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RESEARCH ARTICLE

Cyclodextrin Glucanotransferase Activity of Microbial Strains Isolated from Yam-Cultivation Soils in Northwestern Benin

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Abstract: β-Cyclodextrin, an industrially important oligosaccharide, is produced by microbial enzymes such as cyclodextrin glucanotransferase (CGTase). This study investigated ways to optimize the enzymatic activity of CGTase from *Bacillus cereus*, *Bacillus pumilus*, *Bacillus brevis*, and *Bacillus subtilis* strains isolated from soils in north-western Benin. Cyclization activity of CGTase and the influence of pH, temperature, and metal ions were assessed using conventional assays, together with bioconversion kinetics and cyclodextrin yield. The results revealed several optimal conditions for enhancing β-cyclodextrin biosynthesis, with strong strain-and substrate-dependent variations. *Bacillus pumilus*, *Bacillus brevis*, and *Bacillus subtilis* showed stable activity irrespective of the substrate used, whereas *Bacillus cereus* proved more sensitive to the type of starch. *Bacillus brevis* remained active under acidic conditions (pH 5-6), whereas *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus subtilis* showed optimal activity at neutral to alkaline pH (7-10). All CGTases were active between 37°C and 60°C but became inactive at 70°C. Magnesium stimulated enzymatic activity, while copper inhibited the enzyme at concentrations above 5 mM. β-cyclodextrin biosynthesis was detected after 8 hours of incubation, with higher yields obtained on potato starch. Peak β-cyclodextrin production was achieved with *Bacillus brevis* on potato starch (0.546±0.022 mM), versus 0.043±0.003 mM on soluble starch. For *Bacillus cereus* and *Bacillus pumilus*, potato starch also significantly increased β-Cyclodextrin yields (0.251±0.006 mM and 0.195±0.009 mM, respectively), whereas no significant substrate effect was observed for *Bacillus subtilis*. Optimizing β-cyclodextrin production therefore requires prior knowledge of the pH, temperature, substrate, and metal-ion conditions best suited to the selected strain for efficient CGTase production.

Keywords: Cyclodextrin Glucanotransferase, Enzymatic Activity, β-Cyclodextrin, *Bacillus*

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Introduction

Over the past twenty years, biotechnological research has increasingly turned toward exploiting specific enzymes to improve industrial transformation processes, particularly across various industrial sectors [1]. Enzymes act as biological catalysts, frequently obtained from microorganisms, and speed up biochemical reactions inside living systems while consuming less energy and producing fewer unwanted by-products compared with classical chemical routes [1, 2]. Cyclodextrin glucanotransferases (CGTases), members of the α-amylase family [3], are microbial enzymes that catalyze four key reactions: cyclization, coupling, disproportionation, and hydrolysis. Among these, cyclization corresponds to an intramolecular transglycosylation reaction that converts starch or related substrates into cyclodextrins (CDs) [4]. CDs are a group of cyclic oligosaccharides built from glucopyranose units connected via α-(1,4) linkages [5]. Three natural CD types exist: alpha (α), beta (β), and gamma (γ), each generated by enzymatic breakdown of starch [3]. Of these three forms, β-CD is industrially the most exploited because of its comparatively low water solubility and greater stability [6]. CGTase is therefore the key enzyme responsible for the cyclization of starch into β-CD, and the efficiency of this reaction directly determines the industrial yield of β-CD [7]. β-CD yields and the type of CDs formed are strongly influenced by substrate type and reaction conditions such as pH and temperature, which vary between enzymes from different microbial sources [8]. CDs and their derivatives owe their appeal to structural features that allow them to form inclusion complexes with numerous compounds, with applications spanning food processing, textiles, medicine, analytical chemistry, agriculture, and pharmaceutics. Given this broad range of uses, worldwide demand for β-CD keeps rising [9]. Its production, however, relies predominantly on industrial processes, which represents a high cost for developing countries [10]. Therefore, establishing local production processes using novel indigenous microbial strains and locally available agricultural substrates could reduce production costs, strengthen industrial autonomy, and promote the valorization of natural resources. The synthesis of β-CD is closely dependent on the enzymatic properties of CGTases, particularly their optimal cyclization activity, thermal stability, and pH tolerance [11].

Prior work has demonstrated that such properties can be shaped by several factors, including the nature of the carbon source, metal ion concentration, and the physicochemical conditions of the growth medium [11]. For example, varying the substrate differentially affects CGTase expression and activity depending on the producing strain [12]. Similarly, some metal ions stimulate enzymatic activity while others markedly inhibit it [11]. Although CGTases have been extensively studied, particularly regarding their application in CD production, few studies have focused on their production from Bacillus strains isolated from yam field soils and on their enzymatic activities across different substrates. To our knowledge, no published study has jointly assessed the effects of pH, temperature, metal ions, and thermostability on CGTase catalytic activity for strains isolated from yam-cultivated soils in north-western Benin. These soils, which are rich in organic matter and residual starch, may harbor microorganisms with specific and potentially valuable enzymatic properties. It was therefore hypothesized that Bacillus strains originating from yam field soils could possess distinctive CGTase characteristics related to their adaptation to starch-rich environments. In this context, the present work was undertaken with the aim of optimizing the enzymatic activity of CGTases produced from soluble starch and potato starch by four Bacillus strains for the biosynthesis of β-CD.

Materials and Methods

Materials

The microbial strains used in this study were identified and preserved at the Laboratory of Natural Sciences and Applications of Higher Normal School of Natitingou. These include Bacillus cereus, Bacillus pumilus, Bacillus brevis, and Bacillus subtilis, which were respectively isolated from soil samples collected in Yarikou, Tigninti, central Toucountouna, and Tchakalakou, areas where farmers cultivate yam tubers.

Methods

Partial Production and Purification of CGTase

Strains were each inoculated into 1 mL of sterile distilled water before being transferred into 5 L of liquid growth medium (pH 10.3) containing 2.0% (w/v) soluble starch or potato starch, 0.5% (w/v) beef extract, 0.5% (w/v) yeast extract, 0.1% (w/v) K_2HPO_4, and 0.02% (w/v) $MgSO_4 \cdot 7H_2O$. Cultures were incubated for six days at 37°C with shaking at 150 rpm, after which cells were removed by centrifugation (8,800× g, 15 min, 4°C). The resulting cell-free supernatant was combined with ammonium sulfate and held at 4°C for 24 h. Ammonium sulfate was introduced progressively up to 70% saturation, with gentle stirring maintained at 4°C for 24 h to allow proteins to precipitate. Following a second centrifugation step (8,800 × g, 30 min), the precipitate obtained was resuspended in 10 mM Tris-HCl buffer, pH 8.0 [13], and the resulting solution served as the crude enzyme extract [14].

Cyclization Activity of Cyclodextrin Glucanotransferase (CGTase)

Cyclization activity was assayed by pre-warming a 1% (w/v) starch solution in 50 mM Tris-HCl buffer (pH 8.0) at 50°C for 5 min, then adding 100 µL of crude enzyme extract and allowing the reaction to proceed at 50°C for 20 min. The reaction was terminated with 375 µL of 0.15 M NaOH, followed by 100 µL of 0.02% (w/v) phenolphthalein prepared in 5 mM Na_2CO_3. After standing at room temperature for 15 min, colour development was recorded at 550 nm. One unit of CGTase activity was defined as the amount of enzyme releasing 1 µmol of β-CD per minute under the assay conditions described above [15].

Effect of Temperature and Thermostability on CGTase Activity

The effect of temperature on the cyclization activity of CGTase was investigated at different temperatures ranging from 37°C to 70°C under standard assay conditions. CGTase thermostability was assessed by incubating crude enzyme extracts in 50 mM Tris-HCl buffer (pH 8) at various temperatures (30°C to 70°C) for different time intervals (10 to 60 min). Aliquots of the enzyme were withdrawn and assayed to determine cyclization activity under the standard assay conditions [10, 15].

Effect of Ph on CGTase Activity Stability

CGTase activity was measured across acidic, neutral, and alkaline conditions using the following buffer systems: 50 mM sodium acetate (pH 5.0-6.0), 50 mM Tris-HCl (pH 7.0-8.0), and 50 mM glycine-NaOH (pH 9.0-10.0) [10]. Each assay was run in triplicate, and mean values are reported.

Influence of Metal Ions on CGTase Activity

Metal ion effects were tested following the approach of [8] with minor adjustments: CGTase activity was measured after adding individual ions, the enzyme being pre-incubated for 20 min with each ion at a final concentration of 5 mM or 10 mM; the ions examined were $CaCl_2$, $CuSO_4$, $FeSO_4$, and $MgSO_4$.

B-Cd Production from the Produced CGTase

β-CD was produced through enzymatic bioconversion of starch, using CGTase obtained from the different strains grown on either soluble starch or potato starch. The procedure followed was that described by [16], applied under the same experimental conditions using 50 mM phosphate buffer at pH 7. Briefly, 1 g of starch was dissolved in 100 mL of 50 mM phosphate buffer (pH 7) and heated at 60°C for 10 min. A 1 mL aliquot of CGTase extract was then added to 10 mL of this starch solution, and the mixture was incubated at 55°C, with 1 mL samples withdrawn every 4 h over a 12 h period. Each sample was heated at 100°C for 5 min, cooled, mixed with 3 mL of cold acetone, and held at 2°C for 4 h. The resulting precipitate was recovered by centrifugation (5000 rpm, 15 min), washed with cold acetone, and dried at 40°C. β-CD formation was then assessed using the phenolphthalein method [16].

Assay of the Synthesized β-CDs

β-CD concentration was determined from the decrease in absorbance at 550 nm resulting from the formation of the β-CD-phenolphthalein complex. Briefly, 0.2 mL of β-CD solution was combined with 0.05 mmol/L Tris-HCl buffer (pH 8) and 1 mL of phenolphthalein working solution, and the tube contents were mixed by vortexing, after which absorbance was measured immediately at 550 nm. The phenolphthalein working solution was prepared by diluting 2 mL of 3 mmol/L ethanolic

phenolphthalein to 100 mL with 125 mmol/L Na_2CO_3/$NaHCO_3$ buffer (pH 10.5) [17]. A calibration curve was then generated using β-CD standards ranging from 0 to 2.5 mM, plotting absorbance at 550 nm against concentration.

CGTase activity was measured using crude enzyme extracts. Protein concentration was not determined; therefore, enzyme activity is expressed as U/mL. Comparisons were performed under identical extraction and assay conditions to allow relative evaluation among strains.

Statistical Analysis

Data were initially recorded using Microsoft Excel 365. Statistical analyses were performed using SigmaPlot software version 14.5. Data are expressed as mean ± Standard Error of the Mean (SEM). For comparisons between two groups within the same strain (soluble starch versus potato starch), Student's t-test was applied when data followed a normal distribution; otherwise, the non-parametric Mann Whitney U test was used. When comparisons involved more than two groups, One-Way Analysis of Variance (ANOVA) was performed. Differences were considered statistically significant at $p<0.05$

Results and Discussion

Results

Production and Cyclization Activity of CGTase

In order to evaluate the influence of the carbon source on enzyme production, the cyclization activity of CGTases produced by different microbial strains based on soluble starch and potato starch was measured. The results presented in Table 1 highlight the variations in enzyme activity depending on the strain and the type of starch used.

Table 1: Cyclization activity of CGTases produced from soluble starch and potato starch

Microbial strains	CGTase cyclization activity (U/mL)		p-value
	Soluble starch	Potato starch	
Bacillus cereus	4.805±0.09*	4.469±0.07	0.009
Bacillus pumilus	4.675±0.12	4.537±0.08	0.211
Bacillus brevis	4.595±0.07	4.479±0.07	0.124
Bacillus subtilis	4.576±0.09	4.450±0.05	0.134

Values are expressed as mean ± SEM (n = 3). For each strain, CGTase activity produced using soluble starch was compared with that produced using potato starch. Statistical significance was determined using Student's t-test or Mann Whitney U test as appropriate. * indicates $p<0.05$.

For each Bacillus strain, CGTase cyclization activity produced using soluble starch was compared with that produced using potato starch using Student's t-test. No significant differences were observed for Bacillus pumilus, Bacillus brevis, and Bacillus subtilis ($p>0.05$). In contrast, CGTase produced by Bacillus cereus showed significantly higher activity when soluble starch was used compared to potato starch (t-test, $p = 0.009$).

Effect of pH on CGTase Activity Stability

pH is an important parameter that influences the enzymatic activity of CGTases because it affects their catalytic efficiency. To analyze this parameter, the cyclization activity of CGTases produced by different strains was evaluated at acidic, basic, and neutral pH. Figure 1 illustrates the activity profiles as a function of pH, highlighting differences between microbial species and the carbon substrate used during enzyme production.

The optimal pH for CGTase cyclization activity depends on the microbial species and the substrate used during production. *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus subtilis* (Fig. 1.2) produce CGTases that are active at pH 9-10, whereas *Bacillus brevis* produces an enzyme with maximal activity at pH 5-6. In contrast, *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus brevis* (Fig. 1.1) show active cyclization at pH 9-10, while *Bacillus subtilis* is active at pH 7-8. We observed that the use of soluble starch appears to favor the production of enzymes active under basic pH conditions compared to potato starch.

Effect of Temperature and Thermostability on CGTase Activity

The structure and stability of enzymes, particularly CGTases, are influenced by temperature. To determine the optimal temperature range for their activity, CGTases produced from soluble starch and potatoes were exposed to different thermal conditions. Figure 2 shows the influence of temperature on the cyclization activity of CGTases, highlighting both their maximum yield and their constraints in terms of thermostability.

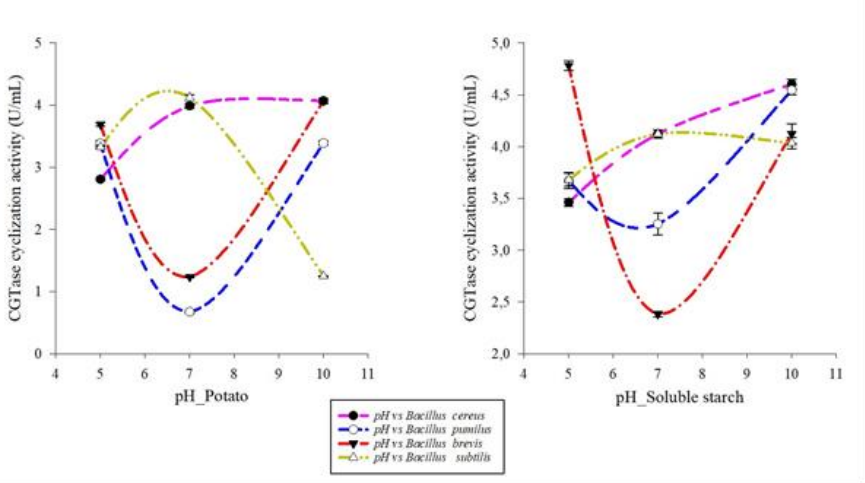


Fig. 1.1. Activity of CGTases produced on potato starch

Fig. 1.2. Activity of CGTases produced on soluble starch

Fig. 1: Effect of pH on the cyclization activity of the produced CGTases

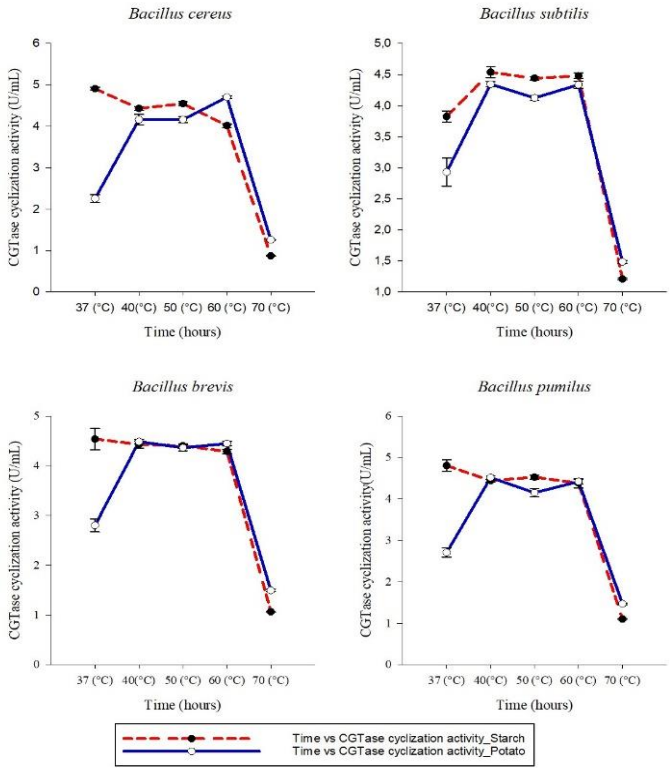
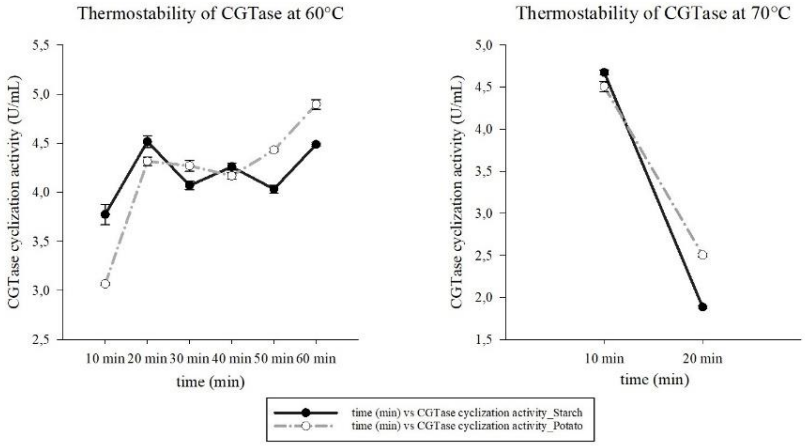


Fig. 2: Effect of temperature on the cyclization activity of CGTases

The optimal temperature for CGTase produced from potato starch was 40 60°C, while for CGTase produced from soluble starch it was 37–60°C for most microbial strains. A loss of enzymatic activity was observed at 70°C, regardless of the strain or substrate used, indicating irreversible thermal denaturation of the enzyme above this temperature. This heat sensitivity highlights the limitations of these CGTases under extreme temperature conditions, despite their good performance within the moderate range of 40–60°C

The thermostability of CGTase produced by Bacillus cereus shows differences depending on the substrate used. At 60°C, the enzymatic activity of CGTase produced from soluble starch was initially higher than that produced from potato starch; however, the latter exhibited an increase in activity after 50 minutes, reaching a maximum value of 4.89 U/mL at 60 minutes. In contrast, at 70°C, enzymatic activities decreased, but CGTase produced with potato starch retained slightly higher activity than that produced with soluble starch. These results highlight the influence of the substrate on enzyme thermal stability, with better heat resistance observed for CGTase produced from potato starch over the long term (Fig. 3).



Fig, 3: Thermostability of CGTase produced by *Bacillus cereus* at 60°C and 70°C

At 60°C, the cyclization activity of CGTase produced from soluble starch remained higher than that of CGTase produced from potato starch throughout the 60-minute incubation. The soluble starch-based CGTase maintained activity between 3.29 and 4.39 U/mL, while the potato starch-based CGTase varied between 3.75 and 4.28 U/mL. This indicates that, contrary to the previous observation for Bacillus cereus, soluble starch provides better thermal stability at 60°C for this strain. At 70°C, a decrease in enzymatic activity was observed; however, CGTase produced from potato starch retained slightly higher activity than the soluble starch-based CGTase after 20 minutes of incubation (Fig. 4).

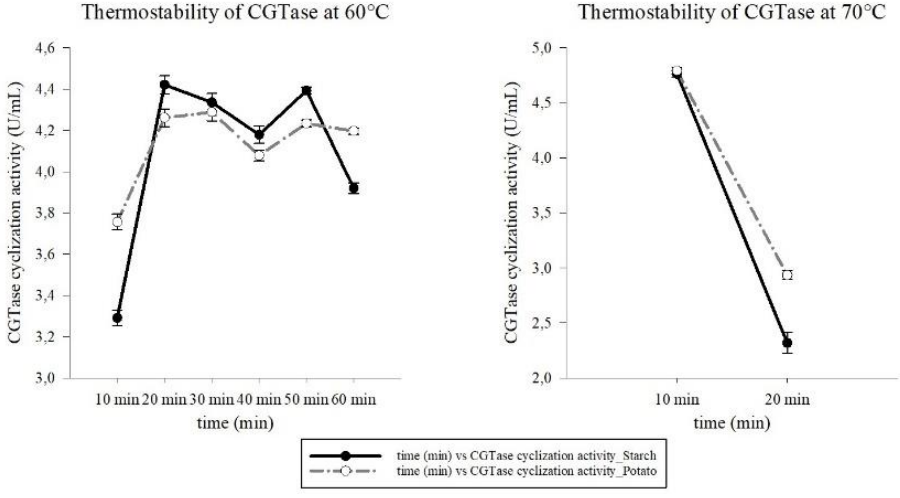


Fig. 4: Thermostability of CGTase produced by *Bacillus pumilus* at 60°C and 70°C

CGTase produced by Bacillus brevis exhibits better thermostability when produced with soluble starch. At 60°C, CGTase from Bacillus brevis with soluble starch remained high (3.47–4.43 U/mL) throughout most of the incubation period compared to potato starch (3.40–4.44 U/mL). At 70°C, cyclization activity decreased for both substrates; however, CGTase produced with potato starch maintained slightly higher activity than that produced with soluble starch (Fig. 5).

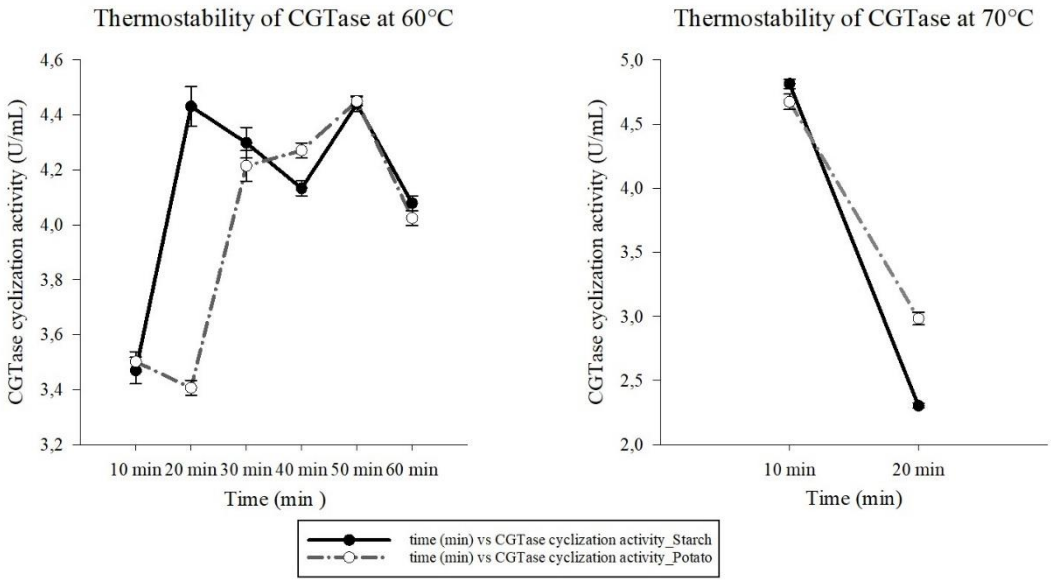


Fig. 5: Thermostability of CGTase produced by Bacillus brevis at 60°C and 70°C

For CGTase produced by Bacillus subtilis, the enzyme produced from soluble starch showed high activity after 20 minutes of incubation, reaching 4.37 U/mL, and remained stable up to 60 minutes. With potato starch, the activity was initially low (2.93 U/mL at 10 min) but gradually increased, reaching 4.18 U/mL after 60 minutes at 60°C. At 70°C, enzymatic activity decreased between 10 and 20 minutes, ranging from 4.89 to 2.6 U/mL and 4.71 to 2.96 U/mL for the two substrates, respectively. However, CGTase produced with potato starch exhibited higher activity than that produced with soluble starch after 20 minutes, indicating better tolerance to extreme heat (Fig. 6).

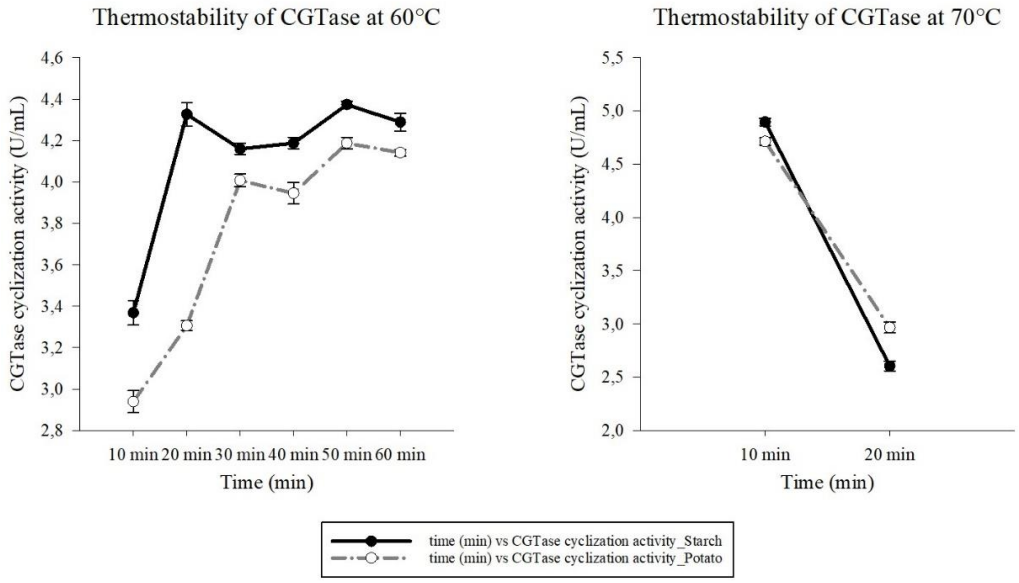


Fig. 6: Thermostability of CGTase produced by Bacillus subtilis at 60°C and 70°C

Analysis of the thermostability of CGTase from the four microbial species showed that the substrate used influenced enzyme thermal stability. At 60°C, CGTase produced from soluble starch generally conferred better thermal stability and maintained high cyclization activity over time for most strains. However, at 70°C, a rapid decline in activity was observed for all strains, although CGTase produced with potato starch retained slightly higher activity.

Effect of Metal Ions on the Enzymatic Activity of CGTase

Metal ions influence enzyme activity by acting either as activators or inhibitors. To examine this influence, the impact of various metal ions was tested on the cyclization activity of CGTases derived from soluble starch. Table 2 reveals significant fluctuations depending on the type of ion and the microbial strain examined.

Table 2: Effect of metal ions on the cyclization activity of CGTase produced from soluble starch

Strains	Conc.	Control	$CuSO_4$	$CaCl_2$	$MgSO_4$	$FeSO_4$
Bacillus cereus	5 mM	4.383±0.04	4.012±0.06	4.043±0.32	5.041±0.01	4.674±0.01
	10 mM		3.781±0.23	4.012±0.02	4.947±0.04	4.397±0.06
Bacillus pumilus	5 mM	4.243± 0.08	3.855±0.02	3.519±0.83	5.104±0.04	4.6±0.02
	10 mM		3.476±0.34	3.617±0.21	5.01±0.01	4.37±0.14
Bacillus brevis	5 mM	4.002± 0.26	3.727±0.05	3.734±0.37	5.025±0.06	4.585±0.08
	10 mM		3.47±0.07	3.92±0.07	4.978±0.01	3.971±0.50
Bacillus subtilis	5 mM	4.215± 0.04	3.715±0.04	4.012±0.02	5.073±0.04	4.734±0.01
	10 mM		3.251±0.7	3.844±0.22	5.01±0.01	4.528±0.21

The results obtained for the effect of metal ions on the cyclization activity of CGTase produced from soluble starch show that copper sulfate ($CuSO_4$) inhibits enzymatic activity in all CGTases from the different microbial species, particularly at 10 mM. Calcium ($CaCl_2$) exhibits a neutral or slightly inhibitory effect for some strains. In contrast, magnesium ($MgSO_4$) enhances enzymatic activity for all strains, with values exceeding 5 U/mL. Ferrous sulfate ($FeSO_4$) has a neutral effect, maintaining activity below that observed with magnesium.

Metal ions influence the activity of CGTases differently depending on their concentration and the substrate used during production. To further this analysis, the effect of various metal salts was studied on enzymes produced from potato starch. Table 3 shows the variations in cyclization activity observed according to the nature of the ions tested and the different bacterial strains.

Table 3: Effect of metal ions on the cyclization activity of CGTase produced from potato starch

Strains	Conc.	Control	$CuSO_4$	$CaCl_2$	$MgSO_4$	$FeSO_4$
Bacillus cereus	5 mM	4.312± 0.0198	3.999 0.075	4.026±0.113	5.136±00	4.779±0.0642
	10 mM		3.843±0.146	3.538±0.614	4.752±0.277	4.513±0.103
Bacillus pumilus	5 mM	4.285±0.0592	3.805 0.199	4.133±0.0384	5.168±00	4.704±0.0846
	10 mM		3.753±0.0537	4.107±0.153	4.963±0.022	4.541±0.0618
Bacillus brevis	5 mM	4.355±0.04	4.025±0.0377	4.147±0.135	5.2±00	4.484±0.102
	10 mM		3.816±0	4.092±0.0191	4.964±0.154	4.659±0.021
Bacillus subtilis	5 mM	4.271± 0.0788	3.868±0.0732	4.065±0.019	5.2±00	4.512±0.0615
	10 mM		3.628±0.0174	3.869±0.183	5.057±0.0669	4.384±0.12

The results regarding the effect of metal ions on the cyclization activity of CGTase produced from potato starch show that copper sulfate ($CuSO_4$) reduces enzymatic activity for all strains, particularly at 10 mM. Calcium chloride ($CaCl_2$) exhibits a moderate or neutral effect on enzymatic activity compared to the controls. In contrast, magnesium sulfate ($MgSO_4$) stimulates activity, with maximum values of 5.2 U/mL at 5 mM for *Bacillus brevis* and *Bacillus subtilis*. Ferrous sulfate ($FeSO_4$) also shows a positive effect on enzymatic activity for all strains, especially at 5 mM, though this effect slightly decreases at 10 mM (e.g., *Bacillus cereus*: 4.779±0.06 U/mL vs. control 4.312±0.02).

Production of β-Cyclodextrin from CGTases Produced by the Identified Strains

The ability of CGTases to convert starch into cyclodextrins is an essential criterion for evaluating their biotechnological potential. In order to analyze the kinetics of β-cyclodextrin production, enzymes from different microbial strains were used to produce β-CD at different time intervals. Figure 7 illustrates the evolution of bioconversion over time and highlights the differences related to the microbial species and the substrate used.

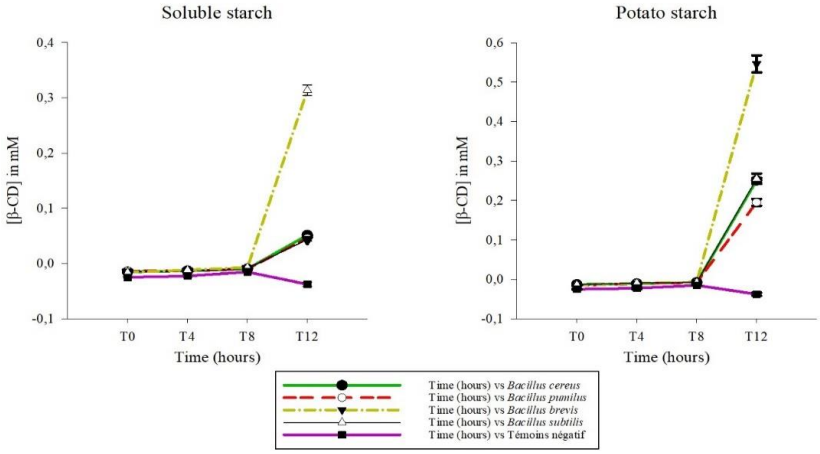


Fig. 7: Kinetics of the bioconversion of starch into β-cyclodextrin by CGTases as a function of time

Bioconversion of starch into β-cyclodextrins by CGTase remained undetectable or negligible from 0 to 8 hours. Bioconversion activity was detected starting from 12 hours of incubation. This bioconversion varied among microbial species and between substrates, with higher production observed using potato starch for all strains tested. The negative control (without enzyme) remained undetectable for both substrates, indicating the absence of β-cyclodextrin production.

The efficiency of bioconversion of soluble starch into β-cyclodextrin depends on both the type of substrate and the microbial strain producing CGTase. Table 4 shows the concentrations obtained for each strain, as well as the differences in yield observed between the two substrates.

Table 4: Biosynthesis of β-Cyclodextrin from commercial starch and potato starch

Strains	β-Cyclodextrin Concentration (mM)		P-value
	Soluble starch	Potato starch	
B. cereus	0.05±0.00	0.25±0.01*	<0.001
B. pumilus	0.05±0.00	0.20±0.01*	0.001
B. brevis	0.04±0.00	0.55±0.02*	<0.001
B. subtilis	0.31±0.01	0.26±0.01	0.100

β-Cyclodextrin concentrations ranged from 0.0435 to 0.314 mM for CGTases produced using soluble starch and from 0.195 to 0.546 mM for CGTases produced using potato starch. For each Bacillus strain, β-cyclodextrin production obtained with soluble starch was compared with that obtained with potato starch using Student's t-test. Statistically significant differences were observed for *Bacillus cereus, Bacillus pumilus*, and *Bacillus brevis* ($p \leq 0.001$), whereas no significant difference was detected for *Bacillus subtilis* (t-test; $p = 0.100$).

Discussion

The present study aimed to optimize the enzymatic activity of CGTases produced by four *Bacillus* strains for β-CD biosynthesis. Statistical analysis using Student's t-test revealed no significant differences in CGTase activity for *Bacillus pumilus, Bacillus brevis,* and *Bacillus subtilis* when enzymes were produced using soluble starch or potato starch ($p>0.05$). This suggests a low sensitivity of these strains to the nature of the carbon substrate used for CGTase production. Similar substrate-independent CGTase activity has been reported for certain Bacillus strains in both recent and

earlier studies [18]. By contrast, the CGTase produced by Bacillus cereus displayed a significant substrate-dependent difference (t-test, p = 0.009), possibly reflecting differential regulation of CGTase expression or variation in enzyme-substrate affinity. CGTase preparations from *Bacillus cereus* have been examined in Nigeria on cassava, sweet potato, and taro starches, and in India on soluble, maize, and rice starches, with concentrations varying accordingly; cassava and potato starch (14.329±0.14 U/mL) were reported as the most favorable substrates for CGTase and CD production [17, 19]. These observations contrast with our results, as the highest concentration was observed with soluble starch (4.805±0.09 U/mL). This discrepancy could be attributed to differences in strain, enzyme production process, or the physicochemical structure of the starches used. The pH optimum for CGTase cyclization likewise varies with the microbial species and the substrate employed during production: the enzyme produced by *Bacillus brevis* shows an acidic optimum (pH 5-6), while enzymes from *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus subtilis* are more active at neutral-to-alkaline pH (7-8 or 9-10), depending on the substrate. These results are consistent with those of [7, 20-23] , who demonstrated that the nature of starch influences the optimal pH of CGTase activity. In addition, other studies confirm that CGTases from alkaliphilic strains such as *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus subtilis* often possess maximum activity at alkaline pH [24, 25]. According to [10, 15] and, some Bacillus species possess the ability to produce CGTases with maximum activity at pH 5. It should be noted that [26]. found that soluble starch favors enzyme production and activity at basic pH, whereas potato starch tends to lower this. Such pH-dependent differences may arise from variations in the enzyme's three-dimensional structure, which could alter active-site configuration depending on pH.

CGTase activity peaked within the 37–60°C range, with a marked loss of activity at 70°C for all strains tested. These results suggest a relatively similar protein structure among the CGTases despite microbial differences. This temperature sensitivity may be explained by the enzyme's tertiary structure, whose non-covalent bonds are likely to break beyond 60-65°C. These findings are consistent with several studies reporting that most *Bacillus*-derived CGTases are active within this moderate temperature range but are poorly resistant to high temperatures [7, 10]. Thermal stability also depended on the substrate used during production and varied with incubation temperature: at 60°C, CGTases from most strains grown on soluble starch retained activity longer, suggesting a conformation more resistant to moderate heat denaturation, in line with earlier findings that thermal stability of CGTases from *Bacillus* species can remain nearly fully active for up to 2 h between 40°C and 70°C, irrespective of the production substrate [8, 10]. By contrast, at 70°C all CGTases lost activity rapidly within 20 min, likely reflecting substantial thermal denaturation. [7] reported a comparable pattern after 20 min of incubation at 65 and 75 °C, underscoring the limited suitability of these CGTases for high-temperature industrial processes despite their good performance under moderate conditions.

Metal ions affected CGTase cyclization activity differently depending on the ion type, its concentration, the strain, and the substrate. The results indicate that magnesium acts as an activator of CGTase activity, with optimal stimulation observed at 10 mM, regardless of the substrate or strain used. In agreement with the literature, where magnesium is commonly described as a cofactor needed for optimal CGTase activity [7, 27]. Ferrous ion (Fe^{2+}) should be used with caution, as our results show a moderately activating effect on certain strains, which is consistent with findings reported by [11, 27, 28]. In contrast, [7, 29] reported that Fe^{2+} acts as a strong inhibitor of enzymatic activity. Conversely, copper behaves as an inhibitor starting from 5 mM, which may be due to partial enzyme denaturation or interactions with active-site residues that prevent substrate binding or disrupt catalysis. This is supported by several studies demonstrating the sensitivity of CGTases to inhibition by metal ions [7, 11, 30]. Calcium exhibited a more complex, concentration-dependent effect, ranging from mild inhibition at low doses to activation at 10 mM, particularly for *Bacillus pumilus.* This suggests an interaction shaped by both the enzymatic and substrate context, a complexity also reported by [11], where calcium could either inhibit or thermally stabilize CGTase depending on conditions. Thus, these results confirm the importance of controlling the ionic composition during the production and industrial use of CGTases for the optimization of yield and stability according to the specific needs of the process. The bioconversion of CGTase to βCDs remained undetectable during the first 8 hours of incubation, before reaching a measurable level from 12 hours. This delay could be the enzymatic adaptation time or a partial activation of the CGTase in the presence of the substrate. This suggests that the enzyme requires a sufficient period of activation or accumulation before reaching measurable activity, reflecting a latency phase in the enzymatic reaction. This initial delay is consistent with the observations of [20, 31] who reported that the formation of β-CDs requires an enzymatic adaptation time and the establishment of a favorable catalytic equilibrium. In addition, the synthesis of β-CD varied depending on the microbial strain and the type of starch used. Potato starch has been shown to be a more effective substrate for most strains, which is consistent with studies showing that its granular structure and amylose content indicate optimal hydrolysis by CGTase [12, 32]. Furthermore, the absence of β-CD in enzyme-free controls confirms that bioconversion is strictly dependent on CGTase activity, eliminating any possibility of

spontaneous or non-enzymatic conversion. These parameters show that the nature of the substrate plays a major role in the efficiency and kinetics of CGTase bioconversion. The concentrations of synthesized β-CDs fluctuate depending on the substrate used and the strain of Bacillus. These measured concentrations range from 0.0435 mM to 0.314 mM for CGTases derived from soluble starch, and from 0.195 mM to 0.546 mM for those derived from potato starch. The difference between substrates, as well as between *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus brevis*, clearly shows the effect of the substrate on βCD yield. These results are confirmed by data from [16, 33], who stated that the best source of starch for CD production varied depending on the source of the enzyme and that potato starch was found to be the best substrate for the enzyme. On the other hand [34], found that tapioca starch was the most suitable substrate. Different starch sources could affect the production of CD, which is likely caused by differences in the structure and properties of starch granules [32]. *Bacillus subtilis* does not show variation depending on the substrate. This result suggests enzymatic regulation independent of the starch source. This could testify to a greater ability of its CGTase to identify different substrates, as shown by [35] on immobilized biocatalytic systems. Finally, the variations between strains, especially between *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus brevis*, could be related to the genetic influence on the specificity of CGTase, confirming that some species favor the production of β-CD more efficiently than others. It would therefore be important to select strains adapted to the substrate to be used to optimize the industrial production of β-CDs. Although this work has contributed to deepening our knowledge of the impact of substrates, pH, temperature, metal ions, and incubation time on the activity of CGTases from various Bacillus strains, some methodological aspects deserve to be deepened for a better understanding of the enzymatic activities of CGTase.

The absence of specific activity (U/mg protein) represents a limitation of this study, as variations in protein content may influence apparent enzyme activity. Future work will focus on enzyme purification and determination of specific activity to allow more accurate comparisons with published CGTases.

Future Research Perspectives

Although this study provides information on certain characteristics of CGTases produced by Bacillus strains isolated from yam-cultivated soils in northwestern Benin, several limitations need to be addressed in future work. In particular, the study did not address the extraction, purification, long-term stability, or detailed kinetic characterization of the produced CGTases. Further work should therefore focus on enzyme purification to determine their specific activities and establish their kinetic parameters (Km and Vmax) from Michaelis–Menten plots. Furthermore, cyclization activities were measured solely by a spectrophotometric assay of the β-cyclodextrin-phenolphthalein complex in a basic medium at 550 nm, an approach considered limited in precision; the use of more advanced analytical techniques, such as High-Performance Liquid Chromatography (HPLC), would allow for more reliable quantification of the produced cyclodextrins. Finally, since the experiments were carried out exclusively at the laboratory scale, scale-up studies using local starch substrates will be necessary to assess the technical and economic feasibility of β-CD production at the pilot or industrial scale.

Conclusion

This study aimed to optimize the enzymatic activity of CGTases produced by four *Bacillus* species isolated from yam cultivation soils in northwestern Benin for the biosynthesis of β-CD. The results show that cyclization activity, thermostability, and bioconversion to β-CD are highly dependent on the microbial strain and culture conditions, including pH, temperature, substrate type, and the presence of metal ions. CGTases produced by *Bacillus pumilus*, *Bacillus brevis*, and *Bacillus subtilis* exhibit relatively stable activity regardless of the type of starch used, while that of *Bacillus cereus* is significantly influenced by the substrate. The CGTases are active within a temperature range of 37 °C to 60 °C but lose their activity at 70°C. Magnesium stimulates enzymatic activity, while copper exerts an inhibitory effect at concentrations above 5 mM. β-CD production is detectable after 8 h of incubation, with overall higher yields on potato starch, with *Bacillus brevis* exhibiting the best performance. These results highlight the biotechnological potential of *Bacillus* strains isolated from yam fields for the valorization of locally available starch resources in Benin. They also indicate that optimizing the industrial production of β-CD relies on a judicious choice of strain-substrate combinations and the precise adjustment of the physicochemical parameters of the production process.

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Author's Contributions

Ténor Dias-Mendel Allode and Cyrille Alode Vodounon: Handling; Data analysis, drafting and finalization of the manuscript.
Atindehou Gabin Dossou, Noël Christi Honzounnon and Akodji Dèfognon Fiacre Marcos Migan: Handling, drafting and finalization of the manuscript.
Sina Orou Abdel and Senou Maximin: Data analysis, drafting and finalization of the manuscript.

Ethics

This study did not involve human participants or animal experimentation. Soil sampling and microbiological analyses were performed in accordance with standard procedures. The authors declare that no ethical considerations arise from the publication of this work.

Conflict of Interest

The authors declare that they have no conflict of interest.

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