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Cyclodextrin Glycosyltransferase Production by the *Bacillus* sp., Subgroup *alcalophilus* using a Central Composite Design

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Abstract: Cyclodextrin glycosyltransferase (CGTase) activity was produced by the *Bacillus* sp., subgroup *alcalophilus* in a culture medium containing cassava starch. A central composite design and response surface methodology were used to study the influence of carbon source (cassava starch), nitrogen sources (yeast extract and tryptone) and sodium carbonate in the production medium. Assays were performed in 300 mL Erlenmeyer flasks containing 100 mL of production medium maintained in a shaker at 150 rpm at 35±1 °C for 72 h of fermentation. The independent variables [0.75% cassava starch, nitrogen sources (0.375% yeast extract and 0.375% tryptone) and 1% Na₂CO₃] produced an enzyme activity of 96.07 U mL⁻¹.

Key words: Cyclodextrin glycosyltransferase, enzyme activity, central composite design, *Bacillus* sp., subgroup, *alcalophilus*, cassava starch

INTRODUCTION

Cyclodextrins (CDS) are products of the enzyme, cyclodextrin glucanotransferase (CGTase) and are cyclic oligosaccharides formed by glycosidic units through α -1,4 bonds (Qi *et al.*, 2004). The most industrially used CDS are α -CD, β -CD and λ -CD, made up of 6, 7 and 8 glucose units (Seon *et al.*, 2009). Cyclodextrin have a truncated conical shape, the outer side of which is hydrophilic due to primary and secondary hydroxyls and the inner cavity is apolar due to the high electron density caused by pairs of free electrons from the oxygens involved in the glycosidic bonds being directed toward the interior of the cavity (Ribeiro and Sera, 2007). The inclusion complex may be formed by molecules of different shapes and sizes, capable of either completely or partially adapting within the apolar cavity, causing changes in undesirable properties of the host molecules (Gornas *et al.*, 2009; Haiyee *et al.*, 2009). The inclusion of CDS in the rigid matrix generally causes changes in the physiochemical properties of the inserted molecules, such as an increase in stability and

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biocompatibility (Moraes *et al.*, 2007). These properties have made CDS increasingly more important for industrial applications and are employed in pharmaceutical, food and agro-industrial products (Aime *et al.*, 2009). The considerable variety of inclusion complexes (Abdel-Shafi and Ai-Shihry, 2009) provides beneficial effects, such as protection from lipid oxidation (Haiyee *et al.*, 2009), reduction or stability of aromas and flavors (Seon *et al.*, 2009), color stability (Provenzi *et al.*, 2006) vitamin stability (Cho *et al.*, 2001) and the protection of active ingredients from oxidation (Szente and Szejtli, 2004) thereby reducing storage costs and increasing the shelf life of food products.

The enzyme CGTase (EC 2.4.1.19) catalyzes starch conversion reactions by means of cycling, coupling and disproportioning in different proportions of α -CD, β -CD and γ -CD formation, depending on culture conditions, environmental factors and the microorganism used in the production (Biwer *et al.*, 2002). Thus, optimizing the production of the enzyme has been the aim of many studies using different starch sources, such as cassava (Higuti *et al.*, 2003).

The present study used the response surface method through a Central Composite Design (CCD) to select carbon and nitrogen sources as well as sodium carbonate variables that may influence the production of CGTase by the microorganism, *Bacillus* sp., subgroup *alcalophilus*. This experiment design is an effective method that uses multifactor productivity measures, thereby economizing time and cost in the investigation of the effect of variables on the results of experiments (Seon *et al.*, 2009).

MATERIALS AND METHODS

Microorganism

The *Bacillus* sp., subgroup *alcalophilus* strain was obtained from the culture collection of the industrial microbiology laboratory of the Biological Sciences Institute of Rio Claro (SP, Brazil) a culture isolated from a cassava starch mill (Ribeirão Bonito, SP, Brazil). This microorganism was characterized by the André Tosello Foundation (Campinas, SP, Brazil).

Identification of the Microorganism

The identification of the microorganism was achieved from the phylogenetic analysis of partial 16S ribosomal DNA (16S rDNA) sequences, which were compared to 16S rDNA sequences from organisms represented in the Ribosomal Database Project (Wisconsin, USA) and Genbank databases. Distance matrices were calculated using the Jukes and Cantor (1969) model and the design of the phylogenetic tree (Fig. 1) based on evolutionary distances was performed using the Neighbor-Joining method (Saitou and Nei, 1987).

Activation of Inoculum

The *Bacillus* sp., subgroup, *alcalophilus* was cultured in a medium containing (w/v) 1% cassava starch, 0.5% yeast extract, 0.5% tryptone, 0.1% K_2HPO_4 and 0.02% $MgSO_4 \cdot 7H_2O$, completed with 10% Na_2CO_3 in Erlenmeyer flasks (250 mL) at 150 rpm and $35 \pm 1^\circ C$ for 56 h, through successive transferences to fresh media at 8 h intervals. The production of the CGTase enzyme was observed by the formation of a yellow halo around the bacterial colony. The viability of microorganism and the possibility of enzyme production were determined in a solid, Nakamura and Horikoshi (1976) medium and analyzed based on color intensity and diameter of the halos formed in the medium.

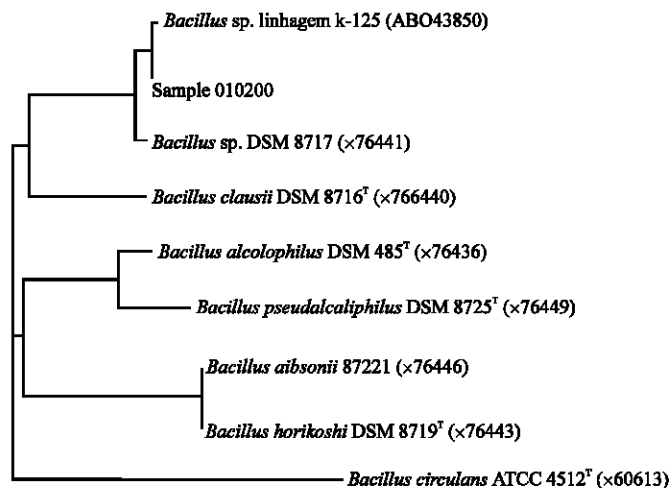


Fig. 1: Phylogenetic tree based on analysis of partial 16S rDNA of sample 010200 and stains of *Bacillus* and *Bacillus circulans* (the latter of which was used as the outgroup). No. following species name correspond to the access acronym Genbank database

Enzyme Production Through Shaker Fermentation

After selecting the parameters that influence CGTase production, such as type and concentration of carbon source (ground cassava, nitrogen sources (yeast extract and tryptone) and Na_2CO_3 , the option was made to use 1% cassava starch (w/v), 0.5% yeast extract (w/v), 0.5% tryptone (w/v) and 1.5% Na_2CO_3 (w/v). These parameters were used as the central point in the CCD. The *Bacillus* sp., Subgroup *alcalophilus* was cultured in 300-mL Erlenmeyer flasks containing 100 mL of the culture medium incubated at 150 rpm at $35 \pm 1^\circ\text{C}$ for 18 h. From this culture, 10 mL (10% v/v) was inoculated in 90 mL of the Nakamura and Horikoshi (1976) culture in 300 mL Erlenmeyer flasks. At the end of fermentation periods of 24, 48 and 72 h, samples were removed and centrifuged at 10,000 rpm for 10 min. The cell-free supernatant was used for the determination of enzyme activity. All assays were performed in duplicate.

Enzyme Activity

Enzyme Activity (EA) was measured based on the phenolphthalein method (Makela *et al.*, 1988). Five milliliter of the supernatant from the enzyme broth and 5.0 mL of 1% starch solution [0.1 g of soluble starch, 1.0 mL of CaCl_2 solution (0.05 M), 1.0 mL of Tris-HCl buffer solution (0.05 M; pH 8.0) and distilled water for a final volume of 10 mL] were placed in a thermostatic reactor at $55 \pm 1^\circ\text{C}$. Samples were removed at 0, 3, 6, 9 and 12 min and inactivated at 100°C for 5 min. Cyclodextrin was quantified as a function of time by adding 2.5 mL of phenolphthalein solution (pH 10.5) to 0.5 mL of the inactivated samples. Absorbance of the final solution was read from a spectrophotometer at 550 nm. The EA was defined as the amount of cyclodextrin produced per minute.

Calculation of Enzyme Activity

Graphs were designed with the slope value of each assay, with a linear adjustment performed on the OriginPro software program (version 7.5). The EA was determined by the following equation:

Table 1: Levels of variables used in the central composite design

Independent variables		Variations and levels				
		$-\alpha$	-1	0	1	$+\alpha$
Optimization of production medium (%)						
Cassava starch	X_1	0.58	0.75	1.00	1.25	1.42
Tryptone and yeast extract	X_2	0.58	0.75	1.00	1.25	1.42
Sodium carbonate (Na_2CO_3)	X_3	0.66	1.00	1.50	2.00	2.44

$$A = \alpha \beta F_{\text{dil}} \cdot 1000 \cdot \frac{V_{\text{enz}}}{V_{\text{reac}}} \quad (1)$$

where, A is EA ($\mu\text{mol}/\text{min}/\text{mL}$); α is the angle coefficient of the standard curve ($\mu\text{mol} \cdot \text{mL}^{-1}/\text{ABS}$); β is the angle coefficient of the experimentally obtained curve (ABS/min); v is the enzyme volume from the reactor ($V_{\text{sample}} (\text{mL})/V(\text{mL})$) and F_{dil} is the dilution factor.

Central Composite Design

The experiments were carried out based on a CCD. To assess the tendency of variables in the CGTase production process, a two-level factor design was used with three variables, two replicates on the central point and six experiments on the axial points (α), totaling 16 experiments. The variables were coded based on the following equation:

$$x_i = (X_i - X_{\text{cp}}) / \Delta X_i \quad (2)$$

where, x_i is the coded value of the independent variable, X_i is the real value of the independent variable, X_{cp} is the real value of an independent variable at the central point and ΔX_i is the step change value.

Table 1 shows the variations and levels of the independent variables analyzed. The Root Mean Square (RSM) was generated from an empirical equation produced by CCD.

Errors in the production measurements were estimated by means of duplicate assays. The results were analyzed with the aid of the Statistica 7 software program (Statistic 7 Stat Soft, USA).

RESULTS AND DISCUSSION

The microorganism was identified as a *Bacillus* sp., from the subgroup *alcalophilus*, still without species definition and therefore not described in the literature. The current classification of species within the genus *Bacillus* and related genera is well established and is based on a combination of numerous experimental approaches (Xu and Côté, 2003). A tree of *Bacillus* 16S rRNA sequences, including 8 type strains of *Bacillus* species, clustered all of the isolates belonging to the previously identified species to the corresponding specie together (Fig. 1). We found that the sample 010200 isolate was distributed in the subgroup *alcalophilus* of the tree (Fig. 1).

Optimization of Significant Medium Components Using Central Composite Design

The CCD in AE was employed to investigate the interactions among the significant factors (carbon source, nitrogen sources and sodium carbonate) and determine the optimal values of the enzyme activity. The design matrix and the corresponding results of AE experiments to determine the effects of three independent variables are shown in Table 2. The best concentrations were 0.75% carbon and nitrogen and 1% for Na_2CO_3 . CGTase production increased from 10.7 ± 0.43 to $96.0 \pm 0.02 \text{ U mL}^{-1}$.

Table 2: Central composite design with real and predicted enzyme activity values

Experiments	Coded variables			Real variables			AE (U mL ⁻¹)
	X ₁	X ₂	X ₃	X ₁	X ₂	X ₃	
1	-1	-1	-1	0.75	0.75	1	96.0±0.02
2	1	-1	-1	1.25	0.75	1	40.3±0.06
3	-1	1	-1	0.75	1.25	1	61.0±1.03
4	1	1	-1	1.25	1.25	1	10.7±0.43
5	-1	-1	1	0.75	0.75	2	43.2±1.46
6	1	-1	1	1.25	0.75	2	38.5±0.34
7	-1	1	1	0.75	1.25	2	42.8±0.04
8	1	1	1	1.25	1.25	2	66.3±0.42
9	-1.68	0	0	0.58	1	1.5	66.1±0.31
10	1.68	0	0	1.42	1	1.5	53.1±0.02
11	0	-1.68	0	1	0.58	1.5	66.9±0.22
12	0	1.68	0	1	1.42	1.5	35.3±0.88
13	0	0	-1.68	1	1	0.66	68.4±0.26
14	0	0	1.68	1	1	2.34	38.1±0.78
15	0	0	0	1	1	1.5	45.8±0.76
16	0	0	0	1	1	1.5	42.4±0.41

X₁: Cassava starch; X₂: Tryptone and yeast extract; X₃: Sodium carbonate

Table 3: Regression results of CGTase production

Coded factor	Parameter	Student's t	Level of significance
Constant	50.93	19.4099	0.0000
X ₁	-7.9886	-2.8111	0.0169
X ₂	-6.6120	-2.3267	0.0401
X ₁ X ₃	15.6265	4.2104	0.0014
X ₂ X ₃	11.5061	3.1002	0.0100

R²: 0.901; R: 0.95; Adjusted R²: 0.80; F_T: 10.16; F_C (4.11): 3.36

The results obtained during the peak EA were analyzed using multiple regression, with the isolated variables, quadratic variables and their interactions as factors. Table 3 shows the results of the significant effects of the variables, together with the coefficient of determination (R²) and F value from the Fisher distribution as the response to CGTase production.

The lowest and the highest values of CGTase activity were obtained when maximal and minimal levels of cassava starch (X₁) and nitrogen sources (X₂) were used, respectively. These results suggested that this variable significantly affect the CGTase production and hence, its activity. The low level of cassava starch (-1.0 in coded value or 0.75%, w/v) and nitrogen sources (-1.0 in coded value or 0.75%, w/v) allowed the strain to produce a higher CGTase activity than the higher level of carbon and nitrogen sources concentration as showed in Table 2. We can observe in Table 2 by indication of software that with a decrease in concentrations of variables X₁ (experiments 9 and 10) and X₂ (experiments 11 and 12) for the same values of X₃, the EA increased.

As can be seen from Table 3, the Student's t-values for factors cassava starch concentration (X₁), nitrogen sources concentration (X₂), interaction between cassava starch and yeast carbonate sodium (X₁X₃) and interaction between nitrogen sources and carbonate sodium (X₂X₃) were above the 1% level of significance. The result of the calculated F (F_C) was greater than the tabulated F (F_T) at the 1% level of significance, thereby allowing the rejection of H₀ at a 1% level of significance. Thus, there is 99% confidence that the model is significant. The coefficient of determination (R²) of 0.80 indicates an adequate adjustment of the experimental data for the EA response based on the proposed empirical equation, that is, 80% of the variability in the data was explained by the equation from the adjusted model.

The model can be applied in screening crucial and critical medium components. The Eq. 3 was obtained from analysis of multiple regression and the significant terms were included in the following equation:

$$EA = 50.93 - 7.98X_1 - 6.61X_2 + 15.62X_1X_2 + 11.5X_2X_3 \quad (3)$$

where, EA is enzyme activity ($U\ mL^{-1}$), X_1 is cassava starch concentration (%), X_2 is concentration of $\frac{1}{2}$ tryptone and $\frac{1}{2}$ yeast extract; and X_3 is Na_2CO_3 concentration.

Based on the experimental results of CGTase production, the quadratic model of CGTase activity as a function of carbon, nitrogen and Na_2CO_3 concentrations generated two linear terms and two interactions, using the Statistic 7 software program.

The variable with positive fitted constant has an enhancer effects towards CGTase production than the one with negative coefficient, which had inhibitory effects. Therefore, the negative sign of the isolated variables (X_1 and X_2) means that a reduction in the concentration of cassava starch and nitrogen sources increases enzyme activity and positive sign of the interactions of variables (X_1X_2 and X_2X_3) means that a reduction in the concentration of cassava starch/carbonate sodium and nitrogen sources/carbonate sodium decrease enzyme activity.

Response surfaces were designed in order to facilitate the visualization of the effect of the independent variables (cassava starch, nitrogen sources and Na_2CO_3) on EA (Fig 2-4).

Production of the CGTase enzyme was achieved by the substitution of the concentrations of nitrogen sources, carbon source and sodium carbonate. The greatest EA was achieved when the soluble starch was substituted with cassava starch and CCD variations were performed on the concentrations of carbon and nitrogen sources and sodium carbonate in the culture medium. Moreover, enzyme production by *Bacillus* sp., subgroup *alcalophilus* was best at the fermentation time of 72 h. Analyzing the response surfaces (Fig2-4), an optimal region for the EA of $96.07\ U\ mL^{-1}$ was determined at the concentrations of cassava starch (0.75 %), nitrogen sources (0.75%) and sodium carbonate(1%).

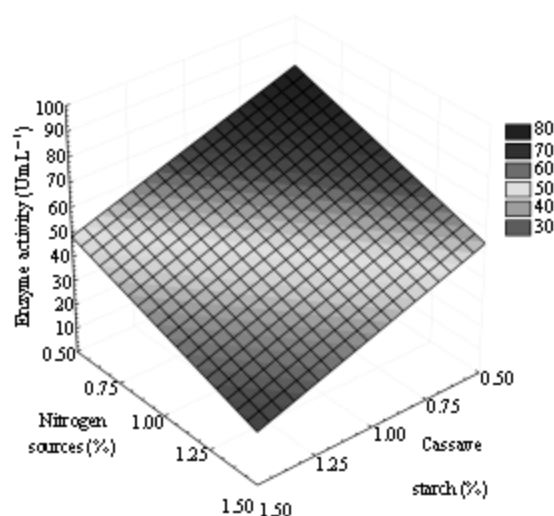


Fig. 2: Response surface of CGTase production as a function of cassava starch concentration and concentration of nitrogen sources

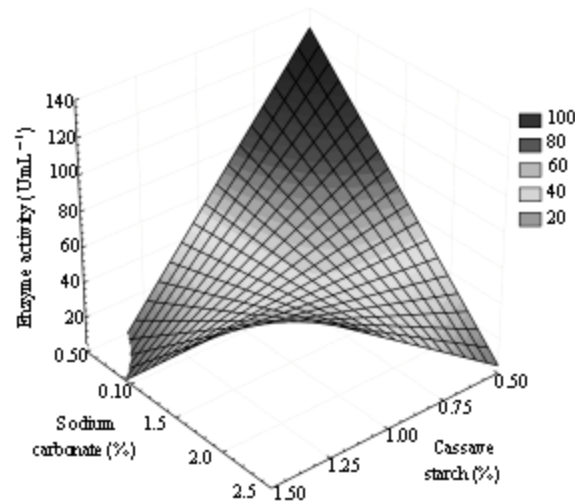


Fig. 3: Response surface of CGTase production as a function of cassava starch concentration and concentration of sodium carbonate

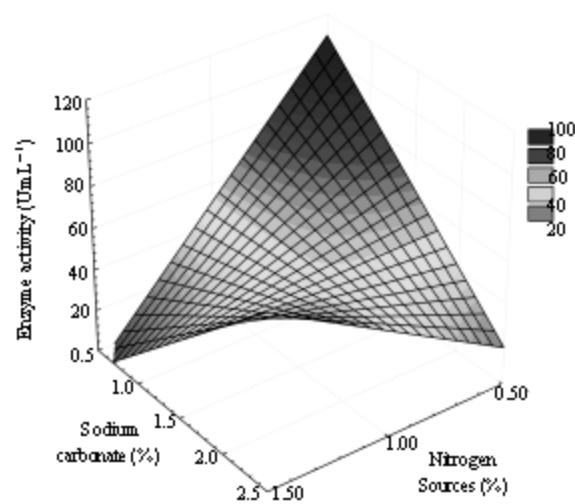


Fig. 4: Response surface of CGTase production as a function of sodium carbonate concentration and concentration of nitrogen sources

The CCD proved an effective method for determining the production of CGTase by the *Bacillus* sp., subgroup *alcalophilus*. The following is the optimized culture medium for intensifying CGTase production (%): cassava starch, 0.75; tryptone, 0.375; yeast extract, 0.375; K_2HPO_4 , 0.1; $MgSO_4 \cdot 7H_2O$, 0.02; Na_2CO_3 , 1 and initial pH of 9.2, which resulted in a 14.28% increase when compared to the original medium.

In a earlier study using the microorganism *Bacillus* sp., subgroup, *alcalophilus* (Freitas *et al.*, 2004), the CGTase activity of 88.6 U mL^{-1} was achieved using 1.5% starch, 1.5% nitrogen and 1% sodium carbonate in 72 h of fermentation.

Adriana *et al.* (2002) achieved an EA of 5.8 U mL^{-1} with optimized concentrations of 1.5 g L^{-1} of cassava starch and 0.4 g L^{-1} ammonium sulfate using CCD. Zain *et al.* (2007) achieved an EA of 80.12 U mL^{-1} with optimized concentrations of 3.3 g L^{-1} of carbon and 0.13 g L^{-1} of nitrogen, analyzed using the response surface method. According to Khairizal *et al.* (2004) optimized values of 1.48 and 1.89% starch and yeast extract during the fermentation for CGTase production yielded an activity of 84 U mL^{-1} , using a mathematical model provided by the response surface method, thereby demonstrating the importance of using an experimental design with variations in concentrations of carbon and nitrogen sources for CGTase production. This happen due to the presence of concentration of some essential nutrients to stimulate CGTase production. The CGTase production was influenced by the level of cassava starch, nitrogen sources and sodium carbonate (Table 3). As shown in response surfaces (Fig. 3-4), the effect of cassava starch, nitrogen sources and sodium carbonate concentration on CGTase production by *Bacillus* sp., subgroup *alcalophilus* was simulated according to the model.

Experimental Validation of the Optimized Conditions

To confirm the model adequacy and the results from an analysis of the response surface, three additional experiments were repeated the optimal medium composition. As a result, the mean value of AE was $93.1 \pm 0.06 \text{ U mL}^{-1}$, which was close to the predicted value (102.96 U mL^{-1}). The correlation between predicted and measured values verifies the model validation, indicating that the model was adequate for obtaining the optimum region in the range of studied parameters.

CONCLUSION

The value of 96.07 U mL^{-1} obtained in the present study had a 6.545% error in relation to the predicted value of 102.96 U mL^{-1} , thereby demonstrating the considerable reliability of the results. The results indicate the potentiality of using milled cassava as a fermentation substrate for the production of CGTase using the microorganism, *Bacillus* sp., subgroup *alcalophilus* and the use of experimental design in the medium optimization to obtain high enzyme activity.

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