

β -Cyclodextrin Production by Simultaneous Fermentation and Cyclization

HERON O. S. LIMA, FLAVIO F. DE MORAES, AND
GISELLA M. ZANIN*

State University of Maringá, Chemical Engineering Department, Av. Colombo,
5790 - BL. E46 - 09, 87020-900, Maringá, PR, Brazil

ABSTRACT

Production of β -cyclodextrin (CD) with high-dextrose equivalent (DE) starch hydrolysates by simultaneous fermentation and cyclization (SFC) gives higher yields than using only the enzyme CGTase, because fermentation eliminates glucose and maltose that inhibit CD production, while at the same time, produces ethanol that increases yield. A 10% (w/v) solution of cassava starch, liquefied with α -amylase, was incubated with CGTase using: only the enzyme, added ethanol (from 1 to 5%), and added yeast, *S. cerevisiae* (12% w/v), plus nutrients, the latter being the SFC process. Reaction conditions were: 38°C, pH 6.0, DE from 2 to 25, and 3.3 mL of CGTase/L. The yield of β -CD has decreased with an increase in DE, and maximum reaction yields were found for DE equal to 3.54, reaching 5.6, 14.7, and 11.5 mM β -CD, respectively. For an increase of DE, of approx 6 times (from 3.54 to 23.79), β -CD yield decreased 6 times for the first, and second reaction media with 3% (v/v) ethanol, and only approx 3 times for SFC (from 11.5 to 3.73 mM), showing that this process is less sensitive to variations in the DE.

Index Entries: Cyclodextrin; CGTase; fermentation; cassava starch.

INTRODUCTION

Cyclodextrins (CDs) are cyclic oligosaccharides that are normally formed by 6 to 8 glucopyranose units, linked by α -1,4 bonds. The most common cyclodextrins are: α -CD (cyclohexamilose), β -CD (cycloheptamilose), and γ -CD (cyclooctamilose). These cyclic maltodextrins are produced through the action of the enzyme cyclodextrin glycosyl transferase (CGTase) upon liquefied starch (1). The ring structure formed is highly hydrophilic on the outside because of the great numbers of hydroxyls, but

* Author to whom all correspondence and reprint requests should be addressed (E-mail: gisellazanin@cybertelecom.com.br).

relatively hydrophobic inside because of the glycosylic bonds and hydrogen atoms that face the interior of the cavity (2). In aqueous solutions, this structure allows the inclusion of nonpolar molecules of suitable size, inside the cyclodextrin cavity. For long and complex molecules it may be its most hydrophobic parts that can be included inside the CDs (3–5). This property of the CDs makes them valuable microencapsulation products at the molecular level, conferring chemical and physical stabilization to the complexed substances. As a consequence of this property, CDs have a broad actual and potential field of application in various industries such as pharmaceutical, food, cosmetics, and agroindustries (6,7).

The production of β -CD from corn and potato starches with the addition of solvents (ethanol among others) was reviewed recently (8–13). There are at least four mechanisms through which a solvent can enhance CD production (12). The solvent could:

1. Affect starch structure and accessibility by opening the starch molecule.
2. Reduce the concentration of CD products by complexation and precipitation, and therefore shift the reaction towards the formation of products.
3. Change the enzyme conformation and improve affinity.
4. Reduce water activity, and therefore decrease hydrolytic reactions that produce small size oligosaccharides, which inhibit CD production.

Ethanol is emerging as a solvent with greater potential of being applied to enhance CD production, because (11–14):

1. Ethanol has no toxicity restriction if CDs produced in this way would be applied in the food or pharmaceutical industries.
2. CDs form inclusion complexes with ethanol and become less susceptible to reverse decomposition reactions.
3. Ethanol helps control microbial contamination and can be easily removed by distillation and be recycled.
4. Fermentation industries have long experience with handling ethanol, and the risks of flammability are safely controlled.

In these processes, reaction yield is affected by the presence of maltooligosaccharides that not only participate in reactions of intermolecular transglycosylation, but also function as inhibitors of the CGTase enzyme. Noteworthy among these saccharides are glucose and maltose. Mori et al. (13) observed that the presence of ethanol and glucose enhances the reverse decomposition of CDs. Therefore, it seemed plausible that, elimination of glucose and addition of ethanol higher CD yields. This is the concept of the simultaneous fermentation and cyclization process (SFC) proposed in this article. The basic idea is to eliminate glucose and maltose by fermentation, because they inhibit CD production, and at the same time produce ethanol that increases CD yield.

Given the great potential of application of the CDs, and the abundance of cassava starch available at low price in Brazil, it was decided to study in this work the production of β -CD through the process of simultaneous fermentation and cyclization (SFC), offering therefore, another alternative end use for a national resource.

OBJECTIVES

The following objectives were set for this work:

1. Production of liquefied cassava starch solutions of a desired dextrose equivalent (DE) value.
2. Determination of the activity profile for Wacker (Munich, Germany) CGTase as a function of pH using liquefied cassava starch as substrate.
3. To study the influence of enzyme dosage on β -CD yield.
4. To determine the influence of the yeast nutrients upon the CGTase.
5. To select compromise conditions for the production of cyclodextrins using simultaneously CGTase, and fermentation by yeast.
6. To study the influence of ethanol on β -CD production.
7. To test the production of CDs using three comparative conditions:
 - a. Only the enzyme CGTase.
 - b. Addition of ethanol (CGTase + ethanol). In this condition ethanol is added to the composition of the substrate solution.
 - c. SFC process (CGTase + yeast + nutrients). This condition constitutes the simultaneous fermentation and cyclization process (SFC), in which ethanol is produced by yeast fermentation at the same time that CDs are produced by the enzyme CGTase.
8. To evaluate the SFC process in comparison with the other conditions.

MATERIALS AND METHODS

Substrate

The substrate is cassava starch (Copagra) at a nominal concentration of 10% (w/v), and actual 9.33% (w/v) dry-solids content, liquefied with Novo Termamyl α -amylase (4.3 μ L of enzyme/g of starch) for different lengths of reaction time at 95°C, pH 6.0, giving maltodextrin solutions of different dextrose equivalent (DE), from 2 to 25. After reaching the DE value desired, the α -amylase was inactivated with 0.27% (v/v) of 1 N HCl and boiling for 10 min. Then the pH of the solution was corrected to 6.0 with 1 N NaOH.

CGTase Enzyme

The CGTase enzyme used was a gift from Wacker. It was obtained from *E. coli* engineered with CGTase from an alkalophilic *Bacillus* sp.1 (15). Specific enzyme activity at 38°C, and pH 6.0 was 70.5 μ mol β -CD/min/mg

of protein, determined as given below. Enzyme concentration in the stock solution was 0.997 mg of protein/mL.

Yeast and Nutrients

Commercial dried baker's yeast from Fleischmann containing *Saccharomyces cerevisiae* cells was used. Following the recommendation of Lima et al. (16) to obtain a well-developed fermentation, the commercial products ammonium sulfate and triple superphosphate were used, as a source of nitrogen and phosphorus, each in the proportion of 0.1% (w/v) of substrate solution. Yeast and nutrients were used together with CGTase in the simultaneous fermentation and cyclization process (SFC).

CGTase Enzyme Activity as a Function of pH

This test was conducted in assay tubes containing a final concentration of 0.5% (w/v) liquefied cassava starch, 0.1 mL CGTase/L, Tris-HCl buffer 0.01 M, and CaCl_2 5 mM, at 38°C, and pH in the range of 5.0 to 8.0.

Influence of Enzyme Dosage on β -CD Yield

For this test, liquefied cassava starch with a DE of 23.5 was used at 38°C, pH 6.0, and the CGTase enzyme was added in one of the following concentrations: 0.16, 3.3, 5, and 6.6 mL of enzyme/L. Samples were taken at regular intervals with the objective of following the production of β -CD during a period of 24 h.

Influence of Yeast Nutrients on β -CD Yields

In this case, the production of β -CD as a function of time was followed in the presence of 0.1% (w/v) of ammonium sulfate and 0.1% (w/v) triple superphosphate that provide the essential nutrients for yeast fermentation. However, during this test, yeast cells were not added, and therefore, there was no fermentation, the objective of the experiment being to test the influence of these nutrients on β -CD yield. Liquefied cassava starch with a DE of 26.06, 10% (w/v) was used at 38°C and pH 6.0, with 1 mL CGTase/L.

Influence of Ethanol on β -CD Yield

This test was conducted with liquefied cassava starch, 10% (w/v), with two DE values, namely 12.88 and 23.37 at 38°C, pH 6.0, and two ethanol concentrations: 3 and 5% (v/v) respectively. CGTase concentration was set to 1 mL/L and the production of β -CD was followed for a period of 24 h.

Production of β -CD by the SFC Process and Other Comparative Conditions

The substrate solution was incubated with either 0.8 or 3.3 mL of CGTase/L in three different test conditions:

1. Only the enzyme CGTase.
2. Addition of ethanol: CGTase + ethanol (1 to 5% v/v).
3. SFC process: CGTase + yeast (12% w/v) + nutrients (0.1% w/v of both ammonium sulfate and triple superphosphate).

Erlenmeyer flasks of 250 mL containing 150 mL of the reaction mixture were incubated and gently shaken in a Dubnoff thermoregulated water bath. The DE of the liquefied cassava starch solution was varied from approx 2 to 25, and reaction conditions were 38°C, pH 6.0. Samples of 0.5 mL were taken at regular intervals, boiled for 10 min, stocked at 4°C, and later assayed for β -CD produced. Each test was run for a period of 24 h.

Assay Methods

Protein concentration was determined by the Coomassie blue method according to Bradford (17). The DE was determined through the reducing-sugar method of Somogyi (18), using glucose as standard. The quantity of β -CD produced in the reaction medium was assayed by the dye-extinction method of Vikmon (19), modified by Mäkelä et al. (20) and Hamon and Moraes (21). The concentration of ethanol produced by fermentation was determined by gas chromatography after ethanol separation by distillation.

RESULTS AND DISCUSSION

Production of Liquefied Cassava Starch Solutions of Desired DE

Figure 1 presents the data on dextrose equivalent (DE) as a function of hydrolysis time. The production of liquefied cassava starch, within the specified conditions, gives DE values that increases linearly with hydrolysis time up to 15 min, and for the whole period tested it can be correlated by the following equation:

$$DE = 1.101 t / (1 + 0.001875 t^{1.649}) \quad (1)$$

valid for the interval $0 \leq t \leq 50$ min, where t is the reaction time, and $r^2 = 0.9986$.

These results were used for producing the other cassava starch solutions of desired DE values as used in this work.

Activity as a Function of pH for Wacker CGTase

Figure 2 shows the results for the activity of Wacker CGTase as a function of pH. It can be seen that optimum activity is observed in the pH range of 6.0 to 7.0; at 38°C. For pH 6.0 the activity value is 70.5 μ mol β -CD/min/mg of protein. These results are in accord with data obtained by Hamon and Moraes (21) with the same enzyme, and Merck soluble starch

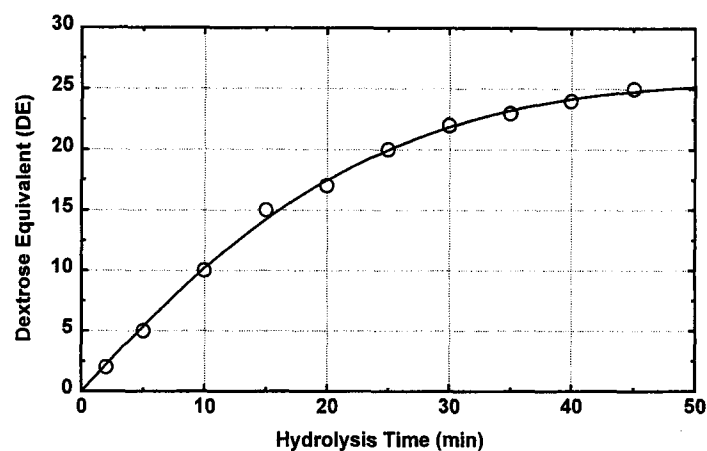


Fig. 1. Dextrose equivalent (DE) as a function of hydrolysis time, 10% (w/v) liquefied cassava starch, at 95°C, pH 6.0, 4.3 μmol of Novo Termamyl/g of starch.

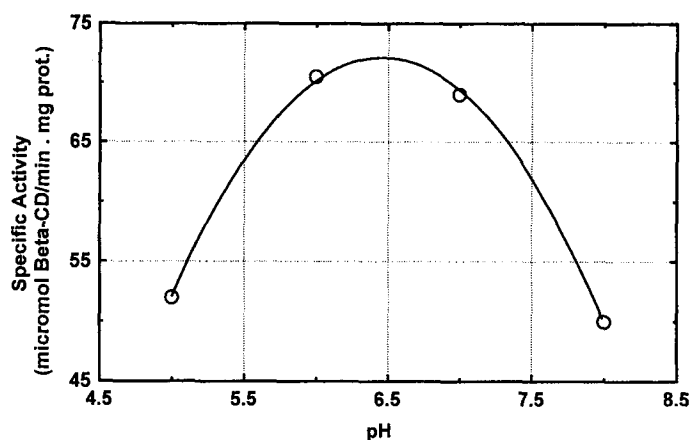


Fig. 2. Activity profile of Wacker CGTase as a function of pH. Reaction conditions: 0.5% (w/v) liquefied cassava starch, 0.1 mL CGTase/L, Tris-HCl buffer 0.01 M and CaCl_2 5mM, at 38°C.

as the substrate. Mäkelä et al. (22) studying the CGTase from alkalophilic bacillus ATCC 21783 also observed maximum activity at pH values of approx 7.0. Collected optimum pH and temperature values for various CGTases are shown in Table 1 for comparison.

Influence of Enzyme Dosage on β -CD Yield

Figure 3 presents the results for β -CD production with different enzyme dosages. It can be seen that β -CD concentration increases with time for a period up to 6 h of reaction, and then the curve flattens, an effect resulting from the reversibility of the reactions that occurs in cyclodextrin

Table 1
Optimum Temperatures and pHs for Various CGTases Produced by Different *Bacillus*

Microorganisms	Optimum pH	Optimum Temperature (°C)	Main CD Produced	Reference
<i>Bacillus macerans</i> (IFO-3490)	6.1-6.2	55	α	23
<i>Bacillus megaterium</i>	5.2-6.2	55	β	23
<i>Bacillus</i> sp (ATCC-21783)	4.5-4.7	50	β	24
<i>Bacillus coagulans</i>	6.5-8.5	65	β	25
<i>Bacillus alkalophilic</i> 290-3	6.0-8.0	60	γ	26
<i>B. thermoanaerobacter</i> sp	5	60	α, β	10
<i>Bacillus</i> sp BE-101	6.0-6.5	45	β	11
<i>Bacillus alkalophilic</i>	6.0-7.0	50	β	21
<i>Bacillus lentus</i>	6.5-8.5	45-55	β	27
<i>Bacillus alkalophilic</i> sp	5.5	50-55	α	28
<i>Brevibacterium</i> sp	10	55	γ	29

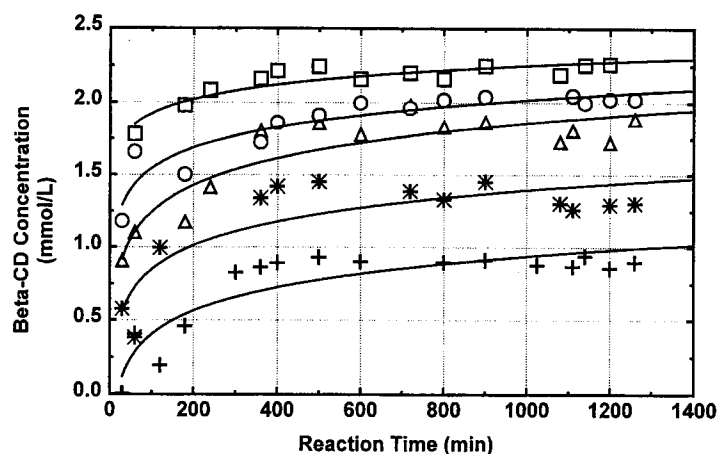


Fig. 3. Production of β -CD as a function of Wacker CGTase enzyme dosage: (+) 0.8 mL/L; (*) 1.6 mL/L; (Δ) 3.3 mL/L; (O) 5.0 mL/L; ($\square$) 6.6 mL/L.

production. The relatively low production of β -CD results from the high DE, as it will become clear with further data. For the same enzyme and using 10% (w/v) Fluka (Buchs, Switzerland) maltodextrin 10, at 50°C, pH 8.0, Hamon and Moraes (21) have obtained about 10 mM β -CD in 24 h.

Influence of Yeast Nutrients on β -CD Yield

The influence of the yeast nutrients, ammonium sulfate, and triple superphosphate, upon CGTase capability of producing β -CD, was shown to be negligible as can be seen in Fig. 4, because in both cases, with or without nutrients, the final concentration reached by β -CD is the same.

Compromise Conditions for the Simultaneous Fermentation and Cyclization Process (SFC)

The results of Fig. 2 have permitted to choose pH 6.0 for the following tests, because in this pH, Wacker CGTase enzyme activity is close to its optimum, and in the SFC tests this pH is not too far from the optimum for fermentation with *Saccharomyces* (16).

Recommended temperature for alcoholic fermentation is within 25 to 36°C, with lower temperatures retarding fermentation and higher temperatures favoring ethanol evaporation (16). However, since Wacker CGTase can work well in temperatures up to 50°C, a compromise temperature was chosen, namely 38°C, for running the subsequent tests that compare the production of β -CD for conditions with or without ethanol and the SFC process.

Enzyme dosage was set for most cases at 3.3 mL of stock Wacker CGTase/L of liquefied cassava starch, because this was considered a good compromise between the yields of β -CD and enzyme expenditure, as seen on Fig. 3.

Influence of Ethanol on β -CD Production

Figure 5 shows that the addition of 5% ethanol increases the production of β -CD from liquefied cassava starch at the DE values tested, namely an increase of 80% for DE = 12.88 and 100% for DE = 23.37. Additional results in this article show that addition of ethanol increases β -CD yield for all DE values, in accordance with results from Lee and Kim (11), and Mattsson et al. (12).

Production of β -CD by the SFC Process and other Comparative Conditions

Figures 6–13 show the evolution of β -CD produced in the three comparative conditions: only the enzyme CGTase, addition of ethanol: CGTase + ethanol, and SFC (simultaneous fermentation and cyclization) process, that is CGTase + yeast + nutrients. It can be seen that the fastest produc-

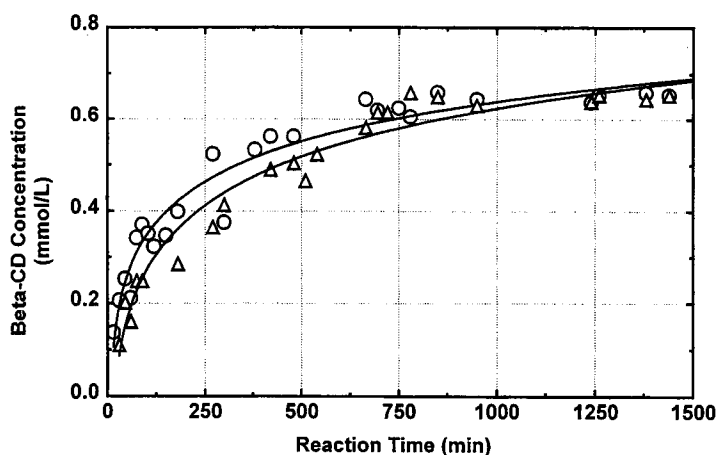


Fig. 4. Production of β -CD as a function of time in the presence of the yeast nutrients; (○) without nutrients; (Δ) with 0.1% (w/v) of triple superphosphate and 0.1% (w/v) ammonium sulfate; 10% (w/v) cassava starch, at 38°C, pH 6.0, 1 mL CGTase/L, and initial DE = 26.06.

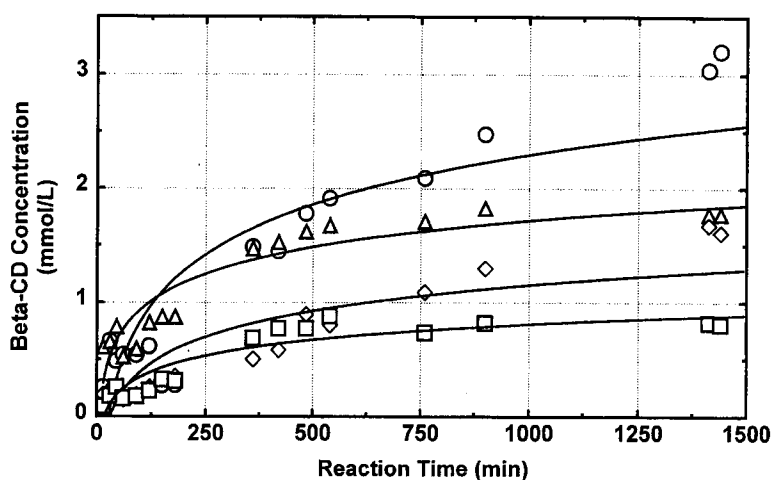


Fig. 5. Production of β -CD as a function of time in the presence and absence of ethanol: (○) DE = 12.88 + 5% (v/v) ethanol; (Δ) DE = 12.88 without ethanol; (◇) DE = 23.37 + 5% (v/v) ethanol; (□) DE = 23.37 without ethanol; 10% (w/v) liquefied cassava starch, at 38°C, pH 6.0, with 1 mL CGTase/L.

tion of β -CD occurs when there is addition of ethanol, and the slowest in the case of the SFC process. The SFC process conditions give generally a higher final β -CD yield than using only CGTase, and sometimes even higher than with addition of ethanol, depending on the quantity of ethanol added. This proves the basic idea of the SFC process.

Data on CD production starting with the same DE shows that fermentation that produces endogenous ethanol in the reaction medium, is more

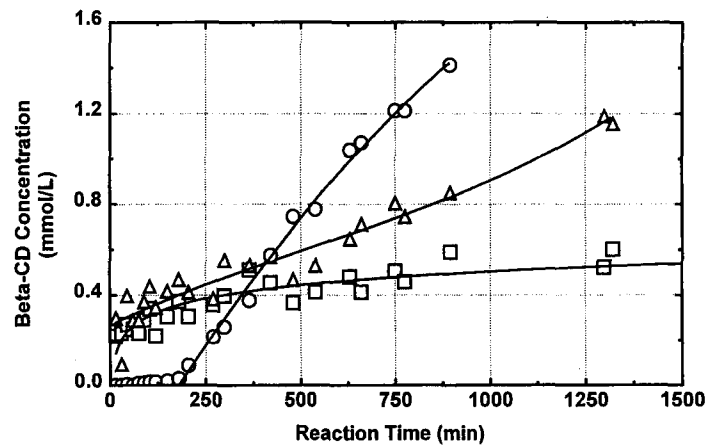


Fig. 6. Production of β -CD with liquefied cassava starch with DE = 23.89; 10% (w/v), at 38°C, pH 6.0 and 0.8 mL CGTase/L: (□) only the enzyme CGTase; (Δ) CGTase + 3% (v/v) ethanol; (○) SFC.

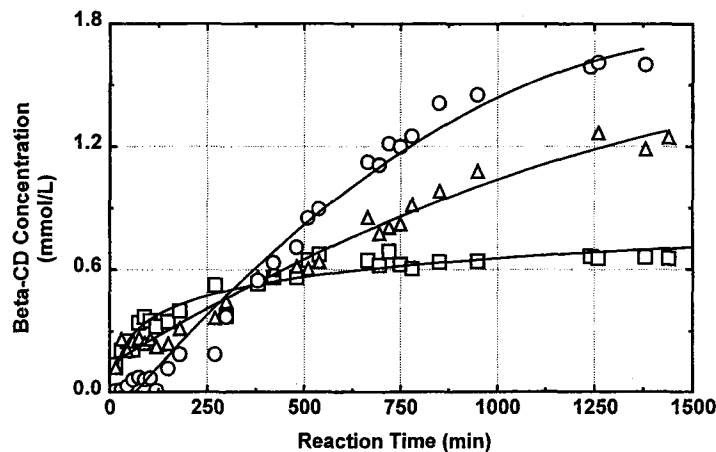


Fig. 7. Production of β -CD with liquefied cassava starch with DE = 26.06; 10% (w/v), at 38°C, pH 6.0 and 0.8 mL CGTase/L: (□) only the enzyme CGTase; (Δ) CGTase + 3% (v/v) ethanol; (○) SFC.

effective for increasing CD yield than the addition of exogenous ethanol to the substrate. Therefore, the elimination of glucose and maltose, which occurs with fermentation, is more important to increase CD yield than the presence of ethanol.

Table 2 allows a rapid comparison of final values for β -CD concentration in the three comparative cases, and the plot of this data clearly shows in Fig. 14 the strong inhibitory effect of high DE on the production of β -CD with liquefied cassava starch solution. These results are in line with those obtained with other starchy materials (30,31).

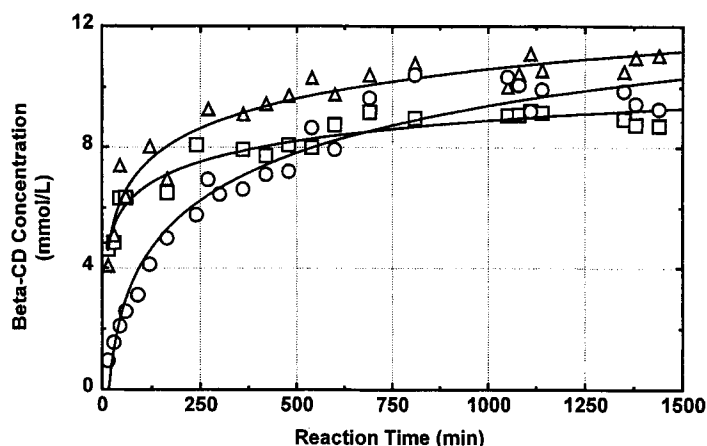


Fig. 8. Production of β -CD with liquefied cassava starch with DE = 2.88; 10% (w/v), at 38°C, pH 6.0 and 0.33% (v/v) CGTase: (□) only the enzyme CGTase; (Δ) CGTase + 1% (v/v) ethanol; (○) SFC.

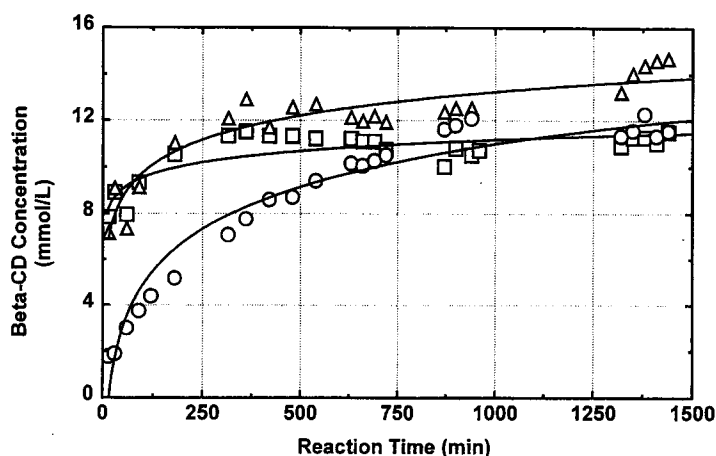


Fig. 9. Production of β -CD with liquefied cassava starch with DE = 3.54; 10% (w/v), at 38°C, pH 6.0 and 0.33% (v/v) CGTase: (□) only the enzyme CGTase; (Δ) CGTase + 3% (v/v) ethanol; (○) SFC.

The yield of β -CD has decreased with an increase in DE values from 2 to 25 in any of the reaction media, and it appears that there is an optimum for DE values between 2 and 10. Maximum β -CD yields for each reaction media were found for DE equal to 3.54, reaching 5.6 and 14.4 mM β -CD for the first and second medium, and 11.5 mM β -CD for the SFC process conditions. For an increase of DE of approx 6 times (from 3.54 to 23.79), β -CD yield decreased 6 times for reaction media one, and two (with 3% v/v ethanol), whereas for the SFC process, this reduction was only approx

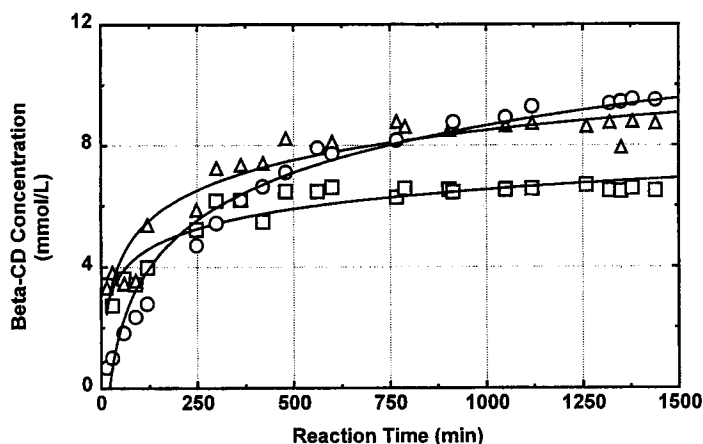


Fig. 10. Production of β -CD with liquefied cassava starch with DE = 7.43; 10% (w/v), at 38°C, pH 6.0 and 0.33% (v/v) CGTase: ($\square$) only the enzyme CGTase; (Δ) CGTase + 3% (v/v) ethanol; ($\circ$) SFC.

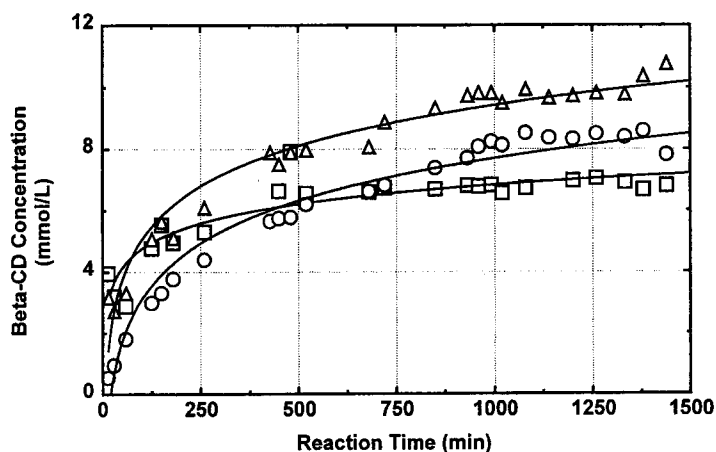


Fig. 11. Production of β -CD with liquefied cassava starch with DE = 10.58; 10% (w/v), at 38°C, pH 6.0 and 0.33% (v/v) CGTase: ($\square$) only the enzyme CGTase; (Δ) CGTase + 5% (v/v) ethanol; ($\circ$) SFC.

3 times (from 11.5 to 3.73 mM), showing that the SFC process is less sensitive to variations in the DE of the liquefied starch solution.

It can also be observed in Fig. 14 that the addition of exogenous ethanol or the SFC process are able to counteract to a certain extent, the inhibitory effects of high DE on β -CD production, but not to the point of producing, for very high DE, the same amount of β -CD as is obtained for example, with very low DE. Therefore, the inhibitory effects of small oligosaccharides during the formation of cyclodextrins are stronger than the enhancement produced by the presence of ethanol.

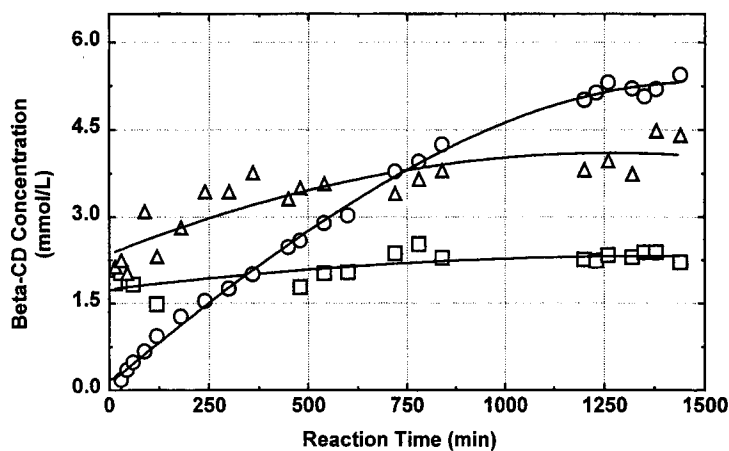


Fig. 12. Production of β -CD with liquefied cassava starch with DE = 18.41; 10% (w/v), at 38°C, pH 6.0 and 0.33% (v/v) CGTase/L: ($\square$) only the enzyme CGTase; (Δ) CGTase + 5% (v/v) ethanol; ($\circ$) SFC.

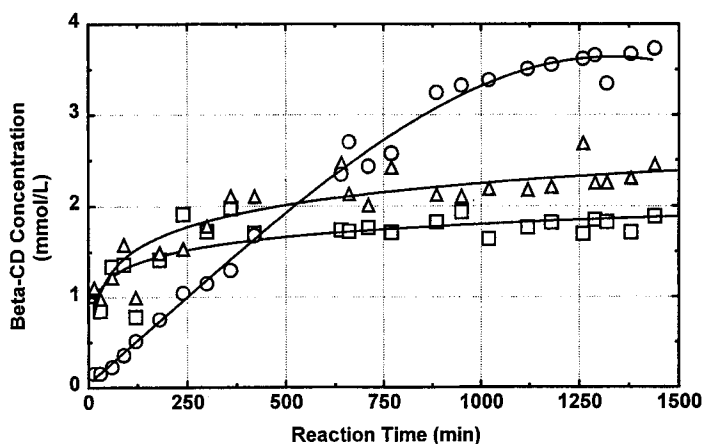


Fig. 13. Production of β -CD with liquefied cassava starch with DE = 23.79; 10% (w/v), at 38°C, pH 6.0 and 0.33% (v/v) CGTase/L: ($\square$) only the enzyme CGTase; (Δ) CGTase + 5% (v/v) ethanol; ($\circ$) SFC.

This work has shown that given a cassava starch solution of high DE, it is possible with the SFC process to produce β -CD at higher yields than using only the enzyme CGTase. For example, for DE = 22.2, the SFC process gives 97% higher yields than using only CGTase, and 52% higher than using CGTase plus 5% v/v ethanol. However, at high DE values the CD yield is low enough to possibly preclude the use of the SFC process for industrial application, unless the high DE solutions are byproduct streams that might gain added value by the presence of a small percentage of CDs.

Table 2
Concentration of CD Produced at 38°C, and pH 6.0, with Various Liquefied Cassava Starch Solutions of Different DE Values, in the Presence and Absence of Ethanol, and for the Simultaneous Fermentation and Cyclization (SFC) Process

Volume of CGTase added (mL/L)	1		0.8		3.3					
Dextrose Equivalent (DE)	12.88	23.37	23.89	26.06	2.88	3.54	7.43	10.58	18.41	23.79
Concentration of β -CD (mM) after 24 h; no ethanol	1.83*	0.82*	0.59*	0.66	8.72	11.5	6.50	6.80	2.38	1.89
Ethanol added (% v/v)	5	5	3	5	1	3	3	5	5	5
Concentration of β -CD (mM) after 24 h; with ethanol	2.48*	1.30*	0.85*	1.25	11.1	14.7	8.74	10.8	4.41	2.45
Ethanol produced (SFC, % v/v)	-	-	1.54	2.18	0.84	1.03	1.18	0.86	0.52	1.68
Concentration of β -CD (mM) after 24 h; SFC.	-	-	1.41	1.61	9.27	11.5	9.51	8.6	5.44	3.73

(*) 15 h of reaction.

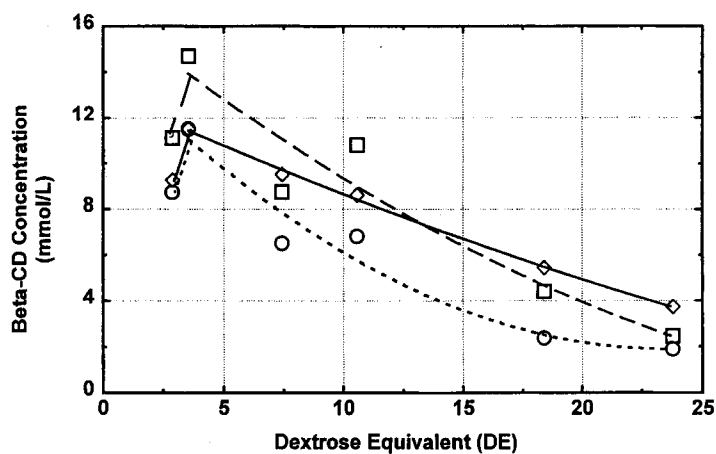


Fig. 14. Maximum yields of β -CD as a function of initial DE of the liquefied cassava starch 10% (w/v), at 38°C, pH 6.0 and 0.33% (v/v) CGTase: (O) only the enzyme CGTase; (□) CGTase + 3% (v/v) ethanol; (Δ) SFC.

The results of this work have show additionally that: cassava starch is a suitable raw material for the production of β -CD, and additional work is needed in this area to try improve CD yield.

ACKNOWLEDGMENTS

The authors are thankful for the financial support received from CAPES/PADCT, CNPq, FINEP, and the State University of Maringá. The companies that supplied materials (Copagra, Novo, and Wacker) are also acknowledged.

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