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ENZYMATIC PRODUCTION OF β-CYCLODEXTRIN FROM JACKFRUIT SEEDS (*Artocarpus intergrifolia* L.)

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Abstract - *β*-Cyclodextrin (*β*-CD) is a compound of great application for pharmaceutical and food industries, and is generally produced by starchy substrates by cyclomaltodextrin glucanotransferase (CGTase) action. The objective of this study was to produce *β*-CD using an alternative source of starch, such as jackfruit seed (*Artocarpus intergrifolia* L.) bran (JSB) by a commercial CGTase. The highest productivity of *β*-CD (52.10 μM/h.g) was obtained from 10 g of JSB in 100 mL of citrate buffer (10 mM / pH 6.0), with 17 % (v/v) of ethanol and 1.34 U/g of CGTase, at 59 °C for 4 hours. These same conditions were applied to starches extracted from the JSB (SJSB) and ginger (SG) and also, potato starch (SP). The SJSB and SG performances were similar to SP, and resulted in productivities around 2.7 times higher in relation to JSB. Thus, it is possible to conclude that both JSB and SJSB are promising substrates for *β*-CD production.
Keywords: Cyclomaltodextrin glucanotransferase; Factorial design; Ginger; Phenolphthalein; Starch.

INTRODUCTION

Cyclodextrins (CDs) are cyclic oligosaccharides composed of *D*-glucose molecules united by linked glycosidic bonds (*α*-1,4) made by the action of cyclomaltodextrin glucantransferase (CGTase) from starch. The best-known CDs are those composed of six (*α*-CD), seven (*β*-CD) and eight (*γ*-CD) *D*-glucose units (Del Valle, 2004; Brewster and Loftsson, 2007; Radu et al., 2016). The structures of CDs (Figure 1) present a conical cylindrical shape, also called a conical trunk, which provides the amphiphilic property to these molecules (Britto et al., 2004). Its outer surfaces present a hydrophilic character, due to the presence of hydroxyls that are oriented to the external cavity, whereas the hydrogen atoms of glucose units are oriented to the internal cavity, thus conferring the lipophilic character of CD molecules (van der Veen et al., 2000). This structural characteristic allows the formation of inclusion compounds with several other molecules (Divakar and Maheswaran, 1997), significantly altering the physico-chemical properties of these included compounds, for example: increasing aqueous solubility and bioavailability of different drugs and protecting or masking flavors/aromas for food production (Azzi et al., 2018; Del Valle, 2004; Malakias et al., 2018).

The cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) are the enzymes capable of acting on amylaceous substrates (such as maize, cassava, rice and potato starches) producing CDs. Among their forms of action (van der Veen et al., 2000), CGTases produce CDs by intramolecular transglycosylation (cyclization) in which the linear

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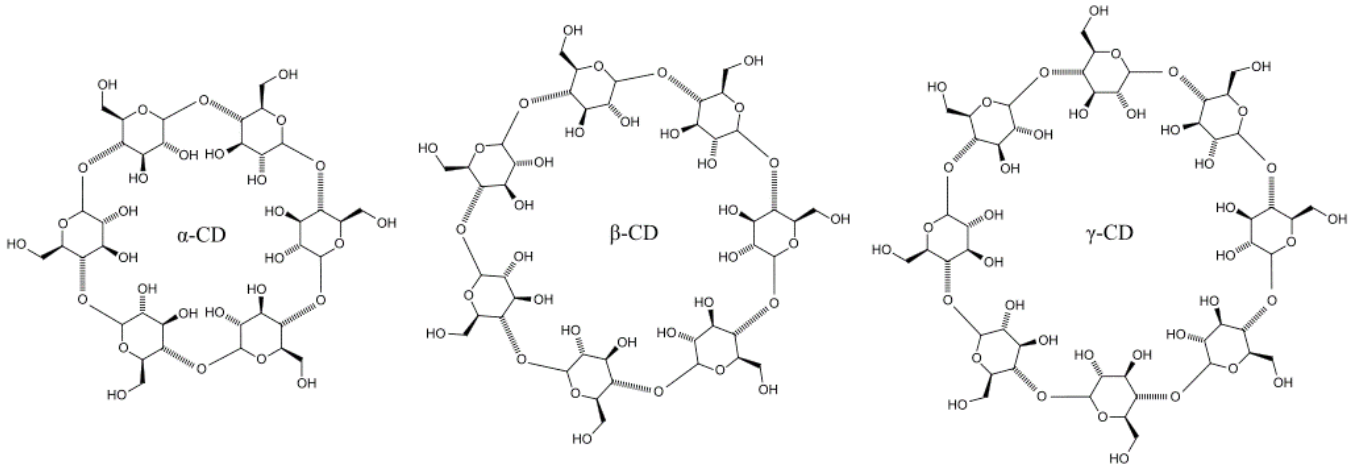

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Figure 1. Chemical structures of the three most common cyclodextrins with six (α-CD), seven (β-CD) and eight (γ-CD) D-glucose units.

chain of starch is cleaved and the reducing end is transferred to the non-reducing end of the same chain, resulting in a cyclical structure (Uitdehaag et al., 2002; Szejtli, 1998).

CGTases and, consequently, CDs can be produced by different microorganisms under cultivation (Cucolo et al., 2006; Rajesh et al., 2015), but the direct application of the enzyme on its substrate is also of great industrial applicability. Mori and coworkers (1995), for example, investigated a purified CGTase of *Brevibacterium* sp. nº 9605 and proposed that, initially, mainly γ-CD is produced by cyclization, followed by β- and, much less, by α-CD; but then they are quickly coupled to maltose or maltotriose (hydrolyzates) to form maltooligosaccharides which leads to a more significant decrease of γ-CD (faster than β- and α-CD). According to these authors, the addition of ethanol is beneficial to CD production since it inhibits the coupling of the hydrolyzates. Additionally, Tardioli et al. (2006) state that organic solvents, such as ethanol, promote the expulsion of water molecules from the active center of CGTases, thus minimizing reactions contrary to the cyclization. However, CDs have been also produced without ethanol addition (Cucolo, 2009; Rajput et al., 2016; Rauf et al., 2008).

Depending on the source of starch and the enzymatic reaction parameters, different yields can be obtained and this is an incentive to search for new substrates for CD production. Bertolini et al. (1998), for example, when working with a commercial CGTase from *Bacillus macerans*, proposed as a substrate cassava meal (a solid residue with a starch content of 70 % w/w) for α- and β-CD production with the intention of reducing the costs of CD production with purified starches. From this perspective, the consumption and processing of jackfruit (*Artocarpus intergrifolia* L.), a tropical fruit, results in the accumulation of its seeds as a residue, but they are a rich source of carbohydrates that can be potentially applied in different bioprocesses (Babitha et al., 2007; Nair et al., 2016). Jackfruit seeds correspond to 8-15 % of the whole fruit weight with variable starch contents (30 - 94 % w/w) and basic characterization of its starch has already been performed (Madruga et al., 2014, Santos et al., 2013, Zhang et al., 2018).

Taking all of that into account, the purpose of the present study was to present the jackfruit seed bran (JSB) as an alternative amylaceous substrate for the production of β-CDs by the action of a commercial CGTase. In order to obtain the best conditions for the process, aiming at an increase in productivity, we investigated the factors: temperature, pH, ethanol and enzyme on β-CD production. Since JSB is a substrate with no pre-treatment, starches extracted from JSB, ginger and potato were also applied to compare productivities.

MATERIALS AND METHODS

Amylaceous sources

Jackfruit (*Artocarpus intergrifólia* L.) seeds were obtained from ripe and well-preserved fruits harvested in the region of Ilhéus/Itabuna (Bahia, Brazil) from September to December of 2017. The pulp and seeds were manually separated and the seeds were ground in a knife mill (Macro/Willye - NL - 226 - 03). The crude bran was sieved (granulometry of 210 μm - 70 Mesh) and then dried with air circulation (Tecnal - TE - 394/2) at 105 °C to constant weight. The obtained material was denominated jackfruit seed bran (JSB) and it was autoclaved (121 °C / 15 min) and stored under refrigeration (5 °C) until the moment of use. The starches from JSB and ginger (*Zingiber officinale*) (also acquired in the region of Ilhéus/Itabuna, Bahia, Brazil) were extracted as described below. In addition,

a standard potato starch (SP) (*Solanum tuberosum*) was purchased (Dinâmica®) for comparison.

Enzymatic synthesis of *β*-CD

Syntheses of *β*-cyclodextrin (*β*-CD) were carried out in erlenmeyers of 250 mL with a useful volume of 100 mL, under orbital agitation of 120 rpm (Incubator Shaker ACB LABOR), with 10 g of the amylaceous substrate in buffer (10 mM, citrate or carbonate) at different pHs, temperature, enzyme concentration and volume of ethanol (Table 1). Cyclomaltodextrin glucanotransferase (CGTase) from *Bacillus licheniformis* (Toruzyme® 3.0 L) was kindly donated by Novozymes (Paraná, Brazil).

Samples of 1.0 mL were collected and then 11.0 mL of hydrochloric acid (0.02 M) added and the mixture was kept in a boiling bath for 5 min and then transferred to an ice bath in order to inactivate the CGTase. Each sample was then centrifuged at 4,000 *g* for 20 minutes and the supernatants were diluted and filtered with a syringe filter membrane (PES 0.45 μm) for further *β*-CD quantification (expressed as mM); the results were also expressed as productivities (μM/h.g) considering the time of reaction and the initial weight of substrate applied.

Table 1. Coded matrix for the first Fractional Factorial Design (FF) 2^{4-1} and Analysis of the Effects. The analyzed factors were: enzymatic activity per gram of jackfruit seeds bran (*GC*, U/g), ethanol (*Et*, % v/v), temperature (*T*, °C) and pH (*pH*); the evaluated response was the concentration of β-cyclodextrins (*βCD*, mM). The experiments were performed under the fixed conditions of 120 rpm and 24 h. Real values for each factor are presented in parentheses.

Matrix					
	Factors				Response
Run	CG (U/g)	Et (% v/v)	T (ºC)	pH	βCD (mM)
1	-1 (0.45)	-1 (7)	-1 (40)	-1 (5)	0.44
2	+1 (1.34)	-1 (7)	-1 (40)	+1 (7)	0.62
3	-1 (0.45)	+1 (21)	-1 (40)	+1 (7)	0.51
4	+1 (1.34)	+1 (21)	-1 (40)	-1 (5)	0.53
5	-1 (0.45)	-1 (7)	+1 (64)	+1 (7)	1.69
6	+1 (1.34)	-1 (7)	+1 (64)	-1 (5)	1.65
7	-1 (0.45)	+1 (21)	+1 (64)	-1 (5)	2.17
8	+1 (1.34)	+1 (21)	+1 (64)	+1 (7)	2.01
9	0 (0.89)	0 (14)	0 (52)	0 (6)	1.37
10	0 (0.89)	0 (14)	0 (52)	0 (6)	1.59
11	0 (0.89)	0 (14)	0 (52)	0 (6)	1.87

Effect Analysis				
Factors	Effect	Stand. Error	t(5)	p-value
Mean/Inter.	1.20	0.08	15.23	<0.01*
Curvat.	0.82	0.30	2.67	0.04*
CG	<0.01	0.16	<0.01	0.99
Et	0.21	0.16	1.31	0.25
T	1.36	0.16	8.58	< 0.01*
pH	0.01	0.16	0.05	0.96

* Statistically significant values at 89% of confidence ($p \leq 0.11$).

Study of the conditions for *β*-CD production

The determination of the best conditions for *β*-CD production from JSB and with the commercial CGTase was carried out by means of Experimental Design (Rodrigues and Iemma, 2014). In the first stage, based on the literature (Balbino et al, 2009; Tardioli et al, 2006), the independent variables (factors) considered were: CGTase activity per gram of JSB (*CG* = 0.45 - 1.34 U/g), volume of ethanol in 100 mL of reaction (*Et* = 7 - 21 % v/v), temperature of reaction (*T* = 45 - 65 ºC) and initial pH of reaction (*pH* = 5.0 - 7.0). The effects of each factor on the dependent variable (response) - concentration of *β*-CD (*βCD*, mM) - in 24 h of synthesis, were analyzed (STATISTIC v.8, Statsoft, USA) with a Fractional Factorial Design (FF) 2^{4-1} with eight factorial runs and three repetitions of the central point, totaling 11 runs (Table 1). Based on the results of the first 2^{4-1} FF, new ranges were defined for the same factors: *CG* = 1.34 - 4.02 U/g; *Et* = 21 - 35 % (v/v); *T* = 55 - 65 ºC and *pH* = 6.0 - 8.0 for a second 2^{4-1} FF (Table 2).

With the information obtained from the two FF, in the third step of the analysis, a Central Composite Rotatable Design (CCRD) 2^3 with eight factorial runs,

Table 2. Coded matrix for the second Fractional Factorial Design (FF) 2^{4-1} and Analysis of the Effects. The analyzed factors were: enzymatic activity per gram of jackfruit seeds bran (*GC*, U/g), ethanol (*Et*, % v/v), temperature (*T*, °C) and pH (*pH*); the evaluated response was the concentration of *β*-cyclodextrins (*βCD*, mM). The experiments were performed under the fixed conditions of 120 rpm and 24 h. Real values for each factor are presented in parentheses.

Matrix					
	Factors				Response
Runs	CG (U/g)	Et (% v/v)	T (ºC)	pH	βCD (mM)
1	-1 (1.34)	-1 (21)	-1 (55)	-1 (6)	0.98
2	+1 (4.02)	-1 (21)	-1 (55)	+1 (8)	1.19
3	-1 (1.34)	+1 (35)	-1 (55)	+1 (8)	1.17
4	+1 (4.02)	+1 (35)	-1 (55)	-1 (6)	1.26
5	-1 (1.34)	-1 (21)	+1 (65)	+1 (8)	0.24
6	+1 (4.02)	-1 (21)	+1 (65)	-1 (6)	0.74
7	-1 (1.34)	+1 (35)	+1 (65)	-1 (6)	0.60
8	+1 (4.02)	+1 (35)	+1 (65)	+1 (8)	1.20
9	0 (2.68)	0 (28)	0 (60)	0 (7)	1.66
10	0 (2.68)	0 (28)	0 (60)	0 (7)	1.28
11	0 (2.68)	0 (28)	0 (60)	0 (7)	1.38

Effect Analysis				
Factors	Effect	Stand. Error	t(5)	p-value
Mean/Inter.	0.92	0.07	13.13	< 0.01*
Curvat.	1.04	0.27	3.86	0.01*
CG	0.35	0.14	2.51	0.05*
Et	0.27	0.14	1.94	0.11*
T	-0.45	0.14	-3.23	0.02*
pH	0.06	0.14	0.40	0.70

* Statistically significant values at 89% of confidence (*p*-value ≤ 0.11).

Table 3. Coded matrix for the Central Composite Rotatable Design (CCRD) 2^3. The analyzed factors were: enzymatic activity per gram of jackfruit seeds bran (*GC*, U/g), ethanol (*Et*, % v/v) and temperature (*T*, °C); the evaluated response was the concentration of *β*-cyclodextrins (*βCD*, mM) in three different time. The experiments were performed under the fixed conditions of pH = 6.0 and 120 rpm. Real values for each factor are presented in parentheses.

Run	Factors			Response			
	CG (U/g)	Et (% v/v)	T (ºC)	βCD (mM)			
				8 h	12 h	16 h	20 h
1	-1 (1.07)	-1 (15.2)	-1 (52.4)	2.12	2.14	2.09	2.00
2	+1 (1.52)	-1 (15.2)	-1 (52.4)	1.85	2.48	2.26	2.00
3	-1 (1.07)	+1 (18.2)	-1 (52.4)	1.90	2.15	2.19	1.98
4	+1 (1.52)	+1 (18.2)	-1 (52.4)	2.05	2.54	2.02	1.93
5	-1 (1.07)	-1 (15.2)	+1 (58.4)	2.00	2.13	2.35	2.10
6	+1 (1.52)	-1 (15.2)	+1 (58.4)	2.66	2.72	2.05	2.20
7	-1 (1.07)	+1 (18.2)	+1 (58.4)	2.05	2.72	2.14	2.61
8	+1 (1.52)	+1 (18.2)	+1 (58.4)	2.06	2.66	2.30	1.86
9	-1.68 (0.89)	0 (17.0)	0 (56.0)	2.02	2.06	1.57	1.83
10	+1.68 (1.79)	0 (17.0)	0 (56.0)	2.37	2.27	2.11	1.59
11	0 (1.34)	-1.68 (14.0)	0 (56.0)	2.26	2.14	2.13	1.72
12	0 (1.34)	+1.68 (20.0)	0 (56.0)	2.23	2.44	1.72	1.70
13	0 (1.34)	0 (17.0)	-1.68 (50.0)	1.82	1.83	1.89	1.66
14	0 (1.34)	0 (17.0)	+1.68 (62.0)	2.39	2.48	2.53	2.22
15	0 (1.34)	0 (17.0)	0 (56.0)	2.53	2.57	2.44	2.08
16	0 (1.34)	0 (17.0)	0 (56.0)	2.33	2.37	2.29	2.00
17	0 (1.34)	0 (17.0)	0 (56.0)	2.16	2.31	2.23	2.00

six axial points and three central points was carried out, totaling 17 runs (Table 3). For this design, with pH fixed at 6.0, the ranges studied for the factors were: *CG* = 0.09 - 0.18 U/g; *Et* = 14.0 - 20.0 % (v/v) and *T* = 50 - 62 ºC. The response *βCD* was analyzed at 8, 12, 16 and 24 h. Finally, with the fixed conditions of *CG* = 1.34 U/g, *Et* = 17 % (v/v) and 8 h, the temperatures of 56, 59 and 62 °C were analyzed by the Tukey's test. After selecting the temperature of 59 °C, the same conditions of *β*-CD production were applied to the starches extracted from jackfruit seed bran (SJSB) and from ginger (SG) and to a standard potato starch (SP); the obtained productivities (with 4 and 8 h of synthesis) were compared.

Extraction of starches from jackfruit seed bran and ginger

The starch extraction was performed based on the methodology described by Schoch and Maywald (1968). Therefore, the amylaceous source (jackfruit seed bran and ginger, separately) was ground in a kitchen blender (Philips-Walita) at its maximum intensity, for 5 minutes. The obtained mass was pressed into a cotton cloth and the liquid colleted; the fibrous residue was returned to the blender and reprocessed for another 3 minutes and again pressed into a cotton cloth. The obtained liquid extract was left under static conditions for 4 h at ambient temperature (~25 ºC) and then centrifuged the 25,000 *g* for 15 minutes; the centrifugation was repeated three to four times, until the medium became free of visible turbidity (mucilage). The starches thus extracted (SJSB for the jackfruit seed bran and SG for ginger) were oven dried (Tecnal - TE - 394/2) at 40 °C until constant weight and applied in the enzymatic synthesis.

Determination of *β*-CD

The concentration of *β*-CD (mM), was calculated indirectly through the inclusion reaction that occurs between *β*-CD and phenolphthalein (PHE), using the colorimetric method already described in the literature (Tardioli et al., 2006; Moriwaki et al., 2009). Briefly, standard samples of *β*-CD (Sigma-Aldrich) containing 0.5 mL de *β*-CD (0 - 1 mM) were mixed with 2.5 mL of a stock solution of PHE (0.06 mM), freshly prepared in carbonate-bicarbonate buffer (0.12 M / pH 10.5) and the absorbance at 550 nm was determined in a UV-Vis spectrophotometer (Shimadzu). The calibration curve was obtained using Equation 1, which requires the concentration of the standard *β*-CD solution ($C_{\beta CD}$, M), the dilution factor in the cuvette (f = 6), the final concentration of PHE in the cuvette ($\alpha = 0.5\times10^{-5}$ M) and the absorbance of the sample (A, abs) and control condition (A_o, abs). Finally, the equilibrium constant ($K_{\beta CD}$ = 15,221 ± 185 M^{-1}) was calculated by the Levenberg-Marquardt iteration algorithm (R^2 = 0.994) using OriginPro® 2017 (OriginLab Corporation), and is in good agreement with published data elsewhere (Tardioli et al., 2006).

$$C_{\beta CD} = f.\alpha\left(1-\frac{A}{A_0}\right)\left(1+\frac{A_0}{\alpha.A.K_{\beta CD}}\right) \quad (1)$$

Determination of activity of CGTase

The activity of CGTase was determined in 100 mL of reaction medium containing 10 g of standard soluble

starch (Dynamic), 89.9 mL of citrate buffer solution (10 mM / pH 6.0), 10 mL of ethanol and 0.1 mL of CGTase solution (Moriwaki et al., 2009). The reaction was followed for 90 min, with samples withdrawn every 10 min and analyzed later for the concentration of *β*-CD. The standard curve was obtained for a concentration range from 3,270 to 6,850 μM of *β*-CD and resulted in an angular coefficient ($\Delta C/\Delta t$) of 89.31 μM/min (R^2 = 0.9998). Equation 2 shows the calculation of the activity of CGTase solution (U = the amount of enzyme required to produce 1.0 μM of *β*-CD per minute under the reaction conditions) as a function of $\Delta C/\Delta t$ and the reaction volume (V_R = mL) and the enzyme volume (V_E = mL). The starting CGTase solution applied presented an activity of 89.31 U/mL.

$$U = \frac{V_E}{\left(\frac{\Delta C}{\Delta t}\right) \cdot V_R} \quad (2)$$

RESULTS AND DISCUSSION

Analysis of the first 2^{4-1} FF

According to the conditions defined for the first Fractional Factorial Design (FF) 2^{4-1}, the effects of the factors on the production of *β*-CD were statistically analyzed with 89 % of confidence (Table 1) - in accordance with the confidence level defined for the second FF. Given the results, the curvature was positive and statistically significant ($p < 0.11$), indicating that the condition of the central points [0.89 U/g, 14 % of ethanol, 52 ºC, pH = 6.0 and 24 h] were favorable to the response, resulting in an average activity of 1.61 ± 0.25 mM (equivalent productivity of 6.71 μM/h.g). The analysis of the effects also indicated that only temperature (*T*) was statistically significant in the analyzed range; the tests performed at the highest temperature (64 °C - experiments 7 and 8 from Tab. 1) resulted in values about 25 to 35 % higher than the central points (at 52 °C).

The increase in productivity with the increase in temperature could be explained by the fact that CGTases from the *Bacillus* genus are relatively thermostable, with optimal temperatures ranging from 60 to 90 °C (Biwer et al., 2002). The increase in temperature in enzymatic processes, however, must be conducted carefully, for this variable also affects greatly the enzymatic stability (half-life). Rather et al. (2015), for example, restricted the temperature to 60 °C because the CGTase used had shown a drastic reduction (about 7 fold) in half-life when the temperature was between 60 to 70 °C and Bertolini et al. (1998) suggested the temperature of 50 °C for no more than 4 h of synthesis and also commented that higher starch concentration (30 % w/v) can help increase the thermal-stability of CGTases. Moreover, the increase in efficiency of the enzymatic action with the increase in temperature should be considered together with the increase in costs necessary to sustain higher temperatures.

For a better understanding of the effects, a few decisions were made for the second FF. The new temperature levels were increased, because of the positive effect that this variable had shown; however, the equipment available at the time limited the higher level of the temperature to 65 °C. The effects of the other factors also presented positive values, although not statistically significant (Tab. 1). However, this information was considered when selecting the new range of values for the enzyme (*CG*), ethanol (*Et*) and *pH*. For the second FF, the highest levels of *CG* and *Et* were selected as the new lowest levels and, in respect to the optimum pH for the enzyme (5.0 - 6.5) as informed by the manufacturer, the central level of *pH* (6.0) was displaced as the lowest level.

Analysis of the second FF 2^{4-1}

As informed before, the analysis of effects from the second FF was performed at 89 % of confidence (Table 2) and, once again, the curvature was statistically significant ($p < 0.11$). In this second FF, the central points [2.68 U/g; 28 % of ethanol; 60 °C; pH = 7.0; 24 h] presented an average activity of 1.44 ± 0.20 mM (equivalent productivity of 6.00 μM/h.g). However, when comparing the central point average and the total average of the experiments from both FF, the response was higher in the first FF, indicating that the levels of the variables in the second FF somehow exceeded the best conditions, which is a valuable information about the maximum levels, since they should be avoided. In addition, both central point conditions revealed important information.

According to the effects of the second FF, *CG* and *Et* presented positive and statistically significant effects ($p < 0.11$), suggesting that it was still possible to increase their levels. Nevertheless, it was chosen not to increase any further these two factors for the reasons explained as follows. The increase in enzyme may also increase the yield due to a greater availability of the biocatalyst, but it also means an increase in costs, and that should be avoided as possible. Concerning ethanol, which is a strong protein denaturant at higher concentration besides its positive effects on cyclization (mentioned earlier in this work), the work of Mori et al. (1995) is relevant, in which the reaction was set at 40 °C since at 50 °C the CGTase denaturation was inevitable due to the ethanol content of 20 % (v/v). Additionally, comparing the results from Tables 1 and 2, it is possible to observe that the decrease in *βCD* values (from the first FF: 0.44 - 2.17 mM to the second FF: 0.24 to 1.66 mM) does not justify increasing once again these two factors. For the next stage of analysis, and combining all the information obtained from the two FF performed, *CG* had its new levels defined to

avoid a process with high costs, but still productive, and the new levels for *Et* were defined with the intention to avoid a possible enzymatic denaturation.

On account of the negative effect of temperature (*T*) observed in the second FF and considering the central point levels of both FF designs (52 and 60 °C), the highest and the lowest levels of the new design were located around these values (50 and 62 °C, respectively). This is an interesting information since the manufacturer of the enzyme recommends its application at 80 - 95 °C however, considering the amount of ethanol employed and the equipment limitation mentioned before, it was considered to be less harmful to CGTase to conduct the reaction in milder temperatures. Meanwhile, since the *pH* was not significant in any of the two FFs (from 5.0 to 8.0, under the conditions analyzed), pH 6.0 was selected, basically due to the central points of the first FF and the fact that this value sits within the range of optimal pH as informed by the enzyme manufacturer. Interestingly, for the same commercial CGTase applied in this present study, Rauf et al. (2008) determined the optimum pH for *β*-CD production as 8.6, with a different substrate, but it is important to note that no pH below 8.0 was investigated by these researchers. For two different CGTase, the pH 7.0 (Mori et al., 1995) and 6.0 (Balbino et al., 2009) were preferred. Another important parameter selected to be investigated in the next step of analysis was the reaction time in order to obtain better productivities; for example, times for *β*-CD production of 20 h (Mori et al., 1995), 4 h (Bertolini et al., 1998; Rauf et al., 2008) and 2 h (Rajput et al., 2016) have been reported, all with purified starches.

Analysis of the 2^3 CCRD

With the results from the Central Composite Rotatable Design (CCRD) 2^3, the analysis was performed this time with 80 % of confidence in order to be able to obtain as much information as possible from the data. According to the results (Tab. 3), the highest average production (2.40 ± 0.13 mM) of *β*-CD was obtained with 12 h, but the highest average productivity (29.32 μM/h.g) was obtained with 8 h. In this time of synthesis, the terms: (*CG*), (*T*), (T^2) and (*CG*)(*T*), for a quadratic model, were statistically significant ($p < 0.20$) (Tab. 4). However, when removing the non-significant terms, and performing the analysis of variance (ANOVA) (data not presented), the model was not approved, not even with 80% of confidence. One of the reasons is explained by the Pure Error, which explains the variability of the factorial runs in comparison to the central points; there was not enough variability between the experiments and the central points (compare the responses of the experiments from 1 to 14 to the central points experiments from 15 to 17 in Tab. 3). This suggests that the design conditions were already within a favorable range and the variations of the factors were not able to promote much differentiation. Without a statistically significant model, no response surface analysis can be performed; subsequently, the analysis of effect (Tab. 4) was once again necessary to make decisions.

Table 4. Effect analysis of the factors: concentration of cyclomaltodextrin glucanotransferase (CGTase) per gram of jackfruit seed bran (*CG*, U/g), volume of ethanol (*Et*, % v/v) and temperature (*T*, °C), on the production of *β*-cyclodextrin (*βCD*, mM) at different times. Data obtained according to the CCRD 2^3 performed.

βCD 8 h	Effect	Stand. Error	t(7)	p-value	βCD 12 h	Effect	Stand. Error	t(7)	p-value
Mean/Interc.	2.35	0.11	20.92	<0.01*	Mean/Interc.	2.40	0.13	18.58	< 0.01*
(CG)	0.17	0.11	1.58	0.16*	(CG)	0.24	0.12	1.95	0.09*
$(CG)^2$	-0.14	0.12	-1.21	0.26	$(CG)^2$	-0.07	0.13	-0.49	0.64
(Et)	-0.09	0.11	-0.86	0.41	(Et)	0.16	0.12	1.33	0.22
$(Et)^2$	-0.11	0.12	-0.91	0.39	$(Et)^2$	0.02	0.13	0.17	0.87
(T)	0.26	0.11	2.51	0.04*	(T)	0.29	0.12	2.43	0.05*
$(T)^2$	-0.20	0.12	-1.76	0.12*	$(T)^2$	-0.07	0.13	-0.54	0.60
(CG).(Et)	-0.06	0.14	-0.42	0.69	(CG).(Et)	-0.15	0.16	-0.95	0.38
(CG).(T)	0.20	0.14	1.44	0.19*	(CG).(T)	-0.05	0.16	-0.32	0.76
(Et).(CG)	-0.13	0.14	-0.96	0.37	(Et).(CG)	0.12	0.16	0.73	0.49
βCD 16 h	**Effect**	**Stand. Error**	**t(7)**	**p-value**	**βCD 20 h**	**Effect**	**Stand. Error**	**t(7)**	**p-value**
Mean/Interc.	2.31	0.15	15.61	< 0.01 *	Mean/Interc.	2.01	0.14	14.39	< 0.01*
(CG)	0.11	0.14	0.81	0.45	(CG)	-0.16	0.13	-1.23	0.26
$(CG)^2$	-0.25	0.15	-1.63	0.15*	$(CG)^2$	-0.08	0.14	-0.59	0.58
(Et)	-0.12	0.14	-0.83	0.43	(Et)	0.01	0.13	0.05	0.96
$(Et)^2$	-0.19	0.15	-1.24	0.26	$(Et)^2$	-0.08	0.14	-0.59	0.58
(T)	0.20	0.14	1.43	0.20*	(T)	0.26	0.13	2.02	0.08*
$(T)^2$	0.01	0.15	0.09	0.93	$(T)^2$	0.08	0.14	0.54	0.60
(CG).(Et)	0.03	0.18	0.17	0.87	(CG).(Et)	-0.23	0.17	-1.32	0.23
(CG).(T)	-0.04	0.18	-0.19	0.85	(CG).(T)	-0.15	0.17	-0.88	0.41
(Et).(CG)	0.05	0.18	0.25	0.81	(Et).(CG)	0.07	0.17	0.38	0.72

* Statistically significant values at 80% of confidence ($p \leq 0.20$).

For CGTase (*CG*) at 8 h, in Table 4, a positive (but small) effect can be observed and, in Table 3, it was not possible to observed a great difference between the highest or the central level of *CG*. In that way, the enzymatic concentration of 1.34 U/g was selected in detriment to 1.78 U/g, considering that even a small reduction in quantity of enzyme might mean a considerable reduction in the process cost. Within the analyzed range, ethanol (*Et*) was not significant at any time (Table 4) and presented low or negative effects; therefore, *Et* was fixed at 17 % (v/v) as the central point. When analyzing the temperature (*T*) at 8 h (Tab. 3), at the highest temperature (62 °C) and the central points (56 °C), it is possible to question whether there is a statistical difference between them (2.39 and 2.42 mM, respectively). For this reason, under the fixed conditions of: *CG* = 1.34 U/g, *Et* = 17 % (v/v), *pH* = 6.0 and *t* = 8 h, *β*-CD productions at 59 and 62 °C were compared to 56 °C.

Univariate temperature analysis

The more appropriate temperature for *β*-CD synthesis was investigated at 56, 59 and 62 °C and the results obtained can be observed in Table 5. The Tukey test for the productions was carried out first at 95 % of confidence, and no statistical difference was observed between the evaluated conditions. However, in order to be able to select a temperature and considering the confidence levels applied previously, the Tukey test was performed again with a lower confidence level (85 %) and a statistically significant difference ($p < 0.15$) was observed between the temperatures of 59 and 62 °C and the lower temperature, 56 °C (Table 5). By following these steps of analysis, the decision between the three temperatures analyzed was made in favor of 59 °C (less energy expenditure than at 62 °C), which resulted in a productivity of *β*-CD of 32.99 μM/h.g. If the confidence level of 95 % had been preferred, the more appropriate choice of temperature would be towards the lower temperature (56 °C).

When analyzing the work of Rauf et al. (2008) it is possible to observe that, for sago starch, the temperature of 65 °C was fixed since it was already known that, above 67 °C, sago starch would start to gelatinize, which means the starch crystalline structure would start to absorb water and, eventually, it would disintegrate irreversibly. On the contrary, Rajput and coworkers (2016), recommend a previous gelatinization to improve the surface/volume ratio for CGTase but, contrarily, the highest productivities were obtained with raw starches. A possible inconvenience related to a previous gelatinization is the negative effect on some mass transport phenomena due to the increase in viscosity. Gelatinization was not induced for JSB in this work and, depending on the specie, for jackfruit seed starches gelatinization temperatures of 56 and 61 °C (Madruga et al., 2014) and 70 °C (Rodrigues et al., 2018) have been reported.

Table 5. Analysis of the enzymatic production of *β*-cyclodextrin (*β*CD, mM), and the equivalent productivities, under the conditions of 1.34 U/g of jackfruit seed bran, 17 % (v/v) of ethanol, pH 6.0, 8 hours of synthesis and 120 rpm at three different temperatures: 56, 59 and 62 °C.

Temperature (°C)	βCD (mM)	Productivity (μM/h.g)
56	2.34 ± 0.19 [a]	29.25
59	2.64 ± 0.18 [b]	32.99
62	2.55 ± 0.05 [b]	31.92

Mean values followed by the same letter are not statistically significant different from each other according to the Tukey test at 85% of confidence.

Production of *β*-CD with starches

Finally, with the conditions defined for the production of *β*-CD [10 g of JSB, 1.34 U/g, 17 % (v/v) of ethanol, 59 °C, pH 6.0, 120 rpm] three other starches were evaluated in order to access the applicability of JSB: the starches extracted from JSB (SJSB) and ginger (SG) and the standard potato starch (SP). Figure 2 represents the concentrations of *β*-CD obtained with these different amylaceous substrates at different times (4 and 8 h). In a way, it was expected to obtain higher yields with more purified (or more concentrated) starches instead of JSB, which was not submitted to any purification/concentration step, and that was proven by the results (Figure 2).

The best productivities were obtained with 4 h of synthesis and for the three starches analyzed similar productivities were obtained (~140 μM/h.g)

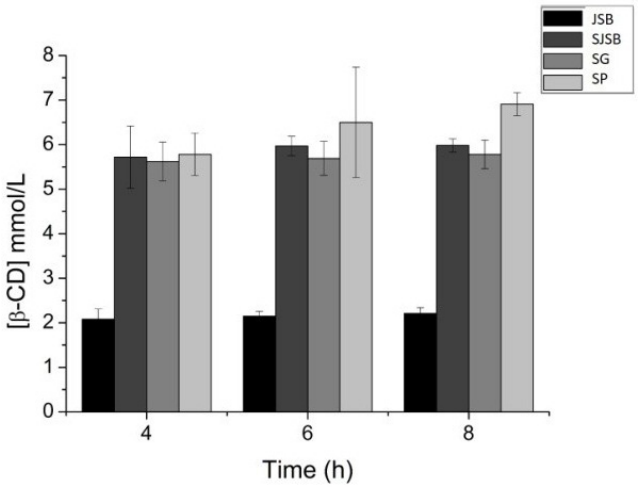

Figure 2. Production of β-cyclodextrin (*βCD*, mmol/L) by cyclomaltodextrin glycanetransferase on jackfruit seed bran (JSB), the starches from this bran (SJSB) and ginger (SG) and a standard potato starch (SP). Syntheses were performed under the conditions of: 1.34 U/g of substrate, 17 % (v/v) of ethanol, 59 °C, pH 6.0 e 120 rpm.

and, in comparison to JSB, their values were about 2.7 times greater. With the increase of time, the *β*-CD concentrations did not increase greatly; after 8 h increases of around 5 % were obtained with JSB and SJSB, 3 % with SG and only with SP occurred an increase of 20 %. Considering JSB, it was also possible to observe that the reduction of time from 8 to 4 h led to a productivity increase from 32.99 to 52.10 μM/h.g. Compared with the productivity from the first FF (6.71 μM/h.g), after this study it was possible to increase productivity by more than seven times with a substrate without any pre-treatment. Based on some results presented in the literature, it is possible to affirm that JSB is a promising substrate for CD production.

For comparison, Bertolini and collaborators (1998) investigated a commercial CGTase of *Bacillus macerans* (undeclared activity) under the reaction conditions of: pH 6.0, 50 °C, 10 % (v/v) of enzyme and 4 h and obtained productivities of *β*-CD of: 2.97 and 1.21 mM/h with 5 % (w/v) of cassava starch and cassava meal, respectively. In this case, it is possible to observe that the productivities of *β*-CD were higher than the productivities obtained in the present study (1.43 mM/h for SJSB and 0.52 mM/h for JSB, respectively), and this may be explained by the type and greater amount of both enzyme and substrates, since the reaction was performed under the same pH but with a lower temperature. Balbino et al. (2009), using a CGTase from *Bacillus macerans* and maize starch, were able to propose the conditions of: pH 6.0, 65 ºC, 15 % (w/v) of substrate, 10 % (v/v) of ethanol, 24 h and around 0.74 U/g, resulting in about 20 mM of *β*-CD (equivalent productivity of 55.56 μM/h.g). In comparison to this study, the reaction conditions applied by Balbino et al. (2009) had the same pH, higher temperature and substrate concentration and lower ethanol and enzyme concentrations and, when compared to the productivity of SJSB (143.09 μM/h.g), their result was less efficient.

The Experimental Design methodology has been applied by other researchers to improve *β*-CD production. For example, Rauf et al. (2008) included agitation as a factor and Rajput et al. (2016) investigated similar factors: enzyme, substrate and temperature; in both works, ethanol was not used and it was possible to perform the optimization of *β*-CD production. The optimum conditions defined by Rauf et al. (2008) were: a much more basic pH (8.6), more concentrated substrate (sago starch = 15 % w/v) and enzyme (equivalent to around 1.91 U/g), a faster agitation (200 rpm) and an equal time of reaction, which led to 8.43 g/L of *β*-CD (considering 100 mL of reaction, an equivalent productivity of 123.79 μM/h.g) a lower response than the one obtained with SJSB. For the work of Rajput et al. (2016), the optimum conditions were: less enzyme (equivalent to 0.032 U/g), more substrate (potato starch = 15 % w/v), lower temperature (55.6 °C), shorter time (2 h) and the same pH, with an impressive production of 24.86 mM (equivalent productivity of 828.80 μM/h.g). In this case, the higher productivity can be associated with the CGTase source (*Microbacterium terrae* KNR 9) and the shorter synthesis time.

CONCLUSIONS

In this study, it was possible to investigate the production of *β*-cyclodextrin (*β*-CD) by the action of a commercial maltodextrin glucanotransferase (CGTase) on jackfruit seed bran (JSB) in order to define the conditions that led to an improvement of seven fold on productivity. Moreover, and under the same parameters of reaction, starches extracted in this work by a simple methodology had similar productivity to a standard potato starch. Therefore, it was possible to produce a compound of interest for pharmaceutical and food industries from a low cost amylaceous material, the choice between JSB and SJSB could be made, when applicable, based on a productivity increase by almost 3 fold due to substrate purity.

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