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Byproducts from cassava industry: alternative substrates for cyclodextrin glycosyltransferase production by alkalophilic *Bacillus trypoxylicola* SM-02

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Resumen

Bioproductos de la industria de la yuca: sustratos alternativos para la producción de ciclodextrina glucosiltransferasa por alcalófilo Bacillus trypoxylicola *SM-02*

En el presente trabajo estudiamos el uso licor de maíz fermentado (LMF), harina de cáscara de yuca (HCY) y aguas residuales de yuca para la producción de ciclodextina glicosiltransferasa (CGTase) por un nuevo aislado alcalófilo de *Bacillus trypoxylicola* SM-02 en fermentación sumergida. Los experimentos se realizaron por Diseño Central Compuesto Rotativo 2^2 totalizando 11 ensayos. La mayor actividad enzimática de 352.53 U/mL se obtuvo con 1.5 g de HCY y 0.6 g de LMF. La temperatura y el pH óptimos fueron 55 ºC y pH 8.0, respectivamente. CGTase mostró una actividad relativa superior al 50% durante 120 min. a la temperatura óptima. Solo el CaCl2 mostró actividad positiva para CGTasa. Los resultados apuntaron a un buen potencial de *B. trypoxylicola* SM-02 para la producción de CGTasa usando substratos residuales.

Palabras clave: Enzima; Subproductos agroindustriales; Bacteria.

Abstract

In the present work was studied the use of cassava peel flour (CPF), corn steep liquor (CSL), and cassava wastewater as substrates to produce cyclodextrin glycosyltransferase (CGTase) from a new alkalophilic isolate of *Bacillus trypoxylicola* SM-02 by submerged fermentation. The experiments were performed as a Central Composite Design 2^2, totalizing 11 assays. An enzymatic activity of 352.53 U/mL was obtained using 1.5 g of CPF and 0.6 g of CSL. The optimum temperature and pH of CGTase was 55 °C and 8.0, respectively. The CGTase depicted a relative activity of more than 50% for 120 min at the optimum temperature. The only salt that positively influenced the CGTase activity was $CaCl_2$. The results are indicative of a potential role of *B. trypoxylicola* SM-02 in the production of CGTase using residual substrates.

Key words: Enzyme; Agro-industrial byproducts; Bacteria.

Introduction

An increasing interest in the study involving cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19), also known as cyclomaltodextrin glucanotransferase, has developed due to its ability to convert the starch molecules to cyclodextrins (CDs) through an intramolecular transglycosylation reaction (cyclization) (Van der Veen *et al.* 2000, Amud *et al.* 2008, Satyawali *et al.* 2017). Cyclodextrin glycosyltransferase is synthesized extracellularly by different microorganisms (Han *et al.* 2014). However, the bacteria belonging to the genus *Bacillus* Cohn, 1872 are most widely studied for CGTase production (Costa *et al.* 2015, Eş *et al.* 2016, Coelho *et al.* 2016). Cyclodextrins are characterized as cyclic oligosaccharides containing six (α-CD), seven (β-CD) and eight (γ-CD) glucopyranose units linked by α-1,4 glycosidic linkages. The CDs are characterized by a ring of truncated cone shape consisting of a hydrophilic exterior and a hydrophobic interior surface and thus are capable of forming inclusion compounds with a wide variety of molecules (Venturini *et al.* 2008, Kfoury *et al.* 2018). The formation of inclusion compounds resulting from the merging of the CD ring and a guest molecule (organic and inorganic) in the liquid or eventually solid medium is known to alter the physical and chemical properties of these compounds such as an increase or decrease in the solubility, stability, color, and smell (Venturini *et al.* 2008). The cyclodextrins have been utilized as aroma and flavor stabilizers and for the elimination of unwanted compounds in the food industry (Arya & Srivastava 2006), to reduce the toxicity of some drugs in the pharmaceutical industry (Nicolazzi *et al.* 2002), and in washing and dyeing processes in the textile industry (Andreaus *et al.*, 2010). These are also employed in removing pollutants from the soil (Szaniszló *et al.* 2005, Venturini *et al.* 2008). In an attempt to reduce the cost of production of CGTase, some residual products found in abundance at low cost and with high nutritional content have been utilized as an alternative. This is important for the CGTase production due to the high cost of both the enzyme and the CDs. The products and by-products derived from cassava have been utilized as substrates for CGTase production. Alves-Prado *et al.* (2002) studied CGTase production using four strains of *Bacillus* sp., subgroup *alcalophilus* by semi-solid-state fermentation using cassava starch. This carbon source was also used in the enzyme production by *Bacillus licheniformis* (Weigmann, 1898) Chester, 1901, *Bacillus* sp. BACNC-1 and BACRP (Bonilha *et al.* 2006, Menocci *et al.* 2008). The residues from the cassava flour industry such as cassava peel and cassava wastewater and waste from the corn production such as corn steep liquor are considered as economically viable substrates with good potential for CGTase production. The cassava wastewater is considered a relatively polluting agent due to the significant amount of soluble sugars and organic matter present in it (high CDO values), besides being potentially toxic due to the presence of cyanide formed from the enzymatic hydrolysis of Lynamarin, a cyanogenic glycoside present in members of Manihot genus as cassava (Kaewkannetra *et al.* 2011, Zevallos *et al.* 2018, Watthier *et al.* 2019). Therefore, cassava wastewater, as a fermentative substrate may help in reducing the environmental impacts produced by inappropriate disposal of this waste besides contributing in reducing the cost of the CGTase production. In this context, the present study aimed to analyze the CGTase production using cassava wastewater and ground cassava peel as carbon sources, and corn steep liquor as a nitrogen source by submerged fermentation using a bacterial isolate obtained from cassava flour factory and identified as *Bacillus trypoxylicola* SM-02 (Coelho *et al.,* 2016).

Material and Methods

Bacterial strain and substrates

Bacillus trypoxylicola SM-02 was isolated from the soil samples containing cassava wastewater from a cassava flour factory in Cruz das Almas county, Bahia, Brazil (Coelho *et al.* 2016). The cassava peel and cassava wastewater used as substrates in the fermentative process were also provided by cassava flour factories of Cruz das Almas county. The bacterial isolate was preserved in 20% glycerol at -10 °C. The cassava peel was washed under running water to remove the excess soil and later dried in a kiln with air circulation at 70 °C for 72 hours. After drying, the cassava peel was ground to obtain a fine powder (Cassava Peel Flour-CPF).

Molecular identification

A pair of universal primers, 8F (5'AGAGTT TGATCCTGGCTCAG3') and 1492R (5'ACGGC-TACCTTGTTACGACTT3'), was used to amplify the 16S rRNA. The sequencing was carried out commercially by Macrogen Co. (Korea). The 16S rRNA sequence was used as a query in BLASTN25 search against the National Center for Biotechnology Information (NCBI) database. MEGA 5.05 software was used to construct an evolutionary model and to generate the maximum likelihood tree. The 16S rRNA sequences used in the phylogenetic analysis were retrieved from the site of List of Prokaryotic Standing Names (LPSN) during (Coelho *et al.* 2016)

Fermentation assays

The pre-inoculum consisted of a basal medium as described by Nakamura & Horikoshi (1976) containing (g/L): soluble starch, 10; yeast extract, 5; peptone, 5; $MgSO_4.7H_2$, 0.2; KH_2PO_4, 1.0; and Na_2CO_3, 10 in distilled water. The culture of *B. trypoxylicola* SM-02 was transferred to 10 mL of pre-inoculum medium and maintained at 35 °C at 150 rpm for 24 hours. For inoculum, the starch was replaced by CPF and the nitrogen sources (yeast extract and peptone) by corn steep liquor-CSL (Sigma®) and was solubilized in cassava wastewater in place of distilled water. After an incubation of 24 hours, the pre-inoculum was transferred to 125 mL Erlenmeyer flasks containing 50 mL of inoculum medium and kept at 35 °C and 150 rpm for 24 hours. Then, an aliquot of 1 mL of the inoculum ($O.D._{600}$= 0.1) was transferred to 250 mL Erlenmeyer flasks containing 25 mL of fermentation medium according to the Central Composite Design 2^2 at 35 ºC and 150 rpm (Table 1). After 72 hours of fermentation, the samples were centrifuged at 5,000 rpm, 4 °C for 30 min. The cell-free supernatant and the biomass were used for the determination of CGTase activity and cellular growth, respectively.

Optimization using Central Composite Design (CCD)

The concentration of CPF and CSL was performed using Response Surface Methodology (RSM) according to Rodrigues & Iema (2009). The CGTase activity (U/mL) and the substrate concentrations were as dependent (response) and independent variables, respectively. A matrix of factorial design 2^2 was constructed using CCD, resulting in 11 assays. Two levels were chosen, one superior (+1) and one inferior (-1), besides the center point (0) and two axial points (+1.41 and -1.41). The central point was used with three repetitions for determining the methodological accuracy (Table 1). This model is represented by a second-order polynomial regression:

$$y = b_0 + b_1 X_1 + b_2 X_2 + b_{12} X_1 X_2 + b_{11} X_1^2 + b_{22} X_2^2$$

where y is the predicted response of CGTase activity; X_1 and X_2 are code forms (cassava peel flour and corn steep liquor, respectively); b_0 refers to the intersection point; b_1 and b_2 are linear coefficients; b_{12} is the coefficient of double interaction; and b_{11} and b_{22} are quadratic coefficients. The values of the studied levels were calculated by:

$$X_n = \frac{(X - X_0)}{X_{+1} - X_{-1}}$$

where, X_n is the encoded value; X is the real value of the independent variable; X_0 is the real value of the central point; X_{+1} is the value of the superior level, and X_{-1} is the value of the inferior level. The results presented by the applied experimental model were assessed through the Statistica software release version 7.1, Stat Soft. Inc., USA.

Enzymatic activity

The CGTase activity was determined by a colorimetric method using cyclodextrin-phenolphthalein complex (CD-PHE) (Suzuki *et al.* 1990). The reaction mixture containing 5.0 mL of crude enzyme extract (cell-free supernatant) and 5.0 ml of 1% soluble starch solution was incubated in a thermostated reactor at 55 °C, pH 8.0. The samples of 0.5 ml from the reaction solution were withdrawn at 0, 3, 6, 9, and 12 minutes and inactivated in boiling water for 5 minutes. Then, 2.5 mL of an alcoholic solution of phenolphthalein (3 mM) diluted in a buffer containing 600 mM Na_2CO_3 and pH 10.5 was added. The absorbance was read at 550 nm. CGTase production was observed by the decreasing intensity of the pink reagent due to the formation of inclusion compounds of CDs with phenolphthalein.

Cellular growth

After fermentation, the biomass was separated by centrifugation at 5000 rpm for 30 minutes at 4 ºC. The biomass was washed by suspending in 5 mL of distilled water and centrifuged. After removing the supernatant, the precipitate was re-suspended,

and the optical density was measured at 600 nm. The cellular growth was quantified by comparing with the standardized curve based on dry mass x optical density.

Determination of Total Reducing Sugar (TRS)

The samples were subjected to an acid hydrolysis in 2 M HCl. After boiling for 20 minutes, the samples were neutralized with 2 M NaOH. The total reducing sugars were determined using 3,5-dinitrosalicylic acid (DNS) method according to Miller (1959).

Determination of total protein

Total protein concentration was determined by Bradford method by adding 0.2 mL of the crude enzyme extract to 2 mL of the Bradford reagent. Absorbance was recorded at 595 nm (Bradford 1976)

Partial characterization of crude enzyme extract

The optimum temperature for CGTase activity was determined by incubating the crude extract containing the enzyme in the range of 45 °C to 70 °C at pH 8.0. The thermal stability was evaluated by incubating the crude enzyme extract at 50 °C, 55 °C, and 60 °C in a thermostated bath for 5 hours. The determination of the optimal pH was performed by using different buffers at 50 mM: glycine-HCl pH 2.0-3.0; sodium citrate pH 3.0-6.0; phosphate pH 6.0-8.0; Tris-HCl pH 8.0-9.0; and glycine-NaOH pH 9.0-10.0. The influence of metal ions was evaluated using the following solutions prepared at 50 mM: $CaCl_2$, $FeCl_2$, NaCl, $ZnSO_4$, EDTA, KCl, $MnCl_2$, $CuSO_4$, $BaCl_2$, $HgCl_2$, and $MgCl_2$. For control, the enzyme activity was performed using the supernatant without the addition of salts to the reaction mixture.

Results

Table 1 shows the matrix of CCD 2^2 with the results of CGTase activity and biomass production using cassava peel and corn steep liquor as substrates, diluted in cassava wastewater after 72 hours of fermentation along with the values predicted by the statistical model.

The experimental conditions resulted in the highest values of CGTase activity at the center point (9, 10, and 11) with an average activity of 352.53 U/mL, using 1.5 g of cassava peel flour and 0.6 g of corn steep liquor. A similar production was observed in the assay 7 (1.5 g of cassava peel flour and 0.2 g of corn steep liquor) resulting in 318.25 U/mL of CGTase activity (Table 1).

However, according statistical analysis of the regression coefficients (Table 2), only the cassava peel flour in the quadratic term was statistically significant at 95% confidence level ($p<0.05$). These results indicated that the only variable that influenced CGTase activity was the carbon source. The nitrogen source (corn steep liquor) did not influence the production of CGTase ($p> 0.05$), which means that the variation in its concentration had no effect on the enzymatic activity within the studied concentration range (0.2 to 1.0 g). The values for F_{calc} of 15.82 and R^2 of 82.71% indicated that the results were highly significant and

Run	Codified values		Real values (g)		CGTase activity (U/mL)		Biomass (g/L)	
	X_1	X_2	Cassava peel flour	Corn steep liquor	Observed values	Predicted values	Observed values	Predicted values
1	–1	–1	1.0	0.32	288.60	247.84	5.28	5.08
2	+1	–1	2.0	0.32	136.67	202.47	6.17	7.58
3	–1	+1	1.0	0.88	73.36	43.35	12.85	11.86
4	+1	+1	2.0	0.88	122.54	199.10	5.63	6.25
5	–1.41	0	0.8	0.60	15.45	72.90	8.51	9.43
6	+1.41	0	2.2	0.60	244.20	150.94	8.59	7.23
7	0	–1.41	1.5	0.20	318.25	307.95	5.90	5.12
8	0	+1.41	1.5	1.00	186.48	160.97	8.64	8.98
9	0	0	1.5	0.60	340.99	352.53	7.24	6.96
10	0	0	1.5	0.60	356.97	352.53	6.97	6.96
11	0	0	1.5	0.60	359.64	352.53	6.67	6.96

X_1= Cassava peel flour; X_2= Corn steep liquor

Tabla 1. Matriz del Diseño Central Compuesto Rotativo 2^2 que muestra valores independientes y respuestas de *B. trypoxylicola* SM–02 en fermentación sumergida a 35 ºC y 150 rpm durante 72 h.

Table 1. Matrix of Central Composite Design 2^2 showing independent values and responses by *B. trypoxylicola* SM–02 in submerged fermentation at 35 ºC and 150 rpm for 72 h.

Factors	Regression coefficient	Standard error	t(5)	*p-value*
Mean	352.53	41.40	8.52	0.000367
Cassava peel flour (*L)	27.59	25.35	1.09	0.326070
Cassava peel flour (**Q)	–120.31	30.18	–3.99	0.010459
Corn steep liquor (L)	–51.97	25.35	–2.05	0.095678
Corn steep liquor (Q)	–59.04	30.18	–1.96	0.107785
Cassava peel flour × corn steep liquor	50.28	35.85	1.40	0.219758

*L=Linear; **Q=Quadratic; R^2=0,8271

Tabla 2. Análisis de regresión de la producción de CGTasa por *B. trypoxylicola* SM–02 en fermentación sumergida a 35 ºC y 150 rpm durante 72 h utilizando aguas residuales de yuca, harina de cascara de yuca y licor de maíz fermentado como sustratos.

Table 2. Regression analysis of CGTase production by *B. trypoxylicola* SM–02 in submerged fermentation at 35 ºC y 150 rpm for 72 h using cassava wastewater, cassava peel flour, and corn steep liquor as substrates.

Factors	Freedom degree	Sum of the squares (SS)	Means squares (MS)	F_{calc}
Regression	3	174414.0	58138.0	15.82[a]
Residue	7	25709.4	3672.8	
Total	10	148704.6		

[a] Significant at 5% probability; R^2: 0.8271

Tabla 3. Análisis de varianza para la producción de CGTasa de *B. trypoxylicola* SM–02 en fermentación sumergida a 35 °C y 150 rpm durante 72 h utilizando aguas residuales de yuca, harina de cascara de yuca y licor de maíz fermentado como sustratos.

Table 3. Analysis of variance for CGTase production by *B. trypoxylicola* SM–02 in submerged fermentation at 35 ºC y 150 for 72 h rpm using cassava wastewater, cassava wastewater, cassava peel flour, and corn steep liquor as substrates.

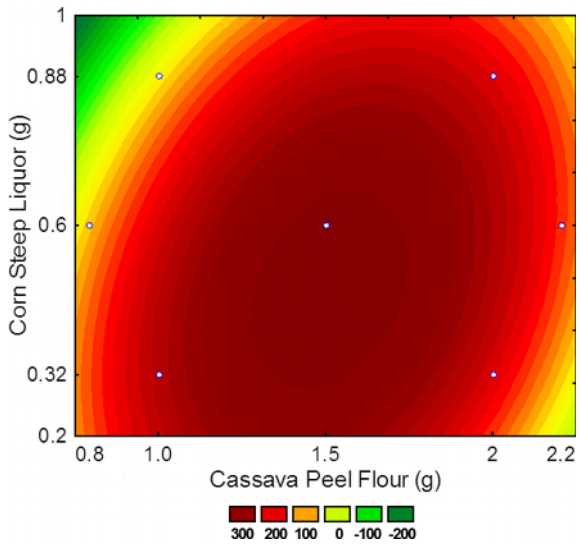

Figura 1. Diagrama de contorno para la producción de CGTasa de *B. trypoxylicola* SM–02 por fermentación sumergida con aguas residuales de yuca, harina cáscara de yuca y licor de maíz fermentado a 35 °C y 150 rpm durante 72 h. La figura muestra desde la producción de CGTasa más pequeña (verde) hasta la mayor (rojo).

Figure 1. Contour plot for CGTase production from *B. trypoxylicola* SM–02 by submerged fermentation using cassava wastewater, cassava peel flour and corn steep liquor as substrates at 35 °C and 150 rpm for 72 h. The figure shows the production of CGTase from the smallest (green) to the largest (red) value.

the correlation between the predicted and observed values was good (Table 3). In this way, it can be affirmed that the results fit well with the model and the coefficient regression values could be used to generate the model equation:

$$Enzymatic\ activity = 352.53 + 27.59\,X_1 - 120.31\,X_1^2 - 51.97\,X_2 - 59.04\,X_2^2 + 50.28\,X_1X_2$$

The equation generated by the model is important because, through it, it is possible to derive the optimal conditions to determine the critical point (Rodrigues & Iema, 2009). The chart of contour curves (Fig. 1) confirmed these results to be within the range studied for the dependent variables, and the center point region presented the highest CGTase activity. Response Surface Methodology is very useful for the modeling and the analysis of problems, where the outcome is influenced by multiple variables and the objective is the response optimization (Maddipati *et al.* 2011). Furthermore, this type of chart allows viewing a wide range of substrate concentrations where the enzyme production remains high. This is of importance when using wastes or agro-industrial byproducts as substrates for fermentation processes, which show considerable variation in its composition.

Under optimized conditions, CGTase production follows the exponential growth phase of *B. trypoxylicola* SM-02 until 72 hours of fermentation after which, there was a downward trend coinciding with the decline of cellular growth. At the same time, almost complete consumption of sugars was noted, which may be attributed to the metabolic activity of the bacteria after 120 hours (Fig. 2).

Partial characterization of enzyme crude extract

Physical and chemical characterization was performed using the crude enzyme extract obtained under optimized fermentation conditions. The enzyme displayed a reduced activity in acidic pH ranging from 2.0 to 6.0. At the pH values 7.0 and 8.0, CGTase exhibited a relative activity of 80% with an optimum pH at 8.0. At pH 9.0, the enzyme activity decreased but remained above 50% until pH 10.0 (Fig. 3).

The optimum temperature of CGTase produced by *B. trypoxylicola* SM-02 was 55 °C with 50% relative activity at 50, 60 and 65 °C and was completely inactivated at 70 °C (Fig. 4A). In relation to thermal stability, the activity of CGTase

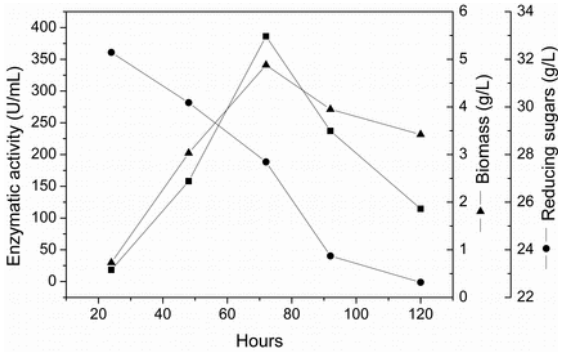

Figura 2. Fermentación sumergida en el transcurso del tiempo por *B. trypoxylicola* SM–02 con aguas residuales de yuca, harina cáscara de yuca y licor de maíz fermentado como substratos a 35 °C y 150 rpm durante 120 h.

Figure 2. Submerged fermentation time-course of *B. trypoxylicola* SM–02 using cassava wastewater, cassava peel flour, and corn steep liquor as substrates at 35 °C and 150 rpm for 120 h.

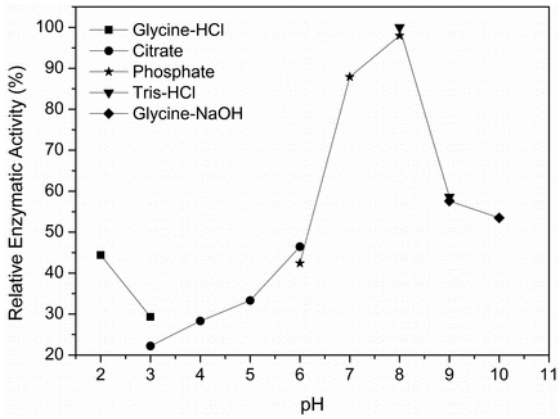

Figura 3. pH óptimo de la actividad CGTasa de *B. trypoxylicola* SM-02 cultivada en aguas residuales de yuca, HCY y LMF como sustratos a 35 ºC y 150 rpm. La fuerza iónica para todos los tampones fue de 50 mM.

Figure 3. Optimum pH of CGTase activity of *B. trypoxylicola* SM-02 grown on cassava wastewater, CPF, and CSL as substrates at 35 °C and 150 rpm. The ionic strength for all buffers was 50 mM.

from *B. trypoxylicola* SM-02 presented a relative activity of 75%, on average, between 30 minutes and 1 hour of incubation, and about 60% after 2 hours. After 3 hours the enzyme still showed a relative activity of about 40% at 55 ºC (Fig. 4B).

Assessing the influence of ions (Table 4), it was observed that enzyme production was positively influenced by $CaCl_2$, which resulted in an increase in the enzyme activity compared with the control. The enzyme was weakly inhibited by $MgCl_2$, $FeCl_2$, NaCl, and $CuSO_4$ reaching an activity almost next to the control, and was strongly inhibited by EDTA, $BaCl_2$ resulting in a drop of enzyme activity to below 75% (Table 4).

Discussion

Although there are reports of the use of the residues from cassava processing, i.e., peel and cassava wastewater in the production of other enzymes such as amylase, protease, and lipase (Barros *et al.* 2013), these residues have not been used for CGTase production. Agro-industry substrates are cited in the literature as best for the CGTase production, compared with the conventional synthetic substrates, whose origin is corn starch. Cucolo *et al.* (2006) described the highest CGTase production using tapioca flour than the soluble starch. Similarly, our group also observed a high CGTase production in cassava flour by *Bacillus* sp. SM-02 (Coelho *et al.* 2016). This shows that the substrates of agricultural origin are considerably efficient for the enzyme production, possibly

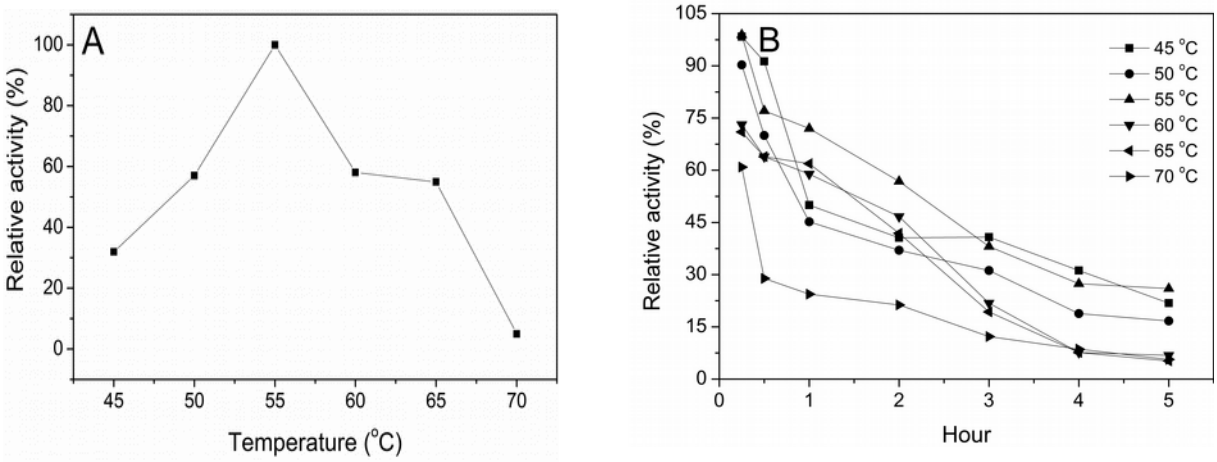

Figura 4. Temperatura óptima (a) y estabilidad térmica (b) de CGTasa producida por *B. trypoxylicola* SM-02 cultivada en aguas residuales de yuca, HCY y LMF como sustratos a 35 ºC y 150 rpm durante 72 h.

Figure 4. Optimum temperature (a) and thermal stability (b) of CGTase produced by *B. trypoxylicola* SM-02 02 grown on cassava wastewater, CPF, and CSL as substrates at 35 °C and 150 rpm for 72h.

due to a complete chemical composition of macro and micronutrients, which supplement the fermentation medium and consequently increase the CGTase production. According to Alves-Prado *et al.* (2008) the concentration of lipids in the starch should be considered, since it is a factor that interferes in the production of CGTase. Cassava starch presents low amount of lipids and does not form amylose-lipid complexes in root and tuber starches, unlike in cereal starches. In addition, less content of amylose (between 17% and 20%) than cereal starch (25 to 30%) may explain the high CGTase production in substrates derived from cassava (Moorthy *et al.* 2006, Weber *et al.* 2009, Senanayake *et al.* 2013).

The nitrogen source is an important element in the CGTase production, especially when starch derived from cassava is used. This is due to the fact that there is a reduction in the enzyme production if this substrate is used without supplementation with peptone and yeast extract (Cucolo *et al.* 2006). Organic nitrogen sources have been found to be most suitable compared to the inorganic ones and peptone and yeast extract are the most used nitrogen sources (Mahat *et al.* 2004, Ibrahim *et al.* 2005, Cucolo *et al.* 2006, Avci & Dömnez 2009). In this work, however, we chose to use the corn steep liquor as an interesting alternative due to its low cost, as it is a residue from the corn processing industry. The corn steep liquor is a nitrogen source widely used in fermentation processes for the production of different substances like enzymes (cellulase, laccase) (Wang *et al.* 2014, Ladeira *et al.* 2015), organic acids (succinic acid, lactic acid) (Lee *et al.* 2000, Xi *et al.* 2013, Wang *et al.* 2015), fuels (butanediol, ethanol) (Maddipati *et al.* 2011, Yang *et al.* 2013) and exopolysaccharides (Sharma *et al.* 2013). Corn steep liquor contains a mixture of reducing sugars and amino acids as well as water-soluble vitamins and minerals and, therefore, serves as an excellent nutrient source (Xiao *et al.* 2012, Xiao *et al.* 2013).

The profile of submerged fermentation time-course of *B. trypoxylicola* SM-02 (Fig. 2) shows that the decrease in the cellular growth and consequently the enzyme activity after 72 hours seems to be related to the low concentration of sugars in the medium. This suggests that it is necessary to maintain a high concentration of sugars in the medium for high enzyme production, which can be obtained with the use of fed-batch fermentation processes that constantly feed the system with the substrates. A similar kinetic behavior was observed in other studies on CGTase production with different species of *Bacillus* such as *Bacillus* sp. H25 and *Bacillus* sp. subgroup *alcalophilus* (E16, H27, and H54) (Alves-Prado *et al.* 2002), where the enzyme synthesis began in the exponential phase; however, the maximum production was achieved in the stationary phase.

B. trypoxylicola SM-02 showed an optimum pH for the enzymatic activity in an alkaline pH range, indicating that this bacterium is alkalophilic (Fig. 3). CGTases produced by different species of *Bacillus* exhibited an optimum pH in the neutral and alkaline range with a few exceptions in the acidic range (Alves-Prado *et al.* 2002, Freitas *et al.* 2004, De Souza *et al.* 2013, Blanco *et al.* 2014). CGTase production in alkaline pH is advantageous because it reduces the tendency of starch gelatinization, which decreases the stickiness of starch at high concentration in the fermentation medium (Goo *et al.* 2014). Although the catalytic properties of CGTase are strongly affected by the composition of the fermentation medium, the optimum temperature between 50 °C and 55 °C is a characteristic of several species of *Bacillus* producing CGTase using different substrates (Gawande *et al.* 2003, Freitas *et al.* 2004, Cucolo *et al.* 2006, Ibrahim *et al.* 2012).

The thermal stability of CGTase from *B. trypoxylicola* SM-02 was very good, remained above of 50% after 2 hours (Fig. 4). This behavior is similar to that described for CGTase de *B. trypoxylicola* SM-02 obtained using cassava flour as a carbon source (Coelho *et al.*, 2016). Thermostability is a further desired property of a CGTase since a higher reaction temperature reduces the viscosity of the starch substrate and can result in higher yields of ciyclodextrin (Sonnendecker & Zimmermann 2019).

The information about ions that affect the enzymatic activity is important for the choice of substrates used in the fermentation process because depending on the composition, they may positively or negatively influence the enzyme synthesis. It is already a well-established fact that some ions act as essential enzymatic cofactors for the process of catalysis in some enzymes. The literature mentions the ions Mn^{2+} and Mg^{2+} as positive inducers of CGTase production by species of *Bacillus*, whereas Cu, Co, and Hg inhibit the enzyme activity. Some ions such as Fe, Ba^{2+} and

Zn displayed both an increase and decrease in the cyclization activity of the enzyme depending on the *Bacillus* species (Freitas *et al.* 2004, Arya & Srivastava 2006, Singh *et al.* 2010, Martínez-Mora *et al.* 2012, Ibrahim *et al.* 2012). For *B. trypoxylicola* SM-02, $CaCl_2$ was the only salt that increased enzymatic activity (Table 4). CGTase belongs to the α-amylase superfamily and these enzymes have regions that contain highly conserved amino acid residues located in or close to the active site, and that act directly on the cleavage of the glycosidic bond, bonding to the substrate, stabilizing the transition state and binding of calcium ions. Some of these enzymes, including CGTase, contain aspartic acid residues responsible for binding calcium ions and, apparently, these conserved sequences are related to the maintenance of the enzyme's catalytic site structure (Janecek, 2002, Matsuura 2002). In addition, Saboury (2002), studying the interaction of *Bacillus amyloliquefaciens* α-amylase (BAA) with divalent calcium observed that the binding of calcium stabilized the enzyme against surfactant and thermal denaturation, preventing spontaneous decrease in biological activity of α-amylase. EDTA, in turn, is a chelating agent that can combine with several mineral ions, including calcium, which may explain the decrease of about 70% of the enzymatic activity in the presence of this compound.

Conclusions

Cassava peel four, cassava wastewater, and corn steep liquor could be considered as promising substrates for CGTase production by *B. trypoxylicola* SM-02 by submerged fermentation. The produced enzyme exhibited attractive features for industrial applications. It has an optimum alkaline pH that provides a lower risk of contamination by most competing microorganisms. Furthermore, the optimum temperature of CGTase (55 °C) produced by *B. trypoxylicola* SM-02 and its good thermostability facilitate the use of this enzyme in industrial processes. The use of cassava wastewater as a substrate for the fermentation processes would also help reduce the environmental impacts caused by the inappropriate disposal of this waste into the environment and the use of cassava peel can generate a new alternative of this residue. It is possible to decrease the CGTase production cost because the carbon sources involved in the study are characterized as abundant and low-cost and, in addition, have a high content of nutrients, allowing the generation of economically viable processes.

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