



Continuous production of β -cyclodextrin from starch by highly stable cyclodextrin glycosyltransferase immobilized on chitosan



Jéssie da Natividade Schöffner, Manuela Poletto Klein, Rafael C. Rodrigues, Plinho Francisco Hertz*

Biotechnology, Bioprocess and Biocatalysis Group, Institute of Food Science and Technology, Federal University of Rio Grande do Sul, Avenida Bento Gonçalves, 9500, P.O. Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:

Received 15 April 2013

Received in revised form 17 July 2013

Accepted 19 July 2013

Available online 24 July 2013

Keywords:

Cyclodextrin glycosyltransferase

Enzyme immobilization

Packed bed reactor

Chitosan

ABSTRACT

Cyclodextrin glycosyltransferase (CGTase) from *Thermoanaerobacter* sp. was covalently immobilized on glutaraldehyde-activated chitosan spheres and used in a packed bed reactor to investigate the continuous production of β -cyclodextrin (β -CD). The optimum temperatures were 75 °C and 85 °C at pH 6.0, respectively for free and immobilized CGTase, and the optimum pH (5.0) was the same for both at 60 °C. In the reactor, the effects of flow rate and substrate concentration in the β -CD production were evaluated. The optimum substrate concentration was 4% (w/v), maximizing the β -CD production (1.32 g/L) in a flow rate of 3 mL/min. In addition, the biocatalyst had good operational stability at 60 °C, maintaining 61% of its initial activity after 100 cycles of batch and 100% after 100 h of continuous use. These results suggest the possibility of using this immobilized biocatalyst in continuous production of CDs.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Cyclodextrin glycosyltransferases (CGTases) (EC 2.4.1.19) is one of the most important groups of amylolytic enzymes. These enzymes catalyze the conversion of starch to cyclodextrins (CDs), which are cyclic oligosaccharides composed of α -(1 $\rightarrow$ 4)-glycosidic-linked residues. The best-characterized forms are α -, β - and γ -CD consisting of six (cyclomaltohexaose), seven (cyclomaltoheptaose) and eight (cyclomaltooctaose) D-glucose units, respectively (Goh, Mahadi, Hassan, Rahman & Illias, 2009). The conformation of CDs is formed by a conical cylinder with a relatively hydrophobic internal cavity and a hydrophilic outer surface (Szejtli, 1982). Therefore, CDs are soluble in water and capable of forming inclusion complexes with a variety of molecules, that are placed in their hydrophobic cavity (Nakai, Yamamoto, Terada & Watanabe, 1987). This feature makes CDs attractive for various applications in diverse fields such as food, chemical, pharmaceutical and textile industries as well as in biotechnology, agriculture and environmental protection (Astray, Mejuto, Morales, Rial-Otero & Simal-Gandara, 2010; Biwer, Antranikian & Heinzle, 2002; Stella & He, 2008).

Cyclodextrins have been recommended for applications in food processing and as food additives with a variety of aims, mainly as stabilizers of components that are sensitive to oxygen and heat- or

light-induced degradation and solubilize food colorings and vitamins (Astray, Mejuto, Morales, Rial-Otero & Simal-Gandara, 2010; Sente & Szejtli, 2004). Immobilization of CGTase has been proposed as a way for reducing CDs production cost, and makes feasible its application in food industries (Sobral, Rodrigues, Oliveira, Olivo, de Moraes & Zanin, 2003).

Immobilization has been seen as a very powerful tool to improve enzyme properties allowing the use of this relatively expensive catalyst in the industry (Garcia-Galan, Berenguer-Murcia, Fernandez-Lafuente & Rodrigues, 2011). There are several benefits of using immobilized enzymes, among them, the increase of stability, activity, specificity and selectivity, the decrease of inhibition by reaction products, the improved operation control, and the easy end of reaction and enzyme recover (Rodrigues, Ortiz, Berenguer-Murcia, Torres & Fernandez-Lafuente, 2013). Moreover, heterogeneous biocatalysts can be used in a variety of reactors, and its choice will depend on the immobilization protocol and support (Garcia-Galan, Berenguer-Murcia, Fernandez-Lafuente & Rodrigues, 2011). In the literature, there are few studies dealing the continuous production of cyclodextrins, with emphasis on the use of packed bed (Lee, Lee & Shin, 1991) and fluidized-bed (Cao, 2005; Tardioli, Zanin & de Moraes, 2000) reactors.

Chitin is a polymer widely abundant in nature and its use is a good example of recycling of byproducts of the food industry for value-added products (Muzzarelli, 1996). Chitosan, the principal derivative of chitin, has desirable characteristics for enzymes immobilization (Muzzarelli, 1980). Chitosan has reactive amino and hydroxyl groups in its linear polyglucosamine

* Corresponding author. Tel.: +55 51 3308 7094; fax: +55 51 3308 7048.

E-mail address: plinho@ufrgs.br (P.F. Hertz).

chains, available to direct reactions with enzymes or to chemical modifications, with glutaraldehyde for example, when the native amino groups from chitosan surface are bound to its aldehyde groups, that after will bind to amino groups from enzyme (Chiou & Wu, 2004; Juang, Wu & Tseng, 2002). Additionally, it exhibits mechanical stability, rigidity, nontoxicity and biocompatibility, important features for applications in food industry (Krajewska, 2004). Crucially, as biodegradable polymers chitin/chitosan materials are eco-friendly, safe for humans and the natural environment (Goycoolea, Argüelles-Monal, Peniche & Higuera-Ciapara, 2000; Muzzarelli, 2012).

Packed bed reactors (PBR) are the most commonly system employed for continuous production with heterogeneous catalysis, which can minimize labor and overhead costs for industrial applications. Compared to other immobilized enzyme reactors, such as stirred reactors, PBR is suitable for long-term and industrial scale production without mechanical shear stress; it is more cost effective than the batch operation because possesses advantages of continuity, easy operation, high stability and lower by-product formation (Chang, Shaw, Yang & Shieh, 2007; Delattre, Michaud & Vijayalakshmi, 2008; Halim, Kamaruddin & Fernando, 2009; Tavernier, Michaud, Wadouachi & Petit, 2009).

Therefore, the main objective of this study was the continuous production of β -CD from soluble starch. For this, a CGTase from *Thermoanaerobacter* sp. (Toruzyme®), was covalently immobilized on chitosan spheres and used in a packed bed reactor, at different operational conditions in order to achieve maximum production.

2. Materials and methods

2.1. Materials

Commercial CGTase from *Thermoanaerobacter* sp. (Toruzyme® 3.0L) was kindly provided by Novozymes A/S (Bagsvaerd, Denmark). Chitosan (from shrimp shells, $\geq 75\%$ deacetylated), phenolphthalein and β -CD were purchased from Sigma–Aldrich (St. Louis, USA). The soluble starch and glutaraldehyde were purchased from Nuclear. All chemical products used were of analytical grade.

2.2. Methods

2.2.1. Preparation and immobilization of CGTase

The solid support was prepared by precipitation using the neutralization method (Chiu, Chung, Giridhar & Wu, 2004) and activated with glutaraldehyde (5% v/v) as previously described (Klein et al., 2012). In order to test the support loading, 10 mL of enzyme solution with different protein concentrations, in sodium phosphate buffer (0.1 M, pH 6.0) was added to 100 chitosan spheres (representing 20, 60, 120 and 200 mg/g of dry support), and incubated, overnight, at room temperature under gentle shaking in an orbital shaker (200 rpm). The immobilized biocatalyst obtained was then sequentially washed with phosphate buffer (0.1 M, pH 6.0), NaCl (1 M) and ethylene glycol (0.2 M) in order to remove the unbound proteins.

Immobilizations yield and efficiency were calculated as described by Sheldon and van Pelt (2013), as follows in Eqs. 1 and 2, respectively:

$$\text{Immobilization yield (\%)} = 100 \times \left(\frac{\text{immobilized activity}}{\text{starting activity}} \right) \quad (1)$$

$$\text{Immobilization efficiency (\%)} = 100 \times \left(\frac{\text{observed activity}}{\text{immobilized activity}} \right) \quad (2)$$

2.2.2. Activity of free and immobilized CGTase and protein quantification

The CGTase activity was assayed following the synthesis of β -CD by the dye-extinction colorimetric method using phenolphthalein, developed by Vikmon (1981) and modified by Kaneko, Kato, Nakamura, & Horikoshi (1987) and Pinto, Flores, Zachia Ayub, & Hertz (2007). Reaction mixture, containing 0.65 mL of soluble starch solution (4% w/v) in 0.1 M sodium phosphate buffer (pH 6.0) and 0.35 mL of properly diluted free enzyme was incubated at 60 °C for 15 min. The reaction was stopped by addition of 4 mL of 0.04 mM phenolphthalein dissolved in 125 mM Na_2CO_3 solution. The absorbance was measured at 550 nm in spectrophotometer (model Ultrospec 3100 pro-UV/Visible, Amersham Pharmacia biotech).

The activity of immobilized preparation was measured by the same method, incubating the spheres with 1.05 mL of phosphate buffer (0.1 M, pH 6.0) and 1.95 mL of starch solution (4% w/v) of soluble starch in the same buffer. Also, the quantification of β -CD in the reactor product was made by adding phenolphthalein solution (2 mL) in 0.5 mL of the samples. A standard curve in the range between 40 and 400 $\mu\text{g/mL}$ was prepared using commercial β -CD. One unit of cyclodextrin glycosyltransferase activity (U) was defined as the amount of enzyme that produces 1 μg of β -CD per minute at 60 °C and pH 6.0 with 4% of soluble starch as a substrate solution. Protein content (mg/mL) of the solutions was determined by the Lowry assay (Lowry, Rosebrough, Farr & Randall, 1951).

2.2.3. Activity of CGTase at different temperature and pH

The effect of temperature on the activity of free and immobilized enzyme was measured at different values (50–95 °C) at pH 6.0. The relative activity was calculated as the ratio between the activity at each temperature and the maximum obtained.

The effect of pH was investigated using different buffers at various pH values (glycine–HCl pH 3.0, sodium acetate pH 4.0 and 5.0, sodium phosphate pH 6.0 and 7.0, tris–HCl pH 8.0, carbonate–bicarbonate pH 9.0 and 10.0) and the temperature fixed at 60 °C, for activity reaction. The relative activity was calculated as the ratio between the activity at each pH and the maximum obtained. A standard curve was prepared with commercial β -CD for each pH value tested.

2.2.4. Operational stability of immobilized CGTase

To investigate the stability of the immobilized enzyme and possibility of reuse, repeated batch reactions were carried out at 60 °C, with 4% of substrate solution, as described above. Between each batch reaction, the support with immobilized enzyme was washed three times with activity buffer to remove possible residues of substrate or product. The reaction cycle time was fixed at 15 min, in order to avoid product inhibition and maintain substrate saturation conditions and, thereafter, repeated with fresh solution of substrate. The activity measured in the first cycle was considered as 100% for calculation of the remaining percent activity after each one.

2.2.5. System setup of the reactor and continuous production of β -CD

The continuous production of β -CD was made in packed bed reactor composed of a water-jacketed glass column (12.0 cm $\times$ 1.2 cm), with temperature control (at 60 °C) and adjustable cylindrical plugs at both ends. In order to retain the enzyme immobilized into the reactor and to ensure the even distribution of reaction mixture across the bed, a glass wool (16 mg, Merck) was placed at the ends. The column was packed with 724 spheres of immobilized enzyme, corresponding to 7.24 mg of protein or 5579.79 U in the reactor. The substrate solution was fed through the column bottom using a peristaltic pump and, to

Table 1
Effect of the protein load in the enzyme immobilization.

Protein concentration applied (mg/g dry support)	Enzyme concentration applied (U/g dry support)	Immobilization yield (%)	Immobilization efficiency (%)
20	252.68	95.8 ± 2.24	6.1 ± 0.25
60	758.05	89.7 ± 4.51	2.6 ± 0.98
120	1516.11	94.2 ± 2.72	1.4 ± 0.16
200	2526.85	47.6 ± 2.15	1.7 ± 0.32

The analysis for immobilization yield and efficiency were performed in triplicate.

avoid substrate precipitation, it was maintained in water bath at 60 °C. Samples of 5 mL were collected in triplicate at the end of the reactor, after reaching the steady state, and analyzed for β -CD production.

To observe the effect of substrate concentration on the β -CD production, the soluble starch solution, with concentrations varying from 0.2% to 10% (w/v) in sodium phosphate buffer (0.1 M, pH 6.0), were pumped into the column in a continuous flow rate of 3 mL/min. Thereafter, to determine the effect of the flow rate, the substrate solution with 4% of soluble starch was pumped into the column in different flow rates (0.4 up to 5 mL/min).

Continuous production of β -CD was evaluated by circulating the substrate solution (0.2% w/v of starch) with a flow rate of 3 mL/min, at 60 °C, during 50 h.

3. Results and discussion

3.1. Effect of protein loading on the immobilization of enzyme

The results of immobilization yield and efficiency are presented in Table 1. The highest immobilization yield and efficiency were obtained at protein concentration of 20 mg/g of dry support. Increasing the protein concentration in the immobilization solution, both yield and efficiency immobilization decreased. The highest immobilization efficiency was 6.1% at that protein concentration, while for higher protein concentrations the immobilization efficiency was below 3%.

The low recovery of the enzyme activity can be due to steric hindrance caused by the process of immobilization that occurs between glutaraldehyde and the terminal amino of the enzyme. Thereby, the immobilization can cause diffusional restrictions of the substrate to the active site of the protein (mainly if a macromolecular substrate, as starch, is used, decreasing the activity of the enzyme after immobilization) (Amud, Presa da Silva, Tardioli, Faria Soares, Moraes & Zanin, 2008; Tardioli, Zanin & de Moraes, 2006). Results of the same magnitude, as the obtained in this work, were also found by other authors for covalent immobilization of CGTase (Martín, Plou, Alcalde & Ballesteros, 2003; Prousoontorn & Pantatan, 2007; Sobral, Rodrigues, De Oliveira, De Moraes & Zanin, 2002).

A way to solve the problem of low efficiency could be changing the immobilization conditions as contact time, pH, buffer and temperature, or even performing the immobilization of the enzyme in the presence of substrate (Cao, 2005). The substrates can protect the enzyme active site against harmful conformational changes or formation of new bonds that denature the enzyme active site during the process. Other possible solution could be the use of larger spacer arm (García-Galan, Berenguer-Murcia, Fernandez-Lafuente & Rodrigues, 2011; Matte, Nunes, Benvenuti, Schöffner, Ayub & Hertz, 2012; Tardioli, Zanin & de Moraes, 2006).

For the next experiments, the immobilization was performed with a protein load of 20 mg/g of dry support.

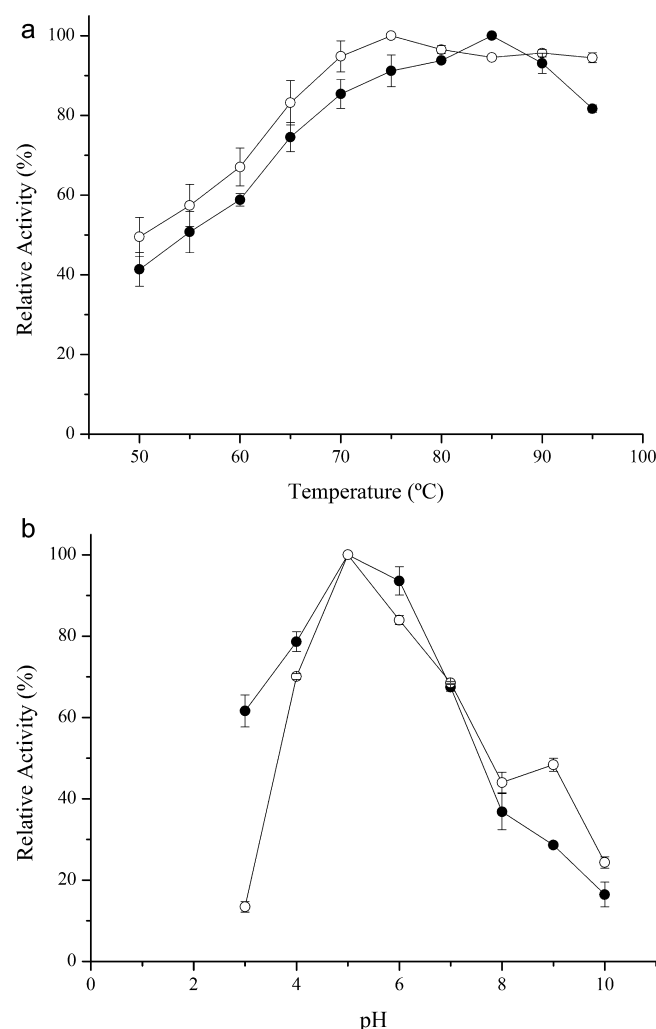


Fig. 1. Effect of the temperature (a) and pH (b) on the activity of free (○) and immobilized (●) CGTase. (a) Activities were assayed at different temperatures using sodium phosphate buffer (0.1 M, pH 6.0); 100% is equivalent to 1302 U/mL and 15796 U/g for free and immobilized CGTase, respectively. (b) Activities were assayed at 60 °C and different pH values; 100% is equivalent to 978 U/mL and 9933 U/g for free and immobilized CGTase, respectively.

3.2. Effects of temperature and pH on CGTase activity

Properties of the enzyme may suffer alterations due to the conformational changes provided by the support after immobilization. Thus, the enzymatic properties of the immobilized CGTase were compared with those of soluble enzyme. The influence of temperature and pH in their relative activities was assayed in the range of 50–95 °C and pH values of 3–10 (Fig. 1). Both biocatalysts showed an optimum pH of 5.0. The free enzyme showed maximum activity between 70 °C and 95 °C, and the immobilized enzyme between 75 °C and 90 °C. These results showed the protein structure after immobilization was similar to the free enzyme. A similar behavior regarding to optimum temperature and pH of immobilized CGTase, without major changes, was found by Martín et al. (2003) in a covalent binding to Eupergit C.

The synthesis of CDs at high temperatures improves the starch solubility and decreases the viscosity, avoiding the use of other enzymes, such as amylases, for pre-hydrolysis and solubilization of starch (Sakinah, Ismail, Hassan, Zularisam & Illias, 2009). Consequently, in the gelatinization temperature, the enzymatic rate of conversion was improved. This peculiarity of thermostable enzymes is ideal for immobilization processes and subsequent use

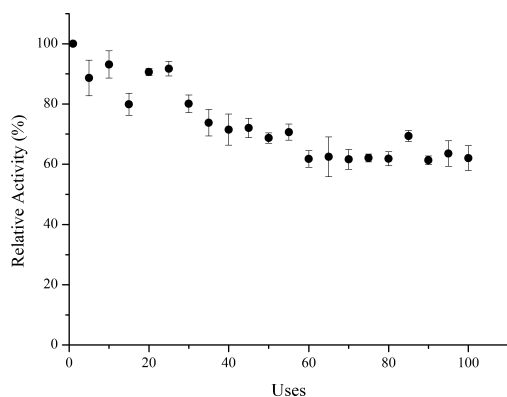


Fig. 2. Operational stability of the CGTase immobilized. Activities were assayed at 60 °C and pH 6.0 (sodium phosphate buffer 0.1 M) with 4% (w/v) of substrate in consecutive batch reactions of 15 min. Analyses were carried out in triplicate. 100% is equivalent to 10001 U/g.

in batch or continuous process, since they present a good operational stability in high temperatures (Bíwer, Antranikian & Heinzle, 2002).

3.3. Operational stability of immobilized CGTase

The operational stability of immobilized CGTase was evaluated under consecutive batch reactions, performing washes between each batch in order to remove remaining substrates and products. The results (Fig. 2) show that 61% of the original activity was retained after one hundred cycles of 15 min. A significant decrease in the enzyme activity was observed only after 60 cycles, with 68% of remaining activity. The great operational stability can be attributed to a protection afforded by this immobilized system and suggested its applicability for continuous production of cyclodextrins.

In a previous work (Matte, Nunes, Benvenuti, Schöffner, Ayub & Hertz, 2012), the same CGTase was immobilized on silica microspheres and retained 60% of its initial activity after 15 cycles of 15 min. The *Paenibacillus macerans* CGTase immobilized on PVC showed a good yield of CD (45% substrate conversion using α -CD) with more than 85% of the initial catalytic activity after 14 cycles (Abdel-Naby, 1999).

3.4. Effect of substrate concentration and flow rate on β -cyclodextrin production

The continuous production of β -cyclodextrin was evaluated in a PBR containing 5579.79 U or 7.24 mg of CGTase immobilized on chitosan spheres. This experiment was carried out at 60 °C and pH 6.0 with different starch concentrations (Fig. 3) and flow rates (Fig. 4). The amount of produced β -CD in the reactor was examined as a function of substrate concentration from 0.2 up to 10% (w/v) of soluble starch, fixing the flow rate at 3 mL/min for all experiments. At low substrate concentration (Fig. 3), the conversion rate for this particular residence time was maximal (7.9%). The increase of the starch concentration until 4% (w/v) led to a higher production rate, when excess substrate caused a decrease in conversion rate. Although at a slower rate, the production increased until a limit that the high substrate concentration caused its decrease, either by increasing the viscosity, which in turn hinders access of substrate to the enzyme, or by the fact that at high concentrations, the starch acts as an inhibitor of the reaction. The maximum β -CD content was 1.32 g β -CD/L with 4% of substrate.

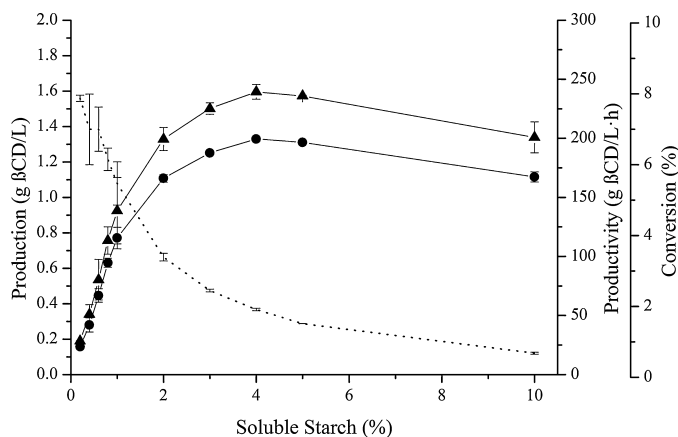


Fig. 3. Effect of the substrate concentration on the production (●), productivity (▲) and conversion (dotted line). Conditions: 7.24 mg of immobilized CGTase (5579.79 U), 60 °C, pH 6.0 (sodium phosphate buffer 0.1 M), flow rate of 3 mL/min corresponding to a residence time of 30 s.

The effects of the flow rate on the β -CD production were also investigated (Fig. 4). For this study, the substrate concentration was fixed at 4% (w/v) of soluble starch. At low flow rates (0.4–1 mL/min), the conversion rate was maximum, producing 1.697 g β -CD/L with a conversion rate of 4.3%. The increase in flow rate led to a residence time in the reactor too short and the synthesis of β -CD reduced around 30%. However, the productivity rose until it reached the maximum of 310 g β -CD/L h at 5 mL/min.

Comparing Figs. 3 and 4, it can be seen that higher amounts of β -CD were formed at the intermediate concentration of substrate and low flow rates. Tardioli and co-workers (2000), using a fluidized reactor with a load of 417 mg of immobilized CGTase (approximately 58 times higher than in this study) and maltodextrin as a substrate, obtained 13.2% of conversion in β -CD, producing 12.76 g/L of β -CD with 13 min of residence time. In this work, using 4% soluble starch as substrate, it was reached, at a residence time of 1 min, values of 3.8% of conversion to β -CD, and production of 1.51 g β -CD/L.

The importance of substrate concentration and residence time was studied by Tavernier et al. (2009) in continuous production of oligoglucuronans by immobilized glucuronan lyase, when it was possible to control the degree of polymerization of oligoglucuronans by varying these parameters.

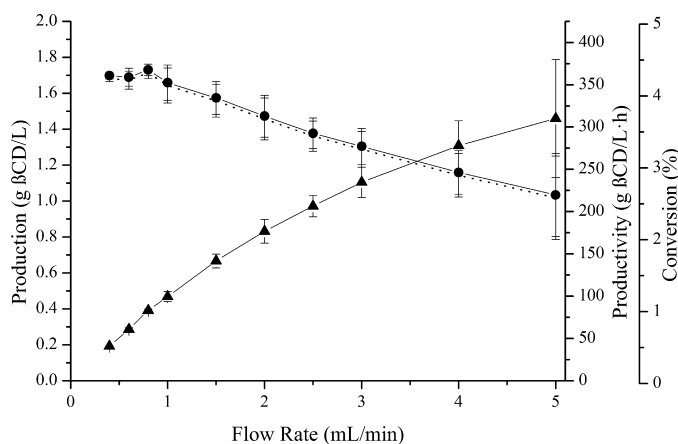


Fig. 4. Effect of the substrate flow rate on the production (●), productivity (▲) and conversion (dotted line). Conditions: 7.24 mg of immobilized CGTase (5579.79 U), 60 °C, pH 6.0 (sodium phosphate buffer 0.1 M) and substrate concentration of 4% (w/v).

3.5. Continuous production of β -cyclodextrin and operational stability of the reactor

To evaluate the operational stability of the PBR, it was continuously used with the same amount of immobilized CGTase. The operational conditions were selected based on the previous results; flow rate was chosen to be an intermediate value between production and productivity (3 mL/min), and the substrate concentration (0.2% w/v) on the higher conversion rate (7.87%). The continuous operation was carried out during 52 h. The production of β -CD decreased after 49 hours, reaching 78% (± 7.03) of the initial activity. This occurred due to the super packing of spheres, causing a reduction of 45% of bed height, and the consequent decrease in the residence time of the substrate from 1.22 min to 0.14 min. However, after the spheres being washed and re-packed to the column, at the same initial conditions, they recovered 100% of their initial activity for at least more 50 h, i.e., although the super packing of chitosan spheres, it was possible to recover the initial activity performing a buffer wash at room temperature.

4. Conclusions

In this work, cyclodextrin glycosyltransferase (CGTase) from *Thermoanaerobacter* sp. was immobilized on glutaraldehyde-activated chitosan spheres, a polymer known for its low propensity for microbial contamination. The immobilized enzyme exhibits no significant changes in their characteristics (optimum temperature and pH) and has a promising operational stability for the continuous production of CDs.

In the packed bed reactor it was possible to achieve a productivity of the 239 g de β -CD/Lh at a substrate concentration of 4%. This ratio can be increased to above 310 g de β -CD/Lh by increasing the flow rate from 3 to 5 mL/min. Moreover, the biocatalyst presented a very high operational stability. It was possible to use the immobilized CGTase for 100 batches retaining around 60% of its initial activity, or in continuous process, to use for 100 h, performing a buffer wash after 50 h, maintaining the same production rate. These results demonstrated that this system may be effective for the scale-up of production of CDs in a packed bed reactor, making possible to control it by changing flow rate and substrate concentration.

Acknowledgment

The authors thank, CNPq, Fapergs, Novozymes A/S for the gift of the Toruzyme.

References

- Abdel-Naby, M. A. (1999). Immobilization of *Paenibacillus macerans* NRRL B-3186 cyclodextrin glucosyltransferase and properties of the immobilized enzyme. *Process Biochemistry*, 34, 399–405.
- Amud, A. E., Presa da Silva, G. R., Tardioli, P. W., Faria Soares, C. M., Moraes, F. F., & Zanin, G. M. (2008). Methods and supports for immobilization and stabilization of cyclomaltodextrin glucanotransferase from *Thermoanaerobacter*. *Applied Biochemistry and Biotechnology*, 146(1–3), 189–201.
- Astray, G., Mejuto, J. C., Morales, J., Rial-Otero, R., & Simal-Gandara, J. (2010). Factors controlling flavors binding constants to cyclodextrins and their applications in foods. *Food Research International*, 43(4), 1212–1218.
- Biwer, A., Antranikian, G., & Heinzle, E. (2002). Enzymatic production of cyclodextrins. *Applied Microbiology and Biotechnology*, 59(6), 609–617.
- Cao, L. (2005). Immobilised enzymes: science or art? *Current Opinion in Chemical Biology*, 9(2), 217–226.
- Chang, S.-W., Shaw, J.-F., Yang, C.-K., & Shieh, C.-J. (2007). Optimal continuous biosynthesis of hexyl laurate by a packed bed bioreactor. *Process Biochemistry*, 42(9), 1362–1366.
- Chiou, S.-H., & Wu, W.-T. (2004). Immobilization of *Candida rugosa* lipase on chitosan with activation of the hydroxyl groups. *Biomaterials*, 25(2), 197–204.
- Chiu, S. H., Chung, T. W., Giridhar, R., & Wu, W. T. (2004). Immobilization of beta-cyclodextrin in chitosan beads for separation of cholesterol from egg yolk. *Food Research International*, 37(3), 217–223.
- Delattre, C., Michaud, P., & Vijayalakshmi, M. A. (2008). New monolithic enzymatic micro-reactor for the fast production and purification of oligogalacturonides. *Journal of Chromatography B*, 861(2), 203–208.
- García-Galan, C., Berenguer-Murcia, A., Fernandez-Lafuente, R., & Rodrigues, R. C. (2011). Potential of different enzyme immobilization strategies to improve enzyme performance. *Advanced Synthesis & Catalysis*, 353(16), 2885–2904.
- Goh, K. M., Mahadi, N. M., Hassan, O., Rahman, R., & Illias, R. M. (2009). A predominant beta-CGTase G1 engineered to elucidate the relationship between protein structure and product specificity. *Journal of Molecular Catalysis B: Enzymatic*, 57(1–4), 270–277.
- Goycoolea, F. M., Argüelles-Moná, W., Peniche, C., & Higuera-Ciampara, I. (2000). Chitin and Chitosan. *Novel Macromolecules in Food Systems*, 265–308.
- Halim, S. F. A., Kamaruddin, A. H., & Fernando, W. J. N. (2009). Continuous biosynthesis of biodiesel from waste cooking palm oil in a packed bed reactor: Optimization using response surface methodology (RSM) and mass transfer studies. *Bioresource Technology*, 100(2), 710–716.
- Juang, R. S., Wu, F. C., & Tseng, R. L. (2002). Use of chemically modified chitosan beads for sorption and enzyme immobilization. *Advances in Environmental Research*, 6(2), 171–177.
- Kaneko, T., Kato, T., Nakamura, N., & Horikoshi, K. (1987). Spectrophotometric determination of cyclization activity of β -cyclodextrin-forming cyclomaltodextrin glucanotransferase. *Journal of the Japanese Society of Starch Science*, 34(1), 45–48.
- Klein, M. P., Nunes, M. R., Rodrigues, R. C., Benvenutti, E. V., Costa, T. M. H., Hertz, P. F., & Ninow, J. L. (2012). Effect of the support size on the properties of beta-galactosidase immobilized on chitosan: Advantages and disadvantages of macro and nanoparticles. *Biomacromolecules*, 13(8), 2456–2464.
- Krajewska, B. (2004). Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. *Enzyme and Microbial Technology*, 35(2–3), 126–139.
- Lee, Y.-H., Lee, S.-H., & Shin, H.-D. (1991). Performance of column type bioreactor packed with immobilized cyclodextrin glucanotransferase for cyclodextrin production. *Journal of Microbiology and Biotechnology*, 1(1), 63–69.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 193(1), 265–275.
- Martin, M. T., Plou, F. J., Alcalde, M., & Ballesteros, A. (2003). Immobilization on Eupergit C of cyclodextrin glucosyltransferase (CGTase) and properties of the immobilized biocatalyst. *Journal of Molecular Catalysis B: Enzymatic*, 21(4–6), 299–308.
- Matte, C. R., Nunes, M. R., Benvenutti, E. V., Schöffner, J. d. N., Ayub, M. A. Z., & Hertz, P. F. (2012). Characterization of cyclodextrin glucosyltransferase immobilized on silica microspheres via aminopropyltrimethoxysilane as a spacer arm. *Journal of Molecular Catalysis B: Enzymatic*, 78, 51–56.
- Muzzarelli, R. A. A. (1980). Immobilization of enzymes on chitin and chitosan. *Enzyme and Microbial Technology*, 2(3), 177–184.
- Muzzarelli, R. A. A. (1996). Chitin. In J. C. Salamone (Ed.), *The polymeric materials encyclopedia* (pp. 295–300). Boca Raton FL, USA: CRC Press, Inc.
- Muzzarelli, R. A. A. (2012). Nanochitins and nanochitosans, paving the way to eco-friendly and energy-saving exploitation of marine resources. In K. M. Jaszewski, & M. Moller (Eds.), *Polymer science: A comprehensive reference* (Vol. 10) (pp. 153–164). Amsterdam: Elsevier BV.
- Nakai, Y., Yamamoto, K., Terada, K., & Watanabe, D. (1987). New methods for preparing cyclodextrin inclusion-compounds. 1. Heating in a sealed container. *Chemical & Pharmaceutical Bulletin*, 35(11), 4609–4615.
- Pinto, F. S. T., Flores, S. H., Zachia Ayub, M. A., & Hertz, P. F. (2007). Production of cyclodextrin glucosyltransferase by alkaliphilic *Bacillus circulans* in submerged and solid-state cultivation. *Bioprocess and Biosystems Engineering*, 30(5), 377–382.
- Prousoontorn, M. H., & Pantatan, S. (2007). Production of 2-O-alpha-glucopyranosyl β -ascorbic acid from ascorbic acid and beta-cyclodextrin using immobilized cyclodextrin glucosyltransferase. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, 57(1–4), 39–46.
- Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, A., Torres, R., & Fernandez-Lafuente, R. (2013). Modifying enzyme activity and selectivity by immobilization. *Chemical Society Reviews*, 42, 6290–6307.
- Sakinah, A. M. M., Ismail, A. F., Hassan, O., Zularisam, A. W., & Illias, R. M. (2009). Influence of starch pretreatment on yield of cyclodextrins and performance of ultrafiltration membranes. *Desalination*, 239(1–3), 317–333.
- Sheldon, R. A., & van Pelt, S. (2013). Enzyme immobilisation in biocatalysis: Why, what and how. *Chemical Society Reviews*, 42, 6223–6235.
- Sobral, K. A., Rodrigues, R. O., De Oliveira, R., De Moraes, F., & Zanin, G. (2002). Immobilization of Cyclodextrin glucosyltransferase (CGTase) from *Bacillus firmus* in commercial chitosan. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, 44(1–4), 383–386.
- Sobral, K. A., Rodrigues, R. O., Oliveira, R. D., Olivo, J. E., de Moraes, F. F., & Zanin, G. M. (2003). Evaluation of supports and methods for immobilization of enzyme cyclodextrin glucosyltransferase. *Applied Biochemistry and Biotechnology*, 105, 809–819.
- Stella, V. J., & He, Q. (2008). Cyclodextrins. *Toxicologic Pathology*, 36(1), 30–42.
- Szejtli, J. (1982). Cyclodextrins and their inclusion complexes. *Starch*, 34, 395–401.
- Szenté, L., & Szejtli, J. (2004). Cyclodextrins as food ingredients. *Trends in Food Science & Technology*, 15(3–4), 137–142.
- Tardioli, P. W., Zanin, G. M., & de Moraes, F. F. (2000). Production of cyclodextrins in a fluidized-bed reactor using cyclodextrin-glycosyl-transferase. *Applied Biochemistry and Biotechnology*, 84–86, 1003–1019.

- Tardioli, P. W., Zanin, G. M., & de Moraes, F. F. (2006). Characterization of *Thermoanaerobacter* cyclomaltodextrin glucanotransferase immobilized on glyoxyl-agarose. *Enzyme and Microbial Technology*, 39(6), 1270–1278.
- Tavernier, M. L., Michaud, P., Wadouachi, A., & Petit, E. (2009). Continuous production of oligoglucuronans by immobilized glucuronan lyase. *Enzyme and Microbial Technology*, 45(1), 48–52.
- Vikmon, M. (1981). Rapid and simple spectrophotometric method for determination of microamounts of cyclodextrins. In J. Szejtli (Ed.), *Proceedings of the first international symposium on cyclodextrins* (pp. 69–74). Dordrecht, Netherlands: D. Reidel Publishing Company.