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ISOLATION, SCREENING, IDENTIFICATION AND OPTIMIZED PRODUCTION OF EXTRACELLULAR CELLULASE FROM BACILLUS SUBTILIS USING CELLULOSIC WASTE AS CARBON SOURCE

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ABSTRACT

The bioconversion of cellulose is important for global stabilization and a sustainable human society. In this study, efficient cellulolytic bacteria were screened and isolated from decayed lignocellulosic waste. Among the isolates, three strains, NASCB-5 , NASCB-8 and NASCB-12 showing higher potential for cellulase production were purified and sub cultured on carboxymethyl cellulose (CMC) agar plates. Among the three isolates NASCB-5 had given maximum enzyme activity.The isolates NASCB-5 were identified as Bacillus subtilis strains by morphological, physiological, biochemical and 16S rRNA gene analysis. The isolated strain produced cellulase enzymes complex, which suggested synergic cellulolytic systems in Bacillus subtilis. Cellulase was produced in cellulase enzyme production media containig waste paper as Carbon source and the culture conditions like temperature, pH, and Incubation time and medium components nitrogen sources were optimized. Optimal concentration of inoculum for enzyme production is 2 % and incubation time is 60 hrs. Optimum temperature and pH of the medium for the cellulase production by Bacillus subtilis was 40 ^{0}C and pH 7.

Keywords: Cellulase, bacteria, molecular identification, 16S rRNA, *Bacillus subtilis*

INTRODUCTION

Lignocellulose is the most abundant carbohydrate in nature (**Lee, 1997**). Lignocelluloses are regarded as the most important renewable resource for bioconversion. Enormous amounts of agricultural, industrial and municipal cellulosic wastes have been accumulating or used inefficiently due to the high cost of their utilization processes (**Oberoi, *et al*., 2008**). It has become considerable economic interest to develop processes for effective treatment and utilization of cellulosic wastes as inexpensive carbon sources. Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization.

Cellulase is the enzyme that hydrolyzes the β-1, 4-glycosidic bonds in the polymer to release glucose units. Cellulase is a multi enzyme system composed of several enzymes with numerous isozymes, which act in synergy.Cellulases (3.2.1.4) have a wide range of industrial applications such as textile, laundry, pulp and paper, fruit juice extraction, and animal feed additives as well as in bioethanol production (**Bhat, 2000**). The cellulases have great potential in saccharification of lignocellulosics to fermentable sugars which can be used for production of bioethanol, lactic acid, and single cell protein (**Maki, *et al*., 2009**).

One major obstacle facing the development of lignocellulosic biofuel is the cellulose hydrolysis. Generally speaking, there is a lack of efficient microorganisms which can produce sufficient amounts of cellulases to efficiently breakdown crystalline cellulose to glucose. Moreover, the bio refining process remains economically unfeasible due to a lack of biocatalysts that can overcome costly hurdles (**Maki, *et al*., 2009**). Therefore, the chance to obtain cheap ethanol will depend on the successful isolation and screening of novel cellulose producing strain.

Cellulases are widely spread in nature, predominantly produced by microorganisms, like molds, fungi and bacteria (**Pérez, *et al*., 2002**). There has been increasing interest in cellulase production by bacteria because of fast growth rate (**Petre, *et al*., 1999**). Although a large number of bacteria can degrade cellulose, only a few of them produce significant quantities of free enzyme capable of completely hydrolyzing crystalline cellulose. Therefore, there has been much research aimed at obtaining new microorganisms producing cellulase enzymes with higher specific activities and greater efficiency.

The objective of our study was to isolate, identify and characterize those isolates displaying the greatest cellulase activity and optimize the cellulase production using waste paper as carbon source for the possible use in large scale bio refining.

MATERIAL AND METHODS

Isolation and screening of cellulolytic microbes

Decayed cellulosic waste, compost, soil ,decayed fruit samples were collected in a sterile container.The samples were serially diluted and spread plated on a CMC agar.The plates were incubated for 2 days 37^{0}C and observed for clear zone around colony. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min (**Bradner *et al*., 1999**). To visualize clear zones formed by cellulase positive strains the plates were destained using 1M NaCl solution. Positive and better zone producing strain was chosen and used for further studies. Positive colony from these CMC agar plates were sub cultured on fresh CMC plate. These plates were used as master plate.

Identification of bacteria

For the identification of strain of interest cultural characteristics, morphological characteristics, and biochemical tests were conducted and identified on the basis of characters as given in Bergey's Manual of Systematic Bacteriology (**Bergey, 1957**) The parameters investigated included Indole test, Methyl red test, Vogues-Proskauer test, Citrate utilization test, Catalase test, Oxidase test, Gelatin test, Motility test, Amylase test, Nitrate reduction test, Carbohydrate fermentation test by standards methods .The various media was prepared in sterile distilled water and pH was adjusted accordingly.

Cellulase Enzyme production and Assay

The Carboxy methyl cellulose (CMC) broth containing 0.2% (w/v) CMC and paper waste as sole carbon/energy source was used for enzymes production. 2% of bacterial culture was inoculated into the 50 ml of the sterile CMC broth. The cultures were incubated in shaking for 3 days at 37ºC. Cultures were harvested by centrifugation at 6000xg for 15 minutes and the cell free culture supernatants used as crude enzyme source.

The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using a dinitrosalicylic acid (DNS) method (**Miller, 1959**). One unit of FP-ase, CMC-ase was defined as the amount

of enzyme, which released µmole of reducing sugar measured as glucose per min under the assay conditions.

Filter paper assay

Filter paper assay (FPase) for total cellulase activity in the culture filtrate was determined according to the standard method. 0.5ml of culture filtrate as enzyme source was added to Whatman No. 1 filter paper strip (1 x 6 cm; 50 mg) immersed in 1ml of 0.5 M Sodium phosphate buffer of pH 7.0. After incubation at 40 ^{0} C for 30 min, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method (**Ghose, 1987**). One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per ml per min.

CMCase assay

CMCase activity was measured using a reaction mixture containing 1 ml of 1% carboxymethyl cellulose (CMC) in 0.5 M sodium phosphate buffer of pH 7.0 with 0.5ml of enzyme supernatant filtrate (**Wood, 1988**) .The reaction mixture was incubated at 50^{0}C for 30 min and the reducing sugar produced was determined by DNS method.Total sugar and protein were also estimated according to Miller (**Miller, 1959**) and Lowry's method (**Huang, 1971***).*

Molecular identification of bacterial isolate

Pure culture of the isolate was grown overnight on nutrient broth for the isolation of DNA. The DNA was isolated from the bacteria using Cell Lysis method and 16S rDNA was amplified by using the primers 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and1492R (5´-GGTTACCTTGTTACGACTT-3´) in a MJ Research PTC Mini-cycler.The amplified 16S rDNA PCR product was sequenced using automated sequencer (Applied Biosystem). The Sequence Similarity Search was done for the 16S rDNA sequence using online search tool called BLAST (http://www.ncbi.nlm.nih.gov/blast/). The unknown organism was identified using the maximum aligned sequence through BLAST search.

Optimization of production parameters

The various process parameters that influence the enzyme production were optimized over a wide range. Process parameters such as incubation period, initial pH , incubation temperature, different nitrogen sources and inoculum size were optimized for maximum enzyme production in triplicates. The most suitable pH of the fermentation medium was determined by adjusting the pH of the culture medium at different levels in the range of pH 3.0- 9.0. In order to determine the effective temperature for cellulase production fermentation was carried out at different temperature in the range of 20 to 50 ^{0}C. The fermentation was carried out up to 120 h, and the production rate measured at 12 h intervals. To detect the appropriate nitrogen source for cellulase production by the isolate, the fermentation medium was supplemented with yeast extract, peptone, glycine and malt extract nitrogen compounds at 0.5% level, thereby substituting the prescribed nitrogen source of the fermentation medium.

RESULTS AND DISCUSSION

Isolation and Screening of the cellulase producing bacteria

Bacteria are well known agents of decomposition of organic matter in general and of cellulosic substrate in particular as reported by. Lynd *et al*, (2002). As bacteria can utilize wide range of cellulosic waste, therefore, interest in the search for cellulase producing novel bacterial species is increasing. The present study was carried out with an aim of isolating, screening and identification of efficient cellulase producing bacteria from natural habitat.

Habitats that are rich with cellulosic substrates are the best sources in which we can isolate cellulolytic microorganisms .Decayed cellulosic waste, compost, soil ,decayed fruit etc were selected as a source for obtaining desirable cellulase producing organisms, because these are rich source of diverse group of cellulolytic microorganisms. Further, its wide availability, ease of processing and cost effectiveness also plays an important role for its selection.The cellulase producing bacteria were isolated from different samples by serial dilution method and spread plating on CMC agar.The isolated were named as NASCB .CMC agar is a selective media and selectively supports the growth of the cellulolytic organisms because cellulase producing organisms can only utilize cellulose as the carbon source.

The screening of the cellulolytic bacterial isolate was performed based on the diameter of clearing zone surrounding the colony on the CMC medium. The appearance of the clear zone around the colony after the addition of Congo red solution was strong evidence that the bacteria produced cellulase in order to degrade cellulose. The diameter of clearing zone for each isolate is shown in Table 1. There are around 15 bacteria were grown on CMC agar Among the 15 isolates, Depending on the diameter of clear zone around the colony, three bacterial stains were identified as efficient cellulase producing bacteria and the isolate NASC B-5 has given highest clear zone diameter and its initial identification was done by gram staining ,colony morphology amd molecular identification based on 16s rRNA gene sequence. The remaining isolate has given clear zone less than 10mm.So that they eliminated from the study. The isolate NASC B-5 has been used for further studies in the enzyme production and their ability to degrade cellulose.

Table 1 The zone of clearance of cellulase enzyme produced by bacterial isolates isolated form soil

Isolates	Organism	Diameter of Clear zone (mm)
NASCB-5	Bacillus sp	25
NASCB-8	Bacillus sp	18
NASCB-14	Pseudomonas sp	12

Morphological Identification of isolate NASCB-5

The isolate NASC B-5 was purified by repeated Sub-culturing on the nutrient Agar medium at regular intervals and stored at 4^{0}C. The isolates were identified based on the morphology and biochemical characteristics (Table 2). The morphology of isolate NASCB-5 is light creamy brown , flat rough colonies with irregular edges ,motile and spreading rapidly on the surface of agar medium.They are Gram positive bacilli in singles with spores centrally positioned. The isolate is oxidase positive, glucose fermenting (with acetoin and gas), sucrose fermenting and are Indole and Methyl red negative, Vogues-Proskauer ,Citrate Catalase, Gelatin ,Amylase and Nitrate reduction test are positive which is the characteristic biochemical properties of *Bacillus subtilis* .Isolate NASC B-5 identified as Bacillus sp.

Table 2 Staining and Biochemical characteristics of the bacterial isolate NASC B-5

SI.NO	Biochemical test	Result
	Gram staining	Gram Positive
	Endo spore staining	+
1	Motility test	+
2	Indole	-
3	Methyl red	-
4	Vogues-Proskauer test	+
5	Citrate utilization test	+
6	Catalase test	+
7	Oxidase test	+
8	Gelatin test	+
9	Amylase test	+
10	Nitrate reduction test	+
12	**Carbohydrate fermentation**	
12	Glucose	+
13	Lactose	-
	Sucrose	+
	Manitol	-

Cellulase enzyme production and assay

Cellulases production was quantitatively determined for isolates grown in the CMC and paper waste as substrate as shown in table 3**.** The FP-ase and CMC-ase activities of *NASC B-5* was higher than other isolates, suggesting that these isolate which have appreciable cellulolytic activity are valuable in the bioconversion process of cellulolytic material.

Table 3 Production of cellulase by bacterial isolates in media with CMC as carbon source

Isolates	CMCase(U/ml)	FPase (U/ml)	Protein (mg/ml)	Sugar(mg/ml)
NASCB-5	0.451	0.231	2.33	0.255
NASCB-8	0.212	0.090	1.02	0.082
NASCB-14	0.120	0.037	0.953	0.011

Table 4 Production of cellulase by bacterial isolates in media with paper waste as carbon source

Isolates	CMCase(U/ml)	FPase (U/ml)	Protein (mg/ml)	Sugar(mg/ml)
NASCB-5	0.542	0.343	2.68	0.255
NASCB-8	0.123	0.056	0.99	0.073
NASCB-14	0.930	0.032	0.84	0.008

Molecular characterization based on 16S rRNA gene

Molecular methods being highly sensitive and selective currently used to identify microorganisms. Environmental conditions may have intense impact on morphological and physiological characteristics, hence the accurate identification of isolates turned out to be more difficult (**Bakri , 2010**) . Therefore, it was suggested that the molecular techniques are more significant for the characterization of the new isolates, allowing grouping the strains. Furthermore, complex studies (microbiological, biochemical and molecular) are essential, when the identification of new isolate is the purpose of the investigation

The isolated bacteria NASC B-5 was identified in species level by molecular technique in which genomic DNA was isolated and 16S gene was amplified with suitable primers and phylogenetic analysis was done. The genomic DNA was isolated and checked on agarose gel, was found to be of high molecular weight and intact.Amplification of 16S rRNA gene was carried out by using universal primer specific to 16S rRNA genes. The gel electrophoresis was performed to analysis the PCR product. The PCR product has a size in a range between 500 to 1000 bp proved that the 16S RNA gene of NASC B-5 was successfully amplified and after purification it was used for sequencing. The obtained sequence of 16S rRNA fragment showed 93% similarity with target sequence to the closely related to sequence of *Bacillus subtilis* The isolates, NASC B-5 was identified as *Bacillus subtilis.*

Phylogenetic analysis of 16S rDNA sequences

Phylogenetic analysis was done using sequences targeting from 16S rRNA regions of bacteria. The sequences were then inputted into a sequence alignment program called Clustal W. An alignment was then done using UPGMA algorithm which finds the relatedness between the isolates assuming that the rate of evolution is constant. The aligned sequences were then uploaded into a program called Tree View which allows us to view the phylogenetic tree produced from the alignment information using the UPGMA algorithm. The phylogenetic analysis revealed similarity with *Bacillus subtilis* .

The phylogenetic Tree in Figure 1 is divided in to two main groups and each group is divided in to sub groups. The phylogenetic analysis of these strains using its 16 S rDNA sequence data showed that strain NASB-5 has highest similarity with *Bacillus subtilis* .Molecular identification of organism exhibit high specificity and sensitivity and can be used for classifying microorganisms at taxonomical level. The molecular characterization can also be a useful tool to phylogenetically relate the organism on the basis of their characteristic morphological features as well as physiological and functional aspects .

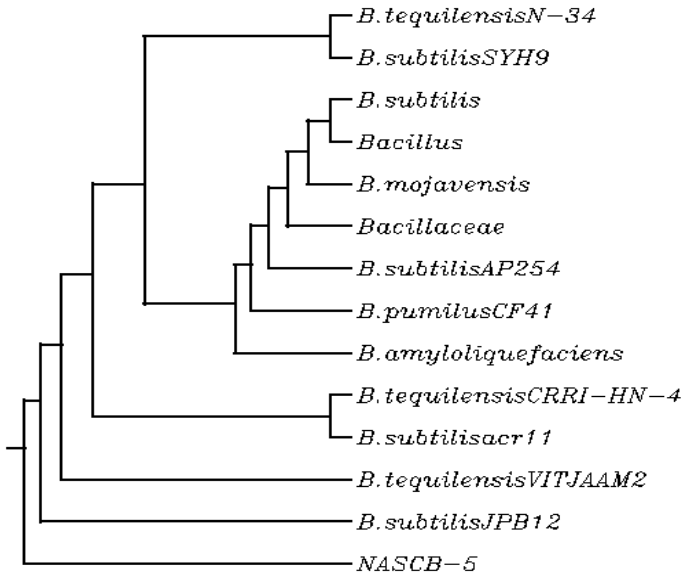

Figure 1 Molecular phylogenetic tree deduced from the sequence of 16S rRNA *of Bacillus subtilis* (NASC B-5) with respect to the closely related sequence available in Gen Bank

Optimization of Cellulase enzyme production

Effect of incubation period on Cellulase production

The of incubation period for enzyme production by *Bacillus subtilis* was detected in the production medium which contain paper waste as carbon source from the first day to fifth day (Figure2).The maximum production was found between 48 to 72 hrs and the major peak of activity was found at 60 hrs (0.72 $\pm$ 0.02U/ml). Incubation beyond the optimum time showed a rapid decline in the enzyme yield, as compared to maximum (0.306 U/ml) at 96hrs. It might be due to the depletion of nutrients in the medium which stressed the bacterial physiology resulting in the inactivation of secretary machinery of the enzymes (**Ariffin, 2006**). Most of the *Bacillus* sp. Are maintaining log phase from 3 h to 12 h of its growth. This variation of log phase timing is based on the nutrient present in the medium and the cultural condition of the organism (**Yang,et al., 1995**).

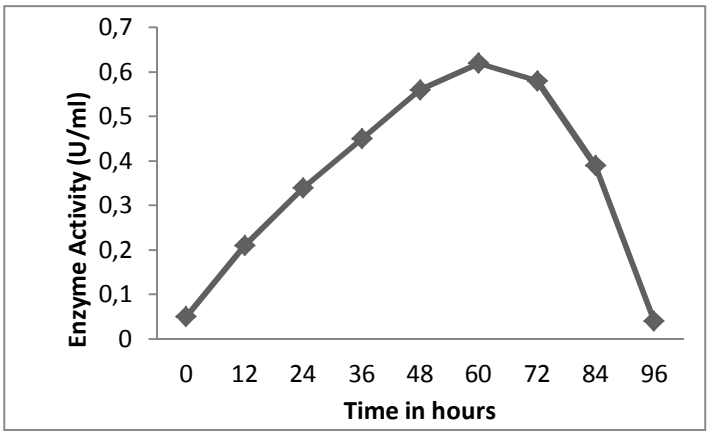

Figure 2 Effect of Incubation time on enzyme production by *Bacillus subtilis* in enzyme production media containing waste paper as Carbon source

Effect of temperature and pH on Cellulase production

Temperature is also an important factor that influences the cellulase yield. Maximum enzyme production by NASC B-5 was found to be between 35 to 45^{0}C (Figure 3).The maximum cellulase production was obtained at temperature 40^{0}C (0.741 $\pm$ 0.02 U/ml) and followed by 35^{0}C. Many workers have reported different temperatures for maximum cellulase production either in flask or in fermentor studies using Bacillus sp. suggesting that the optimal temperature for cellulase production depends on the strain variation of the microorganism . Immanuel et al. (2006) recorded maximum endoglucanase activity in *Cellulomonas*, *Bacillus* and *Micrococcus* sp. at 40^{0}C at neutral pH.

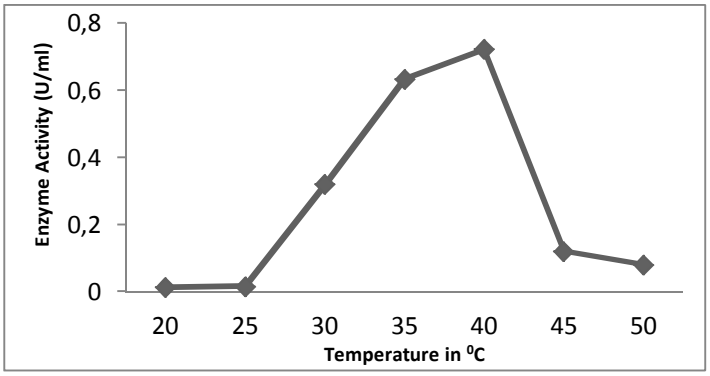

Figure 3 Effect of Temperature on enzyme production by *Bacillus subtilis* in enzyme production media containing waste paper as Carbon source.

Cellulase production at 40^{0}C under various pH was shown in Figure.4 . Hydrogen ion concentration of the production medium strongly affects many enzymatic processes and transport of compounds across the cell membrane. The optimum pH for maximum enzyme production was 7 followed by pH 6. The enzyme activity gradually increased when increasing the pH up to the optimum followed by a gradual fall in activity. Most microorganisms grow optimally within a wide pH range. Immanuel et al. (2006) reported that the cellulolytic enzyme, endoglucanase from *Cellulomonas*, *Bacillus*, and *Micrococcus* sp., isolated from the estuarine coir netting effluents hydrolyzes substrate in the pH range of 4.0 to 9.0, with maximum activity at pH 7.0. Contrary to that, Song et al. (1985) observed optimal cellulase production at pH 9.0 by *Clostridium acetobutylium.*

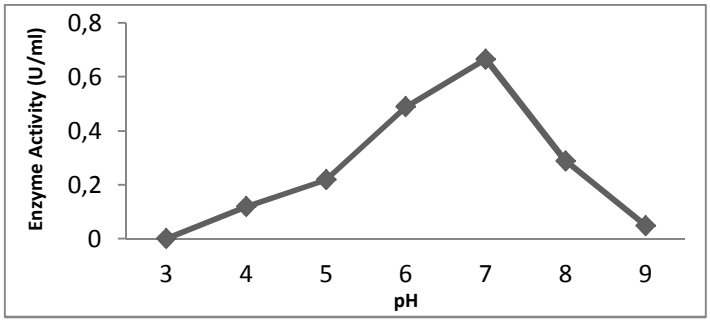

Figure 4 Effect of pH on enzyme production by *Bacillus subtilis* in enzyme production media containing waste paper as Carbon source

Effect of inoculums concentration on Cellulase production

The effect of concentration of inoculum on enzyme production was studied by inoculating different concentration of inoculums ranges from 1% to 3%in CMC broth.The concentration of initial inoculums plays a critical role in enzyme yield in production media.The media was inoculated with different concentration of inoculum and incubated at 40^0C for 72 hrs. The optimum enzyme production observed in inoculums concentration rages from 1.5% to 2.5% of inoculums(Figure.5).The maximum production obtained in 2% (0.687$\pm$0.02U/ml) followed by 2.5% and 1.5% (0.43$\pm$0.02U/ml) .

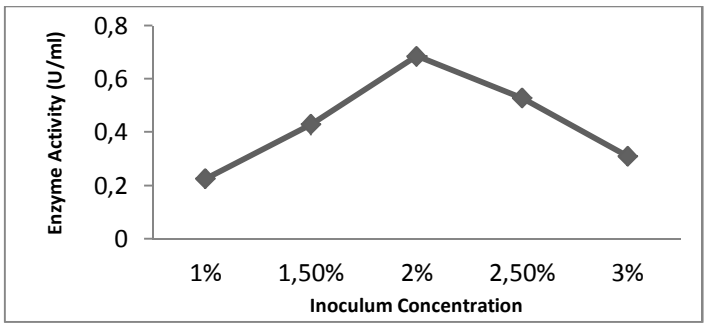

Figure 5 Effect of Inoculum concentration on enzyme production by *Bacillus subtilis* in enzyme production mediia containing waste paper as Carbon source

Effect of Nitrogen source on Cellulase production

The Nitrogen source plays an important role in enzyme production.Its effect on enzyme production by Bacillus subtilis was studied by supplementing different Nitrogen source in to production media.Diffeent Nitrogen tested individually at the concentration of 0.5% Nitrogen in production media and it incubated at 40^0C for 72hrs .The maximum enzyme production observed in media supplemeted with malt extract (0.61 U/ml) as Nitrogen source.(Figure 6). These data were in accordance with the results of Ray et al. (2007) who reported that organic nitrogen sources were more suitable for optimizing the cellulase production by *B. subtilis* and *B. circulans* than inorganic sources.

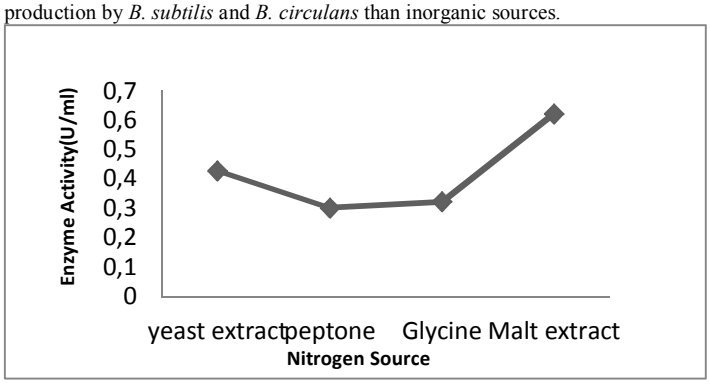

Figure 6 Effect of Nitrogen source on enzyme production by *Bacillus subtilis* in enzyme production meida containing waste paper as carbon source

CONCLUSION

Several microorganisms capable of converting cellulose into simple carbohydrates had been discovered for decades. However, needs for newly isolated cellulytic microbes were still remained. In this study we have isolated and identified efficient cellulase producing bacteria from cellulose rich environment. The bacterial isolate was characterized based on 16S rRNA sequence and was identified as a *Bacillus subtilis*. The isolate *Bacillus* subtilis NASC B 5 showed a potential to produce cellulase using waste paper as a substrate and its enzyme production efficiency was incraesd by optimization of cultural conditions and media components .Since pure commercialized cellulose is too expensive to be used as substrate,waste paper maybe a good alternative for cellulase production from industrial point of view. Isolation, characterization and optimization of cellulose producing bacteria may provide a good starting point for the discovery of such beneficial enzymes for bioconversion of lignocellulosic waste in to ethanol

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