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Production of Prebiotic Galacto-Oligosaccharides from Lactose Using β-Galactosidases from *Lactobacillus reuteri*

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The β-galactosidases (β-Gals) of *Lactobacillus reuteri* L103 and L461 proved to be suitable biocatalysts for the production of prebiotic galacto-oligosaccharides (GOS) from lactose. Maximum GOS yields were 38% when using an initial lactose concentration of 205 g/L and at ∼80% lactose conversion. The product mixtures were analyzed by capillary electrophoresis (CE) and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Disaccharides other than lactose and trisaccharides made up the vast majority of GOS formed. The main products were identified as β-D-Gal*p*-(1→6)-D-Glc (allolactose), β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→6)-Lac, and β-D-Gal*p*-(1→3)-Lac. There were no major products with β1→4 linkages formed. Both intermolecular and intramolecular transgalactosylation were observed. D-Galactose proved to be a very efficient galactosyl acceptor; thus, a relatively large amount of galactobioses was formed. Monosaccharides could be conveniently separated from the mixture by chromatography using a strong cation-exchange resin.

KEYWORDS: ***Lactobacillus*; β-galactosidase; galacto-oligosaccharides; prebiotics; transgalactosylation**

INTRODUCTION

Galacto-Oligosaccharides. In recent years, much investigation has been carried out in the field of pro- and prebiotics as functional foods. Galacto-oligosaccharides (GOS) are used as nondigestible, carbohydrate-based food ingredients in human and animal nutrition. Their ability to promote the proliferation of intestinal bifidobacteria and lactobacilli has been recognized (*1*, *2*). The predominance of bifidobacteria in the colon has been suggested to cause beneficial effects for maintaining human health, providing protection from infection, and facilitating the normal functions of the gut.

Apart from their proposed effects on health, GOS have certain other useful properties. Their stability under acidic conditions during food processing makes them potentially applicable as ingredients for a wide variety of food products. Their excellent taste quality and relatively low sweetness make GOS interesting functional sweeteners. They pass the small intestine without being digested and are therefore of low caloric value. In addition, GOS cannot be metabolized by microorganisms of the oral cavity and are thus not implicated in the formation of dental caries (*1*, *3*−*5*).

Transgalactosylation. Galacto-oligosaccharides are the products of transgalactosylation reactions catalyzed by β-galactosidases when using lactose or other structurally related galactosides as the substrate. β-Galactosidases are generally classified as hydrolases. In fact, hydrolysis of the glycosidic bond is a special case of transgalactosylation in which the galactosyl acceptor is water (*6*). Scheme 1 illustrates the possible lactose conversion reactions catalyzed by β-galactosidases.

Transgalactosylation is thought to involve intermolecular as well as intramolecular reactions. Intramolecular or direct galactosyl transfer to D-glucose yields regioisomers 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 (*5*, *7*, *8*). By intermolecular transgalactosylation, di-, tri-, and tetrasaccharides and eventually higher oligosaccharides are produced. Any sugar molecule in the reaction mixture can be the nucleophile to accept the galactosyl moiety from the galactosyl−enzyme complex. The GOS produced can be regarded as kinetic intermediates as they are also substrates

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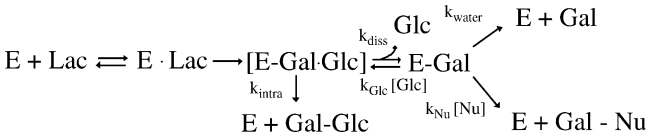
Scheme 1. Hydrolysis and Galactosyl Transfer Reactions, both Intra- and Intermolecular, during the Conversion of Lactose Catalyzed by β-Galactosidases[a]



[a] E, enzyme; Lac, lactose; Gal, galactose; Glc, glucose; Nu, nucleophile (*5*).

for hydrolysis (*5*, *6*, *9*, *10*). For all these reasons, the GOS yield and composition change dramatically with reaction time, are very complex, and can hardly be predicted.

The chemical structure and composition of GOS greatly depend on the enzyme source (*6*, *8*), and as they are supposed to selectively stimulate probiotic bacteria in the gut, we used β-galactosidases from probiotic *Lactobacillus* species for the formation of GOS from lactose in this study. We speculate that lactobacilli possess β-galactosidases producing tailor-made GOS that are particularly advantageous for their own proliferation.

MATERIALS AND METHODS

Materials. 2-Aminopyridine (AP) was purchased from Fluka (Buchs, Switzerland). *o*-Nitrophenyl β-D-galactopyranoside (*o*NPG), sodium cyanoborohydride (95%), and acetic acid were supplied by Sigma (St. Louis, MO). Methanol was supplied by Roth (Karlsruhe, Germany), lactose by Merck (Darmstadt, Germany), and D-galactose by Fluka, and 4-O-β-D-galactopyranosyl-D-galactose and 3-*O*-β-D-galactopyranosyl-D-galactose were obtained as a mixture from Megazyme (Bray, Ireland) and used after further purification. Allolactose [β-D-Gal*p*-(1→6)-D-Glc] was a kind gift of S. Riva (CNR, Milan, Italy). Authentic samples of β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→6)-D-Lac, and β-D-Gal*p*-(1→3)-D-Lac were kindly provided by P. Kosma (Department of Chemistry, BOKU, Vienna, Austria). The galacto-oligosaccharide product Elix'or was supplied by Friesland Foods Domo (Zwolle, The Netherlands) and 4′GOS-P (β1−4-linked galacto-oligosaccharides) by Yakult Honsha (Tokyo, Japan). Glucose oxidase from *Aspergillus niger* and horseradish peroxidase were obtained from Boehringer (Mannheim, Germany).

Enzymes. β-Galactosidases were produced using two strains of *Lactobacillus reuteri*, L103 and L461, obtained from Lactosan Starterkulturen. Enzymes were produced by fermentation on a lactose-based medium and purified to homogeneity by hydrophobic interaction and affinity chromatography (*11*).

Standard β-Galactosidase Assay. β-Galactosidase activity was measured at 30 °C using *o*NPG as the substrate. The reaction was started by adding 20 μL of enzyme sample to 480 μL of 22 mM *o*NPG in buffer [50 mM sodium phosphate buffer (pH 6.5)] and stopped after exactly 10 min by adding 750 μL of 0.4 M Na_2CO_3. The absorbance of *o*NP is measured at 420 nm. One enzyme unit (U_{oNPG}) is defined as the amount of enzyme releasing 1 μmol of *o*NP per minute under the reaction conditions described above. All measurements and experiments were performed at least in duplicate, and the experimental error never exceeded 5%.

Enzyme Assay with Lactose. To determine the β-galactosidase activity with the natural substrate lactose, the assay described in ref *12* was used with slight modifications. Twenty microliters of enzyme sample was added to 480 μL of a substrate solution [600 mM lactose in 50 mM sodium phosphate buffer (pH 6.5)] and incubated at 30 °C for 10 min. The reaction was stopped by boiling the sample for 5 min. The amount of glucose released was measured with an enzymatic assay based on glucose oxidase and peroxidase (*13*). One lactose enzyme unit (U_{Lac}) refers to the amount of enzyme forming 1 μmol of D-glucose per minute under the reaction conditions described above.

Protein Measurement. The amount of protein was determined with the Bio-Rad Coomassie Blue reagent using BSA as the standard.

Monosaccharide Analysis. D-Glucose was measured enzymatically as described above. For the determination of the amount of D-galactose, a lactose/D-galactose test kit from Boehringer was used.

Precolumn Derivatization with 2-Aminopyridine for Capillary Electrophoresis (CE). The procedure for precolumn derivatization described by Petzelbauer et al. (*14*) was employed with some modifications. Ten microliters of a sample (up to 300 nmol of sugars) was dried under vacuum for 1 h at 60 °C using the SPD SpeedVac system (Thermo Savant). Twenty microliters of an aminopyridine solution (1 g of 2-aminopyridine in 470 μL of acetic acid and 600 μL of methanol) was added to the dry sample. The mixture was incubated on a thermo-block at 90 °C for 15 min. After the incubation time, the sample was placed under vacuum in a SPD SpeedVac system for 30 min at 60 °C for evaporating the excess of the reagents. Twenty-five microliters of 59 mg/mL (in 30% acetic acid) cyanoborohydride was added to the sample, and the sample was incubated for 30 min at 90 °C. Finally, the sample was dried under vacuum in a SPD SpeedVac system at 60 °C for 2 h and resuspended in 200 μL of deionized water.

Capillary Electrophoresis Conditions. A capillary electrophoresis system with a UV-DAD detector (Agilent Technologies, Palo Alto, CA) together with a fused silica capillary column (internal diameter of 25 μm) equipped with a bubble cell detection window (bubble factor of 5) was used for carbohydrate analysis. The capillary had a total length of 64.5 cm and an effective length of 56 cm. The capillary was preconditioned before each run by flushing with 50 mM phosphoric acid for 3 min followed by flushing with running buffer (100 mM phosphoric acid titrated with 1 M sodium hydroxide to pH 2.5) for 3 min. The sample was injected into the capillary at the anodic end by a positive pressure of 50 mbar for 5 s. The positive polarity mode and an operating temperature of 30 °C were employed. A current of 20 μA was applied after sample injection and kept constant during the run. The resulting voltage was approximately 23 kV. The detection wavelength was set at 240 nm with a bandwidth of 10 nm.

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). For eluent preparation, MilliQ water, 50% (w/v) NaOH, and NaOAc (Baker, Deventer, The Netherlands) were used. Two different combinations of four eluents were used for effective GOS separation. Eluent A (100 mM NaOH), eluent B (water), eluent C (100 mM NaOH and 1 M NaOAc), and eluent D (100 mM NaOH and 50 mM NaOAc) were mixed to form the following gradients: gradient 1, 100% A from 0 to 20 min and from 0 to 100% D from 20 to 70 min; and gradient 2, 15% A and 85% B from 0 to 70 min. After each run, the column was washed for 10 min with 100% C and re-equilibrated for 15 min with the starting conditions of the employed gradient. Boiled and centrifuged samples (20 μL) were injected via a Spark basic marathon autosampler, and separations were performed at a rate of 1 mL/min. Detection time and voltage parameters were set according to waveform A (*15*).

Thin Layer Chromatography (TLC). TLC was carried out using high-performance TLC silica plates (Kieselgel 60 F245, Merck). An appropriately diluted sample containing ≈20 g/L sugar was applied to the plate (1.2 μL) and eluted twice in ascending mode with an *n*-butanol/*n*-propanol/ethanol/water mixture (2/3/3/2). Thymol reagent was used for detection.

Analysis of Intermolecular Galactosyl Transfer under Defined, Initial-Velocity Conditions. Intermolecular transgalactosylation to lactose was assessed by varying the initial lactose concentration from 4 to 600 mM and incubation with β-galactosidase for 20 min at 30 °C in 50 mM sodium phosphate buffer (pH 6.5) containing 10 mM $MgCl_2$. The reaction was stopped by boiling for 5 min, and the amounts of D-glucose and D-galactose released were measured enzymatically. Galactosyl transfer to D-glucose was assessed by performing assays with 10 mM *o*NPG in buffer [50 mM sodium phosphate buffer

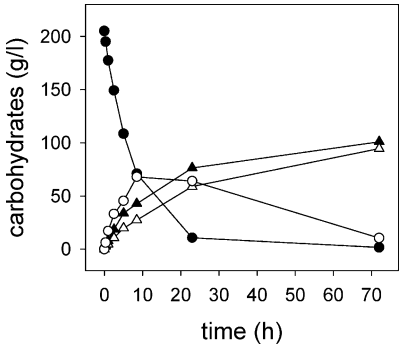


Figure 1. Course of reaction for lactose conversion in a discontinuous batch process. The reaction was carried out at 30 °C, using 50 mM sodium phosphate buffer (pH 6.5), 1 mM $MgCl_2$, and 0.8 $unit_{Lac}$/mL L103 enzyme: (●) lactose, (○) GOS, (▲) glucose, and (△) galactose. The amounts of released glucose and galactose were measured enzymatically; the amounts of lactose and GOS were measured by HPAEC–PAD and CE.

(pH 6.5)] and varying D-glucose concentrations. Reactions were stopped by heat after 10 min, half of the reaction mix was used for the enzymatic assessment of D-galactose, and to the other half was added 0.4 M Na_2CO_3 for measuring the amount of *o*NP released (see Standard *β*-Galactosidase Assay).

GOS Production. To produce GOS, discontinuous conversion reactions were carried out with *β*-galactosidases from L103 and L461 on a 2−20 mL scale. The influence of process parameters was studied by varying the initial substrate concentration (135, 300, and 600 mM lactose), pH (6 and 6.5), temperature (25, 30, and 37 °C), and buffer concentration (50 and 200 mM sodium phosphate buffer containing 1 mM $MgCl_2$). Agitation was applied at 300 rpm.

Removal of Monosaccharides and Fractionation of GOS. Separation of GOS from D-glucose and D-galactose was carried out as previously described (*16*) using the Unibead UBK-530 strongly acidic cation-exchange resin (Mitsubishi Chemical Industries). The freeze-dried and desalted sample was dissolved in water to contain approximately 70% (w/v) sugars, and 3.5 mL of the solution was applied to a column with effective dimensions of 2.5 cm × 200 cm. The operating temperature was 70 °C, and elution was carried out with water at a flow rate of 8.9 mL/min. Fractions of 17.8 mL were collected and analyzed by TLC. Pooled fractions were analyzed by CE and HPAEC−PAD.

RESULTS AND DISCUSSION

Production of GOS. To confirm the potential of the novel *β*-galactosidases described here for the production of GOS, a number of discontinuous conversion reactions were carried out employing 200 g/L lactose as the substrate. **Figure 1** shows substrate conversion and product formation of a typical batch reaction. As lactose is converted, not only D-glucose and D-galactose, the primary hydrolysis products, but also GOS are formed as a result of transgalactosylation catalyzed by the enzyme (Scheme 1). After reaction between 8 and 20 h, a maximum GOS yield of ∼70 g/L is reached. GOS are no end products; they are only transiently formed as they are also subject to hydrolysis which becomes more and more pronounced toward the end of the reaction when the substrate lactose becomes depleted. Therefore, the amount and composition of GOS change dramatically with the degree of substrate conversion which is illustrated in **Figure 2**. Up to ∼80% lactose conversion, the amount of GOS, expressed by their relative concentration (percentage of GOS of total sugars), is constantly rising. After that hydrolysis prevails over synthesis, which eventually leads to a totally hydrolyzed product consisting of equimolar amounts of D-glucose and D-galactose. When taking

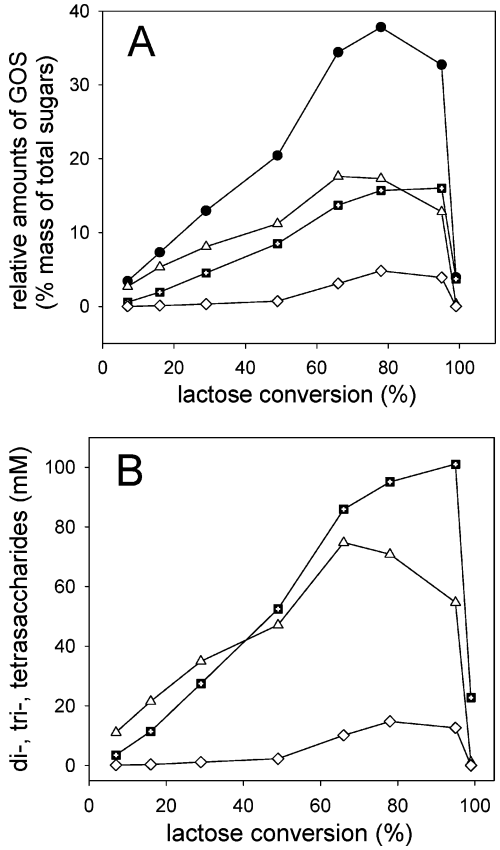


Figure 2. Formation and degradation of GOS during lactose conversion by L103 *β*-galactosidase. The reaction was performed at 30 °C at an initial lactose concentration of 205 g/L (600 mM) in 50 mM sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$: (●) total GOS, () disaccharides, (△) trisaccharides, and (◇) tetrasaccharides.

a look at the size distribution within the GOS mixture, one can see that at the beginning of the reaction trisaccharides dominate (**Figure 2**). This is not surprising as at that time of reaction lactose is by far the most abundant sugar species in the mixture that can act as a galactosyl acceptor. As lactose conversion proceeds, the amount of hydrolysis products D-glucose and D-galactose increases, and via transgalactosylation, disaccharides other than lactose are formed. The GOS produced also act as galactosyl acceptors, resulting in an increasingly complex saccharide mixture. Disaccharides become the dominant species by weight at ∼80% lactose conversion (**Figure 2A**). However, when the molar distribution of different oligosaccharide species is examined, the disaccharides exceed the trisaccharides at a conversion level as low as 40% (**Figure 2B**). This seems surprising as at this time the reaction mixture still contains 1.8 times more lactose and other disaccharide molecules than monosaccharides. It is also striking that disaccharides are formed right from the beginning of the reaction when there are hardly any monosaccharide galactosyl acceptors available. Intramolecular transgalactosylation (*7*) and different transfer rates for different acceptors (*12*, *17*) are to some extent responsible for these phenomena as discussed below in more detail; on the other hand, disaccharides are intermediates of trisaccharide degradation as well (*10*). Amounts of total GOS and di-, tri-, and tetrasaccharides were determined by CE which makes the easy classification of sugar products possible. Using the method described here, the sugars elute in groups depending on their degree of polymerization. **Figure 3** shows a typical electropherogram.

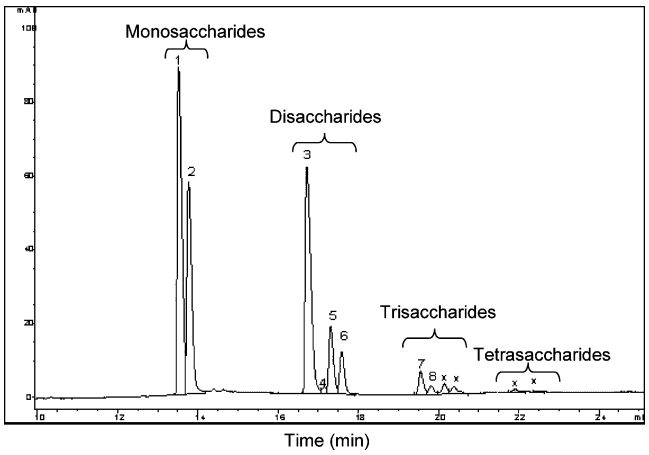


Figure 3. Separation and quantification by capillary electrophoresis of individual GOS produced during the lactose conversion catalyzed by L103 or L461 β-galactosidase. The sample presents a mixture of sugars obtained after the reaction of L103 β-Gal with 205 g/L lactose. The extent of substrate conversion is approximately 67%. The identified compounds are indicated: (1) glucose, (2) galactose, (3) lactose, (4) D-Gal*p*-(1→3)-D-Glc, (5) D-Gal*p*-(1→6)-D-Glc (allolactose) with D-Gal*p*-(1→3)-D-Gal, (6) D-Gal*p*-(1→6)-D-Gal, (7) D-Gal*p*-(1→6)-Lac, and (8) D-Gal*p*-(1→3)-Lac. Products marked with an x are minor components and were not identified. Peaks appearing at ~22 min are tetrasaccharides.

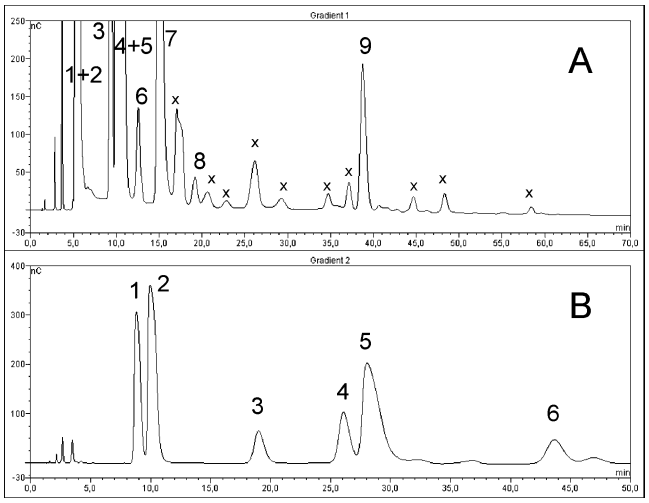


Figure 4. Separation and quantification by HPAEC–PAD of individual GOS produced during the lactose conversion catalyzed by L103 or L461 β-galactosidase using gradient 1 (**A**) and gradient 2 (**B**). The sample presents a mixture of sugars obtained after the reaction of L103 β-Gal with 205 g/L lactose. The extent of substrate conversion is approximately 78%. The identified compounds are indicated: (1) galactose, (2) glucose, (3) D-Gal*p*-(1→6)-D-Gal, (4) D-Gal*p*-(1→6)-D-Glc (allolactose), (5) lactose, (6) D-Gal*p*-(1→3)-D-Gal, (7) D-Gal*p*-(1→6)-Lac, (8) D-Gal*p*-(1→3)-D-Glc, and (9) D-Gal*p*-(1→3)-Lac. Products marked with an x were not identified.

Major Transferase Products and Their Formation during Lactose Hydrolysis. Individual GOS can be separated very effectively when using a Carbopac PA1 column for HPAEC with pulsed amperometric detection as shown in **Figure 4**. With authenticated standards and the standard-addition technique, it was possible to identify the main products of transgalactosylation by L103 and L461 β-galactosidase. They are β-D-Gal*p*-(1→6)-D-Glc (allolactose), β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→6)-Lac, and β-D-Gal*p*-(1→3)-Lac. Two different gradients were necessary for the quantification of the sugar mixture, one optimized for the separation of the whole product spectrum (**Figure 4A**) and one designed to separate D-glucose from D-galactose, and allolactose from lactose (**Figure 4B**). **Figure 5** shows the changes in the concentrations of the major components of the GOS mixture with lactose conversion. At the beginning of the reaction, the trisaccharide β-D-Gal*p*-(1→6)-Lac dominates, with respect to molarity (**Figure 5B**) and even more pronouncedly in percent mass per mass of total GOS (**Figure 5A**). β-D-Gal*p*-(1→3)-Lac is the second most important trisaccharide product. The disaccharides allolactose and β-D-Gal*p*-(1→6)-D-Gal become more and more important toward the end of the reaction, indicating that they are less prone to hydrolysis than the trisaccharides.

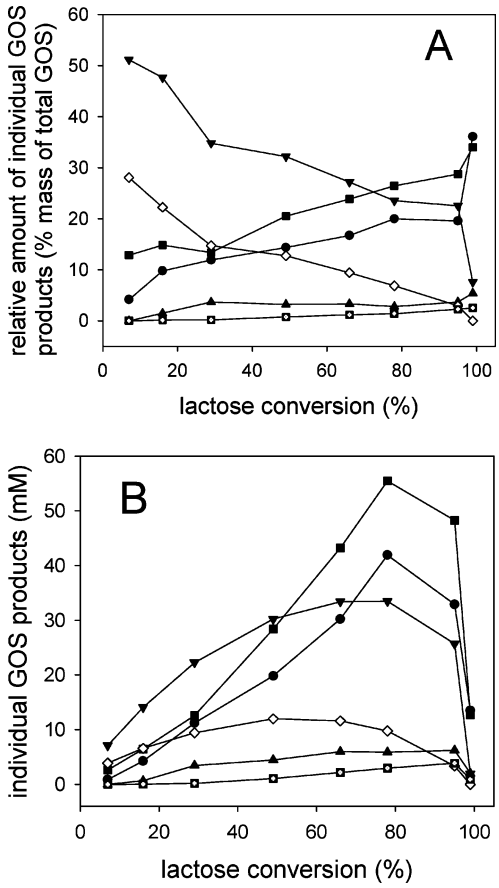


Figure 5. Formation and degradation of individual GOS during lactose conversion by L103 β-galactosidase. The reaction was performed at 30 °C at an initial lactose concentration of 205 g/L in 50 mM sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$: (▼) D-Gal*p*-(1→6)-Lac, (■) D-Gal*p*-(1→6)-D-Glc (allolactose), (●) D-Gal*p*-(1→6)-D-Gal, (◇) D-Gal*p*-(1→3)-Lac, (▲) D-Gal*p*-(1→3)-D-Gal, and () D-Gal*p*-(1→3)-D-Glc.

In general, β-galactosidases of L103 and L461 have a high specificity for the formation of β1→6 linkages as the three identified transglycosylation products β-D-Gal*p*-(1→6)-D-Glc, β-D-Gal*p*-(1→6)-D-Gal, and β-D-Gal*p*-(1→6)-D-Lac make up at least 60% of total GOS during the whole reaction. β1→3 linkages seem to be the second most important and represent approximately 16% of GOS on average. Interestingly, no β-D-Gal*p*-(1→4)-D-Gal could be detected, and the spectrum of 4′GOS-P (Yakult Honsha), which is reported to consist of mainly β1→4 linked oligosaccharides, is very different from our product (**Figure 11**). Looking at the ratio of β1→6 to β1→3 linkages at the level of individual sugar species (**Figure 6**), one can see that the different product couples behave quite differ-

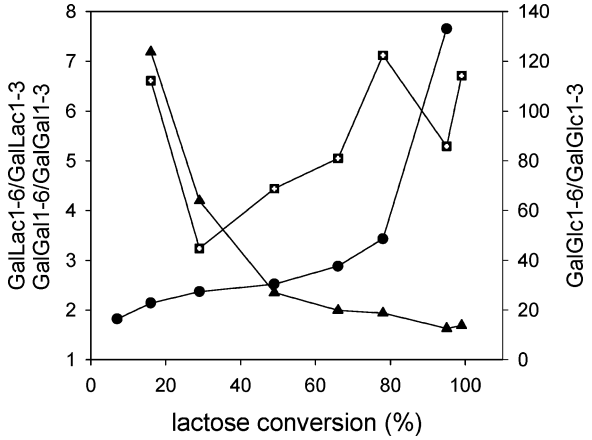


Figure 6. Changes in ratios of 1–6 and 1–3 linked GOS during conversion of lactose. The reaction was performed at 30 °C at an initial lactose concentration of 205 g/L in 50 mM sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$: (●) D-Gal*p*-(1→6)-Lac/D-Gal*p*-(1→3)-Lac, (▲) D-Gal*p*-(1→6)-D-Glc/D-Gal*p*-(1→3)-D-Glc, and (□) D-Gal*p*-(1→6)-D-Gal/D-Gal*p*-(1→3)-D-Gal.

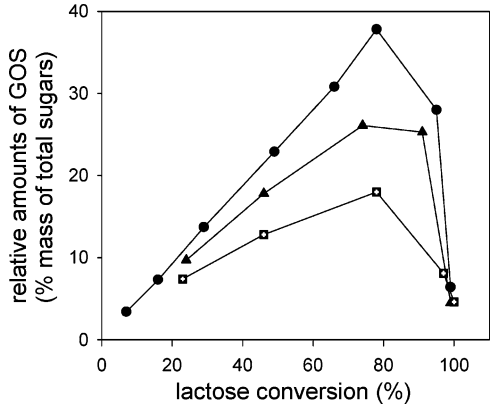


Figure 7. Formation of GOS during lactose conversion at different initial lactose concentrations by L103 β-Gal. The reactions were performed at 30 °C in 50 mM sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ using 205 (●), 103 (▲), and 46 g/L (□) lactose.

ently. While the β-D-Gal*p*-(1→6)-D-Lac/β-D-Gal*p*-(1→3)-Lac ratio increases drastically toward the end of the reaction from ~2 to 8, the β-D-Gal*p*-(1→6)-D-Glc/β-D-Gal*p*-(1→3)-Glc ratio is ~130 at the beginning of the conversion and levels off to 15 at the end. For the β-D-Gal*p*-(1→6)-D-Gal/β-D-Gal*p*-(1→3)-Gal ratio, there is no clear trend; this ratio is between 3 and 7 throughout the whole reaction. It can be concluded that the specificity of the glycosidic bond formed by the *L. reuteri* β-galactosidases strongly depends on the galactosyl acceptor.

Influence of Process Parameters on GOS Production. The influence of lactose concentration, temperature, pH, and phosphate buffer concentration on GOS production by L103 and L461 β-galactosidase was investigated. For all conditions that were chosen, enzyme activity was stable throughout the whole conversion reactions. As found by many other authors as well [reviewed by Prenosil (*9*) and Mahoney (*8*)], the lactose concentration has a significant impact on GOS yield (**Figure 7**). For initial lactose concentrations of 205, 103, and 46 g/L, the maximum GOS yields were 38, 26, and 18%, respectively. Compared to those of other β-galactosidases, a yield of 38% GOS is in the upper range of reported results (*8*). Because of the very narrow pH range (pH 6−6.5) in which the enzyme is most stable (*11*), we were very limited in variation of pH. No significant difference in GOS production and composition could be detected between pH 6 and 6.5 (data not shown). Chockchaisawasdee et al. (*18*) described the use of 0.2 M phosphate buffer as a synthesis buffer, but in our hands, no significant increase in GOS yield occurred when the phosphate buffer concentration was increased from 50 to 200 mM. In fact, it had a slight negative effect on the reaction rate (data not shown). The impact of different process temperatures (25, 30, and 37 °C) on GOS formation was also investigated. Using temperatures higher than 37 °C was not possible due to the lack of enzyme thermostability. Apart from accelerated reaction rates at elevated temperatures, no effect on GOS yield or composition was observed.

Intermolecular Transgalactosylation. The intermolecular transfer of galactose to acceptors other than water typically presents the major pathway for the formation of GOS during lactose hydrolysis (see Scheme 1). As the sugar composition, which is also the acceptor composition, changes constantly during the reaction, an exact prediction of product formation and degradation cannot be made. However, the partitioning of galactosylated enzyme between the reaction with water, and hence hydrolysis, and the reaction with the major acceptors lactose and D-glucose can be studied under defined initial velocity conditions. When complete hydrolysis of the disaccharide lactose occurs, equimolar amounts of D-glucose and D-galactose are formed; therefore, the velocities at which the two sugars are released are identical, and the v_{Glu}/v_{Gal} ratio is 1.0. In the presence of high concentrations of Nu (e.g., a sugar acceptor for the galactose), v_{Glu}/v_{Gal} will increase. Richard et al. (*17*) derived eq 1 from Scheme 1, where the rate constant ratio k_{Nu}/k_{water} is obtained as the slope from the linear correlation of v_{Glu}/v_{Gal} with increasing concentrations of Nu. When *o*NPG was used as the substrate, v_{oNP}/v_{Gal} was measured.

$$v_{Glu}/v_{Gal} = 1 + k_{Nu}[Nu]/k_{water} \quad (1)$$

These rate constant ratios can therefore be used as a measure of the ability of a certain substance to act as a galactosyl acceptor (i.e., nucleophile) which allows an estimation of the level of transgalactosylation products obtained of a known reaction mixture. When the process described here is examined, the main candidates for galactosyl acceptors are the substrate lactose and the hydrolysis products D-glucose and D-galactose. For the first two, the rate constant ratios were determined, giving very similar results for the β-galactosidases from L103 and L461. The k_{Lac}/k_{water} ratios were 1.6 ± 0.1 and 1.7 ± 0.1 M^{-1}, respectively (**Figure 8A**). Interestingly, the rate constants k_{Glc}/k_{water} were 6.7 ± 0.3 and 6.2 ± 0.1 M^{-1}, respectively (**Figure 8B**), indicating that D-glucose is an ~4-fold better galactosyl acceptor than lactose. Therefore, disaccharides other than lactose will make up a large proportion of the obtained GOS mixture. For a known composition of a reaction mixture, one can estimate the relative extent of galactosyl transfer to the substrate (lactose) and the D-glucose product using $(k_{Lac}[Lac]/k_{water})/(k_{Glc}[Glc]/k_{water})$. Unfortunately, k_{Gal}/k_{water} could not be determined as the amount of galactose released cannot be measured in the presence of an excess of free galactose. However, we assume that galactose is an even better galactosyl acceptor than glucose because of the large amounts of galactobioses in the product mixture. **Figure 9** shows the Glc/Gal and GalGlc/GalGal ratios during the reaction. Between 20 and 80% lactose conversion, the galactosyl transfer to glucose is only 1.25 times higher than that to galactose even though there is 1.7 times more glucose than galactose in the reaction mixture. Furthermore, intra-

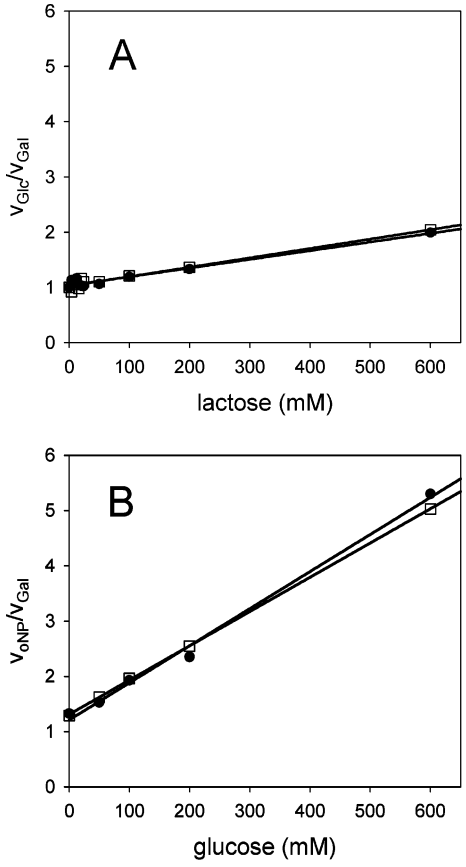


Figure 8. Transgalactosylation activity of L103 and L461 β-Gal as a function of the initial concentration of (**A**) lactose and (**B**) D-glucose. The reactions with L103 β-Gal (●) and L461 β-Gal (□) were carried out for 20 min at 30 °C, using different concentrations of lactose, or different concentrations of D-glucose and 10 mM *o*NPG in 50 mM sodium phosphate buffer (pH 6.5).

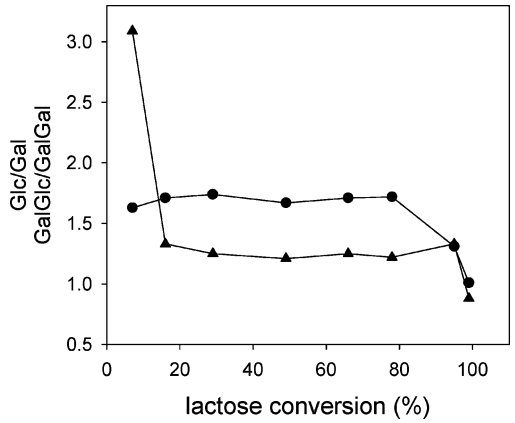


Figure 9. D-Glucose/D-galactose (●) and GalGlc/GalGal (▲) ratios during lactose conversion by L103 β-Gal. The reaction was performed at 30 °C at an initial lactose concentration of 205 g/L in 50 mM sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$.

molecular transgalactosylation to glucose as described in the next paragraph also contributes to the formation of GalGlc reaction products.

Intramolecular Transgalactosylation. A reason disaccharides can occur even at the beginning of lactose conversion in the absence of significant amounts of monosaccharide galactosyl acceptors is the so-called intramolecular or direct transgalactosylation (*7*). In this reaction pathway, the noncovalently enzyme bound glucose is not released into the solution but is directly linked to the galactosyl−enzyme intermediate (Scheme 1). **Figure 9** reveals that at a lactose conversion level of 6% the ratio between GalGlc disaccharides and GalGal disaccharides is more than 2 times higher than later in the reaction. At this point, the reaction mixture is made up of 566 mM lactose (of initially 600 mM), 22 mM glucose, 15 mM galactose, 11 mM GalLac trisaccharides, 2.6 mM allolactose, and 0.8 mM β-D-Gal*p*-(1→6)-D-Gal. If only intermolecular galactosyl transfer took place, the formula $(k_{Lac}[Lac]/k_{water})/(k_{Glc}[Glc]/k_{water})$ (as described before) could be applied. This would give a galactosyl transfer rate ratio of lactose and glucose of 6.8. However, the GalLac/GalGlc ratio as analyzed with HPAEC−PAD is only 4.2, which implies that there must be significant intramolecular transgalactosylation as well. This value of 4.2 represents the whole reaction up to 6% lactose conversion, beginning right at the start of reaction when there is no free glucose available to act as a galactosyl acceptor for intermolecular transgalactosylation which even more strongly indicates considerable intramolecular transgalactosylation. Assuming that transfer to galactose is occurring at the same rate as transfer to glucose as discussed before, intramolecular galactosyl transfer accounts for at least 9% of galactosyl transfer at 6% lactose conversion. Later in the reaction, the influence and extent of intramolecular transgalactosylation cannot be determined due to the increasing complexity of the product spectrum. Each sugar species is galactosylated and hydrolyzed at an unknown specific rate.

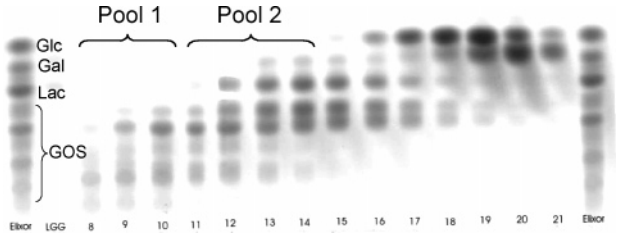


Figure 10. TLC of GOS fractions after separation on a UBK530 column.

Removal of Monosaccharides and Fractionation of GOS. The product of a 20 mL discontinuous lactose conversion reaction [600 mM lactose, 50 mM sodium phosphate buffer (pH 6.5), and 1 mM $MgCl_2$ at 30 °C] stopped at 94% conversion was desalted and applied to a UBK530 column. Fractions were collected and analyzed by TLC, and fractions containing no monosaccharides were pooled in two portions (**Figure 10**). Pool 1 contained ∼79% trisaccharides and 19% tetrasaccharides and pool 2 20% lactose, 56% disaccharides, and 24% trisaccharides. Together, they contain 0.1% of the monosaccharides, 50% of the lactose and disaccharides, 82% of the trisaccharides, and all of the tetrasaccharides of the initially applied sample. In other words, 63% of the total GOS could be separated from the monosaccharides in one simple chromatographic step. In **Table 1** are listed the compositions of the initial sample, the pools, and the commercially available products Elix'or (Friesland Foods Domo) and Oligomate 50 (Yakult Honsha).

Comparison of *Lactobacillus* GOS with Commercially Available GOS Products. We were interested in determining whether the GOS product described here differs from other GOS products already on the market. **Table 1** shows that the L103 product at 78% lactose conversion is more similar to Oligomate 50 than to Elix'or with respect to the distribution of mono-, di-, and oligosaccharides. Both commercial products contain more tetrasaccharides and higher oligosaccharides than the sample obtained in our experiments. When comparing the different products at the level of sugar species as analyzed by

Table 1. Composition of Different GOS Mixtures (% of total sugar)

	Elix'or	Oligomate 50[a]	L103[b]	L461[c]	pool 1[d]	pool 2[e]	pools 1 and 2[f]
monosaccharides	20	37.5	40.2	63.32	0.00	0.44	0.3
lactose	20	11.5	22.0	6.0	1.4	19.9	13.9
disaccharides	19.8	15	15.7	16.27	1.13	55.80	38.2
trisaccharides	23.4	18	17.3	10.78	78.43	23.81	41.4
tetrasaccharides	10.8	12.6	4.8	2.27	19.05	0.00	6.1
pentasaccharides	6	5.4	0	0.00	0	0.00	0.0

[a] Data obtained from ref *5*. [b] Product of a discontinuous conversion reaction using L103 β-Gal and 205 g/L lactose at 30 °C in 50 mM sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ at 78% lactose conversion. [c] Product of a discontinuous conversion reaction using L461 β-Gal and 205 g/L lactose at 30 °C in 50 mM sodium phosphate buffer (pH 6.5) and 1 mM $MgCl_2$ at 94% lactose conversion. [d] Pool 1 after the sample described in footnote c had been applied to a UBK530 chromatographic column. [e] Pool 2 after the sample described in footnote c had been applied to a UBK530 chromatographic column. [f] Mixture of pools 1 and 2.

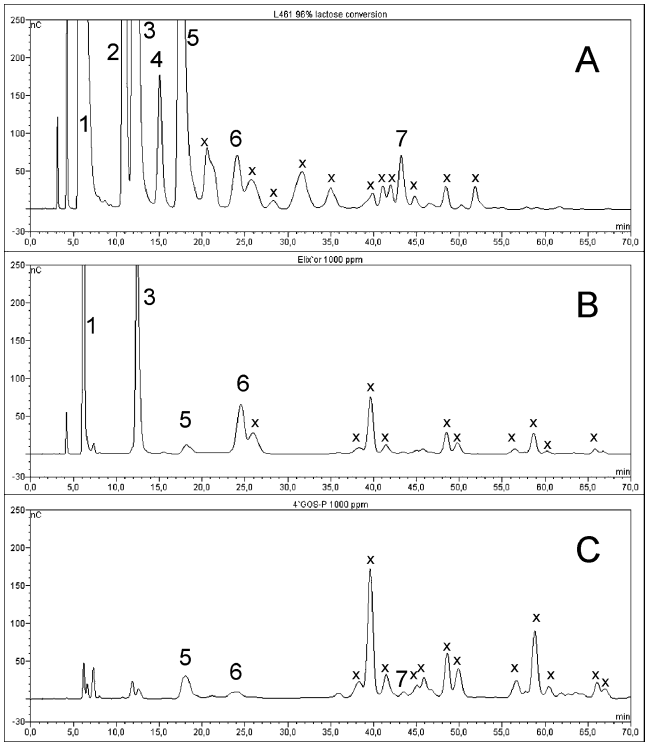


Figure 11. HPAEC–PAD chromatograms of the L461 β-Gal GOS mixture at 96% lactose conversion (**A**), Elix'or (**B**), and 4′GOS (**C**).

HPAEC−PAD, however, one can see that the L103 product and the commercial samples are very different (**Figure 11**). Elix'or shows the same pattern as the 4′GOS-P product, indicating that the main components are β1→4 linked oligosaccharides, whereas our product consists of mainly β1→6 and β1→3 linked saccharides.

All of the commercially available products described here proved to be prebiotic (*1*, *2*). Future experiments with *Lactobacillus* GOS will show whether these can enhance the prebiotic effect and be even more selective for probiotic bacteria as they are produced by an enzyme derived from such a probiotic bacterium. Rabiu and co-workers (*19*) obtained some promising results with GOS synthesized by β-galactosidases from bifidobacteria.

Conclusions. β-Galactosidases from two different isolates of *L. reuteri*, L103 and L461, were used for hydrolysis and transgalactosylation of lactose. Both enzymes are very similar in their properties, with regard to maximum GOS yields, 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 mixture contained a relatively high fraction of non-lactose disaccharides. This results from the fact that both glucose and galactose are better acceptors for galactosyl transfer than lactose. Both enzymes that were studied preferentially form β1→6 and β1→3 linkages in transgalactosylation mode. Recently, Luz Sanz et al. (*20*) investigated the prebiotic potential of a number of disaccharides and found that β-D-Gal*p*-(1→6)-D-Gal, one of the major products of L103 and L461 β-Gal, is a highly prebiotic molecule. Therefore, the novel β-galactosidases from *L. reuteri* should be of considerable interest for the production of prebiotic GOS.

ABBREVIATIONS USED

AP, 2-aminopyridine; CE, capillary electrophoreses; β-Gal, β-galactosidase; GalGal, galactobiose; GalGlc, galactosylglucose; GalLac, galactosyllactose; GOS, galacto-oligosaccharides; HPAEC−PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; Nu, nucleophile; *o*NP, *o*-nitrophenol; *o*NPG, *o*-nitrophenyl β-D-galactopyranoside; TLC, thin-layer chromatography.

LITERATURE CITED

(1) Sako, T.; Matsumoto, K.; Tanaka, R. Recent progress on research and applications of non-digestible galacto-oligosaccharides. *Int. Dairy J.* **1999**, *9*, 69−80.

(2) Vulevic, J.; Rastall, R. A.; Gibson, G. R. Developing a quantitative approach for determining the in vitro prebiotic potential of dietary oligosaccharides. *FEMS Microbiol. Lett.* **2004**, *236*, 153−159.

(3) Crittenden, R. G.; Playne, M. J. Production, properties and applications of food-grade oligosaccharides. *Trends Food Sci. Technol.* **1996**, *7*, 353−361.

(4) Matsumoto, K.; Kobayashi, Y.; Ueyama, S.; Watanabe, T.; Tanaka, R.; Kan, T.; Kuroda, A.; Sumihara, Y. Galactooligosaccharides. In *Oligosaccharides. Production, properties, and applications*; Nakakuki, T., Ed.; Japanese Technology Reviews; Gordon and Breach: Tokyo, Japan, 1993; Vol. 3, pp 90−106.

(5) Nakayama, T.; Amachi, T. β-Galactosidase, enzymology. In *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation*; Flickinger, M. C., Drew, S. W., Eds.; John Willey: New York, 1999; pp 1291−1305.

(6) Boon, M. A.; Janssen, A. E. M.; van't Riet, K. Effect of temperature and enzyme origin on the enzymatic synthesis of oligosaccharides. *Enzyme Microb. Technol.* **2000**, *26*, 271−281.

(7) Huber, R. E.; Kurz, G.; Wallenfels, K. A quantitation of the factors which affect the hydrolase and transgalactosylase activities of β-galactosidase (*E. coli*) on lactose. *Biochemistry* **1976**, *15*, 1994−2001.

(8) Mahoney, R. R. Galactosyl-oligosaccharide formation during lactose hydrolysis: A review. *Food Chem.* **1998**, *63*, 147−154.

(9) Prenosil, J. E.; Stuker, E.; Bourne, J. R. Formation of oligosaccharides during enzymatic lactose: Part I: State of art. *Biotechnol. Bioeng.* **1987**, *30*, 1019−1025.

(10) Smart, J. B. Transferase activity of the β-galactosidase from *Streptococcus thermophilus*. *Appl. Microbiol. Biotechnol.* **1991**, *34*, 495−501.

(11) Nguyen, T.-H.; Splechtna, B.; Steinböck, M.; Kneifel, W.; Lettner, H. P.; Kulbe, K. D.; Haltrich, D. Purification and characterization of two novel β-galactosidases from *Lactobacillus reuteri*. *J. Agric. Food Chem.* **2006**, *54*, 4989−4998.

(12) Petzelbauer, I. I.; Nidetzky, B.; Haltrich, D.; Kulbe, K. D. Development of an ultra-high-temperature process for the enzymatic hydrolysis of lactose. I. The properties of two thermostable β-glycosidases. *Biotechnol. Bioeng.* **1999**, *64*, 322−332.

(13) Kunst, A.; Draeger, B.; Ziegenhorn, J. Colorimetric methods with glucose oxidase and peroxidase. In *Methods of enzymatic analysis*; Bergmeyer, H. U., Bergmeyer, J., Grasszl, M., Eds.; VCH Publishers: Weinheim, Germany, 1988; pp 178−185.

(14) Petzelbauer, I.; Zeleny, R.; Reiter, A.; Kulbe, K. D.; Nidetzky, B. Development of an ultra-high-temperature process for the enzymatic hydrolysis of lactose: II. Oligosaccharide formation by two thermostable β-glycosidases. *Biotechnol. Bioeng.* **2000**, *69*, 140−149.

(15) Dionex. Technical Note 21.

(16) Splechtna, B.; Petzelbauer, I.; Baminger, U.; Haltrich, D.; Kulbe, K. D.; Nidetzky, B. Production of a lactose-free galacto-oligosaccharide mixture by using selective enzymatic oxidation of lactose into lactobionic acid. *Enzyme Microb. Technol.* **2001**, *29*, 434−440.

(17) Richard, J. P.; Westerfeld, J. G.; Lin, S.; Beard, J. Structure−reactivity relationships for β-galactosidase (*Escherichia coli*, lac Z). 2. Reactions of the galactosyl-enzyme intermediate with alcohols and azide ion. *Biochemistry* **1995**, *34*, 11713−11724.

(18) Chockchaisawasdee, S.; Athanasopoulos, V. I.; Niranjan, K.; Rastall, R. A. Synthesis of galacto-oligosaccharide from lactose using β-galactosidase from *Kluyveromyces lactis*: Studies on batch and continuous UF membrane fitted bioreactors. *Biotechnol. Bioeng.* **2005**, *89*, 434−443.

(19) Rabiu, B. A.; Jay, A. J.; Gibson, G. R.; Rastall, R. A. Synthesis and fermentation properties of novel galacto-oligosaccharides by β-galactosidases from *Bifidobacterium species*. *Appl. Environ. Microbiol.* **2001**, *67*, 2526−2530.

(20) Luz Sanz, M.; Gibson, G. R.; Rastall, R. A. Influence of disaccharide structure on prebiotic selectivity in vitro. *J. Agric. Food Chem.* **2005**, *53*, 5192−5199.

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