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Klonierung, Expression, biochemische Charakterisierung und Oligosaccharid-synthese durch β-Galactosidase aus *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 und *Bifidobacterium breve* DSM 20231

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ABSTRACT

ARREOLA SHERYL LOZEL. University of Natural Resources and Life Science Vienna. 2014. **Molecular cloning, expression, biochemical characterization, and oligosaccharide synthesis of β-galactosidases from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 and *Bifidobacterium breve* DSM 20231**

Supervisor: Univ.Prof. Dipl.-Ing. Dr.techn. Dietmar Haltrich

The study focused on the use of *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 and *Bifidobacterium breve* DSM 20213 for the production and characterization of β-galactosidase as well galacto-oligosaccharides (GOS) and hetero-oligosaccharides (HeOS) synthesis. It is anticipated that GOS and HeOS produced by these β-galactosidases will be used for the specific proliferation of these bacterial genera in the gut, thus they can be considered prebiotic. The *lacZ* gene from *L. bulgaricus* was cloned into different inducible lactobacillal expression vectors for overexpression in the host strain *L. plantarum* WCFS1 while the two β-galactosidases, BbreβgalI and BbreβgalII, from *B. breve* were overexpressed in *Escherichia coli* with co-expression of the chaperones GroEL/GroES. The three recombinant β-galactosidases were purified to electrophoretic homogeneity and further characterized. When used for lactose conversion in transferase mode, Lbulβgal, BbreβgalI and BbreβgalII showed very high transgalactosylation activity; the maximum yields of GOS was approximately 50, 33, and 44% of total sugars, respectively when using an initial concentration of ~200 g/L lactose. The predominant transgalactosylation products of BbreβgalI and BbreβgalII are β-D-Gal*p*-(1→6)-D-Glc and β-D-Gal*p*-(1→3)-D-Lac while that of Lbulβgal are β-D-Gal*p*-(1→6)-D-Glc and β-D-Gal*p*-(1→6)-Lac. Lbulβgal, BbreβgalI,

and BbreβgalII were also investigated with respect to their propensity to transfer galactosyl moieties onto lactose, D-glucose and D-galactose, L-fucose, GlcNAc and GalNAc under defined, initial-velocity conditions. Galactosyl transfer from Lbulβgal or BbreβgalII to GlcNAc occurs with a partitioning ratios k_{Nu}/k_{water} that are 2 and 6 times those for the reactions of the galactosylated enzymes with glucose and lactose, respectively. Using lactose as galactosyl donor and GlcNAc as acceptor, Lbulβgal and BbreβgalII synthesized β-D-Gal*p*-(1→6)-GlcNAc as the major product. The structure of this product was confirmed by NMR. These results indicate that these enzymes can be of interest for synthesis of both prebiotic GOS and HeOS.

Klonierung, Expression, biochemische Charakterisierung und Oligosaccharid-synthese durch β-Galactosidase aus *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 und *Bifidobacterium breve* DSM 20231

ZUSAMMENFASSUNG

Im Mittelpunkt dieser Arbeit stand die Anwendung von *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 und *Bifidobacterium breve* DSM 20213 für die Produktion des Enzyms β-Galactosidase, die biochemische Charakterisierung dieser Enzyme sowie die Bildung von Galactooligosacchariden (GOS) und Heterooligosacchariden (HeOS). Während GOS bereits als Präbiotika sehr gut etabliert sind, kann auch angenommen werden, dass HeOS, die mittels dieser Enzyme aus probiotischen Organismen gebildet werden, ebenfalls präbiotische Eigenschaften aufweisen und speziell das Wachstum von Lactobazillen bzw. Bifidobakterien im Darm fördern. Das *lacZ* Gen aus *L. bulgaricus*, welches für die β-Galactosidase kodiert, wurde mittels verschiedener induzierbarer Vektoren in *L. plantarum* WCFS1 überexprimiert, während die beiden β-Galactosidasen aus *B. breve*, BbreβgalI and BbreβgalII, in *Escherichia coli* heterolog produziert wurden, wobei hier Koexpression der Chaperone GroEL/GroES notwendig war. Diese drei rekombinanten β-Galactosidasen wurden anschließend im Detail biochemisch charakterisiert. *Lbulβgal,* BbreβgalI und BbreβgalII zeigten sehr hohe Transgalactosylierungsaktivität mit Laktose als Substrat; die maximalen Ausbeuten an GOS lagen bei etwa 50, 33 und 44% relativ zu den gesamten Zuckern bei Anwendung von ~200 g/L Laktose als Ausgangssubstrat. Die wichtigsten Transgalaktosylierungsprodukte von BbreβgalI und BbreβgalII waren β-D-Gal*p*-(1→6)-D-Glc und β-D-Gal*p*-(1→3)-D-Lac, während

Lbulβgal primär β-D-Gal*p*-(1→6)-D-Glc und β-D-Gal*p*-(1→6)-Lac bildete. Die drei Enzyme wurden ebenfalls bezüglich ihrer Eignung, auf bestimmte Zuckerakzeptoren (D-Galactose, L-Fucose, GlcNAc and GalNAc) Galaktosylreste zu übertragen, untersucht. Diese Eigenschaft wurde mittels des Partitionsverhältnisses k_{Nu}/k_{water} quantifiziert. Für Lbulβgal und BbreβgalII war dieses Verhältnis für GlcNAc als Akzeptor etwa 2 bzw. 6 Mal höher als für Glucose und Lactose, was darauf hinweist, dass GlcNAc einen ausgezeichneter Akzeptor für den Galactosylrest darstellt. Bei Verwendung von Laktose als Galactosyldonor und GlcNAc als Akzeptor konnte schließlich mit beiden Enzymen β-D-Gal*p*-(1→6)-GlcNAc als Hauptprodukt in sehr guten Ausbeuten erhalten. Die Struktur dieses Produktes wurde auch mittels NMR bestätigt.

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To UPLB Institute of Chemistry…

To my family and friends…

MY DEEPEST GRATITUDE

To GOD be the Glory!

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B	Intanon, M.; **Arreola, S. L.;** Pham, N. H.; Kneifel, W.; Haltrich, D.; Nguyen, T. H., Nature and biosynthesis of galacto-oligosaccharides related to oligosaccharides in human breast milk. *FEMS Microbiology Letters* **2014,** *353*, 89-97.	B1
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D	**Arreola, S. L.;** Intanon, M.; Suljic, Jasmina; Kittl, Roman; Ngoc, H. P.; Kosma, P; Haltrich, D.; Nguyen, T.-H., Two β-galactosidases from the human isolate *Bifidobacterium breve* DSM 20213: Molecular cloning and expression, biochemical characterization and synthesis of galacto-oligosaccharides. (Revision submitted to PLOS ONE).	D1
E	**Arreola, S. L.;** Intanon, M.; P.; Kosma, P; Haltrich, D.; Nguyen, T.-H., Biochemical characterization of four β-galactosidases towards formation of galacto- and hetero-oligosaccharides. (In preparation for submission to PLOS ONE)	E1

I. INTRODUCTION[a]

A. Prebiotics and probiotics

Probiotics refer to viable microorganisms which promote or stimulate beneficially the microbial population of the gastro-intestinal tract or GIT. (*1*) Lactic acid bacteria (LAB) and bifidobacteria are the major representatives of probiotic microorganisms and have long been used in the production of a wide range of foods without adverse effects on humans. (*2*) *Bifidobacterium* and *Lactobacillus* species, among LAB, receive special attention in the applications of probiotic products because of their GRAS (generally recognized as safe) status and beneficial effects on human health. (*3*) Among the *Lactobacillus* and *Bifidobacterium* strains used for applications in probiotic products include *L. acidophilus*, *L. amylovorus, L. casei*, *L. crispatus*, *L. gasseri*, *L. johnsonii*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus, B. bifidum, B. longum, B. infantis, B. breve,* or *B. adolescentis*. (*2, 4-7*)

A dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host's health. (*8*) Based on the criteria (*9, 10*) (i) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; (ii) fermentation by intestinal microflora; and (iii) selective stimulation of growth and/or activity of intestinal bacteria associated with health/well-being, only inulin/fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and lactulose

[a] Part of the following publications:

1) Intanon, M.; **Arreola, S. L.;** Pham, N. H.; Kneifel, W.; Haltrich, D.; Nguyen, T. H., Nature and biosynthesis of galacto-oligosaccharides related to oligosaccharides in human breast milk. *FEMS Microbiology Letters* **2014,** *353*, 89-97.
2) **Arreola, S. L.;** Intanon, M.; Ngoc, H. P.; Haltrich, D.; Nguyen, T.-H., Galacto-Oligosaccharides: Recent Progress on Research and Application As Prebiotics In *Galactose: Structure and Function in Biology and Medicine*, Pomin, V. H., Ed. Nova Science Publishers Hauppauge NY, 2014. (**In Press**)

are fulfilling these requirements for prebiotics as documented and proven in several studies, although promise exists for several other dietary oligosaccharides.(*9-12*) Prebiotic oligosaccharides can serve as fermentable substrates for certain members of the gut microbiota, and have been found to modulate the colonic flora by selective stimulation of beneficial bacteria such as bifidobacteria and lactobacilli as well as inhibition of 'undesirable' bacteria. (*13, 14*)

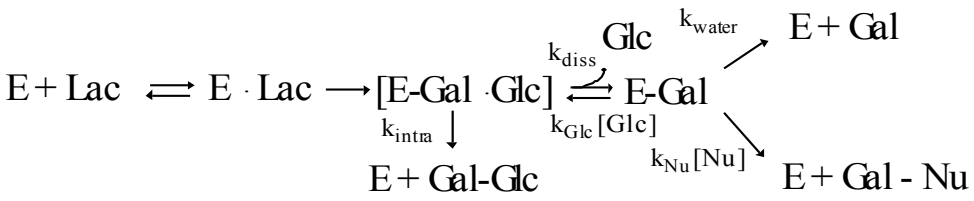
B. Production of galacto-oligosaccharides by β-galactosidases

GOS, the products of transgalactosylation reactions catalyzed by β-galactosidases when using lactose as the substrate, are non-digestible carbohydrates meeting the criteria of 'prebiotics'. GOS are of special interest to human nutrition because of the presence of structurally related oligosaccharides together with different complex structures in human breast milk. (*15-17*) Production of GOS (or sometimes referred to as TOS, transgalactosylated oligosaccharides) typically employs lactose as galactosyl donor and the transfer of the galactosyl moiety of lactose to suitable acceptor carbohydrates or nucleophiles using either glycoside hydrolases (EC 3.2.1.) or glycosyltransferases (EC 2.4.) (*18, 19*). However, due to limited supply, high price and necessity of specific sugar nucleotide as substrate of glycosyltransferases, industrial GOS production favors the use of glycoside hydrolases. (*18*)

Glycoside hydrolases (GH) are classified based on the stereochemical outcome of the hydrolysis reaction; they can be either *retaining* or *inverting* enzymes. Amino acid sequence similarities, hydrophobic cluster analysis, reaction mechanisms, and the conservation of catalytic residues allow classification of β-galactosidases (β-gal; β-D-galactoside galactohydrolase E.C.3.2.1.23; lactase) in the GH families GH1, GH2, GH35, and GH42, indicating their structural diversity. (*19*) GH1 β-glycosidases are

retaining enzymes of which the most commonly known enzymatic activities are myrosinases (thio-β-glucosidases), β-mannosidases, β-galactosidases, phospho-β-glucosidases and phospho-β-galactosidases. The GH2 family to which most of the β-galactosidases belong, comprises the LacZ and LacLM β-galactosidases as isolated and described from *E. coli*, lactic acid bacteria and bifidobacteria. GH1 and GH2 β-galactosidases use only lactose, β-(1→3) and β-(1→6) linked galactosides as their substrates while those belonging to families GH35 and GH42 act on different galactose-containing glycosides including higher oligosaccharides and polysaccharides.(*19*) Owing to the different substrate specificities, β-galactosidases of GH2 and GH42 are often found in the same organism.(*20-22*)

β-Galactosidases catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides such as lactose (*23, 24*) and are found widespread in nature. They catalyze the cleavage of lactose (or related compounds) in their hydrolysis mode thus they are used in the dairy industry to remove lactose from various products. An attractive biocatalytic application is found in the transgalactosylation potential of these enzymes which is based on their catalytic mechanism.(*25*) Retaining β-galactosidases undergo a two-step mechanism of catalysis. First, this mechanism involves the formation of a covalently linked galactosyl-enzyme intermediate. Subsequently, the galactosyl moiety is transferred to a nucleophilic acceptor. Water, as well as all sugar species present in the reaction mixture, can serve as a galactosyl acceptor. Hence, the resulting final mixture contains hydrolysis products of lactose, which are glucose and galactose, unconverted lactose as well as di-, tri- and higher oligosaccharides. (*26, 27*) Scheme 1 illustrates the possible lactose conversion reactions catalyzed by β-galactosidases.



Scheme 1. Hydrolysis and galactosyl transfer reactions during the β-galactosidase catalyzed conversion of lactose. E, enzyme; Lac, lactose; Gal, galactose; Glc, glucose; Nu, nucleophile.

Transgalactosylation involves both intermolecular and intramolecular reactions. Intramolecular or direct galactosyl transfer to D-glucose yields regio-isomers of lactose. The glycosidic bond of lactose (β-D-Gal*p*-(1→4)-D-Glc) is cleaved and immediately formed again at a different position of the glucose molecule before it diffuses out of the active site. This is how allolactose (β-D-Gal*p*-(1→6)-D-Glc), the presumed natural inducer of β-galactosidases in certain microorganisms, is formed even in the absence of significant amounts of free D-glucose (*26, 28*). By intermolecular transgalactosylation, various di-, tri-, tetrasaccharides and eventually higher oligosaccharides are produced. Any sugar molecule in the reaction mixture can be the nucleophile accepting the galactosyl moiety from the galactosyl-enzyme complex, which is formed as an intermediate in the reaction. The GOS produced are kinetic intermediates; they act as substrates for hydrolysis (*28, 29*) hence GOS yield and composition change dramatically with reaction time, and the GOS mixtures thus obtained are very complex and can hardly be predicted.

β-Galactosidases can be obtained from different sources including microorganisms, plants and animals. Microbial sources of β-galactosidase are of great biotechnological interest because of easier handling, higher multiplication rates, and production yield. An extensive list of bacterial and fungal sources of β-galactosidases, as well as the lactose conversion reaction conditions and GOS yields, are given in

(*30*). Table 1 presents some of the commercially available bacterial, fungal and yeast β-galactosidases.

Table 1. Commercially available β-galactosidases

Name	Manufacturer	Microorganism
BioLactase NTL-CONC	Biocon	*Bacillus circulans (31, 32)*
Lactozym pure 6500 L	Novozymes	*Kluyveromyces lactis (32)*
Lactase F "Amano"	Amano Enzyme Inc	*Aspergillus oryzae (32, 33)*
Biolacta FN5	Daiwa Fine Chemicals Co., Ltd.	*Bacillus circulans (33, 34)*
LACTOLES L3	Biocon Ltd., Japan	*Bacillus circulans (33)*
Maxilact	DSM Food Specialties	*Kluyveromyces lactis (35, 36)*
Tolerase	DSM Food Specialties	*Aspergillus oryzae (35, 36)*

β-galactosidases from different species possess very different specificities for building glycosidic linkages, and therefore produce different GOS mixtures. *Kluyveromyces lactis* β-galactosidase produced predominantly β-(1→6)-linked GOS (*37*) while *Aspergillus oryzae* β-galactosidase produced mainly β-(1→6) followed by β-(1→3) and β-(1→4) linked GOS (*38*), *Bacillus circulans* β-galactosidase forms β-(1→2), β-(1→3), β-(1→4), β-(1→6) linked GOS (*39*), whereas β-galactosidases from *Lactobacillus* spp. showed preference to form β-(1→3) and β-(1→6) linkages in transgalactosylation mode. (*28, 40-42*)

A number of studies have focused on the use of the genera *Bifidobacterium* and *Lactobacillus* for the production and characterization of β-galactosidases. (*40-52*) It is anticipated that GOS produced by these β-galactosidases will have better selectivity for growth and metabolic activity of these bacterial genera in the gut, and thus will lead to improved prebiotic effects.(*53*) GOS are produced from lactose by

microbial β-galactosidases employing different enzyme sources and preparations including crude enzymes, purified enzymes, recombinant enzymes, immobilized enzymes, whole-cell biotransformations, toluene-treated cells, and immobilized cells. The enzyme sources, the process parameters as well as the yield and the productivity of these processes for GOS production are summarized in detail in recent reviews.(*17, 30, 54, 55*)

The choice of process technology either for lactose hydrolysis or GOS production depends on the nature of the substrate and the characteristics of the enzyme. The primary characteristic, which determines the choice and application of a given enzyme, is the operational pH range. Acid-pH enzymes, which are mainly from fungi, are suitable for processing of acid whey and whey permeate, while the neutral-pH enzymes from yeasts and bacteria are suitable for processing milk and sweet whey. Depending on the enzyme source, the pH value of the reaction mixture can be very acidic when using β-galactosidases from *A. oryzae* and *Bullera singularis* with optimum GOS yields at pH 4.5 and 3.7, respectively. (*56, 57*) Isobe and others studied the β-galactosidase from an acidophilic fungus, *Teratosphaeria acidotherma* AIU BGA-1, which was stable over the pH range of 1.5 to 7.0 and exhibited optimal activity at pH 2.5-4.0 and 70°C (*58*). Most bacteria and fungi however, observed maximum yield of GOS at neutral pH. (*46*) The highest GOS yields are generally observed when the reaction proceeds to 45 - 90% lactose conversion. (*30*) Furthermore, these reports described the cloning and characterization of these enzymes and studied their transgalactosylation activity in detail, for example β-galactosidase BgbII from *B. adolescentis* showed high preference towards the formation of β-(1→4) linkages while no β-(1→6) linkages were formed.(*59*) In contrast, the β-galactosidase BgbII from *B. bifidum* showed a clear preference for the

synthesis of β-(1→6) linkages over β-(1→4) linkages.(*21*) A recombinant β-galactosidase from *B. infantis* was found to be an excellent biocatalyst for GOS production giving the highest GOS yield of 63%.(*60*)

In order to reduce enzyme costs by avoiding laborious and expensive chromatographic steps for the purification of the biocatalyst, crude β-galactosidase extract from *Lactobacillus* sp. directly obtained after cell disruption and separation of cell debris by centrifugation was used in lactose conversion for GOS production.(*61*) It was reported that there was no obvious difference in the obtained GOS yields using either purified or crude β-galactosidase at 37 °C, and in addition the crude enzyme was found to be equally stable as the purified one. Therefore, crude β-galactosidase extracts are suitable for a convenient and simple process of GOS production. Because of the GRAS status of most *Lactobacillus* spp., it is also safe to use these crude extracts in food and feed applications. The reduction of reaction temperature to 17 °C to limit microbial growth and the use of a cheap lactose source such as whey permeate powder did not have significant adverse effects on the GOS yield.(*61*)

Protein engineering is a powerful approach to favor transgalactosylation over hydrolysis, and hence to improve transgalactosylation yields. A truncated β-galactosidase from *B. bifidum* enhanced the transgalactosylation activity of the enzyme towards lactose and as a result a normal, hydrolytic β-galactosidase was converted to a highly efficient transgalactosylating enzyme.(*62*) A mutagenesis approach was applied to the galactosidase BgaB of *Geobacillus stearothermophilus* KVE39 in order to improve its enzymatic transglycosylation of lactose into oligosaccharides. Exchange of one amino acid, arginine Arg109, in β-galactosidase BgaB to either lysine, valine or tryptophan improved significantly the formation of the main trisaccharide, i.e. 3'-galactosyllactose. The yield of this trisaccharide increased

from 2% to 12%, 21% and 23%, respectively, for these different variants compared to that of the native enzyme.(*63*) Enhancement of the production of GOS was achieved by mutagenesis of *Sulfolobus solfataricus* β-galactosidase LacS. Site-directed mutagenesis was performed to obtain two mutants of LacS, F441Y and F359Q, and the GOS yield was increased by 10.8 and 7.4%, respectively.(*64*) Although protein engineering strategies were successful to enhance transgalactosylation activities of different β-galactosidases it has not been described for β-galactosidases yet that this approach was successful to alter also the linkage type of the GOS products.(*65*)

GOS are manufactured and commercialized mainly in Japan, the United States and Europe. The major manufacturers are Yakult Honsha (Japan) with their product Oligomate, Nissin Sugar Manufacturing (Japan) with Cup-Oligo, Snow Brand Milk Products (Japan) with P7L, GTC Nutrition (United States) with Purimune, Friesland Foods Domo (the Netherlands) with Vivinal GOS, Clasado Ltd.(UK) with Bimuno, and Dairy Food Ingredients (Ireland) with Dairygold GOS.(*17, 30, 66*) Commercial GOS preparations typically are transparent syrups or white powders containing oligosaccharides of different DP, non-converted lactose and the monosaccharides glucose and galactose. They differ in purity of the GOS products and in the linkages of the oligosaccharide chains, which depend on the enzymes used for the GOS productions. Oligomate contains mainly β-(1→6) linked GOS, Vivinal GOS, Cup-Oligo and Purimune contain mainly β-(1→4) linkages, while Bimuno contains mainly β-(1→3) linked GOS.(*30*) Table 2 presents some commercial GOS and the enzymes used in their productions.

Table 2. Composition and enzyme source of commercial GOS

Product	Company	Total GOS (% w/w)	Enzyme Source
Oligomate 55	Yakult Pharmaceutical Industry Co., Ltd. Tokyo, Japan	>55	*Sporobolomyces singularis, Kluyveromyces lactis (67, 68)*
Vivinal® GOS	Friesland Foods Domo, Amersfoort, The Netherlands	~60	*Bacillus circulans(69, 70)*
Purimune™	GTC Nutrition, Colorado, USA	≥ 90	*Bacillus circulans(68, 71)*
Bimuno® GOS	Clasado Ltd., Milton Keynes, England	48-55	*Bifidobacterium bifidum (72, 73)*
Cup-oligo	Kowa Company Ltd., Tokyo, Japan	70	*Cryptococcus laurentii* (*30*, *74*)

C. Physical and biological characterization of GOS

The physico-chemical properties of GOS are of significant interest for their application in the food industries. Generally, GOS are transparent/colorless water-soluble products (80% w/w solubility), and more viscous than high-fructose corn syrup.(*14*, *30*) GOS are stable during treatment at elevated temperature of up to 160 °C and as low as pH 2. They are also stable during long-term storage at room temperature under acidic conditions.(*27*) The caloric value of GOS was estimated to be 1.7 kcal g^{-1}, this is approximately 30-50% of those of digestible carbohydrates such as sucrose.(*27*) GOS are low-calorie sweeteners since they pass through the human small intestine without being digested. GOS are undigested by pancreatic enzymes and gastric juice while passing the small intestine, which makes them suitable for low-calorie diets and for consumption by individuals with diabetes.(*30*) They can be used as humectants because of their high moisture-retaining capacity to prevent excessive drying, hence to keep the foodstuff moist. They can alter the freezing temperature of frozen foods and reduce the amount of coloring due to Maillard reactions in heat-processed foods as relatively fewer reducing moieties are available.(*30, 75*) These

properties enable GOS to be applied in a wide variety of food products. Apart from being used as sweeteners, GOS are nowadays incorporated in a wide range of products such as fermented milk products, breads, jams, snack bars, confectionery, beverages, infant milk formulas, and as sugar replacements.(*14*, *27*)

Prebiotic galacto-oligosaccharides can serve as fermentable substrates for certain members of the gut microbiota, and have been found to modulate the colonic flora.(*13, 14*) The physiological importance and health benefits of prebiotic GOS have been reported extensively in several recent reviews on prebiotics and functional oligosaccharides.(*17, 76*) The biological effects of GOS on human health are discovered in many different dimensions.

Prebiotic effects of GOS depend significantly on the degree polymerization, the linkage types and composition of the GOS mixtures. These differences are known to be important when it comes to GOS assimilation by beneficial bacteria in the colon. It was reported that the administration of a GOS mixture containing the β-(1→3), β-(1→4) and β-(1→6) linkages proved to have a better bifidogenic effect than a mixture containing GOS with β-(1→4) and β-(1→6) linkages alone.(*53*) Furthermore, bifidogenic properties of GOS are dose dependent. It is known that bifidobacteria populations generally increase as the GOS dosage and purity increase. However, it has been shown that even when GOS were administered for many weeks and at high doses, there were still some individuals for whom a bifidogenic response did not occur.(*77*)

D. Human Milk Oligosaccharides (HMO)

Human milk is known as the sole source of nourishment for breast-fed infants and for its promotion of a healthy development of newborns. Human milk is comprised of a complex mixture of oligosaccharides (5-10 g L^{-1} in addition to lactose) that are different in size, linkage and charge.(*78*) HMO are a heterogenic group of about 200 molecular species consisting of mostly neutral and fucosylated oligosaccharides.(*79*) The potential health benefits of HMO have been studied with emphasis on prebiotic effects.(*78*) *In vitro* studies have shown that HMO bind and block the infection of pathogenic bacteria to animal cells by acting as receptor analogues to the intestinal cell glycans.(*80-82*) Studies have shown the ability of HMO to selectively support the growth of specific strains of bifidobacteria thus providing insight on how HMO modulate the infant intestinal microbiota. These results suggest that the prebiotic and bifidogenic effects of HMO are structure-specific and may vary depending on the HMO composition in milk.(*80, 83, 84*). Because of these, prebiotic HMO are of great interest for human nutrition.

HMO are composed of both neutral and anionic species with building blocks of 5 monosaccharides: D-glucose (Glc), D-galactose (Gal), *N*-acetyl-D-glucosamine (GlcNAc), L-fuctose (Fuc), and *N*-acetylneuraminic acid (sialic acid). The basic structure of HMO includes a lactose core at the reducing end which is elongated by *N*-acetyllactosamine units with at least 12 different types of glycosidic bonds, wherein fucose and sialic acid residues are added to terminal positions.(*80, 85*) The terminal lactose is typically elongated by lacto-*N*-biose units (LNB; β-D-Gal*p*-(1→3)-D-GlcNAc) in type I or *N*-acetyllactosamine units (LacNAc; β-D-Gal*p*-(1→4)-D-GlcNAc) in the rarer type II structures. Both LNB and LacNAc are attached via a β-(1→3) linkage to the galactosyl moiety of the terminal lactose, with an additional β-

1,6-linkage in branched HMO. These LNB and LacNAc units can be repeated up to 25 times in larger HMO, forming the core region of these oligosaccharides. A further variation results from the attachment of fucosyl and sialic acid residues. Thus, the simplest structures following this general scheme (apart from certain trisaccharides such as galactosyllactose, fucosyllactose and sialyllactose) are the tetrasaccharides lacto-*N*-tetraose (β-D-Gal*p*-(1→3)-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc; type I), and lacto-*N*-*neo*-tetraose, (β-D-Gal*p*-(1→4)-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc; type II) (*86-89*). Mono- and difucosyllactose, lacto-*N*-tetraose and its fucosylated derivatives as well as sialyllactose and the sialylated forms of lacto-*N*-tetraose are the major HMO in human milk.

E. Hetero-oligosaccharides formation by β-galactosidases

Oligosaccharides produced by heterologous galactosyl transfer are termed as hetero-oligosaccharides or HeOS.(*90*) HMO structures or structurally related compounds can be accessed through different approaches. One such approach that has received some interest is based on β-galactosidase-catalyzed transglycosylation. *Sulfolobus solfataricus* and *Kluyveromyces lactis* β-galactosidases were used to produce lactulose and galactosylated aromatic primary alcohols (*91, 92*), respectively while an array of sugar alcohols and mono- and disaccharides have been shown to act as an acceptor carbohydrate for *Enterobacter cloacae* B5 β-gal (*93*). Moreover, β-gal from *Bacillus circulans, K. lactis* and *L. bulgaricus* were proved to be a suitable biocatalyst for the production of *N*-acetyl-oligosaccharides using lactose and GlcNAc as substrates.(*94-96*) Other galactosyl acceptor carbohydrates that have been reported include mannose, fructose, Fuc, xylose, glucoronic acid, sucrose, maltose, lactulose, maltose-oligosaccharodes, cycloisomaltooctaose or ruboside. (*90*) HeOS from lactose

include compounds which incorporate Fuc, Gal, mannose and GlcNAc e.g. monosaccharides that are frequently present in glycans recognized by bacterial pathogens or bacterial toxins. (*97*)

However, it is challenging to deduce the specificity of the galactosylated enzyme for the reaction with nucleophile. A generally simple equation (Equation 1) has been developed to determine the ability of different nucleophile to act as galactosyl moiety acceptor which in turn allows an estimation of the transgalactosylation products obtained.(*98-101*) Novel HeOS are of interest because of a potentially extended functionality in addition to GOS. The transfer constant ratio k_{Nu}/k_{water} can be obtained by applying the velocity ratio (ν_{oNP}/ (ν_{Gal}) or (ν_{Glc}/ (ν_{Gal}) against acceptor concentration and fitting on the equation using the nonlinear least fit

$$\frac{\nu_{Glc}}{\nu_{Gal}} = 1 + \frac{k_{Nu}}{k_{water}}[Nu] \qquad \text{Equation 1}$$

F. Objectives

The general objective of this study is to clone, express, purify and biochemically characterize the β-galactosidases from *L. delbrueckii* subsp. *bulgaricus* DSM 20081 *and Bifidobacterium breve* DSM 20231 and investigate their ability to produce galacto- and hetero-oligosaccharides in biocatalytic processes. The specific objectives are to:

(1) heterologously express the single-gene encoded β-galactosidase (*LacZ*) from *L. bulgaricus* in *L. plantarum* using pSIP vectors thus, the overexpression of this enzyme in a food grade host,

(2) clone two β-galactosidases, BbreβgalI and BbreβgalII, from *B. breve* and express in *Escherichia coli*,

(3) purify, characterize and compare from *L. bulgaricus* β-galactosidase (Lbulβgal), BbreβgalI and BbreβgalII with respect to the spectrum of GOS produced,

(4) determine the propensity of Lbulβgal, BbreβgalI, BbreβgalII and from *L. reuteri* β-gal (Lreuβgal) to transfer galactosyl moiety to different acceptors (lactose, Glc, Gal, Fuc, GlcNAc and GalNac) under defined and initial velocity conditions,

(5) Optimize conditions for transgalactosylation of Lbulβgal and BbreβgalII using lactose as galactosyl donor and GlcNAc as galactosyl acceptor, and

(6) Identify the major transgalactosylation products of Lbulβgal and BbreβgalII using lactose as galactosyl donor and GlcNAc galactosyl acceptor using NMR.

II. MATERIALS AND METHODS[a]

A. Chemicals and vectors

All chemicals and enzymes were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise and were of the highest quality available. The test kit for the determination of D-glucose was obtained from Megazyme (Wicklow, Ireland). All restriction enzymes, T4 DNA ligase, and corresponding buffers were from Fermentas (Vilnius, Lithunia). The plasmid pET-21a (+) was from Novagen (Darmstadt, Germany) and the plasmid pGRO7 encoding the chaperones GroEL and GroES was purchased from TAKARA Bio Inc.(Shiga, Japan). Galacto-oligosaccharide standards of β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→6)-D-Glc, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→4)-D-Gal, β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Lac, β-D-Gal*p*-(1→4)-D-Lac, β-D-Gal*p*-(1→6)-D-Lac were purchased from Carbosynth (Berkshire, UK) while β-D-Gal*p*-(1→3)-D-GlcNAc (Lacto-*N*-biose I, LNB I) and β-D-Gal*p*-(1→4)-D-GlcNAc (*N*-acetyl-D-lactosamine, LacNAc) were purchased from Dextra Laboratories (Reading, U.K.).

B. Bacterial strains and culture conditions

L.delbrueckii subsp. *bulgaricus*

Bacterial Strains and Culture Conditions. The type strain *L. delbrueckii* subsp. *bulgaricus* DSM 20081 (other collection numbers are ATCC

[a] part of the **(1)** published journal article (Nguyen, T.-T.; Nguyen, H. A.; **Arreola, S. L.;** Mlynek, G.; Djinovic-Carugo, K.; Mathiesen, G.; Nguyen, T.-H.; Haltrich, D., Homodimeric β-Galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081: Expression in *Lactobacillus plantarum* and Biochemical Characterization. *J Agric Food Chem* **2012,** *60*, 1713-1721),

(2) article submitted to PLOS one (**Arreola, S. L.;** Intanon, M.; Suljic, Jasmina; Kittl, Roman; Ngoc, H. P.; Kosma, P; Haltrich, D.; Nguyen, T.-H., Two β-galactosidases from the human isolate *Bifidobacterium breve* DSM 20213: Molecular cloning and expression, biochemical characterization and synthesis of galacto-oligosaccharides. (Revision submitted to PLOS ONE) and

(3) article in preparation for submission to PLOS ONE. (**Arreola, S. L.;** Intanon, M.; P.; Kosma, P; Haltrich, D.; Nguyen, T.-H., Biochemical characterization of four β-galactosidases towards formation of galacto- and hetero- oligosaccharides.

11842; originally isolated from Bulgarian yogurt in 1919(*102*)) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). All bacterial strains used in this study are shown in Table 3. *Lactobacillus* strains were cultivated in MRS media at 37 °C, without agitation. *Escherichia coli* NEB5α (New England Biolabs, Ipswich, MA) was grown at 37 °C in Luria–Bertani (LB) medium with shaking at 120 rpm. When needed, erythromycin was supplemented to media in concentrations of 5 μg mL^{-1} for *Lactobacillus* or 200 μg mL^{-1} for *E. coli*, whereas ampicillin was used at 100 μg mL^{-1} for *E. coli*.

DNA Manipulation. Total DNA of *L. bulgaricus* DSMZ 20081 was isolated using chloroform extraction as described by Nguyen et al.(*43*) with slight modifications. In short, cell pellets from 3 mL overnight cultures were resuspended and incubated at 37 °C for 1 h in 400 μL of 1 mM Tris–EDTA buffer pH 8 (TE buffer) containing 50 μL of lysozyme (100 mg mL^{-1}) and 50 μL of mutanolysin (480 U mL^{-1}). The mixture was subsequently supplemented with 50 μL of 10% SDS and 10 μL of proteinase K (20 mg mL^{-1}) and incubated further at 60 °C for 1 h. After inactivation of proteinase K (at 75 °C for 15 min), 2 μL of RNase (2 mg mL^{-1}) was added to the mixture, and incubation was continued at 37 °C for 30 min. Genomic DNA was extracted and purified by using phenol–chloroform and precipitated with 3 M sodium acetate, pH 3.8, and ice-cold isopropanol. The DNA precipitate was washed with cold (−20 °C) 70% ethanol, and the dried DNA pellets were dissolved in 50 μL of TE buffer, pH 7.5, at room temperature with gentle shaking.

The primers used for PCR amplification of *lacZ* from the genomic DNA of *L. bulgaricus* DSM 20081 (NCBI reference sequence no. NC_008054) (*102*) were supplied by VBC-Biotech Service (Vienna, Austria) and are listed in Table 4. The

appropriate endonuclease restriction sites were introduced in the forward and reverse primers as indicated. DNA amplification was performed with Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland) as recommended by the supplier and using standard procedures.(*103*) The amplified PCR products were purified by the Wizard SV Gel and PCR Clean-up system kit (Promega, Madison, WI). When needed, the PCR fragments were subcloned into the pJET1.2 plasmid (CloneJET PCR cloning kit, Fermentas), and *E. coli* was used as a host for obtaining the plasmids in sufficient amounts before transformation into *Lactobacillus*. All PCR-generated inserts were confirmed by DNA sequencing performed by a commercial provider.

Plasmid construction and transformation. Gene fragments of *lacZ* with or without the His_6-tag were excised from the pJETlacZ plasmid using *BsmB*I and *Xho*I and ligated into the 5.6 kb *Nco*I–*Xho*I fragments of pSIP403 or pSIP409, resulting in the plasmids pTH101, pTH102, pTH103, and pTH104 (Table3). The constructed plasmids were transformed into electrocompetent cells of *L. plantarum* WCFS1 according to the protocol of Aukrust and Blom.(*104*)

Expression of recombinant β-Galactosidase. For the heterologous overexpression of the *lacZ* gene from *L. bulgaricus*, overnight cultures (∼16 h) of *L. plantarum* WCFS1 harboring the expression plasmid pTH101, pTH102, pTH103, or pTH104 were added to 15 mL of fresh MRS medium containing erythromycin to an OD_{600} of ∼0.1 and incubated at 30 °C without agitation. The cells were induced at an OD_{600} of 0.3 by adding 25 ng mL^{-1} of the inducing peptide pheromone IP-673 (supplied by the Molecular Biology Unit, University of Newcastle-upon-Tyne, U.K.). Cells were harvested at an OD_{600} of 1.8–2, washed twice by buffer P (50 mM sodium phosphate buffer (pH 6.5) containing 20% w/v glycerol and 1 mM dithiothreitol), (*105*) and resuspended in 0.5 mL of the same buffer. Cells were disrupted in a bead

beating homogenizer using 1 g of glass bead (Precellys 24; PEQLAB, Germany). Cell-free extracts were obtained after a centrifugation step at 9000 *g* for 15 min at 4 °C.

Fermentation and protein purification. *L. plantarum* WCFS1 harboring pTH101 or pTH102 was cultivated in 1 L fermentations to obtain sufficient material for purification of LacZ. The cultivation conditions and the induction protocol were identical to those of the small-scale cultivations. Expression of *lacZ* was induced at OD_{600} 0.3, and the cells were harvested at OD_{600}~6. After centrifugation as above, cells were disrupted by using a French press (Aminco, Silver Spring, MD, USA), and debris was removed by centrifugation (30000 x *g*, 20 min, 4 °C). The purification of the recombinant enzyme was performed by immobilized metal affinity chromatography using a Ni-Sepharose column (GE Healthcare, Uppsala, Sweden)(*45*) or substrate affinity chromatography (with the substrate analogue *p*-aminobenzyl 1-thio-β-D-galactopyranoside immobilized onto cross-linked 4% beaded agarose; Sigma) as previously described.(*105*) Purified enzymes were stored in 50 mM sodium phosphate buffer (pH 6.5) at 4 °C.

Table 3. Strains and plasmids used for cloning and overexpression of the β-galactosidase gene *Lac*Z from *Lactobacillus delbrueckii* subsp. *bulgaricus*[a]

strains and plasmids	relevant characteristics and purpose	Reference
Strains		
L.. bulgaricus DSM 20081	original source of *lacZ*	DSMZ
Lactobacillus plantarum WCFS1	host strain, plasmid free	(*106*)
E. coli NEB5α	cloning host	New England Biolabs
Plasmids		
pJET1.2	For PCR fragments storage	Fermentas
pSIP403	*spp*-based expression vector, pSIP401 derivative, Em^r, *gusA* controlled by P_{sppA}	(*107*)
pSIP409	*spp*-based expression vector, pSIP401 derivative, Em^r, *gusA* controlled by P_{sppQ}	(*107*)
pTH101	pSIP403 derivative, *gusA* replaced by *lacZ*	this study
pTH102	pSIP403 derivative, *gusA* replaced by *lacZ* carrying C-terminal His_6-tag	this study
pTH103	pSIP409 derivative, *gusA* replaced by *lacZ*	this study
pTH104	pSIP409 derivative, *gusA* replaced by *lacZ* carrying C-terminal His_6-tag	this study

[a]Em^r, erythromycin resistance; *spp*, sakacin P gene cluster; *gusA*, β-glucuronidase reporter gene; *lacZ*, β-galactosidase gene

Table 4. Primers used for cloning and overexpression of the β-galactosidase gene *Lac*Z from *Lactobacillus delbrueckii* subsp. *bulgaricus*[a]

primer	restriction enzyme	sequence (5→3)	ref sequence accession no.
F1	*BsmB*I	GCTG CGTCTC CCATGAGCAATAAGTTAGTAAAAG	NC_008054, GeneID: 4085367
R1	*Xho*I	CGCG CTCGAG TTATTTTAGTAAAAGGGGCTG	NC_008054, GeneID: 4085367
R2	*Xho*I	CGCG CTCGAG TTA*GTGGTGGTGGTGGTGGTG*TTTTAGTAAAAGGGGC	NC_008054, GeneID: 4085367

[a]Restriction sites are underlined, the His_6-tag sequence is shown in italic.

2. *Bifidobacterium breve*

Bacterial strains and culture conditions. *B. breve* DSM 20213, an infant isolate, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain was grown anaerobically at 37 °C in MRS medium. (*108*) *Escherichia coli* DH5α (New England Biolabs, Frankfurt am Main, Germany) was used in the transformation experiments involving the subcloning of the DNA fragments. *Escherichia coli* T7 express (Novagen, Darmstadt, Germany) was used as expression host for the vectors carrying the target DNA fragment encoding β-galactosidases.

Construction of β-galactosidase expression vectors. The Bbreβgal I gene (NCBI Reference No. EFE90149.1) and BbreβgalII gene (NCBI Reference No. EFE88654.1) were amplified using proof-reading Phusion polymerase with the primer pairs 5BbBG1Nde1 (5'-AATACATATGCAAGGAAAGGCGAAAACC-3´), 3BbBG1Not1 (5´-ATAGCGGCCGCGATTAGTTCGAGTGTCACATCC-3´) and 5BbBG2Nde1 (5'-AATACATATGAACACAACCGACGATCAG-3´), 3BbBG2Not1 (5'-ATAGCGGCCGCGATGAGTTCGAGGTTCACGTC-3´), respectively. The forward primers contain *Nde*I and the reverse primers include *Not*I recognition sites (underlined). The template for the PCR reaction was obtained from cells scratched from an MRS agar plate and suspended in the PCR mix. The initial denaturation step at 98°C for 3 min was follow by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 2 min, followed by a final extension step at 72°C for 5 min. The amplified genes were digested with the corresponding restriction enzymes. Subsequently, the gene fragments were ligated into the pET-21a(+) vector without the natural stop codon and in frame with the C-terminal His_6-tag sequence on

the vector, and transformed into *E. coli* DH5α cells. The resulting expression vectors BreβgalI and BreβgalII were transformed into two different hosts, *E. coli* T7 Express and *E. coli* T7 Express carrying the plasmid pGRO7 (*E. coli* T7 Express GRO), for comparison of the expression levels. The correct nucleotide sequences were confirmed by sequencing (VBC-Biotech, Vienna, Austria). The basis local alignment search tool (BLAST) from the National Center for Biotechnology Information BLAST website was used for database searches. The comparison of β-galactosidases from *B. breve* with homologous proteins was carried out using the program ClustalW2 (version 2.0).(*109*)

Heterologous expression of BbreβgalI and BbreβgalII. The expression levels of BbreβgalI and BbreβgalII with and without co-expression of the chaperones GroEL and GroES were compared. To this end, all cultures were grown at 37 ºC in 250 mL of MagicMedia (Invitrogen Corporation, Carlsbad, CA, USA) until an optical density at OD_{600nm} of 0.6 was reached, and then the cultures were incubated further at 20ºC overnight. The co-expression of the chaperons was induced with 1 mg mL^{-1} L-arabinose. The cells were harvested by centrifugation (6,000 × *g*, 30 min, and 4 °C), washed twice with 50 mM sodium phosphate buffer (pH 6.5)and disrupted using a French press (AMINCO, Silver Spring, MD, USA). The resulting homogenate was centrifuged at 25,000 × *g* for 30 min at 4°C to remove the cell debris. The crude extracts were tested for protein concentration and β-galactosidase activity using the standard assay.

Subsequently, the expression of BbreβgalI and BbreβgalII was studied further. Different induction conditions were compared by varying the concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) in LB medium. *E. coli* T7 express GRO cells carrying BbreβgalI and BbreβgalII plasmids, respectively, were grown at 37 ˚C

in 100 mL of LB medium containing 100 µg mL^{-1} ampicillin and 1 mg mL^{-1} L-arabinose for chaperone induction until an optical density at OD_{600nm} of ~0.8 was reached. IPTG was added to the culture medium in final concentrations of 0.1, 0.5, 1.0 mM, respectively, and the cultures were incubated at 18 ˚C for 16 h. The cultures were harvested, washed twice and resuspended in 1 mL of 50 mM sodium phosphate buffer (pH 6.5). Cells were disrupted in a bead beating homogenizer using 0.5 g of glass bead (Precellys®24 Technology; PEQLAB, Germany). The crude extracts obtained after centrifugation (16,000 × *g* for 20 min at 4ºC) were tested for β-galactosidase activity using the standard enzyme assay and protein concentrations.

Fermentation and purification of recombinant β-galactosidases. *E. coli* T7 express GRO cells carrying the plasmids BbreβgalI and BbreβgalII, respectively, were grown at 37ºC in 1 L LB medium containing 100 µg mL^{-1} ampicillin, 20 µg mL^{-1} chloramphenicol and 1 mg mL^{-1} L-arabinose until OD_{600nm} of 0.8 was reached. IPTG (0.5 mM for BbreβgalI and 1 mM for BbreβgalII) was added to the medium and the cultures were incubated further at 18 ºC for 16 h. The cultures were then harvested, washed twice with 50 mM sodium phosphate buffer (pH 6.5) and disrupted by using a French press. Cell debris was removed by centrifugation (25,000 x *g*, 30 min, 4ºC) and the lysate (crude extract) was loaded on a 15 mL Ni-immobilized metal ion affinity chromatography (IMAC) column (GE Healthcare, Uppsala, Sweden) that was pre-equilibrated with buffer A (20 mM phosphate buffer, 20 mM imidazole, 500 mM NaCl, pH 6.5). The His-tagged protein was eluted at a rate of 1 mL min^{-1} with a 150 mL linear gradient from 0 to 100% buffer B (20 mM sodium phosphate buffer, 500 mM imidazole, 500 mM NaCl, pH 6.5). Active fractions were pooled, desalted and concentrated by ultrafiltration using an Amicon Ultra centrifugal filter unit (Millipore,

MA, USA) with a 30 kDa cut-off membrane. Purified enzymes were stored in 50 mM sodium phosphate buffer (pH 6.5) at 4°C for further analysis.

3. Lactobacillus reuteri L103

β-galactosidase from *Lactobacillus reuteri* L103 (Lreuβgal) was overexpressed in *Escherichia coli* and purified according to the method described.(*43*)

C. Molecular weight determination

Native polyacrylamide gel electrophoresis (PAGE), denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and activity staining using 4-methylumbelliferyl β-D-galactoside (MUG) as the substrate were carried out as previously described [(105)] using the Phast System with precast gels (Pharmacia Biotech, Uppsala, Sweden). Gel permeation chromatography was performed on a Superose 12 column (16 × 1000 mm; GE Healthcare) using 20 mM sodium phosphate buffer (pH 6.5)containing 150 mM NaCl, and with the Sigma Gel Filtration Molecular Markers Kit with standard proteins of 12–200 kDa. In addition, pyranose oxidase with a molecular mass of 270 kDa was used as a standard.(*110*)

Size exclusion chromatography - Multi-angle laser light scattering (SEC-MALLS) analysis was performed with a Superdex S200 10/300 GL column (GE Healthcare) equilibrated in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM β-mercaptoethanol and 5 mM EDTA. Sample separations were performed at room temperature with a flow rate of 0.5 mL min^{-1} with an HPLC pump (Agilent Technologies 1260 infinity). Samples (50 μL) were injected as indicated at a concentration of 2.5 mg mL^{-1}. On-line MALLS detection was performed with a miniDawn Treos detector (Wyatt Technology Corp., Santa Barbara, CA, USA) using

a laser emitting at 690 nm. Protein concentration was measured on-line by refractive index measurement using a Shodex RI-101 instrument (Showa Denko, Munich, Germany). Analysis of the data was performed with the ASTRA software (Wyatt Technology).

D. β-Galactosidase activity assays

The measurement of β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) or lactose as the substrates was carried out as previously described.(*105*) When chromogenic *o*NPG was used as the substrate, the reaction was initiated by adding 20 µL of enzyme solution to 480 µL of 22 mM *o*NPG in 50 mM sodium phosphate buffer (pH 6.5) and stopped after 10 min of incubation at 30°C by adding 750 µL of 0.4 M Na_2CO_3. The release of *o*-nitrophenol (*o*NP) was measured by determining the absorbance at 420 nm. One unit of *o*NPG activity was defined as the amount of enzyme releasing 1 µmol of *o*NP per minute under the described conditions.

When lactose was used as the substrate, 20 µL of enzyme solution was added to 480 µL of 600 mM lactose solution in 50 mM sodium phosphate buffer (pH 6.5). After 10 min of incubation at 30 °C, the reaction was stopped by heating the reaction mixture at 99 °C for 5 min. The reaction mixture was cooled to room temperature, and the release of D-glucose was determined using the test kit from Megazyme. One unit of lactase activity was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions.

E. Protein determination

Protein concentration was determined by the method of Bradford (*111*) using bovine serum albumin as the standard.

F. Steady-state kinetic measurement

All steady-state kinetic measurements were obtained at 30 °C using *o*NPG and lactose as the substrates in 50 mM sodium phosphate buffer (pH 6.5) with concentrations ranging from 0.5 to 22 mM for *o*NPG and from 1 to 600 mM for lactose, respectively. The inhibition of *o*NPG hydrolysis by D-galactose and D-glucose as well as that of lactose hydrolysis by D-galactose was investigated as well. The kinetic parameters and inhibition constants were calculated by nonlinear regression, and the observed data were fit to the Henri-Michaelis-Menten equation (SigmaPlot, SPSS Inc., Chicago, IL, USA).

G. pH and temperature dependency of activity and stability

The pH dependency of the recombinant enzymes was evaluated by standard assay with 22 mM *o*NPG in the pH range of 3–10 using Briton-Robinson buffer (20 mM acetic acid, 20 mM phosphoric acid, and 20 mM boric acid titrated with 1 M NaOH to the desired pH). To evaluate the pH stability, the enzyme samples were incubated at various pH values using Britton-Robinson buffers at 37 ^{o}C and the remaining activity was measured at time intervals with *o*NPG as substrate. The temperature optima for hydrolytic activity of BbreβgalI and BbreβgalII with both substrates lactose and *o*NPG were determined at 20–90 °C. The thermostability was evaluated by incubating the pure enzyme in 50 mM sodium phosphate buffer (pH 6.5)

at several temperatures (4, 30, 37, 45, 50 °C). The residual activities were measured regularly with *o*NPG as substrate.

H. Differential Scanning Calorimetery (DSC)

DSC measurements were performed using a MicroCal VP-DSC System (GE Healthcare) controlled by the VP-viewer program and equipped with a 0.137-mL cell. Studies were made with 1 mg mL^{-1} protein samples in 50 mM phosphate buffer (pH 6.5) Samples were analyzed using a programmed heating scan rate of 60 °C h^{-1} in the range of 33–80 °C. For baseline correction, a buffer blank was scanned in the second chamber and subtracted. The heat capacity (C_p) was expressed in kcal mol^{-1} K^{-1}. Data analysis was performed with the MicroCal Origin software (GE Healthcare) and experimental data points were fitted to an *MN2-State Model.*

I. Substrate specificity

Substrate specificity of the recombinant enzymes was determined using various structurally related chromogenic substrates under standard assay conditions as described for *o*NPG. The chromogenic substrates tested were 2-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-β-D-mannopyranoside, 4-nitrophenyl-α-D-galactopyranoside, and 4-nitrophenyl-α-D-glucopyranoside at substrate concentration of 22 mM.

Substrate affinities of the recombinant enzymes towards lactose, β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→6)-D-Glc, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→4)-D-Gal, β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Lac, β-D-Gal*p*-(1→4)-D-Lac, β-D-Gal*p*-(1→6)-D-Lac were also evaluated by incubating each enzyme with ~3 mM of each of the galactosides at 30 °C in 50 mM sodium phosphate buffer (pH 6.5). Specificity of

Lbulβgal and *Bbre*βgalII towards β-D-Gal*p*-(1→3)-D-GlcNAc, β-D-Gal*p*-(1→4)-D-GlcNAc and β-D-Gal*p*-(1→6)-D-GlcNAc was evaluated using ~1 U_{Lac} mL^{-1} incubated with ~12.5 mM of each galactoside at 30 °C in 50 mM sodium phosphate buffer (pH 6.5). Generally, samples were taken after 30 and 60 min and reactions were stopped by incubation at 95 °C for 5 min. The relative activities of the recombinant enzymes towards each galactoside were determined considering the percentage of the hydrolysis (or conversion) of each galactoside under similar reaction conditions.

J. Galacto-oligosaccharide synthesis and analysis

Discontinuous conversion reactions were carried out to determine the transgalactosylation reaction of the Lreuβgal, Lbulβgal, BbreβgalI and BbreβgalII. The influence of process parameters such as temperature and enzyme concentration was also studied. The substrate lactose solution (200 g L^{-1}) was prepared in 50 mM sodium phosphate buffer containing 1 mM Mg^{2+}. Agitation was applied at 300 rpm with a thermomixer (Eppendorf, Hamburg, Germany). Samples were taken at certain time intervals to determine the residual activities and the carbohydrate contents in the reaction mixtures by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

HPAEC−PAD analysis was 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, and Chromeleon version 6.5 (Dionex Corp., Sunnyvale, CA, USA). All eluents were degassed by flushing with helium for 30 min. Separations were performed at room temperature on a CarboPac PA-1 column (4 mm × 250 mm) connected to a CarboPac PA-1 guard column (Dionex) (34). Separation of D-Glc-, D-Gal, lactose and allolactose was carried out with an

isocratic run (45 min) with 15 mM NaOH at 1.0 mL min^{-1}, followed by 25 min elution with 100 mM. For separation of other GOS, eluent A (100 mM NaOH) and B (100 mM NaOH and 150 mM NaOAc) were mixed to form the following gradient: 98% A from 0 to 10 min, 98% A to 52% A from 10 to 40 min, and then 52 % A for another 5 min. The column was washed with 20% B for 10 min and re-equilibrated for 15 min with the starting conditions of the employed gradient.

K. Intermolecular galactosyl transfer under defined initial velocity

Initial velocities were determined using 50 mM sodium phosphate buffer (pH 6.5) at 30 °C using either 10 mM *o*NPGal or 100 mM lactose as substrate. This substrate concentration was a compromise between the practical requirement to measure initial velocity of D-Gal (and/or D-Glc) and to maximize the transfer of D-Gal to the external nucleophile but not to the substrate. The final enzyme concentration used was ≤ 1.0 U mL^{-1}. The relationship between [*o*NP] (or [Glc]) and [Gal] was found to be linear to 30 min. Thus, the standard reaction time of 20 min was used. v_{oNP}, v_{Glc}, and v_{Gal} were measurements of molar concentrations of *o*nP, Glc, and Gal, respectively. Ratio of ν_{oNP} and ν_{Gal} were measured in the presence and absence of glucose concentration varied between from 2.5 to 20 mM.

The intermolecular transgalactosylation to lactose was done using with various initial lactose concentration (9 - 602 mM) while galactosyl transfer to either GlcNAc, GalNAc Fuc were assessed using 100 mM lactose with acceptor concentration varying from 12.5 to 200 mM. Generally, after preincubation of the enzyme for 20 min at 30 °C, the reaction was stopped by heating for 5 min at 95 °C. The rate of formation of *o*NP (ν_{oNP}) was measured using the standard β-galactosidase assay while galactose (ν_{Gal}) or glucose (ν_{Glc}) measurement was carried out by HPLC (Dionex;

MA, USA) using an Aminex HPX-87K column (300 x 7.8 mm; Bio-Rad, Hercules, CA, USA) equipped with refractive index detector. Water was used as mobile phase at a flow rate of 0.80 mL min^{-1} and a column temperature of 80 °C. As general rule, all measurements should not inhibit *o*NP release by 20% compared with the control lacking the acceptor and a linear dependence of the velocity on the acceptor concentration must be observed.

L. *N*-acetyl oligosaccharide production

N-acetyl-oligosaccharide synthesis was carried out using lactose and GlcNAc (or GalNAc) as substrate with either Lbulβgal or BbreβgalII. The influences of temperature (30 and 50 °C), substrate concentrations (0.6 M and 1 M), molar ratios of donor: acceptor (1:2, 1:1 and 2:1), and enzyme concentrations (2.5 and 5.0 U mL^{-1}) were also investigated. Generally, a solution (2.0 mL) containing the substrates was dissolved in 50 mM sodium phosphate buffer (pH 6.5) containing 1 mM Mg^{2+}. The enzyme was added and incubated at the required temperature and agitated at 300 rpm with a thermomixer (Eppendorf, Hamburg, Germany). Aliquots of samples were withdrawn at certain time intervals to determine the residual activities and carbohydrate contents using either HPAEC-PAD as described (*112*) or HPLC system containing UV detector (210) nm using Hypercarb column (0.32 × 150 mm, inner diameter 5 μm). Ammonium formate buffer (0.3% formic acid, pH 9.0) was used as Buffer A, and a gradient was performed from 0 to 35% acetonitrile within 35 min using a Dionex Ultimate 3000 (cap flow, 1 mL min^{-1}). The GlcNAc transgalactosylation yield was determined based on the starting GlcNAc concentration and was calculated using Equation 2.

$$\text{GlcNAc transgalactosylation yield (\%)} = \left(\frac{\text{GlcNAc}_{\text{initial}} - \text{GlcNAc}_{\text{remaining}}}{\text{GlcNAc}_{\text{initial}}}\right) \times 100 \qquad \text{Equation 2}$$

M. Purification of *N*-acetyl-oligosaccharides

For purification and identification of GlcNAc transfer products, a 10-mL discontinuous batch reaction using BbreβgalII (5 U_{Lac} mL^{-1}) was carried out at 30 °C using initial equimolar concentration of lactose and GlcNAc (600 mM each) dissolved in 50 mM sodium phosphate buffer (pH 6.5) with 1 mM Mg^{2+}. Agitation was at 300 rpm rotary shaker. After 4 h, the reaction was stopped by heating at 95 °C. Due to the complex course of transgalactosylation reactions, the reaction mixture was partially purified by gel permeation chromatography on Bio-Gel P2 (2.0 x 100 cm) equilibrated in water containing 5% (v/v) ethanol and 0.0015 % (w/v) NaCl. The elution was followed by UV reading at 210 nm to detect presence of GlcNAc and of transgalactosylation reactions. The fractions containing the desired transgalactosylation product were pooled, freeze-dried, and redissolved in acetonitrile. The complete purification of the transgalactosylation product was obtained by using HPLC system (UV detector at 210 nm) and using Hypercarb column as described above. The peaks corresponding to the products were pooled and dried under pressure and freeze-dried.

N. NMR measurements

NMR spectra were recorded at 27 ^{o}C in 99.9% D_2O with a on a Bruker Avance III^{TM} 600 spectrometer (^{1}H at 600.13 MHz and ^{13}C at 150 MHz) equipped with a BBFO broad-band inverse probe head and z-gradients using standard Bruker NMR software. COSY experiments were recorded using the program cosygpqf with 2048 x 256 data points, respectively, pet t_1-increment. HSQC spectra were recorded using hsqcedetgp with 1024 x 128 data points and 16 scans, respectively, pet t_1-increment .

^{1}H NMR spectra were referenced to internal DSS (δ= 0); ^{1}H NMR spectra were referenced to external 1,4-dioxane (δ= 67.4).

O. Statistical Analysis

All experiments and measurements were performed at least in duplicate, and the data are given as the mean ± standard deviation when appropriate. Student's *t*-test was used for the comparison of data.

III. RESULTS[a]

Plasmid construction and expression of β-galactosidase derived from *L. bulgaricus* in *L. plantarum*

The four expression plasmids termed pTH101, pTH102, pTH103 and pTH104 were constructed based on the plasmids pSIP403 and pSIP409. (*107*) To this end, *gusA*, which originally was used as a reporter gene in the pSIP plasmid series, was replaced by *lacZ*, the gene encoding β-galactosidase from *L. delbrueckii* subsp. *bulgaricus* DSM 20081, both with and without a hexa-histidine tag (Table 3). In these vectors, the transcription of *lacZ* is regulated by the inducible promoters P_{sppA} and P_{sppQ} for the pSIP403 and pSIP409 derivatives, respectively (Figure 1). The constructed plasmids were electroporated into *L. plantarum* WCFS1, and the expression of *lacZ* with the different vectors was subsequently studied in this host, using an inducer concentration of 25 ng mL^{-1}. (*103*, *113*) Induced and non-induced cells were harvested in the late stationary phase (OD_{600} of 1.8–2.0), and the intracellular cell-free extracts were analyzed by SDS-PAGE (Figure 2) and β-galactosidase activity assays (Table 5).

Figure 2 shows unique bands of ~100 kDa in induced *L. plantarum* cells, which clearly demonstrate high expression of *lacZ* regardless of the expression plasmid used. Analysis of the crude cell extracts confirmed these high production yields, with volumetric activities in the range of ~15–23 U mL^{-1} of cultivation

[a] part of the **(1)** published journal article (Nguyen, T.-T.; Nguyen, H. A.; **Arreola, S. L.;** Mlynek, G.; Djinovic-Carugo, K.; Mathiesen, G.; Nguyen, T.-H.; Haltrich, D., Homodimeric β-Galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081: Expression in *Lactobacillus plantarum* and Biochemical Characterization. *J Agric Food Chem* **2012,** *60*, 1713-1721)

(2) revised article submitted to PLOS ONE. (**Arreola, S. L.;** Intanon, M.; Suljic, Jasmina; Kittl, Roman; Ngoc, H. P.; Kosma, P; Haltrich, D.; Nguyen, T.-H., Two β-galactosidases from the human isolate *Bifidobacterium breve* DSM 20213: Molecular cloning and expression, biochemical characterization and synthesis of galacto-oligosaccharides.

(3) article in preparation for submission to PLOS ONE. (**Arreola, S. L.;** Intanon, M.; P.; Kosma, P; Haltrich, D.; Nguyen, T.-H., Biochemical characterization of four β-galactosidases towards formation of galacto- and hetero- oligosaccharides.

medium and specific activities of ~160–200 U mg^{-1} (Table 5). The β-galactosidase activities in *L. plantarum* cells without plasmids were 0. 002 U mL^{-1} and 0.07 U mg^{-1} (data not shown), and hence the enzyme activities obtained can be attributed solely to

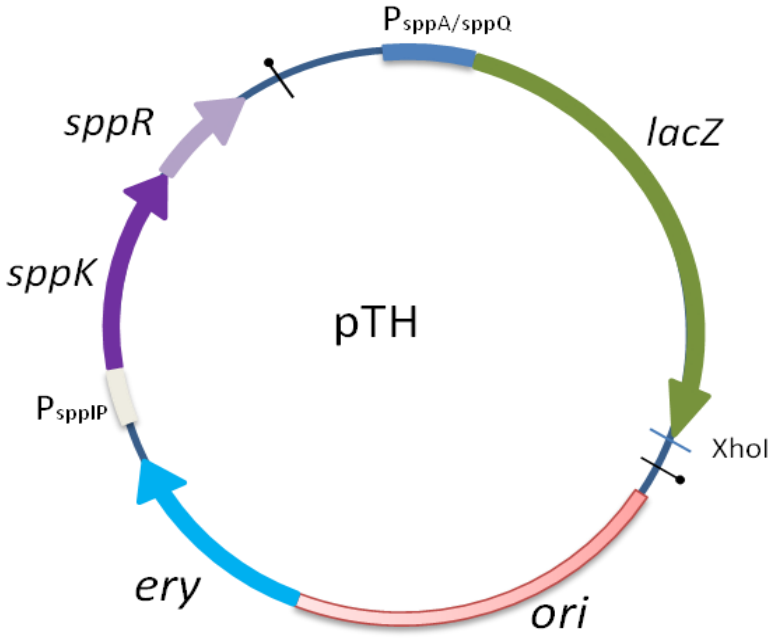


Figure 1. Schematic overview of the pTH plasmids developed in this study. The structural gene *lacZ* (with or without a hexa-histidine tag) is controlled by the inducible promoters P_{sppA} (pSIP403 derivatives) or P_{sppQ} (pSIP409 derivatives). P_{sppIP} is controlling the structural genes of the two-component regulatory system, *sppK*, a histidine kinase, and *sppR*, a response regulator. Ery is indicating the erythromycin resistance marker, and transcriptional terminators are marked by lollypop structures.

the plasmid-encoded LacZ from *L. bulgaricus*. Table 3 shows that the choice of the P_{sppA} promoter (pSIP403 derivatives) or P_{sppQ} promoter (pSIP409 derivatives) did not significantly affect the levels of β-galactosidase activity. Interestingly, activity levels obtained for the His-tagged β-galactosidase were consistently lower than for the non-tagged enzyme. This can, however, mainly be attributed to a decrease in activity of the tagged β-galactosidase (see below) and not to the expression levels.

Non-induced cells of *L. plantarum* harboring the various expression vectors were also cultivated and harvested as above to check for basal expression ('leakage') from the promoters (Table 5). All expression strains show basal β-galactosidase activities that are clearly higher than activities found for non-transformed *L. plantarum* cells, but that are still low compared to the activities obtained upon induction. Cells carrying pSIP409-derived vectors containing P_{sppQ} show lower basal activities than cells harboring pSIP403-derived vectors based on P_{sppA}. As a consequence, the highest induction factors, *i.e.*, the quotient of specific activity obtained for induced and non-induced cells, of roughly 50 were found for the constructs pTH103 and pTH104 carrying P_{sppQ} as the promoter.

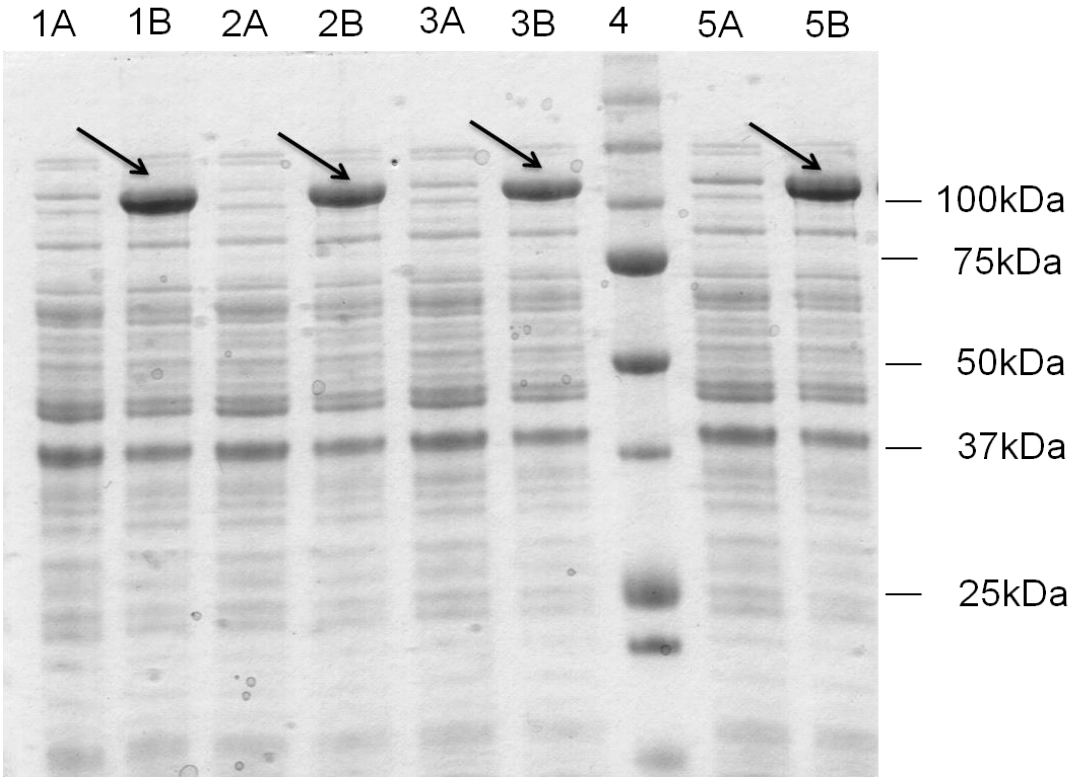


Figure 2. SDS-PAGE analysis of cell-free extracts of non-induced (A) and induced cells (B) of *L. plantarum* WCFS1 harboring pTH101 (lane 1A, 1B), pTH103 (lane 2A, 2B), pTH104 (lane 3A, 3B) and pTH102 (lane 5A, 5B). Lane 4 shows the Precision Plus Protein standard (Biorad). The gel was stained with Coomassie blue.

Table 5. β-Galactosidase activity in cell-free extracts of induced and non-induced cells of *L. plantarum* WCFS1 carrying various expression plasmids[a]

Plasmid	Volumetric activity (U mL^{-1} fermentation broth)		Specific activity (U mg^{-1} protein)		Induction factor[b]
	induced	non-induced	induced	non-induced	
pTH101	22.5 ± 0.8	1.50 ± 0.04	196 ± 3	10.3 ± 1.1	19
pTH102	15.5 ± 0.6	1.62 ± 0.13	158 ± 3	11.7 ± 0.5	13
pTH103	22.0 ± 1.3	0.63 ± 0.03	193 ± 10	4.11 ± 0.18	47
pTH104	18.0 ± 0.5	0.51 ± 0.04	168 ± 4	3.43 ± 0.13	49

[a]Data are expressed as the average ± standard deviation of three independent cultivations

Fermentation and purification of recombinant LbulβgaI

In order to obtain sufficient protein (tagged and non-tagged) for further characterization, *L. plantarum* harboring pTH101 or pTH102 was cultivated on a larger scale, and gene expression was induced as described above. Typical yields obtained in 1-liter laboratory cultivations were approximately 7.5 ± 0.2 g wet biomass and 53 kU of non-tagged (pTH101) and 43 kU of His-tagged (pTH102) β-galactosidase activity. The recombinant enzymes were purified to apparent homogeneity from cell extracts (specific activities in these crude extracts were 193 U mg^{-1} for non-tagged wild-type LacZ and 165 U mg^{-1} for His-tagged LacZ) by single-step purification protocols using either substrate affinity chromatography or immobilized metal affinity chromatography. The specific activity of the purified recombinant enzymes was 306 U mg^{-1} for wild-type, non-tagged LacZ and 251 U mg^{-1} for His-tagged LacZ, respectively, when using the standard *o*NPG assay. Both purification procedures yielded homogenous β-galactosidase as judged by SDS-PAGE (Figure 3A).

Molecular characterization of the l*acZ* gene product, Lbulβgal

Lbulβgal is a homodimer, consisting of two identical subunits of ~115 kDa, as judged by denaturing SDS-PAGE (molecular mass of ~115 kDa as judged by comparison with reference proteins; Figure 3A) and Native PAGE (molecular mass of ~200 kDa; Figure 3B). Gel permeation chromatography and comparison with protein standards of known mass gave a molecular mass of 230 kDa for native LacZ. This compares well to the calculated molecular mass of 114,047 Da deduced for the LacZ subunit from its sequence. Activity staining directly on the Native PAGE gel using 4-methylumbelliferyl β-galactoside as the substrate indicated furthermore that the protein band of ~200 kDa indeed shows β-galactosidase activity (Figure 3B).

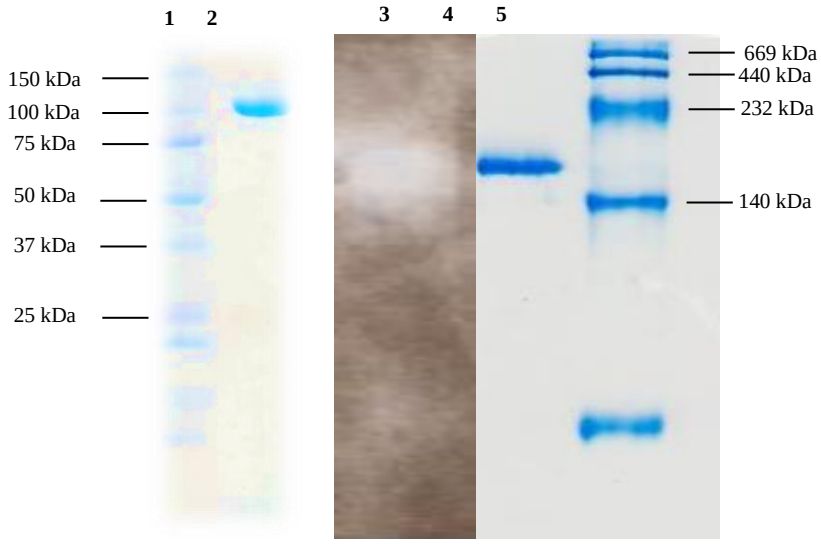


Figure 3. Electrophoretic analysis of purified recombinant β-galactosidase from *L. bulgaricus*: (A) SDS-PAGE (lanes: 1, Precision plus Protein standard ladder (Bio-Rad); 2, purified recombinant enzyme); (B) native-PAGE (lanes: 3, activity staining of β-galactosidase using 4-methylumbelliferyl β –D-galactoside; 4, purified β-galactosidase; 5, high molecular mass protein ladder (Amersham TM HMW, GE Healthcare).

Table 6. Kinetic parameters for recombinant β-galactosidase LacZ from *L. bulgaricus*, both non-tagged and C-terminally His-tagged, for the hydrolysis of lactose and *o*-nitrophenyl β-D-galactopyranoside (*o*NPG)

Substrate	Method for determination of enzyme activity	Kinetic parameter[a]	Non-tagged LacZ	His-tagged LacZ
Lactose	Release of D-glucose	$v_{max,Glc}$ (μmol min^{-1} mg^{-1})	123 ± 5	111 ± 4
		$K_{m,Lac}$ (mM)	19.2 ± 3.8	19.9 ± 3.8
		k_{cat} (s^{-1})	234 ± 13	211 ± 10
		k_{cat}/K_m (M^{-1} s^{-1})	12300	10600
		$K_{i,Gal}$ (mM)	70.7 ± 16.8	nd
*o*NPG	Release of *o*NP	$v_{max,oNP}$ (μmol min^{-1} mg^{-1})	317 ± 6	257 ± 5
		$K_{m,oNPG}$ (mM)	0.919 ± 0.088	1.20 ± 0.11
		k_{cat} (s^{-1})	603 ± 15	492 ± 13
		k_{cat}/K_m (M^{-1} s^{-1})	655000	410000
		$K_{i,Glc}$ (mM)	123 ± 9	nd
		$K_{i,Gal}$ (mM)	9.52 ± 1.54	nd

[a]A molecular mass of 114 and 115 kDa was used to calculate k_{cat} from v_{max} for native and His-tagged LacZ, respectively. nd stands for not determined.

Enzyme kinetics of recombinant Lbulβgal

The steady-state kinetic constants of wild-type, non-tagged and His-tagged LacZ *L. bulgaricus* β-galactosidase are summarized in Table 6. The k_{cat} values were calculated on the basis of the theoretical v_{max} values experimentally determined by nonlinear regression and using a molecular mass of 114 kDa for the catalytically active subunit (115 kDa for His-tagged LacZ). One notable feature of LacZ from *L. bulgaricus* is the relatively high turnover number k_{cat} of 234 s^{-1} measured for the natural substrate lactose. Attaching a His-tag to the C-terminus of LacZ decreases the catalytic rate of the enzyme by 10–18%, depending whether lactose or *o*NPG is used as the substrate. Lbulβgal is not inhibited by its substrates lactose in concentrations of

up to 600 mM or *o*NPG in concentrations of up to 25 mM as is evident from the Michaelis-Menten plots (not shown). This is in contrast to some heterodimeric lactobacillal β-galactosidases of the LacLM type, where distinct substrate inhibition by *o*NPG was observed. (*41*, *44*)

The hydrolysis end products, D-galactose and D-glucose, were found to competitively inhibit the hydrolytic activity of Lbulβgal. Albeit, this inhibition of e.g. D-galactose on cleavage of the natural substrate lactose is only moderate as is evident from the ratio of the Michaelis constant for lactose and the inhibition constant for D-galactose ($K_{i,Gal}/K_{m,Lac}$ = 3.7), and inhibition by D-glucose is even less pronounced as is obvious from the high inhibition constant measured for the hydrolysis of *o*NPG and the high ratio of K_i to K_m for this reaction ($K_{i,Glc}/K_{m,oNPG}$ = 134).

Effect of metal ions on Lbulβgal activity

Various mono- and divalent metal ions were tested with respect to a possible stimulating or inhibitory effect on β-galactosidase activity. These were added in final concentrations of 1–50 mM to the enzyme in Bis-Tris buffer, with results shown in Table 7. The monovalent cations K^+ and Na^+ activated β-galactosidase activity when using this buffer system considerably, for example an almost 12-fold increase in activity was found in the presence of 50 mM Na^+ compared to a blank where no metal ion was added to the enzyme sample. When using 50 mM sodium phosphate buffer pH 6.5, K^+ only resulted in a slight activation of approximately 1.4-fold when added in 10 mM concentrations. The divalent cations Mg^{2+}, Ca^{2+} and Zn^{2+} showed an inhibitory effect when using Bis-Tris buffer, and especially the latter cation inhibited β-galactosidase activity strongly (Table 7). Interestingly, when using 50 mM sodium phosphate buffer instead of Bis-Tris buffer, Mg^{2+} showed an activating effect (150%

relative activity) at concentrations of 1 and 10 mM; this could indicate a synergistic effect with Na^+ present in the buffer. (*114*)

Table 7. Effect of cations on activity of recombinant β-galactosidase LacZ from *L. bulgaricus* in 10 mM Bis-Tris buffer (pH 6.5)[a]

cation	relative activity (%)		
	1 mM	10 mM	50 mM
blank (none)	100	100	100
Na^+	722	1030	1190
K^+	365	536	507
Mg^{2+}	85	31	nd[b]
Ca^{2+}	77	38	nd
Zn^{2+}	3	0.55	nd

[a]Enzyme activity was determined under standard assay conditions 10 mM Bis-Tris buffer (pH 6.5) using *o*NPG as the substrate with the respective cation added to give the stated final concentration. Experiments were performed in duplicates, and the standard deviation was always < 5%.[b] nd, not determined.

Effect of temperature and pH on Lbulβgal activity and stability

The temperature optima of the activity of β-galactosidase from *L. bulgaricus* are 45–50 and 55–60 °C for *o*NPG and lactose hydrolysis, respectively, when using the 10 min assay (Figure 4A). The pH optimum of LacZ activity is pH 7.5 for both substrates lactose and *o*NPG (Figure 4B). Overall, the pH curves show a broad peak with 75% of maximal β-galactosidase activity in the pH range of 6–9 (Figure 4B). Catalytic stability, that is, the length of time the enzyme remains active before undergoing irreversible inactivation, of β-galactosidase from *L. bulgaricus* was measured at a constant pH of 7.0 while the temperature was varied from 37 to 60 °C. In

addition, we tested the effect of different buffers and the addition of cations on stability. Lbulβgal activity showed first-order inactivation kinetics when analyzed in the plot of ln(residual activity) versus time (not shown). Data for the inactivation constants k_{in} and half-life times of activity $\tau_{1/2}$ are summarized in Table 8. Regardless of the temperature, stability was comparable in phosphate buffer without added cation and Bis-Tris buffer containing 10 mM Na^{+}, the metal ion that was found to increase activity significantly. Addition of 10 mM Mg^{2+} to phosphate buffer increased the stability considerably. Under these conditions, Lbulβgal was well stable at 50 °C with a half-life time of >1 day. When the temperature was increased to 60 °C, activity was however lost rapidly.

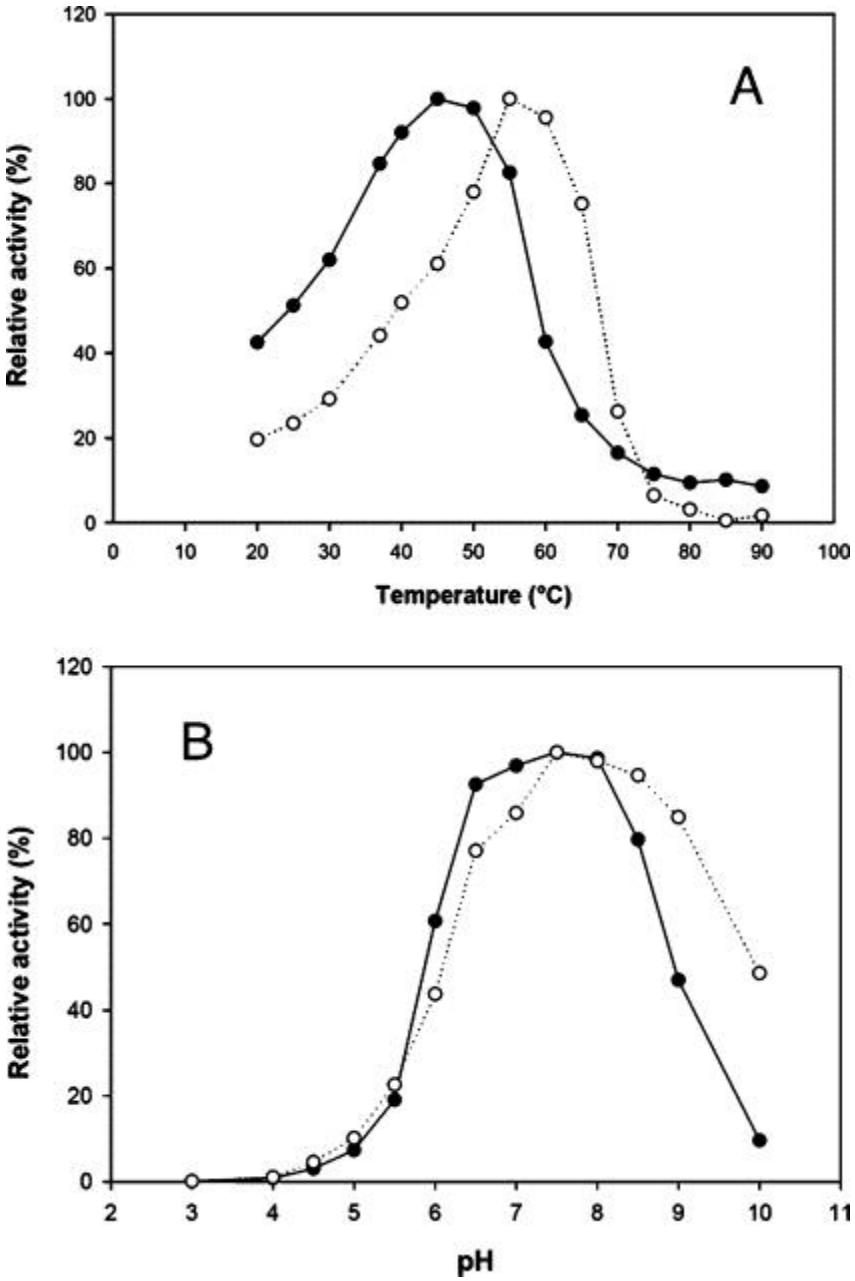


Figure 4. Temperature and pH optima of the activity of recombinant β-galactosidase from *L. bulgaricus*: (○) lactose as substrate; (●) *o*NPG as substrate. Relative activities are given in comparison with the maximum activities measured under optimal conditions (100%) which were 412 and 237 U mL^{-1} with oNPG and lactose as substrate, respectively, when determining the temperature optimum (A) and 680 and 106 U L^{-1} with oNPG and lactose as substrate, respectively for the pH dependence of activity (B).

Table 8. Catalytic stability of recombinant β-Galactosidase from *L. bulgaricus*[a]

Temperature (°C)	Sodium phosphate buffer (pH 7)		Sodium phosphate buffer (pH 7) + 10mM Mg^{2+}		Bis-tris buffer (pH7) + 10mM Na^{+}	
	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)
37	0.0053	145	0.0016	345	0.0084	82.5
50	0.925	0.75	0.026	26	1.12	0.62
60	15.3	0.045	1.0	0.32	16.9	0.041

[a]The inactivation constant k_{in} and half-life time of activity $\tau_{½}$ were calculated at different temperatures and reaction conditions. Both buffer concentrations were 50 mM each. Experiments were performed in duplicates, and the standard deviation was always <5%.

Lactose transformation and synthesis of GOS by Lbulβgal

Lactose conversion and product formation of a typical Lbulβgal-catalyzed reaction, using an initial lactose concentration of 600 mM (205 g L^{-1}) in 50 mM sodium phosphate buffer with 10 mM $MgCl_2$, pH 6.5, and 1.5 U_{Lac} mL^{-1} of β-galactosidase activity at 30°C, are shown in Figure 5A. During the initial reaction phase, GOS are the main reaction products, which are formed together with the primary hydrolysis products D-galactose and D-glucose. The concentration of total GOS reached a maximum of 102 g L^{-1} after 12 h of reaction, when 90% of initial lactose were converted, this corresponds to a yield of almost 50% GOS. Thereafter, the concentration of GOS decreased since these oligosaccharides are not stable end products of a thermodynamically controlled reaction. They are only transiently formed, and serve both as acceptors for galactosyl transfer but also as substrates for further LacZ-catalyzed hydrolysis. This breakdown of GOS, however, proceeds only slowly, most probably because of end product inhibition by D-galactose, which at this point of the reaction is present in notable concentrations, and only approximately 10% of total GOS are degraded when the reaction proceeds for another 12 h. As mentioned, GOS can also serve as acceptor for galactosyl transfer, and hence the

composition and amount of GOS change significantly during the progress of the reaction, as is shown in more detail in Figure 5B. Up to ~90% lactose conversion, the amount of total GOS, expressed by their relative concentration (percentage of GOS of total sugars in the reaction mixture) was increasing almost linearly. At the beginning of the reaction, the trisaccharides β-D-Gal*p*-(1→6)-Lac and β-D-Gal*p*-(1→3)-Lac were formed predominately. This is not unexpected since lactose is the most abundant carbohydrate species in the reaction mixture acting as the galactosyl acceptor during this phase. With further progress of the reaction, the concentrations of D-galactose and D-glucose are increasing steadily. These monosaccharides in turn become important acceptors for the transferase reaction, and disaccharides other than lactose are formed. In addition, disaccharides can be formed through cleavage of the trisaccharides that are predominantly formed during the initial phase of the reaction. Non-lactose disaccharides were prevailing by weight at around 75% lactose conversion and later, with β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→3)-D-Glc as the two main products. In addition to these main GOS components which were identified and quantitated by HPAEC-PAD using authentic standards and the standard addition technique, β-D-Gal*p*-(1→3)-D-Gal and β-D-Gal*p*-(1→6)-D-Gal were identified in the reaction mixtures; these were, however, minor constituents. GOS containing new β-(1→4) linkages could not be identified in these mixtures.

To examine whether the high thermostability of Lbulβgal can be exploited for GOS synthesis, the lactose conversion experiments at higher temperatures, *i.e.*, 40 and 50 °C, using otherwise identical conditions were conducted. Table 9 lists these results for a comparable degree of lactose conversion of ~90%. The reaction mixture showed a very similar composition regardless of the reaction temperature, however the time

needed to obtain 90% lactose conversion was greatly reduced, from 12 h reaction time at 30 °C to 8 h at 40 °C and only 5 h at 50 °C.

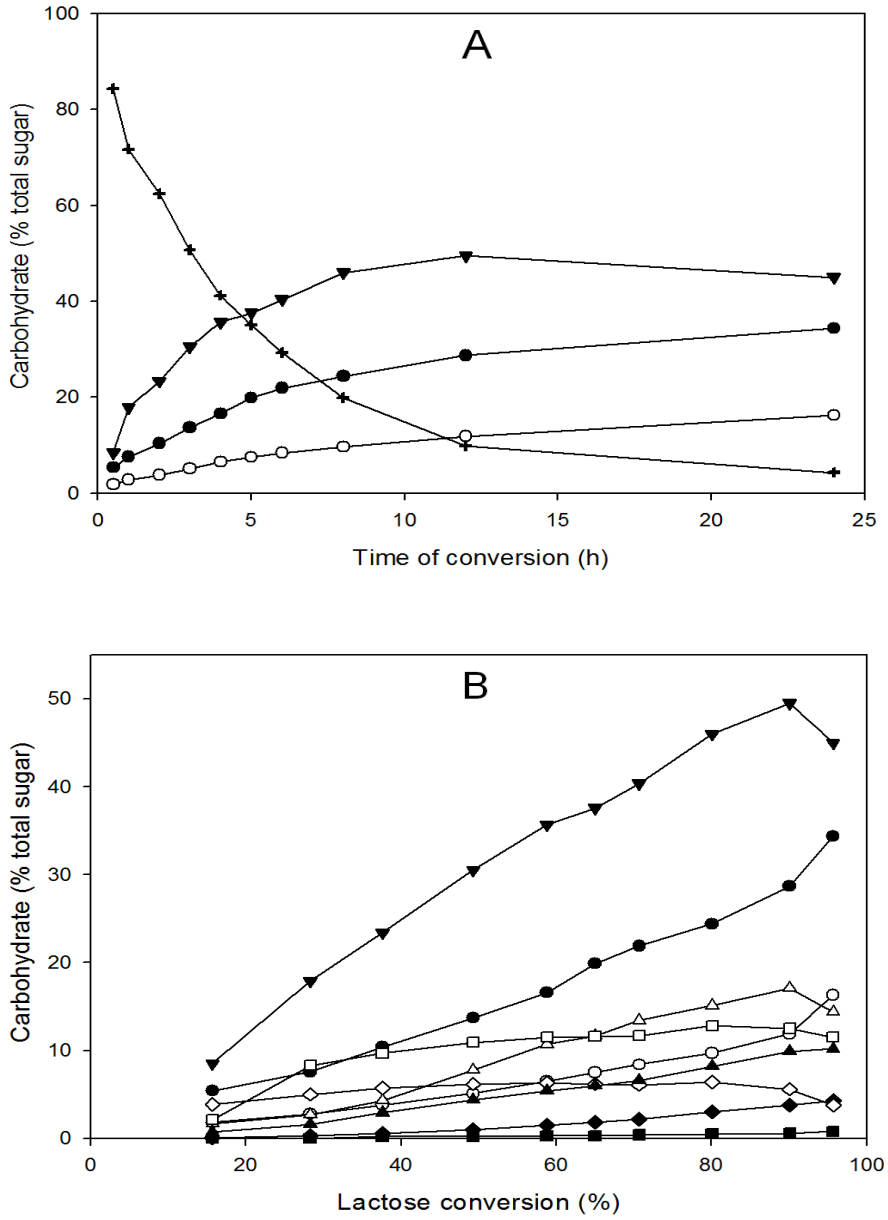


Figure 5. Composition of the sugar mixture during lactose conversion by recombinant β-galactosidase from *L. bulgaricus*. The reaction was carried out at 30°C with an initial concentration of 600 mM of lactose in 50 mM sodium phosphate buffer pH 6.5 in the presence of 10 mM $MgCl_2$ using ~1.5 U_{Lac} mL^{-1} of enzyme. (A) Time course of the conversion. Symbols: (+), lactose; (●), glucose; (○), galactose; (▼) total galacto-oligosaccharides (GOS). (B) Composition of the sugar mixture and individual GOS components at different degrees of lactose conversion. Symbols: (●), glucose; (○), galactose; (▼) total (GOS); (◆), β-D-Gal*p*-(1→3)- D-Glc; (■), β- D-Gal*p*-(1→3)-D-Gal; (◊), β-D-Gal*p*-(1→3)-Lac; (Δ), β-D-Gal*p*-(1→6)-D-Glc; (□), β-D-Gal*p*-(1→6)-Lac; (♦), unidentified GOS. Monosaccharides were measured enzymatically, lactose and GOS were quantified by HPAEC-PAD and CE. Individual sugars are given as the percentage of total sugars (205 g L^{-1}) in the mixtures.

Table 9. Galacto-oligosaccharide components (% w/w of total sugar) of GOS mixtures obtained with β-galactosidase of *L. bulgaricus* at three different temperatures[a]

GOS components	Reaction temperature		
	30 °C	40 °C	50 °C
Glucose	28.7	31.0	32.5
Galactose	11.9	13.5	14.2
Total GOS	49.5	48.7	48.2
β-D-Gal*p*-(1→3)-D-Gal	0.6	0.6	0.6
β-D-Gal*p*-(1→3)-D-Glc	3.8	4	3.9
β-D-Gal*p*-(1→3)-Lac	5.6	5.1	4.5
β-D-Gal*p*-(1→6)-D-Gal	1.0	1.3	1.1
β-D-Gal*p*-(1→6)-D-Glc	17.1	15.5	15
β-D-Gal*p*-(1→6)-Lac	12.5	12.5	13.2
Unknown OS	8.9	9.7	9.9
Lactose conversion	90.1 [b]	93.2 [c]	94.9 [d]

A lactose concentration of 600 mM (205 g/l) and 1.5 U mL^{-1} of β-galactosidase activity (determined with lactose as substrate under standard assay conditions) were used in each experiment. Data are given for the maximal yields obtained during the course of the reaction. Experiments were performed in duplicate, and the standard deviation was always <5%. [b]At 12h.[c]At 8 h. [d]At 5 h.

Expression and purification of recombinant BbreβgalI and Bbreβgal from *B. breve*

The BbreβgalI and BbreβgalII genes were cloned into pET-21a(+). The resulting expression vectors were then transformed into *E. coli* T7 express cells and T7 express cells carrying the plasmid pGRO7. The resulting clones were cultivated under inducing conditions in MagicMedia to compare the expression yields with and without chaperone co-expression. BbreβgalI and BbreβgalII expressed in the strains with chaperones showed a 30- and 14-fold increase in activity compared to the activity obtained from the strains without chaperones, respectively (Table 10). When using these conditions, 193 kU per liter of fermentation broth with a specific activity

of 159 U mg^{-1} of BbreβgalI and 36 kU per liter of fermentation broth with a specific activity of 31 U mg^{-1} of BbreβgalII were obtained.

Table 10. β-Galactosidase activities in cell-free extracts of recombinant *E. coli* expressing *B. breve* β-gal I (BbreβgalI) or β-gal II (BbreβgalII) with and without coexpression of chaperones[a]

Enzyme	Volumetric activity (kU L^{-1} fermentation broth)[b]		Specific activity (U mg^{-1} protein)		Expression factor[c] (fold)
	without chaperones	with chaperones	without chaperones	with chaperones	
BbreβgalI	6.4	193.2	1.8	159.0	30.2
BbreβgalII	2.6	36.5	2.5	31.4	14.0

[a]Values are the mean of two cultivations.
[b]*o*NPG was used to determine enzyme activity.
[c]The expression factors are calculated as the ratios of the volumetric β-galactosidase activities obtained from the expressions with chaperones and without chaperones.

The expression levels of both enzymes increased even further when gene expression was induced using IPTG. The highest yields were obtained when 0.5 mM IPTG was used for induction with 683 kU per liter of fermentation broth for BbreβgalI and 169 kU per liter of fermentation broth for BbreβgalII when 1.0 mM IPTG was used (Table 11), which was an approximately 3.5- and 4.5-fold increase in enzyme yields for BbreβgalI and BbreβgalII, respectively, compared to the expressions in MagicMedia.

The enzymes were purified with a single-step purification using an IMAC column, and the results of representative purification procedures for both enzymes are summarized in Table 12. The purified enzymes were obtained with purification factors of approximately 3.2- and 8.5-fold from the crude extracts with an overall yield of approximately 70% and 50% for BbreβgalI and BbreβgalII, respectively. The specific activities of the purified enzymes were found to be 461 U mg^{-1} of protein for BbreβgalI and 196 U mg^{-1} of protein for BbreβgalII when using the standard *o*NPG assay. The purification procedure yielded a homogenous BbreβgalI and BbreβgalII preparation as judged by SDS-PAGE gel (Figure 6).

Table 11. Effect of isopropyl β-D-1-thiogalactopyranoside (IPTG) as inducer on β-galactosidase expression in *E. coli*[a]

Enzyme	IPTG concentration	Volumetic activity (kU L^{-1} fermentation broth)[b]	Protein (mg mL^{-1})	Specific activity (U mg^{-1})
BbreβgalI	0.1	657.1	7.6	86.5
	0.5	682.7	4.8	142.2
	1.0	567.3	3.5	162.1
BbreβgalII	0.1	98.0	5.8	16.9
	0.5	65.3	3.6	18.1
	1.0	168.6	4.9	34.4

[a]Values are the mean of two cultivations.
[b]*o*NPG was used to determine enzyme activity.

Table 12. Purification of recombinant *B. breve* β-galactosidases[a]

Enzyme	Purification step	Total activity (U)[b]	Total protein (mg)	Specific activity (U mg^{-1})	Purification factor	Recovery (%)
BbreβgalI	crude enzyme	9002	63.0	142.9	1.0	100.0
	IMAC	6274	13.6	461.3	3.2	69.7
BbreβgalII	crude enzyme	2521	109.0	23.1	1.0	100.0
	IMAC	1257	6.4	196.4	8.5	49.9

[a]Values are the mean of two cultivations.
[b]*o*NPG was used to determine enzyme activity.

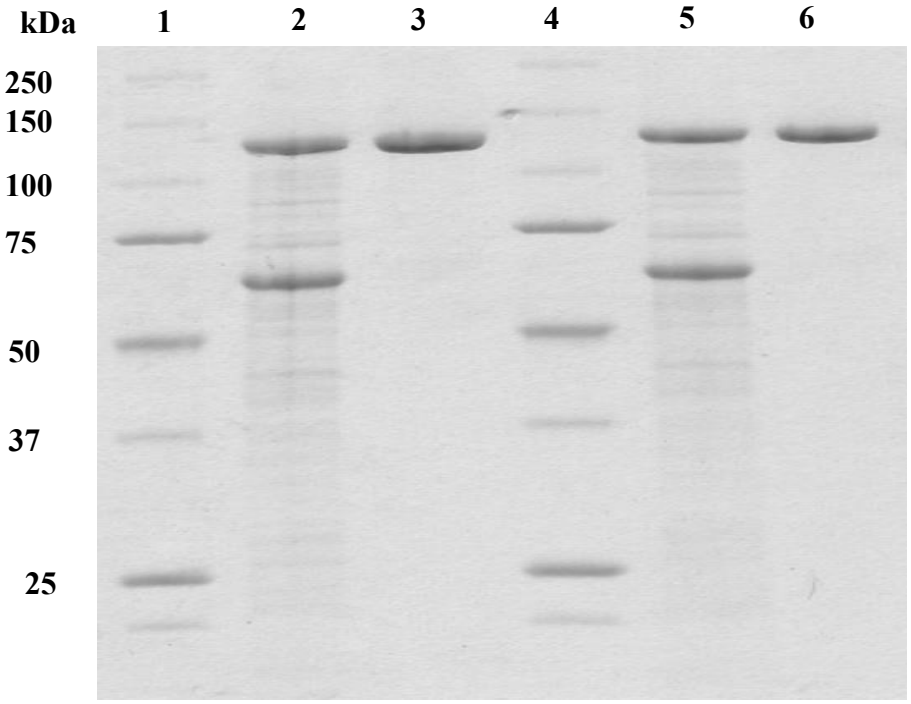


Figure 6. SDS-PAGE analysis of recombinant *β*-galactosidases from *B. breve* stained with Coomassie blue. Lanes 1 and 4 shows the molecular mass marker (Amersham); lanes 2 and 5 are the crude extracts of BbreβgalI and BbreβgalII, lanes 3 and 6 are the purified enzymes of BbreβgalI and BbreβgalII.

Molecular characterization of BbreβgalI and BbreβgalII

Both recombinant β-galactosidases from *B. breve* showed molecular masses of approximately 120 kDa as judged by SDS-PAGE in comparison with reference proteins (Figure 6). Molecular masses of 116,127 and 116,594 Da were calculated for BbreβgalI and BbreβgalII, respectively, based on their DNA sequences. SEC-MALLS analysis revealed that the native molecular masses of BbreβgalI and BbreβgalII are 220 and 211 kDa, respectively. Therefore, it can be concluded that both enzymes are homodimers (Table 13).

Table 13. Structural properties of recombinant β-galactosidases from *B. breve*

Enzyme	No. of amino acids	Molar mass (kDa) determined by		
		DNA sequence	SDS-PAGE	SEC-MALLS
BbreβgalI	1051	116.1	~120	220
BbreβgalI	1045	116.6	~120	211

Kinetic studies of BbreβgalI and Bbreβgal

The steady-state kinetic constants and the inhibition constants of *B. breve* β-galactosidases determined for the hydrolysis of lactose and *o*-nitrophenyl β-D-galactopyranoside (*o*NPG) are summarized in Table 14. The k_{cat} values were calculated on the basis of the theoretical v_{max} values experimentally determined by nonlinear regression and using a molecular mass of 116 kDa for the catalytically active subunit. BbreβgalI and Bbreβgal are not inhibited by their substrates, which are *o*NPG in concentrations of up to 25 mM or lactose in concentrations of up to 600 mM, as it is evident from the Michaelis-Menten plots (not shown).

The catalytic efficiencies (k_{cat}/K_m) for the two substrates, lactose and *o*NPG, indicate that the latter is the preferred substrate of both BbreβgalI and Bbreβgal. The end product D-galactose was found to competitively inhibit the hydrolysis of lactose by both enzymes. This inhibition, however, is only moderate as is obvious from the ratio of the Michaelis constant for lactose and the inhibition constant for D-galactose, which were calculated for both enzymes (BbreβgalI, $K_{i,Gal}/K_{m,Lac}$ = 1.8; BbreβgalI, $K_{i,Gal}/K_{m,Lac}$ = 3.6). D-Galactose was also found to be a competitive inhibitor against *o*NPG with inhibition constants of 15 mM for BbreβgalI and 34 mM for BbreβgalII.

Based on the ratio of K_i to K_m this inhibition is even less pronounced BbreβgalI, $K_{i,Gal}/K_{m,oNPG}$ = 11.5; BbreβgalII, $K_{i,Gal}/K_{m,oNPG}$ = 50.7). *o*NPG was also used as the substrate for studying inhibition by the second end product, D-glucose. Again, glucose is a competitive inhibitor of both enzymes, but this inhibiting effect is not pronounced BbreβgalI, $K_{i,Glc}/K_{m,oNPG}$ = 92; BbreβgalI, $K_{i,Glc}/K_{m,oNPG}$ = 55).

Table 14. Kinetic parameters of two recombinant β-galactosidases (BbreβgalI and BbreβgalII) from *B. breve* for the hydrolysis of lactose and *o*-nitrophenyl β-D-galactopyranoside (*o*NPG)

Substrate	Method for determination of enzyme activity	Kinetic parameter	BbreβgalI	BbreβgalII
Lactose	release of D-Glc	$v_{max,Lac}$ (μmol min^{-1} mg^{-1})	59 ± 2	97 ± 5
		$K_{m,Lac}$	15.3 ± 3.2	7.5 ± 0.9
		k_{cat} (s^{-1})	114 ± 4	188 ± 10
		k_{cat}/K_m (mM^{-1} s^{-1})	7.4 ± 1.9	25 ± 4
		$K_{i,Gal}$	28 ± 9	27 ± 6
*o*NPG	release of *o*NP	$v_{max,oNP}$ (μmol min^{-1} mg^{-1})	486 ± 9	188 ± 3
		$K_{m,oNP}$	1.3 ± 0.1	0.67 ± 0.07
		k_{cat} (s^{-1})	939 ± 7	364 ± 6
		k_{cat}/K_m (mM^{-1} s^{-1})	722 ± 66	543 ± 65
		$K_{i,Gal}$	15 ± 3	34 ± 5
		$K_{i,Glc}$	120 ± 31	37 ± 4

Effects of temperature and pH on BbreβgalI and Bbreβgal activity and stability

Both *o*NPG and lactose were used as substrates to determine the temperature and pH optimum of BbreβgalI and BbreβgalII activity. The pH optimum of BbreβgalI is pH 7.0 for both *o*NPG and lactose hydrolysis (Figure 7A, B). This enzyme is also most stable at pH 7.0, retaining 60% and approximately 30% of its activity when incubated at pH 7.0 and 37°C for 4 and 10 h, respectively (Figure 8). BbreβgalI has a half-life time of activity ($\tau_{1/2}$) of approximately 5 h when incubated at pH 7.0 and 37°C. The pH optimum of BbreβgalII is pH 6.5 for both *o*NPG and lactose hydrolysis (Figure 7A, B). A profile of pH stability was also determined for BbreβgalI and the enzyme is most stable at pH 6.0 – 7.0. The residual activities of this enzyme after 10 h of incubation at pH 6.0, 6.5, and 7.0 at 37°C were 72%, 82%, and 83%, respectively (Figure 8). The stability of both enzymes rapidly dropped at pH values below 6.0 or above 8.0. When incubated at pH 5.0 for 10 min, BbreβgalI showed no residual activity while BbreβgalII retained only 20% of its activity (data not shown).

The optimum temperature of BbreβgalI activity was 50 °C when using both *o*NPG and lactose as substrates under standard assay conditions. In comparison to BbreβgalI, BbreβgalII had higher optima, which were at 55 °C for both *o*NPG and lactose as substrates (Figure 7C, D). Both recombinant enzymes showed a half-life time of activity ($\tau_{1/2}$) of approximately 5 months at 4 °C when stored in sodium phosphate buffer (pH 6.5). Both enzymes also showed their stability at 30 °C with half-life time of activities ($\tau_{1/2}$) of 73 and 109 h for BbreβgalI and BbreβgalII, respectively (Table 15A, B).

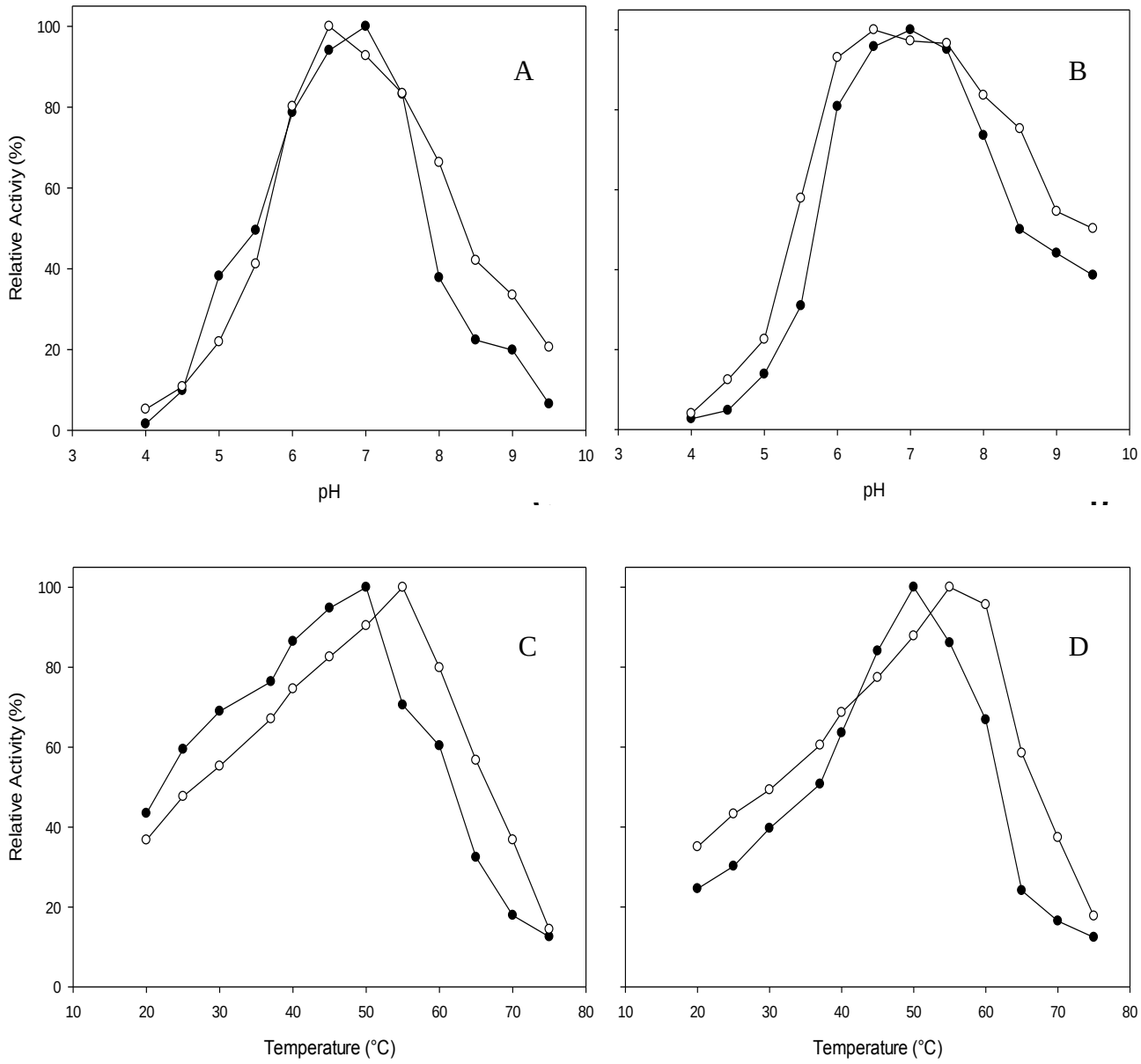


Figure 7. pH (A and B) and temperature (C and D) optimum of β-galactosidase activity for *B. breve* BbreβgalI (●) and BbreβgalII (○) using *o*NPG (A and C) and lactose (B and D) as substrate.

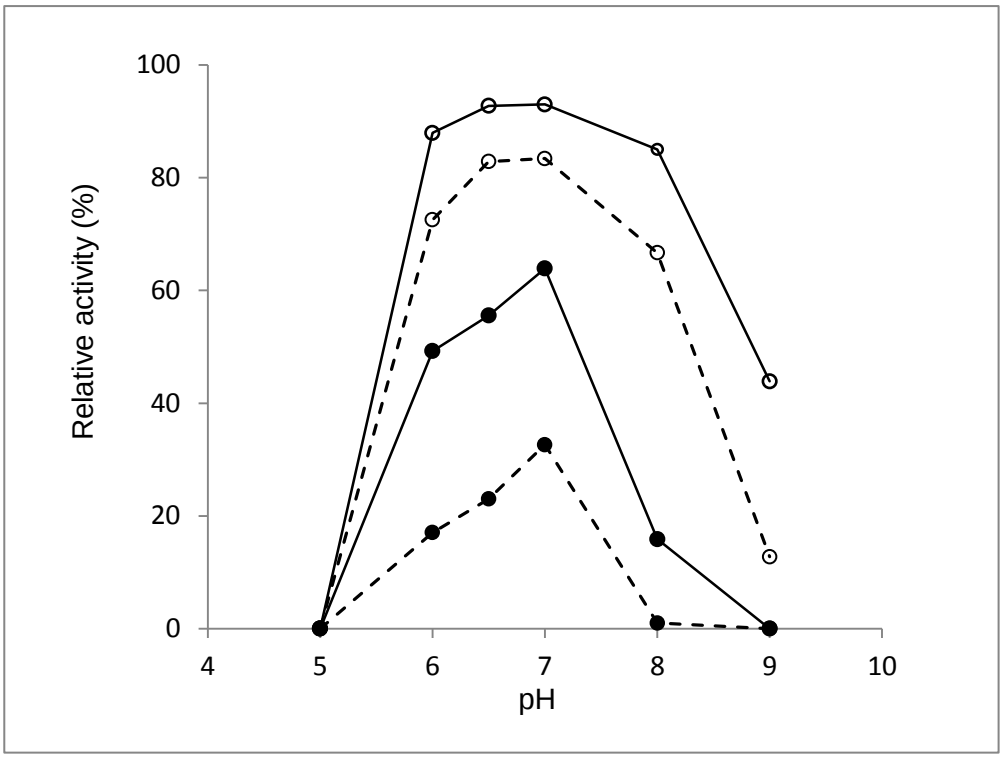


Figure 8. pH stability of the β-galactosidases from BbreβgalI (●) and BbreβgalI (○) incubated at 37 °C in Britton-Robinson buffer over a pH range of pH 5.0 – 9.0 for 4 h (solid lines) and 10 h (dashed lines). The residual activity was measured after 4 h and 10 h (B) and *o*NPG was used as substrate for the enzyme assay.

Thermal stability of both BbreβgalI and BbreβgalII was significantly improved in the presence of $MgCl_2$. Table 15 (A, B) shows the effect of 1 and 10 mM of $MgCl_2$ on the thermostability of BbreβgalI and BbreβgalII at 37 °C and higher. In the presence of 1 mM $MgCl_2$ BbreβgalII showed 19-, 10- and 4-fold increase in its half-life time of activity ($\tau_{1/2}$) at 37, 45 and 50 °C, respectively. A further increase of the Mg^{2+} concentration to 10 mM showed to be less effective with respect to stabilization of this enzyme at the above temperatures. At all conditions tested, BbreβgalII was found to be more stable than BbreβgalI. In the presence of 1 mM $MgCl_2$ the half-life time of BbreβgalII activity ($\tau_{1/2}$) at 50 °C was increased to 1.25 h, compared to 0.12 h without Mg^{2+}. A further increase of the Mg^{2+} concentration to 10 mM was more effective in improving thermostability of BbreβgalII activity.

Thermal denaturation of BbreβgalI and BbreβgalII monitored in DSC

Calorimetric studies on the thermal denaturation of BbreβgalI and BbreβgalII were performed using DSC. Both enzymes showed a single endothermic peak in the DSC scan which fitted very well on the basis of a two-state transition model (Figure 9A, B). The observed melting temperatures T_m, 49.97 and 55.58 °C for BbreβgalI and BbreβgalII in Figure 9A, B, respectively, are in excellent agreement with the optimum temperatures of these two enzymes as shown in Figure 7C, D.

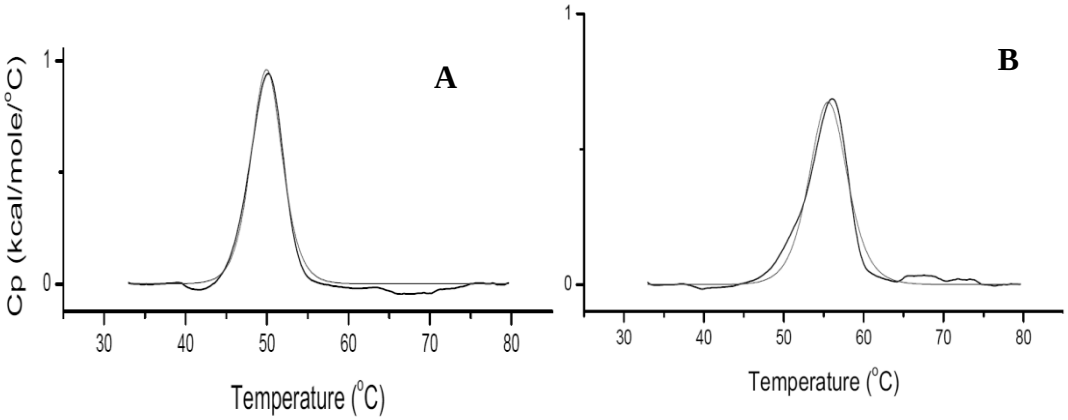


Figure 9. Normalized DSC thermograms of *Bifidobacterium breve* (A) BbreβgalI and (B)BbreβgalII (1 mg mL^{-1}) in 50 mM sodium phosphate buffer, pH 6.5 with a heating rate of 60°C h^{-1} at temperature range from 33 to 80 °C. Fits of experimental data to a two state model are shown with a thinner and smoother line.

Table 15. Stability of *β*-galactosidases from *B. breve* at different temperatures in the absence of $MgCl_2$ as well as in the presence of 1 and 10 mM $MgCl_2$

(A) BbreβgalI

Temperature (°C)	Sodium phosphate buffer, pH 7		Sodium phosphate buffer, pH 7 +1 mM Mg^{2+}		Sodium phosphate buffer, pH 7 + 10 mM Mg^{2+}	
	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)
30	1.00 (± 0.00) × 10^{-2}	73	2.00 (± 0.01) × 10^{-3}	428	3.00 (± 0.00) × 10^{-3}	235
37	0.32 ± 0.03	2	2.00 (± 0.00) × 10^{-2}	37	2.30 (± 0.00) × 10^{-2}	28
45	9.56 ± 0.26	0.07	0.96 ± 0.04	0.72	1.18 ± 0.06	0.59
50	36.7 ± 1.2	0.02	9.00 ± 0.39	0.08	10.80 ± 0.37	0.06

(B) BbreβgalII

Temperature (°C)	Sodium phosphate buffer, pH 6.5		Sodium phosphate buffer (pH 6.5) +1 mM Mg^{2+}		Sodium phosphate buffer (pH 6.5) + 10 mM Mg^{2+}	
	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)
30	4.67 (± 0.06) × 10^{-3}	109	2.58 (± 0.08) × 10^{-3}	268	2.34 (± 0.16) × 10^{-3}	297
37	2.07 (± 0.07) × 10^{-2}	33	3.79 (± 0.01) × 10^{-3}	183	3.78 (± 0.07) × 10^{-3}	183
50	5.70 ± 0.04	0.12	0.55± 0.01	1.25	0.19 ± 0.0.01	3.7

BbreβgalI and BbreβgalII substrate specificity

BbreβgalI and BbreβgalII displayed a narrow substrate range when using chromogenic substances. Both enzymes showed 1% activity (relative to *o*NPG) when using 2-nitrophenyl-β-D-glucopyranoside while no activity (<0.05%) was observed when 4-nitrophenyl-β-D-mannopyranoside, 4-nitrophenyl-α-D-galactopyranoside, or 4-nitrophenyl-α-D-glucopyranoside were used as substrates.

Activities of *B. breve* β-galactosidases with individual galactosides are expressed as a percentage of hydrolysis (or conversion) of each substrate after 30 and 60 min (Table 16). It was found that BbreβgalI shows high hydrolytic activity towards

lactose, β-D-Gal*p*-(1→6)-D-Glc (allolactose), β-D-Gal*p*-(1→3)-D-Lac, β-D-Gal*p*-(1→3)-D-Glc and β-D-Gal*p*-(1→3)-D-Gal and hydrolyzes these substrates at comparable rates. BbreβgalII also showed high activities with lactose, β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→3)-D-Lac but these substrates were hydrolyzed at slightly lower rates than that by BbreβgalI. BbreβgalII hydrolyzes the disaccharides β-D-Gal*p*-(1→3)-D-Glc and β-D-Gal*p*-(1→3)-D-Gal at significantly lower rates than BbreβgalI. Both enzymes show low activity with the disaccharide β-D-Gal*p*-(1→4)-D-Gal and the trisaccharides β-D-Gal*p*-(1→4)-D-Lac and β-D-Gal*p*-(1→6)-D-Lac, which is evident from the slow hydrolysis rates of these substrates. The disaccharide β-D-Gal*p*-(1→6)-D-Gal was hydrolyzed by both enzymes at the same rate, which is approximately 50% of the rate of lactose hydrolysis in the first 30 min of the reactions.

Table 16. Relative activities of *B. breve* β-galactosidases for individual galactosides[a]

Substrate	% Conversion			
	BbreβgalI		BbreβgalII	
	30 min	60 min	30 min	60 min
Lactose	>99	>99	86.3	>99
β-D-Gal*p*-(1→6)-D-Glc	>99	>99	98.3	>99
β-D-Gal*p*-(1→3)-D-Glc	97.7	>99	61.7	88.6
β-D-Gal*p*-(1→3)-D-Gal	98.7	>99	73.1	90.9
β-D-Gal*p*-(1→4)-D-Gal	11.6	12.4	4.7	11.6
β-D-Gal*p*-(1→6)-D-Gal	52.1	79.0	48.2	80.4
β-D-Gal*p*-(1→3)-D-Lac	>99	>99	85.7	97.6
β-D-Gal*p*-(1→4)-D-Lac	22.1	29.1	21.8	26.2
β-D-Gal*p*-(1→6)-D-Lac	10.3	30.4	5.9	14.9

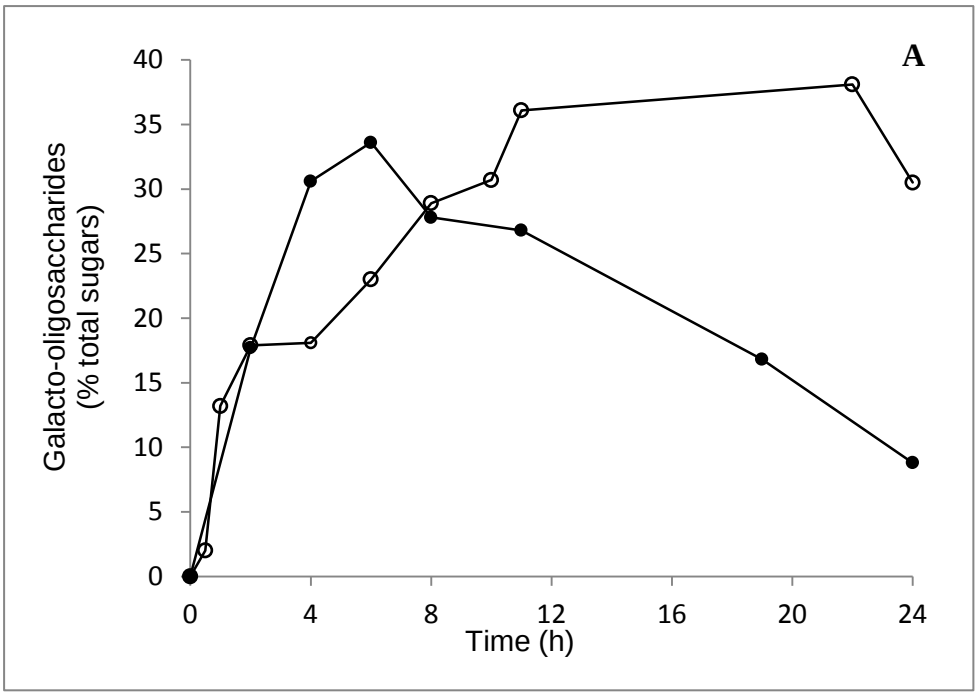
[a]Results are expressed as a percentage of hydrolysis (or conversion) of each substrate after 30 and 60 min.

GOS synthesis by BbreβgalI and BbreβgalII

The transgalactosylation activity of BbreβgalI and BbreβgalII was investigated. Figure 10 shows GOS formation of a typical discontinuous conversion reaction at 30 °C with an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ using 1.5 U_{Lac} mL^{-1} of enzyme. Under these conditions, maximum GOS yields of 33% total sugars after 6 h of reaction at 70% lactose conversion and of 38% total sugars after 22 h of reaction at 96% lactose conversion were obtained with BbreβgalI and BbreβgalII, respectively. It also shows that during lactose conversion using BbreβgalI under these conditions, there is only a slight increase in GOS yield between 85% and 96% lactose conversion. The GOS yield almost reaches its maximum at 36% total sugars after 11 h of reaction at 85 % lactose conversion using BbreβgalII (Figure 10A, B). The amount of GOS expressed as percentage of total sugars is constantly rising up to ~70% and ~90% lactose conversion using BbreβgalI and BbreβgalII, respectively. After these points, at which maximum GOS yields were obtained for both enzymes, the concentration of GOS decreased because they are also subjected to hydrolysis by the β-galactosidases. This is particularly pronounced for BbreβgalI.

The effect of enzyme concentration in discontinuous conversion reactions on GOS yield was investigated. When the concentration of BbreβgalI in the conversion reaction was reduced to 1.0 U_{Lac} mL^{-1}, a slight difference on the maximum GOS yield, which was 30% total sugars at 70% lactose conversion, was observed. Interestingly, when the concentration of BbreβgalII in the conversion reaction was increased to 2.5 U_{Lac} mL^{-1}, maximum GOS yield increased from 38% to 44% of total sugars, which was obtained at 84% lactose conversion, and also the time needed to obtain this maximum GOS yield was reduced to 6 h (data not shown).

Individual GOS can be separated effectively using a Carbopac PA1 column for HPAEC with pulsed amperometric detection as shown in Figure 11A, B. It was possible to identify the main products of transgalactosylation by both BbreβgalI and BbreβgalII. These main transferase products formed and degraded at different lactose conversion are presented in Table 17. The predominant oligosaccharide product was identified as β-D-Gal*p*-(1→6)-D-Glc (allolactose), accounting for approximately 45% and 50% of the GOS formed by transgalactosylation by BbreβgalI and BbreβgalII, respectively, at maximum total GOS yield. β-D-Gal*p*-(1→3)-D-Lac was identified as the second predominantly transferase product at the maximum total GOS yield point, contributing approximately 32% and 16% of the total GOS formed by transgalactosylation by BbreβgalI and BbreβgalII, respectively. Other identified products, including β-D-Gal*p*-(1→3)-Glc, β-D-Gal*p*-(1→3)-Gal, β-D-Gal*p*-(1→6)-Gal, β-D-Gal*p*-(1→6)-Lac and β-D-Gal*p*-(1→4)-Lac, make up approximately 12% and 20% of total GOS (at total GOS maximum yield point) formed using BbreβgalI and BbreβgalII, respectively. 4′-Galactobiose was not detected at all during the course of lactose conversion. It should be noted that the unidentified peaks 8 and 14 were present in detectable concentrations (Figure 11A, B). However, the structure of these components has yet to be determined.



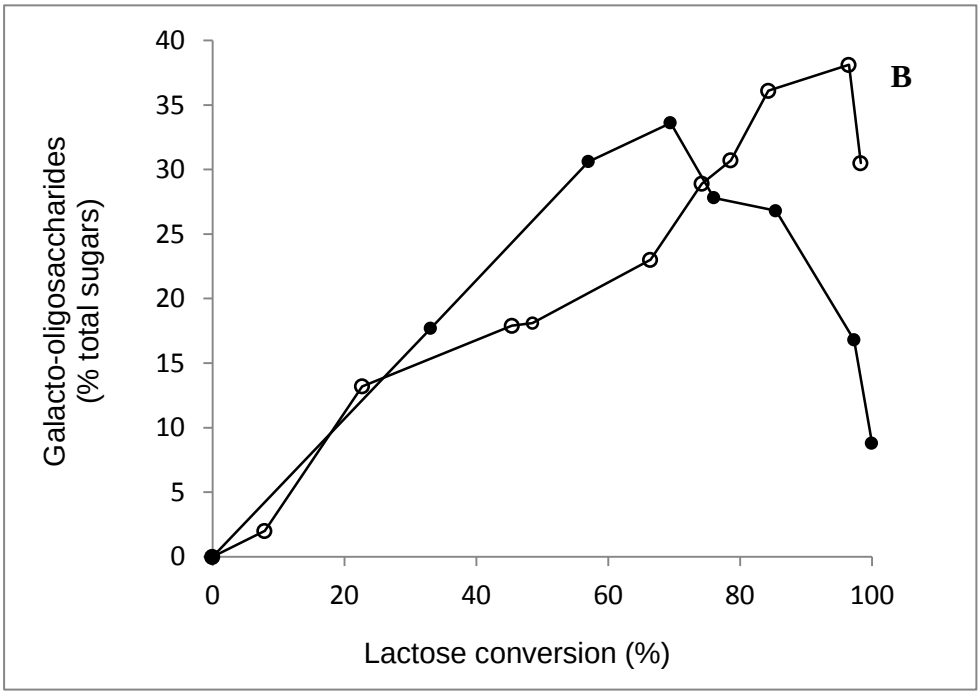


Figure 10. Time course of GOS formation (A) and degradation of GOS during lactose conversion (B) catalyzed by *B. breve* BbreβgalI (●) and BbreβgalI (○). The reaction was performed at 30°C at an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ using 1.5 U_{Lac} mL^{-1}.

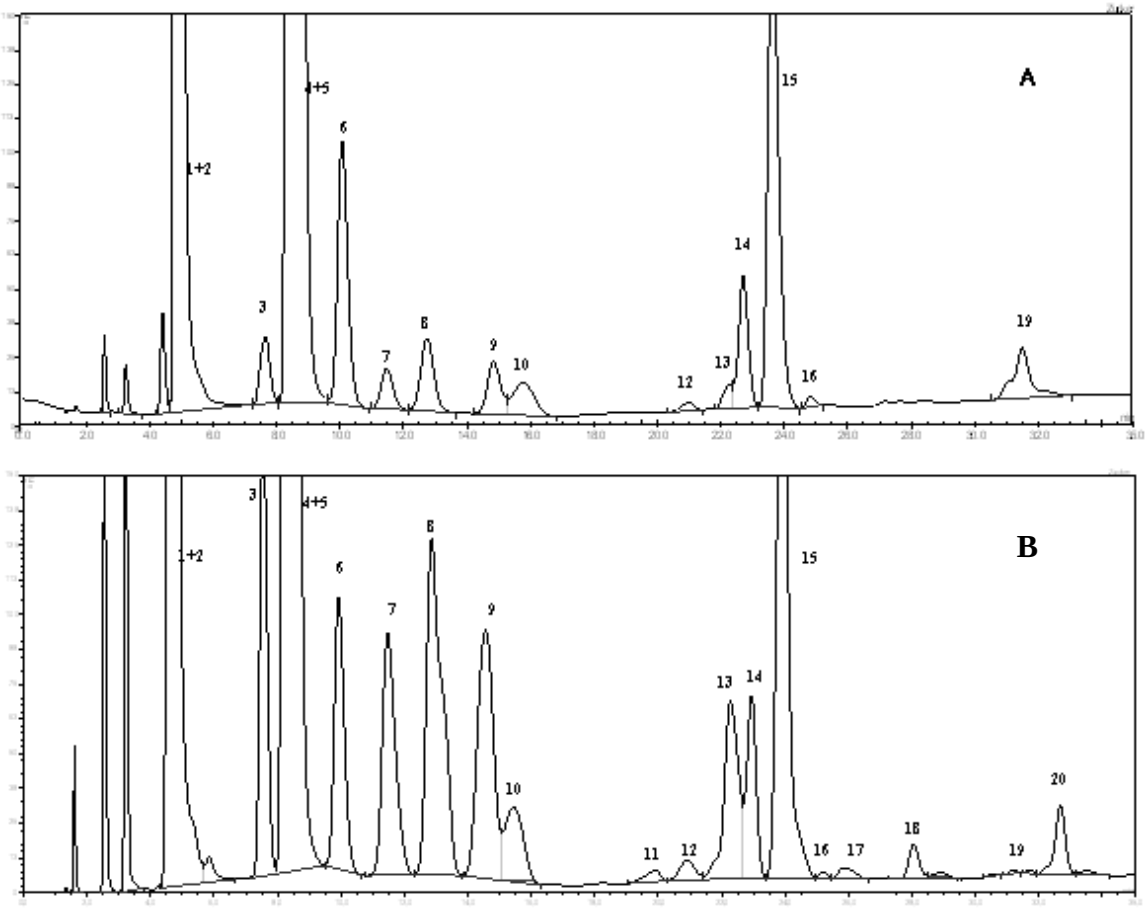


Figure 11. Separation and quantification by HPAEC-PAD of individual GOS produced during lactose conversion catalyzed by (A) BbreβgalI and (B) BbreβgalI. The identified compounds are (1) D-galactose, (2) D-glucose, (3) D-Galp-(1→6)-D-Gal, (4) D-Galp-(1→6)-D-Glc (allolactose), (5) D-Galp-(1→4)-D-Glc (lactose), (6) D-Galp-(1→3)-D-Gal, (7) D-Galp-(1→6)-Lac, (9) D-Galp-(1→3)-D-Glc, (13) D-Galp-(1→4)-Lac and (15) D-Galp-(1→3)-Lac. Peaks 8, 10-12, 14, and 16-20 were not identified.

Table 17. Individual GOS components produced by the transgalactosylation reaction of BbreβgalI (I) and BbreβgalII (II) from *Bifidobacterium breve* DSM 20031 using lactose as substrate.

GOS components[a]	Degree of Lactose Conversion													
	20%		35%		55%		70%		84%		95%		99%	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II
GOS components (g L^{-1})														
D-Gal*p*-(1→6)- D-Glc	5.39	5.59	9.75	10.2	24.0	25.7	27.3	39.2	23.8	45.3	14.0	43.2	6.39	25.0
D-Gal*p*-(1→6)- D-Gal	0.21	0.58	0.35	0.88	0.63	1.94	0.86	2.62	2.22	3.52	4.13	5.11	5.30	8.20
D-Gal*p*-(1→3)- D-Gal	0.95	0.43	1.50	0.74	2.68	1.75	3.41	2.07	4.01	2.90	2.28	3.77	1.32	4.30
D-Gal*p*-(1→3)- D-Glc	0.16	0.60	0.28	1.23	0.76	3.23	1.29	4.45	2.17	5.30	2.03	6.51	1.21	4.95
D-Gal*p*-(1→3)-Lac	1.25	1.42	9.04	3.99	18.6	8.08	19.6	10.4	7.64	14.3	1.55	16.4	<0.01	14.9
D-Gal*p*-(1→4)-Lac	0.08	0.16	0.12	0.38	0.26	1.42	0.44	2.21	0.62	2.75	0.33	3.35	0.04	1.88
D-Gal*p*-(1→6)-Lac	0.22	0.69	0.44	0.97	0.61	2.30	1.42	2.92	2.57	3.73	2.88	4.50	1.51	3.74
Other GOS	0.00	0.00	7.89	0.49	4.58	3.48	6.34	2.24	9.20	9.96	0.0	2.90	0.00	9.25
Total GOS	8.26	9.47	29.4	18.85	52.2	47.9	60.7	66.1	44.6	87.8	27.2	85.7	15.8	72.2
GOS components (mM)														
D-Gal*p*-(1→6)- D-Glc	15.7	16.3	28.5	29.7	70.2	75.1	79.8	115	69.7	132	40.9	126.1	18.7	73.0
D-Gal*p*-(1→6)- D-Gal	0.61	1.69	1.02	2.57	1.84	5.67	2.51	7.65	6.49	10.3	12.1	14.9	15.5	24.0
D-Gal*p*-(1→3)- D-Gal	2.78	1.26	4.38	2.16	7.83	5.11	9.96	6.05	11.7	8.47	6.67	11.0	3.86	12.6
D-Gal*p*-(1→3)- D-Glc	0.47	1.75	0.82	3.59	2.22	9.44	3.77	13.0	6.34	15.5	5.93	19.0	3.53	14.5
D-Gal*p*-(1→3)-Lac	2.48	2.82	17.9	7.91	36.9	16.0	38.9	20.7	15.2	20.5	3.02	32.43	0.0	29.4
D-Gal*p*-(1→4)-Lac	0.16	0.32	0.24	0.75	0.52	2.82	0.87	4.38	1.23	5.45	0.65	6.64	0.08	3.73
D-Gal*p*-(1→6)-Lac	0.44	1.38	0.87	1.93	1.22	4.57	2.81	5.78	5.09	7.39	5.70	8.92	3.00	7.41
GOS components (% mass of total GOS)														
D-Gal*p*-(1→6)- D-Glc	65.3	59.0	33.2	54.0	46.1	53.7	45.1	59.3	53.4	51.6	51.5	50.4	40.5	34.6
D-Gal*p*-(1→6)- D-Gal	2.54	6.12	1.19	4.67	1.21	4.05	1.42	3.96	4.97	4.01	15.2	5.97	33.6	11.4
D-Gal*p*-(1→3)- D-Gal	11.5	4.54	5.11	3.93	5.14	3.65	5.62	3.13	8.98	3.30	8.39	4.40	8.37	5.95
D-Gal*p*-(1→3)- D-Glc	1.94	6.33	0.95	6.53	1.46	6.74	2.13	6.73	4.87	6.03	7.47	7.60	7.67	6.85
D-Gal*p*-(1→3)-Lac	15.1	15.0	30.8	21.2	35.7	16.8	32.3	15.8	17.1	16.3	5.70	19.1	0.0	20.6
D-Gal*p*-(1→4)-Lac	0.97	1.69	0.41	2.02	0.50	2.96	0.73	3.34	1.39	3.13	1.21	3.91	0.25	2.60
D-Gal*p*-(1→6)-Lac	2.66	7.33	1.49	5.17	1.18	4.81	2.34	4.41	5.76	4.24	10.6	5.25	9.60	5.17

[a]D-Gal*p*-(1→4)-D-Gal was not detected at all lactose conversion level (limit of detection = 0.01 g L^{-1}). The reaction was performed at 30°C at an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ using 1.0 ULac mL^{-1} (BbreβgalI) or 2.5U_{Lac} mL^{-1} (BbreβgalII)

Intermolecular galactosyl transfer rate ratio

The propensity of four β-galactosidases, Lbulβgal, Lreuβgal, BbreβgalI, and BbreβgalII to transfer galactosyl moiety to different nucleophiles (such as lactose, Glc, Gal, Fuc, GlcNAc and GalNAc) was determined by measuring the initial velocities and establishing the partitioning ratio, k_{Nu}/k_{water}. Measurements were done at 30 °C in 50 mM sodium phosphate buffer (pH 6.5) by using either *o*NPG or lactose as galactosyl donor. Plots of (ν_{oNP}/ν_{Gal}) or (ν_{Glc}/ν_{Gal}) against [Nu] were linear for a specific range of acceptor concentrations. The F-test at 95% probability level confirmed the validity of the linear fit for the range of [Nu] as shown in Figure 12. Moreover, the goodness of the fit for the lines as represented by r^2 was usually greater than 0.98. In the absence of glucose, all the β-galactosidases completely hydrolyzed *o*NPG; ν_{oNP}/ν_{Gal} was nearly 1.0. BbreβgalII, which has the highest inhibition against glucose ($Ki_{,Glc}$ = 37 ± 4, Table 14), resulted in short range of glucose tested (up to 0.02 M) compared to other β-gal (e.g. *Lreu*βgal, 0.6 M). When k_{Glc}/k_{water} was determined, BbreβgalII showed lowest partitioning ratio (3.91 ± 0.44 M^{-1}) while Lbulβgal showed the highest (9.36 ± 0.56 M^{-1}) as shown in Table 18. When lactose alone was used as the substrate, where the only possible galactosyl acceptors are lactose, and its hydrolysis products, D-Gal and D-Glc, BbreβgalII showed the lowest k_{Lac}/k_{water} ratio (0.53 M^{-1}) while that of Lbulβgal was the highest (2.79 M^{-1}).

The propensity of four β-galactosidases to transfer the galactose to either GlcNAc, GalNAc or Fuc was determined using a fixed lactose concentration (100 mM) and varying nucleophile concentration and the k_{Nu}/k_{water} was determined by plotting the ν_{Glc}/ν_{Gal} against the respective [Nu]. In the absence of any external galactosyl acceptor, ν_{Glc}/ν_{Gal} was found to be ~1.3 using Lbulβgal suggesting high GOS production rate while that of the three β-gals was nearly 1.0 indicating that

hydrolysis of lactose is the preferred reaction. When GlcNAc was added as the galactosyl acceptor, Lbulβgal and BbreβgalII were shown to effectively transfer the galactosyl moiety to GlcNAc rather than to water as shown by the rate constant ratio, k_{GlcNAc}/k_{water} of 16.8 and 5.42, respectively. Lbulβgal can also possibly synthesized GalGalNAc as *N*-acetyl-D-galactosamine can serve as galactosyl acceptor considering

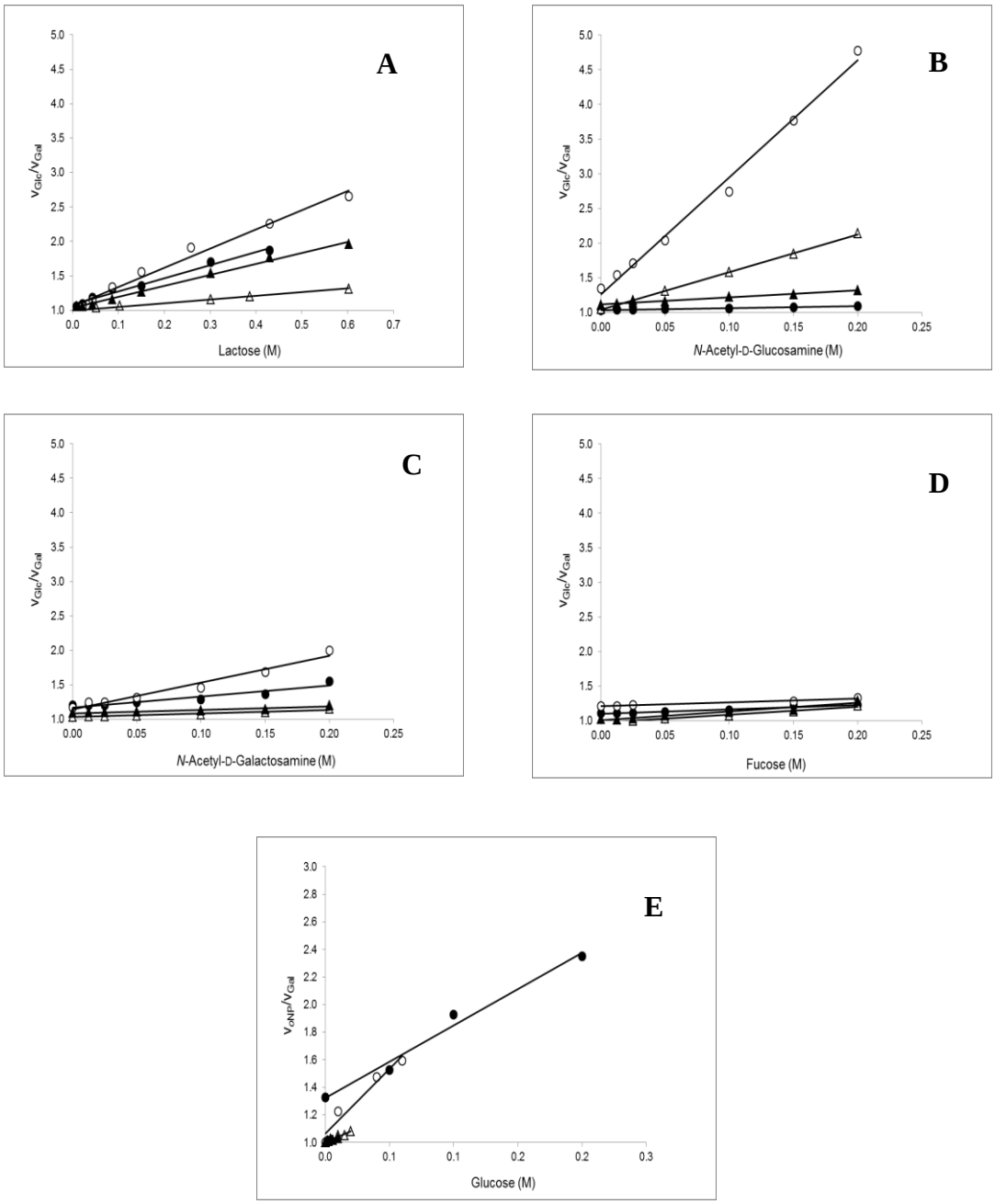


Figure 12. Transgalactosylation activity of *L. reuteri* (●), *L. bulgaricus* (○), *B. breve* β-gal I (▲), and *B. breve* β-gal II (Δ) in the presence of different exogenous nucleophiles. (A) Lactose (B) *N*-acetyl-D-glucosamine (C) *N*-acetyl-D-galactosamine (D) L- fucose (E) D-Glucose.

the obtained k_{GalNAc}/k_{water} ratio (3.21). BbreβgalI, BbreβgalII and Lreuβgal showed $k_{GalNAc}/k_{water} \leq 1.0$. L-fucose, on the other hand, was shown to be weak nucleophile based on the k_{GalNAc}/k_{water} ratio (≤ 1.27). The ratio of k_{Gal}/k_{water} is also an essential kinetic parameter to measure the propensity to transfer the galactosyl moiety to another galactose unit. Unfortunately, k_{Gal}/k_{water} could not be determined because the amount of galactose released cannot be measured in the presence of excess of free galactose.

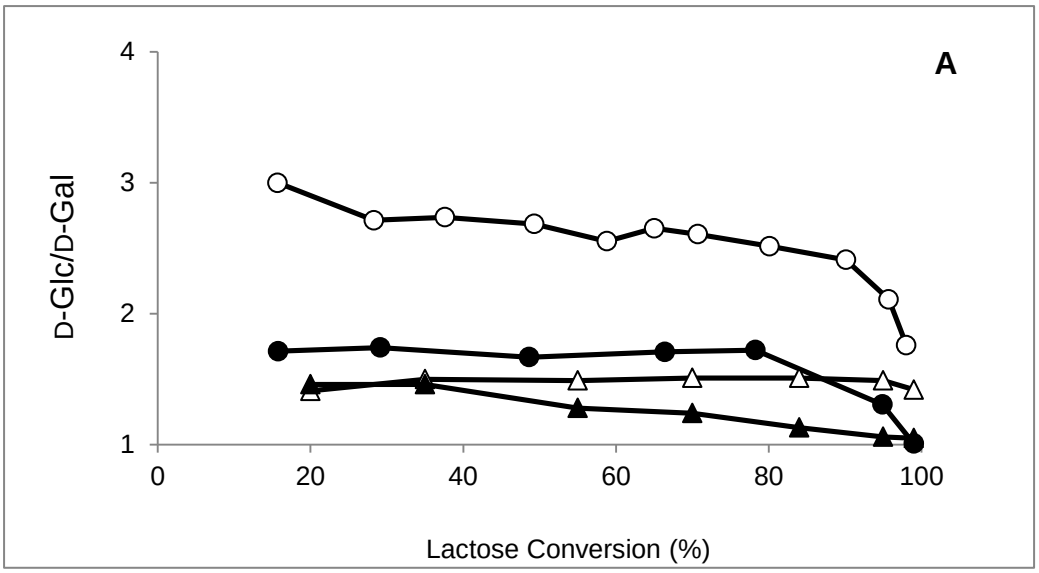
Table 18. Partitioning ratios (k_{Nu}/k_{water}, M^{-1}) for reaction of galactosylated β-galactosidases with exogenous nucleophiles and with water.

β-galactosidase sources	Nucleophile				
	D-Glc[a]	Lactose	GlcNAc	GalNAc	L-fucose
Bifidobacterium breve					
BreβgalI	6.73 ± 0.62	1.61 ± 0.05	1.01 ± 0.03	0.36 ± 0.03	1.27 ± 0.12
BreβgalI	3.91 ± 0.44	0.53 ± 0.02	5.42 ± 0.05	0.39 ± 0.02	1.16 ± 0.05
Lactobacillus bulgaricus	9.36 ± 0.56	2.79 ± 0.15	16.8 ± 0.7	3.21 ± 0.26	0.54 ± 0.05
Lactobacillus reuteri	6.7 ± 0.3*	1.91 ± 0.12*	0.27 ± 0.01	1.07 ± 0.09	0.67 ± 0.06

[a]Measured with 10 mM oNPGal as substrate and calculated from the v_{oNP}/v_{Gal}
* Ref. (*28*)

The transgalactosylation activity of the four recombinant β-galactosidases was compared using ~200 g L^{-1} lactose as substrate in sodium phosphate buffer (pH 6.5), 1 mM $MgCl_2$ at 30 ^{o}C. At all times, the ratio of D-Glc/D-Gal was higher with Lbulβgal when compared with that of Lreuβgal, BbreβgalI and BbreβgalII (Figure 13A); maximum of D-Glc/D-Gal ratio with Lbulβgal was 3.0 at 16% lactose conversion where the trisaccharides β-D-Galp-(1→6)-Lac and β-D-Galp-(1→3)-Lac formed

predominantly (Figure 5B). This ratio decreased to 2.71 and remained constant until lactose conversion is about 70% and decreased dramatically at 90% lactose conversion. The same trend was observed for BbreβgalI where max D-Glc/D-Gal ratio was observed at the initial stage of the reaction and was further decreased as the reaction progressed. For Lreuβgal and BbreβgalII, maximum values of D-Glc/D-Gal ratio are found in a rather broad range of lactose conversion (20-80%) and remained constant until the maximum GOS yield point was reached. At about 98-99% lactose conversion, D-Glc/D-Gal ratio using Lreuβgal and BbreβgalI was nearly 1.0 while with Lbulβgal and BbreβgalII was still 1.76 and 1.42. Maximum GOS yields of 49.5%, 35.3%, 30.3% was achieved with Lbulβgal, Lreuβgal, BbreβgalI and BbreβgalII, respectively (Figure 13B).



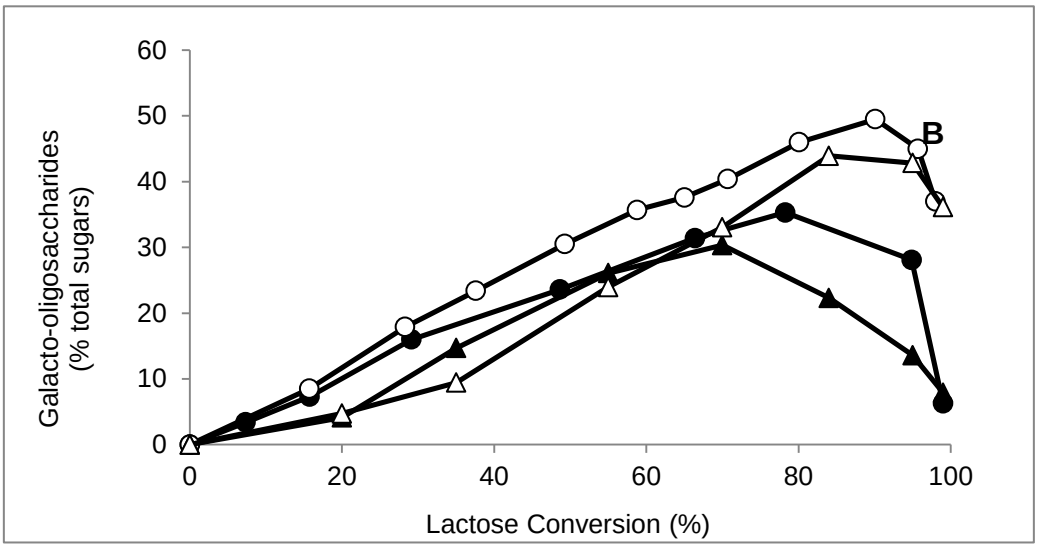


Figure 13. D-Glucose/D-Galactose ratio (A) and galacto-oligosaccharide production (B) during lactose conversion by β-galactosidase from *L. reuteri* (●), *L. bulgaricus* (○), *B. breve* β-gal I (▲), and *B. breve* β-gal II (Δ). The reactions were performed at 30 °C at an initial lactose concentration of ~200 g/L in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$.

***N*-acetyl oligosaccharide production by BbreβgalII and Lbulβgal**

To determine the transgalactosylation activity of BbreβgalII and Lbulβgal using lactose as galactosyl donor and lactose or GlcNAc as galactosyl acceptor, a number of discontinuous conversion reactions were carried with either BbreβgalII or Lbulβgal ($2.5U_{Lac}$ mL^{-1}) using initial concentration of 600 mM lactose in the presence or absence of 600 mM GlcNAc. The reaction temperature was at 30 ^{o}C. The formation of D-Glc, D-Gal, and GOS was monitored at different time intervals. Typical HPLC chromatograms of GOS catalyzed by Lbulβgal and BbreβgalII are depicted in Figure 14A and B, respectively. Figure 15 shows that using lactose alone as the substrate, the maximum D-Glc/D-Gal ratio with Lbulβgal and BbreβgalII is 3.0 and 1.5, respectively while presence of GlcNAc as substrate resulted in maximum D-Glc/D-Gal ratio of 11.0 and 4.6, respectively.

Allolactose is the major intramolecular transgalactosylation product when lactose is used as the substrate alone. Using an equimolar of lactose and GlcNAc as substrate, the maximum allolactose yield was found to decrease significantly with Lbulβgal while no significant change on that was observed with BbreβgalII ($k_{GlcNAc}/k_{Glc} \approx 1.4$) as shown in Figure 16. With BbreβgalII, increase in D-Glc/D-Gal ratio in the presence of GlcNAc as substrate can be also attributed to the inhibition of GalGal formation particularly 6'-galactobiose (Figure 17). Moreover, tri-GOS yield of BbreβgalII decreased significantly from 24 to 13 g L^{-1} when GlcNAc was added to lactose as substrate (Figure 18).

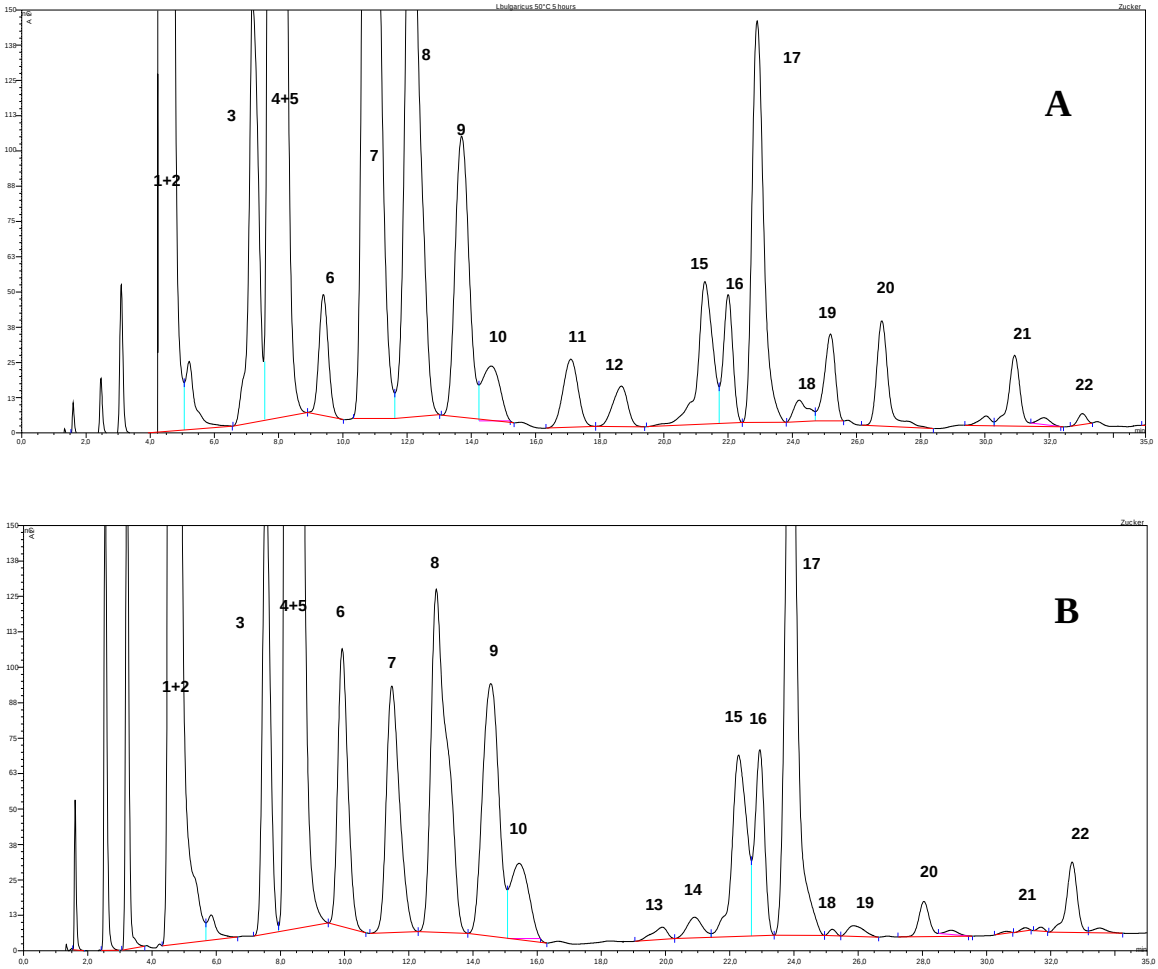


Figure 14. Separation and quantification by HPAEC-PAD of individual GOS produced during lactose conversion catalyzed by (**A**) *L. bulgaricus DSM* 20081 β-gal and (B) *B. breve* β-gal II. The identified compounds are (1) D-Gal, (2) D-Glc, (3) D-Gal*p*-(1→6)-D-Gal, (4) D-Gal*p*-(1→6)-D-Glc (allolactose), (5) D-Gal*p*-(1→4)-D-Glc (lactose), (6) D-Gal*p*-(1→3)-D-Gal, (7) D-Gal*p*-(1→6)-Lac, (9) D-Gal*p*-(1→3)-D-Glc, (15) D-Gal*p*-(1→3)-Lac, (17) D-Gal*p*-(1→4)-Lac. Peaks 8, 10-14, 16, 18-22 were not identified.

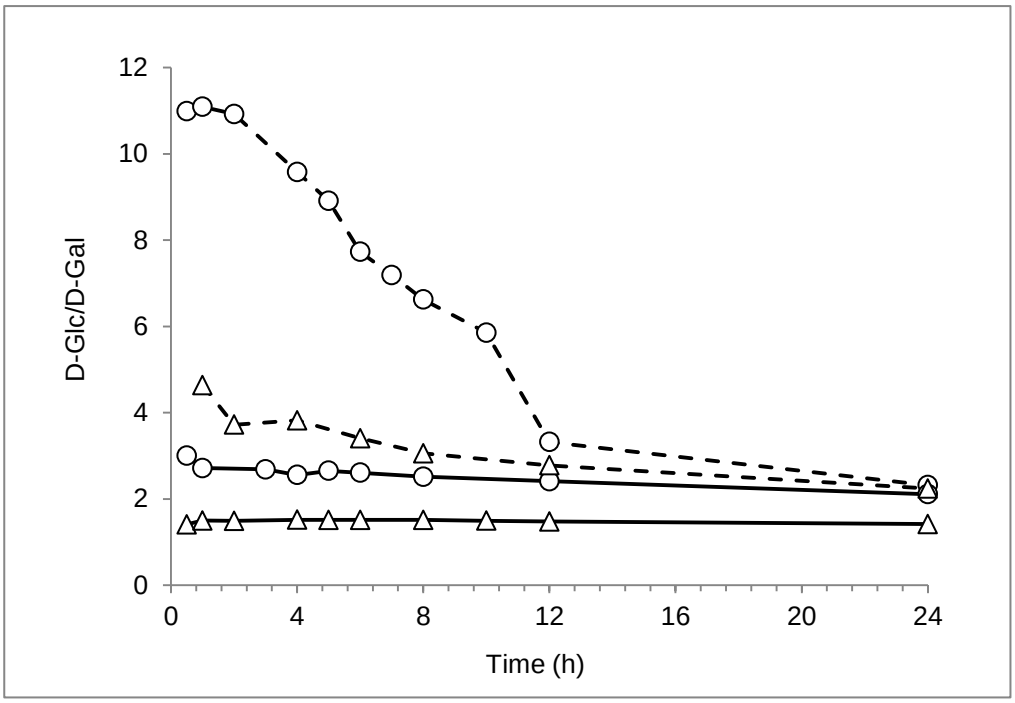


Figure 15. Effect of *N*-acetyl-D-glucosamine on D-Glc/D-Gal ratio during galacto- (solid line) and *N*-acetyl-oligosaccharides (dashed line) production catalyzed by β-galactosidase from *L. bulgaricus* (○) and *B. breve* β-galII (Δ).

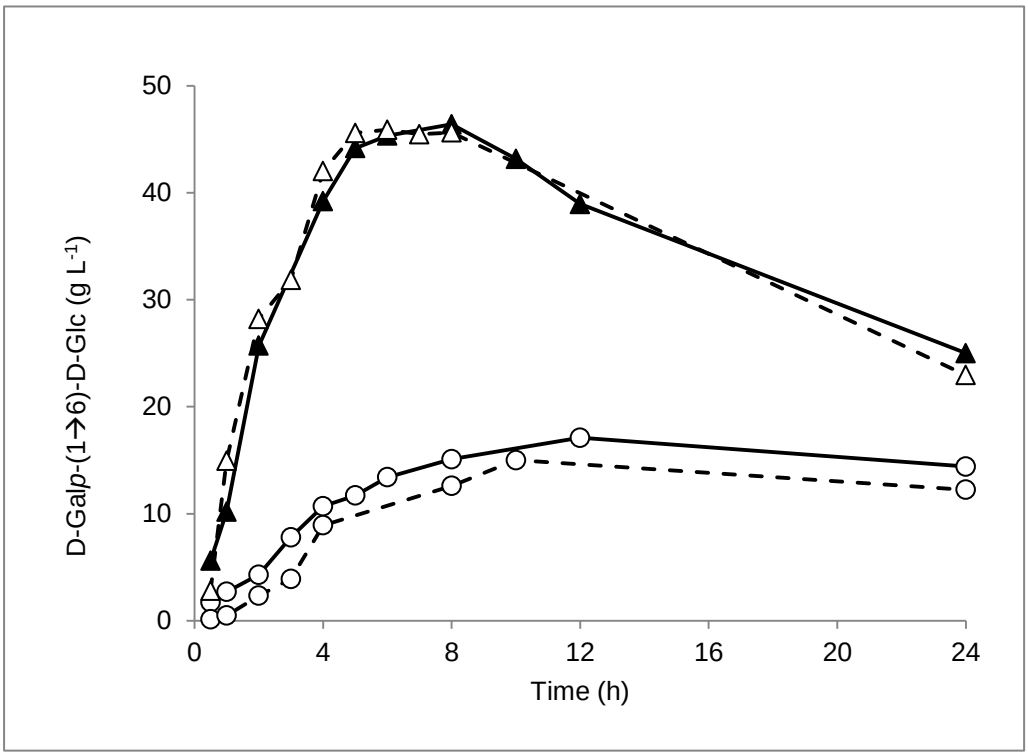


Figure 16. Allolactose (D-Gal*p*-(1→6)-D-Glc) formation during galacto- (solid line) and *N*-acetyl-oligosaccharides (dashed line) production catalyzed by β-galactosidase from *L. bulgaricus* (○) and *B. breve* βgal-II (Δ).

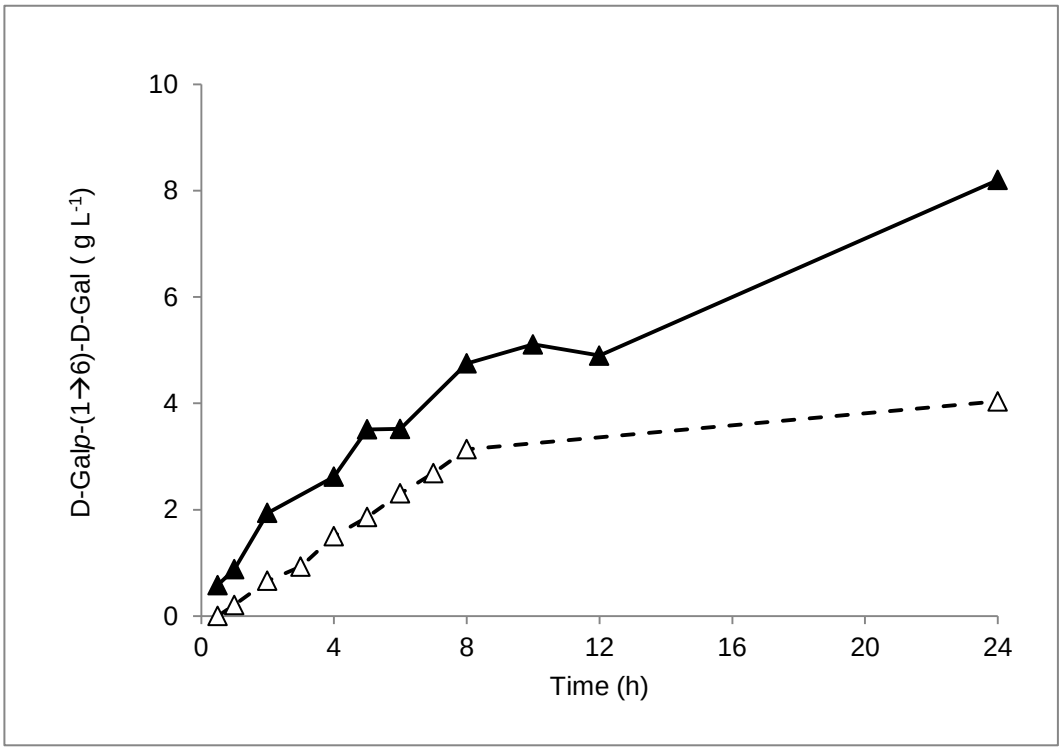


Figure 17. Formation of 6'-galactobiose (D-Gal*p*-(1→6)-D-Gal) during galacto- (solid line) and *N*-acetyl-oligosaccharides (dashed line) production catalyzed by *B. breve* β-galII.

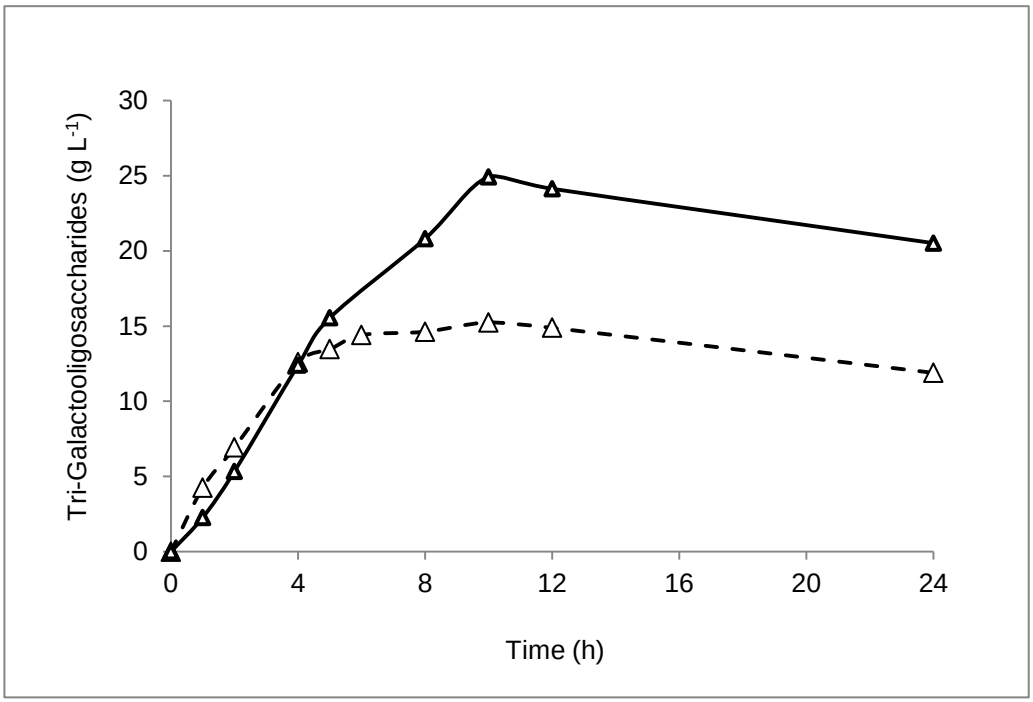


Figure 18. Production of tri-galacto-oligosaccharides by *B. breve* β-gal II in the absence (solid line) and presence (broken line) of *N*-acetyl-D-glucosamine. The reactions were performed at 30 °C at an initial lactose concentration of 600 mM in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$. Initial GlcNAc concentration used was 600 mM.

To optimize GlcNAc transgalactosylation production, a number of discontinuous conversion reactions were carried out varying the enzyme source and concentration, and donor to acceptor ratio. Figure 19 shows GlcNAc transgalactosylation yield at 30 ^{o}C containing initial concentration of 600 mM lactose and 600 mM GlcNAc in sodium phosphate buffer (pH 6.5), 1 mM $MgCl_2$ using 2.5 U_{Lac} mL^{-1} of either Lbulβgal or BbreβgalII. Under these conditions, the maximum molar yields of *N*-acetyl oligosaccharides produced were 40% after 6 h of reaction and 32% after 8 h with Lbulβgal and BbreβgalII, respectively. After these maximum yield points, the yield decreased because the GlcNAc containing products served as substrate for hydrolysis. The effect of BbreβgalII concentration on GlcNAc transgalactosylation yield was also investigated. Increasing the BbreβgalII concentration from 2.5 to 5.0 U_{Lac} mL^{-1} speeded up the reaction reaching the same amount of maximum yield (32%) only after 4 hours. The yields of transgalactosylation reactions were improved by optimizing the molar ratios of the donor to the acceptor (Figure 20). No significant change on the maximum yields was observed when the donor/acceptor ratio is increased from 1:1 to 2:1 either with Lbulβgal (40%) or BbreβgalII (~32%). Lbulβgal which is stable at 50 ^{o}C, was incubated at a solution containing 1M Lactose and GlcNAc each. It was found out that higher yield of 51.4% was observed with Lbulβgal with either 1:1 or 2:1 ratio (Figure 21). Moreover, a significant decrease on yield (25.4%) was observed when the initial lactose concentration is less than the GlcNAc concentration (1:2).

The production of *N*-acetyl-oligosaccharides with lactose and *N*-acetyl-D-galactosamine using Lbulβgal as biocatalyst was investigated. The substrate containing lactose and GalNAc was dissolved in sodium phosphate buffer (pH 6.5) containing 1 mM Mg^{2+} and 2.5 U_{Lac} mL^{-1} and incubated at 30 ^{o}C. Furthermore, the

effect of donor/acceptor molar ratio was also examined. Figure 22 shows that the maximum yield (29.19%) was obtained after 1 h with initial concentration of 0.6 M each of lactose and GalNAc. From the HPLC profile (Figure 23), di- and tri-GalNAc containing oligosaccharides were formed however, were not identified and quantified yet.

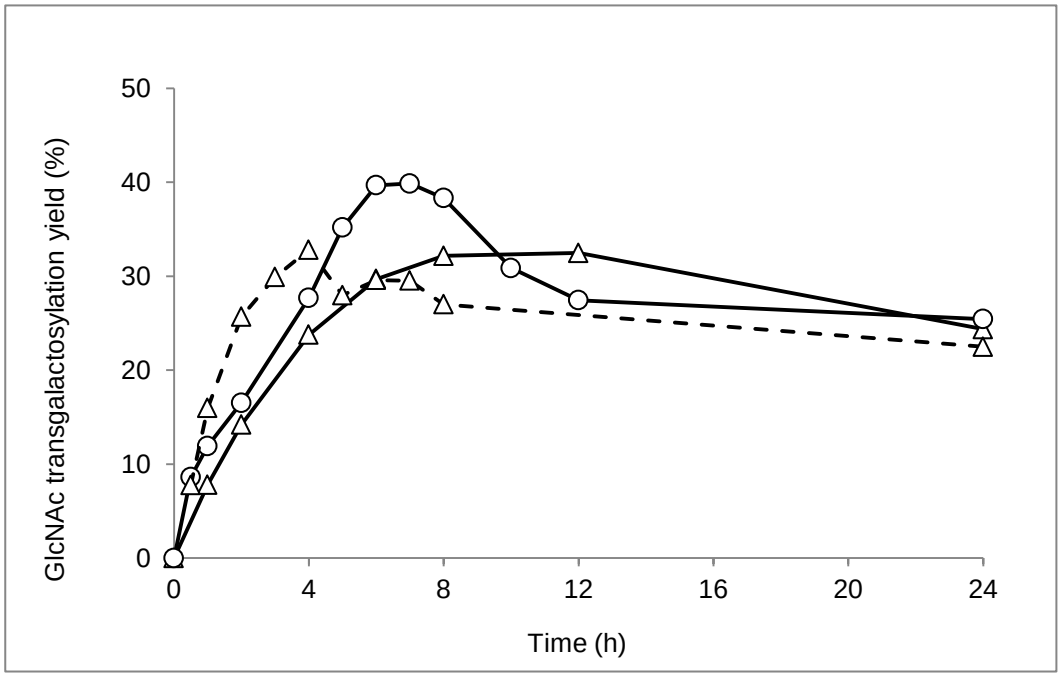


Figure 19 .Comparison of GlcNAc transgalactosylation yield catalyzed by *L. bulgaricus* (○) and *B. breve* β-galactosidases II (Δ). The reaction was performed at 30 °C using initial concentration of 600 mM Lactose: 600 mM GlcNAc in 50 mM sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ varying the amount of enzyme. 2.5 U_{Lac} mL^{-1} (solid line) and 5 U_{Lac} mL^{-1} (broken line).

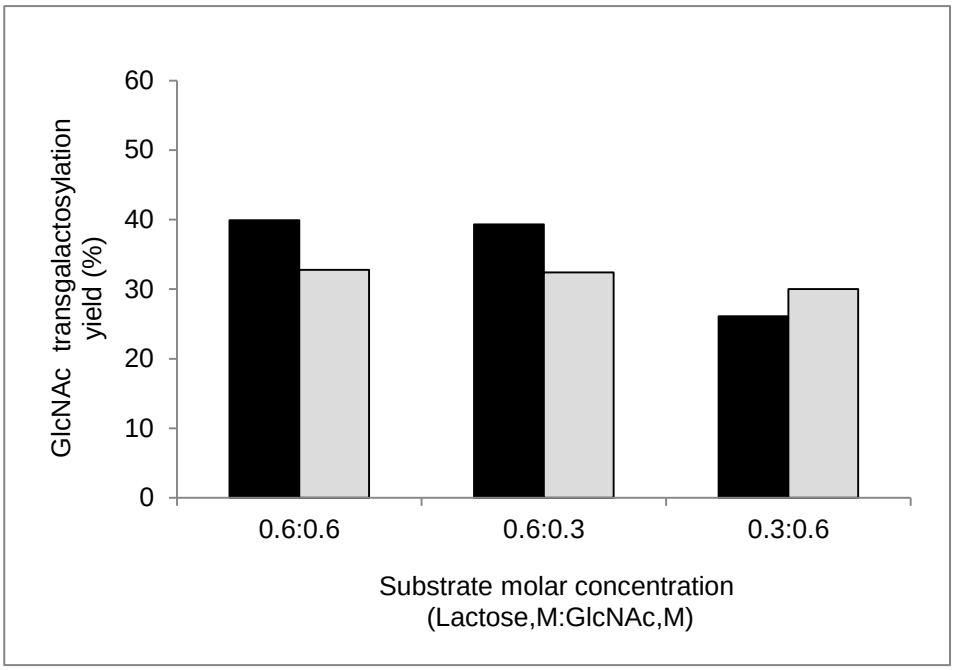


Figure 20. Effect of different lactose and GlcNAc molar ratio on GlcNAc transgalactosylation yield catalyzed by β-galactosidase from *L, bulgaricus* (black) and *Bifidobacterium breve* BbreβgalII (gray). The reaction was done in sodium phosphate buffer (pH 6.5) containing 1 mM Mg^{2+} incubated at 30 °C.

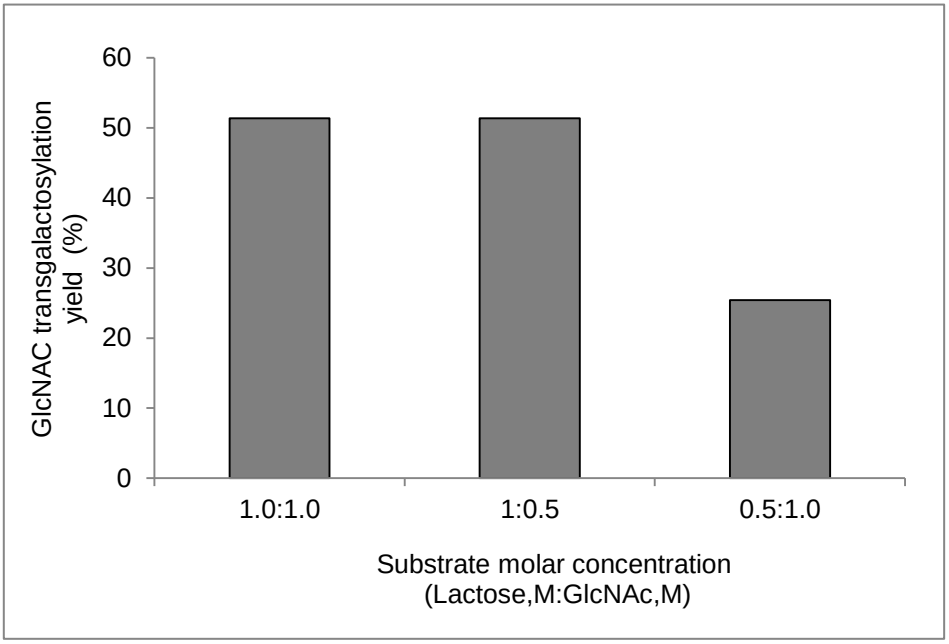


Figure 21. Maximum GlcNAc transgalactosylation yield catalyzed by β-galactosidase from *L, bulgaricus* (black) at different initial lactose and GlcNAc concentration (M), 1:1, 1.0:0.5, 0.5:1.0 The reaction was done in sodium phosphate buffer (pH 6.5) containing 1 mM Mg^{2+} incubated at 50 °C.

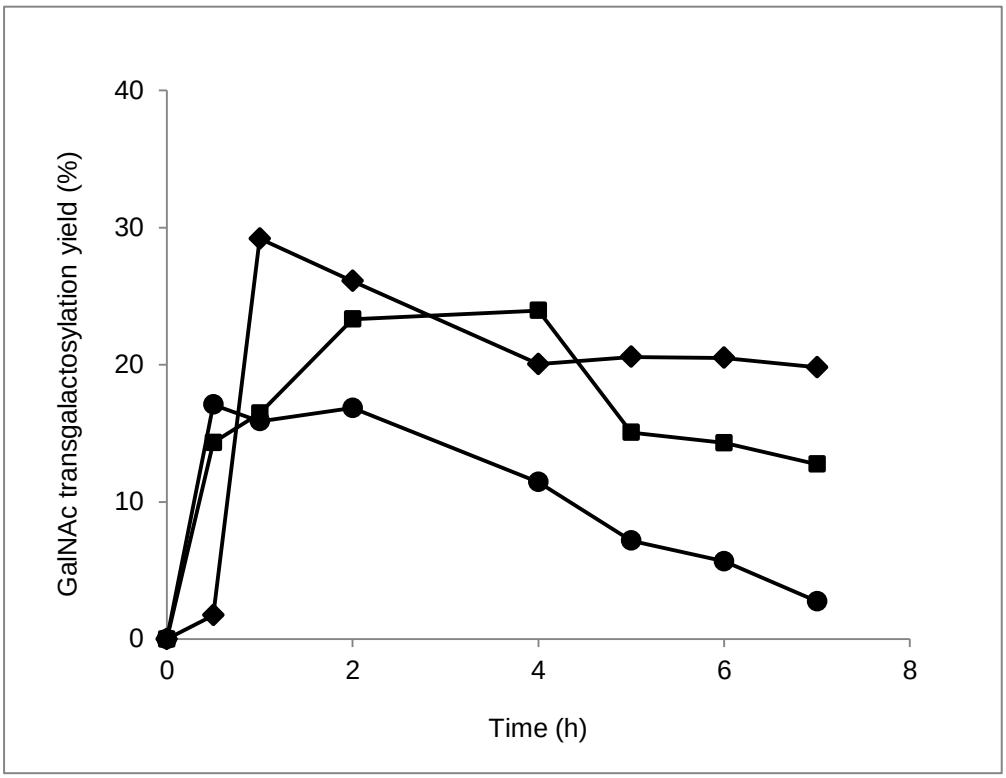


Figure 22. GalNAc transgalactosylation yield at different initial lactose: GalNAc molar ratio catalyzed by β-galactosidase from *L. bulgaricus*. The reaction was done in sodium phosphate buffer (pH 6.5) containing 1 mM Mg^{2+} incubated at 50 °C. Lactose:GalNAc molar concentration (M): 0.6:0.6(■); 0.6:0.3(♦); 0.3:0.6(●)

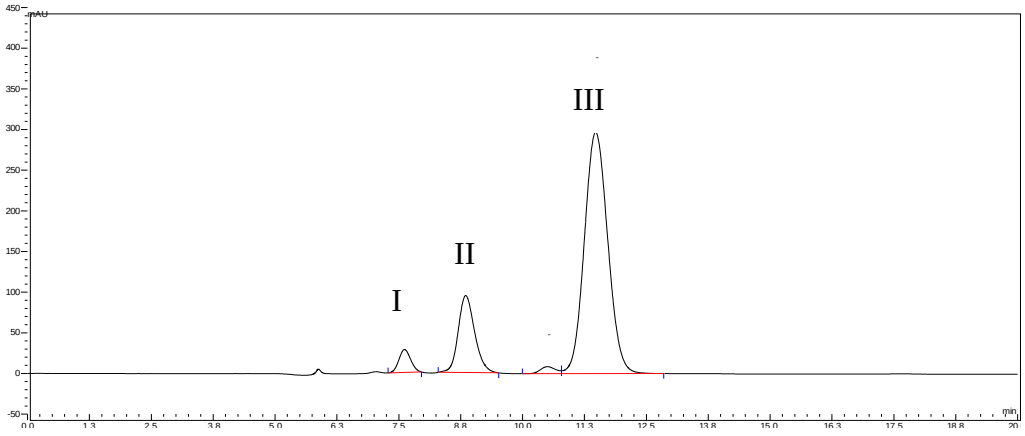


Figure 23. HPLC-UV profile of GalNAc-containing oligosaccharides produced by *L. bulgaricus* β-galactosidase at initial concentration of 600 mM lactose and 600 mM GalNAc in 50 mM sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$.Peaks I and II are the tri- and di-GalNAc containing oligosaccharides, respectively and Peak III is the free GalNAc.

Structural Characterization of GlcNAc transgalactosylation products

The optimum condition for the production of *N*-acetyl oligosaccharides by Lbulβgal were 1M lactose and 1M GlcNAc, 50 ^{o}C and 2.5 U_{Lac} mL^{-1} while that of BbreβgalII was 0.6 M lactose and 0.6 M GlcNAc, 30 ^{o}C and 5.0 U_{Lac} mL^{-1}. Using the mentioned condition, transgalactosylation reaction was done and products were separated using Hypercarb column equipped with UV detector (210 nm). The chromatographic patterns and the compounds synthesized by Lbulβgal and BbreβgalII were found to be similar (Figure 24). To determine the identity of the major GlcNAc transgalactosylation product, a preparative synthesis with BbreβgalII performed using the best of experimental condition, i.e. equimolar of lactose and GlcNac and incubation for 4 h at 30 ^{o}C with 5 U_{Lac} mL^{-1}. After all the purification steps, the major product was found to be β-D-Gal*p*-(1→6)-D-GlcNAc (*N*-acetyl-allolactosamine) identified by the NMR data (Figure 25, derived from an Heteronuclear Single Quantum Coherence or HSQC experiment) which indicated a low-field shift of Carbon 6 of the reducing GlcNAc to 69.4 ppm. The data when corrected for different referencing were in full agreement for published 13C NMR data of *N*-acetyl-allolactosamine.(*115-117*) The retention times of Peaks 2 and 4 were congruent with the retention times of α- and β-anomer of β-D-Gal*p*-(1→4)-D-GlcNAc standard, respectively and the compound eluting at the retention time of β-D-Gal*p*-(1→4)-D-GlcNAc standard was also identified in the HPAEC-PAD analysis (data not shown).

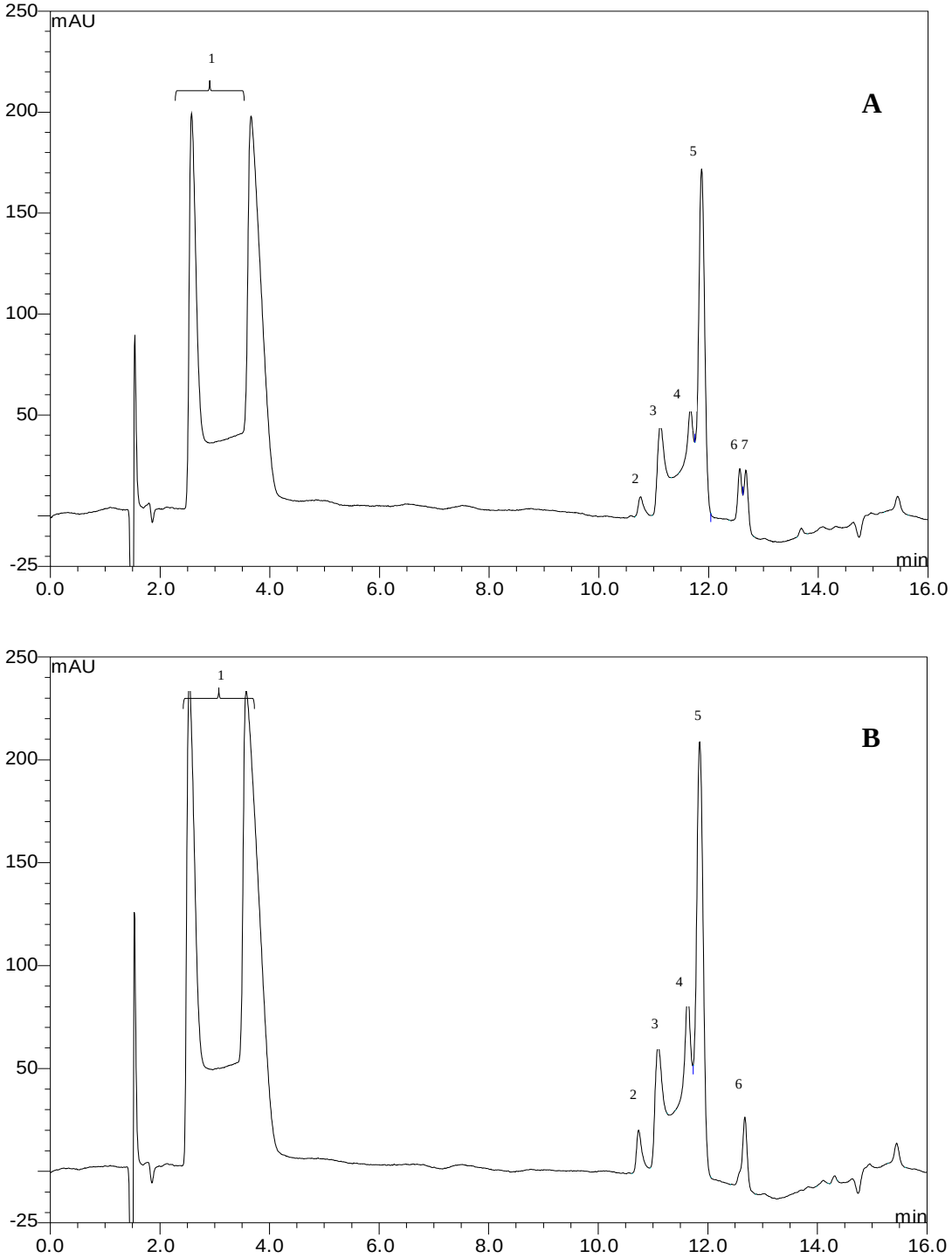


Figure 24. HPLC-UV chromatogram of *N*-acetyl oligosaccharides produced by *L. bulgaricus* (A) and *B. breve* β-galactosidases II (B) using equimolar concentration of lactose and GlcNAc dissolved in 50 mM sodium phosphate buffer (pH 6.5) containing 1 mM $MgCl_2$. Peak Identification: (1) GlcNAc, (3) α-D-Gal*p*-(1→6)-D-GlcNAc (5) β-D-Gal*p*-(1→6)-D-GlcNAc. Peaks 2,4, 6 and 7 were not identified.

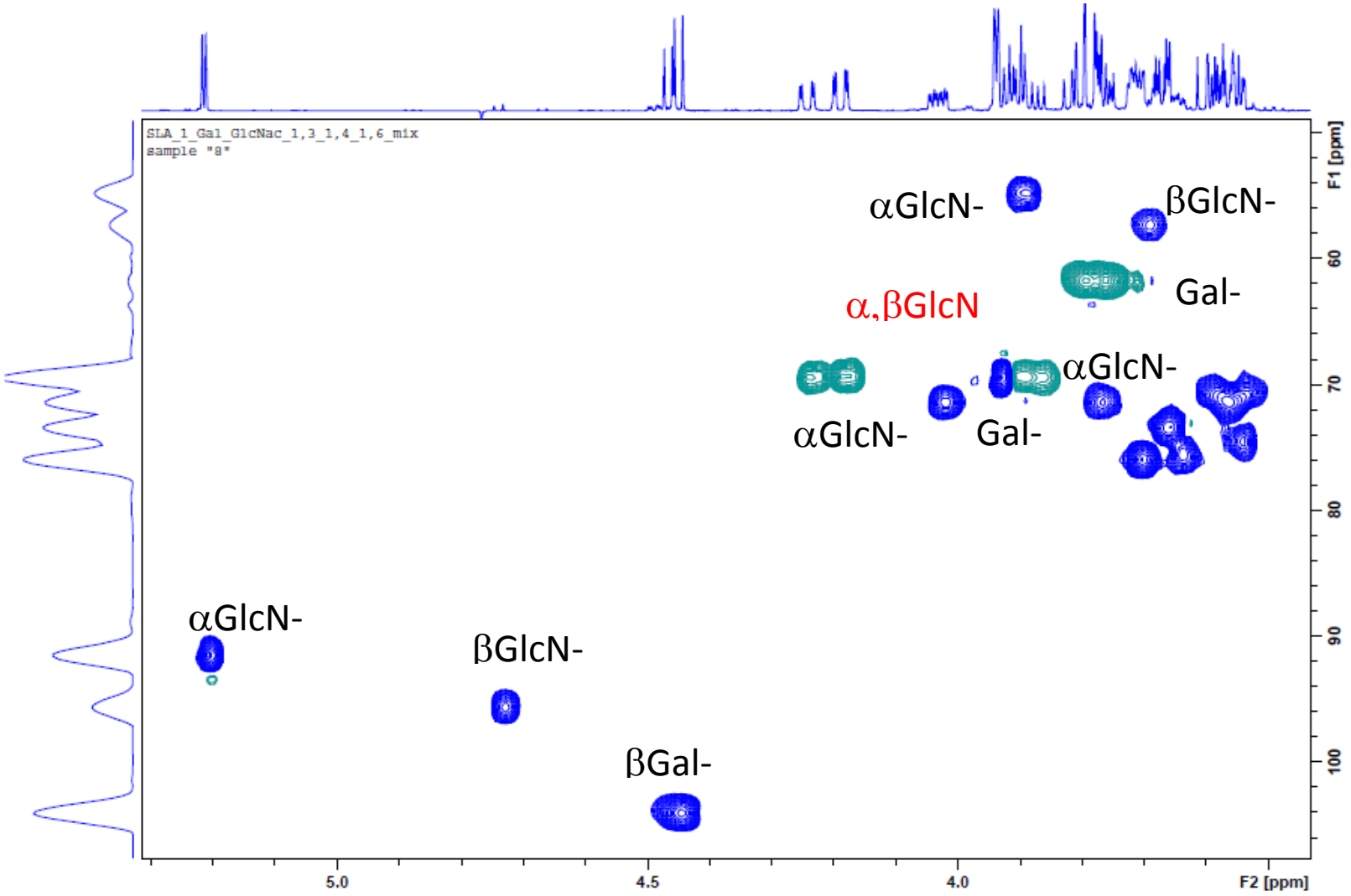


Figure 25. Multiplicity edited HSQC spectrum of β-D-Gal*p*-(1→6)-D-GlcNAc

_N_-acetyl-allolactosamine production by Lbulβgal and BbreβgalII under optimal condition

Using the optimum condition for the production of *N*-acetyl oligosaccharides with Lbulβgal and BbreβgalII, the formation and degradation of *N*-acetyl-allolactosamine was monitored. The maximum total amount of *N*-acetyl-allolactosamine was 41% after 5 h and 24% after 4 h with Lbulβgal and BbreβgalII, respectively (Figure 26). After that, hydrolysis of GlcNAc containing products prevailed. The ability of Lbulβgal and BbreβgalII to hydrolyze lacto-N-biose and its isomers was evaluated to determine preferred substrate (Table 19).

The substrate specificity of Lbulβgal and BbreβgalII significantly differ. Whereas both enzymes acts on β-D-Gal*p*-(1→4)-D-GlcNAc preferentially, BbreβgalII hydrolyzed faster (95.7%) compared to that Lbulβgal (62.5%) after 30 min.

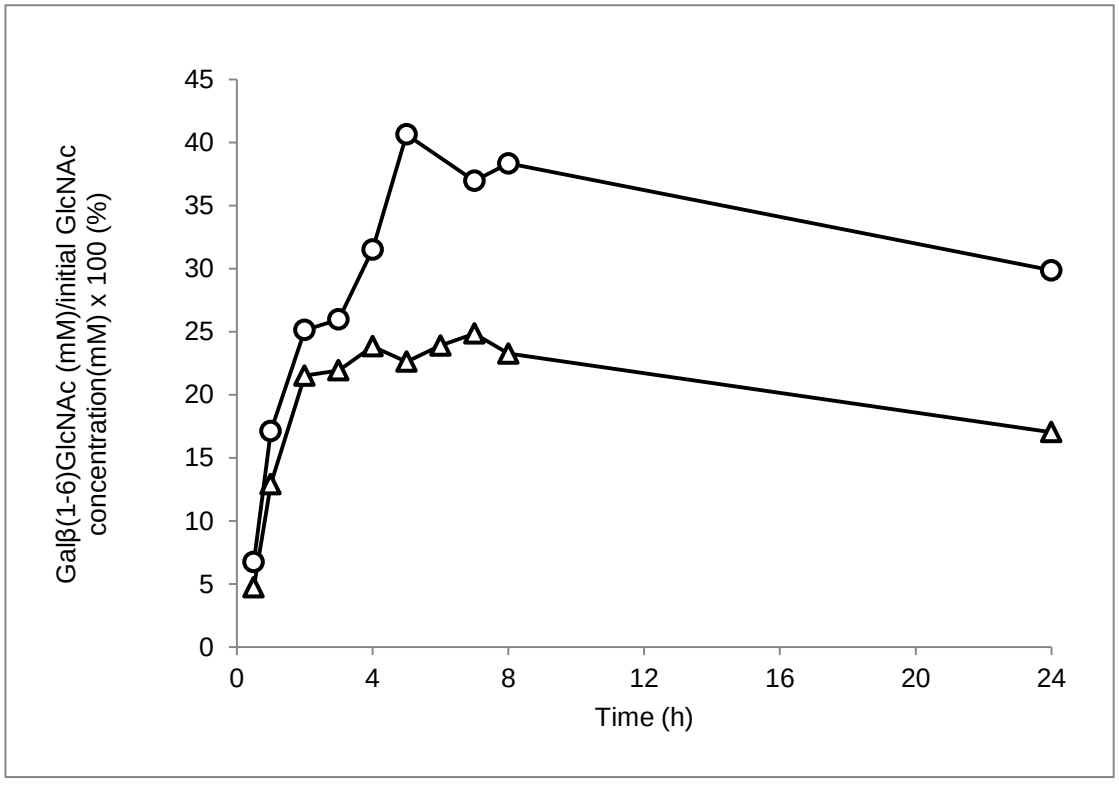


Figure 26. Time course of β-D-Gal*p*-(1→6)-D-GlcNAc production catalyzed by β-galactosidase from *L. bulgaricus* (○) and *B. breve* β-gal II (Δ) using lactose and GlcNAc as substrate with the molar ratio of 1:1.

Moreover, BbreβgalII acted on β-D-Gal*p*-(1→6)-D-GlcNAc faster than β-D-Gal*p*-(1→3)-D-GlcNAc with ~44.5% and 23.2% hydrolysis, respectively. Lbulβgal, although hydrolyzed β-D-Gal*p*-(1→3)-D-GlcNAc preferably than β-D-Gal*p*-(1→6)-D-GlcNAc, its hydrolysis rate is still lower compared to BbreβgalII.

Table 19. Activities of Lbulβgal and BbreβgalII with three different *N*-acetyl-galactoside

Substrate	Percent Hydrolysis			
	Lbulβgal		BbreβgalII	
	30min	60 min	30min	60 min
β-D-Gal*p*-(1→3)-D-GlcNAc	25.0	31.3	23.2	35.3
β-D-Gal*p*-(1→4)-D-GlcNAc	62.5	82.5	95.7	98.5
β-D-Gal*p*-(1→6)-D-GlcNAc	6.82	20.9	44.5	69.8

IV. DISCUSSION[a]

L. bulgaricus is an important member of the LAB because of its wide use as a starter culture together with *Streptococcus thermophilus* for the manufacturing of e.g. yoghurt and other fermented milk products. *L. bulgaricus* rapidly converts lactose into lactic acid during growth in milk resulting in fast acidification, which is important for structural properties of various products together with food preservation. The metabolism of lactose involves two main enzymes, a lactose antiporter permease (LacS) for the uptake of the sugar, and a soluble β-galactosidase (LacZ) for the intracellular cleavage of lactose into glucose and galactose, both encoded in the *lac* operon. β-Galactosidases are frequently found in LAB and *Lactobacillus* spp., not only in milk-associated, species but also in those that are predominantly isolated from other environments such as plants (e.g. from decaying plant materials or fruits) or animals (e.g. from the oral cavity or the intestinal tract) (*118*, *119*). *Lactobacillus* spp. encode most often β-galactosidases that according to the CAZy nomenclature (http://www.cazy.org, (*19*)) belong to glycoside hydrolase family GH2 and GH42. Lactobacilli such as *L. acidophilus* or *L. helveticus* carry two distinct genes encoding β-galactosidases belonging to both GH2 and GH42 (*22*), whereas other strains such as *L. bulgaricus* possess only one β-galactosidase.(*102*) The predominant GH2 β-galactosidases found in lactobacilli are of the LacLM type, heterodimeric proteins of ~105 kDa, which are encoded by the two overlapping genes *lacL* and *lacM*. Several lactobacillal β-galactosidase genes of this type that have been cloned and

[a] part of the **(1)** published journal article (Nguyen, T.-T.; Nguyen, H. A.; **Arreola, S. L.;** Mlynek, G.; Djinovic-Carugo, K.; Mathiesen, G.; Nguyen, T.-H.; Haltrich, D., Homodimeric β-Galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081: Expression in *Lactobacillus plantarum* and Biochemical Characterization. *J Agric Food Chem* **2012,** *60*, 1713-1721),

(2) revised article submitted to PLOS ONE. (**Arreola, S. L.;** Intanon, M.; Suljic, Jasmina; Kittl, Roman; Ngoc, H. P.; Kosma, P; Haltrich, D.; Nguyen, T.-H., Two β-galactosidases from the human isolate *Bifidobacterium breve* DSM 20213: Molecular cloning and expression, biochemical characterization and synthesis of galacto-oligosaccharides. (Revision submitted to PLOS ONE) and

(3) article in preparation for submission to PLOS ONE. (**Arreola, S. L.;** Intanon, M.; P.; Kosma, P; Haltrich, D.; Nguyen, T.-H., Biochemical characterization of four β-galactosidases towards formation of galacto- and hetero- oligosaccharides.

characterized the resulting proteins with respect to their biochemical properties recently include including LacLM from *L. reuteri* (*105*), *L. acidophilus* (*44*), *L. pentosus* (*45*), *L. plantarum* (*41*), and *L. sakei.* (*42*) In addition, di- or oligomeric GH2 β-galactosidases of the LacZ type, encoded by the single *lacZ* gene, are sometimes but not often found in lactobacilli, while they are more frequent in other LAB including *Streptococcus salivarius* or *S. thermophilus* (*120*) or bifidobacteria including *B. bifidum* (*121*) or *B. longum* subsp. *infantis.* (*60*)

When using the sequence of *L. bulgaricus* LacZ (accession number YP_619096) in a Blast search, it shows highest homologies with β-galactosidases from *Lactobacillus ruminis* (ZP_08563149.1; 51% identity), *Lactobacillus animalis* (ZP_08548824.1; 51% identity), *S. salivarius* (YP_004727546.1; 50% identity), *S. thermophilus* (YP_139826.1; 49% identity), *Lactobacillus salivarius* (YP_535272.1; 47% identity), *Bifidobacterium longum* subsp. *infantis* (ZP_03976734.1; 46% identity), *B. longum* subsp. *longum* (YP_004221245.1; 46% identity) and *Bifidobacterium adolescentis* (YP_910468.1; 44% identity). A sequence alignment of three well-studied members of GH2 β-galactosidases, LacZ from *E. coli*, which is the best studied β-galactosidase in this family, LacZ of *L. bulgaricus* and the larger subunit LacL of heterodimeric β-galactosidase from *L. reuteri,* showed some but not very pronounced homology between these members of family GH2, with 33% and 44% identity between *L. bulgaricus* LacZ and *E. coli* LacZ or *L. reuteri* LacL, respectively. Identity between the two latter proteins was 33%. The catalytic nucleophile Glu537 and the general acid/base catalyst Glu461 in *E. coli* LacZ (*122*) are strictly conserved in *L. bulgaricus* LacZ (Glu532 and Glu465, respectively) and *L. reuteri* LacL (Glu536 and Glu468, respectively).

Production levels obtained for β-galactosidase LacZ were rather low in the wild-type strain of *L. bulgaricus* with only ~4000 U of activity per liter of medium (MRS containing 2% lactose) after cultivation at 37°C for 24 h (data not shown), and hence we attempted heterologous overexpression. We therefore cloned the *lacZ* gene into the pSIP-vectors (*107, 113, 123*) using *L. plantarum* WCFS1 as host for over-expression. The *lacZ* gene, both with and without a C-terminal His-tag, was inserted into two different expression vectors, pSIP403 and pSIP409, differing only in the promoters P_{sppA} and P_{sppQ}. The expression yields were well comparable for these two different constructs (Table 5). The background β-galactosidase activity from the chromosomal β-galactosidase genes *lacLM* of *L. plantarum* was negligible when grown on glucose, and hence the β-galactosidase activities detected in these experiments originate only from the plasmid-located *lacZ* gene. Addition of the IP-673 peptide pheromone induced *lacZ* expression considerably (Table 5), however, both promoters showed some low basal expression of the recombinant β-galactosidase, especially P_{sppA}. The differences in promoter leakage resulted in more than twice higher induction factors for the pSIP409 derivatives compared to the pSIP403 ones (Table 5). This is in accordance with our previous studies on the expression of heterodimeric lactobacillal β-galactosidases LacLM using the same pSIP expression vectors. (*103, 124*) Interestingly, the activities obtained for His-tagged β-galactosidase LacZ were always lower by approximately 20–30% (Table 5) despite that both protein versions are produced in comparable levels (Figure 2). The reduced activity is therefore most probably caused by the C-terminally His-tag, since specific activities determined for purified, homogenous non-tagged and His-tagged LacZ (306 and 251 U mg^{-1}) also differ by ~20%.

When cultivating *L. plantarum*/pTH101 on a larger scale we obtained a volumetric activity of 53,000 U L^{-1} of β-galactosidase activity for non-tagged LacZ with a specific activity of 193 U mg^{-1}, this is an increase by more than 13-fold compared to the activity obtain with *L. bulgaricus*. As judged from the specific activity of purified LacZ (306 U mg^{-1}), this value correspond to ~173 mg of recombinant protein produced per liter medium, with 63% of the total soluble intracellular protein in *L. plantarum* amounting for LacZ. These results confirm the excellent applicability of the lactobacillal expression system for producing high amounts of soluble, active recombinant protein, as is also evident from Figure 3. The expression system still carries an antibiotic (erythromycin) resistance gene as selection marker, and therefore the system is not truly food-grade even though the host *L. plantarum* is a GRAS organism (*125*). Recently, we developed pSIP derivatives where the *L. plantarum* the alanine racemase gene (*alr*) was used as an efficient, alternative food-grade selection marker (*124*). The production yields of recombinant proteins with these vectors were in fact yields slightly higher for lactobacillal β-galactosidase of the LacLM type than with the original vectors. The current system used for the expression of *L. bulgaricus lacZ* can thus be easily adapted to true food-grade expression.

Bifidobacterium breve DSM 20213 is an isolate from the infant gut. Three β-galactosidases, two of which belonging to glycoside hydrolase family 2 (NCBI Reference No. EFE90149.1, EFE88654.1) and one to glycoside hydrolase family 42 (NCBI Reference No. EFE89025.1), were found in the genome sequence of *Bifidobacterium breve* DSM 20213 (NCBI Reference Sequence: NZ_ACCG00000000.2). Furthermore, one additional putative β-galactosidase (NCBI Reference No. EFE89763.1) was found in this genome sequence. BbreβgalI and

BbreβgalII are encoded by the corresponding *lacZ* genes (NCBI Reference No. EFE90149.1; EFE88654.1) and belong to glycoside hydrolase family 2 (GH2 family). BbreβgalI and BbreβgalI, were cloned, heterologously expressed in *E. coli* and biochemically characterized. Comparison of amino acid sequences deduced from these two *lacZ* genes revealed 57% of sequence homology. The expression levels of these β-galactosidases in *E. coli* are significantly higher than those of other recombinant β-galactosidases previously reported, e.g. β-galactosidases from *L. reuteri* L103 (110 kU L^{-1} fermentation broth) (*43*), *B. licheniformis* DSM 13 (74 kU L^{-1} fermentation broth (*126*). The values of 683 and 169 KU L^{-1} obtained in simple shaken flask cultures for Bbreβgal I and BbreβgalII, respectively, correspond to values of ~1.5 and 0.86 g of recombinant protein produced per L of medium. Furthermore, ~31% and 18% of the total soluble protein in the cellular extracts of *E. coli* overexpressing the genes encoding BbreβgalI and BbreβgalII, respectively, can be attributed to the recombinant proteins as judged by the specific activities. Co-expression of the chaperones GroEL/GroES significantly boosted expression levels of both β-galactosidases (Tables 10, 11). An explanation might be an increase of correctly folded β-galactosidases. The co-expression with GroEL/GroES has previously been reported for soluble expression of several proteins (*127, 128*) but this is the first report on the co-expression of β-galactosidases with GroES/GroEL.

The β-galactosidase LacZ from *L. bulgaricus* was found to be a homodimeric enzyme. It shows a pH optimum in the neutral area around pH 7, a temperature optimum for the short-time assay and hydrolysis of lactose of around 50 C, and it requires ions such as Na^{+}, K^{+} or Mg^{2+} for activity. Some of these enzyme characteristics have been reported previously for the enzyme isolated from *L. bulgaricus* (*129-132*), which confirms that the properties of recombinantly expressed

LacZ are identical to those of the enzyme isolated from its natural source. Interestingly, we could not confirm some results on β-galactosidase from *L. bulgaricus*, in which pH optimum of 5.0 to 5.5 (*133*), even though the *lacZ* gene used for the heterologous production of that enzyme in *E. coli* was obtained from the identical source (*L. delbrueckii* subsp. *bulgaricus* ATCC 11842, which is identical to the DSM20081 strain used here). These authors used different buffers (sodium acetate and HEPES) and added 1 mM Co^{2+} and Mn^{2+}, while we used Britton-Robinson buffer with these metal ions, which could be an explanation for different results. One noteworthy and remarkable property of β-galactosidase from *L. bulgaricus* is its thermostability when compared to the stability of other lactobacillal β-galactosidases. Its stability and activity is increased by the presence of ions such as Mg^{2+}; this, however, seems common among GH2 β-galactosidases, and is also observed for e.g. the *E. coli* β-galactosidase (*134, 135*) as well as for some β-galactosidases from *Lactobacillus* spp. of the LacLM type (*45, 105*). Another feature distinguishing the LacZ β-galactosidase from *L. bulgaricus* from other lactobacillal β-galactosidases of the LacLM type are its kinetic properties for the natural substrate, lactose. It is characterized by a remarkably high k_{cat} value of 234 s^{-1} as compared to for example 98 s^{-1} for LacLM from *L. plantarum* (*41*), 58 s^{-1} for LacLM from *L. reuteri* (*105*), 50.4 s^{-1} for LacLM from *L. acidophilus* (*44*), or 43 s^{-1} for *L. sakei* (*41*) (all at 30°C), while the Michaelis constants are comparable for these enzymes, with a value of 19 mM for the β-galactosidase from *L. bulgaricus*, and values of 4.04–31 mM for the LacLM β-galactosidases mentioned before. Also the inhibition by the end product D-galactose, as expressed by the K_i value of 71 mM, is comparable or slightly more pronounced than the modest inhibition of the LacLM β-galactosidases from *L. sakei* (180 mM) or *L. reuteri* (89 mM). Because of these kinetic properties together with the

increased thermostability β-galactosidase from *L. bulgaricus* resembles more the LacZ β-galactosidase from *S. thermophilus* (k_{cat} of ~575 s^{-1} at 37°C, K_m of 6.9 mM, K_i of 60 mM for D-galactose (*136*); or k_{cat} of 234 s^{-1}, K_m of 1.1 mM, temperature not specified (*137*)) than other LacLM β-galactosidases from lactobacilli.

On the other hand, the K_m values determined for lactose, 15.3 and 7.5 mM for Bbreβgal I and Bbreβgal II, respectively, are lower compared to the values reported for other β-galactosidases from *Bifidobacterium* spp. including *B. adolescentis* β-gal II (60 mM) (*138*), *B. breve* B24 (95.58 mM) (*52*), *B. bifidum* β-gal I (29.90 mM) and β-gal II (47.13 mM) (*49*), as well as fungal and yeast β-galactosidases that are commonly employed in technological applications, for example *A. oryzae* (36–180 mM), *A. niger* (54–99 mM), *K. fragilis* (15–52 mM) (*139*), *K. lactis* (35 mM) (*140*). These K_m values of *B. breve β*-galactosidases compare favorably with the values reported for β-galactosidases from *B. bifidum β*-gal III (9.56 mM) (*49*), *L. reuteri* (13 mM) (*105*), and *L. crispatus* (14 mM) (*141*). These relatively low K_m values of the *B. breve* β-galactosidases can be an advantage, e.g. when the complete hydrolysis of lactose is desired. The inhibition by the end product galactose is moderate as it is evident from the ratio of K_i to K_m calculated for this competitive inhibitor. This ratio of K_i to K_m represents a specificity constant, which determines preferential binding of the substrate lactose versus that of the monosaccharide end products, hence a high value for this ratio is desirable for efficient hydrolysis of lactose. The *B. breve* β-galactosidases display values for the $K_{i,Gal}/K_{m,Lac}$ ratio of 1.8 and 3.6 for BbreβgalI and BbreβgalI, respectively, indicating low inhibition, and which compare favorably with the values reported for the $K_{i,Gal}/K_{m,Lac}$ ratio for *L. reuteri*, which is 2.9 (*105*), and *L. bulgaricus*, which is 3.7 (*40*). Values for the $K_{i,Gal}/K_{m,Lac}$ ratio reported for example for *B. licheniformis*, *A. oryzae, A. niger* and *K. fragilis* are as low as 0.0055,

0.01, 0.006 and 0.84, respectively, indicating severe inhibition by the end product galactose. (*126, 142*) It was only possible to determine $K_{i,Glc}$ for *o*NPG hydrolysis since the lactase assay using lactose as substrate is based on the determination of the released glucose. Values for the $K_{i,Glu}/K_{m,oNPG}$ ratio measured for *o*NPG hydrolysis for BbreβgalI and BbreβgalII are 92 and 55, respectively, indicating that the inhibition by glucose, the second monosaccharide end product, is even less pronounced than the inhibition by galactose.

The application of β-galactosidases for the synthesis of GOS has recently received considerable attention (*17, 30, 54, 55, 143*), and especially β-galactosidases from food-grade or beneficial probiotic sources are of interest for this application. (*21, 144, 145*) GOS are prebiotic sugars (*9*), and as such they are of interest for a number of food applications. It is therefore of increasing interest to exploit food-grade expression systems based on GRAS organisms to convert lactose to GOS.

The transgalactosylation activity of *L. bulgaricus* LacZ has been described before (*130-132*), but has not been studied in much detail, e.g. the structures of the main transferase products have not been identified. In fact, using β-galactosidase from *L. bulgaricus* for the synthesis of GOS is quite attractive, and we obtained GOS in total yields of ~50% with an initial lactose concentration of 205 g L^{-1}. This initial lactose concentration is based on the solubility of lactose at ambient temperature. This efficacy in producing GOS in high yields seems to be clearly superior to that found for other β-galactosidases from various LAB, which have reported yields ranging from 28% for the LacLM β-galactosidase from *L. plantarum* to 41% for *L. sakei* LacLM and *S. thermophilus* LacZ when using comparable initial concentrations of lactose.(*22, 41, 42*)

On the other hand, highest total GOS yields of 33% and 44% were obtained when BbreβgalI and BbreβgalII were used in discontinuous conversion reactions with an initial lactose concentration of 200g/L. An increase in reaction temperature would help to increase the solubility of lactose, however this was not possible since both enzymes lack sufficient stability above 30°C. The maximum GOS yield obtained with BbreβgalII is comparable to the reported yields obtained with other β-galactosidases from *Bifidobacterium* spp., for example *B. angulatum* (43.8%), *B. bifidum* BB-12 (37.6%), *B. adolescentis* (43.1%)(*51*), and *B. breve* B24 (42%)(*52*), however the lactose conversions for GOS synthesis using these β-galactosidases were performed with initial lactose concentration of 30% (w/w). Additionally, Goulas et al. (*21*) reported a yield of 47% of GOS using BbgIV from *B. bifidum* NCIMB41171 at 40°C and 40% (w/w) initial lactose concentration, while Osman et al.(*146*) obtained a yield of 55% at 65°C and 43% (w/w) initial lactose concentration using the same enzyme. It was found by many authors that the initial lactose concentration has a significant impact on GOS yields. (*24, 26, 28*) When looking at comparable initial lactose concentrations, a recombinant, engineered β-galactosidase from *B. infantis* was reported to be an excellent biocatalyst for the GOS production giving the total GOS yield of 65% at 37°C.(*60*)

The reaction temperature hardly affected the maximum GOS yield or the composition of the GOS mixture. Several studies have shown that transgalactosylation becomes more pronounced compared to hydrolysis at higher temperatures (*147, 148*), which obviously is not the case for the Lbulβgal. An increase in the reaction temperature from 30 °C to 50 °C, accelerated the reaction significantly, and therefore the productivity increased from 8.5 to 19.8 g L^{-1} h^{-1} GOS for the LacZ-catalyzed reaction. Here, the thermostability of Lbulβgal can be an advantage, since

solubility of lactose increases significantly with temperature (e.g., 440 g L^{-1} at 50°C). One may therefore expect improved GOS yields when using Lbulβgal at increased temperatures together with higher substrate concentrations.

Lbulβgal formed GOS structurally similar to those obtained with other β-galactosidases from LAB (*22, 28, 41, 44, 143*), yet proportions of individual components varied to some extent. The predominant oligosaccharide products were identified as β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→6)-Lac, together accounting for approximately 60% of the GOS, indicating that this β-galactosidase has a propensity to synthesize mainly β-(1→6)-linked GOS. β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→3)-D-Gal, and β-D-Gal*p*-(1→3)-Lac were unequivocally identified as reaction products, but were present in lower concentrations. Current commercial GOS products contain structures with predominant β-(1→4)-linkages (*28, 30, 66*), while the lactobacillal enzymes, like Lbulβgal, show a strong tendency to form β-(1→6)-linked transgalactosylation products. On the other hand, the predominant transgalactosylation products were identified as β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→3)-D-Lac, together accounting for more than 75% and 65% of the GOS formed by transgalactosylation by BbreβgalI and BbreβgalII, respectively. Both enzymes show very low activity towards β-D-Gal*p*-(1→4)-D-Gal, and interestingly, this disaccharide was not detected and hence formed at all during lactose conversion by BbreβgalI and BbreβgalII.

The recombinant enzymes Lbulβgal, BbreβgalI and BbreβgalII have shown to have propensity to synthesize β-(1→6) and β-(1→3)-linked GOS. Looking at the ratios of β-(1→6) and β-(1→3) linkages at the level of individual sugar species, one can see that both enzymes show preference towards β-(1→6)-bond formation during intramolecular transgalactosylation. β-Galactosidase BgbII from *B. adolescentis*

showed high preference towards the formation of β-(1→4) linkages while no β-(1→6) linkages were formed (30). In contrast, the β-galactosidase BgbII from *B. bifidum* showed a clear preference for the synthesis of β-(1→6) linkages over β-(1→4) linkages (45). β-D-Gal*p*-(1→3)-D-Lac was also found to be a major product formed by β-GalI from *B. infantis* (26). Apparently, the formation of certain bonds in transgalactosylation mode of β-galactosidases is a specific property of individual enzymes. Moreover, it was shown that a GOS mixture, produced by enzymes from *Bifidobacterium bifidum* and predominately containing β-(1→3) and β-(1→6)-linked oligosaccharides, showed significantly higher prebiotic and bifidogenic effects than did a commercial galacto-oligosaccharide mixture consisting mainly of β-(1→4)-linked sugars.(*53*)

BbreβgalI and BbreβgalII show highest affinities towards lactose, allolactose and β-D-Gal*p*-(1→3)-D-Lac among the substrates tested. It is conceivable that the 'probiotic' β-galactosidases, which rapidly hydrolyze certain galacto-oligosaccharide structures, can preferentially form these glycosidic linkages as well when acting in transgalactosylation mode, and this is again confirmed in this study. Previous studies reported the presence of multiple β-galactosidases in *B. infantis*, *B. adolescentis*, or *B. bifidum* (*21, 59, 60, 121, 138, 149*), and revealed that these enzymes are very different with respect to substrate specificity and regulation of gene expression. Having isoenzymes with different properties might be advantageous for microorganisms because of higher adaptability to changing growth conditions and each isoenzyme may be responsible for either hydrolysis or synthesis reactions. Understanding the role and function of multiple isoenzymes in bacterial physiology can be supported by investigating the biochemical properties and their activities as well as their specificities towards different substrates. The isoenzymes from *B. breve* DSM 20213,

BbreβgalI and BbreβgalII, show only slight differences in their pH and temperature optima using both *o*NPG and lactose as substrates, however, BbreβgalII is generally more stable than BbreβgalI under all conditions tested. In terms of substrate specificity, both enzymes show high hydrolytic activity with lactose, allolactose, and 3′-galactosyl lactose, in addition, BbreβgalII shows significantly lower affinities towards the two disaccharides, β-D-Gal*p*-(1→3)-D-Glc and β-D-Gal*p*-(1→3)-D-Gal compared to BbreβgalII. However, the reason why *B. breve* DSM 20213 possesses two β-galactosidase isoenzymes with relatively similar substrate preferences and biochemical properties remains indistinctively.

Transgalactosylation is described to involve intermolecular as well as intramolecular reactions. Intramolecular or direct galactosyl transfer to D-Glucose yields regio-isomers of lactose. In this reaction pathway the noncovalently enzyme-bound glucose is not released from the active site but linked immediately to the galactosyl enzyme intermediate. Intermolecular transgalactosylation on the other hand involves transfer of galactosyl moiety from the galactosylated enzyme to other nucleophile present thus yield various di-,tri, tetra-GOS. Different transfer rates for different acceptors are to some extent responsible for these phenomena.

The transfer constant k_{Nu}/k_{water} provides a useful tool to measure the ability of a certain substance to act as a galactosyl acceptor *(i.e.* nucleophile) which in turns allows an estimation of the level of transgalactosylation products obtained of a known reaction mixture. During complete hydrolysis, a velocity ratio of $\nu_{Glc}/\nu_{Gal} = 1.0$ for reaction in water, where the formation of *o*-nitrophenol (or D-Glc) and D-Gal are stoichiometric. This ratio however increases as the intermediate is trapped by added nucleophile to form Nu-Gal at the expense of D-Gal. Plots of (ν_{oNP}/ν_{Gal}) or (ν_{Glc}/ν_{Gal}) against [Nu] were linear for a specific range of acceptor concentrations. Deviation

from linearity which occurred mainly at low and high concentrations of nucleophile can be due to competition for binding to the nucleophile binding site of the galactosyl-enzyme intermediate [E-Gal]. (*98, 150*) The k_{Nu}/k_{water} results obtained with lactose (0.53 – 2.79) and *o*NPG (3.91 – 9.36) in this study is comparable with the data published others. (*28, 99*) When k_{Glc}/k_{Lac} was determined (obtained from the ratio of k_{Glc}/k_{water} to k_{Lac}/k_{water}), BbreβgalII showed the highest ratio of 7.4 while Lreuβgal, Lbulβgal and *Bbre*βgalI was at ~3 - 4. These results indicate that by BbreβgalII, D-Glc is ~7-fold better galactosyl acceptor than lactose hence, disaccharides other than lactose will make up the large proportion of the obtained GOS mixture compared with the other three β-gal.

The ratio of GalGlc disaccharides and GalLac trisaccharides (Figure 27), revealed that BbreβgalI and BbreβgalII have GalGlc/GalLac ratio of ~4-5 while that of LbulβgalI and LreuβgalI are at 0.44 at about 20% conversion. At maximum GOS yield point, BbreβgalII showed the highest GalGlc/GalLac ratio (3.5) while that of BbreβgalI, LbulβgalI and LreuβgalI are 2.0, 1.4 and 1.7, respectively. Although it was predicted from k_{Glc}/k_{Lac} that BbreβgalII will have higher GalGlc/GalLac ratio compared with other β-gal, the low GalGlc/GalLac ratio as measured by HPAEC-PAD signifies that there must be a significant intramolecular transgalactosylation as well.

Lbulβgal and BbreβgalII were shown to effectively transfer the galactosyl moiety to GlcNac rather than to water as shown by the rate constant ratio, k_{GlcNAc}/k_{water} of 16.8 and 5.42, respectively. BbreβgalII and Lbulβgal also showed high preference to transfer galactosyl moiety to GlcNAc rather than unto lactose with rate constant ratios, k_{GlcNAc}/k_{Lac} of 10.23 and 6.74, respectively. This would mean that GalGlcNAc would be the preferred products rather than tri-GOS. Figure 19 revealed that

formation of tri-GOS was significantly decreased in the presence of GlcNAc and the maximum D-Glc/D-Gal ratio with Lbulβgal and BbreβgalII is 3.0 and 1.5, respectively while presence of GlcNAc as substrate resulted in maximum D-Glc/D-Gal ratio of 11.0 and 4.6, respectively. This only showed that the galactosyl moiety was preferentially transferred to GlcNAc rather unto Glc or lactose.

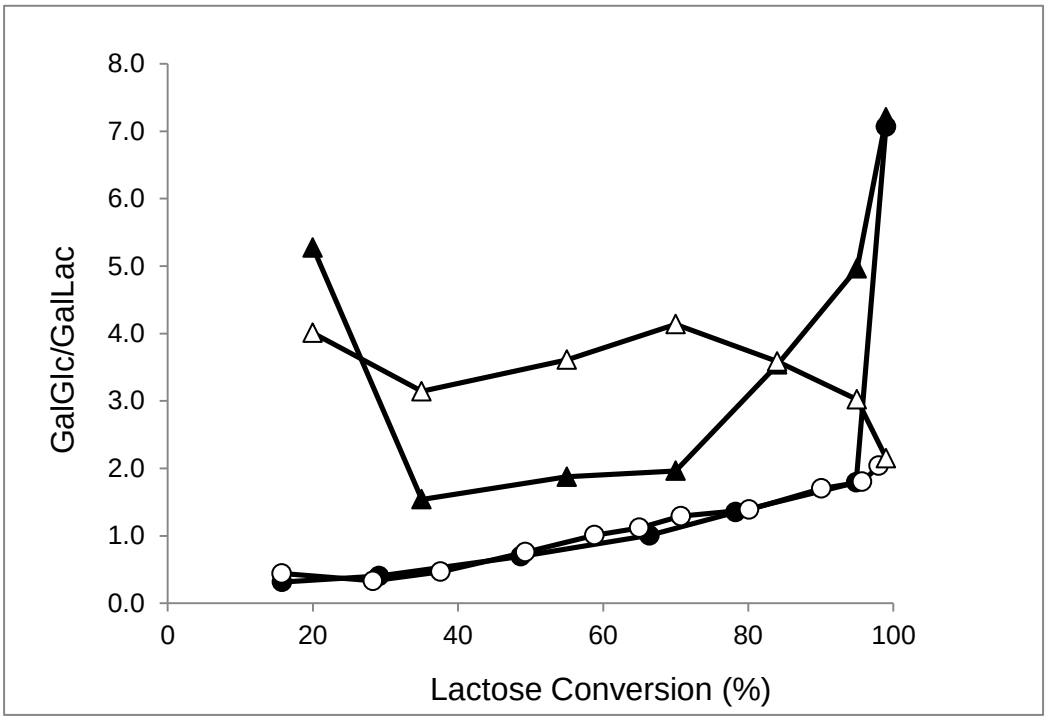


Figure 27. GalGlc/GalLac ratio during lactose conversion by β-galactosidase from *L. reuteri* (●), *L. bulgaricus* (○), *B. breve* β-gal I (▲), and *B. breve* β-gal II (Δ). The reactions were performed at 30 °C at an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$.

The ratio of lactose and GlcNAc presents an additional parameter for the formation of GlcNAc transgalactosylation products. The present study showed high yield when the ratio of lactose to GlcNAc is 1:1 to 2:1. On the other hand, large excess of galactosyl acceptor (GlcNAc) significantly decreased the GlcNAc transgalactosylation yield because of untransformed substrate. High yield is

theoretically obtained if donor and acceptor are present at a molar ratio of 1:1. (*90*) The observed optimum molar ratio of donor/ acceptor is different from previous reports. Optimal molar ratio of donor/acceptor was 3:1 on β-D-galactosyl disaccharide production by β-gal from porcine liver(*151*) while that for the transgalactosylation of Fuc, GlcNAc, GalNAc and mannose was achieved with high yield at a donor:acceptor ratio of 1:1 or an excess of galactosyl acceptor. (*95, 152-154*). The lactulose and lactosucrose yield produced with fructose and sucrose as galactosyl acceptors, respectively was the highest at a molar donor to acceptor ratio in the range of 1:1 to 1:2. (*155-157*)

Lbulβgal and BbreβgalII once again showed to have a propensity to synthesize β-(1→6) linkages; 80% of the GlcNAc transgalactosylation product is *N*-acetyl-allolactosamine. The formation of disaccharides as product of transgalactosylation of galactose and GlcNAc by β-gal of lactobacilli and bifidobacteria has been reported and the linkage preference varies. β-gal from *K. lactis*, *L. bulgaricus* and *Lc. lactis* expressing LacLM of *L. plantarum* synthesized *N*-acetyl-allolactosamine as the major product and LacNAc as minor product. (*94, 96*) Meanwhile, β-gal from *B. bifidum* and *B. circulans* favored LacNAc over *N*-acetyl-allolactosamine.(*117, 158, 159*) *N*-acetyl-allolactosamine was exclusively synthesized by β-gal from *P. multicolor*, *A. oryzae*, *B. longum*.(*117*) Presence of higher DP N-acetyl oligosaccharides in the reaction mixtures of transgalactosylation by using β-gal from *S. soflataricus, A. oryzae* or *E. coli* was also observed however were identified (*152*) Recently, Schwab et al. reported formation of *N*-acetyl-oligosaccharides using lactose and GlcNAc as substrate using *C. mesenteroides* FUA3143, *Lb. ruminis* ChCC8818 and *B. longum* CHCC8700 however they were not identified.(*154*) Sakai et al. (*116*) on the other hand reported that galactosyl transfer to 6'-position can be done by consecutive use of

β-gal from *K. lactis* and *B. circulans* β-galactosidase from *Sulfolobus solfataricus*, β-D-Gal*p*-(1→6)-D-GlcNAc together with an unidentified sugar were the main products starting from a mixture of 1 M lactose and 1 M GlcNAc, while β-D-Gal*p*-(1→4)-D-GlcNAc and β-D-Gal*p*-(1→3)-D-GlcNAc were formed as well, yet in lower concentrations.(*152*) This reaction was also optimized for using β-galactosidase from *Bacillus circulans* as the biocatalyst. This enzyme is known for its propensity to synthesize β-(1→4) linkages (*116*) in its transgalactosylation mode, and hence the main reaction product here was Gal*p*-(1→4)-D-GlcNAc together with smaller amounts of GlcNAc-containing higher oligosaccharides (one tri- and one tetrasaccharides) and β-D-Gal*p*-(1→6)-D-GlcNAc. The total yield was 40% for these GlcNAc-containing oligosaccharides when starting from 0.5 M lactose and GlcNAc each. (*95*) This reaction and the β-galactosidase from *B. circulans* were also compared to the enzyme from *Kluyveromyces lactis* and the reaction conditions were optimized. The latter enzyme was shown to formed predominately β-D-Gal*p*-(1→6)-D-GlcNAc. Again, both enzymes formed a mixture of various di- to tetra-saccharides.(*96*) Since these structures resemble the core of HMO they could be of interest as prebiotic compounds to be added to food.

Lbulβgal and BbreβgalII showed preference to hydrolyze β-D-Gal*p*-(1→4)-D-GlcNAc and can moderately hydrolyze β-D-Gal*p*-(1→3)-D-GlcNAc and β-D-Gal*p*-(1→6)-D-GlcNAc. Among species prevalent in the feces of breast-fed infants only *B. infantis*, which possesses a specialized HMO utilization cluster composed of β-galactosidase, fucosidase, sialidase and β-hexosaminidase is capable of releasing and utilizing monosaccharides from complex HMOs (*160, 161*). In contrast, *B. bifidum* releases monosaccharides from HMOs but is not able to use fucose, sialic acid and GlcNAc. *B. breve* was able to ferment but not release monosaccharides.(*84*)

Moreover, GlcNAc is metabolized both by strains of bifidobacteria and lactobacilli (*22, 160*). The present results shows that Lbulβgal and BbreβgalII can be used to unmasked the type II core structure of HMOs and the synthesized GlcNAc – containing HeOS extend the spectrum of potentially bifidogenic oligosaccharides that can be used as food additives particularly in infant formula. Furthermore, a study on the structure/function relationship of various disaccharides with respect to their prebiotic effect showed that amongst a group of galactose-containing disaccharides, those containing a (1→6)-linkage supported growth of bifidobacteria and lactobacilli best in mixed culture populations *(144)*.

V. CONCLUSIONS AND OUTLOOK

The properties of Lbulβgal differ in some important aspects from those of lactobacillal β-galactosidases of the LacLM type. Its high activity, modest inhibition by the end product D-galactose and high transgalactosylation activity together with its thermostability make this enzyme an attractive biocatalyst for various food-related applications. Lbulβgal, as well as the two GH2 β-galactosidases from *B. breve* DSM 20213, BbreβgalI and BbreβgalII, were found to be very well suited for the production of galacto-oligosaccharides, components that are of great interest because of their use in functional food. Lbulβgal and BbreβgalII showed preference to transfer galactosyl moiety to the 6'- position as shown by the high fractions of β-D-Gal*p*-(1→6)-D-Glc and β-D-Gal*p*-(1→6)-D-GlcNAc during transgalactosylation reactions. Knowledge on the enzymatic formation of the GOS and HeOS from lactose will enable oligosaccharides production with defined linkage type and degree of polymerization to enable studies on structure-function relationships for their prebiotic activity.

VI. LITERATED CITED

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APPENDIX A

Nguyen, T.-T.; Nguyen, H. A.; **Arreola, S. L.;** Mlynek, G.; Djinovic-Carugo, K.; Mathiesen, G.; Nguyen, T.-H.; Haltrich, D., Homodimeric β-Galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081: Expression in *Lactobacillus plantarum* and Biochemical Characterization. *J Agric Food Chem* **2012,** *60*, 1713-1721.

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Homodimeric β-Galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081: Expression in *Lactobacillus plantarum* and Biochemical Characterization

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ABSTRACT: The *lacZ* gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081, encoding a β-galactosidase of the glycoside hydrolase family GH2, was cloned into different inducible lactobacillal expression vectors for overexpression in the host strain *Lactobacillus plantarum* WCFS1. High expression levels were obtained in laboratory cultivations with yields of approximately 53000 U of β-galactosidase activity per liter of medium, which corresponds to ∼170 mg of recombinant protein per liter and β-galactosidase levels amounting to 63% of the total intracellular protein of the host organism. The wild-type (nontagged) and histidine-tagged recombinant enzymes were purified to electrophoretic homogeneity and further characterized. β-Galactosidase from *L. bulgaricus* was used for lactose conversion and showed very high transgalactosylation activity. The maximum yield of galacto-oligosaccharides (GalOS) was approximately 50% when using an initial concentration of 600 mM lactose, indicating that the enzyme can be of interest for the production of GalOS.

KEYWORDS: *β-galactosidase, lactase, transgalactosylation, galacto-oligosaccharides, prebiotics*

INTRODUCTION

Lactic acid bacteria (LAB) and especially lactobacilli are important starter and adjunct cultures in the production of foods that require lactic acid fermentation, notably various dairy products, fermented vegetables, fermented meats, and sourdough bread.[1,2] *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*), a thermophilic Gram-positive bacterium with an optimal growth temperature of 45 °C, is one of the economically most important representatives of the heterogeneous group of LAB, with a worldwide application in yogurt production and in other fermented milk products.[3] *L. bulgaricus* is a homofermentative LAB, and during growth in milk it rapidly converts lactose into lactic acid for food product preservation. The metabolism of lactose in this organism involves two main enzymes, a lactose antiporter permease (LacS) for the uptake of the sugar and a β-galactosidase (LacZ) for the intracellular cleavage of lactose into glucose and galactose, both of which are part of the *lac* operon.[4] *Lactobacillus* spp. encode β-galactosidases that belong to glycoside hydrolase families GH2 and GH42 according to the CAZy nomenclature (http://www.cazy.org).[5] The predominant GH2 β-galactosidases found in lactobacilli are of the LacLM type, heterodimeric proteins of ∼105 kDa, which are encoded by the two overlapping genes, *lacL* and *lacM*. We recently cloned several lactobacillal β-galactosidase genes of this type, including *lacLM* from *Lactobacillus reuteri*,[6] *Lactobacillus acidophilus*,[7] *Lactobacillus pentosus*,[8] *Lactobacillus plantarum*,[9] and *Lactobacillus sakei*,[10] and characterized the resulting proteins with respect to their biochemical properties. In addition, di- or oligomeric GH2 β-galactosidases of the LacZ type, encoded by the single *lacZ* gene, are sometimes, but not often, found in lactobacilli, whereas they are more frequent in other LAB including *Streptococcus salivarius* and *Streptococcus thermophilus*[11] or bifidobacteria including *Bifidobacterium bifidum*[12] or *Bifidobacterium longum* subsp. *infantis*.[13]

β-Galactosidases catalyze the hydrolysis of the β-1,4-D-glycosidic linkage of lactose and structurally related substrates. β-Galactosidases have two main technological applications in the food industry—the removal of lactose from milk and dairy products[14] and the production of galacto-oligosaccharides (GalOS), exploiting the transglycosylation activity of some of these enzymes.[15,16] GalOS are prebiotic sugars, which are defined as a "selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-

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Table 1. Strains and Plasmids Used for Cloning and Overexpression of the β-Galactosidase Gene *lacZ* from *Lactobacillus delbrueckii* subsp. *bulgaricus*[a]

strains and plasmids	relevant characteristics and purpose	ref
strains		
L. delbrueckii subsp. *bulgaricus* DSM 20081	original source of *lacZ*	DSMZ
Lactobacillus plantarum WCFS1	host strain, plasmid free	42
E. coli NEB5α	cloning host	New England Biolabs
plasmids		
pJET1.2	for subcloning and PCR fragment synthesis	Fermentas
pSIP403	*spp*-based expression vector, pSIP401 derivative, Em^r, *gusA* controlled by P_{sppA}	33
pSIP409	*spp*-based expression vector, pSIP401 derivative, Em^r, *gusA* controlled by P_{sppQ}	33
pTH101	pSIP403 derivative, *gusA* replaced by *lacZ*	this study
pTH102	pSIP403 derivative, *gusA* replaced by *lacZ* carrying C-terminal His_6-tag	this study
pTH103	pSIP409 derivative, *gusA* replaced by *lacZ*	this study
pTH104	pSIP409 derivative, *gusA* replaced by *lacZ* carrying C-terminal His_6-tag	this study

[a]Em^r, erythromycin resistance; *spp*, sakacin P gene cluster; *gusA*, β-glucuronidase reporter gene; *lacZ*, β-galactosidase gene.

Table 2. Primers Used for Cloning of the β-Galactosidase Gene *lacZ* from *Lactobacillus delbrueckii* subsp. *bulgaricus*[a]

primer	restriction enzyme	sequence (5→3)	ref sequence accession no.
F1	*Bsm*BI	GCTGCGTCTCCCATGAGCAATAAGTTAGTAAAAG	NC_008054, GeneID: 4085367
R1	*Xho*I	CGCGCTCGAGTTATTTTAGTAAAAGGGGCTG	NC_008054, GeneID: 4085367
R2	*Xho*I	CGCGCTCGAG*TTAGTGGTGGTGGTGGTGGTG*TTTTAGTAAAAGGGGC	NC_008054, GeneID: 4085367

[a]Restriction sites are underlined; the His_6-tag sequence is shown in italic.

being and health".[17] GalOS are complex mixtures of different oligosaccharides, and the spectrum of the oligosaccharides making up these mixtures strongly depends on the source of the β-galactosidase used for the biocatalytic reaction as well as on the conversion conditions used in their production.[15,18] Rabiu et al.[19] and Tzortzis et al.[20] produced various GalOS mixtures using lactose as substrate and β-galactosidases from different probiotic bifidobacteria. Subsequently, they showed that these different mixtures typically resulted in better growth of the producer strain of the enzyme for GalOS production. This concept can serve as the basis for a new generation of functionally enhanced, targeted oligosaccharides and has increased interest in β-galactosidases from beneficial probiotic organisms.[21] Because lactobacilli have traditionally been recognized as potentially health-promoting, probiotic bacteria,[22] GalOS produced by their β-galactosidases can be of interest for nutritional purposes. In the present study we report the heterologous expression of the single-gene encoded β-galactosidase (LacZ) from *L. bulgaricus* in *L. plantarum* using pSIP vectors and, thus, the overexpression of this enzyme in a food grade host. In addition, the β-galactosidase was purified, characterized, and compared to the enzymes of the LacLM type, also with respect to the spectrum of GalOS produced by these different β-galactosidases.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals and enzymes were purchased from Sigma (St. Louis, MO) unless otherwise stated and were of the highest quality available. MRS broth powder was obtained from Merck (Darmstadt, Germany). All restriction enzymes, T4 DNA ligase, and shrimp alkaline phosphatase (SAP) were from Fermentas (Vilnius, Lithuania).

Bacterial Strains and Culture Conditions. The type strain *L. delbrueckii* subsp. *bulgaricus* DSM 20081 (synonym *L. bulgaricus*; other collection numbers are ATCC 11842; originally isolated from Bulgarian yogurt in 1919[23]) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). All bacterial strains used in this study are shown in Table 1. *Lactobacillus* strains were cultivated in MRS media at 37 °C, without agitation. *Escherichia coli* NEB5α (New England Biolabs, Ipswich, MA) was grown at 37 °C in Luria−Bertani (LB) medium with shaking at 120 rpm. When needed, erythromycin was supplemented to media in concentrations of 5 μg/mL for *Lactobacillus* or 200 μg/mL for *E. coli*, whereas ampicillin was used at 100 μg/mL for *E. coli*.

DNA Manipulation. Total DNA of *L. bulgaricus* DSMZ 20081 was isolated using chloroform extraction as described by Nguyen et al.[24] with slight modifications. In short, cell pellets from 3 mL overnight cultures were resuspended and incubated at 37 °C for 1 h in 400 μL of 1 mM Tris−EDTA buffer pH 8 (TE buffer) containing 50 μL of lysozyme (100 mg/mL) and 50 μL of mutanolysin (480 U/mL). The mixture was subsequently supplemented with 50 μL of 10% SDS and 10 μL of proteinase K (20 mg/mL) and incubated further at 60 °C for 1 h. After inactivation of proteinase K (at 75 °C for 15 min), 2 μL of RNase (2 mg/mL) was added to the mixture, and incubation was continued at 37 °C for 30 min. Genomic DNA was extracted and purified by using phenol−chloroform and precipitated with 3 M sodium acetate, pH 3.8, and ice-cold isopropanol. The DNA precipitate was washed with cold (−20 °C) 70% ethanol, and the dried DNA pellets were dissolved in 50 μL of TE buffer, pH 7.5, at room temperature with gentle shaking.

The primers used for PCR amplification of *lacZ* from the genomic DNA of *L. bulgaricus* DSM 20081 (NCBI reference sequence no. NC_008054)[23] were supplied by VBC-Biotech Service (Vienna, Austria) and are listed in Table 2. The appropriate endonuclease restriction sites were introduced in the forward and reverse primers as indicated. DNA amplification was performed with Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland) as recommended by the supplier and using standard procedures.[25] The amplified PCR products were purified by the Wizard SV Gel and PCR Clean-up system kit (Promega, Madison, WI). When needed, the PCR fragments were subcloned into the pJET1.2 plasmid (CloneJET PCR cloning kit, Fermentas), and *E. coli* was used as a host for obtaining the plasmids in sufficient amounts before transformation into *Lactobacillus*. All PCR-generated inserts were confirmed by DNA sequencing performed by a commercial provider.

Plasmid Construction and Transformation. Gene fragments of *lacZ* with or without the His_6-tag were excised from the pJETlacZ plasmid using *Bsm*BI and *Xho*I and ligated into the 5.6 kb *Nco*I−*Xho*I

fragments of pSIP403 or pSIP409, resulting in the plasmids pTH101, pTH102, pTH103, and pTH104 (Table 1). The constructed plasmids were transformed into electrocompetent cells of *L. plantarum* WCFS1 according to the protocol of Aukrust and Blom.[26]

β-Galactosidase Assays. β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) or lactose as the substrates, as described previously.[6] In brief, these assays were performed in 50 mM sodium phosphate buffer of pH 6.5 at 30 °C, and the final substrate concentrations in the 10 min assays were 22 mM for *o*NPG and 575 mM for lactose. Protein concentrations were determined by using the method of Bradford with bovine serum albumin (BSA) as standard.

Expression of Recombinant β-Galactosidase. For the heterologous overexpression of the *lacZ* gene from *L. bulgaricus*, overnight cultures (~16 h) of *L. plantarum* WCFS1 harboring the expression plasmid pTH101, pTH102, pTH103, or pTH104 were added to 15 mL of fresh MRS medium containing erythromycin to an OD_{600} of ~0.1 and incubated at 30 °C without agitation. The cells were induced at an OD_{600} of 0.3 by adding 25 ng/mL of the inducing peptide pheromone IP-673 (supplied by the Molecular Biology Unit, University of Newcastle-upon-Tyne, U.K.). Cells were harvested at an OD_{600} of 1.8−2, washed twice by buffer P (50 mM sodium phosphate buffer, pH 6.5, containing 20% w/v glycerol and 1 mM dithiothreitol),[6] and resuspended in 0.5 mL of the same buffer. Cells were disrupted in a bead beating homogenizer using 1 g of glass bead (Precellys 24; PEQLAB, Germany). Cell-free extracts were obtained after a centrifugation step at 9000*g* for 15 min at 4 °C.

Fermentation and Protein Purification. *L. plantarum* WCFS1 harboring pTH101 or pTH102 was cultivated in 1 L fermentations to obtain sufficient material for purification of LacZ. The cultivation conditions and the induction protocol were identical to those of the small-scale cultivations. Expression of *lacZ* was induced at OD_{600} 0.3, and the cells were harvested at OD_{600} ~6. After centrifugation as above, cells were disrupted by using a French press (Aminco, Silver Spring, MD), and debris was removed by centrifugation (30000*g*, 20 min, 4 °C). The purification of the recombinant enzyme was performed by immobilized metal affinity chromatography using a Ni-Sepharose column (GE Healthcare, Uppsala, Sweden)[8] or substrate affinity chromatography (with the substrate analogue *p*-aminobenzyl 1-thio-β-D-galactopyranoside immobilized onto cross-linked 4% beaded agarose; Sigma) as previously described.[6] Purified enzymes were stored in 50 mM sodium phosphate buffer, pH 6.5, at 4 °C.

Gel Electrophoresis, Gel Permeation Chromatography, and Activity Staining. Native polyacrylamide gel electrophoresis (PAGE), denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), and activity staining using 4-methylumbelliferyl β-D-galactoside (MUG) as the substrate were carried out as previously described[6] using the Phast System with precast gels (Pharmacia Biotech, Uppsala, Sweden). Gel permeation chromatography was performed on a Superose 12 column (16 × 1000 mm; GE Healthcare) using 20 mM sodium phosphate buffer, pH 6.5, containing 150 mM NaCl, and with the Sigma Gel Filtration Molecular Markers Kit with standard proteins of 12−200 kDa. In addition, pyranose oxidase with a molecular mass of 250 kDa was used as a standard.[27]

Characterization of Recombinant β-Galactosidase. Steady-state kinetic data for the substrates lactose or *o*NPG were obtained at 30 °C in 50 mM sodium phosphate buffer, pH 6.5, with concentrations ranging from 0 to 600 mM for lactose and from 0 to 25 mM for *o*NPG. Furthermore, the inhibition of the hydrolytic activity of LacZ by D-glucose as well as D-galactose was investigated by adding these sugars into the assay mixture in concentrations ranging from 10 to 300 mM, and the respective inhibition constants were determined. The kinetic parameters and the inhibition constants were calculated using nonlinear regression, fitting the observed data to the Henri−Michaelis−Menten equation using SigmaPlot (SPSS, Chicago, IL).

The pH dependence of β-galactosidase activity was evaluated in the range of pH 3−10 using Britton−Robinson buffer (containing 20 mM each of phosphoric, acetic, and boric acid adjusted to the required pH with NaOH). The temperature dependence of β-galactosidase activity was assessed by measuring activity in the range of 20−90 °C for 10 min. The catalytic stability of β-galactosidase was determined by incubating the enzyme in 50 mM phosphate buffer (pH 6.5) at various temperatures and by subsequent measurements of the remaining enzyme activity (A) at various time points (t) using the standard *o*NPG assay. Residual activities (A_t/A_0, where A_t is the activity measured at time t and A_0 is the initial activity) were plotted versus the incubation time. The inactivation constants k_{in} were obtained by linear regression of ln(activity) versus time. The half-life values of thermal inactivation $\tau_{1/2}$ were calculated using $\tau_{1/2} = \ln 2/k_{in}$.[28]

To study the effect of various cations on β-galactosidase activity, the enzyme samples were assayed at 30 °C for 10 min with 22 mM *o*NPG (10 mM Bis-Tris, pH 6.5, or 50 mM sodium phosphate buffer, pH 6.5) as the substrate in the presence of various cations added in final concentrations of 1−50 mM. The measured activities were compared with the activity blank of the enzyme solution determined under identical conditions but without added cations using the standard *o*NPG assay. Unless otherwise stated, the nontagged enzyme LacZ was used for these characterization experiments.

Lactose Hydrolysis and Transgalactosylation. The synthesis of galacto-oligosaccharides (GalOS) was carried out in discontinuous mode using purified recombinant, nontagged β-galactosidase from *L. bulgaricus* (1.5 lactase U/mL of reaction mixture). Reaction conditions were 600 mM initial lactose concentration in sodium phosphate buffer (50 mM, pH 6.5) containing 10 mM $MgCl_2$; the incubation temperature was varied from 30 to 50 °C. Continuous agitation was applied at 300 rpm. Samples were withdrawn periodically, and the composition of the GalOS mixture was analyzed by capillary electrophoresis and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), following methods described previously.[29] Individual GOS compounds were identified and quantified by using authentic standards and the standard addition technique.[16,30]

Statistical Analysis. All experiments and measurements were performed at least in duplicate, and the data are given as the mean ± standard deviation when appropriate. Student's t test was used for the comparison of data.

RESULTS AND DISCUSSION

Plasmid Construction and Expression of β-Galactosidase Derived from *L. bulgaricus* in *L. plantarum*. The yields of β-galactosidase activity when using the wild-type strain of *L. bulgaricus* as a producer are rather low; for example, β-galactosidase levels were only ~4000 U of activity/L of medium (MRS containing 2% lactose) after cultivation at 37 °C for 24 h for *L. delbrueckii* subsp. *bulgaricus* DSM 20081. Hence we attempted heterologous overexpression in a food grade organism to obtain higher yields of this biotechnologically attractive enzyme, and we cloned the *L. bulgaricus lacZ* gene into the vectors pSIP403 and pSIP409, which differ only in their promoters.[31−33] The four expression plasmids pTH101, pTH102, pTH103, and pTH104 were constructed by replacing *gusA*, which originally was used as a reporter gene in the pSIP plasmid series, by *lacZ*, both with and without a hexa-histidine tag (Table 1). In these vectors, the transcription of *lacZ* is regulated by the inducible promoters P_{sppA} and P_{sppQ} for the pSIP403 and pSIP409 derivatives, respectively (Figure 1). The expression of *lacZ* with the different vectors was subsequently studied in *L. plantarum* WCFS1 as host, using an inducer concentration of 25 ng/mL of the inducing peptide pheromone IP-673.[25,31] Induced and noninduced cells were harvested in the late stationary phase (OD_{600} of 1.8−2.0), and the intracellular cell-free extracts were analyzed by SDS-PAGE, which showed unique bands of ~100 kDa in induced *L. plantarum* cells (Figure 2) and β-galactosidase activity assays

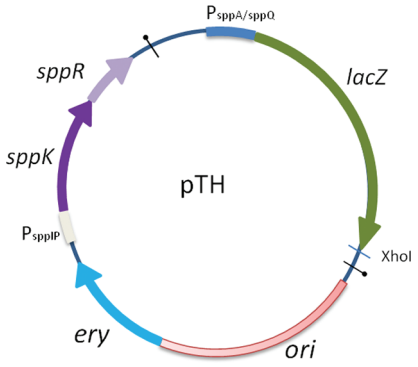

Figure 1. Schematic overview of the pTH plasmids developed in this study. The structural gene *lacZ* (with or without a hexa-histidine tag) is controlled by the inducible promoters P_{sppA} (pSIP403 derivatives) or P_{sppQ} (pSIP409 derivatives). P_{sppIP} controls the structural genes of the two-component regulatory system, *sppK*, a histidine kinase, and *sppR*, a response regulator. *Ery* indicates the erythromycin resistance marker, and transcriptional terminators are marked by lollypop structures.

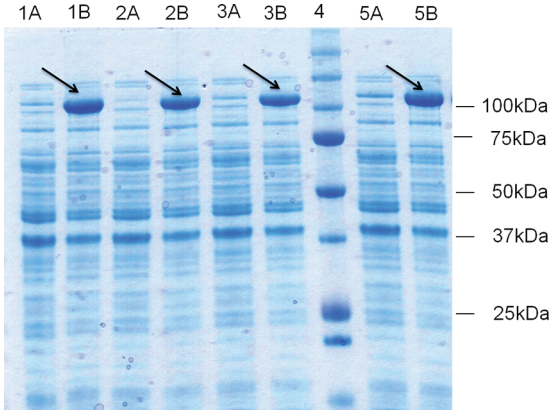

Figure 2. SDS-PAGE analysis of cell-free extracts of noninduced (A) and induced cells (B) of *L. plantarum* WCFS1 harboring pTH101 (lanes 1A, 1B), pTH103 (lanes 2A, 2B), pTH104 (lanes 3A, 3B), and pTH102 (lanes 5A, 5B). Lane 4 shows the Precision Plus Protein standard (Bio-Rad). The gel was stained with Coomassie blue.

(Table 3). Analysis of the crude cell extracts gave volumetric activities in the range of ~15−23 U/mL of cultivation medium and specific activities of ~160−200 U/mg (Table 3). The *β*-galactosidase activities in *L. plantarum* cells without plasmids were diminishing (0.002 U/mL and 0.07 U/mg), and hence the enzyme activities obtained can be attributed solely to the plasmid-encoded LacZ from *L. bulgaricus*. The choice of the P_{sppA} promoter (pSIP403 derivatives) or P_{sppQ} promoter (pSIP409 derivatives) did not affect the levels of *β*-galactosidase activity, because expression yields were well comparable and statistically not different for these constructs.

Noninduced cells of *L. plantarum* harboring the various expression vectors were also cultivated and tested for basal expression ("leakage") from the promoters (Table 3). Cells carrying pSIP409-derived vectors containing P_{sppQ} show significantly lower basal activities than cells harboring pSIP403-derived vectors based on P_{sppA}. As a consequence, the highest induction factors, that is, the quotient of specific activity obtained for induced and noninduced cells, of roughly 50 were found for the constructs pTH103 and pTH104 carrying P_{sppQ} as the promoter.

Interestingly, the activities obtained for His-tagged LacZ were always significantly lower by approximately 20−30% despite both protein versions being produced in comparable levels as judged by SDS-PAGE analysis. The reduced activity is most probably caused by the C-terminal His-tag, because specific activities determined for purified, homogeneous nontagged and His-tagged LacZ (306 and 251 U/mg) also differ by ~20%. The exact mechanism of how the His-tag interferes with the activity is, however, not known.

Fermentation and Purification of Recombinant *β*-Galactosidase LacZ. *L. plantarum* harboring pTH101 or pTH102 was cultivated on a larger scale (1 L cultivation volume), and gene expression was induced in accordance with the previous experiments. Typical yields obtained in 1 L laboratory cultivations were approximately 7.5 ± 0.5 g wet biomass and 53 ± 2 kU of nontagged (pTH101) and 43 ± 2 kU of His-tagged (pTH102) *β*-galactosidase activity. As judged from the specific activity of the crude cell extract (193 U/mg for nontagged LacZ) and that of the purified enzyme, 63% of the total soluble intracellular protein in *L. plantarum* amounts to the heterologously expressed protein, which was produced at levels of ~170 mg recombinant protein/L of medium.

The recombinant enzymes were purified to apparent homogeneity from cell extracts by single-step purification protocols using either substrate affinity or immobilized metal affinity chromatography. The specific activity of the purified recombinant enzymes was 306 U/mg for wild-type, nontagged LacZ and 251 U/mg for His-tagged LacZ, respectively, when using the standard *o*NPG assay. Both purification procedures yielded homogeneous *β*-galactosidase as judged by SDS-PAGE (Figure 3A).

Molecular Characterization of the *lacZ* Gene Product, *β*-Galactosidase LacZ. *β*-Galactosidase from *L. bulgaricus* is a homodimer, consisting of two identical subunits of ~115 kDa, as judged by denaturing SDS-PAGE (molecular mass of ~115 kDa as judged by comparison with reference proteins; Figure

Table 3. *β*-Galactosidase Activity in Cell-free Extracts of Induced and Noninduced Cells of *L. plantarum* WCFS1 Carrying Various Expression Plasmids[a]

	volumetric activity (U/mL fermentation broth)		specific activity (U/mg protein)		
plasmid	induced	noninduced	induced	noninduced	induction factor[b]
pTH101	22.5 ± 0.8	1.50 ± 0.04	196 ± 3	10.3 ± 1.1	19
pTH102	15.5 ± 0.6	1.62 ± 0.13	158 ± 3	11.7 ± 0.5	13
pTH103	22.0 ± 1.3	0.63 ± 0.03	193 ± 10	4.11 ± 0.18	47
pTH104	18.0 ± 0.5	0.51 ± 0.04	168 ± 4	3.43 ± 0.13	49

[a]Data are expressed as the average ± standard deviation of three independent cultivations. The specific *β*-galactosidase activity in cell-free extracts of nontransformed *L. plantarum* was 0.07 U/mg. [b]The induction factors are calculated from the specific *β*-galactosidase activity obtained under inducing conditions divided by the activity under noninduced conditions in cells harvested at OD_{600} of 1.8−2.0.

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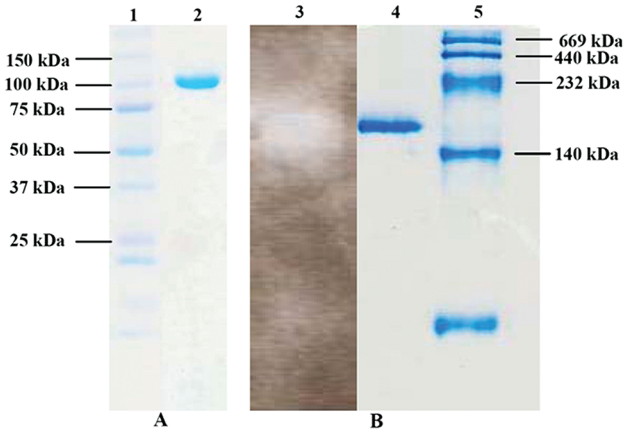

Figure 3. Electrophoretic analysis of purified recombinant *β*-galactosidase from *L. bulgaricus*: (A) SDS-PAGE (lanes: 1, Precision plus Protein standard ladder (Bio-Rad); 2, purified recombinant enzyme); (B) native-PAGE (lanes: 3, activity staining of *β*-galactosidase using 4-methylumbelliferyl *β*-D-galactoside as substrate; 4, purified *β*-galactosidase; 5, high molecular mass protein ladder (GE Healthcare)).

3A) and native PAGE (molecular mass of ∼200 kDa; Figure 3B). Gel permeation chromatography and comparison with protein standards of known mass gave a molecular mass of 230 kDa for native LacZ. This is in good agreement with the calculated molecular mass of 114 047 Da deduced for the LacZ subunit from its sequence. Activity staining directly on the native PAGE gel using 4-methylumbelliferyl *β*-galactoside as the substrate indicated furthermore that the protein band of ∼200 kDa indeed shows *β*-galactosidase activity (Figure 3B).

Enzyme Kinetics. The steady-state kinetic constants for the hydrolysis of the natural substrate lactose as well as for the artificial substrate *o*-nitrophenol *β*-D-galactopyranoside (*o*NPG) together with the inhibition constants for both end products, D-galactose and D-glucose, for *β*-galactosidase from *L. bulgaricus* are summarized in Table 4. The k_{cat} values were calculated on the basis of the theoretical v_{max} values experimentally determined by nonlinear regression and using a molecular mass of 114 kDa for the catalytically active subunit (115 kDa for His-tagged LacZ). *β*-Galactosidase from *L. bulgaricus* is not inhibited by its substrates lactose in concentrations of up to 600 mM or *o*NPG in concentrations of up to 25 mM as is evident from the Michaelis−Menten plots (not shown). The hydrolysis end products D-galactose and D-glucose competitively inhibit the hydrolytic activity of *β*-galactosidase from *L. bulgaricus*, albeit this inhibition of, for example, D-galactose on cleavage of the natural substrate lactose is only moderate as is evident from the ratio of the Michaelis constant for lactose and the inhibition constant for D-galactose ($K_{i,Gal}/K_{m,Lac}$ = 3.7). The inhibition by D-glucose is even less pronounced as is obvious from the high inhibition constant measured for the hydrolysis of *o*NPG and the high ratio of K_i to K_m for this reaction ($K_{i,Glc}/K_{m,oNPG}$ = 134).

Effect of Metal Ions on Enzyme Activity. Various mono- and divalent metal ions were tested with respect to a possible stimulating or inhibitory effect on *β*-galactosidase activity. These were added in final concentrations of 1−50 mM to the enzyme in Bis-Tris buffer (Table 5). The monovalent cations

Table 5. Effect of Cations on Activity of *β*-Galactosidase in 10 mM Bis-Tris Buffer, pH 6.5[a]

	relative activity (%)		
cation	1 mM	10 mM	50 mM
blank (none)	100	100	100
Na^+	722	1030	1190
K^+	365	536	507
Mg^{2+}	85	31	nd[b]
Ca^{2+}	77	38	nd
Zn^{2+}	3	0.55	nd

[a]Enzyme activity was determined under standard assay conditions in 10 mM Bis-Tris buffer, pH 6.5, using *o*NPG as the substrate with the respective cation added to give the stated final concentration. Experiments were performed in duplicates, and the standard deviation was always <5%. [b]nd, not determined.

K^+ and especially Na^+ activated *β*-galactosidase activity when using this buffer system considerably; for example, an almost 12-fold increase in activity was found in the presence of 50 mM Na^+ compared to a blank where no metal ion was added to the enzyme sample. When using the standard 50 mM sodium phosphate buffer, pH 6.5, K^+ only resulted in a slight activation of approximately 1.4-fold when added in 10 mM concentrations. The divalent cations Mg^{2+}, Ca^{2+}, and Zn^{2+} showed an inhibitory effect when using Bis-Tris buffer, and especially the latter cation inhibited *β*-galactosidase activity strongly (Table

Table 4. Kinetic Parameters for Recombinant *β*-Galactosidase LacZ from *L. bulgaricus*, both Nontagged and C-Terminally His-Tagged, for the Hydrolysis of Lactose and *o*-Nitrophenyl *β*-D-Galactopyranoside (*o*NPG)

substrate	method for determination of enzyme activity	kinetic parameter[a]	nontagged LacZ	His-tagged LacZ
lactose	release of D-glucose	$v_{max,Glc}$ (μmol min^{-1} mg^{-1})	123 ± 5	111 ± 4
		$K_{m,Lac}$ (mM)	19.2 ± 3.8	19.9 ± 3.8
		k_{cat} (s^{-1})	234 ± 13	211 ± 10
		k_{cat}/K_m (M^{-1} s^{-1})	12300	10600
		$K_{i,Gal}$ (mM)	70.7 ± 16.8	nd
*o*NPG	release of *o*NP	$v_{max,oNP}$ (μmol min^{-1} mg^{-1})	317 ± 6	257 ± 5
		$K_{m,oNPG}$ (mM)	0.919 ± 0.088	1.20 ± 0.11
		k_{cat} (s^{-1})	603 ± 15	492 ± 13
		k_{cat}/K_m (M^{-1} s^{-1})	655000	410000
		$K_{i,Glc}$ (mM)	123 ± 9	nd
		$K_{i,Gal}$ (mM)	9.52 ± 1.54	nd

[a]Molecular masses of 114 and 115 kDa were used to calculate k_{cat} from v_{max} for native and His-tagged LacZ, respectively.

5). Interestingly, when using 50 mM sodium phosphate buffer instead of Bis-Tris buffer, Mg^{2+} showed an activating effect (150% relative activity) at concentrations of 1 and 10 mM; this could indicate a synergistic effect with Na^+ present in this buffer.[14]

Effect of Temperature and pH on Enzyme Activity and Stability. The temperature optima of the activity of β-galactosidase from *L. bulgaricus* are 45−50 and 55−60 °C for *o*NPG and lactose hydrolysis, respectively, when using the 10 min assay (Figure 4A). The pH optimum of LacZ activity is pH 7.5 for both substrates lactose and *o*NPG (Figure 4B). Overall, the pH curves show a broad peak with 75% of maximal β-galactosidase activity in the pH range of 6−9 (Figure 4B). Catalytic stability, that is, the length of time the enzyme remains active before undergoing irreversible inactivation, of β-galactosidase from *L. bulgaricus* was measured at a constant pH of 7.0 while the temperature was varied from 37 to 60 °C. In addition, we tested the effect of different buffers and the addition of cations on stability. LacZ activity showed first-order inactivation kinetics when analyzed in the plot of ln(residual activity) versus time (not shown). Data for the inactivation constants k_{in} and half-life times of activity $\tau_{1/2}$ are summarized in Table 6. Regardless of the temperature, stability was comparable in phosphate buffer without added cation and Bis-Tris buffer containing 10 mM Na^+, the metal ion that was found to increase activity significantly. Addition of 10 mM Mg^{2+} to phosphate buffer increased the stability considerably. Under these conditions, *L. bulgaricus* LacZ was well stable at 50 °C with a half-life time of >1 day. When the temperature was increased to 60 °C, activity was, however, lost rapidly (Table 6). This effect of ions such as Mg^{2+} on stability and activity seems common among GH2 β-galactosidases and is also observed for *E. coli* β-galactosidase LacZ[34,35] as well as for some β-galactosidases from *Lactobacillus* spp. of the LacLM type.[6,8] Several metal-binding sites were identified in the structure of *E. coli* LacZ, some of which are located in the direct vicinity of the active site. These ions are thought to take directly part in the catalytic mechanism and also to contribute to subunit interaction and hence stabilization of *E. coli* LacZ.[34,35]

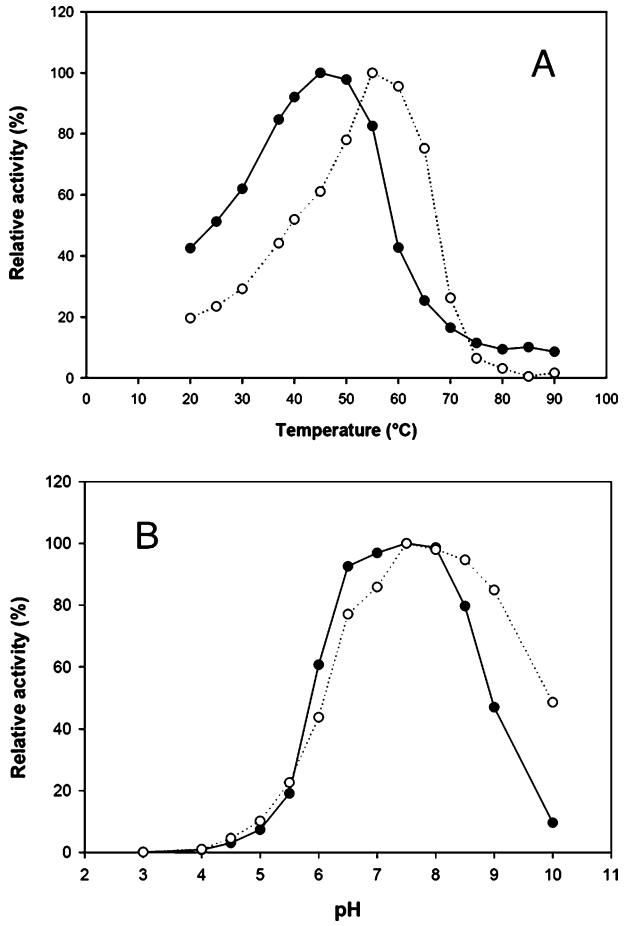

Figure 4. Temperature and pH optima of the activity of recombinant β-galactosidase from *L. bulgaricus*: (○) lactose as substrate; (●) *o*NPG as substrate. Relative activities are given in comparison with the maximum activities measured under optimal conditions (100%), which were 412 and 237 U/mL with *o*NPG and lactose as the substrate, respectively, when determining the temperature optimum (A) and 680 and 106 U/mL with *o*NPG and lactose as the substrate, respectively, for the pH dependence of activity (B).

Lactose Transformation and Synthesis of Galacto-oligosaccharides. The transgalactosylation activity of *L. bulgaricus* LacZ has been described before,[36−38] but has not been studied in much detail; for example, the structures of the main transferase products have not been identified. Lactose conversion and product formation of a typical LacZ-catalyzed reaction, using an initial lactose concentration of 600 mM (205 g/L) in 50 mM sodium phosphate buffer with 10 mM $MgCl_2$, pH 6.5, and 1.5 $U_{lactose}$/mL of β-galactosidase activity at 30 °C, are shown in Figure 5A. During the initial reaction phase, galacto-oligosaccharides (GalOS) are the main reaction products, which are formed together with the primary hydrolysis products D-galactose and D-glucose. The concentration of total GalOS reached a maximum of 102 g/L after 12 h of reaction, when 90% of initial lactose was converted; this corresponds to a yield of almost 50% GalOS. Thereafter, the concentration of GalOS decreased because they are also hydrolyzed by the β-galactosidase. This breakdown of GalOS, however, proceeds only slowly, most probably because of end product inhibition by D-galactose, which at this point of the reaction is present in notable concentrations, and only

Table 6. Catalytic Stability of Recombinant β-Galactosidase from *L. bulgaricus*[a]

	sodium phosphate buffer, pH 7		sodium phosphate buffer, pH 7 + 10 mM Mg^{2+}		Bis-Tris buffer, pH 7 + 10 mM Na^+	
temperature (°C)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)
37	0.0053	145	0.0016	345	0.0084	82.5
50	0.925	0.75	0.026	26	1.12	0.62
60	15.3	0.045	1.0	0.32	16.9	0.041

[a]The inactivation constant k_{in} and half-life time of activity $\tau_{1/2}$ were calculated at different temperatures and reaction conditions. Buffer concentrations were 50 mM each. Experiments were performed in duplicates, and the standard deviation was always <5%.

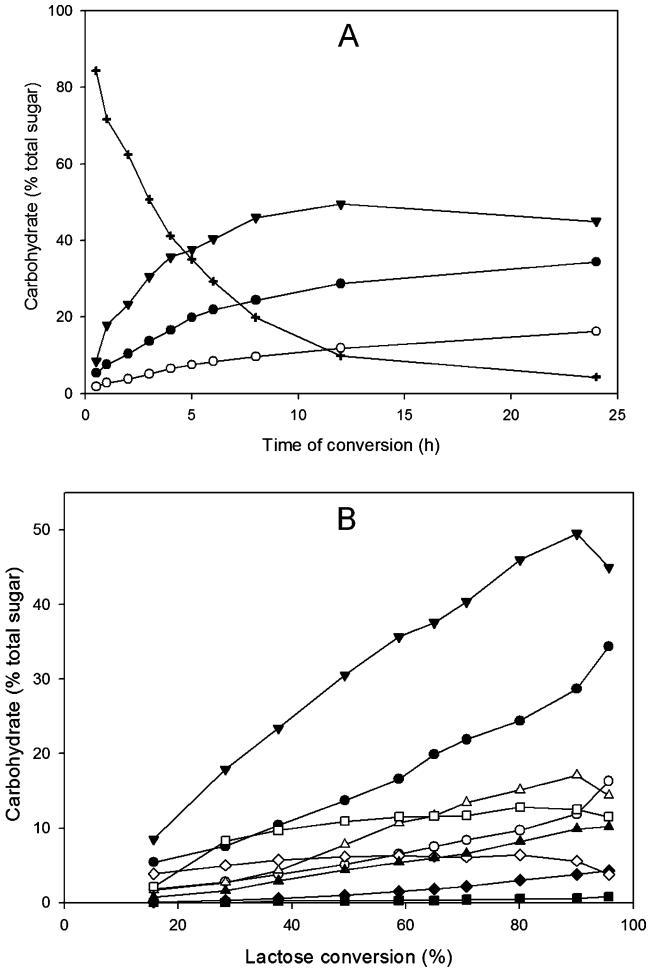

Figure 5. Composition of the sugar mixture during lactose conversion by recombinant β-galactosidase from *L. bulgaricus*. The reaction was carried out at 30 °C with an initial concentration of 600 mM lactose in 50 mM sodium phosphate buffer, pH 6.5, in the presence of 10 mM $MgCl_2$ using ~1.5 $U_{lactose}$/mL of enzyme. (A) Time course of the conversion: (+), lactose; (●), glucose; (○), galactose; (▼) total galacto-oligosaccharides (GalOS). (B) Composition of the sugar mixture and individual GalOS components at different degrees of lactose conversion: (●), glucose; (○), galactose; (▼) total (GalOS); (♦), β-D-Gal*p*-(1→3)-D-Glc; (■), β-D-Gal*p*-(1→3)-D-Gal; (◇), β-D-Gal*p*-(1→3)-Lac; (△), β-D-Gal*p*-(1→6)-D-Glc; (□), β-D-Gal*p*-(1→6)-Lac; (◆), unidentified GalOS. Monosaccharides were measured enzymatically, lactose and GalOS were quantified by HPAEC-PAD and CE. Individual sugars are given as the percentage of total sugars (205 g/L) in the mixture.

approximately 10% of total GalOS are degraded when the reaction proceeds for another 12 h. A detailed analysis of the main transferase products formed is given in Figure 5B. Up to ~90% lactose conversion, the amount of total GalOS, expressed by their relative concentration (percentage of GalOS of total sugars in the reaction mixture) was increasing almost linearly. At the beginning of the reaction, the trisaccharides β-D-Gal*p*-(1→6)-Lac and β-D-Gal*p*-(1→3)-Lac were formed predominately. With further progress of the reaction, the concentrations of D-galactose and D-glucose increased steadily, and these monosaccharides became important acceptors for the transferase reaction; hence, disaccharides other than lactose are formed as well. Non-lactose disaccharides were prevailing by weight at around 75% lactose conversion and later, with β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→3)-D-Glc as the two main products. In addition to these main GalOS components, β-D-Gal*p*-(1→3)-D-Gal and β-D-Gal*p*-(1→6)-D-Gal were identified in the reaction mixtures; these were, however, minor constituents. GalOS containing new β-(1→4) linkages could not be identified in these mixtures. β-Galactosidase from *L. bulgaricus* formed GalOS structurally similar to those obtained with other β-galactosidases from LAB,[7,9,15,16,39] yet proportions of individual components varied to some extent. The predominant oligosaccharide products were identified as β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→6)-Lac, together accounting for approximately 60% of the GalOS, indicating that this β-galactosidase has a propensity to synthesize β-(1→6)-linked GalOS.

To examine whether the high thermostability of *L. bulgaricus* LacZ can be exploited for GalOS synthesis, we also ran the lactose conversion experiments at higher temperatures, that is, 40 and 50 °C, using otherwise identical conditions. Table 7 lists these results for a comparable degree of lactose conversion of ~90%. The reaction mixture showed a very similar composition regardless of the reaction temperature. However, the time needed to obtain 90% lactose conversion was reduced, from 12 h of reaction time at 30 °C to 8 h at 40 °C and only 5 h at 50 °C, and therefore the productivity increased from 8.5 to 19.8 g L^{-1} h^{-1} GalOS for the LacZ-catalyzed reaction at the highest temperature tested. It is interesting to note that the reaction temperature hardly affected the maximum GalOS yield or the composition of the GalOS mixture. Several studies have shown that transgalactosylation becomes more pronounced compared to hydrolysis at higher temperatures.[40,41]

Table 7. Oligosaccharide Components (% w/w of Total Sugar) of GalOS Mixtures Obtained with β-Galactosidase of *L. bulgaricus* at Three Different Temperatures[a]

GalOS component	reaction temperature		
	30 °C	40 °C	50 °C
glucose	28.7	31.0	32.5
galactose	11.9	13.5	14.2
total GOS	49.5	48.7	48.2
β-D-Gal*p*-(1→3)-D-Gal	0.6	0.6	0.6
β-D-Gal*p*-(1→3)-D-Glc	3.8	4	3.9
β-D-Gal*p*-(1→3)-Lac	5.6	5.1	4.5
β-D-Gal*p*-(1→6)-D-Gal	1.0	1.3	1.1
β-D-Gal*p*-(1→6)-D-Glc	17.1	15.5	15
β-D-Gal*p*-(1→6)-Lac	12.5	12.5	13.2
unknown OS	8.9	9.7	9.9
lactose conversion	90.1[b]	93.2[c]	94.9[d]

[a]A lactose concentration of 600 mM (205 g/L) and 1.5 U/mL of β-galactosidase activity (determined with lactose as substrate under standard assay conditions) were used in each experiment. Data are given for the maximal yields obtained during the course of the reaction. Experiments were performed in duplicate, and the standard deviation was always <5%. [b]At 12 h. [c]At 8 h. [d]At 5 h.

In conclusion, the properties of β-galactosidase LacZ from *L. bulgaricus* differ in some important aspects from those of lactobacillal β-galactosidases of the LacLM type. Its high activity, modest inhibition by the end product D-galactose, and high transgalactosylation activity together with its thermostability make this enzyme an attractive biocatalyst for various food-related applications.

AUTHOR INFORMATION

Author Contributions

[‖]T.-T.N. and H.A.N contributed equally to this work.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CAZy, Carbohydrate-Active enZYmes Database; CE, capillary electrophoresis; DTT, 1,4-dithiothreitol; GalOS, galacto-oligosaccharides; GOD, glucose oxidase; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; Lac, lactose; MUG, 4-methylumbelliferyl β-D-galactoside; *o*NP, *o*-nitrophenol; *o*NPG, *o*-nitrophenyl-β-D-galactopyranoside; PMSF, phenylmethanesulfonyl fluoride.

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APPENDIX B

Intanon, M.; **Arreola, S. L.;** Pham, N. H.; Kneifel, W.; Haltrich, D.; Nguyen, T. H., Nature and biosynthesis of galacto-oligosaccharides related to oligosaccharides in human breast milk. *FEMS Microbiology Letters* **2014,** *353*, 89-97.

MINIREVIEW

Nature and biosynthesis of galacto-oligosaccharides related to oligosaccharides in human breast milk

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Abstract

Human milk oligosaccharides (HMO) are prominent among the functional components of human breast milk. While HMO have potential applications in both infants and adults, this potential is limited by the difficulties in manufacturing these complex structures. Consequently, functional alternatives such as galacto-oligosaccharides are under investigation, and nowadays, infant formulae are supplemented with galacto-oligosaccharides to mimic the biological effects of HMO. Recently, approaches toward the production of defined human milk oligosaccharide structures using microbial, fermentative methods employing single, appropriately engineered microorganisms were introduced. Furthermore, galactose-containing hetero-oligosaccharides have attracted an increasing amount of attention because they are structurally more closely related to HMO. The synthesis of these novel oligosaccharides, which resemble the core of HMO, is of great interest for applications in the food industry.

Introduction

Certain oligosaccharides are considered to be beneficial for human and animal hosts due to their ability to stimulate selectively growth and/or activity of one or a limited number of bacteria in the colon. They are classified as 'prebiotics', new functional food ingredients that are of considerable interest. The prebiotic compounds are typically oligosaccharides of various compositions, and galacto-oligosaccharides (GOS), the products of transgalactosylation reactions catalyzed by β-galactosidases when using lactose as the substrate, are nondigestible carbohydrates meeting the criteria of 'prebiotics' (Roberfroid *et al.*, 2010). GOS are of special interest to human nutrition because of the presence of structurally related oligosaccharides together with different complex structures in human breast milk (Sangwan *et al.*, 2011). Several different functions are attributed to these human milk oligosaccharides (HMO). With respect to the influence on the intestinal microbiota, the neutral fraction of HMO seems to be a key factor for the development of the intestinal microbiota typical for breastfed infants and hence for the prebiotic effect. GOS together with inulin/fructo-oligosaccharides (FOS) and lactulose are among the most important and best-studied groups of prebiotic oligosaccharides. At present, these commercially important oligosaccharides with prebiotic status are available mainly in the Japanese, European and USA markets. A mixture of GOS and long-chain FOS was introduced in the market especially for the use in infant formula. This mixture can mimic HMO to some extent and shows a pronounced prebiotic effect; in that it stimulated the development of intestinal microbiota comparable with those found in breastfed infants (Boehm *et al.*, 2008). Hence, biocatalytically produced GOS can be of significant interest for the nutrition of infants. β-galactosidases have also been used to produce hetero-oligosaccharides (HOS) with potentially extended functionality in addition to GOS. Mannose, fructose, *N*-acetylneuraminic acid, glucuronic

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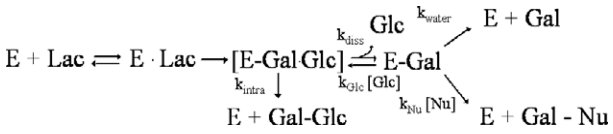
acid and a number of aromatic compounds have been shown to act as galactosyl acceptor for β-galactosidases (Gänzle, 2012). The choice of suitable acceptor and enzyme allows the formation of 'tailor-made' HOS of high interest for applications in the food industry. This article highlights the recent progress on research in microbial production of GOS. The emerging trends in the biosynthesis of the novel oligosaccharides, which are structurally more closely related to HMO, will be reviewed as well.

Microbial production of GOS

Transgalactosylation of lactose using β-galactosidases

β-galactosidases (β-gal; EC 3.2.1.23) catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides (such as lactose). GOS are the products of transgalactosylation reactions catalyzed by β-galactosidases when using lactose or other structurally related galactosides as the substrate. β-galactosidases undergo a two-step mechanism of catalysis. First, this mechanism involves the formation of a covalently linked galactosyl-enzyme intermediate. Subsequently, the galactosyl moiety linked to the nucleophile in the active site is transferred to a nucleophilic acceptor. Water, as well as all sugar species present in the reaction mixture, can serve as a galactosyl acceptor. Hence, the resulting final mixture contains hydrolysis products of lactose, which are glucose and galactose, unconverted lactose as well as di-, tri- and higher oligosaccharides. Scheme 1 illustrates possible lactose conversion reactions catalyzed by β-galactosidases, and structures of some GOS are given in Fig. 1.

β-galactosidases can be obtained from different sources including microorganisms, plants and animals. Microbial β-galactosidases have been isolated and characterized from yeasts, fungi and bacteria. The major industrial enzymes are obtained from *Aspergillus* spp. and *Kluyveromyces* spp. where *Kluyveromyces lactis* is probably the most widely used source (Kim *et al.*, 2004). Microbial sources of β-galactosidase are of great biotechnological interest because of easier handling, higher multiplication rates and production yield. Table 1 presents some of the commercially available bacterial, fungal and yeast



Scheme 1. Hydrolysis and galactosyl transfer reactions, both intra- and intermolecular, during the conversion of lactose catalyzed by β-galactosidases. E, enzyme; Lac, lactose; Gal, galactose; Glc, glucose; Nu, nucleophile.

β-galactosidases. An extensive list of bacterial and fungal sources of β-galactosidases, as well as the lactose conversion reaction conditions and GOS yields, are given in the review by Torres *et al.* (2010).

β-galactosidases from different species possess very different specificities for building glycosidic linkages and therefore produce different GOS mixtures. For example, the β-galactosidase from *K. lactis* produced predominantly β-(1→6)-linked GOS, the β-galactosidase from *Aspergillus oryzae* produced mainly β-(1→3) and β-(1→6) linkages, *Bacillus circulans* β-galactosidase forms mainly β-(1→4)-linked GOS (Rodriguez-Colinas *et al.*, 2014), whereas β-galactosidases from *Lactobacillus* spp. showed preference to form β-(1→6) as well as β-(1→3) linkages in transgalactosylation mode (Splechtna *et al.*, 2006; Nguyen *et al.*, 2012).

Production of GOS

Microbial sources of β-galactosidases for GOS production include crude enzymes, purified enzymes, recombinant enzymes, immobilized enzymes, whole-cell biotransformations, toluene-treated cells and immobilized cells. The enzyme sources, the process parameters as well as the yield and the productivity of these processes for GOS production are summarized in detail in recent reviews (Torres *et al.*, 2010; Sangwan *et al.*, 2011). The highest GOS productivity, 106 g L^{-1} h^{-1}, was observed when β-galactosidase from *A. oryzae* immobilized on cotton cloth was used for GOS production in a packed-bed reactor (Albayrak & Yang, 2002).

Bifidobacteria and lactobacilli have been studied intensively with respect to their enzymes for various different reasons, one of which is their 'generally recognized as safe' status and their safe use in food applications. It is anticipated that GOS produced by these β-galactosidases will have better selectivity for growth and metabolic activity of these bacterial genera in the gut and thus will lead to improved prebiotic effects. A number of studies report the presence of multiple β-galactosidases, for example, in *Bifidobacterium infantis*, *Bifidobacterium adolescentis* or *Bifidobacterium bifidum* (Hung & Lee, 2002; Hinz *et al.*, 2004; Goulas *et al.*, 2009). It was shown that these enzymes are very different with respect to substrate specificity and regulation of gene expression. Furthermore, these reports described the cloning and characterization of these enzymes and studied their transgalactosylation activity in detail; for example, β-galactosidase BgbII from *B. adolescentis* showed high preference toward the formation of β-(1→4) linkages, while no β-(1→6) linkages were formed (Hinz *et al.*, 2004). In contrast, the β-galactosidase BgbII from *B. bifidum* showed a clear preference for the synthesis of β-(1→6) linkages over β-(1→4)

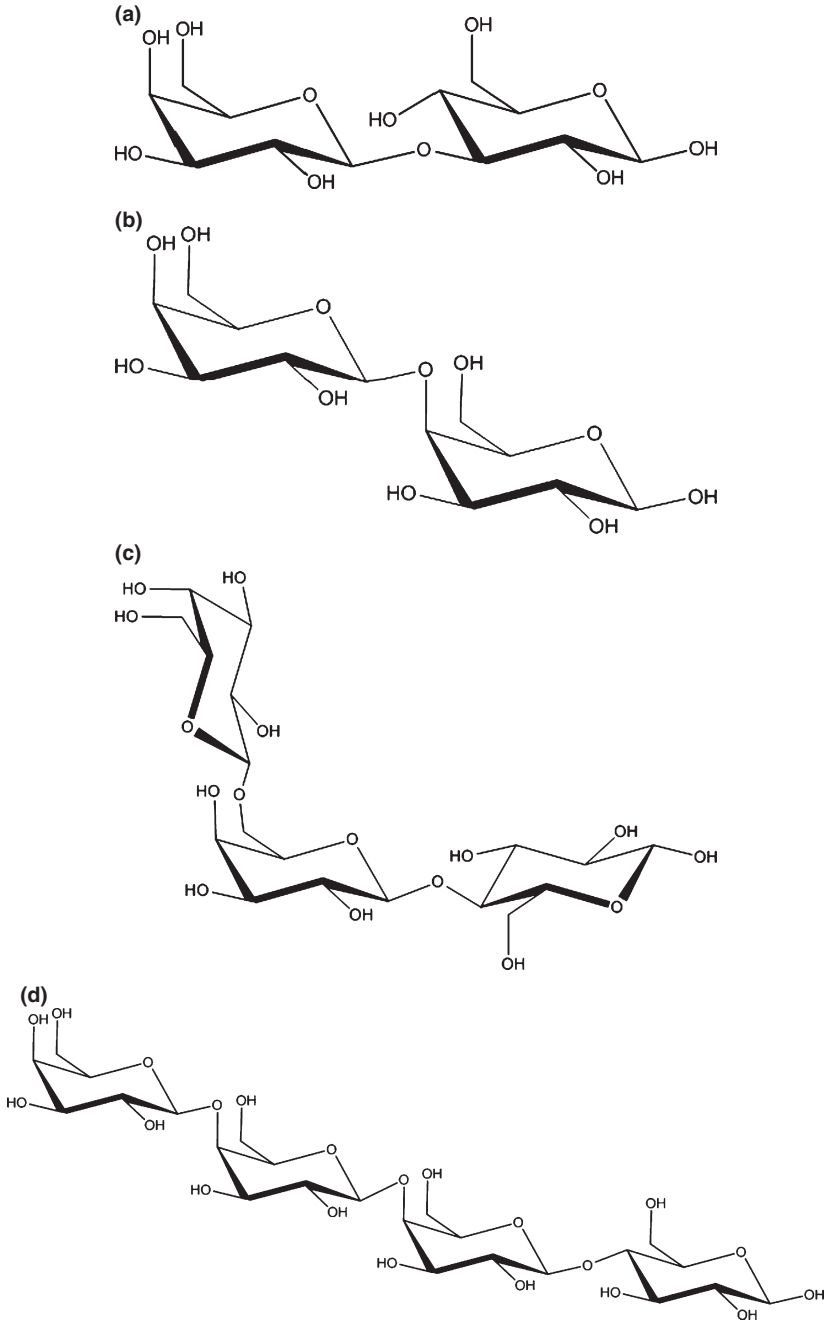


Fig. 1. Structures of some GOS: β-D-Gal*p*-(1→3)-D-Glc (a), β-D-Gal*p*-(1→4)-D-Gal (b), β-D-Gal*p*-(1→6)-Lac (c), β-D-Gal*p*-(1→4)-D-Gal*p*-(1→4)-Lac (d).

Table 1. Commercial β-galactosidases

Name	Manufacturer	Microorganism
BioLactase NTL-CONC	Biocon	*Bacillus circulans* (Rodriguez-Colinas *et al.*, 2014)
Lactozym pure 6500 L	Novozymes	*Kluyveromyces lactis* (Rodriguez-Colinas *et al.*, 2014)
Lactase F 'Amano'	Amano Enzyme Inc	*Aspergillus oryzae* (Rodriguez-Colinas *et al.*, 2014)
Biolacta FN5	Daiwa Fine Chemicals Co., Ltd	*Bacillus circulans*
LACTOLES L3	Biocon Ltd, Japan	*Bacillus circulans*
Maxilact	DSM Food Specialties	*Kluyveromyces lactis*
Tolerase	DSM Food Specialties	*Aspergillus oryzae*

linkages (Goulas *et al.*, 2009). A recombinant β-galactosidase from *B. infantis* (Hung & Lee, 2002) is an excellent biocatalyst for GOS production giving the highest GOS yield of 63% (mass of GOS of the total sugars in the reaction mixture). β-galactosidases of lactobacilli play an important role in a number of commercial processes, for example, milk processing or cheese making. Recent studies of β-galactosidases, especially with respect to their enzymatic and molecular properties, from *Lactobacillus reuteri* or *Lactobacillus bulgaricus* showed that these enzymes are very well suited for the production of GOS (Splechtna *et al.*, 2006; Nguyen *et al.*, 2012). Maximum GOS yields at 30 °C were *c.* 40% when using purified β-galactosidases from *L. reuteri* with initial lactose concentration of 205 g L^{-1} and at *c.* 80% lactose conversion (Splechtna *et al.*, 2006). Purified β-galactosidase from *L. bulgaricus* gave the highest yield of 50% for the lactobacillal enzymes at 90% lactose conversion (Nguyen *et al.*, 2012). To reduce enzyme costs, a crude β-galactosidase extract from *Lactobacillus* sp. directly obtained after cell disruption and separation of cell debris by centrifugation was used in lactose conversion for GOS production (Splechtna *et al.*, 2007b).

Choice of process technology

The choice of process technology either for lactose hydrolysis or GOS production depends on the nature of the substrate and the characteristics of the enzyme. The primary characteristic, which determines the choice and application of a given enzyme, is the operational pH range. Acid pH enzymes, which are mainly from fungi, are suitable for processing of acid whey and whey permeate, while the neutral pH enzymes from yeasts and bacteria are suitable for processing milk and sweet whey. Depending on the enzyme source, the pH value of the reaction mixture can be acidic when using, for example, the β-galactosidase from *A. oryzae* with an optimum GOS yield at pH 4.5 (Iwasaki *et al.*, 1996). The β-galactosidase from an acidophilic fungus, *Teratosphaeria acidotherma* AIU BGA-1, is stable over the pH range of 1.5–7.0 with optimal activity at pH 2.5–4.0 and 70 °C (Isobe *et al.*, 2013). In contrast, the maximum yield of GOS was observed at neutral pH for most bacterial and fungal β-galactosidases. The highest GOS yields are generally observed when the reaction proceeds to 45–90% lactose conversion (Torres *et al.*, 2010).

Studies of thermostable glycoside hydrolases have been conducted in pursuit of GOS production at high temperatures. These include β-glycosidases from *Alicyclobacillus acidocaldarius*, *Thermus thermophilus* KNOUC202 or *L. bulgaricus*, to name a few (Di Lauro *et al.*, 2008; Nam *et al.*, 2010; Nguyen *et al.*, 2012). Cold-active β-galactosidases have also attracted attention because their applications in the industrial processes of lactose hydrolysis and oligosaccharides synthesis can lower the risk of mesophiles contamination. Cold-active β-galactosidases were isolated from different sources such as *Paracoccus* sp. 32d, *Halorubrum lacusprofundi* and *Thalassospira frigidphilosprofundus* (Wierzbicka-Woś *et al.*, 2011; Karan *et al.*, 2013; Pulicherla *et al.*, 2013). Soluble cold-active β-galactosidase from *Paracoccus* sp. 32d was found to efficiently hydrolyze lactose in milk at 10 °C (Wierzbicka-Woś *et al.*, 2011). There has been relatively little research on GOS synthesis at low temperatures by these psychrophilic enzymes.

Reactor set-up is an important factor that can influence both the yield and the composition of the GOS mixtures formed. Continuous GOS production using a continuous stirred tank reactor (CSTR) with an external cross-flow membrane was compared with the batch-wise mode of conversion using β-galactosidase from *L. reuteri*. Marked differences were detected for the two reactor setups. Above 65% lactose conversion, the GOS yield was lower for the CSTR due to a lower content of tri- and tetrasaccharides in the reaction mixture. In the CSTR, β-gal from *L. reuteri* showed up to twofold higher specificity toward the formation of β-(1→6) linked GOS with β-D-Gal*p*-(1→6)-D-Glc and β-D-Gal*p*-(1→6)-D-Gal being the main GOS components formed under these conditions (Splechtna *et al.*, 2007a). A rotating disk membrane bioreactor was compared over batch mode to obtain purified GOS with high yield. It was found that GOS yield and purity were 32.4% and 77%, respectively, in batch mode followed by diafiltration-assisted nanofiltration, while in the immobilized state, they were 67.4% and 80.2% at 105 rad s^{-1} membrane speed. Retention of the monosaccharides that inhibit the enzyme in the reaction volume of batch mode reduced the yield of GOS. On the contrary, simultaneous production and purification of GOS in the rotating disk membrane bioreactor led to a high yield of GOS (Sen *et al.*, 2012).

Compared with soluble β-galactosidases, immobilized β-galactosidases may provide advantages such as high enzyme reusability, higher cell densities in bioreactors, improved enzyme stability, reutilization and continuous operation, and easier separation of the products (Verma *et al.*, 2012). Higher activity but lower thermostability was reported for β-galactosidase immobilized on chitosan nanoparticles than that bound onto macroparticles (Klein *et al.*, 2012). β-galactosidase immobilized in polyvinyl alcohol lenses was more stable and converted more lactose than when immobilized in solgel carriers (Jovanovic-Malinovska *et al.*, 2012). Compared with the corresponding free enzyme systems, immobilization resulted in less product inhibition by glucose and a higher stability at denaturing temperatures (Klein *et al.*, 2012; Verma *et al.*, 2012).

Prediction of GOS production by modeling techniques

Several kinetic mechanisms, either mechanistic, empirical or a combination of both, have been proposed to account for the transgalactosylation reactions and to subsequently define strategies to optimize GOS production. A six-parameter model was developed to describe oligosaccharide production from lactose hydrolysis by β-galactosidase from *B. circulans*; the model considered glucose inhibition, but ignored the formation of tetra- and higher oligosaccharides (Boon *et al.*, 2000). A model of *K. lactis* β-galactosidase describing both hydrolysis and transgalactosylation reactions with glucose and lactose as acceptors fitted well to the experimental data of the time course reactions at various concentrations of lactose (Kim *et al.*, 2004). A pseudo steady-state model for the kinetically controlled synthesis of GOS with *A. oryzae* β-galactosidase was presented by Vera *et al.* (2011). This model predicted substrate and product profiles during GOS synthesis in the temperature range between 40 and 55 °C, providing a useful tool for process scale-up and optimization. The model accounts for the total GOS production and its composition, which is a definite advantage over previously existing models. However, the model tends to underestimate disaccharides consumption and penta-GOS (GOS-5) formation and to overestimate glucose production.

Leveraging the power of protein engineering for GOS production

Protein engineering is a powerful approach to favor transgalactosylation over hydrolysis and hence to improve transgalactosylation yields. A truncated β-galactosidase from *B. bifidum* enhanced the transgalactosylation activity of the enzyme toward lactose, and as a result, a normal, hydrolytic β-galactosidase was converted to a highly efficient transgalactosylating enzyme (Jørgensen *et al.*, 2001). A mutagenesis approach was applied to the galactosidase BgaB of *Geobacillus stearothermophilus* KVE39 to improve its enzymatic transglycosylation of lactose into oligosaccharides. Exchange of one single amino acid, arginine Arg109, in β-galactosidase BgaB to either lysine, valine or tryptophan improved significantly the formation of the main trisaccharide, that is, 3′-galactosyl-lactose. The yield of this trisaccharide increased from 2% to 12%, 21% and 23%, respectively, for these different variants compared with that of the native enzyme (Placier *et al.*, 2009). Enhancement of the production of GOS was also achieved by mutagenesis of *Sulfolobus solfataricus* β-galactosidase, LacS. The GOS yield obtained from two mutants of LacS, F441Y and F359Q, was increased by 10.8% and 7.4%, respectively (Wu *et al.*, 2013). Although protein engineering strategies were successfully applied to enhance transgalactosylation activities of different β-galactosidases, this approach has not yet been reported to alter the linkage type of the GOS products as well.

Biosynthesis of oligosaccharides structurally related to those found in human milk

Mature human milk contains *c.* 7–12 g L^{-1} of free oligosaccharides in addition to lactose, which typically is present in concentrations of 55–70 g L^{-1} (Wu *et al.*, 2011). Currently, up to 200 unique oligosaccharide structures varying from 3 to 22 sugar units have been identified (Kobata, 2010). HMO are composed of the five monosaccharide building blocks D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc) and sialic acid (*N*-acetylneuraminic acid). They can be grouped into neutral and charged oligosaccharides, the latter being sialylated and comprising *c.* 20% of all HMO (Wu *et al.*, 2011). The structures of HMO show typical patterns. Lactose (Gal-β-1,4-Glc) is found at the reducing end of HMO. This terminal lactose is typically elongated by lacto-*N*-biose units (LNB; Gal-β-1,3-GlcNAc) in type I or *N*-acetyl-lactosamine units (LacNAc; Gal-β-1,4-GlcNAc) in the rarer type II structures. Both LNB and LacNAc are attached via a β-1,3-linkage to the galactosyl moiety of the terminal lactose, with an additional β-1,6-linkage in branched HMO.

These LNB and LacNAc units can be repeated up to 25 times in larger HMO, forming the core region of these oligosaccharides. A further variation results from the attachment of fucosyl and sialic acid residues (Fig. 2). Thus, the simplest structures following this general scheme (apart from certain trisaccharides such as galactosyl-lactose, fucosyl-lactose and sialyl-lactose) are the tetrasaccharides lacto-*N*-tetraose, Gal-β-1,3-GlcNAc-β-1,3-Gal-β-1,4-Glc (type I), and lacto-*N*-*neo*-tetraose, Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc (type II; Fig. 3). Urashima listed an HMO classification based on their 13 core structures; of which, the most abundant components are 2′-fucosyllactose (Fuc-α-1,2-Gal-β-1,4-Glc), lacto-*N*-tetraose (Gal-β-1,3-GlcNAc-β-1,3-Gal-β-1,4-Glc) and lacto-*N*-fucopentaose (Fuc-α-1,2-Gal-β-1,3-GlcNac-β-1,3-Gal-β-1,4-Glc) (Urashima *et al.*, 2013). Despite recent modern analytical techniques, HMO identification remains a challenge for researchers.

The composition and content of HMO can vary significantly between different mothers. It varies depending on both their blood group type and the time/length of lactation (Totten *et al.*, 2012). Nevertheless, mono- and difucosyl-lactose, lacto-*N*-tetraose and its fucosylated

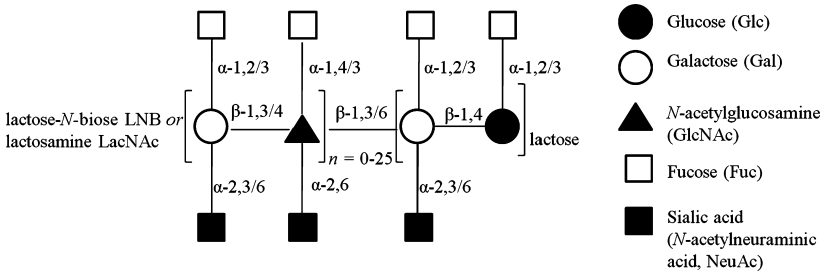


Fig. 2. Schematic structure of HMO.

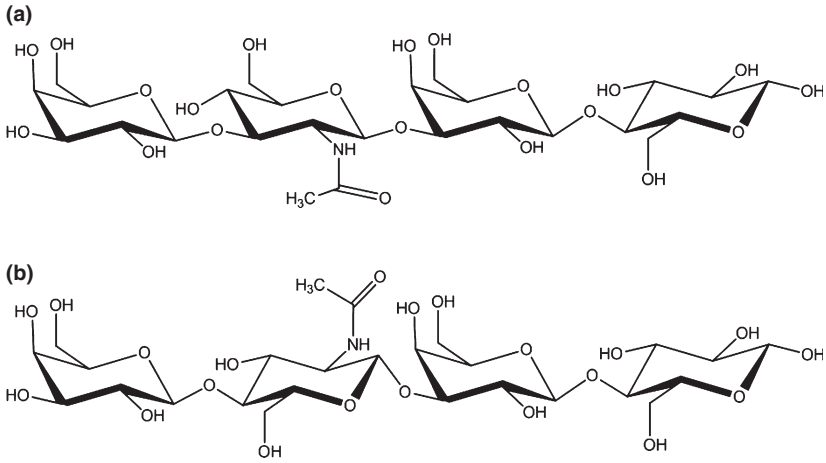


Fig. 3. Some simple structures of HMO: tetrasaccharides lacto-*N*-tetraose, Gal-β-1,3-GlcNAc-β-1,3-Gal-β-1,4-Glc (a), and lacto-*N*-*neo*-tetraose, Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc (b).

derivatives as well as sialyl-lactose and the sialylated forms of lacto-*N*-tetraose are major HMO in human milk. The presence and absence of α-(1,3/4)- and α-(1,2)-fucosylated oligosaccharides indicate the activity of the Lewis (*Le*)- and secretor (*Se*) gene of the mother, with the latter being responsible for the expression of α-(1,2)-fucosyl transferase. Totten *et al.* (2012) described the four possible phenotypes expressed in HMOs and reported that West African populations have higher abundance of Lewis negative and nonsecretors than in European and American populations.

Approaches to biosynthesis of related HMO structures

Considerable interest exists in the efficient production of HMO, which is albeit hampered by the structural complexity as well as the complex mixture of HMO. Many manufacturers are trying to emulate HMO; however, essential ingredients are mostly absent from infant formula due to the lack of industrial production methods. Most often, GOS and/or FOS are added to infant formulas to mimic the effect of HMOs.

The most promising approach toward the production of defined HMO structures seems to be microbial, fermentative methods employing single, appropriately engineered microorganisms. This *in vivo* approach was introduced by the group of Eric Samain. They used a β-galactosidase-negative *Escherichia coli* strain over-expressing a β-1,3-*N*-acetylglucosaminyl-transferase gene from *Neisseria meningitidis*. When feeding lactose to this engineered strain, this disaccharide was taken up by the indigenous β-galactoside permease of *E. coli*. The recombinantly synthesized β-1,3-*N*-acetylglucosaminyl transferase then utilized the intracellular pool of UDP-GlcNAc to transfer GlcNAc residues regiospecifically to lactose, resulting in the formation of the trisaccharides GlcNac-β-1,3-Gal-β-1,4-Glc. This compound was released into the extracellular medium in yields of 6 g L^{-1} (Priem *et al.*, 2002). In a similar manner, an engineered *E. coli* strain, over-expressing an α-1,3-fucosyltransferase from *Helicobacter pylori* and genetically engineered to provide sufficient GDP fucose as the intracellular substrate for the glycosyltransferase, was used to produce various fucosylated HMO from lactose added to the medium (Dumon *et al.*, 2001). An engineered *E. coli* strain over-expressing the α-2,3-sialyltransferase gene from *N. meningitidis*, together with an engineered pathway to provide the activated sialic acid donor CMP-Neu5Ac as the substrate for the glycosyltransferase, produced 3′-sialyl-lactose in concentrations of up to 25 g L^{-1} in high cell density cultivations with continuous lactose feed (Fierfort & Samain, 2008). 6′-sialyl-lactose was efficiently produced when employing α-2,6-sialyltransferase from *Photobacterium* sp.

again in metabolically engineered *E. coli* (Drouillard *et al.*, 2010). In a very recent study, a recombinant *Pasteurella multocida* sialyltransferase exhibiting dual trans-sialidase activities catalyzed trans-sialylation using either 2-*O*-(*p*-nitrophenyl)-α-D-*N*-acetylneuraminic acid or casein glycomacropeptide (whey protein) as the sialyl donor and lactose as the acceptor, resulting in production of both 3′-sialyl-lactose and 6′-sialyl-lactose. The enzyme was capable of catalyzing the synthesis of both 3′- and 6′-sialylated GOS when GOS served as acceptors (Guo *et al.*, 2014). A mutant of the sialidase from the nonpathogenic *Trypanosoma rangeli* expressed in *Pichia pastoris* after codon optimization has been reported to exhibit trans-sialidase activity. The enzyme catalyzed the transfer of sialic acid from cGMP (casein glycomacropeptide) to lactose at high efficiency, giving a yield at the 5 L scale of 3.6 g 3′-sialyl-lactose. The estimated molar trans-sialylation yield was 50% for the 3′-sialyl residues in cGMP without substantial hydrolysis of 3′-sialyl-lactose. Lacto-*N*-tetraose and lacto-*N*-fucopentaoses also functioned as acceptor molecules demonstrating the versatility of this trans-sialidase for catalyzing sialyl-transfer toward different HMO (Michalak *et al.*, 2014).

LNB is a key disaccharide component of HMO such as lacto-*N*-tetraose and lacto-*N*-fucopentaose. LNB can be produced in a purely enzymatic approach, making use of the synthetic capacity of sugar phosphorylases. LNB phosphorylase, together with sucrose phosphorylase, UDP-glucose-hexose-1-phosphate uridylyltransferase and UDP-glucose 4-epimerase produced LNB from sucrose and GlcNAc in the presence of phosphate and catalytic amounts of UDP-Glc in yields of 85% (Nishimoto & Kitaoka, 2007). Recent development to enhance thermostability of galacto-*N*-biose/lacto-*N*-biose phosphorylase by directed evolution yielded a mutant that exhibited 20 °C higher thermostability than the wild type, which is suitable for industrial production of LNB at temperatures higher than 50 °C for faster reaction and prevention of microbial contamination (Koyama *et al.*, 2013).

Galactose-containing HOS

An approach that has received some interest is the synthesis of HOS; of which, some are expected to resemble HMO-like structures, using β-galactosidases. This approach is based on β-galactosidase-catalyzed transglycosylation with lactose as donor (thus transferring galactose onto suitable acceptors) and GlcNAc as acceptor, thus obtaining *N*-acetyl-lactosamine (LacNAc) and its regioisomers. Using this approach and a hyperthermophilic β-galactosidase from *S. solfataricus*, Gal-β-1,6-GlcNAc together with an unidentified sugar were the main products starting from a mixture of 1 M lactose and 1 M GlcNAc, while LacNAc and Gal-β-1,3-GlcNAc were formed as well, yet in lower concentrations (Reuter *et al.*, 1999). This reaction was also optimized for using β-galactosidase from *B. circulans* as the biocatalyst. This enzyme is known for its propensity to synthesize β-1,4-linkages in its transgalactosylation mode, and hence the main reaction product here was LacNAc together with smaller amounts of GlcNAc-containing higher oligosaccharides (one tri- and one tetrasaccharides) and Gal-β-1,6-GlcNAc. The total yield was 40% for these GlcNAc-containing oligosaccharides when starting from 0.5 M lactose and GlcNAc each (Li *et al.*, 2010). Crude cellular extracts of *L. bulgaricus* and *Lactococcus lactis* MG1363 expressing LacLM of *Lactobacillus plantarum* were used as sources of β-galactosidases for the formation of HOS by galactosylation of *N*-acetylglucosamine (GlcNAc) and fucose with the main products identified as Gal-β-(1→4)-GlcNAc, Gal-β-(1→6)-GlcNAc, Gal-β-(1→6)-Gal-β-(1→4)-GlcNAc and Gal-β-(1→6)-Gal-β-(1→6)-GlcNAc (Gänzle, 2012).

Conclusion

HMO yet cannot be commercially produced due to their structural and compositional complexity; however, increased biochemical knowledge on suitable glycosyltransferases may pave the road to microbial, fermentative methods employing single, appropriately engineered microorganisms. The presence of structurally related oligosaccharides together with different complex structures in human breast milk makes GOS attract increasing interests from researchers and manufacturers. The insights into the structures and the production of GOS together with advancement in the area of biotechnology will certainly result in the enhancement of the production of GOS in the future. The use of lactic acid bacteria and *Bifidobacteria* as the sources of β-galactosidases offers substantial potential for the production of GOS and is an interesting approach for the production of new carbohydrate-based functional food ingredients that are of interest in applications such as infant formula. Nowadays, infant formulae are supplemented with GOS to mimic the biological effects of HMO. Some structures of novel galactose-containing HOS resemble the core of HMO, and hence, these novel functionally enhanced, prebiotic oligosaccharides could be of interest for a wide range of applications.

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APPENDIX C

GALACTO-OLIGOSACCHARIDES
RECENT PROGRESS ON RESEARCH AND APPLICATION AS PREBIOTICS

Arreola, S. L.; Intanon, M.; Ngoc, H. P.; Haltrich, D.; Nguyen, T.-H., Galacto-Oligosaccharides: Recent Progress on Research and Application As Prebiotics In *Galactose: Structure and Function in Biology and Medicine*, Pomin, V. H., Ed. Nova Science Publishers Hauppauge NY, 2014. (**In Press**)

Abstract

Prebiotic oligosaccharides have attracted an increasing amount of attention because of their physiological importance and functional effects on human health, as well as their physico-chemical properties, which are of interest for various applications in the food industries. Galacto-oligosaccharides (GOS), one of the major groups of prebiotic oligosaccharides, are formed via the transgalactosylation reaction from lactose. This reaction is catalysed by a number of β-galactosidases (lactases) in addition to their hydrolytic activity. GOS are complex mixtures of different oligosaccharides, and the spectrum of the oligosaccharides making up these mixtures strongly depends on the source of the enzyme used for the biocatalytic reaction as well as on the conversion conditions used in their production. These oligosaccharides are of great interest because of their proven prebiotic (bifidogenic) characteristics. A plethora of GOS is also found in human milk, and these differently substituted oligosaccharides are associated with a number of beneficial effects for the breast-fed infant. This chapter reviews the production, the properties, the biological effects as well as the applications of galacto-oligosaccharides as prebiotics. The chapter also includes emerging trends in the production of novel, galactose-containing hetero-oligosaccharides, which are structurally more closely related to human milk oligosaccharides.

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Introduction

The concept of 'probiotics' with the emphasis on the human host was described as 'a mono- or mixed-culture of live microorganisms which when applied to man or animal affects beneficially the host by improving the properties of the indigenous microflora' [1, 2]. Presently, there is a general acceptance that 'probiotics' refer to viable microorganisms which promote or stimulate beneficially the microbial population of the gastro-intestinal tract (GIT) [3, 4]. It was stated in different reviews that such microorganisms may not necessarily be constant inhabitants of the GIT but they should beneficially affect the health of man and animal [4-7]. Lactic acid bacteria (LAB) and bifidobacteria are the major representatives of probiotic microorganisms [4, 8]. LAB and bifidobacteria have long been used in the production of a wide range of foods without adverse effects on humans [8]. *Bifidobacterium* and *Lactobacillus* species, among LAB, receive special attention in the applications of probiotic products because of their GRAS (generally recognized as safe) status and beneficial effects on human health. Their beneficial roles on the host were summarized by Klaenhammer (1998) [9] including maintenance of the normal microflora, pathogen interference, exclusion and antagonism, immuno-stimulation and immuno-modulation, anti-carcinogenic and anti-mutagenic activities, deconjugation of bile acids, and lactase presentation *in vivo*. A number of *Lactobacillus* and *Bifidobacterium* strains are used for applications in probiotic products and these include for example *L. acidophilus*, *L. amylovorus*, *L. casei*, *L. crispatus*, *L. gasseri*, *L. johnsonii*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *B. bifidum*, *B. longum*, *B. infantis*, *B. breve*, or *B. adolescentis* [8, 10-13].

Since its first introduction [14], the concept of prebiotics has attracted an increasing amount of attention and stimulated both scientific and industrial interests. This concept was later revised [15, 16], and according to an updated definition of the prebiotic concept, 'a dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health' [16]. Based on the criteria [15, 17] (i) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; (ii) fermentation by intestinal microflora; and (iii) selective stimulation of growth and/or activity of intestinal bacteria associated with health/well-being, only inulin/fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and lactulose are fulfilling these requirements for prebiotics as documented and proven in several studies, although promise exists for several other dietary oligosaccharides [15, 17-20].

Prebiotic oligosaccharides can serve as fermentable substrates for certain members of the gut microbiota, and have been found to modulate the colonic flora by selective stimulation of beneficial bacteria such as bifidobacteria and lactobacilli as well as inhibition of 'undesirable' bacteria [4, 21, 22]. Galacto-oligosaccharides (GOS), the products of transgalactosylation reactions catalyzed by β-galactosidases when using lactose as the substrate, are non-digestible carbohydrates meeting the criteria of 'prebiotics'. GOS are of special interest to human nutrition because of the presence of structurally related oligosaccharides together with different complex structures in human breast milk [23-25].

Production of galacto-oligosaccharides

β-Galactosidases and transgalactosylation of lactose

Production of GOS (or sometimes referred to as TOS, transgalactosylated oligosaccharides) typically employs lactose as galactosyl donor and the transfer of the galactosyl moiety of lactose to suitable acceptor carbohydrates or nucleophiles using either glycoside hydrolases (EC 3.2.1.) or glycosyltransferases (EC 2.4.) [26-28]. Glycosyltransferases catalyze glycosidic bond formation employing sugar donors containing a nucleoside phosphate or a lipid phosphate leaving group, and are quite efficient as well as regio-and stereo-selective compared to glycoside hydrolases. However, due to limited supply, high price and necessity of specific sugar nucleotide as substrate of glycoside hydrolases, industrial GOS production favours the use of glycoside hydrolases [26].

Glycoside hydrolases (GH) are classified based on the stereochemical outcome of the hydrolysis reaction; they can be either *retaining* or *inverting* enzymes. Amino acid sequence similarities, hydrophobic cluster analysis, reaction mechanisms and the conservation of catalytic residues allow classification of β-galactosidases (β-gal; β-D-galactoside galactohydrolase E.C.3.2.1.23; lactase) in the GH families GH1, GH2, GH35, and GH42, indicating their structural diversity [28]. GH1 β-glycosidases are retaining enzymes of which the most commonly known enzymatic activities are myrosinases (thio-β-glucosidases), β-mannosidases, β-galactosidases, phospho-β-glucosidases and phospho-β-galactosidases. GH1 β-galactosidases are predominant in the plant kingdom and have been explored for their 3D conformation, active site residues, and mechanism of action with few reports from Archaea and recently also from the Eubacteria *Meiothermus ruber* DSM1279 [29]. The GH2 family, to which

most of the β-galactosidases belong, comprises the LacZ and LacLM β-galactosidases as isolated and described from *E. coli*, lactic acid bacteria and bifidobacteria. GH1 and GH2 β-galactosidases use only lactose, β-(1→3) and β-(1→6) linked galactosides as their substrates while those belonging to families GH35 and GH42 act on different galactose-containing glycosides including higher oligosaccharides and polysaccharides [28]. Owing to the different substrate specificities, β-galactosidases of GH2 and GH42 are often found in the same organism [30-32].

β-Galactosidases catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides (such as lactose) [33-35] and are found widespread in nature. They catalyze the cleavage of lactose (or related compounds) in their hydrolysis mode, and are thus used in the dairy industry to remove lactose from various products. An attractive biocatalytic application is found in the transgalactosylation potential of these enzymes, which is based on their catalytic mechanism [33, 36]. Retaining β-galactosidases undergo a two-step mechanism of catalysis. First, this mechanism involves the formation of a covalently linked galactosyl-enzyme intermediate. Subsequently, the galactosyl moiety linked to the nucleophile is transferred to a nucleophilic acceptor. Water, as well as all sugar species present in the reaction mixture, can serve as a galactosyl acceptor. Hence, the resulting final mixture contains hydrolysis products of lactose, which are glucose and galactose, unconverted lactose as well as di-, tri- and higher oligosaccharides [37, 38]. Scheme 1 illustrates the possible lactose conversion reactions catalysed by β-galactosidases, and structures of some galacto-oligosaccharides are given in Figure 1.

Transgalactosylation is described to involve intermolecular as well as intramolecular reactions. Intramolecular or direct galactosyl transfer to D-glucose yields regio-isomers of lactose. The glycosidic bond of lactose (β-D-Gal*p*-(1→4)-D-Glc) is cleaved and immediately formed again at a different position of the glucose molecule before it diffuses out of the active site. This is how allolactose (β-D-Gal*p*-(1→6)-D-Glc), the presumed natural inducer of β-galactosidases in certain microorganisms, can be formed even in the absence of significant amounts of free D-glucose [37, 39]. By intermolecular transgalactosylation, various di-, tri-, tetrasaccharides and eventually higher oligosaccharides are produced. Any sugar molecule in the reaction mixture can be the nucleophile accepting the galactosyl moiety from the galactosyl-enzyme complex, which is formed as an intermediate in the reaction. The GOS produced are not the product of an equilibrium reaction, but must be regarded as kinetic intermediates as they are also substrates for hydrolysis, and hence transgalactosylation reactions are kinetically controlled [39, 40]. For these reasons GOS yield and composition change dramatically with reaction time, and the GOS mixtures thus obtained are very complex and can hardly be predicted.

Enzyme sources

β-Galactosidases can be obtained from different sources including microorganisms, plants and animals. Microbial sources of β-galactosidase are of great biotechnological interest because of easier handling, higher multiplication rates, and production yield. Table 1 presents some of the commercially available bacterial, fungal and yeast β-galactosidases. Recently, a number of studies have focused on the use of the genera *Bifidobacterium* and *Lactobacillus* for the production and characterisation of β-galactosidases, including the enzymes from *L. reuteri, L. acidophilus, L. plantarum,*

L. sakei, L. pentosus, L. bulgaricus, L. fermentum, L. crispatus, B. infantis, B. bifidum, B. angulatum, B. adolescentis, and B. pseudolongum and B. breve [41-55]. Bifidobacteria and lactobacilli have been studied intensively with respect to their enzymes for various different reasons, one of which is their 'generally recognized as safe' (GRAS) status and their safe use in food applications. It is anticipated that GOS produced by these β-galactosidases will have better selectivity for growth and metabolic activity of these bacterial genera in the gut, and thus will lead to improved prebiotic effects [56]. An extensive list of bacterial and fungal sources of β-galactosidases, as well as the lactose conversion reaction conditions and GOS yields, are given in [57].

Studies of thermostable glycosyl hydrolases have been conducted in pursuit of GOS production at high temperatures. These include β-glycosidases from *Pyrococcus furiosus* (F426Y), *Thermotoga maritima, Penicillum simplicissimum, Saccharopolyspora rectivirgula, Aspergillus niger, Bifidobacterium bifidum, Bacillus stearothermophilus, Alicyclobacillus acidocaldarius, Thermus aquaticus* YT-1, *Thermus thermophilus* KNOUC202, and *L. bulgaricus,* to name a few [47, 58-63]. The GOS yields as influenced by transgalactosylation of lactose at different temperatures were given in recent reviews [57, 64]. Cold-active β-galactosidases have also attracted attention because their applications in the industrial processes of lactose hydrolysis and oligosaccharides synthesis can lower the risk of mesophiles contamination. Cold-active β-galactosidases were isolated from *P. haloplanktis* TAE 79, *Planococcus* sp., *Arthrobacter psychrolactophilus, Arthrobacter* sp. 32c, *Alkalilactibacillus ikkense, Paracoccus* sp. 32d, *Halorubrum lacusprofundi,* and *Thalassospira frigidphilosprofundus* [65-72] and have been reported for their

potential use to hydrolyze lactose in dairy products processed at low temperatures. Soluble cold-active β-galactosidases from *Paracoccus* sp. 32d and *Lactococcus lactis* IL 1403 were found to efficiently hydrolyse lactose in milk at 10°C [71, 73].

It is a well-known fact that β-galactosidases from different species possess very different specificities for building glycosidic linkages, and therefore produce different GOS mixtures. For example, the β-galactosidase from *Kluyveromyces lactis* produced predominantly β-(1→6)-linked GOS [74], the β-galactosidase from *Aspergillus oryzae* produced mainly β-(1→3) and β-(1→6) linkages in their GOS [75], *Bacillus circulans* β-galactosidase forms β-(1→2), β-(1→3), β-(1→4), β-(1→6) linked GOS [76], whereas β-galactosidases from *Lactobacillus* spp. showed preference to form β-(1→3) and β-(1→6) linkages in transgalactosylation mode [39, 43, 45, 47].

Microbial production of GOS

GOS are produced from lactose by microbial β-galactosidases employing different enzyme sources and preparations including crude enzymes, purified enzymes, recombinant enzymes, immobilized enzymes, whole-cell biotransformations, toluene-treated cells, and immobilized cells. The enzyme sources, the process parameters as well as the yield and the productivity of these processes for GOS production are summarised in detail in recent reviews [25, 57, 64, 77]. The highest GOS productivity, 106 g L^{-1} h^{-1}, was observed when β-galactosidase from *Aspergillus oryzae* immobilized on cotton cloth was used for GOS production in a packed-bed reactor [78].

The choice of process technology either for lactose hydrolysis or GOS production depends on the nature of the substrate and the characteristics of the enzyme. The primary characteristic, which determines the choice and application of a given enzyme, is the operational pH range. Acid-pH enzymes, which are mainly from fungi, are suitable for processing of acid whey and whey permeate, while the neutral-pH enzymes from yeasts and bacteria are suitable for processing milk and sweet whey. Depending on the enzyme source, the pH value of the reaction mixture can be very acidic when using β-galactosidases from *A. oryzae* and *Bullera singularis* with optimum GOS yields at pH 4.5 and 3.7, respectively [79, 80]. Isobe and others studied the β-galactosidase from an acidophilic fungus, *Teratosphaeria acidotherma* AIU BGA-1, which was stable over the pH range of 1.5 to 7.0 and exhibited optimal activity at pH 2.5-4.0 and 70°C [81]. The maximum yield of GOS was observed at neutral pH for most bacteria and fungi though [44]. The time required to get maximum GOS depends inversely on the amount of enzyme. The highest GOS yields are generally observed when the reaction proceeds to 45 - 90% lactose conversion [57].

Despite of the importance of LAB, and in particular *Lactobacillus* spp. and bifidobacteria, for food technology and dairy applications, and despite numerous studies on the gene clusters involved in lactose utilization by these bacteria, β-galactosidases from *Lactobacillus* and *Bifidobacterium* spp. have been characterized in detail pertaining to their biochemical properties or investigated for their ability to produce GOS in biocatalytic processes only recently. Previous studies reported the presence of multiple β-galactosidases in *B. infantis*, *B. adolescentis, B. bifidum*, as well as *B. breve* [51, 52, 55, 82-86], and revealed that these enzymes are very

different with respect to substrate specificity and regulation of gene expression. Furthermore, these reports described the cloning and characterization of these enzymes and studied their transgalactosylation activity in detail, for example β-galactosidase BgbII from *B. adolescentis* showed high preference towards the formation of β-(1→4) linkages while no β-(1→6) linkages were formed [85]. In contrast, the β-galactosidase BgbII from *B. bifidum* showed a clear preference for the synthesis of β-(1→6) linkages over β-(1→4) linkages [32]. A recombinant β-galactosidase from *B. infantis* was found to be an excellent biocatalyst for GOS production giving the highest GOS yield of 63% (mass of GOS of the total sugars in the reaction mixture) [51].

β-Galactosidases of lactobacilli play an important role in a number of commercial processes, e.g., milk processing or cheese making [87, 88]. Recent studies of β-galactosidases, especially with respect to their enzymatic and molecular properties, from *L. reuteri*, *L. acidophilus*, *L. plantarum*, *L. sakei*, *L. pentosus*, and *L. bulgaricus* [39, 41-43, 45-47, 89, 90] revealed that these enzymes were found to be very well suited for the production of galacto-oligosaccharides. Maximum GOS yields at 30°C were 38% when using purified β-galactosidases from *L. reuteri* and *L. acidophilus* with initial lactose concentration of 205 g/L and at ~80% lactose conversion [39, 42]. When using purified β-galactosidases from *L. plantarum* and *L. sakei*, the yields at 30°C were 41% with similar initial lactose concentrations and at 77% and 85% lactose conversion, respectively [43, 45]. Purified β-galactosidase from *L. bulgaricus* gave the highest yield of 50% at 90% lactose conversion [47], and on the other hand, purified β-galactosidase from *L. pentosus* gave the lowest yields, 31%, compared to

the above-mentioned β-galactosidases from lactobacilli [46]. In order to reduce enzyme costs by avoiding laborious and expensive chromatographic steps for the purification of the biocatalyst, crude β-galactosidase extract from *Lactobacillus* sp. directly obtained after cell disruption and separation of cell debris by centrifugation was used in lactose conversion for GOS production [90]. It was reported that there was no obvious difference in the obtained GOS yields using either purified or crude β-galactosidase at 37°C, and in addition the crude enzyme was found to be equally stable as the purified one. Therefore, crude β-galactosidase extracts are suitable for a convenient and simple process of GOS production. Because of the GRAS status of most *Lactobacillus* spp. it is also safe to use these crude extracts in food and feed applications. The reduction of reaction temperature to 17°C to limit microbial growth and the use of a cheap lactose source such as whey permeate powder did not have significant adverse effects on the GOS yield [90].

Protein engineering is a powerful approach to favour transgalactosylation over hydrolysis, and hence to improve transgalactosylation yields. A truncated β-galactosidase from *B. bifidum* enhanced the transgalactosylation activity of the enzyme towards lactose and as a result a normal, hydrolytic β-galactosidase was converted to a highly efficient transgalactosylating enzyme [91]. A mutagenesis approach was applied to the galactosidase BgaB of *Geobacillus stearothermophilus* KVE39 in order to improve its enzymatic transglycosylation of lactose into oligosaccharides. Exchange of one amino acid, arginine Arg109, in β-galactosidase BgaB to either lysine, valine or tryptophan improved significantly the formation of the main trisaccharide, i.e. 3'-galactosyl lactose. The yield of this trisaccharide increased from 2% to 12%, 21% and 23%, respectively, for these different variants

compared to that of the native enzyme [92]. Enhancement of the production of GOS was achieved by mutagenesis of *Sulfolobus solfataricus* β-galactosidase LacS. Site-directed mutagenesis was performed to obtain two mutants of LacS, F441Y and F359Q, and the GOS yield was increased by 10.8 and 7.4%, respectively [93]. Although protein engineering strategies were successful to enhance transgalactosylation activities of different β-galactosidases it has not been described for β-galactosidases yet that this approach was successful to alter also the linkage type of the GOS products [94].

Manufacturers of GOS

GOS are manufactured and commercialized mainly in Japan, the United States and Europe. The major manufacturers are Yakult Honsha (Japan) with their product Oligomate, Nissin Sugar Manufacturing (Japan) with Cup-Oligo, Snow Brand Milk Products (Japan) with P7L, GTC Nutrition (United States) with Purimune, Friesland Foods Domo (the Netherlands) with Vivinal GOS, Clasado Ltd. (UK) with Bimuno, and Dairy Food Ingredients (Ireland) with Dairygold GOS [23, 25, 57, 95-97]. Commercial GOS preparations typically are transparent syrups or white powders containing oligosaccharides of different DP, non-converted lactose and the monosaccharides glucose and galactose. They differ in purity of the GOS products and also in the linkages of the oligosaccharide chains, which depend on the enzymes used for the GOS productions. Oligomate contains mainly β-(1→6) linked GOS, Vivinal GOS, Cup-Oligo and Purimune contain mainly β-(1→4) linkages, whilst Bimuno contains mainly β-(1→3) linked GOS [57]. Table 2 presents some commercial GOS and the enzymes used in their productions.

Properties and biological effects of galacto-oligosaccharides

Properties of GOS

The physico-chemical properties of galacto-oligosaccharides are of significant interest for their application in the food industries. Generally, GOS are transparent/colourless water-soluble products (80% w/w solubility), and more viscous than high-fructose corn syrup [22, 57]. GOS are stable during treatment at elevated temperature of up to 160°C and as low as pH 2. They are also stable during long-term storage at room temperature under acidic conditions [38]. The caloric value of GOS was estimated to be 1.7 kcal g^{-1}, this is approximately 30-50% of those of digestible carbohydrates such as sucrose [38]. GOS are low-calorie sweeteners since they pass through the human small intestine without being digested. GOS are undigested by pancreatic enzymes and gastric juice while passing the small intestine, which makes them suitable for low-calorie diets and for consumption by individuals with diabetes [57, 97]. They can be used as humectants because of their high moisture-retaining capacity to prevent excessive drying, hence to keep the foodstuff moist. They can alter the freezing temperature of frozen foods and reduce the amount of colouring due to Maillard reactions in heat-processed foods as relatively fewer reducing moieties are available [57, 97, 98]. These properties enable GOS to be applied in a wide variety of food products. Apart from being used as sweeteners, GOS are nowadays incorporated in a wide range of products such as fermented milk products, breads, jams, snack bars, confectionery, beverages, infant milk formulas, and as sugar replacements [22, 23, 38].

Biological effects

Prebiotic galacto-oligosaccharides can serve as fermentable substrates for certain members of the gut microbiota, and have been found to modulate the colonic flora [4, 21, 22]. The physiological importance and health benefits of prebiotic GOS have been reported extensively in several recent reviews on prebiotics and functional oligosaccharides [25, 99, 100]. The biological effects of GOS on human health are discovered in many different dimensions.

Positive impact on the intestinal bifidobacteria and lactobacilli population

Galacto-oligosaccharides, like other prebiotics, are metabolized selectively in the gastrointestinal tract by beneficial bacteria associated with health benefits and well-being. These carbohydrates can thus positively modulate the colonic microbiota, which exerts an important influence on host health [15, 21, 22]. Bifidobacteria and lactobacilli play an important role in the eco-physiology of the colonic microbiota, although their population sizes and species composition vary among different groups of human population. The growth of these bacteria has been linked to beneficial health effects such as increased resistance to infection, stimulation of the immune system activity, protection against cancer, prophylactic and therapeutic benefits. Bifidobacteria are also known to excrete a range of water soluble vitamins such as folate, nicotinic acid, thiamine, pyridoxine and vitamin B12 [22].

Different methods such as pure culture fermentations of single, selected strains [101, 102] and *in vitro* fermentations of mixed bacterial populations, particularly fecal bacteria, have been used as preliminary screening tools for prebiotic activities [103-105] whereas *in vivo* fermentations of non-digestible carbohydrates in animals and

human subjects have been reported for evaluating the prebiotic effects of different oligosaccharide mixtures [15]. Pure culture fermentations are performed in appropriate basal media supplemented with the respective prebiotics, and the increase in cell numbers is quantified by turbidimetry of the cultures or by viable cell count. pH-controlled batch cultures are, however, better models to investigate the interactions between the gut populations in response to certain carbohydrates. Here fermentation is again based on basal media, with the test carbohydrate being the sole fermentable substrate present, but the use of fecal bacterial populations allows for an investigation of the interactions, competition and cross-feeding during growth on the selected substrate. Changes in fecal bacteria concentrations are monitored using molecular techniques such as fluorescent *in situ* hybridization (FISH) or real-time PCR [106, 107]. Alternatively, in vitro colonic models and ^{13}C labelling of substrates can be used to study the prebiotic activity [108]. The ability of galacto-oligosaccharide uptake generally seems to vary within the genus of *Lactobacillus* and *Bifidobacterium*, and hence different growth rates on various oligosaccharides can be observed. In a recent study, growth of single strains of *Bifidobacterium*, *Lactobacillus* and *Streptococcus* on various trisaccharides including 4´-galactosyl-lactose and 6´-galactosyl-lactose was evaluated, and in general these strains grew faster on the trisaccharides with a β-(1→6)-galactosyl moiety [109]. A plethora of GOS is also found in human milk, and these differently substituted oligosaccharides are associated with a number of beneficial effects for the breast-fed infant. Because of this, GOS are incorporated in infant formula to achieve a bifidogenic effect and to imply a "breast-fed-like" flora [110]. A recent study demonstrated the prebiotic attributes of a purified Vivinal GOS formulation in an *in-vitro* colon model. The authors observed an increase in numbers of lactobacilli and bifidobacteria as well other beneficial bacteria,

with a concomitant decrease in numbers of *Bacteroides* species, *Eubacterium halii*, *Prevotella*, and *Lactococcus* [108].

Prebiotic effects of GOS depend significantly on the degree polymerization, the linkage types as well as the composition of the GOS mixtures, and also vary between individuals. These differences are known to be important when it comes to GOS assimilation by beneficial bacteria in the colon. It was reported that the administration of a GOS mixture containing β-(1→3) as well as β-(1→4) and β-(1→6) linkages proved to have a better bifidogenic effect than a mixture containing GOS with β-(1→4) and β-(1→6) linkages [56]. Furthermore, bifidogenic properties of GOS are dose dependent. It is known that bifidobacteria populations generally increase as the GOS dosage and purity increase. However, it has been shown that even when GOS were administered for many weeks and at high doses, there were still some individuals for which a bifidogenic response did not occur [111].

Protective effect against infections and intoxications

The use of prebiotics to prevent non-antibiotic associated gut diseases is promising. GOS are dietary prebiotic oligosaccharides and increasingly used as new food ingredient, especially in infant formula. Infants are more susceptible to gastrointestinal pathogens than adults. It is well known that a higher number of bifidobacteria in the intestine of breast-fed infants has been associated with a better health compared with formula-fed infants. It was suggested that GOS administration reduced intestinal infections and lowered the incidence of gastroenteritis in healthy infants during the first year of age [112]. GOS are not only effective against enteric infections in infants but also against respiratory infections. A study, in which new-

born infants were fed a mixture of 4 probiotic bacterial strains along with galacto-oligosaccharides, revealed no effect on the incidence of any allergic diseases and it seemed to increase resistance to respiratory infections during the first two years of life [113]. A similar result was found in another study of preterm infants in Finland, which concluded that early supplementation of prebiotics (GOS and polydextrose mixture) or probiotics reduce the risk of virus-associated respiratory tract infections during the first year of life [114].

The use of GOS as functional mimics for a cell-surface toxin receptor is continuing to be developed for treatment or prevention of an acute or chronic disease associated with the adhesion or uptake of a cholera toxin (Ctx), which is a significant cause of gastrointestinal disease globally. Ctx antiadhesive activity of GOS was shown in a study [115], in which GOS fractions containing more than 5% hexasaccharides (DP6) exhibited more than 90% binding to the cell-surface toxin receptor (GM1) and its competitive inhibition was dose dependent. Supplementation of the prebiotic GOS in enteral nutrition was also found to significantly improve the intestinal barrier function in secondary infectious complications associated with severe acute pancreatitis rats, which might be partly attributed to an increase in the population of probiotic bifidobacteria, stimulation of the production of sIgA in the intestinal mucus, decrease in the apoptosis of intestinal epithelial cells, and regulation of the expression of the tight junction protein occluding [116].

Protective mechanisms of GOS against enteric infections and intoxications are associated with the potential to inhibit pathogen infections by blocking or competing for bacterial adhesion sites as well as competitive inhibition of adhesion or uptake of

bacterial toxin in intestinal cells. GOS showed the best adherence inhibition of *Escherichia coli* strain E2348/69 on HEp-2 and Caco-2 cells with the depletion in adherence on both HEp-2 and Caco-2 cells by 65 and 70%, respectively, when compares with FOS, inulin, lactulose, and raffinose [117]. A protective mechanism of GOS against other enteric pathogens such as *Salmonella* and *Listeria* was also reported. Purified GOS, derived from a mixture produced by the enzymatic activity of *Bifidobacterium bifidum*, were reported to reduce *Salmonella enterica* serovar Typhimurium adhesion and invasion both *in vitro* and *in vivo* [118]. It was demonstrated that ~2.5 mg GOS mL^{-1} significantly reduced the invasion of *S.* Typhimurium. The presence of GOS also prevented the adherence or invasion of *S.* Typhimurium to enterocytes, and thus reduced its associated pathology. It was also suggested that this protection appeared to correlate with significant reductions in the neutral and acidic mucins detected in goblet cells, possibly as a consequence of stimulating the cells to secrete the mucin into the lumen [118]. In a recent study, it was demonstrated that galacto-oligosaccharides, obtained from transgalactosylation of lactose (GOS-La) or lactulose (GOS-Lu), and their derivatives caseinomacropeptide hydrolysates (hCMP:GOS-La and hCMP:GOS-Lu) significantly reduced adhesion *of Salmonella enterica* CECT 443 and *Listeria monocytogenes* CECT 935. GOS-Lu and hCMP:GOS-Lu also inhibited the production of IL-1β, inflammatory cytokines, by intestinal cells stimulated by the pathogens tested [119].

Immunomodulation for the prevention of allergies and gut inflammatory conditions

Currently, there is increasing interest in the utilization of prebiotics to modulate the immune system and attenuate inflammations in the colon. Most of these data originate from animal models and were obtained in relation to FOS and its prebiotic effect.

Nevertheless, there are some studies that either suggest or prove an effect of GOS on the immune system, suggesting either a direct or indirect modulatory effect [97].

Immunity consists of the innate and adaptive immune system. The innate immune system is the first line of defence for the body and it comprises a physical barrier such as the skin, phagocytic, inflammatory, dendritic and natural killer (NK) cell, as well as other soluble components such as cytokines and complement proteins. The adaptive immune system response occurs after activation of the innate system. It is more antigen-specific and it involves the T- and B-lymphocytes. Lymphocytes play an important role in this component of the immune system, either by modulating the function of other immune cells, or by directly destroying infected cells [22]. Several animal model studies on the immunomodulatory effects of GOS were reported. A study in mice revealed that $CD25^{+}$ regulatory T-cells have an important role in modulated Flu-vaccine responses induced by orally supplied prebiotic oligosaccharides containing GOS [120]. A recent study investigated the effect of GOS on colitis development and on immune variables in *Smad*3-deficient mice treated with the pathogen *Helicobacter hepaticus*. The results showed that GOS significantly reduced colitis severity in response to *H. hepaticus,* and it was suggested that GOS reduces colitis by modulating the function and trafficking of NK cells and may provide a novel therapeutic strategy for individuals with inflammatory bowel disease [121]. It is known that supplementation of non-digestible oligosaccharides during pregnancy has positive effects on hypertension as well as the metabolism, and may be used to ameliorate pregnancy-related metabolic disturbances. The effects of non-digestible oligosaccharides on the immune system during pregnancy of mice supplemented with a specific mixture of short-chain galacto- and long-chain fructo-

oligosaccharides (ratio of 9:1) was found to elicit a more tolerogenic immune reaction in pregnant mice [122].

In human studies, it has been suggested that GOS play a role in the development of the immune system in infants, and may consequently inhibit the onset of allergy. A specific prebiotic mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (ratio of 9:1) has shown to reduce the incidence of atopic dermatitis at 6 months of age in infants at risk for allergy [123]. The synbiotic effect of several probiotic strains and GOS in preventing allergic diseases was demonstrated as well. High-risk pregnant women received probiotics for 2-4 weeks before delivery and their infants received a probiotic formulation with GOS for 6 months. It was shown that probiotics when taken together with GOS showed no effect on the incidence of all allergic diseases in high-risk children from birth to the age of two but significantly prevented eczema and especially atopic eczema [113].

Trophic effects of short-chain fatty acids (SCFAs) on the colonic epithelium

Short-chain fatty acids (SCFA), which are the end products of saccharolytic fermentations in the gut, are of interest as they are claimed to promote human health or may be antagonistic to intestinal competitors [15, 124]. The principal SCFA that result from carbohydrate fermentations are acetate, propionate and butyrate. SCFA stimulate colonic blood flow as well as fluid and electrolyte uptake [125], and inhibit proliferation and induce apoptosis in colon cancer cells [126]. SCFA affect the synthesis of vitamins and essential amino acids, interaction with training of gut-associated lymphoid tissue; and resistance against colonization by enteric pathogens. Moreover, SCFAs affect colonic epithelial cell transport, energy transduction in

colonocytes, growth and cellular differentiation [22, 127]. These trophic properties have important physiological implications, in addition to maintaining the mucosal defence barrier against invading organism. *In vitro* studies involving fecal microbiota are useful models for studying fermentation activity of oligosaccharides. FOS and GOS (at 10 g L^{-1}) were shown to increase acetate and butyrate formation, with transient accumulation of lactate and succinate [128]. It was also demonstrated that a GOS mixture derived from lactose with 6'-galactosyl-lactose and 4'-galactosyl-lactose as the main components exhibited a bifidogenic effect in fecal slurries similar or slightly higher than the commercial Vivinal®-GOS, and enhanced the production of acetic acid and SCFAs [129].

Mineral absorption

Consumption of GOS significantly increases calcium absorption in humans and GOS play a role in increasing bioavailability of calcium, which is a key to bone mass density [22]. Different studies in rats and human trials have shown positive effects of GOS in promoting calcium absorption. In a study on the dose-response effect of GOS supplementation on calcium and magnesium absorption, mineral retention, bone properties and gut microbiota in growing rats, beneficial effects on calcium and magnesium absorption and retention, femur calcium uptake, bone strength, and bone mineral density were observed. These effects either directly or indirectly were attributed to a decrease in cecal pH, an increase in cecal wall and content weight, and an increase in number of bifidobacteria [130]. A similar study reported that a diet containing GOS stimulated calcium absorption and the ingestion of GOS prevents osteopenia in partially gastrectomized rats [131]. Recently, the dose-response relationship of GOS supplementation on calcium absorption in adolescent girls was

investigated. Significant improvement in calcium absorption with both low and high doses of GOS was observed, but it was not a dose-response relationship [132]. These observations may have particular importance because bone mass accretion is maximal during adolescence [133].

Other health benefits of GOS

Other, additional health benefits of GOS such as effects on serum cholesterol and high-density lipoprotein (HDL) cholesterol levels, improving brain functions, maintenance of brain health, and adjunctive treatment of neuropsychiatric disorders were reported. It is believed that prebiotics can decrease serum cholesterol levels and increase HDL cholesterol levels when used in subjects with initial elevated serum cholesterol levels. However, the role of GOS regarding this health effect is still in question since it was demonstrated that 5.5 g of a B-GOS mixture administration to healthy elderly had no effect upon total serum cholesterol and HDL cholesterol levels [134]. In addition, it was shown that no differences in total cholesterol and low-density lipoprotein (LDL) cholesterol levels exist in infants receiving an infant formula supplemented with GOS and long-chain FOS in comparison with infants receiving a control infant formula [135]. More studies are required in order to demonstrate the underlying mechanisms of GOS in this respect.

Recently, evidence was presented that suggests effects of GOS in improving brain function with possible involvement of gut hormones. A study in rat reported that the effect of GOS on components of central N-methyl-D-aspartate receptor (NMDAR) signalling was greater than FOS, and it may reflect the proliferative potency of GOS on microbiota and increased brain derived neurotropic factor (BDNF) expression.

This evidence suggested further investigations on the utility of prebiotics in the maintenance of brain health and adjunctive treatment of neuropsychiatric disorders [136]. In another recent study, it was reported that GOS or GOS-rich prebiotic yogurt could delay the onset of disease and prolong the lifespan in mice. They can attenuate motor neuron loss as well as muscle atrophy and dysfunction. Furthermore, they possess anti-inflammatory and anti-apoptotic effects through the regulation of related molecules. Altogether, GOS may have therapeutic potential for amyotrophic lateral sclerosis (ALS), and prebiotic yogurt may be considered as a nutritional therapy for this intractable disease [137].

Emerging trends in the production of novel hetero-oligosaccharides

Human milk is known as the sole source of nourishment for breast-fed infants and for its promotion of a healthy development of newborns. Human milk is comprised of a complex mixture of oligosaccharides (5-10 g L^{-1} in addition to lactose) that are different in size, linkage and charge [138]. Human milk oligosaccharides (HMO) are a heterogenic group of about 200 molecular species consisting of mostly neutral and fucosylated oligosaccharides [139]. The potential health benefits of HMO have been studied with a special emphasis on prebiotic effects [138]. Recent studies have reported on the ability of HMO to selectively support the growth of specific strains of bifidobacteria thus providing insight on how HMO modulate the infant intestinal microbiota [140-142]. These results suggest that the prebiotic, bifidogenic effects of HMO are structure-specific and may vary depending on the HMO composition in milk [138, 140-142]. In addition, HMO are also considered as a mechanism to protect infants against exogenous infections [140, 143]. *In vitro* studies have shown that HMO bind and block the infection of pathogenic bacteria to animal cells by acting as

receptor analogues to the intestinal cell glycans [140, 144, 145]. Because of these benefits, prebiotic HMO are of great interest for human nutrition.

HMO are composed of both neutral and anionic species with building blocks of 5 monosaccharides: D-glucose, D-galactose, *N*-acetylglucosamine, L-fucose, and *N*-acetylneuraminic acid (sialic acid). The basic structure of HMO includes a lactose core at the reducing end which is elongated by *N*-acetyl-lactosamine units with at least 12 different types of glycosidic bonds, wherein fucose and sialic acid residues are added to terminal positions [140, 143]. The terminal lactose is typically elongated by lacto-*N*-biose units (LNB; Gal-β-1,3-GlcNAc) in type I or *N*-acetyl-lactosamine units (LacNAc; Gal-β-1,4-GlcNAc) in the rarer type II structures. Both LNB and LacNAc are attached via a β-1,3-linkage to the galactosyl moiety of the terminal lactose, with an additional β-1,6-linkage in branched HMO. These LNB and LacNAc units can be repeated up to 25 times in larger HMO, forming the core region of these oligosaccharides. A further variation results from the attachment of fucosyl and sialic acid residues. Thus the simplest structures following this general scheme (apart from certain trisaccharides such as galactosyl-lactose, fucosyl-lactose and sialyl-lactose) are the tetrasaccharides lacto-*N*-tetraose, Gal-β-1,3-GlcNAc-β-1,3-Gal-β-1,4-Glc (type I), and lacto-*N*-*neo*-tetraose, Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc (type II) (Figure 2) [146-149]. Mono- and difucosyl-lactose, lacto-*N*-tetraose and its fucosylated derivatives as well as sialyl-lactose and the sialylated forms of lacto-*N*-tetraose are major HMO in human milk.

Some of the above-mentioned HMO structures or structurally related compounds can be accessed through different approaches. One such approach that has received some

interest is based on β-galactosidase-catalysed transglycosylation with lactose as donor (thus transferring galactose onto suitable acceptors) and GlcNAc as acceptor, thus obtaining *N*-acetyl-lactosamine (LacNAc) and its regioisomers. Using this approach and a hyperthermophilic β-galactosidase from *Sulfolobus solfataricus*, Gal-β-1,6-GlcNAc together with an unidentified sugar were the main products starting from a mixture of 1 M lactose and 1 M GlcNAc, while LacNAc and Gal-β-1,3-GlcNAc were formed as well, yet in lower concentrations [150]. This reaction was also optimised for using β-galactosidase from *Bacillus circulans* as the biocatalyst. This enzyme is known for its propensity to synthesize β-1,4-linkages in its transgalactosylation mode, and hence the main reaction product here was LacNAc together with smaller amounts of GlcNAc-containing higher oligosaccharides (one tri- and one tetrasaccharides) and Gal-β-1,6-GlcNAc. The total yield was 40% for these GlcNAc-containing oligosaccharides when starting from 0.5 M lactose and GlcNAc each [151]. This reaction and the β-galactosidase from *B. circulans* were also compared to the enzyme from *Kluyveromyces lactis* and the reaction conditions were optimised.The latter enzyme was shown to forme predominately Gal-β-1,6-GlcNAc. Again, both enzymes formed a mixture of various di- to tetra-saccharides [152]. Since these structures resemble the core of HMO they could be of interest as prebiotic compounds to be added to food.

Conclusion

The understanding of the relationship between certain oligosaccharide structures and prebiotic function already had an impact on novel prebiotic products that were brought to the market, and will have a more important impact in the future. The insights into the structures, production and biological effects of prebiotic galacto-

oligosaccharides together with advancement in the area of biotechnology will certainly result in the enhancement of the production of GOS. The presence of structurally related oligosaccharides together with different complex structures in human breast milk makes GOS attract increasing interests from researchers and manufacturers. Since the structures of GOS or novel hetero-oligosaccharides resemble the core of human milk oligosaccharides they could be of interest as prebiotic compounds to be added to food, and intensified research in this field is needed to open the door for commercial production of these novel functionally enhanced, prebiotic oligosaccharides.

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Figure captions

Scheme 1. Hydrolysis and galactosyl transfer reactions, both intra- and intermolecular, during the conversion of lactose catalysed by β-galactosidases. E, Enzyme; Lac, lactose; Gal, galactose; Glc, glucose; Nu, nucleophile.

Figure 1. Structures of some galacto-oligosaccharides

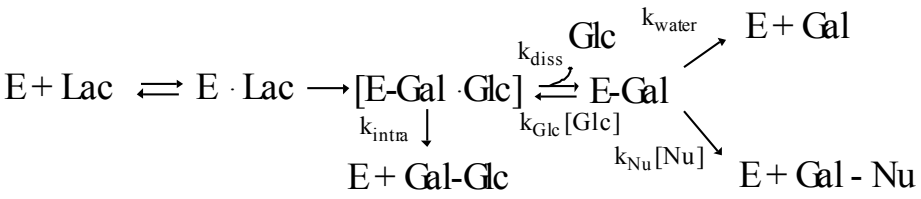
Figure 2. Some simple structures of human milk oligosaccharides: tetrasaccharides lacto-*N*-tetraose, Gal-β-1,3-GlcNAc-β-1,3-Gal-β-1,4-Glc (A), and lacto-*N*-*neo*-tetraose, Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc (B)

Table 1. Commercial β-galactosidases

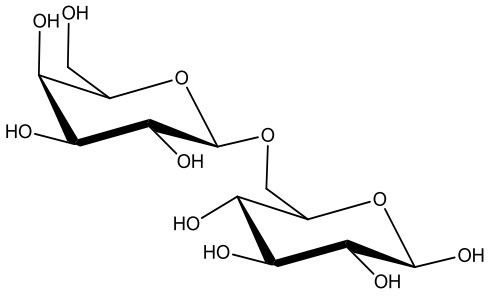
Name	Manufacturer	Microorganism
BioLactase NTL-CONC	Biocon	*Bacillus circulans* [153, 154]
Lactozym pure 6500 L	Novozymes	*Kluyveromyces lactis* [154]
Lactase F "Amano"	Amano Enzyme Inc	*Aspergillus oryzae* [154, 155]
Biolacta FN5	Daiwa Fine Chemicals Co., Ltd.	*Bacillus circulans* [155, 156]
LACTOLES L3	Biocon Ltd., Japan	*Bacillus circulans* [155]
Maxilact	DSM Food Specialties	*Kluyveromyces lactis* [157, 158]
Tolerase	DSM Food Specialties	*Aspergillus oryzae* [157, 158]

Table 2. Commercial galacto-oligosaccharides (GOS) in the market

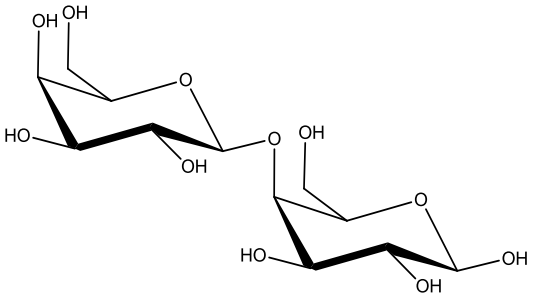
Product	Company	Total GOS (% w/w)	Enzyme Source
Oligomate 55	Yakult Pharmaceutical Industry Co., Ltd. Tokyo, Japan	>55	*Sporobolomyces singularis*, *Kluyveromyces lactis* [159, 160]
Vivinal® GOS	Friesland Foods Domo, Amersfoort, The Netherlands	~60	*Bacillus circulans* [161, 162]
Purimune™	GTC Nutrition, Colorado, USA	≥ 90	*Bacillus circulans* [163, 164]
Bimuno® GOS	Clasado Ltd., Milton Keynes, England	48-55	*Bifidobacterium bifidum* [165, 166]
Cup-oligo	Kowa Company Ltd., Tokyo, Japan	70	*Cryptococcus laurentii* [57, 167]


E + Lac
E · Lac
[E-Gal ·Glc]
E-Gal
Glc
E + Gal
E + Gal-Glc
E + Gal - Nu
k_{water}
k_{diss}
k_{intra}
k_{Glc} [Glc]
k_{Nu} [Nu]

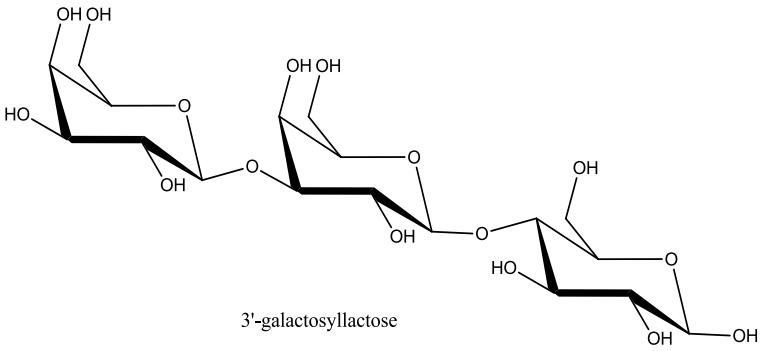
Scheme 1



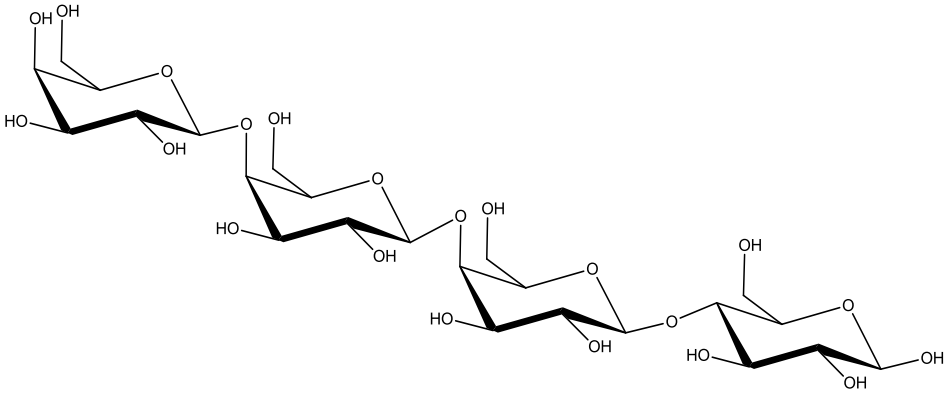
allolactose



4'-galactobiose

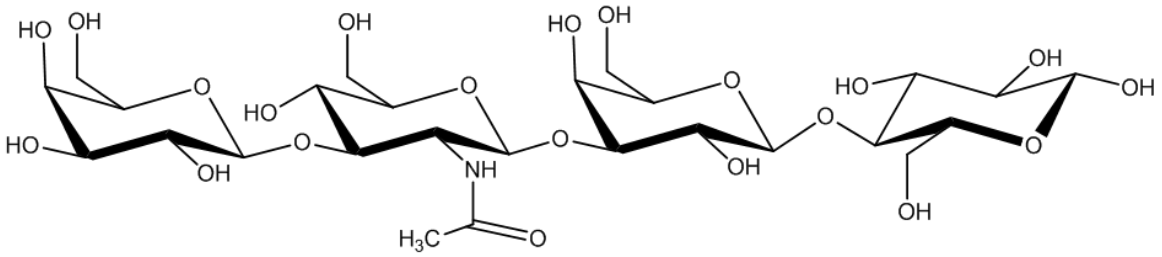


3'-galactosyllactose

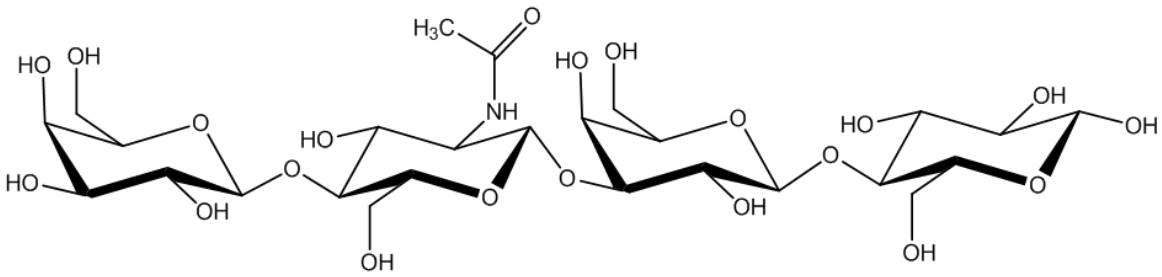


4'-digalactosyllactose

Figure 1.


HO
OH
O
HO
OH
O
OH
HO
O
NH
H3C
O
HO
OH
O
OH
O
HO
OH
OH
O
OH

(A)


H3C
O
HO
OH
O
HO
OH
NH
HO
O
OH
O
HO
OH
O
OH
HO
OH
O
OH

(B)

Figure 2.

APPENDIX D

Two β-galactosidases from the human isolate *Bifidobacterium breve* DSM 20213: Molecular cloning and expression, biochemical characterization and synthesis of galacto-oligosaccharides

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Phone: 43-1-47654 6148. Fax: 43-1-47654 6199.

Abstract

Two β-galactosidases, β-gal I and β-gal II, from *Bifidobacterium breve* DSM 20213, which was isolated from the intestine of an infant, were overexpressed in *Escherichia coli* with co-expression of the chaperones GroEL/GroES, purified to electrophoretic homogeneity and biochemically characterized. Both enzymes β-gal I and β-gal II belong to glycoside hydrolase family 2 and are homodimers with native molecular masses of 220 and 211 kDa, respectively. The optimum pH and temperature for hydrolysis of the two substrates *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) and lactose were determined at pH 7.0 and 50°C for β-gal I, and at pH 6.5 and 55°C for β-gal II, respectively. The k_{cat}/K_m values for *o*NPG and lactose hydrolysis are 722 and 7.4 $mM^{-1}s^{-1}$ for β-gal I, and 543 and 25 $mM^{-1}s^{-1}$ for β-gal II. Both β-gal I and β-gal II are only moderately inhibited by their reaction products D-galactose and D-glucose. Both enzymes were found to be very well suited for the production of galacto-oligosaccharides with total GOS yields of 33% and 44% of total sugars obtained with β-gal I and β-gal II, respectively. The predominant transgalactosylation products are β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→3)-D-Lac, accounting together for more than 75% and 65% of the GOS formed by transgalactosylation by β-gal I and β-gal II, respectively, indicating that both enzymes have a propensity to synthesize β-(1→6) and β-(1→3)-linked GOS. Both enzymes show very low affinity towards β-D-Gal*p*-(1→4)-D-Gal, and this disaccharide was not detected at all during lactose conversions by β-gal I and β-gal II. The resulting GOS mixtures contained relatively high fractions of allolactose, which results from the fact that glucose is a far better acceptor for galactosyl transfer than galactose and lactose, and intramolecular transgalactosylation contributes significantly to the formation of this disaccharide.

INTRODUCTION

The colonic microbiota is composed of more than 400 different species, some of which have been related to health and well-being of the host (1). In practice, the beneficial bacteria that serve as main targets to be increased in number and/or activity by different approaches are bifidobacteria and lactobacilli (2). Members of the genus *Bifidobacterium* are one of the most common organisms found in the human gastro-intestinal tract (3, 4). These species are considered to be important in maintaining human health as they contribute to carbohydrate fermentations in the colon, and their diversity and number provide a marker for the stability of the human intestinal microflora (5). The major *Bifidobacterium* species found in the adult microflora are *Bifidobacterium adolescentis* and *B. longum* while *B. infantis* and *B. breve* are the predominant bifidobacteria in infant intestinal tracts (4, 6).

Prebiotic oligosaccharides can serve as fermentable substrates for certain members of the gut microbiota and have been found to modulate the colonic flora by selective stimulation of beneficial bacteria as well as inhibition of 'undesirable' bacteria (7-9). Galacto-oligosaccharides (GOS), the products of transgalactosylation reactions catalyzed by β-galactosidases when using lactose as the substrate, are non-digestible carbohydrates meeting the criteria of 'prebiotics'. GOS have attracted increasing attention because of the presence of structurally related oligosaccharides together with different complex structures in human breast milk. Therefore the use of GOS in infant formula is nowadays of great interest (10-12).

β-Galactosidases (β-gal; EC 3.2.1.23) catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides (such as lactose) (13-15) and are found widespread in nature. They catalyze the cleavage of lactose (or related compounds) in hydrolysis mode, and are thus used in dairy industry to remove lactose

from various products. An attractive biocatalytic application is found in the transgalactosylation potential of these enzymes, which is based on their catalytic mechanism (13, 16). Microbial β-galactosidases have been isolated and characterized from yeasts, fungi and bacteria (13, 17, 18). The major industrial enzymes are obtained from *Aspergillus* spp. and *Kluyveromyces* spp., where the *Kluyveromyces lactis* enzyme is probably the most widely used (17, 19-22). The use of lactic acid bacteria (LAB) and bifidobacteria as sources of β-galactosidases offers substantial potential for the production of GOS (23). An additional attractive application of β-galactosidases from probiotic bacteria such as lactobacilli or bifidobacteria has been proposed, namely their use in the production of tailor-made prebiotics targeting specifically advantageous and beneficial intestinal microorganisms (24, 25).

Despite the importance of LAB, in particular *Lactobacillus* spp., and bifidobacteria for food technology and dairy applications, and despite numerous studies on the gene clusters involved in lactose utilization by these bacteria, β-galactosidases from *Bifidobacterium* and *Lactobacillus* spp. have only recently been characterized in detail pertaining to their biochemical properties or investigated for their ability to produce GOS in biocatalytic processes (26-41). In this paper, we describe the cloning of two β-galactosidases from *Bifidobacterium breve* DSM 20213, an isolate from the intestines of an infant, and their expression in *Escherichia coli*. Furthermore, biochemical properties of these enzymes and their potential to produce GOS are also presented.

MATERIALS AND METHODS

Chemicals and vectors. All chemicals and enzymes were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise and were of the highest quality available.

The test kit for the determination of D-glucose was obtained from Megazyme (Wicklow, Ireland). All restriction enzymes, T4 DNA ligase, and corresponding buffers were from Fermentas (Vilnius, Lithunia). The plasmid pET-21a (+) was from Novagen (Darmstadt, Germany) and the plasmid pGRO7 encoding the chaperones GroEL and GroES was purchased from TAKARA Bio Inc. (Shiga, Japan). Galacto-oligosaccharide standards of β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→6)-D-Glc, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→4)-D-Gal, β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Lac, β-D-Gal*p*-(1→4)-D-Lac, β-D-Gal*p*-(1→6)-D-Lac were purchased from Carbosynth (Berkshire, UK).

Bacterial strains and culture conditions. *B. breve* DSM 20213, an infant isolate, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain was grown anaerobically at 37°C in MRS medium (42). *Escherichia coli* DH5α (New England Biolabs, Frankfurt am Main, Germany) was used in the transformation experiments involving the subcloning of the DNA fragments. *Escherichia coli* T7 express (Novagen, Darmstadt, Germany) was used as expression host for the vectors carrying the target DNA fragment encoding β-galactosidases.

Construction of β-galactosidase expression vectors. The β-gal I gene (NCBI Reference No. EFE90149.1) and β-gal II gene (NCBI Reference No. EFE88654.1) were amplified using proof-reading Phusion polymerase with the primer pairs 5BbBG1Nde1 (5'-AATACATATGCAAGGAAAGGCGAAAACC-3´), 3BbBG1Not1 (5´-ATAGCGGCCGCGATTAGTTCGAGTGTCACATCC-3´) and 5BbBG2Nde1 (5'-AATACATATGAACACAACCGACGATCAG-3´), 3BbBG2Not1 (5'-ATAGCGGCCGCGATGAGTTCGAGGTTCACGTC-3´), respectively. The forward primers contain *Nde*I and the reverse primers include *Not*I recognition sites

(underlined). The template for the PCR reaction was obtained from cells scratched from an MRS agar plate and suspended in the PCR mix. The initial denaturation step at 98°C for 3 min was follow by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 2 min, followed by a final extension step at 72°C for 5 min. The amplified genes were digested with the corresponding restriction enzymes. Subsequently, the gene fragments were ligated into the pET-21a(+) vector without the natural stop codon and in frame with the C-terminal His_6-tag sequence on the vector, and transformed into *E. coli* DH5α cells. The resulting expression vectors β-gal I and β-gal II were transformed into two different hosts, *E. coli* T7 Express and *E. coli* T7 Express carrying the plasmid pGRO7 (*E. coli* T7 Express GRO), for comparison of the expression levels. The correct nucleotide sequences were confirmed by sequencing (VBC-Biotech, Vienna, Austria). The basis local alignment search tool (BLAST) from the National Center for Biotechnology Information BLAST website was used for database searches. The comparison of β-galactosidases from *B. breve* with homologous proteins was carried out using the program ClustalW2 (version 2.0) (43).

Heterologous expression of β-galactosidases. The expression levels of β-gal I and β-gal II with and without co-expression of the chaperones GroEL and GroES were compared. To this end, all cultures were grown at 37ºC in 250 mL of MagicMedia (Invitrogen Corporation, Carlsbad, CA, USA) until an optical density at OD_{600nm} of 0.6 was reached, and then the cultures were incubated further at 20ºC overnight. The co-expression of the chaperons was induced with 1 mg mL^{-1} L-arabinose. The cells were harvested by centrifugation (6,000 × *g*, 30 min, 4°C), washed twice with 50 mM sodium phosphate buffer, pH 6.5, and disrupted using a French press (AMINCO, Silver Spring, MD). The resulting homogenate was

centrifuged at 25,000 × *g* for 30 min at 4°C to remove the cell debris. The crude extracts were tested for protein concentration and β-galactosidase activity using the standard assay.

Subsequently, the expression of β-gal I and β-gal II was studied further. Different induction conditions were compared by varying the concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) in LB medium. *E. coli* T7 express GRO cells carrying β-gal I and β-gal II plasmids, respectively, were grown at 37˚C in 100 mL of LB medium containing 100 µg mL^{-1} ampicillin and 1 mg mL^{-1} L-arabinose for chaperone induction until an optical density at OD_{600nm} of ~0.8 was reached. IPTG was added to the culture medium in final concentrations of 0.1, 0.5, 1.0 mM, respectively, and the cultures were incubated at 18˚C for 16 h. The cultures were harvested, washed twice and resuspended in 1 mL of 50 mM sodium phosphate buffer, pH 6.5. Cells were disrupted in a bead beating homogenizer using 0.5 g of glass bead (Precellys®24 Technology; PEQLAB, Germany). The crude extracts obtained after centrifugation (16,000 × *g* for 20 min at 4ºC) were tested for β-galactosidase activity using the standard enzyme assay and protein concentrations.

Fermentation and purification of recombinant β-galactosidases. *E. coli* T7 express GRO cells carrying the plasmids β-gal I and β-gal II, respectively, were grown at 37ºC in 1 L LB medium containing 100 µg mL^{-1} ampicillin, 20 µg mL^{-1} chloramphenicol and 1 mg mL^{-1} L-arabinose until OD_{600nm} of 0.8 was reached. IPTG (0.5 mM for β-gal I and 1 mM for β-gal II) was added to the medium and the cultures were incubated further at 18ºC for 16 h. The cultures were then harvested, washed twice with 50 mM sodium phosphate buffer, pH 6.5 and disrupted by using a French press (AMINCO, Silver Spring, MD). Cell debris was removed by centrifugation (25,000 x *g*, 30 min, 4ºC) and the lysate (crude extract) was loaded on a 15 mL Ni-

immobilized metal ion affinity chromatography (IMAC) column (GE Healthcare, Uppsala, Sweden) that was pre-equilibrated with buffer A (20 mM phosphate buffer, 20 mM imidazole, 500 mM NaCl, pH 6.5). The His-tagged protein was eluted at a rate of 1 mL min^{-1} with a 150 mL linear gradient from 0 to 100% buffer B (20 mM sodium phosphate buffer, 500 mM imidazole, 500 mM NaCl, pH 6.5). Active fractions were pooled, desalted and concentrated by ultrafiltration using an Amicon Ultra centrifugal filter unit (Millipore, MA, USA) with a 30 kDa cut-off membrane. Purified enzymes were stored in 50 mM sodium phosphate buffer, pH 6.5 at 4°C for further analysis.

Gel electrophoresis analysis. The purity and the molecular mass of β-galactosidases were determined by SDS-PAGE. The enzymes were diluted to 1 mg protein mL^{-1} and incubated with 2×Laemmli buffer at 90 ºC for 5 min. Protein bands were visualized by staining with Bio-safe Coomassie (Bio-Rad). Unstained Precision plus Protein Standard (Bio-Rad) was used for mass determination.

Size exclusion chromatography - Multi-angle laser light scattering (SEC-MALLS) analysis. Size exclusion chromatography (SEC) was performed with a Superdex S200 10/300 GL column (GE Healthcare) equilibrated in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM β-mercaptoethanol and 5 mM EDTA. Sample separations were performed at room temperature with a flow rate of 0.5 mL min^{-1} with an HPLC pump (Agilent Technologies 1260 infinity). Samples (50 μL) were injected as indicated at a concentration of 2.5 mg mL^{-1}. On-line MALLS detection was performed with a miniDawn Treos detector (Wyatt Technology Corp., Santa Barbara, CA) using a laser emitting at 690 nm. Protein concentration was measured on-line by refractive index measurement using a Shodex RI-101 instrument

(Showa Denko, Munich, Germany). Analysis of the data was performed with the ASTRA software (Wyatt Technology).

β-Galactosidase assays. The measurement of β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) or lactose as the substrates was carried out as previously described (33). When chromogenic *o*NPG was used as the substrate, the reaction was initiated by adding 20 µL of enzyme solution to 480 µL of 22 mM *o*NPG in 50 mM sodium phosphate buffer (pH 6.5) and stopped after 10 min of incubation at 30°C by adding 750 µL of 0.4 M Na_2CO_3. The release of *o*-nitrophenol (*o*NP) was measured by determining the absorbance at 420 nm. One unit of *o*NPG activity was defined as the amount of enzyme releasing 1 µmol of *o*NP per minute under the described conditions.

When lactose was used as the substrate, 20 µL of enzyme solution was added to 480 µL of 600 mM lactose solution in 50 mM sodium phosphate buffer, pH 6.5. After 10 min of incubation at 30°C, the reaction was stopped by heating the reaction mixture at 99°C for 5 min. The reaction mixture was cooled to room temperature, and the release of D-glucose was determined using the test kit from Megazyme. One unit of lactase activity was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions.

Protein determination. Protein concentration was determined by the method of Bradford (44) using bovine serum albumin as the standard.

S**teady-state kinetic measurement.** All steady-state kinetic measurements were obtained at 30°C using *o*NPG and lactose as the substrates in 50 mM sodium phosphate buffer, pH 6.5, with concentrations ranging from 0.5 to 22 mM for *o*NPG and from 1 to 600 mM for lactose, respectively. The inhibition of *o*NPG hydrolysis by D-galactose and D-glucose as well as that of lactose hydrolysis by D-galactose was

investigated as well. The kinetic parameters and inhibition constants were calculated by nonlinear regression, and the observed data were fit to the Henri-Michaelis-Menten equation (SigmaPlot, SPSS Inc., Chicago, IL).

pH and temperature dependency of activity and stability. The pH dependency of the recombinant enzymes was evaluated by standard assay with 22 mM *o*NPG in the pH range of 3–10 using Briton-Robinson buffer (20 mM acetic acid, 20 mM phosphoric acid, and 20 mM boric acid titrated with 1 M NaOH to the desired pH). To evaluate the pH stability of β-gal I and β-gal II, the enzyme samples were incubated at various pH values using Britton-Robinson buffers at 37^{o}C and the remaining activity was measured at time intervals with *o*NPG as substrate. The temperature optima for hydrolytic activity of β-gal I and β-gal II with both substrates lactose and *o*NPG were determined at 20–90°C. The thermostability was evaluated by incubating the pure enzyme in 50 mM sodium phosphate buffer (pH 6.5) at several temperatures (4, 30, 37, 45, 50°C). The residual activities were measured regularly with *o*NPG as substrate.

Differential Scanning Calorimetery (DSC). DSC measurements were performed using a MicroCal VP-DSC System (GE Healthcare) controlled by the VP-viewer program and equipped with a 0.137-mL cell. Studies were made with 1 mg mL^{-1} protein samples in 50 mM phosphate buffer (pH 6.5) Samples were analyzed using a programmed heating scan rate of 60°C h^{-1} in the range of 33–80°C. For baseline correction, a buffer blank was scanned in the second chamber and subtracted. The heat capacity (C_p) was expressed in kcal mol^{-1} K^{-1}. Data analysis was performed with the MicroCal Origin software (GE Healthcare) and experimental data points were fitted to an MN2-State Model.

Substrate specificity. Substrate specificity of the recombinant enzymes was determined using various structurally related chromogenic substrates under standard assay conditions as described for *o*NPG. The chromogenic substrates tested were 2-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-β-D-mannopyranoside, 4-nitrophenyl-α-D-galactopyranoside, and 4-nitrophenyl-α-D-glucopyranoside at substrate concentration of 22 mM.

Substrate affinities of the recombinant enzymes towards some galactosides were also evaluated. An appropriate amount of each enzyme was incubated with ~3 mM of each galactoside (lactose, β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→6)-D-Glc, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→4)-D-Gal, β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Lac, β-D-Gal*p*-(1→4)-D-Lac, β-D-Gal*p*-(1→6)-D-Lac) at 30°C in 50 mM sodium phosphate buffer, pH 6.5. Samples were taken after 30 and 60 min and reactions were stopped by incubation at 95°C for 5 min. The relative activities of the recombinant enzymes towards each galactoside were determined considering the percentage of the hydrolysis (or conversion) of each galactoside under similar reaction conditions.

Galacto-oligosaccharides synthesis and analysis. Discontinuous conversion reactions were carried out to determine the transgalactosylation reaction of the recombinant β-galactosidases from *B. breve*. The influence of process parameters such as temperature (17, 30, 37°C) and enzyme concentration (1, 1.5, 2.5 U mL^{-1}) was also studied. The substrate lactose solution (200 g L^{-1}) was prepared in 50 mM sodium phosphate buffer containing 1 mM Mg^{2+}. Agitation was applied at 300 rpm with a thermomixer (Eppendorf, Hamburg, Germany). Samples were taken at certain time intervals to determine the residual activities and the carbohydrate contents in the reaction mixtures by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

HPAEC−PAD analysis was 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, and Chromeleon version 6.5 (Dionex Corp., Sunnyvale, CA). All eluents were degassed by flushing with helium for 30 min. Separations were performed at room temperature on a CarboPac PA-1 column (4 mm × 250 mm) connected to a CarboPac PA-1 guard column (Dionex) (34). Separation of D-glucose-, D-galactose, lactose and allolactose was carried out with an isocratic run (45 min) with 15 mM NaOH at 1.0 mL min^{-1}, followed by 25 min elution with 100 mM. For separation of other GOS, eluent A (100 mM NaOH) and B (100 mM NaOH and 150 mM NaOAc) were mixed to form the following gradient: 98% A from 0 to 10 min, 98% A to 52% A from 10 to 40 min, and then 52 % A for another 5 min. The column was washed with 20% B for 10 min and re-equilibrated for 15 min with the starting conditions of the employed gradient.

RESULTS

Expression and purification of recombinant β-galactosidases from *B. breve*. The β-gal I and β-gal II genes were cloned into pET-21a (+). The resulting expression vectors were then transformed into *E. coli* T7 express cells and T7 express cells carrying the plasmid pGRO7. The resulting clones were cultivated under inducing conditions in MagicMedia to compare the expression yields with and without chaperone co-expression. β-Gal I and β-gal II expressed in the strains with chaperones showed a 30- and 14-fold increase in activity compared to the activity obtained from the strains without chaperones, respectively (Table 1). When using these conditions, 193 kU per liter of fermentation broth with a specific activity of 159

U mg^{-1} of β-gal I and 36 kU per liter of fermentation broth with a specific activity of 31 U mg^{-1} of β-gal II were obtained.

The expression levels of both enzymes increased even further when gene expression was induced using IPTG. The highest yields were obtained when 0.5 mM IPTG was used for induction with 683 kU per liter of fermentation broth for β-gal I and 169 kU per liter of fermentation broth for β-gal II when 1.0 mM IPTG was used (Table 2), which was an approximately 3.5- and 4.5-fold increase in enzyme yields for β-gal I and β-gal II, respectively, compared to the expressions in MagicMedia.

The enzymes were purified with a single-step purification using an IMAC column, and the results of representative purification procedures for both enzymes are summarized in Table 3. The purified enzymes were obtained with purification factors of approximately 3.2- and 8.5-fold from the crude extracts with an overall yield of approximately 70% and 50% for β-gal I and β-gal II, respectively. The specific activities of the purified enzymes were found to be 461 U mg^{-1} of protein for β-gal I and 196 U mg^{-1} of protein for β-gal II when using the standard *o*NPG assay. The purification procedure yielded a homogenous β-gal I and β-gal II preparation as judged by SDS-PAGE gel (Figure 1).

Gel electrophoresis analysis. Both recombinant β-galactosidases from *B. breve* showed molecular masses of approximately 120 kDa as judged by SDS-PAGE in comparison with reference proteins (Figure 1). Molecular masses of 116,127 and 116,594 Da were calculated for β-gal I and β-gal II, respectively, based on their DNA sequences. Size exclusion chromatography in combination with online multi-angle laser light scattering **(**SEC-MALLS) analysis revealed that the native molecular masses of β-gal I and β-gal II are 220 and 211 kDa, respectively. Therefore, it can be

concluded that both enzymes are dimers and it is likely that they are homodimers consisting of two identical subunits (Table 4).

Kinetic parameters. The steady-state kinetic constants and the inhibition constants of *B. breve* β-galactosidases determined for the hydrolysis of lactose and *o*-nitrophenyl β-D-galactopyranoside (*o*NPG) are summarized in Table 5. The k_{cat} values were calculated on the basis of the theoretical v_{max} values experimentally determined by nonlinear regression and using a molecular mass of 116 kDa for the catalytically active subunit. β-Gal I and β-gal II are not inhibited by their substrates, which are *o*NPG in concentrations of up to 25 mM or lactose in concentrations of up to 600 mM, as it is evident from the Michaelis-Menten plots (not shown). The catalytic efficiencies (k_{cat}/K_m) for the two substrates, lactose and *o*NPG, indicate that the latter is the preferred substrate of both β-gal I and β-gal II.

The end product D-galactose was found to competitively inhibit the hydrolysis of lactose by both enzymes. This inhibition, however, is only moderate as is obvious from the ratio of the Michaelis constant for lactose and the inhibition constant for D-galactose, which were calculated for both enzymes (β-gal I, $K_{i,Gal}/K_{m,Lac}$ = 1.8; β-gal II, $K_{i,Gal}/K_{m,Lac}$ = 3.6). D-Galactose was also found to be a competitive inhibitor against *o*NPG with inhibition constants of 15 mM for β-gal I and 34 mM for β-gal II. Based on the ratio of K_i to K_m this inhibition is even less pronounced (β-gal I, $K_{i,Gal}/K_{m,oNPG}$ = 11.5; β-gal II, $K_{i,Gal}/K_{m,oNPG}$ = 50.7). *o*NPG was also used as the substrate for studying inhibition by the second end product, D-glucose. Again, glucose is a competitive inhibitor of both enzymes, but this inhibiting effect is not pronounced (β-gal I, $K_{i,Glc}/K_{m,oNPG}$ = 92; β-gal II, $K_{i,Glc}/K_{m,oNPG}$ = 55).

Effects of temperature and pH on enzyme activity and stability. Both *o*NPG and lactose were used as substrates to determine the temperature and pH optimum of

β-gal I and β-gal II activity. The pH optimum of β-gal I is pH 7.0 for both *o*NPG and lactose hydrolysis (Figure 2A, B). This enzyme is also most stable at pH 7.0, retaining 60% and approximately 30% of its activity when incubated at pH 7.0 and 37°C for 4 and 10 h, respectively (Figure 3). β-Gal I has a half-life time of activity ($\tau_{1/2}$) of approximately 5 h when incubated at pH 7.0 and 37°C. The pH optimum of β-gal II is pH 6.5 for both *o*NPG and lactose hydrolysis (Figure 2A, B). A profile of pH stability was also determined for β-gal II and the enzyme is most stable at pH 6.0 – 7.0. The residual activities of this enzyme after 10 h of incubation at pH 6.0, 6.5, and 7.0 at 37°C were 72%, 82%, and 83%, respectively (Figure 3). The stability of both enzymes rapidly dropped at pH values below 6.0 or above 8.0. When incubated at pH 5.0 for 10 min, β-gal I showed no residual activity while β-gal II retained only 20% of its activity (data not shown).

The optimum temperature of β-gal I activity was 50°C when using both *o*NPG and lactose as substrates under standard assay conditions. In comparison to β-gal I, β-gal II had higher optima, which were at 55°C for both *o*NPG and lactose as substrates (Figure 2C, D). Both recombinant enzymes showed a half-life time of activity ($\tau_{1/2}$) of approximately 5 months at 4°C when stored in sodium phosphate buffer, pH 6.5. Both enzymes also showed their stability at 30°C with half-life time of activities ($\tau_{1/2}$) of 73 and 109 h for β-gal I and β-gal II, respectively (Table 6A, B).

Thermal stability of both β-galactosidases I and II was significantly improved in the presence of $MgCl_2$. Table 6 (A, B) shows the effect of 1 and 10 mM of $MgCl_2$ on the thermostability of β-gal I and β-gal II at 37°C and higher. In the presence of 1 mM $MgCl_2$ β-gal I showed 19-, 10- and 4-fold increase in its half-life time of activity ($\tau_{1/2}$) at 37°C, 45°C and 50°C, respectively. A further increase of the Mg^{2+} concentration to 10 mM showed to be less effective with respect to stabilization of this enzyme at the

above temperatures. At all conditions tested, β-gal II was found to be more stable than β-gal I. In the presence of 1 mM $MgCl_2$ the half-life time of β-gal II activity ($\tau_{1/2}$) at 50°C was increased to 1.25 h, compared to 0.12 h without Mg^{2+}. A further increase of the Mg^{2+} concentration to 10 mM was more effective in improving thermostability of β-gal II activity.

Thermal denaturation monitored in differential scanning calorimetry (DSC). Calorimetric studies on the thermal denaturation of the two recombinant β-galactosidases were performed using DSC. Both enzymes showed a single endothermic peak in the DSC scan which fitted very well on the basis of a two-state transition model (Figure 4A, B). The observed melting temperatures T_m, 49.97 and 55.58°C for β-gal I and β-gal II in Figure 4A, B, respectively, are in excellent agreement with the optimum temperatures of these two enzymes as shown in Figure 2C, D.

Substrate specificity. The two β-galactosidases from *B. breve* displayed a narrow substrate range when using chromogenic substances. Both enzymes showed 1% activity (relative to *o*NPG) when using 2-nitrophenyl-β-D-glucopyranoside while no activity (<0.05%) was observed when 4-nitrophenyl-β-D-mannopyranoside, 4-nitrophenyl-α-D-galactopyranoside, or 4-nitrophenyl-α-D-glucopyranoside were used as substrates.

Activities of *B. breve* β-galactosidases with individual galactosides are expressed as a percentage of hydrolysis (or conversion) of each substrate after 30 and 60 min (Table 7). It was found that β-gal I shows high hydrolytic activity towards lactose, β-D-Gal*p*-(1→6)-D-Glc (allolactose), β-D-Gal*p*-(1→3)-D-Lac, β-D-Gal*p*-(1→3)-D-Glc and β-D-Gal*p*-(1→3)-D-Gal and hydrolyzes these substrates at comparable rates. β-Gal II also showed high activities with lactose, β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-

Gal*p*-(1→3)-D-Lac but these substrates were hydrolyzed at slightly lower rates than that by β-gal I. β-Gal II hydrolyzes the disaccharides β-D-Gal*p*-(1→3)-D-Glc and β-D-Gal*p*-(1→3)-D-Gal at significantly lower rates than β-gal I. Both enzymes show low activity with the disaccharide β-D-Gal*p*-(1→4)-D-Gal and the trisaccharides β-D-Gal*p*-(1→4)-D-Lac and β-D-Gal*p*-(1→6)-D-Lac, which is evident from the slow hydrolysis rates of these substrates. The disaccharide β-D-Gal*p*-(1→6)-D-Gal was hydrolyzed by both enzymes at the same rate, which is approximately 50% of the rate of lactose hydrolysis in the first 30 min of the reactions.

GOS synthesis. The transgalactosylation activity of *B. breve* β-gal I and β-gal II was investigated. Figure 5 shows GOS formation of a typical discontinuous conversion reaction at 30°C with an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ using 1.5 U_{Lac} mL^{-1} of enzyme. Under these conditions, maximum GOS yields of 33% total sugars after 6 h of reaction at 70% lactose conversion and of 38% total sugars after 22 h of reaction at 96% lactose conversion were obtained with β-gal I and β-gal II, respectively. It also shows that during lactose conversion using β-gal II under these conditions, there is only a slight increase in GOS yield between 85% and 96% lactose conversion. The GOS yield almost reaches its maximum at 36% total sugars after 11 h of reaction at 85 % lactose conversion using β-gal II (Figure 5A, B). The amount of GOS expressed as percentage of total sugars is constantly rising up to ~70% and ~90% lactose conversion using β-gal I and β-gal II, respectively. After these points, at which maximum GOS yields were obtained for both enzymes, the concentration of GOS decreased because they are also subjected to hydrolysis by the β-galactosidases. This is particularly pronounced for β-gal I.

The effect of enzyme concentration in discontinuous conversion reactions on GOS yield was investigated. When the concentration of β-gal I in the conversion reaction was reduced to 1.0 U_{Lac} mL^{-1}, a slight difference on the maximum GOS yield, which was 30% total sugars at 70% lactose conversion, was observed. Interestingly, when the concentration of β-gal II in the conversion reaction was increased to 2.5 U_{Lac} mL^{-1}, maximum GOS yield increased from 38% to 44% of total sugars, which was obtained at 84% lactose conversion, and also the time needed to obtain this maximum GOS yield was reduced to 6 h (data not shown).

Individual GOS can be separated effectively using a Carbopac PA1 column for HPAEC with pulsed amperometric detection as shown in Figure 6A, B. It was possible to identify the main products of transgalactosylation by both β-gal I and β-gal II. These main transferase products formed and degraded at different lactose conversion are presented in Table 8 and Figure 7A, B. The predominant oligosaccharide product was identified as β-D-Gal*p*-(1→6)-D-Glc (allolactose), accounting for approximately 45% and 50% of the GOS formed by transgalactosylation by β-gal I and β-gal II, respectively, at maximum total GOS yield. β-D-Gal*p*-(1→3)-D-Lac was identified as the second predominantly transferase product at the maximum total GOS yield point, contributing approximately 32% and 16% of the total GOS formed by transgalactosylation by β-gal I and β-gal II, respectively. Other identified products, including β-D-Gal*p*-(1→3)-Glc, β-D-Gal*p*-(1→3)-Gal, β-D-Gal*p*-(1→6)-Gal, β-D-Gal*p*-(1→6)-Lac and β-D-Gal*p*-(1→4)-Lac, make up approximately 12% and 20% of total GOS (at total GOS maximum yield point) formed using β-gal I and β-gal II, respectively. 4′-Galactobiose was not detected at all during the course of lactose conversion. It should be noted that the

unidentified peaks 8 and 14 were present in detectable concentrations (Figure 6A, B). However, the structure of these components has yet to be determined.

DISCUSSION

Three β-galactosidases, two of which belonging to glycoside hydrolase family 2 (NCBI Reference No. EFE90149.1, EFE88654.1) and one to glycoside hydrolase family 42 (NCBI Reference No. EFE89025.1), were found in the genome sequence of *Bifidobacterium breve* DSM 20213 (NCBI Reference Sequence: NZ_ACCG00000000.2). Furthermore, one additional putative β-galactosidase (NCBI Reference No. EFE89763.1) was found in this genome sequence. The two β-galactosidases selected for this work, β-gal I and β-gal II, are encoded by the corresponding *lacZ* genes (NCBI Reference No. EFE90149.1; EFE88654.1) and belong to glycoside hydrolase family 2 (GH2 family). β-Galactosidases of the GH2 family generally receive more attention in terms of transgalactosylation activity and GOS formation as enzymes of this family usually show better transgalactosylation properties than enzymes of the GH42 family (45, 46). GH2 β-galactosidases are predominantly found in lactobacilli (33, 35, 38, 39, 47) and it is our interest to study the biochemical and molecular properties of these enzymes from bifidobacteria, an important group of bacteria because of their safe status and beneficial effects on human health.

Bifidobacterium breve DSM 20213 is an isolate from the infant gut. The possibility of rationally targeting prebiotics to specific groups of bacteria such as certain known and approved probiotic strains is a promising prospect. One potential approach to this end is the use of enzymes, such as a β-galactosidase obtained from a probiotic strain, for the synthesis of oligosaccharides (25). β-Galactosidases from

probiotic micro-organisms might produce oligosaccharide structures that have special prebiotic effects, specifically targeting selected probiotic strains (24, 25). Therefore, galacto-oligosaccharides produced by β-galactosidases from *Bifidobacterium breve* DSM 20213 when acting in transgalactosylation mode are expected to selectively promote the growth of certain *Bifidobacterium* spp. in adult and infant human gut.

In this study, two GH2 β-galactosidases of the LacZ type of *B. breve* DSM 20213, β-gal I and β-gal II, were cloned, heterologously expressed in *E. coli* and biochemically characterized. Comparison of amino acid sequences deduced from these two *lacZ* genes revealed 57% of sequence homology. The expression levels of these β-galactosidases in *E. coli* in this present study are significantly higher than those of other recombinant β-galactosidases previously reported, e.g. β-galactosidases from *L. reuteri* L103 (110 kU L^{-1} fermentation broth) (48), *B. licheniformis* DSM 13 (74 kU L^{-1} fermentation broth) (46). The values of 683 and 169 kU L^{-1} obtained in simple shaken flask cultures for β-gal I and β-gal II, respectively, correspond to values of ~1.5 and 0.86 g of recombinant protein produced per L of medium. Furthermore, ~31% and 18% of the total soluble protein in the cellular extracts of *E. coli* overexpressing the genes encoding β-gal I and β-gal II, respectively, can be attributed to the recombinant proteins as judged by the specific activities. Co-expression of the chaperones GroEL/GroES significantly boosted expression levels of both β-galactosidases (Tables 1, 2). An explanation might be an increase of correctly folded β-galactosidases. The co-expression with GroEL/GroES has previously been reported for soluble expression of several proteins (49-51) but this is the first report on the co-expression of β-galactosidases with GroEL/GroES.

Kinetic constants were determined for the two substrates lactose and *o*NPG. The K_m values determined for lactose, 15.3 and 7.5 mM for β-gal I and β-gal II,

respectively, are lower compared to the values reported for other β-galactosidases from *Bifidobacterium* spp. including *B. adolescentis* β-gal II (60 mM) (29), *B. breve* B24 (95.58 mM) (41), *B. bifidum* β-gal I (29.90 mM) and β-gal II (47.13 mM) (32), as well as fungal and yeast β-galactosidases that are commonly employed in technological applications, for example *A. oryzae* (36–180 mM), *A. niger* (54–99 mM), *K. fragilis* (15–52 mM) (52), *K. lactis* (35 mM) (53). These K_m values of *B. breve* *β*-galactosidases compare favorably with the values reported for *β*-galactosidases from *B. bifidum β*-gal III (9.56 mM) (32), *L. reuteri* (13 mM) (33), and *L. crispatus* (14 mM) (54). These relatively low K_m values of the *B. breve* β-galactosidases can be an advantage, e.g. when the complete hydrolysis of lactose is desired.

The inhibition by the end product galactose is moderate as it is evident from the ratio of K_i to K_m calculated for this competitive inhibitor. This ratio of K_i to K_m represents a specificity constant, which determines preferential binding of the substrate lactose versus that of the monosaccharide end products, hence a high value for this ratio is desirable for efficient hydrolysis of lactose. The *B. breve* β-galactosidases display values for the $K_{i,Gal}/K_{m,Lac}$ ratio of 1.8 and 3.6 for β-gal I and β-gal II, respectively, indicating low inhibition, and which compare favorably with the values reported for the $K_{i,Gal}/K_{m,Lac}$ ratio for *L. reuteri*, which is 2.9 (33), and *L. bulgaricus*, which is 3.7 (47). Values for the $K_{i,Gal}/K_{m,Lac}$ ratio reported for example for *B. licheniformis*, *A. oryzae*, *A. niger* and *K. fragilis* are as low as 0.0055, 0.01, 0.006 and 0.84, respectively, indicating severe inhibition by the end product galactose (46, 55). It was only possible to determine $K_{i,Glc}$ for *o*NPG hydrolysis since the lactase assay using lactose as substrate is based on the determination of the released glucose. Values for the $K_{i,Glu}/K_{m,oNPG}$ ratio measured for *o*NPG hydrolysis for β-gal I and β-gal

II are 92 and 55, respectively, indicating that the inhibition by glucose, the second monosaccharide end product, is even less pronounced than the inhibition by galactose.

An attractive biocatalytic application of β-galactosidases is found in the transgalactosylation potential of these enzymes, which is based on their catalytic mechanism. Recombinant GH2 β-galactosidases from the infant isolate *B. breve* are found to be suitable for the production of GOS via transgalactosylation. Highest total GOS yields of 33% and 44% were obtained when β-gal I and β-gal II were used in discontinuous conversion reactions with an initial lactose concentration of 200g L^{-1}. The conversions were performed with this initial lactose concentration based on the solubility of lactose at ambient temperature. An increase in reaction temperature would help to increase the solubility of lactose, however this was not possible since both enzymes lack sufficient stability above 30°C. The maximum GOS yield obtained with β-gal II is comparable to the reported yields obtained with other β-galactosidases from *Bifidobacterium* spp., for example *B. angulatum* (43.8%), *B. bifidum* BB-12 (37.6%), *B. adolescentis* (43.1%) (24), and *B. breve* B24 (42%) (41), however the lactose conversions for GOS synthesis using these β-galactosidases were performed with initial lactose concentration of 30% (w/w). Additionally, Goulas et al. (45) reported a yield of 47% of GOS using BbgIV from *B. bifidum* NCIMB41171 at 40°C and 40% (w/w) initial lactose concentration, while Osman et al. (56) obtained a yield of 55% at 65°C and 43% (w/w) initial lactose concentration using the same enzyme. It was found by many authors that the initial lactose concentration has a significant impact on GOS yields (15, 34, 57). When looking at comparable initial lactose concentrations, a recombinant, engineered β-galactosidase from *B. infantis* was reported to be an excellent biocatalyst for the GOS production giving the total GOS yield of 65% at 37°C (26).

Both enzymes show highest affinities towards lactose, allolactose and β-D-Gal*p*-(1→3)-D-Lac among the substrates tested. It is conceivable that the 'probiotic' β-galactosidases, which rapidly *hydrolyze* certain galacto-oligosaccharide structures, can preferentially *form* these glycosidic linkages as well when acting in transgalactosylation mode, and this is again confirmed in this study. The predominant transgalactosylation products were identified as β-D-Gal*p*-(1→6)-D-Glc (allolactose) and β-D-Gal*p*-(1→3)-D-Lac, together accounting for more than 75% and 65% of the GOS formed by transgalactosylation by β-gal I and β-gal II, respectively. Both enzymes show very low activity towards β-D-Gal*p*-(1→4)-D-Gal, and interestingly, this disaccharide was not detected and hence formed at all during lactose conversion by β-gal I and β-gal II.

Transgalactosylation is described to involve intermolecular as well as intramolecular reactions. Intramolecular or direct galactosyl transfer to D-glucose yields regio-isomers of lactose, and disaccharides are formed right from the beginning of the reaction even when hardly any monosaccharide galactosyl acceptors are available. In this reaction pathway the noncovalently enzyme-bound glucose is not released from the active site but linked immediately to the galactosyl enzyme intermediate. Different transfer rates for different acceptors are to some extent responsible for these phenomena. Figure 8A reveals the ratio between GalGlc disaccharides and GalGal disaccharides at all lactose conversion levels formed during transgalactosylation using β-gal I and β-gal II. This ratio was as high as ≈5 (for β-gal I) or ≈6 (β-gal II) during the initial phase of the reaction (at 20% lactose conversion), at which the concentration of the main hydrolysis products D-Glc and D-Gal, which serves as galactosyl acceptor, are relatively low. This indicates that D-glucose is an excellent galactosyl acceptor, in fact it is a far better acceptor than D-galactose for

galactosyl transfer by both of these two enzymes. Figure 8B shows that D-glucose is also a better galactosyl acceptor than D-lactose when looking at the ratio between GalGlc disaccharides and GalLac trisaccharides. Especially at the beginning of the reaction, this ratio was ≈5 (for β-gal I) or ≈4 (β-gal II) at 20% lactose conversion. This indicates that at least D-Gal*p*-(1→6)-D-Glc is formed by intramolecular transgalactosylation, that is, the D-Gal moiety is transferred onto D-Glc before it can leave the active site of β-galactosidase and another acceptor molecule or water can enter the active site. As β-D-Gal*p*-(1→3)-D-Lac is the second main product during transgalactosylation after D-Gal*p*-(1→6)-D-Glc and when looking at the ratio between GalGal disaccharides and GalLac trisaccharides (figure 8B), it can be concluded that D-lactose is preferred to D-galactose as galactosyl acceptor during intermolecular transgalactosylation.

The recombinant enzymes from *B. breve*, β-gal I and β-gal II, have a propensity to synthesize β-(1→6) and β-(1→3)-linked GOS. Looking at the ratios of β-(1→6) and β-(1→3) linkages at the level of individual sugar species (Figure 9A, B), one can see that both enzymes show preference towards β-(1→6)-bond formation during intramolecular transgalactosylation while β-(1→3) is the more preferred linkage during intermolecular transgalactosylation. β-Galactosidase BgbII from *B. adolescentis* showed high preference towards the formation of β-(1→4) linkages while no β-(1→6) linkages were formed (30). In contrast, the β-galactosidase BgbII from *B. bifidum* showed a clear preference for the synthesis of β-(1→6) linkages over β-(1→4) linkages (45). β-D-Gal*p*-(1→3)-D-Lac was also found to be a major product formed by β-GalI from *B. infantis* (26). Apparently, the formation of certain bonds in transgalactosylation mode of β-galactosidases is a specific property of individual enzymes.

Previous studies reported the presence of multiple β-galactosidases in *B. infantis*, *B. adolescentis,* or *B. bifidum* (26, 28-32, 45), and revealed that these enzymes are very different with respect to substrate specificity and regulation of gene expression. Having isoenzymes with different properties might be advantageous for microorganisms because of higher adaptability to changing growth conditions and each isoenzyme may be responsible for either hydrolysis or synthesis reactions. Understanding the role and function of multiple isoenzymes in bacterial physiology can be supported by investigating the biochemical properties and their activities as well as their specificities towards different substrates. The isoenzymes from *B. breve* DSM 20213, β-gal I and β-gal II, show only slight differences in their pH and temperature optima using both *o*NPG and lactose as substrates, however, β-gal II is generally more stable than β-gal I under all conditions tested. In terms of substrate specificity, both enzymes show high hydrolytic activity with lactose, allolactose, and 3′-galactosyl lactose, in addition, β-gal II shows significantly lower affinities towards the two disaccharides, β-D-(1→3)-galactosyl glucose and β-D-(1→3)-galactobiose compared to β-gal I. However, the reason why *B. breve* DSM 20213 possesses two β-galactosidase isoenzymes with relatively similar substrate preferences and biochemical properties remains indistinctively.

In conclusion, two GH2 β-galactosidases from *B. breve* DSM 20213, β-gal I and β-gal II, were studied in detail regarding their biochemical properties, distribution of oligosaccharides formed, and linkages preferentially synthesized in transgalactosylation mode. Both enzymes were found to be very well suited for the production of galacto-oligosaccharides, components that are of great interest because of their use in functional food. The resulting GOS mixtures contained relatively high fractions of allolactose, which results from the fact that glucose is a far better acceptor

for galactosyl transfer than galactose and lactose, and intramolecular transgalactosylation contributes significantly to the formation of this disaccharide. 3′-Galactosyl lactose was found to be the major trisaccharide in the GOS mixtures. Both enzymes studied form preferentially β-(1→6) and β-(1→3) linkages in transgalactosylation mode. The β-galactosidases from *B. breve* DSM 20213 should be of considerable interest for the production of prebiotic GOS.

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FIGURE CAPTIONS

Figure 1. SDS-PAGE analysis of recombinant *β*-galactosidases from *B. breve* stained with Coomassie blue. Lanes 1 and 4 shows the molecular mass marker (Amersham); lanes 2 and 5 are the crude extracts of β-gal I and β-gal II, lanes 3 and 6 are the purified enzymes of β-gal I and β-gal II.

Figure 2. pH (A and B) and temperature (C and D) optimum of β-galactosidase activity for *B. breve* β-gal I (●) and β-gal II (○) using *o*NPG (A and C) and lactose (B and D) as substrate.

Figure 3. pH stability of the β-galactosidases from *B. breve* β-gal I (●) and β-gal II (○) incubated at 37°C in Britton-Robinson buffer over a pH range of pH 5.0 – 9.0 for 4 h (solid lines) and 10 h (dashed lines). The residual activity was measured after 4 h and 10 h (B) and *o*NPG was used as substrate for the enzyme assay.

Figure 4. Normalized DSC thermograms of *Bifidobacterium breve* (A) β-gal I and (B) β-gal II (1 mg mL^{-1}) in 50 mM sodium phosphate buffer, pH 6.5 with a heating rate of 60°C h^{-1} at temperature range from 33 to 80°C. Fits of experimental data to a two state model are shown with a thinner and smoother line.

Figure 5. Time course of GOS formation (A) and formation and degradation of GOS during lactose conversion (B) catalyzed by *B. breve* β-gal I (●) and β-gal II (○). The reaction was performed at 30°C at an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ using 1.5 U_{Lac} mL^{-1}.

Figure 6. Separation and quantification by HPAEC-PAD of individual GOS produced during lactose conversion catalyzed by (A) *B. breve* β-gal I, (B) *B. breve* β-gal II and. The identified compounds are (1) D-galactose, (2) D-glucose, (3) D-Galp-(1→6)-D-Gal, (4) D-Galp-(1→6)-D-Glc (allolactose), (5) D-Galp-(1→4)-D-Glc (lactose), (6) D-Galp-

(1→3)-D-Gal, (7) D-Galp-(1→6)-Lac, (9) D-Galp-(1→3)-D-Glc, (13) D-Galp-(1→4)-Lac and (15) D-Galp-(1→3)-Lac. Peaks 8, 10-12, 14, and 16-20 were not identified.

Figure 7. Formation and degradation of individual GOS formed by *B. breve* β-gal I (A) and β-gal II (B) during lactose conversion. Reaction conditions: initial lactose concentration of 200 g L^{-1} in 50 mM sodium phosphate buffer (pH 6.5) with 1 mM Mg^{2+} and 30°C and 1.0 U_{Lac} mL^{-1} β-gal I or 2.5 U_{Lac} mL^{-1} β-gal II. Symbols: (●) D-Gal*p*-(1→6)-D-Glc; (■) D-Gal*p*-(1→6)-D-Gal; (▲) Gal*p*-(1→3)-D-Gal; (∆) D-Gal*p*-(1→3)-D-Glc; (□) D-Gal*p*-(1→3)-D-Lac; (○) D-Gal*p*-(1→4)-Lac, (+) D-Gal*p*-(1→6)-D-Lac.

Figure 8. D-Glucose/D-Galactose (solid lines) and GalGlc/GalGal (dashed lines) ratios (A); GalGlc/GalLac (solid lines) and GalGal/GalLac (dashed lines) ratios (B) during lactose conversion by *B. breve* β-gal I (close symbol) and β-gal II (open symbol). The reactions were performed at 30°C at an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$.

Figure 9. Changes in ratios of D-Gal*p*-(1→6)-D-Glc/D-Gal*p*-(1→3)-D-Glc (A) and Gal*p*-(1→3)-D-Gal/D-Gal*p*-(1→6)-D-Gal (solid lines) and D-Gal*p*-(1→3)-D-Lac/D-Gal*p*-(1→6)-D-Lac (dashed lines) during lactose conversion by *B. breve* β-gal I (close symbols) and β-gal II (open symbols). The reactions were performed at 30°C at an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$.

1 **TABLES**

2

Table 1. β-Galactosidase activities in cell-free extracts of recombinant *E. coli* expressing *B. breve* β-gal I or β-gal II with and without coexpression of chaperones[a]

Enzyme	Volumetric activity (kU L^{-1} fermentation broth)[b]		Specific activity (U mg^{-1} protein)		Expression factor[c] (fold)
		with chaperones		with chaperones	
β-gal I	6.4	193.2	1.8	159.0	30.2
β-gal II	2.6	36.5	2.5	31.4	14.0

[a]Values are the mean of two cultivations.

[b]*o*NPG was used to determine enzyme activity.

[c]The expression factors are calculated as the ratios of the volumetric β-galactosidase activities obtained from the expressions with chaperones and without chaperones.

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Table 2. Effect of isopropyl β-D-1-thiogalactopyranoside (IPTG) as inducer on β-galactosidase expression in *E. coli*[a]

Enzyme	IPTG concentration	Volumetic activity (kU L^{-1} fermentation broth)[b]	Protein (mg mL^{-1})	Specific activity (U mg^{-1})
β-gal I	0.1	657.1	7.6	86.5
	0.5	682.7	4.8	142.2
	1.0	567.3	3.5	162.1
β-gal II	0.1	98.0	5.8	16.9
	0.5	65.3	3.6	18.1
	1.0	168.6	4.9	34.4

[a]Values are the mean of two cultivations.

[b]*o*NPG was used to determine enzyme activity.

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Table 3. Purification of recombinant *B. breve* β-galactosidases [a]

Enzyme	Purification step	Total activity (U)[b]	Total protein (mg)	Specific activity (U mg^{-1})	Purification factor	Recovery (%)
β-gal I	crude enzyme	9002	63.0	142.9	1.0	100.0
	IMAC	6274	13.6	461.3	3.2	69.7
β-gal II	crude enzyme	2521	109.0	23.1	1.0	100.0
	IMAC	1257	6.4	196.4	8.5	49.9

[a]Values are the mean of two cultivations.

[b]*o*NPG was used to determine enzyme activity.

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Table 4. Structural properties of recombinant β-gal I and β-gal II from *B. breve*

Enzyme	No. of amino acids	Molar mass (kDa) determined by		
		DNA sequence	SDS-PAGE	SEC-MALLS
β-gal I	1051	116.1	~120	220
β-gal II	1045	116.6	~120	211

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Table 5. Kinetic parameters of two recombinant β-galactosidases (β-gal I and β-gal II) from *B. breve* for the hydrolysis of lactose and *o*-nitrophenyl β-D-galactopyranoside (*o*NPG)

Substrate	Method for determination of enzyme activity	Kinetic parameter	β-gal I	β-gal II
Lactose	release of D-Glc	$v_{max,Lac}$ (μmol min^{-1} mg^{-1})	59 ± 2	97 ± 5
		$K_{m,Lac}$	15.3 ± 3.2	7.5 ± 0.9
		k_{cat} (s^{-1})	114 ± 4	188 ± 10
		k_{cat}/K_m (mM^{-1} s^{-1})	7.4 ± 1.9	25 ± 4
		$K_{i,Gal}$	28 ± 9	27 ± 6
*o*NPG	release of *o*NP	$v_{max,oNP}$ (μmol min^{-1} mg^{-1})	486 ± 9	188 ± 3
		$K_{m,oNP}$	1.3 ± 0.1	0.67 ± 0.07
		k_{cat} (s^{-1})	939 ± 7	364 ± 6
		k_{cat}/K_m (mM^{-1} s^{-1})	722 ± 66	543 ± 65
		$K_{i,Gal}$	15 ± 3	34 ± 5
		$K_{i,Glc}$	120 ± 31	37 ± 4

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Table 6. Stability of *β*-galactosidases from *B. breve* at different temperatures in the absence of $MgCl_2$ as well as in the presence of 1 and 10 mM $MgCl_2$

(A) BbreβgalI

Temperature (°C)	Sodium phosphate buffer, pH 7		Sodium phosphate buffer, pH 7 +1 mM Mg^{2+}		Sodium phosphate buffer, pH 7 + 10 mM Mg^{2+}	
	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)
30	$1.00 (\pm 0.00) \times 10^{-2}$	73	$2.00 (\pm 0.01) \times 10^{-3}$	428	$3.00 (\pm 0.00) \times 10^{-3}$	235
37	0.32 ± 0.03	2	$2.00 (\pm 0.00) \times 10^{-2}$	37	$2.30 (\pm 0.00) \times 10^{-2}$	28
45	9.56 ± 0.26	0.07	0.96 ± 0.04	0.72	1.18 ± 0.06	0.59
50	36.7 ± 1.2	0.02	9.00 ± 0.39	0.08	10.80 ± 0.37	0.06

(B) BbreβgalII

Temperature (°C)	Sodium phosphate buffer, pH 6.5		Sodium phosphate buffer (pH 6.5) +1 mM Mg^{2+}		Sodium phosphate buffer (pH 6.5) + 10 mM Mg^{2+}	
	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)	k_{in} (h^{-1})	$\tau_{1/2}$ (h)
30	$4.67 (\pm 0.06) \times 10^{-3}$	109	$2.58 (\pm 0.08) \times 10^{-3}$	268	$2.34 (\pm 0.16) \times 10^{-3}$	297
37	$2.07 (\pm 0.07) \times 10^{-2}$	33	$3.79 (\pm 0.01) \times 10^{-3}$	183	$3.78 (\pm 0.07) \times 10^{-3}$	183
50	5.70 ± 0.04	0.12	0.55 ± 0.01	1.25	$0.19 \pm 0.0.01$	3.7

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Table 7. Relative activities of *B. breve* β-galactosidases for individual galactosides. Results are expressed as a percentage of hydrolysis (or conversion) of each substrate after 30 and 60 min.

Substrate	% Conversion			
	β-gal I		β-gal II	
	30 min	60 min	30 min	60 min
Lactose	>99	>99	86.3	>99
β-D-Gal*p*-(1→6)-D-Glc	>99	>99	98.3	>99
β-D-Gal*p*-(1→3)-D-Glc	97.7	>99	61.7	88.6
β-D-Gal*p*-(1→3)-D-Gal	98.7	>99	73.1	90.9
β-D-Gal*p*-(1→4)-D-Gal	11.6	12.4	4.7	11.6
β-D-Gal*p*-(1→6)-D-Gal	52.1	79.0	48.2	80.4
β-D-Gal*p*-(1→3)-D-Lac	>99	>99	85.7	97.6
β-D-Gal*p*-(1→4)-D-Lac	22.1	29.1	21.8	26.2
β-D-Gal*p*-(1→6)-D-Lac	10.3	30.4	5.9	14.9

Table 8. Individual GOS components produced by the transgalactosylation reaction of β-gal I (I) and β-gal II (II) from *Bifidobacterium breve* DSM 20031 using lactose as substrate. The reaction was performed at 30°C at an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ using 1.0 U_{Lac} mL^{-1} (β-gal I) or 2.5 U_{Lac} mL^{-1} (β-gal II)

GOS components[a]	Degree of Lactose Conversion													
	20%		35%		55%		70%		84%		95%		99%	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II
GOS components (g L^{-1})														
D-Gal*p*-(1→6)-D-Glc	5.39	5.59	9.75	10.2	24.0	25.7	27.3	39.2	23.8	45.3	14.0	43.2	6.39	25.0
D-Gal*p*-(1→6)-D-Gal	0.21	0.58	0.35	0.88	0.63	1.94	0.86	2.62	2.22	3.52	4.13	5.11	5.30	8.20
D-Gal*p*-(1→3)-D-Gal	0.95	0.43	1.50	0.74	2.68	1.75	3.41	2.07	4.01	2.90	2.28	3.77	1.32	4.30
D-Gal*p*-(1→3)-D-Glc	0.16	0.60	0.28	1.23	0.76	3.23	1.29	4.45	2.17	5.30	2.03	6.51	1.21	4.95
D-Gal*p*-(1→3)-Lac	1.25	1.42	9.04	3.99	18.6	8.08	19.6	10.4	7.64	14.3	1.55	16.4	<0.01	14.9
D-Gal*p*-(1→4)-Lac	0.08	0.16	0.12	0.38	0.26	1.42	0.44	2.21	0.62	2.75	0.33	3.35	0.04	1.88
D-Gal*p*-(1→6)-Lac	0.22	0.69	0.44	0.97	0.61	2.30	1.42	2.92	2.57	3.73	2.88	4.50	1.51	3.74
Other GOS	0.00	0.00	7.89	0.49	4.58	3.48	6.34	2.24	9.20	9.96	0.0	2.90	0.00	9.25
Total GOS	8.26	9.47	29.4	18.85	52.2	47.9	60.7	66.1	44.6	87.8	27.2	85.7	15.8	72.2
GOS components														
D-Gal*p*-(1→6)-D-Glc	15.7	16.3	28.5	29.7	70.2	75.1	79.8	115	69.7	132	40.9	126.1	18.7	73.0
D-Gal*p*-(1→6)-D-Gal	0.61	1.69	1.02	2.57	1.84	5.67	2.51	7.65	6.49	10.3	12.1	14.9	15.5	24.0
D-Gal*p*-(1→3)-D-Gal	2.78	1.26	4.38	2.16	7.83	5.11	9.96	6.05	11.7	8.47	6.67	11.0	3.86	12.6
D-Gal*p*-(1→3)-D-Glc	0.47	1.75	0.82	3.59	2.22	9.44	3.77	13.0	6.34	15.5	5.93	19.0	3.53	14.5
D-Gal*p*-(1→3)-Lac	2.48	2.82	17.9	7.91	36.9	16.0	38.9	20.7	15.2	20.5	3.02	32.43	0.0	29.4
D-Gal*p*-(1→4)-Lac	0.16	0.32	0.24	0.75	0.52	2.82	0.87	4.38	1.23	5.45	0.65	6.64	0.08	3.73
D-Gal*p*-(1→6)-Lac	0.44	1.38	0.87	1.93	1.22	4.57	2.81	5.78	5.09	7.39	5.70	8.92	3.00	7.41
GOS components (% mass of total GOS)														
D-Gal*p*-(1→6)-D-Glc	65.3	59.0	33.2	54.0	46.1	53.7	45.1	59.3	53.4	51.6	51.5	50.4	40.5	34.6
D-Gal*p*-(1→6)-D-Gal	2.54	6.12	1.19	4.67	1.21	4.05	1.42	3.96	4.97	4.01	15.2	5.97	33.6	11.4
D-Gal*p*-(1→3)-D-Gal	11.5	4.54	5.11	3.93	5.14	3.65	5.62	3.13	8.98	3.30	8.39	4.40	8.37	5.95
D-Gal*p*-(1→3)-D-Glc	1.94	6.33	0.95	6.53	1.46	6.74	2.13	6.73	4.87	6.03	7.47	7.60	7.67	6.85
D-Gal*p*-(1→3)-Lac	15.1	15.0	30.8	21.2	35.7	16.8	32.3	15.8	17.1	16.3	5.70	19.1	0.0	20.6
D-Gal*p*-(1→4)-Lac	0.97	1.69	0.41	2.02	0.50	2.96	0.73	3.34	1.39	3.13	1.21	3.91	0.25	2.60
D-Gal*p*-(1→6)-Lac	2.66	7.33	1.49	5.17	1.18	4.81	2.34	4.41	5.76	4.24	10.6	5.25	9.60	5.17

[a] D-Gal*p*-(1→4)-D-Gal was not detected at all lactose conversion level (limit of detection = 0.01 g L^{-1}).

FIGURES

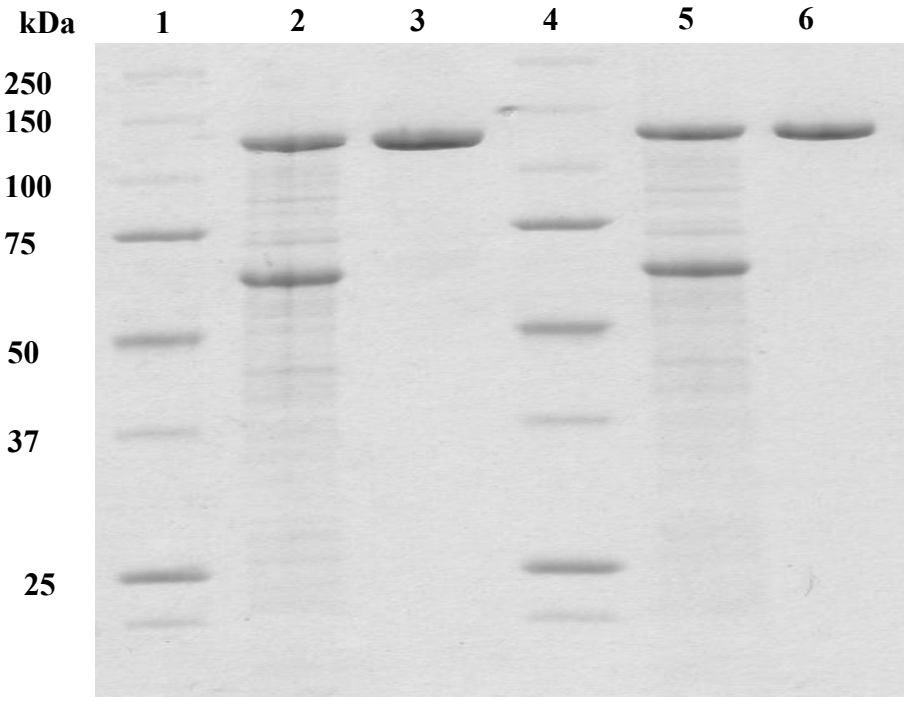


Figure 1.

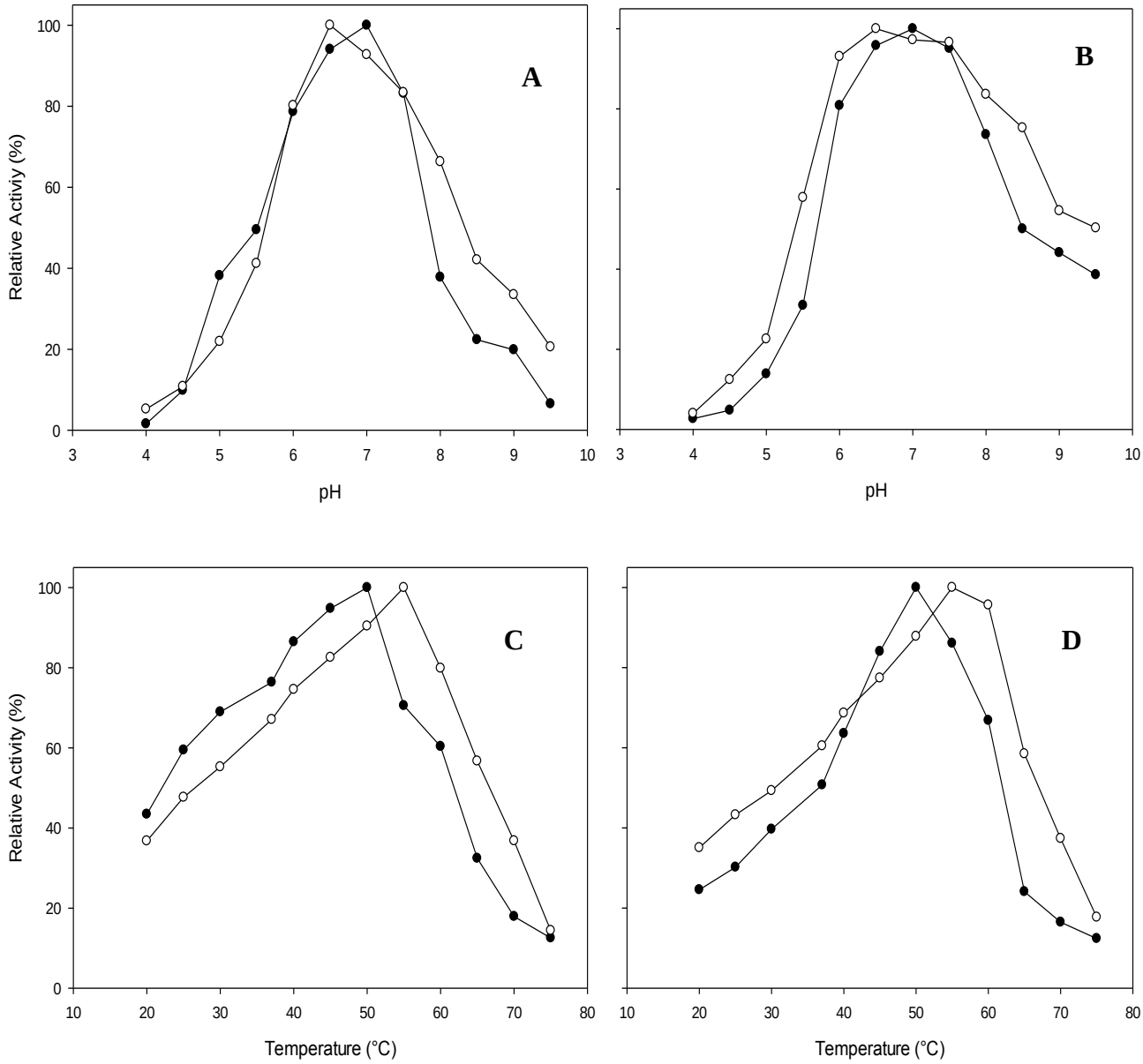

A
B
C
D
Relative Activiy (%)
Relative Activity (%)
pH
pH
Temperature (°C)
Temperature (°C)
0
20
40
60
80
100
3
4
5
6
7
8
9
10
10
20
30
40
50
60
70
80

Figure 2.

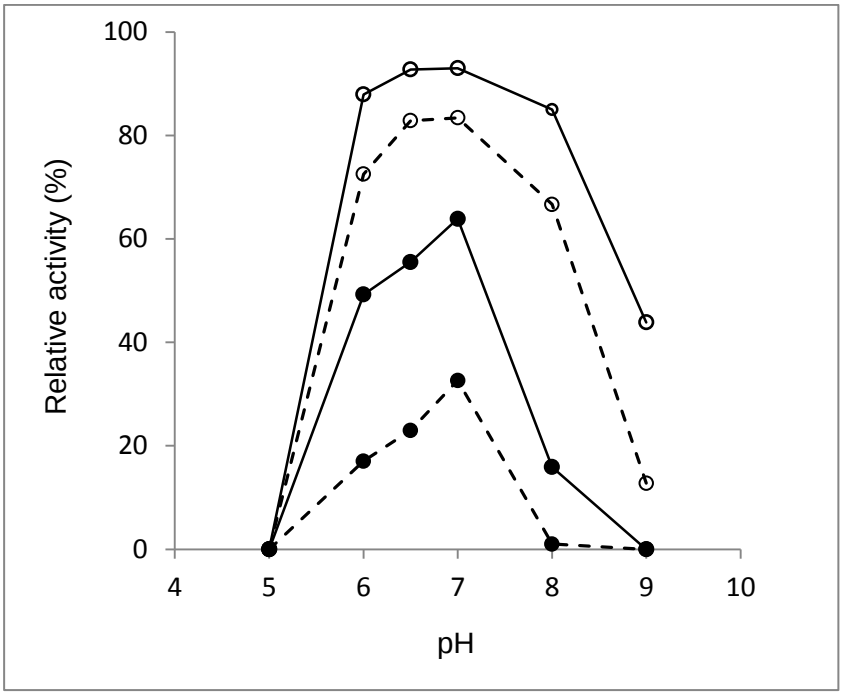


Figure 3.

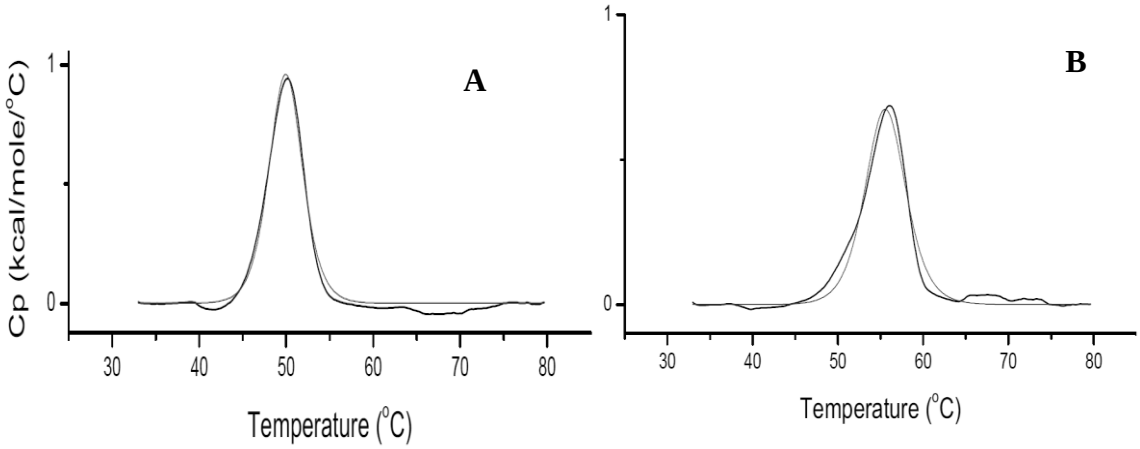
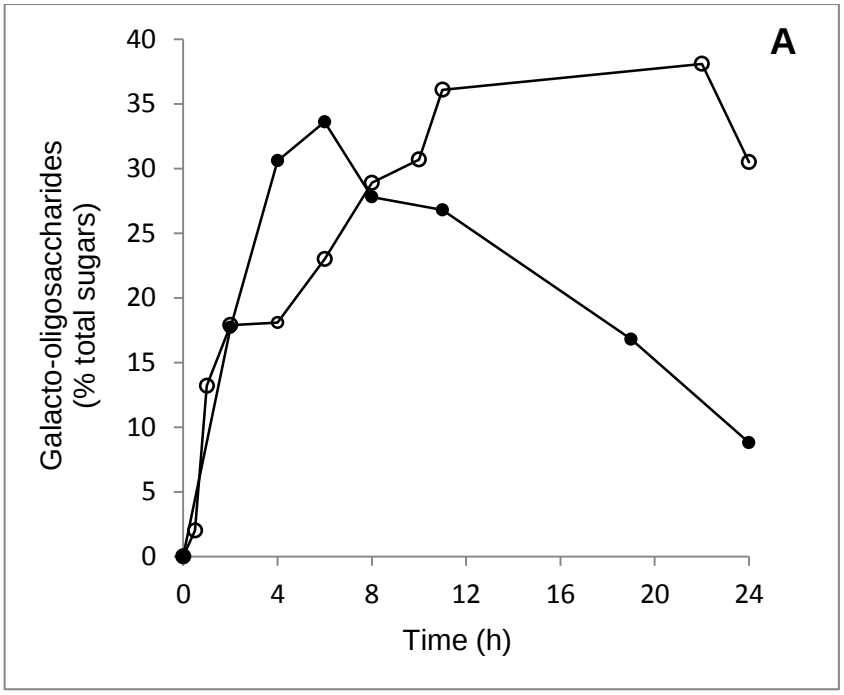


Figure 4.


A
Galacto-oligosaccharides (% total sugars)
Time (h)
0
5
10
15
20
25
30
35
40
0
4
8
12
16
20
24

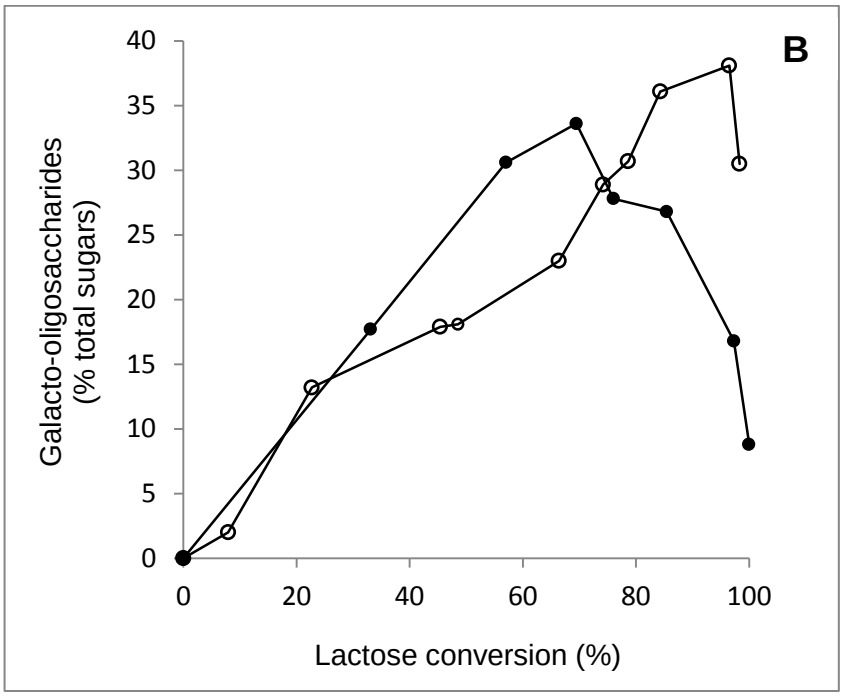

B
Galacto-oligosaccharides (% total sugars)
Lactose conversion (%)
0
5
10
15
20
25
30
35
40
0
20
40
60
80
100

Figure 5.

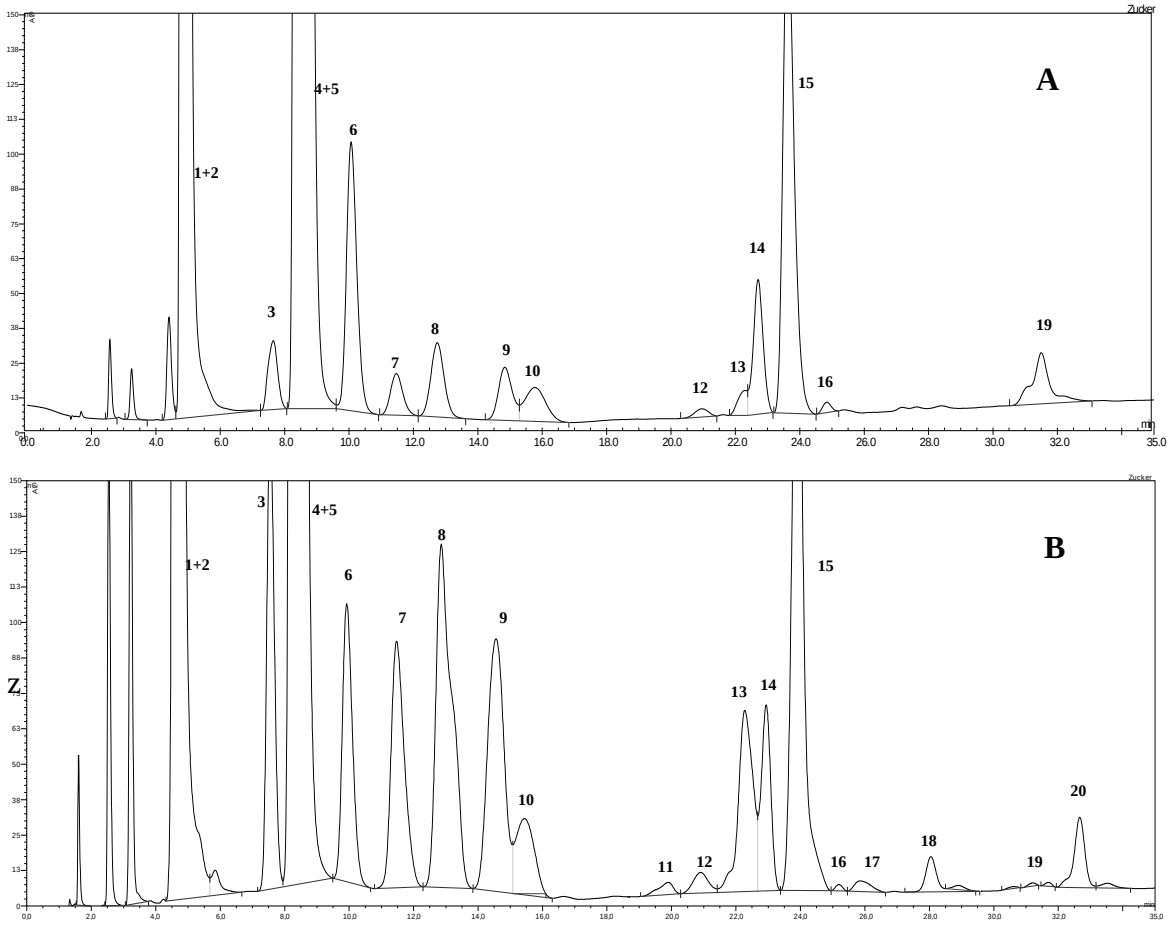
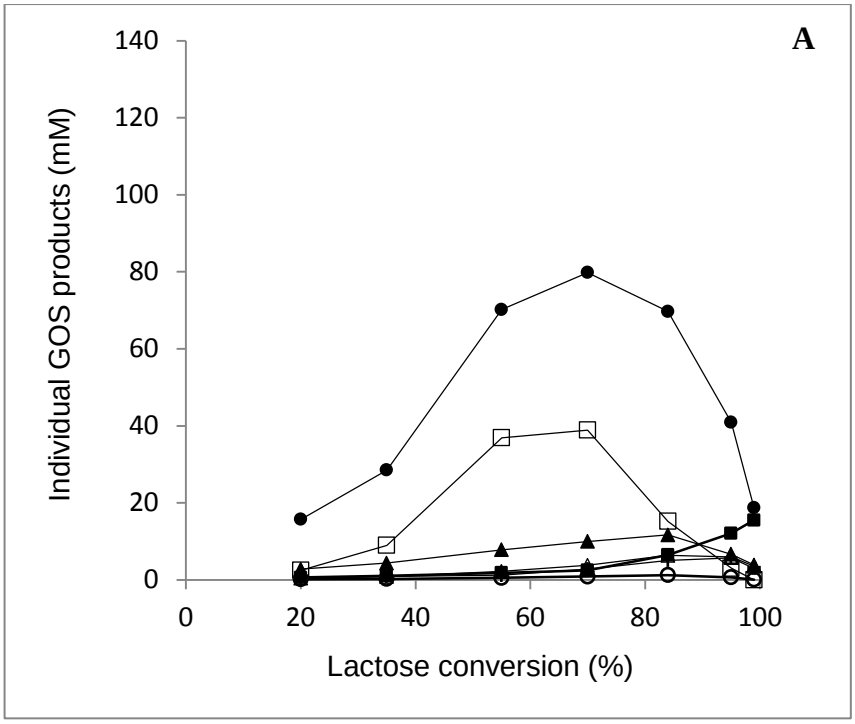

Zucker
A
1+2
3
4+5
6
7
8
9
10
12
13
14
15
16
19
mV
min
Zucker
B
1+2
3
4+5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
mV
min

Figure 6.


A
Individual GOS products (mM)
140
120
100
80
60
40
20
0
0
20
40
60
80
100
Lactose conversion (%)

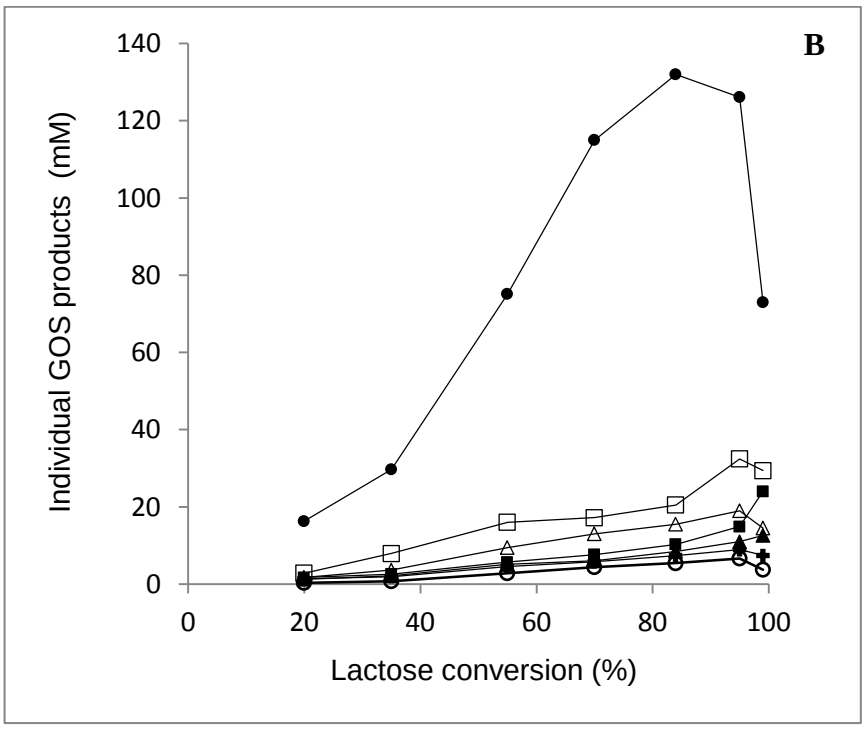
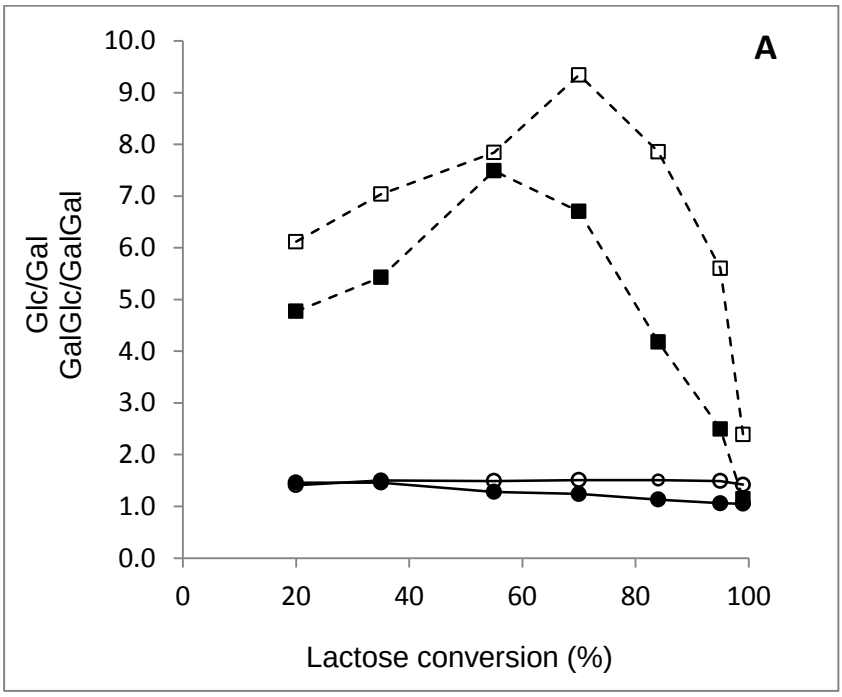

B
Individual GOS products (mM)
140
120
100
80
60
40
20
0
0
20
40
60
80
100
Lactose conversion (%)

Figure 7.


A
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Glc/Gal
GalGlc/GalGal
0
20
40
60
80
100
Lactose conversion (%)

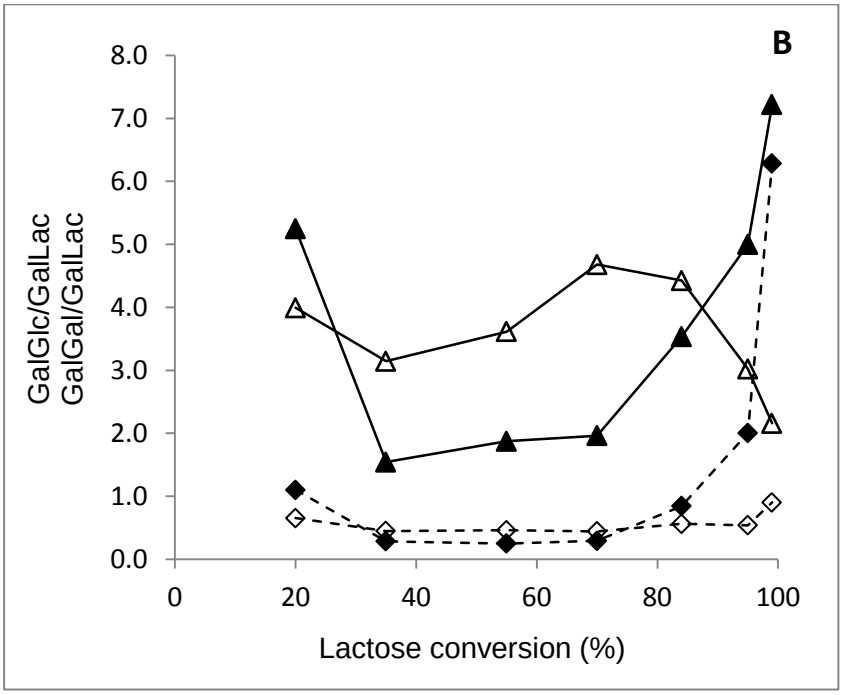
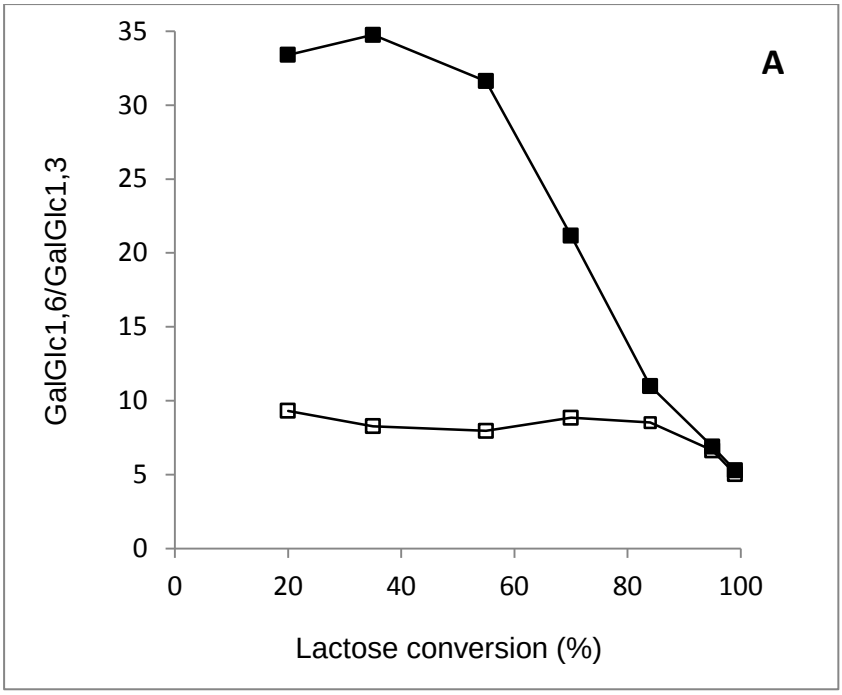

B
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
GalGlc/GalLac
GalGal/GalLac
0
20
40
60
80
100
Lactose conversion (%)

Figure 8.


A
GalGlc1,6/GalGlc1,3
35
30
25
20
15
10
5
0
0
20
40
60
80
100
Lactose conversion (%)

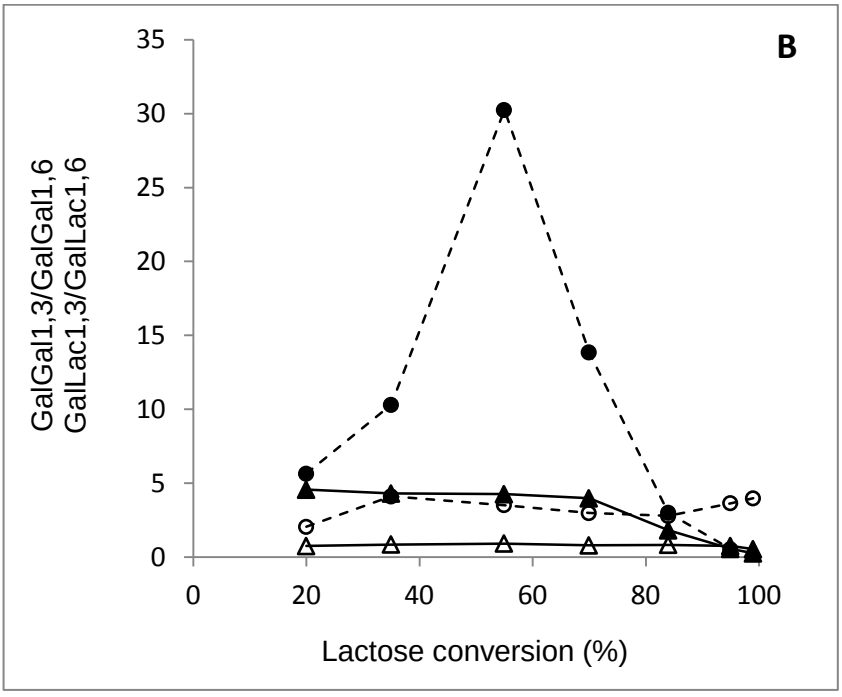

B
GalGal1,3/GalGal1,6
GalLac1,3/GalLac1,6
35
30
25
20
15
10
5
0
0
20
40
60
80
100
Lactose conversion (%)

Figure 9.

APPENDIX E

Biochemical characterization of four β-galactosidases towards formation of galacto- and hetero- oligosaccharides

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Phone: 43-1-47654 6148. Fax: 43-1-47654 6199.

Abstract

The β-galactosidase of *Lactobacillus reuteri* L103 (Lreuβgal), *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 (Lbulβgal), *Bifidobacterium breve* DSM 20281 β-gal I (BbreβgalI), and β-gal II (BbreβgalII), was investigated in detail with respect to its propensity to transfer galactosyl moieties onto lactose, the primary hydrolysis products D-glucose and D-galactose, and certain sugar acceptors such as L-fucose, *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine (GlcNAc) under defined, initial-velocity conditions. The rate constant ratios determined for these different acceptors were used as a measure for the ability of a certain substance to act as a galactosyl acceptor. Galactosyl transfers from Lbulβgal or BbreβgalII to GlcNAc occurs with partitioning ratio k_{Nu}/k_{water} which are 2 and 6 times those for the reactions of the galactosylated enzymes with glucose and lactose, respectively. Using lactose as galactosyl donor and GlcNAc as acceptor, and a substrate molar concentration ratio of 1.0, Lbulβgal and BbreβgalII catalyzed formation of the maximal *N*-acetyl-allolactosamine of 41% after 5 h and 24% after 4h, respectively. Lbulβgal or BbreβgalII may serve as catalyst for the enzymatic synthesis of prebiotic galacto-and hetero-oligosaccharides.

INTRODUCTION

Beta-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23; β-gal**)** has been long known to catalyse the hydrolysis of lactose into glucose and galactose, and transfer of galactosyl moiety to suitable acceptors. During lactose conversion by β-Gal, galactose is covalently linked to the active site of the enzyme and subsequently to water. If lactose is present in excess, β-gal will use lactose, or its hydrolysis products, glucose and galactose, as an alternative galactosyl acceptor to form galacto-oligosaccharides or GOS (Scheme 1). The β-gal source, lactose concentration and working temperature influence the GOS type and yield, and the specific linkages, thus creating a wide array of GOS. (*1*)

Beta-galactosidases have also been used to synthesize heterooligosaccharides (HeOS). *Sulfolobus solfataricus* and *Kluyveromyces lactis* β-galactosidases were used to produce lactulose and galactosylated aromatic primary alcohols (*2*, *3*), respectively while an array of sugar alcohols and mono- and disaccharides have been shown to act as an acceptor carbohydrate for *Enterobacter cloacae* B5 β-gal (*4*). Moreover, β-gal from *Bacillus circulans, K. lactis* and *L. bulgaricus* were proved to be a suitable biocatalyst for the production of *N*-acetyl-oligosaccharides using lactose and *N*-acetyl-D-glucosamine (GlcNAc), as substrates. (*5*-*7*). The choice of galactosyl acceptor and enzyme allows formation of tailor-made HeOS with potential application as food additives (*8*).

Human milk oligosaccharides (HMOs) represent the third most abundant component in breast milk after lactose and fatty acids (*9*). HMOs core structure lactose is cross linked with GlcNAc, L-fucose (Fuc) and *N*-acetylneuraminic acid (NeuAc; sialic acid) via several glycosidic bonds. HMOs are resistant to gastrointestinal digestion in host infants, thus majority of HMOs reach the colon. (*10*,

11). It has been suggested that HMOs act as substrate for infant bifidobacteria and possibly serve as soluble ligand analogs and block pathogen adhesion reducing the incidence of childhood diarrhea and infections (*12-14*)

Applying the transgalactosylation properties of β-galactosidase, it is possible to mimic carbohydrate receptors of bacterial adhesion of the terminal structures of HMOs. While it is challenging to deduce the specificity of the galactosylated enzyme for the reaction with nucleophile, a generally simple equation has been developed to determine the ability of different nucleophile to act as galactosyl moiety acceptor.(*15-18*). The transfer constant ratio k_{Nu}/k_{water} can be determined by applying the velocity ratio (v_{oNP}/ (v_{Gal}) or (v_{Glc}/ (v_{Gal}) against acceptor concentration and fitting on Equation 1 using the nonlinear least fit.

$$\frac{v_{Glc}}{v_{Gal}} = 1 + \frac{k_{Nu}}{k_{water}}[Nu] \qquad \text{Equation 1}$$

In this paper, the propensity of β-galactosidases from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 (*L. bulgaricus,* Lbulβgal), *L. reuteri* (Lreuβgal), *Bifidobacterium breve* DSM 20281 β-gal I (BbreβgalI) and β-gal II (BbreβgalII) to transfer galactosyl moiety to different acceptors such as lactose, Glc, Gal, Fuc, GlcNAc and GalNAc, will be determined. Also, optimization on the enzymatic synthesis of LacNAc and its isomers using Lbulβgal and BbreβgalII will be presented.

MATERIALS AND METHODS

Chemicals. All chemicals and enzymes were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise and were of the highest quality available. The test kit for the determination of D-glucose was obtained from Megazyme (Wicklow, Ireland). All restriction enzymes, T4 DNA ligase, and corresponding buffers were from Fermentas (Vilnius, Lithunia). The plasmid pET-21a (+) was from Novagen (Darmstadt, Germany) and the plasmid pGRO7 encoding the chaperones GroEL and GroES was purchased from TAKARA Bio Inc.(Shiga, Japan). Galacto-oligosaccharide standards of β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→6)-D-Glc, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→4)-D-Gal, β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Lac, β-D-Gal*p*-(1→4)-D-Lac, β-D-Gal*p*-(1→6)-D-Lac were purchased from Carbosynth (Berkshire, UK) while β-D-Gal*p*-(1→3)-D-GlcNAc (Lacto-*N*-biose I, LNB I) and β-D-Gal*p*-(1→4)-D-GlcNAc (*N*-acetyl-D-lactosamine, LacNAc) were purchased from Dextra Laboratories (Reading, U.K.).

Enzyme preparation. β-galactosidase from *Lactobacillus reuteri* L103 (Lreuβgal) was overexpressed in *Escherichia coli* and purified according to the method described (*19*) while the *lacZ* gene encoding β-gal from *L. bulgaricus* was expressed in *L. plantarum* WCFS1 harboring pTH101 and was purified as previously described (*20*). *B. breve* DSM 20231 β-gal I and βgal II were prepared according to the procedure described (Arreola, unpublished).

β-galactosidase assays. The measurement of β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) or lactose as substrates was carried out as described previously (*21*). Briefly, these assays were performed in 50 mM sodium phosphate buffer of pH 6.5 at 30 °C, and the final substrate concentrations in the 10 min assay were 22 mM for *o*NPG and 575 mM for lactose. Protein concentrations

were determined by using the method of Bradford with bovine serum albumin (BSA) as standard.

GOS synthesis. The ability of the four recombinant β-galactosidases to synthesize GOS was compared by carrying out different discontinuous conversion reactions on a 2-mL scale. The activity (U_{Lac}/mL) of the recombinant β-gal used were as follows: *L. reuteri*, 0.8; *L. bulgaricus*, 1.5; *B. breve* β-gal I 1.0; *B. breve* β-gal II, 2.5. Reaction conditions were 600 mM initial lactose concentration in sodium phosphate buffer (50 mM, pH 6.5) containing 1 mM Mg^{2+}, incubation temperature is at 30 ^{o}C with continuous agitation at 300 rpm. At certain time interval, samples were withdrawn and reaction was stopped by heat at 90 ^{o}C for 5 minutes. The composition of the GOS mixture was analyzed by HPAEC-PAD following the method described previously. D-Glc, D-Gal, lactose, and GOS compounds were identified and quantified using external standard technique.

Intermolecular Galactosyl Transfer under Defined Initial-Velocity. Initial velocities were determined using 50 mM sodium phosphate buffer, pH 6.5 at 30 °C using either 10 mM *o*NPGal or 100 mM lactose as substrate. This substrate concentration was a compromise between the practical requirement to measure initial velocity of D-Gal (and/or D-Glc) and maximize the transfer of D-Gal to the external nucleophile but not to the substrate. The final enzyme concentration used was ≤ 1.0 U/mL. The relationship between [*o*NP] (or [Glc]) and [Gal] was found to be linear to 30 min. Thus, the standard reaction time of 20 min was used. v_{oNP}, v_{Glc}, and v_{Gal} were measurements of molar concentrations of *o*nP, Glc, and Gal, respectively. Ratio of v_{oNP} and v_{Gal} were measured in the presence and absence of glucose concentration varied between from 2.5 to 20 mM.

The intermolecular transgalactosylation to lactose was done using with various initial lactose concentration (9- 602 mM) while galactosyl transfer to either GlcNAc, GalNAc and L-fucose were assessed using 100 mM lactose with acceptor concentration varying from 12.5 to 200 mM. Generally, after preincubation of the enzyme for 20 min at 30 °C, the reaction was stopped by heating for 5 min at 95 °C. The rate of formation of *o*NP (v_{oNP}) was measured using the standard β-galactosidase assay while galactose (*v*Gal) or glucose (*v*Glc) measurement was carried out by HPLC (Dionex; MA, USA) using an Aminex HPX-87K column (300 x 7.8 mm; Bio-Rad, Hercules, CA, USA) equipped with refractive index detector. Water was used as mobile phase at a flow rate of 0.80 mL min^{-1} and a column temperature of 80 °C. As general rule, all measurements should not inhibit *o*NP release by 20% compared with the control lacking the acceptor and a linear dependence of the velocity on the acceptor concentration must be observed.

***N*-acetyl Oligosaccharide Production.** *N*-acetyl-oligosaccharide synthesis was carried out using lactose and GlcNAc (or GalNAc) as substrate with either Lbulβgal or BbreβgalII. The influences of temperature (30 and 50 °C), substrate concentrations (0.6 M and 1 M), molar ratios of donor: acceptor (1:2, 1:1 and 2:1), and enzyme concentrations (2.5 and 5.0 U/mL) were also investigated.

Generally, a solution (2.0 mL) containing the substrates was dissolved in 50 mM sodium phosphate buffer, pH 6.5 containing 1 mM Mg^{2+}. The enzyme was added and incubated at the required temperature and agitated at 300 rpm with a thermomixer (Eppendorf, Hamburg, Germany). Aliquots of samples were withdrawn at certain time intervals to determine the residual activities and carbohydrate contents using either HPAEC-PAD as described (*22*) or HPLC system containing UV detector (210) nm using Hypercarb column (0.32 × 150 mm, inner diameter 5 μm). Ammonium formate

buffer (0.3% formic acid, pH 9.0) was used as Buffer A, and a gradient was performed from 0 to 35% acetonitrile within 35 min using a Dionex Ultimate 3000 (cap flow, 1 ml/min). The GlcNAc transgalactosylation yield was determined based on the starting GlcNAc concentration and was calculated using Equation 2.

$$\text{GlcNAc transgalactosylation yield (\%)} = \left(\frac{\text{GlcNAc}_{\text{initial}} - \text{GlcNAc}_{\text{remaining}}}{\text{GlcNAc}_{\text{initial}}}\right) \times 100 \quad \text{Equation 2}$$

Purification of *N*-acetyl-oligosaccharides. For purification and identification of GlcNAc transfer products, a 10-mL discontinuous batch reaction using BbreβgalII (5 U_{Lac}/mL) was carried out at 30 °C using initial equimolar concentration of lactose and GlcNAc (600 mM each) dissolved in 50 mM sodium phosphate buffer (pH 6.5) with 1 mM Mg^{2+}. Agitation was at 300 rpm rotary shaker. After 4 h, the reaction was stopped by heating at 95 °C. Due to the complex course of transgalactosylation reactions, the reaction mixture was partially purified by gel permeation chromatography on Bio-Gel P2 (2.0 x 100 cm) equilibrated in water containing 5% (v/v) ethanol and 0.0015 % (w/v) NaCl. The elution was followed by UV reading at 210 nm to detect presence of GlcNAc and of transgalactosylation reactions. The fractions containing the desired transgalactosylation product were pooled, freeze-dried, and redissolved in acetonitrile. The complete purification of the transgalactosylation product was obtained by using HPLC system (UV detector at 210 nm) and using Hypercarb column as described above. The peaks corresponding to the products were pooled and dried under pressure and freeze-dried.

NMR Measurements. NMR spectra were recorded at 27 ^{o}C in 99.9% D_2O with a on a Bruker Avance III^{TM} 600 spectrometer (^{1}H at 600.13 MHz and ^{13}C at 150 MHz) equipped with a BBFO broad-band inverse probe head and z-gradients using

standard Bruker NMR software. COSY experiments were recorded using the program cosygpqf with 2048 x 256 data points, respectively, pet t_1-increment. HSQC spectra were recorded using hsqcedetgp with 1024 x 128 data points and 16 scans, respectively, pet t_1-increment . ^{1}H NMR spectra were referenced to internal DSS (δ= 0); ^{1}H NMR spectra were referenced to external 1,4-dioxane (δ= 67.4).

Substrate Specificity. Specificity of Lbulβgal and *Bbre*βgalII towards β-D-Gal*p*-(1→3)-D-GlcNAc, β-D-Gal*p*-(1→4)-D-GlcNAc and β-D-Gal*p*-(1→6)-D-GlcNAc was evaluated using ~1 U_{Lac} mL^{-1} incubated with ~12.5 mM of each galactoside at 30 °C in 50 mM sodium phosphate buffer (pH 6.5). Generally, samples were taken after 30 and 60 min and reactions were stopped by incubation at 95 °C for 5 min. The relative activities of the recombinant enzymes towards each galactoside were determined by taking the percentage hydrolysis each galactoside. All measurements are done in duplicate.

RESULTS AND DISCUSSION

This work presents a characterization of β-galactosidases from two lactobacilli strains, *Lreuβgal* and Lbulβgal and two from bifidobacteria (BbreβgalI and BbreβgalII). Although basic and biochemical properties of Lreuβgal and Lbulβgal have been investigated in earlier works (*20*, *21*, *23*), detailed kinetic analyses on toward production of GOS and HeOS based on the transfer constants (k_{Nu}/k_{water}) from Scheme 1 has not been reported.

Hydrolysis against Transgalactosylation Using Lactose as Substrate. The measurement of D-Glc/D-Gal ratio as a function of the reaction time provides a good estimate of as to which extent transgalactosylation (to lactose, D-Glc or D-Gal as acceptors) competes with hydrolysis during lactose conversion. This ratio however,

does not accurately reflect the extent of lactose conversion because a transfer of the galactosyl moiety can occur either via intramolecular or intramolecular reaction.

Using 600 mM initial lactose concentration, the formation of D-Glc and D-Gal was monitored over the entire course of the hydrolysis (Figure 1A). At all times, the ratio of D-Glc/D-Gal was higher for Lbulβgal when compared with that of Lreuβgal, BbreβgalI and BbreβgalII; maximum of D-Glc/D-Gal ratio for Lbulβgal, was at 3.0 at 16% lactose conversion where the trisaccharides β-D-Galp-(1→6)-Lac and β-D-Galp-(1→3)-Lac formed predominantly. This ratio decreased to 2.71 and remained constant until lactose conversion is about 70% and decreased dramatically at 90% lactose conversion. The same trend was observed for BbreβgalI where max D-Glc/D-Gal ratio was observed at the initial stage of the reaction and was further decreased as the reaction progressed. For Lreuβgal and BbreβgalII, maximum values of D-Glc/D-Gal ratio are found in a rather broad range of lactose conversion (20-80%) and remained constant until the maximum GOS yield point was reached. At about 98-99% lactose conversion, D-Glc/D-Gal ratio using Lreuβgal and BbreβgalI was nearly 1.0 suggesting that all lactose and GOS formed were all hydrolyzed. In contrast, D-Glc/D-Gal ratio with Lbulβgal and BbreβgalII was still 1.76 and 1.42, respectively implying that a significant amount of GOS remains in the solution even if lactose conversion is nearly 100%.

The ratio of D-Glc/D-Gal can be related to the GOS yield; Lbulβgal which exhibited highest D-Glc/D-Gal ratio showed the highest GOS yield at all lactose conversion level compared that of the other three β-gal (Figure 1B). The yields, as well as the type of GOS formed, differ significantly among the β-gal studied. Lreuβgal and Lbulβgal yielded the same type of GOS which is different from that of

BbreβgalI and BbreβgalII. Typical HPLC chromatograms of GOS catalyzed by Lbulβgal and BbreβgalII are depicted in Figure 2.

After reaching the maximum GOS yield point, a significant decrease of the ratio was observed for all the β-gal due to degradation of the previously formed products containing D-galactosyl residues. At ~98% lactose conversion, the remaining amount GOS with Lreuβgal and BbreβgalII is ~8% while that of Lbulβgal and BbreβgalII is ~36% GOS.

Partitioning Analysis. The transfer constant k_{Nu}/k_{water} provides a useful tool to measure the ability of a certain substance to act as a galactosyl acceptor *(i.e.* nucleophile) which in turns allows a estimation of the level of transgalactosylation products obtained of a known reaction mixture. During complete hydrolysis, a velocity ratio of ν_{Glc}/ν_{Gal} = 1.0 for reaction in water, where the formation of *o*-nitrophenol (or D-Glc) and D-Gal are stoichiometric. This ratio however increases as the intermediate is trapped by added nucleophile to form Nu-Gal at the expense of D-Gal.

Initial velocities were measured at 30 °C in 50 mM sodium phosphate buffer, pH 6.5. Plots of (ν_{oNP}/ν_{Gal}) or (ν_{Glc}/ν_{Gal}) against [Nu] were linear for a specific range of acceptor concentrations. Deviation from linearity which occurred mainly at low and high concentrations of nucleophile can be due to competition for binding to the nucleophile binding site of the galactosyl-enzyme intermediate [E-Gal]. (*15*)

The F-test at 95% probability level confirmed the validity of the linear fit for the range of [Nu] as shown in Figure 3. Moreover, the goodness of the fit for the lines as represented by r^2 was usually greater than 0.98. The k_{Nu}/k_{water} results obtained with lactose (0.53 – 2.79) and *o*NPG (3.91 – 9.36) in this study are comparable with the data published others. (*16, 23*)

In the absence of glucose, all the β-galactosidases completely hydrolyzed *o*NPG; v_{oNP}/v_{Gal} was nearly 1.0. BbreβgalII, which has the highest inhibition against glucose ($Ki_{,Glc}$ = 37 ± 4), resulted in short range of glucose tested (up to 0.02 M) compared to other β-gal (*e.g. Lreu*βgal, 0.6 M). When k_{Glc}/k_{water} was determined, BbreβgalII showed lowest partitioning ratio (3.91 ± 0.44 M^{-1}) while Lbulβgal showed the highest (9.36 ± 0.56 M^{-1}) as shown in Table 1. On the other hand, when lactose alone was used as the substrate, where the only possible galactosyl acceptors are lactose, and its hydrolysis products, D-Gal and D-Glc, BbreβgalII showed the lowest k_{Lac}/k_{water} ratio (0.53 M^{-1}) while that of Lbulβgal was the highest (2.79 M^{-1}). When k_{Glc}/k_{Lac} was determined (obtained from the ratio of k_{Glc}/k_{water} to k_{Lac}/k_{water}), BbreβgalII showed the highest ratio of 7.4 while Lreuβgal, Lbulβgal and *Bbre*βgalI was at ~3 - 4. These results indicate that with BbreβgalII, D-Glc is ~7-fold better galactosyl acceptor than lactose hence, disaccharides other than lactose will make up the large proportion of the obtained GOS mixture compared with the other three β-gal.

To prove this hypothesis, the concentration GalGlc disaccharides and GalLac trisaccharides was measured at different lactose conversion (Figure 4). At about 20% conversion, BbreβgalI and BbreβgalII have GalGlc/GalLac ratio of ~4-5 while that of LbulβgalI and LreuβgalI are at 0.44. At maximum GOS yield point, BbreβgalII showed the highest GalGlc/GalLac ratio (3.5) while that of BbreβgalI, LbulβgalI and LreuβgalI are 2.0, 1.4 and 1.7, respectively. Although it was predicted from k_{Glc}/k_{Lac} that BbreβgalII will have higher GalGlc/GalLac ratio compared with other β-gal, the low GalGlc/GalLac ratio as measured by HPAEC-PAD signifies that there must be a significant intramolecular transgalactosylation as well.

The propensity of four β-galactosidases to transfer the galactose to either GlcNAc, GalNAc or Fuc was determined using a fixed lactose concentration (100

mM) and varying nucleophile concentration. The k_{Nu}/k_{water} was determined by plotting the the v_{Glc}/v_{Gal} against the [Nu]. In the absence of any external galactosyl acceptor, v_{Glc}/v_{Gal} was found to be ~1.3 using Lbulβgal suggesting high GOS production rate while that of the three β-gal was nearly 1.0 indicating that hydrolysis of lactose is the preferred reaction.

When GlcNAc was added as the galactosyl acceptor, Lbulβgal and BbreβgalII were shown to effectively transfer the galactosyl moiety to GlcNAc rather than to water as shown by the rate constant ratio, k_{GlcNAc}/k_{water} of 16.8 and 5.42, respectively. BbreβgalII and Lbulβgal also showed high preference to transfer galactosyl moiety to GlcNAc rather than unto lactose with rate constant ratios, k_{GlcNAc}/k_{Lac} of 10.23 and 6.74, respectively. This would mean that GalGlcNAc would be the preferred products rather than tri-GOS. To confirm this hypothesis, a discontinuous conversion reaction consisting either BbreβgalII or Lbulβgal ($2.5U_{Lac}$/mL) was incubated in 600mM lactose in the absence and presence of 600 mM GlcNAc. The formation of D-Glc, D-Gal, and tri-GOS was determined at different time intervals. Figure 5 shows that using lactose alone as the substrate, the maximum D-Glc/D-Gal ratio with Lbulβgal and BbreβgalII is 3.0 and 1.5, respectively while presence of GlcNAc as substrate resulted in maximum D-Glc/D-Gal ratio of 11.0 and 4.6, respectively. A 3-fold increase in the D-Glc/D-Gal suggests that the galactosyl moiety was transferred preferentially to GlcNAc rather than into Glc or lactose. Looking at the k_{GlcNAc}/k_{Glc} ratio of Lbulβgal (1.8), it was predicted that, GlcNAc is the preferred galactosyl acceptor than glucose.

Allolactose is the major intramolecular transgalactosylation product when lactose is used as the substrate alone. (*20*) Using an equimolar of lactose and GlcNAc as substrate, the maximum allolactose yield was found to decrease significantly with Lbulβgal while no significant change on that was observed with BbreβgalII

($k_{GlcNAc}/k_{Glc} \approx 1.4$) as shown in Figure 6. The increase in D-Glc/D-Gal ratio in the presence of GlcNAc as substrate can be also attributed to the inhibition of GalGal formation particularly 6'-galactobiose (Figure 7) using BbreβgalII. Moreover, tri-GOS yield of BbreβgalII decreased significantly from 24 to 13 g/L when GlcNAc was added to lactose as substrate (Figure 8). This proved that GlcNAc is the much preferred galactosyl moiety acceptor than lactose.

N-acetyl-D-galactosamine can also serve as galactosyl acceptor when using Lbulβgal based the obtained k_{GalNAc}/k_{water} ratio (3.21). BbreβgalI, BbreβgalII and Lreuβgal showed $k_{GalNAc}/k_{water} \leq 1.0$ suggesting that hydrolysis is the preferred reaction in the presence of GalNAc. L-fucose, on the other hand, was shown to be weak nucleophile based on the k_{GalNAc}/k_{water} ratio (≤ 1.27). The ratio of k_{Gal}/k_{water} is also an essential kinetic parameter to measure the propensity to transfer the galactosyl moiety to another galactose unit. Unfortunately, k_{Gal}/k_{water} could not be determined because the amount of galactose released cannot be measured in the presence of excess of free galactose.

Preparation and synthesis of *N*-acetyl oligosaccharides. To optimize GlcNAc transgalactosylation production, a number of discontinuous conversion reactions were carried out varying the enzyme source and concentration, and donor to acceptor ratio. Figure 9 shows GlcNAc transgalactosylation yield at 30 ^{o}C containing initial concentration of 600 mM lactose and 600 mM GlcNAc in sodium phosphate buffer (pH 6.5), 1 mM $MgCl_2$ using 2.5 U_{Lac}/mL of either Lbulβgal or BbreβgalII. Under these conditions, the maximum molar yields of *N*-acetyl oligosaccharides produced was 40% after 6 h of reaction and 32% after 8 h with Lbulβgal and BbreβgalII, respectively. After these maximum yield points, the yield decreased because the GlcNAc containing products served as substrate for hydrolysis. The effect

of BbreβgalII concentration on GlcNAc transgalactosylation yield was also investigated. Increasing the BbreβgalII concentration from 2.5 to 5.0 U_{Lac}/mL speeded up the reaction reaching the same amount of maximum yield only after 4 hours.

The yields of transgalactosylation reactions were improved by optimizing the molar ratios of the donor to the acceptor (Figure 10). No significant change on the maximum yields was observed when the donor/acceptor ratio is increased from 1:1 to 2:1 either with Lbulβgal or BbreβgalII. When the molar concentrations of the substrates were increased to 1M Lactose and GlcNAc each and the reaction temperature was increased to 50 ^{o}C, higher yield of 51.4% was observed with Lbulβgal with either 1:1 or 2:1 ratio. (Figure 11) Moreover, a significant decrease on yield (25.4%) was observed when the initial lactose concentration is less than the GlcNAc concentration (1:2).

The ratio of lactose and GlcNAc presents an additional parameter for the formation of GlcNAc transgalactosylation products. The present study showed high yield when the ratio of lactose to GlcNAc is 1:1 to 2:1. On the other hand, large excess of galactosyl acceptor (GlcNAc) significantly decreased the GlcNAc transgalactosylation yield because of untransformed substrate. High yield is theoretically obtained if donor and acceptor are present at a molar ratio of 1:1. (*24*) The observed optimum molar ratio of donor/ acceptor is different from previous reports. Optimal molar ratio of donor/acceptor was 3:1 on β-D-galactosyl disaccharide production by β-gal from porcine liver(*25*) while that for the transgalactosylation of Fuc, GlcNAc, GalNAc and mannose was achieved with high yield at a donor:acceptor ratio of 1:1 or an excess of galactosyl acceptor. (*6, 26-28*). The lactulose and lactosucrose yield produced with fructose and sucrose as galactosyl acceptors,

respectively was the highest at a molar donor to acceptor ratio in the range of 1:1 to 1:2. (*29-31*)

The production of *N*-acetyl-oligosaccharides with lactose and *N*-acetyl-D-galactosamine using Lbulβgal as biocatalyst was investigated. The substrate containing lactose and GalNAc was dissolved in sodium phosphate buffer (pH 6.5) containing 1 mM Mg^{2+} and 2.5 U_{Lac}/mL and incubated at 30 ^{o}C. Furthermore, the effect of donor/acceptor molar ratio was also examined. Figure 12 shows that the maximum yield (29.19%) was obtained after 1 h with initial concentration of 0.6 M each of lactose and GalNAc. From the HPLC profile (Figure 13), di- and tri-GalNAc containing oligosaccharides were formed however, were not identified and quantified yet.

Structural Characterization of GlcNAc transgalactosylation products. The optimum condition for the production of *N*-acetyl oligosaccharides using Lbulβgal were 1M lactose and 1M GlcNAc, 50 ^{o}C and 2.5 U_{Lac}/mL while that of BbreβgalII was 0.6 M lactose and 0.6 M GlcNAc, 30 ^{o}C and 5.0 U_{Lac}/mL. Using the mentioned condition, transgalactosylation reaction was done and products were separated using Hypercarb column equipped with UV detector (210 nm). The chromatographic column used can separate the anomeric forms of reducing sugars thus, each oligosaccharide synthesized is represented on the chromatograms by two peaks constituting the α and β forms. The chromatographic patterns and the compounds synthesized by Lbulβgal and BbreβgalII were found to be similar (Figure 14).

To determine the identity of the major GlcNAc transgalactosylation product, a preparative synthesis with BbreβgalII performed using the best of experimental condition, *i.e.* equimolar of lactose and GlcNac and incubation for 4 h at 30 ^{o}C with 5

U_{Lac}/ml. After all the purification steps, the major product was found to be β-D-Gal*p*-(1→6)-D-GlcNAc (*N*-acetyl-allolactosamine) identified by the NMR data (Figure 15, derived from an Heteronuclear Single Quantum Coherence or HSQC experiment) which indicated a low-field shift of Carbon 6 of the reducing GlcNAc to 69.4 ppm. The data when corrected for different referencing were in full agreement for published 13C NMR data of *N*-acetyl-allolactosamine.(*32-34*) The retention times of Peaks 2 and 4 were congruent with the retention times of α- and β-anomer of β-D-Gal*p*-(1→4)-D-GlcNAc standard, respectively and the compound eluting at the retention time of β-D-Gal*p*-(1→4)-D-GlcNAc standard was also identified in the HPAEC-PAD analysis (data not shown). Based on the elution rate, Peaks 6 and 7 can be assigned to trisaccharide GalGalGlcNAc however the identity of these compound are yet to be determined.

***N*-acetyl-allolactosamine production by β-galactosidase from *L. bulgaricus* and *B.breve* β-gal II under optimal condition.** Using the optimum condition for the production of *N*-acetyl oligosaccharides with LbulβgalandBbreβgalII, the formation and degradation of *N*-acetyl-allolactosamine was monitored. The maximum total amount of *N*-acetyl-allolactosamine was 41% after 5 h and 24% after 4h with LbulβgalandBbreβgalII, respectively (Figure 16). After that, hydrolysis of GlcNAc containing products prevailed. The reported *N*-acetyl-allolactosamine yield of *B. circulans* β-gal is lower than that of Lbulβgal but higher than of BbreβgalII. (*35*)

The formation of disaccharides as product of transgalactosylation of galactose and GlcNAc by β-gal of lactobacilli and bifidobacteria has been reported and the linkage preference varies. β-gal from *K. lactis*, *L. bulgaricus* and *Lc. lactis* expressing LacLM of *L. plantarum* synthesized *N*-acetyl-allolactosamine as the major product and LacNAc as minor product. (*7, 36*) Meanwhile, β-gal from *B. bifidum* and *B.*

circulans favored LacNAc over *N*-acetyl-allolactosamine . (*34, 37, 38*) With β-gal from *P. multicolor*, *A. oryzae*, *B. longum* the *N*-acetyl-allolactosamine was exclusively synthesized.(*34*) Presence of higher DP N-acetyl oligosaccharides in the reaction mixtures of transgalactosylation by using β-gal from *S. soflataricus*, *A. oryzae* or *E. coli* was also observed however were identified (*26*) Recently, Schwab et al reported formation of *N*-acetyl-oligosaccharides using lactose and GlcNAc as substrate using *C. mesenteroides* FUA3143, *Lb. ruminis* ChCC8818 and *B. longum* CHCC8700 however they were not identified.(*8*)

Substrate Specificity. The core structures of the HMO are classified into type I and type II based on the disaccharide unit at the reducing end. Type I contain lacto-N-biose I (LNB, β-D-Gal*p*-(1→3)-D-GlcNAc while and type II oligosaccharides have N-acetyllactosamine (LacNAc; β-D-Gal*p*-(1→4)-D-GlcNAc at the non-reducing end. In HMO, type I oligosaccharides are predominant while type II oligosaccharides are the minor components. The ability of Lbulβgal and BbreβgalII to hydrolyze lacto-N-biose and its isomers was evaluated to determine preferred substrate (Table 2).

The substrate specificity of Lbulβgal and BbreβgalII significantly differ. Whereas both enzymes acts on β-D-Gal*p*-(1→4)-D-GlcNAc preferentially, BbreβgalII hydrolyzed faster (95.7%) compared to that Lbulβgal (62.5%). Moreover, BbreβgalII acted on β-D-Gal*p*-(1→6)-D-GlcNAc faster than β-D-Gal*p*-(1→3)-D-GlcNAc with ~44.5% and 23.2% hydrolysis, respectively. Lbulβgal, although hydrolyzed β-D-Gal*p*-(1→3)-D-GlcNAc preferably than β-D-Gal*p*-(1→6)-D-GlcNAc, its hydrolysis rate is still lower compared to BbreβgalII.

Among species prevalent in the feces of breast-fed infants. only *B. infantis*, which possesses a specialized HMO utilization cluster composed of β-galactosidase, fucosidase, sialidase and β-hexosaminidase is capable of releasing and utilizing

monosaccharides from complex HMOs (*14*, *39*). In contrast, *B. bifidum* releases monosaccharides from HMOs but is not able to use fucose, sialic acid and GlcNAc. *B. breve* was able to ferment but not release monosaccharides (*40*). Moreover, GlcNAc is metabolized both by strains of bifidobacteria and lactobacilli (*14*, *41*). The present results shows that Lbulβgal and BbreβgalII can be used to unmasked the type II core structure of HMOs and the synthesized GlcNAc –containing HeOS extend the spectrum of potentially bifidogenic oligosaccharides that can be used as food additives particularly in infant formula.

CONCLUSIONS

Kinetic analyses of galactosylated β-galactosidases from *L. reuteri*, *L. bulgaricus*, *B.bre*ve βgal I and β-gal II with sugar monomers as nucleophile provided a useful characterization of the of the specificities of the enzymes for transgalactosylation. The transgalactosylation of GlcNAc was studied starting from lactose as galactosyl donor and the efficiency and regioselectively of Lbulβgal and BbreβgalII were compared. The reaction was optimized by varying the enzyme concentration and donor/acceptor ratio. Under optimal condition, Lbulβgal and BbreβgalII showed to catalyze β-D-Gal*p*-(1→6)-D-GlcNAc at a very high concentration of 41% (470 mM) and 24% (206 mM) obtained at 5 and 4 h, respectively. HPLC analysis revealed one more di- and two tri- galactosylated GlcNAc but their linkage type could not be identified. These HeOS are of interest because of a potentially extended functionality in addition to GOS.

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FIGURE CAPTIONS

Figure 1. D-Glucose/D-Galactose ratio (A) and galacto-oligosaccharide production (B) during lactose conversion by β-galactosidase from *L. reuteri* (●), *L. bulgaricus* (○), *B. breve* β-gal I (▲), and *B. breve* β-gal II (Δ). The reactions were performed at 30 °C at an initial lactose concentration of ~200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$.

Figure 2. Separation and quantification by HPAEC-PAD of individual GOS produced during lactose conversion catalyzed by (**A**) *L. bulgaricus DSM* 20081 β-gal and (B) *B. breve* β-gal I. The identified compounds are (1) D-Gal, (2) D-Glc, (3) D-Gal*p*-(1→6)-D-Gal, (4) D-Gal*p*-(1→6)-D-Glc (allolactose), (5) D-Gal*p*-(1→4)-D-Glc (lactose), (6) D-Gal*p*-(1→3)-D-Gal, (7) D-Gal*p*-(1→6)-Lac, (9) D-Gal*p*-(1→3)-D-Glc, (15) D-Gal*p*-(1→3)-Lac, (17) D-Gal*p*-(1→4)-Lac. Peaks 8, 10-14, 16, 18-22 were not identified.

Figure 3. Transgalactosylation activity of *L. reuteri* (●), *L. bulgaricus* (○), *B. breve* β-gal I (▲), and *B. breve* β-gal II (Δ) in the presence of different exogenous nucleophiles. (A) Lactose (B) *N*-acetyl-D-glucosamine (C) *N*-acetyl-D-galactosamine (D) L- fucose (E) D-Glucose.

Figure 4. GalGlc/GalLac ratio during lactose conversion by β-galactosidase from *L. reuteri* (●), *L. bulgaricus* (○), *B. breve* β-gal I (▲), and *B. breve* β-gal II (Δ). The reactions were performed at 30 °C at an initial lactose concentration of 200 g L^{-1} in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$.

Figure 5. Effect of *N*-acetyl-D-glucosamine on D-Glc/D-Gal ratio during galacto- (solid line) and *N*-acetyl-oligosaccharides (dashed line) production catalyzed by β-galactosidase from *L. bulgaricus* (○) and *Bifidobacterium breve* β-galII (Δ).

Figure 6. Allolactose (D-Gal*p*-(1→6)-D-Glc) formation during galacto- (solid line) and *N*-acetyl-oligosaccharides (dashed line) production catalyzed by β-galactosidase from *L. bulgaricus* (○) and *Bifidobacterium breve* β-galII (Δ).

Figure 7. Formation of 6'-galactobiose (D-Gal*p*-(1→6)-D-Gal) during galacto- (solid line) and *N*-acetyl-oligosaccharides (dashed line) production catalyzed by *Bifidobacterium breve* β-galII.

Figure 8. Production of tri-galacto-oligosaccharides by *B. breve* β-gal II in the absence (solid line) and presence (broken line) of *N*-acetyl-D-glucosamine. The reactions were performed at 30 °C at an initial lactose concentration of 600 mM in sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$. Initial GlcNAc concentration used was 600 mM.

Figure 9. Comparison of GlcNAc transgalactosylation yield using *L. bulgaricus* (○) and *B. breve* β-galactosidases II (Δ). The reaction was performed at 30 °C using initial concentration of 600 mM Lactose: 600 mM GlcNAc in 50 mM sodium phosphate buffer, pH 6.5 and 1 mM $MgCl_2$ varying the amount of enzyme. 2.5 U_{Lac}/mL (solid line) and 5 U_{Lac}/mL (broken line)

Figure 10. Effect of different lactose and GlcNAc molar ratio on GlcNAc transgalactosylation yield catalyzed by β-galactosidase from *L, bulgaricus* (black) and *Bifidobacterium breve* β-gall II (gray). The reaction was done in sodium phosphate buffer (pH 6.5) containing 1 mM Mg^{2+} incubated at 30 °C.

Figure 11. Maximum GlcNAc transgalactosylation yield catalyzed by β-galactosidase from *L, bulgaricus* (black) at different initial lactose and GlcNAc concentration (M), 1:1, 1.0:0.5, 0.5:1.0 The reaction was done in sodium phosphate buffer (pH 6.5) containing 1 mM Mg^{2+} incubated at 50 °C.

Figure 12. GalNAc transgalactosylation yield at different initial lactose: GlcNAc molar ratio catalyzed by β-galactosidase from *L. bulgaricus*. The reaction was done in sodium phosphate buffer (pH 6.5) containing 1 mM Mg^{2+} incubated at 50 °C.

Figure 13. HPLC-UV profile of GalNAc-containing oligosaccharides produced by *L. bulgaricus* β-galactosidase at initial concentration of 600 mM lactose and 600 mM GalNAc in 50 mM sodium phosphate buffer, pH 6.5 and 1 mM $MgCl_2$.Peaks I and II are the tri- and di-GalNAc containing oligosaccharides, respectively and Peak III is the free GalNAc.

Figure 14. HPLC-UV chromatogram of *N*-acetyl oligosaccharides produced by *L. bulgaricus* (A) and *B. breve* β-galactosidases II (B) using equimolar concentration of lactose and GlcNAc dissolved in 50 mM sodium phosphate buffer (pH 6.5) containing 1 mM $MgCl_2$. Peak Identification: (1) GlcNAc, (3) α-D-Gal*p*-(1→6)-D-GlcNAc (5) β-D-Gal*p*-(1→6)-D-GlcNAc. Peaks 2,4, 6 and 7 were not identified.

Figure 15. Multiplicity edited HSQC spectrum of β-D-Gal*p*-(1→6)-D-GlcNAc

Figure16. Time course of Galβ (1→6)GlcNAc production catalyzed by β-galactosidase from *L. bulgaricus* (○) and *B. breve* β-galactosidases II (Δ). The reaction was using lactose and GlcNAc with the molar ratio of 1:1.

SCHEME

Scheme 1. Hydrolysis and galactosyl transfer reactions during the β-galactosidase catalyzed conversion of lactose. E, enzyme; Lac, lactose; Gal, galactose; Glc, glucose; Nu, nucleophile.

1 **TABLES**

2

Table 1. Partitioning ratios (k_{Nu}/k_{water}, M^{-1}) for reaction of galactosylated β-galactosidases with exogenous nucleophiles and with water.

β-galactosidase sources	Nucleophile				
	D-Glc[a]	Lactose	GlcNAc	GalNAc	L-fucose
Bifidobacterium breve					
β-galI	6.73 ± 0.62	1.61 ± 0.05	1.01 ± 0.03	0.36 ± 0.03	1.27 ± 0.12
β-galII	3.91 ± 0.44	0.53 ± 0.02	5.42 ± 0.05	0.39 ± 0.02	1.16 ± 0.05
Lactobacillus bulgaricus	9.36 ± 0.56	2.79 ± 0.15	16.8 ± 0.7	3.21 ± 0.26	0.54 ± 0.05
Lactobacillus reuteri	6.7 ± 0.3*	1.91 ± 0.12*	0.27 ± 0.01	1.07 ± 0.09	0.67 ± 0.06

[a]Measured with 10 mM oNPGal as substrate and calculated from the v_{oNP}/v_{Gal}
*Ref. (*23*)

3

4

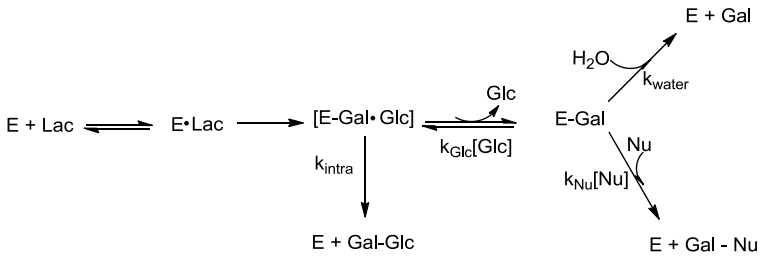
Table 2. Activities of Lbulβgal and BbreβgalII with different galactosides are expressed as percentage of hydrolysis of each substrate after 30 and 60 min

	Percent Hydrolysis			
	Lbulβgal		BbreβgalII	
	30min	60 min	30min	60 min
β-D-Gal*p*-(1→3)-D-GlcNAc	25.0	31.3	23.2	35.3
β-D-Gal*p*-(1→4)-D-GlcNAc	62.5	82.5	95.7	98.5
β-D-Gal*p*-(1→6)-D-GlcNAc	6.82	20.9	44.5	69.8

1

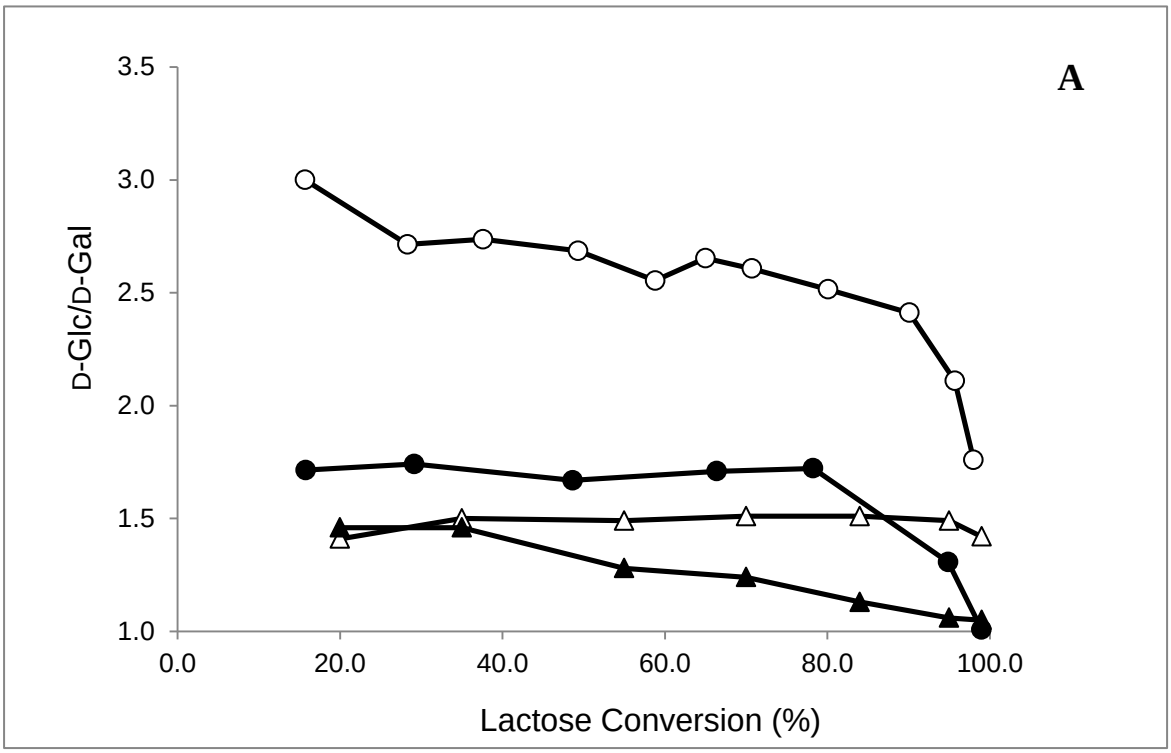
2

SCHEME



Scheme 1.

FIGURES



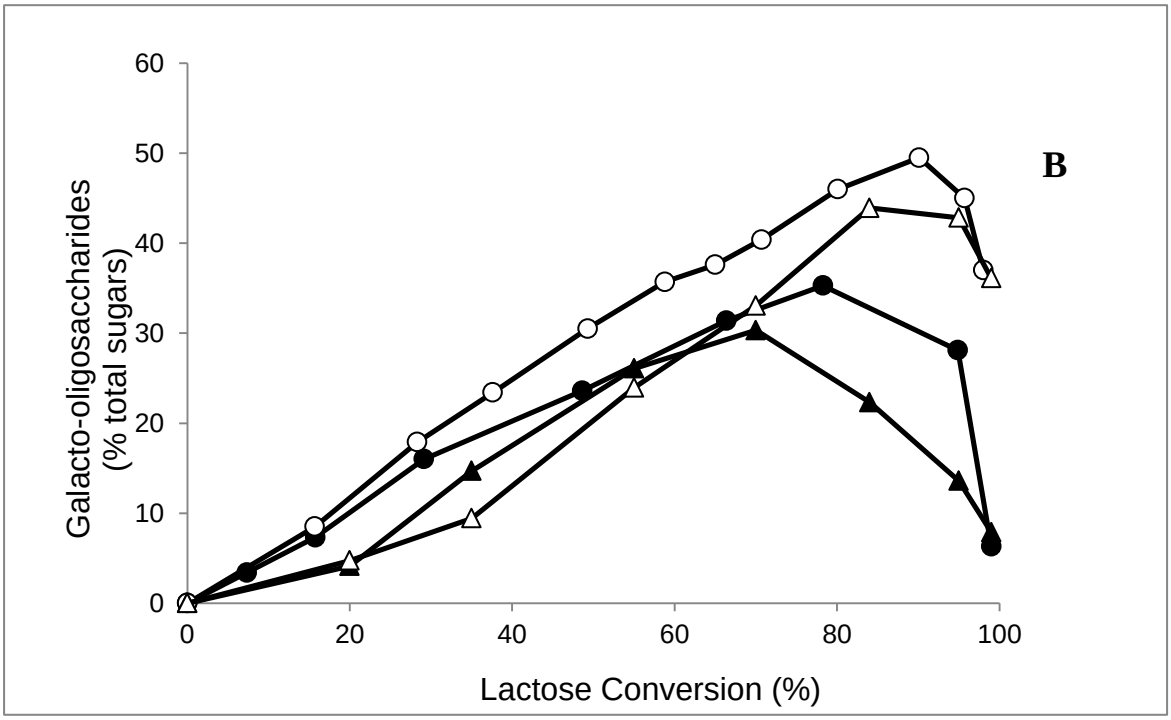


Figure 1.

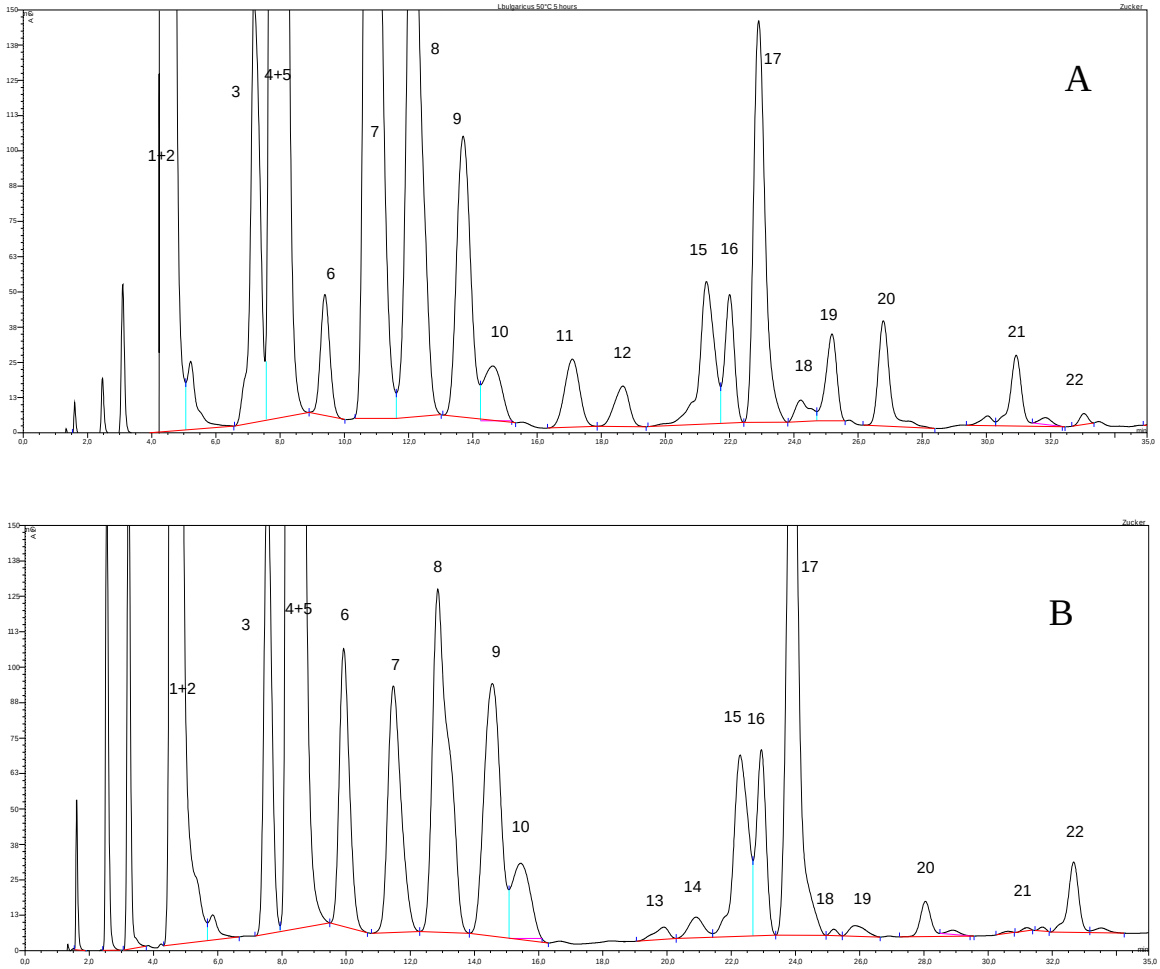
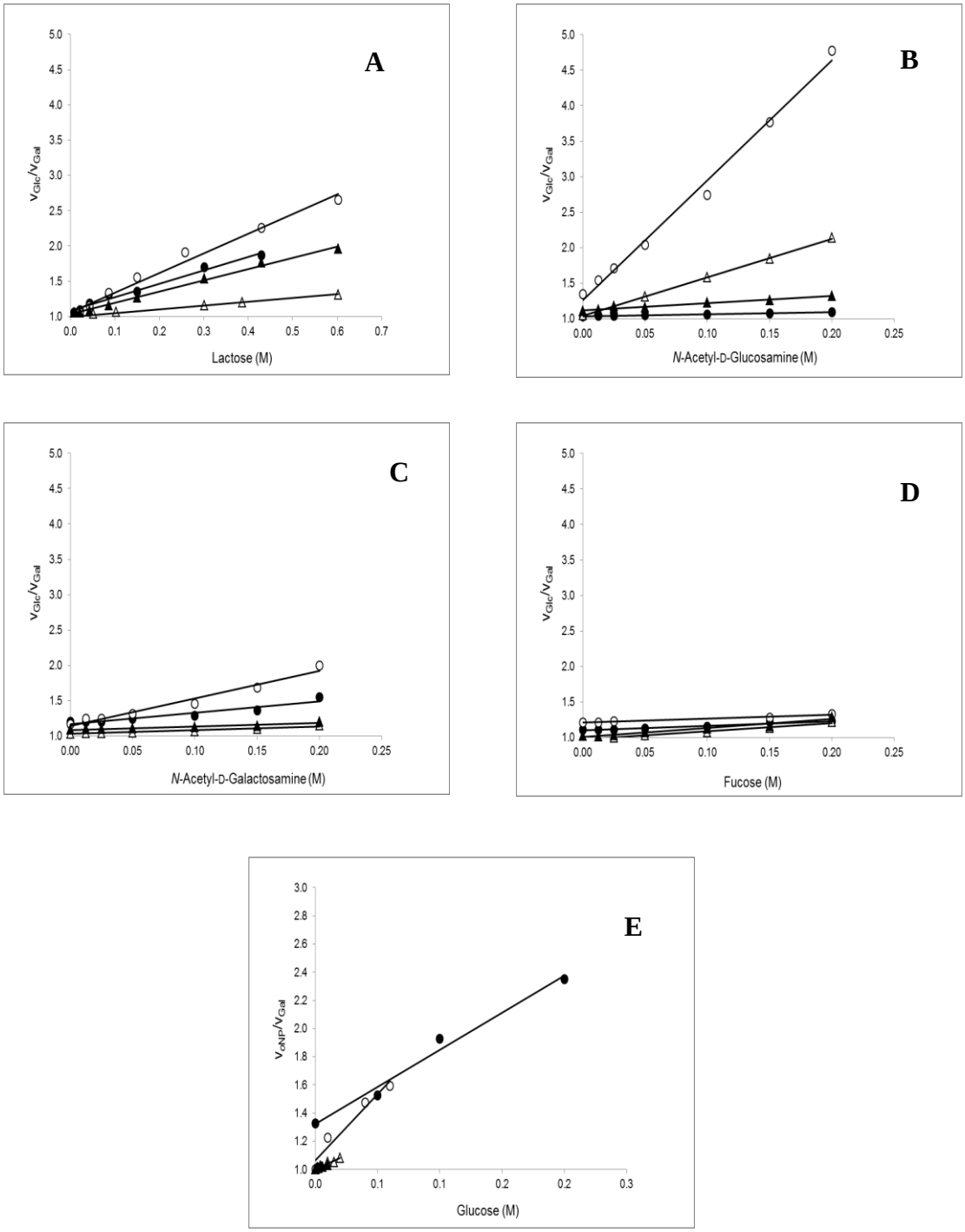

A
1+2
3
4+5
6
7
8
9
10
11
12
15
16
17
18
19
20
21
22
B
1+2
3
4+5
6
7
8
9
10
13
14
15
16
17
18
19
20
21
22

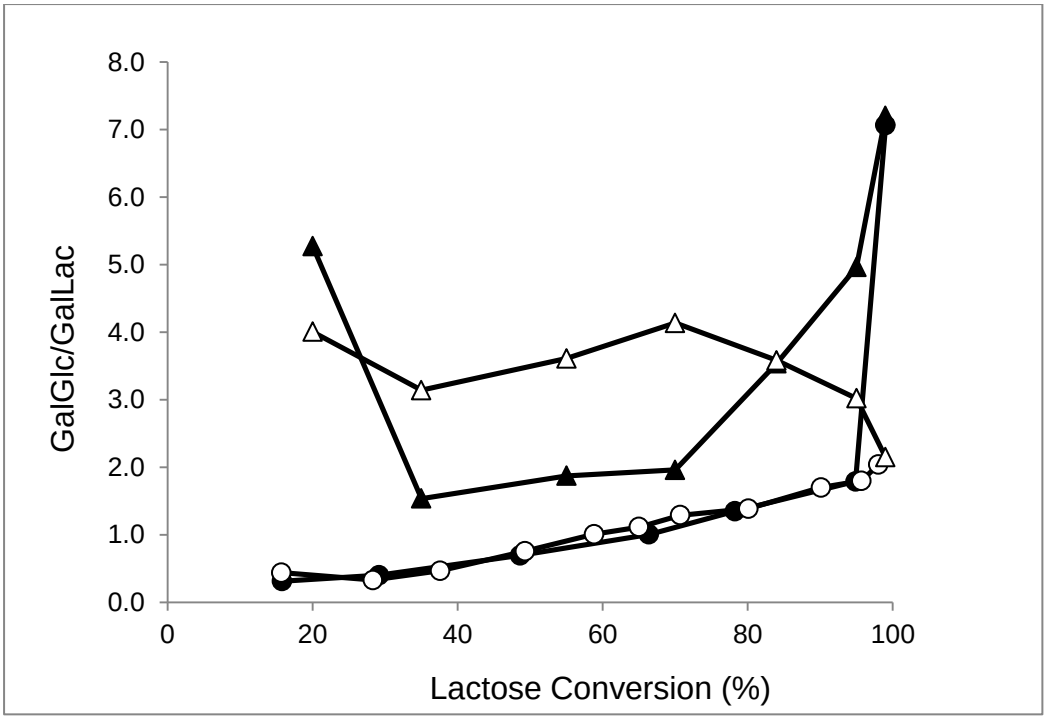
Figure 2.


A
B
C
D
E
v_{Glc}/v_{Gal}
v_{cNP}/v_{Gal}
Lactose (M)
N-Acetyl-D-Glucosamine (M)
N-Acetyl-D-Galactosamine (M)
Fucose (M)
Glucose (M)

1

2 Figure 3.

3



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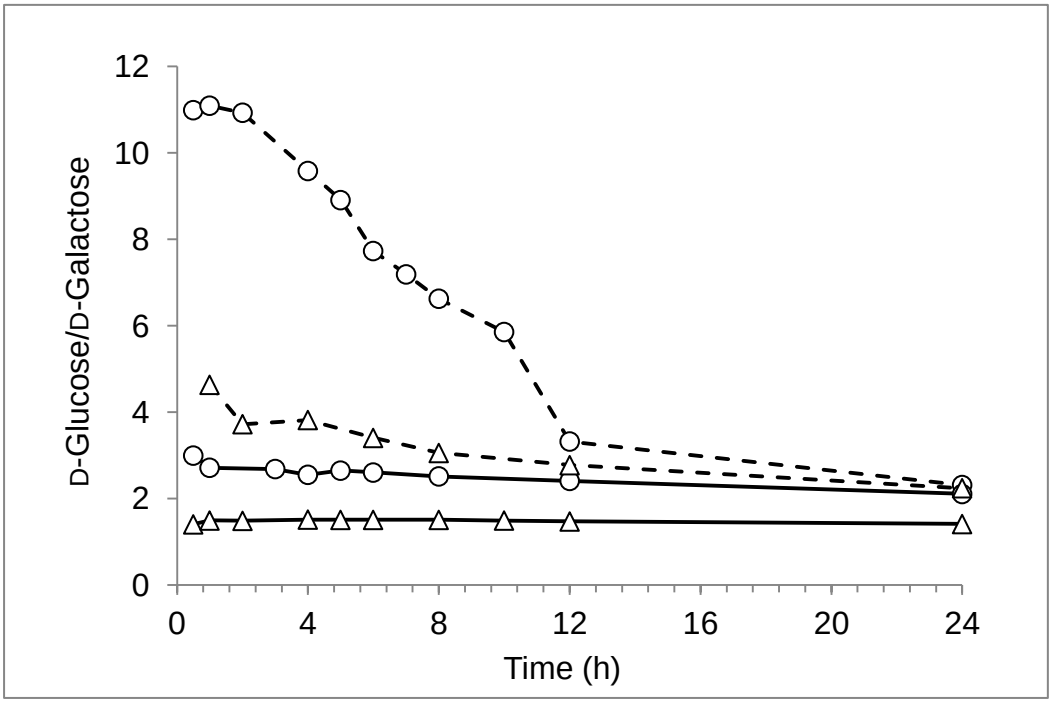

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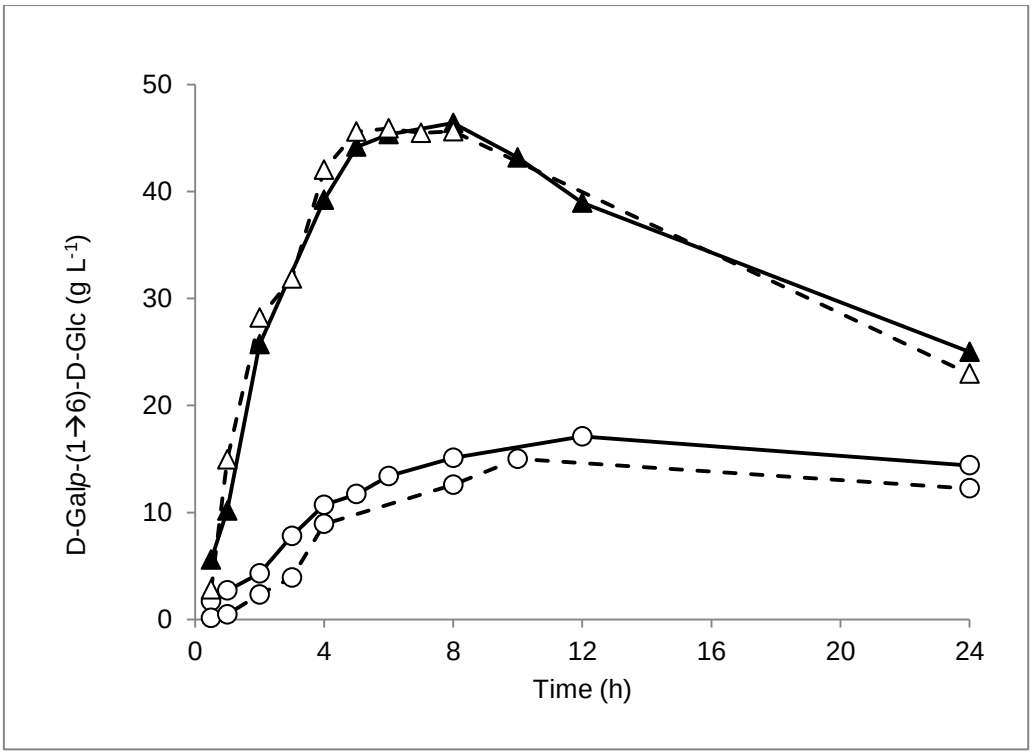

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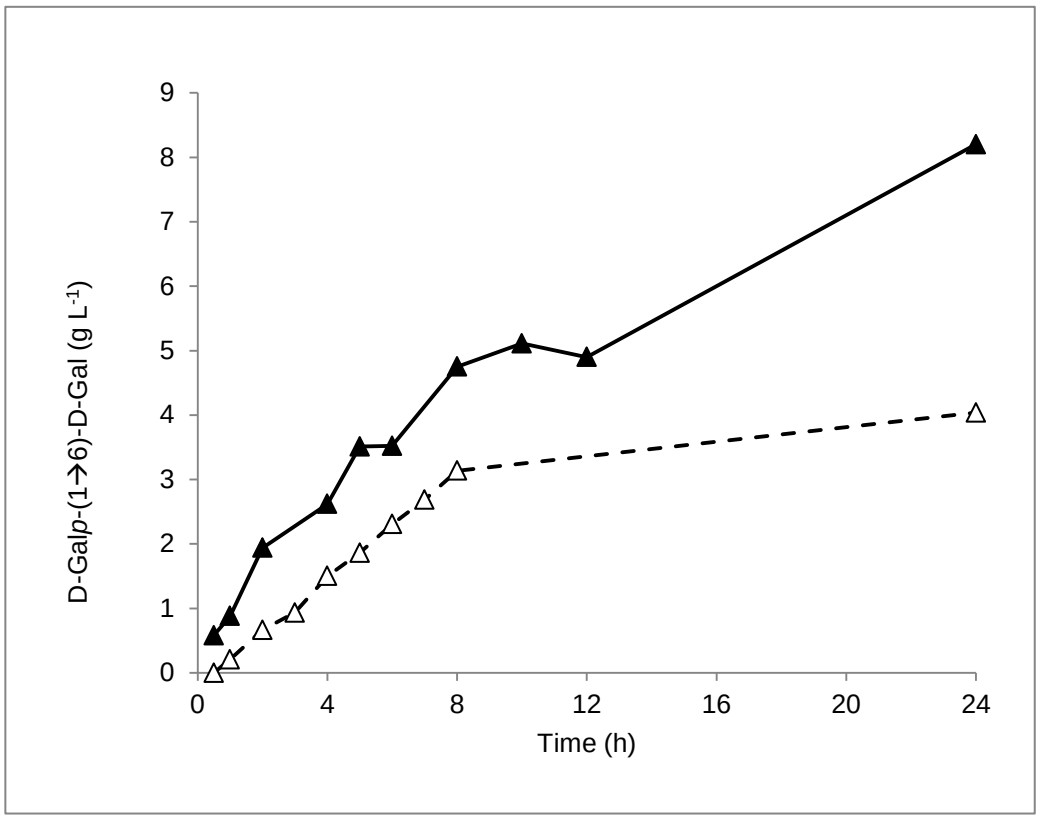

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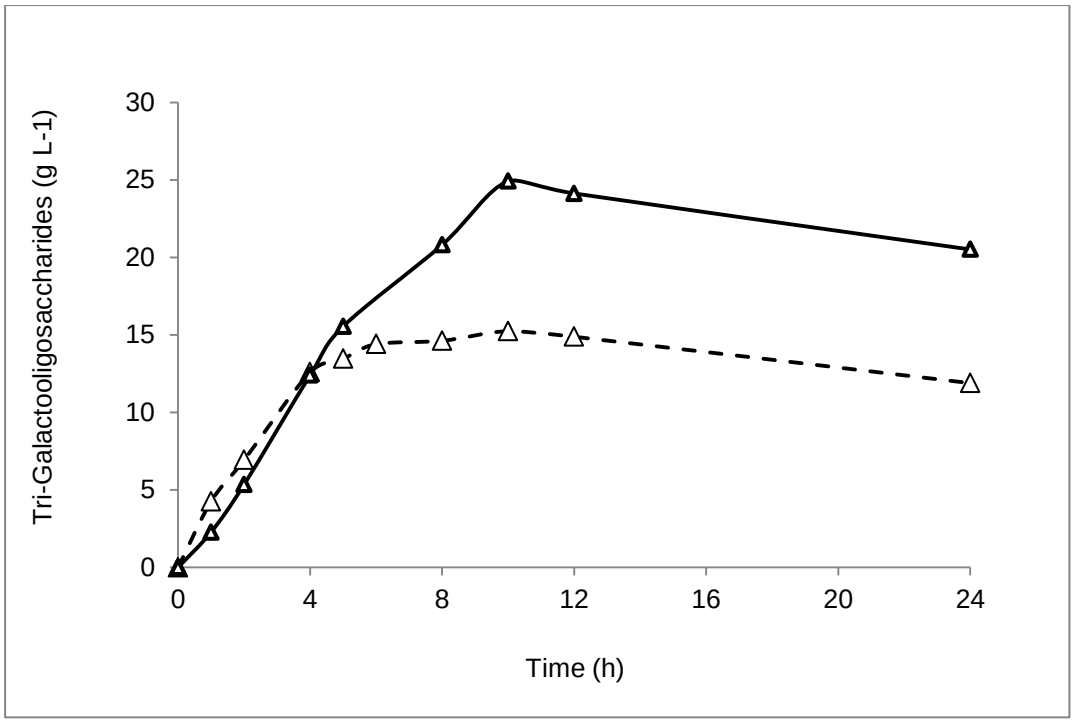

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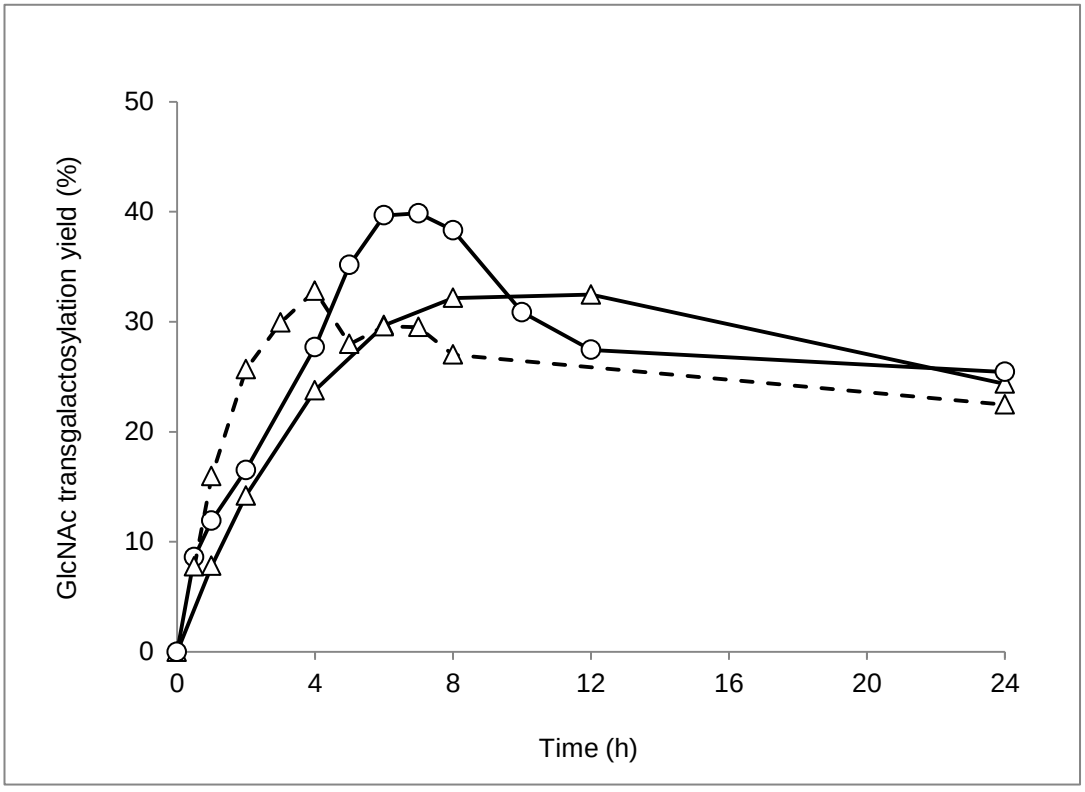

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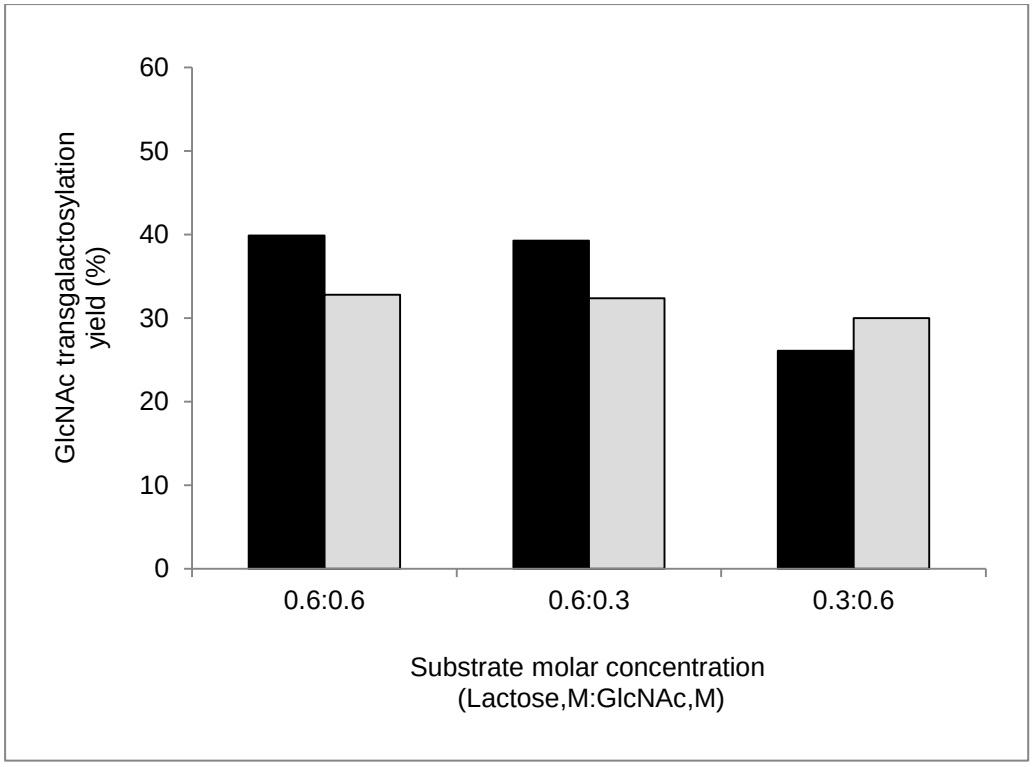

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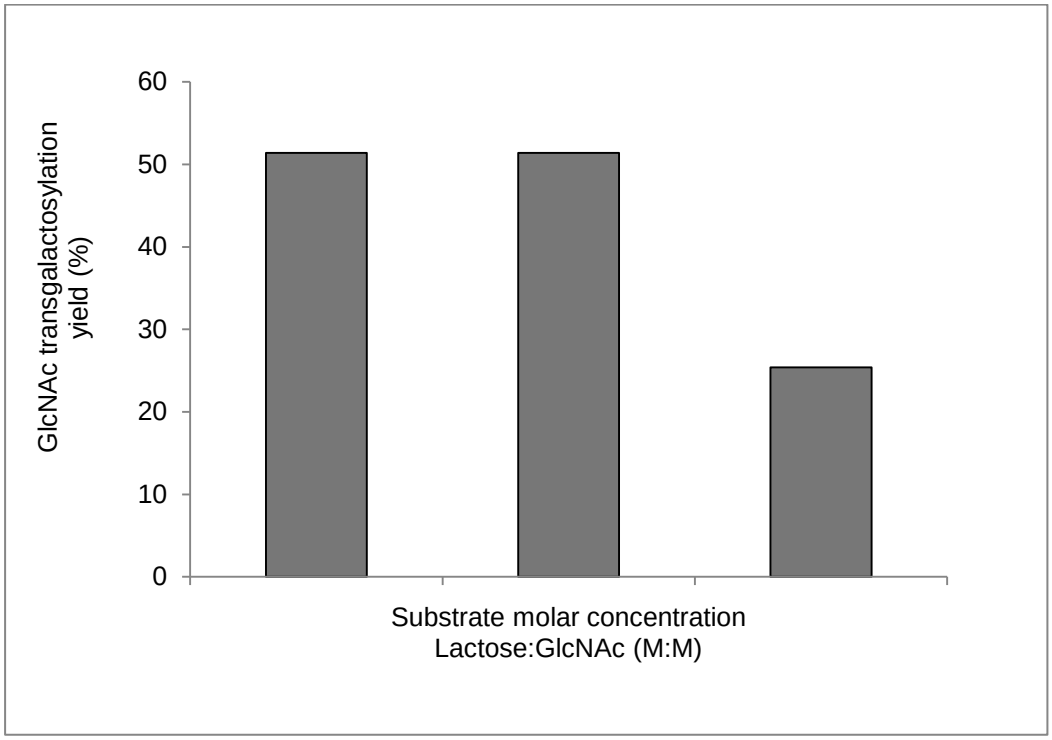

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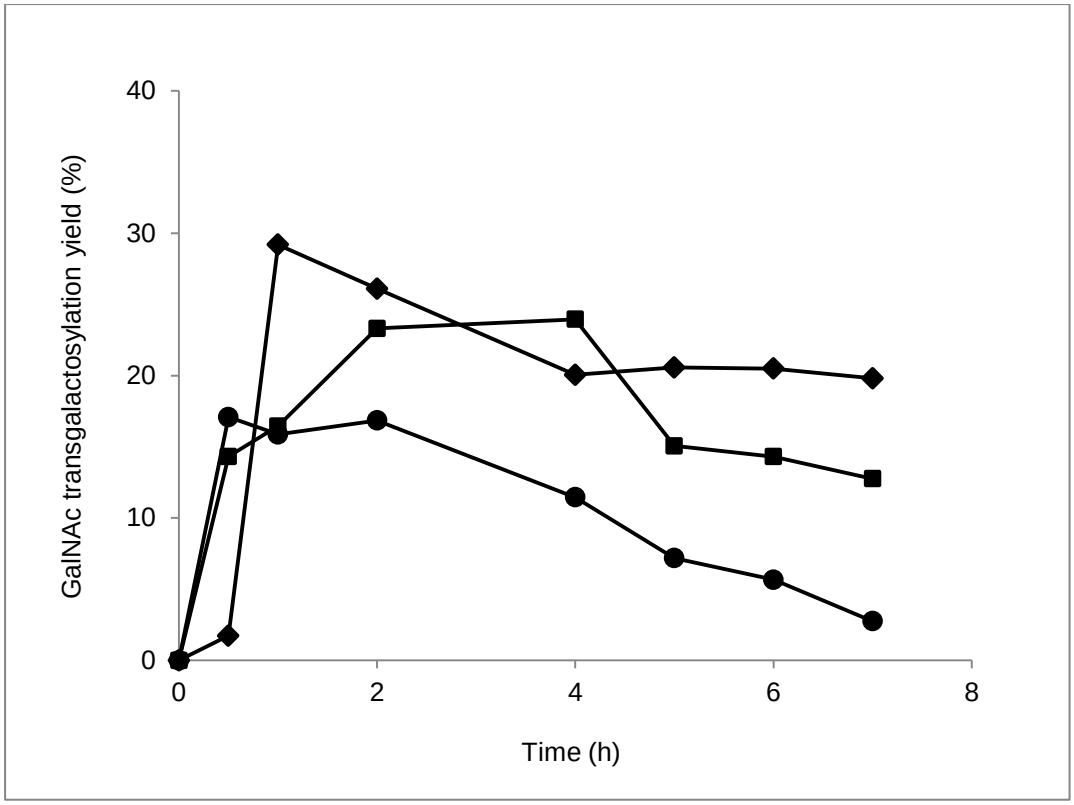

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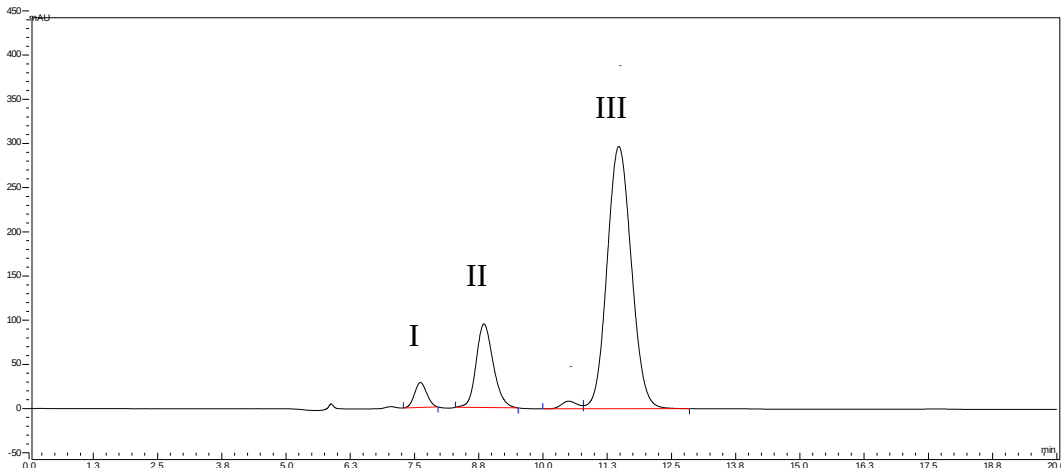

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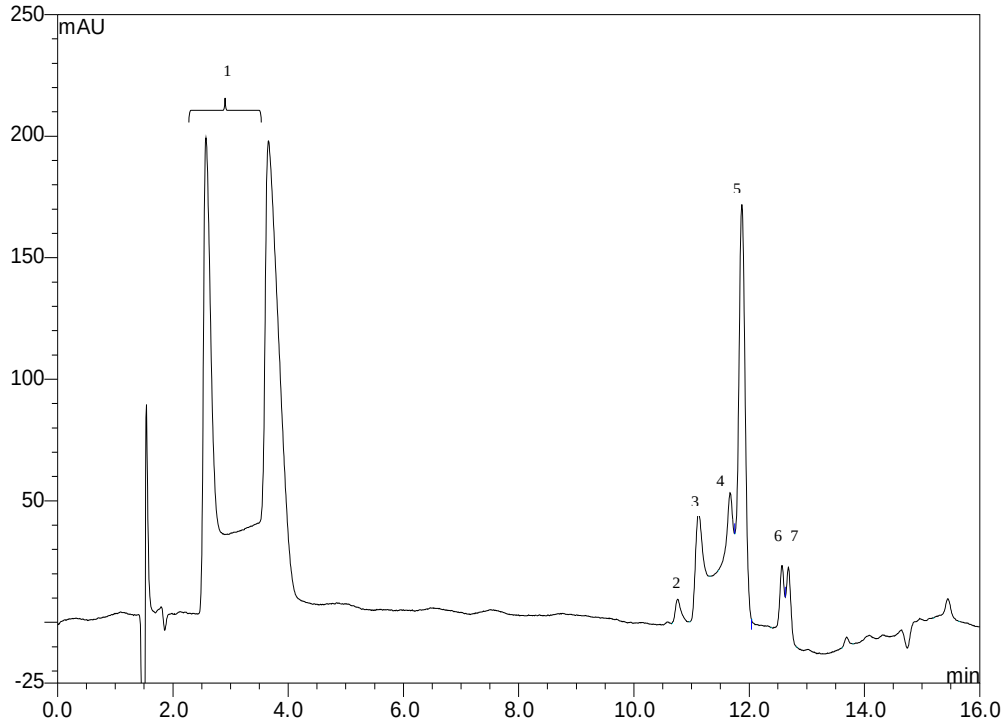
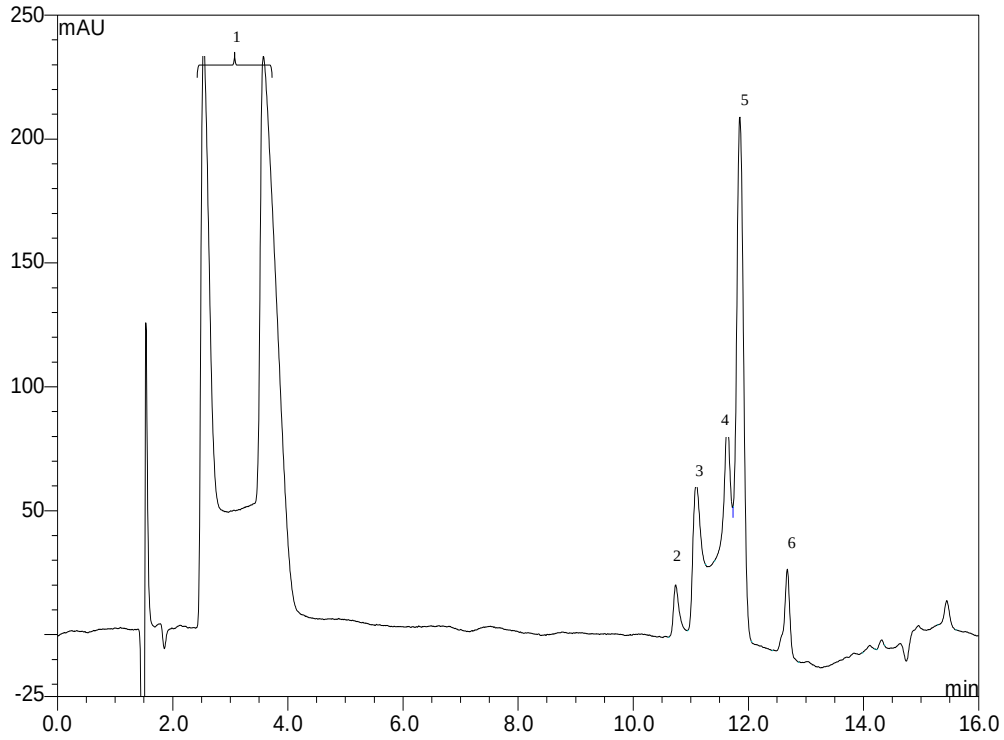

mAU
250
200
150
100
50
-25
1
2
3
4
5
6 7
min
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0


mAU
250
200
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100
50
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1
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3
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min
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16.0

Figure 14.

1

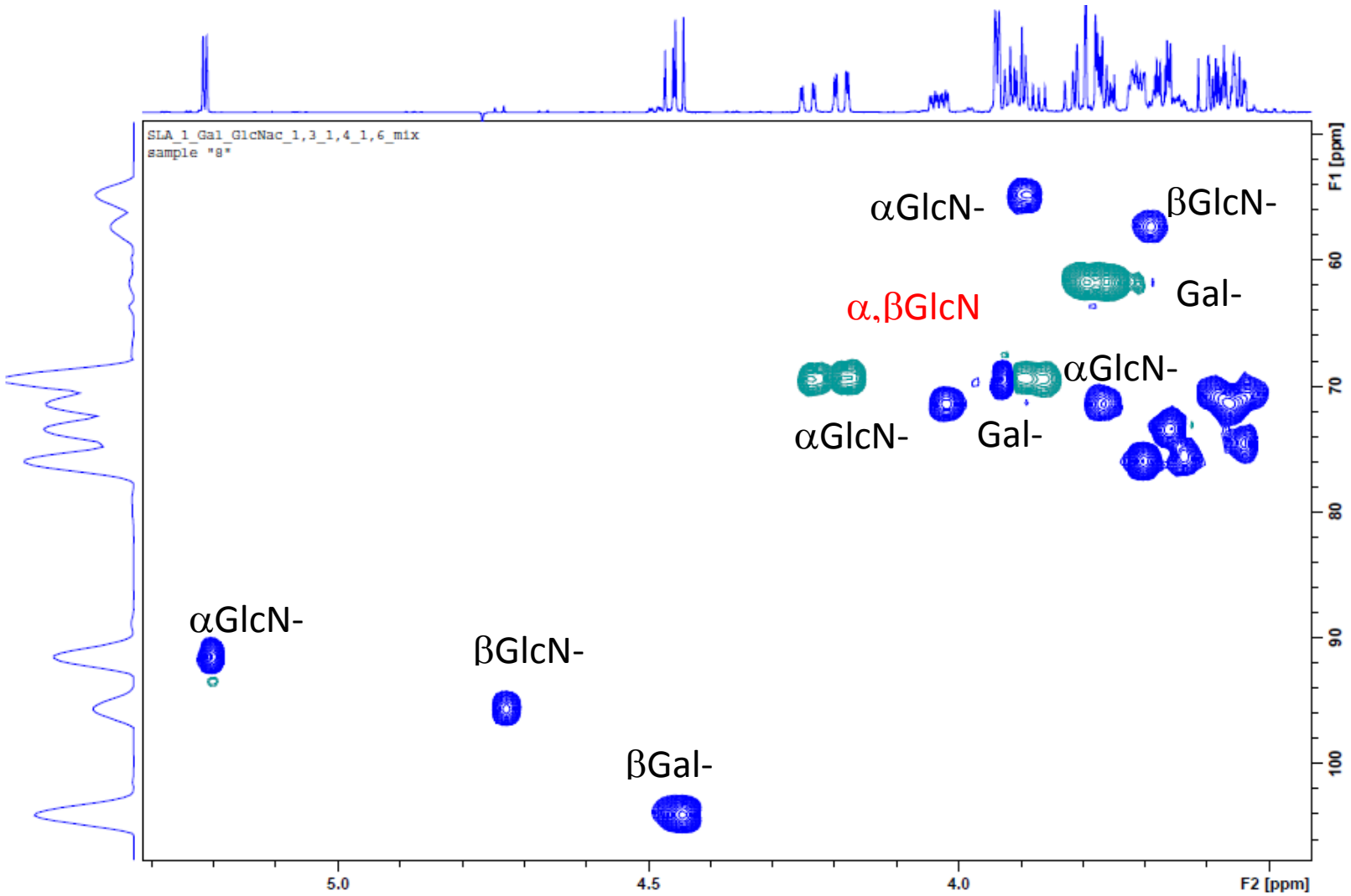
SLA_1_Gal_GlcNac_1,3_1,4_1,6_mix
sample "8"
αGlcN-
βGlcN-
Gal-
α,βGlcN
αGlcN-
αGlcN-
Gal-
αGlcN-
βGlcN-
βGal-
5.0
4.5
4.0
F2 [ppm]
F1 [ppm]
60
70
80
90
100

Figure 15.

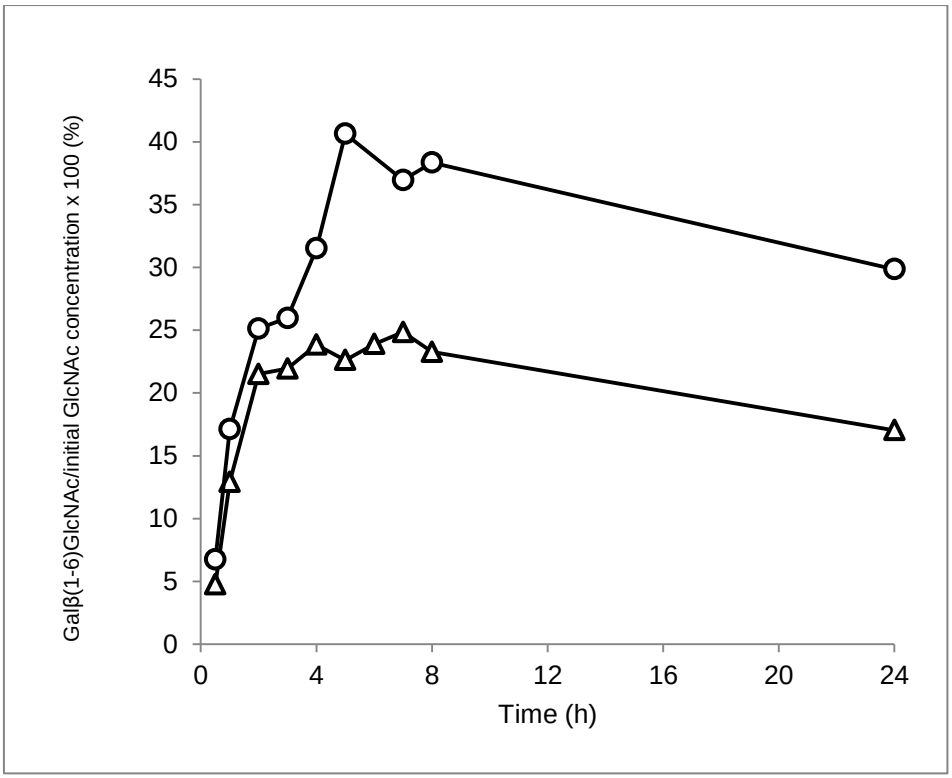
Galβ(1-6)GlcNAc/initial GlcNAc concentration x 100 (%)
45
40
35
30
25
20
15
10
5
0
0
4
8
12
16
20
24
Time (h)

Figure 16.