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Different strategies for cyclodextrin production: Ultrafiltration systems, CGTase immobilization and use of a complexing agent

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ABSTRACT

The study comparatively evaluated diverse strategic models of cyclodextrin (CD) production by the CGTase of *Bacillus firmus* strain 37: continuous production and repetitive batches in ultrafiltration systems; immobilization of CGTase on curdlan and vegetable sponge natural supports; the use of the glycyrrhizin complexing agent to modulate CGTase selectivity in favor of γ-CD production. All strategies had in common the possibility of separation of CGTase from its inhibitory products and its reuse. In the continuous production model, at 48 h of assay, the highest productivity and selectivity for β-CD were obtained, 1.47 mmol/L/h and 92.8%, respectively. Glycyrrhizin was able to modulate the production of γ-CD with selectivity of 61.2% for 30-h batches. The comparative evaluation of the different strategic models for obtaining CDs showed particularities that should be considered, and most of the models studied returned satisfactory yields as well as excellent selectivity.

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides obtained from starch by means of a molecular transglycosylation reaction catalyzed by the enzyme cyclodextrin glycosyltransferase (CGTase). The main CDs are α-, β-, and γ-CD, and have respectively 6, 7, and 8 glucose units joined by α-1,4 glycosidic bonds. The physical-chemical properties of the CDs make them capable of forming inclusion complexes with several other molecules, providing, for example, better solubility and stability, as well as generating a series of technological and industrial applications (Del Valle, 2004; Van Der Veen, Uitdehaag, Dijkstra, & Dijkhuizen, 2000).

CDs were first isolated in 1891, but knowledge of their structure, the study of complex formation properties, the preparation of derivatives, and the characterization of CGTases only developed in the mid 1930s and early 1970s (Szejtli, 1988). From the 1970s, CDs began to be used in the pharmaceutical, food, chemical, and other industries, motivated by the large number of articles and patents in the area, by reliable toxicological studies, and by the production of β-CD on an industrial scale (Szejtli, 1987).

The cyclizing activity of CGTase can be inhibited in the presence of high concentrations of product, which causes a decrease in the production of CDs as its concentration in the reaction medium increases. Thus, in the batch production of CDs, when the reaction mixture is close to its final equilibrium, CGTase becomes insensitive to variations in substrate concentration. The possibility of higher concentrations of enzyme would only cause the equilibrium to settle down more quickly. Therefore, an alternative to improve the yield of CDs is to remove their inhibitory products accumulated in the reaction mixture, which can be obtained by the use of an ultrafiltration system coupled to the reactor containing the reaction medium. Ultrafiltration systems, whether conventional or tangential, allow the use of different production strategies, such as the repetitive batch model or continuous production (Fenelon, Aguiar, Miyoshi, Martinez, & Matioli, 2015; Gastón, Costa, & Ferrarotti, 2015; Gastón, Szerman, Costa, Krymkiewicz, & Ferrarotti, 2009; Gawande & Patkar, 2001; Kim, Lee, & Kim, 1993; Slominska, Szostek, & Grzeskowiak, 2002).

CGTase immobilization is also an important tool, which presents many potential advantages when compared to its use in free form, such as repeated and prolonged use of the immobilized enzymes, and the ability to separate the enzymes from the reaction medium and undertake the continuous production process in simpler reactors (Shuler & Kargi, 2002; Schöffer, Klein, Rodrigues, & Hertz, 2013; Schöffer et al., 2017b). Natural supports have been increasingly targeted for use in immobilization processes. The vegetable sponge is a low cost, easily available, and renewable plant derivative. This matrix is free of toxic

Abbreviations: CDs, cyclodextrins; α-CD, alpha cyclodextrin; β-CD, beta cyclodextrin; γ-CD, gamma cyclodextrin; CGTase, cyclodextrin glycosyltransferase; NMWL, nominal molecular weight limit

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materials, simple to apply and operate, and has high stability during long-term repeated use (Iqbal, Saeed, Edyvean, O'Sullivan, & Styring, 2005). Another possible support is curdlan, a high molecular weight glucose polymer, more specifically a β-1,3-glucan produced by non-pathogenic strains of *Agrobacterium* sp. and recently approved for food use by the United States Food and Drug Administration (US FDA). Its high molecular weight and large number of free hydroxyl groups, available for activation and binding to other molecules, suggest that curdlan is suitable for enzyme immobilization (Saudagar & Singhal, 2004).

The high solubility of γ-CD facilitates the preparation of more concentrated solutions of active molecules. However, α- and β-CD are more frequently produced on an industrial scale, due to the low production of γ-CD by microbial CGTases (Wang, Wu, Chen, & Wu, 2013). One of the ways of achieving a better yield of γ-CD is to prevent its destruction by reversible reactions that may occur during its production. This is possible by the formation of a stable γ-CD complex with a suitable complexing agent, such as glycyrrhizin. This substance is natural, non-toxic, and has a high sweetening power. In addition, it complexes with γ-CD with a specificity of 100% compared to the other CDs (Matioli, Zanin, & Moraes, 2000; Sato, Nagano, Yagi, & Ishikura, 1985; Sato & Yagi, 1991).

Thus, the aim of this work was to evaluate comparatively different strategies of CD production, which have in common the possibility of the separation of CGTase from its inhibitory products in the reaction medium, and consequently its reuse and better exploitation. For all assays, the CGTase from *Bacillus firmus* strain 37 and 5% (w/v) corn starch substrate in the presence of 10% ethanol (v/v) were used under the reaction conditions optimized in a previous study (Fenelon et al., 2015). In the first strategy, two different ultrafiltration systems were used for the production of β-CD in the form of continuous and repetitive batches. The second set of strategies comprised the immobilization of CGTase in two natural supports, curdlan and vegetable sponge, and the evaluation of the production of CDs in repetitive batches. The last strategy adopted was the use of the glycyrrhizin complexing agent to modulate the selectivity of CGTase in favor of γ-CD, increasing its yield in the repetitive batch model in the ultrafiltration system.

2. Materials and methods

2.1. Materials

The α-, β-, and γ-CDs used as standard, and the glycyrrhizin, used as a complexing agent, were purchased from Sigma (St. Louis, MO, USA). Corn starch, used as a substrate in all trials, was food grade (Maizena, Unilever Company) and was purchased at a local market. Maltodextrin (dextrin 10 from corn starch, article 31410), used in determinations of enzyme activity, was obtained from Fluka (Buchs, Switzerland). Curdlan, used as immobilization support, was purchased from Wako Pure Chemical Industries (Osaka, Japan), and the vegetable sponge *in natura* was obtained from a local market. All other chemicals used were of analytical grade.

2.2. Enzyme and reaction medium

The CGTase enzyme used in all experiments was obtained from *B. firmus* strain 37, isolated from cassava cultivation soil by Matioli, Zanin, Guimarães, and Moraes (1998).

For the immobilization strategies, CGTase was purified by biospecific affinity chromatography, according to the methodology described by Moriwaki, Mazzer, Pazzetto, and Matioli (2009). For all other strategies, the CGTase used was semi-purified by the methodology studied by Fenelon et al. (2015).

The enzymatic activity, protein content, and specific enzymatic activity of the samples were determined to standardize the enzyme concentration in relation to the volumes of reaction media for the production of CDs. In all assays, the enzymatic concentration used in the reaction medium was 0.1 U/mL. One unit of enzyme activity (U) was defined as the amount of CGTase producing 1 μmol of β-CD per minute under test conditions.

The general composition of the reaction medium used was: 5% (w/v) corn starch substrate, 10% (v/v) ethanol, 20% (v/v) Tris-HCl 50 mmol/L buffer (pH 8.0), 10% $CaCl_2$ 5 mmol/L solution, and purified water q.s. 100% (Fenelon et al., 2015).

2.3. Production of CDs in 12-h repetitive batches in an ultrafiltration system

The ultrafiltration system for CD production in repetitive batches was previously presented by Fenelon et al. (2015). At that time, 8 repetitive batches were performed, and it was observed that the batches with duration of 12 h offered advantages for the productivity and selectivity of β-CD. However, in the previously tested model, the starch concentration was not adjusted to each new batch, causing an increase in medium viscosity and gradual reduction of the ultrafiltration flow after each batch. In this study, the number of batches was extended and the concentration of substrate in the reaction medium was corrected according to the starch conversion rate in CDs.

Using the semi-purified CGTase from *B. firmus* strain 37 at an enzymatic concentration of 0.1 U/mL of reaction medium, CD production was carried out using 5% (w/v) corn starch substrate in the presence of 10% (v/v) ethanol, pH 8.0, in a system of repetitive batches with a duration of 12 h. Among each batch, the reaction medium was ultrafiltered to remove the CDs and other inhibitory products formed during the reaction. CGTase was recovered and immediately reused in the next batch. The assay was performed in a jacketed glass reactor with a capacity of 50 mL of reaction medium, which remained under constant stirring at 50 °C.

At the end of the first 12-h batch, the entire volume of the reaction medium was transferred to an Amicon® device (Merck Millipore, Darmstadt, Germany) using a peristaltic pump, upon which it was subjected to ultrafiltration through a membrane NMWL of 10 kDa and 44.5 mm in diameter, using nitrogen gas pressure and under stirring. The CGTase of *B. firmus* strain 37 has a molecular weight of approximately 78,000 Da (Matioli, Zanin, & Moraes, 2001) and thus was retained by the membrane. However, α-, β-, and γ-CD, which have weights of 972, 1135, and 1297 Da, respectively, were filtered.

The filtrate was collected and stored for determination of CD concentration. All the retained volume (containing the enzyme) was suspended in a small amount of 50 mmol/L Tris-HCl buffer and, with the aid of the peristaltic pump, transferred back to the reactor. A new 50 mL of production medium was added with the corrected corn starch concentration according to the 25% conversion rate reported by Fenelon et al. (2015). Under the same conditions as the first batch, subsequent reactions of 12 h were carried out, with application of the ultrafiltration procedure at the end of each. Fig. 1 presents the strategy of producing CDs in repetitive batches in an ultrafiltration system. In this model, a single peristaltic pump was used to transfer the reaction medium to the ultrafiltration device, and then to return the unfiltered components (enzyme and substrate) to the reactor, which was fed with a new reaction medium to perform the following batch.

2.4. Production of CDs in a continuous ultrafiltration system

Using the same reaction parameters as described in Section 2.3, the CD production was carried out in a glass jacketed reactor with capacity of 500 mL of reaction medium, coupled to an Ultrafiltration Module (TE-0198, Tecnal, Piracicaba, Brazil) equipped with a hollow fiber column with a 50 kDa NMWL exclusion limit. The reactor was also coupled to a feed system for replacement of the converted substrate. The volume of the reaction medium in the reactor was maintained at 500 mL; however, the total operational volume was 800 mL as 300 mL were circulating between the conduits of the system and the column.