International Journal of Advanced Biotechnology and Research
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 3, Issue 2, 2012, pp 578-584
http://www.bipublication.com

OPTIMIZATION OF PROCESS PARAMETERS FOR THE PRODUCTION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE BY NEWLY ISOLATED BACILLUS SP. TPR71H BY CONVENTIONAL METHOD

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[Received-02/06/2012, Accepted-26/06/2012]

ABSTRACT

The extracellular enzyme cyclodextrin glucanotransferase (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. In addition, CGTase production is highly dependent on the strain, medium composition and culture conditions. Therefore we undertook this study with a newly isolated strain of *Bacillus* sp. TPR71H. CGTase activity produced from *Bacillus* sp. TPR71H was optimised in shake flasks using conventional method. Effects of nutrients, including several carbon, nitrogen and mineral sources, and environmental conditions like pH, temperature were assayed. The selected minimal medium consisted of 1.0 % starch, 0.5 % yeast extract, 0.5 % peptone, 0.1 % disodium hydrogen phosphate, 0.02 % magnesium sulphate and distilled water. The process parameters were optimized by changing one independent variable, while fixing the others at a certain level. Maximum CGTase activity obtained in supernatants was 30.34 U/mL. In this study we have screened conditions for optimal CGTase production by newly isolate bacillus sp. TPR71H. From this study we found that the optimized parameters for the production of CGTase by *Bacillus* sp. TPR71H were Soluble Starch 3%, Yeast Extract 0.5%, K_2HPO_4 0.1%, Inoculum Level 3.5%, Inoculum Age 24h, Incubation Period 36h, rpm 220, Incubation Temperature 32°C and the pH of 7.5.

Key words: CGTase, Optimization, Conventional method, nutritional parameters, environmental parameters.

INTRODUCTION

Screening of appropriate carbon, nitrogen and mineral sources including additional nutrients is one of the most critical stages in the development of an efficient and economic fermentation process [1]. Yield of any microbial

fermentation product can be improved by optimizing the environmental conditions, nutrients and by genetic manipulation of the strain. The methodologies used for screening and optimization of the various fermentation factors fall in to two major categories, those which are classical and statistical [2]. The present study was conducted to improve the production of CGTase by optimizing the various parameters involved in the production using classical (conventional) method. These were optimized by changing one independent variable, while fixing the others at a certain level [3,4].

The medium I described in our earlier finding (5 IJPPS) was taken as a basal medium and it was further optimized to achieve higher yield of CGTase by the isolated *Bacillus* sp. TPR71H. The different process parameters including pH, temperature, inoculum concentration and age of inoculums were optimized. Various nutritional parameters such as nitrogen sources like peptone, yeast extract, ammonium sulphate and sodium nitrite; additional carbon sources like lactose, glucose, soluble starch, galactose, maltose and sucrose were optimized by conventional method i.e., changing one independent variable while fixing all others at fixed levels. Finally, the time course of production was evaluated under the optimized conditions.

MATERIALS AND METHODS

Chemicals and media

All the chemicals used in this study were of analytical grade. Media constituents used in this study were procured from Hi-Media, Mumbai.

Inoculum/ Production medium

Medium for the production of cyclodextrin glycosyltransferase contains the following ingredients soluble starch (1.0%), Yeast extract (0.5%), Peptone (0.5%), Na_2HPO_4 (0.1%), $MgSO_4$ (0.02%), Distilled water up to 100mL and the pH is maintained at 7.0 [6].

Preparation of Inoculum

For the preparation of inoculum, the growth contents of two days old slant culture was suspended into 5mL of sterile distilled water and transferred into 45mL of inoculum medium contained in 250mL Ehrlenmeyer flask. The flask was incubated on a rotary shaker at 30ºC for 24h. 1% of this suspension was transferred to production medium.

Preparation of Production medium

1% (0.5mL) of the above inoculum medium was transferred aseptically to 49.5mL of production medium. The flasks were kept on the rotary shaker at 30ºC. The samples were withdrawn for every 12h up to 72h and centrifuged at 10,000rpm for 10min in order to remove the cells and other insoluble materials. The clear supernatant was used as a crude enzyme for estimation.

Assay of CGTase

Assay of CGTase was carried out according to the method of Kaneko *et al.,* 1987 [7]. The amount of β-cyclodextrin produced was estimated from the standard graph of 0-500μg/mL β-CD concentration against absorbance. One unit of CGTase was defined as the amount of enzyme required to produce 1μmol of β-CD/min. All the experiments were conducted in triplicate and the mean values were calculated.

The reaction mixture containing 1mL of 40mg of soluble starch in 0.1M potassium phosphate buffer (pH 6.0) and 0.1mL of the crude enzyme from the culture and the mixture was incubated in water bath at 60°C for 10 min. The reaction was stopped with 3.5mL of 30mM NaOH. Finally, 0.5mL of 0.02% (w/v) phenolphthalein in 5mM Na_2CO_3 was added and mixed well. After leaving the mixture to stand for 15min at room temperature, the reduction in colour intensity was measured at 550nm. A blank lacking the enzyme is tested simultaneously with each batch of samples.

Optimization of process parameters

Effect of initial pH on CGTase production

In order to understand the influence of initial pH of the medium on CGTase production the medium was adjusted to pH 5.5 to 9.5 with 1N HCl and 1N NaOH. The flasks were inoculated with the 1mL of 24h old culture and kept for incubation at 30ºC for 48h. The samples were withdrawn and analysed for the CGTase activity.

Effect of incubation temperature on CGTase production

The influence of incubation temperature on CGTase production by the isolated bacteria was investigated by incubating the medium in various temperature ranges from 20 to 40ºC.

Effect of inoculum size and age on CGTase production

To study the influence of inoculum size on enzyme production the medium flasks were incubated with various concentrations of inoculum from 0.5 to 3.5mL of 24h culture. To investigate the effect of age of inoculum 18 to 48h old culture was inoculated in the media. After incubation, the samples were withdrawn and estimated for CGTase activity.

Effect of various carbon and nitrogen source on CGTase production:

In order to study the best suitable carbon source for effective CGTase production by the isolated *Bacillus* sp TPR71H various carbon compounds viz soluble starch, xylose, lactose, maltose, sucrose, glucose, mannitol, fructose and galactose were studied at 1%. While in case of nitrogen source, various organic and inorganic nitrogen compounds such as peptone, casein, yeast extract, malt extract, ammonium chloride, ammonium sulphate, ammonium phosphate and sodium nitrate were investigated at 0.5%. Further the best suitable compounds were studied at various concentrations.

Effect of incubation time on CGTase production

Finally, experiments were run under the optimized conditions obtained from the above experiments. The flasks were incubated upto 72h and the samples were withdrawn periodically for every 12h and analysed for the CGTase production.

All experiments were conducted in triplicate and the mean values were considered.

RESULTS AND DISCUSSION

Effect of initial pH on CGTase production

The medium pH plays a vital role in the production of extracellular enzymes. pH influences the growth of the micro-organism as well as secretion of the enzymes from cells to medium. In order to investigate the best suitable pH for CGTase production by the isolated *Bacillus* sp. TPR71H, the medium pH was adjusted from 5.5 to 9.0. Fig 1 shows the effect of pH on CGTase production by the isolated bacteria. The pH profiles shows a bell shape curve, indicating that at narrow pH range the organism is able to produce the higher yields of CGTase. pH 7.5 was found to be optimum for the enzyme production (13.72U/Ml). Above and below this pH, a decreased production of enzyme activity was observed. Initially, the loss of activity under alkaline conditions is low when compared to the acidic concentration. After pH 8 the loss of enzyme activity is high at pH 9.0 and 70 % decrease in enzyme production was observed.

Effect of incubation temperature on CGTase production

Incubation temperature dependent variation in CGTase production was reported in several bacterial species. Keeping this in view, experiments were performed to understand the effect of incubation temperature on CGTase production by the isolated bacterial strain by incubating the fermentation medium at different temperatures ranging from 20 to 40ºC. Figure2 shows that the incubation temperature regulated CGTase production by *Bacillus* sp TPR71H. Parabolic nature of enzyme production curve was noticed with increase in incubation temperature. Maximum enzyme production of 14.5U/ mL was noticed at a temperature of 32^{o}C. Variation of the temperature in either side of this resulted in decrease of CGTase production in cell free broth. The loss of activity is more at the lower temperature when compared to the higher temperatures.

Effect of inoculum size and age on CGTase production

The amount of initial biomass controls the kinetics of growth and several biological metabolic functions leading to the overall biomass and extracellular enzyme production. To study the same, experiments were planned with increasing inoculum concentration from 0.5 to 3.5% and the CGTase activity was monitored. The results indicated that, CGTase production varied with variation in initial inoculum level

(Fig 3). The maximum enzyme production (16.18U/mL) was observed in 3.5% initial inoculum supplemented conditions.

Inoculum of various age periods (18, 24, 30, 36, 42 and 48h) was employed for CGTase production. The optimum inoculum age achieved by this step was 24h with the CGTase production of 16.58U/mL and the results are presented in the figure 4.

Effect of various carbon sources on CGTase production

In submerged fermentation process, the selection of a suitable carbon source for a fermentation process is a critical factor and thus involves the screening of a number of materials for microbial growth and product formation. The carbon source was known to be the determining factor in the rate of CGTase synthesis [8]. The selection was done using industrial grade carbon sources.

In the present study nine substrates like soluble starch, glucose, lactose, maltose, sucrose, galactose, xylose, fructose and mannitol were used for growth and CGTase production by *Bacillus* sp. TPR71H. All the substrates supported growth and CGTase formation by the culture. Soluble starch proved superior to other substrates with the production of 17.27U/mL (Fig 5). Further, medium containing soluble starch alone at different concentrations were tested for CGTase production. A high CGTase titre of 23.65U/mL was obtained in a medium containing soluble starch of 3% alone as a substrate. The results presented in figure 6.

Effect of various nitrogen sources on CGTase production

For the present study eight nitrogen sources like peptone, casein, yeast extract, ammonium chloride, ammonium sulphate, ammonium phosphate, malt extract and sodium nitrite were used for growth and CGTase production by *Bacillus* sp. TPR71H. All the substrates supported growth and CGTase formation by the culture. Yeast extract proved superior to other substrates with the production of 27.64U/mL (Fig 7). Peptone was found to be best nitrogen source next to the yeast extract. The equal amounts of yeast extract and peptone in the medium resulted 24.52U/mL CGTase production which is lesser than the yeast extract alone. Further studies were conducted with the yeast extract alone as a nitrogen source. Medium containing yeast extract alone at different concentrations were tested for CGTase production. A high CGTase titre of 27.81U/mL was obtained in a medium containing yeast extract of 0.5% alone as a substrate. The results were presented in Fig 8.

Effect of various mineral sources on CGTase production

Different types of mineral sources were used to evaluate their influence on CGTase production by *Bacillus* sp. TPR71H strain. In the present study eight mineral sources viz magnesium chloride, sodium chloride, potassium dihydrogen phosphate, potassium phosphate, zinc sulphate, ferrous sulphate, magnesium sulphate and calcium carbonate were tested for effective CGTase production by *Bacillus* sp. TPR71H. All the substrates supported growth and CGTase formation by the culture. Dipotassium phosphate proved superior to other substrates with the production of 30.59U/mL (Fig 9).

Effect of incubation period on CGTase production

The above optimized conditions were tested at the various time intervals for the production of the CGTase. The samples were withdrawn for every 12h and estimated for the enzyme activity. From the figure 10 it was observed that during 12 to 36h, it follows the growth phase, later it attains the stationary phase. At 36h the maximum CGTase production (30.34U/mL) was observed. Further incubation, decreases the enzyme production, it may be due to reduction of nutrients in the medium and formation of protease. The results were presented in figure 10.

CONCLUSION

The optimal production medium for CGTase production by the newly isolated bacterial strain TPR71H was done by submerged fermentation method. For this procedure, we used medium number I which gave the best as it was resulting in a high yield of CGTase volume (9.66U/mL).

This production medium was optimized by conventional method to improve the production of CGTase. These were optimized by changing one independent variable, while fixing the others at a certain level. From this study we found that the optimized parameters for the production of CGTase by *Bacillus* sp. TPR71H were Soluble Starch 3%, Yeast Extract 0.5%, K_2HPO_4 0.1%, Inoculum Level 3.5%, Inoculum Age 24h, Incubation Period 36h, rpm 220, Incubation Temperature 32°C and the pH of 7.5. In this optimized medium the production of CGTase was found to be 30.34 U/mL.

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Figures:

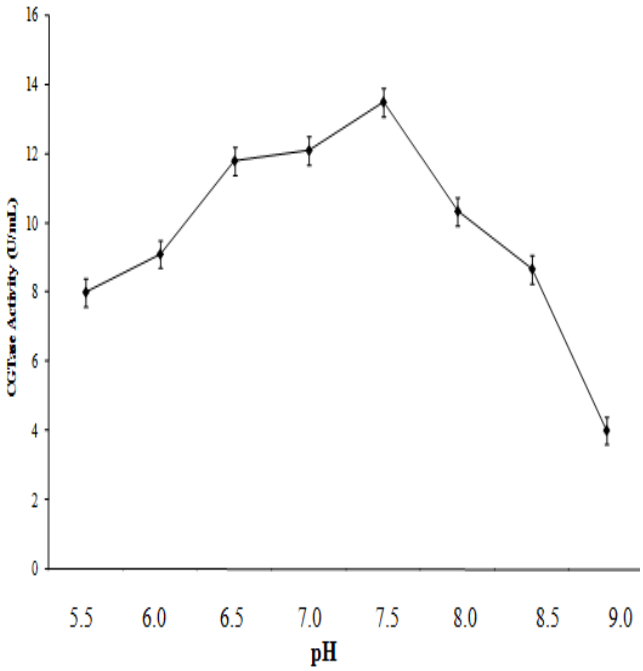

Fig 1. Effect of initial pH on CGTase production by isolated *Bacillus* sp TPR71H

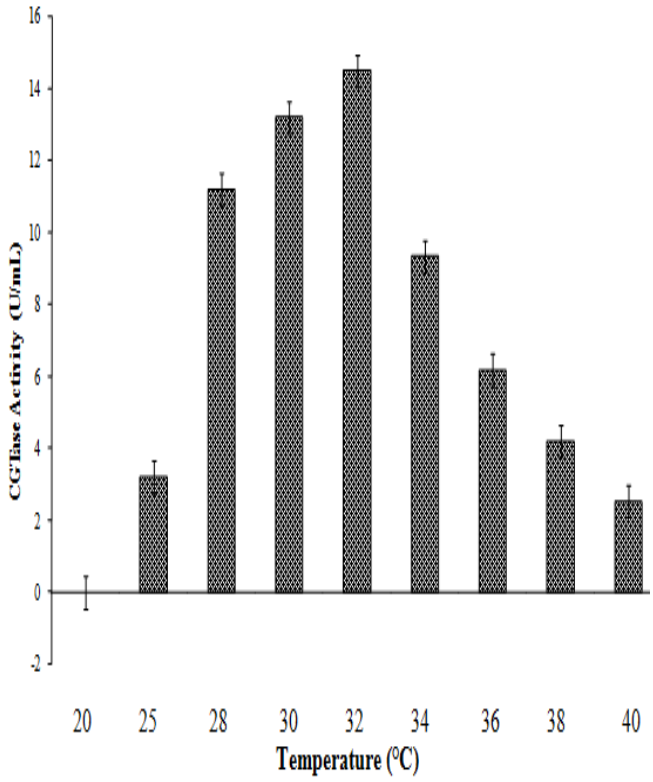

Fig 2. Effect of incubation temperature on CGTase production by isolated *Bacillus* sp TPR71H

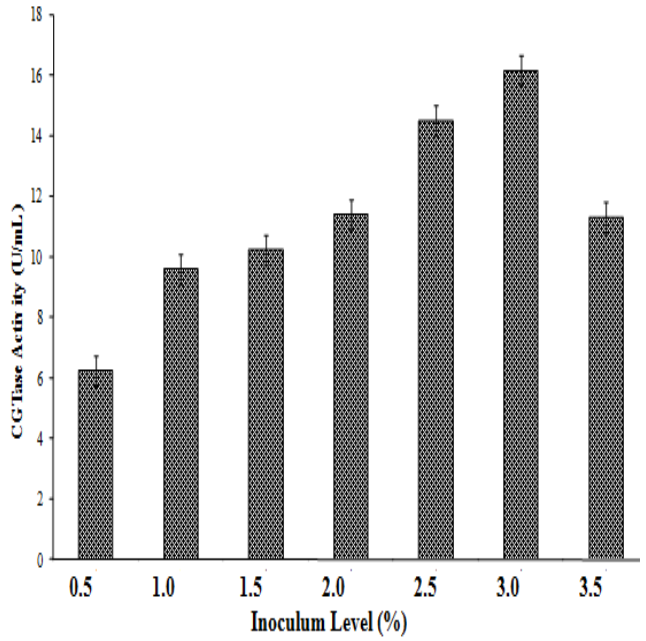

Fig 3. Effect of inoculum Level on CGTase production by isolated *Bacillus* sp TPR71H

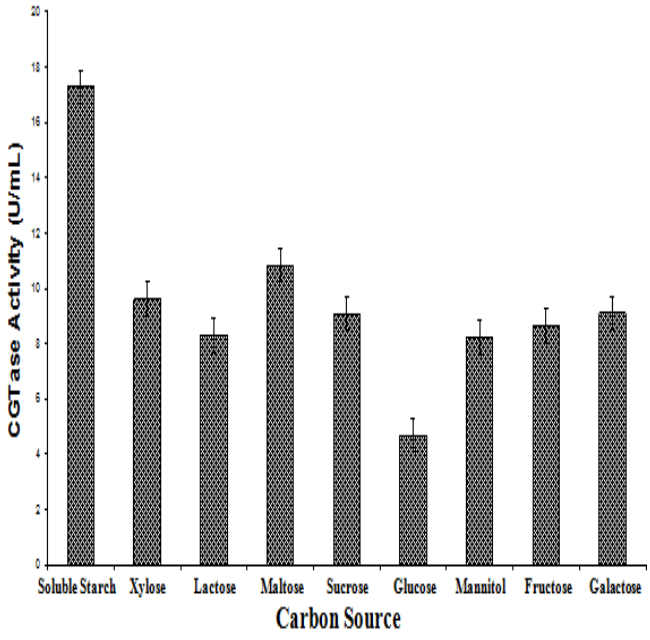

Fig 5. Effect of carbon sources on CGTase production by isolated *Bacillus* sp TPR71H

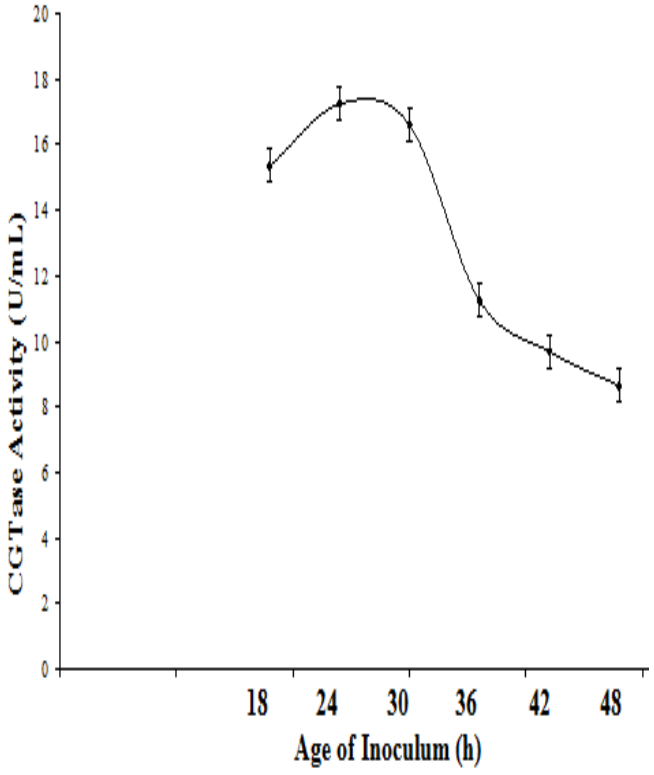

Fig 4. Influence of inoculum age on CGTase production by isolated *Bacillus* sp TPR71H

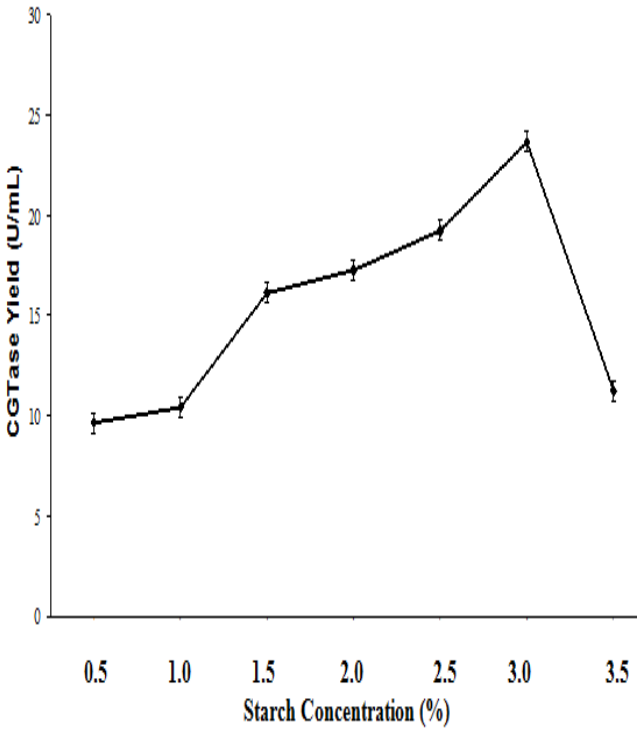

Fig 6. Effect of starch concentration on CGTase production by isolated *Bacillus* sp TPR71H

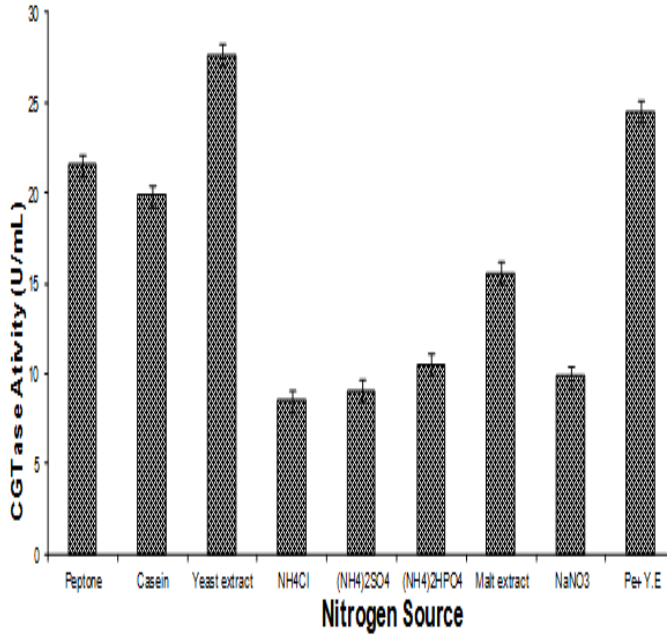

Fig 7. Effect of various nitrogen sources on CGTase production by isolated *Bacillus* sp TPR71H

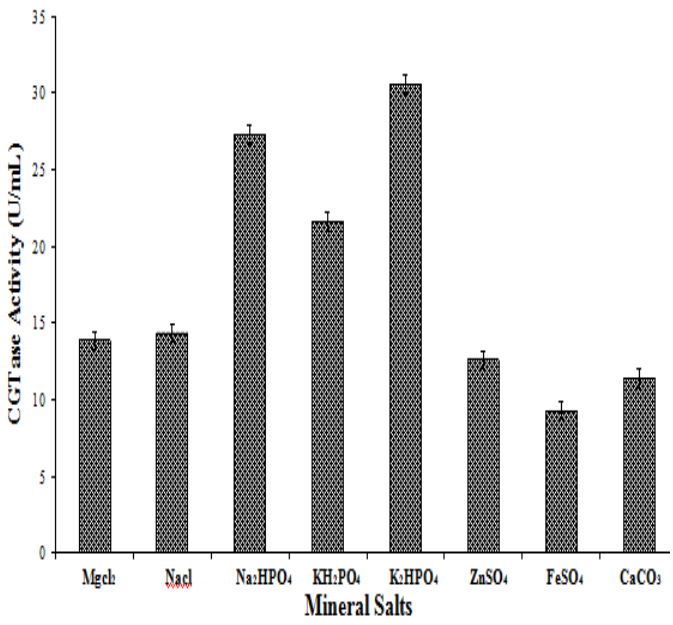

Fig 9. Effect of various mineral salts on CGTase production by isolated *Bacillus* sp TPR71H

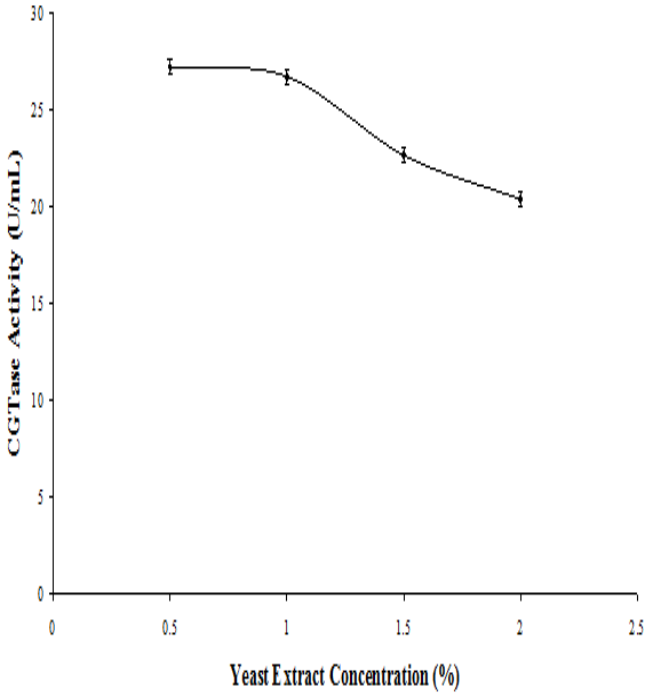

Fig 8. Effect of yeast extract concentration on CGTase production by isolated *Bacillus* sp TPR71H

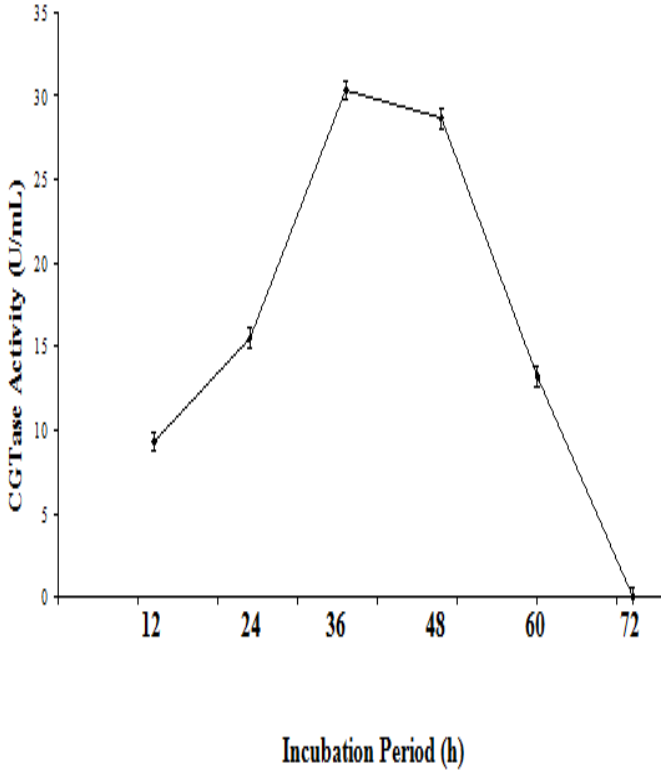

Fig 10. Effect of incubation period on CGTase production by isolated *Bacillus* sp TPR71H.