# Universität für Bodenkultur

## Department für Lebensmittelwissenschaften und –technologie

### Institut für Lebensmittelbiotechnologie


# *Enzymatic synthesis and investigation of novel galacto-oligosaccharides*

**master thesis**


**handed in by**

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## Abstract:

The present work continues the ongoing research of our institute to explore efficient lactose conversion processes. The aim of this study was to create novel galacto-oligosaccharides using a heterologously overexpressed β-galactosidase from *Lactobacillus reuteri* in combination with the following four monomeric sugars present in human milk: L-Fucose, N-Acetyl-neuraminic acid, N-Acetyl-D-galactosamine and N-Acetyl-D-glucosamine. The resulting products are supposed to mimic human milk oligosaccharides and can thus be used to improve the quality of infant formula. Other applications include their usage as valuable prebiotic ingredients in foodstuff and a pharmaceutical utilization. Although the efficiency of the enzyme to use the added sugars as acceptor-molecules for transglycosylation was less than 20 % (w/w), a total of at least five novel galacto-oligosaccharides could be identified by means of HPLC-analysis. Transglycosylation to N-Acetyl-neuraminic acid could not be observed.

## Kurzdarstellung:

Die vorliegende Arbeit führt die Forschungstätigkeit unseres Instituts fort, effiziente Prozesse zur Konvertierung von Laktose zu entwickeln. Ziel des Projekts war es, durch Kombination einer heterolog überexprimierten β-Galactosidase aus *Lactobacillus reuteri* mit folgenden vier in der Muttermilch vorkommenden Zuckern neue Galaktooligosaccharide herzustellen: L-Fucose, N-Acetyl-neuraminsäure, N-Acetyl-D-galactosamin and N-Acetyl-D-glucosamin. Die hergestellten Derivate sind den in der Muttermilch vorkommenden Oligosacchariden ähnlich und können deshalb dafür benutzt werden, die Qualität von Babynahrung zu verbesseren. Darüberhinaus bietet sich deren Anwendung als wertvolle prebiotische Inhaltsstoffe für eine Vielzahl von Nahrungsmitteln an, sogar eine pharmazeutische Anwendung wäre denkbar. Obwohl die Effizienz des eingesetzen Enzyms die eingesetzten Zucker zu transglycosylieren unter den Versuchsbedingung unter 20 % (w/w) lag, konnten insgesamt mindestens fünf neue Galacto-oligosaccharide nachgewiesen werden. Eine Transglycosylierung der N-Acetyl-neuraminsäure konnte nicht beobachtet werden.

*“All things must pass.”*

*George Harrison (1943 – 2001)*

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# 1. Introduction

It was late in the twentieth century, only a little more than 25 years ago, when Karry Mullis invented the PCR** (Polymerase chain reaction). His invention proved to be of enormous relevance for biotechnology, enabling scientists to amplify specific DNA sequences.

Biotechnology is on a constant rise ever since and is believed to be one of the most important scientific fields in the rising twenty-first century. All over the world scientists focus their efforts on developing new applications, products and techniques that were unimaginable some decades ago. Especially heterologously overexpressed enzymes are of particular interest for numerous applications, as enzymatic conversions are very specific and swift.

One among the vast number of enzymes that are of industrial importance is β-galactosidase, the enzyme which hydrolyses lactose. Lactose accumulates in huge amounts as a cheap by-product of the dairy industry, but is costly to dispose of. Therefore strategies to convert surplus lactose to more valuable products are in demand. Intriguingly the enzyme β-galactosidase not only hydrolyses lactose, but also possesses the ability to create new products out of its cleavage products via a transgalactosylation reaction. This feature can be exploited to create a diverse number of galactosylated products.

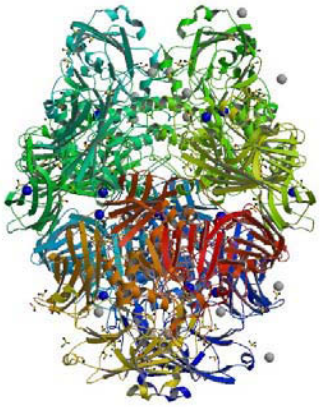
For more than a decade our institute has been investigating the ability of different β-galactosidases to generate oligosaccharides, which can be used as prebiotic ingredients in foodstuff. This thesis continues the ongoing research and reports basic experiments to produce oligosaccharides with a similar composition to those in human breast milk. The final products are supposed to improve the quality of infant formulas, but can generally be used in foodstuff as valuable prebiotic ingredients. As human milk oligosaccharides are potent defence agents, even a medical application of the final products is imaginable.

* In 1972 a research team from the University of Wisconsin already published an article describing this novel technique (Kleppe & Khorana 1972), but their concept did not receive much attention then. It is Karry Mullis, whom this invention is generally credited to and who received a Nobel prize for it in 1992.

## 2. Literature review

### 2.1. β-galactosidase

β-galactosidase (β-gal, lactase, EC 3.2.1.23) is an enzyme that catalyzes hydrolysis and transgalactosylation of lactose and other β-D-galactopyranosides (Prenosil et al. 1987). The enzyme is widespread in nature and can be isolated from many different sources, including microorganisms, plants and animals (Mahoney 1996). Although β-galactosidases from different organisms catalyze the same kind of reaction, there are substantial differences in their constitution: For example, β-gals from *E.coli* strains, which are the best studied β-galactosidases by far, are usually 464-kDa tetramers with identical subunits (Matthews 2005). The β-gal from *Bacillus circulans*, which has been used in a number of recently published reports regarding GOS-synthesis (e.g. Li et al. 2010) is a ~65-kDa monomer and the β-gal of certain *Lactobacillus reuteri* strains was determined to be heterodimeric with two subunits of 35- and 72-kDa (Nguyen et al. 2006). Some micro-organisms possess DNA sequences encoding for several β-galactosidases, such as *Bifidobacterium bifidum* DSM20215 with three different monomeric β-galactosidases (Moller et al. 2001). However, all β-galactosidases can be assigned into four families of glycosyl hydrolases according to sequence similarities (Henrissat 1991).



*3D-structure of the tetrameric beta-galactosidase from E.coli k12 derived by X-ray diffraction with the remarkable resolution of 1.75 Å. Beta-galactosidases from E. coli are best studied by far, while no crystal structure of β-gals from Lactobacillae has been published yet.*
*(source: Protein Data Bank)*

It is worth mentioning that the glycosidic bond of the substrate is very stable with a half life of several million years and enzymes responsible for their cleavage accomplish their task with rate constants up to 1000 $s^{-1}$. Thus, glycosidases are among the most proficient catalysts known so far (Zechel & Withers 2000).

At the present time systematic studies investigating correlations between amino acid sequence homology and enzyme characteristics have not yet been published due to a lack of published amino acid sequences and undefined standard reaction conditions for transglycosylation (Gosling et al. 2010).

The use of β-galactosidase to hydrolyse lactose to D-glucose and D-galactose is of interest from both the nutritional and technological point of view, since the resulting sugars are sweeter than lactose, more readily fermented and get absorbed directly from the intestine (Mahoney 1996). At the present time there are two different relevant

industrial applications for β-galactosidases: the degradation of lactose in diary products e.g. to render them suitable for people suffering form hypolactasia (Shaukat et al. 2010), and the production of prebiotic galacto-oligosaccharides (GOS), a relatively new class of compounds, which are promising ingredients for all kinds of foodstuff (Gosling et al. 2010).

## 2.2. Hydrolysis and transglycosylation

The catalytic mechanism of β-gal has only been determined in studies with enzymes from *E.coli,* but it is likely that many other β-galactosidases use this mechanism as well (Mahoney 1998):
In a first step the enzyme needs to bind a substrate molecule. The substrate molecule must consist of a galactosyl unit linked by a glycosidic bond of beta stereochemistry. There is a broad specificity for the other part of the substrate molecule, but a galactosyl moiety needs to be present for the enzyme to catalyze the reaction (Gosling et al. 2010). Two carboxylic groups were identified on the active side of the enzyme, approximately 5.5 Å apart. One of the carboxylic groups protonates the glycosidic oxygen, which cleaves the glycosidic bond of the substrate. The other carboxylic group acts as a nucleophile and forms a covalent galactosyl-enzyme intermediate. The non-galactosyl part of the substrate diffuses away. In a second step the incoming acceptor molecule is deprotonated and attacks at the anomeric center, displacing the galactosyl from the enzyme. Both bonds of the intermediate structures have an oxocarbenium ion character and because of two inverting steps the overall result leads to a retaining mechanism, which keeps the beta-glycosidic bond in the product (Zechel & Withers 2000).
In addition, it has been found out that mono- and divalent metal ions (such as $Na^+$ and $Mg^{2+}$) support optimal activity and stability for β-galactosidase, but their exact role in the mechanism has not been elucidated yet (Nguyen et al. 2006).

A simplified illustration of this mechanism applied to lactose conversion would look as follows (Mahoney 1998):

(1) enzyme + lactose → enzyme-lactose
(2) enzyme-lactose → galactosyl-enzyme + glucose
(3) galactosyl-enzyme + acceptor → galactosyl-acceptor + enzyme

Where the acceptor is a sugar, the result is the formation of galacto-oligosaccharides via transgalactosylation. Where the acceptor is a water molecule, monomeric galactose and glucose are formed by hydrolysis. Hydrolysis can thus be regarded to be a special instance of galactosyl-transfer to water. A third possibility is intramolecular or direct

transglycosylation: Here the non-galactosyl part of the substrate is re-attached to the galactosyl-part yielding regio-isomers, e.g. allolactose (β-1,6 bond) from lactose (β-1,4 bond) (Cardelle-Cobas et al. 2009).

GOS structures as products from enzymatic conversions differ in saccharide composition, regiochemistry of the glycosidic linkages and the degree of polymerization. Different saccharide compositions are a result of glucose or galactose used as first acceptors. If glucose is used as acceptor, oligosaccharides with $(Gal)_n$-Glu composition will be formed, if galactose is used, $(Gal)_n$-Gal oligosaccharides will be the result. An even more diverse mixture of products can be gained by addition of compounds with OH-moieties to the reaction mixture, since all OH-groups present in the reaction mixture are supposed to be used for transglycosylation. Resulting new products therefore generally have $(Gal)_n$-acceptor structure.

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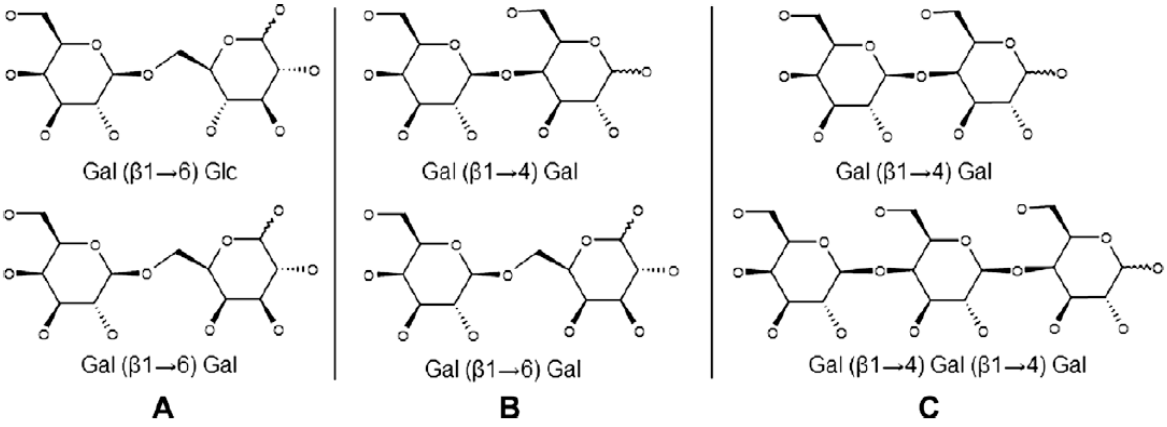


*Examples of how GOS structures can vary (adopted from Gösling et al (2010).*
***A** shows GOS with different saccharide composition, **B** different regiochemistry and **C** different polymerization degrees.*

The mixture of GOS products with different regiochemistry is primarily controlled by the identity of the used enzyme and is probably related to the structure and/or reaction mechanisms of the enzyme (Gosling et al. 2010). The β-1,6 linkage with the primary hydroxyl group of an acceptor-sugar is often reported to be most abundant and there are mechanistic models to support this finding (Juers et al. 2003). To date, no mechanistic explanation has been offered to explain the preferential production of other linkage types by some enzymes, such as β-1,2, β-1,3 or β-1,4. In principle the galactose-moiety can be transferred to any free hydroxyl group of an acceptor-sugar except for the C1 hydroxyl (Mahoney 1998). Because regioisomers show different formation and hydrolysis rates, the reaction time additionally influences the amounts of different GOS present in a batch (Gosling et al. 2010). Some publications even report an influence of the enzyme

concentration on the product spectrum gained by transglycosylation (e.g. Ji et al. 2005)
The degree of polymerization of GOS produced by enzymatic lactose conversion is usually reported to be between two and five (Mussatto & Mancilha 2007), GOS with higher polymerization degrees might be formed as well, but usually in quantities too small to be detected (Mahoney 1998). Quantitatively the amount of different GOS products is dependent on the source of the used enzyme, as well as on the reaction time. For example, before tetrameric GOS can be formed trimeric GOS need to be present as galactosyl acceptors. It has subsequently been shown that oligosaccharides with a higher polymerization degree, such as penta- and hexasaccharides, reach their maximum concentration at the expense of the total GOS yield (Albayrak & Yang 2001).
The newly formed galacto-oligosaccharides are only transient products, because they are substrates for the enzyme and will again be converted. This leads to very complex mixtures of different galacto-oligosaccharides that are almost impossible to predict (Splechtna et al. 2006). In order to predict the formation of GOS over time a number of mechanistic or experimental models have been developed. Mechanistic models strike a balance between a poor fit to experimental data due to oversimplification and inappropriate estimation of a large number of rate constants. Models including more terms are generally able to fit experimental data more closely, but the greater number of degrees of freedom can produce large error margins around individual terms. Empirical models might be useful for optimization of GOS production, especially a method known as response surface methodology has been used with some success (Gosling et al. 2010).
However, under most conditions hydrolysis predominates compared to transglycosylation, because in aqueous solvents $H_2O$ is the most available acceptor-molecule in the mixture. Higher GOS yields can therefore be achieved by decreasing the ratio of acceptor sugars to water by increasing the initial lactose concentration. After a certain time all formed GOS become degraded and the remaining products of the mixture are monomeric galactose and glucose (and eventually additionally added acceptors).
In order to anticipate the competing hydrolysis reaction in GOS synthesis, other solvents than water have been used to carry out the conversion. Mixtures of different organic solvents (Finch & Yoon 1997), as well as ionic liquids (Kaftzik et al. 2002) have been successfully used to improve the yield of GOS-synthesis. Especially third generation ionic liquids might offer an attractive approach for efficient GOS synthesis (Gorke et al. 2010).
GOS yields in aqueous enzymatic synthesis above 50 % (of the initial lactose concentration) are not often exceeded, in most reports the yields are between 30 % and 40 % (Gosling et al. 2010). It is worth mentioning that also the reverse reaction - the formation of lactose from D-galactose and D-glucose - is reported in literature to be catalysed by β-galactosidases (Prenosil et al. 1987). However, the reverse reaction is much slower than lactose hydrolysis or oligosaccharide formation (Mahoney 1996) and is

therefore of no experimental importance.

## 2.3. *Lactobacillus reuteri*

The heterofermentative, Gram-positive bacterium *Lactobacillus reuteri* was recorded in scientific classifications in the beginning of the 20th century, but was falsely grouped as a member of *Lactobacillus fermentum.* This mistake was corrected in the 1960s by the German scientist Gerhard Reuter, who reclassified *Lactobacillus reuteri* as *Lactobacillus fermentum biotype II*. Other German scientists subsequently showed that *L. reuteri* is clearly distinguishable from other biotypes of *L. fermentum* based on several phenotypical and genetic characteristics and thus proposed the organism for a distinct species status. Their proposal was accepted and since 1980 *L. reuteri* is classified as a distinct species in the genus *Lactobacillus* (Casas & Dobrogosz 2000).

*Lactobacillus reuteri* can be found as an inhabitant in the gut of animals, other significant natural habitats outside the host have not been identified so far (Walter et al. 2010). *L. reuteri* shows a very pronounced host specialization, which was shown in experiments, where it was tried to transfer strains of *L.reuteri* from different species to gnotobiotic subjects. The result was that indigenous strains of *L.reuteri* frequently outperformed exogenous strains when competing in the gastrointestinal tract (Oh et al. 2009).

Different strains of *L. reuteri* are widely used as probiotic agents in food and feed and it has been shown that *L. reuteri* can effectively colonize the intestine rapidly after ingestion (Valeur et al. 2004). Several studies suggest different health promoting effects of *L. reuteri* containing probiotics, for example a positive impact on oral health (Nikawa et al. 2004), an increased resistance to pathogens (e.g. (Casas & Dobrogosz 2000) or an ameliorative effect on colitis (Fabia et al. 1993). However, the EFSA (European Food Safety Authority) has been reluctant to allow probiotics containing a certain strain of *L. reuteri* to be marketed with the health-claim "natural defence", since this claim was "not sufficiently defined and no further details were provided in the proposed wording" (EFSA 2010).

*L. reuteri* is also known to form biofilms, which secrete antimicrobial and anti-inflammatory compounds (Jones & Versalovic 2009). The most prominent of those antimicrobial compounds is reuterin (or 3-hydroxypropionaldehyde), which is synthesized from glycerol. Reuterin is an interesting compound for different applications because it is active in a wide range of pH values against bacteria, yeasts, fungi, protozoa and viruses (Cleusix et al. 2008).

When Gerhard Reuter intensively studied the Lactobacillus biota of the human digestive system in the 1960s, he reported *L. reuteri* to be one of the dominant Lactobacilli regulary detectable in humans (Reuter 2001). Several more recent studies (e.g. Molin et

al. 1993) report a very low prevalence of *L. reuteri* in humans, suggesting a massive decline of the *L. reuteri* population size during the past 50 years. The reasons and implications of this disappearance, not limited to *L. reuteri* and also observable for other microbial lineages, are yet unclear, but some scientists associate it with the rapid increase of diseases of civilization, which have arisen without obvious explanations. This "disappearing microbiota" hypothesis might explain the rise and fall of several common diseases in developed countries (Blaser & Falkow 2009).

## 2.4. Prebiotics

There is a huge market potential for products that lead to health improvement without requiring a change of lifestyle (Shields et al. 2003). Therefore, the market for functional foods is on a constant rise and one of the leading trends in today's food industry (Saarela et al. 2002). In particular functional foods targeted towards improving the balance and activity of the gastrointestinal tract are the largest segment of the functional food market in Europe (Siró et al. 2008). In this context pro- and prebiotics have been receiving much attention in recent years (Venter 2007).

Probiotics are "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (Guarner et al. 2008) and their intake has been recommended for many years now in the Western World. Prebiotics on the other hand are a comparatively new class of compounds and have first been defined only 15 years ago (Gibson & Roberfroid 1995). In a redefinition of the original concept a prebiotic is a "selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits upon host well-being and health" (Gibson et al. 2004). Therefore three criteria are to be met in order to classify a compound as prebiotic (Roberfroid 2007):

1. Resistance to gastric acidity, to hydrolysis by mammalian enzymes and to gastrointestinal absorption, so that a significant proportion of the compound is available for fermentation in the intestine.

2. The ability of the intestinal microflora to ferment the compound.

3. The compound stimulates selectively the growth and/or activity of bacteria that are associated with health and well-being.

At the moment there are only two compounds that clearly fulfil the above criteria, fructooligosaccharides (FOS) (including lactulose and the fructopolysaccharide inulin) and galactooligosaccharides (GOS). Several other carbohydrates are mentioned in literature as candidates, but the evidence is too sparse to justify their classification as prebiotics at

the present time (Roberfroid 2007). However, in Japan, which is the leading market for functional food, additional kinds of carbohydrates have already received the status of prebiotics (Sako et al. 1999), (e.g. soybean oligosaccharides, xylo oligosaccharides and isomalto oligosaccharides*). Other promising candidates for prebiotics are certain peptides, proteins and lipids (Venter 2007). Health benefits following the ingestion of prebiotics are directly related to the physiological changes they induce. Demonstrating a direct clinical or health benefit of prebiotics proves to be difficult, because small changes in metabolism may not give rise to evident improvements in health for many years (Cummings & Macfarlane 2002). However, over the years a number of health benefits has been associated with prebiotics, which are summarized in the table below (adapted from (Venter 2007):

| Beneficial effect | Proposed mechanism | Reference |
|---|---|---|
| Alleviation of constipation | - Stimulation of microbial growth and thus stimulation of peristalsis by increased bowel content | Spiller 2001 |
| Increased number of beneficial bacteria in the large intestine | - Prebiotics are a preferred energy source for some beneficial bacteria<br>- Acidification as a result from prebiotic degradation favours growth of beneficial micro-organisms | Cummings et al. 2001 |
| Increased mineral absorption | - Intake of prebiotics lowers pH, which aids the solubilisation of minerals<br>- Prebiotics enlarge the absorption surface by promoting proliferation of enterocytes<br>- Increased capacity of calcium transporters in the colon | Coudray et al. 2003<br>Cummings et al. 2001<br>Venter 2007 |
| Anticarcinogenic effects | - Production of protective metabolites out of prebiotics<br>- Shift of colonic metabolism to saccharolysis with more benign end products | Prasad 1980<br>Pedersen et al. 2007 |
| Modulation of the immune system | - Direct contact of lactic acid bacteria or bacterial products with immune cells<br>- Production of SCFA<br>- Modulation of mucin production | Schley & Field 2002 |
| | - Reduced peak levels of insulin and induction of lipogenic enzymes via | Roberfroid 2000 |

*http://www.medicinalfoodnews.com/vol01/issue3/foshu.htm, information retrieved on December, 1st 2010

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|---|---|---|
| Regulation of lipid metabolism | increased gene transcription<br>- Production of SCFA<br>- Increased bile acid synthesis in the liver because of precipitation and excretion of bile acid to the intestine | Schley & Field 2002<br>Pedersen et al. 2007 |
| Increased production of Vitamin B | - Proliferation of vitamin B producing micro-organisms | Mussatto & Mancilha 2007 |

One extension of the prebiotic concept is the potential use of prebiotics as defence agents due to their anti-adhesive properties. Although a prophylactic approach has not been thoroughly evaluated, human milk oligosaccharides show exemplary efficiency in this regard (Rastall & Maitin 2002).

Interestingly, all attempts to establish a clear dose-effect relation between the amount of ingested prebiotic and the numeral increase of lactic acid bacteria in the faeces have failed so far. Instead it was observed that the initial number of bifidobacteria is inversely correlated with its increase (Rycroft et al. 2001).

Unlike probiotics, which are rather difficult to handle in foodstuffs, prebiotics show some advantages as food components: Prebiotics are usually very stable against oxygen, heat and acids, which is not the case for probiotics (Venter 2007). Additionally prebiotics are cheaper to produce and carry less risk as compared to probiotics. Apart from their health beneficial effects prebiotics can also have functional properties in food, such as giving fat-based spreads and dairy products a creamy mouth feel (Macfarlane et al. 2006).

Prebiotic oligosaccharides are generally well tolerated, having no severe side effects following ingestion. Some individuals report increased frequency of flatulence and excessive consumption might lead to intestinal discomfort and diarrhoea as an effect of transferred water in the large bowel via osmosis and from production of gases by the intestinal microflora (Mussatto & Mancilha 2007).

Although a considerable amount of knowledge regarding prebiotics has been gathered over the years, further research has to be carried out in order to reveal additional key information about prebiotics. However, food containing prebiotics may offer a very straight-forward approach of using diet for improved health (Venter 2007).

## 2.5. Galacto-oligosaccharides (GOS)

As implied by the nomenclature, galacto-oligosaccharides (GOS) are molecules predominantly composed of galactose units with a polymerization degree between two and ten sugar units per molecule. Galacto-oligosaccharides are used as prebiotic food ingredients in human and animal nutrition (Nguyen et al. 2006) and have received a positive response to the GRAS notification (GRAS Notice No. GRN 000236).

In the European Union the EFSA (European Food Safety Authority) has already decided about six health-claims regarding the usage of GOS in food products. Three health-claims have passed EFSA's pre-screening, namely "maintains a healthy normal digestive system," "prebiotic/bifidogenic," and "increases calcium absorption". The other three claims "helps support a healthy immune system in an ageing population", "helps to manage the symptoms associated with irritable bowel syndrome"; and "energizes your immunity boosting bacteria" or "helps boost your body's self-defence" have been rejected because of insufficient scientific data (Torres et al. 2010).

GOS find further application as low-caloric sweetener with an caloric value of approx. 1.7 kcal/g (Sako et al. 1999), about 1/3 of the sweetness of sucrose and their low potential for the development of dental caries (Macfarlane et al. 2006). Galacto-oligosaccharides are furthermore interesting for their application in food industry due to their pleasant taste, their stability to heat and acid, good moisture retention, high solubility and their ability to increase the texture and mouth-feel of products (Venter 2007).

The only adverse effect of GOS known so far is transient osmotic diarrhoea that occurs when an excess of galacto-oligosaccharides is consumed with symptoms very similar to unabsorbed sugar alcohols or lactose (in symptomatic lactose-intolerant individuals). The daily amount of GOS that can be used safely has been estimated to be approximately 0.3 to 0.4 g/kg body weight or about 20 g per human body (Sako et al. 1999).

In principal there are four different ways to obtain galacto-oligosaccharides. Firstly traditional synthesis in liquid media can be used to produce GOS, but such an approach is tedious and low in yield because of extensive protecting-group manipulations between each glycosylation step (Smoot & Demchenko 2009). A recent development and good alternative to traditional synthesis is solid-phase oligosaccharide synthesis, which might be especially useful to synthesize small amounts of desired oligosaccharides and use them as standards in analysis. However, this technique has not yet reached the same efficiency as solid-phase nucleotide synthesis (Seeberger 2009) and the production of large quantities would probably be comparatively expensive. Another possibility to obtain GOS is their isolation from milk, especially goat's milk is an interesting candidate for such an approach (Martinez-Ferez et al. 2006). The fourth and currently most used strategy for large scale GOS-production is β-galactosidase-catalysed conversion of lactose, a

reaction which was first observed in the early 1950's (Wallenfels 1951). Japan has become the largest market for GOS-production with six thousand tonnes of GOS being manufactured in 2005 in Japan alone (Gosling et al. 2010). Depending on reaction conditions and source of the enzyme enzymatic synthesis yields a highly diverse mixture of GOS with a polymerization degree between 2- 8 that are either β-1,2; β-1,3; β-1,4 or β-1,6 linked (Macfarlane et al. 2006). Interestingly, there is evidence that microorganisms grow best on mixtures of GOS, which have been produced by their own β-galactosidases (Rabiu et al. 2001).

The excellent functional and health promoting properties of GOS combined with an increasing demand for products that lead to health improvement without requiring a change of lifestyle (Shields et al. 2003), GOS production is very likely to increase in future and find broad application in all kinds of foodstuff (Mussatto & Mancilha 2007), as virtually all carbohydrate containing food can be fortified (Venter 2007).

## 2.6. Human milk oligosaccharides (HOS)

In the nineteenth century research revealed a major component of human milk to be an unidentified carbohydrate not to be found in cow milk. As this substance was thought to be another form of lactose, it was named gynolactose ( (Mc Veagh & Miller 1997) ( the ancient Greek word *γυναικός* (gynaikos) means "woman's"). It turned out that this substance was not a single carbohydrate, but a complex mixture of human milk oligosaccharides, of which over 150 have already been identified. The oligosaccharide fraction is the third largest solute in human milk (after lactose and lipids) and present in greater quantities than protein (Miller et al. 1994).

All human milk oligosaccharides are assembled from only six different monosaccharides: D-glucose, D-galactose, L-Fucose, sialic acid, N-Acetyl-D-glucosamine and N-Acetyl-D-galactosamine (Jensen 1995). Most interestingly, exactly the same six monosaccharides contribute to the construction of gangliosides in the human brain (Wang & Brand-Miller 2003). This might be linked to the fact that the brain is the fastest growing organ of the infant.

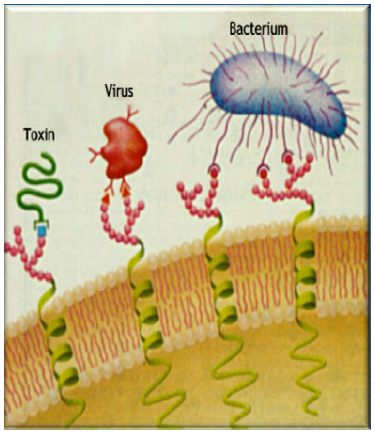
Human milk oligosaccharides (HOS) are synthesized in the Golgi apparatus of cells located inside the female breast, lining the alveoli and smaller ductules. Since lactose molecules are used as precursors for human milk oligosaccharides, there is an inverse relationship between HOS and lactose: When HOS levels are high, lactose is present in relatively small amounts and *vice versa* (Mc Veagh & Miller 1997). Almost all HOS possess a lactose moiety at their reducing end, and, if present, sialic acid or L-Fucose molecules at the nonreducing end (Miller et al. 1994).

Human milk typically contains between 5 and 8 g/l HOS (Gnoth et al. 2000) with a

composition that is distinct from those of other mammals with regard to its high content of diverse fucosylated and sialylated oligosaccharides. The closest match to the pattern of HOS has so far been found in elephant's milk (Kunz et al. 1999). Although goat's milk oligosaccharides do not have the same good similarity, they are much easier to obtain in large scale as a by-product of goat cheese production and might therefore offer an interesting alternative (Martinez-Ferez et al. 2006).

Most of the oligosaccharides in human milk occur in their free form, only a small fraction of approximately 10 % is bound to other components (Miller et al. 1994). The concentration of individual oligosaccharides does not remain static during lactation, but is highly variable between individuals, during lactation and can even change during a single feed. The total oligosaccharide concentration declines over the first months of lactation, with the amount of sialic acid decreasing the most and the amount of L-Fucose the least (Miller et al. 1994).

The changes in the composition of HOS during lactation could simply be interpreted as a consequence of ageing of those cells responsible for HOS synthesis (Viverge et al. 1990). However, they are more often interpreted to be an adoption of the milk composition to fit the needs of the infant (Coppa et al. 1990).



*Toxins, viruses and bacteria bind via glycoconjugates to the mucosal surface of the intestine. HOS with a composition identical to the target binding site can therefore act as decoy and prevent attachment.**

The role of HOS is not that of an energy source, since they are not digested by the infant and thus reach the colon. Here they fulfil a plethora of health promoting functions, only a few of which have already been elucidated. Their first and most obvious function in the colon is to act as prebiotics. In fact, HOS are the main source of carbon and energy for the intestinal flora (Mc Veagh & J.B. Miller 1997). Especially the Gram-positive bifidobacteria and lactobacilli rely on the degradation of N-Acetyl-D-glucosamine containing oligosaccharides, since they need N-Acetyl-D-glucosamine as a component of their cell wall (Hudault et al. 1994).

The effect of HOS on the intestinal flora becomes obvious when the composition of stool from breast-fed and formula fed infants is compared: By the end of the first week of life, bifidobacteria represent 95 % of the total bacterial population in the faeces of breast-fed children, while in formula fed infants their occurrence is below 70 %. Bifidobacteria and other lactobacilli that grow very well on HOS are believed to create an environment with a lower pH, which discourages the proliferation of pathogenic bacteria and enhances mucosal cell proliferation (Lönnerdal 2000).

A low pH furthermore enhances the absorption of micronutrients and trace elements (Venter 2007). A rapid colonization of the infants gut with lactobacilli and bifidobacteria

also occupies nutrients and adhesion sites that would be required for the establishment of harmful micro-organisms (Mc Veagh & Miller 1997).

Another effect of human milk oligosaccharides is that they can prevent pathogen infection by inhibiting their adhesion to the mucosal surface (Kunz & S. Rudloff 2006). Bacterial adhesion is a highly specific receptor-mediated interaction between structures present on the bacterial surfaces and complimentary glycoconjugates on the mucosal surface (Karlsson 1995). HOS with a composition identical to the target binding site of pathogens can therefore act as a decoy or even detach bacteria from cell membranes (Mc Veagh & Miller 1997). Breast-fed infants thus show a reduced susceptibility for a number of pathogen binding related infections in the laryngopharyngeal region and the digestive tract (Bode 2006).

It has been shown that the urine of infants contains small amounts of HOS. This implies that HOS are partially ingested by the infant and undergo systemic circulation (Obermeier et al. 1999). Therefore HOS also protect the genito-urinary system from infections to some degree (Hanson 2004). Another supposed effect of systemic circulation is an alteration of protein-carbohydrate interactions, HOS might thus serve as anti-inflammatory components and thus further contribute to the lower incidence of inflammatory diseases in breast-fed infants (Kunz & Rudloff 2006).

Since the understanding of the whole scope of the functions human oligosaccharides serve is still limited, it is probable that additional features will be elucidated in the near future. HOS might in the future be used by people of all ages for treatment and prevention of infections, since they act as defence agents. One of their biggest advantages is that they are not bactericidal, so pathogens will most likely not become resistant to them. Other advantages are an extreme stability to heat and alcohols, which alleviates sterilization. Furthermore HOS are unlikely to induce any allergic reactions. (Mc Veagh & Miller 1997).

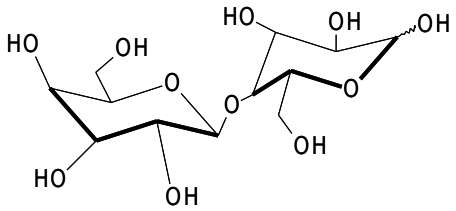
In Japan, infant formulas are already fortified with a small number of certain HOS that are easy to prepare in large scale quantities. As the effects of individual human oligosaccharides are not clear yet, this strategy is not supported by the the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (Agostoni et al. 2004).

* Picture taken from http://south.sanfordhealth.org/classlibrary/Page/Images/files/Bode.pdf

## 2.7. Sugars in Human milk

### 2.7.1 Lactose

Lactose (4-O-β-galactopyranosyl-D-glucopyranose, $C_{12}H_{12}O_{11}$) is a disaccharide consisting of one D-glucose molecule linked to a D-galactose molecule through a β-1,4 glycosidic linkage. The name is derived from the Latin word for milk (*lac, lactis*, n.) and the suffix –*ose*, which denotes sugar molecules in chemistry. Lactose has a molecular weight of 342.3 g/mol and appears as an odourless white solid at room temperature. The solubility of lactose is low compared to other disaccharides, at room temperature sucrose is ten times more soluble than lactose (Gänzle et al. 2008).




Lactose
(Lac)

Dissolved lactose is present in α- and β-forms, depending on the glucose fragment, which can either be in α- or β-pyranose form. In aqueous solutions α- and β-lactose show mutarotation until a ratio of 37:63 is reached. This equilibrium ratio is slightly affected by temperature, but not by pH (Haase & Nickerson 1966). It is worth mentioning that the β-form of lactose is sweeter than the α-form (Belitz 2007).

Solid lactose can be present in two crystalline forms, α-hydrate and β-anhydride, as well as in an amorphous mixture of both forms, depending on the conditions of crystallization The two crystalline forms show notable different physical properties, including different melting points and solubility (Zadow 1984).

Lactose is the main carbohydrate in the milk of almost all mammals with a species dependent approximate concentration between 20 to 100 g/l. One of the reasons why milk contains lactose instead of e.g. glucose as main energy source is that a disaccharide has only half of the osmolarity compared to two unlinked monosaccharides. This is important considering that the newborn receives large amounts of carbohydrates in short periods and monosaccharides would therefore be more likely to cause postprandial stress (Jensen 1995).

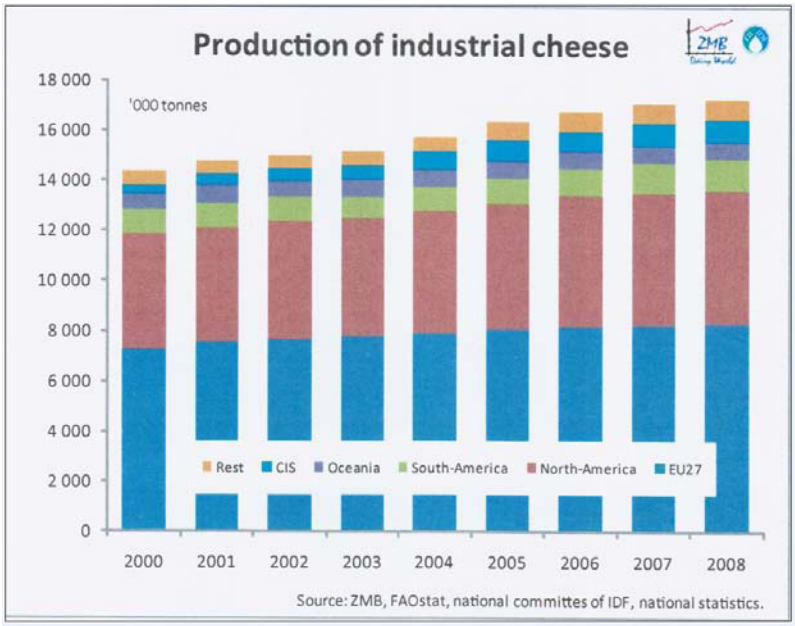
Another interesting point is that lactose is β-1,4 linked, while most other carbohydrate macronutrients in nature are glucans with an α-1,4 linkage. As lactose is found almost exclusively in milk, this particular linkage protects lactose form digestion by a large number of microbes that rely on a glucan-polymer source. Therefore the lactating breast and the infant's gastrointestinal tract are less likely to get infected (Jensen 1995).

Also the galactose-moiety of lactose is supposely not only a source of energy (following

epimerization to D-glucose), but also supplies the rapidly growing brain, which requires large amounts of galactocerebrosides. Although D-galactose can be synthesized in the infant's liver, this function might be underdeveloped in the young (Jensen 1995). However, experiments carried out in the 1960s indicate that no endogenous D-galactose is needed for the synthesis of galactolipids (Varma et al. 1962).

Lactose accumulates in form of whey as a by-product of cheese production. Annual worldwide whey production in 2005 was reported to be around 160 million tons (containing ~5% lactose = 8 million tons of lactose) with the same growth rate as milk and cheese of 1-2 % each year (Smithers 2008).

Some whey finds utilization, e.g. in liquid diary products, as animal feed or as liquid fertilizer, but an enormous amount of whey finds no application and needs to be disposed of. Whey (in particular the lactose fraction of whey) has an exceptionally high demand of oxygen for its decomposition that is about 175-fold higher than that of the typical sewage effluent and should therefore not be discharged into a municipal sewage system (Smithers 2008).



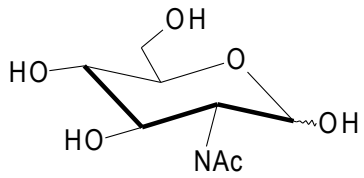

*Lactose accumulates in form of whey during cheese production. As worldwide cheese production is on a constant growth, so is surplus lactose.*

As a consequence most countries have enacted adequate laws to counteract environmental problems associated with lactose (Gänzle et al. 2008). In Austria, sec 1 subs 1 of the applicable water law ("Wasserrechtsgesetz", WRG) prohibits the disposal of lactose containing fluids into the sewage system or into bodies of water (households and research facilities are excluded from this regulation). Therefore, strategies for converting lactose into commercially viable products are in demand, so that lactose does no longer need to be costly disposed of. Although scientific literature of the last 65 years is replete with suggestions and pronouncements to overcome this problem, a commercially viable large scale application has not yet been found and the diary industry is still waiting for a "final solution" of the whey (and thus lactose) problem (Gänzle et al. 2008).

## 2.7.2. N-Acetyl-D-glucosamine (GlcNAc or NAcGA)

N-Acetyl-D-glucosamine (2-Acetamido-2-deoxy-D-glucose, $C_8H_{15}NO_6$) is a derivative of glucose, carrying an acetamide group on the C2 atom instead of a hydroxyl group. NAcGA is the principal building block of chitin as well as of murein and is therefore among the most abundant organic molecules on our planet (Tharanathan & Prashanth 2007). N-Acetyl-D-glucosamine is generally widespread in living matter, especially as a component of oligosaccharides, glycolipids and glycoproteins, which serve a multitude of functions within the organism (El Ashry et al. 2007).

N-Acetyl-D-glucosamine has a taste similar to glucose, but only half its sweetness. Therefore NAcGA has been used in Japan as a sweetening food additive in a variety of products (Takahashi et al. 2009), e.g. fruit juices and 'smoothies', dehydrated instant fruit mixes, sports drinks and iced tea drinks.




N-Acetyl-D-Glucosamine (GlcNAc)

GlcNAc, which can be synthesized in human cells out of glucose, is furthermore believed to act as a nutrient sensor and might thus play a crucial role in insulin resistance of diabetes mellitus patients (Wells et al. 2003). N-Acetyl-D-glucosamine and other glucosamine derivatives are also utilized as over-the-counter drugs for treatment or prevention of osteoarthritis, although the ultimate proof for its effectiveness in this regard has yet to be given as studies frequently yield opposite results (Vlad et al. 2007). Other medical applications of NAcGA include the use against inflammatory diseases of various parts of the digestive system, but again controlled trials to confirm the efficiency are lacking (Salvatore et al. 2000).

Although GlcNAc is considered a safe compound without any relevant toxicity, people allergic to shellfish should be cautious since N-Acetyl-D-glucosamine is usually processed out of chitin derived from seafood (Anderson et al. 2005).

Interestingly, NAcGA is one of the only six different monosaccharides present in human milk (Jensen 1995). In vitro studies clearly demonstrate a prebiotic effect of GlcNAc, when present within an oligosaccharide structure (Kunz & Rudloff 2006). Monomeric N-Acetyl-D-glucosamine is not prebiotic, because it is digestible (Takahashi et al. 2009), but it is worth mentioning that especially the derivative 2-Ethyl-2-acetamido-2-deoxy-D-glucose is reported to exhibit a strong prebiotic effect in vitro (Gyorgy 1953).

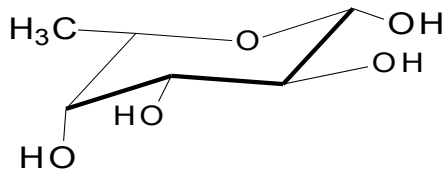
*In vivo* studies about the efficiency of N-Acetyl-D-glucosamine containing

oligosaccharides have not been published yet.

As N-Acetyl-D-glucosamine is derived from chitin, which accumulates in seafood industry as a waste product, it is a cheap compound with a price of 1,1 €/g when bought at Sigma-Aldrich® in 25 g scale*.

### 2.7.3. L-Fucose (Fuc)

L-Fucose (6-deoxy-L-galactose, $C_6H_{12}O_5$) is a hexose deoxy sugar that is present in a variety of organisms. Unlike most other natural occurring hexoses fucose has no hydroxyl group on the C6 atom and – remarkably - fucose is predominantly L-configurated. The epimer D-Fucose is not present in animals, but can be found in some plants (Springer & Williamson 1962). Fucose has a molecular weight of 164.16 g/mol, a melting point between 150 – 153 °C and appears as a white odourless powder at room temperature.




L-Fucose
(Fuc)

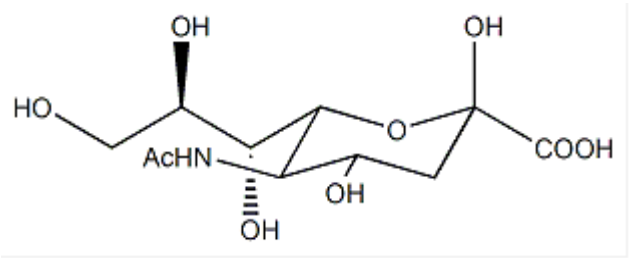
In mammalian cells L-Fucose is a common component of N- and O- linked glycans and glycolipids. Fucosylated glycans are known to play an important role in a variety of biological settings, but the full scope of their functions has not yet been elucidated (Staudacher et al. 1999). Additionally, L-Fucose was shown to suppress allergic contact dermatitis by interfering with lymphokine activity (Hasegawa et al. 1980). Apart from that, L-Fucose was shown to inhibit the growth of certain liver cancer cells *in vitro* (Kuroda 1974). As increased levels of fucosylation can be observed in a number of pathological conditions, certain types of fucosylated compounds can be used as tumor markers and changes in the fucosylation pattern could provide a novel strategy for cancer therapy (Miyoshi et al. 2008). A study conducted in the 1990s revealed increased concentrations of fucosylated haptoglobin and an increased activity of the enzyme fucosyl transferase in heavy smokers and heavy drinkers (Thompson et al. 1991).

L-Fucose is one of only six 6 different monosaccharides to be found in human milk. L-Fucose containing oligosacchardides in breast milk act as defense agents (Jensen 1995). It was shown that such oligosaccharides can inhibit the hemaglutinin activity of the classical strain of *Vibrio cholera* (Holmgren et al. 1983) and can protect against the heat stable enterotoxin of *Escherichia coli* (Newburg et al. 1990). It is supposed that L-Fucose

* Purity >95%, price information retrieved on August, 10th 2010 at www.sigmaaldrich.com

containing oligosaccharides have a structural homology to cell surface carbohydrates and therefore protect the ephitelium from pathogen binding (Newburg 1997). Fucosylation furthermore seems to protect oligosaccharides from degradation within the small intestine (Gnoth et al. 2000). Despite the frequent occurrence in a variety of biological settings, L-Fucose is comparatively expensive with a price of 22.5 €/g when bought at Sigma-Aldrich® in 5 g scale*. However, the German company *Jennewein Biotechnologie GmbH*** reports to have developed a innovative production process that enables them to sell L-Fucose at least five times cheaper compared to Sigma-Aldrich's price (personal E-mail correspondence).

### 2.7.4. N-Acetylneuraminic acid (NeuAc) (sialic acid)




Sialic acid (N-Acetyl-neuraminic acid (NANA))

N-Acetylneuraminic acid (5-Acetamido-3,5-Dideoxy-D-Glycero-D-Galacto-2-Nonulopyranosonic acid, $C_{11}H_{19}O_9$) is one of the many natural occurring derivatives of the sialic acid family. The sialic acid family comprises all 43 naturally occurring derivatives of the nine-carbon sugar neuraminic acid. The sialic acid family can be subdivided into one branch that is N-acetylated (to form N-Acetyl-neuraminic acids) and another branch that is based on N-Glycolyl-neuraminic acids (Wang & Brand-Miller 2003). As sialic acid molecules can be substituted on various positions, a wide variety of isomers can be found in nature (Varki 1992).

N-Acetyl-neuraminic acids are widespread in nature, but rarely occur in free form. Sialic acids can be detected in essentially all tissues of vertebrates and have received their name according to the fluid where NeuAc was first isolated from - the ancient Greek word *σιαλικό* (sialiko) means "from saliva" (Mc Veagh & Miller 1997). Sialic acids are commonly found as components of oligosaccharides, glycoproteins and glycolipids. In oligosaccharides NeuAc usually occupies the terminal nonreducing position of the oligosaccharide chain (Wang & Brand-Miller 2003).

Although not entirely precise, the terms sialic acid and N-Acetyl-neuraminic acid are often used as synonyms in literature about human milk oligosaccharides (Jensen 1995). This is

* Purity >99%, price information retrieved on August, 10th 2010 at www.sigmaaldrich.com
** http://www.jennewein-biotech.de/

because N-Acetyl-neuraminic acid was the only sialic acid to be found in healthy individuals, while N-glycolylneuraminic acid had only been isolated from certain human tumors (Harms et al. 1996). Subsequent studies revealed that both forms are present in healthy individuals, but the amount of N-glycolylneuraminic acid in normal tissues is usually close to the detection limit with less than 1% of total sialic acids (Malykh et al. 2001).

In human milk approximately half of the oligosaccharides that have been characterized so far are sialylated (Mc Veagh & Miller 1997). They are supposed to serve at least two different functions for the offspring: on the one hand they can act as highly specific receptors for a variety of pathogenic organisms and thus prevent infections (Varki 1993). On the other hand sialylated oligosaccharides may serve as a supplier for monomeric N-Acetyl-neuraminic acid. Although the infant is able to synthesize sialic acid in the liver, the amounts that can be produced might not be sufficient to fulfil the demand that arises from the very fast growing brain during the first months of live (Mc Veagh & Miller 1997). Hydrolysis of the sialylated oligosaccharides to the monomeric sialic acid can be achieved by endogenous sialidases or sialidases of bacterial origin. Additionally the possibility of autohydrolysis of the oligosaccharide has been reported, since sialic acids are relatively strong with a p*K*a between 2.2 – 3 (Wang & Brand-Miller 2003). Oligosaccharides containing sialic acid are not reported to exhibit prebiotic effects, but rather seem to protect the oligosaccharide from bacterial degradation, as stated by some authors (György et al. 1974). Other authors report to have noticed a growth-promoting effect of substances in human milk that contain sialic acid (Nakano et al. 2001).

In cells from higher animals and some microorganisms, sialic acid is produced in a long pathway starting from glucose. The importance of sialic acid for the human body is outlined by the fact that severe deficiency has not been observed yet, because it is supposed to be lethal (Mc Veagh & Miller 1997).

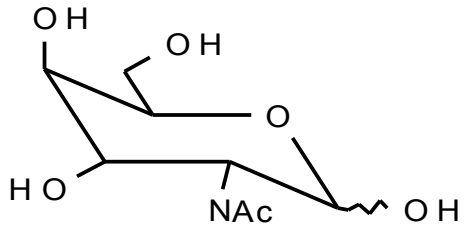
When bought at Sigma-Aldrich®, sialic acid most expensive with a price of 607 €/g*. However, the British company *Carbosynth Limited* offers the same substance purer and 50-times cheaper, 5 g of sialic acid can be purchased for only 60 €**.

### 2.7.5. N-Acetyl-D-galactosamine (GalNAc)

N-Acetyl-D-galactosamine (2-Acetamido-2-deoxy-D-galactose, $C_8H_{15}NO_6$) is a derivative

* Synthetic, Purity >95%, price information retrieved on November, 5th 2010 at www.sigmaaldrich.com
** Synthetic, Purity >98%, price information retrieved on November, 5th 2010 at www.carbosynth.com
*** Purity ~ 98 %, price information retrieved on November, 5th 2010 at www.sigmaaldrich.com

of galactose, whose hydroxyl residue is replaced by an acetamide- moiety. GalNAc is a white odourless solid with a molecular mass of 221.21 g/mol and a melting point between 172 and 173 °C.




N-Acetyl-D-Galactosamine (GalNac)

N-Acetyl-D-galactosamine is one of the only 6 different monosaccharides in human milk and the most expensive of those (~177 €/g if bought in 5g scale from Sigma-Aldrich***). There seems to be some confusion about the identity of of N-Acetyl-D-galactosamine as a discrete component of human milk oligosaccharides, because it is frequently not stated to be a component by some authors (e.g. Bode 2006) but frequently by others (e.g. Jensen 1995 or Kunz & Rudloff 2006).

Knowledge of the biological importance of GalNAc is limited and research has mainly focused on investigations of the corresponding enzyme β-1,4-N-Acetyl-galactosaminyl-transferase, which has been characterized in great detail (Manzella et al. 1996).

The addition of N-Acetyl-D-galactosamine to proteins is a highly protein-specific process, since the transferase responsible for its additions competes with the β-1,4-galactose-transferase, which is expressed in higher levels (Harduin-Lepers et al. 1993).

Proteins glycosylated with oligosaccharides containing GalNAc-4-$SO_4$ at the terminal end are often glycoprotein hormones. Such proteins can be found in all classes of vertebrates in a highly conservated structure which underlines their central role in the biology of glycoprotein hormones (Hooper et al. 1995).

Especially the oligosaccharide sequence $Man_3GalNAc_2$ can frequently be found on glycosylated proteins, often further derivatized with sialic acid or L-Fucose moieties (Du et al. 2009).

Publications regarding the prebiotic properties of N-Acetyl-D-galactosamine containing oligosaccharides are not available, probably due to the high price of this monomeric sugar molecule. However, the precursor D-galactosamine would be much cheaper than GalNac (≈36 €/g if bought in 5g scale from Sigma-Aldrich*) and could probably be N-acetylated smoothly in a three step-synthesis. Although the preparation of N-Acetyl-D-galactosamine from D-galactosamine appears to be uncomplicated, removal of the educt would be imperative since D-galactosamine is known to be a potent liver toxicant (Tran-Thi et al. 1985).

* Purity >99 %, price information retrieved on November, 5th 2010 at www.sigmaaldrich.com

## 2.8. Infant formula

Until the famous German chemist Justus von Liebig patented the first marketed infant formula preparation in 1867, there had not been a promising alternative to breastfeeding. Statistics from the eighteenth century show an appalling mortality rate between 80 and 99.6 per cent among infants who were not breastfed (Anderson et al. 1982).

Liebig's recipe for the „perfect infant food", as he called his mixture, included a mixture of wheat flour, cow's milk and malt flour, cooked with a small amount of potassium bicarbonate. Although a commercial success, Liebig's formula was soon replaced by formulas containing evaporated cow's milk as main ingredient, which are easier to handle (Fomon 2001).

Early infant formulas were absolutely inadequate as the sole source of an infant's nutrition, but deficiency symptoms such as rickets, scurvy and anemia were widespread in infants, irrespective of their source of nutrition (Anderson et al. 1982).

Along with an improving understanding of (infant) nutritional needs, infant formulas have been consequently modified according to scientific knowledge (Fomon 2001). Modern infant formulas contain a variety of added compounds, some of which are obligatory additives and others optional (Pencharz et al. 2005). There was an ongoing discussion whether pro- or prebiotics (or both) should be added to infant formulas (Vandenplas 2002), in the meanwhile some commonly available infant formula in Austria are already fortified with galacto-oligosaccharides (personal observation).

It has been reported that infant formulas containing a certain mixture of oligosaccharides (90 % GOS and 10 % FOS) induce a metabolic activity of the intestinal microflora similar to that in breast-fed infants (high levels of acetate and lactate and a low pH) (Bakker-Zierikzee et al. 2007). Moreover, the composition of the gut microbiota becomes more dominated by bifidobacteria, which is the dominant species in breast-fed infants (Boehm & Moro 2008). Although both galacto- and fructo-oligosaccharides are generally regarded as safe ingredients, their general use in infant formulas is not recommended at the present time due to a lack of scientific data that would justify their addition. (Agostoni et al. 2004).

The addition of probiotics to infant formulas has been tested in treatment of diarrhea and atopic diseases, as well as for establishing a bifidobacteria-dominated intestinal microflora (Bakker-Zierikzee et al. 2007). A major problem of probiotic infant formulas is the heat-sensitivity of the probiotic bacteria that prevent the addition of hot water, which would be required to exclude the presence of foodborne pathogens (Edelson-Mammel & Buchanan 2004).

The preparation of infant formulas in general is often a source of error and therefore a risk for the formula fed infant: In an Italian study only 11 % of infant formula using parents prepared the formula correctly (according to the four WHO criteria: 1. sterilize the bottle at each feed, 2. wash hands with warm water and soap before preparation, 3. use water at ≥ 70 °C, 4. use formula immediately after preparation and discard the remains) (Carletti & Cattaneo 2008).

There is nowadays common agreement in the scientific community that human milk is superior to infant formula and thus breastfeeding should be promoted. But it has not always been like this, especially between the 1940s and the 1970s a massive decrease in breastfeeding is observable, at least partly caused by scientific recommendations (Hendershot 1984). Scientific opinion has changed in the meantime and only in cases of extremely preterm infants and infants with certain metabolic disorders or allergies infant formulas are recommended as first choice (Anderson et al. 1982).

Recently a very interesting article was published in the non-scientific German newspaper DIE ZEIT*. The author criticizes the pressure exerted on women to breast-fed their children in order not to appear as "bad mothers", even if in some cases the mother suffers terrible pain or produces insufficient amounts of milk. Furthermore breast-feeding-schedules are often very difficult to follow for working mothers. The author moreover cites the PROBIT (Promotion of breastfeeding intervention trial)-study (Kramer et al. 2001) that revealed a detrimental effect of breast-feeding for more than 2 months in regard to incidences of allergies. In conclusion the author states that it is probably best for nursing mothers to rely more on their own intuition about how to feed the newborn and less on indecisive scientific data.

However, although human milk is probably irreplaceable as the best nutrition source for infants, current formulas fulfil the classically recognized roles of infant nutrition and can be used safely (Jensen 1995). It is very likely that together with the improved understanding of the role of each individual component in human milk, infant formulas will be adjusted accordingly to better match the requirements of the infant. (Vandenplas 2002).

*DIE ZEIT, Magazin, Ausgabe 41, issued October 10th 2010, title: "Stille Macht" by Silke Hohmann

# 3. Experimental part

## 3.1. Aims of the study

The general objective of this master thesis was to explore the ability of the enzyme ß-galactosidase from *Lactobacillus reuteri* L103 to transglycosylate several acceptor sugars, which are present in human milk and thereby create oligosaccharides similar to human milk oligosaccharides. The specific aims of the study were as follows:

• To purify a heterologously overexpressed ß-galactosidase from *Lactobacillus reuteri* L103, which was expressed in *E. coli* in 2006 and stored frozen at – 26 °C

• To explore the ability of the enzyme to transglycosylate N-Acetyl-D-glucosamine, N-acetyl-D-galactosamine, L-Fucose and N-Acetyl-neuraminic acid (sialic acid) in a variety of experiments

• To screen existing literature regarding GOS production and to compare the results of this master thesis to the literature

• To evaluate and try to improve the existing methods applied in our laboratory for oligosaccharide analysis and to find new methods applicable for GOS analysis

• To determine the effect of short chain alcohols on enzyme activity and stability.

## 3.2.Materials and methods

### 3.2.1. Materials

#### 3.2.1.1. Chemicals

All chemicals were used as received form the suppliers. All $H_2O$ that was used for experiments and analysis was ultrapure, taped from an Ultra Clear basic UV device (SG, Germany).

| Compound (purity) | Supplier |
|---|---|
| 2-aminopyridine (~ 98 %) | Fluka, Germany |
| acetic acid (≥ 99 %) | Fluka, Germany |
| acetone (≥ 99.9 %) | Fluka, Germany |
| aniline (≥ 99 %) | Sigma-Aldrich, Germany |
| betaine monohydrate (> 99 %) | Fluka, Germany |
| 1-butanol (98 %) | Fluka, Germany |
| diphenylamine (>99 %) | Fluka, Germany |
| DMSO (≥99.5 %) | Sigma-Aldrich, Germany |
| DTT (>99 %) | Sigma-Aldrich, Germany |
| ethanol (96 %) | VWR, Austria |
| L-Fucose (99 %) | Sigma-Aldrich, Germany |
| D-fructose (Ph.Eur.) | VWR, Austria |
| D-galactose (≥99 %) | Fluka, Germany |
| D-glucose (≥99.5 %) | Roth, Germany |
| glycerine (99.5 %) | Roth, Germany |
| $H_2SO_4$ (>65 %) | Roth, Germany |
| $H_3PO_3$ (99 %) | Sigma-Aldrich, Germany |
| $H_3PO_4$ (>85 %) | Fluka, Germany |
| imidazole (buffer grade) | AppliChem, Germany |
| KOH (≥86 %) | Fluka, Germany |
| lactose monohydrate (Ph.Eur.) | Roth, Germany |
| methanol (99 %) | Roth, Germany |
| $MgCl_2$ hexahydrate (>99 %) | Fluka, Germay |
| Na-Acetate (99.7 %) | VWR, Austria |
| N-Acetyl-D-galactosamine (~ 98 %) | Sigma-Aldrich, Germany |
| N-Acetyl-D-glucosamine (99 %) | Sigma-Aldrich, Germany |
| N-Acetyl-neuraminic acid (sialic acid) (≥98 %) | Sigma-Aldrich, Germany |
| NaCl, p.a. (>99.5 %) | Merck, Germany |
| NaOH (>99 %) | Merck, Germany |

| | |
|---|---|
| NaOH solution 50 % | Merck, Germany |
| NaPP (≥99 %) | Fluka, Germany |
| *o*NPG (≥98 %) | Sigma-Aldrich, Germany |
| 1- propanol (>99 %) | Fluka, Germany |
| 2- propanol (≥99.5 %) | Roth, Germany |
| sodium-cyanoborohydride (95 %) | Sigma-Aldrich |
| sucrose (>99 %) | Fluka, Germany |
| thymol (>99 %) | Merck, Germany |

### 3.2.1.2. Solutions and buffers

#### Buffers for $His_6$tag purification

Buffer A

20 mM NaPP

0.5 M NaCl

20 mM imidazole, adjusted to pH 6.5 and filtrated prior use (ϕ 0.45 µm)

Buffer B

20 mM NaPP

0.5 M NaCl

500 mM imidazole, adjusted to pH 6.5 and filtrated prior use (ϕ 0.45 µm)

#### Enzyme storage buffer (double concentrated)

5 mM DTT

1 M betaine

20 % (v/v) glycerine

#### Buffers for *o*NPG activity test

Reaction buffer

22 mM *o*NPG in 50 mM NaPP, adjusted to pH 6.5

Stop solution

0.4 M KOH

## Reaction buffer

0.58 M (200 g/l) lactose

1 mM $MgCl_2$ dissolved in 50 mM NaPP, adjusted to pH 6.5

## Enzyme dilution buffer

50 mM NaPP, adjusted to pH 6.5

## Buffers for HPLC

### Dionex pA1 column

Buffer A

100 mM NaOH,filtrated prior use (ϕ 0.45 µm)

Buffer B

100 mM NaOH

1 M Na-acetate, filtrated prior use (ϕ 0.45 µm)

Buffer C = UHQ ($H_20$)

Buffer D

100 mM NaOH

50 mM Na-acetate, filtrated prior use (ϕ 0.45 µm)

### Supelco Gel H column

0.1 % (v/v) $H_3PO_4$, filtrated prior use (ϕ 0.45 µm)

## Buffers for capillary electrophoresis

Washing buffer

100 mM $H_3PO_4$, or 100 mM $H_3PO_3$ degassed prior use (ultrasonic bath)

Running Buffer

50 mM $H_3PO_4$ or 50 mM $H_3PO_3$ adjusted to pH 2.5, degassed prior use (ultrasonic bath)

**TLC buffers**

Running buffer

n-butanol : n-propanol : ethanol : $H_20$
2 : 3 : 3 : 2

Staining buffer A

0.5 % (w/v) thymol dissolved in 5 % (v/v) $H_2SO_4$ in ethanol

Staining buffer B

4 g diphenylamine dissolved in a solution of 4ml aniline, 200 ml acetone and 30 ml 85% $H_3PO_4$

## 3.2.2. Methods

### 3.2.2.1. Protein purification

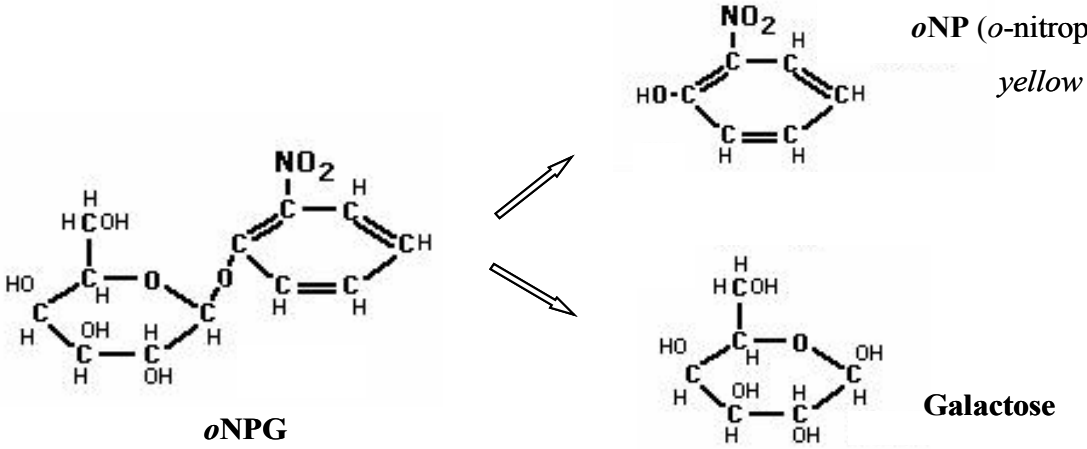
The received crude extracted was centrifuged (25 000 rpm, 4 °C, 25 min) to obtain a clear solution. The specific activity was determined using the standard beta-galactosidase activity assay and 10 ml of the centrifuged crude extract was applied to the equilibrated Sephadex® protein purification column connected to an ÄKTAprime system. After elution of unbound protein an imidazole gradient from 0 to 100 % was set up to detach the target protein from the column and fractions of 4 ml size were collected. All fractions within the target protein peak area were subjected to a Bradford assay as well as to a standard beta-galactosidase activity assay. That fraction with most specific activity was transferred to an Amicon tube with a molecular cutoff of 30.000 kDa and washed 3 times with 10 mM DTT in 50 mM NaPP buffer (4 000 rpm, 4 °C, 25 min). The remaining volume of approx. 750 µl was diluted with the same volume of enzyme storage buffer.

### 3.2.2.2. Standard activity beta-galactosidase assay

The chromogenic compound *ortho*-nitrophenyl-beta-D-galactoside (*o*NPG) is a common synthetic substrate for determination of beta-galactosidase activity. By cleavage of the synthetic substrate, *o*-nitrophenol (oNP) is released and colours the reaction mixture yellowish. Since the intensity of colourization is directly proportional to the amount of substrate converted, measuring absorbance at 420 nm is sufficient to determine enzyme activity.

One enzyme unit (U) is defined as the amount of enzyme releasing 1 µmol of oNP per minute under the specified assay conditions.

To start the reaction 10 µl of enzyme-dilution is added to 240 µl of a solution containing 22 mM *o*NPG dissolved in 50 mM sodium phosphate buffer (pH 6.5). The reaction mixture is incubated for exactly ten minutes at 30 °C and then stopped by adding 375 µl stop solution. The liquid is then transferred to a cuvette and the absorbance is measured at a wavelength of 420 nm. Multiplication of the absorbance with the dilution factor and the factor 2.1186 (derived from standard curve) yields the activity of the enzyme per ml.
All measurements are done in duplicate with less than 5 % deviation of the results.




*o*-nitrophenyl-β-D-galactopyranoside

### 3.2.2.3 Bradford assay

The colorimetric Bradford protein assay is used to estimate the protein concentration of a sample. Combined with the results from the standard beta-galactosidase assay, enzyme activity can be specified in U/mg.
The assay is based on the observation that the dye Coomassie Brilliant Blue G-250 changes the colour of an acidic solution from brown to blue upon binding a protein. As the change of colour is proportional to the amount of protein in the sample, measuring absorbance at 595 nm is sufficient for protein quantification by comparing the results with those from a BSA standard curve.

To test a sample simply 20 µl of the enzyme dilution are mixed with 1 ml of the Bradford reagent. The sample is incubated at room temperature for 10 -15 minutes minutes and then the absorbance is measured. The photometer automatically converts the absorbance

to a result in mg/ml by comparison to the standard curve. Multiplication of the result from the photometer with the dilution factor yields protein concentration per ml of the sample.

### 3.2.2.4. Preliminary experiments

As the duration of the experiments was shortened in comparison to the protocol hitherto used in our laboratory, the amount of enzyme added to the reaction mixture had to be adjusted accordingly. This was accomplished by preliminary experiments:

with 10 % of the acceptor sugar

4 times 30 mg of the respective acceptor sugar were dissolved in 300 µl reaction buffer. The reactions were started by adding 4 different amounts of enzyme in range between 1 µl of undiluted enzyme to 1 µl of a 1:20 dilution. One sample of 100 µl was taken immediately after the enzyme was added and the sample was heated for 5 minutes to deactivate the enzyme. Two more samples were withdrawn and inactivated after three and six hours. All samples were kept on ice until analysis with HPLC-analysis variant 1, which was usually carried out the same day over night. Analysis of the HPLC-chromatograms was followed by another preliminary experiment that was carried out identically, using 4 different enzyme concentrations based on the HPLC-results. The new samples were again subjected to HPLC-analysis variant 1 and the amount of enzyme to be used in the main experiment was chosen according to the second preliminary experiment.

with 20 % of the acceptor sugar

For preliminary experiments with 20 % of the acceptor sugar, 60 mg instead of 30 mg was dissolved in 300 µl reaction buffer. All other steps were carried out in the same way as for the experiments with 10 % acceptor sugar.

with no acceptor sugar (standard batch)

For the standard batch no acceptor sugar was dissolved in reaction buffers. Apart from this all other steps were done likewise. The standard batch was used as a reference reaction in all experiments.

### 3.2.2.5. Transglycosylation experiments

**with 10 % of the acceptor sugar**

150 mg of the respective acceptor sugar was dissolved in 1.5 ml reaction buffer. A volume increase of the solution of approx. 5 % could be observed. The reaction was started by adding the amount of enzyme, which was determined from the preliminary experiments (8.7 U/ml)

The reaction was carried out in a thermal mixer for 6 hours main reaction time (an additional sample was taken after 24 hours) at 37°C and an agitation speed of 800 rpm. An overall number of 15 samples (100 µl each) was withdrawn after 0 h, 20', 40', 60', 90', 120' and 150' as well as after 3 h, 3.5 h, 4 h, 4.5 h, 5 h, 5.5 h, 6 h and 24 h. The enzyme was deactivated by storing the sample at 99.9 °C for 5 minutes.

The samples were used for the following analysis:
10 µl for determination of enzyme activity on oNPG
15 µl for HPLC-analysis variant 1
30 µl for HPLC-analysis variant 2 and TLC
5 µl for CE-analysis
10 µl for HPLC-analysis variant 3
10 µl for enzymatic determination of the glucose content
**Σ** 80 µl

The remaining 20 µl were stored at -26 °C for eventualities.

**with 20 % of acceptor sugar**

300 mg of the respective acceptor sugar was dissolved in 1.5 ml reaction buffer. A volume increase of approximately 10 % was apparent. The reaction was started by adding the same amount of enzyme (8.7 U/ml) as for the main experiments with 10 % acceptor sugar.

The rest of the experiment was identical to the main experiment with 10 % acceptor sugar.

**with 10 % sialic acid**

150 mg sialic acid was dissolved in 1.5 ml reaction buffer. The resulting pH of 1.9 was adjusted to 6.7 by addition of 131 µl 4 M KOH. The rest of the experiment was carried out identically to the other experiments with 10 % acceptor sugar.

### 3.2.2.6. HPLC analysis

HPLC analysis was used in 3 different variants for GOS analysis and observation of the conversion progress. Variants 1 and 2 were done with slight modifications according to Nguyen et al. (2006)**,** variant 3 has not yet been described in literature with regard to GOS analysis.
The software used for all HPLC analysis was Chromeleon version 6.5 (Dionex Corp., Sunnyvale, CA).

**Variants 1 and 2**

HPAEC-PAD analyses were carried out on a Dionex DX-500 system consisting of a GP50 gradient pump, an ED 40 electrochemical detector with a gold working electrode and an Ag/AgCl reference electrode. All eluents were degassed with helium for 20 minutes in order to remove carbon dioxide. Separations were performed at 25 °C on a CarboPac PA-1 column (4 mm; 250 mm) connected to a CarboPac PA-1 guard column (Dionex). Samples (50 µl) were injected via a Spark basic marathon autosampler and separations were performed at a flow rate of 1 mL/min. Detection time and voltage parameters were set to waveform "A" according to Dionex Technical Note 21.

Variant 1 was used for quantification of monosaccharides and lactose. Samples diluted 1:1800 were eluted for 50 minutes in an isocratic eluent of 15 % eluent A and 85 % eluent C. Then the column was washed for 10 minutes with eluent B and re-equilibrated for 15 minutes with 15 % eluent A and 85 % eluent C. The overall analysis time per sample was therefore 75 minutes. A standard curve derived from 5 samples containing the particular sugars in concentrations between 0.0025 g/l and 0.1 g/l was used for quantification .

Variant 2 was used to compare the complex mixture of oligosaccharides formed during the reaction. Samples diluted between 1:20 to 1:30 were eluted using the following

gradient:

For the first 20 minutes 100 % eluent A, then a gradient from 0-100 % eluent D until 70 minutes, then the column was washed for 10 minutes with eluent B and afterwards re-equilibrated for 15 minutes with 100% eluent A. The overall analysis time per sample was therefore 95 minutes.

**Variant 3**

Variant 3 was used for quantification of monosaccharides and identification of products derived from N-acetyl-group containing acceptor sugars. The UV-detector to monitor N-acetyl-groups was therefore set to a wavelength of 210 nm. The analyses were carried out on a Summit system consisting of a P 680 HPLC pump, an ADI-100 automated sample injector, a thermostatic column compartment TCC-100, a Shodex RI 101 RI-detector and an UVD 170 UV-detector.

Separations were performed at 30 °C on a Supelcogel H column (4.6 mm, 250 mm) connected to a Supelcogel H guard-column (4.6 mm, 50 mm).

The samples were diluted to 1:20 for RI-detection and between 1:200 to 1:400 for UV-detection and eluted with 0.1 % phosphoric acid and a flow rate of 0.17 ml/min.

For quantification, standard curves derived from 10 samples (5 standards for RI-detection and 5 standards for UV-detection) containing the particular sugars in concentrations between 10 g/l to 0.05 g/l were used. The analysis time per sample was set to 35 minutes for all samples except those from batches containing N-acetyl-D-galactosamine where the analysis time had to be increased to 40 minutes.

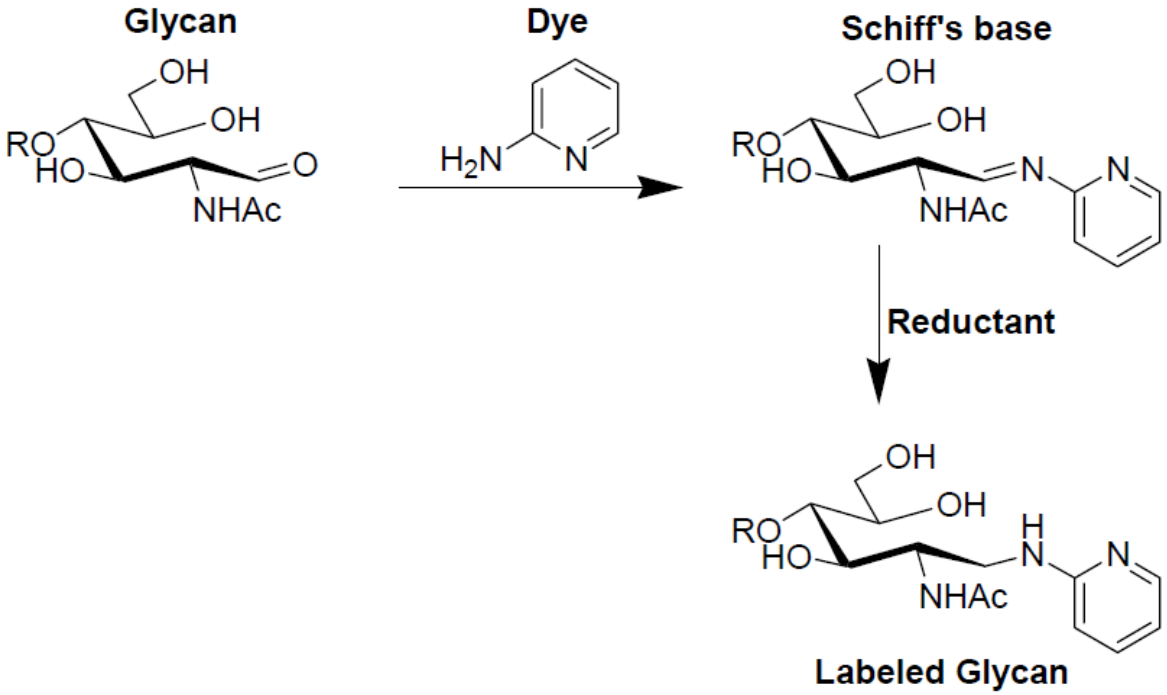
## 3.2.2.7. Thin Layer Chromatography

TLC was carried out at room temperature on HPTLC LiChrospher® Silica gel 60 $F_{254}$s plates. 0.5 µl of 1:10 diluted sample was applied using a micro-pipette and eluted twice in ascending mode with TLC buffer. The dry plate was then sprayed with TLC staining agent B for samples containing compounds with N-acetyl-groups or with TLC staining agent A for samples without N-acetyl-groups. If TLC staining agent B was used, the plates were baked at 180 °C for approximately 7 minutes to visualize spots. For TLC staining agent A the plates were heated to 110 °C and sprayed and heated again if necessary.

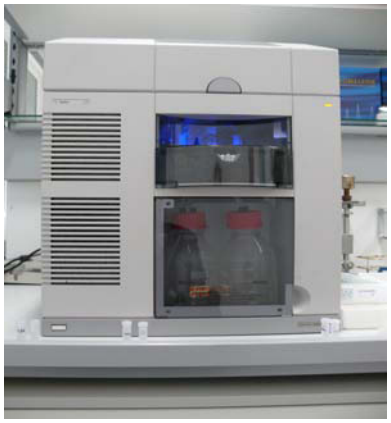
### 3.2.2.8. CE sample preparation and analysis

CE sample preparation and analysis were done according to a protocol in our knowledge data base, which is a slight modification of a derivatization method described by Splechtna et al, 2006.

The derivatization method is a reductive amination that yields chromophoric derivatives, which can be monitored in the UV light of the capillary electrophoresis apparatus. The derivatization is depicted in the following scheme:




5 µl of sample was dried under vacuum at 60 °C using a SPD SpeedVac system (Thermo Savant). The sample was then incubated on a thermoblock at 90 °C for 15 minutes with 20 µl of a solution containing 1 g of 2-aminopyridine in 470 µl of acetic acid and 600 µl methanol. After incubation time, the sample was placed under vacuum in the SPD SpeedVac system for 2h, evaporating the excess of the reagents. 25 µl of a solution containing 59 mg/ml (in 30 % acetic acid) $NaCNBH_3$ was added to the sample and the mixture was incubated for 30 minutes at 90 °C. After incubation, the sample was dried under vacuum in a SPD SpeedVac system at 60 °C for 2 h, then re-suspended in 200µl UHQ and kept on -26 °C until CE analysis.



For CE-analysis, a capillary electrophoresis system with an UV-DAD detector (Agilent Technologies, Palo Alto, USA) together with a fused silica capillary column (internal diameter of 25 µm) equipped with a bubble cell detection window (bubble factor of 5) was used. The capillary had a total length of 64.5 cm and an effective length of 56 cm. The running buffers were either 50 mM $H_3PO_4$ set to a pH of 2.5 or 50 mM $H_3PO_3$ adjusted to pH 2.5. As washing buffers either 100 mM $H_3PO_4$ or 100 mM $H_3PO_3$ were used. The samples were injected into the capillary at the anodic end by a positive pressure of 50 mbar for 5 seconds. The positive polarity mode and an operating temperature of 30 °C were used. The current was set to 15 µA (13 µA in experiments with $H_3PO_4$) and was kept constant during the run. The resulting voltage was approximately 26 kV. Several detection wavelengths were set between 210 and 240 nm, each with a bandwidth of 10 nm.

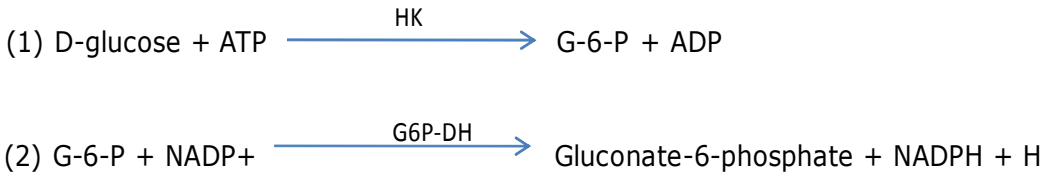
Running and washing buffers were degassed in an ultrasonic bath prior use.

At the end of each CE-analysis day the capillary was washed for 15 minutes with UHQ and stored in empty vials over night.

### 3.2.2.9. Enzymatic analysis of D-glucose

An enzymatic glucose assay from Megazyme™ (HK/G6P-DH format) was used to determine D-glucose. The assay is based on the ability of the enzyme hexokinase (HK) to phosphorylate D-glucose, yielding glucose-6-phosphate (G-6-P). In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH) G-6-P is oxidized to gluconate-6-phosphate with the formation of NADPH out of NADP+. As the amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose, measurement of the increase in absorbance at 340 nm is sufficient for D-glucose determination.

The principle reaction mechanism of this assay can be summarized the following:



Procedure (in order to be more cost efficient all amounts given the manual were divided by four):
500 µl UHQ were mixed with
25 µl sample (dilution factor 1:300), for blank UHQ instead of sample
25 µl imidazole buffer (2 M imidazole, 100 mM $MgCl_2$ ) and
25 µl $NADP^+$/ATP-solution (0.3125 mg $NADP^+$ and 0.916 mg ATP) in a standard cuvette

The absorbance (A1) was measured and the reaction was started by the addition of
5 µl HK/G6P-DH suspension (2.1 U HK and 1.1 U G6P-DH)

The mixture was stirred with a plastic spatula and the absorbance (A2) was measured again after approx. 5 minutes.

The concentration of D-glucose in g/l can then be determined by using the following formula: *(A2 – A1) * 0.6634 * dilution factor*
All measurements were done in duplicate.

### 3.2.2.10. Methods for calculating the degree of lactose conversion, acceptor usage and GOS-production

The calculation is based on the integrals of HPLC analysis from variant 1 and 3.

Degree of lactose conversion: (1 - *lactose at* $t_0$ / *lactose at* $t_x$) * 100

Degree of acceptor usage: (*acceptor at* $t_0$ – *acceptor at* $t_x$ ) / *acceptor at* $t_0$ * 100

Degree of GOS production: (*lactose at* $t_0$ + *acceptor at* $t_0$ ) – (*lactose at* $t_x$ + *galactose at* $t_x$ + *glucose at* $t_x$ + *acceptor at* $t_x$ )

### 3.2.2.11. Method to determine the effect of enzyme concentration on transglycosylation products

As some publications (e.g. Ji et al. 2005) report an effect of enzyme concentration on the pattern of transglycosylation products, it was studied whether such an effect is observable in this project as well.
Additional batches with 10 % acceptor-sugars with only 5 U/ml enzyme were prepared the same way as in the main experiments with 10 % acceptor-sugars and the samples

were analysed with HPLC variant 1 and 2. The results for the degree of lactose conversion from HPLC analysis variant 1 were compared with those from the main experiment with 10 % acceptor sugars. Samples with similar lactose conversion levels were identified and their HPLC-spectra from variant 2 were compared.

### 3.2.2.12. Method to determine the effect of enzyme deactivation methods on transglycosylation products

As the combination of heat, reducing sugars and amino acid might lead to unwanted side reactions that distort an accurate evaluation of the reaction products, the effect of two different enzyme deactivation methods was studied:
In one batch with 15 % (w/v) L-Fucose as added acceptor-sugar samples of 160 µl were taken out at regular intervals. Half the sample was kept at 99.9 °C for 5 minutes, the other half was deactivated by adding 110 µl stop solution and then approx. 15 seconds later neutralized by adding 10 µl 2.5 M citric acid to a pH of 6.65.
The samples were afterwards diluted to 1:1800 and analysed with HPLC-variant 1 and 2. Direct comparison of congruent samples did not yield any differences in peak pattern or relative peak area-ratios.

### 3.2.2.13. Methods to determine the effects of various alcohols on enzyme activity and stability in and without presence of MgCl2

**Enzyme activity**

The enzyme activity was tested in solutions with varying concentrations of methanol, ethanol, 1-propanol and 2-propanol in comparison to the standard enzyme dilution buffer:
A solution containing 50 mM NaPP in 50 % (v/v) of UHQ and 50 % (v/v) of the particular alcohol was prepared and the pH of the solution was set to 6.5.

Nine Eppendorf tubes containing 6.6 mg *o*NPG each were prepared for every alcohol and filled according to the following table:

| Percentage of alcohol | Volume of enzyme dilution buffer | Volume of 50% (v/v) alcohol |
|---|---|---|
| 0% | 1 ml | |
| 5% | 900 µl | 100 µl |
| 10% | 800 µl | 200 µl |
| 15% | 700 µl | 300 µl |
| 20% | 600 µl | 400 µl |
| 25% | 500 µl | 500 µl |
| 30% | 400 µl | 600 µl |
| 40% | 200 µl | 800 µl |
| 50% | | 1 ml |

A volume of 240 µl of each liquid was transferred to a new vial and 10 µl enzyme-dilution (1:25 000) was added. The rest of the experiment was identical to the standard beta-galactosidase activity assay. The experiment was carried out in duplicate.

**Stability test without $MgCl_2$**

As the best activity results were obtained with 20 % (v/v) of methanol and 15 % (v/v) of ethanol, 1-propanol and 2-propanol the stability of the enzyme was tested at these concentrations in comparison to the standard enzyme dilution buffer:

10 µl of enzyme dilution (1:500) was added to 490 µl of the particular alcohol and the mixture was kept at 37°C and 400 rpm.

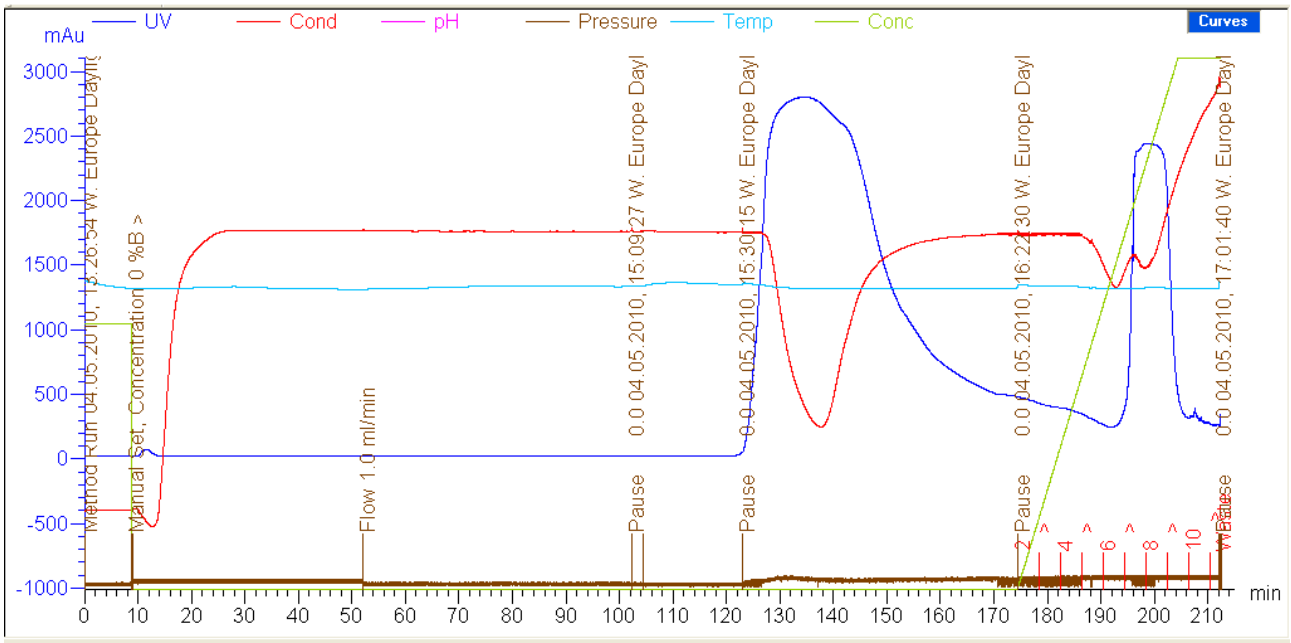
10 µl of the mixture was subjected to a standard beta-galactosidase activity assay in hourly intervals for an overall time of 6 hours.

**Stability test with $MgCl_2$**

The liquids mentioned above were prepared with 10 mM $MgCl_2$ before the enzyme-dilution was added. Precipitation after pH-adjustment was observable in all samples, except for the standard enzyme dilution buffer. The rest of the experiment was carried out identically as the experiment without $MgCl_2$.

# 4. Results and discussion

## 4.1. Protein purification




The blue line represents the UV-absorbency and shows two peaks. The first peak represents all protein that did not bind to the column (flow-through), the second peak shows the target protein. The ascending green line displays an imidazole-gradient that is used to detach the target protein from the column. The red line represents conductivity and is only of minor importance in this case.

| Purification step | Total activity (U/ml) | Total protein (mg/ml) | Specific activity (U/mg) |
|---|---|---|---|
| Centrifuged crude extract | 1671 | 45.95 | 36.37 |
| His-tag column | 8898 | 33.04 | 269.61 |
| Final enzyme solution | 12399 | 42.2 | 293.83 |

## 4.2. Novel transglycosylation products

Experiments with three of the four used acceptor sugars yielded a total of at least five new products as observable by HPLC analysis variants 1 and 3. Experiments with sialic acid did not yield any new peaks or a decrease in the peak area of sialic acid.

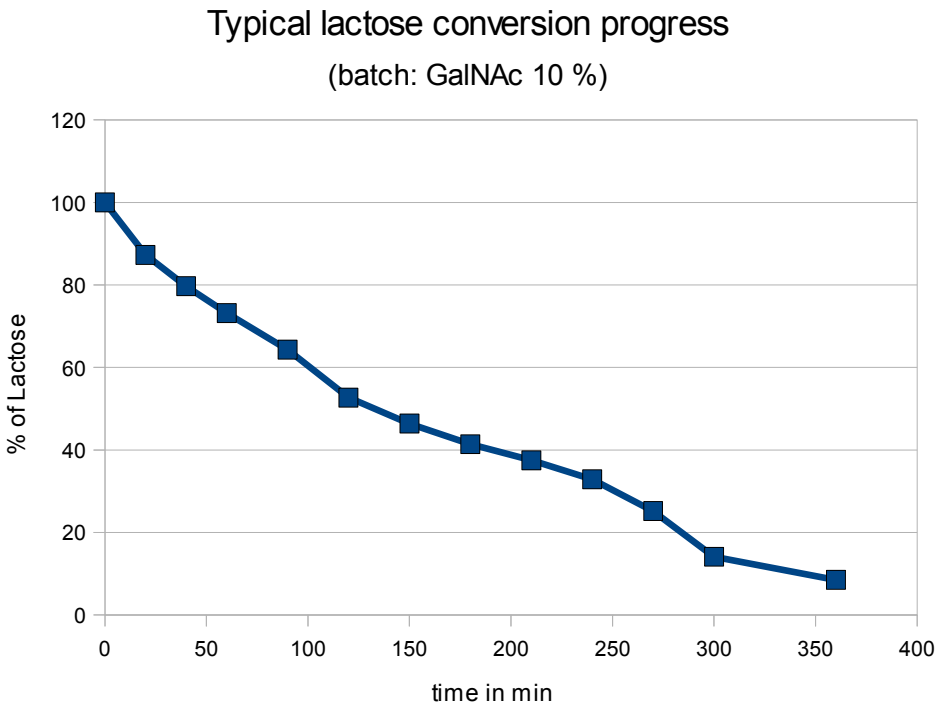
Chromatograms in HPLC variant 1 show a decrease in the peak area for the acceptor sugar and one new peak in experiments with L-Fucose, N-Acetyl-D-galactosamine and N-Acetyl-D-glucosamine (a representative sample of spectra is shown in the Appendix chapter 7*.4. Some HPLC- and CE-spectra*).

The UV-detector in HPLC variant 3 can be used to selectively monitor oligosaccharides with N-Acetyl-groups. Thereby two peaks representing at least two different products were found in experiments with N-Acetyl-D-galactosamine and N-Acetyl-D-glucosamine. The retention time of the new peaks corresponds with the retention time for di- and trisaccharides and the relative peak area of the peaks suggest a strong prevalence of new dimeric products over new trimeric products.

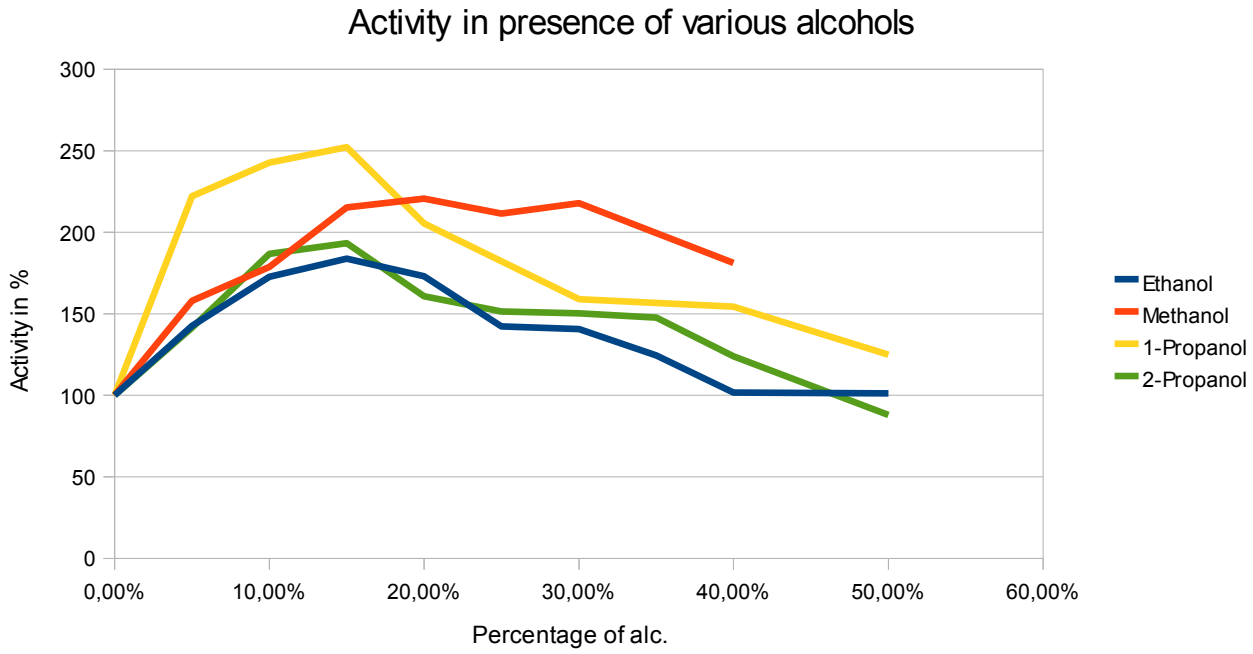
L-Fucose does not carry an N-Acetyl-group so new products formed with this acceptor can not be monitored with an UV-lamp.

Without having standard-compounds for quantification or the possibility to carry out structure determination more detailed information about the new products can not be provided. However, as the used enzyme shows a very distinct preference to create β(1,6) and β(1,3) linked products, it is likely that the majority of new products has this linkage as well.

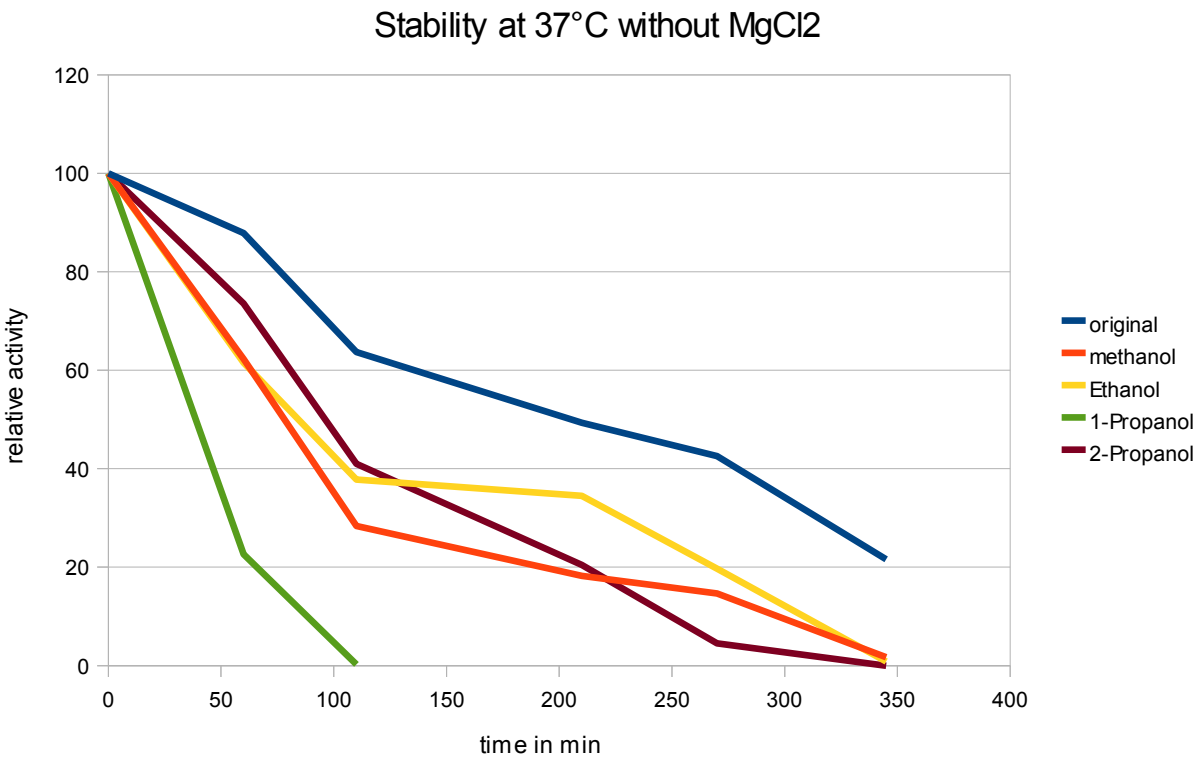
### 4.3. Graphical visualisations



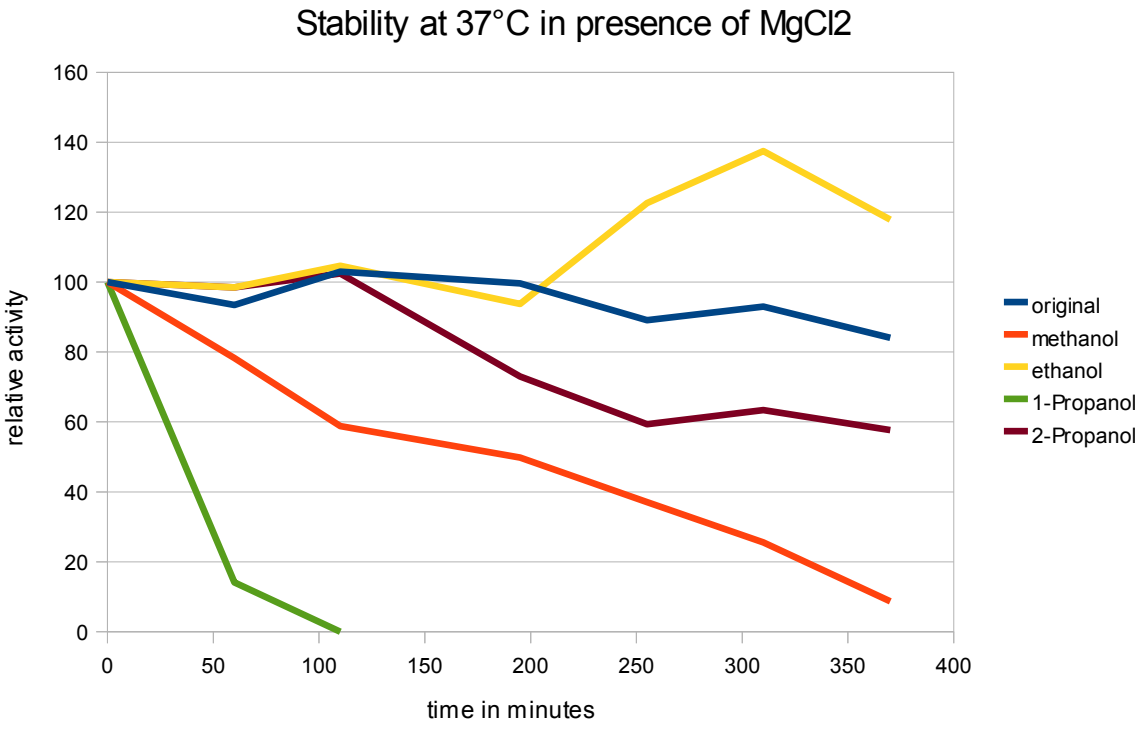

In preliminary experiments the appropriate amount of enzyme was determined to convert approx. 90 % lactose in 6 hours.



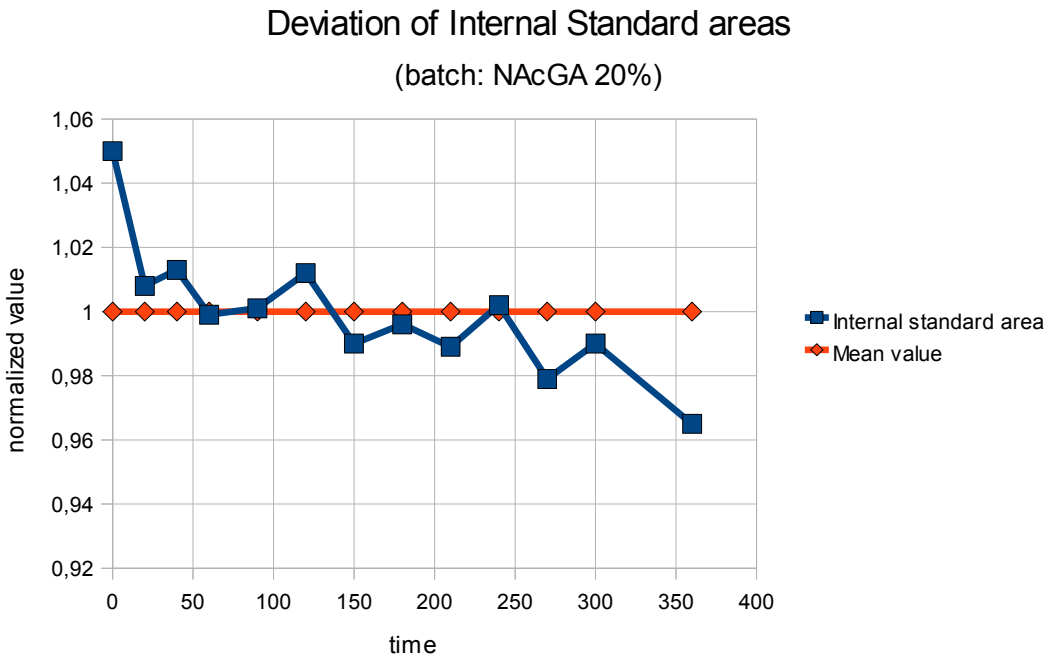

Short chain alcohols considerably increase the activity of the enzyme in a broad range of concentrations as determined with a modified standard activity beta-galactosidase assay.



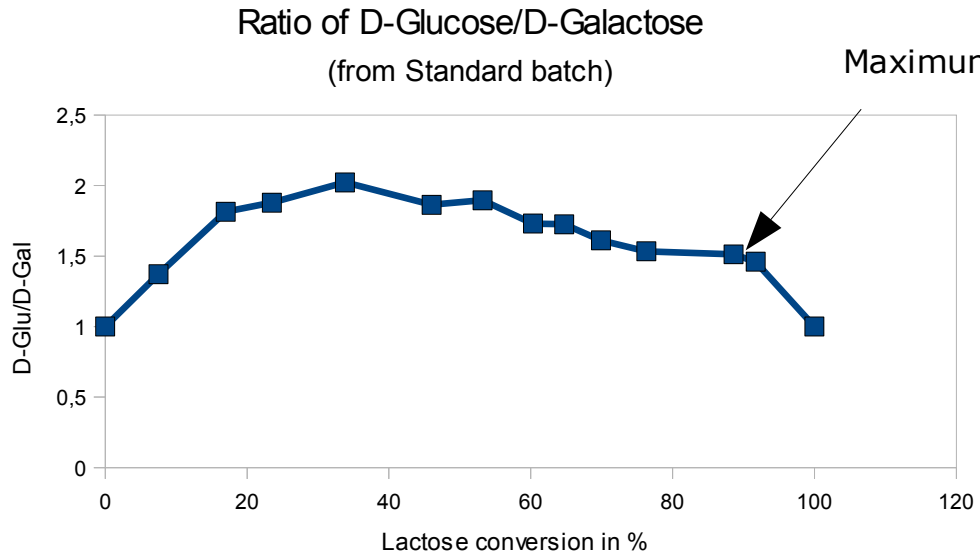

The stability of the enzyme at 37 °C without presence of $MgCl_2$ is not very pronounced.




In presence of $MgCl_2$ the stability of the enzyme is significantly improved for some alcohols. The activity increase in ethanol is probably due to evaporation of the solvent.




The internal standard-technique with L-fucose was applied in this project, showing deviations from the mean value of all internal standard areas between 1-5 % in all batches.




The D-Glucose/D-Galactose ratio is usually a powerful indicator to determine maximum GOS levels. The surprising fact that maximum GOS levels do not correspond with the highest D-Glucose/D-Galactose ratio might indicate a general weakness of the data, but significance in regard to the acceptor usage is nevertheless given.

## 4.4. Tabulated results

### Standard batch

| time | Is (pA1) | Mf (pA1) | Lactose (g/l) + Allolactose (g/l) (see discussion) | | | Glucose (g/l) (see discussion) | | | Galactose (g/l) (see discussion) | | | L. C. (%) | TOS (g/l) | TOS (% original Lactose) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Enzymatic | pA1 original | pA1 modified | Gel H | | | |
| **0'** | 10950 | 1001 | 197.4 | 197.5 | 198.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | 0 | 0 |
| **20'** | 10688 | 1025 | 178.4 | 182.8 | nd | 7.6 | 7.8 | 9.3 | 5.1 | 5.3 | 5.7 | 7.5 | 2.5 | 1.3 |
| **40'** | 10.683 | 1.025 | 159.9 | 163.9 | nd | 13.9 | 14.3 | 15.2 | 8.2 | 8.4 | 7.9 | 17.0 | 11.8 | 6.0 |
| **60'** | 10.731 | 1.021 | 148.0 | 151.1 | nd | 19.1 | 19.5 | 22.1 | 10.8 | 11.0 | 10.4 | 23.5 | 16.8 | 8.4 |
| **90'** | 10.689 | 1.025 | 127.7 | 130.8 | nd | 26.4 | 27.1 | 28.1 | 14.7 | 15.1 | 13.4 | 33.8 | 25.4 | 12.8 |
| **2h** | 10.837 | 1.011 | 105.5 | 106.7 | nd | 31.9 | 32.2 | 36.2 | 17.9 | 18.1 | 17.3 | 46.0 | 41.3 | 20.8 |
| **2.5h** | 10.622 | 1.031 | 89.8 | 92.5 | nd | 38.0 | 39.2 | 41.7 | 21.3 | 22.0 | 20.7 | 53.2 | 44.7 | 22.5 |
| **3h** | 10.660 | 1.027 | 76.4 | 78.5 | nd | 41.5 | 42.7 | 45.8 | 24.7 | 25.4 | 24.7 | 60.3 | 51.8 | 26.1 |
| **3.5h** | 10.916 | 1.004 | 69.4 | 69.7 | nd | 47.9 | 48.1 | 49.5 | 28.7 | 28.8 | 27.9 | 64.7 | 51.8 | 26.1 |
| **4h** | 11.226 | 0.976 | 61.0 | 59.5 | nd | 54.2 | 52.9 | 58.1 | 32.5 | 31.7 | 32.8 | 69.9 | 54.3 | 27.4 |
| **4.5h** | 11.537 | 0.947 | 49.4 | 46.8 | nd | 59.0 | 55.9 | 61.3 | 36.2 | 34.3 | 36.4 | 76.3 | 61.4 | 31.0 |
| **5h** | 11.460 | 0.954 | 23.6 | 22.5 | nd | 59.4 | 56.6 | 63.2 | 38.6 | 36.8 | 37.4 | 88.6 | 82.4 | 41.5 |
| **6h** | 11.457 | 0.955 | 17.1 | 16.4 | nd | 68.0 | 64.9 | 69.5 | 46.4 | 44.3 | 44.5 | 91.7 | 72.8 | 36.7 |
| mean | 10.96 | | | | | | | | | | | | | |

nd: not determined TOS: total galacto-oligosaccharides

L.C.: lactose conversion Mf: multiplication factor Is: Internal standard

Lactose without allolactose

# N-Ac-GA 10 %

| time | Is (pA1) | Mf (pA1) | Lactose (g/l) + Allolactose (g/l) + Gal-N-Ac-GA-product (see discussion) | | | Glucose (g/l) (see discussion) | | | Galactose (g/l) (see discussion) | | | Acceptor sugar (g/l) | | | L. C. (%) | **acceptor usage (%) from Gel H** | TOS (g/l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Enzymatic | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Gel H | | | |
| **0'** | 10.735 | 1.006 | 190.1 | 191.3 | 191.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 87.2 | 87.8 | 92.1 | 0.0 | **0.0** | 0 |
| **20'** | 10.978 | 0.984 | 175.3 | 172.5 | nd | 12.0 | 11.8 | 15.1 | 5.0 | 4.9 | 3.5 | 87.3 | 85.9 | 89.3 | 9.8 | **3.1** | 4.6 |
| **40'** | 10.621 | 1.017 | 154.8 | 157.4 | nd | 17.2 | 17.4 | 18.3 | 8.4 | 8.6 | 7.3 | 84.4 | 85.9 | 88.5 | 17.7 | **3.9** | 11.1 |
| **60'** | 11.028 | 0.979 | 141.1 | 138.2 | nd | 22.2 | 21.8 | 24.7 | 11.4 | 11.2 | 9.1 | 82.5 | 80.8 | 87.9 | 27.8 | **4.6** | 24.1 |
| **90'** | 10.742 | 1.006 | 131.4 | 132.2 | nd | 29.3 | 29.5 | 30.1 | 15.5 | 15.6 | 13.1 | 86.5 | 87.0 | 87.5 | 30.9 | **5.0** | 18.3 |
| **2h** | 10.917 | 0.99 | 107.6 | 106.5 | nd | 35.1 | 34.7 | 37.4 | 19.4 | 19.2 | 17.0 | 85.4 | 84.6 | 86.1 | 44.3 | **6.5** | 36.6 |
| **2.5h** | 10.673 | 1.012 | 87.8 | 88.9 | nd | 39.6 | 40.1 | 38.5 | 22.8 | 23.1 | 21.4 | 85.1 | 86.1 | 86.1 | 53.5 | **6.5** | 44.9 |
| **3h** | 10.881 | 0.993 | 80.7 | 80.1 | nd | 44.4 | 44.1 | 46.5 | 26.6 | 26.4 | 22.9 | 83.8 | 83.2 | 82.7 | 58.1 | **10.2** | 49.8 |
| **3.5h** | 10.851 | 0.996 | 71.3 | 71.0 | nd | 49.1 | 48.9 | 51.3 | 31.1 | 31.0 | 27.2 | 85.1 | 84.7 | 89.1 | 62.9 | **3.3** | 43.2 |
| **4h** | 10.844 | 0.996 | 59.0 | 58.8 | nd | 52.3 | 52.1 | 56.4 | 34.4 | 34.3 | 29.9 | 85.7 | 85.4 | 89.1 | 69.3 | **3.3** | 48.8 |
| **4.5h** | 10.492 | 1.029 | 41.5 | 42.7 | nd | 55.8 | 57.4 | 58.9 | 38.3 | 39.4 | 33.3 | 86.4 | 88.9 | 89.4 | 77.7 | **2.9** | 54.2 |
| **5h** | 10.947 | 0.987 | 35.4 | 35.0 | nd | 61.3 | 60.5 | 65.2 | 44.3 | 43.7 | 37.4 | 91.4 | 90.2 | 90.3 | 81.7 | **2.0** | 53.6 |
| **6h** | 10.743 | 1.006 | 11.4 | 11.5 | nd | 68.6 | 69.0 | 70.2 | 52.7 | 53.0 | 43.9 | 95.7 | 96.2 | 90.1 | 94.0 | **2.2** | 59.5 |
| mean | 10.804 | | | | | | | | | | | | | | | | |

nd: not determined

TOS: total galacto-oligosaccharides

Is: Internal standard

L.C.: lactose conversion

Mf: multiplication factor

Lactose and allolactose without Gal-NAcGA product

Lactose without allolactose and Gal-NAcGA product

# N-Ac-GA 20 %

| time | Is (pA1) | Mf (pA1) | Lactose (g/l) + Allolactose (g/l) + Gal-N-Ac-GA-product (see discussion) | | | Glucose (g/l) (see discussion) | | | Galactose (g/l) (see discussion) | | | Acceptor sugar (g/l) | | | L. C. (%) | **acceptor usage (%) from Gel H** | TOS (g/l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Enzymatic | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Gel H | | | |
| **0'** | 12.777 | 1.051 | 168.6 | 177.2 | 182.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 196.9 | 206.9 | 181.3 | 0.0 | **0.0** | 0.0 |
| **20'** | 13.360 | 1.008 | 151.7 | 152.8 | nd | 6.2 | 6.2 | 8.1 | 4.1 | 4.1 | 3.9 | 193.9 | 195.4 | 177.2 | 13.8 | **2.3** | 18.4 |
| **40'** | 13.204 | 1.019 | 131.6 | 134.1 | nd | 11.5 | 11.7 | 13.4 | 6.6 | 6.7 | 6.7 | 189.6 | 193.3 | 170.4 | 24.3 | **6.0** | 35.5 |
| **60'** | 13.476 | 0.999 | 123.6 | 123.5 | nd | 18.1 | 18.1 | 21.2 | 9.7 | 9.6 | 8.3 | 197.7 | 197.5 | 167.6 | 30.3 | **7.5** | 41.1 |
| **90'** | 13.442 | 1.001 | 110.9 | 111.0 | nd | 24.3 | 24.4 | 25.1 | 12.5 | 12.5 | 10.6 | 190.1 | 190.4 | 163.4 | 37.3 | **9.9** | 49.1 |
| **2h** | 13.299 | 1.012 | 93.9 | 95.0 | nd | 29.9 | 30.3 | 35.6 | 15.2 | 15.4 | 12.8 | 183.6 | 185.8 | 155.0 | 46.4 | **14.5** | 65.4 |
| **2.5h** | 13.600 | 0.990 | 84.7 | 83.9 | nd | 37.7 | 37.3 | 38.4 | 19.3 | 19.1 | 16.7 | 191.0 | 189.0 | 165.9 | 52.7 | **8.5** | 54.8 |
| **3h** | 13.520 | 0.996 | 58.4 | 58.1 | nd | 46.0 | 45.8 | 55.0 | 24.1 | 24.0 | 20.3 | 199.5 | 198.7 | 173.8 | 67.2 | **4.1** | 60.5 |
| **3.5h** | 13.610 | 0.989 | 49.4 | 48.8 | nd | 52.1 | 51.6 | 48.3 | 28.2 | 27.9 | 24.3 | 205.0 | 202.7 | 155.8 | 72.4 | **14.1** | 78.1 |
| **4h** | 13.439 | 1.002 | 37.4 | 37.5 | nd | 51.6 | 51.7 | 58.7 | 28.7 | 28.8 | 26.4 | 191.4 | 191.7 | 156.9 | 78.9 | **13.5** | 86.1 |
| **4.5h** | 13.746 | 0.979 | 30.9 | 30.3 | nd | 57.4 | 56.2 | 54.3 | 33.1 | 32.4 | 29.2 | 199.0 | 194.8 | 160.9 | 82.9 | **11.2** | 81.9 |
| **5h** | 13.601 | 0.990 | 24.8 | 24.5 | nd | 61.3 | 60.7 | 65.0 | 36.5 | 36.2 | 31.0 | 199.0 | 196.9 | 162.3 | 86.2 | **10.5** | 80.0 |
| **6h** | 13.928 | 0.965 | 21.0 | 20.3 | nd | 65.6 | 63.3 | 78.5 | 40.3 | 38.9 | 34.7 | 200.3 | 193.3 | 168.9 | 88.5 | **6.8** | 71.3 |
| mean | 13.46 | | | | | | | | | | | | | | | | |

nd: not determined TOS: total galacto-oligosaccharides Is: Internal standard

L.C.: lactose conversion Mf: multiplication factor

Lactose without allolactose and Gal-NAcGA product

# L-Fucose 10 %

| time | Is (pA1) | Mf (pA1) | Lactose (g/l) + Allolactose (g/l) (see discussion) | | | Glucose (g/l) (see discussion) | | | Galactose (g/l) (see discussion) | | | Acceptor sugar (g/l) (see discussion) | | | L. C. (%) | **acceptor usage (%)** | TOS (g/l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Enzymatic | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Gel H | | | |
| **0'** | 47.57 | 0.939 | 188.7 | 177.3 | 191.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 89.5 | 84.1 | 91.7 | 0.0 | **0.0** | 0 |
| **20'** | 46.2 | 0.97 | 168.1 | 163.0 | nd | 7.5 | 7.3 | 7.9 | 3.8 | 3.7 | 3.5 | 94.2 | 91.3 | 87.6 | 8.0 | **4.4** | 21.9 |
| **40'** | 44.97 | 0.997 | 148.7 | 148.3 | nd | 15.7 | 15.6 | 18.3 | 7.1 | 7.1 | 6.2 | 96.5 | 96.3 | 81.6 | 16.3 | **11.0** | 30.9 |
| **60'** | 44.77 | 1.002 | 127.4 | 127.7 | nd | 18.9 | 18.9 | 24.9 | 8.9 | 8.9 | 8.8 | 93.1 | 93.3 | 82.1 | 28.0 | **10.5** | 45.9 |
| **90'** | 42.59 | 1.05 | 108.2 | 113.6 | nd | 25.6 | 26.9 | 27.1 | 11.9 | 12.5 | 13.2 | 89.5 | 94.0 | 81.3 | 35.9 | **11.3** | 49.2 |
| **2h** | 44.91 | 0.999 | 87.2 | 87.1 | nd | 31.8 | 31.8 | 36.5 | 14.9 | 14.9 | 15.2 | 88.7 | 88.6 | 78.5 | 50.9 | **14.4** | 71.2 |
| **2.5h** | 44.27 | 1.013 | 80.4 | 81.4 | nd | 40.6 | 41.1 | 42.5 | 19.3 | 19.6 | 18.4 | 93.2 | 94.4 | 79.1 | 54.1 | **13.8** | 62.4 |
| **3h** | 44.45 | 1.009 | 62.1 | 62.7 | nd | 41.0 | 41.4 | 54.9 | 20.1 | 20.3 | 20.5 | 86.7 | 87.5 | 78.6 | 64.6 | **14.3** | 80.6 |
| **3.5h** | 44.57 | 1.006 | 55.1 | 55.4 | nd | 46.8 | 47.1 | 48.3 | 23.5 | 23.7 | 24.7 | 88.7 | 89.2 | 80.2 | 68.7 | **12.5** | 77.1 |
| **4h** | 45.13 | 0.994 | 49.1 | 48.8 | nd | 52.8 | 52.5 | 53.6 | 27.5 | 27.3 | 28.1 | 90.8 | 90.2 | 79.4 | 72.5 | **13.4** | 75.5 |
| **4.5h** | 44.82 | 1.001 | 46.2 | 46.3 | nd | 59.0 | 59.1 | 62.1 | 31.8 | 31.8 | 31.8 | 93.2 | 93.3 | 81.8 | 73.9 | **10.8** | 64.6 |
| **5h** | 43.84 | 1.023 | 29.1 | 29.8 | nd | 61.3 | 62.7 | 63.5 | 34.1 | 34.8 | 35.0 | 93.2 | 95.3 | 81.8 | 83.2 | **10.8** | 74.4 |
| **6h** | 44.95 | 0.998 | 21.7 | 21.6 | nd | 69.3 | 69.2 | 73.5 | 40.9 | 40.8 | 43.5 | 94.6 | 94.4 | 87.9 | 87.8 | **4.1** | 64.0 |
| mean | 44.85 | | | | | | | | | | | | | | | | |

nd: not determined

TOS: total galacto-oligosaccharides

L.C.: lactose conversion

Mf: multiplication factor

Is: Internal standard

Lactose without allolactose

# L-Fucose 20 %

| time | Is (pA1) | Mf (pA1) | Lactose (g/l) + Allolactose (g/l) (see discussion) | | | Glucose (g/l) (see discussion) | | | Galactose (g/l) (see discussion) | | | Acceptor sugar (g/l) (see discussion) | | | L. C. (%) | **acceptor usage (%) from Gel H** | TOS (g/l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Enzymatic | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Gel H | | | |
| **0'** | 39.53 | 1.074 | 177.3 | 190.4 | 179.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | 176.2 | 0.0 | **0.0** | 0 |
| **20'** | 42.84 | 0.996 | 165.8 | 165.2 | nd | 7.7 | 7.7 | 6.3 | 3.6 | 3.6 | 3.7 | | | 163.8 | 13.2 | **7.0** | 15.5 |
| **40'** | 43.13 | 0.99 | 148.8 | 147.3 | nd | 12.7 | 12.6 | 11.2 | 6.0 | 5.9 | 7.3 | | | 156.9 | 22.6 | **10.9** | 33.0 |
| **60'** | 41.8 | 1.021 | 142.7 | 145.6 | nd | 18.9 | 19.3 | 14.0 | 8.7 | 8.8 | 9.0 | | | 162.9 | 23.5 | **7.5** | 19.1 |
| **90'** | 42.89 | 0.995 | 119.5 | 118.9 | nd | 27.7 | 27.5 | 28.9 | 12.1 | 12.0 | 10.5 | | | 149.9 | 37.5 | **14.9** | 47.4 |
| **2h** | 42.63 | 1.001 | 107.6 | 107.7 | nd | 34.9 | 34.9 | 32.1 | 15.3 | 15.3 | 12.5 | | | 150.0 | 43.4 | **14.9** | 47.8 |
| **2.5h** | 43.55 | 0.98 | 97.4 | 95.4 | nd | 41.6 | 40.8 | 42.5 | 18.4 | 18.1 | 14.5 | This peak is overloaded in a dilution of 1:1800 | | 153.1 | 49.9 | **13.1** | 48.4 |
| **3h** | 42.4 | 1.007 | 80.2 | 80.8 | nd | 46.4 | 46.7 | 45.3 | 20.9 | 21.1 | 18.4 | | | 162.7 | 57.6 | **7.6** | 44.5 |
| **3.5h** | 42.15 | 1.013 | 68.9 | 69.8 | nd | 52.5 | 53.2 | 55.1 | 24.4 | 24.7 | 21.5 | | | 164.4 | 63.3 | **6.7** | 43.6 |
| **4h** | 42.28 | 1.009 | 61.0 | 61.5 | nd | 56.0 | 56.5 | 58.9 | 26.3 | 26.6 | 22.7 | | | 157.5 | 67.7 | **10.6** | 53.6 |
| **4.5h** | 42.56 | 1.003 | 55.1 | 55.2 | nd | 62.3 | 62.4 | 65.1 | 30.2 | 30.3 | 25.0 | | | 156.4 | 71.0 | **11.2** | 51.4 |
| **5h** | 43.88 | 0.972 | 44.6 | 43.4 | nd | 69.4 | 67.5 | 68.4 | 34.5 | 33.5 | 34.1 | | | 159.1 | 77.2 | **9.7** | 52.3 |
| **6h** | 45.23 | 0.94 | 29.7 | 27.9 | nd | 68.5 | 64.4 | 70.1 | 35.0 | 32.9 | 36.1 | | | 160.3 | 85.3 | **9.0** | 70.3 |
| mean | 42.68 | | | | | | | | | | | | | | | | |

nd: not determined

TOS: total galacto-oligosaccharides

L.C.: lactose conversion

Mf: multiplication factor

Is: Internal standard

Lactose without allolactose

# GalNAc
# 10 %

| time | Is (pA1) | Mf (pA1) | Lactose (g/l) + Allolactose (g/l) (see discussion) | | | Glucose (g/l) (see discussion) | | | Galactose (g/l) (see discussion) | | | Acceptor sugar (g/l) (see discussion) | | | L. C. (%) | **acceptor usage (%) from Gel H** | TOS (g/l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Enzymatic | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Gel H | | | |
| **0'** | 12.74 | 1.018 | 188.6 | 192.1 | 190.7 | 0 | 0 | 0 | 0.0 | 0.0 | 0.0 | 82.5 | 84.0 | 82.0 | 0.0 | **0.0** | 0 |
| **20'** | 12.93 | 1.003 | 167.2 | 167.7 | nd | 9.6 | 9.7 | 9.1 | 4.3 | 4.3 | 3.7 | 81.0 | 81.2 | 76.7 | 12.7 | **6.5** | 14.3 |
| **40'** | 12.58 | 1.03 | 148.7 | 153.1 | nd | 16.2 | 16.7 | 18.1 | 6.8 | 7.0 | 8.0 | 77.3 | 79.6 | 75.4 | 20.3 | **8.1** | 20.5 |
| **60'** | 12.75 | 1.017 | 138.2 | 140.6 | nd | 23.0 | 23.4 | 25.9 | 9.2 | 9.4 | 10.2 | 78.5 | 79.8 | 74.3 | 26.8 | **9.4** | 25.1 |
| **90'** | 12.98 | 0.999 | 123.6 | 123.6 | nd | 32.0 | 31.9 | 35.6 | 12.8 | 12.8 | 13.1 | 78.5 | 78.5 | 71.5 | 35.7 | **12.9** | 33.0 |
| **2h** | 12.74 | 1.018 | 99.4 | 101.2 | nd | 37.0 | 37.6 | 42.6 | 14.9 | 15.2 | 17.0 | 72.2 | 73.5 | 71.7 | 47.3 | **12.6** | 47.0 |
| **2.5h** | 12.61 | 1.028 | 86.7 | 89.1 | nd | 43.1 | 44.3 | 47.2 | 17.8 | 18.3 | 20.7 | 71.4 | 73.4 | 71.6 | 53.6 | **12.7** | 49.3 |
| **3h** | 12.71 | 1.021 | 77.8 | 79.4 | nd | 48.5 | 49.5 | 55.1 | 20.8 | 21.2 | 23.4 | 71.3 | 72.7 | 70.3 | 58.6 | **14.3** | 52.3 |
| **3.5h** | 12.92 | 1.004 | 71.8 | 72.1 | nd | 54.4 | 54.6 | 58.1 | 24.1 | 24.2 | 28.2 | 73.2 | 73.5 | 72.1 | 62.5 | **12.1** | 49.7 |
| **4h** | 13.2 | 0.983 | 64.4 | 63.3 | nd | 62.0 | 60.9 | 67.8 | 28.4 | 27.9 | 32.3 | 76.5 | 75.2 | 74.3 | 67.1 | **9.4** | 46.3 |
| **4.5h** | 13.43 | 0.965 | 50.1 | 48.3 | nd | 64.1 | 61.8 | 62.3 | 30.5 | 29.5 | 33.0 | 74.7 | 72.0 | 73.2 | 74.8 | **10.8** | 59.9 |
| **5h** | 13.53 | 0.957 | 28.2 | 27.0 | nd | 72.9 | 69.7 | 71.8 | 36.5 | 34.9 | 35.9 | 80.3 | 76.9 | 75.1 | 85.9 | **8.4** | 66.0 |
| **6h** | 13.53 | 0.957 | 17.0 | 16.3 | nd | 81.5 | 78.0 | 80.4 | 44.4 | 42.4 | 43.2 | 84.2 | 80.6 | 76.5 | 91.5 | **6.7** | 59.5 |
| mean | 12.97 | | | | | | | | | | | | | | | | |

nd: not determined

TOS: total galacto-oligosaccharides

L.C.: lactose conversion

Mf: multiplication factor

Is: Internal standard

Lactose without allolactose

# GalNAc 20 %

| time | Is (pA1) | Mf (pA1) | Lactose (g/l) + Allolactose (g/l) (see discussion) | | | Glucose (g/l) (see discussion) | | | Galactose (g/l) (see discussion) | | | Acceptor sugar (g/l) (see discussion) | | | L. C. (%) | acceptor usage (%) from Gel H | TOS (g/l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Enzymatic | pA1 original | pA1 modified | Gel H | pA1 original | pA1 modified | Gel H | | | |
| **0'** | 12.43 | 1.025 | 179.5 | 183.9 | 179.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | 183.0 | 0.0 | **0.0** | 0 |
| **20'** | 12.34 | 1.032 | 165.8 | 165.2 | nd | 7.8 | 8.0 | 9.2 | 3.5 | 3.6 | 3.8 | | | 182.1 | 10.2 | **0.5** | 3.1 |
| **40'** | 12.65 | 1.008 | 152.3 | 153.4 | nd | 16.0 | 16.2 | 18.2 | 6.9 | 6.9 | 7.8 | | | 174.4 | 16.6 | **4.7** | 11.2 |
| **60'** | 12.35 | 1.031 | 145.5 | 150.1 | nd | 21.4 | 22.1 | 23.6 | 8.9 | 9.2 | 12.2 | | | 174.0 | 18.4 | **4.9** | 6.7 |
| **90'** | 12.77 | 0.999 | 131.6 | 131.4 | nd | 30.9 | 30.9 | 35.6 | 12.7 | 12.6 | 17.2 | | | 178.5 | 28.6 | **2.5** | 8.7 |
| **2h** | 12.7 | 1.004 | 107.3 | 107.7 | nd | 36.1 | 36.2 | 44.0 | 15.0 | 15.1 | 21.6 | | | 172.2 | 41.5 | **5.9** | 30.9 |
| **2.5h** | 12.92 | 0.987 | 101.0 | 99.7 | nd | 44.6 | 44.0 | 48.2 | 19.1 | 18.8 | 27.3 | This peak is overloaded in a dilution of 1:1800 | | 175.5 | 45.8 | **4.1** | 24.2 |
| **3h** | 13 | 0.98 | 95.2 | 93.3 | nd | 52.9 | 51.8 | 56.8 | 23.5 | 23.0 | 30.8 | | | 169.8 | 49.3 | **7.2** | 24.2 |
| **3.5h** | 12.85 | 0.992 | 85.3 | 84.7 | nd | 58.2 | 57.8 | 58.1 | 26.9 | 26.7 | 35.5 | | | 162.2 | 54.0 | **11.4** | 30.8 |
| **4h** | 12.93 | 0.985 | 73.1 | 72.1 | nd | 60.6 | 59.7 | 68.5 | 29.0 | 28.6 | 40.1 | | | 161.7 | 60.8 | **11.6** | 40.0 |
| **4.5h** | 13 | 0.98 | 68.6 | 67.2 | nd | 70.8 | 69.4 | 70.9 | 35.4 | 34.7 | 42.5 | | | 162.1 | 63.5 | **11.4** | 28.7 |
| **5h** | 13.02 | 0.979 | 29.9 | 29.3 | nd | 75.1 | 73.5 | 73.5 | 39.4 | 38.6 | 47.9 | | | 165.1 | 84.1 | **9.8** | 55.6 |
| **6h** | 12.77 | 0.998 | 16.0 | 16.0 | nd | 74.7 | 74.6 | 80.3 | 40.9 | 40.9 | 57.7 | | | 163.7 | 91.3 | **10.6** | 67.0 |
| mean | 12.75 | | | | | | | | | | | | | | | | |

nd: not determined

TOS: total galacto-oligosaccharides

L.C.: lactose conversion

Mf: multiplication factor

Is: Internal standard

Lactose without allolactose

## 4.5. Discussion

### 4.5.1. Experimental and general considerations

This project is the first in our laboratory that expands conventional enzymatic lactose conversion with the use of acceptor sugars. Standard reaction conditions are generally applicable (except for sialic acid, where the pH had to be adjusted) and the addition of acceptor-molecules had only a minor effect on the overall reaction in regard to conversion velocity and the pattern of GOS obtained. The amount of added acceptors was chosen in % (w/v) of lactose-solution, which was not ideal, as it would have been more meaningful to use molar ratios. Calculating back the percentage values gives an approximate donor:acceptor ratio of 1:1 for the experiments with 10 % (w/v) L-Fucose, a ratio of 1:0.7 for the experiments with 10 % (w/v) N-Acetyl-D-glucosamine and N-Acetyl-D-galactosamine and a ratio of 1:0.5 for the experiment with sialic acid. The molar ratios for the experiments with 20 % (w/v) acceptors are therefore 1:2 for L-Fucose and 1:1.5 for GalNAc and NacGA. An experiment with 20 % (w/v) sialic acid has not been realized, because sialic acid did not dissolve completely in the reaction solution.

Another potential improvement of the experimental design concerns the preparation of the reaction solution. In this project, the appropriate amount of acceptor sugar was weighed out in an Eppendorf tube and then filled up with reaction buffer. This leads to a volume increase of approx. 5 % in experiments with 10 % (w/v) acceptors and approx. 10 % volume increase for the experiments with 20 % (w/v) acceptor sugars. Therefore the absolute concentrations in g/l are lowered, but not the relative molar donor/acceptor ratio. In the standard batch without additional acceptors this phenomenon did not occur, which allows only a limited comparison to the experiments with added acceptor sugars. Ideally lactose, the acceptor sugars and $MgCl_2$ should be weighed out in a measuring flask, which is then filled up with NaPP-buffer. Due to the high-price of some acceptor sugars one would have to use small measuring flasks with volumes of one or two ml, which are not available in our laboratory, but could be purchased at low expenses.

In comparison to the original protocol the reaction time was shortened from 12 hours main reaction time to 6 hours and more samples were taken at more regular intervals. In order to convert the same amount of lactose in a shorter time the enzyme concentration had to be increased. Studying the effect of different enzyme concentrations by comparison of HPLC-spectra at similar lactose conversion levels shows an influence of enzyme concentration on the obtained GOS spectra. This suggests the possibility to optimize enzyme concentration in expense of a well-defined overall reaction time.

Immediately after sample collection the enzyme must be deactivated in order to get clear results for a defined moment of lactose conversion. Sample deactivation can be achieved by addition of a strong alkaline liquid (as done in the *o*NPG-activity test) or by storing the

sample for 5 minutes at 99.9 °C (as done for the experiments). Since the combination of heat, sugars and proteins might lead to unwanted side reactions, which may distort the obtained GOS spectra, the effects of heat deactivation and alkalization were compared. As there is no difference observable in the HPLC-spectra of both deactivation methods, heat deactivation is clearly preferable, because it is more convenient and the neutralization of the alkaline reaction mixture dilutes the sample and forms salts that might compromise analysis. Moreover experiments were conducted that show that there is no need to keep the sample at 99.9 °C for 5 minutes, because the enzyme is sufficiently denatured after 3 minutes. However, again HPLC-spectra do not show any difference between samples deactivated for five minutes or three minutes.

After enzyme deactivation the standard protocol instructs to freeze samples until analysis. This is applicable for standard batches with 580 mM lactose, but as experiments with acceptor sugars involve considerably higher overall concentrations of sugars, it is better to store tightly sealed samples at 4 °C and take the (low) risk of microbial contamination. Thawing frozen samples often resulted in precipitation of sugars and it turned out to be a tedious task to re-dissolve the precipitate in the small sample volumes with a combination of ultrasonic bath, microcentrifuge and vortex-apparatus. Complete re-dissolving of the precipitate is imperative, because once a volume of sample is taken out in presence of a precipitate, all concentrations in subsequent analysis will be incorrect.

In general the added sugars were only poorly used as acceptors for transglycosylation, an acceptor-usage of more than 20 % could never be observed. Comparing this value to the literature (e.g. Li et al. 2010: ~40 % of NAcGA with a β-gal from *Bacillus circulans*; Kraftzik et al. 2002: ~30 % of NacGA with a β-gal from *Bacillus circulans*) reveals a low efficiency in transglycosylation to added acceptor-molecules of the β-galactosidase used in this project. N-Acetyl-neuraminc acid appears not to be of use for transglycosylation, as no new peak was observable under UV-light in HPLC analysis variant 3. This might be because of the considerable higher molecular weight of sialic acid compared to the other acceptors and therefore occurring steric hindrances.

However, transglycosylation reactions have mostly only been reported with N-Acetyl-D-glucosamine so far, so the other three sugars of human milk might be interesting candidates for transglycosylation reactions in view of their novelty value.

Recently, similar experiments as done in this thesis were performed in a master thesis from Canada (Lee 2009). The author used crude extracts of several lactic acid bacteria to form galacto-oligosaccharides with D-mannose, L-Fucose and N-Acetyl-D-glucosamine. Although no quantification of the new derivatives was achieved, their very small peaks suggest a low efficiency in transglycosylation to the added acceptor-molecules. Although this is in good agreement with the present work, the overall data is far too sparse to

disqualify beta-galactosidases from lactic acid bacteria to be used for transglycosylation to added acceptors.

The maximum acceptor-usage does not occur at the same time as the maximum of galacto-oligosaccharide formed. While maximum GOS yields are usually reported at the end of the reaction when the bigger part of lactose is converted, maximum acceptor usage could frequently be observed in the middle of the reaction at about 40 – 60 % of lactose conversion.

The best result was obtained in the experiments with 20 % (w/v) L-Fucose, but since L-Fucose has a smaller molecular weight compared to the other added acceptor-molecules, it remains unclear whether L-Fucose is the most attractive acceptor-molecule or whether the donor/acceptor ratio of 1:2 is most favourable.

It might be possible that different reaction conditions would yield far better results, but as long as the mechanisms for transglycosylation remain unexplored, an experimental approach to optimize reaction conditions seems unpromising without using high-troughput methods of analysis. This can clearly be depicted with a recently published article from the Nanjing Agricultural University (Li et al. 2010): In this publication the effects of only four different reaction temperatures, concentrations of substrates, molar ratios of donor/acceptor and enzyme concentrations were studied. This gives a total of (4 * 4 * 4 * 4 =) 256 different experiments, which have to be done in duplicate (= 512 experiments). The authors do not state how many samples were taken per experiment, but this information can be easily gained by counting the amount of data points they show in their graphics, which is 13. This means they have analysed a total of (512 * 13 =) 6656 samples. We do not have any method available (except TLC and enzymatic analysis), which can process more than about 20 samples/day, so it would take at least (6656 / 20 = ) 332 working days to test all samples in our laboratory. But still the result would be inconclusive since the optimal conditions might be outside the few tested parameters.

The authors in the article mentioned above use two columns (a Waters® Sugar-D and a Shodex® Sugar KS-801) for HPLC-analysis, of which at least one would have been very suitable for this project as well, since it can show the whole spectra of new N-Acetyl-D-glucosamine products.

In principal high concentrations of donors and acceptors are advantageous, since hydrolysis and transglycosylation are competitive reactions and more molecules other than water present in the reaction medium will favour transglycosylation. In a 1 ml standard batch with 580 mM (200 g/l) lactose, $H_2O$ molecules are present in approx. 100-fold excess (~$3.345 * 10^{22}$ $H_2O$ molecules compared to $3.521 * 10^{20}$ lactose molecules). As this disequilibrium can only partly be overcome by increasing substrate concentrations, water is ideally replaced by another solvent. In fact, it was shown that

replacing 25 % (v/v) of water with [MMIM] [$MeSO_4$] doubled acceptor usage from 30 to 60 % (w/w) (Kraftzik et al. 2002).

Another effect of a higher concentration of sugars in the mixture is that more water molecules will be required for hydrate shells, which might then not be fully available as acceptors any more.

As the solubility of solids is temperature-dependent, high temperatures are generally preferable in experiments with additional acceptor molecules, especially because lactose shows pronounced temperature-dependent solubility. In this project experiments with 20 % (w/v) acceptor-sugars were close to the solubility limit, as sometimes precipitation during the experiment was observable. In this case it was tried to re-dissolve the precipitate or at least get a uniform suspension in order to take representative samples. It was impossible to increase the reaction temperature, since the used enzyme has a very limited thermal stability as already determined (Nguyen et al. 2006).

The enzyme used in this project was originally isolated from a *Lactobacillus reuteri* strain found calf. The usage of β-galactosidases from probiotic organisms is based on the observation that microorganism grow best on such GOS-mixtures produced by their own β-galactosidases. As it known that *L. reuteri* shows a very distinct host-specialisa ion, a *Lactobacillus reuteri*-strain isolated from calf can probably not proliferate in the human's gut. It would therefore be interesting to use the enzyme from a human *L. reuteri* strain and compare the obtained spectrum of GOS.

A detailed description of the reaction characteristics has already been reported by Splechtna et al. 2006 and the interested reader is referred to this publication. Only the most important facts shall therefore be summarized as follows:

At the beginning of the reaction, when lactose levels are high, lactose is the preferred acceptor sugar for transglycosylation yielding trisaccharides as easily observable in HPLC analysis variant 2. Even at low concentrations of monosaccharides at the beginning of the reaction, considerable amounts of disaccharides are formed, which can be explained by intramolecular transglycosylation. The newly formed GOS act as both galactosyl donors and acceptors, resulting in an increasingly complex product-mixture. Up to approx. 80 % lactose conversion the amount of GOS is constantly rising, afterwards hydrolysis prevails over synthesis leading to equimolar amounts of D-glucose and D-galactose at the very end of the reaction. D-galactose is preferentially used as an acceptor-molecule compared to D-glucose, which is observable by comparing the amounts of GOS in relation to the abundance of galactosyl-acceptors. The used enzyme shows a very distinct preference to create β(1,6) and β(1,3) linked products.

As the acetyl-moiety at position C-2 of some acceptor sugars is in close proximity to the preferred C-3 position, it would be interesting, whether steric hindrances hamper the formation of β(1,3) linked products. However, without standard-compounds or the

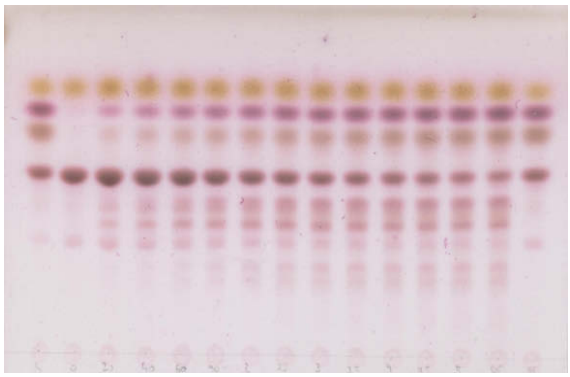
possibility to carry out structure determination any speculation about this is not fruitful. Finally it shall be pointed out that the reaction is very easy to scale up.

### 4.5.2. Methods for analysis

As multiple analysis methods were used during this project, their characteristics shall be individually discussed below:

TLC analysis

Standard TLC analysis is well-known to be easy to realize with almost no equipment required. The most persuasive argument for TLC is the concurrent analysis of all samples from one batch at the same time, while alternative methods for analysis can only process one sample at a time. The HPTLC plates used in this project are comparatively expensive with a price of approx. 20 €/plate and about half a plate is needed to develop all samples from one batch. HPTLC plates have a smaller particle diameter of the solid phase, which enables more efficient separation in a shorter time. Typical parameters for HPTLC-analysis are therefore a plate length of 5 cm and developing times shorter than 20 minutes. As these parameters only yield poor results with the eluents used in this project, the plate length had to be elongated to approx. 9 cm, which results in a developing time of approx. 2 h. In order to improve resolution the plates were eluted twice in ascending mode. The original protocol in our laboratory uses a dipping method for staining, but better results can be achieved with the spraying method that was used for this thesis. Very good results can hereby be achieved with staining agent A, which is unfortunately not appropriate for sugars containing N-Acetyl-groups. In order to stain them staining agent B had to be used, with inferior results compared to staining agent A.



*As lactose conversion proceeds, reaction products accumulate. TLC analysis is sufficient for monitoring the conversion process, quantification or characterization of reaction products is not possible. (Peaks from up to bottom: L-fucose, D-glucose, D-galactose, lactose and GOS)*

Under the conditions used in this project lactose and monomeric sugars are separated well, with $R_f$ values for the monomers between 0.6 and 0.75 and an $R_f$ value for lactose of approx. 0.5. The other conversion products have lower $R_f$ values and are only poorly separated, which inhibits identification of single reaction products.

Quantification with TLC is principally possible, but requires special equipment and well-elaborated methods. It was indeed tried to use TLC for GOS-quantification in a master thesis from Ireland (Manucci, 2009), which was successful for D-glucose and D-galactose, but not for lactose and GOS. However, the TLC development procedure used in this project clearly separates lactose from GOS, which would probably allow lactose quantification as well.

To monitor the N-Acetyl-groups of acceptor-molecules and new products the samples were applied undiluted on the TLC-plates, but only one spot for the acceptor sugar was visible under UV-light after development. Higher concentrations prepared by multiple application of 0.5 µl sample yielded considerably lower $R_f$ values, but still did not reveal any conversion product with a response signal under UV-light. This is probably because the concentration of individual new N-Acetyl-group containing products is too low to be detected using this method.

In general it might be possible to achieve better TLC-results with AMD (automated multiple development)-systems, but the high structural similarity of the reaction products might hamper efficient separation and AMD-systems are still high-priced devices. In conclusion, traditional TLC analysis can only give a vague idea of GOS products and is only of historical importance, since the transglycosylation reaction was first observed on TLC-sheets.

Enzymatic determination of glucose

Enzymatic determinations often require only a photometer and are therefore feasible in most laboratories. Although enzymatic determinations are supposed to be extremely precise, results are sometimes hard to reproduce and some authors from our university even describe the enzymatic approach as "neither sensitive nor reliable" (Zeleny et al. 1997).

In the authors hands the enzymatic determinations using a kit from Megazyme™ gave a satisfactory fit with the data from HPLC-analysis, but in general they tend to slightly overestimate glucose-levels. By dividing all amounts given in the manual by four, the expense is lowered to approx. 0,25 €/sample. The main disadvantage of enzymatic determination in comparison to other analytical techniques is the high expenditure of work required to obtain only one result. Another disadvantage is that the response signal from the photometer is only linear at low glucose concentrations, so dilutions are required. In this thesis all samples subjected to enzymatic analysis were diluted 1:300, which means that a difference of 0.005 absorbance units results in 1 g glucose difference (according to the formula given in the enzyme kit booklet: absorbance * 0.6634 * dilution factor). Results with more precision can therefore be gained by using dilution

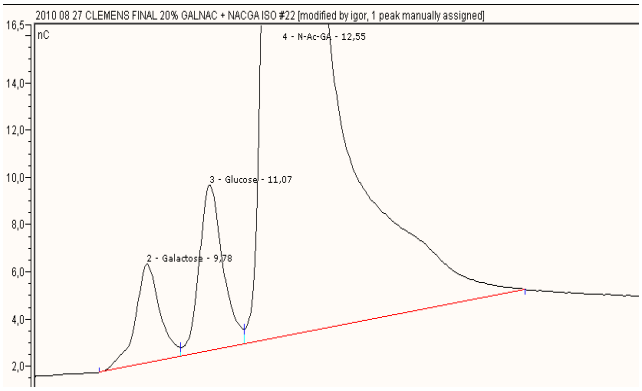
factors as low as possible, which would require two series of measurements for every batch: A first series in a high dilution in order to determine which dilution is best to use for the second series. However, results from HPLC are much easier to gain and a very accurate determination of glucose is only of minor importance for this project, so the enzymatic approach has not been elaborated.

Other enzymatic kits would be commercially available for lactose, D-galactose, N-Acetyl-D-glucosamine and sialic acid. An enzymatic kit for L-Fucose is not available, but several methods for enzymatic determination of L-Fucose have been published (e.g. Morris 1982). For enzymatic determination of N-Acetyl-D-galactosamine neither kits are available, nor publications could have been found.

HPLC analysis variant 1

HPLC analysis variant 1 was conducted on a pA1-column from Dionex™, which is a standard column for monosaccharide analysis. The system is equipped with a sensitive amperiometric detector, which implies that high dilutions (of approx. 1:2000) are necessary for analysis. As high dilutions are time-consuming to prepare and inevitably add a systemic error to all results, the usage of an amperiometric detector had no advantage in variant 1 compared to the approx. 100 times less sensitive RI-detector of HPLC variant 3.

An isokratic elution was used to quantify monosaccharides and lactose. D-galactose and D-glucose are not base-line separated under the conditions used, but quantification is nevertheless possible. By using the standard elution program of our laboratory, all used acceptor sugars are well separated from all other peaks, but lactose, N-Acetyl-D-glucosamine and N-Acetyl-D-galactosamine show intensive tailing, which is probably due to the vastly over-aged column (it carries a sign declaring *best before use: 2000*). The result of tailing is that some peaks do not really have retention times any more, but rather retention periods with 10-15 minutes per peak.




*Tailing as well as insufficient base line recovery hamper quantification of some peaks in HPLC variant 1.*

Furthermore, the base line does not reach ground level again after the two acceptor sugar-peaks mentioned above, which hampers proper quantification. Another effect of

the vastly over-aged column is that low levels of allolactose (one of the reaction products) can not be separated from high levels of lactose efficiently enough for quantification, but the amount of lactose is needed for determination of lactose conversion and the amount of GOS present at a certain time. Therefore, the two unresolved peaks for allolactose and lactose had to be handled as a single lactose peak, leading to a constant underestimation of the lactose conversion rate and GOS-levels. When most of the lactose was converted, the peaks for allolactose and lactose could be separated, so at least the overall conversion rate could be determined.

L-Fucose shows a very short retention time of approximately 5 minutes and is therefore suitable to be used as an internal standard. The internal standard-technique with L-Fucose was applied in this project, showing deviations of all L-Fucose peak areas from the mean value between 1-5 %. The internal standard was therefore rather inappropriate to check whether the dilutions were prepared correctly, but very well suited to show the limitations of the autosampler. In experiments with L-Fucose as an acceptor sugar, fructose was used as an internal standard and proved to be suitable as well as L-Fucose.

In experiments with sialic acid as an acceptor-molecule a new method (described in Dionex™ technical note 41) had to be used in order to detect and quantify sialic acid. As this new method is only applicable for sialic acid and for no other compound in the batch, every sample had to be tested twice: once with the new elution program in order to quantify sialic acid and once more with the standard elution program to quantify the other sugars.

In comparison to the standard batch where no acceptor was added to the reaction mixture, three new peaks are observable using the standard elution program, one for an L-Fucose derivative that appears after glucose and one for an N-Acetyl-D-glucosamine derivative, which is observable as a peak appearing after lactose. A small new peak between lactose and glucose is observable in experiments with N-Acetyl-D-galactosamine. Since no standard compounds are available, it is impossible to determine the identity or quantity of these new peaks, but their relatively small peak area suggest that they do not represent the whole scope of new products.

The observed phenomenon that 1 ppm of the acceptor-sugars L-Fucose and N-Acetyl-D-galactosamine already overloads the column, while 1 ppm of N-Acetyl-D-glucosamine, lactose, D-galactose and D-glucose give a linear response is interesting and might be a problem of the overaged column too. This problem would be very easy to overcome just by preparing higher dilutions, but as HPLC variant 3 is much more appropriate for quantification due to the problems (tailing, no base line recovery) associated with the overused column, running the batches again in a higher dilution was not realized.

Another problem associated with the tailing effect observable for N-Acetyl-D-glucosamine and N-Acetyl-D-galactosamine is that conversion products (in particular Gal β(1,x)NacGA

and Gal β(1,x)GalNAc derivatives) might have retention times close to the tailing monomer-peaks and could thus get superimposed. This apprehension is supported by the results of a colleague who determined the retention times of small chain chito-oligosaccharides using the same conditions as for this project. Superimposition would result in underestimation of the transglycosylation efficiency of the enzyme to the added acceptor sugars.

Finally, it is worth mentioning that with a complete different elution program all of the problems associated with the overaged column could be overcome. The parameters for this elution program are: A: $H_20$; B: 250 mM NaOH; C: 500 mM Na-Acetate, 250 mM NaOH; 0-5 min: 6% A 94 % B; 5-15 min: gradient to 40 % B, 60 % A; 15-30 min: gradient to 60 % A, 40 % B; 30-45 min: 94 % A, 6 % B. Another huge advantage of this program is the considerably shorter time needed per analysis (only 45 minutes instead of 75 minutes). Unfortunately this method has only been found in a very late stage of the project and was only applied exemplary for a few samples.

| substance | area/100 ppm |
|---|---|
| Gal | 150 |
| Glu | 130 |
| GalGal 1-6 | 100 |
| Allolac | 80 |
| Lac | 80 |
| GalGal 1-3 | 90 |
| Tri 1-4,1-6 | 55 |
| ? | 70 |
| GalGlc 1-3 | 100 |
| ? | 70 |
| ? | 70 |
| ? | 70 |
| ? | 70 |
| ? | 70 |
| ? | 70 |
| ? | 70 |
| ? | 70 |
| ? | 70 |
| ? | 70 |
| ? | 70 |
| Tri 1-4,1-3 | 60 |
| ? | 55 |
| ? | 55 |
| ? | 55 |
| ? | 55 |
| ? | 55 |
| ? | 55 |
| ? | 55 |
| ? | 55 |
| ? | 55 |
| ? | 43 |
| ? | 43 |
| ? | 43 |

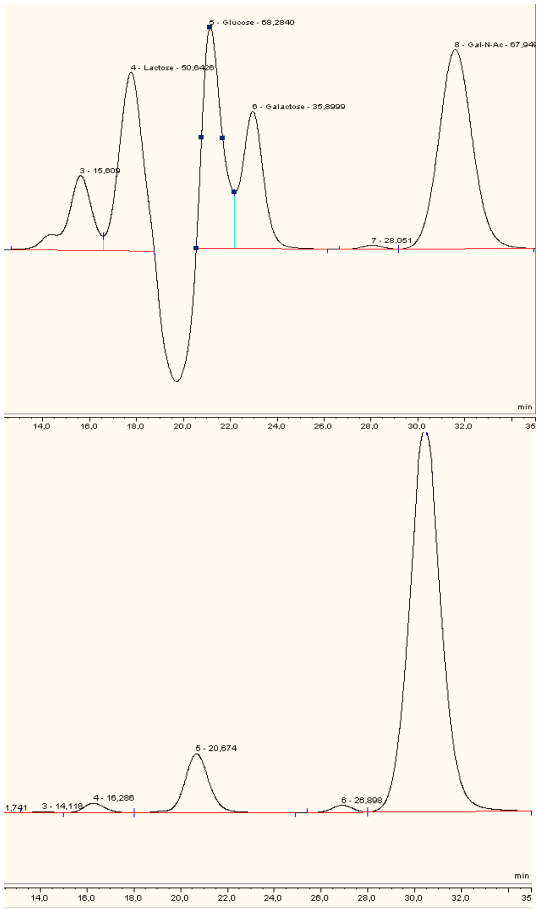
*As only a few compounds have been identified so far, the area-values of all others have to be estimated what inevitably yields inaccurate results in HPLC variant 2.*

HPLC analysis variant 2

HPLC analysis variant 2 is a method using gradient elution, first published in 2006 (Splechtna et al. 2006) and has not been changed in the meantime. Although the focus of variant 2 is clearly to visualize the oligosaccharide fraction, the pA1 column for monosaccharides is used. As we have a pA100 column for oligosaccharides also available in our laboratory it might be useful to develop a protocol for this column in future, especially since there is also a semi-preparative pA100 column available, which could be used to collect fractions. The method for the pA1 column requires a low dilution of only 1:20 leading to a tremendous overload of all peaks, which are visible using analysis variant 1, but allows to monitor those compounds present in quantities too small to be detected with the high dilution from variant 1. A few compounds of the oligosaccharide-products have been identified so far and can be quantified. For all other compounds estimated values have to be used, which is clearly a weak point of this method. Especially since the identified compounds show a high diversity in their

response signal for compounds with the same polymerization degree (e.g. lactose 80 su (square units) for 100 ppm and Gal β(1,6)Gal) 100 su for 100 ppm), while the response signal is estimated to be the same for unidentified compounds with the same polymerization degree. However, as results based on this method have already been published in scientific journals, it might be possible that the errors cancel each other out. As no significant new peaks are observable in the experiments conducted with the new acceptor sugars, quantification based on the results from HPLC variant 2 has not been conducted. Even if new peaks would have been observable, it remains highly unclear whether the estimated numbers are sufficient to use or not.

HPLC analysis variant 3




*Samples in HPLC variant 3 are monitored with both an UV-lamp (below) and an RI-detector (above).*
*The UV-lamp selectively shows N-Acetyl-group containing oligosaccharides. The shift of retention times is due to serial arrangement of the two different detectors.*

HPLC analysis variant 3 was used in order to overcome the quantification problems of the acceptor sugars observed in HPLC variant 1 and to monitor N-Acetyl-group containing oligosaccharides under UV-light. It is a new method of GOS analysis, which has not been reported in literature yet. Since the Dionex™-system used for variant 1 and 2 is not compatible with parts from other suppliers, it was impossible to connect the UV-lamp there. On the other hand Dionex™-columns can not be connected to our HPLC-systems with UV-detectors, because Dionex™-columns require higher pressure. Therefore, a new column had to be chosen. Although the choice to use the Supelcogel H column was not based on solid arguments, the column showed to be suitable for GOS-analysis. The separation mechanism is based on the ability of the solid-phase to interact with the OH-groups of the analytes and separates them according to the amount of OH-groups they carry. This leads to a size dependent separation of oligomers, which elute in inverse relationship to their polymerization degree, while monomers are separated individually.

Peaks in HPLC analysis variant 3 are simultaneously detected with an RI-detector and

with an UV-detector. Since the RI-detector is not very sensitive in comparison to an amperiometric detector, only low dilutions of 1:20 were required for quantification, which can be easily prepared in a one-step dilution. As the UV-detector is approximately 10 – 20 times more sensitive than the RI-detector quantification would require dilutions of approx. 1:300. However, since all peaks for which standard compounds are available can be conveniently quantified with the RI-detector, therefore an additional dilution and quantification is only optional.

As the peaks for the added acceptor sugars are baseline-separated and no problem with the recovery of the baseline is observable, quantification of the acceptor sugars is rendered from a grievous ordeal using the overaged pA1-column to a triviality. By knowing the amount of acceptor sugars and galactose as determined with HPLC analysis variant 3 it was possible to adjust the integration-style for the pA1-column accordingly.

Using the UV lamp to monitor N-Acetyl-group containing oligosaccharides showed two peaks for all experiments when N-Acetyl-group containing acceptor sugars were used, one for at least one dissacharide and one for at least one trisaccharide. The comparison of the respective peak areas suggests a strong prevalence of new disaccharides over new trisaccharides, but an exact quantification of the particular new reaction products is impossible.

The characteristics of the column allow quantification of lactose only at the beginning of the experiment, when no other disaccharides are present in the reaction mixture, but quantification of the monomers is possible throughout the experiment. Unfortunately the glucose peak interferes with the negative injection peak and can therefore not be quantified. This problem can be overcome by skipping the washing step between samples or maybe even by using 0.1 % (v/v) $H_3PO_4$ for washing instead of UHQ-water..

Another advantage of HPLC variant 3 is that less time is need per analysis in comparison to HPLC variant 1.

CE analysis and sample preparation

Capillary electrophoresis is a rather exotic technique for GOS analyses, since very few publications from outside our institute using this method have been published. Although samples require a mandatory derivatization step prior analysis, capillary electrophoresis can be regarded to be a superior method for GOS-analysis. Derivatives with the same polymerization-degree elute in groups and supposed that the derivatization step is quantitative and all derivatives of a certain polymerization degree have the same response signal, quantification of not only groups with the same polymerization degree, but even of individual compounds was achieved.

Other advantages of CE-analysis are a relatively short analysis time needed per sample

(approx. 30 minutes), very small amounts of the analytes required and the (theoretical) possibility to automatize the system.

However in this project capillary electrophoresis was by far the most inefficient method of GOS analysis. After one year with periodic phases of intensive CE-measurements not a single electropherogram could be prepared which was even close to the high-quality electropherograms, which are shown in publications from our institute (e.g. Splechtna et al. 2006, see chapter 7*.4. Some HPLC- and CE-spectra*). Therefore any attempts for quantification were not successful, but at least some qualitative information could be gained. It appears that new GOS products with N-Acetyl-groups are clearly separated from other products, yielding one big peak after the group of disaccharides, but no peak after the group of trisaccharides or higher polymerization degrees. This is in accordance with the analysis of HPLC variant 3 and suggest a prevalence of the formation of new disaccharides over new trisaccharides.

It has been tried in this project to monitor the N-Acetyl-group-containing sugars without derivatization, but even in high concentrations (of 100 g/l) their response signal is too weak to allow quantification or identification of reaction products.

Due to unknown reason it was impossible to analyse more than approx. 20 samples in inferior quality before the capillary became ruined, easily observable by voltage values at the system limit at a very low applied current. The reason for this shortcoming could not be identified and the frequent deterioration of the capillaries was the biggest matter of expense in this project.

With an elaborated protocol at hands, one could easily determine whether the derivatization step is truly quantitative and all compounds with same polymerization degree give the same response signal. The overall amount of sugars during conversion always remains equal (=1) and only the distribution of compounds with different polymerization degree changes, which can be depicted as follows:

$$xA + yB + zC + vD = 1$$

(x, y, z and v → respective peak areas for mono, di-, tri- and tetrasaccharides
A, B, C and D → response signals for mono, di-, tri- and tetrasaccharides (unknown variables))

Having four different electropherograms of the same batch at different conversion levels gives four different equations with four unknown variables each. It is then easy to determine the values for the unknown variables (A, B, C and D) either by using mathematical software or manual calculation. The resulting values must then be applied to a fifth electropherogram, where the respective peak areas are multiplied with the determined values for the unknown variables. If the result after addition of the calculated

numbers is 1 (or very close to 1), the determined numbers can be used for quantification. If the result is not 1, either the derivatization is not quantitative or compounds with the same polymerization degree give different response signals. In this case quantification of unidentified compounds would not be possible. This new method for calculation is only applicable if the volume of the reaction mixture does not change during the conversion progress, which could be possible. However, it would be easy to check this: if one would carry out a conversion in a capillary, even small volume changes would become obvious.

The derivatization step is definitely a crucial point in CE-analysis, especially the formation of the Schiff's base after addition of 2-aminopyridine. Imine bonds are generally not very stable and usually only intermediates, but the phenyl-group of 2-aminopyridine can stabilize the bond via electron displacement to the aromatic ring to some degree. It remains uncertain how stable the intermediate really is, but exposure to high temperatures and low pH-values in the presence of water should probably generally be avoided.

A commercial kit for CE-sample preparation with 2-aminopyridine is also available* for the rather high price of ~ 450 USD per 15 samples. The derivatization method is very similar to our protocol, but the excess of reagents is not removed after the formation of the Schiff's base and DMSO instead of methanol is used as reaction solvent. In the last step of the commercial kit a purification-column is used to remove 2-aminopyridine. In literature complete removal of 2-aminopyridine prior CE-analysis is stated to be mandatory in order not to compromise analysis (Mülhardt 2008), but with our method the peak supposed to be 2-AP is clearly separated from the other peaks with a very short retention time.

*http://www.ludger.com/docs/catalog-and-price-list/Ludger-Price-List-2010-USD.pdf

## 5. Outlook

Most of the publications regarding galacto-oligosaccharide production focus on the synthesis of GOS using various enzymes and reaction conditions, but the analysis of the products is usually neglected by merely giving a rough description of the reaction products or determining only a few compounds (Coulier et al. 2010). This is because in-depth characterization proves to be difficult, for example because of high-priced standards or the great diversity of structural-similar reaction products. It is therefore desirable that the analysis of GOS will become more developed, along with the overall ongoing progress in science. Especially modern combinatory methods of analysis (e.g. HPLC/NMR or TLC/MS) are likely to become more available in the future and offer great potential for GOS-analysis. While at least a dozen of transglycosylation-experiments can be conducted on a single day, the analysis of samples is comparatively time consuming and currently the bottleneck in GOS-research. It is therefore desirable that high-troughput methods of GOS-analysis will be developed in order to greatly improve the opportunities of scientists to identify ideal reaction parameters. Maybe a modern TLC approach is very suitable in this regard, since it is one of the characteristics of TLC-analysis to process multiple sample simultaneously.

GOS-products with different polymerization degrees should be comparatively easy to separate (ideally even during the conversion), since the relative difference of their molecular weight is very pronounced. Especially nanomembrane technology might soon offer an attractive and efficient approach for a convenient size separation of conversion products.

(Automated) solid-phase synthesis of oligosaccharides is hopefully by and by about to become a standard-technique and its widespread application will probably render standard compounds for GOS-analysis much more affordable.

As human milk oligosaccharides have remarkably complex structures, their production by use of only one enzyme with transglycosylation activity is not promising. Several enzymes need to be combined to create products identical or at least very similar to those in human milk. These enzymes could either be glycoside hydrolases with good transglycosylation efficiency or transferases. The use of transferases does not seem very promising at the moment, because they require specific sugar nucleotides as substrates, which are only available at prohibitive prices at the moment (Torres et al. 2010). Especially the glycoside hydrolase chitinase might be a very interesting candidate for a combinatory approach for HOS production, since the enzyme transfers N-acetyl-D-glucosamine units and has, similar to beta-galactosidase, an outstanding cheap substrate that accumulates as a waste product.

But before even thinking about the combination of several enzymes, it is imperative to

first identify a β-galactosidase from a probiotic organism, which shows good transglycosylation activity to added acceptor sugars. As the number of enzymes tested in this regard is still very limited, it is hard to forecast whether such an approach will be successful or not.

Even if it would turn out that native enzymes from probiotic organisms are not suitable for transglycosylation to acceptor-molecules, protein engineering offers vast opportunities.

Another very interesting approach to highly-efficient GOS synthesis is the usage of non-aqueous ionic liquids, since they can not be used as an acceptor for transglycosylation and suppress secondary hydrolysis of the reaction products. As advanced ionic liquids are cheap, biodegradable and in general environmentally friendly, it is likely that their use will increase for all kinds of applications (Gorke et al. 2010), including GOS-synthesis.

## 6. Summary and conclusions

This thesis explores the enzymatic synthesis of novel galacto-oligosaccharides, which were produced by separate addition of four different acceptor-sugars in two different concentrations to a standard reaction mixture for transglycosylation. Since the acceptor-sugars are the same as those to be found in human milk, the new products are supposed to mimic the effects of human milk oligosaccharides. The most important results of the project are summarized below.

- The heterologously overexpressed enzyme from *Lactobacillus reuteri* can be purified very efficiently using established techniques.

- The overall transglycosylation efficiency of the enzyme without the usage of additional acceptor molecules is comparatively good with a maximum of approx. 40 % (w/w) GOS of the initial lactose concentration.

- The transglycosylation reaction to the added acceptor-molecules at standard conditions is comparatively low with less than 15 % (w/w) acceptor usage. The best result could be derived with 20 % (w/v) L-Fucose (14.9 % acceptor usage). Transglycosylation on N-Acetyl-neuraminic acid could not be observed.

- Experiments with 20 % acceptor sugar yielded significantly more new products in absolute numbers but not in relative numbers.

- Three of the four used acceptor-sugars carry an N-Acetyl-moiety that shows UV-absorption. These sugars and their transglycosylation products can thus be selectively monitored with an UV-detector in HPLC analysis. Thereby at least 4 new conversion products could be detected. A fifth new product derived from L-Fucose could be detected with an amperiometric detector.

- Standard methods of analysis are not sufficient to detect the whole scope of the novel galacto-oligosaccharides.

- When N-Acetyl-neuraminic acid is used as an acceptor sugar, standard reaction conditions and standard methods for HPLC analysis can not be applied.

- Short chain alcohols considerably increase the activity of the enzyme over a broad range of concentrations.

# 7. Appendix

## 7.1. Various HOS and their structure (adopted from Jensen 1995)

To depict the high structural complexity of human milk oligosaccharides a short and non exhaustive compilation is given below. More than 150 different HOS have already been identified.

**2`-Fucosyllactose**

Fuc α(1 → 2) Gal β(1 →4) Glc

**3` Fucosyllactose**

Gal β(1 → 4) ↘
Fuc α(1 → 3) ↗ Glc

**Lactodifucotetraose**

Fuc α(1 → 2) Gal β(1 →4) ↘ Glc
Fuc α(1 → 3) ↗

**3`-Sialyllactose**

NANA α(2 → 3) Gal (1 → 4) Glc

**6`-Sialyllactose**

NANA α(2 → 6) Gal β(1 → 4) Glc

**6`- Galactosyllactose**

Gal β(1 → 6) Gal β(1 →4) Glc

**Lacto-N-neohexaose**

Gal β(1 →4) GlcNAc β(1 →6) ↘
Gal β(1 →4) GlcNAc β(1 →3) ↗ Gal β(1 →4)

**Lacto-N-octaose**

Gal β(1 →4) GlcNAc β(1 →3) Gal β(1 →4) GlcNAc β(1 →6) ↘
Gal β(1 →3) GlcNAc β(1 →3) ↗ Gal β(1 → 4) Glc

**Lacto-N-Neooctaose**

Gal β(1 →3) GlcNAc β(1 →3) Gal β(1 →4) GlcNAc β(1 →6) ↘
Gal β(1 →4) GlcNAc β(1 →3) ↗ Gal β(1 → 4) Glc

***iso*-Lacto-N-Octaose**

Gal β(1 →3) GlcNAc β(1 →3) Gal β(1 →4) GlcNAc β(1 →6) ↘
Gal β(1 →3) GlcNAc β(1 →3) ↗ Gal β(1 → 4) Glc

***para*-Lacto-N-Octaose**

Gal β(1 →3) GlcNAc β(1 →3) Gal β(1 →4) GlcNAc β(1 →3) Gal β(1 →4) GlcNAc β(1 →3) Gal β(1 →4) Glc

**Monofucosylmonosialyllactose**

NANA α(2 → 3) Gal β(1 →4) ↘ Glc
Fuc α(1 → 3) ↗

**Lacto-N-Tetraose**

Gal β(1 → 3) GlcNAc β(1 → 3) Gal β(1 → 4) Glc

**Lacto-N-fucopentaose**

Fuc α(1 → 2) Gal β(1 →3) GlcNAc β(1 → 3) Gal β(1 → 4) Glc

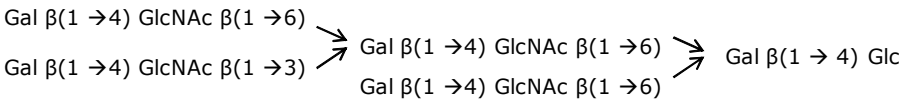
**Lacto-N-difucohexaose**

Fuc α(1 → 2) Gal β(1 →3) ↘
Fuc α(1 → 4) ↗ GlcNAc β(1 → 3) Gal β(1 → 4) Glc

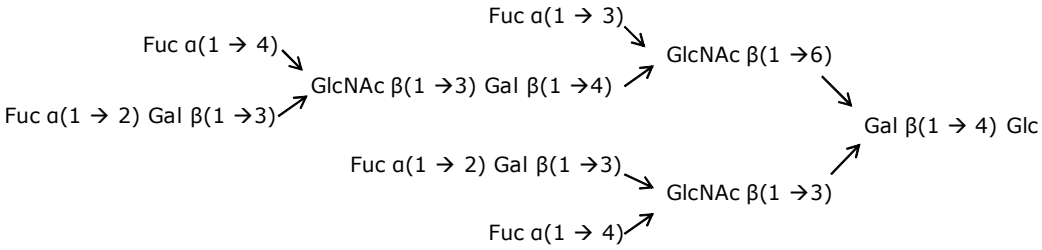
**Sialyllacto-N-tetraose**

NANA α(2 → 6) Gal β(1 → 3) GlcNAc β(1 → 3) Gal β(1 → 4) Glc

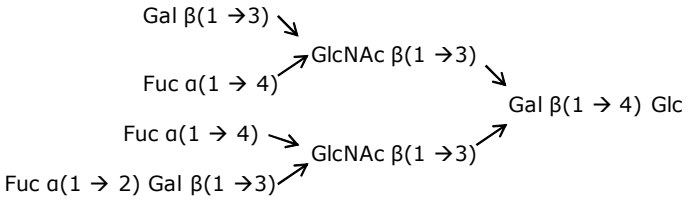
**Lacto-N-decaose**



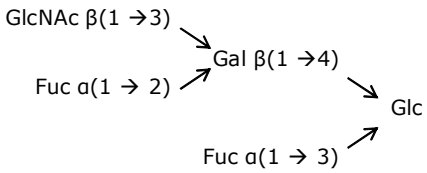

**Pentafucosyl-iso-lacto-N-octaose**



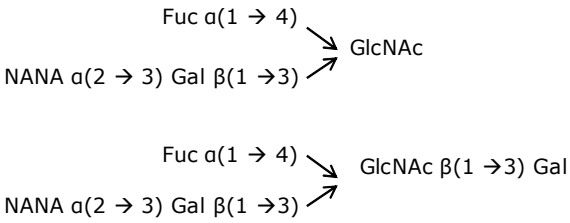

**Trifucosyllacto-N-hexaose**




**A- pentasaccharide**




**Two Oligosaccharides with nonlactose structures**




**Legend:**

| | |
|---|---|
| **Gal: galactose** | **GlcNAc: N-acetylglucosamine** |
| **Glc: glucose** | **GalNAc: N-acetylgalactosamine** |
| **Fuc: fucose** | **NANA: N-acetylneuraminic acid (sialic acid).** |

**The oligosaccharide 6`- Galactosyllactose is one of the very few HOS that can be produced via transglycosylation of a β - galactosidase**

## 7.2. Abbreviations

| Abbreviaton | Full text |
|---|---|
| % | per cent |
| [MMIM] [$MeSO_4$] | 1,3-di-methyl-imidazole-methyl sulphate |
| °C | degree Celsius |
| AMD | Automated multiple development |
| ATP | adenosine triphosphate |
| ß-gal | beta-galactosidase |
| CE | Capillary electrophoresis |
| DMSO | Dimethyl sulfoxide |
| DTT | Dithiothreitol |
| e.g. | exempli gratia (at example) |
| EFSA | European Food Safety Authority |
| FOS | Fructo-oligosaccharides |
| Fuc | L-Fucose |
| g | gram |
| GalNAc | N-Acetyl-D-galactosamine |
| GOS | Galacto-oligosaccharides |
| G6P | glucose-6-phosphate |
| G6P DH | glucose-6-phosphate dehydrogenase |
| h | hour |
| $H_20$ | water |
| $H_2SO_4$ | Sulphuric acid |
| $H_3PO_4$ | Phosphoric acid |
| $H_3PO_3$ | Phosphorous acid |
| HOS | Human milk oligosaccharides |
| HPLC | High-performance liquid chromatography |
| HPTLC | High-performance thin layer chromatography |
| kDA | kilodalton |
| KOH | Potassium hydroxide |
| kV | kilovolt |
| Matr. Nr. | Matrikelnummer (matriculation number) |
| mbar | millibar |
| mg | milligram |
| $MgCl_2$ | Magnesium chloride |
| min | minutes |
| ml | millilitre |
| MS | Mass spectrometry |
| NaCl | Sodium chloride |
| NAcGA | N-Acetyl-D-glucosamine |
| NADPH / NADP+ | Nicotinamide adenine dinucleotide phosphate |
| nm | nanometre |
| NMR | Nuclear magnetic resonance |
| NaPP | Sodium dihydrogen phosphate Dihydrate |
| $NaCNBH_3$ | Sodium cyanoborohydride |
| ATP | Adenosine triphosphate |
| *o*NPG | *ortho*-Nitrophenyl-β-galactoside |
| PCR | Polymerase chain reaction |
| pH | potential of hydrogen |
| Ph. Eur. | Pharmacopoea Europaea |
| RI | refractive index |
| rpm | rounds per minute |
| su | square units |
| TLC | Thin Layer Chromatograpy |
| U | units |
| UHQ | Ultra high quality water |
| USD | US-dollar |
| UV | Ultraviolet |
| (v/v) | volume per volume |
| (w/w) | weight per weight |
| (w/v) | weight per volume |

## 7.3. Acknowledgements

Although more people than mentioned below deserve tribute for being there for me, I would like to express my eternal gratitude to:

• Dietmar Haltrich and Clemens Peterbauer for kindly receiving me in the Institute of Food Biotechnology and allowing me to carry out research more or less independently on such an interesting topic.

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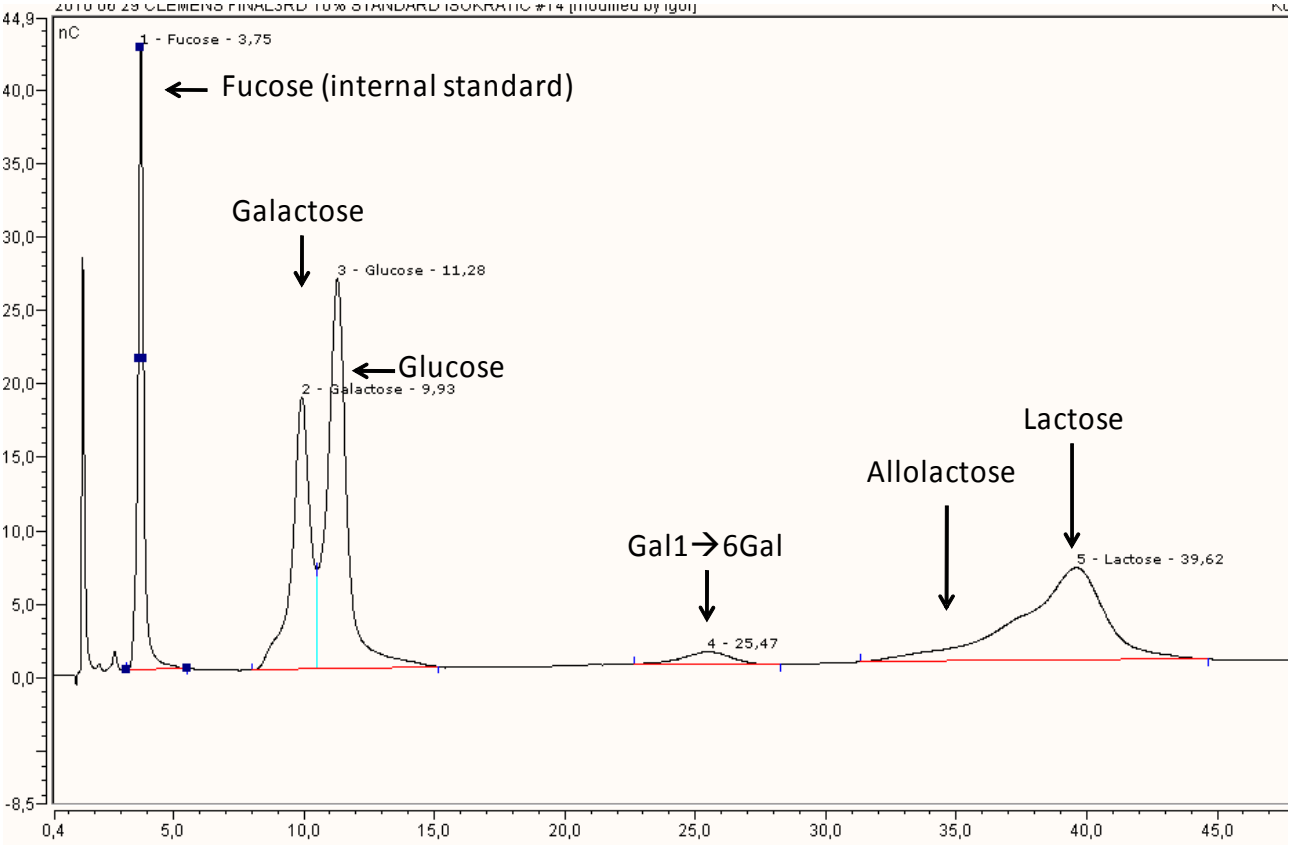
• Cindy Lorenz for her excellent expertise and her gracious assistance in all HPLC-matters.

• Viktoria Hell, who appears like a heaven sent gift for under-experienced junior scientists and who keeps our laboratory in such a good shape.

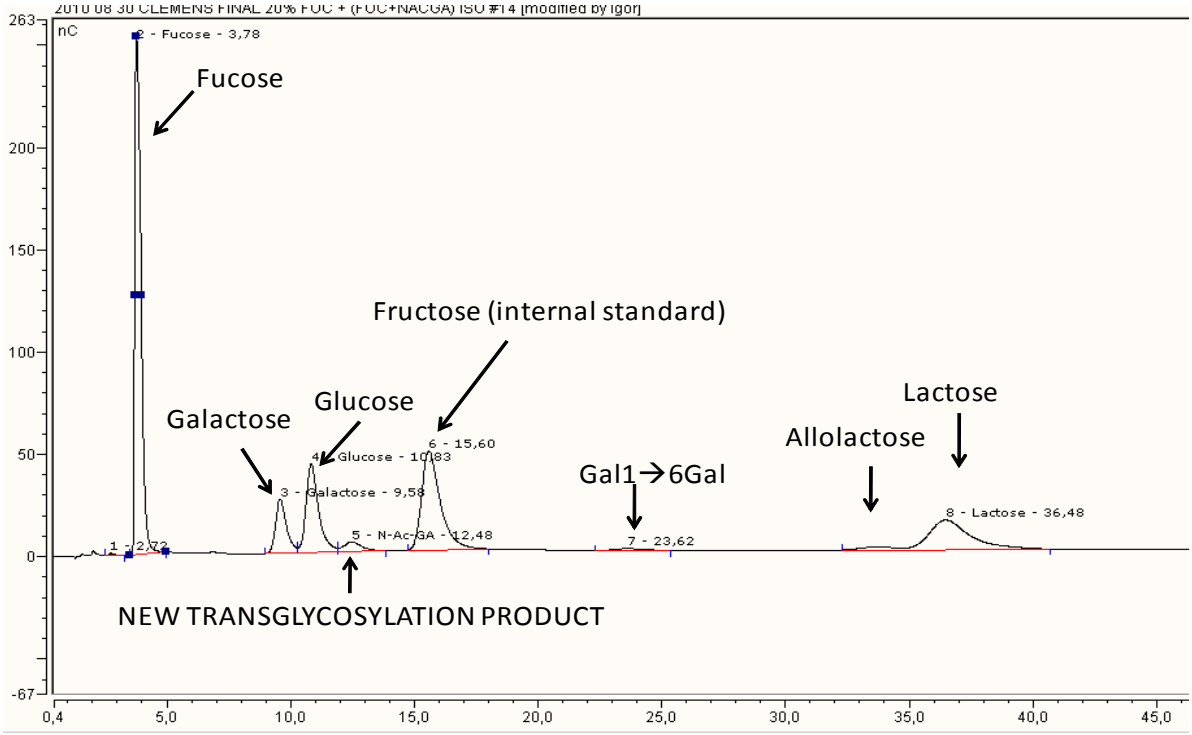
• the rest of our institute for being almost irreplaceable as fine members of the Institute of Food Biotechnology

Unfortunately it is a shortcoming of human perception only to realize how good things are when they are already gone or about to vanish.

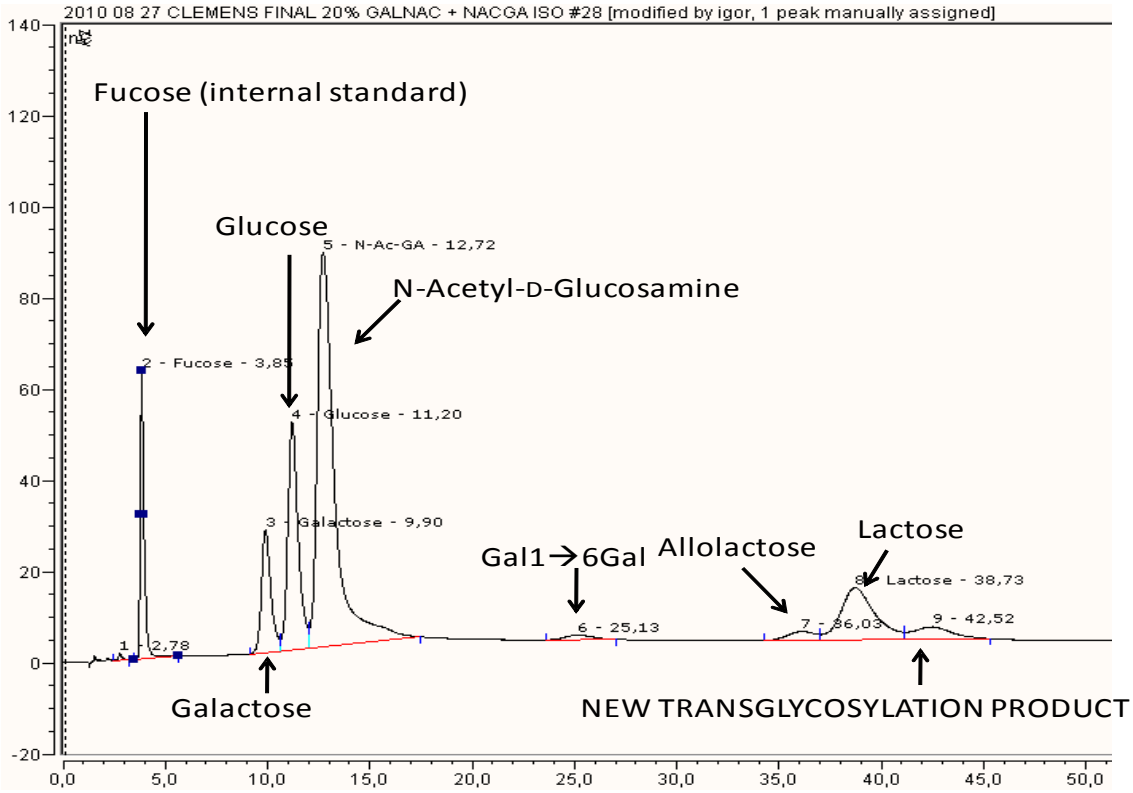
## 7.4. Some HPLC and CE spectra



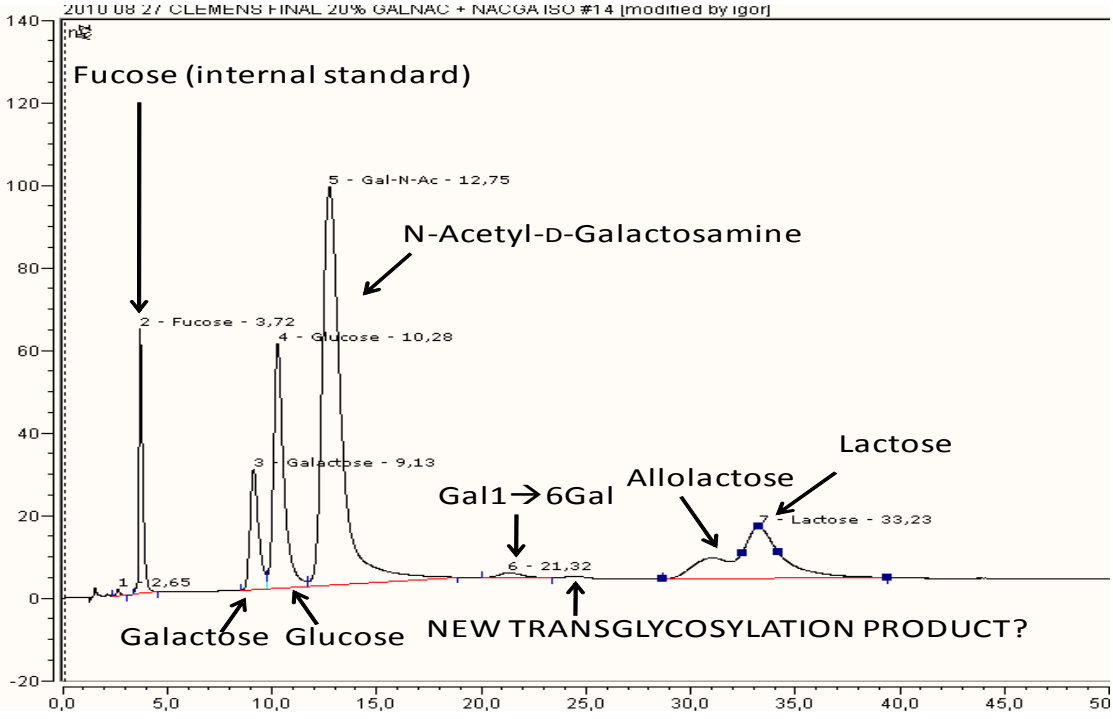

**HPLC variant 1, standard-batch, after 3hs**



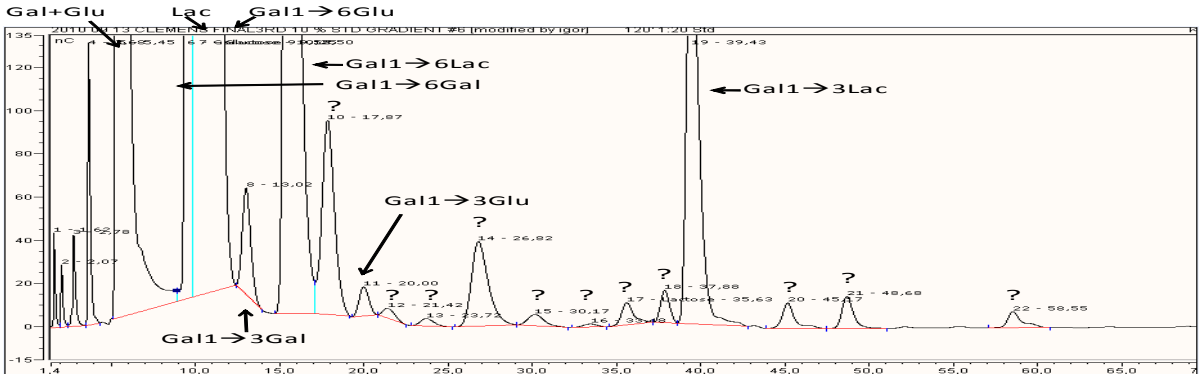

**HPLC variant 1, L-Fucose 20 %, after 3hs**



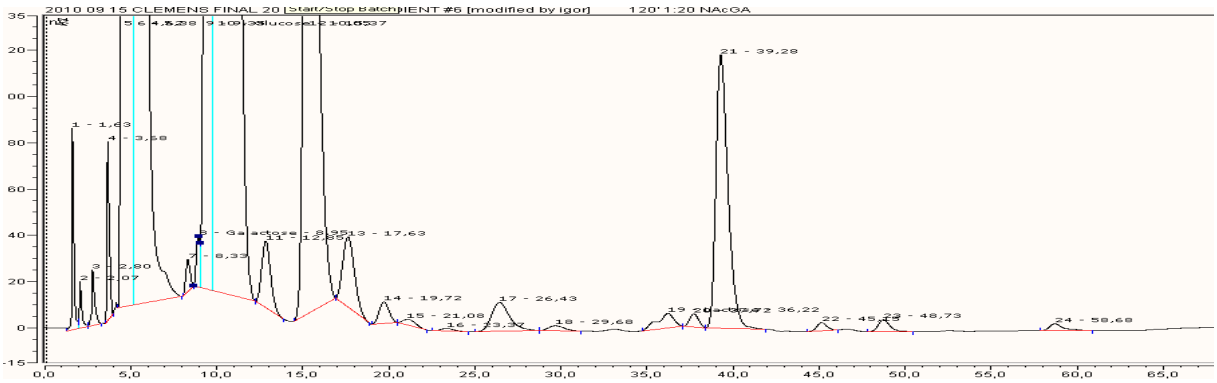

**HPLC variant 1, N-Acetyl-D-Glucosamine, after 3hs**



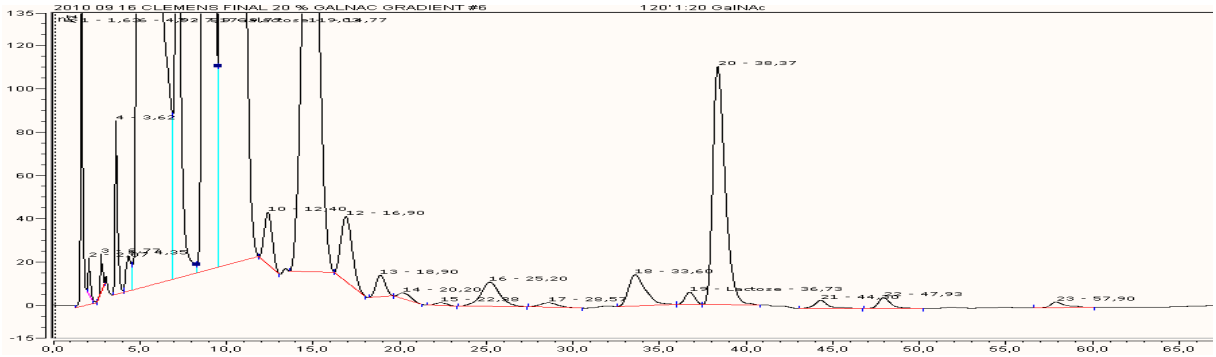

**HPLC variant 1, N-Acetyl-D-Galactosamine, after 3hs**




**HPLC variant 2, standard-batch, after 120'**



**HPLC variant 2, N-Acetyl-D-Glucosamine 20 % batch , after 120'**



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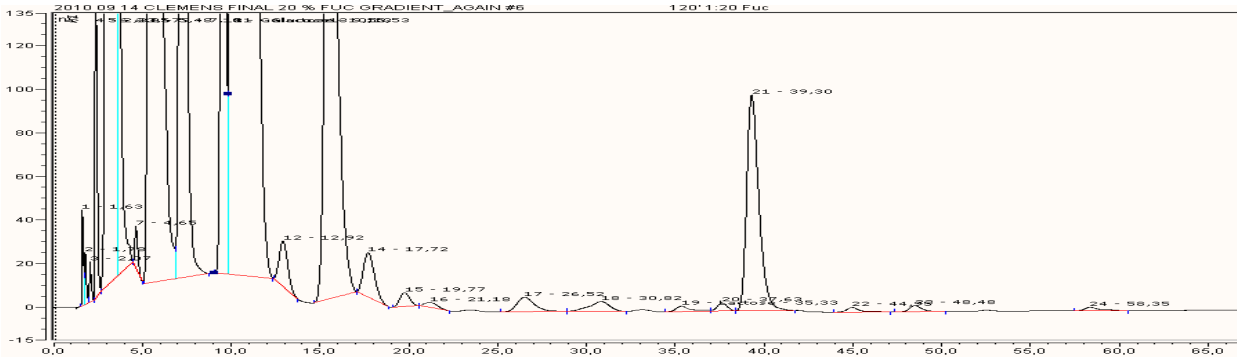
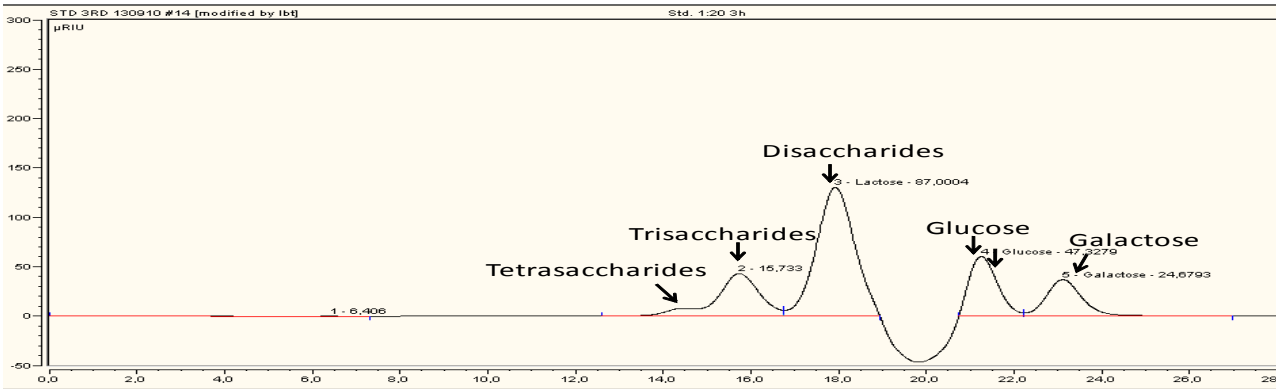
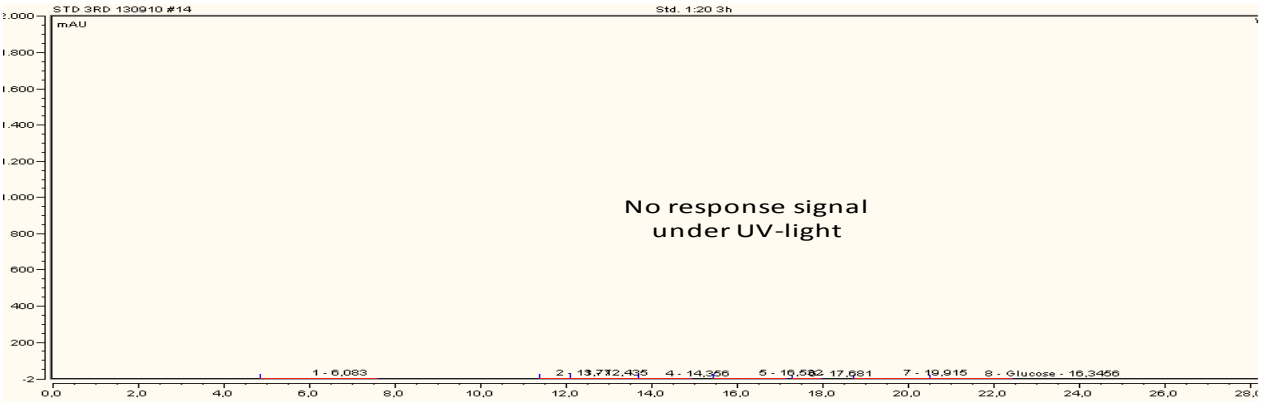


**HPLC variant 2, L-Fucose 20 % batch , after 120'**




**HPLC variant 3, RI-detection, standard-batch, after 3hs**

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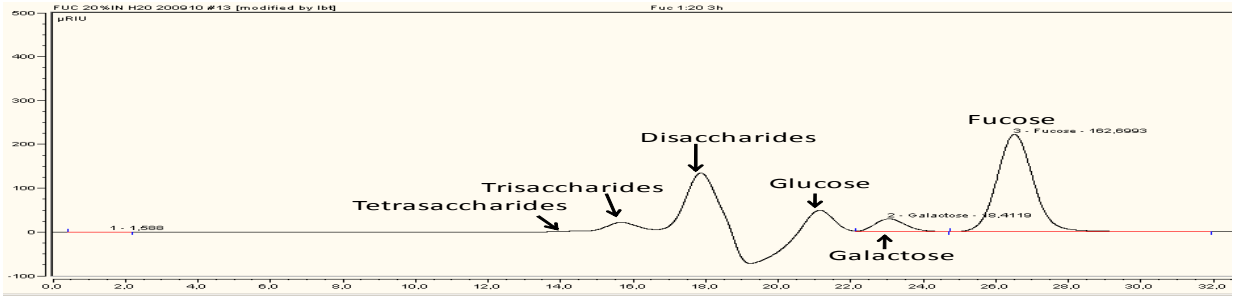
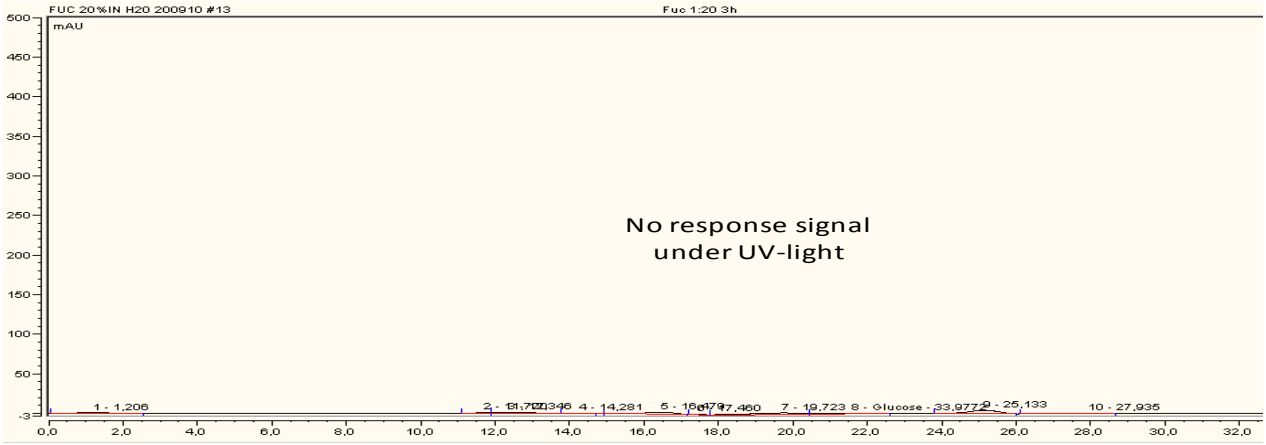



**HPLC variant 3, RI-detection, L-Fucose 20 % batch, after 3 hs**

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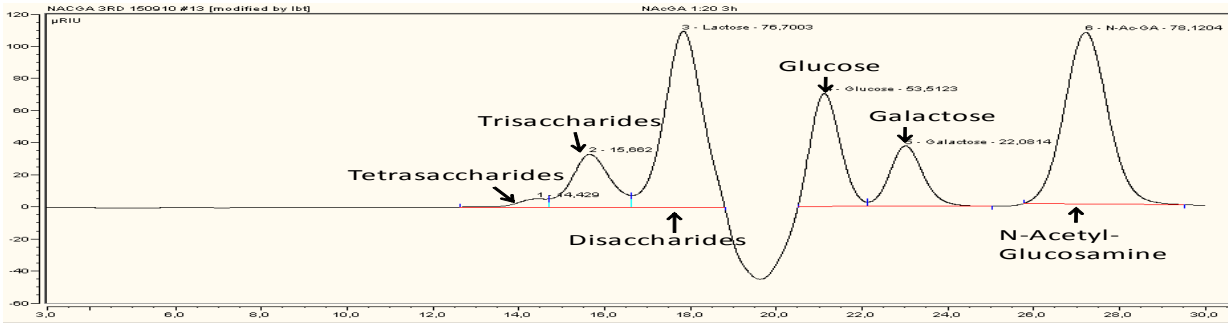
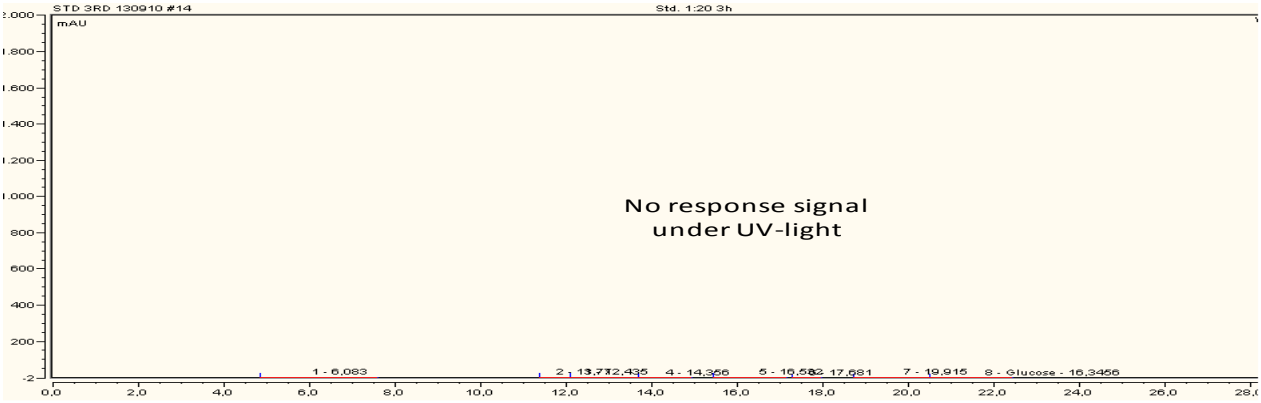



**HPLC variant 3, RI-detection, N-Acetyl-glucosamine 20 % batch, after 3hs**

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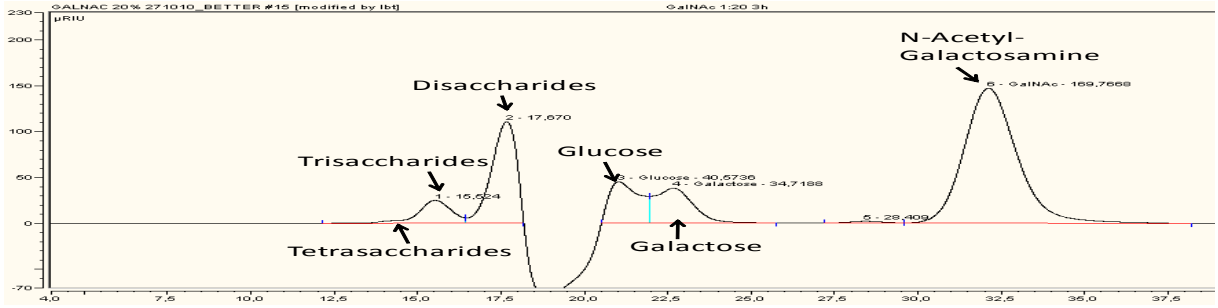


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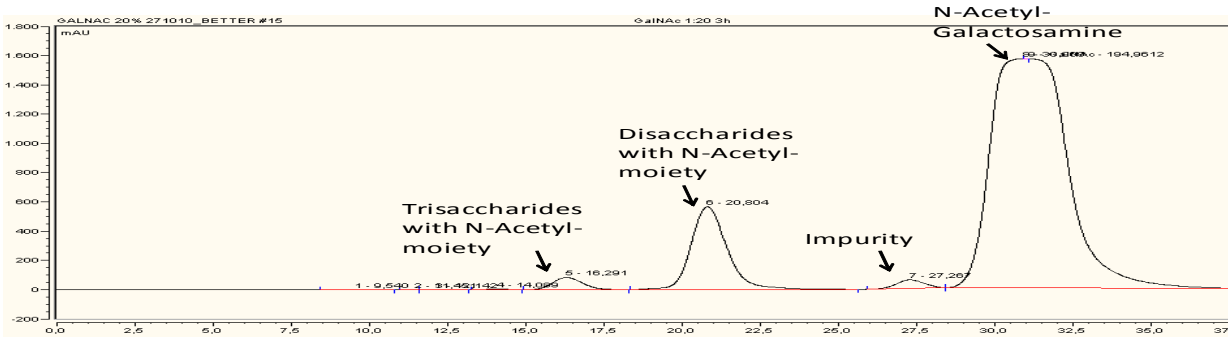


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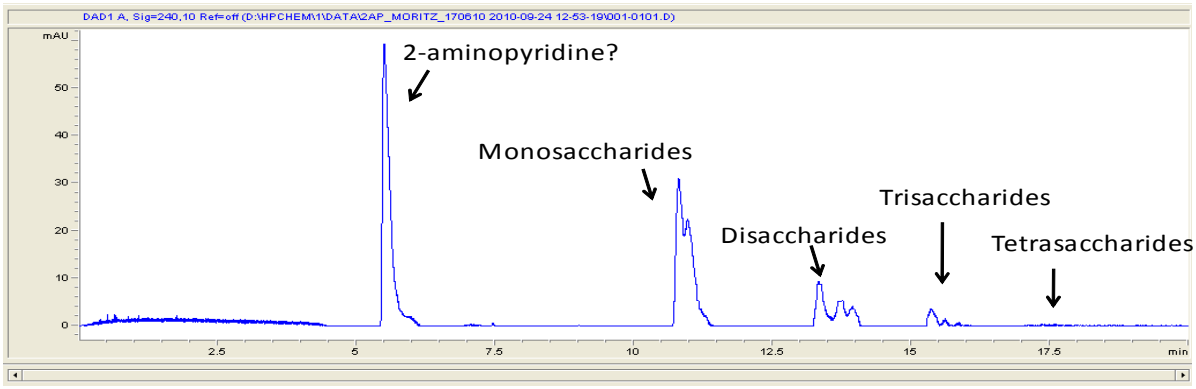
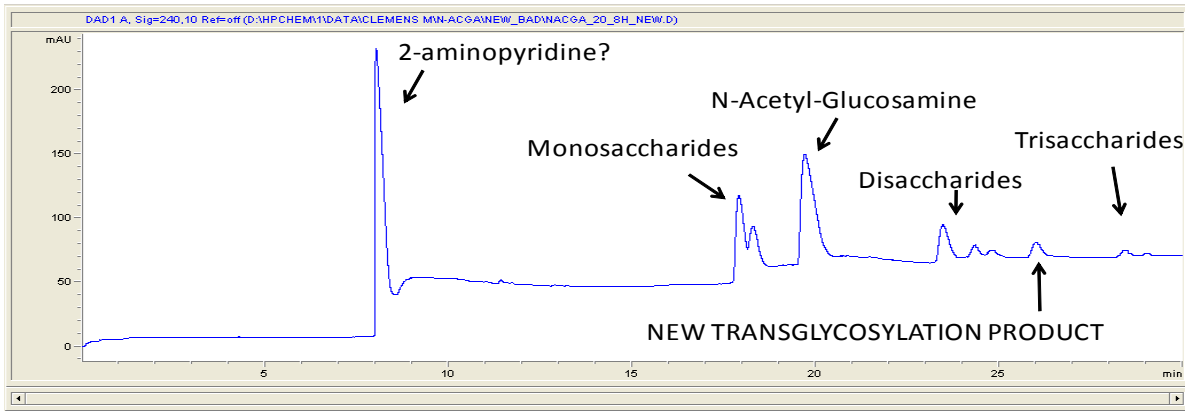
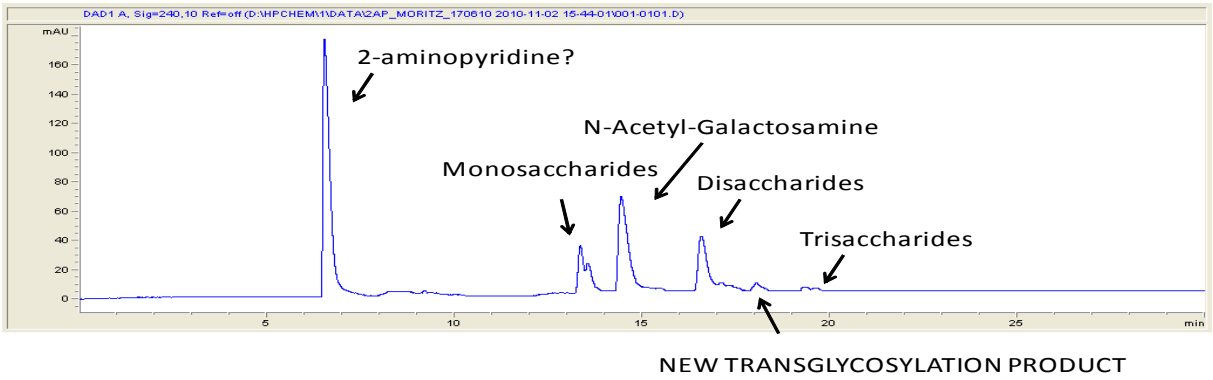


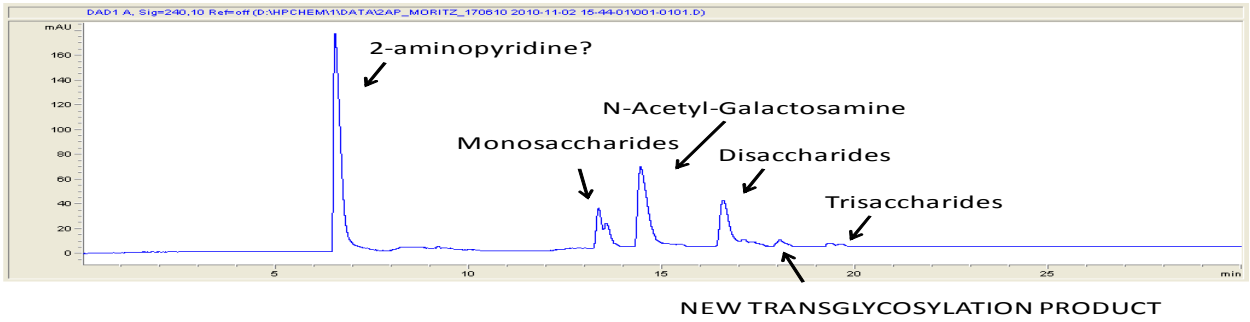

**CE-analysis, Standard-batch, after 2hs**



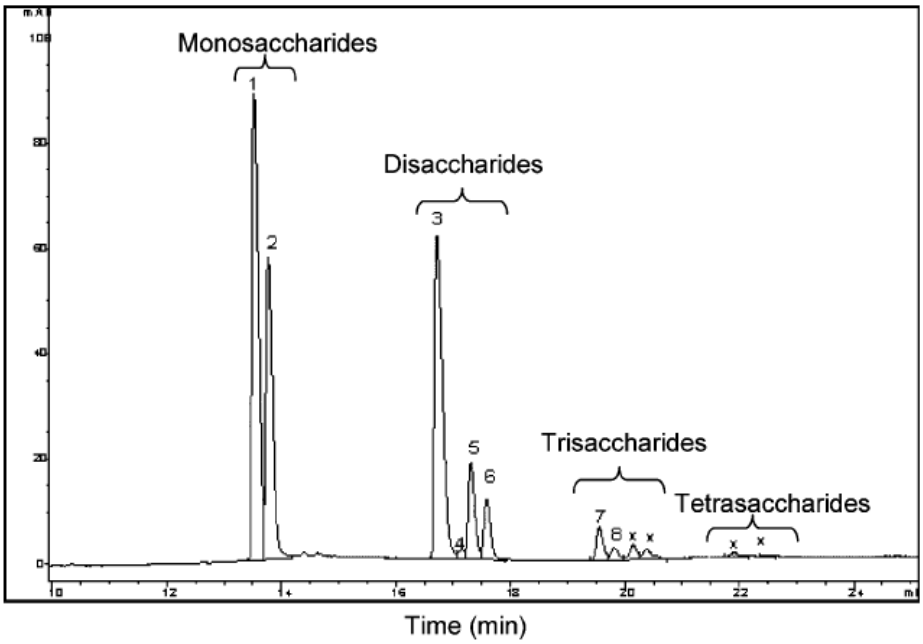

**CE-analysis, L-Fucose-batch, after 2hs**



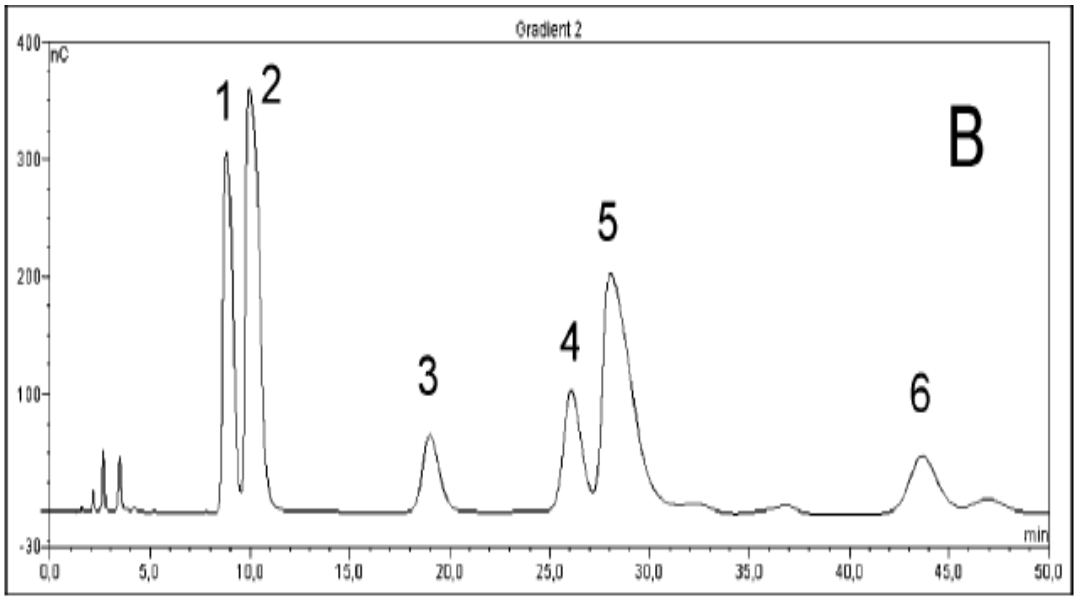

**CE-analysis, N-Acetyl-D-glucosamine, after 2hs**




**CE-analysis, N-Acetyl-D-galactosamine, after 2hs**




**published CE-spectrum from a standard-batch (taken from Splechtna et. al 2006)**




**published HPLC variant 1-spectrum from a standard-batch (taken from Splechtna et. al 2006)**

## 7.5. TLC results

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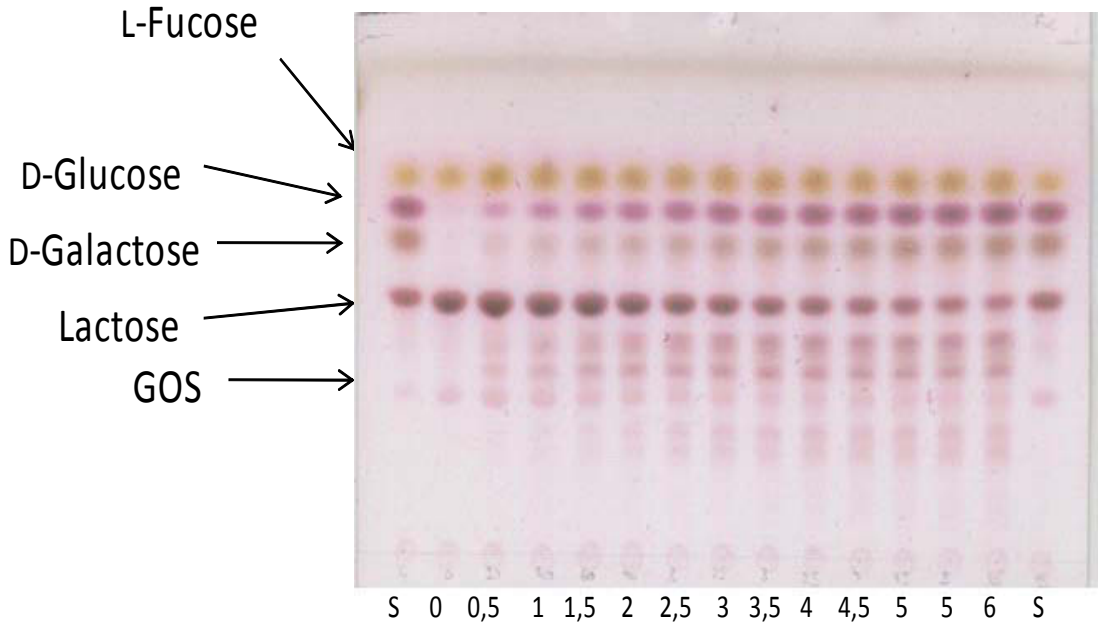


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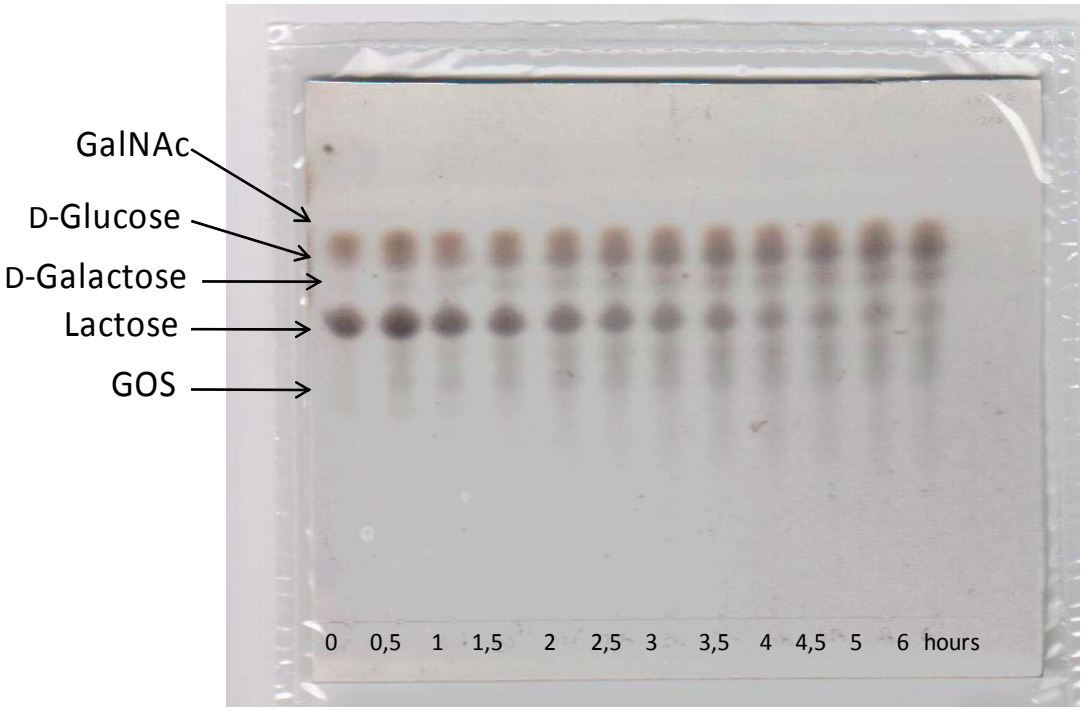

## 7.6. References


Agostoni, C. et al., 2004. Prebiotic oligosaccharides in dietetic products for infants: a commentary by the ESPGHAN Committee on Nutrition. *Journal of Pediatric Gastroenterology and Nutrition*, 39(5), p.465.

Albayrak, N. & Yang, S.T., 2001. Production of galacto-oligosaccharides from lactose by Aspergillus oryzae beta-galactosidase immobilized on cotton cloth. *Biotechnology and bioengineering*, 77(1), pp.8–19.

Anderson, J.W., Nicolosi, R.J. & Borzelleca, J.F., 2005. Glucosamine effects in humans: a review of effects on glucose metabolism, side effects, safety considerations and efficacy. *Food and chemical toxicology*, 43(2), pp.187–201.

Anderson, S.A., Chinn, H.I. & Fisher, K.D., 1982. History and current status of infant formulas. *American Journal of Clinical Nutrition*, 35(2), p.381.

Bakker-Zierikzee, A.M. et al., 2007. Effects of infant formula containing a mixture of galacto-and fructo-oligosaccharides or viable Bifidobacterium animalis on the intestinal microflora during the first 4 months of life. *British Journal of Nutrition*, 94(05), pp.783–790.

Belitz, H., Grosch, W. & Schieberle, P., 2007. *Lehrbuch der Lebensmittelchemie* 6. ed., Springer, Berlin.

Blaser, M.J. & Falkow, S., 2009. What are the consequences of the disappearing human microbiota? *Nat Rev Micro*, 7(12), pp.887-894.

Bode, L., 2006. Recent Advances on Structure, Metabolism, and Function of Human Milk Oligosaccharides. *J. Nutr.*, 136(8), pp.2127-2130.

Boehm, G. & Moro, G., 2008. Structural and functional aspects of prebiotics used in infant nutrition. *Journal of Nutrition*, 138(9), p.1818S.

Cardelle-Cobas, A. et al., 2009. Gas chromatographic-mass spectrometric analysis of galactosyl derivatives obtained by the action of two different [beta]-galactosidases. *Food Chemistry*, 114(3), pp.1099–1105.

Carletti, C. & Cattaneo, A., 2008. Home preparation of powdered infant formula: is it safe? *Acta Paediatrica*, 97(8), pp.1131–1132.

Casas, I.A. & Dobrogosz, W.J., 2000. Validation of the probiotic concept: *Lactobacillus reuteri* confers broad-spectrum protection against disease in humans and animals. *Microbial ecology in health and disease*, 12(4), pp.247–285.

Cleusix, V. et al., 2008. Glycerol induces reuterin production and decreases Escherichia coli population in an in vitro model of colonic fermentation with immobilized human feces. *FEMS Microbiology Ecology*, 63(1), pp.56-64.

Coppa, G.V. et al., 1990. Preliminary study of breastfeeding and bacterial adhesion to uroepithelial cells. *The Lancet*, 335(8689), pp.569–571.

Coudray, C. et al., 2003. Effects of inulin-type fructans of different chain length and type of branching on intestinal absorption and balance of calcium and magnesium in rats. *European journal of nutrition*, 42(2), pp.91–98.

Coulier, L. et al., 2009. In-Depth Characterization of Prebiotic Galacto-oligosaccharides by a Combination of Analytical Techniques. *Journal of agricultural and food chemistry*, 57(18), pp.8488–8495.

Cummings, J.H. & Macfarlane, G.T., 2002. Gastrointestinal Effects of Prebiotics. *British Journal of Nutrition*, 87(Supplement S2), pp.S145-S151.

Du, J. et al., 2009. Metabolic glycoengineering: Sialic acid and beyond. *Glycobiology*, 19(12), pp.1382-1401.

Edelson-Mammel S.G. & Buchanan R.L., 2004. Thermal Inactivation of Enterobacter sakazakii in Rehydrated Infant Formula. *Journal of Food Protection*, 67, pp.60-63.

El Ashry, H., Aly, M. & El Sayed, 2007. Synthesis and biological relevance of N-acetylglucosamine-containing oligosaccharides. *Pure and Applied Chemistry*, 79, p.12.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA); Scientific Opinion on the substantiation of health claims related to *Lactobacillus reuteri* ATCC 55730 and "natural defence" (ID 905) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal 2010*;8(10):1805. [12 pp.]. doi:10.2903/j.efsa.2010.1805. Available online: www.efsa.europa.eu/efsajournal.htm

Fabia, R. et al., 1993. The effect of exogenous administration of *Lactobacillus reuteri* R2LC and oat fiber on acetic acid-induced colitis in the rat. *Scandinavian journal of gastroenterology*, 28(2), pp.155–162.

Finch, P. & Yoon, J.H., 1997. The effects of organic solvents on the synthesis of galactose disaccharides using [beta]-galactosidases. *Carbohydrate Research*, 303(3), pp.339–345.

Fomon, S.J., 2001. Infant feeding in the 20th century: formula and beikost. *Journal of Nutrition*, 131(2), p.409S.

Gänzle, M.G., Haase, G. & Jelen, P., 2008. Lactose: Crystallization, hydrolysis and value-added derivatives. *International Dairy Journal*, 18(7), pp.685-694.

Gibson, G.R. & Roberfroid, M.B., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *Journal of Nutrition*, 125(6), p.1401.

Gibson, G.R. et al., 2004. Dietary Modulation of the Human Colonic Microbiota: Updating the Concept of Prebiotics. *Nutrition Research Reviews*, 17(02), pp.259-275.

Gnoth, M.J. et al., 2000. Human milk oligosaccharides are minimally digested in vitro. *Journal of Nutrition*, 130(12), p.3014.

Gorke, J., Srienc, F. & Kazlauskas, R., 2010. Toward advanced ionic liquids. Polar, enzyme-friendly solvents for biocatalysis. *Biotechnology and Bioprocess Engineering*, 15(1), pp.40–53.

Gosling, A. et al., 2010. Recent advances refining galactooligosaccharide production from lactose. *Food Chemistry*, 121(2), pp.307-318.

Guarner, F. et al., 2008. Probiotics and prebiotics. Available at: evelyntribole.com [Accessed May 10, 2010].

György, P. et al., 1974. Undialyzable Growth Factors for Lactobacillus bifidus var. pennsylvanicus. *European Journal of Biochemistry*, 43(1), pp.29-33.

Gyorgy, P., 1953. A hitherto unrecognized biochemical difference between human milk and cow's milk. *Pediatrics*, 11(2), pp.98-108.

Haase, G. & Nickerson, T., 1966. Kinetic reactions of alpha and beta lactose. I. Mutarotation. Journal of dairy science, 49(2), pp.127-132.

Hanson, L., 2004. Protective effects of breastfeeding against urinary tract infection. *Acta Paediatrica*, 93(2), p.154.

Harduin-Lepers, A., Shaper, J.H. & Shaper, N.L., 1993. Characterization of two cis-regulatory regions in the murine beta 1,4-galactosyltransferase gene. Evidence for a negative regulatory element that controls initiation at the proximal site. *Journal of Biological Chemistry*, 268(19), pp.14348-14359.

Harms, G. et al., 1996. Binding specificity of influenza C-virus to variablyO-acetylated glycoconjugates and its use for histochemical detection ofN-acetyl-9-O-acetylneuraminic acid in mammalian tissues. *Glycoconjugate Journal*, 13(4), pp.621-630.

Hasegawa, S., Baba, T. & Hori, Y., 1980. Suppression of Allergic Contact Dermatitis by alpha-L-fucose. *Journal of Investigative Dermatology*, 75(3), pp.284–287.

Hendershot, G.E., 1984. Trends in Breast-Feeding. *Pediatrics*, 74(4), pp.591-602.

Henrissat, B., 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal*, 280(Pt 2), pp.309-316.

Holmgren, J., Svennerholm, A.M. & Lindblad, M., 1983. Receptor-like glycocompounds in human milk that inhibit classical and El Tor Vibrio cholerae cell adherence (hemagglutination). *Infect. Immun.*, 39(1), pp.147-154.

Hooper, L.V. et al., 1995. Differential Expression of GalNAc-4-sulfotransferase and GalNAc-transferase Results in Distinct Glycoforms of Carbonic Anhydrase VI in Parotid and Submaxillary Glands. *Journal of Biological Chemistry*, 270(11), pp.5985-5993.

Hudault, S. et al., 1994. Relationship between intestinal colonization of Bifidobacterium bifidum in infants and the presence of exogenous and endogenous growth-promoting factors in their stools. *Pediatric research*, 35(6), p.696.

Jensen, R.G., 1995. Handbook of milk composition, San Diego (USA): Academic press.

Jones, S. & Versalovic, J., 2009. Probiotic *Lactobacillus reuteri* biofilms produce antimicrobial and anti-inflammatory factors. *BMC Microbiology*, 9(1), p.35.

Ji, E.S., Park, N.H. & Oh, D.K., 2005. Galacto-oligosaccharide production by a thermostable recombinant beta-galactosidase from Thermotoga maritima. *World Journal of Microbiology and Biotechnology*, 21(5), pp.759–764.

Juers, D.H. et al., 2003. Structural Basis for the Altered Activity of Gly794 Variants of Escherichia coli [beta]-Galactosidase. *Biochemistry*, 42(46), pp.13505–13511.

Kaftzik, N., Wasserscheid, P. & Kragl, U., 2002. Use of Ionic Liquids to Increase the Yield and Enzyme Stability in the β-Galactosidase Catalysed Synthesis of N-Acetyllactosamine. *Organic Process Research & Development*, 6(4), pp.553-557.

Karlsson, K.A., 1995. Microbial recognition of target-cell glycoconjugates. *Current Opinion in Structural Biology*, 5(5), pp.622–635.

Kleppe, R. & Khorana, H.G., 1972. Studies on Polynucleotides. *Journal of Biological Chemistry*, 247(19), pp.6149-6156.

Kramer, M.S. et al., 2001. Promotion of Breastfeeding Intervention Trial (PROBIT): A Randomized Trial in the Republic of Belarus. *JAMA*, 285(4), pp.413-420.

Kunz, C. & Rudloff, S., 2006. Health promoting aspects of milk oligosaccharides. *International Dairy Journal*, 16(11), pp.1341-1346.

Kunz, C. et al., 1999. Lactose-Derived Oligosaccharides in the Milk of Elephants: Comparison with Human Milk. *British Journal of Nutrition*, 82(05), pp.391-399.

Kuroda, Y., 1974. Differential inhibition by fucoses of aggregation of rat hepatoma cells in rotation-mediated cell cultures. *Journal of the National Cancer Institute*, 52(1), pp.161-166.

Li, W. et al., 2010. Synthesis of oligosaccharides with lactose and N-acetylglucosamine as substrates by using β-d-galactosidase from Bacillus circulans. *European Food Research and Technology*, 231(1), pp.55-63.

Lee, V.S., 2009. The use of crude cell extracts of lactic acid bacteria optimized for beta-galactosidase activity to form galactooligosaccharides with lactose, mannose, fucose, and N-acetylglucosamine. ,master thesis, Faculty of Graduate Studies and Research Edmonton, Alberta, available at http://repository.library.ualberta.ca

Lönnerdal, B., 2000. Breast milk: a truly functional food. *Nutrition*, 16(7-8), pp.509–511.

Macfarlane, S., Macfarlane, G. & Cummings, J., 2006. Review article: prebiotics in the gastrointestinal tract. *Alimentary Pharmacology & Therapeutics*, 24(5), pp.701-714.

Mahoney, R., 1996. Lactose: enzymatic modification. In Advanced Dairy Chemistry Vol. 3. London: Chapman and Hall, pp. p.77-126.

Mahoney, R.R., 1998. Galactosyl-oligosaccharide formation during lactose hydrolysis: A review. *Food Chemistry*, 63(2), pp.147-154.

Malykh, Y.N., Schauer, R. & Shaw, L., 2001. N-Glycolylneuraminic acid in human tumours. *Biochimie*, 83(7), pp.623–634.

Manucci,F., 2009. *Enzymatic synthesis of Galactooligosaccharides from whey permeate*. master thesis. Dublin Institute of Technology. Available at: http://arrow.dit.ie/cgi/viewcontent.cgi?article=1021&context=tourmas.

Manzella, S.M., Hooper, L.V. & Baenziger, J.U., 1996. Oligosaccharides Containing β1,4-Linked N-Acetylgalactosamine, a Paradigm for Protein-specific Glycosylation. *Journal of Biological Chemistry*, 271(21), pp.12117-12120.

Martinez-Ferez, A. et al., 2006. Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology. *International Dairy Journal*, 16(2), pp.173-181.

Matthews, B.W., 2005. The structure of E. coli [beta]-galactosidase. *Comptes Rendus Biologies*, 328(6), pp.549-556.

Mc Veagh, P. & Miller, J., 1997. Human milk oligosaccharides: only the breast. *Journal of Paediatrics and Child Health*, 33(4), pp.281-286.

Miller, J.B. et al., 1994. The oligosaccharide composition of human milk: temporal and individual variations in

monosaccharide components. *Journal of pediatric gastroenterology and nutrition*, 19(4), p.371.

Miyoshi, E., Moriwaki, K. & Nakagawa, T., 2008. Biological Function of Fucosylation in Cancer Biology. *J Biochem*, 143(6), pp.725-729.

Molin, G. et al., 1993. Numerical taxonomy of Lactobacillus spp. associated with healthy and diseased mucosa of the human intestines. *Journal of Applied Microbiology*, 74(3), pp.314–323.

Moris, J.B., 1982. Enzymatic assay for subnanomole amounts of L -fucose. *Analytical Biochemistry* 121 (1), pp 129-134

Moller, P.L. et al., 2001. Intra- and Extracellular {beta}-Galactosidases from Bifidobacterium bifidum and B. infantis: Molecular Cloning, Heterologous Expression, and Comparative Characterization. *Appl. Environ. Microbiol.*, 67(5), pp.2276-2283.

Mülhardt, C., 2008. *Der Experimentator: Molekularbiologie/ Genomics*, Springer.

Mussatto, S.I. & Mancilha, I.M., 2007. Non-digestible oligosaccharides: A review. *Carbohydrate polymers*, 68(3), pp.587–597.

Nakano, T., Sugawara, M. & Kawakami, H., 2001. Sialic acid in human milk: composition and functions. *Acta Paediatrica Taiwanica* = Taiwan Er Ke Yi Xue Hui Za Zhi, 42(1), pp.11-17.

Newburg, D.S. et al., 1990. Fucosylated Oligosaccharides of Human Milk Protect Suckling Mice from Heat-Stabile Enterotoxin of Escherichia coli. *The Journal of Infectious Diseases*, 162(5), pp.1075-1080.

Newburg, D.S., 1997. Do the Binding Properties of Oligosaccharides in Milk Protect Human Infants from Gastrointestinal Bacteria? *J. Nutr.*, 127(5), p.980S.

Nguyen, T. et al., 2006. Purification and Characterization of Two Novel [beta]-Galactosidases from *Lactobacillus reuteri*. *J. Agric. Food Chem*, 54(14), pp.4989–4998.

Nikawa, H. et al., 2004. *Lactobacillus reuteri* in bovine milk fermented decreases the oral carriage of mutans streptococci. *International journal of food microbiology*, 95(2), pp.219–223.

Obermeier, S. et al., 1999. Secretion of 13C-labelled oligosaccharides into human milk and infant's urine after an oral [13C]galactose load. *Isotopes in Environmental and Health Studies*, 35(1-2), pp.119-125.

Oh, P.L. et al., 2009. Diversification of the gut symbiont *Lactobacillus reuteri* as a result of host-driven evolution. ISME J, 4(3), pp.377-387.

Pedersen, A., Sandstr\öm, B. & Van Amelsvoort, J.M., 2007. The effect of ingestion of inulin on blood lipids and gastrointestinal symptoms in healthy females. *British Journal of Nutrition*, 78(02), pp.215–222.

Pencharz, P. et al., 2005. Global standard for the composition of infant formula: recommendations of an ESPGHAN coordinated international expert group. *Journal of pediatric gastroenterology and nutrition*, 41(5), p.584.

Prasad, K.N., 1980. Butyric acid: A small fatty acid with diverse biological functions. *Life Sciences*, 27(15), pp.1351-1358.

Prenosil, J.E., Stuker, E. & Bourne, J.R., 1987. Formation of oligosaccharides during enzymatic lactose hydrolysis and their importance in a whey hydrolysis process: Part II: Experimental. *Biotechnology and Bioengineering*, 30(9), pp.1026-1031.

Prenosil, J.E., Stuker, E. & Bourne, J.R., 1987. Formation of oligosaccharides during enzymatic lactose: Part I: State of art. *Biotechnology and Bioengineering*, 30(9), pp.1019-1025.

Rabiu, B.A. et al., 2001. Synthesis and Fermentation Properties of Novel Galacto-Oligosaccharides by {beta}-Galactosidases from Bifidobacterium Species. *Appl. Environ. Microbiol.*, 67(6), pp.2526-2530.

Rastall, R.A. & Maitin, V., 2002. Prebiotics and synbiotics: towards the next generation. *Current Opinion in Biotechnology*, 13(5), pp.490–496.

Reuter, G., 2001. The Lactobacillus and Bifidobacterium microflora of the human intestine: composition and succession. *Current Issues in Intestinal Microbiology*, 2(2), pp.43–53.

Roberfroid, M., 2007. Prebiotics: The Concept Revisited. *J. Nutr.*, 137(3), pp.830S-837.

Roberfroid, M.B., 2000. Prebiotics and probiotics: are they functional foods? *American Journal of Clinical Nutrition*, 71(6), p.1682S.

Rycroft, C.[. et al., 2001. Fermentation properties of gentio-oligosaccharides. *Letters in Applied Microbiology*, 32, pp.156-161.

Saarela, M. et al., 2002. Gut bacteria and health foods--the European perspective. *International Journal of Food Microbiology,* 78(1-2), pp.99-117.

Sako, T., Matsumoto, K. & Tanaka, R., 1999. Recent progress on research and applications of non-digestible galacto-oligosaccharides. *International Dairy Journal*, 9(1), pp.69-80.

Salvatore, S. et al., 2000. A pilot study of N-acetyl glucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease. *Alimentary Pharmacology and Therapeutics*, 14(12), pp.1567-1579.

Schley, P.D. & Field, C.J., 2002. The Immune-Enhancing Effects of Dietary Fibres and Prebiotics. *British Journal of Nutrition*, 87(Supplement S2), pp.S221-S230.

Seeberger, P.H., 2009. Chemical glycobiology: why now? *Nature Chemical Biology*, 5(6), pp.368–372.

Shaukat, A. et al., 2010. Systematic Review: Effective Management Strategies for Lactose Intolerance. *Annals of Internal Medicine*.

Shields, K. et al., 2003. Chitosan for weight loss and cholesterol management. Available at: http://www.ajhp.org/cgi/content/citation/60/13/1310 [Accessed May 10, 2010].

Siró, I. et al., 2008. Functional food. Product development, marketing and consumer acceptance--A review. *Appetite*, 51(3), pp.456-467.

Smithers, G.W., 2008. Whey and whey proteins--From 'gutter-to-gold'. *International Dairy Journal*, 18(7), pp.695-704.

Smoot, J.T. & Demchenko, A.V., 2009. Chapter 5 Oligosaccharide Synthesis: From Conventional Methods to Modern Expeditious Strategies. In Academic Press, pp. 161-250. Available at: http://www.sciencedirect.com/science/article/B7CS1-4WM0HVK-8/2/51a747f181deb8b7811e02a6db109e39 [Accessed May 14, 2010].

Spiller, G.A., 2001. CRC handbook of dietary fiber in human nutrition, CRC Press.

Splechtna, B. et al., 2006. Production of Prebiotic Galacto-Oligosaccharides from Lactose Using β-Galactosidases from *Lactobacillus reuteri*. *Journal of Agricultural and Food Chemistry*, 54(14), pp.4999-5006.

Springer, G.F. & Williamson, P., 1962. Immunochemical significance of l- and d-fucose derivatives. *Biochemical Journal*, 85(2), pp.282-293.

Staudacher, E. et al., 1999. Fucose in N-glycans: from plant to man. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1473(1), pp.216-236.

Takahashi, M. et al., 2009. Lack of chronic toxicity or carcinogenicity of dietary N-acetylglucosamine in F344 rats. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, 47(2), pp.462-471.

Tharanathan, R.N. & Prashanth, 2007. Chitin/chitosan: modifications and their unlimited application potential–an overview. *Trends in food science & technology*, 18(3), pp.117–131.

Thompson, S., Matta, K.L. & Turner, G.A., 1991. Changes in fucose metabolism associated with heavy drinking and smoking: a preliminary report. *Clin Chim Acta*, 201(1-2), pp.59-64.

Torres, D.P. et al., 2010. Galacto-Oligosaccharides: Production, Properties, Applications, and Significance as Prebiotics. *Comprehensive Reviews in Food Science and Food Safety*, 9(5), pp.438–454.

Tran-Thi, T. et al., 1985. Toxicity of D-galactosamine for rat hepatocytes in monolayer culture. *Experimental and Molecular Pathology*, 42(1), pp.89-116.

Valeur, N. et al., 2004. Colonization and Immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the Human Gastrointestinal Tract. *Appl. Environ. Microbiol.*, 70(2), pp.1176-1181.

Vandenplas, Y., 2002. Oligosaccharides in Infant Formula. *British Journal of Nutrition*, 87(Supplement S2), pp.S293-S296.

Varki, A., 1992. Diversity in the sialic acids. *Glycobiology*, 2(1), p.25.

Varki, A., 1993. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology*, 3(2), pp.97-130.

Varma, S.N., Schwarz, V. & Simpson, I.M.N., 1962. The role of dietary lactose in the synthesis of brain galactolipids. *Biochemical Journal*, 85(3), pp.546-549.

Venter, C.S., 2007. Prebiotics: an update. *Journal of Family Ecology and Consumer Sciences*, 35, pp.17–25.

Viverge, D. et al., 1990. Discriminant carbohydrate components of human milk according to donor secretor types. *Journal of pediatric gastroenterology and nutrition*, 11(3), p.365.

Vlad, S.C. et al., 2007. Glucosamine for pain in osteoarthritis: Why do trial results differ? *Arthritis & Rheumatism*, 56(7), pp.2267-2277.

Wallenfels, K., 1951. Enzymatische Synthese von Oligosacchariden aus Disacchariden. *Naturwissenschaften*, 38(13), pp.306–307.

Walter, J., Britton, R.A. & Roos, S., 2010. Host-microbial symbiosis in the vertebrate gastrointestinal tract and the *Lactobacillus reuteri* paradigm. *Proceedings of the National Academy of Sciences*.

Wang, B. & Brand-Miller, J., 2003. The role and potential of sialic acid in human nutrition. *Eur J Clin Nutr*, 57(11), pp.1351-1369.

Wells, L., Vosseller, K. & Hart, G.W., 2003. A role for N -acetylglucosamine as a nutrient sensor and mediator of insulin resistance. *Cellular and Molecular Life Sciences*, 60(2), pp.222-228.

Zadow, J.G., 1984. Lactose: properties and uses. *Journal of dairy science*, 67(11), p.2654.

Zechel, D.L. & Withers, S.G., 2000. Glycosidase Mechanisms: Anatomy of a Finely Tuned Catalyst. *Accounts of Chemical Research*, 33(1), pp.11-18.

## 7.7. Curriculum vitae

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Geburtstag: Montag, 7.3.1983

**Curriculum vitae**

| | |
|---|---|
| 1983 | Geburt in Freistadt (OÖ) |
| 1983 – 1989 | Kindheit in Sautern und Kindergarten in Pitten (NÖ) |
| 1989 – 1993 | Besuch der Volksschule Pitten |
| 1993 – 1997 | Besuch des Clemens Maria Hofbauer Gymnasiums (ehemals Gymnasium der Redemptoristen Katzelsdorf) |
| 1997 – 2002 | Besuch der Handelsakademie Wiener Neustadt; |
| 2002 – 2003 | Zivildienst beim ÖHTB (Österreichisches Hilfwerk für Taubblinde und hochgradig Hör- und Sehbhinderte) |
| 2003 – 2006 | Studium der Biotechnologie am fh-campus wien, Vertiefungsrichtung: Wirkstoffchemie |
| 2006 | Praxissemester an der Háskoli Íslands in Reykjavik, Island; |
| 2007 | Diplomarbeitssemster an der Háskoli Íslands zur Erlangung des akademischen Grades Diplomingenieur (FH) |
| seit 2008 | Studium der beiden Masterstudiengänge "Lebensmittelwissenschaften und -technolgie" and "Biotechnologie" an der Universität für Bodenkultur Wien |
| Jänner 2011 | Abschluss des Masterstudiums "Lebensmittelwissenschaften und -technologie" Erlangung des akademischen Grades Diplomingenieur |

## 7.8. Eigenständigkeitserklärung

„Ich erkläre, dass ich die vorliegende Diplomarbeit selbst verfasst habe und dass ich dazu keine anderen als die angeführten Behelfe verwendet habe. Außerdem habe ich die Reinschrift der Diplomarbeit einer Korrektur unterzogen und ein Belegexemplar verwahrt.
Ich erteile meine Zustimmung, dass die vorliegende Arbeit für wissenschaftliche Zwecke öffentlich zugängig gemacht werden kann."

Clemens Malainer