Food Processing & Nutritional Science

Research Article | Vol 2 Iss 1

Optimization of Galactooligosaccharides Reaction Conditions, Through Commercial Galactosidases

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Received: May 30, 2021; Accepted: June 23, 2021; Published: June 30, 2021

ABSTRACT

Galactooligosaccharides are high value carbohydrates. This work evaluates performance of three fungal β-galactosidases in galactooligosaccharides production. Commercial β-galactosidases from *A. oryzae* (Enzeco) and *A. niger* (Maxilact A4 and EB_GLAN of Megazyme), were used. Initial lactose concentration was 400 g/L for all enzymes. Best conditions for galactooligosaccharides synthesis using Enzeco enzyme were T = 60°C, pH = 4.5, enzyme-lactose ratio (E/S) = 0.5 achieving a final GOS yield of 27% w/w. Best synthesis conditions using Maxilact A4 enzyme were T = 50°C, pH = 5, E/S = 0.5, obtaining a final galactooligosaccharides yield of 24% w/w. Best synthesis conditions using EB-GLAN enzyme were T = 50°C, pH = 5, E/S = 0.5 with a final galactooligosaccharides yield of 25% w/w. Enzeco β-galactosidase presents best conditions for hydrolysis and galactooligosaccharides production than the other two enzymes evaluated. It is remarkable to have information about galactooligosaccharides production parameters for enzymes without information reported a result that could be considered in enzyme selection for this process.

KEYWORDS

Galactooligosaccharides; Fungal enzymes; Enzymatic synthesis; β-galactosidase

INTRODUCTION

Cheese whey is the cheapest source of lactose, raw material in the production of galactooligosaccharides (GOS). The volume of whey produced is 9 L per kg of cheese produced. Cheese producers use whey to feed pigs losing the opportunity to leverage the added value [1]. There is a problem in the environmental field caused by the dumping of whey to rivers and watercourses, increasing the BOD and COD parameters, because of its 7% of organic solids.

Galactosyl-oligosaccharides (GOS) are carbohydrates that have a glucose unit linked to several galactose units [2-6]. A lot of studies have demonstrated the prebiotic properties of these compounds, which are synthesized industrially by enzymatic reactions from lactose [2].

Prebiotic effect of these products, is caused by their capability to be precursors of free fatty acids, then decreasing pH in gut, providing unfavorable conditions for

Citation: Castro German, Optimization of Galactooligosaccharides Reaction Conditions, Through Commercial Galactosidases. Food Proc Nutr Sci 2(1): 34-46.

pathogenic microorganisms growth. GOS are also responsible to feed and fortify the probiotic microorganisms, which live in the human intestine and help to prevent the entry of pathogens [3]. GOS may provide possible solutions to cases of acute diarrheal disease (ADD), one of the highest causes of death in childrens under five years in Colombia [4]. On the other hand, GOS are an alternative for the prevention of carcinogenic cases of digestive system that have become increasingly frequent, as well as in the case of osteoporosis,these products provide conditions for calcium absorption, its consumption could be a treatment of this disease and others associated with calcium deficiency [3,5].

GOS are used as raw material in food processing, has properties such as resistance to high temperatures and low pH, making them more suitable for application in certain foods, for example those which are subjected to severe heat treatments (jams, UHT milk and baked products). Similarly in acidic foods, such as acidic fruit nectars (orange, passion fruit) or fermented milks. These advantages extend the possibilities of use of GOS and are the reasons that explain they are more appreciated than the other prebiotics [6].

GOS are produced by β-galactosidases, widely distributed in nature, have been isolated from a variety of animal, plant and microbial sources [7]; however, only a few are recognized as safe by regulatory agencies, and therefore useful for food or pharmaceutical use [8]. At industrial level, only microbial galactosidases are used, mainly from yeasts (*Kluyveromyces lactis* and *Kluyveromyces fragilis*) and fungal (*A. niger* and *A. oryzae*), the latter having the advantages of a low price and high stability.

Origin of the enzymes largely determines its properties and behavior. Enzymes catalytic action depends on temperature, pH, enzyme and substrate concentration, and the presence of inhibitors [8,9,10]. β-galactosidases have two simultaneous activities: hydrolysis and transgalactosylation [11], former one splits lactose molecule to produce glucose and galactose and latter generates GOS. Adjustment of operating conditions determines which of the two activities prevails [12]. Initial concentration of lactose is one of the most determining factors, at low concentrations hydrolysis is favored due to having more water molecules available to act as nucleophilic acceptors, while at high concentrations of lactose is increased the probability that nucleophilic acceptor be a saccharide [2].

Transgalactosylation reactions allow the synthesis of GOS, lactose being the donor of galactose required in this reaction. At the beginning of the reaction, when lactose concentration is higher, transgalactosylation activity prevails [7, 9]; but as the reaction progresses, the hydrolytic activity becomes predominant, which ultimately leads to the production of glucose and galactose, the latter being a competitive inhibitor of the enzyme [12,13].

Differences in enzyme structure and/or in the coupling mechanism of sugars make it possible to achieve different distributions of synthesis products and therefore vary both the reaction yield and the selectivity of the β-galactosidases used [2,14]. Have been made many studies evaluating different processing conditions like pH, temperature, enzyme concentration [12,15-18]. Two enzymes used in the present research (Maxilact A4 and EBGLAN) do not have results about the effects of the conditions mentioned on the response variables.

This paper evaluates the effect of enzyme type, pH, temperature and enzyme-substrate relationship on hydrolysis and transgalactosylation reactions for three thermostable enzymes of fungal origin. The first one highly referenced in the literature (Enzeco - *A. oryzae*) in GOS production, and the two others with few published results

(Maxilact A4 and EBGLAN both from Megazyme - *A. niger*).

MATERIALS AND METHODS

Materials

Galactosidases of fungal origin were preferred, as they work at higher temperatures than those from yeasts. This condition will become important due to the high concentration of solids necessary for the GOS production process and, therefore, the higher solubility required, which is achieved at higher temperatures. All three enzymes are recognized as GRAS. β-galactosidase from *A. oryzae* was a gift from Enzyme Development Corporation - Enzeco (New York, USA) (A), β-galactosidases from *A. niger* were a gift from Megazyme (Wicklow, Ireland) - EB-GLAN (C) and Interenzimas S.A.S. (Bogotá, Colombia) - Maxilact A4 (B). o-nitrophenyl β-D-galactopyranoside (oNPG), o-nitrophenol (oNP) and HPLC malto-oligosaccharides standards were acquired from Sigma-Aldrich (St. Louis, MO, USA). Lactose and the other chemicals were acquired from Merck (Darmstadt, Germany).

Enzyme assays

o-NPG hydrolisis activity

β-Galactosidase hydrolysis activity assay for fungal lactases is based on a 15-min hydrolysis of an o-nitrophenyl β-D-galactopyranoside (oNPG) substrate at 37°C and pH 4.5 (acetate buffer). One lactase unit (ALU) is defined as the quantity of enzyme that release o-nitrophenol (oNP) at a rate of 1 mmol/min [19]. oNP quantitation was done by spectrophotometry, determining the absorbance at 420 nm and relating it to the concentration with a calibration curve with a correlation coefficient greater than 0.99. Specific activity was also calculated, it is given per mg of protein. Protein content of enzyme preparation was stablished by Kjeldahl method [20].

oNPG hydrolysis kinetics

Kinetic assays of enzymatic hydrolysis were carried out in a cell imaging multi-mode reader, Cytation 3 from Biotek, with 96 well micro-plates. Each one was loaded with 200 µland absorbance was determined at 420 nm. Response variable was the specific hydrolysis activity, defined as µmol of oNP produced per minute of reaction and per g of protein in the enzyme [µmol oNP. g protein^{-1}. min^{-1}]. Five pH values (4, 4.5, 5, 5.5, and 6) (Mcilvaine buffer 0,01 M) were tested, and four enzyme concentrations for each enzyme [21]. Temperature was set at 40ºC and oNPG concentration 2 mM.

The variable E/S* was used, it is the ratio of protein mass in the enzyme to substrate mass (oNPG), using as a reference value 6.6 g protein/g oNPG [21], with Lactozym 6500L enzyme. Additionally, two lower values (2 and 3) and one higher (9,4) were tested for the three enzymes used [22,23].

Lactose hydrolysis activity

β-Galactosidase activity was also determined using lactose 2mM as substrate in McIlvaine buffer 0,01 M, pH 4,5. Enzyme concentration was the same used in activity assay using oNPG. The reaction mixture was placed in a 50 ml stirred flask (120 rpm), 25 ml of working volume. Temperature was kept at 40ºC, reaction time was 15 min, after which a sample was taken and heated at 92ºC for 10 min to stop reaction and subsequent cooling. One unit of lactose activity was defined as the amount of enzyme releasing 1 µmol of glucose per minute under the given conditions. Glucose concentration was determined by HPLC-RI [24] methodology will be described posteriorly.

Lactose hydrolysis kinetics

Lactose hydrolysis kinetics was carried out in the same way as described for lactose hydrolysis activity, but the reaction time was 35 minutes, with periodic sampling. Each of the

samples were heated at 92°C for 10 minutes to stop the reaction and subsequent cooling. The concentration of sugars was determined by HPLC-RI [21,24].

Transgalactosylation activity

Trangalactosylation reaction was carried out in 50 mL Erlenmeyer's with stirring (120 rpm), reaction volume was 25 mL at 40 ° C. Lactose was dissolved in 0.01 M McIlvaine buffer at pH 4.5, according to enzymes technical data sheets. Initial concentration of lactose (Lo) was 400 g/L. Enzyme-lactose ratio, E/S (g of enzyme/100 g of lactose) was 0.5 [12]. Reaction time was 15 minutes, after that enzyme was inactivated by heating in a water bath at 92 ° C for 10 minutes and an immediate cooling. Transgalactosylation activity was defined as the amount of enzyme that catalyzes the transgalactosylation of 1 µmol of GOS per minute at an initial lactose concentration of 40% m/v, pH 4.5 and 40°C. Sugars concentration was determined by HPLC-RI [24].

Transgalactosylation kinetics

oNPG activity was determined periodically, it does not decrease more than 5%. In case of activity losses, it was compensated with a proportional increase in the mass of enzyme in the tests.

Lactose conversion was carried out in 50 mL Erlenmeyer's with stirring (120 rpm), reaction volume was 25 mL at different temperatures (40, 50 and 60°C). Lactose was dissolved in 0.01M McIlvaine buffer at different pH 4.5, 5.0 and 5.5. Initial concentration of lactose (Lo) used was 400 g/L. The enzyme-lactose ratio, E/S was 0.25, 0.5 and 0.75. Reaction was started by adding the respective enzyme according to the lactose concentration and E/S. Reaction was monitored for 80 minutes, sampling every 20 minutes, the enzyme was inactivated by heating in a water bath at 92°C for 10 minutes and immediate cooling.

Response variables were the production of GOS (g/L), calculated by mass balance, as the difference between the initial amount of lactose and the result of the sum of lactose, glucose and galactose in a determined time. The GOS productivity (g/L.min) was defined as the amount of total GOS per volume of reaction medium and reaction time. The yield was defined as the mass of GOS obtained over the initial mass of lactose * 100 (%).

GOS production optimization

Optimization was carried out under the same experimental conditions used in the transgalactosylation tests and according to a Box Behnken experiment design with three factors and three levels, pH (4,5, 5 and 5,5), temperature (40, 50 and 60°C) and E/S (0,25, 0,5 and 0,75).

Analytical methods

Samples taken during the enzyme activity and kinetics tests were diluted 1:20 with deionized water, filtered on 0.22 µm cellulose acetate membranes and stored at 4°C until analysis.

Lactose, glucose, galactose, and GOS concentrations were determined with a Transgenomic CHO-411 column at 75°C, operated by an HPLC system (Thermo) equipped with a Shodex RID-10A detector. The eluent was deionized water at a flow rate of 0.4 mL/ min [24]. The quantitation was done supported by maltooligosaccharides standards.

STATISTICAL ANALYSIS

Enzyme activity tests with oNPG and lactose were done by triplicate. Analysis of variance (ANOVA) was performed on these results, with a significance level of 0.05 using Minitab 16 software.

RESULTS AND DISCUSSION

oNPG hydrolysis activity

Enzymatic activity of the Enzeco and Maxilact A4 enzymes, which are shown in Table 1 are within the values reported in their respective technical sheets. The activity reported in the technical file for the EBGLAN enzyme was determined with pNPG as substrate, then is not comparable with that obtained in this work and may explain the great difference between the values.

Enzyme	Protein (%) w/w	Activity (ALU/g) (theoric)	Activity (ALU/g) (experimental)	Specific activity (ALU/g of protein)	
Enzeco	39	Min. 100000	105659+/-6754	270921+/-17331	**a**
Maxilact A4	2,8	Min. 5000	5562+/-399	198643+/-14250	**b**
EBGLAN	22,6	Min. 54240*	40577+/-5619	179544+/-24863	**b**

Table 1: Hydrolitic activity (ALU/g) for used enzymes. *: this value was done with pNPG reactant.

Enzeco enzyme have the greater specific activity (Table 1), perhaps because it is the only one obtained from *A. oryzae*. This source is more common for industrial application enzymes than *A. niger* [25].

oNPG hydrolysis kinetics

pH effect over oNPG hydrolysis kinetics

Enzymes from A. oryzae and A. niger are characterized by having an appropriate pH for oNPG hydrolysis between 4 and 4.5 [22, 26, 27, 28], a characteristic behavior of fungal enzymes [29]. This agrees with the results presented in Figure 1, it shows the highest oNP concentration at pH 4 for Enzeco and EB GLAN, for Maxilact A4 it is observed the same result at the beginning, but later the behavior changes

Figure 1 shows the hydrolysis kinetics for the three enzymes, Enzeco shows the highest concentration value, 1.94 mM oNP (pH 4, t: 5.5 s), EBGLAN 1.09 mM oNP (pH 4, t: 5.5 s) and Maxilact 0.93 mmol oNP (pH 6, t: 21 s). For almost all pH values, at the beginning oNP concentration rise up fast, but after the first 5 minutes this behavior changes getting a little negative slope. At the beginning of the reaction the active sites of the enzyme are empty, but they are quickly occupied by the oNPG causing the reaction and subsequent release of the oNP; however, the release and subsequent occupation of the active site for a new conversion takes longer due to the continuous decrease in the concentration of the substrate, making it increasingly difficult for it to meet the active sites of the enzyme.

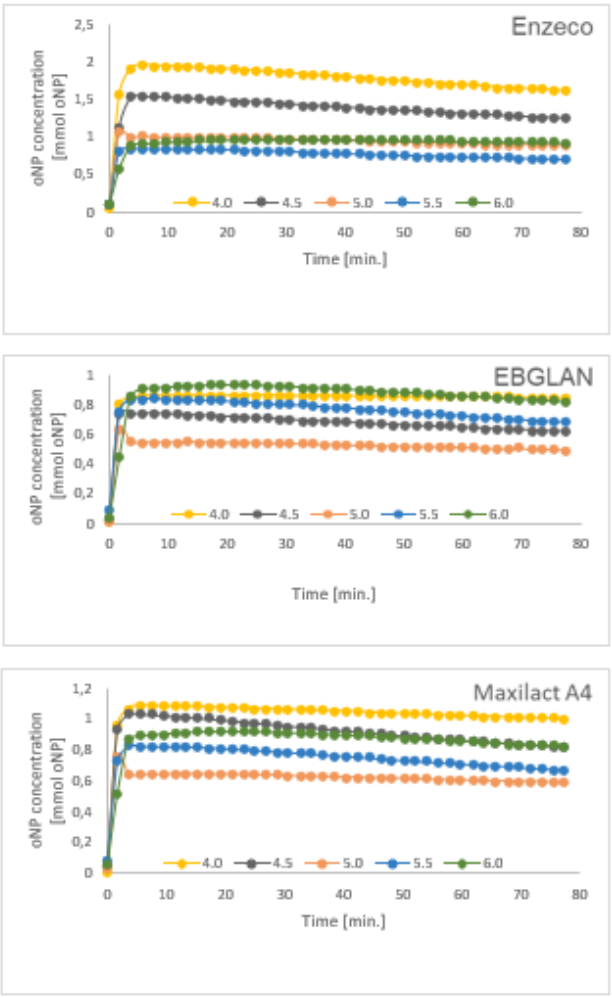

Figure 1: Effect of pH on the concentration of oNP [mmol oNP] for the three enzymes a) Enzeco, b) EB-GLAN, c) Maxilact A4, T = 40ºC, E/S* = 3 g protein/g oNPG.

In Figure 1, the highest oNP concentration values obtained for Enzeco and EBGLAN at pH 4 are observed, these decrease as the pH increases to a minimum value (pH 5.5 and 5, respectively), for higher pH values concentration increases again. For Maxilact A4 the behavior is similar with a minimum value at pH 5, however the maximum concentration of oNP is achieved at pH 6. For this enzyme,

initially the highest concentration of oNP also occurs for low pHs, but after the second sampling time the maximum oNP concentration value is given for pH 6. This behavior is similar to previously reported [30], for a lactase of K. lactis, which after reaching its maximum at pH 6.5, decreases to a minimum at pH 7, and then increases again.

In Figure 1 it is observed how a maximum is reached in the concentration of oNP close to the first 5 min in most cases, then there is a decrease until the end of the test. Apparently, a possible loss in color stability can cause this decrease, as was already observed [31].

Although the methodology used to obtain results of activity and kinetics with oNPG was different, both present the same order of activities for the enzymes used. oNP concentration is directly related with specific hydrolytic activity.

Type of enzyme and E/S* effect over oNPG hydrolysis kinetics

The protein to substrate ratio, E/S*, was adjusted to normalize each enzyme's behavior, based on its active component (protein) and thus be able to compare their specific activities. All the fungal enzymes evaluated exhibit a similar behavior, the three of them presented the lowest enzymatic activity at a ratio of 9.4 g protein / g oNPG, which increases to its maximum value at E/S* = 3 and decreases with smaller proportions (Figure 2). This can be related to the particular way each one of the enzymes receive the substrate in its active sites, it is associated with its structure and morphology, in the case of *A. oryzae* and *A. niger* enzymes, have a morphological similarity of 81% [32].

For Enzeco enzyme the value of the maximum specific activity was 12.52 µmol oNP.mg protein^{-1}.min^{-1}, comparing to 16.73 µmol oNP.mg protein^{-1}.min^{-1} reported for a liquid culture of the *A. oryzae* enzyme purified by ultrafiltration [22]; the difference could be attributed to the fact that this medium may have other enzymes with high hydrolytic activity that can increase its activity.

Reports obtained approximately 3.4 µmol oNP.mg protein-1.min-1 for a lactase from *A. niger* [33], while in this work the enzymes of that fungus, EBGLAN and Maxilact A4, presented values of 6.926 and 6,934 µmol oNP.mg protein-1.min-1, respectively (Figure 2). The difference may be due to the effect of using different buffers, acetate in the former and McIlvaine in this work. Additionally, [33] evaluated the activity of the enzyme extract, with impurities that can affect the activity, while in the present work the activity on the purified commercial enzyme was determined, which may also contribute to the difference found.

According to [10] lactase from *A. oryzae* presents higher values of specific activity than lactase from A. niger, which agrees with this work where the enzyme Enzeco (*A. oryzae*) presented the highest values of specific activity (12.52 µmol oNP. mg protein-1.min-1) against the enzymes EBGLAN and Maxilact A4 (*A. niger*) (6,926 and 6,934 µmol oNP.mg protein-1.min-1).

It is probable that the differences in the conditions of the tests had an effect, since [22] carried out their tests at 30ºC, 1.6 mM oNPG and pH 4.8, [33] did it at 50°C, 100 mM and pH 5, and [10] at 40ºC, 45 mM of oNPG and the optimum pH of each enzyme; conditions all different from those used in the present work (40°C, 2 mM oNPG and pH 4), it makes their comparison difficult.

Lactose hydrolytic activity

Specific hydrolysis activity with lactose for each enzyme were: Enzeco 1.49 +/- 0.065, Maxilact A4 1.30 +/- 0.048 and EBGLAN 1.14 +/- 0.079 [µmol glucose.mg protein^{-1}.min^{-1}] (Figure 3). These results are similar to those obtained with oNPG (Figure 2) in both cases the highest activity is presented for Enzeco. [34] established that despite the fact that lactose is the natural substrate for this

enzyme, there may be substrates that present greater hydrolyzability and therefore greater activity. According to this, oNPG is more hydrolyzable by the evaluated enzymes than lactose, this may explain the wide difference in specific activity when compared to Figure 2.

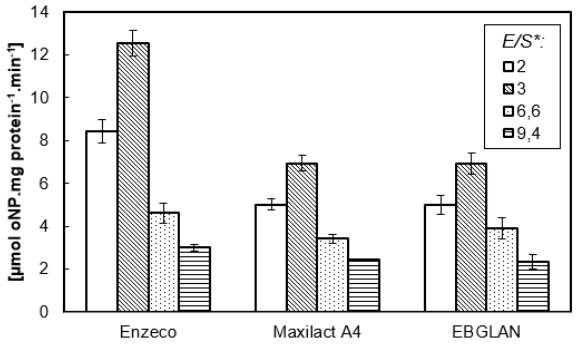

Figure 2: Effect of the type of enzyme and E/S* on the specific hydrolytic activity [µmol oNP.g protein^{-1}.min^{-1}] at T = 40ºC, 15 min., pH 4.

Lactose hydrolytic kinetics

Figure 4 shows the lactose hydrolysis kinetics for the three enzymes, Enzeco enzyme consumes almost all the lactose (99.4%), while EBGLAN 78.6% and Maxilact A4 64.6%. Due to the low concentration of lactose (2 mM or 0.68 g/L) and the high hydrolytic activity of the Enzeco enzyme, close to 100% hydrolysis is achieved, which leads to a high consumption of lactose (Figure 3 and Table 1).

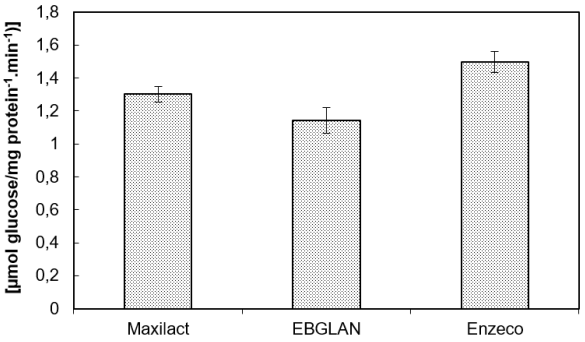

Figure 3: Specific hydrolytic activity with lactose [µmol glucose. mg protein^{-1}.min^{-1}] at T = 40ºC, 15 min., pH 4.

For evaluated enzymes, the production of glucose and galactose is proportional to the hydrolyzed lactose, the lower hydrolysis capacity of EBGLAN and Maxilact A4 (Figure 4) means that less glucose and galactose are obtained, which is consistent with observed behavior in Table 1 and Figure 3.

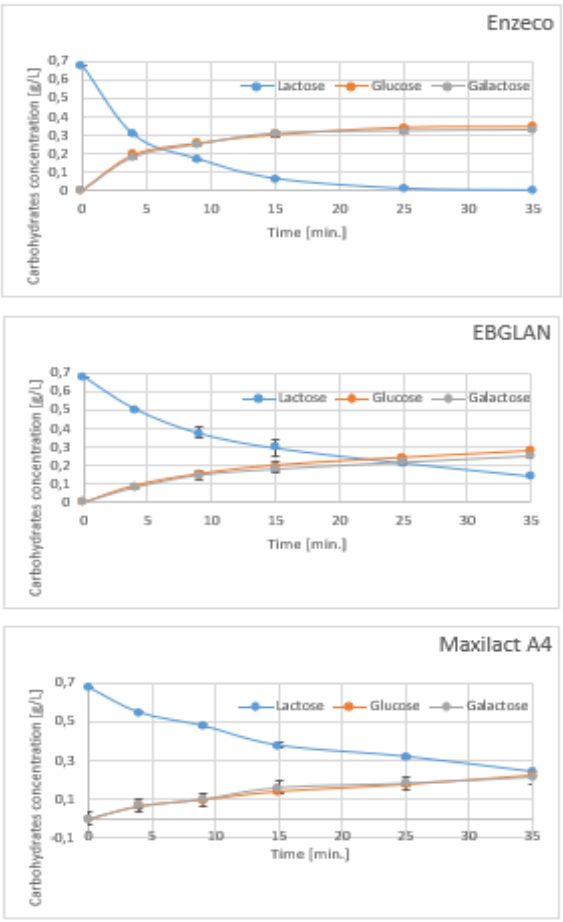

Figure 4: Lactose hydrolysis vs time for a) Enzeco, b) EBGLAN and c) Maxilact A4. pH 4, T = 40 ° C, E/S* = 3 g protein / g oNPG.

Transgalactosylation activity

Specific transgalactosylation activities of EBGLAN do not present significant difference with Enzeco and Maxilact A4, while between the two latter there are significant difference (Table 2), so it is presumed that their common fungal origin determines this behavior [14].

Enzyme	Transgalactosylation activity (µmol GOS.min^{-1}. g enzyme^{-1})	Transgalactosylation specific activity (µmol de GOS.min^{-1}. g protein^{-1})	
Enzeco	31576,10 +/-928	80964,36+/-2379	**a**
Maxilact A4	2050,39+/-113	73228,36+/-4028	**b**
EBGLAN	17721,52+/-644	78413,82+/-2849	**ab**

Table 2: Transgalactosylation activity (µmol of GOS.min-1. g enzyme-1) for an initial lactose concentration of 40% m / v, pH 4.5 and 40° C, for the enzymes used.

The hydrolytic capacity of Enzeco (*A. oryzae*) is greater, either with oNPG or with lactose as a substrate, when compared with EBGLAN and Maxilact A4 (*A. niger*), therefore the origin of the enzyme can make a difference in terms of the hydrolysis reaction in the two cases. This difference is not observed in the transgalactosylation reaction.

Transgalactosylation kinetics

GOS are the result of simultaneous reactions of generation (transgalactosylation) and consumption (hydrolysis) of GOS, this makes it very important to establish the moment of maximum production through the monitoring of kinetic behavior. For the three enzymes, such monitoring was carried out and it was established that in minute 80 the maximum production of GOS was achieved, after this the hydrolysis prevails over the transgalactosylation, causing the decrease in the concentration of total GOS.

High initial concentrations of lactose favor transgalactosylation reactions over hydrolysis ones; In this case, 400 g/L were used, this is the suggested concentration in many works to provide the best conditions for the highest production of GOS [2,14].

Only a percentage of this initial lactose is used, Enzeco and EBGLAN transformed 51% of the initial lactose and Maxilact A4 47% (Figure 5a). Were reported lactose conversion values for A. oryzae enzymes, of 56 and 55% respectively, but with initial lactose concentrations of 500 and 571 g/L at 40ºC [16,17], values greater than that evaluated in this work.

On the other hand, GOS production for Enzeco was 107.69 +/- 3.24 g/L, for Maxilact A4, 96.15 +/- 2.23 g/L and for EBGLAN 99.84 +/- 2.1 g/L (Figure 5d). [32] affirm that the enzymes of *A. oryzae* present higher production of GOS than those corresponding to *A. niger*, that produced 48 g/L of GOS, but with initial concentrations of lactose of 300 g/L [15].

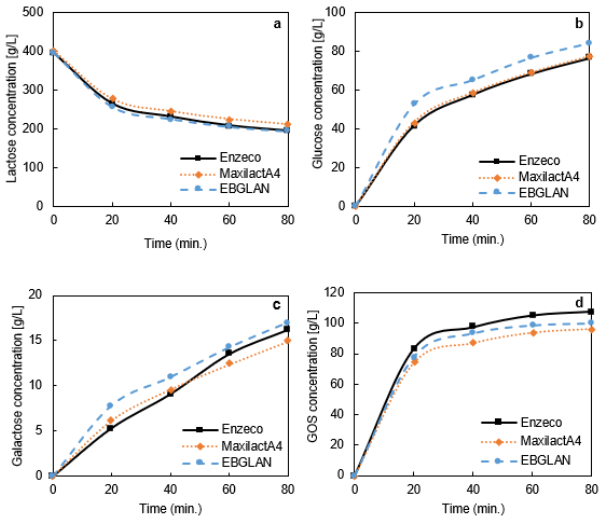

Figure 5: Kinetics of the transgalactosylation reaction for Enzeco, Maxilact A4 and EBGLAN at the best conditions evaluated for each of them. a) Lactose, b) Glucose, c) Galactose and d) GOS.

Maxilact A4 exhibited the lowest hydrolytic capacity of the three enzymes used; As can be seen in Figure 5, lactose consumption is the lowest, as a consequence produces less glucose and galactose, so that despite having the same transgalactosylation activity of the other enzymes, the lower available galactose implied a lower production of GOS. EBGLAN hydrolyzes more lactose than Maxilact A4 and therefore produces more glucose and galactose. The production of GOS does not present significant differences between these two enzymes, probably because although EBGLAN has a greater amount of galactose available for the generation of GOS, such difference is not very large, and since two galactose molecules are required to form a GOS3 molecule and three galactose molecules to form one GOS4, the total GOS generated does not make a significant difference.

The amount of GOS that is produced by the enzymatic reactions with β-galactosidases is the result of a balance between the hydrolysis reaction and the transgalactosylation reaction. Enzeco enzyme showed the

best balance of these activities, to achieve the highest concentration of total GOS (107.69 +/- 3.24 g/L).

Optimization of production, yield and productivity, for the three enzymes.

In Table 3 the reaction conditions shown are pH, temperature, and enzyme and substrate concentrations, which are of great importance for fungal enzymes. The pH and temperature values that report the best results are in the order of 4-5 for pH and 50-60°C for temperature [16-18,35-41].

Enzyme	pH	T (°C)	Lo	P	Y	References
			(g/L)	(g GOS/L*h)	(g GOS/g Lo*100)	
Enzeco - Ao	4,5	55	395	81	27	This work
EBGLAN -	5	55	400	72	24	This work
Maxilact A4 -	5	55	395	75	25	This work
A. niger	7	40	2-30	-	16	[15, 37],
A. oryzae	4,5	40	380	24	31	[16]
A. oryzae	4,5	40	500	-	52	[17]
A. oryzae	4,5-6,5	40-60	100-475	-	10-35	[12]
A. oryzae	4,5	40	43-270	-	22	[18]

Table 3: Productivity and yield of GOS., Lo: Initial concentration of lactose (g/L). P: GOS productivity (g/L*h). Y: GOS yield (%) = GOS concentration (g/L)/Lo.

Temperature can increase the solubility of lactose, which is low at room temperature (220 g/L at 25°C) [42]. For this reason, several investigations have been directed to the discovery and characterization of thermostable enzymes [43-45]. These enzymes allow processes to run at elevated temperatures and thus with a higher initial lactose concentration, which in turn can increase the maximum GOS concentration achieved.

The ability to work at higher temperatures using thermostable enzymes also offers the advantage of limiting the potential for microbial contamination within the process. However, increases in yields should be balanced by the greater loss of enzyme activity at higher temperatures [2]. Maillard reactions between aminic side chains of enzymes and sugars have been shown to become significant by inactivating thermostable enzymes at 80°C or higher [46].

pH affects the kinetics of lactose hydrolysis and GOS synthesis by E. coli β-galactosidases [11]. This suggests that it may be possible to selectively control the rates of GOS synthesis and its degradation by varying the pH of the reaction medium, thus increasing GOS yields. However, this may be a characteristic that varies between different enzymes, similar to the effect of temperature on performance. Many studies have also found similar appropriate pH values for GOS synthesis and its hydrolysis [45-49]. For example, β-galactosidase from Sulfolobus solfataricus, which showed an optimum pH for the synthesis of GOS at 6.0, close to the optimum for the hydrolysis of oNPG at pH 6.5 [45]. Furthermore, according to [16] substrate concentrations for high GOS production are between 20%-50% m/v.

Observing Table 3, in the referenced works with one or more of these conditions different from the suggested values, report lower values of GOS performance [12,15,18]. In the works [16,17], despite working at a low temperature (40ºC), the highest performance is achieved due to the high concentration of lactose used, 57 and 50% m/v, respectively.

This is an indication that the lactose concentration is a very important variable to achieve the maximum production of GOS [2,14,50]. This dependence on the initial concentration of lactose has at least two contributing factors: increased availability of galactosyl saccharide acceptors and decreased availability of water. The former increases the rate of GOS synthesis and the latter decreases the hydrolysis of lactose and GOS, this means that using high initial concentrations of lactose promotes the synthesis of GOS [39,51,52].

Enzyme	GOS (g/L)	Productivity* (g/L min)	Yield % (GOS/Lo)	T opt °C	pH opt	E/S opt
Enzeco	110,5	1,38	28	54,3	4,6	0,39
Maxilact A4	99,9	1,25	25	53,9	4,8	0,54
EBGLAN	95,8	1,2	24	51,7	4,8	0,52

Table 4: Optimization results using Minitab 16 software. *: Productivity taken at 80 min.

Results of the optimization using Box Behnken design are shown in Table 4 and Figure 6, according to these the Enzeco enzyme achieves the highest yield and productivity of GOS (28% and 1.38 g. L^{-1}. min^{-1} respectively), at T = 54.3 ° C, pH = 4.6 and E/S = 0.39. This is a consequence of the good balance of hydrolysis and transgalactosylation activities for this enzyme, as discussed above.

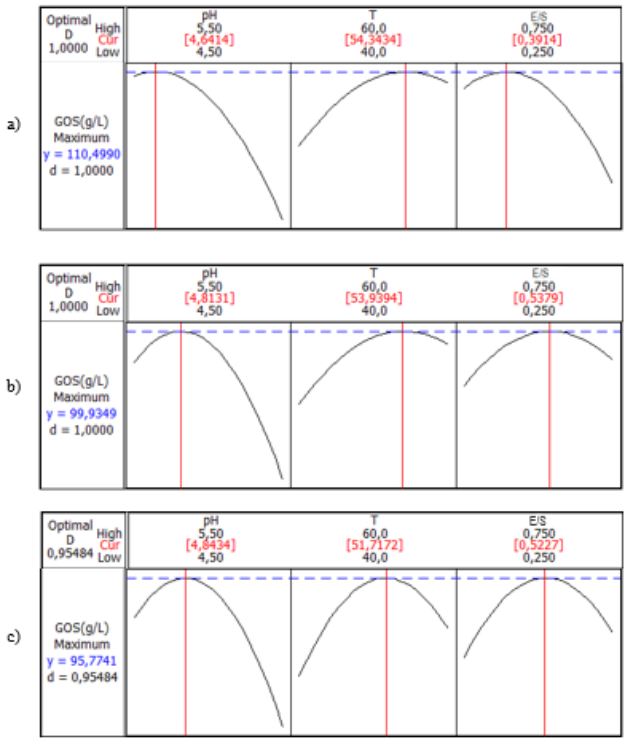

Figure 6: GOS production optimization graph (g/L) with Minitab 16, for the enzymes a) Enzeco, b) Maxilact, c) EBGLAN.

Optimized conditions obtained for Enzeco, observed in Figure 6 were pH 4.6, T = 54ºC and E/S = 0.39, were validated experimentally obtaining a yield of 29.2%, a productivity 1.36 g. L^{-1}.min^{-1} and a production of 107.69 g. L^{-1}, these results do not present significant differences with those optimized by Minitab 16 (Table 4 row 2).

The results of Figure 6 agree with those found in other works [12,16-18], where the enzymes of A. oryzae, are better in the production of GOS and with similar yields to those obtained in this work.

The best performance in kinetic tests for Enzeco enzyme, support the results of higher GOS production for this enzyme.

CONCLUSIONS

The hydrolytic properties were defined for three commercial β-galactosidases, Enzeco, Maxilact A4 and EBGLAN, using oNPG and lactose as substrates. Enzeco enzyme presented the highest specific hydrolytic activity with oNPG, followed by EBGLAN and Maxilact A4 that presented the same value. The specific hydrolytic activity with lactose presented a behavior similar to that obtained with oNPG, Enzeco presented the highest value and the other two enzymes a lower value and similar between them.

The specific transgalactosylation activity was evaluated for the three enzymes and no significant differences were found, which implies a similar capacity for GOS synthesis for them. The production of galactooligosaccharides was evaluated using the three commercial β-galactosidases, achieving GOS production yields of 27%, 25% and 24% w/w for the enzymes Enzeco, Maxilact A4 and EBGLAN, respectively.

The optimized conditions obtained for Enzeco were pH 4.6, T = 54ºC and E/S = 0.39, under those conditions the production, yield and productivity of GOS were 107.7 g/L, 29.2% and 1.36 g. L^{-1}.min^{-1} respectively, in the same order the results optimized by Minitab 16 were 110.5 g/L, 28% and 1.38 g.L^{-1}.min^{-1}, which are not statistically different from the experimental ones.

Enzeco's commercial lactase presents the best results for the hydrolysis tests with ONPG, lactose and GOS production compared to the other two evaluated.

ACKNOWLEDGEMENT

Minciencias, Colombian Ministry of Innovation, Technology and Science; Colfuturo; National University of Colombia, Sede Bogotá; University of La Salle, Bogotá, Colombia.

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