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RESEARCH

Production and purification of cyclodextrin glucanotransferase from *Alkalihalobacillus lehensis* JO-26

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ABSTRACT

Cyclodextrin glucanotransferase (CGTase) is a unique enzyme that helps to convert starch into cyclic oligosaccharides called cyclodextrins. In this study, a cyclodextrin glucanotransferase producing bacteria was isolated from soil samples obtained around Bosso region of Minna, Niger State, Nigeria. The isolate 11D had the highest CGTase enzyme activity and was isolate was biochemically and physiologically characterized and identified as *Alkalihalobacillus lehensis* JO- 26. The production of the enzyme was optimized by using various starches obtained from tubers. Sweet potato starch was found to give the maximum enzyme activity of 24.81 ± 0.150 U/mL. The CGTase enzyme produced by A. *lehensis* JO-26 was purified with 80 % ammonium sulphate precipitation followed by starch adsorption chromatography on DEAE- Sepharose 6B. The purified enzyme had a specific activity of 78.37 U/mL, with 44.64 % recovery and 5.27-fold purification. The maximum enzyme activity was observed at pH 9.0 and temperature of 55 °C. The study suggests the enzyme may be of great importance for industrial applications.

Keywords: Alkalihalobacillus lehensis JO-26, Bosso, cyclodextrin glucanotransferase (CGTase), cyclodextrin, Horikoshi (II) medium, purification

RESUMEN

Producción y purificación de la glucanotransferasa de ciclodextrina de *Alkalihalobacillus lehensis* JO-26. La glucanotransferasa de ciclodextrina es una enzima única que ayuda en la conversión del almidón en oligosacáridos cíclicos llamados ciclodextrinas. En este trabajo, se aisló una bacteria productora de glucanotransferasa de ciclodextrina, a partir de muestras de suelo obtenido en la región Bosso de Minna, estado de Niger, Nigeria. El aislamiento 11D mostró la mayor actividad de enzima CGTasa, y el mismo fue aislado, caracterizado bioquímica y fisiológicamente, y se le identificó como Alkalihalobacillus lehensis JO-26. Luego se optimizó la producción de la enzima, con el uso de varios tipos de almidón de tubérculos. La mayor actividad enzimática (24.81 ± 0.150 U/mL), se obtuvo a partir del almidón de boniato. La enzima CGTasa se purificó a partir de A. lehensis JO-26 mediante precipitación dcon sylfato de amonio al 80 %, seguido de cromatografía de adsorción con almidón en matriz DEAE-Sefarosa 6B. La enzima purificada mostró una actividad específica de 78.37 U/mL, con un 44.64 % de recuperación y una purificación de hasta 5.27 veces. La actividad enzimática máxima se obtuvo a pH 9.0 y 55 °C. Los resultados sugieren que la enzima puede ser de gran interés para aplicaciones industriales.

Palabras clave: Alkalihalobacillus lehensis JO-26, Bosso, glucanotransferasa de ciclodextrina (CGTase), ciclodextrina, medio Horikoshi (II) medium, purificación

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Introduction

Cyclodextrin glucanotransferase (cyclomaltodextrins glucanotransferase, cycloamylase, CGTase) is a unique enzyme produced by some microorganisms that acts on starch and related carbohydrates [1]. The enzyme catalyzes four main reactions namely: cyclization, hydrolysis, coupling and disproportionation [2]. Among them, cyclization has been recognized to be the most important, as it produces non-reducing cyclic molecules known as cyclodextrin through an intramolecular transglycosylation reaction [3].

Cyclodextrins (Cycloamylose) are cyclic homogenous oligosaccharides of 6-12 α-1,4-D-linked glucose residues [4]. They show a unique structure, in which the hydroxyl polar groups are outside with the interior been hydrophobic [5]. This configuration confers it with an ability to encapsulate various guest molecules in its cyclic cavity [6]. Based on this property, Cyclodextrins can form complexes and have found useful industrial applications [7-9]. They are identified based on the number of glucopyranose units: α-cyclodextrins, β-cyclodextrins, γ-cyclodextrins containing 6, 7, and 8 glucopyranose units respectively.

Several *Bacillus* species have been reported to produce CGTase. These include *Bacillus firmus* [10], *Bacillius licheniformis* [11], *Thermoanaerobacter* spp. [12], *Bacillus clarkia* [13], *Bacillus lehensis* [14], *Bacillus circulans* [15], *Bacillus flexus* [16]. There has been also reported the production of cyclodextrins using different starch [17]. However, the efficient industrial application of the enzyme requires both the availability of an advantageous CGTase producing microorganism, and the efficient production and purification of the enzyme.

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Therefore, this work was aimed to isolate and identify a CGTase-producing bacteria, and to produce CGTase. For this purpose, a CGTase producing bacteria was isolated from a cassava processing field at Minna, Niger State, Nigeria, with no previous reports neither for the isolation of alkaliphilic bacteria from cassava processing sites in that area, nor screening for CGTase production.

Materials and methods

Collection of sample and screening of CGTase producers

Soil samples were obtained from cassava processing regions around Mobil area, Bosso, Minna, Niger state, Nigeria (9° 36' 52.08" N, 6° 32' 51.52" E). CGTase-producing organisms were isolated by procedures described by Park *et al*. [18]. A 1-g soil sample was suspended in ten milliliters of sterile, distilled water. Next, 0.1 mL of the supernatant was spread over a Horikoshi (II) agar plates containing 2 % soluble starch, 0.5 % peptone, 0.5 % yeast extract, 0.01 % $MgSO_4$, 0.1 % K_2HPO_4, 2 % agar, 0.03 % phenolphthalein, 0.01 % methyl orange, and 1 % sodium carbonate (autoclaved separately). The plates were incubated at 37 °C for 24-48 h. The formation of a halo area around the colonies on the plate was considered a positive indication of CGTase.

Production of crude CGTase

Bacteria strain was grown in a 250 mL flask containing 50 mL of alkaline Horikoshi (II) medium, pH 10.5 , containing 2 % soluble starch, 0.5 % peptone. 0.5 % yeast extract, 0.1 % KH_2PO_4, 0.02 % $MgSO_4 \cdot 7H_2O$ and 1 % Na_2CO_3 (autoclaved separately). The culture was incubated at 200 rpm for 24 h at 37 °C. This served as the inoculum. Ten percent (w/v) of the bacteria inoculum was used to cultivate the production media in a 500 mL Erlenmeyer flask containing 100 mL of Horikoshi (II) medium [19], at 200 rpm for 72 h. The production broth was centrifuged at 10 000 rpm for 20 min at 4 °C, and the cell free supernatant was used for the determination of the enzyme activity.

CGTase activity

The cycloamylase activity was determined by the phenolphthalein colorimetric method, with slight modification. The reaction mixture consisting of 5.0 mL of crude enzyme solution (cell free supernatant) and 5.0 mL of 1 % soluble starch solution (0.1 g of soluble starch, 1.0 mL of 0.05 M $CaCl_2$ solution, 1.0 mL of 0.05 M Tris-HCl buffer solution, pH 8.0 and distilled water for a final volume of 10 mL) was incubated in a thermostated reactor at 52 °C. Aliquots of the reaction mixture, 0.5 mL each, were collected at 0, 3, 6, 9 and 12 min, and inactivated in boiling water for 5 min. Then, 2.5 mL of an alcoholic 3 mM phenolphthalein solution diluted in 0.6 M Na_2CO_3 buffer, pH 10.5, was added to each inactivated sample. Finally, absorbance was read at 550 nm [20]. The CGTase activity was established by the reduced intensity of the pink reagent due to the formation of cyclodextrins-phenolphthalein-complex. One unit of CGTase activity was defined as the amount of enzyme releasing 1 µmol of β-CD per min under the defined assay conditions. The determination of total protein was carried out according to the Lowry's method [21], using bovine serum albumin as standard.

Characterization of CGTase producing bacteria

The CGTase-producing bacteria were characterized based on their cultural, morphological and biochemical characteristics. The biochemical characterization was done according to the method of Bergey's Manual of Determinative Bacteriology [22].

Molecular identification

The selected bacteria isolate was grown for 24 h in a nutrient broth medium, followed by centrifugation at 10 000 rpm for 20 min at 4 °C. The PCR sequencing preparation followed the protocol described by Neilan [23]. The PCR sequencing preparation mix consisted of 5 µL of 5 × fusion buffer, 1.0 µL of 25 mM $MgCl_2$, 1 µL of 10 mM of dNTPS mix, 1 µL containing 10 pM of primers 27FC (5'-CCAGCAGCCCCGCTAATACG-3') and 1492 RT (5'-ATCGGCTACCTTGTTACGACTTC-3'), and 0.3 µL of Tag DNA polymerase, 1 µL DNA template, completed to 24 µL with PCR sterile distilled water. The PCR was run in a GeneAmp 2720 PCR System Thermal cycler (Applied Biosystem Inc., USA), with a PCR profile consisting of an initial denaturation at 95 °C for 5 min; followed by 35 cycles consisting of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 minute; and a final termination step at 72 °C for 7 mins and chill at 4 °C. PCR products were analyzed by DNA gel electrophoresis in 1.5 % agarose gels and 1 × TAE running buffer, at 120 V for 30 min, followed by UV light visualization. Then, the PCR product was sequenced by the Sanger's method, with the Big DyeTM Terminator v3.1 cycle sequencing kit (Catalog No:4337455; Thermo Fisher Scientific, USA) in an Applied Biosystems 3130 Genetic Analyser (Hitachi LTd, Japan). The DNA sequence obtained was searched in the BLASTN database of the National Center for Biotechnology Information (NCBI). Finally, the maximum likelihood tree based by the neighbor joining method [24], using the MEGA 5.05 software for sequence comparison.

Molecular weight determination of CGTase using SDS-PAGE

The molecular weight of the enzyme was determined by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), as described by Al-Sharawi *et al*. [25]. Briefly, 20 µL of the sample was loaded in the well and electrophoresed in Tris-Glycine electrophoresis buffer at 100 V for 2 h. Next, the gel was removed from the cassette and stained with Coomassie brilliant blue R-250 for 1-2 h and completely destained using destaining solution [26].

Effect of pH and temperature on production of CGTase

The culture was incubated with agitation in an orbital shaker at 200 rpm, at 37 °C for 72 h. Subsequently, cells were centrifuged at 10 000 rpm, at 4 °C for 20 min, and the activity of CGTase was estimated in the supernatant by phenolphthalein assay.

The effect of pH and incubation temperature on the CGTase production was investigated. Ten percent

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(v/v) seed inoculum of the selected bacteria strain was inoculated in 50 mL production medium, pH 8.0 to 11.0. The culture was incubated with agitation in an orbital shaker at 200 rpm, at 37 °C for 72 h. Subsequently, cells were centrifuged at 10 000 rpm, at 4 °C for 20 min, and the activity of CGTase was estimated in the supernatant by phenolphthalein assay. The process was repeated for the determination of pH with pH of the production medium set at 10.0. The effect of temperature on the production was determined at 30, 35, 37 and 40 °C for 72 h, on an orbital shaker at 200 rpm.

CGTase purification

The CGTase was purified using ammonium sulphate precipitation and starch adsorption chromatography in a DEAE Sepharose 6B column. The crude enzyme was subjected to 80 % ammonium sulphate precipitation, under continuous stirring at 4 °C for 1 h. After the dissolution of the salt, the ammonium sulphate solution was centrifuged at 10 000 rpm at 4 °C for 20 min. The protein pellet was resuspended in 40 mL of 5 mM HCl-Tris buffer, pH 8. The precipitated enzyme was dialyzed against several changes of distilled water using a cellulosic bag [14]. The dialyzed enzyme was chromatographed on a DEAE Sepharose 6B column stabilized with 30 mM Tris-HCl chromatography buffer, pH 8, containing 5 mM $CaCl_2$.

A total volume of 5 mL of the dialysate sample was applied to the column. The column was washed with Tris-HCl buffer pH 8.0 containing 10 mg/mL β-CD. Fractions of 4 mL were collected at a flow rate of 20 mL/h from the column. The enzyme was eluted with chromatography buffer containing 10 mg/mL of β-CD at a rate of 0.5 mL/min, and fractions of 4 mL were collected.

Characterization of purified CGTase

The optimal pH and temperature of the purified enzyme was determined following the procedure described by Goel and Nene [19]. The CGTase activity was carried out by pre-incubating the purified enzyme with the buffers for each pH, for 30 min. The buffers used were: sodium acetate for pH 4.25, 5.0 and 6.0; sodium phosphate for pH 6.0, 7.0 and 8.0; Tris-HCl for pH 8.0-10.0; glycine NaOH for pH 9.0 and 10.0, and sodium carbonate pH 10.0 and 11.0. The optimum temperature for CGTase activity was determined by incubating the purified enzyme at 20, 30, 40, 45, 50, 55, 60, 65, 70 and 80 °C, and enzyme activity assayed as earlier described. The thermal stability was evaluated by incubating the purified enzyme at 40, 50, 55, 60 and 70 °C in a thermostated water bath for 4 h at 1-h intervals.

Results

Isolation and screening of CGTase producing bacteria

Eleven isolates were obtained from the samples screened on phenolphthalein and methyl orange dyes Horikoshi (II) medium. Among the isolated bacteria, the bacteria coded 11B showed the highest enzymatic production of CGTase (Figure 1). The isolate produced a significant yellowish halo region around the wells of the plate (Figure 2). The molecular and genetic identification of the bacteria through 16S rRNA gene sequencing followed by BLAST analysis of the consensus sequence, revealed the isolate to be and *Alkalihalobacillus lehensis* JO-26, accession number MF321817.1. The PCR results showing the positive amplification of

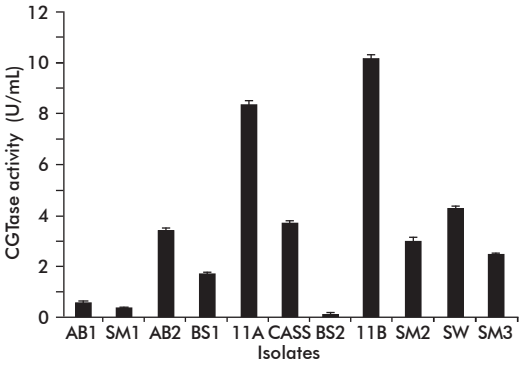

Figure 1. Cyclodextrin glucanotransferase (CGTase) activity of different bacterial isolates from a cassava processing field at Minna, Niger State, Nigeria.

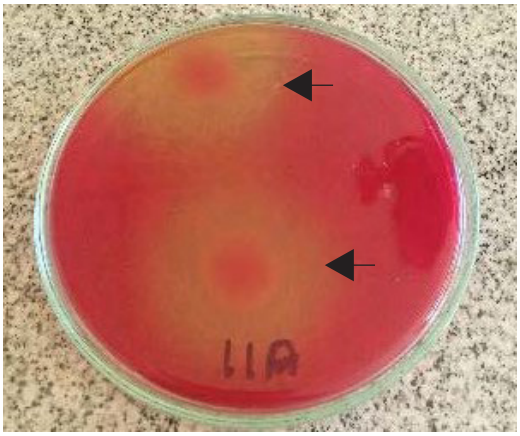

Figure 2. Agar plate of Horikoshi (II) medium showing two bacterial colonies with positive activity for CGTase production. Arrows indicate the halo zone of two colonies of bacterial isolate 11A.

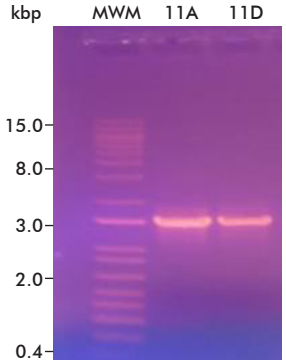

Figure 3. Acrilamide gel electrophoresis of PCR products amplifying the CGTase gene from genomic DNA od bacterial isolates with positie CGTase enzyme activity. MWM: Molecular weight marker of the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Catalog No. 4337455; ThermoFisher Scientific, USA).

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the CGTase are shown in Figure 3. The phylogenetic screening of the isolates through 16S RNA sequencing showed similarities between *Alkalihalobacillus* species such as *A. miscanthi*, *A. patogoniensis* and *A.clausii* (Figure 4). CGTase produced by *A. lehensis* JO-26 was stained with Coomassie brilliant blue (Figure 5) and the molecular weight of the bands was determined by the molecular weight marker, which ranged 11-180 kDa.

Effect of different experimental condition on CGTase activity of A. *lehensis* strain JO-26

Enzyme activity of 13.78 ± 0.69 U/mL was observed for *A. lehensis* at 72 h (Figure 6A). The optimum pH for production of CGTase by *A. lehensis* JO-26 was 10.0 (Figure 6B). An optimal temperature of 37 °C was identified for CTGase production by *A. lehensis* JO-26 (Figure 6C).

CGTase production from tuber starches

The production medium was supplemented with starches obtained from different tubers, and the enzyme activity was estimated. The highest CGTase activity of 24.98 ± 0.485 U/mL was observed with the medium supplemented with sweet potato starch. This was closely followed by the medium supplemented with cocoyam starch (15.28 ± 0.627 U/mL). Enzyme activities of 14.8 ± 0.09 U/mL and 8.34 ± 0.14 U/mL were observed for medium containing cassava and yam starch, respectively, after 72 h of fermentation (Figure 6C).

Purification of CGTase by A. *lehensis* strain JO-26, and effect of pH and temperature on the CGTase activity

Purification of CGTase from *A. lehensis* JO-26 increased the yields of CGTase 5.27-fold, with a 44.07 % yield and the specific activity increased from 14.86 to 78.37 U/mg (Table).

Effect of pH and temperature on purified CGTase

The effect of pH on enzyme activity was studied by diluting with different buffers at varying pH and then incubating with the substrate. Maximum enzyme activity was observed at pH 9.0 (Figure 7). Regarding the enzyme activity, a maximum was observed at pH of 9.0, but a decline ocurred at pH 10.0 (Fiure 8). The optimum temperature of the enzyme was observed at 55 °C (Figure 9).

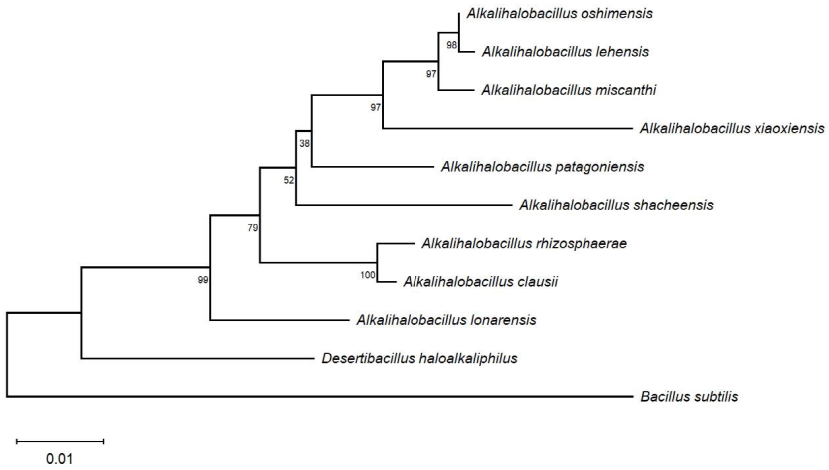

Figure 4. Phylogenetic relationship of the *Alkalihalobacillus lehensis* bacterial isolate, identified by PCR sequencing from isolates showing cyclodextrin glucanotransferase (CGTase) activity. Bacteria were isolated from a cassava processing field at Minna, Niger State, Nigeria.

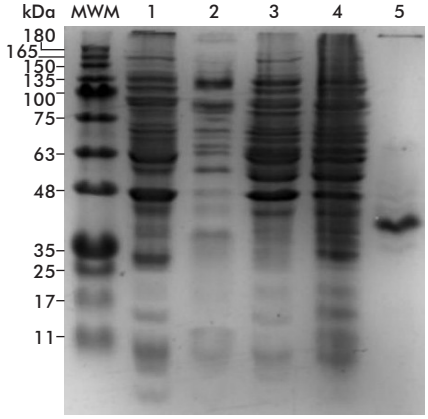

Figure 5. SDS-PAGE analysis of purification steps of the cyclodextrin glucanotransferase (CGTase) from a *Alkalihalobacillus lehensis* bacterial isolate from a cassava processing field at Minna, Niger State, Nigeria. The enzyme was purified by ammonium sulphate precipitation, followed by starch adsorption chromatography with DEAE Sepharose 6B. Lanes: 1, Crude preparation; 2, dyalisate; 3, ammonium sulphate precipitate; 4, Crude enzyme; 5, fraction purified by starch adsorption chromatography. MWM: True Color High Range Protein Molecular Weight Marker 10-250 kDa (catalog Number S2600; Sinapse Biotechnologia, Brazil).

*D*iscussion

CGTase converts starch into cyclodextrins, a type of industrially useful compounds with the ability to form inclusion complexes that alter their physical and chemical properties. In this study, alkalophilic bacteria with the ability to produce cyclodextrin glucanotransferase were isolated from soils obtained from Bosso region in Niger State, Nigeria. The soils were screened on Horikoshi (II) medium containing phenolphthalein and methyl orange. Out of the isolates obtained, two bacteria produced enzyme activity through its halo zone significantly present on the PMO plates. This could be due to the formation of complex between the cyclodextrin and phenolphthalein, thereby leading to an intensity in the color of the phenolphthalein [19]. The results of biochemical tests along with Gram's staining and colony observation suggested the strain belonged to the *Bacillus* genus.

The generic identification of the bacterial isolate validated by 16 S RNA gene sequencing and BLAST analysis of the consensus sequence revealed that the two isolates with the highest CGTase-producing activity showed highest similarity with *Alkalihalobacillus lehensis* JO-26 (Genbank accession number MF 321817.1). The MEGA X- software used to analyze the phylogenetic relationship between the isolate and other *Bacillus* species showed a cluster of the strain with different species of *Alkalihalobacillus*, such as *A. miscanthi*, *A. xiaoxiensis*, *A. shacheensis*. Several reports have found bacterial strains among

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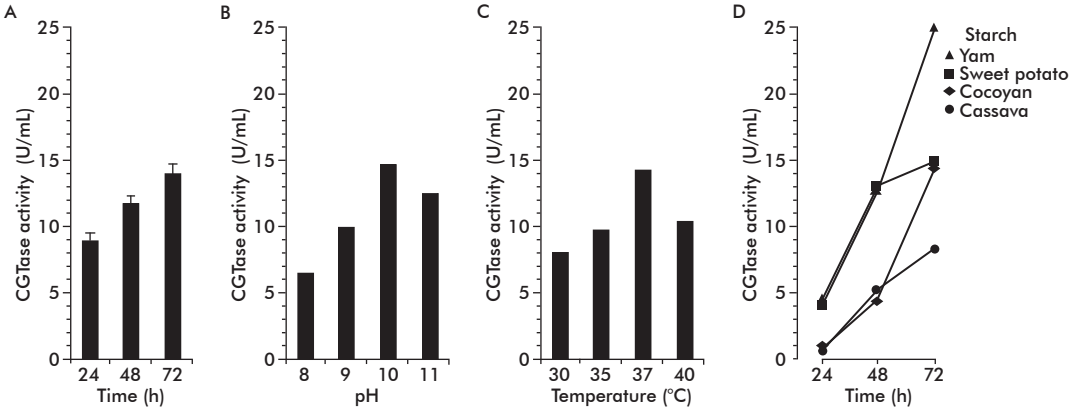

Figure 6. Effect of *Alkalihalobacillus lehensis* JO-26 growing parameters on the activity of cyclodextrin glucanotransferase (CGTase). A) Incubation time. B) pH. C) Temperature. D) Starch substrate.

the the highest producers of CGTase, such as *B. firmus* [27], *B. megaterium* [28], *B. circulans* [15], *B. flexus* [16], *B. clarkii* [13].

The two bacterial isolates *Alkalihalobacillus oshimensis* wahaha MZJ-1 and *A. lehensis* JO-26 were further optimized with various starch from tubers by replacing the soluble starch in the production medium with the tuber starches. *A. lehensis* JO-26 had activity of 24.81 ± 0.150 U/mL with sweet potato starch, and was further selected for purification and optimization studies. Biwer *et al*. [29] reported that starch and amylopectin are preferred substrates for CGTase production. Sweet potato starch has clusters of amylopectin which comprises 5-10 amylopectin chains per cluster, it therefore seems that sweet potato starch plays a vital role in cyclodextrin glucanotransferase production [30]. In this sense, other starch sources, such as potato starch, has also been reported to be good substrates for the production of CGTase [31], which could be a source for further optimization.

Then, the effect of temperature and pH on the production of CGTase was studied. Temperature is a critical factor that influences enzyme production. The optimum temperature was observed at 37 °C for *A. lehensis* JO-26. Optimum temperature for the production of CGTase was 30 °C for *Paenibacillus* sp. L55 and 37 °C for *E. aurantiacum* [32]. Other reports on CGTase production by *Paenibacillus* have mentioned the optimum temperature for CGTase production in the range 30-37 °C [33]. Our results further confirm 37 °C as the optimal temperature for optimal production of CGTase.

Regarding pH, it was determined that pH 10. 0 was optimal for CGTase production by *A. lehensis* JO-26. The pH affects production medium, influencing the movement of chemicals throughout the cell membranes and also on chemiosmotic energy generation [34]. The results are in agreement with similar reports, which indicated pH 9.0 for *B. alkalophilis* and pH 10.0 for *B. licheniformis* [35]. Rosso *et al*. [36] informed an optimal pH 8.3 for for CGTase production by *B. circulans* DF 9R, and Savergave *et al*. [10] established optimal pH 9.0 for *B. firmus* CGTase production using a bioreactor [10].

Once the enzyme was optimally produced, it was partially purified by ammonium sulphate precipitation and DEAE-Sepharose column chromatography. Then,

Table. Total activity (U/mL), protein concentration (mg/mL), yield (%) and purification fold in each step of the purification of the cyclodextrin glucanotransferase (CGTase) enzyme produced by *Alkalihalobacillus lehensis* strain JO-26

Purification step	Activity (U/mL)	Protein concentration (mg/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	31.25	2.103	14.86	100	1
Ammonium sulphate precipitation	20.69	0.626	33.05	66.21	2.22
DEAE Sepharose	13.95	0.178	78.37	44.64	5.27

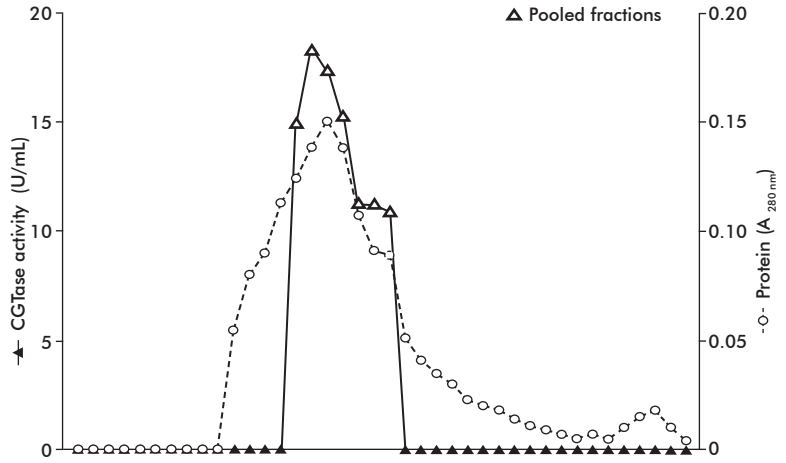

Figure 7. Elution profile of CGTase from *Alkalihalobacillus lehensis* strain JO-26 on DEAE Sepharose 6B Effect of *Alkalihalobacillus lehensis* JO-26.

the *A. lehensis* CGTase was obtained with a specific activity of 78.37 U/mg and a purification fold of 5.27, and a yield of 44.64 %. Its partial purification in a recovery of 87.3 % activity and 40-fold purification [37]. In this regard, More *et al*. [38] reported a yield of 49.44 % from *Bacillus halodurans*, using starch adsorption. A purification fold of 6.1 has been reported for CGTase from *B. flexus* SV1 [16]. Also, a purification fold of 21 and yield of 26.40 % was obtained from *B. agaredhenes* [39]. Our analysis by SDS-PAGE of the CGTase gave an estimated molecular weight of

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52 kDa. Molecular weight of most CGTase is found within 50-110 kDa [25]. Differences in the genetic background of the different microorganisms could account for variations in the molecular weight among the different CGTases [31].

When studying the effect of pH on enzyme activity, an optimum pH of 9.0 was obtained, with a broad active range for pH 4.0-11.0. This was in agreement with pH values for a CGTase isolated from *Bacillus* sp ND1 and *Bacillus agaradhaaerens* LS-3C [40]. Moreover, Moriwaki *et al*. [41] working on CGTase from *Bacillus sphaericus* found that the enzyme activity was retained at alkaline pH 8.0, for up to 4 h at 50 °C. The high activity of CGTase at alkaline pH makes it generally preferred for industrial uses in processes requiring extreme pH conditions [29].

Regarding the optimum temperature for CGTase from *A. lehensis* JO-26, it was determined that enzyme activity was optimal at 55 °C, with an active range of 45-65 °C. Similarly, an optimal temperature of 60 °C was reported for CGTase from *B. flexus* sv1 [16]. In this sense, Ibrahim *et al*. [42] found that CGTase from A*mphibacillus* sp. NPST-10 had a broad temperature range of 45-75 °C. A slightly higher optimal temperature of 70 °C has also been characterized for the CGTase from *Bacillus* sp. N227 [43]. Mostly, an optimal range of 55-65 °C is common to alkalophiles [25, 44, 45].

In conclusion, the isolate *A.lehensis* JO-26 is a promising candidate for CGTase production. The isolated CGTase enzyme was found to be active at alkaline pH and at a broad temperature range. In addition, the gene could be isolated and expressed in other organisms for maximal production or even genetically modified in order to improve CGTase activity yields and performance. This could support industrially significant biotechnological applications of the enzyme, further improving the molecule's properties and applications for a wide array of resulting products. Further characterization of the enzyme is recommended to scale up the industrial application of the enzyme.

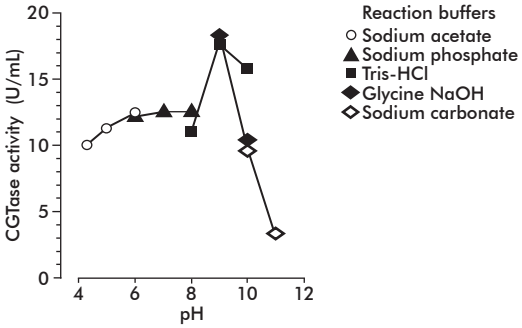

Figure 8. Effect of pH and enzyme reaction buffers on the activity of CGTase from *Alkalihalobacillus lehensis* strain JO-26.

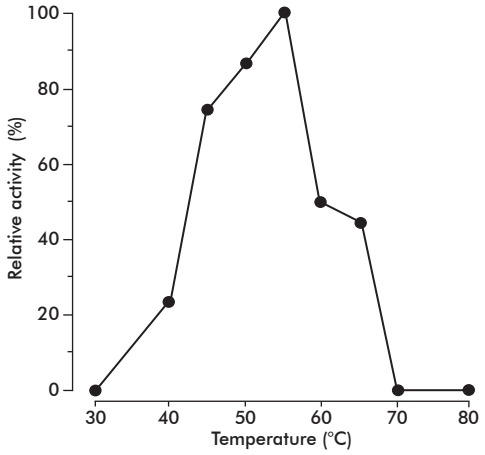

Figure 9. Effect of temperature on the activity of CGTase from *Alkalihalobacillus lehensis* strain JO-26.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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