ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Microbiology Stream

Production and Optimization of cellulase enzyme under Submerged and Solid State Fermentation from *Trichoderma* Isolates

By

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A Thesis submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfilments of the Requirements for the Degree of Master of Science in Microbiology

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October, 2011

Acknowledgments

First, I would like to express my heartfelt gratitude to my instructor and research advisor Dr. Tesfaye Alemu for his consistence and constructive guidance, critical and continuous evaluation over the year, during the course of this project.

I would like to thank Addis Ababa University, for providing me financial and technical support. I would like to thank Hawassa University (WGCFNR) for giving me the opportunity for the study.

My thanks goes to Ato Dawit Tewolde (MSc.), Melaku Zemedkun (PhD student in AAU), Molisa Jida (PhD student in AAU) for their valuable suggestions. I would like to thank Mss Hymanot Abebe (MSc.), Researcher in AAU, for her providing me wheat bran and cotton seed for solid state fermentation.

My sincere thanks is also forwarded to Zenebech Aytenew, Nigat Mekonnen, Brtukan Tamerea, Tigist Mengesha, Tirunesh Shiferaw and Beletu Mekonen, for their technical support during my laboratory work.

Finally, I would like to thank my family, for their consistent encouragement and help.

Table of contents page

List of figures **page**

List of tables **Page**

Abbreviations

µl= Microliter

BB= Barley bran

CBD= Cellulose binding domain

CD=Catalytic domain

CM= Cotton seed

CMCase= Carboxymethyl cellulase

CP= Coffee pulp

Da=Dalton

DNS= Dinitrosalicylic acid

EC= Enzyme Commission

Fig= Figure

M= molar

Min= minutes

ml= milletter

mM= Milli molar

OD= Optical Density

OPEC= Organization of Petroleum Exporting Countries

RA=Relative activity

rpm= Revolution per minute

RS= Rice bran

RT=Room temperature

SmF= Submerged state fermentation

SSF= Solid state fermentation

WB=Wheat bran

WS= Wheat straw

PGE= Polyethylene glycol

Abstract

Pure cellulose and agricultural wastes were used as sole carbon sources for the production of cellulase enzymes by *Trichoderma* isolates in both SmF and SSF, respectively. These isolates (seven) were obtained from Mycology laboratory, Faculty of Life science, Addis Ababa University. Carboxymethyl cellulose (CMC) and Congo red were used to screen four isolates for their ability to produce cellulase enzymes. Cellulase production was assayed by measuring the amount of glucose released in μmol/ml/min by using the DNS assay method at 540nm. To maximize the cellulase production, the critical parameters like carbon source, nitrogen source, cellulose concentration, temperature and pH were optimized under SmF. The highest cellulase enzyme activity was observed after 12 days of incubation on media containing, yeast extract (1%), cellulose concentration (1%) and pH (5.5) from all isolates under SmF whereas under SSF maximum cellulase enzyme was recorded between 5-11 days incubation. WB, RB and WS were found to suitable for high cellulase production whereas CS, CP and BB relatively showed the least cellulase production in SSF. *T*richoderma isolates AUT1 produce the highest CMCase on WS (5.68U/g), AUT5 on rice bran (8.15U/g), AUT2 and AUT4 on wheat bran, their enzymatic activities were 4.92U/g and 7.01U/g, respectively. Enzyme synthesis was repressed in the presence of glucose and fructose while it was induced in the presence of maltose and lactose in SmF. The maximum amount of cellulase was observed between 55% to 65% moisture contents. It is evident from the present study, solid state fermentation was better than liquid state fermentation for the production of cellulase by *Trichoderma* isolates using agricultural wastes. The cellulase enzymes produced by *Trichoderma* isolates were active at temperature ranges of 40-60^{o}C and pH values 4.5-6.5.

Key words:- *Agricultural wastes, Carboxymethyl cellulase, Cellulase activity, DNS methods and Congo red*

1. Introduction

Cellulose is the most abundant biopolymer in nature and constitutes a large pool of carbon source for the microorganisms responsible for the decomposition of organic matter in soil (Shankar *et al.,* 2011). Cellulose together with hemicelluloses, pectin and lignin are mostly found in the cell wall of green plants and are important components for agricultural wastes. These agricultural wastes are disposed on the earth improperly and cause environmental pollutions (Brijwani *et al.,* 2010).

Enzymes which catalyze the hydrolysis of cellulose into disaccharides, glucose unites, simpler sugar, are generally known as cellulases or cellulolytic enzymes. Cellulase is a family of O-glycoside enzymes that hyrolyse β-1,4-glycosidic bonds of native cellulose and other related cello-oligosaccharide derivatives. Cellulases are among the most extensively studied enzymes. They are distributed throughout the biosphere such as plants, animals and microorganisms. Cellulases are chiefly produced by microorganisms and they are distributed throughout the world (Chinedu and Okochi, 2003).

Cellulolytic enzymes are well known for their biotechnological potential in various industries including food industries, animal feed industries, brewing and wine making, agriculture biomass refining, pulp and paper industries, textile and laundry industries and ethanol production (Bhat, 2000). However, the cost of cellulase production and optimization profoundly influences the economics of the entire production process. Currently, these enzymes account for approximately 20% of world enzyme market. Commercially speaking, the main production organisms are strains of *Trichoderma reese*i (Murphy and Horgan, 2005).

Trichoderma are filamentous fungi belonging to a group of largely asexually reproducing soil fungi. They are very effective soil and rhizosphere colonizers with high biodegradation potential (Chet and Baker, 1981 and Kubicek, 2004). They are thought to be cellulase producers, and their crude enzymes are commercially available. *Trichoderma* have received widespread industrial interest because of their ability to produce extracellular lignocellulose-degrading hydrolyses in large amounts (Panddy *et al*., 1994; Panddy, 1992 and Chet and Inbar, 1994). *Trichoderma* sp. have been known to produce cellulases for a long time and many of the enzymes have been characterised: the endo-cellulase of *Trichoderma is* possibly the most studied enzyme (Wiley, 1981).

Enzyme based cellulose processing industries have considerable economic significance through upgrading agricultural products to qualities of demand (Wiley, 1981). In Ethiopia, attempts were made to study the potential of cellulolytic fungi for different biotechnological activities. Fassil (1983) isolated and characterized aerobic cellulose degrading microorganisms from different habitats, decomposing plant matter, arable soil, garden soil and manure. It has been found that *A. niger*, *cheatomium* sp., *Curvularia* sp., *Helminthosporium* sp., *Rhizopus* sp., *Starchybotrys* sp., *Trichoderma* sp., and *Zyorrhynchus* sp., were the most abundant cellulase producing microorganisms. The effect of teff straw or corn stalk on cellulase and biomass production by *Trichoderma* spp. BDCC-1, *Pencillium* spp. BDCC-1 and *Cladosporium* spp. BDCC-3 were studied and compared with those of cellulosic substrates (Ameha, 1994). Berhanu, (1990) studied the production conditions, release and activity of cellulases and β-glucosidase from *Cladosorium* spp. BDCC-3. In order to fill the existing gaps of research, therefore, experiments were designed to examine the cellulase from *Trichoderma* spp. on both submerged and solid state fermentation using agricultural wastes.

Enzymes could be produced by submerged state fermentation (SmF) and solid-state fermentation (SSF) (Fig. 2). SmF involves the production of enzymes by microorganisms in a liquid nutrient media whereas SSF is a fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients in absences of free flowing liquid. Generally, SSF holds tremendous potential for the production of enzymes (Pandey, 1992).

Agro-industrial wastes are very important sources for the preparation of SSF. However, naturally agro-industrial wastes are heterogeneous in composition which affects the decomposition of wastes by microorganisms. In addition to this, many factors like temperature, pH, and moisture content are very important for microbial growth and enzyme production during SSF process. Therefore, it is a prerequisite to design a set of optimal process operating conditions to achieve high enzyme production (Pandey, 1992) .

2. Objectives

2.1. General objective

- Production and optimization of cellulase enzyme from *Trichoderma* isolates under Submerged and solid state fermentation

2.2. Specific objectives

- To optimize cultivation conditions for maximum cellulase enzyme production,
- To evaluate the effect of different carbon and nitrogen sources on the production of cellulase enzymes,
- To characterize the crude enzymes and determine optimum working conditions,
- To screen and select agro-industrial wastes for cellulase production,

3. Literature Review

3.1. Cellulases

The group of hydrolytic enzymes implicated in the bioconversion of celluloses are known as cellulases or cellulase-systems. The cellulase-system comprises three major highly specific enzymes namely; the endo-glucanases, the exo-glucanases and β-glucosidases. These enzymes are non-constitutive and are produced by many microorganisms such as bacteria, actinomycetes and fungi. The cellulase-systems of fungal origin are the most abundant and widely studied. Among the fungi, *Trichoderma*, a softwood rotting fungus, is the most potent cellulase-system producer (Sukumaran *et al.*, 2005).

Based on the amino acid sequence and possibly secondary structure predicted by hydrophobic cluster analysis, cellulases are classified in to glycoside hydrolase (GH) families (Henrissat, 1991). The enzyme cellulase, a multi enzyme complex made up of several proteins, catalyses the conversion of cellulose to glucose in an enzymatic hydrolysis (Zahri *et al.*, 2005; Aneja, 2005).

Cellulases are extracellular enzymes which released in to the growing media. However, β-glucosidase occurred into three places: cell membrane, intracellular and extracellularly. All cellulytic enzymes share the same chemical specificity for β-1,4-glycosidic bonds ,which they cleave by a general acid catalyzed hydrolysis. Most cellulolytic hydrolyses are proteins of 30-40 KDa molecular mass with acidic optima between 2.5 and 4.5 (Baldrian, 2008).

Cellulases are inducible enzymes and their syntheses is strongly repressed by soluble sugars or other easily metabolizeble substrates. Therefore, cellulose and Sophorose are natural inducers whereas glucose is catabolite represser (Zahri *et al.*, 2005; Aneja, 2005). However, the mechanism of induction by cellulose is still not clear because being large insoluble macromolecules, cellulose cannot go through the plasma membrane directly (Zahri *et al.*, 2005; Aneja, 2005). Some other inducers are also known as lactose and dilute acid-treated hydrolysate.

A study conducted by Ul-Huque (1992) selection-screening techniques have resulted in the production of catabolite repression resistant mutant strains and at the same time hyper production of either one or all the component enzymes in the cellulase complex. The

cellulase enzymes derived from a thermophilic anaerobic *Clostridium thermicellum* has been reported to resistant to end product inhibition and repression.

Enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulase enzymes on to the surface of the cellulose, the biodegradation of cellulose to fermentable sugars and the desorption of cellulase (Zhang *et al*., 2006). Enzymatic hydrolyses of cellulose is preferred for industrial purposes due to the high yields of desired hydrolytic products with minimal by-products, environmentally safe and cost effective. Effective utilization of cellulosic material through bioprocesses will be an important key to overcome the shortage of foods, feed and fuels, and reduction of waste materials, which the world may face in the near future, because of the explosive increase in human population and industrialization.

3.2 Microbial Sources of Cellulases

3.2.1 Fungi

Cellulases are produced in nature by various terrestrial and marine organisms (Szakacs *et al*., 2006). They are distributed throughout the world, such as plants, animals and microorganisms. Cellulase enzymes produced chiefly by microbial sources, like bacteria, actinomycetes, filamentous fungi and protozoans, which catalyze the cellulose. The most important sources for industrial cellulases production are filamentous fungi, found in the soil, plants, found in the marine environments (Bhat, 2000).

Although microorganisms capable of complete degradation of native cellulose are widespread in the soil environment, this ability appears to be confined to small number of species, which are predominantly fungi. The potential cellulase producing fungal genera include *Bulgaria, Chaetomium and Trichoderma* (Ascomycetes), *Coriolus, Phaenerochaete, Coriolus, Schizophyllum* (Basidiomycetes), *Aspergillus, Geotrichum and Penicillium* (Deuteromycetes) (Lynd *et al*., 2002).

Cellulolytic microbes are primary carbohydrate degraders and are generally unable to use proteins and lipids as energy sources for growth. Thus, a large number of bacteria, actinomycetes, and filamentous fungi produce cellulase to degrade cellulose (Sukumaran *et al*, 2005). The ability to secret large amounts of extracellular protein is characteristic of certain fungi and such strains are most suited for production of higher level of extracellular cellulases (Sukumaran *et al*, 2005).

A large number of fungi produce extracellular cellulases. The ability to fully solubilise the crystalline cellulose is restricted to relatively few of these (Gow and Gadd, 1996). These organisms grow under suitable condition to produce cellulase, *T. viride* and *T. reesei* produce active cellulase when grown in solid and submerged culture, *Asperigillus niger* produces highly active cellulase when grown in liquid media by both surface and submerged culture methods. *Pencillium*, capable of dissolving cotton completely, *Stachy, Botsysatra, Pcsaotia, Palmarum, Merulius, Lnerymans, Polyspores, Neurospora* have been reported to produce cellulase.

***Trichoderma*: Diversity and Ecology**

Trichoderma are filamentous fungi commonly found in the soil community that are facultative saprophytes. The genus *Trichoderma* currently consists of more than 89 species, which are usually cosmopolitan and typically soil borne or wood decaying anamorph used widely as a bioconversion of lingocellulolytic wastes in to value added products such as enzymes, amino acids, organic acids (Kubicek, 2004). *T. reesei* as a producer of industrially used cellulases, *T. aggressivium* as a competitor of the commercial mushroom *Agaricus bisporus*. *Trichoderm* species as toxin producing indoor moulds and some strains even acting as human pathogens have been widely studied and classified under Kingdom: Fungi, Phylum: Ascomycota, Class: *Euascomycetes*, Order: *Hypocreales*, Family: *Hypocreaceae* and Genus: *Trichoderma* (indonesiaforest.webs.com).

Trichoderma are ubiquitous fungus that is widely distributed in the soil, plant material, decaying vegetation, and wood. Although it is commonly considered as a contaminant. *Trichoderma* are highly successful colonizers of their habitats, which is reflected both by their efficient utilization of the substrate and high secretion capacity of hydrolytic enzymes (Papavizas, 1985). They are commonly found on cellulosic materials and can thus often be found wherever decaying plant material is available as well as in the rhizosphere of plants where they can induce systemic resistance against pathogens and use a wide range of compounds as sole carbon and nitrogen sources (Papavizas, 1985).

The genus *Trichoderma* has 89 species. The most commonly studied spp. are: *T. harzianum*, *T. koningii*, *T. longibrachiatum*, *T. reesei*, *T. pseudokoningii*, and *T. viride*. Morphological features of the conidia and phialides help in differentiation of these species from each other. *T. reesei*, a mitosporic green mould, was recognized during the world war II based on a single

isolate from the Solomon Islands and since then used in industry for production of cellulases. It is believed to be an anamorph (asexual stage) of the common pan tropical ascomycete (Hypocrea) (Druzhinina *et al.*,2010).

Colonies of *Trichoderma* grow rapidly and mature in 5 days. At 25°C and on potato dextrose agar, the colonies are woolly and become compact in time. From the front, the colour is white. As the conidia are formed, scattered blue-green or yellow-green patches become visible. These patches may sometimes form concentric rings. They are more readily visible on potato dextrose agar compared to Sabouraud dextrose agar. Reverse is pale, tan, or yellowish (indonesiaforest.webs.com).

Septate hyaline hyphae, conidiophores, phialides, and conidia are observed. *Trichoderma longibrachiatum* and *Trichoderma viride* may also produce chlamydospores. Conidiophores are hyaline, branched, and may occasionally display a pyramidal arrangement. Phialides are hyaline, flask-shaped, and inflated at the base. They are attached to the conidiophores at right angles. The phialides may be solitary or arranged in clusters. Conidia are one-celled and round or ellipsoidal in shape. They are smooth- or rough-walled and grouped in sticky heads at the tips of the phialides. These clusters frequently get disrupted during routine slide preparation procedure for microscopic examination. The colour of the conidia is mostly green (indonesiaforest.webs.com).

Trichoderma spp. are known as producer of many enzymes. These enzymes include: chitinases, proteases, glucanases, cellulases and xylanase. Among these cellulases are extensively studied and involved in several industrial applications and commonly found in the cellulolytic materials. *T. harzianum* and *T. reesei* are the potential cellulase producing fungi and the most frequently used species for the production of cellulases for complete hydrolysis of cellulosic substrates into its monomeric glucose, which is a fermentable sugar important for ethanol production (Zahri *et al.*, 2005; Aneja, 2005). *Trichoderma* spp. involved in several applications some of these are described in the next session. .

Bio-control: *Trichoderma* is a potent bio-control agent and used extensively for post-harvest disease control. It has been used successfully against various plant pathogenic fungi belonging to various genera, viz. *Fusarium*, *Phytophthora*, *Sclerotium*. The mechanisms are antibiotics production, myco-parasitism, nutrient competition and hydrolytic enzyme production (Papavizas , 1985).

Plant Growth Promoter: *Trichoderma* strains solubilise phosphates and micronutrients. The application of *Trichoderma* strains with plants such as grasses increases the number of deep roots, thereby increasing the plant's ability to resist drought (Papavizas , 1985).

Trichoderma spp. is important participants in the nutrient cycle. They aid in the decomposition of organic matter and make available to the plant many elements normally inaccessible. Yedidia *et al*. (2001) noted that the presence of the fungus increased the uptake and concentration of a variety of nutrients (copper, phosphorus, iron, manganese and sodium) in the roots of plants grown in a liquid medium. These increased concentrations indicated an improvement in plant active-uptake mechanisms.

Biochemical Elicitors of Disease Resistance: *Trichoderma* strains are known to induce resistance in plants. Three classes of compounds that are produced by *Trichoderma* and induce resistance in plants are now known. These compounds induce ethylene production, hypersensitive responses and other defence related reactions in plant cultivates (Papavizas , 1985).

Transgenic Plants: Introduction of endo-chitinase gene from *Trichoderma* into plants such as tobacco and potato plants has increased their resistance to fungal growth. Selected transgenic lines are highly tolerant to foliar pathogens such as *Alternaria alternata, A. solani*, and *Botrytis cinerea* as well as to the soil-borne pathogen, *Rhizectonia* spp. (Papavizas , 1985).

Bioremediation: *Trichoderma* strains play an important role in the bioremediation of soil that are contaminated with pesticides and herbicides. They have the ability to degrade a wide range of insecticides: organochlorines, organophosphates and carbonates (Papavizas , 1985).

Bio-conversion: Bioconversion of lignocellulosic wastes could make a significant contribution to the production of organic chemicals. They serve as the production of bio-fuel, enzymes, other high-value added bio-products (Howard *et al.*, 2003). A diverse spectrum of lignocellulolytic microorganisms, mainly fungi and bacteria have been isolated and identified over the years and this list still continues to grow rapidly. Already by 1976 an impressive collection of more than 14,000 fungi which were active against cellulose and other insoluble fibres were collected. Despite the impressive collection of lignocellulolytic microorganisms only a few have been studied extensively and mostly *T. reesei* and its mutants are widely

employed for the commercial production of hemicellulases and cellulases . *T. reesei* might be a good producer of hemi-cellulolytic and cellulolytic enzymes but is unable to degrade lignin (Howard *et al*.,2003).

Much research efforts have been done on lowering the cost of enzymes. The approaches which have been considered in lowering enzyme cost falls into three groups: (1) Screening for organisms with novel enzymes; (2) Strain improvement of existing industrial organisms and enzyme engineering (Vu *et al*., 2011), (3) production and operations related factors such as choice of substrate, culturing conditions, recycling of enzymes and redesigning of processes (Howard *et al*., 2003).

Moosavi-Nasab and Majdi-Nasab (2007) produced cellulase by growing *T. reesei* on sugar beet pulp as a substrate. They showed that cellulose in sugar beet pulp has a potential to be used as a substrate to produce cellulase. The effect of cellulose concentration on cellulase production was investigated; an increase in cellulose concentration causes a decrease in cellulase activity. The maximum cellulase production was obtained after 4-6 days of cultivation. They also compared the production of cellulase in the medium containing sugar beet pulp or pure crystalline cellulose. The pure crystalline cellulose resulted in a lower cellulase than the sugar beet pulp.

(Vyas and Vyas, 2005) studied the production of cellulase by co-culturing *T. viride, Aspergillus terreus* and *A. nidulans*. The combination of *T. viride* and *A. terreus* showed higher cellulase activity as compared to other two combinations. The advantage of mixed culture is more pronounced in SSF condition because the colonization of substrate may be accomplished better in symbiotic association i.e. each species having its own niche for growth and substrate degradation.

Andade and Marbach (2003) investigated cellulase production by *Trichoderma* spp. using agro-industrial by-products as substrates and observed the influence of the concentration of agricultural wastes on cellulase production. Wheat bran and peptone were the best sources of carbon and nitrogen for the production of cellulase, respectively. Generally, high cellulase enzymes were achieved at high concentration of carbon sources and low amount of nitrogen sources.

Juhasz *et al*. (2005), examined the production of cellulases and hemicellulases from *T. reesei* on various carbon sources: on a cellulose substrate (Solka Floc), on pre-treated corn stover, on pre-treated hardwood and softwood, i.e. willow and spruce, respectively. The pre-treated corn stover showed higher cellulolytic activities than on Solka Floc, while the activities obtained on steam pre-treated spruce and willow were considerably lower.

Higher cellulase activities could be obtained by pre-treatments of the cellulosic materials to reduce the association of cellulose with other plant cell-wall polymers. Bio-conversion of cellulosic materials into useful, higher value products normally require multi-step processes which include: 1) Pre-treatment (Mechanical, Chemical and Biological), 2) Hydrolysis of the polymers to produce readily metabolizable molecules (Hexose and pentose sugars), 3) Bio-utilization of these molecules to support microbial growth or to produce chemical products and 4) the separation and purification (Pandey, 1992).

Malik *et al*. (2010) studied the effect of untreated and pre-treated sugarcane bagasse with different physical and chemical agents for the production of cellulolyic enzymes by *T. viride.* Among all treatments, steam pre-treatment at 200ºC gave the maximum cellulases yield than (CMCase 1.57 U/ml/min, FPase 0.921 U/ml/min) untreated bagasse (CMCase 0.084 U/ml/min, FPase 0.0138 U/ml/min).

Pothiraj *et al*. (2006) produced cellulase enzymes from cassava wastes using different fungal cultures such as *Rhizopus stolonifer*, *Aspergillus niger* and *Aspergillus terreus*. *R. stolonifer* was the most efficient in bio-converting cassava waste into fungal protein (9%) compared to *A. niger* and *A. terreus*. The highest cellulase activity was observed on the 10^{th} day in *R. stolonifer* mediated fermentation.

Baig, (2005) produced cellulase by growing *T. lingorum* on banana agro-wastes comprising pseudo-stem and leaves as a substrate. Banana leaves and soy peptone was found to be the best carbon and nitrogen sources for cellulase production, respectively. The optimum pH and temperature of the medium was found to be 5.6-5.8 and 45^{o}C, respectively.

Hussein *et al*. (2011) isolated 17 fungal isolates from soil and investigated some factors that affect the cellulase production from *Alternaria alternata* and *Aspergillus wentii*. It has been reported that glucose and cellulose were the most effective as a carbon source while, urea was

the best nitrogen source for cellulases production. Initial pH 5.0 and incubation temperatures at 25ºC or 35ºC were achieved high cellulases production.

3.3 Classification of Cellulases

Based on the site of secretion, cellulase can be classified in to two classes: complexed and non-complexed. Complexed cellulases are found in anaerobic bacteria and anaerobic fungi whereas non-complexed cellulase systems are mostly found in filamentous fungi and actinomycete and released in to the surface of the substrate. Organisms that produce non-complexed cellulase systems are most often used in the industrial production of cellulolytic enzymes, because the secreted enzymes can easily be harvested (Goldschmidt, 2008).

According to their mode of action, cellulolytic enzymes fall into one of two main groups, endogluconase or cellobiohydrolase (Gow and Gadd, 1996). The complete degradation of cellulose to glucose requires the action of at least three types of enzymes (Gow and Gadd, 1996): endo-β-1,4 -glucanase, exo-β-1,4-glucanase (cellobiohydrolase) and β-glucosidase (Zahri *et al*., 2005; Aneja, 2005; Miettinen-Oinonen, 2007). The mode of action and their industrial application would be discussed on the next sections.

Endoglucanase

Endoglucanase (EG, EC 3.2.1.4):- CMCase, one of the members of cellulase complex, cleaves the internal glycosidic bonds of cellulosic chains yielding celloligosaccharides and acts synergistically with exo-glucanases and β-D-glucosidases during the solubilisation of cellulosic material (Zhang *et al*., 2006). These enzymes are generally inactive towards crystalline cellulose and cellobiose. One important point to note is that endoglucanases do not directly contribute to the generation of soluble saccharides from insoluble cellulose (Mosier *et al*., 1999; Bhat and Hazlewood, 2003). Four genes, coding for the two endoglucanases(EG I and EG II) and the two cellobiohydrolases (CBH I and CBH II) have been isolated from *T.reesei* (Aneja, 2005).

CMCase has versatile applications; widely used in textile industry and in laundry detergents; used in the pulp and paper industry for various purposes; facilitates fermentation of biomass into bio-fuels and even to treat Phytobezoars, a form of cellulose bezoars found in human stomach. It has also great potential for utilization in the food industry; for coffee processing, extraction and clarification of juices, extraction of oils from the oilseeds and olive plant (Bhat, 2000).

Exo-glucanase

Exo-glucanase (CBHs):- like endoglucanases the CBHs are highly active on amorphous and swollen cellulose, but degrade crystalline and cello-oligosaccharides rather poorly (Bhat and Hazlewood, 2003). CBHs release cellobiose, dimer of glucose, from the terminal ends of cellulose. It typically accounts for 80% of the amount of enzyme cellulases produced in *Trichoderma* fermentation. Recent kinetics studies and high resolution structural data confirmed that there are two classes of CBHs, CBH I and CBH II. CBH I attacks the non-reducing ends of cellulose, and CBH II attacks the reducing ends. Most members of *Trichoderma (T. reesei)* produce two CBHs which release cellobiose from both ends (Aneja, 2005).

β-Glucosidase

β-glucosidase (cellobiase):- β-*glucosidase*, hydrolases cellobiose to glucose, supplying the fungus with an easily-metabolisable carbon source. There are two types of β-glucosidase (BGL I and BGL II). *β*-glucosidase is found in three places, cell membrane, intracellular and extracellular. Many *Aspergillus* members have been reported to contain multiple β-glucosidases with a large variety in molecular mass. β-glucosidase is an important step because cellobiose inhibits the action of many cellulase components. Some β-glucosidase showed activity towards H_3PO_4-swollen CM-celluloses, but most β-glucosidase are inactive towards these and other polymeric substances such as Avicel, filter paper and cotton (Bhat and Hazlewood, 2003).

3.4 Cellulolytic Domains

Most fungal cellulases are organized in to two structurally independent domains; a catalytic core domain (CD), and a cellulose-binding domain (CBD). These two domains are usually interconnected via a short flexible linker (Bhat and Hazlewood, 2003). The catalytic core domain is the part of the cellulase where the hydrolysis of the cellulose chain takes place. This domain is the largest part of the enzyme. It varies greatly in size between different cellulases (Bhat and Hazlewood, 2003).

The cellulose-binding domain is a small wedge-shaped domain consisting of approximately 35 amino acids. The function of the CBD is to bind on the surface of cellulose, and serve as an "anchor" for the enzyme, keeping it strongly adsorbed to the cellulose surface (Wilson and Irwin, 1999). This reduces the need for strong binding of the catalytic domain to the cellulose, and thereby enables the enzyme to have a higher rate of turnover. The two domains in the

enzyme are interconnected via a flexible linker. The linker is usually very rich in threonines, serines, and prolines, and it is heavily glycosylated. The role of the linker is probably to keep the two domains apart, and to restrict their movements with respect to one and another, and the glycosylation of the linker probably makes it less flexible, and probably decreases its sensitivity to proteolytic enzymes.

3.5 Hydrolytic Mechanism of Cellulases

The cellulolytic enzymes have two different enzymatic mechanisms by which they can hydrolyze the glycosidic bonds in cellulose; the retaining and the inverting mechanisms (Davies and Henrissat, 1995).

3.5.1 Retaining Mechanism

The retaining glycoside hydrolase mechanism leads to a net retention of the configuration at the anomeric carbon (C1) of the substrate after cleavage. This is performed via a double displacement mechanism, i.e., the hydrolysis of a glycosidic bond creates a product with the same configuration at the anomeric carbon as the substrate had before hydrolysis (Fig. 1a). The catalytic machinery of these enzymes involves two catalytic carboxylate residues that usually sit at opposite sides of the sugar plane. These are glycosylation and deglycosylation. Glycosylation is a double displacement reaction and a general acid-catalysed leaving group, form a glycosyl-enzyme intermediate. Deglycosylation, the first carboxylate residue now functions as a general base that activates an incoming nucleophile by stealing a proton from it. This activated nucleophile then hydrolyses the glycosyl-enzyme intermediate(Davies and Henrissat, 1995).

3.5.2 Inverting Mechanism

The inverting glycoside hydrolase mechanism (Fig. 1b) leads to a net inversion of the configuration at the anomeric carbon (C1) of the substrate after cleavage. This is performed via a single nucleophilic displacement mechanism, that is the hydrolysis of a beta-glycosidic bond creates a product with the alpha-configuration, and vice-versa. The catalytic machinery of these enzymes involves two catalytic carboxylates. These two carboxylate residues provide a general acid-catalyzed leaving group departure, and a general base-assistance to nucleophilic attack by a water molecule from the opposite side of the sugar ring (Davies and Henrissat, 1995).

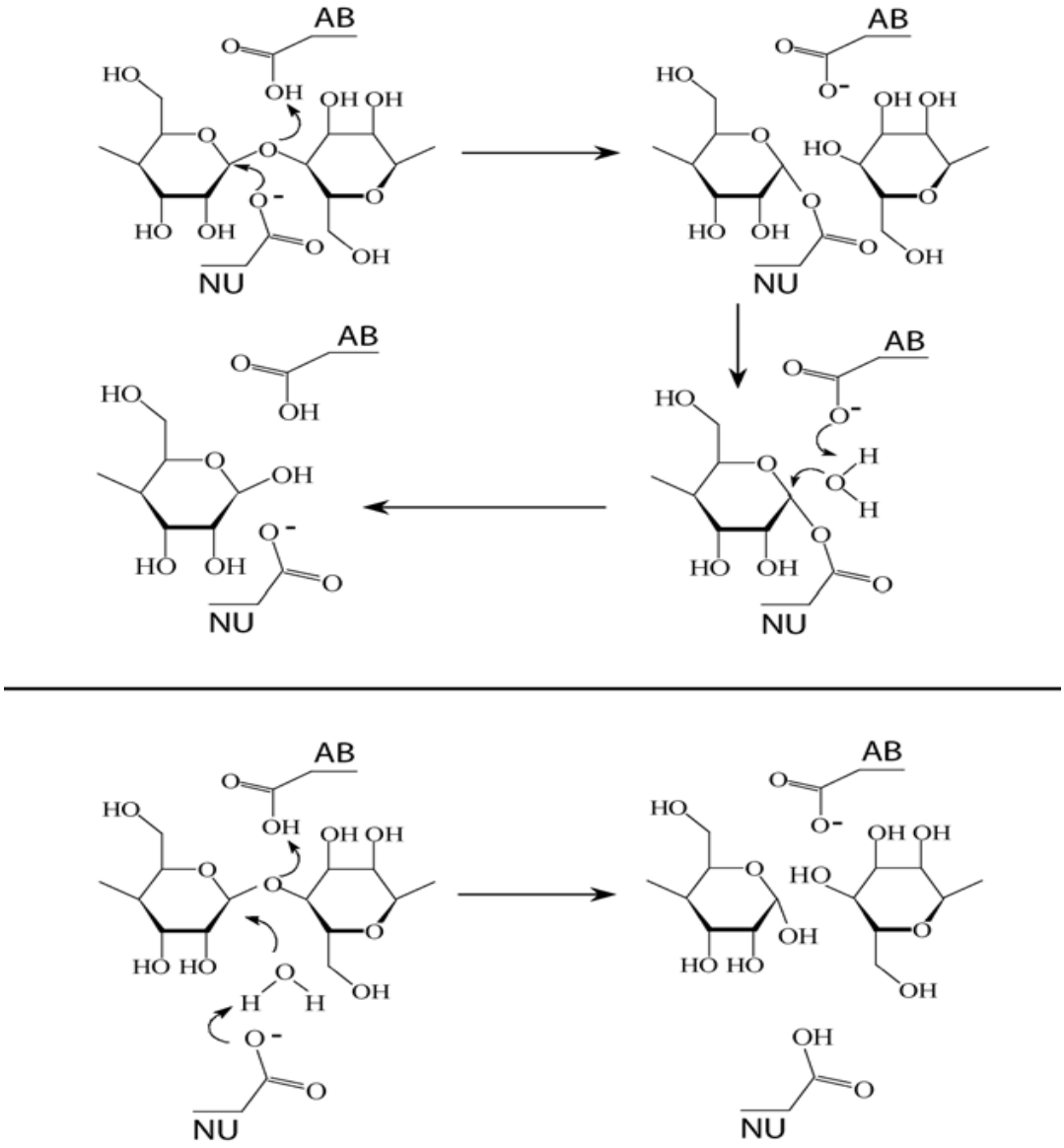

Figure 1. Hydrolytic mechanism of cellulases: The two enzyme mechanisms observed for cellulases: (a) the retaining mechanism and (b) the inverting mechanism. In the retaining mechanism the configuration at the anomeric carbon will be in b-configuration after hydrolysis, i.e., the configuration is retained. The distance between the two catalytic carboxylates in the retaining enzymes is ~5.5 Å. In the inverting mechanism the configuration at the anomeric carbon will be changed from b to a configuration upon hydrolysis. The distance between the two catalytic carboxylates in the inverting enzymes usually varies between 6.5 and 9.5 Å ((Davies and Henrissat, 1995).

3.6 Factors Affecting Cellulase Production

Cellulose digestion is affected by temperature, pH, chemical composition and concentration of cellulose, the nature of microorganism and the presence of carbon sources and nitrogen sources.

3.6.1 Temperature

Cellulolytic microorganisms grow well in a wide range of temperature and thereby produce enzymes. Most cellulase producing microorganisms have their own optimum temperature for cellulase production.

3.6.2 pH

According to Shafique *et al.*(2009) pH was the key factor that affect the production of cellulases enzymes from *T. harzianum* under solid state fermentation and plate screening medium. Fungus mostly preferred acidic media whereas bacterial mostly needs neutral and alkaline pH condition. At high pH the fungal growth and enzyme production would be less. In contrary to this, bacteria showed best growth and enzyme production at high pH.

3.6.3 Chemical composition of cellulose

Cellulose almost never occurs alone in nature but is usually associated with other plant substances. This association may affect its natural degradation. Cellulose fibrils are embedded in a matrix of other polymers, primarily including hemicelluloses, pectin, and proteins (Leschine, 1995). Mechanisms of cellulose degradation by cellulases enzyme has not been fully established because of the high crystallinity and water insolubility of cellulose fibers (Yamada *et al*., 2005).

The greatest challenges of the past decades still with us, the exploration of economic enzymatic saccharification of cellulose. The three main problems are (1) the present need for a long cultivation time to obtain maximal saccharification or cellulase activity, (2) the repression by glucose and intermediate products of cellulase synthesis, and (3) the difficulty of cultivation at high concentration of cellulose in view of fluid dynamic constrains.

3.6.4 Cellulose Concentration

Cellulose concentration is also affects the growth and production of cellulase enzymes by microorganisms (Moosavi-Nasab and Majdi-Nasab, 2007). Most of the time, the optimum cellulase production was obtained when the concentration cellulose approximately 1%. Above or below this value the production of cellulase would become decreased.

The complete degradation of cellulose is required at least three types of cellulase enzymes: endocellulase, exocellulase and cellobiase. However, these enzymes has been isolated and characterized from only a small group of microorganisms. The complete systems of cellulase enzymes has been isolated and characterized from *T. reesei*. Thus, cellulose is completely digested by *T. reesei*. *Trichoderma* is a good producer of endo and exo-cellulase but poor in B-glucosidase (Aneja, 2005).

Moreover, most cellulolytic microorganisms lack efficient ligninase systems, and have problems degrading lignin. It is only some bacidomycetes (white-rot fungi) that have such efficient systems (Leschine, 1995). The complex nature of lignin makes its direct degradation by enzymes a difficult task. The degradation of lignin is less well characterized, and there are conflicting opinions on how the different lignin-degrading enzymes act and cooperate. Most likely aromatic radicals, produced by extracellular peroxidases from the lignin-degrading organism are involved.

3.6.6 Carbone and Nitrogen Sources

Carbone and nitrogen are very essential sources for the growth and production of enzymes. Cellulases are inducible enzymes, which are always needs an inducer. Therefore, Cellulose is a natural inducer. There are other Carbon sources that induces cellulases production; sophomers, lactose, CMC and sucrose whereas glucose and end products repress the production of cellulase enzymes (Kubicek *et al*.,1993).

Successful scale-up strategy demands optimization of critical parameters that influence microbial growth and product formation. Often optimization of multiple parameters is an arduous and time consuming task. In this work, we aimed optimization of media for the production of cellulase from *Trichoderma* isolates using SSF and SmF at different temperature, pH and moisture contents. And also the effect of different carbon and nitrogen sources were evaluated for the production of cellulase enzymes (Pothiraj and Eyini, 2007).

3.7 Application of Cellulase Enzyme

Cellulases have varies industrial applications. The main use of cellulases is in textile industry (biostoning and biofinishing), in laundry detergent industry, in food and feed industry, bio-ethanol production and in pulp and paper industry (Bhat, 2000). Today, the enzymes are commonly used in many industrial applications, and the demand for more stable, highly active and specific enzymes is growing rapidly (Bhat, 2000).

3.7.1 Food industry

Biotechnology of cellulases and hemicellulases began in early 1980s, first in animal feed followed by food applications. Subsequently, these enzymes were used in the textile, laundry as well as in the pulp and paper industries. The main activities of cellulases in food industry are extraction, clarification and stabilization of fruit juices and vegetables, productions of fruit nectars and purees and isolation and separation of starch and gluten from wheat flour. Cellulases enzymes in food industry decreased viscosity of pulp mush, increased filtration rate and reduced processing time, increased juice yield and improved extraction of valuable components. Currently, these enzymes account for approximately 20% of the world enzyme market, mostly from *Trichoderma* and *Aspergillus* (Bhat, 2000).

3.7.2 Feed industry

Applications of cellulases in the feed industry have received considerable attention because of their potential to improve feed value and performance of animals. Pre-treatment of agricultural silage and grain feed by cellulases can improve its nutritional value. There are four reasons for using enzymes in animal feed: 1) to break down anti-nutritional factors; 2) to increase the availability of starches, proteins and minerals enclosed within fiber- rich cell walls; 3) to break down specific chemical bounds in raw materials which are not usually broken down by animals' own enzymes, thus releasing more nutrients; and, 4) to supplement the enzymes produced by young animals (Bhat, 2000). Enzyme feed additives for ruminants are concentrated extracts obtained from fungal fermentations (*T. longibrchiatum, Aspergillus niger* or *A. oryzae*) and bacterial (*Bacillus spp*.).

3.7.3 Textile industry

Cellulases are widely used in the textile industry for the manufacture and finishing of cellulose-containing materials. They have been successfully used for the bio-stoning of jeans and bio-polishing of cotton and other cellulosic fabrics. Most cotton or cotton blend garments, during repeated washings, tend to become fluffy and dull. This is mainly due to the presence of partially detached micro-fibrils on the surface of garments that can be removed by cellulases in order to restore a smooth surface and original colour to the garment (Bhat, 2000). Enzymatic bio-polishing is a novel biological process for textiles made from cellulosic fibres such as cotton, rayon etc. The controlled treatment with acidic cellulases improves softness and water absorbance of the fibres, strongly reduces the tendency for pill formation and provides a clearer surface with less fuzz (McAuliffe *et al*., 2007).

The use of cellulases in general in textile industry confers a verity of advantages: enzymes are easily to use and treatments can be adapted to run on existing equipment and at different stages of textile wet processors; mild treatment conditions can be employed; enzymes can be completely biodegradable and will not accumulate in the environment; enzymes are an economical option as they save chemicals and energy and can reduce processing times (Bhat, 2000; Miettinen-Oinonen, 2007).

3.7.4 Detergent Industry

Use of cellulases along with protease and lipase in the detergents is a more recent innovation in this industry. The industrial application of alkaline cellulases as a potential detergent additive is being actively pursued with a view to selectively contacting the cellulose within the interior of fibres and removing soil in the inter-fibril spaces in the presence of the more conventional detergent ingredients. Nowadays, liquid laundry detergent containing anionic or non-ionic surfactant, citric acid or a water-soluble salt, protease, cellulase and a mixture of propanediol and boric acid or its derivative have been used to improve the stability of cellulases. As most of the cellulose fibers in the modern textile industry enzymes are used increasingly in the finishing of fabrics and clothes are arranged as long, straight chains some small fibers can protrude from the yarn or fabric. The cellulases are applied to remove these rough protuberances for a smoother, glossier and brighter coloured fabric (Bhat, 2000).

3.7.5 Paper and Pulp industry

Interest in the application of cellulases in the pulp and paper industry has increased considerably during the last decade. Cellulases have potential in the pulp and paper industry. The mechanical pulping processes such as refining and grinding of the woody raw material lead to pulps with high content of fines, bulk and stiffness. While in contrast, bio-mechanical pulping using cellulases resulted in substantial energy savings (20-40%) during refining, and improvements in hand-sheet strength properties. Moreover, cellulases increase the alkali solubility of treated pulp and deinking of different types of paper wastes. The wide spectrum of industrial uses for cellulases establishes a need for commercial cellulase products containing different cellulase components and functioning optimally in different pH and temperature ranges (Bhat, 2000).

3.7.6 Ethanol production

Ethanol is a natural component of alcoholic beverages and its use has seen continued growth since the late 1970s, when it was used as a product extender due to gasoline shortages caused by the OPEC (Organization of Petroleum Exporting Countries) oil embargoes. As a result,

production of ethanol from renewable carbohydrate materials has been attracting worldwide interest and research has been directed to the production of ethanol by immobilized microorganisms using continuous culture (Goksungur, and Zorlu, 2001).

Ethanol can be produced from carbohydrates such as sugar, starch, and cellulose by fermentation using yeasts or other organisms. World production of ethanol in 2005 was about 12 billion gallons (Renewable Fuels Association). Although many countries produce ethanol from a variety of feed stocks, Brazil and the United States are the major producers of ethanol in the world, each accounting for approximately 35% of global production. In 2005, Brazil produced 4.2 billion gallons of ethanol, up from 4 billion gallons in 2004. Production of ethanol in Brazil utilizes sugar and molasses from sugarcane as a primary feedstock. In addition to Brazil, production of ethanol from sugarcane is currently underway in several other countries including Australia, Columbia, India, Peru, Cuba, Ethiopia, Vietnam, and Zimbabwe (Shapouri and Salassi, 2006).

Annual production of cellulose is estimated to be 40 to 50 million tons. Much of the cellulose in nature exists as wastes (Gautam *et al.*, 2010). Therefore, cellulolytic microorganism play an important role in the biosphere by reducing cellulose and they also convert cellulose waste into various economically important products like monomeric sugars, single cell proteins or microbial biomass proteins, sugars, alcohol and Bio-ethanol, compost, antibiotics, enzymes, to everyday use for man. Thus, the necessity to achieve large-scale, cost-effective production of active preparations of *Trichoderma* has been increased (Attitalla and Salleh, 2010; Xu *et al*., 2007). In the previous time, ethanol could be produced from starchy materials, however, these materials are used for human foods and animal feeds.

The major draw backs of bio-conversion of cellulytic material into high quality products such as enzymes, proteins, single cell proteins, organic acids and alcohol are: - low ethanol yield, low tolerance of the organisms to ethanol and high sensitivity to end products (Goldschmidt, 2008). *Clostridium thermocellum* is one of the most promising candidates for cellulose binding proteins with the native cellulolytic strategy. This is because it has the ability to convert cellulose directly into ethanol and it is one of the most extensively studied cellulolytic anaerobes. However, *C. thermocellum* lacks the ability to convert pentose sugars (Demain and Newcomb, 2005).

Other Applications

Apart from these common applications, cellulases are also employed in formulations for removal of industrial slime, in research for generation of protoplast, and for generation of antibacterial chitooligosaccharides, which can be used in food preservation, immuno-modulation and as a potent antitumor agent (Sukumaran *et al*., 2005).

3.8 Agro-industrial wastes

Agro-industrial wastes are renewable form of resources generated round the year all over the world. Tremendous amounts of agro-based wastes/ by-products are generated every year over the globe and their improper management causes environmental pollution. The elimination of wide range of pollutants and wastes is an absolute requirement to promote a sustainable and friendly environment. Biotechnology plays a major role in the removal of contaminants and is taking advantage of the greater adaptability of microorganisms to degrade/convert such compounds.

A study conducted by Wen *et al*. (2005) (cited in Rashid *et al*., 2009) cellulase enzymes provide a key opportunity for achieving tremendous benefits of biomass utilization through the bioconversion of the most abundant cellulosic wastes into the simplest carbohydrate monomer, glucose. However, enzyme cost is estimated to represent approximately 50% of the total hydrolysis process cost. A study conducted by Lee (1981) (cited in Howard *et al*., 2003) puts the enzyme cost into stark monetary terms.

The production of cellulases using various substrates and nutrients by microorganisms has been reported. Most of the fungi produce several enzymes with similar cellulolytic activity. *Trichoderma* releases at least four types of endoglucanases, two cellobiohydrolases and three endoxylanases. Rashid *et al*. (2009) already studied the production of cellulase enzymes using the POME as a basal medium.

Cellulase enzyme production with expensive media constituents- celluclast, glucose, yeast extract, peptone, urea, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, $FeSO_4$, $MnSO_4$, $CoCl_2$, $CaCl_2$ etc have been reported by many researchers (Rashid *et al*., 2009). Therefore, by conducting systematic screening studies using agricultural wastes as a media, optimum composition of the co-substrates can be selected in an effort to reduce the production cost of cellulase enzyme production, since agricultural bio-products is considered as a waste with minimal cost.

In the developing country this wastes found in large amount and are not properly disposed. Most of these wastes are emerged from agriculture. Ethiopia is one of the developing country and 80% of the population of Ethiopia is agrarian. The most important agricultural wastes are wheat bran, wheat straw, teft straw, coffee husk/pulp, etc. Thus, microbial utilization of the inexhaustible cellulosic biomass could help solve modern waste disposal problems, help alleviate shortages of food and animal feed and diminish man's dependence on fossil fuels by providing a convenient and renewable source of energy in the form of ethanol (Cowling and Kirk, 1976).

In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues such as sugarcane bagasse, sugar beet pulp, coffee pulp/husk, apple pomace etc (Pothiraj and Eyini, 2007). Many researchers have been used different agricultural wastes for the production of cellulase enzymes by growing different microorganisms. Wheat bran for *Alternaria alternata* and *Aspergillus wentii* (Hussein *et al*., 2011), banana agro-waste for *T. lignorum* (Baig, 2005), Sugar beet pulp for *T. reesei* (Moosavi-Nasab1 and Majdi-Nasab, 2007), groundnut shell for *Aspergillus terreus, A. nidulans* and *T. viride* (Vyas A and Vyas, 2005). Abo-State *et al*. (2010) have studied the production of cellulase of *T. viride* and *Aspergillus spp* in solid state fermentation. The solid cultures were prepared by growing the fungus on wheat straw, wheat bran, rice straw and corn cob. The highest cellulases were produced on the agriculture wastes in the order wheat straw> wheat bran> rice straw > corn cob.

One of the significant applications of agro-industrial wastes like bagasse and wheat bran is biotechnological production of enzymes. Several processes have been developed that utilize these raw materials for the production of bulk chemicals and value-added fine products such as ethanol, Single cell protein, mushrooms, enzymes, organic acids, amino acids and biologically active secondary metabolites. Utilization of agro-industrial residues in bioprocesses on the one hand provides alternative substrates and on the other hand helps in solving pollution problems, which their disposal may otherwise cause, havoc to vegetation (Pothiraj and Eyini, 2007). Therefore, the production of cellulase from *Trichoderma* isolates may involve in varies industrial application such as in feed industry, textile and detergent industry, paper industry and ethanol production.

3.9. Methods of Fermentation

3.9.1 Submerged State Fermentation

Submerged state fermentation involves the production of enzymes by microorganisms in a liquid nutrient media. Fungal cultures adopt different growth patterns when cultivated in liquid and solid substrates. Under SmF conditions, they are exposed to hydrodynamic forces, Research studies on fungal growth patterns in SmF is well investigated for a number of industrially important fermentations. In liquid environments, fungi grow as pellets or free mycelia, depending on the genotype of the strain and culture conditions (Papagianni, 1995).

3.9.2 Solid State Fermentation

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. Some of the substrates that are commonly used for SSF include; sugar cane bagasse, wheat bran, rice bran, maize bran, wheat straw, saw dust, corn flour, wheat flour and pre-treated willow (Pandey *et al.*,1994).

The use of SSF for enzyme production offers several advantages over 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. The most significant problem of SSF is the high heterogeneity, which makes difficult to focus one category of hydrolytic processes, and lead to poor trials of modeling, labour intensive, lack of uniformity in the substrate, limitation on the choice of the type of the SSF (Pandey,1992).

In general, the major factors that affect microbial synthesis of enzymes in a SSF system include, selection of a suitable substrate and microorganism, pre-treatment of the substrate, particle size (inter-particle space and surface area) of the substrate, thus may involve screening of several agro-industrial residues (Doelle *et al.*,1997). The production of cellulase could be improved by mutation under SSF (Vu *et al.*, 2011).

3.10. Methods of Extraction

Several-stages are required for the extraction and purification of enzymes from microorganisms (Fig. 1). For small molecules like citric acid and penicillin, organic solvents such as butanol, butly acetate or large amines are used. For larger and more complex bio-molecules (enzymes), aqueous two-phase extraction can be applied. These systems are composed of an aqueous mixture of a salt ($MgSO_4$) and polymer (polyethylene glycol, PEG) or two polymer (PEG, dextral). In certain concentration ranges these separate in to two phases. The dense bottom phase contains most of the salt, whereas the top phase mainly contains the polymer (Coline and Biorn, 2006).

Cellulases are either cell-bound or extracellular (Krairitthichai and Thongwai, 2003). Most commercially enzymes are extracellular, and the first step in their isolation is separation from the solution. The extraction of intracellular enzymes from microorganisms are very difficult since they contain hard cell well, which do not destruct easily and often required several chemicals that reduce the enzyme activity (Chinedu and Okochi, 2003).

The fungal sources are predominantly used for the production of commercially important cellulase. However, they take longer growth period for maximum enzyme production. As compared to fungi the bacterial cellulase producers are less effective. But they produce enzymes within a short period of time and can be genetically engineered effectively (Ponnambalam *et al.*, 2011).

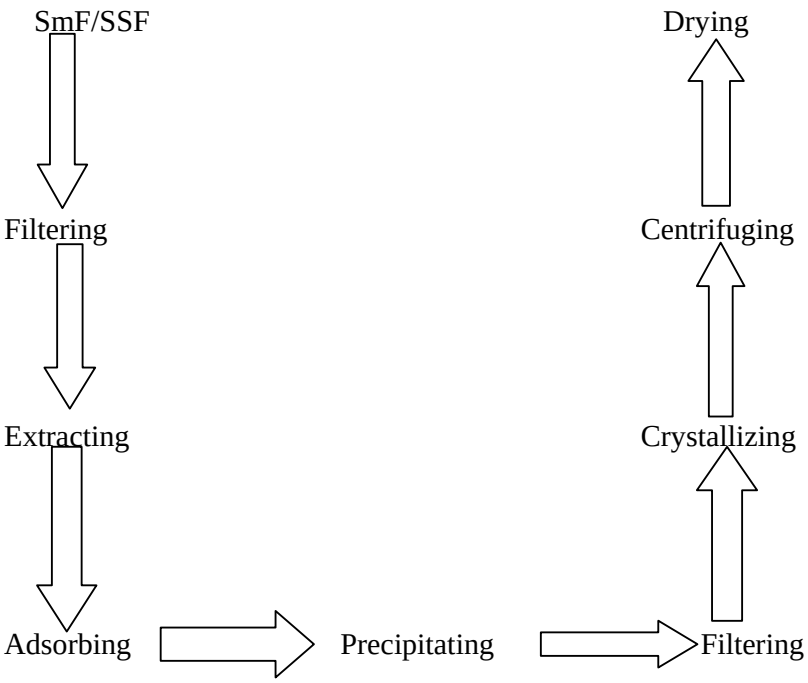

Figure 2. General overview of enzyme and antibiotic extraction (Coline and Biorn, 2006).

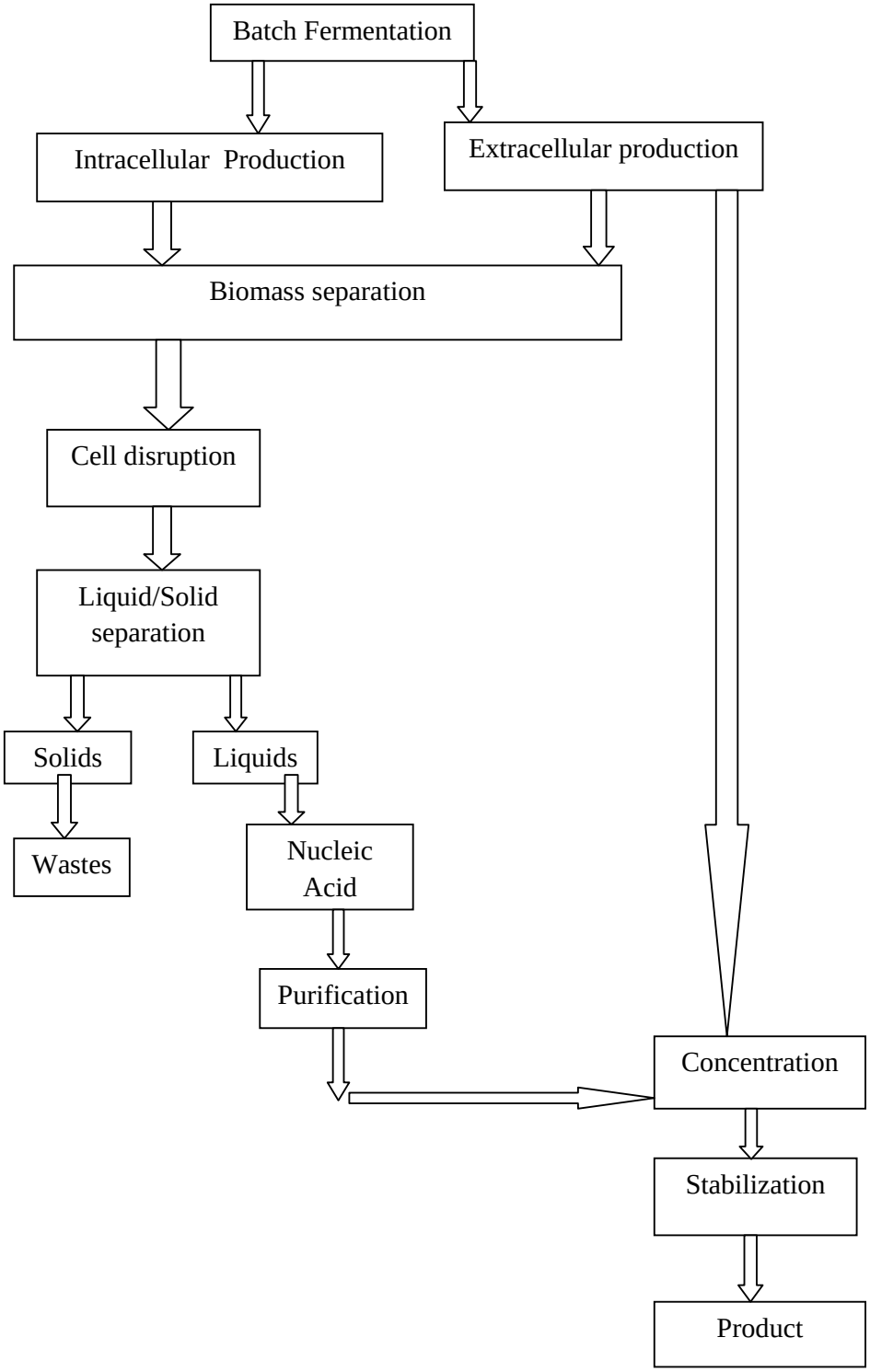

Figure 3. Flow diagram of fungal enzyme production (Murphy and Horgan, 2005)

4. Materials and Methods

4.1. Fungal Isolates

Seven fungal isolates used in this study (*Trichoderma* isolates) were obtained from Mycology Laboratory, Faculty of Life Science, Addis Ababa University. All *Trichoderma* isolates used in this study were previously isolated from soil collected from Jimma and Gera areas. All the isolates were designated as AUT1-7 which stands for Addis Ababa University *Trichoderma*. Screening the potential cellulase producing *Trichoderma* isolates, enzyme extraction and enzyme assaying were conducted.

Table 1. Designation of the isolates

Isolates	Designation
Addis Ababa University *Trichoderma*1	AUT1
Addis Ababa University *Trichoderma*2	AUT2
Addis Ababa University *Trichoderma*3	AUT3
Addis Ababa University *Trichoderma*4	AUT4
Addis Ababa University *Trichoderma*5	AUT5
Addis Ababa University *Trichoderma*6	AUT6
Addis Ababa University *Trichoderma*7	AUT7

4.2 Preparation of Inoculum

Potato dextrose agar (PDA, oxiod) was prepared according to the manufacturers recommendation, sterilized at 121^0C for 15 minutes and poured into the Petri dishes (pH 5.6). Using core borer a plug (5mm diameter) of the preserved *Trichoderma* isolates were placed at the centre of the PDA and incubated aerobically at 30^oC for 7 days. After 7 days of cultivation on PDA the isolates were transferred into CMC (carboxymethylcellulose) containing media for screening the potential cellulase producing *Trichoderma* isolates.

4.3 Screening of Cellulase Producing *Trichoderma* Isolates

To screen the potential cellulase producing *Trichoderma* isolates, enrichment procedure was done in minimal medium comprising of g/L: Na_2NO_3; 2g, K_2HPO_4; 1g, $MgSO_4$ $7H_2HO$; 0.5g, KCl; 0.5g, CMC;5g and peptone;2g with 15g agar, pH 5.5 (Aneja, 2005). After incubation for

5 days at 30°C in CMC agar media, the plates were flooded with 0.1% Congo Red for 15 min. Again the plates were destained with 1M NaCl for 30min. The isolates that showed a clearing zone around the colony were selected as cellulase producing fungi.

4.4 Culture Condition for Cellulase Production

4.4.1 Liquid State Fermentation

Cellulase production was carried out by using cellulose as sole carbon source in SmF. The composition of the medium was in g/L: (Na_2NO_3; 2g, K_2HPO_4; 1g, $MgSO_4$ $7H_2HO$; 0.5g, KCl; 0.5g, Cellulose;5g and peptone;2g) (Aneja, 2005). An Erlimmenary flask (250ml) containing the above media was autoclaved at 121^oC for 15 min. The cellulose medium was inoculated with two plugs (5mm diameter) of *Trichoderma* isolates from 7 days old culture and incubated on a shaker (Orbital shaker, Gerhardt, Bonn) at 121rpm. After 12 days of cultivation the culture filtrates were filtered off (What man filter paper No.1) and transferred into Eppendrof tube to centrifuge (Hermle, Germany), at 10,000 rpm for 15min to remove cell debris. The supernatants were used to determine the cellulolytic activity by the standard assay method (Ghose, 1987). Autoclaving, centrifugation, filtration, composition of the media and assaying were the same in all circumstances unless otherwise stated.

4.5 Optimization of Cellulase Production

4.5.1 The Effect of Temperature on cellulase production under SmF

An Erlimmenary flask (250ml) containing cellulose broths (Na_2NO_3; 0.2%, K_2HPO_4; 0.1%, $MgSO_4$ $7H_2HO$; 0.05%, KCl; 0.05%, Cellulose;0.5% and peptone; 0.2%) (Aneja, 2005) was inoculated with two plugs of 7 old days culture of *Trichoderma* isolates and incubated at different temperature (15^oC, 20^oC, 25^oC, 30^oC, 35^oC and 40^oC) to find out the effect of temperature on cellulase production by these isolates. After 12 days of incubation the culture broths were filtered off (Whatman No.1 filter paper) and transferred into Eppendrof tube to centrifuge (Hermle, Germany), at 10,000 rpm for 15min to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS (dinitro salisalic acid reagent) method (Ghose, 1987). The OD was measured using UV-Spectrophotometer (JENWAY, 6405UV/Vis. Spectrophotometer, UK) at 540nm.

4.5.2 The Effect of pH on cellulase production under SmF

In order to determine the effect of pH on cellulase production under submerged state fermentation, the Erlimmenary flasks (250ml) containing cellulose broth (Na_2NO_3; 0.2%, K_2HPO_4; 0.1%, $MgSO_4$ $7H_2HO$; 0.05%, KCl; 0.05%, Cellulose;0.5% and peptone; 0.2%)

(Aneja, 2005) were adjusted to pH (3.5, 4.5, 5.5, 6.5, 7.5 and 8.5) by using 1N NaOH and 1N HCl before fungal inoculation. After 12 days of cultivation at room temperature on a orbital shaker at 121 rpm, the culture broths were filtered off and transferred to Eppendrof tube to centrifuge at 10,000 rpm for 15min to remove cell debris. The supernatants were used to assay cellulase enzyme by using DNS method (Ghose, 1987). The OD was measured at 540nm.

4.5.3. The effect of Cellulose Concentration on cellulase production under SmF

To determine the effect of cellulose concentration for the production cellulase in SmF, different concentrations of cellulose were prepared in an Erlimmenary flask (250ml) starting from 0.5 to 2% with an interval of 0.5, pH 5.5 (Ul-Huque, 1992). The media was autoclaved at 121^{o}C for 15min. *Trichoderma* isolates were inoculated on the cellulose broths. After 12 days of cultivation at room temperature on a shaker, the culture broths were filtered off and transferred into Eppendrof tube to centrifuge at 10,000 rpm for 15min to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS method (Ghose, 1987). The OD was measured at 540nm.

4.5.4. The Effect of Carbon Sources on cellulase production under SmF

The effect of carbon sources on the production of cellulase enzyme from *Trichoderma* isolates were evaluated by adding 0.2% of different carbon sources (glucose, fructose, maltose and lactose) on a growing media containing 0.3% cellulose, 0.2% $NaNO_3$, 0.2% K_2HPO_4, 0.05% $MgSO_4$ $7H_2O$, 0.05% KCl, and 0.2% peptone, pH 5.5. The control was prepared containing 0.5% cellulose and $NaNO_3$, 0.2% K_2HPO_4, 0.05% $MgSO_4$ $7H_2O$, 0.05% KCl, and 0.2% peptone, pH 5.5. The autoclaved Erlenmeyer (250ml) flasks containing cellulose broth were inoculated with two plugs of *Trichoderma* isolates and incubated at room temperature on a shaker. After 12 days of cultivation, the culture broths were filtered off and transferred into Eppendrof tube to centrifuge at 10,000 rpm for 15 minutes to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS method (Ghose, 1987). The OD was measured at 540nm.

4.5.5. The Effect of Nitrogen Sources on cellulase production under SmF

The effect of nitrogen sources on the production of cellulase enzyme by *Trichoderma* isolates was determined by replacing peptone with 1% of other nitrogen sources. The nitrogen sources were used peptone, ammonium sulphate, sodium nitrate and yeast extract. After autoclaved at 121^{o}C for 15min the Erlenmeyer flasks (250ml) were inoculated with two plugs of *Trichoderma* isolates and incubated at room temperature for 12 days on a shaker, pH 5.5.

The culture broths were filtered off and transferred into Eppendrof tube to centrifuge at 10,000 rpm for 15min to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS method (Ghose, 1987). The OD was measured at 540nm.

4.5.6 Shaker and Static Condition for the Production of Cellulase

The Erlimmenary flasks containing cellulose broths (Na_2NO_3; 0.2%, K_2HPO_4; 0.1%, $MgSO_4$ $7H_2HO$; 0.05%, KCl; 0.05%, Cellulose;0.5% and peptone; 0.2%) (Aneja, 2005) was autoclaved at 121^oC for 15min. The flasks were inoculated with *Trichoderma* isolates and incubated on a static and shaker (121rpm) conditions. In both cases the content was incubated at room temperature, pH 5.5. After 12 days of incubation, the culture broths were filtered off and transferred to Eppendrofe tube to be centrifuged at 10,000 rpm for 15min to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS method (Ghose, 1987).

4.5.7 Time Course of Enzyme Production

The optimum time course for cellulase production by *Trichoderma* isolates in SmF was determined by incubating the isolates at room temperature on a shaker over a period of 14 days. An Erlenmeyer flask (250ml) containing minimal medium using cellulose as sole carbon source, pH 5.5, were inoculated with two plugs of *Trichoderma* isolates. Samples were withdrawn from the culture broths at 2 days intervals over a period of 14 days. The supernatants that resulted following filtration and centrifugation at 10,000 rpm for 15min to remove mycelia, were assayed to determine reducing sugars using DNS method (Ghose, 1987).

4.6. Solid State Fermentation

Wheat straw, wheat bran, barley bran, rice bran, coffee pulp and cotton seed were used for the production of cellulase enzyme from *Trichoderma* isolates under SSF. To ten gram of each cellulose source was separately transferred into 250ml Erlenmeyer flask capacity, 10 ml stock mineral salt solution (K_2HPO_4 0.05g, $MgSO_4.7H_2O$ 0.02g, NH_4NO_3 0.1g, $CaCl_2.2H_2O$ 0.01g and 1ml of 1% $FeCl_3$) was added and autoclaved at 121^oC for 15 minute (Ul-Huque, 1992). After 12 days of incubation at 30^oC, the culture was extracted by adding 100 ml distilled water, filtered using (What man No.1 filter paper) and centrifuged at 10,000rpm for 15min. The supernatants were used to assay enzyme activity (Ghose, 1987).

4.6.1 Effect of Additives for Cellulase Production under SSF

Wheat straw, rice bran and wheat bran were supplemented with different carbohydrates as carbon source at a concentration of 5% (w/w) and nitrogen sources at a concentration of 1%

(w/w) and the effect of these additives on the level of cellulase production were evaluated. The enzyme was extracted on the optimum time of growth and its activity was measured following the standard assay procedure (Ghose, 1987).

4.6.2 Moisture Content of the Solid Media

The effect of moisture contents on enzyme production was studied by varying the percentage of water in the medium from 45% to 80% (with an interval of 10%). All the liquid added in to the flask and original moisture content of the wheat bran (6.2%), rice bran (6.5%) and wheat straw (6.1%) was taken in to consideration in calculating the percentage of water in the medium. After 12 days of incubation at 30^{o}C, the culture was extracted by adding 100 ml distilled water, filtered using (What man No.1 filter paper) and centrifuged at 10,000rpm for 15min. The supernatants were used to assay enzyme activity (Ghose, 1987).

4.7 Analytical Methods

4.7.1 Cellulase Activity Assay

CMCase was assayed by using a modified method described by Mandels *et al*., in 1976 (cited in Dashtban *et al*., 2010). The activity was determined by mixing 0.1ml of enzyme solution with 0.9ml of 0.5% CMC in 50mM of sodium acetate buffer in a 14ml of test tube, pH 5, vortexed for 1min, incubated for 30 minutes at 50^{o}C. The reaction was stopped by adding 2ml of dinitrosalicyclic acid (DNS) reagent in the above mixture. The mixture was boiled for 15min (95-100^{o}C) in a boiling water bath and cooled in cold water. The formation of reducing sugars was measured by DNS reagents (Ghose, 1987).

One unite of cellulase activity was defined as the amount of enzyme produced 1µm of glucose/ minute under the specified assay conditions. An appropriate blank which contained 0.1mL of distilled water instead of crude enzyme extract was also run along with the test. The control was prepared by adding the crude enzyme extract after the addition of DNS reagent on the substrate. The equation of Y=[0.517x + 0.049]mg/ml was used to made the standard curve.

1) Y=[0.517x + 0.049]mg/ml
2) Y=[0.517x + 0.049]*1000/180] µmol/ml (Ghose, 1987)

Where, Y=the amount of glucose equivalents liberated from CMC hydrolysis

X=Optical density measured at 540nm

4.7.2 Crude Enzyme Characterization

For the determination of optimum temperature and temperature stability, activity was determined by carrying out the above standard assay at several temperature values. To determine optimum temperature for cellulase enzyme activity, the reaction mixture was incubated for 30min in the temperature range of 20^{o}C-80^{o}C (with an interval of 10^{o}C). Temperature stability, each crude enzyme was incubated at different temperature from 20^{o}C-80^{o}C (with an interval of 10^{o}C) for 30min before the addition of substrate and the residual activity was measured following the standard assay conditions. A simultaneously prepared enzyme-buffer mix was stored at 4^{0}C for 30min to be used as a control (Ariffin *et al*., 2006).

Optimum pH for cellulases activity was determined by assaying the cellulase enzyme at different pH values (3 to10). The buffers used were citrate phosphate buffer (pH 3.0 to 7.0), Tris buffer (7.0 to 9.0) and Glycine-NaOH buffer (pH 9-10) (with an interval of 1 in all pH ranges). For the determination of pH stability, 100µL of enzyme was mixed with 450µL buffers of varying pH and incubated at room temperature for 30min. Residual activity were measured following the standard assay conditions (Ariffin *et al*., 2006).

4.8 Statistical Analysis

All experiments and enzyme assays were performed in duplicates, statistically evaluated by excel and SPSS (version 16) and results have been presented as mean ± SEM. (standard mean error).

5. Results

5.1. Screening of Cellulase Producing Isolates

All *Trichoderma* isolates were screened for cellulase activity using CMC agar (selective agar). All isolates of *Trichoderma* were positive for CMCase. However, isolates were differing in their ability to produce cellulose degrading enzymes (Table 2). The isolate (AUT5) was showed the highest hallow zone on the CM-cellulose agar media (75mm) whereas AUT7 showed the least clear zone diameter (9mm).

Table 2. Preliminary screening of cellulolytic *Trichoderma* isolates on CMC media

Isolates	Clear zone (mm) Mean	Enzyme activity (U/ml)
AUT1	32	0.4
AUT2	30	0.41
AUT3	15	0.12
AUT4	54	0.37
AUT5	75	0.33
AUT6	10	0.11
AUT7	9	0.14

It is evident from Table (2) AUT1, AUT2, AUT4 and AUT5 were the most efficient isolates selected according to the high clear zone diameter when grown in CMC agar. They showed clear zone of 75mm, 54mm, 32mm and 30mm on CM-cellulose agar media, respectively, and were selected for further studies. Moreover, this experiment was confirmed again by DNS method. Isolate AUT5 showed the highest clear zone (75mm) but it showed only 0.33U/ml cellulolytic activity on the broth culture using DNS methods. Similarly, isolates AUT1 and AUT2 showed the highest enzymatic activities, 0.4U/ml and 0.41U/ml, respectively whereas isolates AUT3, AUT6 and AUT7 displayed small amount of cellulolytic activities, 0.12, 0.11 and 0.14U/ml, respectively.

5.3 Optimization of Cellulase Production

Several factors affect the production of cellulase enzymes by *Trichoderma* isolates. Among the factors determined in this study were temperature, carbon sources, nitrogen sources, pH, cultivation condition and cellulose concentration under SmF. In addition to this, agricultural wastes, its moisture level and different additives were optimized under SSF.

5.3.1 The Effect of Temperature on Cellulase Production under SmF

To study the effect of temperature on cellulase production from *Trichoderma,* the isolates were incubated at different temperatures (15^{o}C, 20^{o}c, 25^{o}c, 30^{o}c, 35^{o}c, 40^{o}C). The maximum cellulase enzyme activity recorded at 30^{o}C for all isolates (AUT1, AUT2, AUT4 and AUT5) (Fig.4). However, their enzymatic activity on cellulosic media at 30^{o}C was different. Isolate AUT2 gave the highest cellulase enzyme activity (0.544±0.011U/ml) on cellulose broth at 30^{o}C pH 5.5. Similarly, isolates AUT1, AUT4 and AUT5 produced 0.5025±0.0075U/ml, 0.4995±0.0045U/ml and 0.4285±0.0115U/ml cellulase enzyme activities at 30^{o}C and pH values 5.5, respectively. The enzyme activity increased as the temperature increase up to 30^{o}C then after it began to decrease when the temperature raising above 30^{o}C.

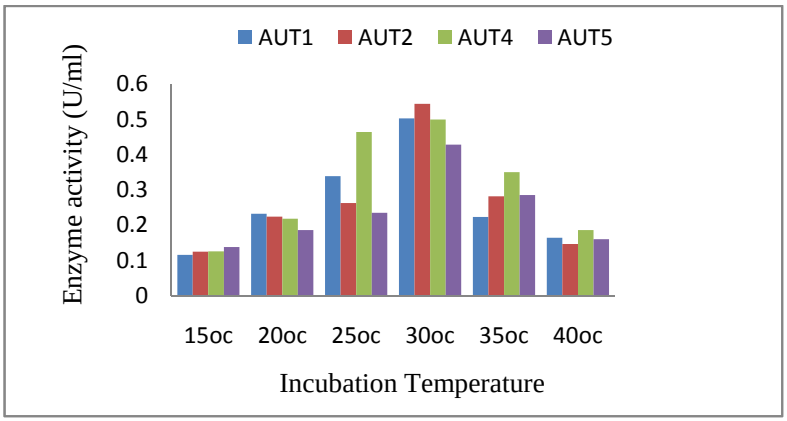

Figure 4. The effect of incubation temperature on the production of cellulase enzymes from *Trichoderma* isolates.

5.3. The Effect of pH on Cellulase Production under SmF

Fig. 5. shown that the effect of pH on the crude cellulase enzyme production from *Trichoderma* isolates. The optimal pH for cellulase enzyme production for all isolates was 5.5. The cellulase enzyme activities for isolates AUT1, AUT2, AUT4 and AUT5, at PH 5.5 were 0.3905±0.0015, 0.3935±0.0045, 0.5805±0.0085 and 0.314±0.0U/ml, respectively. Isolates AUT4 showed the highest cellulase enzyme activity at pH 5.5 whereas isolate AUT5 showed the least cellulase enzyme activity.

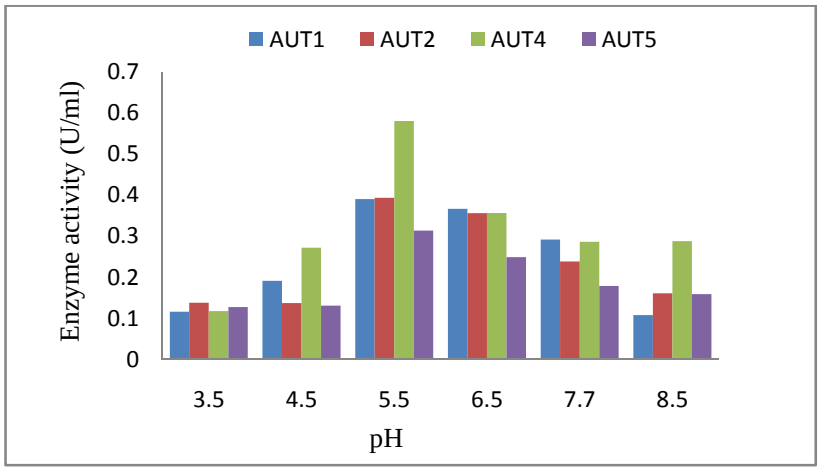

Figure 5. The effect of pH on the production of cellulase from *Trichoderma* isolates.

5.5 The Effect of Cellulose Concentration on Cellulase Production under SmF

Fig. 6 shown that when the concentrations of cellulose increase the enzyme activity also increased until the concentration reached at 1%. Maximum cellulase production was obtained at 1% cellulose concentration by all *Trichoderma* isolates. Further increased in cellulose concentration beyond the level of 1% the production of cellulase enzyme begun to decrease. The enzyme activities of the isolates AUT1, AUT2, AUT4 and AUT5 at 1% were 0.625±0.005, 0.609±0.004, 0.785±0.005 and 0.215±0.005U/ml, respectively.

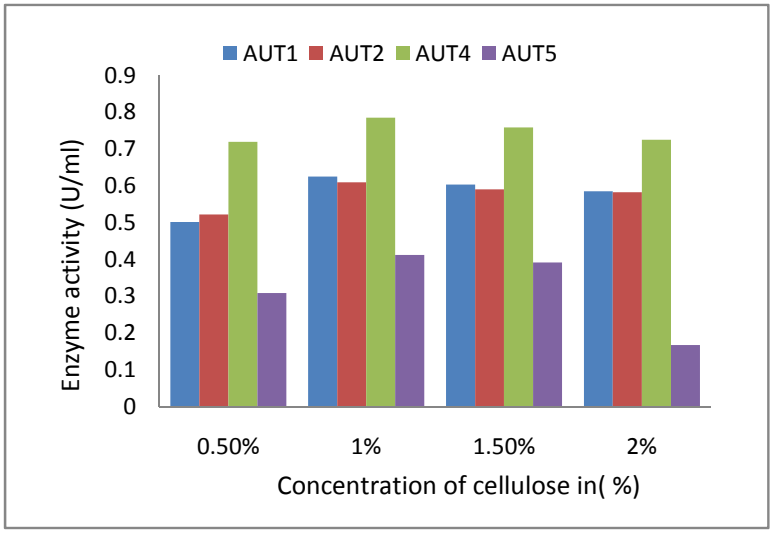

Figure 6. Effect of cellulose concentrations on cellulase production by *Trichoderma* isolates

5.6 Time Course of Enzyme Production

Fig. 7 demonstrates the production of cellulase enzymes by isolates over a period of 14 days. Initially, the activity was low in each case. The highest cellulase production were recorded after 12 days of incubation by all isolates. Their enzyme activities for AUT1, AUT2, AUT4 and AUT5 were 0.3975±0.0055, 0.4033±0.0053, 0.3705±0.0045 and 0.3195±0.0045U/ml, respectively. Isolate AUT2 produced large amount of cellulase enzymes whereas isolate AUT5 produced small amount of cellulase enzymes upon 12 days incubation.

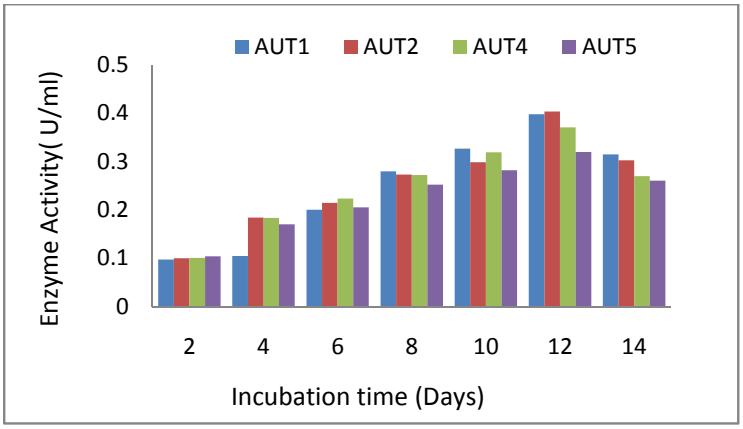

Figure 7.Time course of cellulase production for AUT1,AUT2, AUT4,and AUT5.

5.7 The Effect of Carbon Sources on Cellulase Production under SmF

As depicted in Fig. 8 the carbon source in the medium was affect considerably in the synthesis of cellulolytic enzymes by *Trichoderma* isolate in liquid culture. Lactose and maltose were found to induce the production of cellulase by the four isolates but glucose and fructose reduced the production of cellulase. Lactose was a good inducer of cellulase production followed by maltose.

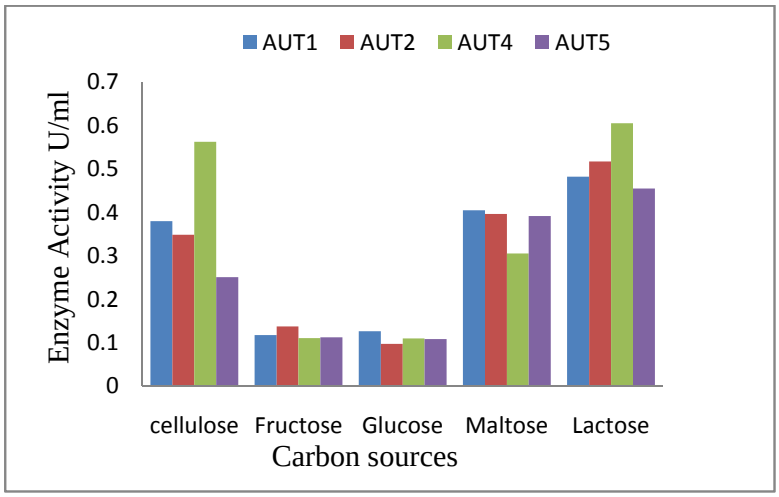

Figure 8. The effect of carbon sources on the production of cellulase enzyme from *Trichoderma* isolates.

5.8 The Effect of Nitrogen Sources on Cellulase Production under SmF

The nitrogenous sources showed to influence the production of cellulase enzymes by *Trichoderma* isolates under SmF. It is evident from (Fig. 9) all *Trichoderma* isolates showed the highest cellulase enzyme production on yeast extract supplemented medium the least cellulase enzyme production was recorded on sodium nitrate medium. However, in the absence of nitrogen sources that was used as a control, the isolates were grown in the broth, but cellulase enzyme activity was not detected.

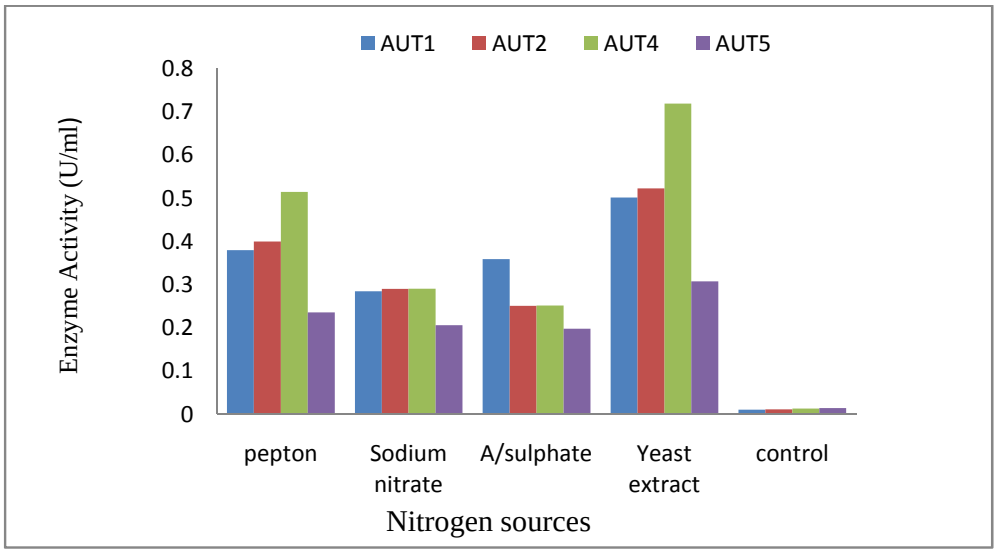

Figure 9. The effect of nitrogen sources on the production of cellulase enzymes from *Trichoderma* isolates

5.9 Static and Shaking Condition

The formation of cellulase in the shaking state was much higher than in static condition (Table 3.). CMCase activity obtained in the static culture of *Trichoderma* isolate (AUT4) was only 0.164 ± 0.01U/ml and the activity was increased to 0.467 ± 0.0015U/ml in agitated culture. This was approximately 3 times higher than static condition. Therefore, in agitated condition the enzymatic activity was much higher than in static condition.

Table 3. The effect of cultivation condition on the production of cellulase enzymes

	Enzyme activity U/ml		
Isolates	Static	Shaker	SH/ST
AUT1	0.115 ± 0.0075	0.339 ± 0.002	2.9 times
AUT2	0.148 ± 0.008	0.334 ± 0.006	2.3 times
AUT4	0.164 ± 0.01	0.467 ± 0.0015	2.8 times
AUT5	0.139 ± 0.0025	0.246 ± 0.0005	1.8times

5.10 Solid State Fermentation for Cellulase Production

Different agricultural wastes were used as substrates for the production of cellulase by *Trichoderma* isolates. Under SSF, wheat straw, wheat bran and rice bran showed maximum cellulase production after 12 days of incubation at 30^{o}C (Table 4). Maximum cellulase production was recorded by AUT1 (5.68±0.06U/g) on wheat straw, AUT2 (4.92±0.16U/g) and AUT4 (7.01±0.055U/g) on wheat bran and AUT5 (8.15±0.065U/g) on rice bran. The minimum cellulase production was observed by AUT2 (1.05±0.08U/g) on CS and by AUT5 (1.26±0.02U/g) on WS. Generally, CS, BB and CP were showed the least amount of cellulase production from *Trichoderma* isolates. Finally, wheat bran for isolates AUT2 and AUT4, wheat straw for isolate AUT1 and rice bran for isolate AUT5 were selected to optimized moisture contents and additives for cellulase enzyme production under SSF (Section 5.11 and 5.12).

Table 4. Growing of *Trichoderma isolates* on different solid substrates for the production of cellulase

Isolates	Enzyme Activity U/g			
	AUT1	AUT2	AUT4	AUT5
WB	4.98 ± 0.17	4.92±0.16	7.01±0.055	5.99±0.055
WS	5.68±0.06	2.95±0.065	6.62±0.105	1.26±0.02
RB	5.32±0.17	4.08±0.065	5.95±0.1	8.15±0.065
CS	2.43±0.065	1.05±0.08	5.81±0.015	6.26±0.105
CP	2.13±0.04	3.72±0.06	4. 68±0.04	3.81±0.055
BB	3.49±0.055	4.42±0.045	2.80±0.03	1.66±0.075

NB: WB-wheat bran, WS-wheat straw, RB- rice bran, CS-cotton seed, CP-coffee pulp and BB-barley bran

5.11. The Effect of Additives on Cellulase production under SSF

Under SSF the effect of different additives were evaluated for the production of cellulase enzymes by *Trichoderma* isolates. It is evident from (Table 5) cellulose and lactose showed the highest cellulase enzyme production whereas maltose, glucose and fructose showed the least amount of cellulase production as compared to the control. Lactose was a good inducer of cellulase production followed by cellulose. Cellulase enzyme was not produced in the presence of fructose and maltose by isolates AUT1, AUT4 and AUT5. However, cellulase production by AUT2 was not significantly affected by the presence of any carbon sources. The enzymatic activity of cellulase on the control was higher than in the media containing maltose, glucose and fructose but less than in the media containing cellulose and lactose (Table 5).

In addition to this, the production of cellulase enzymes from *Trichoderma* isolates were also affected by the presence of nitrogen sources. The combination of wheat straw and sodium nitrate showed highest cellulase production by isolate AUT1 (5.54±0.05U/g). However, the combination of rice bran and peptone showed the highest cellulase production by isolate AUT5 (8.955±0.135 U/g); and peptone and wheat bran showed highest cellulase production by AUT2 and AUT4, their enzyme activities were 6.46±0.11, 6.795±1.465U/g, respectively.

Table 5. The effect of carbon and nitrogen sources on the production of cellulase enzymes under SSF.

Isolates		Enzyme activity U/g			
		AUT1	AUT2	AUT4	AUT5
Carbon sources	Control	3.68±0.06	4.92±0.16	4.01±0.055	3.15±0.065
	Glucose	1.1±0.03	3.345±0.015	2.615±0.035	1.93±0.04
	fructose	None	6.425 ± 0.225	None	None
	Maltose	None	5.82 ±0.055	None	None
	Lactose	4.005 ±0.05	5.335 ±0.08	5.59 ±0.07	5.57±0.09
	Cellulose	3.94 ±0.05	4.99 ±0.06	5.95 ±0.06	4.665 ±0.07
Nitrogen sources	Yeast extract	2.875±0.075	5± 0.09	5.925±0.035	3.725±0.035
	Sodium nitrate	5.54±0.05	5.195±0.085	4.905±0.085	3.81±0.15
	Ammonium/S	4.365±0.035	5.645±0.055	6.11±0.03	5.735±0.095
	Peptone	2.645±0.055	6.46±0.11	6.795±1.465	8.955±0.135

5.12. The Effect of Moisture Level

The cellulase enzyme produced from *Trichoderma* isolates were affected by the moisture content of the substrate as indicated in Fig. 10. The maximum amount of cellulase were recorded between 55% to 65% moisture contents. At 65% moisture content, isolates AUT5 and AUT1 were showed maximum enzyme activity (7.34U/g) and (5.37 U/g), respectively where as isolates AUT2 and AUT4 showed maximum enzyme activity at 55% moisture content and their enzyme activities were (5.92U/g) and (4.65U/g), respectively. Moisture contents < 45% or > 70% were not suitable for high cellulase enzyme production. As depicted in Fig 10, isolate AUT1 did not show any enzyme activity at the moisture contents 45% and 80%, isolates AUT4 and AUT5 were not showed any enzymatic activities at the moisture level 80%.

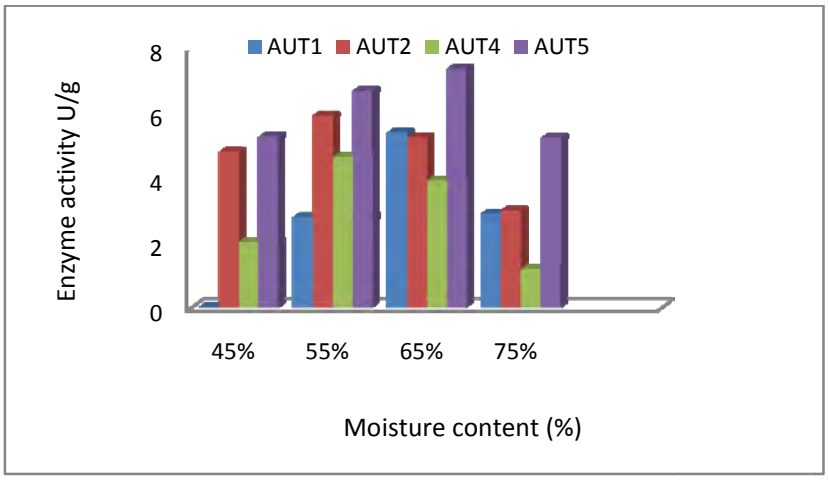

Figure 10. The effect of moisture level on the production cellulase from *Trichoderma* isolates.

Table 6 shows the time course of maximum cellulase enzyme production under SSF showed variation depending up on the substrate and isolates used. The isolate AUT4 showed maximum enzyme peak after 5 days incubation on wheat bran at 30^{o}C; isolate AUT2 showed maximum enzyme production at 9 days incubation at 30^{o}C; isolates AUT1 and AUT5 showed maximum cellulase enzyme activities after 11 days incubation on wheat straw and rice bran, respectively. Isolate AUT5 did not show any enzyme activity in the first five days incubation. However, isolate AUT4 showed maximum cellulase enzyme activity in the first 5 days of incubation.

Table 6. Time course of cellulase enzyme production under SSF

Isolates/days	Enzyme activity U/g					
	3	5	7	9	11	13
AUT1	2.745±0.045	4.19±0.12	4.305±0.125	3.92±0.03	5.47±0.06	3.73±0.07
AUT2	2.52±0.06	3.725±0.095	6.68±0.04	8.305±0.065	7.355±0.055	6.995±0.095
AUT4	7.47±0.43	9.96±0.03	9.12±0.18	8.15±0.87	7.165±0.115	7.33±0.08
AUT5	None	None	1.49±0.03	5.744±0.056	7.675±0.035	7.185±0.075

5.13. Crude Enzyme Characterization

5.13.1 Optimum Temperature for Activity of cellulase enzymes

The CMCase enzyme activity of crude cellulase after being subjected to different incubation temperature recorded on Fig 11. The optimal temperature for the crude cellulase was found to be 40^{o}c for isolate AUT1, 50^{o}C for isolate AUT2 and 60^{o}C for isolates AUT4 and AUT5.

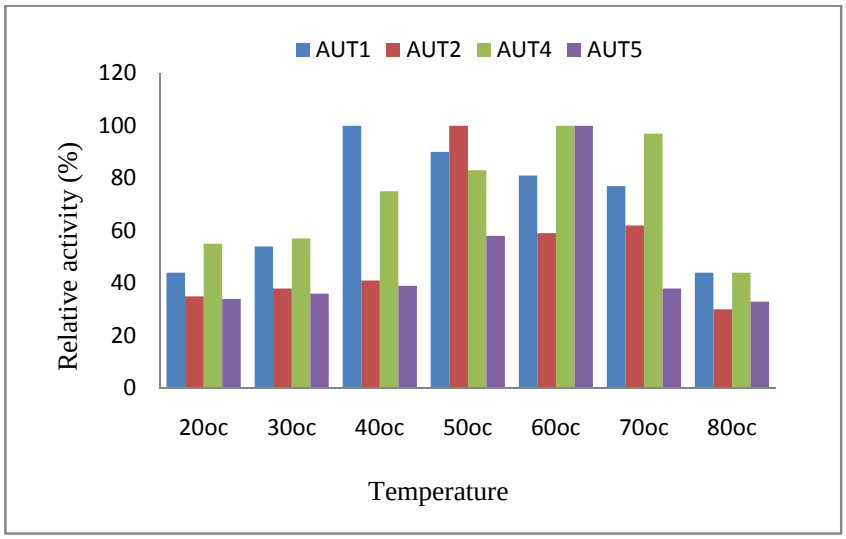

Figure 11.Temperature profile of *Trichoderma cellulase*; assayed at different temperature values and pH 5. The experiments were done in duplicates.

5.13.2 Temperature Stability of cellulase enzymes

The effect of temperate on the enzyme stability was determined by maintaining the enzyme at various temperature ranging from 20^{o}C-80^{o}C for 30min. The cellulases were stable below 40°C. However, cellulase activities from AUT1, AUT2, AUT4 and AUT5 were maintained at 91%, 61%, 52% and 54% after 30min of incubation at 40^{o}c, respectively. Cellulases from all isolates subjected to 60^{o}C and above retained less than 50% of their original activity (Fig. 12).

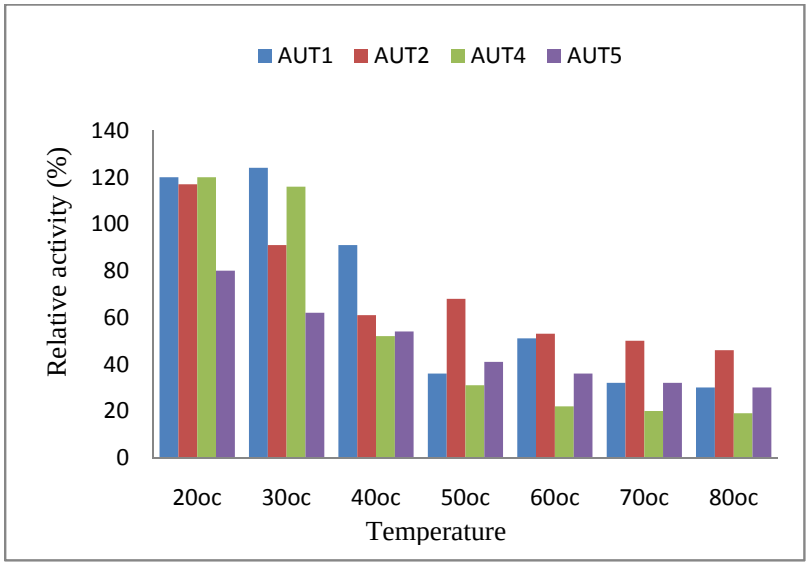

Figure 12. The effect of temperature on the stability of cellulase enzyme

5.13.3 Optimum pH for Activity of Cellulase Enzyme

The effect of pH on the activity of enzyme was investigated by determination of the activity at various pH values. Results (Fig. 13) obtained indicated that a sharp optimum pH of 5.0 was found for *Trichoderma* isolates and exhibited more than 80% of its activity in the pH range of 4.5-6.5.

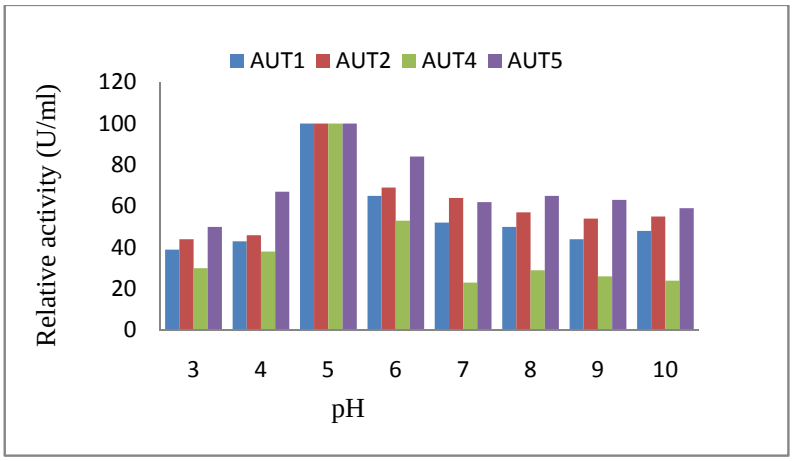

Figure 13. pH profile of *Trichoderma* isolates cellulase at 50^{o}C.

5.13.4 pH Stability of Cellulase Enzyme

The stability of cellulase enzymes extracted from *Trichoderma* isolates was determined by incubating them at pH values from 3 to 10 at room temperature for 30min. *Trichoderma* cellulases were stable in a broad pH range with maximum stability in the pH range of 4.5 - 6.5 (Fig. 14). However, the enzyme decreased its activity at pH values above 7.5. More than 80% of the residual relative activity of the enzyme were retained in the pH ranges of 4.5-6.5. At pH lower than 3 and higher than 11, the activities were lost completely.

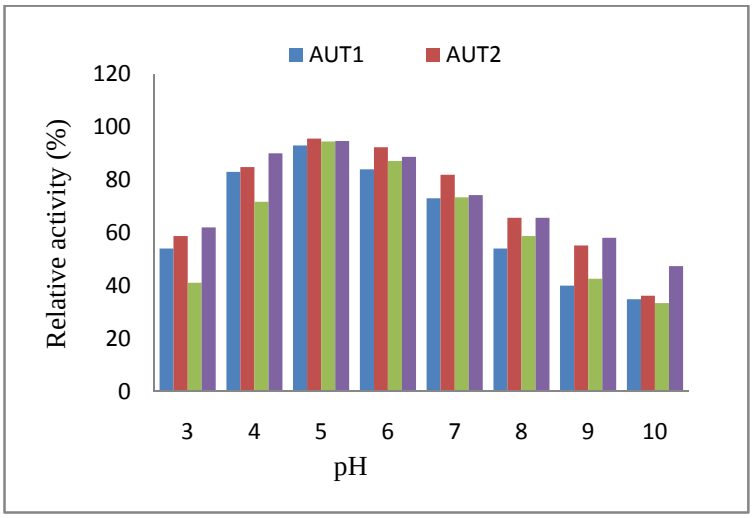

Figure 14. The effect of pH on the stability of *Trichoderma* cellulase

6. Discussion

All isolates used in this study showed carboxymethyl cellulase (CMCase) activity (Table 2). Cellulase production by *Trichoderma* isolates was affected by varying temperature, pH, medium and various growth factors. Thus, the effect of different agricultural wastes and additives were also evaluated for the production of cellulase enzymes in SSF. In this study, the highest cellulase production was shown by isolate AUT4 while the least cellulolytic activity was displayed by isolate AUT5.

In the present study, the *Trichoderma* isolates were able to grow over a broad range of temperature 15-40^{o}C. However, maximum cellulase production was obtained at 30^{o}C. This might be due to better growth of the isolates at this temperature. This result is considerably similar to what was reported by Shafique *et al.* (2009) who indicated that the optimum temperature for maximum cellulase production for *T. reesei* was 30 ± 2^{o}C. However, the results appeared to contradict previous results reported by Gautam *et al*. (2010) who showed that the optimum temperature for cellulase production under SmF is between 40-50^{o}C for *T. viride* and Li *et al*. (2009) who observed that the optimum temperature for cellulase enzyme production from *T. viride* was 50^{o}C.

The pH level of the medium is one of the most important parameters that influence the growth of an organism and thereby enzyme production. In the present study, *Trichoderma* isolates were able to grow at a wider pH ranges between 3.5-8.5. However, the optimum pH for cellulase production by *Trichoderma* isolates was 5.5 (Fig. 5). This result is considerably similar to what was reported by Li *et al*. (2009) who showed that the optimum pH for cellulase production from *T. viride* was at 5. Similarly, Voragena *et al*. (1988) have reported that the optimum pH for maximum cellulase production from *T. viride* was in the range between 4.0 and 5.5. Optimum pH for fungal cellulase varies from species to species. Both high acidic and high basic pH shows negative effects, but a medium with low acidic pH, 5.5 was ideal for enzyme production. This might be due to the fact that fungal cultures require slightly acidic pH for their growth and enzyme biosynthesis (Haltrich *et al*., 1996)..

In the present study, the media containing lactose and maltose induced the production of cellulase whereas the media having glucose and fructose repressed the production of cellulase by *Trichoderma* isolates in SmF (Fig. 9). In agreement with this study Szakacs *et al*., (2006)

and Baig, (2005) have reported that glucose and fructose represses the production of cellulase activity and lactose, avicel and CMC induced the production of cellulase by *Trichoderma* spp. In contrast to the present study Coban and Biyik (2011) reported that glucose gave the highest yield, followed by fructose, sucrose and ethanol.

Similarly, under SSF, glucose and fructose repressed the production of cellulase enzymes whereas cellulose and lactose induced the production of cellulase from *Trichoderma* isolates. Sun *et al.* (2010) reported that supplementation of lactose to the apple pomace favoured marked cellulase production by *Trichoderma* spp. However, the presence of maltose, fructose and glucose induced the production of cellulase enzyme by isolate AUT2. On the other hand, fructose and maltose completely inhibited the production of cellulase by isolates AUT1, AUT4 and AUT5 (Table 5).

The nitrogen source used in the medium is one of the major factors affecting cellulase production. The highest amount of cellulase activity was recorded in SmF containing yeast extract as sole nitrogen source whereas the least cellulase production were observed in SmF containing sodium nitrate (Fig. 9). Similarly, Ahamed and Vermette (2008) revealed that yeast extract yielded the highest CMCase activity by *T. reesei* RUT-C30. In contrast to the present study, the maximum production of cellulase by *T. viride* was observed in the medium having $NaNO_3$ as the nitrogen source (Khare and Upadhyay, 2011). Showing that organic compounds stimulated higher cellulase yields compared with inorganic compounds. It is believed that simple and organic nitrogen sources like peptone have a stimulatory effect on both the growth rate and cellulase synthesis and thereby shortening the lag phase of the culture and there is an assumption that ammonium salts may have an inhibitory effect on cellulase production.

The result of SSF showed that organic nitrogen sources (peptone and yeast extract) was a good nitrogen source for cellulase production by isolates AUT2 (wheat bran), AUT4 (wheat bran) and AUT5 (rice bran). However, isolate AUT1 produced maximum amount of cellulase in the media containing inorganic nitrogen (sodium nitrate) as the sole nitrogen sources on wheat straw (Table 5). Similarly, Coban and Biyik (2011) reported that organic nitrogen source gave the highest yield of cellulase enzymes under SSF. Contrary to this study, Wahid *et al.* (2011) reported that ammonium sulfate was found to be the best nitrogen source for the production of cellulase enzymes by *T. reesei* RUT C-30 from solid state fermentation of oil

palm followed by calcium nitrate, urea, peptone and yeast extract. This was most probably due to ammonia, as it was transported into the cell in the form of metabolite nitrate, nitrite, urea and amino acids which triggered the synthesis of protein and cellulase (Mikes *et al*., 1994).

The result clearly showed that addition of cellulose at 1% was optimal for cellulase production (Fig. 6). The production of cellulase raised with increasing the concentration of cellulose up to 1% (W/V). Furthermore increasing the concentration of cellulose beyond the level of 1% resulted in lower cellulase production by *Trichoderma* isolates. This is probably due to the high viscosity of the medium, which decreases the oxygen supply to the cells. High viscosity leads to retard cell division, resulted in low production metabolism and cellulase excretion (Ul-Haque, 1992). It was also reported that the optimal cellulose concentration for high cellulase production for *A. niger* was 1% (Gautam *et al*., 2010). The present study considerably well agreement with the study conducted by Rashid *et al*., 2009 who reported that the cellulase production by *T. reesei* was reached optimum at 0.5-1.5% cellulose concentration.

The production of cellulase enzyme from *Trichoderma* isolates in shaking state was much higher than in stationary condition under SmF. Shaking state was 2-3 times higher than static condition (Table 3). In agreement with the present work Moussa and Tharwat (2007) reported that Shaking cultures give higher yields of cellulases compared with static ones by *Sclerotium rolfsii*. A study conducted by Fritsche in 1999 (cited in Korish, 2003) production of cellulase in shaking condition becomes higher, because of good oxygen supply, the cultures mix well to the substrates and significant increase in the time of contact and area between the fungal cells and substrates. In the static culture, a layer of mycelium grow at the top of the culture while the substrate remained at the bottom of the flask, which significantly reduced the contact time and area between the fungal cells and substrates.

A number of agricultural wastes have been employed for the cultivation of *Trichoderma* isolates to produce cellulase enzymes. Wheat bran, rice bran and wheat straw gave the highest cellulase production by *Trichoderma* isolates (Table 4). The isolate AUT1 produced maximum amount of cellulase on wheat straw (5.68±0.06U/g), AUT5 on rice bran (8.15±0.065U/g), AUT2 and AUT4 on wheat bran (4.92±0.16U/g and 7.01±0.055U/g), respectively (Table 4). Balaraju *et al*. (2010) stated that the production of cellulase enzymes

in SSF by *Oudemansiella radicata* was maximum when using wheat bran, followed by rice bran. It might be due to the fact that wheat bran contains adequate amount of nutrients like proteins 1.32%, carbohydrates 69%, fats 1.9%, fibers 2.6%, ash 1.8% Ca 0.05%, Mg 0.17%, P 0.35%, K 0.45%, S 0.12%, various amino acids and porosity for oxygen supply (Balaraju *et al*., 2010). However, this work appeared to contradict with the previous results reported by Ravindran *et al.* (2010) that showed cotton seed under SSF condition gave maximum enzyme production at high alkaline pH by *Chaetomium* spp. This may be due to the adsorption of enzymes and the formation of enzyme-substrate complexes that are critical to enzymatic hydrolysis of cellulose Arifolu and Ogel, 2000 (cited in Abo-State *et al.*, 2010).

In the present study, optimum cellulase production was obtained ranging between 55-65% moisture contents (Fig. 10). This result is considerably similar to what was reported by Sun *et al*. (2010) who showed that the optimum moisture content for endo-glucanase production from *Trichoderma* spp. on apple pomace under SSF was 70%. Cellulase production was found to decrease above 70% moisture content. These may be due to higher moisture level decreases porosity, promotes stickiness, change substrate structure, and reduce aeration, which results in lowered oxygen transfer and reduces growth of organism. By contrast, lower moisture content (<45%) reduces solubility of the nutrients, which then become inaccessible to organism (Sun *et al*., 2010).

Incubation time is one of the most important factors affecting the growth and the production of cellulase enzyme by *Trichoderma* isolates under SmF and SSF (Fig. 7 and Table 6). Maximum amount of cellulase enzymes from these isolates (four) were recorded on the 12^{th} day in SmF using pure cellulose. Under SSF, isolates AUT1 and AUT5 showed maximum cellulase production after 11 days of incubation whereas AUT4 and AUT2 showed maximum cellulase enzyme production after 5 and 9 days of incubation, respectively (Table 6). Masbah *et al*. (1983) working on *T. koningii* reported that the cellulase activity reached a plateau after 16 days of incubation under SSF. Khare and Upadhyay (2011) who have reported that the maximum production of cellulases by *T. viride* was observed after 6 days of incubation. While in a study by Sun *et al*. (2010), enzyme activity from apple pomace by *Trichoderma* sp. was maximum at 120 h in SmF. This is probably due to the cease of the growth, the release of simpler sugar and proteases into the medium during the later growth phase (Ishaque and Kluepfel, 1980). Therefore, it is believed that proper cultivation time allows maximum microorganism growth and product formation to a certain degree in a fermentation system.

The time of maximum cellulase production was higher when incubated on pure cellulose compared to agricultural wastes.

In the present study, the cellulase was optimally active in the pH range of 5.5-6.5 and a temperature of 40-60^{o}C; highly stable below 40^{o}C and pH ranges 4.5-6.5 (Fig. 11,12,13 and 14). Most of the fungal cellulase has an optimum pH of 4.0–6.0. Besides, the wide range of optimum pH (pH 5–9) (Kansoh *et al*., 1999). *Trichoderma* and *Aspergillus sp.* has an optimum temperature of 40 - 55°C (Kansoh et al., 1999). Similarly, CMCase by *B. pumilus EB3* was optimally active at pH 6.0 and temperature 60°C. The CMCase also retained its activity over a wide pH range (pH 5.0–9.0) and temperature range (30-70°C) (Ariffin *et al*., 2006). Cellulases which are active in the acidic pH range (pH 4.8-6) are considered to be suitable for industrial application such as stone washing denim (pH 4-7), paper industry (pH 5), animal feed supplement (acidic pH) and textile industry (Temp. 40-50^{o}C and pH 5) (Tolan and Foody, 1999).

7. Conclusions

- This study indicated that *Trichoderma* isolates are capable of producing high activities of CMCase.
- Solid state fermentation provide better yield than liquid state fermentation for cellulase enzyme production
- Wheat straw, wheat bran and rice bran were good substrate for cellulase enzyme production by *Trichoderma* isolates. From economical and environmental point of view agricultural wastes are better substrate for cellulase enzyme production
- The optimum temperature and pH for cellulase enzyme production from *Trichoderma* isolates were 30^{o}C and 5.5, respectively; and yeast extract and peptone were the preferred nitrogen source under SmF and SSF, respectively
- *Trichoderma* cellulase was optimally active in the pH range of 5.5-6.5 and a temperature of 40-60^{o}C; highly stable below 40^{o}C and pH ranges 4.5-6.5

8. Recommendations

- Agro-industrial wastes should be evaluated for the production of cellulase enzymes for bio-ethanol production
- Cellulase enzymes produced from *Trichoderma* isolates should further be purified
- Studies should be required to determine the inhibitory concentration of simple sugars for the production of cellulase by *Trichoderma* isolates.
- Cellulase enzymes should be evaluated for industrial purpose such as textile industry, animal feed and ethanol production.

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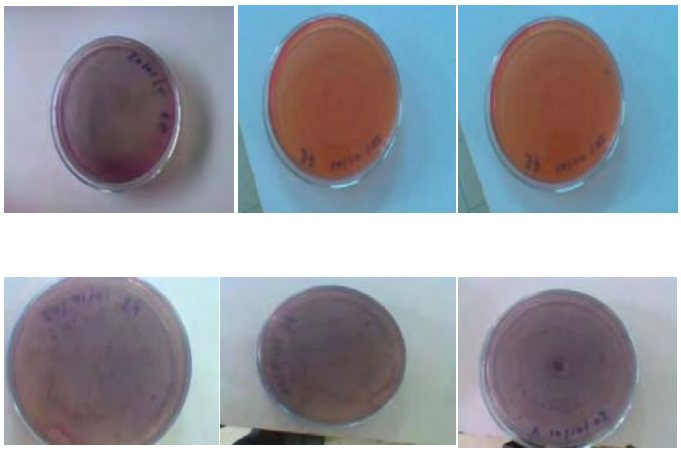
10. Appendix

Screening of cellulase producing Trichoderma isolates by Congo Red reagents



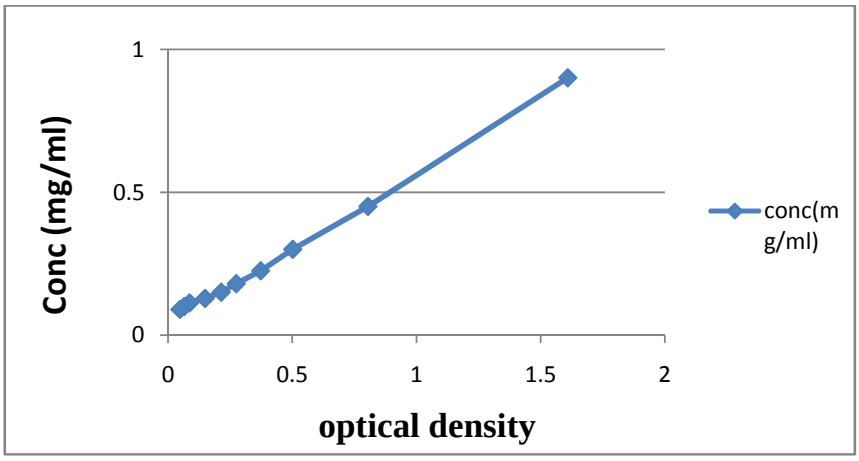


Figure 1. Standard curve of DNS method using glucose

$Y=[0.517x + 0.049]mg/ml$

Where, Y= Concentration of released glucose

X= Optical density (OD)

Declaration

I, the undersigned, declared that this thesis is my original work, has not been presented for a degree in any University and that all sources of materials used for the thesis have been duly acknowledged.

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Date: October,2011