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Production of lactose-free galacto-oligosaccharide mixtures: comparison of two cellobiose dehydrogenases for the selective oxidation of lactose to lactobionic acid

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Abstract—Galacto-oligosaccharides, complex mixtures of various sugars, are produced by transgalactosylation from lactose using β-galactosidase and are of great interest for food and feed applications because of their prebiotic properties. Most galacto-oligosaccharide preparations currently available in the market contain a significant amount of monosaccharides and lactose. The mixture of galacto-oligosaccharides (GalOS) in this study produced from lactose using recombinant β-galactosidase from *Lactobacillus reuteri* contains 48% monosaccharides, 26.5% lactose and 25.5% GalOS. To remove efficiently both monosaccharides and lactose from this GalOS mixture containing significant amounts of prebiotic non-lactose disaccharides, a biocatalytic approach coupled with subsequent chromatographic steps was used. Lactose was first oxidised to lactobionic acid using fungal cellobiose dehydrogenases, and then lactobionic acid and monosaccharides were removed by ion-exchange and size-exclusion chromatography. Two different cellobiose dehydrogenases (CDH), originating from *Sclerotium rolfsii* and *Myriococcum thermophilum*, were compared with respect to their applicability for this process. CDH from *S. rolfsii* showed higher specificity for the substrate lactose, and only few other components of the GalOS mixture were oxidised during prolonged incubation. Since these sugars were only converted once lactose oxidation was almost complete, careful control of the CDH-catalysed reaction will significantly reduce the undesired oxidation, and hence subsequent removal, of any GalOS components. Removal of ions and monosaccharides by the chromatographic steps gave an essentially pure GalOS product, containing less than 0.3% lactose and monosaccharides, in a yield of 60.3%.

Keywords: β-Galactosidase; Transgalactosylation; Cellobiose dehydrogenase; Galacto-oligosaccharides; Prebiotics; Lactobionic acid; Laccase

1. Introduction

During the last years, galacto-oligosaccharides (GalOS) were reported to be beneficial for human health, and they are now recognised as prebiotics.[1,2] GalOS, non-digestible oligosaccharides, are not hydrolysed or absorbed in the upper intestinal tract, they pass on to the colon where they are then fermented selectively by beneficial intestinal bacteria, which implicate a balanced

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid); CDH, cellobiose dehydrogenase; DCIP, 2,6-dichloro-indophenol; GalOS, galacto-oligosaccharides; *o*NPG, *o*-nitrophenyl-β-D-galactopyranoside; *o*NP, *o*-nitrophenol; TLC, thin-layer chromatography; CE, capillary electrophoresis; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection

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and advantageous microbiota.[3–6] GalOS are the products of transgalactosylation reactions catalysed by β-galactosidases when using lactose or other structurally related galactosides as the substrate. In aqueous systems transgalactosylation has to compete with hydrolysis, and therefore GalOS mixtures always contain considerable amounts of unreacted lactose and monosaccharides.[7–9] The removal of those sugars can be essential not only for the purity of the final product[10] but also for microbiological studies on the prebiotic effects of these GalOS mixtures. Prebiotic functionality is often studied by looking at the relative fermentability of these sugars, either in pure or mixed microbial cultures. Evidently, these studies will be biased if these mixtures contain high amounts of D-glucose, D-galactose and lactose,[4,11] and therefore methods to remove these monosaccharides and lactose are of significant interest.[10]

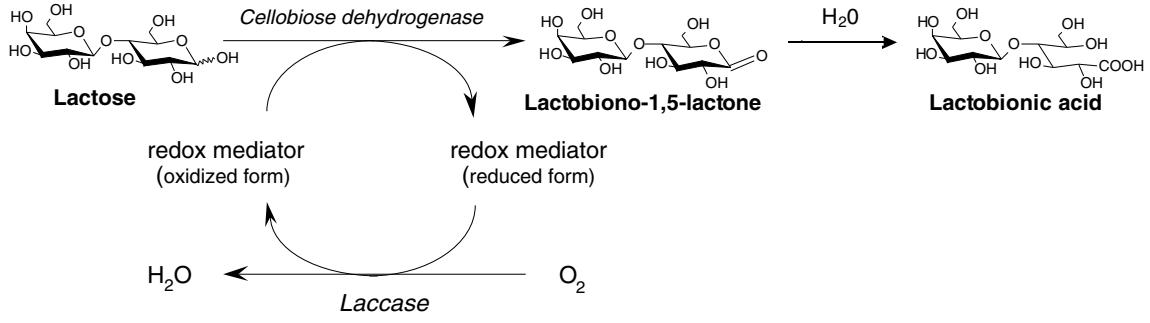
Recently, we introduced a novel approach for the removal of lactose, which is based on the selective enzymatic oxidation of lactose into lactobionic acid using fungal cellobiose dehydrogenase [CDH; EC 1.1.99.18, cellobiose:(acceptor) 1-oxidoreductase].[12] Based on sequence homologies and phylogenetic analysis, CDHs have been classified as members of class-I, represented by the basidomycete CDHs, and class-II, comprising the structurally more complex ascomycete CDHs.[13,14] CDH members of these two classes are quite similar in their biochemical and kinetic properties, but also show some distinct differences. In general, CDH is rather specific for their carbohydrate substrates, oxidising cellobiose, cello-oligosaccharides, lactose and only very few other sugars.[15,16] Monosaccharides are very poor substrates for class-I CDHs while class-II CDHs are more relaxed pertaining to their substrate specificity, and can show considerable activity with monosaccharides such as D-glucose.[13,17] To employ CDH in the biocatalytic oxidation of lactose, a reaction system composed of CDH, a redox mediator (electron acceptor) such as dichloro-indophenol (DCIP), and laccase was used. Scheme 1 depicts how the continuous regeneration of the oxidised form of the redox mediator is achieved by the oxygen-dependent, laccase-catalysed reoxidation of its reduced form so that it is continuously available for the CDH-catalysed reaction.[18,19]

In this study, the approach of removing lactose from GalOS mixtures containing amongst others significant amounts of non-lactose disaccharides was studied in more detail. We compared for the first time a class-I and a class-II CDH with respect to their specificities for various components present in complex GalOS mixtures and hence for their suitability of the suggested application. It was of special interest whether and which components of the complex GalOS mixtures will be oxidised by these CDHs in addition to lactose since these oxidised sugars will then be removed by the subsequent chromatographic steps.

2. Results and discussion

2.1. Galacto-oligosaccharide production

Discontinuous GalOS production was performed at 23 °C using 5 U/mL of recombinant β-galactosidase from *Lactobacillus reuteri* L103 expressed in *Escherichia coli* with an initial lactose concentration of 206.5 g/L (602 mM). The reaction was stopped at 73% lactose conversion yielding a product mixture containing 48% (w/w) monosaccharides, 26.5% (w/w) lactose, 9.8% (w/w) non-lactose disaccharides, 14.7% (w/w) trisaccharides and 1.0% (w/w) tetrasaccharides as analysed by capillary electrophoresis (CE). A quantitative analysis of this GalOS mixture together with its main components is given in Table 1. As was shown previously, (β-galactosidase) from *L. reuteri* has a preference for forming β-(1→3) and β-(1→6) bonds in its transgalactosylation mode, and hence the main GalOS products were identified by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) to be β-D-Gal*p*-(1→6)-D-Glc (allolactose), β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Glc,



Scheme 1. Reaction scheme of the cellobiose dehydrogenase-catalysed oxidation of lactose to lactobionic acid using laccase for the regeneration of the oxidised form of the redox mediator.

Table 1. Composition of the GalOS mixture after the transgalactosylation reaction of lactose (206.5 g/L) with recombinant β-galactosidase from *L. reuteri* L103

Name	Structure	Concentration [g/L]	Composition [% w/w]
Glucose		62.4	30.2
Galactose		36.8	17.8
Lactose	β-D-Gal*p*-(1→4)-D-Glc	54.7	26.5
Allolactose	β-D-Gal*p*-(1→6)-D-Glc	10.1	4.9
	β-D-Gal*p*-(1→6)-D-Gal	7.7	3.7
	β-D-Gal*p*-(1→3)-D-Gal	1.7	0.8
	β-D-Gal*p*-(1→3)-D-Glc	0.7	0.3
6′-Galactosyl lactose	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc	17.8	8.6
3′-Galactosyl lactose	β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc	4.8	2.3
Tetrasaccharides		2.1	1.0
Unidentified		7.8	3.8

Table 2. Composition of GalOS before and after prolonged oxidation (reaction time of 93 h) using CDH from different sources

	Composition of GalOS [% w/w]				Total loss [%]
	Before oxidation		After oxidation		
(A) CDH from M. thermophilum					
Disaccharides	38.3		59.1		1.5
β-D-Gal*p*-(1→6)-D-Glc		19.1		30.0	
β-D-Gal*p*-(1→6)-D-Gal		14.6		22.9	
β-D-Gal*p*-(1→3)-D-Gal		3.3		4.2	
β-D-Gal*p*-(1→3)-D-Glc		1.3		2.0	
Trisaccharides	42.9		19.7		70.7
β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc		33.8		9.6	
β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc		9.1		10.1	
Tetrasaccharides	4.0		5.1		19.5
Unidentified	14.8		16.1		30.2
Peak 21 min		0.6		1.4	
Peak 23 min		2.3		1.2	
Peak 46.5 min		1.4		3.1	
(B) CDH from S. rolfsii					
Disaccharides	38.3		50.0		1.7
β-D-Gal*p*-(1→6)-D-Glc		19.1		25.4	
β-D-Gal*p*-(1→6)-D-Gal		14.6		19.4	
β-D-Gal*p*-(1→3)-D-Gal		3.3		3.6	
β-D-Gal*p*-(1→3)-D-Glc		1.3		1.6	
Trisaccharides	42.9		29.4		48.5
β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc		33.8		17.6	
β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc		9.1		11.8	
Tetrasaccharides	4.0		4.4		17.5
Unidentified	14.8		16.2		17.2
Peak 21 min		0.6		1.5	
Peak 23 min		2.3		3.4	
Peak 46.5 min		1.4		3.5	

Lactose was completely oxidised and is not shown here.

β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc (3′-galactosyl lactose) and β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc (6′-galactosyl lactose).

2.2. Oxidation of lactose to lactobionic acid using cellobiose dehydrogenases

Two different CDHs from the basidomycete *Sclerotium* (*Athelia*) *rolfsii* and the ascomycete *Myriococcum thermophilum* were compared for the oxidation of lactose in preliminary experiments. In these, the reaction was not stopped when lactose was oxidised completely but was continued for an extended period of time to investigate whether the additional components formed during the transgalactosylation reaction will be oxidised in addition to lactose, and how specific CDH is with respect to the substrates that have structures closely related to lactose. It should be mentioned that most of the components

found as main products in the GalOS mixtures are not available as pure substances, which hampers the detailed specificity studies of CDH. These reactions were carried out in parallel in 6-well plates for 93 h at 25 °C (Figs. 1 and 2). At the beginning of the reaction a rapid conversion of lactose accompanied by a decrease in the pH of the reaction mixture was observed. The pH, which initially was around 6, was allowed to drop to 4.0 and was then kept constant at this value. After 510 min lactose was almost quantitatively oxidised to lactobionic acid regardless of the CDH used (Figs. 1 and 2), and no by-products were detectable as judged by HPLC analysis. During the first 330 min of the reaction, when the lactose concentration in the reaction mixture was still >35 mM, CDH from *S. rolfsii* almost exclusively oxidised

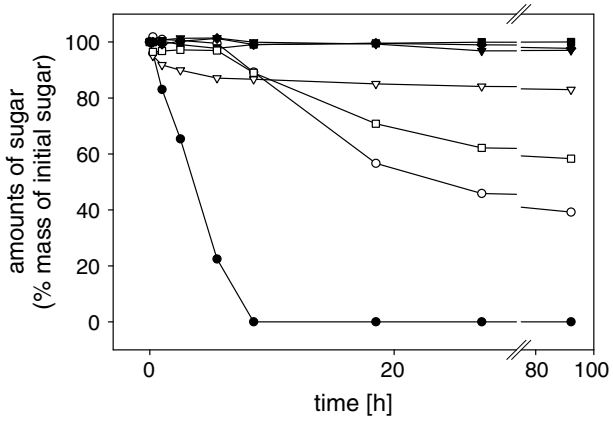


Figure 1. Enzymatic oxidation of lactose and some other components of the GalOS mixture using CDH from *S. rolfsii* in the reaction system shown in Scheme 1. Symbols: lactose (full circle), β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc (open circle), β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc (full triangle), β-D-Gal*p*-(1→3)-D-Gal (open triangle), unidentified GalOS from the analysis by HPAEC-PAD (gradient 1) with a retention time of ~21 min (full square), ~23 min (open square), ~46.5 min (full diamond), respectively.

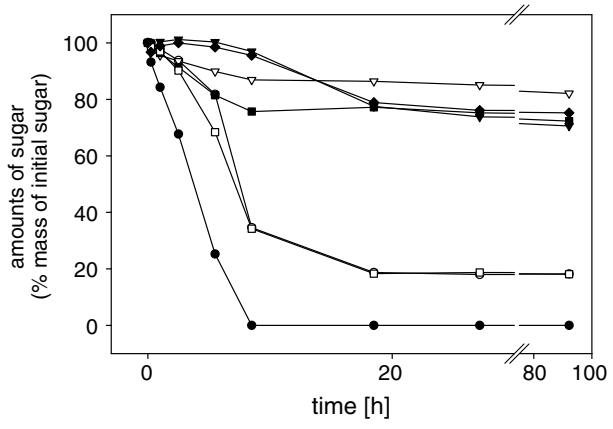


Figure 2. Enzymatic oxidation of lactose and some other components of the GalOS mixture using CDH from *M. thermophilum* in the reaction system shown in Scheme 1. Symbols as in Figure 1.

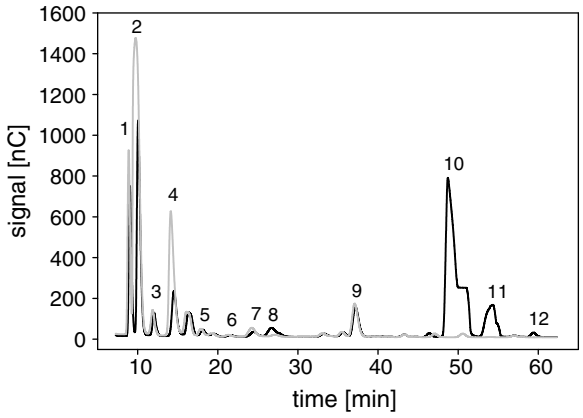


Figure 3. HPAEC-PAD chromatograms of the GalOS mixture before oxidation using CDH from *S. rolfsii* (grey line) and after 92 h of reaction time (black line). Numbering refers to (1) β-D-Gal*p*-(1→6)-D-Gal, (2) lactose + β-D-Gal*p*-(1→6)-D-Glc (allolactose), (3) β-D-Gal*p*-(1→3)-D-Gal, (4) β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc, (5) β-D-Gal*p*-(1→3)-D-Glc, (6) and (7) unidentified GalOS, (9) β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, (10) lactobionic acid, (8), (11) and (12) oxidised sugars.

lactose while β-D-Gal*p*-(1→3)-D-Gal was the only other concomitantly oxidised compound, but this disaccharide was converted only to a very low extent. At the latter phase of the reaction, when the lactose concentration approached the Michaelis constant of *S. rolfsii* CDH (2.4 mM), this CDH utilised the trisaccharide 6′-galactosyl lactose [β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc] and another unidentified GalOS (retention time ~23 min as analysed by HPAEC-PAD; Fig. 3) as its substrates. The trisaccharide 3′-galactosyl lactose [β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc] and two other unidentified GalOS (retention time ~21 min and ~46.5 min as analysed by HPAEC-PAD; Fig. 3), which were oxidised by CDH of *M. thermophilum* (see below), were very poor substrates for CDH of *S. rolfsii* with less than 2% of these sugars converted over the entire reaction time (Fig. 1, Table 2). No other component of the complex GalOS mixture was oxidised by CDH from *S. rolfsii* during the reaction time of 93 h as judged by HPAEC-PAD analysis.

When CDH from *M. thermophilum* was used in this reaction system, 6′-galactosyl lactose and an unidentified GalOS (retention time of ~23 min as analysed by HPAEC-PAD; Fig. 4) were oxidised from the very beginning of the reaction concomitantly with lactose (Fig. 2). These two sugars were almost completely oxidised during the reaction time of 93 h, while lactose, the preferred substrate of CDH in this reaction mixture, was completely converted within 10 h (Table 2). 3′-Galactosyl lactose and two other unidentified GalOS (retention times of ~21 min and ~46.5 min as analysed by HPAEC-PAD; Fig. 4) were converted at rates that are >10-fold higher compared to the reaction using CDH from *S. rolfsii*. In general, CDH from *S. rolfsii*

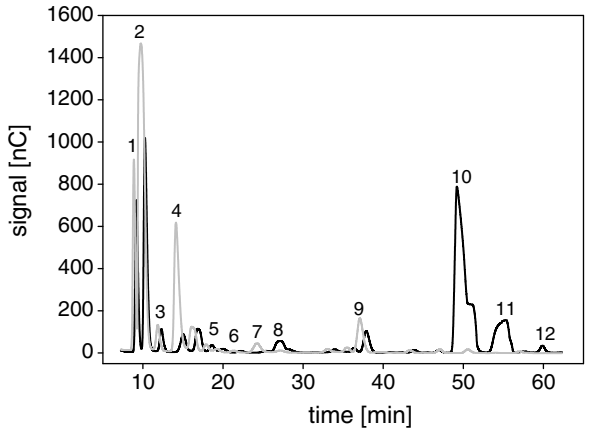


Figure 4. HPAEC-PAD chromatograms of the GalOS mixture before oxidation using CDH from *M. thermophilum* (grey line) and after 92 h of reaction time (black line). Numbering as in Figure 3.

is more specific of the two oxidoreductases compared in this study, and hence is better suited for the proposed application of selective lactose oxidation. This CDH only oxidises other GalOS once lactose in the reaction mixture is almost depleted and its concentration approaches the K_m-value of 2.4 mM determined for this enzyme.[20] Careful control of the reaction, that is termination when lactose conversion is almost quantitative, will thus minimise the undesired oxidation of other components of the reaction mixture and their removal from the GalOS product in the subsequent chromatographic steps.

Cellobiose dehydrogenases are known to convert cellobiose [β-D-Glc*p*-(1→4)-D-Glc] and higher cello-oligosaccharides [β-D-Glc*p*-(1→4)-β-D-Glcp_n-(1→4)-D-Glc; $n = 1$–4] as their proposed in vivo substrates. These substrates, which only vary in the number of glucose moieties, are typically oxidised at comparable rates while the Michaelis constant increases for cellotriose or higher oligosaccharides.[13,20] This indicates that the active site can accommodate larger oligosaccharides sufficiently well as was also shown for the structure of CDH from *Phanerochaete chrysosporium* with a bound substrate analogue.[21,22] Furthermore, it is known that the prerequisites for good CDH substrates are a glucosyl moiety at the reducing end of the sugar substrate and a β-(1→4) glycosidic linkage adjacent to this terminal glucose.[15,16] These structural requirements are also reflected by the specificities of the two CDHs for the components of the GalOS mixture found in this study. 6′-Galactosyl lactose is in agreement with these rules hence it is a substrate of both CDHs, albeit it is oxidised at significantly lower rates than lactose. Interestingly, 3′-galactosyl lactose is of the β-(1→4) glycosidic linkage type and it carries a terminal glucosyl moiety, yet it is not oxidised by CDH from *S. rolfsii* and is only a rather poor substrate of CDH from *M. thermophilum*. Presumably, the β-(1→3)-linked sugar moiety at the non-reducing end of this trisaccharide hinders its accommodation in the active site. In accordance with the abovementioned minimal structural requirements for good CDH substrates, β-D-Gal*p*-(1→3)-D-Glc, allolactose [β-D-Gal*p*-(1→6)-D-Glc], β-D-Gal*p*-(1→3)-D-Gal and β-D-Gal*p*-(1→6)-D-Gal, which are among the prominent transgalactosylation products of β-galactosidase from *L. reuteri*, are either not oxidised by CDH at all or are very poor substrates as in the case of β-D-Gal*p*-(1→3)-D-Gal.

CDH from *S. rolfsii* (0.84 U/mL), which was found to be more specific with respect to lactose oxidation, was then used for the large-scale conversion starting with 5 L of GalOS mixture. The reaction was stopped after 9 h when lactose conversion was complete. Depletion of lactose was amongst others evident from an almost complete lack of oxygen consumption by the regenerative enzyme laccase, an accompanied increase in the redox potential of the reaction mixture, as well as by a sharp decrease in the consumption of Na_2CO_3 which had to be added during the reaction to maintain the pH of the reaction mixture by titrating lactobionic and other sugar acids formed (Fig. 5). Analysis by HPAEC-PAD showed that lactose was quantitatively oxidised to lactobionic acid. In addition, three galactooligosaccharide components, β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc, β-D-Gal*p*-(1→3)-D-Gal and the unidentified GalOS with a retention time of ~23 min, were oxidised to some extent so that the total loss of GalOS was 16.7%.

2.3. Removal of ions by ion-exchange chromatography

This lactose-free sugar mixture obtained after CDH-catalysed oxidation contained 270 mM of anions and 163 mM of cations, and was applied to a strong cation resin (Lewatit S 2528) coupled with a medium basic anion resin (Lewatit S 4328). Although the theoretical capacity of the anion-exchange material was 1.75 equiv/L, lactobionic acid was not completely removed after one run. After three consecutive chromatographic runs, 99.7% of lactobionic acid was removed from the mixture.

2.4. Removal of monosaccharides

The deionised GalOS mixture after the ion-exchange chromatographic steps was concentrated to a total sugar concentration of 60%. D-Glucose and D-galactose were then separated from the GalOS mixture to an extent of 99.7% and 99.2%, respectively, by using the strong cation-exchange material UBK-530 (Na^+ form resin) which functions also as a size-exclusion material. Thin-layer chromatography (TLC) of the eluting fractions was used to monitor the separation. Fractions rich in GalOS but low in monosaccharides were combined to give the final product. As almost complete removal of

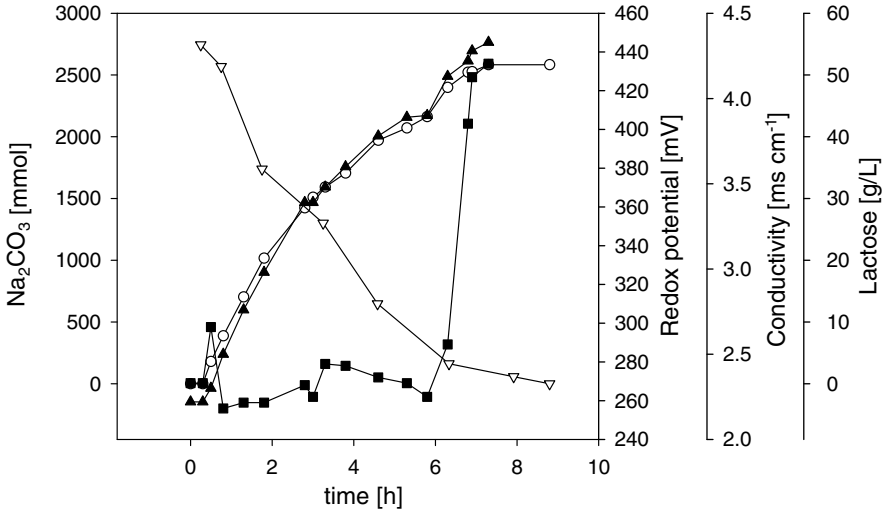


Figure 5. Enzymatic oxidation of lactose into lactobionic acid using a sugar solution (206.5 g/L) containing lactose (26.5%), monosaccharides (48%) and GalOS (25.5%). The reaction mixture consisted of CDH from *S. rolfsii* (0.84 U/mL), laccase (1.75 U/mL), DCIP (2.5 mM), and acetic acid to obtain an initial pH of 4. It was flushed with pure oxygen to supply the substrate for laccase. The pH was controlled by the addition of 1 M sodium carbonate. Symbols: lactose concentration (open triangle), redox potential (full square), conductivity (full triangle), Na_2CO_3 consumption (open circle).

monosaccharides was one of the criteria for pooling the fraction of this chromatographic procedure, approximately 60% of the non-lactose disaccharides (27% of total sugar) was lost during this separation step. The final GalOS product was of very high purity containing 99.1% GalOS, 0.6% lactobionic acid, 0.1% D-glucose, 0.1% D-galactose and 0.1% lactose. The overall recovery of GalOS during the entire process of separation was 60.4% of the total GalOS formed in the transgalactosylation reaction, or 15.4% of the lactose initially employed to form GalOS. The final GalOS mixture essentially free of lactose, glucose and galactose can be obtained in very high purity of 99.1% GalOS. This pure GalOS mixture should be of great advantage for studies on the prebiotic effect of GalOS.

3. Experimental

3.1. Chemicals

All chemicals used were of analytical grade and of the highest purity available. Acetic acid and *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) were supplied by Sigma (St. Louis, MO, USA). 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,6-dichloro-indophenol (DCIP), D-glucose, D-galactose and D-lactose were purchased from Fluka (Buchs, Switzerland), 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 and NMR identification. Allolactose [β-D-Gal*p*-(1→6)-D-Glc] was synthesised as previously reported.[23] 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). Glucose oxidase from *Aspergillus niger* and horseradish peroxidase were obtained from Boehringer Mannheim (Mannheim, Germany).

3.2. Enzymes

The β-galactosidase used for the production of the galacto-oligosaccharide mixture originated from *L. reuteri* L103 (Lactosan GmbH & Co KG, Kapfenberg, Austria) and was overexpressed in *E. coli* BL21 Star (DE3).[24,25] The recombinant enzyme was purified to apparent homogeneity with a specific activity of 235 U/mg protein using immobilised metal affinity chromatography. Cellobiose dehydrogenase (CDH) from *S.* (*Athelia*) *rolfsii* CBS 191.62 was prepared as described by Baminger et al.[20] The enzyme was purified as previously reported to a specific activity of 23.4 U/mg protein.[19,20] CDH from the ascomycete fungus *M. thermophilum* was prepared as previously reported using the fungal wild-type strain.[26] The enzyme preparation was purified to a specific activity of 10.7 U/mg protein. Laccase from *Trametes pubescens* MB 89 was prepared as described by Galhaup et al.[27,28] and purified to a specific activity of 64.5 U/mg protein.

3.3. Enzyme activity assays

The assay for β-galactosidase activity was essentially as described by Splechtna et al.[29] The activity of β-galactosidase was determined at 30 °C using chromogenic *o*NPG as the substrate and measuring the absorption

of *o*-nitrophenol (*o*NP) formed at 420 nm. One unit of *o*NPG activity is defined as the amount of enzyme releasing 1 μmol of *o*NP per minute under the assay conditions described above. The assay mixture for determining cellobiose dehydrogenase (CDH) activity contained 0.3 mM DCIP and 30 mM lactose in 100 mM sodium acetate buffer, pH 4.0. The reduction of DCIP was continuously monitored at 30 °C and 520 nm ($\varepsilon_{520} = 6.8\ mM^{-1}\ cm^{-1}$) over 3 min.[30] One unit of CDH activity is defined as the amount of enzyme that reduces 1 μmol of DCIP per minute under these conditions. Laccase activity was measured using 1 mM ABTS ($\varepsilon_{420} = 43.2\ mM^{-1}\ cm^{-1}$) in 100 mM acetate buffer, pH 4.0. The oxidation of ABTS was followed at 30 °C and 420 nm over 3 min. One unit of laccase activity is the amount of enzyme needed to oxidise 1 μmol of ABTS per minute under the above reaction conditions.

Protein was determined by the method of Bradford[31] with the BioRad Coomassie Blue reagent using bovine serum albumin as the standard.

3.4. Sugar analysis

D-Galactose was determined using a commercial D-galactose/lactose test kit (Megazyme). D-Glucose was measured enzymatically using an assay based on glucose oxidase and peroxidase.[32] Galacto-oligosaccharides were analysed using thin-layer chromatography (TLC), capillary electrophoresis (CE) and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).[29] TLC was carried out using high-performance TLC silica plates (Merck, Darmstadt, Germany). A capillary-electrophoresis system with a UV-DAD detector (Agilent Technologies, Palo Alto, CA, USA) and a fused silica capillary was used. HPAEC-PAD analysis was carried out on a Dionex DX-500 system (Dionex, Sunnyvale, CA, USA) and the CarboPac PA-1 column (4 × 250 mm) at 27 °C.[29]

3.5. Galacto-oligosaccharide production

Discontinuous GalOS production was carried out at a 5-L scale using purified β-galactosidase at 23 °C in a stirred tank reactor (total volume of 6 L).[33] The conversion was performed with 5 U/mL of β-galactosidase and 602 mM lactose dissolved in 50 mM sodium phosphate buffer (pH 6.0) containing 2 mM $MgCl_2$. When the desired degree of lactose conversion was reached, the reaction mixture was heated to 98 °C to inactivate β-galactosidase and clarified by centrifugation.

3.6. Oxidation of lactose to lactobionic acid

Two different cellobiose dehydrogenases, originating from *S. rolfsii* and *M. thermophilum*, were compared for the oxidation of lactose to lactobionic acid. In 5-mL batchwise reactions directly using the GalOS mixtures after the transgalactosylation reaction, each CDH was applied at 4.0 U/mL to oxidise lactose. Laccase was employed in 5-fold excess over CDH activity to re-oxidise ABTS in the presence of molecular oxygen (air). The following reactant concentrations were used: GalOS mixture in 50 mM sodium phosphate buffer pH 6.0, 1 mM ABTS, 4 U/mL CDH and 20 U/mL laccase. Sodium carbonate (1 M) was used during the reactions to maintain a constant pH value of 4.0. The reactions were performed in 6-well plates at 25 °C under agitation to increase the oxygen transfer. At different time points, 150-μL samples were taken, heated to 98 °C and centrifuged. The clear supernatant was analysed for sugars with HPAEC-PAD.

CDH from *S. rolfsii* was used to oxidise non-converted lactose in the 5-L GalOS sample obtained in the β-galactosidase-catalysed transgalactosylation reaction. This sugar solution (total sugar concentration of 206.5 g/L) contained 48% monosaccharides, 26.5% lactose and 25.5% GalOS. The reaction was performed in a 6-L reactor at 30 °C. CDH from *S. rolfsii* (0.84 U/mL), laccase (1.75 U/mL) and DCIP (2.5 mM) was added to this GalOS mixture. The initial pH was set to 4.0 using acetic acid (1 M), and then controlled at this by the addition of 1 M sodium carbonate. The reactor was flushed with pure oxygen to supply the co-substrate for laccase. A constant dissolved oxygen tension of 100% air saturation (approximately 0.25 mM O_2) was maintained by using the oxygen control unit of the bioreactor (Applikon Pilot System ADI 1075; Schiedam, The Netherlands).

3.7. Removal of ions by ion-exchange chromatography

After the enzymatic oxidation step, the GalOS product solution was centrifuged and filtered to remove insolubles, and then applied onto two ion-exchange chromatography columns in series for the removal of ions. The first column (XK 50/60 GE Healthcare, Uppsala, Sweden) contained 955 g of the strong cation-exchange material Lewatit S 2528 (Bayer AG, Leverkusen, Germany) and was connected to a second column (INdEX 140/500, GE Healthcare), containing 2015 g of the anion-exchange material Lewatit S 4328 (Bayer AG). The binding capacities of the two resins were 1.75 equiv/L and 1.4 equiv/L, respectively. The cation resin was conditioned using 2 M HCl for 3 column volumes (CV) and washed with water (5 CV), while the anion resin was activated with 20% acetic acid (3 CV) and washed with water (5 CV). The complete GalOS sample of 5.25 L was applied in one step. Elution was done with water at a flow rate of 20 mL/min. Fractions of 100 mL were collected and analysed using HPAEC-PAD and conductivity measurement.

3.8. Removal of monosaccharides by size exclusion

The GalOS mixture after the ion-exchange chromatography steps was concentrated to 60% total sugar (w/v) and applied batchwise to a size-exclusion chromatography column using the strongly acidic cation-exchange resin UBK-530 from Diaion (Mitsubishi Chemical Corporation, Tokyo, Japan).[34] Samples (50 mL each) were applied to a column (50/3000) which was equipped with a double jacket for thermostatting at 70 °C and connected to a RI detector. Water was used as eluent at a flow rate of 8 mL/min. Fractions of 35 mL were collected, and analysed for their sugar composition using TLC. Fractions without monosaccharides were combined and gave the final product, which was subsequently concentrated and lyophilised.

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