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Isolation, purification and characterization of a novel CGTase from alkalophilic *Bacillus lehensis* SV1

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ABSTRACT

A Cyclodextrin glycosyl transferase (CGTase) E.C. 2.4.1.19 enzyme is capable of converting starch and related substrates into α, β and γ cyclodextrins in different ratio. They are currently seen as highly interesting industrial enzymes because of their broad substrate specificity. A positive alkalophilic strain was isolated from the soil sample capable of producing CGTase as *Bacillus lehensis* SV1 and characterized by 16s rDNA studies and Phylogenetic analysis. The CGTase activity was assayed by phenolphthalein method using starch as substrate. CGTase was purified by ion-exchange and gel filtration chromatography. The purified CGTase was a monomer showed a molecular mass of 40±1 kDa as estimated by SDS-PAGE and a 42.6-fold purification with a 29.03% yield. The optimum pH is at two pH range 5.0 and 8.0 and temperature 60^{o}C. The K_m and V_{max} was found to be 1.03mg/ml and 0.241mg/min respectively. Metal ions like $MgSO_4$ and $FeSO_4$ exhibited highest activity and $MgSO_4$ and $CaCl_2$ inhibited CGTase at higher concentrations. The isolated CGTase can be used in development of drug delivery agents and inclusion complexes.

KEYWORDS

CGTase;
Bacillus lehensis;
β-cyclodextrins.

INTRODUCTION

Cyclodextrin glycosyl transferase is a unique extracellular enzyme that can degrade starch and related substrates to form Cyclodextrin (CD) as products[1]. Cyclodextrin glycosyltransferase (1,4-a-D-glucan-4-aglycosyltransferase)(CGTase; E.C. 2.4.1.19) is produced by *Thermoanaerobacterium*[2] and some species of Bacillus, such as *Bacillus megaterium*[3], *Bacillus macerans*[4,5], *Bacillus stearothermophilus*[6], *Bacillus Klebsiella*[7], *Bacillus firmus*[8] and *Bacillus lentus*[9].

Cyclodextrins (CD) are non-reducing cyclic structures consisting of 6, 7 or 8 glucose residues, joined by α- 1, 4 linkages, forming α, β and Ò CD respectively[10]. Their cyclic structure provides an enclosed

space in which specific molecules form inclusion complexes. Hence they are regarded as being superior molecular complexation agents. They can be synthesized enzymatically by cyclodextrin glycosyltransferase or 1, 4- α -D-glucopyranosyltransferase (CGTase). CGTase is a unique enzyme able to convert starch and related sugars into CD via a cyclization reaction. Most of these enzymes produce β -CD as the main product[11]

The β -CD is reported to be more suitable for industrial use, since inclusion complexes can be prepared easily and are very stable due to their low solubility in water[12]. Due to this property, the CDs are applicable in medicine, pharmaceutical, food, cosmetic industries, agriculture and for environmental protection[13-17]. We have recently reported, new strain of CGTase producing bacteria *Bacillus lehensis* SV1 classified based on 16s rRNA analysis. The aim of the present study was to isolate an alkaliphilic, CGTase-producing bacteria; purify and characterize CGTase which would be useful in the context of industrial applications.

MATERIALS AND METHODS

Microorganisms

Alkaliphilic, CGTase producing bacteria, identified by 16s rDNA and Phylogenetic studies as *Bacillus lehensis* SV1, was isolated from soil from the root region of pumpkin. Samples of 1g of soil were suspended in 10ml of sterile distilled water. After soil sedimentation, 0.1 ml of supernatant was used as inoculum.

Screening

Screening was carried out by the rapid screening method[18]. Horikoshi II agar medium containing (w/v) 1% soluble starch, 0.5% peptone, 0.5% yeast extract 0.1% potassium-di- hydrogen phosphate, 0.02% magnesium sulfate, 0.02% phenolphthalein, 0.01% methyl orange, 1% sodium carbonate and 1.5% agar-agar. The medium was adjusted to pH 10.5 so as to isolate alkaliphilic bacteria[19,20]. 0.1ml of the soil supernatant was inoculated on to the solid medium by the spread-plate method. The plates were incubated at 37°C for 24-72 hours and colonies that surround by yellowish halo were considered as CGTase producers and were selected for further study.

Culture medium

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, potato starch, hydrolyzed starch and amylopectin as carbon source and peptone, yeast extract as nitrogen and sodium carbonate source in individual flasks. Soluble starch and peptone gave the best result and so culture medium of following composition was used for enzyme production: 1% soluble starch, 1% peptone, 0.1% potassium-dihydrogen phosphate, 0.02% magnesium sulfate, 1% sodium carbonate with pH 10.5.

Enzyme assay

CGTase assay was measured by mixing 0.5 ml of 4% soluble starch in 0.05M potassium phosphate buffer pH 8.0 and 0.5 ml of enzyme CGTase was incubating at 60°C for 10 minutes. The reaction was stopped by boiling for 5 minutes Then 2.5 ml of phenolphthalein solution was added. The mixture was left at room temperature for 15 minutes and the reduction in color intensity was measured at 550 nm in relation to blank sample containing a mixture of water and phenolphthalein. One unit of CGTase was defined as the amount of enzyme that formed 1μmol of β-CD/ml/min.

Protein assay

The cell protein content was determined according to Lowry method using Bovine Serum Albumin (BSA) as standard[21].

Partial purification of enzyme

The cell-free extract was used for partial purification of enzyme. The extract was subjected to ammonium sulfate 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.

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Purification by starch adsorption

Starch adsorption was carried out as follows[22]: 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.

Purification of CGTase

Purification of CGTase was carried out by the method of Ferroti et al[22]. The culture filtrate was first filtered and centrifuged at 5000 rpm, supernatant was then subjected to ammonium sulfate precipitation. The precipitate obtained was dialyzed and lyophilized and then loaded onto a DEAE-Cellulose anion exchange column 1.5 × 18 cm, equilibrated with 10mM sodium acetate buffer (pH 4.5), with a linearly increasing NaCl concentration gradient (0 to 0.5M) in the same buffer. The six fractions containing CGTase activity were pooled, concentrated, and dialyzed overnight against same buffer. Gel filtration chromatography was performed using sephadex G-100 column 2.0 × 40 cm. The DEAE-purified sample was loaded on to the column and 3mL fraction was collected. The eluted active fractions were dialyzed and protein content was determined by Bradford's method[23] with crystalline bovine serum albumin as the standard.

Biochemical characterization

Polyacrylamide gel electrophoresis

To determine the purity of the protein and its molecular weight, sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with a 12% polyacrylamide gel and protein was visualized by staining the gel with silver staining[24] for determining homogeneity and relative molecular mass using standard molecular weight markers (Genei India Pvt. ltd). Native PAGE was performed at room temperature with 12% gel. After electrophoresis, the slab gels were subjected to the activity staining for the CGTase and it was fixed with a solution containing 10% (v/v) acetic acid and 40% methanol (v/v).

The SDS-discontinuous buffer system was performed based on the method of Laemmli[24]. A 12% resolving gel was used to separate the proteins in our sample. After completion of electrophoresis, the gel was subjected to silver staining. The molecular weight marker used contained Myosin, Rabbit Muscle (205,000), Phosphorylase b (97,400), Bovine Serum Albumin (66,000), Ovalbumin (43,000), and Carbonic Anhydrase (29,000). The CGtase Zymogram was performed using a discontinuous buffer system with 12% resolving gel[25].

Effect of temperature on activity and stability of enzyme

The enzyme was incubated at different temperatures between 30-80°C and the activity at optimum temperature was determined by Phenolphthalein assay method. Thermal stability was determined by incubating enzyme at temperatures between 30-80°C for 1 hour. Activity was determined by phenolphthalein assay method.

Effect of pH on activity and stability of enzyme

The optimum pH of the enzyme. Was determined by using buffers ranging from 3-10. Stability of the enzyme was determined by incubating 0.5ml of enzyme in buffer of different pH range for 60 min and activity was determined by phenolphthalein assay method.

Effect of metal ions on the activity of CGTase

The enzyme was incubated with different divalent cations like Fe^{2+},Ca^{2+}, Mg^{2+},Mn^{2+},Cu^{2+},Zn^{2+} and activity was measured using starch as substrate

Kinetic parameters

The K_m and V_{max} was determined by incubating 0.5ml of enzyme with starch substrate (soluble, corn and potato) at different concentrations (2-20mg/ml) in phosphate buffer of pH 8. The assay was performed according to standard enzyme assay. K_m and V_{max} was determined by Line weaver Burk Plot[26].

Effect of group specific reagents and inhibitors on enzyme activity

The enzyme was incubated with different com-

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pounds like $HgCl_2$ 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) to know the active site aminoacid in the enzyme.

RESULTS AND DISCUSSION

Isolation of CGTase producing bacteria

Alkalophilic CGTase producing bacteria was isolated from soil sample present in the root region of pumpkin, suspended in saline and the supernatant was spread on the surface of plate containing Horikoshi II agar medium. The plates were incubated at 37°C for 24-72 hours and colonies that were surrounded by yellowish halo due to phenolphthalein–cyclodextrin (CD) complex (Figure 1) were treated to isolate pure bacterial culture and selected for further study. The bacteria was identified by 16s rDNA sequence data (1512 bp) and phylogenetic tree analysis that, the isolated strain has close resemblance with *Bacillus lehensis* MLB-2 with the similarity of 99%. Therefore, the organism is named as *Bacillus lehensis* SV1.

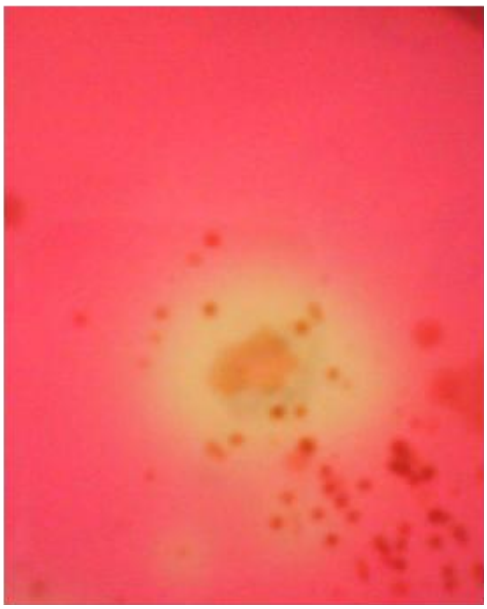

Figure 1

Effect of carbon and nitrogen sources

B. lehensis SV1 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 peptone as nitrogen sources. These were selected as the components of the submerged fermentation medium so as to optimize enzyme production.

Time course of 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 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 day wise and 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 (shown in Figure 2). 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.

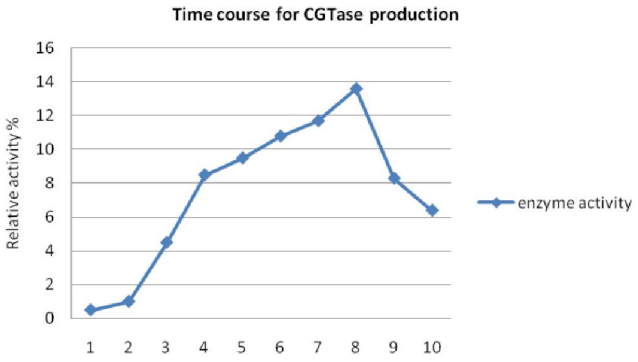

Figure 2

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

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starch adsorption method, followed by ion exchange chromatography on DEAE cellulose and Gel filtration chromatography on sephadex G-100, the specific activity was 165.2 U/mg and the fold purification and yield were 42.6 and 29.03 % respectively. Other methods of purification[27-33] 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 followed by Ion exchange and Gel filtration was appreciable, particularly in light of the ease of the purification technique.

The purified enzyme was homogenous showing a single band on SDS-PAGE (Figure 3). On comparison with standard molecular weight markers, the apparent molecular weight was found to be approximately 40 ± 1 kDa. It was also found that the enzyme was a monomer through SDS-PAGE under reducing conditions (Figure 4). Previous studies[34,35] 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.

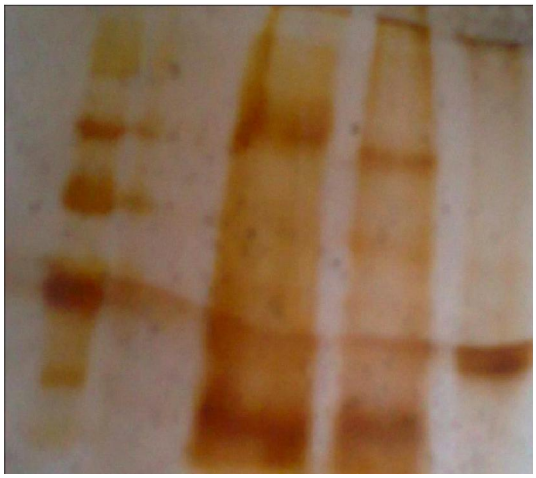

Figure 3

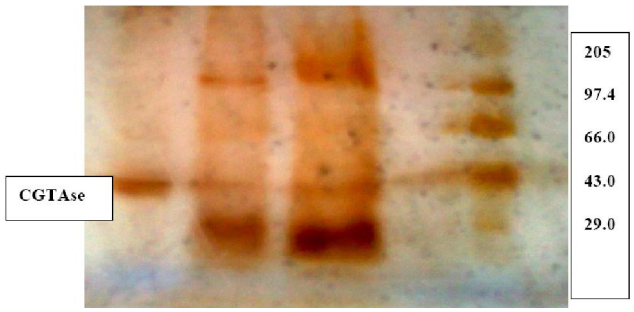

Figure 4

TABLE 1

Fraction	Volume (ml)	Total Activity (µmol/min)	Total Protein (mg)	Specific Activity (U/mg)	Fold Purification	Yield
Cell Free Extract	100	89.38	15.72	5.69	1	100%
Acetone Precipitate	10	61.14	4.02	15.2	2.67	68.4%
Purified by starch adsporption	5	44.19	3.8	98.64	17.34	49.44%
Ion Exchange chromatography	3.5	32.3	3.1	114.4	36.02	20.10
Gel filtration chromatography	3.0	38.1	3.0	165.2	42.6	29.03

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 the enzyme has degraded starch, as (shown in Figure 5). 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.

Figure 5

Enzyme characteristics

Kinetic parameters

The Km parameter is correlated to the affinity of

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the enzyme for its substrate and it depends on the sources of the enzyme and substrate. The km and Vmax values for purified CGTase from *B.lehensis* SV1 with soluble starch as substrate were found to be 1.03mg/ml and 0.241 mg/min respectively, as determined by the LB-plot shown in Figure 6. There have been several reported Km values from various CGTases when soluble starch was used as the substrate, such as CGTase from *Bacillus sp*. G1, 0.15 mg/mL[41]; *B. firmus*, 1.21 mg/mL[8]; *K. pneumoniae* AS-22, 1.35 mg/mL[7]. This shows that the isolated CGTase has a relatively high affinity for soluble starch.

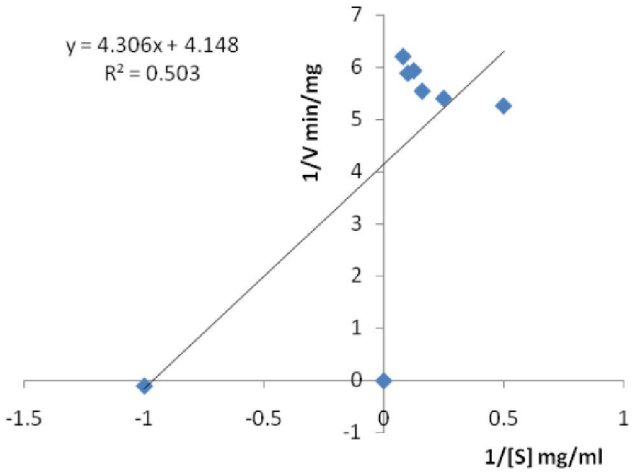

Figure 6

Effect of temperature

The effect of temperature was determined for purified enzyme. The optimum temperature of *B.lehensis* SV1 was found to be 60°C using soluble starch as substrate (Figure 7). Previous studies have reported similar results in alkaliphilic *Bacillus pseudalcaliphilus* 20RF[36], *Bacillus sp*.[37] and *Bacillus sp. G1*[36]. In comparison *Bacillus sp*. C26 was active at 65°C, *K.Pneumoniae* AS-22 at 45°C[7], *B.alkalophilic* CGII at 55°C[37] and broad range of activity between 45°C-70°C[20]. The enzyme was stable in the temperature range of 30°C -50°C (shown in Figure 6) similar to *Bacillus sp*. C26[7].

Effect of pH

The effect of pH on enzyme activity was determined for partially purified enzyme. The optimum pH of *B.lehensis* SV1 was found to be both pH 5 and 8 using soluble starch as substrate (Figure 8). Previous studies have reported CGTase activity from Bacillus sp showed two peaks at pH 5 & 8.5[38]. In *Bacillus firmus*[8], *B. firmus* NCIM 5119[39] pH optima was 6 and 9. The established two pH optima may have been caused by the subtle structural change due to alkaline pH. Probably, substrate-binding modes of the enzyme may be different[37]. CGTase from alkaliphilic *Bacillus sp*. G-825-6 exhibited two pH optima 6 & 9[16].

The enzyme was stable between pH 6-8. Previous studies have shown in *K.Pneumoniae* AS-22, the enzyme was stable between pH 6-9[7]. In *Bacillus sp* C26 the enzyme was stable over the wide pH range of 7-9[22] and in alkaliphilic *Bacillus pseudalcaliphilus* 20RF the enzyme was stable in the pH range of 5-11[37].

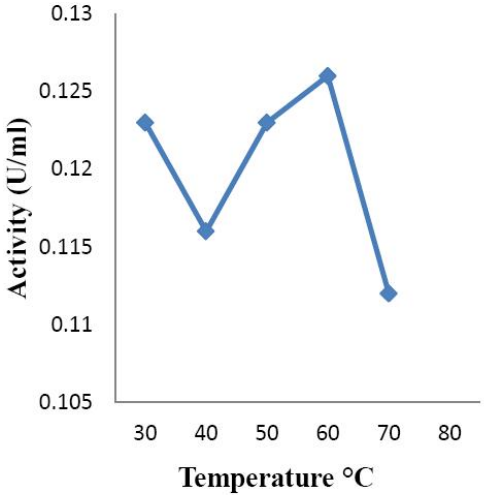

Figure 7

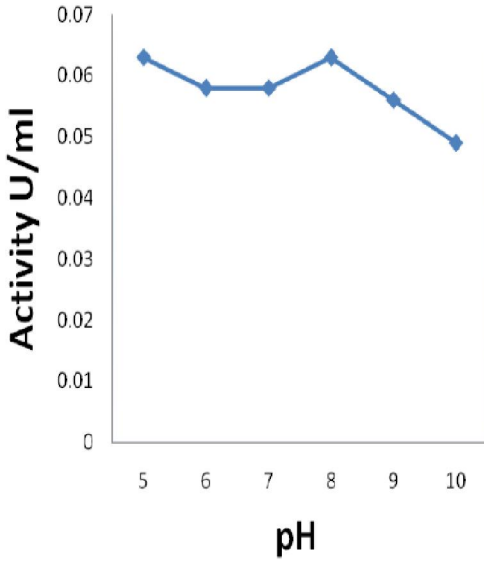

Figure 8

Effect of metal ions

CGTase activity was measured in presence of di-

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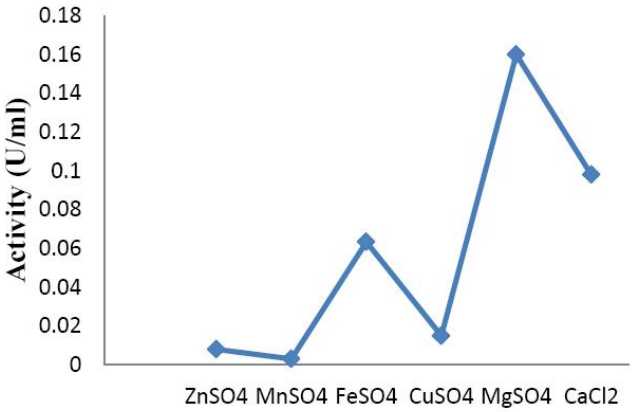
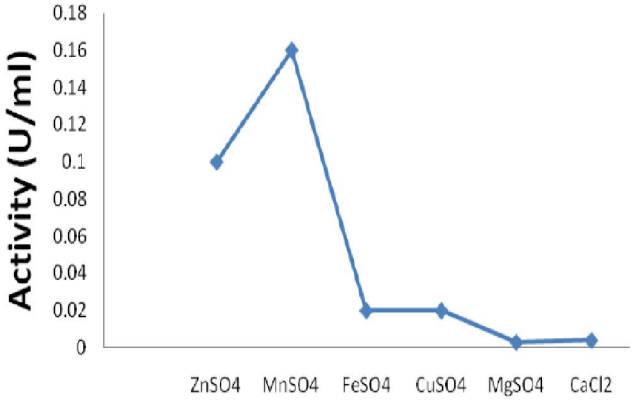
valent metal ions such as $ZnSO_4$, $MnSO_4$, $FeSO_4$, $CuSO_4$, $MgSO_4$ & $CaCl_2$ at 2mM and 10mM concentration (Figure 9). At 2mM concentration, the highest activity was observed in presence of $MgSO_4$ and $FeSO_4$[7] in *B.lehensis* SV1(Figure 9). At high concentration of 10mM $MgSO_4$ and $CaCl_2$ inhibits the enzyme. Presence of Metal ions such as Mg^{2+} and Fe^{2+} increased CGTase production. Magnesium was essential for bacterial growth and iron for CGTase production as reported in case of *Bacillus firmus*[8], *B.stearothermophilus B.autolyticus and Bacillus sp* respectively[38,39]. At 10mM concentration MgSO4 and $CaCl_2$ was found to inhibit the activity of CGTase. (Figure 10). In comparison Cu^{2+} has a significant inhibitory effect on CGTases from *Bacillus* AL-6[40], *B. firmus*[8], *B. agaradhaerens*[41]. Unlike the enzyme from *B. pseudalcaliphilus* 20RF which retained 90% of its activity in the presence of Cu^{2+}[42].



Metal ions at 2mM concentration

Figure 9



Metal ions at 10mM concentration

Figure 10

Effect of group specific reagents and potential inhibitors

EDTA, Urea, NAI, NaN_3, SDS, STT and TLCK activated the enzyme while IAA, TPCK and PMSF showed inhibitory effects. $HgCl_2$ had no effect on enzyme activity (Figure 11). 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[43].

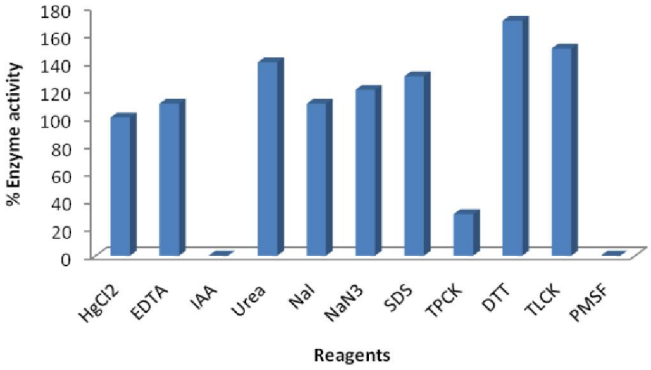


Figure 11

CONCLUSION

The CGTase producing alkalophilic Bacteria was isolated and identified as *Bacillus lehensis* SV1. The culture conditions for growth and production were optimized based on carbon, nitrogen and Sodium carbonate source. The enzyme purification was achieved by acetone precipitation, ion-exchange, gel-filtration chromatography and starch adsorption methods. The purified CGTase was a monomer showed a molecular mass of 40±1 kDa as estimated by SDS-PAGE and a 46.6-fold purification with a 29.03% yield. The present enzyme from *B.lehensis* has a temperature optimum at 60°C, stable between 30°C-50°C, pH optima 5 & 8, stable in pH range of 6-8, High activity in presence of Mg^{2+} and Fe^{2+} metal ions, inhibition by Mg^{2+} and Ca^{2+} and Km of 1.08mg/ml relatively high affinity for soluble

starch. The present organism can be used for CD production, in development of drug delivery agents and inclusion complexes.

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