Production, Purification and Characterization of Cellulases from *Humicola* Sp.

By

Tasleem Khan

216-FBAS/MSBT/F-15

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University, Islamabad

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Researcher

Tasleem khan

216-FBAS/MSBT/F-15

Supervisor

Dr.Syed Ali Imran Bokhari

Assistant Professor

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University, Islamabad

(2015- 2017)

"In the name of ALLAH The Most Gracious and The Most Beneficial"

Department of Bioinformatics and Biotechnology

International Islamic University, Islamabad

Dated: _____

FINAL APPROVAL

It is certified that we have read the final thesis submitted by **Mr. Tasleem Khan** and it is our Judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the MS Degree in Biotechnology.

COMMITTEE

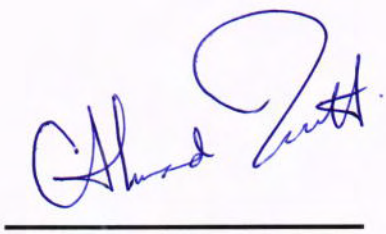
External Examiner

Dr. Ahmad Bilal

Deputy Director

Ministry of Science & Technology

Islamabad, Pakistan

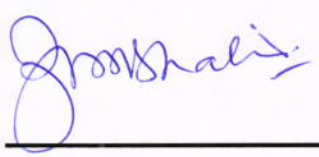


Internal Examiner

Prof. Dr. Zafar Mahmood Khalid

Bioinformatics & Biotechnology

International Islamic University Islamabad



Department of Bioinformatics and Biotechnology

International Islamic University, Islamabad

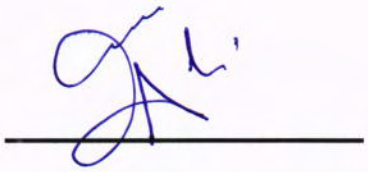
Supervisor

Dr. Syed Ali Imran Bokhari

Assistant Professor

Department of Bioinformatics and Biotechnology

International Islamic University, Islamabad



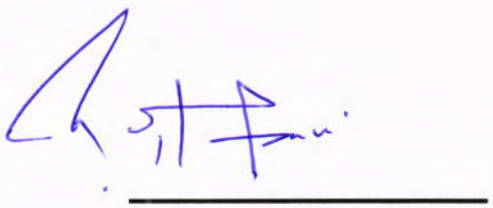
Chairman

Dr. Asif Mir

Associate Professor

Department of Bioinformatics and Biotechnology

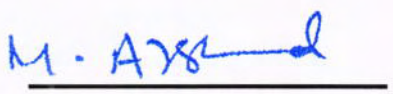
International Islamic University, Islamabad



Dean, FBAS

Professor Dr. Arshad Zia

International Islamic University, Islamabad



A thesis submitted to Department of Bioinformatics and Biotechnology,
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This humble effort is

Dedicated

To

My beloved

Parents,

Friends, Family

And

Teachers

Who inspired me for higher ideals of Life

DECLARATION

I hereby solemnly declare that the work **"Production, Purification and Characterization of Cellulases from *Humicola* Sp."** presented in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated: _____

Tasleem Khan
216-FBAS/MSBT/F-15

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Tasleem Khan

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ABBREVIATIONS

Gly	Glycine
Ser	Serine
Ala	Alanine
C-terminal	Carboxyl-terminal
N-terminal	Amino-terminal
ATPase	Adenosine triphosphatase
MFP	Membrane fusion protein
Dsb	Disulfide bond
mM	Millimolar
EDTA	Ethylenediaminetetra acetic acid
PMSF	Phenylmethylsulfonyl fluoride
h	Hours
w/w	Weight/weight
W/V	Weight/volume
v/v	Volume/ volume
U/mg	Unit/milligram
U/ml	Unit/milliliter
SDS	Sodium Dodecyl Sulfate
PAGE	Polyacrylamide Gel Electrophoresis
kDa	Kilodalton
DEAE	Diethyl aminoethyl
min	Minutes
FAMEs	Fatty acid methyl esters
rRNA	Ribosomal Ribonucleic Acid
bp	Basepair
DAP	Diammonium phosphate
aa	Amino acids

ABSTRACT

Cellulose is a vital naturally occurring polymers on global earth and cellulose constitute almost 90% of bio-waste substances. It is a major living source and cheap energy reservoir based on energy. There are a lot of nutritional and biochemical processes that perform degradation of cellulose, hemicellulose and lignocellulosic materials. The degradation of cellulose by bacteria and fungi have been extensively studied.

Cellulase, is a group of enzymes having complex structure and this class of enzymes are equally valuable in health, medicines, agriculture demining of fabrics, detergents paper and pulp industries. Cellulases are the world's most abundant enzymes, consisting of natural polymers 'cellulose 'and lignocellulose into reducing sugars and used in many industrial and biotechnological applications like biofuel and bioethanole production etc. Most of the fungi involved one or more cellulolytic enzymes including endoglucanase, exoglucanase and β- glucosidase.

The current research was conducted for the production, purification and characterization of cellulase enzyme from *Humicola specie*. Many optimized parameters are used for the growth of *Humicola fuscoatra.* Optimized parameters like incubation time, inoculums size, different carbon and nitrogen sources, different pH ranges and temperature were used step by step in Vogel's medium for the isolation and purification of these enzymes. The result showed that 10 % inoculum size, 48 h of incubation time, DAP as nitrogen source, medium pH- 7 and 45°C temperature were the most favorable requirements for the production of cellulases by *Humicola fuscoatra* specie under submerged fermentation (SmF). Crude extract was subjected to ammonium sulphate precipitation using 20 to 80 % saturation. Among all of these saturations 40 % was the most suitable for purification of cellulase enzyme. Characterization was performed for the purified cellulase and checked various kinetic parameters like pH and temperature. The effect of pH range (4.0-8.0) and temperature (20-65°C), and different concentration of carbon sources on purified cellulase were checked. The CMCase enzyme production at pH - 7 was 24.64 , Exoglucanase production was 17.49 and protein estimation was 769.20 µg /ml.

The result showed that CMCase production was 15.42 U/ml at 45°C , Exoglucanase production was 13.39 U/ml and protein estimation was 848.47 µg/ ml from all of the mentioned temperature (25 -65°C).

INTRODUCTION

1. INTRODUCTION

Cellulases are complex enzyme system that breaks down the bond linkages in cellulose fibers, lignocellulose and other carbohydrate based materials. These enzymes are produced by many microorganisms such as plants, animals and other living entities. Fungi and bacteria are the first organisms producing cellulases. They have different classes due to their varying structure and sequence of amino acids linked in them (Henrissat, 1991). Cellulases contain various hydrolytic binding units and modules that are placed at the two terminals N and C terminus. The cellulose degradation is performed by these enzymes naturally.

For the breakdown of cellulose into its respective units, the microorganism's secrete extraordinary content of these enzymes. Cellulases play important role and are used commercially for production of various food items and detergents. These enzymes facilitate digestion of feed straw of different animals. They are considered as the world's best commercial market enzymes in different industries. (Bayer *et al.*,2007, Zaldivar*et al.*, 2001).

Biotechnological prospective of cellulases in many industries such as food, feed straw, medicine and drug has been a driving force for intense research focus on cellulases for past several decades. However, application of cellulases for breakdown of cellulosic wastes for production of renewable biofuel has a major research focus in recent years in view of rapidly diminishing fossil fuel reserves and world's major source of energy supply, because of serious environmental concerns including greenhouse gas emissions, and rapid fluctuations in market prices of petroleum products. Among several energy resources, bioethanol generated from lignocellulosic biomass (LB) has emerged as one of the strongest candidate for renewable transport fuel resource. Abundant quantum of LB generated from agricultural and forestry represents an inexpensive, and underutilized renewable feedstock.

Cellulose fibers breakdown enzymes are produced by using optimized growth parameters. They are produced by solid state as well as submerged fermentations (Lynd *et al.*, 2002). Among all of these microorganisms, fungi have the best potential to produce cellulases. Organisms which generates the cellulase enzymes are mainly *T. reesei* which

has the ability to generate these enzymes. A lot of filamentous fungi also produced these enzymes in global market.

In 1980, the major work done was started on cellulases and hemicellulases which were used in food items and different ingredient of diet.(Godfrey and West, 1996). Today, these enzymes account for around 20% of the world enzyme market (Mantyla *et al.*,1998) mostly from *Trichoderma* and *Aspergillus.* In 1930, xylanases and pectinases were used in food markets largely, and majorly the utilization of these important enzymes, cellulases and xylanases showed significant values in different fields of life. The world market account for 20 % of these enzymes.

Cellulases are the enzymes which convert the most naturally occurring polymer cellulose into reducing sugars and are used in many industrial applications like bioethanol and biofuel (Bhat, 2000). Many microorganisms degrade cellulose but fungi are the main agents of degradation of cellulose and used for commercial production of cellulases (Bhat,2000). Endoglucanases hydrolyze the biopolymers to produce its derivative oligosaccharide residue. Similarly, β-glucosidase convert cellubiose or other oligosaccharide into glucose unit (Bhatia *et al.*,2002). The synergistic mode of action of these enzymes are responsible for degradation of various cellulosic biomasses into its residues. Among all of the organisms, fungi species of *Trichoderma and Aspergillus* are well known for cellulolytic potential (Lynd *et al.*,2002). The cellulase enzyme production of *Trichoderma reesei* has greater limitation of being devoid of β-glucosidase activity (Rosgaard *et al.*, 2006).

Cellulases are the multicomponent enzymes which are responsible for conversion of lignocellulose and other waste substances in simple saccharide units. In 1960, the initial work was done by protein surface cross linking reactions. Mostly amino acids and their amino groups were used for surface attachment of protein. Cellulose have a crystal like structure having long chain of polymers of glucose units. The enzyme cellulase degrades these celluloses and lignocelluloses and many bio-waste materials forming various useful industrial substances and cosmetics(Li et al., 2006; Gao *et al.*, 2008).Cellulose which are naturally occurring energy source nowadays. In terrestrial environments cellulose plays the role of primary source for photosynthesis and major sustainable energy reservoir in the environment (Zhang and Lynd, 2004). The production

of cellulases and amylases shows unique qualities and generated by optimized growth conditions. Basidiomycetes are a type of fungi which causes a rapid degradation of wood and carbohydrate component (Eriksson *et al.,* 1990).

Rice bran is the major carbon source generated in Agriculture and Industrial sector. It totally accounts for 50% of the dry weight of the processed raw material. It is cheaper carbon and nitrogen source in order to decrease the production costs. In global market, several million metric tons of this residue is produced annually. Rice bran is a poor animal food having low protein content. Rice bran does not find any major commercial application till now and most of this by product is generally predisposed of in an open area, leading to potentially severe environmental complications. Given this situation, it is necessary to look for processes that allow the controlled elimination of this residue or its industrial reutilization.

Bacteria and fungi are equally important in production of cellulases enzymes among all of the microorganism fungi are the best agent for production of these targeted enzymes and *T. reeseae* is also one of them producing cellulose enzyme (Amouri & Gargouri, 2006). *Aspergillus niger* and *T. reesae* are the fungi which have the ability of generation of cellulases. For complete breakdown of cellulosic substrate into simpler sugar units *T. herzianum* is the best one specie.

In degradative processes enzymes plays vital role, these enzymes are produced by microorganisms which are required in large quantities and having larger commercial demands. For other cellular activities a variety of enzymes are necessary which helps especially in growth of the microorganisms (Solomon *et al.*, 1999). In nature cellulose is founded in long polymer form of glucose units connected by covalent bonds. Enzymes are very necessary and required for all metabolic activities to cells and bodies of microorganisms. They are used in different fields of life such as in production of single cell protein, wines, animal feed stock and high level of glucose (Solomen *et al.*,1999). In environment enzymes improve accessibility of food, feed stuff and other useful wastes. They are used for a long time. These enzymes are necessary in each and every industry almost. For example, medicine, health, food, feed straw, detergents and agriculture. Cellulases improve the digestion of nutrients and used in energy sectors (Bhatti *et al.,* 2013).

Cellulases have been widely used in food and pharmaceutical industries and also for conniving environmental pollution (Niaz*et al.*, 2011). Endoglucanases carboxymethylcellulases and beta glucosidases (ß-1,4-D- glucan-4-glucanohydrolases, EC 3.2.1.4) are a complete cellulase system. The other members of cellulases include Exoglucanase (EC. 3.2.1.91) and cellobiases (EC. 3.2.1.21), which act in synergy (Jabbar *et al.*, 2008). Cellulose is the major component of plant biomass. It is a linear, un-branched homopolysaccharide comprising of glucose residue joined together by ß 1-4 glyosidic linkages and its molecules are varying widely in length and are usually arranged in bundles or fibrils (Walsh, 2002).

Enzyme blend has the characteristics to modify the taste and flavor properties of different food items. For production of freshly food items these companies use different enzymes so as to improve flavor and taste of foods. In developed countries like UK, USA and Japan they used these enzymes to improve citrus foods, fruits and best salads. Next xylanases and cellulases mixture remove the extreme unpleasantness in fruits and citrus peels (Roe and Bruemmer, 1977). Worked on the degradation of cellulose materials which is a main factor this was done by enzymes because the process was cheap, easily accessible, economic and purification was also cheap and simple. Extra ordinary research has been conducted so that to produce larger amount of cellulases, hemicellulases by using bio-waste materials.

Utilization of agricultural and industrial wastes for the production of cellulase enzymes from microorganisms has also been found anattractive way to resolve the environmental pollution problems. The present study describes the production of CMCase by an *Aspergillus* species using wheat straw as a carbon source. Carboxymethyl cellulase is the major enzyme of the cellulases complex as it initiates the attack on cellulose. Researchers have been obtained major interests in cellulases because of their utilization in several industries (Jamil *et al.*, 2005). In recent years the interest in cellulases, hemicellulases,xylanases and pectinases has increased due to their application in the production of bio- fuel energy and bio-ethanol gases.

The hydrolysis of cellulosic and other bio wastes through a complex enzymatic system may yield a reasonably yield greater amount of food products by utilization of low cost energy during fermentation techniques. Researchers have been made greater

efforts and findings have been made by enzyme suppliers and industrialist to improve accessible enzymes as a result, thermophiles species are being insistently pursue to provide novel enzymes that are extremely thermostable depending on the environmental habitat of the native organism. Major interest has been used for production of cellulases enzyme. They are utilized anywhere in food and fabric industries for production of bioethanol and bio gas (Bayer*et al.*, 2007). It was struggled to optimized the hydrolytic performance to be enhanced and made this technique cheap and viable for marketing values (Sheehan and Himmel, 1999). For complete breakdown of cellulosic wastes three types of enzymes named as Exoglucanase, endo glucanase and beta glucosidase are used. These enzymes are used for hydrolysis of cellulose. They used different enzymes like endo and Exoglucanase for proving maximum synergy of it. This process was costly and un healthy with respect to energy demands. In order to seek for transparency, the functionally based kinetic model for enzymatic hydrolysis of pure cellulose by individual enzymes has been developed (Zhang and Lynd, 2006).

Haakana *et al.*, 1997) although not for active cellulose enzyme systems.Later on, a congregation of an efficient cellulome has been obtained through yeast. This cellulome has the characteristics of endoglucanase and Exoglucanase. The structure of cellulosome as breakdown units called domains linked by CBD.The hydrolytic domain is linked by a unit called dockerin unit which is responsible for assemblage of cellulosome in a complex enzyme system. The use of exhaustive raw material and process yield, there is necessary a fermentation technique which is economically easy and feasible. The measuring units are very critical for extra amount of products. For the production of novel and valuable cellulases which have greater demands in industry, we used optimized conditions in many of the fermentation processes for cellulose production by using fungus *Aspergillus niger* explained and worked on the kinetic mechanisms of partially impure enzymes known as Exoglucanase and endo glucanase from the fungus *Aspergillus niger*.

During fermentation, optimized growth parameters and dietary requirements such as incubation time, aeration facility, development and growth conditions were to be enhanced so that to gained larger products formation and items of foods. The role in the growth of microorganisms and subsequent product formation Cellulosic and

Lignocellulosic insubordination, that retards the fermentable saccharides units through enzymes. The combination of lignin, pectin, cellulose made the plant cell wallto get the better amount of glucose products initial steps and measuring kinematics should be improved from feed stocks (Chandra *et al.*, 2007).

Nowadays global demands forthe production of biofuels on large scale from living sources, which is the need for economical and global energy sources. The fossil fuel should be used here so as to utilized and get much environment healthy fuels. Biofuels are used in the united states. They are attractive means of energy sources for sustainable developments. From corn derived starches net fuels energy production and lower input energy costs are consumed (Brennan*et al.*, 1996. The production of 10 Mt ethanol from wood is projected by the year 2010, leaving the presently used starch-based process far behind. Moreover, direct fermentation of biomass to solvents by bacteria seems to be economically viable and efficient and could ultimately be recognized without the addition of external enzymes, if there used cellulolytic microorganisms of standard qualities (Zaldivar *et al.*, 2001. In near future, cellulase and its group enzyme system will be used for the degradation of urban and agricultural wastes to get valuable end products like biofuels. These enzymes will expedite the conventional fermentation techniques and improve fodder quality and to facilitate flavor and syrup extraction. In fact, Yamada stated that 45 tons of cellulases are more worthy than 170 million yen. The wide range of substrate hydrolysis exist for cellulose conversion and give us ethanol (Cooney *et al.*, 1978).

Bhat,(2000), Anish *et al.*, (2007).Kibblewhite*et al.*, (1995) used these enzymes for the betterment of drainage system of homes and industries. They are used in different factories and mills to improve this system of drainage. They are used to peel off fibers and fibrils on the colloidal surface substances which causes harmful issues of drainage in mills. Therefore, cellulases and hemicellulases play significant role in this functioning (Kantlinen et al (1995).

2. AIMS AND OBJECTIVES

- Optimization of carbon and nitrogen sources for cellulases production from *Humicola* sp.
- Optimization of growth temperature and media pH for cellulases production from*Humicola* sp.
- Partial purification of cellulases from *Humicola* sp.
- Calculation of various kinetic parameters of partially purified cellulases.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Bayer, (1994) performed his research work on filamentous fungal strains of *Trichoderma reesei* for cellulase enzyme production. *T. reesei* has the ability of producing larger quantities of extracellular cellulases. This species degrades various cellulosic and lignocellulosic biomasses. Cellulases are produced by many organisms like bacteria and fungi, having diverse features. At commercial scale cellulases are usually used in medicines, fabrics, textile industry,bio stoning and bio finishing of cotton. *T.reesei* has the ability of producing homologous and heterologous cellulases. *T.reesei* performed production of endoglucanase II, cellubiohydrolases I and II (CBHI and CBHII). The cellulase genes were transformed under powerful genes of *T. reesei* cbhl promoter. The level of production of endoglucanase produced by transformation techniques was compared with the copy number of the eglII expression gene cassette. The data showed that CBHI was increased up to 1.6 fold and CBHII production was noted 3, 4 fold compared with the host strains to that of parental strains. Further, same amount of CBHII protein were produced using the selected genes cbhl and cbh2 promotors. This research revealed that novel neutral cellulases has the potential of bio stoning. Cellulase production from thermophilic fungus *Melanocarpus albomyces* was proved to be active in secretion of dye from indigo dyed denim. The cellulase p roduction from recombinant *Melanocarpus albomyces* fungus (Ma cel45 A) performed best production at neutral pH in the bio finishing of of denim fabrics.

Bhat, (2000) performed the valuable research on cellulase enzyme. They worked on cellulases, hemicellulases and pectinases and has pointed out vital scientific knowledge in biotechnological research. Recently cellulases, cellobiohydrolyses and β D galactopyranosidases are produced by many filamentous fungi under solid state fermentation as well as submerged fermentation. In fact, the marketing demand of these enzymes is growing more rapidly day by day in different fields of life like health, pharmaceuticals, food, textile, animal feed, pulp and paper industry as well as wine production. Cellulases and their related enzymes are also widely used in agriculture sector.

Botella *et al.,* (2005) produced several hydrolytic enzymes like cellulases pectinases and xylanases by using *Aspergillus awanori* as a source of microorganism

under solid state fermentation process. The major biowaste in the wine industry were used as carbon source for *Aspergillus* sp and produced these hydrolytic enzymes. They conducted 7 days' experiments and the enzyme extracts were obtained in different interval of time. They pointed out grape pomace and maize straw a good substrate for cellulase enzyme production.

Naim and Jamil., (2007) used many fungal species for the production of different enzymes such as cellulases, cellobiohydrolyses and xlanases. In this study endoglucanase production was performed from thermostable fungus *Chaetomium thermophile*. The carbon sources were added in the Vogel's medium. Xylane, corn cobs, glucose and carboxy methyl cellulose was used for the fungus growth for 5 days at 180 rpm in shaking incubator. CMCase were produced in lesser amount by the selected fungus *Chaetomium thermophile*. The two carbon sources xylane and carboxy methyl cellulose produced the enzyme in larger amount while glucose produces it in less amount. The optimized conditions used here were pH 5.0 , temperature 50 °C, incubation period 120 h and substrate 1 % for the selected fungal species. The partially purified endoglucanase unit was 0.064 U/ml. the protein estimated was 0.103 mg/ml and specific activity was noted 0.615 IU/mg.

Ahmed *et al.,* (2009) highlighted the structure and morphology of plant cell wall. According to him the cell wall of plant are constituted by cellulose, hemicelluloses and lignin. Among all these, cellulose is the major component of plant cell wall. Cellulose are degraded by cellulose degrading enzymes which give opportunity for biomass utilization. The filamentous fungi including *Trichoderma harzianum* yielded cellulases, Exoglucanase (EXG), Endoglucanase (EG), and β D galactosidase (BGL) were obtained from the selected sp of fungi. The optimal suitable conditions for this fungus was 5.5 pH, temperature 28 °C and 120 h of incubation period. For the better yield of cellulase production different carbon sources such as glucose, CMC (carboxy methyl cellulose), corn cobs, wood chip, wheat bran, and xylane were selected and used. The data obtained through these carbon sources are noted it shows that CMC produces large amount of cellulase production while glucose suppressed the production of cellulase. This fungus was cultured at 28°C for 120 hr with 1 % CMC. Cellulases and their related enzymes were then purified from the culture filtrate of *Trichoderma harzianum* by precipitation of

Ammonium sulphate further followed bygel filteration on sephadex G250, G50. Specific activities of these enzymes were checked after purification of the enzymes. Exoglucanase, endoglucanase and β D galactosidase showed maximum activities of49.22, 0.63 and 0.35 respectively.

Gori and Malana., (2010) concluded that the extracellular cellulase enzymes have a major role in biomass utilization. Cellulase enzyme can be produced through many bacterial and fungal species. To fulfill the marketing demand of cellulases scientists are now days working to optimize the growth parameters for cellulase producing species. Here in this current study cellulase enzyme was produced under solid state fermentation process by using selected *Aspergillus* species. For maximum production of cellulase wheat straw were used as a carbon source. Temperature 35 °C, pH 4.0 were the optimized conditions for this enzyme production. Carboxymethyle cellulase enzyme activity was 4% wheat straw, 0.125 %cane molasses and 1.5 % yeast sludge.

Jonathan *et al.*, (2010) highlighted production of Cellulosic ethanol and other feed stocks are a good source of vital bioethanol and biogas. The recent techniques to obtain carbohydrates from cellulose due to formation of plant biomass. For the source of metabolic glucose Insects secretes various symbiotic enzymes to proficiently utilized the lignin and cellulose materials. However, conventional biological and biochemical techniques have been used to identify and characterize these enzymes, the improvement of genomic and proteomic research work are expected to allow new insights into insect digestion of cellulose.This information which is highly relevant to the design of improved industrial processes of biofuel bioethanol production and to identify potential new targets for development of insecticides. This review describes the miscellaneous methodologies and techniques used to detect, quantify, purify, clone and express cellulolytic enzymes from insectsand elaborate their significance and limitations.

Leonid *et al.*,(2011) performed worked on the production bioethanol from lignin and cellulose. Cellulose is the major agent of manufacturing the cell wall of plants and it produces larger amount of ethanol and fuels. The cellulose hydrolyzing bacteria are used to produced cellulases and cellubiohydrolases. A genomic based researched was conducted for the identification of cellulases enzymes. Here, we find out the range of cellulases and intend a genomic approach to defeat this limited access.

Milala *et al.*, (2011) conducted research on *Rhizopus stolonifer* and produced cellulase enzyme production. The enzyme was then purified by Ammonium sulphate precipitation method. The carbon sources used here were millet stalk and saw dust for the growth of the organism and so as to induce enzyme production. Cellulase activity was determined by consuming cellulose and substrate at 25°C and pH 5 at room temperature 28°C and pH 5 after time interval of 192 h. The enzyme unit for millet stalk and saw dust were 25.3U/ml and 33.00 U/ml respectively. The enzyme was then partially purified by Ammonium sulphate precipitation of the 72 h. From the current study it was cleared that *Rhizopus stolonifer* was the ability of secreting cellulase enzyme in the culture medium containing these two carbon sources. The data obtained also showed that proper pretreatment processes of millet stalk and feed substrate may enhances higher yield of cellulase.

Mrudulwa and Murugammal, (2011) conducted his research work using *Aspergillus niger* on solid state fermentation for cellulase enzyme. The technique found best was solid state as compare to submerged. The highest enzyme production was noted after 72 h of incubation period in solid state and 96 h in submerged fermentation. CMCase activities founded in solid state fermentation (SSF) were 8.89 U per gram of dry mycelia bran while in Smf CMCase activities were found 3.29 U per ml cultural broth. The nutritional and physical requirements were availed to obtained highest level of enzyme. The parameters of fermentation like pH, temperature, substrate, carbon and nitrogen sources were optimized. The pH 6, temperature 30°C were optimized for high amount of cellulase production. The lactose, peptone and coir waste as substrate were used and showed maximum productivity both in solid and submerged fermentation(Smf). The moisture ratio of 1:2 (w/v) was reported for highest production of cellulase under solid state fermentation (SSF).

Sasi *et al.*, (2012) collected different water sample from different places from the east coast of India. They isolated so many *Aspergillus* species and selected five of them. These species were identified by using lactophenole cotton blue staining method. For the production of cellulase enzyme, *Aspergillus flavus* was selected and tested in modified park,s agar for carbon sources, good optimized facility of aeration, heat, pH, nitrogen compounds and incubation time. For The purification of the enzyme a salt method known

as ammonium sulphate precipitation was used then it was forwarded to HPLC. SDS PAGE was then runned for its molecular weight determination. The current result showed that cellulase production is higher at pH 8 and temperature 30°C on the seven days of incubation period. The result showed that the enzyme production rate was maximum on 30°C after an incubation time of 7 days and this was also cleared that the carbon source rice bran showed high level of cellulase production.

Soares *et al.*, (2012) worked on cellulases production from the waste of coir pith by the help of *Aspergillus nidulans*. $AJSUO_4$ and the conversion of coir pith into bio fertilizer for the production of *Solanum lycopersicum*. NaoH is used for the feed stock material. The experiment was fascinated under coir pith with best suitable conditions of 60 % moisture content, temperature 40 °C and pH 5, under solid state fermentation process. This experiment was runned for 11 days for optimum production of cellulase enzyme. Further, the newly freshed enzyme was purified by a salt method known as ammonium sulphate precipitation method, ion exchange chromatography and dialysis. *Aspergillus nidulans* $AJSUO_4$ was seen to utilized and yielded endoβ glucanase, exo β glucanase and β glucosidase. The type of cellulase enzyme was produced by waste coir pith and converted into bio fertilizers using *Azobactor Chrooccum* and several bacillus species.

Charitha *et al.*, (2012) worked on cellulase enzyme production by different fungal strains of *Aspergillus niger* against cellulosic and lignocellulosic wastes like wheat bran, rice husk,dab gross and corn cobs at varying conditions of pH 7.0, temperature 45°C and incubation period of 2-8 days. Production of cellulases was done by dinitrosalicyclic acid and filter chromatography method. In the assay of DNS method, the enzymes produced was 3.81IU at a maximum temperature of45 °C. The partial purification of cellulase enzyme produced by *Aspergillus niger*. *The*Vogel's medium had two larger protein of molecular weight of 33 and 24 kDa respectively.

Riaz *et al.*, (2014) demonstrated the various biochemical and biophysical factors on cellulase enzyme using local fungal species using submerged fermentation techniques CMCase was produced at maximum level .The energy of activation for the enzyme production $E_a(p)$ was 108 kj ml and kinetic parameters ie ΔHp and ΔSp were 106.047 kj mol- and 117.589 kj mol- and -36.29 kj mol respectively. The result was unique because

for the first time kinetic and thermodynamic aspect of CMCase production by *Humicola insolens* was evaluated.

Bajaj *et al.,* (2014) performed research on cellulases production their industrial applications including saccharification of cellulosic and lignocellulosic biomass. In this study fungal strains of *Sporotrichum* thermophile LAR5 were used because it was cheap and easily accessible for cellulase enzyme production. The substrate wheat bran produces maximum cellulase enzyme production (2000IU/L). Maize bran and rice husk also produces best enzyme production. The optimum temperature for cellulase enzyme production was noted 60°C -70°C although the temperature 80°C – 90°C may also fever cellulase production. The thermostability of cellulases were notedat 50 -60°C for 30 min but it gradually reduces as the time and temperature increases. The optimum pH for cellulase enzyme is 5 although best activity was observed in a range of acidic and alkaline pH (3 -10). Cellulase enzyme activity was higher in acidic range and it reduces at alkaline pH. Further, the cellulase enzyme showed higher activity on acidic pretreated maiz and rice straw and have the potential for cellulosic and lignocellulosic biomass transformation.

Kaur *et al.,* (2015) highlighted that the desired enzyme cellulase were produced from the fungus *Humicola fuscoatra* by using solid state fermentation studies using different growth conditions like pH, temperature, incubation time, inoculum size so as to optimize those conditions which enhances cellulase enzyme production. The capability of cellulase and their related enzymes to enhance the digestibility of paddy strawwas also checked. Cellulase enzyme production was maximum at optimized conditions like pH 6.0 mandel media at temperature 45°C. For maximum enzyme production there founded 1×10^7 spores /ml of inoculum size. Enzyme production is related the time of incubation, when time is increased enzyme production enhances usually from 2-6 days and it gradually reduces with less incubation period. With the increase in incubation period and enzyme concentration, the content of detergent fibers, Acid detergents, cellulose and hemicelluloses reduced gradually with sudden increase in lignin and silica content. The concentration of cellulose, hemicelluloses, ADF, and NDF decreased by 10.8, 23.4, 5.9, and 9.2 % respectively. Although silica and lignin content increased by 6.4 and 9.7 % respectively as compared to control at 2μ mole enzyme concentration after 36 hof

pretreatment. This study showed that the enzyme produced from *Humicola fuscoatra* has the ability of increasing digestibility of paddy straw and hence utilization of paddy straw for different goals.

Pandey *et al.*, (2015) analyzed that eight species of *Trichoderma* produced considerable amount of cellulase production under solid state fermentation. Different carbon sources were used like sucrose, maltose, wheat bran, corn cobs, dab grass wood chips and filter papers. From all of these sources fungal species *T. herazianum* produced highest yield of cellulase. Various optimized parameters were also reported for this study. The optimum temperature, pH and thermal stability of isolated enzymes were also analyzed. Enzyme production was maximum at temperature 30-40°C and pH 4-6 for cellulase. It has been investigated that *Trichoderma* species have the highest capability of producing cellulase enzyme.

Mahalakshmi and Jayalakshmi, (2016) worked on cellulase production from selected strains of fungal sp *Aspergillus niger*. They used different substrates such as rice bran, corn cobs, and ground nut cakes etc for production of cellulase enzyme. The solid state fermentation was carried out for the production of extracellular enzyme production from *Aspergillus niger*. Incubation period of 6 days and 37°C was optimized for the purpose of high yield of cellulase enzyme. Among all of these substrate groundnut oil cake was declared a best and high yield enzyme production for *Aspergillus niger*.

MATERIALS AND METHODS

3. MATERIAL AND METHODS

3.1 Chemicals

All the chemicals like agar technical, glucose, yeast extract, malt extract, trisodium citrate, Ammonium sulphate, Ammonium nitrate, potassium di hydrogen phosphate, magnesium sulphate, avicel, Carboxy methyl cellulose, sodium hydroxide, potassium tartrate, dinitro Sali syclic acid etc were used in this research project. The glassware such as beakers, pipettes, flasks, blue tips, spreader, petri dishes, test tubes, microscopic slides, cover slips and falcon tubes were used.

3.1.1 Apparatus

Apparatus such as water bath, spectrophotometer, microscope, autoclave, laminar flow hood, and shaking as well as static incubator were used for this study.

3.2 Streaking method

The streaking was conducted in a laminar hood. The laminar hood was sterilized by 70 % ethanol concentration used in the experiment. Equipments and glassware used were PDA media, petri plates, pipits, spatula and spreader was decontaminated for 15 mints at 121 °C under pressure of 15 Pascal (pa).The antibiotics streptomycin was used for un wanted species so as to decontaminate the media before pouring it in petri plates. 25 ml media were poured in plates and waited for half an hour until it solidified. Now the plates were labelled and fungal colony was picked by wire loop and streaked in culture plates. The culture plates were incubated in shaking incubator at different temperature ranges of 40 °C, 45 °C and 50°C. The plates were observed after every 24 hours. *Humicola fuscoatra* growth was observed after 72 hours especially.

3.3 Identification of fungal species

The isolated fungi were obtained from stock culture of bioinformatics and biotechnology department lab. The fungal strains were sub cultured with the usage of incinerated wire loop and purified fungi was then checked by the help of light microscope in such a way that a drop of pure water usually distilled water, fungal spores, incinerated wire loop were kept on the slide. The slip was then covered and sealed. The desired fungi were then identified by physiological properties and shape of hyphae. During research work performed from production to purification and optimization it was checked that

Humicola sp. Culture growth was not so rapid but slow and completed after 72 hours usually.

3.4 Slant formation and streaking

For obtaining inoculum production fresh slants of desired fungi *Humicola fuscoatra* was prepared in aseptic conditions. PDA media was prepared and poured in fresh slants. The media was poured according to slant size and then fungal culture was picked by wire loop and was streaked in zig zag fashion. Then the temperature was provided and incubation time of 4-6 days. The fungal spores were grown and slants were full of fungal spores. The newly growing slant was then transferred to best growing conditions of 45°C. After five days of incubation a complete fungal growth was obtained on the slant.

3.5 Spore suspension

. It was prepared by taking 0.9 percent saline water in 5-6 days old slant followed by gentle scratching of slant surface with sterilized wire loop and for production of inoculum size 1-2 ml of spore suspension were utilized using suitable nitrogen and carbon sources.

3.6 Carboxy Methyl Cellulose (CMC) preparation

It was prepared by taking 0.5 g CMC dissolved in 50 ml distilled water. It was then mixed properly through continuous stirring and kept in refrigerator for later usage.

3.7 1% Avicel preparation

1 % avicel was prepared by adding 0.5g cellulose dissolved in 50 ml distill water. The chemical was mixed by keeping on stirrer for proper mixing into distilled water and then it was kept in refrigerator for onward usage.

3.8 Enzyme assay reagents for Cellulase

The reagents used in enzyme assay were 0.1 M citrate buffer, Enzyme solution, DNS reagent 1 ml, Carboxy methyl cellulose (CMC) 1 %, Avicel 1 %.

3.8.1 Enzyme assay for Cellulase

Enzyme activity was determined by using submerged fermentation medium using dinitro salicylic acid (DNS) Shamala *et al.*, (1985).

The extracellular CMCase as well as Exoglucanase Enzyme assay was performed by taking 0.5 ml CMC, 0.5 ml citrate buffer, 0.5 ml enzyme solution in a 100 ml flask, the mixture was put in the incubator at 50°C for 30 mints in water bath. After 30 mints 1 ml DNS was added in the reaction mixture and boiled for 5 mints to stop further reaction. The OD was checked on 550 nm on spectro photometer.

For Exoglucanase assay, two tubes were taken one was control and the other was test. In control tube 1 ml citrate buffer, 0.5 ml substrate avicel, and 0.5 ml distilled water while in test tube 1 ml citrate buffer, 0.5 ml Exoglucanase substrate avicel and 0.5 ml crude enzyme was taken. Both tubes were kept in water bath at 45°C for 30 minutes. The dinitrosalicyclic acid (DNS) was added and was boiled for 5 minutes. After boiling, was checked optical density (OD) by spectrophotometer at 550 nm wavelength.

3.8.2 Protein assay for Cellulase

The protein assay was performed by taking two tubes. One tube for control containing 1 ml Bradford reagent, 100 micoliter distilled water. The second tube for test containing 1 ml Bradford reagent, 100 microliters crude enzyme. Then both test and control tubes were kept in water bath 50°C for 30 minutes, and then were checked optical density (OD) by spectrophotometer at 595 nm wavelength.

3.9 Different parameter used for Cellulase production

For the production of cellulase enzyme from *Humicola fuscoatra* followimg factors were studied one by one in this research work.

3.9.1 Time course optimization for Cellulase production

The incubation time influences metabolic processes like enzyme production, enzyme inhibition, and protein denaturation. Hence in this study different incubation time was tested for the production of maximum cellulase enzyme.

3.9.2 Optimization of inoculum size for Cellulase production

The inoculum size plays vital role in the production of cellulase enzyme under submerged fermentation of *Humicola fuscoatra.* So here in this study different inoculum sizes (5 %, 10 %, 15 %). These concentrations were used for optimization of inoculum size.

3.9.3 Carbon sources selection for Cellulase production

Different carbon sources were checked for optimization study. Four substrate were taken for our desired work for this purpose. i.e wheat brane, rice husk, wood chips and corn cobs. These substrates were dried under shadow and ground into a fine piece by electrical instrument. The media was autoclaved here Vogel's medium was used then taken 2.5 ml of inoculum of *Humicola fuscoatra.* It was introduced in 250 ml flasks. After this the flasks were labelled and moved to refrigerator. The enzyme and protein assay was observed through spectrophotometer after 12, 24, 36, 48, and 72 hours. From all of these mentioned substrates wheat bran was selected a good one for production of cellulase enzyme it is because enzyme showed high values 9.4 IU.

3.9.4 Nitrogen sources selection for Cellulase production

Different nitrogen sources were used to check enzyme assay ie. Ammonium sulphate, Ammonium nitrate, Urea, and DAP. Nitrogen source were added and used according to calculated measurement scale by the following ratio 0.17% Ammonium sulphate, 0.42% Ammonium nitrate, 0.32% Urea and 0.707% DAP in Vogel media (Roe, 2001). The formula of Vogel's media is noted in chart form. All the accessible substrates autoclaved in 250 ml and 2.5 ml spore suspension was presented. In each 12-hour chemical measure was checked for compound movement. From all of these four utilized substrates the best activity was noted in DAP.

Table 3.1 Cellulase enzyme production Vogel's media chart on carbon sources for *Humicola fuscoatra*

Chemical	**Amount**
Glucose	1 %
Tri. sodium citrate	0.5 %
KH_2PO_4	0.5 %
$(NH_4)_2SO_4$	0.4 %
NH_4NO_3	0.2 %
Yeast extract	0.2 %
$MgSO_47H_2O$	0.02 %

3.9.5 Optimization of temperature for Cellulase production

Humicola fuscoatra was cultured in different temperature ranges like 35°C , 40°C , 45 °C and 50°C against substrate respectively. The growth appears at the 45°Cafter time duration of 48 hour was Black to white after some time below and at very high temperature, the growing rate of the fungus was reduced and affected.

3.9.7 Inoculum optimization for Cellulase

For inoculum optimization four different parameter like 2.5 ml, 4.5 ml,6.5 ml and 8.0 ml was added in Vogel's medium in different flasks. Enzyme activity was checked after each 12, 24, 36, 48, and 60 hours. From all of these four different parameters enzyme activity was highest at 4.5 ml inoculum concentration.

3.9.8 Optimization of pH for Cellulase

On the optimized substrates of carbon and nitrogen. The activity of the enzyme was measured at a pH after 12, 24 , 36 ,48, and 60 h. The enzyme activity was highest at a pH range of 7.0.

3.10 Partial purification of Cellulase enzyme

We refreshed 100 ml enzyme production and after 48 hour filtered it properly. Then we took 7 flasks and added 2ml crude enzyme product in each flask. For the purpose of purification, a known concentration of ammonium sulphate was added in the solution for 24 hours. We added 20 %, 30 %, 40 %, 50 %, 60 %,70 % and 80 % in each flask respectively. Then this solution was carried for centrifugation. The centrifugation was performed at 4000 rpm for 5 minute. After this the protein and enzyme assays were checked. From all of these concentrations 20 %, 30 % and 50 % were selected Ammonium sulphate concentration table shown in figure (Bokhari *et al.*, 2009).

3.11 Characterization of Cellulase

To study the potential aspect of *Humicola fuscoatra* cellulase production different parameters were tested in control and tested one. i.e. Effect of substrate concentration on enzyme assay, effect of pH, effect of incubation time and effect of inoculum size on cellulase enzyme. For optimization of different parameter from crude enzyme unit calculated in the presence of undiluted enzyme while characterization of cellulase for different parameter of purified enzyme.

3.11.1 Influence of substrate concentration on partially purified Cellulase

The influence of substrate concentration on *Humicola fuscoatra* cellulase enzyme, different concentrations like 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml, 0.5 ml, 0.6 ml, 0.7 ml, 0.8 ml, and 0.9 ml carboxy methyl cellulase were used on optimized temperature 45°C. Maximum enzyme activity was observed at 0.9 ml CMC.

3.11.2 Influence of temperature on Cellulase enzyme activity

The influence of temperature on *Humicola fuscoatra* cellulase was executed in incubator at 150 rpm for 15 minutes. Cellulase enzyme assay was checked on different ranges of temperature at constant time which include 25°C, 30°C, 35°C, 40°C ,45°C, 50°C, 55°C, 60°C and 65°C. Every 15 minutes through submerged fermentation assay of enzyme was checked. High enzyme unit was gained at 45°C.

3.11.3 Influence of pH on purified Cellulase

The experiment was started in the presence of different buffers at maximum temperature 45 °C and for time interval of 30 mints. The different buffers of pH 4.0, pH 4.4, pH 4.8, pH 5.2, pH 5.8, pH 6.2, pH 6.8, pH 7.0 and pH 7.8. Maximum activity of cellulase was observed at pH 7.0.

3.11.4 Influence of incubation time on Cellulase enzyme activity

The influence of incubation time on cellulase enzyme activity of *Humicola fuscoatra* at optimized conditions was checked and noted i.e from time interval of 5 mints to 50 mints. Results showed that highest enzyme activity was observed at incubation time of 30 mints at 45°C.

RESULTS

4. RESULTS

4.1 Isolation and screening of thermophilic fungal species

The thermophilic fungal species like *Humicola grisea* and *Humicola fuscoatra* were obtained from stock culture of Biotechnology and Bioinformatics dept lab. Although the fungal culture was refreshed again and again on PDA slant media for further usage in our research ahead.

4.2 Identification of fungal species

The Species of this fungal culture was identified by our senior Fiaz Ahmed, MS Biotechnology but for further verification It was identified again. Different fungal species were observed and identified by morphological and microscopic features of the spores and mycelial structure (Watanabe, 2002). *Humicola* species growing rate is totally different from other fungal growth. These fungal species growths completed in approximately 5-7 days of incubation period. After 48 h of growth, the fungal culture looks whitish in appearance. With the passage of time spore formation starts and the color gradually changes into black. The microscopic images of these fungi were exclusively observed.

4.3 Optimization of different parameters for Cellulase production

Different conditions were studied to get most extreme generation of cellulase protein from pure *Humicola fuscoatra.*

4.3.1 Time course optimization for Cellulase production

The time course and incubation time is utmost important steps for maximum yield of enzyme production by *Humicola fuscoatra* sp. The incubation time affects different metabolic processes like enzyme production, enzymatic inhibition, promotion and protein denaturation. Therefore, here in this study different incubation time from 12-72 h were checked for the production of high yield of cellulase enzyme using wheat bran as a carbon source. The other carbon sources rice husk, wood chips and corn cobs were also used. Maximum enzyme production was noted by using a carbon source wheat bran. The CMCase enzyme unit for wheat bran, rice husk, wood chip and corn cobs were 9.73 U/ ml, 5.23 U/ ml, 6.88 U/ ml and 8.22 U/ ml respectively. The highest protein production was 1055 µg/ ml at the time interval of 48 h.

Table 4.1 Wheat bran time course optimization for production of Cellulase

S.no	Incubation time (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein µg/ ml
1	12	3.95	3.31	244.30
2	24	4.39	3.79	263.90
3	36	5.29	4.42	369.05
4	48	9.73	5.69	1055.71
5	60	6.44	3.09	529.11
6	72	4.81	2.89	410.61

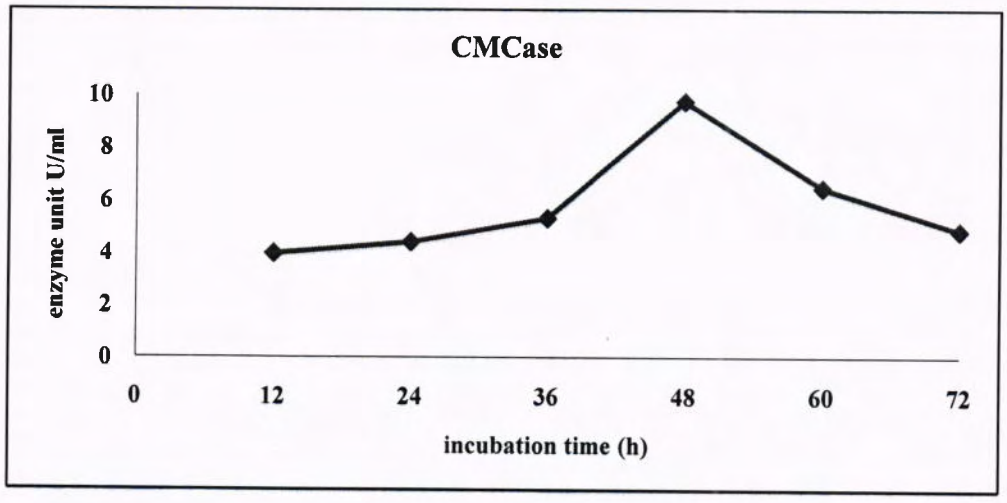

Figure 4.1 Wheat bran time course optimization for CMCase enzyme

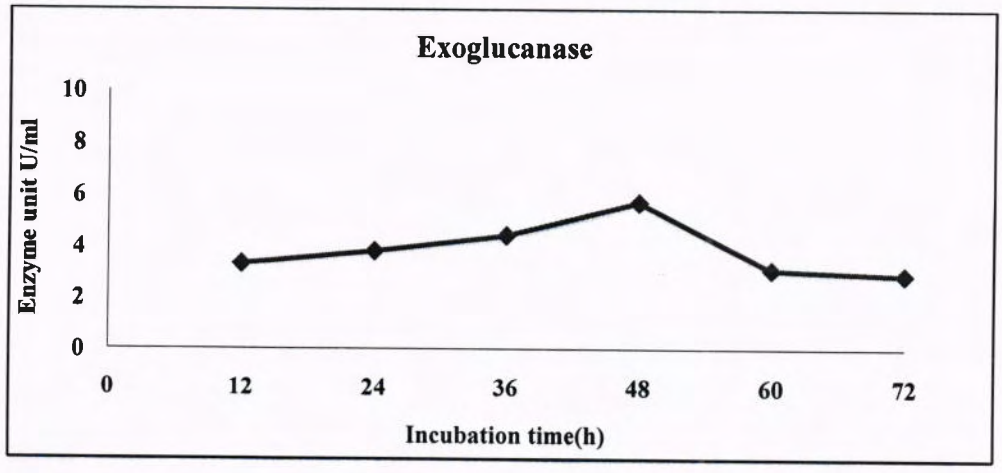

Figure 4.2 Wheat bran time course optimization for Exoglucanse enzyme

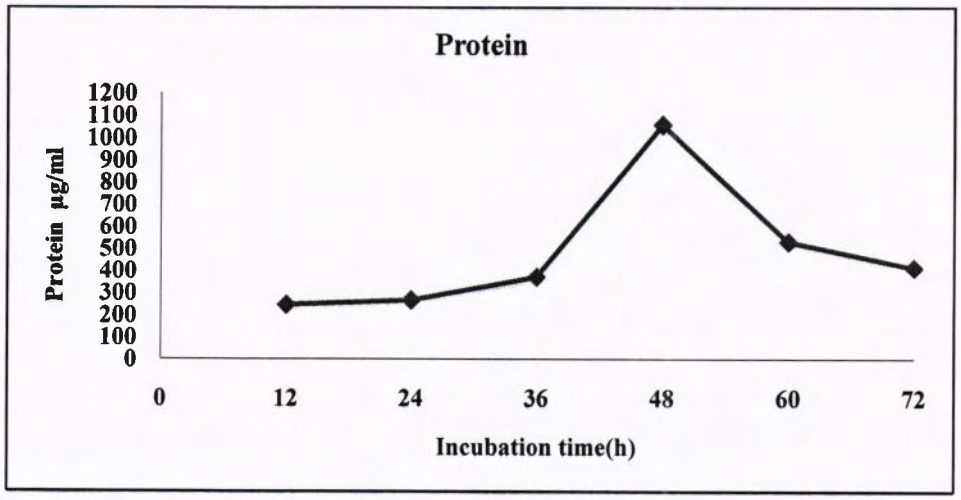

Figure 4.3 Wheat bran time course optimization for extracellular protein production

Table 4.2: Rice husk time course optimization for Cellulase enzyme

S.no	Incubation time (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein µg/ ml
1	12	3.63	2.91	202.70
2	24	4.41	3.34	241.42
3	36	4.90	4.13	372.65
4	48	14.01	4.78	678.11
5	60	5.01	3.88	473.39
6	72	3.16	2.49	279.19

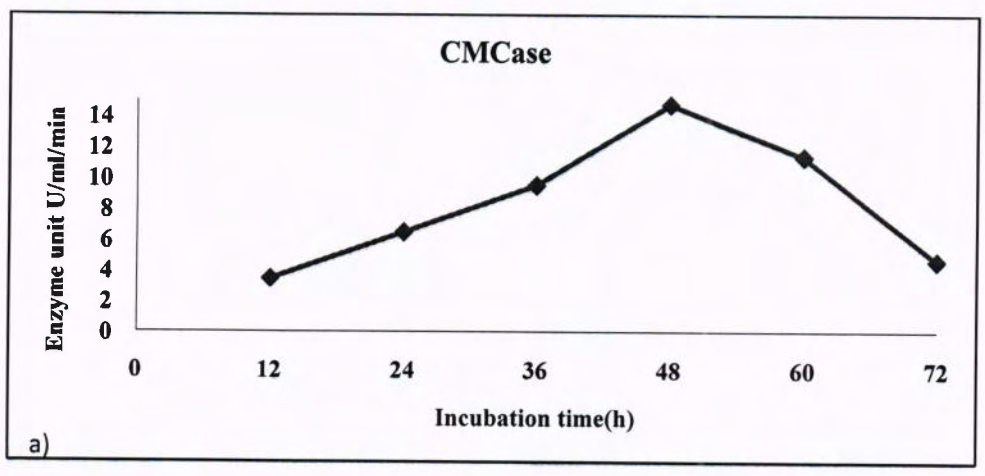

Fig 4.4 Rice husk time course optimization for CMCase enzyme

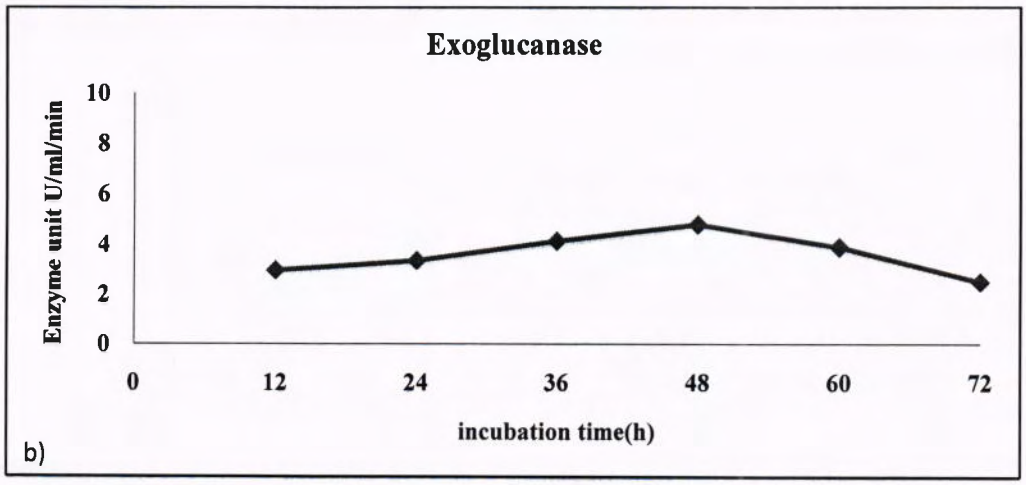

Figure 4.5 Rice husk time course optimization for Exoglucanase enzyme

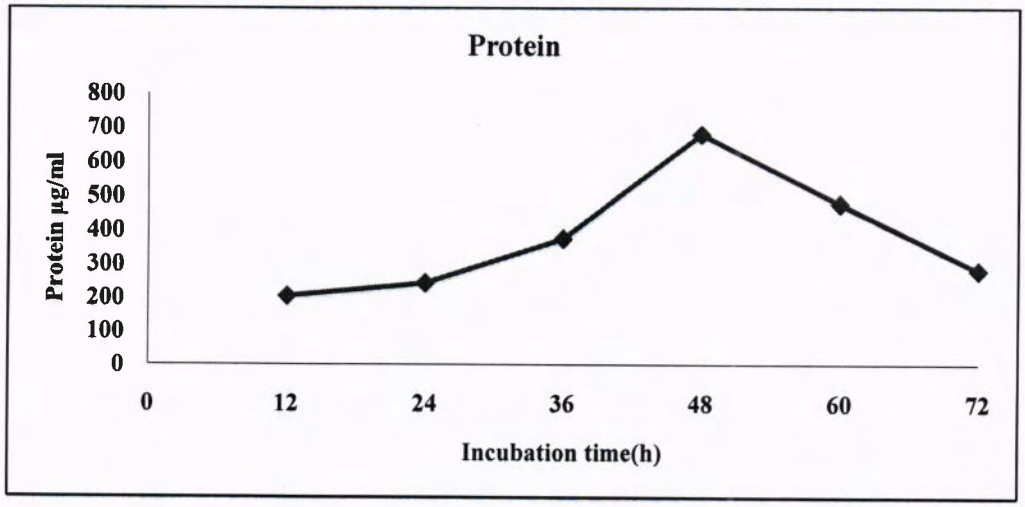

Figure 4.6 Rice husk time course optimization for extracellular protein production

Table 4.3 Wood chip time course optimization for Cellulase enzyme production

S.no	Incubation time(h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ ml
1	12	4.74	3.78	239.20
2	24	5.83	5.01	212.47
3	36	6.74	6.02	444.09
4	48	11.2	7.35	659.27
5	60	7.44	4.88	331.87
6	72	3.51	2.55	211.89

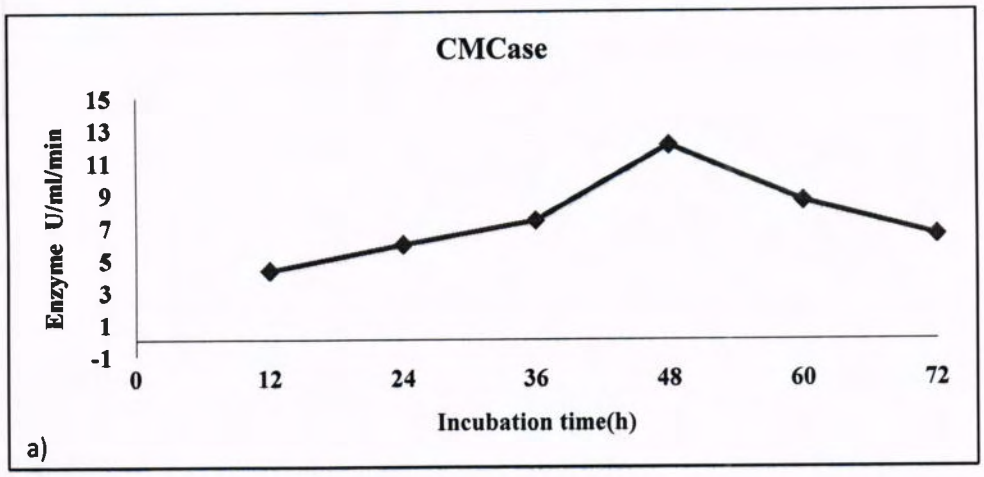

Figure 4.7 Wood chip time course optimization for CMCase enzyme

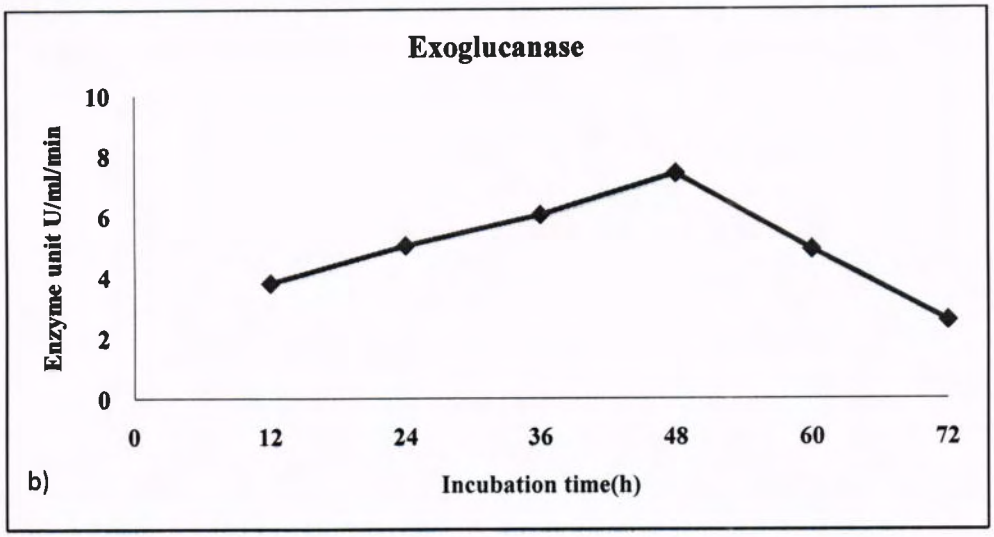

Figure 4.8 Wood chip time course optimization for Exoglucanase enzyme

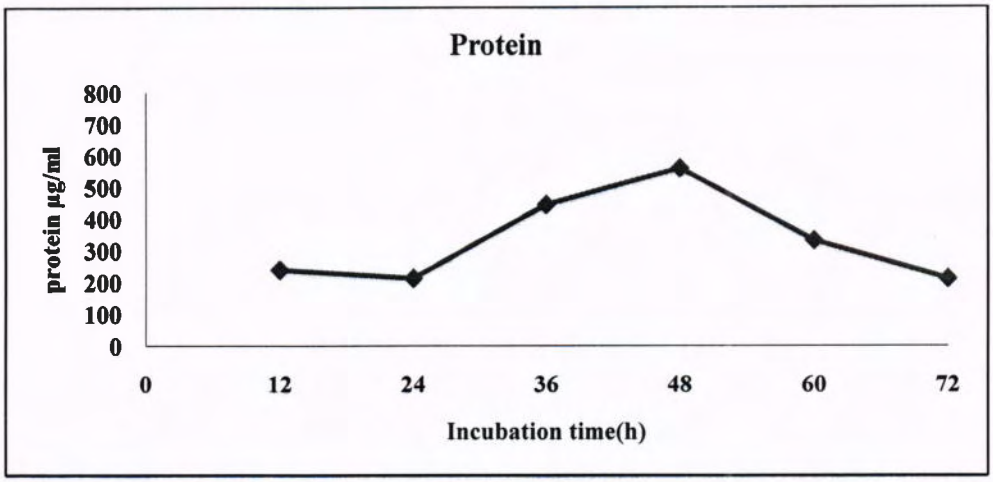

Figure 4.9 Wood chip time course optimization for extracellular protein production

Table 4.4 Corn cobs time course optimization for Cellulase enzyme

S.no	Incubation time (h)	CMCase U/ml	Exoglucanase U/ml	Protein µg/ ml
1	12	3.45	2.91	202.51
2	24	6.44	4.39	242.46
3	36	7.50	6.66	320.61
4	48	8.70	7.95	1070.76
5	60	6.33	5.43	267.26
6	72	4.59	4.91	691.21

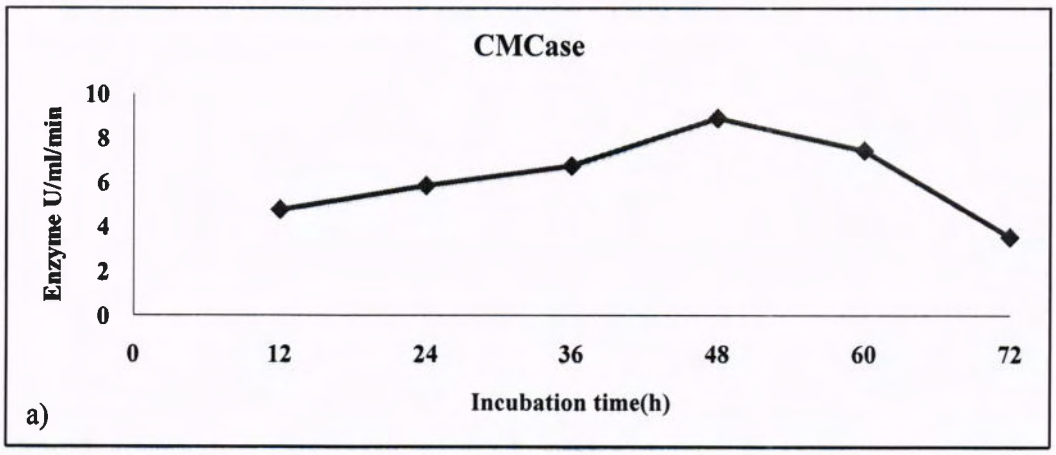

Figure 4.10 Corn cobs time course optimization for CMCase enzyme

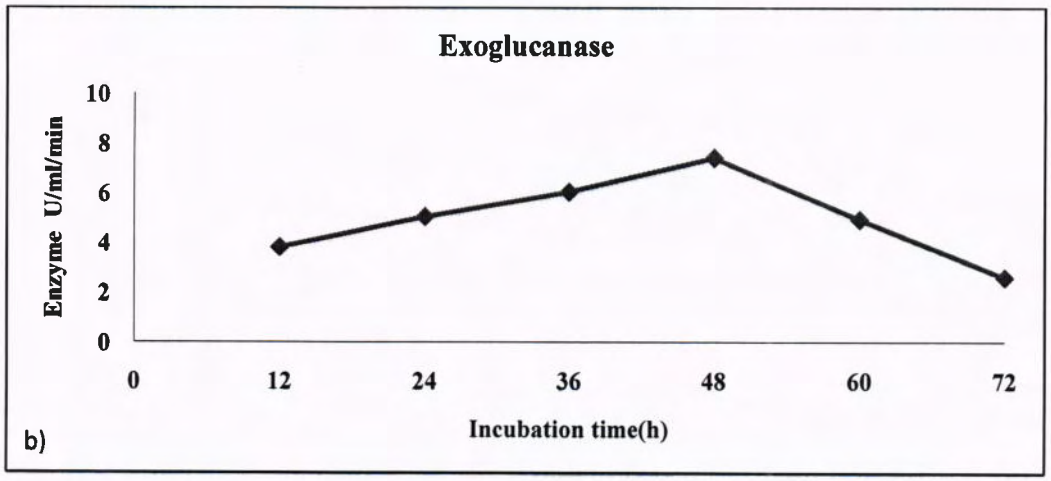

Figure 4.11 Corn cobs time course optimization for Exoglucanase enzyme

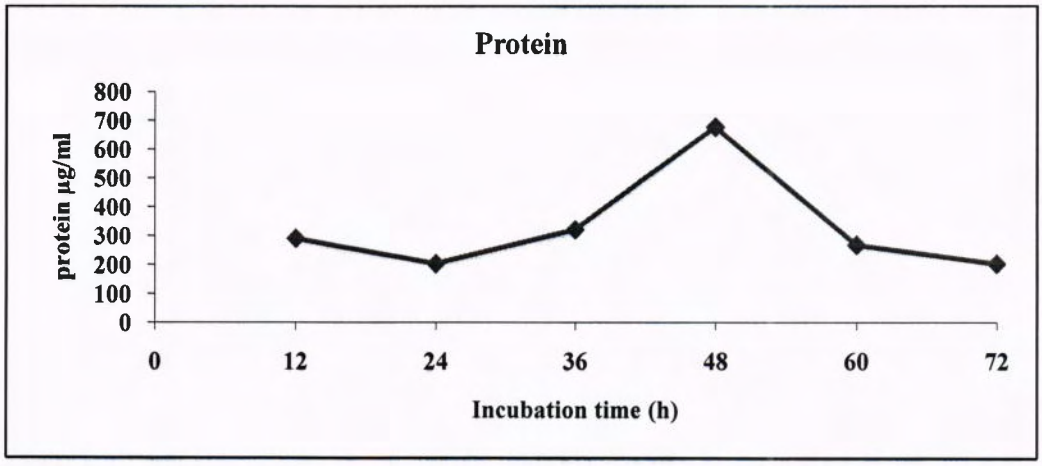

Figure 4.12 Corn cobs time course optimization for extracellular protein production

4.4 Optimization of inoculum size for Cellulase production

Different inoculums size was checked on production of cellulase by *Humicola fuscoatra* sp. The inoculums size has key role in production of this enzyme because inoculums size effects rate of enzyme production activity mainly. Here 5 %, 10 %, 15 % inoculums sizes were checked and the protein and enzyme unit were calculated against each concentration. 5 %, 10 %, 15 % inoculums size gave 9.38 U/ml, 10.02 and 8 .42 U/ ml CMCase activity and7.71 U/ ml, 9.44 U/ ml and7.35 U/ ml endoglucanase enzyme units respectively. The highest protein calculated at the time of 48 h for 5 %, 10 %, and 15 % was 301.50 µg/ ml, 233.61µg/ ml and 119.20 µg/ ml respectively.

Table 4.5 5 %optimization of inoculums size for Cellulase

S.no	Incubation time (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein µg/ ml
1	12	5.76	3.55	248.09
2	24	6.26	4.71	341.52
3	36	7.74	5.44	209.81
4	48	13.40	11.40	501.50
5	60	6.31	5.50	271.01
6	72	4.53	3.11	204.40

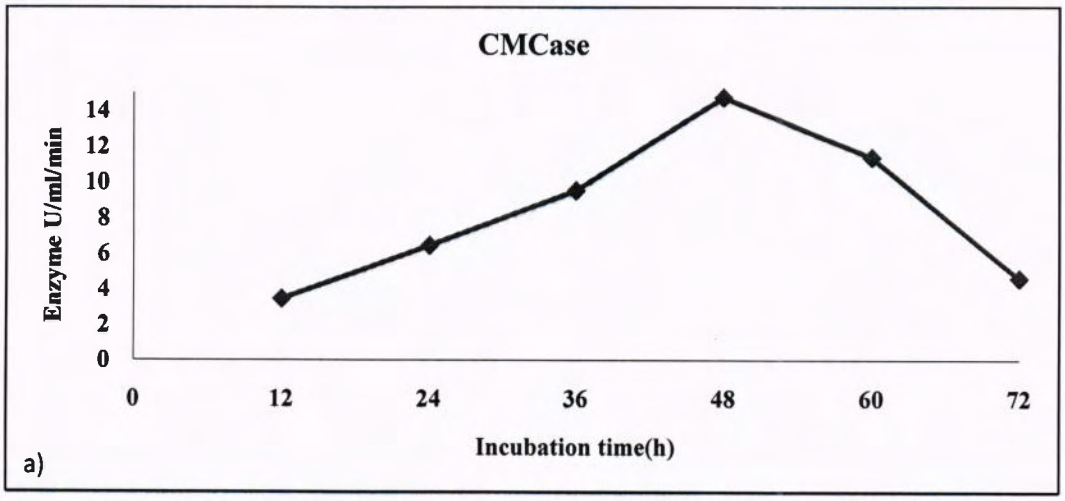

Figure 4.13 5% inoculamoptimization for CMCase enzyme

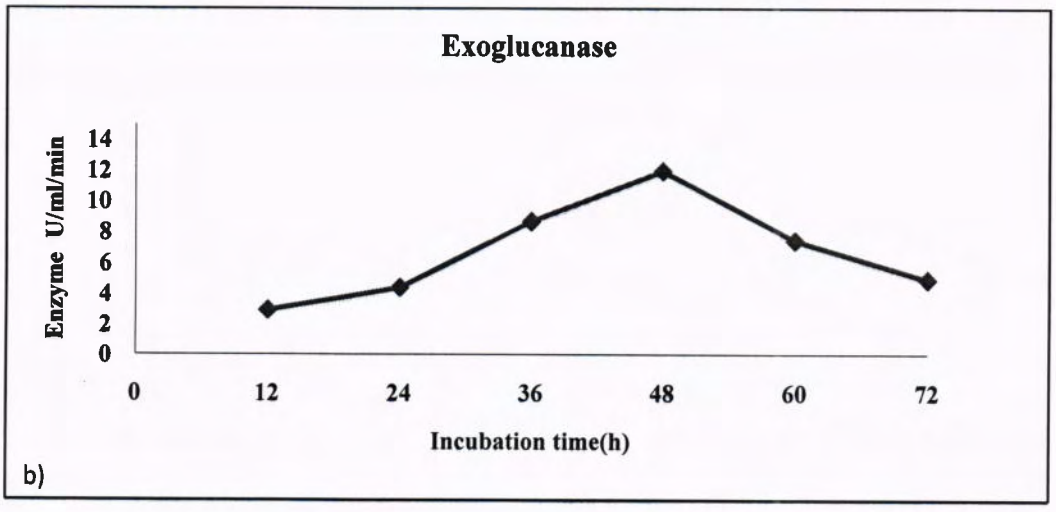

Figure 4.14 5% inoculums optimization for Exoglucanase enzyme

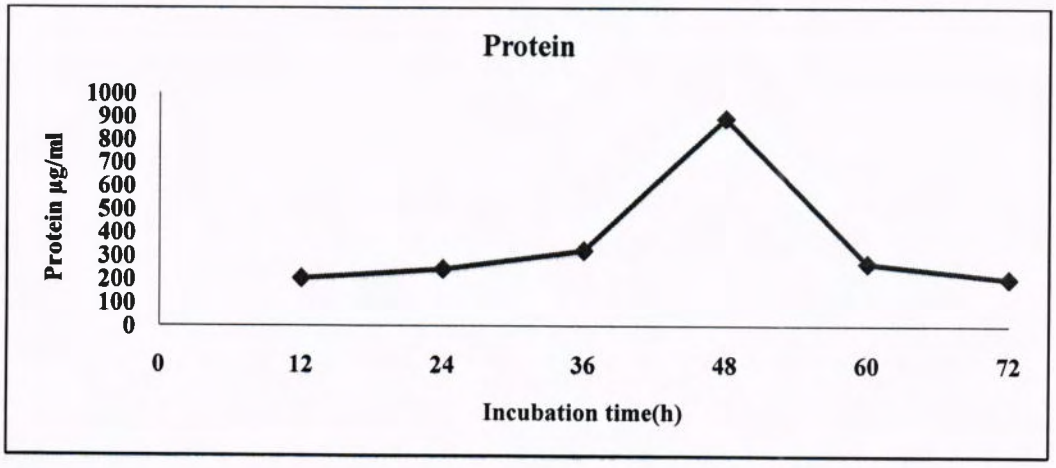

Figure 4.15 5 % inoculum optimization for extracellular protein production

Table 4.6 10 % inoculum optimization for Cellulase enzyme

S.no	Incubation time(h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ ml
1	12	4.81	3.42	250.60
2	24	5.31	4.53	285.72
3	36	7.55	5.90	369.33
4	48	14.20	12.27	233.61
5	60	6.71	4.72	295.01
6	72	3.24	3.60	202.10

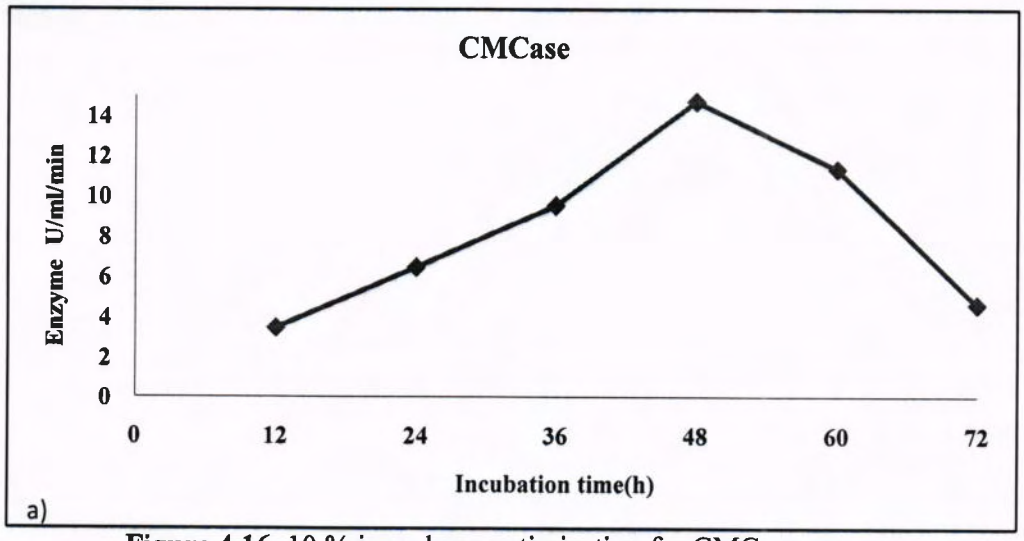

Figure 4.16 10 % inoculams optimization for CMCase enzyme

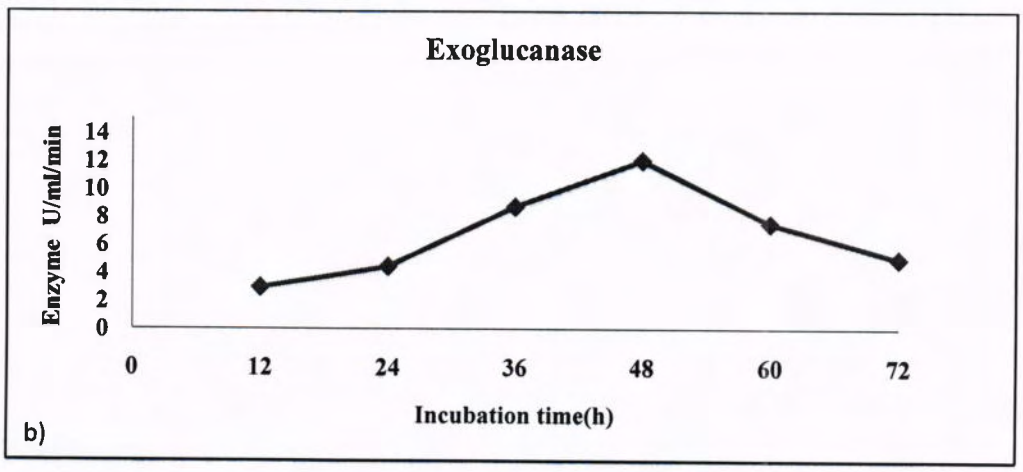

Figure 4.17 10 % inoculum optimization for Exoglucanase enzyme

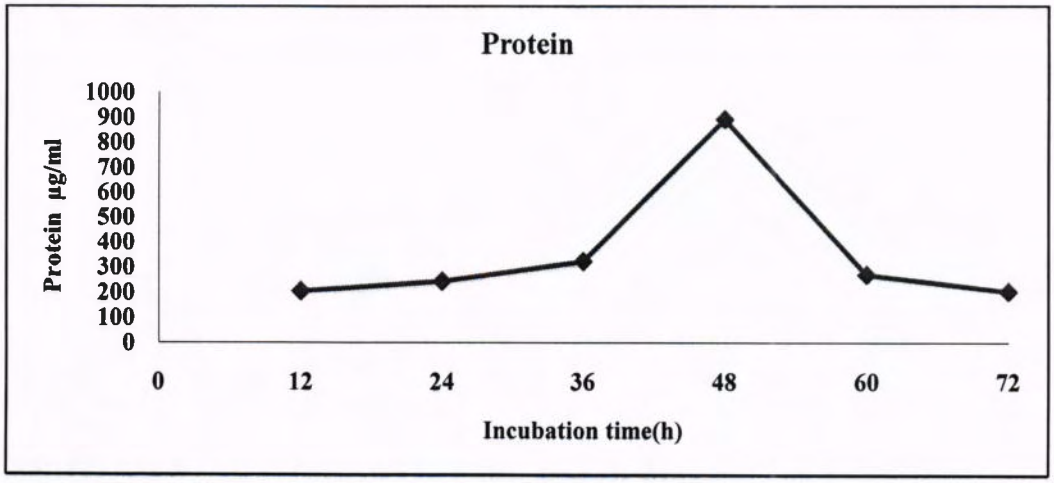

Figure 4.18 10 % inoculum optimization for extracellular protein production

Table 4.7 15 % inoculam optimization for Cellulase production

S.no	Incubation time(h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ ml
1	12	3.33	2.79	250.51
2	24	4.43	3.42	261.39
3	36	5.29	5.91	373.90
4	48	9.86	8.91	619.20
5	60	6.91	5.09	288.71
6	72	4.26	4.09	262.19

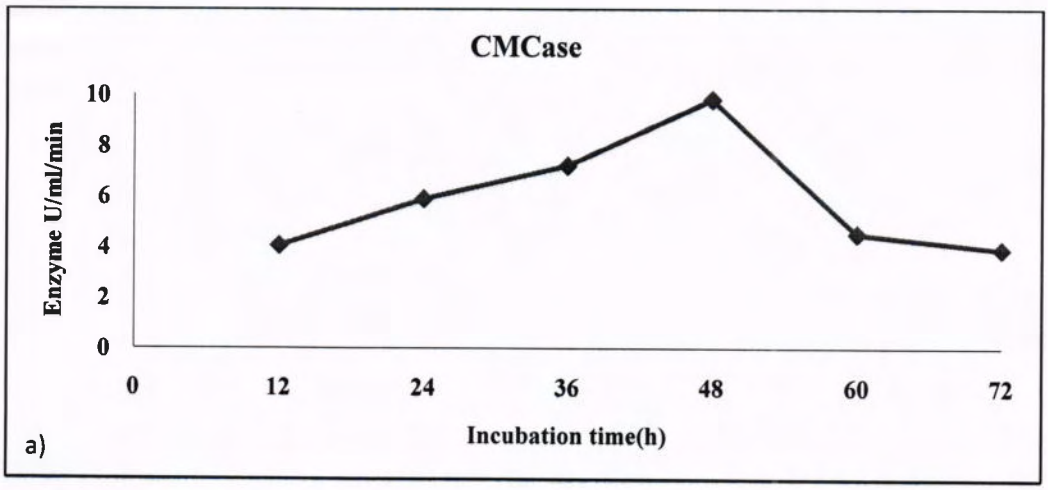

Figure 4.19 15 % inoculams optimization for CMCase enzyme

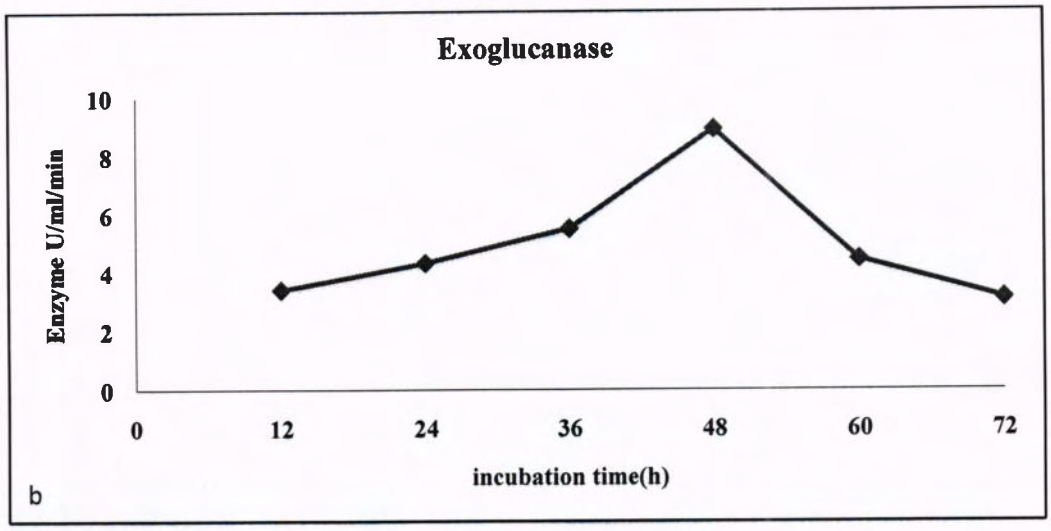

Figure 4.20 15 % inoculams optimization for Exoglucanase enzyme

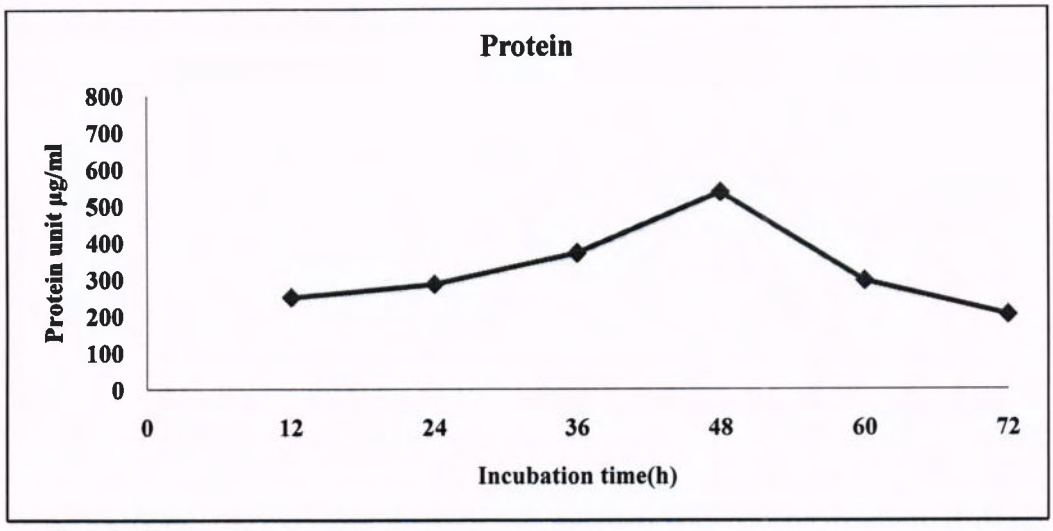

Figure 4.21 15 % inoculums optimization for extracellular protein production

4.5 Selection of carbon sources for production of Cellulase

Humicola fuscoatra were grown on various carbon sources such as wheat bran, rice husk, wood chip and corn cobs at a temperature of 45°C for a specific time of 5 days. After every 12 h the enzyme assay and protein assay was done for each substrate to investigate cellulase enzyme production on different interval of time i.e.12 h, 24 h, 36 h, 48 h and 60 h respectively. From all of these chosen carbon substrates cellulase enzyme

shows a unit of maximum value and selected highest activity taking average of all units after 48 h. The results of cellulase enzyme and protein production at various carbon substrate. The highest enzyme unit was produced on wheat bran at 48 h was 5.44 U/ ml /min while highest protein estimation was observed at 48 h was 3.98 mg/ ml.

Table 4.8 Carbon source wheat bran optimization for Cellulase production

S.no	Time of incubation (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ ml
1	12	7.33	5.79	250.51
2	24	11.42	13.42	261.39
3	36	23.39	26.91	373.9
4	48	27.42	28.35	619.2
5	60	18.91	22.09	288.71
6	72	10.26	13.09	262.19

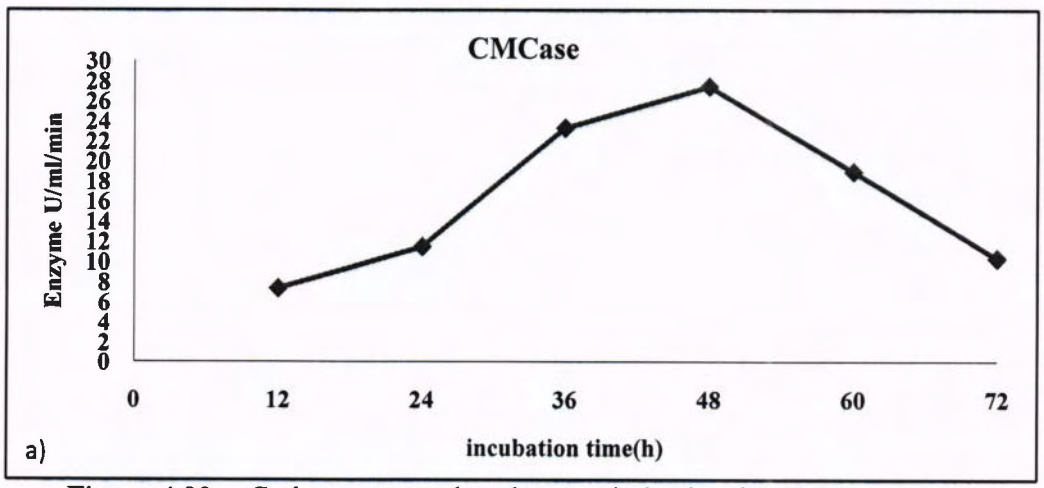

Figure 4.22 Carbon source wheat bran optimization for CMCase enzyme

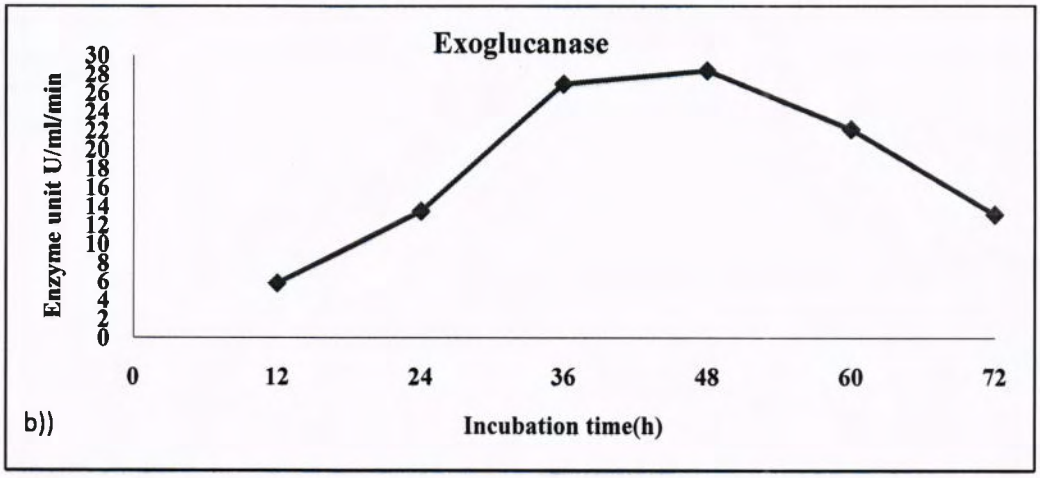

Figure 4.23 Carbon source wheat bran optimization for Exoglucanse enzyme

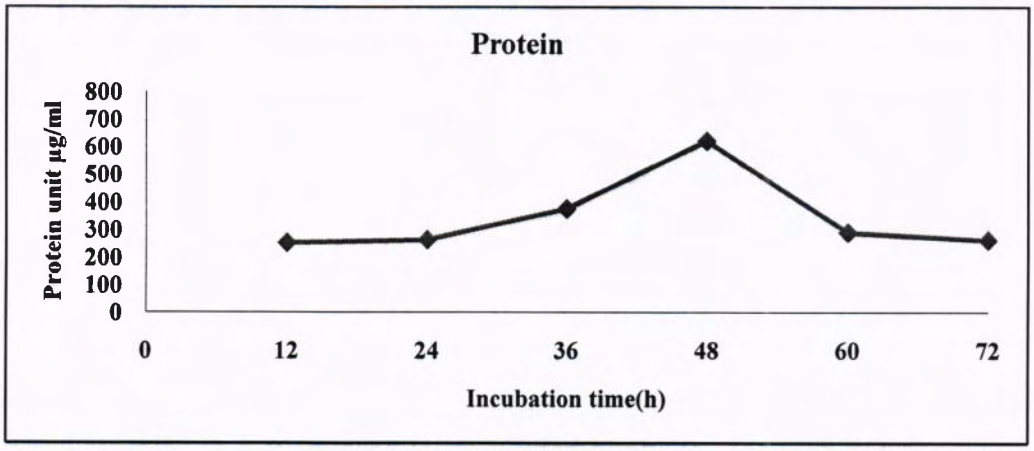

Figure 4.24 Carbon source wheat bran optimization for extracellular protein production

Table 4.9 Carbon source rice husk optimization for Cellulase production

S.no	Time of incubation (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ ml
1	12	3.05	2.94	289.51
2	24	6.09	4.39	202.46
3	36	9.51	8.66	320.61
4	48	14.85	14.02	676.23
5	60	11.33	7.43	267.26
6	72	4.59	4.91	201.21

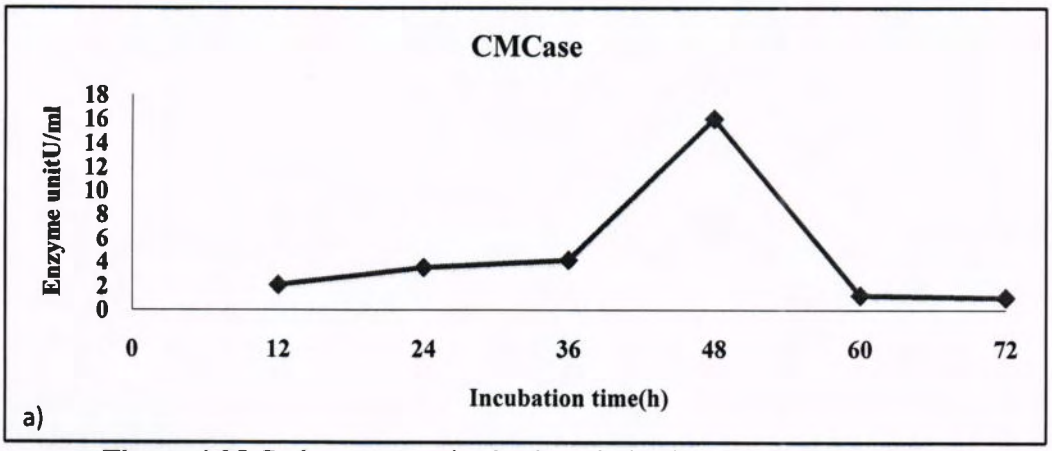

Figure 4.25 Carbon source rice husk optimization for CMCase enzyme

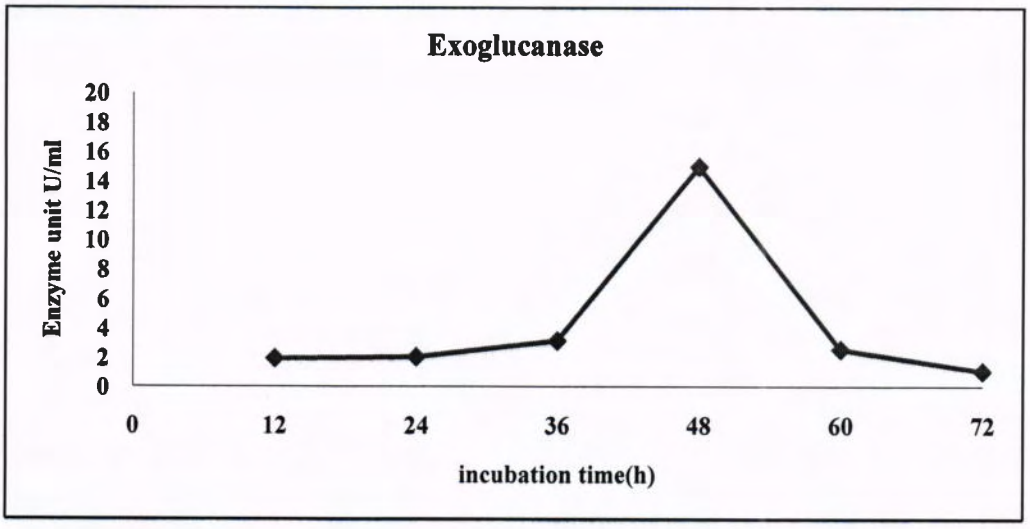

Figure 4.26 Carbon source rice husk optimization for Exoglucanase assay

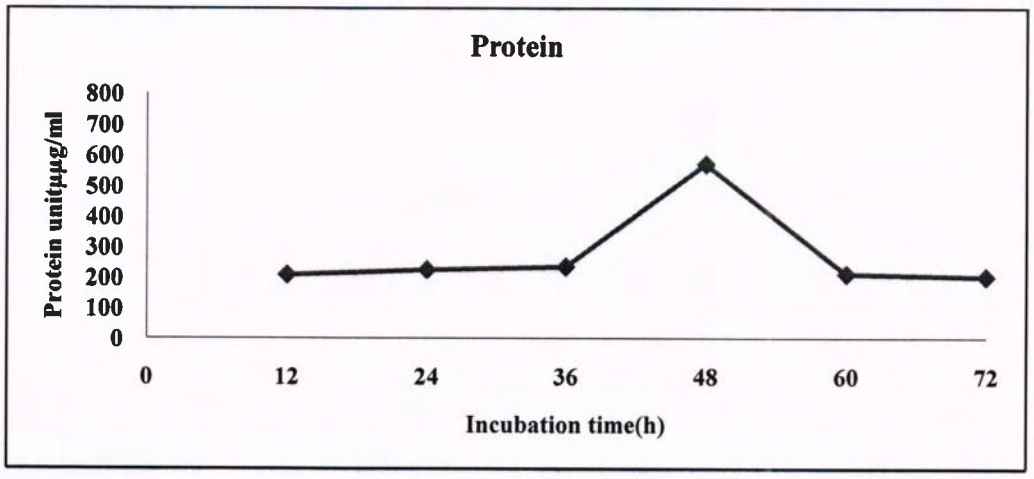

Figure 4.27 Carbon source rice husk optimization for extracellular protein production

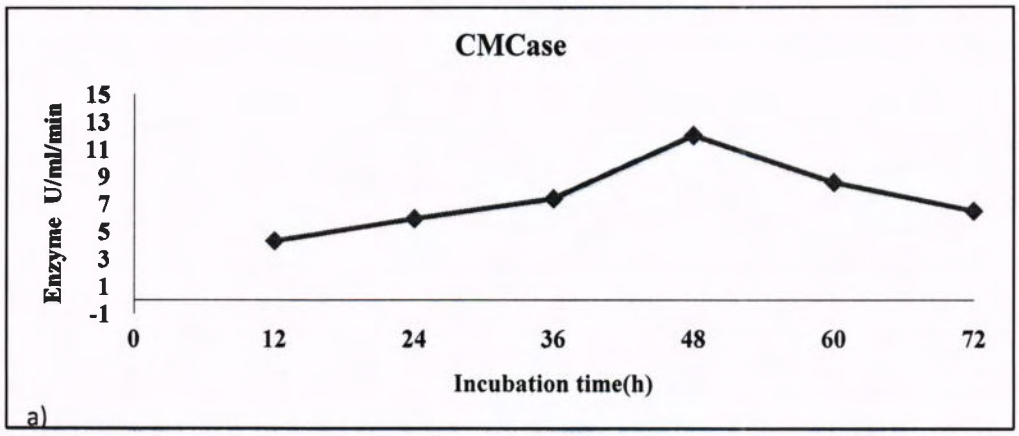

Figure 4.40 Nitrogen source Ammonium sulphate optimization for CMCase enzyme

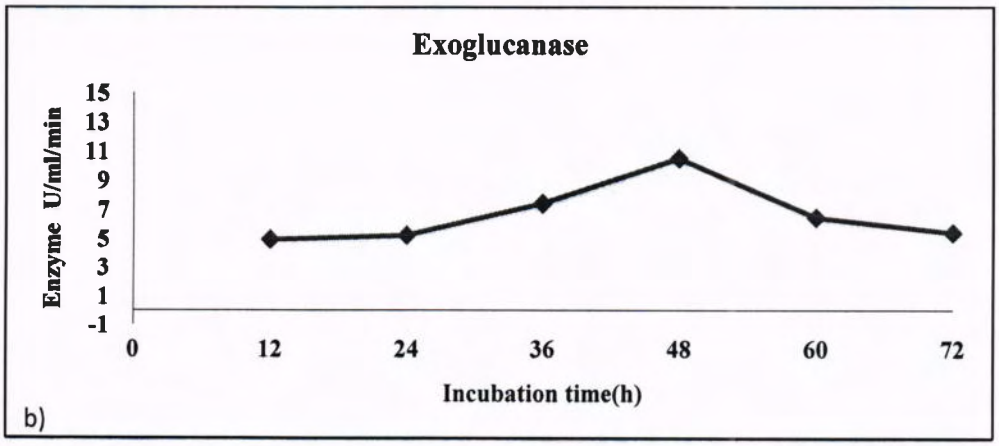

Figure 4.41 Nitrogen source ammonium sulphate optimization for Exoglucanase enzyme

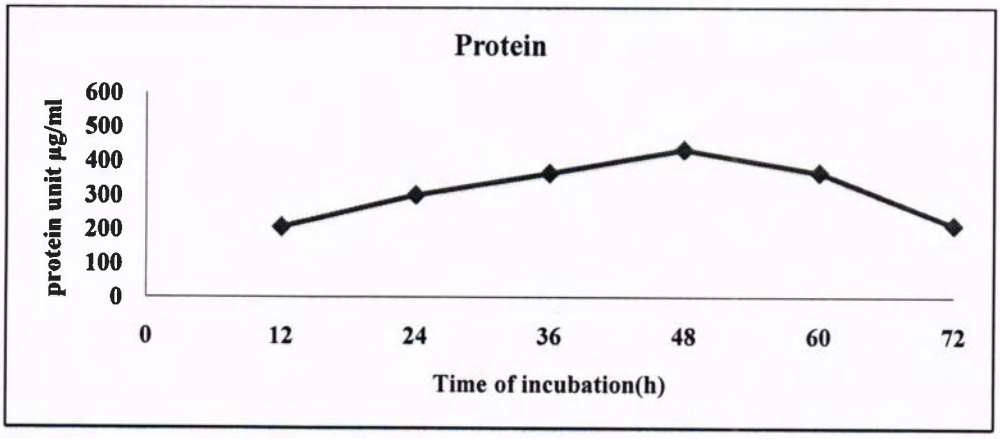

Figure 4.42 Nitrogen source ammonium sulphate optimization for extracellular protein production

Table 4.15 Nitrogen source ammonium nitrate optimization for Cellulase production

S.no	Incubation time (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ ml
1	12	2.94	2.69	266.20
2	24	4.38	3.44	279.50
3	36	6.48	7.22	370.35
4	48	8.28	9.38	404.80
5	60	5.32	6.21	312.78
6	72	3.66	3.12	390.50

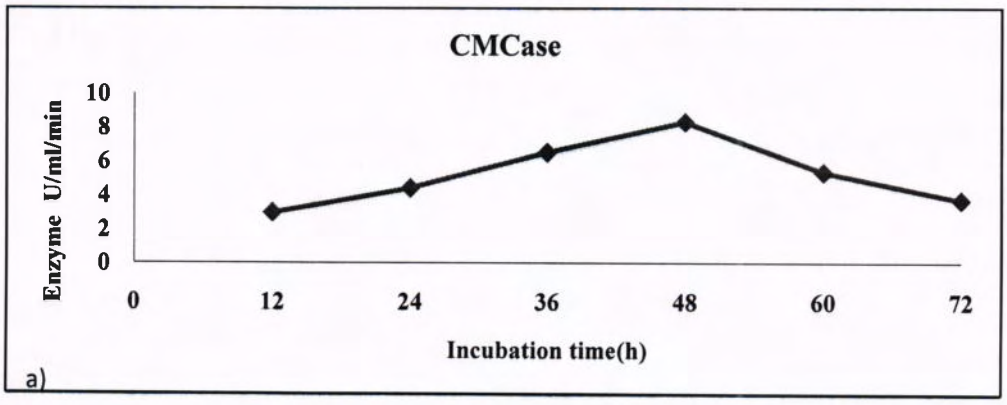

Figure 4.43 Nitrogen source ammonium nitrate optimization for CMCase enzyme

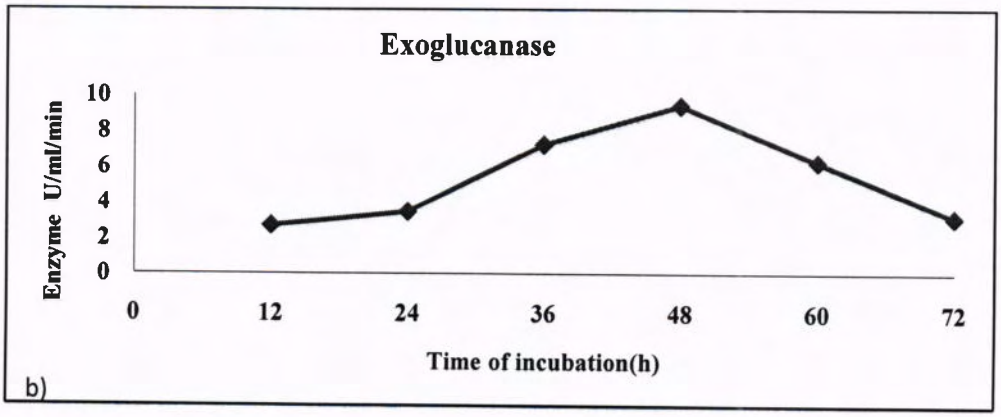

Figure 4.44 Nitrogen source Ammonium nitrate optimization for Exoglucanse enzyme

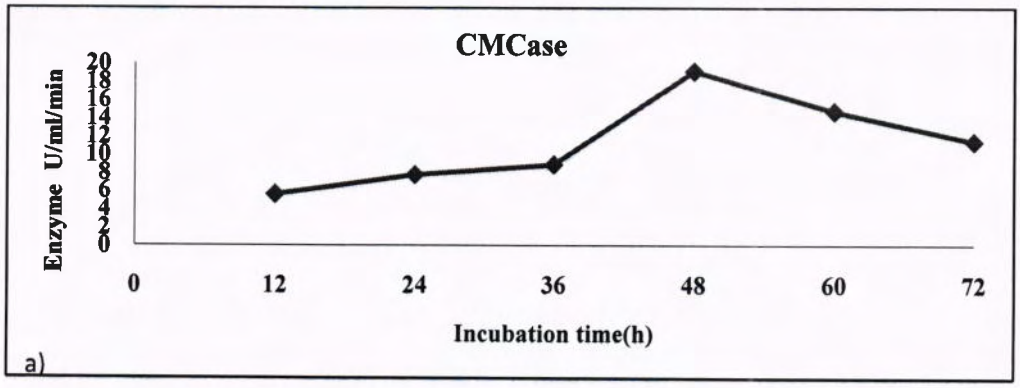

Figure 4.46 pH optimization (pH 5) for CMCase enzyme

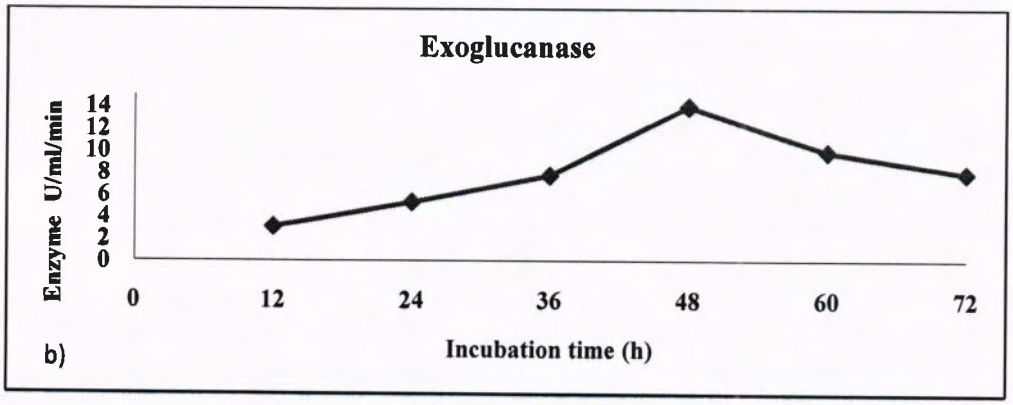

Figure 4.47 pH optimization (pH 5) for Exoglucanse enzyme

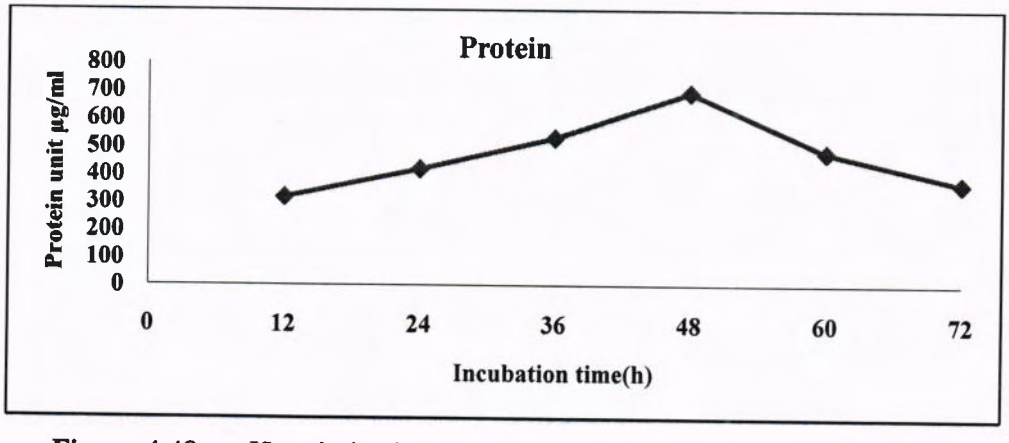

Figure 4.48 pH optimization (pH 5) for extracellular protein production

Table 4.17 pH optimization (pH 6) for production of Cellulase

S.no	Time of incubation(h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ ml
1	12	4.90	2.90	365.20
2	24	5.98	4.36	411.50
3	36	9.59	6.56	550.50
4	48	21.25	16.76	661.07
5	60	16.65	11.39	479.21
6	72	10.64	8.74	620.08

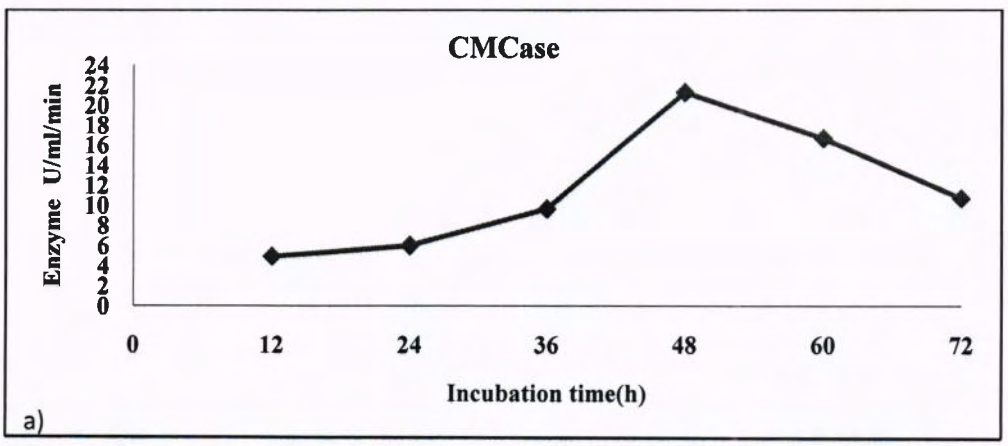

Figure 4.49 pH optimization (pH 6) for CMCase enzyme

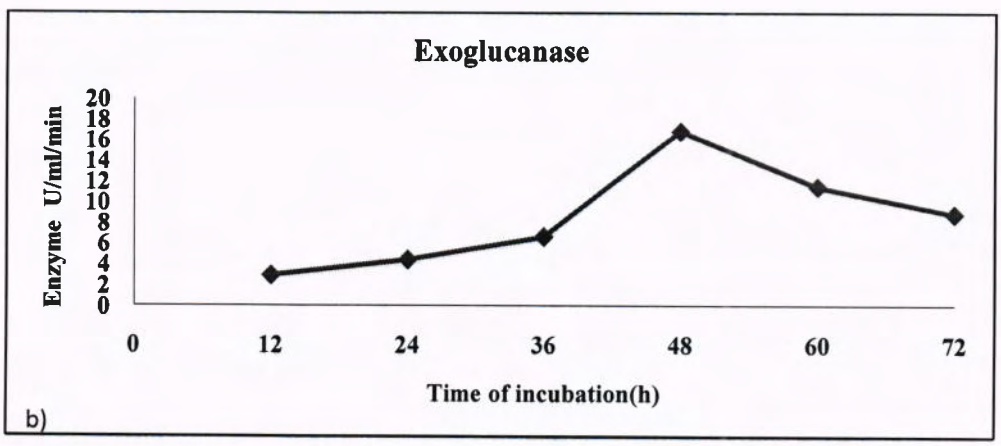

Figure 4.50 pH optimization (pH 6) for Exoglucnase enzyme

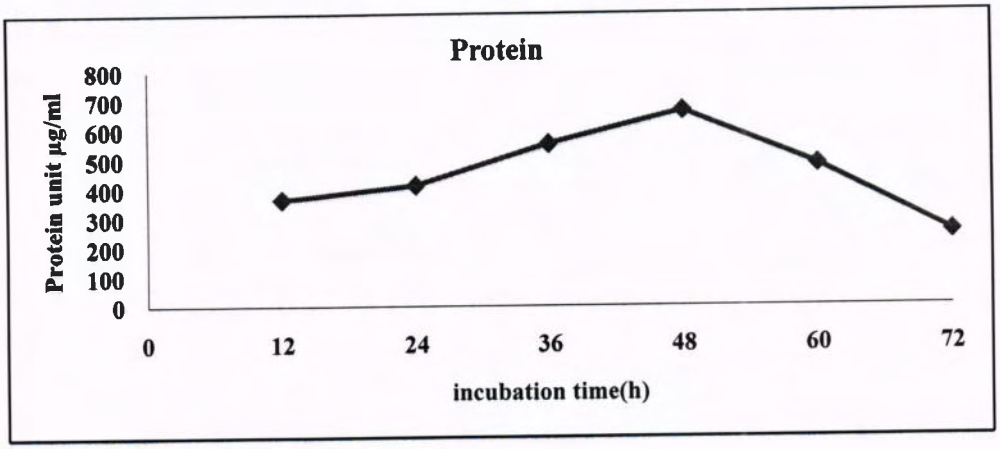

Figure 4.51 pH optimization (pH 6) extracellular protein production

Table 4.18 pH optimization (pH 7) for production of Cellulase

S.no	Incubation time (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ ml
1	12	6.96	5.89	311.31
2	24	8.85	7.64	465.70
3	36	14.73	11.73	680.19
4	48	24.64	17.49	769.20
5	60	18.31	12.31	400.09
6	72	11.71	10.50	219.81

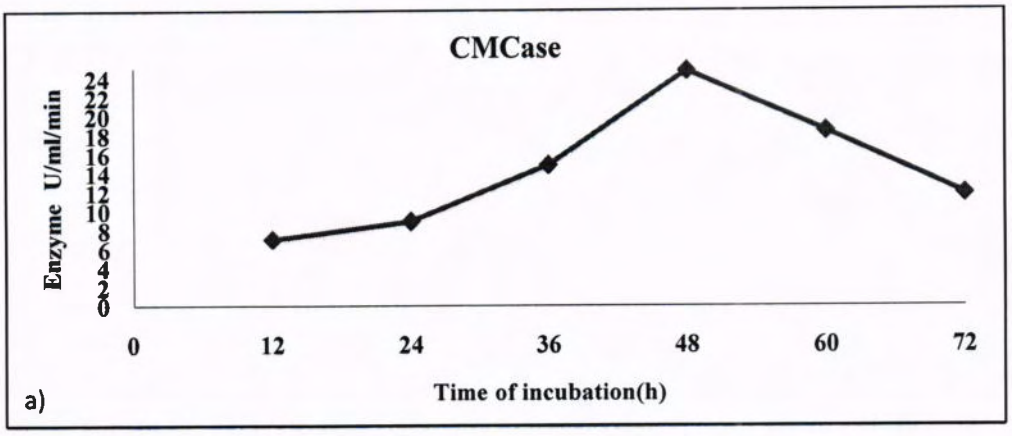

Figure 4.52 pH optimization (pH 7) for CMCase enzyme

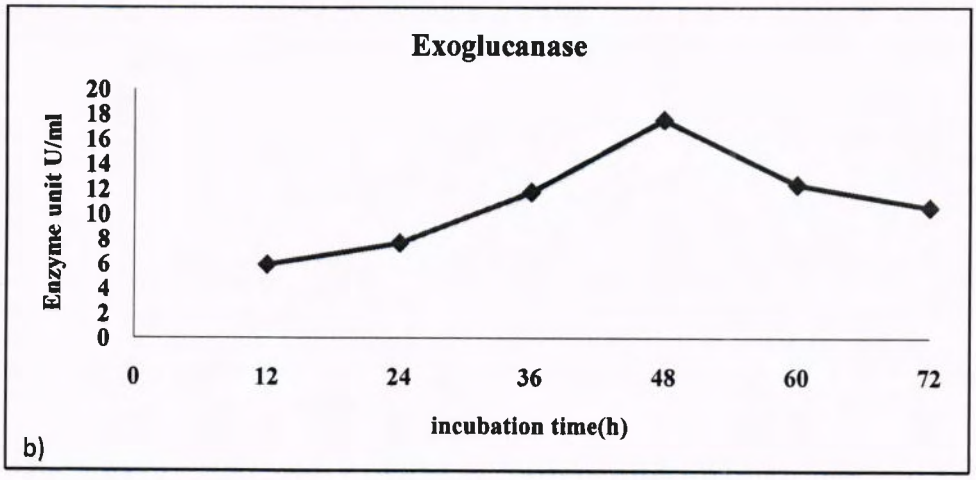

Figure 4.53 pH optimization (pH 7) for Exoglucanase enzyme

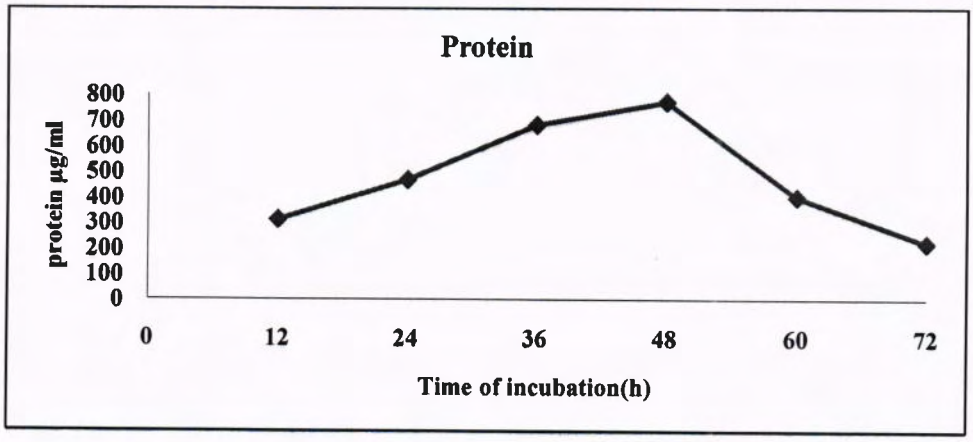

Figure 4.54 pH optimization (pH 7) for extracellular protein production

Table 4.19 pH optimization (pH 8) for Cellulase production

S.no	Incubation time (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ ml
1	12	3.66	2.96	203.50
2	24	6.75	4.95	371.02
3	36	7.93	6.78	450.21
4	48	9.50	8.79	680.82
5	60	7.56	6.21	460.64
6	72	5.50	4.77	271.08

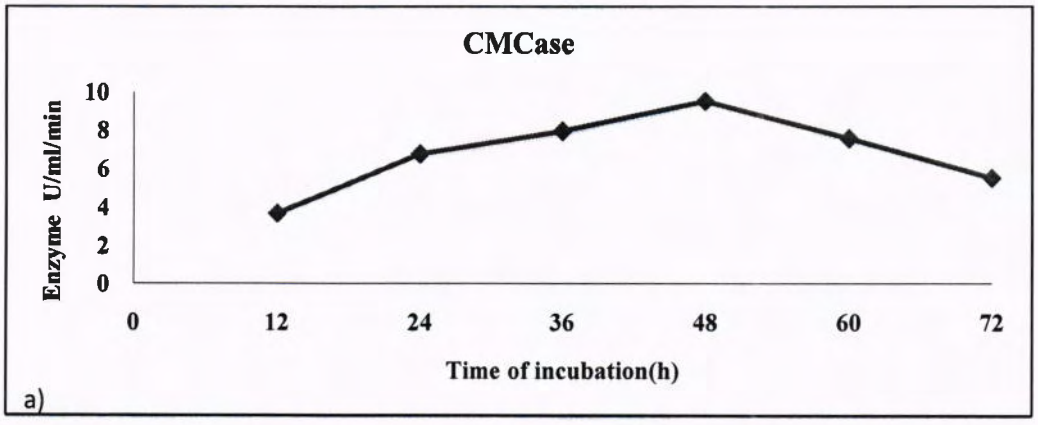

Figure 4.55 Table: pH optimization (pH 8) for CMCase enzyme

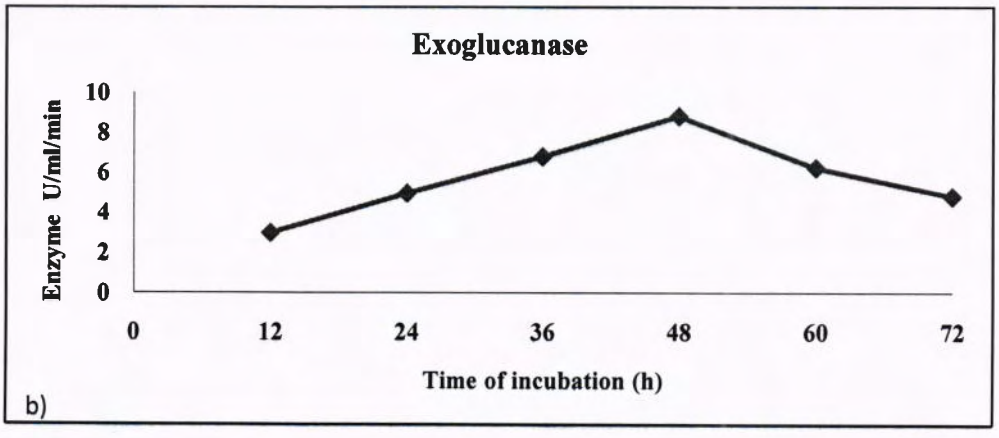

Figure 4.56 pH optimization (pH 8) for Exoglucanse enzyme

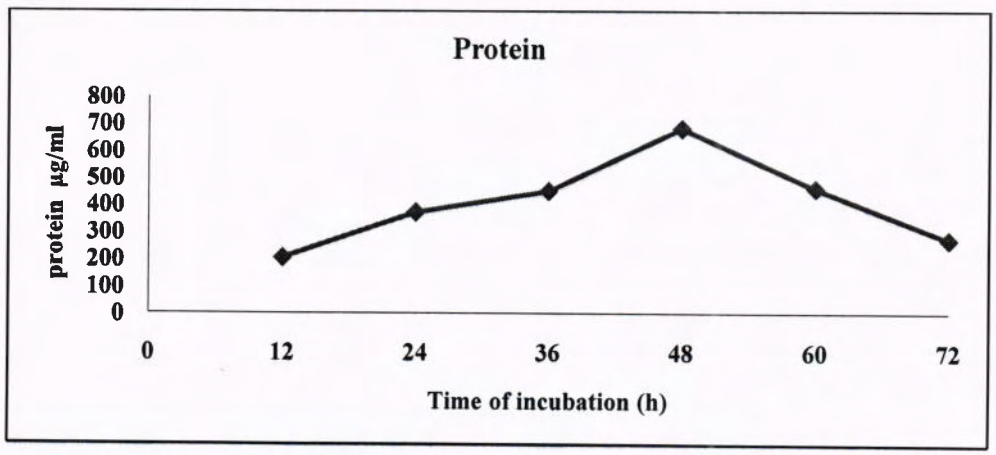

Figure 4.57 pH optimization (pH 8) for extracellular protein production

Table 4.10 Carbon source wood chip optimization for Cellulase production

S.no	Incubation time (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein unit µg/ml
1	12	7.28	6.78	260.20
2	24	8.74	9.78	290.31
3	36	9.38	9.29	398.41
4	48	15.42	13.39	848.08
5	60	11.37	11.54	478.55
6	72	7.91	17.21	339.13

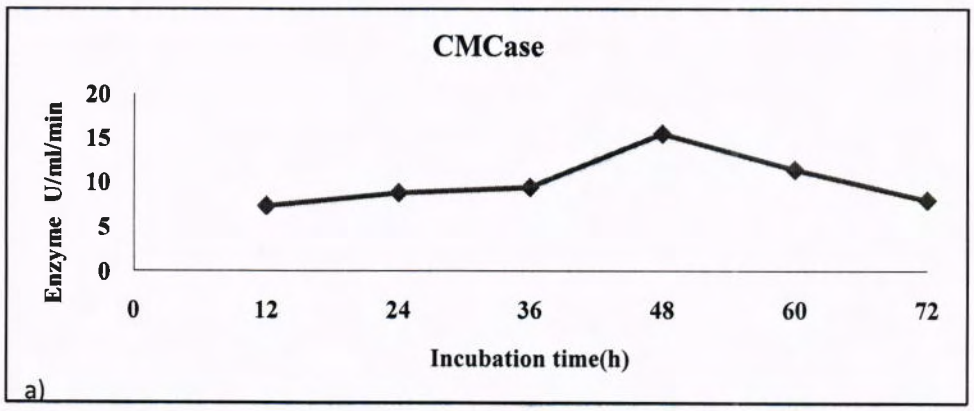

Figure 4.28 Carbon source wood chip optimization for CMCase enzyme

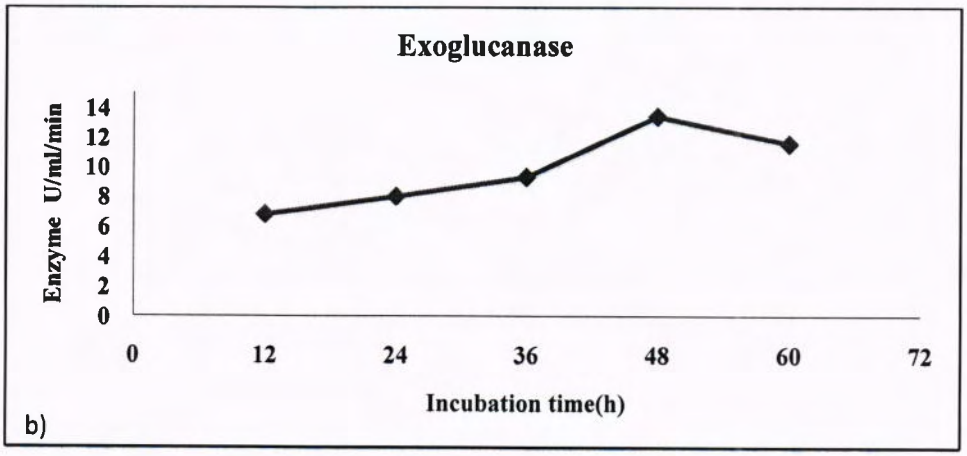

Figure 4.29 Carbon source wood chip optimization for Exoglucanase enzyme

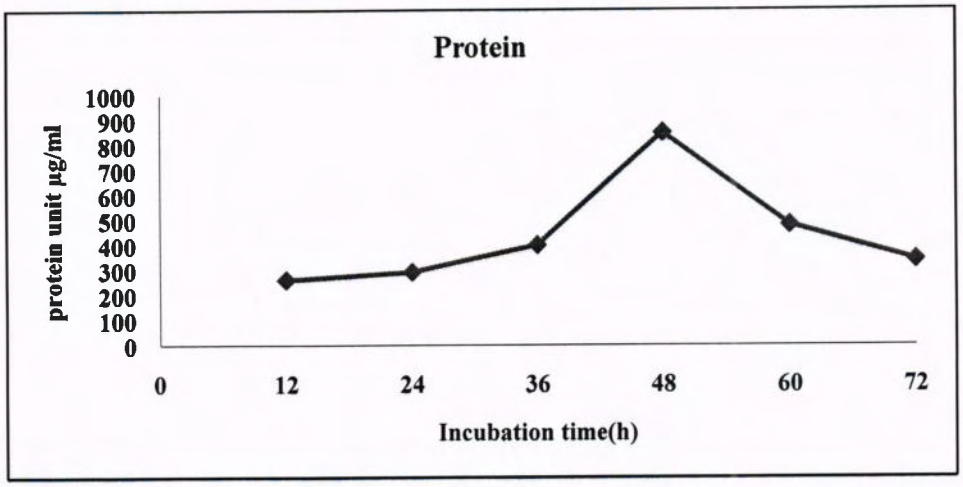

Figure 4.30 Carbon source wood chip optimization for extracellular protein production

Table 4.11 Carbon source corn cobs optimization for Cellulase production

S.no	Incubation time (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ml
1	12	5.26	4.97	230.5
2	24	7.66	7.65	280.31
3	36	9.13	8.78	320.54
4	48	10.64	9.95	535.41
5	60	8.66	7.83	360.22
6	72	6.24	5.91	215.33

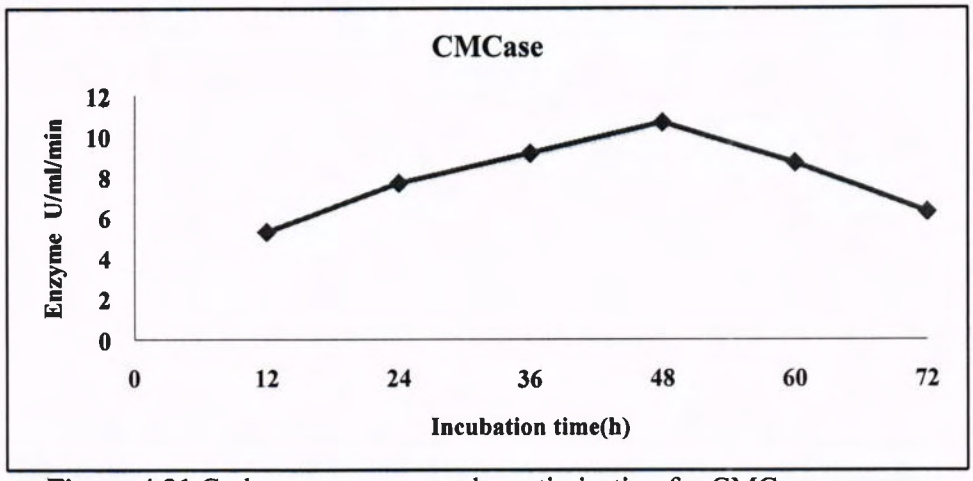

Figure 4.31 Carbon source corn cobs optimization for CMCase enzyme

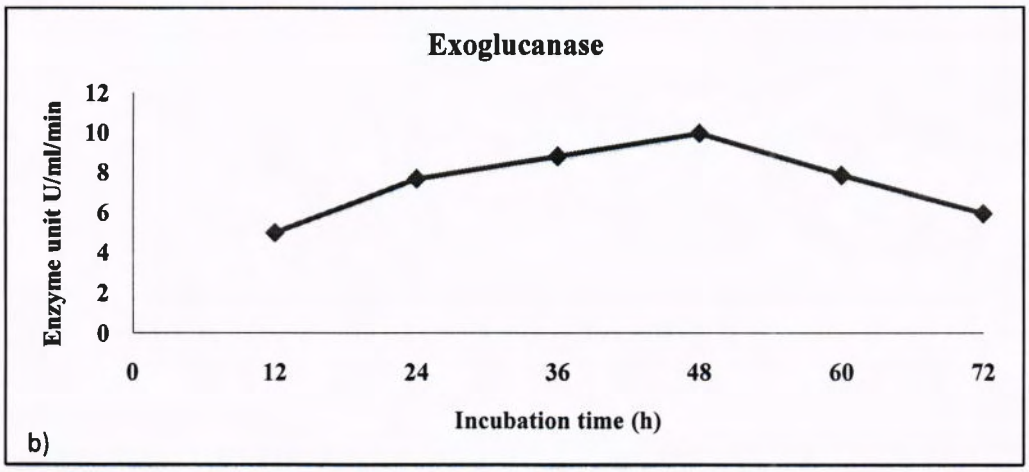

Figure 4.32 Carbonsource corn cobs optimization for Exoglucanase enzyme

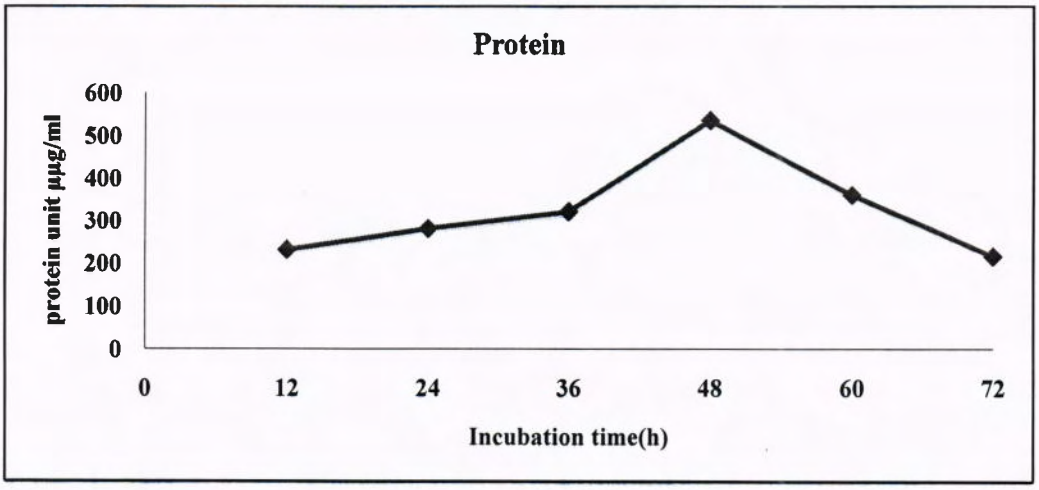

Figure 4.33 Carbon source corn cobs optimization for extracellular protein production

4.6 Selection of nitrogen sources for Cellulase production

Different nitrogen sources were selected for the growth of *Humicola fuscoatra.* The nitrogen sources used here in this research are ammonium nitrate, ammonium sulphate, Urea and DAP with a selected carbon source (wheat bran). *Humicola fuscoatra* was grown in the presence of all these mentioned sources for 5-7 days. After every 12 h the nitrogen source was observed forcellulase and protein production showed that highest enzyme unit 20.48 U/ ml/min was produced on DAP at 48 h showed highest protein unit produced on DAP at 48 h was noted 881.31mg/ ml.

Table 4.12 Nitrogen source urea optimization for Cellulase production

s.no	Time of incubation(h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay μg/ml
1	12	4.98	3.68	210.59
2	24	6.29	5.98	360.20
3	36	8.93	7.49	474.32
4	48	16.27	12.26	766.64
5	60	9.41	6.20	212.35
6	72	4.13	3.12	209.51

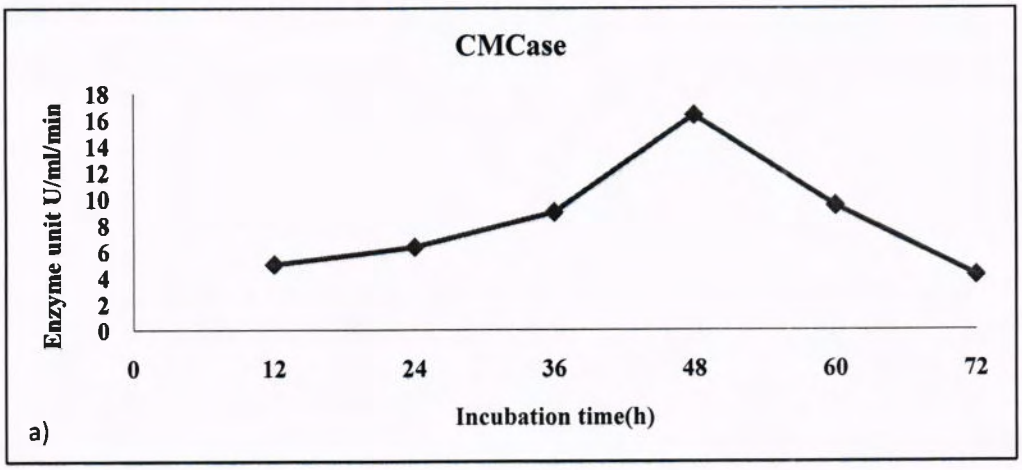

Figure 4.34 Nitrogensource urea optimization for CMCase enzyme

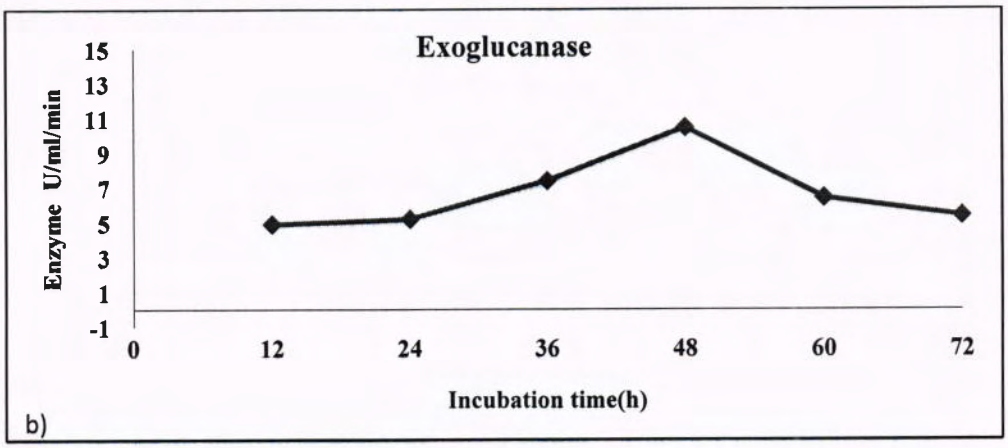

Figure 4.35 Nitrogen source urea optimization for Exoglucanase enzyme

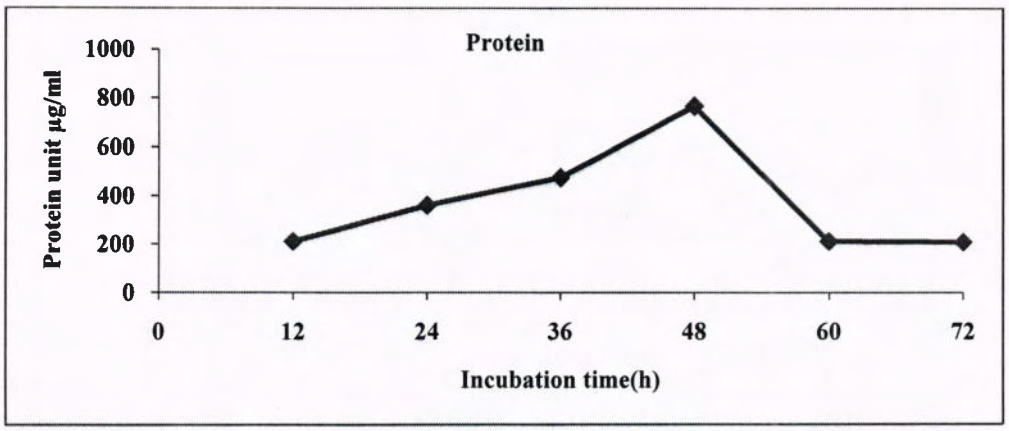

Figure 4.36 Nitrogen source urea optimization for extracellular protein production

Table 4.13 Nitrogen source DAP optimization for Cellulase production

S.no	Incubation time (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ml
1	12	5.49	4.50	250.10
2	24	7.94	6.99	348.25
3	36	16.46	14.94	580.44
4	48	20.48	18.53	881.38
5	60	9.42	12.11	329.11
6	72	3.87	2.56	273.21

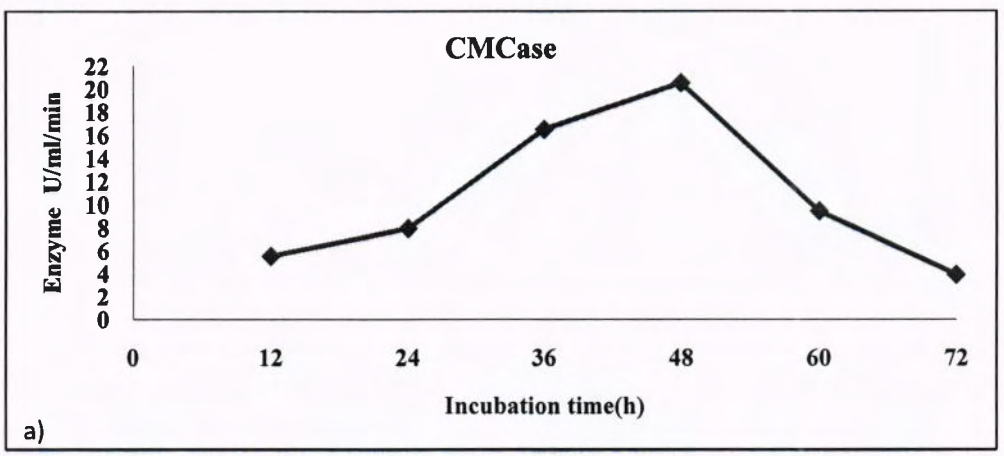

Figure 4.37 Nitrogen source DAP optimization for CMCase enzyme

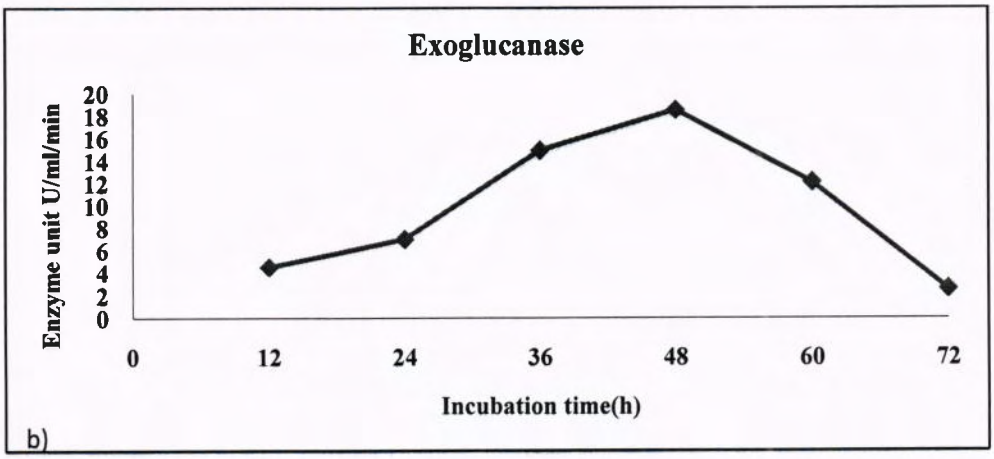

Figure 4.38 Nitrogen source DAP optimization for Exoglucanase enzyme

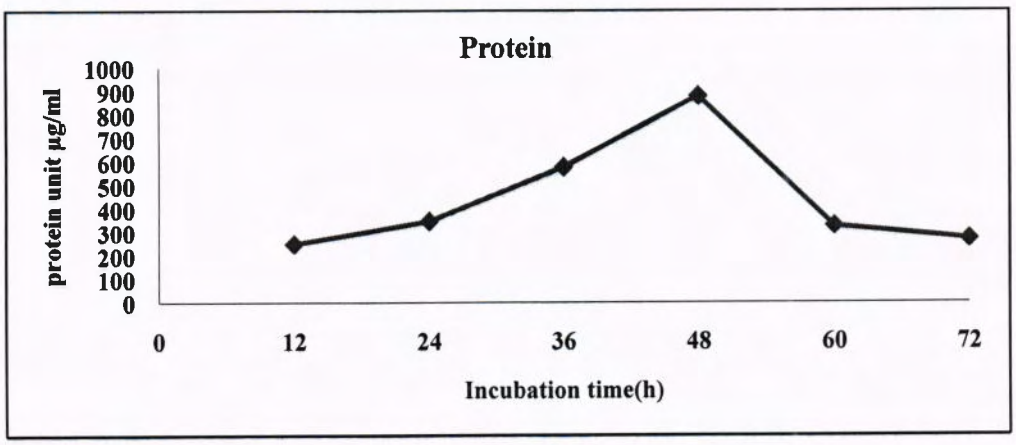

Figure 4.39 Nitrogen source DAP optimization for extracellular protein production

Table 4.14 Nitrogen source Ammonium sulphate optimization for Cellulase production

S.no	Incubation time (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ ml
1	12	4.29	4.91	205.10
2	24	5.89	5.21	298.50
3	36	7.37	7.39	363.21
4	48	11.95	10.46	431.29
5	60	8.53	6.42	365.41
6	72	6.45	5.38	211.68

4.8 Optimization of temperature for Cellulase enzyme production

For optimization of temperature on cellulase enzyme production from *Humicola fuscoatra* we checked different temperature ranges like 35°C, 40°C, 45°C and 50°C. *Humicolafuscoatra*was grown on these above mentioned temperatures for a pick value of the enzyme. The production was carried out for 72 h and maximum enzyme unit and protein production was calculated at 48 h after checking 12 h of time interval. The highest enzyme unit 59.51 U/ ml produced at 45°C and that highest protein unit was 92.20 mg/ ml produced at 45 °C after 48 h.

Table 4.20 Temperature optimization (35°C) for Cellulase production

S.no	Time of incubation(h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ ml
1	12	5.79	4.29	220.55
2	24	7.92	7.56	245.78
3	36	8.34	7.89	328.24
4	48	9.45	9.02	486.75
5	60	7.62	7.01	292.45
6	72	5.48	5.78	208.49

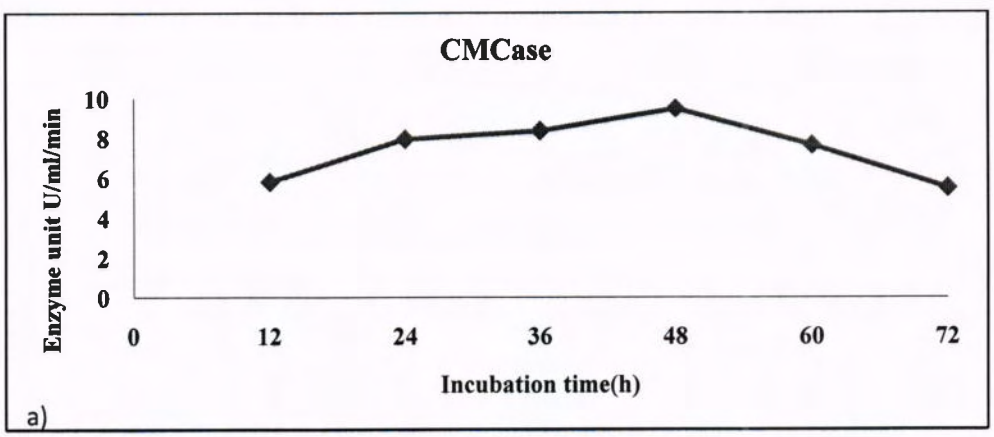

Figure 4.58 Temperature optimization (35°C) for CMCase enzyme

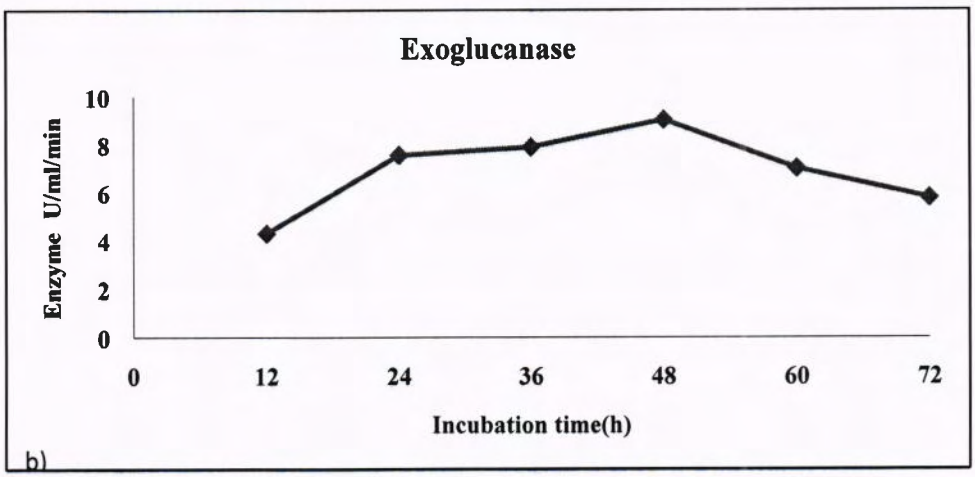

Figure 4.59 Temperature optimization (35°C) for Exoglucanase enzyme

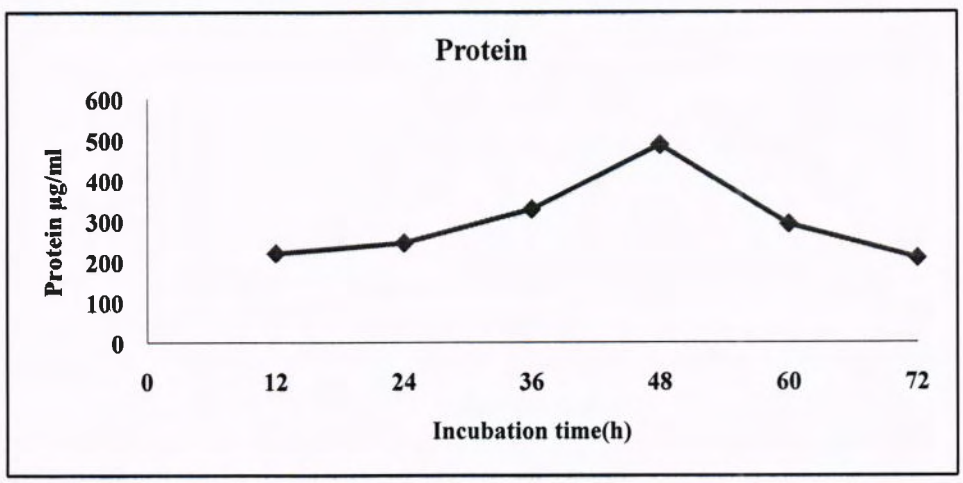

Figure 4.60 Temperature optimization (35°C) for extracellular protein production

Table 4.21 Temperature optimization (40°C) for Cellulase production

S.no	Time of incubation(h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein assay µg/ ml
1	12	6.21	5.31	214.12
2	24	7.84	8.12	264.78
3	36	9.79	9.39	320.29
4	48	10.49	10.22	499.31
5	60	8.78	9.71	317.50
6	72	7.14	6.88	226.27

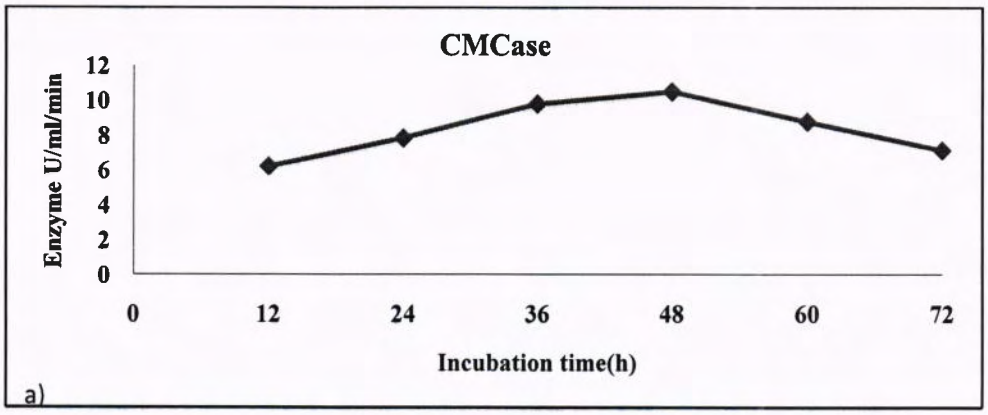

Figure 4.61 Temperature optimization (40°C) for CMCase enzyme

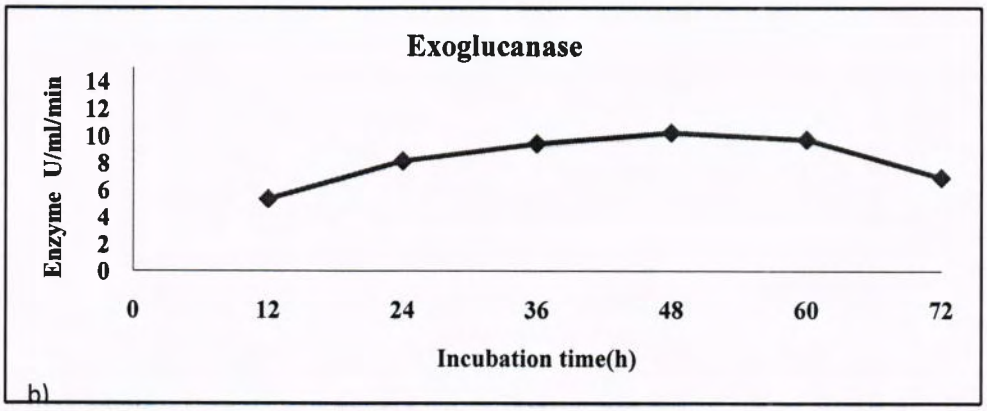

Figure 4.62 Temperature optimization (40°C) for Exoglucanase enzyme

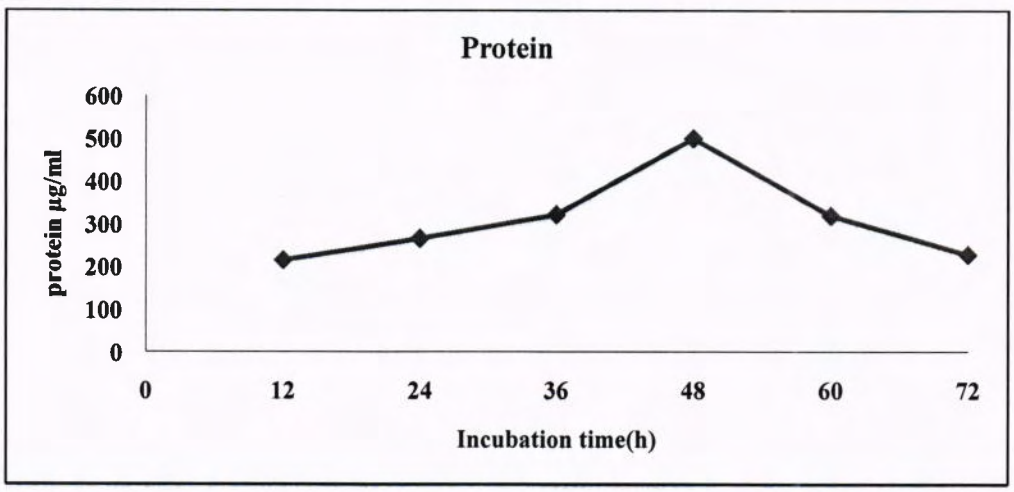

Figure 4.63 Temperature optimization (40°C) for extracellular protein production

Table 4.22 Temperature optimization (45°C) for Cellulase production

S.no	Incubation time (h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein unit µg/ ml
1	12	7.28	6.78	260.20
2	24	8.74	7.98	290.74
3	36	9.36	9.29	398.41
4	48	15.42	13.39	848.47
5	60	11.37	11.54	478.73
6	72	7.91	7.21	339.12

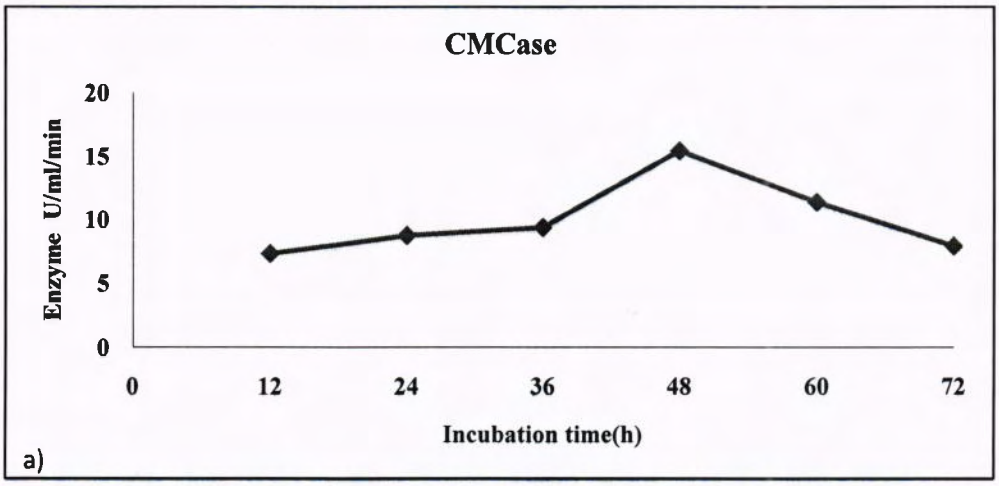

Figure 4.64 Temperature optimization (45°C) for CMCase enzyme

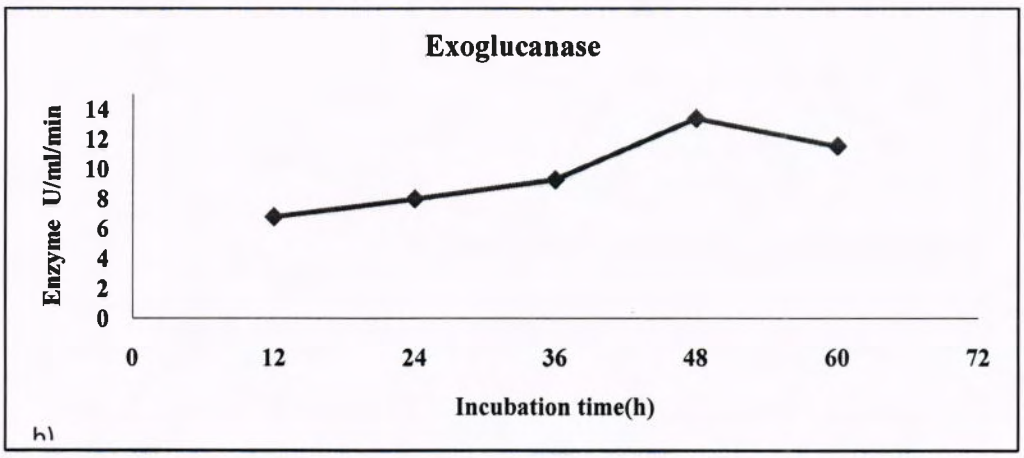

Figure 4.65 Temperature optimization (45°C) for Exoglucanase enzyme

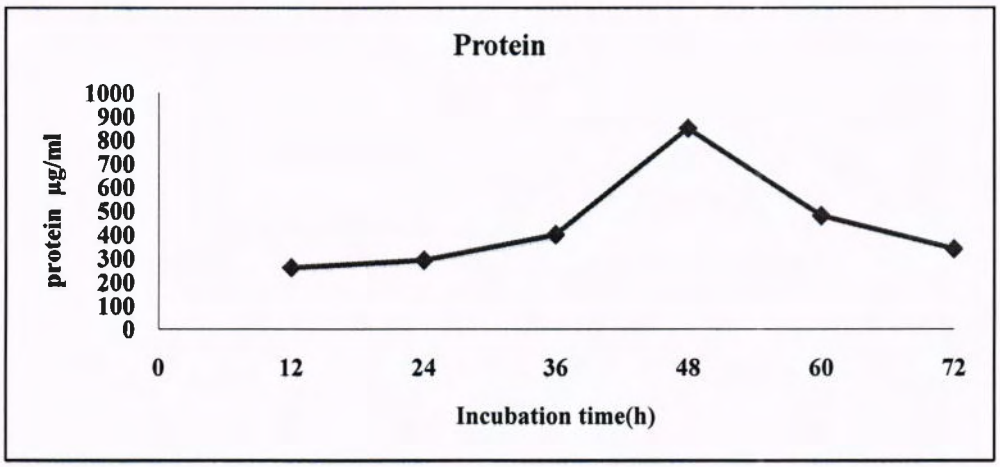

Figure 4.66 Temperature optimization (45°C) for extracellular protein production

Table 4.23 Temperature optimization (50 °C) for Cellulase production

S.no	Time of incubation(h)	CMCase U/ml/min	Exoglucanase U/ml/min	Protein µg/ ml
1	12	5.26	4.97	230.50
2	24	7.66	7.65	280.31
3	36	9.13	8.78	320.54
4	48	10.64	9.95	535.41
5	60	8.66	7.83	360.22
6	72	6.24	5.90	215.33

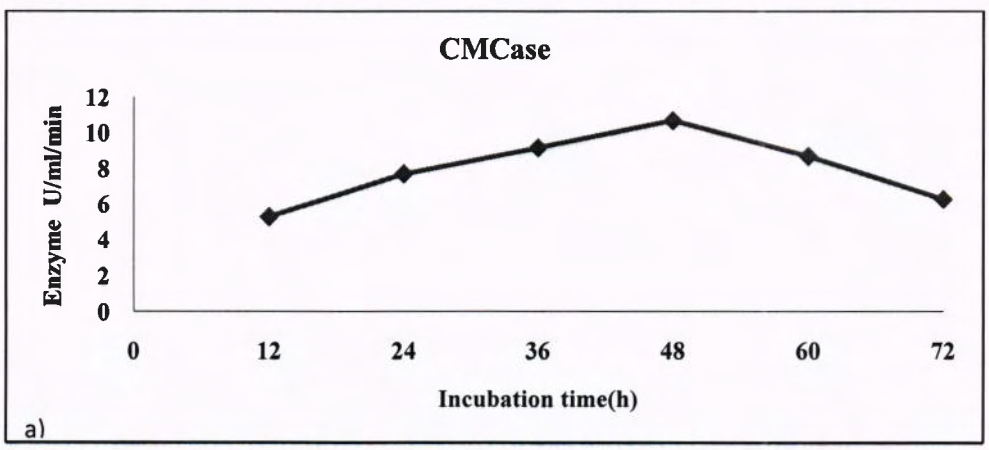

Figure 4.67 Temperature optimization (50 °C) for CMCase enzyme

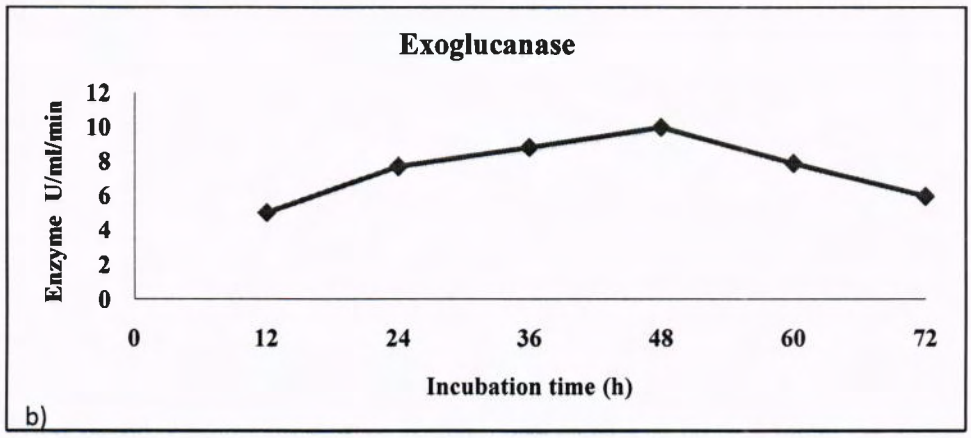

Figure 4.68 Temperature optimization (50 °C) for Exoglucanase enzyme

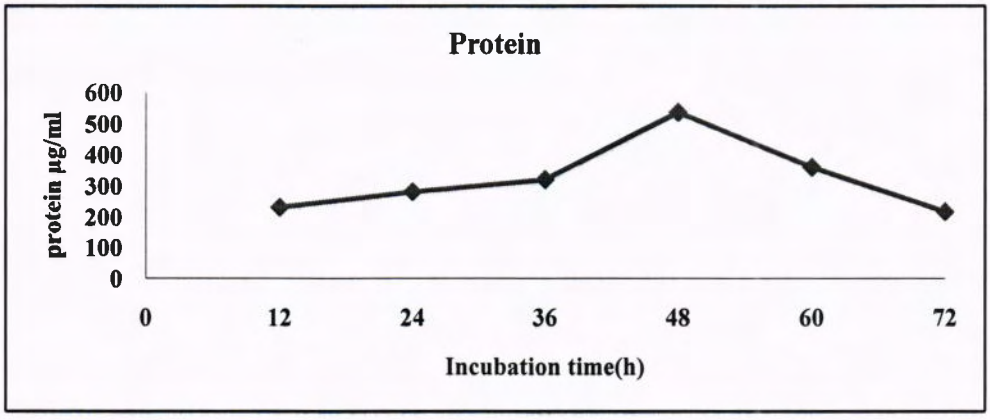

Figure 4.69 Temperature optimization (50 °C) for extracellular protein production

4.9 Partial purification of Cellulase enzyme

The Purification of impure cellulase enzyme was performed by ammonium sulphate precipitation techniques now obtaining the required facilitating conditions for *Humicola fuscoatra.* The different concentrations of ammonium sulphate concentration different concentrations like 20 %, 30 %, 40 %, 50 %,60 %, 70 % and 80 % were added in 100 ml crude enzyme preparation. The crude enzyme was divided in 7 test tubes so as to occupy 2 ml in each tube and then added the mentioned concentration of ammonium sulphate and kept for overnight. After performing centrifugation, the protein and enzyme units were calculated for crude preparation of enzyme. After this we selected 20 %, 30 % and 50 % concentrations for further use. then we added these concentrations of

ammonium salt and kept in fridge for 4 h and performed centrifugation and obtained supernatant and pellet values. In supernatant we added 5.6 g ammonium sulphate while suspended the pellet in 10 ml citrate buffer. For supernatant we again performed centrifugation and obtained supernatant and pellet values The pellet values were also checked andsaved.

Table 4.24 The enzyme precipitation from *Humicola*sp on various concentration of Ammonium sulphate salt

Ammonium sulphate salt concentration %	CMCase U/ml/min	Exoglucanase U/ml/min	Protein µg/ml
20	10.90	8.65	581.20
30	16.66	13.42	670.59
40	19.71	17.59	879.64
50	22.56	21.98	963.82

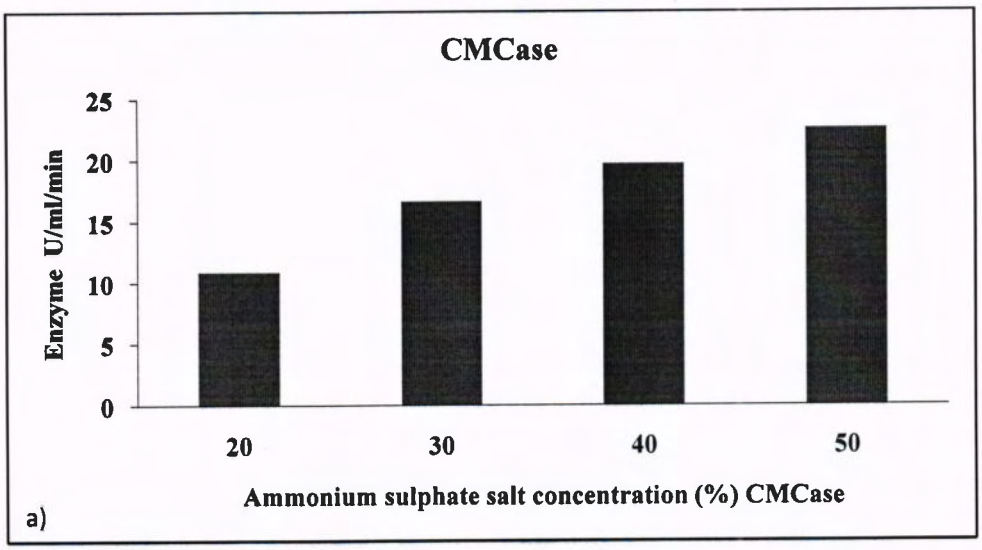

Figure 4.70 Ammonium sulphate salt concentration for CMCase enzyme

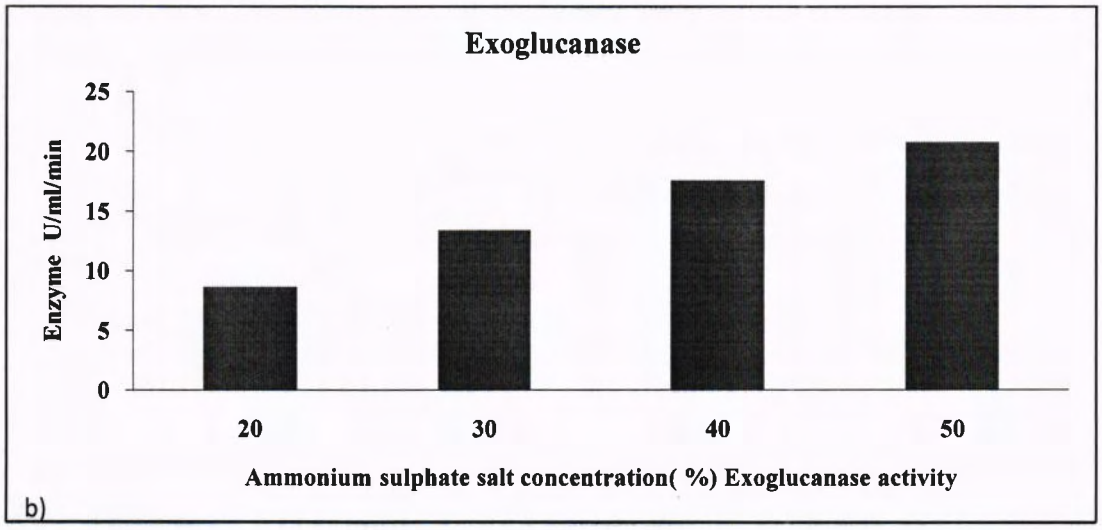

Figure 4.71 Ammonium sulphate salt concentration for Exoglucanase enzyme

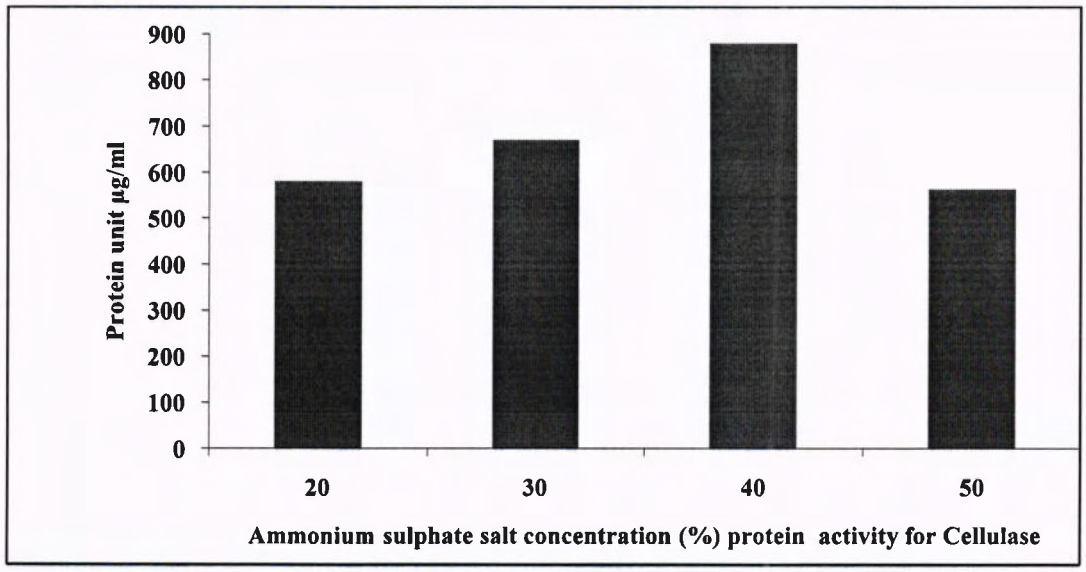

Figure 4.72 Ammonium sulphate salt concentration for Cellulase enzyme

4.10 Characterization of purified Cellulase of *Humicola fuscoatra*

The partially purified enzyme obtained from *Humicola* sp. was used to find out different kinetic parameters as following.

4.10.1 Effect of CMC concentration on partialy purified Cellulase

The effect of CMC concentration on enzymatic activity of *Humicola Fuscoatra* was observed on optimized temperature 45°C. Different CMC concentrations tested from 0.1 ml to 0.9 ml as shown in table 4. Results showed that the highest pick value of the

enzyme at 0.9 ml was larger of all concentrations. with the increase in CMC concentration maximum enzyme unit was calculated at 0.9 ml i .e 34.96 (U/ ml/ min). Table 4.3 data was plotted in the form of line weaver –Burk plot figure. The K_m and V_{max} values for cellulase were calculated as 0.6 gm/ ml and 315.5 U/ml /min.

Table 4.25 Effect of different CMC concentration on Cellulase enzyme

CMC concentration (gm/ ml)	Cellulase Enzyme unit (U/ml/min	1/ CMC concentration (gm/ ml)	1/ Enzyme unit (U/ml/min
0.1 ml	6.71	10	0.14903
0.2 ml	12.76	5	0.07837
0.3 ml	17.57	3.33	0.05692
0.4 ml	17.78	2.5	0.05624
0.5 ml	19.64	2	0.05086
0.6 ml	23.38	1.66	0.04277
0.7 ml	25.58	1.42	0.03909
0.8 ml	28.23	1.25	0.03542
0.9 ml	34.96	1.11	0.0286

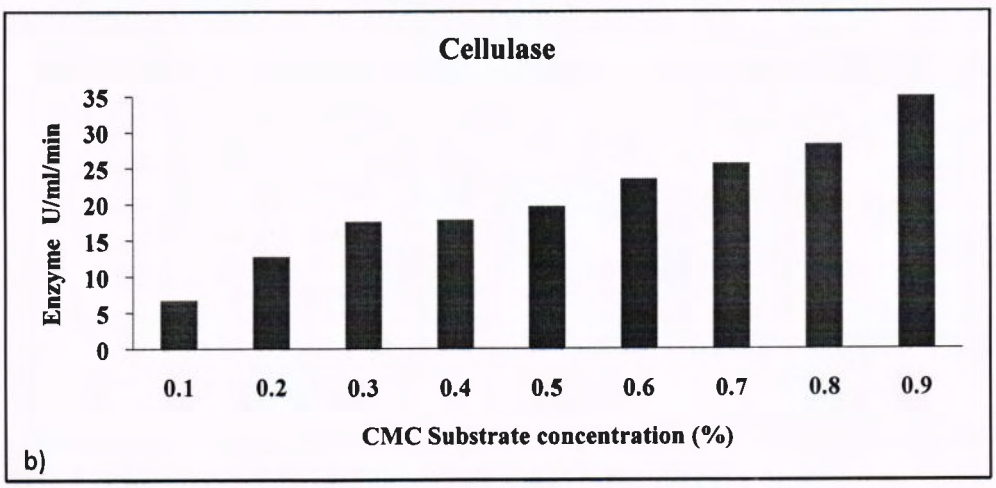

Figure 4.73 Effect of different CMC concentration for Cellulase enzyme

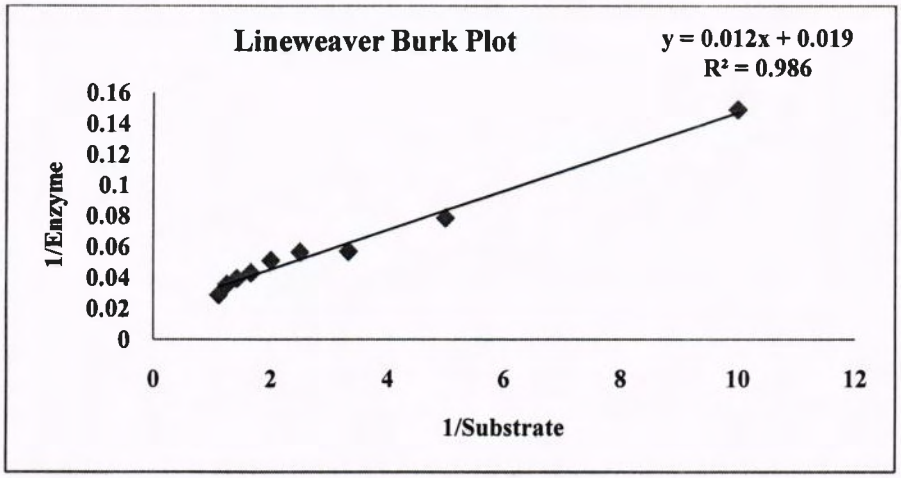

Figure 4.74 Line weaverburk plot for Cellulase enzyme

4.11 Influence of pH on Cellulase enzyme assay

The influence of pH on cellulase enzyme from *Humicola fuscoatra* activity was observed on various pH buffers from pH 4.0 to pH 7.5. the effect of Ph 6.5 revealed that highest cellulase assay was obtained at a pH of 5 after the incubation time of 15 mints.

Table 4.26 Effect of pH on partialy purified Cellulase enzyme

pH	Enzyme activity (U/ml/min)
4.0	15.14
4.4	16.37
4.8	19.18
5.2	24.35
5.8	25.28
6.2	31.32
6.5	38.50
7.00	34.17
7.5	18.80

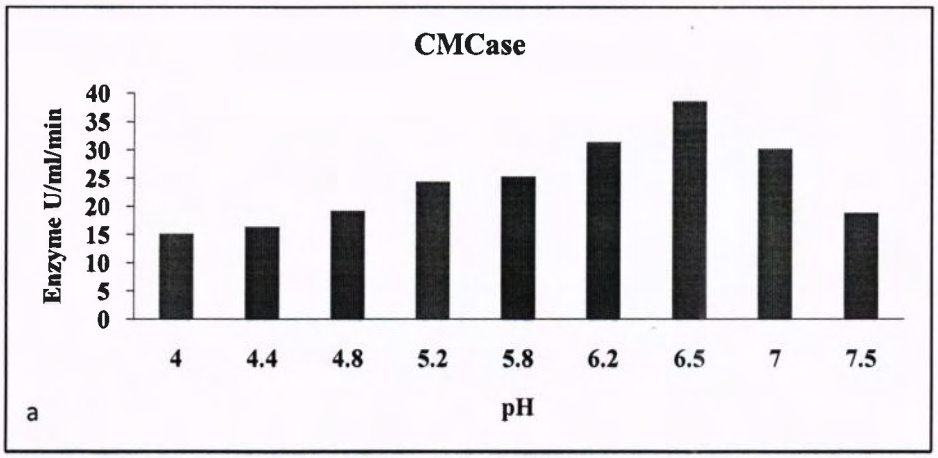

Figure 4.75 Effect of pH on CMCase enzyme

4.12 Influence of temperature on Cellulase enzyme activity

The influence of temperature on cellulase assay from *Humicola Fuscoatra* was evaluated on different values of temperature. This was observed from 25°C to 65°C after the time interval for 15 mints. Results showed that highest activity of cellulase was noted at 45°C.

Table 4.27 Effect of temperature on Cellulase enzyme

Temperature °C	Enzyme unit (U/ml/min
25	4.32
30	6.31
35	9.45
40	19.27
45	39.41
50	32.25
55	21.15
60	15.52
65	9.55

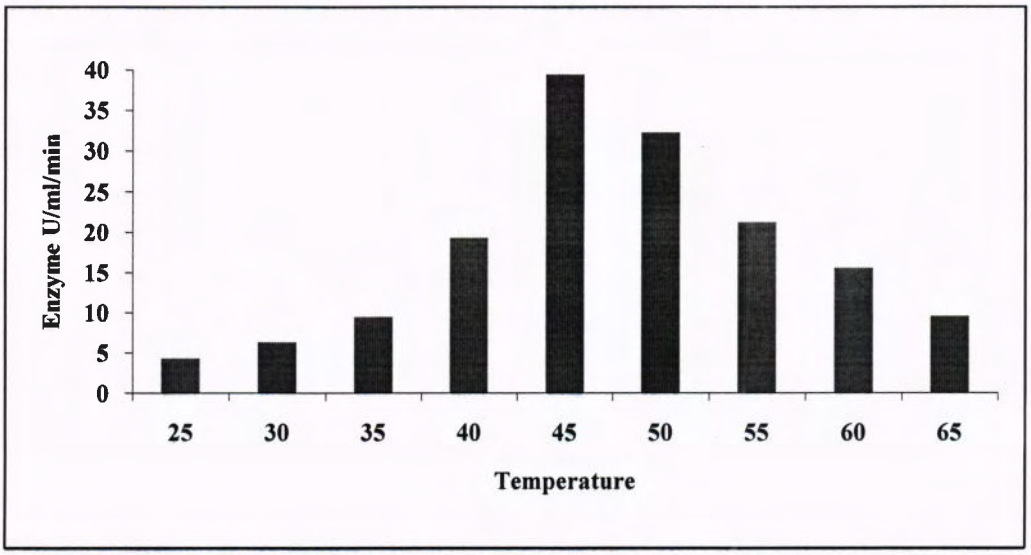

Figure 4.76 Effect of temperature on Exoglucanase enzyme

4.13 Influence of incubation time on Cellulase enzyme activity

The influence of incubation time on cellulase enzyme activity may also varies. The unit value of *Humicola fuscoatra* on optimized conditions was noted i.e. from time interval of 5 mints to 50 mints. Results showed that highest enzyme activity was observed at incubation time of 30 mints at 45°C.

Table 4.28 Effect of incubation time on Cellulase enzyme activity

Incubation time (mints)	Enzyme unit (U/ml/min)
5	5.33
10	10.40
15	15.12
20	19.17
25	22.47
30	33.34
35	30.03
40	28.95
45	22.20
50	18.15

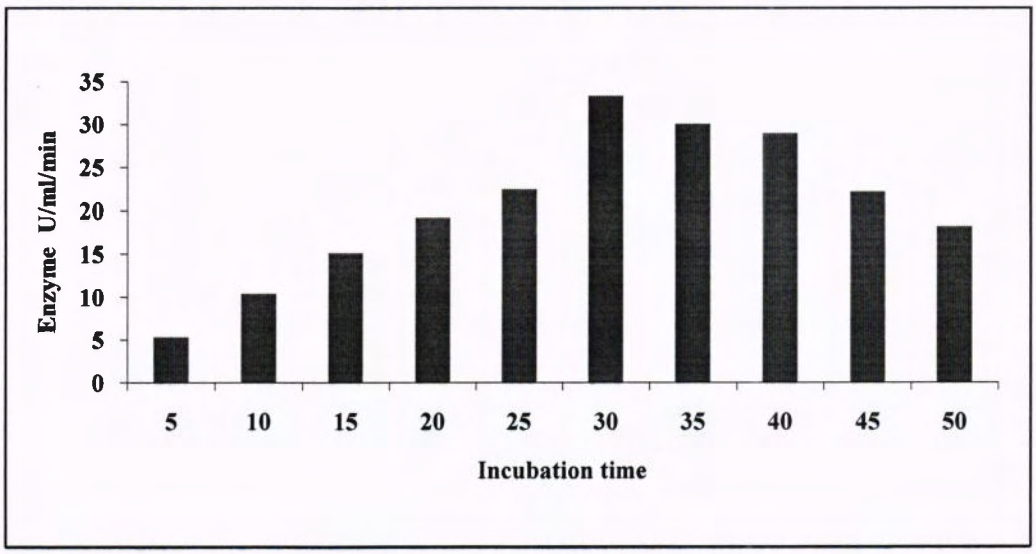

Figure 4.77 Effect of incubation time on CMCase enzyme

DISCUSSION

5. DISCUSSION

Enzymes are biological catalysts which speeds up the chemical reactions inside the cell or body. They are highly specific in nature, means that they catalyzed the specific chemical or biochemical reactions not all the reactions. They also lower the activation energy of the molecules which are necessary for life. There are 2000 different types of enzymes, each of which shows specifity for chemical reactions. Cellulases are the class of enzymes that hydrolyse cellulose and lignocellulose bio wastes. These enzymes are synthesized by various types of microorganisms such as bacteria and fungi. Fungi are the major source of production of cellulase enzyme.*Trichoderma* is well known specie for the production of cell wall hydrolyzing enzymes, Bajaj *et al.*, (2013).

Cellulases are defined as the enzymes that breaks down β -1,4 linkages in cellulose chains and fibers. These enzymes are generated by many microorganismslike protozoans, plants, and animals. Different families of the enzymes have different catalytic groups and different arrangements of amino acids so they have various shapes and morphology (Henrissat, 1991). Cellulases are the enzymes which converts world's most plentiful biopolymers, cellulose, lignin and hemicelluloses into reducing sugars and used in many industrial applications like biofuel and bioethanol production etc., (Bhat, 2000). Many microorganisms are used for the degradation of cellulose to produce cellulases but Fungi are the best living entities which performed the breakdown of cellulose. they are majorly distributed in nature and used for commercial production of cellulases. Most of the fungi intricate one or more cellulolytic enzymes including endoglucanase, Exoglucanase and β- glucosidase (Bhat, 2000). Cellulase and their group enzymes are mainly used in many industrial applications, especially in health, medicines, agriculture, drugs, detergents, in laundry for color care, food industry, fiber modification, animal feed, textile, paper and pulp industry(Bhatia*et al.*,2001).

Tahtamouni *et al.*, 2006 reported that Cellulase and its group enzymes are produced from plants, animals, insects and microbial sources. By fermentation techniques microbial and industrial enzymes have the vast advantages of being capable to high level production for global and commercial level. A large number of bacteria, fungi, and algae are available for their capability to produce cellulytic enzymes like cellulases.

Streptomyces sp. is one of the best known enzyme producers of them (El-Sersy *et al.*, 2010). Good optimizing conditions with favourite environment are provided to microorganisms to produce cellulases. and these conditions were optimized by maintaining different carbon and nitrogen sources in the medium maintained at pH 6 - 6.5 and temperature 50°C under solid state fermentation. So many studies were carried out such as studying their biochemical and biophysical characteristics. Such optimized conditions were carboxy methyl cellulose (CMC). Magnesium sulphate, potassium phosphate, Ammonium nitrate and liquid medium for high level of cellulase enzyme production by *Streptomyces* sp grown at 30°C for nearly 96 h of incubation period.

*Humicola*are species of thermophilic fungi belongs to hypomycetes and chaetomicae family usually isolated from soil wastes. There is large no of species of *Humicola*mostly 20 species among them are freely founded and familiar.They are most important species having strongly cellulytic fungi, although *Humicola greasea* are non-pathogenic but sometimes it causes diseases in humans which is known as peritonitis as well as hypersensitivity pneumonitis. *Humicola fuscoatra* was identified morphologically as the causative organism but still not confirmed molecularly. However, it was successfully treated with antifungal therapy. In The present study work was done on the production, and purification of cellulases from *Humicola fuscoatra.* The next objective was to perform characterization of different parameters on *Humicola.* Here differenttypes of growth parameters and fermentation conditions such as time cource, inoculum size, effect of pH, temperature, aeration and carbon nitrogen sources.. First of all, the enzyme was subjected to 20 to 80 % ammonium sulphate precipitation concentrations. The characterization was performed after purification of crude cellulase enzyme extract. The partially purified cellulase enzyme characterization was performed for different types of kinetic parameters like influence of pH, temperature, substrate concentration incubation time was tested. The main purpose of this current research was to optimized various conditions for cellulase production on maximum level for further commercial uses at industrial scale.

Riaz *et al.*, (2014) worked on *Humicolainsolen* at a temperature of 45°C, to generate maximum level of cellulase.It is reported that CMCase was produced by

Humicola insolens on 2% CMC at 45°C and using various inoculum densities. Pick value of CMCase production i.e., 0.79 ± 0.04 was observed at 8% inoculum size. CMCase was produced by *Humicola insolen* on different concentrations of carbon sources like. CMC at 45°C and pH 5.0 using optimized 8% i.e 12.41 mg cells mL-1 inoculum size. 5% CMC showed maximum CMCase production i.e. 2.0± 0.110 and total protein at that time was 0.131±0.007and specific activity was 15.45. Javed *et al.*, (2011) and Huma *et al.*, (2012) studied the effect of pH on CMCase production by *Humicola insolens* and was observed at pH,4.0, 4.5, 5.0, 5.5 and 6.0 at 45°C, keeping all the optimized conditions same. Highest CMCase production was 2.0±0.1 U mL-1 at pH 5.0 and the extracellular protein at the time of maximum CMCase production was 0.13 mg mL-1, while specific activity was 15.45. The μ and td were 0.046 and 15.0, respectively. The *Humicola insolens* was grown at different temperatures ranges i.e., 36°C, 39°C, 42°C and 45°C for the optimal production CMCase using pre optimized conditions. Maximum CMCase production i.e. 1.99±0.121 was observed at 45°C. Total protein at the time of maximum CMCase production was 0.129±0.007, while specific activity was 15.42.

In this current study, different inoculums size such as 5, 10, and 15 % were checked for the maximum production of cellulases. All these percentages were used but the result showed that 10% inoculums size created best and highest enzyme production and according to fateh *et al.,* 2011 same like result produced at 10 % inoculums size. In my research project incubation time was performed from 12 to 72 hours while maximum result showed at 48 h CMCase production was 14.70, Exoglucanase enzyme was 11.69 and protein estimation was 107.71μg/ml. According to Luiza Jecu, 2010 the best cellulase enzyme production was obtained by using citrate buffer of pH 5.0 9.85 IU/ml and in contrast with me there produced highest enzyme production at pH 7.0. The CMCase enzyme production was 24.64 , Exoglucanase production was 17.49 and protein estimation was 769.20 μg /ml.

Ahmed *et al.*, 2005 reported that the medium pH and temperature is a vital and most necessary factor which effects on the creation of enzyme production. The temperature for the enzyme production for *Aspergillus* species was optimized. Maximum CMCase activities were achieved at 35 °C and in my research work I optimized the

temperature 45°C for *Humicola* species. The result showed that CMCase production was 15.42 U/ml, Exoglucanase production was 13.39 U/ml and protein estimation was 848.47 µg/ ml. there is a clear difference between these two data. According to Gouri and Malana, (2010) reported that urea was the most favorable nitrogen source for cellulase enzyme production for *Aspergillus* species while in contrast with my research data, there was optimized DAP a good nitrogen source for the creation of cellulase enzyme by *Humicola Fuscoatra* utilizing DAP as a nitrogen source. Exoglucanase and protein production. After 48 h of incubation time CMCase production was 20.48 U/ml, Exoglucanase production was 18.53 and protein production was 881.38 µg/ ml.

According to Naim and Jamil, (2007) they used different carbon sources and produced highest enzyme unit by using corn cobs 28.15 U/ ml and in my research study four types of carbon sources like rice husk, dab grass, wood chip and wheat bran forhighest enzyme. the Carboxymethyle cellulase production was 15.99 U/ ml, Exoglucanase production was noted 14.94 and protein production was 568.90 µg/ ml for *Humicola Fuscoatera* after 48 h of incubation period. (Javed et al., 2011; Huma *et al.*, 2012) studied the influence of CMC concentration on partially impure enzyme cellulase. The cellular protein content found was larger by using 8 % density at 45°C. Various time course was taken and checked for extracellular protein, CMCase activity and biomass content. The optimum substrate concentration optimized was 5% which was used in further experiments. In contrast to my research work, influence of PH, temperature, substrate conc, and incubation time were checked. The cellulase enzyme production from *Humicola fuscoatra* bysubmerged method of fermentation. The yield of cellulase was so much greater 40.17 U/ml at temperature 45°C 39.41 U/ml and incubation period of 30 minutes was 33.34 U/ml.

Conclusion and future prospects

The result obtained here identified that carbon source wheat bran, nitrogen source DAP, optimum medium pH 7.0 and fermentation temperature 45°C for 48 h yielded maximum production of cellulase (U/ml/min). The production of cellulases by *Humicola fuscoatra* under submerged fermentation was evaluated. CMC served as a powerful inducer that enhanced the cellulases yields where avicel repressed cellulases activities. Scientists also found edlh genes in this specie which could be implanted in plants to control global warming in future.

REFERENCES

6. REFERENCES

Ahmed S. and Ashraf, M. Production of Industrially important enzymes using molecular approaches; cellulases and xylanases. In: Genetic resources and Biotechnology II, Volume Two, eds by D. Thangadurai, T. Pullaiah, Pedro A.

Ahmed, S., Bashir, A., Saleem, H., Saadia, M., & Jamil, A. (2009). Production and purification of cellulose-degrading enzymes from a filamentous fungus *Trichoderma harzianum*. Pakistan Journal of Botany, 41(3), 1411-1419.

Amouri, B., & Gargouri, A. (2006). Characterization of a novel β-glucosidase from a Stachybotrys strain. *Biochemical Engineering Journal*, *32*(3), 191-197.

Anish, R., Rahman, M. S., & Rao, M. (2007). Application of cellulases from an alkalothermophilic Thermomonospora sp. in biopolishing ofdenims. *Biotechnology and Bioengineering*, *96*(1), 48-56.

Anuradha Jabasingh, S., Varma, S., & Garre, P. (2014). Production and purification of cellulase from Aspergillus nidulans AJSU04 under solid-state fermentation using coir pith. *Chemical and biochemical engineering quarterly*, *28*(1), 143-151.

Bahkali, A. H. (1996). Influence of various carbohydrates on xylanase production in Verticillium tricorpus. *Bioresource Technology*, *57*(3), 265-268.

Bajaj, B. K., Sharma, M., & Rao, R. S. (2014). Agricultural residues for production of cellulase from *Sporotrichum thermophile* LAR5 and its application for saccharification of rice straw. *J Mater Environ Sci*, *5*(5), 1454-1460.

Baker, R. A., & Bruemmer, J. H. (1989). Quality and stability of enzymically peeled and sectioned citrus fruit.Balatti. Regency publications, New Delhi. 20

Ballav, S., Dastager, S., & Kerkar, S. (2011). Biotechnological significance of Actinobacterial research in India. *Recent Research in Science and Technology*, *4*(4).

Bayer, E. A., Lamed, R., & Himmel, M. E. (2007). The potential of cellulases and cellulosomes for cellulosic waste management. *Current opinion in Biotechnology*, *18*(3), 237-245.

Bayer, E. A., Morag, E., & Lamed, R. (1994). The cellulosome—a treasure-trove for Biotechnology. *Trends in biotechnology*, 12(9), 379-386.

Bergquist, P., Te'o, V., Gibbs, M., Cziferszky, A., de Faria, F., Azevedo, M., & Nevalainen, H. (2002). Expression of xylanase enzymes from thermophilic microorganisms in fungal hosts. *Extremophiles*, *6*(3), 177-184.

Bhat, M. K. (2000). Cellulases and related enzymes in biotechnology. *Biotechnology Advances*, 18(5), 355-383.

Bhatia, Y., Mishra, S., & Bisaria, V. S. (2002). Microbial β-glucosidases: cloning, properties, and applications. *Critical Reviews in Biotechnology*, *22*(4), 375-407.

Bhatti, H. N., Batool, S., & Afzal, N. (2013). Production and characterization of a novel β-glucosidase from *Fusarium solani. International Journal of Agriculture & Biology*, *15*(1).

Bisaria, V. S. (1991). Bioprocessing of agro-residues to glucose and chemicals. *Bioconversion of Waste Materials to Industrial Products.*, 187-223.

Botella, C., De Ory, I., Webb, C., Cantero, D., &Blandino, A. (2005). Hydrolytic enzyme production by *Aspergillus awamori* on grape pomace. *Biochemical Engineering Journal*, *26*(2), 100-106.

Brennan, M. B. (1996). Enzyme Discovery Heats Up (Vol 74, Pg 33, 1996). *Chemical & Engineering News*, *74*(44), 6-6.

Chandra, R. P., Bura, R., Mabee, W. E., Berlin, D. A., Pan, X., & Saddler, J. N. (2007). Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics?. In *Biofuels* (pp. 67-93). Springer Berlin Heidelberg.

Chen, Q., Jin, Y., Zhang, G., Fang, Y., Xiao, Y., & Zhao, H. (2012). Improving production of bioethanol from duckweed (*Landoltia punctata*) by pectinase pretreatment. *Energies*, *5*(8), 3019-3032.

Chesson, A. (1987). Supplementary enzymes to improve the utilization of pig and poultry diets. *Recent Advances in Animal Nutrition, 1987*, 71-89.

Cooney, C. L., Wang, D. I. C., Wang, S. D., Gordon, J.,& Jiminez, M. (1978). Simultaneous cellulose hydrolysis and ethanol production by a cellulolytic anaerobic bacterium. In *Biotechnol. Bioeng. Symp.;(United States)*(Vol. 8).

Coutinho, P. M. (2004). Carbohydrate-active enzymes server. *http://afmb. cnrs-mrs. fr/~ cazy/CAZY/index. html.*

Couto, S. R., & Sanromán, M. A. (2006). Application of solid-state fermentation to food

industry—a review. *Journal of Food Engineering*, *76*(3), 291-302..

Devi, M. C., & Kumar, M. S. (2017). Production, optimization and partial purification of cellulase by Aspergillus niger fermented with paper and timber sawmill industrial wastes. *Journal of microbiology and biotechnology research*, *2*(1), 120-

El-Sersy, N. A., Abd-Elnaby, H., Abou-Elela, G. M., Ibrahim, H. A., & El-Toukhy, N. M. (2010). Optimization, economization and characterization of cellulose produced by marine *Streptomyces ruber*. *African Journal of Biotechnology*, *9*(38), 6355-6364.

Eriksson, K. E. L., Blanchette, R., & Ander, P. (2012). *Microbial and Enzymatic Degradation of Wood and Wood Components*. Springer Science & Business Media.

Fennington, G., Lupo, D., & Stutzenberger, F. (1982). Enhanced cellulase production in mutants of Thermomonospora curvata. *Biotechnology and bioengineering*, *24*(11), 2487-2497.

Guarna, M. M., Lesnicki, G. J., Tam, B. M., Robinson, J., Radziminski, C. Z., Hasenwinkle, D., ... & Kilburn, D. G. (1997). On-line monitoring and control of methanol concentration in shake-flask cultures of Pichia pastoris. *Biotechnology and bioengineering*, *56*(3), 279-286.

Godfrey, T., & West, S. (1996). The application of enzymes in industry. *Industrial enzymology, 2nd edn. The Nature Press, New York*, 512.

Gao, J., Weng, H., Zhu, D., Yuan, M., Guan, F., & Xi, Y. (2008). Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. *Bioresource Technology*, *99*(16), 7623-7629.

Gilkes, N. R., Claessens, M., Aebersold, R., Henrissat, B., Meinke,A., Morrission, H. D., ... & Miller, R. C. (1991). Structural and functionalrelationships in two families of β-1, 4-glycanases. *The FEBS Journal*, *202*(2), 367-377.

Gori, M. I., & Malana, M. A. (2010). Production of carboxymethyl cellulase from local isolate of Aspergillus species. *Pak. J. life soc. sci*, *1*, 1-6.

Guo, H., Ingolia, N. T., Weissman, J. S., & Bartel, D. P. (2010). Mammalian microRNAs Predominantly act to decrease target messenger RNA levels. Nature, 466 (7308), 835.

Haakana, H., Miettinen-Oinonen, A., Joutsjoki, V., Mäntylä, A., Suominen, P., &Vehmaanperä, J. (2004). Cloning of cellulase genes from *Melano carpusalbomyces* and their efficient expression in *Trichoderma reesei. Enzyme andMicrobial Technology*, *34*(2), 159-167.

Hashmi, A. S. (2008). Fermentation of Yeast Sludge with Brevibacterium flavum to Enhance Lysine Concentration. *J. Chem. Soc. Pak*, *30*(4), 643.

Henrissat B (1991). A classifi cation of glycosyl hydrolases based on amino acid sequence similarities . Biochem. J. 280 (Pt 2): 309 – 316 .

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal*, *280*(2), 309-316.

Ibrahim, N. A., El-Badry, K., Eid, B. M., & Hassan, T. M. (2011). A new approach for biofinishing of cellulose-containing fabrics using acid cellulases. *Carbohydrate Polymers*, *83*(1), 116-121.

Javed, M. R., Rashid, M. H., Mukhtar, Z., Riaz, M., Nadeem, H., Huma, T., & Ashiq, N. (2011). Kinetics and thermodynamics of high level-glucosidase production by mutant derivative of *Aspergillus niger* under submerged growthconditions. *African Journal of Microbiology Research*, *5*(17), 2528-2538.

Kantelinen, A., Jokinen, O., Sarkki, M. L., Pettersson, C., Sundberg, K., Eckerman, C., & Holmbom, B. (1995, June). Effects of enzymes on the stability of colloidal pitch. In *Proc. 8th Int Symp Wood and Pulping Chemistry*(Vol. 1, pp. 605-12).

Kaur, B., Bhatia, S., & Phutela, U. (2015). Production of cellulases from *Humicola fuscoatra* MTCC 1409: Role of enzymes in paddy straw digestion. *African Journal of Microbiology Research*, *9*(9), 631-638.

Kibblewhite, R. P., Bawden, A. D., & Brindley, C. L. (1995). TMP fibre and fines qualities of thirteen radiata pine wood types. *Appita journal*, *48*(5), 367-377.

Kirk, O., Borchert, T. V., & Fuglsang, C. C. (2002). Industrial enzymeapplications. *Current opinion in biotechnology*, *13*(4), 345-351.

Kumar, V., Shahid, M., Srivastava, M., Singh, A., Pandey, S., & Maurya, M. K. (2015).

Screening of Trichoderma species for virulence efficacy on seven most predominant phytopathogens. *African Journal of Microbiology Research*, *9*(11), 793-799.

Li, Y. H., Ding, M., Wang, J., Xu, G. J., & Zhao, F. (2006). A novel thermos acidophilic endoglucanase, Ba-EGA, from a new cellulose-degrading bacterium, Bacillus sp. AC-1. *Applied Microbiology and Biotechnology*, *70*(4), 430-436.

Lynd, L. R., Weimer, P. J., Van Zyl, W. H., & Pretorius, I. S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and molecular biology reviews*, *66*(3), 506-577.

Mahalakshmi, N., & Jayalakshmi, S. (2016). Research article cellulase production by *Aspergillus niger* under solid state fermentation using agro industrial wastes. *Int J Adv Multidiscip Res*, *3*, 78-83.

Mansfield, S. D., Wong, K. K., De Jong, E., & Saddler, J. N. (1996). Modification of Douglas-fir mechanical and kraft pulps by enzyme treatment. *Tappijournal*, *79*(8), 125-132.

Milala, M. A., Shugaba, A., Zanna, H., & Appollos, B. (2011). Isolation and Partial purification of cellulase from Rhizopus stolonifer.

Mrudula, S., & Murugammal, R. (2011). Production of cellulase by *Aspergillus niger* under submerged and solid state fermentation using coir waste as asubstrate. *Brazilian Journal of Microbiology*, *42*(3), 1119-1127.

Naika, G. S., & Tiku, P. K. (2010). Characterization of functional intermediates of endoglucanase from Aspergillus aculeatus during urea and guanidine hydrochloride unfolding. *Carbohydrate research*, *345*(11), 1627-1631..

Naim, S., & Jamil, A. (2007). Production of endoglucanase from a thermophilic fungus. *Pak. J. Agri. Sci*, *44*, 1.

Niaz, M., Iftikhar, T., & Rashid, M. H. (2011). Carboxyl group modification of *Gymnoascella citrina* glucoamylase: Cross-linking with hydrophobic nucleophile enhanced thermostability and thermophilicity. *Clinical Biochemistry*, *44*(13), 93-94.

Park, S. R., Cho, S. J., Kim, M. K., Ryu, S. K., Lim, W. J., An, C. L.,& Yun, H. D.(2002). Activity enhancement of Cel5Z from Pectobacterium chrysanthemi

Reesei Cellulases and Other Hydrolases (TRICEL '93), vol. 8, Espoo, Finland, 1993.

Park, S. R., Cho, S. J., Kim, M. K., Ryu, S. K., Lim, W.J., An, C. L.,& Yun, H. D.(2002). Activity enhancement of Cel5Z from Pectobacterium chrysanthemi PY35 by removing C-terminal region. *Biochemical and biophysical research communications*, *291*(2), 425-430.

Pere, J., Siika-aho, M., Buchert, J., & Viikari, L. (1995). Effects of purified Trichoderma reesei cellulases on the fiber properties of kraft pulp. *Tappi journal (USA)*.

Pretel, M. T., Lozano, P., Riquelme, F., & Romojaro, F. (1997). Pectic enzymes in fresh fruit processing: optimization of enzymic peeling of oranges. *Process Biochemistry*, *32*(1), 43-49.

Riaz, H., Jabbar, A., Rashid, M. H., Riaz, S., &Latif, F. (2014). Endoglucanase Production by Humicola insolens: Effect of physiochemical factors on growth kinetics and thermodynamics. *International Journal of Agriculture & Biology*, *16*(6).

Roe, B., & Bruemmer, J. H. (1977). Treatment requirements for debittering and fortifying grapefruit and stable storage of the product. In *Proc Fla State Hortic Soc* (Vol. 90, pp. 180-2).

Rosgaard, L., Pedersen, S., Cherry, J. R., Harris, P., & Meyer, A. S. (2006). Efficiency of new fungal cellulase systems in boosting enzymatic degradation of barley straw lignocellulose. *Biotechnology Progress*, *22*(2), 493-498.

Sadhu, S., & Maiti, T. K. (2013). Cellulase Production by Bacteria: A Review.Sasi, A., Ravikumar, M., & ManthiriKani, S. (2012). Optimization, production and purification of cellulase enzyme from marine Aspergillus flavus. *African Journal of Microbiology Research*, *6*(20), 4214-4218.

Sasi, A., Ravikumar, M., & ManthiriKani, S. (2012). Optimization, production and purification of cellulase enzyme from marine Aspergillus flavus. *African Journal of Microbiology Research*, *6*(20), 4214-4218.

Shallom, D., & Shoham, Y. (2003). Microbial hemicellulases. *Current opinion in microbiology*, *6*(3), 219-228.

Sheehan, J., & Himmel, M. (1999). Enzymes, energy, and the environment: a strategic

perspective on the US Department of Energy's research and development activities for bioethanol. *Biotechnology Progress*, *15*(5), 817-827.

Singh, A., Srivastva, M., Pathak, N., Rastogi, S., & Srivastava, A. K. (2012). Evaluation of Antagonastic Activity and Shelf Life Study of Trichoderma viride (01PP 8315/11). *Advances in Life Sciences*, *1*(2), 138-140.

Soares, I., Távora, Z., Barcelos, R. P., & Baroni, S. (2012). Microorganism-produced enzymes in the food industry. In *Scientific, Health and Social Aspects of the Food Industry*. InTech.

Soares, I., Távora, Z., Barcelos, R. P., & Baroni, S. (2012). Microorganism-produced enzymes in the food industry. In *Scientific, Health and Social Aspects of the Food Industry*. InTech..

Sohail, M. U. H. A. M. M. A. D., Ahmad, A. Q. E. E. L., & Khan, S. A. (2011). Production of cellulases from Alternaria sp. MS28 and their partial characterization. *Pak. J. Bot*, *43*(6), 3001-3006.

Solomon, B. O., Amigun, B. A. M. I. K. O. L. E., Betiku, E. R. I. O. L. A., Ojumu, T. V., & Layokun, S. K. (1999). Optimization of cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 grown on bagasse. *JNSChE*, *16*, 61-68.

Srivastava, N., Rawat, R., Singh Oberoi, H., & Ramteke, P. W. (2015). A review on fuel ethanol production from lignocellulosic biomass. *International Journal of Green Energy*, *12*(9), 949-960.

Sukharnikov, L. O., Cantwell, B. J., Podar, M., & Zhulin, I. B. (2011). Cellulases: ambiguous nonhomologous enzymes in a genomic perspective. *Trends in biotechnology*, *29*(10), 473-479.

Sun, Y., & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource technology*, *83*(1), 1-11.

Tahtamouni, M. E. W., Hameed, K. M., & Saadoun, I. M. (2006). Biological control of *Sclerotinia sclerotiorum* using indigenous *chitinolytic actinomycetes* in Jordan. *The Plant Pathology Journal*, *22*(2), 107-114.

Tuncer, M., Kuru, A., Isikli, M., Sahin, N., & Celenk, F. G. (2004). Optimization of extracellular endoxylanase, endoglucanase and peroxidase production by Streptomyces sp. F2621 isolated in Turkey. *Journal of Applied*

Microbiology, *97*(4), 783-791.

Walsh, G. (2002). Industrial Enzymes: Proteases and Carbrohydrases. *Proteins, Second Edition*, 327-369.

Wyman, C. E. (2007). What is (and is not) vital to advancing cellulosic ethanol. *TRENDS in Biotechnology*, *25*(4), 153-157.

Zaldivar, J., Nielsen, J., & Olsson, L. (2001). Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Applied Microbiology and Biotechnology*, *56*(1), 17-34.

Zhang, Y. H. P., & Lynd, L. R. (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulose systems. *Biotechnology and Bioengineering*, *88*(7), 797-824.

Zhang, Y. H. P., & Lynd, L. R. (2006). A functionally based model for hydrolysis of cellulose by fungal cellulase. *Biotechnology and Bioengineering*, *94*(5), 888-898.