EFFECT OF CARBON AND NITROGEN SOURCE ON CELLULASE PRODUCTION BY *Aspergillus terreus* MTCC 7600

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ABSTRACT

Cellulases are the enzyme that hydrolyze cellulose,it is a linear polymer of anhydro glucose units linked together by β-1, 4- glycosidic bondproduced by different fungi, bacteria, and protozoan. In a time of increasing energy prices, use of freely available biomass as a resource is one of the prime focus in current scientific community. In the present study the effect of carbon and nitrogen sources were investigated on the cellulase production by *Aspergillus terreus* MTCC 7600. Orange peel, wheat bran, rice straw, corn meal, soybean meal and mustard meal were used as a sole source of carbon and nitrogen .Out of these sources the maximum yield of cellulase was obtained on 6^{th} day of incubation using wheat bran and soybean meal as a substrate that is of about (0.26±0.006) then the yield obtained on 12^{th} and 18^{th} day having high specific activity (1.26±0.006). The enzyme was found to be stable at temperature 40ºC and pH5. As the cellulase enzyme possess high enzyme activity and they are also resistant to environmental stress conditional they can be used in a wide range of industrial applications.

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Deeksha Agrawal

DECLARATION

I hereby declare that this thesis entitled ***"EFFECT OF CARBON AND NITROGEN SOURCE ON CELLULASE PRODUCTION BY Aspergillus terreus"*** is an authentic record of my own work carried out at School of Biotechnology and Biosciences, **Lovely Professional University, Phagwara,** for the partial fulfillment of the award of Master of Technology in Microbiology under the guidance of Dr. Loveleen Kaur, School of Biotechnology and Biosciences, Lovely Professional University, Phagwara.

This work is my original and has not been submitted for any degree/diploma in this or any other University. The information furnished in this dissertation is genuine to the best of my knowledge and belief.

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CERTIFICATE

This is to certify that **Deeksha Agrawal (1130960)** has completed Dissertation project report (BTY 731), entitled "**Effect of carbon and nitrogen source on cellulase production by *Aspergillus terreus*"** under my guidance and supervision. To the best of my knowledge, the present work is the result of their original investigation and study. No part of the report has ever been submitted for any other degree at any university.

This report is fit for submission and the partial fulfilment of the conditions for the award of M.Sc. in Microbiology**.**

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CHAPTER 1

INTRODUCTION

In a time of increasing energy prices, use of freely available biomass as a resource is one of the prime focus current scientific community. In recent years, use of renewable, economical, and readily available agricultural residue for the production of numerous products that has been attracting attention. Maximum quantities of agro residue are obtained from agricultural practices, forests, and industrial processes, mainly from agro-allied based industries such as paper and pulp, textile and timber industries (Hammad *et al.,* 2014). Agro residues such as rice straw, wheat bran, corn stover, sugar cane bagasse, corn cobs, soybean meal, mustard meal, corn meal, sunflower meal etc. are used as substrate in solid state fermentation (Nandy *et al.,* 2012). The utilization of these substrates for cultivation of microbes tends to produce organic acids, cellular proteins, enzymes, biologically important secondary metabolites, mushrooms, prebiotic oligosaccharides and also used as a source of fermentable sugars for the production of ethanol (Sanchez, 2009). Particularly, in these bioprocesses the microbial enzymes can be the products themselves.Enzymes are very important products obtained for human requirements through microbial sources. In the areas of industrial, food and environmental biotechnology, a wide range of industrial processes utilize enzymes at some stage or other (Pandey *et al.,* 1999).

Cellulase is an enzyme produced by different fungi, bacteria, and protozoan. Cellulases are that enzyme that hydrolyze cellulose, is a linear polymer of anhydro glucose units linked together by β-1, 4- glycosidic bond (Maurya *et al.,* 2011). Cellulose degrading enzymes system is a complex of three major types of enzymes that exhibit higher collective activity and degrade cellulose, a phenomenon known as synergism (Iqbal *et al.,* 2010). These enzymes occur in different forms in the preparation of enzymes and they act synergistically in the cellulose saccharification (Oberoi *et al.,* 2008). One major component enzyme of the cellulase complex is Endo-β-D-glucanase (CMCase). It catalyzes the hydrolysis of cellulose by breaking the sugar residues within the molecule. Exo-β-D-glucanase (cellodextrinases) and β-

glucosidase (glucoside glucohydrolases) converts cellulose into glucose and hence they are used on large scale (Shobana *et al.,* 2013). Cellulase has high interest in industries because it has wide applications such as starch processing, malting and brewing, grain alcohol fermentation, and animal feed production, extraction of fruit and vegetable juices, as well as manufacture of pulp, paper and textiles (Adsul *et al.,* 2007; Kaur *et al.,* 2007).

Cellulases can be produced by solid-state fermentation (SSF) or submerged fermentation (SSF), which is well employed in the enzyme production, is a fermentation process wherein the solid material acts as both a nitrogen source and physical support. SSF is defined as a process of fermentation in which microorganisms grow on solid materials in the absence of free liquid and the moisture necessary for growth of microorganisms exists in adsorbed state or composite with solid matrix (Krishna, 2005). Both bacteria and fungi are known to produce cellulases using complex cellulosic substrates, however, fungal enzymes are generally complete comprising of all the cellulosic activities. SSF has several advantages over submerged fermentation, it improves the yield of enzyme production, high volumetric productivity, and requirement of energy was low, low operating expenses and low sterility requirements (Murugammal, 2011). The high volumetric productivity of SSF also leads to lower water requirements and reduces the volume of residue generated. In the process of solid state fermentation the solid substrate not only produces the nutrients to the culture, but also presents as a harbor for the microbial cells (Kassebullha *et al.,* 2006). During fermentation moisture content of the medium changes as a result of evaporation and metabolic activities, thus optimum level of moisture for the substrate is the most important factor for the production of enzymes (Grover *et al.,* 2013). In addition, the agro-industrial by-products and crop residues can be used as carbon sources at a lower cost (Kim *et al.,* 2013). Filamentous fungi are the most commonly used microorganisms in SSF because they are able to grow on solid materials with low water contents. There are several reports describing use of agro industrial residues such as wheat bran, rice straw, orange peel, wheat straw, sugarcane baggase, corn cob, soy bean, rice bran as substrates for the production of cellulose (Sanchez, 2009). These residues are produced in high quantity throughout

the year. These substrates can be used as carbon and/or nutrient source, as a solid support in solid state fermentation process for the production of many valuable compounds (Martins *et al.,* 2012).

However, commercial enzyme productions are preferred using filamentous fungi, because the enzyme levels are produced by these cultures are higher than those obtained from yeast and bacteria. Different fungi are used for cellulase production such as *Trichoderma, Aspergillus, Neurospora, Sporotrichum, Pseudomonqs etc*. *Aspergillus* and *Trichoderma* spp. are well known efficient for cellulase production (Mrudula, 2011). The genera *Trichoderma* and *Aspergillus* are thought to be producers of cellulase, crude enzymes produced by these microorganisms are commercially available for the use of agriculture (Romero *et al.,* 1999). Among these *Trichoderma* produces relatively large amount of Endo-β-glucanase and Exo-β-glucanase with low quantity of β-glucosidase whereas *Aspergillus* produces relatively high amount of Endo-β-glucanase and β-glucosidase but low levels of Exo-β-glucanase production (Rajeev *et al.,* 2009).

Almost all fungi of genus *Aspergillus* synthesize cellulase, therefore *Aspergillus* have advanced potential to dominate the industry of enzymes. They have been broadly studied for their capacity to secrete high levels of enzymes that degrade cellulose (Zhou *et al.,* 2008).

Aspergillus terreus, also known as *Aspergillus terrestrius*, is a fungus (mold) found worldwide in soil, plant debris, and indoor air environment (Griff *et al.,* 2005). *Aspergillus* is a cosmopolitan, ubiquitous, filamentous fungus and found in nature. While a teleomorphic state has been described only for some of the *Aspergillus* sp., others are found to be mitosporic, without any known production of sexual spore (Anwar *et al.,* 2013). The genus *Aspergillus* includes over 185 species. Around 20 species have so far been found as causative agents of opportunistic infections in man. However, *Aspergillus terreus* is among the other species less commonly isolated as opportunistic pathogens (Velegraki *et al.,* 213). *Aspergillus* is known to be a thermophilic fungus, which means that it can tolerate heat and can grow at body temperature or higher temperature. It is also known to be a xerophilic fngus and can obtain moisture from the air if humidity at 60% or higher than this. Until recently, it

is thought to be strictly asexual, now *A. terreus* is known to have an ability of sexual reproduction (Arabatzis *et al.,* 2013).

This present study aimed to study the effect of carbon and nitrogen source on cellulase production from *Aspergillus terreus* MTCC 7600 by solid state fermentation using agroindustrial residues. *Aspergillus terreus* is a saprotrophic fungus. It in habitats soil and other places. *Aspergillus terreus* is known to have many industrial applications including production of organic acids as well as enzymes and also the production of secondary metabolites including lovastatin, a drug used for lowering serum cholesterol. Solid state fermentation has many advantages. SSF is simple process, cost effective, reduces pollution, also resembles the habitat of few fungi.

CHAPTER 2
TERMINOLOGY

Enzymes: Enzymes are proteins or biological molecules that serve as catalyst and help complex reactions occur everywhere in life.

Cellulase: Cellulase is an enzyme that is being produced by fungi, bacteria and protozoans. It catalyzes cellulolyisis, the decomposition of cellulose.

Fermentation: It is a metabolic process that converts sugar to acids, gases and alcohol.

Solid State Fermentation: It is defined as the fermentation process that occurs in the absence or near absence of water.

Submerged Fermentation: It utilizes free flowing liquid substrates such as molasses, liquid broth and corn steep liquor. The enzymes and bioactive compounds are secreted in the fermentation broth.

Enzyme Activity: It is defined as a unit required for the amount of a particular enzyme. One unit is defined as the amount of the enzyme that produces a certain amount of enzymatic activity, *i.e.,* the amount that catalyzes the conversion of 1 micromole of substrate per minute.

Specific Activity: It is the activity of an enzyme per milligram of total protein. It expressed in terms of micromole/min/mg.

UV Visible Spectroscopy: It refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectra region. This means it uses light in the visible and near ultraviolet and near infrared ranges that directly affects the perceived color of the chemicals involved.

Centrifugation: It is a process which involves the use of the centrifugal force for the sedimentation of heterogeneous mixtures or to separate two immiscible liquids.

CHAPTER 3
REVIEW OF LITERATURE

3.1: Substrates used for the enzyme production:

Rao *et al.,* (2013) stated that the main causes of environmental pollution are industrial and agricultural residues. Conversion of these residues into useful products may decrease the strength of the problems caused by these residues. Agro industrial residue such as sugarcane bagasse, wheat bran, rice bran, corn cob, wheat straw , rice straw, wheat straw are used for enzyme production and these substrates are cheapest and available throughout the year in India (Okafor *et al*., 1987, Mitra *et al.,* 2004). Wheat bran has been most commonly used in many processes.

Economically, other than food stuffs, the most important industrial material that is affected by microorganisms was wood products and cellulose (Wainright, 1992, Jing *et al.,* 1987).

Singh *et al.,* (2008) stated that with respect to environmental affects, continued development of biosustainable and renewable resource technology is of great importance. A large amount of agro-industrial residue containing high lignocellulosics and starch content is generated every year all over the World. Lignocellulose depicts the most abundant renewable organic resource in soil and is the major constituent of biomass (Sanchez, 2009). It consists of cellulose, hemicellulose, polymers, and lignin that are chemically bonded by covalent cross-linkages and non-covalent forces (Perez *et al.,* 2002).

Pandey *et al.,* (1999) stated that for the process of Solid State Fermentation, agro-industrial residues are generally preferred and normally considered the best substrates. A number of these substrates had employed for the culturing of microorganism to produce enzymes host. Karmakar *et al.,* (2010) stated that the major part of plant biomass is cellulose. Therefore, the residues generated from agricultural fields, agro industries and forests comprise a large amount of underutilized or unutilized cellulose. These residues generally cause pollution problem to the environment (Abu *et al.,* 2000). Nowadays these residues are considered as the major renewable natural resource (Acharya *et al.,* 2008) and are

converted to valuable products such as chemicals, biofuels, improved animal feeds, cheap energy sources of fermentation, and human nutrients (Howard *et al.,* 2003).

Grigogorevski *et al.,* (2009) stated that for large scale production of fuels and chemicals, lignocellulosic residues obtained from forestry and agriculture had great potential as they are cheap. The biodegradation of cellulose to soluble sugars is only feasible after the action of cellulolytic pools obtained by cellulolytic microorganisms (Wingren *et al.,* 2003). In current years, more scientific attention is given to this process because of its environmental and economical significance.

Zoppas *et al., (*2013) stated that agroindustrial residues are rich in lignocellulosic materials that are certainly obtained by industrial and agricultural activities. The following could be mentioned as being among the lignocellulosic residues produced in volume by the Brazilian agroindustrial activity: sugarcane bagasse, sugarcane bark, sugarcane straw, corn cobs, corn straw, rice bran, rice straw, wheat bran, wheat chaff, wood scraps and cotton residue are used for their study. 20 to 60% cellulose, 15 to 30% lignin and 20-30% hemicelluloses present in agricultural residues. In the world, the available quantity of these substrates is very large (Pauli *et al.,* 1998 and Bon *et al.,* 2008).

Jadhav *et al.,* (2013) stated that the enzyme cellulase is responsible for the degradation cellulose. The enzyme cellulase is made of endoglucanase, β-glucosidases and cellobiohydrolases (Hammad *et al.,* 2010). They all act synergistically to convert complex carbohydrates that are present in lignocellulosic residues into glucose efficiently (Holker, 2004). Cellulase is used to cleave cellulose into oligosaccharides compounds (Chellapandi and Himanshu, 2008), of which CMCase cleaves bonds at random and liberates cellobiosyl units from the non reducing end of cellulose chains (Bhat and Bhat, 1997). In different industrial applications, cellulases are sold in large amounts, for example in animal feed production, in starch processing, malting and brewing, grain alcohol fermentation, pulp and paper industry etc (Dogaris *et al.,* 2009). There is developing market for cellulase in the area of saccharification of agricultural residues for technology of bioethanol and in the field of detergent industry (Singhania *et al.,* 2009).

3.2: Cellulase producing Fungus:

Table 1: List of fungi which produce cellulase and the substrates for their growth:

S.No	Fungus	Substrate	Reference	Year
1	*Aspergillus fumigatus*	Wheat Bran, Rice Straw	Paul *et al.,*	2013
2	*Aspergillus fumigatus*	Oil Palm Trunk	Ang *et al.,*	2013
3	*Termitomyces clypeatus*	Mustard Straw and Mustard Stalk	Pal *et a.,*	2013
4	*Chaetomium sp.*	Mangrove leaves and Mangrove wood	Ravaindran *et al.,*	2012
5	*Aspergillus flavus*	Wheat Bran	Gomathi *et al.,*	2012
6	*Aspergillus terreus*	*Saccharum spontaneum*	Ahmed *et al.,*	2012
7	*Acremonium cellulolyticus*	Wheat Bran	Kanna *et al.,*	2011
8	*Aspergillus niger*	Orange Peel	Mrudula *et al.,*	2011
9	*Aspergillus niger*	Coconut shell	Coelho *et al.,*	2010
10	*Aspergillus fumigatus*	Wheat Bran, Rice Straw	Sherief *et al.,*	2010
11	*Aspergillus heteromorphus*	Wheat Straw	Singh *et al.,*	2009
12	*Aspergillus niger*	Bagasse from sugarcane	Aguiar *et al.,*	2008
13	*Trichoderma longibrachiatum*	Sweet Orange	Omojasola *et al.,*	2008
14	*Aspergillus phoenicis*	Dairy Manure	Wen *et al.,*	2005
15	Trichoderma reesei	Corn Cob	Liming *et al.,*	2004
16	*Aspergillus terreus*	Sugarcane Bagasse	Garg *et al.,*	2004
17	*Trichoderma lignorum*	Banana	Baig *et al.,*	2004
18	*Fusarium oxysporum*	Corn Stover	Panagiotou *et al.,*	2003

3.3: Cellulase:

Zoppas *et al.* (2008), stated that enzymes are proteins that revealed catalytic activity. The enzyme complex molecular structure consists of one part of protein, but it can be connected to other molecules such as carbohydrates and lipids. Enzymes are present in all living cells, which exercise the function of catalysts of the reactions that compose the anabolic and catabolic pathways of cellular metabolism (Alberton, 2004). Cellulases are the complex mixture of enzymes produced by cellulolytic microorganisms. These enzymes are commonly linked by β-1, 4 linkage and they are required for the complete solubilization of cellulose (Bon *et al.,* 2008).

Venkatramanan *et al.,* (2014) stated that during the growth of microorganisms on cellulosic materials, cellulases which are inducible enzymes are synthesized. Different types of cellulases are required for the complete enzymatic hydrolysis of cellulosic materials which include endoglucanase, β-D glucosidase and exocellobiohydrolase (Mehmet, 2010). The β 1-4 bonds present in the cellulose molecule is hydrolyzed by the endoglucanase and cellobiose unit is released by exocellobiohydrolase. Bacteria and fungi play an important role in the degradation of cellulose (Lalitha, 2011). The cellulose using microorganisms includes filamentous fungi, aerobic and anaerobic mesophilic bacteria, alkalophilc and thermophilic bacteria, actinomycetes and certain protozoa (Mir, 2011, Fadel, 2000). In general, fungi are well known agents responsible for decaying organic matter, and in particular, for decomposition of cellulosic materials.

CMCase had broad range of applications in animal feed, textile, fuel, processing of food; residue management, paper and pulp industry, chemical industries, medical/pharmaceutical industry, genetic engineering, protoplast production and pollution treatment (Tarek and Nagwa, 2007).

Sandhu *et al.,* 2013 stated that cellulose is a huge natural polymer on earth most influencing agricultural residue. This biomass is a renewable and huge resource with great potential for bioconversion to valuable byproducts. It can be degraded by the enzyme cellulase produced by cellulolutyic bacteria. This enzyme possesses various industrial applications and now they considered as major group of industrial enzyme.

Dabhi*et al.,* (2014) stated that cellulose is made of repeated units of cellobiose that contain two anhydrous glucose rings joined by a β-1, 4 glycosidic bonds) (Klemm *et al.,* 1988) and is a high molecular weight linear hompolymer.

3.4: Cellulase Production:

Shobana *et al.,* (2013) isolated *Aspergillus fumigatus* for the production of cellulase. *Aspergillus fumigatus* was tested for its ability to produce cellulase production. Production of cellulase was examined in agro industrial residue such as rice bran, coconut coir pith, rice husk and wheat bran. *Aspergillus fumigatus* showed highest activity of enzyme in rice bran. In this study, the optimum parameters for the organism were measured under altering conditions such as pH, temperature and concentration of substrates. The maximum cellulase production was observed at temperature of 25^0C , pH of 4 and substrate concentration of 5 gm for *Aspergillus fumigatus.*

Hammad *et al.,* (2010) isolated twenty nine strains of fungal from agro industrial residues. Wheat straw, wheat bran, rice straw, rice bran and corn cobs were selected as cheap, renewable agro industrial residues for solid state fermentation. Different strains of *Aspergillus* and *Trichoderma viride* were grown on the agro industrial residues and activity of CMCase, FPase, Avicelase and soluble protein were determined. *Trichoderma viride* gave the maximum activity of CMCase on the substrate wheat straw (555U/ml), whereas the maximum FPase (141U/ml) and Avicelase (46U/ml) were observed on wheat bran. The isolated strain *Aspergillus* MAM-F35 produces the maximum CMCase (487 U/ml), FPase (79 U/ml) and Avicelase (35 U/ml) on wheat straw.

Milala *et al.,* (2008) used fermentation feed substrates such as rice husk, millet straw, guinea corn and saw dust were used for the assay of activity of cellulase by *Aspergillus candidans*. These substrates were pretreated with 5% NaOH and sterilized. From the studies, they observed that rice husk produced maximum activity of cellulase of 7.50followed by millet straw (6 IU) and guinea corn stalk (5.84 IU). At pH 5, rice husk and millet straw showed high activity of enzyme, whereas at pH 3 and 4, guinea corn stalk and saw dust respectively gave high activity of enzyme.

Narra *et al.,* (2012) found that rice straw can be use as an efficient substrate for cellulase production by solid state fermentation with *Aspergillus terreus*. Substrate concentration, moisture ratio, inoculum size and initial pH were optimizing using response surface methodology. The predicted filter paper activity under optimized parameters was found to be 9.73 U/g. Hydrolysis of the biomass pretreated with 0.125% to 1% NaOH for 24h at room temperature was performed using crude cellulase preparation and it was found that the treatment with 0.5% NaOH at room temperature for 24hr was the most efficient treatment method for saccharification. Under the optimized conditions, rice straw yielded 676 mg reducing sugars per gram of substrate at a cellulase loading of 9 FPU g(-1) substrate.

Shahriarinour *et al.,* (2011) used Response surface methodology (RSM) to evaluate the effects of dissolved oxygen tension (DOT) and initial pH on the carboxymethylcellulase (CMCase) production, filter-paper hydrolase (FPase), and β-glucosidase by *Aspergillus terreus* in a 2 L stirred tank bioreactor. Delignified oil palm empty fruit bunch (OPEFB) fibre can be used as the main substrate under submerged fermentation. Growth of *A. terreus* and the production of three main components of cellulase were optimized by central composite design (CCD) design. Statistical analysis of results shown that the individual terms of these two variables (DOT and pH) have significant effects on production and the growth of all components of cellulase. Maximum growth (13.07 g/L) and cellulase activity (CMCase = 50.33 U/mL, FPase = 2.29 U/mL and β-glucosidase = 15.98 U/ml) was obtained when the DOT and initial culture pH were set at 55% and 5.5, respectively. A high proportion of β-glucosidase to FPase (8:1) in cellulase of *A. terreus* was beneficial for efficient hydrolysis of cellulosic materials.

Hui *et al.,* (2010) isolated *Aspergillus terreus* from rotting bagasse. The cultural and nutritional requirements for maximum cellulase production by the organism either in the free or immobilized statesusing cellulose as sole carbon source were similar, except an increase in the temperature optimum from 30 to 40°C, which occurred upon immobilization. In the freestate, the maximum filter paper hydrolase, carboxymethylcellulase and glucosidase activities produced were 2.1, 13.6, and 3.2

U/ml, respectively, while in the immobilized state, the levels are 1.8, 12.0, and 2.4 U/ml.

Holker *et al.,* (2004)*;* Esterbauer *et al.,* (1991) stated that Soybean hulls accounts for 5-8% of the 96 million metric tons soybean crop of 2006 in USA (Mielenz *et al.,* 2009). Soybean hulls have rich cellulosic composition including cellulose and hemicellulose (Brijwani *et al.,* 2010).

3.5: Solid State Fermentation:

Aguilar *et al.,*(2008) explained that xylanase (EC 3.2.1.8), though not part of the group, complements the cellulolytic enzyme system as it is needed to elicit complete and efficient hydrolysis of the lignocellulosic biomass, which has an appreciable amount of hemicellulose or xylan (Brijwani *et al.,* 2010; Brijwani, 2011). It has been widely accepted that Solid State Fermentation (SSF) is an attractive means to produce cellulase economically because of its lower capital investment and lower operating cost (Cen and Xia 1999). Further, the ability of SSF to minimize catabolite repression has been already described for several enzymes

Garg *et al.,* (1981) studied the protein production by *Aspergillus terreus* GN1 growing on 1.0% alkali-treated bagasse is studied under various cultural conditions. The maximum protein content of 20.1% and protein recovery of 11.2% was obtained with an initial pH of 4.0, with 1/5 (v/v) inoculum in continuously shaken cultures grown for seven days. They found to be the highest crude protein percent also corresponded with highest carboxymethylcellulase and filter paper enzyme activities.

Hussein *et al.,* (2013) studied that the production of cellulase under the optimum fermentation conditions from nontoxic *Asperigllus flavus* NRRL in Cellulose Powder Medium (CPM). The maximum production of cellulase by *A. flavus* NRRL 5521 reaching (0.11 IU mL^{-1} min^{-1}) is achieved at 10% rice straw, inoculum size of 7%, 48 h of incubation period, initial pH of growth medium 7.0 and yeast extract as a nitrogen sources at a concentration of 0.33 g N L^{-1}.

3.6: Effect of Carbon and Nitrogen Source on Cellulase Production:

Rajendra *et al.,* (1998) stated that culture filtrate obtained from *Aspergillus* isolate exhibited good activities against the filter paper as substrate. The optimum temperature and pH values for growth were found to be 3^{o}C and 6.5, respectively. However, the optimum temperature and pH for the activities of the cellulose enzymes were recorded as 44^{o}C and 4.5 respectively. Production of the enzymes in liquid medium reached its maximum level (9 units/ml) on the 15th day of incubation with an incubation period of up to 21 days. Out of various carbon and nitrogen sources tested, carboxymethyl-cellulose (medium viscosity) and $(NH)_2Fe\ (SO)_2.6H20$ were found to be the best for cellulose enzyme production, whereas cellulose powder and NH_3enhanced mycelia growth.

Padmavathi *et al.*, (2012) isolated two marine fungi, *Aspergillus terreus* and *Mucor plumbeus* for the production of cellulase using submerged fermentation technique. Ten different substrates *viz.*, wheat bran, rice bran, leaves of bamboo, peepal, banana, sugarcane, maize, eucalyptus and lantana, ragi straw were collected from different parts of rural Bangalore and were used for the production of cellulase. *Aspergillus terreus* and *Mucor plumbeus* gave best enzyme activity on Lantana leaves of 213.3IU/ml and 206 IU/ml, respectively. Various parameters such as nitrogen source, carbon source, pH, temperature and incubation time were studied for the cellulases production. The saccharification degree was also assayed on the basis of amount of reducing sugar released. The saccharification percentage with respect to lantana leaves in presence of *Aspergillus terreus* and *Mucor plumbeus* were determinedas 56% and 28% respectively.

Sethi *et al.,* (2013) isolated Cellulase-producing bacteria from soil and identified them as *Pseudomonas fluorescens, Bacillus subtilis*, *E. coli*, and *Serratia marcescens*. Optimization of the fermentation conditions such as pH, temperature, carbon sources, and nitrogen sources was carried out. The optimum conditions found for cellulase production were 40°C at pH 10 with glucose as carbon source and ammonium sulphate as nitrogen source and coconut cake for stimulating the production of cellulase. Among bacteria, *Pseudomonas fluorescens* is the best

cellulase producer among the four followed by *Bacillus subtilis*, *E. coli*, and *Serratia marscens.*

Sakhti *et al.,* (2011) isolated *Aspergillus niger* the soil and used it for cellulase production. Optimization of cellulose production was done by using various physical (temperature, pH, salinity, and incubation time) and chemical parameters (carbon sources and nitrogen sources). Cellulose production was maximum at the temperature 20^0C, pH 6.0 after 48hrs. When fructose was used as a carbon source malt extract was used as a nitrogen source. The cellulose enzyme extract was partially purified and its protein fraction was subjected to SDS-PAGE which revealed two protein bands with the molecular weights of about 83kD and 53kD.

3.7: Effect of pH and Temperature:

Bundela *et al.,* (2008) isolated *Trichoderma viride* form the municipal solid residue and also optimized the physicochemical properties and nutritional parameters for production of cellulase. Result showed that as pH value increased, enzyme activity also increased. It was also noted that the activity of enzyme was stable at pH in the range of 5.0 to 8.0 and at temperature between 50^0C to 80^0C. Many researchers have reported various temperatures for maximum production of cellulase either in flask or in fermentor studies using *Trichoderma* sp. indicating that the optimal temperature for production of cellulase also depends on the strain variation of the microorganism (Murao et al., 1988, Lu et al., 2003).

Kiranmayi *et al.,* (2011) recorded the effect of temperature and pH on enzyme activity by growing the *Aspergillus* at different pH ranges from 4 to 9 and temperatures ranges from 25^0C to 55^0C. With commercial cellulose as substrate the optimal temperature and pH was recorded. pH at 5 considered as optimal pH for the enzyme activity and there was a significant decrease in the activity of enzyme with the increase in pH. Akiba *et al.,* (1995) reported that optimum pH for maximum cellulase activity was 4. Acharya *et al.,* (1995) and Sohail *et al.,* (2009) have also reported that maximum activity of cellulase at pH 4.

CHAPTER 4
RATIONALE AND SCOPE OF THE STUDY

Cellulase is an enzyme produced by fungi, bacteria and protozoans. Cellulases are the enzymes that breakdown cellulose to glucose or short chain of polysaccharides or oligosachharides. Cellulase is used in many industries for the production of enzymes, primary and secondary metabolites. It is used in the food processing industries. Cellulase is also used in textile industry, paper and pulp industry and also in the fermentation of biomass to biofuels. Cellulases are even used for pharmaceutical applications. The importance of utilization of microbial cellulase in environment is enhanced by the ruminants as a source of dietary proteins. It is also an integral component of composting and anaerobic digestion. Cellulase was produced by using different agroindustrial residues in solid state fermentation. Different agroindustrial residues such as wheat bran, orange peel, rice straw, corn meal, soybean meal and mustard meal etc were used. Most of these were used as animal feed. Major problem is burning of agroindustrial residues which cause pollution in the environment. However, they should not be considered as wastes as they are rich in proteins, sugars and minerals. Agroindustrial residues can be used as carbon, nutrient or solid support in solid state fermentation for the production of valuable products. These sources are very cost effective, easily available throughout the year and have the potential to enhance the production of enzyme.

The scope of this research is to study the effect of different agroindustrial residues used as carbon and nitrogen source to enhance the production of cellulase by *Aspergillus terreus* MTCC 7600

CHAPTER 5
OBJECTIVES OF STUDY

The topic of present study is Effect of carbon and nitrogen source on cellulase production by *Aspergillus terreus* MTCC 7600. Keeping in mind the importance of cellulase production by *Aspergillus terreus* MTCC 7600, the work was planned to meet the following objectives:

1. To study the effect of carbon sources on cellulase production by *Aspergillus terreus* MTCC 7600
2. To study the effect of nitrogen sources on cellulase production by *Aspergillus terreus* MTCC 7600
3. To study the effect of temperature, pH and heat stability of crude enzyme

CHAPTER 6
MATERIALS AND RESEARCH METHODOLOGY

6.1: Materials:

Different equipments required for project work: Incubator, Laminar Air Flow, Autoclave, Water Bath, Centrifuge, Air Dryer, and Rotary Shaker used in this work were availed from Laboratories of School of Biotechnology and Biosciences, Lovely Professional University, Phagwara.

Glass wares, chemicals and reagents used in this work were procured from CDH, Loba Chemie and HiMedia, School of Biotechnology and Biosciences, Lovely Professional University, Phagwara.

6.2: Microorganisms:

Aspergillus terreus MTCC 7600 was procured from Microbial Type Culture Collection, Chandigarh. The fungus was maintained on Potato Dextrose Agar (PDA) media. The media was prepared and autoclaved at 121^0C for 15 minutes. Media was then transferred to plates under sterilized condition and remains it to solidify. Afterwards, quadrant streaking was done aseptically. Incubate plates at 28^0C for 3-4 days. Fungus was sub cultured after every 7 days.

Composition of PDA (HiMedia):

Materials	**Grams/Litre**
Potatoes, infusion form	200.000
Dextrose	20.000
Agar	15.000
Final pH (at 25^0C)	5.6±0.2

6.3: Macroscopic and Microscopic Study:

The *Aspergillus terreus* MTCC 7600 was grown on Potato Dextrose Agar plates and incubate plates for 4 to 5 days at 28^0C.. Macroscopic study of growth of fungus was done by observing its growth characteristics, color of colony on surface as well as reverse.

Aspergillus terreus MTCC 7600 was microscopically identified after staining with Lactophenol Cotton Blue (LCB). Place a drop of lactophenol cotton blue onto clean grease free slide and add loopfull culture, the culture was mixed with lactophenol cotton blue. Place a coverslip over the suspension in such a manner that there should not be any bubble formation. The slides were then observed under microscope at 10X, 40X and 100X (Oil immersion).

6.4: Radial Growth of *Aspergillus terreus* MTCC 7600:

For the study of radial growth of *Aspergillus terreus* MTCC 7600, potato dextrose agar was made. From the plates of equal sized (8mm) bits of agar covered with fungal mycelium was cut by using borer. The agar bit containing fungal culture was transferred to the centre of freshly prepared potato dextrose agar plates. The plates were incubated at 28ºC and their radial growth pattern was observed at an interval of 24 hours for 7 days.

6.5: Enzyme Production using Solid State Fermentation:

6.5.1: Substrate Preparation:

Wheat Bran (WB) and Rice Straw (RS) were collected from local market of Jalandhar (Punjab). They were sun dried for 2-3 days to remove any moisture content and sealed in air tight plastic bags for further use.

Orange Peel (OP) was collected and subjected to sun drying for about 15 days. The orange peel left after drying was then grinded and sealed in air tight container.

Soybean Meal, Corn Meal and Mustard Meal were collected from the local industries of Jalandhar (Punjab). Meals were sun dried for 4-5 days and sealed in air tight plastic bags for further use.

6.5.2: Basal medium:

Solid-State fermentation of orange peel, wheat bran, rice straw, soybean meal, corn meal and mustard meal were done by using basal medium as the moistening agent for the substrates. The pH of the media was adjusted to 6.0.

Materials	Composition
NaCl	0.5%
KH_2PO_4	0.5%
$MgSO_4.7H_2O$	0.1%
$CaCl_2$	0.05%
NH_4NO_3	0.5%
Peptone	0.1%
$MnSO_4.7H_2O$	0 .001%
$ZnSO_4.7H_2O$	0.001%
$FeSO_4.7H_2O$	0.005%
$CoCl_2.6H_2O$	0.0002%

6.5.3: Inoculum Preparation:

The spores from 7 to 8 days old culture maintained in PDA slants were wetted by adding 9ml distilled water and 1ml tween-80. The spores were scratched with a sterilized inoculating needle and the tubes were shaken gently. The supernatant containing spores were serially diluted aseptically and 2 ml of the suspension was then used as inoculum for solid state studies having spore count of 3.4×10^7 spores/ml.

6.5.4: Solid-State Fermentation:

Ten gram of solid substrates *viz.*, wheat bran, orange peel and rice straw used as carbon sources were transferred to 250 ml of Erlenmeyer flasks. They were moistened with basal salt solution. The Erlenmeyer flasks were plugged with cotton plugs and sterilized by autoclaving at 121ºC for 15 minutes. After cooling to room temperature substrate was then inoculated with 2 ml of spore suspension and the solid-state fermentation assemblies were then incubated at 28 ±2 ºC.

Five gram of wheat bran was supplemented with five gram of soybean meal, mustard meal and corn meal were transferred to 250 ml of Erlenmeyer flasks. They

were moistened with basal salt solution. The flasks were plugged with cotton plugs and sterilized by autoclaving at 121ºC for 15 minutes. After cooling to room temperature substrate was then inoculated with 2 ml of spore suspension and the solid-state fermentation assemblies were then incubated at 28 ±2 ºC.

Production of cellulase in Solid-State Fermentation was assayed after 6, 12 and 18 days of incubation time.

6.5.5: Enzyme Extraction:

30ml of Sodium Citrate buffer was added to each flask after 6 days of incubation period, then shaken at rotary shaker for 1hour and filtered with the help of muslin cloth. The filtrate was centrifuged at 5000rpm for 15 minutes. After centrifugation supernatant was collected and used as crude enzyme. The same procedure was repeated for 12 and 18 days of incubation.

6.6: Measurement of Enzyme Activity and Protein Content:

6.6.1: Reagent Preparation:

6.6.1.1: Preparation of DNS (Dinitro Salicylic acid) reagent:

DNS reagent was prepared by dissolving 1 g DNS, 1 g Sodium hydroxide (NaOH), 0.05 g Sodium Sulphite and 0.2 g of Phenol and making up the volume to 100 ml with distilled water. 20 % Sodium Potassium Tartrate solution was prepared separately in distilled water.

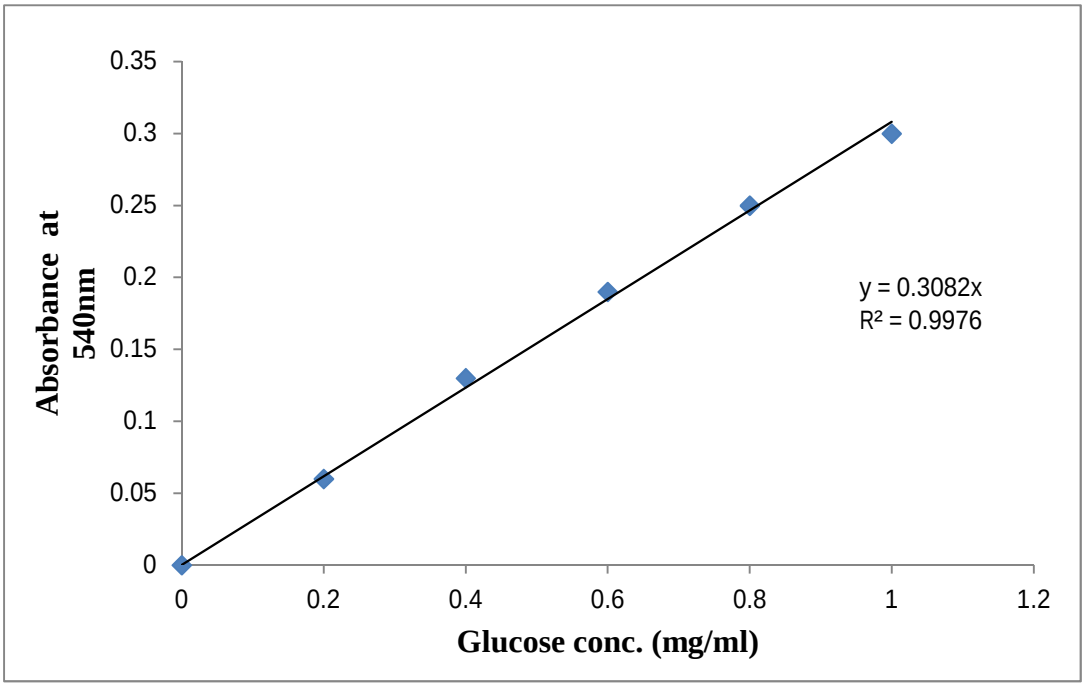
6.6.1.2: Carboxy Methyl Cellulase (CMC) Solution:

1 % of CMC solution was prepared by dissolving 1gm of CMC in 0.2 M Sodium Citrate Buffer at pH 5.

6.6.1.3: Glucose Standard Curve:

Stock solution of glucose of concentration 1mg/ml was made and further dilutions ranging from 0.1 to 0.6 mg/ml were made. 3 ml of DNS was added in each test tube. Test tubes were covered with aluminum foil and placed in boiling water bath for 10-15 minutes. Thereafter, the tubes were taken out and 1 ml of sodium

potassium tartrate was added. Test tubes were allowed to cool down to room temperature and absorbance was read at 540nm (Miller, 1959).

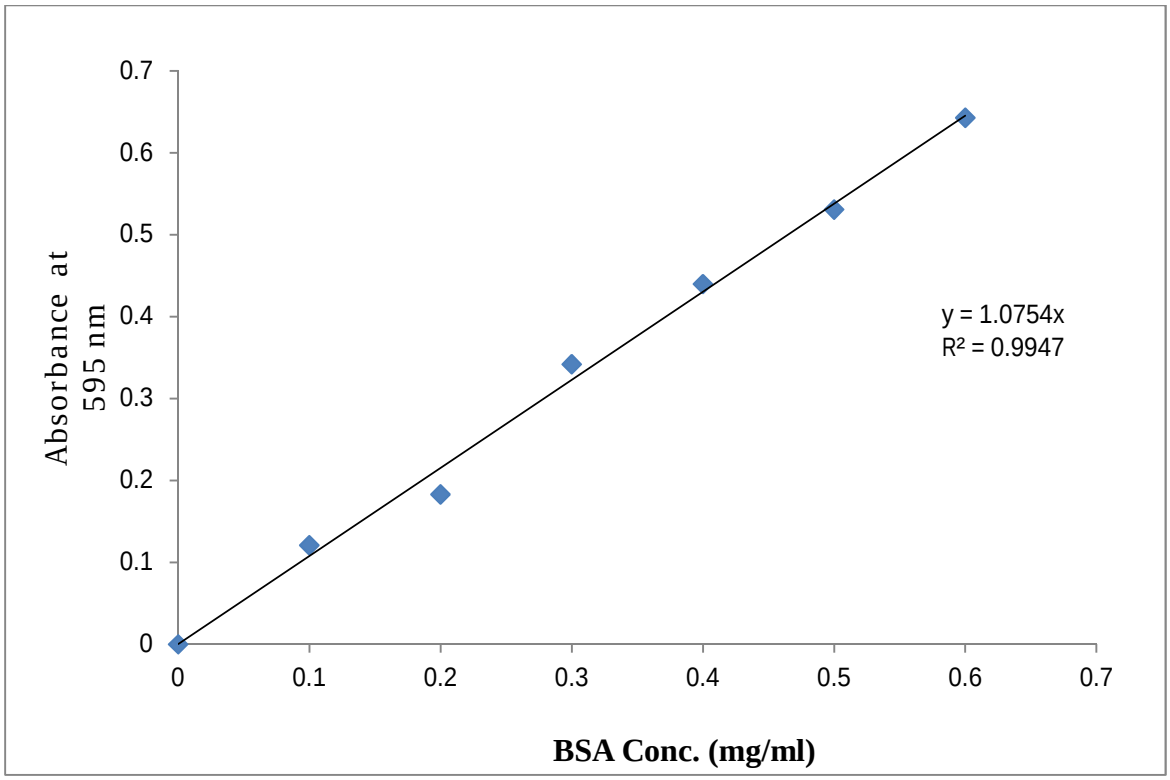


6.6.1.4: Preparation of Bradford Reagent:

10 mg of Coomassie brilliant blue G-250 dye was dissolved in 5 ml of 95% ethanol. Then 10 ml of 85% o-phosphoric acid was added and the final volume was made upto 100 ml by adding distilled water.

6.6.1.5: Protein Standard Curve:

Stock solution of protein was prepared by taking 0.1gm of BSA in 100 ml of distilled water. Various concentrations of standard protein solution from stock solution were made as 0.1 mg/ml to 0.6 mg/ml into series of test tubes containing 1 ml of distilled water. Then 1 ml volume was made by adding distilled water. 4 ml of Bradford reagent was added to each tube and kept for 30 minutes at room temperature. After this using UV-Visible Spectrophotometer, absorbance of each concentration was taken at 595 nm.



6.6.2 Determination of Enzyme Activity:

CMCase activity in crude enzyme was determined according to Mandels *et al.,* 1969 in which 0.5 ml of enzyme (dilutions of crude enzyme extract (CEE)- 1:50 or 1:100) were reacted with 0.5 ml of CMC solution (1% CMC in 100 mm Citrate buffer) for 60 minutes at 50°C and the reaction was stopped by adding DNS reagent and contents of the test tubes were boiled for 15 minutes. Absorbance was read at 540nm. Absorbance was then compared with the standard graph plotted by reacting known concentration of glucose (0.2 mg/ml to 1 mg/ml) with DNS reagent and plotting a graph between concentration of glucose (X axis) and OD at 540nm (Y axis). One unit CMCase activity is defined as amount of enzyme that releases 1 micromole of glucose per minute under standard reaction conditions.

Formula for calculating enzyme activity = $\frac{\text{Amount of sugar released}}{180} \times \frac{1}{60} \times \frac{5}{0.5} \times 10^3$ (Units/ml)

6.6.3: Determination of Protein Content:

Amount of protein in crude enzyme was determined by Bradford's method of protein estimation, in which 0.5ml of crude enzyme, 0.5ml of distilled water was reacted with 4ml of Bradford's reagent and the absorbance was read at 595nm. Absorbance was compared with the standard curve prepared by reacting known concentration of protein ranging from 20 µg/ml to 100 µg/ml with the Bradford's reagents and plotting a graph between concentration of protein BSA (X axis) and OD at 595 nm (Y axis).

Formula for calculation of specific activity = $\frac{\text{Enzyme activity (U/mg)}}{\text{Total Protein}}$

6.7: Plate Assay Method using Congo red:

For the plate assay method, the cellulase from *Asprgillus terreus* was used. 1% CMC Czapek Dox Agar media was prepared, autoclaved at 121^0C for 15 minutes. Afterwards, under sterilized conditions media was poured into plates having diameter of 8mm, remains it to solidify. Later, the wells were made with the help of cork borer. 10 microlitres of cellulase was poured into wells in agar plates and plates were incubated for 24 hours at 28^0C. After 24 hours, plates were flooded with 0.1 % Congo red and kept for 15 to 20 minutes followed by washing with 1 M NaCl for 15 to 30 minutes.

Composition of 1% CMC Czapek-Dox Medium:

Materials	**Grams/Litre**
Sucrose	30.0
$NaNO_3$	2.0
K_2HPO_4	1.0
$MgSO_4$	0.05
KCl	0.5
$FeSO_4$	0.01
Carboxy Methyly Cellulose	1.0
Agar-agar	20.0

6.8: Enzyme Stability Assay:

6.8.1: Temperature Stability:

For the determination of the stable temperature of the cellulase enzyme, the enzyme was incubated with substrates for 30 minutes at different temperatures ranges from 30^0C to 80^0C.

6.8.2: pH Stability:

For the determination of the stable pH of the cellulase enzyme, the enzyme was mixed with substrates at different pH levels ranges from 3 to 8. These substrates were prepared in different buffer solutions: Sodium Acetate (pH 3 to 4.5), Sodium Citrate (pH 5 to 5.5) and Sodium Phosphate Buffer (pH 6 to 9). Incubate the enzymes for 10 minutes at 50^0C.

6.8.3: Heat Stability:

For the determination of heat stability, the enzyme was incubated in the standard buffer at 30^0C, 40^0C, 50^0C, 60^0C and 70^0C for 15 minutes.

Then the activity was determined by DNS method.

CHAPTER 7
RESULTS AND DISCUSSION

7.1: Morphology Study:

The culture of fungus was maintained on Potato Dextrose Agar plates at 28±2^{o}C by frequent subculturing and colony morphology and microscopic examination was carried out.

7.1.1: Colony Morphology:

On Potato Dextrose Agar, from Plate 1 it has been observed that colonies have the potential to grow rapidly and they have smooth margins. In some cases, they are capable to be floccose possessing hair like soft tufts. The colonies are rounded and crumpled from centre to margin. In the medium, *Aspergillus terreus* emits yellow pigment. On ageing, the yellow pigment produced by fungus starting to diffuse out and colored the media yellow. It has been observed that the color of *Aspergillus terreus* on the surface ranges from Cinnamon to Brown while on the reverse side of petriplate it appears to be White to Brown. The color in the centre is brown and white at periphery.

Plate: 1 Petriplate showing colony morphology of *Aspergillus terreus* MTCC 7600 with suppressed white grown with brownish centre.

7.1.2: Microscopic Features:

Fungal hyphae were stained with alctophenol cotton blue and observed under microscope at 10X, 40X, 100X (Plate 2). Under the microscope *Aspergillus terreus* MTCC 7600 shows septate and hyaline hyphae with conidial heads which bear small twist of phialides. Conidial heads are compact and densely columnar. *Aspergillus terreus* has smooth conidiophores with small conidia, smooth walled and globose shaped.

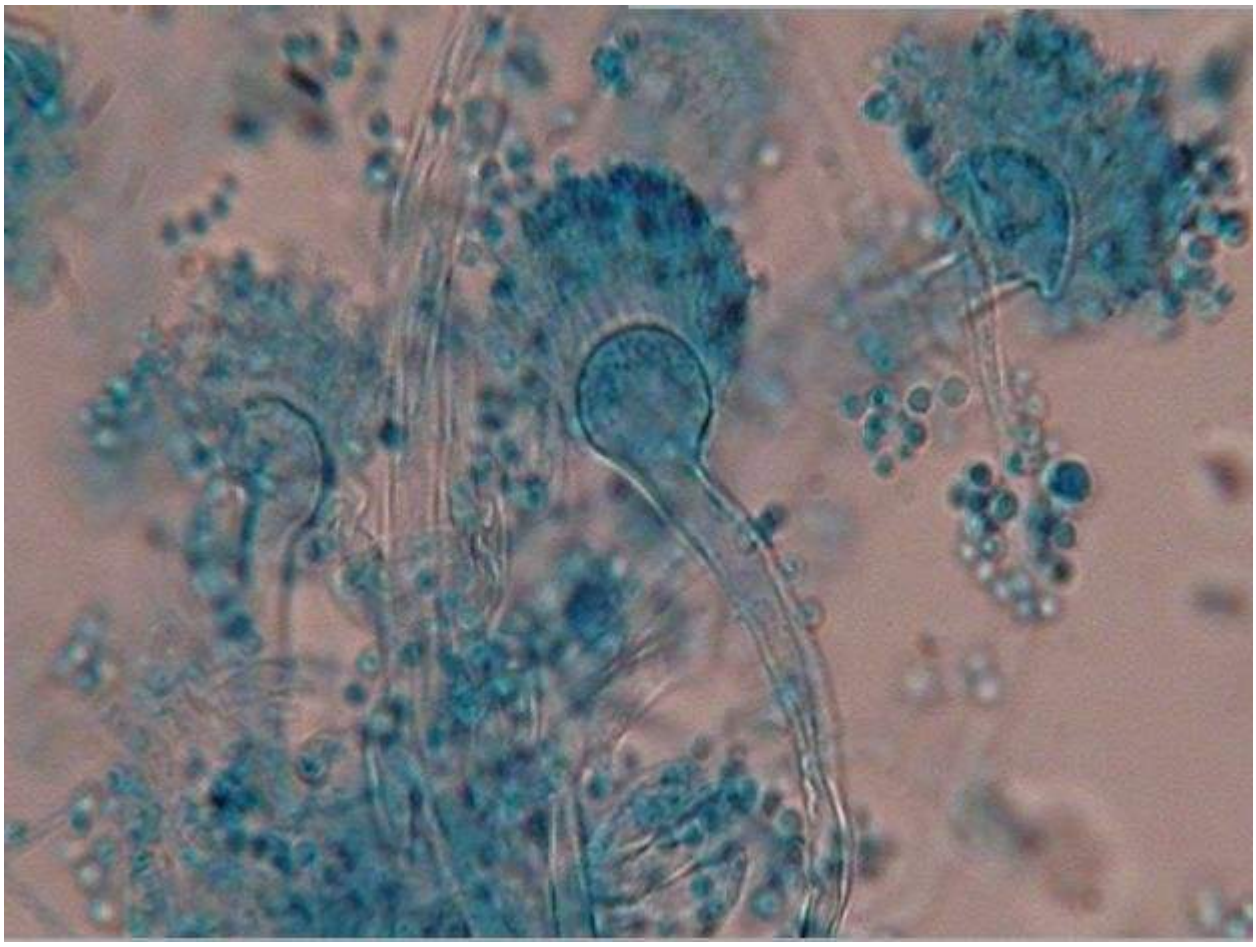


Plate 2: Microscopic view of *Aspergillus terreus* MTCC 7600 at 40X showing conidia and conidiophores

7.1.3: Radial Growth Study:

Aspergillus terreus MTCC 7600 was cultured on Potato Dextrose Agar medium at 28±2^{o}C, colony diameter was recorded weekly from the edge of the initial inoculum to study the growth rate. Rapid growth was observed till 5 days of incubation. *Aspergillus terreus* MTCC 7600 has a granular. Percentage growth rate of 83.6, 50.2 and 30.6 was observed after 2, 3 and 4days respectively. Rapid growth rate was observed for first 3 days and after 4 days marginal growth was observed which shows significant difference. The entire plate was filled with white-brown surface mycelium after 7 days.

Table 2: Measurement of growth of *Aspergillus terreus* MTCC 7600 by colony diameter with time (hour):

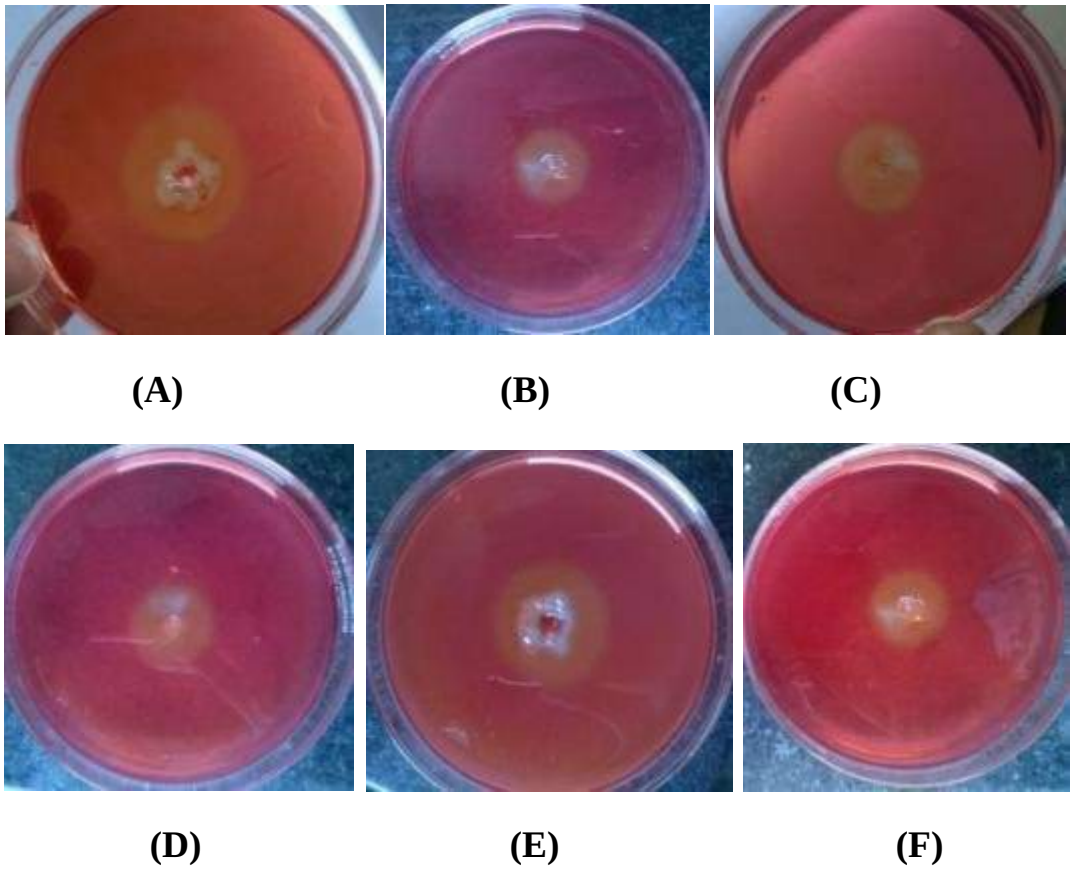
Time (Hours)	Diameter (mm)*	% Increase in growth/day
24	11.6	0.0
48	21.3	83.6
72	32.0	50.2
96	41.8	30.6
120	49.6	18.6
144	56.9	14.7
168	60.4	6.15
CD@5%	1.25	

Temperature of incubation-28±2^{o}C

Medium used- Potato Dextrose Agar

*****Average of three replicates

7.2: Qualitative determination of crude cellulase extract produced by *Aspergillus terreus* MTCC 7600:



(A) **(B)** **(C)**

(D) **(E)** **(F)**

Plate 5: Qualitative determination of crude cellulase extract produced by *Aspergillus terreus* MTCC 7600 on: (A) Wheat Bran (B) Orange Peel (C) Rice Straw (D) Wheat Bran + Corn Meal (E) Wheat Bran + Soybean Meal (F) Wheat Bran + Mustard Meal

The crude enzyme extract obtained from cellulase from *Aspergillus terreus* MTCC 7600 was used for the qualitative determination of cellulase using Congo red. After incubation of 24 hours; plates were flooded with 0.1% Congo red dye followed by 1 M NaCl. Yellow zones were obtained against dark background after 24 hour. Soybean meal showed maximum diameter of zone of decolorization (17 mm) followed by corn meal (14 mm), wheat bran (13 mm), mustard meal (9 mm), rice straw (7 mm) and orange peel (6 mm). All substrates showed significant differences from one another. From result it has been observed that all the substrates exhibited cellulase activity.

Table 5: Qualitative determination in terms of zone diameter on crude cellulase extract produced on different agroindustrial residues by *Aspergillus terreus* MTCC 7600

Substrate	***Zone Diameter (mm)**
Wheat Bran	13
Orange Peel	6
Rice Straw	7
Wheat Bran + Corn Meal	14
Wheat Bran + Soybean Meal	17
Wheat Bran + Mustard Meal	9
CD@5%	2.06

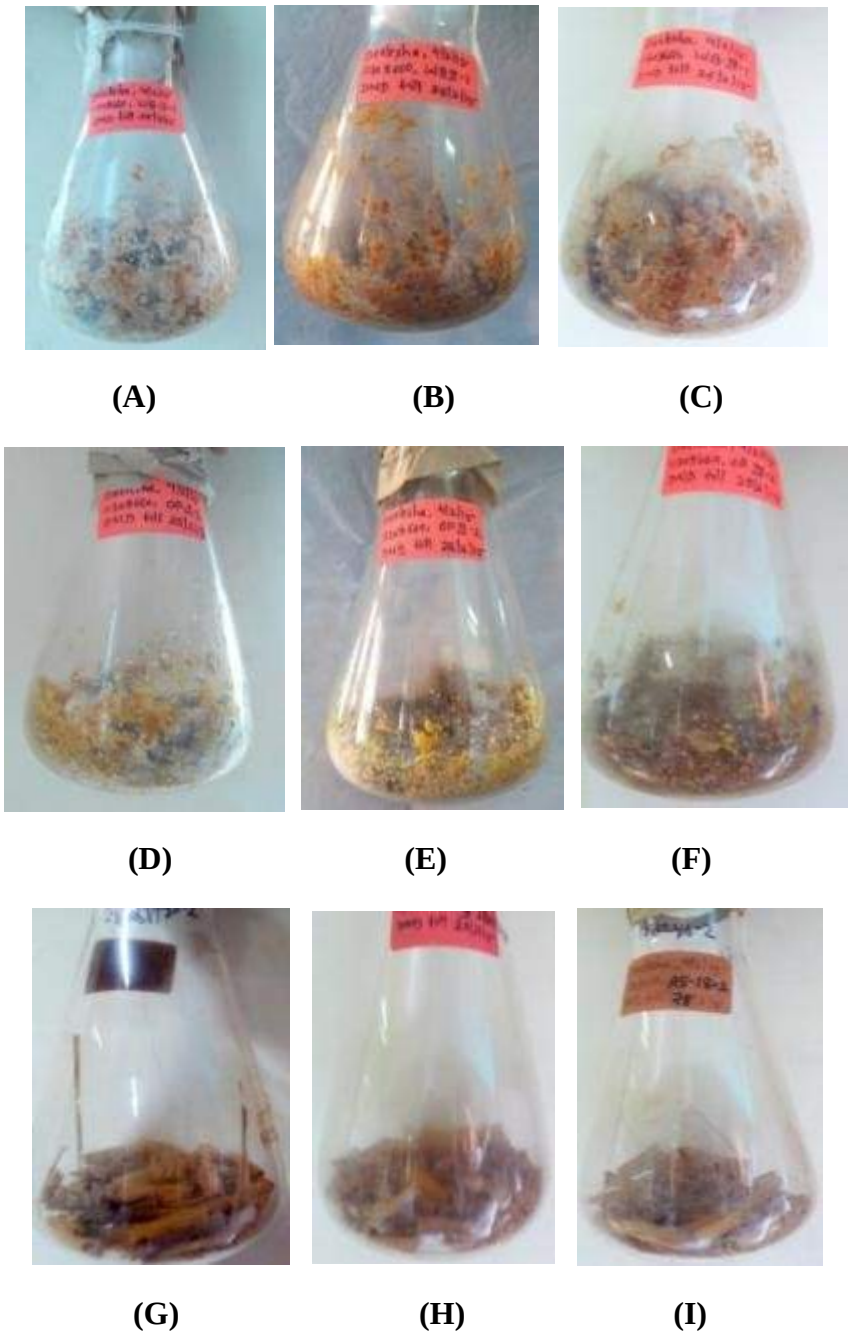
*Average of two replicates

This assay is a well developed procedure for cellulase activity and has been used with variations (Teather and Wood, 1982; Rohrmann and Molitoris, 1992). Zinc chloride can also be used for staining instead of Congo red dye (Sass, 1958).

7.3: Effect of carbon source on cellulase production by *Aspergillus terreus* MTCC 7600:

Aspergillus terreus MTCC 7600 was grown on different agroindustrial residues *viz.*, wheat bran, orange peel and rice straw used as carbon sources for the production of cellulase at 28 ± 2^0C. From plate 3, it has been observed that maximum growth of *Aspergillus terreus* MTCC 7600 was obtained on wheat bran on the 6^{th} day of incubation and less growth was observed on rice straw. Brown color mycelia were observed on different substrates used as carbon sources. After 18 days of incubation,

the substrate seems to become dry which shows less growth of *Aspergillus terreus* MTCC 7600. Yellow color w diffuses with increase in incubation time.



(A) **(B)** **(C)**

(D) **(E)** **(F)**

(G) **(H)** **(I)**

Plate 3: Growth of *Aspergillus terreus* MTCC 7600 on (A) Wheat Bran (Day 6), (B) Wheat Bran (Day 12), (C) Wheat Bran (Day 18), (D) Orange Peel (Day 6), (E) Orange Peel (Day 12), (F) Orange Peel (Day 18), (G) Rice Straw (Day 6), (H) Rice Straw (Day 12), (I) Rice Straw (Day 18).

Aspergillus terreus MTCC 7600 was found to degrade different cellulosic agroindustrial residues in solid state fermentation conditions (Table 2). Out of all, the carbon sources used, wheat bran showed highest cellulase activity. Figure 3 shows the activities of cellulases produced by *Aspergillus terreus* MTCC 7600 growing on different agroindustrial residues that can be used as substrates by solid state fermentation. The enzyme activity was found to be calculated on 3rd day (Appendix 1) , it gave less enzyme activity which increases to maximum day 6. On 6th day it has been observed that wheat bran gave the higher production of cellulase that is 0.26±0.006 IU/mg followed by rice straw (0.21±0.006 IU/mg) and orange peel (0.16±0.006 IU/mg) on the 6th day of incubation. Wheat bran (0.12±0.005IU/mg) showed less enzyme activity on the 18th day of incubation followed by rice straw (0.11±0.006 IU/mg) and orange peel (0.08±0.006 IU/mg). Results indicate that as time increases, cellulolytic activity increases till day 6 and decreases thereafter (6 Days> 12 Days> 18 Days).

Table 3: Effect of agroindustrial residues as carbon source on cellulase production by *Aspergillus terreus* MTCC 7600 by Solid State Fermentation with respect to time

Substrate	6 Days			12 Days			18 Days		
	Enzyme Activity (U/ml)	**Protein Content (mg/ml)**	***Specific Activity (IU/mg)**	**Enzyme Activity (U/ml)**	**Protein Content (mg/ml)**	***Specific Activity (IU/mg)**	**Enzyme Activity (U/ml)**	**Protein Content (mg/ml)**	***Specifc Activity (IU/mg)**
Wheat Bran	26.75±0.45	102.85±0.55	0.26±0.006	19.88±0.092	92.49±0.81	0.22±0.003	11.12±0.27	92.98±2.20	0.12±0.005
Orange Peel	13.42±0.29	84.37±4.86	0.16±0.006	7.55±0.44	65.30±2.19	0.11±0.003	1.92±0.037	23.93±1.36	0.08±0.006
Rice Straw	22.41±0.49	109.58±2.25	0.21±0.006	15.67±0.49	94.99±1.29	0.17±0.003	9.89±0.19	90.43±2.99	0.11±0.006
CD@5%	-	-	0.24	-	-	0.12	-	-	0.19

*Average of two replicates. Temperature of fermentation is 28±2^{o}C at pH 5, Buffer- Sodium Citrate

CD@5%: substrates: 0.009

No of days: 0.009

Substrates X No. of days: 0.02

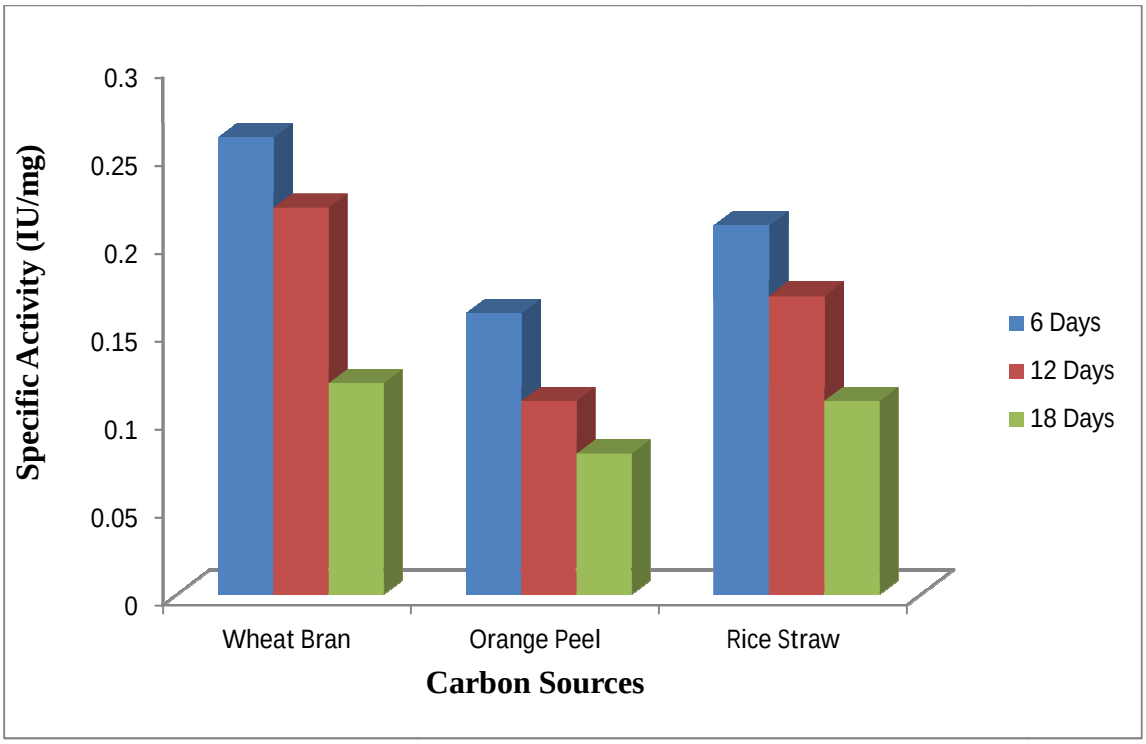


Figure 1: Effect of agroindustrial residues as carbon source on cellulase production by *Aspergillus terreus* MTCC 7600 in Solid State Fermentation with respect to time

It has been observed that wheat bran showed highest enzyme activity (0.26±0.006) on the 6th day of incubation, it may be due to wheat bran having sufficient amount of nutrients and even in the moist state wheat bran was found to remain loose, therefore, it provides a large surface area (Fenikosava *et al*, 1960). Dhillon *et al.*, (1989) obtained the highest enzyme activity after 7 days of incubation time when *Aspergillus terreus* was grown on rice straw, Muarya *et al.*, (2012) showed the highest cellulase activity of 2.29 U/ml at 70% moisture content. Mekala *et al.*, (2008) observed that at high level of moisture (70%) the substrate stops the penetration of oxygen and low level of moisture retards the growth or activity of enzyme. And it was reported by many authors that *Aspergillus spp.* has highest cellulase production (Park and Asqueiri, 1992 and Madamwar, 1997). Production rate was higher in solid state fermentation than in submerged fermentation condition (Da Silva *et al.*, 2005) and incubation time required for the production of enzyme in solid state fermentation was shorter than in submerged fermentation (Jiafa *et al.*, 1993).

7.4: Effect of Supplementation of Nitrogen Sources on Cellulase Production by *Aspergillus terreus* MTCC 7600:

For the production of cellulase, *Aspergillus terreus* MTCC 7600 was grown on different agroindustrial residues used as nitrogen sources such as corn meal, soybean meal and mustard meal were supplemented in wheat bran. Brown color mycelia were observed on different agroindustrial residue used as nitrogen source. Dark brown color and light brown color growth was observed when soybean meal and mustard meal was supplemented in wheat bran, respectively.

(A)

(B)

(C)

(D)

(E)

(F)

(G) **(H)** **(I)**

Plate 4: Growth of *Aspergillus terreus* MTCC 7600 on (A) Corn Meal (Day 6), (B) Corn Meal (Day 12), (C) Corn Meal (Day 18), (D) Soybean Meal (Day 6), (E) Soybean Meal (Day 12), (F) Soybean Meal (Day 18), (G) Mustard Meal (Day 6), (H) Mustard Meal (Day 12), (I) Mustard Meal (Day 18).

In solid state fermentation, substrates will acts as carrier as well as carbon and nitrogen sources for growth of fungus. *Aspergillus terreus* MTCC 7600 was found to degrade different agroindustrial residues used as nitrogen source under solid state fermentation (Table 3). Different agroindustrial residues (corn meal, soybean meal and mustard meal) as nitrogen source were supplemented in wheat bran for the production of cellulase in solid state fermentation, of which soybean meal showed highest activity of cellulase (1.22±0.006 IU/mg) on the 6^{th} day of incubation followed by corn meal (0.94±0.003 IU/mg) and mustard meal (0.78±0.006 IU/mg). Figure 4 shows that on the 18^{th} day, substrates gave less enzyme activity. On the 18^{th} day soybean meal gave highest enzyme activity as compared to corn meal and mustard meal (Table 3). This indicates that as the time increases, enzyme activity decreases (6^{th} Day >12^{th} Day > 18^{th} Day). Mustard meal showed least enzyme activity of 0.22±0.003 IU/mg on the 18^{th} day. This shows significant differences from one another.

Table 4: Effect of supplementation of nitrogen source in wheat bran on cellulase production by *Aspergillus terreus* MTCC 7600by solid state fermentation with respect to time

Substrate	**6 Days**			**12 Days**			**18 Days**		
	Enzyme Activity (U/ml)	**Protein Content (mg/ml)**	***Specific Activity (IU/mg)**	**Enzyme Activity (U/ml)**	**Protein Content (mg/ml)**	***Specific Activity (IU/mg)**	**Enzyme Activity (U/ml)**	**Protein Content (mg/ml)**	***Specifc Activity (IU/mg)**
Corn Meal	38.45±0.015	41.16±0.142	0.94±0.003	26.14±0.009	32.24±0.267	0.72±0.001	20.16±0.009	42.47±0.791	0.48±0.008
Soybean Meal	42.50±0.012	34.84±0.155	1.22±0.006	29.13±0.095	27.22±0.006	1.07±0.003	24.31±0.046	37.75±0.918	0.64±0.003
Mustard Meal	31.15±0.012	39.93±0.280	0.78±0.006	24.41±0.075	43.21±0.089	0.57±0.003	11.31±0.012	52.63±0.652	0.22±0.033
CD@5%	-	-	0.17	-	-	0.11	-	-	0.35

*Average of two replicates, Temperature of fermentation- 28^{o}C, pH 5, Buffer- Sodium Citrate

CD@5% substrates: 0.01

No. of days: 0.01

Substrates X No. of Days: 0.

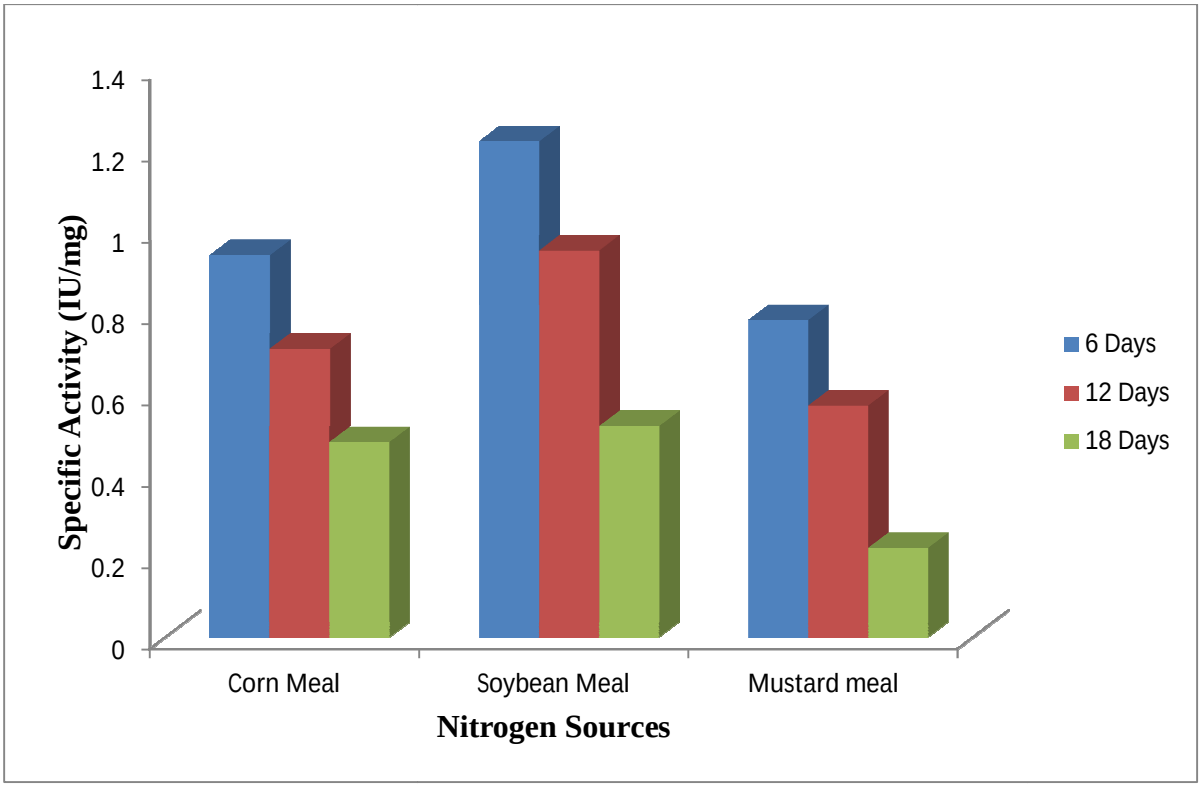


Figure 2: Effect of supplementation of nitrogen source in wheat bran on cellulase production by *Aspergillus terreus* MTCC 7600

The cellulase production was significantly affected by different agro industrial residues used as nitrogen sources. According to Xiao *et al.*, (2007) soybean meal produces more cellulase than sucrose and yeast extract. Addition of organic nitrogen source resulted in increased growth rate and production of enzyme (Sun *et al.*, 1999). According to Mrudula *et al.*, (2011) maximum cellulase activity was observed when peptone was supplemented as nitrogen source in wheat bran (4% w/v). Similar results were obtained by Enari *et al.*, (1977) who reported that maximum cellulase was produced when peptone used as nitrogen source in solid state fermentation. Thakur *et al.*, (2015) uses soybean meal for the production of protease by *Aspergillus oryzae*,

7.5: Study of factors affecting enzyme activity:

7.5.1: Effect of Temperature on the cellulase activity:

For the determination of optimum temperature for cellulase activity, the cellulase activity was estimated by performing enzyme assay at various temperatures ranges from 30^0C to 70^0C. The optimum temperature of *Aspergillus terreus* MTCC 7600 cellulase activity was observed at 40^0C (92.2 %). The enzyme retained about 88% activity at 50^0C which fell at70^0C to 56.4% (Figure 5). This shows significant differences from one another. Thus, increase in temperature resulted in decrease in cellulase activity (Table 5).

Table 6: Effect of temperature on cellulase activity by *Aspergillus terreus* MTCC 7600:

Temperature (^{0}C)	***Enzyme Activity (%)**
40	92.2
50	88.7
60	69.6
70	56.4
80	48
CD@5%	1.53

***Average of two replicates**

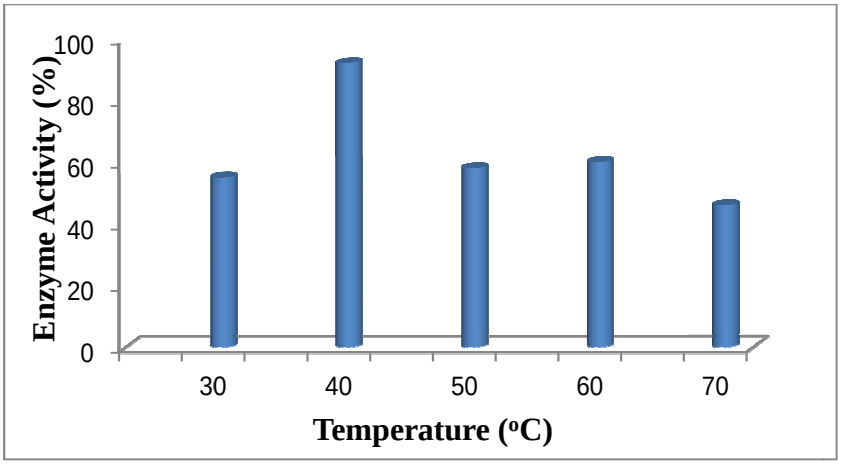


Figure 3: Effect of temperature on cellulase activity by *Aspergillus terreus* MTCC 7600

According to Coral *et al.,* (2002), optimum temperature was estimated to be around 40^0C. Alva *et al.,* (2007) also recorded highest cellulase activity at 30^0C. These values are lower than the commercial cellulase production (Deerland Cellulase 4000). It is an enzyme produced from *Aspergillus niger* and shows highest cellulase activity with β glucosidase and hemicellulase. The temperature optimum for this cellulase was found to be 60^0C (Demerdash, 1992).

Since the enzyme produced from *Aspergillus terreus* MTCC 7600 retained about 88% of activity at 50^0C, so it can be exploited for industrial processes which take place at elevated temperatures.

7.5.2: Effect of pH on the cellulase activity:

For the determination of optimum pH of cellulase, cellulase activity was estimated in three buffered solutions *viz.,* Sodium Acetate (pH 3 to 4.5), Sodium Citrate (pH 5 to 5.5) and Sodium Phosphate Buffer (pH 6 to 7). It was observed that value of pH range from 3 to 7 for enzyme production. Figure 6 showed the optimum pH for cellulase activity at pH 5 (82.3%). 70.2 %, 79.1% and .7% activity retained at pH 4, 6 and 7 respectively. Less activity (38.4%) was observed at pH 3 (Figure 6). This confirms that enzyme would be activated at acidic pH and was useful for cellulase activity. It has been observed that it shows significant differences.

Table 7: The effect of pH on cellulase activity of *Aspergillus terreus* MTCC 7600:

pH	***Enzyme Activity (%)**
3	38.4
4	70.2
5	82.3
6	79.1
7	78.7
CD@5%	0.91

***Average of two replicates.

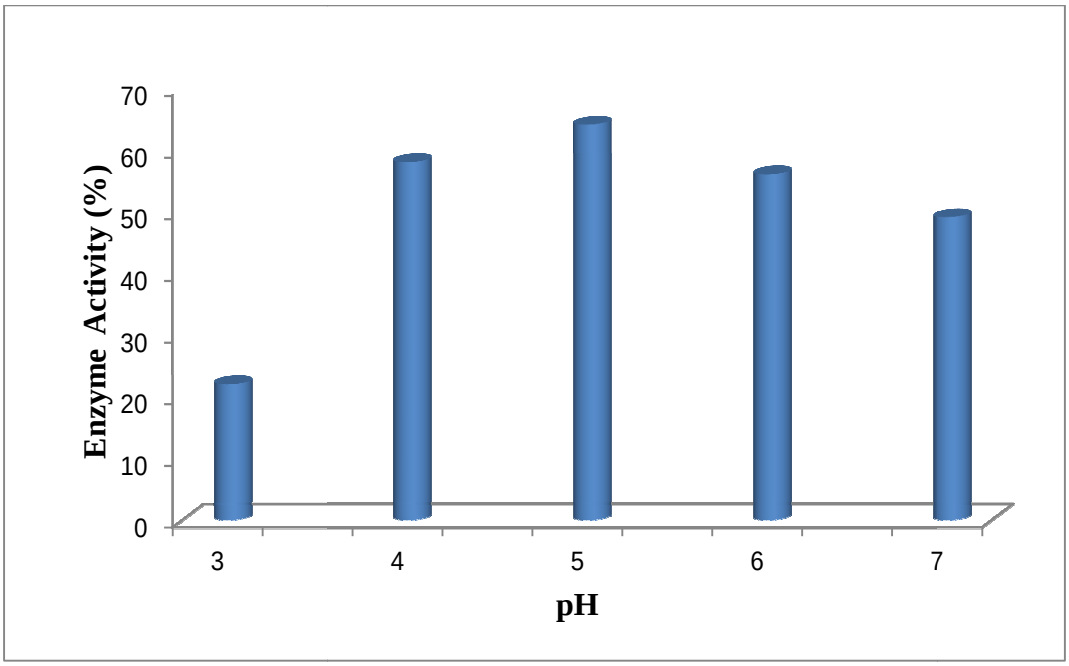


Figure 4: Effect of pH on cellulase activity of *Aspergillus terreus* MTCC 7600

According to Osama *et al.,* (2012) the maximum activity of cellulase from *Aspergillus terreus* DSM 826 was produced at pH 4.5 and 5 when rice straw and sugarcane baggase were used as source of carbon. Similar results were obtained by Coral *et al.,* (2002) who found that optimum pH for activity of cellulase enzyme between 4 and 4.5. Many of the fungal cultures favour acidic pH for the growth and biosynthesis of enzymes (Haltrich *et al.,* 1996). Optimal pH varies from species to species for fungal cellulases, therefore, in most cases the optimum pH ranges from 3 to 6 (Niranjane *et al.,* 2007).

7.5.3: Heat stability of cellulase:

The heat stability of the enzyme was observed by heating the enzyme at various temperatures ranges from 40^0C to 90^0C for 15 minutes. The results obtained are outlined in Table 8, 39.2% of the original activity was retained for 15 minutes at 90^0C and 93.5% of enzyme activity was retained at 40^0C (Figure 7). Studies on heat stability indicate that enzyme was stable upto 50^0C. This shows significant differences from one another. However, as temperature increases from 50^0C to 60^0C till 90^0C, the activities rapidly decreased.

Table 8: Heat stability of cellulase by *Aspergillus terreus* MTCC 7600:

Temperature	Enzyme Activity (%)
40	93.5
50	86.7
60	68.6
70	56.5
80	48.8
90	39.2
CD@5%	1.4

*Average of two replicates.

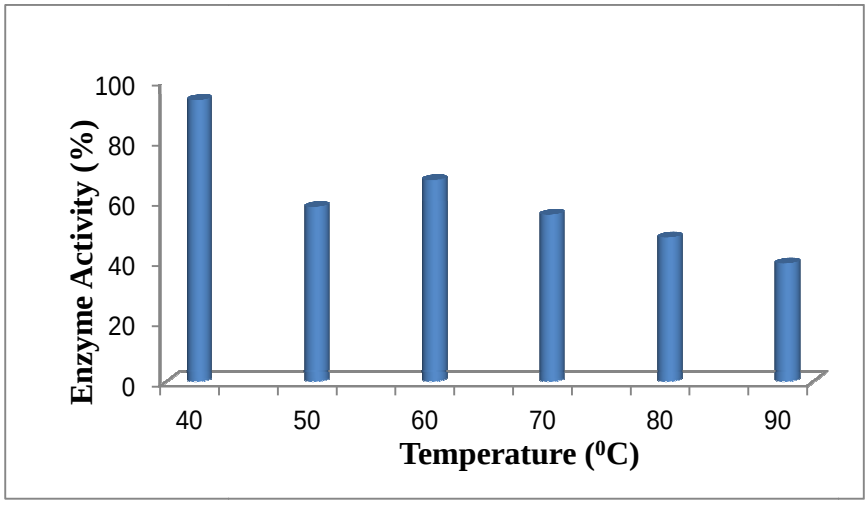


Figure 5: Heat stability of cellulase of *Aspergillus terreus* MTCC 7600

Result indicates that at low temperature enzyme activity was high and high temperature shows less activity because at high temperature, denaturation of enzymes occurred. With increase in temperature enzymes starts to inactivate. According to Betini JHA *et al.*, (2009) the enzyme produced by *Aspergillus oryzae* was stable upto 50^{o}C for more than 1 hour and at 60^{o}C it was found that the enzyme activities get diminished very rapidly.

CHAPTER 8

CONCLUSION AND FUTURE SCOPE

On the basis of the present study it was concluded that *Aspergillus terreus* have the ability to degrade agroindustrial residues. Fungal strains used for the production of enzyme has many benefits such as the extracellular enzymes are produced, making easier for the process of extraction. Dinitrosalicylic acid (DNS) method was used for the estimation of reducing sugars. The present study was aimed for studying the effect of carbon and nitrogen source for the production of cellulase by *Aspegillus terreus* MTCC 7600 using agroindustrial residues under solid state fermentation. Different agroindustrial residues such as wheat bran, orange peel, rice straw, soybean meal, corn meal and mustard meal as carbon and nitrogen source were used for the production of cellulase. Highest enzyme activity was observed on 6^{th} day which decreased with increase in incubation time. Wheat bran (0.26±0.006 U/mg) as carbon source gave maximum activity of enzyme on the 6^{th} day of incubation and soybean meal supplemented as nitrogen source in wheat bran showed highest activity of enzyme (1.22±0.006) on the 6^{th} day of incubation. In both cases, increase in incubation time results in decreased enzyme activity (6^{th} day> 12^{th} day> 18^{th} day). Enzymes are very sensitive to temperature and pH. Therefore, the selection of optimum temperature and pH is necessary for the production of cellulase. In this study, the effect of pH and temperature on cellulase produced by *Aspergillus terreus* MTCC 7600 was studied. The optimum temperature and pH for cellulase was found to be 40^0C and 5 respectively. Further increase in temperature results in decreasing enzyme activity. The enzyme was found to be stable upto 90^0C at which 39.2% of total activity was retained. Maximum activity was retained at 40^0C.

It can be concluded that *Aspergillus terreus* MTCC 7600 can be industrially utilize for the synthesis of cellulase and studies for the strain improvement can be carried out to increase enzyme production. Using inexpensive carbon and nitrogen sources are the great advantages from the practical and economical point of view for various industries including textile, biofuel production, brewing, food and feed industry, pulp and paper and agriculture.

CHAPTER-9

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CHAPTER 10

APPENDIX

Appendix 1: Effect of carbon sources on cellulase production by *Aspergillus terreus* MTCC 7600 on 3rd day:

Substrate	***Specific Activity (IU/mg)**
Wheat Bran	0.18±0.006
Orange Peel	0.08±0.006
Rice Straw	0.15±0.011

*****Average of two replicates.