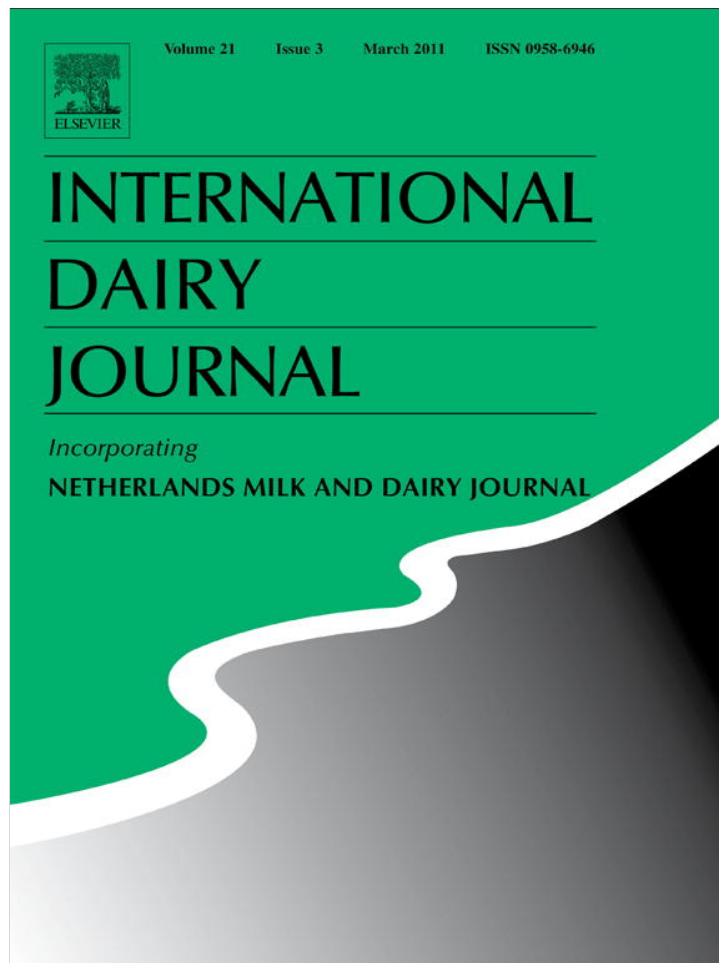


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Characterization of galactooligosaccharides produced by β -galactosidase immobilized onto magnetized Dacron

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ABSTRACT

Bioconversions using enzymes immobilized in magnetic supports present significant advantages due to the easy separation of the enzyme from the reaction mixture and the simplicity and low cost of the support preparation. The characterization of the oligosaccharide mixture obtained by the action of β -galactosidase covalently attached, via glutaraldehyde, to a hydrazide–Dacron–magnetite composite is presented. The fractionation of the oligosaccharide mixture by high performance liquid chromatography, followed by the analysis of the purified compounds by mass spectrometry and nuclear magnetic resonance spectroscopy permitted the identification of glucose, galactose, lactose and a hexose disaccharide containing a 1→6 linkage. Also, the following GOS were identified: β -D-Galp-(1→6)- β -D-Galp-(1→4)-Glc, β -D-Galp-(1→4)-[β -D-Galp-(1→6)]-Glc and β -D-Galp-(1→6)- β -D-Galp-(1→6)- β -D-Galp-(1→4)-Glc. When GOS yield (26.2%) and kinetics of biotransformation of lactose by the Dacron immobilized β -galactosidase were compared with values obtained for the enzyme immobilized in other magnetic supports, similar behaviour was observed.

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1. Introduction

The production of galactooligosaccharides (GOS), like other oligosaccharides, is becoming of increasing interest, as their beneficial effects on human health have been recognized by several authors (Boehm & Stahl, 2007; Maischberger et al., 2008). Oligosaccharides can be produced from natural polysaccharides (e.g., inulin, starch) by chemical or enzymatic hydrolysis, and can also be synthesized from smaller saccharides or by reverse hydrolysis reactions. In addition, reverse hydrolysis reactions, using glycosidases and glycosyltransferases may be employed (Mussatto & Mancilha, 2007).

When lactose is used as the starting material, a group of non-digestible carbohydrates known as GOS are synthesized by the action of β -galactosidase. GOS contain one or no glucose molecule and one to six galactose molecules bound by different glycosidic

bonds such as β -(1→2), β -(1→3), β -(1→4) and β -(1→6). Mixtures of oligosaccharides of various chain length and glycosidic bonds are formed during production (Alander et al., 2001; Ito et al., 1993; Tannock et al., 2004). Considerable amounts of unreacted lactose and monosaccharides are always present as transgalactosylation has to compete with hydrolysis (Mahoney, 1998; Maischberger et al., 2008).

Optimization of the best conditions for the production of GOS by the action of β -galactosidases requires the screening of enzymes that favour transgalactosylation and the selection of the corresponding reaction conditions (Cheng, Duan, & Sheu, 2006; Gänzle, Haase, & Jelen, 2008). These conditions and the enzymes used will establish the linkage between the galactose units, the efficiency of transgalactosylation and the components in the final products. Glycosidic bonds between two galactose units are mainly β -(1→4) bonds when β -galactosidases derived from *Bacillus circulans* (Mozaffar, Nakanishi, Matsuno, & Kamikubo, 1984) or *Cryptococcus laurentii* (Ozawa, Ohtsuka, & Uchida, 1989) are used, and β -(1→6) bonds when enzymes derived from *Aspergillus oryzae* or *Streptococcus thermophilus* (Ito et al., 1990) are used.

β -Galactosidase for lactose hydrolysis or GOS production has been immobilized on different magnetic supports, including

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polysiloxane–polyvinyl alcohol magnetic particles (mPOS–PVA) (Neri et al., 2008, 2009a), magnetic poly(glycidylmethacrylate and methylmethacrylate) particles (Bayramoglu, Tunali, & Arica, 2007), magnetic polysiloxane–polyaniline particles (mPOS–PANI) (Neri et al., 2009b) and thermo-sensitive magnetic hydrogel microspheres (Kondo & Fukuda, 1997). As for non-immobilized enzymes, the predominant reaction products are trisaccharides, namely 4'- or 6'-galactosyllactose, and longer oligosaccharides consisting of 4 or more monosaccharide units (Albayrak & Yang, 2002; Li, Xiao, Lu, & Li., 2008; Shin, Park, & Yang, 1998). Substantial amounts of trans-galactosylated disaccharides are also produced in these reactions (Ishikawa et al., 1995; Ito et al., 1990). As expected, the immobilization process also plays an important role on the overall composition of the final GOS mixture (Sako, Matsumoto, & Tanaka, 1999).

In the present study, the chemical characterization of a GOS mixture obtained by the action of β -galactosidase covalently attached, via glutaraldehyde, to a hydrazide–Dacron–magnetite composite is presented. Bioconversion process kinetics and yield were evaluated and compared with previously obtained data for β -galactosidase immobilized in different magnetic supports.

2. Materials and methods

2.1. Hydrazinolysis of Dacron and magnetization

Hydrazinolysis of Dacron (Terphane, Inc., Cabo, Brazil) and magnetization were obtained according to Carneiro-Leão, Oliveira, and Carvalho Jr. (1991), with the modifications implemented by Amaral, Carneiro-da-Cunha, Carvalho Jr., and Bezerra (2006). The magnetic hydrazide–Dacron obtained was filtered under vacuum and washed exhaustively with distilled water until pH 7.0, dried at 105 °C overnight and sieved (<100 μ m).

2.2. β -Galactosidase immobilization

Magnetic hydrazide–Dacron particles (100 mg) were treated with glutaraldehyde according to Neri et al. (2009b). Activated magnetic hydrazide–Dacron particles were washed with distilled water 10 times and incubated overnight with 10 mL of β -galactosidase from *A. oryzae* (0.72 mg mL⁻¹) (Sigma, Tokyo, Japan). The magnetic- β -galactosidase–Dacron preparation was then washed 10 times with 20 mM citrate–phosphate buffer, pH 4.5 and kept in this buffer at 4 °C until use.

2.3. GOS production

The kinetics of GOSs formation were studied as follows: lactose (Sigma, Munich, Germany) solutions of increasing concentration (5, 10, 20, 30, 40 and 50%, w/v) prepared in 20 mM citrate–phosphate buffer solution, pH 4.5, were added (10 mL) to the immobilized enzyme preparation (100 mg) and samples were taken from 5 to 720 min and analyzed for sugar content by high performance liquid chromatography (HPLC).

The reaction kinetics were studied at four different temperatures (30, 40, 50, and 60 °C). GOS yield value was obtained by dividing the total weight of GOS produced by the weight of lactose consumed and multiplying by 100. The reaction medium at different conditions for GOS production was under orbital stirring at 20 rpm.

2.4. Sugar analysis by HPLC

Sugar analysis were determined according to Neri et al. (2009a), except that the column used was substituted from MetaCarb 67H to MetaCarb 87H (Varian Inc., Palo Alto, CA, USA). The response of the

refractive index detector was recorded and integrated using the Star Chromatography Workstation software (Varian Inc.). The accuracy of the analysis was verified by determining mass balance of the sugars.

2.5. Retention of activity

The retention of activity of the immobilized enzyme was evaluated by incubation for another 10 times. The immobilized enzyme derivative was washed five times with 20 mM citrate–phosphate buffer, pH 4.5, between each successive use.

2.6. GOS fractionation

Fractionation of the different trisaccharides and tetrasaccharides was achieved using the methodology of Dias et al. (2009). The HPLC system (Jasco, Tokyo, Japan) was further equipped with a Prevail Carbohydrate ES column (5 μ m, 250 \times 4.6 mm, Alltech, Deerfield, IL, USA). A mixture of acetonitrile and 0.04% ammonium hydroxide in water (70/30, v/v) was used as eluent at a flow rate of 0.9 mL min⁻¹. Reaction solution (20 μ L 20%, w/v, initial lactose concentration, pH 4.5, 40 °C, after 29 h) was injected. The refractive index (RI) signal was recorded and four fractions (3, 4, 5 and 6) were collected manually. The fractions were analyzed by mass spectrometry and, fractions 4, 5 and 6 were analyzed by nuclear magnetic resonance (NMR) spectroscopy.

2.7. Analysis of GOS by mass spectrometry

Positive-ion mass spectra (MS) using electrospray ionization (ESI), ESI-MS and product ion spectra (ESI-MS/MS) of the ions observed in the ESI-MS, were obtained using an LXQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). For ESI analysis, oligosaccharide fractions were dissolved in methanol/water/formic acid (50:50:0.1, by vol). Samples were introduced at a flow rate of 5 μ L min⁻¹ and the voltage applied was 5.5 kV. Nitrogen was used as the nebulizing and the drying gas. The heated capillary was kept at 350 °C. In each experiment, the ion transmission parameters were optimized automatically to improve the detection of the analyte of interest. Full scan mass spectra ranging from m/z 100 to 1500 were acquired in the positive mode. In the MS^{*n*} ($n = 2, 3$) experiments, collision energy varying 20 and 25 of normalized collision energy.

2.8. Analysis of GOS by NMR

The NMR structural characterization of the GOS mixture was done as described by Dourado, Cardoso, Silva, Gama, and Coimbra (2006). ¹H and ¹³C NMR spectra were recorded in D₂O on a Bruker Avance 500 spectrometer (Wissembourg, France) operating at 500.13 and 125.77 MHz, respectively, the chemical shifts expressed in δ (ppm) values relative to sodium trimethylsilyl-2,2,3,3-d₄-propionate (TSP-d₄) as external reference. Lactose was used as a reference compound for the structural elucidation of GOS.

2.9. Statistical analyses

Results correspond to the mean of three independent experiments. Evaluation of the experimental data indicated that the error associated with each data point did not exceed 5%.

Table 1
Comparison of GOS yield produced by free and immobilized enzymes.

Source of enzyme	GOS yield				Reaction conditions		Reference	
	%		(g L ⁻¹)		% (°C)	pH		
<i>Aspergillus oryzae</i>	26.2	131.0	26.1	130.1	50	40	4.5	This study
	26.0	129.9	26.1	130.1	50	40	4.5	Neri et al., 2009a ^a
	25.7	128.6	26.1	130.1	50	40	4.5	Neri et al., 2009b ^b
Pectinex Ultra SP-L	25.5	51.0	22.6	45.2	20	40	4.5	Gaur et al., 2006 ^c
	15.8	47.4	12.8	38.4	30	55–60	4.0–5.0	Aslan & Tanrıseven, 2007 ^d
<i>Bacillus</i> sp.	41.0	147.6	43.0	154.8	36	55	5.0	Cheng et al., 2006 ^e

^a Magnetic polysiloxane–polyvinyl alcohol.
^b Magnetic polysiloxane–polyaniline particles.
^c Covalent coupling to chitosan.
^d Eupergit C.
^e Chitosan.

3. Results and discussion

3.1. GOS formation

In the present study, the activity of the β -galactosidase from *A. oryzae* immobilized on magnetized Dacron with 31 mg of protein g⁻¹ of support (which was the maximum immobilization capacity of the support) resulted in the production of two types of GOSs, tri- and tetrasaccharides (fractions 4, 5 and 6), with maximum concentrations of 20.2% and 5.9% (w/v), respectively after 4 h. Only one type of GOS was obtained by Gaur, Pant, Jain, and Khare (2006) with the same enzyme immobilized in chitosan, and these were trisaccharides with maximum concentrations of 25.5% after 12 h. The prevalence of trisaccharides is in accordance with the literature data reported for the synthesis of GOSs by β -galactosidase from various origins (Albayrak & Yang, 2002; Hsu, Lee, & Chou, 2007; Li et al., 2008).

Table 1 presents values for maximum GOS concentration (g L⁻¹) and corresponding GOS yield (%) obtained using free and immobilized enzyme in different supports. It is clear that no significant differences were observed in GOS yield when *A. oryzae* β -galactosidase was used in its free form or immobilized in different magnetic supports. These results confirm the excellent accessibility of the substrate to the immobilized enzyme when magnetic supports are used for its immobilization, suggesting that events such as diffusion, mass transfer, steric and conformational changes do not interfere with the interaction with the enzyme substrate and do not reduce enzyme activity (Bayramoglu et al., 2007; Neri et al., 2009a, 2009b).

In light of this, not surprisingly, the results concerning the kinetics of GOS formation, the effect of lactose initial concentration

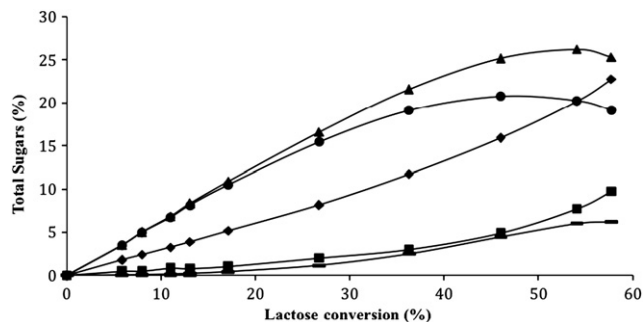


Fig. 1. Formation and degradation of GOS during lactose conversion by immobilized *A. oryzae* β -galactosidase on magnetized Dacron. The reaction was performed at 40 °C and pH 4.5 and initial lactose concentration of 50%: ▲, GOS; ◆, glucose; ■, galactose; ●, trisaccharides; ■, tetrasaccharides. Results varied within 1 and 5%.

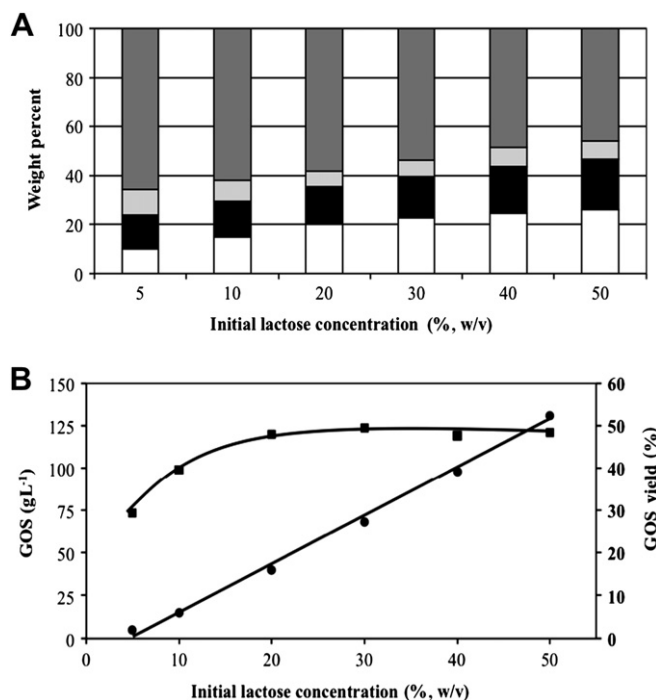


Fig. 2. Effect of the initial lactose concentration on the composition of saccharide mixture (□, GOS; ■, glucose; ■, galactose; ■, lactose) at (A) the maximum yield of GOS and (B) the relationship expressed in terms of (●) total GOS (g L⁻¹) and (■) maximum GOS yield. Production catalyzed by immobilized *A. oryzae* β -galactosidase on magnetized Dacron (40 °C, pH 4.5). Results varied within 1 and 5%.

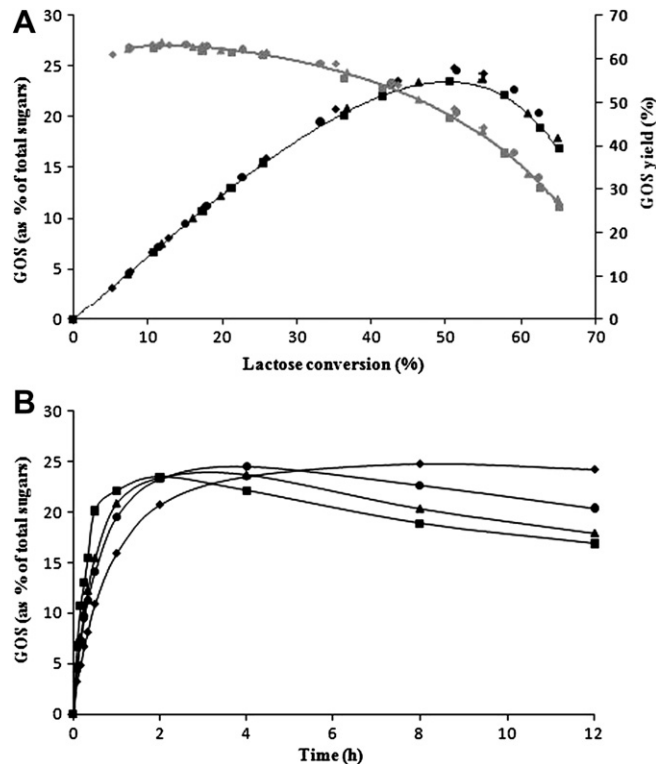


Fig. 3. Effect of temperature (◆, 30 °C; ●, 40 °C; ▲, 50 °C; ■, 60 °C) on the GOS production (black symbols, expressed as a percent of total sugars; grey symbols, percentage GOS yield) from the hydrolysis of 40% (w/v) lactose catalyzed by immobilized *A. oryzae* β -galactosidase on magnetized Dacron: (A) GOS production versus lactose consumption; (B) time course of GOS production (◆, 30 °C; ●, 40 °C; ▲, 50 °C; ■, 60 °C). Results varied within 1 and 5%.

and temperature on GOS production and yield are similar to those reported for the same enzyme immobilized in other magnetic supports mPOS–PVA and mPOS–PANI authors to define these abbreviations (Neri et al., 2009a, 2009b). Figs. 1–3 represent the effect of these parameters on GOS production. Overall, the maximum GOS content in the product changed from 10.1% at 34% lactose conversion (initial lactose concentration 5%) to 26.2% at 54.1% lactose conversion (initial lactose concentration 50%). Moreover, as the initial lactose concentration increased from 5% to 50%, the trisaccharide content doubled, from 9.4% to 20.7%, while tetrasaccharides increased by 6.9-fold, from 0.9% to 6.2%. Temperature (between 30 and 60 °C) had no relevant effect on GOS yield while higher initial rates of GOS production were obtained as temperature was increased. However, no differences on the amount of total GOS produced were observed for temperatures between 40

and 60 °C after 2h of reaction, a similar behaviour to that described by Martínez-Villaluenga, Cardelle-Cobas, Corzo, Olano, and Villamiel (2008) for β -galactosidase from *Kluyveromyces lactis*.

3.2. Re-use of magnetized DACRON on lactose hydrolysis

One of the key advantages of enzyme immobilization on magnetic support is its straight forward re-utilization (Kondo & Fukuda, 1997). However, the successful application of this advantage requires that the enzyme retains its activity for as many uses as possible. For the particular case of Dacron, the re-use was assayed for up to 10 cycles at 25 °C with lactose (20%, w/v) as substrate. The enzyme retained 90% of its initial activity after ten uses (date not shown), a result similar to what has been previously reported (Neri et al., 2009a, 2009b). Other supports evaluated include entrapped

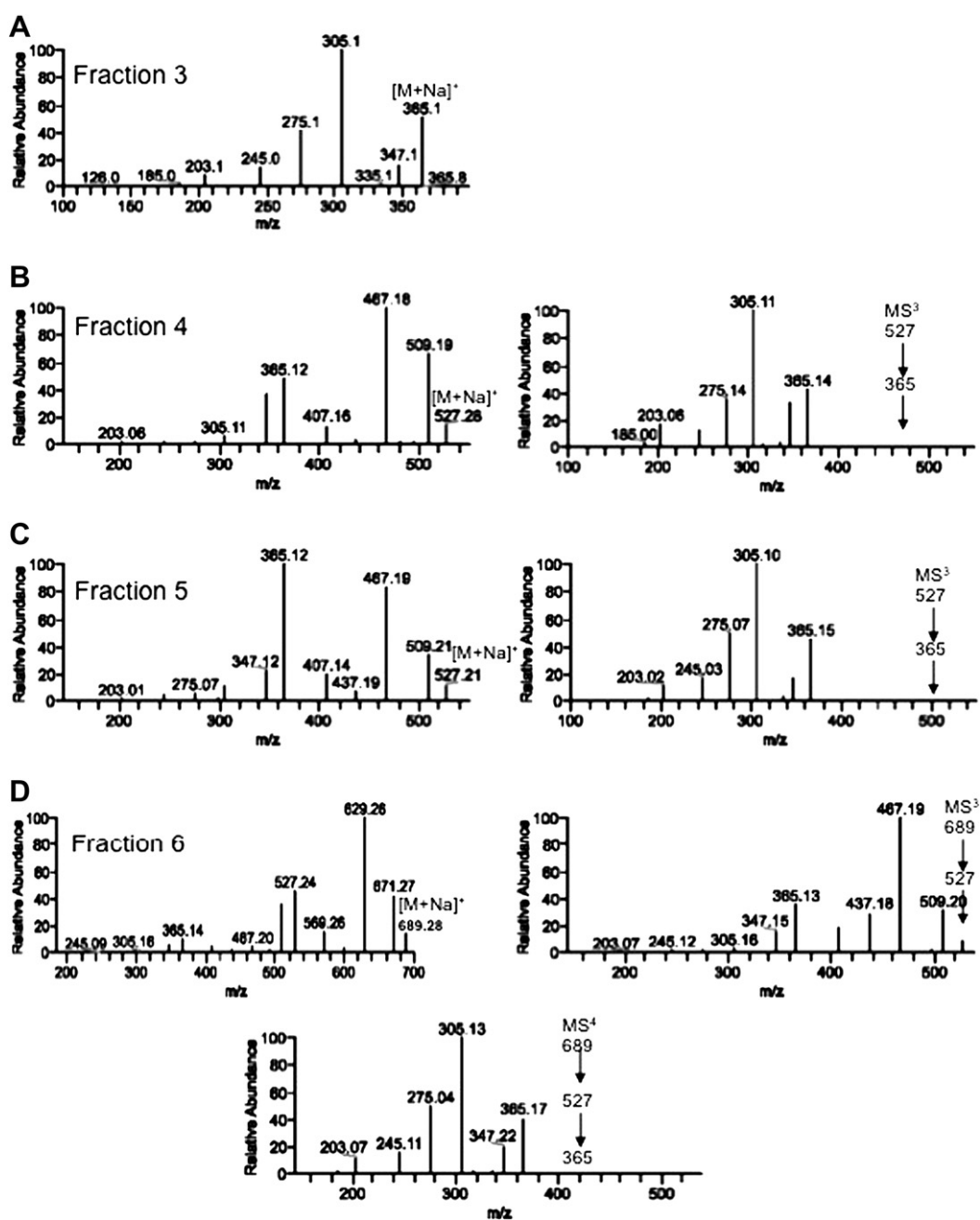


Fig. 4. ESI-MS spectra of the oligosaccharide that eluted respectively in: (A) fraction 3 (m/z 365); (B) fraction 4 (m/z 527); (C) fraction 5 (m/z 527); (D) fraction 6 (m/z 689).

crosslinked concanavalin A- β -galactosidase a retention of 95% activity after seven repeated uses (Haider & Husain, 2007) was observed, IgG-cellulose immobilized β -galactosidase 46% of the initial activity remained after 10 uses (Haider & Husain, 2009) and chitosan (Gaur et al., 2006) observed a decrease of more than 20% in the enzyme activity was observed after four uses.

Overall, these results confirm the efficiency of magnetic supports for the immobilization of β -galactosidase.

3.3. Characterization of GOS

The oligosaccharides that eluted in fractions 3, 4, 5 and 6 were characterized by ESI-MS. In the corresponding ESI-MS spectra it was possible to identify the peaks corresponding to the $[M + Na]^+$ ions of the eluted oligosaccharides, which permitted the determination of molecular weights. When analyzing these results, it must be considered that the presence of one peak does not confirm that only one oligosaccharide was eluted, as different carbohydrates of the same molecular weight can be present. The ESI-MS spectrum of fraction 3 showed an ion at m/z 365, corresponding to a hexose disaccharide, while the ESI-MS spectra of fractions 4 and 5 showed an ion at m/z 527, suggesting that trimers of hexoses were eluted in these peaks. For fraction 6, an ion at m/z 689 was identified, allowing the identification of tetramers of hexoses.

ESI-MS/MS experiments were conducted for the $[Hex_n + Na]^+$ ion ($n = 2-4$) in each fraction, to confirm their monosaccharide composition. The ion at m/z 365 ($[Hex_2 + Na]^+$) of the disaccharide eluted in fraction 3 fragmented by the loss of a hexose residue (-162 Da), yielding an ion at m/z 203 (Fig. 4A), that corresponded to an $[Hex + Na]^+$ ion. Also, the presence of the product ions at m/z 305, 275 and 245 resulted from the cross ring fragmentation of two glucose units linked by a 1 \rightarrow 6 type linkage, as previously described (Zaia, 2004). This information is in agreement with the results inferred by the HPLC elution pattern, that suggest a different structure between the lactose (fraction 2), which has two hexose units linked by a 1 \rightarrow 4 linkage, and that of the disaccharide eluting in fraction 3. This, according with the MS/MS results, has two hexose units linked by a 1 \rightarrow 6 linkage.

The ESI-MS/MS spectra of the trisaccharides, with $[M + Na]^+$ ion at m/z 527, eluting in fractions 4 and 5 (Fig. 4B and C) showed major product ions formed by the loss of one and two hexose units (ions at m/z 365 and 203, respectively). This fragmentation pattern allowed the conclusion that the trisaccharides were composed of three hexose units. Also, the ESI-MS/MS spectra of these trisaccharides present in fractions 4 and 5 were slightly different, suggesting that they have dissimilar structures. For the GOS eluted in fraction 4, the presence of major fragment ions at m/z 467 and m/z 407, that were formed by the loss of 60 Da ($-C_2H_4O_2$) and 120 Da ($-C_4H_8O_4$), suggested the presence of a 1 \rightarrow 4 linkage, most probably at the reducing end. Also, the sequential fragmentation (MS^3) of the product ion at m/z 365 ($[Hex_2 + Na]^+$) showed product ions due to cross ring cleavages by losses of 60, 90 and 120 Da, indicating that these two monomers are linked by a 1 \rightarrow 6 linkage. Together, the MS/MS results from the compound in fraction 4 suggested that this must have a 1 \rightarrow 4 and a 1 \rightarrow 6 linkage, the first at the reducing end. In opposition, the ESI-MS/MS spectrum of the trisaccharide in fraction 5 (Fig. 4C) showed a small ion at m/z 437 along with ions at m/z 467 and 407. Although this fragment ion suggests the presence of a 1 \rightarrow 6 linkage at the reducing end of the molecule, its low abundance makes this hypothesis unproven. Nevertheless, the presence of a 1 \rightarrow 6 linkage in this trisaccharide was confirmed by the MS^3 spectra of its product ion at m/z 365. Thus, although MS/MS analysis did not allow the correct assignment of

the type of linkages present in this trisaccharide, it indicated that the two trisaccharides had distinct structures.

With respect to the tetrasaccharide eluted in fraction 6, the fragmentation of its $[M + Na]^+$ ion at m/z 689 was obtained by the loss of one, two and three hexose units, forming the product ions $[Hex_3 + Na]^+$, $[Hex_2 + Na]^+$, $[Hex + Na]^+$ at m/z 527 and 365 respectively. This data confirmed that the compound was a tetramer of hexose units. Moreover, the analysis of the MS^3 spectra of the referred product ions (data not shown) indicated that this tetramer had a 1 \rightarrow 4 linkage at the reducing end and two other 1 \rightarrow 6 linkages.

The NMR analysis of fractions 4, 5 and 6 confirmed the results from the mass spectrometry analysis and permitted the determination of the detailed structure of the eluted compounds. It should

Table 2

1H and ^{13}C chemical shifts (δ , ppm) of D_2O solutions of the synthesized galactooligosaccharides (fractions 4–6).

Galactooligosaccharide residues	Chemical shifts ^a						
		1	2	3	4	5	6
Fraction 4							
T- β -Galp ^b	1H	4.49	3.56	3.68	3.98	3.95	4.08
		$(J = 7.9$ and 1.6 Hz)					
6- β -Galp-(1 \rightarrow c)	^{13}C	106.2	73.9	75.3	71.3	76.9	71.9
	1H	4.47	3.56	3.66	3.95	3.72	3.78
4- β -GlcP		$(J = 8.0$ Hz)					
	^{13}C	106.0	73.7	75.5	71.5	78.0	63.9
4- α -GlcP	1H	4.67	3.31	3.66	3.68	3.63	3.97
		$(J = 8.0$ Hz)					
4- α -GlcP	^{13}C	98.6	76.6	77.4	82.1	77.6	63.0
	1H	5.23	3.60	3.83	3.68	3.97	3.89
4- α -GlcP		$(J = 3.7$ Hz)					
	^{13}C	94.7	73.9	74.5	82.3	72.8	62.9
Fraction 5							
T- β -Galp ^d	1H	4.46	3.56	3.66	3.93	3.72	3.79
		$(J = 7.8$ and 5.4 Hz)					
T- β -Galp ^c	^{13}C	106.0	73.5	75.4	71.6	78.0	63.9
	1H	4.51	3.56	3.66	3.93	3.72	3.79
4,6- β -GlcP		$(J = 7.8$ Hz)					
	^{13}C	105.7	73.8	75.6	71.6	78.1	63.9
4,6- α -GlcP	1H	4.70	3.32	3.68	3.80	3.96	4.22
		$(J = 5.7$ Hz)					
4,6- α -GlcP	^{13}C	98.8	76.5	76.6	80.8	77.2	70.4
	1H	5.23	3.60	3.84	3.77	3.91	4.29
4,6- α -GlcP		$(J = 3.7$ Hz)					
	^{13}C	94.8	74.0	74.2	80.8	73.7	70.4
Fraction 6							
T- β -Galp ^b	1H	4.51	3.56	3.66	3.97	3.71	3.78
		$(J = 7.7$ Hz)					
6- β -Galp-(1 \rightarrow b,*	^{13}C	106.0	73.6	75.7	71.5	78.0	63.9
	1H	4.46	3.56	3.67	3.97	3.93	4.06
6- β -Galp-(1 \rightarrow c,*	1H	4.47	3.56	3.67	3.92	3.91	4.06
		$(J = 7.8$ Hz)/(J = 7.7 Hz)					
4- β -GlcP	^{13}C	106.2	73.7	75.3	71.3	76.8	72.0
	1H	4.68	3.31	3.66	3.68	3.63	3.96
4- α -GlcP		$(J = 8.0$ Hz)					
	^{13}C	98.6	76.6	77.4	82.0	77.6	63.1
4- α -GlcP	1H	5.23	3.61	3.85	3.68	3.97	3.89
		$(J = 3.7$ Hz)					
4- α -GlcP	^{13}C	94.7	73.9	74.5	82.3	72.9	62.9

^a Assignments done in accordance to the data in the proton (1H NMR), carbon (^{13}C NMR), homonuclear shift correlation with presaturation during relaxation delay (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple quantum coherence (HMBC) spectra.

^b Residue linked to the O-6 of Galp (an asterisk indicates that most of the chemical shifts of these two sugar residues were not possible to discriminate).

^c Residue linked to the O-4 of GlcP (an asterisk indicates that most of the chemical shifts of these two sugar residues were not possible to discriminate).

^d Residue linked to the O-6 of GlcP.

be noted that the NMR spectra of each fraction only presented signals corresponding to one compound, suggesting that single GOSs were collected in fractions 4, 5 and 6. For each oligosaccharide, the ^1H and ^{13}C chemical shifts were assigned by the interpretation of their ^1H NMR, ^{13}C NMR, HSQC, COSY and HMBC spectra (data not shown) and by further comparison with those of lactose and to the literature (Bock, Pedersen, & Pedersen, 1984; Kimura, Matsumoto, Ishihara, Harada, & Miyagi, 1995; Yanahira et al., 1995). Accordingly, the compound eluted in fraction 4 showed characteristic ^1H and ^{13}C chemical shifts for T- β -Galp ($\delta_{\text{H}-1} = 4.47$ ppm, $J = 8.0$, $\delta_{\text{C}-1} = 106.0$ ppm), 4- β -GlcP ($\delta_{\text{H}-1} = 4.67$ ppm, $J = 8.0$ Hz, $\delta_{\text{C}-1} = 98.6$ ppm) and 4- α -GlcP monomers ($\delta_{\text{H}-1} = 5.23$ ppm, $J = 3.7$ Hz, $\delta_{\text{C}-1} = 94.7$ ppm) (Table 2). Also, the remaining ^{13}C resonances in its ^{13}C NMR spectrum at 106.2, 73.9, 75.3, 71.3, 76.9 and 71.9 ppm, as their corresponding ^1H resonances in the ^1H NMR spectrum [4.49 ($J = 7.9$ and 1.6 Hz), 3.56, 3.68, 3.98, 3.95 and 4.08 ppm], indicated the presence of an internal 6- β -Galp-(1 \rightarrow residue). Thus, these results permitted the conclusion that the compound eluted in fraction 4 was a β -D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)-GlcP.

The trisaccharide in fraction 5 was identified as β -D-Galp-(1 \rightarrow 4)-[β -D-Galp-(1 \rightarrow 6)]-GlcP. In accordance to this, the C-6 and H-6 resonances of the GlcP sugar unit (70.4 ppm and 4.29/4.22 ppm for the α and β anomers) were deshielded compared with those of lactose (62.8/62.9 ppm and 3.72/3.81 and 3.69 ppm) and two T- β -Galp sugar units ($\delta_{\text{H}-1} = 4.51$ ppm, $J = 7.8$ Hz; $\delta_{\text{H}-1} = 4.46$ ppm, $J = 7.8$ and 5.4 Hz) were detected.

NMR data from fraction 6 indicated the presence of the tetrasaccharide β -D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)-GlcP. The chemical shifts of the two internal β -galactosyl residues could only be discriminated for their H-1/C-1 signals ($\delta_{\text{H}-1} = 4.46$ ppm, $J = 7.8$ Hz; $\delta_{\text{C}-1} = 106.2$ ppm) for the 6- β -Galp-(1 \rightarrow sugar unit linked to the O-6 of a β -Galp) and ($\delta_{\text{H}-1} = 4.47$ ppm, $J = 7.7$ Hz; $\delta_{\text{C}-1} = 106.3$ ppm) for the 6- β -Galp-(1 \rightarrow sugar unit linked to the O-4 to the GlcP residue), as these two sugar units have a similar chemical environment.

4. Conclusion

A magnetic and immobilized preparation of β -galactosidase from *A. oryzae* capable of producing GOS with yields comparable to other systems was synthesized by using an inexpensive and simple methodology. It also allowed for a straight forward and rapid separation of the reaction medium from the catalyst, making possible an effective control of the reaction time. Moreover, it retained 90% of its initial activity after ten runs. Fractionation of the GOS mixture allowed the identification of glucose, galactose, lactose and one hexose disaccharide with a 1 \rightarrow 6 linkage. The GOS were identified as two trisaccharides (β -D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)-GlcP and β -D-Galp-(1 \rightarrow 4)-[β -D-Galp-(1 \rightarrow 6)]-GlcP) and one tetrasaccharide (β -D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)-GlcP). The composition of the obtained GOS mixture and, above all, its chemical and structural composition will contribute not only to the development of advanced fractionation/separation processes of the different GOS but will also play an important role in the understanding of the mechanisms associated with the prebiotic properties of the GOS.

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