OPTIMIZATION OF CULTIVATION CONDITIONS FOR PRODUCTION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE BY *BACILLUS FLEXUS* ISOLATED FROM ALKALINE METEORITE CRATER LONAR LAKE, INDIA

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Abstract: The extracellular enzyme cyclodextrin glycosyltransferase (CGTase) synthesizes cyclic malto-oligosaccharides called cyclodextrins (CDs) from starch and related glucans. CGTases are produced by a variety of bacteria, mainly *Bacillus* species, by submerged culture in complex medium. CGTases differ in the amount and types of CDs produced. Production of CGTAse in Horikoshi medium of pH 8 was studied at laboratory lavel using *Bacillus flexus* isolated from Lonar meteorite Crater Lake. The *Bacillus flexus* was confirmed by partial 16s r-RNA sequencing & by growth on Hichrome *Bacillus* agar and by using biochemical tests following Bergey's manual of Systematic Bacteriology. The screening & confirmation of CGTase production was done by Yellow zone on Horikoshi medium containing phenolphthalein and methyl orange. Production of Cgtase was carried out using different carbon sources like Rice bran, Wheat bran, Potato peels, Tapioca waste and effects of different Physical & chemical factors like incubation period, pH, temperature, metal ions, on activity of CGTase was studied. The maximum production was obtained at 30°C and at pH 8.0. The enzyme was partially purified by organic solvent precipitation using different (v/v) concentrations of acetone. The activity was checked spectrophotometrically by using β – Cyclodextrin as standard. The enzyme partially purified by acetone was further purified by ammonium sulphate precipitation. The partially purified enzyme showed maximum of 50.9659 U/ml/min activity at temperature 40°C, 50.59643 U/ml/min activity at pH 8.5.

Keywords: *Bacillus flexus*, Lonar lake, CGTase, Alkaliphilic, Cyclodextrin.

Introduction: Rhizobacteria are present in the soil in an average of about 10^8 cells per gram and from the soil, they are transferred to various associated environment including plant, foods, animals, marine and fresh water habitat [1]. The extremophilic bacterial species from soil has developed unique metabolic and physiological function that ensure survival in extreme habitat but also offer a potential for the production of different industrially important products like antibiotic, lactic acid, ethanol, acetone, butanol and certain enzymes like, amylases, invertases, CGTase (Cyclodextrin Glycosyl transferase), proteases, Xylanase, Lipases, etc.

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is a member of α-amylase family (family 13 of glycosyl hydrolases). Although CGTase is closely related to α-amylase, α-amylase usually catalyze hydrolysis reaction using water as acceptor whereby CGTase preferably catalyze transglycosylation reactions in which glucosyl residues are used as acceptor in forming cyclodextrins (CDs) as the main product. CGTase is a multifunctional enzyme, besides cyclization it also display intermolecular transglycosylation (coupling, disproportionation) and hydrolytic activity on starch and CDs[2].

In sum, it has four functions: cyclization, coupling, disproportionation and hydrolysis [3], [4]. The most common CGTase production belongs to *Bacillus* genus, as well as other archaea[3].

Currently, bacteria are still regarded as an important source of CGTases. Since the discovery of *Bacillus macerans* as the first source that is capable of producing CGTases, a wide variety of bacteria have been determined as CGTase producers, namely aerobic mesophilic bacteria, aerobic thermophilic, anaerobic thermophilic and aerobic alkalophilic bacteria. Various genera of bacteria that are known as CGTase producer includes *Bacillus*, *Klebsiella*, *Pseudomonas*, *Brevibacterium*, *Thermoanaerobacterium*, *Corynebacterium*, *Micrococcus*, and *Clostridium* [5]. Most CGTases produce a mixture of α-, β- and γ-CD in different ratios, depending on the origin of the CGTase as well as the reaction conditions [4]. CGTase is classified into three different types, α-CGTase, β-CGTase and γ-CGTase according to the major CD produced. Therefore, in the present study, different parameters for the production of cyclodextrin Glycosyltransferase by the isolated *Bacillus flexus* were studied. *Bacillus flexus* was isolated from Alkaline Meteorite Crater Lake Lonar, India (Data not published).

Material and Methodology:

Basal medium and Bacterial isolate used: Bacterial isolate isolated from soil samples collected from Alkaline Meteorite Crater Lake Lonar, India is used in this study and Identified by biochemical and 16S rRNA gene sequencing as *B. flexus*[6]. The gene sequence for this isolate has been deposited to the NCBI GenBank Database under accession number (Data not published). Basal Horikoshi medium II

used for cultivation of bacteria in this study consist of Solution 1: Starch 1.0 % (w/v), peptone 0.5% (w/v), yeast extract 0.5% (w/v) K_2HPO_4 0.1% (w/v), $MgSO_4$ 0.02% (w/v). Solution 2: Na_2CO_3 1.0% (w/v) autoclaved separately [7]. Samples were harvested at predetermined time intervals for the measurement of CGTase production and activity.

Screening of the Isolated *Bacillus flexus* species for CGTase production: The isolate, *B. flexus* was screened for CGTase production using a screening technique, in which the isolates were streaked over the plates of Horikoshi media II with phenolphthalein indicator and Methyl orange stain and plates were incubated at 27-30°C for 24-48 hrs, and observed for the zone of hyıdrolysis of starch. Hydrolysis of starch is indicated by yellow zone around the colonies of isolate, which is indicative of production of CGTase around the colonies [7].

Production of CGTase by *Bacillus flexus* (BI56A): The basal Horikoshi medium II was used for production of CGTase. Ten percent (v/v) of bacterial inoculum of isolated *B. flexus* was inoculated in the 100 ml basal Horikoshi medium II into 250 ml conical flask and incubated at 30°C for 48 hrs with continuous orbital shaking at 130 rpm. The cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C. the supernatant was assayed for CGTase activity and used as crude enzyme solution.

Assay of enzyme activity of CGTase by Phenolphthalein assay method: Cyclization activity of CGTase enzyme samples (from isolates BI56A) was determined by the phenolphthalein method [8]. To 1.25 ml of 4.0% soluble starch, 0.25 ml purified CGTase was added. The reaction mixture was incubated for 30 min at 60°C. The reaction was stopped by boiling for 5 min and 1.0 ml of the reaction mixture was incubated with 4.0 ml of phenolphthalein solution. The decrease in phenolphthalein absorption at 550 nm reflected the amount of CD in the reaction which was quantified from calibration curve. One unit of activity was defined as the amount of enzyme able to produce 1 mole of β-CD per minute under the appropriate condition. Monitoring CGTase activity on agar plate was performed by pouring mixture of methyl orange and phenolphthalein on Horikoshi medium [7] or LB plate in the presence of 1% soluble starch.

Optimization of parameters for CGTase production: In the present study, the effect of cultural condition and media composition on the CGTase production by *B. flexus* was performed by using one-factor-at-a-time method, in which holding all the factors steady except the one which was studied. The investigated factors include several nitrogen and carbon sources with their various concentrations, time period of incubation, intial pH, and various fermentation temperatures. For each of factor, three sets of experiment were conducted and the mean of the values was reported.

Result and Discussion:

Effect of incubation period on CGTase production: Researchers from different countries studied the effect of incubation period reported that *Bacillus* species showed good growth and CGTase production in the incubation period 24 to 48 hrs of incubation. In the present study, the effect of incubation period was studied (with reference to agro-industrial waste: potato peels, wheat bran, rice bran and tapioca flour as sole source of carbon) by incubating the production medium for 5 days and the samples were taken periodically & it was seen that the isolate *B. flexus* showed increasing production and specific activity of CGTase from 6 hrs till 48 hrs and then the production and specific activity of CGTase decreased continuously. The specific activity of CGTase was low in early hours with all the agro-industrial wastes used and it increased steadily till 30-48 hours with all the agro-industrial wastes used and then decreased till the end of incubation with all the agro-industrial wastes used. So form the results it was seen that optimum incubation period of the isolate *B. flexus* for the production of CGTase was 30-48 hrs. it was also seen that the change in the carbon source does not affect the incubation period required for a moderate activity of the CGTase, but with tapioca flour the production and activity of CGTase was maximum and with wheat bran it was minimum (Fig. 1).

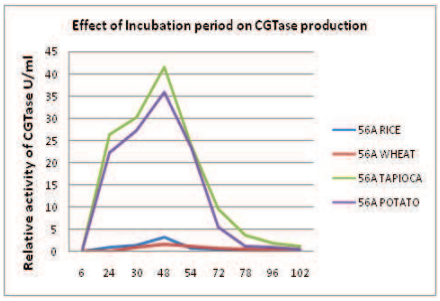

Fig. 1 Effect of incubation period on CGTase production

Some researchers reported that *Bacillus* species TPR71H showed the maximum CGTase production at 36h. Further incubation, decreases the enzyme production, it may be due to reduction of nutrients in the medium and formation of protease[9]. Some researchers studied the Time course of CGTase production for *B. circulans* and observed maximal growth at 24 hours and slows down later on, enzyme production increases sharply after on, between 24 and 48 hours culture [10]. This pattern has not been frequently observed, except for *B. firmus*[5].

Effect of starch concentration on CGTase production: While studying production of CGTase, the effect of the starch concentration (the sole source of carbon in the production medium) on production of CGTase was studied and it was seen that, the isolate *B. flexus* showed increasing production of CGtase when the starch concentration is in the range of 0.5% to 1.5 %, with specific activity maximum at 1.5 % starch concentration but as the starch concentration increased above 1.5% till 2.5 % the CGTase production decreased with specific activity minimum at 2.5 % starch concentration. The relative activity of CGtase also showed the range of 0.5 to 1.5 % starch concentration for maximum activity (Fig. 2).

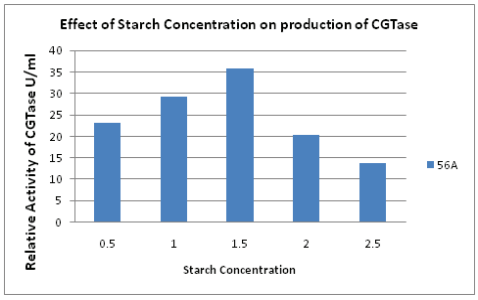

Fig. 2 Effect of starch concentration on CGTase production

From these results of Production of CGTase, we can see that, Starch at concentration ranging from 0.5% to 1.5% is suitable for CGTase production. So, for further studies Starch and yeast extract at concentration of 1.5% is used for production of CGTase. Some researchers studied effect of tapioca starch concentration on the β-CD production with the amount of CGTase fixed at 12 U/g starch. The total amount of β-CD produced increased with an increasing concentration of tapioca starch[11]. The highest amounts of β-CD production was obtained at 6% and 7% tapioca starch with the amount of CGTase fixed at 12 U/g starch and concluded that CGTase from *Bacillus* sp. C26 is more suitable for CD production from tapioca starch than the CGTases from *Bacillus* sp. G1[12] and *B. circulans* DF 9R [13] because the *Bacillus* sp. C26 CGTase produced highest amounts of β-CD at higher concentration of starch (6% starch concentration) compared to the CGTases from those *Bacillus* (5% starch concentration).

Effect of carbon source on CGTase production: Researchers from different countries studied the effect of different carbon sources reported that starch is very efficient carbon source for CGT*ase* production. In the present study different starch sources from agroindustrial wastes (as sole source of carbon) from Nanded region were used for production of CGT*ase.* While studying the effect of carbon source, isolate *B. flexus* showed efficient utilization of tapioca starch and potato starch with maximum specific activity of CGTase, and showed poor utilization of rice and wheat starch with minimum specific activity of CGTase. Though the isolate *B. flexus* can act on various starch sources, the yield and initial rate of β-CD production from tapioca starch were highest compared to the others (Fig. 3).

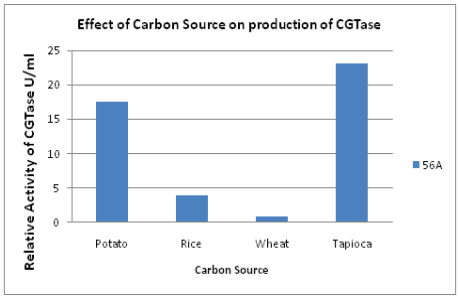

Fig. 3 Effect of carbon source on CGTase production

Some researchers reported that cassava supplementation produces a high growth rate for *B. licheniformis*, it induces low CGTase activity [14]. Soluble starch was found as a best carbon source for *B. firmus* and *B. macerans*[15]. However studies carried out by Some researchers showed that 0.5% (w/v) glucose was found to be the most suitable substrate for CGTase production [16].

Some researchers observed that CGTase produced β-CD from tapioca starch rapidly and achieved a maximum concentration within 24 hrs[11]. The highest β-CD production from tapioca starch was also observed in CD production by CGTase from *Bacillus* sp. G1 [12], while soluble starch was the best substrate for CGTase from *K. pneumonia* AS-22[17].

Influence of different nitrogen sources on CGTase production: The influence of different nitrogen sources on CGTase production was studied by supplementing the basal medium with peptone and yeast extract as sole nitrogen source. Among the nitrogen sources in the basal medium, yeast extract was the most effective additives resulting in the increase of the enzyme production (Fig. 4, Fig. 5).

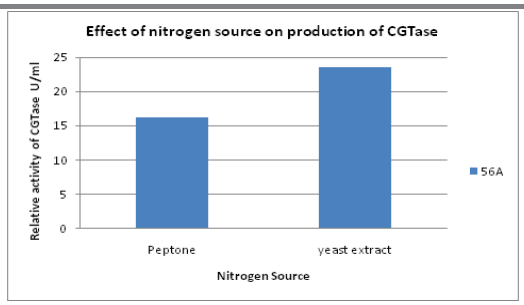

Fig. 4 Influence of different nitrogen sources on CGTase production

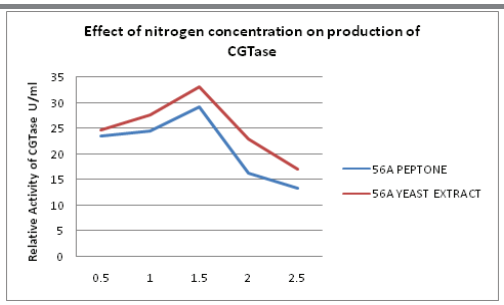

Fig. 5 Influence of different nitrogen concentration on CGTase production

To evaluate the effect of yeast extract, different concentrations was added to production medium (0.5 to 2.5 %). Maximum CGTase production by the isolate *B. flexus* was found in medium containing 1.5 % yeast extract (Fig. 5).

Researchers from different countries observed that 1% yeast extract gave the highest CGTase yield, and this was selected as the optimal nitrogen source for CGT ase production from *Bacillus* sp. C26[18]. This result was different from the CGT ase production of alkalophilic *Bacillus* sp. TS1-1 as the highest activity of CGTase was observed at high concentration of yeast extract of 1.89% [19].

Effect of pH and temperature on CGTase production: The effect pf pH and temperature on CGTase enzyme production by the isolate *B. flexus* was studied by growing culture at different temperature (10, 20, 30, 40, 50, 60, 70°C) and initial pH between 4 and 10. A high level of CGTAse activity was obtained in the culture medium with pH 8 (Fig. 6) and optimum temperature at 40°C (Fig. 7).

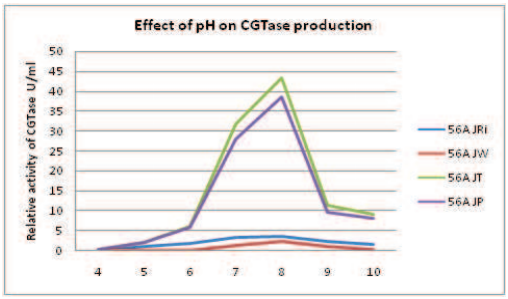

Fig. 6 Effect of pH on CGTase production

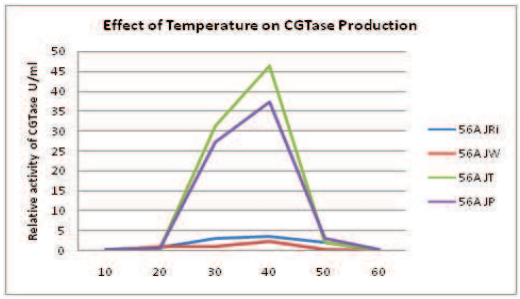

Fig. 7 Effect of Temperature on CGTase production

Some researchers reported that while studying the effect of temperature and pH on CGTase activity the CGTase activity was significantly higher at 37°C than 47 & 27°C during 16 to 36 h of incubation and pH 8 showed maximum CGTase activity at 24h of incubation [14]. But the difference in CGTase activity among the different pH ranges is insignificant. The CGTase activity of *Bacillus* sp G1 was found to be optimal at pH 6[20].

Some researchers observed that the pH optima of the enzyme were 7.5 for the cyclisation activity and 8.0 for the hydrolysis activity. The temperature optima for the cyclisation and hydrolysis activity were 50°C and 60°C respectively [21]. A broad range of temperature between 45°C and 70°C was already reported [22][23].

Effect of metal ions on CGTase production: The effect of metal ions on CGTase production by the isolate *Bacillus flexus* was studied (with reference to agroindustrial waste: potato peels, wheat bran, rice bran and tapioca flour as sole source of carbon). It was seen that, the production and activity of CGTase was more in presence of Ca^{++} and Mg^{++} ions as $CaCl_2$ and $MgSO_4$ as compared to other metal ions (Fig. 8).

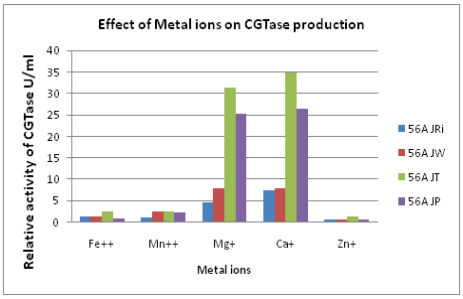

Fig. 8 Effect of metal ions on CGTase production

Some researchers studied the effect of cations on the production of CGTase was studied by substituting

different metal ions in the media. All metal ions inhibited the activity of CGTase. Mg^{2+}showed moderate inhibition but Fe^{2+} and Zn^{2+} were the strong inhibitors of CGTase activity [14]. But it was also reported that Mg^{2+} and Ca^{2+} showed little activation on CGTase activity [24]. Some researchers reported that, the purified enzyme was strongly inhibited by Zn^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+} and Fe^{3+}[25]. CGTase from *Brevibacterium* sp. No. 9605 [26] and *Bacillus halophilus* INMIA-3849 [27] were strongly inhibited by Zn^{2+}. On the other hand, CGTase from *Brevibacterium* sp. No. 9605 [26] and *Bacillus agaradhaerens*[28] were strongly inhibited by Cu^{2+}. This observation suggests that the anion part of the metals also play a significant role in stabilizing the CGTase.

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