Research Article Open Access

Characterization of important new industrial enzymes CGTase and their application in the transglycosylation of stevia glycosides

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Submission Date: September 9th, 2025, **Acceptance Date:** December 1st, 2025, **Publication Date:** December 4th, 2025

Please cite this article as: Ghochikyan V., Avetisova G., Soghomonyan T., Manukyan L., Keleshyan S., Melkonyan L. Characterization of important new industrial enzymes CGTase and their application in the transglycosylation of stevia glycosides. *Functional Food Science* 2025; 5(12): 717 – 732. DOI: https://doi.org/10.31989/ffs.v5i12.1773

ABSTRACT

Background: *Stevia rebaudiana* is a key source of natural sweeteners, but its glycosides, such as stevioside, have limitations in terms of sweetness and taste. Cyclodextrin glycosyltransferase (CGTase), an enzyme that catalyzes cyclodextrin formation, has potential to enhance stevia glycosides via transglycosylation. *Alkalophilic Bacilli*, which thrive in high-pH environments, have emerged as a promising source of CGTase. The present study focuses on the following objectives: firstly, the isolation and identification of CGTase-producing alkalophilic Bacilli; secondly, the characterization of the enzyme; and thirdly, the investigation of its application in improving stevia glycoside production, with a view to potentially leading to more efficient and sustainable sweeteners.

Objective: is to isolate and identify CGTases-producing new strains and to characterize the enzymes in terms of molecular weight, temperature, and pH optimization, heat stability, metal tolerance, and hydrolytic and cyclizing activities.

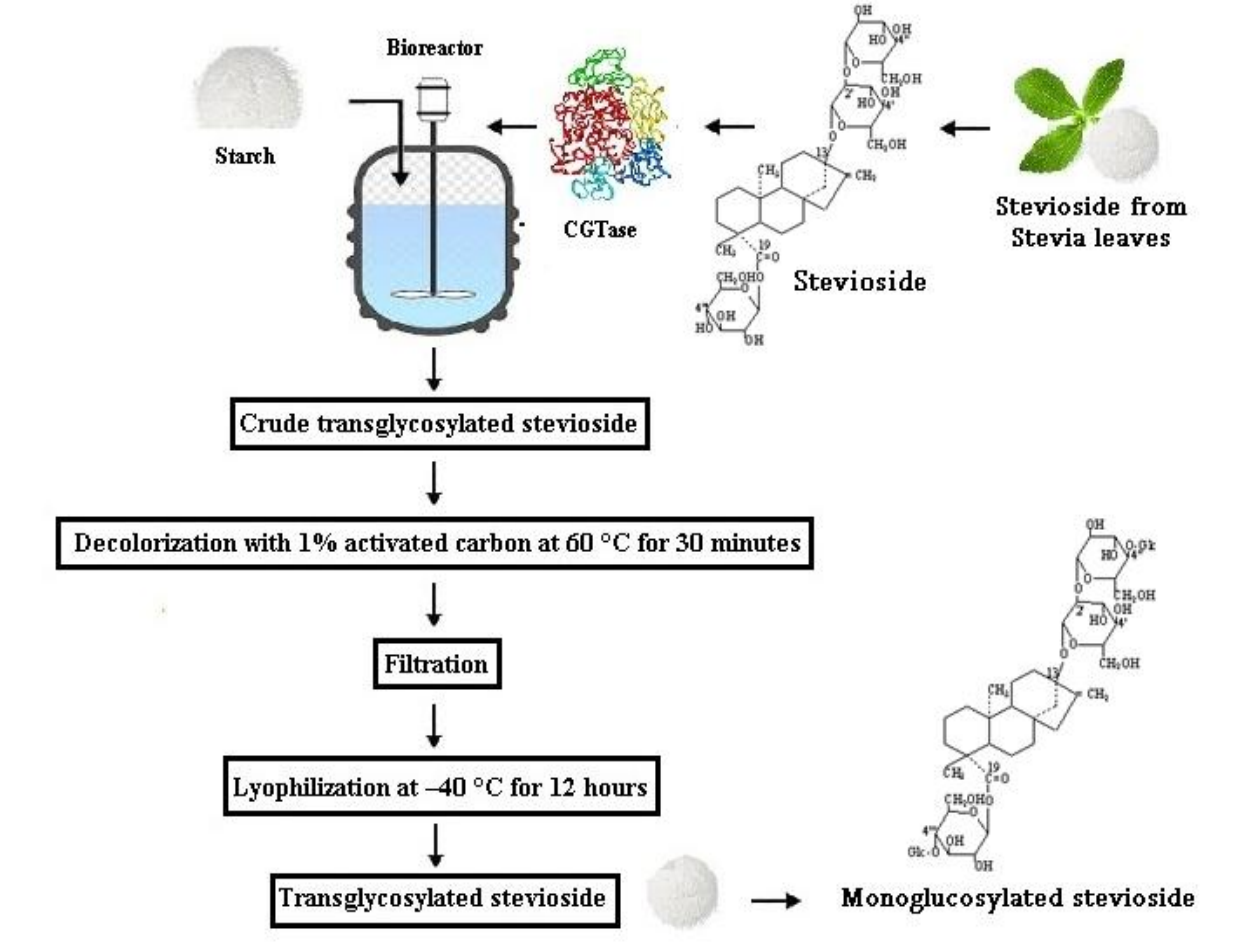
Methods: The "Harikoshi" medium was used for the isolation and cultivation of CGTase-producing strains. To identify the strains under discussion, a molecular taxonomic investigation was undertaken. The purified enzymes were characterized using SDS-PAGE, electrophores method. The biochemical characteristics of the enzymes were determined using standard accepted methods.

Results: Based on 16S rRNA analysis, strains A-12 and A-19 isolated from saline soils were identified as *Halalkalibacter akibai* and *Salipaludibacillus agaradhaerens*, respectively. The maximum CGTase activity for strain A-12 was observed at 60°C, while for A-19 it was at 65°C. The pH optima for A-12 and A-19 CGTases were determined to be 8.5 and 9, respectively. In optimal conditions, the enzyme activities were 115U/mL (A-12) and 140U/mL (A-19). Subsequently,

optimal conditions were developed for the transglycosylation of Stevia glycosides and the decolorization of the obtained products.

Conclusion: The purification and characterization of a novel CGTase from alkalophilic Bacilli has the potential to significantly impact the development of more efficient and sustainable stevia-based sweeteners. This research contributes to the potential industrial application of CGTases for improving the quality of natural sweeteners, with implications for the food and pharmaceutical industries. The study demonstrated that CGTases produced by alkalophilic cultures can function as effective biocatalysts in the enzymatic transglycosylation of stevia glycosides using starch as a donor.

Keywords: Alkalophilic Bacilli, Cyclodextrin glycosyltransferase (CGTase), stevia glycosides, transglycosylation, stevioside modification, sustainable sweeteners



Graphical Abstract: Biocatalytic Transglycosylation of Stevia Glycosides by Novel Alkalophilic CGTases

INTRODUCTION

The growing global demand for natural, low-calorie sweeteners in food technology, nutrition, and medicine stems from increasing consumer awareness of metabolic and lifestyle-related diseases. Among these sweeteners, diterpenoid glycosides such as stevioside and rebaudioside A, derived from Stevia rebaudiana Bertoni, have attracted considerable attention due to their

intense sweetness—approximately 250–300 times that of sucrose—and diverse pharmacological benefits, including hypoglycemic, hypolipidemic, anti-inflammatory, diuretic, and gastroprotective activities [1]. However, their application remains limited by undesirable sensory characteristics, particularly bitterness and a lingering aftertaste [2-3].

Structurally, steviol glycosides share a common steviol backbone but differ in the number and linkage of glycosidic residues at the C13 and C19 positions. Modifying these positions through enzymatic glycosylation has been shown to substantially improve the sweetness profile and reduce bitterness [4]. Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is a multifunctional enzyme that catalyzes the intramolecular and intermolecular transfer of α-(1,4)-linked glucose units from starch to acceptor molecules, yielding glucosylated derivatives with enhanced solubility and improved sensory perception [5-6]. The number and positioning of these transferred glucose units critically determine the resulting sweetness intensity and overall flavor profile [7]. Recent studies have identified alkaliphilic and halophilic microorganisms as valuable sources of highly active and stable CGTases suitable for industrial processes [8]. For instance, *Shouchella oshimensis* P-106 produces a β-CGTase that operates efficiently under alkaline conditions (pH 9.0, 30°C) with minimal α- and γ-CGTase activity, demonstrating remarkable substrate specificity [9]. Similarly, *Bacillus* sp. T1 exhibits strong catalytic activity and stability in alkaline environments, emphasizing the biotechnological potential of these enzymes [10].

Particularly noteworthy is the CGTase from *Bacillus agaradhaerens* (now *Salipaludibacillus agaradhaerens*) LS 3C, isolated from an Ethiopian soda lake. The purified enzyme (~110 kDa, monomeric) displays extreme pH stability (5.0–11.4), with optimal activity at pH 9.0 and 55°C, and predominantly produces β-cyclodextrins. Gene sequence analysis revealed a 679 aa enzyme (~76 kDa mature form) showing high homology to other *B. agaradhaerens* strains but also unique substitutions in conserved residues, marking it as an "intermediary enzyme" within the GH13 family. Immobilized variants of this enzyme demonstrated enhanced operational stability, underscoring their potential for continuous industrial biocatalysis [11].

Most recently, a novel CGTase from *Alkalihalobacillus oshimensis* CGMCC 23164 (designated CGTase-13) exhibited exceptional transglycosylation efficiency toward stevioside and rebaudioside A, achieving conversion rates of 86.1% and 90.8%, respectively, surpassing all reported commercial enzyme systems to date. The resulting glucosylated products exhibited significantly improved sensory profiles compared to those generated by Toruzyme® 3.0 L, including reduced bitterness, smoother sweetness onset, and higher sweetness intensity [12].

Building upon these advances, the present study focuses on the systematic characterization of cyclodextrin glucanotransferases derived from two novel alkaliphilic bacterial isolates—*Halalkalibacillus akibai* A12 (MDC 3522) and *Salipaludibacillus agaradhaerens* A19 (MDC 3523). The research aims to elucidate the biochemical and catalytic properties of these CGTases, including enzyme kinetics, pH and temperature optima, stability profiles, and substrate specificity. Moreover, their transglycosylation performance in modifying steviol glycosides is evaluated under variable operational parameters, providing a foundation for their potential application in producing improved, natural, low-calorie sweeteners with desirable organoleptic attributes.

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METHODS

Isolation and Cultivation of Alkalophilic CGTase-Producing Strains: Alkalophilic bacterial strains capable of producing cyclodextrin glycosyltransferase (CGTase) were isolated from saline–alkaline soils collected in the Armavir region of the Republic of Armenia. The sampling area is characterized by semi-arid climate conditions, light-textured alkaline soils with elevated sodium carbonate content, and moderate salinity, which provide a favorable ecological niche for alkaliphilic and halotolerant microorganisms.

Soil samples were suspended in sterile distilled water (1:3, w/v), thoroughly mixed, and allowed to sediment. The supernatant was subsequently used as an inoculum for enrichment cultures. Enrichment was performed under aerobic conditions at 28–37 °C for 4–5 days using a medium containing starch as the sole carbon source.

For subsequent cultivation, Horikoshi medium was prepared with the following composition (% w/v): starch 1.0; peptone 0.5; yeast extract 0.5; NaCl 0.5; KH_2PO_4 0.1; $MgSO_4 \cdot 7H_2O$ 0.02; Na_2CO_3 1.0. The medium was sterilized by autoclaving at 121 °C for 15 min, and the pH was adjusted to 9.0 prior to inoculation. Cultivation was carried out at 37 °C with shaking at 220 rpm [13].

Pure cultures were obtained by streak plating from the enrichment cultures onto solidified Horikoshi medium in Petri dishes and subsequently maintained under alkaline conditions for further screening and enzyme characterization.

Morphological and Microscopic Characterization: Bacterial cells were stained using the Gram method. Spore staining was performed following the Ozhesko method, while flagella staining was carried out according to Leifson's method as modified by Peshkov [14]. Morphological features were observed using a Leica DM500 trinocular microscope (×1000 magnification) equipped with Leica EC3 digital camera software (×10 magnification) (Leica Microsystems GmbH, n.d.). The preliminary identification of bacterial cultures was performed according to [15].

Molecular Characterization (16S rRNA Gene Sequencing): Genomic DNA was extracted from pure bacterial cultures using the phenol–chloroform method [16]. The 16S rRNA gene was amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [17]. Purified PCR products were subjected to high-throughput sequencing using the Illumina MiSeq platform (Illumina, USA). The raw reads were processed by quality filtering and assembly with the QIIME2 pipeline [18]. The resulting consensus sequences were compared with reference sequences available in the NCBI GenBank database using the BLASTn algorithm.

Fermentation in the Bioreactor: Scale-up fermentation was performed in a 10-liter bench-top bioreactor (Labfrez BIOF-10L, China). The working volume was 6 liters. The fermentation medium was the same as described above and sterilized in situ before inoculation. Operational parameters: agitation speed 800rpm, temperature 30°C, aeration rate 1.5vvm, pH maintained at 9.0, antifoam added manually. Inoculation volume was 10% (v/v). Fermentation lasted 24-30 hours. Samples were taken to monitor OD600, pH, and enzyme activity. The culture broth was centrifuged at 10,000×g for 15 min at 4°C to obtain the cell-free supernatant.

Ultrafiltration and Ammonium Sulfate Fractionation: The cell-free supernatant was subjected to ultrafiltration using a Millipore Pellicon 2 Mini tangential flow filtration system equipped with 100kDa and subsequently 50kDa polyethersulfone (PES) membranes. Sequential ultrafiltration enabled stepwise concentration and partial purification of the extracellular enzyme fraction. The

retentates were collected and used for further purification [19].

For protein fractionation, the concentrated enzyme solution was precipitated with ammonium sulfate. Solid ammonium sulfate was slowly added to the enzyme solution under constant stirring at 4°C to reach defined saturation levels (30%, 50%, and 70%). After each saturation step, the mixture was equilibrated for 2 h and then centrifuged at 10,000×g for 20 min at 4°C. The resulting pellets were resuspended in 50mM Tris-HCl buffer (pH-8.0) and dialyzed overnight against the same buffer to remove residual salt. The dialyzed fractions were subsequently assayed for protein concentration and enzyme activity. [20]

Protein Concentration Determination: Protein content was determined by the Bradford method based on the absorbance of the enzyme-Coomassie Brilliant Blue complex at 595nm, using bovine serum albumin (BSA) as a standard [21].

SDS–PAGE Analysis: The purity and apparent molecular weights of the proteases were assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) conducted on 12% polyacrylamide gels, according to the method of Laemmli [22]. Molecular weight estimation was performed using a prestained ROTI®Mark Tricolor Protein Marker (10–245kDa, Carl Roth GmbH, Germany).CGTase Cyclization Activity Assay: Cyclization activity was assayed using the following reaction mixture: 2mL of 2% potato starch solution in the appropriate buffer and 0.5mL of crude enzyme. Incubation at 50°C. At time intervals (0.5-1.0min), 600µL samples were withdrawn and mixed with 900µL of phenolphthalein or bromocresol green reagent. One unit of activity was defined as the amount of enzyme producing 1µmol of β-cyclodextrin per minute [23].

CGTase Transglycosylation Activity Assay: The reaction mixture contained 4U of CGTase, 10 mg soluble starch, 50 mM sucrose, and 10 mM $CaCl_2$ in 1 mL of 0.1M phosphate buffer. Incubated at 50°C for 15 minutes. Reaction was stopped by boiling, centrifuged at 8000×g for 15 minutes. HPLC determined Maltosyl-fructose. One unit was defined as the amount forming 1µmol maltosyl-fructose per minute [24].

Concentration of Stevia Glycoside Solutions: Solutions were concentrated using a vacuum rotary evaporator (Laborota 4000/HB/G3, Heidolph, Germany).

Decolorization of Stevioside Derivatives: The obtained stevioside derivative solutions were subjected to a decolorization step prior to further analysis. Activated charcoal (1% w/v; Norit®, France) was added to the concentrated glycoside solution, and the suspension was incubated at 60 °C with constant stirring for 30 minutes. After treatment, the mixture was filtered through Whatman No.1 filter paper to remove the charcoal together with adsorbed colored impurities. The clarified and decolorized solutions were then used for subsequent analyses and assays.

Thermal Stability Assay: 10mg of CGTase enzyme was dissolved in 1mL saline and incubated at different temperatures (30-90°C) for 60 minutes. Residual activity was determined [25].

pH Stability Assay: 10mg of enzyme was dissolved in buffer solutions (pH 5.0-10.0 citrate-phosphate, pH 6.5-9.0 phosphate, and 0.5-1.0% Na_2CO_3), incubated for 60 min, and residual activity measured [26].

Statistical Analysis: Statistical analysis results are presented as mean values of three to five independent experimental measurements, with mean-squared deviations (error bars). Calculations were performed using Microsoft Excel 16, and the probability values were determined using SPSS 16.0.

RESULTS

Isolation and Preliminary Characterization: After three successive purifications by streaking on solidified Horikoshi medium, two alkalophilic cyclodextrin glycosyltransferase (CGTase)-producing strains (designated A-12 and A-19) were successfully isolated in pure culture. Their morphological, physiological, and biochemical characteristics were examined.

Morphological Characteristics: Strain A-12. The cells are Gram-positive, motile rods measuring 0.5-0.6×2.0-3.6µm, capable of forming terminally located oval spores that cause cell swelling. On agar medium, strain A-12 forms circular, bright-yellow, greasy, and glossy colonies with a smooth surface, flat profile, even margins, and a homogeneous structure. In liquid culture, strain A-12 develops a dense, smooth, and spreading pellicle.

Strain A-19. The cells are Gram-positive, motile rods measuring 0.5-0.7×2.0-3.5µm, producing terminally located oval spores that markedly distend the cell. On agar medium, strain A-19 forms circular, white, opaque colonies with a rough surface, convex profile, wavy margins, and a homogeneous internal structure. In liquid culture, strain A-19 produces a thin, smooth, ring-shaped, spreading pellicle.

Molecular Identification: Initial identification based on morphological and physiological features suggested that both isolates belonged to the genus Bacillus. However, subsequent 16S rRNA gene sequencing provided a more precise taxonomic assignment. Strain A-12 was identified as *Halalkalibacter akibai* (GenBank accession no. PQ 867136), whereas strain A-19 was identified as *Salipaludibacillus agaradhaerens* (GenBank accession no. PQ 867137).

Physiological and Biochemical Characterization: The examination of the isolated alkalophilic cultures revealed that they exhibited generally similar physiological and biochemical characteristics. Specifically, they demonstrated adverse reactions for indole production, dihydroxyacetone formation, and urease activity, while showing positive reactions for catalase production, acetylmethylcarbinol (Voges–Proskauer test), and casein degradation (Table 1).

Table 1. Morphological and physiological–biochemical characteristics of alkalophilic CGTase-producing cultures A-12 and A-19.

	A-12	A-19
Cell morphology	Rod-shaped, 0.5-0.6 × 2.0-3.6µm, Gram-positive, motile, peritrichous flagellation	Rod-shaped, 0.5-0.7 × 2.0-3.5µm, Gram-positive, motile, peritrichous flagellation
Spore shape	Oval	Oval
Spore position	Terminal	Terminal
Sporangium swelling	+	++
Catalase	+	+
Dihydroxyacetone production	–	–
Voges–Proskauer reaction	+	+
Lecithinase reaction	–	–
Nitrate reduction	+	–
Starch hydrolysis	+	+
Citrate utilization	–	+
Casein degradation	+	+
Gelatin liquefaction	+	+
Acid production from glucose, arabinose, sucrose, mannitol	+ + + +	+ + + +

	A-12	A-19
Gas production from glucose, arabinose, sucrose, mannitol	– – – –	– – – –
Growth at pH 5.7-7.0	–	–
Growth at 30°C, 37°C,50°C, 65°C	+ + – –	+ + – –
Phenylalanine deamination	–	–
Growth at NaCl 5%, 7%, 10%, 15%	+ + + +	+ + – –
Urease production	–	–
Indole production	–	–
H_2S production	–	–
Tyrosine assimilation	–	–

Both strains demonstrated largely similar morphological and biochemical profiles, characterized by rod-shaped, Gram-positive, motile cells with terminal oval spores. They were catalase-positive, casein- and starch-degrading, and capable of acid production from multiple carbohydrates. However, differences were observed in nitrate reduction, citrate utilization, and tolerance to higher NaCl concentrations, indicating subtle physiological divergence between strains A-12 and A-19.

Enzyme Production in Bioreactor: Both alkalophilic strains, *Halalkalibacter akibai* A-12 and *Salipaludibacillus agaradhaerens* A-19, were cultivated under controlled optimal growth conditions in a 10L bench-top bioreactor with a working volume of 6L. Growth proceeded for 30h under optimized parameters (30°C, pH 9.0, 800rpm, 1.5 vvm aeration), after which enzyme activity in the culture supernatant was measured. The results demonstrated that strain A-12 achieved an extracellular CGTase activity of 21U/mL, while strain A-19 reached 33U/mL. These findings indicate that both strains are efficient enzyme producers under alkaline fermentation conditions, with strain A-19 exhibiting comparatively higher productivity.

Ultrafiltration and Ammonium Sulfate Fractionation: The culture supernatant obtained after fermentation was subjected to sequential ultrafiltration using a Millipore Pellicon 2 Mini tangential flow filtration system. Initially, the broth was concentrated threefold with a 100kDa polyethersulfone (PES) membrane, allowing the removal of high-molecular-weight impurities. The permeate was then further concentrated threefold using a 50kDa PES membrane, resulting in the enrichment of the extracellular enzyme fraction while reducing low-molecular-weight contaminants. After ultrafiltration, the CGTase activity of the concentrated culture fluids reached 115 U/mL for strain A-12 and 140 U/mL for strain A-19, indicating a substantial increase in enzyme enrichment compared with crude broth.

To further purify the enzyme, the concentrated preparation was subjected to ammonium sulfate precipitation. Solid ammonium sulfate was gradually added to the enzyme solution at 4°C with continuous stirring. The enzyme activity was primarily recovered in the 50-70% saturation range, where the majority of contaminating proteins remained soluble at lower salt concentrations. After centrifugation, the precipitated protein fractions were resuspended in Tris-HCl buffer (50 mM, pH-8.0) and dialyzed to remove residual ammonium sulfate. The dialyzed fraction retained significant CGTase activity, confirming that the enzyme was efficiently enriched within the 50–70% saturation fraction.

Tables 2 and 3 below summarize stepwise purification parameters for CGTase produced by strains A-12 and A-19. Calculations are based on a crude protein concentration of in 6,000mL culture supernatant and measured enzyme activities at each purification step.

Table 2. Purification of CGTase from strain A-12 by ultrafiltration and ammonium sulfate fractionation

Purification step	Volume (mL)	Total activity (U)	Total protein (mg)	Protein (mg/mL)	Specific activity (U/mg)	Recovery (%)
Fermentation broth	6000	156,000	55,200	9.20	2.83	100.0
Ultrafiltration (100→50kDa)	1,150	132,250	20,700	18.00	6.39	84.8
$(NH_4)_2SO_4$ fraction (50-70%)	200	86,000	3,040	15.20	28.29	55.1

Table 3. Purification of CGTase from strain A-19 by ultrafiltration and ammonium sulfate fractionation

Purification step	Volume (mL)	Total activity (U)	Total protein (mg)	Protein (mg/mL)	Specific activity (U/mg)	Recovery (%)
Fermentation broth	6000	222,000	58,200	9.70	3.81	100.0
Ultrafiltration (100→50kDa)	1350	189,000	22,950	17.00	8.24	85.1
$(NH_4)_2SO_4$ fraction (50-70%)	200	133,000	3,308	16.54	40.21	59.9

SDS–PAGE Analysis: The molecular weights of the purified enzymes were estimated by SDS–PAGE. As shown in Figure 1, the major protein bands corresponding to enzymes A-12 and A-19 migrated at approximately 43kDa and 41kDa, respectively. Lane M contained the Roti Mark tricolor protein marker. Lanes 1 and 2 (A-12 and A-19 after ultrafiltration, respectively) showed partially purified preparations with fewer background bands than the crude culture supernatant. Lane 3 (A-19 culture supernatant) showed a heterogeneous protein mixture, reflecting the presence of numerous extracellular components before purification. Lanes 4 and 5 (A-12 and A-19 after ammonium sulfate fractionation, respectively) demonstrated further enrichment of the enzymes, with distinct single bands corresponding to the expected molecular sizes.

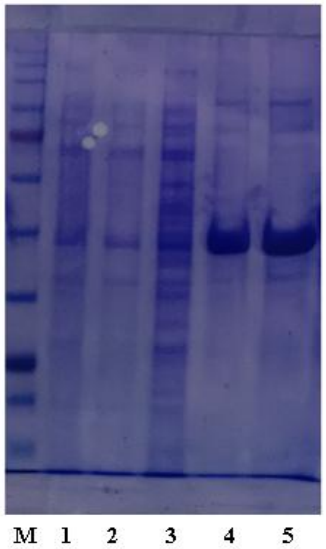

Figure 1. SDS–PAGE analysis of enzymes A-12 and A-19. Lane M: Roti Mark tricolor protein marker; Lane 1: A-12 enzyme after ultrafiltration; Lane 2: A-19 enzyme after ultrafiltration; Lane 3: A-19 culture supernatant; Lane 4: A-12 enzyme after ammonium sulfate fractionation; Lane 5: A-19 enzyme after ammonium sulfate fractionation.

These results confirm that the applied purification strategy effectively enriched the target enzymes, yielding preparations with dominant bands consistent with the typical molecular weights of cyclodextrin glycosyltransferases.

Biochemical Characterization of Enzymes A-12 and A-19Temperature optimum: The effect of temperature on enzyme activity was evaluated within the range of 30-90°C for the preparations obtained from strains A-12 and A-19 (Figure 2). Enzyme activities were relatively low below 40°C, reaching only 59% and 54% of the maximum activity for A-12 and A-19, respectively. A progressive increase was observed between 45°C and 55°C, with both enzymes showing pronounced increases in activity.

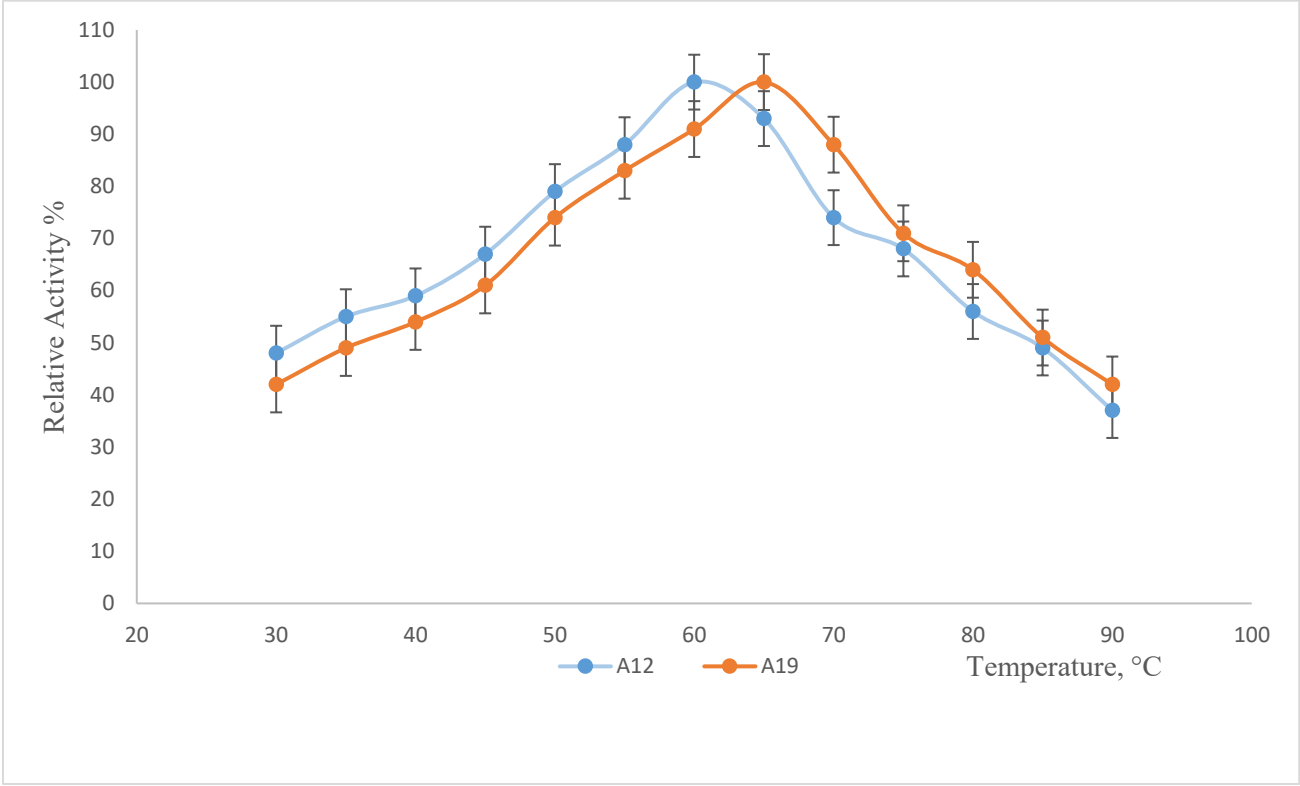

Figure 2. Effect of temperature on the relative activity of enzymes from strains A-12 and A-19. *Enzyme activities were measured in the range of 30-90°C, and the maximum activity observed for each strain was set as 100%.

The enzyme from strain A-12 displayed maximal activity at 60°C (100%), after which activity gradually decreased, retaining 56% at 80°C. In contrast, the enzyme from strain A-19 exhibited its maximum activity at 65°C (100%) and maintained comparatively higher activity at 70°C (88%). A sharp decline in activity was recorded above 75°C for both enzymes.

These results demonstrate that the enzyme produced by strain A-12 has an optimum temperature of 60°C, whereas the enzyme from strain A-19 shows a higher optimum at 65°C and exhibits broader thermal stability.

pH optimum: The effect of pH on the activity of enzymes produced by strains A-12 and A-19 was examined over the pH range 5.0-10.0 (Figure 3). Both enzymes showed relatively low activity under acidic conditions (pH 5.0-6.0), with activities not exceeding 62%. A gradual increase was observed as the pH approached neutral values, with A-12 and A-19 retaining 76% and 71% of their maximal activities at pH 7.0, respectively.

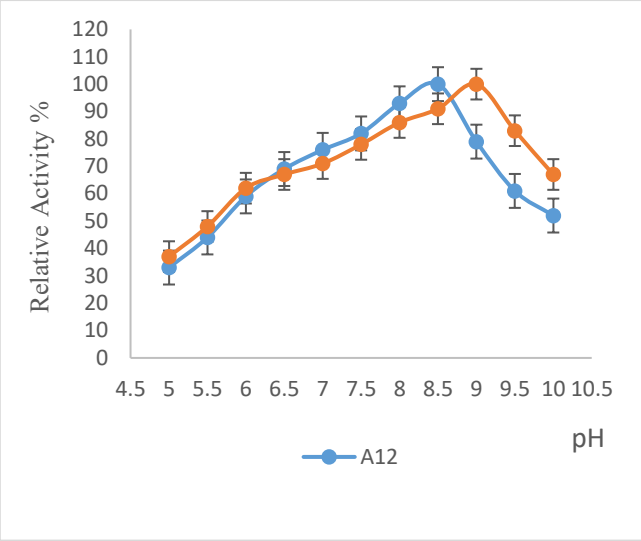

Figure 3. Effect of pH on the relative activity of enzymes from strains A-12 and A-19. *Enzyme activities were determined over the pH range of 5.0-10.0, with the maximum activity for each strain considered as 100%.

The highest activity for the enzyme from strain A-12 was observed at pH 8.5, where it reached 100% of its relative activity, followed by a decline at higher pH values. In contrast, the enzyme from strain A-19 exhibited maximum activity at pH 9.0, with 100% relative activity, and maintained relatively high activity at pH 9.5 (83%).

Overall, these results indicate that the enzyme from strain A-12 is optimally active at pH 8.5, whereas the enzyme from strain A-19 displays an alkaline optimum at pH 9.0 and broader stability under alkaline conditions.

Thermal Stability: The thermal stability of enzymes from strains A-12 and A-19 was evaluated over the temperature range of 40-90°C (Figure 4). Both enzymes retained more than 90% of their initial activity up to 45°C. At 50°C, the residual activities decreased to 86% for A-12 and 84% for A-19, followed by a gradual decline at higher temperatures.

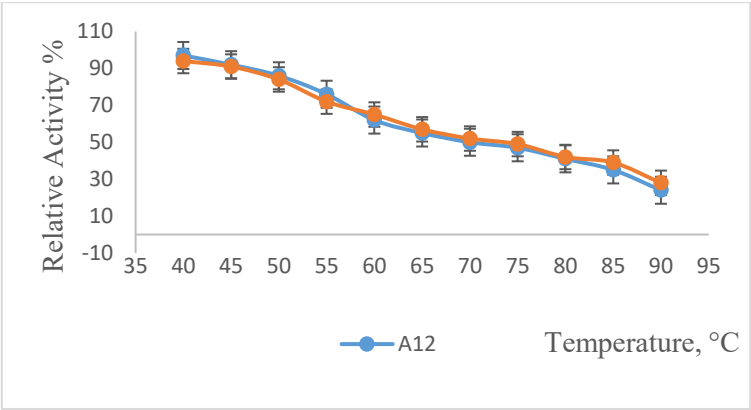

Figure 4. Thermal stability of enzymes from strains A-12 and A-19. *Residual activities were measured after incubation at different temperatures (40-90°C), with activities at 40°C considered as 100%.

At 60°C, the enzymes preserved approximately 62% (A-12) and 65% (A-19) of their activities, indicating moderate thermostability. Further temperature increases resulted in progressive loss of activity, with both enzymes maintaining around 50% of their initial activity at 70°C. At 80°C, the residual activities dropped

to 41% (A-12) and 42% (A-19). At 90°C, only 24% and 28% of the activity was retained for A-12 and A-19, respectively.

These results demonstrate that both enzymes are moderately thermostable, with residual activity detectable even at 90°C. Strain A-19 showed slightly higher resistance at elevated temperatures compared to A-12.

Effect of pH on Enzyme Thermal Stability: The dependence of enzyme thermal stability on pH was evaluated by incubating enzyme preparations for 20 min at the temperature corresponding to 50% inactivation in 20 mM buffer solutions spanning pH 2.0-12.0. Following incubation, the samples were rapidly cooled to 4°C in a water bath, and the residual activity was measured under standard assay conditions at pH-6.0.

As shown in Figure 5, both enzymes A-12 and A-19 displayed distinct pH-dependent stability profiles. At highly acidic conditions (pH 2.0-3.5), the residual activity of both enzymes remained below 40%. A gradual increase in stability was observed as the pH approached neutrality, with enzyme A-12 reaching maximal residual activity (100%) at pH-8.5, while enzyme A-19 retained the highest activity (100%) at pH-9.0. Both enzymes exhibited broad stability within the near-neutral to slightly alkaline range (pH 6.0-9.5), maintaining over 70% of their activity. In contrast, at more alkaline conditions (pH 11.0-12.0), stability decreased significantly, with residual activities falling to 37% and 44% for A-12 and A-19, respectively.

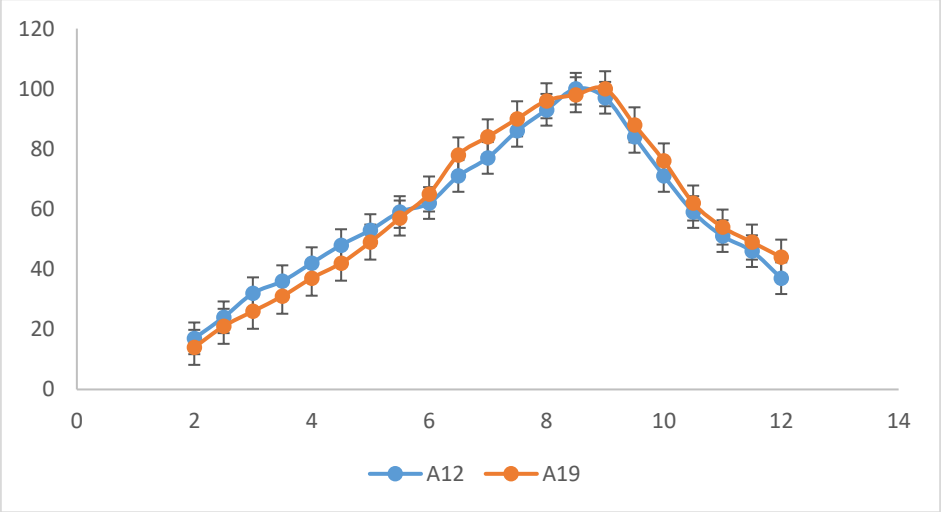

Figure 5. Effect of pH on the thermal stability of enzymes A-12 and A-19.

Overall, these findings indicate that both enzymes demonstrate maximum thermal stability under near-neutral to mildly alkaline conditions, with enzyme A-12 being most stable at pH-8.5, and enzyme A-19 at pH-9.0.

Effect of Metal Ions on CGTase Activity: The influence of various metal ions on CGTase activity produced by alkalophilic strains A-12 and A-19 was systematically investigated to assess the enzyme's structural stability and catalytic dependence on divalent cations.

Calcium (Ca^{2+}) markedly enhanced enzyme activity, restoring it to 100% in both strains compared to 68% and 65% in the metal-free control. Magnesium (Mg^{2+}) also showed a strong stimulatory effect, maintaining 96% and 87% relative activity in strains A-12 and A-19, respectively. The activation by Ca^{2+} and Mg^{2+} is consistent with previous reports indicating that these ions stabilize enzyme conformation by neutralizing negatively charged residues and promoting the integrity

of the catalytic domain, especially around the conserved calcium-binding loop typical of GH13-family CGTases [2].

In contrast, transition metal ions such as Cu^{2+}, Ni^{2+}, and Co^{2+} exhibited notable inhibitory effects. Cu^{2+} caused severe activity loss, reducing residual activity to 10–17%, which can be attributed to its high affinity for thiol groups and potential to induce oxidative damage to catalytic residues. Similarly, Co^{2+} and Ni^{2+} decreased enzyme activity to 51–57% and 46–52% (estimated), respectively, likely by interfering with the active-site geometry and substituting essential structural ions.

Zinc (Zn^{2+}) moderately affected CGTase activity, possibly due to its partial competition with catalytic metal sites, whereas Fe^{2+} and Al^{3+} ions exhibited strong inhibitory effects, reducing enzyme activity to 31–16%. Heavy metals such as Ag^{+} and Hg^{2+} completely abolished CGTase activity, reflecting their known ability to irreversibly bind to cysteine and histidine residues critical for catalytic function.

Overall, the results demonstrate a distinct metal-ion dependence pattern: Ca^{2+} and Mg^{2+} act as essential stabilizers and activators, while Cu^{2+}, Ni^{2+}, and Co^{2+} are potent inhibitors, disrupting enzyme structure and activity. This behavior is characteristic of alkalophilic CGTases, where enzyme performance relies on maintaining the structural coordination between catalytic residues and stabilizing metal ions.

Table 4. Effect of metal ions on CGTase activity in alkalophilic cultures

Metal Ion	Concentration (mM)	Strain A-12 (%)	Strain A-19 (%)
None	-	68	65
Ca^{2+}	1.0	100	100
Mg^{2+}	1.0	96	87
Zn^{2+}	1.0	70	76
Co^{2+}	1.0	51	57
Fe^{2+}	1.0	33	40
Cu^{2+}	1.0	10	17
Ba^{2+}	1.0	16	11
Al^{3+}	1.0	14	16
Ag^{+}	1.0	0	0
Hg^{2+}	1.0	0	0

Stevioside Transglycosylation and Organoleptic Properties: Cyclodextrin glycosyltransferases (CGTases) from the new alkalophilic strains A-12 and A-19 were applied to the transglycosylation of stevioside using starch as a donor substrate under optimized conditions. The enzymatic reactions led to the formation of modified steviol glycoside derivatives with altered structural and sensory profiles.

Qualitative and sensory outcomes: Native stevioside is associated with bitterness scores averaging 6-7 on a 9-point hedonic scale. The modified glycosides showed reduced bitterness, with average scores of 2-3, reflecting a substantial decrease in lingering aftertaste [27-28].

Overall organoleptic improvement: Sensory evaluation conducted with assessing participants indicated that the modified steviol glycosides exhibited a noticeably smoother and more sucrose-like sweetness profile. Most participants described the glucosylated derivatives as having a more pleasant, balanced taste, with reduced bitterness and a faster onset of sweetness compared to the unmodified compound.

Overall, the sensory feedback demonstrated that CGTase-mediated transglycosylation effectively mitigates the characteristic bitterness of steviol glycosides, leading to an improvement in flavor harmony

and overall palatability. These results highlight the potential of the obtained derivatives as enhanced natural sweeteners suitable for applications in food and pharmaceutical formulations [29-30].

DISCUSSION

The two newly isolated alkalophilic strains, *Halalkalibacter akibai* A-12 and *Salipaludibacillus agaradhaerens* A-19, exhibited phenotypic and biochemical traits characteristic of Bacillus-related CGTase producers. Morphological and biochemical profiling revealed minor differences such as nitrate reduction and salt tolerance, distinguishing these isolates from previously reported alkalophiles. Both strains effectively secreted CGTase under alkaline fermentation, with A-19 demonstrating higher enzyme productivity and stability.

Sequential purification via tangential flow ultrafiltration and ammonium sulfate fractionation enriched the enzyme preparations, increasing specific activity and purity. SDS–PAGE confirmed molecular masses of approximately 43 kDa for A-12 and 41 kDa for A-19, consistent with CGTases of *Bacillus* origin. The enzymes exhibited optimal activity under moderately high temperatures and alkaline pH values (A-12 at 60 °C, pH 8.5; A-19 at 65 °C, pH 9.0). These properties underline their robustness for biotechnological processes that require stability under harsh conditions.

Importantly, the CGTases demonstrated distinct responses to metal ions. While Ca^{2+} and Mg^{2+} ions enhanced enzymatic activity, heavy metals such as Cu^{2+}, Ni^{2+}, and Co^{2+} showed clear inhibitory effects—an observation aligning with prior reports on metal-ion-dependent conformational changes in α-amylase-like enzymes. This balance between activation and inhibition reflects the structural adaptability of these biocatalysts, which could be harnessed for controlled industrial applications.

From an applied perspective, these CGTases hold promise for the modification of natural sweeteners such as stevioside through transglycosylation. The resulting glycosides exhibited smoother and more sucrose-like sweetness, reducing bitterness and improving overall flavor perception. These findings align with the functional food framework by promoting bioactive compound modification to enhance both nutritional and sensory qualities of products [31].

Overall, this study contributes novel insights into alkalophilic CGTase diversity, demonstrating the first isolation of Halalkalibacter akibai and Salipaludibacillus agaradhaerens from Armenian saline-alkaline soils. Their stability, substrate versatility, and ability to improve bioactive compounds through enzymatic transglycosylation highlight new directions in functional food biotechnology and enzyme-based flavor enhancement [32].

CONCLUSION

Two novel alkalophilic bacterial strains, *Halalkalibacter akibai* A-12 and *Salipaludibacillus agaradhaerens* A-19, were successfully isolated from saline-alkaline soils of Armavir Province, Armenia, expanding the known diversity of CGTase-producing Bacillus-related species. Both strains produced stable extracellular CGTase enzymes under alkaline fermentation, with A-19 showing superior yield and thermostability.

These CGTases demonstrated efficient transglycosylation of stevioside, leading to smoother sweetness and reduced bitterness—offering a sustainable enzymatic strategy for producing high-quality, natural sweeteners. Their thermostable and alkali-tolerant nature makes them valuable biocatalysts for applications in food and pharmaceutical formulations [32-33].

The novelty of this research lies in the discovery of two previously unreported alkalophilic producers from unique Armenian habitats and their demonstrated ability

to generate functionally improved bioactive compounds. This work bridges microbial enzyme technology with functional food science, supporting the design of next-generation low-calorie sweeteners that contribute to metabolic health and consumer acceptability [34].

Competing Interests: The authors have no financial interests or conflicts of interest.

Author's Contributions: Conceptualization, V. G., Methodology, G. A., L. Manukyan., T. S., Software, T. S.., Validation, L. Melkonyan., S. K.., Resources, V. G., Writing—review & editing, V. G., L. Melkonyan., T.S., All authors have read and agreed to the published version of the manuscript.

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