OPTIMIZATION FOR THE PRODUCTION OF CELLULASE ENZYME FROM FUNGUS ISOLATED FROM MUNICIPIAL WASTES

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CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University,

Kirtipur, Kathmandu, Nepal

Garima Bista

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By

Garima Bista

Registration number: 5-3-28-55-2013

Supervisor

Assistant Professor

Mrs. Jarina Joshi

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LIST OF ABBREVIATIONS

BT	Biotechnology
$C_2H_5O_6$	Glucose
$CaCl_2$	Calcium Chloride
$CoCl_2$	Cobalt Chloride
CBH	Cellobiohydrolases
CBM	Carbohydrate binding module
CMC	Carboxymethyl Cellulose
DNS	3, 5 Dinitrosalicylic acid
KH_2PO_4	Dipotassium Hydrogen Phosphate
$KNaC_4H_4O_6.4H_2O$	Potassium Sodium Tartarate
$MgSO_4$	Magnesium Sulphate
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
$Na_3C_6H_5O_7$	Sodium Citrate
$(NH_4)_2SO_4$	Ammonium Sulphate
SmF	Submerged fermentation
SSF	Solid state fermentation
$ZnSO_4$	Zinc Sulphate

ABSTRACT

Cellulases (EC 3.2.1.4) catalyze the hydrolysis of 1, 4-β-D-glycosidic linkages in cellulose and play a significant role in nature by recycling this polysaccharide which is the main component of plant cell wall. This enzyme has enormous potential in industries and is used in food, beverages, textile, laundry, paper, pulp industries etc. Therefore, there has been much research aimed at obtaining new microorganisms producing cellulase enzymes with higher specific activities and greater efficiency.

The study was aimed to reduce the production cost of cellulase by isolating good strain and optimizing the production medium. The research was specifically designed to assess cellulase production from fungus isolated from waste water, moist soil, rice straw, cow dung, rotten dried leaf and goat manure. A total of 9 fungal cultures were isolated, out of which 4 isolates (D3, S6, G4 and S5) showed cellulase activity done in a plate assay method using basal salt media incorporated with 1% CMC. Among the 4 isolates, D3 (isolated from dung sample) showed the highest cellulase activity with largest zone of hydrolysis of 18 mm. From morphological and microscopic test, the fungal strain (D3) was expected to be *Apergillus* species. The assay of cellulase in term of CMCase was performed by measuring the release of reducing sugar (DNS method). Optimization of process parameters was carried out for the isolate (D3) to maximize enzyme yield. On optimization, *Aspergillus* species showed maximum enzyme activity at pH 6 (1.783 IU/ml) and temperature 30°C (3.333 IU/ml) at 120 hours of incubation. Further, bioreactor had been used for the study of cellulase production from isolate D3. Under optimized condition of agitation at 200 rpm and aeration of 1 l/m, isolate D3 showed the highest production in the 6^{th} day (144 hours of incubation) with enzymatic activity of 8.966 IU/ml and 11.166 IU/ml.

Keywords: Fungi, *Aspergillus* species, Cellulase, Municipal wastes, Submerged state fermentation

CHAPTER 1

INTRODUCTION

1.1 Background

Cellulose hydrolysis to glucose requires the effort of exoglucanases, endoglucanases and the β glucosidases. Exoglucanases, which is also referred to as 1, 4-β-D-glucancellobiohydrolase, are usually active on crystalline cellulose and cleave into disaccharide units either from reducing or non-reducing end. Endoglucanases (1, 4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4) are more active against the amorphous regions of cellulose and can also hydrolyze substituted celluloses such as carboxymethyl cellulose (CMC) and hydroxyethyl cellulose (HEC) internally. β Glucosidases (EC 3.2.1.21) is an enzyme that split cellobiose (sugar) and other soluble oligosaccharides to glucose (M.K. Bhat and S Bhat, 1997). These enzymes have various potential applications in the production of fuel, chemical, food, textile, animal feed, pharmaceutical industries and in waste management.

Municipal waste management is one of the key topics in environmental protection (Vergara SE and Tchobanoglous G, 2012). In England, around 23.7 million tons of household waste was generated in 2009/10, which equates to 1036 kg per household (Keeling C, 2011). The current technologies for municipal waste treatment are incineration, landfill, composting and anaerobic digestion. Recently, anaerobic conversion of municipal waste into biogas has attracted growing interest as a promising method to reduce environmental impact and to generate renewable fuel at the same time. However, some municipal wastes contain over 50% lignocellulosic content (Li *S et al*., 2012) and anaerobic digestion may not represent the most efficient process. An alternative approach is to hydrolyze the lignocellulosic component in the municipal waste into simple sugars and then ferment these sugars into ethanol. It was estimated that around 152 l of ethanol could be generated from a ton of processed municipal waste.

The enzymatic hydrolysis of lignocellulose to sugars requires various mixtures of enzymes that include mainly cellulase. Cellulase can be produced by various bacteria such as *Bacillus subtilus, Bacillus circulans* (Ray Ak *et al.,* 2007), *Bacillus sphaericus* JS1 (Singh J *et al.,* 2004), *Cellulomonas flavigena* (Rajoka MI, 2004) and fungi like *Aspergillus niger* (Guruchandran V and Sasikumar C, 2010) and *Trichoderma reesei*. Among all of these cellulase producing bacteria and fungi*, Aspergillus niger* and the mesophilic and filamentous fungi named *Trichoderma reesei* have attracted most attention due to their high cellulase productivity, easy safe use in industry and the availability of their whole genome sequences. Both submerged fermentation and solid state fermentation (SSF)

have been used in cellulase production. Although municipal waste can contain a high lignocellulosic content, there has been limited research into the use of municipal waste for cellulase production. Other research demonstrated the possibility of using municipal waste to produce cellulase via solid state fermentation (SSF) but by using raw (not autoclaved) municipal waste (Stutzenberger FJ, 1971; Gautam *et al.,* 2011). Cellulolytic fungi isolated from this source have been used to convert cellulosic materials into valuable compounds such as ethanol and organic acid. Some of these fungi are mesophiles whereas others are thermotolerant. Usually the thermotolerant elaborate enzymes are more active at high temperature and more thermostable than enzymes produced by their mesophilic counterparts. The biological degradation of cellulose has a great importance in the activity of living system. Many cellulolytic waste products which otherwise are inedible not potable for human consumption are converted into useful products by the aid of microorganisms. Cellulolytic organism can convert cellulose to various economically important products and monomeric sugars, single cell protein, antibiotics and compost for the use of people. The utilization of cellulosic material has now contributed to the production of food and energy (A.E. Humphery *et al*., 1977).

Microbial enzymes have the enormous advantage of being able to be produced in large quantities by established fermentation techniques. Enzyme production is closely controlled in microorganisms and therefore, to improve its productivity, these controls can be exploited and modified. To establish a successful fermentation process, it is necessary to make the microorganism for overproduction of the desired metabolite. An elaborate investigation is therefore required to establish the optimum condition to scale up enzyme production in an individual fermentation process. Several researchers have shown that the production costs of cellulase are very much tightly associated with the productivity of enzyme producing microbial strains (P.S. Chahal *et al.,* 1992; K. Reczey *et al.,* 1996; P.F. Omojasola and O.P. Jilani, 2008). Such methods would provide assistance to alleviate deficit of food and animal feeds, solve modern waste disposal problem and diminish man's dependence on fossil fuels by providing a convenient and renewable source of energy in the form of glucose which can then be used for the production of various organic acids, ethanol and several other chemicals (J. H. J. R. Makoi and P. A. Ndakidemi, 2008).

The present study was aimed to examine maximum production of cellulase enzyme using cellulase producing fungal strain from waste samples to mitigate high cost of cellulase for degradation of organic wastes.

1.2 Current studies

The plant kingdom produces lignocellulosic biomass to the tune of 10–50 billion tons per annum worldwide (Sticklen, 2006). This renewable biomass can be hydrolyzed to pentose and hexose sugars that serve as building blocks for various industrial products (Chandrakant and Bisaria, 1998; Lynd *et al.,* 2005; Bevan and Franssen, 2006; Bayer *et al.,* 2007; Hatti-Kaul *et al.,* 2007; Madhavan *et al.,* 2012; Seiboth *et al.,* 2012). For this, enzymatic methods to hydrolyze the plant biomass are preferred due to non-inhibitory by-products and nontoxic effluents. Hydrolytic enzymes like cellulases convert lignocelluloses to sugars that can be fermented by various microbes to biofuels and other value-added products. The relatively high cost of these enzymes remains a major barrier to their commercial application in any bioindustry, although significant reduction in the cost of these enzymes has been made in recent years. The focus areas of research have been to improve the efficiency of known enzymes, identify new and more active enzymes, find optimized enzyme mixes for pretreated lignocelluloses and reduce the cost of enzyme production (Merino and Cherry, 2007).

For the conversion of plant biomass into various bioproducts, a significant bottleneck is enzymatic hydrolysis of lignocelluloses to soluble sugars. These sugars are metabolized through various natural and engineered pathways toward the products of interest. The success of projected biorefinery processes depend to a large extent on the economics of hydrolytic enzyme production. Presently, mesophilic fungal strains like *Trichoderma reesei* and *Aspergillus niger* produce cellulases at an industrial scale. Various recombinant cellulases have been successfully expressed in industrial strains which can improve the economics due to their high specificities for targeted bioproducts. In addition, designer cellulosomes and xylanosomes are expected to make the hydrolytic enzymes more effective because of efficient surface binding and direct action on lignocelluloses (Biswas *et al.,* 2014).

1.3 Research hypothesis

Because of diverse nature, fungal species of Nepal may have adapted in different ways and some of these species may be an efficient cellulase producer. Study of cellulase from such organism to produce potent cellulase enzyme and characterization of such enzyme may be applicable for variety of industrial purposes.

1.4 Objectives

1.4.1 General objectives

To select potent cellulolytic fungus from waste samples and optimize cellulase enzyme productivity.

1.4.2 Specific objectives

1. To screen potent cellulolytic fungus from cellulosic waste samples.
2. To identify the fungus by morphological study.
3. To optimize cellulase enzyme production efficiency using potent strain.
4. To develop more promising protocols for large scale production.

1.5 Rationale

Cellulose is the most common organic polymer representing about 1.5 x 10^{12} tons of the annual biomass production through photosynthesis and is the most abundant and renewable biopolymer on earth and the dominating waste material from agriculture. Cellulase provides a key opportunity for achieving tremendous benefits of biomass utilization. The growing interest toward the conversion of lignocellulosic biomass into fermentable sugars has generated an additional request for cellulases and their related enzymes. Bioconversion of lignocellulose is initiated primarily by microorganisms which are capable of degrading lignocellulosic materials. However, in the present scenario, the main requisite of these enzymes-based bioconversion technologies are reaction conditions and the production cost of the related enzyme system. Therefore, much research should be aimed at obtaining new microorganisms producing cellulase enzymes with higher specific activities and greater production efficiency.

Fungi have evolved to be the most powerful and prevalent biomass degrading organisms in nature exhibiting a diverse range of lifestyles for the turnover of lignocellulosic material on earth. Given their significant activity and ability to be readily produced at high titers on the industrial scale, fungal cellulase cocktails are an excellent starting point for industrial biorefinery applications. Indeed, industrial-scale lignocellulosic biorefineries, mostly slated to produce bioethanol at this point are beginning to come online worldwide at this time of this review.

1.6 Scope

Life on Earth depends on photosynthesis, which results in production of plant biomass having cellulose as the major component. The carbon cycle is closed primarily as a result of the action of cellulose-utilizing microorganisms present in soil and the guts of animals. Thus, microbial cellulose utilization is responsible for one of the largest material flows in the biosphere and is of interest in relation to analysis of carbon flux at both local and global scales. The importance of microbial cellulose utilization in natural environment is further enhanced by the status of ruminants as a major source of dietary protein. Finally, microbial cellulose utilization is also an integral component of widely used processes such as anaerobic digestion and composting.

Plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity. Cellulosic materials are particularly attractive in this context because of their relatively low cost and plentiful supply. The central technological impediment to more widespread utilization of this important resource is the general absence of low-cost technology for overcoming the recalcitrance of cellulosic biomass. A promising strategy to overcome this impediment involves the production of cellulolytic enzymes, hydrolysis of biomass, and fermentation of resulting sugars to desired products in a

single process step via a cellulolytic microorganism or consortium. Such “consolidated bioprocessing” (CBP) offers very large cost reductions if microorganisms can be developed that possess the required combination of substrate utilization and product formation properties.

CHAPTER 2

LITERATURE REVIEW

2.1 Lignocelluloses

Lignocellulose refers to the complex of lignin and cellulose present in the cell walls of woody plants. It is actually the plant dry matter (biomass) and hence called as the lignocellulosic biomass. Most abundant available raw material on earth, it is used for the production of biofuels, mainly bio-ethanol. These lignocelluloses are structural polysaccharides of plants and are composed of cellulose (~50%), hemicellulose (~30%), and lignin (~20%). These lignocelluloses are widely distributed among the vascular plants. Cellulose and hemicellulose are polysaccharides composed of simple sugars whereas, lignin is a complex network of aromatic alcohols. Hemicelluloses and lignin provide an amorphous matrix to which crystalline cellulose microfibrils are distributed. Cellulose microfibrils are stabilised by intra and inter-molecular hydrogen bonds and are surrounded by hemicellulosic polymers. The cellulose-hemicellulose matrices are protected by lignin, an amorphous insoluble organic polymer which supports the tissues of vascular plants and prevent the microbial attack on internal cellulosic structures (Howard R *et al.,* 2003).

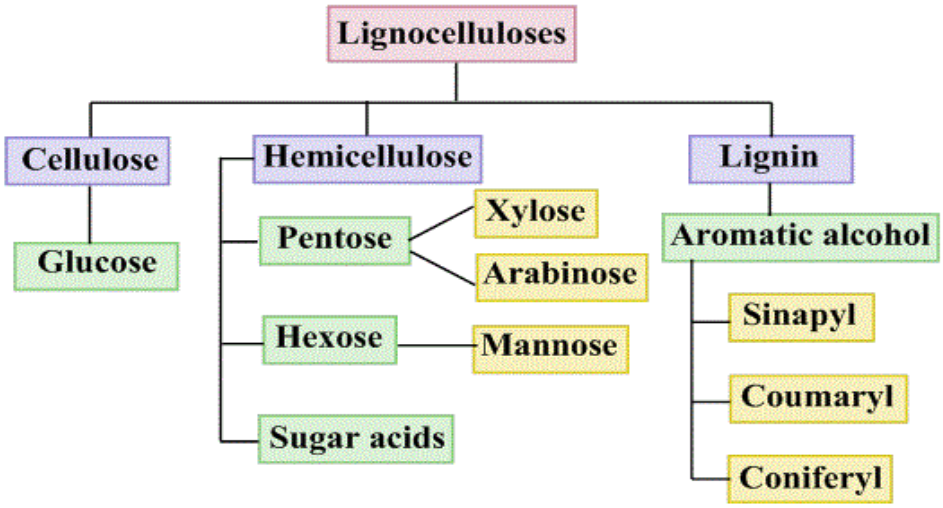

Figure 1: Schematic representation showing the contents present in the lignocelluloses.

2.1.1 Lignin

Lignin is the most complex polymer made up of phenylpropaneunits such as coumaryl alcohol, coniferyl alcohol and sinapyl alcohol linked in a three-dimensional structure which makes it difficult to degrade. Lignin component of soft woods contain more than 90% of coniferyl alcohol and that of hardwood is composed of coniferyl and sinapyl

alcohol in varying proportions (Harmsen P. *et al.*, 2010). They provide rigidity to the plant body and help in liquid transport and prevention of microbial attack.

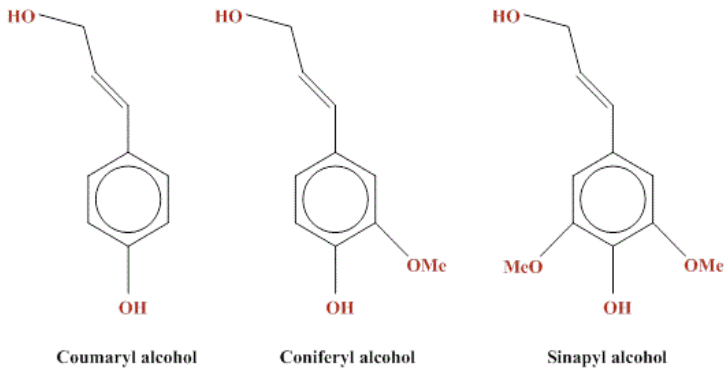

Figure 2: Three phenylpropane monomers in lignin.

2.1.2 Hemicellulose

Hemicellulose is the second most abundant part (15-35%) of lignocellulosic biomass and are heterogeneous polymers of sugar acids, pentoses (including arabinose and xylose) and hexoses i.e. the glucose, galactose and the mannose. These are highly branched polymers and lack crystalinity. In nature, the composition of hemicellulose is uneven which depends on the nature of plant source. In case of soft woods (e.g., gymnosperm) like spruce and pine, hemicellulosic part is composed mainly of mannan, especially glucomannan and galactoglucomannan whereas, secondary walls of hardwood (e.g., angiosperm) and herbaceous plants constitutes chiefly of xylans.

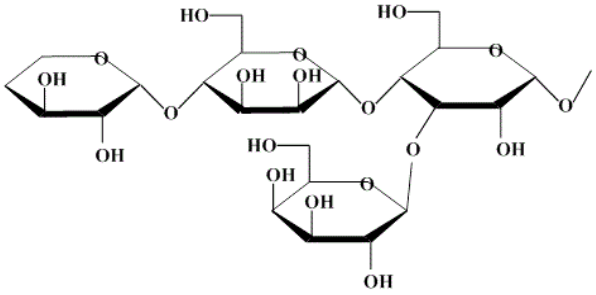

Figure 3: Structure of hemicelluloses

2.1.3 Cellulose

Cellulase is a group of enzyme that is produced chiefly by the fungi, bacteria and protozoans. These are the type of enzymes which hydrolyze cellulose on acting together. Cellulose is the main component in the plant cell walls and is most widely distributed on earth. As the most abundant carbohydrate, cellulose is one of the most important cabon sources in the biosphere. Cellulose is a polysaccharide with the formula $(C_6H_5O_{10})n$, where n ranges from 500 to 5,000 depending on the source of polymer consisting of a linear chain of several to over ten thousand β(1→4) linked D glucose units. Due to the strong hydrogen bonds that are found between cellulose chains, the strength helps in

the formation of cellulose base fiber through the association of hydrogen bonds. These fibers are composed of long parallel chains of these molecules. The chains are interlinked with each other by hydrogen bonds between the hydroxyl groups of adjacent molecules leading to the formation of crystalline regions of cellulose. A parallel orientation of adjacent chains is also favored by the intermolecular hydrogen bonds. Although, an individual hydrogen bond is relatively weak, many of such bonds acting together can impart great stability to certain conformations of large molecules. Reduction in the density during the pretreatment process results in the downfall of combination which increases the gap between the molecules, variation in the orientation and the formation of amorphous regions of cellulose (Filson and Dawson-Andoh, 2009). The intramolecular hydrogen bonds also exist in between cellulose chains. The geometry of the short carbon hydrogen bonds minimizes the distance between layers and therefore Vander Waals forces are maximized. These bundles are then crystallized into fibers by the same side-to-side hydrogen bonding and layer to layer Vander Waals forces. Hence, due to its robust crystal structure, cellulose is resistant to degradation (Perez and Samain, 2010).

Cellulose is one of the most abundant organic sources of food, fuel and chemicals. However, its usefulness is dependent upon its hydrolysis to glucose. Acid and high temperature degradation is unsatisfactory in that the resulting sugars are decomposed. Enzymatic degradation (cellulase) is one of the most effective means of degrading cellulose into its useful components. Although cellulases are distributed throughout the biosphere, they are most prevalent in fungal and microbial sources.

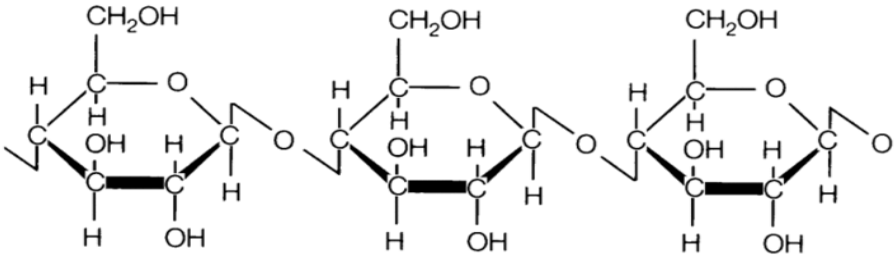

Figure 4: Structure of cellulose

2.2 Cellulase and their mode of action

Cellulase includes group of enzymes which hydrolyze cellulose including exoglucanase, endoglucanase and β-glucosidase (i.e the cellulase complex). The catalytic modules of cellulases have been classified into numerous families based on their amino acid sequences and crystal structures (Henrissat, 1991). Cellulase molecules generally have a catalytic domain, cellulose binding domain and the connecting bridge (linker). Cellulases contain noncatalytic carbohydrate-binding modules (CBMs) and other functionally known or unknown modules which may be located at the N- or C-terminus of a catalytic

module. It actually subdivides β-1, 4 endoglucanases (EG I, II, III and V), β-1, 4-cellobiohydrolases (CBH I and II), xylanases (XYN I and II), β-glucosidase, a-Larabinofuranosidase, acetyl xylan esterase, β-mannanase and α-glucuronidase (Lenting and Warmoeskerken, 2001). To hydrolyze and metabolize insoluble cellulose, the microorganisms must secrete the cellulases (possibly except BG i.e. the β-glucosidase) that are either free or cell-surface-bound. As a result, cellulose can be usefully degraded to glucose with this enzyme in synergistic action. A large number of bacteria, fungi and actinomycetes are known to degrade cellulose. In addition to the cellulase hydrolysis of cellulose into glucose and other active ingredients, plant cell contents improve the extraction rate by increasing the permeability of plant cell walls. So, cellulose is widely used in plant based raw materials for industrial as well as agricultural production.

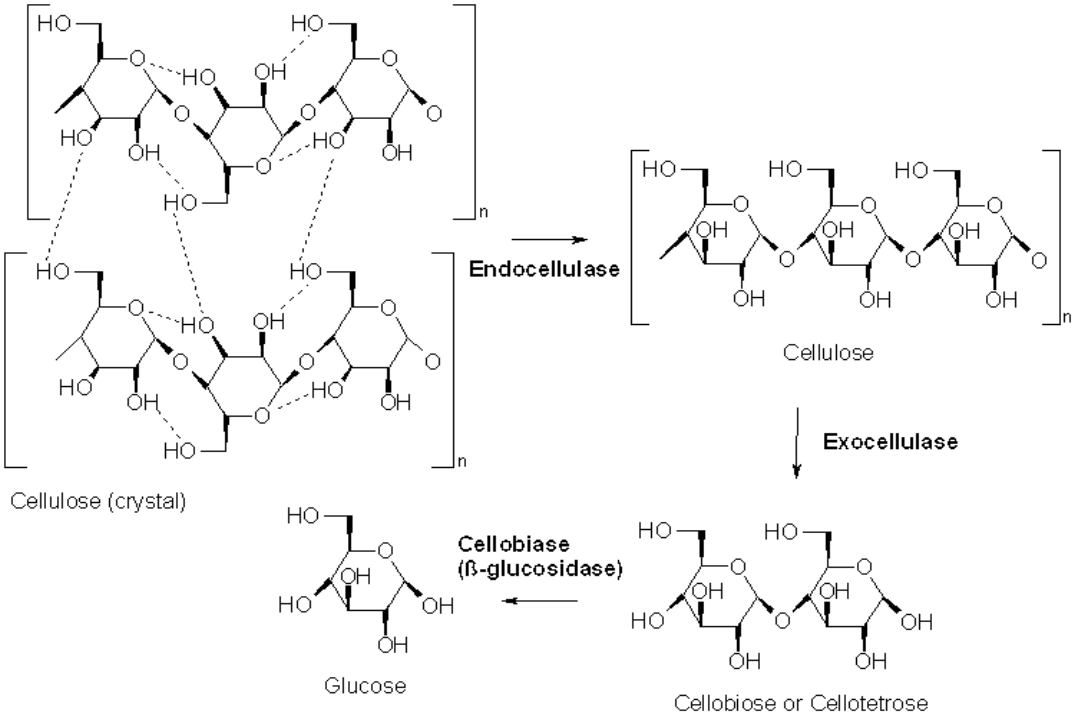

Figure 5: The three types of reaction catalyzed by cellulases- 1. Breakage of the noncovalent interactions present in the amorphous structure of cellulose (endocellulase). 2. Hydrolysis of chain ends to break the polymer into smaller sugars (exocellulase). 3. Hydrolysis of disaccharides and tetrasaccharides into glucose (β-glucosidase).

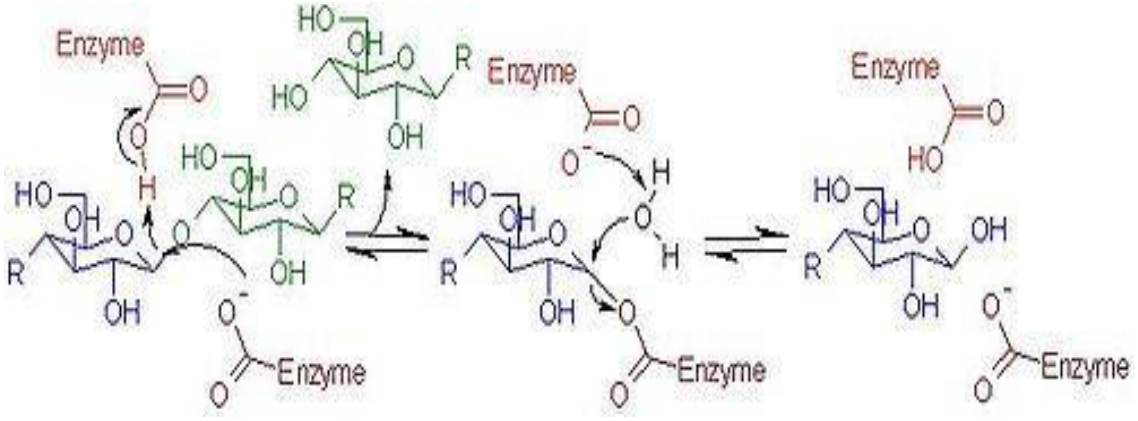

Figure 6: βeta-glucosidase activity of cellulase

Cellulase activity is determined by its effect on microcrystalline cellulose with respect to glucose formation**.** Cellulase activity assays can be classified into initial rate assays and end-point assays (Zhang *et al.,* 2009). The former is the same as regular enzyme assays on soluble substrates. The latter is specifically designed for cellulase. Taking filter paper activity as an example, it requires the release of a fixed amount of soluble sugars (i.e., 4 mg glucose measured by the DNS assay with glucose as reference) within a fixed time (i.e., 1 hour) from 50 mg of Whatman No. 1 filter paper. The enzyme solution needs a series of dilution for this assay.

Also, cellulase samples can be individual samples or their mixture; the substrates can be soluble or insoluble; the substrates can be soluble cellulose derivatives (e.g., carboxymethylcellulose [CMC]), or pure cellulose (microcrystalline, regenerated amorphous cellulose, bacterial microcrystalline cellulose, filter paper) or pretreated biomass. So many variations in experimental conditions from the enzymes to substrates, the complicated relationship between physical heterogeneity of the cellulosic materials and the complexity of cellulase enzyme systems (synergy and/or competition) result in great challenges in cellulase activity assays (Ghose, 1987; Wood and Bhat, 1988; Zhang and Lynd, 2006; Zhang *et al.,* 2009).

2.2.1 Endocellulase

Endoglucanase or CMCase act by cleaving internal β-glycosidic bonds in the cellulose chain thereby making chain ends accessible to cellobiohydrolase. Different endoglucanases are produced by archaea, bacteria, fungi, plants and animals with different catalytic modules belonging to families 5–9, 12, 44, 45, 48, 51 and 74. Fungal endoglucanases in general possess a catalytic module with or without a CBM (carbohydrate binding module), while bacterial endoglucanases may possess multiple catalytic modules, CBMs and other modules with unknown function. The catalytic modules of most endoglucanases have a cleft/grove-shaped active site which allows the endoglucanases to bind and cleave the cellulose chain to generate glucose, soluble

cellodextrins or insoluble cellulose fragment. However, some endoglucanases can act processively based on their ability to hydrolyze crystalline cellulose and generate the major products as cellobiose. These enzymes are inactive against crystalline celluloses such as cotton or avicel (Sajith *et al.,* 2016).

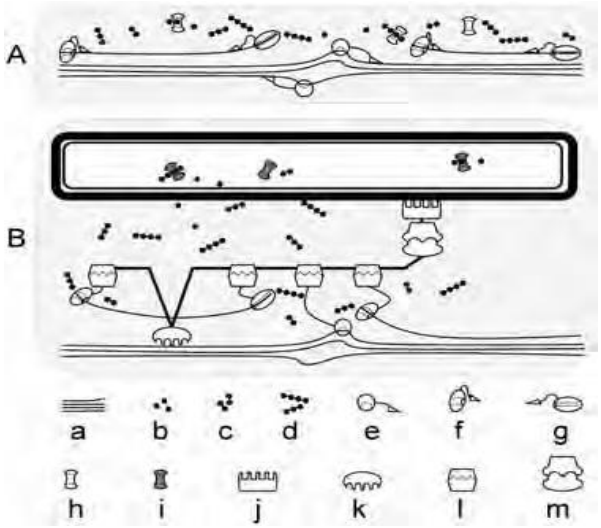

Figure 7: Schematic representation of the hydrolysis of cellulose by noncomplexed (A) and complexed (B) cellulase systems. a, cellulose; b, glucose; c, cellobiose; d, oligosaccharides; e, endoglucanase with carbohydrate-binding module (CBM); f, exoglucanase (acting on reducing ends) with CBM; g, exoglucanase (acting on nonreducing ends) with CBM; h, β -glucosidase; i, cellobiose/cellodextrinphosphorylase; j, S-layer homology module; k, CBM; l, type-I dockerin–cohesion pair; m, type-II dockerin–cohesin pair.

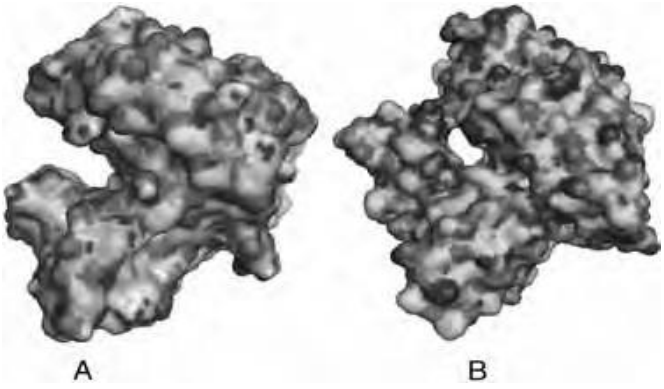

Figure 8: Crystal structures of family 6 endoglucanase and exoglucanase. (A) The structure of endoglucanase Cel6A of *Thermobifida fusca* (PDB code: 1TML), which exhibits a deep cleft at the active site. (B) The structure of exoglucanase Cel6A of *Humicola insolens* (PDB code: 1BVW) in which the active site of it bears an extended loop that forms a tunnel.

2.2.2 Cellobiases or beta-glucosidases

Cellobiases hydrolyzes cellobiose and short chain cellooligosaccharides to glucose but has no effect on cellulose. While it rapidly hydrolyzes cellobiose and cellotriose, its rate of attack decreases markedly with an increasing degree of polymerization. This is in contrast to exo-β-1, 4 glucanas, which acts preferentially on longer cellooligosaccharides. BGs (β Glucosidases) degrade cellobiose which is a known inhibitor of CBH (Cellobiohydrolases) and endoglucanase. Different BGs (β Glucosidases) are produced by various archaea, bacteria, fungi, plants and animals with different catalytic modules belonging to families 1, 3 and 9. Based on either experimental data or structural homology analysis, the stereochemistry of families 1 and 3 BGs (β Glucosidases) is of the retaining type while the stereochemistry of family 9 BG (β Glucosidases) is of the inverting type. Most of the time aerobic fungi produce extracellular BGs and anaerobic bacteria keep their BGs (β glucosidases) in cytoplasm. BGs (β Glucosidases) have active site which is pocket-shaped allowing them to bind the nonreducing glucose unit and clip glucose off from cellobiose or cellodextrin.

2.2.3 Exoglucanases

Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains liberating either cellobiose or glucose as major products. The exocellulases or exoglucanases are also known as cellodextrinases and act on the terminals of oligosaccharide chains generated by endocellulases releasing glucose or cellobiose. Thus, cellulases are capable of breaking the glycosidic bonds of cellulose microfibrils resulting in the release of oligosaccharides and improving the digestibility of cellulose (Dillon, 2004). Exoglucanases effectively work on microcrystalline cellulose by peeling cellulose chains from the microcrystalline structure (Teeri, 1997). CBH (Cellobiohydrolases) is the most-studied exoglucanase and are produced by many bacteria and fungi with catalytic modules belonging to families 5, 6, 7, 9, 48 and 74 glycoside hydrolases. Aerobic fungal CBHs (Cellobiohydrolases) are in families 6 and 7 only, aerobic bacterial CBHs are in families 6 and 48, anaerobic fungal CBHs are in family 48 and anaerobic bacterial CBHs are in family 9 as well as 48. In other words, family 7 CBHs only originate from fungi and family 48 CBHs mostly originate from bacteria. The most significant topological feature of CBHs' catalytic module is the tunnel structure which is formed by two surface loops (Figure 8 B). The tunnel may cover the entirety (e.g., family 7 CBH) or part of the active site (e.g., family 48 CBH). Figure 8 shows the crystal structures of family 6 endoglucanase and exoglucanase. These two enzymes share the similar folding. However, the active site of endoglucanase is the deep cleft structure while it is a tunnel for exoglucanase. The tunnel shaped active site of exoglucanase system enables the enzyme to hydrolyze cellulose in a unique processive manner (Koivula *et al.,* 2002; Vocadlo and Davies, 2008). The glycoside hydrolase family

48 exoglucanases are widely believed to play important roles in crystalline cellulose hydrolysis mediated by bacterial cellulase systems which is somewhat similar to that of the *Trichoderma* CBHI (Cel7A). Family 48 exoglucanases is dominant catalytic components of cellulosomes such as *Clostridium cellulolyticum* CelF (Reverbel-Leroy *et al.,* 1997), *Clostridium cellulovorans* ExgS (Liu and Doi, 1998), *Clostridium jusui* CelD (Kakiuchi *et al.,* 1998), and *Clostridium thermocellum* CelS (Kruus *et al.,* 1995; Wang *et al.,* 1994) or as key noncomplexed cellulase components, such as *Cellulomonas fimi* CbhB (Shen *et al.,* 1995), *Clostridium stercorarium* Avicelase II (Bronnenmeier *et al*., 1991), *Thermobifida fusca* Cel48 (Irwin *et al*., 2000), *Paenibacillus barcinonensis* BP-23 Cel48C (Sánchez *et al.,* 2003) and *Ruminococcus albus* 8 Cel48A (Devillard *et al.,* 2004). For example, *C. thermocellum* CelS accounts for ~30% of the weight of the cellulosome isolated from the Avicel-grown culture but its level decreases to ~10% of the weight of that isolated from cellobiose-grown culture implying that CelS plays a key role for crystalline cellulose hydrolysis (Bayer *et al.,* 1985; Zhang and Lynd, 2005).

2.2.4 Oxidative cellulases

These enzymes depolymerize cellulose by radical reactions, as for instance cellobiose dehydrogenase (acceptor).

2.2.5 Cellulose phosphorylases

Cellulose phosphorolysases depolymerize cellulose using phosphates instead of water.

Avicelase has almost exclusively exo-cellulase activity since avicel is a highly micro-crystalline substrate.

Within the above types of cellulases, there are also progressive (also known as processive) and nonprogressive types. Progressive (processive) cellulase will continue to interact with a single polysaccharide strand while the non progressive cellulase will interact once then disengage and engage another polysaccharide strand.

Cellulase action is considered to be synergistic as all three classes of cellulase can yield much more sugar than the addition of all three separately. Aside from ruminants, most animals (including humans) do not produce cellulase in their bodies and can only partially break down cellulose through fermentation limiting their ability to use energy in fibrous plant material.

2.3 Measurement of cellulase activity

As the native substrate, cellulose is a water-insoluble polymer and the traditional reducing sugar assays using this substrate can not be employed for the measurement of cellulase activity. Analytical scientists have developed a number of alternative methods.

2.3.1 Viscometric substrates

The enzymatic activity of cellulase has been estimated by the decrease of viscosity of hydroxyethylcellulose solution as a function of incubation time. The decrement is a function of the applied shear force (Joos *et al.,* 1969). A viscometer is a device that can be used to measure the decrease in viscosity of a solution containing a cellulose derivative that is water soluble, for example: carboxymethyl cellulose (CMC) upon incubation with a cellulase sample. The decrease in viscosity is directly proportional to the cellulase activity. While such assays are very sensitive and specific for endo-cellulase (exo-acting cellulase enzymes produce little or no change in viscosity), they are limited by the fact that it is hard to define activity in conventional enzyme units (i.e. the micromoles of substrate that is hydrolyzed or product produced per minute).

2.3.2 Cellooligosaccharide substrates

Oligosaccharides are widely used as chromatographic standards for HPLC/HPAEC-PAD analysis or as defined substrates in enzyme characterisation studies. The lower DP cello-oligosaccharides (DP2-6) are sufficiently soluble in water to act as viable substrates for cellulase enzymes (Telke *et al.,* 2013). However, as these substrates are themselves reducing sugars, they are not suitable for use in traditional reducing sugar assays because they generate a high blank value. However, their cellulase mediated hydrolysis can be monitored by HPLC (High performance liquid chromatography) or IC (Ion chromatography) methods to gain valuable information on the substrate requirements of a particular cellulase enzyme.

2.3.3 Reduced cellooligosaccharide substrates

Borohydride reduced oligosaccharides are also available in certain classes for the specific purpose of successfully performing reducing sugar assays in which the native oligosaccharides themselves being reducing sugars, can produce a high blank absorbance value thereby decreasing the sensitivity of the assay. Cello-oligosaccharides can be chemically reduced through the action of sodium borohydride to produce their corresponding sugar alcohols. These compounds do not react in reducing sugar assays but their hydrolysis products do. This makes borohydride reduced cello-oligosaccharides valuable substrates for the assay of cellulase.

2.3.4 Dyed polysaccharide substrates

Dyed polysaccharide substrates are used for the evaluation and identification of enzyme activities (McClearry, 1980). These substrates can be subdivided into two classes:

2.3.4.1 Insoluble chromogenic substrates

High purity dyed and crosslinked insoluble chromogenic substrates are used for the identification of enzyme activities in research, microbiological enzyme assays and in

vitro diagnostic analysis. An insoluble cellulase sustrate such as AZCL-HE-cellulose absorbs water to create gelatinous particles when placed in solution which is gradually depolymerised and solubilised by the action of cellulase. The reaction is terminated by adding an alkaline solution to stop enzyme activity and the reaction slurry is filtered or centrifuged. The colour in the filtrate or supernatant is measured to determine the enzyme activity.

2.3.4.2 Soluble chromogenic substrates

Soluble chromogenic substrates for the assay of enzyme are advantageous for rapid analysis of large amount of samples and also permit the evaluation of activities of enzymes (Biely *et al*., 1985). The enzyme activity can be measured by incubating cellulase sample with a water-soluble substrate such as azo-CM-cellulose. The reaction is then terminated and high molecular weight partially hydrolysed fragments are precipitated from solution with an organic solvent such as ethanol or methoxyethanol. The suspension is then mixed thoroughly, centrifuged and the colour in the supernatant solution (due to small soluble dyed fragments) is measured. With the help of a standard curve, the activity can be determined.

2.3.5 Enzyme coupled reagents

New reagents have been developed recently that allow the specific measurement of endo-cellulase (McCleary *et al.,* 2014; Mangan *et al.,* 2014). It involves the use of functionalised oligosaccharide substrates in the presence of an ancillary (supporting) enzyme. In the enzyme coupled reagents, a cellulase enzyme is able to recognise the trisaccharide fragment of cellulose and cleave its unit making the ancillary enzyme present in the reagent mixture (β-glucosidase) to hydrolyse the fragment containing the chromophore or fluorophore. The assay is then terminated by the addition of a basic solution that stops the enzymatic reaction and deprotonates the liberated phenolic compound to produce the phenolate species. The cellulase activity of a given sample is directly proportional to the quantity of phenolate liberated, which can be measured using a spectrophotometer. The acetal functionalisation on the non-reducing end of the trisaccharide substrate prevents the action of the ancillary β-glucosidase on the parent substrate.

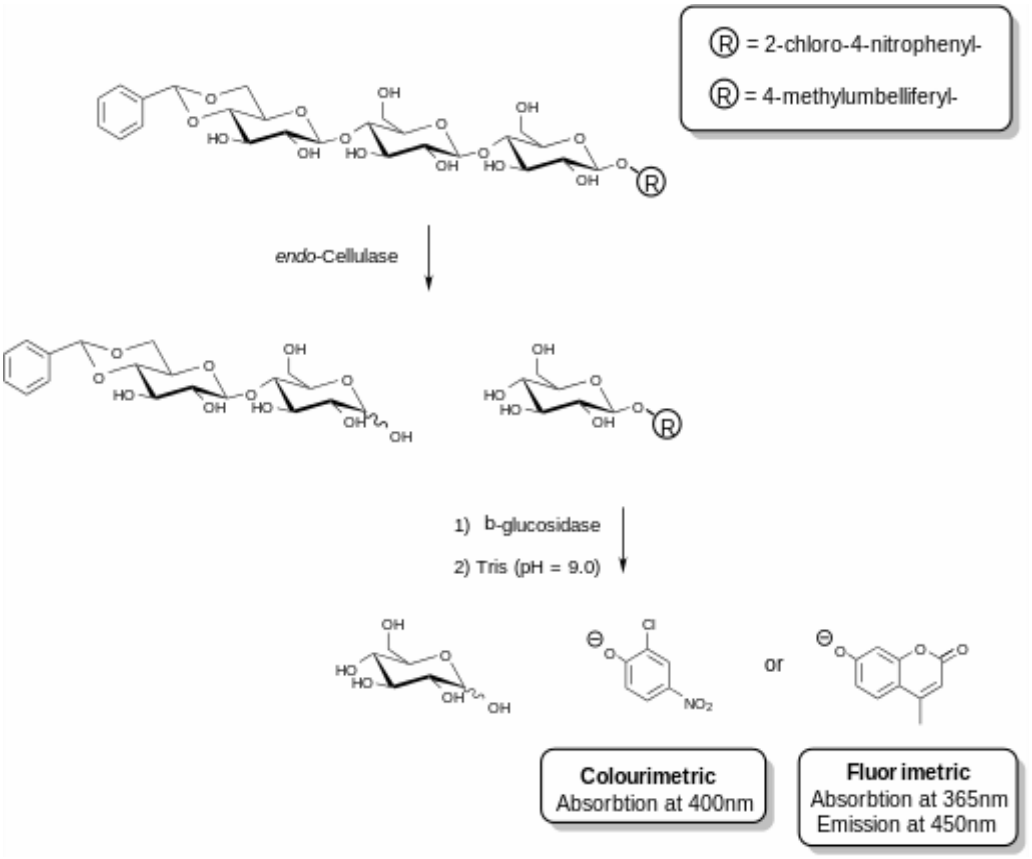

Figure 9: Colourimetric and fluorimetric cellulase substrates in the presence of ancillary β-glucosidase for the specific measurement of endo-cellulase activity.

2.4 Industrial Applications of Cellulases

Cellulases have a wide range of enormous potential applications in biotechnology and many thermo stable endoglucanase appeared to have a great potentiality for industrial use. In most of the cases they are used with hemicelluloses, pectinases, ligninase and allied enzymes. Details of most promising applications are discussed below.

2.4.1 Cellulase in food processing industries

Enzyme infusion has the potential of producing fruit and vegetable juices which is very important from commercial standpoint. The production of fruit and vegetable juices requires methods for extraction, clarification and stabilization. The production of juice from wholefruits involve crushing to pulp mash which after mechanical processing (pressing, centrifuging and filtering) resulted into a clear fruit juice and a solid phase called pomace (Galante *et al.,* 1998). Use of macerating enzymes (composed of cellulase and pectinase) not only improves the cloud stability and texture of nectars and purees, but also decreases their viscosity rapidly. Moreover, texture, flavor and aroma properties of fruits and vegetables can be improved by reducing excessive bitterness of citrus fruits by infusion of enzymes such as pectinases and β-glucosidases.

2.4.2 Cellulase in wine and brewery industries

Bioconversion of cellulosic materials to bioalcohol involves a multistep process which first uses cellulolytic enzymes for hydrolysis of polymers to pentose/hexose sugars and fermentation followed by distillation of these sugars into ethanol. In the beer wort production, Pajunen (1986) opined that the enzyme preparation from *Trichoderma* was the best as judged by its cost and performance ratio. In an earlier study, Oksanen *et al*. (1985) observed that the endoglucanase II and the cellobiohydrolase (CBH) II of the *Trichoderma* cellulase system were responsible for a maximum reduction in the degree of polymerisation and wort viscosity. Significant and reproducible improvements in grape pressability, settling rate and total juice yield were achieved using a combination of macerating enzymes which were noticeable only with a correct balance of pectinolytic, cellulolytic and hemicellulolytic enzymes. Using three varieties (Soave, Chardonnay and Sauvignon) of white grapes from Northern Italy, Galante *et al*. (1998) assessed the performance of Cytolase 219 (a commercial enzyme preparation derived from *Trichoderma* and *Aspergillus* containing pectinase, cellulase and the hemicellulase) in wine making. They reported a 10-35% increase in the extraction of the first wine must, a 70-180% increase in the must filtration rate, significant improvement in wine stability, 50–120 min decrease in pressing time, 30-70% decrease in must viscosity and 20-40% energy saving during cooling of fermenters.

2.4.3 Cellulase in textile industries

Enzymes have been used in the leather industry for many years and more recently have been introduced into modern textile industries. The textile industries take advantage of both complete and individual cellulase components to achieve partial cellulose hydrolysis and improve fabric properties where the cellulase would act upon the fibre to reduce the cell wall thickness and would make the fibre more flexible and collapsible. Commercial cellulases have also been shown to enhance the whiteness, brightness and color characteristics of cotton fabrics (Csiszar *et al*., 1998).

2.4.4 Cellulase in detergent industries

Cellulase preparations capable of modifying cellulose fibrils can improve color brightness, feel and dirt removal from the cotton blend garments. The industrial application of alkaline cellulases as a potential detergent additive is being actively pursued with a view to selectively contact the cellulose within the interior of fibers and remove soil in the interfibril spaces in the presence of the more conventional detergent ingredients. Cellulose fibers in the modern textile industry enzymes are used increasingly in the finishing of fabrics and clothes. The cellulases are applied to remove the rough perturbance that protrudes out from the fabric and produces a smoother, glossier and brighter-colored fabric (Kuhad *et al.,* 2011).

2.4.5 Applications of cellulase in pulp and paper industries

The use of cellulases along with xylanase and ligninase in the pulp and paper industries has increased considerably during last decade. Refining of primary or secondary fibers can generate small particles (fines) that can reduce the drainage rate of pulps during paper making operations. Cellulases seem to preferentially attack and hydrolyze the fines produced during the refining operation and therefore improve the pulp's drainage property. Cellulase and hemicellulases helps in modification of coarse mechanical pulp and handsheet strength properties, partial hydrolysis of carbohydrate molecules and the release of ink from fibre surfaces which results into deinking of recycled paper. Cellulases have also been used to remove ink from papers and to enhance papermaking properties of recycled fibers.

2.4.6 Application of cellulase in animal feed

Cellulases have potential application in animal feed industry consumed by poultry, pigs, ruminants as well as pet and fish farming. The successful use of these enzymes in animal diet is to eliminate Anti-Nutritional Factors (ANF) present in grains or vegetables and degrade certain cereal components in order to improve the nutritional value of feed and to supplement animals own digestive enzymes (e.g., proteases, amylases and glucanases). Moreover, cellulases and hemicellulases are responsible for partial hydrolysis of lignocellulosic materials, dehulling of cereal grains, hydrolysis of β-glucans and better emulsification and flexibility of feed materials which results in the improvement in the nutritional quality of animal feed (Chesson, 1987; Cowan, 1996; Galante *et al*., 1998). Cellulases, hemicellulases and pectinases are responsible for the expression of preferred genes in ruminant and monogastric animals for high feed conversion efficiency.

2.4.7 Application of cellulases in research and development

Mixture of different cellulase along with hemi-cellulase and pectinase have immense potential and application in research and development area for controlling plant diseases and enhancing plant growth. A cocktail of different cellullases, hemicellulases and pectinases results in the solubilisation of fungal or plant cell wall to produce protoplast (Beguin and Aubert, 1994; Bhat and Bhat, 1997). Cellulases and related enzymes are used in the biocontrol of plant pathogens and different plant diseases by inhibiting the germination of spores of the plant pathogens.

2.4.8 Application in waste utilization

Cellulose is the major part of plant biomass. The wastes generated from forests, agricultural fields and agro industries contain a large amount of unutilized or

underutilized cellulose. The wastes generally accumulate in the environment causing various problems of pollution (Abu *et al*., 2000). Nowadays, these wastes are converted into valuable products such as enzymes, sugar, biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds and human nutrients which are accomplished by cellulase.

2.5 Cellulase production by fermentation

A fermentor (bioreactor) is a closed vessel with adequate arrangement for aeration, agitation, temperature and pH control and drain or overflow vent to remove the waste biomass of cultured microorganisms along with their products. It is used for commercial production in fermentation industries and is a device in which a substrate of low value is utilized by living cells or enzymes to generate a product of higher value. Fermentors are extensively used for food processing, fermentation, waste treatment, etc.

Aeration system is one of the most critical part of a fermentor. In a fermentor with a high microbial population density, there is a tremendous oxygen demand by the culture but oxygen being poorly soluble in water, hardly transfers rapidly throughout the growth medium. It is necessary, therefore, that elaborate precautions are taken using a good aeration system to ensure proper aeration and oxygen availability throughout the culture. However, sparger and impeller are used to ensure proper aeration in fermentor. The sparger is typically just a series of holes in a metal ring or a nozzle through which filter-sterilized air (or oxygen-enriched air) passes into the fermentor under high pressure. The air enters the fermentor as a series of tiny bubbles from which the oxygen passes by diffusion into the liquid culture medium. The impeller (also called agitator) is an agitating device necessary for stirring of the fermenter. The stirring mixes the gas bubbles and the microbial cells through the liquid culture medium ensuring uniform access of microbial cells to the nutrients. The size and position of the impeller in the fermentor depends upon the size of the fermentor. In tall fermentors, more than one impeller is needed if adequate aeration and agitation is to be obtained. Ideally, the impeller should be 1/3 of the fermentors diameter fitted above the base of the fermentor. The number of impeller may vary from size to size to the fermentor. Baffles in fermenters are normally incorporated to prevent vortex and to improve aeration efficiency. They are metal strips roughly one-tenth of the fermentors diameter and attached radially to the walls.

In any microbial fermentation, it is necessary not only to measure growth and product formation but also to control the process by altering environmental parameters as the process proceeds. For this purpose various devices are used in a fermentor.

Agitation speed is one of the important factor in the fermentation process since it helps to increase the amount of dissolved oxygen in the cultivation medium. Excessive

agitation speed, however, would produce greater mechanical forces or hydrodynamic shear stress which can lead to damage of fungal mycelila and pellets thus leading to cell destruction and lower enzyme production. The enzyme production was greatly effected by higher agitation rates. The enzyme production dropped at higher agitation rates. Agitation speed of the culture broth has a variety of effects on microorganisms including rupture in the cell wall, change in the morphology of filamentous microorganisms, variation in the efficiency and rate of growth and also variation in the rate of formation of the desired product.

Commercial production of an enzyme has been growing day by day due to the demand of novel biocatalyst and to expand the market of an enzyme, enzymes are produced in large volume and large number of products. Enzyme production is the most powerful successor central to the modern biotechnology industry. The production of the enzyme is comparable to the methods used for the manufacture of other industrial products. The general steps in enzyme production can be described in following steps:

2.5.1 Selection of source of enzyme

The primary step in the production of enzyme is to select the best source from which an enzyme is easily produced in a short period of time. Animal, plant, fungal and microbial sources are mostly used as strain as these can produce biological active enzymes. Half of the enzymes sources use fungi and yeast as the main source, over 1/3 use bacteria while remaining use animal and plants. But, microorganisms are preferred as the best source of enzyme as they can be cultured easily in enlarged quantities in relatively short period of time. The microorganisms can be grown easily and can be easily manipulated to produce the enzyme of need.

2.5.2 Isolation and improvement of strain

Microorganisms producing desired enzymes are screened from their natural habitat. The aim of isolating suitable organisms is for the production of an enzyme in high amount and other metabolites in low amount, completion of fermentation in a short time and for the utilization of low-cost culture medium by the microorganism. The selected microorganisms are then improved for the enzyme production by appropriate methods. Mutagenesis such as chemical mutagens and UV rays are used to induce mutations. The strain can also be genetically engineered to produce large amount of enzyme and thus selected and improved strain is preserved for future use.

2.5.3 Preparation of inoculum

The main objective of the preparation of inoculum is to obtain organisms in an optimal state which is more compactable with inoculation into cell culture, media and tissue culture. The aim is to achieve a high biomass in a large quantity for the use as an

inoculum. This process has many advantages in the industrial microbiology to produce enzymes, beverages, toxins, drugs, proteins, vitamins, food etc. The inoculum should be free from contamination and must be in an active stage of growth and size. The selected and improved organisms are multiplied in broth medium to obtain inoculum of spores, mycelium and bacterial cells to be used in an industrial process.

2.5.4 Formulation and preparation of media

The culture medium is formulated in such a way that it contains all the nutrients to support adequate growth of microorganisms that will ultimately result in a good quality of enzyme production. The ingredients of the medium should be readily available at low cost and are nutritionally safe. An ideal medium should contain a chief source of carbon, nitrogen, amino acids, growth promoters, trace elements and little amount of salt. A typical medium should have carbohydrates (molasses, barley, corn, wheat, etc.) and protein (soybean, cotton seed, peanut, whey, etc.). The pH of the medium should be kept optimum for good microbial growth and enzyme production. The constituents are mixed in appropriate proportion, autoclaved or filtered for optimum production yield.

2.5.5 Production process

It involves the sterilization of medium, inoculation of culture and the fermentation process. Industrial production of an enzyme is mostly carried out by submerged state fermentation and to the lesser extent by solid state fermentation. Submerged culture technique yields more with less contamination and is thus a preferred method. However, fungal enzymes such as amylase, cellulase, protease and pectinase are produced by solid state fermentation. The medium is sterilized by using suitable sterilization technique. The fermentation is started by inoculating the medium with sufficient amount inoculum. The growth conditions (temperature, pH, oxygen supply, nutrient supply) are maintained at the optimum level. The foam formed is minimized by using the antifoaming agent. The production of an enzyme is mostly carried out by batch fermentation rather than by continuous fermentation process. The bioreactor is left for 2-7 days in most production cases. Most of the enzymes are produced when the exponential phase is complete but in few cases, they are produced during exponential phase.

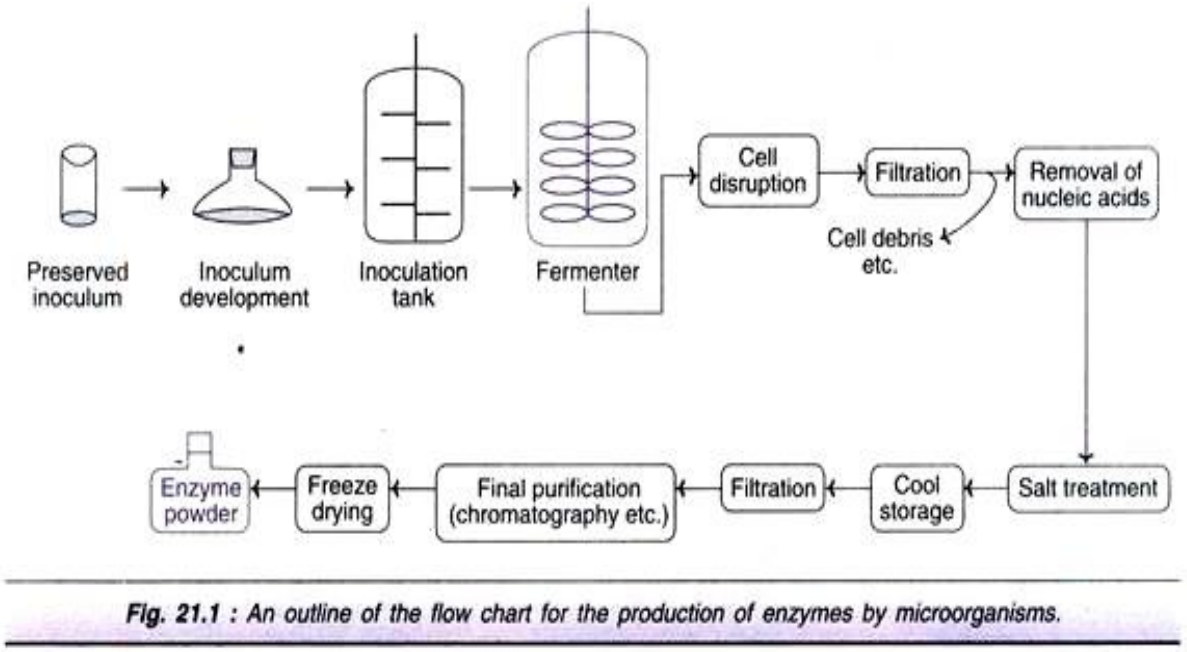

Fig. 21.1 : An outline of the flow chart for the production of enzymes by microorganisms.

Figure 10: General outline of production of enzyme using microorganism.

2.5.6 Recovery and purification of enzymes

The enzyme produced may be extracellular or intracellular. The enzyme may be obtained in crude or purified forms, solid or liquid form. The step involved during the down streaming process depends upon the nature of enzyme and degree of purity desired. The recovery of all extracellular enzymes is easy compared to intracellular. The recovery of intracellular enzymes requires additional steps of the disruption. The various methods employed for extraction of intracellular enzymes are:

2.5.6.1 Physical method

It includes ultrasonication, osmotic shock, heat shock, high pressure, grinding with glass and others.

2.5.6.2 Chemical method

It includes treating with alkalies, organic solvents, detergents etc.

2.5.6.3 Enzymatic method

It includes treatment with lysozyme and others.

The steps after extraction of intracellular enzyme are similar with both types of enzymes and includes

Removal of cell debris by filtration or centrifugation.

a. Removal of nucleic acid by their precipitation.
b. Precipitation of enzyme using salts (eg; ammonium sulfate), organic solvents eg; ethanol, isopropanol, acetone etc.

c. Concentration of enzyme by liquid -liquid partition.

d. Separation by the chromatographic technique such as ion exchange, size exclusion and hydrophobic interaction chromatography.

e. Drying by evaporation, freeze dryers and packing of the enzyme to obtain powdered enzyme.

For obtaining liquid enzyme, the enzyme obtained after separation of biomass and removal of nucleic acid is preserved and stabilized by using preservatives and stabilizers.

The most commonly used technology for the large scale production of enzymes (Submerged fermentation) is generally carried out in the presence of free flowing liquid, in which soluble substrates are dispersed. However, compared to liquid medium, filamentous fungi like *Aspergillus, Penicillium* and *Trichoderma* normally produce large quantity of cellulase in solid medium. In submerged fermentation (SmF), various factors such as pH, temperature, substrate concentration, inducer, medium composition, etc. influence the production of cellulase significantly. However, submerged fermentation requires long fermentation time (i.e., gestation period) with less production of enzymes. Among microorganisms, bacteria are most commonly used for the production of enzymes by submerged fermentation (Smitha R *et al.,* 2013; Sreedevi S *et al.,* 2013) but, some species of fungi such as *Aspergillus, Penicillium, Trichoderma*, etc. are also being cultivated under submerged fermentation (SmF) for the production of cellulase.

The cellulolytic activities of fungi may differ from species to species depending upon the medium as well as the culture conditions. Therefore, the formulation of suitable fermentation strategies is the benefical factor for deciding the efficiency of a fungus in terms of the production of cellulase. Various synthetic or natural carbon sources are used in submerged fermentation (SmF) to obtain the better productivity. For instance, Acharya *et al.,* (2008) studied the production of cellulase by *A. niger* on pretreated (by alkali) sawdust and the maximum cellulase activity that was observed was in between 4 and 4.5 pH, at 120 rpm and temperature at 28°C with peptone as the sole nitrogen source. Similarly, Karthikeyan *et al.,* (2011) investigated the production of cellulase by *Penicillium* strain K-P in liquid medium by supplementing various carbon and nitrogen sources at varying pH and temperature with the fungus showing maximum cellulase activity in the presence of fructose and ammonium nitrate on day 5 with pH of 3.0 and temperature of 30°C. Production of cellulases was studied by *A. niger* in the presence of various carbon and nitrogen sources at varying pH and it was found that the maximum production of cellulase was on Czapek-Dox medium supplemented with 1% CMC (Carboxy methyl cellulose) or sawdust at pH 5 (Narasimha G *et al.,* 2006). From the reports, it is clear that the production of cellulase was mainly observed under acidic pH

and temperature in between 20-30°C with the addition of the ability of the fungi in utilizing the carbon and the nitrogen sources present in the liquid medium.

Solid state fermentation (SSF) is regarded as one of the most important approach employed in the industries for the increased production of different enzymes. Solid state fermentation uses culture substrates with low water levels (reduced water activity) which is particularly appropriate for mould. The methods used to grow filamentous fungi using solid state fermentation allow the best reproduction of their natural environment. The medium is saturated with water but little of it is free-flowing. The solid medium comprises both the substrate and the solid support on which the fermentation takes place. The substrate used is generally composed of vegetal byproducts such as beet pulp or wheat bran. In the recent years, solid state fermentation (SSF) is gaining more interest as a suitable strategy for the recycling of nutrient-rich wastes such as lignocelluloses. Solid state fermentation (SSF) facilitates not only the possibilities for the bioconversion of agro-residues to value-added products but also it enables the efficient recycling of lignocellulosic materials with the expenditure of less energy. In the earlier days, solid state fermentation (SSF) was considered as feasible only for the fermentation of food or food-associated products but further studies showed several benefits of this technology such as high enzyme yield at low cost, smaller vessels, lower water consumption, reduced wastewater treatment costs and lower energy consumption (no need to heat up water, poor mechanical energy input due to smooth stirring), use of agricultural waste as substrate and wider range of additional enzyme activities than found in submerged sate fermentation (Robinson T *et al*., 2001; Couto and Sanraman, 2006). Hence, solid state fermentation (SSF) is an attractive means to produce cellulase economically, because of its lower capital investment and operating cost (Xia and Cen, 1999).

Due to the discrete nature of solid state fermentation (SSF), the physico-chemical characteristics of substrate such as crystallinity, bed porosity and enormous surface area can influence the production of cellulolytic enzyme system in fungal cultures. In solid state fermentation (SSF), the operating conditions like temperature, pH and moisture content are vital factors influencing the microbial growth and production of cellulase. Availability of oxygen in the open space between substrate particles (i.e., porosity) and generation of heat are the major challenges in solid state fermentation (SSF) which have to be addressed properly (Thibault J *et al.,* 2000; Oostra J *et al.,* 2001). Agricultural wastes such as brans of wheat and rice, corn stover, straws of wheat and rice, sugarcane baggase, sawdust, etc. are the most commonly used substrates for the production of cellulase. For instance, Liu *et al.,* (2011a) investigated that the production of cellulase on different lignocellulosic substrates such as straws of rice, wheat and cotton, corn stover and corncob using *Aspergillus fumigatus* Z5, the corncob supported the maximum

production of endoglucanase. *P. echinulatum* 9A02S1 showed the maximum production of cellulolytic enzymes on the medium containing a mixture of pretreated sugarcane bagasse and wheat bran (Camasssola and Dillon, 2007). Dutta *et al.,* (2008) studied the production of cellulases from *P. citrinum* using brans of wheat and rice and rice straw as substrate. All these substrates supported the maximum production of cellulases. *Aspergillus niger* MS82 efficiently utilized the lignocellulosic substrates like grass, bagasse and corncob with variable cellulase activities (Sohail M *et al.,* 2009). *A. flavus* BS1 competently utilized different lignocellulosic substrates and supported higher levels of cellulase activity (Sajith S *et al.,* 2014). *A. flavus* Linn NSPR 101 showed the production of cellulase on various natural substrates like bagasse, corncob and sawdust (Ojumu TV *et al.,* 2003).

Table 1: Demonstration of the production of cellulase on various solid substrates by different species of fungi and the activities expressed in gram dry fermented substrates (gds).

Fungi	**Substrate**	**Cellulase(U/gds)**	**Reference**
A. flavus BS1	tapioca flour and sawdust	5408.5	Sajith *et al.,* 2014
A. fumigates	rice straw and wheat bran	14.7	Sherief *et al.,* 2010
A.niger	wheat bran	3.2	Chandra *et al.,* 2007
A. niger	coir waste	3.3	Mrudula and Murugammal, 2011
A. niger	saw dust	3.9	Devi and Kumar, 2017
A. niger MS82	grass	100	Sohail *et al.,* 2009
A. terreus M11	corn stover	581	Gao *et al.,* 2008 b
A. fumigates Z5	corn stover	526.3	Liu *et al*., 2011 a
Aspergillus spp. MAM-F35	wheat straw	487	AboState *et al.,* 2010

niger USM Al	sugarcane bagasse and palm kernel cake	3.2	Lee *et al.,* 2011
Fomitopsis sp. RCK2010	wheat bran	84	Deswal *et al.,* 2011
P. citrinum	rice bran	2	Dutta *et al.,* 2008
P. fellutanum	lactose	81	Kathiresan and Manivannan, 2010
P. echinalutum 9AO2S1	sugarcane bagasse and wheat bran	282.4	Camassola and Dillon, 2007
R. oryzae	water hyacinth	450	Karmakar and Ray, 2011
T. reesei	sugarcane bagasse and palm kernel cake	2.2	Lee *et al.,* 2011
T. reesei. LW1	corn straw and wheat bran	452.5	Wang *et al*., 2005
T. reesei NRRL 11460	sugarcane bagasse	154.6	Singhania *et al.,* 2006
T. reesei RUTC30	sugarcane bagasse	121	da Silva Delabona *et al.,* 2012
T. harzianum	CMC	150	Rubeena *et al.,* 2013
T. viride	CMC	173	Neethu *et al.* ,2012

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 MATERIALS

All the reagents were purchased from Himedia, India Pvt. LTD and all the chemicals were of reagent grade.

3.2 METHODOLOGY

3.2.1 Collection of Sample

Municipal waste samples were collected from different localities of Kathmandu and Lalitpur valley as shown in table 2. The solid samples were taken by the means of sterilized spatulas and packed in zip locked sterile plastic bags. The liquid sample was taken by dipping the sterilized bottle few centimetres deep below the water. The samples were then brought to the laboratory for further study. They were stored at 4°C until use.

Table 2: Samples collected from the different localities of Kathmandu and Lalitpur valley

S. No.	**Samples**	**Location**
a..	Goat manure	Kirtipur, Kathmandu
b.	Dried rotten leaf	Garden of Central Department of Botany, TU
c.	Cow dung	Dhobighat, Lalitpur
d.	Waste water	Bagmati river, Balkhu, Kathmandu
e.	Moist Soil	Sangam basti, Lalitpur
f.	Rice straw	Sanepa, Lalitpur

3.2.2 Isolation of Fungi

In case of solid samples, one gram of sample was transferred to aliquots of 9 ml sterile distilled water in test tube and in case of water samples, one ml of the samples was transferred to aliquots of 9 ml distilled water in test tubes. It was then shaken vigorously for 15 minutes. The suspension was then subjected to serial dilutions from the test tubes and spreaded to the appropriate potato dextrose agar plates in dublicate. The plates were then incubated for 5 days at 28°C (Gautam *et al.,* 2011). The well grown spreaded single colonies were picked up and subcultured on potato dextrose agar slants.

3.2.3 Screening for Cellulase Enzyme

Municipal waste samples associated fungi were tested for their ability to produce the hydrolytic enzymes: cellulase in a plate assay method using 1% carboxymethyl cellulose in a basal salt media (pH 5.2) incubating at 28°C. 0.1% Congo red solution was added and counterstained with 1 M NaCl for 15–20 min. The zone of cellulose hydrolysis appeared as a clear area around the colony.

3.2.4 Identification of organism by Lactophenol Cotton Blue Staining (LCBS)

1. A strip of transparent tape was cut and placed between ends of thumb and index finger with the sticky side out and a loop was made by extending outwards by closing the thumb and the index finger.

2. The culture plate was opened with the opposite hand and the tape was pressed against the colony of interest.

3. A drop of Lactophenol Blue was placed on a clean glass slide and pressed.

4. The tape was smoothed back on the slide by opening fingers and using gauze.

5. Another drop of Lactophenol Blue was placed on top of the tape and a large coverslip was kept on top of the slide.

6. It was then examined under the microscope.

3.2.5 Optimization of Culture Conditions for Enzyme Production

3.2.5.1 Effect of pH on enzyme production

To determine optimal pH, isolate D3 (from cow dung sample) was cultivated in a 150 ml flask containing 50 ml optimized medium with pH ranging from 4.0 to 8.0 at the interval of 1. The pH of the medium was adjusted by using 1 N HCl and 1 N NaOH. The flasks were then kept in stationary state at 25°C, 30°C, 35°C, 40°C, 45°C and 50°C for 7 days. Enzyme assay was carried out by DNS method at 24 hours interval for 7 days continuously. The process was carried out in triplicate.

3.2.5.2 Effect of temperature on enzyme production

In order to determine the optimize temperature for cellulase production by the isolate D3, fermentation was carried out in the range of 5°C intervals at 25°C, 30°C, 35°C, 40°C, 45°C and 50°C for 7 days. Enzyme assay was carried out by DNS method at 24 hours interval for 7 days continuously. The process was carried out in triplicate.

3.2.5.3 Effect of agitation on enzyme production

To determine the effect of agitation, the basal media were kept in the bioreactor, mixed well and autoclaved at 121°C for 30 minutes at 15 lbs pressure. After cooling, bioreactor was inoculated with 1% spore suspension of isolate D3 and mixed thoroughly. The production was carried out at optimized condition (30°C temperature and pH 6) at 100 rpm, 150 rpm and 200 rpm for 8 days. Cell growth was monitored every 24 hr to evaluate the increase or decrease in the concentration of cellulase. The culture was taken after incubating at every 24 hrs and centrifuged at 5000 rpm for 20 min. Enzyme assay was carried out by DNS method at 24 hours interval for 8 days continuously. The process was carried out in triplicate.

3.2.5.4 Effect of aeration on enzyme production

To determine the effect of aeration, the basal media were kept in the bioreactor, mixed well and autoclaved at 121°C for 30 minutes at 15 lbs pressure. After cooling, bioreactor was inoculated with 1% spore suspension of isolate D3 and mixed thoroughly. The production was carried out at optimized condition (30°C temperature and pH 6) at 200 rpm with varied aeration (0.5 l/min, 1.0 l/min and 2.0 l/min) for 8 days. Cell growth was monitored every 24 hour to evaluate the increase or decrease in concentration of cellulase. The culture was taken after incubating at every 24 hrs and centrifuged at 5000 rpm for 20 min. Enzyme assay was carried out by DNS method at 24 hours interval for 8 days continuously. The process was carried out in triplicate.

3.2.6. Enzyme Assay

The CMCase activity was measured by incubating 0.5 ml of culture supernatant with 0.5 ml of 1% CMC prepared in 0.05 M sodium citrate buffer (pH 4.8). The reducing sugars liberated were estimated by the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959). The enzyme reaction was stopped by the addition of 3 ml DNS reagent to the above reaction mixture, boiled in capped glass tubes for 5 min, cooled and then the optical density was measured at 540 nm. The CMCase activity was determined using a calibration curve for D-glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1 µg of glucose per minute. CMCase activity was calculated by the formula (Chakraborty and Mahajan, 2014);

$$\boldsymbol{Units}\left(\frac{\boldsymbol{IU}}{\boldsymbol{ml}}\right) = \frac{(\boldsymbol{\mu g\ of\ glucose\ released})\ \boldsymbol{x}\ (\boldsymbol{Total\ assay\ volume})\boldsymbol{x}\ \boldsymbol{Dilution\ factor}}{(\boldsymbol{Volume\ of\ enzyme\ used})\ \boldsymbol{x}\ (\boldsymbol{Duration\ of\ incubation\ in\ minute})}$$

CHAPTER 4

RESULTS

4.1 Isolation and screening of organism

Samples (goat manure, dried rotten leaf, cow dung, waste water, moist soil and rice straw) were diluted serially, spreaded in PDA plates and incubated at 28°C for 4 days. Serially diluted sample plates of rotten dried leaf, waste water, moist soil and rice straw showed growth at 10^{-3} dilution while cow dung and goat manure showed growth at 10^{-4} dilution. The fungal colonies were further subcultured to isolate pure culture.

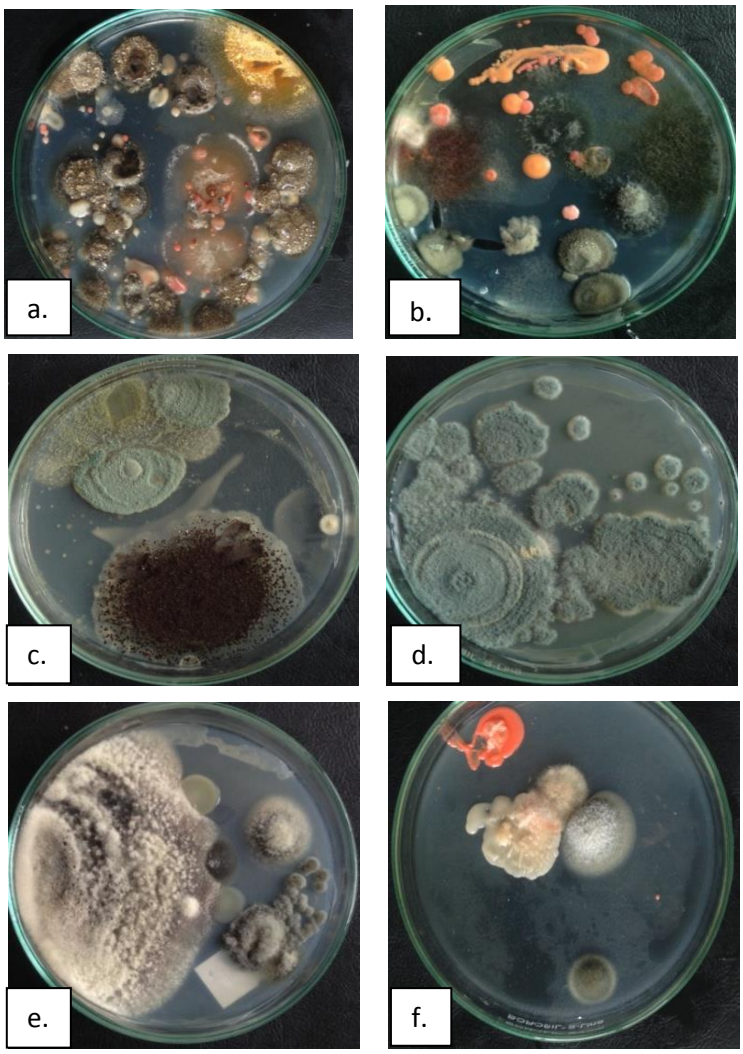

Figure 11: Mixed culture of microorganism from samples a. Goat (10^{-4}), b. Leaf (10^{-3}), c. Dung (10^{-4}), d. Water (10^{-3}), e. Soil (10^{-3}), f. Straw (10^{-3}) on Potato Dextrose Agar (PDA) plates.

Table 3: Number of isolates of different samples

S. No.	Samples	Isolates
1.	Rotten dried leaf	1
2.	Waste water	1
3.	Goat manure	3
4.	Moist Soil	1
5.	Rice straw	1
6.	Cow dung	2

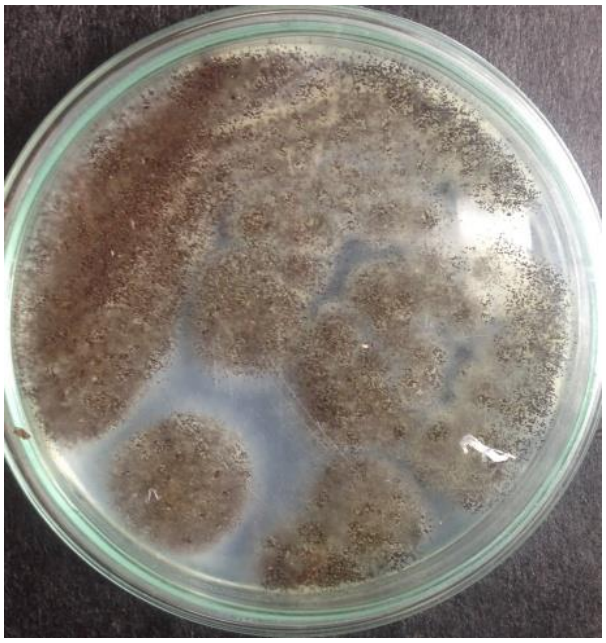

Figure 12: Pure culture of microorganism isolated from the sample of cow dung (D3) in Potato Dextrose Agar (PDA) agar plates.

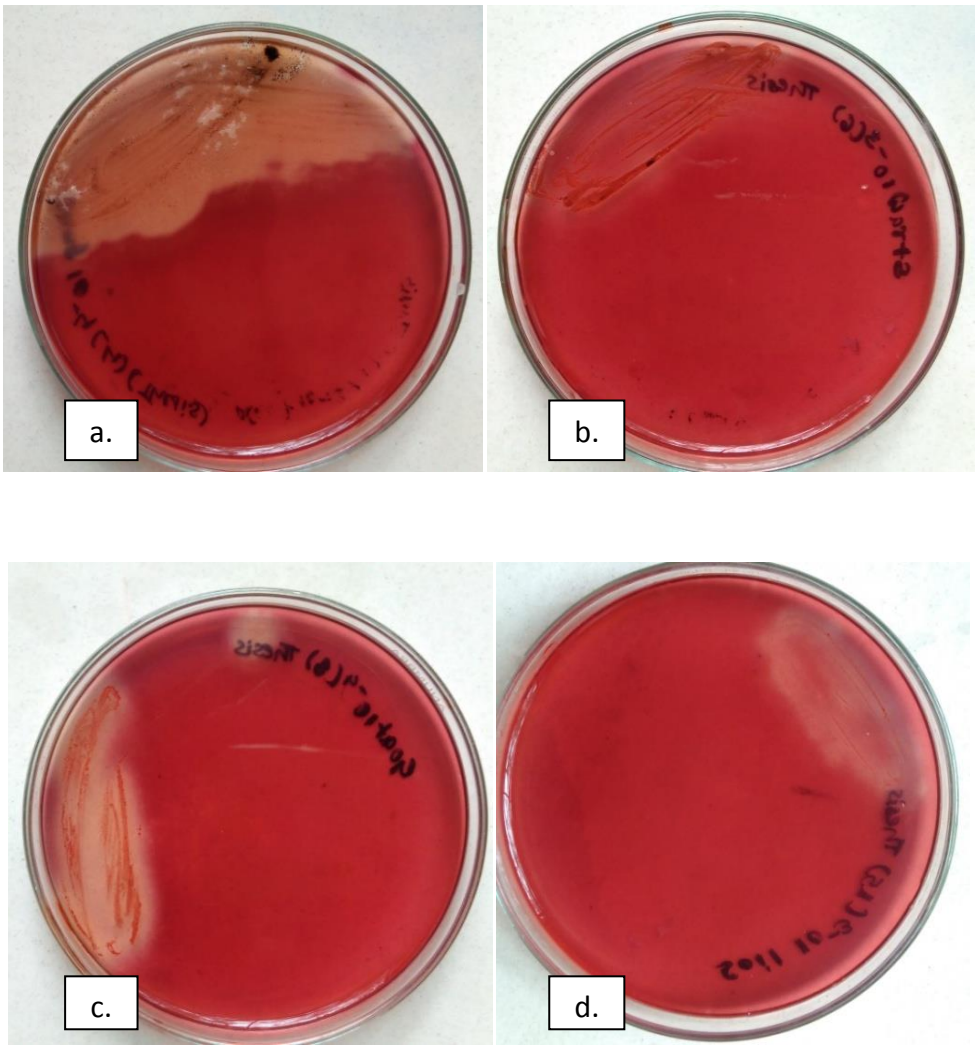

Figure 13: Screening of isolates for cellulase activity a. Dung (D3), b. Straw (S6), c. Goat (G4) and d. Soil (S5).

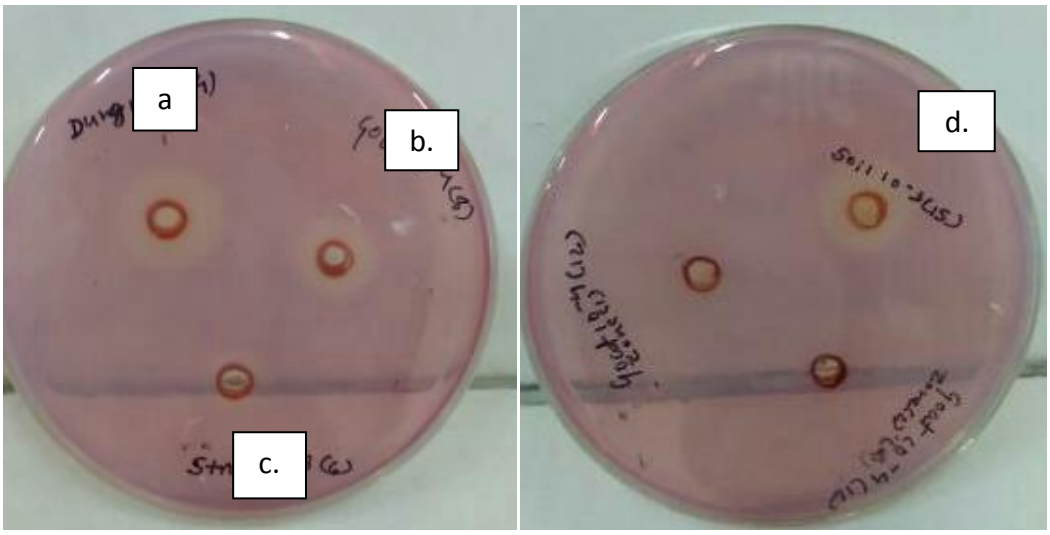

Figure 14: Zone of CMC hydrolysis of a. Dung (D3), b. Goat (G4), c. Straw (S6) and d. Soil (S5)

Table 4: Measurement of zone of hydrolysis

S. No.	**Samples**	**Zone (mm)**
a.	D3	18 mm
b.	G4	12 mm
c.	S6	8 mm
d.	S5	17 mm

The isolate of D3 (cow dung) showed the largest zone of inhibition (18 mm) which was selected for further optimization study. The isolate S5 was not selected for the further optimization study as the fungus strain was presumably of *Aspergillus flavus* that produces aflatoxins (poisonous carcinogens).

4.2 Morphology of isolate D3

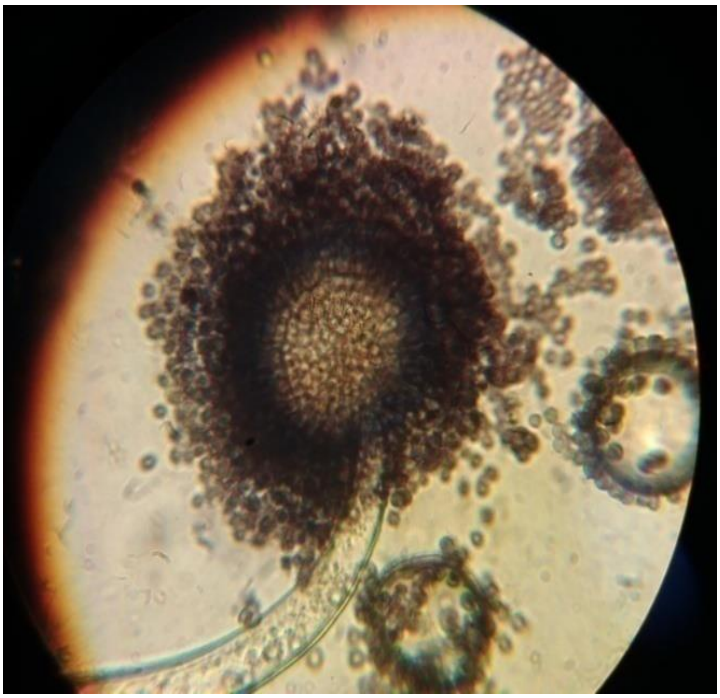

Figure 15: Microscopic view of isolate D3 (cow dung sample) observed under the microscope using the lactophenol cotton blue staining technique.

4.3 Optimization of culture conditions for enzyme production

4.3.1 Optimization of pH

The effect of pH on cellulase production was observed from pH ranging from 4.0 to 8.0 at the interval of 1 (Figure 16). Enzyme assay was carried out by DNS method at the interval of every 24 hours for 7 days continuously. The fungal cellulase production increased gradually from pH 4 and reached maximum at pH 6. Cellulase exhibited highest enzymatic activity at pH 6.0 with the activity of 1.783 IU/ml. Enzyme activity seemed to decrease gradually as the pH value increased above 6.0.

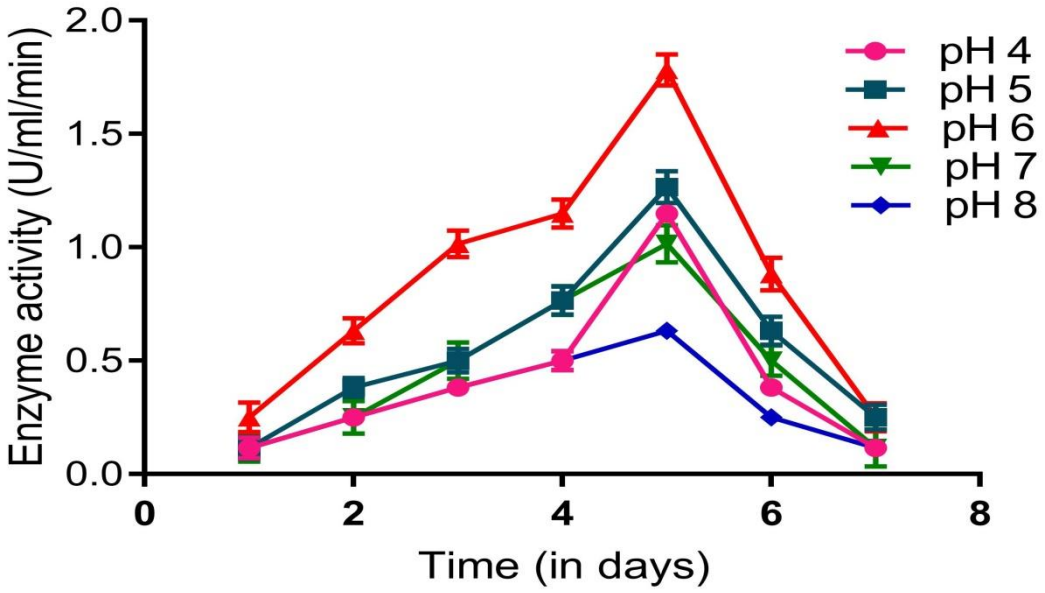

Figure 16: Effects of pH on cellulase production by isolate (D3).

4.3.2 Optimization of temperature

The temperature optimization was done at 5°C intervals ranging from 25°C to 50°C. Enzyme assay was carried out by DNS method at the interval of every 24 hours for 7 days continuously. The optimum temperature for fungal cellulase was found to be 30°C with enzyme activity 3.333 IU/ml (Figure 17). The activity decreased as the temperature was further increased above 30°C.

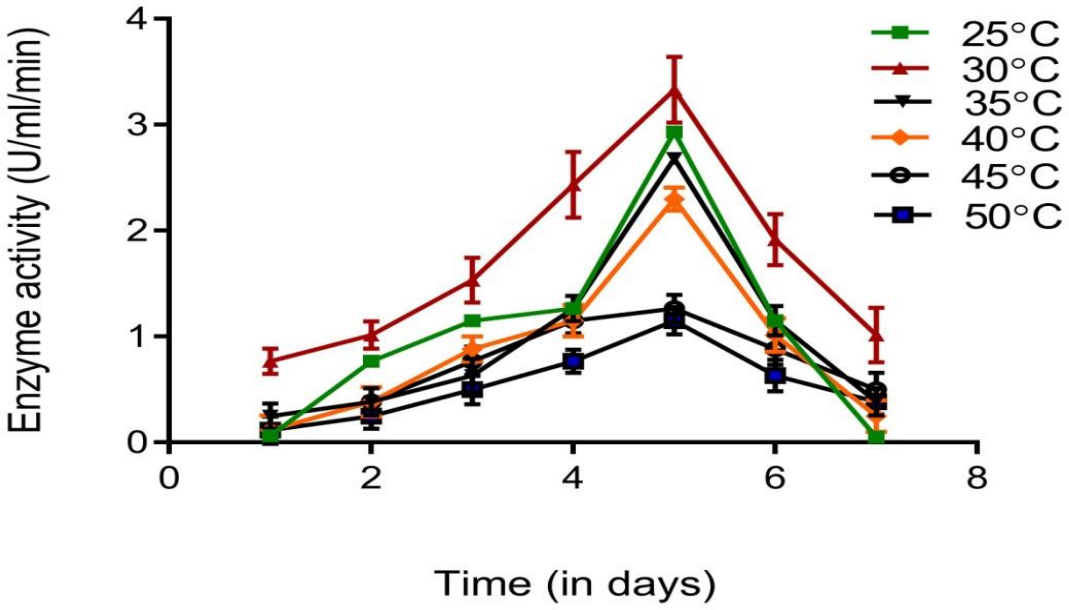

Figure 17: Effect of temperature on cellulase production by isolate (D3).

4.3.3 Optimization of agitation

The optimization of agitation was done at 100 rpm, 150 rpm and 200 rpm with the help of Electrolab Fermenter. Cellulase activity was measured by DNS method at the interval of every 24 hours upto 8 days continuously. It was found that the maximum cellulase activity (8.966 IU/ml) was observed at 200 rpm on the 6th day. From the 6th day onwards cellulase activity decreased.

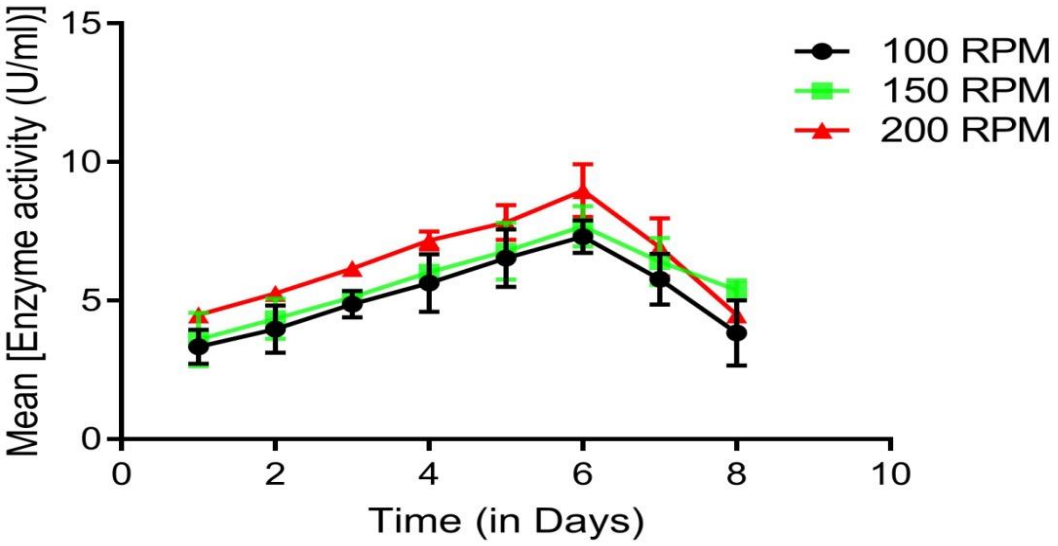

Figure 18: Effect of agitation on cellulase production by isolate (D3).

4.3.4 Optimization of aeration

Aeration optimization was carried out at 0.5 l/min, 1l/min and 2 l/min with the help of Electrolab Fermenter. Cellulase activity was measured by DNS method at the interval of 24 hours upto 8 days. It was found that the maximum cellulase activity (11.166 IU/ml) was observed at 1 l/min (200 rpm) on the 6th day. Cellulase activity seemed to decrease after the 6th day.

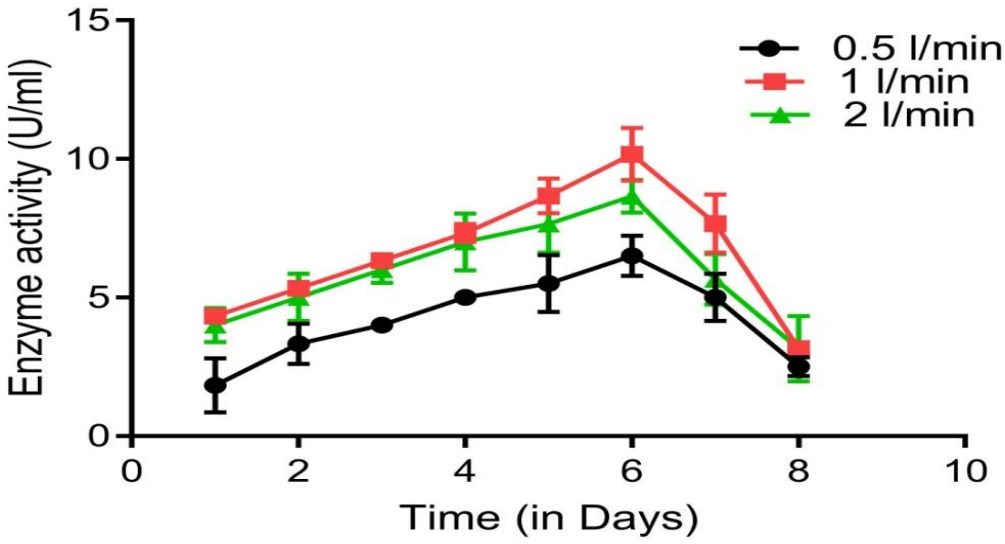

Figure 19: Effect of aeration on cellulase production by isolate (D3).

CHAPTER 5

DISCUSSION

Lignocellulose is the most abundant biopolymer available on earth. Hydrolysis of lignocellulose into fermentable sugars, sugar acids and phenolics is the pre-requisite for its successful exploitation as substrates for the large scale production of industrially significant value added products. Though remarkable collection of lignocellulolytic microorganisms has been brought to limelight, only a few, especially fungi have been studied extensively as they secrete copious lignocellulolytic enzymes extracellularly.

Figure 11 shows the mixed culture of microorganism isolated from the samples of a. goat manure, b. rotten dried leaf, c. cow dung, d. waste water, e. moist soil and f. rice straw in Potato Dextrose Agar (PDA) plates. These samples were used for the study as the fungi grew well on the substrate and also proved to be inexpensive lignocellulosic material for the production of cellulase enzymes (Abdullah *et al.,* 2016). A total of 9 fungal cultures were isolated from the above samples using the potato dextrose agar plates and then screened for cellulase activity which was done by CMC hydrolysis on CMC agar plates. Upon incubation, the growth of fungal culture was observed on plates and the plates were flooded with Congo red dye. The zone of CMC hydrolysis was considered as cellulase producing fungus (Ram *et al.,* 2014) and the isolate with the largest zone (D3) was selected for further optimization study.

Using of CMC as the sole carbon source plays a pivotal role for achieving the highest level of cellulase production because CMC induces cellulase gene expression (Khatiwada *et al.,* 2016). Screening of fungi for the cellulase activity was carried out by the hydrolysis of substrate incorporating in the basal salt medium. After the incubation period, enzyme activities were detected by the appearance of zones either by substrate clearances or coloration and discoloration around the fungal colonies (Gautam *et al.,* 2011). Figure 13 shows CMC agar plated with microorganisms stained with congo red and fixed with either 1 N HCl and 1 N NaOH. Both of these chemicals stop fungal growth and enhance the contrast between the halo and the background. Halo formation on CMC agar plates results from cleavage of CMC into fragments smaller than cellohexaose to which Congo red does not bind. Generally, in this method, the Congo red interacts with intact β (1-4)-D-glucans in carboxymethyl cellulose. Moreover, halos could result from cleavage of CMC into fragments small enough to be washed out of the plates during the staining procedure. In either case, only endoglucanase activity would be expected to produce a zone of hydrolysis (Sazci *et al.,* 1986). All fungi used in this work effectively produced true cellulolytic activity. Among the fungi studied here, isolate D3 produced largest zone (18mm) which indicated that it yielded higher production of cellulases as compared to

other isolates and hence converted more cellulose to glucose. For the above test, isolate D3 showed the highest zone of 18 mm (Table 4) around the colony which was then selected for further optimization study.

The isolate D3 was observed under the microscope using the lactophenol cotton blue staining technique (Figure 15). Lactophenol Cotton Blue Stain (LCBS) is formulated with lactophenol (which serves as a mounting fluid) and cotton blue. Organisms suspended in the stain are killed due to the presence of phenol. The high concentration of the phenol deactivates lytic cellular enzymes thus the cells do not lyse. Lactic acid preserves the fungal structures and the cotton blue serves as an acid dye that stains the chitin present in the cell walls of fungi (Leck A., 1999). On staining with cotton blue, the organism that was identified was presumably of *Aspergillus* species. Microscopically, *Aspergillus* species can be identified by its hyaline, septate hyphae. Asexual conidiophores can be identified by being long and globose at the tip, with what appears to be a hymenial layer of structures, each "ejecting" its own spore. Macroscopically, this fungus can be identified growing on substrate producing colonies of yellow to white hyphae, turning black with the formation of conidia (MA Moslem *et al.,* 2010).

The pH of the fermentation medium is reported to impact the growth of any microbial strain and consequent metabolic product formation (Okaiyeto *et al.,* 2016). In addition, many enzymatic processes and the transport of various components across the cell membrane are strongly affected by the pH of the medium (Fatokun *et al.,* 2016). An optimum pH is required to maintain the three-dimensional shape of the active site of enzymes and the change in pH results in a loss of functional shape of the enzyme due to alterations in the ionic bonding of the enzyme. Increase or decrease in the pH of the medium resulted in declined enzyme production. Hence, it is essential to optimize pH for maximum cellulase production. The result (Figure 16) showed that the maximal production for cellulase was observed at pH 6 after 120 hours of incubation with enzyme activity of 1.783 IU/ml. A further increase in pH reduced the cellulase activity which was probably due to the proteolytic inactivation of the cellulase. This value is in accordance with Akiba *et al.*, (1995) who reported that the optimum pH for cellulase production by *A. niger* was between 6.0 and 7.0. The results obtained in our study are also in agreement with Gautam *et al.* (2011) who observed the highest production of cellulases by *Aspergillus niger* at pH 6.5. Hence, it is suggested that slightly acidic pH values favoured cellulase production.

Incubation at different temperature plays an important role in the growth, development and metabolic activities of a microorganism. The temperature is found to influence extracellular enzyme secretion possibly by changing the physical properties of the cell membrane (Rahman *et al.,* 2005). Since enzyme is a secondary metabolite produced

during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield (Sabu *et al.,* 2002). Hence, considering all these factors, it is essential to optimize temperature for maximum cellulase production. The results of the test made at different temperatures value showed that the optimal temperature for endoglucanase acitivity produced by isolate D3 was 30°C after 120 hours of incubation with enzyme activity of 3.333 IU/ml (Figure 17). At high temperature, the results showed that the enzyme activity was decreased when the temperature increased above 40°C. Temperature above and below the optimum level inhibited the cellulase activity by the microorganism probably due to inhibition of the multienzyme complex system of the cell (Sohail *et al.,* 2009). At low temperature, substrate transport across the cell is suppressed and lower product yields are attained. Similarly, at higher temperature, the thermal denaturation of enzymes of the metabolic pathway could result in decreased enzyme production (M.I. Rajaoka, 2004). It is also reported that the different temperatures for maximum cellulase production either in flask or in fermenter studies using *Aspergillus* species suggested that the optimal temperature for cellulase production also depends on the strain variation of the microorganisms. Some investigators have even found maximum cellulase production at higher temperatures and others recorded highest yield at lower temperatures. Bansal *et al.* (2012) reported that *A. niger* NS-2 exhibited a wide range of temperature for its growth and cellulase production on agriculture and kitchen waste residues and highest yields of CMCase, FPase and β-glucosidase were obtained at 30 °C after 96 h. The optimum temperature for cellulase production by *A. fumigates* was reported to be 32 °C (Gilna & Khaleel, 2011).

Dissolved oxygen tension and stirrer speed are important parameters for performing desired fermentation of fungal strain. The availability of oxygen is a major parameter to be considered for effective microbial cell growth rate. Agitation is directly related to oxygen transported from the gas phase to liquid phase followed by oxygen uptake by individual microbial cell (Najafpour, 2007). Therefore, the effect of different aeration and agitation on growth of microorganisms and enzyme yield were investigated in a bioreactor. The microorganisms differ in their oxygen requirement. Oxygen tends to act as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The rate of the agitation speed could influence the extent of mixing and affect the nutrient availability as well (Venugopal *et al.,* 2007). Agitation speed has affected many enzyme activities in different strains of bacteria and fungi as well as microalgae. Agitation provides adequate mixing, mass, heat transfer and also improve dissolved oxygen in the culture medium. At lower agitation speed, insufficient oxygen in the culture medium usually affects the microbial growth, whereas higher agitation speeds sometimes also lower the enzyme production (Seth M and Chand S, 2000).

Higher agitation speed develop shear forces among the suspended microbial cells in the culture medium and the production drops due to cell damages which results from cell collision. Shear forces also can effect the fungal cell which can lead to the changes in morphology by damaging the external and internal cell structures, variation in fungal growth and yield formation (Darah *et al.,* 2011; Zhu *et al.,* 2012). Therefore, the optimal agitation speed is necessary to be determined in order to obtain maximal enzyme production. The result indicated that for all different mixing rates used in the fermentation process, the enzyme production increased rapidly and reached maximum level at 200 rpm at 144 hours (6^{th} day) of incubation with activity of 8.966 IU/ml (Figure 18). The 200 rpm of stirring speed was found to be most conducive for cell growth. This is probably due to aeration of the culture medium which was increased and dissolved oxygen in the media was sufficient. Nutrient uptake by fungus increased resulting in increased cellulase production. However, the different strains of fungus demonstrated different optimal agitation speeds for maximizing the enzyme production yield in submerged fermentation. Increasing agitation intensity enhances gas-liquid mass transfer as well as the mechanical forces that act on the fungal cells (Cui *et al*., 1998). The latter effect can be seen from the reduced cell growth at higher agitation (for eg. 500 rpm) which could be attributed to shear stress and heterogenous mixing effects (Nadeem *et al*., 2009). During the lag and the exponential growth phase, oxygen uptake rate generally increased, followed by a constant rate during stationary growth phase (Garcia-Ochoa *et al.,* 2000). Increase in CMCase activity was observed till the 6^{th} day. CMCase activity increased as agitation speed was increased from 100 to 200 rpm. Increment to higher speed (for eg. 500 rpm) resulted in decrement of cellulase activity which may be the result of the formation of high shear zones close to the impellers caused by high impeller speed with subsequent physical damage to the cells and a reduction in process productivity (Badino *et al.,* 2001). Cellulase activity was also observed to decrease nearing the end of fermentation possibly as a result of nutrient exhaustion and cell lysis. A study on cellulase deactivation in a stirred tank bioreactor concluded that: (1) the extent of deactivation increased with an increase in agitation speed, (2) the extent of deactivation for cellulase and its three components differed significantly and that (3) exoglucanase (FPase) contributes to the major decrease in cellulolytic activity in the initial stage of shearing (Gunjikar *et al*., 2001).

Effects of aeration was determined by keeping the agitation rate fixed at 200 rpm while the aeration was varied. At 200 rpm agitation speed, improvement in cellulase activity was observed when aeration rates increased from 0.5 to 1.0 l/min. Increment in the aeration rate more than 1.0 l/min resulted in reduced enzymatic activity. The optimum cellulase activity of 11.166 IU/ml was obtained at 1.0 l/min aeration rate. This indicated that both low and high aeration rate had more negative effect on cellulase activity. The

higher enzyme inactivation in highly aerated culture may be due to irreversible oxidation of amino acid residues of the enzyme structure (Cabiscol *et al.,* 2000). However, it has been reported that improving aeration rate has a positive effect on enzyme activity from aerobic microorganism (Bakri *et al.,* 2002; Jafari *et al.,* 2007; El-Enshasy *et al.,* 2008). In this study, elevated aeration rate were accompanied by a corresponding increase in biomass, with highest activity observed at 1.0 l/min (Figure 19 shows enzyme activity of 11.166 IU/ml). Xu and Yun (2004) stated that aeration results in better mixing of the exopolysaccharides from *Paecilomyces tenuipes* C240 (a type of enthomopathogenic fungus) in a stirred-tank fermenter. This in turn helps to maintain a concentration gradient between the interior and exterior of the cell allowing better diffusion of nutrients to the cells. Reddy *et al*. (2002) reported that combining high flow rate with low agitation resulted in impeller flooding which occurred when the impeller is surrounded by air column causing air flow pattern in the vessel to be dominated by air flow up the stirrer shaft. Improper contact between impeller and liquid then results in poor mixing, reduced air dispersion and diminished oxygen transfer rates. Enzymatic activity dropped at the seventh day of fermentation.

CHAPTER 6

SUMMARY

Even though a large chunk of lignocelluloses are formed annually, its bulkiness on earth does not accumulate due to the swift action of microorganisms on it. They efficiently degrade these organic materials so as to provide themselves with carbon and energy source for their growth and allow the recycling of carbon back into the ecosystem. Although a large number of microbes can thrive on lignocelluloses, a few of them produce the complete battery of enzymes necessary for the breakdown of lignocelluloses into simpler molecules for further aerobic or anaerobic catabolism. In nature, a wide variety of bacteria and fungi are evolved to produce lignocellulolytic enzymes as a part of ecological recycling. Fungi are mainly exploited for the production of lignocellulolytic enzymes on large scale for use in industry as they grow attached to the solid substrates with limited moisture content, i.e. natural solid-state fermentation (SSF). It has been studied extensively because their elongated hyphen creates mechanical pressure on the cellulose structure causing them to produce large amounts of cellulase. Most fungal cellulases have the capability to digest cellulose. Fungi such as *Penicillium* species, *Aspergillus* species and *Trichoderma* species are cellulase producers. Enzyme produced by these microorganisms is commercially available for agricultural and industrial uses. Improvement of microbial strains for the over-production of industrial products has been the hallmark of all commercial fermentation processes. Such improved strains can reduce the cost of the process and may also possess some specialized desirable characteristics.

The current study was aimed at isolation of promising cellulase producing fungus, its identification and optimization of cultural conditions for production of cellulolytic enzymes. The cellulolytic activity of the culture was studied by standard CMC (Carboxy methyl cellulose) and Congo red plate assay method. One of the fungal isolate (D3) identified as one of the *Aspergillus* species based on cultural, morphological and microscopic characteristics was selected for further study as it effectively produced cellulase and showed largest zone of inhibition (18mm). Cellulase production with *Aspergillus* species in liquid state fermentation was highest at pH 6 (1.783 IU/ml) and 30°C (3.333 IU/ml) with incubation time of 5 days. Agitation (200 rpm) and aeration (1 l/m) maintained at 30°C and pH 6 at 144 hours of incubation gave higher yields of cellulase with enzyme activity of 8.966 IU/ml and 11.166 IU/ml respectively. It is possible that still there may be many more isolates to be discovered which have enormous potential to produce cellulase. Isolates exhibiting high cellulase activity can prove to be of immense help to humans in production of pharmaceutical and many more products.

Municipal waste in the form of cellulose which is the most renewable biomass in the atmosphere can be shown to be used in the production of valuable enzyme by *Aspergillus* species. The municipial wastes could provide an economical advantage as a solid substrate as well as carbon source for production of cellulase enzyme by using fungus strain. The exploration of sustainable substrates, microorganisms and fermentation strategies are to be effectively evolved so as to achieve higher productivity, quality and economic feasibility. Moreover, further studies should be accelerated for the expansion of cellulase research by manipulating the ability of microorganism via gene/protein engineering for the effective utilization of biomass facilitating better methods for bioconversion as well as solid waste management.

CHAPTER 7

CONCLUSIONS

Cellulases account for a significant share of the world's industrial enzyme market. The growing concerns about depletion of crude oil and the emissions of greenhouse gases have motivated the production of bioethanol from lignocellulose, especially through enzymatic hydrolysis of lignocelluloses materials i.e. sugar. However, costs of cellulase for hydrolysis of pretreated lignocellulosic materials need to be reduced and their catalytic efficiency should be further increased in order to make the process economically feasible. Engineering cellulolytic enzymes with improved catalytic efficiency and enhanced thermostability is important to commercialize lignocelluloses biorefinery. Individual cellulase can be enhanced by using either rational design or directed evolution. However, improvements in cellulase performance have been incremental and no drastic activity enhancement has been reported to date. The further improvement on cellulase performance needs the better understanding of cellulose hydrolysis mechanisms as well as the relationship of cellulase molecular structure, function and substrate characteristics.

A good strain of fungus was isolated using the municipal wastes and then further optimized for the large scale production of cellulase enzyme. The research work gives a brief idea about the fungus and cellulase enzyme produced by it. Aeration and agitation rates were found to affect cell growth and cellulase production. Low aeration (0.5 l/min) and low agitation rate (100 rpm) affected cell growth and cellulase production. Balance between aeration and agitation rate is thus essential for the optimum yield of cell biomass and cellulase production.

RECOMMENDATIONS

a. To characterize the *Aspergillus* species by molecular method.
b. To study the effect of metal ions, inducers and inhibitors for cellulase production.
c. To immobilize cellulase enzyme and study its activity.
d. To develop effective protocols for large scale production of cellulase enzyme.

REFERENCES

Abdullah, J. J., Greetham, D., Pensupa, N., Tucker, G. A., & Du, C. (2016). Optimizing Cellulase Production from Municipal Solid Waste (MSW) using Solid State Fermentation (SSF). *J. Fundam. Renew. Energy Appl*, **6**: 206.

Abu, E. A., Onyenekwe, P. C., Ameh, D. A., Agbaji, A. S., & Ado, S. A. (2000, October). Cellulase (EC 3.2. 1.3) production from sorghum bran by Aspergillus niger SL1: An assessment of pretreatment methods. In *Proceedings of the International Conference on Biotechnology: Commercialization and Food security, Abuja, Nigeria* (pp. 153-159).

Acharya, P. B., Acharya, D. K., & Modi, H. A. (2008). Optimization for cellulase production by Aspergillus niger using saw dust as substrate. *African Journal of Biotechnology*, ***7***(22).

Akiba, S., Kimura, Y., Yamamoto, K., & Kumagai, H. (1995). Purification and characterization of a protease-resistant cellulase from Aspergillus niger. *Journal of fermentation and bioengineering*, **79**(2): 125-130.

Badino, A. C., Facciotti, M. C. R., & Schmidell, W. (2001). Volumetric oxygen transfer coefficients (k L a) in batch cultivations involving non-Newtonian broths. *Biochemical engineering journal*, **8**(2): 111-119.

Bakri, Y., Jacques, P., Shi, L. K., & Thonart, P. (2002). Influence of a new axial impeller on KLa and xylanase production by Penicillium canescens 10-10c. *Applied biochemistry and biotechnology*, **98**(1-9): 1037-1048.

Bayer, E. A., Setter, E., & Lamed, R. (1985). Organization and distribution of the cellulosome in Clostridium thermocellum. *Journal of bacteriology*, **163**(2): 552-559.

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

Béguin, P., & Aubert, J. P. (1994). The biological degradation of cellulose. *FEMS microbiology reviews*, **13**(1): 25-58.

Bevan, M. W., & Franssen, M. C. (2006). Investing in green and white biotech. *Nature Biotechnology*, **24**(7): 765-767.

Bhat, M. K., & Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. *Biotechnology advances*, **15**(3): 583-620.

Biely, P., Mislovičová, D., & Toman, R. (1985). Soluble chromogenic substrates for the assay of endo-1, 4-β-xylanases and endo 1, 4-β-glucanases. *Analytical biochemistry*, **144**(1): 142-146.

Biswas, R., Persad, A., & Bisaria, V. S. (2014). Production of cellulolytic enzymes. *Bioprocessing of Renewable Resources to Commodity Bioproducts*, 105-132.

BRONNENMEIER, K., RÜCKNAGEL, K. P., & STAUDENBAUER, W. L. (1991). Purification and properties of a novel type of exo-1, 4-β-glucanase (Avicelase II) from the cellulolytic thermophile Clostridium stercorarium. *The FEBS Journal*, **200**(2): 379-385.

Cabiscol Català, E., Tamarit Sumalla, J., & Ros Salvador, J. (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. *International Microbiology, 2000, vol. 3, núm. 1, p. 3-8*.

Camassola, M., & Dillon, A. J. P. (2007). Production of cellulases and hemicellulases by Penicillium echinulatum grown on pretreated sugar cane bagasse and wheat bran in solid-state fermentation. *Journal of Applied Microbiology*, **103**(6): 2196-2204.

Chahal, P.S., Chahal, D.S. and André, G., 1992. Cellulase production profile of Trichoderma reesei on different cellulosic substrates at various pH levels. *Journal of fermentation and bioengineering*, **74**(2): pp.126-128.

Chakraborty, A., & Mahajan, A. (2014). Cellulase activity enhancement of bacteria isolated from oil-pump soil using substrate and medium optimization. *Am. J. Microbiol. Res*, **2**(2): 52-56.

Chandra, M. S., Viswanath, B., & Reddy, B. R. (2007). Cellulolytic enzymes on lignocellulosic substrates in solid state fermentation by Aspergillus niger. *Indian Journal of Microbiology*, **47**(4): 323-328.

Chandrakant, P., & Bisaria, V. S. (1998). Simultaneous bioconversion of cellulose and hemicellulose to ethanol. *Critical reviews in biotechnology*, **18**(4): 295-331.

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

Couto, S. R., & Sanromán, M. A. (2006). Application of solid-state fermentation to food industry—a review. *Journal of Food Engineering*, **76**(3): 291-302.

Cowan, W. D. (1996). Animal feed. *Industrial Enzymology. 2nd ed. London*, 360-71.

Csiszar, E., Szakacs, G., & Rusznak, I. (1998). Bioscouring of cotton fabrics with cellulase enzyme.

Cui, Y. Q., Van der Lans, R. G. J. M., & Luyben, K. C. A. M. (1998). Effects of dissolved oxygen tension and mechanical forces on fungal morphology in submerged fermentation. *Biotechnology and Bioengineering*, **57**(4): 409-419.

da Silva Delabona, P., Farinas, C. S., da Silva, M. R., Azzoni, S. F., & da Cruz Pradella, J. G. (2012). Use of a new Trichoderma harzianum strain isolated from the Amazon rainforest

with pretreated sugar cane bagasse for on-site cellulase production. *Bioresource technology*, **107**: 517-521.

Darah, I., Sumathi, G., Jain, K., & Lim, S. H. (2011). Influence of agitation speed on tannase production and morphology of Aspergillus niger FETL FT3 in submerged fermentation. *Applied biochemistry and biotechnology*, **165**(7-8): 1682-1690.

Deswal, D., Khasa, Y. P., & Kuhad, R. C. (2011). Optimization of cellulase production by a brown rot fungus Fomitopsis sp. RCK2010 under solid state fermentation. *Bioresource technology*, **102**(10): 6065-6072.

Devi, M. C., & Kumar, M. S. (2017). Production, optimization and partial purification of cellulase by Aspergillus niger fermented with paper and timber sawmill industrial wastes. *Journal of microbiology and biotechnology research*, **2**(1): 120-128.

Devillard, E., Goodheart, D. B., Karnati, S. K., Bayer, E. A., Lamed, R., Miron, J. & Morrison, M. (2004). Ruminococcus albus 8 mutants defective in cellulose degradation are deficient in two processive endocellulases, Cel48A and Cel9B, both of which possess a novel modular architecture. *Journal of Bacteriology*, **186**(1): 136-145.

Dutta, T., Sahoo, R., Sengupta, R., Ray, S. S., Bhattacharjee, A., & Ghosh, S. (2008). Novel cellulases from an extremophilic filamentous fungi Penicillium citrinum: production and characterization. *Journal of industrial microbiology & biotechnology*, **35**(4): 275-282.

El Enshasy, H., Abuoul-Enein, A., Helmy, S., & El Azaly, Y. (2008). Optimization of the industrial production of alkaline protease by Bacillus licheniformis in different production scales. *Aust. J. Basic Appl. Sci*, **2**(3): 583-593.

El-Hadi, A. A., El-Nour, S. A., Hammad, A., Kamel, Z., & Anwar, M. (2014). Optimization of cultural and nutritional conditions for carboxymethyl cellulase production by Aspergillus hortai. *Journal of Radiation Research and Applied Sciences*, **7**(1): 23-28.

Fadzilah, K., & Mashitah, M. D. (2010). Cellulases production in palm oil mill effluent: Effect of aeration and agitation. *Journal of Applied Sciences (Faisalabad)*, **10**(24): 3307-3312.

Fatokun, E. N., Nwodo, U. U., & Okoh, A. I. (2016). Classical optimization of cellulas and xylanase production by a marine Streptomyces species. *Applied Sciences*, **6**(10): 286.

Filson, P. B., & Dawson-Andoh, B. E. (2009). Sono-chemical preparation of cellulose nanocrystals from lignocellulose derived materials. *Bioresource Technology*, **100**(7): 2259-2264.

Galante, Y. M., De Conti, A., & Monteverdi, R. (1998). Application of Trichoderma enzymes in the food and feed industries. *Trichoderma and Gliocladium*, **2**: 327-342.

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

García-Ochoa, F., Castro, E. G., & Santos, V. E. (2000). Oxygen transfer and uptake rates during xanthan gum production. *Enzyme and microbial technology*, **27**(9): 680-690.

Gautam, S. P., Bundela, P. S., Pandey, A. K., Khan, J., Awasthi, M. K., & Sarsaiya, S. (2011). Optimization for the production of cellulase enzyme from municipal solid waste residue by two novel cellulolytic fungi. *Biotechnology research international*, 2011.

Ghose, T. K. (1987). Measurement of cellulase activities. *Pure and applied Chemistry*, **59**(2): 257-268.

Gunjikar, T. P., Sawant, S. B., & Joshi, J. B. (2001). Shear Deactivation of Cellulase, Exoglucanase, Endoglucanase, and β-Glucosidase in a Mechanically Agitated Reactor. *Biotechnology progress*, **17**(6): 1166-1168.

Guruchandran, V., & Sasikumar, C. (2010). Cellulase production by aspergillus niger fermented in sawdust and bagasse. *Journal of Cell & Tissue Research*, **10**(1).

Hammad, A. I., Swelim, M., & Gannam, R. B. (2010). Enhanced production of cellulase (S) by Aspergillus spp. isolated from agriculture wastes by solid state fermentation. *American-Eurasian Journal of Agricultural and Environmental Science*, **8**(4): 402-410.

Harmsen, P. F. H., Huijgen, W., Bermudez, L., & Bakker, R. (2010). *Literature review of physical and chemical pretreatment processes for lignocellulosic biomass* (No. 1184). Wageningen UR Food & Biobased Research.

Hatti-Kaul, R., Törnvall, U., Gustafsson, L., & Börjesson, P. (2007). Industrial biotechnology for the production of bio-based chemicals–a cradle-to-grave perspective. *Trends in biotechnology*, **25**(3): 119-124.

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

Howard, R. L., Abotsi, E. L. J. R., Van Rensburg, E. J., & Howard, S. (2003). Lignocellulose biotechnology: issues of bioconversion and enzyme production. *African Journal of biotechnology*, **2**(12): 602-619.

Humphrey, A. E., Moreira, A., Armiger, W., & Zabriskie, D. (1977, January). Production of single cell protein from cellulose wastes. In *Biotechnol. Bioeng. Symp.;(United States)* (Vol. 19, No. 7). Univ. of Pennsylvania, Philadelphia.

Irwin, D. C., Zhang, S., & Wilson, D. B. (2000). Cloning, expression and characterization of a family 48 exocellulase, Cel48A, from Thermobifida fusca. *The FEBS Journal*, **267**(16): 4988-4997.

Jafari, A. R., Sarrafzadeh, M. H., Alemzadeh, I., & Vosoughi, M. (2007). Effect of stirrer speed and aeration rate on the production of glucose oxidase by Aspergillus niger. *Journal of Biological Sciences*, **7**(2): 270-275.

Joos, P., Sierens, W., & Ruyssen, R. (1969). The determination of cellulase activity by viscometry. *Journal of Pharmacy and Pharmacology*, **21**(12): 848-853.

Kakiuchi, M., Isui, A., Suzuki, K., Fujino, T., Fujino, E., Kimura, T. & Ohmiya, K. (1998). Cloning and DNA Sequencing of the Genes Encoding Clostridium josui Scaffolding Protein CipA and Cellulase CelD and Identification of Their Gene Products as Major Components of the Cellulosome. *Journal of bacteriology*, **180**(16): 4303-4308.

Kalogeris, E., Christakopoulos, P., Katapodis, P., Alexiou, A., Vlachou, S., Kekos, D., & Macris, B. J. (2003). Production and characterization of cellulolytic enzymes from the thermophilic fungus Thermoascus aurantiacus under solid state cultivation of agricultural wastes. *Process Biochemistry*, **38**(7): 1099-1104.

Kang, S. W., Park, Y. S., Lee, J. S., Hong, S. I., & Kim, S. W. (2004). Production of cellulases and hemicellulases by Aspergillus niger KK2 from lignocellulosic biomass. *Bioresource technology*, **91**(2): 153-156.

Karthikeyan, N., Sakthivel, M., & Palani, P. (2011). Screening, Identifying of Penicillium KP Strain and Its Cellulase Producing Conditions. *Journal of Ecobiotechnology*, **2**(10).

Kathiresan, K., & Manivannan, S. (2010). Cellulase production by Penicillium fellutanum isolated from coastal mangrove rhizosphere soil. *Research Journal of Microbiology*, **5**(11): 1160-1164.

Keeling, C. (2011). *Canterbury Region Waste Data Report 2009/2010*. Environment Canterbury.

Khatiwada, P., Ahmed, J., Sohag, M. H., Islam, K., & Azad, A. K. (2016). Isolation, Screening and Characterization of Cellulase Producing Bacterial Isolates from Municipal Solid Wastes and Rice Straw Wastes. *Journal of Bioprocessing & Biotechniques*, **6**: 280-285.

Koivula, A., Ruohonen, L., Wohlfahrt, G., Reinikainen, T., Teeri, T. T., Piens, K. & Sinnott, M. L. (2002). The Active Site of Cellobiohydrolase Cel6A from Trichoderma reesei: The Roles of Aspartic Acids D221 and D175. *Journal of the American Chemical Society*, **124**(34): 10015-10024.

Kruus, K., Wang, W. K., Ching, J., & Wu, J. H. (1995). Exoglucanase activities of the recombinant Clostridium thermocellum CelS, a major cellulosome component. *Journal of bacteriology*, **177**(6): 1641-1644.

Kuhad, R. C., Gupta, R., & Singh, A. (2011). Microbial cellulases and their industrial applications. *Enzyme research*, *2011*.

Leck, A. (1999). Preparation of lactophenol cotton blue slide mounts. *Community Eye Health*, **12**(30): 24.

Lee, C. K., Darah, I., & Ibrahim, C. O. (2011). Production and optimization of cellulase enzyme using Aspergillus niger USM AI 1 and comparison with Trichoderma reesei via solid state fermentation system. *Biotechnology Research International*, *2011*.

Lenting, H. B. M., & Warmoeskerken, M. M. C. G. (2001). Mechanism of interaction between cellulase action and applied shear force, an hypothesis. *Journal of biotechnology*, **89**(2): 217-226.

Li, S., Zhang, X., & Andresen, J. M. (2012). Production of fermentable sugars from enzymatic hydrolysis of pretreated municipal solid waste after autoclave process. *Fuel*, **92**(1): 84-88.

Liu, C. C., & Doi, R. H. (1998). Properties of exgS, a gene for a major subunit of the Clostridium cellulovorans cellulosome. *Gene*, **211**(1): 39-47.

Liu, D., Zhang, R., Yang, X., Wu, H., Xu, D., Tang, Z., & Shen, Q. (2011). Thermostable cellulase production of Aspergillus fumigatus Z5 under solid-state fermentation and its application in degradation of agricultural wastes. *International Biodeterioration & Biodegradation*, **65**(5): 717-725.

Lynd, L. R., Van Zyl, W. H., McBride, J. E., & Laser, M. (2005). Consolidated bioprocessing of cellulosic biomass: an update. *Current opinion in biotechnology*, **16**(5): 577-583.

Madhavan, A., Srivastava, A., Kondo, A., & Bisaria, V. S. (2012). Bioconversion of lignocellulose-derived sugars to ethanol by engineered Saccharomyces cerevisiae. *Critical reviews in biotechnology*, **32**(1): 22-48.

Makoi, J. H., & Ndakidemi, P. A. (2008). Selected soil enzymes: examples of their potential roles in the ecosystem. *African Journal of Biotechnology*, **7**(3).

Mangan, D., McCleary, B. V., Liadova, A., Ivory, R., & McCormack, N. (2014). Quantitative fluorometric assay for the measurement of endo-1, 4-β-glucanase. *Carbohydrate research*, **395**: 47-51.

McCleary, B. V. (1980). New chromogenic substrates for the assay of alpha-amylase and (1→ 4)-β-D-glucanase. *Carbohydrate research*, **86**(1): 97-104.

McCleary, B. V., Mangan, D., Daly, R., Fort, S., Ivory, R., & McCormack, N. (2014). Novel substrates for the measurement of endo-1, 4-β-glucanase (endo-cellulase). *Carbohydrate research*, **385**: 9-17.

Merino, S. T., & Cherry, J. (2007). Progress and challenges in enzyme development for biomass utilization. In *Biofuels* (pp. 95-120). Springer, Berlin, Heidelberg.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*, **31**(3): 426-428.

Mo, H., Zhang, X., & Li, Z. (2004). Control of gas phase for enhanced cellulase production by Penicillium decumbens in solid-state culture. *Process Biochemistry*, **39**(10): 1293-1297.

Moslem, M. A., Mashraqi, A., Abd-Elsalam, K. A., Bahkali, A. H., & Elnagaer, M. A. (2010). Molecular detection of ochratoxigenic Aspergillus species isolated from coffee beans in Saudi Arabia. *Genetics and Molecular Research*, **9**(4): 2292-2299.

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

Nadeem, M., Qazi, J. I., & Baig, S. (2009). Effect of aeration and agitation rates on alkaline protease production by Bacillus licheniformis UV-9 mutant. *TURKISH JOURNAL OF BIOCHEMISTRY-TURK BIYOKIMYA DERGISI*, **34**(2): 89-96.

Narasimha, G., Sridevi, A., Buddolla, V., Subhosh, C. M., & Rajasekhar, R. B. (2006). Nutrient effects on production of cellulolytic enzymes by Aspergillus niger. *African Journal of Biotechnology*, **5**(5): 472-476.

Neethu, K., Rubeena, M., Sajith, S., Sreedevi, S., Priji, P., Unni, K. N., ... & Benjamin, S. (2012). A novel strain of Trichoderma viride shows complete lignocellulolytic activities. *Advances in Bioscience and Biotechnology*, **3**(08): 1160.

Ojumu, T. V., Solomon, B. O., Betiku, E., Layokun, S. K., & Amigun, B. (2003). Cellulase Production by Aspergillus flavus Linn Isolate NSPR 101 fermented in sawdust, bagasse and corncob. *African Journal of Biotechnology*, **2**(6): 150-152.

Okaiyeto, K., Nwodo, U. U., Okoli, S. A., Mabinya, L. V., & Okoh, A. I. (2016). Implications for public health demands alternatives to inorganic and synthetic flocculants: bioflocculants as important candidates. *Microbiologyopen*, **5**(2): 177-211.

Oksanen, J., Ahvenainen, J., & Home, S. (1985, January). Microbial cellulase for improving filterability of wort and beer. In *JOURNAL OF THE INSTITUTE OF BREWING* (Vol. 91, No. 3, pp. 130-130). 33 CLARGES STREET, LONDON, ENGLAND W1Y 8EE: INST BREWING.

Omojasola, P. F., & Jilani, O. P. (2008). Cellulase production by Trichoderma longi, Aspergillus niger and Saccharomyces cerevisae cultured on waste materials from orange. *Pakistan Journal of Biological Sciences*, **11**(20): 2382-2388.

Oostra, J., Le Comte, E. P., Van den Heuvel, J. C., Tramper, J., & Rinzema, A. (2001). Intra-particle oxygen diffusion limitation in solid-state fermentation. *Biotechnology and bioengineering*, **75**(1): 13-24.

Pajunen, E. (1986). Optimal use of-glucanases in wort production. In *EBC-Symposium on wort production, Monograph XI, Maffliers, France* (pp. 137-48).

PÉrez, S., & Samain, D. (2010). Structure and engineering of celluloses. *Advances in carbohydrate chemistry and biochemistry*, **64**: 25-116.

Rahman, R. N. Z. A., Geok, L. P., Basri, M., & Salleh, A. B. (2005). Physical factors affecting the production of organic solvent-tolerant protease by Pseudomonas aeruginosa strain K. *Bioresource technology*, **96**(4): 429-436.

Rajoka, M. I. (2004). Influence of various fermentation variables on exo-glucanase production in Cellulomonas flavigena. *Electronic journal of Biotechnology*, **7**(3): 07-08.

Ram, L., Kaur, K., & Sharma, S. (2014). Screening isolation and characterization of cellulase producing micro-organisms from soil. *International Journal of Pharmaceutical Science Invention*, **3**(1): 12-18.

Ray, A. K., Bairagi, A., Ghosh, K. S., & Sen, S. K. (2007). Optimization of fermentation conditions for cellulase production by Bacillus subtilis CY5 and Bacillus circulans TP3 isolated from fish gut. *Acta Ichthyologica et Piscatoria*, **1**(37): 47-53.

Reczey, K., Szengyel, Z. S., Eklund, R., & Zacchi, G. (1996). Cellulase production by T. reesei. *Bioresource Technology*, **57**(1): 25-30.

Reddy, V., Reddy, P., Pillay, B., & Singh, S. (2002). Effect of aeration on the production of hemicellulases by T. lanuginosus SSBP in a 30 l bioreactor. *Process Biochemistry*, **37**(11): 1221-1228.

Reverbel-Leroy, C., Pages, S., Belaich, A., Belaich, J. P., & Tardif, C. (1997). The processive endocellulase CelF, a major component of the Clostridium cellulolyticum cellulosome: purification and characterization of the recombinant form. *Journal of bacteriology*, **179**(1): 46-52.

Robinson, T., Singh, D., & Nigam, P. (2001). Solid-state fermentation: a promising microbial technology for secondary metabolite production. *Applied microbiology and biotechnology*, **55**(3): 284-289.

Romero, M. D., Aguado, J., González, L., & Ladero, M. (1999). Cellulase production by Neurospora crassa on wheat straw. *Enzyme and Microbial Technology*, **25**(3): 244-250.

Rubeena, M., Neethu, K., Sajith, S., Sreedevi, S., Priji, P., Unni, K. N. & Benjamin, S. (2013). Lignocellulolytic activities of a novel strain of Trichoderma harzianum. *Advances in Bioscience and Biotechnology*, **4**(02): 214.

Sabu, A., Sarita, S., Pandey, A., Bogar, B., Szakacs, G., & Soccol, C. R. (2002). Solid-state fermentation for production of phytase by Rhizopus oligosporus. *Applied Biochemistry and Biotechnology*, **102**(1): 251-260.

Sajith, S., Priji, P., Sreedevi, S., & Benjamin, S. (2016). An overview on fungal cellulases with an industrial perspective. *J Nutr Food Sci*, **6**(461): 2.

Sajith, S., Sreedevi, S., Priji, P., Unni, K. N., & Benjamin, S. (2014). Production and partial purification of cellulase from a novel fungus, Aspergillus flavus BS1. *Annals of microbiology*, **64**(2): 763-771.

Sanchez, M. M., Pastor, F. I., & Diaz, P. (2003). Exo-mode of action of cellobiohydrolase Cel48C from Paeni bacillus sp. BP-23. *The FEBS Journal*, **270**(13): 2913-2919.

Sazci, A., Erenler, K., & Radford, A. (1986). Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with the dinitrosalicyclic acid reagent method. *Journal of Applied Microbiology*, **61**(6): 559-562.

Seiboth, B., Herold, S., & Kubicek, C. P. (2012). Metabolic engineering of inducer formation for cellulase and hemicellulase gene expression in Trichoderma reesei. In *Reprogramming microbial metabolic pathways* (pp. 367-390). Springer Netherlands.

Seth, M., & Chand, S. (2000). Biosynthesis of tannase and hydrolysis of tannins to gallic acid by Aspergillus awamori—optimisation of process parameters. *Process Biochemistry*, **36**(1): 39-44.

Shen, H., Gilkes, N. R., Kilburn, D. G., Miller, R. C., & Warren, R. A. J. (1995). Cellobiohydrolase B, a second exo-cellobiohydrolase from the cellulolytic bacterium Cellulomonas fimi. *Biochemical journal*, **311**(1): 67-74.

Sherief, A. A., El-Tanash, A. B., & Atia, N. (2010). Cellulase production by Aspergillus fumigatus grown on mixed substrate of rice straw and wheat bran. *Research Journal of Microbiology*, **5**(3): 199-211.

Singh, J., Batra, N., & Sobti, R. C. (2004). Purification and characterisation of alkaline cellulase produced by a novel isolate, Bacillus sphaericus JS1. *Journal of Industrial Microbiology and Biotechnology*, **31**(2): 51-56.

Singhania, R. R., Sukumaran, R. K., Pillai, A., Prema, P., Szakacs, G., & Pandey, A. (2006). Solid-state fermentation of lignocellulosic substrates for cellulase production by Trichoderma reesei NRRL 11460.

Smitha, R. B., Jisha, V. N., Sajith, S., & Benjamin, S. (2013). 403401. Dual production of amylase and δ-endotoxin by Bacillus thuringiensis subsp. kurstaki during biphasic fermentation. *Microbiology*, **82**(6): 794-800.

Soares Júnior, F. L., Dias, A. C. F., Fasanella, C. C., Taketani, R. G., Lima, A. O. D. S., Melo, I. S., & Andreote, F. D. (2013). Endo and exoglucanase activities in bacteria from mangrove sediment. *Brazilian Journal of Microbiology*, **44**(3): 969-976.

Sohail, M., Siddiqi, R., Ahmad, A., & Khan, S. A. (2009). Cellulase production from Aspergillus niger MS82: effect of temperature and pH. *New Biotechnology*, **25**(6): 437-441.

Sreedevi, S., Sajith, S., & Benjamin, S. (2013). Cellulase producing bacteria from the wood-yards on Kallai river bank. *Advances in Microbiology*, **3**(04): 326.

Sticklen, M. (2006). Plant genetic engineering to improve biomass characteristics for biofuels. *Current opinion in biotechnology*, **17**(3): 315-319.

Stutzenberger, F. J. (1971). Cellulase production by Thermomonospora curvata isolated from municipal solid waste compost. *Applied microbiology*, ***22***(2), 147-152.

Teeri, T. T. (1997). Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *Trends in Biotechnology*, **15**(5): 160-167.

Telke, A. A., Zhuang, N., Ghatge, S. S., Lee, S. H., Shah, A. A., Khan, H., ... & Kim, S. W. (2013). Engineering of family-5 glycoside hydrolase (Cel5A) from an uncultured bacterium for efficient hydrolysis of cellulosic substrates. *PLoS One*, **8**(6): e65727.

Thibault, J., Pouliot, K., Agosin, E., & Pérez-Correa, R. (2000). Reassessment of the estimation of dissolved oxygen concentration profile and KLa in solid-state fermentation. *Process Biochemistry*, **36**(1): 9-18.

Venugopal, T., Jayachandra, K. A., & Appaiah, A. (2007). Effect of aeration on the production of endopectinase from coffee pulp by a novel thermophilic fungi, Mycotypha sp. strain no. AKM1801. *Biotechnology*, **6**(2): 245-250.

Vergara, S. E., & Tchobanoglous, G. (2012). Municipal solid waste and the environment: a global perspective. *Annual Review of Environment and Resources*, **37**: 277-309.

Vocadlo, D. J., & Davies, G. J. (2008). Mechanistic insights into glycosidase chemistry. *Current opinion in chemical biology*, **12**(5): 539-555.

Wang, J. S., Wang, J., & Gulfraz, M. (2005). Efficient Cellulase Production from Corn Straw by Trichoderma Reesei LW1 through Solid State Fermentation Process. *Ethnobotanical Leaflets*, *2005*(1): 7.

Wang, W. K., Kruus, K., & Wu, J. H. D. (1994). Cloning and expression of the Clostridium thermocellum celS gene in Escherichia coli. *Applied microbiology and biotechnology*, **42**(2): 346-352.

Wood, T. M., & Bhat, K. M. (1988). Methods for measuring cellulase activities. *Methods in enzymology*, **160**: 87-112.

Xia, L., & Cen, P. (1999). Cellulase production by solid state fermentation on lignocellulosic waste from the xylose industry. *Process Biochemistry*, **34**(9): 909-912.

Xu, C. P., & Yun, J. W. (2004). Influence of aeration on the production and the quality of the exopolysaccharides from Paecilomyces tenuipes C240 in a stirred-tank fermenter. *Enzyme and microbial technology*, **35**(1): 33-39.

Zhang, Y. H. P., & Lynd, L. R. (2005). Regulation of cellulase synthesis in batch and continuous cultures of Clostridium thermocellum. *Journal of bacteriology*, **187**(1): 99-106.

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

Zhang, Y. P., Hong, J., & Ye, X. (2009). Cellulase assays. *Biofuels: methods and protocols*, 213-231.

Zhu, H., Liu, W., Tian, B., & Zhang, S. (2012). Fluid flow induced shear stress affects cell growth and total flavone production by Phellinus igniarius in stirred-tank bioreactor. *Chiang Mai J Sci*, **39**(1): 69Ã75.

APPENDIX

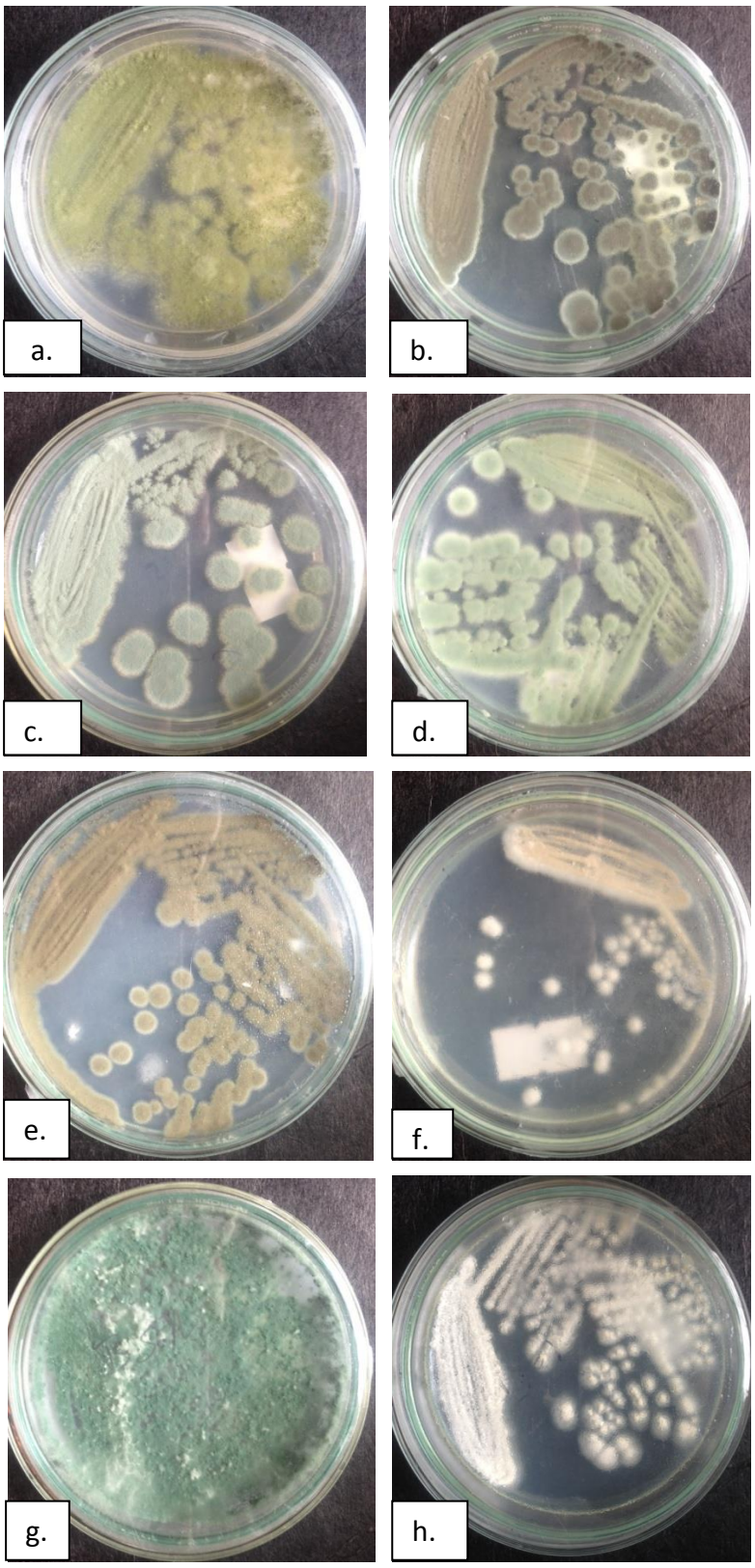

Figure 12: Pure culture of fungus isolated from the sample of a. Leaf (L1), b. Water (W2), c. Goat (G4), d. Soil (S5), e. Straw (S6), f. Dung (D7), g. Goat (G8) and h. Goat (G9) in Potato Dextrose Agar (PDA) agar plates.

Composition of PDA

Ingredients	**g/l**
Potatotes, infusion form	200.00
Dextrose	20.00
Agar	15.00

Final pH (at 25°C) – 5.6±0.2

Basal salt composition (g/500ml)

KH_2PO_4	1 gm
$(NH_4)_2SO_4$	0.7 gm
$CaCl_2$	0.15 gm
$MgSO_4.7H_2O$	0.15 gm
$CoCl_2.6H_2O$	1 gm
CMC	1%
$MnSO_4.H_2O$	0.078 gm
$FeSO_4.7H_2O$	0.25 gm
$ZnSO_4.7H_2O$	0.07 gm

Preparation of Congo red

0.1 gram Congo red was weighed and dissolved in few ml distilled water and was made to a total volume of 100 ml by adding distilled water.

Preparation of Dinitrosalicylic Acid (DNSA) reagent

1. 1 g of Dinitrosallicylic Acid

2. 30 g of Sodium Potassium Tartarate

3. Sodium Hydroxide (2M) 20mls

100 ml of DNSA reagent was prepared by mixing 30 g of Sodium Potassium Tartarate, 1 g of DNSA and 20 ml of 2 M NaOH. The total volume was made up to 100 ml by adding distilled water.

Standard curve of Glucose