



## Influence of culture medium pH on the production of CGTase by *Bacillus firmus* Strain No. 37

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**ABSTRACT.** The enzyme cyclomaltodextrin-glucanotransferase (CGTase) is a transglucosidase able to convert corn starch into cyclodextrin (CD). CDs are widely applied in industry given the ability to form inclusion complexes with a great variety of organic molecules. Regarding the optimum pH of CGTase, values reported in the literature vary according to the enzyme producing microorganism, being 8.0 the optimum pH of CGTase produced by *Bacillus firmus* Strain No. 37. This work studied the influence of the pH of culture medium with different concentration of nutrients on the production of the enzyme CGTase by *Bacillus firmus* Strain No. 37. For this purpose, the microorganism was grown in three culture media with different concentrations of carbon and nitrogen. The pH control was performed by adding sodium carbonate. The fermentation process was analyzed by the following methods: Bradford (1976) method to determine soluble proteins, DNS method to analyze sugars, and the method of complexation with  $\beta$ -CD to analyze the enzyme activity. The best result for CGTase enzyme activity was  $0.22 \text{ U mL}^{-1}$ , obtained with medium containing 2.0% soluble corn starch and yeast extract, and pH 8.3.

**Keywords:** starch, enzyme activity, cyclodextrin, fermentation, optimum pH.

## Influência do controle de pH em meio de cultivo de *Bacillus firmus* Cepa No. 37 para a produção de CGTase

**RESUMO.** A enzima ciclomaltodextrina-glucano-transferase (CGTase) é uma transglicosidase capaz de converter amido em ciclodextrinas (CDs). As CDs são amplamente aplicadas industrialmente pela capacidade de formar complexos de inclusão com uma variedade de moléculas. No que se refere ao pH ótimo da CGTase, os valores relatados na literatura variam de acordo com as espécies do micro-organismo produtor da enzima, sendo 8,0 o pH ótimo da CGTase produzida por *Bacillus firmus* Cepa No. 37. O objetivo deste trabalho foi estudar a influência do controle de pH no meio de cultivo de *Bacillus firmus* Cepa No. 37 com diferentes concentrações de nutrientes para produzir a enzima CGTase. Para isso, o micro-organismo foi cultivado em três meios de cultura contendo diferentes concentrações de fonte de carbono e nitrogênio. O controle de pH foi realizado com adição de carbonato de sódio. As análises utilizadas para avaliar o processo fermentativo foram método de Bradford (1976) para determinar proteínas solúveis, o método DNS para análise de açúcares e o método de complexação com  $\beta$ -CD para análise da atividade enzimática. O melhor resultado de atividade da enzima CGTase foi  $0,22 \text{ U mL}^{-1}$ , obtida em meio contendo 2,0% de amido de milho e de extrato de levedura e pH 8,3.

**Palavras-chave:** amido, atividade enzimática, ciclodextrinas, fermentação, pH ótimo.

### Introduction

The enzyme CGTase is responsible for catalyzing the formation of cyclodextrin from starch or related sugars. Cyclodextrins (CDs) are non-reducing cyclic oligosaccharides, comprising D-glucose residues linked by  $\alpha$ -1, 4 glycosidic bonds. The most common are  $\alpha$ -,  $\beta$ - and  $\gamma$ -CD containing 6, 7 and 8 glucosyl residues, respectively. The attraction of these cyclic, torus-shaped oligosaccharides arises from their ability to form inclusion complexes with a wide variety of organic molecules. This property has been used for

stabilization and solubilization of various substances of interest to pharmaceutical, cosmetic and food industry, as well as in bioconversions and separation processes (STARNES, 1990).

CGTase is produced mainly by strains of *Bacillus* (GOEL; NENE, 1995; MARTINS; HATTI-KAUL, 2002; MATIOLI et al., 2000; TOMITA et al., 1993; YIM et al., 1997), however production by species of *Klebsiella* (BENDER, 1977), *Brevibacterium* (MORI et al., 1994), *Aspergillus* (LEE; CHEN, 1997) and thermophilic *Archaea* (TACHIBANA et al., 1999) has also been reported.

Concerning the optimum pH to CGTase, the values reported in the literature vary according to the species of microorganism that produce the enzyme. According to Matioli et al. (1998), the  $\beta$ -CGTase produced by *Bacillus firmus* Strain No. 37 was stable at 60°C and pH 8.0.

Preliminary cultivation of *Bacillus firmus* Strain No. 37 for producing the enzyme CGTase showed that high concentration of soluble corn starch has increased the consumption of sugar by the microorganism with a consequent reduction of pH in the culture medium. The drop in pH values below 8.0 resulted in low levels of activity of the enzyme CGTase.

In this paper, we report the cultivation of *Bacillus firmus* Strain No. 37 for the production of CGTase in culture media with different concentrations of carbon and nitrogen source, under controlled pH.

## Material and methods

**Enzyme.** The enzyme CGTase was produced by *Bacillus firmus* Strain No. 37. Initially, the microorganism was seeded in Petri dishes containing semi-solid medium, whose composition was in w v<sup>-1</sup>: 1.0% soluble corn starch, 0.5% peptone, 0.5% yeast extract, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.02% MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.0% Na<sub>2</sub>CO<sub>3</sub> and 1.5% agar. The plates were incubated at 37°C for 72 hours in order to start the multiplication of cells that were in a vegetative state. After this period, the cell mass present in the plates was scraped with the aid of an inoculating loop and aseptically transferred to the pre-inoculum, whose composition was similar to the semi-solid medium, except agar. In the pre-inoculum was added 2 mL of  $\alpha$ -amylase diluted so that part of the soluble corn starch was pre-saccharified. The pre-inoculum volume was 500 mL, placed in flasks with a capacity of 1000 mL. Then, the pre-inoculum was kept in a rotary shaker at 37°C, 150 rpm for 48 hours. After that, an aliquot of 10% v v<sup>-1</sup> of the medium was removed from the pre-inoculum and inoculated in the culture media. The cultivation was carried out at 37°C for three days, with agitation of 150 rpm. 20 mL-samples were taken from every 10 to 14 hours, alternatively. After centrifugation of the samples, liquid fractions (enzymatic medium) were stored under refrigeration for later analysis of pH, total reducing sugars, soluble proteins and enzyme activity.

**Composition of culture media.** Culture media used in this study had different concentrations of soluble corn starch and yeast extract as listed in Table 1.

**Table 1.** Composition of culture media<sup>1</sup>.

Components (% w v <sup>-1</sup> )	Medium PI	Medium AC#1	Medium IC#1
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.02	0.02	0.02
K <sub>2</sub> HPO <sub>4</sub>	0.1	0.1	0.1
Peptone	0.5	0.5	0.5
Yeast extract	0.5	2.0	4.0
Soluble corn starch	1.0	2.0	4.0
Na <sub>2</sub> CO <sub>3</sub>	1.0	1.0	1.0
$\alpha$ -amylase volume (mL)	2.0	-	-
Medium volume (mL)	500	500	500
Capacity of the flask (mL)	1000	1000	1000

**pH reading and pH control.** To read and to control the pH of culture media, it was used the Tecnal pHmeter Model TEC-2. The pH control was done punctually, out of 14 in 14 hours, as often as the samples were taken from. In each pH reading below 8.0, it was added sodium carbonate, sterilized in a stove at 120°C for 4 hours, until the return of pH to the range of 8.0 to 8.5.

**Determination of sugars.** The concentration of total reducing sugars was determined by the increase in absorbance at 600 nm of a solution containing the enzymatic medium and the DNS reagent. Firstly, however, the samples were subjected to a prior process of acid hydrolysis. After hydrolysis, it was done the neutralization of the sample and reaction with DNS (2, 5-dinitrosalicylic acid), as described by DNS method (MILLER, 1959).

**Determination of proteins.** To measure the soluble proteins content of enzymatic medium, it was used the colorimetric method of Bradford, which uses the Coomassie blue dye (BRADFORD, 1976), with reading at 595 nm and measurement done in duplicate.

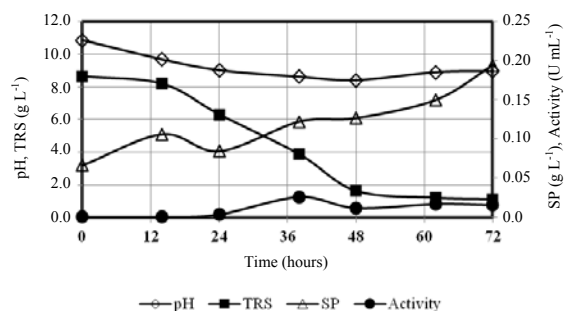
**CGTase activity.** CGTase activity assays were performed in dextrin solution 1% (w v<sup>-1</sup>) in Tris-HCl 0.05 M CaCl<sub>2</sub> and 50 mM, pH 8.0 and 50°C. The dosing tube containing 1.0 mL of 1% dextrin (w v<sup>-1</sup>) was placed in a water bath heated to 50°C. 1.0 mL of enzymatic medium was added to substrate in each tube. After the reaction time, ranging from 5 to 30 minutes, the tubes were taken to a bath at 100°C for 5 min. to inactivate the enzyme. The *blank* was prepared in a tube containing 1.0 mL of enzymatic medium, which was subjected to a bath at 100°C for 5 min. to inactivate the enzyme before adding the substrate dextrin 1% (w v<sup>-1</sup>). After water bath at 100°C, the samples were cooled to room temperature. The  $\beta$ -CD produced in the reaction between the enzyme CGTase and substrate dextrin was determined by the

<sup>1</sup>Culture medium PI containing low concentrations of carbon and nitrogen source (1.0% w v<sup>-1</sup> soluble corn starch and 0.5% w v<sup>-1</sup> yeast extract) and  $\alpha$ -amylase for soluble corn starch saccharification (2.0 mL). Culture medium AC#1 containing intermediate concentrations of carbon and nitrogen source (2.0% w v<sup>-1</sup> soluble corn starch and 2.0% w v<sup>-1</sup> yeast extract). Culture medium IC#1 containing high concentrations of carbon and nitrogen source (4.0% w v<sup>-1</sup> soluble corn starch and 4.0% w v<sup>-1</sup> yeast extract). Culture media AC#1 and IC#1 do not contain  $\alpha$ -amylase.

extinction of the color of phenolphthalein at 550 nm, which occurs by complexation with  $\beta$ -CD. The CGTase enzyme activity was expressed as  $\text{U mL}^{-1}$ , in which one unit (U) is the amount the CGTase that catalyzes the production of one micromole of  $\beta$ -CD per minute under the reaction conditions.

## Results and discussion

With the fermentation at  $37^\circ\text{C}$  for 3 days, the pH of the medium PI, which contained the lowest concentrations of soluble corn starch and yeast extract, remained in the range of 8.0 to 10.0 over the cultivation, without the addition of sodium carbonate for pH control (Figure 1). The consumption of total reducing sugars occurred gradually, reaching a concentration of  $2.0 \text{ g L}^{-1}$  with 48 hours of culture. The Figure 1 illustrates that in the period from 14 to 48 hours, in which the sugar concentration was 8.0 to  $2.0 \text{ g L}^{-1}$ , pH decreased from 9.6 to 8.6. Figure 1 also showed that the concentration of soluble proteins in the medium PI fluctuated in the first 24 hours of culture, showing an upward trend after this period. The maximum concentration of soluble protein was  $0.19 \text{ g L}^{-1}$  at 72 hours of culture. The values of CGTase enzyme activity were low throughout the fermentation, and the maximum activity of  $0.026 \text{ U mL}^{-1}$  obtained at 38 hours of culture, as shown in Figure 1.

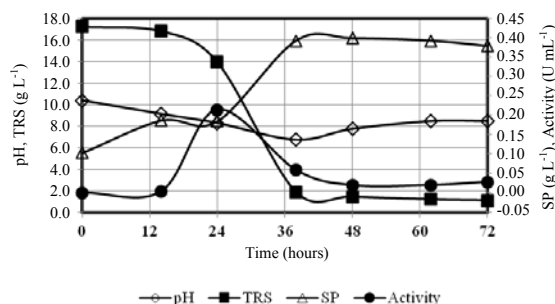


**Figure 1.** Variation in pH, total reducing sugars (TRS), soluble proteins (SP) and enzyme activity over time in the medium PI.

Unlike the behavior observed in medium PI, the pH of the medium AC#1 suddenly dropped by 38 hours of cultivation, from 8.3 to 6.8 (Figure 2). This result showed that the punctual pH control was not effective, since from 24 to 38 hours the pH gradually decreased due to the consumption of total reducing sugars.

Also according to the Figure 2, there was the consumption of total reducing sugars, an increase in CGTase activity of the enzyme and the concentration of soluble proteins present in the AC # 1. In the period from 14 to 38 hours, the

concentration of total reducing sugars decreased from  $16.8$  to  $1.9 \text{ g L}^{-1}$ .



**Figure 2.** Variation in pH, total reducing sugars (TRS), soluble proteins (SP) and enzyme activity over time in the medium AC#1.

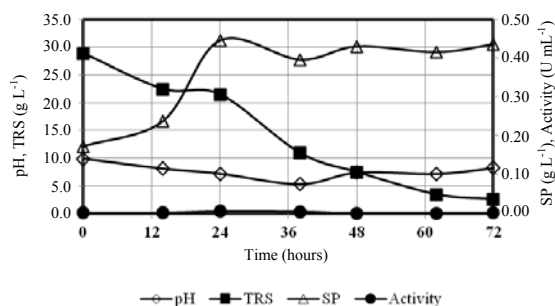
Within 24 hours of culture, the CGTase enzyme activity reached the maximum value of  $0.22 \text{ U mL}^{-1}$ ; by this time the pH was 8.3. The concentration of soluble proteins showed an upward trend within 48 hours of culture, reaching a value of  $0.40 \text{ g L}^{-1}$ , twice the value obtained in the medium PI. Thereafter, the protein concentration remained almost constant until the end of the cultivation (Figure 2).

Matioli et al. (1998) studied the production and purification of CGTase from *Bacillus firmus* Strain No. 37 and obtained an enzymatic activity of  $0.12 \text{ U mL}^{-1}$ . The culture medium components were similar to that used in the medium AC#1, except the yeast extract concentration that was four times lower, 0.5%. This difference between the yeast extract concentration in the medium prepared by Matioli et al. (1998) and medium AC#1 may explain the high value of enzyme activity obtained in medium AC#1,  $0.22 \text{ U mL}^{-1}$ .

Moriwaki et al. (2007) reported the production and characterization of a new cyclodextrin glycosyltransferase from *Bacillus firmus*, strain 7B. The enzymatic activity observed in the cell-free supernatant was  $0.19 \text{ U mL}^{-1}$ , similar result to that obtained in the medium AC#1 with *Bacillus firmus* Strain No. 37.

Similar to observed in the medium AC # 1, in the medium IC#1 the pH decreased from the baseline of 9.8 to 5.3 in 38 hours of culture (Figure 3). The punctual addition of sodium carbonate was not effective in maintaining the pH in the alkaline range, since the pronounced consumption of total reducing sugars between 24 and 38 hours resulted in pH drop to values below 7.0. In the same period that the pH decreased from 9.8 to 5.8, the concentration of total reducing sugars was  $28.8$  to  $10.9 \text{ g L}^{-1}$ . Also according to Figure 3, the activity of the enzyme CGTase showed almost undetectable values throughout the culture. The concentration of soluble proteins in the medium IC#1 increased up to 24 hours, reaching a value of

0.45 g L<sup>-1</sup>, slightly higher than in the medium AC#1. By the end of the cultivation, the concentration of soluble proteins in the medium IC#1 fluctuated in the range 0.40 to 0.45 g L<sup>-1</sup>.



**Figure 3.** Variation in pH, total reducing sugars (TRS), soluble proteins (SP) and enzyme activity over time in the medium IC#1.

## Conclusion

In conclusion, besides controlling pH in the range 8.0 to 8.5 in the medium for producing the enzyme CGTase, it is required availability of intermediate concentrations of carbon and nitrogen source to the microorganism *Bacillus firmus* Strain No. 37, for a satisfactory production of the enzyme of interest, CGTase. The medium AC#1, which contained 2.0% soluble corn starch and 2.0% yeast extract, presented the maximum value of CGTase enzyme activity, 0.22 U mL<sup>-1</sup>, 24 hours of culture, and at pH 8.3.

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