Isolation and Screening of Efficient Ligninolytic Fungi

By

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2013

CERTIFICATE I

This is to certify that this thesis entitled, **ìIsolation and Screening of Efficient Ligninolytic Fungiî**, submitted for the degree of **Master of Science** in the subject of **Microbiology** to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Miss Tanvi Bhatia**, Admn. No.**2011BS98M**, under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE-II

This is to certify that this thesis entitled, **ìIsolation and Screening of Efficient Ligninolytic Fungiî**, submitted by **Miss Tanvi Bhatia**, Admn. No.**2011BS98M** to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, in partial fulfillment of the requirements for the degree of **Master of Science** in the subject of **Microbiology** has been approved by the Studentís Advisory Committee after an oral examination on the same.

MAJOR ADVISOR **HEAD OF THE DEPARTMENT**

DEAN, POST-GRADUATE STUDIES

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CHAPTER - I

INTRODUCTION

Lignin, next to cellulose and hemicelluloses, is the third most abundant compound in plant biomass. It is resistant to microbial degradation because of its high molecular weight and presence of various biologically stable linkages. It provides mechanical strength and rigidity to vascular plants. Lignin is the most recalcitrant organic compound among all other naturally produced organic compounds. When vascular plants die or drop litter, lignified organic carbon is incorporated into the top layer of the soil. This recalcitrant material has to be broken down and recycled by microorganisms to maintain the earthís carbon cycle.

It was highlighted that, microbial degradation of lignin had potential advantages over the prevailing chemical degradation, due to energy and environmental concerns (Keller *et al*., 2003). Also microbial lignin degradation is drawing attention as an alternative to pulping or enzymatic hydrolysis of lignocellulosic materials for production of various biofuels and other products.

Lignin biodegradation has diverse effects on soil physical and chemical structure and effect soil quality. The microbial degradation of litter results in the formation of humus, and ligninolysis probably facilitates this process by promoting the release of aromatic humus precursors from the litter. These precursors include incompletely degraded lignin, flavanoids, terpenses, lignans, condensed tannins, and uberins (Hudson, 1986). The disadvantage of slow lignin decomposition is that decomposing lignocellulose supports high populations of microorganisms that may produce phytotoxic metabolites. High microbial populations in undecomposed litter also compete with crop plants for soil nitrogen and other nutrients (Lynch and Harper, 1985). However, degrading lignocellulosic biomass by current available microorganism is still far to meet the industrial demands (Shary *et al*., 2007; Afrida *et al*., 2009). Thereby, isolation of new microbial strains for degradation of lignin is still essential.

Microbial degradation of lignin is very important and economical in comparison to chemical removal of lignin by environmentally hazardous acids or alkalis and, therefore it is emerging as a good alternative. Among various microorganisms, fungi are more efficient candidates for lignin degradation, due to their ability of deriving energy not through photosynthesis but via decomposition of

organic matter. Typically, molds secrete extracellular hydrolytic enzymes, mainly from the hyphal tips. These enzymes degrade complex biopolymers such as starch, cellulose and lignin into simpler substances, which can be absorbed by the hyphae. In comparison to other microflora, molds play a major role in the decomposition of organic materials, enabling the recycling of nutrients throughout ecosystems. It is reported that fungi belonging to different genera, such as *Aspergillus fumigatus, A. japonicus, A. niger, A. terreus* and *Penicillium simplicissimum*, were capable of degrading both aromatic and carbohydrate components of water-soluble lignocarbohydrate complexes (LCC) or Kraft lignin (Milstein *et al*., 1984; Zeng *et al*., 2006).

Lignin degrading fungi are either white rot fungi (degrades lignin) or brown rot fungi (degrades cellulose and hemicelluloses). White rot fungi (basidiomycetes) are reported to be the best lignin degrading fungi. (Gilberton, 1980; Abdel- Raheem and Shearer, 2002; Urairuj *et al*., 2003). The most well known white rot fungi, which have been studied extensively to establish for the mechanism of lignin degradation are *Phanerochaete chrysosporium* and *Tramestes versicolor* (Urairuj *et al*., 2003). However, because of slow growth rates; easily counteracted by other lower microorganisms; damageable enzymes and production costs for them, the direct use of single white rot fungi and their enzymes in industrial processes is still very difficult.

Lignin biodegradation performed by these fungi is a multistep process mediated by enzymes of the ligninolytic complex such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) (Nagai *et al*., 2007). They have developed a unique, nonspecific degradation system that functions in the extra cellular environment. Laccase (benzenediol: oxygen oxidoreductases) belongs to the family of multicopper that catalyzes the oxidation of various aromatic substances. Catalytic properties and broad substrate specificity of laccase have a great potential in varied industrial applications (Givaudan *et al*., 1993; OíMalley *et al*., 1993; Edens *et al*., 1999). Although Laccase were isolated and reported from bacteria (Thurston, 1994), most studies on laccase were reported from fungal organisms (Hernandez *et al*., 1994; Perumal and Kalaichelvan, 1996; Vasconcelos *et al*., 2001; Mechichi *et al*., 2006; Zhang *et al*., 2006). Lignin peroxidase appears to be a key enzyme in the oxidation of nonphenolic phenylpropanoid units which lead to polymer fragmentation (Hammel *et al*., 1985). Manganese peroxidase, one of the important enzymes of fungi, oxidizes Mn^{2+} to Mn^{3+}, which in turn may attack phenolic structures in lignin as long as it is stabilized by suitable metal chelators secreted by fungi (Zhang *et al*., 2006).

This ligninolytic system of white-rot fungi is also directly involved in the degradation of various xenobiotic compounds and dyes (Hofrichter, 2002; Songulashvili *et al*., 2007). Through intensive study of ligninolytic fungi, it has been determined that these organisms produce extracellular enzymes with very low substrate specificity, enabling them to mineralize a wide range of highly recalcitrant organopollutants that are structurally similar to lignin (Hofrichter, 2002).

To isolate and identify the ligninolytic fungi from various sources and to investigate and determine the activities of laccase, manganese peroxidase and lignin peroxidase, the present investigation was planned with the following objectives:

1. To isolate lignin degrading fungi
2. To screen the efficacy of lignin degrading fungal isolates.

CHAPTER - II

REVIEW OF LITERATURE

Lignin, which is one of the most widely distributed natural organic polymers, is very slowly decomposed in the environment due to its complex chemical structure. There are methods to degrade lignin chemically, but it harms the environment (Keller *et al*., 2003). There was a breakthrough in the field of lignin biodegradation in 1983 when fungal ligninases and their hydrogen peroxide requirement were described by Tien and Kirk (1983). When vascular plants die or drop litter, lignified organic carbon is incorporated into the top layer of the soil. This recalcitrant material has to be broken down and recycled by microorganisms to maintain the earthís carbon cycle (Hammel, 1997). Conditions like low pH, lack of rainfall do not favor the biological breakdown of lignocelluloses and leads to pronounced accumulations of litter in soil (Spurr and Barnes, 1980). High temperature, high moisture content, high oxygen availability and high palatability of the litter to microorganisms favor decomposition. The more highly lignified litter is, the less digestible it is, and the more its decomposition depends on the unique organisms that can degrade lignocelluloses (Hammel, 1997).

2.1 Lignin structure

Lignin, the second-most abundant biopolymer on Earth and a heterogeneous polymer in lignocellulosic residues, is the only naturally synthesized polymer with an aromatic backbone (Wei *et al*., 2009). The complex structure of lignin makes it the recalcitrant material. It is formed in vascular plant cell walls by the oxidative coupling of several related phenylpropanoid precursors: coniferyl alcohol, sinapyl alcohol, and *p*-hydroxycinnamyl alcohol. These precursors form the guaiacyl- (G), syringyl- (S) and *p* -hydroxyphenyl (H) subunits in the lignin molecule, respectively (Martinez *et al*., 2005). The subunits ratio and consequently the lignin composition vary between different plant groups (Wong, 2009). Peroxidases or laccases in the plant cell wall oxidize these monomers by one electron, yielding transient resonance-stabilized phenoxy radicals that then polymerize in a variety of configurations. The possible ways that the precursors can couple can be portrayed on paper simply by drawing the conventional resonance forms of the phenoxy radicals, and then by linking the most important of these in various pair wise

combinations. Lignin consists primarily of the intermonomer linkages shown in Fig.1, and that the arylglycerol-,-aryl ether structure circled in the figure is quantitatively the most important of these, constituting over 50% of the polymer. Lignin is covalently associated with hemicelluloses in the cell wall via numerous types of linkages. Among the most important are ether bonds between the benzylic carbon of lignin and the carbohydrate moiety, ester bonds between the benzylic carbon of lignin and uronic acid residues, and lignin-glycosidic bonds. The matrix of lignin and hemicellulose encrusts and protects the cellulose of the plant cell wall (Jeffries, 1990).

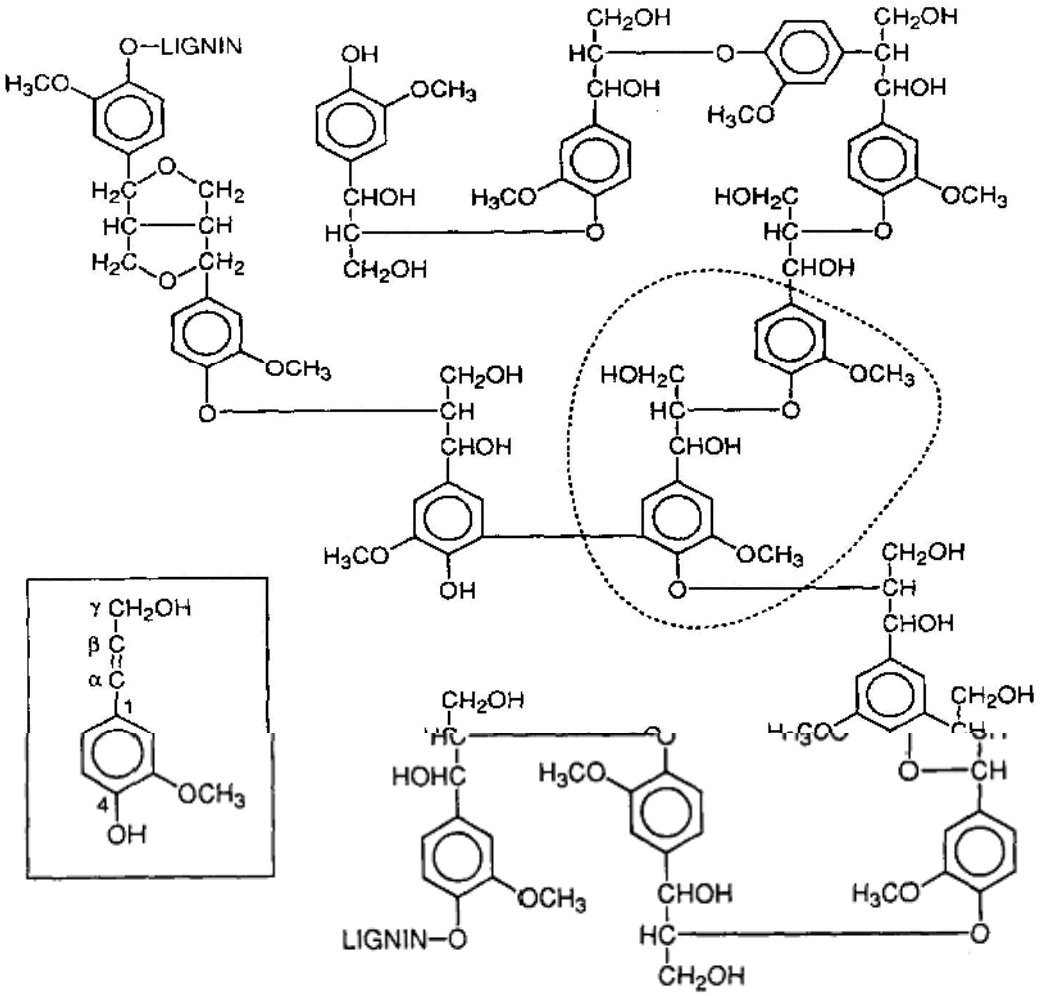

Fig. 1. Common structures of softwood lignin, with an example of the major arylglycerol-ß-aryl ether structure circled. The inset shows coniferyl alcohol, the phenylpropanoid building block of softwood lignin.

Fungi that degrade lignin face several problems (Hammel, 1997). As the polymer is extremely large and highly branched, ligninolytic mechanisms must be extracellular and further it is interconnected by stable ether and carbonñcarbon

bonds. These mechanisms must be oxidative rather than hydrolytic and since lignin consists of a mixture of stereo irregular units, fungal ligninolytic agents have to be much less specific than typical biological catalysts. Moreover its insolubility in water limits its bioavailability to ligninolytic systems and dictates that ligninolysis is a slow process.

2.2 Lignin degrading microorganisms

A number of bacteria and fungi enzymatically break lignin compounds. Among the fungi, basidiomycetes constitute the most conspicuous group of fungi in the environment and comprise very different ecological groups such as white-rot, brown-rot, and leaf litter fungi (Songulashvili *et al*., 2007). Major research involving bioconversion and biodegradation of lignin has been focused on wood-rot fungi, particularly white-rot fungi, as compared to other ligninolytic organisms (Ohkuma *et al*., 2001). White-rot fungi, belonging to the basidiomycetes, are capable of mineralizing lignin efficiently. Many white-rot fungi are natural inhabitants of soil litter. However, the direct use of single white rot fungi and their enzymes in industrial processes is not recommended due to their slow growth rate, easily counteraction by other microorganisms, damageable enzymes and high production cost (Yang *et al*., 2011). Many white rot fungi can simultaneously attack lignin, hemicelluloses and cellulose whereas others preferentially work on lignin in a selective manner. For example, while *Ceriporiopsis subvermispora* (Guerra *et al*., 2004), *Dichomitus squalens* (Fackler *et al*., 2006), *Physisporinus rivulosus* (Hilden *et al*., 2007) and *Phlebia* spp. (Fackler *et al*., 2006; Arora and Sharma, 2009) selectively attack lignin. *Heterobasidium annosum* (Daniel *et al*., 1998), *Trametes versicolor* (Tanaka *et al*., 1999), *P. chrysosporium* (Sanchez, 2009) and *Irpex lacteus* (Xu *et al*., 2009) simultaneously degrade all cell wall components. Besides lignin degradation, the ligninolytic system produced by white-rot fungi can also mineralize a wide variety of environmental organo- pollutants including chlorophenols, dioxins and polycyclic aromatic hydrocarbons (Bumpus *et al*., 1985 and Mileski *et al*., 1988).

Many reports emphasized decolorization of several synthetic and natural dyes used in dye stuff, pulp and paper, pharmaceutical and other industries by white rot fungi (Balan and Monteiro, 2001 and Gill *et al*., 2002). It has been determined that ligninolytic fungi produce extracellular enzymes with very low substrate specificity, enabling them to mineralize a wide range of highly recalcitrant organopollutants that are structurally similar to lignin (Hofrichter, 2002).

Generally, most of basidiomycetes have an optimal temperature for growth of about 26-30ºC. Though there are rare reports but thermotolerant basidiomycetes

have been more beneficial in pulping and bleaching processes and other biotechnological applications (Khanongnuch *et al*., 2004). They isolated and characterized 113 thermotolerant wood rotting fungi from northern Thailand. When cultivated in the liquid basal medium containing 0.02% Poly R-478 dye at 37ºC at 150 rpm *Coriolus versicolor* strain RC3 degraded Poly R-478 dye completely within 5 days at 42^{O}C. This was found to be more active over the well researched lignin degrading white rot fungi, *P. chrysosporium*. Lignin degradation by the fungi is suggested to play a role in sudden death syndrome (SDS) in soybean (Lozovaya, 2007).

Wood biodegradation by certain ascomycetes was first described in detail and designated as ìsoft rotî by Savory (1954). Nilsson and team, (1989) demonstrated that, some higher ascomycetes, particularly *Daldinia concentrica,* degraded Aspen wood with the same intensity as *Trametes versicolor,* a basidiomycete typically classified as white-rot fungus. The ascomycete, *Chrysonilia sitophila* could degrade rice hull and *Pinus radiata* bark products and also produce ligninolytic and cellulolytic enzymes (Ferraz *et al*., 1991). Whereafter, different *Penicillium* strains was described as potential degraders or was able to degrade compounds with related lignin structures (Rodriguez *et al*., 1994).

A fungus strain *Aspergillus* sp. F-3 from a group of fungal strains which were isolated from forest soil in Dalian of China degraded 65% of alkali lignin (2,000 mg l^{-1}) after 8 days of incubation at 30^{0}C at pH 7. The removal of color was up to 100% on8th day. The biodegradation of lignin by *Aspergillus* sp. F-3 favored initial pH 7.0. Further, it decomposed alkali lignin more effectively when co-cultured with white-rot fungus (Yang *et al*., 2011).

Huiju Gao and coworkers, (2011) isolated an ascomycete producing ligninolytic enzymes and characterized its lignin degradation capability. Among 20 isolates, GHJ-4 was isolated from decayed wood of Salix matsudana Koidz in Mount Tai, China, by different indicator compounds assay. The capability of utilizing several lignin model compounds and decoloration of aromatic dyes by GHJ-4 strain revealed its ligninolytic potentiality.

2.3 Lignin degrading enzymes

Electron microscopy of the early stages of the fungal degradation of wood showed that oxidative ligninolytic enzymes are too large to penetrate into the wood cell wall micropores (Srebotnik *et al*., 1988). Thus, it has been suggested that prior to the enzymatic attack, low-molecular weight diffusible reactive oxidative compounds must initiate changes to the lignin structure (Srebotnik *et al*., 1988; Tanaka *et al*., 1999). Fungi degrade lignin by secreting enzymes collectively termed

ìligninasesî. Ligninases can be classified as either phenol oxidases (laccase) or heme peroxidases [lignin peroxidase (LiP), manganese peroxidase (MnP)] (Martinez *et al*., 2005). In general, laccases use molecular oxygen as electron acceptors while peroxidases use hydrogen peroxide as a co-substrate (Mai *et al*., 2004).

It has been shown that the genome of *P. chrysosporium* contains ten lignin peroxidase and five manganese peroxidase genes (Martinez *et al*., 2004) and therefore *P. chrysosporium* produces several lignin peroxidase and manganese peroxidase isoenzymes but no laccase (Singh and Chen, 2008). Although lignin peroxidase is able to oxidize the non-phenolic part of lignin (which forms 80- 90% of lignin composition), it is absent from some lignin degrading fungi (Wang *et al*., 2008).

2.3.1 Phenol oxidases (laccases) (benzenediol:oxygen oxidoreductases, EC 1.10.3.2)

Laccases are glycosylated blue multi-copper oxidoreductases (BMCO) that use molecular oxygen to oxidize various aromatic and nonaromatic compounds through a radical catalyzed reaction mechanism (Claus, 2004; Baldrian, 2006). Initially discovered in the Japanese lacquer tree *Rhus vernicifera* (Yoshida, 1883), laccases have since been found in many other plants and insects (Claus, 2004). However, for the most part, laccases have been found and studied in white-rot fungi, such as *Trametes* sp. strain AH28-2 (Xiao *et al*., 2003), *T. versicolor* (Necochea*et al*., 2005), *Pleurotus ostreatus* D1 (Pozdniakova *et al*., 2006), *Cerrena unicolor* strain 137 (Michniewicz *et al*., 2006), *Cyathus bulleri* (Salony *et al*., 2006), *Trametes pubescens* (Shleev *et al*., 2007) and *Lentinus tigrinus* (Ferraroni *et al*., 2007). Laccase production using a liquid culture has also been reported in brown-rot fungi, including *Coniophora puteana* (Lee *et al*., 2004). Also, ascomycetes such as *Chaetomium thermophile* (Ishigami and Yamada, 1986), *Neurospora crassa* (Germann, 1988), *Magnaporthe grisea* (Iyer and Chattoo, 2003), *Myrothecium verrucaria* 24G-4 (Sulistyaningdyah *et al*., 2004) and *Melanocarpus albomyces* (Hakulinen *et al*., 2006) are able to produce laccases. In addition by application of gene-specific PCR primers, laccase genes were detected in a few different fungal species including *Pycnoporus cinnabarinus*, *Pycnoporus coccineus*, *Pycnoporus sanguineus*, *Cyathus* sp. and also in xylariaceous ascomycetes *Xylaria* sp. and *Hypoxylon* sp. (Pointing *et al*., 2005). The physiological roles of fungal laccases include pigment production, growth inhibition of plant pathogens and as well as degradation of lignocellulosic materials (Thurston, 1994). Their capacity to remove

xenobiotic substances and produce polymeric products makes them a useful tool for bioremediation purposes.

2.3.2 Lignin peroxidases (LiP) (1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol:hydrogenperoxide oxidoreductases, EC 1.11.1.14)

Lignin peroxidases (LiPs) were the first ligninolytic enzymes to be discovered in *P. chrysosporium* (Glenn *et al.,* 1983; Tien and Kirk, 1983). Since then, more lignin peroxides have been found in different *P. chrysosporium* strains (Tien and Kirk, 1984; Renganathan *et al*., 1985) and other white-rot fungi, such as *T. versicolor* (Johansson, 1993). Lignin peroxidases are heme-containing glycoproteins and play a central role in the biodegradation of lignin (Piontek *et al*., 2001). Lignin peroxidases catalyze the H_2O_2-dependent oxidative depolymerization of a variety of non-phenolic lignin compounds (diarylpropane), ,-*O*-4 non-phenolic lignin model compounds and a wide range of phenolic compounds (e.g. guaiacol, vanillyl alcohol, catechol, syringic acid, acteosyringone) with redox potentials up to 1.4 V (Wong, 2009). In addition, LiP genes were detected in a few different fungal species including *Panus* sp., *P. coccineus*, *P. sanguineus* and *Perenniporia medulla-panis* (Pointing *et al*., 2005).

2.3.3 Manganese peroxidases (MnP) (Mn(II):hydrogen-peroxide oxidoreductases, EC 1.11.1.13)

Manganese peroxidase are extracellular glycoproteins and are secreted in multiple isoforms which contain one molecule of heme as iron protoporphyrin IX (Asgher *et al*., 2008). Since the discovery of MnP in *P. chrysosporium* (Glenn and Gold, 1985), more manganese peroxidase have been found in other basidiomycetes, such as *Bjerkandera* sp. (Palma *et al*., 2000), *Agaricus bisporus* (Lankinen *et al*., 2001), *Lenzites betulinus* (Hoshino *et al*., 2002), *Phanerochaete flavido-alba* (de la Rubia *et al*., 2002), *Panus tigrinus* (Lisov *et al*., 2003) and *Nematoloma frowardii b19* (Hilden *et al*., 2008). The presence of manganese peroxidase can increase the degree of dye decolorization. Chagas and Durant, (2001) in particular found that manganese peroxidase was the main enzyme involved in dye decolorization by *P. chrysosporium*.

Some wood-degrading fungi contain all three classes of lignin-modifying enzymes, while certain others contain only one or two of these enzymes (Hatakka, 1994; Dhouib *et al*., 2005). The production of ligninolytic enzymes is observed as a colourless halo around microbial growth (Dhouib *et al*., 2005). So, the lignin degradation capability of microbes is initially screened indirectly on solid media containing different indicator compounds.

2.4 Fungal ligninolytic mechanisms

In most fungi that have been examined, ligninolysis occurs during secondary metabolism under nutrient limitation. The fungus avoids synthesizing and secreting metabolically expensive ligninolytic agents/ enzymes, when easily metabolizable substrates are more accessible than lignocellulose are present. The limiting nutrient for fungal growth in most woods and soils is probably nitrogen, and most laboratory studies of ligninolytic fungi have been done in nitrogen-limited culture media (Kirk and Farrell, 1987). However, a few ligninolytic fungi, e.g. some species of *Bjerkandera,* are ligninolytic even when sufficient nitrogen is present (Kaal *et al.,* 1993). Several mechanisms have displayed one fundamental similarity that is on the generation of lignin free radicals which, subsequently undergo a variety of spontaneous cleavage reactions (Hammel, 1997).

2.4.1 Laccase

Laccases are blue copper oxidases that catalyze the one-electron oxidation of phenolics and other electron-rich substrates. Laccases contain multiple copper atoms which are reduced as the substrates are oxidized. After four electrons have been received by a laccase molecule, the laccase reduces molecular oxygen to water, returning to the native state. The action of laccase on lignin resembles that of Mn(III) chelates that are produced during the action of manganese peroxidase, in that phenolic units are oxidized to phenoxy radicals, which can lead to degradation of some structures (Kawai *et al.,* 1988).

2.4.2 Lignin Peroxidase

Lignin peroxidase contains ferric heme and operates via a typical peroxidase catalytic cycle (Kirk and Farrell, 1987; Gold *et al*., 1989). Lignin peroxidase is oxidized by H_2O_2 to a two-electron deficient intermediate, which returns to its resting state by performing two one-electron oxidations of donor substrates. However, lignin peroxidases are more powerful oxidants than typical peroxidases are, and consequently oxidize not only the usual peroxidase substrates such as phenols and anilines, but also a variety of non-phenolic lignin structures and other aromatic ethers that resemble the basic structural unit of lignin (Kersten *et al.,* 1990). The H_2O_2-dependent oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol) to veratraldehyde is the basis for the standard assay used to detect lignin peroxidase in fungal cultures (Kirk *et al.,* 1990).

The lignin peroxidase-catalyzed oxidation of a lignin substructure begins with the abstraction of one electron from the aromatic ring of the donor substrate, and the resulting species, an aryl cation radical, then undergoes a variety of postenzymatic reactions. (Kersten *et al.,* 1985; Shoemaker *et al.,* 1985; Hammel *et*

al., 1986; Kirk and Farrell, 1987). For example, dimeric model compounds that represent the major arylglycerol-,-aryl ether lignin structure undergo C- -C, cleavage upon oxidation by lignin peroxidase (Kirk *et al,* 1986). These results strongly support a ligninolytic role for lignin peroxidase, because C- -C, cleavage is a major route for ligninolysis in many white rot fungi (Kirk and Farrell, 1987) (Fig. 2.). But lignin peroxidase, like other enzymes, is too large to enter the pores in sound wood (Srebotnik *et al.,* 1988). To initiate ligninolysis directly, it must therefore act at the surface of the secondary cell wall. Fungal attack of this type is indeed found, but electron microscopic observations also indicate that white rot fungi can remove lignin from the interior of the cell wall before they have degraded it enough for enzymes to penetrate. It has been proposed that lignin peroxidase might circumvent the permeability problem by acting indirectly to oxidize low molecular weight substrates that could penetrate the lignocellulosic matrix and act themselves as oxidants at a distance from the enzyme (Harvey *et al.,* 1986), but no convincing candidate for a diffusible lignin peroxidase-dependent oxidant of this type has emerged so far.

Lignin peroxidase is the only fungal oxidant known that can efficiently mimic, *in vitro, the* C- -C, cleavage reaction, a characteristic of ligninolysis by white rot fungi such as *Phanerochaete chrysosporium.* LiP must therefore be considered an important ligninolytic agent, but it may act in concert with other, smaller oxidants that can penetrate and open up the wood cell wall.

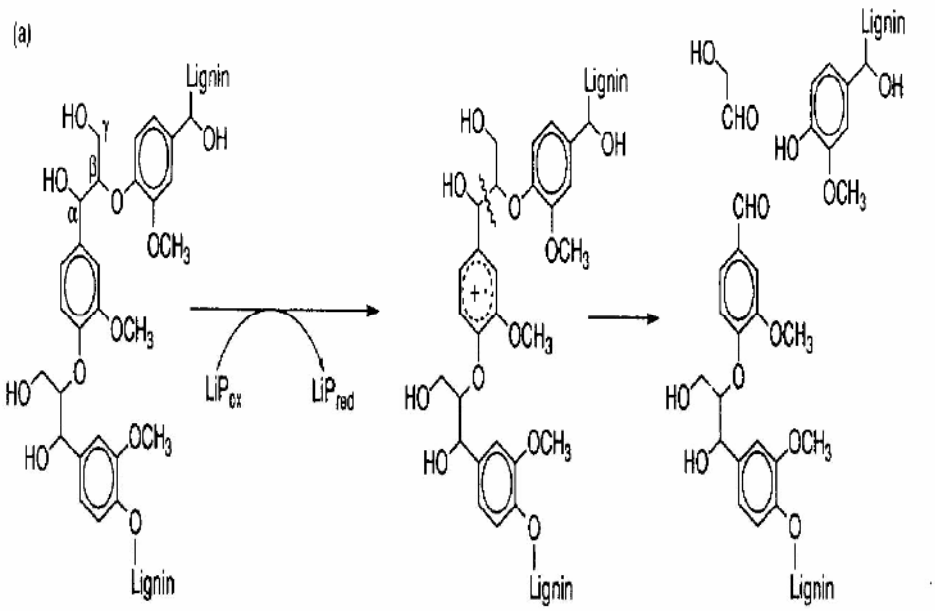

Fig.2. Cleavage of a recalcitrant internal non-phenolic arylglycerol beta-aryl ether lignin structure by oxidized lignin peroxidase

2.4.3 Manganese peroxidase

Manganese peroxidase occur in most white rot fungi, and are similar to conventional peroxidases, except that Mn(II) is the obligatory electron donor for reduction of the one-electron deficient enzyme to its resting state, and Mn(III) is produced as a result (Wariishi *et al.,* 1992). This reaction requires the presence of bidentate organic acid chelators such as glycolate or oxalate, which stabilize Mn(III) and promote its release from the enzyme. The resulting Mn(III) chelates are small, diffusible oxidants that can act at a distance from the manganese peroxidase active site. They are weak oxidizing therefore, consequently unable to attack the recalcitrant non-phenolic structures that predominate in lignin. However, Mn(III) chelates do oxidize the more reactive phenolic structures that make up approximately 10% of lignin. These reactions result in a limited degree of ligninolysis via C‑ -aryl cleavage and other degradative reactions (Fig. 3.) (Wariishi *et al,* 1991; Tuor *et al.,* 1992). There is an interesting possibility that manganese peroxidase generated Mn(III) might cleave phenolic lignin structures in this fashion to facilitate later attack by the bulkier but more powerful oxidant i.e. lignin peroxidase (Hammel, 1997).

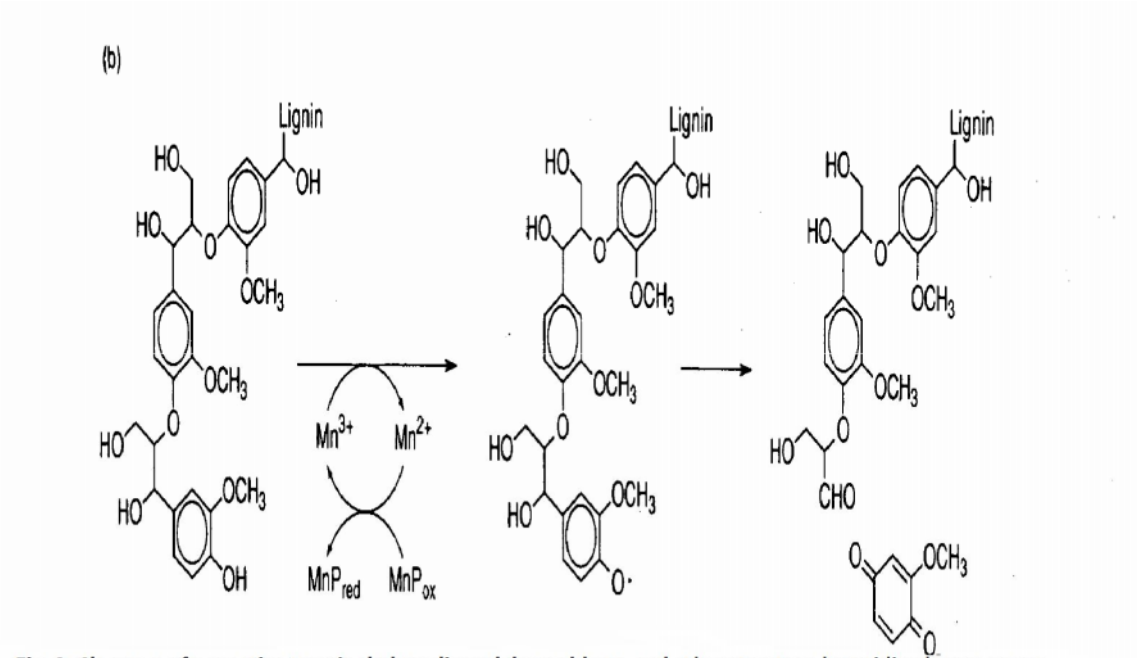

Fig. 3. Cleavage of a reactive terminal phenolic arylglycerol-beta-aryl ether structure by oxidized manganese peroxidase

These enzymes enable the fungi to degrade natural complex aromatic polymers of lignin as well as complex aromatic polymers, such as pesticides, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs). They also degrade recalcitrant environmental pollutants such as crude oil wastes, textile effluents, organochloride agrochemicals and pulp effluents which are a cause of serious environmental pollution (Mtui and Nakamura, 2004; Kiiskinen *et al*., 2004).

CHAPTER - III

MATERIAL AND METHODS

The present investigation was carried out in Soil Microbiology Laboratory, Department of Microbiology, CCS Haryana Agricultural University, Hisar. The following materials and methods were used during the present research work.

3.1. Chemicals

The chemicals and media ingredients were of AR and GR grade manufactured by Glaxo Laboratories Limited, Mumbai, E. Merck Limited, Worli, Mumbai, CDH Bioscience (P) Limited, New Delhi and Hi-media Laboratories (P) Limited, Mumbai. Guaiacol was from Sisco Research Laboratories Pvt. Limited, Mumbai, India.

3.2. Composition of various media

3.2.1. Malt extract agar (MEA) medium

Components	Quantity (g/L)
Malt extract	30.0
Mycological peptone	5.0
Agar-agar	20.0
pH	7.0

The malt extract agar media was also modified in four ways to measure the zone of clearance by the fungal isolates.

3.2.2. Modified malt extract agar (MEA) medium I

Components	Quantity (g/L)
Malt extract	30.0
Tryptone	5.0
Agar-agar	20.0
pH	7.0

3.2.3. Modified malt extract agar (MEA) medium II

Components	Quantity (g/L)
Malt Extract	30.0
Peptone	5.0
$MnSO_4$	0.2
Agar-agar	20
pH	7.0

3.2.4. Modified malt extract agar (MEA) medium III

Components	Quantity (g/L)
Malt Extract	30.0
Peptone	5.0
$CuSO_4$	0.5
Agar-agar	20
pH	7.0

3.2.5. Modified malt extract agar (MEA) medium IV

Components	Quantity (g/L)
Malt Extract	30.0
Peptone	5.0
$MnSO_4$	0.2
$CuSO_4$	0.5
Agar-agar	20
pH	7.0

For screening of ligninolytic fungi, 0.04% aniline blue and 0.02% tannic acid were added to all the above media.

3.3. Sterilization of media and glassware

Different glassware were sterilized by dry heat in an oven at 180 ± 2^0C for 2 h. Culture media and water were sterilized by autoclaving at 15 lbs psi pressure for 15 minutes at 121^{O}C.

3.4 Isolation and screening of fungal cultures for production of lignin degrading enzymes

3.4.1. Collection of samples

Soil samples were collected from various ecological niches like dumping sites, biogas slurry compost, leaf litter from various parks or gardens, paper and pulp mills and mushroom waste of CCS HAU, Hisar, and Yamunanagar for isolation of fungal cultures.

3.4.2. Isolation of fungal cultures

Ligninolytic fungi were isolated by two methods:

3.4.2.1.Dilution plating method

Ligninolytic fungi were isolated from various soil samples by dilution plating technique on malt extract agar medium. The inoculated malt extract agar plates were incubated at 28±2 ^{0}C for 7 days.

3.4.2.2. Enrichment culture technique

Ligninolytic fungi were also isolated from various soil samples by enrichment culture technique in malt extract broth. Hundred ml of malt extract broth in 250 ml conical flask was inoculated with 10 g of soil sample. The flasks were incubated at 28$\pm$2^{0}C on rotary shaker at 140 rpm. Samples were withdrawn at an interval of 7 days and up to 1 ml were poured in petri plates along with malt extract agar media and incubated at 28$\pm$2^{0}C for 7 days.

Fungal isolates thus obtained were purified by at least 4 to 5 subculturing on fresh malt extract agar plates. All the isolated fungal cultures were maintained on malt extract agar (MEA) slants at (4±1^{o}C).

3.4.3. Screening of ligninolytic fungi

Various fungal isolates were screened for ligninolytic activity using malt extract agar media containing 0.04% aniline blue dye. The culture spots were applied on MEA (with dye) plates using inoculation needle and incubated at 28±2^{o}C for 72 hours. Ligninolytic enzyme producers decolorized the dye on the plates and a zone of clearance was formed. Zone efficiency was calculated by taking the ratio of clear zone diameter to colony diameter.

3.4.4. Determination of ligninolytic enzymes activity of the selected fungal isolates

For ligninolytic enzymes production, 100 ml of malt extract broth taken in 250 ml Ehrlenmeyer conical flask and inoculated with approximately 10^7 spores/ml from 7 days old slant of fungal isolates. The flasks were then incubated at 28$\pm$2^{0}C on rotary shaker at 140 rpm. The clear culture filtrate obtained by filtration was used for determining ligninolytic enzymes activity (Mandels, 1969).

3.4.4.1. Determination of laccase activity

0.1 M Phosphate buffer (pH 6.0)

For preparing phosphate buffer, stock solutions of following molarity were prepared:

A. 0.2 M solution of KH_2PO_4 (27.22 g in 1000 ml)

B. 0.2 M solution of K_2HPO_4 (34.84 g in 1000 ml)

A known volume of 87.7 ml of A and 12.3 ml of B were mixed and diluted to make the total volume 200 ml with distilled water and pH was set at 6.0 with pH meter.

0.02 M buffered guaiacol

A known volume of 1.24 g (1.10 ml) of guaiacol and 500 ml of phosphate buffer were mixed.

Two ml of culture filtrate was taken in cuvette and placed in UV Spectrophotometer at 495 nm to adjust light absorbance at zero. The cuvette was taken out and dispensed with 2 ml of guaiacol solution and immediately transferred back to spectrophotometer. Change in light absorption was recorded for every 30 seconds. The change in absorbance between 30 and 150 seconds was taken and results were expressed as change in absorbance/minute. An increase in activity by 0.001 in 60 seconds was taken as one unit of laccase activity.

3.4.4.2.Determination of manganese peroxidase activity

0.1 M Phosphate buffer (pH 5.8)

For preparing phosphate buffer stock solutions of following molarity were prepared:

A. 0.2 M solution of KH_2PO_4 (27.22 g in 1000 ml)

B. 0.2 M solution of K_2HPO_4 (34.84 g in 1000 ml)

A known volume of 92 ml of A and 8 ml of B were mixed and diluted to make the total volume 200 ml with distilled water and pH was set at 5.8 with pH meter.

0.05 M buffered guaiacol

A known volume of 0.6207 g (0.55 ml) of guaiacol and 100 ml phosphate buffer were mixed.

0.4 M hydrogen peroxide

A known concentration of 1.36 g (1.23 ml) H_2O_2 was mixed in 100 ml distilled water.

2 mM manganese sulfate

$MnSO_4$, 30.2 mg was dissolved in 100 ml distilled water.

The culture filtrate, 2.0 ml, was put in cuvette and placed in UV Spectrophotometer at 465 nm to adjust light absorbance at zero. The cuvette was taken out and dispensed in a mixture of 2.0 ml buffered guaiacol, 0.2 ml of manganese sulfate and 0.2 ml H_2O_2. The change in light absorption was recorded for every 30 seconds. The change in absorbance between 30 and 150 seconds was taken and results were expressed as change in absorbance/minute. An increase in activity by 0.001 in 60 seconds was taken as one unit of manganese peroxidase activity.

3.4.4.3. Determination of lignin peroxidase activity

0.1 M phosphate citrate buffer (pH 3)

For preparing phosphate buffer, stock solutions of following molarity were prepared:

A. 0.1 M solution of citric acid (19.213 g in 1000 ml)
B. 0.2 M solution of Na_2HPO_4 (35.6 g in 1000 ml)

A known volume of 79.5 ml of A and 20.5 ml of B were mixed.

10 mM veratryl alcohol

A known volume of 0.168 g (0.15 ml) of veratryl alcohol and 100 ml distilled water were mixed.

10 mM hydrogen peroxide

A known concentration of 34.0147 mg (0.03 ml) H_2O_2 and 100 ml distilled water were mixed.

The culture filtrate, 0.5 ml, was put in cuvette and placed in UV Spectrophotometer at 310 nm to adjust light absorbance at zero. The cuvette was taken out and dispensed in a mixture of phosphate citrate buffer 1.5 ml, veratryl alcohol 1.0 ml, H_2O_2. The change in light absorption was recorded for every 30 seconds. The change in absorbance between 30 and 150 seconds was taken and results were expressed as change in absorbance/minute. An increase in activity by 0.001 in 60 seconds was taken as one unit of lignin peroxidase activity.

3.4.5. Morphological characterization of isolated fungal isolates

Wet mounts of isolated fungi were prepared in water and lactophenol blue and all the fungi were characterized on the basis of their hyphae and arrangement of spores.

Composition of Lactophenol

Components	Quantity
Phenol crystals	20 g
Lactic acid	16 ml (20 g)
Pure glycerin	31 ml (40 g)
0.05% cotton blue	20 ml

CHAPTER - IV

EXPERIMENTAL RESULTS

Lignin is an aromatic polymer synthesized from phenylpropanoid precursors. The composition and percentages of these polymers vary from one plant species to another. Moreover, the composition within a single plant varies with age, stage of growth, and other conditions. It is present in the cellular cell wall, conferring structural support, impermeability, and resistance against microbial attack and oxidative stress. Structurally, lignin is an amorphous heteropolymer, non-water soluble and optically inactive. This complex structure of lignin makes its degradation very difficult.

Fungi play an important role in the biodegradation of various substrates rich in lignin and cellulose. Therefore, it is obvious that methods using these ligninolytic fungi for biotechnological applications are quite promising (Daljit and Arora, 1995; Petre *et al*., 2005). The aim of the present investigation was to isolate lignin degrading fungi and to determine the activity of ligninolytic enzymes and their abilities to degrade this complex.

4.1 Isolation and screening of fungal cultures for production of lignin degrading enzymes

4.1.1. Isolation and study of morphological characters of fungal isolates

Fungi were isolated from different soil samples collected from various ecological niches of HAU, Hisar and nearby areas. A total of 24 mutually distinct fungi were isolated which are shown in Table 1.

Table 1: List of various fungal isolates and their site of isolation

S.no	Isolates	Ecological niches
1	HST1	Vermicompost (HAU)
2	HST2	Mushroom waste (HAU)
3	HST3	Mushroom waste (HAU)
4	HST4	Mushroom waste (HAU)
5	HST5	Vermicompost (HAU)
6	HST6	Mushroom waste (HAU)
7	HST7	Biogas slurry (HAU)
8	HST8	Mushroom waste (HAU)
9	HST9	Leaf and Litter (Hisar)
10	HST10	Vermicompost (HAU)
11	HST11	Mushroom waste (HAU)
12	HST12	Compost (HAU)

13	HST14	Vermicompost (HAU)
14	HST15	Waste from dumping site (Hisar)
15	HST16	Compost (HAU)
16	HST17	Compost (HAU)
17	HST18	Waste from dumping site (Hisar)
18	HST19	Biogas slurry (HAU)
19	HST20	Waste from dumping site (Hisar)
20	HST21	Biogas slurry (HAU)
21	HST22	Compost (HAU)
22	HST23	Mushroom waste (HAU)
23	HST24	Compost (HAU)
24	YST1	Paper and pulp waste (Yamunanagar)

The morphological characters of the isolates revealed that their colony form varied from circular to irregular, colony size varied from small to large and margins were entire to undulate with varying spore color as shown in Table 2.

Table 2: Morphological characters of the fungal colonies on malt extract agar plates

S.no	Fungal isolate	Form of colony	Size of colony	Margin	Colour
1	**HST1**	Circular	Large	Entire	Creamy mycelium
2	**HST2**	Circular	Large	Entire	Brown spores
3	**HST3**	Filamentous	Large	Entire	Whitish grey filaments
4	**HST4**	Irregular	Moderate	Undulate	Green spores
5	**HST5**	Filamentous	Moderate	Entire	Bluish white filaments
6	**HST6**	Filamentous	Large	Entire	White mycelium
7	**HST7**	Filamentous	Large	Entire	Black filaments
8	**HST8**	Circular	Moderate	Entire	Black spores
9	**HST9**	Circular	Large	Entire	Green spores
10	**HST10**	Circular	Large	Entire	Whitish brown spores
11	**HST11**	Irregular	Large	Entire	Greenish white mycelium
12	**HST12**	Circular	Moderate	Entire	Creamy mycelium
13	**HST14**	Filamentous	Large	Entire	Black spores
14	**HST15**	Circular	Large	Entire	Green spores
15	**HST16**	Irregular	Large	Undulate	Pinkish green mycelium
16	**HST17**	Filamentous	Small	Entire	Creamy mycelium
17	**HST18**	Circular	Small	Entire	White mycelium
18	**HST19**	Irregular	Large	Entire	Black spores
19	**HST20**	Filamentous	Large	Undulate	Grey mycelium
20	**HST21**	Filamentous	Moderate	Undulate	White filaments
21	**HST22**	Irregular	Large	Entire	Black spores
22	**HST23**	Circular	Large	Entire	Black spores
23	**HST24**	Circular	Moderate	Entire	Greenish yellow mycelium
24	**YST1**	Irregular	Large	Undulate	Creamy pink spores

4.1.2 Screening of fungal isolates for clear zone formation

Screening of all the fungal isolates having ligninolytic activity was done on malt extract agar media containing aniline blue dye (0.04%) and tannic acid (0.02%) and their zone of clearance was measured after seven days of incubation at 28±2^{O}C and out of 24 mutually distinct isolates, 16 were found to be ligninolytic and the zone of clearance varied from 1.03 to 1.20 as shown in Table 3 with HST15 isolated from garbage dumping site showing the largest zone of clearance of 1.20.

Table 3: Zone of clearance formed by the isolates on malt extract agar plates

S.no	Fungal isolates	Zone of clearance (A/B)*
1	**HST1**	1.03
2	**HST2**	1.03
3	**HST3**	1.02
4	**HST4**	1.05
5	**HST5**	1.05
6	**HST6**	1.04
7	**HST7**	1.05
8	**HST8**	1.04
9	**HST9**	1.12
10	**HST10**	1.05
11	**HST11**	1.10
12	**HST12**	1.03
13	**HST14**	1.09
14	**HST15**	1.20
15	**HST16**	1.08
16	**YST1**	1.05

*zone of clearance =A/B where A stands for total diameter (zone + colony) and B stands for colony diameter

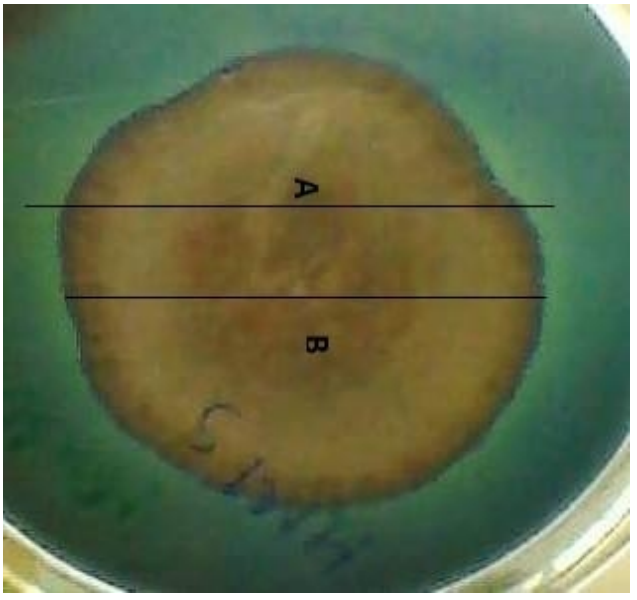

Fig. 4. Measurement of zone of clearance (A/B)

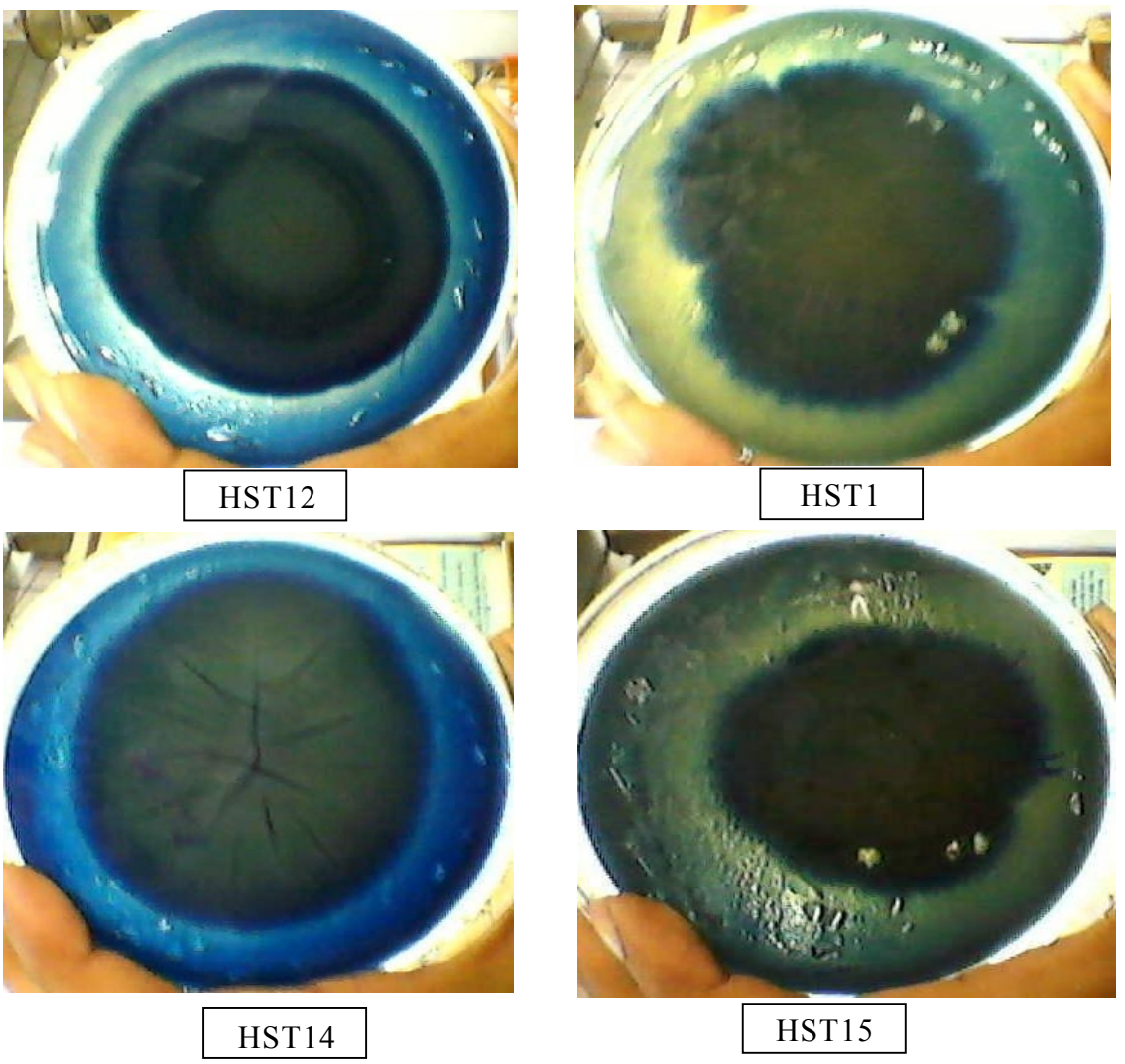

Fig. 5: Malt extract agar plates showing zone of clearance of some fungal isolates

The malt extract media was modified by adding different compounds containing tryptone instead of peptone (medium I), $MnSO_4$ (medium II), $CuSO_4$ (medium III) and $MnSO_4$ and $CuSO_4$ (medium IV) and the zone of clearance of isolates were measured as shown in Tables 4, 5, 6 and 7.

Table 4: Zone of clearance formed by the isolates on modified malt extract agar plates (medium I)

S.no	Fungal isolates	Zone of clearance
1	HST1	_
2	HST2	_
3	HST3	_
4	HST4	_
5	HST5	_

6	**HST6**	1.06
7	**HST7**	_
8	**HST8**	_
9	**HST9**	_
10	**HST10**	_
11	**HST11**	_
12	**HST12**	_
13	**HST14**	1.04
14	**HST15**	1.05
15	**HST16**	_
16	**YST1**	_

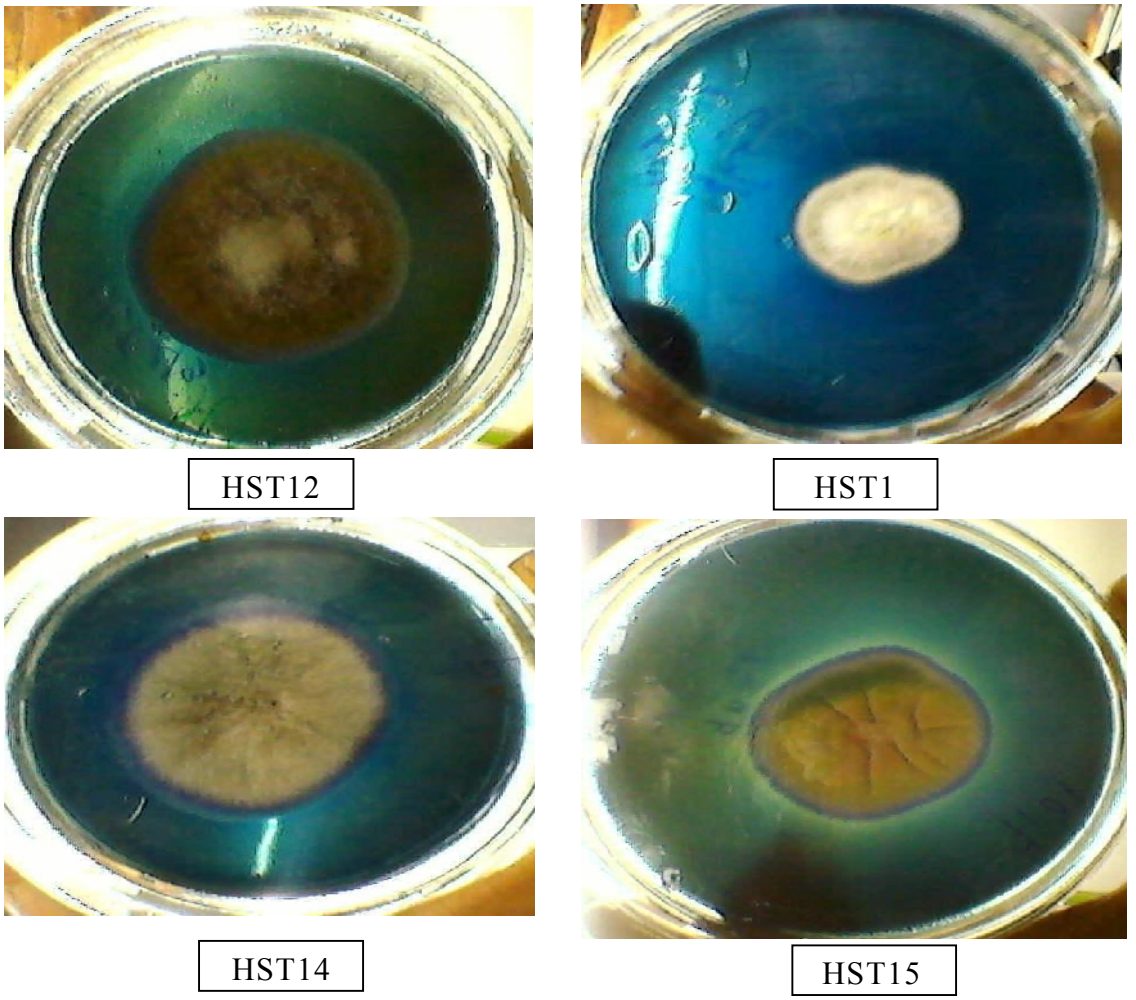

Fig. 6: Modified malt extract agar plates (medium I) showing zone of clearance of some fungal isolates

Table 5: Zone of clearance formed by the isolates on modified malt extract agar plates (medium II)

S.no	Fungal isolates	Zone of clearance
1	HST1	_
2	HST2	1.2
3	HST3	1.1
4	HST4	1.2
5	HST5	_
6	HST6	_
7	HST7	_
8	HST8	1.4
9	HST9	_
10	HST10	1.09
11	HST11	_
12	HST12	_
13	HST14	_
14	HST15	_
15	HST16	1.05
16	YST1	1.2

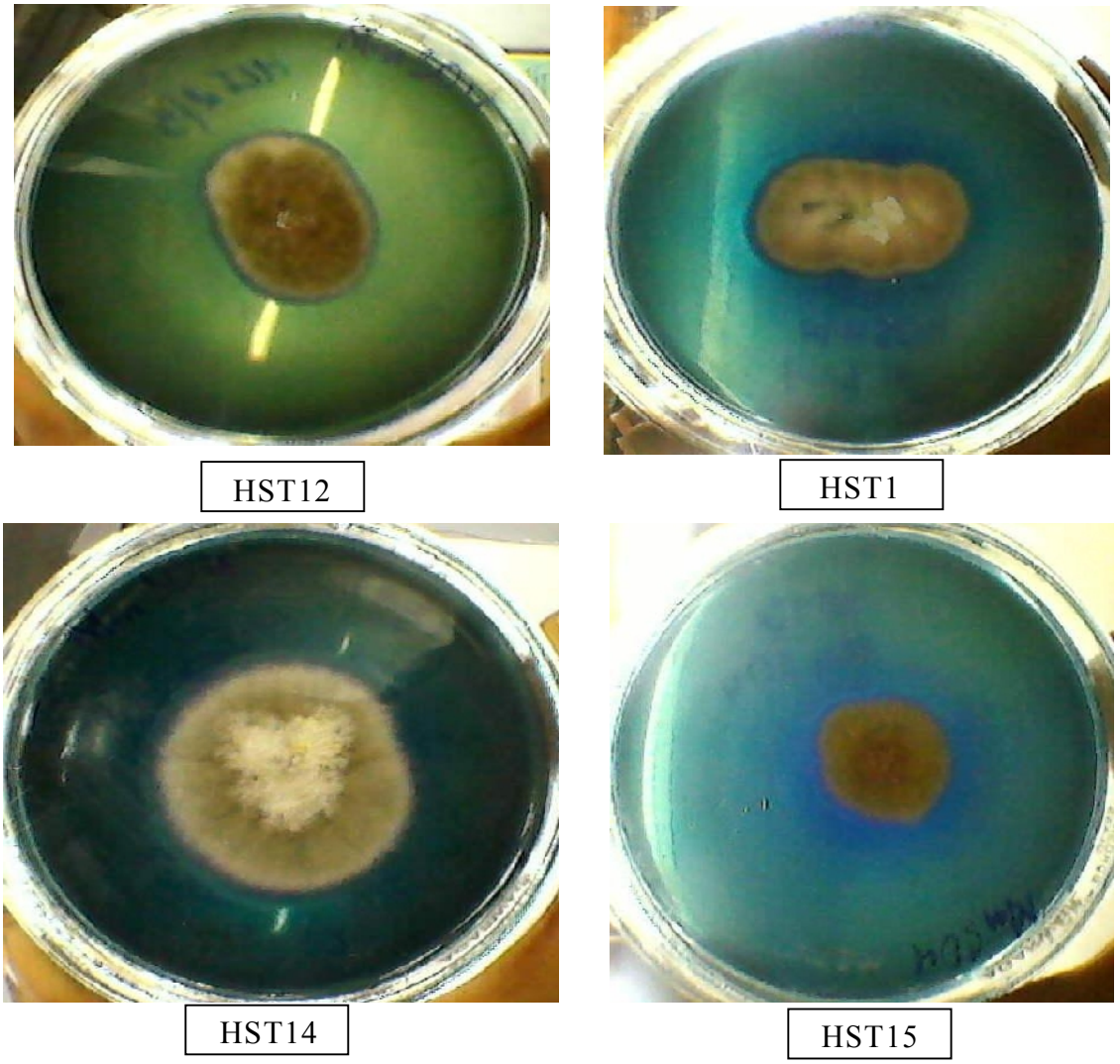

Fig. 7: Modified malt extract agar plates (medium II) showing zone of clearance of some fungal isolates

Table 6: Zone of clearance formed by the isolates on modified malt extract agar plates (medium III)

S.no	Fungal isolates	Zone of clearance
1	HST1	_
2	HST2	_
3	HST3	_
4	HST4	_
5	HST5	_
6	HST6	_
7	HST7	_
8	HST8	_
9	HST9	1.03
10	HST10	_
11	HST11	_
12	HST12	_
13	HST14	1.03
14	HST15	_
15	HST16	_
16	YST1	_

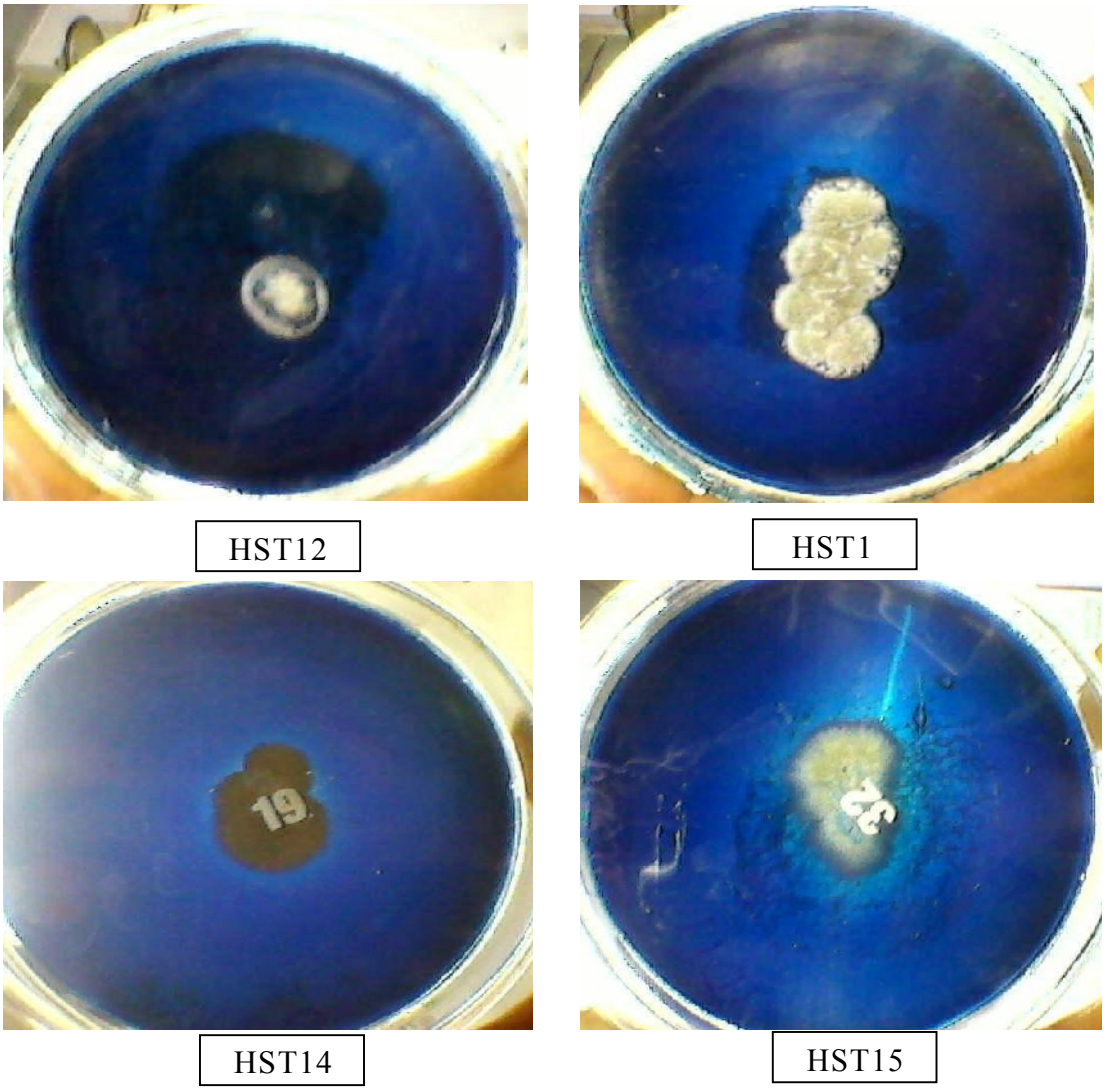

Fig. 8: Modified malt extract agar plates (mediumIII) showing zone of clearance of some fungal isolates

Table 7: Zone of clearance formed by the isolates on modified malt extract agar plates (medium IV)

S.no	Fungal isolates	Zone of clearance
1	HST1	1.07
2	HST2	_
3	HST3	_
4	HST4	_
5	HST5	_
6	HST6	_
7	HST7	_
8	HST8	1.1
9	HST9	1.01
10	HST10	_
11	HST11	_
12	HST12	_
13	HST14	_
14	HST15	_
15	HST16	_
16	YST1	1.06

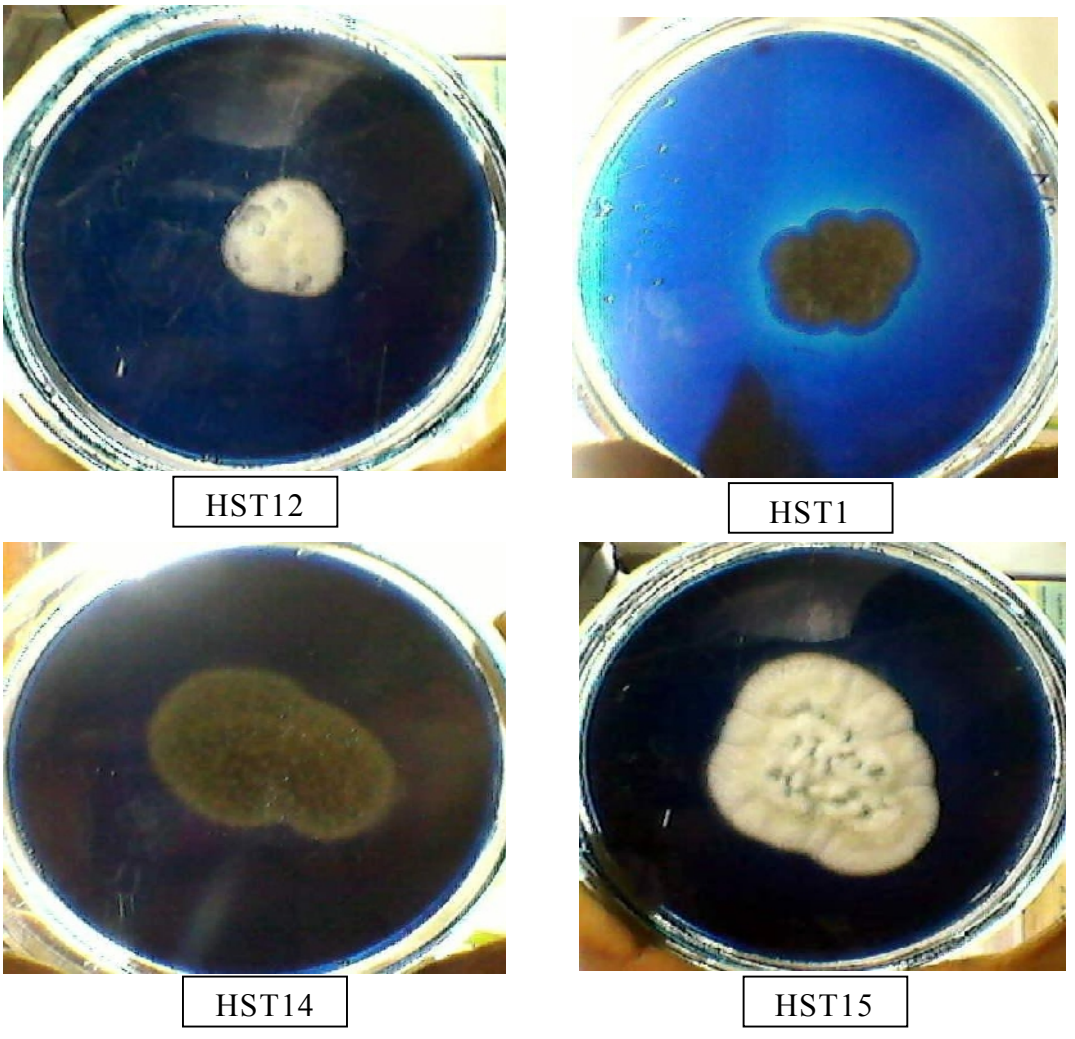

Fig. 9: Modified malt extract agar plates (mediumIV) showing zone of clearance of some fungal isolates

Although the isolates were cultured on modified media, but it was found that the isolates showed better zone of clearance on normal malt extract agar medium.

4.2 Activity of lignin degrading enzymes of fungal isolates

A total of five cultures were selected for measuring enzyme activity on the basis of their zone of clearance on malt extract agar medium.

4.2.1 Laccase activity of fungal isolates

In liquid malt extract medium, Laccase activity of the isolates ranged from 0.75 to15.5 U/ml. The isolate HST 9 showed highest laccase activity (15.5 U/ml) followed by HST16 (8.50 U/ml), HST15 (6.00 U/m), HST11 (2.00 U/ml) and HST14with minimum laccase activity (0.75 U/ml) as shown in Table 8.

Table 8: Laccase activity of selected fungal isolates

S.no	Fungal isolate	Laccase activity (U/ml)
1	**HST9**	15.50
2	**HST11**	2.00
3	**HST14**	0.75
4	**HST15**	6.00
5	**HST16**	8.50

4.2.2 Manganese peroxidase activity of fungal isolates

In liquid malt extract medium, manganese peroxidase activity of the isolates ranged from 0.75 to 4 U/ml. The isolate HST 9 had highest manganese peroxidase activity, 4U/ml followed by HST11, 3.00 U/ml, HST15, 1.60 U/ml, HST16, 1.50 U/ml and HST14 with minimum manganese peroxidase activity (0.75U/ml) as shown in Table 9.

Table 9: Manganese peroxidase activity of selected fungal isolates

S.no	Fungal isolate	Manganese peroxidase activity (U/ml)
1	**HST9**	4.00
2	**HST11**	3.00
3	**HST14**	0.75
4	**HST15**	1.60
5	**HST16**	1.50

4.2.3 Lignin peroxidase activity of fungal isolates

In liquid malt extract medium, lignin peroxidase activity of the isolates ranged between 5 to 21 U/ml. The isolate HST15 had highest lignin peroxidase activity (21.0 U/ml) followed by HST16 (13.0 U/ml), HST11 (10.0 U/ml), and HST9 and HST14 had minimum lignin peroxidase activity (5 U/ml) as shown in Table 10.

Table 10: Lignin peroxidase activity of selected fungal isolates

S.no	Fungal isolate	Lignin peroxidase activity (U/ml)
1	**HST9**	5.0
2	**HST11**	10.0
3	**HST14**	5.0
4	**HST15**	21.0
5	**HST16**	13.0

4.3 Morphological characterization of selected fungal isolates

Morphological studies were done for the above five isolates. Wet mounts were prepared in lactophenol blue and water and observed under the microscope. On the basis of their hyphae, spores and sporangiophore, they were identified as shown in Table 11.

Table 11: Identification of selected fungal isolates

S.no	Fungal isolate	Fungi identified	
1	**HST9**	Ascomycetes	Possibly *Aspergillus*
2	**HST11**	Basidiomycetes	Possibly *Kreigeria*
3	**HST14**	Basidiomycetes	Possibly *Helicogloea*
4	**HST15**	Ascomycetes	Possibly *Aspergillus*
5	**HST16**	Ascomycetes	Possibly *Aspergillus*

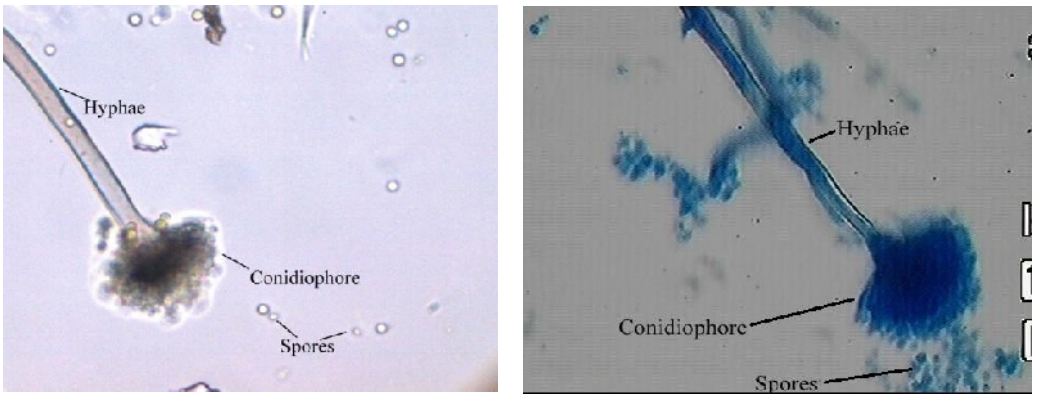

Fig. 10: HST9, isolated from leaf and litter waste

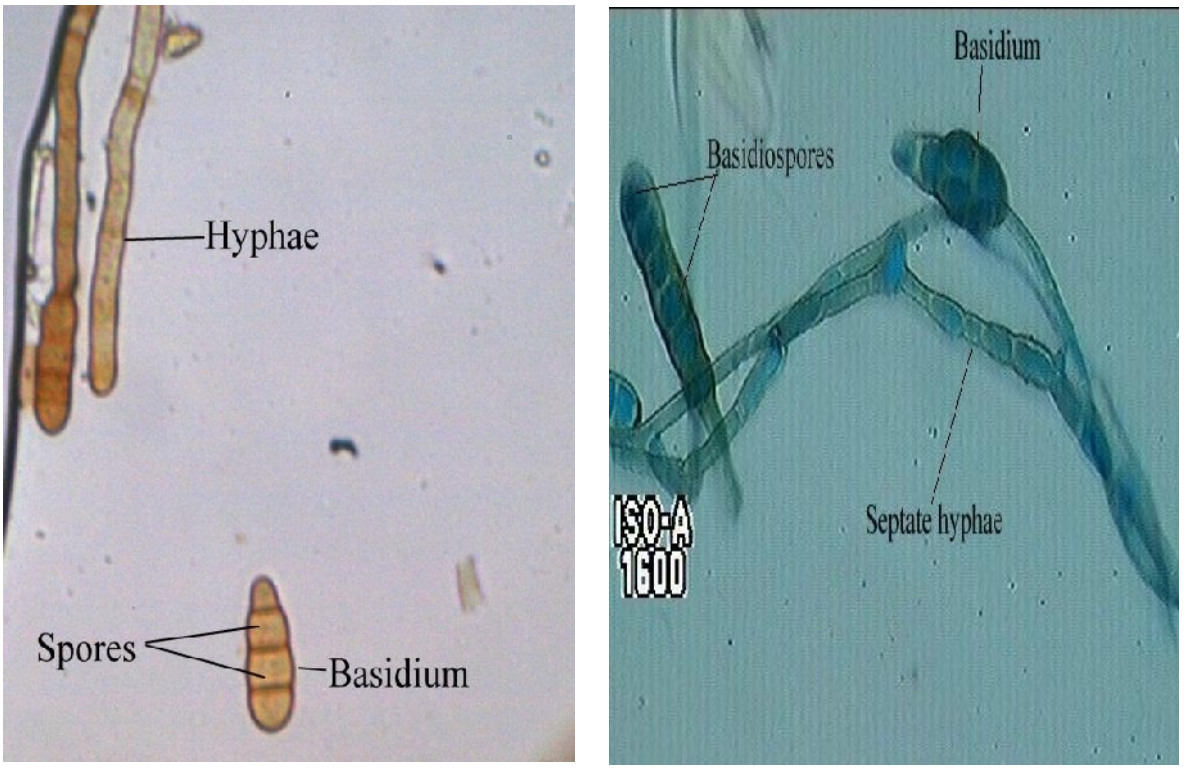

Fig. 11: HST11, isolated from mushroom waste

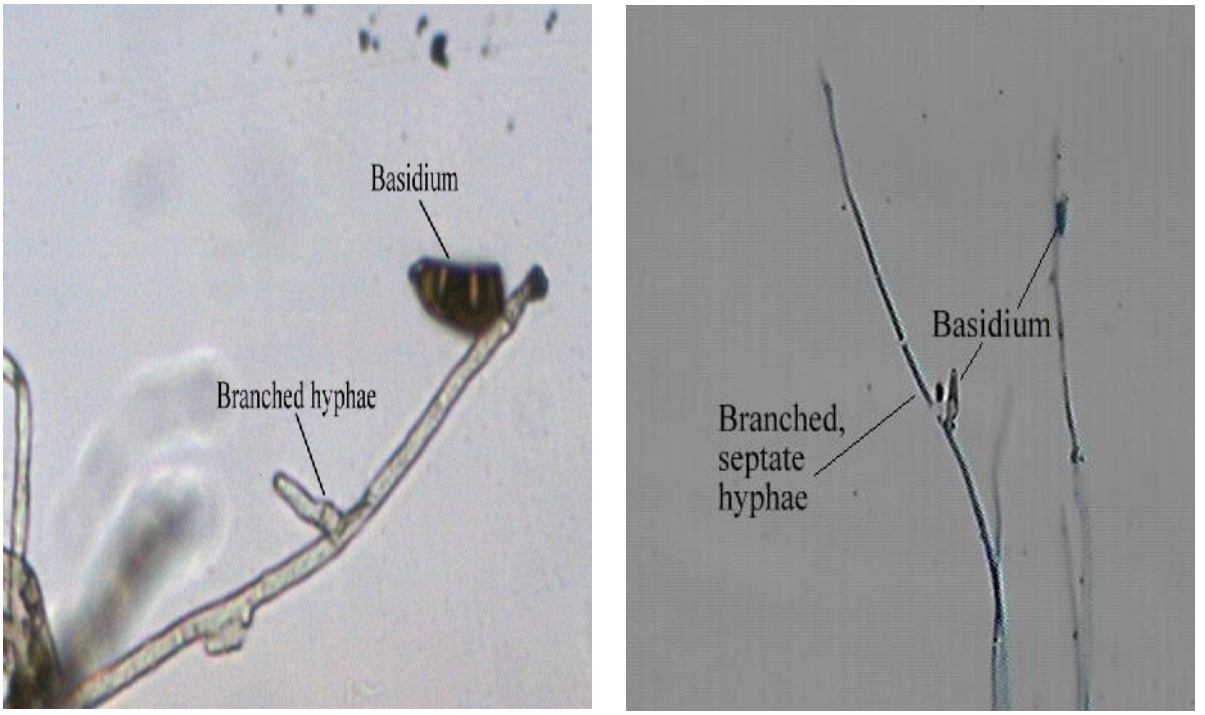

Fig. 12: HST14, isolated from Vermicompost

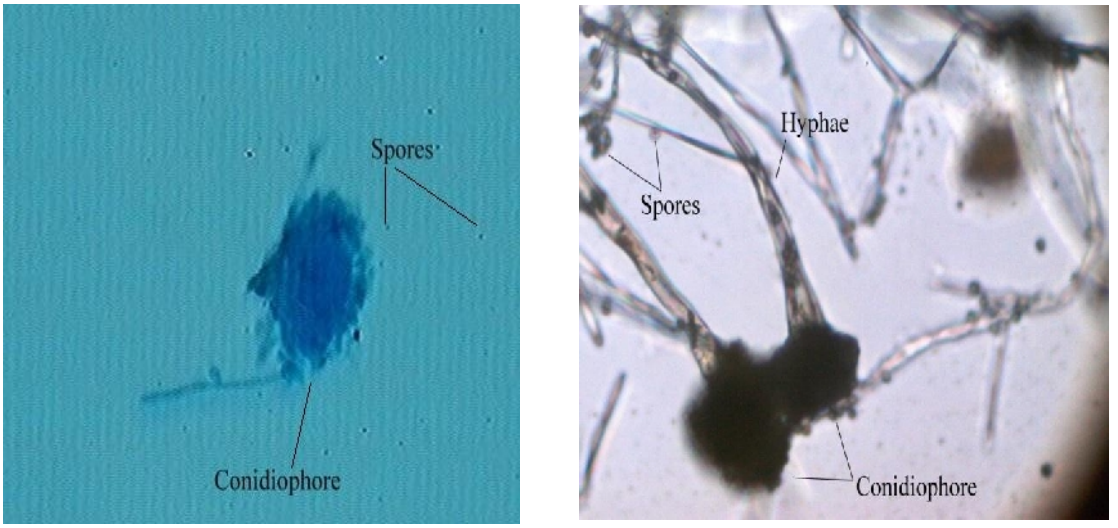

Fig. 13: HST15, isolated from garbage dumping site

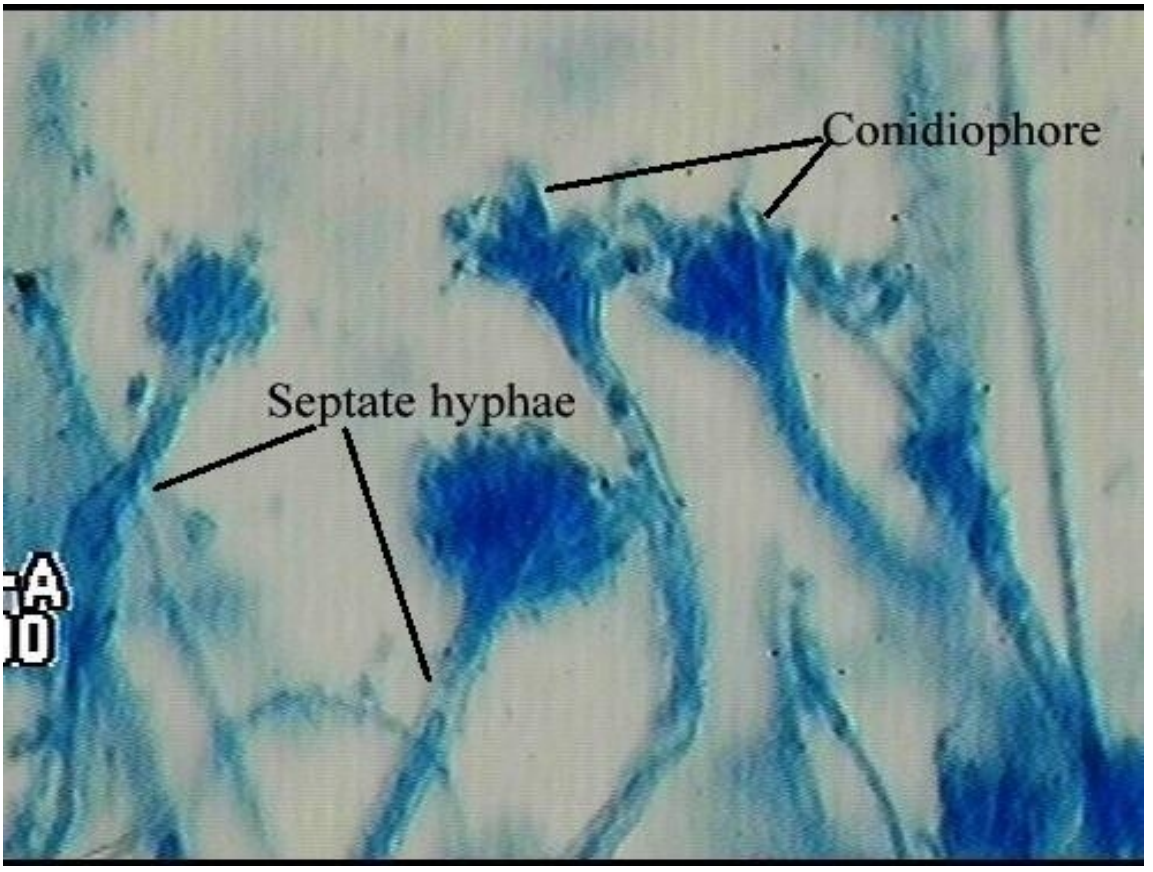

Fig. 14: HST16, isolated from compost soil

CHAPTER - V

DISCUSSION

Lignin is considered to be the main hurdle in the degradation of lignocellulosic biomass. This macromolecule plays a vital role in providing mechanical support to bind plant fibers together. It also decreases the permeation of water through the cell walls of the xylem, thereby playing an intricate role in the transport of water and nutrients. It is the only naturally synthesized polymer with an aromatic backbone (Wei *et al*., 2009). Finally, lignin plays an important function in a plantís natural defense against degradation by impeding penetration of destructive enzymes through the cell wall. Although lignin is necessary to trees, it is undesirable in most chemical paper making fibers and is removed by pulping and bleaching processes. Among all the components of lignocellulosic material, lignin is the most resistant to microbial degradation (Himmel *et al*., 2007; Sanchez, 2009). During the present investigation the isolation of efficient ligninolytic fungi was done.

A large amount of plant waste is being continuously accumulated on the Earth. Lignocelluloses in nature derive from wood, grass, agricultural residues, forestry wastes and municipal solid wastes. The cellulose, hemicelluloses and lignin content in typical plant waste is 40-50, 15-35 and 20-40%, respectively and the plant litter layer has 20-50% lignin (Area and Cheradame, 2011). A part of the waste goes into the soil and their microbial degradation starts which depend upon various physical factors such as temperature, moisture, aeration and also on efficiency of different microorganisms. Therefore during the present investigation, isolation of efficient ligninolytic fungi was done from soil samples which were collected from various ecological niches like leaf litter waste, garbage dumping sites, mushroom waste, paper and pulp waste, compost, Vermicompost, biogas slurry from HAU and other nearby areas.

Although lignin resists attack by most microorganisms, but a large number of white-rot fungi can degrade lignin efficiently (Abbas *et al*., 2005; Wong, 2009). Over 600 species of basidiomycetes have been found to be ligninolytic converting lignin to CO_2, by secreting extracellular laccase, lignin peroxidase and manganese ñ dependent peroxidase isozyme (Kumar and Gupta, 2006).

A total of 24 mutually distinct fungi were isolated from different soil samples by serial dilution method and enrichment culture method on malt extract agar media. Morphological characters of all the fungal colonies on malt extract agar plates showed a large variation in colony morphology like colony - size (small to large), form (circular to irregular), margin (entire to undulate) and color (creamy mycelium to green spores) varied from one isolate to another (Table-2). The fungal isolates were screened for ligninolytic activities by observing their ability to form clear zone on malt extract agar medium containing tannic acid and aniline blue and the zone diameter varied from 1.03 to 1.20 with HST15 showing largest zone of 1.20 (Table 3). In a similar study, 88 fungal isolates from different algae, sea grasses and decaying wood samples collected from Abou-keer, Alexanderia, Egypt were isolated and screened. Results obtained from both qualitative and quantitative assay showed that the marine fungal isolate *Trematosphaeria mangrovei* measured the highest zone diameter and colony diameter in agar plate screening test with guaiacol Mabrouk *et al.,* 2010). Fungal strains were isolated and screened for their ligninolytic activity based on their ability to oxidize dyes, poly R-478 and remazol brilliant blue. The color removals in 57 samples were in the range of 11.5 to 38.4% in poly- R dye and 11.1 to 72.0 % in remazol brilliant blue Selvam *et al*., 2012).

The zone of clearance was also seen on modified malt extract agar medium by adding tryptone instead of peptone, manganese sulfate (because for the enzyme manganese peroxidase, Mn(II) is the obligatory electron donor), copper sulfate (the enzyme laccase contain multiple copper atoms which are reduced as the substrates are oxidized) and both manganese sulfate and copper sulfate, respectively keeping in view that these compounds can enhance the efficiency of fungi to degrade lignin and the zone of clearance was measured. But it was observed during the present investigation that the zone formation was comparatively poor in modified media (Table 4, 5, 6 and 7) and hence it was not enhancing the ligninolytic activity.

Microbial degradation of lignin is carried out by the activities of various enzymes. Certain white rot fungi have been found to be ligninolytic due to the secretion of certain extracellular enzymes. The main enzymes of fungi taking part in lignin degradation are lignin peroxidase, manganese peroxidase and laccase (Tanaka *et al*., 2009). Liquid culture assay was used to isolate efficient ligninase producing fungi for degradation of lignin. HST15 isolate had 21.0 U/ml lignin peroxidase activity. HST9 showed 15.5 U/ml laccase activity and 4.00 U/ml manganese peroxidase activity, out of the five selected ligninolytic fungal isolates. Rest four isolates had comparative low activity due to less enzyme production (Table 8, 9 and 10). Kalmis *et al*., (2008) studied ligninolytic enzyme activities of different fungal species (six

commercial and 13 wild) in solid and liquid culture media. It was postulated that, among the wild strains, only *Pleurotus ostreatus*-1 (MCC45), *P. ostreatus*-2 (MCC40), *Pleurotus eryngii*-1 (MCC25) and *P. eryngii*-2 (MCC26), and commercial strains *P. ostreatus*, *P. sajor-caju*, *P. eryngii* presented lignin peroxidase (LiP) activity. Dashtban and coworkers, (2010) described ligninolytic enzyme families produced by fungi that are involved in wood decay processes, their molecular structures, biochemical properties and the mechanisms of action which render them attractive candidates in biotechnological applications. These enzymes include phenol oxidase (laccase) and heme peroxidases [lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP)]. Accessory enzymes such as H_2O_2-generating oxidases and degradation mechanisms of plant cell-wall components in a nonenzymatic manner by production of free hydroxyl radicals (ïOH) are also discussed. Huiju Gao *et al*. (2011) isolated an ascomycete producing ligninolytic enzymes and characterized its lignin degradation capability. Among 20 isolates, GHJ-4 was isolated from decayed wood of Salix matsudana Koidz in Mount Tai, China, by different indicator compounds assay. The capability of utilizing several lignin model compounds and decoloration of aromatic dyes by GHJ-4 strain revealed its ligninolytic potentiality. The taxonomy of the fungi was *Paraconiothyrium variabile*.

On morphological characterization of five selected fungi, it was found that HST9, HST15 and HST16 were ascomycetes and HST11 and HST14 were identified to be basidiomycetes on the basis of their hyphae, spores and sporangiophores. Sivakami *et al*., (2012) isolated a lignolytic fungus from Chennai which was able to produce laccase and lignin Peroxidase. The organism was identified as *Pleurotus ostreatus* using 28 S rRNA sequence analysis.

CHAPTER - VI

SUMMARY AND CONCLUSION

1. Twenty four mutually distinct fungal species were isolated from soil and different ecological niches.
2. Out of these, 16 isolates were screened, which were showing ligninolytic activity on the basis of formation of zone of clearance on malt extract agar media containing aniline blue dye.
3. The zone of clearance varied from 1.20 to 1.03 with fungal isolate HST15 showing the largest zone of clearance of 1.20 on malt extract agar plates.
4. Four different modified media containing tryptone instead of peptone (medium I), $MnSO_4$ (medium II), $CuSO_4$ (medium III) and $MnSO_4$ and $CuSO_4$ (medium IV) were used to measure zone of clearance. The zone of clearance on modified media was poor as compared to that of normal malt extract agar medium.
5. Best five fungal isolates were selected for measuring the enzyme activity in malt extract broth on the basis of their zone of clearance.
6. The activity of laccase varied from 0.75 to 15.50 U/ml, manganese peroxidase varied from 0.75 to 4.00 U/ml and that of lignin peroxidase varied from 10.0 to 42.0 U/ml.
7. The isolate, HST9 had highest laccase activity (15.5 U/ml) and manganese peroxidase activity (4.00 U/ml). However, highest lignin peroxidase activity was measured in HST15 (42.0 U/ml).
8. As lignin peroxidase enzyme is considered as the main enzyme in ligninolysis, therefore, HST15 was scored as the best isolate.
9. Morphological characterization of the best five isolates revealed three as ascomycetes and two as basidiomycetes.

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ABSTRACT

Title of Thesis	:	**Isolation and screening of efficient ligninolytic fungi**
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Lignin, the most abundant aromatic biopolymer on earth, is extremely recalcitrant to degradation. By linking to both cellulose and hemicellulose, it creates a barrier to any chemicals or enzymes and prevents the penetration of lignocellulolytic enzymes into the interior lingocellulosic structure. Lignin is the major hurdle in the degradation of lignocellulosic biomass. It is degraded by an enzyme complex containing three enzymes namely laccase, manganese peroxidase and lignin peroxidase that are collectively known as ligninases. These enzymes are produced by several microorganisms, commonly by fungi but most of them have high production cost and no efficiency in enzyme production and its enzyme activity. Therefore in the present investigation, a total of 24 mutually distinct fungi were isolated from different ecological niches such as leaf and litter waste, garbage dumping site, mushroom waste, compost, vermicompost, paper and pulp waste and biogas slurry. Out of these 24 fungal isolates, 16 fungal cultures were screened as ligninolytic fungi by observing the formation of zone of clearance on malt extract agar plates containing aniline blue dye and tannic acid. The zone of clearance ranged from 1.03 to 1.20 Isolate HST15 formed the largest zone of clearance of 1.20. The fungal isolate, HST9 was showing highest laccase (15.5 U/ml) and Manganese peroxidase (4 U/ml) activity among the isolates while maximum lignin peroxidase activity was observed in HST15 (21U/ml). On the basis of lignin peroxidase activity, HST15 was selected as the best ligninolytic fungal isolate. The selected fungal isolates were morphologically identified on the basis of their hyphae, sporangiophore and spores. Among the five best fungal isolates, three were identified to be ascomycetes and two were basidiomycetes.

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