Journal of Molecular Catalysis B: Enzymatic 72 (2011) 206–212

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Influence of reaction conditions on the selectivity of the synthesis of lactulose with microbial β-galactosidases

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ARTICLE INFO

Article history:
Received 18 March 2011
Received in revised form 2 June 2011
Accepted 8 June 2011
Available online 15 June 2011

Keywords:
Lactulose
Prebiotic
β-Galactosidase
Transgalactosylation
Galacto-oligosaccharides

ABSTRACT

Commercial β-galactosidase preparations from *Bacillus circulans*, *Kluyveromyces lactis* and *Aspergillus oryzae* were evaluated as catalysts for the synthesis of lactulose. Among them, the enzyme from *A. oryzae* was selected for further studies. The effect of reaction conditions was then studied on product composition during the kinetically controlled synthesis of lactulose by transgalactosylation with *A. oryzae* β-galactosidase. Product composition was not affected by pH, temperature, total initial concentration of sugar (lactose plus fructose) and enzyme to substrate ratio within the ranges studied. However, lactose to fructose ratio strongly influenced product composition being then possible to control the lactulose to galacto-oligosaccharide ratio within ample margins. Maximum lactulose yield (0.282 g of lactulose per g initial lactose) was obtained using 1/8 lactose to fructose molar ratio, 50% (w/w) total initial sugars, 40 °C, pH 4.5 and enzyme to initial lactose ratio equivalent to 200 IU/g.

1. Introduction

Lactulose (4-O-β-D-galactopyranosyl-D-fructose) is a non-digestible oligosaccharide (NDO) widely recognized as prebiotic [1–3]. Lactulose was the first lactose derived NDO marketed and sold as a laxative for the treatment of acute and chronic constipation [4]; however, its potential as prebiotic has renewed the interest in its production [5,6]. Lactulose is currently produced by alkaline isomerization catalysis [7]; however, the chemical process has several drawbacks since reaction is poorly specific, side reactions occurring so that low product yields are obtained, colored by-products are formed and intense purification is required [8,9]. Recently, the enzymatic synthesis of lactulose has been arisen as an interesting technology for lactulose production, both in terms of process and environmental considerations. However, the enzymatic process requires further research to be assessed as an industrially viable option.

Lactulose can be produced by a kinetically controlled reaction of transglycosylation from lactose using fructose as galactosyl acceptor and β-galactosidase [10–13] or β-glycosidase [14] as catalysts. β-Galactosidase catalyzes the hydrolysis of lactose to glucose and galactose and it is used extensively in food and pharmaceutical products as lactose remover. However, under appropriate conditions β-galactosidases can catalyze the galactosyl transfer of a β-galactoside to a hydroxyl containing acceptor moiety different than water, so synthesis instead of hydrolysis occurs [15]. Since lactose and fructose are present in the reaction medium for lactulose synthesis, both of them can act as acceptors rendering galacto-oligosaccharides (GOS) and lactulose, respectively [11]. The synthesis of lactulose is schematically represented in Fig. 1.

The purpose of this work is to evaluate the effect of selected variables (source of β-galactosidase, total initial concentration of sugars, lactose to fructose molar ratio, enzyme to initial lactose ratio, temperature and pH) on product composition during the kinetically controlled enzymatic synthesis of lactulose so that, by controlling them, a certain pre-established product composition (lactulose to GOS ratio) may be obtained at will. Even though the simultaneous formation of GOS and lactulose may be considered as a disadvantage from the standpoint of product purity, GOS are valuable because they are prebiotics as well and a mixture of both may lead to the production of a NDO of increased prebiotic index [16,17]. In fact, we have some evidences indicating that mixtures of those NDOs have a better prebiotic index than lactulose or GOS alone, so for this reason the synthesis will be assessed in terms of the selectivity of the reaction of transgalactosylaction.

Among β-galactosidases, that from *Aspergillus oryzae* outstands, because it is a very active and stable commodity enzyme, having GRAS status; moreover, this enzyme has a high transgalactosylation activity [18]. However, enzymes from yeast and bacteria

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doi:10.1016/j.molcatb.2011.06.007

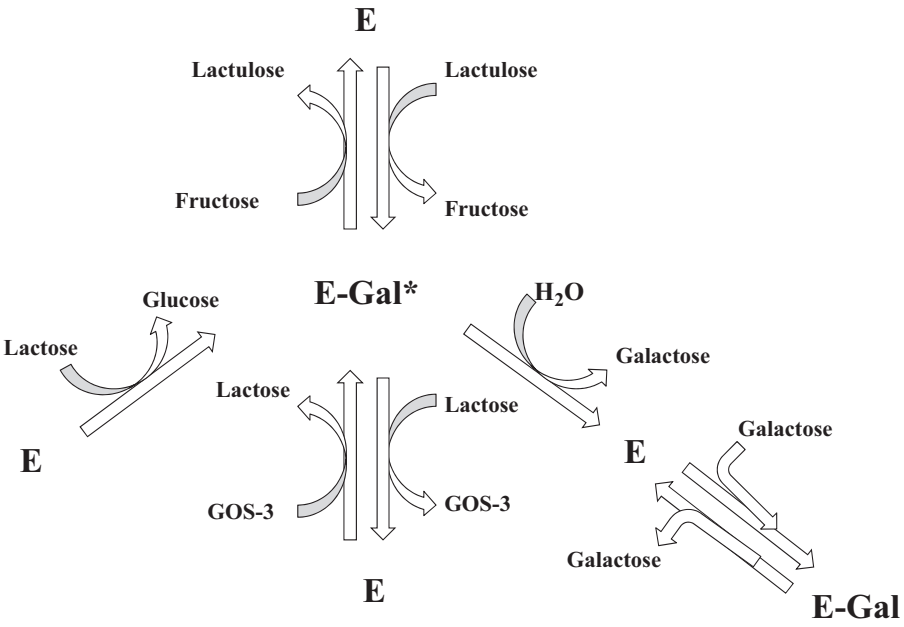

Fig. 1. Mechanism of synthesis of lactulose and GOS with β-galactosidase.

have been proven as lactulose producers as well and therefore β-galactosidases from fungal, yeast and bacterial origin were considered in this study.

2. Materials and methods

2.1. Materials

D(+) lactose monohydrate, *o*-nitrophenol (*o*-NP) and *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPG) and GOS standards were supplied by Sigma (St. Louis, MO, USA). Lactulose was provided by Discovery Fine Chemicals (Wimborne, UK). All other reagents were analytical grade and provided either by Sigma or Merck (Darmstadt, Germany).

Three commercial β-galactosidases from different origin were initially tested. A commercial β-galactosidase preparation of *A. oryzae*, marketed under the trade name Enzeco® Fungal Lactase Concentrate, was kindly donated by Enzyme Development Corporation, New York, USA and used in all experiments. The enzyme preparation had a specific activity of 196,000 IU/g, being one international unit of activity (IU) equivalent to the amount of enzyme hydrolyzing 1 μmol of *o*-NPG per minute at pH 4.5, 40 °C and 30 mM *o*-NPG. Lactozym 3000 L HP G from *Kluyveromyces lactis* was a product from Novozymes kindly supplied by Novo Nordisk Brasil; the enzyme has a specific activity of 3000 IU/mL, being in this case one international unit of activity (IU) equivalent to the amount of enzyme hydrolyzing 1 μmol of *o*-NPG per minute at pH 6.5, 40 °C and 15 mM *o*-NPG. Lactoles L3 from *Bacillus circulans* was a product from Daiwa Kasei K.K (Amano, Japan); the enzyme has a specific activity of 2420 IU/mL, being in this case one international unit of activity (IU) equivalent to the amount of enzyme hydrolyzing 1 μmol of *o*-NPG per minute at pH 5.5, 45 °C and 15 mM *o*-NPG.

2.2. Hydrolytic activity over o-NPG

Initial hydrolysis rates of *o*-NPG were determined by measuring *o*-NP release during the first 5 min of reaction. *o*-NP was determined by absorbance at 420 nm in a Jenway 6715 spectrophotometer, using an extinction coefficient of 253.5 M^{-1} cm^{-1} for pH 4.5, 628.35 M^{-1} cm^{-1} for pH 5.5 and 1640.16 M^{-1} cm^{-1} for pH 6.5 under the assay conditions. The assays were carried out in triplicate with less than 5% difference among samples.

2.3. HPLC analysis of the reaction products

During the stage of selection of the enzyme source, glucose, galactose, fructose, lactulose, GOS and lactose were determined by HPLC using an evaporative light scattering detector (ELSD) model 2000 ES (Alltech) at 92 °C and a nitrogen flow-rate of 3 L/min. Samples were eluted in a Luna-NH2 (Phenomenex) column (250 mm × 4.6 mm) at 40 °C and flow-rate of 1 mL/min. The initial mobile phases was acetonitrile/water 80/20 during the first 25 min, a gradient to acetonitrile/water 70/30 was performed for 10 min and finally a gradient to acetronitrile/water 80/20 for the last 10 min. The processing and analysis of the results was made in the software Varian Star LC, Workstaion 6.41. Standards of galactose, glucose, fructose, lactose, lactulose, 4β-galactobiose, 4β-galactosyllactose and 3α-4β-3α galactotetraose were used to determine their retention times and check the linear range of the measurements.

In the following stages, substrates and products of synthesis were analyzed in a Jasco RI 2031 HPLC equipment, provided with refractive index detector, an isocratic pump (Jasco PU2080) and autosampler (Jasco AS 2055), using BP-100 Ca^{++} columns (300 mm × 7.8 mm) for carbohydrate analysis (Benson Polymerics, Reno, USA). Samples were eluted with milli-Q water at a flow-rate of 0.5 mL/min. Column and detector temperatures were 80 and 40 °C respectively. During synthesis, 0.5 mL samples were taken at regular intervals and the reaction was stopped by adding 0.5 mL of 200 mM NaOH solution and then diluted and filtered through 0.22 μm Durapore membranes (Filterpore, Chile) prior to assay. Chromatograms were integrated using the software ChromPass. The composition of samples was determined by assuming that the area of each peak is proportional to the weight percentage of the respective sugar on the total sugar mass [18–21]. The accuracy of this assumption was checked by a material balance according to Boon et al. [19]. Standards of galactose, glucose, fructose, lactose, lactulose, 4β-galactobiose and 3α-4β-3α galactotetraose were used to determine their retention times and check the linear range of the measurements.

2.4. Selection of the enzymatic source

Lactulose synthesis was initially performed with the three biocatalysts at 40 °C, 50% (w/w) total initial sugars concentration, lactose to fructose molar ratio of 1200 IU/g lactose at the corresponding pH optima (4.5 for the *A. oryzae*, 5.5 for the *B. circulans* and 6.5 for the *K. lactis* β-galactosidase). Reactions were carried out in 150 mL Erlenmeyer flasks by dissolving 32.76 g of lactose and 17.4 g of fructose (molar ratio of 1/1) in 40 g of 100 mM McIlvaine citrate–phosphate buffer at the corresponding pH. Substrates were dissolved by heating the solution at a temperature over 95 °C (no degradation of sugars was observed) and then, after cooling to the reaction temperature, 10 g of a properly diluted enzyme solution was added to start the reaction. Time course of the reaction was followed to determine the maximum lactulose concentration attained. Product composition is expressed as the percentage of each component in the reacted mixture. It was determined by analyzing the amounts of lactulose, GOS-2, GOS-3 and GOS-4 synthesized, numbers meaning the monosaccharide units of each galacto-oligosaccharide. The assays were carried out in duplicate, with standard deviations never exceeding 5%. The quantification of sugars was carried out in a HPLC with evaporative light scattering detector, because ELSD detector is more sensitive than refractive index detector and it allows the separation and quantification of the different types of disaccharides (GOS-2) and lactulose present in the reaction media, which is in this case required.

2.5. Effect of variables on the synthesis of lactulose with the selected biocatalyst

Syntheses were carried out with *A. oryzae* β-galactosidase in magnetically stirred 150 mL Erlenmeyer flasks. Sugars were dissolved in 100 mM citrate–phosphate buffer at the corresponding pH of reaction by previously heating over 95 °C and then adjusting to the corresponding reaction temperature. Afterwards, 10 g of enzyme solution in 100 mM McIlvaine citrate–phosphate buffer at the corresponding pH of reaction were added.

An experimental design was conducted to select the most relevant variables in the synthesis of lactulose with β-galactosidase from *A. oryzae*, which was the one selected as β-galactosidase source. Temperature, pH, enzyme to initial lactose ratio, total initial sugars and lactose to fructose molar ratio were chosen as variables to be tested.

In order to evaluate the effect of pH in the range from 2.5 to 8.5, the remaining variables were kept constant: 40 °C, 200 IU/g, 50% (w/w) total initial sugars concentration and 1/1 lactose to fructose molar ratio. To test the effect of temperature, syntheses were carried out in the range from 40 to 60 °C, the remaining variable being kept constant in the values above at pH 4.5. To assess the effect of total initial sugars concentrations, syntheses were conducted in the range from 40 to 60% (w/w), other variables were kept constant as above at a temperature of 40 °C. To determine the effect of the enzyme to initial lactose ratio syntheses were conducted in the range from 40 to 600 IU/g, other variables being kept constant as above at 50% (w/w) total sugars concentration. Finally, the effect of lactose to fructose molar ratio was evaluated in the range from 4/1 to 1/8, other operational variables being kept constant as above. The assays were carried out in duplicate with less than 5% difference among samples.

The following evaluation parameters were defined:

- Lactose conversion (X_{Lactose}), representing the fraction of initial lactose transformed during the reaction.

$$X_{\text{Lactose}} = \frac{g\ lactose_{\text{inital}} - g\ lactose}{g\ lactose_{\text{inital}}} \quad (1)$$

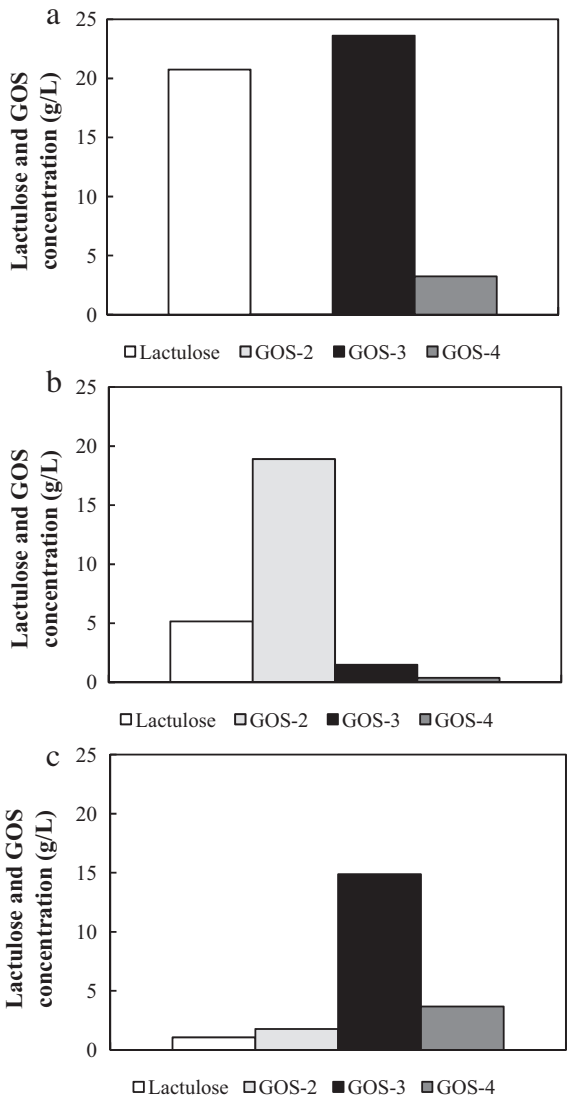

Fig. 2. Product composition in the reaction of lactulose synthesis at 40 °C, 50% (w/w) total initial sugars concentration, lactose/fructose molar ratio of 1/1, enzyme to initial lactose ratio of 200 IU/g β-Galactosidase from: (a) *A. oryzae* at pH 4.5, (b) *K. lactis* at pH 6.5 and (c) *B. circulans* at pH 5.5. Difference between duplicates was less than 5% in all cases.

- Lactulose yield ($Y_{\text{Lactulose}}$), representing the fraction of initial lactose converted into lactulose at maximum concentration of lactulose in the reaction mixture.

$$Y_{\text{Lactulose}} = \frac{g\ lactulose}{g\ lactose_{\text{inital}}} \quad (2)$$

- GOS yield (Y_{GOS}), representing the fraction of initial lactose converted into GOS at maximum concentration of lactulose in the reaction mixture.

$$Y_{\text{GOS}} = \frac{g\ \text{GOS}}{g\ lactose_{\text{inital}}} \quad (3)$$

- Specific productivity of lactulose ($\pi_{\text{Lactulose}}$), representing the amount of lactulose produced per unit of biocatalyst mass (m_{biocat}) and unit time at maximum concentration of lactulose in the reaction mixture.

$$\pi_{\text{Lactulose}} = \frac{g\ lactulose}{m_{\text{biocat}} \cdot time} \quad (4)$$

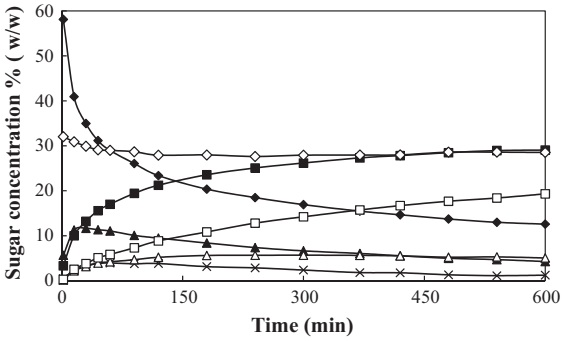

Fig. 3. Time course of the synthesis of lactulose from lactose and fructose at molar ratio of 1/1, 50% (w/w) total initial sugars concentration, enzyme to initial lactose ratio of 200 IU/g, 50 °C and pH 4.5 in 100 mM McIlvaine citrate–phosphate buffer. ♦, lactose; ◊, fructose; ■, glucose; □, galactose; △, lactulose; ▲, trisaccharides (GOS-3); ×, tetrasaccharides (GOS-4). Difference between duplicates was less than 5% in all cases.

- Specific productivity of GOS (π_{GOS}), representing the amount of GOS produced per unit of biocatalyst mass (m_{biocat}) and unit time at maximum concentration of lactulose in the reaction mixture.

$$\pi_{GOS} = \frac{g\,GOS}{m_{biocat} \cdot time} \quad (5)$$

- -Selectivity of transgalactosylation reaction, representing the molar ratio of lactulose to total GOS.

$$Selectivity = \frac{mole\ of\ lactulose}{mole\ of\ GOS} \quad (6)$$

3. Results and discussion

3.1. Selection of the biocatalyst

Results of the synthesis of lactulose with the three biocatalysts under predetermined conditions are presented in Fig. 2. Product composition was strongly dependent on the enzyme source. The tendency to easily accept other nucleophiles than water in the active center and to properly transfer the galactosyl moiety to fructose greatly differs among β-galactosidases from different origins [22]. The β-galactosidase from *A. oryzae* produced both lactulose and GOS, mostly GOS-3 and GOS-4 (Fig. 2a). The enzyme from *K. lactis* produced mainly GOS-2 with lesser amounts of lactulose (Fig. 2b). The enzyme from *B. circulans* produced mainly GOS-3 with small amounts of lactulose (Fig. 2c). Based on these results, the enzyme form *A. oryzae* was selected for matching the conditions required for this study.

A typical time course of the reaction of synthesis of lactulose with *A. oryzae* β-galactosidase is presented in Fig. 3, where substrates and products profiles are shown. A series of experimental variables (pH, temperature, enzyme to substrate ratio, total initial sugars concentration and lactose to fructose ratio) were further examined in terms of their effect in the composition of the reaction mixture.

3.2. Effect of pH

Fig. 4 shows the effect of pH on the synthesis of lactulose, where the concentrations of lactulose and total GOS synthesized are plotted against lactose conversion. As seen, product composition is not affected by pH; however, reaction rate is and a significant decrease is obtained at pH 6.5 or over. Table 1 shows this effect, expressed as maximum specific lactulose productivity ($\pi_{Lactulose}$) and specific GOS productivity (π_{GOS}). A similar behavior has been reported for the synthesis of GOS with free and immobilized *A. oryzae* β-galactosidase where, at same lactose conversion, pH affected the

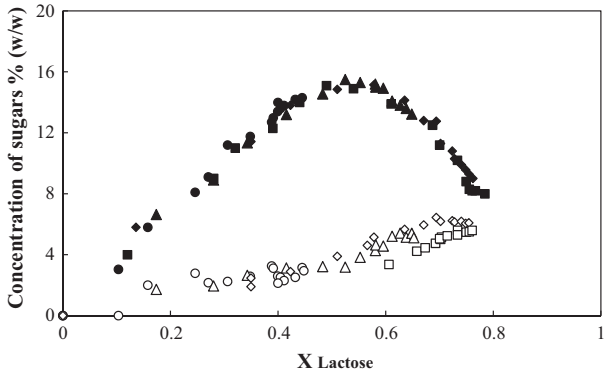

Fig. 4. Effect of pH in the synthesis of lactulose from lactose and fructose with *A. oryzae* β-galactosidase. Lactose to fructose molar ratio of 1/1, 50% (w/w) total initial sugars concentration, enzyme to initial lactose ratio of 200 IU/g and 50 °C. GOS total ■, pH 2.5; ♦, pH 4.5; ▲, pH 6.5; ●, pH 8.5. Lactulose □, pH 2.5; ◊, pH 4.5; △, pH 6.5; ○, pH 8.5. Difference between duplicates was less than 5% in all cases.

reaction rate but not the oligosaccharide distribution according to its chain length [20,21]. This effect has also been observed for permeabilized cells of *K. lactis*, where the rate of synthesis of lactulose decreased significantly at high pHs [10]. Furthermore, $Y_{Lactulose}$ and Y_{GOS} were not significantly affected by pH as reported for the synthesis of GOS [20,21] and lactulose using β-galactosidases from other sources [10,11].

3.3. Effect of temperature

Effect of temperature in the synthesis of lactulose is presented in Fig. 5. No effect of temperature on product composition at the same lactose conversion was observed within the range studied. A similar behavior has been reported for the synthesis of GOS with lactose as substrate [15,20,21]. $Y_{Lactulose}$ and Y_{GOS} were not significantly affected by temperature as reported for the synthesis of GOS [20,21] and lactulose using β-galactosidases from other sources [10,11]. However, as in the case of pH, $\pi_{Lactulose}$ and π_{GOS} were affected by temperature increasing in the whole range considered (see Table 1). Another important aspect is that the enzyme remained active during the synthesis at 60 °C, which was unexpected considering the thermal stability of *A. oryzae* enzyme under non-reactive conditions [18,21]. However, it is known that the stability of *A. oryzae* β-galactosidase [18] and other glycosidases

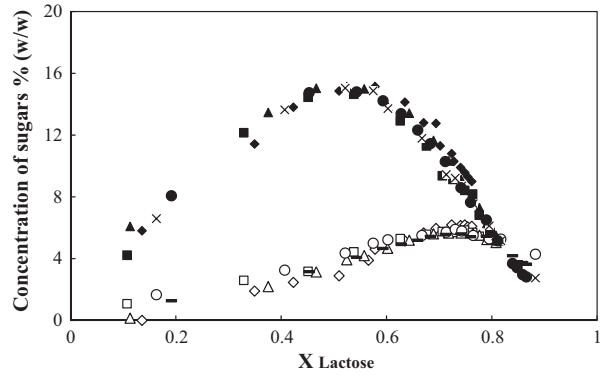

Fig. 5. Effect of temperature in the synthesis of lactulose from lactose and fructose with *A. oryzae* β-galactosidase. Lactose and fructose molar ratio of 1/1, 50% (w/w) total initial sugars concentration, enzyme to initial lactose ratio of 200 IU/g and pH 4.5 in 100 mM McIlvaine citrate–phosphate buffer. GOS total ♦, 40 °C; ■, 45 °C; ▲, 50 °C; ×, 55 °C; ●, 60 °C. Lactulose ◊, 40 °C; □, 45 °C;△, 50 °C; ○, 55 °C; —, 60 °C. Difference between duplicates was less than 5% in all cases.

Table 1
Effect of operational variables on specific productivity of lactulose ($\pi_{Lactulose}$) and GOS (π_{GOS}) in the synthesis of lactulose with β-galactosidase from *A. oryzae*.

Variable	Level	$\pi_{Lactulose}$ (g h^{-1} mg enz^{-1})	π_{GOS}[a] (g h^{-1} mg enz^{-1})
pH	2.5	0.0088	0.0148
	4.5	0.0078	0.0128
	6.5	0.0040	0.0101
	8.5	0.0025	0.0119
Temperature (°C)	40	0.0078	0.0128
	45	0.0107	0.0171
	50	0.0133	0.0242
	55	0.0 179	0.0295
	60	0. 0211	0.0322
Initial sugar concentration (% w/w)	40	0.0079	0.0110
	50	0.0078	0.0128
	60	0.0064	0.0126
Enzyme/lactose ratio (IU/g lactose)	40	0.0073	0.0125
	200	0.0078	0.0128
	400	0.0079	0.0131
	600	0.0070	0.0124
Lactose/fructose molar ratio	4/1	0.030	0.0183
	2/1	0.040	0.0123
	1/1	0.0078	0.0128
	1/2	0.0128	0.0113
	1/4	0.0180	0.0092
	1/6	0.0234	0.0062
	1/8	0.0414	0.0085

[a] At maximum concentration of lactulose on the reaction mixture.

[23,24] is highly improved under reactive conditions as sugars are frequently enzyme stabilizers.

3.4. Effect of the enzyme to substrate ratio

Fig. 6 shows that there is no effect of the enzyme to lactose ratio on product composition along lactose conversion during the synthesis of lactulose. As expected, reaction rate increased with the increase in the enzyme to lactose ratio, as already reported for the synthesis of lactulose with recombinant *Escherichia coli* bearing the gene of the thermostable β-galactosidase from the hyperthermophilic bacterium *Sulfolobus solfataricus* [11]. Also $Y_{Lactulose}$ was not significantly affected by the enzyme to substrate ratio, remaining around 0.09 g lactulose/g initial lactose for the whole range considered. This finding is in agreement with previously reported results for the synthesis of GOS with β-galactosidase of *A. oryzae* from lactose as substrate [25]. Furthermore, $\pi_{Lactulose}$ and π_{GOS} were not significantly affected by increasing the enzyme to lactose ratio (see Table 1).

3.5. Effect of the total initial concentration of sugars

As seen in Fig. 7, there was no effect of the total initial concentration of sugars on product composition in the reaction of synthesis of lactulose. Despite this, the concentration of products increased with the initial concentration of sugars, producing a maximum lactulose concentration of 39.2, 64.2 and 91.6 g kg^{-1} of water at 40, 50 and 60% (w/w) of total initial concentration of sugars, respectively. Lee et al. [10] reported an increase in the level of synthesis of lactulose with permeabilized cells of *K. lactis* when increasing the initial concentration of sugars at constant lactose to fructose ratio, very much in the same way as in the case of the cloned thermostable β-galactosidase from *S. solfataricus* already referred [11]. $Y_{Lactulose}$ and Y_{GOS} were slightly affected by the total initial concentration of sugars, increasing at higher initial sugars concentration. This behavior is similar than those previously reported for the synthesis of GOS [15,20,21]. However, $\pi_{Lactulose}$ decreased and π_{GOS} increased at higher initial sugars concentration (see Table 1). This

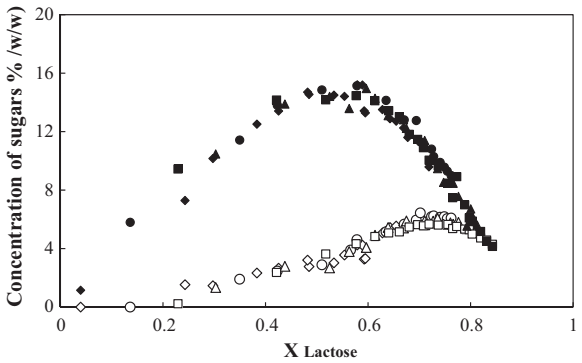

Fig. 6. Effect of the enzyme to lactose ratio in the synthesis of lactulose from lactose and fructose with *A. oryzae* β-galactosidase. Lactose to fructose molar ratio of 1/1, 50% (w/w) total initial sugars concentration, 40 °C and pH 4.5 in 100 mM McIlvaine citrate–phosphate buffer. GOS total ♦, 40 IU/g; ●, 200 IU/g; ▲, 400 IU/g; ■, 600 IU/g. Lactulose ◊, 40 IU/g; ○, 200 IU/g; △, 400 IU/g; □, 600 IU/g. Difference between duplicates was less than 5% in all cases.

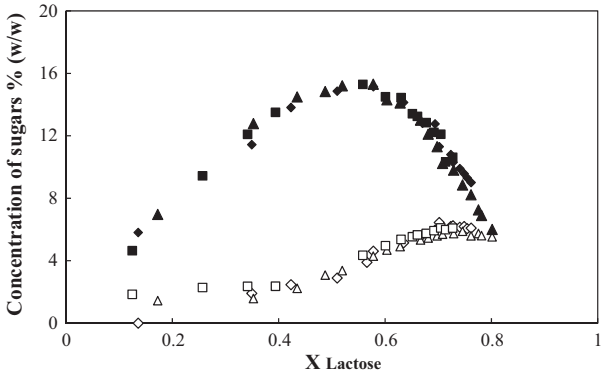

Fig. 7. Effect of the total initial sugars concentration in the synthesis of lactulose from lactose and fructose with *A. oryzae* β-galactosidase. Lactose and fructose at molar ratio of 1/1, enzyme to initial lactose ratio of 200 IU/g, 40 °C and pH 4.5 in 100 mM McIlvaine citrate–phosphate buffer. GOS total ▲, 40% (w/w); ♦, 50% (w/w); ■, 60% (w/w). Lactulose △, 40% (w/w); ◊, 50% (w/w); □, 60% (w/w). Difference between duplicates was less than 5% in all cases.

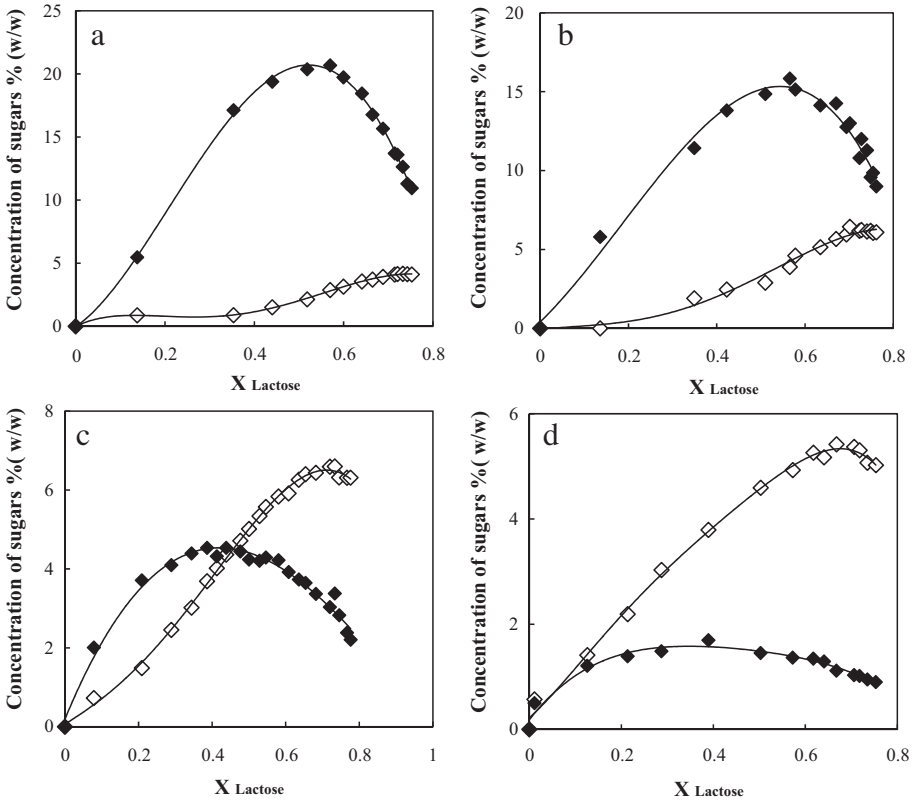

Fig. 8. Effect of lactose to fructose molar ratio on product composition in the synthesis of lactulose from lactose and fructose with *A. oryzae* β-galactosidase at 50% (w/w) total initial sugars concentration, enzyme to initial lactose ratio of 200 IU/g, 40 °C and pH 4.5 in 100 mM McIlvaine citrate–phosphate buffer. ◊, lactulose; ♦, total GOS. Lactose to fructose molar ratio (a) 2/1; (b) 1/1; (c) 1/4; (d) 1/8. Difference between duplicates was less than 5% in all cases.

may be the consequence of an increase in the viscosity of the reaction media which produces a decrease on the reaction rate due to mass transfer limitations [20]; it may also be the consequence of a decrease in the reaction rate produced by the low water activity at high sugar concentrations [26].

3.6. Effect of the lactose to fructose ratio

Fig. 8 presents the results on the effect of lactose to fructose ratio on product composition along the reaction of synthesis of lactulose. Product composition was strongly affected by this variable and, as shown in Fig. 8, there is an inverse correlation between the lactose to fructose ratio and the lactulose to total GOS ratio in the product. Lactulose/total GOS molar ratios four times higher than those reported in the four previous sections were obtained. Fig. 9 shows that the effect of lactose to fructose ratio is stronger over GOS than lactulose synthesis. When lactose to fructose ratio was higher than 1/1, synthesis of GOS was favored in detriment of lactulose. At ratios between 1/2 and 1/4 two distinct zones were observed: at low substrate conversion synthesis of GOS was still predominant, while at higher conversions lactulose synthesis outweighed GOS synthesis. At ratios below 1/6, lactulose synthesis prevailed at all substrate conversions. This is consistent with the work by Adamczak et al. [27] where they reported an increase in lactulose concentration at higher fructose to lactose ratios. However they merely increased fructose concentration while keeping lactose concentration constant, so they actually increased the total initial concentration of sugars. According to the work reported using β-galactosidase from *S. solfataricus*, high concentration of lactulose was obtained in the range of molar lactose to fructose ratio from 2/1 to 1/2, but at ratios over 4/1 and under 1/4 lactulose concentration was severely reduced [11]. Furthermore, at lower lactose to

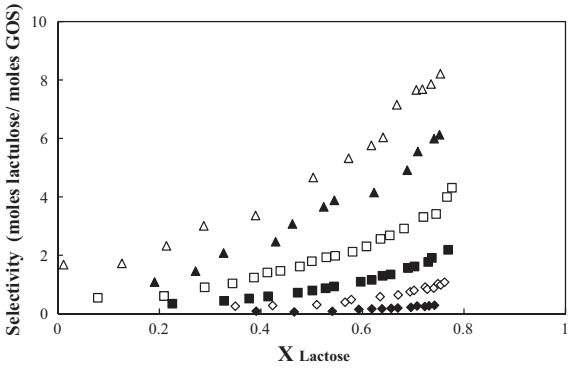

Fig. 9. Effect of lactose to fructose molar ratio on the selectivity of *A. oryzae* β-galactosidase in the synthesis of lactulose from lactose and fructose at 50% (w/w) total initial sugars concentration, enzyme to initial lactose ratio of 200 IU/g, 40 °C and pH 4.5 in 100 mM McIlvaine citrate–phosphate buffer. Lactose to fructose molar ratio ♦, 4/1; ◊, 1/1; ■, 1/2; □, 1/4; ▲, 1/6; △, 1/8. Difference between duplicates was less than 5% in all cases.

fructose ratios $\pi_{Lactulose}$ and $Y_{Lactulose}$ synthesis were increased and π_{GOS} decreased, as shown in Table 1.

Table 2 summarizes the effect of the molar ratio of lactose to fructose in $Y_{Lactulose}$ and Y_{GOS} (evaluated at the maximum concentration of lactulose). As shown, the lactulose to total GOS molar ratio can be varied within ample margins by tuning the initial lactose to fructose molar concentrations. This allows controlling the proportion of both components at will so as to obtain a mixture of oligosaccharides of an increased prebiotic effect. Preliminary results indicate that mixtures of lactulose and GOS in certain proportions have a better prebiotic index than the oligosaccharides alone.

Table 2
Effect of lactose to fructose molar ratio on the yield of lactulose ($Y_{Lactulose}$) and galacto-oligosaccharides (GOS) (Y_{GOS}) and selectivity (moles of lactulose/mole of total GOS) in the synthesis of lactulose from lactose and fructose. At 50% (w/w) total initial sugars concentration, enzyme to initial lactose of 200 IU/g, 40 °C and pH 4.5 in 100 mM McIlvaine citrate–phosphate buffer.

Lactose/fructose molar ratio	$Y_{Lactulose}$[a] (g lactulose/ g lactose)	Y_{GOS}[a] (g GOS/ g lactose)	Selectivity[a] (moles lactulose/mole total GOS
4/1	0.030	0.181	0.26
2/1	0.052	0.136	0.52
1/1	0.095	0.164	0.91
1/2	0.146	0.129	1.77
1/4	0.205	0.105	3.01
1/8	0.282	0.058	7.15

[a] At maximum concentration of lactulose on the reaction mixture.

4. Conclusions

Selectivity of the reaction of synthesis of lactulose with β-galactosidase was strongly dependent on the enzyme source, being the one from *A. oryzae* superior in terms of the lactulose to GOS production ratio, being therefore selected for further studies. The selectivity of the selected enzyme was not influenced by operational variables like pH, temperature, initial total sugars concentration and enzyme to substrate ratio within the ranges studied. However, product composition was highly dependent on the lactose to fructose initial molar ratio, being then possible to tune up the selectivity of the reaction by manipulating that variable. This may have a significant impact provided that lactulose and GOS prove to have a synergistic effect in terms of prebiotic index.

Acknowledgements

Work funded by Grant 110005 Fondecyt, Chile and performed within the framework of CYTED-D Enznut and NovelProbio networks. Project BIO2010-20508-C04-01 from Spanish Ministry of Science and Innovation also supported this research. We acknowledge the generous donations of enzymes from Enzyme Development Corporation and Novo-Nordisk.

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