d] *assumed that 19% of enzyme preparation is protein, equal to Biolacta N5*

In these calculations, we took into account a running time of 2151 h (equal to the half-life time of the enzyme in the PBR at 38% (w/v) lactose) and we took into account the adaptation of the flow rate to obtain an outgoing product with a constant GOS content because of enzyme inactivation. Not included is the cleaning time in between repeated batches. If this is taken into account, the comparable PBR would be somewhat smaller and its size would be more comparable to the size of the batch reactor.

If the batch reaction with a final GOS content of 35 dm% from Table 5 is considered in a virtual reactor with a volume of 1 $m^3$, the volumetric productivity of this reaction would be 0.042 $m^3 \cdot h^{-1}$. A hypothetical PBR with the same productivity averaged over its whole running time should only have a volume of 16 L. This is 63 times smaller than the volume of the hypothetical batch reactor. The large difference in reactor volume of the PBR between the two cases (1.5 $m^3$ vs. 16 L) is due to the huge difference in enzyme dosage, and therewith running time, of the batch reaction.

Thus, depending on the enzyme dosage and running time of a batch reaction, the volume of the PBR can be similar in size or much smaller compared to the volume of the batch reactor and have the same volumetric productivity. The smaller volume of the reactor would lead to a reduction in capital and operating costs.

A few aspects should be taken into account when choosing the desired running time for GOS production with immobilized Biolacta N5 in a PBR. In these calculations the half-life time of the enzyme was chosen as a guide. A shorter running time would decrease the enzymatic productivity, but the overall activity of the enzyme would be higher. *Vice versa,* a longer running time would increase the enzymatic productivity, but the average volumetric productivity in time would decrease because of a lower enzyme activity in the end of the reaction.

If a higher temperature is chosen, a higher substrate concentration is possible that will lead to a higher initial enzyme activity. However, the enzyme stability will be lower, which results in a shorter running time. The eventually chosen conditions will be a trade-off between substrate concentration, temperature, and running time.

It is possible to increase the productivities of the PBR with immobilized enzyme even further. Compared to the activity retention of immobilized enzyme reported by Hernaiz and Crout (2000), the activity retention of 31% that we found is rather low. This might be because of multipoint attachment of the enzyme to the carrier. Multi-point-attachment enhances the enzyme stability (Fernandez-Lafuente et al. 1995; Mateo et al. 2000; Sheldon 2007) and therefore allows for longer use of the biocatalyst and thus ample compensation for the initial loss of activity, but lowers the activity. The actual influence of a higher stability and a lower activity retention on the enzymatic and

volumetric productivity of GOS production with immobilized β-galactosidase should be investigated to consider the use of multipoint attachment.

## Conclusions

Biolacta N5 can be well immobilized on Eupergit C 250L and used for GOS production in a packed-bed reactor (PBR). The stability of the enzyme is high: the enzyme half-life time at a substrate concentration of 38% (w/v) is approximately 90 days. The enzymatic productivity of immobilized enzyme in the PBR is more than six-fold higher than the productivity of free enzyme in a batch reactor. For an equal volumetric productivity of the PBR to that of a batch reactor, the reactor volume can be much smaller, depending on the enzyme dosage and running time of the batch reactor.

The carbohydrate profiles of immobilized enzyme in the PBR and free enzyme in the batch reactor plotted against residence time are overall similar, but those of immobilized enzyme in the PBR show slightly more hydrolysis (galactose production) and slightly less GOS production than those of free enzyme in the batch reactor. Most likely this is the result of diffusion limitation. Based on the enzymatic as well as the volumetric productivity of both reactor systems, GOS production with the PBR using immobilized Biolacta N5 is an efficient alternative to GOS production in the traditional batch reactor with free enzyme.

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# Chapter 7

General discussion

## Introduction

The overall aim of this thesis is to better understand the mechanism of the β-galactosidase preparation from *Bacillus circulans*, Biolacta N5, in galacto-oligosaccharide (GOS) synthesis. This enzyme is more effective in GOS production than β-galactosidases from other sources. We therefore aimed to obtain more knowledge on the various β-galactosidase isoforms present in Biolacta N5 that we could use in optimizing the GOS production from lactose with Biolacta N5. Optimization of the GOS production should result in a process that results in a higher oligosaccharide yield and a lower lactose content.

During the purification of Biolacta N5, which is described in Chapter 2, we found that the preparation consists of four active fractions, β-gal-A, β-gal-B, β-gal-C, and β-gal-D. At low lactose concentrations, they behave differently in lactose hydrolysis and GOS synthesis activity. Studying the thermodynamics of lactose conversion with isothermal titration calorimetry confirmed these differences. However, the selectivity of the isoforms hardly differed at high substrate concentrations, which are typically used for GOS production. GOS formation rates of the isoforms indicate that β-gal-A and β-gal-B are the best isoforms for GOS production.

In Chapter 3, small carbohydrates that were added to the *o*NPG activity assay were found to be used as acceptor by Biolacta N5 which yielded an increased *o*NP formation rate. Molecular crowding results in a higher affinity of Biolacta N5 for the substrate *o*NPG. In chapter 4, the effect of reactants (galactose, glucose, lactose, and oligosaccharides) on the *o*NPG converting activity of various β-galactosidases was quantified. Biolacta N5 was well capable of using lactose as a substrate, which results in a rapid synthesis of the enzyme-galactose complex; water was a poor acceptor for the enzyme-galactose complex, which results in a low hydrolysis rate; while galactose was used as acceptor instead of inhibitor, resulting in a high rate of transgalactosylation. Among the isoforms of Biolacta N5, β-gal-A was found to have the highest rate constants for using lactose (and oligosaccharides) as a substrate; however, also its hydrolysis rate was highest.

Chapter 5 describes a method for the determination of the enzyme stability in buffer and in systems with initially 5.0 and 30% (w/w) lactose. Usually the stability of enzymes is measured in diluted aqueous solutions, which we found underestimates the stability at elevated lactose concentrations. The stability at high substrate concentrations shows that Biolacta N5 can be used at higher temperatures than is presumed based on aqueous measurements. This will result in a higher enzyme activity.

Since free Biolacta N5 retains approximately 50% of its activity after a batch reaction of 24h at an initial lactose concentration of 30% (w/w) and can be used for a longer running time, an alternative method to produce GOS was investigated in Chapter 6. Biolacta N5 immobilized on Eupergit C 250L can be used for GOS production in a packed-bed reactor (PBR): the stability of the immobilized enzyme is much higher than that of the free enzyme, and the final product compositions of both processes are similar.

In this chapter, we go deeper in optimization of the overall process, based on changes in the enzyme and 'substrate' composition. Moreover, we discuss the sustainability of GOS production batch and continuous mode.

## Process optimization

### *Isoforms of Biolacta N5*

β-Gal-B, β-gal-C, and β-gal-D all share the same precursor, namely β-gal-A (Song et al. 2011a). The isoforms are thought to be truncated through protease activities into the shorter amino acid sequences that are still active. Recently, Song et al. (2013) reported that the discoidin (DS) domain that has a high affinity for the binding of galactosyllactose, is responsible for a high hydrolysis rate of trisaccharides. This domain is only present in β-gal-A (Figure 1), which was found to show high hydrolytic activity towards galactosyllactose at low substrate concentrations. The effect of the DS domain at high substrate concentrations, which are typically used in industrial GOS production, was not assessed. We found hardly any differences among the selectivities of the isoforms at high lactose concentrations (chapter 2). Because more lactose is available for the active sites of the enzymes, more GOS is synthesized, also for the isoforms without DS domain, β-gal-B, β-gal-C, and β-gal-D. The ratio between the rate of GOS synthesis and the rate of GOS hydrolysis is much higher at high lactose concentrations than at low lactose concentrations. A higher lactose concentration increases the synthesis of GOS, but does not seem to affect the hydrolysis of GOS. Therefore, the effect of missing the DS domain is negligible at high lactose concentrations. The DS domain seems to play an important role only at low lactose concentrations.

On the other hand, β-gal-A and β-gal-B do have a higher net GOS formation rate at high lactose concentrations than β-gal-C and β-gal-D. Since β-gal-B is lacking the DS domain as well as β-gal-C and β-gal-D, this higher net GOS formation rate cannot be caused by the presence of this domain. The higher net GOS formation rate must be due to the difference between the amino acid sequence of β-gal-B and the amino acid

sequence of β-gal-C (in Figure 1 represented by lacking of the third (seen from left) domain called 'e' in the amino acid sequence (Song et al. 2011b). Removal of this part of the amino acid sequence might affect the structure and folding of the isoforms in such a way that β-gal-C and β-gal-D are less accessible for lactose.

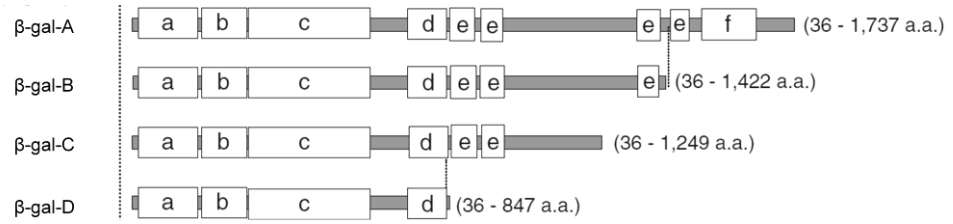


**Figure 1. Domains of the amino acid sequences of the β-galactosidase isoforms of *B.circulans* (adapted from Song et al. (2011b)). (f) represents DS domain.**

## *Avoiding protease activity*

Since β-gal-A and β-gal-B have the highest GOS formation rate at high lactose concentrations, these isoforms are optimal for GOS production. One option is to isolate these isoforms from the others and to use them separately in GOS production. However, a significant part of the enzyme activity is then lost since β-gal-C and β-gal-D are not used at all.

A better option would be to prevent the formation of β-gal-C, and β-gal-D, e.g. by inhibiting the protease activity. We did several tests with protease inhibitors during lactose conversion with Biolacta N5. However, the carbohydrate profiles in time were similar to those without protease inhibitor, which indicates that the protease is already inactive before lactose conversion.

Thus, the protease has to be removed or inactivated already from the *Bacillus circulans* culture broth during production of Biolacta N5 to prevent as much formation of β-gal-C and β-gal-D as possible. Production of any desired β-gal-B may then be achieved with the removed protease in a controllable way.

An alternative to removing the protease from the culture broth could be cloning of the desired amino acid sequence and expressing the corresponding enzyme in a host organism, like Song et al. (2011b) reported. If only β-gal-A and/or β-gal-B are expressed in a host organism and the protease is not, the precarious removal of the protease can be avoided.

*GOS production with additives*

The use of additives during *o*NPG conversion with Biolacta N5 can lead to higher enzyme activity. Small carbohydrates can be used by the enzyme as acceptor molecules. It is investigated whether the addition of other acceptors leads to a higher GOS yield. Gosling et al. (2011) did a similar study and measured the GOS yield and rate of trisaccharide formation during lactose conversion with added sucrose. Their study shows that the maximum GOS yield and formation rate both increase with the addition of sucrose. However, the productivity calculated over the total amount of initial disaccharides is lower with the addition of sucrose (i.e. the yield and rate at 20% (w/w) lactose are higher than the yield and rate at 10% (w/w) lactose plus 10% (w/w) sucrose). Li et al. (2009) found that the production of lactosucrose increased with increasing the initial sucrose concentration, but they do not report the yields of galactosyllactose or total trisaccharide. Here, the addition of trehalose was investigated during lactose conversion into GOS. 15% (w/w) trehalose was added to a system with 15% (w/w) lactose.

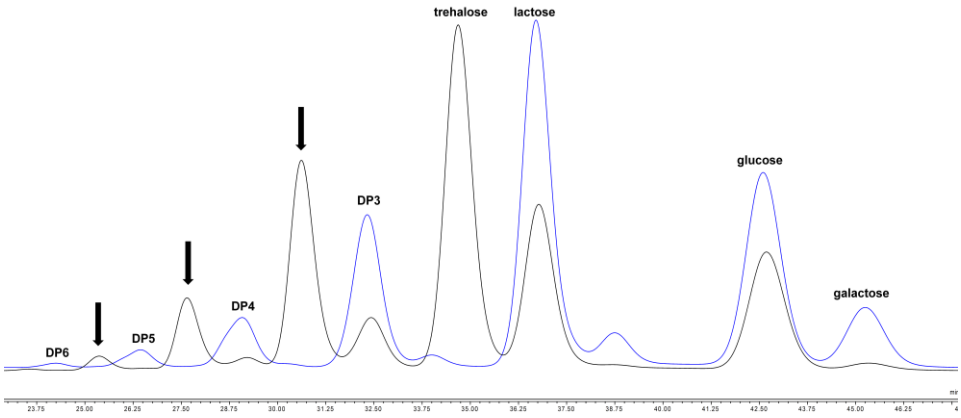


**Figure 2.** HPLC chromatogram of the carbohydrate composition after 24 hours of conversion of 15% (w/w) lactose (blue line) and a mixture of 15% (w/w) lactose and 15% (w/w) trehalose (black line) with Biolacta N5 at 25 °C and pH 6.0. Peaks marked with DP3, DP4, DP5 and DP6 are the peaks indicating trisaccharides, tetrasaccharides, pentasaccharides, and hexasaccharides regularly present after lactose conversion. The arrows indicate the peaks that are not detected in the chromatogram of conversion of only lactose.

Many peaks are observed in the HPLC chromatograms that were not observed during conversion of only lactose, which is shown in Figure 2. An experiment with only trehalose with Biolacta N5 shows that trehalose cannot be used as substrate. This implies that trehalose facilitates the oligosaccharide production by acting as acceptor for the enzyme. This does imply that other oligosaccharides are produced than with only lactose.

The product composition during disaccharide (lactose and trehalose) conversion of a system with 15% (w/w) lactose and 15% (w/w) trehalose was compared to that of systems with 15% (w/w) and 30% (w/w) lactose.

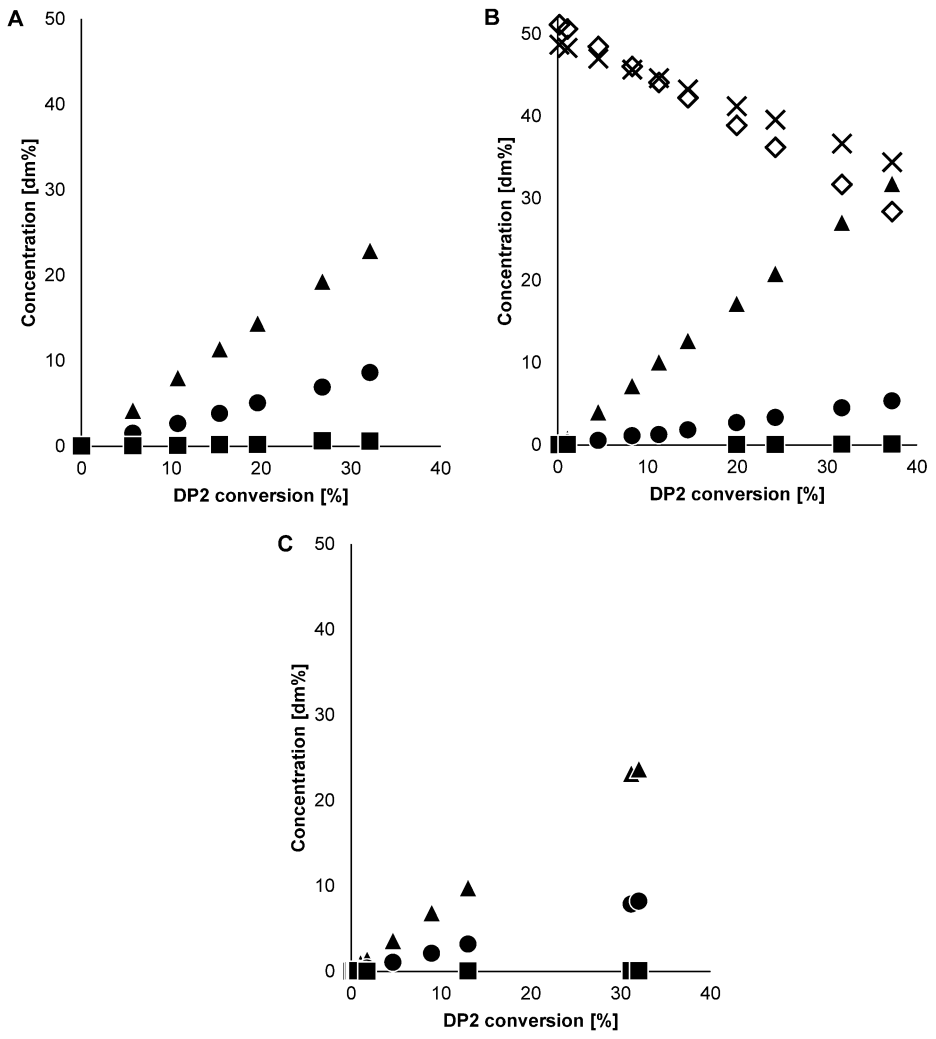


**Figure 3.** Production of carbohydrates as a function of the total disaccharide (lactose + trehalose) conversion with Biolacta N5 at (A) 15% (w/w) lactose, (B) 15% (w/w) lactose plus 15% (w/w) trehalose, and (C) 30% (w/w) lactose initially at 25ºC and pH 6.0. Symbols: ■ galactose; ● glucose; ◇ disaccharides other than trehalose, including lactose; ▲ total oligosaccharide; and **X** trehalose.

Figure 3 shows that the product composition starting with 15% (w/w) lactose was similar to the product composition with 30% (w/w) lactose at the same level of lactose conversion, except for slightly more galactose due to slightly more hydrolysis. The total

production of oligosaccharides starting with an 1:1 mixture of lactose and trehalose was found to be somewhat higher than starting with only lactose, whereas the glucose production was somewhat lower. A lower production of glucose is most likely due to a smaller chance of lactose entering the enzyme's active site than in the system with 30% (w/w) lactose, because trehalose is competing. That means a lower production of the enzyme-galactose complex. However, once the enzyme-galactose complex is formed, a disaccharide is more likely to act as acceptor than a monosaccharide since less monosaccharides are present.

Adding small carbohydrates that cannot act as substrate but can act as acceptor can lead to a higher GOS yield, and leads to a new composition of the final oligosaccharide mixture. This raises many possibilities for the production of various types of oligosaccharides that may be health beneficial as well. Furthermore, when half of the initial lactose content is replaced by another carbohydrate, this reduces the amount of lactose in the final product with 50%. If a lower ratio of lactose vs. acceptor carbohydrate is chosen, the lactose content in the final product might be further reduced. The lower lactose content may be a step forward in making the product available for lactose intolerant people. Of course, an acceptor carbohydrate should be chosen that results in a prebiotic product.

### *GOS production from milk*

GOS are often added to dairy products as a prebiotic ingredient. This is a complex process: GOS are first produced from lactose that was originally purified from milk. Is it not possible to produce GOS in milk or any other dairy product from the present lactose, so that many steps can be circumvented? Various studies describe the production of GOS in milk (Gosling et al. 2009; Mozaffar et al. 1985; Rodriguez-Colinas et al. 2012). However, only relatively low levels of GOS have been reached. Much higher GOS levels are found in lactose conversion with high lactose concentrations. The lactose concentration of milk is only approximately 5%, which is too low to obtain a high GOS yield. GOS production in milk is not the way to make the product available for people that suffer from lactose intolerance, since only galactose and glucose are present in the final product at 100% conversion of the lactose.

Higher GOS yields in milk can be achieved if the milk is concentrated prior to enzyme addition. However, a side effect of GOS production in milk might be the production of many more varying products than when GOS is produced from purified

lactose, since other components (than the reactants produced from purified lactose) that are present in milk can be used as acceptor.

## Process efficiency - Exergy analysis

The increased productivity with immobilized Biolacta N5 that was found in Chapter 6 is highly advantageous. However, when comparing two (or more) possible production systems, the sustainability of each system has to be assessed, next to the productivity and the production costs. Sustainability is a very important topic nowadays. To assess the sustainability of a system, exergy analysis can be performed. Exergy can be used to describe the potential or available work used in a system or stream (Boom 2011). Since the available work is always reduced (according to the second law of thermodynamics), exergy will always be lost (Boom 2011).

In this section, exergy analyses are performed on the packed-bed reactor with immobilized enzyme from chapter 6 and on a batch reactor with free enzyme. The first reaction uses a lactose concentration of 33% (w/w) (38% (w/v)) in a 16 L reactor at 50ºC, for the latter one two cases are considered: 30% (w/w) lactose in a 1 $m^3$ reactor and 60% (w/w) lactose in a 390 L reactor, both at 60ºC. These conditions result in the same volumetric productivity. Various unit operations are considered which are shown in the flow chart in Figure 4.

In the system with 30% (w/w) lactose, the lactose is dissolved at the reaction temperature. In the other systems, the lactose is dissolved at the temperature necessary to dissolve this concentration of lactose. Steam is used to heat the solutions. In the batch reactor at 60% (w/w) lactose and the PBR at 33% (w/w) lactose, the solution is cooled down to the reaction temperature with cooling water before the reaction takes place. In the batch systems, the reaction starts when enzyme is added. During the reaction, the temperature remains constant at the reaction temperature using steam. After the reaction, both batch reactions are cooled down to 45ºC and the system at 60% (w/w) dry matter is diluted to 45% (w/w) dry matter prior to filtration. In both batch reactions, the enzyme is removed by filtration during which the temperature remains constant at 45ºC. In the system of the PBR, filtration is not necessary since the enzyme is immobilized. Finally, the solution is concentrated to 75% dry matter by evaporation. Steam is used to heat the solution and evaporate the excess moisture. Filtration and evaporation after the batch reaction take place in continuous mode, the other steps in batch mode. Dissolving the lactose before the reaction in the PBR takes place in batch mode, all the other steps in continuous mode.

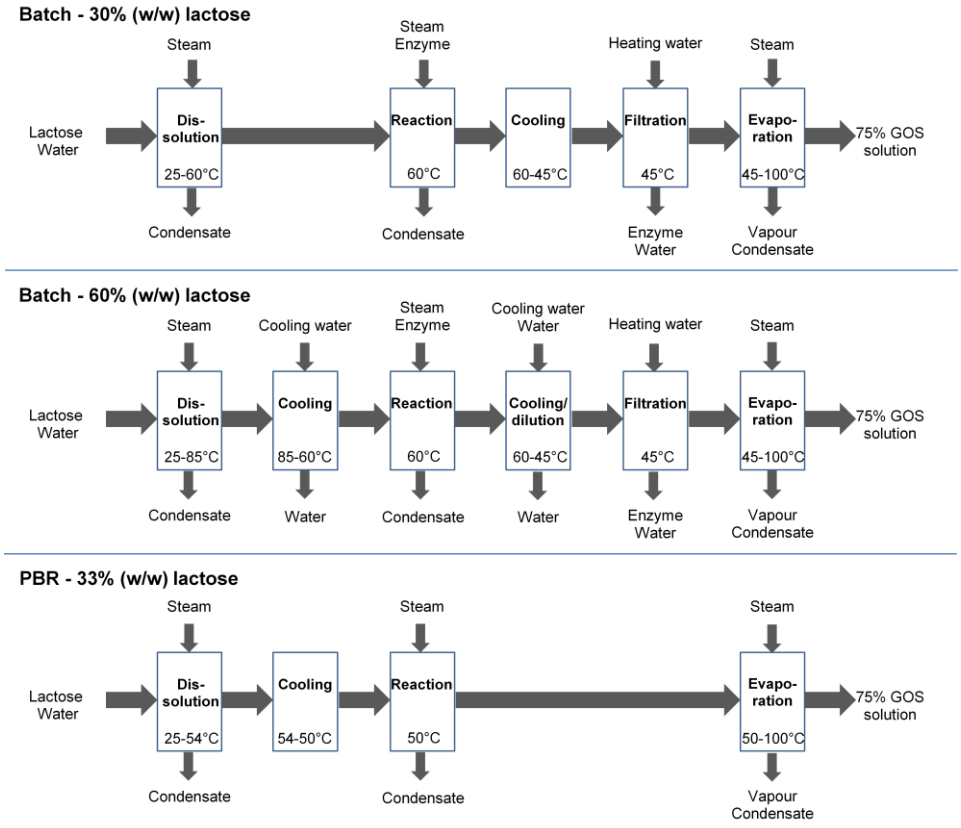


**Figure 4.** Flow charts of GOS production in a batch reactor at 30% (w/w) lactose initially, batch reactor at 60% (w/w) lactose initially; and PBR at 33% (w/w) lactose.

Figure 5 shows the sizes of the masses used in each unit operation. Because of the high initial concentration of the system with 60% (w/w) lactose, the total mass (including water) is much smaller. Therefore, less mass has to be heated in various steps and less water and steam has to be added for heating.

In this exergy analysis, the main types of exergy that have to be taken into account are thermal exergy and chemical exergy. The thermal and chemical exergy of each stream are calculated as described by Boom (2011) and they are visualized in Figure 6. The reference state used is 25ºC and 1 bar.

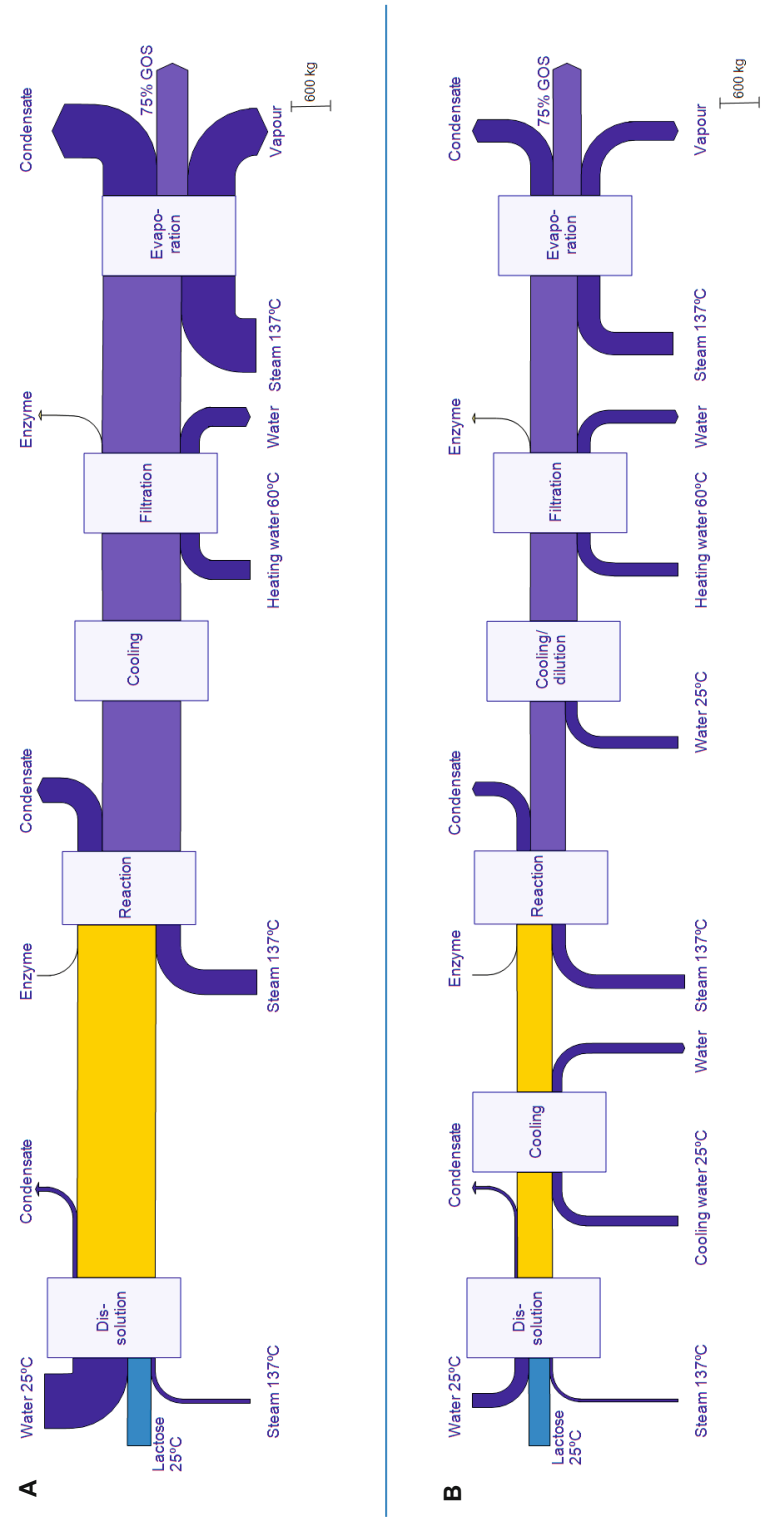


**Figure 5.** Sankey diagrams of masses in the GOS production with a (A) batch reactor at 30% (w/w) lactose initially and 60ºC, (B) batch reactor at 60% (w/w) lactose initially and 60ºC; and (C) PBR at 33% (w/w) lactose and 50ºC. All three diagrams have the same scale.

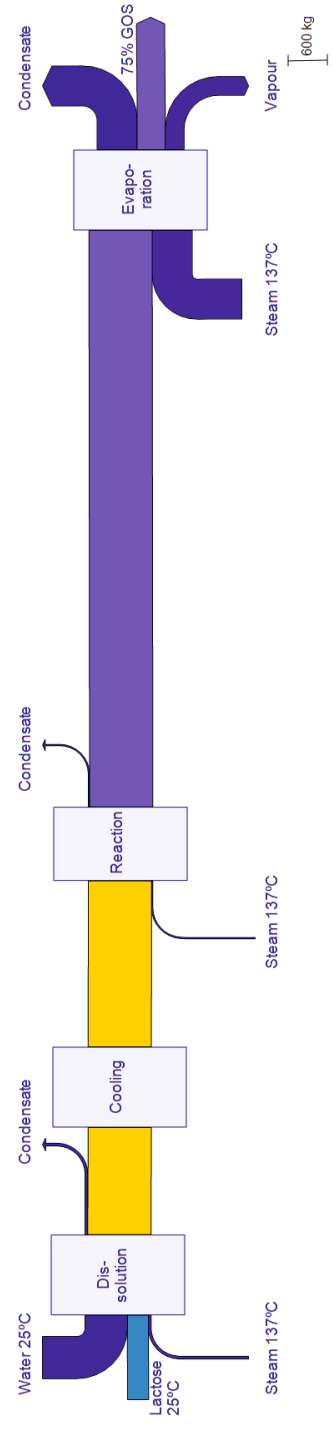


**Figure 5 continued.**

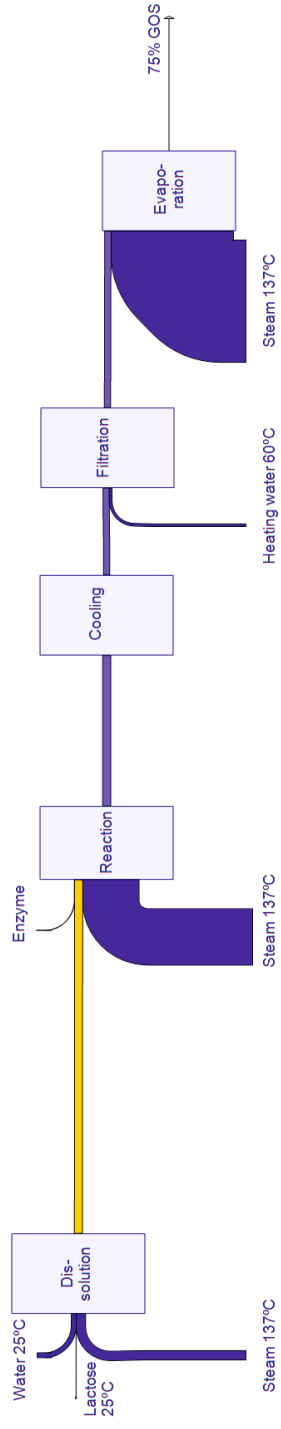


**Figure 6.** Grassmann diagrams of exergy flows in GOS production in a (A) batch reactor at 30% (w/w) lactose initially, (B) batch reactor at 60% (w/w) lactose initially and 60°C; and (C) PBR at 33% (w/w) lactose and 60°C. The chemical exergy of lactose/oligosaccharides (which is 5900, 5300, and 5300 MJ for the respective processes) is neglected in these diagrams. All three diagrams have the same scale.

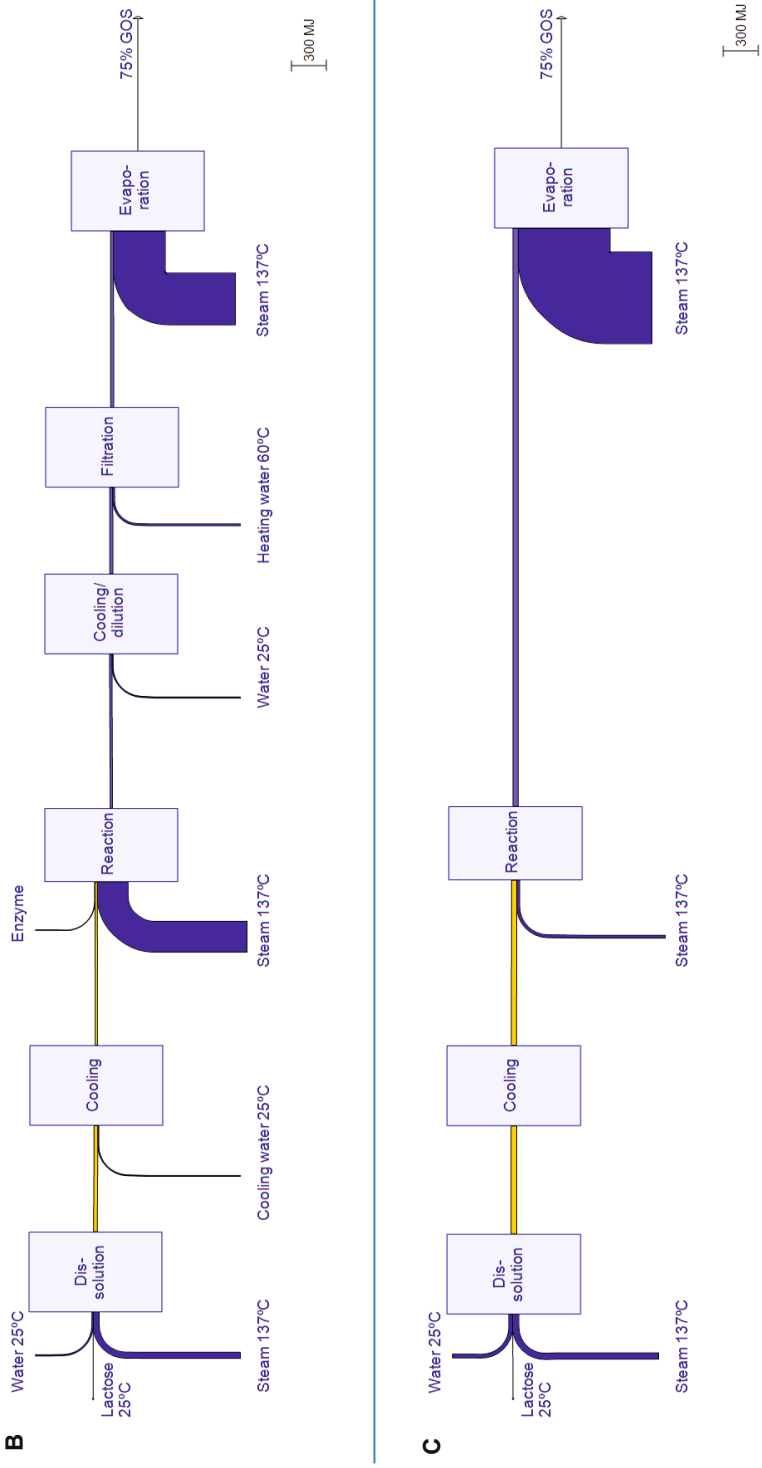


**Figure 6 continued.**

**Table 1.** Total exergy input and exergy loss over the whole process and in each unit operation (including chemical exergies of the components).

| | Total exergy input [MJ] | Total exergy loss [%] | Exergy loss dissolution [MJ] | Exergy loss cooling [MJ] | Exergy loss reaction [MJ] | Exergy loss cooling/ dilution [MJ] | Exergy loss filtration [MJ] | Exergy loss evaporation [MJ] |
|---|---|---|---|---|---|---|---|---|
| 30% (w/w) batch | 7486 | 21 | 51 | - | 452 | 14 | 21 | 1028 |
| 60% (w/w) batch | 6059 | 12 | 30 | 18 | 247 | 2.8 | 14 | 434 |
| 33% (w/w) PBR | 6144 | 14 | 35 | 3.0 | 23 | - | - | 771 |

Figure 6 shows the exergy input and output of each unit operation. Table 1 shows the exergy input and exergy loss over the whole GOS production process as shown in Figure 6. During GOS production with a batch reactor at 30% (w/w) lactose, almost a quarter of the total exergy input is lost. Approximately 1400 MJ less exergy is used in the two other cases, which is a reduction of almost 20%. Overall, a batch reactor with 60% (w/w) lactose initially seems to have a similar performance in terms of exergy as a PBR with 33% (w/w) lactose.

At low substrate concentrations, more energy has to be used to heat up the solution and to keep a constant temperature than at a high substrate concentration. Besides, more water has to be evaporated to obtain an equal final dry matter content. We did not take any possible reuse into account of the various 'waste' warm water or steam flows. Figure 7 shows how much energy is actually wasted in each step in the form of water and vapor. If these energy flows would be reused, this could substantially reduce the exergy loss. However, this re-use in a batch process is not trivial.

An advantage of a PBR over a batch reactor is that the reactor volume can be much smaller. To apply a constant temperature over the reactors during the reaction, less energy has to be added to the PBR than to the batch reactor. Besides, the running time of the PBR can be much longer and cleaning does not have to be applied that often. In this way, the exergy loss is lower since fewer chemicals for cleaning have to be used. Besides, filtration is necessary to remove the enzyme from the GOS solution when using free enzyme in a batch reactor. This step can be circumvented when using immobilized enzyme, which results in a smaller exergy loss. On the other hand, the immobilization of the enzyme requires the use of many chemicals. This is not taken into account in this case study. The usage of these chemicals will increase the exergy loss. An extra advantage may be, that the process with the immobilized enzyme is a continuous process; re-use of energy in such a system by direct heat-exchange is much simpler than a pure batch process.

## 7 Conclusions

This research reported in this thesis has yielded better insight in the mechanism of GOS synthesis with β-galactosidases. GOS production with Biolacta N5 is more effective than with β-galactosidases from other sources, because of three main reasons: the enzyme's affinity for using lactose as substrate is higher, Biolacta N5 is hardly inhibited by galactose, and it uses galactose very well as acceptor. The β-galactosidase isoforms in Biolacta N5 differ in GOS synthesis and lactose hydrolysis under aqueous conditions, which was confirmed with ITC. ITC is a very sensitive and accurate

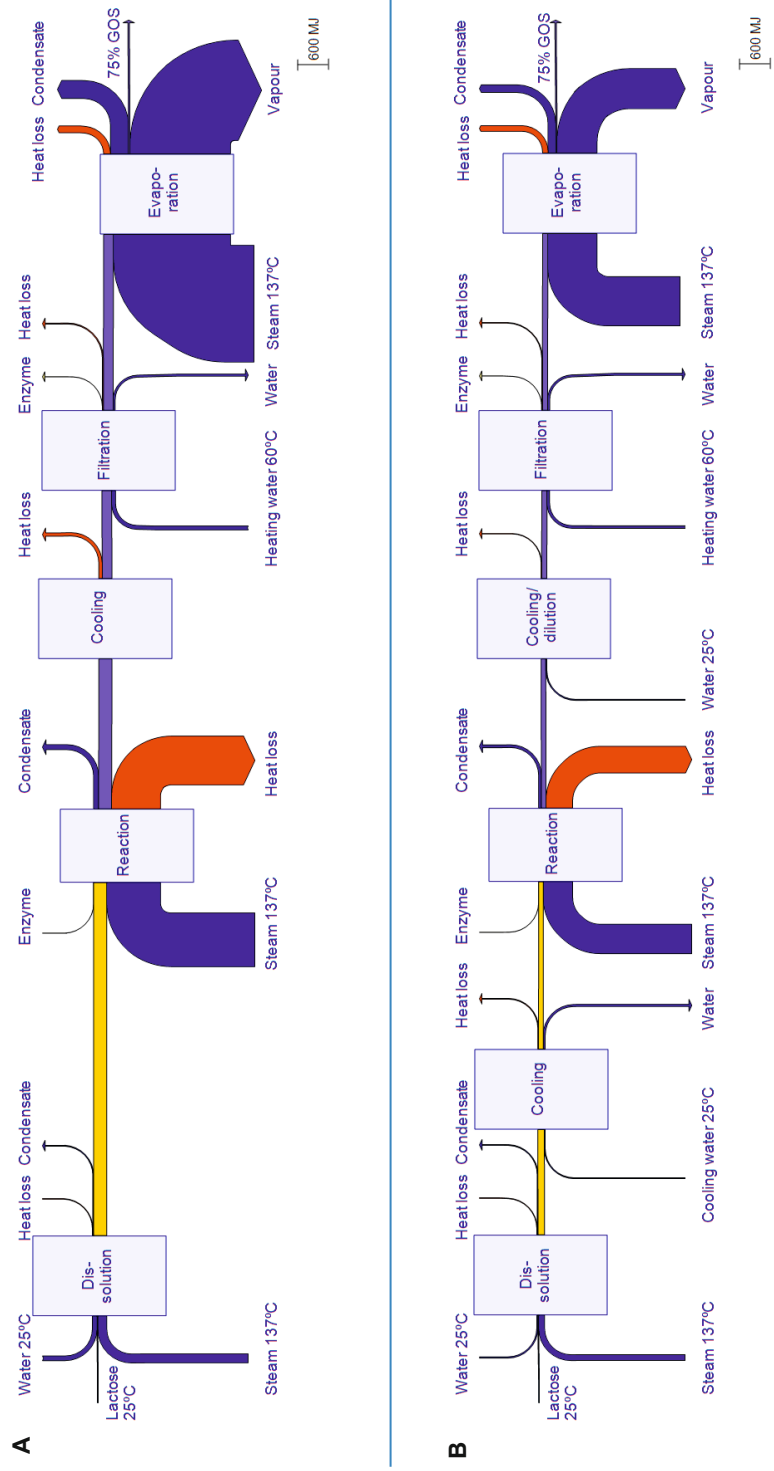


**Figure 7.** Sankey diagrams of energy used in the GOS production with a (A) batch reactor at 30% (w/w) lactose initially and 60°C, (B) batch reactor at 60% (w/w) lactose initially and 60°C; and (C) PBR at 33% (w/w) lactose and 50°C. All three diagrams have the same scale.

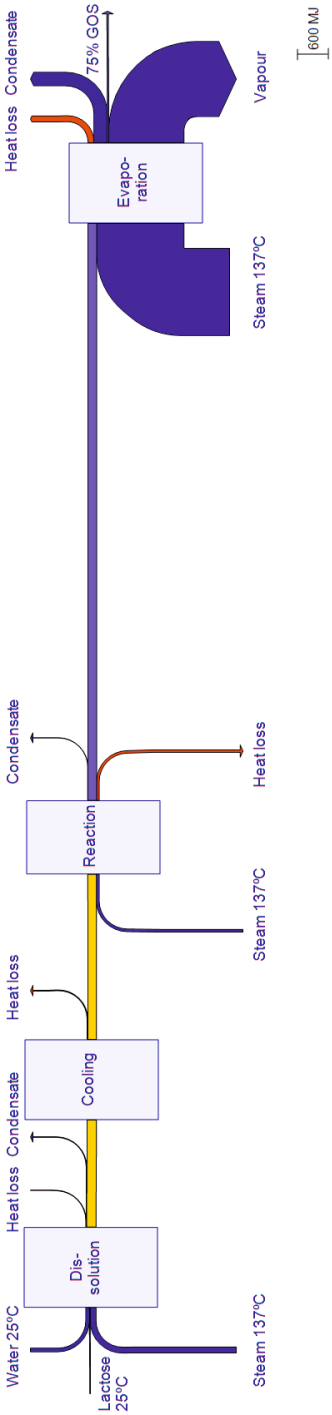


**Figure 7 continued.**

technique; however, the interpretation of the results for complex reactions is rather precarious.

The isoforms did not show differences in their selectivities when using high substrate concentrations. β-Gal-A and β-gal-B were found to have the highest GOS formation rates when using these, industrially relevant, conditions in contrast to what was expected based on aqueous conditions. The drawback of β-gal-A is that is has the highest hydrolysis rate as well.

A GOS production process with a higher enzymatic productivity was found when Biolacta N5 was immobilized and the immobilized enzyme was used in a PBR. Also the sustainability of GOS production in a PBR with immobilized enzyme at a lactose concentration of 33% (w/w) can be, in terms of exergy, much better than that in a batch reactor with free enzyme at 30% (w/w) lactose initially.

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## Summary

## Samenvatting

## Summary

Galacto-oligosaccharides (GOS) are generally enzymatically synthesized with β-galactosidases. GOS are of interest because of their prebiotic effects on human health. They are mainly applied in infant nutrition, because of their resemblance to human milk oligosaccharides, but they are also applied in e.g. dairy products and beverages.

β-Galactosidases synthesize GOS from lactose through transgalactosylation: instead of only using water as acceptor (as in hydrolysis), they can use carbohydrates as acceptor. In this way, GOS with a degree of polymerization up to ten can be formed. The ratio of hydrolysis over transgalactosylation depends on the substrate concentration, temperature, and the source of the enzyme.

A β-galactosidase preparation from *Bacillus circulans*, called Biolacta N5, is known to produce high GOS yields compared to enzymes from other sources. The aim of this thesis was to obtain more insight on the mechanism of GOS production with Biolacta N5 and to investigate how the GOS production process can be optimized.

Biolacta N5 consists of four β-galactosidase isoforms, β-gal-A, β-gal-B, β-gal-C, and β-gal-D, which were purified and characterized in chapter 2. At low substrate concentrations, these isoforms differ in hydrolysis and transgalactosylation activity. β-Gal-D seems the best isoform for GOS production, followed by β-gal-C and β-gal-B, and β-gal-A showed the least GOS formation. By studying the thermodynamics of lactose conversion with isothermal titration calorimetry (ITC), the differences in behavior were confirmed, although the interpretation of the results of ITC was quite difficult dealing with a complex mixture of reactions. In contrast to the selectivity at low substrate concentrations, the selectivity of the isoforms hardly differed at high lactose concentrations. These conditions are usually used for industrial GOS production. Only β-gal-A produced slightly more galactose. The initial GOS formation rates indicated that β-gal-A and β-gal-B are the best isoforms for GOS production.

In chapter 3, the effect of high concentrations was further studied on the behavior of the complete Biolacta N5 preparation. High concentrations of reacting and non-reacting carbohydrates were added to the *o*NPG activity assay with Biolacta N5. Small carbohydrates were found to act as acceptor in the reaction, which resulted in an increased reaction rate. The rate of the limiting step of the reaction, i.e. the binding of the galactose residue with the acceptor, is increased, and therewith the release of the product is faster. At the same time, the additives cause molecular crowding, which results in a higher affinity between the enzyme and the substrate.

In chapter 4, a kinetic model was developed to quantify the effects of lactose, glucose, galactose, and oligosaccharides on the *o*NPG converting activity of the β-galactosidases from *B.circulans*, *Aspergillus oryzae* and *Kluyveromyces lactis*. Using

multiple substrates simultaneously yields more information than using only lactose or *o*NPG, because of the competition between the substrates. Three main differences were found that explain why Biolacta N5 produces higher GOS yields than other β-galactosidases: (i) it had a higher reaction rate constant of using lactose or oligosaccharides as substrate relative to water as acceptor (so it had a very low relative hydrolysis rate); and (ii) it also had a high reaction rate with galactose as acceptor, whereas (iii) the other two enzymes are strongly inhibited by galactose. The reaction rate constants indicate that β-gal-A is the most active isoforms in GOS production; however, also its hydrolysis rate is highest. Many of the rate constants increase with increasing molecular weight of the isoforms.

Chapter 5 reports on the stability of Biolacta N5 at various temperatures in buffer, and in systems with initially 5.0 and 30% (w/w) lactose. Samples were taken in time and analyzed for *o*NPG converting activity. The *o*NPG converting activity was corrected for the presence of lactose, glucose, galactose, and oligosaccharides with the mechanistic model from chapter 4. The stability, expressed with the half-life time, of the enzyme was found to strongly increase with initial lactose concentrations. At high substrate concentration, higher temperatures can be used for GOS production than was presumed feasible based on stability measurements in diluted solutions.

Biolacta N5 is still active after one batch run of GOS production, but in a batch process the enzyme is wasted after the reaction. For this reason, the use of immobilized enzyme in a continuous packed bed reactor (PBR) was investigated in terms of productivity in chapter 6. The carbohydrate composition of the product in both systems was comparable. The half-life time of the immobilized enzyme at a lactose concentration of 33% (w/w) and 50ºC was approximately 90 days. The enzymatic productivity using immobilized enzyme in a PBR may be six times higher than that using free enzyme in a batch reactor. When striving for an equal volumetric productivity of both systems, the volume of a PBR can be much smaller than that of a batch reactor, depending on the enzyme dosage and running time of the one batch.

Chapter 7 discusses various alternatives for process optimization. One option for a higher GOS productivity is to use an enzyme preparation that contains only β-gal-A and β-gal-B. A somewhat higher oligosaccharide yield can be obtained when initially using a mixture of lactose with a better acceptor molecule. This results in a changed oligosaccharide composition and less lactose in the final product. The sustainability of GOS production in a PBR with immobilized enzyme and 33% (w/w) lactose seems to be similar in terms of exergy to that in a batch reactor with free enzyme and 60% (w/w) lactose.

## Samenvatting

Galacto-oligosachariden (GOS) worden meestal enzymatisch gesynthetiseerd met β-galactosidases. Productie van GOS is interessant vanwege de prebiotische werking van GOS op de menselijke gezondheid. GOS worden vooral toegevoegd aan kindervoeding omdat ze vergelijkbaar zijn met de oligosachariden die in borstvoeding voorkomen, maar ze worden ook toegevoegd aan bijvoorbeeld zuivelproducten en dranken voor volwassenen.

β-Galactosidases vormen GOS vanuit lactose via transgalactosylering: naast het gebruik van water als acceptor (dat hydrolyse veroorzaakt) kunnen ze ook koolhydraten die aanwezig zijn in de oplossing, als acceptor gebruiken. Op deze manier kunnen GOS met een polymerisatiegraad tot tien gevormd worden. De verhouding tussen hydrolyse en transgalactosylering is afhankelijk van de substraatconcentratie, de temperatuur en de herkomst van het enzym.

Een β-galactosidase preparaat van *Bacillus circulans*, genaamd Biolacta N5, staat bekend om zijn hoge GOS-opbrengst vergeleken met enzymen van andere herkomst. Het doel van het onderzoek beschreven in dit proefschrift was om meer kennis te vergaren over het mechanisme van Biolacta N5 om GOS te produceren en om te bestuderen hoe het productieproces van GOS verder verbeterd kan worden.

Biolacta N5 bestaat uit vier isovormen, β-gal-A, β-gal-B, β-gal-C en β-gal-D. Hun opzuivering en karakterisering is beschreven in hoofdstuk 2. Bij lage substraatconcentraties verschillen de isovormen in hydrolyse- en transgalactosyleringsactiviteit. β-Gal-D lijkt de beste isovorm voor GOS-productie, gevolgd door β-gal-C en β-gal-B; terwijl β-gal-A het minst geschikt is voor GOS-productie. De verschillen in het gedrag van de isovormen werden bevestigd door met isotherme titratiecalorimetrie (ITC) te kijken naar de thermodynamica van lactoseomzetting. Daarbij moet vermeld worden dat de interpretatie van de ITC-resultaten lastig is, omdat we te maken hebben met een complex mengsel en verschillende reacties. Experimenten bij hoge lactoseconcentraties, die gewoonlijk gebruikt worden voor industriële GOS-productie, laten weinig verschillen zien in de selectiviteit van de isovormen. Alleen β-gal-A geeft een iets hogere productie van galactose. De initiële vormingssnelheid van GOS geeft een indicatie dat β-gal-A en β-gal-B de beste isovormen zijn voor de productie van GOS.

In hoofdstuk 3 is het effect van hoge concentraties op het gedrag van het complete Biolacta N5 preparaat verder bestudeerd. Hoge concentraties koolhydraten werden aan het *o*NPG-activiteitsassay met Biolacta N5 toegevoegd. Kleine koolhydraten bleken als acceptor te fungeren, hetgeen tot een hogere reactiesnelheid leidde. De snelheidsbepalende stap in de reactie, de binding van het galactoseresidu aan de

acceptor, is sneller en daarom is de productvorming sneller. Tegelijkertijd veroorzaakten alle toegevoegde koolhydraten, zowel de reagerende als niet-reagerende, 'molecular crowding'. Dat leidde tot een sterkere affiniteit tussen het enzym en het substraat.

In hoofdstuk 4 is een kinetisch model ontwikkeld om de effecten van lactose, glucose, galactose en oligosachariden op de activiteit voor *o*NPG-omzetting van de β-galactosidases van *B.circulans*, *Aspergillus oryzae* en *Kluyveromyces lactis* te kwantificeren. Het gebruik van meerdere substraten levert meer informatie op dan wanneer alleen lactose of alleen *o*NPG gebruikt zou worden, omdat het een competitief effect heeft. De drie grootste verschillen tussen de β-galactosidases die verklaren waarom Biolacta N5 een hogere GOS-opbrengst geeft dan de andere β-galactosidases, zijn: Biolacta N5 heeft, ten opzichte van de reactiesnelheidsconstante voor het gebruik van water als acceptor, een hogere reactiesnelheidsconstante voor het gebruik van lactose of oligosachariden als substraat (het heeft relatief gezien een zeer lage hydrolysesnelheid); en Biolacta N5 heeft ook een hogere reactiesnelheid voor het gebruik van galactose als acceptor, terwijl de andere twee enzymen sterk geremd worden door galactose. De reactiesnelheidsconstanten geven aan dat β-gal-A in de productie van GOS de actiefste isovorm is. Echter ook de hydrolysesnelheid van β-gal-A is het hoogste. Veel van de reactiesnelheidsconstanten nemen toe met het toenemen van het molecuulgewicht van de isovormen.

Hoofdstuk 5 gaat in op de stabiliteit van Biolacta N5 bij verschillende temperaturen in buffer en in systemen met initieel 5.0 en 30% (w/w) lactose. Tijdens het experiment werden monsters genomen waarvan de activiteit voor *o*NPG-omzetting gemeten werd. De activiteit voor *o*NPG-omzetting werd met het mechanistische model uit hoofdstuk 4 gecorrigeerd voor de aanwezigheid van lactose, glucose, galactose en oligosachariden. De stabiliteit/halfwaardetijd van het enzym nam sterk toe met het verhogen van de initiële lactoseconcentratie. Bij hoge substraatconcentraties kunnen daarom hogere temperaturen gebruikt worden voor de productie van GOS dan tot nu toe werd aangenomen. Aannames waren tot dusver gebaseerd op stabiliteitsbepalingen in waterige systemen.

Ondanks dat Biolacta N5 nog erg actief is na een GOS-productiebatch, wordt het enzym naderhand als afval beschouwd. Daarom hebben we in hoofdstuk 6 het gebruik van geïmmobiliseerd enzym in een gepakte kolom (PBR) onderzocht. De productsamenstelling van beide systemen was vergelijkbaar. De halfwaardetijd van het geïmmobiliseerde enzym in een 33% (w/w) lactoseoplossing bij 50ºC was ongeveer 90 dagen. De enzymatische productiviteit van een PBR met geïmmobiliseerd enzym kan daarom zes keer hoger zijn dan die van een batch reactor met vrij enzym. Daarnaast kan het volume van een PBR veel kleiner zijn dan dat van een batchreactor voor het bereiken

van dezelfde volumetrische productiviteit. Dit is afhankelijk van de enzymdosis en de lengte van een batchreactie.

In hoofdstuk 7 worden verschillende alternatieven voor procesoptimalisering besproken. Een mogelijkheid voor een hogere GOS-productie is het gebruik van een enzympreparaat dat alleen β-gal-A en β-gal-B bevat. Daarnaast kan een iets hogere opbrengst worden behaald als er een mengsel van lactose met een beter acceptormolecuul gebruikt wordt in plaats van alleen lactose. Dit zal wel resulteren in een aangepaste oligosacharidesamenstelling en minder lactose in het eindproduct. Als laatste is de duurzaamheid van GOS-productie in een PBR met geïmmobiliseerd enzym bij 33% (w/w) lactose vergeleken met die van GOS-productie in een batch reactor met vrij enzym bij 60% (w/w) lactose. Beide systemen zijn vergelijkbaar in termen van exergieverliezen.

# Dankwoord

# Acknowledgements

## Dankwoord

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## Curriculum vitae

## Publications

## Overview of the completed training activities

## Curriculum vitae

Anja Warmerdam was born in Blijham, The Netherlands, on the 24th of January 1985. After the primary school in Blijham, she followed secondary education at the Dollard College in Winschoten, where she received her VWO diploma in 2003. In 2003, she left Blijham and started studying Biotechnology at Wageningen University. She followed both the BSc and MSc in Biotechnology with the specialization in Process Technology. She performed two theses at the chair group of Food and Bioprocess Engineering: the BSc thesis was on the enzymatic hydrolysis of starch gelatinized at high pressure; the MSc thesis was on dynamic interfacial tension effects during emulsification in microreactors with Y-junctions. Her internship was performed at the Department of Chemical and Biochemical Engineering at the Technical University of Denmark (DTU) in Lyngby, Denmark. Here, she studied the enzymatic release, purification, and identification of a potentially bioactive pectin polymer from black currant residue. After she received her MSc degree in November 2008, she started her PhD project on the enzymatic conversion of lactose into galacto-oligosaccharides at the chair group of Food Process Engineering at Wageningen University within the framework of the Carbohydrate Competence Center (CCC). The results obtained during this project are described in this thesis.

## Publications

Warmerdam A, Paudel E, Jia W, Boom RM, Janssen AEM. 2013. *Characterization of β-galactosidase isoforms from Bacillus circulans and their contribution to GOS production.* Applied Biochemistry and Biotechnology 170(2):340-358.

Warmerdam A, Wang J, Boom RM, Janssen AEM. *Effects of carbohydrates on the oNPG converting activity of β-galactosidases.* Submitted for publication.

Warmerdam A, Zisopoulos FK, Boom RM, Janssen AEM. *Kinetic characterization of β-galactosidases.* Submitted for publication.

Warmerdam A, Boom RM, Janssen AEM. *β-Galactosidase stability at high substrate concentrations.* Submitted for publication.

Warmerdam A, Benjamins F, de Leeuw TF, Broekhuis AA, Boom RM, Janssen AEM. *Galacto-oligosaccharide production with immobilized β-galactosidase in a packed-bed reactor vs. free β-galactosidase in a batch reactor.* Submitted for publication.

Steegmans MLJ, Warmerdam A, Schroën CGPH, Boom RM. 2009. *Dynamic interfacial tension measurements with microfluidic Y-junctions.* Langmuir 25(17): 9751–9758.

# Overview of completed training activities

## *Discipline-specific*

| | |
|---|---|
| Enzyme technology, lignocellulose, and non-starch polysaccharides (DTU) | 2008 |
| Advanced course biocatalysis (BSDL) | 2009 |
| Reaction kinetics in food science (VLAG) | 2009 |
| Process economics and cost engineering (OSPT) | 2010 |
| Summer course on glycosciences (VLAG) | 2010 |
| CCC-dagen, Groningen | 2010-2012 |
| Netherlands process technology symposium (NPS) | 2010-2011 |
| Advanced food analysis (VLAG) | 2010 |
| Sustainability analysis in food production (VLAG) | 2011 |
| GOS workshop (FrieslandCampina) | 2012 |
| 6$^{th}$ International congress on biocatalysis, Hamburg | 2012 |
| Starch round table (CCC) | 2012 |

## *General*

| | |
|---|---|
| PhD competence assessment (WGS) | 2009 |
| Afstudeervak organiseren en begeleiden (DO) | 2009 |
| PhD week (VLAG) | 2009 |
| Project and time management (WGS) | 2011 |
| Scientific writing (Language Services) | 2012 |
| Career assessment (WGS) | 2012 |
| Voice matters - voice and presentation skills training (WGS) | 2012 |
| Career perspectives (WGS) | 2012 |

## *Optional*

| | |
|---|---|
| Food Process Engineering Symposium | 2009-2012 |
| Weekly Food Process Engineering group meetings | 2009-2012 |
| Organizing PhD study tour to the USA | 2010 |
| Food Process Engineering PhD study tour to the USA | 2010 |
| Food Process Engineering PhD study tour to Finland and the Baltic States | 2012 |

Cover: galacto-oligosaccharides mixture where each yellow hexagon represents a glucose residue and each blue hexagon represents a galactose residue.

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