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Screening, Partial Purification and Stability Studies of Cyclodextrin glycosyl transferases from *Bacillus amyloliquefaciens* Strain L4-6

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Abstract: Cyclodextrin glycosyl transferases (CGTases) are produced by a wide range of bacteria, mainly *Bacillus* species by submerged fermentation. CGTase production is highly dependent on the strain, medium composition and culture conditions. The present investigation was undertaken with an objective to screen, isolate, identify the microorganism producing CGTase and its purification with improved production and purification fold. Among the 37 colonies screened for production of CGTase, 07 isolates were selected [RS 1-7 having zone diameter in the range 18-27 mm and optimum exhibited by RS-4 (Sample B-27 mm)] based on their hydrolytic zones, microscopic charecteristics and CGTase production capabilities and further streaked onto Horikoshi-phenolphthalein (PHP) plates for futher studies. The isolated sample **B** subjected to morphological and strain level characterization by 16S r-DNA based molecular technique was found to be *Bacillus amyloliquefaciens* strain L4-6 (GenBank Accession Number: KC464454.1) based on nucleotide homology and phylogenetic analysis. The purified enzyme was stable at a wide pH range from 4 to 12 and was optimally active at pH 8. The enzyme exhibited optimum activity at a temperature of 60°C was stable for 1 h. With increase in substrate concentration from 40-200 µg/ml, the enzyme activity of CGTase (µg β-CD /min/ mg protein) was not enhanced significantly, which increases proportionally with an increase in substrate concentration (200-300 µg/ml) and reached the plateau stage. A potent CGTase was obtained with a purification fold, specific activity and yield value of 41.65, 3000.68 U/mg and 2.23 % respectively by ammonium salt precipitation, followed by Sephadex G-25 and DEAE Cellulose coloumn chromatography. The apparent molecular weight of the CGTase was found to be 81 kDa by SDS PAGE.

Key words: Cyclodextrin glycosyl transferases, *Bacillus amyloliquefaciens* strain L4-6, enzyme activity, specific activity, stability, molecular mass.

Introduction

Cyclodextrin glycosyl transferase (E.C. 2.4.1.19; CGTase) is a bacterial enzyme belonging to the family of α amylase specifically known as glycosyl hydrolase family 13 and converts starch into cyclodextrins [1] which are closed ring structures having six or more glucose units joined by means of α -1,4 glucosidic bonds [2,3]. CGTase is a common starch degrading enzyme producing by many bacteria species in particular of the Bacillus genus (i.e. *B.circulans, B.macerans, B. stearothermophillus*) [5,6] and archaea. Three major type

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of cyclodextrin (CD) are produce by CGTase depending on number of glucose units are α-CD, β-CD and γ-CD that were also recognized safe by FDA [7,8]. Cyclodextrin are hydrophobic inside and hydrophilic outside, they form complexes with hydrophobic compounds and enhance the solubility and bioavailability of such compounds [9,10]. This is of high interest for pharmaceuticals as well as dietary supplement [11] in which hydrophobic compounds shall be delivered. α-CD has been used as a dietary fiber in European union. European assessment report confirms that consumption of α-CD can reduce blood sugar peaks following a high starch meal. Weight loss supplements are marketed from α-CD which claims to bind to fat and an alternative to other anti obesity medications. α-CD complexes with certain carotenoid food colorants have been shown to intensify color,increase water solubility and improve slight stability. The strong ability of complexing fragrances can also be used for another purpose [11]. On January 23,2013, a formal clinical trial to evaluate HPβ CD cyclodextrin therapy as a treatment for Niemann-Pick disease, type C was announced by scientists from the NIH's National Center for Advancing Translational sciences and the Eunice Kennedy Shriver National institute of child Health and Human Development. Cyclodextrins can solubilize hydrophobic drugs in pharmaceutical applications, and cross link to form polymers used for drug delivery [11]. The ability to forming complexes with hydrophobic molecules has led to their usage in supramolecular chemistry. Particularly it was used to synthesize certain mechanically interlocked molecular architectures such as rotaxanes and catenanes. In 2013 α-CD is found to be able to selectively form second sphere coordination complex with tetrabromourate anion from transition-metal anion mixtures, and thus is used to selectively recover gold from various gold bearing materials in an environmentally benign manner. β-CD are used to produce HPLC column allowing chiral enantiomers separation. So the present objective to search for a novel bacterium from local habitat for the production of cost effective, eco friendly, thermostable and a potent CGTase.

Materials and method

Isolation and primary screening of CGTase producing bacteria

Soil sample was collected from railway sidings in a sterile container and processed for further biological studies. Soil sample collected was suspended in sterile saline and the solid particles were allowed to settle and then the saline sample were diluted. Further 100 µl of sample were added in Horikoshi agar plate containing, starch 1.0 % (w/v), peptone 0.5 % (w/v), yeast extracts 0.5 % (w/v), K_2HPO_4 0.1 % (w/v), $MgSO_4$ 0.02 % (w/v), Na_2CO_3 1.0 % (w/v), Water 100 mL and pH 8 [12,13]. The plates were incubated at 37°C for 24 hrs. to obtain bacterial colonies with yellowish clearance zone that showed zone of hydrolysis, the organism exhibiting biggest diameter were selected and streaked onto Horikoshi phenolphthalein plates for several times [14]. All the strains selected for liquid culture studies were stored in 10 % Glycerol solution at -20°C. For routine cultivation of the strains, Horikoshi agar plates were used.

Secondary screening

Inoculum preparation

Different strains selected for liquid culture studies were plated on Horikoshi agar plates and incubated at 37°C for 24 hours. A loopful of culture from this fresh plate was used to inoculate a 250 ml flask containing 50 ml of Horikoshi broth for inoculum production. After 24 hours of incubation at 37°C and 200 rpm in a constant temperature rotary shaker, 10 ml of this was transferred to another 500 ml flask containing 90 ml of Horikoshi broth, having the composition of 100 ml, for enzyme production [15]. The flask was incubated for 24 hours at 37°C and 200 rpm in a rotary shaker. Medium composition was kept constant from inoculum to enzyme production stage.

Sample preparation

Inoculums prepared as mentioned above, was transferred 10 % v/v level to 500 ml flasks containing 100 ml of production media. The production flasks were incubated at 37°C and 200 rpm in a rotary shaker.

After 24 hours the culture was centrifuged at 10000 rpm for 10 minutes at 4°C. Culture supernatant from this step was used to estimate enzyme activity. This culture supernatant was used as enzyme in all further studies.

Enzymatic assay

The CGTase activity was estimated by using starch as a substrate, using a slightly modified method [5] Reaction mixture containing 100 µl (160 mg/ml) starch (1.6 % w/v solution in assay buffer) and 200 µl of assay buffer (50 mMtris HCL buffer) were taken in test tubes. Reaction was initiated by addition of 200 µl of suitably diluted enzyme solution and the above mixture was incubated at 60°C for 10 minutes. After 10 minutes the reaction was terminated by the addition of 100 µl of 80 mM NaOH .Then 2 ml of 125 mM of sodium carbonate buffer with phenolphthalein was added to the above mixture in order to determine the amount of degradation of starch by the enzyme. And after 15 mins the absorbance 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 10-200 mg/ml of β-CD in 50 mMTris-HCl (pH 8).

Identification of the Bacterial Isolate of sample

The bacterial isolate sample B showing highest enzyme activity was subjected to different morphological, biochemical, physiological characteristics as well as 16S r-DNA technique for strain level identification. The results of the morphological, biochemical and physiological tests were put into Bergey's Manual of Determinative Bacteriology [16] and bacterial identification software's like PIB win version 2.0 and ABIS online for accurate identification. The isolate Sample B was exposed to temperature range of 4°C - 100°C, pH range of 3-12 and sodium chloride concentrations range of 2-10 % for evaluation of physiological characteristics. The antibiogram pattern [17] of the isolate Sample B was tested for sensitivity against different antibiotic discs (Himedia, Mumbai) viz., Erythromycin (15µg), Streptomycin (25µg), Ciprofloxacin (30 µg), Ampicillin (10 µg) + Solbactum (10 µg), Ceftriaxone (30 µg) + Tazobactum (10 µg), Cotrimoxazole (25 µg), Amikacin (30 µg), Gentamicin (30 µg), Cefotaxime (30 µg), Ofloxacin (2 µg), Chloramphenicol (50 µg), Nalidixic acid (30 µg) and Polymyxin (300 U).

16S rDNA gene sequencing of sample B

DNA was isolated from the culture. Its quality was evaluated on 1.2 % Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA.The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

Phylogenetic analysis of the strain

The 16S rDNA gene sequence of the strain sample B was used as a query to search for homologous sequence in the nucleotide sequence databases by running BLAStn program. The evolutionary history was inferred using the Neighbor-Joining method [18]. The bootstrap consensus tree inferred from 500 replicates [19] is taken to represent the evolutionary history of the taxa analyzed [19]. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method [20] and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+ Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option. Phylogenetic analyses were

conducted in MEGA4 [21].

Characterization studies

Effect of temperature on enzyme activity and stability

To check the effect of temperature on enzyme activity, enzyme assay was performed at different temperatures in the range of 40°C-80°C [14.22,23] and using 50 mM tris HCL buffer at pH 8, 125 mM sodium carbonate buffer with phenolphthalein at pH 10.5 and 80 mM NaOH.

Effect of substrate concentration on enzyme activity

To check the effect of substrate concentration on enzyme activity, enzyme assay was performed at different subatrate concentration level in the range of 40 mg/ml -400 mg/ml and using 50 mM trisHCl buffer at pH 8, 125 mM sodium carbonate buffer with phenolphthalein at pH 10.5 and 80 mM NaOH.

Kinetic parameters of CGTase

Kinetic studies of the purified CGTase were investigated by measuring initial rates of CGTase reaction at different concentrations of soluble starch in 50 mMTris-HCl buffer (pH 8.0) at 60°C. The kinetic parameters, K_m and V_{max}, were estimated using Michaelis-Menten equation and double reciprocal plot known as Lineweaver-Burk plot [24].

Protein estimation

Ten dilutions of a protein standard (i.e. BSA) was prepared, which is representative of the protein solution to be tested. The linear range of the assay for BSA is 1.2 to 10.0 µg/ml. 800 µl of each standard and sample solutions were pipetted in to a clean eppendorf. Protein solutions were assayed in duplicate. 500 µl of dye reagent concentrate was added to each tube and was vortexed. Samples were incubated at room temperature for 10 minutes. Absorbance was measured at 700 nm. Graph was plotted from the observed data. Protein concentration was determined according to the method described by the method of Lowry's [25] with bovine serum albumin as the standard.

Purification studies of CGTase

Ammonium sulphate precipitation

Salt precipitation of CGTase, using ammonium sulphate [26,27] was carried out at 50 % saturation level. The required amount of ammonium sulphate was dissolved in the supernatant, collected previously by centrifuging the culture broth to separate enzyme supernatant from cell pellet. Addition of salt was carried out on magnetic stirrer very slowly to ensure constant stirring under chilled condition. After all ammonium sulphate was dissolved, the mixture was stirred for another 2 hours to allow complete equilibration between dissolved and aggregated protein. The whole mixture was left undisturbed at 4°C overnight [26,23]. Next day it was centrifuged at 6000 rpm at 4°C for 25 minutes to convert the precipitated protein into pellet. The supernatant was decanted. Then the precipitate was dissolved in minimal volume of 50 mM tris HCL buffer of pH 8. Enzyme activity was calculated by CGTase assay and the concentration of protein was determined by folin catechu assay method.

Sephadex G-25 column

One gm of sephadex G-25 was taken and dissolved in in 100 ml of distilled water for overnight. Kept in refrigerator. Next day it was loaded in 10 ml of disposable syringe. The column was washed with distilled water for 4-5 times, then it was again washed with 50 mM trisHCl buffer till spectro reading comes negative at 280 nm. This is to verify whether the column is free from contamination or not. Sephadex G- 25 column was used for desalting out the salts from the proteins. Elutions from the column were taken for CGTase activity test and protein determination.

Concentration of protein with sucrose

The elutions collected from the sephadex G-25 column was subjected to molecular porous membrane tubing and was kept over sucrose overnight at 4°C for concentrating the protein present in the elution .

DEAE column (Diethlyaminoethyl cellulose)

Five gm of dry DEAE was taken and 10 ml of distilled water was added and allowed it to settle

for 25-30 mins. Measured volume is the column volume (CV) to be used for measuring the washing solutions [27,28]. The column was first washed with 0.1 M NaOH containing 0.5 M NaCl, then it was again washed with 0.1 M HCl containing 0.5 M NaCl. Again it was washed with distilled water until pH is 5 or greater .Again it was washed with 1M NaCl and the pH of the slurry should be maintained to 7-8 with NaOH. At this stage the column is again passed with 10X buffer of our choice. The dialysed solution which was concentrated on sucrose plate was applied to the DEAE column, equilibrated with 50 mM trisHCl buffer. Elution was performed with a liner 200 ml gradient of 0.5M-1M NaCl in 50 mM trisHCl buffer at a flow rate of 30 ml per hr. The active fractions from the column were pooled and CGTase activity and protein concentration were determined.

Native-SDS PAGE

CGTase was electrophoresed on a 7.5 % native polyacrylamide gel and stained [6.24] for both protein and enzyme activity detection. For activity staining the gel was soaked in 10 ml of 2 % w/v soluble starch in 0.1 mol. Phosphate buffer, pH 7.0 at 40°C for 30 min. it was then quickly rinsed several times with distilled water. 10 ml of Iodine staining reagent (0.2 % w/v Iodine in 2 % w/v potassium iodide) was added for colour development at room temperature, the clear band on the blue background indicates Starch-Degrading activity

Results

Isolation and primary screening of CGTase producing bacteria

A total of 37 colonies were isolated from the samples. Out of 37 isolates, 7 were selected based on their microscopic characteristics. They were examined for CGTase production. Among 7selected bacterial isolates RS-1, RS-2, RS-3, RS-4, RS-5, RS-6, RS-7 having diameter (18-27 mm).Bacterial colonies of railway siding (RS-4) with yellowish clearance zone which showed the biggest diameter (27 mm) shown in Fig. 1. was selected and streaked onto Horikoshi-phenolphthalein (PHP) plates for further work. It was named as sample B.

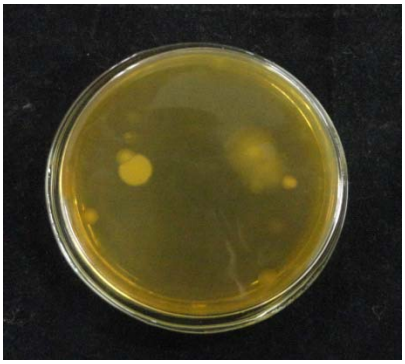

Figure 1. Plate showing highest zone of hydrolysis

Identification of bacterial isolate of sample B

From the gram staining photo micrograph shown in Fig. 2 of isolated microorganism it was concluded that,the microorganism is gram positive in nature having size ranging from 1.73 mm - 2.25 mm.

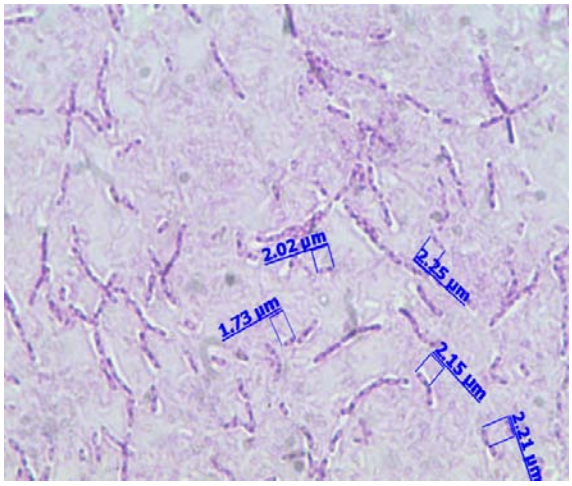

Figure 2. Gram staining photo micrograph of the isolated organism

16S rDNA gene sequencing of sample B

When DNA quality was evaluated on 1.2 % Agarose Gel, a single band of high-molecular weight DNA has been observed. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel. Consensus sequence of 1264bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program

Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA4. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1264 positions in the final dataset. The culture, which was labeled as Sample-B was found to be *Bacillus amyloliquefaciens* strain L4-6 (GenBank Accession Number: KC464454.1) based on nucleotide homology and phylogenetic analysis shown in Fig. 3 and 4.

Characterization studies

Effect of temperature on enzyme activity and stability

CGTase showed significant activity in a wide temperatures range, 40°C-80°C, showing maximal enzyme activity at 60°C. The relative enzyme activities at 65, 70, 75 and 80°C were found to be 89 %, 83.4 %, 71.8 % and 43 % respectively as represented in Fig. 5.

Effect of substrate concentration on enzyme activity

With increase in substrate concentration from 40-200 mg/ml the specific activity of CGTase (mg b-CD/min/mg protein) was not enhanced significantly. However the specific activity of en-

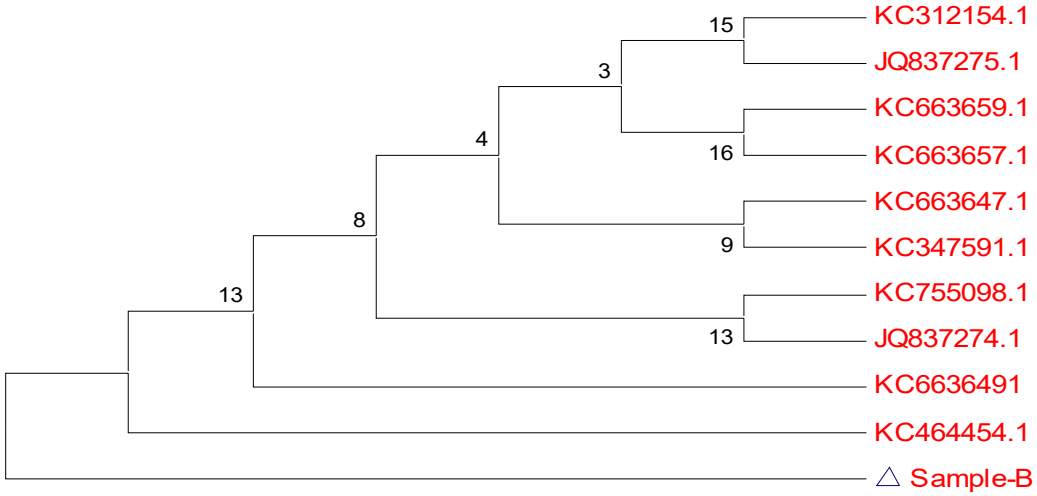

Figure 3. Evolutionary relationships of 11 taxanomy

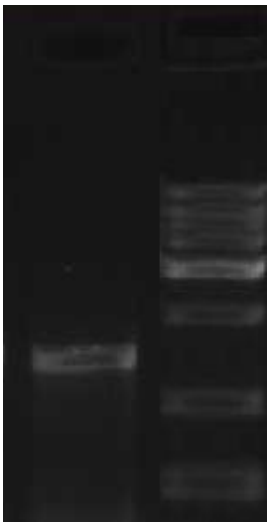

Figure 4. [Gel Image 1]

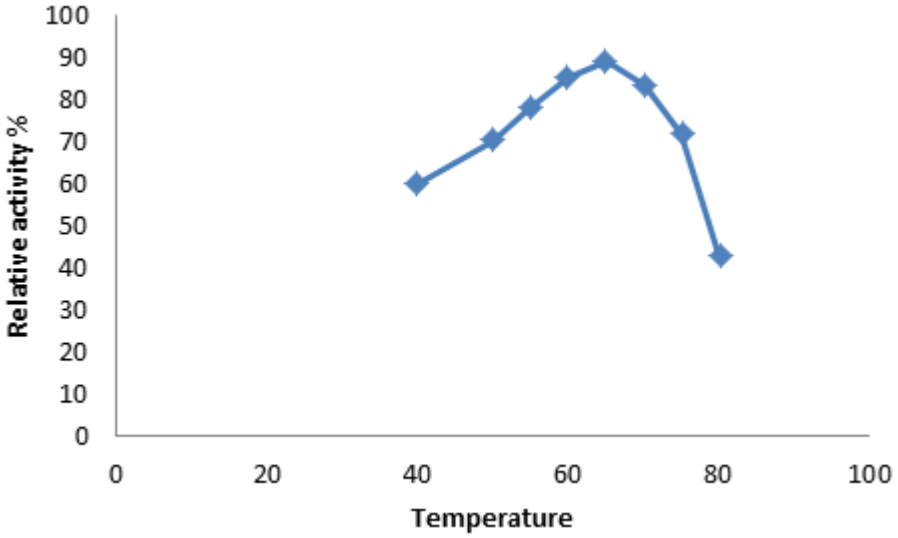

Figure 5. Effect of temperature on *Bacillus amyloliquefaciens strain L4-6* CGTase activity

zyme increased with an increase of substrate concentration (200-300 mg/ml) proportionally and then reached the plateau stage as represented in Fig. 6.

Kinetic parameters of CGTase

The kinetic parameters, K_m and V_{max}, were estimated using Michaelis-Menten equation and double reciprocal plot known as Lineweaver-Burk plot. The K_m and V_{max} values using soluble starch as a substrate were estimated to be 25 mg/mL and 144 mg β-CD/min/mg protein respectively. Fig. 7 shown the low value of K_m indicates the high affinity of *Bacillus amyloliquefaciens* strain L4-6 CGTase toward the substrate.

Protein estimation

Protein content of the purified enzyme was found to be 0.277 mg.

Purification of CGTase

The elution profile of *Bacillus amyloliquefaciens* L4-6 by Sephadex G 25 column was carried out. Thus the CGTase obtained from *Bacillus amyloliquefaciens* L4-6 was purified to 41.65 fold, with specific activity and yield value of 3060.68 (U/mg) and 2.23 % respectively. Specific activity, yield percentage and fold purification CGTase from *Bacillus amyloliquefaciens* at each purification step was shown in Table 1.

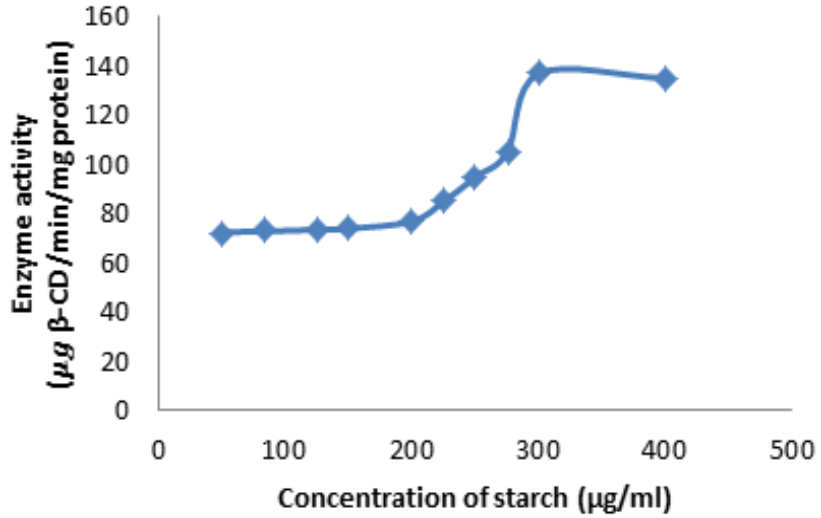

Figure 6. Effect of substrate concentration on enzyme activity

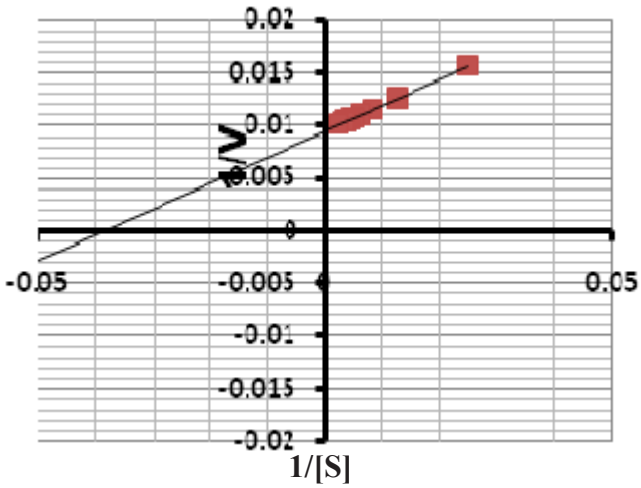

Figure 7. Estimation of kinetic constants

Table I. Purification of CGTase from *Bacillus amyloliquefaciens*

Sample	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude	518	38057.46	73.47	100	1
Ammonium sulphate ppt.	12.5	1254.625	100.37	3.295	1.366
Sephadex G-25	6.4	1109.31	173.33	2.915	2.35
DEAE	0.277	850	3000.68	2.230	41.65

Native-SDS PAGE

The results of the protein profiling by SDS-PAGE of the crude supernatant showed fused bands while fractions obtained with Sephadex G-25 after ammonium sulpahte precipitation and subsequent dialysis and concentration with sucrose showed only 3 bands of CGTase with enhanced purity. A single band indicating the high purity and homogeneity of the isolated enzyme with an approx. molecular weight of 81 kDa was observed. The overall purification of CGTase (Sp activity 3060.68 U/mg) from *Bacillus amyloliquefaciens* L4-6 was about 41.65 fold with a 2.23 % yield.

Characterization studies

Effect of temperature on enzyme activity

Enzyme strain of *Bacillus aminoliqueficiances* L4-6 CGTase was observed to exhibit maximum acti-vity at 60°C. It got rapidly denatured at 70°C and above.

Effect of pH on enzyme activity

Strain *Bacillus aminoliqueficiances* L4-6 alkaline CGTase was optimally active at pH 8. Apart from it, this enzyme was observed to be active over a wide range of pH values, making it a versatile candidate to diverse application. More that 70 % of enzyme activity was observed at all pH values, in the range 8.0 to 9.5. It shows a very poor activity at pH 10 and above.

Thermal stability studies

When culture supernatant of Strain *Bacillus aminoliqueficiances* L4-6 was incubated at 37°C, 50°C and 60°C for varied periods of time, this enzyme was found to have a half life of 96 hours, 8 hours and 15 minutes respectively. Enzyme stability of this enzyme for extended periods at these temperatures would be an added advantage for there industrial application.

pH stability studies

Strain *Bacillus aminoliqueficiances* L4-6 was found to be quite stable with a wide range of pH values from 7.5 to 10. Maximum stability was observed at pH 7.5, 8.0, 8.5 and 10.0 after 1 hour of enzyme incubation in respective buffers. But

more than 80 % of maximum stability was retained on incubation at all ph range of 7.5-11.0.pH is one important factor in deciding usefulness of alkaline CGTase for a particular industrial application.

Kinetic constants determination for strain *Bacillus aminoliqueficiances* L4-6

Starch substrate was used for strain *Bacillus aminoliqueficiances* L4-6 for determination of Km and V_{max} by using Lineweaver-Burk plots. Strain *Bacillus aminoliqueficiances* L4-6 exhibited Km value of 25 µg/ml and V_{max} value of 144 U/ml.

Production and purification of strain Bacillus aminoliqueficiances L4-6

Strain *Bacillus aminoliqueficiances* L4-6 was found to be more stable, high yielding and thus production and successive purification of it was carried out. Enzyme production was carried out in a conical flask of 500 ml total volume and working volume of 200 ml. Thus from here a part of culture broth was centrifuged and the supernatant was subjected to ammonium sulphate precipitation follwed by sephadex G-25 column pass to remove excess salt from the concentrated enzyme. Then the eluted fractions were loaded onto dialysed membrane for concentrating the protein with the help of sucrose. The concentrated protein was then loaded into DEAE coloumn .The enzyme was found to bind to the matrix and eluted at 0.35- 0.5M NaCl gradient in manual runs where it was step gradient approach and the same enzyme was eluted at 250- 300 mM NaCl linear gradient .

Determination of molecular weight

Ion exchange eluted fractions having maximum enzyme activity were loaded onto NATIVE-PAGE. Gel was stained with potassium iodide solution. When the mixture of CGTase and sample buffer were loaded without boiling, it was observed to have a molecular weight of 80 kD ,by when the sample buffer and CGTase.Ion exchange eluted fractions having maximum enzyme activity were loaded onto SDS-PAGE. Gel was stained with Coomassie blue R-250.When the mixture of protease and sample buffer were loaded without boiling), it was observed to have a molecular weight of 66kD, by when the sample buffer and protease were boiled before loading the mixture onto the gel, it was observed to exhibit a Mr of 22kD. This protease was apparently a trimeric protein. Molecular weight of protease was compared with Lower molecular weight marker and standard BSA solution. Moreover a single band of enzyme was observed on SDS-PAGE both for boiled and unheated fraction, thus it was concluded that the enzyme was purified to homogeneity in single step purification.

Discussion

Among the 37 colonies screened for production of CGTase, RS-4 (Sample B-27 mm) was selected based on their hydrolytic zones. The isolated sample B was found to be *Bacillus amyloliquefaciens strain* L4-6 (GenBank Accession Number: KC464454.1). The purified enzyme was stable at a wide pH range from 4 to 12 and was optimally active at pH 8 . The enzyme exhibited optimum activity at a temperature of 60°C. The relative enzyme activities at 65, 70, 75 and 80°C were found to be 89 %, 83.4 %, 71.8 % and 43 % respectively as represented in Fig. 5 and it was stable for 1 h. As represented in Fig. 6 with increase in substrate concentration from 40-200 µg/ml, the enzyme activity of CGTase (µg β-CD /min/ mg protein) was not enhanced significantly, which increases proportionally with an increase in substrate concentration (200-300 µg/ml) and reached the plateau stage. A potent CGTase was obtained with a purification fold, specific activity and yield value of 41.65, 3000.68 U/mg and 2.23 % respectively by ammonium salt precipitation, followed by Sephadex G-25 and DEAE Cellulose coloumn chromatography. The apparent molecular weight of the CGTase was found to be 81 kDa by SDS PAGE.

Conclusion

A potent cyclodextrin glucanotransferase (CGTase) was produced from *Bacillus amyloliquefaciens* L4-6 Gene Bank Acession no. KC464454.1 was purified up to 41.65 fold with a 2.23 % yield by ammonium salt precipitation,

Sephadex G-25 and DEAE Cellulose coloumn chromatography. The purified enzyme was stable at pH range from 4 to 12 and was most active at pH 8. The temperature optimum for its activity was 60°C, and the enzyme was stable for 1 hour. The apparent molecular weight of the CGTase was found to be 81 kDaby Native-SDS PAGE.

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