

Optimization of Cyclodextrin Glycosyltransferase Production from Sorghum

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Abstract

A central composite experimental design was used to evaluate optimal conditions of the medium component, pH and temperature for cyclodextrin glycosyltransferase production from sorghum by *Bacillus circulans* (American Type Culture Collection). Sorghum concentration, initial pH and temperature were optimized using the response surface method. Maximal CGTase production of 216.94 U.mL⁻¹ was obtained after 48 hours of fermentation when the concentrations of the medium were 0.16% sifted sorghum, 0.5% triptone, 0.5% yeast extract, 1% sodium carbonate, 0.1% K₂HPO₄, 0.02% MgSO₄·7 H₂O, initial pH of 8.0 and temperature of 35°C under rotational shaking. Further studies aimed at increasing the fermentation scale using optimal conditions for the high enzymatic production and high volumetric productivity are needed to improve the fermentation process described in the present study.

Keywords: Sorghum; Response surface methodology; Optimization of production; Cyclodextrin glycosyltransferase

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides with a variable number of glucose units joined by α linkages -1,4, the most common features of which are 6, 7 and 8 glucose units with diameters of about 5.2 Å (α -CD), 6.6 Å (β -CD) and 8.4 Å (γ -CD) [1] and sufficiently hydrophilic externally to make CDs water soluble [2]. CDs solubilize and modify drugs, foods, cosmetics, etc. due to the formation of inclusion complexes [3]. Cyclodextrin glycosyltransferase (CGTase) EC 2.4.1.19 (α -1,4-glucan 4-glycosyltransferase) is a monomeric enzyme with a molecular weight of around 75 kDa [4]. CGTase has an amino acid sequence similar to that of the enzyme α -amylase and therefore belongs to the amylase family. This enzyme catalyzes the conversion of starch into CDs through a reversible intramolecular transglycosylation reaction, cyclization, hydrolysis and coupling to the starch [5].

CDs production involves starch treatment with CGTase produced by bacteria [6]. The selection of carbon sources is a critical factor in the fermentation process for CGTase production. The wrong carbon source may cause catabolite repression of the enzyme production and reduce the growth rate of the microorganism [7]. Sorghum is a starch-rich grain grown widely in Brazil. The increase in sorghum crops is due to the capacity of the plant to adapt to conditions of water deficiency and recycle nutrients in the soil. Nitrogen fixation in sorghum crops has been addressed in recent studies [8]. According to the National Sorghum Producers, sorghum has a low production cost – at least 20 percent less than other major grain crops. Thus, the use of sorghum as a source for downstream products has an economic benefit. The sorghum has been used as a carbon source for products as ethanol [9] and Lactic acid except for CGTase production are not yet known [10].

Sorghum use as a carbon source in the media may lower the cost of CGTase production. The aims of the present study were to evaluate the use of sorghum as a substrate for the production of CGTase using the microorganism *Bacillus circulans* and optimize its concentration and other production variables, such as temperature and initial pH, in an experimental design.

Materials and Methods

Analysis of starch in Sorghum

The starch in sorghum was calculated as the difference between total sugars and reducing sugars: Sorghum starch = total sugars – reducing sugars.

For the analysis of total sugars, 2 g of sorghum were transferred to a 100-mL Erlenmeyer flask; 1 ml of sodium hydroxide 10% (v/v) was added and autoclaved (1 atm at 120°C) for 1 hour. At room temperature, 10 ml of hydrochloric acid were added and autoclaved again (1 atm at 120°C) for 30. At 25°C, the excess hydrochloride acid was neutralized with sodium hydroxide 40% (v/v), transferred to a 500-mL volumetric flask and the volume was completed with distilled water. The resultant solution was stirred and filtered with dry filter paper (90 mm). Two hundred fifty μ L of filtered solution were added to 250 μ L of 3,5-dinitro salicylic acid (DNS), boiled for 5 min. and cooled. Absorbance of the final solution was read using a spectrophotometer at 540 nm. The standard curve was made with glucose P.A.

For the analysis of reducing sugars, 2 g of sorghum was transferred to a 500-mL volumetric flask; the volume was completed with distilled water, stirred and filtered with dry filter paper (90 mm). Two hundred fifty μ L of the solution volume were added to 250 μ L of DNS, boiled for 5 min. and cooled in an ice bath. Absorbance of the final solution was read using a spectrophotometer at 540 nm. The standard curve was made with glucose P.A.

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Microorganism

Bacillus circulans was acquired from the culture collection of the Industrial Microbiology Laboratory of the Biological Sciences Institute of Rio Claro (SP, Brazil) and was originally purchased from the American Type Culture Collection.

Activation of inoculum: *Bacillus circulans* was cultured in a medium (100 mL) containing (w/v) 1% soluble 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 a 300-mL Erlenmeyer flask at 150 rpm and 35°C. Successive transferences were performed at eight-hour intervals for 72 h. The centrifuged broth of the last peal was centrifuged and stored with 40% glycerol at -80°C.

Enzyme production

The parameters affecting CGTase production, such as the carbon source (sorghum), Nakamura and Horikoshi (1976) medium, temperature and initial pH, were studied using a central composite design [11]. *Bacillus circulans* was cultured in 300-mL Erlenmeyer flasks containing 100 mL of modified Nakamura and Horikoshi (1976) medium incubated at 150 rpm for 24, 48 and 72 h. The production broth was centrifuged at 10,000 rpm for 10 min. and the cell-free of supernatant was used for the determination and enzyme activity.

Enzyme activity (EA) was measured using the phenolphthalein method [12]. Five mL 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 (0.05M), 1.0 mL of Tris-HCl buffer solution (0.05M; pH 8.0) and distilled water for a final volume of 10 mL] were placed in a thermostatic reactor at $55 \pm 1^\circ C$. Samples were withdrawn at zero, three, six, nine and 12 min. and inactivated at 100°C for five 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 in a spectrophotometer at 550 nm. EA was defined as the amount of cyclodextrin produced per min. and determined by Equation 1.

$$A = \alpha \cdot \beta \cdot F_{dil} \cdot 1000 \cdot (V_{reactor} / V_{enzymatic}) \quad (1)$$

in which A is enzyme activity ($\mu\text{mol}/\text{min.}/\text{mL}$); α is the slope of the standard curve ($\mu\text{mol. mL}^{-1}/\text{ABS}$); β is the slope of the experimental curve ($\text{ABS}/\text{min.}$); V is the enzyme volume of the sample; and Fdil is the dilution factor.

Statistical analysis

The Statistic 7.0 program (Stat Soft, Tulsa, OK, USA) was used for the experimental design and multiple regression analysis of the data. The response surface methodology was employed to determine the interaction between process variables, optimize the results and minimize the variability in the results. Multiple regression analysis was performed and the equation representing the model was statistically analyzed with the coefficient of determination (R). The F-test and t-test established the statistical significance of the proposed model.

Carbon source selection

The selection of the carbon source was performed by substituting the carbon source in the Nakamura and Horikoshi culture medium with sour and sweet cassava (starches that differ in acidity), sorghum and a commercial soluble starch [11].

Central composite design

Experiments were performed to determine the influence of the

variables on the enzyme production process using two experimental central composite designs. The variables of the first and second planning performed in shake flasks were $X_1 \equiv$ sorghum starch (%), $X_2 \equiv$ initial pH and $X_3 \equiv$ temperature. The levels of the variables studied were placed in coded form in Table 1, using the following encoding equation for each experimental design:

$$xi = (Xi - X_{cp}) / \Delta Xi \quad (2)$$

in which xi is the coded value of the independent variable; Xi is the real value of the independent variable; X_{cp} is the real value of an independent variable at the central point; and ΔXi is the step change value. Table 1 displays the variations and levels of the independent variables analyzed. The root mean square was generated from an empirical equation produced by the central composite design.

Results and Discussion

The choice of sorghum grain was determined after preliminary testing with different carbon sources in the Nakamura and Horikoshi culture medium: sour and sweet cassava, sorghum in nature and soluble starch. Sorghum starch proved to be the best carbon source for CGTase production. This is due the fact that the sorghum grain (ranging in size from 6 to 30 μm) exhibits 100% amylopectin [13] and CGTase breaks the α -1,4 starch bonds in order to catalyze the transference of amylopectin into CDs [14]. A number of different several carbon sources have been employed to reduce CGTase production costs. Cassava starch is cheaper than commercial potato starch and has been used to optimize the production of CGTase from *Bacillus lehensis* [15]. Kulprecha et al. (2009) used corn and rice as carbon sources to produce CGTase [16].

Table 2 displays the test results for the total and reducing sugars and starch concentration with different sorghum grain sizes (based on mesh scale): non-sifted sorghum seed and 1.19, 0.71, 0.5 and 0.064 meshes. Total and reducing sugars, starch and enzyme activity were tested at 24-hour intervals for 72 hours.

Table 2 also displays the relationship between sorghum grain size, percentage of starch and enzyme activity, using each different grain size as substrate. The same amount of grain has different starch concentrations depending on seed size. Starch and glucose concentrations were greatest in the non-sifted grain. A direct correlation was found between grain size and enzyme activity. The

Variables	Range and levels				
	- α	-1	0	1	+ α
Sorghum (X_1)	0.33	1	0.8	1	1.17
pH (X_2)	6.32	7	8	9	9.98
Temperature (X_3)	33.3	34	35	36	36.7

Table 1: Experimental range and levels of independent variables used in central composite design.

Sample (mesh)	Total sugar (g L^{-1})	Reducing sugar (g L^{-1})	Starch		
			Non-reducing sugar (g L^{-1})	Starch (%)	EA (U mL^{-1})
0.06	1.19	0.21	0.98	24.40	190.99
0.5	1.27	0.31	0.96	24.00	191.68
0.71	1.34	0.37	0.97	24.20	172.65
1.19	1.45	0.4	1.05	25.40	186.84
SG*	1.51	0.48	1.03	26.80	227.49

*Non-sifted sorghum grain

Table 2: Total and reducing sugars, starch concentration and enzyme activity with different sizes of sorghum grain.

highest concentration of starch from sorghum corresponds to the greatest enzyme activity. This is due to capacity of *Bacillus circulans* to use the non-sifted grain easily, possibility due to size of the starch molecule and/or its position on the grain.

Culture conditions regarding sorghum concentration, pH and temperature for CGTase production were optimized using the response surface methodology. Table 3 displays the design matrix in coded and real variables and respective results (enzyme activity).

The greatest CGTase production was 216.94 U.mL⁻¹, obtained with 0.162% sorghum and pH 8 at 35°C (Table 3, experiment 15). Multiple regression analysis was performed on the data. Equation 3 describes the experimental data.

$$EA = 205.26 - 11.15X_1 + 3.44X_2 + 24.82X_3 + 7.81X_1X_2 + 15.74X_1X_3 - 23.24X_2X_3 - 1.59X_1^2 - 11.64X_2^2 - 42.55X_3^2 \quad (3)$$

The quadratic model relates three linear terms, three quadratic terms and three factorial interactions, in which Y is the predicted response (EA) and X₁, X₂ and X₃ are the coded values of sorghum, initial pH and temperature, respectively.

Table 4 displays the Student's t-distribution and p-values that describe the significant probability (less than α = 0.1). The results demonstrate the significant effect of the variables X₁ (sorghum), X₂ (initial pH) and X₃ (temperature). The isolated variables X₂ and X₃ and quadratic variables X₁², X₂² and X₃² have positive coefficients, meaning that an increase in their values results in an increase in enzyme yield. The isolated variable X₁ and the X₂X₃ interaction have negative coefficients, meaning that an increase in their values results in a reduction in enzyme production.

The non-significant terms X₁X₂ and X₁X₃ were discarded and the reduced Equation 4 was generated to represent the CGTase production model.

$$EA = 209.19 + 5.83X_1 - 8.62X_2 - 24.07X_3 + 8.60X_2X_3 - 27.22X_1^2 - 52.00X_2^2 - 20.37X_3^2 \quad (4)$$

ANOVA of the quadratic regression model demonstrated in Eq. 4 was highly significant, which is evident from the results of Fisher's F-test, which had a very low probability value [(p model > F) = 0.0000]. The adjusted model was checked by the coefficient of determination (R²) and the multiple correlation coefficient (R). The R² value of 0.9841 demonstrates the high degree of significance of the model and indicates that the independent variables explained 98% of the variability in enzyme activity, with only 2% not explained by the model. The high R value (0.9951) demonstrates strong agreement between the experimental and predicted values regarding CGTase production.

The response surface, 3D graph and contour plot representation of the regression equation demonstrate the interaction of the variables at their optimal levels for maximal response (Figure 1).

The rounded response surfaces propose optimal variables. The response surface for CGTase production represents different combinations of two variables while keeping the other variable at the zero level. The three variables X₁, X₂ and X₃ had a significant effect on CGTase production at a 90% confidence level. An algorithm was used in the Maple 15 program to calculate the stationary point for the production of CGTase. The optimal concentration of sorghum, initial pH and temperature CGTase production from *Bacillus circulans* were 0.16% (Figures 1d and 1e), 8.06 (Figures 1d-1f) and 34.78°C, respectively (Figures 1e-1f). Predicted maximal enzymatic activity was 212.2 U.mL⁻¹, which is very close to the 211.40 U.mL⁻¹ obtained experimentally. Thus, the experimental design proved effective for the optimization of substrate concentration and experimental conditions for maximal CGTase production [17,18].

Sorghum, initial pH and temperature were the relevant variables controlling CGTase production (Figures 1a-1c) and their strong interaction for CGTase production was expected. As different CGTase

Experiments	Coded variables			Real variables			EA (U.mL ⁻¹)
	X ₁	X ₂	X ₃	X ₁	X ₂	X ₃	
1	-1	-1	-1	0.152	7	34	135.63
2	-1	-1	1	0.152	7	36	70.58
3	-1	1	-1	0.152	9	34	115.21
4	-1	1	1	0.152	9	36	76.46
5	1	-1	-1	0.172	7	34	157.77
6	1	-1	1	0.172	7	36	90.99
7	1	1	-1	0.172	9	34	118.33
8	1	1	1	0.172	9	36	94.11
9	-1.68	0	0	0.145	8	35	130.44
10	1.68	0	0	0.178	8	35	140.13
11	0	-1.68	0	0.162	6.32	35	85.11
12	0	1.68	0	0.162	9.68	35	45.32
13	0	0	-1.68	0.162	8	33.32	194.45
14	0	0	1.68	0.162	8	36.68	114.87
15	0	0	0	0.162	8	35	216.94
16	0	0	0	0.162	8	35	201.71
17	0	0	0	0.162	8	35	205.87
18	0	0	0	0.162	8	35	208.98
19	0	0	0	0.162	8	35	211.40

EA = enzyme activity; X₁ = sorghum grain (%); X₂ = pH; X₃ = Temperature (°C)

Table 3: Central composite rotational design for optimization of carbon source, pH and temperature for production of CGTase by *Bacillus circulans* and respective enzyme activity.

Factor		Standard error	Computed t-value	p-value
Intercept			209.1972	3.145447
X_1^d	0.092503	0.030230	5.8333	1.906315
X_2^e	-0.136800	0.030230	-8.6267	1.906315
X_3^f	-0.381770	0.030230	-24.0745	1.906315
X_1^2	-0.033085	0.030230	-2.7248	2.489621
X_2^2	0.019431	0.030230	1.6002	2.489621
X_3^2	0.104507	0.030230	8.6067	2.489621
X_1X_2	-0.440251	0.030815	-27.2299	1.905937
X_1X_3	-0.840758	0.030815	-52.0016	1.905937
X_2X_3	-0.329493	0.030815	-20.3794	1.905937

Table 4: Coefficients estimated by linear regression ($R^2=0.968$; adj. $R^2=0.937$).

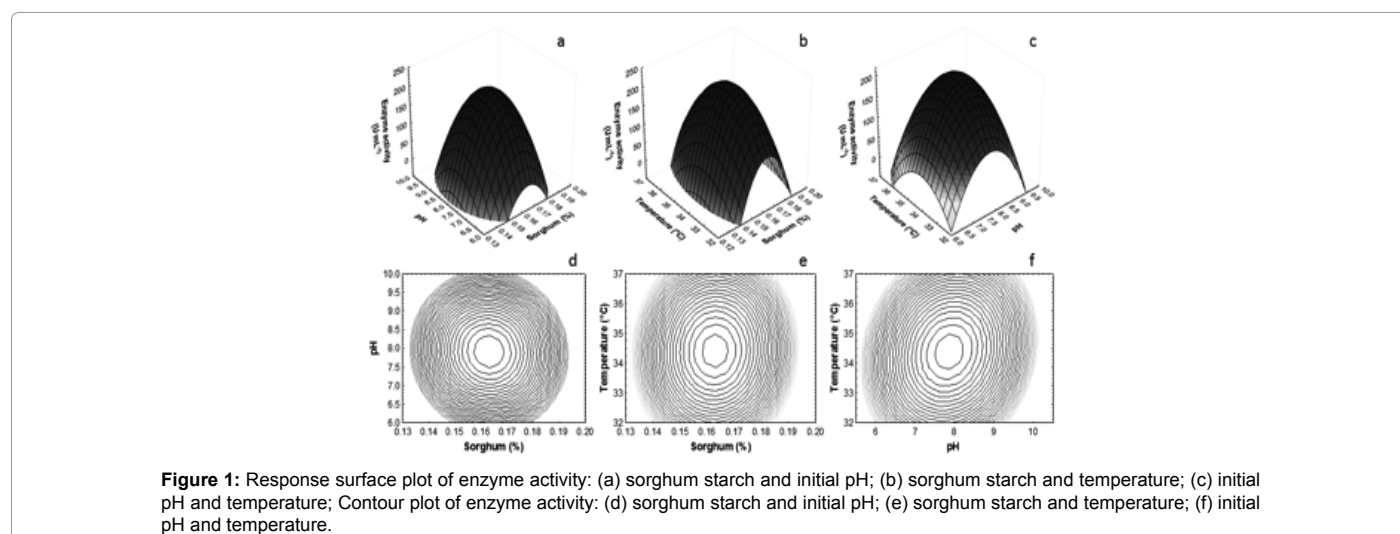


Figure 1: Response surface plot of enzyme activity: (a) sorghum starch and initial pH; (b) sorghum starch and temperature; (c) initial pH and temperature; Contour plot of enzyme activity: (d) sorghum starch and initial pH; (e) sorghum starch and temperature; (f) initial pH and temperature.

bacteria require different carbon concentrations for growth, sorghum at higher and lower concentrations may inhibit CGTase production from *Bacillus circulans* due to catabolite repression or a lack of cell nutrients.

Sorghum starch is an inexpensive alternative to soluble starch. Starting materials account for 60 to 80% of the cost of enzyme production by fermentation [19]. The substitution of a soluble starch with 0.75% cassava starch achieves the highest CGTase yield at 72 h [20]. Alves-Prado et. al. found that, when associated with wheat, cassava can replace soluble starch as the carbon source in CGTase production, achieving 80.50 U.mL⁻¹ of enzyme activity with E16 do *Bacillus* sp. The effect of the carbon source on CGTase production was positive in the present study, as sorghum is rich in starch and contains other elements, such as protein [8]. Previous studies have also investigated CGTase production by *Bacillus circulans* varying pH to increase CGTase production [21]. The use of Na₂CO₃ is reported to control pH for microorganism growth, as CGTase production was enhanced with the increase in Na₂CO₃ concentration to reach pH 8 [22]. The positive effect of pH and temperature on CGTase production from *Bacillus circulans* has also been described by Rosso et al. who report optimal conditions of initial pH 8.3 and 37°C [23].

The greatest enzyme activity for CGTase production was 216.94 U.mL⁻¹ under optimized conditions obtained after 48 hours of culture with 0.16% sorghum, 0.5% triptone, 0.5% yeast extract, 1% sodium carbonate, 0.1% K₂HPO₄ and 0.02% MgSO₄·7 H₂O at an initial pH of 8 and temperature of 35°C. Thus, the use of sorghum for fermentation

by *Bacillus circulans* is viable and produces a considerable amount of CGTase. This study focused on high production in a rotational shaker. Further studies using sorghum as the carbon source with a scale increase in the fermentation process are needed to reduce the costs of CGTase production.

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