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Detailed Analysis of Galactooligosaccharides Synthesis with β-Galactosidase from *Aspergillus oryzae*

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ABSTRACT: The synthesis of galactooligosaccharides (GOS) catalyzed by β-galactosidase from *Aspergillus oryzae* (Enzeco) was studied. Using 400 g/L of lactose and 15 U/mL, maximum GOS yield, measured by HPAEC-PAD, was 26.8% w/w of total carbohydrates, obtained at approximately 70% lactose conversion. No less than 17 carbohydrates were identified; the major transgalactosylation product was 6′-*O*-β-galactosyl-lactose, representing nearly one-third (in weight) of total GOS. In contrast with previous reports, the presence of at least five disaccharides was detected, which accounted for 40% of the total GOS at the point of maximum GOS concentration (allolactose and 6-galactobiose were the major products). *A. oryzae* β-galactosidase showed a preference to form β(1→6) bonds, followed by β(1→3) and β(1→4) linkages. Results were compared with those obtained with β-galactosidases from *Kluyveromyces lactis* and *Bacillus circulans.* The highest GOS yield and specific productivity were achieved with *B. circulans* β-galactosidase. The specificity of the linkages formed and distribution of di-, tri-, and higher GOS varied significantly among the three β-galactosidases.

KEYWORDS: *glycosidase, galactooligosaccharides, prebiotics, transglycosylation, β-galactosidase, oligosaccharides*

INTRODUCTION

β-Galactosidase (β-D-galactoside galactohydrolases, EC 3.2.1.23) catalyzes the hydrolysis of the galactosyl moiety from the nonreducing end of various oligosaccharides. This enzyme is profusely utilized in the dairy industry due to its ability to hydrolyze lactose from milk.[1] In addition, β-galactosidases can catalyze transgalactosylation reactions, in which a galactose moiety is transferred to a nucleophilic acceptor different from water, potentially any sugar present in the reaction medium, thus forming different oligosaccharides. Transgalactosylation is a kinetically controlled reaction, caused by the competition between the reactions of hydrolysis and synthesis. The predominance of synthesis over hydrolysis depends mainly on the origin of the β-galactosidase,[2] the initial sugar concentration,[3] and the water thermodynamic activity.[4,5] If lactose is the initial substrate, transgalactosylation results in the production of galactooligosaccharides (GOS) formed by a mixture of di-, tri-, and even higher oligosaccharides characterized by the presence of a terminal glucose and the remaining saccharide units being galactose, and disaccharides comprising two units of galactose.[6,7]

Apart from being noncariogenic, GOS are nondigestible carbohydrates that modulate the colonic microbiota, promoting a healthy balance.[6] Among other derived effects, GOS reduce the level of blood serum cholesterol, improve mineral absorption, and prevent colon cancer development. In fact, GOS constitute the major part of oligosaccharides in human milk.[8−10] The properties of GOS depend significantly on their chemical composition, structure, and degree of polymerization.[11] Depending on the origin of the β-galactosidase, yield and composition of GOS vary significantly.[12−15] The most studied β-galactosidases are those from *Kluyveromyces lactis*,[16−18] *Aspergillus oryzae*,[19−22] *Aspergillus niger*,[23] *Bifidobacterium* sp.,[24] and *Bacillus circulans*.[25] It is well determined that the chemical structure of the synthesized oligosaccharides (composition, number of hexose units, and types of linkages between them) may affect their fermentation pattern by probiotic bacteria in the gut.[11,16]

The β-galactosidase from *A. oryzae* is a monomeric enzyme whose molecular mass and isoelectric point are 105 kDa[26] and 4.6,[27,28] respectively. Its optimum temperature is in the range from 45 to 55 °C[29,30] and shows an optimum pH of 4.5 with *o*-nitrophenyl-β-D-galactopyranoside and 4.8 with lactose as substrates.[26]

The β-galactosidase from *A. oryzae* has been applied to the synthesis of different transgalactosylated products such as GOS,[20−22] lactulose,[19,31] and galactosyl-polyhydroxyalcohols,[32,33] using both soluble and immobilized enzyme preparations. The β-galactosidase from *A. oryzae* has been immobilized by different strategies including entrapment in alginate,[34] covalent attachment onto various carriers,[35−38] or combined ionic adsorption and cross-linking.[39]

In this work, we have studied the transgalactosylation capacity of a commercial β-galactosidase preparation from *A. oryzae.* A detailed kinetic study of the transglycosylation reaction with lactose was performed, including the structural identification of the synthesized GOS. Results were compared with those obtained with the β-galactosidases from *K. lactis* and *B. circulans.*

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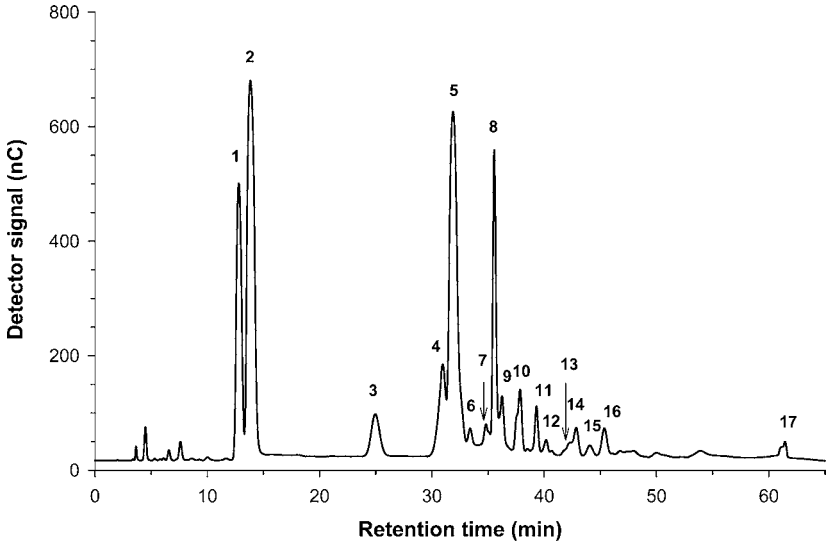

Figure 1. HPAEC-PAD analysis of the reaction of lactose with *A. oryzae* β-galactosidase. The chromatogram corresponds to the reaction mixture after 7 h. Reaction conditions: 400 g/L lactose, 15 U/mL of β-galactosidase, 0.1 M citrate-phosphate buffer (pH 4.5), and 40 °C. Peak assignation for GOS is described in Table 1. Peaks 1, 2, and 5 correspond to galactose, glucose, and lactose, respectively.

MATERIALS AND METHODS

Materials. β-Galactosidase from *Aspergillus oryzae* (ENZECO Fungal Lactase Concentrate, batch no. 21929) was kindly donated by Enzyme Development Corporation, EDC (New York, USA). Glucose, galactose, lactose monohydrate, and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were from Sigma-Aldrich. 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). All other reagents and solvents were of the highest available purity and were used as purchased.

Activity Assay. The enzymatic activity toward *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was measured at 25 °C following *o*-nitrophenol (ONP) release at 420 nm using a microplate reader (Versamax, Molecular Devices). The reaction was started by adding 10 μL of the enzyme (properly diluted) to 190 μL of 45 mM ONPG solution in 0.1 M citrate-phosphate buffer (pH 4.5). The increase in absorbance at 420 nm was recorded continuously during 2 min. The molar extinction coefficient of ONP under assay conditions was 230 M^{-1} cm^{-1}. One international unit of activity (U) was defined as that corresponding to the hydrolysis of 1 μmol of ONPG per min under the above conditions.

Production of Galacto-Oligosaccharides from Lactose. The reaction mixture (25 mL) contained 400 g/L of lactose (34.7% w/w) dissolved in 0.1 M citrate−phosphate buffer at pH 4.5. The biocatalyst was added to adjust the β-galactosidase activity in the reaction mixture (measured with ONPG) to 15 U/mL. The mixture was incubated at 40 °C in an orbital shaker (Vortemp 1550) at 200 rpm. At different times, 200 μL aliquots were sampled from the reaction vessel, and the reaction was stopped by incubating the samples in a Thermo Shaker TS-100 (Boeco) at 96 °C. Samples were filtered using 0.45 μm cellulose filters (National Scientific) coupled to Eppendorf tubes and centrifuged for 5 min at 6000 rpm. For each sample, two dilutions with water (1:400 and 1:4000) were done before HPAEC-PAD analysis. GOS synthesis was evaluated by two parameters: GOS yield and GOS specific productivity. GOS yield represents the mass of total GOS obtained during the synthesis per unit mass of initial lactose, and GOS specific productivity represents the mass of total GOS produced per unit mass of protein added and per unit of reaction time.

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 × 250 mm Carbo-Pack PA-1 column (Dionex) connected to a 4 × 50 mm CarboPac PA-1 guard column was used at 30 °C. Eluent preparation was done with Milli-Q water and 50% (w/v) NaOH (Sigma-Aldrich). The initial mobile phase was 15 mM NaOH for 28 min; then, a gradient from 15 mM to 200 mM NaOH was performed in 7 min, and finally, 200 mM NaOH was maintained for 25 min. The flow rate of the mobile phase was 1 mL/min along the analysis. Peaks were analyzed using Chromeleon software. The identification of the different carbohydrates was done based on commercially available standards or products purified in our laboratory as described in previous papers.[15,40] For those compounds whose standards were not available, quantification was performed on the basis of the calibration curve of standards with the same degree of polymerization.

Purification of GOS by Semipreparative HILIC. For the isolation of unknown GOS in the mixture, the reaction was stopped after 15 min. The biocatalyst was inactivated by boiling the solution for 10 min. The reaction mixture was filtered, and the filtrate was purified by semipreparative hydrophilic interaction chromatography (HPLC-HILIC). Carbohydrates eluted in the order of increasing degree of polymerization. A quaternary pump (Delta 600, Waters) coupled to a Kromasil-NH_2 column (5 μm, 10 × 250 mm, Merck) was used. The column temperature was kept constant at 30 °C. The acetonitrile/water 72/28 (v/v) mixture was degassed with helium and used as the mobile phase at a flow-rate of 8 mL/min for 40 min. Peaks were detected using an evaporative light-scattering detector DDL-31 (Eurosep) equilibrated at 60 °C. A three-way flow splitter (model Acurate, Dionex) and a fraction collector II (Waters) were employed. The fractions containing the main peaks were pooled, and the solvent was removed by rotary evaporation.

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

Nuclear Magnetic Resonance (NMR). The structure of the oligosaccharides was elucidated using a combination of 1D (^1H, ^{13}C) and 2D (COSY, TOCSY, NOESY, and 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 $^1H/X$ probe with a gradient in the Z axis, at a temperature of 298 K.

Table 1. Composition of GOS Synthesized by β-Galactosidase from *Aspergillus oryzae*

peak	type	compound	short name	contribution to total GOS concentration (g/L)
3	disaccharide	Gal-β(1→6)-Gal	6-galactobiose	11.6
4	disaccharide	Gal-β(1→6)-Glc	allolactose	18.4
6	disaccharide	Gal-β(1→3)-Gal	3-galactobiose	2.3
7	disaccharide	Gal-β(1→4)-Gal	4-galactobiose	3.2
8	trisaccharide	Gal-β(1→6)-Gal-β(1→4)-Glc	6′-*O*-β-galactosyl-lactose	31.8
9	trisaccharide[a]	unknown	unknown	9.3
10	disaccharide	Gal-β(1→3)-Glc	3-*O*-β-galactosyl-glucose	7.2
11	tetrasaccharide	Gal-β(1→6)-Gal-β(1→6)-Gal-β(1→4)-Glc[b]	6′-*O*-β-(6-galactobiosyl)-lactose	4.5
12	trisaccharide[a]	unknown	unknown	1.7
13	trisaccharide[a]	unknown	unknown	2.3
14	trisaccharide	Gal-β(1→4)-Gal-β(1→4)-Glc	4′-*O*-β-galactosyl-lactose	6.7
15	trisaccharide[a]	unknown	unknown	1.7
16	trisaccharide	Gal-β(1→3)-Gal-β(1→4)-Glc[c]	3′-*O*-β-galactosyl-lactose	4.4
17	tetrasaccharide	unknown	unknown	2.6

[a]Proposed on the basis of their retention times. [b]Proposed structure based on enzyme specificity and previous bibliography. [c]Proposed structure based on NMR data.

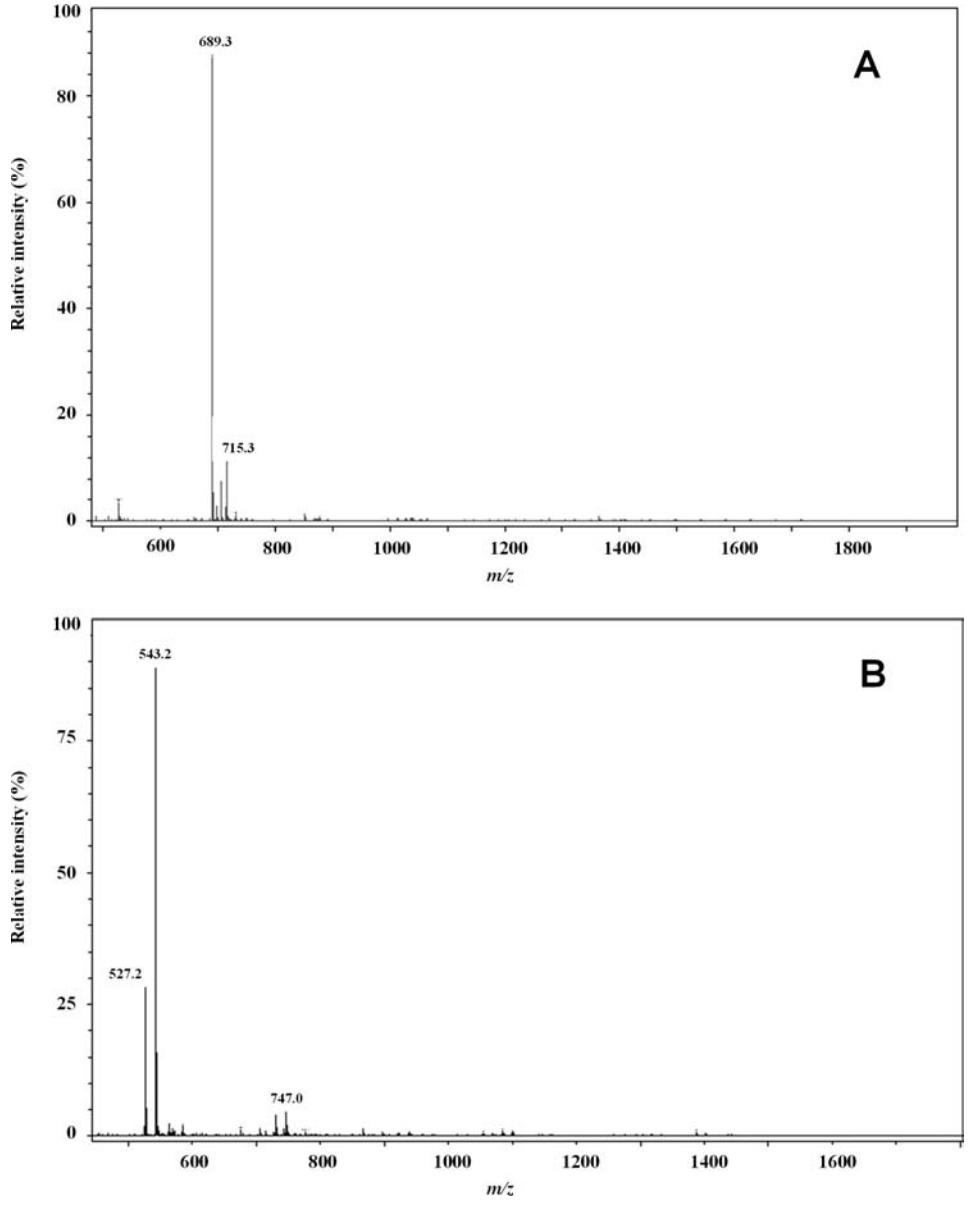

Figure 2. Mass spectra of peaks 11 (A) and 16 (B) in the HPAEC-PAD chromatogram.

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RESULTS AND DISCUSSION

GOS Specificity of *A. oryzae* β-Galactosidase. The synthesis of GOS catalyzed by a preparation of commercially available β-galactosidase from *A. oryzae* (Enzeco Fungal Lactase) was studied using 400 g/L lactose and 15 U/mL. First, the product selectivity of this enzyme was analyzed by HPAEC-PAD. Figure 1 shows the HPAEC-PAD chromatogram of the reaction mixture obtained when the maximum GOS concentration is reached, identifying the presence of at least 17 products in the reaction mixture. Peaks 1, 2, and 5 correspond to galactose, glucose, and lactose, respectively. In addition to commercial standards, several GOS purified as previously reported by us with a β-galactosidase from *K. lactis*[15] allowed us to identify in the chromatogram the disaccharide 3-*O*-β-galactosyl-glucose (peak 10) and the trisaccharide 6′-*O*-β-galactosyl-lactose (peak 8). Table 1 shows the peak assignments for most of the products in the HPAEC-PAD chromatogram, as well as their contribution to total GOS concentration.

The main galactooligosaccharide synthesized by this enzyme was the trisaccharide 6′-*O*-β-galactosyl-lactose, representing almost one-third (in weight) of total GOS produced at the point of maximum yield. Several disaccharides with different glycosidic bonds were also identified, involving two units of galactose (galactobiose) or one of glucose and one of galactose. The major disaccharide produced was 6-*O*-β-galactosyl-glucose

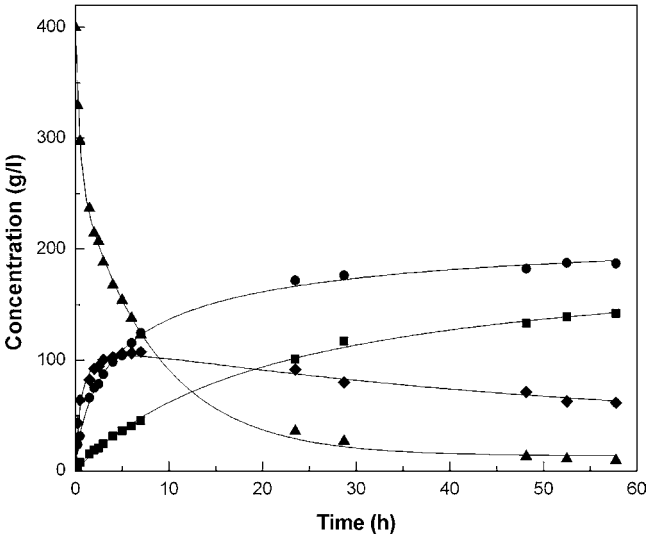

Figure 3. Kinetics of GOS synthesis catalyzed by β-galactosidase from *A. oryzae*. Reaction conditions: 400 g/L lactose, 15 U/mL of β-galactosidase l, 0.1 M citrate-phosphate buffer (pH 4.5), and 40 °C. (▲) Lactose; (●) glucose; (■) galactose; (◆) total GOS.

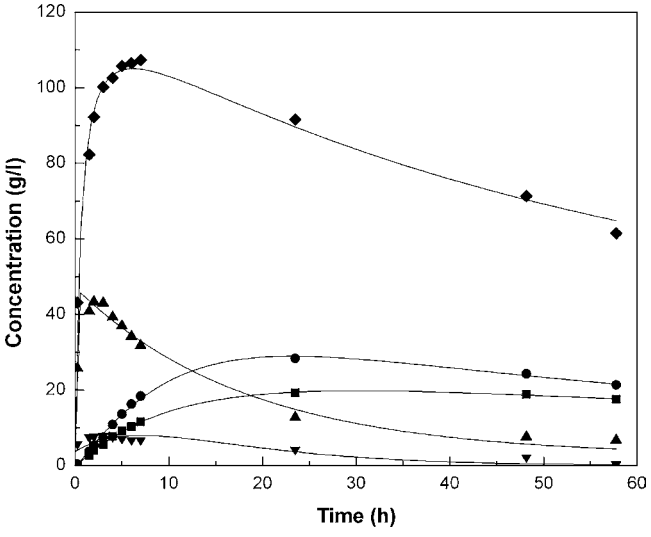

Figure 4. Analysis of GOS formed by β-galactosidase from *A. oryzae* as a function of time. Reaction conditions: 400 g/L lactose, 15 U/mL of β-galactosidase, 0.1 M citrate-phosphate buffer (pH 4.5), and 40 °C. (◆) Total GOS; (●) allolactose ; (■) 6-galactobiose; (▲) 6′-*O*-β-galactosyl-lactose; (▼) 4′-*O*-β-galactosyl-lactose.

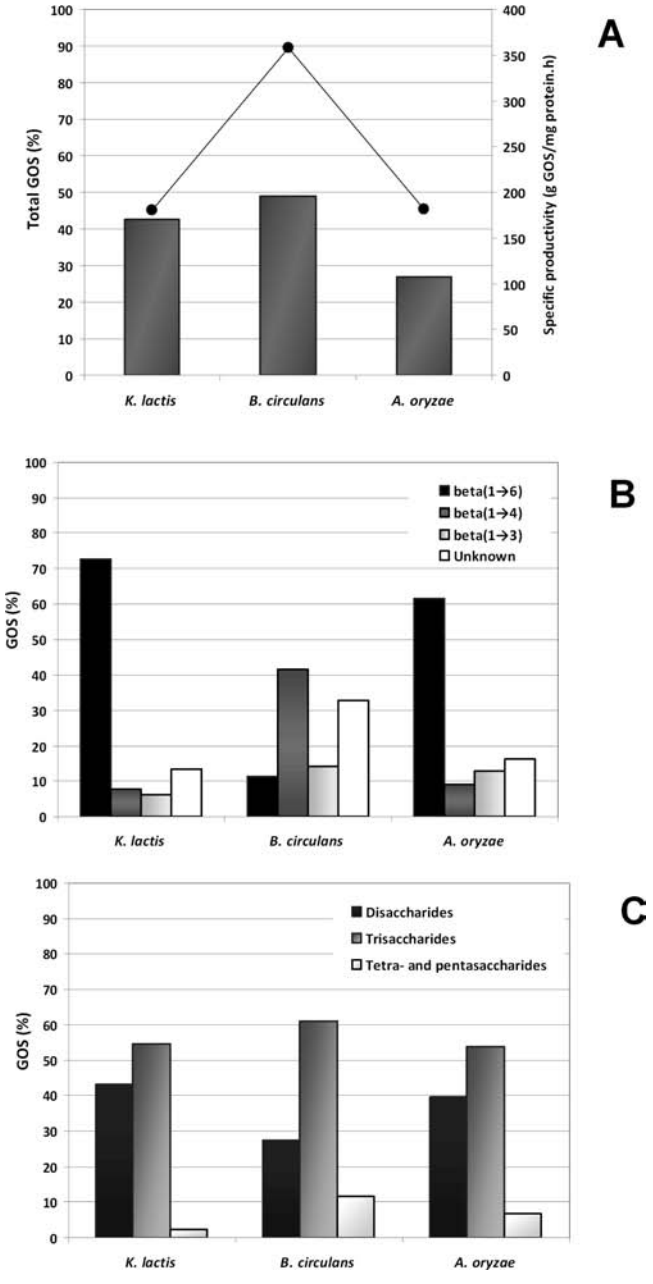

Figure 5. Comparison of the β-galactosidases from *A. oryzae*, *K. lactis*, and *B. circulans*: (A) maximum yield of GOS (bars) and specific productivity (●); (B) distribution of products with β(1→6), β(1→3), and β(1→4) linkages at the point of maximum GOS concentration; and (C) distribution of di-, tri-, and higher oligosaccharides at the point of maximum GOS production.

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(allolactose), representing 18.4% of the total GOS synthesized at the point of maximum yield.

Two unknown reaction products (peaks 11 and 16) were partially purified by semipreparative HILIC for their identification. The mass spectrum of peak 11 indicated that it was a tetrasaccharide (Figure 2A). The purity of this compound was not enough for an unambiguous structure determination; however, based on the preference of this enzyme for the formation of β(1→6) bonds and the high concentration of 6′-*O*-β-galactosyl-lactose in the reaction medium (which may serve as acceptor), we propose that this peak corresponds to Gal-β(1→6)-Gal-β(1→6)-Gal-β(1→4)-Glc. With respect to peak 16, its mass spectrum indicated that it corresponded to a trisaccharide (Figure 2B). The purity of this compound was not enough for a structural determination; however, the NMR spectra showed the presence of a β(1→3) linkage. As the main contaminants in such a fraction were identified in HPAEC-PAD and contained basically β(1→4) and β(1→6) bonds, these results suggested that peak 16 could correspond to the trisaccharide 3′-*O*-β-galactosyl-lactose. On the basis of their retention times, peaks 9, 12, 13, and 15 were assumed to be trisaccharides (although it should not be ruled out that one of them could correspond to a tetrasaccharide, as peak 11 showed an unexpectedly low retention time) and peak 17 a tetrasaccharide or a higher oligosaccharide.

Toba et al. were the first to report the characterization of some of the tri-, tetra- and pentasaccharides synthesized by *A. oryzae* β-galactosidase.[41] In particular, they mentioned the presence of three trisaccharides, 6′-*O*-β-galactosyl-lactose, 3′-*O*-β-galactosyl-lactose, and Gal-β(1→4)-Gal-β(1→6)-Glc (the latter was never identified in the chromatograms of the present work); two tetrasaccharides, Gal-β(1→6)-Gal-β(1→6)-Gal-β(1→4)-Glc and Gal-β(1→6)-Gal-β(1→3)-Gal-β(1→4)-Glc; and one pentasaccharide, Gal-β(1→6)-Gal-β(1→6)-Gal-β(1→6)-Gal-β(1→4)-Glc. Srisimarat and Pongsawasdi also identified 6′-*O*-β-galactosyl-lactose among the synthesized GOS, using in this case *A. oryzae* β-galactosidase colyophilized with β-cyclodextrin.[42] Neri et al. characterized several of the GOS obtained with a preparation of *A. oryzae* β-galactosidase covalently immobilized onto a hydrazide−Dacron−magnetite composite.[37] In particular, they identified three of the GOS previously characterized by Toba et al.: the trisaccharides 6′-*O*-β-galactosyl-lactose and Gal-β(1→4)-Gal-β(1→6)-Glc, and the tetrasaccharide Gal-β(1→6)-Gal-β(1→6)-Gal-β(1→4)-Glc. The authors also mentioned the presence of a disaccharide containing a β(1→6) bond, but no further information on its structure was given.

Kinetics of GOS Synthesis. Figure 3 illustrates the kinetics of GOS production at 40 °C using 400 g/L lactose and an enzyme concentration of 15 U/mL. A maximum GOS concentration of 107 g/L (26.8% w/w of the total amount of sugars) was obtained at 7 h of reaction time, corresponding to approximately 70% of lactose conversion. Beyond 7 h of reaction, hydrolysis was the predominant reaction resulting in a slow decrease of GOS concentration to values close to 60 g/L, whereas monosaccharide concentration increased up to 330 g/L. The difference in concentration between glucose and galactose decreased after the point of maximum GOS yield as a consequence of the increase in the hydrolysis to the transgalactosylation ratio.

Before comparing these results with other related studies, it is important to consider the effects of several operational parameters on GOS production. Regarding the influence of pH and temperature on GOS synthesis, it has been found that even though both parameters affect reaction rates, they do not affect maximum GOS concentration.[37,43] However, it has been proved that the increase in lactose concentration has a strong positive effect on maximum GOS yield attained.[21,44] Therefore, initial lactose concentration should be considered for a sound comparison with other studies. Albayrak and Yang, working with *A. oryzae* β-galactosidase at initial lactose concentration of 500 g/L, obtained a GOS yield of 27% (w/w), of which approximately 70% were trisaccharides.[20] In agreement with these results, Neri et al., using an initial lactose concentration of 500 g/L and 40 °C, obtained a GOS yield of 26.1% (77.4% trisaccharides and 22.6% tetrasaccharides).[37] No significant differences were observed when employing the free or immobilized enzyme. Huerta et al. reported a GOS yield of 29% using an initial lactose concentration of 546 g/L. GOS composition was approximately 66% trisaccharides, 24% tetrasaccharides, and 10% of pentasaccharides, without significant differences when using the soluble or the glyoxyl-agarose immobilized enzyme.[36] Gaur et al. reported that, when starting with a 200 g/L lactose solution, *A. oryzae* β-galactosidase formed only trisaccharides, with a maximum concentration of 22.6% (w/w) for the soluble enzyme and 25.5% (w/w) for a chitosan-immobilized biocatalyst.[35] Guleç et al. reported the production of mainly trisaccharides, with a maximum GOS concentration of 20.8% (w/w) using 320 g/L lactose solution and 55 °C.[30]

One of the main contributions of the present work was the quantification of the main product 6′-*O*-β-galactosyl-lactose and the identification and quantification of five disaccharides in the reaction mixture, two of them containing a β(1→6) bond, the other two with a β(1→3) bond, and one with a β(1→4) bond, representing 27.9, 8.8, and 3.0% of total GOS, respectively.

Our results show that the contribution of disaccharides to total GOS was quite significant, a finding that is normally disregarded for *A. oryzae* β-galactosidase because disaccharides are generally masked by lactose when using common analytical methods. At the point of maximum GOS concentration (7 h, Figure 3), the amounts of di-, tri-, and higher polymerization degree oligosaccharides (expressed in weigh percentage referred to the total sugars) were 10.7, 14.5, and 1.8%, respectively. In this context, the closely related β-galactosidase from *A. niger* is also able to synthesize at least five disaccharides other than lactose, including two galactodisaccharides (3- and 6-galactobiose) and three lactose isomers resulting from the transfer of a galactosyl group to the C-2, C-3, or C-6 of a released glucose.[23]

Considering that the degree of polymerization[45,46] and the glycosidic linkages present in GOS[11] affect the fermentation pattern by the gut microbiota, the kinetics of formation of the main oligosaccharides represents useful information to control product properties at will. Taking into account only the two major disaccharides (allolactose and 6-galactobiose) and trisaccharides (6′-*O*-β-galactosyl-lactose and 4′-*O*-β-galactosyl-lactose), Figure 4 illustrates the kinetic analysis of the four products formed, related to total GOS in the mixture. The time at which maximum concentration is reached varied drastically according to the polymerization degree of the product, corresponding to 2 and 23 h for tri- and disaccharides, respectively. The profile of allolactose formation indicates that in the case of this β-galactosidase, the mechanism involved in its production is mainly an intermolecular reaction, as this disaccharide reaches its maximum concentration when the

amount of free glucose accounts for 43% of total sugars. In the case of other sources of β-galactosidases, intra- and intermolecular mechanisms may be implicated.[3,14]

Comparison of GOS Formation with Respect to β-Galactosidases from Other Origin. Significant differences in maximum GOS concentration, specific productivity, and product specificity were found in the GOS synthesis reaction when using β-galactosidases from *A. oryzae* (this work), *K. lactis*,[15] and *B. circulans*.[40]

The GOS yield and specific productivity, at the point of maximum GOS concentration, is represented in Figure 5A for each of the three enzymes. The synthesis of GOS with *B. circulans* β-galactosidase resulted in the highest GOS yield and specific productivity, with values almost doubling the ones obtained with *A. oryzae* β-galactosidase. Even though *K. lactis* β-galactosidase produces more than 40% of GOS, the specific productivity of the commercial enzyme Lactozym 3000L is low when compared to that of *B. circulans* preparations and is similar to the one obtained with the *A. oryzae* β-galactosidase.

Product specificity also varied depending on the origin of the enzyme, as illustrated in Figure 5B and C. The specific features of *A. oryzae* β-galactosidase indicate a tendency to form preferably β(1→6) bonds, followed by β(1→3) and β(1→4) linkages. In the case of *K. lactis* β-galactosidase, the enzyme also exhibits a clear tendency to synthesize β(1→6) bonds, whereas the *B. circulans* counterpart prefers the formation of β(1→4) bonds. Regarding product distribution (Figure 5C), the *B. circulans* enzyme would be the best choice for a product enriched in trisaccharides, as they represent more than 60% of the total GOS. However, *K. lactis* and *A. oryzae* β-galactosidases would be preferable if the target is a product with a significant content in di- and trisaccharides.

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Notes

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ABBREVIATIONS USED

GOS, galactooligosaccharides; ONPG, *o*-nitrophenyl-β-d-galactopyranoside; HPAEC-PAD, high performance anion-exchange chromatography coupled with pulsed amperometric detection; HILIC, hydrophilic interaction chromatography; NMR, nuclear magnetic resonance; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight

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