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# Removal of lactose in crude galacto-oligosaccharides by β-galactosidase from *Kluyveromyces lactis*

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ABSTRACT

In order to remove the residual lactose in crude galacto-oligosaccharides (GOS), different commercial soluble β-galactosidases from *Kluyveromyces lactis* (Lactozym Pure 6500L, Maxilact L2000, Lactase NL and Biolactasa-NL) and reaction conditions (temperature, total carbohydrate concentration and enzyme:substrate mass ratio) were evaluated. To select the best biocatalyst, the hydrolytic activity on *o*-NPG and thermal stability of all enzymes were evaluated in the absence and presence of three cations ($Co^{2+}$, $Mg^{2+}$, $Mn^{2+}$) at different concentrations. The enzyme source, cation and cation concentration were selected to obtain the highest hydrolytic activity and thermal stability. Then lactose hydrolysis of raw GOS was assessed varying the temperature (30 °C–45 °C), total carbohydrate concentration (10%-50%) and enzyme:substrate mass ratio (50 IU $g^{-1}$–400 IU $g^{-1}$) and considering the lactose percentage decrease as response parameter ($D_L$). Lactase NL was selected as the best enzyme, with a hydrolytic activity of 286 IU $mg^{-1}$ and a half-life of 9 h at 35 °C in the presence of 1 mM $Mn^{2+}$. The best reaction conditions for lactose hydrolysis employing the selected enzyme were 35 °C, 50% initial carbohydrate concentration and 135 IU $g^{-1}$. At such conditions of lactose hydrolysis, 70% reduction of lactose in raw GOS was obtained, with an increase of 48% in monosaccharides and of 30% in GOS. This pre-hydrolytic step is a key aspect for the subsequent purification of GOS by nanofiltration or selected bioconversion, in which monosaccharides can be removed efficiently producing GOS of high purity.



## 1. Introduction

GOS are one of the few non-digestible oligosaccharides that are properly considered as prebiotics [1], and are increasingly being used as health-promoting factors in functional foods [2–5]. The β-galactosidases from *Aspergillus oryzae* and *Bacillus circulans* are typically used for transgalactosylation reactions because of their favorable balance between transgalactosylation and hydrolytic activities [6,7]. However, the enzymatic synthesis of GOS is a kinetically-controlled reaction where GOS yields rarely exceed 50% [7]; for that reason, a significant amount of unreacted lactose and monosaccharides (glucose and galactose) remains at the end of reaction [8]. This residual lactose limits the use of GOS in the elaboration of foods for lactose intolerants, whereas glucose and galactose increase the caloric value and decrease the prebiotic potential of the product [9]. Purification of raw GOS is then a requirement for most of their applications and becomes a critical issue in terms of production cost [10].

The most employed strategy for GOS purification is chromatography, but this is an expensive and difficult to scale-up operation [11], so that other alternatives have been proposed, such as yeast bioconversion and nanofiltration [9,12,13]. Both are rather simple and scalable operations that are highly effective in the removal of monosaccharides, but not lactose, from raw GOS [14]. The incorporation of a lactose hydrolysis step, after GOS synthesis, would eliminate most of the remaining lactose in crude GOS, thus favoring the subsequent removal of monosaccharides by either nanofiltration or bioconversion, yielding a GOS of high purity. β-Galactosidases from *Kluyveromyces* species have a high hydrolytic activity and have been successfully used in the dairy industry for lactose hydrolysis in the formulation of lactose-free foods [15–17].

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The purpose of this work was the evaluation of a lactose hydrolysis step with β-galactosidase from *Kluyveromyces lactis* as a pretreatment before the subsequent purification of raw GOS. The goal was to achieve a substantial reduction of the lactose content in the product without diminishing the amount of total GOS thus favoring the subsequent removal of monosaccharides. In a previous work, analyzing the lactose removal in milk [18], it was observed that the enzyme from *K. lactis* was able to synthesize GOS (up to 7.0 g/L) during the typical production of lactose-free milk. Interestingly, the *K. lactis* β-galactosidase displayed a low tendency to hydrolyze the formed GOS, which occurred only at high lactose conversion. In other words, *K. lactis* β-galactosidase exhibits a notable preference to hydrolyze lactose compared with GOS products. This could be related with the fact that this enzyme synthesizes mostly GOS with β(1 → 6) linkages, which are more resistant to enzymatic hydrolysis than β(1 → 4) bonds. Based on these findings, β-galactosidase preparations from *K. lactis* were selected to assess the reduction of the lactose content in the raw GOS without significantly altering the amount of total GOS.

## 2. Experimental procedures

### 2.1. Materials

β-Galactosidase from *A. oryzae* (Enzeco® fungal lactase concentrate) was kindly donated by Enzyme Development Corporation, New York, USA. The enzyme had a specific activity of 196,000 $IU_H\,g^{-1}$, where one international unit of hydrolytic activity ($IU_H$) is equivalent to the amount of enzyme hydrolyzing 1 μmole of *ortho*-nitrophenyl-β-galactoside (*o*-NPG) per minute at pH 4.5, 40 °C and 30 mM *o*-NPG. β-Galactosidase from *K. lactis* (Lactozym Pure 6500L, with 146 $IU_H\,mL^{-1}$) was a gift from Novozymes Latin America Ltda. (Araucária, Brazil); β-galactosidase from *K. lactis* (Maxilact L2000, with 240 $IU_H{\cdot}mL^{-1}$) was kindly donated by DSM Food Specialties (The Netherlands); β-galactosidase from K. *lactis* (Lactase NL, with 783 $IU_H\,mL^{-1}$) was a gift from Enzyme Development Corporation (New York, USA); β-galactosidase from *K. lactis* (Biolactasa-NL, with 573 $IU_H\,mL^{-1}$), was supplied by Biocon (Barcelona, Spain). One international unit of activity ($IU_H$) for all β-galactosidases from *K. lactis* was defined as the amount of enzyme hydrolyzing 1 μmole of *o*-NPG per minute at pH 6.5, 40 °C, 1.6 mM $MgCl_2$ and 30 mM *o*-NPG. Glucose, galactose, NaOH, *ortho*-nitrophenol (*o*-NP) and *o*-NPG were purchased from Sigma-Aldrich, USA. Food-grade lactose was purchased from Milkaut S.A, Argentina. Manganese chloride hydrate, potassium phosphate dibasic and monobasic were purchased from Merck, Germany. Citric acid, sodium hydroxide, magnesium chloride hydrate and cobalt chloride hydrate were purchased from Lobachemie, India.

### 2.2. Analyses

Protein was determined according to Bradford method [19]. Enzyme hydrolytic activity was determined on *o*-NPG as artificial substrate, following *o*-NP release at 405 nm and 40 °C. Enzyme hydrolytic activity of all *K. lactis* β-galactosidases was measured in $IU_H$, 1 $IU_H$ being defined as the amount of enzyme producing 1 μmole of *o*-NP per minute at pH 6.5 and 40 °C using 45 mM *o*-NPG as substrate. Thermal stability was determined by incubating 5 $mg\,mL^{-1}$ of enzyme in 100 mM citrate phosphate buffer pH 6.5 at 35 °C; aliquots were taken at different times and the remaining activity was determined after convenient dilution of the sample. The thermal stability is expressed in terms of the enzyme inactivation rate constants ($k_D$) and the haft-life time ($t_{1/2}$), calculated using a one-stage first-order enzyme thermal inactivation model. Substrate and product analyses were carried out using two systems: one HPLC delivery system equipped with refractive index detector that allows determining glucose, galactose, disaccharide and oligosaccharides, and one high-performance anion-exchange chromatographic system with pulsed amperometric detector (HPAEC-PAD) that allows a more precise determination of the product species including the type of linkage between sugar units. HPLC analysis with refractive index detector used a Inertsil NH2, −5 μ column for carbohydrate analysis (GL Sciences Inc., Japan) and acetonitrile/Mili Q water 60% v/v at a flow of 1 $mL\,min^{-1}$ as eluent. Both, the chromatographic column and detector were maintained at 40 °C. To determine retention times and linear measuring range, glucose, lactose and raffinose were employed as standards. The chromatograms were integrated using the software Chrom Jasco Pass. HPAEC-PAD analysis was carried out according to Rodriguez-Colinas et al. [20] with small modifications, in a ICS3000 Dionex system consisting of a SP gradient pump, an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode, and an autosampler (model AS-HV). All eluents were degassed by flushing with helium. A pellicular anion-exchange 4 × 250 mm Carbo-Pack PA-1 column (Dionex) connected to a 4 × 50 CarboPac PA-1 guard column (Dionex Corp, Sunnyvale, Canada) was used at 30 °C. Eluent preparation was performed with Milli-Q water and 50% (w/v) NaOH. The initial mobile phase was 15 mM NaOH applied at 1.0 $mL\,min^{-1}$ for 28 min. A gradient from 15 to 200 mM NaOH was performed in 7 min at 1.0 $mL\,min^{-1}$, and 200 mM NaOH was maintained for 25 min. A gradient from 200 to 15 mM NaOH was performed in 7 min at 1.0 $mL\,min^{-1}$, and 15 mM NaOH was maintained for 9 min. The peaks were analyzed using Chromeleon software. Identification of the different carbohydrates was done on the basis of commercially available standards or previously purified GOS according to the laboratory protocol developed by Rodriguez-Colinas et al. [21,22].

### 2.3. GOS synthesis

Enzymatic synthesis of GOS was carried out according to Vera et al. [23], employing *A. oryzae* β-galactosidase at 100 $IU_H\,g^{-1}$ lactose, 50 °C, pH 4.5 and 50% w/w lactose concentration; reaction was conducted for 2 h. Substrate and product analyses were carried out by HPAEC-PAD chromatography.

### 2.4. Selection of enzyme source and cation based on activity and thermal stability under non-reactive conditions

The enzyme source was selected from four commercial preparations of soluble β-galactosidase from *K. lactis* (Lactozym Pure 6500L, Maxilact L2000, Lactase NL and Biolactasa-NL), evaluating their hydrolytic activity on *o*-NPG and thermal stability under non-reactive conditions (buffer plus cofactor). Since several authors have reported that the ionic environment has an important effect on β-galactosidase hydrolytic activity and stability at non-reactive condition [24], different cations were evaluated. $Mg^{2+}$ has been used with most β-galactosidases based on the fact that it has been proved to be a cofactor of *Escherichia coli* β-galactosidase [25–27]. The effect of ions on *K. lactis* β-galactosidase has been reported in a few cases and agreement exists that $Co^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ as reducing agents increase the enzyme activity or protect it from thermal deactivation [28–31]. However, it is still unclear which is the best cation and its optimum concentration for *K. lactis* β-galactosidase activity and stability, so four commercial preparations of β-galactosidase from *K. lactis* were characterized in terms of hydrolytic activity and thermal stability using $Co^{2+}$, $Mg^{2+}$, $Mn^{2+}$ as cations in a concentration range from 0 to 1 mM. Cation, cation concentration and β-galactosidase were selected on the basis of hydrolytic activity and thermal stability determined as described above. Once the best cation and cation concentration