

## PRODUCTION OF CGTASE BY A *BACILLUS ALKALOPHILIC* CGII STRAIN ISOLATED FROM WASTEWATER OF A MANIOC FLOUR INDUSTRY

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### ABSTRACT

CGTase production by a new strain of *Bacillus alkalophilic* CGII isolated from Brazilian wastewater of manioc flour industry was examined. The growth medium used was composed by 1.5% starch, 1.5% nitrogen and 1% Na<sub>2</sub>CO<sub>3</sub>. Higher activity was obtained with starch, maltodextrin and galactose. When glucose was added to the medium, no enzyme production was observed. High enzyme activity and growth were reached when aeration was increased (88.6 U/mL). The enzyme characterization showed an optimum pH and temperature 8.0 and 55°C for starch hydrolyses, respectively. Mg<sup>+</sup> and Ca<sup>++</sup> showed small activation; however, Hg<sup>+</sup> and Cu<sup>+</sup> showed a strong enzyme inhibition.

**Key words:** *Bacillus alkalophilic* CGII; CGTase; β-cyclodextrin; starch hydrolysis; enzyme activity

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### INTRODUCTION

The enzyme CGTase (cyclodextrin glycosyltransferase) converts starch into cyclic maltooligosaccharide known as cyclodextrin (1,20,21,27). Cyclodextrin (CD) may contain six (α-CD, cyclohexamilose cG<sub>6</sub>), seven (β-CD cycloheptamilose cG<sub>7</sub>), or eight (γ-CD, cyclooctamilose cG<sub>8</sub>) units linked by α-1,4 glycosidic bonds (3,31). The main difference among them is the apolar cavity size and solubility in water (2). The CDs have the capability to form inclusion complexes with a variety of molecules, modifying their physical chemical properties. In general, this is the reason why CDs are being widely used in food, pharmacy and cosmetics industries (2,23,26,27). Several microorganisms are CGTase producers, but the genus *Bacillus* is the most frequently cited in the literature (1,3,5,9,11,12,13, 14,15,17,18,19,20,21,24,25,30). In this work an alkalophilic CGTase producer microorganism, characterized as *Bacillus alkalophilic* CGII was isolated from wastewater of manioc flour industry. The main objective of the work was

to improve the production of CGTase and to characterize the enzyme partially.

### MATERIALS AND METHODS

#### Isolation of the CGTase producer microorganism

To isolate the CGTase producer microorganism, the method described by Park *et al.* (22) was used. Soil samples of 1g were suspended in 50 mL of sterilized water and 0.1 mL of the suspensions was inoculated in plates containing culture medium prepared with 2.0% soluble starch (Ecibra); 0.5% polypeptone (Oxoid); 0.5% yeast extract (Oxoid); 0.1% K<sub>2</sub>HPO<sub>4</sub> (Synth); 0.02% MgSO<sub>4</sub> x 7H<sub>2</sub>O (Synth) 1.0% Na<sub>2</sub>CO<sub>3</sub> (Mallinckrodt); 2.0% Agar (Difco); 0.03% phenolphthalein (Synth) and 0.01% methyl orange (Merck), pH 10.5, and incubated at 37°C. Light zones were observed around the CGTase producer colonies due to phenolphthalein inclusion in the medium by the cyclodextrin produced. The CGTase producer colonies were transferred into tubes containing the

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same culture medium but without phenolphthalein and methyl orange.

### CGTase production

The selected strain was cultivated in flasks containing 200 mL of culture medium and incubated at 37°C during 18 hours at 200 rpm. This culture was used to inoculate (10% V/V) 2L of culture medium, in a fermentator (5 L capacity) containing 2 mL of antifoaming agent. The incubation was done at 37°C, 200 rpm and aeration 1.5 vvm. Samples were drawn periodically to analyze the production of the enzyme, the pH and the enzymatic activity. To improve the enzyme production, many attempts were made, such as substitution of starch by different carbohydrates, and variation in nitrogen and Na<sub>2</sub>CO<sub>3</sub> concentration. After enzyme production, the culture was centrifuged, and 3% of starch was added to the supernatant containing the enzyme for adsorption and desorption in phosphate buffer 10mM (pH 7.0) containing NaCl 2M, acetone 3V and ammonium sulphate 70%. The enzyme was dialyzed in distilled water during 12 hours and stored under refrigerated conditions.

### Enzymatic activity

Cell-free enzymatic activity was measured according to Mäkela *et al.* (16) and Goe and Nene (8). Five mL of the supernatant and 5.0 mL of a 1% starch solution [0.1 g of soluble starch; 1.0 mL CaCl<sub>2</sub> (0.05 M) and pure water for a total volume of 10 mL] were mixed in a thermostatic reactor at 50°C. Samples were taken periodically from the reactors, and inactivated in water at 100°C for 5 minutes. The concentration of cyclodextrin was measured by addition of 2.5 mL of a 3mM phenolphthalein solution (5.0 mL of 0.6 M Na<sub>2</sub>CO<sub>3</sub> buffer, pH 10.5, and the volume completed with 2.5 mL of distilled water in a volumetric flask) to 0.5 mL of the inactivated samples. The absorbance of the final solution was analyzed in spectrophotometer at 550 nm. A unit of the enzymatic activity was defined as the quantity of enzyme that produces 1 µmol of β-CD per minute under standard conditions.

## RESULTS AND DISCUSSION

### Isolation of the microorganism

During the selection of CGTase producer microorganisms, four strains of alkalophilic *Bacillus* were isolated. The strain that showed highest activity β-CD production was characterized as *Bacillus alkalophilic* CGII.

### CGTase production

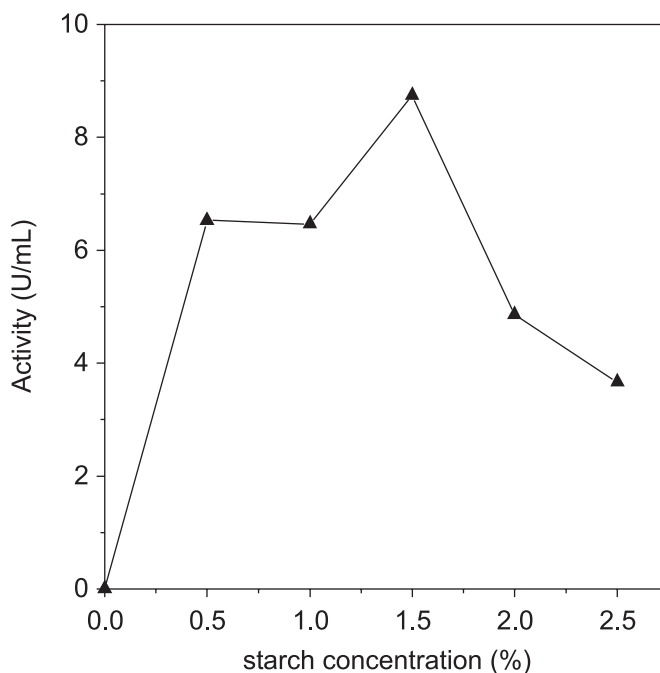
Table 1 shows the effect of the carbon source on the enzyme synthesis after 48 hours of fermentation. According to the results, the microorganism grew very well in all studied carbon sources, but the growth was slower in mannitol and glucose. Higher specific activities were obtained with starch, maltodextrin

and galactose. When glucose was added to the medium, enzyme production was not observed. Fig.1 shows the effect of starch concentration, showing that the highest activity was reached at 1.5%.

**Table 1.** Effect of carbohydrate source on the synthesis of CGTase by *B. alkalophilic* after 48 hour-fermentation.

Carbon source	Initial pH	Final Mass	Dry (mg/mL)	Activity Mass (U/mL)	Specific Activity (U/mg)
Starch	10.15	8.59	7.80	37.48	12.00
Maltodextrin	10.15	9.14	5.28	15.70	7.40
Fructose	10.00	8.74	8.36	8.90	2.66
Lactose	10.12	9.04	7.38	6.51	2.20
Galactose	10.03	9.18	4.80	6.31	3.28
Xylose	9.95	8.77	6.30	6.20	2.46
Wheat bran	10.02	8.99	nd	5.66	nd
Sucrose	10.15	8.67	4.49	4.23	2.35
Sorbitol	10.16	9.33	4.29	2.97	1.73
Manitol	10.15	8.98	3.68	2.25	1.52
Maltose	10.08	8.64	6.80	1.54	0.57
Glucose	10.05	8.59	3.75	0.00	0.00

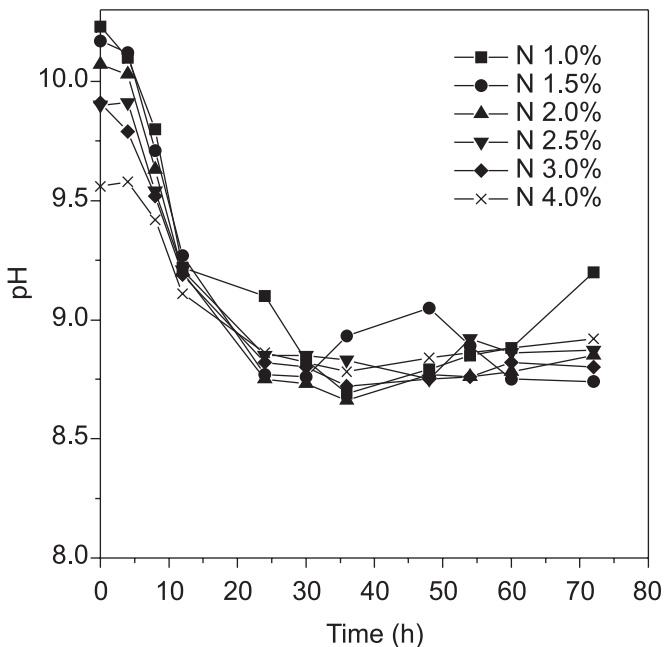
nd: not determined.



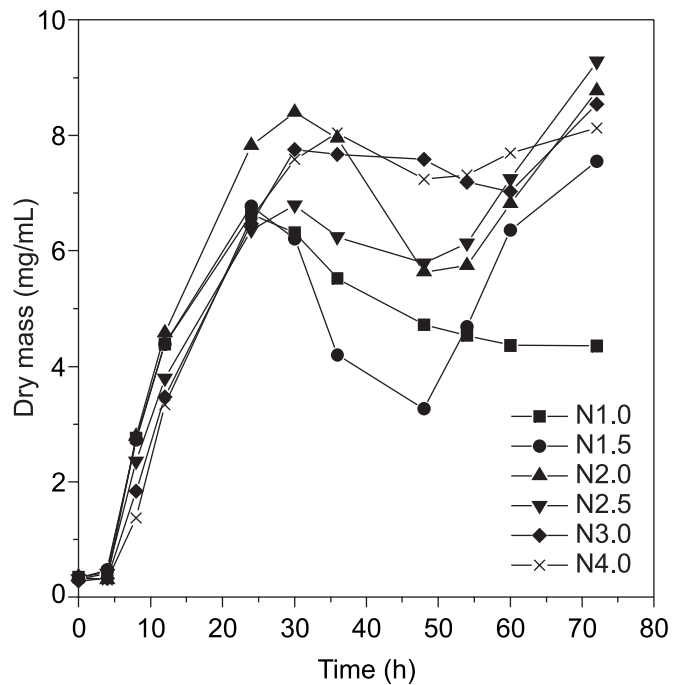
**Figure 1.** CGTase activity as a function of the starch concentration.

Fig. 2 shows the effect of nitrogen concentration (yeast extract and peptone) on the cellular growing and enzyme production. It is important to note that the initial pH is lower for higher nitrogen concentration present in the medium. However; the  $\Delta$ pH was not altered at any concentration. It was verified that, the maximum growth occurred at about 24-30 hours in all concentrations. A diauxic phase was observed at lower concentrations (Fig. 3). At the same time as the nitrogenous concentration increases, the exponential phase becomes longer. The 1.5% nitrogenous concentration was chosen for the next assays because in this concentration it was possible to achieve a better enzymatic activity in a short period of time, that is, at around 36 hours of fermentation, as shown in Fig. 4.

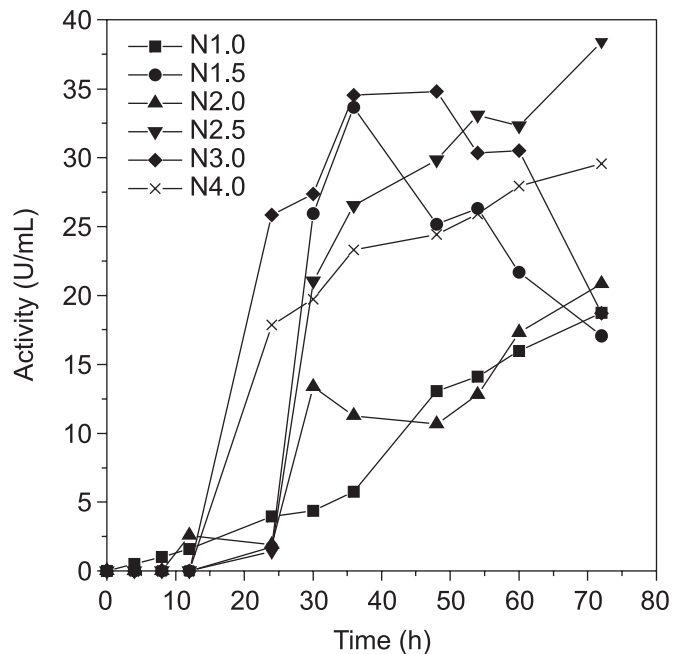
The effect of  $\text{Na}_2\text{CO}_3$  on cellular growth and enzyme production was studied varying the concentration from 0.25% to 1.5%. As shown in Fig. 5, the difference between the starting and final pH in all concentrations tested, did not change, that is, the  $\Delta$ pH was kept almost constant. Regarding to cellular growth, it was observed a larger exponential phase at higher concentrations of  $\text{Na}_2\text{CO}_3$  and an enhanced diauxic at lower concentrations (Fig. 6). At higher concentrations of  $\text{Na}_2\text{CO}_3$  the fermentation time to obtain a good enzymatic activity was the same as for lower concentrations (Fig. 7). Based in this fact, the 1.0%  $\text{Na}_2\text{CO}_3$  concentration was chosen for the other assays, because the addition to the culture medium is only to increase its pH, and to buffer the medium for the growth of a alkalophilic microorganism.



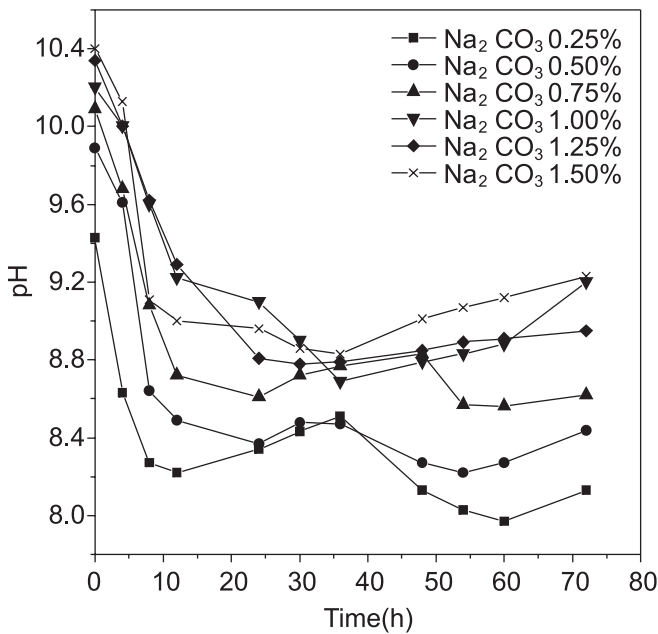
**Figure 2.** pH as a function of nitrogen concentration: (■) 1%; (●) 1.5%; (▲) 2.0%; (▼) 2.5%; (◆) 3.0%; (X) 4.0%.



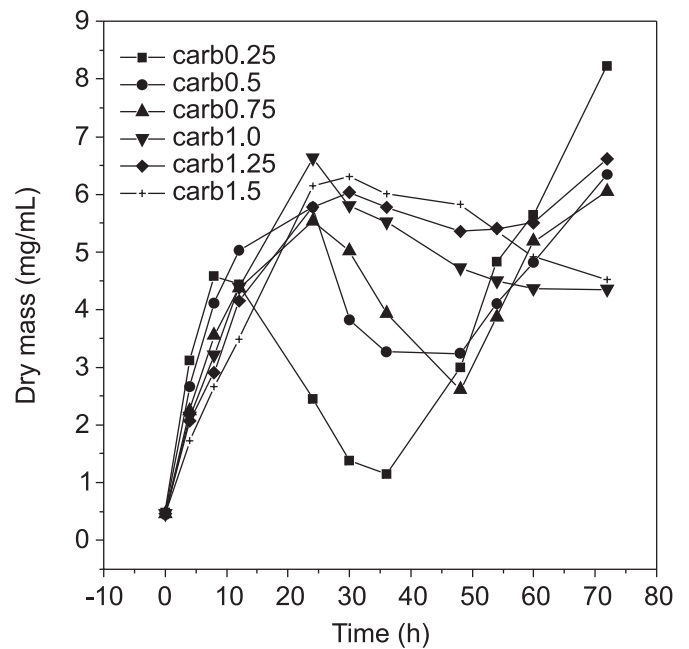
**Figure 3.** Dry mass as a function of nitrogen concentration: (■) 1%; (●) 1.5%; (▲) 2.0%; (▼) 2.5%; (◆) 3.0%; (X) 4.0%.



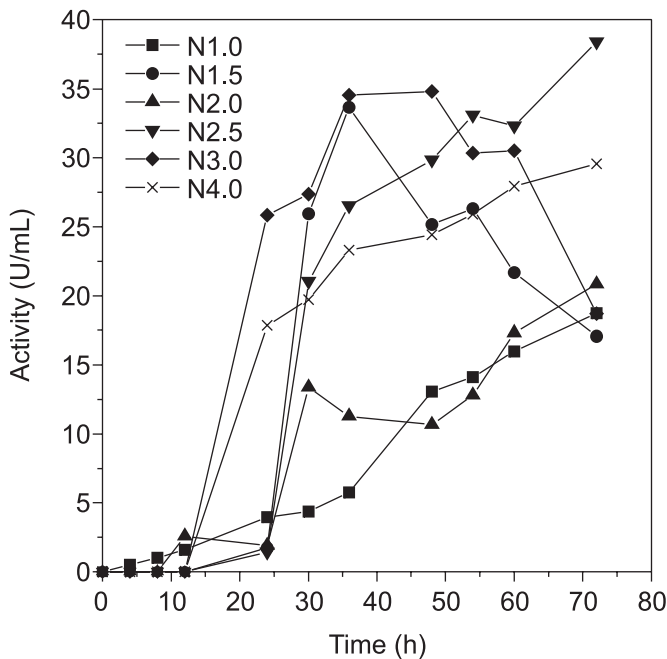
**Figure 4.** Enzymatic activity as a function of nitrogen concentration: (■) 1%; (●) 1.5%; (▲) 2.0%; (▼) 2.5%; (◆) 3.0%; (X) 4.0%.



**Figure 5.** pH as a function of  $\text{Na}_2\text{CO}_3$  concentration: (■) 0.25%; (●) 0.5%; (▲) 0.75%; (▼) 1.0%; (◆) 1.25%; (X) 1.5%.



**Figure 6.** Dry mass as a function of  $\text{Na}_2\text{CO}_3$  concentration: (■) 0.25%; (●) 0.5%; (▲) 0.75%; (▼) 1.0%; (◆) 1.25%; (X) 1.5%.

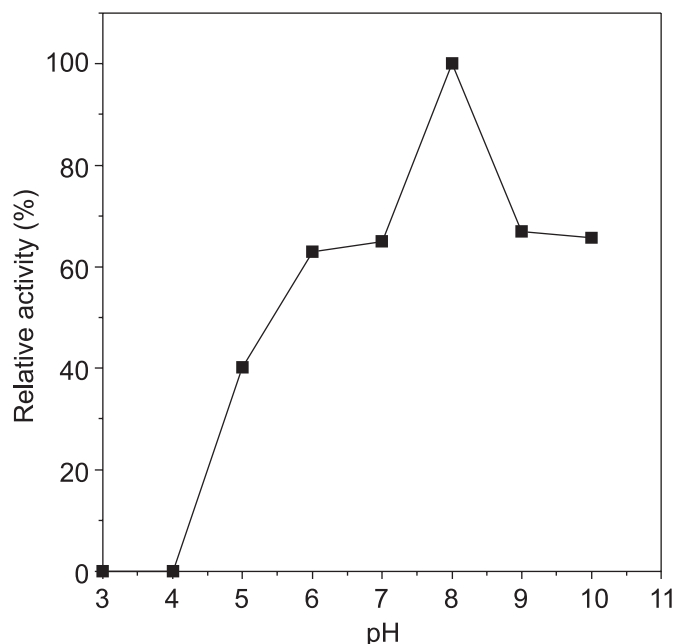


**Figure 7.** Enzymatic activity as a function of  $\text{Na}_2\text{CO}_3$  concentration: (■) 0.25%; (●) 0.5%; (▲) 0.75%; (▼) 1.0%; (◆) 1.25%; (X) 1.5%

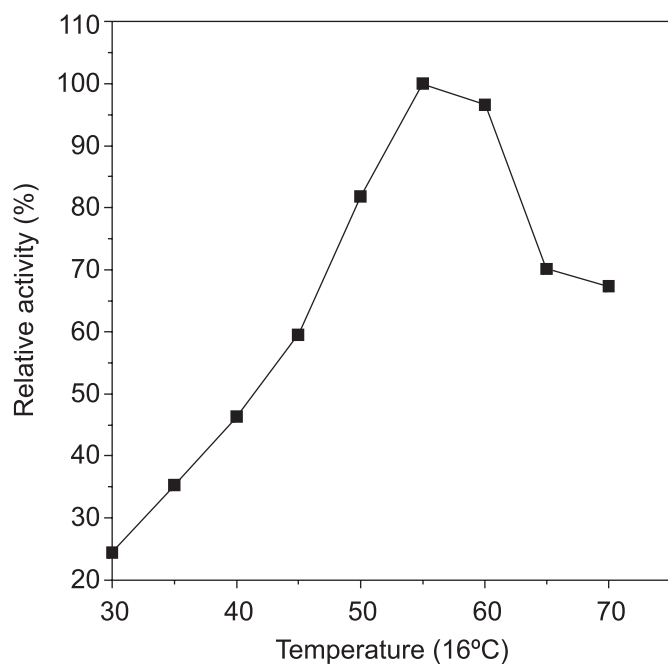
It was observed that the use of antifoaming at 0.05% (v/v) did not influence the enzymatic production, and that the aeration of 1.5 vvm allowed a production of 88.6 U/mL.

### Enzyme characterization

For partial enzyme characterization in the supernatant three concentration methods were used: a) precipitation with ammonium sulphate (70%); b) precipitation with 3V acetone; c) adsorption with starch and elution with NaCl 2N. The specific activity was determined. Precipitation with ammonium sulphate and acetone showed the best yields. The enzyme obtained by these two methods was submitted to chromatography in DEAE-Trysacryl, achieving a purification of 11.7 times. SDS-PAGE of the active fraction showed three proteic bands, and one of them was CGTase. Other studies of characterization were made with the partially purified enzyme. Figs. 8 and 9 show the effect of pH and temperature on the enzymatic activity, respectively. The optima pH and temperature for starch hydrolysis were 8.0 and 55°C respectively. Thatai *et al.* (29) determined a pH of 6.6 and temperature of 65°C and Gawande *et al.* (7) found the optimal temperature of 65°C for CGTase from *Bacillus firmus*. Table 2 shows the results obtained with various metallic ions and EDTA.  $\text{Mg}^+$  and  $\text{Ca}^+$  showed little activation. However,  $\text{Hg}^+$  and  $\text{Cu}^+$  showed a strong inhibition, while the remaining ions showed weak inhibition. These results contrast with the ones obtained by Fujita *et al.* (6) who verified a strong inhibition for  $\text{Zn}^{2+}$ ,  $\text{Fe}^{2+}$



**Figure 8.** Enzymatic activity as a function of pH, using the following buffers 50 mM citrate-phosphate (pH 3.0), 50 mM sodium acetate (pH 4.0–6.0), 50 mM Tris-HCl (pH 7.0–9.0) and 50 mM carbonate/bicarbonate (pH 10.0).



**Figure 9.** Enzymatic activity as a function of temperature: Conditions: pH 8.0; 1% starch solution containing  $\text{CaCl}_2$  0.05 M.

**Table 2.** Effect of the ions and EDTA on the enzymatic activity.

Ions (5mM)	Relative activity (%)
Control	100
$\text{Mg}^{2+}$	109
$\text{Ca}^{2+}$	105
KCl	92
$\text{Fe}^{2+}$	90
EDTA	88
$\text{Co}^{2+}$	87
$\text{Mn}^{2+}$	80
$\text{Zn}^{2+}$	61
$\text{Cu}^{2+}$	33
$\text{Hg}^{2+}$	6

and  $\text{Cu}^{2+}$  and a stability in the presence of  $\text{Hg}^{2+}$  for the enzyme of *Bacillus* sp. AL-6.

The results indicate that *B. alkalophilic* CGII is a good CGTase producer when compared to the other microorganisms. Further studies are needed in order to optimize the enzyme production and the quantification of  $\beta$ -cyclodextrin production by HPLC analysis and to explore the biotechnological potential of this enzyme.

## RESUMO

### Produção de CGTase por *Bacillus alkalophilic* CGII isolado de água residuária de uma fecularia de mandioca

Estudou-se a produção de CGTase por uma nova cepa de *Bacillus alkalophilic* CGII, isolada de água residuária de uma fecularia de mandioca, durante cultivo em meio composto de 1,5% de amido, 1,5% de fonte de nitrogênio e 1%  $\text{Na}_2\text{CO}_3$ . A atividade enzimática foi alta quando se utilizou amido, maltodextrina e galactose como fontes de carbono. Quando se utilizou glicose no meio de cultivo não se observou produção da enzima. Atividade enzimática alta (88,6 U/mL) e melhor crescimento foram obtidos quando se aumentou a aeração. A caracterização da enzima mostrou um pH ótimo de 8,0 e temperatura ótima de 55°C sendo que a enzima sofreu uma pequena ativação por  $\text{Mg}^+$  e  $\text{Ca}^{++}$ . A enzima foi fortemente inibida por  $\text{Hg}^+$  e  $\text{Cu}^+$ .

**Palavras-chave:** *Bacillus alkalophilic* CGII, CGTase,  $\beta$ -ciclodextrina, hidrólise do amido, atividade enzimática

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