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Isolation, Purification and Biochemical Characterization of CGTase from *Bacillus halodurans*.

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Summary

A novel Cyclomaltodextrin glucanotransferase (CGTase) producer, *Bacillus halodurans* was isolated from soil obtained from sugarcane fields. CGTase was produced in bulk through submerged batch fermentation in Horikoshi's Media II. Soluble starch was used as carbon source and a combination of yeast extract and peptone were used as nitrogen source in the media, along with MgSO₄·7H₂O, K₂HPO₄ and Na₂CO₃, as they were found to be ideal for CGTase production. The enzyme was purified through acetone precipitation and starch adsorption methods, which proved to be simple and efficient methods of purification. Starch adsorption purified sample was found to be homogenous on performing SDS-PAGE and the yield of the method was 49.44% with fold purification of 17.34. The enzyme had appreciable affinity for starch with a Km of 1.1mM and a turnover number of 10.9s⁻¹ and was found to have an apparent molecular weight of ≈33 KDa. CGTase had two pH optima at pH 7.0 and pH 9.0 and a temperature optimum of 60°C. There was no effect of metal-chelating agents on enzyme activity indicating that the enzyme is not a metalloenzyme, however it is a metal-activated enzyme as activity was enhanced by Mn²⁺. Inhibitory effects of group specific reagents indicate that serine and histidine residues may be involved in enzyme activity. The microorganism isolated grows in a wide range of temperatures, pH and salt concentrations, which are useful attributes in industrial applications requiring versatile organisms. The enzyme isolated also has appreciable activity at higher temperature and pH and is easily purified; making it valuable for use in industry.

Keywords – CGTase; Cyclomaltodextrin glucanotransferase; *Bacillus halodurans*, β-cyclodextrin

INTRODUCTION

Cyclomaltodextrin glucanotransferase (CGTase) with EC number 2.4.1.19 is a bacterial enzyme that is classified under the α-amylase family or the glycosyl hydrolase family 13 (Park et al, 2000) due to their similar structural domains for starch hydrolysis. However, CGTase exhibits cyclization, disproportionation and coupling activities in addition to dextrinizing activity (Kelly et al, 2009b). The enzyme is produced from a variety of organisms including *Bacillus* sp., *Paenibacillus* sp., *Klebsiella* sp., *Xanthomonas* sp., *Vibrio* sp., *E.coli.*, *Geobacillus* sp., *Thermococcus* sp., *Thermoanaerobacterium* sp., etc (Khairizal et al, 2004; Tonkova, 1998). It is largely an extracellular enzyme although it has been localized in the cytoplasm of *Escherichia coli* and intracellularly in *Paenibacillus macerans*. However, *Bacillus* spp. are the most commonly isolated organisms that produce CGTase and so the enzyme is most well-characterized in this genus. CGTase producers can be isolated from various sources such as soil, waste, plantations, hot springs and even deep-sea mud (Georganta et al, 1993).

CGTases produce α-, β- and γ-cyclodextrins (CDs) which are cyclic compounds containing 6, 7 and 8 glucose units respectively, bound by α-1,4-bonds. They also produce larger CDs and oligosaccharides. CGTases are classified as α-, β- and γ-CGTase based on their primary cyclodextrin product. CDs have been known for their industrial applications in the food, textile, cosmetic, agricultural, chemical and pharmaceutical industries due to their ability to form inclusion complexes with small hydrophobic molecules (Astray et al, 2009; Cal and Centkowska, 2008; Martin del Valle, 2004). Inclusion of organic or inorganic molecules in CDs changes their properties like solubility, reactivity, heat and light susceptibility, etc (Wind et al, 1998). Among the three CDs, β-CD is the most useful due to cavity diameter of 7-8Å (Popova and Pishtiyski, 2001) and also due to the ease of separation of β-CD (Lee et al, 1992).

The purpose of the present study was to isolate a versatile and alkaliphilic CGTase-producing bacteria, capable of producing mainly or specifically β-CGTase. β-CGTase thus produced would prove to be easily purified and characterized in order to ascertain its applicability in the industry.

MATERIALS AND METHODS

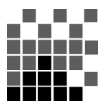
Microorganisms and Culture Conditions

Alkaliphilic, CGTase producing bacteria was isolated from soil samples obtained from sugarcane plantations in Marathahalli, Bangalore. The supernatant obtained by suspending 1g of soil in 10 ml of water and allowing it to sediment was used as inoculums during screening.

Screening was carried out by the rapid screening method of Park et al (1989). Horikoshi's medium II containing 1.5% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.01% MgSO₄·7H₂O, 0.1% K₂HPO₄, 1% Na₂CO₃, 0.02% phenolphthalein, 0.01% methyl orange and 2% agar was utilized. The medium was adjusted to pH 10.5 so as to isolate alkaliphilic bacteria. 0.1ml of the soil supernatant was inoculated on to the solid medium by the spread-plate method. Isolated colonies around which a yellow hollow zone was observed were considered CGTase producers and were selected for further study. The bacteria was identified as *Bacillus halodurans* according to the Bergey's manual.

The appropriate carbon and nitrogen sources were selected by varying the components of the Horikoshi's medium II. The different components used were corn starch, wheat starch, potato starch, hydrolyzed starch and amylopectin as carbon source and peptone, tryptone and yeast extract as nitrogen source in individual flasks. Soluble starch and a combination of yeast extract and peptone gave the best result and so culture medium of following composition was used for enzyme

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production: 1.5% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% K_2HPO_4 , 1% Na_2CO_3 , pH 10.5.

Enzyme Production

The enzyme was produced in bulk through submerged fermentation at 37°C using the modified Horikoshi's medium II at pH 10.5. 1ml of broth (Horikoshi's medium II) inoculated with a loopful of pure culture and allowed to grow at 37°C for 48 hours served as inoculum for submerged fermentation. The broth was harvested on the 8th day and was centrifuged at 10,000 rpm for 10 min in order to obtain the cell-free extract; the supernatant served as the source of CGTase.

Cyclodextrin Quantification

Qualitative analysis of CDs was done by the plate assay method. 2% agar was mixed with 0.035mM methyl orange and 0.04mM phenolphthalein in 125mM Na_2CO_3 respectively for assay of α - and β -CDs. The gel was poured onto a glass plate, a well was punched and a solution of enzyme pre reacted with 4% starch at 60°C was loaded. A zone of clearance around the well was considered an indication of production of respective CDs by the enzyme.

Production of α -CD by the enzyme was assayed by the method of Lejeune et al (1989) slightly modified by Gawande et al (1998).

Production of β -CD by the enzyme was assayed by the phenolphthalein method (Goel and Nene, 1995). 650 μ l of 4% starch in 0.1M phosphate buffer (pH 7.0), 250 μ l of 0.1M phosphate buffer (pH 7.0) and 100 μ l of the enzyme solution were incubated for 15min at 60°C. 4ml of phenolphthalein (0.04mM phenolphthalein in 125mM Na_2CO_3) was then added to the reaction mixture and the absorbance was measured at 540nm. Blank was prepared as before by inactivating the enzyme before addition of substrate. Reduction in the intensity of pink colour was considered a positive test for the production of β -CD. One unit of CGTase activity is defined as the amount of enzyme able to produce 1 μ mol of β -CD per min.

The γ -CD forming activity was determined by the bromocresol green (BCG) method of Kato and Horikoshi (1984) with some modifications.

Protein Assay

The concentration of protein in the enzyme solutions were assayed by the Bradford's method of protein estimation (Bradford, 1976). Bovine serum albumin (in the range of 10 μ g -100 μ g) was used as standard.

Enzyme Purification

The cell-free extract was used for partial purification of enzyme. The extract was subjected to ammonium sulphate precipitation. The salt concentration was raised from 0-70% stepwise and each fraction, obtained by centrifugation and resuspension in minimum quantity of buffer, was tested for CGTase activity after dialysis to remove the salt.

The cell-free extract was also subjected to acetone precipitation. In this method, 4 times the volume of chilled acetone was added to the extract and it was allowed to precipitate overnight at -20°C. A pellet was obtained by centrifugation at 10,000rpm for 10minutes. The pellet was dissolved in a minimum quantity of 0.1M phosphate buffer (pH 7.0). This acetone precipitate was used for the characterization of the enzyme.

Starch adsorption was carried out as follows (Ferrarotti et al, 1996): acetone precipitated sample (10ml) was mixed with 3% starch and ammonium sulphate concentration was raised to 20% saturation at 4°C. The solution was gently stirred for 60 min. The starch (on which the CGTase should adsorb) was collected by centrifugation at 4000g for 20 min at 4°C. The pellet was washed twice with 10 mM phosphate buffer (pH 7.0). 1 mM solution of β -CD (used to extract adsorbed CGTase) in 10 mM phosphate buffer (pH 7.0) was added under mechanical stirring at 37 °C for 30 min. This was then centrifuged (27000g for 10 min at 4 °C) and the supernatant was dialysed overnight against 10 mM phosphate buffer (pH 7.0) at 4°C.

Enzyme Characterization

Polyacrylamide Gel Electrophoresis: The SDS-discontinuous buffer system based on the method of Laemmli (1970). A 12% resolving gel was used; stained by silver staining. The molecular weight marker used contained Phosphorylase b (97.4 kDa), Serum albumin (66.2 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (31 kDa), Trypsin inhibitor (21.5 kDa) and Lysozyme (14.4 kDa).

Zymogram (Manchenko, 1994) was performed using a discontinuous buffer system with 12% resolving gel.

Kinetic Parameters: The effect of substrate concentration on CGTase was determined using the standard phenolphthalein assay method as described before using starch concentrations varying from 0.01% to 10%. The K_m and V_{max} values were then determined from Lineweaver-Burke plot (Lineweaver and Burke, 1934).

Effect of Temperature: The influence of temperature on CGTase activity was studied over the temperature range of 30°C to 90°C. 650 μ l of 4% starch was preincubated with 250 μ l of 0.1M phosphate buffer (pH 7.0) at the respective temperature for 5 minutes. 100 μ l of enzyme was then added to the test tubes and they were incubated for another 15min at the same temperature as preincubation. 4ml of phenolphthalein (0.04mM phenolphthalein in 125mM Na_2CO_3) was then added and the absorbance was checked at 540nm. Separate blanks were run for each of the temperatures with inactivated enzyme.

Effect of pH: The CGTase activity was studied over the pH range of 4.5 to 11.0 using different buffers: 0.2M citric acid- Na_2HPO_4 buffer (pH 4.5-5.0), 0.2M sodium phosphate buffer (pH 5.5-8.0), 0.2M Tris-HCl buffer (pH 8.5), 0.2M glycine-NaOH buffer (pH 9.0 to 11.0).

The assay was performed by preincubating 100 μ l of enzyme with 1 ml of each of the pH buffers at room temperature. 650 μ l of 4% starch was then added to each test tube and incubated for 15 min at 60°C. 4ml of phenolphthalein (0.04mM phenolphthalein in 125mM Na_2CO_3) was then added to each and absorbance was read at 540nm. Separate blanks were run for each of the pHs with inactivated enzyme.

Effect of Metal Ions: Effect of different divalent metal ions on CGTase was analysed. Effect of Fe^{2+} , Cu^{2+} , Zn^{2+} , Ca^{2+} , Mg^{2+} , Mn^{2+} were tested. The metal ions were prepared at 2mM and 10mM concentrations. Each of these ions at both concentrations were assayed separately. 500 μ l of 4% starch and 500 μ l of each metal ion were taken in test tubes and 100 μ l of the enzyme was added. The tubes were incubated at 60°C for 15 min, following which 4ml of phenolphthalein (0.04mM phenolphthalein in 125mM Na_2CO_3) was added and the absorbance was checked at 540nm. Separate blanks were run for each of the metal ions at both concentrations with inactivated enzyme.



Effect of Group Specific Reagents and Potential Inhibitors: Effect of Mercuric chloride (HgCl_2), Ethylenediaminetetraacetic acid (EDTA), Iodoacetic acid (IAA), Urea, N-Acetylimidazole (NAI), Sodium azide (NaN_3), Sodium dodecyl sulphate (SDS), *N-p*-Toluenesulfonyl-L-phenylalanine chloromethylketone (TPCK), Dithiothreitol (DTT), *N-p*-toluenesulfonyl-L-lysine chloromethylketone (TLCK) and phenyl methyl sulphonyl fluoride (PMSF) were tested. 100 μl of the enzyme was pre-incubated with 250 μl of 0.1M phosphate buffer (pH 7.0) and 200 μl of each of the reagents individually. 650 μl of substrate was then added and incubated at 60°C for 15 minutes, following which 4ml of phenolphthalein (0.04mM phenolphthalein in 125mM Na_2CO_3) was added and the absorbance was checked at 540nm. Separate blanks were run for each of the group specific reagents with inactivated enzyme.

RESULTS AND DISCUSSION

Microorganisms and Culture Conditions

Isolation yielded a number of colonies on agar plates and those colonies that showed a yellow hollow zone around them after screening on phenolphthalein-methyl orange agar medium were considered positive for CGTase production and were selected for further studies. The organism was previously named *B. alcalophilus* subsp. *halodurans* (Boyer et al, 1973) and some strains were formerly assigned to *B. lentus* type III (Gordon and Hyde, 1982). The ability of the organism to survive in a wide range of temperature, pH and salt concentration may be useful attributes in industrial applications requiring versatile organisms.

B. halodurans was inoculated into media containing various carbon and nitrogen sources and it was found by the phenolphthalein assay that maximum CGTase production occurred in the presence of soluble starch as carbon source and in the presence of a combination of yeast extract and peptone as nitrogen sources. These were selected as the components of the submerged fermentation medium so as to optimize enzyme production.

Enzyme Production

The organism was grown in submerged fermentation medium described earlier. Submerged fermentation was used as it has been found to be the most successful and simple method of producing



Figure 1. *B. halodurans* colony showing CGTase activity

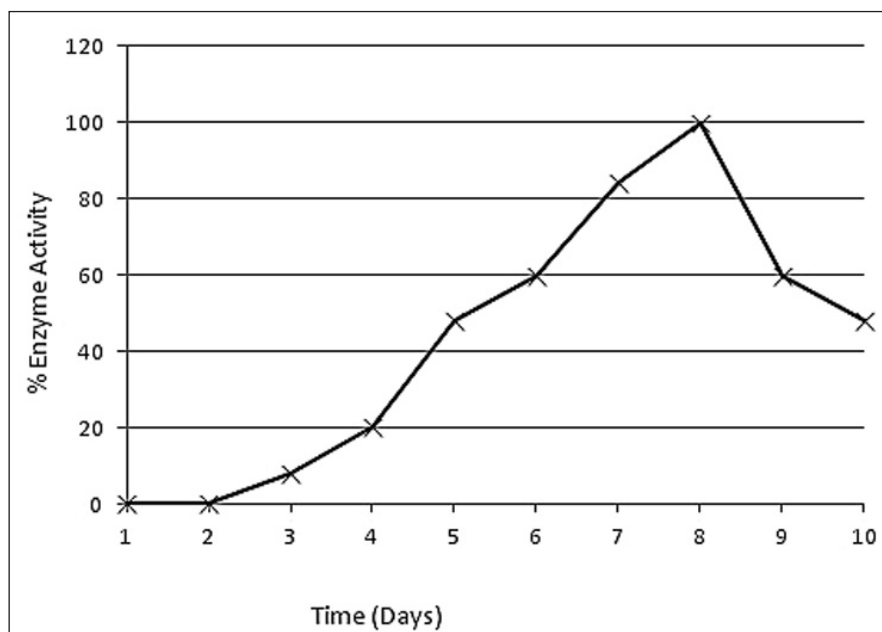


Figure 2. Percentage enzyme activity

Table 1. Purification of CGTase from *B. halodurans*

Fraction	Volume (ml)	Total Activity ($\mu\text{mol/ml}$)	Total Protein (mg)	Specific Activity (U/mg)	Fold Purification	Yield
Cell Free Extract	100	89.38	15.72	5.69	1.00	100%
Acetone Precipitate	10	61.14	4.02	15.20	2.67	68.4%
Purified by starch adsorption	2	44.19	0.448	98.64	17.34	49.44%

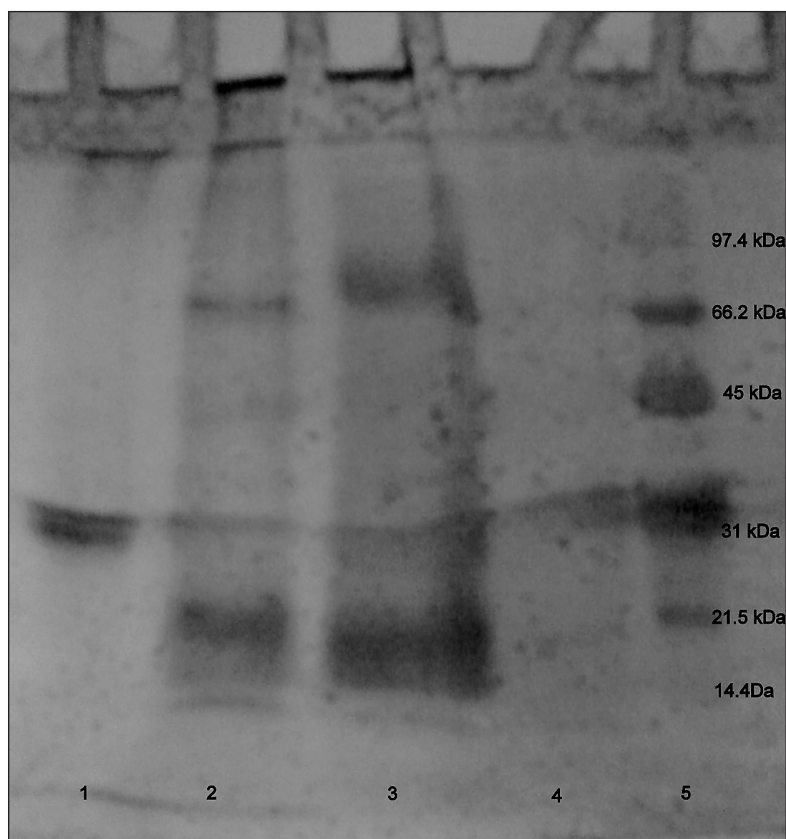


Figure 3. SDS-PAGE analysis during enzyme purification [1-Purified Sample; 2-Partially Purified Sample (Trial 1); 3-Partially Purified Sample (Trial 2); 4-Cell-Free Extract; 5-Molecular Weight Markers]

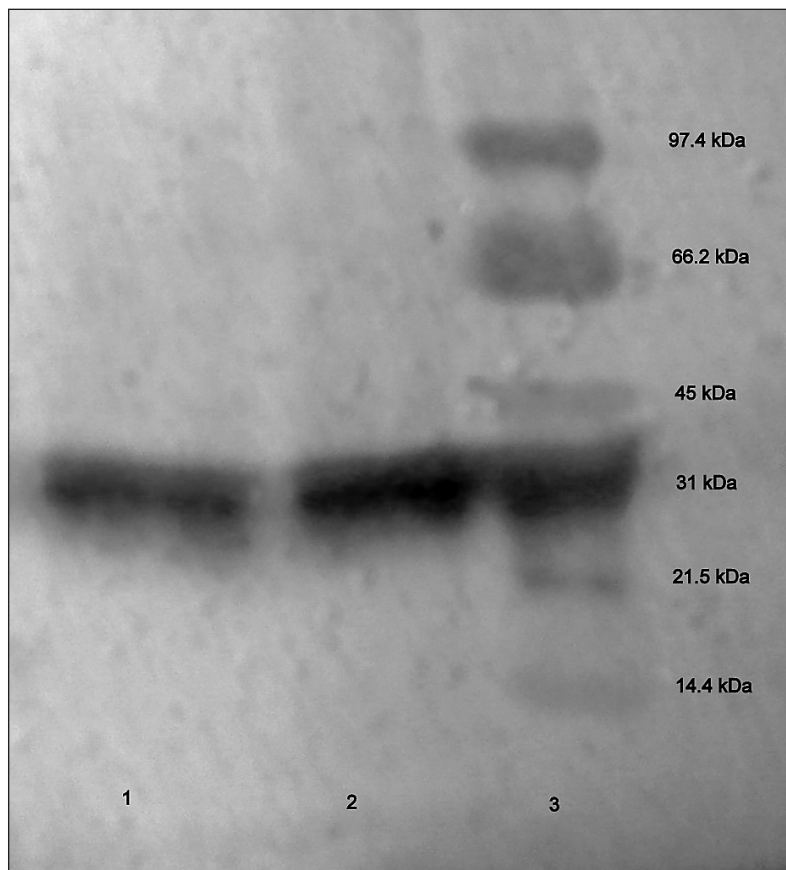


Figure 4. SDS-PAGE analysis of CGTase under reducing and non-reducing conditions [1-Purified Sample (reduced); 2-Purified Sample (non-reduced); 3-Molecular Weight Markers]

an extracellular enzyme in bulk. Solid state fermentations have also been deemed a successful strategy for enzyme production, but many studies have not been conducted on the same in context of CGTase. The enzyme was assayed qualitatively every day by the plate assay method and when a positive result was observed, the enzyme was quantitatively assayed by the phenolphthalein, methyl orange and bromocresol green assay methods described before.

Cyclodextrin Quantification

The maximum enzyme activity in terms of the production of β -CD was seen on the 8th day after inoculation with the organism, as assayed by the phenolphthalein method. The broth was harvested on this day and cell free extract was obtained by removing the cells through centrifugation. This extract was used for further studies on the enzyme.

It was found that the organism did not produce α - or γ -CDs by the methods used in this study. This is significant because production of a single CD as a product would reduce the purification steps involved in separating the three CDs for industrial use.

Enzyme Purification

The enzyme was partially purified by acetone precipitation and further purified by the starch adsorption method. The total activity, specific activity, fold purification and yield of each fraction are as shown in Table 1. After purification to homogeneity with the starch adsorption method, the specific activity was 98.64 U/ml and the fold purification and yield were 17.34 and 49.44% respectively. Other methods of purification (Moriwaki et al, 2007; Pishitski et al, 2008; Rosso et al, 2002; Wind et al, 1995; Savergave et al, 2008; Szerman et al, 2007; Vassileva et al, 2007) that have been utilized include affinity chromatography, ion exchange chromatography, ultrafiltration, gel filtration, etc. On comparison with previous studies, it was found that the yield obtained through starch adsorption was appreciable, particularly in light of the ease of the purification technique.

The purified enzyme was homogenous showing a single band on SDS-PAGE. On comparison with standard molecular weight markers, the apparent molecular weight was found to be approximately 33kDa.

It was also found that the enzyme was a monomer through SDS-PAGE under reducing conditions. Previous studies (Akimaru et al, 1991; Abeyan et al, 2002) have reported CGTases having molecular weights in the range of 33kDa to 200kDa. Both monomeric and dimeric forms of CGTases have been reported. Dimeric forms reported have a higher molecular weight of approximately 70kDa and above.

Zymogram: A zymogram was performed to demonstrate the activity of the enzyme through a native PAGE. The gel was stained with iodine-potassium iodide solution and clear bands were visualized in all the lanes at the position in the gel where

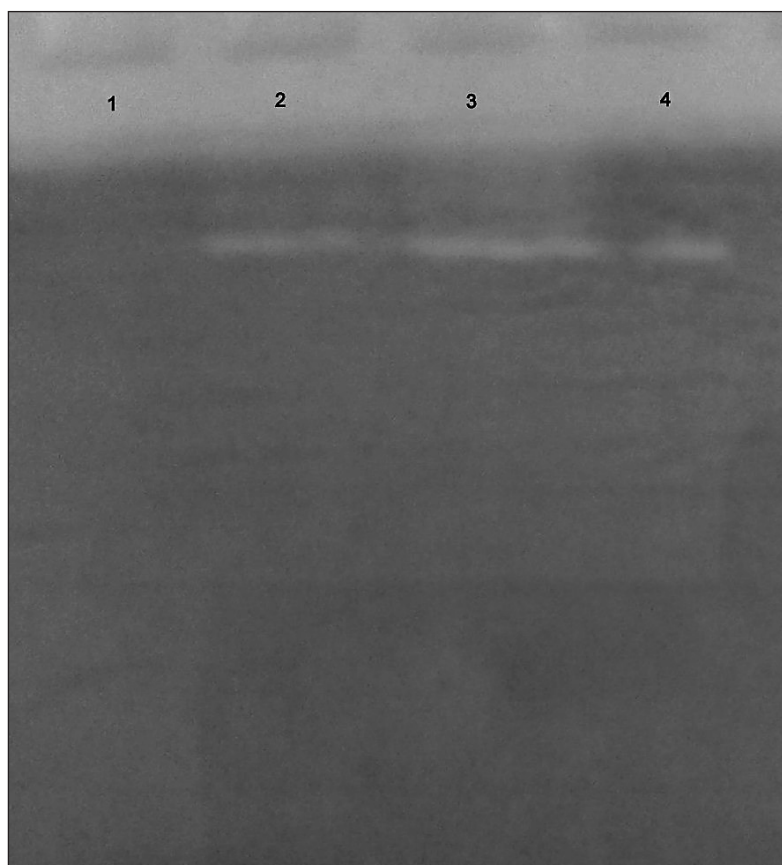


Figure 5. Zymogram [1-Cell-Free Extract; 2-Partially Purified Enzyme (Trial 1); 3-Purified Enzyme (Trial 2); 4-Partially Purified Enzyme]

the enzyme has degraded starch. Zymogram displayed clearance zones where CGTase was present. The DNS method was used to rule out presence of any other amylolytic enzymes in the sample

Enzyme Characterisation

Kinetic Parameters: The K_m and V_{max} values for partially purified CGTase with soluble starch as substrate were found to be 1.1mM and 0.442 μ mol/min respectively, as determined by the LB-plot. Previous reports have shown that the K_m values range from 0.05mM to 15.54mM (Bovetto et al,

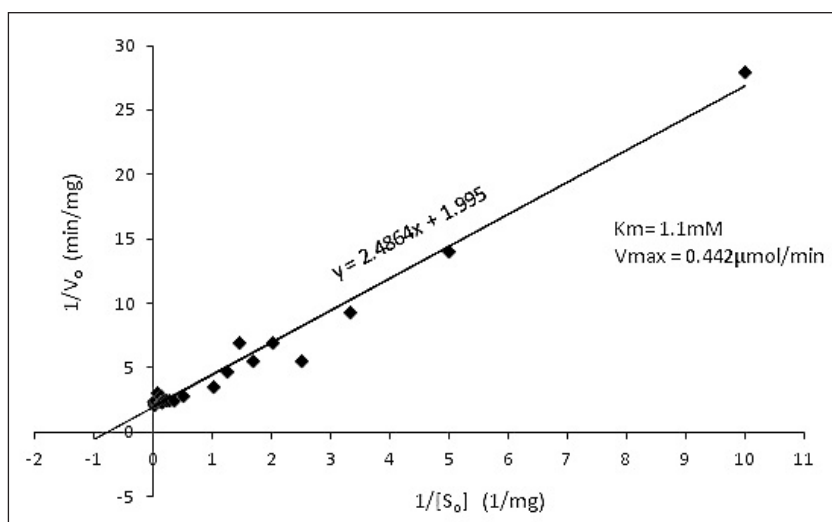


Figure 6. Lineweaver-Burk Plot

1992; Jung et al, 2007; Nakamura et al, 1994). This shows that the isolated CGTase has a relatively high affinity for starch.

K_{cat} was found to be 10.853s⁻¹. Previous studies (Kelly et al, 2009b; Nakamura et al, 1994; Gawande et al, 1999; Jeang et al, 1999; Yamamoto et al, 1999) have reported values ranging from 0.003 to 329s⁻¹.

Effect of Temperature and pH: The effect of temperature and pH on enzyme activity was determined for partially purified enzyme. The optimum temperature was found to be 60°C using soluble starch as substrate. Previous studies (Tachibana et al, 1999; Yoon et al, 2009) show optima between 23°C and 110°C. The enzyme remained active in the tested temperature range of 30-80°C.

The enzyme had two pH optima at pH 7.0 and pH 9.0. The pH optima reported in previous papers (Kanai et al, 2004; Kelly et al, 2009a) range from pH 4.0 to pH 10.3. The enzyme remained active in the tested pH range of pH 5.0 to pH 11.0. The broad ranges of temperature and pH stability may be useful in industrial applications.

Effect of Metal ions: It was found that Mn²⁺ activates CGTase while all the other ions inhibit CGTase to different levels. The effect was checked at 2mM and 10mM metal ion concentration. In presence of divalent metal ions like Cu²⁺, Mg²⁺, Fe²⁺, Ca²⁺ and Zn²⁺, the enzyme was inhibited to different levels while Mn²⁺ activated the enzyme. Previous reports (Akimaru et al, 1991; Abelyan et al, 2002; Kelly et al, 2009a) have shown that various CGTases are affected by metal ions differently. The same ions act as inhibitors in some cases and as activators in others. Many studies show that CGTase have conserved sites for Ca²⁺ and the ion has a stabilizing effect on the enzyme, improving its thermal stability. However, CGTase isolated in this study was inhibited by Ca²⁺. It has been reported that various metal ions like Ba²⁺, Ca²⁺, Mn²⁺, Co²⁺, Hg²⁺, Fe²⁺, K⁺, Mg²⁺, Na⁺, Sr²⁺, Zn²⁺, etc act as cofactors for CGTase. Since the CGTase isolated in this study was activated by Mn²⁺, it is possible that the metal ion acts as a cofactor for the enzyme.

Effect of Group Specific Reagents and Potential Inhibitors: EDTA, Urea, NAI, NaN₃, SDS, DTT and TLCK activated the enzyme while IAA, TPCK and PMSF showed inhibitory effects. HgCl₂ had no effect on enzyme activity. On studying the effect of group specific reagents, it was found that IAA, TPCK and PMSF have an inhibitory effect on CGTase. IAA and TPCK act on histidine and cysteine residues by alkylation. Since it has been reported in previous studies that histidine is an essential amino acid for activity of CGTase, it is possible that our result was due to inactivation of the histidine. Inactivation was also seen in case of PMSF which acts on serine residues which has also been implicated as an important amino acid for enzyme activity in previous studies (Tonkova, 1998).

HgCl₂-Mercuric Chloride; EDTA-Ethylenediaminetetraacetic acid; IAA-Iodoacetic

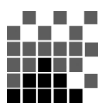


Table 2. Effect of Group Specific Reagents and Potential Inhibitors on CGTase Activity

Reagent	% Enzyme Activity
HgCl ₂	100
EDTA	120
IAA	0
Urea	160
NAI	160
NaN ₃	140
SDS	140
TPCK	40
DTT	160
TLCK	140
PMSF	0

acid; NAI-N-Acetylimidazole; NaN₃-Sodium azide; SDS-Sodium dodecyl sulphate; TPCK-N-p-Toluenesulfonyl-L-phenylalanine chloromethylketone; DTT-Dithiothreitol; TLCK- N-p-toluenesulfonyl-L-lysine chloromethylketone; PMSF-phenyl methyl sulphonyl fluoride.

CONCLUSION

In this study, the purification and characterization of a CGTase from *Bacillus halodurans* has been reported. Purification to homogeneity was achieved by acetone precipitation and starch adsorption with a yield of 49.44% and 17.34 fold purification. The purified enzyme was a monomer and its molecular weight was found to be 33kDa. The purification procedure was simple and easily applicable under industrial conditions. The present enzyme can be used in the pH range of around pH 6.0 to pH 10.5 and temperature range of 40 to 70°C. The enzyme was reasonably stable in presence of divalent cations and was unaffected by a variety of group specific reagents and potential inhibitors.

The present organism can be used for CD production without any additives and also the relatively high affinity for starch is of interest for industrial application. Alkaline CGTases have high commercial value and immobilization of the enzyme would enhance stability and usability of the enzyme. Fed batch systems of fermentation and strain improvement could also be performed to increase the yield of CGTase.

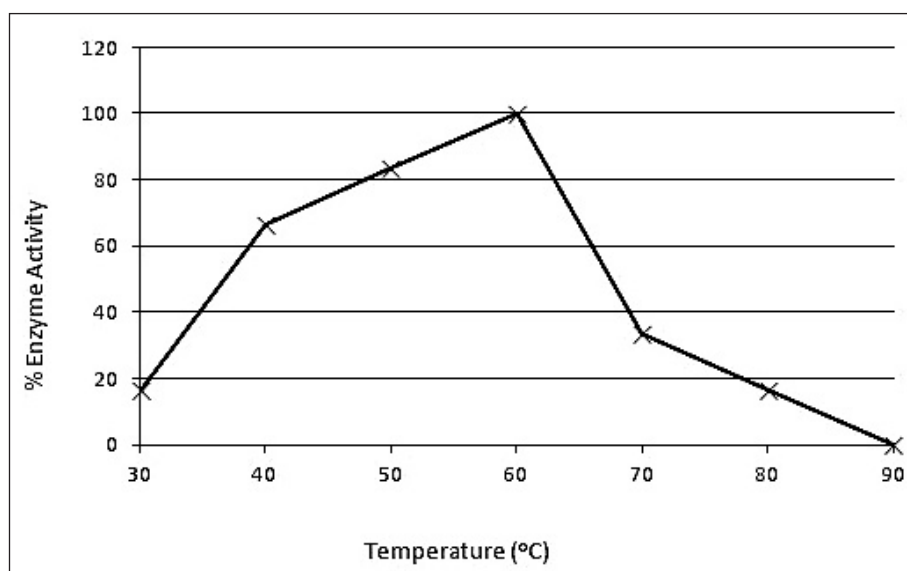


Figure 7. Effect of temperature on CGTase activity

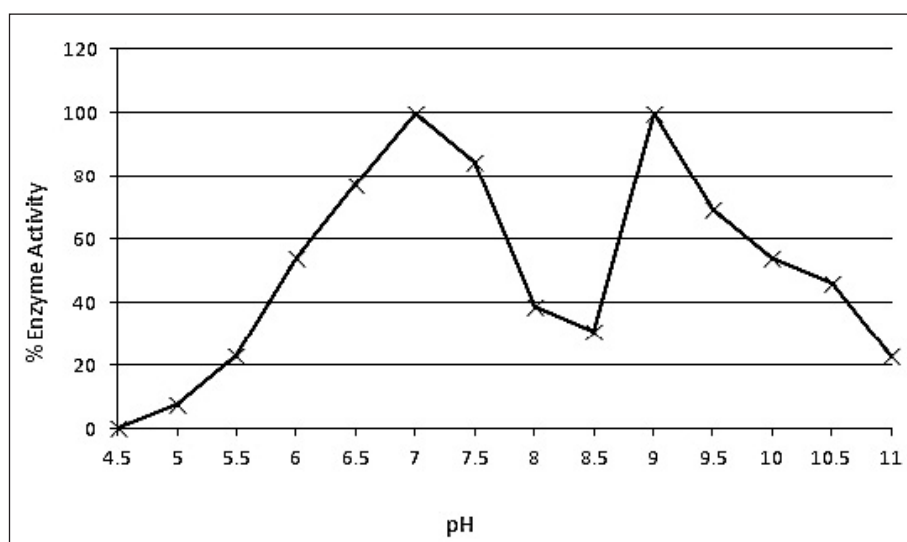


Figure 8. Effect of pH on CGTase activity

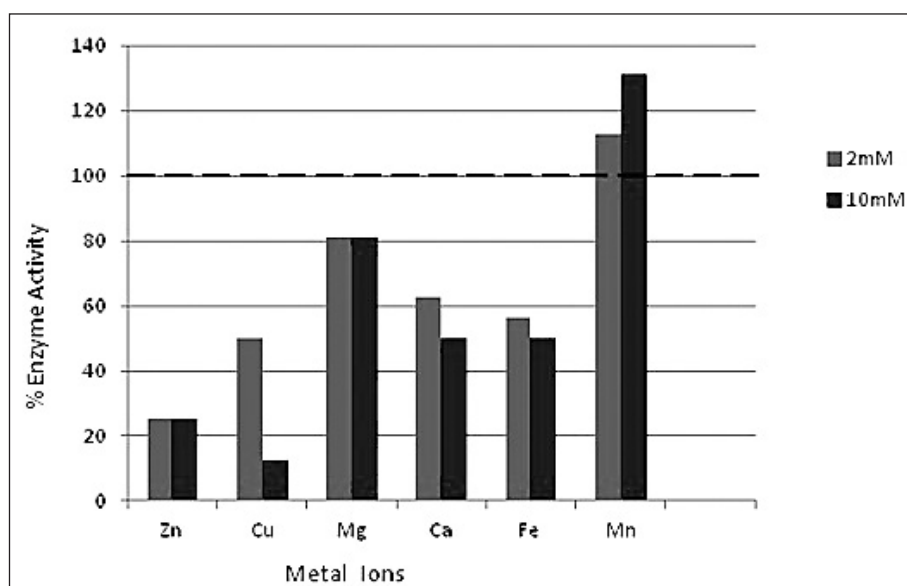


Figure 9. Effect of divalent metal ions on CGTase activity



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