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Recovery of starch from cassava bagasse for cyclodextrin production by sequential treatment with α-amylase and cyclodextrin glycosyltransferase

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ABSTRACT

This work aimed at the enzymatic recovery of starch from cassava bagasse using α-amylase and the production of cyclodextrins (CDs) by cyclodextrin glycosyltransferase (CGTase). Cassava bagasse containing 50% (w/w) starch (dry basis) was hydrolyzed by α-amylase until a maximum starch recovery as a maltooligosaccharide solution. Afterwards, this solution was used for the production of CDs using CGTase as biocatalyst. Optimal conditions for starch extraction using α-amylase were 60 °C and 0.15 mL enzyme/kg dry bagasse, with 93% starch recovery. Several operational parameters were evaluated to optimize the CD yields, such as starch dextrose equivalent (DE), temperature and CGTase loading. The starch DE did not significantly influence the production of CDs, resulting in a similar yield for all experiments (43%). The highest productivity of CDs (93.6 mg of CDs per enzyme Unit) was obtained using an enzyme loading of 2.5 U/g starch for 1 h at 60 °C and pH 6.0. After 6 h of reaction at 60 °C and pH 6.0, the CD yield reached a value of 50%. These findings did indicate that cassava bagasse is a promising feedstock for the production of CDs using this bi-enzymatic system.

1. Introduction

Cassava (*Manihot esculenta Crantz*) is cultivated in tropical and subtropical regions. In 2017, its world production reached 292 million tonnes, being the three largest producers, Nigeria (Africa), Brazil (South America) and Thailand (Asia) with average productions (1994–2017) of 41.5, 23.1 and 22.8 million tonnes (FAO, 2019). For example, in 2017, the Brazilian cassava crop production was approximately 18.9 million tonnes, which represent 72% of the South America production (approximately 26.2 million tonnes) (FAO, 2019). In Brazil, mainly in the South region, most of the cassava production is destined to produce cassava starch, which has a large national and international market (SEAB, 2016).

The industrial extraction of starch from cassava, conventionally by mechanical process, generates high amounts of liquid and solid residues. Among them, the main residue is a fibrous material called cassava bagasse (Leonel et al., 1998; Martinez et al., 2018; Pinheiro et al., 2018). In the cassava processing by scraping to recover the starch, although it has an intense demand for energy and water (Chavalparit and Ongwandee, 2009), high amount of starch (approximately 30.% (w/w) wet basis) is lost in the bagasse or pulp (Leonel et al., 1998; Pandey et al., 2000). Despite of the large amounts of waste produced yearly (approximately 930 kg of bagasse with 85% moisture per tonne of processed root, according to Martinez et al. (2018)), cassava starch industries have not found useful applications for this waste. Usually, it is used for animal feed purposes, or in some cases discarded inadequately, causing serious environmental concerns (Martinez et al., 2018; Pandey et al., 2000; Pinheiro et al., 2018). In 2009, Lacerda et al. (2009) reported that cassava bagasse was donated to farmers for animal feeding, disposed in the factory courtyard, or sold for U$ 5 per tonne.

Thus, cassava bagasse is a starch-rich fibrous residue, containing around 50–60 wt% (dry basis) of starch (Rattanachomsri et al., 2009; Sriroth et al., 2000). This organic-rich material serves as a suitable substrate for growth of microorganisms (Carta et al., 1999; Pandey et al.,

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2000). Specially, filamentous fungi have been widely cultivated for the production of several products, such as organic acids, flavor and aroma compounds (Bramorski et al., 1998; Kolicheski et al., 1997).

In order to reduce environmental problems and add value to the cassava bagasse, the starch could be recovered and used to produce several starch-derived products, such as maltose, maltodextrins, cyclodextrins, glucose syrups, etc. (Araujo-Silva et al., 2018; Lacerda et al., 2009; Pinheiro et al., 2018). Nevertheless, due to its entrapment in the cellulose-hemicellulose matrix, physical or biological (using enzymes) methods need to be applied (Virunanon et al., 2013). Several studies report the advantages of using multi-enzyme systems for this purpose. Shamala and Sreekantiah (1986) improved the alcohol production from cassava starch through saccharification with a multi-enzymatic preparation of cellulase, D-xylanase, β-D-glucosidase, α-amylase, amyloglucosidase, and pectinase. Sriroth et al. (2000) treated the cassava bagasse by sonication or incubation with a multi-enzyme mixture of cellulase and pectinase. Both methods improved the efficiency of starch extraction by disrupting the complex structure of polysaccharides associated with and entrapping starch granules.

Compared with disrupting of the lignocellulosic complex for release of starch granules from cassava bagasse, treatment with α-amylase also allows efficient recovery of starch, mainly as maltooligosacharides (Lacerda et al., 2009; Valeriano et al., 2018), called maltodextrins, which are starch hydrolysates having a dextrose equivalent between 3 to 20% (Kennedy et al., 1995). This product is widely used in food industries due to its functional properties, such as low hygroscopicity, bland flavor, extremely low sweetness, high viscosity, and the ability to retard ice crystal growth in ice creams, ice milk, and other frozen novelties (Guzmán-Maldonado et al., 1995). In addition, maltodextrins from cassava bagasse can also be used as raw material for the production of cyclodextrins (Pinheiro et al., 2018), which are higher value-added products.

Cyclodextrins (CDs) are non-reducing cyclic oligosaccharides, consisting of glucose units linked to each other by α−1,4-glycosidic linkages. Natural CDs are produced by cyclodextrin glycosyltransferase action on starch and consist of 6, 7 or 8 units of glucose, namely α-, β- and γ-CD, respectively (Szejtli, 1990). CDs have a truncated cone-shaped structure, showing a hydrophilic external surface and a hydrophobic internal cavity, which is capable of encapsulating a wide variety of organic molecules, improving physical properties (Marques, 2010). Consequently, CDs are widely used in the pharmaceutical industry to increase the solubility of drugs and to improve their stability in the presence of light and heat (Loftsson and Duchêne, 2007), in the food industry, as complexing agents to reduce bitter tastes and unpleasant odors (Astray et al., 2009), and in the cosmetic industry to reduce the volatility of fragrances and to convert liquids or oils into powder formulations (Buschmann and Schollmeyer, 2002).

Although the demand for cyclodextrins can increase, its high production cost is still prohibitive (Pinheiro et al., 2018). In this context, raw materials of low cost would be an alternative to reduce the process costs (Pinheiro et al., 2018). Thus, agro-industrial wastes with high starch content become promising sources for the production of cyclodextrins due to their low cost and abundance. In this context, this work describes a process for the enzymatic recovery of residual starch from cassava bagasse and its use in the production of cyclodextrins utilizing α-amylase and cyclodextrin glycosyltransferase. Thermostable α-amylases produced from *Bacillus amyloliquefaciens* are used in a wide number of industrial processes such as food, fermentation, textiles and paper industries, because of their stability for perform liquefaction process at high temperatures (100–110 °C) (Souza and Magalhães, 2010). In addition to α-amylases, the CGTase from *Thermoanaerobacter* sp. also has significant starch liquefying capacity and is extremely stable at high temperatures (85–90 °C) (Norman and Jorgensen, 1992). This enzyme produces a mixture of CDs with similar quantities of α- and β-CDs and lower amounts of γ-CD (Norman and Jorgensen, 1992).

2. Material and methods

2.1. Material

CGTase from *Thermoanaerobacter* sp. (Toruzyme® 3.0 L) containing 212 U/mL of activity and 7.1 mg/mL of protein, α-amylases (BAN® 480 L and Termamyl® 120 L, containing 480 KNU–B/kg and 120 KNU/g of activity, respectively, and density of 1.20–1.25 g/mL at 25 °C) and AMG 300L™ (300 AGU/mL of activity and 1.2 g/mL of density at 25 °C) were from Novozymes (Araucaria, Brazil). Dextrin 10, soluble starch and phenolphthalein were purchased from Sigma-Aldrich Co. (St. Louis, MO). GOD-PAP colorimetric kit was acquired from Doles (Goiania, Brazil). Cassava bagasse was supplied by cassava starch manufacturers located in the State of São Paulo, Brazil (Fecularia Flor de Lotus and Syral-Haloteck). All other chemicals (analytical grade) were purchased from Synth (São Paulo, Brazil) and Quemis (São Paulo, Brazil).

2.2. Physico-chemical characterization of cassava bagasse

The starch content in the cassava bagasse was determined by enzymatic extracting using an excess of α-amylase, followed by total hydrolysis with amyloglucosidase (AMG). Eight grams of cassava bagasse (wet basis) were suspended in 400 mL of distilled water in a glass reactor at 90 °C. A volume of 5 mL of α-amylase (Termamyl 120 L®) was added and the suspension was mechanically stirred at 250 rpm for 2 h. After this reaction time, hydrolyzed starch was separated from the solid fraction by vacuum filtration. For total hydrolysis into glucose, the pH of a volume of 100 mL of hydrolyzed starch was adjusted to 4.8, and subjected to enzymatic hydrolysis at 60 °C for 2 h using 1 mL of a solution of AMG 300L™, corresponding to 5 mL AMG 300L™/kg starch. The produced glucose was determined using a GOD-PAP colorimetric kit and the starch content was determined multiplying the glucose content by 0.9 (factor conversion from free glucose to linked-starch glucose). The hemicellulose, cellulose and lignin contents in the solid material remaining after the extraction of starch were determined using the methodology for sugarcane bagasse described by Gouveia et al. (2009). One gram of solid material (~10% moisture) was hydrolyzed by incubation in 10 mL of H_2SO_4 (72%, v/v) for 7 min at 45 °C under vigorous stirring. After, the acid concentration was adjusted to 4% (v/v) and the hydrolysis continued by autoclaving this mixture for 30 min. After this time, the solid and liquid fractions were separated by filtration.

The cellulose and hemicellulose content were determined measuring the concentration of carbohydrates, organic acids and sugar degradation products (furfural and hydroxymethylfurfural) by liquid chromatography. Acid-soluble lignin was spectrophotometry determined at 280 nm and insoluble lignin was quantified as the difference in weight between the solid remaining after acid hydrolysis (dry basis) and the mass of ash after calcination at 800 °C.

The protein content in the bagasse was determined using the Kejldahl method described by Cotta et al. (2007). The content of material extracted in 95% ethanol (v/v) was quantified by Soxhlet extraction.

2.3. Influence of temperature on the liquefaction of the cassava bagasse starch

A suspension of cassava bagasse (40 g/L, dry basis) in 50 mM sodium citrate buffer pH 6.0 was heated at 85 °C for 10 min, afterwards, cooled and transferred to a thermostatized reactor at 60, 70 or 80 °C. The hydrolysis was performed using an enzyme loading of 0.15 mL of α-amylase (BAN® 480 L)/kg of dry bagasse (0.3 mL BAN® 480 L/kg of starch contained in the cassava bagasse) for 5 min under 1000 rpm mechanical stirring. The enzymatic reaction was stopped by reducing the pH to 3.0 using 5 M HCl solution. After, the hydrolyzed starch was separated from the solid fraction by vacuum filtration. The dextrose equivalent (mass of TRS/starch mass) for starch hydrolysates were determined according to Fig. 1, using the DNS method (Miller, 1959) for

the quantification of the total reducing sugars (TRS) and GOD-PAP colorimetric kit for quantification of glucose (after hydrolysis of starch with AMG).

2.4. Determination of the starch (as maltodextrins) content recovered from the cassava bagasse

Five milliliters of the starch hydrolysate (~1.8%, w/v) were diluted in 50 mM sodium citrate pH 4.8 to a final volume of 50 mL and subjected to enzymatic hydrolysis catalyzed by 0.5 mL of a solution of AMG 300L™ (equivalent to 5 mL AMG 300L™/kg starch) for 2 h at 60 °C and pH 4.8. The glucose produced by saccharification by AMG was determined using the GOD-PAP colorimetric kit. The starch amount recovered from the cassava bagasse as a mixture of maltodextrins was calculated by multiplying the glucose content by a factor of 0.9 (conversion factor from glucose to starch).

2.5. Dextrinization of the starch from the cassava bagasse

Starch hydrolysates with different dextrose equivalent (DE) were prepared by hydrolyzing cassava bagasse starch with α-amylase (0.3 mL BAN® 480 L/kg starch) for different reaction times (3, 7, 10 and 20 min). All hydrolysis assays were carried out at the optimal temperature for the starch removal previously described above.

The starch dextrinization was monitored by measuring DE (mass of TRS/starch mass), using the DNS method (Miller, 1959) for the quantification of the total reducing sugars (TRS).

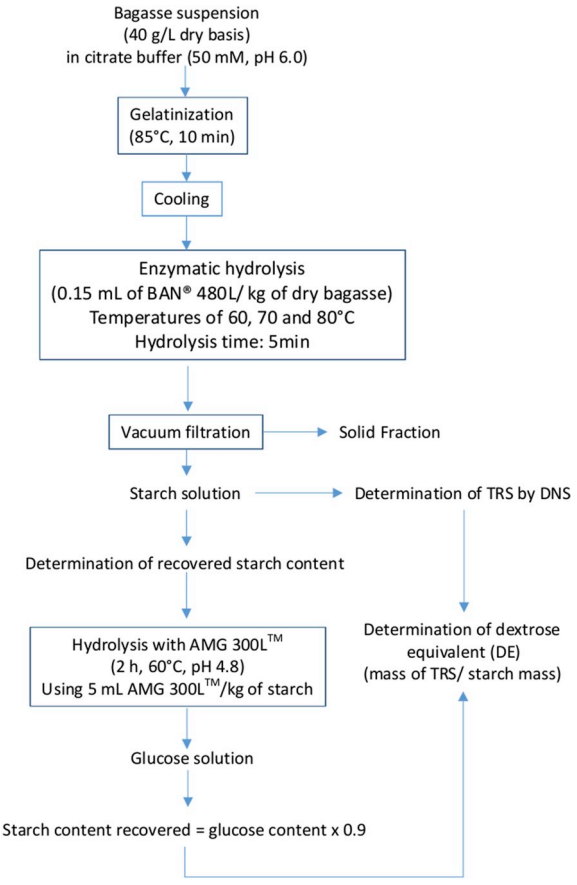

Fig. 1. Flowchart of liquefaction process and determination of Dextrose Equivalent (DE) and starch content recovered.

2.6. Influence of the dextrose equivalent on the cyclodextrin production

After dextrinization of the starch from cassava bagasse with α-amylase, the pH of the starch hydrolysates with different DE values was adjusted to 6.0 for the cyclodextrin production. The set of experiments of production of cyclodextrins (CDs) was performed in stirred glass batch reactors thermostatized at 85 °C. A volume of 50 mL of hydrolyzed starch solutions (12.1–18.5 g/L) was placed in the reactor and after reaching the desired temperature, CGTase was added. The cyclization reaction was performed using an enzyme loading of 20 U CGTase/g starch. After 5 h, the reaction was stopped by adding 2 mL of 5 M HCl, followed by heating in boiling water for 10 min to inactivate the CGTase. Assays using commercial starch (20 g/L) with several DE values were used as standard to compare the influence of DE on the CD production. The concentrations of α-, β- and γ-CD produced after 5 h of reaction were determined by liquid chromatography according to the methodology described by Rojas et al. (2015).

2.7. Influence of the enzyme loading and temperature on the cyclodextrin production

The influence of the enzyme loading and temperature on the production of CDs from cassava bagasse starch hydrolysates was evaluated in the range of 2.5–20 U CGTase/g starch and 40–60 °C, respectively. For the enzyme loading (2.5, 5, 10 and 20 U/g starch), the cyclization reactions were carried out at 60 °C for 6 h in a batch reactor using 50 mL of 18.5 g/L hydrolyzed starch solution (DE of 2.4) in 50 mM citrate buffer pH 6.0. For the effect of the temperature assays, the cyclization reactions were carried out under the same conditions described above (i.e., 6 h, pH 6.0, starch concentration of ~1.8%, m/v), using the best enzyme load previously determined. Aliquots of 1 mL were withdrawn at different reaction times (0, 0.5, 1, 3, and 6 h). The action of CGTase was stopped by adding 20 μL of 3 M HCl, followed by heating in boiling water. Produced CDs was determined by HPLC according to Rojas et al. (2015).

2.8. Standard determination of CGTase activity

CGTase activity was determined by measuring initial rates of β-CD production using 0.5% m/v dextrin 10 solution in 10 mM sodium citrate buffer pH 6.0 as substrate. 100 μL of enzyme (diluted 50-fold) were added to a reactor containing 50 mL of dextrin solution. The reaction was carried out at 60 °C and samples of 1 mL were withdrawn at 2 min intervals for 10 min. The CGTase was inactivated by the addition of 20 μL of 3 M HCl in the samples, followed by incubation in boiling water for 5 min. The β-CD produced was quantified by the colorimetric method of phenolphthalein described by Vikmon (1982).

2.9. Analytical methods

The concentrations of α, β and γ-CDs were determined using the chromatographic method described by Rojas et al. (2015). The analyzes were performed in an Alliance E2695 liquid chromatograph (Waters, Milford, USA) equipped with an evaporative light scattering detector (ELS 2424, Waters). The column was a NUCLEODUR®C18 (150 mm × 4.6 mm, 5 μm), at 30 °C, using a linear gradient of acetonitrile (phase B) and water containing 1% (v/v) acetic acid (phase A) at a flow rate of 0.3 mL/min as following: 0 min–100% phase A, 20 min–80% phase A and 20% phase B, 20.1 min–100% phase A, and 30 min–100% phase A (Rojas et al., 2015).

Carbohydrates (glucose, xylose, arabinose, etc.) and organic acids were quantified by HPLC in an Alliance E2695 liquid chromatograph (Waters, Milford, USA) equipped with a refractive index detector (2414, Waters). The compounds were separated in a HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA), at 45 °C, using 5 mM sulfuric acid as the mobile phase, at a flow rate of 0.6 mL/min.

3. Results and discussion

3.1. Chemical characterization of the cassava bagasse

Table 1 shows the chemical composition of cassava bagasses from two Brazilian starch industries used in this study. The cassava bagasse was composed mainly by starch, representing approximately 50 wt% (dry basis) of its composition. It has been reported a starch composition (determined as carbohydrates) in the cassava bagasse from 32.6 to 79.4% (Lacerda et al., 2009; Martinez et al., 2018; Pandey et al., 2000; Pinheiro et al., 2018; Sivamani et al., 2018; Valeriano et al., 2018). Differences in the composition of cassava bagasses can be attributed to differences in the industrial extraction process of the starch and the peeling processing of the root, as well as the root type or crop characteristics. In addition to starch, the cassava bagasses also contained high amounts of other carbohydrate polymers, such as cellulose and hemicelluloses (19.4–22.7% and 12.7–14.9%, respectively), which are source of fermentable sugars (Xu et al., 2014). Several studies have reported the use of starch from cassava bagasse for the production of ethanol (Klinpratoom et al., 2015; Kunhi et al., 1981; Ma et al., 2011; Martinez et al., 2018; Rattanachomsri et al., 2009). Furthermore, the hemicellulose fraction has also been used in the production of oligosaccharides (Kurdi and Hansawasdi, 2015).

Due to the low protein content (in our study 1.8–2.1%, 0.3–1.6% previously reported by Pandey et al. (2000), and 0.49 to 0.9 previously reported by Martinez et al. (2018)), cassava bagasse has been mainly used in animal feed as an energy source (Sriherwanto et al., 2009). Moreover, due to its high content of insoluble fiber, cassava bagasse has also been evaluated for human consumption (Raupp et al., 1999).

According to Pandey et al. (2000), the lower ash content in the cassava bagasse (0.7–1.5%) compared to that in other materials (e.g., 17.5% and 11.0% for rice straw and wheat bran, respectively) makes it advantageous in bioprocesses involving microbial cultures. Consequently, this residue has been widely used as substrate for growth of filamentous fungi, aiming at the production of aromas, citric acid, and fumaric acid (Carta et al., 1999; Kolicheski et al., 1997).

3.2. Recovery and dextrinization of the starch from cassava bagasse

In this work, starch from cassava bagasse was recovered as a maltodextrin solution in a single process using a commercial thermostable α−amylase. A previous step of gelatinization of a suspension of cassava bagasse (40 g cassava bagasse/L, corresponding to 20 g theoretical starch in the cassava bagasse/L, in dry basis) was carried out aiming to increase the accessibility of glycosidic linkages by the α-amylase. These treatments allowed high removal of starch without disruption of fibrous matrix (Fig. 2a and b). The results of starch recovery from cassava bagasse as a function of the hydrolysis temperature (60–80 °C) are shown in Table 2. High yields of starch recovery (82–93%) compared to the maximum theoretical one could be achieved by hydrolyzing α-1,4 glycosidic linkages. These starch yields were higher than those previously reported by Sriroth et al. (2000), who had recovered 40% of starch (intact granules) by cleaving the lignocellulosic complex of the cassava bagasse by sonication and enzymatic treatment using a mixture of cellulase and pectinase.

Although in our work the starch from cassava bagasse is not recovered as intact granules, the starch hydrolysate could represent an extra profit for the starch industries, because maltodextrin syrup has several direct applications in food industries, as well as it may be used as substrate for the production of other value-added products, such as cyclodextrins, maltose, glucose and fructose syrups.

Although the α-amylase has an optimal temperature between 70 and 80 °C, the highest starch extraction (92.8%) was obtained at 60 °C. Thus, this temperature was selected for further studies because lower temperature can represent an energy saving in large scale processing.

In the starch liquefaction process, by controlling the reaction time and/or by adjusting the enzyme dosage, it is possible to achieve different dextrose equivalent (DE) value. In an industrial process, starch slurry with 40% of solids requires around 1 h to reach DE values from 10 to 12, whereas DE values from 15 to 16 are reached after 2 h (Guzmán-Maldonado et al., 1995). Thus, the influence of the reaction time for maximum recovery of starch from cassava bagasse during amylase hydrolysis, as well as to achieve high DE value was evaluated.

The results of DE and percent of recovery of starch from cassava bagasse as a function of the hydrolysis time at 60 °C are shown in Table 3. It can be seen that the heat treatment of the bagasse at 85 °C for 10 min is enough to extract 59.4% of the initial starch. Further treatment with α-amylase increases the extraction of starch to approximately 90% after a very short reaction time (3 min). However, in order to obtain starch hydrolysates (maltodextrins) with higher DE, a higher hydrolysis time is required. This study was performed because although the percentage of extraction of starch remains around 90%, the DE value of the starch hydrolysates may influence the production of CDs.

3.3. Batch production of CDs

The production of CDs was evaluated using solutions of hydrolyzed starch extracted from cassava bagasse at different amylase hydrolysis time. As a reference, solutions of hydrolyzed commercial starch having different dextrose equivalent (DE) were employed.

Fig. 3 shows the production of CDs from soluble commercial starch and from that extracted from cassava bagasse with different DE values, catalyzed by Toruzyme®. It was previously reported that this CGTase is capable of catalyzing the cyclization reaction from non-hydrolyzed starch at 85 °C in a very short reaction time (10 min). However, time need to be controlled because this enzyme at this temperature also exhibits high hydrolytic activity (Norman and Jorgensen, 1992). Production of CDs from soluble starch (without treatment with α-amylase) catalyzed by Toruzyme® was also reported by Kim et al. (1997). However, in our work, treatment with α-amylase was employed to recover the maximum amount of starch from the cassava bagasse. This previous treatment with α-amylase increased the yield of CDs (from 35% to 42.4% using non-hydrolyzed commercial starch and starch from cassava bagasse hydrolyzed with a DE of 2.4, respectively).

Fig. 3 shows similar pattern in the production of the different CDs (α-, β- and γ-CD) using both commercial starch and starch extracted from cassava bagasse, i.e., 0.7–1.0 g/L of γ-CD, 4.0–5.5 g/L of β-CD and 1.8–3.3 g/L of α-CD. The maximum yield of about 42% of CDs (α-, β- and γ-CD) was obtained using the solution of maltodextrins (~1.8%, w/v, DE 2.4) extracted from cassava bagasse. Total conversion of starch is not achieved using only CGTases, even when it operates under optimal conditions of pH and temperature. This may be explained by the enzyme specificity, which does not hydrolyze α−1,6 glycosidic linkages from the amylopectin (Rendleman, 1997). Besides, the enzyme can also be inhibited by the end-products, CDs can be degraded by coupling and disproportionation reactions also catalyzed by CGTases. Moreover, CGTases cannot act in low molar mass substrates (Pinheiro et al., 2018).

Table 1
Chemical composition of cassava bagasses.

Component	Composition (wt.%, dry basis)	
	Cassava bagasse I[a,c]	Cassava bagasse II[b,c]
Starch	47.1 ± 1.6	50.5 ± 1.1
Cellulose	19.4 ± 1.2	22.7 ± 0.6
Hemicellulose	12.7 ± 0.6	14.9 ± 1.0
Lignin	9.4 ± 0.7	10.6 ± 1.5
Ash	1.5 ± 0.1	1.4 ± 0.0
Proteins	1.8 ± 0.2	2.1 ± 0.2
Extractives	3.0 ± 0.3	3.2 ± 0.2

[a] Supplied by Fecularia Flor de Lotus (Candido Mota, SP, Brazil).
[b] Supplied by Syral Haloteck (Palmital, SP, Brazil).
[c] Cassava bagasses had average moisture of 86%.

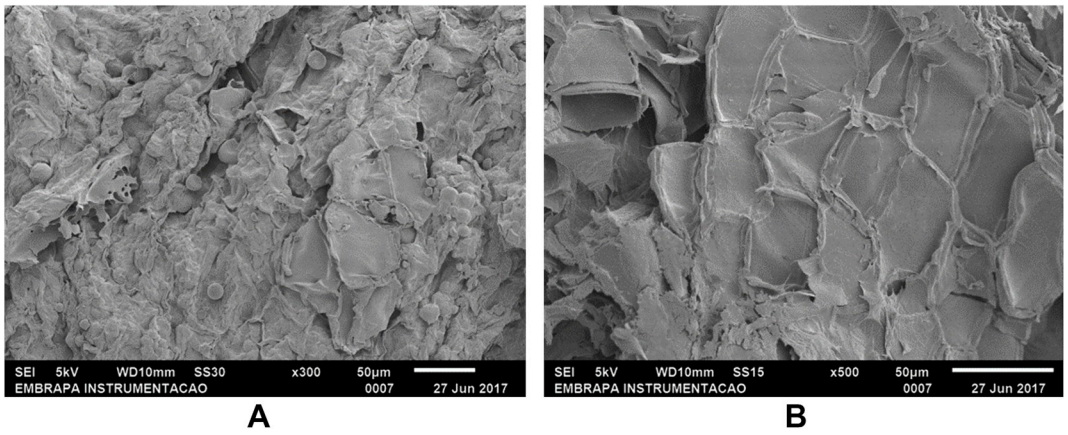

Fig. 2. Scanning electron micrographs of: (a) In nature cassava bagasse and (b) cassava bagasse treated by gelatinization and α−amylase.

Table 2
Percent recovery of starch from cassava bagasse as function of the temperature after 5 min hydrolysis.

Temperature (°C)	Concentration of hydrolyzed starch (g/L)	Dextrose equivalent of hydrolyzed starch	Starch recovery (%)
60	18.6 ± 0.3	3.2	92.8
70	16.7 ± 0.7	6.0	82.0
80	16.0 ± 1.2	3.1	78.4

Table 3
Dextrose equivalent (DE) and percent recovery of starch from cassava bagasse as a function of the hydrolysis time at 60 °C using α-amylase.

Time (min)	Concentration of hydrolyzed starch (g/L)	DE of hydrolyzed starch	Starch recovery (%)
0	12.1	2.40	59.4
3	18.5	2.44	90.6
7	18.3	4.09	89.7
10	18.2	6.62	89.2
20	18.5	10.25	90.6

Similar findings were reported by Pinheiro et al. (2018), who produced CDs from cassava bagasse (40 g/L, corresponding to around 25 g carbohydrates/L) at 70 °C and pH 6.0 using only the Toruzyme® (0.05%, v/v). After 12 h reaction, the CD production reached the following concentrations: 3.0 g/L α-CD, 5.0 g/L β-CD and 1.45 g/L γ-CD, representing 38% conversion. However, when the temperature was decreased to 60 °C the conversion dropped to 11.23%. When using only CGTase to produce CDs from starch, high temperature is required to solubilize the starch and reduce the medium viscosity, improving the gelatinization of the substrate (Pinheiro et al., 2018). Thus, the pre-hydrolysis using α-amylase employed in this work allows researchers to carry out the process at lower temperatures. But only a more elaborate process economic calculation, including various factors, can point out which of the two process alternatives are more attractive for industrial application. These factors are reactor time, use and cost of enzymes, CDs yields and prices, energy consumption, etc.

3.4. Ratio of CDs produced by Toruzyme®

Toruzyme® produced mostly α− and β−CDs (Fig. 3) using commercial starch or starch from cassava bagasse after 5 h reaction at 85 °C using 20 U/g starch with a DE of 2.4. The initial rates of production of α−, β−, γ−CD were also determined at 40 °C using 1.0 U/g starch to give a linear fit of the CD concentration vs. time data (Fig. 4). From these linear fits, it was determined that Toruzyme® produced a CDs ratio of 5 (α−CD): 3 (β−CD): 1 (γ−CD) using cassava bagasse starch. This ratio was close to the one reported by Calsavara et al. (2011), who produced cyclodextrin from a suspension of cornstarch granules (15%, w/v) containing 10% (v/v) ethanol, using the same CGTase, giving a α−CD:

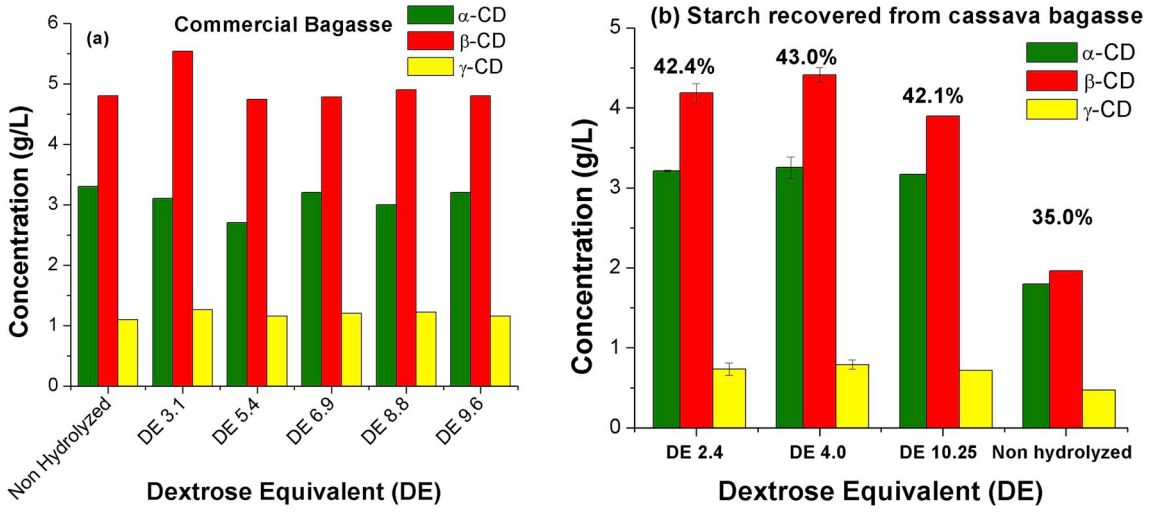

Fig. 3. Production of CDs from (a) commercial starch and (b) starch extracted from cassava bagasse with different dextrose equivalent (DE) values. Reaction conditions: 20 g/L starch, 85 °C, Toruzyme® 20 U/g starch, 5 h reaction.

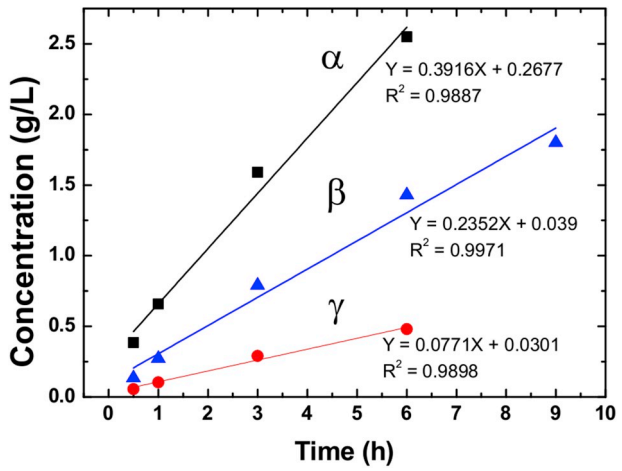

Fig. 4. Linear fit of initial rates of CD production at 40 °C, pH 6.0, from cassava starch (DE ~2.4 and ~18.5 g/L) catalyzed by soluble Toruzyme® (1.0 U/g starch).

β−CD: γ−CD ratio of 4.3:2:1. These results show that at the beginning of the cyclization reaction the α-CD production is favored, however with the progress of the reaction the production of α− and β−CD become very close.

According to Brunei et al. (1998), the degree of polymerization (DP) of the polysaccharide substrate may influence the production of a particular type of cyclodextrin when using the Toruzyme®. The authors reported that this CGTAse produces mainly β-CD from substrates with DP between 3 and 6. When the substrates have DP > 7, α-CD and β−CD are produced in the same ratio. On the other hand, when maltose (DP of 2.0 (Guzmán-Maldonado et al., 1995),) is used as substrate, this CGTase mainly produces α-CD. The substrate used in our work had a DE of 2.4, which correspond to a DP of around 50 (as a rule of thumb, DE × DP = 120 (Kearsley and Dziedzic, 1995),). Thus, the production of α- and β-CD at similar amounts agrees with the findings of Brunei.

3.5. Evaluation of process parameters for batch production of CDs

Fig. 5(a–c) shows the profiles of the cyclization reaction as function of the enzyme loading at 60 °C and pH 6.0 for 6 h in batch assays, using hydrolyzed 1.8% (w/v) starch as substrate. For an enzyme loading of 20 U/g substrate, the maximum concentration of CDs (α−, β− and γ−CDs) was reached after 1 h reaction, after which a slight decrease was observed. This behavior can also be observed for an enzyme loading of 10 U/g substrate, mainly for α-CD. Probably, this is caused by the coupling reaction producing linear oligosaccharides from the opening of the CDs rings, thus decreasing the CDs yield. Similar behavior for the production of CDs was also previously reported (Kawamoto et al., 2001; Tardioli et al., 2006).

The maximum yield of CDs (approximately 50%) was achieved after 6 h of reaction using an enzyme loading of 2.5 U/g starch.

Furthermore, after 1 h reaction, the productivity of CDs (defined as mg of α-, β- and γ-CDs per enzyme unit) was 93.6 mg/U/h, using 2.5 U/g starch at 60 °C and pH 6.0. This productivity was higher than the ones reported by Tardioli et al. (2006) and Kim et al. (1997) using soluble starch (23.9 mg CDs/U/h) and corn starch (12.68 mg CDs/U/h), respectively. Thus, the enzyme loading of 2.5 U/g of starch was select to evaluate the influence of the temperature on the production of CDs from starch recovered from cassava bagasse.

Batch assays were performed at 40, 50 and 60 °C using an enzyme loading of 2.5 U/g starch to analyze the effect of temperature in the process. The results showed (Fig. 6 a-c) that the cyclization reaction at 60 °C yielded the highest CDs concentration, reaching a yield of CDs of approximately 50%, whereas at 40 °C the yield was around 35%. The temperature of 60 °C can be used for the production of CDs catalyzed by Toruzyme® because this enzyme have been reported to be stable at this temperature (Calsavara et al., 2011; Norman and Jorgensen, 1992). In fact, Pinheiro et al. (2018) evaluated the production of CDs from starch of the cassava bagasse at 70 °C and pH 6.0, reaching a conversion of 48.5%, but in this case, the reaction time was 12 h and 10% ethanol was used in the reaction medium in order to increase de starch conversion.

4. Conclusions

Cassava bagasse is a rich source of starch (50 wt%, dry basis). Despite having current low commercial value as animal feed, this residue could be used for the production of high-value products, such as cyclodextrins. This study showed that a bi-enzymatic process using α-amylases and CGTase could be utilized to transform starch from cassava bagasse in CDs. The first treatment with amylase permitted to extract up to 93% of the starch contained in cassava bagasse as maltodextrins. The further use of this material and CGTase permitted to efficiently produce cyclodextrins with a maximum yield of around 50% at 60 °C and pH 6.0, by using Toruzyme® at an enzyme loading of 2.5 U/g starch. This result shows that this residue is a promising source of starch for the production of high value-added products.

Further improvements of these results may be achieved following different strategies. For example, the problems of the 1,6 glycosidic linkages from the amylopectin (Rendleman, 1997) may be overcome by treating the starch with other amylolitic enzymes, such as isoamylase or pullulanase (Pishtiyski and Zhekova, 2006). These enzymes hydrolyze α−1,6 glycosidic bonds releasing oligosaccharides capable of being used by CGTases. Indeed, an increase of 10% in the conversion of corn starch (treated with pullulanase) to CDs has been reported by Pishtiyski and Zhekova (2006). To avoid the enzymatic destruction of the produced CDs, Tardioli et al. (2006), proposed to use a batch operation system

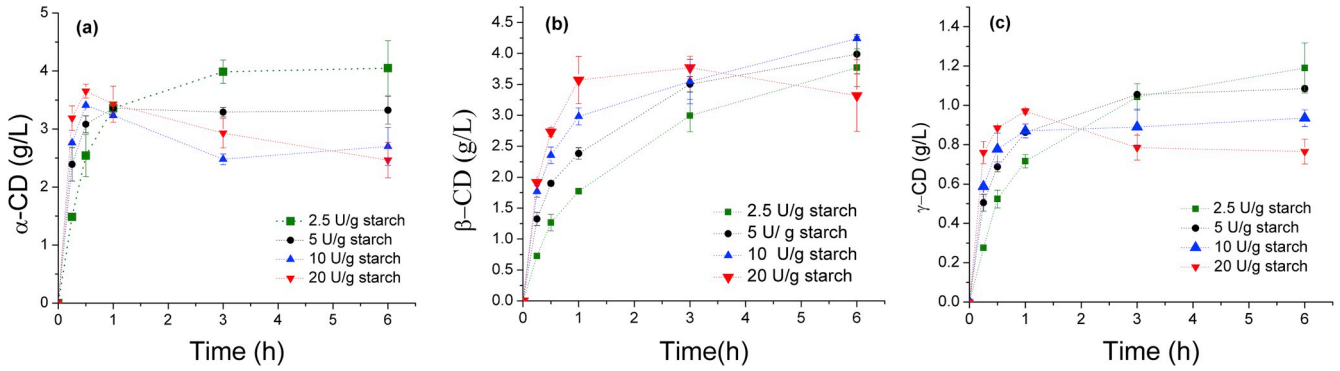

Fig. 5. Production of CDs, (a) α-CD, (b) β-CD and (c) γ-CD, from starch recovered from cassava bagasse (DE of 2.4 and ~18.5 g/L) at 60 °C, pH 6.0, catalyzed by Toruzyme®.

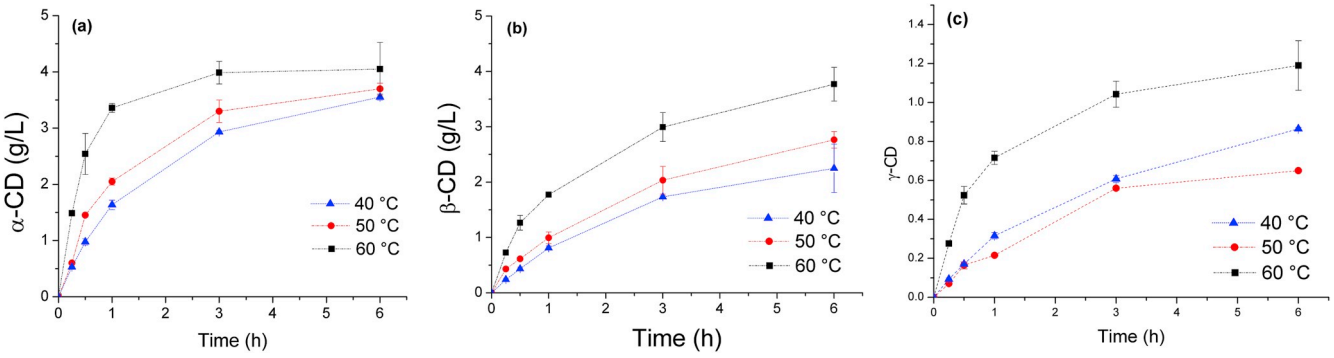

Fig. 6. Production of CDs, (a) α-CD, (b) β-CD and (c) γ-CD, from starch recovered from cassava bagasse (DE of 2.4 and ~18.5 g/L) at 40, 50, and 60 °C (pH 6.0) using soluble Toruzyme®, 2.5 U/g starch.

with continuous removal of the product to avoid CD degradation by coupling and disproportionation CGTase reactions, thus increasing the yield of CDs. These topics are currently in study in our laboratory.

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