SCREENING, ISOLATION AND IDENTIFICATION OF LIGNOCELLULOLYTIC ENZYME PRODUCING ENDOPHYTES FROM BANANA PITH AND PSEUDOSTEM

A PROJECT REPORT

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BONAFIDE CERTIFICATE

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ABSTRACT

Endophytes are microorganisms that live within plant tissue via a symbiotic harmonious system without harming the hosts. To colonize plant tissue, endophytes must be able to utilize available substrate. This is accomplished by the production of extracellular enzymes that can act upon the available substrates. The aim of this project is to identify such endophytes colonizing the pith and pseudostem of the banana plant (*Musaceae*). Since these plant parts contain high amount of lignocellulose, the presence of lignocellulolytic enzyme producing microorganisms can be speculated. In our project, the banana (Kadhali variety) pith and pseudostem were screened for the presence of lignocellulolytic enzyme producing endophytes. The bacterial isolates obtained were identified using morphology, biochemical methods and Bruker's taxonomy employing MALDI-TOF MS. The fungal isolates were identified by their morphology. High enzyme producers were identified using suitable assays. Bioethanol production from lignocellulosics (second-generation biofuels) is a potential area where such high lignocellulolytic enzyme producing endophytes can be effectively employed.

Keywords: Endophytes, lignocellulolytic, extracellular, second-generation biofuels

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LIST OF ABBREVIATIONS

MALDI-TOF MS	Matrix assisted Laser Desorption Ionization – Time of Flight Mass Spectroscopy
Gt	Giga tonnes
kDa	Kilodalton
E.C.	Enzyme Commission
sp.	Species
spp.	Subspecies
GHs	Glycoside hydrolases
CEs	Carbohydrate esterases
LiP	Lignin peroxidase
MnP	Manganese dependant peroxidase
pH	Hydrogen potency
pI	Isoelectric potential
Mn	Manganese
CMC	Carboxymethyl cellulose
CBM	Cellulolysis Basal Media
XBM	Xylanolysis Basal Mediua
LBM	Lignin Modifying Enzyme Basal Media
ABTS	2,2-azino-bis (3-ethyl benzthiuazoline)- 6-sulfonate
mm	Millimetre
PDA	Potato Dextrose Agar
^{0}C	Degree Celsius
w/v	Weight by volume
M	Molar
cm	Centimetre
ml	Millilitre
MR-VP	Methyl Red – Voges Proskauer
rpm	Rotations per minute
µg	Microgram
O.D	Optical density
nm	Nanometre
µl	Microlitre
mol	Mole
IUPAC	International Unit for Pure and Applied Chemistry
rRNA	Ribosomal Ribonucleic Acid
DNS	Dinitro Salicylic Acid reagent

CHAPTER 1
INTRODUCTION

1.1 GENERAL

India is the largest producer of banana accounting for about 16.8 million metric tons per annum. Tamil Nadu ranks first in banana production state wise followed by Maharashtra (www.indiastat.com/agriculture, 2009). Banana is currently the fourth most important food in the world today (after rice, wheat and maize). Banana or the Musa species, belonging to the family *Musaceae*, is a large perennial herb about 6.6 to 30 feet in height. It grows in different types of environment including tropical regions and has many uses which range from the edible bananas to fiber and ornamental plants. History records banana to have been a staple of human diet since ancient times. The banana plant is a source of food, beverages, medicines, flavorings, cooked foods, fermentable sugars, silage, fragrant, rope, cordage, garlands, shelter, clothing, and many ceremonial and religious uses.

Banana is a monocotyledonous angiosperm. Musa species are grouped according to ploidy, the number of chromosome sets they contain and the relative proportion of Musa acuminate (A) and Musa balbisiana (B) in their genome. Most of the edible bananas are either derived solely from *Musa accuminata* or are a hybrid between two wild diploid species, *M.accuminata Colla* and *M.balbisiana Colla*; which contribute to A and B genomes respectively. Hybridization and polyploidy of the A and B genomes have given rise to the various diploid, triploid and tetraploid bananas and various other varieties have been developed by hybridization of these genomes (Simmonds N W, 1962; Robinson J C, 1996).

1.2 NEED FOR STUDY

Generally it is the fruit of the plant that is utilized most while other parts such as banana peel, sheath, pseudostem, male bud, and pith are either not utilized completely or discarded. It is possible to make use of this waste in a very efficient way.

The pseudostem of the banana plant is a clustered cylindrical aggregation of leaf stalk bases. It is made of lignocellulosic fibers. The lignocellulosic content is predominantly of three components: cellulose, hemicellulose and lignin. The banana pseudostem, which is one among the main two agro- waste of the banana industry, contains good amount of cellulose and starch (Katongole *et al*., 2008). The outer covering of pseudostem is mostly cellulosic material while core or pith is rich in polysaccharides and other trace elements but lower in lignin content (Cordeiro *et al*., 2004). The detailed composition of the various plant parts is given in the following table.

Table 1.1 Composition of banana plant parts (Cordeiro *et al*., 2004, 2007)

PLANT PART	COMPOSITION (Expressed in terms of % molar proportion)		
	CELLULOSE	HOLOCELLULOSE	LIGNIN
PETIOLES/ MIDRIB	31.0	62.7	18.0
PSEUDOSTEM	34.0 – 40.0	60 - 65	12.0
LEAF BLADE	20.4	32.1	24.3
LEAF SHEATHS	37.3	49.7	13.3
FLORAL STALK	15.7	20.3	10.7

Large amounts of lignocellulosic waste are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro-industries and they pose an environmental problem. India generates a huge amount of lignocellulose waste that mainly comes from agricultural industries and other agro-based industries. Lignocellulose waste can be potentially used for various value added products including chemicals, bio fuels, and nutrients.

Such a transformation of lignocellulosic waste can be effected by the use of lignocellulolytic enzymes, which include cellulases that degrade cellulose, hemicellulases that catalyze the breakdown of hemicellulose molecules and lignases that degrade lignin. These lignocellulolytic enzymes therefore have numerous applications and significant biotechnological potential for application in a wide variety of industrial processes such as fuel, chemicals, waste management, agriculture and animal feed and as well as in the manufacture of a number of valuable products from lignocellulosic wastes.

Table 1.2 Lignocellulose contents of common agricultural residues and wastes (Howard *et al*., 2003)

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Paper	85-99	0	0-15
Wheat straw	30	50	15
Rice straw	32.1	24	18
Leaves	15-20	80-85	0
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Cotton seeds hairs	80-95	5-20	0
Fresh bagasse	33.4	30	18.9
Grasses	25-40	25-50	10-30

1.3 LIGNOCELLULOSE

Cellulose is the most abundant biopolymer on earth. Cellulose is a simple linear polysaccharide containing about 10,000 molecules of glucopyranose linked to each other. It accounts for 20 to 30% of the dry weight of most plant cell walls (McNeil *et al*., 1984). It is a structural carbohydrate that is responsible for strength and flexibility.

The hemicelluloses, of which a major portion is composed of xylans, constitute 20 – 35% by weight of wood and agricultural residues. Hemicellulose macromolecules are polymers of pentoses (xylose and arabinose), hexoses (mostly mannose) and a number of sugar acids. Hemicelluloses are complex heteropolymers made of glucuroxylans , arabinoglucuronoxylans, glucomannans, arabinogalactans and galactomannans (Haltrich *et al*., 1996; Sunna and Antranikian, 1997; Kulkarni *et al*., 1999; Subramaniyam S. and Prema P., 2002). Xylan forms covalent linkage with lignin and non-covalent linkage with cellulose and maintains the structural integrity of the plant cell wall (Kulkarni *et al*., 1999; Collins *et al*., 2005; Polizeli *et al*., 2005). Structure of xylan varies among different plant species.

Lignin is aromatic, 3-dimensional, amorphous, hydrophobic plant polymer derived from the random coupling of phenylpropanoid precursors: coniferyl, *p*-coumaryl and sinapyl alcohols (Eriksson *et al*., 1990).Lignin is an integral cell wall constituent, which provides plant strength and resistance to microbial and enzymatic degradation (Argyropoulos and Menachem, 1997). After cellulose, lignin is the second most abundant renewable biopolymer in nature. An essential part of the plant cell wall, it imparts rigidity and protects the easily degradable cellulose from attack by pathogens.

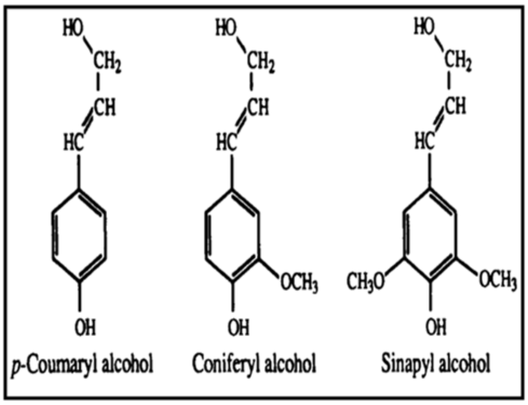


Figure 1.1 Phenylpropanoid precursors that make up lignin

An impressive collection of lignocellulolytic micro organisms have been isolated and identified and some are being used in potential applications. Lignocellulolytic organisms are microorganisms that produce enzymes capable of degrading lignocellulose or rather the components of lignocellulose. The list of such organisms includes chiefly fungi, on which extensive work has been done, and the less prolific, bacteria.

Table 1.3 The major components of lignocellulose and enzymes involved in their degradation.

	Cellulose	Hemicellulose	Lignin
% of wood mass	40 - 50	25 – 40	20 – 35
Monomer	D-anhydroglucopyranose	Xylose Mannose Plus other pentoses and hexoses	Coniferyl alcohol P-coumaryl alcohol Sinapyl alcohol
Polymeric structure	β - 1 - O – 4 linked linear chains	β - 1 - O – 4 linked linear chains with substituted side chains	Dehydrogenative polymerization to an amorphous polymer
Major enzymes involved in depolymerization	Endoglucanase (E.C.3.2.1.4) Cellobiohydrolase (E.C.3.2.1.91) β-glucosidases (E.C.3.2.1.21)	Endoxylanase β-xylosidase (and other hydrolases)	Lignin peroxidase (E.C.1.11.1.7) Manganese dependant peroxidase (E.C.1.11.1.7) Laccase (E.C.1.101.3.2)

1.4 ENDOPHYTES

Endophytes are defined as “All organisms inhabiting plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to the host” (Petrini, 1991). Endophytes may have developed intimate relationships with their hosts during evolution and may be host- or even tissue-specific. The mutualistic relationship between the endophytes and the host plants results in benefits for both sides via a harmonious symbiotic system. In the mutualistic association, the host plants are protected from pest and pathogenic fungi and in return, the host plant provided nutrition to the fungi (Azevedo et al., 2000; Saikkonen et al., 2004). To colonize plant tissue endophytes must be capable to utilize the available substrate in the surrounding environment. Endophytes have developed many significant and novel characteristics to enable them to make use of available compounds, promoting stable symbiosis. The novel characteristics acquired by the endophytes are in terms of their ability to secrete a wide range of extra cellular enzymes that permit colonization and growth.

Given the capability of endophytes to adapt to a plant tissue, there is every possibility that the pith and pseudostem of the banana plant can harbor endophytes. The pseudostem and pith, being highly lignocellulosic in nature, endophytes colonizing them must therefore secrete enzymes to utilize lignocellulosic substrates.

In this study we have isolated lignocellulolytic endophytic bacteria and fungi from banana pith and pseudostem. These isolates were identified using suitable methods, and were screened for lignocellulolytic enzyme activity.

1.5 OBJECTIVES

- Screening for Lignocellulolytic enzyme producing endophytes from banana pith and pseudostem.
- Isolation and identification of Lignocellulolytic enzyme producing endophytes from banana pith and pseudostem.
- Quantitative Screening for high Lignocellulolytic enzyme producing endophytes.

CHAPTER 2
REVIEW OF LITERATURE

2.1 LIGNOCELLULOSIC BIOMASS

Plant biomass is the most abundant renewable bioresource on earth and it is considered to play the same role in the coming years as oil did in the 20th century (Lynd L.R. *et al*., 2002). Lignocellulose is the major component of plant biomass, making up about half of the matter produced by photosynthesis. It consists of cellulose, hemicellulose, and lignin. It is a major structural component of woody and non-woody plants. Plant biomass comprises on an average 23% lignin,40% cellulose and 33% hemicellulose by dry weight (Sa-Pereira *et al*., 2003). Annually, 830 Gt of renewable plant biomass is formed consisting mainly of cellulose and hemicelluloses (Rauscher *et al*., 2006).Plant biomass is an alternative natural source for chemical and feed stocks with a replacement cycle short enough to meet the demand of the world fuel market (Kulkarni *et al*., 1999).

2.2 BANANA PLANT

The banana plant is one of the most useful plants, as almost every part of it can be used for some purpose. Banana plant parts are useful as color absorbers, insecticides, anti oxidants and in the preparation of various functional foods, wine, alcohol, biogas etc. The fruit serves as an ideal and low cost food source. Cultivated in over 130 countries, especially those along the tropics, it is one of the most widely grown tropical fruits. Most edible bananas are derived either solely from *Musa accuminata* or a hybrid between two wild diploid species, *M.accuminata Colla* and *M.balbisiana Colla* which contribute to A and B genomes respectively. Polyploidy and hybridization of the A and B genomes give rise to diploid (AA, BB, AB), triploid (AAA, ABB, AAB, BBB) and tetraploid (AAAA, AAAB, ABBB, AABB) bananas. Many other varieties also exist naturally or can be developed by hybridization of these genomes.

The banana fruit is highly nutritious and easily digestible. It is rich in potassium and calcium, and low in sodium content (Wall M.M, 2006). The sugar content of the fully mature banana is quite high making it an ideal substrate for wine making (Cheirsilp B and Umsakul K, 2008).

Parts of the banana plant generally considered as waste have many potential applications. Only the fruit is commercialized, while the pseudostem, leaves and rachis are discarded (Sagarpa, 2007). These banana waste materials are rich in nutrients and minerals (Cordeiro N *et al*., 2004), can be used to generate energy through decomposition (Clarke W.P *et al*., 2008) and can as well as be used as a good composting material (Ultra V.U *et al*., 2005). Plant parts which can serve this purpose include the banana peel, banana leaves and sheath, and the banana pseudostem, pith and male bud. These plant parts can also be used to make various eatables and paper products. The banana peel is a rich source of starch, total dietary fiber, crude protein and fat. The banana peels contain a good amount of lignin (6-12%), pectin (10-21%), cellulose (7.6-9.6%), hemicelluloses (6.4-9.4%), and galactouronic acid. The banana peel is made use of in wine production (Faturoti B.O *et al*., 2006), ethanol production (Tewari H.K *et al*., 1986) and as substrate for bio gas production. Banana leaves are a good source of lignin, higher than the banana pseudostem. It is used extensively for weaving baskets, mats, food wrappers, plates, and coverings. Banana fibers can be used as natural sorbents, bioremediation agents, as well as in handicrafts and textiles.

Fibers from the pseudostem are used in making fiber based products, given that pseudostem fiber bundles have higher specific strength modulus and low strength at break. The pseudostem is a cluster of cylindrical aggregation of leaf stalk bases. The pseudostem is utilizable for a variety of purposes, the reason being its composition. With high amount of holocellulose and low amount of ash and lignin, they can help cater to a variety of industrial demands. The outer covering of the pseudostem consist mostly of cellulose, while the core or pith is rich in polysaccharides and other trace elements, but lower in lignin

2.3 LIGNOCELLULOSE

Lignocellulose is one part of the complex biomass that requires to be released before being employed in bio process. There is great potential for utilization of this plentiful and renewable resource for diverse applications such as production of energy, liquid fuels, pulp and paper, upgraded ruminant feed, single cell protein, novel chemicals and solvents, adhesives, asphalt and a host of other value added substances, by developing appropriate technologies (Wood, 1985; Fermor, 1993; Kamra and Zadrazil, 1988; Pointing, 2001). It is estimated that 10 to 100 billion metric tonnes of lignocellulosic biomass is produced annually by plants from photosynthesis (Bassham, 1975; Kuhad and Singh, 1993). Almost 50% of waste originating from agro industrial processes, forestry and municipal sources is comprised of lignocellulose (Ishaq and Chahal, 1991). Large amount of lignocellulosic waste pose an environmental pollution problem.

Bioconversion of lignocellulosic waste could make a significant contribution to the production of organic chemicals. Lignin can serve to produce aromatic compounds, whereas low molecular mass aliphatic compounds can be derived from ethanol, which in turn can be produced by the fermentation of sugars generated from the break down on cellulose and hemicellulose.

A number of high value bio products, could also been manufactured from lignocellulosic waste. Potential products that are highly significant here are vanillin and gallic acid. Vanillin can be used for various purposes in the chemical and pharmaceutical industries. Xylitol, derived from hemicellulose, has many applications especially as sweetener instead of sucrose, in teeth hardening and remineralization as well as in chewing gums and toothpaste formulations (Roberto *et al*., 2003; Parajó *et al*., 1998). Hemicelluloses also serve as a source of xylose, from which furfural can be derived. Furfural is used in the manufacture of furfural-phenol plastics, varnishes and pesticides (Montané *et al*., 2002).

Lignocellulose is a complex of polysaccharide micro fibers, most often formed by cellulose and hemicellulose filaments and covered by lignin layers. The lignin stabilizes the complex structure by protecting the polysaccharides against attack by hydrolytic enzymes and other external factors (Leonowicz *et al*., 1999).

The lignocellulosic biomass is composed chiefly of cellulose (35-50%), followed by hemicellulose (20-35%), and lignin (10-25%) (Sun and Cheng, 2002). The remaining fraction is made up of proteins, essential oils and ash. The structure of these materials, cellulose fibers embedded in a lignin polysaccharide matrix, is very complex and the native biomass is generally resistant to enzymatic hydrolysis. The composition of lignocellulose is significant, as it is a factor that determines the nature of wood. Softwoods generally have higher lignin content than in hard woods, whereas hemicellulose content of hard woods is higher than the soft woods.

2.3.1 Cellulose

The cellulose molecule is a homopolymer of glucose units that are linked to each other by β-1,4-glucosidic units, although the true stereochemical unit of cellulose is cellobiose (β-1,4-D-glucosyl-D-glucose). By definition, cellulose is a straight chain polymer of hydrogen bonded β-1,4- linked D-glucans which accounts for 20 to 30% of the dry weight of most primary cell walls (McNeil *et al.*, 1984). Hydrolysis of cellulose yields glucose and cellodextrins as products (Gilbert *et al.*, 1983). The cellulose molecule is a polymer with a degree of polymerization of up to about 15,000 monomeric units. In immature plants, cellulose is mainly of amorphous type. But as plants grow older, cellulose becomes more ordered and crystalline in nature, becoming less degradable than the amorphous form. The crystallinity of cellulose can vary from 20% in primary cell wall to 70% in secondary cell wall. Crystalline cellulose is highly resistant to microbial attack and enzymatic hydrolysis, whereas amorphous cellulose is degraded at a much faster rate (Eriksson *et al.*, 1990).

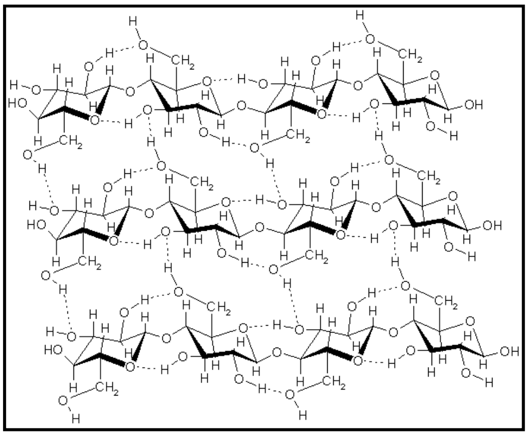


Figure 2.1 Structure of Cellulose

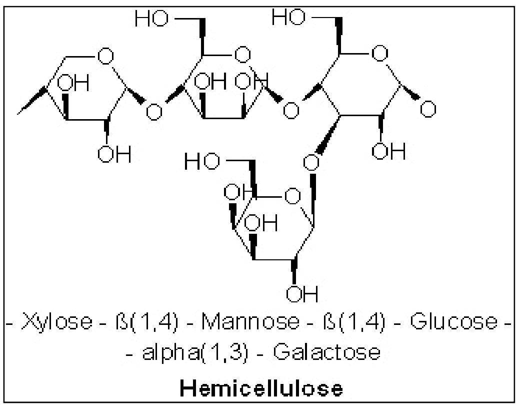


Figure 2.2 Structure of Hemicellulose

2.3.3 Lignin

Lignin is one of the most abundant, complex, aromatic, renewable biopolymer present on the earth. It is a natural polymer consisting of arylpropyl units linked primarily with β-O-4 bond, but also with various other C-C, and C-O linkages. They are highly branched polymeric molecules consisting of phenyl-propane-based monomeric units linked together by different types of bonds such as alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds. Lignin is composed mainly of three cinamyl alcohol precursors. They are p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. The relative proportions of these three constituent units in lignin vary not only with the plant species but also with the plant tissues and location of the lignins within the plant cell wall. The molecular weight of the lignins may be 100 kDa or more. It is found in the highest concentration in the middle lamella, but is most abundant in the secondary walls of vascular plants. It comprises 20-30% of woody plant cell walls and forms a matrix surrounding the cellulose and hemicelluloses. Lignin gives plants rigidity and binds plant cells together, imparting resistance towards impact, bending and compression. It plays an essential role in water and nutrient transport by acting as a barrier to permeation of water across cell walls of xylem tissue. Lignified tissues also prevent invasion of pathogenic microorganisms.

2.3.2 Hemicellulose

Hemicellulose is second to cellulose in abundance on earth, and represents a major source of renewable organic matter. It is structurally more complex than cellulose. By definition, hemicelluloses are a group of homo- and hetero- polymers consisting largely of anhydro-β-(1,4)-D-xylopyranose, mannopyranose, glucopyranose, and galactopyranose main chains with a number of substituents. The major hemicellulose components in hardwood are xylan-based and those in softwood are mannan-based. The principal sugar components of these hemicellulose heteropolysaccharides are: D-xylose, D-mannose, D-glucose, D-galactose, L-arabinose, D-glucuronic acid, 4-O-methyl-D- glucuronic acid, D-galactouronic acid, and to a lesser extent L-rhamnose, L-fucose, and various O-methylated sugars. They usually have a degree of polymerization of 100-200 (Kuhad *et al*.,1997). Most hemicelluloses are built up by β-1,4-linkages between their backbone sugars apart from the galactose-based hemicelluloses, which are characterized by β-1,3-linkages. The mannan hemicelluloses, galactoglucomannans, and glucomannans, present in softwoods and hardwoods, are branched heteropolysaccharides.

Hemicelluloses are stereoregular and have side groups consisting of sugars, sugar acids, and acetyl esters. The acetyl ester groups are bound to the linear polymer chains of various plant hemicellulose polysaccharides. Certain substituents of hemicelluloses render their structure non-crystalline or poorly crystalline so that they exist more as a gel than as oriented fibers. The most abundant hemicellulose is xylan, which comprises about 20-25% of hardwoods and 7-12% of softwoods. It is located predominantly in the secondary cell walls of angiosperms and gymnosperms (Timmel, 1967; Blanchette *et al*., 1988, 1989). Xylan has homopolymeric backbone chains of 1,4-linked β-D-xylopyranose units. The backbone consists of O-acetyl, α-L-arabinofuranosyl, α-1, 2-linked glucuronic, or 4-O-methyl glucuronic acid substituents (Kuhad *et al*.,1997).

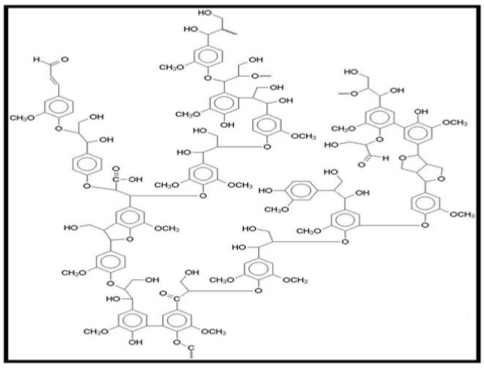


Figure 2.3 Structure of Lignin

2.4 PROBLEMS WITH LIGNOCELLULOSE UTILIZATION

Lignin is most recalcitrant to degradation whereas cellulose is more resistant to hydrolysis than hemicellulose. Lignin forms a physical seal around the cellulose and hemicellulose components of lignocellulose, forming an impenetrable barrier preventing the entry of solutions and enzymes. To efficiently process lignocellulosic waste, the lignin structure must first be modified, because this tri-dimensional phenylpropane-based polymer prevents the access to the cellulose and hemicellulose polymers. Lignin is difficult to dissolve without destroying it and some of its subunits. Alkaline (Chahal, 1992) and acid (Nguyen, 1993; Grethlein and Converse, 1991) hydrolysis methods have been used to degrade lignocellulose. Weak acids tend to remove lignin but result in poor hydrolysis of cellulose whereas strong acid treatment occurs under relatively extreme corrosive conditions of high temperature and pH. Unspecific side-reactions occur which yield non-specific by-products other than glucose, promote glucose degradation and thereby reduce its yield. Some of the unspecific products can be deleterious to subsequent fermentation. For example, acid hydrolysis of lignocellulosic materials produces several inhibitory compounds, such as sugar and lignin degradation products, compounds derived from the lignocellulosic structure and metal ions. Similarly, a large variety of compounds including aromatic, phenolic and aldehydic compounds are released from lignin.

Lignin degradation is an oxidative process and requires the presence of oxygen at a partial pressure equal to that in the natural atmosphere . It is essential to maximize the rate and specificity of lignin molecule degradation while avoiding polysaccharide consumption to promote the delignification of lignocellulosic substrates.

2.5 KEY TO SUCCESSFUL BIOTRANSFORMATION OF LIGNOCELLULOSE

1. *Delignification* to liberate cellulose and hemicellulose from their complex with lignin,
2. *Depolymerisation* of the carbohydrate polymers to produce free sugars,
3. *Bio-utilization* of mixed sugars (glucose, xylose, arabinose, mannose and galactose) by fermentation,
4. *Downstream processing* for separation and purification of products or byproducts and
5. *Recycling* of the by-products or wastes generated

2.6 LIGNOCELLULOLYTIC ENZYMES

2.6.1 Cellulases

Cellulases are enzymes responsible for the hydrolysis of cellulose. They are composed of a complex mixture of enzyme proteins that have different specificities to hydrolyze glycosidic bonds, which are the bonds that connect sugar units. The various enzymes of the cellulase complex act synergistically on the plant fiber to degrade cellulose completely. Cellulases are consortia of free inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials. Cellulases can be divided into three major enzyme activity classes (Goyal *et al*., 1991; Rabinovich *et al*., 2002a). These are endoglucanases (E.C.3.2.1.4), cellobiohydrolase (E.C.3.2.1.91) and β-glucosidase (E.C.3.2.1.21).

Cellulolytic microorganisms play an important role in the biosphere by breaking down cellulose into various economically important products like monomeric sugars, single cell proteins or microbial biomass proteins, compost, antibiotics etc. Cellulase research has been mostly concentrated in fungi but there is increasing interest in cellulase production by bacteria (Nakamura K.and Kppamura K., 1982).

Endoglucanases include β-1,4-D-glucan glucanhydrolase, endo-β-1,4-glucanase, carboxymethylcellulase (CMCase), endo-1,4-β-mannanases as well as exo-1,3-β-glucanases and other enzymes. Endoglucanases break the β-1,4-linkages of glucan polymer, cleaving the polymer in small chains with one reducing end releasing cellodextrins, cellobiose and glucose. They initiate the attack randomly at multiple internal sites in the amorphous region of the cellulose molecule. This opens up sites for subsequent attack by the cellobiohydrolases (Wood T M, 1991). Endoglucanases cannot degrade crystalline cellulose efficiently.

Cellobiohydrolase, also known as exoglucanase, is a major component of the fungal cellulase system and accounts for 40-70% of the total cellulase proteins and is capable of hydrolyzing highly crystalline cellulose (Esterbauer *et al*., 1991). It acts by removing monomers and dimers from the end of the glucose chain. β-glucosidases catalyze the hydrolysis of cellobiose and cello-oligosaccharides into glucose residues and releases glucose and aromatic residues on catalytic degradation of substrate. Endoglucanase and cellobiohydrolases work synergistically to hydrolyze cellulose.

2.6.2 Hemicellulases

Hemicellulases are multidomain proteins (Henrissat and Davies, 2000; Prates *et al*., 2001). These enzymes generally contain structurally discrete catalytic and non-catalytic modules. Hemicellulases are either glycoside hydrolases (GHs) which hydrolyse glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyze ester linkages of acetate or ferulic acid side groups.

Xylanases are glycosidases (o-glycoside hydrolases, EC 3.2.1.8) which catalyze the hydrolysis of endo-β-1,4-D-xylopyranosyl linkages in xylan in a random manner and are extensively used in paper pulp, food and animal feed industry. Conversions of hemicelluloses to valuable products by xylanases hold strong promise for the degradation of a variety of unutilized or underutilized agricultural residues for industrial applications including hydrolysis of lignocelluloses to fermentable sugars for fuel ethanol production, bread making, and clarification of beer and fruit juices.

Xylanase production has been reported in bacteria, yeast and fungi. Enzymatic hydrolysis of xylan is catalyzed by different xylanolytic enzymes such as endo-1,4-β-xylanase,β-xylosidase,α-glucuronidase,α-arabinofuranosidase, and esterase. Among these endo 1,4-β-xylanase (E.C.3.2.1.8) and β-xylosidase are the most important enzymes where the first attacks the main internal chain linkages and the second releases xylosyl residues by endwise attack of xylo-oligosaccharides.

2.6.3 Ligninases

The breakdown of lignin by fungi occurs aerobically through the use of a family of extracellular enzymes collectively termed "lignases". The lignin degrading system faces three major challenges. Firstly, the lignin polymer is large and so ligninolytic systems have to be extracellular. Secondly, the structure of lignin is composed of inter-unit carbon-carbon and ether bonds, hence oxidative degradative mechanism is required instead of hydrolytic mechanism. Thirdly, lignin is stereo-irregular; therefore the system must be much less specific in order to degrade lignin.

The enzymatic degradation of lignin is carried out chiefly by two families of lignolytic enzymes: laccases and peroxidases. Laccases are phenol oxidases and the peroxidases include lignin peroxidase (LiP) and manganese dependant peroxidase (MnP) (Krause *et al*., 2003; Malherbe and Cloete, 2003). Other enzymes that are considered to play roles in the biodegradation of lignin include glyoxal oxidaseglucose oxidase veratryl alcohol oxidase (Bourbonnais and Paice, 1988), methanol oxidase (Nishida and Eriksson, 1987) and oxido-reductase (Bao and Renganathan, 1991).

Lignin peroxidase (E.C. 1.11.1.14) (LiP) has been isolated from several white rot fungi. The active site of the enzyme contains heme moiety which is dependent on H_2O_2 and operates via a typical peroxidase catalytic mechanism. The extracellular N-glycosylated LiP has molecular masses between 38 and 47 kDa and has acidic pIs and pH optima (Gold and Alic, 1993). LiP can efficiently result in C_α-C_β cleavage and extracellular cleavage of aromatic rings that is characteristic of ligninolysis by white-rot fungus. It characterizes a variety of oxidations (Tein and Kirk, 1983): (i) C_α - C_β cleavage of β-1 and β–O-4 lignin models; (ii) oxidation of benzyl alcohols; (iii) oxidation of phenols leading to radical coupling; (iv) hydroxylation of certain benzylic methelene groups; and (v) intradiol cleavage of phenyl glycol structures. The predominant reaction in oxidation of lignin models is the cleavage of C_α - C_β bond (Hammel *et al*., 1986).

Manganese peroxidase (E.C.1.11.1.13) is one of the most common lignin degrading peroxidases produced by majority of wood-decaying fungi and by many litter-decomposing fungi (Hofrichter, 2002). It is an extracellular heme containing peroxidase with a requirement for Mn^{2+} as its reducing substrate. These are glycolsylated proteins with an iron protoporphyrin IX (heme) prosthetic group (Glenn and Gold, 1985; Nie *et al*, 1999). Their molecular weights range between 32 and 62.5 kDa (Hofrichter, 2002) and are secreted in multiple isoforms (Urzua *et al*., 1995). The haem 'Fe' of the native enzyme is ferric and it oxidizes a variety of organic compounds but only in the presence of Mn (II). Mn-peroxidase is unique in its ability to oxidise Mn (II) to Mn (III).

Laccases (E.C.1.10.13.2) is an N-glycosylated extracellular blue copper oxidase, widespread in nature (Mayer and Staples, 2002) and are produced by most of the white-rot fungi. The molecular weights range between 40-90 kDa (Reinhammer, 1984; Call and Mucke, 1997). They have acidic pIs and pH optima. Laccases contain four copper atoms in their active site. They catalyze four consecutive 1-electron oxidations, and then transfer the electrons to molecular oxygen, reducing it to water, and returning the enzyme to its native state (Kuhad *et al*., 1997). They catalyze the oxidation of a variety of phenolic compounds as well as diamines and aromatic amines with concomitant reduction of molecular oxygen to water (Thurston, 1994). Laccases are widely distributed in higher plants (Huang *et al*., 1991) and fungi (Thurston, 1994).

2.7 LIGNOCELLULOLYTIC ENZYME PRODUCING MICROORGANISMS

A diverse spectrum of lignocellulolytic microorganisms that are chiefly fungi (Baldrian and Gabriel, 2003; Falcon *et al*., 1995) and bacteria (McCarthy, 1987; Zimmermann, 1990; Vicuna, 1988) have been isolated and identified over the years and this list continues to grow rapidly. Only a few in this impressive list have been studied extensively and among these only a selected few like *Trichoderma reesei* have been used in the commercial production of lignocellulytic enzymes, cellulases and hemicellulases (Esterbauer *et al*., 1991; Jorgensen *et al*., 2003; Nieves *et al*., 1998). Amongst the organisms possessing ligninolytic capability, the most notable one is the white-rot fungi belonging to the basidiomycetes which are the most efficient and extensive lignin degraders (Gold and Alic, 1993). Of this list, *Phanerochaete chrysosporium* is the best studied lignin-degrading fungus producing plentiful amounts of a unique set of ligninolytic enzymes. Other white-rot fungi such as *Daedalea flavida*, *Phlebia fascicularia*, *P.floridensis* and *P.radiate* can selectively degrade lignin. Bacteria are generally less prolific lignin degraders and the list includes bacteria belonging to the genera *Cellulomonas*, *Pseudomonas* and the actinomycetes *Thermomonospora* and *Microbispora* as well as bacteria with cell surface-bound cellulase complexes like *Clostridium thermocellum* and *Rumniococcus* (Vicuna, 1988; McCarthy, 1987; Miller (Jr) *et al*., 1996; Shen *et al*., 1995; Eveleigh, 1987). Hemicellulases have been reported mainly from bacteria (Gilbert and Hazlewood, 1993; Sunna and Antranikian, 1997), fungi (Sunna and Antranikian, 1997), actinomycetes (Ball and McCarthy, 1989; Beg *et al*., 2000), and yeast (Hrmova *et al*., 1984; Liu *et al*., 1999).

2.7.1 Microorganisms producing cellulose degrading enzymes

A large number of microorganisms including fungi, bacteria and actinomycetes have the ability to produce cellulose degrading enzymes. But only few of them produce the necessary enzymes for degradation of crystalline cellulose. Of the organisms mentioned, it is the fungi that have been most studied with respect to cellulose degradation and production of cellulolytic enzymes. Cellulolytic fungi include brown-rot fungi, soft-rot fungi and white-rot fungi. Brown-rot fungi degrade cellulose rapidly and the most studied for cellulolytic activities are *Poria placenta*, *Lanzites trabeum*, *Tyromyces palustris* and *Coniophora puteana* (Eriksson *et al*., 1990). Soft-rot fungi mainly degrade polysaccharides and the best known of the soft-rot fungi that produces a complete set of cellulases is *Trichoderma viride* (Bisaria *et al*., 1989). Other soft-rot fungi capable of cellulose degradation are *Aspergillus niger*, *Chaetomium cellulolyticum*, *Fusarium oxysporium*, *Neurospora crassa* and *Pencillium pinophilum.* White-rot fungi have the ability to degrade lignin as well as other lignocellulosic components (Eriksson, 1981).

The most studied white-rot fungi is *Phanerochaete chrysosporium* and other important fungi are *Sporotrichum thermophile* and *Coriolus versicolor.* Fungi present in the rumen secrete cellulases that can solubilize cellulose efficiently (Wood, 1991). Typical fungi of this category are *Sphaeromonas communis*, *Piromyces communis, Neocallimastrix frontalis* and *N.patriciarum.* Bacteria are also capable of degrading cellulose but their cellulolytic enzyme systems are not directly comparable to those of fungi. Cellulases are often produced in small amounts by bacteria. The most studied bacteria in this respect are *Clostridium, Cellulomonas, Bacillus and Pseudomonas.* Some important members of actinomycetes that degrade cellulose are mesophilic species of *Streptomyces* and thermophillic species of *Thermomonospora* and *Thermoactinomyces* (Stutzenberger *et al*., 1986; Calza *et al*.,1985).

2.7.2 Microorganisms producing hemicellulose degrading enzymes

Hemicellulases are widespread in nature as they are produced by a variety of microorganisms and also higher plants and animals. The collection of hemicellulolytic microorganisms includes mainly fungi (Baldrian and Gabriel, 2003; Falcon *et al*., 1995) and bacteria (McCarthy, 1987; Zimmermann, 1990; Vicuna, 1988). Hemicellulases are produced both constitutively and inductively.

Fungi are known to be better producers of hemicellulases than bacteria. Fungal hemicellulases have been studied in detail and those of *A.niger* have been the best characterized (Eriksson *et al*., 1990). Brown-rot fungi and white-rot fungi are actively involved in the degradation of hemicellulose. White-rot fungi have effective hemicellulase systems since they are capable of depleting all the structural components of wood, ultimately degrading all wood cell wall components. Xylanase production has been reported in bacteria, yeasts, and fungi. Production and characterization of xylanases from *Trichoderma*, *Aspergillus* and *Fusarium* strains have been reported (Dekker, 1985; Singh *et al*., 1995). *Trichoderma reesei* and *Aspergillus nidulans* have been found to be rich sources of endoxylanases (Gupta *et al*., 2000; Beg *et al*., 2000; Lappalainen *et al*., 2000; Taneja *et al*., 2002; Kapoor and Kuhad, 2002). Xylanase producing microorganisms also include thermophilic fungi of which strains of *Thermomyces lanuginosus* were reported to be among the best cellulase-free xylanase producers in nature (Singh *et al*., 2000; Damaso *et al*., 2002). *Trichoderma koningii* (Li *et al*., 2000), *Aspergillus phoenicis* (Rizzatti *et al*., 2001) and *Pyrodictium abyssi* have been reported for the production of xylosidases. Anaerobic fungi, inhabitants of the alimentary canal of herbivorous animals, can also produce hemicellulolytic enzymes. These fungi can only degrade the structural polysaccharides and cannot utilize the lignin moieties. The most studied of these are *Neocallimastix frontalis*, *N. Patriciarum*, *Piromyces communis* and *Caecomyces communis* (Wubah *et al*., 1993; Mountfort, 1987). Yeast species that can produce xylan-degrading enzyme include some species of *Aureobasidium*, *Cryptococcus* and *Trichosporon*.

2.7.3 Microorganisms producing lignin degrading enzymes

Various ligninolytic enzymes work in synergism to decay the wood (made of lignocellulose, lignin being the major component in wood) efficiently resulting in the establishment of a complex system. Tien and Kirk (1983) had reported the discovery of an extracellular enzyme, now known as lignin peroxidase, from *Phanerochaete chrysosporium* which catalyzed the cleavage of lignin model compounds and spruce and birch lignins in the presence of H_2O_2. Manganese peroxide was first reported by Kuwahara and co-workers (1984) in the lignolytic culture of *P.chrysosporium*. Wood-rot fungi utilize one or the other cell wall constituents preferentially, resulting in wood decay, known as wood rot. There are three types of wood rot, i.e., soft-rot, brown-rot and white-rot, which are based on the component utilized. Only the white-rot fungi have the potential to completely degrade all the three major components of wood.

Soft-rot fungi only partially digest lignin. They prefer carbohydrates and modify lignins to a limited extent. There are over 300 species of known soft- rots, which are either the members of Ascomycetes or Deuteromycetes (Kuhad *et al*., 1997). Typical examples of soft-rot fungi include *Chaetomium*, *Paecilomyces* and *Fusarium* (Rayner and Boddy, 1988, Eriksson *et al*., 1990, Blanchette, 1995).

Brown-rot fungi primarily degrade carbohydrates leaving behind brownish modified lignin, but do not degrade the lignin. The lignin remains more or less intact and becomes modified with brown and crumbly matrix appearance (Eriksson *et al*., 1990; Blanchette, 1995; Highley and Dashek, 1998). *Poria*, *Polyporus* and *Coprinus* are some examples of brown –rot fungi (Buswell and Odier, 1987; Rayner and Boddy, 1988; Erikkson *et al*., 1990; Straatsma *et al*., 1994; Blanchette, 1995; Dix and Webster, 1995).

White-rot fungi can degrade all the components of wood, i.e., cellulose, hemicellulose and lignin. These fungi belong mostly to Basidiomycetes, but some have been identified as belonging to Ascomycetes (Erikkson *et al*., 1990) such as *Xylaria hypoxylon* and *Xylaria polymorpha* (Deacon, 1997; Liers *et al*., 2006). Some other laccase producing ascomycetes are *Rhizoctonia solani* (Wahleithner *et al*., 1996), *Aspergillus nidulans* (Law and Timberlake, 1980), *Podospora anserina* and *Neurospora crassa* (Tamura and Inoue, 1989).

On the basis of degradation of cellulose, hemicellulose and lignin at different rates and extent, the white-rot fungi have been divided into classes of simultaneous or non-selective degraders of lignin along with wood polysaccharides andoror selective or sequential lignin degraders. Simultaneous degraders remove hemicellulose and lignin more or less simultaneously with cellulose, whereas in selective delignification, lignin and hemicelluloses are removed ahead of the cellulose (Eriksson *et al*., 1990; Blanchette, 1995). The reason for the preference for hemicellulose in simultaneous degradation along with lignin is the close spatial relationship of lignin and hemicellulose, encrusting the cellulose microfibrils within the cell wall (Kerr and Goring, 1975; Koshijima *et al*., 1989; Jeffries, 1990). This selective removal of lignin-hemicellulose matrix starting at the lumen surfaces is considered to expose additional enzyme-accessible surfaces, thus allowing the enzymes to act into the wall (Kirk and Cullen, 1998). Selective lignin degraders remove lignin and leave the cellulose intact. Lignin degradation by these fungi is thought to occur during secondary metabolism and typically under nitrogen starvation.

Wood-rot fungi can be divided into three groups based on their ligninolytic enzyme patterns (Hatakka, 1994): 1. LiP, MnP and laccase producing fungi, 2. MnP and laccase producing fungi, and 3. LiP and laccase producing fungi. The most common group among the white-rot fungi is the MnP and laccase producing group.

The litter-decomposing fungi (LDFs) are those basidiomycetous and ascomycetous fungi that, together with bacteria, other fungi and animal population, participate in the decomposition of leaf litter (Dix and Webster, 1995). The basidiomycetous LDFs mostly belong to the families *Agaricaceae*, *Coprinaceae*, *Strophariaceae* and *Tricholomataceae* (Steffen, 2003).

2.8 ENDOPHYTES:

Endophytes are bacterial or fungal microorganisms that colonize healthy plant tissue intercellularly andoror intracellularly without causing any apparent symptoms of disease.Such organisms exhibit complex interactions with their hosts. During a long coevolutionary process with their hosts, endophytes have developed many significant and novel characteristics in the form of a variety of tolerance mechanisms towards host metabolites. In order to maintain a stable symbiosis, endophytes secrete varieties of extracellular enzymes that may help in the degradation of macromolecular compounds or conversion of toxic substances into other substances in order to increase their adaptability and contribute to colonization and growth.

Plants strictly limit the growth of endophytes and endophytes use many mechanisms to adapt to their environments. The ability to accomplish biotransformation with the help of specific enzymes to bring about the transformation could allow them to survive and reproduce.Endophytes can synthesize biologically active substances similar to the secondary metabolites produced by the host plants. These bioactive substances are produced as a part of the long-term symbiotic relationships that endophytes experience with their host plants.

Endophytes and host plants have a relationship that is both mutualistic and antagonistic. Endophyte-host interactions are based on mutual exploitation. As a result of their coevolution with endophytes, hosts have received benefits such as increased resistance to herbivores, pathogens, flooding stress and drought and have also acquired enhanced competitive abilities (Clay and Schardl, 2002). Endophytes also benefit from these interactions as they obtain nutrients from their hosts. Endophytes decompose some plant metabolites with ectoenzymes in order to obtain enough nutrition and energy to survive in the plant tissues.Endophytes colonize the host's roots and can help improve the host's mineral supply (Schulz *et al*., 2002).

Endophytes survive the likely harsh environment within plants either by the strong tolerance they have towards host metabolites or they have the potential to bring about biotransformation .Biotransformation is defined as the chemical alteration of an exogenous substance by or in a biological system. The compound maybe inactivated by the alteration or an active metabolite maybe produced from an inactive parent compound. Biotransformation reactions mediated by endophytes can result in detoxification effects toward toxic metabolites produced by host plants, stereoselective biotransformation and biodegradation.

Endophytes can produce a wide array of extracellular enzymes including proteinases, lipases, cellulases, pectinases, phenol oxidase and lignin catabolic enzymes (Oses *et al*., 2006; Bischohh *et al*., 2009). These enzymes are necessary for penetration and colonization of host plants by endophytes. Endophytes, with the aid of extracellular enzymes, exploit plant residues and utilize various components present therein (Kudanga and Mwenje, 2005; Lumyong *et al*., 2002).

The decomposition of the plant residues depends on the effectiveness of the enzymes respectively. Oses *et al*., (2006) found that four endophytic fungi, belonging to the basidiomycete class, isolated from Chilean tree species *Drimys winteri* and *Prumnopitys andina* were able to develop a non-selective white rot wood decay pattern. Endophytic fungi, *Alternaria*, *Phoma* and *Phomopsis*, isolated from different-aged surface-sterilized pods of *Colophospermum mopane*, showed lignocellulolytic enzyme activity. Endophytes that have potential lignocellulolytic capabilities may significantly accelerate the decay of pods and could thus allow effective germination of seeds in an arid environment when conditions are favourable (Jordaan *et al*., 2006). A strain of the endophyte *Phomsis*, screened from the inner bark of *Bischofia polycarpa*, accelerated the decomposition of peanut straw . Presence of large number of lignocellulose degradation strains in endophytes have been proven in a number of studies. These include *Xylaria* sp., *Geniculosporium*, *Coccomyces* sp., *Monotospora* sp. (Koide *et al*, 2005; Osono and Takeda, 2001, 2002). Endophytic fungi are being considered for cleaning up the environment, with most studies focusing on the white rot-fungi (Marco-Urrea *et al*., 2008)

2.9 APPLICATIONS OF LIGNOCELLULOLYTIC ENZYMES

Lignocellulose degradation holds great value for biotechnological conversion of lignocellulosic materials into value-added products. Lignocellulolytic enzymes are used in various industries such as bioconversion, chemical and pharmaceutical, detergents, food, environment, fodder, textile and paper and pulp industries.

Cellulases have significant biotechnological potential in various industries including food, animal feed, brewing and wine making, agriculture, biomass refining, pulp and paper, and textile and laundry. Hemicellulases are used in food and beverage additives to improve the properties of dough and bakery products as well as clarification of wines. Biotechnological applications of xylanases include bioconversion of lignocellulosic materials to fermentative products, clarification of juices, improvement of consistency of beer and digestibility of animal feedstock. Xylanases also find use in the pulp and paper industry. Ligninolytic enzymes have applications in various processes such as oxidation of organic pollutants, pulp delignification, stabilization of fruit juices, biosensors development, biofuel cells, textile biofinishing, environmental protection processes, beverage processing, animal feed stuffs, bioleaching systems, cosmetics, enzyme immunoassays and wastewater detoxification, denim stone washing, detergent manufacturing and transformation of antibiotics and steroids (Tien and Kirk, 1998).

2.9.1 Paper and pulp industries

Enzymatic deinking is a promising biotechnological application of cellulases in the pulp and paper industry. Cellulases alone, or in combination with other enzymes like xylanases, can be used for deinking different types of paper waste. Cellulases and xylanases release toner particles that facilitate flotation and subsequent steps that include aggregation using high temperatures and then vigorous dispersion for size reduction.

Endoglucanases and endoxylanases have been shown to improve optical and strength properties of paper from enzymatically deinked pulp. Biopulping provides significant energy savings. Biomechanical pulping using white-rot fungi was originally applied as pretreatment of wood chips to modify lignin and extractives. Mixture of cellulases and hemicellulases have been used for bio-modification of fiber properties with the aim of improving drainage and beatability in the paper mills before or after beating of pulp. Endoglucanases I and II are useful in these fiber modification applications. Endoglucanase II can significantly reduce the pulp viscosity to low concentrations without causing any structural damage to the fibers.

Hemicellulases are used in the treatment of cellulosic pulps to remove residual xylans. Xylanases, mannases and their accessory enzymes selectively degrade the hemicellulose portion in pulps without affecting the cellulose. Xylanase pretreatment has a lot of advantages over bleaching of the pulp. Since xylan does not form a tightly packed structure, it is more accessible to hydrolytic enzymes. The removal of xylan by xylanases leads to decrease in energy demand during bleaching and also leads to reduction in adsorbable organic halogen (AOX) and dioxin concentration due to reduced chlorine requirement to achieve a given brightness. Lignin peroxidase was reported to be effective in decolorizing kraft pulp mill effluents.

2.9.2 Textile industry

Cellulases are used for the biostoning of jeans and biopolishing of cotton and other cellulosic fabrics. Enzymatic stonewashing allows up to 50% higher jean load and provides the desired soft finish. Enzymatic biopolishing is a novel biological finishing process for textiles made from cellulosic fibers. Cellulase preparations, rich in endoglucanases, are best suited for biopolishing, enhancing fabric look, feel and colour without the need for chemical coating of fibers. They help remove short fibers, surface fuzziness, creates a smooth and glossy appearance, and improves colour brightness, hydrophilicity and moisture absorbance.

Cellulases can improve the cleaning power of detergents. Alkaline cellulase from *Bacillus* sp., in addition to the detergent, can remove soil in the inter-fibre spaces. Cellulases have been shown to effectively facilitate the abrasion of indigo dye from fibre surfaces. Xylanases are used in the pretreatment of low quality jute fibre, which is hard due to higher hemicellulose content, selectively removing xylan and without affecting the fibre strength.

2.9.3 Agriculture and Environment

Various enzyme preparations consisting of different combinations of cellulases, hemicellulases and other enzymes like pectinases have potential applications in agriculture for enhancing growth of crops and controlling plant diseases. Fungal β-glucanases are capable of controlling diseases by degrading cell walls of plant pathogens. Cellulolytic fungal species of *Trichoderma*, *Geocladium*, *Chaetomium* and *Penicillium* are known to play a key role in seed germination, rapid plant growth, flowering and crop yields.

New approaches to combat environmental contaminants are adopted by utilizing bacteria, fungi and their respective genetically modified variants that can produce enzymes capable of degrading the pollutants. Ligninolytic cultures of *Phanerochaete chrysosporium* and *Trametes versicolor* have been reported to degrade PCB, anthracene, fluorine, phenanthrene, benzopyrene and dichloroaniline (Morgan *et al*., 1991; Field *et al*., 1991; Collins *et al*., 1996; Baldrian *et al*., 2000). White-rot fungi have been studied for dye decolorization or degradation (Reddy, 1995). Laccases and peroxidases have been found to be suitable for the treatment of wastewater from the textile industry (Murugesan *et al*., 2003; Zille *et al*., 2003). Laccases can degrade a variety of synthetic dyes. Bleaching of industrial effluents and dye colors by white-rot fungi and their ligninolytic enzymes is a promising biotechnological application. They can be used to decolorize and degrade organic chlorine-containing material from pulp mill wastewaters (Eriksson and Kirk, 1985). Organopollutants such as 2, 4, 6 – trinitrotoluene (TNT), polychlorinated biphenyls (PCB), organochlorines, PAHs and wood preservatives were shown to be degraded by white-rot fungi (Pointing, 2001). Laccase has applications in the decolorization of industrial dyes (Rodriguez *et al*., 1999)

Manganese peroxidase has potential environmental application and it is utilized to degrade pollutants as it oxidizes a wide range of substrates, including several phenolic compounds, high molecular weight chlorolignins and nylon.

Ligninolytic fungi such as *Pleurotus pulmonarius* and *Pleurotus ostreatus* are useful in the biodegradation of toxic compounds (Pointing, 2001; Lau *et al*., 2003; Hestbjerg, 2003). *Pleurotus* species have been shown to degrade lignocellulosic agro-industrial wastes.

2.9.4 Food

Fungal cellulases have been shown to improve the yield of sugars from unmodified malts in the brewing process. In the production of alcohol from cassava, cellulases from *Trichoderma* increase ethanol yields by breaking down cellulose into glucose. Cellulases are used to improve and speed up colour extraction by pectinases from fruit skins. Xylanases are useful for applications in food processing. Xylanase pretreatment of cereal and millet flours can reduce processing times in fermentation of traditional foods. Other applications of xylanases are in clarifying juices and wine, for extracting coffee and plant oils, for improving nutritional properties of silage, for macerating plant cell walls, for producing food thickeners and for providing different texture to bakery products (Subramaniyam and Prema, 2002). They have been used to enhance the recovery of starch from wheat flours. In the baking industry, xylanases are used in improving desirable texture, loaf volume and shelf-life of bread.

2.9.5 Chemical Industry

Lignin degrading enzymes have been used for the detoxification of endocrine-disrupting chemicals. This application is based on the fact that endocrine disrupters like bisphenol A, nonyl phenols and phthalic esters are similar to natural hormones, being phenolic compounds, and they are therefore good substrates for ligninolytic enzymes. Laccases from white-rot fungi can be used to treat alkyl phenols, bisphenols, and natural and synthetic estrogens adsorbed on soil.

2.9.6 Biosensors

A biosensor is a device that detects, transmits and records information regarding a physiological or a biochemical change. Laccase-containing biosensors have been developed for immunoassays, glucose determination and determination of aromatic amines and phenolic compounds. A carbon based biosensor modified with a crude enzymatic extract of the *Pleurotus ostreatus* fungi as a source for the enzyme laccase has been proposed for determining the concentration of catecholamines in pharmaceutical formulations. Laccase catalyzes the oxidation of adrenaline and dopamine in the corresponding quinones and the current obtained in the electrochemical reduction of each of the products is related to the concentration of catecholamines. Cellulose binding domains of cellulases can be fused to heterologous proteins and used as an affinity tag in purification or immobilization of fusion proteins.

2.9.7 Bioconversion

Enzymatic saccharification of lignocellulosic substances produces monosaccharides, which can be subsequently fermented to a variety of products such as ethanol, other alcohols, organic acids, single cell protein and lipids (Kuhad *et al*., 1997). Glucanases are added either during mashing or primary fermentation to hydrolyze glucan, reduce the viscosity of wort and improve the filterability.

Bioethanol, a cost-effective way to reduce greenhouse gas emissions and gasoline use in transport, can be produced from agricultural residues such as cereal straws and corn stalks. Complete cellulolytic and hemicellulolytic enzyme systems are required to achieve maximum hydrolysis of complex substrates to yield monomeric sugars, from which bioethanol can be produced.

Various xylose-rich hemicellulosic materials can serve as abundant and cheap source for production of xylitol, which is used as a natural food sweetener, by fermentation. Hemicellulases can be used for the enzymatic synthesis of oligosaccharides. Controlled degradation of lignin by white-rot fungi or actinomycetes can give valuable aromatic products. Synthesis of conducting polyaniline and study of naphthol oxidation, both being substances with wide applications, are also areas where laccases have been used.

The white rot Basidiomycete *Phanerochaete chrysosporium* has been used for the saccharification of lignocellulosic agro-waste of paddy straw, wheat straw, sugarcane bagasse ,cotton stalks and coffee pulp, as this fungus is able to completely degrade cellulose, hemicellulose and lignin. *Pleurotus* species have also been used to improve digestibility of lignocellulosic biomass.

2.9.8 Feed

Fiber-degrading enzymes, such as cellulase and xylanase, have potential application in the animal feed industry where they are used for improving feed utilization, milk yield and body weight gain (Beauchemin *et al*., 2001, 2003).

Cellulases are used to improve the silage of cattle feed. Improvement of nutritional value has been reported on pretreatment of agricultural silage and grain feed by xylanases (Beg *et al*., 2001). Xylanases reduce the viscosity and increase absorption by breaking down the non-starch polysaccharides in high fiber rye- and barley- based feeds. Most commercial xylanases are produced by *Trichoderma*, *Bacillus*, *Aspergillus*, *Penicillium*, *Aureobasidium*, and *Talaromyces* spp. (Godfrey *et al*., 1996). Lignocellulosic waste from pulp and paper industries and dairy and agricultural industries is the potential substrate for use in the production of single cell protein (SCP) for feed and food purpose (Kuhad *et al*., 1997). The potential to convert cellulose to SCP by growing microorganisms directly on cellulosic substrate for microbial protein production and by facilitating production of extracellular cellulase enzyme that can be used for saccharification of cellulosic substrates represents an efficient waste recycling process. Organisms used for producing SCP generally are bacteria *Cellulomonas* and *Lactobacillus*; molds *Aspergillus, Penicillium, Chaetomium* and *Polyporus* and the yeasts *Saccharomyces, Candida* and *Rhodotorula* (Kuhad *et al*., 1993, 1997).

CHAPTER 3
MATERIALS AND METHODS

3.1 MATERIALS

- Banana pith and Pseudostem (Kadhali Variety).
- 70% ethanol.
- Gram's Crystal violet.
- Gram's Iodine.
- Gram's decolorizer.
- Gram's Safranin.
- Carboxymethyl cellulose.
- Xylan (Beech wood Xylan) (SIGMA ALDRICH).
- Lignin (SIGMA ALDRICH).
- Kovács oxidase reagent.
- MR-VP broth.
- Methyl red solution.
- Voges-Proskauer reagents (Barritt's reagent A and Barritt's reagent B).
- Kovács Indole reagent.
- McIlvaine's Buffer (pH 7.2) for Bacterial assay.
- Citrate buffer (pH 4.8) for fungal assay.
- DNS reagent.
- 2,2-azino-bis (3-ethylbenzthiazoline)-6-sulfonate (ABTS)
- Guaiacol
- Hydrogen Peroxide
- Acetate Buffer (pH 5.0)
- Tri chloro acetic acid

Table 3.1 Materials for Isolation, Screening and Staining

CATEGORY	MEDIA	COMPOSITON
ISOLATION	Potato Dextrose Agar	
SCREENING	Cellulolysis Basal Media (CBM)	Yeast Extract, Soya Peptone, Agar, Carboxy Methyl Cellulose
	Xylanolysis Basal Media (XBM)	Yeast Extract, Soya Peptone, Agar, Xylan
	Lignin Modifying Enzyme Basal Media (LBM)	Yeast Extract, Soya Peptone, Agar, Lignin, Glucose
STAINING	CBM	Congo red and Sodium Chloride
	XBM	Potassium Iodide and Iodine
	LBM	Ferric Chloride {$FeCl_3$} and Potassium Ferricyanide {K_3 [Fe (CN) $_6$]}

Table 3.2 Inoculum and Production media composition

INOCULATION MEDIA	PRODUCTION MEDIA		
	CELLULASE	HEMICELLULASE	LIGNINASE
Tri Sodium Citrate	Tri Sodium Citrate	Tri Sodium Citrate	Potassium dihydrogen phosphate(KH_2PO_4)
Potassium dihydrogen phosphate(KH_2PO_4)	Potassium dihydrogen phosphate(KH_2PO_4)	Potassium dihydrogen phosphate(KH_2PO_4)	Magnesium sulphate ($MgSO_4$)
Sodium nitrate($NaNO_3$)	Sodium nitrate($NaNO_3$)	Sodium nitrate($NaNO_3$)	Calcium chloride ($CaCl_2$)
Ammonium sulphate ($(NH_4)_2SO_4$)	Ammonium sulphate ($(NH_4)_2SO_4$)	Ammonium sulphate ($(NH_4)_2SO_4$)	Ferrous sulphate
Magnesium sulphate ($MgSO_4$)	Magnesium sulphate ($MgSO_4$)	Magnesium sulphate ($MgSO_4$)	Sodium nitrate($NaNO_3$)
Peptone	Peptone	Peptone	Copper sulphate
Yeast extract	Yeast extract	Yeast extract	Lignin and Glucose
Glucose	Carboxymethyl cellulose	Rice Bran	Yeast extract

3.2 METHODS:

3.2.1 Isolation of endophytes from banana pith and pseudostem

i. The banana pith and pseudostem samples were surface sterilized by dipping in 70%.
ii. Autoclaved forceps and knifes were used for cutting the samples into small pieces of 10mm*10mm dimensions under aseptic conditions.
iii. Sufficient numbers of cut pieces (4 pieces) were placed in two distinct set of PDA plates (90mm diameter), one containing antibiotic cephalosporin (250 milligram) to inhibit bacterial growth and other without antibiotic.
iv. The plates were incubated at 33°C for 24 - 48 hours.
v. After 2 days of incubation, colonies observed around the periphery of the pith and within the pseudostem were carefully isolated and were transferred to PDA slants under aseptic conditions.
vi. The slants were incubated at 33°C for 24 - 48 hours.

3.2.2 Morphological identification

Simple staining Procedure:

i. A clean microscopic slide was taken. A drop of autoclaved distilled water was placed on it.
ii. A loopful of bacterial culture was taken, and was smeared on the slide.
iii. The smear was then heat fixed and few drops of a simple stain; crystal violet was added and was allowed to remain for 2 minutes.
iv. The excess stain was washed off and was allowed to air dry.
v. The slide was then observed under medium (40X) and high power (100X, Oil Immersion) bright field microscope.

Gram Staining Procedure:

i. A clean microscopic slide was taken. A drop of autoclaved distilled water was placed on it.
ii. A loopful of bacterial culture was taken, and was smeared on the slide. The smear was then heat fixed and few drops of a primary stain, Crystal violet was added and was allowed to remain for 2 minutes. The excess stain was washed off
iii. Few drops of Gram's iodine, which acts as mordant was added and was allowed to remain for a minute.
iv. Few drops of Gram's decolorizer was added, and was allowed to remain for 5 – 10 seconds. It was then immediately washed off with water.
v. Counter stain, Safranin was added and was allowed to remain for 2 minutes. The excess stain was washed off and was allowed to air dry
vi. The slide was then observed under medium (40X) and high power (100X, Oil Immersion) bright field microscope.

3.2.3 Screening for lignocellulolytic enzyme producers

Cellulase Screening (Stephen B. Pointing, 1999)

i. Suitable Cellulolysis Basal Media (CBM) was prepared in sufficient quantity, autoclaved and was poured onto petriplates (90mm diameter).
ii. The CBM plates were inoculated under aseptic conditions and were incubated at 33°C for 24 - 48 hours.
iii. After 2 days of incubation, the plates were flooded with 2% w/v aqueous Congo red solution for 15 minutes and washed with distilled water. The plates were then flooded with 1M NaCl for 15 minutes to destain and then to visualize clearance zones.
iv. The colony diameter and the clearance zone diameter were noted down.

Xylanase Screening (Stephen B. Pointing, 1999)

i. Suitable Xylanolysis Basal Media (XBM), was prepared in sufficient quantity, autoclaved and were poured onto petriplates (90mm diameter).
ii. The XBM plates were inoculated under aseptic conditions and were incubated at 33°C for 24 - 48 hours.
iii. After 2 days of incubation, the plates were flooded with 0.25% w/v aqueous I_2 and KI solution for 5 minutes and washed with distilled water to visualize clearance zones.
iv. The colony diameter and the clearance zone diameter were noted down.

Ligninase Screening (Stephen B. Pointing, 1999)

i. Suitable LME Basal Media (LBM) was prepared in sufficient quantity, autoclaved and was poured onto petriplates (90mm diameter).
ii. The LBM plates were inoculated under aseptic conditions and were incubated at 33°C for 24 - 48 hours.
iii. After 2 days of incubation, the plates were flooded with 1% w/v aqueous solution of $FeCl_3$ and $K_3[Fe(CN)_6]$ for 5 minutes and washed with distilled water to visualize clearance zones.
iv. The colony diameter and the clearance zone diameter were noted down.

3.2.4 Biochemical identification

Oxidase test:

i. Oxidase test discs were placed on 24 hours old bacterial culture plates and were observed for significant colour development.

Lactose fermentation test:

i. 30ml each of autoclaved lactose fermentation media was poured into 10 test tubes.
ii. Two sets of tubes of lactose oxidative-fermentative medium were inoculated by stabbing half way to the bottom or ¼-inch from the bottom with the isolate.
iii. One set of the tubes was overlaid with a thick layer of mineral oil. This overlay prevents the diffusion of oxygen into the medium and creates an anaerobic condition in the tube.
iv. The tubes were incubated at 35˚C for 48 hours.

Indole test:

i. 30ml each of autoclaved tryptone broth was poured into 5 test tubes.
ii. The test tubes were inoculated with a small amount of pure culture.
iii. They were incubated at 35˚C for 48 hours.
iv. After 2 days of incubation, 5 drops of Kovács Indole reagent was added to the tubes.

MR-VP test:

i. 2.5ml each of autoclaved MR-VP broth was poured into 10 test tubes.
ii. The tube containing MR-VP broth was inoculated with light inoculum under aseptic conditions.
iii. The test and the control cultures were incubated at 35˚C for 48 hours.

iv. After 2 days of incubation, to one set of 5 test tubes, 5 drops of methyl red reagent was added and the color change if any was observed.

v. To the another set of 5 tubes approximately 0.6ml of Barritt's reagent A and 0.2ml of Barritt's reagent B were added.

vi. The tubes were carefully shaken for 30 seconds to 1 minute to expose the medium to atmospheric oxygen.

vii. The tubes were allowed to stand for at least 30 minutes and any color change was observed.

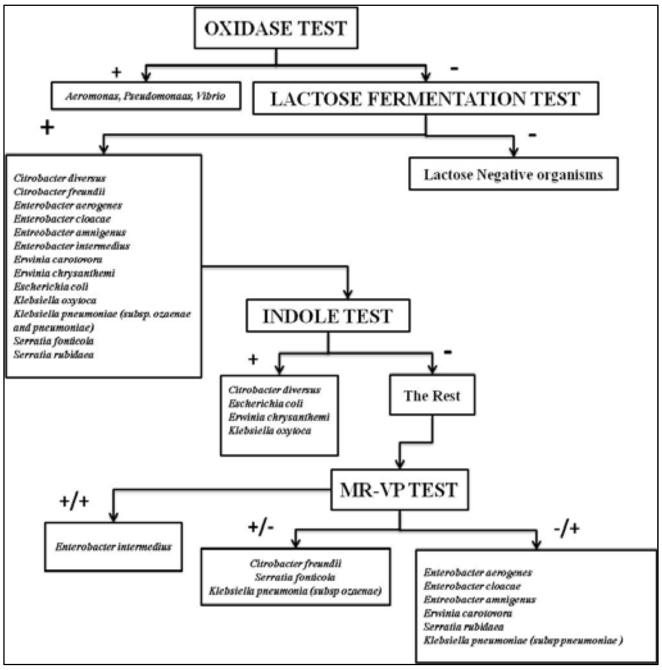


Figure 3.1 Biochemical Identification flowchart

Motility test:

i. Using a sterile needle, a good amount of culture was stabbed into the medium to within 1cm of the bottom of the tube. Care was taken so that the needle was in the same line as it entered when removed from the medium.

ii. They were incubated at 35˚C for 18 hours or until growth was evident.

3.2.5 Enzyme production and harvesting:

Inoculation media preparation:

i. Eight 250ml conical flasks containing 50ml of inoculation media were autoclaved.

ii. Five bacterial isolates and 3 fungal isolates were inoculated in the inoculum media under aseptic conditions.

iii. The flasks were then incubated at 30˚C for fungal and 37°C in the case of bacterial cultures in an orbitary shaker at 120 rpm for 48 hours.

Production media preparation:

i. Eight 250 ml conical flasks containing 50ml of production media were autoclaved.

ii. 1ml of cell culture from the inoculation media was transferred aseptically to the 50 ml of production media.

iii. The flasks were then incubated at 30˚C for fungi and 37°C in the case of bacterial cultures in an orbitary shaker at 120 rpm for 48 hours.

Enzyme harvesting:

i. After 72 hours, the production media broth was centrifuged at 4°C and 10,000 rpm for 10 minutes and the clear supernatant was stored under refrigeration to avoid proteolysis of the desired enzyme.

3.2.6 Assay procedure:

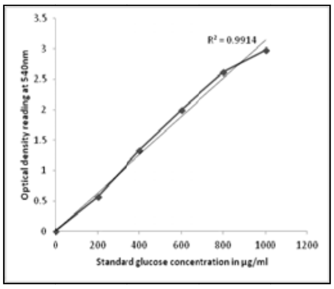


Figure 3.2 Glucose Calibration Curve

Table 3.3 Glucose Standard Calibration Table

Reagents`	Spectro Zero	S1	S2	S3	S4	S5
Volume of Standard Glucose solution (ml)	-	0.2	0.4	0.6	0.8	1.0
Concentration of Standard Glucose (µg/ml)	-	200	400	600	800	1000
Volume of Distilled Water (ml)	2.0	1.8	1.6	1.4	1.2	1.0
Volume of Buffer (ml)	1.0	1.0	1.0	1.0	1.0	1.0
Volume of DNS Reagent (ml)	3.0	3.0	3.0	3.0	3.0	3.0
Incubate in a boiling water bath for 15-20 minutes						

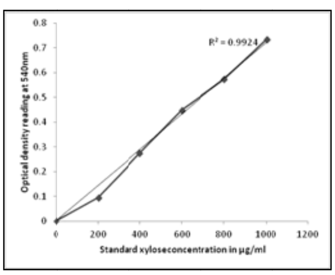


Figure 3.3 Xylose Calibration Curve

Table 3.4 Xylose Standard Calibration Table

Reagents	Spectro Zero	S1	S2	S3	S4	S5
Volume of Standard Xylose solution (ml)	-	0.2	0.4	0.6	0.8	1.0
Concentration of Standard Xylose(µg/ml)	-	200	400	600	800	1000
Volume of Distilled Water (ml)	2.0	1.8	1.6	1.4	1.2	1.0
Volume of Buffer (ml)	1.0	1.0	1.0	1.0	1.0	1.0
Volume of DNS Reagent (ml)	3.0	3.0	3.0	3.0	3.0	3.0
Incubate in a boiling water bath for 15-20 minutes						

Table 3.5 Carboxymethyl cellulase assay for endo-β-1, 4-glucanase

Measurement of Cellulase Activities,IUPAC,1987								
Reagents	**B7**		**F1**		**F2**		**F4**	
	T	**C**	**T**	**C**	**T**	**C**	**T**	**C**
Volume of Buffer (ml)	1.0	-	1.0	-	1.0	-	1.0	-
Volume of Substrate (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Volume of enzyme (ml)	1.0	-	1.0	-	1.0	-	1.0	-
Incubate for 60 minutes at 37°C (for Bacteria) and 50° (for fungi)								
Volume of DNS Reagent (ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Volume of enzyme (ml)	-	1.0	-	1.0	-	1.0	-	1.0
Incubate in a boiling water bath for 15-20 minutes								
O.D Reading at 540nm (Average of Duplicates)	0.632	0.301	1.514	0.793	1.637	0.826	1.065	0.690

Table 3.6 Assay for endo-β-1, 4-xylanase

Measurement of Hemicellulases Activities,IUPAC,1987								
Reagents	**B7**		**F1**		**F2**		**F4**	
	T	**C**	**T**	**C**	**T**	**C**	**T**	**C**
Volume of Buffer (ml)	1.0	-	1.0	-	1.0	-	1.0	-
Volume of Substrate (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Volume of enzyme (ml)	1.0	-	1.0	-	1.0	-	1.0	-
Incubate for 60 minutes at 37°C (for Bacteria) and 50° (for fungi)								
Volume of DNS Reagent (ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Volume of enzyme (ml)	-	1.0	-	1.0	-	1.0	-	1.0
Incubate in a boiling water bath for 15-20 minutes								
O.D Reading at 540nm (Average of Duplicates)	0.499	0.241	0.051	0.046	0.604	0.138	0.801	0 .102

Table 3.7 Assay for Fungal Laccase using ABTS

Reagents	**Blank**	**F1**	**F2**	**F4**
Volume of Buffer (µl)	990	940	940	940
Volume of Substrate (µl)	10	10	10	10
Volume of enzyme (µl)	0	50	50	50
Incubate at 37°C for 15 minutes				
Volume of Arresting agent (µl)	50	50	50	50
O.D Reading at 420nm	0.000	0.544	0.544	0.447

CHAPTER 4
RESULTS AND DISCUSSION

4.1 ISOLATION OF ENDOPHYTES FROM BANANA PITH AND PSEUDOSTEM

7 bacterial isolates and 5 fungal isolates were obtained from banana pith and pseudostem samples that were placed on PDA plates.

4.2 MORPHOLOGICAL IDENTIFICATION

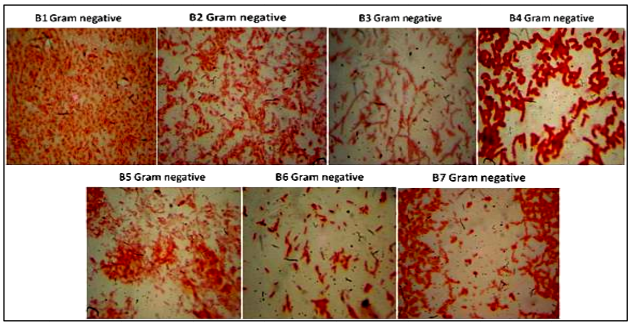


Figure 4.1 Gram Staining Results

Table 4.1 Simple Staining and Gram Staining Results

ISOLATE	MORPHOLOGICAL CHARACTERISTICS
B1	Gram Negative medium rods
B2	Gram Negative medium rods
B3	Gram Negative thin, long rods
B4	Gram Negative oval shaped rods
B5	Gram Negative short rods
B6	Gram Negative thin rods
B7	Gram Negative short rods

4.3 SCREENING FOR LIGNOCELLULOLYTIC ENZYME PRODUCERS

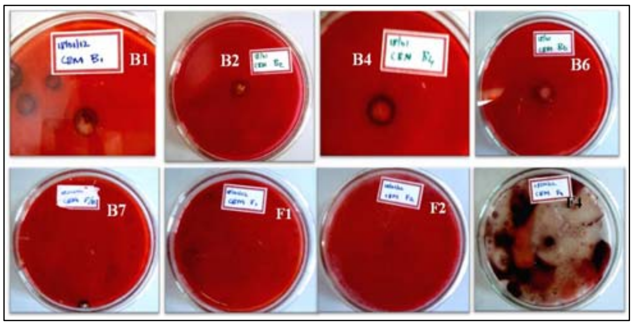


Figure 4.2 CBM agar clearance after Congo red staining

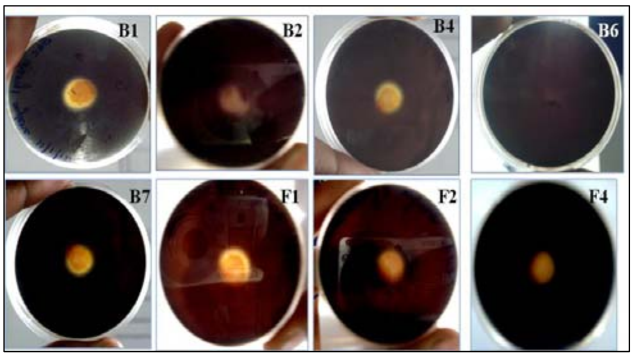


Figure 4.3 XBM agar clearance after iodine and potassium iodide staining

Table 4.2 Qualitative screening for lignocellulolytic enzyme activity

BASAL MEDIA & PRODUCTION MEDIA	BACTERIAL ISOLATES SHOWING GOOD RESULTS (In terms of ratio of clearance zone to colony diameter)		FUNGAL ISOLATES SHOWING GOOD RESULTS	
	Isolate	Ratio(Average of Duplicates)	Isolate	Average Ratio
CELLULOLYSIS BASAL MEDIUM	B1	2.625	F1	1.465
	B2	2.140	F2	3.000
	B4	2.750	F4	4.400
	B7	4.400		
XYLANOLYSIS BASAL MEDIUM	B1	3.000	F1	Only growth observed, zone of clearance not measured
	B2	5.000	F2	
	B6	5.000	F4	
	B7	4.000		
LIGNIN MODIFYING ENZYME BASAL MEDIA	B2 B4 B6	Only growth observed, zone of clearance not measured	F1 F2 F4	Only growth observed, zone of clearance not measured

Based on the above reported qualitative screening assay, only 5 bacterial and 3 fungal isolates (B1,B2,B4,B6,B7,F1,F2,F4) were carried for further work, since they have shown significant lignocellulolytic enzyme activity.

4.4 BIOCHEMICAL IDENTIFICATION

4.4.1 Oxidase test:

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase, an enzyme sometimes called indophenols oxidase. In the presence of an organism that contains the cytochrome oxidase enzyme, the reduced colorless reagent becomes an oxidized colored product.

No color change was observed, indicating that these gram negative rods belong to Enterobacteriaceae family.

4.4.2 Lactose fermentation test:

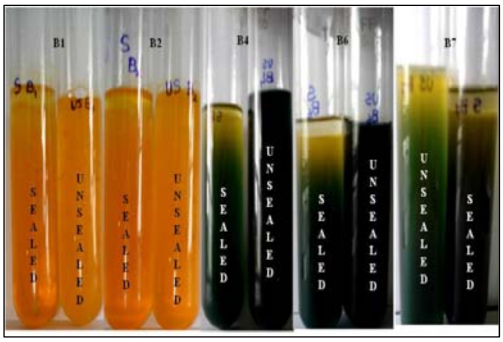


Figure 4.4 Lactose Fermentation Results

The oxidative-fermentative test determines if certain gram negative rods metabolize lactose by fermentation or aerobic respiration (oxidatively). During the anaerobic process of fermentation, pyruvate is converted to a variety of mixed acids depending on the type of fermentation. The high concentration of acid produced during fermentation will turn the bromothymol blue indicator in OF media from green to yellow in the presence or absence of oxygen.

Certain non-fermentating gram-negative bacteria metabolize lactose using aerobic respiration and therefore only produce small amount of weak acids during the Kreb's cycle and Entner Doudoroff (glycolysis). The increased concentration of lactose in the medium enhances the production of these weak acids to a level that can be detected by bromothymol blue indicator. To further enhance the detection of these weak acids, this medium contains a reduced concentration of peptones. This reduces the production of amines from the metabolism of amino acids, therefore reducing the neutralizing effect of these products. Dipotassium phosphate buffer is added to further promote acid detection. Bacteria giving this reaction in OF media are oxidative.

Isolates B1 and B2 were lactose fermentors, where as B4, B6 and B7 where lactose non-fermentors.

4.4.3 Indole test:

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. It is used as part of IMViC procedures, a battery of tests designed to distinguish among members of the family Enterobacteriaceae.

Tryptophan amino acid can undergo deamination and hydrolysis by bacteria that express tryptophanase enzyme which catalyzes the following reaction.

Tryptophan + Water = Indole + Pyruvic acid + Ammonia

A positive indole test is indicated by the formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent. If the culture is indole negative, the reagent layer will remain yellow or be slightly cloudy.

All 5 bacterial isolates B1, B2, B4, B6, and B7 showed negative results.

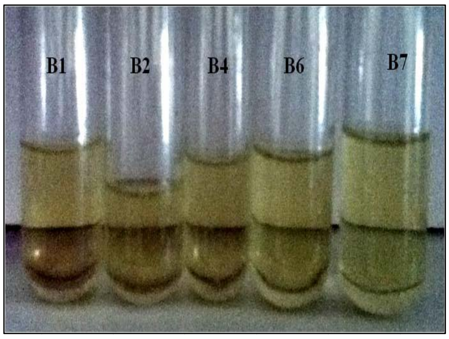


Figure 4.5 Indole Test Results

4.4.4 MR-VP test:

The paired MR-VP tests were used to distinguish between members of the family Enterobacteriaceae.

Escherichia coli and other members of the low-ratio organisms described by Clark and Lubes ferment sugars by the mixed acid pathway resulting in low ratio of CO_2 to H_2 gas produced by fermentation. The mixed acid pathway gives 4 mol of acid products (mainly acetic and lactic acid), the large quantity of acid produced causes a significant decrease in the pH of the culture medium. In contrast, *Enterobacter aerogenes* and other members of the high-ratio organisms (those that produce a high ratio of CO_2 toH_2 from the fermentation of glucose) ferment sugars via the butanediol fermentation pathway, producing only 1 mol of acid per mol of glucose. This pathway results in lower degree of acidification of the culture medium. The pH indicator methyl red (p-dimethylaminobenzene-O-carboxylic acid) has been found to be suitable to measure the concentration of hydrogen ions between pH 4.4 (red) and 6.0 (yellow).

When the culture medium turns red after addition of methyl red, because of a pH at or below 4.4 from the fermentation of glucose, the culture has positive result for the MR test. A negative MR test is indicated by a yellow color in the culture medium, which occurs when less acid is produced (pH is higher) from the fermentation of glucose.

All 5 bacterial isolates B1, B2, B4, B6, and B7 showed negative MR test.

Bacteria fermenting sugars via the butanediol pathway produce acetoin (i.e., acetyl methyl carbinol or 3-hydroxybutanone) as an intermediate which can be further reduced to 2,3-butanediol.

$$2 \text{ pyruvate} = \text{acetoin} + 2CO_2$$

$$\text{Acetoin} + NADH + H^+ = 2, 3 \text{ Butanediol} + NAD^+$$

In the presence of KOH the intermediate acetoin is oxidized to diacetyl, a reaction which is catalyzed by α-napthol. Diacetyl reacts with the guanidine group associated with the molecules contributed by peptone in the medium, to form a pinkish-red-colored product. The α-napthol in the Barritt's reagent serves as a color intensifier.

All 5 bacterial isolates B1, B2, B4, B6, and B7 showed positive VP test.

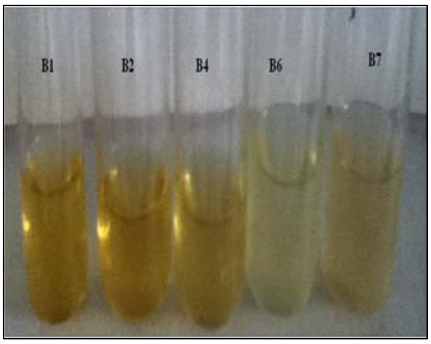


Figure 4.6 Methyl Red Test Results

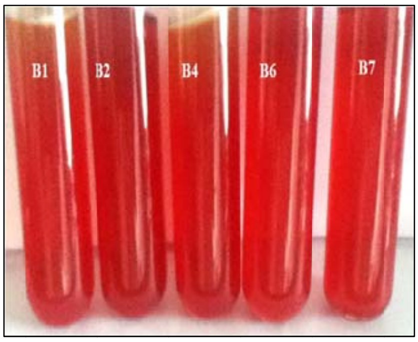


Figure 4.7 Voges-Proskauer Test Results

4.4.5 Motility test:

Hazy growth away from the stab line is a positive indication of motility.

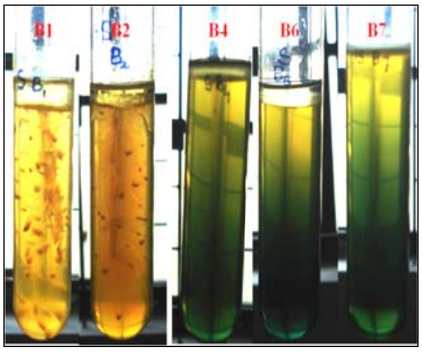


Figure 4.8 Motility Test Results

Isolates B1 and B2 were found to be motile and isolates B4,B6 and B7 were non-motile.

4.5 MOLECULAR CHARACTERIZATION OF ISOLATES

4.5.1 Bacterial Identification using MALDI-TOF MS Analysis:

Protein imaging of the 5 bacterial isolates using MALDI-TOF MS analysis, and Bruker taxonomic matching pattern revealed a quite few possible organisms for each isolate. Biochemical identification results and the MALDI-TOF flex analysis results were correlated and the best possible organism was finalized. Further characterization can be obtained by 16S rRNA sequencing.

Table 4.3 Bacterial Identification

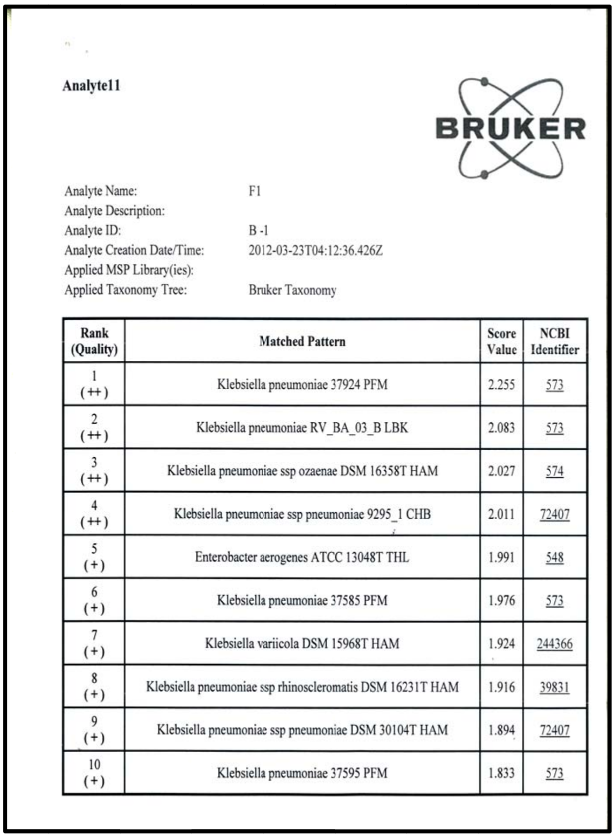
ISOLATE	Bruker Daltonics Flex Analysis Score Value	IDENTIFIED BACTERIA
B1	1.991	*Enterobacter aerogenes*
B2	1.686	*Enterobacter aerogenes*
B4	1.377	*-Could not be identified-*
B6	1.677	*Klebsiella pneumoniae spp pneumoniae*
B7	1.725	*Klebsiella variicola*

4.5.2 Fungal Identification:

Identification of fungal isolates based on morphological examination was carried out by Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi and the following results were obtained.

Table 4.4 Fungal Identification

ISOLATE	IDENTIFIED FUNGUS
F1	*Nigrospora oryzae*
F2	*Aspergillus sydowii*
F4	*Aspergillus niger*



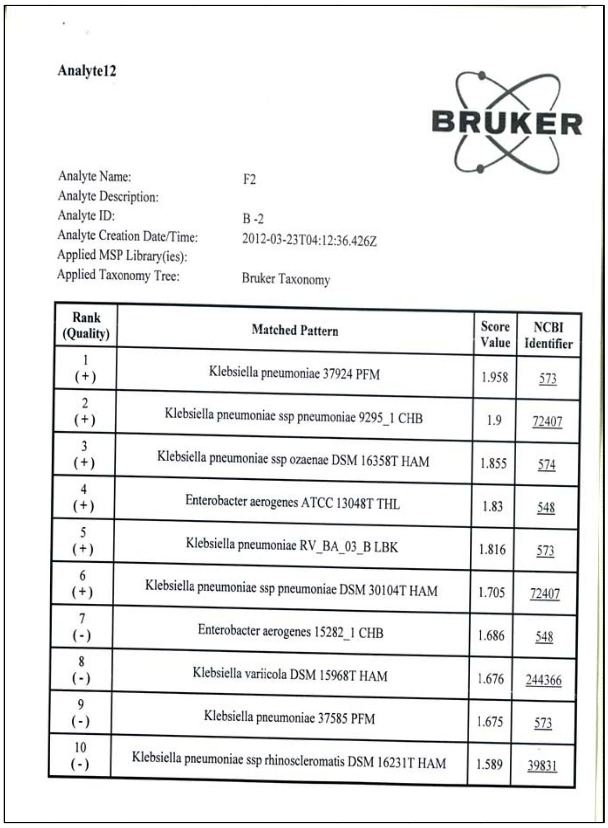
Analyte11

BRUKER

Analyte Name: F1
Analyte Description:
Analyte ID: B -1
Analyte Creation Date/Time: 2012-03-23T04:12:36.426Z
Applied MSP Library(ies):
Applied Taxonomy Tree: Bruker Taxonomy

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (++)	Klebsiella pneumoniae 37924 PFM	2.255	573
2 (++)	Klebsiella pneumoniae RV_BA_03_B LBK	2.083	573
3 (++)	Klebsiella pneumoniae ssp ozaenae DSM 16358T HAM	2.027	574
4 (++)	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.011	72407
5 (+)	Enterobacter aerogenes ATCC 13048T THL	1.991	548
6 (+)	Klebsiella pneumoniae 37585 PFM	1.976	573
7 (+)	Klebsiella variicola DSM 15968T HAM	1.924	244366
8 (+)	Klebsiella pneumoniae ssp rhinoscleromatis DSM 16231T HAM	1.916	39831
9 (+)	Klebsiella pneumoniae ssp pneumoniae DSM 30104T HAM	1.894	72407
10 (+)	Klebsiella pneumoniae 37595 PFM	1.833	573

Figure 4.9 Bruker analysis score value for B1



Analyte12

BRUKER

Analyte Name: F2
Analyte Description:
Analyte ID: B -2
Analyte Creation Date/Time: 2012-03-23T04:12:36.426Z
Applied MSP Library(ies):
Applied Taxonomy Tree: Bruker Taxonomy

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+)	Klebsiella pneumoniae 37924 PFM	1.958	573
2 (+)	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	1.9	72407
3 (+)	Klebsiella pneumoniae ssp ozaenae DSM 16358T HAM	1.855	574
4 (+)	Enterobacter aerogenes ATCC 13048T THL	1.83	548
5 (+)	Klebsiella pneumoniae RV_BA_03_B LBK	1.816	573
6 (+)	Klebsiella pneumoniae ssp pneumoniae DSM 30104T HAM	1.705	72407
7 (-)	Enterobacter aerogenes 15282_1 CHB	1.686	548
8 (-)	Klebsiella variicola DSM 15968T HAM	1.676	244366
9 (-)	Klebsiella pneumoniae 37585 PFM	1.675	573
10 (-)	Klebsiella pneumoniae ssp rhinoscleromatis DSM 16231T HAM	1.589	39831

Figure 4.10 Bruker analysis score value for B2

Analyte3

BRUKER

Analyte Name: A3
Analyte Description:
Analyte ID: B-4
Analyte Creation Date/Time: 2012-03-23T07:46:34.989Z
Applied MSP Library(ies):
Applied Taxonomy Tree: Bruker Taxonomy

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (-)	Arthrobacter mysorens DSM 12798T DSM	1.377	257984
2 (-)	Salmonella sp (enterica st Anatum) 11 LAL	1.33	58712
3 (-)	Staphylococcus aureus ssp aureus DSM 20491 DSM	1.328	46170
4 (-)	Staphylococcus aureus ssp aureus DSM 3463 DSM	1.265	46170
5 (-)	Proteus mirabilis 13210_1 CHB	1.195	584
6 (-)	Clostridium novyi 1082_ATCC 17861T BOG	1.192	1542
7 (-)	Prevotella corporis DSM 18810T DSM	1.169	28128
8 (-)	Lactobacillus fuchuensis DSM 14340T DSM	1.169	164393
9 (-)	Bacillus thioparans CIP 109765T CIP	1.16	370439
10 (-)	Salmonella sp (choleraesuis) 08 LAL	1.16	591

Figure 4.11 Bruker analysis score value for B4

Analyte14

BRUKER

Analyte Name: F4
Analyte Description:
Analyte ID: B -6
Analyte Creation Date/Time: 2012-03-23T04:12:36.411Z
Applied MSP Library(ies):
Applied Taxonomy Tree: Bruker Taxonomy

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (-)	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	1.677	72407
2 (-)	Klebsiella variicola DSM 15968T HAM	1.657	244366
3 (-)	Klebsiella pneumoniae 37924 PFM	1.61	573
4 (-)	Klebsiella pneumoniae ssp ozaenae DSM 16358T HAM	1.413	574
5 (-)	Klebsiella pneumoniae RV_BA_03_B LBK	1.371	573
6 (-)	Klebsiella pneumoniae ssp pneumoniae DSM 30104T HAM	1.361	72407
7 (-)	Klebsiella pneumoniae ssp rhinoscleromatis DSM 16231T HAM	1.295	39831
8 (-)	Arthrobacter oxydans IMET 10684T HKJ	1.285	1671
9 (-)	Enterobacter aerogenes 15282_1 CHB	1.285	548
10 (-)	Escherichia coli MB11464_1 CHB	1.273	562

Figure 4.12 Bruker analysis score value for B6

Analyte15

BRUKER

Analyte Name: F5
Analyte Description:
Analyte ID: B -7
Analyte Creation Date/Time: 2012-03-23T04:12:36.411Z
Applied MSP Library(ies):
Applied Taxonomy Tree: Bruker Taxonomy

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+)	Klebsiella variicola DSM 15968T HAM	1.725	244366
2 (-)	Klebsiella pneumoniae RV_BA_03_B LBK	1.532	573
3 (-)	Bacillus mojavensis DSM 9205T DSM	1.526	72360
4 (-)	Bacillus subtilis ssp spizizenii DSM 15029T DSM	1.516	96241
5 (-)	Bacillus subtilis ssp subtilis DSM 10T DSM	1.428	135461
6 (-)	Klebsiella pneumoniae 37924 PFM	1.375	573
7 (-)	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	1.368	72407
8 (-)	Bacillus subtilis DSM 5552 DSM	1.358	1423
9 (-)	Rhizobium rhizogenes B166 UFL	1.315	359
10 (-)	Lactobacillus paracasei ssp paracasei DSM 20312 DSM	1.303	47714

Figure 4.13 Bruker analysis score value for B7

ICAR

Indian Type Culture Collection
Identification / Culture Supply Services
Division of Plant Pathology
Indian Agricultural Research Institute
New Delhi- 110 012

IDENTIFICATION REPORT

Ref.nil 2463 Dated: - 24-3-12

I.D. No.	Ref.No.	Source	Fungus	Identified By
8698.12	F1	natural sources	*Nigrospora oryzae*	
8699.12	F2		*Aspergillus sydowii*	
8700.12	F4		*A.niger*	Dr. T.Prameela Devi Dr. Nita Mathur

for (T. PRAMEELA DEVI)
Sr. Scientist (ITCC)

- See ITCC catalogue on **www. iari.res.in/divisions/plant_pathology/ITCC-2007.pdf**
- Kindly acknowledge ITCC in every research correspondence related to these fungi.
- The minimum time required for the identification of
- **Oomycetes, Ascomycetes and Coelomycetes- 4-6 weeks**
- **Zygomycetes and Hyphomycetes- 3-4 weeks**

Figure 4.14 Fungal identification results

4.6 LIGNOCELLULOLYTIC ENZYME ASSAY

Table 4.5 endo-β-1, 4-glucanase Enzyme Activity

Isolate	Organism	Enzymatic Activity (IU/ml)
B7	*Klebsiella variicola*	6.6266±1.770
F1	*Nigrospora oryzae*	14.4344±0.094
F2	*Aspergillus sydowii*	16.2362±0.210
F4	*Aspergillus niger*	7.5075±0.103

Table 4.6 endo-β-1, 4-xylanase Enzyme Activity

Isolate	Organism	Enzymatic Activity (IU/ml)
B7	*Klebsiella variicola*	21.2345±8.45
F1	*Nigrospora oryzae*	0.4115±0.07
F2	*Aspergillus sydowii*	38.3539±0.87
F4	*Aspergillus niger*	57.5308±9.74

Table 4.7 Fungal Laccase Enzyme Activity

Isolate	Organism	Enzymatic Activity (IU/ml)
F1	*Nigrospora oryzae*	6.2832
F2	*Aspergillus sydowii*	6.2832
F4	*Aspergillus niger*	5.1628

CHAPTER 5
CONCLUSION

7 bacterial and 5 fungal isolates were obtained from around the banana pith and pseudostem samples placed on PDA medium. Qualitative screening was employed to evaluate the lignocellulolytic enzyme producing ability of those isolates. Based on the zone of clearance obtained, 5 bacterial and 3 fungal isolates were considered for further work. These bacterial isolates were subjected to MALDI-TOF MS analysis, and Bruker taxonomic matching pattern, and the identification was done based on the best score value in correlation with the results of the biochemical tests. The fungal isolates were identified based on morphology.

Measurement of cellulase activity, specifically Endo-β-1, 4-glucanase enzyme assay was performed using CMC (low viscosity) and the cellulase from fungal isolate 1 (F1) identified as *Nigrospora oryzae* was observed to have the best activity amongst all. The activity of endo-β-1, 4-xylanase enzyme was found to be highest in fungal isolate 4 (F4), which was identified as *Aspergillus niger*. *Nigrospora oryzae* (F1), and *Aspergillus sydowii* (F2) were found to possess the highest laccase (Ligninolytic enzyme) activity.

FUTURE WORK

The media have to be optimized for high lignocellulolytic enzyme production by the endophytic isolates obtained. Partial purification of the crude enzyme needs to be carried out, and various parameters that affect lignocellulolytic enzyme activity have to be evaluated and further optimization of these parameters for high enzyme activity is to be performed.

APPENDIX

I. POTATO DEXTROSE AGAR:

Dissolve 3.9 gram of Potato Dextrose Agar in 100 ml of distilled water and mix well and autoclave at 121°C for 15 minutes.

II. CELLULOLYSIS BASAL MEDIA:

Dissolve the following ingredients in 100 ml of distilled water and mix well and autoclave at 121°C for 15 minutes.

Yeast Extract (0.1%)	0.1gram
Soya Peptone (0.1%)	0.1 gram
Agar (1.6%)	1.6 gram
Carboxymethyl Cellulose (4%)	4.0 gram

III. XYLANOLYIS BASAL MEDIA:

Dissolve the following ingredients in 70 ml of distilled water and mix well and autoclave at 121°C for 15 minutes.

Yeast Extract (0.1%)	0.07 gram
Soya Peptone (0.1%)	0.07 gram
Agar (1.6%)	1.12 gram
Beech wood xylan (4%)	2.80 gram

IV. LIGNIN MODIFYING ENZYME BASAL MEDIA:

Dissolve the following ingredients in 100 ml of distilled water and mix well and autoclave at 121°C for 15 minutes.

Yeast Extract (0.1%)	0.1 gram
Soya Peptone (0.1%)	0.1 gram
Agar (1.6%)	1.6 gram
Lignin (0.25%)	0.25 gram
Glucose (2% w/v)	2.00 gram

V. SCREENING MEDIA STAINING SOLUTIONS:

Congo Red Solution (2% w/v)	Dissolve 2 gram in 100 ml of distilled water
Sodium Chloride (1M)	Dissolve 5.84 gram in 100 ml of distilled water
Potassium Iodide (0.25% w/v)	Dissolve 0.25 gram in 100 ml of distilled water
Iodine (0.25% w/v)	Dissolve 0.25 gram in 100 ml of distilled water
Ferric Chloride (1% w/v)	Dissolve 2 gram in 100 ml of distilled water
Potassium Ferricyanide(1% w/v)	Dissolve 2 gram in 100 ml of distilled water

VI. BIOCHEMICAL IDENTIFICATION:

Oxidase Test Reagent:

Kovács oxidase reagent (1%) is prepared by dissolving 0.1 gram of N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride in 10 ml of distilled water. Store refrigerated in a dark brown bottle no longer than 1 week.

Lactose Fermentation Media:

Hugh and Leifson's OF Basal medium is prepared by dissolving the following ingredients and bringing the final volume to 1000 ml. The pH should be adjusted to 7.1 prior to autoclaving.

Peptone	2.00 gram
Sodium chloride	5.00 gram
Lactose	10.0 gram
Bromothymol blue	0.03 gram
Agar	3.00 gram
Dipotassium phosphate	0.30 gram

Indole Test Media:

Dissolve 2.0 gram of tryptone and 1.0 gram of sodium chloride in 200 ml of distilled water and autoclave at 121°C for 15 minutes.

MR-VP test Media and Reagent:

Dissolve the following ingredients in 200 ml of distilled water and mix well and autoclave at 121°C for 15 minutes.

Buffered peptone	1.40 gram
Dipotassium phosphate	1.00 gram
Dextrose	1.00 gram

Commercial reagents for biochemical identification:

Methyl red solution	Dissolve 0.1 gram of methyl red in 300 ml of ethanol (95%) and make upto 500 ml with deionized water
Barritt's reagent A	5% (wt/vol) α-napthol in absolute ethanol
Barritt's reagent B	40% (wt/vol) KOH in deionized water
Kovács Indole reagent.	Dissolve 10.0 grams of p-dimethylaminobenzaldehyde (DMAB) in 150 ml Isoamyl alcohol, and 50 ml of concentrated Hydrochloric acid to it.

VII. INOCULUM MEDIA PREPARATION:

Dissolve the following ingredients in 1000 ml of distilled water and mix well and autoclave at 121°C for 15 minutes.

Tri Sodium Citrate	5.0 gram
Potassium dihydrogen phosphate	5.0 gram
Sodium nitrate	2.0 gram
Ammonium sulphate	4.0 gram
Magnesium sulphate	0.2 gram
Peptone	1.0 gram
Yeast extract	2.0 gram
Glucose	10 gram

VIII. PRODUCTION MEDIA PREPARATION:

Dissolve the following ingredients in 995 ml of distilled water and mix well and autoclave at 121°C for 15 minutes. Add 5 ml of separately autoclaved trace element solution.

Cellulase Production Media	**Hemicellulase Production Media**	
Tri Sodium Citrate	Tri Sodium Citrate	5.0 gram
Potassium dihydrogen phosphate	Potassium dihydrogen phosphate	5.0gram
Sodium nitrate	Sodium nitrate	2.0 gram
Ammonium sulphate	Ammonium sulphate	4.0 gram
Magnesium sulphate	Magnesium sulphate	0.2 gram
Peptone	Peptone	1.0 gram
Yeast extract	Yeast extract	2.0 gram
Glucose	Rice Bran	10 gram

Ligninase Production Media

Potassium dihydrogen phosphate	1.00 gram
Magnesium sulphate	0.50 gram
Yeast extract	0.10 gram
Copper sulphate	1.00 milligram
Ferrous sulphate	1.00 milligram
Lignin	9.00 gram
Glucose	1.00 gram
Calcium chloride	0.01 gram
Sodium nitrate	0.20 gram

Trace Element Solution Preparation:

Dissolve the following in 95 ml of distilled water. To this add 1 ml of chloroform for preservation.

Citric acid	5.00 gram
Zinc Sulphate	5.00 gram
Ferrous Ammonium Sulphate	1.00 gram
Copper sulphate	0.25 gram
Manganese sulphate	0.05 gram
Sodium molybdate	0.05 gram
Boric acid (Anhydrous)	0.05 gram

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