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Alkalihalobacillus lehensis M136, a Novel Alkaliphilic, Cyclodextrin Glucanotransferase (CGTase)-producing Isolate from Manleluag Hyperalkaline Spring in Pangasinan, Philippines

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A cyclodextrin glucanotransferase (CGTase)-producing bacterium was isolated from Manleluag hyperalkaline spring, Pangasinan, the first to be reported from a hyperalkaline environment in the Philippines. The isolate, M136, had the highest CGTase enzyme activity (46.01 U/mL) out of the forty-one similar bacterial isolates and exceeds the CGTase activity of the reference strain, *Paenibacillus* sp. JCM 9143. Using whole genome analysis, M136 was identified as *Alkalihalobacillus lehensis*. The CGTase enzyme produced by *Alkalihalobacillus lehensis* M136 was partially purified with 73.97% recovery and post-purification activity of 49.52 U/mL. The enzyme has an estimated molecular weight of 78.64 kDa and an isoelectric point of 4.72. Enzyme characterization revealed that it was stable at 30–80 °C (optimal activity at 70 °C), pH 4.0–10.0 (optimal activity at pH 6.0), and in the presence of different metal ions and reagents such as zinc, calcium, magnesium, and manganese, ethylenediaminetetraacetic acid, iodoacetic acid, sodium dodecyl sulfate, and phenylmethylsulfonyl fluoride.

Keywords: *Alkalihalobacillus lehensis* M136, alkaliphiles, cyclodextrin glucanotransferase, enzyme characterization, hyperalkaline spring, partial purification

INTRODUCTION

Alkaliphiles are groups of microorganisms that can grow well at pH values exceeding pH 9, and often in the pH range of 10–13 (Preiss *et al.* 2015). These are one of the extremophile classes with many applications, such as in detergent formulation, textile, leather processes, and pulp and paper production, as well as in the production of different important biochemicals, such as carotenoids, bioactive substances, siderophores, and biosurfactants (Mamo and Mattiasson 2020). In addition, alkaliphiles are reliable and major sources of enzymes. Microbial enzymes catalyze reactions that can withstand harsh chemical and physical conditions, as well as undesired by-products and toxic effluents (Chinnathambi 2015).

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One of the extracellular enzymes produced by alkaliphiles is cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19), a member of the glycoside hydrolase family 13 (Janecek *et al.* 2003; Lee *et al.* 2013; Zhou *et al.* 2020). This extracellular enzyme degrades starch and other 1,4-linked α-glucans to cyclodextrins by cyclization reaction (Mahat *et al.* 2004; Ramli *et al.* 2010). Cyclodextrins are closed-ring structures composed of 6, 7, and 8 glucosyl units referred to as α, β, and ϒ-cyclodextrin, respectively (Higuti *et al*. 2004). In nature, alkaliphilic microorganisms secrete CGTase to monopolize starch as substrate, converting it to cyclodextrins that cannot be utilized by competitive microorganisms (Leemhuis *et al.* 2010). From an industrial point of view, cyclodextrins are used to form inclusion complexes with hydrophobic organic and inorganic compounds, which in turn have numerous applications in food, pharmaceutical, cosmetics, agricultural, and environmental protection (Mahat *et al.* 2004). These highly valued, modified starches can change the physical and chemical properties of encapsulated guest compounds and, hence, increase the solubility and stability, reduce volatility, improve control of the release of drugs, and mask odors and flavors (del Valle 2004). Recently, CGTase was also reported to exhibit antimicrobial activity and play a major role in the biocontrol of fungal pathogens (Zhou *et al.* 2020). The cyclodextrin produced from the bioconversion of starch using CGTase can be tapped and investigated in future studies for other potential uses, such as a virucidal agent, destruction material for virus protein/ lipid shell, and vaccine adjuvant (Jones *et al.* 2020; Jicsinszky *et al.* 2021). Furthermore, CGTase is known to be utilized in the synthesis of various flavonoid glycosides and other transglycosylated products using various acceptors, such as sugar fatty acid esters, polyols, phenolic compounds, capsaicinoids, saponins, and vitamins (Lim *et al.* 2021; Lorthongpanich *et al.* 2022).

Here, we report the isolation, screening, and identification of a CGTase-producing isolate from Manleluag hyperalkaline spring in Mangatarem, Pangasinan, Philippines, as well as the partial purification, characterization, and analysis of the CGTase. To the best of our knowledge, this is the first report of alkaliphilic bacteria from a peculiar environment in the Philippines with CGTase activity.

MATERIALS AND METHODS

Sample Collection in Manleluag Hyperalkaline Spring

The sampling site Manleluag Spring National Park is located in Mangatarem, Pangasinan, Philippines (N 15º 42' 16" E 120º 16' 52"). Sediment and water samples were taken in February, May, August, and November 2017, totaling four sample collections. At each sampling point, the pH and temperature were recorded, and the average values were computed. The samples were immediately transported in an ice chest to the laboratory after collection.

The sediment and water samples were also submitted to the Earth Materials Science Laboratories of the National Institute of Geological Sciences, University of the Philippines Diliman, for major metal analyses using a microwave plasma atomic emission spectrometer.

Isolation of Alkaliphilic Bacteria from Water and Sediment

To maximize the efficiency of isolating microorganisms from the water samples, two set-ups were done: [1] 4 L of the water was filtered using two 0.22-µm nitrocellulose membrane filters of 47 mm diameter. After filtration, nitrocellulose membrane filters were aseptically placed together in 250 mL Horikoshi I broth (per L of medium: 10 g glucose, 5 g polypeptone, 5 g yeast extract, 1 g K_2, HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 50 mL 20% Na_2CO_3, and pH 10.4). These were incubated at 30 °C for 24 h with continuous shaking at 100 rpm to dislodge the cells from the membrane. The samples were plated onto Horikoshi I agar plates, which were incubated for at least 48 h at 37 °C. In the second setup, [2] the nitrocellulose membrane filter was placed directly on top of the Horikoshi I agar base plate after filtration and was then incubated at 37 °C for at least 24 h.

Similar to the water samples, sediment samples were processed in two setups. In brief, [1] 10 g of sediments were transferred aseptically to 250 mL of Horikoshi I broth and incubated at 30 °C with shaking at 100 rpm for 24 h. The samples were then plated onto Horikoshi I agar plates and were incubated for 48 h at 37 °C. In the second setup, [2] 10 g of the sediment was transferred to 90 mL of Horikoshi I broth without glucose. The samples were also plated onto Horikoshi I agar plates and incubated at 37 °C for 24 h.

All isolates were routinely purified and described in terms of cultural and morphological characteristics. These were preserved in 25% glycerol stocks stored at –20 °C and at -80 °C.

Qualitative Screening for CGTase-producing Alkaliphilic Isolates by Using the Phenolphthalein Plate Assay

Mass screening of the CGTase-producing isolates was performed following the method of Kamble and Gupte (2014). Six hundred seventy-two (672) isolates were inoculated in duplicates on alkaline Horikoshi II agar (per liter of medium: 10 g soluble starch, 5 g peptone, 5

g yeast extract, 10 g K_2,HPO_4, 0.2 g $MgSO_4$•$7H_2O$, 10 ml 20% Na_2CO_3, 0.3 g phenolphthalein, 20 g agar), adjusted to pH 10 using 20% (w/v) sodium carbonate. Plates were incubated at 37 °C for 72 h.

The yellowish zone of clearing formed around the colony was measured in mm and the ratio of a zone of clearing (Cz) was computed as follows (Mahmoudabadi *et al.* 2010):

$$Cz = \frac{colony\ diameter}{colony\ diameter\ zone\ of\ clearing} \quad (1)$$

Paenibacillus sp. JCM 9143 (Japan Collection of Microorganisms) served as a reference strain for this assay (Park *et al.* 1989). This is equivalent to *Bacillus circulans* Jordan (ATCC 21783).

To detect the overall top CGTase-producing alkaliphilic isolates, 20 isolates with low Cz values were chosen from the mass-screened CGTase-producing isolates and subjected to another qualitative phenolphthalein plate assay with an inoculum level normalized to an optical density reading of 0.3 or equivalent to 10^7 cells per mL.

Quantitative Phenolphthalein Assay of the CGTase-producing Alkaliphilic Isolates

Following the method of Ibrahim *et al.* (2012) with slight modifications, the cyclization activity of CGTase was determined by measuring the β-cyclodextrin (β-CD) forming activity. The top five isolates in the qualitative assay were inoculated in 50 mL of Horikoshi II broth containing soluble starch but without phenolphthalein. These were then incubated for 48 h at 30 °C with orbital shaking for 150 rpm. Cells and other insoluble materials were removed by centrifugation at 6000 rpm for 20 min at 4 °C. The cell-free supernatant was filtered through a 0.45-µm pore size membrane and was used as a crude enzyme solution for assaying enzyme activity.

Starch solution (1% w/v, 750 µL) was prepared in a 50 mM Tris-HCl buffer (pH 8.0) and pre-incubated at 50 °C for 5 min. A crude enzyme sample (100 µL) obtained from the supernatant was added to the reaction mixture and incubated for 20 min at 50 °C. The reaction was stopped by the addition of 375 µL of 0.15 M sodium hydroxide. Next, 100 µL of 0.02% (w/v) phenolphthalein in 5 mM sodium carbonate was added to the reaction and the optical density (OD) was measured at 550 nm after 15 min incubation at room temperature. One unit (U) of CGTase activity was defined as the amount of enzyme releasing one (1) µmol of β-CD per minute under the defined assay conditions. A standard curve was generated by preparing 80-800 µM of β-CD in 50 mM Tris-HCl (pH 8.0). Only the top five isolates were considered.

Biochemical Profile and Identification Using the Biomerieux Vitek 2 Identification System

The top isolate was submitted to the PNCM (Philippine National Collection of Microorganisms) at the National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños, for further analyses. VITEK 2 System *Bacillus* identification card (BCL card) was used to determine substrate utilization and/or assimilation. The card contained 46 substrates for measuring carbon source utilization, enzymatic activities, inhibition by 6.5% sodium chloride, and resistance to antibiotics (kanamycin, oleandomycin, and polymyxin B).

Whole Genome Analysis of the Top CGTase-producing Alkaliphilic Isolate

The selected top CGTase-producing isolate based on quantitative CGTase activity was identified through whole genome sequencing. The putative isolate was grown in 50 mL Horikoshi I broth for 48 h at 37 °C and was submitted to the Philippine Genome Center DNA Sequencing Core Facility (Quezon City, Philippines) for sequencing.

As reported by Manalang *et al.* (2019), the genomic DNA was extracted using the Purelink Genomic DNA Extraction Kit (Life Technologies Corporation, Carlsbad, CA, USA). A paired-end library was generated using Nextera XT Library Preparation Kit and sequenced with Illumina MiSeq v3 2 x 300-cycle kit (Illumina, Inc., San Diego, CA, USA).

The quality trimming was done using the Trimmomatic tool (Bolger *et al.* 2014), while *de novo* assembly was carried out using SPAdes (v. 3.11.1) (Bankevich *et al.* 2012). An assembly improvement software was also used to scaffold and gap-fill the assembly, and its quality was analyzed using QUAST 4.6 (Gurevich *et al.* 2013). The taxonomic classification of the top isolate was determined by using MiGA (Microbial Genome Atlas), with NCBI (National Center for Biotechnology Information) RefSeq and Prokaryotic databases (Rodriguez-R *et al.* 2018). Subsequently, contigs were reordered using Mauve Contig Mover with *B. lehensis* G1 NZ (Genbank accession number CP003923) (Darling *et al.* 2004) as the reference genome. Lastly, annotation was done using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova *et al.* 2013), as well as the Rapid Annotations using Subsystems Technology (RAST) version 2 RASTtk pipeline (Aziz *et al.* 2008; Brettin *et al.* 2015).

Purification of CGTase from the Top Alkaliphilic Isolate

Ammonium sulfate fractionation was performed, according to the method by Arce-Vazquez *et al.* (2016). Fractional precipitation was conducted, using 60, 70, and 80% (w/v) of ammonium sulfate. The enzyme was

collected by centrifugation at 10,000 rpm for 20 min at 4 °C. The precipitate was dissolved in a 50 mM Tris-HCl buffer at pH 8.0.

Following the protocol from Thombre (2012), 5 mL of the dissolved protein was dialyzed using a seamless cellulose tubing with a cut size of 14,000 Da (Sigma-Aldrich, USA) against distilled water for 6 h.

Quantitative Evaluation of Partially Purified CGTase

The total CGTase activity (U/mL) was also determined by following the previously described quantitative phenolphthalein assay. Bradford assay was also done to determine total protein concentration (mg/mL).

After the data for CGTase activity and the protein concentration were obtained, the specific activity (U/mg), yield (%), and purification fold were computed (Bender 2006).

Characterization of the Partially Purified CGTase based on Temperature, pH, and Presence of Metal Ions and Inhibitors

Based on the protocol by Ibrahim *et al.* (2012), the effect of temperature on CGTase activity was observed over a temperature range from 30–90 °C. The quantitative phenolphthalein assay was carried out, with 750 µL enzyme pre-incubated at different temperatures for 5 min. Blanks were treated the same way as the samples. The activity of the enzyme without pre-incubation was defined as 100%.

The CGTase activity was also examined over a range of pH (4.0–12.0) by using different buffers added in 1% (w/v) of soluble starch. For pH 4.0–6.0, 50 mM of sodium acetate was used, whereas 50 mM Tris-HCl was used for pH 7.0 and 8.0. As for pH 9.0 and 10.0, 50 mM of glycine-NaOH buffer was utilized, while 50 mM of carbonate-bicarbonate buffer was used to attain pH 11.0 and 12.0. The partially purified enzyme was pre-incubated in these buffers for 5 min, and the succeeding steps of the same assay protocol were followed. Considering the enzyme activity without pre-incubation at the best pH of 100%, a plot of the pH profile of the relative activity *versus* pH was generated.

To determine the effect of various metal ions and reagents on the CGTase activity, test ions (Fe^{2+} Cu^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}) and inhibitors [ethylenediaminetetraacetic acid (EDTA), mercuric chloride, iodoacetic acid, urea, sodium azide, sodium dodecyl sulfate (SDS), phenylmethylsulfonide (PMSF), and 2-mercaptoethanol] were normalized to a final concentration of 1 mM prior to analysis. Soluble starch (1% w/v) was added to each metal ion and inhibitor. The partially purified CGTase was pre-incubated in the metal ion and inhibitor solutions for 5 min. CGTase activity was calculated, wherein 100% was expressed as the relative percentage of the enzyme activity obtained in the absence of the metal ions and reagents.

Analysis of CGTase Amino Acid Sequences

The number of amino acid residues, molecular weight, and isoelectric point calculations of the deduced amino acid sequence of the CGTase of the top isolate was performed using ExPASy ProtParam (Gasteiger *et al.* 2005). The open reading frame (ORF) of the *cgt* gene was determined by submitting the nucleotide sequences in the ORF finder of NCBI, with default search parameters. The domains and motifs within the protein sequences were identified using SMART (Ponting *et al.* 1999). The amino acid sequences were also submitted to BLASTP (NCBI) with default settings to search for the sequence that had the highest percent identity. Further information about CGTase was accessed in UniProtKB (EMBL-Bank/GenBank/DDBJ databases). To predict and analyze the protein structure, the amino acid sequence was submitted to Phyre2, set at a normal modeling mode (Kelley *et al.* 2015).

Statistical Analysis

The data from the phenolphthalein assay (U/mL) was subjected to a one-way analysis of variance using SigmaPlot–Statistical Analysis Software v.14.5 (Systat, USA). Pairwise comparison using the Holm-Sidak method was done to compare the mean enzymatic activity of the isolates, with an overall significance level of 0.05.

RESULTS

Sample Collection in Manleluag Hyperalkaline Spring

The recorded temperatures in all quarters of sample collection across all sampling points were from 31–34 °C, whereas the pH ranged from 9.87–11.30. The major metal analyses also revealed that the elements calcium (sediments: 1.35–34.85%; water: 3.90–33.70 ppm), magnesium (sediments: 0.36–4.74%; water: 0.10–6.80 ppm), iron (sediments: 0.88–12.68%; water: 0.23–29.70 ppm), sodium (sediments: 0.16–1.68%, water: 15.00–19.50 ppm), and potassium (sediment: 0.02–0.15%, water: 0.30–3.70 ppm) were detected in the samples obtained from the hyperalkaline spring all throughout the four sampling periods.

Characterization of the Alkaliphilic Isolates from Quarters 1–4 Sample Collection

The alkaliphilic isolates differed in terms of opacity, varying from transparent, translucent, to opaque. The

isolates' form was either round or irregular. Most of the isolates were shiny or glistening. Pigmentation also differed: white, yellow, and orange with varying intensities. The majority of the isolates were entire and undulated, whereas the elevation was observed to be hilly, raised, convex, and flat. Almost all the isolates were Gram-positive and rod-shaped cells. A total of 672 isolates were obtained from the Manleluag hyperalkaline spring.

Qualitative and Quantitative Phenolphthalein Assay Screening of CGTase-producing Alkaliphilic Isolates

The alkaliphilic CGTase producers were screened by qualitative phenolphthalein screening (Figure 1). A total of 41 isolates were positive to produce CGTase. Twenty (20) isolates were chosen based on the lowest Cz values and subjected to another round of screening with normalized cell concentration. Five isolates were further analyzed by quantitative phenolphthalein assay involving spectrophotometric measurement of the cyclization activity. Isolate M136 exhibited the highest CGTase activity of 46.00 U/mL ($p < 0.001$) (Figure 2).

Characterization and Draft Genome Sequence Analysis of Top CGTase-producing Alkaliphilic Isolate

Isolate M136 was described as yellow, smooth, shiny, translucent, umbonate colonies with an entire margin (1–3 mm) (Figure 3). The isolate appeared as Gram-positive rods arranged in single, chains, or clusters, with 1 µm in length and 2–3 µm in width. The biochemical profile of isolate M136 is presented in Table 1. This isolate showed assimilation of beta-xylosidase, L-aspartate arylamidase, phenylalanine arylamidase, L-pyrrolidonyl arylamidase, tyrosine arylamidase, ellman, alpha-mannosidase, and beta-glucosidase. M136 is susceptible to kanamycin and oleandomycin and can also hydrolyze esculin. Referring to the database of Vitek 2 Compact Identification System for BCL card, the identification results reflected the closest phenotypic match, which was *Bacillus sporothermodurans* / *Bacillus clausii.*

As previously reported by Manalang *et al.* (2019), the top isolate was identified as *Alkalihalobacillus lehensis*. The draft genome revealed 24 scaffolds and 29 contigs, which also included five gaps. The total genome size was 3,985,437 bp, with a G+C content of 39.80%. The genome was similar to *Bacillus lehensis* G1 NZ (CP003923) with a 99.12% average nucleotide identity.

Through the NCBI PGAP, 3,923 protein-coding sequences, 14 rRNAs, 72 tRNAs, and five non-coding RNAs were predicted to be present in the genome of the strain. The presence of genes such as CGTase, protease, and chitinase was detected in the draft genome of *A. lehensis* M136 by RASTtk annotation.

The whole-genome sequence of *A. lehensis* M136 was deposited at DDBJ/ENA/GenBank under the accession number RQRY00000000 and SRA accession number PRJNA506874.

Quantitative Evaluation of Partially Purified CGTase

CGTase from *A. lehensis* M136 was partially purified up to 1.91-fold by dialysis, with a 73.97% recovery (Table 2). The CGTase activity was observed to be 49.52 U/mL. The protein content decreased from 0.45 to 0.17 mg/mL,

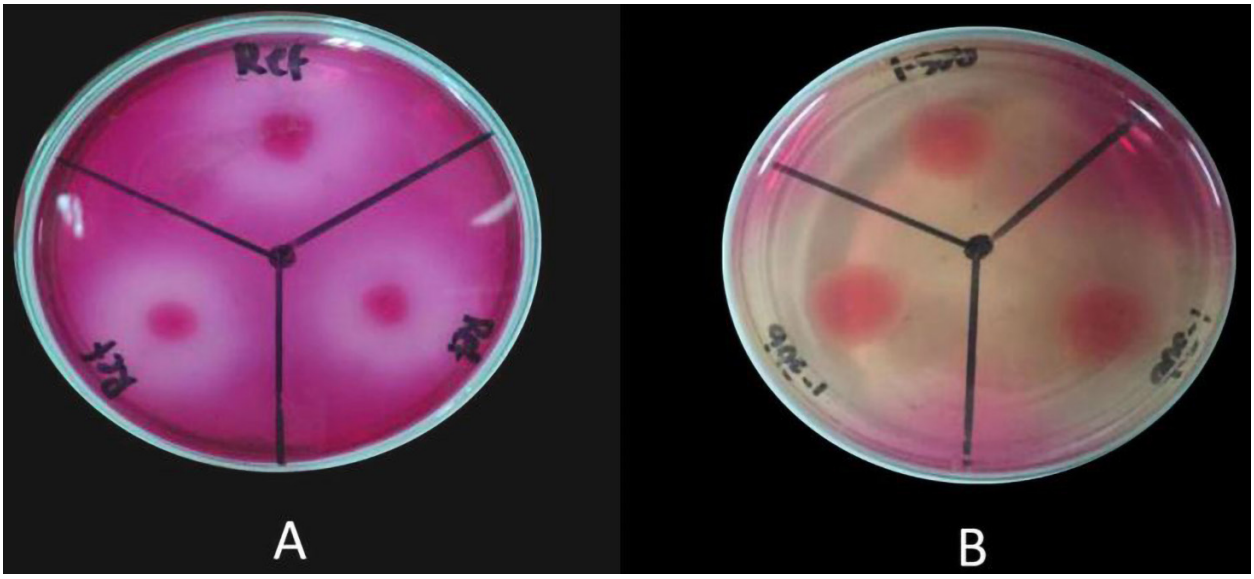

Figure 1. Clearing zones (Cz) of *Paenibacillus* sp. (JCM 9143) as reference strain (A) and isolate M136 (B), exhibited on qualitative phenolphthalein assay.

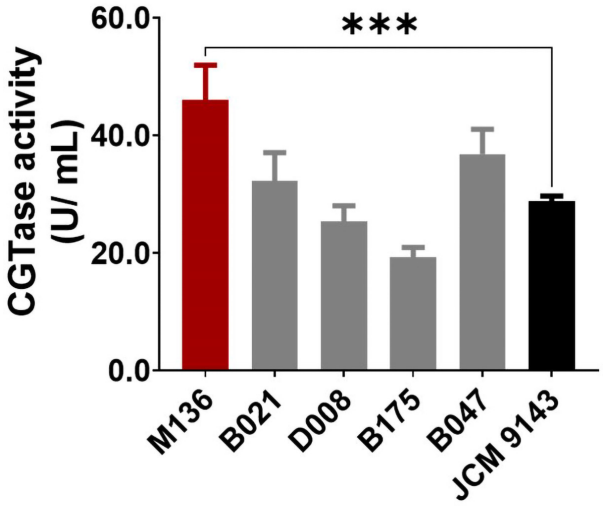

Figure 2. Enzymatic activity (U/mL) of the top five alkaliphilic CGTase-producers based on the quantitative phenolphthalein assay ($p < 0.001$). JCM 9143 was used as the reference strain.

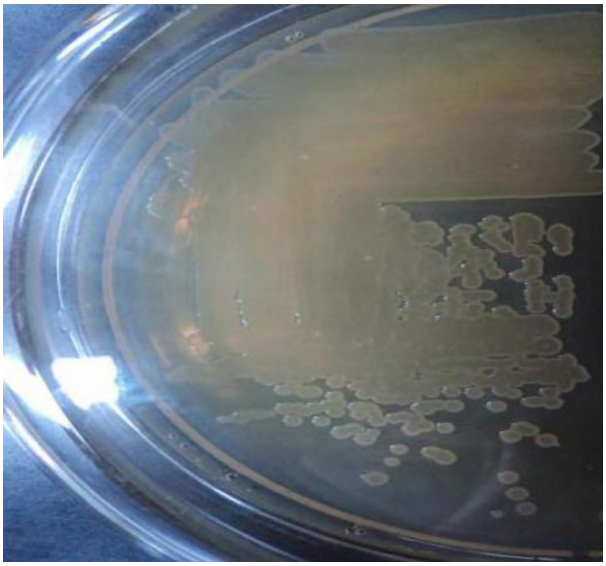

Figure 3. Alkaliphilic isolate M136 on Horikoshi I plate incubated at 37 °C for 48 h.

Table 1. Biochemical tests used to characterize M136 with the BCL card of VITEK®2 system.

Substrates for assimilation tests	Result[a]	Substrates for assimilation tests	Result
Beta-xylosidase	+	D-mannitol	–
L-lysine-arylamidase	–	D-mannose	–
L-aspartate arylamidase	+	D-melezitiose	–
Leucine arylamidase	–	N-acetyl-D-glucosamine	–
Phenylalanine arylamidase	+	Palatinose	–
L-proline arylamidase	–	L-rhamnose	–
Beta-galactosidase	–	Beta-glucosidase	+
L-pyrrolidonyl-arylamidase	+	Beta-mannosidase	–
Alpha-galactosidase	(–)	Phosphoryl choline	–
Alanine arylamidase	–	Pyruvate	–
Tyrosine arylamidase	+	Alpha-glucosidase	–
Beta-n-acetyl-glucosaminidase	–	D-tagatose	–
Ala-phe-pro arylamidase	–	D-trehalose	–
Cyclodextrin	–	Inulin	–
D-galactose	–	D-glucose	–
Glycogene	–	D-ribose	–
Myo-inositol	–	Putrescine assimilation	–
Methyl-A-D-glucopyranoside acidification	–	Growth in 6.5% NaCl	–
Ellman	+	Kanamycin resistance	(–)
Methyl-D-xylosidase	–	Oleandomycin resistance	–
Alpha-mannosidase	+	Esculin hydrolysis	+
Maltotriose	–	Tetrazolium red	–
Glycine arylamidase	–	Polymyxin B resistance	–

[+] positive reaction; [–] negative reaction; [(+)] weak positive, reaction slightly below detection threshold; [(–)] weak negative, reaction slightly above detection threshold

Table 2. Summary of the enzyme activity (U/mL), protein concentration (mg/mL), specific activity (U/mg), yield (%), and fold in each step of the purification of the enzyme CGTase produced by *A. lehensis* M136.

Purification step	Volume (mL)	Enzyme activity (U/mL)	Protein concentration (mg/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
Cell-free Supernatant	50	66.94	0.45	149.75	100.00	1.00
70% ammonium sulfate Precipitation	10	59.35	0.24	247.31	88.67	1.65
dialysis	5	49.52	0.17	286.23	73.97	1.91

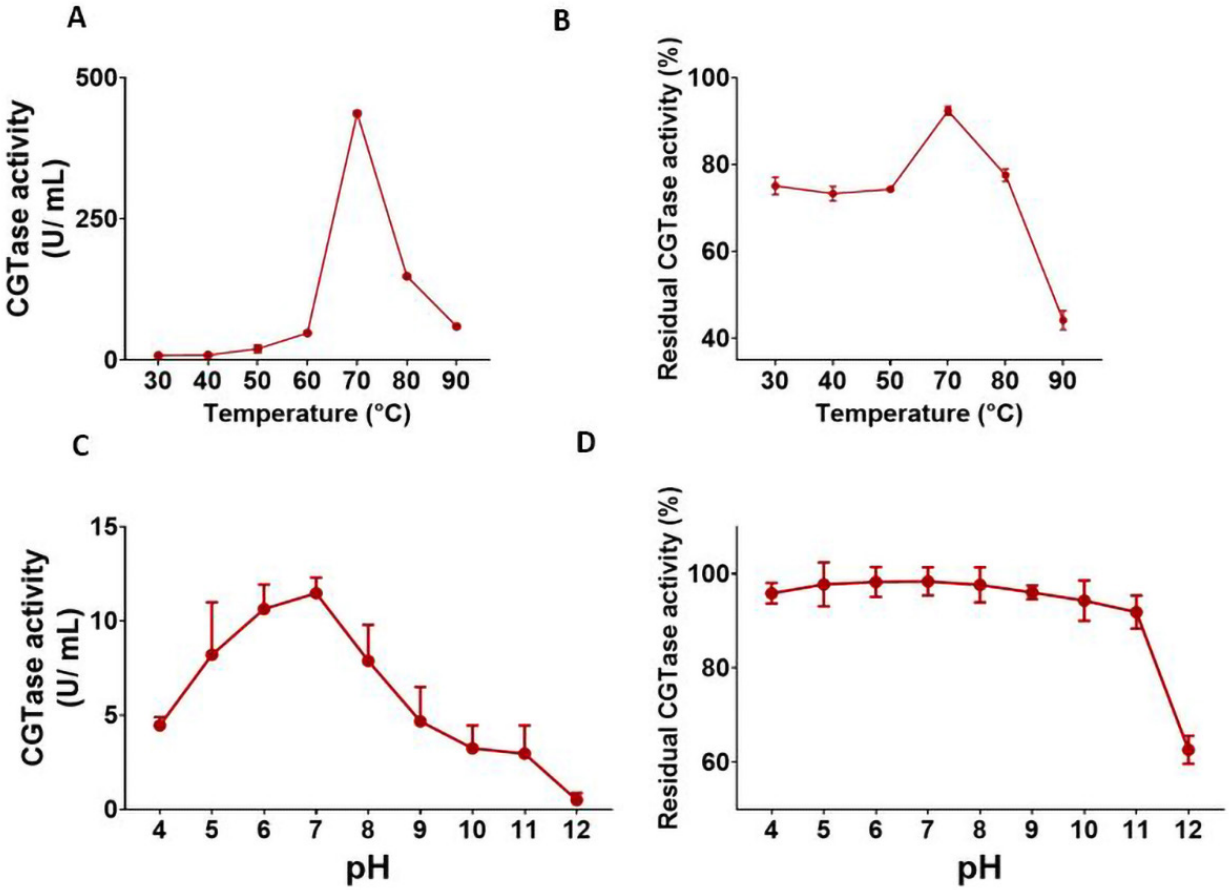

Figure 4. Characterization of partially purified CGTase enzyme from *A. lehensis* M136. The optimum enzyme activity was observed at 70 °C (A) and thermostability was reduced at ≥ 80 °C (B). The effect of pH showed peak enzyme activity at pH 7 (C) and residual enzyme activity at pH 11 (D).

whereas the specific activity increased from 149.75 to 286.23 U/mg.

Characterization of the Partially Purified CGTase Based on Temperature, pH, and Presence of Metal Ions and Reagents

The temperature profile of the CGTase enzyme from M136 was investigated by determining the effect of different temperatures (30–90 °C) on the optimum activity and stability of the enzymes. As shown in Figure 4A, the optimum enzyme activity for *A. lehensis* M136 (436.78 U/mL) was achieved at 70 °C, and the activity decreased upon reaching ≥ 80 °C. The reference strain, JCM 9143, was reported to be optimally active at 65 °C (Vassileva et al. 2007).

The effect of the different temperatures on the stability of the partially purified CGTase was also examined. Based on Figure 4B, the range of temperature in which the CGTase enzyme activity was stable at 30–70 °C, with relative activities ranging from 75.08–92.36%.

Based on Figure 4C, the optimum enzymatic activity

of the partially purified *A. lehensis* M136 CGTase is at pH 7.0 (914.60 U/mL). The optimum pH for the highest enzymatic activity in JCM 9143 is pH 6.0. The stability of the CGTase from *A. lehensis* M136 in a similar pH range was also investigated. As shown in Figure 4D, the enzyme retained 80–96% of its initial activity in a pH range of 4.0–10.0 but was less stable at pH 11.0 and 12.0.

The effect of metal ions, such as Fe^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, and Mn^{2+} on the stability of the CGTase activity of *A. lehensis* M136 was also examined (Figure 5A). The residual activity of the enzyme was lowest in the presence of Fe^{2+} (51.02%), while it was found to be stable with Zn^{2+}, Ca^{2+}, Mg^{2+}, and Mn^{2+}.

The effect of various reagents on CGTase activity was determined by performing an assay under standard conditions, together with the following reagents: mercuric chloride, EDTA, iodoacetic acid, urea, sodium azide, SDS, PMSF, and 2-mercaptoethanol. Based on Figure 5B, enzyme activity was low in the presence of mercuric chloride (48.61%) and 2-mercaptoethanol (49.10%), whereas it was completely inhibited by urea.

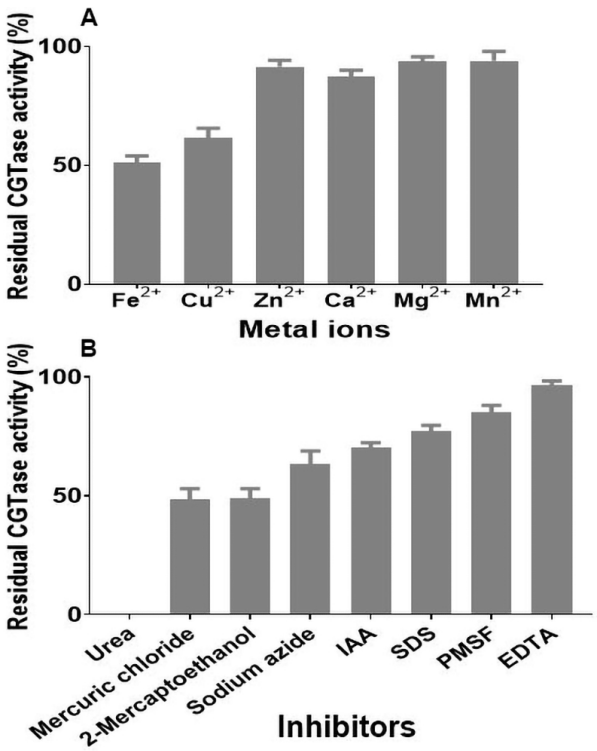

Figure 5. The effect of metal ions (A) and inhibitors (B) on the enzyme activity of partially purified CGTase from *A. lehensis* M136.

Deduced Amino Acid Sequence Analysis of CGTase from *A. lehensis* M136

The deduced amino acid sequence of the CGTase gene is shown in Figure 6A. Analysis of the sequence showed that there was a single ORF of 2,109 bp encoding 703 amino acids. Based on Interpro annotation, the domain IPT (highlighted in gray), alpha-amylase C-terminal (highlighted in green), carbohydrate-binding module family 20 (highlighted in pink), glycosyl hydrolase family 13 catalytic domain (highlighted in red), calcium-binding site (highlighted in yellow) and the signal peptide (highlighted in blue) are present. The CGTase from *A. lehensis* M136 has a molecular weight of 78.64 kDa and a theoretical isoelectric point of 4.72. It also had an instability index of 22.37, indicating that the enzyme was stable (Gasteiger *et al.* 2005).

The domain architecture of the enzyme is shown in Figure 6B. Amino acids in position 30-160 correspond to domain A1, 161-224 to domain B, 225-428 to domain A2, 429-516 to domain C, 517-600 to domain D, and 601-703 to domain E.

Figure 6C illustrates the predicted protein folding of the CGTase. Domain A contains an active site situated at the bottom of $(\beta/\alpha)_8$-barrel, whereas domain B is described as a ring region that binds to the substrate. Domain C is a β-sandwich structure that functions in the binding of maltose. As for the domain D, it has a β-sheet-like structure with an unknown function. Domain E contains a raw-starch binding motif (Zheng *et al.* 2019).

DISCUSSION

In this study, we were able to isolate alkaliphilic bacteria producing an industrially important enzyme from water and sediment samples of Manleluag hyperalkaline spring. The pH and temperature data recorded during the sample collection were almost similar to the values by Woycheese *et al*. (2015), which reported that the Manleluag hyperalkaline springs had a pH of 10.0–11.3 and temperature of 33–36 °C. Hyperalkaline springs with a pH range of 10.9–12.0 are characterized to have a low Mg and high Na, K, Ca, and Cl^- concentrations (Giampouras *et al.* 2019). The high pH is due to the deep carbonate precipitation isolated from atmospheric carbon dioxide.

The surface mixing in the serpentinizing seep of Manleluag hyperalkaline spring also allows redox potential to increase, contributing to the range of metabolic options for microorganisms in the environment, as well as the abundance of organic matter (Woycheese *et al.* 2015). This feature of the Manleluag hyperalkaline spring

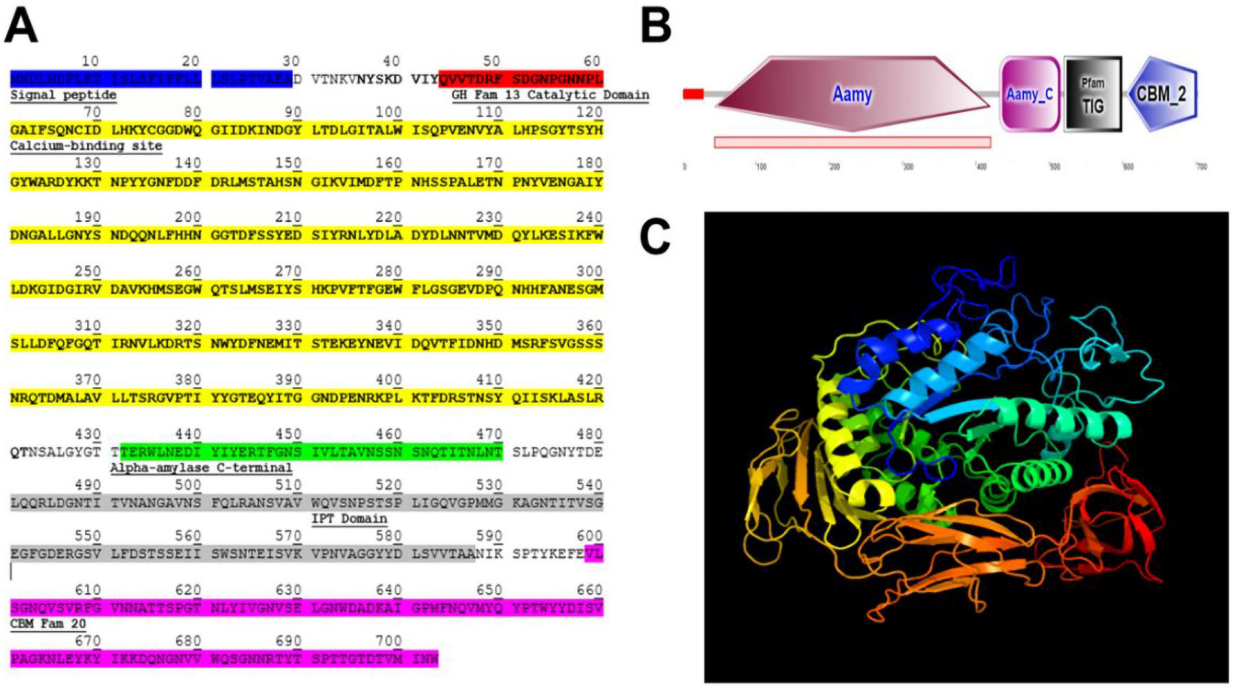

Figure 6. Characterization of CGTase enzyme produced by *A. lehensis* M136: (A) annotated amino acid sequences of the *A. lehensis* M136 CGTase; (B) diagram of the predicted domains and intrinsic features of CGTase from *A. lehensis* M136; (C) predicted stereo view of CGTase from *A. lehensis* M136.

could also contribute to the diversity of the alkaliphilic bacteria isolated from water and sediment samples. The majority of the isolates obtained from the Manleluag hyperalkaline spring have unique pigmentation, which could indicate that the alkaliphilic isolates can produce useful metabolites, such as carotenoids (Preiss *et al.* 2015; Khalikova *et al.* 2019).

Six percent of the total alkaliphilic isolates showed clearing zones around the margins and a reduction of pink reaction brought about by phenolphthalein and sodium carbonate in the quantitative and qualitative screening assays. In the qualitative screening assay, a positive CGTase activity was exhibited by the formation of a yellowish zone of clearing around the colonies against a red-violet background. On the other hand, a reduction in the intensity of the pink color was an indication of a positive result for the production of β-CD in the quantitative assay. Phenolphthalein was transformed into a colorless dianion inside the cavity of the by-product cyclodextrin (Park *et al.* 1989; Ibrahim *et al.* 2012). These results indicated CGTase production by the alkaliphilic isolates. The top isolate, which is M136, was selected based on the highest enzymatic activity. With soluble starch as substrate, the enzymatic activity of *A. lehensis* M136 CGTase is almost close to the *Bacillus cereus* RJ-30 CGTase (54 U/mL) and *Paenibacillus pabuli* (35 U/mL), as studied by Jamuna *et al.* (1993) and Jemli *et al.* (2008), respectively.

Based on the Vitek 2 Compact Identification System, the identification results, which had low discrimination at 50% probability, indicated that the M136 is not yet included in the database. Therefore, an identification through whole genome analysis was deemed necessary. It was found that the top CGTase-producing isolate is novel and identified as *Alkalihalobacillus lehensis* M136. It was confirmed that the gene encoding for CGTase is present in *A. lehensis* M136 genome. Based on the bioinformatics analyses, *A. lehensis* M136 possesses carbohydrate metabolism *via* cyclodextrins (CM-CD) pathway, an unusual pathway for carbohydrate metabolism that ensures survival in starch-poor environments by "monopolizing substrate availability or mitigating the toxicity of surrounding organic substrates and volatiles" (Centeno-Leija *et al.* 2022). Stress tolerance genes which include operons for cobalt-zinc-cadmium resistance genes and different antibiotic resistance genes were also found in the RAST annotation of the genome. Similar genes were reported in *Exiguobacterium* sp. AB2, which was also isolated from the Manleluag hyperalkaline spring (Cabria *et al.* 2014).

The crude CGTase from *A. lehensis* M136 was subjected to partial purification. During purification, as the amount of protein decreases, the specific activity increases (Blanco *et al.* 2014). Enzyme activity was maintained following the precipitation process. The final yield of the enzyme could be attributed to the losses from the number of purification

steps (*i.e.* during enzyme centrifugation of the broth and precipitation process by ammonium sulfate followed by dialysis). In other studies, partial purification of CGTase resulted in a recovery of 87.3% activity and 40-fold purification (Mora *et al.* 2012) and a 42.6-fold purification with 29.03% yield (Vidya *et al.* 2012).

Enzyme characterization is considered a useful tool for knowing the properties of CGTase (Blanco *et al.* 2014). This provides information on potential uses, since it may greatly reduce the need for costly pH and temperature readjustments before enzyme addition (Gessesse and Bashe 1997). Highly productive strains are also required in industries to minimize production costs (Torimiro and Okonji 2013).

The temperature profile of the CGTase enzyme from *A. lehensis* M136 can provide information on enzyme stability during heating or storage, and in environments with varying pH conditions (Shim *et al.* 2004). This shows the increase in the thermodynamic reaction rate at an optimum temperature, followed by the decrease in the thermal denaturation of CGTase (Blanco *et al.* 2014). According to Ibrahim *et al.* (2012), temperature optimum in the range of 55–65 °C had been previously reported for most CGTases, whereas Lee *et al.* (2013) stated that CGTases from various strains have optimal activity at a range of 35–65 °C. It was also reported by Blanco *et al.* (2014) that the optimum temperatures for CGTase range from 30–90 °C. The CGTase from *B. lehensis* MLB2 has an optimum temperature of 60 °C (Elbaz *et al.* 2014), the same as the CGTase from *B. halodurans* (More *et al.* 2012). On the other hand, CGTase from *B. oshimensis* has optimal activity at 37 °C (Pol and Gupte 2016), 55 °C from *B. lehensis* CGII, and 40 °C from *Paenibacillus illinoisensis* ZY-08 (Lee *et al.* 2013). The temperature range obtained from this study can provide the temperature profile. Based on the results, the enzyme is stable at 30–80 °C and optimal at 70 °C. These data are higher than the conventional processes of typical temperatures around 20–40 °C. The advantages of performing processes at higher temperatures can reduce contamination, improve the reaction rate of enzymatic conversion, lower viscosity, and lead to the higher solubility of substrates (Biwer *et al.* 2002). Moreover, CGTase is used in the production of transglycosylated products requiring a temperature range of 30–80 °C (Lim *et al.* 2021).

CGTases from alkaliphiles showed optimal cyclizing activities in a pH of 5.0–10.0 depending on the bacterial strains producing these enzymes (Ibrahim *et al.* 2012). In the study of Pol and Gupte (2016), the CGTase from *Bacillus oshimensis* has optimal activity at pH 8.0. The CGTase from *Bacillus halodurans* is active at pH 9.0 (More *et al.* 2012), and *Paenibacillus illinoisensis* ZY-08 at pH 7.4 (Lee *et al.* 2013).

These results indicate that the production of cyclodextrins using the *A. lehensis* M136 CGTase is possible at a pH range of 4.0–10.0. Moreover, the pH range obtained provides for the performance of the cyclodextrin production process without the need to maintain the pH at a constant level. The established pH range for enzymatic conversion of cyclodextrin using CGTase is at pH 4.5–8.5 (Biwer *et al.* 2002), whereas production of transglycosylated products is conducted at a pH range of 4.5–7.0 (Lim *et al.* 2021). The CGTase from *A. lehensis* M136 is stable at a pH range of 4.0–10.0 (optimal activity at pH 6.0).

Overall, pH and thermal stability are important parameters to consider in the industrial production of cyclodextrins (Blanco *et al.* 2014). CGTases having a stable activity at these parameters are preferred in the industry because they could be utilized in processes requiring extreme conditions (Reddy *et al.* 2017). The first step in cyclodextrin production is starch liquefaction. This is performed at high temperatures. Thus, the thermal and pH stability of CGTase is important in cyclodextrin yield. A wide pH and temperature ranges are for the benefit of high cyclodextrin yield (Vassileva *et al.* 2007).

CGTases are affected by metal ions in such a way that the latter could serve as activators or inhibitors (More *et al.* 2012). This could be attributed to the enzyme's conserved sites for certain ions. Metal ions also maintain the structural conformation of the enzyme and stabilize the binding of the substrate and enzyme complex (Suntornsuk *et al.* 2005; Pol and Gupte 2016). According to Rashid *et al.* (2002), the presence of Ca^{2+} can enhance the CGTase activity and elevate the optimum temperature. Likewise, it improves the thermal stability of the enzyme (More *et al.* 2012). As an activator metal, Ca^{2+} protects the enzymes against thermal denaturation by maintaining an active configuration of the enzymes at high temperatures (Bashir *et al.* 2014). It should also be noted that the actual Ca^{2+} concentration detected in the Manleluag hyperalkaline spring water where *A. lehensis* M136 was isolated from was 21.1 ppm, the highest among all the collection sites and periods. This could indicate that the Ca^{2+} present supports the activity of CGTase. Similarly, Zn^{2+} was also reported to exert a stimulatory effect on the CGTase from *Paenibacillus macerans* (Ibrahim *et al.* 2012).

The low activity of CGTase in the presence of Fe^{2+} suggests the presence of histidine and tyrosine residues in the active center. Similar inhibitory effects of Fe^{2+} were reported for CGTase from *Microbacterium terrae* KNR 9 (Rajput *et al.* 2016) and *Bacillus oshimensis* (Pol and Gupte 2016). According to Ibrahim *et al.* (2012), the inhibitory effect of ions could be accounted for by the metal-catalyzed oxidation of amino acid residues important to the enzyme activity. Having a low CGTase

activity due to the presence of Fe^{2+} is also observed in the mentioned species. Histidine and tyrosine residues in the active site have important roles in cyclization efficiency and in transition state stabilization (Tonkova 1998; Ibrahim *et al.* 2012). Such a feature can also be observed in *Bacillus circulans* strain 251 (Penninga *et al.* 1995).

Based on the study of Vassileva *et al.* (2007), JCM 9143 or *Bacillus circulans* Jordan (ATCC 21783) also has a relatively low CGTase activity in the presence of Fe^{2+}.

Stable enzyme activity is considered an important factor for enzyme application in blends with different substances (Blanco *et al.* 2014). Therefore, it is essential to examine the effect of these substances on enzyme activity.

The inhibition brought about by the different reagents used in the study could possibly be due to the alteration of the structural conformation of CGTase and an indication that the presence of serine and histidine residues may be involved in the enzyme activity (More *et al.* 2012). Mattsson *et al.* (1995) reported histidine functions as a subsite for the substrate binding in the CGTase, based on their study using PCR-mediated techniques. Serine is a part of the catalytic triad, which generates a nucleophilic potential for covalent catalysis (Dodson and Wlodawer 1998).

2-mercaptoethanol was found to inhibit the CGTase activity of *Amphibacillus* sp. NPST-10. EDTA, on the other hand, does not exert an inhibitory effect on the CGTase from *A. lehensis* M136. This suggests that the CGTase from *A. lehensis* M136 is not a metalloenzyme since more than 90% of CGTase activity is retained with EDTA; however, it could be considered as a metal-activated enzyme since it was enhanced by some metal ions (More *et al.* 2012). Similar observations were also reported for CGTase from alkaliphilic *Bacillus pseudoalcaliphilus* 20RF (Ibrahim *et al.* 2012) and *Paenibacillus illinoisensis* ZY-08 (Lee *et al.* 2013).

According to Sivapragasam and Abdullah (2015), purifying CGTase is often a complicated task due to heterogeneity, complexity, and instability, thus obtaining it requires downstream processing. For instance, CGTase purification may utilize high-affinity coordination binding between divalent metal ions. There is selective elution from the resin by elution buffer using various reagents. Moreover, it is important to determine the stability of CGTase in the presence of various reagents and metal ions, since it could influence the solubility of hydrophobic acceptors in transglycosylation reactions (Lim *et al.* 2021). Therefore, the findings in this study with regards to the presence of metal ions and different reagents can provide information on the appropriate conditions for purification.

Previous studies reported that the molecular weight of CGTase is 66 kDa for *Bacillus oshimensis* (Pol and Gupte 2016), 33 kDa for *Bacillus halodurans* (More *et al.* 2012), 81.27 kDa for *Bacillus lehensis* CGII (Blanco *et al*. 2014), 74 kDa for *Paenibacillus illinoisensis* ZY-08 (Lee *et al.* 2013), 74 kDa for *Paenibacillus campinasensis* (Zheng *et al.* 2019), and 27.72 kDA for *Microbacterium terrae* KNR 9 (Rajput *et al.* 2016). For the isoelectric point, Rajput *et al.* (2016) also reported the isoelectric point of CGTase isolated from *M. terrae* KNR 9, which was 4.2. The deduced amino acid sequence of CGTase from *A. lehensis* M136 revealed the highest identity (99.15%) with CGTase from *Bacillus* sp. 1-1 (P31746). Based on the results generated from ExPASy ProtParam (Gasteiger *et al.* 2005), *Bacillus* sp. 1-1 (P31746) CGTase also has 703 amino acids, with a molecular weight of 78.88 kDa and a theoretical pI of 4.77.

The metal-binding sites, particularly for calcium, are located at Asp 52, Asn 54, Asn 57, Asn 58, Gly 76, Asp 78, Asn 161, Ile 212, Asp 221, and His 255. Binding sites for substrates, on the other hand, are in His 162, Arg 249, His 349, Asp 393, and Arg 397. The active sites are Asp 251 (nucleophile) and Glu 279 (proton donor). The conserved amino acid in the acceptor binding site that determines CGTase cyclization (Lee *et al.* 2013), specifically Asp 230, was also identified. The CGTase from *A. lehensis* M136 has at least one calcium-binding site, which is important in stabilizing the enzyme.

According to Suraini *et al.* (2007), the N-terminal domains A to C of CGTase have structural similarities with the α-amylase domains. The TIG domain is characterized to have an "immunoglobulin-like fold." This domain is found in cell surface receptors and in intracellular transcription factors that involve DNA binding (Aravind and Koonin 1999; Bork *et al.* 1999).

The Aamy domain is defined as the alpha-amylase domain. This indicates that the CGTase from *A. lehensis* M136 belongs to family 13 of glycosyl hydrolases and represents the catalytic domain that consists of an "eight stranded α/β barrel containing the active site, interrupted by approximately 70 amino acid calcium-binding domain protruding between beta-strand 3 and alpha-helix 3, and a carboxyl-terminal Greek key beta-barrel domain" (Abe *et al.* 2005). On the other hand, the Aamy_C represents the alpha-amylase C-terminal domain. This is composed of three domains: a triosephosphate isomerase (TIM) barrel containing active site residues and chlorine-binding site (domain A); a long loop region in between the third beta-strand and the alpha-helix of domain A that has calcium-binding sites (domain B); and a C-terminal beta-sheet domain that shows variability in sequence and length between amylases (domain C) (Pujadas and Palau 2001). Lastly, the CBM_2 represents the starch binding domain. It consists of seven beta-strands forming an open-sided distorted beta-barrel (Klein and Schulz 1991). This is also

defined as a "contiguous amino acid sequence within a carbohydrate-active enzyme with a discrete fold having carbohydrate-binding activity."

CONCLUSION

CGTase-producing bacteria have been successfully isolated from the water and sediment samples of the Manleluag hyperalkaline spring in Pangasinan, Philippines. Isolate M136, which is identified as *Alkalihalobacillus lehensis* by whole-genome sequence analysis, shows the highest CGTase activity. The enzyme is partially purified and characterized by determining its stability and optimum pH and temperature, as well as the stability in the presence of various metals and reagents. The results suggest that the enzyme possesses stable and useful attributes with the potential for different industrial applications. Additional studies can be done to determine the optimum fermentation conditions (*e.g.* carbon and nitrogen sources and concentration, pH, temperature) to increase CGTase yield and activity. Chromatography methods coupled with mass spectrometry can also be used to purify the crude CGTase to homogeneity. Optimization of the extracellular secretion of CGTase from *A. lehensis* M136 using recombinant DNA technology can also strengthen the foundation for further industrial production and application. Analyzing the product specificity (α-, β- or γ- cyclodextrin) can also provide a more extensive characterization of the enzyme for potential industrial applications, since CGTases that predominantly synthesize one type of cyclodextrin have a great commercial significance, given that the separation of one type of cyclodextrin from a mixture of products is more time-consuming, costly, and tedious. Gathering experimental data pointing to changes in enzyme structure when exposed to stress, such as circular dichroism, is also possible. Lastly, a comparison of the CGTases from *A. lehensis* M136 and JCM 9143 will be most useful.

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STATEMENT ON CONFLICT OF INTEREST

The authors declare that they have no competing interests during the conduct of this study.

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