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ORIGINAL ARTICLE

Bacillus lehensis—an alkali-tolerant bacterium isolated from cassava starch wastewater: optimization of parameters for cyclodextrin glycosyltransferase production

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Abstract A Gram-positive, endospore-forming, alkali-tolerant bacterial strain, designated CGII, was isolated from the wastewater of a cassava flour mill in the state of São Paulo, Brazil and submitted to phylogenetic studies and biochemical tests. The 16S rRNA gene sequence indicated the highest degree of genomic similarity with MLB2 strains of *Bacillus lehensis* (100%). A two-level central composite rotatable design was then employed to optimize the medium composition and culture conditions for the production of the enzyme cyclodextrin glycosyltransferase (CGTase) in shake-flask and bioreactors. CCTase activity was measured under different production conditions, such as culture medium, agitation and aeration. Highest enzyme production by *B. lehensis* was achieved in 72 h with a maximal activity of 134.05 U mL^{-1}. The response surface method demonstrated that the proposed model achieved a good level of agreement with experimental data, with a correlation coefficient of 0.910, thereby confirming the adequate reliability of the model.

Keywords Cyclodextrin glycosyltransferase · *Bacillus lehensis* · Cassava starch wastewater · CGTase production

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Introduction

There has been increasing interest in alkaliphilic microorganisms due to their ability to grow under extreme conditions and the use of their enzymes in biotechnological applications. The genus *Bacillus* includes species characterized as alkaliphilic, many of which have been studied with a view to finding industrial applications (Horikoshi et al. 1991)

Cyclodextrins (CDs) produced by the enzyme cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) have broad, non-toxic applications in the pharmaceutical, cosmetic and food industries (Del Valle 2004). CGTase is an enzyme that catalyzes four associated reactions: cyclizing, coupling, disproportionation and hydrolyzing (Vassileva et al. 2005). For industrial applications, the cyclizing reaction of CGTases converts starch and related α-1,4-glucans into CDs (Terada et al. 2001; Vassileva et al. 2005). CGTase production depends on the control of parameters in the medium and the environment, which are fundamental to achieving high CGTase activity and, consequently, greater CD production (Albers and Muller 1992). CGTases with different properties are produced by bacteria belonging mainly to *Bacillus* strains (Table 1), and the amino acid sequence of several CGTases has been elucidated from the DNA sequence (Goh et al. 2009).

There are several ways to reduce the costs of CGTase production, such as changing fermentation conditions (agita-

Table 1 Several important producers of cyclodextrin glycosyltransferase (CGTase) and enzymatic activity

Producer	CGTase activity (U mL^{-1})	Reference
Bacillus megaterium	56.1	Ahmed et al. 2010
Alkalophilic *Bacillus* sp.	56.0	Kuo et al. 2009
Bacillus sp. TS1-1	84.0	Mahat et al. 2004
Bacillus firmus	7.05	Gawande et al. 1998
Bacillus G1	54.9	Ibrahim et al. 2005
Bacillus stearothermophilus HR1	14.2	Rahman et al. 2004
Bacillus lehensis	18.9	Yap et al. 2010

tion and aeration) to improve diffusion of the substrate in the medium, or using a new microorganism such as *Bacillus lehensis*. The accessibility of cassava starch and cassava flour mills in Brazil is an incentive for CGTase production, as cassava starch constitutes a low-cost substrate.

High yield CDs offer new applications, such as steroid complexes, allowing new uses of this important compound by forming inclusion structures in the aqueous or molecular phase and complexing organic molecules into cavities known as host–guest complexes (McMullan et al. 1973; Banerjee et al. 2004). Conventional mechanisms of organic molecule complexes, such as solution, suspension, maceration, co-precipitation and freeze drying, are currently the most used (Al-Soufi et al. 2003).

Response surface methodology (RSM) is a statistical model generally used to study the combined effect of certain variables and to optimize conditions in a multivariable system. In the present study, RSM supported on a rotational central composite design (RCCD) was used for the optimization of CGTase production by a previously isolated and identified strain of *Bacillus lehensis*, designated as CGII.

Materials and methods

Characterization of the isolated bacterium

This paper describes an alkaliphilic ellipsoidal spore-forming bacterium, designated CGII, isolated from wastewater samples from a cassava flour mill in the city of Ribeirão Bonito, Brazil (1,100 m above sea level; temperature: 30°C). Characterization of CGTase produced by the strain indicated optimal activity at pH 9.2.

Colony morphology was observed by studying the growth of the strain on tryptic soy agar at 35°C for 24 h. Colony characteristics (color, form, surface and texture), and cell morphology was investigated by means of light microscopy (Bel Photonics) at a magnification of ×1,000 (shape, rearrangement, presence of spores, spore shape). Motility was checked using the method described by Skerman (1967). The Gram reaction was determined using a Gram-staining kit according to the manufacturer´s instructions. Growth at different NaCl concentrations (2.5, 10 and 15%) was studied as described by Holt (1994) and Koneman et al. (1992)

The strain CGII was studied for citrate decarboxylation, decomposition of different substances, production of metabolites. Assimilation of substrates (Table 3) was performed according to Gordon et al. (1973).

Bacterial DNA was extracted and purified according to a protocol recommended by Genomic Prep Cells and Tissue DNA Isolation kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The gene was amplified symmetrically with primers p27f and p1525f. PCR was performed by adding 0.5 g DNA; 1.5 μl p27f primer; 1.5 μl p1525r primer and 18 μl ultrapure water. The sequence was resolved in a sequencer (ALFexpress, Pharmacia), 16S rRNA sequences were edited and aligned using BioEdit program (Hall 1999) and a neighbour-joining tree was constructed by the program MEGA 4 (Tamura et al. 2007).

Production of CGTase

Bacillus lehensis grown and maintained in a nutrient medium (Nakamura and Horikoshi 1976) was cultivated for 72 h on 100 mL culture medium in a shaker (150 rpm), at 35±0.1°C, and an initial pH of 9.2 adjusted with sterile sodium carbonate (1 g L^{-1}).

A central composite rotational design (CCRD) was used in the optimization of CGTase production. The experiments were performed in 500 mL Erlenmeyer flasks containing 100 mL production medium kept on a rotary shaker at an agitation rate of 150 rpm for 72 h at 35±1°C. The production medium consisted of the same salts used in the growth medium, with the addition of cassava starch (0.58–1.42 g L^{-1}, w/v), nitrogen sources (0.58 to 1.42 g L^{-1}, w/v) and sodium carbonate (0.66–2.34 g L^{-1}, w/v). After choosing the best culture medium composition for CGTase production, a second CCRD was employed to optimize agitation (100–250 rpm) and aeration rate (0.5–2.25 vvm). The CCRD was carried out in a 5-L stirring tank reactor (Bio-t-mini Zeta, Rapperswil, Switzerland) containing 2 L production medium. Fermentations were run for 72 h at 35±1°C and samples were withdrawn every 24 h for 72 h. All experiments were carried out in triplicate.

CGTase assay

Enzymatic activity was measured based on the discoloration of phenolphthalein solutions, following the method of Makela et al. (1988). The enzyme was incubated in 1% (w/v) starch potato in 50 mM Tris-HCl buffer, pH 8.0, at 55°C. Reaction samples were withdrawn at 0, 3, 6, 9 and 12 min, and the enzymes inactivated at 100°C for 5 min. Total CD content was determined by absorption at 550 nm in a solution containing phenolphthalein (3 mM) and carbonate sodium buffer (600 mM). One unit of CGTase activity was defined as the amount of enzyme that produces 1 μmol β-CD min^{-1}.

Experimental design

The first experimental CCRD was carried out to identify and optimize nutrients in the production medium (cassava starch, nitrogen sources and sodium carbonate) that had a significant effect on CGTase production. For two factors, this design was made up of a full 2^3 factorial design, with eight cube points augmented with three replications of the center points and six star points, i.e., points with an axial distance to the center of ± α (1.68) for one factor, while the other factor is at level 0. To estimate the optimal point, a second-order polynomial function was fitted to the experimental results. Thus, the influence of all experimental variables, factors and interaction effects on the response was investigated. The objective of the second experiment was to obtain a more precise estimate of the optimal operating conditions for the factors involved. Thus, a central composite circumscribed 2^2 experimental design was used, with two variables (agitation and aeration rate), four star points (±α=1.41) and three replicates at the center point, resulting in a total of 11 experiments. The independent variables, experimental range and levels investigated for both CCRDs are given in Table 2.

Table 2 Experimental range and levels of independent variables used in central composite design

Independent variables		Range and levels				
		−α	−1	0	1	+α
Production medium optimization (g L^{-1})						
Cassava starch	Y_1	3.3	5.0	7.5	10	11.7
Nitrogen sources	Y_2	3.3	5.0	7.5	10	11.7
Sodium carbonate	Y_3	5.8	7.5	10.0	12.5	14.2
Optimal operating conditions						
Aeration (vvm)	Z_1	1.29	1.5	2	2.5	2.7
Agitation (rpm)	Z_2	79.5	100	150	200	220.5

Statistical data analysis

The software package Statistica v. 7.0 (StatSoft, Tulsa, OK) was used for the experimental design, data analysis and quadratic model building. Response surfaces were generated to understand the interaction of different variables. The optimal points for the variables were obtained using the Maple v. 9.5 program (Waterloo Maple, Ontario, Canada).

Results and discussion

Strain identification and characterization

Strain CGII is Gram-positive, aerobic, motile, rod-shaped, spore-forming, and positive for catalase and oxidase. The results shown in Table 3 were confirmed for lecithinase and arginine, and decomposition of starch, nitro-phenylglycoside, nitro-phenylphosphate, nitro-phenylcellobioside, sodium chloride and sodium acetate, growth on NaCl (2.5, 10 and 15%) and glycoside, lactose, rhamnose, isoleucine, lysine, maltotriose, methyl-β-glycoside, leucine, proline and fructose assimilation.

The 16S rRNA sequence was deposited with the National Center for Biotechnology Information (NCBI) GenBank under the accession number HQ399547. The sequence was aligned with those of *Bacillus* reference species available in the NCBI GenBank database and compared with closely related sequences retrieved from the NCBI GenBank database. A clade was formed with *Bacillus lehensis*, with a bootstrap value of 100%, and *Bacillus oshimensis*, with 98%.

The evolutionary history was inferred using the neighbor-joining method of phylogenetic analyses conducted in MEGA 4 (Tamura et al. 2007), and the position in the 16S rRNA *Bacillus* tree is shown in Fig. 1. The optimal tree with the sum of branch length=0.11179654 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site.

First central composite design

Table 4 shows the results obtained from the (CCRD) regarding the variables studied: concentrations of cassava starch (Y_1), nitrogen source (50% tryptone and 50% yeast extract) (Y_2) and sodium carbonate (Y_3). The initial CGTase activity in medium consisting of 15 g L^{-1} soluble starch, 5 g L^{-1} peptone, 5 g L^{-1} yeast extract, 1 g L^{-1} K_2HPO_4 and 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$ was 53.17 g L^{-1}. The highest enzyme production obtained on central composite

Table 3 Physiological and biochemical characteristics of strain CGII isolate from the wastewater of a cassava flour mill in Brazil

Characteristic	Result[a]	Characteristic	Result[a]
Decarboxylation of:		Assimilation of:	
Citrate	–	Arabinose	–
Decomposition of:		Ribose	–
Tyrosine	–	Glycoside	+
Casein	–	Galactose	–
Tetrazolium red	–	Inositol	–
Urea	–	Lactose	+
Esculin	–	Maltose	–
Starch	+	Mannitol	–
Gelatin	–	Raffinose	–
Tween	–	Rhamnose	+
Potassium thiocyanate	–	Salicin	–
Potassium gluconate	–	Sorbitol	–
Phosphate	–	Trehalose	–
Nitro-phenyl glycoside	+	Tagatose	–
Nitro-phenyl phosphate	+	Isoleucine	+
Nitro-phenyl cellobioside	+	Lysine	+
Sodium chloride	+	Maltotriose	+
Sodium acetate	+	Methyl-β-glycoside	+
Glycerol	–	Leucine	+
Nitro-phenyl maltoside	–	Proline	+
Growth on:		Ornithine	–
NaCl	–	Inulin	–
Production of:		Glucosaminide	–
Nitrate reductase	–	Inositol	–
Lecithinase	+	Glucose	–
Phenylalanine desaminase	–	Fructose	+
Indol	–	Arabinose	–
Tryptophan	–	Amylopectin	–
Arginine	+	Saccharose	–
Potassium ketogluconate	–	Dulcitol	–
Oleandomycin	–	Sorbose	–
Mandelic acid	–	Gentiobiose	–
Erythritol	–		

[a] - Negative; + positive

design was 97.38 U mL^{-1} (as seen in run 17). Thus, an increase in enzyme activity was established after the use of experimental design.

The adjusted empirical model obtained for enzyme activity, containing only the significant parameters ($P \leq 0.05$) determined using a Student's t-test, is represented by Eq. 1 (EA_1).

$$EA_1 = 97.17 - 5.55Y_1 + 7.73Y_2 - 8.04Y_3 - 15.02{Y_1}^2 - 18.15{Y_2}^2 - 17.99{Y_3}^2 \quad (1)$$

Table 5 displays Student's *t*-distribution, corresponding values, estimated parameters and the coefficient of correlation (R^2). The probability (P) values were used as a tool to check the significance of each of the coefficients. A larger magnitude of the t-test and smaller P-value denote greater significance of the corresponding coefficient (Dobbins 1994). The goodness-of-fit of the model was checked by an R^2 of 96 (Gawande and Patkar 2001). In this case, the R^2 value (0.968) for Eq. 1 indicates that the sample variation for enzyme activity of 96.8% was attributed to the independent variables and only 3.2% of the total variation cannot be explained by the model. The adjusted R^2 of 0.937 was also satisfactory for confirming the significance of the model.

The results show that the independent variables Y_1 and Y_3 had a significant effect, as demonstrated by the negative coefficient (Table 5), with a decrease in their concentration

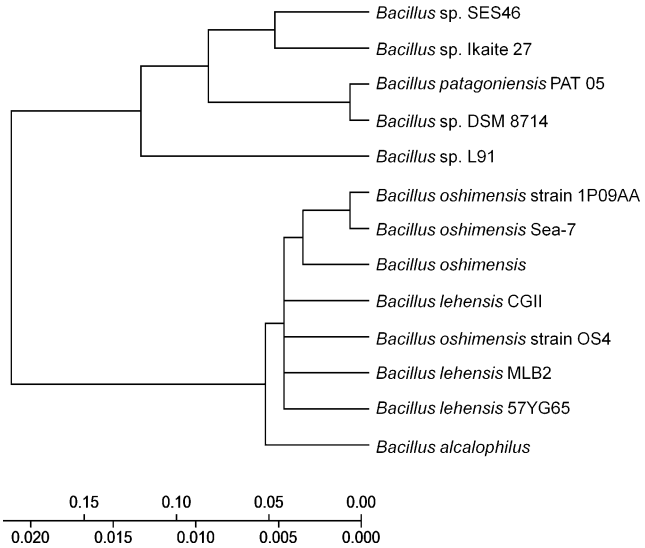

Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strains CGII and the species of *Bacillus*. *Bar* 0.005 and 0.05 replacements per nucleotide position

leading to an increased yield. The use of a high concentration of a carbon source (Y_1) led to catabolite repression and reduced enzyme yield, whereas high concentrations of Na_2CO_3 (Y_3) induced repression and cell death.

Table 4 Central composite rotational design (CCRD) for optimization of three variables in experimental values for enzymatic activity by isolated *Bacillus lehensis* CGII

Run	Y_1[a]	Y_2[b]	Y_3[c]	Enzymatic activity (g L^{-1})
1	5	5	7.5	53.12
2	5	5	12.5	36.89
3	5	10	7.5	57.55
4	5	10	12.5	44.27
5	10	5	7.5	38.37
6	10	5	12.5	20.66
7	10	10	7.5	56.07
8	10	10	12.5	36.89
9	3.3	7.5	10	69.65
10	11.7	7.5	10	48.25
11	7.5	3.3	10	32.31
12	7.5	11.7	10	67.88
13	7.5	7.5	5.8	63.45
14	7.5	7.5	14.2	37.62
15	7.5	7.5	10	95.32
16	7.5	7.5	10	97.34
17	7.5	7.5	10	97.38

[a] Cassava starch concentration

[b] Nitrogen source concentration

[c] Sodium carbonate concentration

The results show that variable Y1 (cassava starch) had a significant effect, based on *P*-values lower than 0.05. This variable has a positive coefficient—an increase in its concentration results in increased production yield. The non-significant terms (Y_1Y_2, Y_1Y_3 and Y_2Y_3) were discarded.

An algorithm in the Maple v. 9.5 program was applied to calculate the stationary point (P_0) for CGTase production. The l values (l_1= −14.51, l_2= −18.54 and l_3= −18.01) indicate that these responses have a maximal point, as they have equal and negative signs. In this case, the optimal medium composition for CGTase production by the isolated *B. lehensis* was 6.96 g L^{-1} cassava starch, 8.07 g L^{-1} nitrogen sources and 9.45 g L^{-1} sodium carbonate, with a maximal predicted production of 98.87 g L^{-1}. As expected, this value was very close to that obtained in experiments 15, 16 and 17, as the variable of the maximization point (enzymatic activity) was close to the central point.

The response surface plot is a graphical representation of the regression equation. It is plotted to understand the interactions of the medium components and optimum concentration of each component (cassava starch, nitrogen sources and sodium carbonate) required for CGTase production (Fig. 2a–c).

The range of greater enzymatic activity (97.38 g L^{-1}) is located close to the central point, between 6 and 8.5 g L^{-1} cassava starch, with 7–9 g L^{-1} nitrogen sources and 9–10 g L^{-1} sodium carbonate (Fig. 2a). The convex response surfaces suggest that there are well-defined optimal variables. If the surfaces are rather symmetric and flat near the optimum, the optimized values may not vary widely from single variable conditions.

The nitrogen sources and sodium carbonate are the dominant variables in CGTase production (Fig. 2c). Hence, a strong interaction between them during fermentation is inevitable. Maximal enzymatic activity occurred when the nitrogen sources and sodium carbonate were near 8 g L^{-1} and 9.4 g L^{-1}, respectively. Therefore, there were no benefits to CGTase production with greater or lesser nitrogen sources and sodium carbonate concentrations.

Second central composite design

The design matrix of the variable in coded units is displayed together with experimental results in Table 6. The highest enzymatic activity achieved in the verification experiment was 134.05 U mL^{-1}, which was very close to the value predicted by the model (135.41 U mL^{-1}), with 2 vvm and 150 rpm (as seen in run 11). The response surface quadratic model was performed in the form of analysis of variance (ANOVA) and the results are summarized in Table 7.

Table 5 Model coefficient estimated by linear regression ($R^2 = 0.968$, adjusted $R^2 = 0.937$)

Factor	Coefficient	Standard error	Computed *t*-value	*P*-value
Intercept	97.168	3.605	26.950	0.000
X_1[a]	−5.552	1.693	−3.279	0.013
X_2[b]	7.729	1.693	4.565	0.003
X_3[c]	−8.043	1.693	−4.750	0.002
X_1^2	2.765	2.212	1.249	0.251
X_2^2	−0.922	2.212	−0.417	0.689
X_3^2	0.185	2.212	0.083	0.935
X_1X_2	−15.022	1.863	−8.061	0.000
X_1X_3	−18.153	1.863	−9.741	0.000
X_2X_3	−17.997	1.863	−9.657	0.000

[a] Cassava starch
[b] Nitrogen sources
[c] Sodium carbonate

ANOVA of the quadratic regression model demonstrates that the model is very significant, as is evident from the results of Fisher's *F*-test ($F_{(5.5)}=S_m^2/S_S^2=11.25>Ft_{(5.5)}=5.05$), with a low probability value [$(P_{model} > F) < 0.009$], which indicates that the model is significant. The goodness-of-fit of the model was checked by determination coeffi-

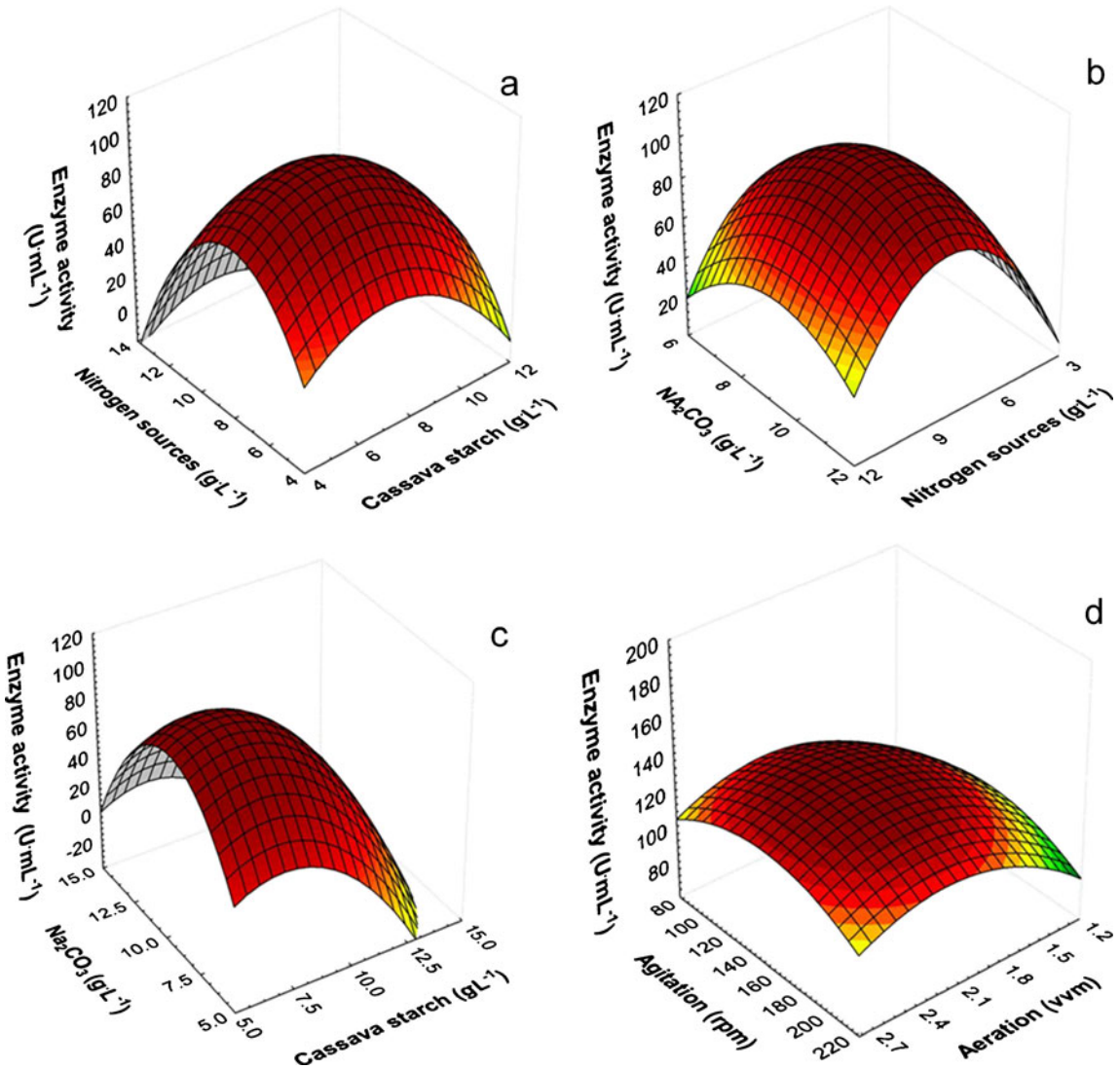

Fig. 2 Response surface plot showing the simultaneous effect on enzymatic activity of **a** cassava starch and nitrogen sources concentration; **b** nitrogen sources and Na_2CO_3 (sodium carbonate) concentrations; **c** cassava starch and Na_2CO_3 concentrations; and **d** aeration and agitation

Table 6 Average results for enzymatic activity by isolated *B. lehensis* CGII under different aeration and agitation conditions

Run	Z_1[a]	Z_2[b]	Enzymatic activity (U mL^{-1})
1	1.50	100	57.16
2	1.50	200	52.48
3	2.50	100	83.52
4	2.50	200	64.68
5	1.29	150	38.92
6	2.70	150	86.88
7	2.00	79.5	39.36
8	2.00	220.5	84.84
9	2.00	150	130.36
10	2.00	150	133.22
11	2.00	150	134.05

[a] Aeration (vvm)

[b] Agitation (rpm)

cient (R^2) and the multiple correlation coefficient (R). In this case, the value of R^2 (0.918) indicates that the sample variation of 91.8% for CGTase was attributed to the independent variables, and only 8.2% of the total variation cannot be explained by the model.

The adjusted R^2 of 0.856 was also satisfactory for confirming the significance of the model. The determination of the significant parameters was performed through a hypothesis test (Student's *t*-test), with a 5% level of significance. The lack of a fit test (0.028), which measures the fitness of the model, results in a significant F value.

Parameters with a level of significance higher than this value were dismissed. The empirically adjusted equation (EA_2), which represents enzymatic activity, is described in Eq. 2:

$$EA_2 = 133.93 + 13.29Z_1 - 35.03{Z_1}^2 - 35.42{Z_2}^2 \qquad (2)$$

in which EA_2 is the predicted response (enzymatic activity) and Z_1 and Z_2 are the coded values of the test variables aeration and agitation, respectively.

In Eq. 2, the isolated variable Z_1 and quadratic variables ${Z_1}^2$ and ${Z_2}^2$ significantly influence the process. A positive Z_1 and negative ${Z_1}^2$ and ${Z_2}^2$ demonstrate that an increase in aeration and a reduction in agitation lead to an increase in enzymatic activity. The coordinates of the stationary points for CGTase production were calculated using the Maple v. 9.5 program: $Z_1 = 0.186$ and $Z_2 = 0.062$. The l values referring to aeration and agitation indicate that these responses have a maximal point, as they have equal and negative signs. Aeration and agitation were 2.1 vvm and 153 rpm, respectively, on the optimization point from the codified variable values for Z_1 (0.1865) and Z_2 (0.0625). The maximal predicted value of enzymatic activity was 135.41 U mL^{-1}. To confirm the adequacy of the model for predicting maximal enzymatic activity, three additional experiments were performed in a fermentor with this optimal medium composition. The mean value of CGTase production was 136.63 U mL^{-1}, which is in excellent agreement with the predicted value (135.41 U mL^{-1}), with a difference of just 0.9%. Thus, the model was proved adequate. Figure 2a–c displays CGTase production under optimized conditions. The final medium composition optimized with RSM was 6.96 g L^{-1} cassava starch, 8.07 g L^{-1} nitrogen sources, 9.45 g L^{-1} sodium carbonate, 1 g L^{-1} K_2HPO_4 and 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$, with 2.1 vvm of aeration, 153 rpm of agitation and a control pH of 9.2.

The surface plot (Fig. 2d) demonstrated an increasing trend for CGTase enzyme with intermediate values of aeration (1.8–2.2 vvm) and agitation (140–160 rpm). Higher or lower levels of aeration and agitation did not favor enzyme production.

Kulpreecha et al. (2009) used various carbon sources such as rice, corn and soluble starch for the synthesis of CGTase. In the present study, we report the use of cassava starch for the first time in fermentor studies for the synthesis of CGTase and as a carbon source in the medium. Cassava starch has previously been used for CGTase production only as a carbon source (Blanco et al. 2009).

The complex mixture of tryptone and yeast extract used as a nitrogen source in the medium led to an increase in enzymatic activity and influenced the fermentation, as supported by the response surfaces. The pH range for CGTase production is 6–10 (Matioli et al. 2000). Sodium carbonate was used to control the pH of the medium (9.2) for the best enzymatic activity. In all tests, the best enzyme activity was achieved in 72 h of fermentation. Highest values of enzymatic activity (134.05 U mL^{-1}) were obtained when the batch run was carried out at 150 rpm (Table 6). At higher agitation rates (> 150 rpm), there was a

Table 7 Analysis of variance for quadratic model ($R^2 = 0.918$; adjusted $R^2 = 0.856$)

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	Probe >*F*
Model	12,503.40	5	2,500.68	11.25	0.009
Residual	1,110.85	5	222.17		
Lack-of-fit	2,744.44	5	548.88	34.68	0.028
Pure error	31.65	2	15.82		
Total	13,614.24				

decrease in cell growth, stress generated and enzymatic activity. However, a decrease in agitation in relation to central point (150 rpm) led to lower enzyme production. The cumulative effect of all influencing variables for CGTase production was similar to the observations reported by Zain et al. (2007) and Mcmullan et al. (1973) in the cultivation of alkaliphilic *Bacillus* sp. and *Bacillus circulans* ATCC. Enzymatic activity values (130.36, 133.22 and 134.05 U mL^{-1}) were highest when the cells grew at 2 vvm (Table 6). In the bioreactor, enzymatic activity diminished when high aeration rates (2.5 and 2.7 vvm) were used together with agitations of 100, 150 and 200 rpm. Rates less than the central point also resulted in a decrease of enzymatic activity, perhaps caused by the higher O_2 demand required by the bacterial cells.

After the RSM optimization process, three different fermentation times were used to check the kinetics of CGTase production in the fermentor. The maximal CGTase production using the shake-flask method was 97.38 U mL^{-1} and increased to 134.05 U mL^{-1} in the fermentor in 72 h. The present paper reports the use of cassava starch as an alternative carbon source in a fermentor for CGTase synthesis. De Freitas et al. (2004) used a range of carbon sources, such as rice, corn and soluble starch, for the synthesis of CGTase (de Freitas et al. 2004). Moreover, lower concentrations of nitrogen sources were required for optimal CGTase production. RSM revealed that nitrogen sources and cassava starch were the limiting nutrients for the synthesis of CGTase, whereas, in fermentor studies, dissolved oxygen and agitation speed affected the nutrient supply for the synthesis of CGTase. In the trials, oxygen deprivation was responsible for lower CGTase synthesis. *Klebsiella pneumoniae* AS-22 has been studied for CGTase production, with combined aeration and three agitation intervals; the synthesis of CGTase was better with higher levels of agitation (Gawande and Patkar 2001).

Although other components of the culture medium, such as nitrogen sources, also remain cheaper for the synthesis of CGTase in fermentation flasks using RSM, nitrogen sources and cassava starch were found to be the limiting nutrient for the synthesis of CGTase during fermentation studies using RSM, in which dissolved oxygen and agitation proved to affect the use of these nutrients with regard to CGTase synthesis. CGTase production increased under ideal conditions of agitation and aeration with controlled pH. These results suggest that the CGII strain isolated from wastewater efficiently synthesizes CGTase using cassava starch as a carbon source and a small amount of nitrogen sources. This study demonstrated the production of CGTase using a cheap nutrient, nitrogen sources and sodium carbonate. Moreover, the strain isolated can use starchy industrial waste for CGTase production, making this process cheaper and more efficient for application in large-scale industrial production.

A scale-up CGTase fermentation using the optimized medium was carried out in the bioreactor (3 L). After 72 h of fermentation, production reached 134.05 U mL^{-1} while maximum CGTase production in shake-flask was 97.38 U mL^{-1}.

Conclusion

The best result for CGTase production (134.05 U mL^{-1}) under optimized conditions was obtained after 72 h with 6.96 g L^{-1} cassava starch, 8.07 g L^{-1} nitrogen sources, 9.45 g L^{-1} sodium carbonate, 1 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2.1 vvm aeration and 153 rpm agitation. Thus, cassava starch proved to be a potential carbon source for CGTase production by *B. lehensis*. The results indicated that production increased with the aeration and agitation rate; an overall increase of 38% was achieved.

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References

Ahmed EM, El-Refai HA (2010) Cyclodextrin glucosyltransferase production by *Bacillus megaterium* NCR: evaluation and optimization of culture conditions using factorial design. Indian J Microbiol 50:303–308. doi:10.1007/s12088-010-0009-x

Albers E, Muller BW (1992) Complexation of steroid-hormones with cyclodextrin derivatives—substituent effects of the guest molecule on solubility and stability in aqueous-solution. J Pharm Sci 81:756–761

Al-Soufi A, Cabrer PR, Jover A, Budal RM, Tato JV (2003) Determination of second-order association constants by global analysis of h-1 and c-13 nmr chemical shifts. Application to the complexation of sodium fusidate and potassium helvolate by beta- and gamma-cyclodextrin. Steroids 68:43–53

Banerjee R, Chakraborty H, Sarkar M (2004) Host-guest complexation of oxicam saids with beta-cyclodextrin. Biopolymers 75:355–365. doi:10.1002/Bip.20147

Blanco KC, Lima CJB, Oliveira PAPLV, Pião ACS, Contiero J (2009) Cyclodextrin glicosiltransferase production by *Bacillus* sp. subgroup alcalophilus using a central composite design. Res J Microbiol 4:450–459

de Freitas TL, Monti R, Contiero J (2004) Production of CGTase by a *Bacillus* alkalophilic CGII strain isolated from wastewater of a manioc flour industry. Braz J Microbiol 35:255–260

Del Valle EMM (2004) Cyclodextrins and their uses: a review. Process Biochem 39:1033–1046. doi:10.1016/S0032-9592(03)00258-9

Dobbins DC (1994) The use of parametric statistics in biological treatability studies. J Air Waste Manage Assoc 44:1226–1229

Gawande BN, Patkar AY (2001) Purification and properties of a novel raw starch degrading-cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* as-22. Enzyme Microb Technol 28:735–743

Gawande BN, Singh RK, Chauhan AK, Goel A, Patkar AY (1998) Optimization ofcyclomaltodextrin glucanotransferase production from *Bacillus firmus*. Enzyme Microb Technol 22:288–291

Goh KM, Mahadi NM, Hassan O, Rahman RNZRA, Illias RM (2009) A predominant beta-CGTase g1 engineered to elucidate the relationship between protein structure and product specificity. J Mol Catal B Enzym 57:270–277. doi:10.1016/j.molcatb.2008.09.016

Gordon R, Webster JM, Hislop TG (1973) Mermithid parasitism, protein turnover and vitellogenesis in desert locust, *Schistocerca gregaria* (Forskal). Comp Biochem Physiol 46:575–593

Hall TA (1999) Bioedit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/nt. Nucleic Acids Symp Ser 41:95–98

Holt JG (1994) Bergey's manual of determinative bacteriology. Williams and Wilkins, Philadelphia

Horikoshi S, Mccune BK, Ray PE, Kopp JB, Sporn MB, Klotman PE (1991) Water-deprivation stimulates transforming growth-factor-beta-2 accumulation in the juxtaglomerular apparatus of mouse kidney. J Clin Invest 88:2117–2122

Ibrahim HM, Yusoff WMW, Hamid AA, Illias RM, Hassan O, Omar O (2005) Optimization of medium for the production of β-cyclodextrin glucanotransferase using Central Composite Design (CCD). Process Biochem 40:753–758

Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC Jr (1992) Koneman's color atlas and textbook of diagnostic microbiology. Lippincott-Raven, Philadelphia

Kulpreecha S, Boonruangthavorn A, Meksiriporn B, Thongchul N (2009) Inexpensive fed-batch cultivation for high poly(3-hydroxybutyrate) production by a new isolate of *Bacillus megaterium*. J Biosci Bioeng 107:240–245. doi:10.1016/j.jbiosc.2008.10.006

Kuo CC, Lin CA, Chen JY, Lin MT, Duan KJ (2009) Production of cyclodextrin glucanotransferase from an alkalophilic *Bacillus* sp. by pH-stat fed-batch fermentation. Biotechnol Lett 31:1723–1727. doi:10.1007/s10529-009-0071-9

Mahat MK, Illias RM, Rahman RA, Rashid NAA, Mahmood NAN, Hassan O, Aziz SA, Kamaruddin K (2004) Production of cyclodextrin glucanotransferase (CGTase) from alkalophilic *Bacillus* sp. TS1-1: media optimization using experimental design. Enzyme Microb Technol 35:467–473

Makela M, Mattsson P, Pintamokenttala K, Korpela T (1988) Nonchromatographic cyclodextrin assays: evaluation of sensitivity, specificity, and conversion mixture applications. J Agric Food Chem 36:83–88

Matioli M, Zanin G, Moraes FF (2000) Ciclodextrinas e suas aplicações em: Alimentos, fármacos, cosméticos, agricultura, biotecnologia, química analítica e produtos gerais. Eduem, Maringá, Brazil

McMullan RK, Saenger W, Fayos J, Mootz D (1973) Topography of cyclodextrin inclusion complexes. Classification of crystallographic data of alpha-cyclodextrin inclusion complexes. Carbohydr Res 31:37–46

Nakamura N, Horikoshi K (1976) Characterization and some cultural conditions of a cyclodextrin glycosyltransferase-producing alkalophilic *Bacillus* sp. Agric Biol Chem 40:753–757

Rahman RA, Illias RM, Nawawi MGM, Ismail AF, Hassan O, Kamaruddin K (2004) Optimisation of growth medium for the production of cyclodextrin glucanotransferase from *Bacillus stearothermophilus* HR1 using response surface methodology. Process Biochem 39:2053–2060

Skerman VBD (1967) A guide to the identification of the genera of bacteria. Williams and Wilkins, Baltimore

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 101:11030–11035. doi:10.1073/pnas.0404206101

Tamura K, Dudley J, Nei M, Kumar S (2007) Mega4: Molecular evolutionary genetics analysis (mega) software version 4.0. Mol Biol Evol 24:1596–1599. doi:10.1093/molbev/msm092

Terada Y, Sanbe H, Takaha T, Kitahata S, Koizumi K, Okada S (2001) Comparative study of the cyclization reactions of three bacterial cyclomaltodextrin glucanotransferases. Appl Environ Microbiol 67:1453–1460

Vassileva A, Beschkov V, Ivanova V, Tonkova A (2005) Continuous cyclodextrin glucanotransferase production by free and immobilized cells of bacillus circulans atcc 21783 in bioreactors. Process Biochem 40:3290–3295. doi:10.1016/j.procbio.2005.03.022

Yap PW, Ariff AB, Woo KK, Hii SL (2010) Production of cyclodextrin glycosyltransferase (CGTase) by *Bacillus lehensis* S8 using sago starch as carbon source. J Biol Sci 10:676–681. doi:10.3923/jbs.2010.676.681

Zain WSWM, Illias RM, Salleh MM, Hassan O, Rahman RA, Hamid AA (2007) Production of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. ts1-1: optimization of carbon and nitrogen concentration in the feed medium using central composite design. Biochem Eng J 33:26–33. doi:10.1016/j.bej.2006.09.024