

Original Research Article

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Production of Cyclodextrin Glycosyltransferase by Newly Isolated Alkalophilic Strain of *Leucobacter* Species

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ABSTRACT

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Cyclodextrins are cyclic oligosaccharides having applications in various industrial and pharmaceutical companies. Cyclodextrin glycosyltransferase is the enzyme that converts starch into cyclodextrins (CDs). We screened 45 strains of CGTase positive isolates from soil samples collected from different regions of tropical forest of Kerala, India. A new strain of *Leucobacter* species which produce CGTase was isolated and the culture conditions and media components were optimized for the enhanced production of the enzyme, which synthesize α and β cyclodextrins. The organism is found to be alkalophilic as its optimum pH is above 10.

Introduction

Cyclodextrin glycosyltransferase (EC.2.4.1.19) is an extra cellular enzyme, which converts starch into a group of cyclic oligosaccharides consisting of 6-12 units of glucose units joined by the α -1, 4 –linkages known as cyclodextrins (CDs). Starch molecules are cleaved into short linear oligosaccharides chains and its new reducing ends are joined, transforming it into non-reducing cyclic molecules. The cyclodextrins have a short cylindrical structure, whose interior is hydrophobic consisting of Hydrogen and carbon atoms and exterior is hydrophilic with hydroxyl groups projecting outwards. Because of this special characteristic feature, they are

capable of forming inclusion complexes with a variety of water immiscible organic and inorganic molecules (Abdel-Naby *et al.*, 2011). Thus cyclodextrins are extensively used in guest – host interaction to make high efficient inclusion compounds and have tremendous application in pharmaceutical industries, textile industries, cosmetics, food and agriculture industries. CDs help to increase the solubility in water, increase the stability, increase the controlled release of drugs, flavors, fragrance, mask bad odors and tastes and increase the longevity of volatile compounds (Atanasova *et al.*, 2008; Hamoudi *et al.*, 2011; Marçon *et al.*, 2009; Sian H., 2005 and Wang *et al.*, 2011).

There are three different types of CDs such as α , β and γ CDs consisting of six, seven and eight glucose units respectively. The CGTase responsible for the production of each of these CDs is also different. Most of the bacterial CGTase produce all the three types of CDs together. The bacterial CGTase are also classified into α -, β - and γ -CGTase based on the major type of CDs they synthesize. Enzymes that synthesize predominantly one type of cyclodextrin have great commercial importance as the separation of one type of CDs from the mixture is not economical (Jemli *et al.*, 2007). The quality of CD produced depends mostly on the type of organisms and the purification methods. Therefore new strains of organisms for the CGTase production of CGTase very important for the high quality CD production as it received great importance in the various industrial sectors (Schmid G, 1989).

CGTase with varying properties are produced to a large scale in a complex medium by bacteria mainly belonging to *Bacillus* species by submerged culture (Andriana *et al.*, 2002). Alkalophilic microorganism is also known to produce this unusual enzyme that can be used in industrial processes for the production of cyclodextrins. All known CGTases (Bart *et al.*, 2000) produces a mixture of cyclodextrins when incubated with starch. Considering the importance of cyclodextrins and the need for new enzymes with higher efficiency for the synthesis CDs, we attempted to screen out new strain of CGTase producing organisms from its natural sources. In our present study we report the isolation of a new strain of *Leucobacter* species which produce CGTase and cyclodextrin and the optimization of media and culture conditions for the enhanced production of CGTase, which synthesize α and β cyclodextrins.

Materials and Methods

Microorganism

We have isolated a new strain of CGTase producing bacteria from the soil of tropical rain forest in Kerala. The isolated organism PK-8 was identified as *Leucobacter* species by morphological and biochemical methods. The organism was screened and isolated in Horikoshi agar plate and the same media was used for the storage and further culturing of the organism.

Media Composition and Culture Conditions

Bacteria was grown in Horikoshi media consisting of 1.0% soluble starch(w/v), 0.05% yeast extract, 0.5% peptone, 0.1% K₂HPO₄, 0.02% MgSO₄.7H₂O, 0.03% phenolphthalein, 0.01% Methyl orange, 1% Na₂CO₃ and 1.5% agar (for solid media) and pH was adjusted to 10.5 (11). Culture plates were incubated at 37 °C for 72 hours. Horikoshi phenolphthalein (PHP) plate was used for detecting the CGTase producing organisms. The morphological characteristics of the isolated strain were determined by Gram's Method. The bacterial strain of *Leucobacter* species was grown in 20 ml of sterile Horikoshi medium (Park *et al.*, 1989). The culture was incubated at 35°C, constantly agitated at 120 rpm for 18 hours. Overnight grown cultures of 0.5 optical density at 660 nm was used as inoculum in all the fermentation experiments.

Production of Crude Enzyme

The organism was inoculated to 100 ml of Horikoshi broth of pH 10.5 in 500ml Erlenmeyer flask. The media was inoculated with 1 ml of 5 % (v/v) of bacterial inoculum of 0.5 OD at 660 nm.

The bacterial culture was incubated at 35°C in an incubator shaker for 6 days. Samples were withdrawn at specific intervals of time, centrifuged at 5000 rpm for 10 minutes and the supernatant was assayed for CGTase activity and used as crude enzyme solution.

CGTase Assay Method

CGTase activity was determined by using phenolphthalein assay (Kaneto *et al.*, 1987). Reaction mixture consist of 1 ml of starch solution (20 mg of soluble starch in 100 ml of 10 mM phosphate buffer - pH 6.6) and 0.1 ml enzyme solution. The mixture was incubated at 65 °C for 20 minutes in a water bath. Subsequently 0.5 ml of 0.02 % (w/v) phenolphthalein in 5 mM Na₂CO₃ solution was added to the reaction mixture and mixed thoroughly. After 15 minutes, the reduction in color intensity was measured at 550 nm. For each batch of samples blanks lacking the CGTase were analyzed simultaneously. As a standard, the soluble starch and enzyme were replaced by 0.5 mg of β – CD and 0.1 ml water respectively. A calibration curve was made using β – CD in 100mm phosphate buffer at pH6.6. One unit of enzyme activity was defined as the quantity of enzyme that produces 1 μ mol of β cyclodextrin per minute under standard conditions.

Cell Biomass Determination

Cell biomass was determined by estimating the dry weight of the media free cells separated from the cultures. Traces of starch present in the cell culture was removed by treating it with 0.1ml (1 unit) of α -amylase to hydrolyze the residual starch to soluble sugars before pelleting the cells by centrifugation. The cell pellet was washed with distilled water and dried in an oven at 95 °C for 24 hr.

Protein Estimation

Protein content of samples was estimated by following the method developed by Lowry *et al.*, 1951.

Optimization of Culture Conditions for CGTase Production

Nutritional components of the media such as carbon sources, nitrogen sources, metallic ions and microelements were optimized by adopting the search technique by varying one factor at a time. Effects of physical factors such as temperature, pH and agitation speed affecting the production of CGTase were also determined. All the experiments were conducted in 500 ml Erlenmeyer flasks. All the experiments were carried out in triplicate with a control.

Chemicals

All chemicals, biochemicals and media constituents used in the study were of analytical grade (unless stated otherwise). Soluble starch, sodium carbonate and phenolphthalein were purchased from Merck. Rice starch, corn starch, tapioca starch, potato starch and sago starch were of industrial grade. Yeast extract and peptone were purchased from SRL. Other media constituents used were purchased from Hi-media.

Results and Discussion

Isolation of CGTase Producing Alkalophilic Bacteria

About 45 bacterial isolates were obtained by the screening of soil samples collected from different places. The isolates showed halo zones around their margin, suggesting possibility of CGTase production. From this 8 positive isolates were propagated in

alkaline liquid medium and CGTase activity was measured in the culture supernatants (Fig: 1). One of these isolates designated as PK-8 showing the highest CGTase production (4.056 U/ml), was selected for further screening. This isolate showed the highest clearance zone of diameter 3 cm in solid alkaline phenolphthalein medium in 24 hrs as a qualitative measurement. Gram staining showed strain PK-8 was gram positive rod shaped with tapering ends. Colonies were circular, raised, golden yellow pigmented. The organism was identified as *Leucobacter* species.

Optimization of the Culture Conditions

Fermentation processes are considerably affected by various factors such as selection of a suitable strain, substrate and process parameters (Pandey *et al.*, 2001). In the present study, optimum temperature, agitation speed and pH for CGTase production were standardized. Different carbon sources like cassava starch, rice starch, potato starch, corn starch, sago starch, wheat starch, soluble starch and glucose were tried as substrates to increase the enzyme production. Different nitrogen sources such as ammonium salts, tryptone, yeast extract, was studied by supplementing them in the production media. Also effect of percentage of carbon and inorganic and organic nitrogen sources were also done to analyze their influence on CGTase production. Effect of metallic ion concentration was carried out by the addition of CaCl₂, MgSO₄, CuSO₄, ZnSO₄, FeSO₄, and MnSO₄ to the production media. All the nutritional and physical factors influencing the production of CGTase were standardized by varying one parameter at a time.

Effect of Inoculum Size on CGTase Production

The effect of initial biomass controls the

kinetics of growth and several biological metabolic functions leading to the overall biomass and extracellular enzyme production. The results of experiments conducted with increasing inoculum concentration from 1% to 10% and the CGTase activity showed that, CGTase production varied with variation in initial Inoculum level (Fig:2). A maximum enzyme production of 24.73 U/ml and specific activity 8.49 U/mg was observed in 4% initial inoculum supplemented conditions.

Effect of Initial pH on CGTase Production

The initial pH and further pH variation in the culture media plays an important role in the production of CGTase and growth of organism. In order to investigate the optimum pH for CGTase production by the bacteria, media with pH adjusted from 4 to 11 was used for the growth of the organism. Fig:3 show the effect of pH on CGTase production by the isolated bacteria. The organism is able to produce enzyme with maximum activity in a narrow pH range from pH 8, 9 and 10. A pH of 10.3 was found to be optimum for the enzyme production with 100.405 U/ml.

Effect of Carbon Sources on CGTase Production

A suitable carbon source is very important for a fermentation process and is very critical if it forms the direct substrate for the product formation. The carbon source was known to be the determining factor in the rate of CGTase synthesis. Since the production of CGTase did not require the presence of specific inducer, production of the enzyme depends mostly on the substrate used (Kabaivanova *et al.*, 1999). A number of starch types were selected as carbon source, since starch is the main substrate for

the synthesis of cyclodextrin and the secretion of CGTase. These starch sources were screened for selecting the best one for the maximum production of CGTase in the medium. In the present study eight different carbon sources such as tapioca starch, rice starch, potato starch, corn starch, sago starch, soluble starch, wheat starch and glucose were used to check the production of CGTase by isolated strain of *Leucobacter* species. The results are presented in Fig 4 and in Table 1.

It was observed that the bacteria can grow well in media supplemented with most of the types of starch as carbon sources. Studies indicated that Potato starch, corn starch, sago starch, and soluble starch are the most suitable sources for enzyme synthesis. Media with Sago starch showed maximum enzyme activity (116.12 U/ml and Specific activity of 37.34 U/mg) and media supplemented with potato starch showed enzyme activity close to that of sago starch (115.75 U/ml and specific activity of 35 U/mg) on the 5th and 4th day of incubation respectively.

The quality of starch is a key factor in the production of cyclodextrins as evidenced by the earlier works also. Wheat starch gave low yield of CD because of its high amylase content (Szerman *et al.*, 2007). Simple sugar glucose did not produce significant result as summarized in Table: 1 and in Fig: 4. But this is in contradictory to a report available for *B. stearothermophilus*, where glucose was found to be the most suitable substrate (Stefanova *et al.*, 1999). However in earlier reports (Jin-Bong *et al.*, 1990; Posci *et al.*, 1988) soluble starch was more suitable for cyclodextrin production by *B. stearothermophilus*. Xylose and glucose were found to be best for *B.cereus* (Jamuna *et al.*, 1993), but the production was enhanced with addition of starch (Thatai *et al.*, 1999).

Different concentrations of soluble starch on the synthesis of CGTase was tested and results obtained showed an increase in CGTase activity corresponding to the increase in concentration of soluble starch. A high CGTase titre of 123.3 U/ml and a specific activity of 41.8 U/ mg was obtained in a medium containing soluble starch of 2.5% as carbon source on the 4th day of incubation. The results are presented in the Figure 5.

Soluble starch concentration above 2.5% i.e. 3% and 5% in the media showed a drastic decrease in enzyme production. Increased soluble starch concentration resulted in the increment of both CGTase activity and biomass production. However when the concentrations of starch in the feed stream exceed beyond 2.5 % (w/v), the CGTase activity dropped significantly. High concentration of substrate will reduce the CGTase production due to the increment of viscosities of the culture that lead to poor oxygen uptake. Above a certain concentration of carbon substrate, when other nutrients are kept constant, catabolic repression may occur (Gawande *et al.*, 2001). This may occur due to limitation of other media components in the culture medium. Results of alkalophilic *Bacillus* sp. (ATCC 31007) reported that soluble starch concentration higher than 20-30 g/l, resulted in low enzyme production. 3% soluble starch was the best for CGTase production by *Bacillus* sp TPR71 H (Ravinder *et al.*, 2012). On the other hand 0.1% sago starch was found suitable for CGTase production by *Bacillus lehensis* S8 (Yap *et al.*, 2010).

In this study both hydrolyzed and raw forms of different starches such as corn starch, potato starch, sago starch, cassava starch and rice starch were used as substrates for CGTase production. Sago starch was the best among the hydrolyzed starches while

corn starch was the best substrate as raw starch. In contrast, for *Klebsiella pneumoniae* As-22 corn starch was not a suitable substrate. It is known that physical treatment makes the starch accessible to CGTase action, while raw starch is inaccessible to the enzyme (Gawande *et al.*, 2001).

Effect of Nitrogen Sources on CGTase Production

Six different inorganic nitrogen sources like $\text{CH}_3\text{COONH}_4$, $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl , NaNO_3 , $(\text{NH}_4)_2\text{HSO}_4$, and NH_4NO_3 were used for studying the effect of inorganic nitrogen sources on CGTase production by *Leucobacter* species. Cell density was high in the media with $(\text{NH}_4)_2\text{SO}_4$, $(\text{NH}_4)_2\text{HSO}_4$, NH_4NO_3 and ammonium acetate at 24 h of incubation and low at NaNO_3 supplementation. Maximum CGTase activity reached at the 4th day of incubation in all media. Among the inorganic nitrogen sources $(\text{NH}_4)_2\text{HSO}_4$ supplied media gave optimum enzyme activity of 63.47 U/ml and a specific activity of 18.9 U/mg, which was low when compared with medium lacking inorganic nitrogen sources, and this indicated that this organism does not need any inorganic nitrogen source in the production medium.

Different types of organic nitrogen sources like yeast extract, peptone, yeast extract and peptone, beef extract, malt extract and tryptone were used to evaluate the growth and CGTase production by the strain of bacteria. All the organic nitrogen sources supported the synthesis of CGTase formation by the culture. Cell density was high in the media with yeast extract and peptone, which also proved superior to other substrates with the production of 360 U mL^{-1} of the enzyme (Fig: 6). Yeast extract alone was found to be best organic nitrogen

source next to yeast extract and peptone (355 U/ml). Tryptone containing medium resulted 352 U/ml CGTase production. Results show that almost all the organic nitrogen sources proved favorable for enzyme production and maximum yield were obtained in the 24 h of incubation. Further studies were conducted selecting yeast extract and peptone as nitrogen source and its effects were compared. Different concentrations varying from 0.5% to 3% were tested for CGTase production. A high CGTase titre of 392 U/ml of CGTase activity and 140 U/mg of specific activity was obtained in a medium containing yeast extract and peptone of 0.5% as substrates. The results were presented in Fig: 7.

In this study enzyme production using inorganic nitrogen sources was found to be low when compared with organic nitrogen sources. It was reported that the best nitrogen source for the production of CGTase was peptone (Illias *et al.*, 2002). But Mahat *et al.* (2004), observed that sago starch and yeast extract have a significant effect on CGTase production. In another study it was reported that the best nitrogen source for CGTase production was yeast extract (1% w/v) but went low when ammonium nitrate was used in the production medium (Yap *et al.*, 2010). But earlier it was found that CGTase production was high in 0.5% yeast extract media which indicates that yeast extract might have some inducer substance or micro nutrients to increase the CGTase production (Ravinder *et al.*, 2012).

Effect of Various Mineral Sources on CGTase Production

The influence of mineral salts on the production profile of CGTase was investigated by supplementing different metal ions in 10 mM concentration in the

media. The results presented in Fig: 8 shows that all metal ions inhibited the activity of CGTase. Mg²⁺ showed moderate inhibition but Fe²⁺, Ca²⁺ and Zn²⁺ were the strong

inhibitors of CGTase production. This is in accordance with earlier reports (Higuti *et al.*, 2003).

Table.1 Yield of CGTase production using different type of starches.

Sources of starch	CGTase activity(U/ml).
Sago starch	116
Soluble starch	115.8
Tapioca starch	16
Corn starch	67
Rice starch	19
Wheat starch	5
Glucose	0.4
Potato starch	102

Fig.1 CGTase production by different isolates in culture filtrates

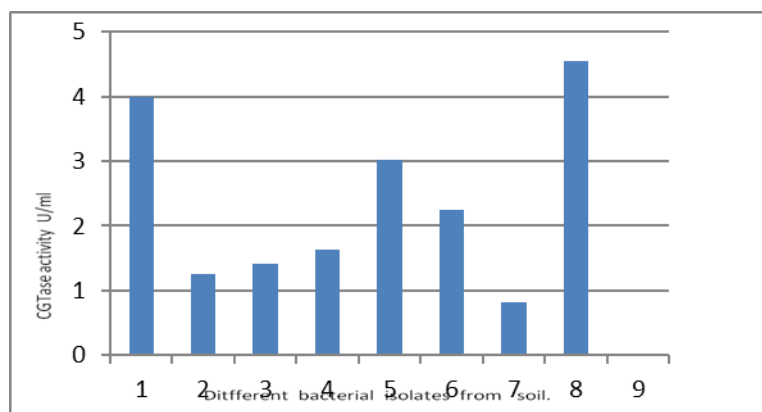


Fig.2 Influence of inoculum size on CGTase production

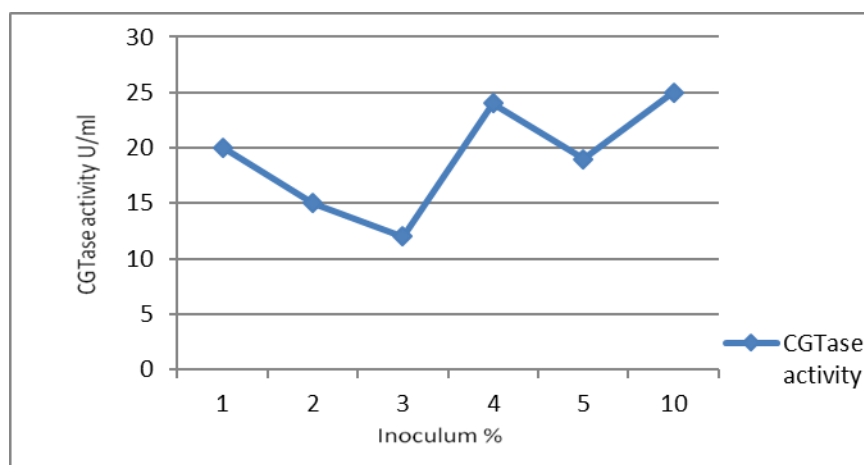


Fig.3 Effect of pH on CGTase production.

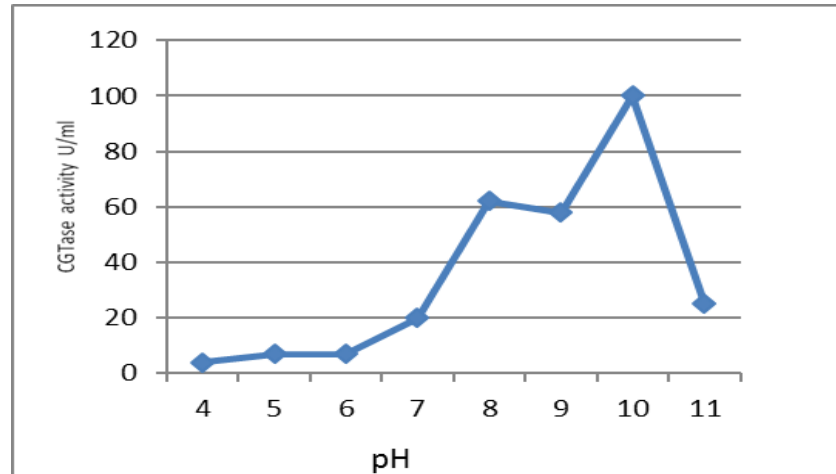


Fig.4 Effect of different carbon sources on CGTase production by *Leucobacter* sps.

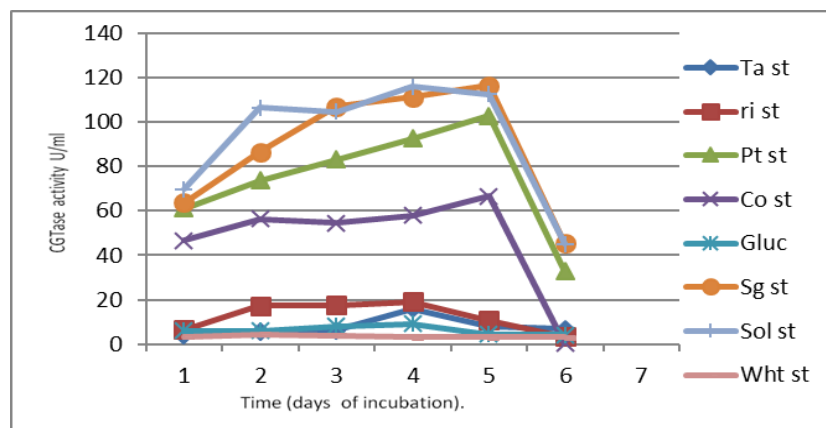


Fig.5 Effect of soluble starch in CGTase production.

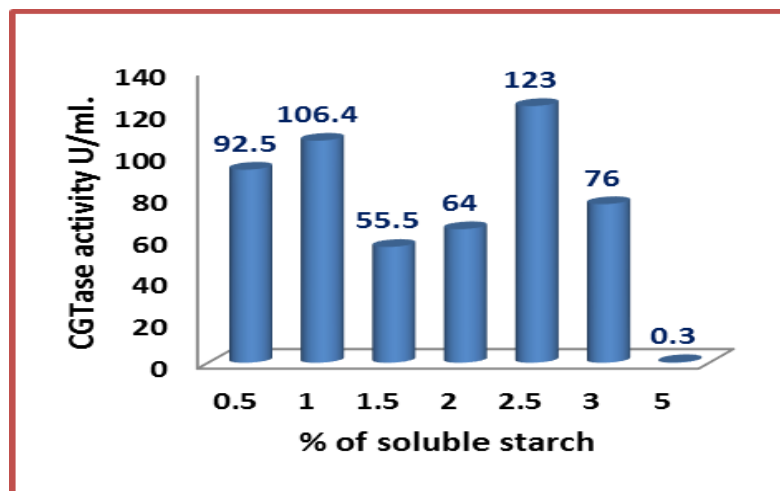


Fig.6 Influence of different nitrogen sources on CGTase production.

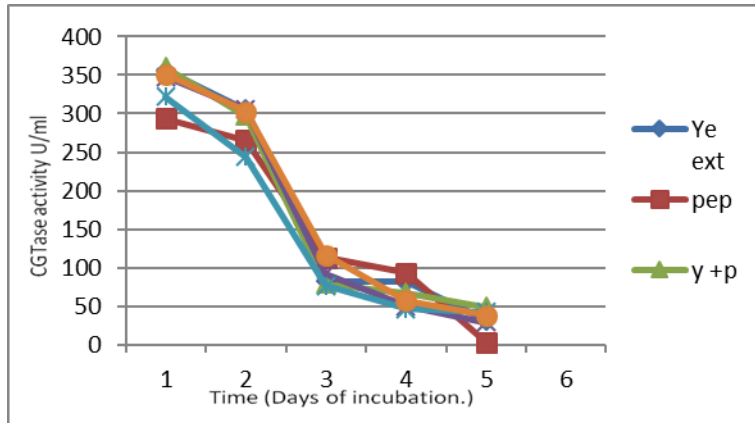


Fig.7 Influence of yeast extract and peptone concentration.

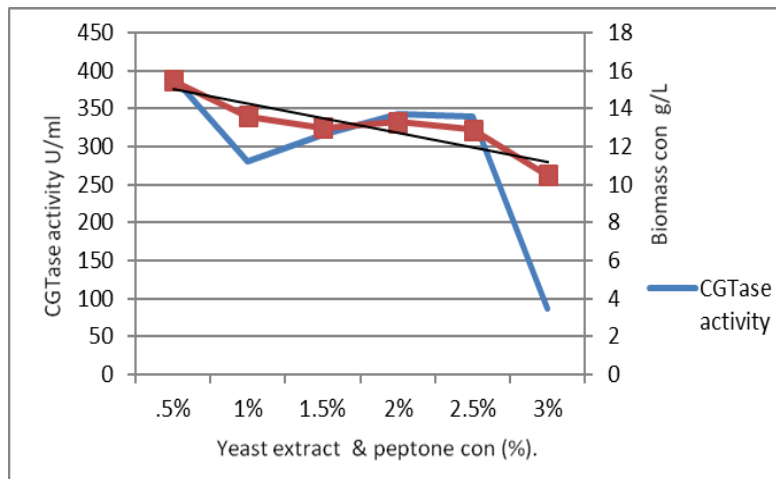


Fig.8 Influence of metal ions on CGTase production.

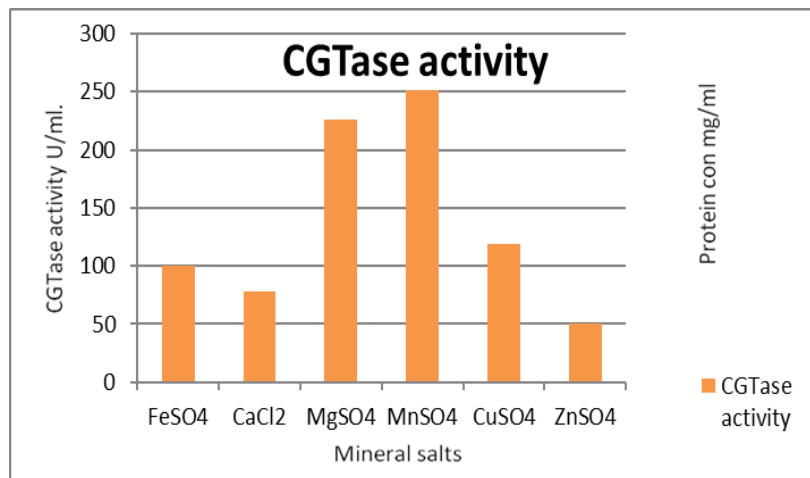


Fig.9 Impact of incubation temperature on CGTase production by *Leucobacter* sps.

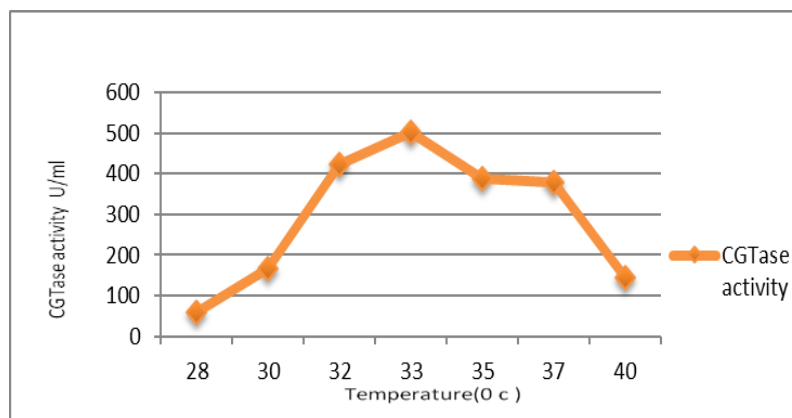
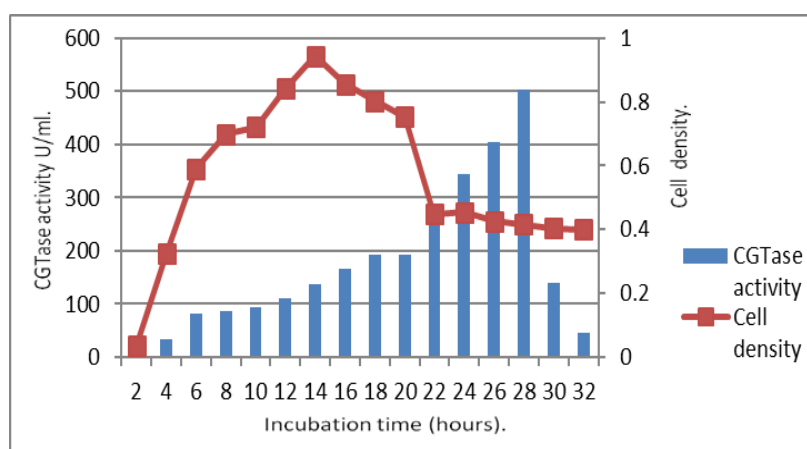


Fig.10 Growth and production profile of CGTase by *Leucobacter* species.



In contrast to the earlier reports (Freitas *et al.*, 2004) Mg²⁺ and Ca²⁺ showed little activation of CGTase activity. The effect of metal ions on enzyme activity seems to be depend on the enzyme source (Higuti.*et al.*, 2003 a). The induction of CGTase activity may vary with the nature of organisms (Sivakumar *et al.*, 2011).

Effect of Incubation Temperature on CGTase Production

In an attempt to understand the relation between production of CGTase and the influence of incubation temperature, isolated culture *Leucobacter* species was grown at different temperatures ranging from 28 °C to 40 °C. Figure 9 shows parabolic nature of

the enzyme production curve with increase in incubation temperature. Maximum enzyme production of 502.7 U/ml and a specific activity of 186 U/mg were noticed at a temperature of 33 °C. Variation of temperature in either side of this resulted in decrease of CGTase production. The loss of activity is more at the lower temperature when compared to higher temperatures.

Different ranges of optimum temperatures were reported for CGTase production. The optimum temperature ranges from 50 °C for *Bacillus firmis* (Moriwaki *et al.*, 2007), 70°C for *Bacillus amyloliquefaciens* (Yu *et al.*, 1988), 80°C for *Bacillus stearotherophilus* (Chung *et al.*, 1998)

and 85°C for Thermo anaerobacter (Tardioli *et al.*, 2006). In the present study it is 33 °C.

Growth Profile and CGTase Production by *Leucobacter* Species

The above optimized conditions were tested at various time intervals for the production of CGTase. Submerged fermentation was carried out using 2.5% (w/v) soluble starch as sole carbon source. The biomass of PK-8 species increased exponentially and reached its maximum value after 24 and 25 h of incubation (Fig: 10). The CGTase production was 550 U/ml when growth of microbes entered the late stationary phase. Maximum CGTase activity of 550U/ml with specific activity of 212 U/mg was attained after 27hr. with maximum biomass of 11.5 g/L. From figure10, it was observed that during 6 to 15 hours it follows a growth phase, later it attains a stationary phase from 16 to 20 hours of incubation. From 21 to 28 h enzyme activity increases and further incubation decreased the enzyme production. It may be due to the reduction of nutrients in the medium and formation of protease, which may degrade the CGTase. The results indicate the production of CGTase by the culture was non-growth associated, and maximum CGTase activity was observed during the late stationary phase.

Production of CGTase enzyme followed a similar enzyme production pattern (Fig: 10). The enzyme synthesis begins from the early exponential phase. However the maximum CGTase activity as measured by dextrinizing activity was obtained at 24 to 28 h of cultivation. From the literature CGTase production by *Bacillus cereus* was at its peak during the 16 to 20 hour of incubation period (Jamuna *et al.*, 1993). While the alkalophilic species *B. circulans* exhibits enzyme activity after 40 h of growth with a long lag period (Makela *et al.*,

1987). Production of CGTase was observed only at the end of the stationary phase for alkalophilic *Bacillus* sp. G1 (Tien S.F., 2001) and *Bacillus agar adherens* reaches stationary phase after 11 h of incubation with maximum enzyme production of 0.31U/ml after 25 h of cultivation (Rita *et al.*, 2002).

From the above comparisons, CGTase production by *Leucobacter* species is growth independent as the enzyme production was maximum at the late stationary phase of the cell growth. It is reported that extracellular CGTase are not involved in the degradation of starch in the exponential growth phase of bacteria, because they are usually released into the culture medium when all the starches has been consumed (Nogrady *et al.*, 1995). The enzyme has probably attached to the cell membrane during the exponential phase, it was retained between the cell membrane and the cell wall during the early stationary phase and was only released into the culture medium in the late stationary phase and during the cell lysis.

In conclusion, Optimization of production medium for CGTase synthesis by the newly isolated bacterial strain *Leucobacter* species was done by submerged fermentation method. Horikoshi medium II which resulted in a high yield of CGTase was further optimized for maximum production of CGTase by conventional method, by changing one independent variable, while fixing others at a certain level. From this study the optimum concentration of carbon and nitrogen sources were observed to be 2.5% (w/v) and 0.5% (w/v), respectively which lead to the highest CGTase activity of 360 U/ml. This showed an increment of 80% to the CGTase production after the optimization of CN ratio in the feeding medium compared to the initial production, which only gave 4.5 U/ml.

The final composition of the optimized medium to get maximum CGTase by newly isolated bacteria identified as *Leucobacter* species is consists of soluble starch 2.5%, yeast extract 0.5%, peptone 0.5%, K₂HPO₄ 0.2%, MgSO₄.7H₂O 0.01%, with incubation period of 28 h, incubation temperature 33 °C and pH 10.3 and agitation speed of 130 rpm. In this optimized medium and culture conditions, the production of CGTase was found to be 550 U/ml. The enzyme production was found to be non-growth associated and maximum production occurs during the late stationary phase of cell growth.

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