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Full Length Research Paper

An alkaliphilic cyclodextrin glycosyltransferase from a new *Bacillus agaradhaerens* WN-I strain isolated from an Egyptian soda lake: Purification and properties

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Alkaliphilic bacteria were isolated from soil and water samples obtained from Egyptian soda lakes in the Wadi Natrun area. Screening for cyclodextrin glycosyltransferase- producing alkaliphilic bacteria resulted in the isolation of 15 positive strains. Strain WN-I was selected as the best producer of CGTase. 16S rDNA sequence analysis and DNA-DNA hybridization identified the isolate as *Bacillus agaradhaerens*. The enzyme was purified to homogeneity up to 21 fold by starch adsorption and anion exchange chromatography with a yield of 26.40%. The pure enzyme was a monomer with an estimated molecular weight of 85 kDa. The enzyme was stable, at 25°C, over a pH range of 5.0 to 11, with a maximum activity at pH 9.0. The enzyme activity exhibited an optimum temperature of 55°C and was stable at 40°C for at least 1 h. Thermal stability was improved in the presence of maltodextrin, starch or $CaCl_2$. The enzyme was slightly stimulated by $CaCl_2$, KC1 and $BaCl_2$ but was completely inhibited in the presence of $FeCl_2$ and strongly inhibited by $ZnCl_2$ and $CoCl_2$ and to a lower extent by $CuCl_2$, EDTA, 2-mercaptoethanol, and dithiothritol. The enzyme produced mainly β-CD (71.20% of the total cyclodextrin amount). The enzyme had higher cyclyzation activity (1.9 fold higher) toward Paselli starch than soluble starch.

Key words: Alkaliphiles, soda lakes, cyclodextrin glycosyltransferase, *Bacillus agaradhaeren,* purification, 16S rDNA.

INTRODUCTION

Naturally occurring alkaline environments, such as carbonate springs, alkaline soils, and lakes, are characterized by their highly basic pH values (pH 8 to 11). They are characterized by the presence of a high concentration of sodium carbonate formed by evaporative concentration (Van den Burg, 2003). Soda lakes are widely distributed over all the world, however as a result of their inaccessibility, few have been explored from a microbiological point of view (Grant and Jones, 2000).

The Egyptian soda lakes in the Wadi Natrun area (30° 15´ N, 30° 30´ E) are an excellent example of these hitherto unexplored alkaline ecosystems. Alkaliphilic microorganisms, in particular *Bacillus* species, have attracted much interest in the past few decades because of their ability to produce extracellular enzymes that are active and stable at high pH values (Van den Burg, 2003; Antranikian, 2005). The unusual properties of these enzymes offer a potential opportunity for their utilization in processes demanding such extreme conditions. The enzyme cyclodextrin glycosyltransferase (EC 2.4.1.19) converts starch and related carbohydrates to non-reducing cyclic maltooligosaccharides called cyclodextrins (CDs) comprised of D-glucose residue

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Figure 1. Wadi Natrun soda lakes in northern Egypt.

linked by α-1, 4 glucosidic bonds. The most common types of CDs are α-, β-, and γ-CD containing 6, 7 and 8 glucosyl-residues respectively (Szerman et al., 2007). CDs are doughnut-shaped molecules with a hydrophilic outer surface and a relatively hydrophobic cavity. With such structural features, cyclodextrins and their derivatives can associate with various organic molecules to form inclusion complexes. This property has been used to stabilize and solubilise various substances of interest to the cosmetic, pharmaceutical, food industries, and bioconversion and separation processes (Martin Del Valle, 2009; Otero-Espinar et al., 2010). As the separation of different CDs is costly and time-consuming, CGTases that synthesises predominantly one type of CD are of great interest (Biwer et al., 2002; Li et al., 2007; Astray et al., 2009). Most CGTases from alkaliphilic

bacteria convert starch into β-CD as the predominant product, however a mixture of other CD forms are still produced in varying ratios. In this work, we presented characterization and identification of CGTase producing alkaliphilic strain, complete purification biochemical characterization of the purified CGTase, and some studies on cyclodextrins production by the purified CGTase.

MATERIALS AND METHODS

Soil and water samples

Sediment and water samples were collected from soda lakes in Wadi Natrun in northern Egypt (Figure 1). Wadi Natrun extends in a northwest by southeast direction between latitudes 30° 15´ north and longitude 30° 30´ east. The bottom of the Wadi Natrun area is 23 m below sea level and 38 m below the water level of Rosetta branch of the Nile. The lowest part of the depression, encircled by contour zero, covers an area of about 272 km^2 (Taher 1999). Sediment and water samples were collected from four different Wadi Natrun soda lakes including Hamara, Bani salama, Dawood, and Elbida lakes. Samples were stored in sterile containers at 4°C, and were transferred to Germany within two weeks. Chemical analysis of the soil samples from the Wadi Natrun soda lakes performed by the Institute of Agricultural Analysis and Research (LUFA)(Rostock, Germany), indicated that the samples were rich in Na^+ (20.3%) and CO_3^{2+} (7.09%) along with low content of P (0.1%), K (0.11%), Ca (0.79%), Mg (1.43%), Cd (0.03 mg/kg), Cr (3 mg/kg), Pb (2 mg/kg), Zn (6 mg/kg) and Hg (0.02 mg/kg). The pH of the sediment and water samples ranged from 9.5 to 12 according to the site of collection.

Isolation of CGTase producers

Isolation of CGTase-producing alkaliphilic bacteria was carried out using rich alkaline agar medium containing 0.02% (w/v) phenolphthalein or 0.03% (w/v) bromocresol green to screen for β- and γ-CGTases, respectively (Park et al., 1989). The alkaline agar medium (pH 10.5) contained soluble starch (10 g/l; Merck), yeast extract (5 g/l; Difco), casamino acids (5 g/l; Difco), peptone (5 g/l; Difco), NaCl (50 g/l), Na_2CO_3 (15 g/l), agar (15 g/l) and 300 µl trace elements solution. The trace element solution contained: $CaCl_2.2H_2O$ (1.7 g/l), $FeSO_4.7H_2O$ (1.3 g/l), $MnCl_2.4H_2O$ (15.4 g/l), $ZnSO.7H_2O.7H_2O$ (0.25 g/l), H_3BO_3 (2.5 g/l), $CuSO_4.5H_2O$ (0.125 g/l), Na_2MoO_4 (0.125 g/l), $CoNO_3.6H_2O$ (0.23 g/l) and 2.5 ml 95 to 97 H_2SO_4. The Na_2CO_3 and trace elements solutions were autoclaved separately before addition to the medium. Sediment and water samples were suspended and serially diluted in a 10% (w/v) NaCl solution prepared in 50 mM glycine-NaOH buffer, pH 10. Aliquots (100 µl) of different dilutions were plated on the alkaline agar medium and incubated at different temperatures, for several days. Formation of halo zone around the colonies, resulting from the production of bromocresol- or phenolphthalein-cyclodextrin inclusion complexes, was an indication of CGTase activity.

Bacterial identification by 16S-rDNA sequencing and DNA-DNA hybridization

Bacterial DNA was extracted using Invisorb Spin DNA Micro kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. 16S-rDNA was amplified by polymerase chain reaction (PCR) using universal forward primer 16F27 and reverse primer 16R1525 (Lane, 1991). The amplified 16S-rDNA was cloned into pGEM®-T vector (Promega, Madison, USA), and transformed into *Escherichia coli*. Plasmid DNA was isolated from positive clones using the Qiaprep® Spin Miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Plasmid specific primers USP and RSP and the prokaryote specific forward primers, 16F530, 16F926 and reverse primers 16R519 and 16R907 (Lane, 1991), were used as infra-red dye labelled dideoxyoligonucleotides. 16S-rDNA was sequenced using the Cycle Reader™ Auto DNA sequencing kit (MBI Fermentas, St. Leon-Rot, Germany) and a LI-COR® automated DNA sequencing machine (MWG-Biotech) according to manufacturer's instructions. The 16S-rDNA gene sequence of the isolates was aligned with reference 16S-rDNA sequences of the European Microbiological Laboratory (EMBL), GenBank (gb, Germany) and the data base of Japan (dbj) using the BLAST algorithm (Altschul et al., 1997) available from the NCBI (National Centre for Biotechnology information) website. DNA-DNA hybridization between the CGTase producing isolate and the type strain, *Bacillus agaradhaerens* DSMZ 8721, was performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as described by Huss et al. (1983).

CGTase production

The same alkaline medium used for the strains isolation, without phenolphthalein, was used for the enzyme production by the selected isolate. A 5 ml liquid medium containing glass tube was inoculated with the selected isolate and incubated overnight at 37°C under orbital shaking (180 rpm). This culture was used to inoculate a 500 ml shaking flask containing 95 ml of the same medium and cultivated under the same conditions for approximately 48 h. Preliminary assay was carried out and 2 ml aliquots were withdrawn at 2 h interval and were centrifuged at 10 000 *g* for 10 min at 4°C. Enzyme production increased gradually until the 30th hour, and remained constant until the end of the assay. Cells and insoluble material were removed by centrifugation at 6 000 *g* for 15 min at 4°C, and cell-free supernatant was filtered through a 0.45 µm pore-size membrane filter and was used as the source of crude CGTase.

Enzymes assay

CGTase activity was measured as β-CD forming activity according to a method described by Mäkelä et al. (1988). 750 µl of a 5% (w/v) maltodextrin solution prepared in 50 mM glycine-NaOH buffer, at pH 9 was pre-incubated at 55°C for 5 min. 25 µl of enzyme sample was added and after incubating for 10 min at 55°C, the reaction was quenched by adding 375 µl of 0.15 M NaOH. Subsequently, 100 µl of 0.02% (w/v) phenolphthalein prepared in 5 mM Na_2CO_3 was added, and after standing 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 µmol of β-CD per min under the defined assay conditions. A calibration curve was made using 0.0055-0.22 µmol of β-CD solution in 50 mM glycine-NaOH buffer, at pH 9, as a standard. Protein concentration was determined according to the method described by Bradford (1976) with bovine serum albumin as the standard protein.

Purification of CGTase

The cell free supernatant was used as a crude CGTase enzyme

preparation and was purified in two steps, including corn starch adsorption and ion exchange chromatography. Corn starch and ammonium sulphate were added to 80 ml of the cell-free supernatant to a concentration of 5% (w/v) and 1 M, respectively, and kept at 4°C with continuous agitation 1 h to allow enzyme adsorption. The mixture was then centrifuged at 5 000 *g* for 10 min and the pellet was washed twice with 40 ml cold 1 M ammonium sulphate solution to remove unbound proteins. In order to elute the adsorbed CGTase from the corn starch, the residue was incubated with 20 ml of 50 mM Tris-HCl buffer (pH 8.0), containing 1 mM β-CD, for 30 min at 37°C with shaking followed by centrifugation (eluate 1). The elution with the same buffer (8 ml) was repeated once (eluate 2). The eluates were pooled (28 ml) dialysed against 50 mM Tris-HCl buffer (pH 8.0) at 4°C. The eluate was concentrated using an Amicon ultrafiltration membrane kit (10 kDa cut-off membrane). 5 ml of the concentrated sample was applied to a MonoQ HR 5/5 column connected to an FPLC system (Pharmacia, Freiberg, Germany) and pre-equilibrated with 50 mM Tris-HCl buffer at pH 8. The column was washed with the same buffer and proteins were eluted using a linear 0 to 1 M NaCl gradient in the same buffer at a flow rate of 1.5 ml/min. Fractions (2 ml) were collected and absorbance was monitored at 280 nm. Fractions containing CGTase activity were pooled and dialyzed against 50 mM Tris-HCl buffer overnight at 4°C.

Homogeneity and estimation of molecular weight

The purity and the molecular weight of the enzyme were determined using SDS-PAGE according to Laemmli (1970) using the PHAST-system (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Protein bands were visualized by silver staining. The molecular weight of the purified protein was estimated on 12% SDS-PAGE using broad range molecular weight standards (Fermentas, St. Leon-Rot, Germany). In addition, the molecular weight of the native protein was estimated by gel filtration Hiload TM 16/60 superdex-200 column with a bed volume of 120 ml (Amersham Pharmcia Biotech, Upplsala, Sweden). The gel was equilibrated with 240 ml of 50 mM Tris-HCl buffer (pH 7.6), and calibrated using gel filtration standard proteins mixture (Amersham Pharmcia Biotech, Upplsala, Sweden). Samples were applied to the column connected to a FPLC and proteins were eluted using the same buffer at a flow rate of 1 ml/min and fraction actions of 0.75 ml each were collected using LKB collector (Pharmacia Biotech, Sweden). Calibration curve was done by plotting the logarithms of the molecular weight of the standard proteins against its partition coefficient (K_{av}) values.

Zymogram staining

A direct specific method for CGTase activity staining was developed in this work (instead of the indirect method using iodine solution). The enzyme samples were applied to 10% native PAGE. After gel electrophoresis, the gel was washed twice with distilled water and twice with 50 mM glycine-NaOH buffer (pH 10). Then, the gel was immersed in solution containing 1% (w/v) soluble starch, 0.02% (w/v) phenolphthalein and 1.5% (w/v) agar in 50 mM glycine-NaOH buffer (pH 10) and after agar solidification, it was incubated for 1 h at 50°C. The CGTase activity was seen as a colourless band on a red background due to the formation of stable colorless CD-phenolphthalein inclusion complex. For the detection of starch degrading activity, the native gel was immersed in cooled melted solution of 1% (w/v) soluble starch and 1.5% (w/v) agar in 50 mM glycine-NaOH buffer (pH 10), and was incubated for 1 h at 50°C.

After that, iodine solution was poured on the gel-agar. The starch-degrading activity was seen as a clear band on dark blue background.

Analysis of the end products of starch degradation by action of the purified CGTase

The enzyme reaction was carried out at 50°C, pH 9.0 using 1% (w/v) of soluble and 1% (w/v) Paselli starch and at defined time intervals it was stopped by placing the samples in a boiling water bath for 5 min. The reducing sugars in the reaction mixture were measured by the method reported by Waffenschmidt et al. (1987) and the starch consumption followed the method described by Krisman (1962). For quantitative analysis of CDs, the produced linear oligosaccharides were initially hydrolyzed by glucoamylase. For this, the starch hydrolysate were cooled and 50 µl was mixed with 5 µl (2 U) of glucoamylase and 45 µl 0.4 M sodium acetate buffer, at pH 5, and incubated for 1 h at 40°C. Then, the reaction was stopped by placing the samples in a boiling water bath for 5 min. The mixtures were filtered through a 0.45 µm membrane filter. The filtered sample (30 µl) without linear oligosaccharides hydrolysis and samples after hydrolysis were analysed by the high performance liquid chromatography (HPLC) system using Aminex-HPX-42-A column (300 by 7.8 mm; Bio-Rad, Hercules, Calif.). CDs and linear sugars were eluted with degassed distilled water at flow rate of 0.6 ml/min. The flow cell was set at 80°C and the products were detected by a refractive index detector (LaChrom, L7490 Merck-Hitachi, Ltd. Tokyo, Japan). Calibration curve was done using 1.0, 2.5, 5.0, 7.5 and 10 mM of α-, β- and γ-cyclodextrin.

RESULTS AND DISCUSSION

Isolation of aerobic CGTase producing alkaliphilic bacteria

Isolation of CGTase producing alkaliphilic bacteria using alkaline agar medium containing 0.02% (w/v) phenol-lphthalein, resulted in the isolation of 15 isolates showing halo zones around their margins, but no halos were observed around colonies grown in medium containing bromocresol green. The positive isolates were propagated in liquid medium and the CGTase activity was measured. One of these isolates, designated as WN-I (W for Wadi; N for Natrun), showing the highest CGTase activity in alkaline liquid medium, was selected for further study.

Characterization and identification of strain WN-I

The CGTase producing isolate, WN-I, grown on alkaline agar medium, showed white rhizoid colonies with filamentous margins which adhered to the agar. No soluble pigment was produced. Culture grown in alkaline liquid medium for 24 h showed motile short rod-shaped cells (Figure 2) about 3 µm in length and 0.25 µm in diameter. Gram positive cells with spherical endospores existed as single, paired, or short chains. Strain WN-I

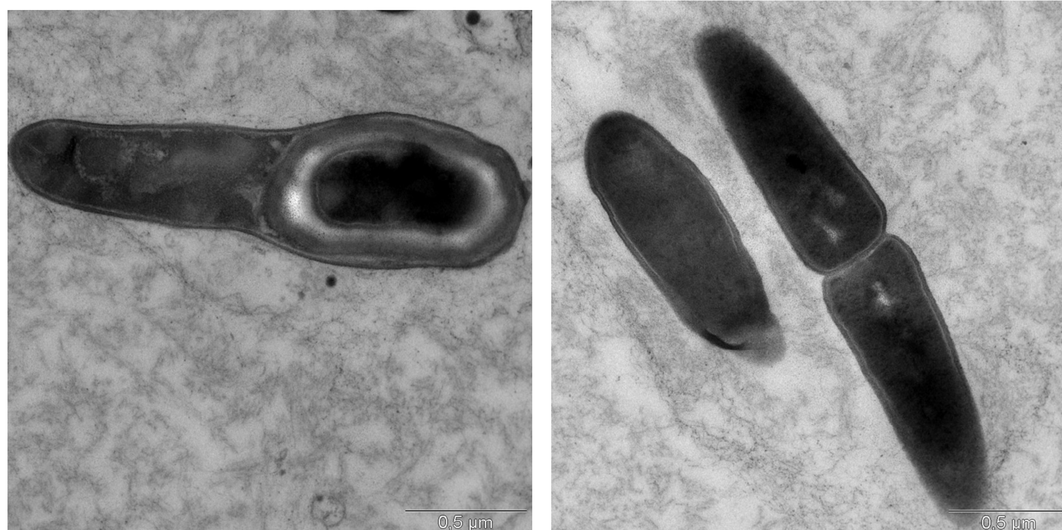

Figure 2. Transmission electron micrographs of strain WN-I.

Table 1. Purification of CGTase from *B. agaradhaerens* WN-I.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (Umg^{-1})	Yield (%)	Purification fold
Crude enzyme	48.80	15.52	3.14	100	1
Corn starch adsorption	22.06	1.14	19.35	45.20	6.16
MonoQ column	12.86	0.194	66.28	26.35	21.10

was able to grow in NaCl concentrations up to 15% (w/v), and in pH 8 to 11, but there was no growth at pH 7 after 48 h incubation at 37°C, indicating it was moderately a halophilic alkaliphilic bacterium. It showed growth at 25, 37 and 45°C but no growth was observed at 50°C after 48 h incubation period. In order to determine the phylogenetic position of strain WN-I, 16S rDNA analysis was performed. Comparative sequence analysis of 16S rDNA gene of strain WN-I and other 16S rDNA available in the database indicated that WN-I had the highest similarity (98.35%) to *Bacillus agaradhaerens* DSMZ 8721. DNA-DNA hybridization between strain WN-I and the type strain, *B. agaradhaerens* DSMZ 8721, was performed by the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen' (DSMZ). Strain WN-I showed 85.1% (95.2) DNA-DNA similarity (in 2 x SSC at 63°C) to *B. agaradhaerens* DSM 8721. When the recommendation of the *ad hoc* committee (Wayne et al., 1987) on a threshold value of 70% DNA-DNA similarity for definition of bacterial species was considered, strain WN-I was a new strain of *B. agaradhaerens* and it was named *B . agaradhaerens* WN-I.

Purification of the CGTase

The CGTase from *B. agaradhaerens* WN-I was purified using two steps procedures including starch adsorption and anion-exchange chromatography and the results are summerized in Table 1. The elution profile of the anion gel chromatography (Figure 3) showed two main peaks; the first one corresponding to the purified CGTase and the second one exhibited α-amylase activity. These purification protocols resulted in purification of CGTase 21.10-fold with a yield of 26.40%. The purified enzyme gave a single protein band by SDS-PAGE (Figure 4A).

A fast specific CGTase activity staining, rather than the non-specific starch/iodine staining, was developed and optimized as described in the materials and methods section. CGTase and starch degrading activity staining of native PAGE indicated that the cell-free supernatant contained two alkaline starch degrading enzymes, including alkaline CGTase in addition to alkaline α-amylase which were successfully purified from each other (Figure 4B). Different separation procedures have been applied for obtaining purified CGTases. In most cases,

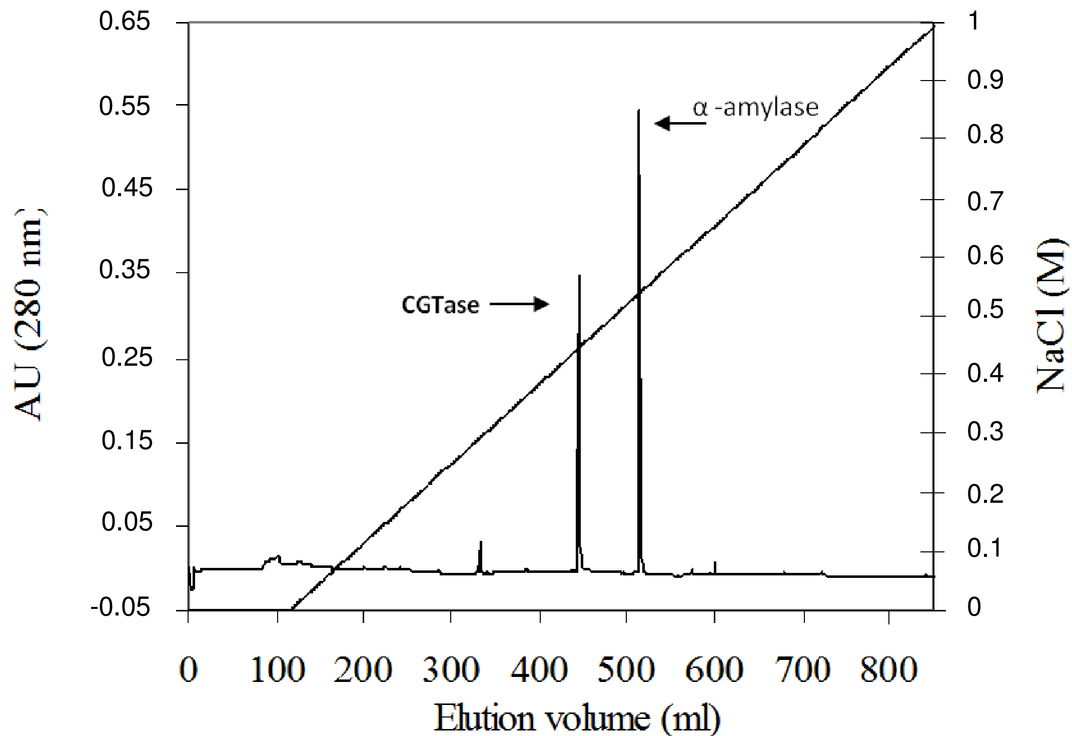

Figure 3. Elution pattern of *B. agaradhaerens* WN-I. CGTase elution from MonoQ ion-exchange column by gradient elution with 0 to1 M NaCl in 50 mM Tris-HCl buffer (pH 8), at flow rate of 1.5 ml/min; 2 ml fractions were collected.

three or four steps of purification, including ultrafiltration, gel filtration, starch adsorption and ion exchange chromatography (Rahman et al., 2006; Charoensakdi et al., 2007; Alves-Prado et al., 2008; Savergave et al., 2008) or ammonium sulfate precipitation and two steps ion exchange chromatography are used (Doukyu et al., 2003; Cao et al., 2005; Ong et al., 2008).

Properties of *B. agaradhaerens* WN-I CGTase

Molecular weight of the enzyme

The molecular weight of the purified denaturated CGTase by SDS-PAGE was estimated to be 85 kDa (Figure 4A) and this value was quite close to the established molecular weight of 89 kDa (Figure 5) of the purified native enzyme by gel filtration on a superdex-200. The purified CGTase from *B. agaradhaerens* WN-I gave a single band of protein by SDS-PAGE and a single protein peak by gel filtration, which indicated that the CGTase was monomeric in nature. Most of the reported CGTases from alkaliphiles are monomeric with molecular weight between 69 and 80 kDa (Cao et al., 2005; Sian et al., 2005; Hirano et al., 2006; Alves-Prado et al., 2007; Atanasova et al., 2011). CGTases with a lower molecular weight between 33, 36, and 59 kDa such as those from *Bacillus coagulans* (Akimura et al., 1991), *Bacillus lentus* (Sabioni and Park 1992), and *Bacillus sphaericus* strain 41 (Moriwaki et al. 2009), respectively were reported. Martins and Hatti-Kau (2002) reported the characterization of CGTase from *B. agaradhaerens* LS-3C, isolated from Ethiopian Soda Lake, with molecular weight of 110 kDa.

Effect of temperature on CGTase activity and stability

The temperature profile of the enzyme was estimated by measurement of the cyclizing activity at various temperatures at pH 6.0 (Figure 6A). The enzyme was optimally active at 55°C. The relative activities at 60 was 76.2% and the increase of temperature to 65°C rapidly decreased enzyme activity to 23.8%. Temperature optima in the range of 55 to 65°C have been reported for CGTases from alkaliphiles (Cao et al., 2005; Sian et al., 2005; Hirano et al., 2006; Alves-Prado et al., 2007; Atanasova et al., 2011).

The effect of temperature on the stability of the CGTase at various pH was investigated. The enzyme solution, in

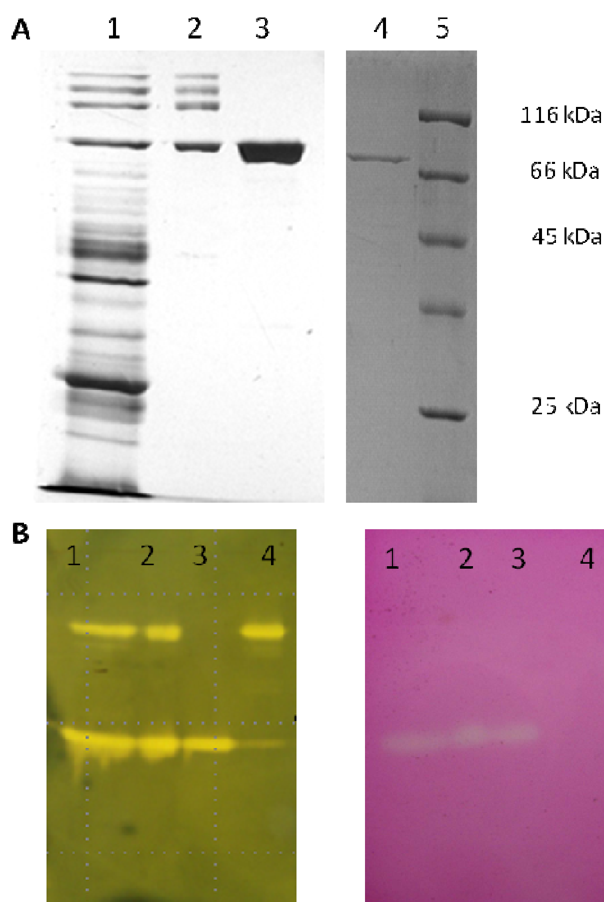

Figure 4. A, SDS-PAGE analysis of various purification steps of the *B. agaradhaerens* WN-I CGTase. Lane 1, Culture supernatant; lane 2, eluate from corn starch; lanes 3 and 4, purified CGTase (MonoQ fractions); lane 5, marker proteins. Proteins were heated with the denaturing buffer at 95ºC for 5 min and separated on 12 % polyacrylamide slab gel. Protein bands were detected by silver staining. B, Native PAGE. Left, starch degrading activity staining; lane 1, crude enzymes (supernatant); lane 2, corn starch fraction; lane 3, purified CGTase; lane 4, purified α-amylase; Right, CGTase activity staining; lane 1, crude enzymes (supernatant); lane 2, corn starch fraction; lane 3, purified CGTase; lane 4, purified α-amylase.

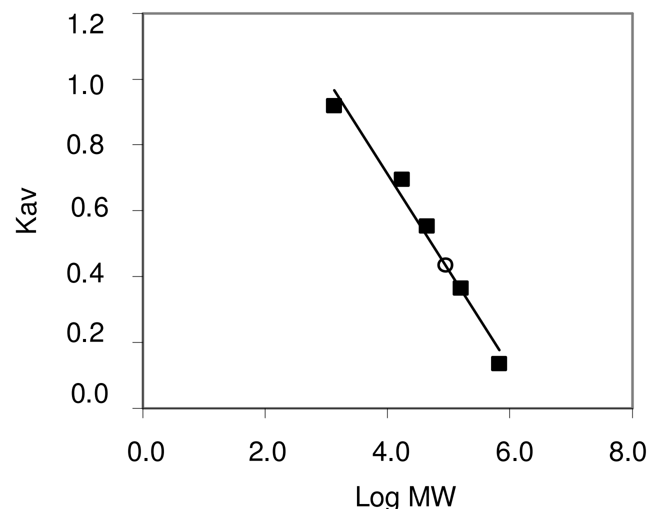

Figure 5. Calibration curve of standard proteins (■) and purified CGTase (o). Standard proteins were applied to Hiload TM superdex 200 16/60 column. Proteins were eluted using 50 mM Tris-HCl buffer (pH 7.6) at a flow rate of 1 ml/min. Log MW, Logarithm of molecular weight (kDa); K_{AV}, partition coefficient.

different buffers (0.1 M) at pH 6.0 (sodium acetate), pH 8.0 (Tris-HCl) and pH 10.0 (glycine-NaOH), respectively, was incubated for 1 h at various temperatures prior to determination of the residual activity under the standard assay conditions. The results presented in Figure 6B indicated that the enzyme was stable up to 40°C at all the tested pH levels. However, at higher temperature, the enzyme was more resistant to thermal denaturation at pH 8 (Tris-HCl). Interestingly, at the same conditions, CGTase from *B. agaradhaerens* LS-3C was totally inactive in Tris-HCl buffer (pH 8.0) at 40°C (Martins and Hatti-Kau, 2002). Earlier reports showed the CGTase to be more resistant to thermal denaturation in the presence of its substrate, product, and calcium ions (Gawande et al., 1999; Alves-Prado et al., 2007; Atanasova et al., 2011). The effect of these additives on the *B. agaradhaerens* WN-I CGTase was studied in glycine buffer at pH 9.0. It was found that the thermal stability of *B. agaradhaerens* WN-I CGTase was enhanced by calcium ion, starch (1%) and maltodextrins (1%) at 45°C by about 1.8, 1.9, and 1.9 fold, respectively (Figure 6C). Structural analysis of the CGTases showed the presence of two calcium ions, one near the N-terminal end and the other near the active site region; provide stability to the conformational structure of flexible regions of the protein molecule including the active site (Harata et al., 1996).

Effect of pH on CGTase activity and stability

The influence of pH on the CGTase activity was established by the determination of the enzyme activity at varying pH values ranging from 5.0 to 11.0 at 50°C using the standard assay conditions. Different suitable buffers were used including 50 mM sodium acetate (pH 5.0 and 6.0), 50 mM sodium phosphate (pH 7.0, 7.5 and 8.0), 50 mM glycine-NaOH buffer (pH 8.5, 9.0, 9.5, 10.0) and 50 mM carbonate-bicarbonate buffer (pH 10.5 and 11.0), respectively. Optimal activity of the purified CGTase was seen at pH 9.0 in 50 mM glycine-NaOH buffer and the enzyme showed low activity at pH 5.0 and 11.0 (Figure 7A). It should be noted that the reported CGTases from

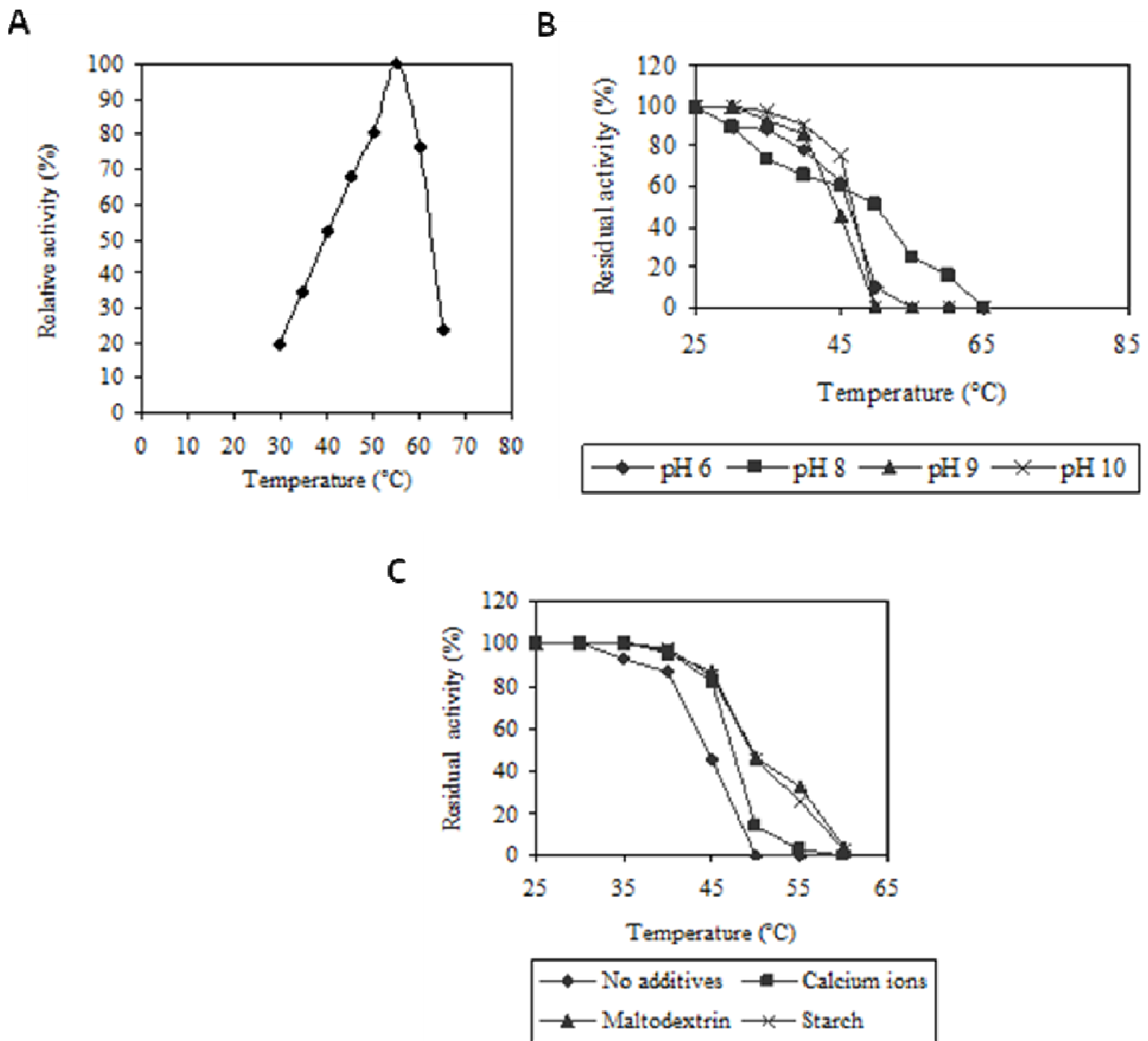

Figure 6. A, Effect of temperature on the activity *B. agaradhaerens* WN-I CGTase. The enzyme activity corresponding to 100% was 5.31 U/ml. Standard deviation of the relative activities were in the range of 0.2 to3.0%. B, Effect of temperature on the stability of *B. agaradhaerens* WN-I CGTase in different pH values. The CGTase prepared in different buffers (pH 6 to 11) was preincubated for 1 h at different temperatures, prior determination of the residual activity under the standard assay conditions. The enzyme activity corresponding to 100% was 5.31 U/ml. Standard deviation of relative activities were in the range of 0.1 to 3.0%.

alkaliphiles show an optimal cyclizing activity in pH range from 5.0 to 10.0 depending on the bacterial producer (Sian et al., 2005; Hirano et al., 2006; Alves-Prado et al., 2007; Moriwaki et al., 2009). However, some CGTase exhibited two pH peaks at pH 6.0 and 9.0, such as CGTase from *Bacillus* sp. 7–12 (Cao et al., 2005), *Bacillus firmus* (Higuti et al., 2003), *B. firmus* NCIM 5119 (Gawande et al., 1999) and *Bacillus pseudalcaliphilus* 20RF (Atanasova et al., 2011).

The pH stability of the *B. agaradhaerens* WN-I CGTase was determined by pre-incubation of the enzyme in buffers of various pH values for 1 h at 25°C prior to the determination of the residual activities measured under standard assay conditions. The activity of the untreated enzyme was taken as 100%. The enzyme retained 94 to100% of its initial activity in a wide range of pH, between pH 6.0 and 11.0 (Figure 7B).The purified CGTase was highly stable at pH 5.0 and 11.0 maintaining

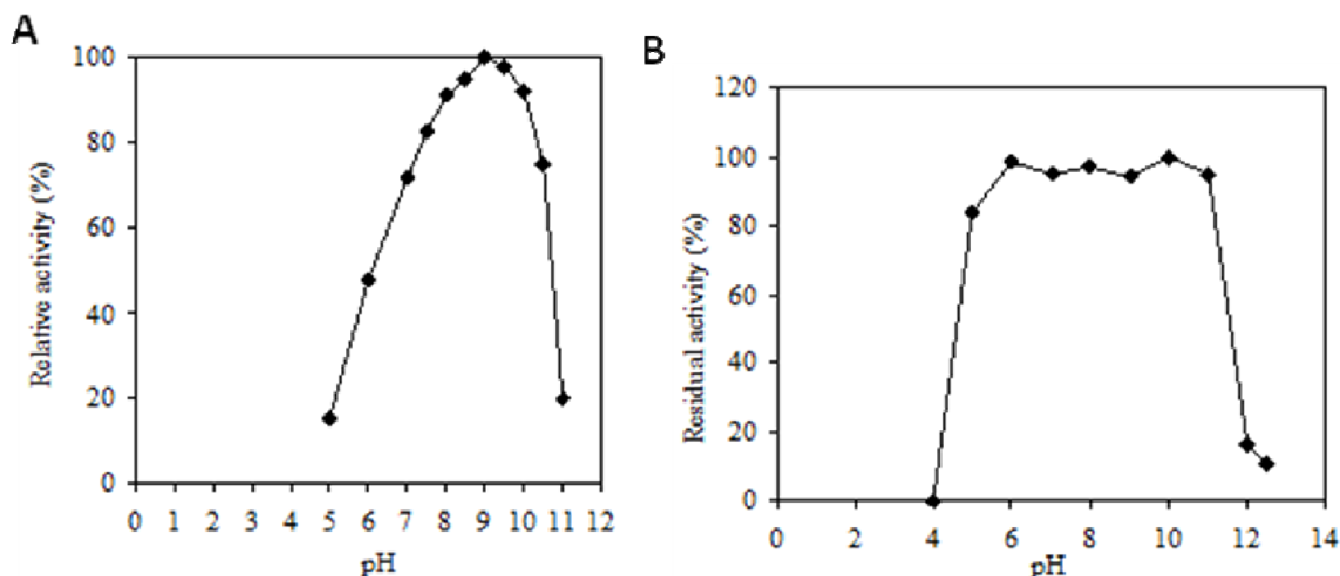

Figure 7. A, Effect of pH on the activity of *B. agaradhaerens* WN-I CGTase. Enzyme activity was measured under the standard assay conditions at various pH values at 55°C. The CGTase activity corresponding to 100% was 4.74 U/ml. Standard deviations of relative activities were in the range of 1 to 4%. B, Effect of pH on the stability of *B. agaradhaerens* WN-I CGTase. Standard deviations of the residual activities were in the range of 0 to 3.5%.

Table 2. Effect of various ions and reagents on the activity of *B. agaradhaerens* WN-I CGTase.

Salt/reagent	Residual activity (%)	
	1 mM	10 mM
None	100.00	100.00
$CaCl_2$	104.67	115.34
KCl	113.70	112.0
NaCl	96.57	87.37
$CuCl_2$	86.60	47.47
$CoCl_2$	21.00	0.00
$ZnCl_2$	28.97	11.83
$MgSO_4$	91.90	74.76
$FeSO_4$	0.00	0.00
$MnCl_2$	94.39	90.96
$BaCl_2$	106.94	109.15
α-CD	104.41	96.56
β-CD	76.00	ND
γ- CD	82.02	62.14
Dithiothritol	68.77	65.30
EDTA	67.40	40.19
Mercaptoethanol	87.34	85.00

(1) Standard deviation, 0.2-3.5; (2)100% enzyme activity was 4.88 U/ml; ND, not determined.

83.3 and 94% residual activities, respectively and could be successfully applied in the range of pH from 5.0 to 11.0. In comparison to other reported CGTases from alkaliphiles, *B. agaradhaerens* WN-I enzyme showed a very wide pH range for stability (after 1 h pre-incubation), slightly higher than alkaliphilic *B. pseudalcaliphilus* 20RF CGTase (Atanasova et al., 2011). However, *B. agaradhaerens* WN-I CGTase was much more stable than other alkaliphilic *B. sphaericus* strain 41 CGTase, where at pH 5.0, the activity retained was 34.2%, while in the range of pH 8.0 to 10.0 the average retention of activity was 16.8% (Moriwaki et al., 2009).

Effect of various reagents and metal ions on CGTase activity

In order to establish the effect of various metal ions and reagents, the enzyme was pre-incubated with 1 and 10 mM of the compounds in 50 mM glycine buffer (pH 9.0) for 1 h at 25°C, and then the residual cyclizing activity was measured under the standard assay conditions (Table 2). A complete loss of activity was established in the presence of Fe^{2+} and Co^{2+}. In addition, significant loss of enzyme activity was observed in the presence of zinc followed by copper salts, especially at high concentrations (10 mM). These results is similar to that reported by Atanasova et al. (2011), who indicated a loss of alkaliphilic *B. pseudalcaliphilus* 20RF CGTase activity in the presence of 15 mM Zn^{2+}, followed by Ag^{+}, Fe^{2+} and

Table 3. Quantitative analysis of the end products of soluble starch hydrolysis by action of the purified *B. agaradhaerens* WN-I CGTase.

Time (h)	Soluble starch (%)	Reducing sugar (mM)	α-CD (mM)	γ-CD (mM)	β-CD (mM)	Total CDs (mM)	Soluble starch conversion (%)	α-CD (%)	γ-CD (%)	β-CD (%)
0	0.94	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.49	0.46	0.12	0.00	1.54	1.66	18.88	7.49	0.00	92.51
1.5	0.38	1.28	0.37	0.23	1.57	2.17	24.66	17.05	10.66	72.29
2	0.30	1.29	0.42	0.08	1.85	2.34	26.59	17.78	3.32	78.90
3	0.24	1.41	0.73	0.29	2.51	3.53	39.98	20.72	8.10	71.18
6	0.15	2.38	0.88	0.16	2.36	3.40	38.62	25.93	4.71	69.36
8	0.23	5.46	1.16	0.44	2.94	4.54	51.56	25.61	9.77	64.62

Table 4. Quantitative analysis of the end products of Paselli starch hydrolysis by action of the purified *B. agaradhaerens* WN-I CGTase.

Time (h)	Paselli starch (%)	Reducing sugar (mM)	α-CD (mM)	γ-CD (mM)	β-CD (mM)	Total CDs (mM)	Paselli starch conversion (%)	α-CD (%)	γ-CD (%)	β-CD (%)
0	0.96	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.17	0.80	0.28	0.28	0.00	1.17	1.45	16.46	19.20	0.00	80.80
0.5	0.57	0.50	0.34	0.00	1.28	1.62	18.40	21.11	0.00	78.89
1	0.35	0.90	0.71	0.07	2.14	2.92	33.15	24.36	2.45	73.19
2	0.19	1.57	1.23	0.28	2.72	4.22	47.95	29.06	6.59	64.35
3	0.15	1.69	1.62	0.59	3.67	5.88	66.77	27.54	10.06	62.39
6	0.09	2.79	1.38	0.75	3.62	5.75	65.27	24.00	13.04	62.96
8	0.06	6.14	1.62	0.95	4.36	6.93	78.60	23.39	13.65	62.96

Co^{2+} ions (Atanasova et al., 2011). Unlike the presented results, the CGTases from *Bacillus* AL-6 (Fujita et al., 1990), *B. firmus* (Yim et al., 1997) and *B. firmus* (Higuti et al., 2003) activity was not inhibited by 1 to 2 mM Fe^{2+}. Furthermore, the potent inhibitory effect of Co^{2+} on the *B. agaradhaerens* WN-I CGTase was in contrast to that reported by Martins and Hatti-Kaul (2002), where cobalt had no effect on the activity of the CGTase from *B. agaradhaerens* LS-3C. Moreover, the CGTase from *B. halophilus* INMIA-3849 was stabilized in the presence of Fe^{2+} or Co^{2+} (Abelyan et al., 1995). The inhibitory effect of ions is attributed to the metal catalyzed oxidation of amino acid residues essential to the enzyme activity. The most probable candidates would be tryptophan which is known to be present in the active site and specific tyrosine and histidine residues which are ascribed an important role in the cyclisation efficiency and in the transition state stabilization (Uitdehaag et al., 1999). The enzyme was slightly inhibited in the presence of manganese and magnesium ions. The reducing agents, 2-mercaptoethanol and dithiothreitol (DTT), resulted in significant CGTase inhibition. The enzyme activity was also inhibited significantly in the presence of EDTA, a metal chelating agent, suggesting that this CGTase could be metalloenzyme. The CGTase activity was slightly stimulated in the presence of Ca^{2+}, which appeared to be a characteristic feature of different bacterial CGTases (Hirano et al., 2006; Alves-Prado et al., 2007). In contrast to *B. agaradhaerens* LS-3C CGTase reported by Martins and Hatti-Kaul (2002), where Ba^{2+} had inhibitory effect, it had slight stimulatory effect on the activity of CGTase from *B. agaradhaerens* WN-I. Generally, the effect of metal ions and reagents on the activity of CGTases depends on the bacterial producer.

Among the CDs, 1 mM of β- and γ-CD showed slight inhibitory effect on the enzyme activity with residual activity of 76 and 82.02%, respectively. Some authors reported the total inhibition of CGTase by the reaction products. The CGTase from *B. megaterium* showed a total loss of activity in the presence of 12 mg/ml of β-CD (Zhekova et al., 2008), as did the CGTase from *B. agaradhaerens* LS-3C in the presence of 2 mg/ml of β-

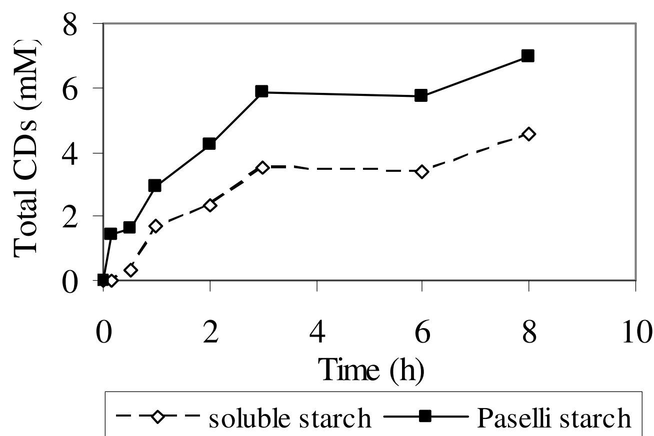

Figure 8. Total CDs production by action of purified CGTase on soluble starch and Paselli starch. Purified CGTase was incubated with 1% (w/v) of soluble starch and Paselli starch, respectively, in 50 mM glycine-NaOH buffer (pH 9), at 50°C. Samples were removed in different time intervals for analysis.

CD (Martins and Hatti-Kaul 2003).

Kinetic parameters

Kinetic constants, K_m and V_{max} values of the purified CGTase were by measuring the initial rates of the reaction at different concentrations of soluble starch or maltodextrins in glycine buffer solution (50 mM, pH 10) at 50°C using Lineweaver–Burk plot (Mathews, 1990). The K_m and V_{max} values using soluble starch were estimated to be 15.4 ± 3.2 mg/ml and 39.2 ± 2.5 µmol/min, respectively. In addition, The K_m and V_{max} values obtained with maltodextrin as the substrate were 20.8 ± 3.0 mg/ml and 102.0 ± 4.5 µmol/min, respectively. K_m values ranging from 1.77 to 5.7 mg/ml and V_{max} from 43 to 1027 µmol-/min have been previously reported for *Bacillus* sp. CGTases (Doukyu et al., 2003; Cao et al., 2005; Ong et al., 2008; Gastón et al., 2009).

Cyclodextrin production by the purified CGTase

The production yield and ratio of the different CDs formed by CGTases are dependent not only on the microbial source producing the enzyme, but also on the nature of the substrate and conditions of biotransformation, such as temperature, pH and time (Thiemann et al., 2004; Moriwaki et al., 2009). Analysis of the end products of the soluble starch degradation by *B. agaradhaerens* WN-I CGTase indicated that the purified enzyme produced different oligosaccharides, G3, G4, G5, and G6 in addition to CDs, indicating hydrolytic activity in addition to the cyclization one. A maximum yield of CDs with about 40% conversion of soluble starch into CDs was obtained after 3 h and the product ratio at that point was 20.72% α-CD, 71.18% β-CD and 8.1% γ-CD (Table 3). Using Paselli starch, the maximum yield of CDs with 66.77% conversion of Paselli starch into CDs was obtained after 3 h and the product ratio was 27.54% α-CD, 62.39% β-CD and 10.06% γ-CD (Table 4). Comparison between the total CD production by action of the purified CGTase on 1% (w/v) soluble starch and Paselli starch showed that the total amount of CD was 1.5-1.8-fold higher using Paselli starch than that in the case of soluble starch (Figure 8). Paselli starch is potato starch predigested with α-amylase. Its average degree of polymerization, that is the average number of glucose units per molecule, is about 50, where soluble starch is more highly polymerized (Van der Veen et al., 2000), indicating that the *B. agaradhaerens* WN-I CGTase had higher activity toward smaller substrate. The profile of CD production by the purified CGTase from *B. agaradhaerens* WN-I was slightly different from that of the *B. agaradhaerens* LS-3C CGTase, which produced mainly β-CD (89%) with minority of α-CD and no production of γ-CD (Martins and Hatti-Kaul, 2002). In addition to the cyclization activity, the purified *B. agaradhaerens* WN-I CGTase had significant hydrolytic activity toward soluble starch (12.1 U/mg) with cyclization to hydrolytic activity ratio of 1: 0.4, respectively. These results are consistent with those reported by Gawande and Patkar (2001). Depending on the most abundant form of cyclodextrin that CGTase produces, the enzyme is sometime classified as α-, β and γ-CGTase (Tonkova, 1998). By this classification, *B. agaradhaerens* WN-I CGTase could be considered as β-CGTase. Of the three kinds of CDs, β-CD is of most practical use because its inclusion complexes are easily prepared and stable. The size of the β-CD polar cavity is optimum for many molecules such as drugs and preservatives; furthermore β-CD is easily separated from the reaction mixtures due to its low solubility in water (Doukyu et al., 2003).

Conclusion

In this study, purification and characterization of a CGTase from a new *B. agaradhaerens* strain (WN-I) isolated from Egyptian Soda Lakes was reported. Enzyme purification to homogeneity was achieved by starch adsorption and ion exchange chromatography with a recovery of 26.40% activity and 21-fold purification. The purified enzyme was monomer and its molecular weight was estimated to be 85 kDa. The applied purification procedure is easily feasible under industrial conditions. In comparison to other CGTases obtained from alkaliphiles, the present CGTase from the new *B. agaradhaerens* WN-I could be effectively used for the conversion of raw starch into cyclodextrins in a wide pH range, from 6.0 to

11.0 and temperatures 50 to 60℃. Moreover, the enzyme was more heat resistant in the presence of starch, maltodextrin and calcium ion. The enzyme showed a significant stability in the presence of various metal ions and reagents after 1 h min incubation at 25℃. The purified CGTase from *B. agaradhaerens* WN-I could be used for an efficient cyclodextrin production without any additives which is of an industrial interest. The achieved high conversion of an soluble starch into cyclodextrins (40%) with predominant formation of β-CD (71.8%) and α-CD (20.72%) and negligible quantity of γ-CD (8.1%) after 3 h of reaction at 50℃ and pH 9.0 makes *B. agaradhaerens* WN-I CGTase industrially desired for cyclodextrin manufacture. The total amount of CD was 1.5 to 1.8-fold higher using Paselli starch than that in the case of soluble starch. Based on the most abundant form of cyclodextrin that CGTase produces, *B. agaradhaerens* WN-I CGTase could be considered as β-CGTase. Finally, *B. agaradhaerens* WN-I CGTase showed some different properties from *B. agaradhaerens* LS-3C CGTase that were previously reported by Martins and Hatti-Kaul (2002), and further analysis at the molecular level including CGTase gene cloning and sequencing is on progress for further characterization.

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