CHARACTERIZATION OF THERMOSTABLE AND ALKALI-TOLERANT CELLULASE FROM ENDOPHYTIC FUNGUS *BARTALINIA SP*

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT

FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE IN MICROBIOLOGY

Under the guidance of

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CERTIFICATE

This is to certify that the dissertation entitled "*characterization of thermostable and alkali tolerant cellulase from endophytic fungus Bartalinia sp*" submitted by Rajnish Yadav (Registration No. 301105015) in partial fulfillment of the requirement for the award of degree of Master of Sciences in Microbiology, to Thapar University, Patiala is a record of student's own work carried out by him under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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CANDIDATE'S DECLARATION

I, hereby declare that the work which is being presented in this dissertation entitled "*characterization of thermostable and alkali-tolerant cellulase from endophytic fungus Bartalinia sp*" submitted by the undersigned in partial fulfillment of the requirements for the award of the degree of Master of Science in Microbiology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own independent and original research work carried out during the period of six months from Jan 2013 to July 2013, under the supervision of Dr. M. S. Reddy, Professor & Head, Department of Biotechnology & Environmental Sciences, Thapar University. The matter embodied in this dissertation has not been submitted in part or full to any other university or institute for the award of any other degree or certificate.

Dated: 15/7/13

Place: Patiala

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ABBREVIATIONS

Å = Angstrom

µl = Microliter

β = beta

CBD = Cellulose binding domain

CD = Catalytic domain

CDB = Czapek Dox Broth

CMC = Carboxymethyl cellulose

CMCase = Carboxymethyl cellulase

DNS = 3, 5- Dinitrosalicylic acid

Dp = Degree of polymerization

EC = Enzyme Commission

EI = Enzymatic Index

Fig. = Figure

FPase = Filter paper assay

GYP = Glucose Yeast Peptone

M = molar

MES = 2-(N-morpholino) ethanesulfonic acid

Min = minutes

Mg = milligram

ml = milliliter

mM = Milli molar

nm = nano meter

OD = Optical Density

RA=Relative activity

RS= Rice straw

RB = Rice bran

SmF = Submerged state fermentation

SSF = Solid state fermentation

U/ml/min = Units per millilitre per minute

WB =Wheat bran

WS = Wheat straw

ABSTRACT

Endophytic fungi are untapped reservoir and not much has been exploited for industrially important enzymes. In this study an endophytic fungus *Bartalinia sp*. isolated from leaf litter, Western Ghats, India was used for studying the cellulase enzyme production and its characterization. It has been considered that some endophytic fungi have evolved a biochemical system for the degradation of cellulosic substrates and the basis of this study is that some endophytic can change their lifestyle to saprobes. This may be correlated to the enzymes that fungi produce. *Bartalinia sp.* was screened for cellulase and effect of pH, temperature along with carbon and nitrogen sources on cellulase activity were evaluated from the crude extract. Also agricultural wastes like wheat straw, rice straw, eucalyptus leaves, bamboo leaves, rice bran, bagasse were used for evaluating their effect on cellulase activity. Furthermore, pH change and biomass produced was monitered and zymographic analysis was also done. The result obtained indicated that cellulase activity was affected by the change in temperature, pH along with carbon and nitrogen sources. Optimum activity of CMCase obtained at 100° C was startling and enzyme is stable both at acidic and alkaline pH. From the study it was revealed that cellulase produced by *Bartalinia sp*. was thermostable and alkali-tolerant.

CHAPTER – I

1. INTRODUCTION

With the increase in demand for energy resources which can provide sustainable development led us to focus on alternative source of energy other than fossil fuels. Country like India which is developing at a much faster rate needs a huge amount of energy resources but it's over dependence on other countries for its energy and oil is an obstacle for its fast growing economy. For a long term solution of energy (also food) problem, lignocellulosic or cellulosic material can serve as good alternative. In India more than 60% population are still engaged in agriculture and it generates huge amount of cellulosic waste which can be exploited as a resource for energy. Cellulose is the most abundant organic molecule present on earth and also considered as renewable source of energy. It consists of linear chains of β-linked glucose with general formula of $(C_6H_{10}O_5)_n$. Cellulose along with hemicelluloses and lignin are mostly found in the cell wall of green plants and are important components for agricultural wastes like wheat straw, rice straw, bagasse, leaves etc. These agricultural wastes are disposed on the earth improperly and cause environmental pollutions (Brijwani *et al.,* 2009). To utilize these materials and to avoid waste pollution, one of the most important approaches is to find applicable cellulase and hemicellulase to hydrolyze the lignocellulosic biomass. We can exploit these natural substrates by microbial technology i.e., degradation of cellulosic biomass by microbial enzymes for different products that could be used directly or indirectly as resource for energy, food etc. Decomposition of cellulose is a complex process which is carried out by microorganisms like fungi, bacteria and actinomycetes (Alexander, 1961). In general, fungi are the initial and principal decomposing microorganisms followed by bacteria and actinomycetes. The cellulolytic fungi include several ascomycetes like *Penicillium, Fusarium, Trichoderma, Aspergillus* (Mandel's, 1981) along with some basidiomycetes. Cellulases from these fungi are being used for degradation of cellulosic biomass. Some endophytes have also been found to be cellulase producing and occur as saprobes and this might be related to the enzymes they produce. According to Promputtha *et al.,* 2010, some endophytic fungi posses capability to produce enzymes that degrade cellulose and lignin .This is also a possible strategy that allows some endophytes to decay host tissue and persist as saprobes after host senescence. Cellulases are inducible enzymes which act synergistically to hydrolyse the cellulosic biomass into simpler sugars. These are categorize

into three major enzyme activity classes: endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Cellulase enzyme could be produced by both submerged and solid state fermentation. In submerged state fermentation liquid medium containing soluble substrates are used whereas solid state involves non soluble substrates. According to Pandey 1992, solid state holds tremendous potential for cellulase production. Cellulases have enormous applications in various industries. It can be used in the textile industry for bio-stoning and bio-finishing of cellulosic fibres. Furthermore, in the food industry, cellulases can be used for extraction and clarification of fruit and vegetable juices, and in the animal feed industry, these enzymes can promote an increase in the nutritive quality of feed (Almeida *et al.,* 2011). In recent decades it has been widely used for the saccharification of cellulosic mass for bioethanol production. Now a day's focus has been shifted from fungi to endophytic fungi for cellulase enzyme, as endophytic fungi are viewed as an outstanding source of bioactive natural products and enzymes because there are so many of them occupying literally millions of unique biological niches (higher plants) growing in so many unusual environments. Endophytic fungi can be isolated from leaves, stems, roots or some other parts of plant. In the past two decades, many valuable bioactive compounds and enzymes with antimicrobial, insecticidal, cytotoxic and anticancer activities have been successfully discovered from the endophytic fungi. Also the rapid progress in rDNA technology has made it possible to genetically engineer microorganism for the rapid production and development of enzymes and also we can study the regulation of particular gene which are industrially important.

The very factors which are restricting the commercial use is the high cost of cellulases, lack of effectiveness of enzyme at varied pH, susceptibility to inhibitory by-products, thermostability, low production etc. Improving the above mentioned factors while reducing the cost of production, is thus one of the key challenges that must be surmount to make cellulases commercially feasible.

1.1 OBJECTIVES OF THE PROJECT

- Optimization of growth conditions for cellulase activity.

- Evaluation of pH and temperature on cellulase activity.

- Evaluation of carbon and nitrogen sources on cellulase activity.

CHAPTER - II

2. LITERATURE REVIEW

Since forty years, many researchers worked on isolation, characterization, estimation and application of cellulases from fungal and bacterial sources but fungal cellulases were found to be more feasible over the bacterial cellulases as they produce cellulase extracellularly and in much more amount. The fungus *Trichoderma reesi* which is being used for the commercial production of cellulase enzyme by some of the manufacturer's is one of the best studied cellulase system.

2.1 ENDOPHYTIC FUNGI

Since the discovery of endophytes in Darnel, Germany in 1904, various investigators have defined endophytes in different ways which is usually dependent on the perspective from which the endophytes were being isolated and subsequently examined. Bacon and White, 2000 gave an inclusive and widely accepted definition of endophytes: "Microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects". Endophytic fungi are the microbes that live symbiotically within plant species. They colonize the internal plant tissues beneath the epidermal cell layers, living within the intercellular spaces of the tissues and it seems that they may penetrate the living cells (Strobel, 2003). Although fungal hyphae can be readily observed with a microscope in the roots, stems, and leaves of their plant hosts, only in the past several decades ecologists have recognized their potential to alter host growth and physiology, internal biochemistry, reproduction, and population dynamics (Cheplick and Stanley, 2009). Most of endophytic fungi belong to the Ascomycetes and anamorphic fungi (Huang *et al*., 2001), but some endophytic isolates may fail to produce reproductive structures even after several months. These isolates can be encouraged to sporulate on medium containing stripes or extract of host plant (Matsushima, 1971).

Fungal endophytes can grow inter-and intra-cellulary as well as endo-and epi-phytically (Schulz and Boyle, 2005). Currently, endophytes are viewed as an outstanding source of bioactive natural products because of their unique biological niches (higher plants) growing

in so many unusual environments. Thus, it would appear that these bio-typical factors can be important in plant selection since they may govern the novelty and biological activity of the products associated with endophytic fungi. Also the symptomless nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggests they can also be aggressive saprophytes or opportunistic pathogens (Selim, 2012). Both fungi and bacteria are the most common microbes existing as endophytes. The most frequently isolated endophytes are the fungi. It turns out that the vast majority of plants have not been studied for their endophytes. Thus, enormous opportunities exist for the recovery of novel fungal forms, taxa, and biotypes. Hawksworth and Rossman, 1987 estimated there may be as many as 1 million different fungal species, yet only about 100,000 have been described. As more evidence accumulates, estimates keep rising as to the actual number of fungal species. For instance, Dreyfuss and Chappela, 1994 estimate there may be at least 1 million species of endophytic fungi alone. It seems obvious that endophytes are a rich and reliable source of genetic diversity and novel, undescribed species. This fact alone helps eliminate the problems of dereplication in compound discovery.

Since the discovery of bioactive compound paclitaxel (taxol) from endophytic fungi *Taxomyces andreane* in 1993 led many scientists to focus on untapped reservoir of endophytic fungi. In the past two decades, many valuable bioactive compounds and enzymes with antimicrobial, insecticidal, cytotoxic and anticancer activities have been successfully discovered from the endophytic fungi. These bioactive compounds could be classified as alkaloids, terpenoids, steroids, lignans, phenols and lactones. (Xu L, 2008) During the long period of co-evolution, a friendly relationship was gradually set up between each endophytic fungus and its host plant. The host plant can supply plenteous nutriment and easeful habitation for the survival of its endophytes. On the other hand, the endophytes would produce a number of bioactive compounds for helping the host plants to resist external biotic and abiotic stresses, and benefiting for the host growth in return (Silvia *et al*, 2007; Rodriguez, 2009). Some endophytic fungi have developed the ability to produce the same or similar bioactive substances as those originated from the host plants. This is beneficial for us to study the relations between the endophytes and their host plants, and to develop a substitutable approach for efficiently producing these scarce and valuable bioactive compounds (Gunatilaka *et al.*, 2006; Zhou L *et al.*, 2009).

Till now very less work has been done on endophytic fungi for cellulase production but in recent years, scientists are exploring the endophytic microbes for their wide range of beneficial products (Strobel and Daisy, 2003) and there is urgent need to focus on these untapped reservoirs. Fig. 1 below shows the variance in growth of different endophytic fungi.

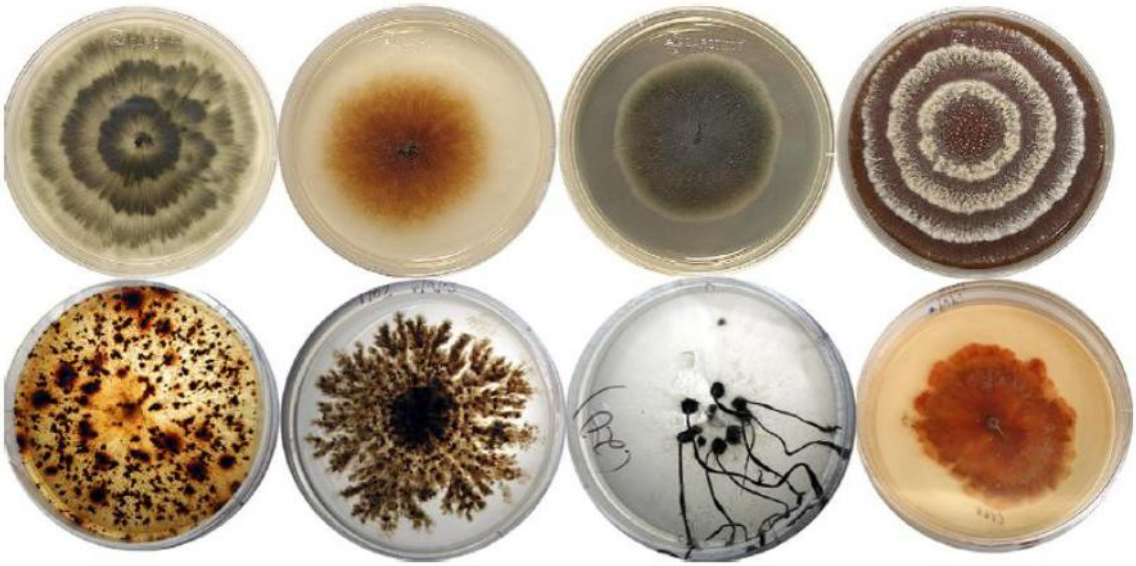

Fig.1-Assortment of Ascomycetous endophytic fungi recovered from foliage of angiosperms and conifers in North America and Panama, Adapted from: (Arnold, 2007).

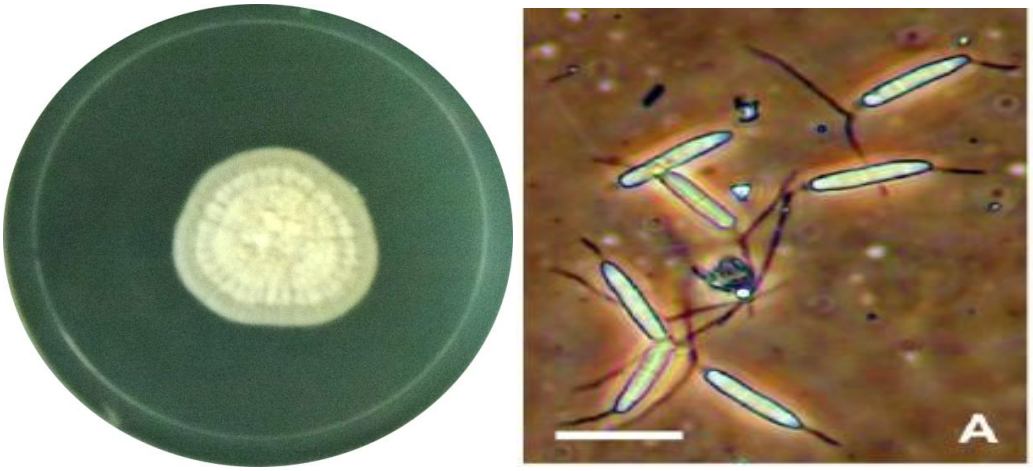

Fig.2- (A) Agni fungus, *Bartalinia sp.* (Class: Sordariomycetes, Order: Xylariales). (Suryanarayanan *et al.*, 2011)

Endophytes may be a novel and good producers of enzymes like xylanase and the production of extracellular cellulase and hemicellulases other than xylanases. They are widespread but usually limited to organisms derived from selected hosts or even host tissues (Leuchtmann *et al.,* 1992; Suto *et al.,* 2002). Choi *et al.,* 2005, screened the endophytic fungi for their ability to produce lignocellulases, amylase, cellulase, ligninase, pectinase and xylanase. Endophytic fungi are able to produce many enzymes (Firakova *et al.,* 2007), so they could be used as biocatalysts in the chemical transformation of natural products and drugs, due to their ability to modify chemical structures with a high degree of stereospecificity and to produce known or novel enzymes that facilitates the production of compounds of interest. According to Promputtha *et al*., 2010, endophytes produce degrading enzyme for the decomposing of leaves once leaves senesce in order to survive as saprobes. The endophytes which have the capability to produce degrading enzymes should have an important role as litter decomposers, but they do not decompose host tissue in the living host. So the degrading enzymes are an important factor affecting the process of endophytes becoming saprobes.

Table 1- Some of the cellulase producing endophytic fungi

S.No.	ENDOPHYTIC FUNGI	REFRENCES
1	*Fusarium sp.*	Maria *et al.,* 2005
2	*Periconia sp.*	Piyanun *et al.,* 2008
3	*Acremonium sp.*	Almeida *et al.,* 2011
4	*Colletotrichum sp.*	Prabuddh *et al.,* 2011
5	*Alternaria sp.*	Prabuddha *et al.,* 2011
6	*Ascocoryne sarcoides*	Gianoulis *et al.,* 2012
7	*Chaetomium sp.*	Luisa *et al.,* 2012
8	*Penicillium glabrum,*	Luisa *et al.,* 2012
9	*Philaophora finlandia*	Caldwell *et al.,* 2000

2.2 LIGNOCELLULOSIC BIOMASS

Lignocellulosic biomass from crops, whose main components are polysaccharides polymers as cellulose and hemicellulose (75-80%) is an important renewable energy source. Cellulose is the primary product of photosynthesis in terrestrial environments, and the most abundant renewable bioresource produced in the biosphere (~100 billion dry tons/year) (Holtzapple, 1993; Jarvis, 2003; Zhang and Lynd, 2004b). It is the most abundant polysaccharide present on earth and also the main component of plants cell wall. It consists of linear chains of β-linked glucose with general formula of $(C_6H_{10}O_5)_n$. Cellulose, which commonly accounts up to 40% of plant biomass is totally insoluble in water (Lederberg, 1992) and vary widely in length and are usually arranged in bundles or fibrils (Walsh, 2002). Within the bundles, cellulose molecules can occur in crystalline or amorphous structures (Walter, 1998). While β-1, 4-linked glucose is the chemical repeating unit, the structural repeat is β-cellobiose (Varrot *et al.*, 2003), Figure 3. In cellulose, glucose chains are tightly bound to each other by Van der Waals forces and hydrogen bonds (inter and intra-molecular) into crystalline structures called elementary fibril (consisting of around 40 glucan chains), about 40 Å wide, 30 Å thick and 100 Å long (Bidlack *et al.*, 1992).

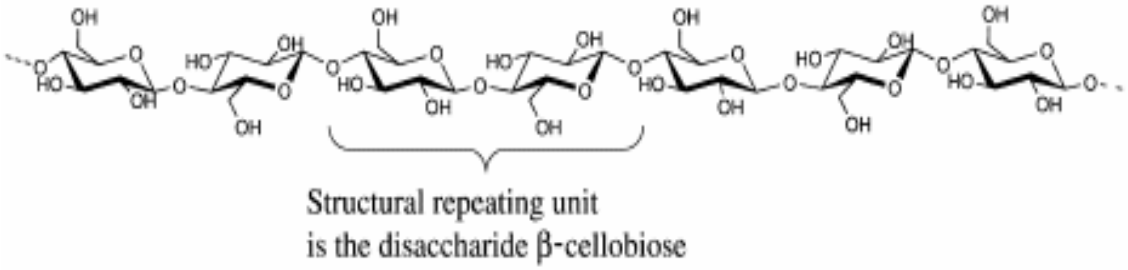

Fig.3 : Chemical structure of cellulose. Linear β-1,4-linked glucose is the chemical repeating unit, while the structural repeat is β-cellobiose, and consequently each glucoside is oriented at 180° in respect to its neighbours. (Varrot *et al.*, 2003).

But a recent investigation by Ding and Himmel, 2006, suggested that the cellulose microfibril in plant cell walls is composed of 36 cellulose chains with a total of six sheets only. Of these 36 chains, only the inner chains are crystalline in nature, whereas the outer chains are non-crystalline. Native cellulose has the degree of polymerization (DP) up to 10000 β-anhydroglucose residues (Hon and Shiraishi, 1991). Cellulose exists in seven crystal

structures (polymorphs) designated as celluloses Iα, Iβ, II, III_I, III_{II}, IV_I, and IV_{II} (O'Sullivan, 1997). In nature, cellulose Iα and Iβ are the most abundant crystal forms. Iα polymorph is meta-stabile, and thus, more reactive than Iβ. No pure sample of Iα have been found in nature. The percentage of Iα polymorph in crystalline cellulose varies from 70 % in bacterial cellulose (O'Sullivan, 1997), 64 % in cellulose isolated from algae *Valonia ventricosa*, to 20 % in ramie and cotton cellulose (Yamamoto and Horii, 1994). The co-existence of two polymorphs of native cellulose, which have different stabilities, may imply that the part of the Iα polymorph within the microfibril is most prone to the enzymatic attack.

After cellulose, hemicellulose is the organic cellulosic material which found to be the most abundant on earth. It also has enormous potential like cellulose to be used as the renewable source for biofuel production (Sanchez and Cardona, (2008). Hemicelluloses are branched heterogeneous polymers consisting of many different sugar monomers like: D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose, L-rhamnose (hexoses), 4-O-methyl-D-glucuronic acid, D-glucuronic acid and D-galacturonic acid (Hon and Shiraishi, 1991). The average degree of polymerization of hemicellulose varies between 70 and 200 (Fengel and Wegener, 1983).

Many agricultural by-products from agricultural activities and agro based processing litter the environment and constitute waste problems. Agro wastes such as rice straw, wheat bran, corn stover, sugarcane bagasse, pomace, corn cobs etc. are used as substrate in solid state fermentation (Padmavathi *et al.*, 2012). The most important processing challenge in the production of biofuel is pretreatment of these biomass. Lignocellulosic biomass is composed of three main constituents namely hemicellulose, lignin and cellulose. Pretreatment methods refer to the solubilization and separation of one or more of these components of biomass. It makes the remaining solid biomass more accessible to further chemical or biological treatment (Demirbas, 2005). The pretreatment is done to break the matrix in order to reduce the degree of crystallinity of the cellulose and increase the fraction of amorphous cellulose. The most suitable form for enzymatic attack (Sanchez, 2008). Pretreatment is undertaken to bring about a change in the macroscopic and microscopic size and structure of biomass which makes the lignocellulosic biomass susceptible to quick hydrolysis with increased yields of monomeric sugars (Mosier, 2005).

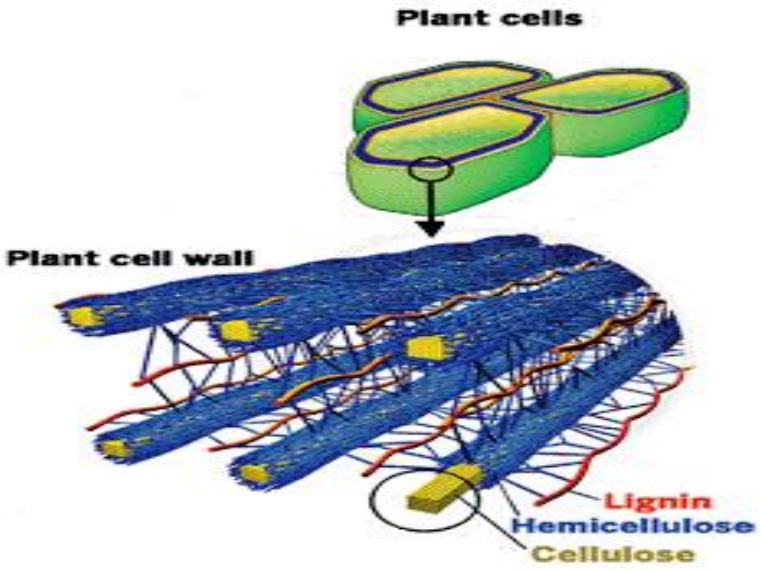

Fig. 4. The constituents of lignocelluloses

Fig 5. Alkali treated Agro-wastes : Bamboo leaves(A), Rice straw(b), Bagasse(C), Rice bran(D), Eucalyptus leaves(E), Wheat straw(F).

2.3 CELLULASE

Cellulases refer to a group of enzymes which act in a concerted manner to hydrolyze cellulose into soluble sugars and provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen *et al.,* 2005 and Lee *et al.,* 2008). Cellulases are inducible enzymes, which are synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo, 2001). Therefore, cellulose and sophorose are natural inducers whereas glucose is catabolite represser (Zahri *et al.*, 2005 and Aneja, 2005). However, the mechanism of induction by cellulose is still not clear because being large insoluble macromolecules, cellulase cannot go through the plasma membrane directly (Zahri *et al.,* 2005 and Aneja, 2005). Some disaccharides also act as inducer, such as lactose, cellobiose, sucrose (Dashtban, 2011). Cellulases, responsible for the hydrolysis of cellulose are composed of a complex mixture of enzyme proteins with different specificities to hydrolyze glycosidic bonds. The complete enzymatic hydrolysis of cellulosic materials needs different types of cellulase *(*Lee *et al.,* 2008*).* Cellulases can be divided into three major enzyme activity classes on the basis of their mode of action (Goyal *et al.,* 1991 and Rabinovich *et al.,* 2002, a and b). These are:

1.Endoglucanase or endo-1, 4-β-glucanase (EC 3.2.1.4),
2.Cellobiohydrolase or exo-1, 4-β-glucanase (EC 3.2.1.91) and
3.β-glucosidase or β-D-glucoside glucohydrolases (EC 3.2.1.21).

Endoglucanases, often called carboxymethyl cellulases (CMCase), are proposed to initiate attack randomly at multiple internal sites in the amorphous regions of the cellulose fibre opening-up sites for subsequent attack by the cellobiohydrolases (Wood, 1991). Cellobiohydrolase, often called an exoglucanase, is the major component of the fungal cellulase system accounting for 40-70% of the total cellulase proteins and can hydrolyze highly crystalline cellulose (Esterbauer *et al.,* 1991). B-glucosidase hydrolyze glucose dimers and in some cases cello-oligosaccharides to glucose. It has been found that most cellulolytic hydrolyses are proteins of 30-40 KDa molecular mass with acidic optima between 2.5 and 4.5 (Baldrian, 2008). Cellulase from different sources have notable difference and differences were mainly seen in their polypeptide characteristics such as carbohydrate content, isoelectric points, catalytic activity, substrate specificity, moleculer weight, amino acid composition etc.

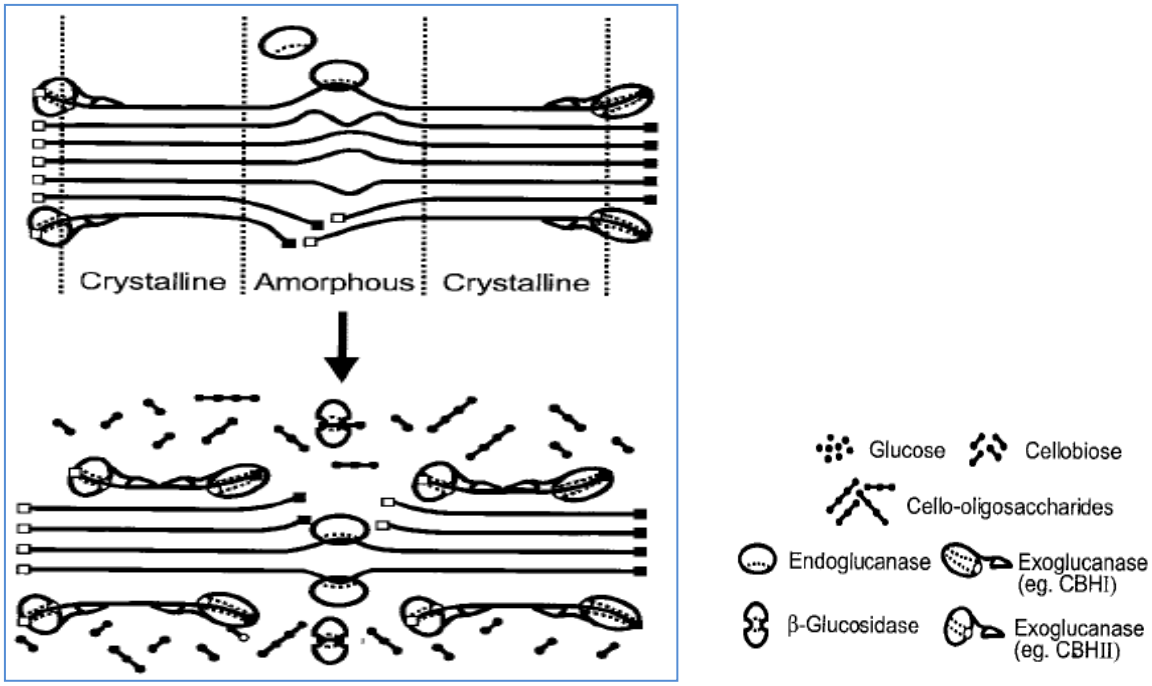

Fig.6. The hydrolysis of amorphous and microcrystalline cellulose by non-complexed cellulase systems. The solid squares represent reducing ends and the open squares non reducing ends. Amorphous and crystalline regions are indicated (Lynd *et al.,* 2002).

Most commonly, cellulases consist of one catalytic domain (CD) and one carbohydrate binding module (CBM), which is usually joined to the CD by a relatively long (30-44 amino acids), often glycosylated, linker peptide. Additionally, a few microorganisms with multi-catalytic domain enzymes and at the same time possessing several binding modules, have been identified (Zverlov *et al.*, 1998; Gibbs *et al.*, 1992). Cellulase act on glycosidic bond by acid catalysis which involves either inverting or retaining mechanism.

In inverting mechanism charged environment of the catalytic site activates the water molecule which acts as nucleophile while acidic amino acid residue donates the proton. This is called inverting because the charge moiety becomes protonated while the proton donor becomes the charged moiety that activates the water molecule for the next reaction. This mechanism leads to net inversion of the configuration at the anomeric carbon.

Rretaining mechanism involves a formation of covalently bound intermediate through nucleophilic attack of the charged amino acid on the glycosyl bond. This mechanism leads to net retention of the configuration at the anomeric carbon. Both the methods are shown in fig. 7.

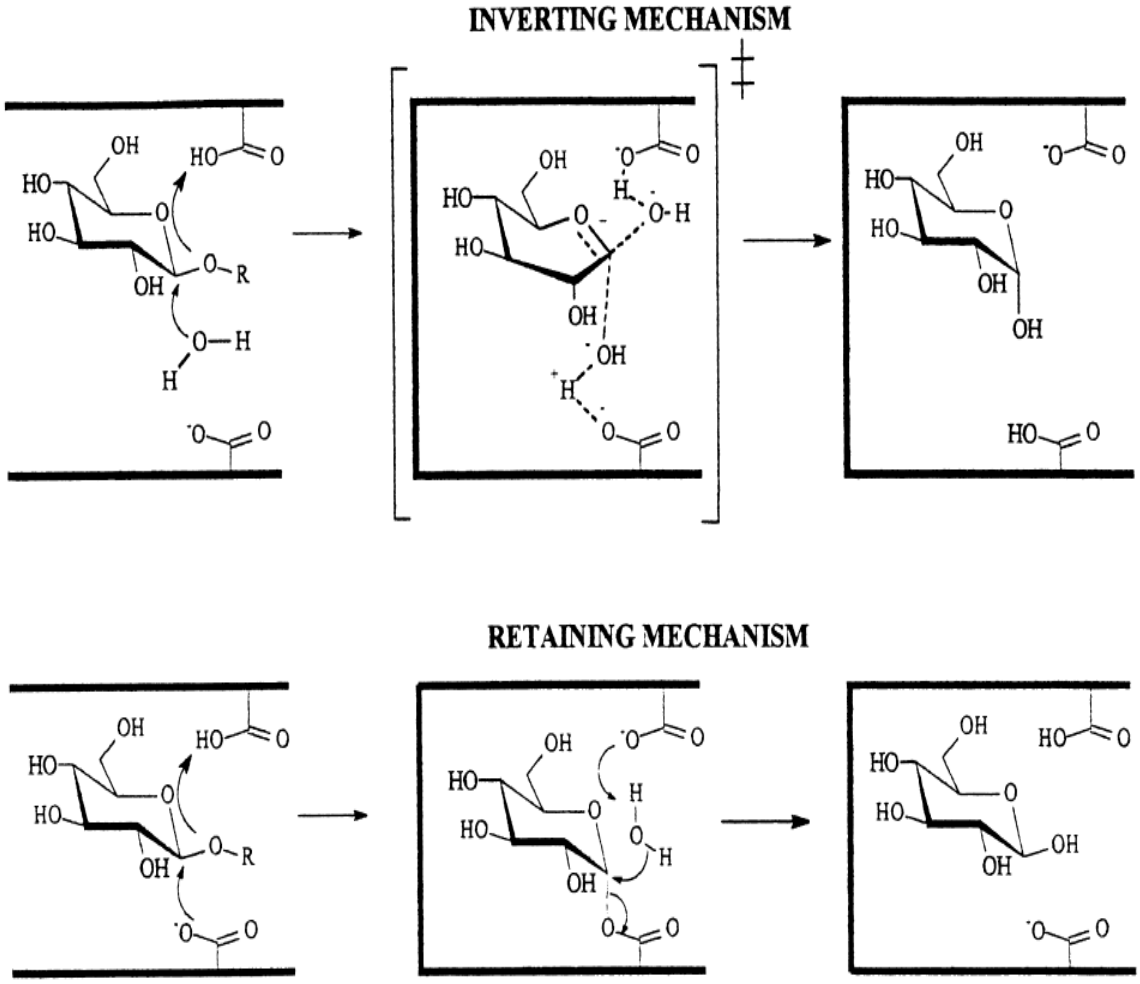

Fig. 7. General scheme for the reaction mechanisms of inverting and retaining glycosidehydrolases. Both pathways pass through transition states. (Withers, 2001).

2.3.1 APPLICATIONS

Cellulases have enormous applications in different industries and this is the third most abundant enzyme produced in the world.

Table 2. Applications of cellulase in various industries (Kuhad *et al.,* 2011)

Agriculture	Plant pathogen and disease control; generation of plant and fungal protoplasts; enhanced seed germination and improved root system; enhanced plant growth and flowering; improved soil quality; reduced dependence on mineral fertilizers
Bioconversion	Conversion of cellulosic materials to ethanol, other solvents, organic acids and single cell protein, and lipids; production of energy-rich animal feed; improved nutritional quality of animal feed; improved ruminant performance; improved feed digestion and absorption; preservation of high quality fodder
Detergents	Cellulase-based detergents; superior cleaning action without damaging fibers; improved color brightness and dirt removal; remove of rough protuberances in cotton fabrics; antiredeposition of ink particles
Food	Release of the antioxidants from fruit and vegetable pomace; improvement of yields in starch and protein extraction; improved maceration, pressing, and color extraction of fruits and vegetables; clarification of fruit juices; improved texture and quality of bakery products; improved viscosity fruit purees; improved texture, flavor, aroma, and volatile properties of fruits and vegetables; controlled bitterness of citrus fruits
Pulp and Paper	Biomechanical pulping; enzymatic deinking; reduced chlorine requirement; improved fiber brightness, strength properties, and pulp freeness and cleanliness; improved drainage in paper mills; production of biodegradable cardboard, paper towels, and sanitary paper
Textile	Biostoning of jeans; biopolishing of textile fibers; improved fabrics quality; improved absorbance property of fibers; softening of garments; improved stability of cellulosic fabrics; removal of excess dye from fabrics; restoration of colour brightness

2.3.2 CELLULASE ENZYME CHARACTERIZATION

There are various factors which affects cellulase production or cellulase activity, but the major ones are temperature , pH, composition and concentration of cellulose, carbon and nitrogen sources. According to Shafique *et al.,* 2009, pH was the key factor that affects the production and activity of cellulases from *Trichoderma harzianum* under solid state fermentation and plate screening medium. Cellulose concentration is also affects the growth and production of cellulase enzymes by microorganisms (Moosavi and Majdi, 2007). The optimum cellulase production was obtained at 1% or above concentration of cellulosic material. Carbon and nitrogen are very essential sources for the growth and production of enzymes. As cellulases are inducible enzymes, so cellulosic carbon source is prerequisite. There are other carbon sources that induces cellulases production; sophorose, lactose, CMC and sucrose whereas glucose and end products repress the production of cellulase enzymes (Kubicek *et al*.,1993; Dashtban *et al.,* 2011, Narasimha *et al.,* 2012). Submerged and solid state fermentation are basically used for the production of cellulase enzyme. The use of solid state fermentation (SSF) for enzyme production offers several advantages over submerged fermentation (SmF). Some of the advantages include high productivity, low Production costs, low risk of contamination due to the inability of many organisms to grow at low water activity and it doesn't required complex machinery, equipment and control systems. Nevertheless, SSF has some limitations. (Pandey, 1992). Solid state fermentation (SSF) is defined as any fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients in absences of free flowing liquid (Pandey, 1992). SSF holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used as the enzyme source (Pandey *et al.,* 1994). Agro-industrial residues are generally considered the best substrates for the SSF processes. The production of cellulase could be improved by mutation under SSF (Vu *et al*., 2011)

Cellulosic substrates like wheat bran, (Kang *et al.,* 2004) wheat straw (Yang *et al.,* 2004) rice straw, corn cob (Abostate *et al.,* 2010), cotton flower shell, groundnut shell, wheat and sorghum straw (Mohite and Magar, 2010), water hyacinth blend (Usama and Din, 2008) saw dust (Acharya *et al*., 2008) and so many type of substrates are used by researchers for the production and characterization of cellulases. Abostate *et al.,* 2010 reported that among the

isolated strains of *Aspergillus* species, maximum CMCase activity was seen on rice straw. He further suggests that the highest cellulases were produced on wheat straw and lowest on corn cob. Kang *et al.,* 2004, and Yang *et al*., 2004, both have also reported the potential of wheat straw as a substrate for cellulose fermentation.

The optimum pH and temperature of the crude extract obtained from *Trichoderma sp. IS-05* were 3.0 and 60° C, respectively (Jackeline, 2011). According to Picart *et al.,* 2007, two *Penicillium sp.* showed optimum temperature and pH of carboxymethyl cellulase activity at 65° C and pH 4.5, respectively. Activity remained stable after incubation at 60° C and pH 4.5 for 3 h.

Zhekova *et al.,* 2012, detected maximal CMCase activity at pH value 5.0 from enzyme extract of *Trichoderma reesei NRRL 3652*. About 70 % relative activities were achieved at the neighbouring pH points – 4.0 and 6.0. The optimal temperature value for maximal CMCase activity was in the range of 55-60 °C. Vries and Visser, 2001 also reported that many *Aspergillus* cellulases have optimum pH near to 4.8

Bjerkandera adusta and *Pycnoporus sanguineus* showed maximum activity at pH-5 and at temperature 60° C but they remain active even at alkaline pH and temperature of 70° C (Rosa *et al.,* 2009). Also according to Harnpicharnchai *et al.,* 2008) endophytic *Perconia sp*. cellulase shows maximum activity at temperature 70° C and at pH optimum of 5 to 6.

Acharya *et al.,* 2008, worked on pH optimization for cellulase production and they found that among range of pH values of 4.0 to 6.0, the maximum cellulase yield was recorded at pH 4.0 (0.0925 I.U.). Akiba *et al.,* 1995, also reported the optimal pH values from *Aspergillus niger* in the range of 6.0 to 7.0. Their report shows resemblance with the previous report of McCleary and Glennie-Holmes, 1985. Also Gao *et al.,* 2008 found maximum cellulase activity at pH range of 3-5 and at temperature of 70° C from *Aspergillus terrus M11*. Cellulases and Hemicellulases from Endophytic *Acremonium* Species shows maximum enzyme activity at a pH range of 5-7 and at temperature 65° C (Almeida *et al.,* 2011).

CHAPTER – III

3. MATERIALS AND METHOD

3.1 FUNGAL CULTURE

One fungal culture used in this study is *Bartalinia sp.* obtained from TIFAC-CORE Laboratory, Thapar University, Patiala. It was previously isolated from leaf litter obtained from private land in the semi-arid zone of the Nilgiris, Western Ghats, Southern India. Screening of isolate for cellulase was done along with crude enzyme extraction, characterization and enzyme activity

3.2 PREPARATION OF INOCULUM

Potato dextrose agar plates was prepared according to manufacturer instructions (Himedia, Mumbai), sterilized at 121°C for 15 minutes and poured into the Petri dishes (pH 5.6). With the help of blade (11mm) a small clump of the preserved isolate was cut and placed at the centre of the potato dextrose agar (PDA) plate and incubated aerobically at 28°C for 7 days. After 7 days of cultivation on potato dextrose agar the isolate was transferred onto CMC containing media for screening.

3.3 SCREENING FOR CELLULASE PRODUCTION

To screen the isolate for potential cellulase production, a small clump was transferred onto YP (GYP medium without glucose) medium containing 0.5% CMC as the sole source of carbon and incubated for 3 to 5 days at 28°C on CMC agar media. After incubation, plates were flooded with 0.2% aqueous Congo red and kept for 3-5 min followed by destaining with 1M NaCl for 15 min. Appearance of yellow zone around the fungal colony in an otherwise red medium indicated cellulase activity (Rohrmann and Molitoris, 1992.) This halo was measured for subsequent calculation of the enzymatic index (EI) using the below mentioned expression:

$$EI = \frac{\text{diameter of hydrolysis zone}}{\text{Diameter of colony}}$$

3.4 CULTURE CONDITION FOR CELLULASE PRODUCTION

3.4.1 SUBMERGED CULTURE

An Erlimmenary flasks (250 ml) containing all the media components of Czapek dox agar (appendix) were mixed and final volume was made 100 ml using distilled water and was autoclaved at 121°C for 15 min. These flasks were then inoculated with two clumps of the fungus from 7 days old culture and incubated in a shaker at 120 rpm. The crude extract obtained was used to determine the cellulolytic activity by the standard assay method (Ghose, 1987).

3.5 CELLULASE ACTIVITY ASSAY

For cellulase activity assay different reagents were used. They are mentioned in the appendix -1 along with their composition**.** Reagent was stored in brown coloured bottle at 4°C. One unit **(**U) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of glucose from the appropriate substrates per minute under the assay conditions.

3.5.1 METHOD

1. Crude enzyme extract was prepared from the culture supernatant by filtration with Whatman filter paper 42.

   Cellulase activity was determined with CMC by assay method developed by Ghose, 1987 (IUPAC) with slight modification. In this method reducing sugar formed by the hydrolysis of cellulosic substrate was estimated by DNS (3, 5- Dinitrosalicylic acid) and the colour formed was measured spectrophotometrically at 540 nm.
2. The substrate for this reaction was made by dissolving 1% CMC in distilled water and kept for overnight at room temperature.
3. The assay mixture contained 1 ml of substrate, 1 ml of buffer (0.1M Citrate buffer), pH 5, (Appendix)} and 1 ml of diluted enzyme (0.5 ml enzyme + 0.5 ml of distilled water).

4. A control was also incubated against the above mixture which contained all the above components except substrate CMC.
5. This reaction mixture was incubated at 50º C for 60 min.
6. The enzyme activity was stopped by adding 3 ml of DNS and again incubated for 15 minutes at 95°-100° C
7. Just after 15 minutes incubation period, 1 ml Rochelle salt was added.
8. After cooling of reaction mixture absorbance was measured at 540 nm against blank made up of buffer and distilled water.
9. The standard graph was prepared by using 2 mg/ml glucose stock solution in 0.1M Citrate buffer of pH-5 (Appendix), and absorbance taken at 540 nm.
10. For Filter paper assay (FPase) Whatman n0. 1 filter paper of dimension 1×6 cm (50 mg) was used as substrate instead of CMC.

For calculating the cellulase activity the absorbance value of controls were subtracted from their corresponding test. CMCase and FPAse were calculated by using the formula described by Ghose, 1987. According to her absorbance values of sample was translated into glucose using standard.

$$\text{CMCase} = \frac{0.185}{\text{Critical enzyme concentration}} \text{ U/ml}$$

$$\text{FPase} = \frac{0.37}{\text{Enzyme concentration to release 2.0 mg glucose}} \text{ U/ml}$$

3.6 CHARACTERIZATION OF ENZYME

3.6.1 OPTIMUM CELLULASE PRODUCTION TIME

The effect of incubation time on cellulase activity of fungus in submerged fermentation (SmF) was determined by incubating the fungus at 28° C over a period of 15 days. Erlenmeyer flasks (250ml) containing sterilised Czapek dox broth (CDB) medium, pH 7.2, having marking of 5 days, 10 days and 15 days were inoculated with two clumps of fungus in each flask from the culture maintained on PDA plates. Experiment was carried out in triplicates. Crude extract was obtained from the culture broths by filtering the broth through filter paper (Whatman 42) after 5 days, 10 days and 15 days of incubation period. The crude extract that resulted was assayed by using 1% CMC and reducing sugars were determined using DNS method (Ghose, 1987). Here citrate buffer of pH-5 was used and reaction mixture was incubated at temperature 50° C. Also the biomass produced and change in pH of media after 5, 10, and 15 days were recorded.

3.6.2 pH

The incubation time which gave the highest cellulase activity was selected for further study. In order to determine the most effective pH for cellulase activity, the submerged state fermentation was carried out in the Erlenmeyer flask (250 ml) containing sterilised Czapek dox agar (CDB) medium, pH 7.2, was inoculated with two clumps of isolate. After 10 days of incubation at 28°C in shaking condition (120 rpm), the culture broth was filtered off with the filter paper. The crude extract obtained was then used to assay cellulase enzyme with buffers of different pH which are mentioned in table-3 by using DNS method (Ghose, 1987). CMCase activity was determined by comparing the O.D with standard curve of glucose. The relative cellulase activity was calculated in percentage by assuming the highest activity as 100%.

Table 3 - The different buffers used for estimating optimum pH were

S. No.	BUFFERS	pH RANGE
1	Sodium acetate Buffer	3-4
2	Citrate Buffer	5
3	Potassium phosphate buffer	6-7
4	Tris-HCL	8
5	Glycine-NaOH	9-10

3.6.3 TEMPERATURE AND THERMOSTABILITY

For determining the enzyme activity at different temperatures, the reaction mixture containing crude enzyme extract, substrate and MES buffer (pH-5) was incubated for 60 min in the temperature range of 30°-100° C (with an interval of 10° C). A simultaneously prepared enzyme-buffer mix i.e. without substrate was stored corresponding to each temperature range for 60 minute to be used as a control (Ariffin *et al*., 2006). For determining of thermo stability reaction mixture was further incubated for 120, 180 and 240 minutes at 100° C.

3.6.4 CARBON SOURCES

In order to evaluate the effect of carbon sources on cellulase activity from the fungal crude extract 1% of different carbon sources (fructose, maltose, lactose, malt extract and CMC) in 100 ml of Czapek dox broth (CDB) media in place of sucrose was used. The effect of carbon sources was compared with medium containing 1% sucrose. All the flasks were then autoclaved at 121° C a1 15 psi for 15 minutes and on cooling each broth was inoculated with two clumps of *Bartalinia sp.* and incubated at 28°C. After 10 days of incubation, the culture broths were filtered off and crude enzyme extract obtained from different carbon sources were used to assay cellulase enzyme activity at 100° C, pH-5 (MES Buffer) by using DNS method (Ghose, 1987). MES buffer is used because of its high buffering capacity at higher temperature. Also the biomass produced and changes in pH of media were recorded.

3.6.5 NITROGEN SOURCES

The effect of nitrogen sources on the cellulase enzyme activity from the fungus was evaluated by adding 0.3% of different nitrogen sources sources (peptone, yeast extract, beef extract, casein, ammonium chloride) in a CDB media in place of sodium nitrate. All the flasks were then autoclaved at 121° C a1 15 psi for 15 minutes and on cooling, the broths were inoculated with two clumps of given isolate and incubated at 28° C. After 10 days of incubation, the culture broths were filtered off and crude enzyme extract obtained from different nitrogen sources were used to assay cellulase activity at 100° C, pH-5 (MES Buffer) by using DNS method (Ghose, 1987). Also the biomass produced and changes in pH of media were recorded.

3.7 SOLID STATE FERMENTATION

3.7.1 SUBSTRATE PREPARATION

Wheat straw, Rice straw, Sugarcane bagasse, Rice bran, Eucalyptus leaves and bamboo leaves were used for the production of cellulase enzyme from the isolates under solid state fermentation (SSF). The above mentioned lignocellulosic substrates were undergone pretreatment processes before being used for the cellulase production.

STEPS:

1) Physical petreatment: The above mentioned substrates were washed with water to remove all residual sugar, dried, chopped and milled to 40 mesh powder.
2) Chemical pretreatment: After physical treatment substrates were treated with 2% NaOH (alkaline pretreatment). For that, substrates were kept in different beakers fully immersed in NaOH solution and left for 12 hours undisturbed at room temperature.
3) After the alkali treatment substrates were washed thoroughly with tap water followed by distilled water till pH becomes neutral.
4) Now the treated substrates were dried in oven (70°-80°c) or in bright sunlight for further use.

3.7.2 INOCULUM PREPARATION

For inoculums preparation, suspension of fungal culture was made in Mandel's and Waber's media. Before making suspension, fungal culture was grown in 100 ml of Potato dextrose broth and was sterilised at 121° C at 15 psi for 15 min, cooled and inoculated with clumps from the fungal culture maintained on PDA plates and incubated at 28°C for 7 days. After incubation, media was filtered through autoclaved Whatman filter paper-42 and accumulated biomass on filter paper was transferred to 250-ml Erlenmeyer flask containing 100 ml Mandel's and Waber media (sterilised at 121°C at 15 psi for 15 min). With the help of tissue homogenizer, the biomass was homogenized in an Erlenmeyer flask containing media which was used as a mother culture for cellulase production. All the above steps were carried out in aseptic conditions

3.7.3 SOLID STATE FERMENTATION CULTIVATION SYSTEM

Fermentation was performed in 250 ml Erlenmeyer flasks containing 5g of each pretreated substrate with 15 ml moisture level The moistening agent used was Mandel's and waber's medium (1969). The flasks were then sterilised at 121°C at 15 psi for 15 min, cooled and inoculated with the prepared mother culture at inoculums concentration of 1 ml per gram of substrate in each case and the flasks were placed in an incubator shaker for 60 minutes at 250 rpm for proper mixing of the substrates with inoculums. After proper mixing, flasks were incubated at 28°C for 10 days. Triplicates were used for each treatment

3.7.4 ENZYME EXTRACTION

Fivefold of autoclaved distilled H_2O was added to the SSF medium of each flask after cultivation and then flasks were placed on a rotator shaker for 1 hour at 300 rpm. The enzyme extracts from different substrates were obtained by filtering the mixtures through nylon cloth followed by centrifugation for 15 minutes at 12000 rpm to remove fungal and substrate residues. The obtained clarified extract were checked for cellulase activity (Shereif *et al*., 2010)

3.8 ZYMOGRAPHY

Proteins present in the supernatant were precipitated by mixing 1 part of supernatant to 4 part of chilled acetone and kept for 1 hour at - 20° C. After that, centrifugation was done to fully precipitate the proteins and acetone was discarded. Pellets were left for few minutes for air drying. Pellets were then dissolved in minimum volume of autoclaved distilled water.

CMCase zymograms were prepared using SDS-10% (Laemmli, 1970) polyacrylamide gels containing 0.2% CMC. CMC was incorporated into the separating gel prior to the addition of ammonium persulphate to avoid polymerisation. After electrophoresis, the gel was washed in solution A for 1 hour and further in solution B for 1 hour respectively in order to remove the SDS. Staining was done with Coomassie Blue R dye in methanol-acetic acid-water solution (4:1:5, by volume) for 1 hour and destained in the same solution without dye. In order to see cellulase activity, renaturation of the enzyme proteins was carried out by leaving the gel in Solution C at 4° C overnight. The gel was then transferred onto a glass plate, sealed in a film, and incubated at 37°C for 4-5 hour. The gel was then stained in a solution of 1% Congo red for 30 min, and destained in 1M NaCl for 15 min. Clear zone around the bands indicated CMCase activity (Ratanakhanokchai et al. 1999).

Table 4 - Solutions for zymography:

Solution A	Sodium phosphate buffer, pH 7.2, containing isopropanol 40%
Solution B	Sodium phosphate buffer, pH 7.2
Solution C	Sodium phosphate buffer, pH 7.2, containing 5 mM β-mercaptoethanol and mM EDTA

3.9 STATISTICAL ANALYSIS

All experiments were performed in triplicates. The data were analyzed by analysis of variance and the means were compared with Tukey's test at $P < 0.05$. All the analyses were performed by using Graph Pad Prism 5.1 software and Costat software.

CHAPTER – IV

4. RESULTS AND DISCUSSION

The main aim of this investigation is to characterize the process parameters for maximum cellulase activity by thermotolerant *Bartalinia sp.* So, in this study, various parameters were used to investigate optimum fermentation conditions for highest cellulase activity.

4.1 SCREENING OF FUNGAL CULTURE FOR CELLULASE

In the present study *Bartalinia sp*. was screened for cellulase production on CMC-agar plate and found to be positive by formation of halo zone that result from degradation of the CMC as shown in Fig-8. The halo zone produced by hydrolysis of cellulose is directly related to the region of action of cellulolytic enzymes, since the dye only remains attached to regions where there are β-1,4-D-glucanohydrolase bonds (Lamb and Loy, 2005). According to Ten *et al*., 2007 the diameter of the halo zone is useful for selection of strains that can efficiently degrade polysaccharides such as cellulose, xylan, and amylose.

Fig. 8. Screening of fungal culture on CMC agar plate for cellulolytic activity

The Enzymatic index was found to be 18mm. The enzymatic index (EI) can be used as a simple and rapid methodology to select strains within the same genus that have potential for the production of enzymes (Ruegger and Tornisielo, 2004).

4.2 DETERMINATION OF CELLULSAE ACTIVITY

To describe the overall (absolute, non-specific) potential of the produced cellulase complex to hydrolyze cellulose. CMCase of crude extract taken from shake flask was determined at different pH, temperature, carbon and nitrogen sources. Endoglucnase (CMCase) activity of the *Bartalinia* cellulases was determined against carboxymethyl cellulose (CMC). Activities were calculated by comparing the absorbance with standard of glucose. A standard was made from stock solution of 2 mg/ml of glucose as shown in Fig-9.

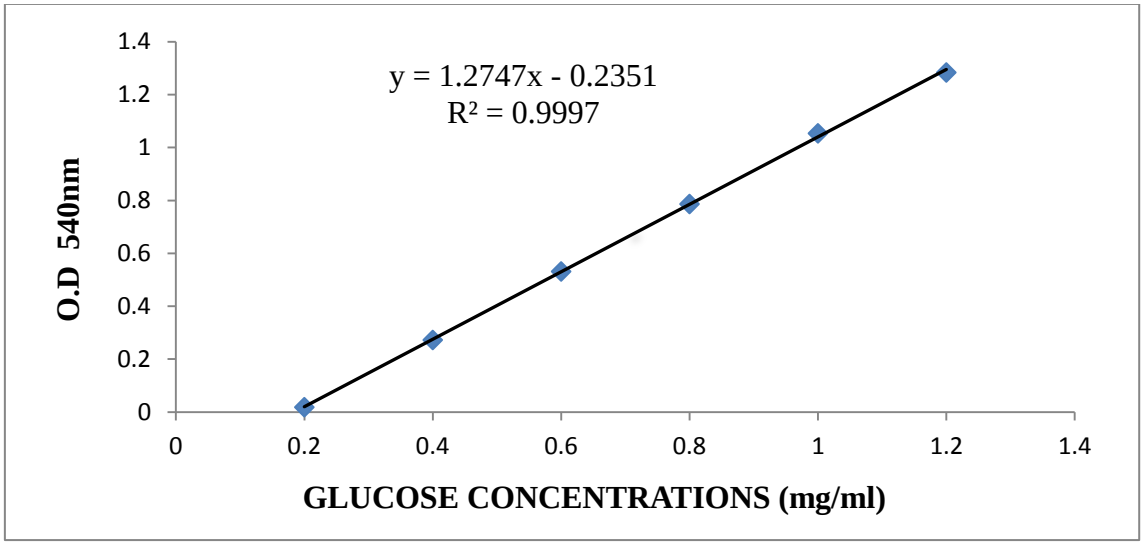

Fig. 9. Standard curve of D- glucose at 540nm

4.3 DETERMINATION OF OPTIMUM CELLULASE PRODUCTION TIME

Fermentation period was an important parameter for enzyme production. CMCase activity was observed over a period of 15 days. The maximum CMCase activity (0.1229 U/ml) was observed in 10th day of incubation as shown in Fig- 10. The incubation period is directly related to the production of enzyme and other metabolic up to a certain extent. At longer period of time, the cellulose activity decreased which might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes. Moreover reducing sugar formed by the action of cellulase also affects the cellulase production and its activity. The biomass production was

more after 15 days of incubation. No considerable changes were found between initial and final pH, although it was found to be low then the initial pH. Very few endophytic fungi were studied for cellulase production and effect of incubation period on cellulase activity but many other filamentous fungi were studied and it has been reported in many research work that maximum cellulase production takes place after 7 days of incubation. According to Garg, 1981 the highest CMCase activity had been recorded after 7 days of incubation for *Aspergillus terrus*. Also maximum cellulase activity by *Trichoderma reesei* (QM9414 mutant) was found on 9th day of incubation period (Despande *et al.*, 2008). The results obtained were in agreement with Gulab and Ashok, 2013, who reported that *Aspergillus flavus, Aspergillus niger, Alternaria alternata, Curvularia lunata, Colletotrichum gloeosporioides, Fusarium equiseti* and *Penicillium digitatum* were showed maximum cellulase activity on 10th day.

Table 5. Effect of incubation time on enzyme activity and pH.

S. NO.	INCUBATION TIME	CMCase ACTIVITY (U/ml) ± SD*	CHANGE IN pH ± S.D*
			INITIAL pH-7.2
1	5	0.1105 ± 0.005 **b**	6.71 ± 0.004 **a**
2	10	0.1229 ± 0.001 **a**	6.46 ± 0.003 **b**
3	15	0.1196 ± 0.002 **a**	6.4 ± 0.003 **b**

*Mean ± Std. deviation is shown in table (n = 3)

The values sharing a common letter within the column are not significant at $P < 0.05$

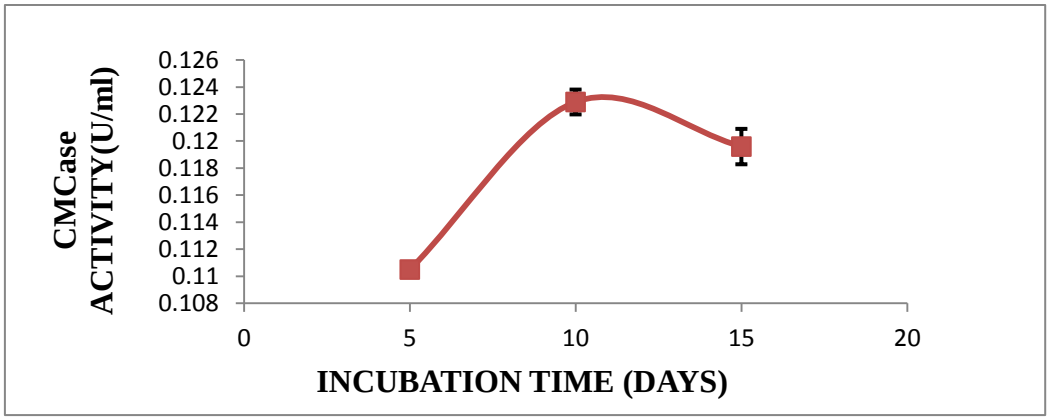

Fig. 10. Graph showing effect of incubation time on CMCase activity

Table 6 - Biomass produced with incubation time

S. No.	INCUBATION TIME (days)	BIOMASS PRODUCED ± S.D*
1	5	0.0500g ± 0.004 c
2	10	0.0946g ± 0.002 b
3	15	0.1156g ± 0.004 a

*Mean ± Std. deviation is shown in table (n = 3)

The values sharing a common letter within the column are not significant at $P < 0.05$

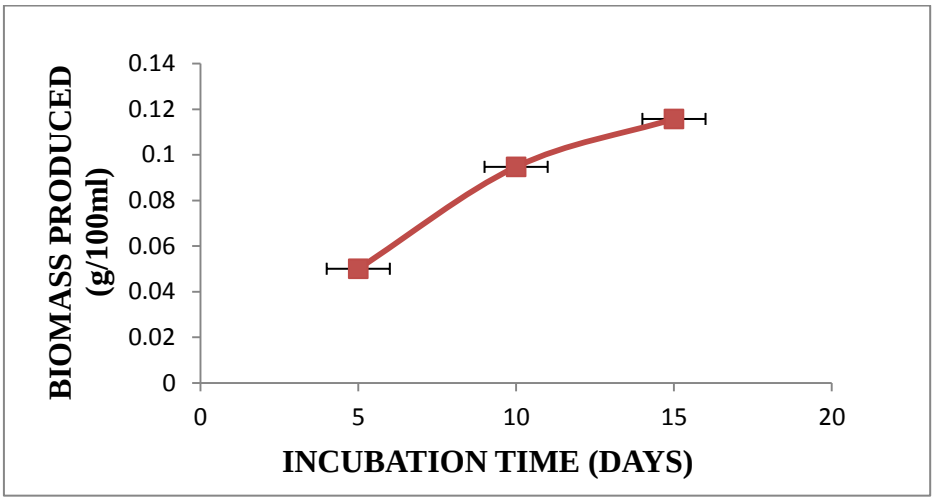

Fig. 11. Graph showing increase in biomass with the incubation time

4.4 pH

Bartalinia sp. showed the highest cellulase activity at pH-5 and it was quite active at wider range of pH (3-10) and retains its activity even at alkaline pH of 10. It was found that pH range 5-8 is most suitable for enzyme activity. Elshafei *et al.,* 2008 reported that CMCase of *A. terreus* DSM 826 gave a broad range of activity within pH 3-6. The results obtained are in agreement with those obtained from where CMCase activity has optimum pH of 4.5-5.5 *(*Farinas, *et al.,* 2010*)*. It was reported that the optimal pH for cellulase from *Aspergillus niger* was between 6.0 and 7.0 (Akiba *et al.,* 1995). Further Gautam *et al.,* 2011 observed that the enzyme activity from *Aspergillus niger and Trichoderma sp*. was stable at pH range of 5.0–8.0.

Table 7 - Relative CMCase activity obtained at different pH

S. No.	pH VALUE OF BUFFER	Relative CMCase ACTIVITY (%)
1	3	64.2
2	4	71.2
3	5	100
4	6	95.5
5	7	94.16
6	8	86.11
7	9	83.83
8	10	81.4

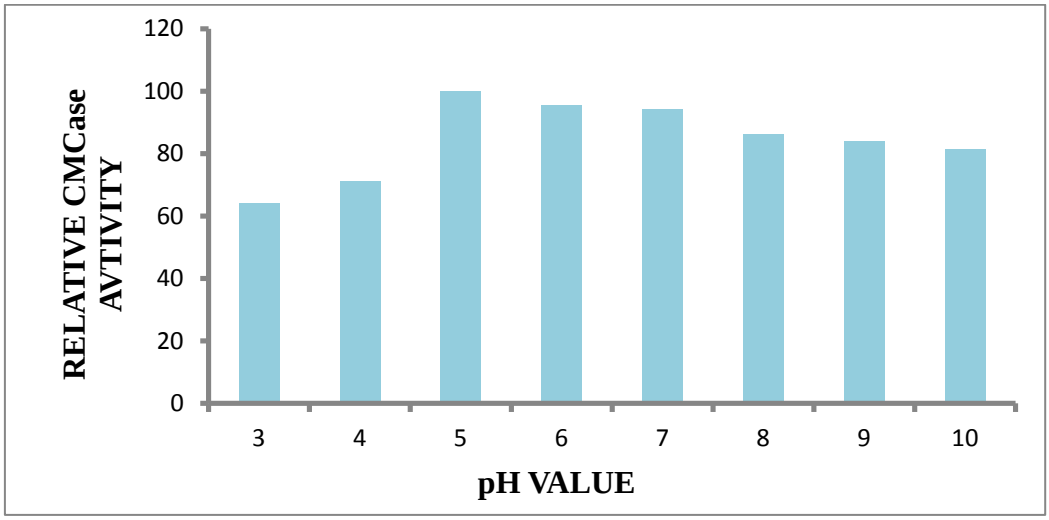

Fig. 12. Graph showing relative CMCase activity at different pH

4.5 TEMPERATURE AND THERMOSTABILITY

To study the effect of temperature on cellulase enzyme activity, crude extract was incubated at different temperatures (30-100° C). The enzyme activity increases as the temperature increases and the optimum cellulase enzyme activity recorded at 100° C which was very astonishing (0.1983 U/ml). As far as thermostability was concerned CMCase activity began to decrease when the incubation time increases from 60 minutes to 120, 180 and 240 minutes. The most interesting and astonishing feature was the maximum CMCase activity at 100°C and thermostability of enzyme at such a higher temperature. Although CMCase activity starts

decreasing with the increase of incubation time from 1 h to 2 h and further to 3 and 4 h but it has been found that enzyme is quiet thermostable.

Table 8 - Effect of temperature on CMCase activity

S.No.	TEMPERATURE	CMCASE ACTIVITY (U/ML) ± S.D*
1	30°	0.0985 ± 0.0008 **f**
2	40°	0.1015 ± 0.001 **f**
3	50°	0.1225 ± 0.001 **e**
4	60°	0.1309 ± 0.002 **d**
5	70°	0.1337 ± 0.0007 **d**
6	80°	0.1474 ± 0.002 **c**
7	90°	0.1780 ± 0.001 **b**
8	100°	0.1983 ± 0.001 **a**

*Mean ± Std. deviation is shown in table (n = 3)

The values sharing a common letter within the column are not significant at $P < 0.05$

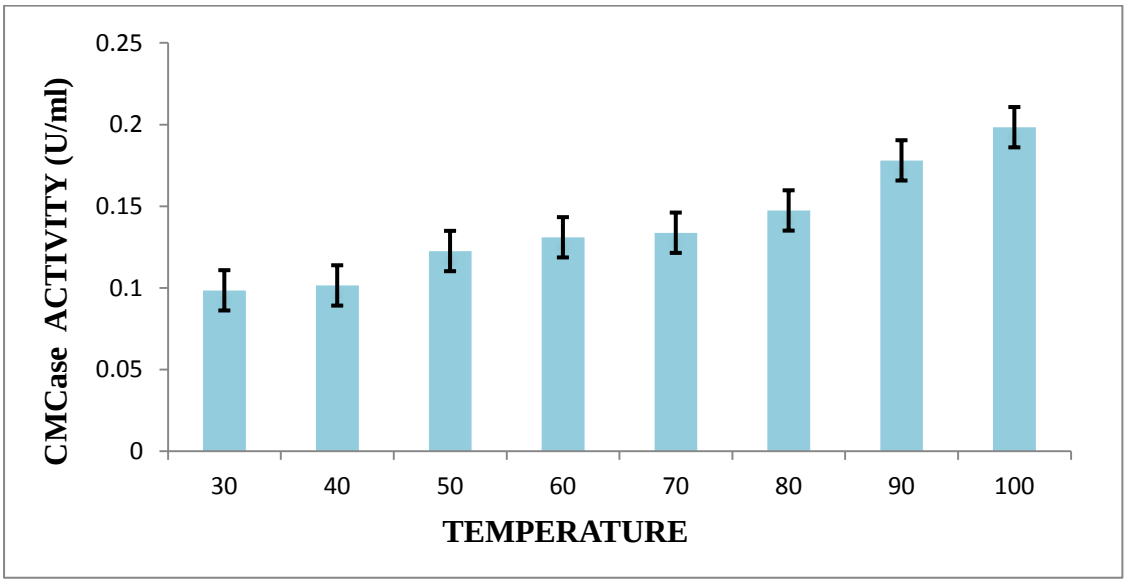

Fig. 13. Graph showing CMCase activity at different temperature

There is no endophytic fungi have been reported till date which shows its maximum activity at 100° C. An unusual feature of this mesophilic fungi can be justified as it shows exceptional exhibition of heat resistance and its saprotrophic nature which was the findings of

Suryanarayanan *et al.*, 2011 acclaim its enzyme activity at higher temperature and cellulase production. Also this might be due to its very well adaptations to surviving fires (Suryanaryanan *et al.*, 2011) .The spores of this fungi are found to be stable for 2 hours at temperature 115° C and therefore it is also known as Agni Fungi (Suryanaryanan *et al.*, 2011). Piyanun, 2008 reported that endophytic fungi *Periconia sp*. exhibit high activity at 70° C and almost retains its 60% of maximal activity after 1.5 h at 70°C. Further author also reported that *Periconia sp*. showed optimal pH of 5 and 6 for cellulase activity and retains 100% activity after incubation for 2 h at pH-8 i.e. highly stable at basic pH.

Table. 9 - Thermostability of cellulase with time

S.No.	INCUBATION TIME (h)	CMCase (U/ml) ± S.D*
1	1	0.1983 ±0.001 **a**
2	2	0.1046 ± 0.003 **b**
3	3	0.0849 ± 0.006 **c**
4	4	0.0585 ± 0.001 **d**

*Mean ± Std. deviation is shown in table (n = 3)

The values sharing a common letter within the column are not significant at $P < 0.05$

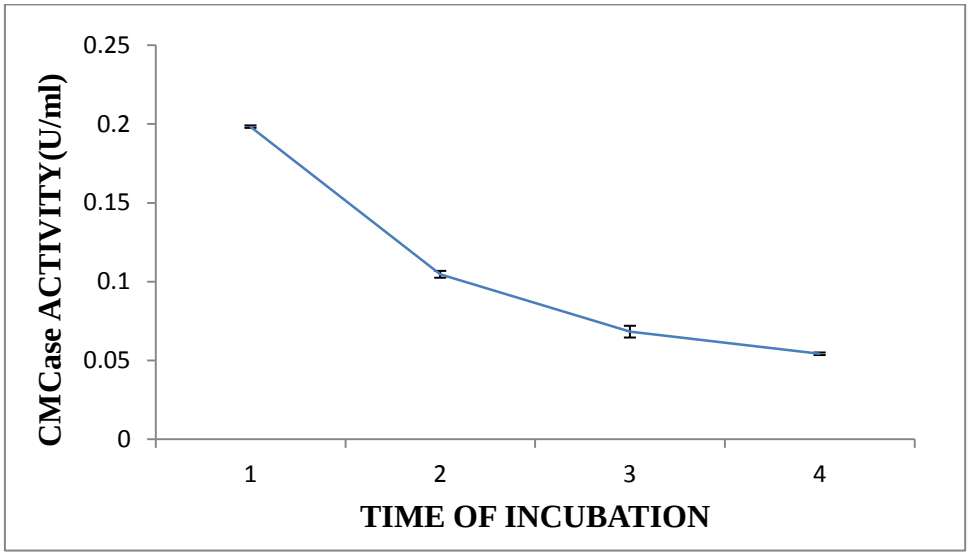

Fig. 14. Graph showing thermostability of cellulase with time

4.6 CARBON SOURCES

As depicted in Fig.15 the cellulase activity was considerably affected by different carbon sources. Among the five carbon sources maximum CMcase activity was shown by maltose followed by lactose whereas minimum activity was shown by fructose. FPAse was also determined and maximum FPase activity was obtained on CMC followed by maltose and lactose and minimum for fructose. Czapek dox broth (CDB) having 1% sucrose served as control. The maximum biomass was obtained from medium having fructose as carbon source whereas minimum was obtained on medium having lactose. No considerable difference was seen between initial and final values of pH. According to Gautam *et al.,* 2011 cellulose and CMC were found to be best for higher cellulase activity followed by sucrose and maltose and at a concentration of 1%. This finding is similar to our present findings. Dashtban, 2011 reported that lactose showed maximum FPase among the carbon sources he tested on *Hypocrea jecorina.* Narasimha *et al.,* 2006 also reported CMC as best carbon source for CMCase activity

Table 10-Effect of carbon sources on CMCase and FPase activity along with pH and biomass

S. No.	**CARBON SOURCES (1%)**	**CMCase U/ml** ± S.D*	**FPASE U/ml** ± S.D*	**PH CHANGE Initial pH-7.2**	**BIOMASS PRODUCED**
1	Maltose	0.1725 ± 0.004 **a**	0.2233 ± 0.003 **c**	6.61	0.066g
2	Lactose	0.1539 ± 0.005 **ab**	0.1898 ± 0.003 **d**	6.82	0.041g
3	CMC	0.1535 ± 0.004 **ab**	0.2450 ± 0.17 **bcd**	6.94	0.032g
4	Malt extract	0.1326 ± 0.10 **bc**	0.2625 ± 0.12 **ab**	6.53	0.037g
5	Fructose	0.1126 ± 0.12 **bc**	0.1558 ±0.004 **e**	6.83	0.078g
6	Sucrose	0.1389 ± 0.02 **c**	0.2830 ± 0.009 **a**	6.58	0.059g

*Mean ± Std. deviation is shown in table (n = 3)

The values sharing a common letter within the column are not significant at $P < 0.05$

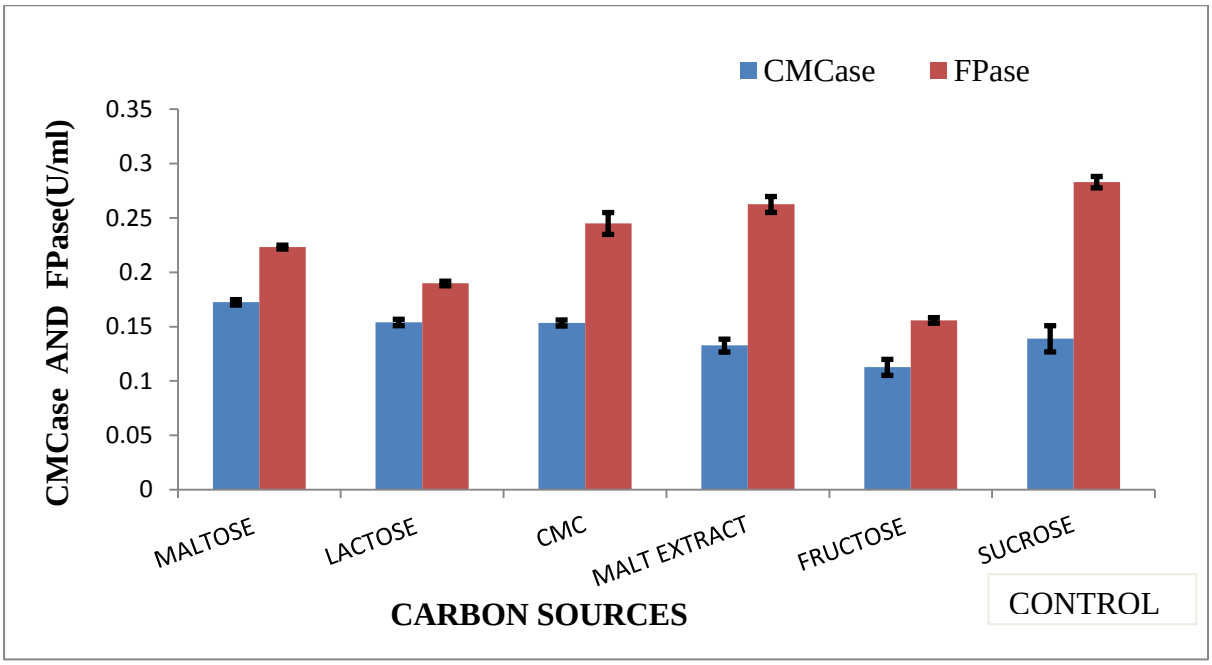

Fig. 15. Graph showing effect of different carbon sources on CMCase and FPase activity

4.7 NITROGEN SOURCES

The nitrogenous sources showed to influence the cellulase activity by *Bartalinia sp.* under submerged condition. It is evident from (Fig.16) the highest CMCase activity obtained from yeast extract (0.1636 U/ml) supplemented CDB medium and minimum cellulase enzyme activity was recorded on ammonium chloride (0.1107 U/ml) supplemented medium. Highest FPase was observed on beef extract (0.1227 U/ml) followed by yeast extract and minimum FPase was obtained from ammonium chloride extract. CDB having 0.3% sodium nitrate served as control. These outcomes were similar to the findings of Thomas *et al.*, 2011, who reported maximum enzyme activity with casein. According to Gao *et al.*, 2008, maximum cellulase activity was obtained on Yeast extract by *Aspergillus terreus*. Similarly, Ahamed and Vermette, 2008, reported that Yeast extract yielded the highest CMCase activity by *Trichoderma reesei* RUT-C30.

The maximum biomass was obtained from Beef extract medium whereas minimum was from casein. This means beef extract favours the growth of the *Bartalinia sp.* No considerable difference was seen between initial and final values of pH.

Table 11 - Effect of nitrogen sources on CMCase and FPase activity, pH change & biomass

S. No.	NITROGEN SOURCES	CMCase U/ml ± S.D*	FPase U/ml ± S.D*	PH CHANGE	BIOMASS PRODUCED
				Initial pH-7.2	
1	Yeast extract	0.1636 ± 0.012 **a**	0.1826 ± 0.007 **b**	6.70	0.084g
2	Casein	0.1248 ± 0.004 **c**	0.1714 ± 0.017 **bc**	6.67	0.027g
3	Beef extract	0.1227 ± 0.007 **bcd**	0.1973 ± 0.002 **b**	6.66	0.092g
4	Peptone	0.1026 ± .002 **d**	0.1234 ± .003 **c**	6.80	0.063g
5	Ammonium chloride	0.1107 ± .008 **cd**	0.1273 ± 0.003 **c**	6.30	0.036g
6	Sodium nitrate	0.1353 ± .003 **b**	0.2717 ± 0.04 **a**	6.58	0.059g

*Mean ± Std. deviation is shown in table (n = 3)

The values sharing a common letter within the column are not significant at $P < 0.05$

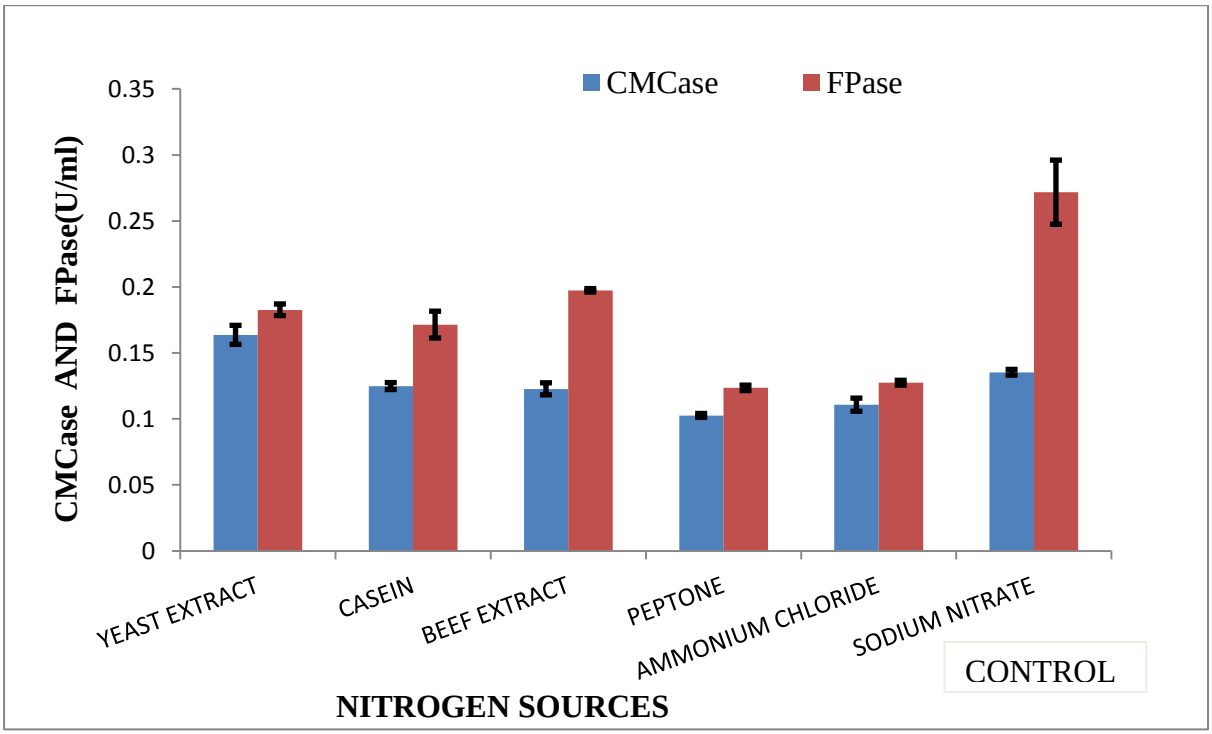

Fig. 16. Graph showing effect of different nitrogen sources on CMCase and FPase activity

4.8 ACTIVITY ON INSOLUBLE SUBSTRATE

Different agricultural wastes were used as substrates for the production of cellulase by *Bartalinia sp.* Crude extract of enzyme from Eucalyptus leaves showed highest CMCase as well as FPAse after 10 days of incubation at 28° C (±2) (Table 12) followed by Rice bran. This might be attributed to lignocellulosic nature of eucalyptus leaves. Moreover, *Bartalinia sp.* had been isolated from leaf litter so it might possible that this fungus was very well adapted in deriving its carbon source from these kinds of cellulosic substrates. Nevertheless efficiency of enzyme activity also depends on the bare composition of the raw material, accessibility to various components and their chemistry and physical association (Gao *et al.,* 2008). Minimum enzyme activity was seen on sugarcane bagasse. This variation may be attributed to the chemical nature and nutrient availability of used substrate. Also pre-treatment methods have great impact over the weakening of cellulosic structure and removal of lignin. As far as activity difference is concerned between soluble and natural carbon sources, submerged fermentation with soluble carbon substrates have advantage over solid state fermentation.

Also the monitoring of pH change and biomass produced is also important, because according to Soccol, 1992, the fungus has a capacity for growth, albeit limited, under extreme conditions of acidity and alkalinity. These features are extremely important for fermentation processes, because they show that under these conditions the vast majority of the bacteria responsible for the contamination of the fermentation processes are inhibited. Also it may led us conclude that whether our fungus is secreting any acid or alkaline compound or not.

Table 12 - Effect of natural carbon sources on CMCase and FPase activity

S.NO	**NATURAL CARBON SOURCES**	**CMCase (U/gds)** ± S.D*	**FPase (U/gds)** ± S.D*
1	Eucalyptus leaves	0.0368 ± 0.001 **a**	0.0384 ±0.001 **a**
2	Rice bran	0.0261 ± 0.001 **b**	0.0294 ± 0.0005 **b**
3	Wheat straw	0.0221 ± 0.001 **c**	0.0218 ±0.001 **c**
4	Rice straw	0.0205 ± 0.0003 **cd**	0.0237 ± 0.0003 **c**
5	Bamboo leaves	0.0181 ± 0.0006 **de**	0.0222 ±0.005 **c**
6	Sugarcane Bagasse	0.0168 ± 0.0003 **e**	0.0184 ± 0.001 **d**

*Mean ± Std. deviation is shown in table (n = 3) The values sharing a common letter within the column are not significant at $P < 0.05$

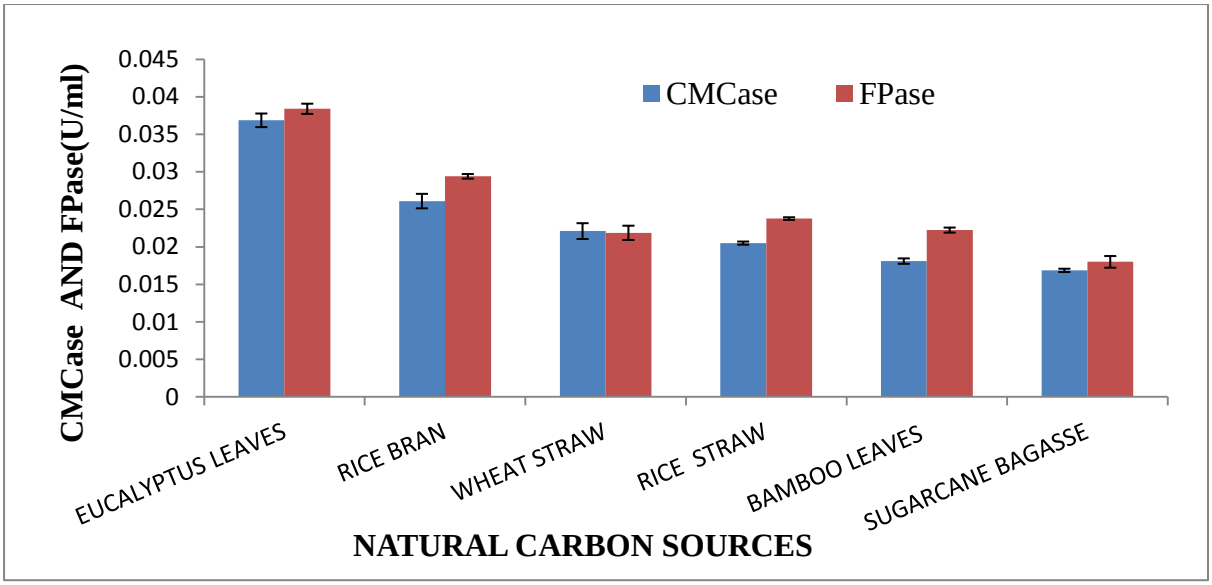

Fig. 17. Effect of Natural carbon sources on CMCase and FPase

4.9 ZYMOGRAPHY

The qualitative analysis for the enzyme was done using Zymography.

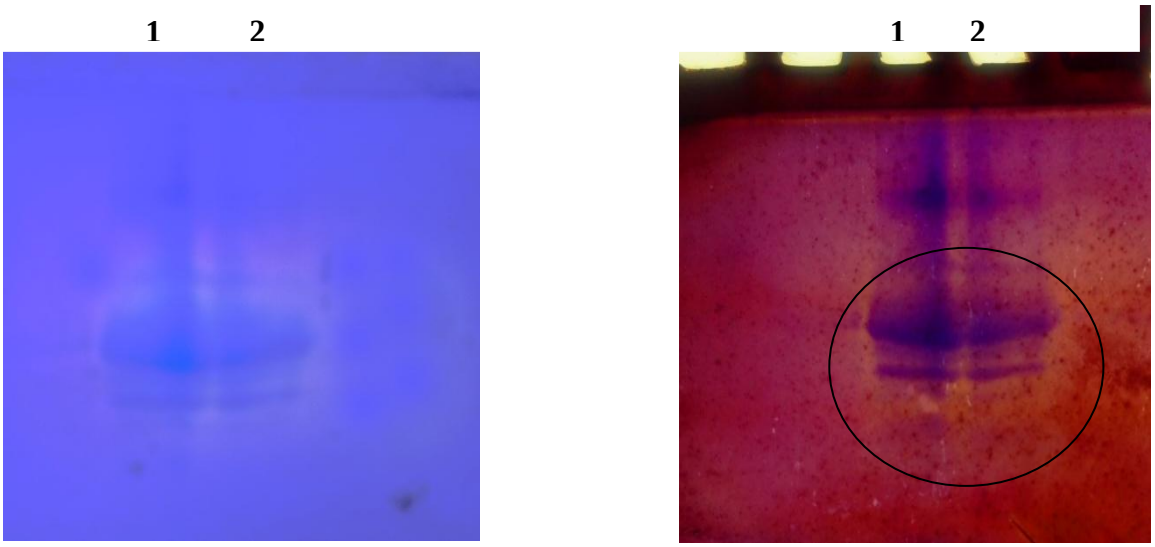

Fig.-18 (a) SDS-PAGE: Cellulase protein bands in lane 1 and 2 were observed after Coomassie brilliant blue staining **(b)** Activity staining with 1% congo red was done after removal of SDS and renaturation of enzyme. The activity of CMCase enzyme was observed around the protein bands as yellow hydrolysis zone.

When the SDS-PAGE gel was stained with Congo-red solution hydrolysis zone was observed showing the activity of cellulase enzyme present in the crude protein extract. The bands were not very clear this might be due to over staining or due to the use of crude enzyme extract

CONCLUSION

The fungi as enzyme sources have many advantages and the enzymes produced by them are normally extracellular, making easier for downstream process. The development of economically feasible technologies for cellulase production and for the enzymatic hydrolysis of cellulosic materials will enable to utilize the large quantities of biomass such as the residues of both food industries and agriculture. Thus the present investigation was selected to conduct an extensive study on cellulases from *Bartalinia sp.* The process development is the key step in fermentation processes. The study related to process development involves optimization of different fermentation conditions (physical and nutritional) towards enhancement of cellulolytic enzymes production. Eucalyptus leaves was selected as a best substrate for cellulase production using *Bartalinia sp.* The optimum temperature, pH and incubation time for cellulase enzyme production from *Bartalinia sp.* were 100° C, pH 5-6 and 10 days respectively. Yeast extract and beef extract were the preferred nitrogen source under submerged fermentation (SmF). Further qualitative analysis for the enzyme was done by using zymography showing the activity of cellulase enzyme present in the crude protein extract precipitated with acetone. The cellulase of *Bartalinia sp.* was thermostable and also alkali-tolerant, maintaining almost 50% of the initial activity when kept for 2 h at 100º C. Due to the inducible, alkali-tolerant and thermotolerant nature of cellulase, the *Bartalinia sp.* used in present study can be exploited for the industrial production of cellulases. Further investigations are required to make use of the full potential of this organism for cellulase production by conducting studies using pure enzyme and by employing genetic, biochemical, and microbial engineering techniques.

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APPENDIX – I

1.1 COMPOSITION OF YP MEDIUM (g/L):

INGREDIENTS	QUANTITY
Yeast extract	0.1g
Peptone	0.5g
Agar	16g
Water	1000mL
CMC	5g
pH	6-6.5

1.2 COMPOSITION OF CDB MEDIUM (g/L):

INGREDIENTS	QUANTITY (g/L)
Sucrose	30
Sodium nitrate	3
Dipotassium phosphate	1
Magnesium sulphate	0.5
Potassium chloride	0.5
Ferrous sulphate	0.010
pH	7.2+0.3

1.3 BUFFERS FOR PH OPTIMIZATION

0.1 M SODIUM ACETATE (100 ML)

pH	0.1M Acetic acid	0.1M Sodium Acetate
3	98.23 ml	1.77 ml
4	84.7 ml	15.3 ml

0.1 M CITRATE BUFFER pH-5 (100 ml)

Solution A: 0.1M sodium bicarbonate	35 ml
Solution B: 0.1M sodium carbonate, decahydrate	65 ml

0.1 M POTASSIUM PHOSPHATE (FINAL VOL.100 ml USING DISTILLED WATER)

pH	1M K_2HPO_4	1M KH_2PO_4
6	1.32 ml	8.68 ml
7	6.15 ml	3.85 ml

0.1 M TRIS HCL (100 ML) (pH-8)

2.42 g Tris base + 1.5 ml of 1N HCl in 100 ml distilled water.

0.1 M GLYCINE – NAOH (100 ML)

Solution (a): Dissolve .750g of Glycine and .585g of NaCL in water and make up to 100ml

Solution (b): 0.1M Sodium Hydroxide

pH	**Solution (a)ml**	**Solution (b)ml**
9.0	8.8	1.2
10.0	6.0	4.0

1.4 MES BUFFER (100 ML, pH-5)

25 mM MES	0.082g
10 mM Sodium acetate	0. 488 g

APPENDIX 2

PREPARATION OF STANDARD

GLUCOSE CONCENTRATION (mg/ml)	GLUCOSE FROM STOCK SOLUTION	DILUTION	BUFFER (ml)
0.2	100 µl	900 µl	1
0.4	200 µl	800 µl	1
0.6	300 µl	700 µl	1
0.8	400 µl	600 µl	1
1	500 µl	500 µl	1
1.2	600 µl	400 µl	1

PREPARATION OF DNS (DINITROSALICYLIC ACID)

COMPONENT	QUANTITY
DNS	10 (g/litre)
Phenol	2 (g/litre)
Sodium sulphite	0.5 (g/litre)
Sodium hydroxide	10 (g/litre)
Distilled water	1000ml

ROCHELLE SALT OR SODIUM POTASSIUM TARTARATE tetrahydrate (40%)

It was prepared by dissolving 40g Rochelle salt in 100ml distilled water.

SUBSTRATE

1% CMC substrate solution was made by dissolving 1g CMC in 100ml distilled water

COMPOSITION OF MANDEL AND WABER'S MEDIA

COMPONENT	QUANTITY(g/L)
$(NH_4)_2SO_4$	1.4
KH_2PO_4	2.0
$CaCl_2.2H_2O$	0.3
$MgSO_4.7H_2O$	0.3
$FeSO_4.7H_2O$	0.005
$MnSO_4.H_2O$	0.0016
$ZnSO_4.7H_2O$	0.0014
$CoCl_2.6H_2O$	0.002
Peptone	0.1
Tween-80	0.1
Distilled water	1