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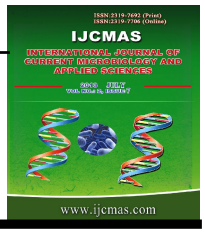

Original Research Article

Production of cyclodextrin glycosyl transferase by *Bacillus megaterium*

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A B S T R A C T

Keywords
Cyclodextrin; CGTase; *Bacillus megaterium*

Cyclodextrin glycosyl transferase (CGTase) fermentation was carried out using a *Bacillus megaterium* isolated from soil. Different fermentation parameters were tried to optimize the CGTase production. Among the carbon and nitrogen sources tried, maltose was found as the best carbon source and yeast extract was the best nitrogen source for CGTase production. Ca^{2+} influences the CGTase production and Zn^{2+} inhibits the enzyme. High CGTase activity was observed at 24 h of incubation in the medium with initial pH 9 and at temperature 27°C.

Introduction

Cyclodextrins (CD) are cyclic homogeneous oligosaccharides of glucose residues composed of 6-8 D-glucose units linked by α-1,4 glycosidic bonds synthesized from starch by the cyclization reaction of a bacterial enzyme cyclodextrin glycosyl transferase (CGTase E.C 2.4.1.19). CD molecules have a unique structure of hydrophobic cavity and hydrophilic surface. Because of this feature, CDs can be easily form inclusion complex with a wide variety of organic compounds (Gawande and Patkar, 2001; Mahat *et al*., 2004) and increasing their water solubility and stability. These properties made CDs as important materials as molecular encapsulator for applications in food, pharmaceutical, dairy and cosmetic industries (Volkova *et al.*, 2000). Some of the physical and chemical changes caused by complexation with CD are solubilisation of lipophilic compounds, stabilization of reactive compounds, removing bad smell and taste, fixation of volatile compounds and controlled release of compounds (Astray *et al*., 2009). In the typical process for CD production by CGTase the starch slurry is gelatinized by heat treatment and liquefied using CGTase or α-amylase and the liquefied starch is cyclized by CGTase to produce CD (Biwer *et al.*, 2002; Li *et al*., 2007). All known CGTase produce α, β and γ-CDs from starch in different ratios depending on the nature of CGTase and the reaction conditions.

CGTase producing bacteria can be found in various places such as soil, waste, plantation, hot springs and even in deep

sea mud. CGTase is produced by different types of bacteria. Major CGTase producers belong to the genus *Bacillus*. However, *Klebsiella pneumonia*, *Micrococcus luteus*, *Thermococcus*, *Brevibacterium* sp. and hyperthermophilic archaea are reported as CGTase producers (Szerman, 2007; Mori, 1999; Tachibana, 1999).

The consumption of CD is increasing worldwide at a higher rate (McCoy, 1999). Use of CDs has increased annually around 20-30%, of which 80-90% is used in food industries. In food industry, CDs are used to produce low-cholesterol butter, utilized for flavour stabilization and delivery in chewing gum, flavored tea, lemon and grapes fruit candies (Pszczola, 1998). Cyclodextrin is used in fruit juice beverages to mask vitamin odor and to mask bitterness in beverages. It is used to convert acetic acid to powder. It also increases the solubility of mustard sauce (Sivakumar and Banu, 2011). However, the cost of production is the limiting factor for the extensive industrial applications of CD (Biwer *et al.,* 2002). Hence many efforts have been made to improve the production of these cyclic oligosaccharides. Production of CGTase could be improved by manipulating fermentation conditions such as pH, temperature, concentrations of nutrients and compositions of the production media. Hence this study was taken with an objective to standardize the fermentation conditions of CGTase.

Materials and Methods

CGTase fermentation

B. megaterium isolated from soil was cultured on a medium containing (g/l) soluble starch 20, peptone 5, yeast extract 5, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.2, Na_2CO_3 10, phenolphthalein 0.3, methyl orange 0.1 and agar 20 and incubated at 37°C. Colonies with a clearing zone around them in the phenolphthalein containing media were identified CGTase positive. Colony with a prominent clearing zone was selected for further studies. Above medium without phenolphthalein, methyl orange and agar was used to prepare inoculum. 5% of the inoculum was transferred to the production medium (g/l: soluble starch 15, yeast extract4, KH_2PO_4- 1.5, $MgSO_4.7H_2O$- 0.1, $FeSO_4.7H_2O$- 0.1, 20% KCl- 12 and NaCl -9) and incubated at 37°C. Specific volume of fermented broth was collected at specific time intervals to analyze CGTase activity.

Assay of cyclizing activity of CGTase

The cyclizing activity of CGTase was determined using the phenolphthalein method (Goel and Nene, 1995) by measuring the production of CD on the basis of its ability to form a colourless inclusion complex. Cell free culture medium was centrifuged at 10000 rpm for 20 min at room temperature and supernatant containing cyclodextrin glycosyl transferase was collected. 5ml of 1% starch solution (0.1g of soluble starch, 1ml of 0.05mM $CaCl_2$ and water to make a total volume of 10 ml) and 5ml of the supernatant were mixed and incubated at 50°C. To measure the concentration of cyclodextrin, samples were collected periodically, inactivated at 100°C for 5 minutes. To 0.5ml of inactivated samples, 2.5ml of 3mM phenolphthalein solution (5ml of 0.6mM Na_2CO_3 buffer, pH 10.5 and the volume completed with 2.5ml of distilled water in a volumetric flask) was added. The absorbance of the final solution was measured in

spectrophotometer at 550nm. A unit of enzymatic activity was defined as the quantity of enzyme that produces one µmol of cyclodextrin per minute under standard conditions.

Effect of different factors on CGTase production

Factors like carbon source, nitrogen source, cations, temperature and pH affecting the production of CGTase were optimized by varying one factor at a time. The effect of temperature was studied by conducting fermentations at 27, 37 and 47°C. Similarly effect of initial pH was studied at pH 6, 7, 8 and 9. Different carbon sources like cassava, starch, maltose and glucose were tried as substrates to analyze their influence on CGTase production. Effect of nitrogen sources such as ammonium salts, tryptone, urea and yeast extract was studied by supplementing them in the production media. Effect of cation concentration on CGTase production was studied by the addition of $CaSO_4$, $MgSO_4$, $MnSO_4$, $FeSO_4$ and $ZnSO_4$ to the production media. The experiments were conducted in 500ml Erlenmeyer flasks. All the experiments were carried out in three sets with a control.

Statistical analysis

The mean values and standard deviations were calculated from the data obtained from three different experiments. Analysis of variance was performed by one way ANOVA procedures followed by Tukey HSD Post Hoc tests using PASW statistics 18. Statistical differences at $p < 0.05$ were considered to be significant. Coefficient of determination (R^2) was derived to determine the relationship between two variables.

Results and Discussion

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 factors such as carbon source, nitrogen source, cations, temperature and pH influencing the CGTase production were standardized by varying one parameter at a time.

Effect of carbon source

High cell density of *B. megaterium* was observed in cassava medium followed by starch, glucose and maltose containing media. Cell density reached a significant maximum level at 24h in all media except starch medium (Fig. 1). High CGTase activity was observed at 24h of incubation in maltose medium than starch medium (Fig. 2). Linear regression studies reveals that a reasonable coefficient exist between incubation and cell density in soluble starch medium (R^2=0.725). Relationship between incubation time and CGTase was high only in glucose medium (0.544). Relationship between cell density and CGTase in cassava medium (R^2=0.285) was higher than the other carbon sources (Fig.3). In general there was no high correlation between cell density and CGTase activity. Though different types of starch can be used as substrates, potato starch is the commonly used substrate for CD production. Maize and wheat starches were given low yield of CD because of its high amylose content (Szerman *et al*., 2007). Cassava starch is a good raw material because of its high amylopectin and low liquefaction temp (Fennema, 1996). Sago palm starch was also used as a substrate for CGTase production (Charoenlap *et al*., 2004).

Figure.1 Effect of different carbon sources on cell density of *B. megaterium*

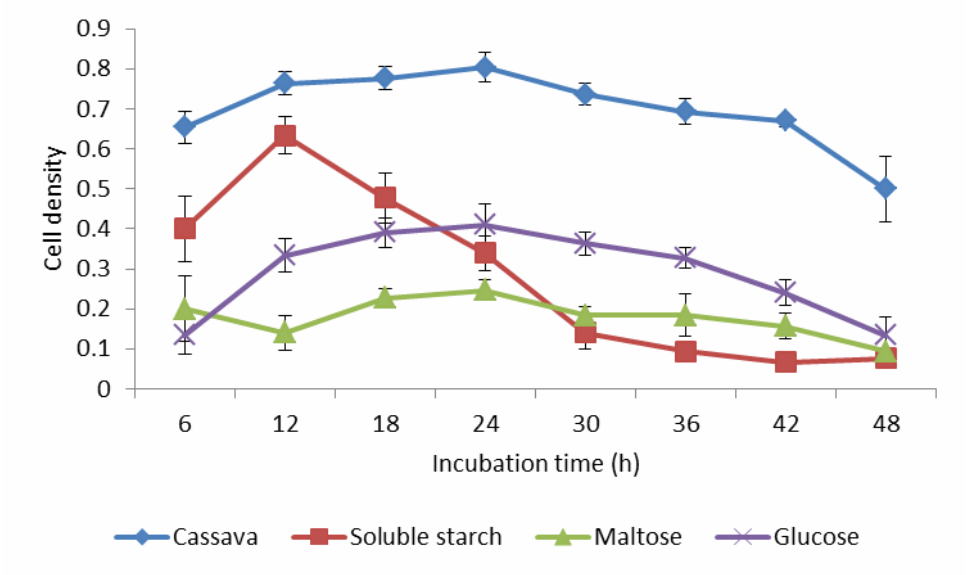


Figure.2 Effect of carbon sources on CGTase activity at different time intervals

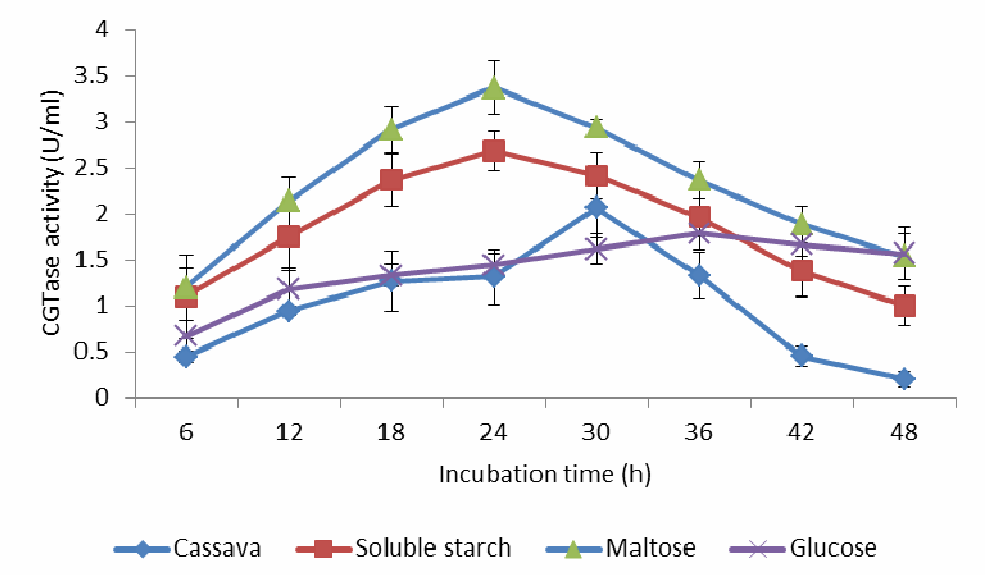
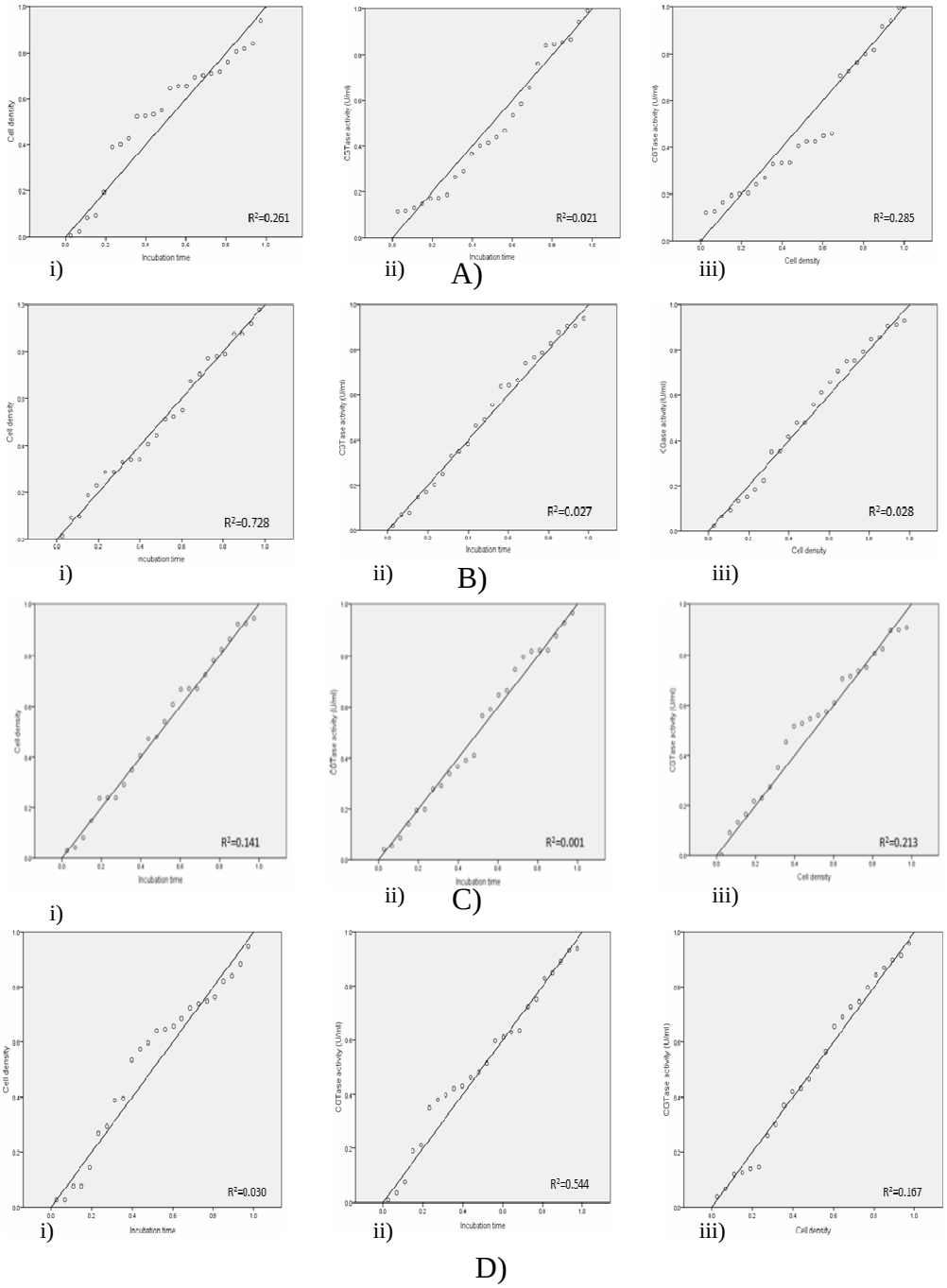


Figure.3 Coefficient of determination of i) incubation vs cell density ii) incubation vs CGTase activity and iii) cell density vs CGTase activity in different carbon sources A)cassava, B)soluble starch, C)maltose and D)glucose.



In this study, though cassava medium gave a good growth of *B. megaterium*, high CGTase activity was observed in maltose medium. In earlier studies different substrates were tried for CGTase production. Both hydrolysed and raw forms of different substrates such as maltodextrin, corn starch, potato starch and cassava starch were used as substrates for CGTase production. Corn starch was the best among the hydrolysed starches while cassava starch was the best substrate as raw starch. In contrast, for *Klebsiella pneumonia* As-22 corn starch was not a suitable substrate (Gawande and Patkar, 2001). It is known that physical treatment makes the starch susceptible to CGTase action, while raw starch is inaccessible to the enzyme (Gawande and Patkar, 2001). 3% soluble starch was the best substrate for CGTase production by *Bacillus* sp. TPR71H (Ravinder *et al*., 2012). On the other hand 0.1% of sago was found suitable for CGTase production by *Bacillus lehensis* S8 (Yap *et al*., 2010).

Effect of nitrogen source

Cell density was high in media with yeast extract and urea. Minimum cell density was observed in ammonium sulphate medium at 24 h of incubation. The cell density reached a maximum at 30 h of incubation in urea and yeast extract supplemented media (Fig. 4). High CGTase activity was found in yeast extract supplemented medium and low in tryptophan supplementation. Maximum CGTase activity reached at 24 h of incubation in all media except medium supplemented with ammonium sulphate (Fig. 5). The value of R^2 is low between incubation time and cell density, incubation time and CGTase in all nitrogen sources. The relationship between cell density and CGTase activity is generally higher than the media supplemented only with carbon source and a maximum value of coefficient of determination found in yeast extract medium (R^2=0.784) (Figure.6).

It was reported that the yeast extract (1%w/v) was the best substrate for the CGTase production but CGTase production went low when ammonium nitrate was used in the production medium (Yap *et al*., 2010). But Ravinder *et al* ., (2012) found that CGTase production was high in 0.5% yeast extract media indicates that yeast extract might have some inducer substance or micronutrients to increase the CGTase production.

Effect of cation

Effect of cations on CGTase production was carried out by the addition of $CaSO_4$, $MgSO_4$, $FeSO_4$ and $ZnSO_4$ to the production media. Maximum CGTase activity was observed at 24 h in $CaSO_4$, $MnSO_4$, $FeSO_4$ and $ZnSO_4$ supplemented media. But in $MgSO_4$ medium maximum activity was found at 30h. Addition of $CaSO_4$ increases 10% higher CGTase activity than the control. But FeSO4, $MnSO_4$ and $MgSO_4$ addition results in 81.3, 81 and 70.7% of CGTase activity respectively. CGTase activity was highly inhibited by Zn^{2+} (54.1%). So Ca^{2+} would be the best cation for the CGTase activity. Thatai *et al*., (1999) reported that Zn^{2+}, a known inhibitor of CGTase, enhanced the activity by 150% and the presence of $CaCl_2$ increased the production of CGTase marginally. Nevertheless, in this work also $CaCl_2$ seemed to increase the production of CGTase slightly. In most cases, calcium ions are required to stabilize CGTase at higher temperature where it will act as a

Figure.4 Effect of different nitrogen sources on cell density of *B. megaterium*

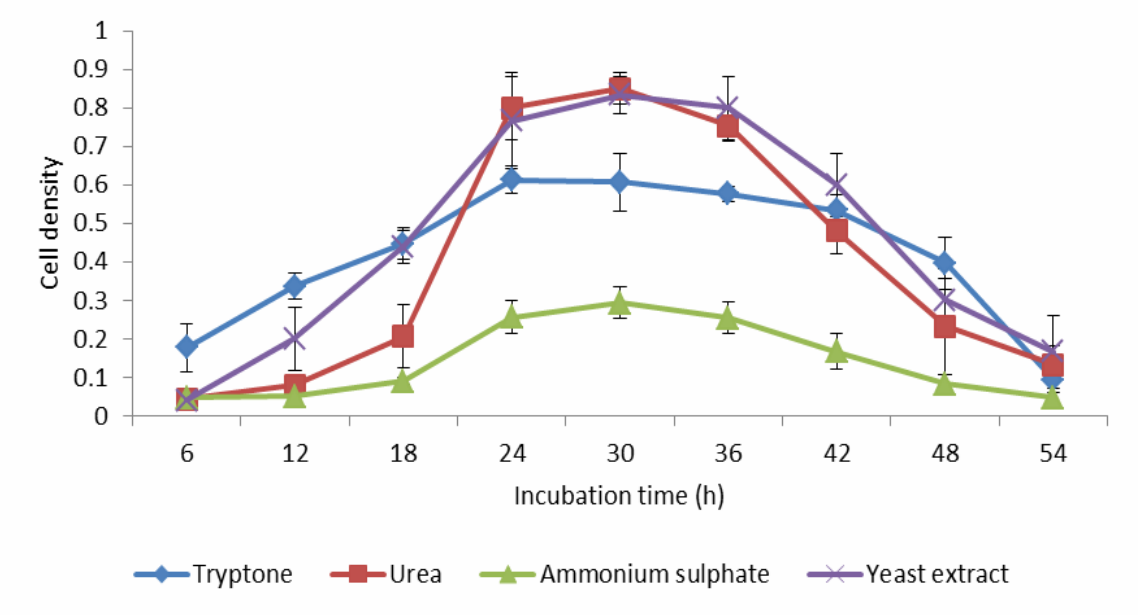


Figure. 5 Influence of different nitrogen sources on CGTase activity

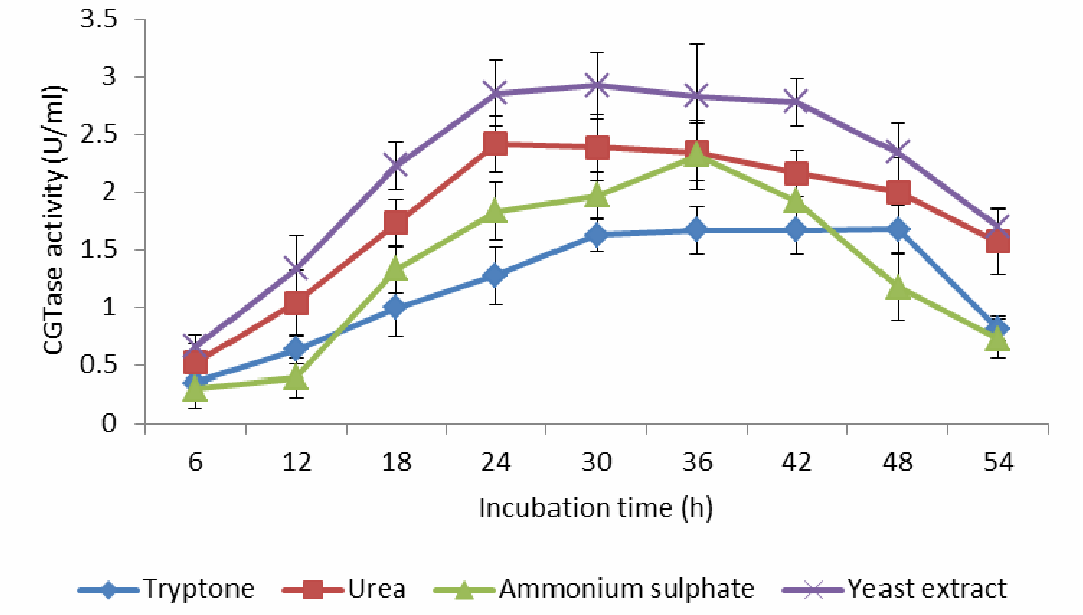


Figure.6 Coefficient of determination of i) incubation vs cell density ii) incubation vs CGTase activity and iii) cell density vs CGTase activity in different nitrogen sources A) tryptone, B) urea, C) ammonium sulphate and D) yeast extract.

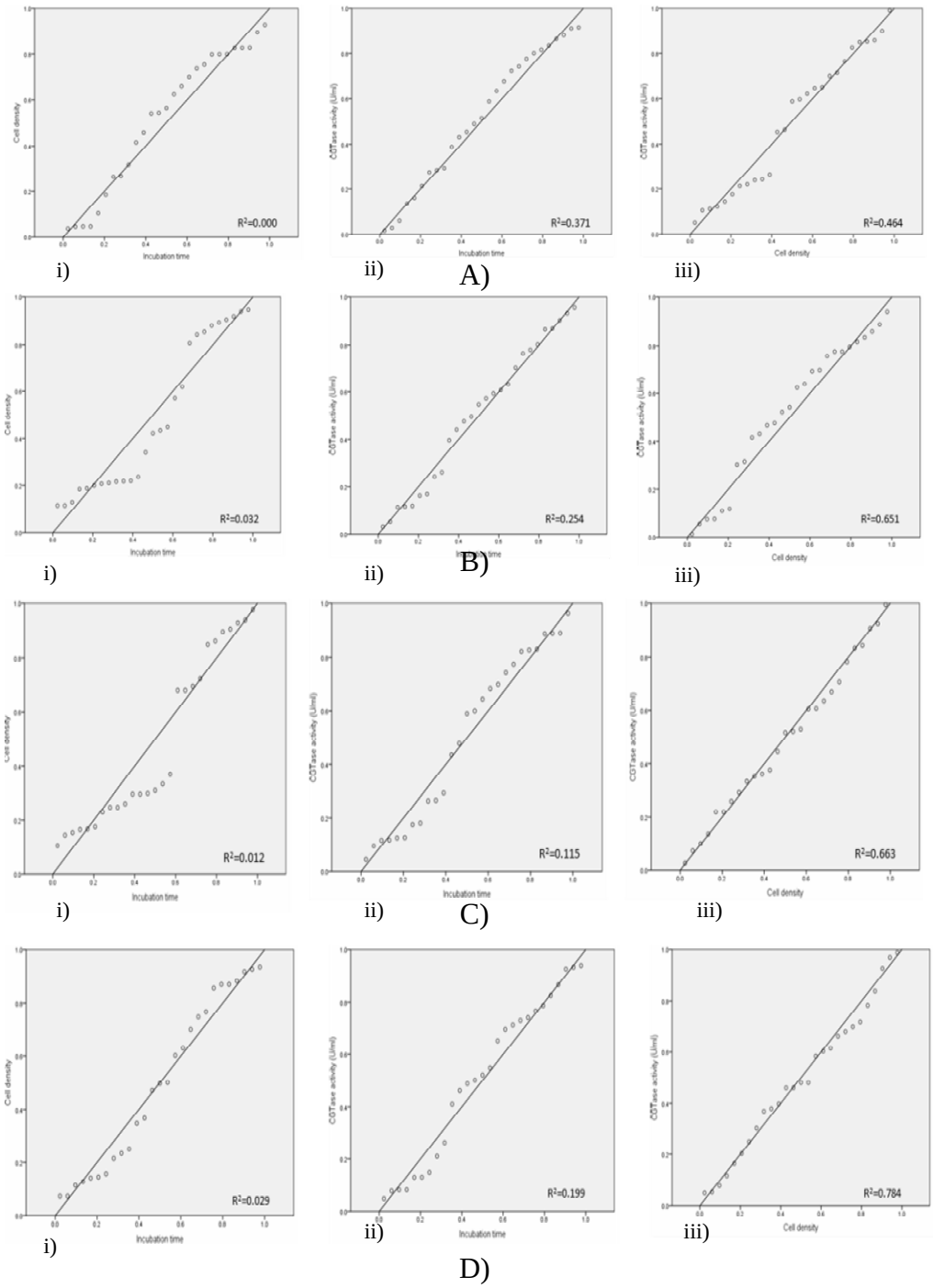


Figure.7 Effect of cation concentration on CGTase activity

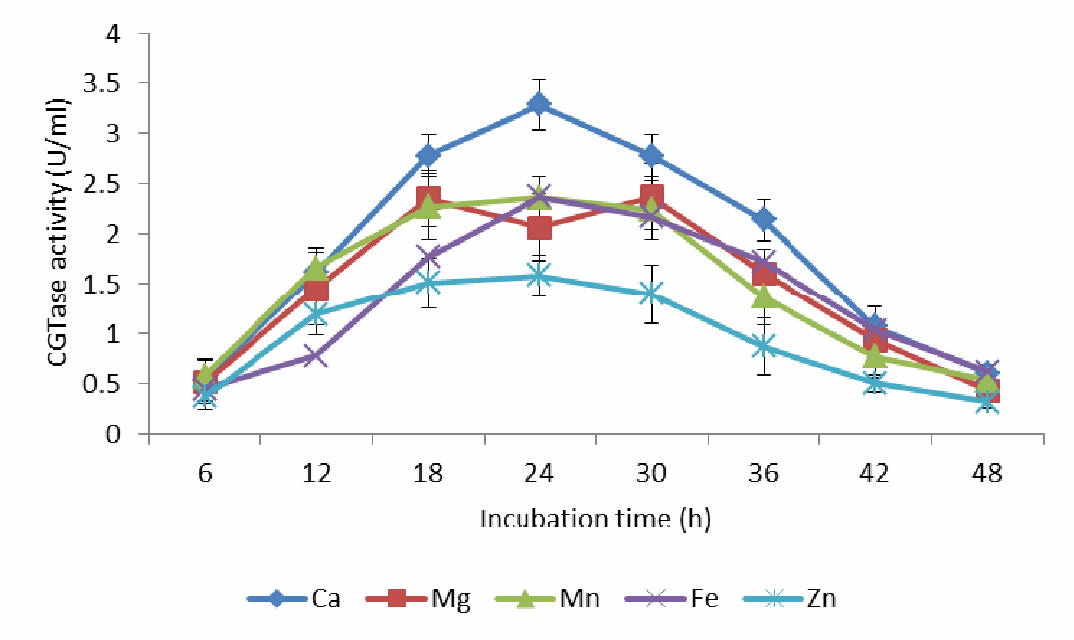


Figure.8 Effect of temperature on CGTase activity at different incubation time

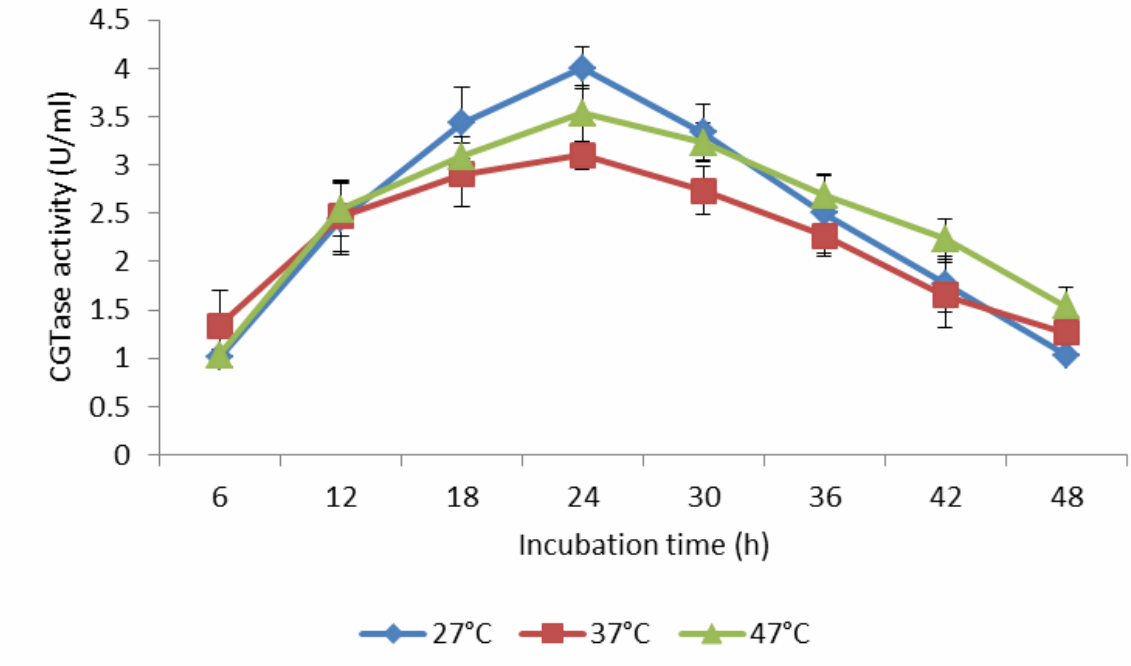
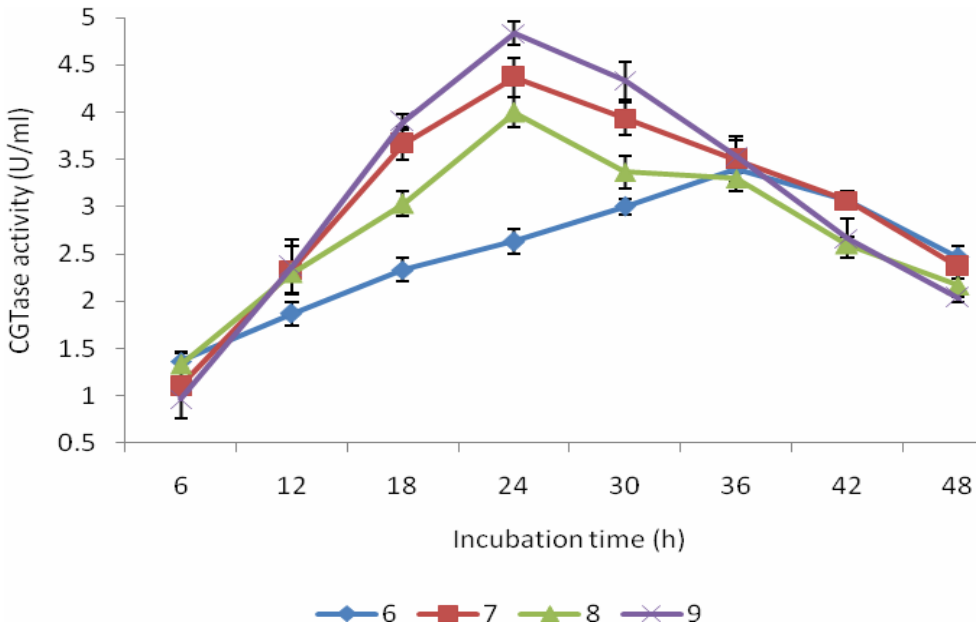


Figure.9 Effect of pH on CGTase activity at different incubation time



protective agent but are not involved in increasing the reaction rate of enzyme. However the concentration of $CaCl_2$ might affect the CGTase production and therefore a suitable concentration should be used if increase in activity is to be achieved. In contrast Freitas *et al.*, (2004) reported that Mg^{2+} and Ca^{2+} showed little activation on CGTase activity. The induction of CGTase activity by cations may vary with the nature of organisms (Sivakumar and Banu, 2011).

Effect of temperature and pH

CGTase activity was high at 24h in all the three tested temperature ranges. Activity of CGTase was high at 27°C than 37 and 47°C. Though it is *Bacillus*, it prefers low temperature for CGTase activity. Different range of optimum temperature was reported for CGTase. The optimum temperature ranges from 50° for *B. firmis* (Moriwaki *et al*., 2007), 70°C for *B.amyloliquefaciens* (Yu et al., 1998), 80°C for *B. stearothermophilus* (Chung et al., 1998) and 85°C for *Thermoanaerobacter* (Tardioli *et al*., 2006). The pH value maintained at the beginning of fermentation is important for product and biomass formation. Maximum CGTase activity was observed at pH9 at 24 h of incubation.

Neutral and slightly acidic pH decreases the enzyme activity indicates that the CGTase prefers slightly alkaline pH for its optimum activity. The optimum pH for CGTase activity of *Bacillus firmis* 7B was 6. The optimum pH varies depending on the nature of microorganism producing CGTase (Moriwaki *et al*., 2007). The reported range of optimum pH is quite wide for CGTase, varying from 4 (Yu *et al.*, 1988) to 12 (Horikoshi *et al.*, 1996).

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