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A new low molecular mass alkaline cyclodextrin glucanotransferase from *Amphibacillus* sp. NRC-WN isolated from an Egyptian soda lake

Samar Z.R. Al-Sharawi[1] · Abdelnasser S.S. Ibrahim[2,3] ✉ · Einas H. El-Shatoury[1] · Hassan M. Gebreel[1] · Ahmad Eldiwany[2]

1 Ain Shams University, Faculty of Science, Department of Microbiology, Cairo, Egypt

2 National Research Center, Department of Chemistry of Natural and Microbial Products, Cairo, Egypt

3 King Saud University, College of Science, Department of Botany and Microbiology, Riyadh, Saudi Arabia

✉ Corresponding author: ashebl@ksu.edu.sa
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Abstract

Background: Cyclodextrin glucanotransferase (CGTase) is one of the most industrially important enzymes used in the commercial production of cyclodextrins (CDs). Alkaliphilic bacteria have attracted much interest in the last few decades because of their ability to produce extracellular enzymes that are active and stable at high pH values. Here, we report the isolation of a new CGTase from alkaliphilic bacteria collected from Egyptian soda lakes and describe the purification and biochemical characterization of this CGTase.
Results: Screening for CGTase-producing alkaliphilic bacteria from sediment and water samples collected from Egyptian soda lakes located in the Wadi Natrun valley resulted in the isolation of a potent CGTase-producing alkaliphilic bacterial strain, designated NRC-WN. Strain NRC-WN was belonging to genus Amplibacullus by 16S rDNA sequence analysis (similarity: ca. 98%). Among the tested nitrogen and carbon sources, peptone (0.15%, w/v) and soluble starch (0.4%, w/v) allowed maximal CGTase production by *Amphibacillus* sp. NRC-WN. CGTase was successfully purified from *Amphibacillus* sp. NRC-WN up to 159.7-fold through a combination of starch adsorption and anion exchange chromatography, resulting in a yield of 84.7%. SDS-PAGE analysis indicated that the enzyme was purified to homogeneity and revealed an estimated molecular mass of 36 kDa, which makes it one of the smallest CGTases reported in the literature. The purified enzyme exhibited maximum activity at 50ºC and was stable up to 70ºC, retaining 93% of its initial activity after treatment for 1 hr. Furthermore, Ca^{2+} ions (10 mM) significantly enhanced the thermal stability of the CGTase. The purified enzyme was active and stable over a wide pH range, showing maximal activity at pH 9.5. The enzyme was significantly stimulated by Zn^{2+}, Ca^{2+} and Co^{2+} but was completely inhibited in the presence of Fe^{3+} and mercaptoethanol. The K_m and V_{max} values of the purified CGTase were estimated to be 0.0434 mg/ml and 3,333.3 mg β-CD/ml/min, respectively. β-CD was the predominant product of starch degradation by the *Amphibacillus* sp. NRC-WN CGTase, followed by α-and γ-CDs.
Conclusions: A new low molecular mass alkaline CGTase was purified from a newly identified alkaliphilic *Amphibacillus* sp. NRC-WN isolate from the Egyptian soda lakes. The enzyme showed promising thermal and pH stability and a high affinity toward starch as a natural substrate.

Keywords: *Amphibacillus* sp.; cyclodextrin glycosyltransferase (CGTase); cyclodextrins; Wadi Natrun soda lakes.

INTRODUCTION

Cyclodextrin glycosyltransferase (CGTase, 1,4-α-D-glucan:1,4-α-D-glucopyranosyltransferase, cyclizing, EC 2.4.1.19) is a member of the α-amylase family of glycosyl hydrolases (Avci and Dönmez,

2009). Although amylases usually hydrolyze glucosidic bonds in starch molecules, CGTase primarily catalyzes transglycosylation reactions, acting as a unique enzyme capable of converting starch and related substrates into cyclodextrins (CDs) (Avci and Dönmez, 2009; Moriwaki et al. 2009). CDs are non-reducing cyclic structures consisting of 6, 7 or 8 glucose residues joined by α-(1,4) linkages to produce α-, β- and γ-cyclodextrin, respectively. CD molecules are doughnut shaped and exhibit a hydrophilic outer surface and a relatively hydrophobic cavity (Martin Del Valle, 2004).

With these structural features, cyclodextrins and their derivatives can accommodate various organic molecules to form inclusion complexes. Because each guest molecule is individually surrounded by a cyclodextrin, the molecule is micro-encapsulated from a microscopic point of view (Astray et al. 2009). This phenomenon can lead to advantageous changes in the chemical and physical properties of the guest molecules, such as stabilization of light- or oxygen-sensitive substances, fixation of volatile substances, protection against microbial degradation, improvement of solubility, modification of liquid substances to powders and masking of unpleasant smells and tastes (Savergave et al. 2008; Astray et al. 2009; Otero-Espinar et al. 2010). For these reasons, CDs have been used to stabilize and solubilize various substances of interest to the cosmetic, pharmaceutical and food industries as well as for bioconversion and separation processes (Martin Del Valle, 2004; Li et al. 2007; Atanasova et al. 2009; Otero-Espinar et al. 2010). Because the separation of different CDs is costly and time-consuming, CGTases that predominantly synthesize one type of CD are of great interest (Biwer et al. 2002; Li et al. 2007; Astray et al. 2009).

Alkaliphilic bacteria have attracted much interest in the last few decades due to the great impact of these alkaliphiles through their valuable, commercially important enzymes. The unusual properties of these enzymes offer the potential opportunity for them to be utilized in processes demanding extreme conditions (Horikoshi, 1999; Antranikian et al. 2005; Moriwaki et al. 2009; Atanasova et al. 2011). Soda lakes and soda deserts, the natural habitats of alkaliphiles, represent the major types of naturally occurring highly alkaline environments, in which the indigenous microflora are subjected to a number of extreme ecological pressures. These ecosystems represent the most stable of the high pH environments on Earth, where high levels of carbonate minerals can generate pH values >11.5 (Horikoshi, 1999; Van den Burg, 2003; Antranikian et al. 2005). In this work, we describe the isolation of a new CGTase-producing alkaliphilic bacterial strain from Egyptian soda lakes and the purification and biochemical characterization of its CGTase.

MATERIALS AND METHODS

Isolation of CGTase-producing alkaliphilic bacteria

Soil, sediment and water samples were collected from different Wadi Natrun soda lakes, in northern Egypt, including Bani Salama, Dawood and Elbida lakes. Wadi Natrun Valley, which contains alkaline inland saline lakes, is an elongated depression approximately 90 km northwest of Cairo (the capital of Egypt). The average length of the valley is approximately 60 km, and its average width is approximately 10 km. The bottom of the Wadi Natrun valley is below sea level and below the water level of the Rosetta branch of the Nile (Taher, 1999). The features of the Wadi Natrun area create an ecosystem that serves as a rich source for the isolation of halophilic and alkaliphilic bacteria (Horikoshi, 1999). The samples were collected in sterile tubes, kept in a refrigerator at 4ºC and transferred to the National Research Center laboratories (Cairo, Egypt) within a few hours of collection.

The collected samples were screened for aerobic alkaliphilic CGTase-producing bacteria using direct and indirect methods (Larsen et el. 1998; Martins et al. 2001). The direct method for screening for CGTase activity was carried out using Horikoshi II agar medium containing 0.02% (w/v) phenolphthalein. This method depends on the fact that the CGTase released to the surrounding alkaline medium converts starch to cyclodextrins that form inclusion complexes with phenolphthalein, resulting in a colour change that is detected as a halo zone around the colonies of the CGTase-producing strains (Larsen et al. 1998). The Horikoshi II agar medium (pH 10.5) contained soluble starch (10 g/l), yeast extract (5 g/l), polypeptone (5 g/l), $MgSO_4$ x $7H_2O$ (0.2 g/l), K_2HPO_4 (1 g/l), NaCl (5 g/l), Na_2CO_3 (10 g/l) and agar (15 g/l).

The samples were serially diluted up to 10^{-5}, and 0.5 ml of each dilution was spread on the surface of Horikoshi II agar medium containing 0.02% (w/v) phenolphthalein. The plates were incubated for 2-5 days at 37ºC, and the colonies that were surrounded by a halo zone resulting from the cyclodextrin-dye complex were selected for further examination. In screening for CGTase producers using the indirect method, alkaliphilic bacteria with starch-degrading enzymes were first isolated using Horikoshi II agar medium (without phenolphthalein). After 2-5 days of incubation at 37ºC, the plates were stained with an iodine solution to detect starch hydrolysis, which appears as a clear zone around growing bacteria (Martins et al. 2001). Next, the starch-degrading enzyme-producing strains were further screened for CGTase activity using Horikoshi II agar medium containing phenolphthalein (0.02%, w/v), as described above.

Bacterial identification

For identification of the selected CGTase-producing alkaliphilic bacteria, 16S rDNA sequences were amplified from the bacteria via PCR using the universal bacterial forward primer 16F27 (5'-AGA GTT TGA TCC TGG CTC AG-3') and a reverse primer, 16R1525 (5'-AAG GAG GTG ATC CAG CCG CA-3'), derived from the *E. coli* 16S rDNA sequence (Lane, 1991). The 50 µl reaction mixture contained at least 100 ng of genomic DNA, 0.2 µM each primer and PCR Supermix High Fidelity (Taq&Go, Qbiogene, Illkirch, France). The amplification reactions were performed in a Peltier PTC-200 thermal cycler with the following conditions: step 1, 5 min of initial denaturation at 95ºC; step 2, 10 cycles consisting of 30 sec at 95ºC, 30 sec at 52ºC and 1.5 min at 70ºC; step 3, 20 cycles consisting of 30 sec at 95ºC, 30 sec at 52ºC and 1.5 min at 70ºC; and step 4, a 5 min final extension at 70ºC. The obtained PCR products were analyzed through 0.8% (w/v) agarose gel electrophoresis. The amplified 16S rDNA products were sliced out of the agarose gels with a sterile razor blade, and the DNA was purified from the agarose using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. The purified DNA was sequenced in an ABI 377 automated sequencer using the PRISM Ready Reaction Kit (Applied BioSystem, Foster City, CA). The sequence data were analyzed through comparison with 16S rDNA gene sequences from bacteria available in the GenBank database, and the nearest relative of the organism was determined through BLAST searches.

CGTase production and optimization

CGTase production by the selected bacterial strain was examined in Horikoshi II liquid medium. A loopful of the bacterial culture from an agar plate was inoculated into 5 ml of Horikoshi II liquid medium in a 50 ml glass tube and incubated at 37ºC overnight. This culture was used to inoculate a 1 L Erlenmeyer flask containing 195 ml of Horikoshi liquid medium. The culture was incubated at 37ºC for 48 hrs under static conditions. At the end of the incubation period, the culture was centrifuged at 12,000 g for 10 min at 4ºC. The cell-free supernatant was used as a source of crude CGTase. For optimization of CGTase production by the selected strain, the effect of different nitrogen and carbon sources on the production of the enzyme was investigated. The mixture of peptone and yeast extract in the Horikoshi II medium was substituted with yeast extract, peptone or casein on an equal nitrogen basis. In addition, various concentrations of the determined best nitrogen source for CGTase production were also investigated, ranging from 0.05 to 0.5% (w/v). Soluble starch was substituted as the carbon source in the Horikoshi II medium by other carbon sources, including dextrin, glucose, fructose and sucrose. Different concentrations of the best carbon source were further studied in the range of 0.0 to 1%.

CGTase assay

CGTase activity was measured via a colorimetric assay method described previously (Mäkelä et al. 1988). For this assay, 100 µl of an enzyme sample was added to 900 µl of a 1% (w/v) soluble starch solution prepared in 50 mM glycine-NaOH buffer (pH 10), pre-incubated at 50ºC for 5 min. The reaction mixture was incubated at 50ºC for 30 min, and the reaction was then halted by adding 3.5 ml of a NaOH solution (30 mM). Subsequently, 500 µl of 0.02% (w/v) phenolphthalein prepared in a 50 mM Na_2CO_3 solution was added, and the mixture was allowed to stand at room temperature for 15 min. The colour intensity was measured at 550 nm. One unit of CGTase activity was defined as the amount of enzyme releasing 1 µM β-CD per min under the defined assay conditions. A calibration curve was generated using 2-8 mM solutions of β-CD in glycine-NaOH buffer (50 mM, pH 10). The protein concentration was determined according to the Lowry method (Lowry et al. 1951) using bovine serum albumin (BSA) as the standard.

CGTase purification

For CGTase purification, 500 ml of production medium was inoculated with the selected CGTase-producing strain and then incubated at 37ºC for 48 hrs under static conditions. This culture was subsequently centrifuged at 12,000 g for 10 min to obtain the cell-free supernatant (crude enzyme). CGTase was purified from the cell-free supernatant through a combination of starch adsorption and anion exchange chromatography, as follows.

Starch adsorption. Ammonium sulphate and corn starch were added to the crude enzyme at concentrations of 1 M and 5% (w/v), respectively, and the mixture was maintained at 4ºC with constant moderate mixing for 1 hr to allow the CGTase to adsorb to the corn starch (Martins and Hatti-Kaul, 2002). The mixture was then centrifuged at 3,000 g for 10 min. Next, the starch pellet was washed twice with 1 M cold ammonium sulphate to remove unbound proteins. The adsorbed CGTase was eluted from the corn starch by incubating the pellet in 70 ml of Tris-HCl buffer (50 mM, pH 8) containing 1 mM β-CD at 37ºC in a shaking water bath for 30 min, followed by centrifugation at 12,000 g for 10 min. The elution step was repeated with 30 ml of the elution buffer, and the obtained eluates were pooled and dialyzed against the same buffer at 4ºC overnight.

Anion exchange chromatography. The eluate from the previous step was concentrated through lyophilization. The dried materials were then suspended in a small volume of Tris-HCl buffer (50 mM, pH 8), and 5 ml of the concentrated enzyme samples was applied to a DEAE-Cellulose column that had been pre-equilibrated with 50 mM Tris-HCl buffer (pH 8) (Martins and Hatti-Kaul, 2002). The column was washed with the same buffer, and the proteins were eluted through the stepwise addition of 50 ml of a 5-30% NaCl solution prepared in the same buffer at a flow rate of 1.5 ml/min. Fractions (5 ml) were collected, and protein levels and CGTase activity were measured. The fractions that displayed CGTase activity were pooled and dialyzed overnight against 50 mM Tris-HCl buffer, pH 8, at 4ºC. The purity and the molecular mass of the enzyme were determined via SDS-PAGE according to the method of Laemmli (1970).

Properties of the purified CGTase

Effect of temperature. The influence of temperature on the catalytic activity of the purified CGTase was determined by measuring the enzyme activity at various temperatures ranging from 25 to 75ºC at pH 9, under standard assay conditions. The thermal stability of the purified CGTase was investigated via pre-incubation of the enzyme at different temperatures (25-80ºC) at pH 9 for different time periods, ranging from 15 to 60 min. The residual activity was then measured using defined standard assay conditions. All of the experiments and enzyme assays were performed in triplicate, and the mean values are reported.

Effect of pH. The effect of pH on enzyme activity was examined by measuring the CGTase activity at different pH values at 50ºC using different buffers. These buffers included a 50 mM sodium acetate buffer (pH 5-6), 50 mM sodium phosphate buffer (pH 7-8), 50 mM glycine-NaOH buffer (pH 8.5-10) and 50 mM sodium hydrogen orthophosphate/sodium hydroxidebuffer (pH 10.5-12). The pH stability of the purified CGTase was determined by incubating the enzyme for 1 hr at room temperature in different buffers with pH levels of 4 to 12, prior to determination of the residual activity under standard assay conditions. The experiments and enzyme assays were carried out in triplicate, and the mean values were recorded.

Effect of various additives on the activity of the CGTase. The purified CGTase was incubated with 1 mM and 10 mM concentrations of various metal ions and additives, including NaCl, $CaCl_2$, $MgCl_2$, $FeCl_3$, KCl, ZnSO4, CuSO4, $MnCl_2$, $CoCl_2$, EDTA, SDS and β-mercaptoethanol in 50 mM glycine-NaOH buffer, pH 9, for 1 hr at room temperature. Aliquots of these mixtures were used to measure the residual enzyme activity under standard assay conditions. CGTase activity measured without any additives was used a control. All of the experiments were performed in triplicate, and the mean values were recorded.

Kinetic parameters. The kinetic constants K_m and V_{max} were determined for the purified CGTase by measuring the initial rates of the reaction using different concentrations of soluble starch (0-60 mg/ml)

in glycine-NaOH buffer (50 mM, pH 9) at 50ºC. K_m and V_{max} were estimated with the Michaelis-Menten equation and a double reciprocal Lineweaver-Burk plot (Mathews and Van Holde, 1990). All of the experiments were carried out in triplicate, and the mean values were recorded.

Cyclodextrin production and analysis. The purified CGTase (100 µl) was added to 1.9 ml of a 1% (w/v) soluble starch solution in 50 mM glycine-NaOH buffer, pH 9.5, followed by incubation at 50ºC. Aliquots (200 µl) of the reaction mixture were collected at different time intervals, and the reaction was halted by placing the reaction mixture in a boiling water bath for 5 min. Different cyclodextrins were analyzed via thin-layer chromatography (TLC) on silica gel-60 pre-coated aluminum sheets (Merck, Darmstadt, Germany, 20 x 20 cm) as previously described (Liebl et al. 1992). Briefly, samples of the reaction mixtures were mixed with 2 volumes of methanol and centrifuged. Then, 15 µl of the starch hydrolysates was applied to the TLC sheet. A mixture of n-propyl alcohol/ethyl acetate/water (7:1:2, v/v/v) was used as a developing solvent. Saccharides were detected by spraying the air-dried sheets with the staining reagent, containing ethanol/acetic acid/sulphuric acid/anisaldehyde (9:0.1:0.5: 0.5, v/v/v/v). Carbohydrates were revealed by heating the plates for 10 min at 120ºC and were visualized as dark green spots (Liebl et al. 1992).

RESULTS AND DISCUSSION

Isolation of CGTase-producing alkaliphilic bacteria

Screening of the sediment and water samples collected from various Wadi Natrun soda lakes for CGTase-producing alkaliphilic bacteria using direct or indirect methods resulted in the isolation of a potent CGTase-producing alkaliphilic bacterium, designated NRC-WN (Figure 1). A 16S rDNA gene sequence analysis was performed to determine the phylogenetic position of the NRC-WN strain. Comparative sequence analysis of 16S rDNA sequences from the NRC-WN strain and the bacteria available in the database indicated that the NRC-WN strain showed the highest similarity (98.0%) to various strains of *Amphibacillus* sp. Therefore, the isolated alkaliphilic bacterium was designated *Amphibacillus* sp. NRC-WN, and its 16S rDNA gene sequence was deposited in GenBank under accession number KF021606. The genus *Amphibacillus* was first identified by Niimura et al. (1990) currently comprises several species (Zhilina et al. 2001; An et al. 2007; Wu et al. 2010a; Wu et al. 2010b; Hirota et al. 2012). In addition, there are several strains of *Amphibacillus* sp. that have been isolated from various environmental niches that are not yet fully identified at the species level, including *Amphibacillus* sp. Blan-31 (Smith et al. 2009), *Amphibacillus* sp. E-112 (Mwirichia et al. 2010), *Amphibacillus* sp. KSUCr3 (Ibrahim et al. 2012b), *Amphibacillus* sp. NPST-10 (Ibrahim et al. 2012a) and *Amphibacillus* sp. SL117 (Sahay et al. 2012).

CGTase production by *Amphibacillus* sp. NRC-WN

CGTase production by *Amphibacillus* sp. NRC-WN was investigated using Horikoshi II liquid medium. To optimize enzyme production, the effects of different nitrogen and carbon sources were investigated. The results indicated that among the nitrogen and carbon sources tested, peptone (0.15%, w/v) and soluble starch (0.4%, w/v) induced maximal CGTase production by *Amphibacillus* sp. NRC-WN (data not shown). These results were in agreement with those reported for CGTase production by *Bacillus* sp. TPR71H (Ravinder et al. 2012) and *Bacillus macerans* (Pocsi et al. 1998), where soluble starch was found to be the best carbon source for the production of CGTase. Different types of microbial strains require different nitrogen levels to support the production of CGTases. Peptone has been reported to be the best nitrogen source for achieving maximum CGTase production in other bacteria (Gawande and Patkar, 2001; Ibrahim et al. 2005). However, Kitcha et al. (2008) observed maximum CGTase production by *Bacillus* sp. C26 using 1% yeast extract.

Purification of CGTase from *Amphibacillus* sp. NRC-WN

CGTase was successfully purified from *Amphibacillus* sp. NRC-WN in two steps: corn starch adsorption and anion exchange chromatography. The elution profile obtained through anion gel chromatography showed several protein peaks, the largest of which corresponded to CGTase (Figure 2). This purification protocol resulted in up to 159.7-fold purification of CGTase from *Amphibacillus* sp. NRC-WN, corresponding to a yield of 84.7%. The purified enzyme showed a single

protein band under SDS-PAGE, indicating that the enzyme was purified to homogeneity (Figure 3). Various purification protocols have been applied previously for CGTase purification, including a combination of ultrafiltration, gel filtration, starch adsorption and ion exchange chromatography (Alves-Prado et al. 2008; Savergave et al. 2008), or ammonium sulphate precipitation followed by two steps of ion exchange chromatography (Ong et al. 2008).

Properties of *Amphibacillus* sp. NRC-WN CGTase

Molecular mass. The molecular mass of the purified CGTase was estimated to be 36 kDa through SDS-PAGE and native gel electrophoresis (Figure 3), which makes it one of the smallest CGTases reported in the literature. Ibrahim et al. (2012a) recently described a CGTase from the *Amphibacillus* sp. NPST-10 strain with a molecular mass of 92 kDa. Most CGTases display a molecular mass between 60 and 110 kDa (Martins and Hatti-Kaul, 2003; Hirano et al. 2006; Avci and Dönmez, 2009; Atanasova et al. 2011). However, a few CGTases with a lower molecular mass have been reported, such as a 33 kDa CGTase from *Bacillus coagulans* (Wang et al. 1995) and a 56 kDa CGTase from *Bacillus sphaericus* strain 41 (Moriwaki et al. 2009).

Effect of temperature on CGTase activity and stability. The effect of temperature on the activity of the *Amphibacillus* sp. NRC-WN CGTase is shown in Figure 4A. The enzyme exhibited significant activity over a wide temperature range of 45 to 55ºC, with maximal enzyme activity being observed at 50ºC, which appeared to be the optimum temperature for the activity of this enzyme. The relative activity at 55ºC was approximately 70%, but increasing the reaction temperature to 60ºC or 65ºC was associated with a dramatic decrease in relative enzyme activity, to 31% or 0.0%, respectively. CGTase from *Amphibacillus* sp. NPST-10 showed similar optimum temperature (Ibrahim et al. 2012a). The optimal temperatures for CGTase activity have been reported to be in the range of 50 to 65ºC (Sian et al. 2005; Hirano et al. 2006; Alves-Prado et al. 2008; Atanasova et al. 2011). However, a CGTase with a higher optimum temperature has been reported from thermophilic bacteria (Kitcha et al. 2008; Avci and Dönmez, 2009).

For evaluation of the thermal stability of the purified *Amphibacillus* sp. NRC-WN CGTase, the enzyme was pre-incubated at different temperatures (25-80ºC) for various time periods (15-60 min). The results shown in Figure 4B indicate that the enzyme is stable up to 70ºC, retaining 93% of its initial activity after 1 hr. However, treatment of the enzyme at 80ºC resulted in complete CGTase denaturation. The thermal stability of *Amphibacillus* sp. NRC-WN CGTase was further investigated in the presence of Ca^{2+} ions at final concentrations of 1 mM and 10 mM. As shown in Figure 4C, the addition of Ca^{2+} resulted in significant enhancement of the enzyme's thermal stability, particularly at the higher Ca^{2+} concentration (10 mM), with retention of 12% or 82% of the initial activity being observed after treatment of the enzyme for 2 hrs at 70ºC in the absence or presence of Ca^{2+}, respectively. The enhancement of enzyme thermostability by Ca^{2+} ions is attributed to the stability of the conformational structure of the flexible regions of the CGTase molecule, including the active site (Martins and Hatti-Kaul, 2003).

Effect of pH on CGTase activity and stability. The pH profile of the purified *Amphibacillus* sp. NRC-WN CGTase was estimated by measuring enzyme activity at varying pH levels ranging from 4.0 to 12.0 at 50ºC under standard assay conditions. The enzyme was active over a wide pH range, from pH 6 to 11, showing relative activities of 35-100%, with maximal activity being observed at pH 9.5 (Figure 5A). However, the enzyme showed no activity at pH 4-5. These results differ from those reported for the CGTase found in *Amphibacillus* sp. NPST-10, which exhibits maximal activity at pH 8 and relative activities of 27.2% and 55.0% at pH 4 and 5, respectively (Ibrahim et al. 2012a). CGTases with other optimal pHs have been reported, displaying pH values ranging from pH 5 to 10, depending on the enzyme (Avci and Dönmez, 2009; Moriwaki et al. 2009; Li et al. 2010; Ibrahim et al. 2012a). The pH stability of the *Amphibacillus* sp. NRC-WN CGTase was determined via pre-incubation of the enzyme in buffers with various pH level for various time periods (1-4 hrs) prior to the determination of residual activities. It was found that the CGTase was stable over a wide pH range following treatment for up to 4 hrs at 50ºC. The enzyme retained 92-94% and 76-82% of its initial activity over a pH range of 6.5-10 after treatment for 1 hr or 4 hrs, respectively (Figure 5B).

Effect of additives on CGTase activity. The influence of certain metal ions and additives on the activity of the purified CGTase is summarized in Table 1. The addition of a 1 mM concentration of most metal ions had no significant effect on enzyme activity. However, the addition of the metal ions at a

final concentration of 10 mM resulted in significant effects on CGTase activity. K^+ showed a significant inhibitory effect on the activity of the enzyme, and Fe^{3+} completely inhibited the activity of the CGTase. These results differ from those found in previous studies by other researchers, where Fe^{3+} ions were shown to have an activation effect on the activity of CGTases from *Brevibacterium* sp. no. 9605 and *Bacillus* sp. G1 (Mori et al*.* 1994; Sian et al. 2005). On the other hand, it was found that Zn^{2+}, Ca^{2+} and Co^{2+} significantly enhanced the activityof the *Amphibacillus* sp. NRC-WN CGTase. Atanasova et al. (2011) also reported a decrease of alkaliphilic *B. pseudalcaliphilus* 20RF CGTase activity in the presence of Co^{2+} and Zn^{2+}. However, this result differed from the findings of Martins and Hatti-Kaul (2002), who observed that Co^{2+} had no effect on the activity of the CGTase of *B. agaradhaerens* LS-3C. In addition, this result was contrasted with what has been found for the CGTase of *Amphibacillus* sp. NPST-10, which is significantly inhibited by Zn^{2+} and Co^{2+} (Ibrahim et al. 2012a).

The inhibitory effect of metal ions on CGTase activity is attributed to the metal-catalyzed oxidation of amino acid residues that are essential for the activity of the enzyme (Uitdehaag et al. 1999). As shown in Table 1, treatment with the reducing agent 2-mercaptoethanol or the denaturing compound SDS resulted in complete inhibition of CGTase activity. EDTA (a metal chelating agent) caused significant inhibition of the enzyme activity, suggesting that the *Amphibacillus* sp. NRC-WN CGTase could be a metalloenzyme. These findings contrast with the results found for the *Amphibacillus* sp. NPST-10 CGTase, for which EDTA has no effect on enzyme activity. Thus, the effect of metal ions and reagents on the activity of CGTases generally depends on the bacterial strain involved.

Table 1. Effect of some metals and additives on the activity of the purified *Amphibacillus* sp. NRC-WN CGTase. The reported values are the mean values of triplicate experiments.

Metals	Relative activity (%)	
	1 mM	10 mM
Control (no additives)	100	100
NaCl	100.2	98.3
$CaCl_2$	99.7	132.9
$MgCl_2$	99.1	92.5
$FeCl_3$	99.6	0.0
KCl	100.2	14.1
$ZnSO_4$	100.2	144.5
$CuSO_4$	99.8	99.3
$MnCl_2$	99.4	99.4
$CoCl_2$	100.3	125.4
EDTA	99.468	79.8
SDS	90.89	0.0
β-Mercaptoethanol	0.0	0.0

Kinetic properties. The kinetic constants K_m and V_{max} were estimated for the purified *Amphibacillus* sp. NRC-WN CGTase from Lineweaver-Burk plots (Figure 6). The K_m and V_{max} values obtained using soluble starch as a substrate were estimated to be 0.0434 mg/ml and 3,333.3 mg β-cyclodextrin/ml/min, respectively. The low K_m value indicates the high affinity of the *Amphibacillus* sp. NRC-WN CGTase for the substrate, thus allowing a high reaction velocity at low substrate concentrations. K_m values of up to 5.7 mg/ml and a V_{max} in the range of 43 to 1027 µmol/ml/min have been previously reported for various CGTases (Cao et al. 2005; Moriwaki et al. 2007; Ong et al. 2008; Gastón et al. 2009, Ibrahim et al. 2012a).

Cyclodextrin production

The production of cyclodextrins from the degradation of soluble starch through the action of the purified *Amphibacillus* sp. NRC-WN CGTase was analyzed via TLC. The results shown in Figure 7 indicatedthat β-CD was the predominant product of the *Amphibacillus* sp. NRC-WN CGTase, followed by α- and γ-CD. Because the separation of different CDs is costly and time consuming, a CGTase that predominantly synthesizes one type of CD is of interest. While most CGTases produce mixtures of all types of CDs, CGTases from alkaliphilic bacteria convert starch into β-CD as the major product, though still in a mixture with the other CDs in different ratios (Martins and Hatti-Kaul, 2002; Atanasova et al. 2009; Atanasova et al. 2011). However, a few enzymes have been reported to primarily act as α-CD or γ-CD producers (Wu et al. 2010b; Mathew and Adlercreutz, 2012).

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REFERENCES

AUERBACH, A.B.; NORINSKY, R.; HO, W.; LOSOS, K.; GUO, Q.; CHATTERJEE, S. and JOYNER, A.L. (2003). Strain-dependent differences in the efficiency of transgenic mouse production. *Transgenic Research*, vol. 12, no. 1, p. 59-69. **[CrossRef]**

BAGIS, H.; ODAMAN MERCAN, H. and DINNYES, A. (2004). Exposure to warmer postoperative temperatures reduces hypothermia caused by anaesthesia and significantly increases the implantation rate of transferred embryos in the mouse. *Laboratory Animals*, vol. 38, no. 1, p. 50-54. **[CrossRef]**

BRINSTER, R.L.; CHEN, H.Y.; TRUMBAUER, M.E.; YAGLE, M.K. and PALMITER, R.D. (1985). Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 82, no. 13, p. 4438-4442. **[CrossRef]**

COVARRUBIAS, L.; NISHIDA, Y. and MINTZ, B. (1986). Early postimplantation embryo lethality due to DNA rearrangements in a transgenic mouse strain. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 83, no. 16, p. 6020-6024. **[CrossRef]**

DAY, R.B. and LINK, D.C. (2012). Regulation of neutrophil trafficking from the bone marrow. *Cellular and Molecular Life Sciences*, vol. 69, no. 9, p. 1415-1423. **[CrossRef]**

DOYLE, T.C.; BURNS, S.M. and CONTAG, C.H. (2004). Techno Review: *In vivo* bioluminescence imaging for integrated studies of infection. *Cellular Microbiology*, vol. 6, no. 4, p. 303-317. **[CrossRef]**

FIELDER, T.J.; BARRIOS, L. and MONTOLIU, L. (2010). A survey to establish performance standards for the production of transgenic mice. *Transgenic Research*, vol. 19, no. 4, p. 675-681. **[CrossRef]**

FIELDER, T.J. and MONTOLIU, L. (2011). Transgenic production benchmarks. In: PEASE, S. and SAUNDERS, T.L. eds. *Advanced Protocols for Animal Transgenesis. An ISTT Manual*. Springer Berlin Heidelberg, p. 81-97. **[CrossRef]**

FILIPIAK, W.E. and SAUNDERS, T.L. (2006). Advances in transgenic rat production. *Transgenic Research*, vol. 15, no. 6, p. 673-686. **[CrossRef]**

HEYDTMANN, M. and ADAMS, D.H. (2009). Chemokines in the immunopathogenesis of hepatitis C infection. *Hepatology*, vol. 49, no. 2, p. 676-688. **[CrossRef]**

KOBAYASHI, Y. (2006). Neutrophil infiltration and chemokines. *Critical Reviews™ In Immunology*, vol. 26, no. 4, p. 307-316. **[CrossRef]**

KOBAYASHI, Y. (2008). The role of chemokines in neutrophil biology. *Frontiers in Bioscience*, vol. 13, no. 13, p. 2400-2407. **[CrossRef]**

NAGY, A.; GERTSENSTEIN, M.; VINTERSTEN, K. and BEHRINGER, R. (2003). Production of transgenic mice. In: INGLIS, J. ed. *Manipulating the Mouse Embryo. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, p. 289-358.

PAPADAKIS, K.A. (2004). Chemokines in inflammatory bowel disease. *Current Allergy and Asthma Reports*, vol. 4, no. 1, p. 83-89. **[CrossRef]**

PRAVTCHEVA, D.D. and WISE, T.L. (1995). A postimplantation lethal mutation induced by transgene insertion on mouse chromosome 8. *Genomics*, vol. 30, no. 3, p. 529-544. **[CrossRef]**

PRAVTCHEVA, D.D. and WISE, T.L. (2003). Transgene instability in mice injected with an *in vitro* methylated *Igf2* gene. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 529, no. 1-2, p. 35-50. **[CrossRef]**

ROCHE, J.K.; KEEPERS, T.R.; GROSS, L.K.; SEANER, R.M. and OBRIG, T.G. (2007). CXCL1/KC and CXCL2/MIP-2 are critical effectors and potential targets for therapy of *Escherichia coli* O157:H7-associated renal inflammation. *The American Journal of Pathology*, vol. 170, no. 2, p. 526-537. **[CrossRef]**

RUMBO, M.; SIERRO, F.; DEBARD, N.; KRAEHENBUHL, J.P. and FINKE, D. (2004). Lymphotoxin β receptor signaling induces the chemokine CCL20 in intestinal epithelium. *Gastroenterology*, vol. 127, no. 1, p. 213-223. **[CrossRef]**

SCHLAPP, G.; GOYENECHE, L.; FERNÁNDEZ, G.; MENCHACA, A. and CRISPO, M. (2011). Use of tolfenamic acid in recipient mice given at the moment of surgical embryo transfer. *Transgenic Research*, vol. 20, no. 5, p. 1183. **[CrossRef]**

SIERRO, F.; DUBOIS, B.; COSTE, A.; KAISERLIAN, D.; KRAEHENBUHL, J.P. and SIRARD, J.C. (2001). Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 24, p. 13722-13727. **[CrossRef]**

SIRARD, J.C.; DIDIERLAURENT, A.; CAYET, D.; SIERRO, F. and RUMBO, M. (2009). Toll-like receptor 5- and lymphotoxin β receptor-dependent epithelial *Ccl20* expression involves the same NF-κB binding site but distinct NF-κB pathways and dynamics. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, vol. 1789, no. 5, p. 386-394. **[CrossRef]**

VINADER, V. and AFARINKIA, K. (2012a). A beginner's guide to chemokines. *Future Medicinal Chemistry*, vol. 4, no. 7, p. 845-852. **[CrossRef]**

VINADER, V. and AFARINKIA, K. (2012b). The emerging role of CXC chemokines and their receptors in cancer. *Future Medicinal Chemistry*, vol. 4, no. 7, p. 853-867. **[CrossRef]**

WALL, R.J. (2001). Pronuclear microinjection. *Cloning and Stem Cells*, vol. 3, no. 4, p. 209-220. **[CrossRef]**

WOLPE, S.D.; SHERRY, B.; JUERS, D.; DAVATELIS, G.; YURT, R.W. and CERAMI, A. (1989). Identification and characterization of macrophage inflammatory protein 2. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 2, p. 612-616. **[CrossRef]**

WU, X.; LEE, V.C.; CHEVALIER, E. and HWANG, S.T. (2009). Chemokine receptors as targets for cancer therapy. *Current Pharmaceutical Design*, vol. 15, no. 7, p. 742-757. **[CrossRef]**

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Figures

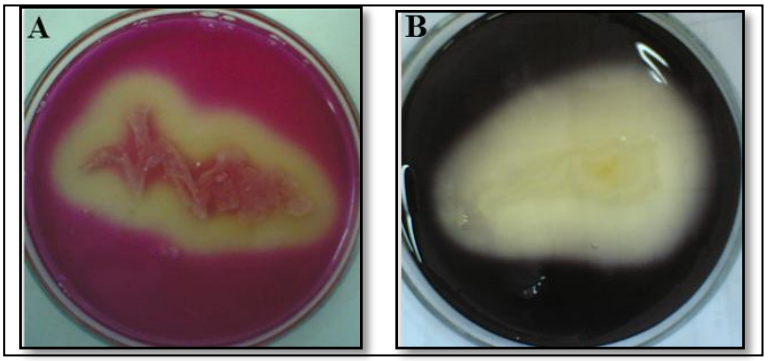

Fig. 1 *Amphibacillus* sp. NRC-WN grown on (A) Horikoshi agar medium containing 0.02% phenolphthalein, revealing β-CGTase production. The detection of CGTase-producing alkaliphilic bacteria was based on the secretion of CGTase by bacterial strains into the medium and the subsequent production of cyclodextrins (CDs). The CDs form inclusion complexes with phenolphthalein, resulting in a color change that is detected as a halo zone around the positive colonies (Larsen et al. 1998). **(B)** NRC-WN grown on Horikoshi agar medium (without phenolphthalein), followed by plate staining with an iodine solution, revealing starch hydrolysis.

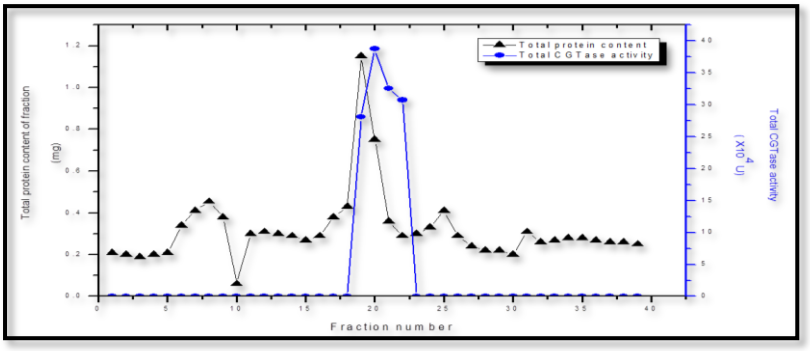

Fig. 2 Elution pattern of *Amphibacillus* sp. NRC-WN CGTase from an ion exchange column. Protein elution was carried out through the stepwise addition of 0-30% NaCl prepared in 50 mM Tris-HCl buffer (pH 8), at flow rate of 1.5 ml/min. Fractions of 5 ml were collected and assayed for CGTase activity and protein content.

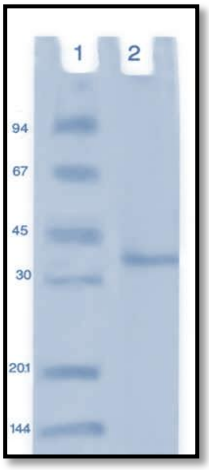

Fig. 3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified CGTase. Lane 1: Molecular mass standard (kDa). Lane 2: purified CGTase. Protein bands were visualized via Coomassie staining.

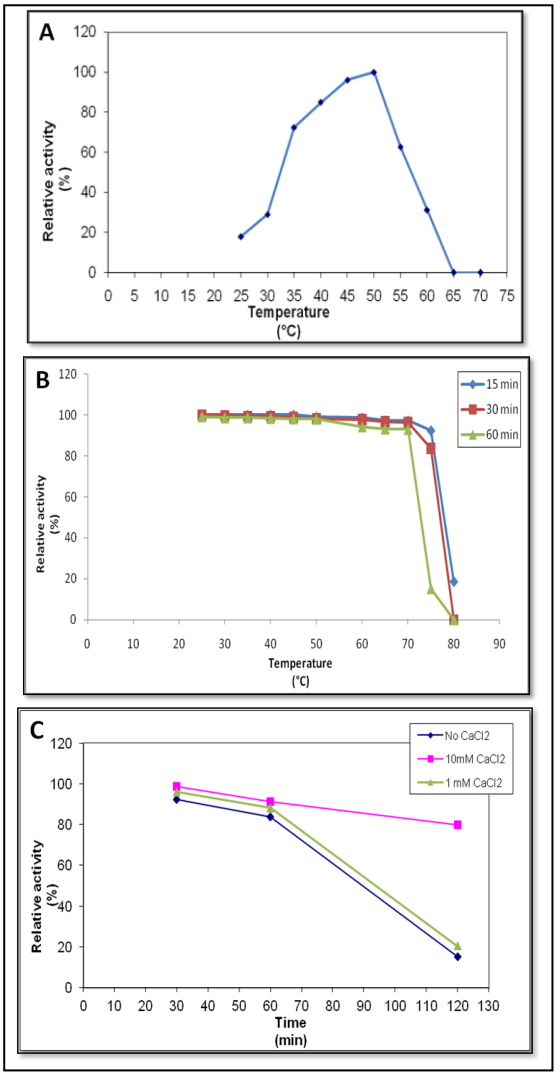

Fig. 4 Effect of temperature on the activity of the purified *Amphibacillus* sp. NRC-WN CGTase (A); thermal stability of CGTase (B); effect of temperature on the stability of the CGTase in the presence and absence of Ca^{2+} ions at 75ºC (C). All experiments and enzyme assays were performed in triplicate, and the mean values are reported. Standard deviations of the relative activities were in range of 0.3-3.0%.

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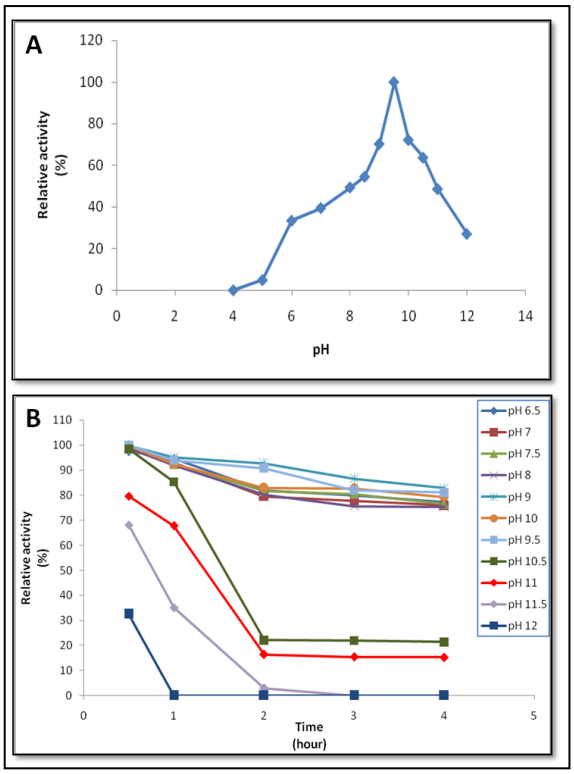

Fig. 5 Effects of pH on the activity of the purified *Amphibacillus* sp. NRC-WN CGTase (A) and enzyme stability (B). All experiments were performed in triplicate, and the mean values are reported. Standard deviations of the relative activities were in range of 0.2-2.6%.

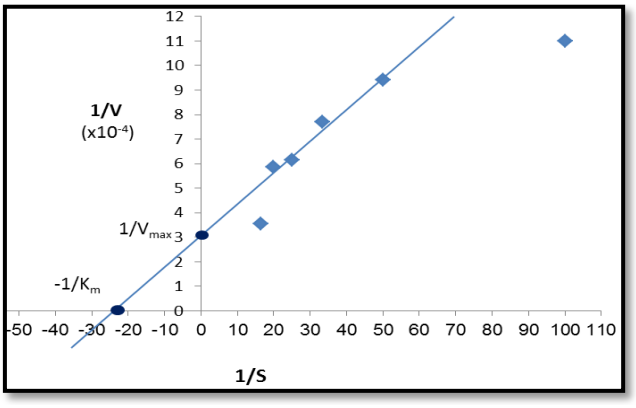

Fig. 6 Lineweaver-Burk plots of the purified CGTase from *Amphibacillus* sp. NRC-WN obtained using soluble starch as substrate. S: Substrate concentration. V: Enzyme velocity (specific activity). All experiments were performed in triplicate, and the mean values are reported.

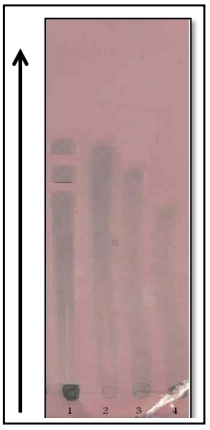

Fig. 7 Thin layer chromatography of the end products of soluble starch degradation through the action of the purified CGTase. Lane 1: Sample following the reaction of the purified CGTase with soluble starch at 50°C for 1 h under standard CGTase conditions, lane 2: α-CD standard, lane 3: β-CD standard and lane 4: γ-CD standard.