International Food Research Journal 24(1): 353-358 (February 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Immobilization of fungi β-galactosidase on celite to produce galactooligosaccharides during lactose hydrolysis

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Article history

Received: 25 January 2016
Received in revised form: 8 March 2016
Accepted: 16 March 2016

Keywords

β-galactosidase
Prebiotic
Transgalactosylation reaction
Celite
Covalent binding;
Semicontinuous process

Abstract

Galactooligosaccharides were synthetized from lactose in a fixed-bed reactor using β-galactosidase from *Aspergillus oryzae* covalently coupled to celite. Properties of immobilized β-galactosidase were characterized and compared with those of the soluble enzyme. The optimum pHs for soluble and immobilized β-galactosidase's activity were 4.6 and 4.0, respectively. The optimum temperature for the free enzyme was 40°C, yet this value was 10°C higher when characterizing the immobilized enzyme. Immobilized β-galactosidase had a high stability when stored in buffer at 4°C for 270 days and had a good operational stability when used 10 times repeatedly. Oligosaccharides were obtained in fixed-bed reactor with a productivity of 3.5 g/L.h from 400 g/L (w/v) lactose solution for 12 hours and lactose was 84.74% hydrolyzed for 24 hours.

Introduction

Functional foods are generally defined as foods or food ingredients which impart a health benefit above and beyond the nutritional value expected from food (Goslin *et al*., 2010; Jovanovic-Malinovskaab and Kuzmanova, 2014; Fai and Pastore, 2015). The group of foods that contains galactooligosaccharides (GOS) is an example of functional foods, which, after *in vitro* animal and human studies, have been established as prebiotic ingredients (Torres *et al*., 2010; Fai *et al.*, 2015).

GOS have many beneficial effects, such as improving lactose tolerance and digestibility of milk products; preventing pathogenic, autogenic diarrhea and constipation; increasing absorptions of different minerals in the intestine; reducing toxic metabolites, undesirable enzymes and serum cholesterol; depressing blood pressure, among others. Therefore, their application as a food additive for health purposes has led to an increase in their commercial demand (Zheng *et al.*, 2006; Gobinath and Prapulla, 2014).

Moreover, the stability under acidic conditions of GOS during food processing makes them potentially applicable as ingredients for a wide variety of food products. Their excellent taste quality and relatively low sweetness make GOS interesting functional sweeteners. They pass the small intestine without being digested and are, therefore, of low caloric value. In addition, GOS cannot be metabolized by microorganisms of the oral cavity and are thus not implicated in the formation of dental caries (Crittenden and Playne, 1996; Manera *et al*., 2010; Gänzle, 2012; Sangwan *et al*., 2014).

GOS could be produced by β-galactosidase (EC.3.2.1.23) treatment of high concentrations of lactose in a transgalactosylation reaction. This transgalactosylation reaction is an intermediary step when galactose units are polymerized to a glucose end unit to form GOS of several polymerization degrees. However, as the reaction continues, all sugars, including GOS, will be hydrolyzed to glucose and galactose monosaccharides (Matella, Dolan and Lee, 2006; Gänzle, 2012; Intanon *et al*., 2014; Fai *et al*., 2015).

β-Galactosidases are present in a wide variety of sources including plants, animals and microorganisms. As for most reactions catalysed by a biological entity, there are many modes in which GOS producing reactions can be performed using

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free or immobilized enzymes, or even whole cells (Goslin *et al*., 2010; Panesar, Kumari and Panesar, 2010; Vera *et al*., 2012).

The use of immobilization technology is of significant importance from an economic point of view since it makes reutilization of the enzyme as well as continuous operation possible and can also help to improve the enzyme stability (Klein *et al.*, 2013). The classification of the immobilization methods is related to the properties of the original enzyme, the type of support used and the methods of support activation as well as enzyme attachment (Worsfold, 1995; Rodrigues *et al*., 2013). Adsorption and covalent coupling are two of the most used techniques, each one with their own merits and demerits. Adsorption is simple but causes problems of leaching; covalent coupling overcomes this, but harsh conditions lead to undesirable loss of activity at times (Worsfold, 1995; Panesar, Kumari and Panesar, 2010; Liu, *et al.*, 2012; Rodrigues *et al.*, 2013).

Some previous research has shown a promising potential to synthesis GOS by different types of β-galactosidase immobilization techniques and undoubtedly these investigations are important steps in commercial and fundamental enzymology (Gaur *et al.*, 2006; Matella, Dolan and Lee, 2006; Grosová, Rosenberg and Rebros, 2008; Li *et al*., 2008; Klein *et al*., 2013; Fai *et al*., 2014). The present study demonstrates an appropriate immobilization method for β-galactosidase on celite, which can be applicable in oligosaccharide synthesis in fixed bed reactors.

Materials and Methods

Material

Aspergillus oryzae β-galactosidase (EC 3.2.1.23) and (p-aminophenyl) trimethoxysilane were obtained from Sigma Co., USA. A glucose oxidase kit was purchased from Labtest, Brazil. All the other reagents used were of analytical grade.

Enzyme adsorption on celite support

Two mg of β-galactosidase (23.96 U/mg) were mixed with celite (1 g, suspended in 10 mL of 0.1 M acetate buffer, pH 4.5) and incubated overnight at 20ºC under constant shaking. The preparation was repeatedly washed with buffer until no protein and enzyme activity were detected in the wash, when analyzed as described in sections 2.5 and 2.6. The celite-bound enzyme was suspended in 5 mL of the same buffer and used as an immobilized preparation for further studies (Gaur *et al*., 2006).

Immobilization support preparation for covalent binding

Celite was heated at 370ºC in an oven for 3h to remove organic matter (the oven was heated to the temperature, before the celite was treated), followed by silanization with a 0.5% (v/v) γ-aminopropyltriethoxysilane (APS) aqueous solution at pH 3.3. The support was then incubated at 75ºC/3h, further washed thoroughly with deionized water and dried at 105ºC/15 h. The silanized support was then activated with glutaraldehyde 2.5% (v/v) in buffer acetate 0.2 M, pH 5.0 for 45 minutes. The support was exhaustively washed with deionized water, which was followed immediately by the immobilization procedure. This methodology was adapted from Zanin and Moraes (1998) and Melo *et al*. (2005).

Celite covalent binding enzyme

β-galactosidase (2 mg) was mixed with celite (1.0 g suspended in 10 mL of 0.1 M acetate buffer, pH 4.5) and incubated overnight at 20ºC under constant shaking. The preparation was repeatedly washed with buffer until the wastewater was protein and enzyme activity free. The celite-bound enzyme was suspended in 5 mL of the same buffer and used as an immobilized preparation for further studies (Gaur *et al*., 2006).

Protein estimation

Protein was determined by the Bradford method (1976) using bovine serum albumin (BSA) as the protein standard (Gaur *et al*., 2006).

β -Galactosidase assay

Activity of free and immobilized β-galactosidase was estimated by the Food Chemical Codex method (Food Chemical Codex, 1991), using o-nitrophenyl-β D-galactopyranoside (ONPG) as the substrate. Enzyme activity was defined as the amount of enzyme that liberated 1 μmol of o-nitrophenol (ONP) per minute under the standard assay conditions. An extinction coefficient for ONP of 4.3 mM was calculated and used.

Effect of pH and temperature on enzyme activity

Optimum temperature and pH were determined by individually changing the conditions of the β-galactosidase activity assay: pH from 4 to 9 and temperature from 30 to 70ºC. An acetate buffer solution (0.2 M) was used for solutions at pH values between 4 and 6. Phosphate buffers (0.2 M) were used for values above this range (Tanriseven and Dogan, 2002).

Determination of kinetic parameters Km and Vmax

Free and immobilized β-galactosidase were used to measure the kinetic using ONPG as the substrate. Km and Vmax were determined using the Lineweaver–Burk plot method (Zhou and Chen, 2001).

Synthesis of galactooligosaccharides

Fixed-bed reactor studies were carried out in a jacketed glass column with lactose recycle in order to produce galactooligosaccharides. The immobilized β-galactosidase, obtained as previously described, was transferred to a jacketed column (2×8 cm) and to 40% lactose solution in 0.2 M acetate buffer (w/v), pH 4. This lactose solution circulated through a fixed-bed reactor in a recycle process using a MasterFlex® L/S peristaltic pump. Temperature of the column was maintained at 45°C using an ultrathermostate Quimis® Q-14 M2 bath (precision temperature ±0.1°C). Sample aliquots (1 mL) were collected at various times from 2 to 24 hours and kept in a boiling water bath for 10 min. Oligosaccharides formed were analyzed as described in section 2.10.

Estimation of oligosaccharides

The identification and quantification of sugars (lactose, glucose, galactose and GOS) was carried out by ion exchange chromatography with pulsed amperometric detection (HPLC-PAD). A Dionex (USA) chromatograph, supplied with a Carbopac PA1 (4 x 250 mm) column, a PA1 (4 x 50 mm) guard column, with a GP50 gradient pump, ED40 electrochemical detector and PEAKNET software (Dionex , USA) were used for the analyses. Sugars were eluted with 20 mM sodium hydroxide, at a flow rate of 1.0 mL/min at room temperature. Before injection, the samples were diluted with water and filtered through 0.22 µm filters (Manera *et al*., 2010; Fai *et al*., 2015).

Results and Discussion

β-galactosidase adsorbed on celite and activated with glutaraldehyde retained nearly 11.3 and 27% of the original activity, respectively. As it can be observed, enzymatic activity of covalent binding immobilization in this study was 2.5 fold higher when compared to the adsorption technique used. For this reason, we chose to continue this study using only the covalent immobilization technique. Table 1 compares the results of free and celite covalent binding enzyme in terms of enzymatic activity and kinetic parameters.

It is interesting that Vmax was less affected than Km upon immobilization in the present study. The change in affinity between the enzyme and its substrate is caused by lower affinity between the substrate and the active site of the immobilized enzyme (Haider and Husain, 2009). Due to the covalent binding, the Km value increased when compared to soluble β-galactosidase. The change of Vmax points to conformational changes in the enzyme during immobilization.

Table 1. Characterization of free and immobilized *Aspergillus oryzae* β-galactosidase. Values represent the means of triplicate sets.

Parameters	Free enzyme	Covalent binding enzyme
Enzymatic activity (U/mg)	11.98 ± 1.73	3.23 ± 0.65
pH optima	4.6 ± 0.00	4.0 ± 0.00
Temperature optima (°C)	40 ± 0.00	50 ± 0.00
Km (mM)	2.34 ± 0.49	9.12 ± 1.66
Vmax (µmol ONP/min)	43.47 ± 1.54	15.08 ± 0.29

One of the limitations associated with enzyme industrial application is their high cost and instability under operational conditions. The overall process becomes cost-effective if the preparation shows higher efficiency, operational stability and reusability (Gaur *et al*., 2006). Celite covalent binding enzyme, in the present research, was reused for 10 cycles without significant loss in the enzymatic activity (retained at least 86% of its activity) and after 270 days of storage, suspended in 0.1 M acetate buffer, pH 4.5 at 3.5°C, retained 85% of its residual enzymatic activity.

For kinetic parameters, pH, thermal, storage, and operational stability, both free and immobilized enzyme were determined using ONPG. It is assumed that both reactions (synthesis and hydrolyze) are usually the same for these enzymatic parameters (Huber, Kurz and Wallenfels, 1976; Becerra *et al*., 2001; Gänzle, 2012).

It is worthy highlighting that glycosidases, in general, are known for their capability to carry out hydrolytic catalysis and transfer reactions, where the sugar residue forming the glucone part of the substrate can be transferred to water or any other hydroxyl acceptor sugars groups. It has been established that the same enzyme catalyzes hydrolytic, synthetic and transfer reactions when it comes to β-galactosidase. These observations can be explained assuming that the acceptor and aglycone occupy the same position in the enzyme molecule (Wallenfels and Malhotra, 1961).

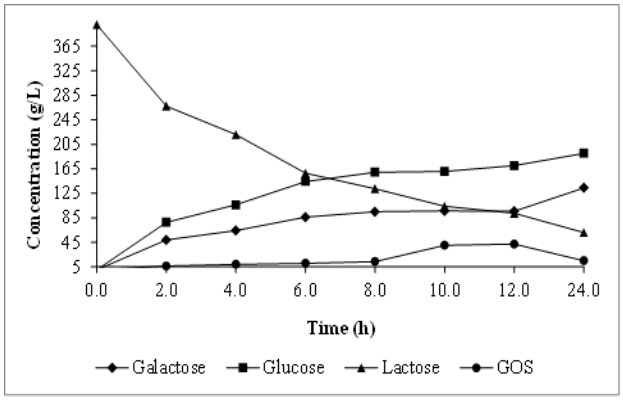

Figure 1. Time course of GOS synthesis in the fixed-bed reactor with lactose recycle in the first cycle, from 40% (w/w) lactose, pH 5.0, 45ºC. CV% = range of 0.44 to 4.80

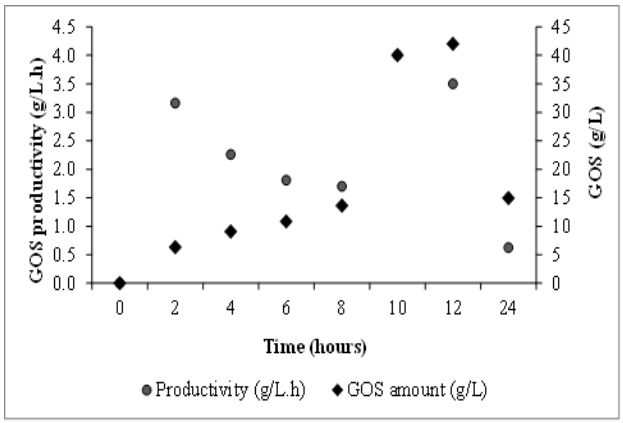

Figure 3. Kinetics of GOS synthetized and productivity catalyzed by immobilized enzyme in the fixed-bed reactor in the first cycle, from 40% (w/w) lactose, pH 5.0, 45ºC. CV% = range of 0.44 to 4.60

It is precisely for these reasons that β-galactosidase has received particular interest since it can mediate the transgalactosylation reaction for the preparation of GOS, just as hydrolytic reaction of lactose for the preparation of low-lactose milk and dairy products.

The GOS production and lactose hydrolysis was monitored and is shown in Figure 1 and Figure 2, respectively. The present study showed a maximum GOS production of 42 g/L, after 12 hours of reaction, showing a productivity of 3.5 g/L.h as seen in Figure 3. The maximum productivity of GOS obtained under these conditions was 4.0 g/L.h after 10h. The total lactose conversion to glucose, galactose and GOS were 76.93 and 84.74% after 12 and 24 hours, respectively. Packed bed reactor was reused for 10 cycles.

Our results also indicate that extending incubation time does not necessarily increase GOS amount as seen in Figure 3. This result is mainly due to the GOS formation as an intermediary step of β-galactosidase hydrolysis, which is dominant near the beginning of the reaction (Matella, Dolan and Lee, 2006).

The mechanism outlined for β-galactosidase

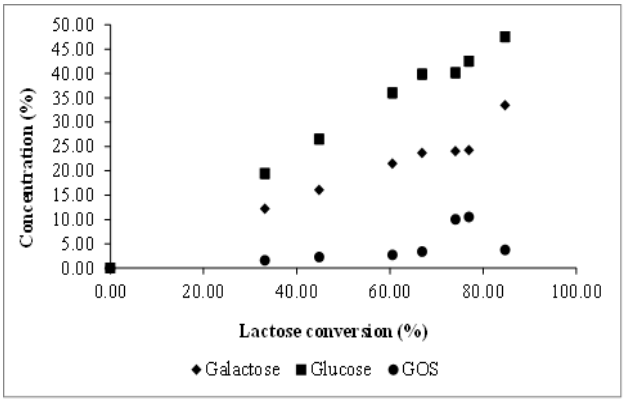

Figure 2. Lactose conversion, and other sugars concentration, catalyzed by immobilized enzyme in the fixed-bed reactor in the first cycle, from 40% (w/w) lactose, pH 5.0, 45ºC. CV% = range of 0.44 to 4.77

indicates that the enzyme will transfer galactose to nucleophilic acceptors containing a hydroxyl group. If the transference happens with water, galactose will happen as the reaction product; if it happens with another sugar, di-, tri- and higher galactosyl-saccharides, collectively termed oligosaccharides, will be obtained instead. In turn, these products happen to be the substrates for the enzyme, being slowly hydrolyzed (Mahoney, 1998; Gänzle, 2012).

These results concur with numerous researchers that have also synthesized GOS using immobilized β-galactosidases from different microorganisms (Albayrak and Yang, 2002; Chockchaisawasdee *et al.*, 2005; Gaur *et al*., 2006; Splechtna, Nguyen and Haltrich, 2007; Manera *et al*., 2010; Urrutia *et al*., 2012).

Pectinex Ultra SP-L, a commercial enzyme preparation obtained from *Aspergillus aculeatus,* containing β-galactosidase activity, was immobilized onto Eupergit C and produced from 30 % (w/v) lactose by 24 h reaction 38.4 and 47.40 g/L of GOS for free and immobilized enzyme, respectively, corresponding to a productivity of 1.6 and 1.97 g/L/h (Aslan and Tanriseven, 2007).

Gaur *et al.* (2006) studied *A. oryzae* β-galactosidase covalently coupled to chitosan and aggregated by glutaraldehyde and reported a 17.3 and 4.6% oligosaccharide yield respectively, within 2 h in a 20% (w/v) lactose solution. In their work the maximum oligosaccharide productivity was 17 and 4.6 g/L.h for the chitosan immobilized enzyme preparation and cross-linked aggregates, respectively.

Fai *et al.* (2014) reported that *A. oryzae* β-galactosidase covalently immobilized on glutaraldehyde-treated chitosan powder could also be used for the production of GOS and to hydrolyze lactose. In this study maximum productivity of 14.42 g/L.h from 400 g/L (w/v) lactose solution after 2

hours of reaction was achieved. Lactose was 44 % hydrolyzed in 12 hours.

In another research GOS were continuously produced using Bullera singularis β-galactosidase immobilized in chitosan beads in a packed bed reactor with a productivity of 4.4 g/L.h from 100 g/L lactose and 6.5 g/L.h from 300 g/L lactose solution (Shin, Park and Yang, 1998).

Conclusions

The production of GOS using *Aspergillus oryzae* β-galactosidase covalently coupled to celite was described in this paper. Immobilized β-galactosidase had high stability and good operational stability, which points to its excellent potential to be used as support. Oligosaccharides obtained in fixed-bed reactor reach a maximum productivity of 4.0 g/L.h from 400 g/L (w/v) lactose solution in 12 hours. The maximum 42 g/L-GOS-production was achieved after 12 hours of reaction and the total lactose conversion to glucose, galactose and GOS were 76.93 and 84.74% after 12 and 24 hours, respectively. Thus, the process can be an interesting alternative for GOS production and lactose hydrolyze.

Acknowledgments

The authors are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Universidade Estadual de Campinas (Unicamp) for financial support.

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