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**Selective synthesis of galactooligosaccharides containing $\beta(1\rightarrow3)$
linkages with - β 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 pH 6.0 and 40 °C. 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 \rightarrow 3)-Gal- β (1 \rightarrow 4)-Glc (3'-*O*- β -galactosyl-lactose), Gal- β (1 \rightarrow 6)-Glc (allolactose), Gal- β (1 \rightarrow 3)-Glc (3-galactosyl-glucose), Gal- β (1 \rightarrow 3)-Gal (3-galactobiose) and the tetrasaccharide Gal- β (1 \rightarrow 3)-Gal- β (1 \rightarrow 3)-Gal- β (1 \rightarrow 4)-Glc. In general, the *B. bifidum* β -galactosidase showed a tendency to form β (1 \rightarrow 3) linkages followed by β (1 \rightarrow 6), and more scarcely β (1 \rightarrow 4) .

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

INTRODUCTION

β -Galactosidases (β -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, β -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, β -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}

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 β -galactosidases from probiotic microorganisms are consumed faster by probiotic species than those produced by other β -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 β -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 $\beta(1\rightarrow3)$ and $\beta(1\rightarrow6)$ linkages,²⁵ whilst $\beta(1\rightarrow4)$ and $\beta(1\rightarrow6)$ bonds prevail in commercial GOS.^{6,33} 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 β -galactosidases.³⁶

In the present work, we evaluated the transgalactosylation activity of a novel commercial β -galactosidase from *Bifidobacterium bifidum* (Saphera) developed to obtain lactose-free products. A detailed chemical characterization of the synthesized GOS was performed.

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- β -D-galactopyranoside (ONPG) were from Sigma-Aldrich. The standards 3-*O*- β -galactosyl-galactose (3-galactobiose), 4-*O*- β -galactosyl-galactose (4-galactobiose), 6-*O*- β -galactosyl-galactose (6-galactobiose), 6-*O*- β -galactosyl-glucose (allolactose) and 4'-*O*- β -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- β -D-galactopyranoside (ONPG) as substrate. The activity was measured at 40 °C following *o*-nitrophenol (ONP) release at 405 nm using a microplate reader (Versamax, Molecular Devices). The reaction was started by adding 10 μ L of the enzyme solution (properly diluted) to 190 μ L 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 °C 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 μ mol 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 μ L of a properly diluted enzyme solution with 450 μ L 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 °C and

70 °C. The reaction was stopped by adding 500 μ L 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 °C by performing the enzyme assay in kinetic mode at different pH values (between 2.0 and 9.0), under the conditions described above. The molar extinction coefficient of *o*-nitrophenol for each pH was determined (pH 2.0, 378.65 $\text{M}^{-1} \text{cm}^{-1}$; pH 3.0, 383.85 $\text{M}^{-1} \text{cm}^{-1}$; pH 4.0, 441.98 $\text{M}^{-1} \text{cm}^{-1}$; pH 5.0, 495.38 $\text{M}^{-1} \text{cm}^{-1}$; pH 6.0, 661.65 $\text{M}^{-1} \text{cm}^{-1}$; pH 7.0, 2043.5 $\text{M}^{-1} \text{cm}^{-1}$; pH 8.0, 4397.83 $\text{M}^{-1} \text{cm}^{-1}$; pH 9.0, 4403 $\text{M}^{-1} \text{cm}^{-1}$). 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 β -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 μ L 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 µm cellulose acetate filters (National Scientific) at 6000 rpm 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 °C 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-NH₂ column (5 μm, 10 x 250 mm, Merck) was used. The column temperature was kept at 25 °C. 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 NH₄OH as ionizing phase) in positive reflector mode.

Nuclear Magnetic Resonance (NMR)

The structure of the oligosaccharides was determined by using a combination of 1D (¹H, ¹³C) and 2D (COSY, NOESY, TOCSY, HSQC, HSQC-TOCSY, HMBC) NMR techniques. The samples were dissolved in D₂O, using TSP-d₄ [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 ¹H-¹³C heteronuclear correlations (HSQC, HSQC-TOCSY, HMBC), values of 5 ppm

and 1-2K points, for the ^1H dimension, and 70 ppm and 384 points for the ^{13}C

dimension, were used. For the ^1H - ^1H 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* - galactosidase

The hydrolytic activity of commercial *Bifidobacterium bifidum* β -galactosidase (Saphera), measured with *o*-nitrophenyl- β -D-galactopyranoside (ONPG) as substrate, was 1506 ± 0.1 U/mL, determined at 40 °C and pH 6.8, which are the optimal conditions reported for several β -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 β -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 smaller 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 β -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 pH 6.0. 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 °C (Fig. 2b). At pH 3.0 and 11.0 the inactivation of the enzyme was very fast. In this context, the β -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* -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 \rightarrow 3)-Gal- β (1 \rightarrow 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 1H -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 \rightarrow 3)-Gal- β (1 \rightarrow 3)-Gal- β (1 \rightarrow 4)-Glc] in a non-ambiguous manner. Fig. 5 shows the similarity of the 1H -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- β -galactosyl-lactose and the disaccharide 3-O- β -galactosyl-glucose, respectively, using purified standards from *K. lactis* β -galactosidase reactions.⁴² With commercial standards it was also possible to identify peak 12 as the trisaccharide 4'-O- β -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 \rightarrow 6)-Glc, allolactose; peak 9, Gal- β (1 \rightarrow 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 β (1 \rightarrow 3) and β (1 \rightarrow 4) disaccharide

fragments were found, with similar proportions. For the Gal-Glc linkages, the $\beta(1\rightarrow4)$ position was predominant, but minor $\beta(1\rightarrow3)$ as well as minute fractions of $\beta(1\rightarrow2)$ linked moieties were also detected. Therefore, the mixture within peak 13 is composed of the Gal- $\beta(1\rightarrow3)$ -Gal- $\beta(1\rightarrow4)$ -Glc and Gal- $\beta(1\rightarrow4)$ -Gal- $\beta(1\rightarrow4)$ -Glc trisaccharides as major products, together with the Gal- $\beta(1\rightarrow3)$ -Gal- $\beta(1\rightarrow3)$ -Glc, Gal- $\beta(1\rightarrow4)$ -Gal- $\beta(1\rightarrow3)$ -Glc analogues as minor components. Such assignments 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- $\beta(1\rightarrow3)$ -Gal- $\beta(1\rightarrow6)$ -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 $\beta(1\rightarrow3)$ linkages [Gal- $\beta(1\rightarrow3)$ -Gal- $\beta(1\rightarrow3)$ -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 $\beta(1\rightarrow3)$ linkages, with less participation of $\beta(1\rightarrow4)$ and $\beta(1\rightarrow6)$ bonds, in the GOS mixture obtained with β -galactosidase from *B. bifidum* NCIMB41171. However, Rabiou et al. identified, in addition to $\beta(1\rightarrow3)$ linkages, several $\beta(1\rightarrow6)$ and $\beta(1\rightarrow4)$ 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 °C 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. Rabiou 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 an increase in lactose concentration reported a higher yield of GOS using whole cells *B. bifidum* NCIMB41171 with β -galactosidase activity. 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 $\beta(1\rightarrow3)$ linkages followed by $\beta(1\rightarrow6)$. However, only a small proportion of products containing new $\beta(1\rightarrow4)$ linkages were observed. It is worth noting that the GOS synthesis using *B. bifidum* β -galactosidase resulted in a high amount of disaccharides (approx. 40.4 g/L), with allolactose, 3-O- β -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 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 $\beta(1\rightarrow4)$ linkages. The profile obtained with *B. bifidum* β -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 β -galactosidases. However, the predominance of $\beta(1\rightarrow3)$ 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 $\beta(1\rightarrow3)$ bonds are metabolized by probiotic bacteria faster than similar GOS containing other linkages, in particular $\beta(1\rightarrow4)$.²⁹

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,

296 total correlation spectroscopy; HSQC, heteronuclear single quantum correlation; HMBC,
297 heteronuclear multiple bond correlation; TSP-d4, 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid
298 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, prior to any incubation, using the standard activity assay.

Figure 3. HPAEC-PAD analysis 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 \rightarrow 3)-Gal- β (1 \rightarrow 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. ^1H -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 \rightarrow 3)-Gal- β (1 \rightarrow 3)-Gal- β (1 \rightarrow 4)-Glc(α/β). For peak 15 (B) the major product is Gal- β (1 \rightarrow 3)-Gal- β (1 \rightarrow 4)-Glc(α/β). For peak 13 (C) a mixture of trisaccharides (according to MS) containing all the possibilities for Gal- β (1 \rightarrow 3/1 \rightarrow 4)-Gal- β (1 \rightarrow 4/1 \rightarrow 3/1 \rightarrow 2)-Glc(α/β) is obtained. For the three peaks, small

492 amounts of oligosaccharides with the reducing Glc residue glycosylated at position 2 have been
493 found (signals at 5.4-5.5 ppm).

494 **Figure 6.** GOS formation vs. lactose conversion using β -galactosidase from *B. bifidum*
495 (Saphera). Experimental conditions: 400 g/L lactose in 0.1 M sodium phosphate buffer (pH 6.0),
496 1.5 U/ml (ONPG units), 40 °C.

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 °C.

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.

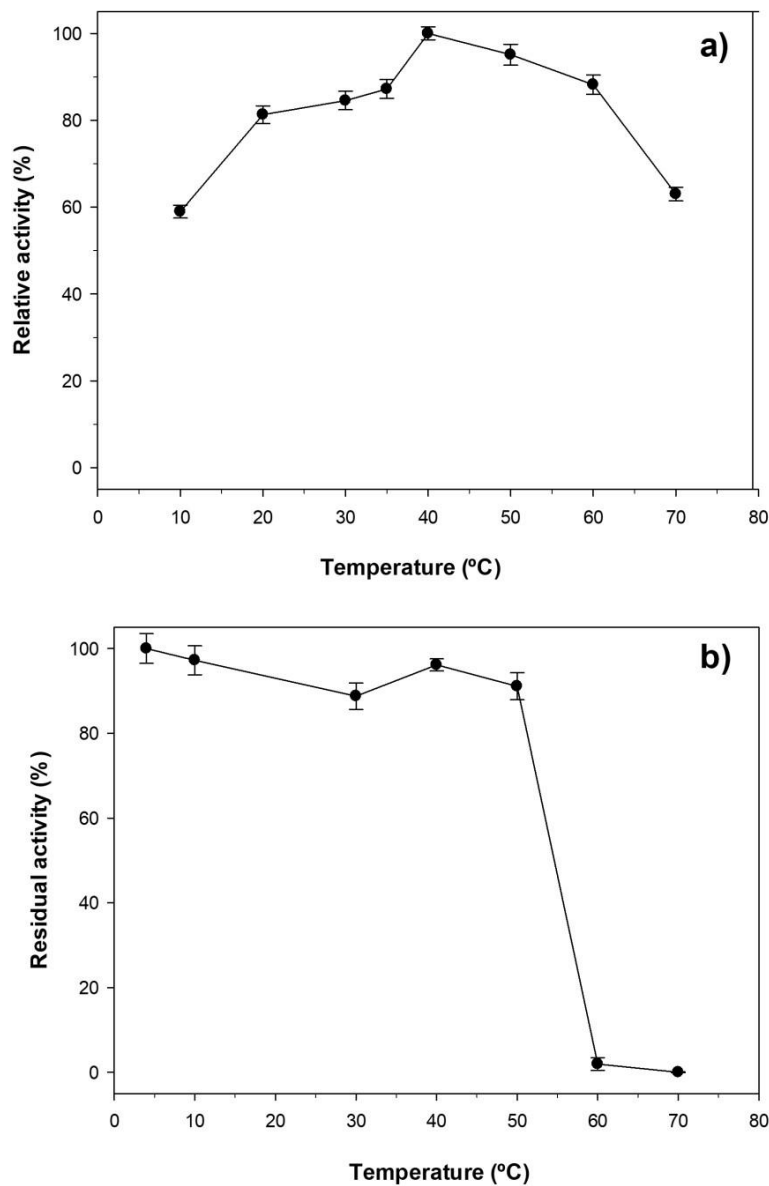


Figure 1. Effect of temperature on a) activity and b) stability of β -galactosidase from *B. bifidum*. For the activity assay, 0.1 M phosphate buffer (pH 6.8) was used. For the thermostability assay, the enzyme was incubated in the same buffer at different temperatures for 1 h. Residual activity was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

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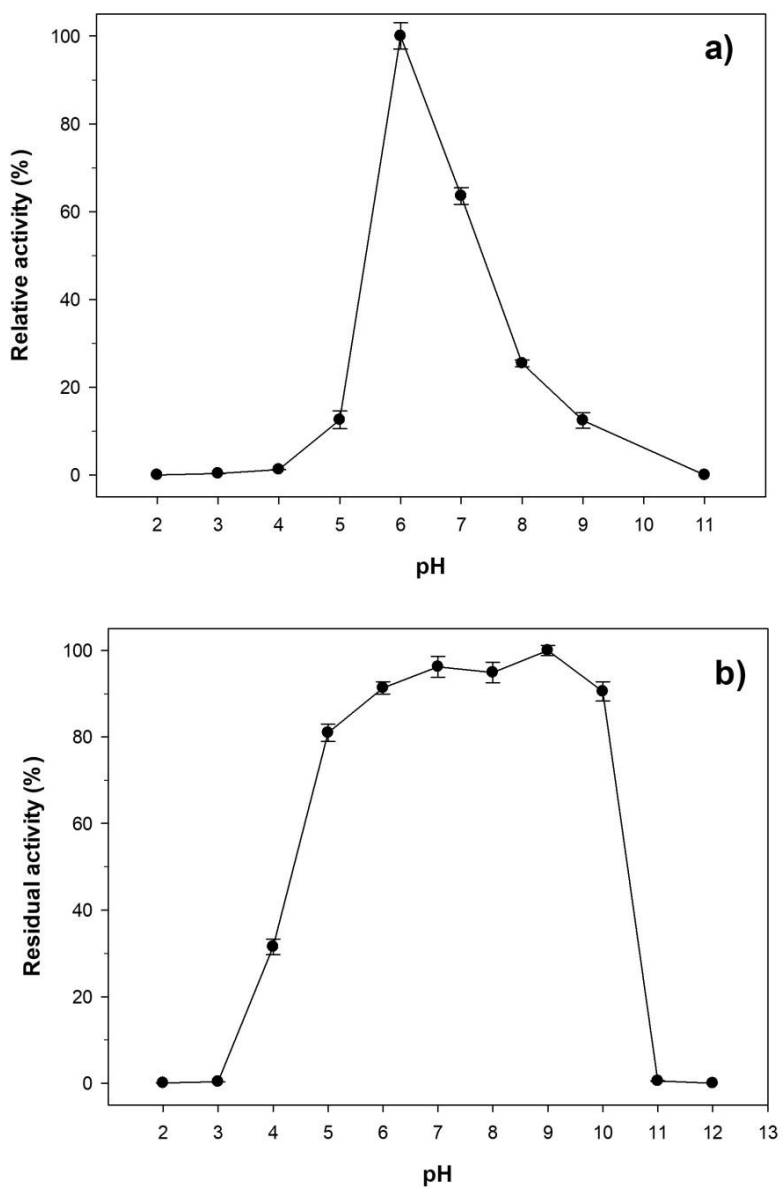


Figure 2. Effect of pH on a) activity and b) stability of β -galactosidase from *B. bifidum*. For the activity assay, 0.1 M Britton & Robinson buffer at different pH was used and the activity measured at 40 °C. For the stability assay, the enzyme solution was incubated under different pH values at 40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

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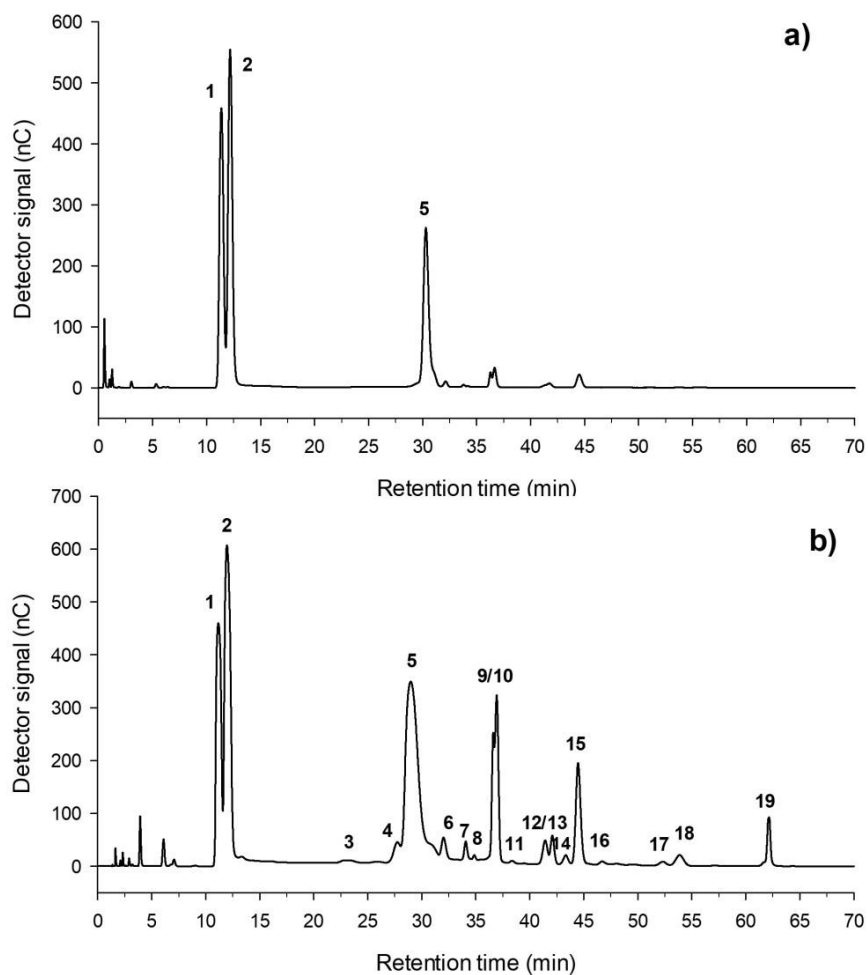


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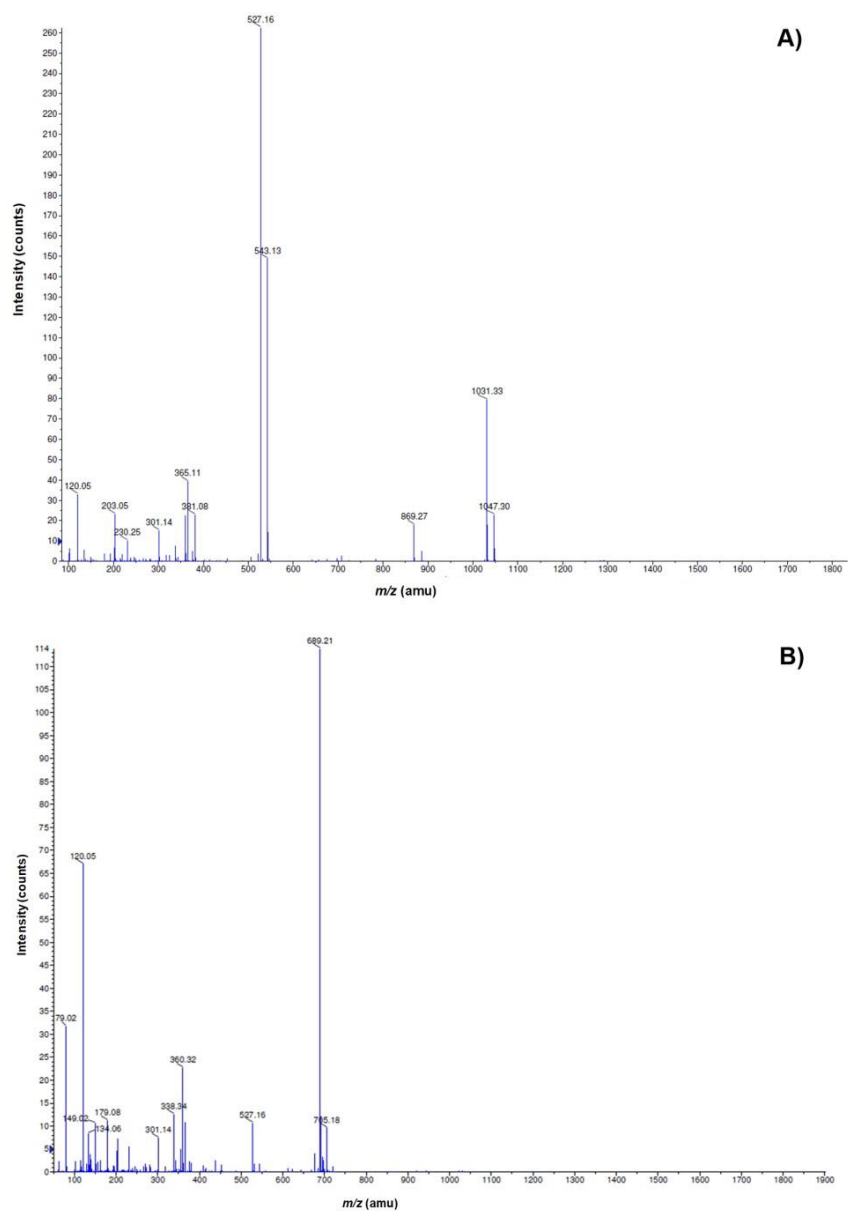


Figure 4. ESI-TOF mass spectra (positive mode) of: A) Peak 15; B) Peak 19.

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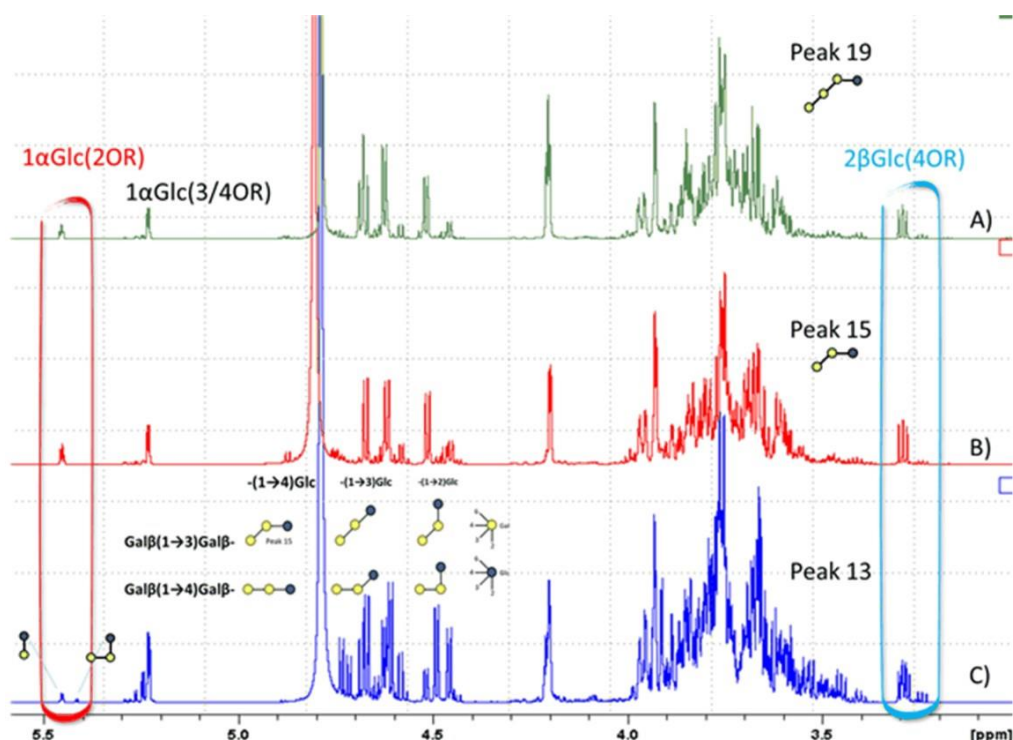


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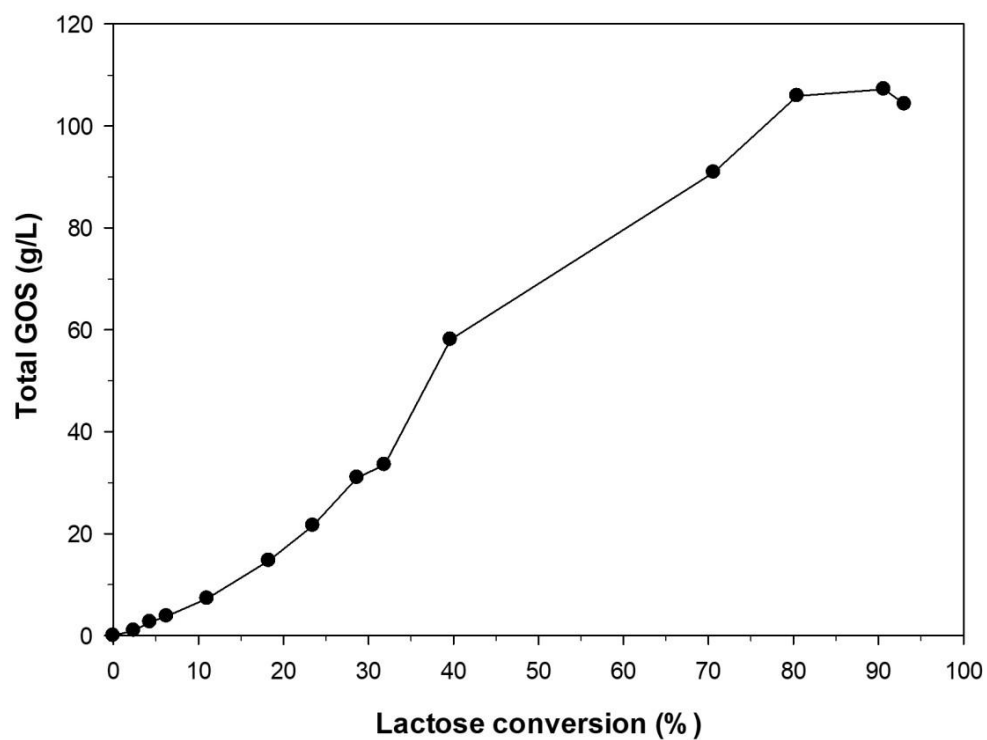


Figure 6. GOS formation vs. lactose conversion using β -galactosidase from *B. bifidum* (Saphera). Experimental conditions: 400 g/L lactose in 0.1 M sodium phosphate buffer (pH 6.0), 1.5 U/ml (ONPG units), 40 °C.

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