JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Comparison between Discontinuous and Continuous Lactose Conversion Processes for the Production of Prebiotic Galacto-oligosaccharides Using β-Galactosidase from *Lactobacillus reuteri*

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Galacto-oligosaccharide (GOS) formation from lactose in discontinuous and continuous modes of conversion was investigated using β-galactosidase (β-gal) from *Lactobacillus reuteri*. A continuous stirred tank reactor (CSTR) with an external crossflow membrane was set up, and continuous GOS production was analyzed and compared to the batchwise formed GOS product. 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 2-fold 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. This could be used to synthesize more defined GOS products.

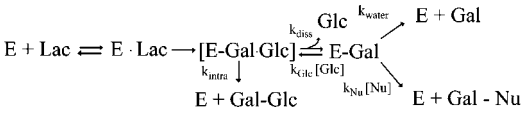
KEYWORDS: **β-Galactosidase; galacto-oligosaccharides; prebiotics; transgalactosylation; membrane reactor; *Lactobacillus***

INTRODUCTION

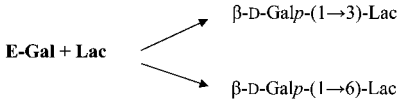
Intensified research efforts in recent years confirm the major importance of the microbial flora in the gastrointestinal tract for human health. Ingestion of prebiotic oligosaccharides can increase the number of desirable bacteria such as bifidobacteria and lactobacilli in the colon (*1*−*4*). Among the oligosaccharides, galacto-oligosaccharides (GOS) are one of the top prebiotics produced commercially (*4*, *5*). Galacto-oligosaccharides have attracted increasing attention because of their presence with different complex structures in human breast milk; therefore, the use of GOS in infant milk formulas is nowadays of great interest (*5*, *6*). In addition, GOS are also incorporated in a wide range of products such as fermented milk products, breads, jams, confectionery, and beverages (*4*).

It is our interest to study β-galactosidases from *Lactobacillus* spp. for the production of galacto-oligosaccharides because it was suggested that β-galactosidases from probiotic microorganisms might produce galacto-oligosaccharide structures that have special prebiotic effects, specifically targeting selected probiotic strains (*2*, *7*). The enzymatic transformation of lactose by β-galactosidases involves the hydrolysis of the substrate into D-galactose and D-glucose and the synthesis of GOS, a complex mixture of various galactose-rich di- and oligosaccharides of different structures, through the transgalactosylation reaction (**Scheme 1**). The amount and composition of the GOS products depend on the source of enzyme, the lactose concentration, and

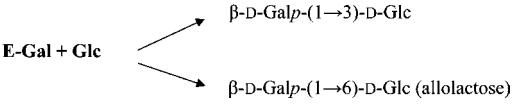
Scheme 1. Hydrolysis and Galactosyl Transfer Reactions, both Intra- and Intermolecular, during the Conversion of Lactose Catalyzed by β-Galactosidases (*18*, *26*)[a]



If Nu is Lac, trisaccharides are formed such as:



If Nu is Glc, disaccharides are formed such as:



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

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10.1021/jf070643z CCC: $37.00
Published on Web 07/14/2007

the reaction conditions in the process. There are a number of papers on the optimization of a GOS production process applicable for industry (*8*−*17*), with most of these studies focusing on commercially available β-galactosidases from fungi and yeasts. Our previous studies focused on discontinuous lactose conversion processes for the production of prebiotic GOS using novel β-galactosidases from *Lactobacillus* spp. (*18*−*20*). These discontinuous modes of operation still prevail in the scientific literature on transgalactosylation processes, because of their ease of operation, possible microbial contaminations in long-term continuous processes especially under realistic reaction conditions at moderate temperatures, or membrane fouling that might occur in systems where enzyme retention is based on a suitable membrane. Continuous systems, however, can offer a more efficient usage of (expensive) enzymes. In addition, different modes of operation might result in different reaction mixtures and an altered GOS composition (*21*) and are therefore of interest both from a fundamental and from an applied point of view. Only a few studies, however, compare continuous and discontinuous processes of transgalactosylation with respect to GOS composition or productivity (*10*, *21*).

In this study, we set up a continuous stirred tank reactor (CSTR) with an external crossflow ultrafiltration membrane. In doing so, we were able to investigate the transgalactosylation properties of β-galactosidase from *Lactobacillus reuteri* under steady-state conditions and compare the obtained GOS product to the one produced in discontinuous batch mode under otherwise identical conditions. In a steady state, the concentrations of possible galactosyl acceptors stay constant over the entire reaction time and, therefore, more defined GOS mixtures are to be expected (*21*), whereas, in discontinuous systems, the composition and concentration of possible galactosyl acceptors are changing constantly.

MATERIALS AND METHODS

Materials. 2-Aminopyridine and D-galactose were purchased from Fluka (Buchs, Switzerland), *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) was from Sigma (St. Louis, MO), and D-lactose monohydrate was supplied by Merck (Darmstadt, Germany). 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 Dr. S. Riva (CNR, Milano, 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 Dr. P. Kosma (Department of Chemistry, BOKU, Vienna, Austria).

Enzyme. The β-galactosidase was produced using a strain of *L. reuteri* obtained from Lactosan Starterkulturen (Kapfenberg, Austria). This strain, originating from calf, was deposited in the culture collection of the supplier under the strain number L103. The organism was cultivated on an 825 L scale using a lactose-based medium. Cells were harvested by centrifugation and homogenized in 50 mM sodium phosphate buffer (pH 6.0). Cell debris was removed by centrifugation, and the crude enzyme extract containing 93 U_{oNPG}/mL of β-galactosidase and 13 mg/mL protein was obtained. The enzyme was purified from the crude extract to apparent homogeneity by hydrophobic interaction and affinity chromatography as previously described (*22*).

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. Absorbance of *o*NP was 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.

Enzyme Assay with Lactose. To determine the β-galactosidase activity with the natural substrate lactose the assay described by Petzelbauer et al. (*23*) was used with slight modifications. Twenty microliters of enzyme sample was added to 480 μL of 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. Glucose released was measured with an enzymatic assay based on glucose oxidase and peroxidase (*24*). 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.

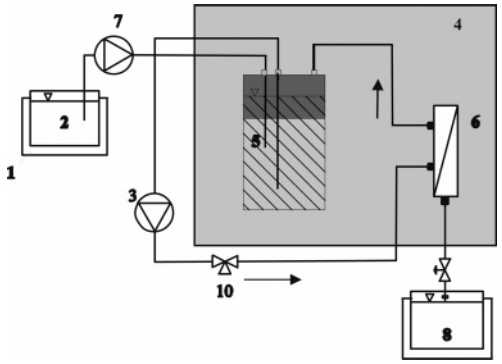

Figure 1. Schematic diagram of the bioreactor with external crossflow membrane: (1) ice bath; (2) substrate reservoir; (3) peristaltic pump; (4) water bath; (5) bioreactor; (6) crossflow membrane; (7) piston pump; (8) outlet stream of the product; (10) sample valve.

Protein Measurement. Protein was determined according to the method of Bradford (*25*) with the Bio-Rad Coomassie Blue reagent using bovine serum albumin as the standard.

Monosaccharide Analysis. D-Glucose was measured enzymatically as described above. For the determination of D-galactose a Lactose/D-Galactose test kit from Boehringer Mannheim (Mannheim, Germany) was used.

Oligosaccharide Analysis. Capillary electrophoresis (CE) and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) were used for the analysis of galacto-oligosaccharides (*18*). A CE system with a UV-DAD detector (Agilent Technologies, Palo Alto, CA) together with a fused silica capillary (internal diameter of 25 μm) equipped with a bubble cell detection window (bubble factor = 5) was used for carbohydrate analysis. Carbohydrate samples were derivatized with 2-aminopyridine for CE analysis as given in detail in ref *18*.

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 (Dionex Corp., Sunnyvale, CA). Separations were performed at room temperature on a CarboPac PA-1 column (4 × 250 mm) connected to a CarboPac PA-1 guard column (Dionex) (*18*).

Discontinuous GOS Production. Discontinuous conversion reactions were carried out with purified β-galactosidase from *L. reuteri* on a 2−20 mL scale. The experiments were routinely performed with 13 U_{oNPG}/mL of β-gal at 37 °C using 600 mM lactose dissolved in 50 mM sodium phosphate buffer (pH 6.0) containing 1 mM $MgCl_2$. Agitation was applied at 300 rpm with a thermomixer (Eppendorf, Hamburg, Germany).

Continuous Conversion of Lactose in an Ultrafiltration Membrane Reactor. A CSTR was set up following the description by Petzelbauer et al. (*21*); the total working volume of this reactor was 21 mL. It was equipped with an external crossflow filtration unit (Vivaflow 50 polyethersulfone membrane module; Sartorius, Göttingen, Germany) with a cutoff of 10 kDa and a filtration surface of 50 cm^2 (**Figure 1**). Due to the tubes connecting the reactor and the ultrafiltration unit, the membrane reactor had a void volume of 6 mL. A water bath was used to attain temperature control (37 ± 1 °C). We selected a closed reactor system in which transmembrane pressure is created by internal pressure. Mixing was achieved by means of recirculation of the reactor volume. To prevent blocking of the ultrafiltration unit, it was necessary to operate the peristaltic pump at a minimum volumetric flow rate of 50 mL/min for the circulation of the reaction mixture to obtain the necessary crossflow velocity. Starting the piston pump for

feeding fresh substrate raised the transmembrane pressure required for the permeate flow. Whereas low molecular weight products and unreacted substrate passed freely through the membrane, the enzyme was retained completely. The enzyme reactor was operated at an initial substrate concentration of 135, 300, or 600 mM lactose. Lactose was dissolved in 50 mM sodium phosphate buffer (pH 6.0) containing 1 mM $MgCl_2$ to improve enzyme stability (*22*) and 0.05% sodium azide to prevent microbial growth in these long-term experiments.

To start the enzyme reactor, it was filled with substrate solution already containing the enzyme and thermostated at 37 °C. The CSTR was run using 30−45 U_{oNPG}/mL of homogeneous β-galactosidase. Substrate was fed continuously by using a piston pump (Amersham-Pharmacia LKB pump P-500) to maintain exact flow rates of 6−48 mL/h. Samples were taken periodically from inside the reactor to control the stability of the enzyme activity under reaction conditions. To this end, the peristaltic pump was stopped, and two clamps were attached to each side of the sample valve, which was opened to enable withdrawal of samples. To monitor conversion, samples were taken on the product side of the crossflow filtration unit.

RESULTS AND DISCUSSION

Discontinuous GOS Production. The formation of GOS during discontinuous lactose conversion by β-galactosidase from *L. reuteri* was previously discussed in detail (*18*, *19*). In the present study, discontinuous GOS production was performed at 37 °C and pH 6.0 by employing 600 mM lactose as the substrate. Under these conditions and at 80% lactose conversion, the GOS yield, expressed by the relative concentration (percentage GOS of total sugars), is 36%. Monosaccharides and unconverted lactose make up 64% of the total sugars. The main products of transgalactosylation by β-gal from *L. reuteri* 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 (*18*). Thus, the enzyme in its transgalactosylation reaction displays a strong preference for catalyzing the formation of both β-(1→6) and β-(1→3) glycosidic linkages.

Continuous GOS Production in an Ultrafiltration Membrane Reactor. To compare discontinuous and continuous modes of operation for transgalactosylation we examined the performance of β-gal from *L. reuteri* for GOS production in a CSTR. Because of the application of a fixed dilution rate a steady state is created in the CSTR through which the concentration levels of substrate and products available for the enzymatic reaction stay constant. When compared to a batch reaction, these more defined reaction conditions can generate a better understanding for the transgalactosylation characteristics of the β-galactosidase applied. Several experiments for continuous GOS production at 37 °C were run, in which both substrate concentration and dilution rate were varied, and long-term stability as well as GOS production and the spectra of GOS formed were investigated. At 50% lactose conversion the total GOS contents in the permeate were 10, 19, and 24%, respectively, when using 135, 300, and 600 mM lactose as the substrate. Results of a representative experiment that was run for 6 days using a 600 mM lactose solution are illustrated in **Figure 2**. Even though enzyme activity measured in samples taken from inside the reactor declined to some extent (data not shown), the conversion level stayed constant. This observation likely reflects the adsorption of the enzyme to surfaces in the reactor, for example, the ultrafiltration membrane, but not enzyme inactivation, which was also reported by Petzelbauer et al. (*21*).

An important parameter for enzymatic reactions in a CSTR is the dilution rate. In **Figure 3** lactose conversion and

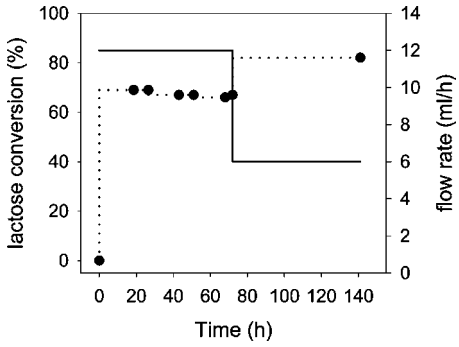

Figure 2. Continuous lactose conversion in a 21 mL CSTR operated at 37 °C using 28 U_{oNPG}/mL β-galactosidase from *Lactobacillus reuteri* and 600 mM lactose in 50 mM sodium phosphate buffer (pH 6.0) + 1 mM $MgCl_2$ + 0.05% sodium azide. Circles are levels of lactose conversion, and the solid line represents the flow rate (mL/h).

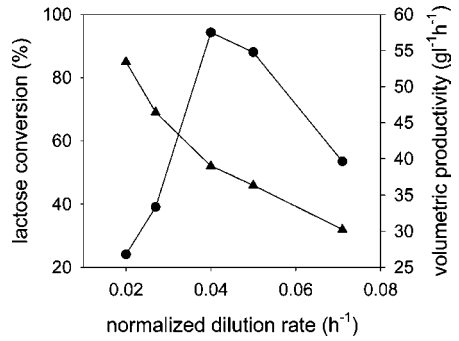

Figure 3. Lactose conversion (triangles) and volumetric productivities, calculated as grams of GOS produced per liter of reactor volume and hour (circles), with β-galactosidase from *Lactobacillus reuteri* at 37 °C in a CSTR as a function of normalized dilution rate (dilution rate multiplied by the reciprocal enzyme concentration in kU/L). The substrate solution was 600 mM lactose in 50 mM sodium phosphate buffer (pH 6.0) + 1 mM $MgCl_2$ + 0.05% sodium azide.

volumetric productivity in grams of GOS per liter of reactor volume and hour were plotted as functions of the normalized dilution rate, which was introduced to consider the intrinsic relationship between the dilution rate and the enzyme activity in the CSTR (*21*) and which is the actual dilution rate multiplied by the reciprocal enzyme concentration (in kU/L reactor volume). Conceivably, lactose conversion decreases with increasing dilution rates. However, the productivity of the reactor rises rapidly between 0.02 and 0.04 h^{-1}, reaches a maximum, and begins to fall again at 0.05 h^{-1}. In other words, optimum productivity is reached at 50% lactose conversion. For simple enzymatic reactions productivity increases with dilution rate due to increased substrate saturation and diminishing product inhibition of the enzyme. In the case of GOS production by β-gal from *L. reuteri* these phenomena are counteracted by the 4 times better transgalactosylation rate to D-glucose than to lactose (*18*), which becomes prominent at higher lactose conversion levels as higher concentrations of glucose are available as galactosyl acceptors for the transgalactosylation reaction.

Influence of the Type of Enzyme Reactor on GOS Yield and Composition. The importance of the enzyme reactor type on GOS composition and yield has been reported recently for two hyperthermophilic enzymes (*21*), yet hardly any other studies have looked in detail at the spectrum of GOS produced with respect to the reactor type employed. Here, comparison of the batch reactor and the CSTR was made pertaining to the

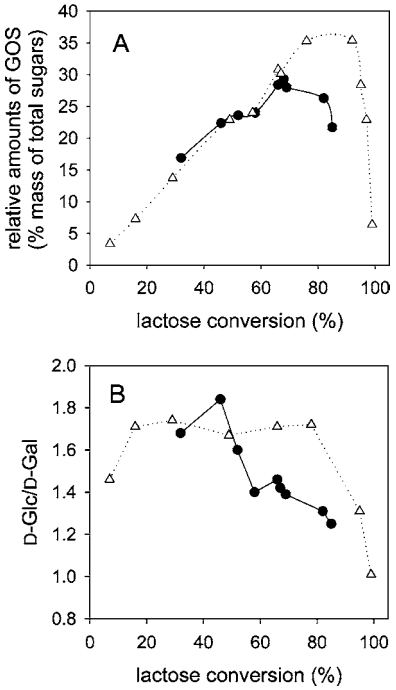

Figure 4. (**A**) Production of galacto-oligosaccharides (GOS) during the conversion of 600 mM lactose at 37 °C using β-galactosidase from *Lactobacillus reuteri* in the CSTR (circles) and in batchwise reaction (triangles). (**B**) Comparison of the ratio of D-glucose to D-galactose observed in a CSTR (circles) and batchwise reaction (triangles) and its dependence on lactose conversion at 37 °C. The experiments were carried out with β-galactosidase from *L. reuteri* in 600 mM lactose dissolved in 50 mM sodium phosphate buffer (pH 6.0) + 1 mM $MgCl_2$. In the case of CSTR 0.05% sodium azide was added to prevent microbial contamination.

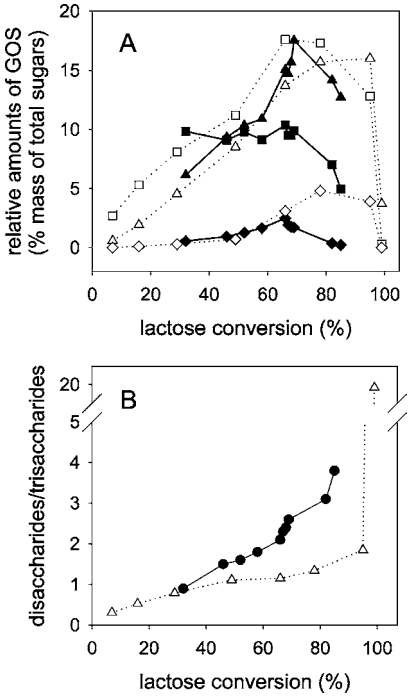

Figure 5. (**A**) Production of di- (triangles), tri- (squares), and tetrasaccharides (diamonds) during the conversion of 600 mM lactose at 37 °C using β-galactosidase from *Lactobacillus reuteri* in the CSTR (solid symbols) and in batchwise reaction (open symbols). (**B**) Comparison of the ratio of molar concentrations of disaccharides other than lactose to trisaccharides observed in a CSTR (circles) and batchwise reaction (triangles) and its dependence on lactose conversion at 37 °C. The experiments were carried out with β-galactosidase from *L. reuteri* in 600 mM lactose dissolved in 50 mM sodium phosphate buffer (pH 6.0) + 1 mM $MgCl_2$. In the case of CSTR 0.05% sodium azide was added to prevent microbial contamination.

amount of GOS produced at identical levels of lactose conversion, the composition of GOS, and the relative proportion of individual components obtained under these different modes of operation. Marked differences were found for the two reactor setups due to the fact that samples taken during a batch reaction reflect the history of all components for the elapsed reaction time, whereas the composition of the product mixture of the CSTR represents a steady state.

Influence on GOS Yield. **Figure 4A** shows the GOS yield at different levels of lactose conversion for the batch reactor and the CSTR. Up to a lactose conversion level of 65% the yields are comparable. However, the maximum yield of GOS in the batch reactor (36% of total sugars) at 80% lactose conversion cannot be reached in the CSTR, which has a maximum yield of 30% at 70% lactose conversion. This is also evident from the ratio of D-glucose to D-galactose, a very powerful indicator of the transgalactosylation reaction. Typically, this ratio is larger than one under conditions where GOS are formed as it reflects the fact that D-galactose is transferred to suitable acceptor molecules. It changes over the course of the lactose conversion reaction and becomes one when the hydrolysis of lactose and all oligosaccharides is complete. This ratio was similar for a batchwise stirred tank reactor (STR) and the CSTR at lower levels of lactose conversion and then dropped for the CSTR below the ratio obtained in the discontinuous reaction beginning at 50% lactose conversion (**Figure 4B**). These observations are contrary to what Petzelbauer et al. (*21*) found for the thermostable β-glycosidase from *Sulfolobus solfataricus*. In their case GOS yield was higher in the CSTR than in the STR.

Influence on GOS Composition. When analyzing the GOS mixtures in more detail, we found some distinct differences between the individual components of the reaction products of the two reactor types. Significantly fewer trisaccharides are obtained in the GOS mixtures in the CSTR at above 40% lactose conversion, and as a result of that there are also fewer tetrasaccharides than under discontinuous reaction conditions. The concentration of disaccharides, however, is found in an equal range with that of the batch product, and their concentration is even higher in the CSTR at a conversion level of 70% (**Figure 5A**). **Figure 5B** illustrates the ratio of disaccharides to trisaccharides for the STR and the CSTR, which was significantly higher for the CSTR at almost all lactose conversion levels considered. Recently, we compared the transgalactosylation rate for β-galactosidase from *L. reuteri* to acceptors such as glucose and lactose, and it was found that the transgalactosylation rate to glucose was 4 times higher than that to lactose (*18*). The concentration of glucose builds only slowly under discontinuous mode of operation and, therefore, is available as an acceptor of the galactosyl moiety in significant concentrations only at later phases of the reaction, resulting in the formation of disaccharides (**Scheme 1**). In contrast, glucose is readily available as an acceptor under steady-state conditions in the continuously operated reactor throughout the entire reaction. Because glucose is a far better acceptor than lactose, more of the galactosyl moieties are transferred onto this monosaccharide sugar and a higher fraction of disaccharides are formed in continuous mode of operation.

Analyzing the GOS composition on the level of sugar species reveals another distinct difference between the two modes of

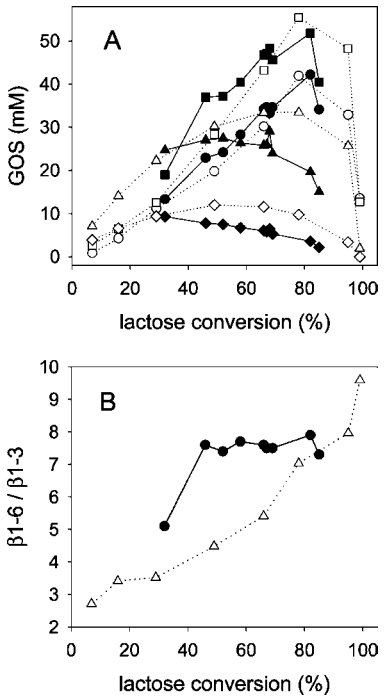

Figure 6. (**A**) Production of individual galacto-oligosaccharides (GOS) during the conversion of 600 mM lactose at 37 °C using β-galactosidase from *Lactobacillus reuteri* in the CSTR (solid symbols) and in batchwise reaction (open symbols): β-D-Gal*p*-(1→6)-D-Gal (circles), β-D-Gal*p*-(1→6)-D-Glc (squares), β-D-Gal*p*-(1→6)-Lac (triangles), β-D-Gal*p*-(1→3)-Lac (diamonds). (**B**) Comparison of the ratio of identified β-(1→6)-linked GOS to identified β-(1→3)-linked GOS observed in a CSTR (circles) and batchwise reaction (triangles) and its dependence on lactose conversion at 37 °C. The experiments were carried out with β-galactosidase from *L. reuteri* in 600 mM lactose dissolved in 50 mM sodium phosphate buffer (pH 6.0) + 1 mM $MgCl_2$. In the case of CSTR 0.05% sodium azide was added to prevent microbial contamination.

operation of the enzyme reactor. **Figure 6A** compares the four major products formed in discontinuous and continuous modes. More β-D-Gal*p*-(1→6)-D-Glc was formed under continuous reaction conditions, especially at lactose conversion levels below 60%, again indicating the higher availablity of the excellent acceptor D-glucose under steady-state conditions. On the contrary, less β-D-Gal*p*-(1→3)-Lac and β-D-Gal*p*-(1→6)-Lac are formed in the CSTR at lactose conversion levels between 50 and 80%. During the batch reaction the ratio of the sum of the identified β-(1→6)-linked GOS to the sum of the β-(1→3)-linked GOS increases steadily (**Figure 6B**), suggesting a higher rehydrolysis rate for the latter compounds. This could also explain the significantly higher β-(1→6) to β-(1→3) ratio of products formed for the steady states in the CSTR, where an almost constant high value for this ratio of 8 was observed for lactose conversion of 40−80%.

Conclusions. To elucidate the transgalactosylation properties of β-galactosidase from *L. reuteri* in more detail, a discontinuous enzyme reactor and a CSTR with an external crossflow ultrafiltration membrane were designed. In contrast to the discontinuous process, steady states are built up in the CSTR, presenting constant conditions for transgalactosylation for each dilution rate applied. Using this approach, it is possible to produce more defined GOS mixtures with increased disaccharide and β-(1→6)-linked GOS contents. Therefore, more defined GOS mixtures are obtained by CSTR at certain dilution rates, which can be used to investigate the prebiotic effect of certain disaccharides and higher oligosaccharides, or β-(1→6)- and β-(1→3)-linked GOS when compared to mixtures obtained by discontinuous conversions.

ABBREVIATIONS USED

CE, capillary electrophoresis; CSTR, continuous stirred tank reactor; DTT, dithiothreitol; β-gal, β-galactosidase; Gal, galactose; Glc, glucose; GOS, galacto-oligosaccharides; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; Lac, lactose; *o*NP, *o*-nitrophenol; *o*NPG, *o*-nitrophenyl-β-D-galactopyranoside; STR, stirred tank reactor.

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Received for review March 6, 2007. Revised manuscript received June 6, 2007. Accepted June 11, 2007. This research work was supported by the Research Centre Applied Biocatalysis (Kompetenz-Zentrum Kplus Angewandte Biokatalyse, Graz, Austria).

JF070643Z