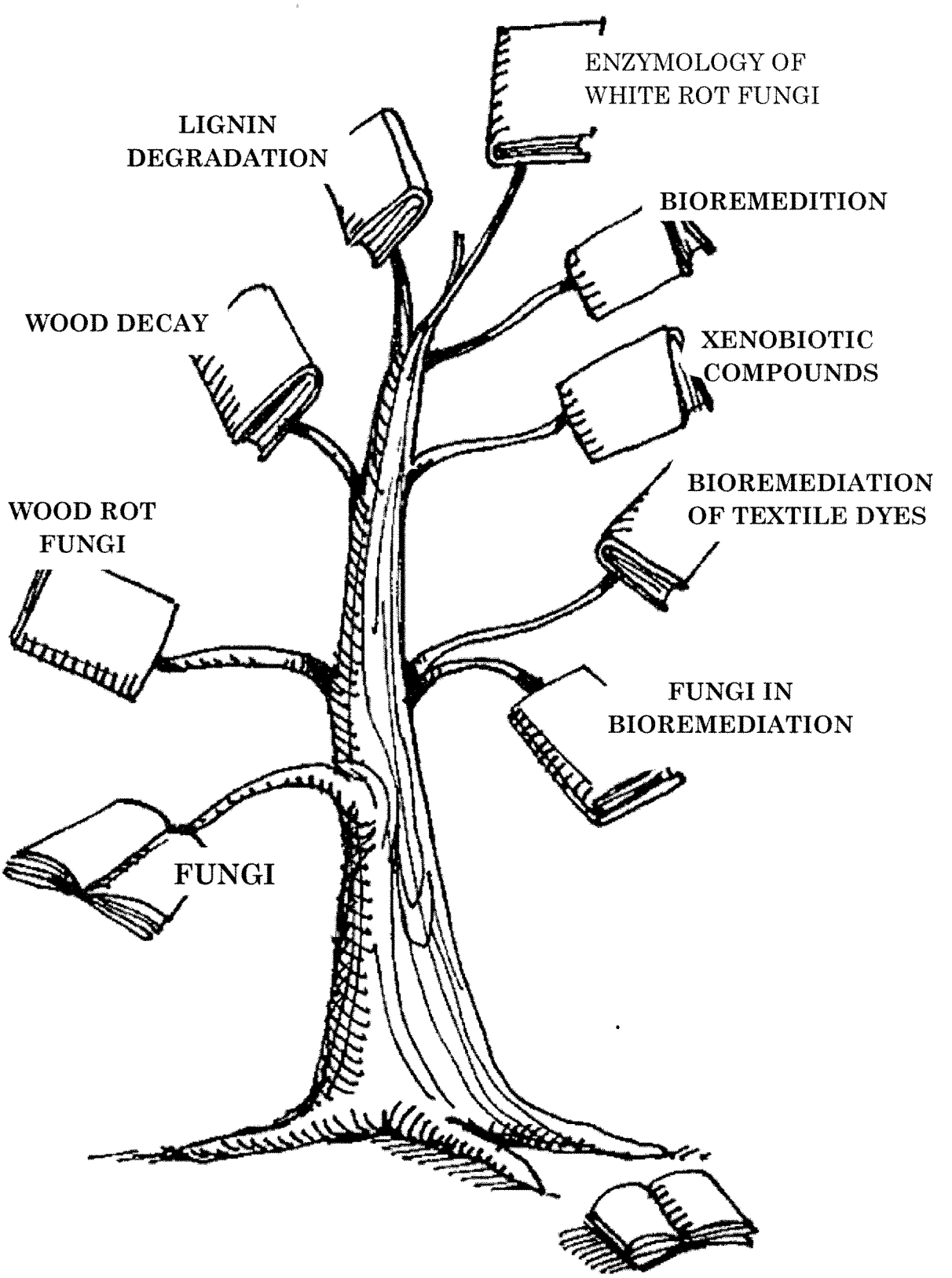
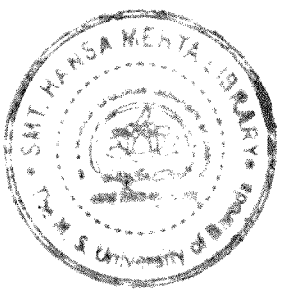
LITERATURE REVIEW AND OBJECTIVES





INTRODUCTION

1.1 Fungi:

Fungi physiology:

Fungi in general are heterotrophic, diverse and wide spread organisms comprised of a dense collection of hyphae in the stalk and head which forms an absorption network called mycelium. Hyphae are often strengthened by tiny hollow tubes of plasma membrane and chitin filled with cytoplasm. Their reproduction is complex and reflects the heterogeneity in lifestyles. Fungi can survive in adverse environmental conditions such as extreme acidic and alkaline pH, and extreme low and high temperature. They reproduce sexually or asexually, depending on conditions in the environment. The microscopic spores produced by them have very diverse shapes to suit their specific functions. They are often produced very quickly even in a matter of days or even hours after the initial colonization on the substrate (Parand, 2001). The spores are produced in enormous number and are dispersed by wind, water, or animals. Spores can often survive for long periods, sometimes even years of unfavourable conditions such as freezing, starvation or dry out (Parand, 2001).

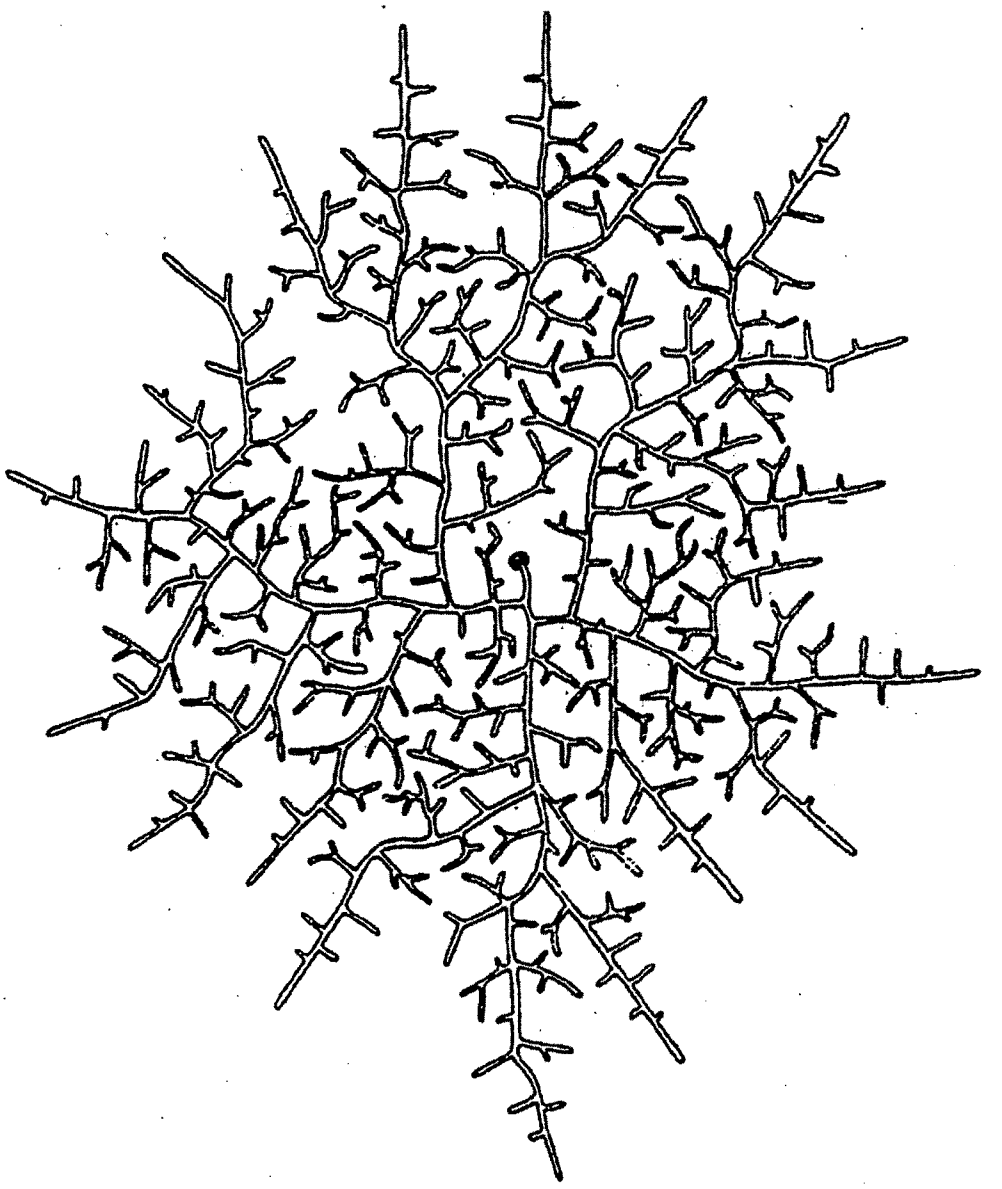


Figure 1.1: Young colony of fungi arising from a spore, note the large number of hyphal tips (Kendrick, 1992).

Fungi are the most important group of organisms contributing to wood decay (Pekka, 2000). Most wood inhabiting fungi are strictly saprotrophic and utilise dead wood as their food base. The saprophytic fungi, especially polypores, are extremely significant in their role as decomposers in global ecosystem and carbon recycling (Pekka, 2000). The main energy sources for fungal growth are wood cell wall polysaccharides although many wood inhabiting fungi and bacteria are also able to modify lignin (Kirk and Farrell, 1987).

Wood rot fungi:

As fungi are heterotrophic in nature, they rely solely on carbon fixed by the other organisms for metabolism and obtain their nutrition from the surrounding environment by extracellular digestion. They are essential for the well being of the most terrestrial ecosystems because they breakdown organic materials and recycle vital nutrients. Fungi have typical enzymes that break down a large variety of complex molecules into smaller organic compounds which allow them to get the needed carbon and energy required for their growth. This remarkable versatility of these enzymes contributes to fungi's ecological success. As decomposers; they play indispensable role in nutrient recycling, especially as saprotrophs and symbionts. They are also imperative in degrading organic matter to inorganic molecules, which can then re-enter anabolic or metabolic pathways in plants or other organisms.

Wood is one of the most abundant and renewable source of energy on earth; it is difficult to degrade wood because of the presence of highly intricate compounds. It is composed of many chemical components and carbohydrates, and each individual component of wood has received specific attention due to its commercial importance. Wood is mainly comprised of three polymeric constituents called lignocellulosics–lignin, cellulose, and hemicellulose. These components constitute about 95% of the dry matter of the wood, out of which cellulose and hemicellulose contribute about 70%, while lignin accounts for 20-30%, depending upon the species (Parand, 2001). Wood rot fungi are able to fragment lignin, cellulose, and hemicellulose, and to further metabolize the fragments (Kirk and Cullen, 1998). The strategy of many wood rot fungi is to exploit the retained wood relatively slowly, and their mycelia being characterized as slow growing, stress tolerant, combative and defensive (Rayner and Baddy, 1988; Holmer and Stenlid, 1997).

Decomposition of the wood is essential to the global nutrient cycle by three main reasons:

1. To digest lignin in order to gain access to energy rich cellulose: Wood contains only small amount of easily usable substrates like sugars and starch. From the three principal components of wood, cellulose (40-50% dry weight, composed of long, straight chains of β-1,4-linked glucose) and hemicellulose (25-40% dry weight, consisting of mixed polymers of glucose, mannose, xylose, arabinose, etc.) are comfortably available to the organisms while lignin (20-35% dry weight, a complex 3-dimensional polymer consisting of three types of phenyl-propane units) is highly resistant to enzymatic attack, and it encrusts the other wall materials or forms chemical complexes with them, which prevents them from getting degraded easily.
2. To release the enormous amount of carbon locked up in the wood of land plants.
3. Low nitrogen content: Nitrogen is an essential element for the growth of all living organisms. Wood typically has a very low content of nitrogen, about 0.03-0.10%, indicates that wood decay fungi have an efficient mechanism for nitrogen metabolism and reuse (Merrill and Cowling, 1966; Levi *et al.*, 1968; Highley, 1987). They are very efficient at both scavenging and re-cycling nitrogen, but some have additional strategies.

Fungi are the organisms that can completely decompose the most organic compounds including lignin, a major component of wood which is very difficult to break down or digest. However, the only organisms capable of mineralizing lignin efficiently are Basidiomycetous white rot fungi and related litter-decomposing fungi (Kirk and Cullen, 1998). Decomposition of wood by fungi is achieved by releasing extracellular enzymes that digest cellulose, hemicellulose and lignin.

Wood decay fungi are of increasing biotechnological interest, because their enzyme systems can detoxify pollutants and delignify agricultural wastes, thus leaving cellulose as a potentially cheap commercial substrate for the industrial fermentations. Fungi capable of selective delignification may have diverse uses, e.g. biopulping of wood in

the paper industry, bioremediation of chemical wastes, and improving the digestibility of lignocellulosic food sources for animal consumption (Eriksson and Kirk, 1985; Adaskavege *et al.*, 1995).

Depending on the type of decay, various physical, chemical and morphological changes occur in wood, which are characteristics to taxonomic group of fungi. These decay patterns have been well characterized and provide useful insights to elucidate deterioration in living trees and wood in service. On the basis of micro-morphological and chemical characteristics of decay produced by these fungi, wood degrading fungi are classified into:

a. Soft rot fungi

b. Brown rot fungi

c. White rot fungi

Soft rot fungi:

Soft rot fungi can degrade all three of the structural components of wood (lignin, cellulose and hemicellulose), although the major food sources are cellulose and hemicellulose. Fungi that cause soft rot have generally been attributed to Ascomycota and Deuteromycota fungi (Blanchette, 1992). They typically occur in wood of high water and nitrogen content. Soft rot in wood often appears brown and can be confused with decay caused by brown rot fungi.

Soft rot differs from other types of wood decay in their pattern of development, which involves T-branching or L-bending and hyphal tunnelling inside lignified cell walls. This distinctive mode of attack was described in the mid-19th century by Schacht (1863) and was elucidated by Savory (1954), who proposed the term soft rot. In addition to cavity formation, soft rot fungi frequently form discrete notches/erosion troughs of cell wall erosion. These notches/erosion troughs are very much similar to and indistinguishable from white rot. Therefore, they are categorised into two distinct soft rot types: type 1 and type 2 (Corbett, 1965; Hale and Eaton, 1985 a, b). Type 1 is characterized by erosion of the secondary wall

forming cavities within the S_2-layer of the plant cell wall. Morphology of these cavities varies depending on the fungus and the substrate (Nilsson, 1985), whereas type 2 is used to describe an erosion of the entire secondary wall. Moreover, the middle lamella is not degraded in type 2 (in contrast to cell wall erosion by white rot fungi), but may be modified in advanced stages of decay. Large strength losses in wood can be associated with soft rot attack (Schwarze and Fink, 1998). Cavities formed in the wood as well as extensive cellulose degradation can result in extremely poor strength. As decay progresses, extensive carbohydrate loss occurs and lignin concentrations increase in the residual wood. In advanced stage, wood become soft and when dry, wood is more brittle.

Brown rot fungi:

Brown rot is caused by members of Basidiomycota, which depolymerise cellulose and hemicellulose rapidly during incipient stages of wood colonization (Rayner and Boddy, 1988; Eriksson *et al.,* 1990; Green and Highley, 1997; Schwarze, 2007). Considerable losses in wood strength occur very early in the decay process, often before decay characteristics are visually evident. Brown rot fungi mainly decompose the cellulose and hemicellulose components in wood, but they can also modify the lignin to a limited extent (Rayner and Boddy, 1988; Eriksson *et al.,* 1990; Green and Highley, 1997; Schwarze, 2007). The chemical and structural analysis from the brown rotted wood suggests that, these fungi produce extracellular compounds which are able to penetrate deep into the wood cell wall structures and participate in degradation reactions (Anne-Christine, 1996). These compounds break the bonds between the fibril structures and depolymerise wood polysaccharides, causing only a limited weight loss at the initial stage of decay (Messner *et al.,* 1984; Highley *et al.,* 1985; Murmanis *et al.,* 1987; Enoki *et al.,* 1990). The initiators of both cellulose and lignin breakdown are suggested to be small molecular weight compounds that can readily diffused from the hyphae, penetrate into the wood cell and start decay (Evans *et al.,*1994; Wood, 1994; Goodell *et al.,*

1997; Shimada *et al.*, 1997). Brown rot fungi penetrate from the cell lumen where the hyphae are in close connection with the S_3 layer (Highley 1987; Viitanen and Ritschkoff, 1991). The hyphae of brown rot fungi grow within the cell lumina on the surface of the S_3 layer but cause little alteration in this layer (Schwarze *et al.*, 1998). In brown rot, the decay process is thought to affect the S_2 layer of the wood cell wall first, while the porosity of the S_3 layer remains relatively unchanged during the decay process or remains intact until the late phase of decay (Highley and Murmanis, 1985, 1987; Kuo *et al.*, 1988). However, the S_1 and S_2 layers become extensively degraded owing to the removal of the polysaccharides (Liese, 1970; Rayner and Boddy, 1988; Eriksson *et al.*, 1990).

Both cellulose and lignin decay mechanisms rely on radical formation, low pH and the production of organic acids. They cause increased alkali solubility of lignin, and the decay is enhanced by high oxygen tension, all of which indicate a crucial involvement of radicals, especially in the early stages of decay (Kirk, 1975; Jin *et al.*, 1990). The radicals formed by brown rot fungi can remove methoxyl groups from lignin and produce methanol, and thus they leave a residue that consists mainly of modified lignin (Eriksson *et al.*, 1990; Jin *et al.*, 1990). Demethoxylation of methoxyl groups and aromatic hydroxylation result in increased phenolic hydroxyl groups, which make lignin more reactive. Brown rot fungi are lacking oxidative phenoloxidase or peroxidase activities required for efficient lignin degradation (Pekka, 2000). However, they have developed an efficient mechanism for cellulose hydrolysis, which probably involves low molecular weight chelators, oxalates, and hydroxyl radicals produced by Fenton reaction (Goodell *et al.*, 1997). The hydroxyl radical is a strong oxidant being able to degrade cellulose, and may affect lignin substructures (Backa *et al.*, 1992; Gierer *et al.*, 1992). Usually, it has been assumed that all brown rot fungi use the same mechanism in wood decay, and that decay involving a Fenton type catalytic system producing hydroxyl radicals attack wood components (Hatakka, 2001). The rotted wood becomes brown in colour and rather than fibrous, both

longitudinal and transverse cracks appear, giving the wood a cubed appearance which break off readily into brown powder. Since large losses of wood strength result from brown rot, living trees with this decay can be hazardous and wood in service may fail. They have been much less investigated than white rot fungi in spite of their enormous economic importance in the destruction of wood (Hatakka, 2001).

White rot fungi:

Wood rotting Basidiomycetous fungi that cause white rot in wood are the most efficient lignin degraders in nature (Otjen and Blanchette, 1985; Kirk and Farrell, 1987; Eriksson *et al.,* 1990; Anagnost, 1998; Boudet, 2000; Luna *et al.,* 2004; Lagaert *et al.,* 2009; Koyani *et al.,* 2010) and they are perhaps nature's major agents for carbon recycling of lignified tissues. There is no any other organism feasibly which can mineralize fully lignified tissue (Kirk and Cullen, 1998). The white rot fungi are obligate aerobes, deriving their nourishment from the biological combustion of wood and associated materials using molecular oxygen as terminal electron acceptor (Kirk, 1998). Taxonomically, they are a group of heterogeneous higher fungi, characterized by their unique ability to depolymerise and mineralize lignin using a set of extracellular ligninolytic enzymes (Hatakka, 2001).

White rot fungi possess the unique ability to efficiently metabolize the large amount of lignin components of wood and leave the cellulose and hemicellulose less affected. In order to break down lignin, they require a co-metabolisable carbon source, which are obtained by the breakdown of cellulose and hemicellulose (Pekka, 2000). Typically, white rot appears as a spongy, stringy, or laminated structure in affected wood. They often cause bleaching of normal wood coloration and appear whiter than normal. Various strains of white rot fungi adapt different wood decay mechanisms and the amount of lignin: hemicellulose: cellulose decayed by them can also be different. Accordingly, two gross patterns of decay exhibited by the white rot fungi are: 1) Simultaneous decay, in which

degradation of cellulose, hemicellulose and lignin occur simultaneously. Many white rot fungi colonize cell lumina and cause cell wall erosion. This type of rot is referred to as non-selective or simultaneous rot (Blanchette, 1995); 2) Selective decay or preferential delignification, where species preferentially remove lignin from wood leaving pockets of white, degraded cells that consist entirely of cellulose (Blanchette, 1984b, 1995; Otjen and Blanchette, 1987).

There are also fungi that are able to produce both types of attack in the same wood (Blanchette, 1984a, b, 1991; Eriksson *et al.,* 1990). Degradation is usually localized to cells colonized by fungal hyphae and substantial amounts of undecayed wood remain even after an advanced decay has occurred. A progressive erosion of the cell wall occurs when components are degraded simultaneously or a diffuse attack of lignin may occur by species that preferentially remove lignin. Strength losses are not significant until late stages of decay. The fungi that decay the sapwood and dark/heartwood turning it white. The white rot fungi causing delignification are found to be very promising when applied to the pulp and paper industry.

In nature, white rot fungi are more commonly found on angiosperms than on gymnosperms wood (Gilbertson, 1980). Usually, syringyl (S) units of lignin are preferentially degraded, whereas guaiacyl (G) units are more resistant to degradation (Hatakka, 2001; Koyani *et al.,* 2010). Basic research on lignin degradation, e.g. its mechanisms, physiology, enzymology, and molecular biology, has been mainly carried out with the corticoid fungus *Phanerochaete chrysosporium* (Kirk and Farrell, 1987; Eriksson *et al.,* 1990; Gold and Alic, 1993). White rot fungi are the most active degraders of the complex aromatic plant polymer lignin to CO_2 (Buswell *et al.,* 1987; Kirk *et al.,* 1987; Boominathan, 1992).

1.2 Lignin:

Lignin (Latin: lignum = wood) is the most abundant and widely distributed renewable aromatic polymer on the terrestrial earth and the

second most abundant renewable biopolymer in nature, after cellulose (Boerjan *et al.*, 2003). About 27-30% of the dry materials in softwoods and between 20-26% in hardwoods consist of lignin. It is one of the major structural components of wood tissue which binds the wood fibres together and imparts the desired strength, rigidity and elasticity to the plant tissue to support the tree. Lignin is deposited as an encrusting and protecting material on the cellulose/hemicellulose matrix; it sets up a complex and acts as a kind of glue that cements the fibrous cell walls together (Steffen, 2003). Lignin is found in all vascular plants, a major fraction being distributed throughout the secondary walls of woody cells (Eriksson *et al.*, 1990). This macromolecule has a critical function of minimizing the water permeation across cell walls of xylem tissue, playing an intricate role in the transport of water and nutrients. Additionally, it hinders the degradation of wall polysaccharides, thus acting as a major line of defence against pathogens, insects and organisms (Wainhouse *et al.*, 1990; Boudet, 2000; Lagaert *et al.*, 2009). From all natural chemical compounds, lignin is found to be extremely recalcitrant due to its molecular architecture which cannot be degraded anaerobically. It is mineralised in an obligate aerobic oxidative process, and its degradation yields no net energy gain (Pointing, 2001), as it cannot be degraded as a sole source of carbon and energy (Field *et al.*, 1993). The physiological importance of lignin biodegradation is the destruction of the matrix it forms, so that the microorganism can gain better access to the real substrates: hemicellulose and cellulose (Field *et al.*, 1993; Canet *et al.*, 2001), from where it obtains energy.

1.3 Lignin structure:

Lignin is an amorphic, three dimensional, aromatic, optically inactive, and unique branched polymer. It is difficult to determine the molecular weight of lignins as they are highly polydisperse materials (Argyropoulosand, 1997). It is a natural product arising from an enzyme initiated dehydrogenative polymerization of a mixture of p-hydroxy-cinnamyl alcohols, despite the presence of numerous asymmetric carbons.

The dynamic structure of lignin is built up of dimethoxylated (syringyl, S), monomethoxylated (guaiacyl, G) and non-methoxylated (p-hydroxyphenyl, H) phenylpropanoid units, derived from the corresponding p-hydroxy-cinnamyl alcohols called the precursors of lignin or monolignols (Figure 1.2).

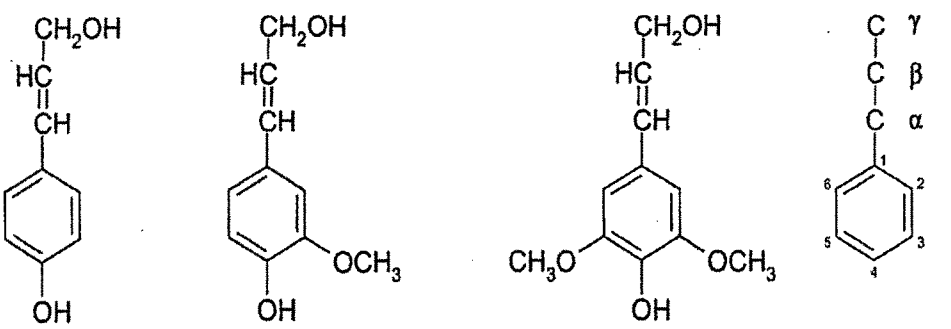


Figure 1.2: Precursors of lignin: From left to right: p-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol, and a model for the numeration of the carbon skeleton (Sjostrom, 1977).

In lignin, these phenyl propanoid precursors are linked together through a variety of bonds, e.g. aryl-ether, aryl-aryl, carbon-carbon bonds (Adler, 1977). Unlike other naturally occurring biopolymers, lignin does not contain identical, easily hydrolysable, repeating linkages that occur at regular intervals (Brown, 1985). In the final polymer, they form phydroxyphenyl, guaiacyl, and syringyl type units, respectively (Pekka, 2000). Structurally, lignin in gymnosperms (softwood lignin), angiosperms (hardwood lignin) and grasses have different monolignols compositions (Sjostrom, 1993; Pekka, 2000). Softwood lignin is referred to as guaiacyl lignin owing to the polymerisation of coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol) units, whereas hardwood lignin consists mainly of guaiacyl and sinapyl structures and grass lignin contains coumaryl structures in addition to the former two types (Martinez, 2005). Instead, lignin is a highly irregular, three dimensional polymers that have no precise chemical structure but have a series of substructures, which occur

at a random basis and they differ from species to species and from plant to plant (Kirk and Farrell, 1987). Ralph (2007) refined the softwood and hardwood lignin structure which is as follow:

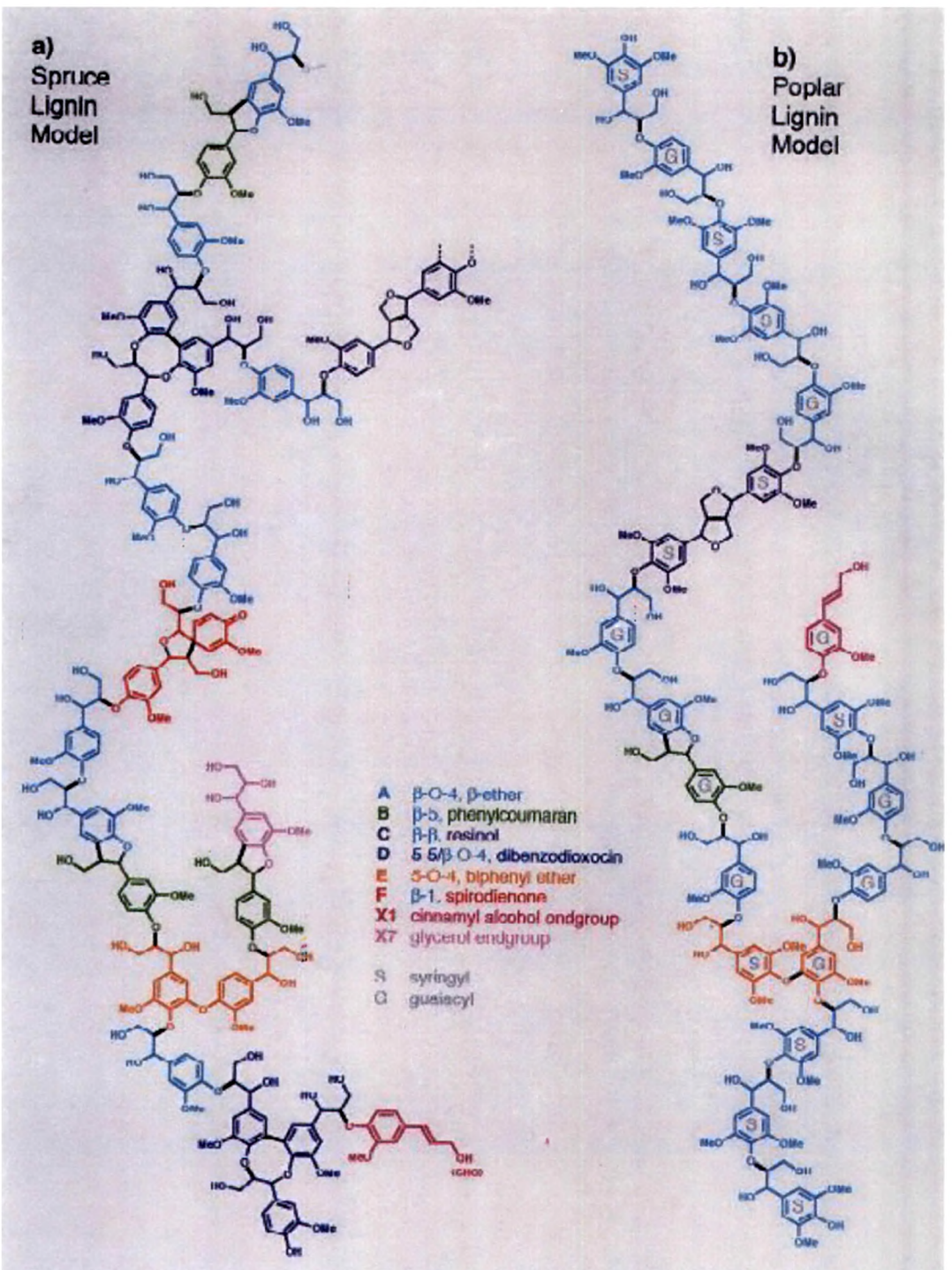


Figure1.3: Lignin polymer model for softwoods (Spruce) and hardwoods (Poplar) (Ralph, 2007).

A general response to fungal infection of plants is a formation of lignin and lignin related compounds, such as suberin, in the vicinity of the invading hyphae (Pekka, 2000). It is unclear; if the monomer composition of this newly formed defence lignin differs from the lignin in the native xylem tissue (Nicole *et al.,* 1992). Lignin contains chiral carbons in both L and D configurations reflected as irregular stereo irregularity of the polymer. The stereo irregularity of lignin makes it resistant to attack by enzymes and protects the easily degradable cellulose from attack by pathogens. Due to the unique structure, lignin is highly resistant and forms a barrier to microbial attack and degradation of wood. In general, only white rot fungi are considered to be efficient degraders of lignin (Kirk and Farrell, 1987; Griffin, 1994, Lonsdale *et al.*, 2008; Lundell *et al.*, 2010).

1.4 Lignin degradation by white rot fungi:

Lignin degradation by white rot fungi has been intensively studied during the last thirty years in relation to biotechnological applications such as biopulping, biobleaching, treating of pulp mill effluents, and soil bioremediation (Akhtar *et al.,* 1992, 1998; Lamar *et al.,* 1992; Messner and Srebotnik, 1994). Although white rot Basidiomycetous and related litter decomposing fungi are the most efficient degraders of lignin, mixed cultures of fungi, Actinomycetes, and bacteria can also mineralize lignin (Tuomela *et al.,* 2000; Hatakka, 2001; Steffen, 2003). Some mycorrhizal fungi were also shown to degrade lignin but the efficiency falls far behind than that of white rot fungi (Trojanowski *et al.,* 1984; Haselwandter *et al.,* 1990). The white rot fungi degrade wood by a simultaneous attack of lignin and cellulose/hemicellulose or selectively degrade far more lignin than polysaccharides (Eriksson *et al.,* 1990; Kuhad *et al.,* 1997). Overall lignin degradation by white rot fungi is believed to be a co-metabolic process requiring a carbon source other than lignin, e.g. parts of the cellulose/hemicellulose of wood are consumed. So far, no organism has

been found to use macromolecular lignin as a sole carbon source (Kirk and Farrell, 1987; Hatakka, 2001).

Lignin degradation generally occurs at the secondary growth phase of fungi, as in the early stages fungi deplete the nutrients. Although nitrogen (Keyser *et al.*, 1978), carbon or sulphur (Jeffries *et al.*, 1981) depletion triggers the production of ligninolytic enzymes, it's not a rule that the mechanism remains same every time (Pekka, 2000). When a greater number of fungal species were analysed, it has been found that a regulatory mechanism of lignin degradation was differed from species to species (Hatakka and Uusi-Rauva, 1983; Leatham and Kirk, 1983; Eriksson *et al.*, 1990; Hatakka, 1994).

During the process of lignin degradation, main challenges faced by the white rot fungi are:

- As lignin does not contain hydrolysable linkages and its structure is comprised of inter unit carbon-carbon and ether bonds, the degradative mechanism must be oxidative rather than hydrolytic (Kirk, 1998).

- As lignin polymer is stereo irregular, the ligninolytic enzymes must be less specific as compared to the hydrolytic enzymes involved in cellulose or hemicellulose degradation. This approach is extremely effective as a minimum amount of protein (enzyme) is synthesised by the organism to cleave the polymer (Evans and Hedger, 2001).

- As lignin is large heterogeneous polymer, it is impossible for any fungi to absorb lignin and degrade it by intracellular enzymes. Therefore, ligninolytic enzymes are extracellularly excreted by the degrading fungi that initiate oxidation of lignin in the extracellular environment (Mester and Tien, 2000).

Thus, white rot fungi have developed very non-specific mechanisms to degrade lignin extracellularly (Barr and Aust, 1994).

1.5 Enzymology of white rot fungi:

To meet the challenges of lignin degradation, white rot fungi produce extracellular peroxidases and phenoloxidases that act non-specifically via generation of free radicals, which are unstable and undergo a variety of spontaneous cleavage reactions (Pekka, 2000). Degradation of lignin can result in the formation of water soluble compounds and in mineralization, i.e. in the formation of CO_2 (Hatakka and Uusi-Rauva, 1983; Dorado *et al.,* 1999). Physiological conditions for lignin degradation, as well as secretion patterns of the ligninolytic enzymes, vary among different fungal species (Hatakka, 1994). Various authors have tried to establish correlations between ligninolytic enzymes and lignin degradation (Käärik, 1965; Ander and Eriksson, 1976, 1977; Eriksson *et al.,* 1990; Hatakka, 1994).

A breakthrough in the enzymology of lignin biodegradation occurred in 1983-84 when the first extracellular enzymes involved in the degradation of lignin were discovered (Glenn *et al.,* 1983; Tien and Kirk, 1983; Kuwahara *et al.,* 1984). However, many of the enzymes necessary for lignin degradation were not characterized before the beginning of the 1980s when virtually only laccase had been known (Hatakka, 2005). Since the discovery of two important peroxidases *viz.* Lignin peroxidases (LiPs) in 1983 and Manganese peroxidases (MnPs) in 1984, these nonspecific extracellular enzymes fascinated for their considerable industrial applications such as biological pulping of paper, fibre bleaching, and bioremediation of xenobiotic compounds such as poly-aeromatic hydrocarbons, certain textile dyes and other thousands of environmentally detrimental chemicals (Kirk and Farrell, 1987).

As mentioned earlier that lignin structure differs from species to species and from various sources (Kirk and Farrell, 1987), white rot fungi also produce different lignin degrading enzymes to cope up with the structural variation in lignin (Hatakka, 1994). The major fungal enzymes acting directly or indirectly on lignin are Lignin peroxidases (LiPs),

Manganese peroxidases (MnPs), and laccases (Kirk, 1998). There is evidence that all three enzymes can act with low molecular weight mediators to bring about lignin oxidation (Kirk and Cullen, 1998). Some white rot fungi produce all three enzymes, some only two, whereas a few fungi apparently produce only one depending on the species and the environmental conditions (Eriksson *et al.*, 1990; Orth *et al.*, 1993; Hatakka, 1994). Various ligninolytic fungi produce different combinations of these enzymes, but not all of these three major enzymes are needed to degrade lignin, suggesting that there is more than one ecologically successful strategy for the degradation of lignin (Hatakka, 1994, 2001).

Radical chemistry is an essential part of lignin biodegradation. Before the finding of lignin peroxidases, it was assumed that the generation of H_2O_2 and other easily diffusible activated oxygen species, such as hydroxyl radicals ($OH^{\bullet}$), superoxide anion radical ($O_2^{\bullet-}$), and singlet oxygen ($^1O_2$) might be responsible for fungal decay of lignin and lignocellulose (Hall, 1980). The involvement of hydroxyl radicals and singlet oxygen was discounted later (Kirk and Farrell, 1987). The key step in lignin degradation by laccase or the ligninolytic peroxidases (LiP and MnP) involves the formation of free radical intermediates, which are formed when one electron is removed or added to the ground state of a chemical (Reddy and Mathew, 2001). Such free radicals are highly reactive and rapidly give up or accept an electron from another chemical, which triggers oxidation or reduction of neighbouring compounds. These radicals can carry out a variety of reactions including benzylic alcohol oxidation, carbon-carbon bond cleavage, hydroxylation, phenol dimerization/ polymerisation and demethylation (Pointing, 2001).

The peroxidases use hydrogen peroxide generated by glyoxal oxidase, glucose oxidase, and cellobiose oxidase or by non-enzymatic systems via Fenton reaction (Pekka, 2000) to promote the oxidation of lignin to free radicals which later on undergo spontaneous reactions with oxygen or water, which leads to depolymerisation. The depolymerisation of lignin by nonspecific extracellular peroxidases is sometimes called

enzymatic combustion (Kirk *et al.*, 1987). Almost all white rot fungi produce MnPs and laccases, but only some fungi produce LiPs, the only enzyme capable of attacking nonphenolic lignin substructures.

Lignin peroxidase (LiP):

Lignin peroxidases are extracellular enzymes that oxidize methoxyl substituents on non-phenolic aromatic structures by abstracting 1-electron and generating cation radicals that undergo further chemical decomposition. They were first characterised by Tien and Kirk (1983) as "ligninases", thereafter another ligninolytic peroxidase was found that required manganese (Kuwahara *et al.*, 1984; Tien and Kirk, 1984). LiP operates via a typical catalytic cycle (Pekka, 2000). It is a heme-containing peroxidase with an unusually high redox potential, where hydroxyl radical ($OH^{\bullet}$) is reactive agent. This extracellular enzyme requires hydrogen peroxide as an oxidant. LiPs have molecular masses of approximately 40 kDa, and have acidic pH optima (Kirk, 1998). They contain a single ferric protoporphyrin IX heme moiety and operate via a typical peroxidase catalytic cycle (Figure 1.4). Thus, LiP is oxidized by H_2O_2 to a 2-electron deficient intermediate termed Compound-I, which returns to its resting state by performing two 1-electron oxidations of donor substrates: the 1-electron deficient intermediate is termed as Compound-II (Kirk, 1998). With excess H_2O_2, Compound-II can be transformed to Compound-III which is an inactive form of the enzyme (Tien, 1987; Gold *et al.*, 1989).

The key characteristic of LiP is, its substrates which are non-phenolic aromatic rings and have carbon-carbon bonds. Therefore, non-specificity of LiPs enables them to oxidize a variety of xenobiotic compounds that have some structural similarity to the lignin substructures. LiPs are more powerful oxidants than typical peroxidases and consequently, oxidize not only the usual peroxidase substrates-phenols and aromatic amines, but also a variety of other aromatic ethers and polycyclic aromatics with appropriate ionization potentials (Kersten *et al.*, 1990).

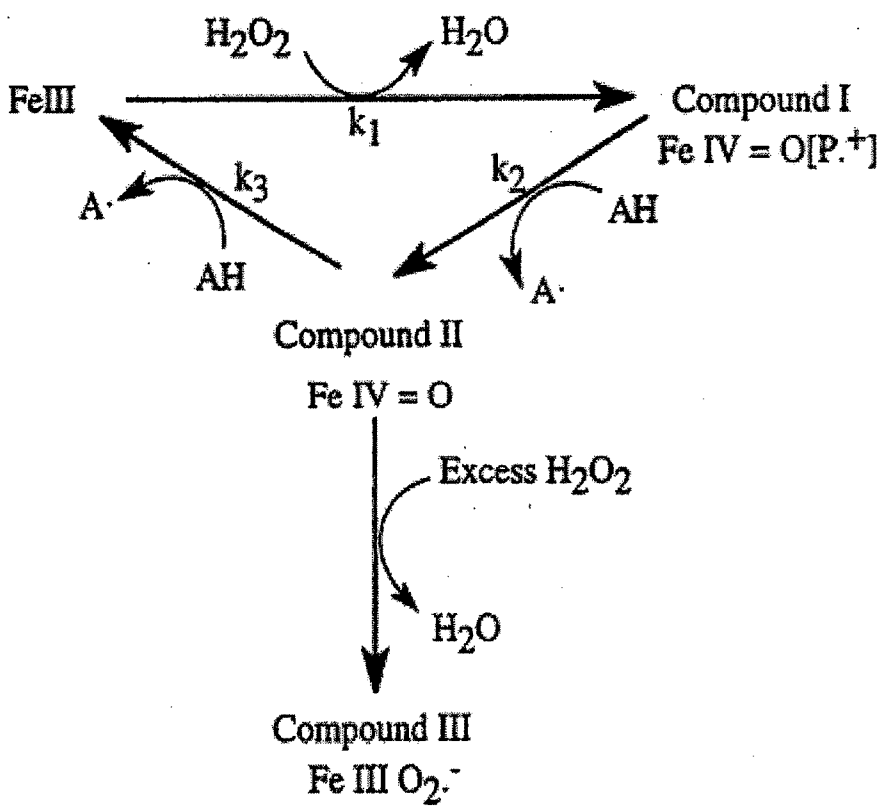


Figure 1.4: Catalytic cycle of lignin peroxidase (Gold *et al.*, 1989).

LiP-catalysed oxidation results in aryl cation radicals that can react as a radical and as a cation, forming a wide variety of degradation fragments (Higuchi, 1989). Although LiP has been regarded for a long time, is the central enzyme influencing delignification. However, recent studies with other fungi and natural woody substrates have indicated that other enzyme systems may be equal effective and efficiently utilised by white rot fungi in lignin biodegradation (Pekka, 2000; Valaskova and Baldrian, 2006; Kova *et al.*, 2009; Marco-Urrea *et al.*, 2009).

***Manganese Peroxidase (MnP)*:**

Manganese peroxidase (MnP), which is exclusively produced by some Basidiomycetes, was first discovered by Kuwahara *et al.*, (1984) and described by Glenn and Gold, (1985). MnP is the most common lignin

degrading peroxidase. Studies with different white rot fungi have shown that MnP appears to be more common than LiP (Orth *et al.*, 1993; Hatakka, 1994; Vares and Hatakka, 1997). MnP has an essential role in the depolymerisation of lignin (Wariishi *et al.*, 1991) and chlorolignin (Lackner *et al.*, 1991), as well as in the demethylation of lignin and bleaching of pulp (Paice *et al.*, 1993). Moreover, the enzyme mediates initial steps in the degradation of high molecular weight lignin (Perez and Jeffries, 1992). It has been shown that MnP in the presence of suitable organic acids is even able to mineralize lignin and lignin model compounds to considerable amounts (Hofrichter *et al.*, 1999). MnP is also a heme containing peroxidase where manganese ion (Mn^{2+}) is reactive agent. They are H_2O_2 dependent extracellular enzymes which act on the phenolic substrates. They are diffusible strong oxidants that can penetrate the substrate. MnPs are glycosylated as LiPs, but less versatile and slightly larger than LiPs. MnP as the name implies, requires manganese to complete its catalytic cycle and its main function appears to be oxidation of chelated Mn^{+2} and Mn^{+3} (Aitken and Irvine, 1990).

Manganese Peroxidases (MnP) have same conventional peroxidase catalytic cycle as LiP, but with utilization of Mn(II) as the substrate (Kirk, 1998). Compound-I of MnP (the intermediate produced by the 2-electron oxidation by H_2O_2) can oxidize phenolic substrates or Mn(II), but compound-II of MnP apparently is reduced only by Mn(II) (Wariishi *et al.*, 1988; Kirk and Cullen, 1998). The Mn(II) must be chelated by bidentate organic acid chelators such as glycolate or oxalate (Cui and Dolphin, 1990; Kishi *et al.*, 1994), which stabilize ion Mn(III)) and promote its release from the enzyme (Glenn and Gold, 1985; Glenn *et al.*, 1986; Wariishi *et al.*, 1988; Kishi *et al.*, 1994; Kirk, 1998). The resulting Mn(III) chelates are diffusible oxidants to promote lignin degradation. However, Mn(III) chelates are not strong oxidants, attack on phenolic structures and oxidize them rather than the non-phenolic units of lignin (Wariishi *et al.*, 1991; Tuor *et al.*, 1992; Kirk and Cullen, 1998).

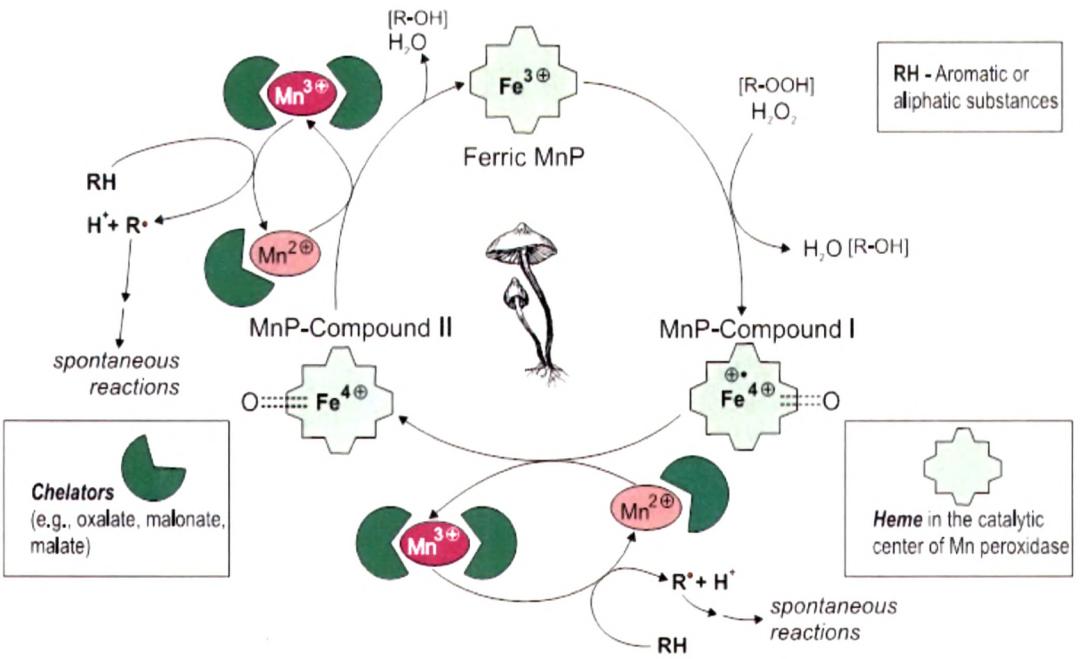
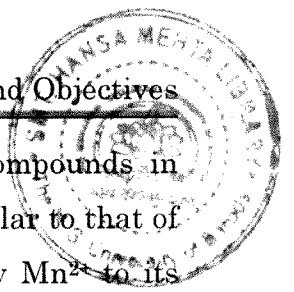


Figure 1.5: The catalytic cycle of manganese peroxidase (Wariishi *et al*,. 1988; Wariishi *et al*., 1992; Kuan *et al*., 1993; Kishi *et al*., 1994; Kirk and Cullen, 1998).

As shown in the catalytic cycle of MnP (Figure 1.5), it starts with the binding of H_2O_2 to the reactive ferric enzyme. Hydrogen peroxide is produced by the fungus using other enzymes (GLOX, AAO) or by MnP in the oxidation of glutathione (GSH), NADPH, and dihydroxy malic acid (Paszczynski *et al.,* 1985). Two electrons from heme are fulfilled to the cleavage of the oxygen-oxygen bond, forming the MnP compound-I. This activated state of the heme centre is able to form a radical complex and to remove an electron from the Mn^{2+} donor resulting in the formation of a highly reactive Mn^{3+} ion (Steffen, 2003). Therefore, formed MnP compound-II is also able to oxidize a Mn^{2+} ion (Kishi *et al.,* 1994). This step closes the cycle and the input of one H_2O_2 results in the formation of two H_2O and two Mn^{3+} (chelated; Wariishi *et al.,* 1992). This Mn^{3+} or chelated Mn^{3+} is in turn able to oxidize various monomeric and dimeric phenols, as well as carboxylic acids, thiols and unsaturated fatty acids forming radicals thereof (Hofrichter, 2002). Forrester *et al.,* (1988) even showed that



suitably chelated Mn^{3+} was able to oxidize lignin model compounds in absence of the enzyme. The catalytic cycle of MnP is very similar to that of LiP differing only in that compound-II is readily reduced by Mn^{2+} to its native form (Wariishi *et al.,* 1989).

The resulting phenoxy radicals can undergo a variety of reactions, which may result in polymer cleavage between the aromatic nuclei and Cα in a propane side chain (Wariishi *et al.,* 1991; Tuor *et al.,* 1992). LiPs have been able to oxidise chelated Mn(II) in the presence of H_2O_2 (Popp *et al.,* 1990; Khindaria *et al.,* 1995). The redox potential of the MnP-Mn system is lower than that of LiP and preferably oxidizes phenolic substrates (Vares, 1996). It seems unlikely that the MnP catalyzed reaction of phenolic units in lignin to phenoxy radicals can result in extensive depolymerisation. Yet white rot fungi that lack LiP but have MnP, have been shown to degrade non-phenolic lignin substructures (Jensen *et al.,* 1996).

Researchers have shown that the production of diffusible peroxyradicals by MnP can occur by another mechanism than via chelates of Mn(III), may affect lignin structure (Kirk, 1998). Moreover, MnP is able to liberate CO_2 directly from lignin substructures (Hofrichter *et al.,* 1999). MnP is one of the most common lignin degrading peroxidases produced by the majority of wood decaying fungi and by many litter decomposing fungi (Hofrichter, 2002). This extracellular enzyme is usually 40-50 kDa (max. 38-62 kDa) in mass (Hofrichter, 2002).

***Laccase*:**

Laccases are blue, copper containing glycosylated polyphenol oxidases that exist as monomers, homidimers or homotetromers (Solomon *et al.,* 1996). They are produced by higher plants and fungi, but it is also found in molds, black yeasts, and some bacteria (Bollag and Leonowicz, 1984; Thurston, 1994; Yaropolov *et al.,* 1994; Mayer and Staples, 2002; Claus, 2003). Fungal laccases have been studied over a hundred years.

The molecular mass for laccases of Basidiomycetes varies between 50 and 70 kDa which is usually smaller than that of plant laccases (Thurston, 1994; Yaropolov *et al.*, 1994) and the acidic pH ranges between 3-4 (Hatakka, 1994).

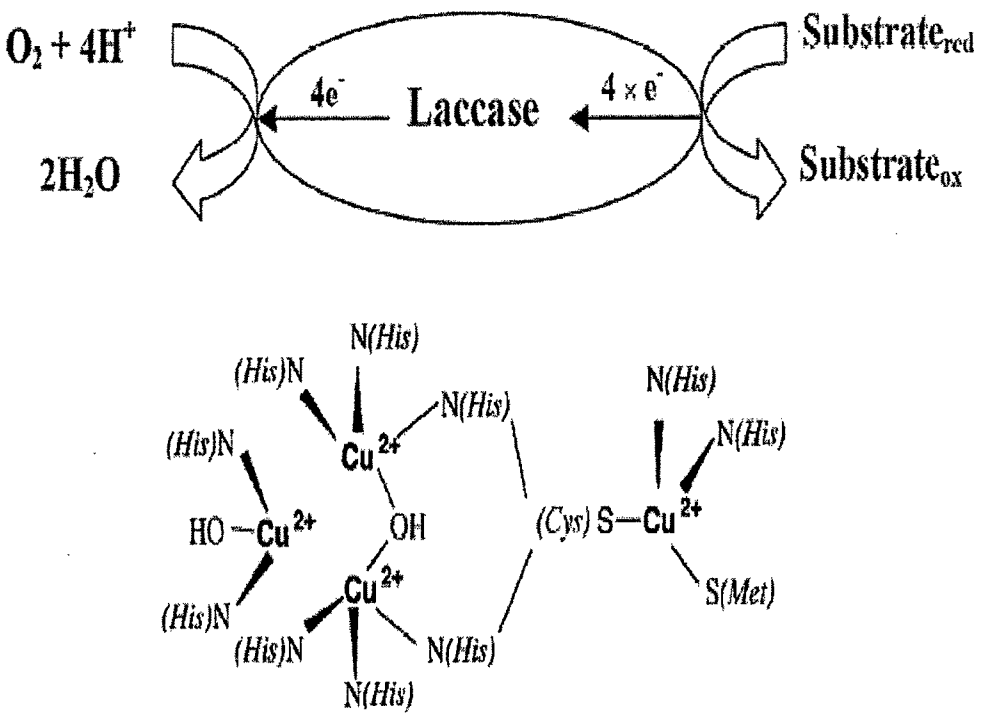


Figure 1.6: Schematic diagram of catalytic reaction of laccase and proposed active site structure (Woonsup, 2006).

Laccases catalyze the reduction of molecular oxygen to water by four consecutive 1-electron oxidation steps of a phenolic substrate (Figure 1.6), aromatic amines and other electron rich substrates, and returning the enzyme to its native state (Kirk, 1998). Like Mn(III) chelates, they oxidize the phenolic units in lignin to phenoxy radicals (Kawai *et al.*, 1988) and ultimately result into degradation of structures. Their active site is constituted by four copper atoms and they utilise molecular oxygen as an oxidant for the oxidation of varieties of phenols and other aromatic compounds to corresponding reactive quinines (Coll *et al.*, 1993; Ikehata *et*

al., 2004). Multi-copper oxidases catalyze the oxidation of wide range of phenols and other substrates with reduction of dioxygen to water.

Fungal laccases contribute to several processes such as lignin degradation, sporulation, pigment production, fruiting body formation and plant pathogenesis (Thurston, 1994; Mayer and Staples, 2002). Although the contribution of laccase to lignin degradation by white rot fungi had long been speculated, its role in ligninolysis was less clear than those of Lignin peroxidase (LiP) and Manganese peroxidase (MnP), partly because its low redox potential did not seem to be suitable for the oxidation of non-phenolic lignin structures (Ikehata *et al.*, 2004). However, in the presence of a mediator, laccases are capable of oxidation of non-phenolic compounds (Eggert *et al.*, 1996a). The interest in laccases for biotechnological applications increased with the discovery of their ability to oxidize high redox potential substrates in the presence of synthetic mediators (Bourbonnais and Paice, 1990), which allows the degradation of xenobiotic compounds (Rodriguez *et al.*, 2004a) and chlorine free bleaching of paper pulp (Camarero *et al.*, 2004).

These groups of enzymes produced by white rot fungi are highly versatile in nature and they find applications in a wide variety of industries. Ligninolytic enzymes are promising to replace the conventional chemical processes of several industries (Maciel *et al.*, 2010). The biotechnological significance of these enzymes has led to a drastic increase in the demand for these enzymes in the recent time (Table 1.1).

Food industry	
Laccase	Phenolic remotion from the food and beverage; Ascorbic acid determination; Sugar beet pectin gelation.
Lignin peroxidase	Source of natural aromatics; Production of vanillin.
Manganese peroxidase	Production of natural aromatic flavours.
Pulp and paper industry	
Laccase	Depolymerisation of lignin, Delignify wood pulps, Bleaching of kraft pulps.
Lignin peroxidase	Decolouriment of kraft pulp, Mill effluents.
Manganese peroxidase	Kraft pulp bleaching.
Textile industry	
Laccase	Textile dye degradation and bleaching.
Lignin peroxidase	
Manganese peroxidase	
Bioremediation	
Laccase	Biodegradation of xenobiotics, Polycyclic Aromatic Hydrocarbons (PAH) degradation.
Lignin peroxidase	Degradation of azo, heterocyclic, reactive and polymeric dyes; Mineralization of environment contaminants, Xenobiotic and pesticides degradation.
Manganese peroxidase	PAH's degradation, Synthetic dyes, Bleach from paper producing plants, Dichloro Diphenyl Trichloro-ethane (DDT), Poly Chlorinated Bi-phenyl (PCB), Tri Nitro Toluene (TNT).
Nanotechnology, Medical and Pharmaceutical and Cosmetics	
Laccase	Polymers production; Coupling of phenols and steroids; Medical agents; Carbon-nitrogen bonds construction; Complex natural products synthesis; Personal hygienic products; Biosensors and bio reporters.
Lignin peroxidase	Functional compounds synthesis, Bioelectro catalytic activity at atomic resolution, Cosmetics and dermatological for skin.
Manganese peroxidase	Acrylamide polymerization, Polymer styrene degradation, Direct electron transfer (DET).

Table 1.1: Enzymes applications in different sectors (Maciel *et al.*, 2010).

1.6 Bioremediation:

Bioremediation is a combination of two words – "Bio" and "Remediate". It can be defined as any process that uses microorganisms or their enzymes to return the environment altered by contaminants to its original condition. Bioremediation is the ability of certain microorganisms, heterotrophic bacteria and fungi, to degrade hazardous organic materials to innocuous compounds such as carbon dioxide, methane, water, inorganic salts, and biomass (Anderson, 1995). As an ecological point of view, bioremediation is the use of microorganisms to degrade, sequester, or remove environmental contaminants (Milton and Nagabhushanam, 2005).

Bioremediation is potentially an environmentally safe, biologically effective, and low cost method to clean up xenobiotics from the environment. The goal of bioremediation is to reduce pollutant levels at least to undetectable, non-toxic or acceptable levels, i.e. within limits set by regulatory agencies (Pointing, 2001) or ideally completely mineralize the organopollutants to carbon dioxide. From an environmental point of view, the total mineralization is desirable as it represents complete detoxification (Gan and Koskinen, 1998). According to Alexander (1981), mineralization is nothing but the complete biologically mediated break down of organic compounds into water, CO_2 and other inorganic residue, which is also termed as biodegradation. However, bioremediation also provides a technique for discarding pollutants by enhancing the same biodegradation processes that occur in nature. Microorganisms use an organic substance as a source of carbon and energy which ultimately results into breakdown or decay of the material. Bioremediation involves detoxifying hazardous substances instead of merely transferring them from one medium to another. It is an urgent need of our planet for protection and restoration from toxic contaminants (Milton and Nagabhushanam, 2005). This process is essential to recycle the waste, so that the elements in them can be used again. Nature has mechanisms for

self renewal and this existing mechanism of bioremediation plays the vital biotechnological role to eradicate environmental contaminations.

Many natural processes like erosion, oil seepage, volcanic eruptions, photochemical conversions etc., introduce many xenobiotic compounds into the environment. In addition, human actions also enhance the level of xenobiotic compounds in the environment by introducing several synthetic xenobiotics. In some cases these releases are deliberate and well regulated (e.g. industrial emissions) while in other cases they are accidental (e.g. chemical or oil spills). Enormous quantities of organic and inorganic compounds are added to the environment each year. Many of these compounds being highly persistence in nature lead to removal of various elements from the biogeochemical cycles. Generally, these chemicals have unusual chemical to physical properties which make them refractory to the biodegradation. Many of these xenobiotic compounds are toxic in nature. The increasing concentration of these toxic compounds in the organisms and biomagnifications along the food chains, finally affect the human beings. With increasing environmental and ecological concern, degradation of these xenobiotics has become an area of intense research.

Many physical, chemical and biological processes have been and are being tried for removal of xenobiotics. Removal of the pollutants from the environment through physical and chemical methods is much more expensive than the equivalent bioremediation process. In addition, bioremediation treatment often does not require heating, it requires relatively inexpensive inputs, such as nutrients, and usually does not generate residuals requiring additional treatment or disposal. Perhaps the most attractive feature of bioremediation is the reduced impact on the natural ecosystems, which should be more acceptable to the public (Zhang and chiao, 2002). Extensive biodegradative capabilities of microorganisms using biological approach have been flourishing in many cases. The complexity of microbial mechanisms for degradation of organopollutants as well as the time period before microbial degradation starts, requiring weeks to months, has made the technology slow to emerge as a viable

method of remediation (Nerud *et al.,* 2003). However, for effective bioremediation, the pollutant must be amenable to metabolic transformation, products must be safe to ecological world, environmental conditions should be favourable for microbial activity and the process should be cost effective and ecofriendly. It becomes apparent that more detailed studies of the principles of biodegradation and the development of efficient methods of decontamination are needed to solve the hazardous waste problem (Nerud *et al.,* 2003). However, there are number of advantages of bioremediation, which may be employed in areas which cannot be reached easily without excavation. Bioremediation, although promising, is not effective for all chemicals and hence, combined chemical, biological and physical techniques will be required for complete solution to the problem of persistent chemicals. It is very clear that development of remediation technologies is lagging far behind the rate and types of xenobiotic introduction into environment.

1.7 Xenobiotic compounds:

Xenobiotic means 'foreign to life', it is a term applied to many recalcitrant organic chemicals. These compounds are generally the unnaturally produced chemicals and are human made chemicals which are present in the environment at high or low concentrations.

The rapid expansion and increasing sophistication of the chemical industries in the past century and particularly over the last thirty years has increased the amount and complexity of xenobiotic compounds in nature. The list of xenobiotic compounds is very long and some of them are directly applied to nature in the form of pesticides or fertilizers, some others are released as industrial waste products (effluents). The xenobiotics would also include a wide variety of dumping such as plastics, detergents and oil spills, either inadvertent or deliberate. Some very much common xenobiotic compounds include:

Heavy Metals like Arsenic, Cadmium, Chromium, and Lead in industry or in households, Mercury, Zinc etc., are generally found in very trace

amount in waste effluent but are very much toxic. Environmental contamination with the pesticides (like DDT, Aldrin, Chlordane, Endosulfan, Endrin, Heptachlor, Diazinon etc.) can lead to pollution of surface water and groundwater.

Poly Chlorinated Biphenyls (PCBs) are organic chemicals that formerly had widespread use in electrical transformers and hydraulic equipments. This class of chemicals is extremely persistent in the environment and has been proven to bio-concentrate in the food chain, thereby leading to environmental and human health concerns.

Polycyclic Aromatic Hydrocarbons (PAHs) are ubiquitous environmental pollutants derived from various manmade and natural resources (Wilson and Jones, 1993; Kastner, 2000). They consist of two or more fused benzene rings in linear, angular, or cluster arrangements (Blumer, 1976). By definition, they contain only carbon and hydrogen, although in a broader sense heterocyclic PAH containing N, S and O atoms are also considered to be PAHs (Kastner, 2000). Polycyclic Aromatic Hydrocarbons include a family of organic pollutants such as: Naphthalene, Fluorene, Anthracene, Phenanthrene, Pyrene, Chrysene, Benzo (a) pyrene etc. Beside PAH, another group of pollutants is synthetic dyes which have created ground water pollution problems in the areas where such industries are located.

Dyes:

Historically, natural dyes were used for painting and dyeing of the surroundings, their skins and their clothes until the mid of 19th century. In 1856, the English chemist W.H. Perkin, in the course of attempts to synthesize quinine, discovered and patented a black-bluish substance with excellent dyeing properties, which later on became known as mauveine, mauve, aniline purple and Tyrian purple. This pioneering synthesis of mauveine by W. H. Perkins started the era of synthetic dyes. In the following years, many other dyes were introduced but the process was strongly stimulated by Kékulé's discovery of the molecular structure of

benzene in 1865 and further research followed a less empirical and more systematic approach. In the beginning of the 20th century, the synthetic dyes had almost completely supplanted the natural dyes (Welham, 2000).

Dye classification:

All the aromatic compounds possess the ability of absorbing electromagnetic energy or radiation and differ in the specific wavelength absorbance. The molecules having the ability of absorbing light with wavelengths in the visible range (~350-800 nm) are coloured. The dyes are molecules with delocalized electron systems with conjugated double bonds that contain two groups: the chromophore and the auxochrome. The chromophore is a delocalised electron system with conjugated double bonds. The most important chromophores are -C=C-, -C=N-, -C=O, -N=N-, $-NO_2$ and -NO groups. The auxochrome is an electron withdrawing or electron donating substituents that cause or intensify the colour of the chromophore by altering the overall energy of the electron system. The most important auxochromes are - NH_2, $-NR_2$, -NHR, -COOH, $-SO_3H$, -OH and $-OCH_3$ groups (De Las Marias, 1976; Van Der Zee, 2002). Based on chemical structure or chromophore, 20–30 different groups of dyes can be discerned. Azo (monoazo, disazo, triazo, polyazo), anthraquinone, phthalocyanine and triarylmethane dyes are quantitatively the most important groups. According to colour index (C.I.), vast array of commercial dyes are classified on the basis of colour, structure and application method. This colour index is edited or revised every three months since 1924 by the "Society of Dyers and Colourists" and the "American Association of Textile Chemists and Colourists". Each dye is assigned to a C.I. generic name determined by its application and colour. Various attractive forces or bonds like Van der Waals, hydrogen, ionic or covalent are established between the dyes with fibres by increasing relative strength of the bond (Ingamells, 1993; Guaratini and Zanoni, 2000; Rocha, 2001). According to the application categories dyes can be classified as seen in Table 1.2.

APPLICATION CLASSES	CHARACTERISTICS
Acid dyes	▪ Highly water-soluble due to the presence of sulphonic acid groups. ▪ Form ionic interactions between the protonated functionalities of the fibres ($-NH^{3+}$) and the negative charge of the dyes. ▪ It also forms Van der Waals, dipolar and hydrogen bonds. ▪ The most common structures are azo, anthraquinone and triarylmethane.
Reactive dyes	▪ Form covalent bonds with -OH, -NH or -SH groups in cotton, wool, silk and nylon. ▪ The problem of coloured effluents associated to the use of these dyes is due to the hydrolysis of the reactive groups that occurs during the dyeing process. ▪ The most common structures are azo, metal complex azo, anthraquinone and phthalocyanine.
Direct dyes	▪ Their flat shape and length enables them to bind along-side cellulose fibres and maximize the Van-der-Waals, dipole and hydrogen bonds. ▪ Only 30% of the 1600 structures are still in production due to their lack of fastness during washing. ▪ The most common structures are almost always sulphonated azo dyes.
Basic dyes	▪ Basic dyes work very well on acrylics due to the strong ionic interaction between dye functional groups such as $-NR^{3+}$ or $=NR^{2+}$ and the negative charges in the copolymer. ▪ The most common structures are azo, diarylmethane, triarylmethane and anthraquinone.

Mordant dyes	▪ Mordants are usually metal salts such as sodium or potassium dichromate. ▪ They act as "fixing agent" to improve the color fastness. ▪ They are used with wool, leather, silk and modified cellulose fibres. ▪ The most common structures are azo, oxazine or triarylmethane.
Disperse dyes	▪ Non-ionic structure, with polar functionality like - NO_2 and –CN that improve water solubility, Van-der-Waals forces, dipole forces and the colour. ▪ They are usually used with polyester. ▪ The most common structures are azo, nitro, anthraquinones or metal complex azo.
Pigment dyes	▪ These insoluble, non-ionic compounds or salts, representing 25% of all commercial dye names, retain their crystalline or particulate structure throughout their application. ▪ The most common structures are azo or metal complex phthalocyanines.
Vat dyes	▪ Vat dyes are insoluble in water, but may become solubilized by alkali reduction (sodium dithionite in the presence of sodium hydroxide). ▪ The produced leuco form is absorbed by the cellulose (Van-der-Waals forces) and can be oxidized back, usually with hydrogen peroxide, to its insoluble form. The most common structures are anthraquinones or indigoids.
Ingrain dyes	▪ The term ingrain is applicable to all dyes formed in situ, in or on the substrate by the development, or coupling, of one or more intermediate compounds and a diazotized aromatic amine.

	▪ In the Colour Index the sub-section designated Ingrain is limited to tetra-azaporphin derivatives or precursors.
Sulphur dyes	▪ Sulphur dyes are complex polymeric aromatics with heterocyclic sulphur containing rings representing about 15% of the global dye production. ▪ Dyeing with sulphur dyes (mainly on cellulose fibres) involves reduction and oxidation processes, comparable to vat dyeing.
Solvent dyes	▪ Non-ionic dyes that are used for dyeing substrates in which they can dissolve as plastics, varnish, ink and waxes. They are not often used for textile processing. ▪ The most common structures are diazo compounds that undergo some molecular rearrangement, triarylmethane, anthraquinone and phthalo-cyanine.
Other dye classes	▪ Food dyes are not used as textile dyes. Natural dyes use in textile processing operations is very limited. ▪ Fluorescent brighteners mask the yellowish tint of natural fibres by absorbing ultraviolet light and weakly emitting blue light. ▪ Not listed in a separate class in the Colour Index. Many metal complex dyes can be found (generally chromium, copper, cobalt or nickel). ▪ The metal complex dyes are generally azo compounds.

Table 1.2: Color Index application classes (Christie, 2001).

Azo dyes:

Azo dyes are the largest group of synthetic dyes and pigments with industrial application due to their relatively simple synthesis and almost unlimited number and types of substituents (McCurdy, 1991). The worldwide production of these organic dyes is currently estimated about

4,50,000 tons/year, with almost 50,000 tons/year lost in effluent during application and manufacture (Lewis, 1999).

Azo dyes contain at least one N=N double bond and make many different structures possible. Monoazo dyes have only one N=N double bond, while diazo, triazo and polyazo dyes contain two, three or more N=N double bonds, respectively. The azo groups are generally connected to benzene and naphthalene rings, but can also be attached to aromatic heterocyclic or enolizable aliphatic groups (Zollinger, 2003). The general structure of the azo dye molecule can be seen in Figure 1.7.

$$Ar — N = N — R$$

Figure 1.7: General structure of azo dyes (where R can be an aryl, heteroaryl or - CH = C (OH) - alkyl derivative).

The lateral groups present are responsible for the different shades and intensities of colour. The colour of the dye depends on its chemical structure while different shades of the dye rely on the physical properties of it. Azo dyes range in shade from greenish yellow to orange, red, violet and brown. However, the important disadvantage, limiting their commercial application, is that most of them are red and none are pure green (Øllgaard *et al.*, 1999).

Synthesis of most azo dyes takes place by coupling of diazonium salts with aromatic amines, phenols, naphthols or aliphatic enols in the *para* position in respect to the amino or hydroxyl group (Zollinger, 2003). These diazonium salts are formed by diazotization of a primary aromatic amines.

Dyes and environmental concern:

There are thousands of dyes manufactured and used in number of industries. Textile, pharmaceutical, printing, food, cosmetic, leather and paper industries are the main users of the dyes and they produce a huge amount of effluent which is usually resistant to biological treatment. The

abandoned enhancement of the dyes in the environment causes the severe problem to the human lives. Some of the dyes are non-toxic, but some of them are converted into toxic or carcinogenic compounds when released into the environment. Due to the fact that the dyes are synthesized to be chemically and photolytically stable, they are highly visible (some can be detected in concentration < 1 mg/l) and persistent in natural environments (Nigam *et al.*, 2000; Rieger *et al.*, 2002). In fact they are xenobiotic compounds because they do not exist as natural products and therefore contain structural elements that cannot be synthesized biochemically (Stolz, 2001; Rieger *et al.*, 2002). Consequently, the release of potentially hazardous dyes in the environment can be an ecotoxic risk and can affect man through the food chain (Van der Zee, 2002). There are many different methods for the treatment of dye wastes and their removal. Usually, treatment with any of the single techniques would not be sufficient for complete degradation because each technique has its own limitation. Dye removal strategies consist therefore, mostly of a combination of different techniques (Van der Zee, 2002). The treatment techniques broadly fall into three categories: Physical, Chemical and Biological.

Physical techniques:

- Membrane filtrations
- Ion exchange
- Coagulation and flocculation
- Sorption
- Ultrasound
- Precipitation
- Flotation
- Ion pair extraction
- Ultrasonic mineralization

Chemical techniques:

- Chlorination
- Bleaching

- Electrolysis
- Ozonation
- Fenton's reagents
- Photochemical
- Sodium hypochlorite (NaOCl)
- Wet air oxidation (WAO)

Biological techniques:

- Bacterial
- Fungal
- Yeasts
- Algae
- Plants

Biological and chemical methods involve the destruction of the dye molecule, while physical methods usually transfer the pollutant to another phase (Ramalho, 2005). Major disadvantage of the physico-chemical technique is its high cost, low efficiency and limited versatility. It has been suggested that enhanced microbial decolourisation may provide a less expensive and more environmentally acceptable alternative to chemical treatment (Selvam *et al.*, 2003). Biological treatment involves either aerobic or anaerobic degradation. Aerobic treatment is ineffective for the degradation of xenobiotic compounds like dyes, but the anaerobic treatment is cost effective and includes enzymatic action.

1.8 Fungi in bioremediation:

According to the principle of microbial infallibility proposed by Alexander (1965), microorganisms have the capability of degrading all naturally occurring compounds. Microorganisms also have the ability to degrade many of the xenobiotic compounds and some of the compounds are still found resistant to this biological degradation. Actual bioremediation is attained only when the degradative product is mineralized completely and reutilised in nature.

Several Basidiomycetes fungi have the characteristic of degrading lignocellulose and use this inexpensive and abundant material as a nutrient source. Metabolization of lignin and its components by fungi is one of the credible evolutions for the degradation of aromatic xenobiotics and/or environmental pollutants. The ability of co-oxidization and transformation of various hazardous chemicals by fungi has aroused interest in using them in bioremediation. Extracellular enzymes produced by the white rot fungi are the key components of the lignin degrading systems, which facilitate degradation of undegradable chemicals such as lignin or an extremely diverse range of very persistent or toxic environmental pollutants (Barr and Aust, 1994). However, not all fungi produce all these enzymes simultaneously, and it has often been difficult to find a distinct correlation between the pollutant degradation and production of enzymes even if the enzymes have been detected (Nerud *et al.*, 2003).

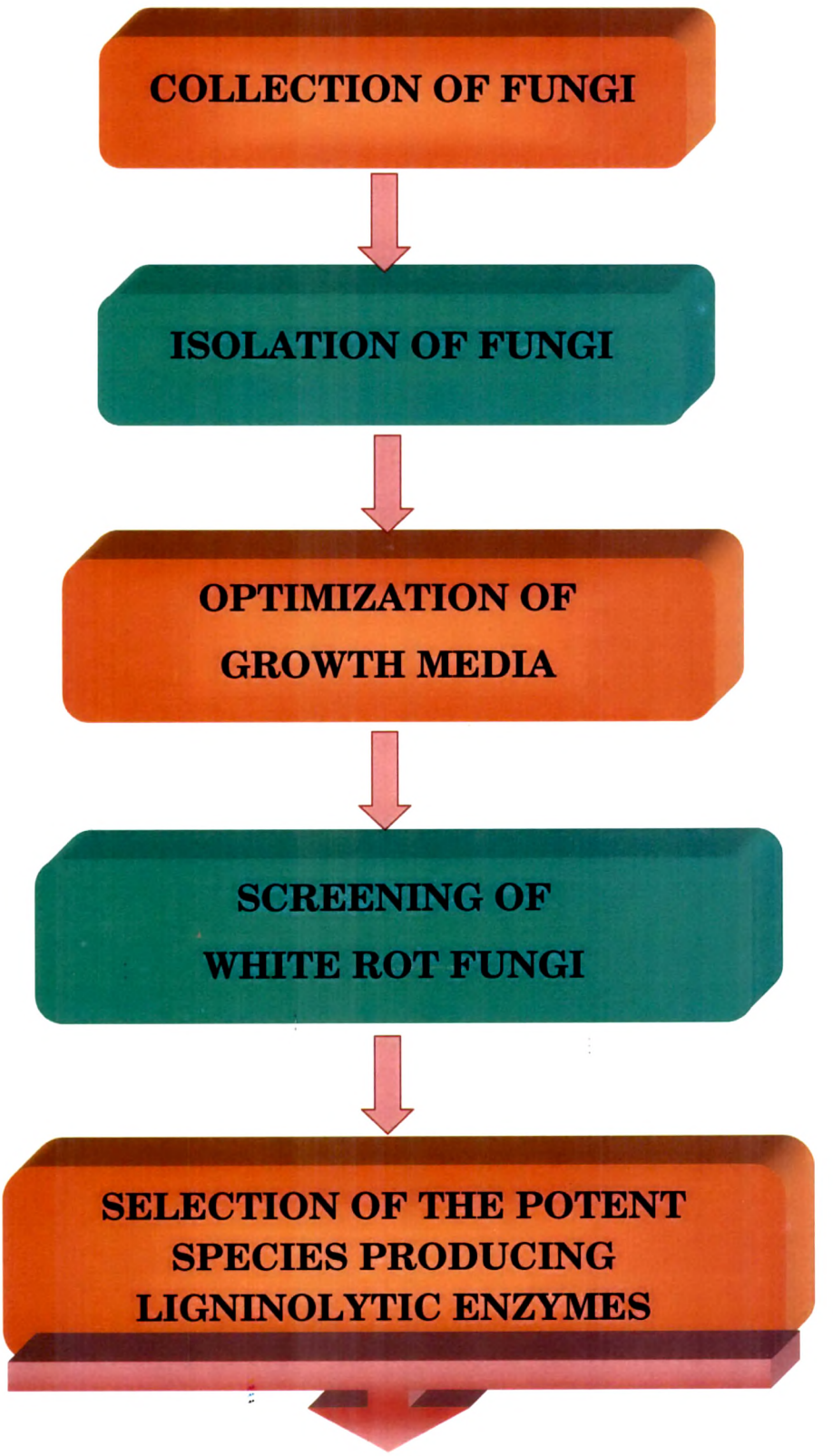
White rot fungi can metabolize compounds including clorophenols, polycyclic aromatic hydrocarbons (PAH), chloroanilines and pesticides such as methoxychlor and DDT (Hammel, 1992). The xenobiotic oxidations of white rot fungi are not rapid or efficient, but they are very nonspecific (Haglund, 1999). The ability of white rot fungi to degrade an exceptionally diverse group of very resistant or toxic environmental pollutants has raised the interest in using white rot fungi in bioremediation research (Aust and Barr, 1994; Moreira *et al.*, 2000; Yesilada *et al.*, 2003; Munari *et al.*, 2008). Thus white rot fungi and their enzymes are plausible source not only for some industrial processes like biopulping and biobleaching, but also in bioremediation of wide range of xenobiotics compounds.

Perusal of literature and information provided in the above discussion indicates that white rot Basidiomycetes play pivotal role in timber damage by degrading highly complex natural molecule like lignin. Though, every year 20-80% of the world timber is destroyed by white rot fungi, the enzymes produced by these fungal species not only have potential biotechnological application in various industrial products but

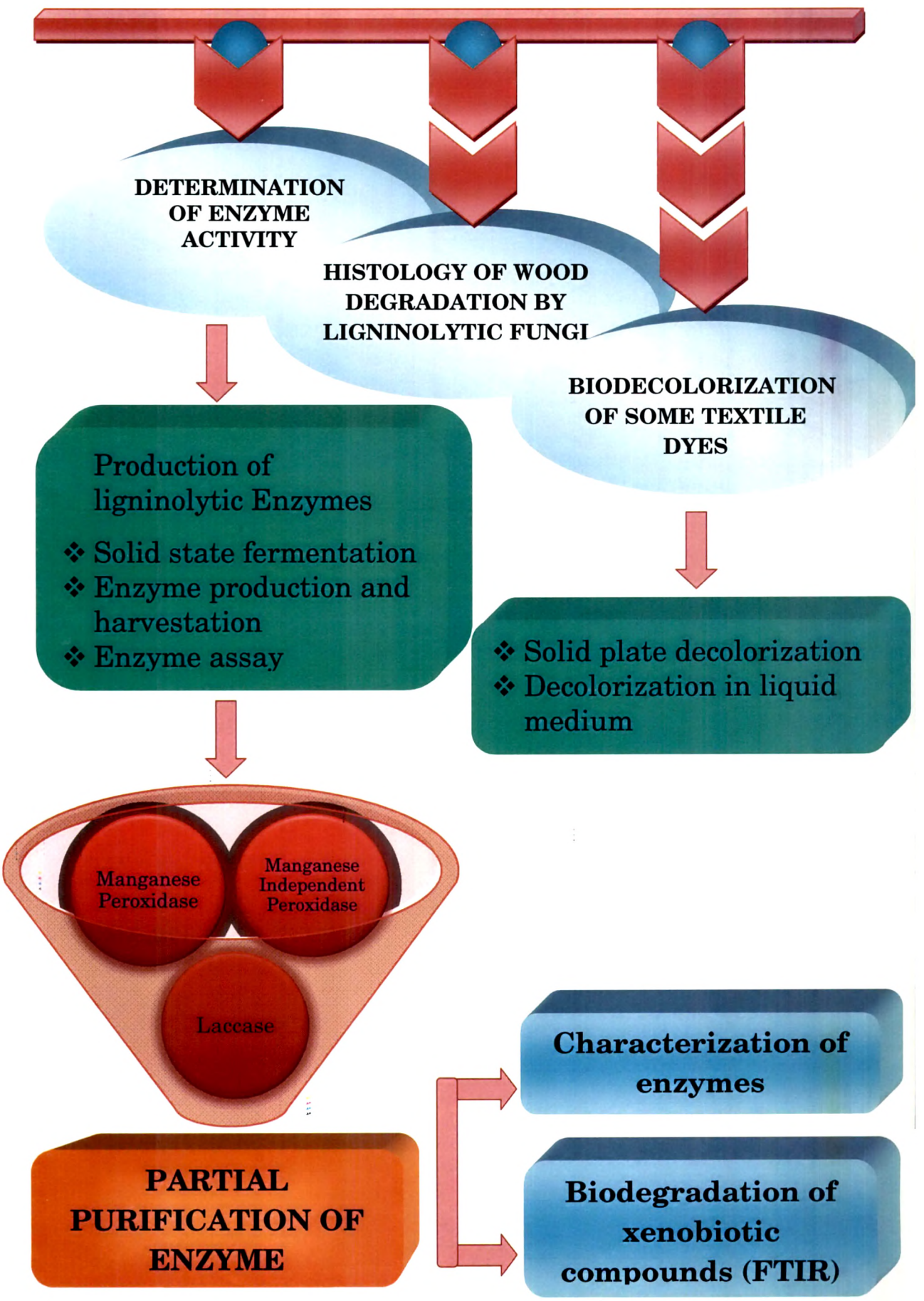
also play crucial role in bioremediation of non-degradable and xenobiotic compounds. Therefore, main objectives of the present study are as follows:

OBJECTIVES

- Isolation, purification and characterization of rot fungi.
- Optimisation of growth media.
- Optimisation of its degrading ability.
- Screening of pure cultures for the production of various lignocellulosic enzymes.
- Production of enzymes by Solid State Fermentation.
- Isolation, purification (complete/partial) and characterization of different enzymes.
- To study enzyme kinetics (effect of temperature, pH, substrate concentration and inhibition studies etc.).
- Molecular characterization of enzymes through electrophoresis.
- *In vitro* testing of isolated fungi for wood decay.



Schematic outline of the experimental work of the thesis.


DETERMINATION OF ENZYME ACTIVITY
HISTOLOGY OF WOOD DEGRADATION BY LIGNINOLYTIC FUNGI
BIODECOLORIZATION OF SOME TEXTILE DYES
Production of ligninolytic Enzymes
❖ Solid state fermentation
❖ Enzyme production and harvestation
❖ Enzyme assay
❖ Solid plate decolorization
❖ Decolorization in liquid medium
Manganese Peroxidase
Manganese Independent Peroxidase
Laccase
PARTIAL PURIFICATION OF ENZYME
Characterization of enzymes
Biodegradation of xenobiotic compounds (FTIR)