Colorimetric Determination of α and β-cyclodextrins and Studies on Optimization of CGTase Production from *B. firmus* Using Factorial Designs

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ABSTRACT

Cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase) production from B. firmus, isolated from soil of Curitiba, PR, was optimized in shake flask using an experimental design approach. The CGTase was produced when the carbon source was starch and β-CD, but when simple sugars such as glucose, galactose, lactose, sucrose, and maltose were used, there was no enzyme production. CGTase production was the same with either organic nitrogen or inorganic nitrogen source. CGTase activity decreased 2-fold when incubation temperature was increased from 28 to 37 °C, and decreased 2.1- fold when the initial pH was lowered from 10.3 to 7.4. The colorimetric determinations of α- and β -CD were analyzed as a non-linear relationship and the equilibrium constant for α -CD/methyl orange and β-CD/phenolphthalein complexes were 7.69×10^3 L/ mol and 2.33×10^3 L/ mol, respectively.

Key words: Cyclodextrin glycosyltransferase, *Bacillus firmus,* cyclodextrins

INTRODUCTION

Cyclodextrin glycosyltranferase (CGTase EC 2.4.1.19) is a bacterial enzyme that converts starch and other 1,4-linked α-glucans to cyclodextrins (α, β and γ-CDs in varying proportions) (French, 1957; Thoma and Stewart, 1965). They have the ability to form inclusion complexes with organic and inorganic compounds, which have numerous applications in biotechnology, and in the food and pharmaceutical industries (Szejtli, 1986; Pszczola, 1988; Matioli,

2000). The interaction of cyclodextrins (CD) with an acid-base indicator gives a stable 1:1 complex, and the equilibrium concentration can be monitored in solution by its absorbance characteristic (Barcza and Buvari-Barcza, 1989). The method based on complexation of phenolphatalein is easy to carry out, sensitive and relatively specific to ß-CD (Cramer, 1952). The decolorization of the indicator is probably due to the destruction of the planar conjugated structure of the phenolphthalein molecule (Nakatani and Hiromi, 1984). An α-CD determination method, highly reproducible and

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selective was described by Lejeune et al. (1989). It involves the formation of an inclusion complex between the α-CD and methyl orange under conditions of low pH and low temperature.

The use of process optimization of various fermentation parameters has been made in attempt to obtain an optimal growth of bacteria and maximum CGTase synthesis (Jamuna et al., 1993; Gawande and Patkar, 1999; Stefanova et al., 1999). In previous works we studied the CGTase production with different sources and concentrations of industrial starch in a basal medium in order to optimize CGTase activity (Higuti et al., 2003b).

We have now analyzed the colorimetric determination of α- and β - CD as a non-linear relationship and studied the optimization of the CGTase production from *B. firmus* using a two-level factorial design.

MATERIAL AND METHODS

Microorganisms

The microorganism used was isolate number 31 of strains of alkalophilic CGTase-producing bacteria, identified as *Bacillus firmus* by morphological, physiological and biochemical tests (Higuti et al., 2003a). It was grown at 28 °C and 120 rpm for 24 h (New Brunswick rotary shaker). The sterilized control culture medium contained 2% soluble starch (corn starch Sigma), 0.5% peptone, 0.5% yeast extract, 0.02% $MgSO_4.7H_2O$, 0.1% K_2HPO_4, pH 10.3. The cells were removed by centrifugation (4000 x g; 10 min), and the supernatant that contained the enzyme, substrates and products was used for further assays.

Enzyme assay

CGTase activity was assayed by the Lejeune et al. (1989) method slightly modified by Gawande and Patkar (1999). A 1.0 mL amount of 1% soluble starch prepared in 50 mmol/L phosphate buffer, pH 7.0, was added with 0.1 mL of properly diluted enzyme and incubated at 40 ºC for 10 min. Enzyme reaction was stopped by immediately cooling the tubes in chilled water followed by addition of 0.1 mL of 1.2 mol/L HCl. Then 2 mL of methyl orange solution (final concentration 0.035 mmol/L) was added to the reaction mixture and the tubes were

maintained at 15 ºC for 30 min. The absorbance was measured at 507 nm and its decrease in absorbance (with respect to a control tube without enzyme) was correlated with the amount of α-CD. One unit of enzyme was defined as the amount of enzyme that produced 1 micromol of α-CD per min under standard conditions.

Measurement of β-CD

The concentration of β-CD was analyzed by the decrease in absorbance at 550 nm due to phenolphtalein-CD complex formation (Goel and Nene, 1995 and modifications reported by Matioli, 1997). To 0.2 mL of β-CD solution in 0.05 mmol/L Tris-HCl buffer, pH 8.0, 1.0 mL of the phenolphthalein working solution was added. The tube was mixed by vortexing and the absorbance was measured immediately. The working solution was prepared by diluting 2 mL of 3 mmol/L phenolphthalein in ethanol (stock solution) to 100 mL with 125 mmol/L Na_2CO_3/$NaHCO_3$ buffer, pH 10.5.

Statistical analysis

The colorimetric determination of α- and β – cyclodextrins was based on molecular complexation of dyes that undergoes spontaneous decolorization. Thus, the absorbance difference was analysed to obtain information on the Kd of the reaction:

Dye (D) + CD ==== Complex (C)
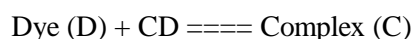
Kd is given by [D] [CD]/ [C], where braquets denotes concentration of the indicated species. Since [Dt] = [D] + [C], and [CDt] = [CD] + [C], Kd can be written as:

Kd = {[Dt] – [C]} [CD] / [C]

Assuming that the δA is directly proportional to [C] and δAmax as the limiting δA under [CDt] >>[C], we can obtain the approximate equation:

δA = (δAmax) [CDt] / (Kd + [CDt])

The fit of δA versus [CDt] at the appropriated wavelength gives Kd and δAmax, using Statistica 5.5 (StatSoft).

RESULTS AND DISCUSSION

The colorimetric methods are suitable for determination of CD concentration and seldom require preliminary steps for the removal of acyclic carbohydrates (Makela et al., 1987). Deviation of linearity could be well explained by the theory of complex formation. Phenolphtalein-β-CD complex was only linear when correlated to β-CD concentration for up to 0.17 mmol/L (Goel and Nene, 1995). The formation of the inclusion complex causes a decrease in absorbance of the methyl orange solution that is only linearly correlated with the cyclomalto hexaose concentration in the range of 0.25 optical density unit and 0.30 mmol/L cyclomaltohexaose, with limited interference by linear oligosaccharides and other cyclodextrins (Lejeune et al., 1989). The enzyme CGTase hydrolyses starch to produce CDs as its main reaction products and the maltooligosaccharides present in the enzyme hydrolyzates do not interfere in the analysis of CDs by the phenolphtalein method (Goel and Nene, 1995).

The experimental data were analyzed in the light of a non-linear relationship that can be easily used with the aid of a statistical package for computers (Figures 1 and 2). The equilibrium constant for α-CD and β-CD complexes were 7.69×10^3 L/mol and 2.33×10^3 L /mol, respectively. The original procedure of Vikmon (1981) for phenolphthalein assay for the determination of β - CD required minor modifications to assure reproducibility. This phenolphthalein assay was analyzed for the susceptibility to interferences by mono, oligosaccharides and mannitol (Table 1). Very little influence was observed in spite of their high concentrations.

Studies on optimization of the CGTase production were performed in shake flasks using a 2^{k-1} statistical experimental design approach. The results are illustrated in Table 2. The CGTase was produced when the carbon source was starch and β-CD, but when a small sugar such as glucose, galactose, lactose, sucrose, and maltose were used there was no enzyme production. Very little differences were observed with different starch sources. CGTase was not produced by *B. firmus* when simple sugars were used as carbon source. Simple sugars favoured CGTase synthesis by *B. cereus* (Jamuna et al. 1993) but not by *K. pneumoniae* (Gawand and Patkar, 1999). CGTase

production was the same with either organic or inorganic nitrogen source. CGTase activity decreased 2-fold when incubation temperature was increased from 28 to 37 °C, and decreased 2.1- fold when the initial pH was lowered from 10.3 to 7.4 (not shown). Enzyme production was lower in the absence of magnesium sulfate.
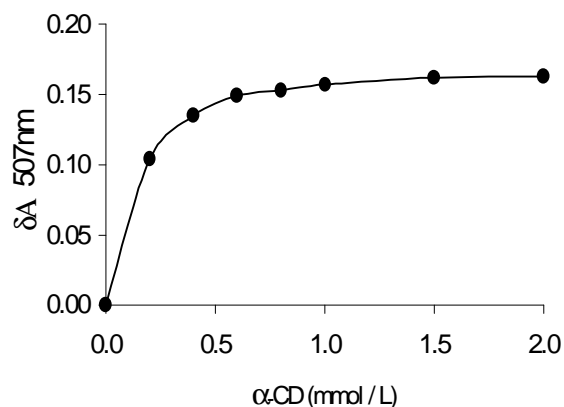

Figure 1 - Differential absorbance at 507 nm of methyl orange as a function of the concentration of α - CD.

The experimental data fitted the equation:
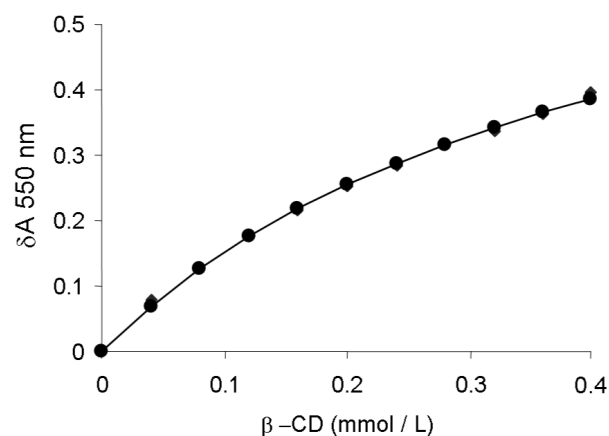$$\delta A = (0.177 \times [\alpha\text{-CD}])/(0.130 + [\alpha\text{-CD}])$$

Figure 2 - Differential absorbance at 550 nm of phenolphtalein as a function of the concentration of β - CD.

The experimental data fitted the equation:
$$\delta A = (0.800 \times [\beta\text{-CD}])/(0.428 + [\beta\text{-CD}])$$

Sugar at 0.1 and 0.2% final concentration was added to 0.2 mmol/L β - CD solution in 0.05 mmol/L Tris-HCl buffer, pH 8.0
The data represent the percentage of the absorbance related to the absorbance obtained in the absence of the sugar.

Similar results have been reported by Gawande and Patkar (1999) for *K. pneumoniae*.
The CGTase activity in U/mL observed with isolate 31 of *B. firmus* was slightly lower than the activity described by Gawande et al. (1998).

Table 1 - Effect of sugar on colorimetric determination of β-CD by phenolphthalein

	Sugar concentration	
	0.10%	**0.20%**
glucose	102.6	99.1
fructose	95.7	96.1
galactose	100.7	116
mannose	93.1	101.5
arabinose	103.3	112.4
xylose	98.4	99.1
mannitol	93.1	101.5
melibioze	110.1	99.3
lactose	95.2	98.1
cellobiose	123.4	130.3
melezitose	102.6	98.9

Table 2 - Effect of carbon and nitrogen sources on enzyme production

C source (2 %)	CGTase (U/mL)	N source (0.5 %)	CGTase (U/mL)	Mineral salts	CGTase (U/mL)
Corn starch	0.22	Peptone	0.15	Mg_4SO_4 (0.02%)	0.21
Manioc starch	0.18	Casein Hydrolysate	0.14		
				K_2HPO_4 (0.1%)	0.22
Potato starch	0.21	$NaNO_3$	0.15		
β-CD	0.37				

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RESUMO
A produção de ciclodextrina glicosiltransferase (EC2.4.1.19, CGTase) de *B. firmus* isolada de solo de Curitiba, PR, foi otimizada com o uso de modelo estatístico fatorial. Foi estudado o efeito de componentes no meio básico bem como pH inicial e temperatura na produção da enzima. O modelo de fatorial 2^{k-1} foi usado. A atividade da CGTase foi monitorada pelo método colorimétrico com alaranjado de metila. Houve produção da CGTase quando a fonte de carbono era amido e β-CD, mas quando galactose, lactose, sacarose e maltose foram usadas, não houve nenhuma produção de enzima. A produção de CGTase foi a mesma com a fonte de nitrogênio orgânico ou inorgânico. A atividade da CGTase diminuiu 2 vezes quando a temperatura de incubação foi aumentada de 28 a 37 °C, e diminuiu 2,1 vezes quando o pH inicial foi abaixado de 10,3 a 7,4. As determinações colorimetricas de α e β - CD foram analisadas como regressão não linear e a constante de equilíbrio para os complexos α-CD/alaranjado de metila e β-CD/fenolftaleina foram 7,69 x 10^3 L/mol e 2,33 x 10^3 L/mol, respectivamente.

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