0273-2289/1559–0291 (Online)/07/136–140/27–40/$30.00

Optimization of Cyclodextrin Glucanotransferase Production From *Bacillus clausii* E16 in Submerged Fermentation Using Response Surface Methodology

Heloiza Ferreira Alves-Prado,[1,2] Daniela Alonso Bocchini,[1,3] Eleni Gomes,[1] Luis Carlos Baida,[4] Jonas Contiero,[2] Inês Conceição Roberto,[5] and Roberto Da Silva*,[1]

[1]Biochemistry and Applied Microbiology Laboratory—IBILCE/UNESP, São José do Rio Preto, SP, Brazil, E-mail: dasilva@ibilce.unesp.br; [2]Biology Institute—UNESP, Rio Claro, SP, Brazil; [3]Chemistry Institute—UNESP, Araraquara, SP, Brazil; [4]Computation Sciences and Statistic Department—IBILCE/UNESP, São José do Rio Preto, SP, Brazil; and [5]Biotechnology Department—School of Engineering of Lorena—EEL/USP—CP: 116, Lorena, SP, Brazil

Abstract

Cyclodextrin glucanotransferase production from *Bacillus clausii* E16, a new bacteria isolated from Brazilian soil samples was optimized in shake-flask cultures. A 2^4 full-factorial central composite design was performed to optimize the culture conditions, using a response surface methodology. The combined effect among the soluble starch concentration, the peptone concentration, the yeast extract concentration, and the initial pH value of the culture medium was investigated. The optimum concentrations of the components, determined by a 2^4 full-factorial central composite design, were 13.4 g/L soluble starch, 4.9 g/L peptone, 5.9 g/L yeast extract, and initial pH 10.1. Under these optimized conditions, the maximum cyclodextrin glucanotransferase activity was 5.9 U/mL after a 48-h fermentation. This yield was 68% higher than that obtained when the microorganism was cultivated in basal culture medium.

Index Entries: Alkalophillus *Bacillus clausii*; CGTase production; cyclodextrin glucanotransferase; experimental design; submerged fermentation; response surface methodology.

Introduction

Cyclodextrin glucanotransferase (CGTase) (EC 2.4.1.19) is an extracellular bacterial enzyme, used in the production of cyclodextrin from starch. Cyclodextrins (CDs) are cyclic maltoligosaccharides made up of six, seven, or eight D-glucose units, named α-, β-, or γ-cyclodextrin, respectively *(1)*.

*Author to whom all correspondence and reprint requests should be addressed.

The internal cavities of CDs are hydrophobic and the external surface hydrophilic, so they can encapsulate a wide variety of hydrophobic molecules, potentially improving their properties. The modified properties make CDs suitable for numerous applications in the food, cosmetic, and pharmaceutical industries. For example, CDs can be used to capture undesirable tastes or odors, to stabilize volatile compounds, to increase water solubility of hydrophobic substances, or to protect a substance against unwanted modifications *(2–6)*. They are commonly produced from starch through four reactions catalyzed by the CGTase:

1. Intramolecular transglucosylation or cyclization reaction, in which a linear oligosaccharide chain is cleaved and the new reducing-end sugar is transferred to the nonreducing-end sugar of the same chain.
2. Coupling reactions, in which a cyclodextrin ring is cleaved and transferred to an acceptor maltooligosaccharide substrate.
3. Intermolecular transglucosylation or disproportionation, in which a linear maltooligosaccharide is cleaved and the new reducing-end sugar is transferred to an acceptor maltooligosaccharide substrate.
4. A weak hydrolyzing activity on starch *(6,7)*.

CGTase producers are mainly alkalophilic *Bacillus* sp., but they are also reported as *Klebsiella* sp., *Micrococcus* sp., *Brevibacterium* sp., *Paenibacillus* sp., *Themoanaerobacter* sp., *Thermoactinomyces* sp., and others *(2)*. Some studies have shown the improvement of CGTase production through changes in the composition of the culture medium *(8–14)*.

Traditional methods of optimization involve changing one independent variable, whereas fixing the others at a certain level. This single-factor search is laborious, time-consuming, and incapable of reaching the true optimum condition as it fails to reveal interactions among variables. Response surface methodology (RSM), described first by Box and Wilson, is an experimental strategy for seeking the optimum conditions for a multivariable system *(15,16)*. It has been successfully used to optimize the medium ingredients and to operate conditions in enzyme production and many other bioprocesses *(8,9,13,14,17–20)*. In earlier studies we isolated a new CGTase producer, first identified as *Bacillus* sp. subgroup *alcalophilus* E16 *(21)* and we also identified some factors affecting the production of CGTase. In the work reported here, these factors were investigated, using a full-factorial central composite design and RSM, with the aim to improve the CGTase production by optimizing the culture medium.

Materials and Methods

Bacterial Strain Isolation and Identification

The strain E16 was isolated from a soil crop sample and its microbial properties were investigated by classical taxonomy as described in Bergey's Manual *(22)*. Based on these results, it was identified as a *Bacillus*

sp. strain E16. Molecular techniques based on partial sequence of 16S ribosomal RNA (rRNA) analysis were applied for specie identification. The genomic DNA of strain E16 was isolated and the 16S ribosomal DNA was amplified by polymerase chain reaction using forward primer p27 and reverse primer p1525. These primers are homologous to preserved regions of the 16S rRNA gene of bacteria. The amplified product polymerase chain reaction was purified and sequenced. The 16S ribosomal DNA sequence was aligned with sequences obtained from Ribosomal Database Project (Wisconsin, WI; http://www.cme.msu.edu/RDP/html) and GenBank (http://www.ncbi.nlm.nih.gov/).

The sequences with high similarity and alcalophilic *Bacillus* sp. were compiled into NEXUS files after alignment in CLUSTAL W program *(23)*. The phylogenetic relationships among selected sequences were estimated by using the maximum parsimony method, using a branch-and-bound algorithm as implemented in PAUP v.4.0b10 *(31)*. Branch support was calculated by bootstrap analysis consisting of 500 replicates. The distance matrix used in this work was constructed by "p" method. DNA sequences of *Clostridium butyricum* strain MW (AJ002592), *B. clausii* strain z a w3 (AY066000), *B. clarkii* DSM 8720 (X76444), *B. clausii* strain Y76-A (AB201796), *B. clausii* DSM 8716 (X76440), *Bacillus* sp. strain ikaite 27 (AJ431332), *B. pataginiensis* PAT 05 (AY258614), *Bacillus* sp. DSM 8714 (X76438), *B. alcalophilus* YB380 (AF078812), *B. horti* (D87035), *Bacillus* sp. strain NER (AJ507321), *Bacillus* sp. TS1-1 (AY751538), *Bacillus* sp. G1 (AY754340), *B. oshimae* K-11 (AB188090), *B. pseudalcaliphilus* DSM 8725 (X76449), *B. alcalophilus* DSM 485T (X76436), *B. horikoshii* DSM 8719 (X76443), *B. circulans* ATCC4513 (AY647299), *B. litoralis* IB-B4 (AJ309561), *B. galactosidilyticus* (AJ535638), and *B. thermoamylovorans* (AJ586361) were obtained from the GenBank DNA sequence library.

Materials

β-Cyclodextrin, maltodextrin, and phenolphthalein were purchased from Sigma (St. Louis, MO). Yeast extract was obtained from Difco (Detroit, MI) and peptone was obtained from Biobrás (Montes Claros, MG, Brazil). Soluble starch was obtained from Mallinckrodt (Paris, France). Other chemicals of analytical grade were obtained from Merck (Darmstadt, Germany).

Cultivation Medium

Stock culture of the strain E16 was maintained at 5°C on agar slant with the same composition as the following basal liquid culture medium plus 1.5% agar *(24)*. The basal liquid culture medium was made up of soluble starch 10.0 g/L, peptone 5.0 g/L, yeast extract 5.0 g/L, K_2HPO_4 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, Na_2CO_3 10.0 g/L (separately sterilized), and pH 10.0 *(25)*. For the optimization experiments the culture medium contained various quantities of soluble starch, peptone, and yeast extract

concentrations; and the initial pH was varied in accordance with the experimental design. Bacteria was transferred by loop to a flask with basal liquid medium and cultured for 24 h to produce the inoculum. The optimization experiments were carried out in 125-mL Erlenmeyer flasks each containing 20 mL of one of the culture media. These flasks were seeded with 0.1 mL from the 24 h-old culture containing 2.6×10^9 cells/mL, and were incubated for 48 h on a rotary shaker at 37°C and 150 rev/min. After a 48-h fermentation, the contents of each flask was centrifuged at 10,000*g* at 5°C for 15 min, and CGTase activity was measured in the supernatant.

CGTase Assay

CGTase activity was measured as β-CD forming activity based on phenolphthalein method *(26)* with slight modifications as described in Alves-Prado et al. *(27)*. One unit of CGTase activity was defined as the amount of enzyme that produces 1 μmol of β-CD per minute.

Experimental Design and Optimization

The software "Statistica" (version 5.0), from StatSoft Inc., was used for design experiments and for regression and graphical analysis of the data obtained. The dependent variable selected for this study was the CGTase activity, expressed in U/mL, and the independent variables chosen were the concentrations of soluble starch, peptone, and yeast extract, plus the initial pH of the culture medium. These variables and the value ranges were chosen based on previous experiments changing one independent variable and fixing the other variables at a certain level *(28)*. In order to determine the optimal conditions for the production of CGTase by *B. clausii* E16, a 2^4 full-factorial central composite design with five coded levels was implemented, resulting in twenty-six sets of experiments. Eq. 1 shows the code of independent variables used in the statistical analysis.

$$x_i = \frac{X_i - X_0}{\Delta X_i} \tag{1}$$

where x_i is the independent variable coded value, X_i is the independent variable real value, X_0 is the independent variable real value of the center point, and ΔX_i is the step change.

The ranges and levels of the variables investigated in this study are given in Table 1. In this table, the studied variables are coded as X_1 (initial pH), X_2 (soluble starch), X_3 (peptone), and X_4 (yeast extract). The five levels are the results of previous runs based on a path of steepest ascent analysis of which (–1) and (+1) points are the minimum and maximum points, respectively. The (0) point is the central point, which was determined from the arithmetic mean between the minimum and maximum points, for each studied

Table 1
Experimental Ranges and Levels of the Independent Process Variables Used in the 2^4 Full-Factorial Central Composite Design

Independent variable	Symbol	Ranges and levels				
		–1.483	–1	0	1	+1.483
pH	X_1	9.34	9.62	10.2	10.78	11.06
Soluble starch (g/L)	X_2	11.3	12.5	15.0	17.5	18.7
Peptone (g/L)	X_3	3.8	4.4	5.6	6.8	7.4
Yeast extract (g/L)	X_4	3.7	4.2	5.3	6.4	6.9

variable. The ($-\alpha = -1.483$) and ($+\alpha = +1.483$) points are levels which result in a star configuration, determined by the mentioned statistic software.

The estimated response surface ($\hat{y}$) was expressed by the second degree Eq. 2:

$$\begin{aligned}\hat{y} = {} & b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{12}X_1X_2 \\ & + b_3X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 \\ & + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + \varepsilon\end{aligned} \tag{2}$$

where $\hat{y}_i$ represents the response variable, b_0 is the intercept, b_1, b_2, b_3, and b_4 are the linear terms, b_{11}, b_{22}, b_{33}, and b_{44} are the quadratic terms, b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, and b_{34} are the cross-product terms, X_1, X_2, X_3, and X_4 represent the variables studied, initial pH, soluble starch concentration, peptone concentration, and yeast extract concentration, respectively, and ε is the random error.

Results and Discussion

Identification of the Isolated Strain

The cells were grown in nutrient agar in alkaline condition (pH 9.5) at 35°C for 24 h. The cells were rod-shaped with stained positive gram and not motile, the spores were ellipsoidal and subterminal. The colonies were circular with entire margins of light yellow color, and approximate size of up to 2.0 mm. The microorganism was strictly aerobe and positive for catalase and oxidase reaction and negative for nitrate to nitrite reduction. The cellular growth at 30, 35, and 40°C was observed on nutrient medium in pH 6.8. These characteristics comprehend the *Bacillus* sp., a phylogenetic analysis with 16S rRNA sequence was necessary for specie identification. So, the strain E16, was first identified as *Bacillus* sp. subgroup *alcalophillus* in accordance to Nielsen *(29)*.

The aim of the phylogenetic analysis was to determine the relationships among some alcalophilic bacilli sequences. A parsimonious tree was

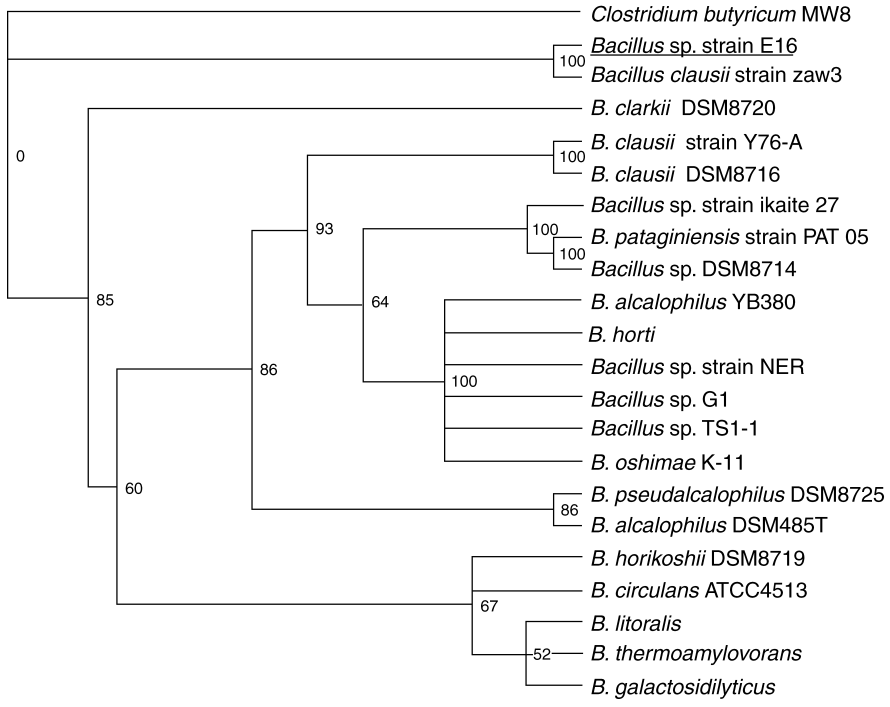

Fig. 1. Phylogenetic tree of the alkalophilic isolates based on 16S rRNA gene sequence using the neighbor-joining method. *C. butyricum* strain MW8 (AJ002592) was used as the outgroup. The accession numbers of the additional 16S rRNA sequences used are as follows: *B. clausii* strain z a w3 (AY066000), *B. clarkii* DSM 8720 (X76444), *B. clausii* strain Y76-A (AB201796), *B. clausii* DSM 8716 (X76440), *Bacillus* sp. strain ikaite 27 (AJ431332), *B. pataginiensis* PAT 05 (AY258614), *Bacillus* sp. DSM 8714 (X76438), *B. alcalophilus* YB380 (AF078812), *B. horti* (D87035), *Bacillus* sp. strain NER (AJ507321), *Bacillus* sp. TS1-1 (AY751538), *Bacillus* sp. G1 (AY754340), *B. oshimae* K-11 (AB188090), *B. pseudalcaliphilus* DSM 8725 (X76449), *B. alcalophilus* DSM 485T (X76436), *B. horikoshii* DSM 8719 (X76443), *B. circulans* ATCC4513 (AY647299), *B. litoralis* IB-B4 (AJ309561), *B. galactosidilyticus* (AJ535638), and *B. thermoamylovorans* (AJ586361).

obtained by the analysis of the 22 species (Fig. 1). From a total of 762 characters, 365 were phylogenetically informative. The consistency index was 0.8108 and the retention index was 0.7969. Two clades, one major containing *B. clarkii* DSM 8720, *B. clausii* strain Y76-A, *B. clausii* DSM 8716, *Bacillus* sp. strain ikaite 27, *B. pataginiensis* PAT 05, *Bacillus* sp. DSM 8714, *B. alcalophilus* YB380, *B. horti*, *Bacillus* sp. strain NER, *Bacillus* sp. TS1-1, *Bacillus* sp. G1, *B. oshimae* K-11, *B. pseudalcaliphilus* DSM 8725, *B. alcalophilus* DSM 485T, *B. horikoshii* DSM 8719, *B. circulans* ATCC4513, *B. litoralis* IB-B4, *B. galactosidilyticus*, and *B. thermoamylovorans* (bootstrap value 85) and the other with the *B. clausii* strain zaw3 and *Bacillus* sp. E16 (bootstrap value 100) were highly resolved. These results agree with those observed on the nucleotides sequence. Based on these results, it is suggested that the

Bacillus sp. strain E16 be named *B. clausii* strain E16, owing to the low divergence shown on the distance between matrixes (3.8%). This sequence was submitted to GenBank and its access number is DQ924973.

Experimental Design and Optimization

The identification of the major factors affecting the experimental response is the first step in determining the optimum conditions for enzyme production. The culture medium concentrations of the components K_2HPO_4, $MgSO_4 \cdot 7H_2O$, soluble starch, peptone and yeast extract, and initial pH of the culture medium were studied previously. Based on these trial runs using a factorial design and a path of steepest ascent, the soluble starch, peptone and yeast extract concentrations, and the initial culture medium pH were identified as important factors in CGTase production *(28)* and were therefore selected for further study of the optimization of CGTase production.

In this study, these variables were statistically optimized with the help of a quadratic model consisting of 2^4 trials plus a star configuration ($\alpha = \pm 1.483$) and two replicates at the center point. The design of this experiment is given in Table 2, together with the experimental results. The highest CGTase activity (4.82 U/mL) was observed in run number 26 and this run corresponded to the center point. The average CGTase activity obtained on the center points was 4.66 U/mL, where the factors soluble starch, peptone, and yeast concentrations and initial medium pH were 15.0 g/L, 5.6 g/L, 5.3 g/L, and 10.2, respectively. This activity was 30.8% higher than that observed in the control run, where the factors were those used in the basal culture medium.

The regression obtained after analysis of variance gives the production of CGTase from *B. clausii* strain E16 as a function of the different initial pH (X_1), soluble starch concentration (X_2), peptone concentration (X_3), and yeast extract concentration (X_4). These terms were included in the following second-order polynomial equation where the mathematical model representing the CGTase activity ($\hat{y}$) in the experimental region studied can be expressed by Eq. 3:

$$\begin{aligned}\hat{y} = {} & 4.472 - 0.296X_1 - 0.363X_2 - 0.384X_3 - 0.181X_4 - 0.362X_1X_2 \\ & + 0.281X_1X_3 + 0.001X_1X_4 - 0.082X_2X_3 - 0.162X_2X_4 \\ & - 0.184X_3X_4 - 0.0674X_1^2 - 0.278X_2^2 - 0.436X_3^2 - 0.362X_4^2\end{aligned} \quad (3)$$

The regression model was generated by Statistica software consisting of 1 offset, 4 linear, 4 quadratic, and 6 interaction terms. Table 3 shows a regression analysis of the estimates and hypothesis tests for the coefficients of regression, which were displayed in Eq. 3. At the 5% probability level, the linear and quadratic coefficients of initial pH (X_1) and peptone concentration (X_3), the linear coefficient of soluble starch concentration (X_2), and the interaction of initial pH and soluble starch concentration (X_1X_2)

Table 2
Experimental Design and Results of the 2^4 Full-factorial Central Composite Design

Runs	Coded levels X_1 X_2 X_3 X_4	Uncoded levels pH	Soluble starch (g/L)	Peptone (g/L)	Yeast extract (g/L)	Enzymatic activity (U/mL) Observed	Predicted
1	–1 –1 –1 –1	9.62	12.5	4.4	4.2	3.85	3.58
2	–1 –1 –1 +1	9.62	12.5	4.4	6.4	4.17	3.96
3	–1 –1 +1 –1	9.62	12.5	6.8	4.2	2.33	2.45
4	–1 –1 +1 +1	9.62	12.5	6.8	6.4	1.85	2.09
5	–1 +1 –1 –1	9.62	17.5	4.4	4.2	3.89	3.75
6	–1 +1 –1 +1	9.62	17.5	4.4	6.4	3.35	3.44
7	–1 +1 +1–1	9.62	17.5	6.8	4.2	2.63	2.95
8	–1 +1 +1 +1	9.62	17.5	6.8	6.4	1.83	1.92
9	+1 –1 –1 –1	10.78	12.5	4.4	4.2	3.23	3.14
10	+1 –1 –1 +1	10.78	12.5	4.4	6.4	3.95	3.52
11	+1 –1 +1 –1	10.78	12.5	6.8	4.2	3.37	3.14
12	+1 –1 +1 +1	10.78	12.5	6.8	6.4	2.64	2.79
13	+1 +1 –1 –1	10.78	17.5	4.4	4.2	2.22	1.86
14	+1 +1 –1 +1	10.78	17.5	4.4	6.4	1.70	1.58
15	+1 +1 +1 –1	10.78	17.5	6.8	4.2	1.97	2.19
16	+1 +1 +1 +1	10.78	17.5	6.8	6.4	1.01	1.17
17	–1.48 0 0 0	9.34	15.0	5.6	5.3	3.65	3.43
18	+1.48 0 0 0	11.06	15.0	5.6	5.3	2.12	2.55
19	0 –1.48 0 0	10.20	11.3	5.6	5.3	3.97	4.40
20	0 +1.48 0 0	10.20	18.7	5.6	5.3	3.54	3.32
21	0 0 –1.48 0	10.20	15.0	3.8	5.3	3.12	4.08
22	0 0 +1.48 0	10.20	15.0	7.4	5.3	3.70	2.94
23	0 0 0 –1.48	10.20	15.0	5.6	3.7	3.68	3.92
24	0 0 0 +1.48	10.20	15.0	5.6	6.9	3.47	3.44
25	0 0 0 0	10.20	15.0	5.6	5.3	4.49	4.42
26	0 0 0 0	10.20	15.0	5.6	5.3	4.82	4.42
Control[a]	–	10.0	10.0	5.0	5.0	3.55	–

[a]basal medium composition.

were found to be significant for the enzyme activity. Similar to our results, the quadratic coefficients for sago starch concentration, peptone from casein concentration as well as the interaction sago starch concentration, and initial pH were significant for the CGTase activity from *B. stearothermophilus* HR1 *(14)*.

The statistical significance of this second-order polynomial model equation (Table 4) was evaluated by performing the *F*-test on the analysis of variance (ANOVA) estimates of mean squares, which showed that this regression is statistically significant ($p = 0.004$) at 95% confidence level.

Table 3
Results of Regression Analysis Using the 2^4 Full-factorial Central Composite Design

Term	Standard errors	Coefficient	$T_{(11)}$ value	*p*-value
Mean	±0.28	4.472	15.974[a]	–
X_1	±0.23	–0.297	–2.592[a]	0.0250
X_1^2	±0.33	–0.674	–4.054[a]	0.0019
X_2	±0.23	–0.363	–3.174[a]	0.0088
X_2^2	±0.33	–0.278	–1.673	0.1225
X_3	±0.23	–0.384	–3.359[a]	0.0064
X_3^2	±0.33	–0.436	–2.619[a]	0.0238
X_4	±0.23	0.181	–1.412	0.1856
X_4^2	±0.33	–0.362	–2.175	0.0523
X_1X_2	±0.56	–0.362	–2.799[a]	0.0173
X_1X_3	±0.56	0.281	–2.178	0.0521
X_1X_4	±0.56	0.001	0.007	0.9943
X_2X_3	±0.56	–0.082	0.632	0.5403
X_2X_4	±0.56	–0.162	–1.292	0.2226
X_3X_4	±0.56	–0.184	–1.422	0.1827

Table 4
Analysis of Variance (ANOVA) for the Regression Model Representing CGTase Activity

Source	SS	DF	MS	*F*-value	*p*-value
Model	20,727	14	1480	554	0004
Residue	2940	11	0267	–	–
Lack of fit	2889	10	0289	550	0321
Pure error	0052	1	0052	–	–
Total	23,669	25	–	–	–

R2, 0.88; SS, sum of squares; DF, degrees of freedom; MS, mean square.

The model fitted the data well and gave a good coefficient of determination ($R^2 = 0.88$), explaining 88% of the variability in the response, the rest (12%) being explained by the residues.

The response surface described by model equation ($\hat{y}$) to estimate dependence of CGTase activity on the variables soluble starch concentration (X_2) and initial pH of medium (X_1) is shown in Fig. 2. This dependence suggests that when the soluble starch concentration is increased, the initial pH value should be decreased to obtain results that tend to maximize CGTase production. For the other studied variables, peptone and yeast extract, there was no significant interaction at the 5% probability level. So, for these variables, the concentrations keep at around the 0 point concentration (central point).

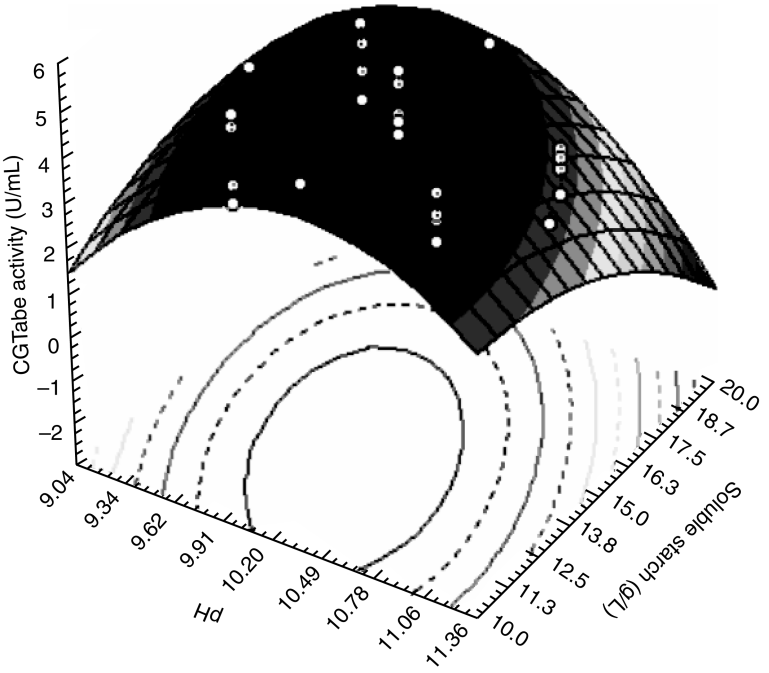

Fig. 2. Response surface described by the model $\hat{y}$, which represents CGTase activity (U/mL) produced by *B. clausii* strain E16 after a 48-h fermentation, as a function of soluble starch concentration and initial pH of growth medium.

The second-order polynomial model was significant in the region studied, the optimum coded values (optimum point) of the variables X_1, X_2, X_3, and X_4 being determined by Eq. 4, as proposed by Myers *(30)*:

$$x_0 = \mathbf{B}^{-1} \cdot \frac{\mathbf{b}}{2} \tag{4}$$

where x_0 is the vector of optimum values; **B** is a matrix of the estimated coefficients of quadratic terms and **b** is a vector of the estimated coefficients of linear terms.

The optimum coded values obtained from the experimental data by Eq. 4 are shown here as components of x_0 (Eq. 5):

$$x_0 = \begin{bmatrix} -0.170_{\text{pH}} \\ -0.616_{\text{soluble starch}} \\ -0.548_{\text{peptone}} \\ -0.527_{\text{yeast extract}} \end{bmatrix} \tag{5}$$

The coded values presented above correspond to initial culture medium pH 10.1; soluble starch 13.5 g/L; peptone 4.9 g/L; yeast extract 5.9 g/L. Under these conditions, the model predicts CGTase activity to be at a high

value of 4.64 U/mL, with a possible range from 4.09 to 5.19 U/mL at the 5% probability level. The decrease of CGTase production at higher concentrations of carbon source (soluble starch) was also found in *B. firmus* (Gawande et al., 1998) and *B. stearothermophilus* HR1 *(14)*, similar to our results.

The canonic form was obtained from the second-order polynomial model ($\hat{y}$) as follows:

$$\hat{y} = 4.64 - 0.786w_{\text{pH}}^2 - 0.518w_{\text{soluble starch}}^2 - 0.282w_{\text{peptone}}^2 - 0.165w_{\text{yeast extract}}^2 \quad (6)$$

In this equation, all of the coefficients are negative, indicating that the optimum point found is the maximum for the surface and thus the adjusted response surface model produces the highest results. Equation 6 may also indicate that any change in its values will cause a fall in CGTase activity. The greatest interference is caused by changes in initial pH and soluble starch concentration. The changes in peptone and yeast extract concentrations cause the least interference on CGTase activity.

Figures 3A and 3B are comparisons of growth and activity in the optimized culture medium found in this study with those achieved in the basal medium proposed by Nakamura and Horikoshi *(25)*. Optimized medium produces the highest CGTase activity (5.9 U/mL) after a 48-h fermentation as analyzed in this study. The biomass production peaked between 32 and 40 h (Fig. 3A). However, with basal medium, the highest CGTase activity was 3.5 U/mL after 48-h fermentation, and the maximum biomass production was obtained in a 24-h fermentation (Fig. 3B). These results indicated that it was possible to increase the CGTase activity by 68%, using the optimized medium, in relation to the original basal culture medium. Another important point was that the CGTase activity on base medium after a 48-h fermentation was similar to that obtained on optimized medium after a 24-h fermentation. This shorter time fermentation for the optimized medium can compensate the increase of the medium components and can lead to a decrease in production costs, because the equipments and energy sources are used only half of time when compared with the basal medium for obtaining the same CGTase activity.

Other authors have successfully improved enzyme production, using RSM. Gawande et al *(9)* working with *B. firmus*, obtained the maximum CGTase activity of 7.05 U/mL after a 80-h fermentation. The optimum composition of the culture medium was found at the central point of the 2^3 full-factorial design, made up of corn starch 21.0 g/L, yeast extract 23.0 g/L, and pharmamedia 22.0 g/L. According to the authors, these results led to an increase of about 20-fold in CGTase activity, compared with the basal medium. Gawande and Patkar *(8)* applied two-level fractional factorial designs to optimize medium composition, in the production of α-CD-specific CGTase from *K. pneumoniae* AS-22. The optimized medium resulted in ninefold higher production of CGTase than in the basal

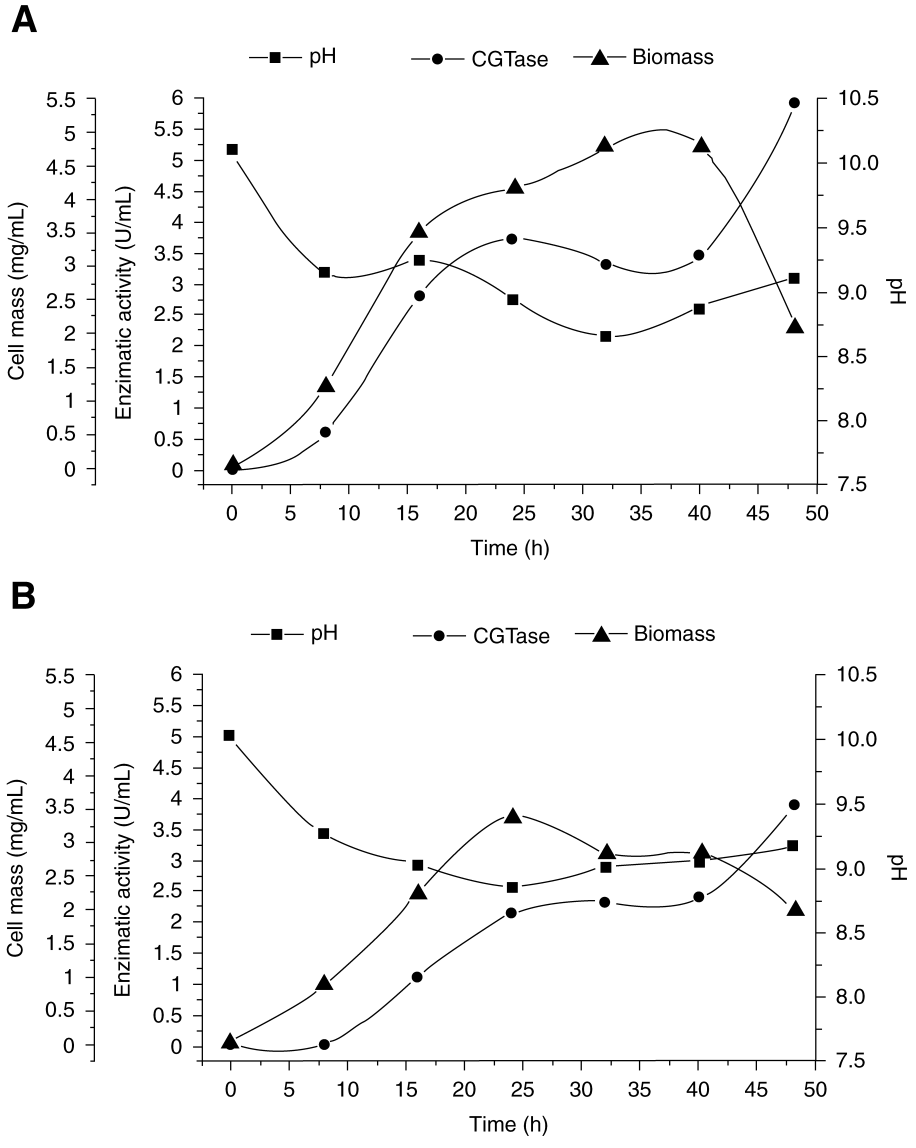

Fig. 3. CGTase production in **(A)** optimized medium and **(B)** basal medium by *B. clausii* strain E16 at 37°C. (—■—) pH; (—●—) enzymatic activity; (—▲—) biomass.

medium. The optimum composition of the nutrient medium was dextrin 49.3 g/L, peptone 20.6 g/L, yeast extract 18.3 g/L, ammonium dihydrogen orthophosphate 6.7 g/L, and magnesium sulfate 0.5 g/L. The CGTase production from *B. stearothermophilus* HR1 was analyzed through RSM, an optimized culture medium was performed, which was made up of sago starch 16.02 g/L, peptone from casein 20.0 g/L, K_2HPO_4 1.4 g/L, $CaCl_2$ 0.2 g/L, and initial pH 7.54. The maximum CGTase activity obtained was 14.20 U/mL *(14)*. Ibrahim et al. *(13)* studied the optimal concentration of the culture medium for CGTase production from *Bacillus* sp. G1 using

a 2^5 central composite design. In this case, the optimized medium increased the CGTase activity by 53% over the basal medium. The optimum composition of the culture medium was tapioca starch 40.0 g/L, peptone 20.0 g/L, $MgSO_4 \cdot 7H_20$ 4.0 g/L, and Na_2CO_3 10 g/L at pH 9.9.

Comparing the literature results, in relation to U/g of substrate, it was observed that better CGTase production was obtained by *Bacillus* sp. G1 (1372.5 U/g of tapioca starch) followed by *B. stearothermophilus* HR1 (886.4 U/g of sago starch), *B. clausii* strain E16 (437.0 U/g of soluble starch), *B. firmus* (434.1 U/g of corn starch), and *K. pneumoniae* AS-22 (335.7 U/g of dextrin). So, better conditions for CGTase production from *B. clausii* strain E16 could be obtained, if other variables such as starch source, inoculum quantities, and time were optimized.

Conclusion

A new CGTase producer was isolated and identified as *B. clausii* strain E16 and the compounds medium for better CGTase production was studied. The 2^4 full-factorial central composite design, with independent variables soluble starch, peptone and yeast extract concentrations, and culture medium initial pH were used to optimize the culture medium composition for CGTase production from *B. clausii* strain E16. The optimized medium resulted in a more than 68% higher CGTase production, compared with that in the basal medium. Optimum conditions found in this work were: soluble starch 13.4 g/L, peptone 4.9 g/L, yeast extract 5.9 g/L, K_2HPO_4 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, and initial pH 10.1 wherein this pH was obtained using Na_2CO_3 13.0 g/L sterilized separately. The study was carried out in shaker flasks at a 48-h fermentation. These results demonstrated that it is possible to apply statistical design to CGTase production, with a strategy RSM that is relatively simple, and saves both time and material.

Acknowledgments

The authors thank FAPESP for financial support.

References

1. Bender, H. (1986), *Adv. Biotechnol. Process.* **6,** 31–71.
2. Alves-Prado, H. F., Gomes, E., and DaSilva, R. (2002), *Bol. SBCTA.* **36,** 43–54.
3. Martin del Valle, E. M. (2004), *Process Biochem.* **39,** 1033–1046.
4. Hedges, A. R. (1998), *Chem. Rev.* **98,** 2035–2044.
5. Szejtli, J. (1997), *J. Mater. Chem.* **7,** 575–587.
6. Tonkova, A. (1998), *Enz. Microbial Technol.* **22,** 678–686.
7. Van der Veen, B. A., Uitdehaag, J. C. M., Dijkstra, B. W. E., and Dijkhuizen, L. (2000), *Biochim. Bioph. Acta* **1543,** 336–360.
8. Gawande, B. N. and Patkar, A. Y. (1999), *Biotechnol. Bioeng.* **64,** 168–173.
9. Gawande, B. N., Singh, R. K., Chauhan, A. K., Goel, A., and Patkar, A. Y. (1998), *Enz. Microbial Technol.* **22,** 288–291.
10. Ramakrishna, S. V., Saswathi, N., Sheela, R., and Jamuna, R. (1994), *Enz. Microbial. Technol.* **16,** 441–444.

11. Turnes, R. E. and Bahar, S. (1996), *Acta Científ. Venez.* **47,** 133–137.
12. Matioli, G., Zanin, G. M., and Moraes, F. F. (2002), *Appl. Biochem. Biotechnol.* **98–100,** 947–961.
13. Ibrahim, H. M., Yusoff, W. M. W., Hamid, A. A., Illias, R. M., Hassan, O., and Omar, O. (2005), *Process Biochem.* **40,** 753–758.
14. Rahman, R. A., Illias, R. M., Nawawi, M. G. M., Ismail, A. F., Hassan, O., and Kamaruddin, K. (2004), *Process Biochem.* **39,** 2053–2060.
15. Box, G. E. P., Hunter, W. G., and Hunter, J. S. (1978), *Statistics for Experimenters. An Introduction to Design, Data Analysis and Model Building*. John Wiley, New York.
16. Barros Neto, B., Scarminio, I. S., and Bruns, R. E. (1996), *Planejamento e Otimização de Experimentos*. 2nd ed., Editora da UNICAMP, Campinas.
17. Dey, G., Mitra, A., Banerjee, R., and Maiti, B. R. (2001), *Biochem. Eng. J.* **7,** 227–231.
18. Haltrich, D., Preiss, M., and Steiner, W. (1993), *Enz. Microbial. Technol.* **15,** 854–860.
19. Bocchini, D. A., Alves-Prado, H. F., Baida, L. C., Roberto, I. C., Gomes, E., and Dasilva, R. (2002), *Process Biochem.* **38,** 727–731.
20. Lee, S. L. and Chen, W. C. (1997), *Enz. Microbial. Technol.* **21,** 436–440.
21. Alves-Prado, H. F., Gomes, E. and DaSilva, R. (2002), *Brazilian J. Food Technol.* **98,** 189–196.
22. Sneath, P. H. A. (1986), In: Bergey's Manual of Systematic Bacteriology. Section 13, Williams &Wilkins, Baltimor, pp. 1105–1139.
23. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994), *Nucleic Acid Res.* **22,** 4673–4680.
24. Nakamura, N. and Horikoshi, K. (1976), *Agric. Biol. Chem.* **40,** 1785–1791.
25. Nakamura, N. and Horikoshi, K. (1976), *Agric. Biol. Chem.* **40,** 753–757.
26. Mäkelä, M. J., Korpela, T. K., Puisto, J., and Laakso, S. V. (1988), *Agric. Food Chem.* **36,** 83–88.
27. Alves-Prado, H. F., Gomes, E., and DaSilva, R. (2006), *Appl. Biochem. Biotechnol.* **129–132,** 234–246.
28. Alves-Prado, H. F. (2000), *M.Sc. Dissertation,* UNESP, Rio Claro, Brazil, pp. 144.
29. Nielsen, P., Fritze, D., and Priest, F. G. (1995), *Microbiology* **141,** 1745–1761.
30. Myers, R. H. (1971), Response surface methodology. Allyn and Bacon, Boston.