

Effects of monovalent cations (Na^+ and K^+) on galacto-oligosaccharides production during lactose hydrolysis by *Kluyveromyces lactis* β -galactosidase

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The influence of cations, Na^+ and K^+ on the oligosaccharides produced in the process of transglycosylation during the hydrolysis of lactose using β -galactosidase from *Kluyveromyces lactis* has been investigated. The enzyme exhibited higher hydrolytic activity in the presence of K^+ than in the presence of Na^+ regardless of the anion present (acetate or phosphate), thus 96% of lactose was hydrolyzed after 3h of reaction. Formation of di- and trisaccharides also proceeded faster in presence of K^+ than of Na^+ , however, the highest levels were reached in presence of Na^+ . Transgalactosylation was favoured at high concentrations of sodium buffer and, on the contrary, hydrolysis of both lactose and oligosaccharides was favoured at low salt concentrations. After 48h of reaction in 1M sodium acetate buffer a yield of 39.5% of GOS was achieved (disaccharides 18.4% and trisaccharides 21.1%). The study of influence of Mg^{2+} revealed that a concentration of 1mM was necessary to obtain a 90% of hydrolysis of lactose and a 16% of disaccharides was obtained after 8h of reaction; however, a maximum yield of trisaccharides of 19% was attained using the highest concentration of Mg^{2+} (2mM) These results could be applied to improve the GOS formation by β -galactosidase from *K. lactis*.

β -galactosidase, *Kluyveromyces lactis*, lactose, oligosaccharides, sodium, potassium.

1. Introduction

Oligosaccharides are one of the largest components of human milk that can reach concentrations as high as 12 g/L (1). Human milk is the first source of oligosaccharides whose important role as prebiotic soluble fibres promoting colonization of beneficial bacteria in the large intestine of breast-fed infants is well known (2). As a consequence, formula-fed infants develop an intestinal flora significantly different from the flora of breast fed infants. Due to the limited availability of human milk, it is necessary to use alternative sources of prebiotic carbohydrates to supplement infant formulae in order to provide a beneficial prebiotic effect on the gastrointestinal tract flora of infants.

Although the hydrolysis of lactose by β -galactosidase (β -gal) is mainly used to produce low-lactose dairy products, the enzyme may also catalyze the synthesis of galactooligosaccharides (GOS) in which one or more galactose moieties are linked to the lactose or galactose by different glycosidic bonds. The structure of the GOS has some similarities to the core molecules of human milk oligosaccharides therefore GOS have gained interest in research and industrial applications (3).

GOS are mainly constituted by di-, tri-, and tetrasaccharides and eventually higher oligosaccharides. However, the distribution of the oligosaccharides formed and linkages preferentially synthesized depends on several factors including the reaction conditions and the source of the enzyme (4-6). Since the composition of the oligosaccharide mixture may affect their prebiotic properties (7), determination of factors affecting composition of synthesized GOS may be necessary for selecting appropriate experimental conditions. Transglycosylation during lactose hydrolysis may **takes** place by intermolecular and intramolecular transfer reactions (8). Intramolecular reaction

is a direct internal transfer of galactose from the 4 position to another position of the glucose moiety, without first releasing the glucose from the active site, and intermolecular means that the β -gal transfers the galactose moiety to another carbohydrate molecule present in solution. Moreover, the major β -gals from all different species are able to hydrolyze galactosyl-lactose so that, while transgalactosylation occurs early in the reaction, the oligosaccharides formed are subsequently hydrolyzed (9). As a result, the extent of transferase products formed depends on the interaction among the different enzymatic activities.

Since the first report by COHN and MONOD (10) about the influence of metal ions on β -gal activity, most studies have focused on the role of cations on lactose hydrolysis (11-14), and the studies on the transferase reactions have been performed on a limited number of β -gals (8, 15, 16). These studies have shown that β -gals are specific in their requirements so that the effect of cations on GOS production varies widely depending on the source from which the enzyme has been isolated.

The aim of the present work was to evaluate the influence of monovalent cations (Na^+ and K^+) on the oligosaccharide mixture produced by the enzymatic hydrolysis of lactose using commercial β -gal from *Kluyveromyces lactis* (Lactozym 3000 L HP G).

2. Material and methods

2.1 Enzyme characterization

β -Gal activity of Lactozym was measured following the method reported by MARTINEZ-VILLALUENGA, *et al.* (5).

2.2 Enzymatic synthesis of GOS

Batch assays for GOS production were carried out at 40 °C using reaction mixtures composed of lactose (250 mg/mL) and Lactozym (9 U/mL) at pH 6.5 in different buffers depending on the study. To evaluate the influence of monovalent cations (Na^+ , K^+) on the enzymatic activities, phosphate and acetate buffers (0.05 M containing 1 mM MgCl_2) were prepared. To study the influence of concentration of sodium acetate on hydrolytic and transgalactosidase activity of Lactozym, different concentrations (0.05, 0.5 and 1 M) were assayed. The potassium-sodium interaction was evaluated using 0.05 M potassium phosphate buffer containing 1 mM MgCl_2 and different amounts of sodium acetate (0.1, 0.2 and 0.5 M). To evaluate the effect of cation Mg^{2+} concentration on the enzymatic activities of β -gal, different assays were carried out using 0, 0.2, 1 and 2 mM Mg^{2+} in 0.05 M sodium phosphate. Reactions were performed in duplicate and withdrawn at different times between 1 and 48 h.

2.3 Gas chromatographic (GC) analysis

Synthesized GOS were analyzed as trimethylsilylated oximes by GC-FID following the method of CARDELLE-COBAS *et al.* (17).

3. Results

Transgalactosylation of lactose using Lactozym resulted in formation of di- and trisaccharides which were previously characterized by GC-MS (18).

3.1 Influence of cation and anion type

The effect of the nature of cations, K^+ and Na^+ , as well as anions, phosphate and acetate, on the hydrolytic and transglycosylation activities of Lactozym using 0.05 M buffer concentration is shown in Fig. 1. The enzyme exhibited higher hydrolytic activity in the presence of K^+ than in the presence of Na^+ regardless of the type of anion present, thus a 96% of lactose was hydrolyzed after 3 h of reaction in presence of K^+ while only a 56% of hydrolysis was achieved when sodium buffers were used (Fig. 1a). COHN and MONOD (10) also found a high hydrolytic activity of β -galactosidase in presence of K^+ .

The transgalactosidase activity of Lactozym was also affected differently by these cations. Formation of disaccharides proceeded faster in presence of K^+ , regardless of the anion assayed (phosphate or acetate) reaching the maximum value of 15.8% of total sugars after 3 h of reaction when 96% of lactose was hydrolyzed and then decrease rapidly up to values near 10%. In presence of Na^+ the formation of disaccharides was much slower but after 24 h a yield of 18% was obtained (Fig. 1b).

Similar effects were observed on the formation of trisaccharides, thus a maximum value of 11.1% of the total sugar was achieved in presence of K^+ (Fig. 1c) after 2 h of time course reaction when about 90% of lactose was hydrolyzed and then trisaccharide content rapidly decreased to reach values near to the 5% of the total sugar; at this time lactose was completely hydrolyzed. However, in presence of Na^+ , the maximum amount of trisaccharides produced was 17.1% of the total sugar when about 85% of lactose was hydrolyzed. For longer reaction times, these carbohydrates decreased slowly up to 14.0% of total sugar.

Fig. 2 shows the hydrolysis of lactose and formation of di- and trisaccharides in 0.05 M potassium phosphate buffers added with sodium acetate at three concentrations 0.1, 0.2 and 0.5 M. Increasing concentrations of Na^+ resulted in a decrease of both hydrolytic and transgalactosidase activities (Fig. 2a); however the formation of di- and trisaccharides (Fig. 2 b and c, respectively) reached higher values as the concentration of Na^+ increased. Maximum values of GOS were reached with sodium phosphate buffer in absence of K^+ after 24 h of reaction.

3.2 Influence of buffer concentration

Fig. 3 shows the effect of sodium acetate concentration on hydrolysis and transglycosylation of lactose. As it can be observed in Fig. 3a, hydrolysis of lactose was hardly affected by the buffer concentration and only a small decrease in the rate at the highest concentration studied (1 M) was observed. After 24 h of reaction, the remaining lactose was 10% at 1 M sodium acetate buffer concentration and values close to 5% were obtained at 0.5 M and 0.05 M buffer concentrations.

The formation of disaccharides (Fig. 3b), was similar at 0.05 and 0.5 M buffer concentrations and lower at 1 M whereas trisaccharides (Fig. 3c), were formed at similar rate at 0.5 and 1 M being lower at 0.05 M. The main differences due to buffer concentration were observed when only 10-20% of initial lactose remained in solution. Under these conditions, hydrolysis

of trisaccharides increased as buffer concentration decreased. Moreover, the hydrolysis of the oligosaccharides formed was hampered at the highest tested buffer concentrations.

Consistent with the above, the relationship glucose/galactose (Glc/Ga) (Fig. 3d) reveals higher values with increasing concentration of the buffer. Transgalactosylation reaction is favoured at high concentrations of buffer, and on the contrary, hydrolysis of both lactose and new sugars, is favoured at low salt concentrations. Thus, after 48 h of reaction in sodium acetate buffer 1 M total GOS synthesized was 39.5% (disaccharides 18.4% and trisaccharides 21.1%).

3.3 Influence of sodium-magnesium interaction

It is well known that magnesium is a cofactor for the action of many enzymes (19-21). In order to study the effect of Mg^{2+} on the enzymatic activities of β -gal, different experiments at Mg^{2+} concentrations ranging from 0 to 2 mM in 0.05 M sodium phosphate were performed (Fig. 4). As it can be observed, the presence of Mg^{2+} ~~magnesium~~ in sodium buffer is important for the action of the β -gal, since in absence of Mg^{2+} only 12% of lactose was hydrolyzed after 24 h and the reaction did not progress further (Fig. 4a). When adding increasing concentrations of Mg^{2+} the hydrolysis of lactose was accelerated. The maximum rate of hydrolysis and disaccharide formation was reached at 1 mM Mg^{2+} concentration (Fig. 4b). The formation of trisaccharides (Fig. 4c) showed a somewhat different behaviour since the maximum rate and yield (19.4%) were reached after 8 h of reaction with a 2 mM Mg^{2+} .

The effect of different cations depends strongly on the origin of the enzyme; HILL and HUBER (13), observed that Na^+ was an activator cation of β -gal from *E. coli*, while Mg^{2+} was a competitive inhibitor. GARMAN *et al.* (9), found an enhancement of activity of the β -gal from some species of lactic acid bacteria when Mg^{2+} was present in buffer of reaction.

Unlike these results, in the reaction carried out in potassium buffer the presence of magnesium was not essential for the enzymatic activities (data not shown).

4. Conclusions

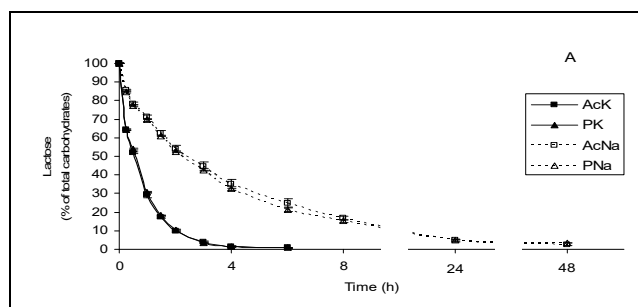
In this work, a study of influence of cations on hydrolytic and transgalactosylase activity of β -galactosidase from *K. lactis* (Lactozym) is presented. The results show that the formation of di- and trisaccharides in the presence of Na^+ is higher than in the presence of K^+ . Under the assayed conditions, using potassium buffer is possible to reach a GOS production of up to 25.1%, whereas in sodium buffer a yield of 39.5% of total sugars was attained. The presence of Na^+ favours both intra- and intermolecular transglycosylation and inhibits the hydrolysis activity of this β -gal, however it must be considered that Mg^{2+} is necessary to the enzyme will be active in presence of sodium buffers. Therefore, present results could be applied to improve the GOS formation by Lactozym).

Acknowledgments

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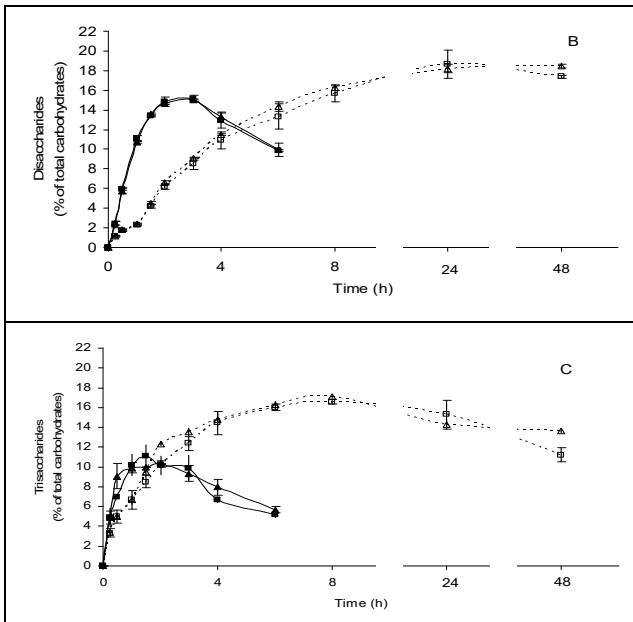
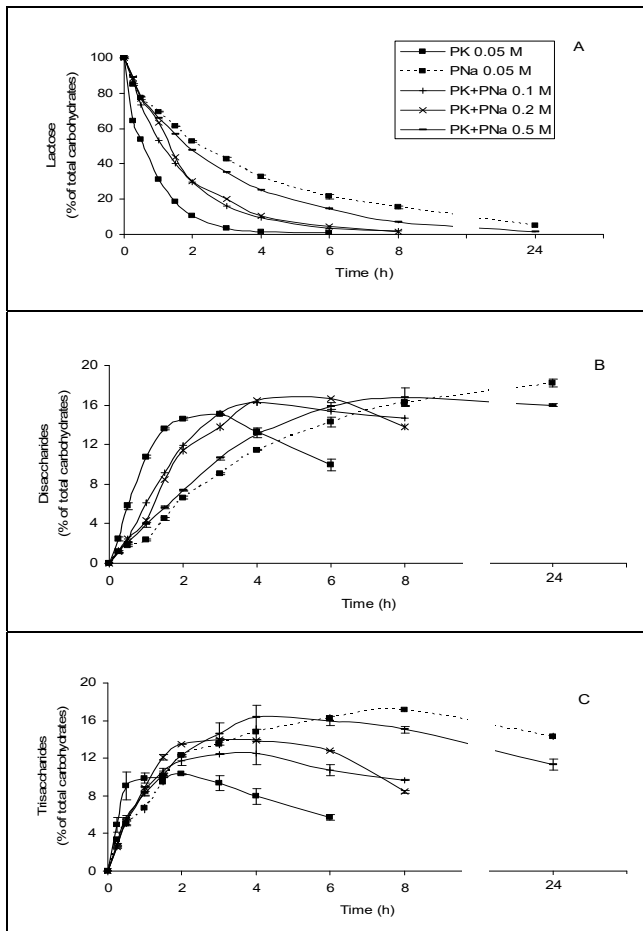


Fig. 1: Hydrolysis of lactose (A); and di- (B) and trisaccharide formation (C) in reaction mixtures (40 °C) containing 250 mg/mL lactose and 9 U/mL of β -galactosidase from *K. lactis* in 0.05 M potassium acetate or phosphate, and sodium acetate or phosphate buffers, pH 6.5. AcK: Potassium Acetate; PK: Potassium Phosphate; AcNa: Sodium Acetate; PNa: Sodium Phosphate.



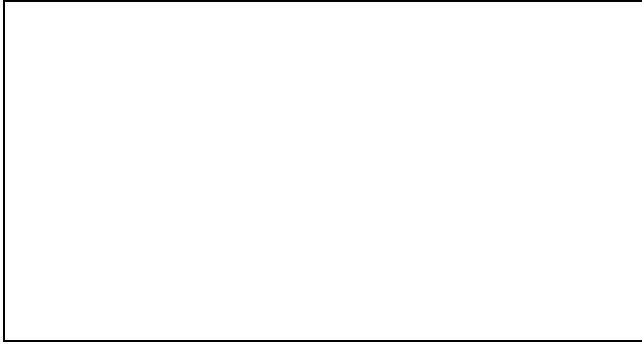


Fig. 2. Hydrolysis of lactose **(A)**; and di- **(B)** and trisaccharide formation **(C)** in reaction mixtures (40 °C) containing 250 mg/mL lactose and 9 U/mL of β -galactosidase from *K. lactis* in 0.05 M sodium phosphate; potassium phosphate (pH 6.5) with 0, 0.1, 0.2 and 0.5 M of Na^+ . PK: Potassium Phosphate; PNa: Sodium Phosphate.

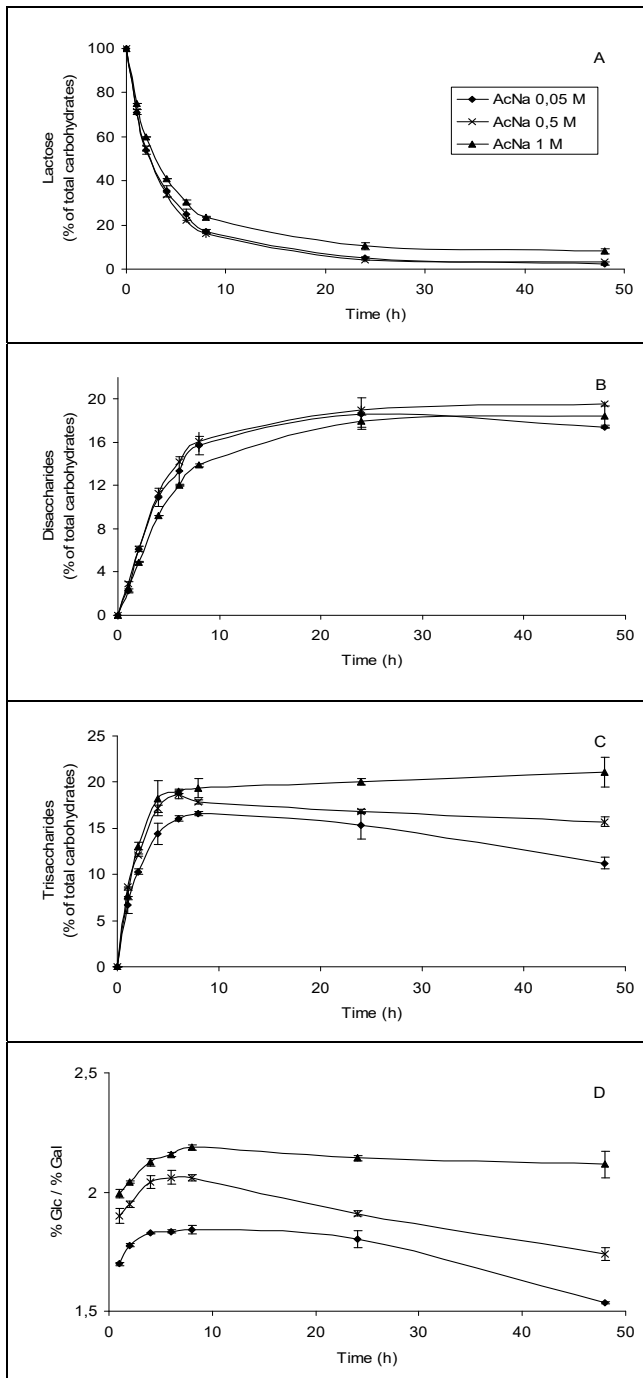


Fig. 3. Influence of buffer concentration (pH 6.5), sodium acetate 0.05, 0.5 and 1 M, on hydrolysis of lactose (A); di- (B) and trisaccharide formation (C) and glucose/galactose ratio (D) in reaction mixtures (40 °C) containing 250 mg/mL lactose and 9 U/mL of β -galactosidase from *K. lactis*.

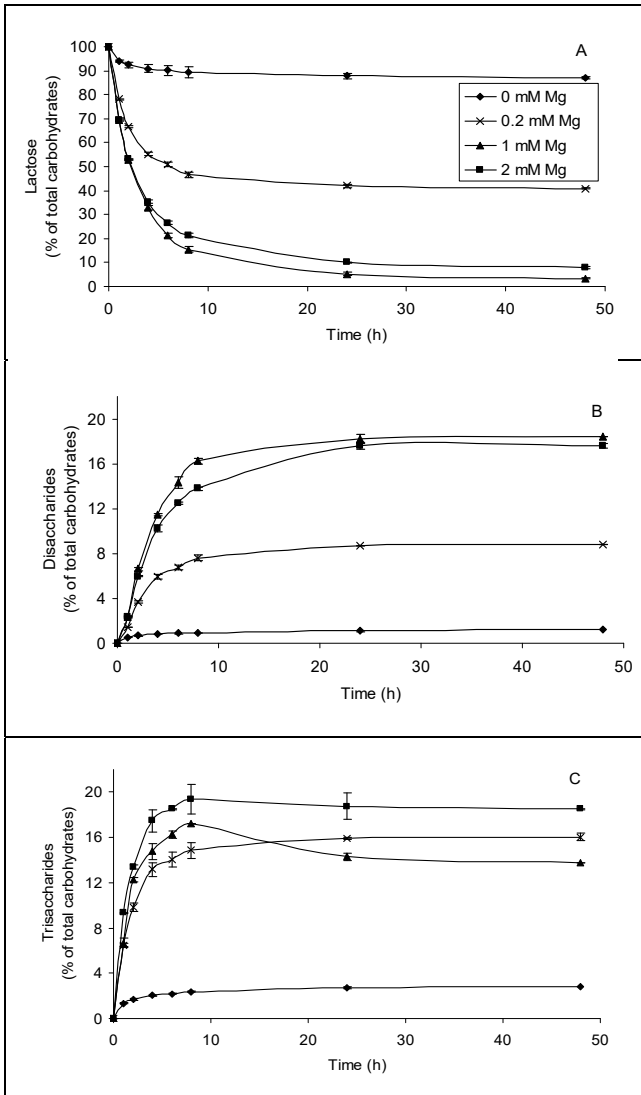


Fig. 4. Influence of Mg^{2+} concentration (0, 0.2, 1 and 2 mM) on hydrolysis of lactose (A); and di- (B) and trisaccharide formation (C) in reaction mixtures (40 °C) containing 250 mg/mL lactose and 9 U/mL of β -galactosidase from *K. lactis* in 0.05 M sodium phosphate (pH 6.5).