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Optimisation of synthesis of oligosaccharides derived from lactulose (fructosyl-galacto-oligosaccharides) with β-galactosidases of different origin

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ABSTRACT

Batch synthesis of fructosyl-galacto-oligosaccharides from lactulose was performed with commercial β-galactosidase preparations from *Aspergillus oryzae*, *Kluyveromyces lactis* and *Bacillus circulans*. The enzyme from *A. oryzae* produced the highest yield and specific productivity of synthesis, being selected for further studies. Optimization of fructosyl-galacto-oligosaccharides synthesis was carried out using response surface methodology, considering temperature and initial sugar concentration as variables and yield and specific productivity as response parameters. Maximum yield of 0.41 g g^{-1} fructosyl-galacto-oligosaccharides was obtained at 70 °C and 60% w/w lactulose concentration, while maximum specific productivity of 1.2 g h^{-1} mg^{-1} was obtained at 70 °C and 40% w/w lactulose concentration.

1. Introduction

Prebiotics are increasingly being considered as health-promoting food components (Wang, 2009). Most prebiotics are non-digestible oligosaccharides (NDO) and, among them, galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), inulin and lactulose have been conclusively proven to exert prebiotic effects (Rycroft, Jones, Gibson, & Rastall, 2001), while other NDO are considered as health-promoting agents complying with some, but not all, of the requirements to be considered as prebiotics (Gänzle, Haase, & Jelen, 2008; Klewicki, 2007; Li et al., 2009). Prebiotic effects on the colonic microbiota depend on the chemical structure of the oligosaccharides (number and type of monomers; type, position and conformation of the glycosidic linkages) (Hernández-Hernández, Montañes, Clemente, Moreno, & Sanz, 2011; Martínez-Villaluenga et al., 2008; Olano & Corzo, 2009). Therefore, evaluating new NDO structures is an open field of research as some of them may lead to better prebiotics (Cardelle-Cobas et al., 2011).

β-Galactosidases are important biocatalysts for industry, traditionally used for their hydrolytic activity to reduce the lactose content in foods and process wastewaters (Illanes, 2011; Tuure & Korpela, 2004), and, more recently, as catalysts for transgalactosylation reactions leading to the synthesis of GOS, lactulose and lactosucrose (Albayrak & Yang, 2002; Guerrero, Vera, Plou, & Illanes, 2011; Kim, Park, & Oh, 2006; Lee, Kim, & Oh, 2004; Li et al., 2009).

The mechanism of the reaction catalyzed by β-galactosidase was described more than 50 years ago as a transglycosylation reaction in which the enzyme catalyzes the transfer of a galactose moiety in a non reducing β-galactoside (donor) to an acceptor containing a hydroxyl group (Prenosil, Stuker, & Bourne, 1987). However, the potential technological value of such a reaction began to be explored more than three decades later for the synthesis of transgalactosylated oligosaccharides, once these compounds acquired interest as potential prebiotics. Among them, GOS and lactulose stand out for their scientifically proven prebiotic condition. GOS are composed of a variable number of galactose units (usually from two to ten) and a terminal glucose unit, mostly β-(1 → 4)- and β-(1 → 6)- linked; lactulose is a disaccharide (4-O-β-D-galactopyranosyl-D-fructose). In the synthesis of GOS, lactose plays both the role of donor and acceptor of the galactosyl residue, so forming trisaccharides which in turn can act as acceptors forming tetrasaccharides and so on (Albayrak & Yang, 2002; Vera, Guerrero, & Illanes, 2011). In the enzymatic synthesis of lactulose, lactose is the galactose donor, fructose acting as acceptor, but since lactose can also act as acceptor, a mixture of lactulose and GOS will be produced (Guerrero et al., 2011; Kim et al., 2006; Lee et al., 2004).

The synthesis of GOS and lactulose is strongly determined by the origin of the β-galactosidase (Guerrero et al., 2011; Kim et al., 2006; Lee et al., 2004; Sanz-Valero, 2009), so that product composition, yield and specific productivity will vary accordingly. Most studies have been focused in increasing yield that, as said above, is strongly dependent on the enzyme source, since it results from

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the balance between transgalactosylation and hydrolytic activities (Bucke, 1996; Kasche, 1986). Another key variable is the initial lactose concentration; high concentrations favoring synthesis over hydrolysis so increasing yield (Albayrak & Yang, 2002; Guerrero et al., 2011; Sanz-Valero, 2009). Maximum allowable concentration of lactose is determined by its solubility, which is strongly dependent on temperature and so this also becomes a key variable (Vera, Guerrero, Conejeros, & Illanes, 2012). Temperature also influences the ratio of transglycosylation to hydrolysis rates (Gosling, Stevens, Barber, Kentish, & Gras, 2010). Other operational variables, such as pH and enzyme concentration, have been shown to have little influence on GOS and lactulose yields, though they affect volumetric productivity (Guerrero et al., 2011; Vera et al., 2011a). Specific productivity of GOS and lactulose synthesis, though being pH and temperature dependent (these variables affect transgalactosylation and hydrolysis rates differently), is not affected by the enzyme to substrate ratio (Bucke, 1996; Guerrero et al., 2011; Kasche, 1986; Vera, Guerrero, Illanes, & Conejeros, 2011).

It has been recently demonstrated that β-galactosidase can accept lactulose as donor and acceptor of transgalactosylated galactose, leading to the synthesis of galacto-oligosaccharides derived from lactulose (Martínez-Villaluenga et al., 2008). These oligosaccharides represent a new type of compounds that are likely to have improved prebiotic activity compared to GOS and lactulose (Cardelle-Cobas et al., 2011; Martínez-Villaluenga et al., 2008; Olano & Corzo, 2009). Some authors (Cardelle-Cobas, Martinez-Villaluenga, Villamiel, Olano, & Corzo, 2008), have suggested that the definition of GOS should be extended to include this new type of NDO that differs from them only in the terminal sugar moiety (fructose instead of glucose or galactose), and have described them as fructosyl-galactooligosaccharides (fGOS).

The synthesis of fGOS with β-galactosidases is a little-explored field, in which the effect of key variables on yield and specific productivity of synthesis has not been clearly established and whose prebiotic effect is still to be scientifically confirmed (Cardelle-Cobas et al., 2011).

The mechanism proposed for the synthesis of fGOS holds that one molecule of lactulose binds to the enzyme while one molecule of fructose is released, the galactosyl-enzyme complex so formed reacts with another molecule of lactulose leading to the formation of the trisaccharide (fGOS-3), which in turn can also act as acceptor of the galactosyl-enzyme complex leading to fGOS-4 and so on, extending the length of the oligosaccharide chain. Released galactose, as a consequence of lactulose hydrolysis, usually acts as a competitive inhibitor of β-galactosidases (Jurado, Camacho, Luzón, & Vicaria, 2002), which stems from the fact that the galactose moiety is the one recognized by the enzyme.

Since the origin of the enzyme is one of the most important variables in the synthesis of transgalactosylated oligosaccharides, three different sources of β-galactosidases, namely *Aspergillus oryzae*, *Kluyveromyces lactis* and *Bacillus circulans* were evaluated as catalysts for the synthesis of fGOS. All of them are readily available commercial preparations previously used as catalysts for the synthesis of GOS. In this way, product distribution, substrate into product yield (Y_{fGOS}) and specific productivity (π_{fGOS}) of fGOS synthesis can be compared and, based on the results obtained, the best enzyme preparation may be selected for the optimization of fGOS synthesis. The selected enzyme may be characterized in terms of the effect of lactulose and fructose on its hydrolytic activity, determining the Michaelis constant for lactulose and possible inhibition constant by fructose; additionally, the effect of galactose and fructose on the reaction of transgalactosylation can be determined so as to assess their impact on the synthesis of fGOS. The synthesis of fGOS should be able to be optimized using response surface methodology, considering temperature and initial concentration of lactulose as variables and Y_{fGOS} and π_{fGOS} as evaluation parameters.

2. Materials and methods

2.1. Materials

Lactulose (4-*O*-β-D-galactoypyranosyl-D-fructose) was provided by Discovery Fine Chemicals (Wimborne, UK); *o*-nitrophenol (*o*-NP), *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPG) and GOS standards (4β-galactobiose and 3α-4β-3α galactotetraose) were supplied by Sigma (St. Louis, MO, USA. D-(+)-galactose, D-fructose and all other reagents were analytical grade and provided either by Sigma or Merck (Darmstadt, Germany).

Three commercial β-galactosidases from different origin were tested. A commercial β-galactosidase preparation of *A. oryzae* (AOG), marketed under the trade name Enzeco® Fungal Lactase Concentrate, was kindly donated by Enzyme Development Corporation (New York, USA). The enzyme preparation had a specific activity of 196,000 IU g^{-1}, where one international unit of activity (IU_H) is equivalent to the amount of enzyme hydrolyzing 1 μmole of *o*-NPG per minute at pH 4.5, 40 °C and 30 mmol L^{-1} *o*-NPG. β-Galactosidase from *K. lactis* (KLG) traded as Lactozym Pure 6500 L was kindly supplied by Novozymes Latin America S.A. (Araucária, Brazil); the enzyme had a specific activity of 4836.3 IU g^{-1}, where one international unit of activity (IU_H) is equivalent to the amount of enzyme hydrolyzing 1 μmole of *o*-NPG per minute at pH 6.5, 40 °C, 1.6 mmol L^{-1} $MgCl_2$ and 30 mmol L^{-1} *o*-NPG. β-Galactosidase from *B. circulans* (BCG) traded under the name Biolactasa-NTL was a product from Biocon (Barcelona, Spain); the enzyme had a specific activity of 3182.7 IU g^{-1}, where one international unit of activity (IU_H) is equivalent to the amount of enzyme hydrolyzing 1 μmole of *o*-NPG per minute at pH 6, 40 °C and 30 mmol L^{-1} *o*-NPG. The enzymes were stored at 4 °C and remained fully active throughout the work.

2.2. HPLC analysis of the reaction products

Substrates and products of synthesis were analyzed in a Jasco RI 2031 HPLC machine, provided with a refractive index detector, an isocratic pump (Jasco PU2080) and autosampler (Jasco AS 2055), using BP-100 Ca^{++} columns (300 × 7.8 mm) for carbohydrate analysis (Benson Polymerics, Reno, USA). Samples were eluted with mili-Q water at a flow-rate of 0.5 mL min^{-1}. Column and detector temperatures were 80 and 40 °C respectively. Chromatograms were integrated using the software ChromPass. Composition of samples was determined by assuming that the area of each peak is proportional to the weight percentage of the respective sugar. The accuracy of this assumption was checked by a material balance according to Boon, Janssen, and van der Padt (1999). Standards of galactose, fructose, lactulose, 4β-galactobiose and 3α-4β-3α galactotetraose were used to determine their retention times and check the linear range of the measurements.

2.3. Selection of the enzyme

Syntheses of fGOS were conducted at 40% w/w lactulose, 40 °C, enzyme to substrate ratio (E/S) of 200 IU_H g^{-1} and at the optimum pH for each enzyme (4.5 for AOG, 6 for BCG and 6.5 for KLG). In the case of KLG, $MgCl_2$ was added as cofactor at 1.6 mmol L^{-1} concentration. Reactions were carried out in 100 mL Erlenmeyer flasks by dissolving 40 g of lactulose into 50 g of 100 mmol L^{-1} McIlvaine citrate–phosphate buffer at the corresponding pH. Substrate was dissolved by heating the solution at a temperature of 95 °C (no degradation of sugars was detected by HPLC analysis) and then, after cooling to the reaction temperature, 10 g of a properly diluted enzyme solution was added to start the reaction. During synthesis, 0.5 mL samples were taken at regular intervals and the reaction