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Optimization of conditions for galactooligosaccharide synthesis during lactose hydrolysis by β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 L HP G)

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Abstract

A study on optimisation of the conditions for galactooligosaccharide (GOS) formation during lactose hydrolysis, produced by Lactozym 3000 L HP G, was carried out. The synthesis was performed during times up to 300 min at 40, 50 and 60 °C, pH 5.5, 6.5 and 7.5, lactose concentration 150, 250 and 350 mg/mL and enzyme concentration 3, 6 and 9 U/mL. The product mixtures were analysed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). During the hydrolysis of lactose, besides glucose and galactose, galactobiose, allolactose and 6′ galactosyl lactose were also formed as a result of transgalactosylation catalysed by the enzyme. The effect of the reaction conditions was different in the formation of di- and the trisaccharide. Thus, the optimal conditions for galactobiose and allolactose synthesis were 50 °C, pH 6.5, 250 mg/mL of lactose, 3 U/mL of enzyme and 300 min, whereas the best reaction conditions for 6′ galactosyl lactose production were 40 °C, pH 7.5, 250 mg/mL of lactose, 3 U/mL of enzyme and 120 min. These results show the possibility to obtain reaction mixtures with Lactozym 3000 L HP G, with different composition, depending on the assayed conditions.

Keywords: Galacto-oligosaccharides; Lactose; Lactozym 3000 L HP G

1. Introduction

Galacto-oligosaccharides (GOS) are non-digestible oligosaccharides, comprised of 2–20 molecules of galactose and one glucose (Miller & Whistler, 2000), which are recognised as prebiotics because they can stimulate the proliferation of lactic acid bacteria and bifidobacteria in the human intestine (Sako, Matsumoto, & Tanaka, 1999). Other health benefits, such as reduction of the level of cholesterol in serum, colon cancer prevention and enhancement of calcium absorption have been also described (Perugino, Trincone, Rossi, & Moracci, 2004; Sako et al., 1999; Tuohy, Rouzaud, Brück, & Gibson, 2005). The confirmed health claims of GOS have significantly increased the public demand for foods containing GOS, particularly in Japan and Europe (Gaur, Pant, Jain, & Khare, 2006). For this reason, a lot of attention has been paid to GOS production, especially via enzymatic transgalactosylation since chemical synthesis of GOS is very tedious (Sears & Wong, 2001). GOS can be synthesised from lactose when this sugar acts as the acceptor and transgalactosylation is catalysed by the enzyme β-galactosidase (EC 3.2.1.23). However, if this acceptor is a water molecule, galactose is released through a hydrolysis reaction (Crittenden & Playne, 1996). The ratio of transferase and hydrolase activities of the enzyme affects the amount and nature of the formed oligosaccharides, since the enzyme source, the concentration and nature of the substrate and the reaction conditions

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(pH, temperature and time) are the main affecting factors (Gaur et al., 2006; Kim, Ji, & Oh, 2004; Mahoney, 1998). Although hydrolysis of synthesised oligosaccharides competes with transgalactosylation, the latter can be favoured at high lactose concentration, elevated temperature and lower water activity (Gaur et al., 2006). Hence, a deep knowledge of the time course of the reaction is required to determine the point of maximum yield of the desired products.

The yeast *Kluyveromyces lactis* is an important commercial source of β-galactosidases (β-D-galactohydrolase, EC 3.2.1.23) (Tello-Solis et al., 2005), with Lactozym 3000 L HP G being one of the most used enzymatic preparations. This enzyme, for its huge hydrolytic activity, has been used to produce lactose-free milk products. Moreover, during this hydrolysis the production of GOS via transgalactosylation has also attracted interest during the last few years. Thus, although a number of studies have been reported on the synthesis of GOS using β-galactosidase from *K. lactis*, most are focused on the formation of trisaccharides and small amounts of higher molecular weight oligosaccharides (Boon, Janssen, & Van't Riet, 2000; Bridiau, Taboubi, Marzouki, Legoy, & Maugard, 2006; Chockchaisawasdee, Athanasopoulos, Niranjan, & Rastall, 2005; Hung & Lee, 2002; Matsumoto et al., 1993) and there are few references on the formation of disaccharides other than lactose (Cheng et al., 2006; Maugard, Gaunt, Legoy, & Besson, 2003). As it is known, disaccharides such as lactulose possesses an important prebiotic character (Tuohy et al., 2002), therefore it is necessary to gain more insight on the formation not only of trisaccharides but also on the disaccharide fraction during transgalactosylation reaction. The present study deals with factors (temperature, pH, time and substrate and enzyme concentration) affecting the formation of the main disaccharides and trisaccharides during lactose hydrolysis, using β-galactosidase from *K. lactis* (Lactozym 3000 L HP G).

2. Materials and methods

2.1. Materials

Lactose monohydrate was supplied by Scharlau (Spain) and D-glucose by Sigma–Aldrich Co. (Germany). D-Galactose, melezitose monohydrate and *o*-nitrophenyl β-D-galactopyranoside (oNPG) were purchased from Fluka (Steinheim, Germany) and galactobiose (β-D-Galp-(1 → 6)-Gal) from Sigma (Steinheim, Germany). Soluble commercial preparation of β-galactosidase from *K. lactis* (Lactozym 3000 L HP G), was kindly provided by Novozymes A/S (Bagsvaerd, Denmark).

2.2. Enzyme characterisation

β-Galactosidase activity was measured using *o*NPG as the substrate. The hydrolysis of *o*NPG was assayed at 40 °C, using *o*NPG at 0.5 g L^{-1} in buffer solution (50 mM potassium phosphate buffer, containing 1 mM $MgCl_2$, pH 6.5). The hydrolysis products are galactose and *o*-nitrophenol (*o*NP) and the enzymatic mechanism is similar to that of lactose hydrolysis. Samples, of 1 mL, were withdrawn at different times. The reaction was stopped by adding 1 mL of 0.5 N H_2SO_4 to 0.44 mL of sample. Afterwards, 1.5 mL of 1 M $NaCO_3$ was added to develop the yellow colour due to the presence of *o*NP. This colour was measured spectrophotometrically at a wavelength of 420 nm. Lactozym 3000 L HP G expressed a β-galactosidase activity of 3205 U. One enzyme unit is defined as the amount of enzyme releasing 1 μmol of *o*NP per mL per minute at 40 °C, pH 6.5.

The amount of soluble protein was performed according to Smith et al. (1985) using the BCA™ Protein Assay Kit procured by Pierce (Illinois, USA). Bovine serum albumin (BSA) was used as standard. Specific activity (U/mg) of the enzyme was calculated from the relation of the activity units (U/mL) over protein concentration (mg/mL). The soluble protein content in the commercial enzyme extract was 36.3 mg/mL. Therefore, the enzyme expressed a specific activity of 88 U/mg.

2.3. Enzymatic synthesis of GOS

Lactose solutions were prepared in 50 mM potassium phosphate buffer containing 1 mM $MgCl_2$. Different reaction conditions were assayed: temperature (40, 50 and 60 °C), pH (5.5, 6.5, 7.5), lactose (150, 250 and 350 mg/mL) and enzyme (3, 6 and 9 U/mL) concentrations. Experiments were carried out in duplicate. Reactions were performed in eppendorfs incubated in an orbital shaker at 300 rpm. Samples were withdrawn at specific time intervals (30, 60, 120, 180, 240 and 300 min) and immediately immersed in boiling water for 5 min to inactivate the enzyme. The samples were stored at −18 °C for subsequent analysis.

2.4. Chromatographic determination of carbohydrates

The carbohydrate composition of the reaction mixtures was determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), on a ICS2500 Dionex system consisting of GP50 gradient pump and ED50 electrochemical detector with a gold working electrode and Ag/AgCl reference electrode. Data acquisition and processing was performed with Chromeleon version 6.7 software (Dionex Corporation, Sunnyvale, CA). For eluent preparation, Milli-Q water, 50% (w/v) NaOH and NaOAc (Fluka, Germany) were used. All eluents were degassed by flushing with helium for 25 min.

Separations were performed following the Van Riel and Olieman method (1991) with some modifications. Elution was at room temperature on a CarboPac PA-10 column (4 mm × 250 mm) connected to a CarboPac PA-10 (4 mm × 50 mm) guard column. Eluent A (12.5 mM NaOH), eluent B (8 mM NaOAc), eluent C (125 mM

NaOH) and eluent D (125 mM NaOH and 500 mM NaOAc) were mixed as follows: 70% A and 30% B from 0 to 3.5 min; 26% A, 70% B and 4% C from 16.5 to 35 min. Finally, 50% A and 50% B were used to wash the column for 10 min and re-equilibrated with the starting conditions of the employed gradient for 20 min. Detection time and voltage parameters were set as follows: $E_1 = 0.1$ V ($t_1 = 400$ ms), $E_2 = 2.0$ V ($t_2 = 10$ ms), $E_3 = 0.6$ V ($t_3 = 60$ ms), $E_4 = -0.1$ V ($t_4 = 500$ ms).

Samples and standard solutions were filtered through a Millipore FH (0.45 μm) (Bedford, MA) membrane before injection. Twenty microliters were injected using an autosampler and separations were performed at a rate of 1 mL/min. Quantification of each sugar was performed by external calibration using standard solutions of galactose, glucose, lactose and melezitose. The regression coefficients of the curves for each standard was always greater than 0.99. The yield of GOS was expressed as percentage of carbohydrate weight formed per weight of lactose initially present.

2.5. Purification of GOS by gel permeation chromatography

Fractionation of reaction mixtures was performed by gel permeation on a column (100 × 2.5 cm) of Bio gel P-2 (<45 μm) at 5 °C. Degassed water was used as the eluent at a flow rate of 15 mL/h. The separated carbohydrates were collected by a fraction collector and fractions of 5 mL containing GOS, as demonstrated by HPAEC-PAD, were freeze-dried for further analysis.

2.6. Characterisation of the main GOS

The fraction containing the main synthesis product was analysed by mass spectrometry and NMR in order to confirm the molecular weight and chemical structure of the galactooligosaccharide. The mass spectrum was recorded using a quadrupole HP 1100 mass detector in the electrospray positive mode (API-ES). The mass spectrometer was operating with 4000 V needle potential, 330 °C gas temperature, drying gas flow of 10 L/min and 40 psi nebuliser pressure. Scan m/z range was from 100–1500.

The chemical structure of the main trisaccharide was identified by ^{13}C NMR and ^{1}H NMR. Both spectra were recorded in D_2O (Fluka, Steinheim, Germany) at 400 MHz with a Varian MERCURY 400 spectrometer operated at 293.1 K. Chemical shifts were measured by reference to acetone ($\delta = 30.5$ for ^{13}C NMR and $\delta = 2.225$ for ^{1}H NMR).

3. Results and discussion

3.1. GOS production by Lactozym 3000 L HP G

Fig. 1 shows the HPAEC-PAD profile, of the products formed from lactose by Lactozym 3000 L HP G under the following conditions: pH 6.5, 40 °C, using 250 mg/mL of lactose and 3 U/mL of enzyme. During hydrolysis of lactose (peak 5) in D-galactose (peak 1) and D-glucose (peak 2), GOS are also formed (peaks 3, 4 and 6) as a result of transgalactosylation catalysed by the enzyme. With authentic standards and the spike technique, it was possible to identify peak 3 as the disaccharide 6′ galactobiose (β-D-Galp-(1 → 6)-D-Gal). Splechtna et al. (2006) following a similar chromatographic method obtained a similar pattern of disaccharides produced by β-galactosidases from *Lactobacillus reuteri* and these authors identified peak 4 with an authentic standard as allolactose (β-Galp-1–6-D-Glc). Maugard et al. (2003) and Cheng et al. (2006) found disaccharides in the reaction mixtures of transgalactosylation, using β-galactosidase, from *K. lactis* but no identification was done. As observed, the most abundant GOS formed corresponded to peak 6. For this reason, purification by gel permeation chromatography was attempted to find an enriched fraction in this oligosaccharide, in order to characterise it. MS analysis of this fraction showed that the main compound corresponded to a trisaccharide with an intense m/z 527 ion. ^{1}H NMR and ^{13}C NMR spectroscopy revealed chemical shifts of peak no. 6 (Table 1) which were coincident with those reported in the literature for the trisaccharide 6′ galactosyl lactose (D-Galp-(1 → 6)-D-Lac), obtained with β-galactosidases from *Cebus apella* (Urashima et al., 1999) and *Aspergillus acuelatus* (Del Val & Otero, 2003). Identification of 6′ galactosyl lactose (D-Galp-(1 → 6)-D-Lac) in the reaction mixture, after the treatment of lactose by β-galactosidase of *K. lactis*, confirms the results of Bridiau et al. (2006), who, in a study on galactosylation of aromatic compounds, found this trisaccharide derived from the action of the same enzyme. Chromatogram profile of GOS products showed in the present work differed from those reported by other authors (Maugard et al., 2003; Chen, Ou-Yang, & Yeh, 2003), in which disaccharides were the main GOS product. These differences can

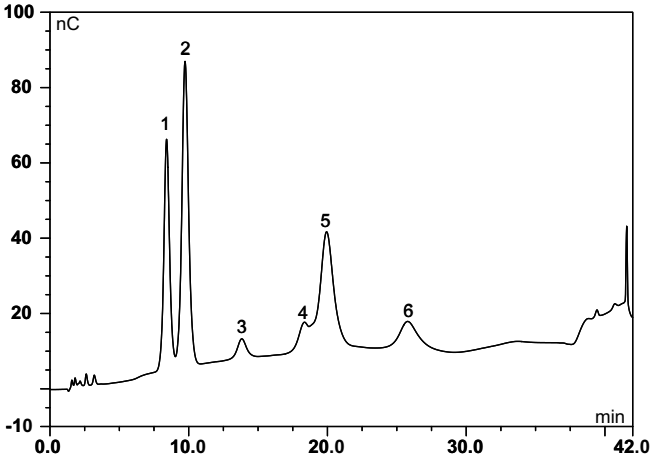

Fig. 1. HPAEC-PAD carbohydrate profile obtained from lactose hydrolysis produced by Lactozym 3000 L HP G at pH 6.5, 40 °C, 250 mg/mL of lactose and 3 U/mL of enzyme. The identified compounds are indicated: (1) galactose, (2) glucose, (3) 6′ galactobiose, (4) allolactose, (5) lactose, and (6) 6′ galactosyl lactose.

Table 1
^{1}H NMR and ^{13}C NMR chemical shifts of purified 6′ galactosyl lactose (β D-Galp-(1 → 6)-Lac)

	Glcα	Glcβ	Galβ1–4	Galβ1–6
^{1}H NMR				
H1	5.26	4.71	4.51	4.52
H2	3.59	3.55	3.61	3.57
H3	3.91	3.64	3.72	3.70
H4	3.69	3.65	4.00	3.98
H5	3.84	3.63	3.80	3.74
H6a	3.99	4.11	3.93	3.75
H6b	3.88	3.85	3.73	3.77
^{13}C NMR				
C-1	92.8	96.7	104.1	104.4
C-2	71.8	75.0	71.8	71.8
C-3	73.4	75.7	73.6	74.7
C-4	80.2	80.5	69.6	69.6
C-5	71.8	76.1	75.6	76.1
C-6	61.2	62.0	69.5	62.0

be explained to the different reaction conditions used by these authors.

The data described above supports previous results (Chockchaisawasdee et al., 2005) which seem to indicate that transgalactosylation reaction catalysed by Lactozym 3000 L HP G produces mainly GOS with linkages β1 → 6. It is known that linkages β1 → 6 are cleaved very fast by β-galactosidase from bifidobacteria (Dumortier, Brassat, & Boaquelet, 1994; Rowland & Tanaka, 1993) and therefore, GOS produced by Lactozym 3000 L HP G could exhibit a high prebiotic potential.

3.2. Optimisation of the reaction conditions for GOS synthesis

Given the interest of the GOS produced by means of Lactozym 3000 L HP G, an optimisation of the enzymatic synthesis was carried out in order to obtain the maximum GOS yields of the reaction since temperature, pH, time and substrate and enzyme concentration were the main studied factors.

3.2.1. Effect of temperature

To determine the influence of temperature on the main di- and trisaccharide produced by Lactozym 3000 L HP G, experiments were performed at 40, 50 and 60 °C at an initial lactose concentration of 250 mg/mL, 3 U/mL of enzyme and pH 6.5, following a time course of reaction of up to 300 min.

Fig. 2 shows trisaccharide (6′ galactosyl lactose) and disaccharides (galactobiose + allolactose) yields (a) and the remaining lactose content (b) during the time course of reaction. Maximum 6′ galactosyl lactose yields were achieved at 40 and 50 °C after 60 min of the reaction (~70% lactose conversion, Fig. 2b), however, disaccharide formation was at a maximum at 50 °C, after 300 min (maximum lactose hydrolysis, Fig. 2b), probably due to the hydrolysis of formed trisaccharides (Mahoney, 1998; Splechtna et al., 2006).

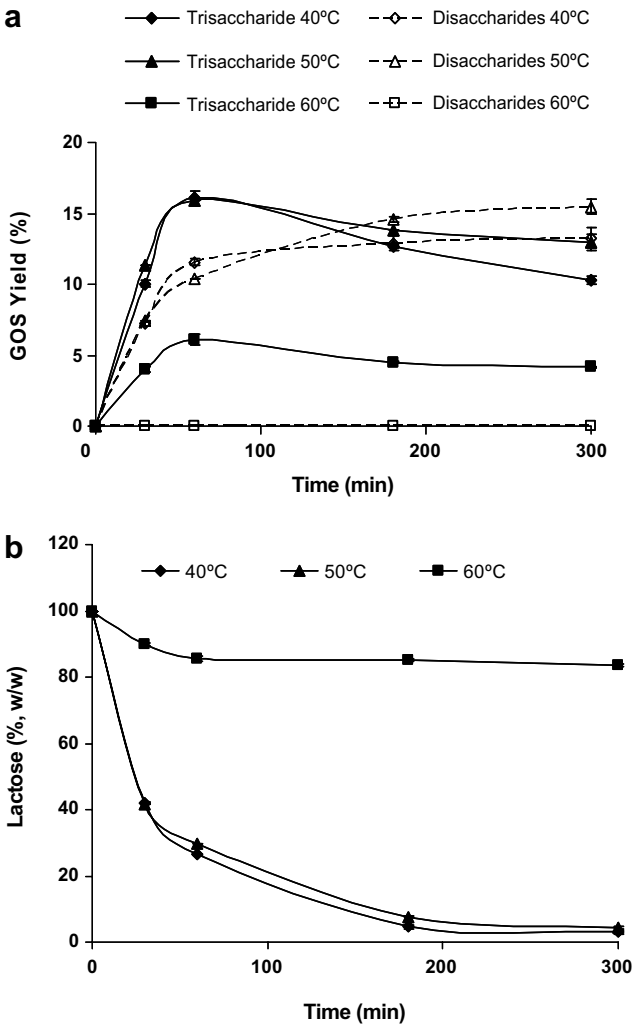

Fig. 2. Effect of temperature on trisaccharide (6′ galactosyl lactose) and disaccharide (galactobiose + allolactose) yields (a) and the remaining lactose content (b) during the time course of reaction performed at 40, 50 and 60 °C at an initial lactose concentration of 250 mg/mL, 3 U/mL of enzyme and pH 6.5 during 300 min (error bars indicate standard deviations).

At 60 °C lactose conversion and GOS formation was markedly lower compared with at 40 °C and 50 °C. At this temperature the yield of 6′ galactosyl lactose deeply decreased and no formation of disaccharides was detected probably due to the denaturation of the enzyme at this temperature.

A similar effect of temperature on total GOS content was observed by Boon et al. (2000) and Hsu, Lee, and Chou (2007) who used β-galactosidases from *Bacillus circulans* and *Bifidobacterium longum* BCRC 15708, respectively, as biocatalysts of the transgalactosylation reaction.

3.2.2. Effect of pH

The effect of pH on the GOS synthesis was investigated at 40 °C by varying pH values from 5.5 to 7.5, at an initial lactose concentration of 250 mg/mL, and 3 U/mL of enzyme.

Fig. 3 shows trisaccharide (6′ galactosyl lactose) and disaccharide (galactobiose + allolactose) yields (a) and the remaining lactose content (b) during the time course of reaction. Maximum trisaccharide yield was observed at pH 6.5 (15.9%) and 7.5 (17.1%) after 60 and 120 min, respectively (lactose conversion close to 80%, Fig. 3b), and extended times of reaction led to trisaccharide degradation. Maximum disaccharides yields were observed after 120 min and the highest values (15%) were recorded at pH 6.5. Prolonged reaction time gave rise to slight degradation of 6′ galactobiose and allolactose. The rapid degradation of 6′ galactosyl lactose, at pH 6.5 at shorter reaction times, and the rises in the formed disaccharides could suggest a favoured hydrolytic activity of the enzyme under this pH. Assays carried out at pH values of 5.5 led to, almost, enzyme inactivation as it can be observed from trisaccharide, disaccharide and lactose content. The optimal pH for GOS production with β-galactosidase from *K. lactis* was in agreement with optimal pH range (6.5–7.5) for lactose hydrolysis (Kim, Lim, & Kim, 1997; Rogalski, Dawidowicz, & Leonowicz, 1994; Tello-Solis et al., 2005).

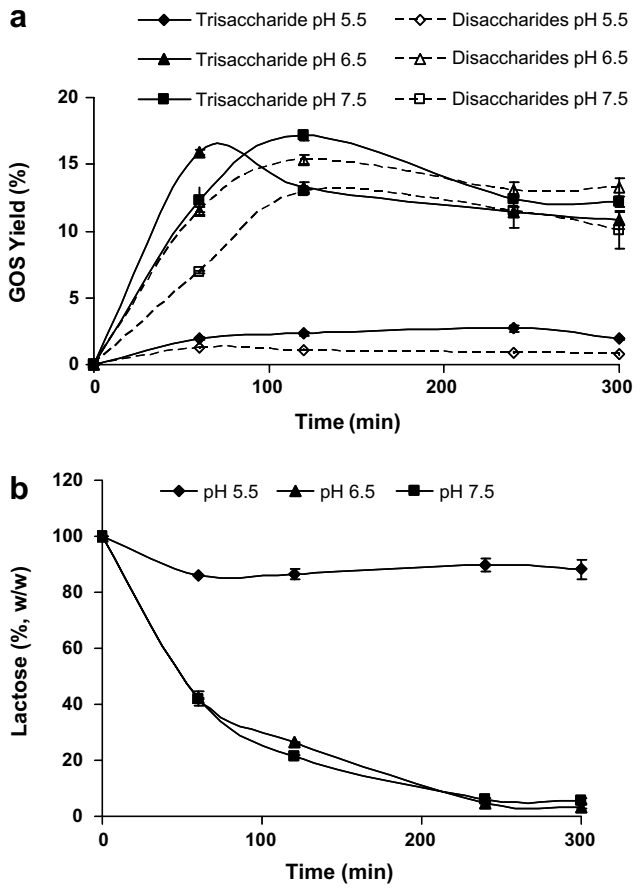

Fig. 3. Effect of pH on trisaccharide (6′ galactosyl lactose) and disaccharides (galactobiose + allolactose) yields (a) and the remaining lactose content (b) during the time course of reaction performed at 40 °C, initial lactose concentration of 250 mg/mL, 3 U/mL of enzyme and pH values from 5.5 to 7.5 (error bars indicate standard deviations).

3.2.3. Effect of substrate concentration

It is well known that one of the main affecting factors for transgalactosylation reaction is the initial lactose concentration (Boon et al., 2000; Cho, Shin, & Bucke, 2003; Huber, Kurz, & Wallenfels, 1976; Jørgensen, Hansen, & Stougaard, 2001; Wierzbicki & Kosikowski, 1972). In this work, to determine the influence of substrate concentration on the GOS production, assays were performed at 40 °C, 3 U/mL of enzyme, pH 7.5 using 150, 250 and 350 mg lactose/mL.

Fig. 4 shows trisaccharide (6′ galactosyl lactose) and disaccharide (galactobiose + allolactose) yields (a) and the remaining lactose content (b) during the time course of reaction.

For initial lactose concentrations of 150, 250 and 350 mg/mL, the maximum trisaccharide yields were 14.8%, 17.1%, 15.9%, after 120 min, respectively. Transgalactosylation significantly increased with lactose concentrations

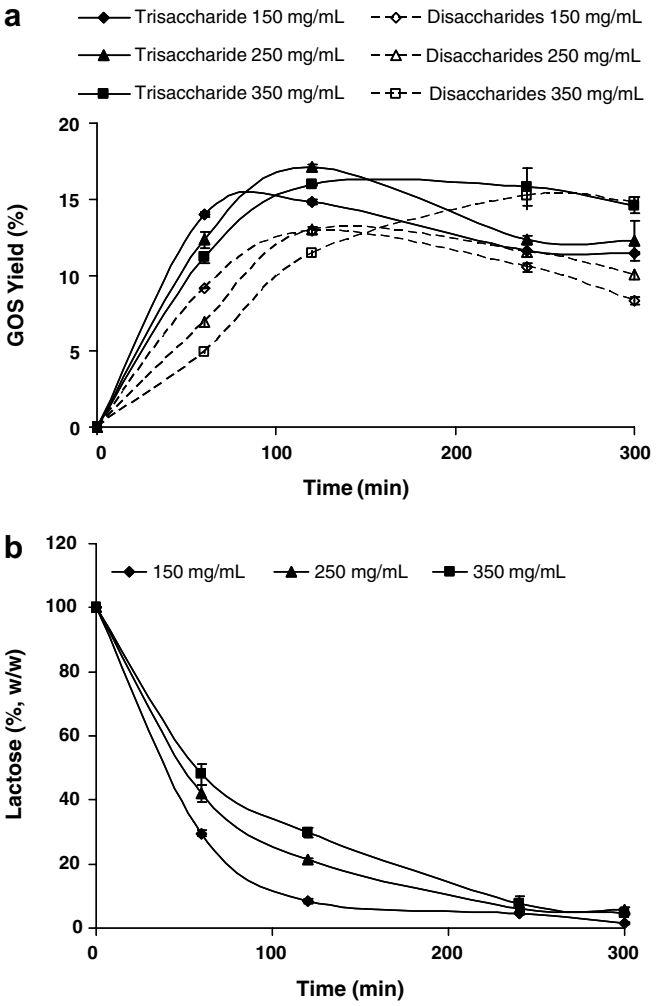

Fig. 4. Effect of lactose concentration on trisaccharide (6′ galactosyl lactose) and disaccharide (galactobiose + allolactose) yields (a) and the remaining lactose content (b) during the time course of reaction performed at 40 °C, 3 U/mL of enzyme, pH 7.5 and 150, 250 and 350 mg lactose/mL (error bars indicate standard deviations).

from 150 to 250 mg/mL due to the fact that, at a low lactose concentration, hydrolysis is favoured since the amount of hydroxyl groups of carbohydrates is lower as compared to those of water, which act as acceptors of galactose. The higher hydrolysis rate observed with 150 mg/mL led to a rapid degradation of the trisaccharide content initially formed. Further increases in initial lactose concentrations from 250 mg/mL to 350 mg/mL after times longer than 120 min led to a considerable increase in the yield of 6′ galactosyl lactose. These observations are consistent with results showed in Fig. 4B, where lactose is converted more rapidly with lower substrate concentration.

Regarding disaccharides, maximum yields (~13%) were detected after 120 min for both initial lactose concentrations 150 and 250 mg/mL. The highest disaccharide yields (15.2%) were found at initial lactose concentration of 350 mg/mL, at longer reaction time's which seems to be due to a slower transgalactosylation reaction, since no high trisaccharide hydrolysis was observed.

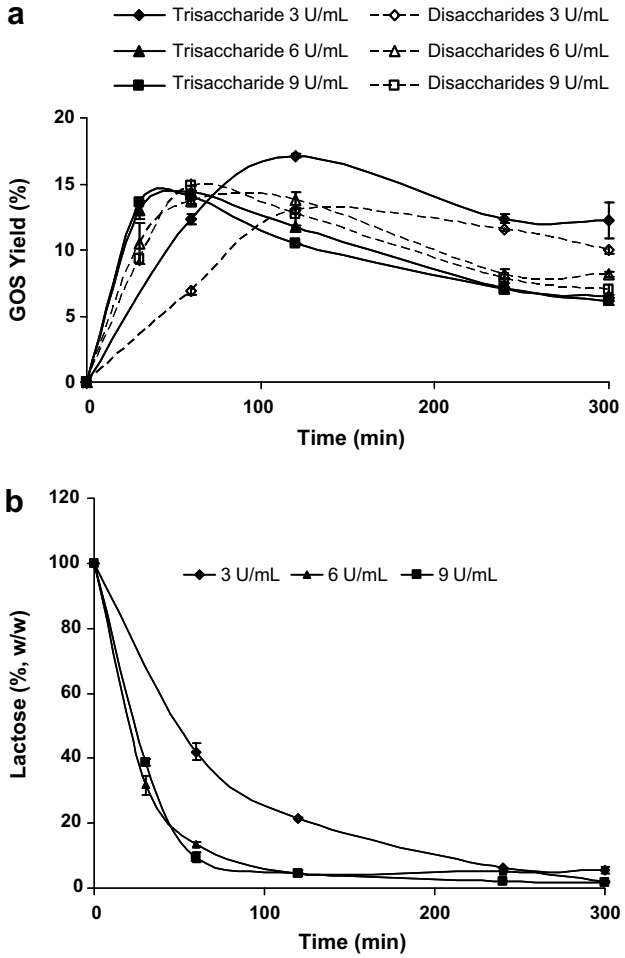

Fig. 5. Effect of enzyme concentration on trisaccharide (6′ galactosyl lactose) and disaccharides (galactobiose + allolactose) yields (a) and the remaining lactose content (b) during the time course of reaction performed at 40 °C, initial lactose concentration of 250 mg/mL, pH 7.5 and 3, 6 and 9 U/mL of enzyme concentration (error bars indicate standard deviations).

3.2.4. Effect of enzyme concentration

To determine the effect of the enzyme concentration on GOS production, experiments were performed at 40 °C at different enzyme concentration 3, 6 and 9 U/mL at initial lactose concentration of 250 mg/mL and pH 7.5.

Fig. 5 illustrates trisaccharide (6′ galactosyl lactose) and disaccharide (galactobiose + allolactose) yields (a) and the remaining lactose content (b) during the time course of reaction. Increases of enzyme concentration brought maximum trisaccharide yields in a shorter reaction time although the highest (17.1%) trisaccharide production was observed with addition of 3 U/mL of enzyme. Moreover, higher trisaccharide hydrolysis (after 120 min) and lactose conversion was observed when 6 and 9 U/mL of enzyme were added. The trisaccharide amounts dominated with the lowest enzyme concentration tested but higher enzyme concentrations led to rises in disaccharide amounts which dominate the GOS mixture when 9 and 6 U/mL of enzyme were added. The highest disaccharide content (15%) was found after 60 min when synthesis was performed with 9 U/mL of enzyme.

4. Conclusions

In this work, the presence of 6′galactobiose (Galp-(1–6)-β-D-Gal), allolactose (Galp-(1–6)-β-D-Glc) and 6′ galactosyl-lactose (Galp-(1–6)-β-D-Galp-(1–4)-D-Glc) have been confirmed in the reaction mixture after the action of the β-galactosidase from *K. lactis*. The results of this work demonstrate the effect of temperature, pH, substrate, enzyme concentration and time on the main disaccharides and trisaccharide formed. The optimal reaction conditions for 6′ galactosyl lactose (D-Galp-(1 → 6)-D-Lac), production with Lactozym 3000 L HP G, were those suitable for the transgalactosylation reaction 40 °C, pH 7.5, 250 mg/mL of lactose, 3 U/mL of enzyme and 120 min. The reaction mixture was composed of 17% of 6′ galactosyl lactose, 13% disaccharides (6% of 6′ galactobiose + 7% allolactose), lactose (21%) and 49% monosaccharides. On the other hand, the best reaction conditions found for disaccharide (6′ galactobiose + allolactose) production by Lactozym 3000 L HP G, as the main GOS present in the reaction mixture were 50 °C, pH 6.5, 250 mg/mL of lactose, 3 U/mL of enzyme and 300 min. This reaction mixture was composed of 12.9% of 6′ galactosyl lactose, 15.5 disaccharides (5.1% of 6′ galactobiose + 10.3% allolactose), lactose (4.3%) and 66.2% monosaccharides. More research is needed to evaluate the potential prebiotic effect of the different di- and trisaccharide fractions.

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