ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

ISOLATION, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF CELLULASE PRODUCING FUNGI

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Manuscript Info

Manuscript History:

Received: 22 August 2015
Final Accepted: 19 September 2015
Published Online: October 2015

Key words:

Cellulase,,Aspergillus sp., 18S rRNA, organic polymer.

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Abstract

Cellulose is the most abundant organic polymer which may be hydrolyzed by enzyme cellulase into smaller sugar components including glucose subunits which can be used for production of ethanol, organic acids and other chemicals. Five different isolates were isolated from collected samples and one was shown maximum zone of hydrolysis which was identified as *Aspegillus sp.* based on morphological, microscopic and molecular level. The best conditions like pH, carbon source, nitrogen source, and temperature and incubation period were also observed for cellulase producing fungi. Incorporation of fructose as carbon source, ammonium sulphate as nitrogen source and pH 6.0 gave best enzyme production by *Aspergillus sp.* The highest enzyme activity was observed on 6th day of incubation at temperature 28ºC. The unknown isolates were identified by amplifying and sequencing of 18S rRNA gene. After amplification the length of amplified DNA was obtained as 532bp and amplified PCR product was compared with sequence obtained from NCBI. A Phylogenetic tree was formed on the bases of comparison.

INTRODUCTION

Earth is facing an energy disaster because of using nonrenewable energy resources such as coal, oil, and gas. The supplies of these resources are not limitless but they will be consumed in a restricted period of time. On renewable energy resources different branches of science are conducting investigation. In this regard biomass is the one of the most important resources and is a renewable organic matter generated from photosynthesis by plants and algae **(Charitha and Kumar, 2012).** Hence utilization of biomass as an energy resource allows us not only to conserves nonrenewable sources of energy, but also to protect biodiversity. In addition, microbiological science gives some information to improve the efficiency of the biomass and it can help in plant waste transformation into productive biomass. This valuable biomass is lost due to lack of satisfactory technology for processing into productive bioresources. Straw of grain is mainly composed of cellulose which is a high molecular weight insoluble polymer of glucose and unable to decompose by animal enzymes **(Rafidah *et. al.*, 2010).** It is a great substrate for microbiological fermentation processes that allows homogeneous, high-quality, and inexpensive cellulolytic enzymes which is a key to the biological degradation of cellulose. **(Padmavathi *et. al.*, 2012)**. Various fungi and other organisms are the basic sources of enzymes that are able hydrolyze these polysaccharides into smaller units. *Trichoderma*, *Aspergillus*, and *Penicillium* constitute a group of microorganisms that produce the cellulase which hydrolyzes cellulose. Extracellular enzymes can be produced in liquid or solid media, although the use of solid media enables rapid screening of large populations of fungi, which is the best way to detect specific enzymes. **(Doolotkeldieva and Bobusheva, 2011)**

On earth cellulose is the most common organic compound having formula $(C_6H_{10}O_5)_n$. It is a polysaccharide consisting of a linear chain of several hundred to over 10000 β (1→4) linked D-glucose units. This degree of polymerization depends on source and treatment method **(Tian *et. al.*, 2011; Sukumaran *et. al.*, 2005).** It is present in 33% of plants matter. It is mainly converting from energy crops into biofuels such as cellulosic ethanol (***).*** Cellulase is the enzyme used to hydrolyze the cellulose and is produced by fungi, bacteria, protozoans (**Khan and Singh, 2011)** and other types of organisms, such as termites and also by microbial intestinal symbionts of other termites (**Tokuda and Watanabe, 2007).**These enzymes can either be free or grouped in a multicomponent enzyme complex (cellulosome) found in anaerobic cellulolytic bacteria. Enzymatic hydrolysis is an economic process in the conversion of cellulose to easily fermentable low cost sugars. From early 1980s biotechnology of cellulase and hemicellulases began, first in animal feed and then in food applications (**Ali *et. al.*, 2011).**

Due to complex nature and wide spread industrial applications of microbial cellulases, it have become the central biocatalysts. This enzyme is composed of separately folding, functionally and structurally distinct units called modules, which form cellulases module. It was synthesized by a large variety of microorganisms during their growth on cellulosic materials including both fungi and bacteria. But among them, the most generally studied cellulase producing microorganisms are *Cellulomonas, Clostridium, Aspergillus, Trichoderma* and *Thermomonospora* **(Kuhad *et. al.*, 2011)**. Fungal cellulases are structurally simpler than bacterial cellulase systems i.e. cellulosomes. Fungal cellulases usually have two separate domains: a catalytic domain (CD) and a cellulose/carbohydrate binding module (CBM) which is made up of approximately 35 amino acids and both these are joined by a short polylinker region which is rich in serine and threonine at the N-terminal.

For cellulase production fungi have good capability as compared to bacteria because bacterial cellulase generally lacks one of the three cellulase activities, that is FPase activity and the application of bacteria in producing cellulase is not widely used **(Ariffin *et. al.*, 2006).** Among all fungal strains, *Aspergillus* sp .have been acknowledged to attain all components of cellulases complex. **(Lakshmi and Narasimha, 2012).** Other than *Aspersillus sp, Trichoderma* is also the best cellulase producing microorganism. Moreover these microorganisms produced the crude enzyme which is commercially presented for agricultural and industrial uses. Commonly bacteria produced the cellulases constitutively, whereas fungal produced only in the presence of cellulose. The ability to produce extra cellular cellulolytic enzymes is widespread in fungi and these enzyme systems have been most extensively studied **(Jalil and Othman, 1998.)**

The main aim and objective of this study was to isolate the fungi which was capable of producing cellulase enzyme and then screen it for cellulase production. The isolate which shown maximum hydrolysis of cellulose degradation then used for biochemical and molecular characterization. The optimum conditions like pH, carbon source, nitrogen source, and temperature and incubation period were also observed for cellulase producing fungi.

MATERIALS AND METHODS

Collection of samples: Different soil samples were collected from four different localities of Punjab. These samples were taken from Khanna paper mill, Amritsar, Wahid Sugar Ltd. Phagwara, Bhalla paper mill, Lambha pind.Site1 and site 2. It was taken by means of spatulas and collected in sterilized polythene bags. The samples were then brought to the laboratory for further study.

Isolation of Fungi: Fungi were isolated from soil sample using standard microbiological techniques. A sample of 1 gram was placed in a graduated cylinder, sterilized distilled water was added to make a total of 10 ml. Serial dilution was done till 10^{-6}. 1 ml of desired dilution (10^{-5} and10^{-6}) was transferred aseptically into Czapak Dox Agar (CDA) containing 1% carboxymethyyl cellulose (CMC) (**Fouzia and Khan, 2011)** and added 30mg/1000ml of streptomycine to inhibit bacterial growth (**Kumar and Mahalingam, 2011)**. Plates were incubated at 28°± 2ºC for 4-6 days. After incubation, small portion of mycelium from each fungal colony was transferred into Potato Dextrose Agar (PDA) slants for further studies.

Screening: Fungi were tested for their ability to produce the hydrolytic enzymes. Mandels and Reese medium was prepared and autoclaved (**Vyas and Vyas, 2005).** Then supplemented this medium with CMC/cellulose was used for congo red test. After solidification of medium, wells were made which are filled with crude enzyme of test organisms. Petri plates were then incubated at 28^0 ± 2°C for 2-3 days (**Devi and Kumar, 2012)** .After incubation the Petri plates were flooded with 0.1% aqueous solution of Congo Red. After 20 min the plates were counterstained with 1M NaCl solution for further 20 min. After 20 min a pale reddish zone around the well was observed. (**Khan and Singh, 2011; Gautam *et. al.*, 2011)**

Identification of fungi: Strains presenting large clearing zones in congo red test were identified by lactophenol blue dye on the basis of cultural characteristics and morphological characteristics. Placed one drop of dye on clean slide and add inoculum of fungi and then covered it with cover slip. The slides were observed under microscope at magnification of 40X.

Production of Cellulase Enzyme: The fungal colonies showing largest zone of decolorization in congo red test were selected for cellulase production on Mendal and Reese broth containing 1% cellulose/CMC as a sole carbon source. Inoculate it with a spore suspension at 28° ± 2°C for 6 days. After appropriate incubation period, sample was withdrawn and filtered by Whatman No. 1 filter paper **(Rajmane and Korekar, 2012)**. Then finally centrifuged at 5000 rpm for 20 min and the supernatant were used as source of crude enzyme.

Enzyme Assay: The potential fungal isolates were assayed enzymatically for cellulases. The assay methods employed were:

a) Exoglucanase activity(FPase): Filter paper activity (FPase) for total cellulase activity in the culture filtrate was used as enzyme source were added to Whatman No. 1 filter paper strip (1 × 6 cm; 50mg) immersed in 1 ml of 0.1M citrate buffer of pH 4.8.Incubate this reaction mixture at 50º± 2ºC for 60 min **(Lee and Ibrahim, 2011)**. After incubation, the reaction was stopped by adding 3 ml of DNS reagent and then the reaction mixture was boiled for exactly 5 minutes in vigorously boiling water bath. The tubes were cooled at room temperature and the absorbance was measured at 540nm. Enzyme activity was represented in term of International Units (IU) known as Filter Paper Units (FPU) which is micromoles (µm) of glucose released / min. / ml.

b) Endoglucanase Activity (CMCase): Endoglucanase activity (CMCase) was measured using a reaction mixture containing 0.5 ml of 1% carboxymethyl cellulose (CMC) in 0.1M citrate buffer (pH 4.8) and 0.5 ml of filtrate crude enzyme (**Gautam *et. al.*, 2010)**. The reaction mixture was incubated at 50º± 2ºC for 30 min. After incubation, the reaction was stopped by adding 3 ml of DNS reagent and then the reaction mixture was boiled for exactly 5 minutes in vigorously boiling water bath. The tubes were cooled at room temperature and the absorbance was measured at 540nm. Enzyme activity was presented in term of International Units (IU), which is micromole (µm) of glucose release / min. / ml.

c) β-glucosidase Activity: β-glucosidase activity was measured using a reaction mixture containing 0.5 ml of 1% salicin prepared in citrate buffer (pH 4.8), 0.5 ml of enzyme sample. The reaction mixture was incubated at 50^0 ± 2ºC for 30 min. After incubation, the reaction was stopped by adding 3 ml of DNS reagent and the reaction mixture was boiled for exactly 5 min in boiling water bath. The tubes were cooled in cold water and the absorbance was then measured at 540 nm. Enzyme activity is presented in term of International Units (IU) which is micromole (µm) of glucose released / min / ml.

Biochemical Characterization and optimization:

Effect of pH on Activity of Cellulase Enzymes: The effect of pH was determined by measuring activity at standard assay over a pH range 3-8. Substrates CMC (1%) for endoglucanase, filter paper (50mg) for exoglucanane and salicin (1%) for β-glucosidase activity was prepared in two buffer viz. citrate buffer (pH 3-5) and Potassium Phosphate buffer (pH 6-8). Enzyme of test organism was mixed with substrate and cellulase assay was performed. Cellulase activity was determined in IU/ml.

Effect of Temperature on Activity of Cellulase Enzymes: The activity was determined by carrying out the standard assay at various temperatures between 30-70^0 ± 2°C for test organism. Enzyme mixed with substrate CMC for endoglucanase activity, filter paper for exoglucanase activity and salicin for β-glucosidase activity prepared at buffer of optimum pH activity obtained. Endoglucanase, exoglucanase and β-glucosidase activity was carried out at different temperature range of 30-70^0 ± 2ºC. Cellulase activity was determined in IU/ml.

Effect of pH on Enzyme Production: To determine optimal pH, the strain was cultivated in flask containing Mendal and Reese medium with different pH ranges from 3.0 to 8.0. The pH of the medium was adjusted by using 1N HCl or NaOH. **(Azzaz *et. al.*, 2012)**. The flasks were kept in 150 rpm rotary shaker at 28º± 2°C for 6 days. At 6^{th} day of incubation, filtered the medium by Whatman No.1 filter paper and then centrifuged at 5000 rpm for 20 min. The supernatant was separated from pellet as crude enzyme. Then the exoglucanase activity of cell free filterate as enzyme was determined in IU/ml.

Effect of Carbon Sources on Enzyme Production: Effects of various carbon compounds namely, cellulose, glucose, sucrose, fructose and galactose were used for studying (**Gautam *et. al.*, 2011).** The Mendal and Reese medium was distributed into different flasks of different carbon sources and these flasks were kept in rotary shaker at 28º ± 2°C for 6 days. At 6th day of incubation, filtered the medium by Whatman No.1 filter paper and then centrifuged at 5000 rpm for 20 min. The supernatant was separated from pellet as crude enzyme. Then the exoglucanase activity of cell free filterate as enzyme was determined in IU/ml.

Effect of Nitrogen Sources on Enzyme Production: In the present study, we aim to detect the appropriate nitrogen source for cellulase production The Mendal and Reese medium was supplemented with different nitrogen source namely, beef extract, yeast extract, sodium nitrate, ammonium nitrate, ammonium sulphate and ammonium chloride (**Gautam *et. al.*, 2011).** After cultivating with inoculum, the flasks were kept in rotary shaker at 28º ± 2°C for 6 days of cultivation. At 6th day of incubation, filtered the medium by Whatman No.1 filter paper and then centrifuged at 5000 rpm for 20 min. The supernatant was separated from pellet as crude enzyme. Then the exoblucanase activity of cell free filterate as enzyme was determined in IU/ml.

Effect of Incubation Period on Enzyme Production: Incubation period was an important parameter for enzyme production. In this study, Mendal and Reese medium was incubated up to 8 days and production rate was measured at 1 day interval (**Gautam *et. al.*, 2011*).*** After each day, filtered the medium by Whatman No.1 filter paper and then centrifuged at 5000 rpm for 20 min. The supernatant was separated from pellet as crude enzyme. Then the exoglucanase activity of cell free filterate as enzyme was determined in IU/ml.

Effect of Temperature on Enzyme Production: In order to determine the effective temperature for cellulase production, the fungus strain was cultivated in Mendal and Reese medium at 28º ± 2°C and 37º ± 2°C ratatory shaker at 150 rpm for 6 days. At 6th day of incubation, filtered the medium by Whatman No.1 filter paper and then centrifuged at 5000 rpm for 20 min. The supernatant was separated from pellet as crude enzyme. Then the exoglucanase activity of cell free filterate as enzyme was determined in IU/ml.

Molecular Characterizations:

- The very first step executed was isolation of genomic DNA from the culture provided by the scientist. Hence, evaluation of the quality was done on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed.
- Fragment of 18S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 750 bp was observed when resolved on Agarose Gel which was continued with purification of PCR amplicon to remove the undue contaminants.
- Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with DF and DR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.
- Consensus sequence of 732bp rDNA gene was generated from forward and reverse sequence data using aligner software.
- The 18S rDNA gene sequence was used to carry out BLAST with the nr database of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

RESULTS AND DISCUSSION

Isolation of cellulase Producing fungi: The fungi were isolated from the samples collected from paper and sugarcane mills for cellulase production. Five different strains of fungi namely B, W1, W2, W3LG and W3DG were isolated from collected samples. They were obtained in the mixed culture plate and were sub cultured by point inoculation. Table1 below have shown the details of isolated fungi.

Table.1: *Detail of sample collection and fungal isolates.*

S. No.	Sampling location	No. of fungus isolated	Name of isolated fungus
1.	Bhalla paper mill, Site 1 and Site 2	1	B

2.	Khanna paper mill	0	-
3.	Wahid Sugar Ltd	4	W1, W2, W3LG and W3DG

Screening of Isolates for Cellulase Production: All the five cultures were screened by congo red test for their cellulase producing potential and the hydrolysis zones were observed. Table 2 and below have shown the results of screening.

Table.2: *Screening for cellulase.*

S. No.	**Isolate**	**Cellulase producing potential of isolates**
1	B	-
2	W1	+
3	W2	++
4	W3LG	+++
5	W3DG	+

Identification of the Isolate Showing Maximum Hydrolysis: The isolated strain showing maximum hydrolysis were carefully identified by morphological characteristics including color of the colony and growth pattern studies and examined under the microscope with magnification of 40X. The isolate with maximum zone was identified as *Aspegillus sp.* By 18S rRNA sequencing, it was also found that the strain was *Aspergillus sp*. with GenBank Accession Number **HF937386.1**

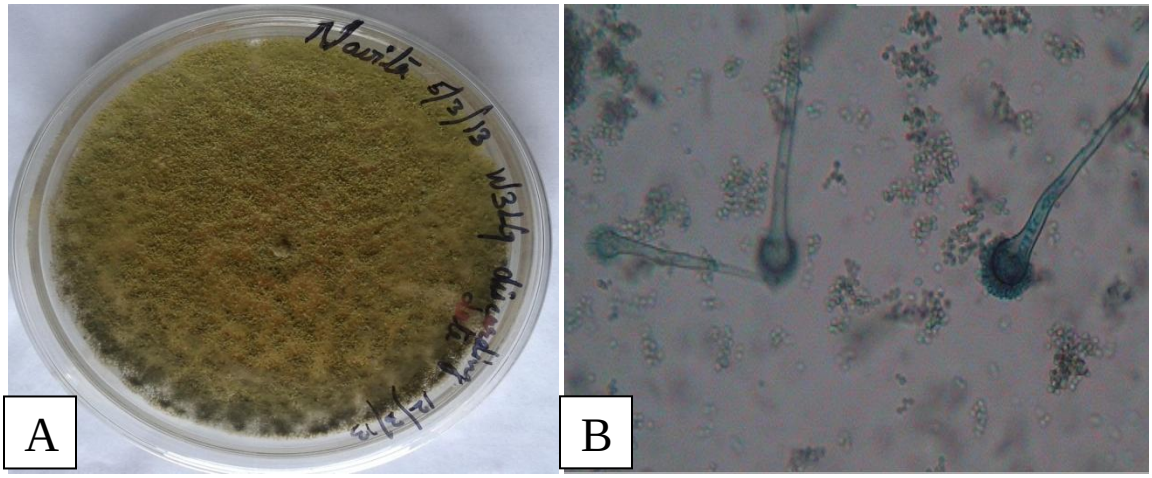

Fig1: (A) *Morphology and characteristic of fungal colony* **(B)** *Microscopic View*

Enzyme Assay: Enzyme activity was measured for all three types of cellulase enzyme and the maximum activity was observed for endoglucanase (CMCase) i.e. 0.26 IU/ml as the figure2 shown. Endoglucanase enzyme catalyzes the breakage of non-covalent interactions present in the crystalline structure of cellulose. Hence it gave highest value of activity than endoglucanase and β-glucosidase. β-Glucosidase gave minimum activity because it hydrolysed the cellobiose (dimmer of cellulose).

Table3: *Enzyme activity for three different types of cellulase*

Enzyme Assay	**Activity (IU/ml)**
Endoglucanase (CMCase)	0.26825

Exoglucanase (FPase)	0.136
β-Glucosidase	0.0296

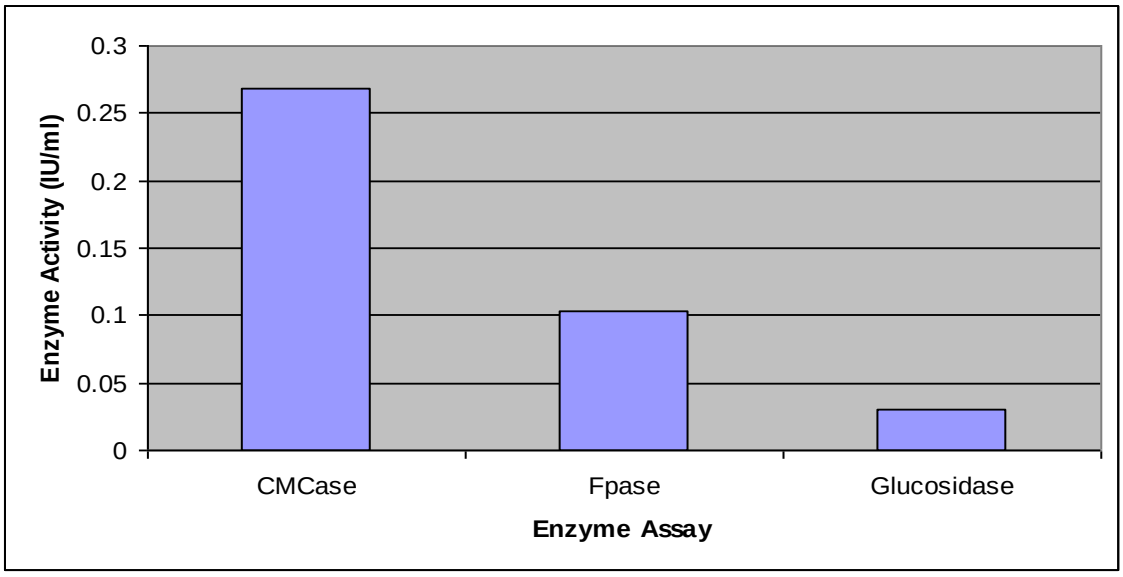

Fig2: *Enzyme activity for three different types of cellulase.*

Biochemical Characterization of cellulase Producing fungi:

Effect of pH on enzyme Activity: Buffer with different pH had a strong influence on cellulase activity. As the table 4 and figure 3 showed that at the fungal isolates gave maximum activity at pH value ranging between 4.0-5.0.That mean pH 5 was the best pH for cellulase production. **Sadaf *et. al*., 2005** also reported that most of the strains of *Aspergillus sp*. were shown maximum activity at pH range from 4-4.8.

Table4: *Enzyme Activity at different pH.*

pH of buffer	**Activity (in IU/ml)**
3	0.0795
4	0.1739
5	0.1924
6	0.1369
7	0.1168
8	0.1054

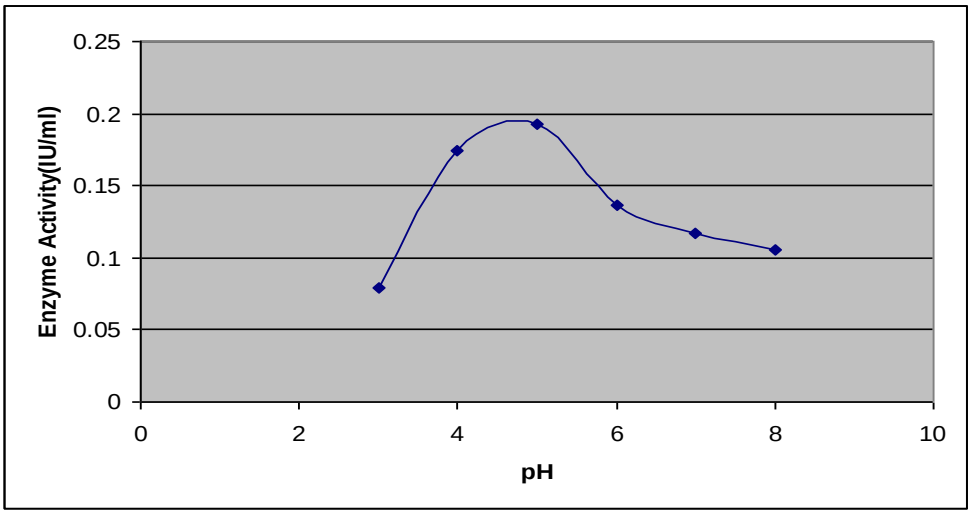

Fig3: *Effect of pH on enzyme activity*

Effect of temperature on Enzyme Activity: The reaction mixture of crude enzyme, citrate buffer, filter paper as substrate and DNS was incubated at different temperatures ranging from 30º to 70º C for 60 min. The highest activity was observed at 50º C temperature. Hence it was the best temperature for exoglucanase activity.

Table5: *Enzyme Activity at different temperature*

Temperature (ºC)	**Enzyme Activity (IU/ml)**
30	0.01253
40	0.01473
50	0.01992
60	0.01767
70	0.01409

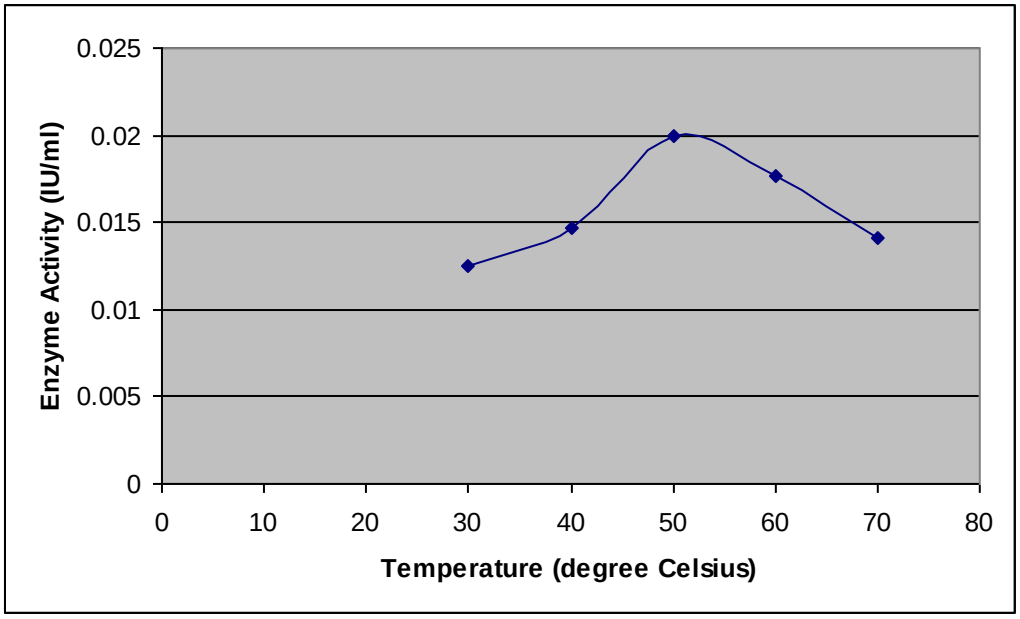

Fig4: *Effect of temperature at Enzyme Activity.*

Effect of pH on Enzyme Production: The fungal strain has exhibited different responses of enzyme production to variation in pH of medium. To estimate the effects of pH value in substrate on cellulase synthesis, the pH value were adjusted by the addition of HCl or NaOH to 4.0, 5.0 6.0, 7.0 and 8.0.The results shown in table 6 and figure 5 showed that the production of exoglucanase activity 0.116 IU/ml was found maximum at pH 6.0. The enzyme activity gradually increased when increasing the pH up to the optimum pH and then followed by a gradual full in activity. The data has been supported by **Gautam *et. al.*, 2011** who have reported the production of exoglucanase activity 1.76 U/mL by *A. niger* was found between 6.0-7.0 and the optimum pH for cellulase activity were found at pH 6.5 whereas **Azzaz *et. al.*, 2012** observed that the cellulase production by *A.niger* in varying pH of medium showed highest values of cellulase activity 0.094 U/ml at pH 6.0. When the pH level of medium increased the enzyme production was decreased. The enzyme system within the same species may vary, depending on the strain under study. An observation reported by **Sindhu *et. al., 2012*** for cellulase production from *Penicillium* species SBSS 30 produced significant levels of cellulase in the range 5.0 to 8.0, with optimum production at pH 6.0. pH below 5.0 and above 8.0 was not favorable for growth and sporulation of fungus, hence there was a decrease in cellulase production at acidic and alkaline conditions.

Table 6: *Enzyme activity at different pH of medium*

pH of media	Activity(IU/ml)
3	0.0795
4	0.10453
5	0.1036
6	0.11655
7	0.10638
8	0.1054

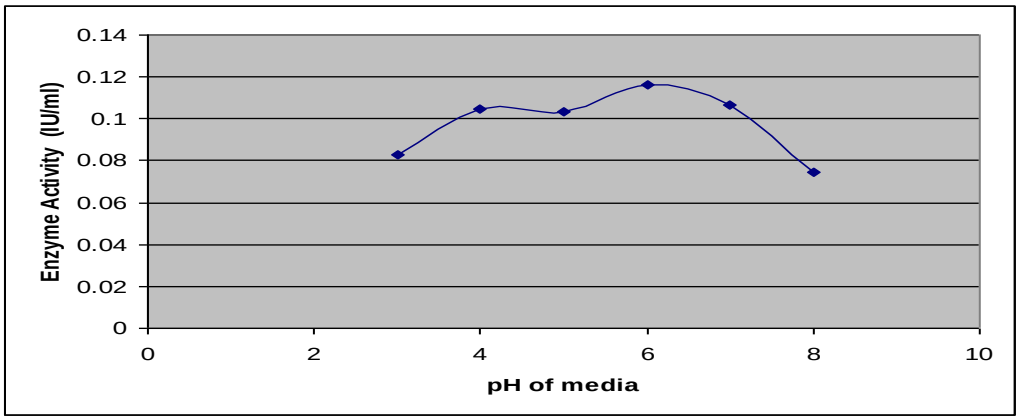

Fig5: *Effect of pH of medium on enzyme Production*

Effect of different Nitrogen sources on Enzyme Production: The data shown in table7 and figure6 revealed that among the six different nitrogen sources, the ammonium sulfate was found to be the best nitrogen source producing the highest level of exoglucanase activity 0.054 IU/ml followed by sodium nitrate, yeast extract, ammonium nitrate, beef extract and ammonium chloride. This data indicating that the inorganic nitrogen source was the best one for enzyme production by *Aspergillus sp.* This results were identical to **Inuwa and Ezekiel, (2010)** who reported that ammonium sulphate ($(NH_4)_2SO_4$) and ammonium dihydrogen phosphate ($NH_4H_2PO_4$) were good nitrogen sources for cellulase synthesis by *A. niger* YL128. It might be due to the reason that ammonium compounds are the better nitrogen compounds for protein and enzyme synthesis as compared to other nitrogen sources whereas **Azzaz *et. al.*, (2012)** indicated that the source of nitrogen should be organic for better result. They reported that meat extract was the best for enzyme production by *A. niger*. All these differences in enzyme production might be due to use of different materials such as different cultural practices, solid matrix or different organisms. In another study of **Tong**

and Rajendra, (1992) who observed that sodium nitrate, sodium nitrite and ammonium nitrate were found to be the best nitrogen sources for mycelial growth whereas ferrum ammonium sulphate and ammonium molybdate were not appropriate and peptone did not support for the growth of *Aspergillus* sp.

Table7: *Enzyme activity at different nitrogen sources.*

Nitrogen Source	Activity(IU/ml)
Ammonium sulfate	0.05365
Ammonium nitrate	0.03238
Sodium nitrate	0.0444
Ammonium chloride	0.02313
Yeast Extract	0.03423
Beef extract	0.02683

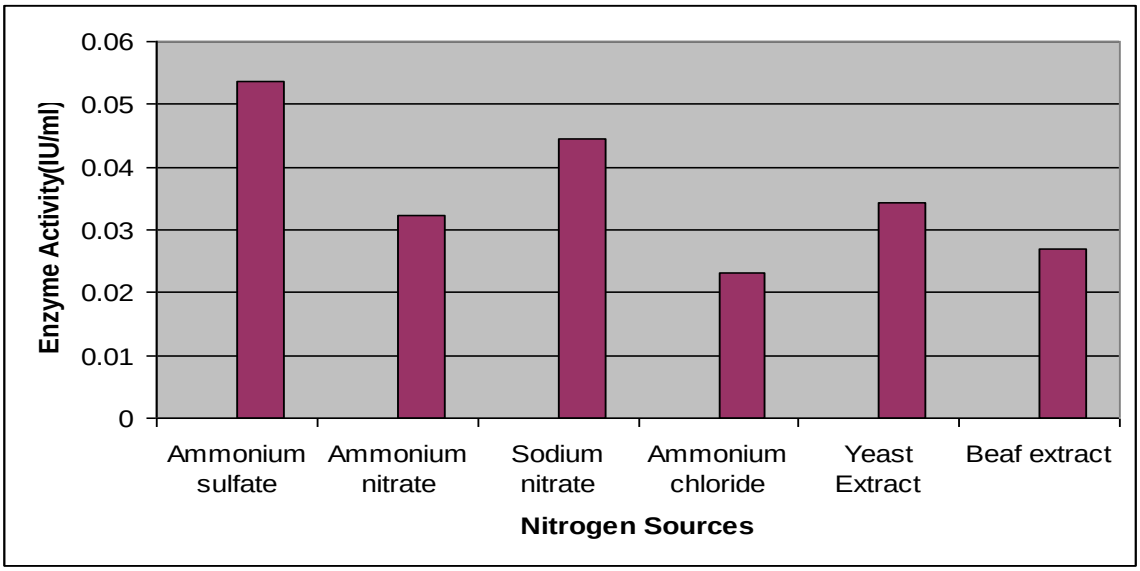

Fig6: *Effect of different nitrogen sources on enzyme production*

Effect of different Carbon Sources on Enzyme Production: Carbon sources play a vital role in the cell metabolism and synthesis of cellulase. Carbon sources experienced for production of cellulase enzyme were cellulose, sucrose, fructose, dextrose and galactose. Among the different carbon sources used fructose was the best carbon source for enzyme production as shown in Table8 and figure7 followed by dextrose, cellulose, sucrose and galactose. But **Gautam *et. al.*, (2011)** observed that the maximum production of enzyme was obtained in culture containing 1.0% sucrose by *A. niger* and *Trichoderma* sp. followed by glucose, cellulose, maltose, and CMC.

Table8: *Enzyme activity at different Carbon sources*

Carbon Source	Activity(IU/ml)
Cellulose	0.01295
Fructose	0.0148
Sucrose	0.01295

Galactose	0.01203
Dextrose	0.01388

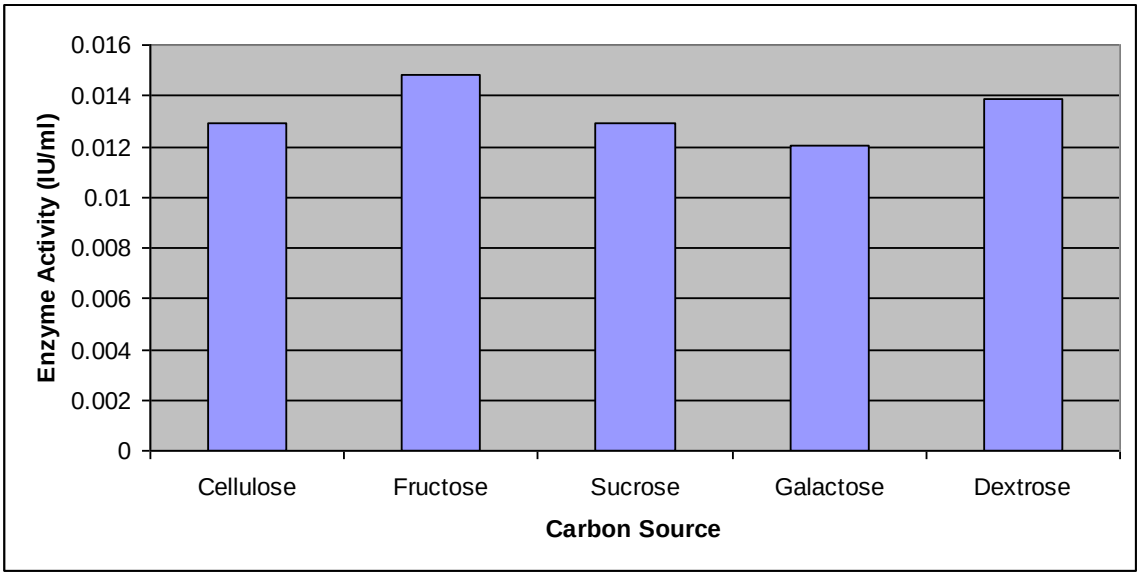

Fig7: *Effect of Carbon sources on enzyme production*

Effect of incubation Period on Enzyme Production: Fungus strain was inoculated into medium in 150mL conical flask and incubated at 28ºC ± 2ºC for a period of 7 days. The cellulase activity was measured at regular intervals. Though, the maximum yield of exoglucanase activity i.e. 0.0536 IU/ml was obtained after 4 days. As table9 and figure8 was shown that the maximum activity of enzyme production was recorded at 6th day of incubation. **Gautam *et. al., (*2011**) found that the best incubation period for enzyme production by *Trichoderma* sp. and *A. niger* was recorded on 5th and 4th day respectively. The incubation period is directly interrelated to the metabolic activities and production of enzyme up to a certain degree. According to the **Azzaz *et. al*., (2012)** the highest cellulase activity was recorded after 3rd day of incubation by *A. niger* grown on wheat straw. It might be due to the depletion of essential nutrients in the medium which created the stressed conditions for fungal strains consequentially inactivation of secretary machinery of the enzymes.

Table9: *Enzyme activity at different incubation time.*

Incubation Time (days)	**Activity(IU/ml)**
2	0.0481
4	0.0518
6	0.0536
8	0.01135

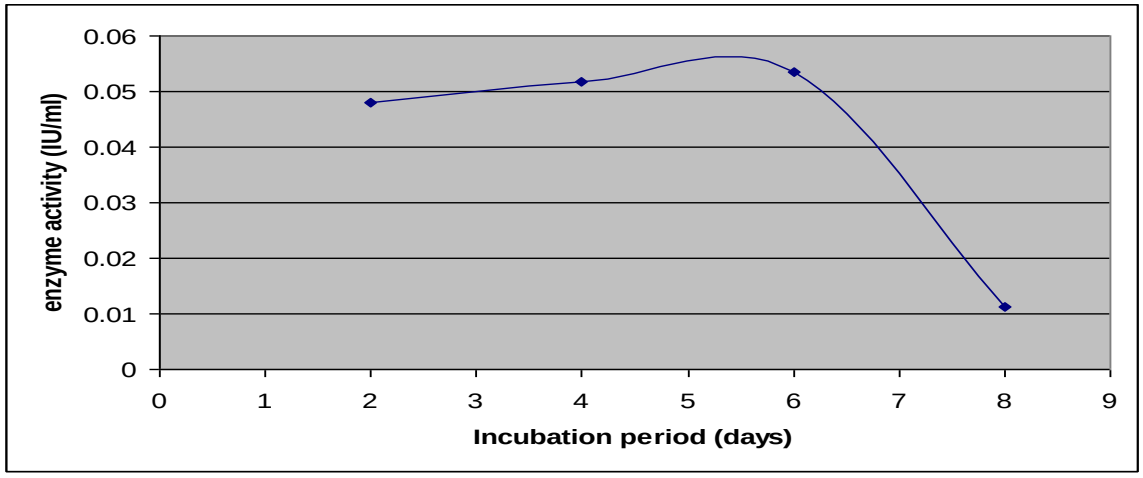

Fig8: *Effect of incubation period on Enzyme Production*

Effect of temperature on Enzyme Activity: Medium temperature plays an important role in cellulase production. The effect of temperature on cellulase activity was determined by incubating the flask at two different temperatures of 28º ± 2°C and 37º ± 2°C and the best temperature for exoglucanase production was 28ºC. The enzyme activity was decreased when the temperature increased and it might be due to thermal denaturation of enzyme. According to **Kumari *et. al.*, (2011); Inuwa and Ezekiel, (2010),** the maximum activity of the cellulase enzyme was seen at 30°C. Many workers have reported different temperatures for maximum cellulase production in flask using *Aspergillus* sp. and *Trichoderma* sp. suggesting that the optimal temperature for cellulase production also depends on the strain variation of the microorganism.

Table10: *Enzyme activity at different temperature of incubation of medium*

Temperature (ºC)	**Activity(IU/ml)**
28±2ºC	0.0536
37±2ºC	0.0407

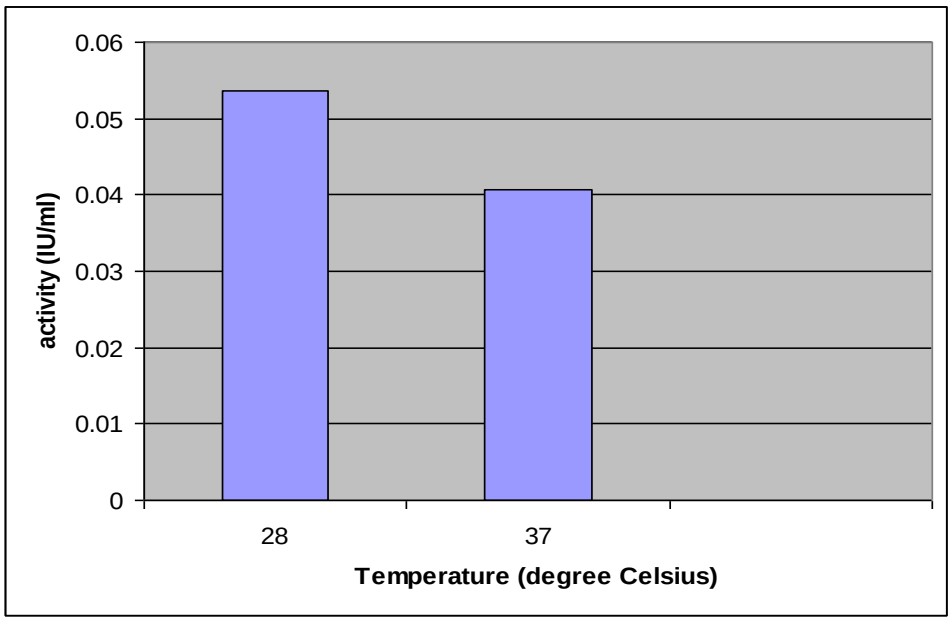

Fig9: *Effect of Temperature on Enzyme Production*

Molecular Characterization of Cellulase producing fungi

Identification: The culture, which was labeled as Sample-W3LG was found to be Aspergillus sp. CCF 4149 genomic DNA containing 18S rRNA gene (GenBank Accession Number: HF937386.1) based on nucleotide homology and phylogenetic analysis.

Gel Image of 18SrDNA amplicon

(Sample: **Sample- W3LG)**

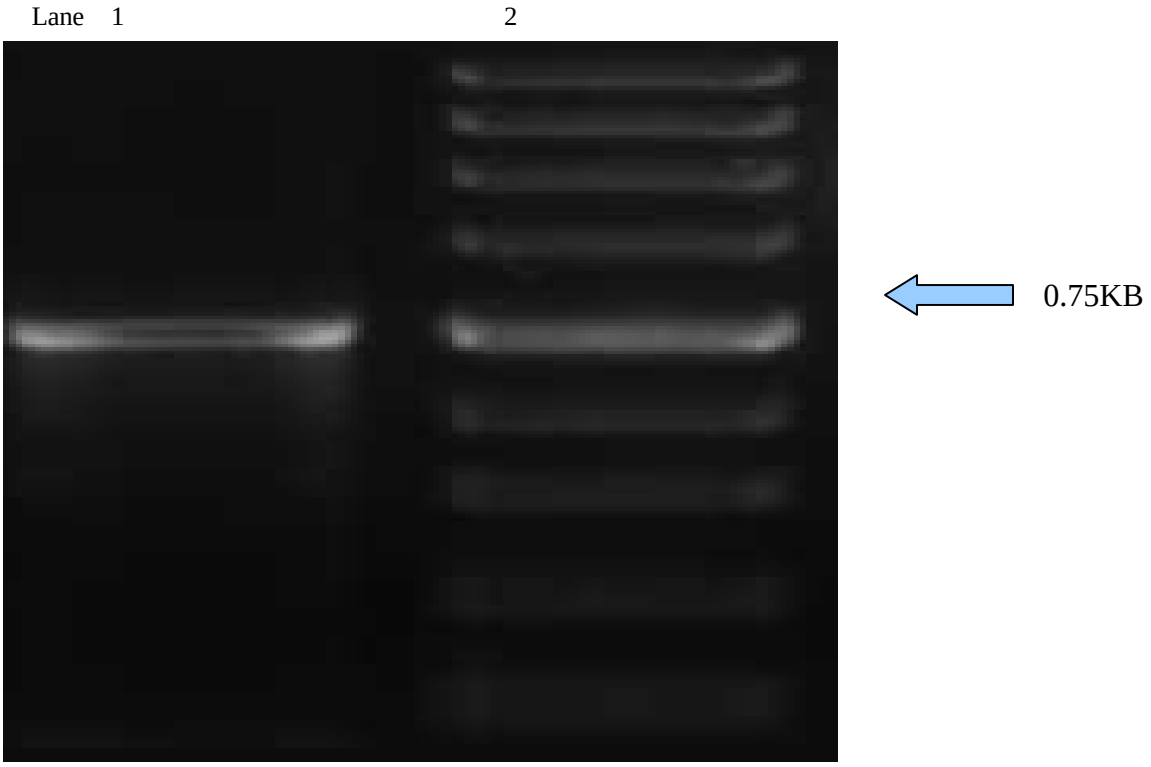

Fig10: *Gel image of 18S rDNA amplicon*

Where: Lane 1 represents the 18S rDNA amplicon band
Lane 2 represents DNA marker

Details of Sequences Generated:

Forward sequence: (484bp)

TCATAGTACGCATCGATACGTTACCAGCGCGTTCCTCGGTCCGGGCAGGCCGATCGCATTGCACCCTC
GGCTATAAGACACCCCGAGAGGTGATACATTCCGAGGGCCTTTGACCGGATGCCCGCCCAAACCGACG
CTGGCCCGCCCACGGGGAAGTACACCTCCCACCCGTGTCTATTGTACCTTGTTGCTTCGGCGGGCCCGT
ATCGCCGTTTCCGAACGGCCGCCGGGGAGGCCTCGCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAA
CATGAACTCTGTTCTGGAAGTATGCAGTCTGAGTTTGATTACCATAATCAATTAAAACTTTCAACAACG
GATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
AGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATCGTAGCGCAGTA

Reverse Sequence: (493bp)
GCTATCAGCGAAATGCGATAAGTAATGTGAATTGCAGGCTATAGCTAAATTCAGTGAATCATCGAGTC
TTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAA
GCACGGCTTGTGTGTTGGGCCCCCGTCCCCGGTTCTCCCCGGGGACGGGCCCGAAAGGCAGCGGCGGC
ACCGCGTCCGATCCCAGTTCTCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGC
CAGCCGACACCCCCAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTA
AGCATATACCGGCACGAGTGCCGGCTGAAACGCGGACCCCGAACTGATCGATCGCGATTACTAT

Aligned Sequence: (732bp)
GCATCGATACGTTACCAGCGCGTTCCTCGGTCCGGGCAGGCCGATCGCATTGCACCCTCGGCTATAAG
ACACCCCGAGAGGTGATACATTCCGAGGGCCTTTGACCGGATGCCCGCCCAAACCGACGCTGGCCCGC
CCACGGGGAAGTACACCTCCCACCCGTGTCTATTGTACCTTGTTGCTTCGGCGGGCCCGCCGTTTCCGA
ACGGCCGCCGGGGAGGCCTCGCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACTCTGTTC
TGGAAGTATGCAGTCTGAGTTTGATTACCATAATCAATTAAAACTTTCAACAACGGATCTCTTGGTTCC
GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA
GTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCT
CAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCCGGTTCTCCCCGGGGACGGGCCCGAAAGGCAGCGGC
GGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCCAGC
CGACACCCCCAACTTT
ATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATACCGGCACGAGT
GCCGGCTGAAACGCGGACCCCGAACTGATCGATC

BLAST data:
Alignment view using combination of NCBI GenBank

Distribution of 200 Blast Hits on the Query

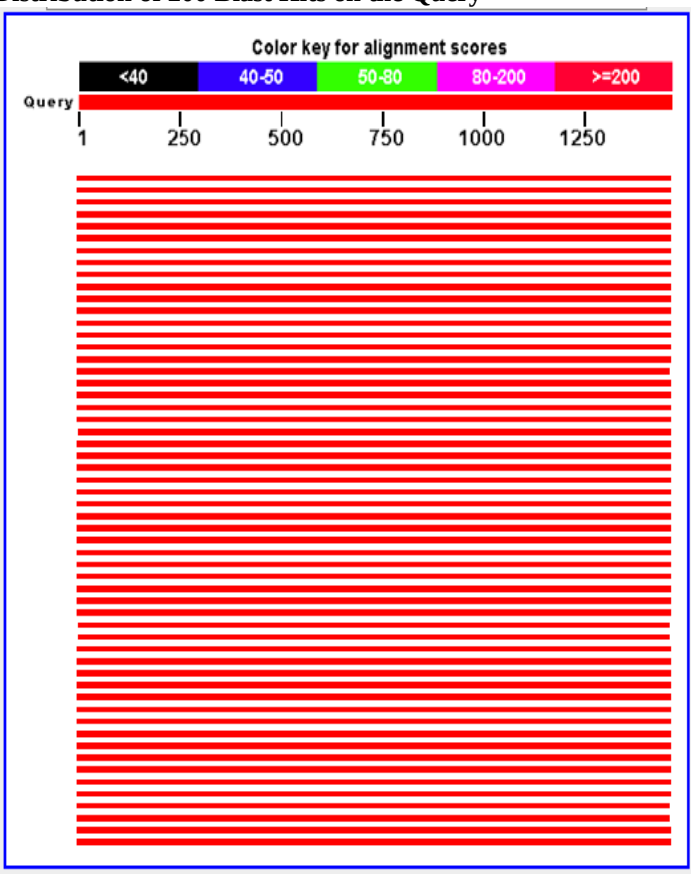

Fig11: *BLAST of aligned nucleotide sequence (732 bp) of fungal isolate*

Sequence Producing Significant Alignments

Table 11: *Description of Sequence Alignment*

Accession	Description	Max score	Total score	Query coverage	Max identity
HF937386.1	**Aspergillus sp. CCF 4149 genomic DNA containing 18S rRNA gene**	**990**	**1193**	**92%**	**100%**
HF937385.1	Aspergillus sp. CCF 4190 genomic DNA containing 18S rRNA gene	979	1182	92%	99%
AB185255.1	Neosartorya glabra genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2	963	963	92%	99%
HE578066.1	Neosartorya hiratsukae genomic DNA containing ITS1	955	955	92%	99%
FR733873.1	Neosartorya hiratsukae 18S rRNA gene (partial), ITS1	955	955	92%	99%
FR733872.1	Neosartorya hiratsukae 18S rRNA gene (partial)	955	955	91%	99%
AB185272.1	Neosartorya glabra genes for 18S rRNA	952	952	91%	99%
HF545008.1	Aspergillus turcosus genomic DNA containing ITS1	941	932	91%	99%
EF669984.1	Neosartorya stramenia isolate NRRL 4652 18S	937	928	91%	99%
AF459733.1	Neosartorya stramenia NRRL 4652 18S	937	928	91%	99%

Dendogram:

File Display Average Caption Help

(A,B) 0.0 0.00

	1	2	3	4	5	6	7	8	9	10	11
1. W3LG		0.000	0.003	0.003	0.005	0.005	0.005	0.004	0.005	0.005	0.005
2. gi|480538421|emb|HF937386.1|Aspergil...	0.000		0.003	0.003	0.005	0.005	0.005	0.004	0.005	0.005	0.005
3. gi|480538420|emb|HF937385.1|Aspergil...	0.004	0.004		0.002	0.004	0.004	0.004	0.003	0.004	0.005	0.005
4. gi|71061072|dbj|AB185255.1|Neosartor...	0.006	0.006	0.002		0.003	0.003	0.003	0.003	0.004	0.005	0.005
5. gi|343772143|emb|HE578066.1|Neosarto...	0.012	0.012	0.008	0.006		0.000	0.000	0.004	0.004	0.005	0.005
6. gi|315131447|emb|FR733873.1|Neosarto...	0.012	0.012	0.008	0.006	0.000		0.000	0.004	0.004	0.005	0.005
7. gi|315131446|emb|FR733872.1|Neosarto...	0.012	0.012	0.008	0.006	0.000	0.000		0.004	0.004	0.005	0.005
8. gi|71061089|dbj|AB185272.1|Neosartor...	0.008	0.008	0.004	0.006	0.008	0.008	0.008		0.005	0.005	0.005
9. gi|409970894|emb|HF545008.1|Aspergil...	0.013	0.013	0.010	0.008	0.010	0.010	0.010	0.013		0.005	0.005
10. gi|152212162|gb|EF669984.1|Neosartor...	0.013	0.013	0.013	0.012	0.013	0.013	0.013	0.015	0.012		0.000
11. gi|18252601|gb|AF459733.1|AF459733 Ne...	0.013	0.013	0.013	0.012	0.013	0.013	0.013	0.015	0.012	0.000	

Fig12: *Dendogram of fungal isolate.*

Phylogenetic Tree:

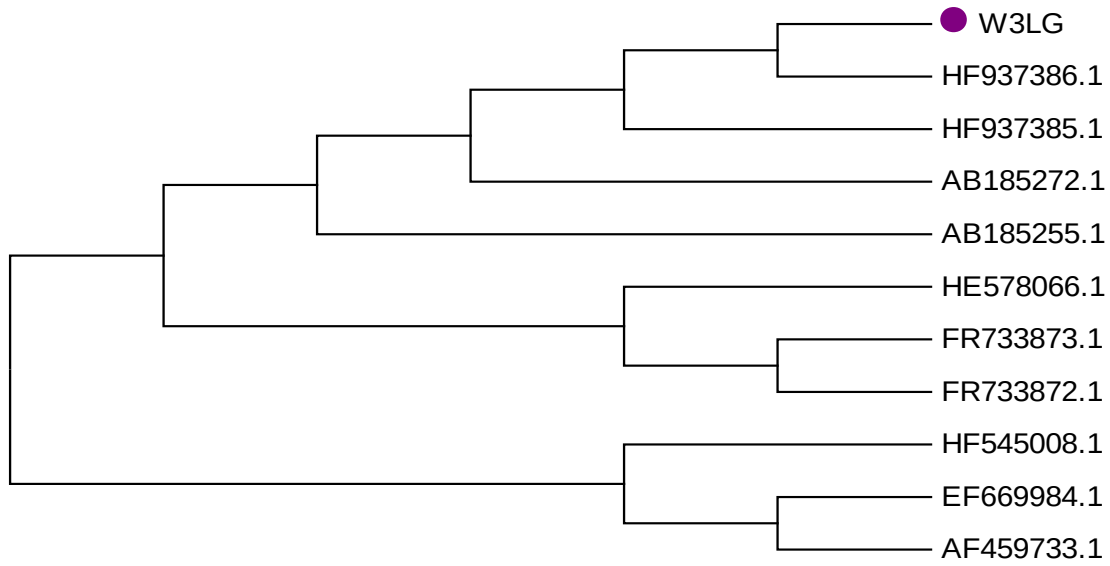

Fig13: *NJ tree showing the phylogenetic relationships among the fungal strains based on 18S rDNA sequences.*

Evolutionary relationships of eleven taxa: The evolutionary history was inferred using the Neighbor-Joining method **(Saitou and Nei, 1987)**. The optimal tree with the sum of branch length = 0.02803329 was shown in fig.

The evolutionary distances were computed using the Kimura 2-parameter method (**Tamura *et. al.*, 2007)** and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 526 positions in the final dataset. Phylogenetic analyses were conducted in MEGA.4 **(Felsenstein, 1985).**

CONCLUSION

Cellulose is the most abundant organic polymer which may be hydrolyzed by enzyme cellulase into smaller sugar components including glucose subunits and these smaller subunits can be used for production of ethanol, organic acids and other chemicals. For achieving enormous benefits of biomass consumption cellulase provide a key opportunity. Cellulase has massive prospective in industries and is used in textile, detergent, paper and pulp, beverages, laundry, food industries etc. Therefore, there has been much research for obtaining new cellulase producing microorganisms with higher activities and better efficiency.

In the present study, five fungal isolates namely B, W1, W2, W3LG and W3DG were isolated from soil samples collected from paper and sugarcane mill and screened for cellulase production. The isolated strain showing maximum zone of hydrolysis of cellulose was later identified as *Aspergillus sp.* Identification of the isolate was examined on the basis of its colony characters, microscopic examination and recognition at molecular level. Cellulase production by this fungus was measured by using enzyme assays.

After comparing three cellulase enzyme system, endoglucanase were gave highest value of activity than exoglucanase and β-glucosidase. For enzyme activity the best temperature was 50ºC and pH was at range of 4.0-5.0.The isolated *Aspergillus sp.* produce cellulase enzyme at pH 6.0. From various nitrogen sources tested, ammonium sulfate was found to be best for cellulase enzyme production because of maximum activity of 0.053 IU/ml. The highest activity reached 0.0148 IU/ml was obtained by using fructose as carbon source. The maximum enzyme production was observed as 0.0536 IU/ml on 6^{th} day of incubation and at 28ºC temperature of fungal growth. Fungal growth was repressed by FeO while CuO was not had any effect on fungal growth.

It was examined on the basis of its colony characters, microscopic examination and recognition of isolate at molecular level based on DNA coding for 18S rRNA. The unknown isolates were identified by amplifying and sequencing of 18S rRNA gene. After amplification the length of amplified DNA was obtained as 532bp and amplified PCR product was compared with sequence obtained from NCBI. A Phylogenetic tree was formed on the bases of comparison.

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Abstract Published:

Navita Pathania, Meenakshi Devi, Rachna Chaudhary and Pachouri U. C. (2012**)** "Cellulase Production: The Need of Present Era" An extended abstract was presented in 3rd Bhartiya Vigyan Sammelan and Expo on "Science for Global Development" October 11-14, organized by Vigyan Bharti, Punjab Technical University, Lovely professional University and Punjab State Council of Science and Technology India.