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Full Length Research Paper

Biochemical characterization of thermostable cellulase enzyme from mesophilic strains of actinomycete

Khwaja Salahuddin[1]*, Ram Prasad[2], Suresh H. Gor[1], Manish D. Visavadia[3], Virendra K. Soni[3] and M. Dilshad Hussain[4]

[1]Department of Botany, Bahauddin Science College, Junagadh-362001, Gujarat, India.
[2]Amity Institute of Microbial Technology, Amity University Uttar Pradesh, Noida-201303, UP, India.
[4]Department of Zoology, Bahauddin Science college, Junagadh-362001, Gujarat, India.
School of Studies in Zoology and Biotechnology,Vikram University,Ujjain-456010, India.

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A few mesophilic strains of actinomycete were used for detection, extraction and characterization of cellulase enzymes. These strains responded to produce all the three components of cellulase complex (endoglucanase, exoglucanase and β-glucosidase) in balanced quantities. Cellulase activity was determined on solid medium supplemented with 1% carboxy methyl cellulose (CMC). Production of cellulase was detected by the formation of clear or transparent zone around colonies. The greater size of transparent zone has been found proportional to the higher capabilities of the strains for enzymes. The extraction of cellulase enzyme was done in liquid basal medium. The assay of cellulase was observed by measuring the release of reducing sugar (RS) by DNS method. All the three components of cellulase viz. endoglucanase, exoglucanase and β -glucosidase were assayed in terms of CMCase, FPase and cellobiase, respectively and expressed in International units (IU). These strains were further tested for their ability to produce cellulase complex enzyme by growing on a defined substrates as well as on delignified cellulosics. The optimization for β-glucosidase enzyme was carried out by studying the various parameters viz. effect of pH, incubation period and nitrogen sources.

Key words: Cellulase, actinomycete, optimization, reducing sugar, carboxy methyl cellulose.

INTRODUCTION

Cellulose is the most abundant organic compound in the world and consists of glucose units linked by β-1,4-glycosidic bonds in a linear mode. Microbial enzymes are preferred and present a wide spectrum of characteristics that enable them indispensable for quite specific applications (Boing, 1999). Actinomycetes constitute a formidable group of industrially important microorganisms that have been explored for the production of thermo stable enzymes. Cellulase is widely used for a variety of novel biotechnological applications such as food, wine, agriculture, textile, detergent, animal feed, pulp and paper industries, as well as research and development (Ryu and Mandel, 1980; Bhat, 2000). The demand for more stable enzymes in many industrial applications is growing rapidly today. For instances, as the process of composting agricultural waste progresses, thermophilic and mesophilic microbes decompose cellulose and lignin (Thambirajah et al., 1995). Many mesophilic and thermophilic bacteria and fungi have been investigated with respect to the bioconversion of agricultural and forest biomass into fuels and valuable chemicals (Wang et al., 1993; Wittmann et al., 1994).

In the current industrial processes, cellulolytic enzymes are employed in detergents, causing colour brightening and softening, stoning of jeans, pre-treatment of cellulosic biomass to improve nutritional quality of the forage and in the pre-treatment of industrial wastes (Van wyk et al., 2001; Zhou et al., 2001; Jang and Chen, 2003). Cellulases have also been considered to play a

*Corresponding author. E-mail: khwajasalahuddin@yahoo.com or Salahuddin212@gmail.com. Tel: +91 09067721514. Fax: +91 285 2675731.

critical role in the generation of potentially sustainable energy sources such as glucose, ethanol, hydrogen and methane (Bayer et al., 2007).

Apart from actinomycete, other microorganisms such as *Cellulomonas, Cytophaga, Clostridium, Corynebacterium, Polyanguim*, and *Geobacillus* have been found capable of secreting cellulase enzyme (Gilker et al., 1991; Grange et al., 2010).

Among the fungi, *Trichoderma viridiae* (*Trichoderma reisei*) and *Aspergillus niger* are the potent sources. Cellulase enzymes required for the hydrolysis of cellulose include endoglucanase or 1,4-β-D-glucan-4-glucano-hydrolases (EC 3.2.1.4), exoglucanases (including 1,4-D-glucan glucanohydrolases (EC 3.2.1.74) and 1,4-β-D glucan cellobiohydrolases (EC 3.2.1.91) and β-glucosidases or β- glucoside glucohydrolases (EC 3.2.1.21) (Matsui et al., 2000; Lee et al., 2002). The co-operative action of all the three components is required for the complete hydrolysis of cellulose to glucose. The microbial decomposition of cellulose has been studied extensively since, the resulting product, glucose, is a material easily utilized by various microorganisms in fermentation industry (Bisaria and Ghosh, 1981; Bequin and Aubert, 1994).

The complete conversion of cellulose to glucose employing cellulase enzyme has been hampered by low yield and high cost of microbial enzymes. Yield of any microbial enzyme can be improved in two ways either by search of newly active producers in nature or improving the wild type strain. The strain improvement can be performed by different methods such as mutagenesis, recombination and genetic engineering. Cellulase is an extra cellular enzyme and was found to be thermo stable in nature.

Its thermo stability can be increased by pH, temperature, oxidizing agents, organic solvents, substrate or ions so it has greater importance in industries (Harchand and Singh, 1997). Thus, the study was carried out to investigate the capabilities of the mesosphilic strains of actinomycete to produce cellulase enzyme at higher temperature on different substrates as well as to characterize by optimizing the growth conditions.

MATERIALS AND METHODS

Microorganism

Soil is the most favorable hub for the microbial population especially the actinomycete. Soil samples were collected from rhizoplane of herbaceous plants of Muzaffarpur district, Bihar, India. The mesophilic strains of actinomycete were isolated on different media by using serial dilution technique. Czapek-Dox-Agar medium ($NaNO_3$, 2.0 g; K_2HPO_4, 1.0 g; $MgSO_4$, 0.5 g; KCl, 0.5 g; $FeSo_4.7H_2O$, 0.001 g; sucrose, 30 g; agar, 15.0 g and distilled water, 1 L) was found to be exclusive medium for the luxuriant growth of actinomycete. On the basis of cultural characteristics, two mesophilic strains such as KS-22 and KS-44 were singled out and subjected to higher temperature (45°C) for further investigation.

Detection of enzyme

Firstly, Czapek-Dox agar medium containing carboxy methyl cellulose (CMC) as carbon source was prepared and subjected to autoclaving at 121°C for 15 min. The sterilized medium was poured on sterile petri plate and allowed to solidify. Later, the microorganism was inoculated on petri plate. Then the inoculated plate was subjected to incubation at 45°C in an inverted position for 3 to 5 days. The plates were flooded with 1% aqueous solution of hexadecyltrimethyl ammonium bromide. This reagent precipitated intact CMC in medium. Now the clear or transparent zones around the colonies of actinomycete were formed which indicated the degradation of CMC by the production of cellulase enzyme (Plumns, 1993). The same procedure was done at 45°C and enzyme was detected at this temperature proven enough to be thermo stable.

Extraction of enzyme

Cellulase enzyme was extracted in crude form. For this, 100 ml of Czapek Dox broth containing 1% CMC was prepared. The broth was sterilized at 121°C for 15 min. 5 ml of bacterial spore suspension containing 1×10^5 spores ml^{-1} was prepared and inoculated into flask and then subjected to shaking incubation at 200 rpm for 5 days at 45°C. After incubation, flask was harvested and contents were filtered through Whatman No.1 filter paper. The filtrate was centrifuged at 10,000 × *g* for 20 min to remove spores. The supernatant was collected in the form of crude enzyme and used for estimation of cellulolytic enzymes.

Estimation of enzyme

All the three components of cellulase enzyme viz. endoglucanase, exoglucanase and β-glucosidase were assayed in terms of different substrates at higher temperature. These enzymes were estimated by the standard procedure (Ishaque and Kluepfel, 1982; Jin et al., 2008; I-Son et al., 2010). Endoglucanase (CMCase) enzyme was assayed in terms of carboxyl methyl cellulose activity. A mixture of 5 ml of 1% CMC, 1 ml of citrate buffer (pH 6.0) and 0.5 ml of enzyme were incubated at 45°C for 4 h. The reaction mixture was mixed with 1 ml of 3,5-dinitrosalicylic acid (DNS) and then kept in boiling water bath for 5 min. Then, the tube was cooled under tap water. The amount of reducing sugars was measured in the form of absorbance at 575 nm by UV- spectrophotometer. Exoglucanase (FPase) enzyme was assayed in terms of filter paperase activity. A mixture of 50 mg Whatman No.1 filter paper (5x6 cm strip), 1 ml of citrate buffer (pH 6.0) and 0.5 ml of enzyme were incubated at 45°C for 4 h. 1 ml of reaction mixture was mixed with 1 ml of DNS and then kept in boiling water bath for 5 min. Then, the tube was cooled under tap water. The amount of reducing sugars (RS) was measured in the form of absorbance at 575 nm by UV – spectrophotometer (Ghosh, 1987). β-glucosidase (cellobiase) enzyme was assayed in terms of p-nitrophenyl- β-D- glucopyranoside (PNPG ase) activity. A mixture of 1 ml of PNPG, 1 ml of citrate buffer (pH 5.0) and 1 ml of enzyme were incubated at 37°C for 4 h. 1 ml of reaction mixture was mixed with 1 ml of DNS and then kept in boiling water bath for 5 min and then the tube was cooled.

The amount of p-nitrophenol liberated was determined spectrophotometrically at 400 nm. Enzyme activity for all the three enzymes is expressed in international units (IU). The IU is the amount of reducing sugar (glucose) released per ml of enzyme extract per unit time. The IU of endoglucanase and exoglucanase was expressed in glucose h^{-1} ml^{-1} while the same for β-glucosidase enzyme was denoted in pNPG h^{-1} ml^{-1}.

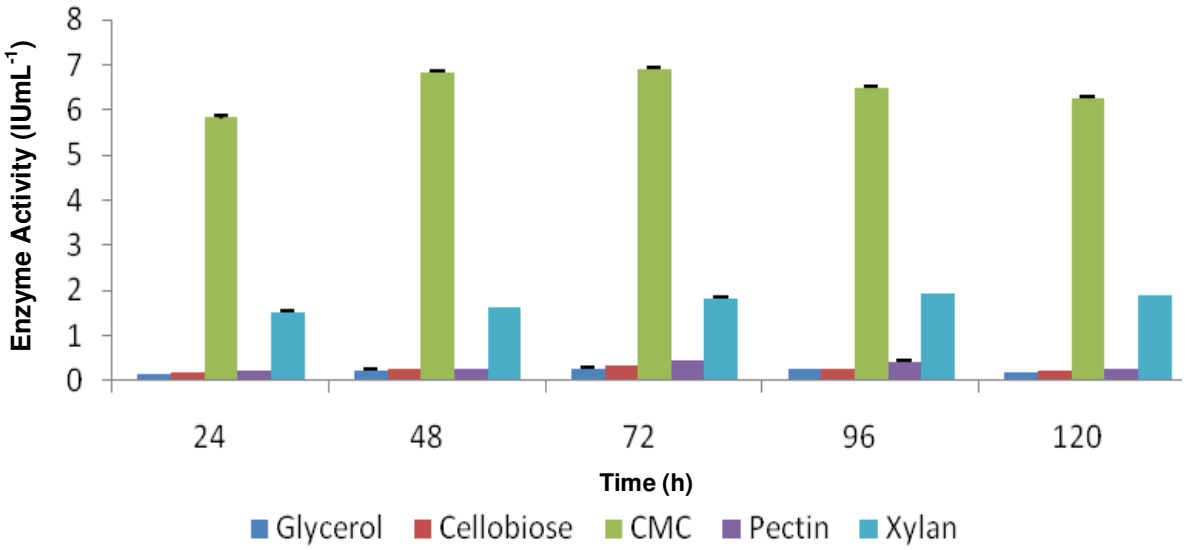

Figure 1a. Cellulase (exoglucanase) production by strain KS-22 on defined substrates.

Culture conditions

To observe the effect of different culture conditions, the present investigation was conducted using basal medium at varying incubation time (24, 48, 72, 96 and 120 h) and pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0). The effect of each factor on enzyme production and reducing sugars was monitored. The effect of different nitrogen sources on cellulase production was investigated in broth medium. Four nitrogen sources such as ammonium sulphate, sodium nitrate, calcium nitrate and peptone were amended to the basal medium.

RESULTS AND DISCUSSION

Cellulase production by mesophilic strains could easily be detected on agar media. However, the ability of these strains to produce hydrolysis zone in cellulase agar plates correlates with cellulase production in shake flask.

The mesophilic strain of actinomycete such as KS-22 was tested for its ability to produce cellulase complex enzyme by growing on various defined substrates such as glycerol, cellobiose, CMC, pectin and xylan (Figures 1a, b and c). The strain KS-22 showed its ability to produce exoglucanase, endoglucanase and β-glucosidase on different incubation periods. The strain KS-22 showed maximum exoglucanase enzyme production on xylan substrate. Glycerol was found to be poor inducer. The moderate production was noticed on CMC and pectin. Cellobiose did not produce the exoglucanase enzyme at all. The maximum enzyme activity was noticed on the 4th day of incubation and afterwards decreased slightly. The production of endoglucanase enzyme was found to be higher on CMC substrate by strain KS-22. Glycerol, cellobiose and pectin were found to be poor inducers. The moderate production was noticed on xylan. The maximum activity of enzyme was observed at 96 h of incubation. The strain KS-22 showed maximum β-glucosidase enzyme activity on CMC substrate. Cellobiose, pectin and xylan were found to be poor inducers. Glycerol did not induce the production of β -glucosidase at all.

Another mesophilic strain KS-44 was also tested for its ability to produce cellular enzyme on defined substrate (Figures 2a, b and c). The strain KS-44 showed maximum yield of exoglucanase enzyme on CMC substrate. The moderate amount of enzyme was noticed on glycerol, pectin and xylan but cellobiose did not induce the production of exoglucanase enzyme. Maximum yield of endoglucanase was noticed on CMC substrate by the strain KS-44. The other substrates such as glycerol, cellobiose, pectin and xylan were found to be moderate inducers of enzyme. The strain KS-44 showed maximum production of β-glucosidase enzyme on cellobiose. The poor induction was found on CMC, pectin and xylan but glycerol did not induce its yield of β -glucosidase enzyme. The exoglucanase, endoglucanase and β-glucosidase activities exhibited a regular increase with the rise in incubation period up to 96 h and later on these activities declined on the 5th day. The decrease in enzyme production could partly be due to accumulation of reducing sugars, which are known to exert a repressive effect on the cellulose system.Actinomycete strains exhibited higher production of exoglucanase and endoglucanase on CMC. This may be attributed to the fact that CMC is a soluble form of cellulose (Linder et al. 1996) but maximum production of β-glucosidase was found out on cellobiose. From this differential response of actinomycete strains shown towards induction, it appears that β-glucosidase synthesis may be regulated in a manner different from exo - and endoglucanase (Gong and Tsao, 1979; Lao and Wilson, 1994; George et al.

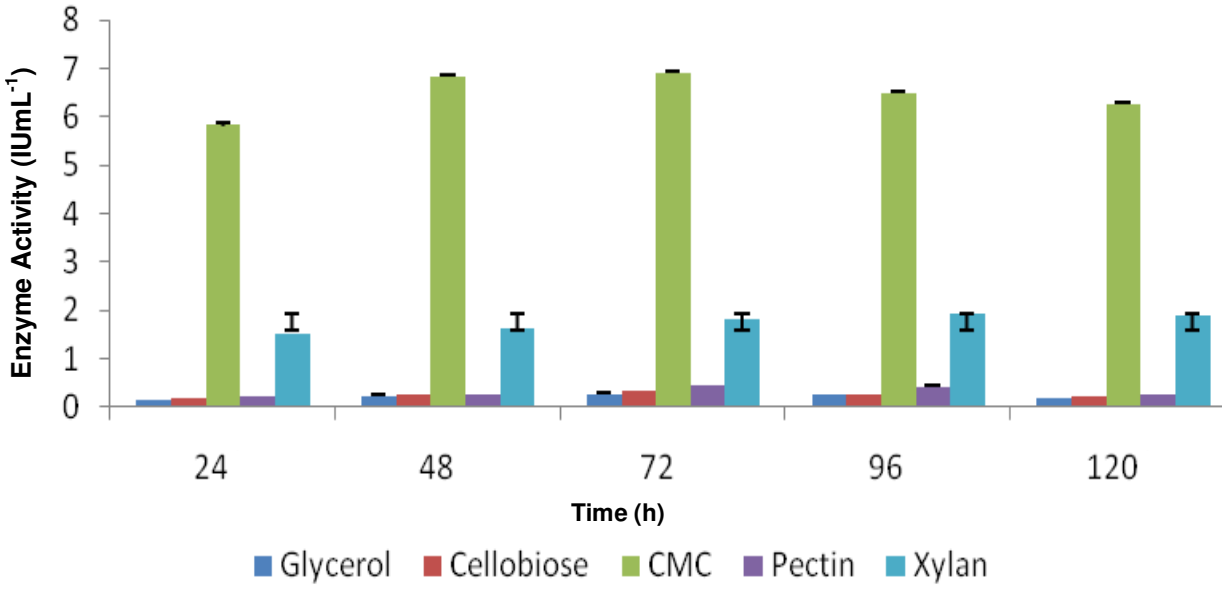

Figure 1b. Cellulase (endoglucanase) production by strains KS-22 on defined substrates.

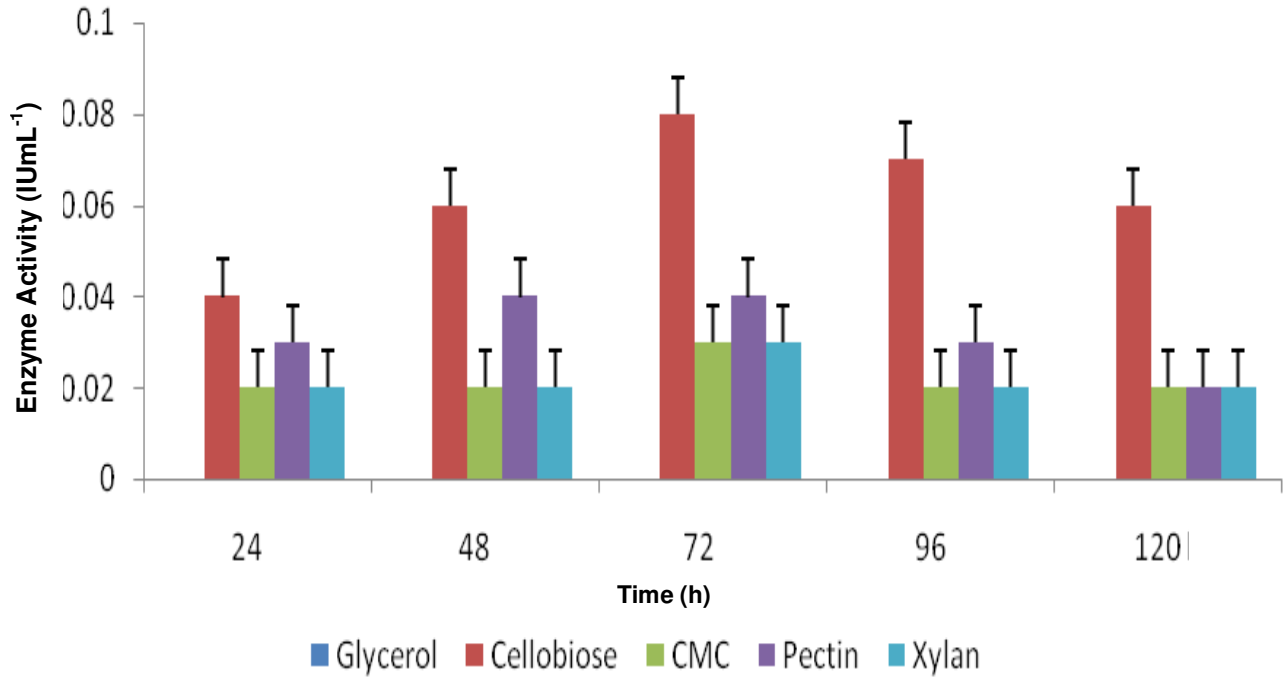

Figure 1c. Cellulase (β-glucosidase) production KS-22 on defined substrates.

2001). The mesophilic strains of actinomycete such as KS-22 and KS-44 were further tested for their ability to produce cellulase complex enzyme by growing on several delignified lignocellulosics viz. wheat straw, rice straw and sugarcane bagasse (Figures 3 and 4a, b and c). These strains showed their ability to exoglucanase, endoglucanase and β-glucosidase on different incubation periods ranging from 24 to 120 h (Godden et al. 1989).

The strains KS-22 and KS-44 showed that maximum exoglucanase activities were on rice straw at 96 h of incubation. Exoglucanase enzyme was low in amount at 24 h and afterwards reached maximum at 96 h and finally attained the stationary phase at 120 h of incubation. The moderate amount of exoglucanase enzyme was produced on wheat straw but the minimum mount was found on sugarcane bagasse. Maximum endoglucanase

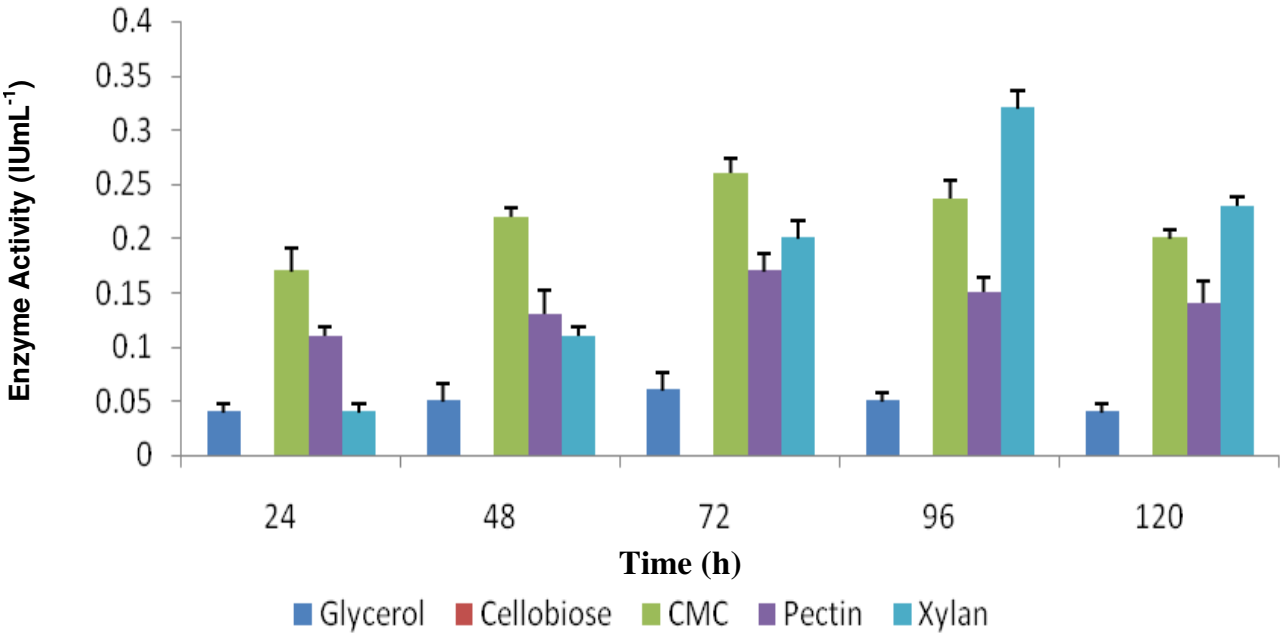

Figure 2a. Cellulase (exoglucanase) production by strain KS-44 on defined substrates.

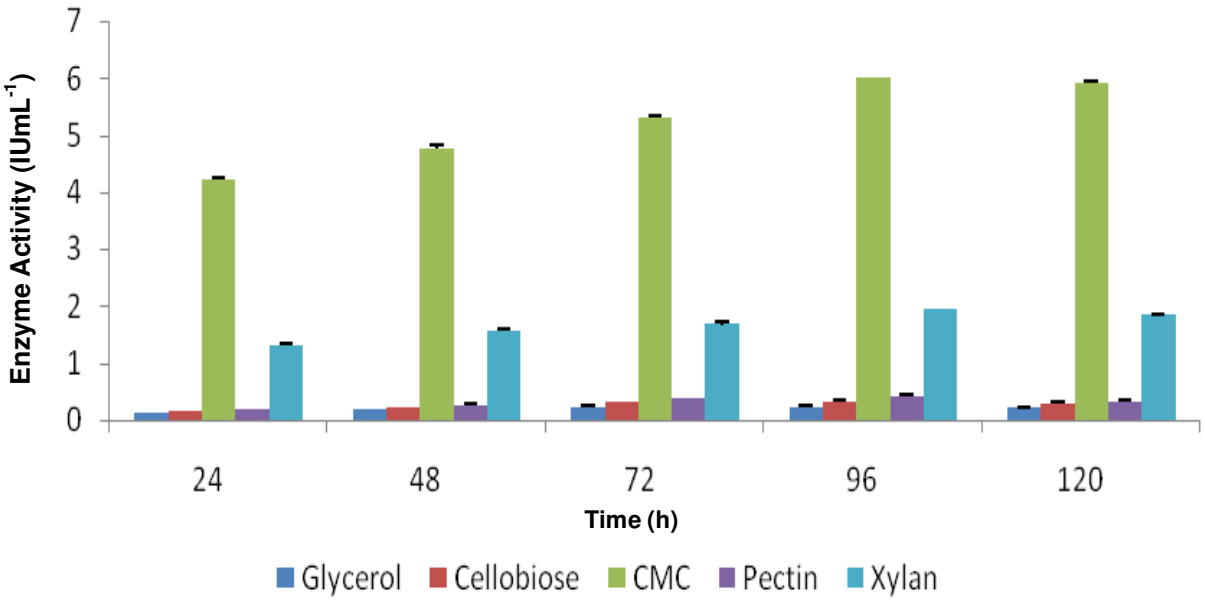

Figure 2b. Cellulase (endoglucanase) production by strain KS-44 on defined substrates.

enzyme was produced on wheat straw. The moderate amount of enzyme was found on rice straw but sugarcane bagasse produced minimum amount. Both strains KS-22 and KS-44 produced maximum β -glucosidase enzyme on rice straw. The moderate amount was noticed on wheat straw.

The minimum yield of β -glucosidase enzyme was found on sugarcane bagasse. It was clear that rice straw was found to be the best inducer for exoglucanase and β -glucosidase enzyme but wheat straw was noticed to be the best inducer for endoglucanase enzyme (Zhou et al. 2008). The yield of cellulase complex by the strains of actinomycete was inducible and the induction was caused by any substrate with β -1, 4- glucan linkage, though the degree of induction varied with the substrate (Wilson et al. 1995).

Parametric optimization was carried out to ascertain the maximum activity of β -glucosidase enzyme by strain KS–

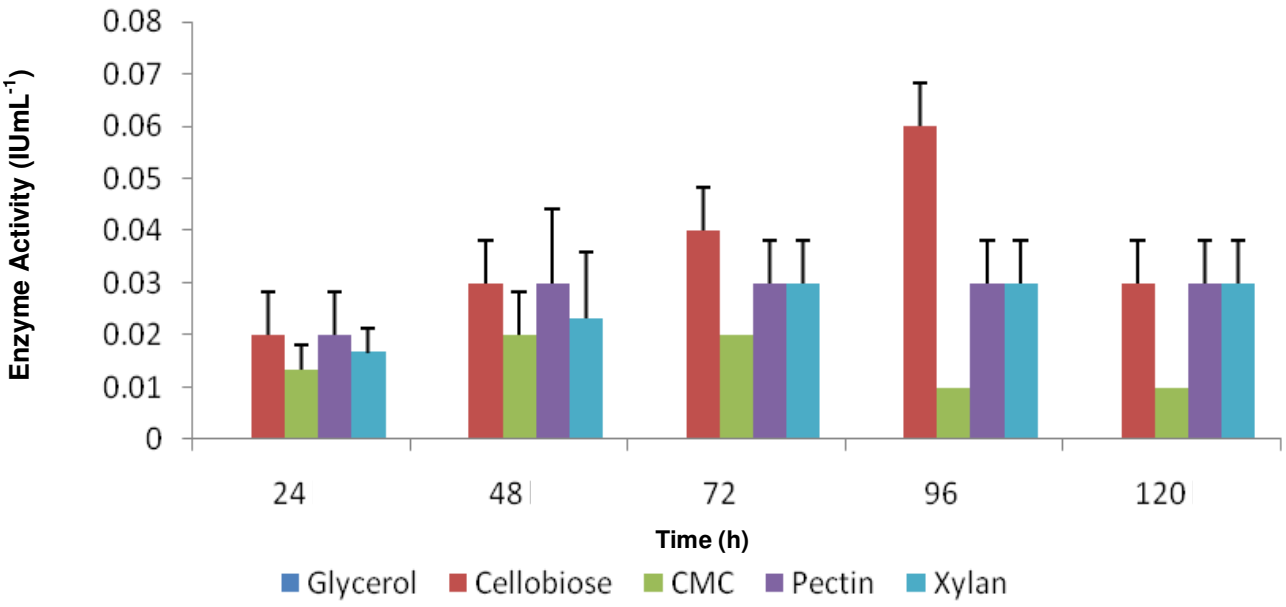

Figure 2c. Cellulase (β-glucosidase) production by strain KS-44 on defined substrates.

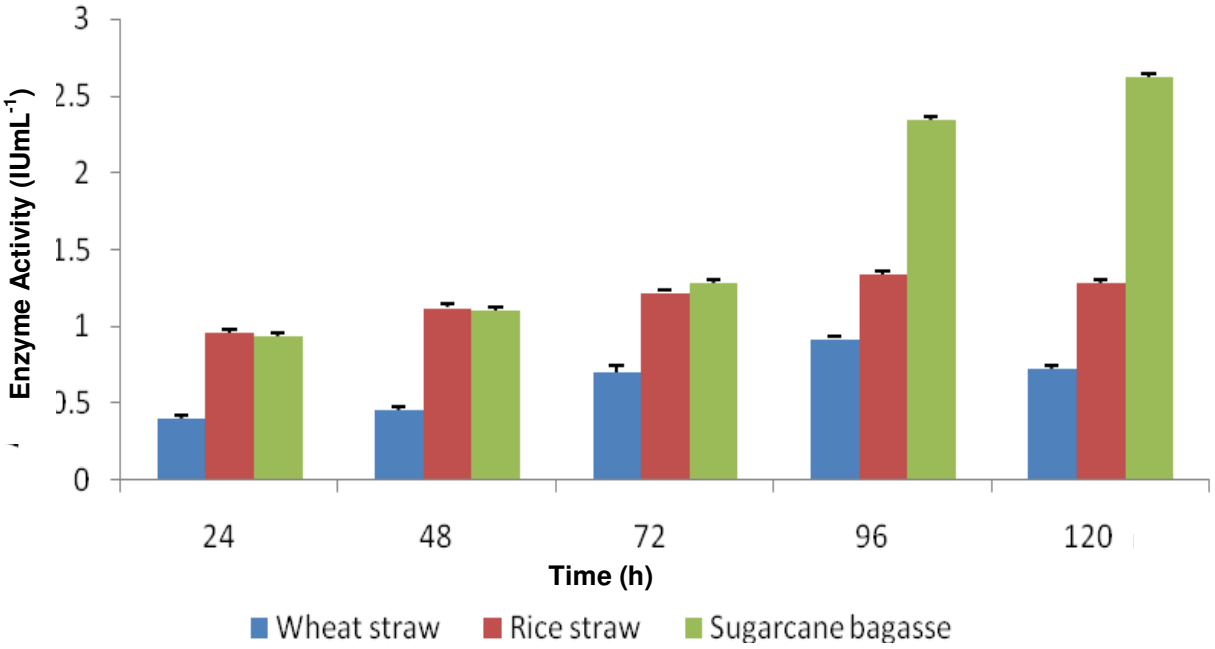

Figure 3a. Cellulase (exoglucanase) production by strain KS-22 on delignified lignocellulosics.

22 (Turner et al. 2004). An important criterion for evaluating the activity of enzyme was pH (Figure 5) under assay condition. The production of β-glucosidase enzyme was found to be maximum at pH 6.0 on 72 h of incubation but showed no activity on 96 h of incubation at any pH. Its production started decelerating with either rise or falls in pH from optimum level. The effect of different nitrogen sources such as sodium nitrate, ammonium sulphate, calcium nitrate and peptone was seen on different incubation periods by the mesophilic strain KS-44 (Figure 6).

It was noticed that sodium nitrate produced maximum amount of β-glucosidase when subjected to 72 h of incubation but no activity of enzyme was observed on 96 h of incubation. The other nitrogen sources such as ammonium sulphate, calcium nitrate and peptone were

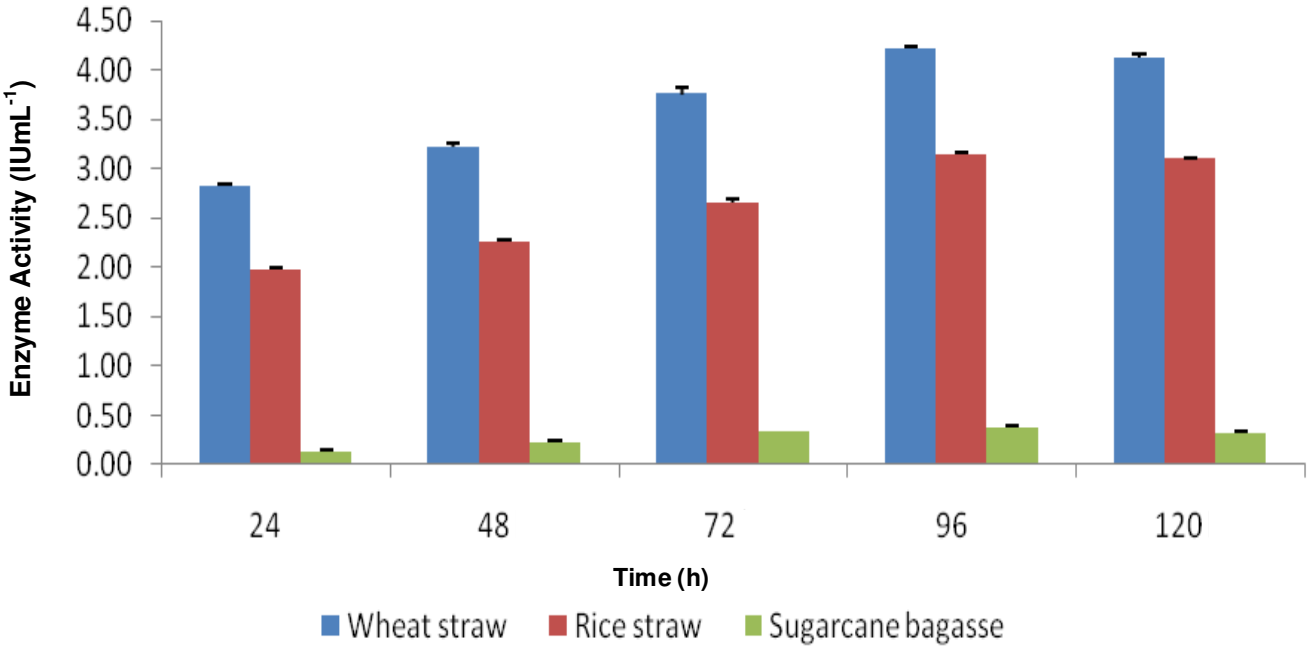

Figure 3b. Cellulase (endoglucanase) production by strain KS-22 on delignified lignocellulosics.

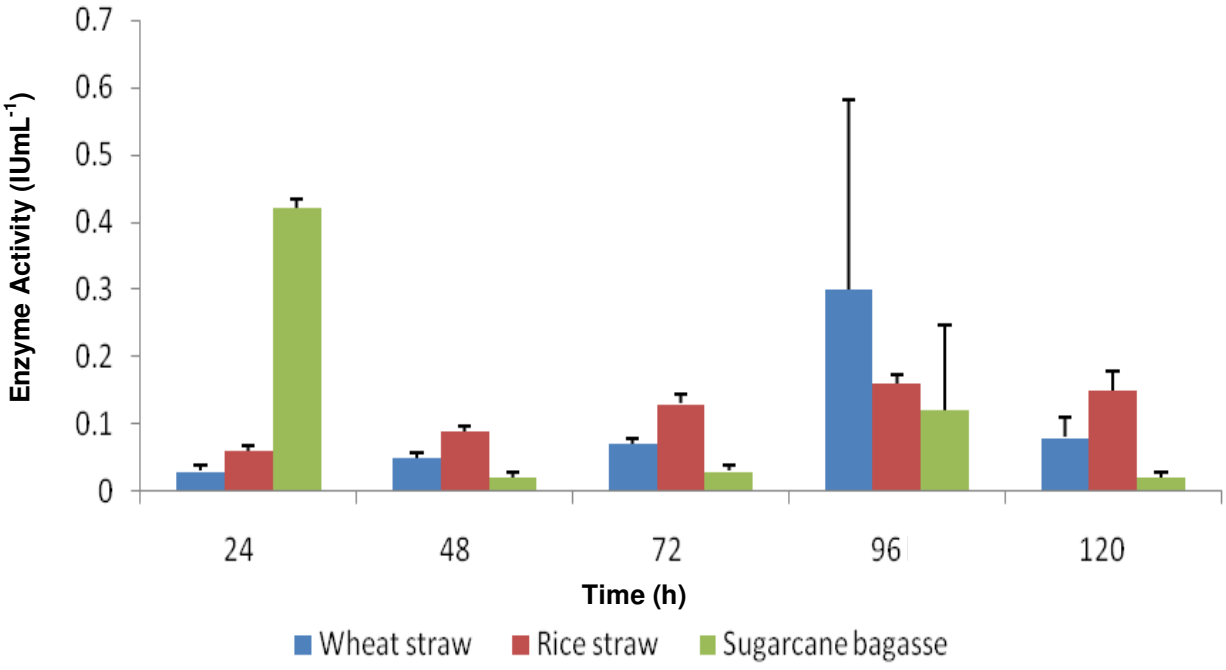

Figure 3c. Cellulase (β glucosidase) production by strain KS-22 on delignified lignocellulosics.

found to be either poor or moderate inducers of enzyme on 72 h of incubation. The result from parametric optimization confirmed the inducing nature of β - glucosidase enzyme. The results suggest that various factors influenced to a great extent the production and activity of all three components of cellulase complex from the mesophilic strains of actinomycete which will be useful for future studies. The major goals for the future cellulase research would be reduction in the cost of cellulase product and improvement of the performance of cellulase to make them more effective so that less enzyme is needed.

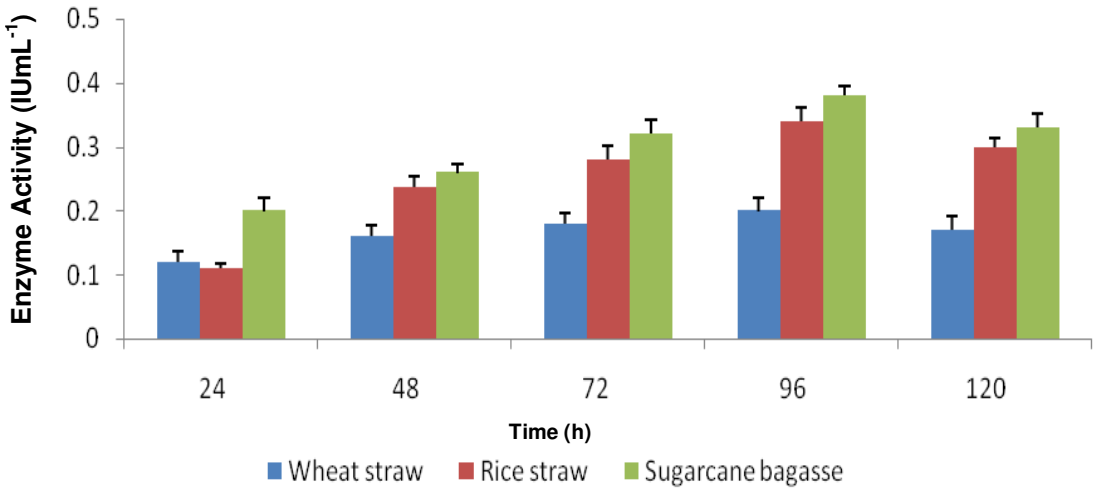

Figure 4a. Cellulase (exoglucanase) production by strains KS-44 on delignified lignocellulosics.

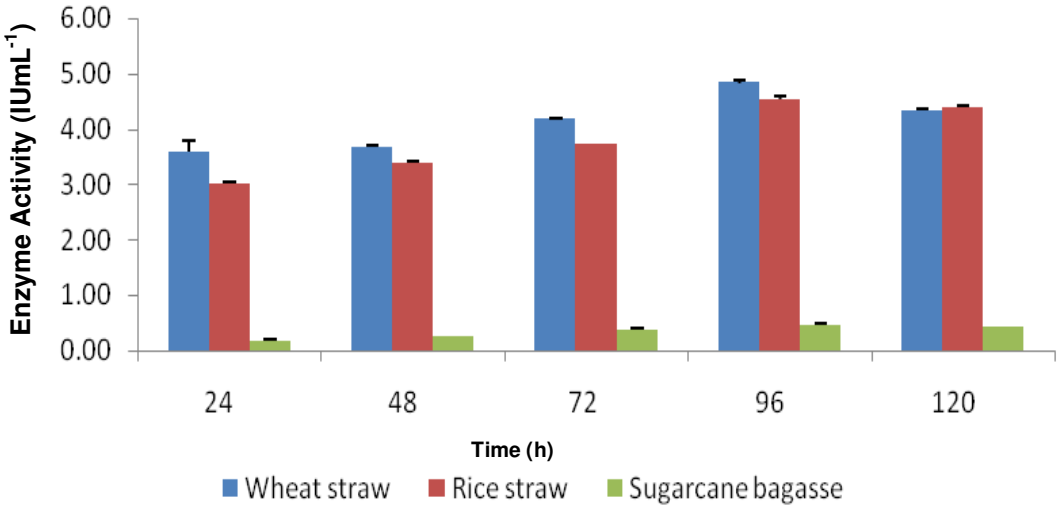

Figure 4b. Cellulase (endoglucanase) production strain KS-44 on delignified lignocellulosics.

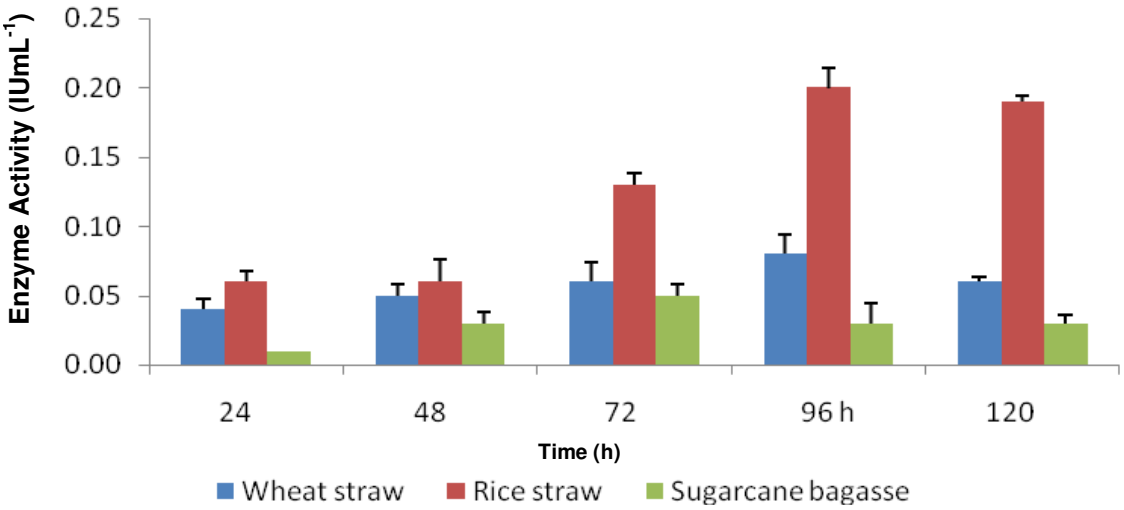

Figure 4c. Cellulase (β glucosidase) production by strain KS-44 on delignified lignocellulosics.

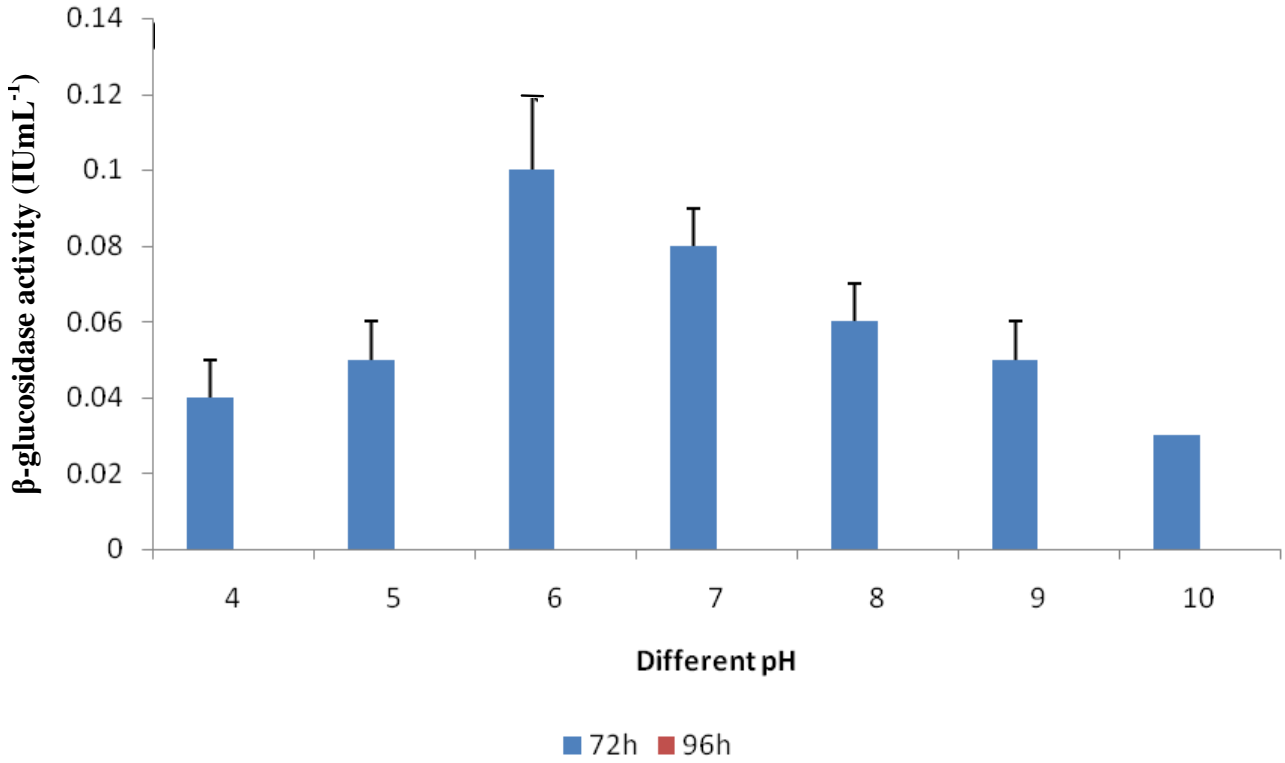

Figure 5. Effect of pH on the activity of β glucosidase of strain KS-22.

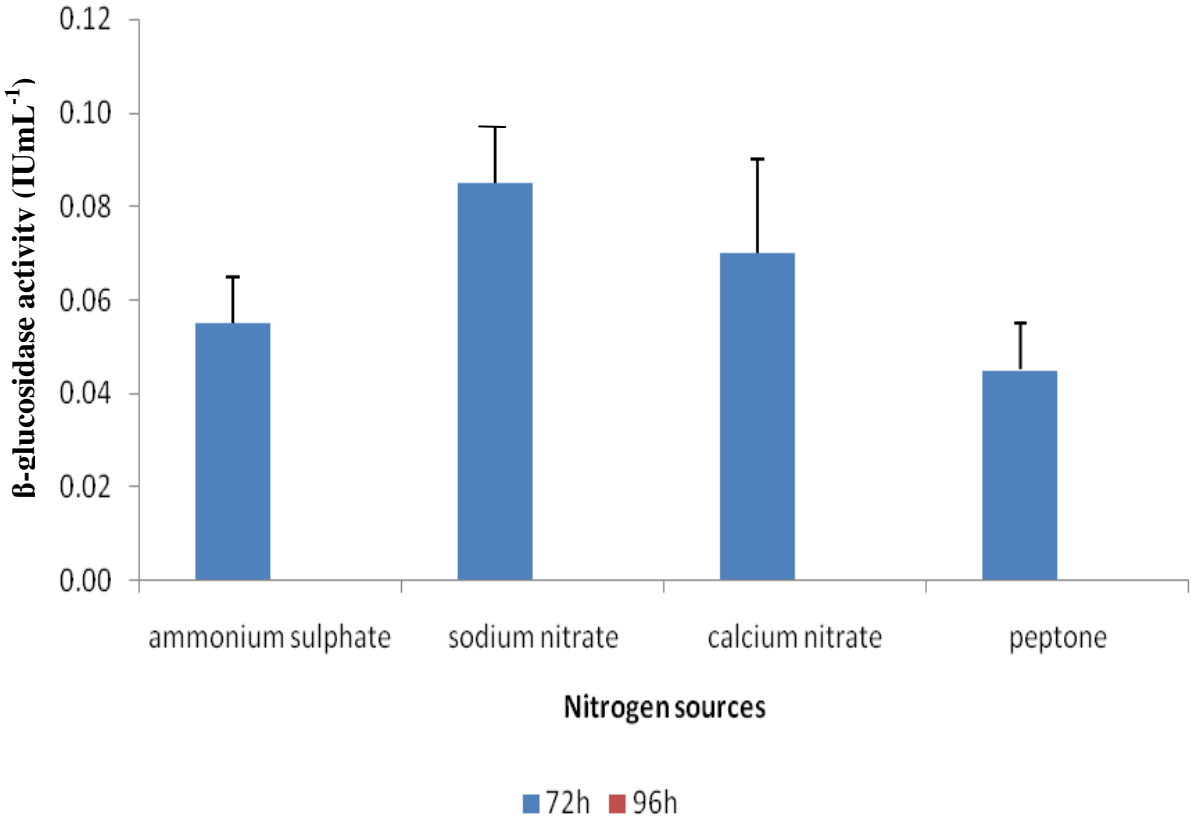

Figure 6. Effect of nitrogen sources on the activity of β glucosidase of strain KS-44.

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