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Screening, optimization and production of a novel β- cyclodextrinase by *Bacillus flexus* MSBC 2

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Abstract

CGTase (Cyclodextrin Glycosyl Transferase), is an important starch modifying enzyme catalyzing conversion of starch and related polysaccharide into cyclodextrins (CDs), which have important applications in numerous industries. In the present study, a potent CGTase producing bacteria was isolated from corn field soil. The bacteria was identified by microscopic and biochemical tests along with 16s rRNA to be as *Bacillus flexus* MSBC 2. From plate assay it was seen that only β-CD was produced. Submerged fermentation parameters for CGTase production was optimized and the best process parameters after optimization was found to be incubation period 5 days, pH 10.0, temperature 37 °C, peptone with yeast extract as best nitrogen source and 2% corn starch as best carbon source . A 3.0 fold increase in enzyme production was observed after optimizing the cultural conditions. The bacterium is specifically producing β- CGTase which is of high commercial value in industries as generally bacterial CGTases produce mixture of cyclodextrins.

Keywords: CGTase, *Bacillus flexus* MSBC 2, β- cyclodextrin.

Introduction

Modified starches like cyclodextrins, maltodextrin derivatives have important commercial applications. Thus, starch modifying enzymes are of great importance, of which Cyclodextrin GlycosylTransferace (CGTase) is one such enzyme. CGTase (2.4.1.19) belongs to α- amylase, family of Glycosyl hydrolase 13 (GH 13) (3). It produces cyclodextrins (CDs) via cyclization reaction an intramolecular transglycosylation reaction. CGTases generally produce mixture of CDs namely; α -, β- and γ- CD depending on the number of glucose units i.e. 6, 7 and 8. Thus, CGTase producing one type of CD are of commercial importance (1).

CDs are α-1,4 linked glucopyrunose units, non reducing, torus shaped, it possesses hydrophobic interior and hydrophilic exterior,(4) which can form inclusion complexes with organic and inorganic guest molecules altering their physical and chemical properties. As a result of this CDs have numerous applications in pharmaceutics, textile, cosmetics, agriculture, food, supramolecular chemistry, environmental protection, membrane and analytical chemistry (5).

Due to its wide applications, CGTase producing micro-organisms are been screened for enzyme of desirable traits suitable for industrial applications (1). Most common producers of CGTase are members of *Bacillus* sp. (6, 7 & 8). CGTase is inducible in nature (2) and thus, its production is affected by the

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nature of substrate used in the fermentation medium and its properties depend on the source of micro-organism (9). Also, the enhancement of enzyme production is found to be influenced by physical and nutritional parameters, thus evaluating these parameters are important for enhancing metabolic production and microbial growth (10 & 11). Numerous strategies like computational techniques and genetic engineering are also used to enhance the production of CGTase (6, 8 & 11). This study presents the screening of *Bacillus flexus* MSBC 2, which is a potent producer of CGTase and the optimization of cultural conditions for maximum enzyme production.

Materials and Methods

Isolation screening and identification of β-CGTase producing bacteria: Soil sample was collected from sugarcane and corn fields, Maddur, Karnataka. 1.0 g of the soil sample was suspended in 10 mL of distilled water, serially diluted sample was screened according to rapid screening method described by Park *et al*. (3), where 100μL of serially diluted suspension was plated on to Horokoshi media –II (HM-II), pH was adjusted to 10.0 in order to isolate alkaophilic bacteria producing CGTase and incubated at 37 °C for 24 - 36 h. The media composition was as follows 1.5% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.01% $MgSO_4$, 0.02% phenolphthalein, 0.01% methyl orange, 1.0% Na_2CO_3 (autoclave separately). Bacteria showing maximum zone of clearance was selected for further studies.

The selected strain was characterized as per Bergey's Manual of Determinative Bacteriology and further confirmed with 16s rRNA technology.

Qualitative analysis for the type of CD produced: The bacterial culture was inoculated onto the Petri plates containing HM II media with dyes 0.035 mM congo red, 3 mM phenolphthalein with 0.035 mM methyl orange and 0.035 mM bromocresol green respectively for 24 - 36h at 37 °C, to check the production of α-, β-, and γ- CD (12).

Preparation of inoculum: The selected strain was sub cultured on to Horikoshi media II (pH 10.0), in the slants excluding indicators at 37°C for 24h, a loop full of culture was then transferred in to HM-II broth excluding indicators, the culture showing cell density of 0.18 OD at 600nm was used as standard inoculum.

***Determination of CGTase activity and protein content*:** CGTase assay was carried out as per slightly modified phenolphthalein method (13), 4% soluble starch in 0.1M phosphate buffer, pH 7.0 was prepared by heating it in boiling water bath for 3 min. To 650 μL of substrate, 250 μL of 0.1 M phosphate buffer pH 7.0 was added along with 100 μL of enzyme. This reaction mixture was incubated at 60 °C for 15 min. 4 mL of 0.04 mM phenolphthalein prepared in 125 mM sodium carbonate, was added immediately after incubation period. Blank contained 0.1 mL of distilled water excluding enzyme. The decrease in absorbance was read at 540 nm. One unit (U) of enzyme activity is, the amount of enzyme required to liberate 1μmol of β - CD per minute under standard assay conditions. Standard curve was plotted from known β- CD concentrations of 0-500 μg/mL. Protein content was determined according to the method described by Lowry *et al*. (14).

***Optimization of cultural parameters*:** Various factors influencing CGTase production was optimized by standardizing one factor, keeping all other variables constant, optimized parameter was then incorporated into the experiment for optimization of next factor. Post incubation, supernatant obtained by centrifuging broth at 8,500 rpm (4 °C) for 10 min served as crude enzyme. All the experiments were carried out in triplicates. CGTase assay of optimized process parameters was carried out as per slightly modified

phenolphthalein method as described above (13).

· Time course of CGTase production:

2% inoculum was added into the HM-II broth(pH 10.0), exclusive of indicators and incubated at 37 °C, 2 mL of cultural filtrate was withdrawn every 24h for a period of seven days. The filtrate obtained was centrifuged at 10,000 rpm for 10 min at 4 °C, supernatant obtained served as crude enzyme source and CGTase activity was assayed as mentioned earlier.

· Effect of incubation temperature on *Bacillus flexus* MSBC 2 for CGTase production:

Culture medium (pH 10.0) were inoculated with 2% inoculum and incubated at different temperatures i.e., 20 °C, room temperature - 28±2 °C, 37 °C, 45 °C and 55 °C. CGTase activity was checked on the optimum day of enzyme production.

· Effect of initial pH of the medium on enzyme production:

Effect of pH was evaluated by adjusting pH to 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 with a digital LI 120 pH meter (ELICO) before sterilization. pH 10.0 served as control. After sterilization at 121 °C for 15 min, media was inoculated with 2% inoculum and incubated at 37 °C for 5 days and CGTase activity was assayed according to slightly modified phenolphthalein method (13).

· Influence of different nitrogen sources on CGTase production by *Bacillus flexus* MSBC 2:

Different nitrogen sources (1%, w/v): peptone, yeast extract, casein, urea, ammonium nitrate, ammonium chloride, beef extract and ammonium sulphate were used. Peptone along with yeast extract served as control. Media was inoculated with 2% inoculums, pH 10.0 and incubated at 37°C. The best nitrogen source was optimized by evaluating the enzyme activity on 5th day.

· Influence of different carbon sources and carbon source concentration on CGTase production by *Bacillus flexus* MSBC 2:

Various carbon sources such as corn starch, potato starch, amylopectin, wheat starch and starch hydrolysate were tested, by replacing it with soluble starch which served as control. Media was inoculated with 2% inoculum and incubated at 37 °C, peptone along with yeast extract as nitrogen source, pH 10.0. Enzyme production was assessed on 5th day.

Varying concentrations of corn starch i.e. 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% was taken and enzyme activity was checked on the 5th day.

Statistical Analysis

Experiments were carried out in triplicates and one way ANOVA was performed for statistical analysis using GraphPad Prism v6.

Results and Discussion

***Isolation, screening and identification of* β-*CGTase producing bacteria*:** Serially diluted soil samples from corn and sugarcane fields were subjected to screening according to rapid screening method (3).Numerous colonies showing distinct yellow hallo was observed on 3rd day.The colonies were then individually plated onto the media and the culture showing maximum zone of clearance was selected for further studies. CGTase producers can be isolated form soil regions of corn root, oat cultures, sugarcane and hyper saline soda lake (15,16). The isolate was identified as *Bacillus* sp. from microscopic examination and the strain was further confirmed as *Bacillus flexus* MSBC 2 through 16s rRNA sequencing.

Plate assay for the type of CD produced: Isolated stain *Bacillus flexus* MSBC 2 was plated on to the medium containing respective indicators namely methyl orange,

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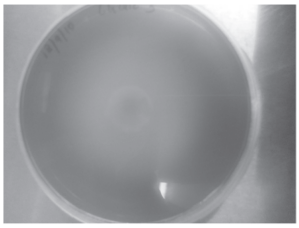

Fig. 1. Screening and isolation of CGTase producing bacteria. Bacteria screened and isolated through rapid screening method showing highest zone of clearance.

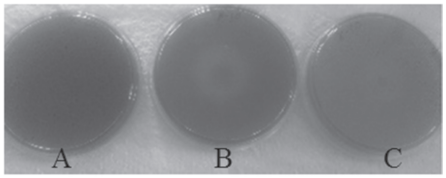

Fig. 2. Plate assay for the type of CD produced. (A) Bromocresol green for γ- CD (B) Phenolphthalein for β-CD (C) methyl orange α- CD.

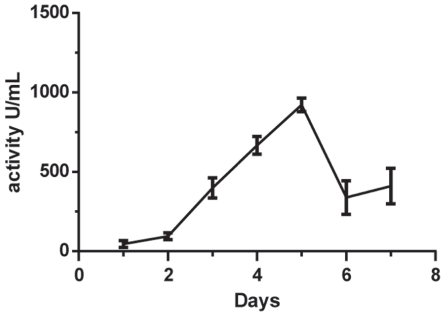

Fig. 3. Time course for optimum enzyme production by *Bacillus flexus* MSBC 2. . CGTase production on different days grown in HMII media with pH 10.0 and inoculated 2% inoculums and incubated at 37 °C. 5th day displayed optimum activity. Results obtained were statistically significant at $p < 0.05$.

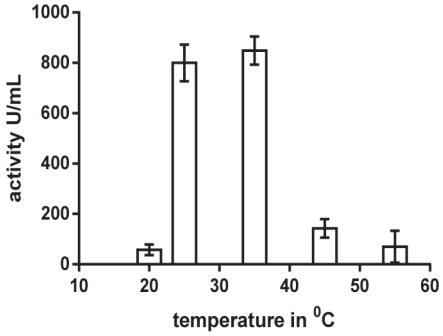

Fig. 4. Effect of incubation temperature on production of CGTase. HM II (pH 10.0) media was inoculated with 2% inoculum and incubated at respective temperatures. 37 °C displayed optimum enzyme activity and as temperature increased enzyme production declined. The values represented differ significantly from each other at $p < 0.05$.

phenolphthalein and bromocresol green for α- β- and γ- CD. Zone of clearance was observed on the plate containing phenolphthalein indicating that only β- CD was produced.
Fig. 2. Plate assay for the type of CD produced. (A) Bromocresol green for γ- CD (B) Phenolphthalein for β-CD (C) methyl orange α- CD.

Optimization of cultural parameters: Time course: CGTase activity was assayed for every 24 h after incubation to identify the optimum incubation period for maximum enzyme production. The enzyme production was observed on the 2nd day and optimum production was observed on 5th day. Depending

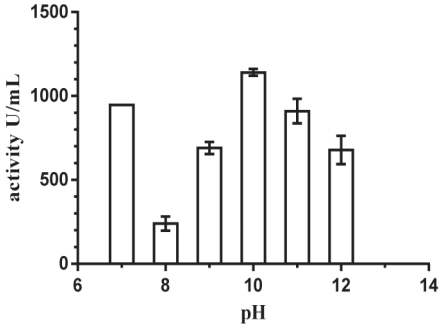

Fig. 5. Effect of initial pH on CGTase production. HM II media was set to respective pH, inoculated with 2% inoculum and incubated at 37 °C for 5 days. pH 10.0 served as control. The values represented differ significantly from each other at $p < 0.05$.

on the source and environmental conditions from which microorganism is obtained time course for enzyme production may vary, *Bacillus firmus* obtained from soil oat culture displayed optimum activity on 5th day (1) whereas, *Bacillus* TS1-1 displayed highest activity at 24 h (6).

***Effect of temperature on CGTase production*:** Incubation temperature plays an important role in enzyme production as it affects the growth rates of micro-organisms. Influence of incubation temperature on CGTase production was studied. Maximum enzyme production was observed at RT and 37 °C. Optimum production was displayed at 37 °C, which was statistically significant ($p < 0.05$). There was a decline in enzyme production when temperature increased beyond 37 °C. Studies conducted by Sivakumar *et al*.,(20) on *Bacillus megaterium* showed similar results where 27 and 37 °C were optimum for enzyme production. The values represented differ significantly from each other at $p < 0.05$.

***Effect of pH of the medium on alkaline protease production*:** Initial pH of medium is an important factor in deciding growth of microorganism either directly or indirectly (17) and enzyme activity, as any change in pH may result in change in shape of substrate and/or enzyme (18).The initial pH of the medium had significantly ($p< 0.05$) influenced the enzyme production. There was an increase in the production of enzyme as pH increased, also the growth of the organism and enzyme production was observed at neutral pH, this may be due to the ability of micro-organism to grow at neutral pH conditions. However, enzyme production was maximum at pH 10.0. There was a significant decrease observed when the pH was further increased to 11.0 and 12.0. pH 6.0 was found to be optimum for *Bacillus* sp. G1(19) for high enzyme production.

***Effect of nitrogen sources on CGTase production*:** Influence of different nitrogen

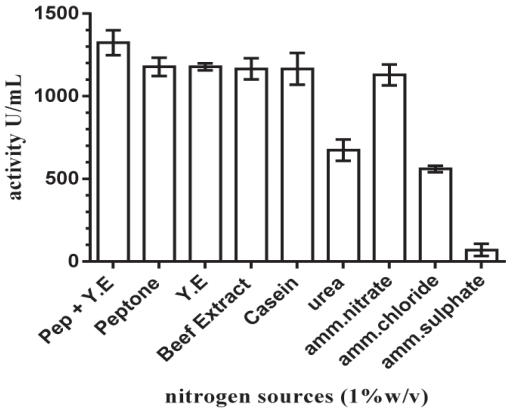

Fig. 6. Effect of nitrogen sources (1.0% w/v) on CGTase production by *Bacillus flexus* MSBC 2. The fermentation media at intial pH of 10.0 was inoculated with 2% inoculums incubated at 37 °C for 5 days. Peptone with yeast extract served as control. The values represented differ significantly from each other $p < 0.05$.

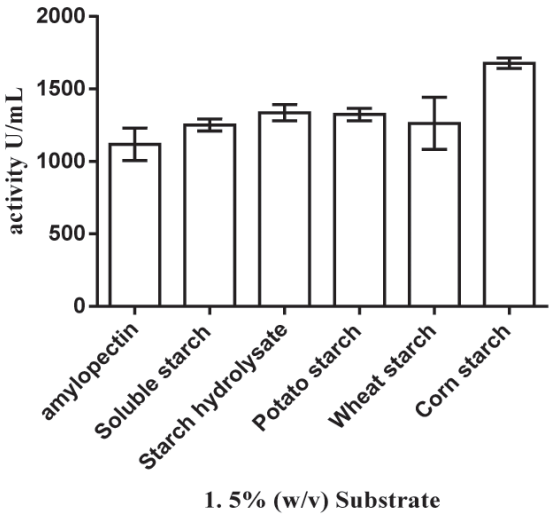

Fig. 7. Influence of various carbon sources (1.5% w/v) on enzyme production by *Bacillus flexus* MSBC 2. Organism was able to utilize various carbon sources and optimum activity was observed in the medium containing corn starch. Values obtained were statistically significant from each other at $P <0.05$.

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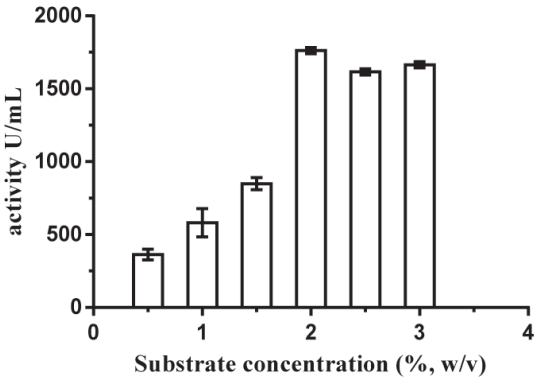

Fig. 8. Substrate of 1.5% served as control and 2% corn starch was found to be the optimum. The results analysed through graph pad prism v6 were statistically significant from each other at $p < 0.05$.

sources on enzyme production were tested, wherein the bacteria was able to use all the nitrogen sources except for ammonium sulphate and ammonium chloride. Significant activity was obtained in the media supplemented with peptone and yeast extract ($p < 0.05$). Generally studies show that yeast extract is found to be the best nitrogen source for CGTase production.

***Effect of substrate and carbon source CGTase production*:** The type of CD produced depends on the substrate and source of enzyme, thus influence of various carbon sources (1.5% w/v) were tested for maximum enzyme production. Corn starch displayed optimum CGTase activity and results obtained were statistically significant at $P_{0.05}$. However, sufficient enzyme activity was observed in the media containing other carbon sources indicating that micro-organism can utilize a wide range of substrates for CGTase production. Corn starch in hydrolysed form was found to best substrate in study conducted by Sivakumar and Shakila(20) where as it was a poor substrate for *Klebsiella pneumonia* As-22 (20,21). This may be due to inducible nature of enzyme production, thus choice of appropriate substrate is necessary for inducing enzyme production by micro-organism.

The effect of altering substrate concentration on CGTase production has been tested and depicted in the **Fig. 8**. The optimum enzyme production was observed at 2.0% concentration which was statistically significant at $P_{0.05}$. Similar results was obtained with *Bacillus firmus* strain No.37 (22), where 2% corn starch resulted in optimum yield of the enzyme.

Fig. 8. Different substrate concentration were analysed for highest CGTase production.

Conclusion

The isolated bacterium, *Bacillus flexus* MSBC 2 was able to use wide range of carbon sources and could grow in alkaline environment which is best suited for industrial processes. Plate assay showed production of only â- CD, which can be used for the commercial production. Further the optimization of fermentation parameters have resulted in a 3.0 fold increase in enzyme production.

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