Continuous Production of Cyclodextrins in an Ultrafiltration Membrane Reactor, Catalyzed by Cyclodextrin Glycosyltransferase From *Bacillus circulans* DF 9R

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Cyclodextrins (CDs) are cyclic oligosaccharides of wide industrial application, whose synthesis is catalyzed by Cyclodextrin glycosyltransferase (CGTase) from starch. Here, CDs were produced using CGTase from Bacillus circulans DF 9R in continuous process and an ultrafiltration membrane reactor. The batch process was conducted as a control. This method allowed increasing the yield from 40 to 55.6% and the productivity from 26.1 to 99.5 mg of CD per unit of enzyme. The method also allowed obtaining a high-purity product. The flow rate remained at 50% of its initial value after 24 h of process, improving the results described in the literature for starch hydrolysis processes. CGTase remained active throughout the process, which could be explained by the protective effect of the substrate and reaction products on CGTase stability. In addition, batch processes were developed using starches from different sources. We concluded that any of the starches studied could be used as substrate for CD production with similar yields and product specificity. © 2015 American Institute of Chemical Engineers *Biotechnol. Prog.*, 31:695–699, 2015
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Introduction

Cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19) is a member of the glycoside hydrolase family 13, also known as the α-amylase family.[1] This enzyme converts starch into mixtures of cyclic, linear, and limit dextrins. Cyclodextrins (CDs) are the most important products of CGTase; small concentrations of glucose, maltose, and maltotriose and negligible amounts of other linear maltooligosaccharides are also obtained. Limit dextrins are insoluble and branched polysaccharides, generated by the action of CGTase on amylopectin, because this enzyme is unable to attack the α 1–6 linkages that give rise to the ramifications of this starch fraction.[2]

CDs are nonreducing maltooligosaccharides with a hydrophilic surface and a hydrophobic central cavity. The most common types are α, β, and γ-CDs, which consist of 6, 7, or 8 glucose residues, respectively, linked by α-1,4 bonds.[3] Because these molecules can form inclusion complexes with many compounds, they are widely used in the food, pharmaceutical, cosmetic, and chemical industries.[4–6] In the last years, there has been a progressive increase in the number of publications and patents related to the production of CDs.[7] Because the purification of α- and γ-CDs considerably increases the cost of production, 97% of the CDs used in the market are β-CDs.[5] Research has been conducted with the aim to enhance the performance of the CD production process and to change the specificity of CGTases to increase the concentration of a particular CD.[8] Generally, the enzymatic conversion of starch is performed in batch reactors, using high concentrations of substrate. However, our previous studies[2] have shown that CGTase from *Bacillus circulans* DF 9R is inhibited by starch concentrations higher than 20 mg/mL. We have also shown that CDs and other reaction products such as maltose and maltotriose inhibit CGTase activity. Because of this, it is interesting to develop systems with immobilized enzymes that allow the separation of the products as they are formed. Among these systems, the enzyme membrane reactor is highlighted because it enables the localization of the enzymes within a defined area and the preservation of its catalytic activity, thus allowing reuse. Furthermore, when the reaction products are removed, their inhibitory effects on the activity of the enzyme are decreased.[9] On the other hand, membrane fouling and enzyme activity decay are responsible for strong limitations in the performance of enzyme membrane reactors.[10] Regarding CD production, some researchers have used ultrafiltration membrane reactors, but could not prolong the processing time due to the inactivation of the enzyme or membrane fouling.[11–13]

In a previous study, we described the optimization of CD production from cassava starch as substrate catalyzed by CGTase from *B. circulans* DF 9R in a batch process.[14] The purpose of the present study was to optimize the conditions for the bioconversion of starch into CD using a reactor coupled to an ultrafiltration membrane able to retain the substrate and enzyme and allow the passage of the reaction products of smaller size. We also compared the production of CDs using starches from different sources as substrate.

Additional Supporting Information may be found in the online version of this article.

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Materials and Methods

Reagents

α, β, and γ-CDs; soluble potato, corn, rice, and wheat starches; glucose, maltose, and maltotriose were obtained from Sigma Chemical Co., MO. Food-grade cassava starch was obtained from a local supplier. The other chemicals used were AR grade from Merck, Darmstadt, Germany.

CGTase production

The experiments were performed using CGTase obtained from *B. circulans* DF 9R and purified by affinity chromatography on α-CD coupled to Sepharose-4B.[15] The strain is deposited at the Microbial Culture Collection, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (Buenos Aires, Argentina), catalog number CCM-A-29: 1290 from the WFCC. The strain was cultured in a minimum saline medium with starch (MAS) consisting of 1.5% cassava starch, 0.4% $(NH_4)_2SO_4$, 100 mM phosphate buffer pH 7.6, 0.002% $MgSO_4$, and 0.002% $FeSO_4$.[16]

CGTase cyclizing activity

The cyclizing activity of CGTase was determined according to the phenolphthalein method,[17] measuring β-CD production spectrophotometrically at 550 nm on the basis of its ability to form a colorless inclusion complex with this dye. One unit of CGTase is defined as the amount of enzyme that catalyzes the production of 1 μmol of β-CD per min under the reaction conditions.

Colorimetric determination of CD

α-CD concentration was determined by the decrease in absorbance at 507 nm because of the formation of a complex between methyl orange and α-CD.[18] β-CD concentration was determined according to the method described in the section CGTase cyclizing activity. γ-CD concentration was determined measuring the absorbance at 630 nm because of the formation of an inclusion complex with bromocresol green.[19]

Analysis of products by HPLC

The CDs obtained were analyzed by HPLC using a Könik KNK-500 apparatus, with a column for carbohydrate analysis (LiChrospher® 100 NH_2 – 5 μm) at 30°C. Samples (20 μL, 20–40 mg of carbohydrate/mL) were injected and oligosaccharides were eluted with acetonitrile:water (70:30), at a flow rate of 1.0 mL/min. Sugars were detected with a Shimadzu RID-10A differential refraction detector. α, β, and γ-CD, glucose, maltose, and maltotriose were used as standards for HPLC analysis.

Effect of reaction products on CGTase stability

To evaluate the effect of reaction products on CGTase stability, thermal inactivation was performed at 56°C in sodium phosphate buffer 25 mM pH 6.4 with addition of maltose, β-limit dextrins, or α, β, or γ-CD. It is known that each of these compounds individually are not attacked by the enzyme.[2] A solution of purified CGTase, with 3.1 U/mL in the same buffer, was used as control. The concentrations of maltose and CD were 10-fold higher than the K_I of each compound, which are sufficient to ensure saturation conditions. Three different concentrations of β-limit dextrins were tested (1.0, 2.0, and 5.0 mg/mL). Aliquots were taken at different times during a period of 140 min and the residual activity was determined.

CD production in batch

For the production of CDs in batch, we reproduced the conditions described by Szerman et al.[14] A suspension of 5.0% cassava starch in phosphate buffer 25 mM pH 6.4 was pretreated for 3 min at 95°C to gelatinize the substrate. Then, 15 U of purified CGTase per gram of starch was added and the mixture was incubated at 56°C for 4 h at 100 rpm. Subsequently, the reaction mixture was centrifuged for 15 min at 25,000*g* and the supernatant was ultrafiltered through YM10 membranes to separate the CDs produced and to retain molecules larger than the cut-off value of 10 kDa. All the experiments were performed at least three times. The reaction products contained in the ultrafiltrate were analyzed by colorimetric reactions and HPLC.

CD production in continuous operation

The continuous production of CD was performed using as reactor a 3-L BioFlo 110 New Brunswick Scientific, USA, coupled to an Ultrafiltration cell, model 8200, Amicon, USA, with Amicon YM10 membrane (diameter 63.5 mm, NMWL 10 kDa). To 1 L of reaction medium consisting of 2.0% liquefied cassava starch solution in phosphate buffer 25 mM pH 6.4, 15 U of purified enzyme per gram of starch was added. The incubation conditions were 56°C and 100 rpm. The initial flow rate was 2.0 mL/min using a vacuum pump. Fractions containing the products whose molecular weights were below 10 kDa were collected at different times during a period of 24 h. As the solution products were separated, the reactor was fed automatically, completing the volume to 1 L with fresh solution of starch in phosphate buffer through the action of a level sensor and a peristaltic pump. The reaction products were analyzed by colorimetric reactions and HPLC.

Results and Discussion

CD production in batch

The conditions for CD production from cassava starch in a batch process using CGTase from *B. circulans* DF 9R were those optimized by Szerman et al.[14]

The highest yield was obtained using 5.0% gelatinized cassava starch and 15 U of purified enzyme per gram of starch in phosphate buffer 25 mM pH 6.4. The mixture was incubated for 4 h at 56°C and 100 rpm. To compare the performance of the process and the CD profile obtained using starches from various sources such as potato, corn, rice, and wheat, several batches were performed in the conditions described above. CDs were determined by HPLC analysis and chromatograms showed elution profiles similar to those obtained using cassava starch as substrate (Supporting Information). On the basis of those chromatograms, we estimated the yield of CD production for each starch. The analysis of variance showed no significant differences in the efficiency of conversion of starch to total CD $(P < 0.001)$ between the different starches and no differences in the α-:β-:γ-CD relationship (Table 1). Therefore, starches from different sources as those tested could be used as substrate to produce CDs

with similar yields. These results disagree with the statement by Biwer et al.,[20] who claimed that the yield and selectivity of the process depend on the type of substrate. Other researchers have described the use of starches from different sources to obtain CDs. Although potato starch is the most commonly used, corn and wheat starch have also been used. However, the latter contain a high proportion of amylose to amylopectin ratio, which leads to decreased production of CD compared with that obtained with potato starch.[14] Although cassava starch is one of the least frequently used, it seems to be a good substrate because of its high content of amylopectin and low liquefaction temperature. Charoenlap et al.[21] optimized CD production using starch from *Cycas revoluta*, a plant species from Southeast Asia commonly known as Sago palm. Moriwaki et al.[22] used a strain of *Bacillus firmus* to transform starch from various sources into CD. These authors obtained the best results with corn starch and concluded that starches from different sources can affect the production of CD and that this could be due to differences in the physical structure of the starch granules and their properties. Ibrahim et al.[23] used starches from various sources, including corn, potato, sago, rice, and cassava and obtained the highest CD conversion with potato starch (38.7%). These authors found no differences in the α-:β-:γ-CD relation obtained by varying the starch source. Zhekova and Stanchev[24] achieved 45.88% corn starch conversion to CD by working with CGTase from *Bacillus megaterium*. Urban et al.[25] compared the conversion of starch to CD by CGTase from *Paenibacillus macerans* CCM 2012 and obtained a yield of 22.24% with amaranth starch and 14.56% with corn starch.

Continuous process for CD production in an ultrafiltration membrane reactor

In a previous work in our laboratory, we showed that the activity of CGTase from *B. circulans* DF 9R is inhibited by high concentrations of substrate and reaction products.[2] CDs behaved as competitive inhibitors, maltose as an uncompetitive inhibitor, and maltotriose showed a mixed inhibition pattern. Therefore, we attempted to increase the yield of conversion from starch to CD by lowering the substrate concentration and the removal of products from the reaction mixture as they are formed. To reach this goal, we developed a continuous process to obtain CDs using a reactor coupled to an ultrafiltration cell. This approach also allows the reuse of the enzyme. The membrane reactor was designed in our laboratory and its features are shown in Figure 1. The ultrafiltration membrane used is 63.5 mm in diameter and has a cut-off of 10 kDa. The cut-off value was selected on the basis of the reports of other authors who found that lower cut-off values lead to a reduction in the permeate flux.[12] This membrane retains the enzymes whose molecular weight is 74.47 kDa and allows the passage of CDs whose molecular weight is on average 1 kDa. To prevent membrane clogging, a system of magnetic agitation was located on its surface. The reaction solution used was 2% cassava starch because, at concentrations higher than that, the enzyme was inhibited.[2] Fractions were collected at different times and the volume and CD concentrations were determined. After 24 h reaction, the concentration of each CD had values similar to those obtained at the initial stages. This demonstrated that the enzyme remained active and could be used for a longer time period (Figure 2).

Table 1. CD Production From Starches of Different Sources

Starch	Yield (%)*	Ratio α-:β-:γ-CD
Cassava	39.2 ± 1.6	1.0:1.7:0.4
Potato	40.2 ± 2.5	1.0:1.8:0.4
Corn	38.0 ± 2.0	1.0:1.8:0.4
Rice	37.7 ± 1.4	1.0:1.8:0.4
Wheat	37.6 ± 2.4	1.0:1.8:0.4

*Relative to the mass of starch added to the reaction medium.

The process yield was 55.6 ± 0.2%, higher than that obtained with the same starch in the batch process (39.2 ± 1.6%). The productivity calculated was 99.5 mg of CD per unit of CGTase for the continuous process and 26.1 mg of CD per unit of CGTase for the batch process. Regarding product specificity, the concentrations of β- and γ-CD obtained in the continuous process were slightly lower than those obtained in the batch process. The α-:β-:γ-CD relationship throughout the process was 1.0:1.4:0.3. An additional advantage of this system is the high purity of the final product, because the enzyme and the unreacted substrate are retained within the reactor.

Son et al.[13] obtained 49.7% yield with a CGTase from *Bacillus macerans,* using a stirred tank equipped with an ultrafiltration membrane, but the production system developed was repeated-batch. Gawande and Patkar[11] compared the production of CDs in batch and continuous process at 40°C, catalyzed by α-CGTase from *Klebsiella pneumoniae*

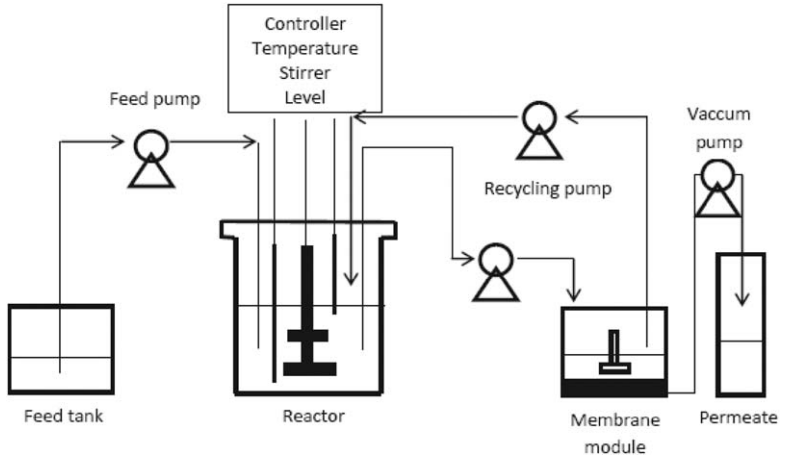

Figure 1. Graphic representation of a reactor coupled to an ultrafiltration membrane.

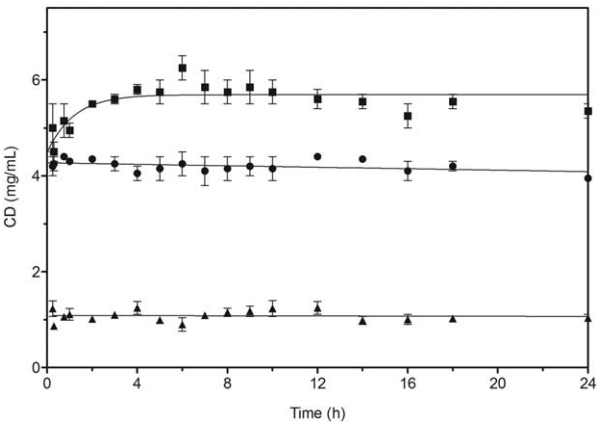

Figure 2. Concentrations of α-CD (square), β-CD (circle) and γ-CD (triangle) determined in the fractions collected along the process for 24 h using a reactor coupled to an ultrafiltration membrane.

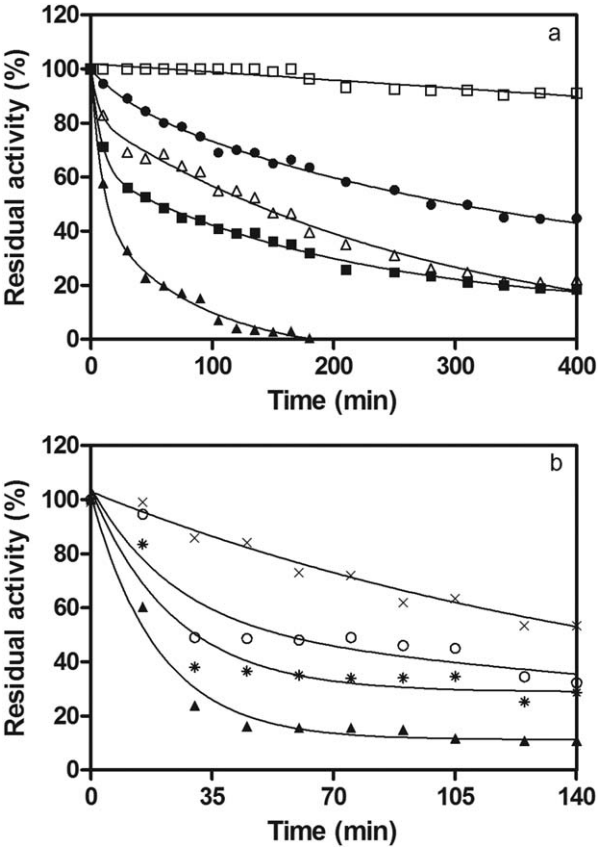

Figure 3. Thermal stability of CGTase at 56°C in sodium phosphate buffer 25 mM pH 6.4 without (triangle) and with addition of carbohydrates: (a) Maltose (square), α-CD (open square), β-CD (circle) and γ-CD (open triangle). Concentrations were 10-fold higher than the *Ki* of each compound. (b) β-limit dextrins at three concentrations (mg/ml) 1.0 (asterisk), 2.0 (open circle) and 5.0 (plus sign). Aliquots were taken at different times and the residual activity was determined.

AS-22 and found that the yield was 15% lower in the continuous process. The authors attributed these results to a possible inactivation of the enzyme during its circulation or adsorption to the membrane. In fact, in that work, the residual activity after 5 h process was 50%. Slominska et al.[12] also observed a decrease in enzyme activity during the process and attributed this observation to the entrapment of the enzyme in the membrane. According to Rios et al.,[10] enzyme activity decay could be due to several phenomena such as catalyst leakage, enzyme denaturation under the effects of pH or temperature, shear effects or adsorption/deposit at the wall. However, CGTase from *B. circulans* DF 9R maintained nearly 100% of its initial activity after 24 h at 56°C. Stability studies showed that the enzyme was mainly stabilized by CD and β-limit dextrins (Figure 3). Although the CDs are removed from the reactor via the ultrafiltration membrane, limit dextrins become concentrated. Moreover, we observed that limit dextrins had little inhibitory effect on CGTase activity, even at high concentrations. A concentration of β-limit dextrins 10-fold higher than that of starch showed a 10% decrease in the inhibitory effect on the β-cyclizing activity.[2] We observed that starch and its hydrolysis products also have stabilizing effect on the enzyme. However, this effect is difficult to quantify because the substrate is attacked by the enzyme and its composition varies during the assay. Another point to consider is the decrease in the flow rate throughout the process (Figure 4). Although the flow rate decreased to a value of about 50% in the first 300 min of the process, its development was not precluded. According to Rios et al.[10] a pre-hydrolysis step before continuous operation minimizes fouling troubles in starch hydrolysis reactions. This would be due to the decrease in the viscosity of the solution. Starch liquefaction before use as substrate may have contributed to preventing membrane fouling and may be the reason for the maintenance of the flow rate during the production of CDs.

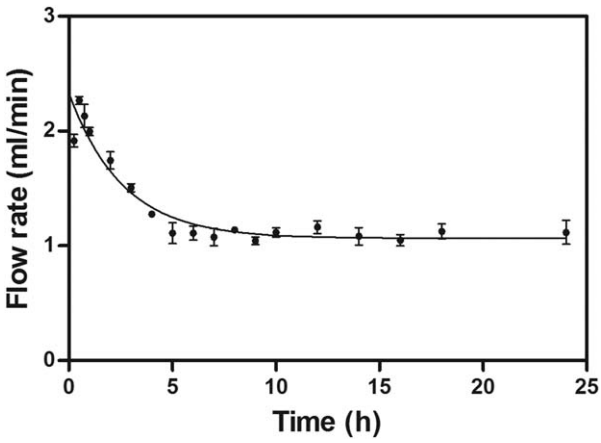

Figure 4. Flow rate throughout the continuous process for CD production in ultrafiltration membrane reactor.

Conclusions

CD production from cassava starch catalyzed by CGTase from *B. circulans* DF 9R was conducted in batch and continuous operation in an ultrafiltration membrane reactor. The yield obtained in each case was 39.2 and 55.6%, respectively. The productivity was 26.1 mg of CD per unit of enzyme for the process in batch and 99.5 mg of CD per unit of enzyme for the continuous process. The flow rate remained at 50% of its initial value after 24 h of process, improving the results described in the literature for starch hydrolysis processes. These results could be due to the pre-hydrolysis of starch. CGTase remained active for at least

24 h, which could be explained by the protective effect of the reaction products on the enzyme stability demonstrated by thermal inactivation assays. The batch tests performed using potato, corn, rice, and wheat starch as substrate showed that these polysaccharides are also suitable substrates for CD production, showing yields close to 40% and similar product specificity.

Acknowledgments

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