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Galactooligosaccharide synthesis from lactose solution or skim milk using the β-galactosidase from *Bacillus circulans*

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ABSTRACT

The synthesis of galactooligosaccharides (GOS) catalyzed by a novel commercial preparation of β-galactosidase from *Bacillus circulans* (Biolactase™) was studied and the products characterized by MS and NMR. Using 400 g/l lactose and 1.5 enzyme units per ml, the maximum GOS yield, measured by HPAEC-PAD analysis, was 165 g/l (41 % w/w of total carbohydrates in the mixture). The major transgalactosylation products were the trisaccharide Gal-β(1→4)-Gal-β(1→4)-Glc and the tetrasaccharide Gal-β(1→4)-Gal-β(1→4)-Gal-β(1→4)-Glc. The GOS yield increased to 198 g/l (49.4 % w/w of total carbohydrates) using a higher enzyme concentration (15 U/ml), which minimized the enzyme inactivation under reaction conditions. Using skim milk (with a lactose concentration of 46 g/l), the enzyme also displayed transgalactosylation activity: maximum GOS yield accounted for 15.4% (7.1 g/l), which was obtained at 50% lactose conversion.

Keywords: glycosidase, galacto-oligosaccharides, prebiotics, transglycosylation, beta-galactosidase, oligosaccharides.

1. INTRODUCTION

β-Galactosidases (β-D-galactoside galactohydrolases, EC 3.2.1.23) catalyze the hydrolysis of the galactosyl moiety from the non-reducing end of various oligosaccharides. These enzymes have attracted attention from dairy industries due to their ability to remove lactose from milk.[1] In addition, β-galactosidases catalyze transgalactosylation reactions in which lactose (as well as the released glucose and galactose) serve as galactosyl acceptors yielding a series of di, tri- and higher oligosaccharides called galactooligosaccharides (GOS).[2,3] GOS are non-cariogenic, reduce the level of cholesterol in serum, prevent colon cancer and exhibit prebiotic properties. In fact, GOS constitute the major part of oligosaccharides in human milk.[4-6] The properties of GOS depend significantly on their chemical composition, structure and degree of polymerization.[7] Depending on the origin of β-galactosidase, the yield and composition of GOS vary notably.[8-11] The most studied β-galactosidases are those from *Kluyveromyces lactis,*[12-14] *Rhizopus oryzae,*[15] *Bifidobacterium sp.*[16] and *Bacillus circulans.*[17]

Regarding the β-galactosidase from *Bacillus circulans,* different isoforms have been reported in the commercial preparation Biolacta (Daiwa Kasei). At least three isoforms with different behaviour in GOS production were characterized: β-galactosidase-1 showed very low transglycosylation activity,[18] β-galactosidase-2 contributed most significantly to GOS synthesis,[18,19] and β-galactosidase-3 was able to produce GOS with β(1→3) bonds.[20] More recently, Song et al.[21] described four isoforms with different molecular size in Biolacta: β-gal-A (189 kDa), β-gal-B (154 kDa), β-gal-C (134 kDa) and β-gal-D (91 kDa). The transferase activity of β-galactosidase from *B. circulans* has been applied to the synthesis of lactosucrose,[22] N-acetyl-lactosamine[23] and other galactosylated derivatives.[24] Interestingly, the enzyme is able to catalyze the galactosylation of different acceptors in the presence of organic

cosolvents up to 50% v/v.[25] The *B. circulans* β-galactosidase has been also immobilized on different supports.[19,26] However, only partial analysis of the GOS formed in the transglycosylation reaction with lactose has been performed,[19] probably due to the complexity of the reaction mixture derived from the presence of several isoforms with different regiospecificity.

In this work, we have studied the transgalactosylation activity of a novel commercial β-galactosidase preparation from *Bacillus circulans* (Biolactase™). A detailed kinetic study of the reaction with lactose was performed, including the structural characterization of the synthesized GOS. It is well reported that oligosaccharides formed by the same monosaccharides with the same anomeric configuration, but differing in the glycosidic bonds between them, exhibit different fermentation patterns.[7]

In addition, skim milk as lactose source was further investigated to assess the effect of lactose concentration on transglycosylation and to explore the *in situ* formation of GOS in dairy products.

2. EXPERIMENTAL PROCEDURES

Materials

Biolactase™ (batch no. MB-878) is a liquid β-galactosidase preparation from *Bacillus circulans* produced by Kerry Ingredients and Flavours (http://www.kerry.com) that was supplied by Biocon (Spain). Glucose, galactose, lactose monohydrate and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were from Sigma-Aldrich. 3-Galactobiose, 4-galactobiose, 6-galactobiose, 6-*O*-β-galactosyl-glucose (allolactose) and 4-*O*-β-galactosyl-lactose were from Carbosynth (Berkshire, UK). Skim milk "Hacendado" was purchased from Mercadona supermarket (Spain). All other reagents and solvents were of the highest available purity and used as purchased.

Activity assay

The enzymatic activity towards *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was measured at 40 ºC following *o*-nitrophenol release at 405 nm using a microplate reader (Versamax, Molecular Devices). The reaction was started by adding 10 µl of the enzyme (conveniently diluted) to 190 µl of 15 mM ONPG in 0.1 M sodium acetate buffer (pH 5.5). The increase of absorbance at 405 nm was followed in continuous mode during 5 min. The extinction molar coefficient of *o*-nitrophenol at pH 5.5 was determined (537 M^{-1} cm^{-1}). One unit (*U*) of activity was defined as that corresponding to the hydrolysis of 1 µmol of ONPG per min.

SDS-PAGE. SDS-PAGE was performed on 8% polyacrylamide gels and the proteins were stained with colloidal Coomassie Blue (Protoblue Safe, National Diagnostics) diluted with ethanol. HMW-SDS calibration quit (53-220 kDa) was from GE Healthcare Bio-Sciences. LMW-SDS calibration quit (15-150 kDa) was from Novagen.

Thermostability of *B. circulans* β-galactosidase

The enzyme (approx. 5.5 U/ml) was incubated at different temperatures (40-60 ºC) in 0.1 M sodium acetate buffer (pH 5.5). Aliquots were harvested at different times, and the remaining activity towards ONPG was determined as described above (after convenient dilution of the enzyme).

Production of galacto-oligosaccharides from lactose solution

The reaction mixture (20 ml) contained 400 g/l lactose (34.7% w/w) in 0.1 M sodium acetate buffer (pH 5.5). The biocatalyst (Biolactase) was then added to adjust the β-galactosidase activity in the reaction mixture to 1.5 U/ml or 15 U/ml. The mixture was incubated at 40 ºC in an orbital shaker (Vortemp 1550) at 200 rpm. At different times, 200 µl aliquots were harvested from the reaction vessel and mixed with 800 µl of 0.4 M Na_2CO_3 (pH 11.0) in order to stop the reaction. Samples were filtered using 0.45 µm cellulose filters (National Scientific) coupled to eppendorf tubes by centrifugation during 5 min at 6000 rpm. For each sample, two dilutions with water (1:400 and 1:4000) were done for HPAEC-PAD analysis.

Production of galacto-oligosaccharides from skim milk

Biolactase was added to skim milk (20 ml) to adjust the β-galactosidase activity in the reaction vessel to 1.5 U/ml. The mixture was then incubated at 40 ºC in an orbital shaker (Vortemp 1550) at 200 rpm. At different times, 200 µl aliquots were harvested from the reaction vessel and mixed with 800 µl of 0.4 M Na_2CO_3 (pH 11.0) in order to stop the reaction. Samples were filtered, conveniently diluted and analyzed as described elsewhere.

HPAEC-PAD analysis

Product analysis was carried out by high performance anion-exchange chromatography

coupled with pulsed amperometric detection (HPAEC-PAD) on a ICS3000 Dionex system consisting of a SP gradient pump, an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode, and an autosampler (model AS-HV). All eluents were degassed by flushing with helium. A pellicular anion-exchange 4 x 250 mm Carbo-Pack PA-1 column (Dionex) connected to a 4 x 50 CarboPac PA-1 guard column was used at 30 °C. Eluent preparation was performed with MilliQ water and 50% (w/v) NaOH (Sigma-Aldrich). The initial mobile phase was 15 mM NaOH at 1.0 ml/min for 28 min. A gradient from 15 mM to 200 mM NaOH was performed in 7 min at 1.0 ml/min, and 200 mM NaOH was maintained for 25 min. In order to increase the sensitivity of the detector at low NaOH concentrations, a Dionex PC10 postcolumn delivery system with 0.2 M NaOH was used at 96 psi. The peaks were analyzed using Chromeleon software. Identification of the different carbohydrates was done based on commercially available standards or purified in our laboratory as described in a previous paper.[11]

Purification of GOS by semipreparative HILIC

For the isolation of unknown GOS in the mixture, the reaction was stopped when GOS yield reached the maximum value. The biocatalyst (Biolactase) was inactivated by boiling the solution for 10 min. The reaction mixture was filtrated and the solution was purified by semipreparative hydrophilic interaction chromatography (HPLC-HILIC). A quaternary pump (Delta 600, Waters) coupled to a LiChrospher-NH2 column (5 µm, 10 x 250 mm, Merck) was used. The column temperature was kept constant at 30 ºC. Acetonitrile/water 90/10 (v/v), degassed with helium, was used as mobile phase (flow rate 6.25 ml/min) for 8 min. Then, a gradient to acetonitrile/water 80/20 (v/v) was performed in 3 min, and this eluent was maintained during 6 min. Finally, a gradient from the latter mobile phase to acetonitrile/water 75/25 (v/v) was performed in 3 min, and maintained for 15 min. Peaks were detected using an

evaporative light-scattering detector DDL-31 (Eurosep) equilibrated at 60 °C. A three-way flow splitter (model Acurate^{TM}, Dionex) and a fraction collector II (Waters) were employed. The fractions containing the main peaks were pooled and the solvent was eliminated by rotary evaporation.

Mass spectrometry

Samples were analyzed by MALDI-TOF mass spectrometry (Bruker, model Ultraflex III TOF-TOF) using 2,5-dihydroxybenzoic acid doped with sodium iodide as matrix, in positive reflector mode.

Nuclear Magnetic Resonance (NMR)

The structure of the oligosaccharides was elucidated using a combination of 1D (^{1}H, ^{13}C) and 2D (COSY, TOCSY, NOESY, HSQC) NMR techniques. The spectra of the samples, dissolved in deuterated water (ca. 10 mM), were recorded on a Bruker AVANCE DRX500 spectrometer equipped with a tunable broadband ^{1}H/X probe with a gradient in the Z axis, at a temperature of 298 K. Chemical shifts were expressed in ppm with respect to the 0 ppm point of DSS, used as internal standard. COSY, TOCSY, NOESY and HSQC standard pulse sequences were provided by Bruker. COSY, TOCSY (80 ms mixing time), and NOESY (500 ms mixing time) experiments were performed with a minimum of 8, 16 and 48 scans, respectively, with 256 increments in the indirect dimension and with 1024 points in the acquisition dimension. The spectral widths were 9 ppm in both dimensions. The HSQC experiment (16 scans) also used 256 increments in the indirect dimension and with 1024 points in the acquisition dimension. The spectral width was 120 ppm in the indirect dimension and 9 ppm in the acquisition one.

3. RESULTS AND DISCUSSION

3.1. GOS specificity of *Bacillus circulans* β-galactosidase

A novel commercial preparation of β-galactosidase from *Bacillus circulans* (Biolactase) was studied. SDS/PAGE gel (Fig. 1) showed the presence in Biolactase of different proteins with molecular mass in the range 75-200 kDa, which according to Song et al.[21] makes probable the existence of various β-galactosidase isoforms in the enzyme preparation. This was also confirmed by native PAGE with 4-methylumbelliferyl-β-D-galactopyranoside, which showed various active bands in that range (data not shown). The volumetric activity of Biolactase towards *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was 2740 U/ml. Protein concentration was 15.4 mg/ml, and the specific activity accounted for 18.3 U/mg protein. We studied in detail the synthesis of galactooligosaccharides (GOS) catalyzed by Biolactase using 400 g/l lactose and 1.5 U/ml (β-galactosidase activity towards ONPG), in particular the selectivity of the bonds formed. It is well reported that the chemical structure of the obtained oligosaccharides (composition, number of hexose units and types of linkages between them) may affect their fermentation pattern by probiotic bacteria in the gut.[7,12]

Fig. 2 shows the HPAEC-PAD chromatogram of the reaction mixture at the point of maximum GOS concentration. Peaks *1*, *2* and *4* corresponded to galactose, glucose and lactose, respectively. As illustrated in the chromatogram, the two main products present in the reaction mixture were peaks *10* and *16*. Using a commercial standard, peak *10* was identified as the trisaccharide 4-galactosyl-lactose [Gal-β(1→4)-Gal-β(1→4)-Glc]. Peak *16* was purified by semipreparative HILIC, as the sugar concentration in samples for HPAEC-PAD analysis was too low for an efficient scaling-up. The mass spectrum of peak *16* showed that it was a tetrasaccharide (data not shown). The analysis of the NMR spectra permitted to assess the presence of five anomeric signals. Further inspection of the spectra allowed us to assign three of them as coming from the Gal residues, while the other two aroused from the Glc moiety (α

and β anomers). From the combination of the information derived from COSY, TOCSY, NOESY and HSQC (Fig. 3A), most of the relevant ^{1}H and ^{13}C resonance signals belonging to the different residues could be assigned. The key information on the substitution pattern was extracted from the analysis of the HSQC experiment. The three cross peaks for the three Gal H4/C4 atom pairs were first identified. Their distinct chemical shifts permitted to distinguish that two of them belonged to glycosylated Gal O-4 atoms, while the third one was non-substituted at O-4. From the cross peaks pattern in the TOCSY experiment it was possible to identify the corresponding intra-residual Gal H-1 anomeric signals for each Gal H-4. Then, the sequential connectivity of the sugar chain moieties was derived in a straightforward manner from the inter-residual cross peaks in the NOESY experiment. On this basis, the NMR data for peak *16* are consistent with a molecule which shows two galactosyl moieties β-(1→4)-linked to O-4 at the galactose unit of lactose, resulting in the tetrasaccharide Gal-β(1→4)-Gal-β(1→4)-Gal-β(1→4)-Glc.

Commercially available standards and other GOS purified in our previous work with the β-galactosidase from *Kluyveromyces lactis*[11] allowed us to identify in the chromatograms the disaccharides allolactose [Gal-β(1→6)-Glc] (peak *3),* 4-galactobiose [Gal-β(1→4)-Gal] (peak *5)* and Gal-β(1→3)-Glc (peak *7),* as well as the trisaccharide 6-galactosyl-lactose [Gal-β(1→6)-Gal-β(1→4)-Glc] (peak *6).* We also purified peak *14* by semipreparative HILIC, whose mass spectrum indicated that it was a trisaccharide. In this case, the NMR spectra displayed four anomeric signals: two of them coming from the Gal residues and the two other coming from the Glc moiety (α and β). Following the same methodology described above, the combined analysis of the HSQC (Fig. 3B) and TOCSY spectra permitted to distinguish the existence of one terminal non-reducing Gal residue, one O-4 substituted Gal moiety, along with a terminal reducing Glc unit, substituted at O-3. Thus, the NOESY spectrum permitted to assess the sequential connectivity, indicating that peak *14* indeed corresponded to the

trisaccharide Gal-β(1→4)-Gal-β(1→3)-Glc. Peaks *8*, *9, 11, 12, 13* and *15* remained unknown.

It is worth emphasizing that the two major products synthesized by *B. circulans* β-galactosidase [the trisaccharide 4-galactosyl-lactose and the tetrasaccharide Gal-β(1→4)-Gal-β(1→4)-Gal-β(1→4)-Glc] contained only β-(1→4) bonds. This result goes further in the control of regioselectivity by an appropriate selection of the enzyme. Yanahira *et al*. were the first in performing structural analysis of the GOS formed by *B. circulans* β-galactosidase (Biolacta from Daiwa Kasei); they reported that the main product was 4-galactosyl-lactose, but the formation of tetrasaccharides was not mentioned.[27] In such paper, several disaccharides and trisacccharides were purified and characterized; the authors reported the presence of various GOS with β-(1→2) bonds,[27] which may correspond to some of the unknown peaks in our study. Recently, Song *et al.* analyzed the GOS production by the different isoforms of *B. circulans* β-galactosidase.[28] Although the authors found significant differences in total GOS yield, the structural analysis of the synthesized compounds was not reported.

The product specificity of *B. circulans* β-galactosidase contrasts with that of the *K. lactis* counterpart. The latter exhibits a tendency to synthesize β-(1→6) bonds: the main products in the reaction mixture are the disaccharides 6-galactobiose [Gal-β(1→6)-Gal] and allolactose [Gal-β(1→6)-Glc], as well as the trisaccharide 6-galactosyl-lactose [Gal-β(1→6)-Gal-β(1→4)-Glc].[11,12] Another difference between both enzymes deals with the formation of disaccharides, because *B. circulans* β-galactosidase yields a moderate amount of allolactose (peak *3*), 4-galactobiose (peak *5*) and 3-galactosyl-glucose (peak *7*), whereas the *K. lactis* enzyme is able to use efficiently free galactose and glucose as acceptors yielding 6-galactobiose and allolactose, respectively, in notable yields.[11]

3.2. Kinetics of GOS synthesis

Table 1 summarizes the evolution of carbohydrate composition of the reaction mixture

employing 400 g/l lactose and 1.5 U/ml Biolactase as biocatalyst. The contribution of the main components (glucose, galactose, lactose and the two major transglycosylation products with β(1→4) bonds), as well the rest of synthesized GOS considered as a pool, are reported. It has been broadly demonstrated that the maximum GOS yield is basically determined by the intrinsic enzyme properties (transgalactosylation to hydrolysis ratio) as well as substrate concentration.[29] In our work, the maximum GOS yield (41.3%, corresponding to a concentration of 165 g/l) was observed at 77.5 h, when lactose conversion accounted for 70%. This value is in the upper range of GOS yields reported (40-45%),[8,30,31] and is similar to that described for the isoform β-galactosidase-2 from *B. circulans*.[21] However, GOS yields are generally lower than those reported for other related prebiotics such as fructooligosaccharides (approx. 65%) using similar strategies.[32,33]

The GOS concentration changes substantially with reaction time because GOS are simultaneously synthesized and hydrolyzed by β-galactosidases.[3,8] In consequence, the time at which reaction is harvested has a crucial influence on GOS yield.[6] Although the concentration of the two main transgalactosylation products (containing only β(1→4) bonds) reached a maximum followed by a progressive decrease (Table 1), the GOS production maintained nearly constant after 70 h (Fig. 4A) because the synthesis of other GOS increased with time. In particular, two disaccharides with β(1→6) and β(1→3) bonds (allolactose and Gal-β(1→3)-Glc, respectively) contributed significantly to the GOS concentration at the latter stages of the reaction. This effect could be explained by the hydrolytic activity of the enzyme, as β-galactosidases are specific for the cleavage of β(1→4) bonds compared with β(1→3) or β(1→6) bonds present in other GOS.

Fig. 4A shows the progress of total GOS formation, and Fig. 4B their distribution based on their polymerization degree. It is worth noting that the remaining lactose at 400 h was still high (23%) compared with similar processes using other glycosidic enzymes.[9,11,13]

This effect could be caused by an inactivation of the β-galactosidase during the process. The experiment depicted in Fig. 4 was carried out at 1.5 U/ml, which is a lower enzyme concentration than the typically used with β-galactosidases (3-12 U/ml).[12,13,34] In fact, at higher enzyme concentrations, reactions are faster and it is less probable to detect any effect of enzyme inactivation on reaction progress. To analyze the possible stability effect, we added fresh enzyme after 440 h (Fig. 4A) and confirmed that the lactose diminished up to 31 g/l (7.8 %) accompanied by a smooth increase of the GOS formed, especially in the contribution of disaccharides (Fig. 4B).

3.3. Effect of enzyme concentration on GOS formation

Fig. 5 illustrates the thermostability of *B. circulans* β-galactosidase. As shown, the enzyme inactivated very fast between 50-60 °C and most of its activity was lost in less than 2 hours. At 40 °C the β-galactosidase lost nearly half of its activity in 10 hours, and from that point the activity decay was slower. Taking into account that enzyme stability is enhanced in the presence of sugars,[35] the operational conditions (high concentration of lactose) are favourable for stability of β-galactosidases.[36] However, the high concentration of residual lactose after 400 h using 1.5 U/ml seems to be related with the inactivation of the enzyme at long reaction times.

To minimize the inactivation effect, we performed the GOS synthesis at 10-fold higher enzyme concentration (15 U/ml). It has been widely reported that, working under kinetic control conditions, enzyme concentration has no effect on the maximum GOS yield as long as no enzyme inactivation takes place,[37] and it only exerts a marked influence on the reaction time at which the maximum oligosaccharide concentration is achieved.[13,37]

Fig. 6A shows that the maximum GOS production at 15 U/ml was achieved in 6.5 h, with a yield of 198 g/l. This value corresponds to 49.4% (w/w) of total sugars (Table 2), which

is higher than the value obtained at 1.5 U/ml (165 g/l, 41.3%). Interestingly, the remaining lactose at the end of the reaction (10 g/l, 2.5% of total carbohydrates) is significantly lower than the obtained at 1.5 U/ml. This confirms that the stability of *B. circulans* β-galactosidase is only moderate under typical GOS formation conditions; at 1.5 U/ml, the reaction is stopped before reaching the final composition.

Table 2 summarizes the evolution of the sugar composition of the mixture with time. Again the two major products were those with only β(1→4) bonds, but once formed they were fastly hydrolyzed by the β-galactosidase, enriching the mixture with GOS that contained other bonds. Fig. 6B illustrates the distribution of di-, tri- and tetrasaccharides with time. After 30 h, the contribution of disaccharides was very significant and even surpassed that of trisaccharides. Allolactose, 3-, 4- and 6-galactobioses, and Gal-β(1→3)-Glc were identified in notable amounts in the HPAEC-PAD chromatograms.

3.4. GOS production in skim milk

Most reports on treatment of milk with β-galactosidases focus on the hydrolytic activity to obtain lactose-free products.[38,39] The production of GOS in milk has been scarcely investigated, probably because the lactose concentration in milk (around 5% w/v) is not appropriate for an optimal transglycosylation to hydrolysis ratio.[40] To overcome this limitation, Chen *et al.* developed a multi-step process applying ultrafiltration to separate lactose from milk proteins, followed by concentration of the permeate and further biotransformation with β-galactosidases.[41]

A positive aspect for the direct biotransformation of lactose in milk into GOS is that the pH of milk (approx. 6.7) is not far from the optimum pH of most β-galactosidases. In particular, the β-galactosidase from *Bacillus circulans* is a good choice for dairy products treatment (whole or skim milk, whey, etc.) as it presents a notable activity at pH 6.7 and is not

inhibited by calcium cations.[18,42]

Gosling *et al.* assayed the *B. circulans* β-galactosidase preparation Biolacta for GOS production in milk in the temperature range 4-60 °C.[43] They observed that GOS yield increased with temperature -as described in other transglycosylation studies-[44] but neither kinetic nor structural analysis of the synthesized GOS was reported. Greenberg *et al.* reported that GOS accounted for 25% of total sugars in milk (quantified by paper chromatography) using the β-galactosidase from *Streptococcus thermophilus*.[45]

We performed a detailed analysis of GOS formation in skim milk using Biolactase preparation. Initial lactose concentration was 46 g/l, measured by HPAEC-PAD. Fig. 7A illustrates the kinetics of GOS synthesis at 40 °C, where the typical pattern with a maximum GOS concentration followed by a progressive disappearance of GOS was observed due to the competition that is established between hydrolysis and transglycosylation (kinetic control).[42,46] This is clearly represented in Fig. 7B that shows how the glucose and galactose concentrations tend to converge at the end of the process. Maximum GOS yield (7.1 g/l, 15.4% of the total carbohydrates present in milk) was obtained at 2 h, when the lactose conversion accounted for 52%. Mozaffar *et al.* reported a maximum amount of GOS close to 5.5% of total sugars, which was obtained at 39% conversion of lactose, using a purified β-galactosidase from *B. circulans.*[42]

Fig. 8 shows the HPAEC-PAD chromatogram close to the point of maximum GOS concentration. As shown, the main peaks were the same as those described using 400 g/l lactose. The main GOS formed was the trisaccharide Gal-β(1→4)-Gal-β(1→4)-Glc (peak *10*). The main difference between both experiments (skim milk *vs.* 400 g/l lactose) was the absence of several minor peaks when milk was the lactose source.

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Table 1. Composition of the reaction mixture (weight percentage referred to the total amount of carbohydrates) using 400 g/l lactose and 1.5 U/ml β-galactosidase from *Bacillus circulans* (Biolactase)[a].

Reaction time (h)	Gal (%)	Glc (%)	Lact (%)	Gal-β(1→4)-Gal-β(1→4)-Glc (%)	Gal-β(1→4)-Gal-β(1→4)-Gal-β(1→4)-Glc (%)	Other GOS (%)	Total GOS (%)
0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
1.0	0.2	2.9	92.1	4.5	0.1	0.2	4.8
2.0	0.4	5.7	85.6	7.6	0.4	0.3	8.3
3.0	0.4	6.2	79.1	13.2	0.7	0.4	14.3
6.0	0.6	8.7	67.4	20.2	2.1	1.0	23.3
9.0	0.8	8.5	65.6	19.7	3.2	2.2	25.1
12.0	1.3	16.1	58.9	17.1	3.6	3.0	23.7
22.0	2.4	22.4	45.9	17.9	4.7	6.7	29.3
32.5	2.8	24.5	38.4	20.0	4.9	9.4	34.3
47.0	3.5	26.8	34.3	18.1	4.8	12.5	35.4
56.5	3.0	26.2	32.3	20.3	4.7	13.5	38.5
71.0	3.3	26.4	30.3	20.4	4.5	15.2	40.1
77.5	3.8	25.6	29.3	22.1	2.7	16.5	41.3
94.5	4.2	27.4	29.3	18.4	2.8	17.9	39.1
103.0	4.3	28.0	28.7	17.5	2.5	19.0	39.0
277.0	4.9	29.8	26.7	14.2	2.0	22.4	38.6
319.0	5.7	30.0	23.0	14.8	3.3	23.2	41.3
418.0	7.9	29.6	22.6	12.4	3.0	24.5	39.9

[a] Experimental conditions: 0.1 M sodium acetate buffer (pH 5.5), 40 °C.

Table 2. Composition of the reaction mixture (weight percentage referred to the total amount of carbohydrates) using 400 g/l lactose and 15 U/ml β-galactosidase from *Bacillus circulans* (Biolactase)[a].

Reaction time (h)	Gal (%)	Glc (%)	Lact (%)	Gal-β(1→4)-Gal-β(1→4)-Glc (%)	Gal-β(1→4)-Gal-β(1→4)-Gal-β(1→4)-Glc (%)	Other GOS (%)	Total GOS (%)
0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
0.5	0.9	12.5	56.7	25.5	3.2	1.2	29.9
1.0	2.0	16.2	47.6	26.4	5.1	2.7	34.2
2.0	4.0	19.1	39.9	23.1	5.6	8.3	37.0
6.5	5.9	18.0	26.7	13.8	3.3	32.2	49.4
10.5	9.4	31.5	17.2	7.4	1.7	32.9	41.9
26.0	17.0	37.1	6.9	4.4	0.2	34.5	39.1
30.5	16.2	33.2	6.8	4.6	0.6	38.6	43.9
46.0	15.1	35.4	5.0	4.7	0.6	39.1	44.5
54.5	15.9	37.7	4.5	4.1	0.0	37.8	42.0
70.5	18.2	40.3	3.3	3.3	0.0	34.9	38.2
78.0	18.4	39.3	4.6	2.0	0.0	35.7	37.7
143.0	18.3	41.3	2.4	1.3	0.0	36.6	37.9
151.0	21.7	39.4	4.8	1.8	0.0	32.4	34.1
175.0	23.9	42.6	2.4	1.3	0.0	29.8	31.1

[a] Experimental conditions: 0.1 M sodium acetate buffer (pH 5.5), 40 °C.

Figure Legends

Fig. 1. SDS-PAGE of β-galactosidase from *B. circulans* (Biolactase). Lane 1: marker proteins (53-220 kDa); Lane 2: Biolactase diluted 1:200; Lane 3: Biolactase diluted 1:1000; Lane 4: marker proteins (15-150 kDa).

Fig. 2. HPAEC-PAD analysis of the reaction of lactose with *B. circulans* β-galactosidase (Biolactase). The peaks correspond to: (*1*) Galactose; (*2*) Glucose; (*3*) Allolactose; (*4*) Lactose; (*5*) 4-Galactobiose; (*6*) 6-Galactosyl-lactose; (*7*) 3-Galactosyl-glucose; (*10*) 4-Galactosyl-lactose; (*14*) Gal-β(1→4)-Gal-β(1→3)-Glc; (*16*) Gal-β(1→4)-Gal-β(1→4)-Gal-β(1→4)-Glc; (*8,9,11,12,13,15*) Other GOS (unknown). The chromatogram corresponds to the reaction mixture after 77.5 h with Biolactase.

Fig. 3. 2D-NMR Heteronuclear Single Quantum Coherence (HSQC) analysis of two galactooligosaccharides obtained in the reaction of lactose with *B. circulans* β-galactosidase: (A) the tetrasaccharide Gal-β(1→4)-Gal-β(1→4)-Gal-β(1→4)-Glc, (B) the trisaccharide Gal-β(1→4)-Gal-β(1→3)-Glc. Only the most relevant signals are assigned and labelled.

Fig. 4. Kinetics of GOS formation at 1.5 U/ml using 400 g/l lactose catalyzed by β-galactosidase from *B. circulans* (Biolactase): (A) Formation of total GOS; (B) GOS distribution as a function of polymerization degree. Reaction conditions: 0.1 M sodium acetate buffer (pH 5.5), 40 °C. Dashed line: addition of a fresh batch of enzyme after 440 h.

Fig. 5. Thermoinactivation of *B. circulans* β-galactosidase at different temperatures in 0.1 M sodium acetate buffer (pH 5.5). Residual activity was determined at the indicated times using the ONPG assay.

Fig. 6. Kinetics of GOS formation at 15 U/ml using 400 g/l lactose catalyzed by β-galactosidase from *B. circulans* (Biolactase): (A) Formation of total GOS; (B) GOS distribution as a function of polymerization degree. Reaction conditions: 0.1 M sodium acetate buffer (pH 5.5), 40 °C.

Fig. 7. Kinetics of GOS formation by Biolactase using skim milk: (A) Total GOS production; (B) Progress of glucose and galactose concentrations. Experimental conditions: 1.5 U/ml, 40 °C.

Fig. 8. HPAEC-PAD analysis of the reaction of skim milk with *B. circulans* β-galactosidase (Biolactase) at the point of maximum GOS concentration (2 h). The peaks correspond to: (*1*) Galactose; (*2*) Glucose; (*4*) Lactose; (*5*) 4-Galactobiose; (*7*) 3-Galactosyl-glucose; (*10*) 4-Galactosyl-lactose; (*16*) Gal-β(1→4)-Gal-β(1→4)-Gal-β(1→4)-Glc.

Fig 1

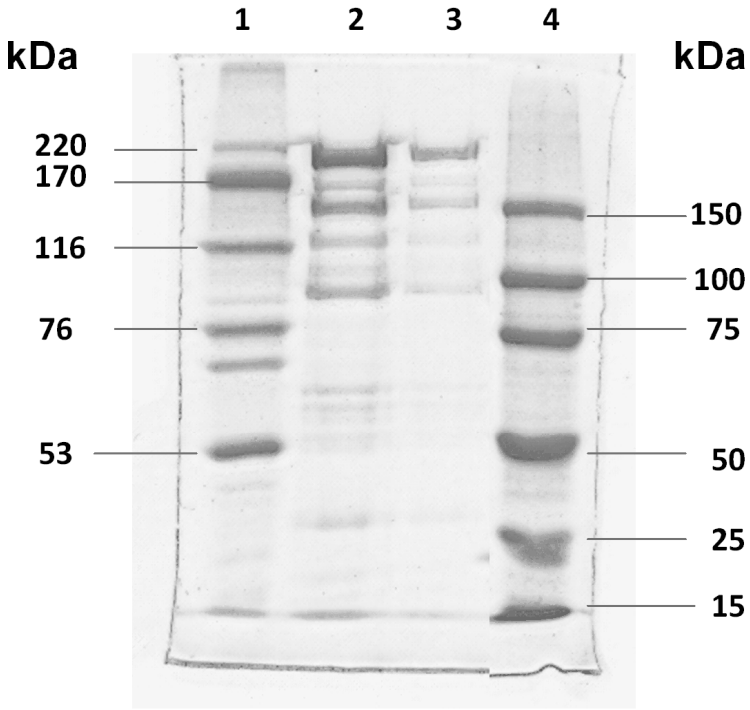
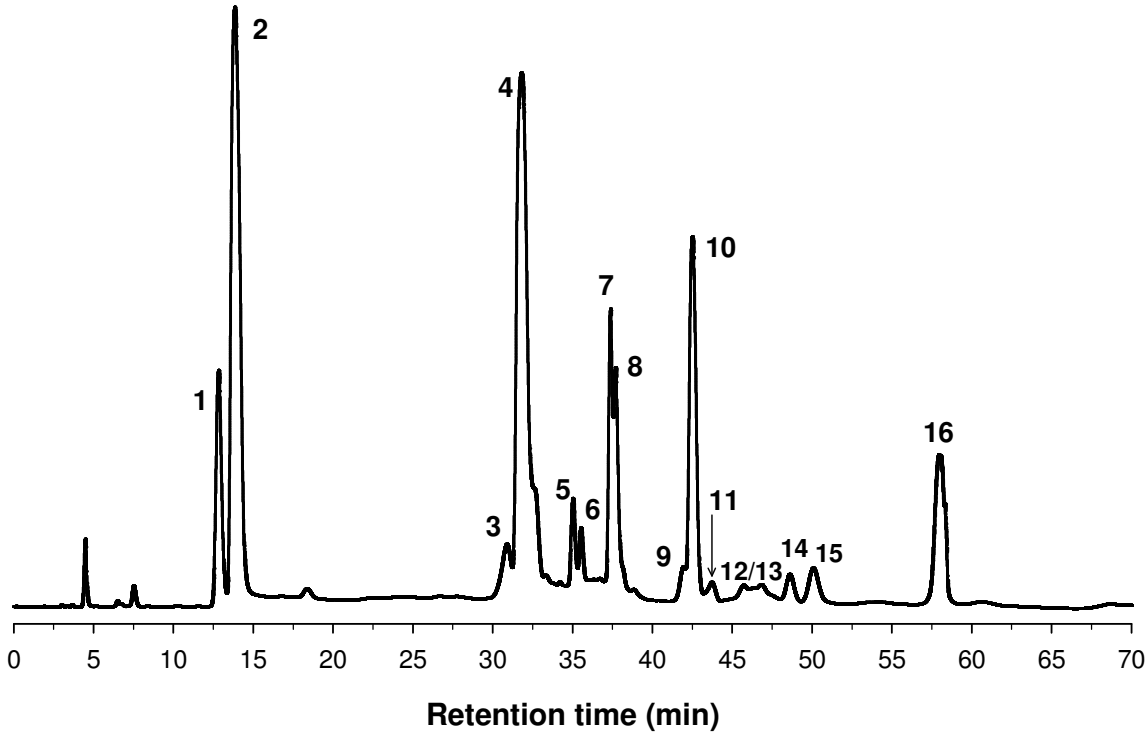


Fig 2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11
12/13
14
15
16
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20
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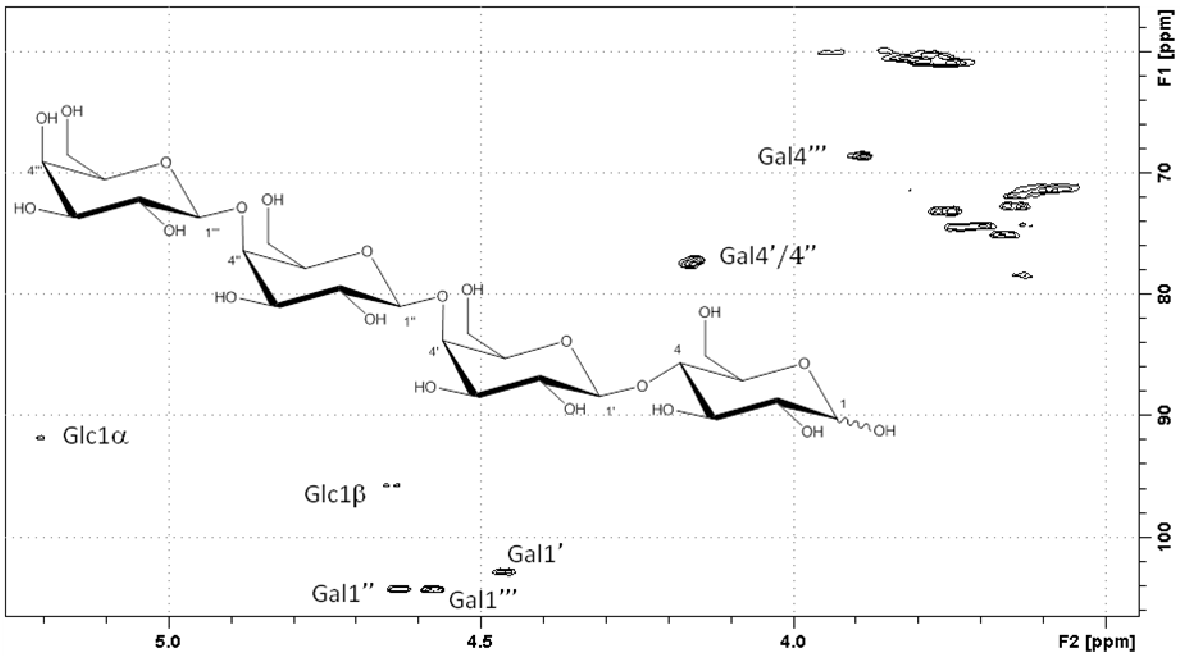
30
35
40
45
50
55
60
65
70
Retention time (min)

Fig 3

A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Gal4'/4''
Glc1α
Glc1β
Gal1'
Gal1''
Gal1'''
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F2 [ppm]
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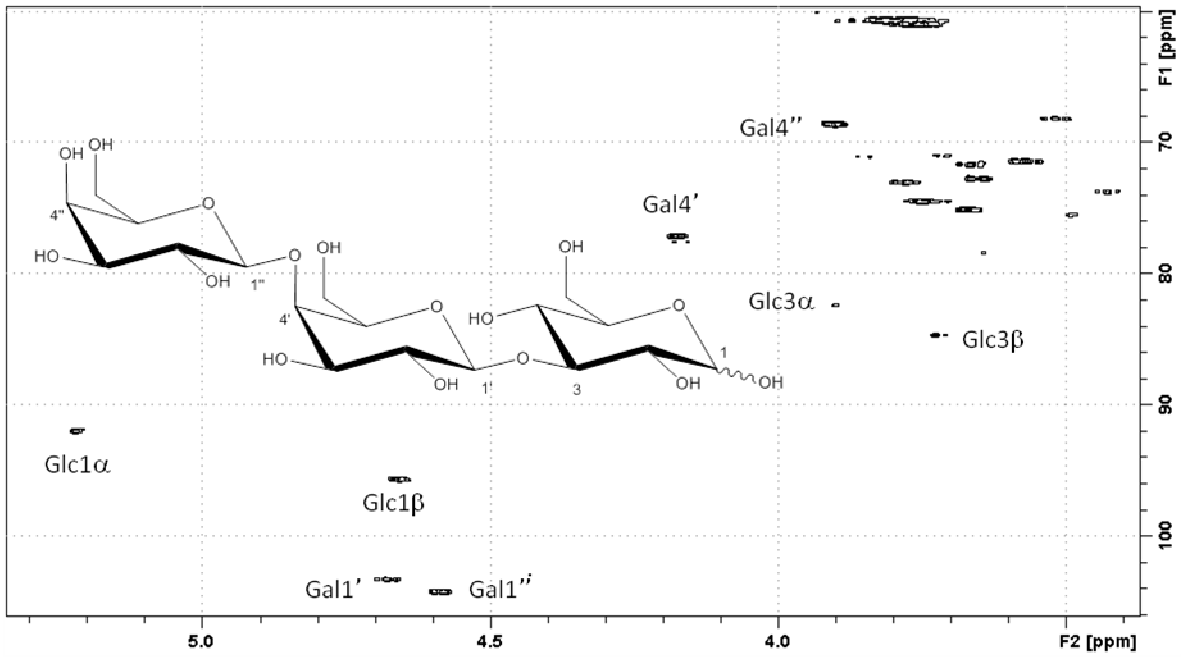
4.5
4.0
70
80
90
100

B


Gal4''
Gal4'
Glc3α
Glc3β
Glc1α
Glc1β
Gal1'
Gal1''
F1 [ppm]
F2 [ppm]
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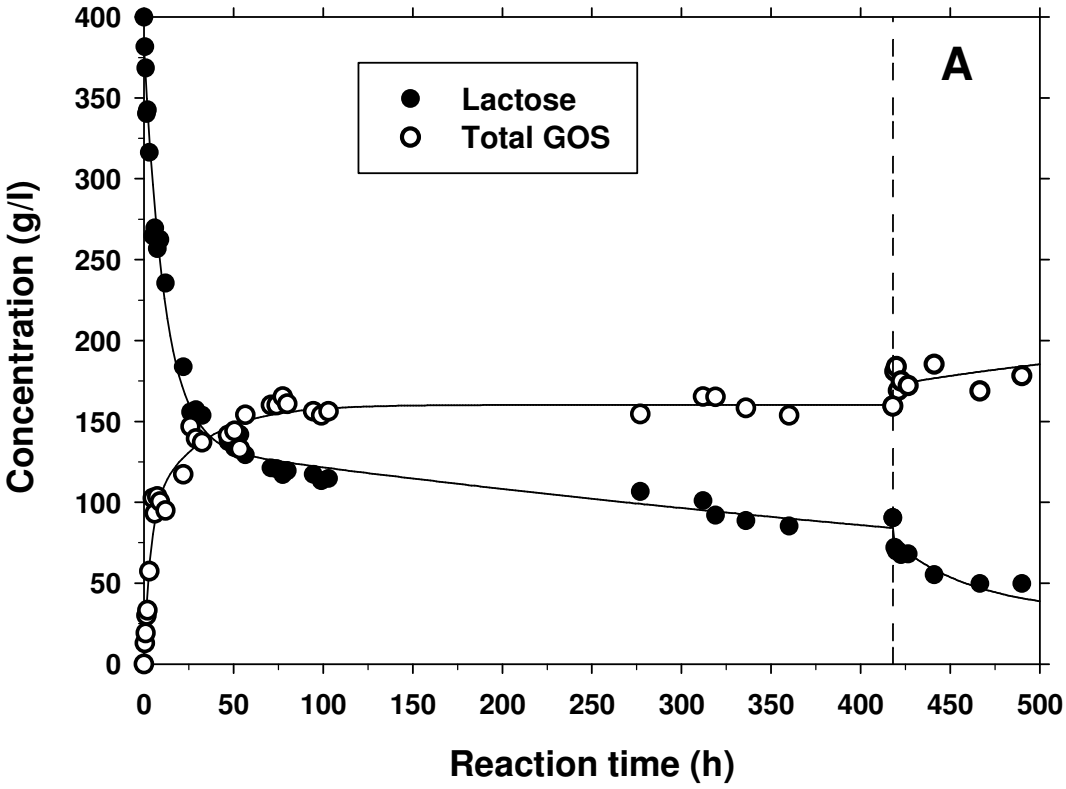
4.5
4.0
70
80
90
100

Fig 4


A
Lactose
Total GOS
Concentration (g/l)
Reaction time (h)
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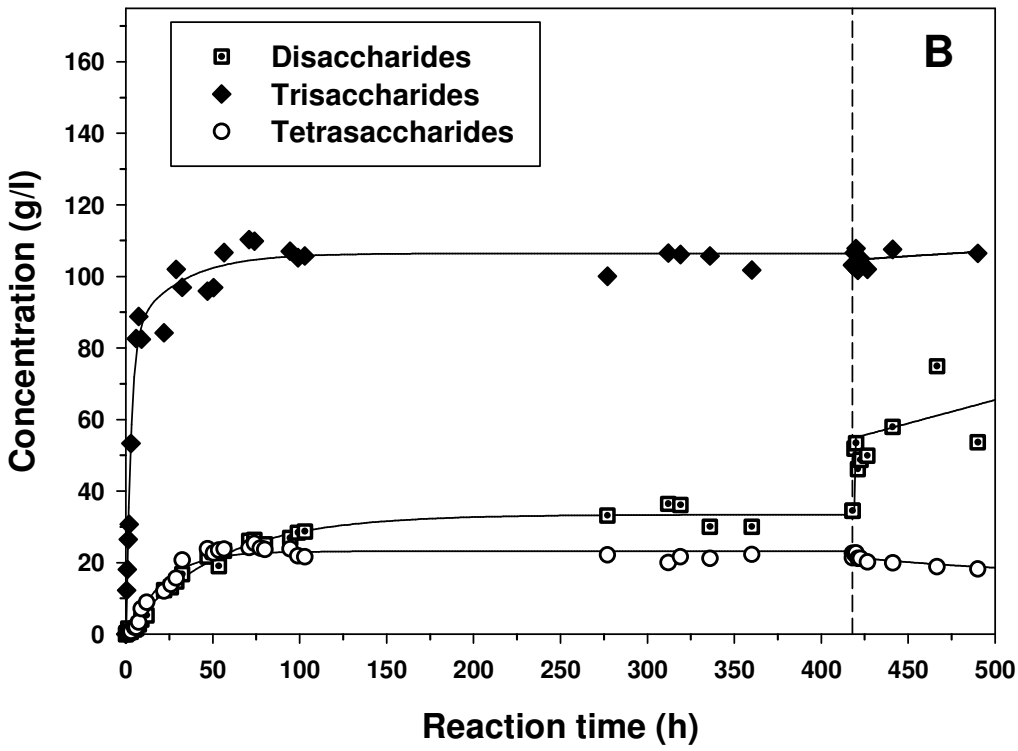
50
100
150
200
250
300
350
400
450
500


B
Disaccharides
Trisaccharides
Tetrasaccharides
Concentration (g/l)
Reaction time (h)
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60
80
100
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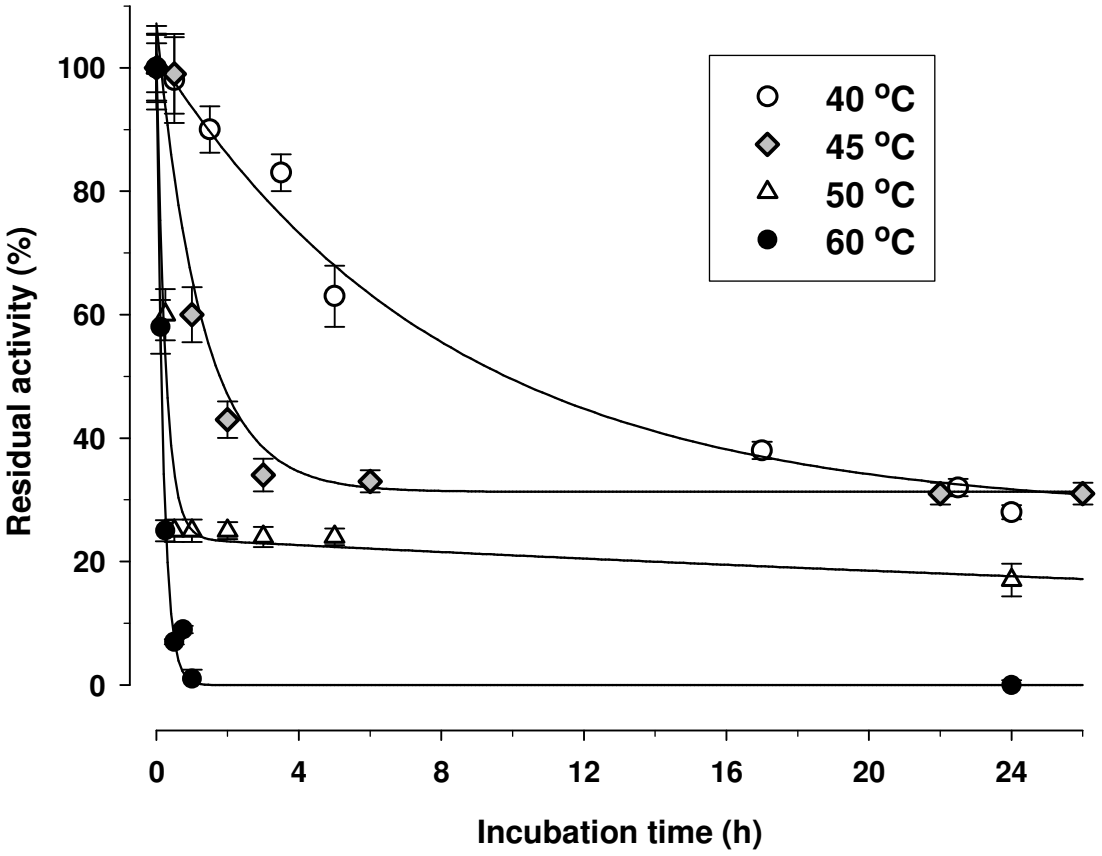
50
150
200
250
300
350
400
450
500

Fig 5


Residual activity (%)
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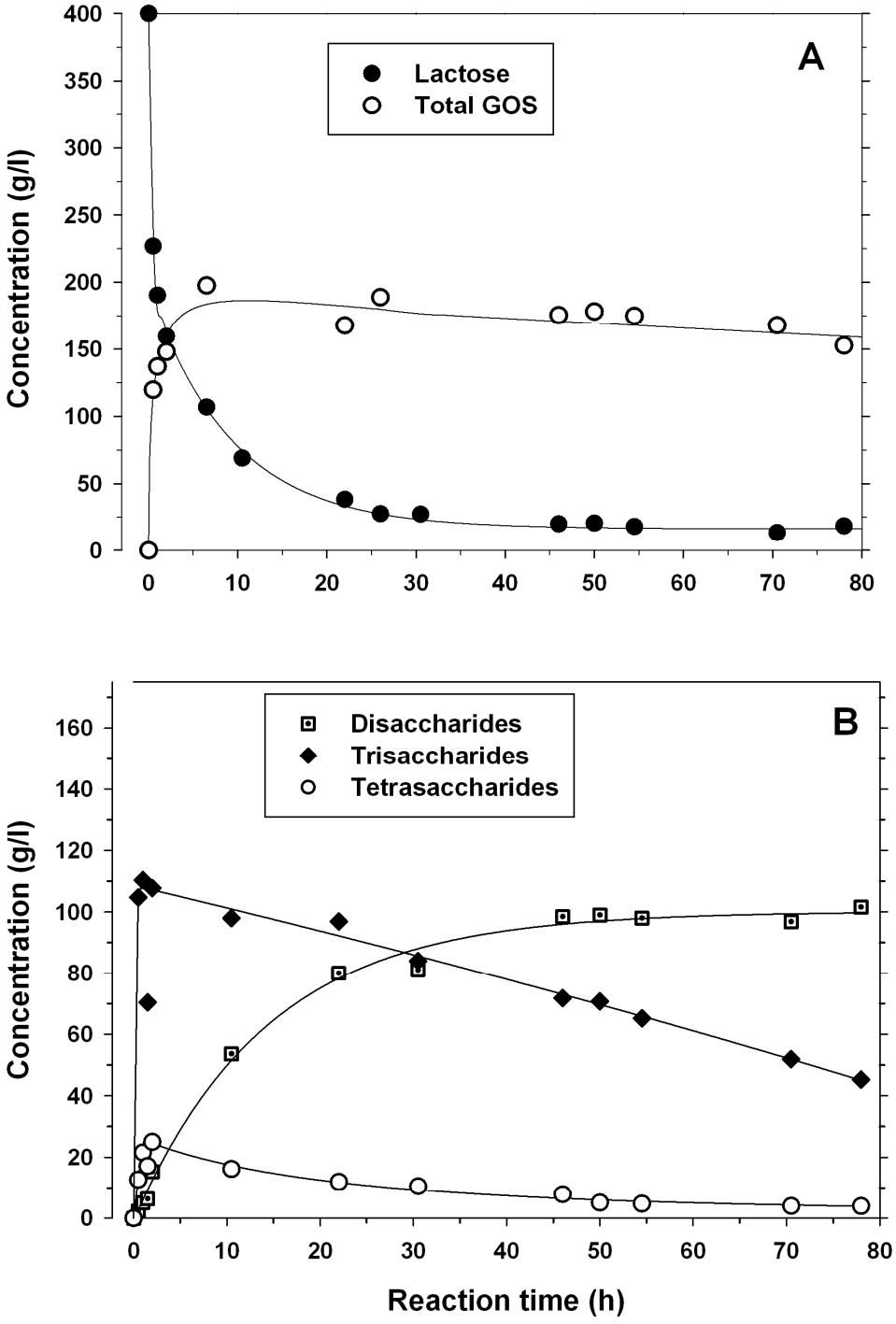
80
60
40
20
0
0
4
8
12
16
20
24
Incubation time (h)
40 °C
45 °C
50 °C
60 °C

Fig 6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Lactose
Total GOS
Concentration (g/l)
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250
200
150
100
50
0
0
10
20
30
40
50
60
70
80
B
Disaccharides
Trisaccharides
Tetrasaccharides
Concentration (g/l)
160
140
120
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20
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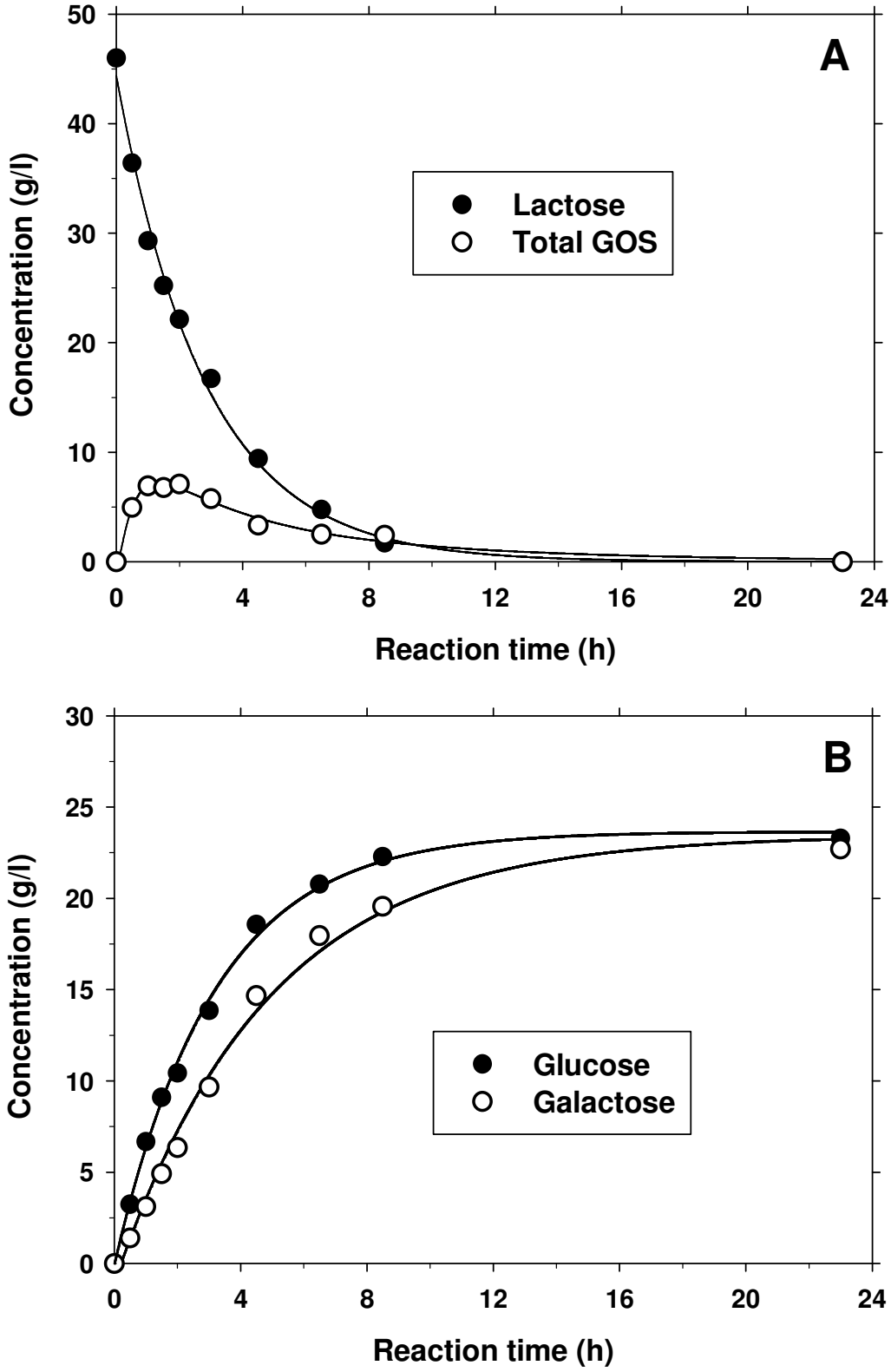
0
10
20
30
40
50
60
70
80
Reaction time (h)

Fig 7


A
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Total GOS
Concentration (g/l)
Reaction time (h)
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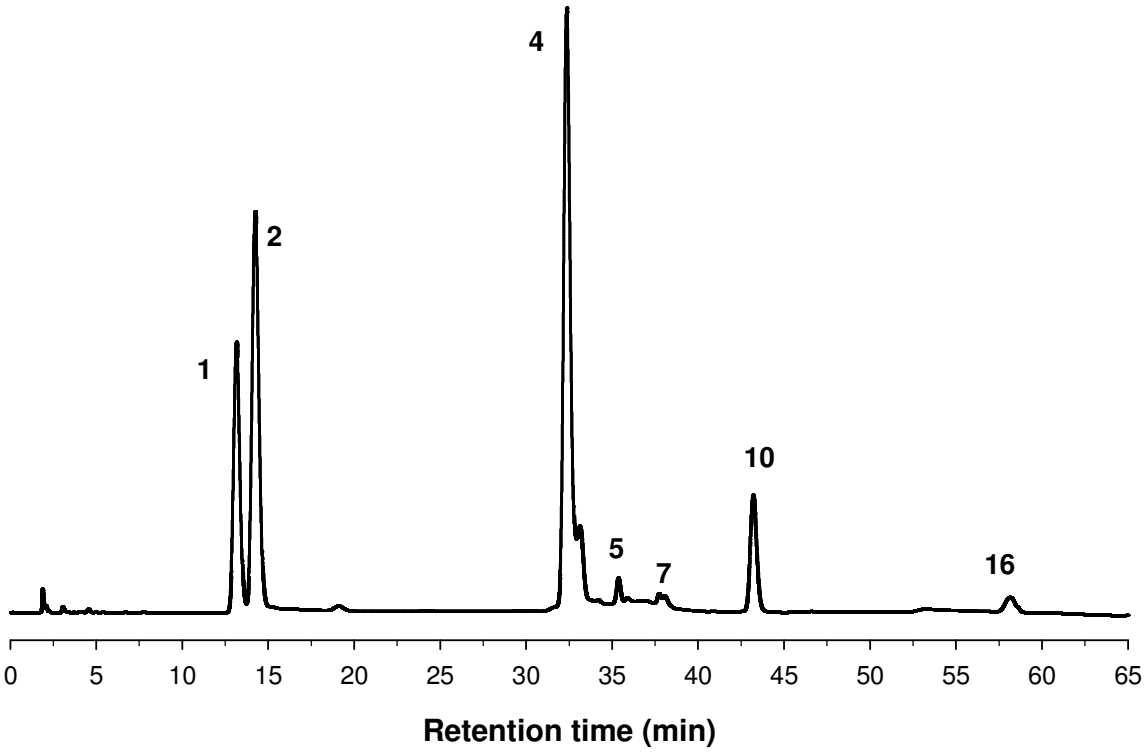
20
30
40
50
0
4
8
12
16
20
24
B
Glucose
Galactose
Concentration (g/l)
Reaction time (h)
0
5
10
15
20
25
30
0
4
8
12
16
20
24

Fig 8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50
55
60
65
Retention time (min)