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RESEARCH ARTICLE

Bioremediation of phenolic compounds by higher fungi - A review.

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Abstract

The white-rot fungi produce an unusual enzyme system, characterized by a specialized group of peroxidases, that catalyzes the degradation of the complex plant polymer lignin. This ligninolytic system shows a high degree of nonspecificity and oxidizes a very large variety of compounds in addition to lignin. Among these compounds are numerous environmental pollutants. Thus, the white-rot fungi show considerable promise as bioremediation agents for use in the restoration of environments contaminated by xenobiotic molecules. The ligninolytic enzymes of white-rot fungi have a broad substrate specificity and have been implicated in the transformation and mineralization of organopollutants with structural similarities to lignin.

Introduction:-

Bioremediation as defined by the American Academy of Microbiology is "the use of living organisms to reduce or eliminate environmental hazards resulting from accumulations of toxic chemicals or other hazardous wastes" (Gibson and Sayler, 1992). Bioremediation uses living organisms to clean up contaminated soil or water. Despite its broad definition, bioremediation usually refers specifically to the use of microorganisms. Bioremediation is a combination of two words – bio, short for biological, and remediation, which means to remedy. The use of fungi to clean up the environment, known as mycoremediation, is also considered a type of bioremediation. Bioremediation is not a new technology. This is evident in that humankind has practiced composting, sewage treatment and fermentation since the beginning of recorded history. All of these processes utilize microbial processes in a degradation process. The modern use of bioremediation began with the opening of the first biological sewage treatment plant in Sussex, UK, in 1891. The use of this technology in cleaning up pollutant spills is gaining popularity. Over the past ten years an increase in the types of contaminants to which bioremediation is applied has been evident.

Biological treatment technologies for the remediation of soils and groundwater contaminated with organopollutants are widely used for their environmentally friendly impact combined with low cost compared to other treatment alternatives (Sasek et al., 2003). An alternative to the biostimulation of indigenous microflora, the so-called practice of bioaugmentation, can favor contaminant degradation when dealing with historically and heavily contaminated sites (Vogel, 1996). Sites contaminated by recalcitrant organic compounds have often been shown to be characterized by the concomitant presence of heavy metals (Bouchez et al., 2000). In such a difficult case, the use of filamentous fungi (WRF, in particular) may give some advantages over bacterial bioaugmentation (Juhasz and Naidu, 2003; Sasek, 2003; D'Annibale et al., 2006). Biofilm reactors have become a focus of interest in the field of bioremediation. Numerous studies have been performed in using biofilm reactors for bioremediation of aromatic pollutants (digioia et al., 2009; Baraldi et al., 2008). Applications of biofilm reactors in biodegradation of xenobiotic compounds have been extensively reviewed (Farhadian et al., 2008). The majority of mycoremediation studies have been performed on artificially contaminated soils spiked with organic pollutants (Pointing, 2001; Sasek, 2003).

Lignin:-

Lignin is formed by removal of water from sugars to create aromatic structures. These reactions are not reversible. There are many possible monomers of lignin, and the types and proportions depend on the source in nature. Lignin can be defined as a hard material embedded in the cellulose matrix of vascular plant cell walls that functions as an important adaptation for support in terrestrial species. Lignin is a phenolic polymer, which is formed through dehydrogenative polymerization of p-hydroxycinnamyl alcohols (p-coumaryl, coniferyl and sinapyl alcohols) (monolignols). The synthesis is thought to initiate by the enzymatic dehydrogenation of monolignol followed by coupling of a resulting resonance-stabilized monolignol radical (Fig.1). The dehydrogenation is a one-electron transfer reaction catalysed by peroxidases or laccases in the presence of hydrogen peroxide (H_2O_2) or oxygen (O_2), respectively (Adler, 1977; Boerjan et al., 2003; Ralph et al., 2004). Lignin is deposited as an encrusting and protecting material on the cellulose/hemicellulose matrix, and it sets up a complex and acts as a kind of glue that cements the fibrous cell walls together.

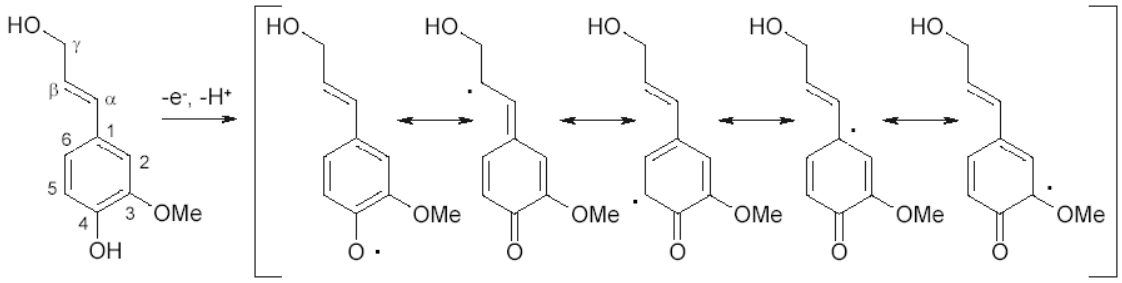

Fig.1:- Dehydrogenation of coniferyl alcohol, modified from Adler (1977).

Lignin is synthesized by higher plants from phenyl propanoid precursors by polymerization of radicals. Plant laccases are suggested to be involved in the lignification process (Monties and Fukushima, 2001). Precursors are produced by plants from Ltyrosine and L-phenylalanine which are synthesized from carbohydrates by the shikimic acid metabolic pathway (Higuchi et al., 1977). Lignin polymerization takes place in cell walls after the polysaccharides have been deposited, and is initiated by enzymatic oxidation of the precursors to phenoxy radicals. These radicals can couple with each other and with the growing lignin polymer in numerous ways to form a complex cross-linked network. This random polymerization of lignin gives it a very complex and irregular structure (Fig.2).

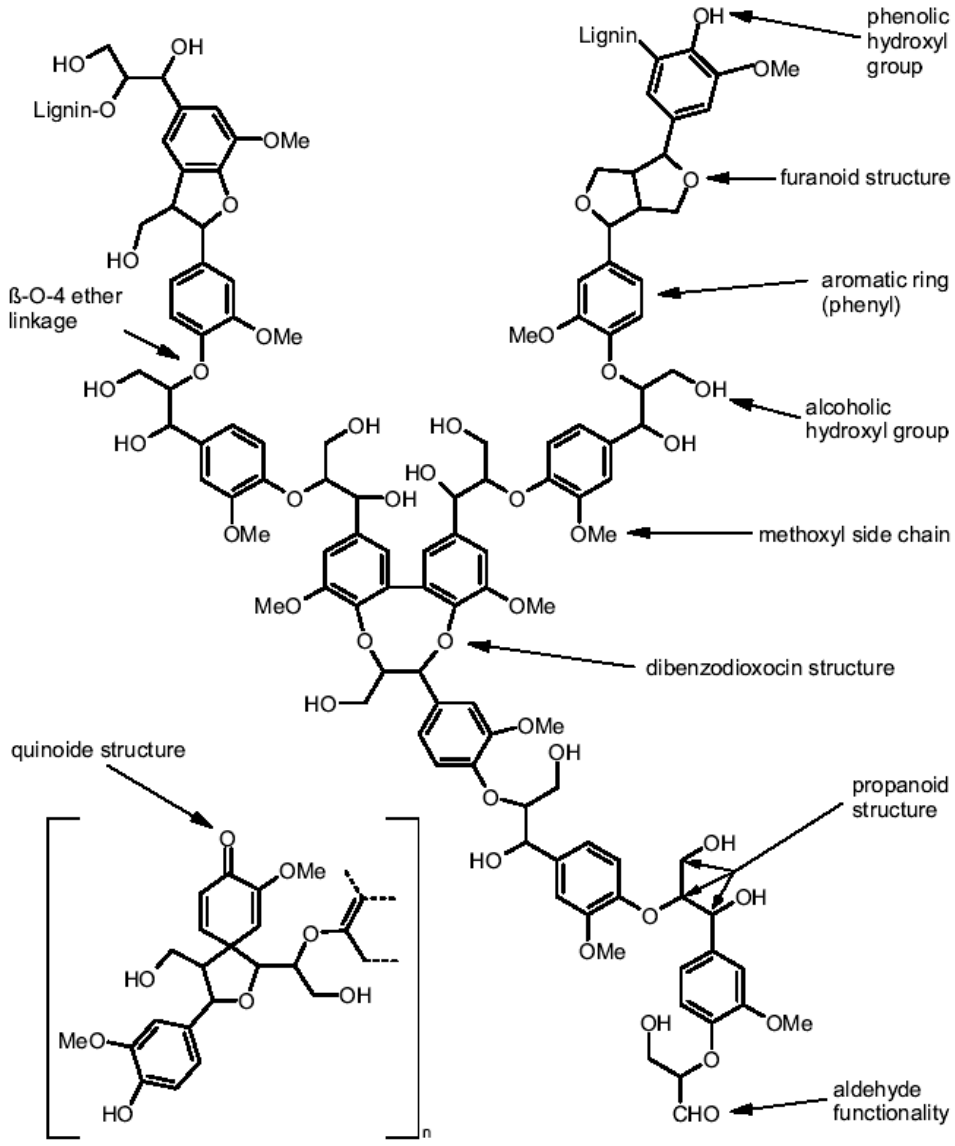

Fig.2:- Lignin model after Brunow and coworkers (Brunow, 2001) including a structure called dibenzodioxocin (Karhunen et al., 1995a, b).

Lignin molecules:-

Lignin is a very large, highly crosslinked and stereochemically complex polymer that is biosynthesized by the polymerization of phenylpropanoid precursors. There are three of these precursors, differing in the number of methoxyl groups on the aromatic ring (Fig.3). The ratio between syringyl and guaiacyl subgroups has been used as a comparative parameter between plant species (Monties and Fukushima, 2001). Guaiacyl lignin is mainly found in softwoods (24-33% of dry biomass), guaiacyl-syringyl lignin (16-25%) in hardwoods and grasses contain guaiacyl-syringyl-p-hydroxyphenol lignin (< 20%; Sjöström, 1977). The methylation of phenolic groups and thus the methoxyl content is recognized as an essential criterion for lignin characterization (Brown, 1985). The O-methyl transferase is the key enzyme in determining the composition of lignin. Gymnosperm, angiosperm, and grass transferases catalyze different conversions leading to different precursors. This explains the occurrence of different types of lignin and relates the O-methyl transferases to the evolution of lignin.

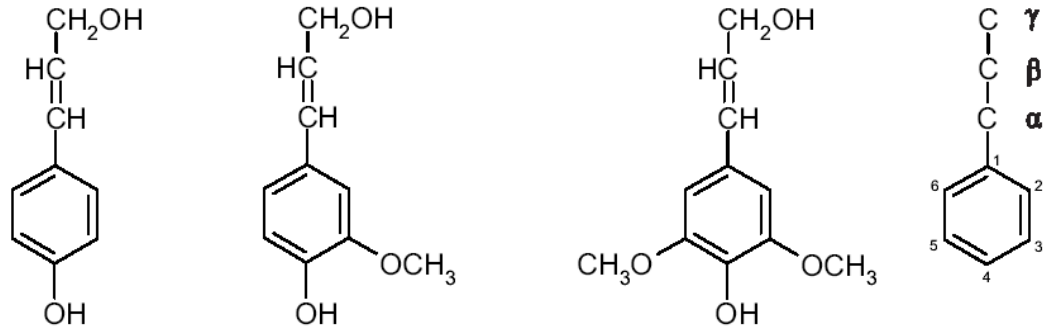

Fig.3:- Precursors of lignin. From left to right: p-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol, and a model for the numeration of the carbon skeleton (Sjöström, 1977).

Mechanism of lignin biodegradation:-

The only organism known to extensively degrade lignin are fungi (Kirk and Farrell, 1987). Because lignin is an insoluble polymer, the initial steps in its biodegradation must be extracellular. Fungi have, in contrast to bacteria, indeed extracellular enzyme systems. Overall lignin degradation by WRF 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). Because of the random polymerization process that forms it, lignin has a complex and irregular structure. The diversity of the inter unit linkages and the irregularity of their arrangement make it difficult for a ligninolytic fungus to produce enzymes that could recognize and cleave all of them. The solution that has evolved in the WRF is to produce enzymes of low specificity that initiate, but do not direct, oxidative reactions in lignin Kirk and Farrell (1987) have termed this process enzymatic combustion: the enzyme activates the lignin to overcome an energy barrier and begin a thermodynamically favored oxidative fragmentation without further control of the reaction pathway by the enzyme. The chemical changes produced by WRF in lignin include oxidative cleavage of the propanoid side chains and also demethylation and oxidation cleavage of aromatic rings (Chen and Chang, 1985). Lignin biodegradation does not proceed by an orderly removal of the peripheral subunits. Instead it also involves oxidation of the aromatic rings and side chains in the interior of the polymer, increasing the solubility of the polymer core at the same time as fragments of varying size are set free. The disorderly nature of this degradation agrees with the concept of enzymatic combustion. A general scheme for lignin biodegradation, which involves the oxidative reactions catalysed by lip, mnp and Lac, is shown in Fig.4.

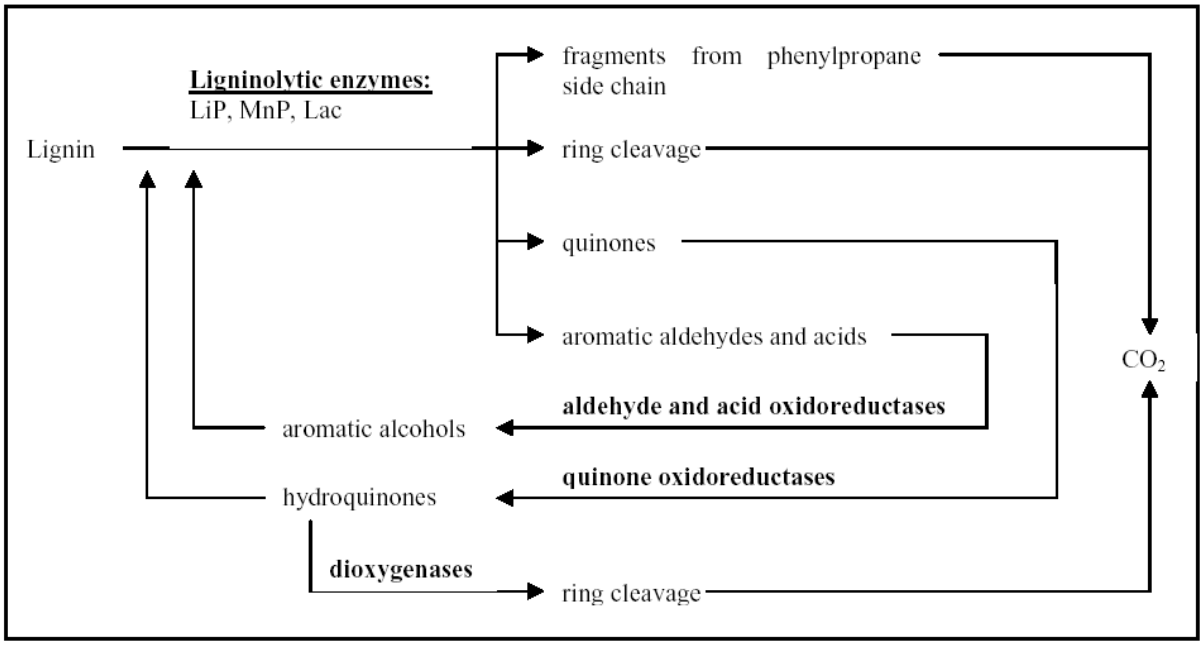

Fig.4:- Proposed schemes for the biodegradation of lignin (Redrawn from Leisola and Garcia 1989).

The model fungus for lignin degradation is Phanerochaete chrysosporium (Kirk 1984) but recently certain other fungi have been thoroughly studied (Ceriporiopsis subvermispora, Phlebia radiata, Pleurotus eryngii; Lundell 1993; Martínez et al., 1994; Hatakka, 2001). Results obtained using P. Chrysosporium identified two extracellular peroxidases that were found to be the most important enzymes involved in the degradation process. These enzymes are lignin peroxidase (lip) and manganese peroxidase (mnp). Laccase was found much earlier than mnp or lip and its activity was assumed to be involved in lignin degradation (Leonowicz et al., 1999). More enzymes are expected to be found such as versatile or hybrid peroxidases, which are modifications of mnp or lip (Mester and Field 1998, Ruiz-Duenas et al. 2001).

Basidiomycetous fungi:-

From an eco-physiological point of view, basidiomycetes that form macroscopic fruiting bodies can be broadly classified into wood-decaying, mycorrhiza-forming, and litter-decomposing fungi (Fig.5). Wood-decomposing fungi colonizing dead or dying tree trunks and stumps utilize cellulose while modifying the hemicellulose and lignin constituents cause either brown-rot or more commonly, white-rot via the utilization of hemicellulose and cellulose during the degradation of lignin. Wood rot fungi are important degraders of the major plant polymers lignin, cellulose and hemicellulose in the biosphere (Boominathan and Reddy, 1992; Kirk and Farrell, 1987; Reddy and D'Souza, 1994). WRF completely mineralize these polymers to CO_2, whereas Brown-rot fungi efficiently decompose cellulose and hemicellulose components of wood but mineralize lignin only to a limited extent (Kirk and Farrell, 1987).

Mushrooms are cultured world wide for their taste, nutritional attributes and potential applications in industries (D'Annibale et al., 2005). In addition, they have many medicinal uses and are good agents of bioremediation (Adenipekun and Fasidi, 2005; Nwanze et al., 2006). Mushrooms are highly nutritious containing protein (19-35%), low fat content (1.3-2%), relatively large amounts of carbohydrate (51-88%) and fiber (4-20%) in dry fruit mushroom (Adejoye et al., 2006).

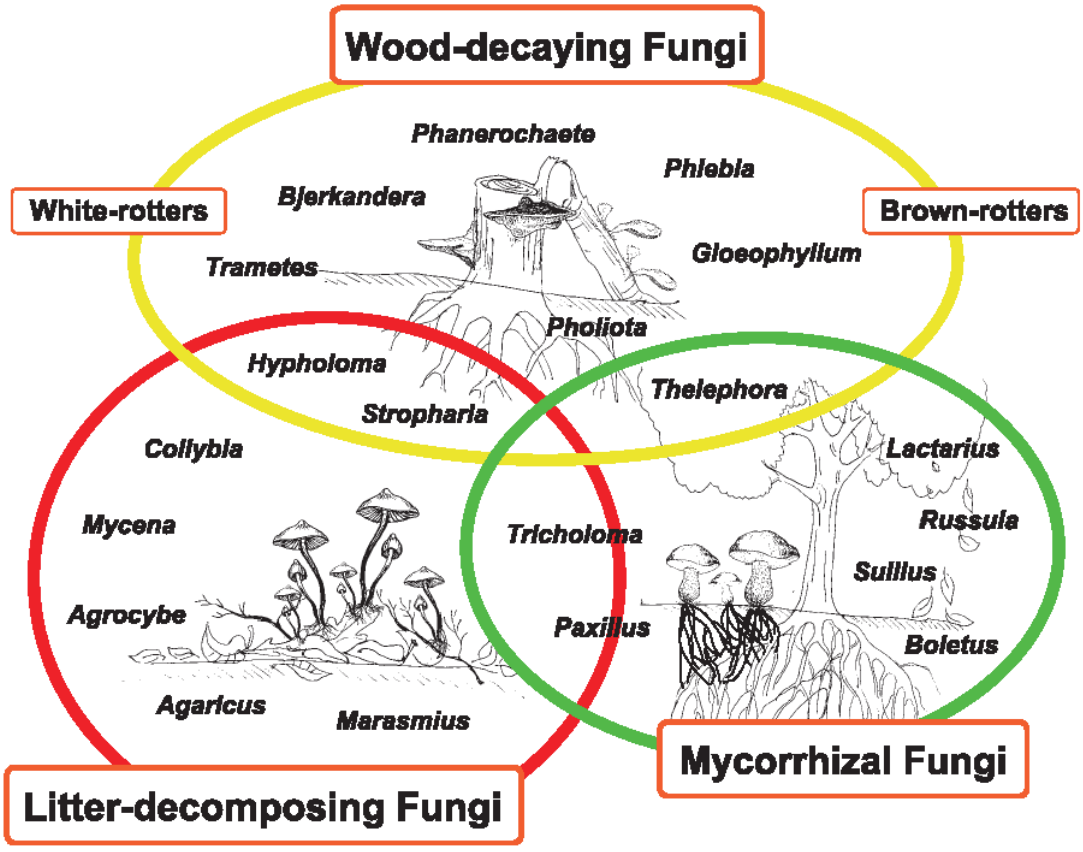

Fig.5:- Ecophysiological division of basidiomycetous fungi into three partially overlapping groups according to their habitat and lifestyle

White-rot fungi (WRF):-

White-rot fungi play an essential role in the decomposition of dead trees, especially in the degradation of lignin. The only organisms reported to degrade lignin efficiently are the WRF that under natural conditions mostly colonize dead or living wood (Eriksson et al., 1990). WRF attack the lignin component of wood and leave the cellulose and hemicellulose less affected. Those WRF that degrade lignin rather than cellulose, are called selective degraders. Selective lignin degraders are especially interesting from the standpoint of biotechnological applications, since they remove lignin and leave the valuable cellulose intact. Lignin degradation by these fungi is thought to occur during secondary metabolism and typically under nitrogen starvation. However, a wide variety of lignin degradation efficiency and selectivity abilities, enzyme patterns and substrates enhancing lignin degradation are reported from these fungi (Hatakka, 2001; Hofrichter, 2002). To be able to degrade the complex lignin molecule, these fungi use various extracellular enzymes with low specificity and strong oxidative activity. Due to the low specificity of the enzymes, WRF also have an ability to degrade a wide variety of environmental pollutants. Many studies are currently focused in the feasibility of using WRF for treatment of compounds, which represent toxicity and an environmental concern, such as phenolic compounds. Recent studies show that fungi with high ligninolytic activity are capable of degrading pollutants including polycyclic aromatic hydrocarbons, polychlorinated biphenyls (pcbs), dioxines, DDT, industrial dyes etc. WRF have therefore been expected to be good candidates for bioremediation of contaminated soil, air and water. Most known WRF are basidiomycetes, although a few ascomycete genera within the Xylariaceae are also capable of white-rot decay (Eaton and Hale, 1993).

In selective WRF the wood secondary cell wall is delignified diffusively starting from the lumen, followed with the delignification of the middle lamella. As WRF are capable of selective lignin degradation prefers hemicelluloses as carbon source, the wood cell walls are enriched with cellulose (Blanchette, 1991). Selective delignification can occur incompletely throughout wood substrate or merely in small, localized areas of complete lignin removal, which is called white pocket rot. In late stages of decay cellulose is also degraded and thus selective lignin degradation is usually limited to early stages of decay (Adasgavek et al., 1995). Selectivity of white-rot decay is dependent also on the physical and chemical environment in wood such as temperature; oxygen, nitrogen, and wood moisture content (Adasgavek et al., 1995) and varies also between wood species (Blanchette et al., 1988). In addition to wood polymers, several WRF are able to degrade wood extractives (Hatakka et al., 2003; van Beek et al., 2007).

Brown-rot fungi (BRF):-

Brown-rot fungi (BRF) belong to wood rotting basidiomycetes, they are distinguished from WRF by their imperfect ability to degrade lignin and different mode of cellulose degradation (Kirk and Highley, 1973; Blanchette et al., 1990). BRF cause wood darken, shrink and break into brick-shaped pieces that crumble easily in to brown powder. BRF drastically depolymerize cellulose and seriously decrease the strength of wood compared with WRF (Kajisa et al., 2004). Chemical analysis of brown-rotted wood indicates that BRF do not utilize lignin to an appreciable extent (Cowling 1961; Kirk and Highley, 1973). The main effect of the fungi on lignin is demethylation of aryl methoxyl groups (Kirk and Adler, 1970), although oxidative changes occur, including some cleavage of aromatic rings (Kirk, 1975). Cowling (1961) also showed that lignin decayed by P. Placenta had appreciably greater solubility in water and 1% naoh than did lignin in sound wood. Haider and Trojanowski (1980) demonstrated that brown-rot fungi can be induced to metabolize lignin to some extent. They found that isolated lignins were degraded to carbon dioxide to a limited extent by BRF in liquid culture.

Litter decomposing fungi (LDF):-

Fungi that colonize soil-litter, in particular litter-decomposing fungi (LDF), include basidiomycetes and ascomycetes living in the upper most portion of the soil and in the humus layer of forests and grasslands. In general, the decomposition of litter is brought about by combined activities of bacterial, fungal and animal populations, but basidiomycetous LDF are particularly important organisms because of their production of a wide range of ligninocellulolytic enzymes (Dix and Webster, 1995). Basidiomycetous litter-decomposers most commonly belong to the order Agaricales, but there are also basidiomycetes in other orders, e.g. Boletales and Poriales. Additionally many macroscopic fruiting body forming ascomycetes (e.g. Gyromitra spp.) Can be considered as LDF in a broader sense. Because LDF include saprotrophic basidiomycetes, nearly all constituents of the litter are open to degradation

by these fungi. The lignocellulosic complex in particular includes lignin that is attacked by a number of enzymes including mnp and laccase. The ability to break down lignin and cellulose enables some of the LDF to function as typical WRF in soil (Hofrichter, 2002), LDF can also produce other hydrolytic and oxidative enzymes, e.g. Lepista nuda produces phosphatase, protease, cellulase, β-xylosidase, β-glucosidase, and phenol oxidase (Colpaert and vanlaere, 1996). LDF seem to release nitrogen during the decomposition of leaf litter (Colpaert and vantichelen, 1996) but tend to accumulate different metals and heavy metals (Rajarathnam et al., 1998). As such, it is clear that the impact of this fungal group is extremely important in forest and grassland ecosystems. Without the activity of LDF we, and forests, would in time be buried by cast off leaves and branches. Litter is often colonized by LDF during the final stage of decay and therefore the accumulation of recalcitrant material (mainly the lignin component of litter) is minimized. This makes LDF one of the most active degraders of tree leaf litter that has major implications for recycling of carbon in soil (Dix and Webster, 1995).

Mycorrhizal fungi (MF):-

Mycorrhizal fungi form a symbiotic relationship with the roots of trees and other plants and provide them with better access to water and nutrients in return for host carbon assimilates. Until recently, they were believed not to exhibit the saprotrophic capabilities of litter-decomposing or wood-decaying fungi, although genes of ligninolytic enzymes and their expression have now been detected (Chen et al., 2003). LDF and MF coexist and interact in soils. There is an indication that some mycorrhizal fungi, such as Paxillus involutus, could be facultative mycorrhiza formers that switch between a saprotrophic and symbiotic habit and being thus able to degrade lignin to some extent (Haselwandter et al., 1990). Mycorrhizal fungi are responsible for many of the fruit bodies found in the forest. Mycorrhizas occur on most plants and in most soils throughout the world, and the majority of fine roots found on trees in natural ecosystems are mycorrhizal. The fungi that form mycorrhizas are a special group of soil fungi that are beneficial to plants. These fungi are multi-purpose, accomplishing many essential functions for their plant partner, the most important being their role in the absorption of nutrients from the soil. Over 5,000 species of fungi have been recorded as forming ectomycorrhizas. Most ectomycorrhizal fungi (EMF) are Basidiomycetes and Ascomycetes, typically producing large, fleshy fruit bodies such as mushrooms, toadstools, puffballs, truffles, coral fungi, cup fungi and resupinate fungi.

Ligninolytic enzymes:-

Enzymes are defined as substances that alter a reaction's rate and a reaction's activation energy without being present in the reaction products (Uhlig, 1998). They are naturally produced by nearly every known organism in order to aid processes such as digestion, metabolism and cell synthesis (Madigan et al., 2003). The hydrophobicity of the lignin (and similar organic compounds), may suggest to think that, not only the enzymes, which tend to be water soluble (Collins and Dobson, 1997; Gadd, 2001), are the only ones involved in lignin degradation. These enzymes probably generate oxidising agents in order to carry out their function (Collins and Dobson, 1997). The original belief that ligninolytic enzyme production is a unique prerogative of white-rot basidiomycetes is being denied by an increasing number of studies reporting the presence of these enzymes in other fungal taxonomic groups (Ayed et al., 2004).

Table-1:- Extracellular ligninolytic enzymes involved in lignin degradation (Hatakka, 2001)

Enzyme	Cofactor	Substrate, mediator	Main effect or reaction
Laccase	O_2	As mediators hydroxybenzotriazole, ABTS	Phenols are oxidized to phenoxyl radicals; mediator Radicals
Aryl alcohol oxidase, AAO		Aromatic alcohols (anisyl, veratryl alcohol)	O_2 reduced to H_2O_2
Lignin peroxidase, lip	H_2O_2	Veratryl alcohol	Aromatic ring oxidized to Cation radical
Manganese peroxidase, mnp	H_2O_2	Mn^{2+}, organic acids as chelators, thiols, Unsaturated lipids	Mn^{2+} oxidized to Mn^{3+}; Further oxidationof phenolic Compounds to phenoxyl Radicals
Versatile peroxidase, VP (hybrid peroxidases)	H_2O_2	Same or similar Compounds as lip and mnp	Same effect on aromatic and Phenolic compounds as lip And mnp

Lignin breakdown is thought to occur by concomitant action of ligninolytic enzymes. Table-1 summarizes the ligninolytic enzymes and their substrates and reactions. The main extracellular enzymes participating in lignin

degradation are heme-containing lignin peroxidase (ligninase, lip, EC 1.11.1.14), manganese peroxidase (mnp, EC 1.11.1.13) and Cu-containing laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) (Hatakka, 2001). A new group of ligninolytic heme-containing peroxidases, combining structural and functional properties of the lip and mnp, are the versatile peroxidase (VP). The VP is capable of oxidation of Mn^{2+} and phenolic compounds, as well as nonphenolic aromatic compounds such as veratryl alcohol. These types of peroxidase were isolated from the white-rot fungi Pleurotus eryngii (Camarero et al., 1999), Pleurotus ostreatus (Cohen et al., 2001), Bjerkandera adusta (Heinfling et al., 1998; Wang et al. 2003) and Bjerkandera sp. (Mester and Field, 1998). Lentinula edodes Mn-dependent peroxidase also oxidizes veratryl alcohol (D'Annibale et al., 1996), while mnp from Panus tigrinus is able to degrade nonphenolic lignin model compounds (Maltseva et al., 1991). In addition, enzymes involved in hydrogen peroxide production such as glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO) (EC 1.1.3.7) are considered to belong to the ligninolytic system.

Laccase:-

Laccases (EC 1.10.3.2) are multicopper oxidases produced by most WRF that oxidize a wide range of aromatic compounds, having high activity on substituted phenols (Thurston, 1994; Canas et al., 2007). Laccases are member of the blue copper oxidase enzyme family characterized by having four cupric ions coordinated such that each of the known magnetic species is associated with a single polypeptide chain. The copper binding domains are highly conserved in the blue copper oxidases (Thurston, 1994). The crystal structure of the depleted laccase from Coprinus cinereus has provided a useful model for the structure of the laccase active site (Ducros et al., 1998). Laccase have been implicated in pigmentation (Coll et al., 1993), fruiting body formation (De Varies et al., 1986), pathogenicity (Williamson, 1997) and lignin degradation (Hatakka, 1994). However only a few of these functions have been experimentally proven.

Like manganese peroxidase, it normally oxidizes only those lignin model compounds with a free phenolic group, forming phenoxy radicals. However in the presence of the artificial substrate ABTS (2,2´-azinobis(3-ethylbenzthiazoline-5-sulphonate) or some other synthetic mediators, laccase can also oxidize certain non-phenolic compounds, veratryl alcohol and Mn^{2+} (Collins and Dobson, 1997). Laccase was first detected in the Japanese lac tree Toxicodendron verniciflua. Later, it was found in certain other plants and fungi, but is also found in molds, black yeasts and some bacteria (Thurston, 1994; Mayer and Staples, 2002; Mikolasch and Schauer, 2009). Laccase is produced by most WRF (Hatakka, 1994), but normally not in Phanerochaete chrysosporium (Kirk and Farrell, 1987) under ligninolytic conditions but it is secreted when cellulose is present as a carbon source (Dittmer et al., 1997). Laccase has the capability to both depolymerize and polymerize lignin model compounds (Eriksson et al., 1990). Despite the fact that laccase was the first enzyme found to have a function in the degradation of lignin.

The biological roles of laccase are diverse (Mayer and Staples, 2002). Together with lignin peroxidase, manganese peroxidase, and manganese independent peroxidases, it is a ligninolytic enzyme (Leonowicz et al., 2001; Martínez et al., 2005). As a result of laccase oxidation, radicals (cationic) can be generated in lignin, and these can cause subsequent aliphatic or aromatic C–C bond cleavage and lignin depolymerization (Hatakka, 1994; Bourbonnais et al., 1995; Leonowicz et al., 1999; Barreca et al., 2003). Laccases are divided into "low-redox potential" and "high-redox potential" laccases depending on the structure and properties of the copper center. The high-redox potential laccases occur mainly in basidiomycetes, especially WRF (Gutierrez et al., 2006; Quaratino et al., 2007; Hernandez-Luna et al., 2008), the low-redox potential laccases seem to be widely distributed in molds (Jung et al., 2002), bacteria, insects, and plants. However, detailed studies by Klonowska et al. (2002, 2005) have shown that the WRF Trametes sp. C30 possesses not only the high-redox potential laccase (LAC1) but also two additional low-redox potential laccases (LAC2 and LAC3) with minor activity (Mikolasch and Schauer, 2009, Fig.6).

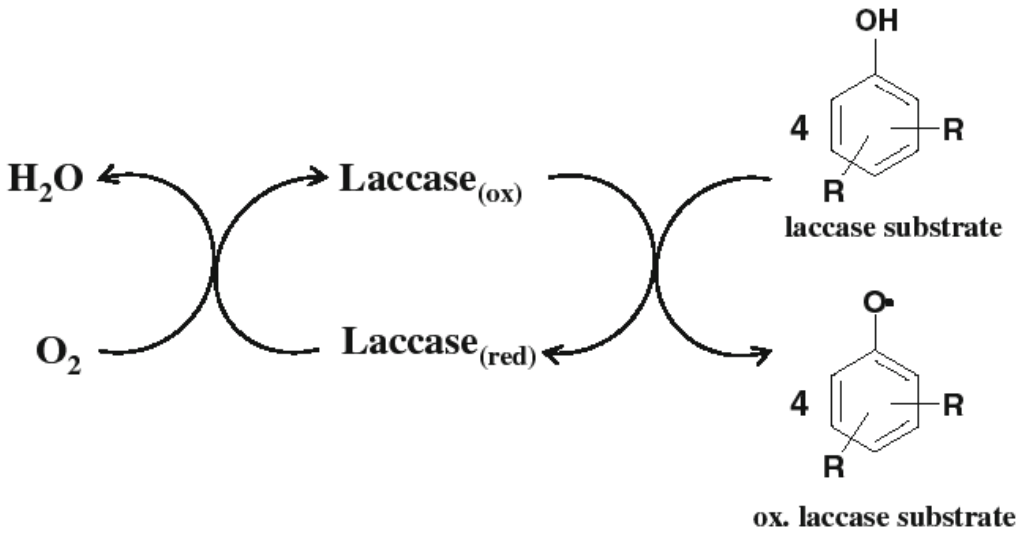

Fig. 6:- Catalytic cycle of laccase- catalyzed substrate oxidation (Mikolasch and Schauer, 2009)

Aryl-alcohol oxidase (AAO):-

Aryl-alcohol oxidase (AAO; EC 1.1.3.7) activity was described for the first time in the fungus Polystictus versicolor (a synonym of Trametes versicolor) in 1960 (Farmer et al., 1960). Since then, AAO has been detected and characterized in other white-rot basidiomycetes including Pleurotus species (Guillén et al., 1990), Bjerkandera adusta (Muheim et al., 1990), and some ascomycetous fungi (Kim et al., 2001). WRF are responsible for lignin degradation, and AAO participates in this process by generating hydrogen peroxide in the redox-cycling of aromatic fungal metabolites (Gutierrez et al., 1994) involving also mycelial dehydrogenases (Guillén et al., 1994). The reaction of AAO (Fig.7) is initiated by the oxidative dehydrogenation of the substrate (reductive half-reaction), and is completed by flavin reoxidation by molecular oxygen with the production of hydrogen peroxide (oxidative half-reaction).

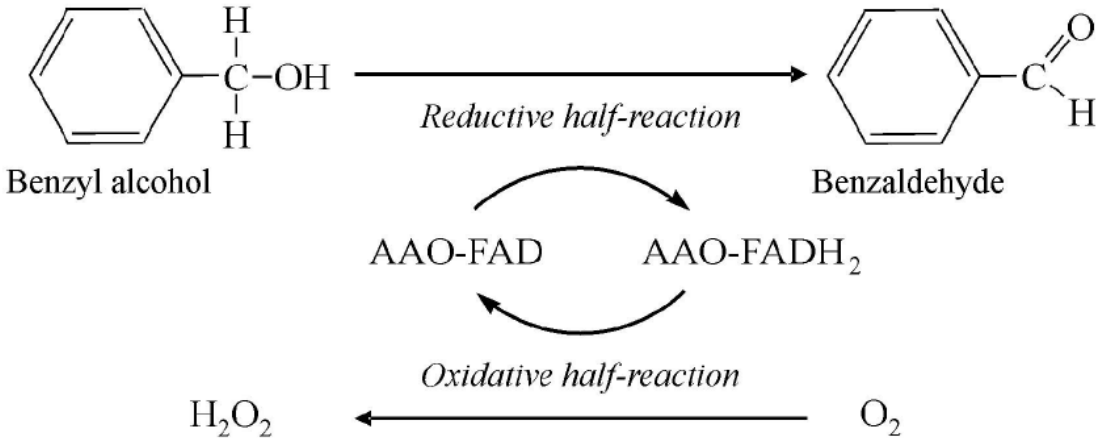

Fig.7:- The catalytic cycle of AAO (Ferreira et al., 2005)

Lignin peroxidase (lip):-

One of the best known ligninolytic enzymes is lignin peroxidase (ligninase; lip; EC 1.11.1.14) which was discovered a little earlier than mnp (Kirk and Farrell, 1987; Kirk and Cullen, 1998). Lip is a glycoprotein that contains one mole of iron protoporphyrin IX as a prosthetic group. It has a series of isoenzymes with molecular weight of 41,000-

42,000 (Kirk and Farrell, 1987; Gold et al., 1989). The enzyme can be assayed by the oxidation of veratryl alcohol (VA) to veratraldehyde at 310 nm (Tien and Kirk, 1984). Fungi that were identified as producing lip include P. Chrysosporium, T. Versicolor, Pleurotus ostreatus, Phlebia tremellosus etc. Lip has a relatively high redox potential and has no substrate specificity. Lip has been shown to oxidise phenolic and non-phenolic lignin related compounds as well as a wide variety of model lignin and related compounds (Barr and Aust, 1994). Among the oxidation reactions catalysed by lip are the cleavage of the Cα -C_β and aryl Cα bonds, ring cleavages in β-O-4 compounds, aromatic ring opening, demethylation and phenolic oxidation (ten Have and Teunissen, 2001). All of these reactions are involved in the same mechanism in which the oxidised enzyme intermediates lip (I) and lip (II) catalyse the initial one-electron oxidation of the substrate to yield an aryl cation radical, followed by a series of non-enzymatic reactions to yield the final products (Gold et al., 1989).

The catalytic cycle of lip is illustrated in Fig.8 (Gold et al., 1989). The native enzyme reacts with H_2O_2 forming the two-electron oxidised intermediate, lip (I), which then oxidizes the lignin substrate (RH) to produce the one-electron oxidized intermediate lip (II) and a substrate radical ($R^{\cdot}$). The lip (II) is then reduced back to the resting enzyme state by oxidizing a second substrate compound while the free radical can undergo a range of reactions. With excess H_2O_2 lip (II) can be converted to an inactive form of the enzyme lip (III) (Tien, 1987, Gold et al., 1989).

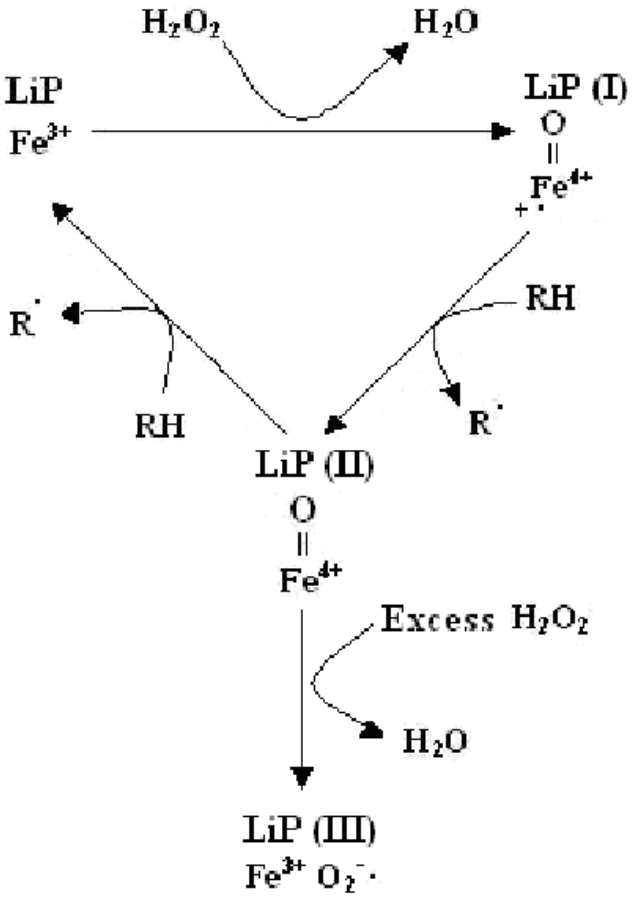

Fig.8:- The catalytic cycle of Lignin peroxidase (Gold et al., 1989)

Manganese peroxidase (mnp):-

Manganese peroxidase (mnp EC 1.11.1.13), which is exclusively produced by some basidiomycetes, was first discovered shortly after lip from Phanerochaete chrysosporium by Kuwahara et al. (1984) and described by Glenn and Gold (1985). Mnp is an extracellular heme containing peroxidase with a requirement for Mn^{2+} as its reducing substrate. Manganese alone can also regulate the production of mnp in Phlebia radiata (Moilanen et al., 1996). Mnp oxidizes Mn^{2+} to Mn^{3+}, which then in turn oxidizes phenolic structures to phenoxyl radicals (Gold et al., 1989). The Mn^{3+} formed is highly reactive and complexes with chelating organic acids such as oxalate or malate (Kishi et al.,

1994), which are produced by the fungus (Hofrichter et al.; 1999, Mäkelä et al., 2002). With the help of these chelators, Mn^{3+}-ions are stabilized and can diffuse into materials such as wood. The redox potential of the mnp-Mn system is lower than that of lip and preferably oxidizes phenolic substrates (Vares, 1996). The phenoxyl radicals produced can further react with the eventual release of CO_2.

The catalytic cycle of mnp (Fig.9) starts with the binding of H_2O_2 to the reactive ferric enzyme. H_2O_2 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). The cleavage of the oxygen-oxygen bond requires the transfer of two electrons from the heme, forming the mnp compound I. This activated state of the heme center 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. The so 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.

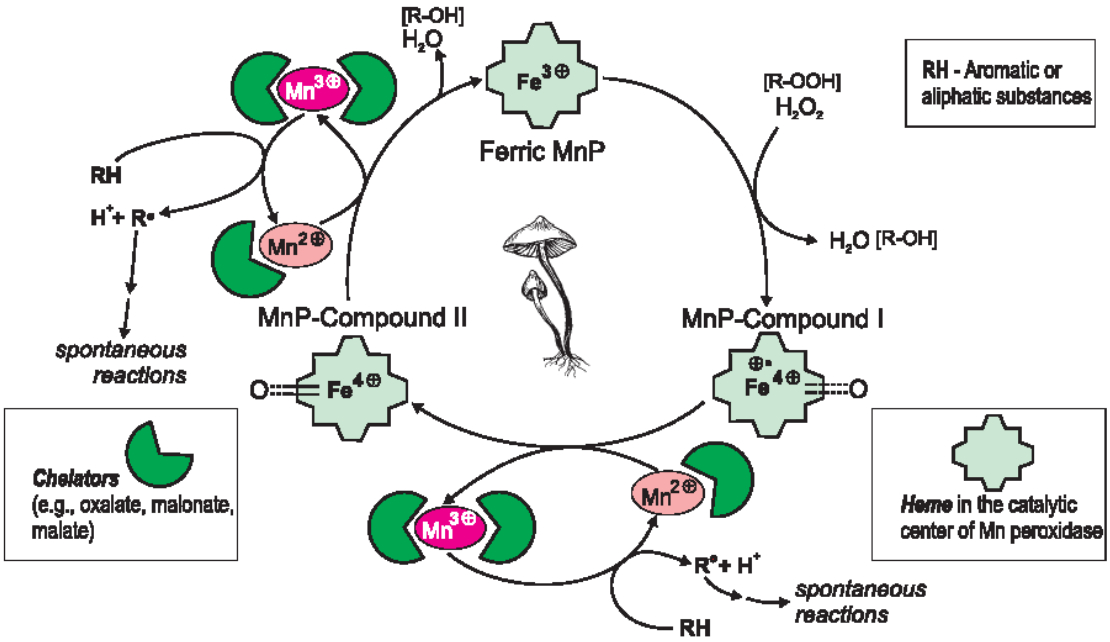

Fig.9:- The catalytic cycle of manganese peroxidase (Wariishi et al., 1988, Wariishi et al., 1992; Kishi et al., 1994; Kirk and Cullen, 1998).

Versatile peroxidase (VP):

Versatile peroxidase (VP; EC 1.11.1.16) is a novel heme peroxidase type described in fungi from the genera Pleurotus and Bjerkandera, whose biochemical, molecular and structural aspects are being thoroughly investigated (Martínez et al., 1996; Mester and Field, 1998; Ruiz-Duenas et al., 1999; Camarero et al., 1999; Banchi et al., 2003; Munteanu et al., 2005). VP (syn. Hybrid peroxidase, manganese- lignin peroxidase) is a new ligninolytic enzyme, combining catalytic properties of manganese peroxidase, oxidation of Mn (II), lignin peroxidase (Mn-independent oxidation of non-phenolic aromatic compounds) and plant peroxidase (oxidation of hydroquinones and substituted phenols).

In the catalytic cycle (Fig.10), the ferric resting peroxidase experiments a two-electron oxidation by peroxide to yield compound I, which is reduced back in to one electron reactions by two molecules of substrate (AH_2) that are oxidized to the corresponding radical ($AH^{\cdot}$). If peroxidase is immobilized on a graphite electrode, the electrode can substitute the electron donor substrates in the common peroxidase cycle. This process is usually referred to as direct electron transfer (ET).

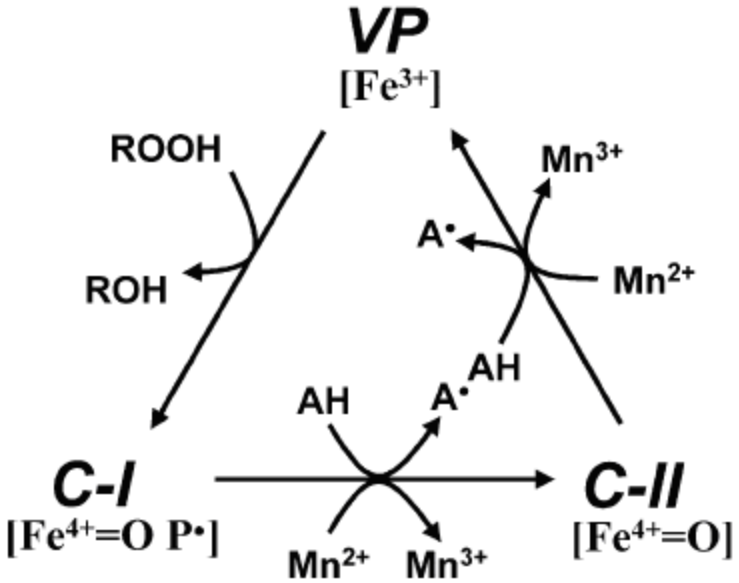

Fig.10:- The catalytic cycle of VP (Ruiz-Duenas et al., 1999)

Phenolic compounds:-

Phenolic compounds are a large and diverse group of molecules, which includes many different families of aromatic secondary metabolites in plants. Most phenolic compounds are used as raw materials in oil refineries, pulp and paper manufacturing plants, resin and cake manufacturing, steel and pharmaceutical industries that generate wastewaters containing phenols and phenolic compounds (Christoskova et al., 2001; Chen et al., 2004; Nuhoglu et al., 2005; Sanchez et al., 2007).

Phenol:-

Phenol is a basic structural unit for a variety of synthetic organic compounds. It is a white crystalline solid with molecular weight of 94.1 g/mol and formula of C_6H_5OH (ATSOR, 1989; US Environmental Protection Agency, 1990, Fig.11). It has a very strong odour (acrid odour) with an odour threshold of 0.04 ppm (Amoore and Hautala, 1983) and a sharp burning taste. It is soluble in most organic solvents and its solubility in water is limited at room temperature, however above 68°C it is entirely water-soluble. It is moderately volatile at room temperature (evaporates more slowly than water) and quite flammable (Calabrase and Kenyon, 1991). It is obtained from coal tar and is widely used as disinfectant for industrial and medical applications. It also serves as a chemical intermediate for the manufacture of nylon 6, other man made fibers, epoxy and other phenolic resins and as a solvent for petroleum refining. Phenol is also used as general purpose disinfectant and can also appear as degradation product of other chlorinated xenobiotics (Bollag et al., 1986). Natural sources of phenol include forest fire, natural run off from urban area where asphalt is used as the binding material and natural decay of lignocellulosic material. The presence of phenol in water imparts carbolic odor to receiving water bodies and can cause toxic effects on aquatic flora and fauna. Fungal strains are variable to degrade phenol. Tricholoma cutaneum, Candida sp. And Rhodotorula sp. Are able to utilize phenol as sole source of carbon and energy. Strains of Penicillium (Scow et al., 1990; Hofrichter et al., 1993) and WRF (Valli and Gold, 1991) have also been shown to metabolize phenol.

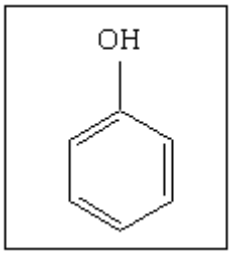

Fig.11:- Chemical structure of Phenol

Chlorophenols:-

Chlorophenols (CP) constitute a series of 19 compounds consisting of mono-, di-, tri-, tetrachloro isomers and one pentachlorophenol (PCP). Generally, higher chlorinated phenols and their salt forms are used in wood preservation industry and in surface treatments for fresh-cut logs and lumber against sapstain fungi and moulds. The lower cps serves as intermediates in the production of higher cps and various pesticides. Cps are a group of toxic compounds that have been widely used as biocides to control bacteria, fungi, algae, mollusks, insects, slime and other biota (Fig.12). Cps that enter non-target upland, wetland, and aquatic environments associate with colloidal and particulate matter and, if not photodegraded, eventually settle onto surface soils (Shiu et al., 1994). The chlorinated phenols have been recognized one of the major environmental problems because they have been widely found in surface water and in wastewater, in particular, in pulp mill effluents (Schellenberg et al., 1984). Chlorinated phenols are considered to be relative persistent in the environment and they are highly toxic. Biodegradation of chlorophenols is dependent on number and position of chlorine substituents; the rate of biodegradation decreases with increase in number of chlorine substituents. Metabolism of cps is not unusual in fungi. WRF capable of degrading cps attracts considerable attention as an effective biotreatment tool in aqueous effluents (Michizae et al., 2001). The degradation pathways of several cps by the WRF P. Chrysosporium have been determined in vitro (Valli and Gold, 1991; Reddy and Gold, 2000).

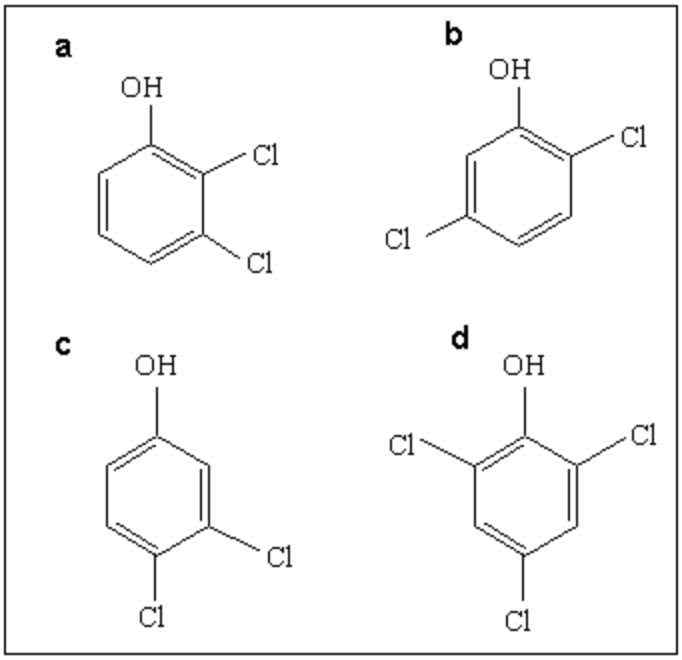

Fig.12:- Chemical structure of 2,3-DCP (a), 2,5-DCP (b), 3,4-DCP (c) and 2,4,6-TCP (d) used in this study

Nitrophenols:-

Nitrophenols (nps) are among the most important and versatile industrial organic compounds with applications as pesticides, pharmaceuticals, pigments, dyes, and rubber chemicals (Ye et al., 2004, Fig.13). Nps, consisting of a huge class of nitroaromatics are known to accumulate in soil through hydrolysis of organic insecticides, such as parathion or methylparathion as well as through the direct use of other nitrophenol derivatives as herbicides (Teramoto et al, 2004). Nps belong to the family of nitro compounds. 2-NP and 4-NP develop in the mixture with nitrating of phenol. Nps are used in the chemical, pharmaceutical and armaments industry as intermediate products with the production of dyes, leathers, rubber, pesticides, fungicides and ammunition. They have poisonous effect on the nervous system of organisms.

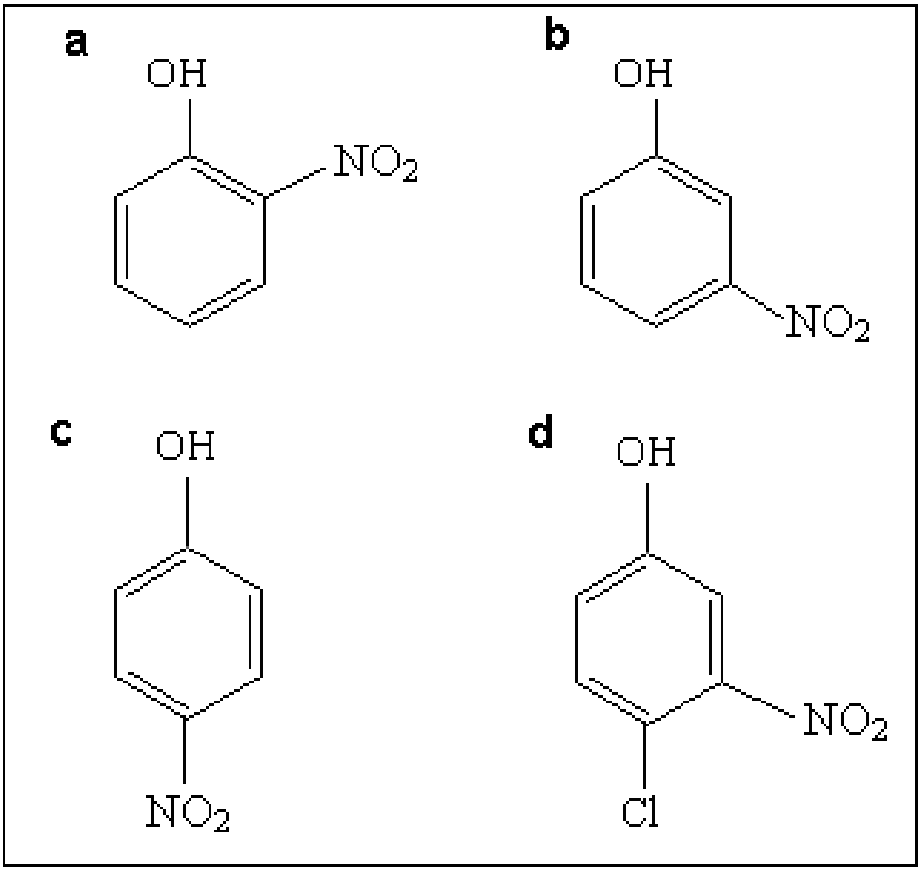

Fig.13:- Chemical structure of 2-NP (a), 3-NP (b), 4-NP (c) and 4-Cl-3-NP (d) used in this study

Degradation of phenolic compounds:-

The focus of this thesis is biodegradation of phenolic compounds. Applications to bioremediate organic pollutants that have been reported to date have focused on the use of bacteria rather than fungi. Although both groups of microorganisms have a number of unique advantages that are applicable to their use in bioremediation strategies, it is likely that the ease of culture of bacteria and the greater level of understanding of bacterial genetic sequences are the factors that are ultimately responsible for the more widespread application of bacteria in bioremediation strategies. Bacteria are also reported to be more amenable to genetic modification and are capable of metabolizing chlorinated organic compounds (Kumar et al., 1996; Bouwer and Zehnder, 1993).

Chlorinated compounds have been detected as pollutants in many contaminated sites. The presence of these compounds is a consequence of their use by the chemical industry for a wide variety of applications, and also due to their formation during the degradation of polychlorinated compounds such as pcbs (Johri et al., 1999). Many of the pathways involved in the biodegradation of chlorinated compounds by microorganism share similar metabolites, and single enzymes are often able to catalyse the degradation of a number of different compounds. Chlorinated catechols for example are key intermediates in the degradation of chlorinated aromatic compounds, and the enzyme toluene 4-monoxygenase has activity against TCE, toluene, ethylbenzene, acetanilide, 2-phenylethanol and phenol (Timmis et al., 1994; mcclure et al., 1991; Yen et al., 1991). The complete degradation of polychlorinated compounds such as pentachloroethane and pcbs requires the sequential occurrence of anaerobic and aerobic reactions (Wackett et al., 1994). One of the most problematic phenolic compound is 2,4,6-trinitrotoluene (TNT) that is often present at sites used to manufacture and handle munitions. Nitroaromatic compounds can be biodegraded under both aerobic and anaerobic conditions (Holliger et al., 1997). Most of the work on biodegradation of phenolic compounds has been carried out on bacteria. Although bacteria are fast growing and can respond to a changing environment by populations utilizing the energy source present, there are important advantages of using fungi instead of bacteria for biodegradation. Many of the pollutants are toxic to the organisms that are supposed to degrade them.

Degradation of phenolic compounds by basidiomycetous fungi:-

There are more than 1,500 different species of WRF. In addition, there are thousands of other fungal species loosely categorized as Brown-rots, dry rots, litter rots, soft rots, mycorrhiza, terricolous, and so on. Most of these species have never been studied for mineralization of phenolic or xenobiotic compounds and represent a large potential for biodegradation research. The first studies on xenobiotic degradation by WRF were carried out with Phanerochaete chrysosporium. It was shown that this basidiomycete is able to degrade DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane), lindane (g-hexachlorocyclohexane), polycyclic aromatic hydrocarbons (PAH) and dioxins (Aust and Benson, 1993; Bumpus et al., 1985; Cameron et al., 2000). Several species have been investigated for their bioremediation capability of phenolic compounds, such as Phanerochaete chrysosporium (Leatham et al., 1983; Paszczynski and Crowford, 1995), Trametes versicolor (Bezalel et al., 1996), Pleurotus spp. (Novotny et al., 1999; Law et al., 2003), Phlebia radiata (Van Aken et al., 1999), Panus tigrinus (Leontievsky et al., 2002), Lentinus squarrosulus, Lentinula edodes (Cai et al., 1993), Ganoderma lucidum (Sridhran et al., 1993), Gleophyllum striatum (Niemenmaa et al., 2008), Gleophyllum trabeum (Niemenmaa et al., 2008), Bjerkandera adusta (Kotterman et al., 1998; Rubilar et al., 2007), Irpex lacteus (Novotny et al., 2000), Volvariella volvacea (Cai et al., 1993) etc. These allow the basidiomycetous fungi to oxidise a wide range of recalcitrant organic compounds, for instance polyaromatic hydrocarbons (pahs) (Muncnerova and Augustin, 1994), polycyclic aromatic hydrocarbons (Bogan and Lamar, 1996), polychlorinated biphenyls (Xu, 1996), polychlorinated dibenzo(p)dioxins, and the pesticides DDT and lindane (Fujita et al., 2002). It has also been reported that P. Chrysosporium is able to mineralize di-, tri-, tetra- and pentachlorophenol (PCP) (Choi et al., 2002). PCP has been used as wide spectrum pesticides and wood preservatives throughout the world. This chemical is currently banned in most countries; however soil contamination continues to be a problem.

Bioremediation is a viable alternative to conventional remediation techniques, but the success of the methodology depends on many factors, both biotic and abiotic. These factors span over a great range, from physico-chemical properties of the contaminant to genetics of the biodegrading microorganisms. Gathering of knowledge about these factors is important as it will increase the accuracy of predicting the outcome of a bioremediation strategy. Two strategies can be discerned; a “top-down” approach used by engineers, mainly relying on stimulating the intrinsic population in order to reduce contaminant levels, and a “bottom-up” approach employed by many researchers, investigating in detail the properties of a single biodegrading microorganism, or a single factor at a polluted site. When these two approaches can be fully integrated, the power of bioremediation as a treatment strategy will greatly increase.

Two particular white-rot species that have received considerable attention are Pleurotus ostreatus and Trametes versicolor. They are both efficient at mineralizing polyaromaric hydrocarbons (Bezalel et al., 1996) and at degrading polychlorinated biphenyls (Zeddel et al., 1993). P. Ostreatus is better able to colonize soils than P. Chrysosporium (Novotny et al., 1999), and although it is a successful lignin-degrading species, it does not exhibit lip activity (Hatakka, 1994; Bennet et al., 2002). The main mechanism of biodegradation employed by WRF is the lignin degradation system of enzymes. The fact that fungal enzymes work extracellularly allows them to access many of the non polar, non soluble xenobiotic compounds that intracellular process (Levin et al., 2003). However the potential of species like Gleophyllum Striatum and Gloeophyllum trabeum in the degradation of xenobiotics was explored recently. Gloeophyllum spp. Are BRF that produces several low molecular weight phenolate compounds and quinones, which have ability to produce hydroxyl radicals in Fenton reaction (Jensen et al., 2001; Niemenmaa et al., 2008). Trametes versicolor was found to be the most efficient lignin degrader in soil, and its PCP degrading capability was also examined. Because some organisms produce toxic metabolites from pollutants (Leštan and Lamar, 1996), the ultimate fate of PCP was also studied, and T. Versicolor was found to be a promising fungus for bioremediation. B. Adusta is very promising for the ability to degrade and decolorize xenobiotic compounds. These processes are accompanied by peroxidase biosynthesis and a decrease in the levels of free radicals and phenolics. This phenomenon is obvious in light of the fact that WRF frequently synthesize ligninolytic enzymes. It is possible that the B. Adusta produces not only ligninolytic enzymes but also cellulase and hemicellulase complexes hydrolyzing other wood compounds (cellulose, hemicelluloses).

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