Carbohydrate Polymers 95 (2013) 465–470

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

High stability of immobilized β-D-galactosidase for lactose hydrolysis and galactooligosaccharides synthesis

Manuela P. Klein[a,b], Lucas P. Fallavena[b], Jéssie da N. Schöffer[b], Marco A.Z. Ayub[b], Rafael C. Rodrigues[b], Jorge L. Ninow[a], Plinho F. Hertz[b,*]

[a] Chemical and Food Engineering Department, Federal University of Santa Catarina, ZC 88040-900 Florianópolis, SC, Brazil
[b] Enzymology Laboratory, Food Science and Technology Institute, Federal University of Rio Grande do Sul, Av. Bento Gonçalves 9500, P.O. Box 15090, ZC 91501-970 Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 27 November 2012
Received in revised form 30 January 2013
Accepted 21 February 2013
Available online 4 March 2013

Keywords:
Continuous enzymatic packed-bed reactor
β-D-Galactosidase
Chitosan macroparticles
Galactooligosaccharides
Lactose hydrolysis

ABSTRACT

β-D-Galactosidase from *Kluyveromyces lactis* was immobilized on glutaraldehyde-activated chitosan and used in a packed-bed reactor for the continuous hydrolysis of lactose and the synthesis of galactooligosaccharides (GOS). The biocatalyst was tested for its optima pH and temperature, thermal stability in the presence of substrate and products, and operational stability. Immobilization increased the range of operational pH and temperature, and the enzyme thermal stability was sharply increased in the presence of lactose. Almost complete lactose hydrolysis was achieved for both milk whey and lactose solution at 37 °C at flow rates up to 2.6 mL min^{-1}. Maximal GOS concentration of 26 g L^{-1} was obtained at a flow rate of 3.1 mL min^{-1}, with a productivity of 186 g L^{-1} h^{-1}. Steady-state operation for 15 days showed the reactor stability concerning lactose hydrolysis.

1. Introduction

Lactose (β-D-galactopyranosyl-(1→4)-D-glucopyranose), the main sugar of milk, can be hydrolyzed by β-D-galactosidase liberating D-glucose and D-galactose, making possible the consumption of milk and other dairy products by lactose-intolerant people (Haider & Husain, 2009). In the presence of highly concentrated lactose, this enzyme can also produce galactooligosaccharides (GOS), by transferring galactosyl residues to lactose molecules. The GOS produced (for example, Gal (β 1→4) Gal (β 1→4) Glc), usually has the structure Gal_n–Glc, where *n* indicates the degree of polymerization, which is typically 1–5 (Gosling, Stevens, Barber, Kentish, & Gras, 2010; Mussatto & Mancilha, 2007). GOS are non-digestible oligosaccharides, which are used as prebiotics food ingredients. The regular consumption of GOS can promote the growth and metabolism of intestinal bifidobacteria, microorganisms that are associated with positive health effects (as the reduction of the level of cholesterol, anticarcinogenic properties, and production of vitamins), when applied in human and other animals diets (Grosova, Rosenberg, & Rebros, 2008). Therefore, the enzyme β-D-galactosidase, in its free or immobilized forms, has an important application in the food industry for lactose hydrolysis and for GOS synthesis.

For the industrial-scale applications of immobilized enzymes, where large amounts of biocatalyst are required, the immobilization protocol must be simple and preferably make use of inexpensive materials as supports (Garcia-Galan, Berenguer-Murcia, Fernandez-Lafuente, & Rodrigues, 2011). Moreover, concerning food applications, nontoxicity and biocompatibility are also required. In this context, chitosan [(1→4)-2-amino-2-deoxy-β-D-glucan], which is a natural polyaminosaccharide derived from chitin [(1→4)-2-acetamido-2-deoxy-β-D-glucan], a by-product of the seafood processing industry, is both safe and cheap (Krajewska, 2004; Muzzarelli, 1980). Another unique property of chitosan is its low propensity for microbial contamination, a problem associated with the immobilization of β-D-galactosidase for lactose hydrolysis (Benhabiles et al., 2012). Chitosan is soluble in acid solutions, and can be precipitated in alkaline pH, thus chitosan particles can be readily obtained dripping an acid chitosan solution into an alkaline coagulation solution (Krajewska, 2004). Prior to enzyme immobilization, chitosan particles can also be easily activated using various agents such as glutaraldehyde (Muzzarelli, 1980), genipin (Chiou, Hung, Giridhar, & Wu, 2007; Muzzarelli, 2009), glycidol or epichlorohydrin (Rodrigues, Mendes, Adriano, Gonçalves, & Giordano, 2008) in order to generate specific groups for enzyme attachment.

* Corresponding author. Tel.: +55 51 3308 7094; fax: +55 51 3308 7048.
E-mail address: plinho@ufrgs.br (P.F. Hertz).

0144-8617
http://dx.doi.org/10.1016/j.carbpol.2013.02.044

Reactors configuration for continuous operations is another important aspect in designing industrial enzymatic processes. Packed-bed reactors (PBRs) are widely and successfully used in many industrial processes. Some of the advantages of using this type of reactor are the reuse of the enzyme without the need of a prior separation; the continuous production of the desired product; the easiness of handling substrates of low solubility by the use of large volumes containing low concentrations of substrate; and the long-term runs and industrial-scale operations. Therefore, PBRs are more cost effective than batch operations (Chang, Shaw, Yang, & Shieh, 2007; Halim, Kamaruddin, & Fernando, 2009).

The main objective of this research was to set up a packed-bed reactor filled with chitosan-immobilized β-D-galactosidase for the continuous hydrolysis of lactose and the synthesis of galactooligosaccharides. The immobilized enzyme was also characterized for its operational optima pH and temperature, and biocatalyst thermal stability, assayed in the presence of substrates and products. Process performance was evaluated in terms of lactose hydrolysis and GOS synthesis as a function of flow rate of substrate feeding. Finally, continuous lactose hydrolysis was carried out in order to evaluate the operational stability of the PBR under steady-state operation.

2. Materials and methods

2.1. Materials

Whey powder was obtained from a local supplier (Elegê Laticínios S.A., Teutônia, Brazil), *Kluyveromyces lactis* β-D-galactosidase was the liquid formulation of Maxilact LX 5000, with a declared activity of ≥5000 NLU/g (DSM Food Specialties, Heerlen, Netherlands). Chitosan (from shrimp shells, ≥75% deacetylated), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), D-glucose, D-galactose, lactose, and raffinose (β-D-fructofuranosyl α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside) were obtained from Sigma–Aldrich (St. Louis, USA). A D-glucose determination kit was purchased from Labtest Diagnóstica SA (São Paulo, Brazil). All solvents and other chemicals were of analytical grade.

2.2. Methods

2.2.1. Preparation of β-D-galactosidase immobilized on chitosan macroparticles

β-D-galactosidase was covalently immobilized on glutaraldehyde-activated chitosan macroparticles as described in a previous work (Klein et al., 2012). The amount of protein attached on chitosan macroparticles was 46.2 mg g^{-1} of dry support, presenting an activity of 230 U g^{-1} of dry support. The chitosan-β-D-galactosidase derivative was stored at 7 °C in 0.1 M of phosphate–potassium buffer (pH 7.0) containing $MgCl_2$ 1.5 mM (activity buffer).

2.2.2. Activity of free and immobilized β-D-galactosidase

Free β-D-galactosidase activity was assayed by incubating the diluted enzyme (50 μL) with 0.5 mL of activity buffer containing ONPG (10 mM) at 37 °C during 2 min. For immobilized β-D-galactosidase, 1.5 mg (dry support) was incubated with 1 mL of activity buffer containing ONPG (10 mM) at 37 °C during 2 min. The reactions were stopped with the addition of borate buffer pH 9.8. Released *o*-nitrophenol (ONP) was spectrophotometrically determined at 415 nm. One unit of β-D-galactosidase activity (U) was defined as the amount of enzyme that hydrolyze 1 μmol of ONPG to *o*-nitrophenol per minute under the conditions previously stated. Protein content of the enzyme solutions was determined by the Lowry assay.

2.2.3. Optima pH and temperature for free and immobilized β-D-galactosidase

The optimum operational pH of β-D-galactosidase activity was studied by monitoring enzyme activity of both free and immobilized preparations in 0.1 M of phosphate–potassium buffer (pH 5.7–8.0) containing $MgCl_2$ 1.5 mM, at 37 °C, while the optimum temperature was determined by measuring the activity between 10 °C and 70 °C at pH 7.

2.2.4. Thermal stability of the immobilized β-D-galactosidase

Thermal stability of the immobilized enzyme was evaluated incubating the biocatalyst in sealed tubes in a thermostatically controlled water bath at 55 °C. Thermal stability was performed under the following conditions: activity buffer, different concentrations of lactose (50 g L^{-1} and 400 g L^{-1}), D-glucose (200 g L^{-1}), D-galactose (200 g L^{-1}), or D-glucose plus D-galactose (200 g L^{-1} of each sugars). Samples were withdrawn at different time intervals and placed in ice bath to stop the thermal inactivation instantaneously. The remaining enzyme activity was determined as described above.

2.2.5. Packed-bed reactor setup

The column type packed-bed reactor (12 cm × 3 cm) was composed of a water-jacketed glass column, flow-rate controller, and water bath. The reactor was packed with 4 g (dry weight) of chitosan-immobilized β-D-galactosidase 2 mm diameter macroparticles, corresponding to approximately 920 U of β-D-galactosidase total activity. The substrate solution was fed through the bottom of the column using a peristaltic pump. The topside and the bottom of the column were fitted with a sintered glass disc (4 mm thick). The total volume of the packed-bed reactor was 29 mL.

2.2.6. Lactose hydrolysis

The hydrolysis of lactose was performed by flowing through the reactor either buffered lactose solution or whey, both containing 50 g L^{-1} of sugar. The lactose solution was prepared in activity buffer, while the whey solution was prepared by suspension of whey powder in distilled water and adjusting the pH to 7. Previously to starting the reaction, the packed-bed reactor was washed with activity buffer. Flow rates were tested from 0.26 mL min^{-1} to 3.4 mL min^{-1}. Samples were collected after the steady state was reached and analyzed for D-glucose formation. Previously to the D-glucose quantification, samples of hydrolyzed whey were diluted and filtered through a 0.22 μm acetate cellulose membrane in order to remove proteins that may cause interference in the analysis. The hydrolysis was carried out at two different temperatures, 37 and 7 °C, in order to simulate real industrial conditions and to avoid possible microbial contaminations.

2.2.7. Continuous synthesis of galactooligosaccharides (GOS)

GOS synthesis was performed by flowing through the reactor buffered lactose solution at high concentration (400 g L^{-1}). Flow rates were tested from 1 to 15 mL min^{-1} for GOS synthesis at 37 °C. Samples were collected after steady state was reached and the reaction product was analyzed by HPLC.

2.2.8. Analytical procedures

Lactose and products from the transgalactosylation reaction (GOS, D-galactose and D-glucose) were analyzed by HPLC (Shimadzu, Tokyo, Japan) equipped with refractor index and Aminex HPX-87C (300 mm × 7.8 mm) column. Ultra-pure water was used as eluting solvent at a flow rate of 0.6 mL min^{-1}, at 85 °C. The concentration of saccharides was calculated by interpolation from external standards. Standards were used for lactose, D-glucose, and D-galactose. GOS concentration was calculated as raffinose equivalents from an external raffinose standard, as described by Gosling,

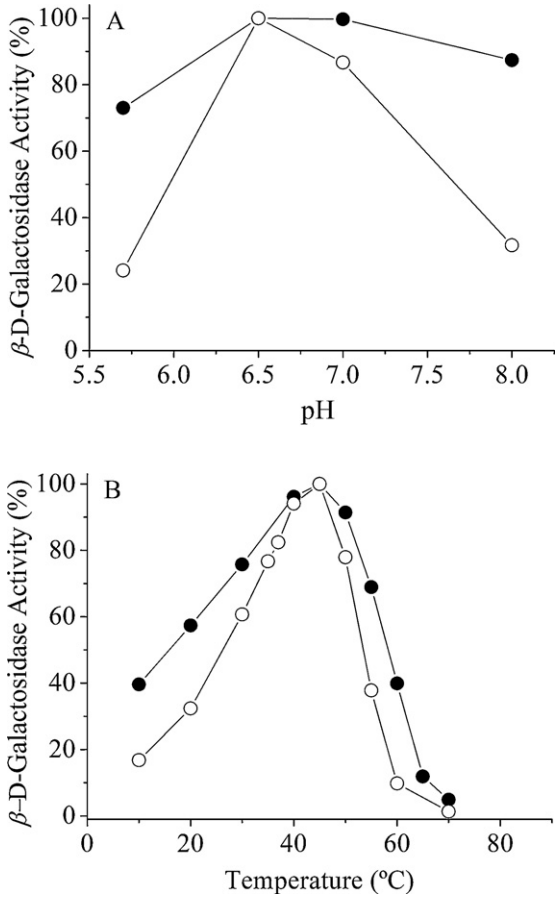

Fig. 1. Effect of pH (A) and temperature (B) on the activity of free β-D-galactosidase (○) and chitosan-immobilized β-D-galactosidase (●).

Stevens, Barber, Kentish, and Gras (2011). The commercial product Vivinal GOS® (Friesland Foods, Netherlands) was used to compare the retention time of the obtained GOS, and its yield (%) was defined as the percentage of GOS produced compared with the weight of initial lactose in the reaction medium.

2.2.9. Operational stability of the packed-bed reactor during lactose hydrolysis

The operational stability of the PBR under steady-state regime was evaluated during 15 continuous days at 37 °C using a flow rate of 2.6 mL min^{-1} containing 50 g L^{-1} of lactose. Aliquots were periodically collected and analyzed for D-glucose formation.

3. Results and discussion

3.1. Properties of the immobilized β-D-galactosidase

3.1.1. Optima pH and temperature

The effect of pH on the relative activity of the free and immobilized β-D-galactosidase was evaluated in the range of 5.7–8.0 (Fig. 1A). After immobilization on chitosan macroparticles, the optimum pH of β-D-galactosidase was enhanced from 6.5 to a broader range between 6.5 and 8.0. Even at acidic pH, the enzyme activity was remarkably enhanced, with more than 70% remaining at pH 5.5, compared to less than 25% for the free form, suggesting the strong stabilization effect on enzyme molecules, provided by the covalent binding to the support. Bayramoglu, Tunali, and Arica (2007), in their studies with *Escherichia coli* β-D-galactosidase immobilized onto magnetic poly(GMA–MMA) beads, found a shift of optimum pH of the enzyme from 7.5 to 7 after immobilization. The authors

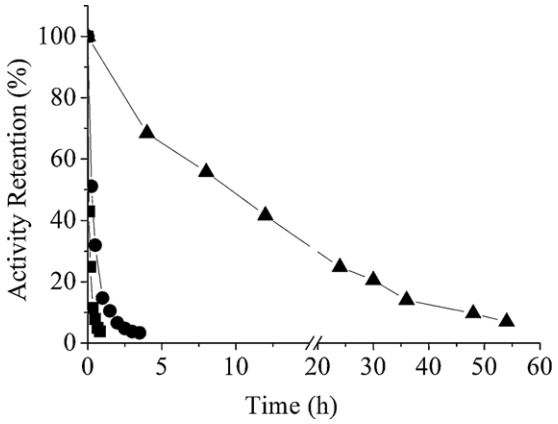

Fig. 2. Thermal stability at 55 °C of chitosan-immobilized β-D-galactosidase in the presence of 400 g L^{-1} lactose solutions (▲), 50 g L^{-1} lactose solutions (●), and activity buffer (■).

reported that this effect was probably due to the basic nature of the amino functionalized surface of the magnetic beads. Similarly, β-D-galactosidase from *K. lactis* immobilized by adsorption on a mixed-matrix membrane containing zirconium dioxide presented a shift in the optimum pH from 6.5 to 7 (Jochems et al., 2011). The authors suggested that this change was due to the microenvironment in the vicinity of the membrane. The support, indeed, can change the pH value around the enzyme catalytic site by changing the concentration of the charged species (e.g. hydrogen and hydroxyl ions) in respect to the bulk solution (De Maio et al., 2003).

Changes in activity related to temperature of immobilized β-D-galactosidase were investigated in the range of 10–70 °C (Fig. 1B). The free and immobilized forms presented maximum activities at 45 °C, while the immobilized enzyme was more active in a wider range of temperatures. Similarly to the changes in pH, the wider range of temperatures with higher activities can be attributed to the effect of immobilization, the protection offered by the chitosan macroparticles to the enzyme. Song, Lee, Kang, and Kim (2010) reported similar results and a broader range of activity was observed for pH and temperature after immobilization of *K. lactis* β-D-galactosidase pretreated with lactose on the surface of silica gel using glutaraldehyde as linker. According to Pereira-Rodríguez et al. (2012), the tetramer of β-D-galactosidase from *K. lactis* is an assembly of dimers, with higher dissociation energy for the dimers than for its tetrameric structure, causing an equilibrium between the dimeric and tetrameric forms of the enzyme when in solution. The binding of the enzyme to the support can prevent the dissociation of subunits of either forms (Fernandez-Lafuente, 2009), consequently causing it structure to be stabilized. Although immobilized enzymes are heterogeneous catalysts, the widened range for pH and temperature can configure another advantage of using immobilized enzymes over the use of their free forms by allowing their applications under different conditions, improving industrial uses.

3.1.2. Thermal stability

Thermal inactivation is an important, limiting factor for prolonged use of enzymes in industrial processes. Inactivation in the presence of substrate and products (reactive conditions) should be evaluated, since this reflects the reaction in an enzyme reactor. Fig. 2 depicts the thermal stability of chitosan-immobilized β-D-galactosidase under different reactive conditions (substrate type and concentration) at 55 °C.

Activity of the immobilized enzyme under nonreactive conditions (in activity buffer) was approximately 43% after 5 min of incubation at 55 °C, which is consistent with the fact that *K. lactis*

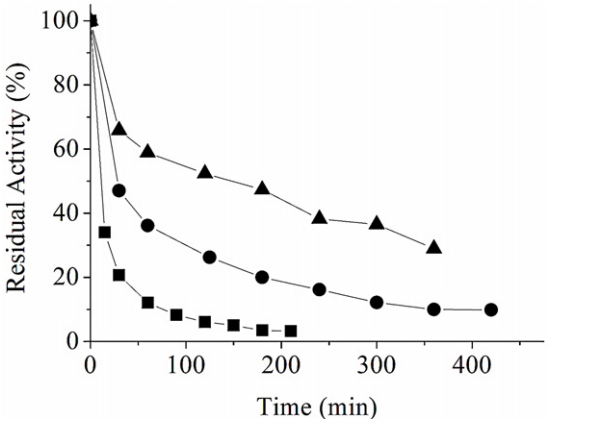

Fig. 3. Thermal stability at 55 °C of chitosan-immobilized β-D-galactosidase in the presence of mixture of D-glucose and D-galactose (each 200 g L^{-1}) (▲), 200 g L^{-1} D-galactose solution (●), and 200 g L^{-1} D-glucose solution (■).

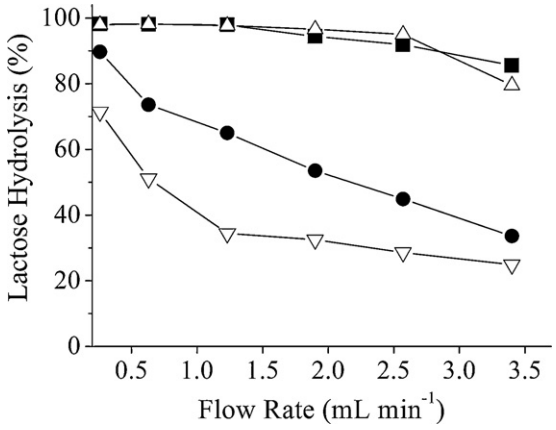

Fig. 4. Effect of the flow rate on the lactose hydrolysis by chitosan-immobilized β-D-galactosidase under different conditions: whey at 37 °C (△), 50 g L^{-1} buffered lactose solution at 37 °C (■), whey at 7 °C (▽), and 50 g L^{-1} buffered lactose solution at 7 °C (●).

β-D-galactosidase is relatively thermolabile. According to Jurado, Camacho, Luzon, and Vicaria (2004), experiments conducted at 30 °C and 35 °C, it was found that this β-D-galactosidase preserved practically all its activity after 3 h of reaction, while at higher temperatures it was virtually deactivated.

In order to assay operational conditions, it was evaluated the thermal stability of the immobilized enzyme in the presence of lactose 50 g L^{-1}, which is the average concentration of lactose present in milk and whey. In the same way, thermal stability test was carried out in presence of whey and lactose 400 g L^{-1} (the concentration used in the GOS synthesis). The results showed that in 50 g L^{-1} of lactose the enzyme retained around 51% of its activity after 15 min at 55 °C, while in 400 g L^{-1} the activity was considerably improved (100% after 15 min and approximately 55.8% after 8 h of incubation) at the same temperature, suggesting that lactose stabilizes the enzyme. According to Jurado et al. (2004), Jurado, Camacho, Luzon, and Vicaria (2005) and Ladero, Santos, and Garcia-Ochoa (2006), lactose (and possibly D-galactose) can stabilize the overall structure of the enzyme by the coupling of the substrate into the active site.

The products generated during the reaction can also play some important role in the enzyme stability. Binding reaction products and other ligands to a protein is a simple way to stabilize its conformation, which is a widely used strategy for stabilizing proteins and enzymes during various operations including enzyme purification and enzyme immobilization (Illanes, Altamirano, & Zuniga, 1996).

Then, in an effort to accurately investigate the effect of substrate and products in the enzyme stability, the thermal stability in the presence of products (D-glucose and D-galactose) was analyzed. As can be seen in Fig. 3, when only D-glucose (200 g L^{-1}) was present, the chitosan-immobilized β-D-galactosidase presented around 50% of its activity after 11 min of incubation; the stability was not significantly increased compared to the inactivation under nonreactive conditions (4.8 min). For *K. lactis* β-D-galactosidase, D-glucose is known to be a non-competitive inhibitor (K_i^{gli} = 794 mM) (Cavaille & Combes, 1995), which means that lactose and D-glucose will independently bind at different sites of the enzyme.

When inactivation was performed in presence of D-galactose (200 g L^{-1}), the β-D-galactosidase residual activity was about 47% after 30 min of incubation, which was higher than that obtained when D-glucose was used in the same concentration. D-Galactose is a competitive inhibitor for this enzyme (K_i^{gli} = 42 mM) (Cavaille & Combes, 1995) and has a much higher inhibitory power than D-glucose. Since the only difference between D-glucose and D-galactose lies on the binding site to the protein, it can be suggested that the protector effect promoted by D-galactose comes from the binding of this sugar to the active site of the enzyme. It was recently reported the three-dimensional structure of *K. lactis* β-galactosidase and the complex structure of the molecule when D-galactose is at the active site, showing that a tryptophan residue, responsible for the binding of D-glucose in the active site of β-galactosidase from *E. coli*, is missing from the active site of *K. lactis* β-galactosidase (Pereira-Rodríguez et al., 2012).

When inactivation was carried out in the presence of D-glucose and D-galactose, both at 200 g L^{-1}, the enzyme stability was improved with 47% of activity still remaining after 180 min of incubation at 55 °C, suggesting the stabilizing effect of osmolytes such as sugars, amino acids, and trehalose (Sampedro, Cortes, Munoz-Clares, Fernandez, & Uribe, 2001). Stabilization would be achieved by an increase in the surface tension of the solution, causing the exclusion of the saccharides from the protein domain and reducing backbone movements away from the fully folded protein form (Bromberg, Marx, & Frishman, 2008; Butler & Falke, 1996; Lin & Timasheff, 1996; Sampedro et al., 2001). The stabilizing effect also depends on the type of carbohydrate used (Sola-Penna & Meyer-Fernandes, 1998).

The analyze of the sugars during inactivation in the presence of lactose 400 g L^{-1} showed that lactose was present during all experiment, with the enzyme probably constantly hydrolyzing lactose up to the moment that it was inhibited by the high concentration of produced D-galactose. After 54 h of inactivation at 55 °C, 63 g L^{-1} of lactose still remained.

3.2. Effect of flow rate on lactose hydrolysis in a packed-bed reactor

Since lactose proved to have the major protecting effect on this β-D-galactosidase, operations under conditions in which the enzyme is constantly catalyzing lactose hydrolysis and avoiding product inhibition could be interesting to test. The use of PBRs for lactose hydrolysis and GOS synthesis could be advantageous over batch operations. Substrates (lactose solution or whey) were continuously pumped at different flow rates (0.26–3.4 mL min^{-1}) through the PBR. Fig. 4 shows the lactose conversion at 37 °C and 7 °C for whey and lactose solution at various flow rates.

At 37 °C, for both lactose solution (50 g L^{-1}) and whey, more than 90% of lactose hydrolysis was reached at a flow rate of 2.6 mL min^{-1} (residence time, 11.3 min). Ansari and Husain (2010), reported 95% of lactose hydrolysis during a 10-days steady-state operation of a PBR filled with β-D-galactosidase adsorbed on concanavalin A-cellulose. However, their operation was conducted at slow flow

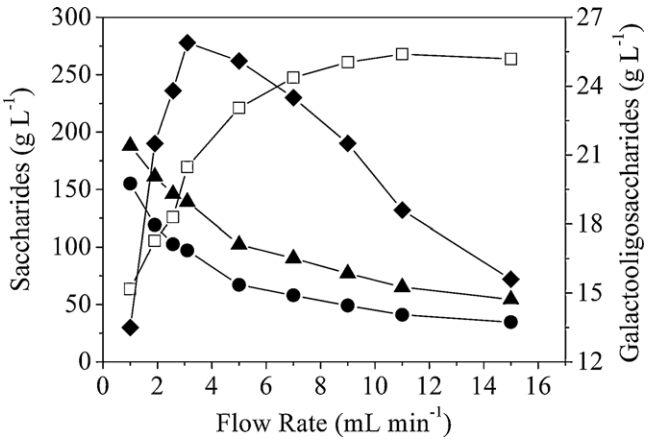

Fig. 5. Effect of flow rate on GOS synthesis by chitosan-immobilized β-D-galactosidase, using 400 g L^{-1} buffered lactose solution, pH 7 at 37 °C. D-Glucose (▲), D-galactose (●), lactose (□), and galactooligosaccharides (♦).

rate of 0.166 mL min^{-1}. A pilot scale module (108 cm × 14 cm), packed with *K. lactis* β-D-galactosidase immobilized on cotton fabric, resulted in 30.23% hydrolysis of lactose from milk, at 37 °C and residence time of 11.8 min (Li, Zhou, & Chen, 2007).

Increasing the substrate flow rate to 3.4 mL min^{-1} caused the lactose conversion to drop to 86 and 80% for lactose solutions (50 g L^{-1}) and whey, respectively. This result can be explained by the short residence time of the substrate inside the reactor.

In order to simulate industrial conditions and reduce the possibility of microbial contamination, lactose hydrolysis was also carried out at 7 °C. As expect, the degree of lactose hydrolysis was reduced for all flow rates when compared with the reaction at 37 °C. For lactose solution (50 g L^{-1}), approximately 90% of lactose was hydrolyzed at a flow rate of 0.26 mL min^{-1}, while for whey 72% of hydrolysis was achieved under same conditions. It is possible that, at 7 °C, the difficulty of mass transfer caused by whey proteins is more pronounced than at 37 °C, which explain the lower lactose conversion observed.

3.2.1. Continuous synthesis of galactooligosaccharides

Fig. 5 shows the changes in the concentration of D-glucose, D-galactose, lactose, and GOS as a function of the substrate flow rate, under steady state operation. The maximum GOS concentration of 26 g L^{-1} was achieved using a substrate flow rate of 3.1 mL min^{-1} (residence time of 9.4 min), corresponding to a yield of 6.5% in GOS and 58% of lactose conversion. At lower flow rates (1–2.6 mL min^{-1}), lower concentrations of GOS were obtained, probably due to the hydrolysis of the synthesized GOS, because the longer residence times (11–29 min) allows for its subsequent hydrolysis by the enzyme. Higher than 3.1 mL min^{-1} flow rates (5–15 mL min^{-1}) causes the residence time to be insufficient for best synthesis, reducing GOS concentration.

Comparatively, Nakkharat and Haltrich (2007) reported 16 g L^{-1} of GOS in a packed-bed reactor containing β-D-galactosidase from *Talaromyces thermophilus* immobilized on Eupergit C, with 50% lactose conversion and 200 g L^{-1} of initial lactose concentration. Neri et al. (2009), worked with β-D-galactosidase from *Aspergillus oryzae* immobilized on magnetic polysiloxane-polyvinyl alcohol, obtaining 103.4 g L^{-1} of GOS, with lactose conversion of 47% and 500 g L^{-1} of lactose, in a batch reaction. Although *K. lactis* β-D-galactosidase is known to show low transgalactosylation activity and thermal stability during the production of galactooligosaccharides compared to *A. oryzae* enzyme (Park & Oh, 2009), when applied in a PBR, the immobilized enzyme was able to work continuously, with high productivities of galactooligosaccharides.

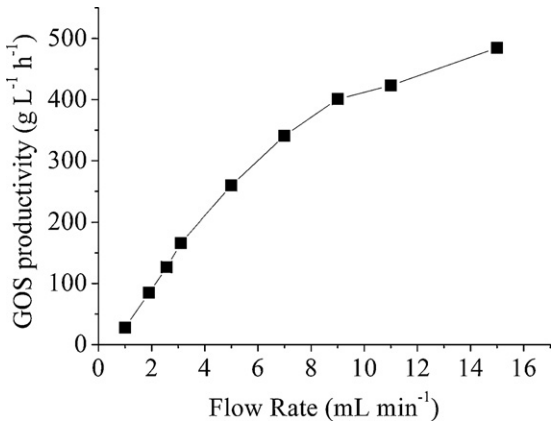

Fig. 6. Effect of flow rate on GOS productivity. Experiments were performed using 400 g L^{-1} buffered lactose solution, pH 7 at 37 °C.

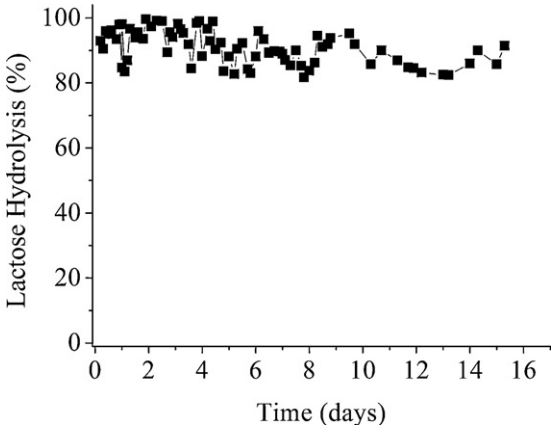

Fig. 7. Operational stability of the PBR filled with chitosan-immobilized β-D-galactosidase, operated continuously using 50 g L^{-1} buffered lactose solution, pH 7 at 37 °C and flow rate of 2.6 mL min^{-1}.

The GOS productivity in the PBR related to the operational flow rate is shown in Fig. 6. It increased to a maximum of 484.5 g L^{-1} h^{-1} at 15 mL min^{-1}. Comparatively, Shin, Park, and Yang (1998), reported oligosaccharide productivity of 4.4 g L^{-1} h^{-1} with lactose solutions of 100 g L^{-1} in PBR with chitosan-immobilized *Bullera singularis* β-D-galactosidase (970 U g^{-1} resin). The higher GOS productivity so far reported, 6000 g L^{-1} h^{-1}, was obtained by Albayrak and Yang (2002), using *A. oryzae* β-D-galactosidase immobilized on cotton cloth by polyethyleneimine and applied in a PBR fed with lactose (400 g L^{-1}) at 2.5 mL min^{-1}.

3.3. Stability of immobilized β-D-galactosidase in the packed-bed reactor

The operational stability of a system is an important parameter in an industrial process, since it directly affects the costs (Nie, Xie, Wang, & Tan, 2006). Fig. 7 shows the operational stability of the immobilized enzyme in the PBR. The reactor was operated continuously at 37 °C at a flow rate of 2.6 mL min^{-1}, mantaining 90% of lactose hydrolysis for longer than 15 days.

4. Conclusions

One of the main objectives in immobilized enzyme technology is to increase the enzyme stability, thus allowing the obtained derivative to be repeatedly used, inclusive at different process conditions, such as in continuous reactors. The use of PBRs for

lactose hydrolysis and GOS synthesis may replace batch reactors, with several cost reductions and operation advantages, including reduced reaction inhibition by substrate and products. In the present study, using chitosan macroparticles, a relatively low cost and easily accessible support, improvements in the operational range of pH and temperature of the enzyme were observed as a consequence of the immobilization process. Furthermore, for the first time it was clearly shown that, the combination of continuous flow with a high content of lactose can sharply increase the stability of *K. lactis* β-D-galactosidase. Thus, Maxilact LX 5000, which is generally used for lactose hydrolysis as a free enzyme, could be advantageously employed in its covalent immobilized form to the hydrolysis of lactose and production of GOS in a continuous PBR.

Acknowledgements

The authors wish to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), for the scholarships of the first and third authors. This work was funded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS).

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