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Synthesis of galacto-oligosaccharides by β-galactosidase from *Aspergillus oryzae* using partially dissolved and supersaturated solution of lactose

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ABSTRACT

The effect of enzyme to substrate ratio, initial lactose concentration and temperature has been studied for the kinetically controlled reaction of lactose transgalactosylation with *Aspergillus oryzae* β-galactosidase, to produce prebiotic galacto-oligosaccharides (GOS). Enzyme to substrate ratio had no significant effect on maximum yield and specific productivity. Galacto-oligosaccharide syntheses at very high lactose concentrations (40, 50 and 60%, w/w, lactose monohydrate) were evaluated at different temperatures (40, 47.5 and 55 °C). Within these ranges, lactose could be found as a supersaturated solution or a heterogeneous system with precipitated lactose, resulting in significant effect on GOS synthesis. An increase in initial lactose concentration produced a slight increase in maximum yield as long as lactose remained dissolved. Increase in temperature produced a slight decrease in maximum yield and an increase in specific productivity when supersaturation of lactose occurred during reaction. Highest yield of 29 g GOS/100 g lactose added was obtained at a lactose monohydrate initial concentration of 50% (w/w) and 47.5 °C. Highest specific productivity of 0.38 g GOS h^{-1} mg enzyme^{-1} was obtained at lactose monohydrate initial concentration of 40% (w/w) and 55 °C, where a maximum yield of 27 g GOS/100 g lactose added was reached. This reflects the complex interplay between temperature and initial lactose concentration on the reaction of synthesis. When lactose precipitation occurred, values of yields and specific productivities lower than 22 g GOS/100 g lactose added and 0.03 g GOS h^{-1} mg enzyme^{-1} were obtained, respectively.

1. Introduction

Galacto-oligosaccharides (GOS) are non-digestible lactose derived oligosaccharides containing from one to seven galactose units linked mostly by β1-4 and β1-6 bonds and a terminal glucose residue; also galactose dimers are considered as GOS [1]. GOS are synthesized form lactose in a kinetically controlled reaction catalyzed by β-galactosidase (β-D-galactoside galactohydrolase E.C.3.2.1.23). This reaction has gained interest in recent years because GOS have consistently being proved as prebiotics [2] and also as interesting functional food ingredients [3], representing an opportunity for cheese whey upgrading. Worldwide annual whey production is estimated between 150 and 200 million tons, with an increase rate of 2% per year [4]. Traditionally, cheese producers have considered whey as a nuisance, but this vision has changed because of the upgrading potential of whey major components (lactose and whey proteins). Lactose *per se* has a reduced field of application because it is barely sweet, poorly soluble and a significant part of the world population suffers from lactose intolerance [5]. Its enzymatic conversion into GOS represents a significant added value and utilizes a GRAS status moderately priced enzyme with which food industry is well acquainted [6,7].

Enzymatic synthesis of GOS is a kinetically controlled reaction, being therefore highly dependent on the enzyme properties. The origin of the enzyme has a marked influence in substrate conversion, productivity and product distribution [8]. Even though β-galactosidases from many organisms have been isolated and tested [9], quite a few can be used in food applications, and among them the ones from *Aspergillus oryzae*, *Bacillus circulans* and *Kluyveromyces lactis* are the most suitable for GOS synthesis. The β-galactosidase from *A. oryzae* outstands because of its high specific activity, high thermal stability and low cost. However, lactose conversion into GOS is rather low [10], so that optimization of reaction conditions aimed to increase it is a key issue in this process. Most significant operational parameters are temperature, enzyme to substrate ratio, initial lactose concentration, pH and mode of reactor operation. Temperature and pH, within the ranges compatible with enzyme activity, have no significant effect on the synthesis yield and product distribution but significantly affect productivity of GOS synthesis [10–12]. The effect of enzyme to substrate ratio on yield and productivity has not been clearly established [13–16]. Lactose initial concentration, independently from the enzyme source, is a most important variable, concentrations higher than 30% (w/v) being required to favor synthesis over

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Nomenclature

E	mass of enzyme preparation added (mg)
E/S	enzyme to initial lactose ratio (IU_T/g)
GOS	mass of total GOS (g)
L	mass of lactose during reaction (g)
L_0	mass of initial lactose concentration (g)
t	time (h)
X	conversion (%)
Y	yield (g GOS/100 g lactose added)
π	Specific productivity (g GOS mg enz^{-1} h^{-1})

hydrolysis so increasing yield [8]. Lactose solubility is rather low at temperatures compatible with enzymatic catalysis, being 250 g l^{-1} at 40 °C [17]. However, supersaturated solutions of lactose can be easily obtained in a metastable condition [18] enabling to obtain higher yield during the synthesis of GOS. Despite this, lactose solutions are unstable over 2.1 times saturation concentration [18], being the synthesis of GOS barely characterized under these conditions. Several modes of reactor operation have been studied for GOS synthesis: packed bed and stirred tank reactors operated continuously, batch, recycle batch and membrane reactors among others. Continuous stirred tank reactors are the less adequate and lower yields are obtained at the same substrate conversion than in batch or continuous plug flow reactors [19–21] where yields are alike [15,16,19–21], which is to be expected from kinetic considerations.

The purpose of this work is the determination of the effect of enzyme to substrate ratio, very high lactose concentration (over 40%, w/w) and temperature in the yield and specific productivity of the synthesis of GOS in batch mode with β-galactosidase from *A. oryzae*.

2. Materials and methods

2.1. Materials

β-Galactosidase from *A. oryzae* (Enzeco® Fungal Lactase) was kindly donated by Enzyme Development Corporation, EDC (New York, USA). The enzyme had a pH optimum between 4.5 and 5.0 and optimum temperature of 55 °C with respect to its hydrolytic activity. The enzyme was stored at 4 °C and remained fully active throughout the work. D (+) lactose monohydrate, D (+) galactose, D (+) glucose and 4β-galactobiose were supplied by Sigma. All other reagents were analytical grade and purchased from Sigma or Merck.

2.2. Analyses

Lactose and reaction products were determined using a Jasco RI 2031 HPLC equipment, with refractive index detector, isocratic pump (Jasco PU2080) and autosampler (Jasco AS 2055), using BP-100 Ag^+ column (300 mm × 7.8 mm) for carbohydrate analysis (Benson Polymerics, Reno, NV, USA). Samples were diluted and filtered through 0.22 μm Millipore Durapore membranes prior to assay by HPLC and 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. Chromatograms were integrated using the software ChromPass. The composition of the samples was determined by assuming that the area of each peak is proportional to the weight percentage of the respective sugar on the total sugars mass [22]. Standards of galactose, glucose, galactobiose and lactose were used to determine their retention times and check the linear range of the measurements. The concentrations of galacto-oligosaccharides (GOS-3, GOS-4, GOS-5 and GOS-6) in g l^{-1} were estimated using an average refractive index considering the values obtained for glucose, galactose and lactose [13,20].

2.3. Transgalactosylation activity

One international unit of β-galactosidase transgalactosylation activity (IU_T) was defined as the amount of enzyme that catalyzes the transglycosylation of 1 μmol of galactose per minute at 40% (w/w) initial lactose concentration, pH 4.5 and 40 °C. The transgalactosylated galactose was determined according to Vera et al. [11].

2.4. Effect of enzyme to initial lactose ratio on GOS synthesis

Reactions of synthesis were carried out at different enzyme to initial lactose ratios (E/S) in the range from 5 to 300 IU_T/g in 150 ml Erlenmeyer flasks magnetically stirred at 550 rpm. During this stage lactose monohydrate initial concentration and temperature were kept constant at 40% (w/w) and 40 °C, respectively. Forty grams of lactose monohydrate were dissolved in 50 g of 100 mM citrate–phosphate buffer at the corresponding pH by heating the solution at temperatures over 95 °C and then, after cooling to reaction temperature, 10 g of properly diluted enzyme solution were added to start the reaction. No lactose degradation occurred during heating. 0.5 ml samples were taken at regular intervals and reaction was stopped by adding 0.5 ml of 200 mM NaOH. Reactions were conducted for a period of time long enough to attain maximum yield (0.25–30 h). All experiments were done in triplicate, differences among replicas never exceeding 5%. The following parameters are defined:

- Conversion (X): represents the percentage of initial lactose reacted during the synthesis.

$$X = \frac{L_0 - L}{L_0} \cdot 100 \quad (1)$$

- Yield (Y): represents the mass of total GOS obtained during the synthesis per unit mass of initial lactose. For the purposes of this work it was evaluated at maximum GOS concentration.

$$Y = \frac{\text{GOS}}{L_0} \quad (2)$$

- Specific productivity (π): represents the mass of total GOS produced per unit mass of enzyme preparation added and per unit of reaction time. For the purposes of this work it was evaluated at maximum GOS concentration.

$$\pi = \frac{\text{GOS}}{E \cdot t} \quad (3)$$

2.5. Effect of initial lactose concentration and temperature on GOS synthesis

A 3^2 factorial design was used to evaluate the effect of initial lactose concentration and temperature on GOS synthesis. Lactose monohydrate initial concentrations were 40, 50 and 60% (w/w), and temperatures were 40, 47.5 and 55 °C. Experimental setup and procedure was the same as already described in Section 2.4. Syntheses were carried out at constant E/S of 100 IU_T/g considering that no significant effect of E/S on yield and specific productivity was previously determined in the experiments presented in Section 3.1. Lactose crystallization occurred during synthesis when working at 60% (w/w) at all temperatures, and at 50% (w/w) at 40 °C; in those cases, samples were filtered through a 0.45 μm membrane prior to enzyme inactivation. Reactions were conducted for 10 h, beyond the point of maximum yield. All experiments were done in triplicate; differences among replicas never exceeded 5%.

2.6. Statistical analysis

Results were analyzed using the statistical tool in *MS Excel 2007*. An ANOVA test was done to determine the statistical significance of the effects of initial lactose concentration and temperature and their interactions

3. Results and discussion

3.1. Effect of enzyme to initial lactose ratio on GOS synthesis

Results obtained with *A. oryzae* β-galactosidase on the effect of enzyme to initial lactose ratio (E/S) on GOS yield and specific productivity, are summarized in Fig. 1. Results show that, within the range studied, E/S has no significant effect on yield and specific productivity, which is in agreement with results reported by Chockchaisawasdee et al. [13]. However, other works suggest a possible effect of E/S both on yield and productivity [14,16]. In a kinetically controlled reaction, maximum yield is determined by the kinetic parameters of the enzyme and not by the enzyme concentration that only affects the rate at which such yield is attained [23]. This is so, because an increase in enzyme concentration increases the rate of GOS formation as well as the rate of hydrolysis. Yield and specific productivities obtained were 28 ± 0.5 g of GOS/100 g of lactose added and 0.18 ± 0.009 g of GOS mg^{-1} enz h^{-1}, respectively. Yield is within the range of values reported for the synthesis of GOS with *A. oryzae*