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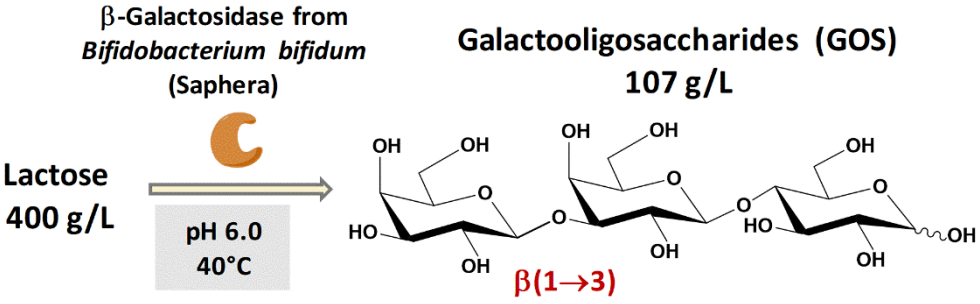

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Selective synthesis of galactooligosaccharides containing β(1→3) linkages with Beta-galactosidase from *Bifidobacterium bifidum* (Saphera)

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ABSTRACT

The transglycosylation activity of a novel commercial β-galactosidase from *Bifidobacterium bifidum* (Saphera) was evaluated. The optimal conditions of operation of this enzyme, measured with *o*-nitrophenyl-β-D-galactopyranoside, were 40 °C and pH around 6.0. Although at low lactose concentrations the character of this enzyme was basically hydrolytic, an increase of lactose concentration to 400 g/L resulted in a significant formation (107.2 g/L, 27% yield) of prebiotic galactooligosaccharides (GOS). The maximum amount of GOS was obtained at a lactose conversion of approximately 90%, which contrasts with other β-galactosidases, for which the highest GOS yield is achieved at 40-50% lactose conversion. Using HPAEC-PAD, semipreparative HPLC-HILIC, MS, 1D and 2D NMR, we determined the structure of most of the GOS synthesized by this enzyme. The main identified products were Gal-β(1→3)-Gal-β(1→4)-Glc (3´-*O*-Beta-galactosyl-lactose), Gal-β(1→6)-Glc (allolactose), Gal-β(1→3)-Glc (3-galactosyl-glucose), Gal-β(1→3)-Gal (3-galactobiose) and the tetrasaccharide Gal-β(1→3)-Gal-β(1→3)-Gal-β(1→4)-Glc. In general, the *B. bifidum* β-galactosidase showed a tendency to form β(1→3) linkages followed by β(1→6), and more scarcely β(1→4).

Keywords: **Galactooligosaccharides, Prebiotics, Transglycosylation, β-Galactosidase, Bifidobacteria, Glycosidases.**

INTRODUCTION

Beta-Galactosidases (Beta-D-galactoside galactohydrolases, EC 3.2.1.23) catalyze the hydrolysis of different oligosaccharides with a galactosyl moiety located at the nonreducing end.[1,2] The dairy industry is taking advantage of this reaction to remove lactose from milk to obtain lactose-free products.[3,4] Due to the high amount of people suffering from lactose intolerance, the demand for lactose-free products is steadily increasing.[5] Apart from its hydrolytic activity, Beta-galactosidases are also able to catalyze transgalactosylation reactions in which lactose –or other carbohydrates– act as acceptor molecules of galactosyl units yielding di-, tri, tetra- or even higher oligomers called galactooligosaccharides (GOS).[6-9] GOS chemically resemble the oligosaccharides present in human milk (HMOS) –which are structurally more complex–, and both families exhibit prebiotic properties.[10-12] More precisely, they improve the gastrointestinal microbiota by promoting the growth and/or the activity of bifidobacteria and lactobacilli,[13] which are regarded to have a beneficial effect on human health.[14,15] In addition to their prebiotic activity, several studies describe that GOS are non-cariogenic, diminish the serum cholesterol level, prevent colon cancer, enhance the digestibility of milk and improve the lactose tolerance.[16,17] Therefore, Beta-galactosidases from generally recognized as safe (GRAS) microorganisms have attracted the attention of the dairy industries for enriching their products with GOS and developing novel functional foods.[13,18] According to their sequences and structures, most Beta-galactosidases belong to families GH1, GH2, GH35, GH42 and GH59 of glycosyl hydrolases.[1,9]

The yield and the composition of the synthesized GOS are influenced by the source of the enzyme and several reaction parameters including lactose concentration, pH, temperature, and water activity.[19-21] The percentage of GOS in the mixtures may vary in a wide range between 15-55 % (*w/w*) depending on such conditions.[22-25] The health promoting properties of GOS depend on their chemical composition, structure and degree of polymerization, which are highly influenced by the origin of the β-galactosidase.[10] The chemical structure of the synthesized oligosaccharides (number of hexose units, types of linkages between them and carbohydrate composition) may affect their fermentation pattern by probiotic bacteria in the human intestine.[26,27] Apparently, GOS mixtures

synthesized by Beta-galactosidases from probiotic microorganisms are consumed faster by probiotic species than those produced by other Beta-galactosidases.[28-30]

Bifidobacterium species are one of the most common organisms in the human gastrointestinal tract.[30,31] It has been demonstrated that GOS produced by Beta-galactosidases from *Bifidobacterium* species have a different structure and significantly higher prebiotic potential compared with commercial mixtures of GOS,[28,32] probably due to the prevalence of β(1→3) and β(1→6) linkages,[25] whilst β(1→4) and β(1→6) bonds prevail in commercial GOS.[6,33] β-galactosidases from *Bifidobacterium* species typically belong to families GH2 and GH42.[1,25] Interestingly, GOS synthesis catalyzed by β-galactosidases from bifidobacteria is not inhibited by the presence of glucose or galactose,[34,35] which are commonly recognized as inhibitors of other Beta-galactosidases.[36]

A novel commercial β-galactosidase from *Bifidobacterium bifidum* (Saphera®) was recently released to the market for obtaining lactose-free products. At moderate lactose concentrations (as those typically found in milk, whey permeate, kephir, etc.), this enzyme is described to synthesize a low amount of GOS compared with other β-galactosidases. In the present work, we have demonstrated that this property can be modulated by controlling lactose concentration. A detailed chemical characterization of the synthesized GOS was also performed. Saphera is an easily available preparation, which could facilitate its broad use for GOS synthesis at high scale.

MATERIALS AND METHODS

Materials

The commercial β-galactosidase Saphera® (Novozym 46091) from *Bifidobacterium bifidum* was kindly supplied by Novozymes A/S (Bagsværd, Denmark). Lactose and *o*-nitrophenyl-Beta-D-galactopyranoside (ONPG) were from Sigma-Aldrich. The standards 3-*O*-Beta-galactosyl-galactose (3-galactobiose), 4-*O*-Beta-galactosyl-galactose (4-galactobiose), 6-*O*-Beta-galactosyl-galactose (6-galactobiose), 6-*O*-Beta-galactosyl-glucose (allolactose) and 4'-*O*-Beta-galactosyl-lactose were from Carbosynth (Berkshire, UK). The rest of standards were synthesized following previous works developed in our laboratory.[22,37,38] All other reagents and solvents were of the highest available purity and used as purchased.

Determination of β-galactosidase activity

The assay of β-galactosidase activity was performed using *o*-nitrophenyl-Beta-D-galactopyranoside (ONPG) as substrate. The activity was measured at 40 ºC following *o*-nitrophenol (ONP) release at 405 nm using a microplate reader (Versamax, Molecular Devices). The reaction was started by adding 10 μL of the enzyme solution (properly diluted) to 190 μL of 15 mM ONPG in 0.1 M phosphate buffer (pH 6.8). The increase of absorbance at 405 nm was followed continuously at 40 ºC during 5 min. The molar extinction coefficient of *o*-nitrophenol at pH 6.8 was 1627 M^{-1} cm^{-1}. One unit of activity (*U*) was defined as that corresponding to the hydrolysis of 1 μmol of ONPG per min under the above specified conditions.

Optimum temperature and pH

The optimum temperature for the β-galactosidase activity was determined by mixing 50 μL of a properly diluted enzyme solution with 450 μL of 15 mM ONPG in 0.1 M phosphate buffer (pH 6.8). The mixture was incubated for 10 min at different temperatures between 10 ºC and 70 ºC. The reaction was stopped by adding 500 μL of 0.4 M Na_2CO_3 and the absorbance at 405 nm was measured in endpoint mode using a microplate reader (Versamax, Molecular Devices). The optimum pH for the β-galactosidase activity was measured at 40 ºC by performing the enzyme assay at different pH values (between 2.0

and 9.0), under the conditions described above, measuring the slope of *o*-nitrophenol release with time. The molar extinction coefficient of *o*-nitrophenol for each pH was determined (pH 2.0, 378.65 M^{-1} cm^{-1}; pH 3.0, 383.85 M^{-1} cm^{-1}; pH 4.0, 441.98 M^{-1} cm^{-1}; pH 5.0, 495.38 M^{-1} cm^{-1}; pH 6.0, 661.65 M^{-1} cm^{-1}; pH 7.0, 2043.5 M^{-1} cm^{-1}; pH 8.0, 4397.83 M^{-1} cm^{-1}; pH 9.0, 4403 M^{-1} cm^{-1}). To minimize the buffer effect, solutions of 15 mM ONPG were prepared using 0.1 M Britton & Robinson buffer,[39] adjusted to the required pH. All the experiments were performed in triplicate and the error was expressed as the standard deviation of the three measurements.

pH and thermal stability of β-galactosidase

In order to evaluate the thermostability of the β-galactosidase, the enzyme was incubated in 0.1 M phosphate buffer pH 6.8 for 1 hour at different temperatures ranging from 4 ˚C to 70 ˚C. The remaining activity towards ONPG was determined by performing enzymatic activity measurements as described above. The evaluation of the stability towards pH was performed incubating enzyme samples at 40 ˚C for 3 hours at various pH values (between pH 2.0 and pH 12.0) using the Britton & Robinson buffer. The remaining activity was measured as described above.

Production of galactooligosaccharides from lactose

Lactose (400 g/L 34.7% *w/w*) was dissolved in 20 mL of 0.1 M phosphate buffer (pH 6.0). The biocatalyst was then added to adjust the Beta-galactosidase activity in the reaction mixture to 1.5 U/mL (ONPG units). The mixture was incubated at 40°C in an orbital shaker (Vortemp 1550) at 200 rpm. At different times, 750 µL aliquots were harvested from the reaction vessel. The enzyme was inactivated by incubating the samples in a Thermomixer (Eppendorf) for 10 min at 96 °C. Samples were then filtered using micro-centrifuge filter tubes, with 0.45 m cellulose acetate filters (National Scientific) at 4000 x g for 5 min. The samples were diluted with water (1:400 and 1:4000) and then analyzed using HPAEC-PAD.

HPAEC-PAD analysis

Products were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a ICS3000 Dionex system (Dionex Corp., CA) consisting of a SP gradient pump, an autosampler (model AS-HV) and an electrochemical detector with a gold working electrode and Ag/AgCl as reference

electrode. 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 mm CarboPac PA-1 guard column was used at 30 °C. Eluents were prepared with Milli-Q water and 50% (w/v) NaOH (Sigma-Aldrich). The initial mobile phase was 15 mM NaOH at 1.0 mL/min for 12 min. A mobile phase gradient from 15 to 200 mM NaOH was performed in 15 min at 1.0 mL/min, and it was maintained for 25 min. The peaks were analyzed by using Chromeleon software. The identification of the different carbohydrates was performed on the basis of commercially available standards or products purified in the laboratory. For those compounds whose standards were not available at sufficient amount, quantification was done based on the calibration curve of standards with the same degree of polymerization.

Purification of GOS

For the purification of unidentified GOS, the reaction was carried out as described above and stopped after 22 h by heating the solution for 10 min at 96 ºC to inactivate the biocatalyst. The reaction mixture was filtered using 0.45 µm paper filters and purified by semipreparative hydrophilic interaction chromatography (HPLC-HILIC). A quaternary pump (Delta 600, Waters) coupled to a Lichrosorb-NH_2 column (5 µm, 10 x 250 mm, Merck) was used. The column temperature was kept at 25 ºC. The acetonitrile/water 75/25 (*v/v*) mobile phase was conditioned with helium and used as at a flow-rate of 6.25 mL/min for 43 min. Peaks were detected by using an evaporative light-scattering detector DDL-31 (Eurosep) equilibrated at 60 °C. A three-way flow splitter (model Accurate, Dionex) and a fraction collector II (Waters) were employed. The fractions containing the main peaks were collected and the solvent was removed by rotary evaporation.

Mass spectrometry

The MS analysis of purified galactooligosaccharides was assessed using a mass spectrometer with hybrid QTOF analyzer (model QSTAR, Pulsar i, AB Sciex). The sample was analyzed by direct infusion and ionized by electrospray (with methanol containing 1% of sodium iodide as ionizing phase) in positive reflector mode.

Nuclear Magnetic Resonance (NMR)

The structure of the oligosaccharides was determined by using a combination of 1D (^{1}H, ^{13}C) and 2D (COSY, NOESY, TOCSY, HSQC, HSQC-TOCSY, HMBC) NMR techniques. The samples were dissolved in D_2O, using TSP-d_4 [3-(trimethylsilyl)propionic-2,2,3,3-d4 acid

sodium salt] as chemical shift reference. Chemical shifts were expressed in parts per million with respect to the 0 ppm point of TSP. The spectra were measured in a Bruker 800 AVIII spectrometer equipped with a 4-channels cryo TCI probe, with gradients in the Z axis, at a temperature of 298K. For the ^{1}H-^{13}C heteronuclear correlations (HSQC, HQSC-TOCSY, HMBC), values of 5 ppm and 1-2K points, for the ^{1}H dimension, and 70 ppm and 384 points for the ^{13}C dimension, were used. For the ^{1}H-^{1}H homonuclear experiments (COSY, NOESY, ROESY), 5 ppm windows were used in both dimensions with a 1-2K x 384 point matrix. For the NOESY experiments, mixing times of 500-600 ms were used. The spinlock time was set to 300 ms for ROESY and 60/100 ms for HSQC-TOCSY experiments. The standard pulse sequences used were provided by Bruker.

RESULTS AND DISCUSSION

Effect of pH and temperature on activity and stability of Bifidobacterium bifidum Beta-galactosidase

The hydrolytic activity of commercial *Bifidobacterium bifidum* β-galactosidase (Saphera), measured with *o*-nitrophenyl-Beta-D-galactopyranoside (ONPG) as substrate, was 1506 ± 0.1 U/mL, determined at 40 ºC and pH 6.8, which are the optimal conditions reported for several Beta-galactosidases from *Bifidobacteria.*[34,35,40]

In order to determine the optimum conditions of operation of this novel enzymatic preparation, a series of activity assays were performed at a fixed pH value (6.8) and different temperatures. The optimum temperature of the Beta-galactosidase from *B. bifidum* (Saphera) was found to be 40 ˚C (Fig. 1a). Furthermore, the thermostability was analyzed measuring the residual activity the enzyme in 0.1 M phosphate buffer (pH 6.8) after 1 h incubation. The β-galactosidase showed a rapid inactivation at temperatures above 50 ˚C (Fig. 1b). The thermal stability was significantly lower than that reported for other related enzymes, such as β-glycosidases from *Sulfolobus solfataricus* and *Pyrococcus furiosus.*[41] However, the β-galactosidase from *B. bifidum* showed a higher thermostability compared to Beta-galactosidases from other *Bifidobacterium* species like *B. breve.*[25]

The optimum pH of this preparation was also studied (Fig. 2a). The highest activity with ONPG was obtained at in the range pH 5.7-6.4. Regarding the stability of the enzyme at different pH values, the results showed that the β-galactosidase displayed good stability in the pH range 5.0-10.0, after 3 hours of incubation at 40 ºC (Fig. 2b). At pH 3.0 and 11.0 the inactivation of the enzyme was very fast. In this context, the Beta-galactosidase from *Bifidobacterium breve* was rapidly inactivated at pH 9.0.[25] Our results fit well with those described by Tzortis *et al.* using whole cells of *B. bifidum* NCIMB 41171, for which the optimum pH and temperature were 6.8-7.0 and 40 ˚C, respectively.[34] The related β-galactosidase from *B. longum* showed optimal pH and temperature of 5.8-6.8 and 45°C, respectively.[35]

GOS specificity of B. bifidum Beta-galactosidase

The ability of *B. bifidum* β-galactosidase to synthesize galactooligosaccharides (GOS) was assessed. First, we followed by HPAEC-PAD the hydrolysis of lactose at 160 g/L under the optimal conditions (pH 6.0, 40 °C). As shown in Fig. 3a, the main reaction catalyzed by this enzyme under these conditions was the hydrolysis. However, the formation of several transgalactosylation products was observed.

We further increased the lactose concentration to 400 g/L to promote transfer reaction. The HPAEC-PAD chromatogram (Fig. 3b) showed at least 19 peaks of which numbers *1*, *2* and *5* corresponded to galactose, glucose and lactose, respectively. The main reaction products corresponded to peaks *10*, *15* and *19*. We were able to purify peaks *15* and *19* by semipreparative HILIC for their characterization. For peak *15,* the major signal in the mass spectrum in positive mode (Fig. 4A) was at *m/z* 527.16, corresponding to the M+[Na]$^+$ ion of a trisaccharide. The 1D and 2D NMR spectra indicated that the structure of the compound fitted well with the trisaccharide 3'-O-β-galactosyl-lactose [Gal-β(1→3)-Gal-β(1→4)-Glc], which was previously identified and characterized in our laboratory studying the GOS synthesis with β-galactosidase from *Aspergillus oryzae.*[22]

The main signal in the mass spectrum of peak *19* (Fig. 4B) was at *m/z* 689.21, corresponding to the M+[Na]$^+$ ion of a tetrasaccharide. The ^{1}H-NMR-based information for the major product of this peak was fairly similar to that recorded for peak *15*, although it clearly displayed one additional anomeric proton (Figs. 5A and 5B). The analysis demonstrated that the Gal rings were not substituted at position 4, and only 3-Gal glycosylated moieties could be found. As for peak *15,* the existing Glc ring was glycosylated position 4. Therefore, the structure of the compound was determined to be 3´-O-β-(3-galactobiosyl)-lactose [Gal-β(1→3)-Gal-β(1→3)-Gal-β(1→4)-Glc] in a non-ambiguous manner. Fig. 5 shows the similarity of the ^{1}H-NMR spectra of peaks *15* and *19*. Both positions α- and β- at the reducing end were unambiguously identified for these compounds by the HSQC spectrum.

Peaks *8* and *9* were identified as the trisaccharide 6'-*O*-Beta-galactosyl-lactose and the disaccharide 3-*O*-Beta-galactosyl-glucose, respectively, using purified standards from *K. lactis* Beta-galactosidase reactions.[42] With commercial standards it was also possible to identify peak *12* as the trisaccharide 4'-O-Beta-galactosyl-lactose and several

disaccharides with different glycosidic bonds, involving two units of galactose (peak *3,* 6-galactobiose; peak *6,* 3-galactobiose; peak *7,* 4-galactobiose) or galactose-glucose (peak *4,* Gal-β(1→6)-Glc, allolactose; peak *9*, Gal-β(1→3)-Glc).

One more reaction product (peak *13*) was also partially purified by semipreparative HPLC. The mass spectrum showed that the main signal corresponded to a trisaccharide. Although the purity of this sample was not enough for a precise structure determination, the identity of the components of the mixture was further studied. From the NMR data, different glycosylation positions could be detected. For the Gal-Gal linkages, both Beta(1→3) and Beta(1→4) disaccharide fragments were found, with similar proportions. For the Gal-Glc linkages, the Beta(1→4) position was predominant, but minor Beta(1→3) as well as minute fractions of Beta(1→2) linked moieties were also detected. Therefore, the mixture within peak *13* is composed of the Gal-β(1→3)-Gal-β(1→4)-Glc and Gal-β(1→4)-Gal-β(1→4)-Glc trisaccharides as major products, together with the Gal-β(1→3)-Gal-β(1→3)-Glc, Gal-β(1→4)-Gal-β(1→3)-Glc analogues as minor components. Such assignations were performed on the basis of previous publications.[43-46] Additionally, small amounts of products with the Glc moiety glycosylated at position 2 were present.

In a recent paper, using the β-galactosidase activity from several *Pantoea anthophila* strains, we were able to identify peak *14* as 3'-*O*-galactosyl-allolactose (Gal-β(1→3)-Gal-β(1→6)-Glc).[47] Regarding peak *10*, one of the most abundant in the GOS mixture, we believe that it could be composed by three galactosyl residues bound by β(1→3) linkages [Gal-β(1→3)-Gal-β(1→3)-Gal, 3-galactotriose]. This assumption is based on the enzyme specificity, the relative retention times and the presence of a significant amount of 3-galactobiose, which could serve as galactosyl acceptor. However, we were not able to isolate it with enough purity for NMR analysis. Peaks *11*, *16*, *17* and *18* remained unidentified, but all of them were minor components of the mixture.

Our results fit well with those obtained using other *B*. *bifidum* strains. Depeint et al.[48] and Goulas et al.[49] reported the presence of β(1→3) linkages, with less participation of β(1→4) and β(1→6) bonds, in the GOS mixture obtained with β-galactosidase from *B*. *bifidum* NCIMB41171. However, Rabiu et al. identified, in addition to β(1→3) linkages, several β(1→6) and β(1→4) bonds.[28] This partially agrees with our findings, but it is well studied

that there are more than one β-galactosidase in *Bifidobacteria* strains, and the synthesized oligosaccharides may vary.[40,49]

Progress of GOS formation

The progress of GOS formation was followed by HPAEC-PAD. Experimental conditions were 400 g/L lactose in 0.1 M sodium phosphate buffer (pH 6.0), 40 ºC and 1.5 U/mL (ONPG units). Table 1 shows the concentrations of the main reaction products at different times. Under the above conditions, the point of maximum GOS production (107.2 g/L, 27% of total sugars in the mixture) was obtained at 50.5 h. At this point, the rest of components in the mixture were glucose (38%), galactose (26%) and lactose (9%). It is worth noting that the time required to get the maximum GOS yield depends inversely on the amount of enzyme; however, as occurs in this kind of kinetically-controlled reactions,[50-52] the GOS concentration at this point is not affected by the amount of biocatalyst. Rabiu et al. reported GOS yields between 24.7-47.6% employing 30% (w/w) lactose and five different species of bifidobacteria, including *B. bifidum* BB-12 (37.6% yield).[28] Tzortzis et al. demonstrated that using whole-cells of *B. bifidum* NCIMB41171 the maximum GOS yield was 35% employing 55% (w/w) lactose.[34]

At the point of maximum GOS concentration, the most abundant GOS corresponded to 3'-galactosyl-lactose (17.7 g/L), allolactose (16.2 g/L), 3-O-β-galactosyl-glucose (9.0 g/L), and 3-galactobiose (8.2 g/L), apart from unidentified peak *10*. These results confirm that the enzyme has a clear tendency to form β(1→3) linkages followed by β(1→6). However, only a small proportion of products containing new β(1→4) linkages were observed. It is worth noting that the GOS synthesis using *B. bifidum* Beta-galactosidase resulted in a high amount of disaccharides (approx. 40.4 g/L), with allolactose, 3-O-Beta-galactosyl-glucose and 3-galactobiose as the main components of this fraction. In this context, Böger et al. recently reported that most probiotic strains of gut bacteria exhibited a preference for DP2 galactooligosaccharides.[53]

Fig. 6 represents the profile of GOS concentration as a function of lactose conversion. It is remarkable, compared with other β-galactosidases, that the GOS concentration increased progressively until 80% of initial lactose has disappeared. Then, the yield of GOS was nearly invariable until approximately 93% of lactose was consumed. These results contrast with those obtained with *Bacillus circulans*[37] and *Aspergillus oryzae*[22] β-

galactosidases, for which the maximum concentration of GOS was achieved at approximately 40-50% of lactose conversion. Thereafter, GOS are hydrolyzed by the enzyme, in particular those containing β(1→4) linkages. The profile obtained with *B. bifidum* Beta-galactosidase resembles that of *Kluyveromyces lactis* lactase, as the maximum GOS yield was also achieved at approximately 95% of lactose conversion.[42] In this context, Hsu et al., using a β-galactosidase from *B. longum*, reported a maximum GOS yield of 32.5%, which obtained at approximately 60% lactose conversion, and was followed by a hydrolysis of the synthesized GOS.[35]

In conclusion, the commercial preparation Saphera gave rise to a moderate GOS yield (27%) compared with other Beta-galactosidases. However, the predominance of β(1→3) linkages in the synthesized GOS has several advantages with respect to other commercial GOS mixtures. First, the production of GOS with Saphera can be easily controlled because GOS hydrolysis only takes place when approximately 95% of the initial lactose has disappeared. Second, it has been reported that GOS containing β(1→3) bonds are metabolized by probiotic bacteria faster than similar GOS containing other linkages, in particular β(1→4).[29] There is a general consensus that GOS synthetized with β-galactosidases from probiotic strains exert a more marked effect on the growth of these probiotic strains.[28,34,48]

ABBREVIATIONS

GOS, galactooligosaccharides; HMOS, human milk oligosaccharides; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; GRAS, generally recognized as safe; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; HPLC, high-performance liquid chromatography; HILIC, hydrophilic interaction liquid chromatography; MS, mass spectrometry; QTOF, quadrupole time of flight; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; NOESY, nuclear overhauser effect spectroscopy ; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum correlation; HMBC, heteronuclear multiple bond correlation; TSP-d4, 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt; DP, degree of polymerization.

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FIGURE CAPTIONS

Figure 1: Effect of temperature on a) activity and b) stability of β-galactosidase from *B. bifidum*. For the activity assay, 0.1 M phosphate buffer (pH 6.8) was used. For the thermostability assay, the enzyme was incubated in the same buffer at different temperatures for 1 h. Residual activity was referred to the initial activity of the enzyme.

Figure 2: Effect of pH on a) activity and b) stability of β-galactosidase from *B. bifidum*. For the activity assay, 0.1 M Britton & Robinson buffer at different pH was used and the activity measured at 40 ˚C. For the stability assay, the enzyme solution was incubated under different pH values at 40 ˚C for 3 h. Residual activity was referred to the initial activity of the enzyme.

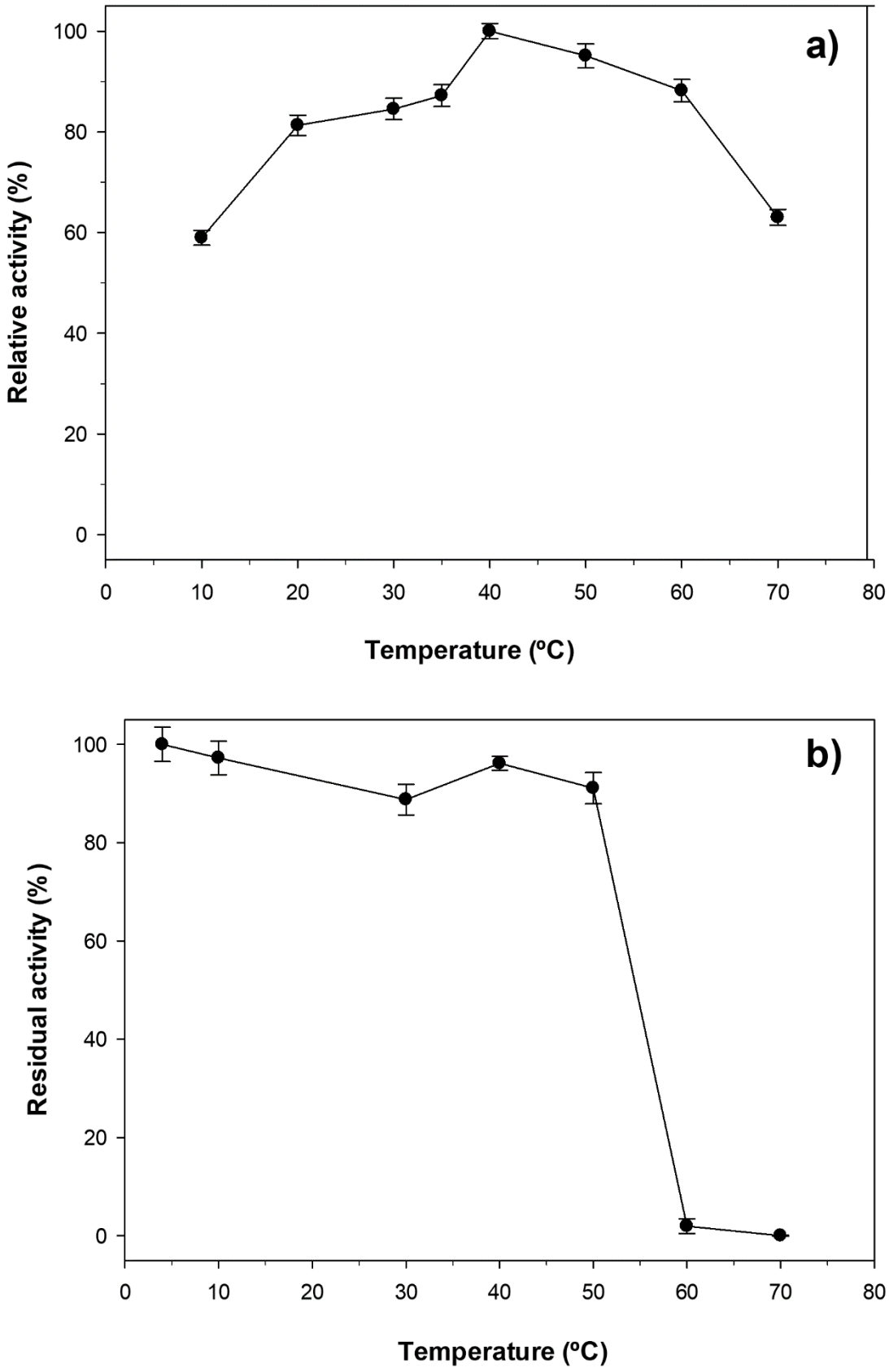
Figure 3: HPAEC-PAD analysis (22 h) of the reaction of lactose with *B. bifidum* β-galactosidase (Saphera) under optimal conditions (pH 6.0, 40 °C) at two lactose concentrations: a) 160 g/L; b) 400 g/L. Peaks assignation: (1) Galactose; (2) Glucose; (3) 6-Galactobiose; (4) Allolactose; (5) Lactose; (6) 3-Galactobiose; (7) 4-Galactobiose; (8) 6'-Galactosyl-lactose; (9) 3-O-β-Galactosyl-glucose; (12) 4´-Galactosyl-lactose; (13) Gal-β(1→3)-Gal-β(1→3)-Glc; (14) 3'-Galactosyl-allolactose; (15) 3´-Galactosyl-lactose; (19) 3´-O-β-(3-Galactobiosyl)-lactose; (10,11,16-18) unidentified.

Figure 4: ESI-TOF mass spectra (positive mode) of: A) Peak 15; B) Peak 19.

Figure 5: ^{1}H-NMR spectra of the reaction products corresponding to peaks *13*, *15* and *19*. The major product for peak *19* (A) corresponds to the tetrasaccharide Gal-β(1→3)-Gal-β(1→3)-Gal-β(1→4)-Glc(α/β). For peak *15* (B) the major product is Gal-β(1→3)-Gal-β(1→4)-Glc(α/β). For peak *13* (C) a mixture of trisaccharides (according to MS) containing all the possibilities for Gal-β(1→3/1→4)-Gal-β(1→4/1→3/1→2)-Glc(α/β) is obtained. For the three peaks, small amounts of oligosaccharides with the reducing Glc residue glycosylated at position 2 have been found (signals at 5.4-5.5 ppm).

Figure 6: GOS formation vs. lactose conversion using β-galactosidase from *B. bifidum* (Saphera). Experimental conditions: 400 g/L lactose in 0.1 M sodium phosphate buffer (pH 6.0), 1.5 U/ml (ONPG units), 40 ºC.

Figure 1 **Go back to Fig. 1**



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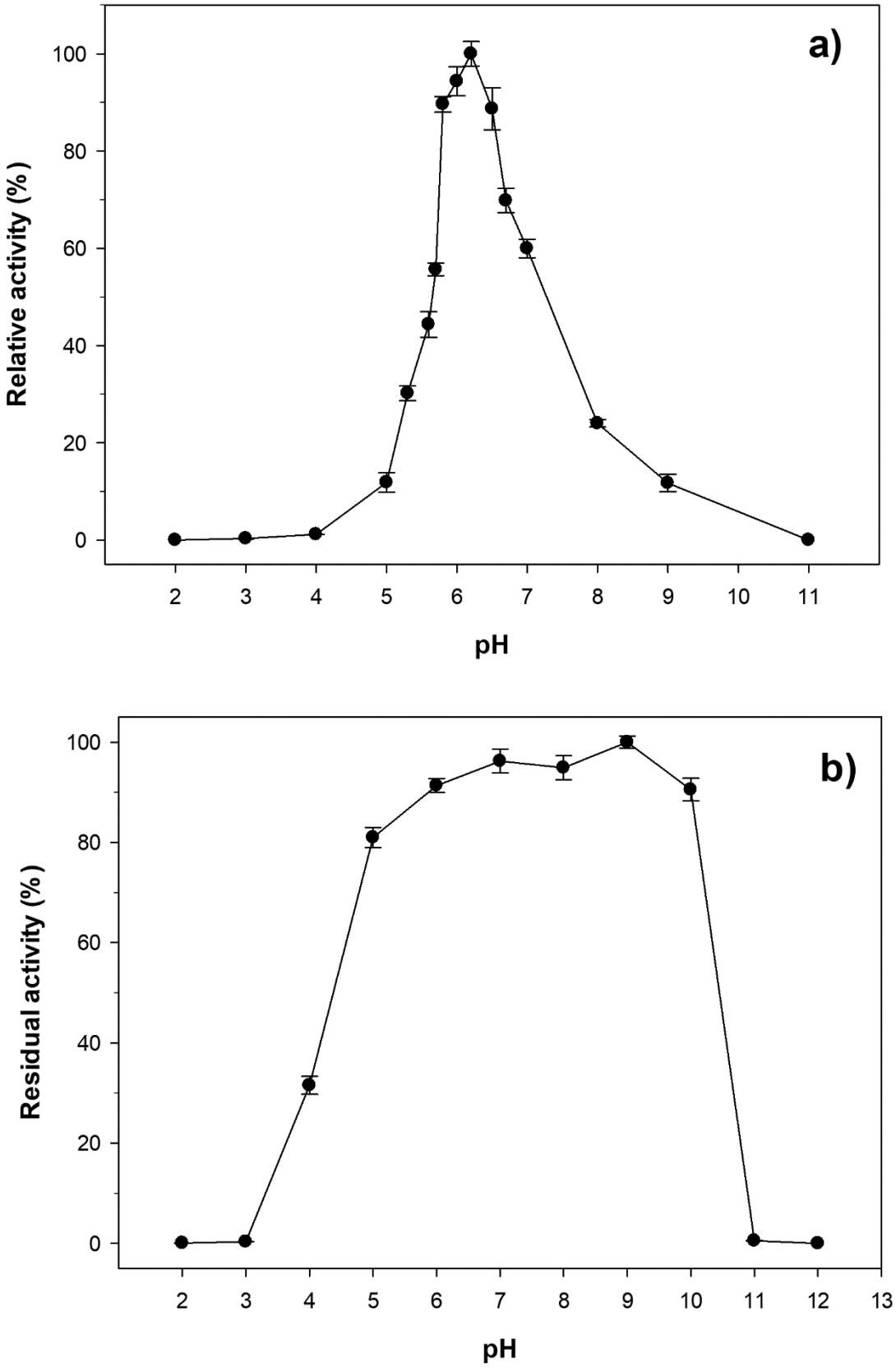
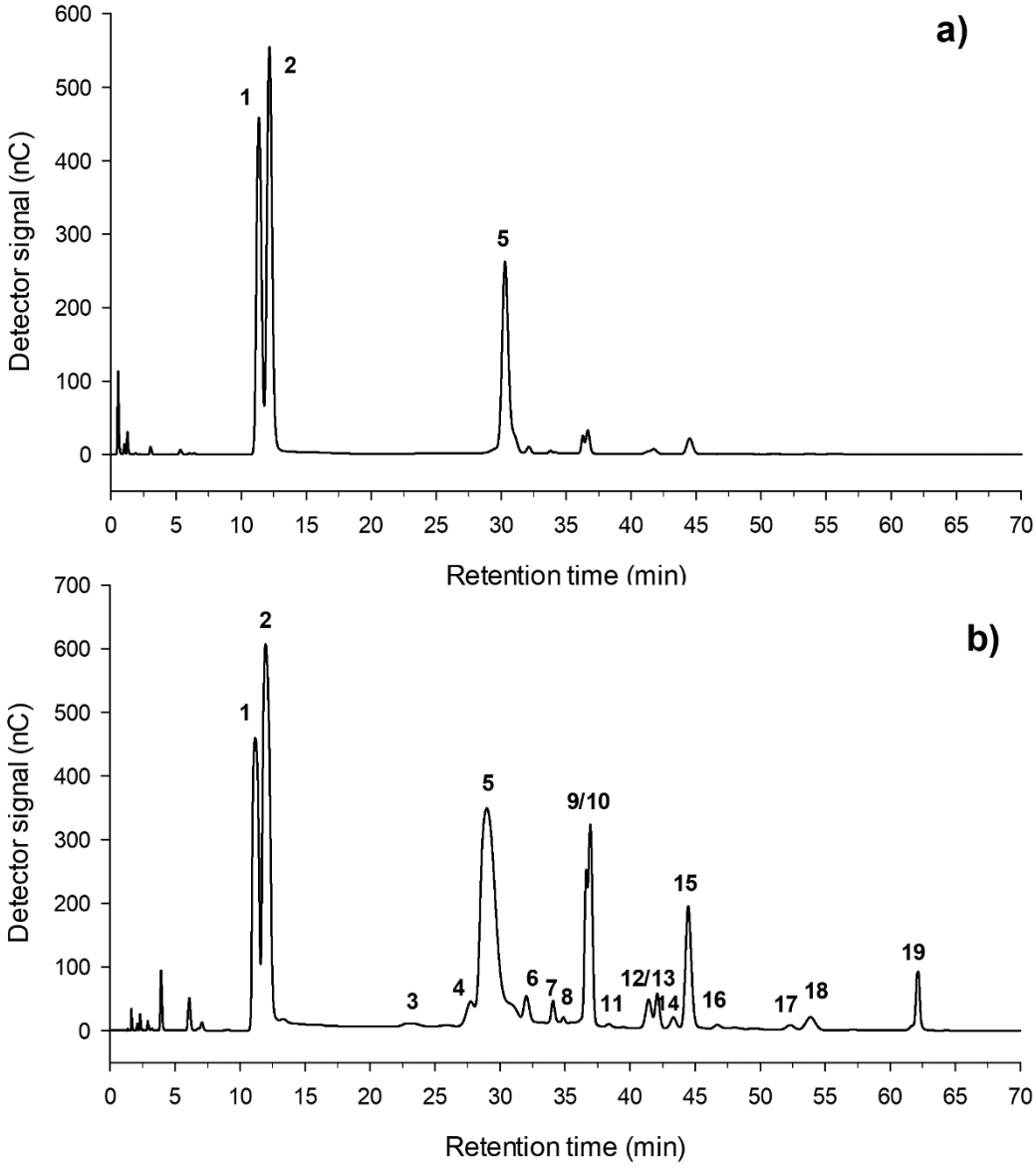


Figure 3 Go back to Fig. 3



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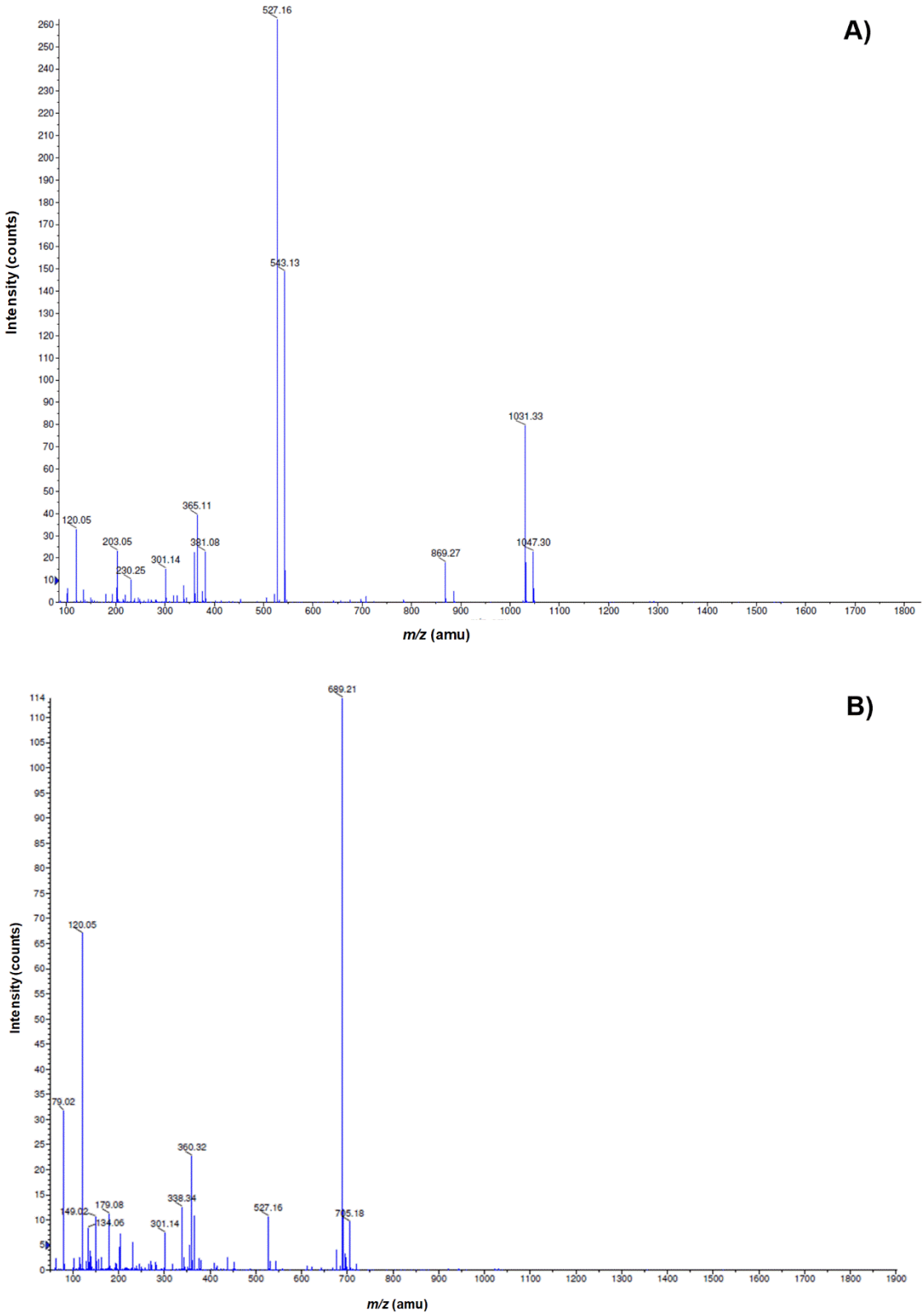

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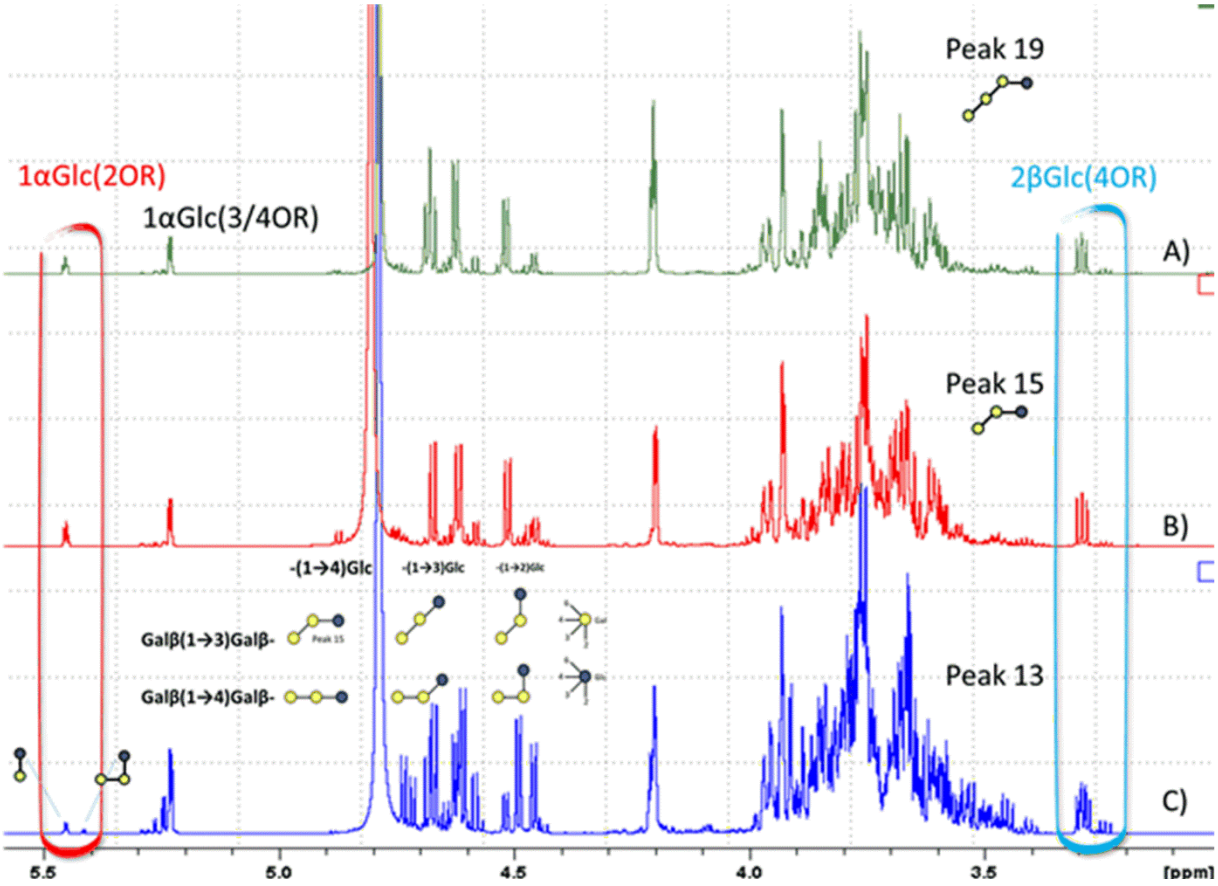

Figure 6 **Go back to Fig. 6**

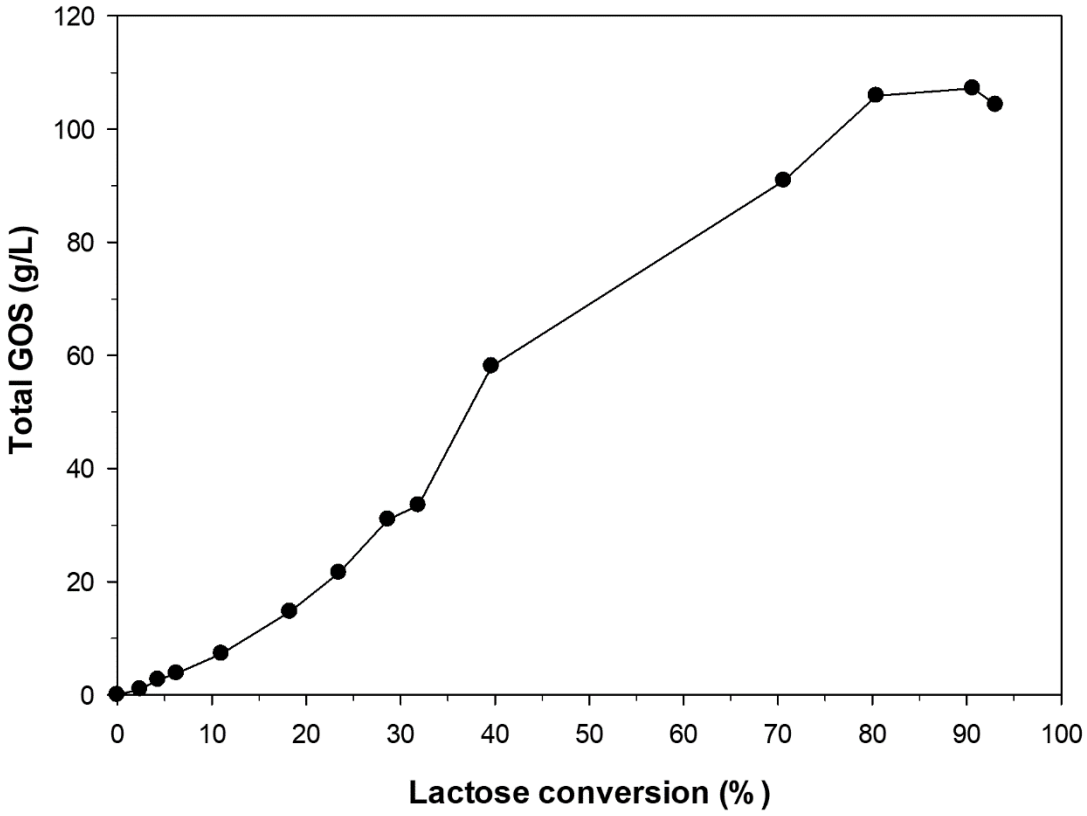

Table 1. Carbohydrate composition (g/L) of the reaction mixture using β-galactosidase from *B. bifidum* (Saphera). Experimental conditions: 400 g/L lactose in 0.1 M sodium phosphate buffer (pH 6.0), 1.5 U/ml, 40 ºC.

Reaction time (min)	Gal (g/L)	Glc (g/L)	Lact (g/L)	Gal-6-Gal (g/L)	Gal-6-Glc (g/L)	Gal-3-Gal (g/L)	Gal-4-Gal (g/L)	Gal-3-Glc (g/L)	Gal-4-Lact (g/L)	Gal-3-Lact (g/L)	Gal-3-Gal-3-Lact (g/L)	Other GOS (g/L)	GOS_{TOT} (g/L)
0	0.0	0.0	400.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	4.1	4.6	390.4	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	1.0
20	6.6	8.0	382.7	0.0	0.0	0.0	0.0	0.1	0.6	1.6	0.1	0.3	2.7
30	9.7	11.6	374.9	0.0	0.0	0.0	0.0	0.3	0.7	2.3	0.1	0.4	3.8
60	16.1	20.8	355.8	0.0	0.0	0.0	0.0	0.8	1.3	3.8	0.3	1.2	7.3
120	25.4	33.0	326.9	0.0	0.0	0.1	0.1	2.2	2.0	6.6	0.8	3.0	14.7
180	30.6	41.7	306.1	0.0	0.0	0.0	0.2	3.8	2.5	8.7	1.2	5.2	21.6
240	34.6	49.1	285.3	0.0	0.0	0.2	0.3	6.0	3.3	11.2	1.9	8.1	31.0
300	38.8	55.3	272.4	0.0	0.0	0.2	0.4	6.5	3.2	11.8	2.2	9.2	33.5
390	40.9	59.6	241.4	0.0	0.6	0.4	0.5	7.6	3.5	12.2	2.6	30.7	58.1
1320	74.1	117.5	117.5	1.1	4.8	3.2	2.3	12.1	4.7	20.8	6.3	35.6	90.9
1830	84.6	131.2	78.2	1.4	7.5	4.6	3.2	13.4	4.1	20.7	7.1	44.0	105.9
3030	103.4	152.0	37.4	2.2	16.2	8.2	4.8	9.0	2.6	17.7	5.4	41.1	107.2
4200	109.2	158.8	27.7	2.8	23.3	10.9	5.5	7.9	1.8	12.2	3.5	36.3	104.3

Gal: Galactose; Glc: Glucose; Lact: Lactose; Gal-6-Gal: 6-galactobiose; Gal-6-Glc: Allolactose; Gal-3-Gal: 3-galactobiose; Gal-4-Gal: 4-galactobiose; Gal-3-Glc: 3-galactosyl-glucose; Gal-4-Lact: 4'-galactosyl-lactose; Gal-3-Lact: 3'-galactosyl-lactose; Gal-3-Gal-3-Lact: 3´-(3-galactobiosyl)-lactose; GOS_{TOT}: Total GOS in the mixture

Go back to Table 1