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REVIEW

LIGNINOLYTIC SYSTEM AND ITS POTENTIAL APPLICATIONS

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INTRODUCTION

Lignin is one of the most abundant biopolymers on earth. It is estimated that the planet currently contains $3x10^{11}$ metric tons of lignin with an annual biosynthetic rate of approximately $2x10^{10}$ tons (Argyropoulos and Menachem, 1997). Lignin constitutes approximately 30 per cent of the dry weight of soft woods and about 20 per cent, the weight of the hardwoods. Research on lignin and its biodegradation has accelerated greatly during the last 10 years, mainly because of the substantial potential application of bioligninolytic systems in pulping, bleaching, converting lignin to useful products and treating wastes (Kirk and Farrell, 1987). Though its complex structure is difficult to degrade, it is the same structure which yields a variety of economically important by products on degradation. In our review, the aspects of lignin degradation and its possible uses in different industries and bioremediation are discussed.

Lignin : the structure and function

Lignin is an integral cell wall constituent of all vascular plants including herbaceous species. The presence of lignin within the cellulosic fibre wall, mixed with hemicellulose creates a naturally occurring composite material which imparts support, strength and rigidity to trees and plants. Lignin is a heterogeneous three dimensional aromatic polymer containing various biochemically stable carbon - carbon and ether linkages between monomeric phenyl propane units. It is formed in plant cells by the enzyme mediated polymerization of three substituted cinnamyl alcohols *viz.*, p-coumaryl, coniferyl and sinapyl alcohol (Sarkanen and Ludwig, 1971). These phenols are synthesized from carbohydrates through shikimate pathway by deamination, ring hydroxylation, phenolic methylation and carboxyl reduction. Single electron oxidation of the cinnamyl alcoholic hydroxyls within the lignifying cell walls produce radicals which exist in mesomeric forms. These couple randomly and condense with each other/with radicals on the growing lignin polymer. The cells involved in lignification possessed all the enzymes necessary for its precursor synthesis (Gross, 1979). Because of the involvement of free radicals, the structure of lignin differs from species to species depending on the oxidation state of the precursor molecule.

Lignin biodegradation

The knowledge on lignin degradation was far from adequate when compared to the degradation of other polymers because of its unique structure. The structure was elucidated only in late 60's (Freudenberg, 1968) and these structural features dictate unusual constraints on biodegradative systems responsible for initial attack. They must be extracellular, non specific and nonhydrolytic. Moreover, lignin is a 600 - 1000 kD molecule which is also too big to enter into cells. Also some of the linkages are unstable, particularly those involving C-α linkages (Kirk and Farrell, 1987). Several agents are believed to participate in lignin degradation including hydrogen peroxide and certain peroxidases called ligninases. In recent years, studies on lignin biodegradation have been accelerated and accumulated due to the use of laboratory synthesized dehydrogenative polymerizate lignins (DHP).

Ligninolytic organisms

Lignin is degraded to different extents by different micoorganisms, of which wood rotting fungi are the most effective, white rot fungi in particular. These predominantly degrade wood from deciduous trees (angiosperms) where they simultaneously attack on lignin, cellulose, and

Table 1. Lignin degrading brown and soft rot fungi and bacteria (Kuhad *et al.*, 1997)

Brown rot fungi	Actinomycetes
Fomitopsis pinicola	*Arthrobacter* sp.
Gleophyllum trabeum	*Microbispora* sp.
Poria placenta	*Nocardia* sp.
Lentinus lepedus	*Streptomyces badius,*
	S.cyaneus, S.setonii
Phaliota adiposa	*S.viridosporus*
Spongiporus sinuosus	*Thermomonospora mesophila*
Tyromyces palustris	
Soft rot fungi	**Other bacteria**
Chaetomium globosum	*Acinetobacter* sp.
Daldinia concentrica	*Xanthomonas* sp.
Lecythophora hoffmannii	*Pseudomonas* sp.
Petrillidium boydii	*Achromobacter* sp.
Pialophora mutabilis	*Aerobacter* sp.
	Erwinia sp.

hemicelluloses, but a few are rather specific (Kuhad, Singh and Eriksson, 1997). Extensive work on lignin degradation exists on the white rot fungi, *Phanerochaete chrysosporium*. The basidiomycetes invade the lumen of wood cells, where they secrete enzymes that decompose and remove the polysaccharides leaving behind a brown modified lignin residue. Studies indicate that a progressive depolymerisation occurs and releases a wide array of low molecular weight fragments. Fragments of kD to predominate (Leisola *et al.*, 1983).

Neither rapid nor extensive bacterial degradation, even under highly aerobic conditions has been reported. Some of the important ligninolytic organisms are listed in Table 1. Ligninolytic *Streptomyces* sp. was reported to degrade pine needles (Kalaichelvan and Ramasamy, 1989) and they possess catabolic plasmids aiding lignin degradation (Sivakumar *et al.*, 1995).

Enzymology

In the complex ligninolytic enzyme system, peroxidases, laccases and H_2O_2 producing oxidases are the most studied. Peroxidases (Lip and MnP) and laccase are defined as phenol oxidases. The reactions catalysed by these enzymes are very similar. They oxidise phenolic compounds, thereby creating phenoxy radicals, while non phenolic compounds are oxidised to the corresponding cation radicals (Gold and Alic, 1993). While all lignin related phenolic compounds are oxidised by phenol oxidases, the different enzymes cleave different substrate ranges for the non phenolic ones. Some of the important ligninases and their mode of action are presented in Table 2.

Ligninase/lignin peroxidase (LiP)

LiP (ligninase, EC 1.11.1.14) was first discovered in *P. chrysosporium* and seems to constitute a major component of the ligniolytic system. These are the extracellular H_2O_2 oxidising enzymes. LiPs catalyze hydrogen peroxide dependent one-electron oxidation of a variety of lignin-related aromatic compounds resulting in the formation of aryl cation radicals. The primary reaction product of LiP with H_2O_2 is the two electron oxidized state compound I, LiPI. Like the horse radish peroxidase (HRP), LiPI is reduced back to the native enzyme *viz* two single electron steps with compound II, LiPII as an intermediate. In the process, the aromatic reducing substrate is oxidized to an aryl cation radical (Ar).

$$LiP + H_2O \text{ ----------> } LiPI + H_2O$$

$$LiP + Ar \text{ ----------> } LiPII + Ar^+$$

$$LiPII + Ar \text{ ----------> } LiP + Ar$$

LiP catalyses a large variety of reactions, e.g. cleavage of β-O-4 ether bonds and C α-C bonds in dimeric lignin model compounts - the basis for the depolymerisation reactions catalysed by LiP. The enzyme also catablyses decarboxylation of phenyl acetic acids, oxidation of aromatic C α alcohols to C α - oxo compounds, hydroxylation, quinone formation and aromatic ring opening. Eh potential in part determines whether an aromatic nuclei, a substrate for LiP. Strong electron-withdrawing group such as an α - carbonyl group tend to deactivate aromatic nuclei, whereas alkoxy groups tend to activate them. There is intense research in LiP since a role for LiP is envisaged in the degradation and detoxification of synthetic aromatic compounds and other xenobiotics like PCB.

Renganathan and Gold (1986) characterised the peroxide oxidised forms by ESR. They demonstrated the formation of compound I (the two electron oxidised form) and compound II (the one electron oxidised form). Compound I was converted to compound II by one electron substrate

Table 2. Ligninases and their mode of action

Types of enzymes involved	Mode of action
Lignin peroxidase (LiP)	The native Fe^{3+} enzyme is first oxidised by H_2O_2 to compound I, and then as one electron reduction of compound I with veratryl alcohol brings the enzyme back to its native form and the catalytic cycle is maintained.
Manganese peroxidase (MnP)	Acts as on phenolic substrates using Mn^{2+} / Mn^{3+} is an intermediate redox couple. The enzyme oxidises Mn II to Mn III which diffuses from the enzyme surface. MnP has also been shown to produce H_2O_2 in the oxidation of glutathione, NADPH and dihydroxy malic acid.
	Catalyse a single electron abstraction from phenolic hydroxylic groups, a substrate common in lignin to produce phenoxy radicals which will *via* non enzymatic steps, result in quinone formation or in polymerisation or depolymerisation of the substrate.
Laccase	Laccase catalyses demethoxylation reactions of terminal phenolic units. Also degrades dimers and α-4 dimers *via* Cβ-o oxidation alkyl - aryl cleavage, and Cα - Cβ cleavage. The non phenolic lignin substructures were oxidised in the presence of a suitable redox mediator, ABTS.
H_2O_2 producing enzymes Intracellular enzymes like glucose 1 oxidase, glucose 2 oxidase, methanol oxidase and fatty acyl CoA oxidase	Glucose oxidase oxidises the product to D gluconolactone and D arabino 2 hexulose. Methanol oxidase oxidises methanol to formaldehyde and H_2O_2. Glyoxal oxidase (GLOX) oxidises several aldehydes and a hydroxy carbonyl compounds which occur as secondary metabolites.

such as phenol, or by 0.5 equivalent of the two electron substrate, veratryl alcohol. In this text, they resemble the horse radish peroxidase (HRP) in many of its properties but it has a higher oxidation potential than HRP.

Manganese peroxidases (MnP)

Manganese peroxidases catalyse the H_2O_2-dependent mediation of lignin and lignin derivatives and a variety of phenolic model compounds. It has been demonstrated that Mn(II) is the preferred substrate for MnP. The enzyme oxidizes Mn(II) to Mn(III) which diffuses from the enzyme surface and in turn oxidizes the phenolic substrates. Organic acids such as oxalate and malonate activate the MnP system by chelating Mn(II) to form soluble complexes with high redox potentials (200 mV) by facilitating the dissociation of Mn(III) from the enzyme.

$$MnP + H_2O_2 \text{ -------> } MnPI + H_2O$$

$$MnPI + Mn(II) \text{ -------> } MnPII + MnP(III)$$

$$MnPII + Mn(II) \text{ -------> } MnP + MnP(III) + H_2O$$

The catalytic cycles of both LiP and MnP are similar to that of HRP. The important difference between these peroxidases is in the nature of the reducing substrate. LiP catalyses the oxidation of nonphenolic lignin model compounds such as veratryl alcohol to veratryl aldehyde. Therefore the unique feature of this enzyme is that it is able to oxidize aromatic compounds with redox potentials beyond the reach of HRP and many other peroxidases. Kinetic results also indicate a 'Ping-Pong' mechanism in which H_2O_2 first catalyses the enzyme and the oxidised enzyme intermediate (Compound I) reacts with veratryl alcohol. The enzyme has an extremely low pH optimum for a peroxidase and its pH dependence apparently controlled by the pH of the catalytic cycle.

Many white rot fungi produces veratryl alcohol which is known to be cofactor involved in thetransformation of lignin, lignin model compounds and xenobiotics by LiP. Mn nutrition was shown to strongly influence the endogenous veratryl alcohol levels in the culture fluids of *Bjerkandera* and *P. chrysosporium*.

Laccase and other phenol oxidising enzymes

The enzyme laccase is widely distributed ir many lignin degrading white rot fungi which has

led to speculation that laccase plays a role in wood and pulp delignification. Bourbonnais *et al.* (1995) envisaged a new role of laccase in lignin biodegradation and their associated activity with Mn peroxidases. Basically, laccases catalyse a single electron abstraction from phenolic hydroxylic groups, a substrate common in lignin to produce a phenoxy radical which will *via* non-enzymatic steps, result in quinone formation or in polymerisation or depolymerisation of the substrate. The substrate range is fairly broad and includes polyphenols, methoxy substituted monophenols and aromatic amines (Thurston, 1994). The substrate range can be extended to non phenolics by inclusion of a mediator such as ABTS. The oxidation of milled wood lignin, demethylation and formation of carboxyl were observed (Bourbonnais *et al.*, 1995).

Laccase oxidises non phenolic compounds with low ionisation potential. It has been widely reported that unlike other white rot fungi *P. chrysosporium* BKM F1767 produces extracellular laccase in a defined medium containing cellulose and either 2.4 or 24mM tartarate. Of late, the practical use of lignin peroxidases has been questioned for biobleaching resulting in a renewed interest in laccase (Archibald, 1992). Positive character of laccase in polymerising the simple phenolics was exploited for the synthesis of humic acid related material (Ramasamy *et al.*, 1994).

Other enzymes

Crude cell extracts from the ligninolytic cultures produce H_2O_2 in the presence of added glucose. Kelley and Reddy (1986) concluded that glucose oxidase is the primary source of H_2O_2. The glyoxal, methyl glyoxal and other hydroxyl, carbonyl and dicarbonyl compounds serve as substrates for the ligninases.

Physiology of lignin degradation

In the case of white rot fungi, lignin is degraded only during secondary metabolism, whose onset is triggered by depleting cultures for nutrient nitrogen, carbon, or sulfur. Nitrogen limitation is most commonly used for ligninase expressions. But the lignin degradation by other white rot fungi are not stimulated by N limitation. Concurrent to the onset of ligninolytic activity, there was an increase in the levels of intracellular levels of cyclic AMP but the relation is yet to be established with ligninase expression (Gold and Alic, 1993). Lignin metabolism requires a cosubstrate such as glucose with increasing levels of oxygen. The presence of MnO_2 is also important in lignin degradation.

The heme proteins separated from the extracellular fluid of *P. chrysosporium* BKMF-1767 grown in defined low N medium containing dimethyl succinate (DMS) buffer have been arbitrarily designated as H1, H2,....H10. The 'H' refers to the heme protein. H1, H2, Hy, H7, H8 and H10 are shown to have LiP activity as typically assayed spectrometrically by measuring the oxidation of veratryl alcohol to veratraldehyde in the presence of H_2O_2 (Tien and Krik, 1983). H3, H4, H5, H9 were shown to have MnP activity as measured by the oxidation of Mn(II) to Mn(III) in the presence of H_2O_2 (Glenn and Gold, 1983).

It was established that the LiP isozymes are related to each other by peptide mapping analysis. Isozymes H1 and H3 fall into one class; H6, H7 and H8 belong to a second class and isozyme H10 belongs to a third class. The enzymes are also glycosylated with a pI ranging from 3.2 to 4.0 and molecular masses ranging from 38 to 43 kDa. Each enzyme also contains 1 mol of iron heme per mol of protein (Glenn and Gold, 1983). The MnP has also been purified to electrophoretic homogeneity. The enzyme exists as a series of glycosylated isozymes with Mr ranging from 45 to 47 kDa. Each isozyme also contains 1 mol of heme per mol of protein.

The increase in total activity was caused by increase in more than one isozyme. H1 and H2 increased the most as a resut of additives. The effect of veratryl alcohol on H6, H7, H8 and H10 was not as great as on H1 and H2. Several nutritional and cultural parameters are important for lignin degradation like a) the presence of a cometabolisable substrate, b) high oxygen tension, c) growth as mycelial mats rather than as submrged pellets in agitated cultures, d) choice of the buffer, e) correct levels of certain minerals and trace elements and f) growth limiting levels of nutrient nitrogen.

Regulation of LiP production

The production of LiPs and MnPs is completely suppressed under conditions of excess N and C in the medium. In defined low-nitogen liquid medium, MnP activity generally peaks on day 4 and LiP activity on day 6. The manganous levels in the medium has a dramatic effect on the production of both LiPs and MnPs. With 12 ppm Mn(II) in medium, high levels of LiP and MnP were noticed but at 40-100 ppm no LiP production occurred whereas, the MnP production increased. On the other hand in 0 ppm of Mn(II), no MnPs were produced but the LiPs were expressed. The presence of Mn(II) selectively expresses MnPs rather than LiPs at excess concentration and it is absolutely needed for expression of *mnp* genes. An enzymatic fraction responsible LiP isozyme termed LiP dephosphorylating (LpD) fraction was purified. Incubation of the LpD fraction with ^{32}P labeled H2, H6, H8 and H10 isozymes separated from nitrogen limited cultures resulted in the formation the dephosphorylated isozymes H1, Ha, Hb and Hc respectively. Dephosphorylation did not significantly change the catalytic properties of the LiP isozymes with veratryl alcohol as a substrate. LiP dephosphorylation is therefore suggested to be a post translation modification process catalysed extracellularly by the LpD activity (Rothschild, Hadar and Dosoretz, 1997).

Other studies demonstrated that the production of LiP vary greatly with strain and culture condition employed and in turn suggested that the expression of different *lip* genes is regulated in response to different environmental conditions such as acetate buffering as against DMS buffering; shaking and agitation during the growth, etc.

The effect of the oxygen level on the formation of the ligninolytic enzyme system in a nonimmersed liquid culture under different C/N ratios was studied. LiP activity was seen if exposed to air when C/N ratio was low. They also do not secrete extracellular polysaccharides. Under high N condition, LiP production was 1800 U/l if exposed to pure oxygen and 1300 U/l in cultures exposed to air, with H1 and H2 being the main isozymes. The formation of MnP was generally less affected by the O_2 level and C/N ratio on the synthesis of glyoxal oxidase paralelled their effects on LiP synthesis except in the case of high N which totally suppressed glyoxal oxidase activity (Rothschild, Hadar and Dosoretz, 1995).

The lignin degradation is also markedly suppressed in the presence of the OH scavenging agents like the benzoate, butylated hydroxy toluene and mannitol. This was confirmed by the non pleiotrophic glucose oxidase negative mutants of *P. chrysosporium* where there was lack of lignin degradation (Kelly, Ramasamy and Reddy, 1986). But in the revertant mutants, lignin degrading ability was restored (Ramasamy, Kelly and Reddy, 1985).

Potential applications of ligninolytic systems

The increase of oil price in the seventies has prompted much research on potential applications for lignin as a readily available renewable source and as a replacement for petrochemical derivatives. The development of techniques for the production of novel engineered materials based on lignin components could open new possibilities for the manufacture of value added products from lignins (Kalaichelvan and Ramasamy, 1990).

Lignins in industries

As a potential source of chemicals and energy, more than 50 million tons of lignin are produced per year by the pulp and paper industries. These are called as sulfate or kraft lignins from the alkaline pulping process and as lignin sulfonates from sulfite pulping process. Most of the kraft lignin which has a calorific value of 23.4 MJ/kg is burnt as an energy source and for recovery of pulping chemicals. Part of the energy generated can be used for evaporation of kraft black liquor. Organosolv lignins are obtained by extraction of wood with organic solvents like ethanol or *n*-butanol. Steam exploded lignins are obtained by treatment of wood at high temperatures and pressures for a short period of time followed by a sudden decompression. Relatively unmodified and low molecular weight lignins without large amount of impurities are obtained by these methods and these could provide a suitable starting material for chemically or enzymatically catalysed lignin modifications. Organosolv lignins have been used for the production of polurethane foams and phenol-formaldehyde resins.

Table 3. **Effects of a wild and mutant strain of *Trametes versicolor* on hardwood kraft pulp brightness and lignin content with or without added MnP (Addleman *et al.*, 1995)**

Trial	Brightness	Kappa no.	Laccase activity	MnP activity (AU/L)
No fungus	39.1	11.0	0	0
MnP alone	38.9	10.7	0	350
Wild	55.0	8.8	66	790
Mutant	41.3	11.0	4	0
Mutant+MnP	46.9	9.5	8	780

From lignosulfonates and kraft lignins, both low molecular and high molecular weight products can be obtained for a multitude of uses. Some examples of applications which have been described in patents include drilling mud additive, rubber additive, dyestuff filler, ink with lignin based colorant, lignin derivative surfactant, cationic lignin amine surface active agent, lignin-epoxysuccinate adduct as dispersant and metal complexing agent, lignin reinforced polymer, polyvinyl alcohol and lignin sulfonate containing adhesive, synthetic lignin-polyisocyanate resin, polyoxyalkylene ether polyols from lignin, isocyanate lignin products, co- polymerisable colorant in polyesters, lignin use in pharmaceuticals, lignosulfonate phenoplasts, acid-polymerised lignosulfonate as extender, polymerisable lignin derivatives, veneer cross-binder, binders for glass fiber insulation, lignin sulfonate-phenol-formaldehyde glue system, clarification of aqueous solutions with polymeric lignins, lignin - diepoxide flocculant, lignin - urea composition for flame proofing, ligninamine asphalt emulsifier, soil conditioner and fertilizer and slow release pesticides.

Biopulping and biobleaching

White rot fungi and their ligninolytic enzymes have potential application in biopulping and biobleaching (Reid and Paice, 1994; Akhtar, Blanchatte and Kirk, 1997; Bajpai and Bajpai, 1997). It seems to be effective in reducing the refinery energy requirement by 20-30 per cent. The removal of lignin from wood for the production of cellulose fibres is an important step in the paper making process. The pulp and paper industry produces vast amounts of chemically modified lignin as by-products or waste materials. Potential applications of lignin biodegrading systems can be seen for the removal of lignin in wood chips or pulps in the production of cellulose fibres. Similarly biological treatment of effluents from the pulping and bleaching processes which contain highly coloured and toxic lignin compounds would reduce the relase of large amounts of harmful chemicals into the environment.

Laccase has been shown to demethylate kraft pulp and non-phenolic compounds and recently laccase has been shown to be present in high amounts along with Mn-peroxidases in cultures of *Trametes versicolor* with abilities to bleach kraft pulp (Boubonnais *et al.*, 1995). Addleman *et al.*, (1995) proved that laccase component is necessary to bleach kraft kiquor with the help of mutation studies. The fact that the mutant also lacked MnP (Mn peroxidases) might mean that there is a common control for both enzymes.

Four white rot isolates were checked for their bleaching ability against *P. chrysosporium* a known pulping organism. The ratio of weight loss to lignin loss was used as an indication of the ability of the organisms to produce pulp without too much damage to other components. Attempts have also been made to bleach kraft pulp with ligninoytic systems. A ligninolytic fungi IZU-154 has been successfully employed for further bleaching of oxygen bleached kraft pulp. They showed that there is marked increase in brightness index by 17 and 22 points by a three day and five day treatment respectively and the kappa number was decreased from 10.1 to 6.4 by five day treatment. Significantly, the yield loss was less than 1 per cent making the fungus suitable for biobleaching process. Recently *Trametes versicolor* has been shown to increase the kraft pulp brightness (Table 3) and the involvement of laccase and Mn peroxidase has been established (Addleman *et al.*, 1995).

Though documental evidences are available on the use of ligninolytic system for decolorisation, the role played by ligninolytic enzyme, lignin peroxidase (LiP), has been questioned. Archibald (1992) suggested that Lip has no significant role in *T. versicolor* pulping system. But another oxidase type enzyme capable of degrading 2-keto-4-methiol butyric acid (KMB) appeared unique to ligninolytic

Table 4. **Dye decolorisation by *Phanerochacte chrysosporium* in N limited condition (Glen and Gold, 1983)**

per cent decolorisation after Dye	1h	6h	24h	48h	5d
Azure B	90	97	100	100	100
Tropeolin O	78	91	95	96	96
Orange II	57	67	91	98	100
Congored	58	87	93	95	97

systems in pulp decolorisation. He also arrived at this conclusion since the addition of biobleaching culture filtrate to a purified LiP proved to be inhibitory. So the application of whole cell system is now suggested along with hemicellulase bleaching during pulp bleaching until the exact system of decolorisation is worked out. The combined treatment will considerably lessen the chlorolignin content and also in considerable energy savings of about 20-30 per cent to offset the cost of biological treatment.

Waste water treatment

In India, paper and pulp industry constitutes an important segment of industrial scene. The Indian paper mills have an installed capacity of about 3.0 million tonnes per annum. Bajpai and Bajpai (1992) examined the possibility of using various white rot fungi on raw effluent. They found that *Trametes versicolor* B7 is capable of reducing 84 per cent of the colour within 4 days. Use of glucose in *T. versicolor* treatment up to 5 g/L increased the colour reducing capacity of *T. versicolor* but additional nitrogen sources did not enhance the colour reduction. The inoculum load at 2 g/µc resulted in 84 per cent colour reduction. They also found that a pH of 4.5-5.0 is suitable for optimum colour reduction. In addition, 38 per cent of the the chemical oxygen demand (COD) was reduced in 48 h in a batch fermentation.

They Mycor (mycelial colour removal) reactor was developed to treat the effluent from the kraft pulp process. The reactor used a fixed film of *P. chrysosporium* immobilised on the surface of the reactor's rotating discs to degrade such compounds as the chlorinated derivatives of guiacol and vanillin and dichlorobenzoate (Huynh, Chang and Joyce, 1985). Besides removing about 80 per cent colour in the spent liquor, about 70 per cent of the organically bound halogen as chloride was also liberated. Efficiency was further improved by the addition of benzyl alcohol or sorbitol. Lewandowski, Armenante and Pak (1990) also developed a reactor for the degradation of a model toxicant 2-chlorophenol by *P. chrysosporium*. They found that immobilisation of alginate beads improved the efficiency by a factor of 40 over a mechanically stirred fermentor. Under well mixed conditions, this reactor and also packed bed system employing a a silica based porous support for the fungus achieved degradation of 2 chlorophenol at feeds upto 520 ppm.

Biodegradation of azo and heterocyclic dyes

Glenn and Gold (1983) first established that ligninolytic cultures of *Phanerochaete chrysosporium* decolourise several polymeric dyes. More recently, decolourisation of the azo dyes Orange II, Tropeolin O. Congo red. Acid red 114. Acid red 88, Biebrich Scarlet. Direct blue 15. Chrysophenine, Tetrazine. and yello and triphenylmethane dyes Basic green 4, Crystal violet, Brilliant green, Cresol red, Bromophenol blue and pararosa anilines has been demonstrated by Crawford *et al.* (1982). It was observed that low nitrogen level was better that optimum nitrogen for colour removal (Table 4).

Purified LiP could achieve a 80 per cent reduction in colour of Azure B, 42 per cent in Orange II, 20 per cent in tropeolin but no effect was noticed in Congo Red, a complex heterocyclic dye (Paszczynski *et al.*, 1992). Decolorisation however demonstrates only transformation of the chromophoric group of the dye and not complete degradation of the dye. Spadaro, Gold and Renganathan (1992) proved that ligninase and manganese peroxidase from *Phanerochaete chrysosporium* were involved in the degradation. They formed a new concept of enhancing the degradability of azodyes by linking selected, readily degradable substitutents into the chemical structure of recalcitrant dyes.

Degradation of xenobiotics

P. chrysosporium has been shown to degrade and mineralise a wide variety of agricultural and industrial pollutants (Higson, 1991) mainly due to its ligninase system. This enzyme has been shown

to catalyse the initial oxidation of several persistent xenobiotics including PAH, chloroaromatic compounds, nitrogluenes and dioxane (Valli, Warishi and Gold, 1992). The system has limited solubility in water and is not readily available in soil to intracellular metabolism (Higson, 1991).

In remediating PAH contaminated sites (Bogan and Lamar, 1995) the degradation is initiated by free radicals mechanisms: PAH-s with ionisation potentials at or less approximately 7.55 eV are substrates for direct one electron oxidation by LiP, while those higher than this threshold are apparently acted on by radical species formed during MnP dependent lipid peroxidation reactions (Moen and Hammel, 1994). For the degradation of endosulfan, it utilised oxidative and hydrolytic pathways for metabolism. Piperonyl butoxide, a known cytochrome P 450 inhibitor significantly inhibited the oxidation of endosulfan to endosulfan sulphate and enhanced the hydrolysis to endosulfan diol. Judging the inactivity of extracellular fluid and partially purified lignin peroxidases revealed that for metabolism of this compound, it does not involve the action of extracellular peroxidases (Kullman and Matsumura, 1996).

Removal and degradation of pentachlorophenol by *P. chrysosporium* in static cultures was studied using ammonium lignosulfonate (LS), a waste product from paper mill in which 72-75 per cent lignin was removed. The PCP dehalogenation occurred despite the fact that LiP activity was inhibited by LS. Maximum mineralisation was observed after 13 days and the intermediates were mostly the organic halides. The study on mRNA of 10 known lignin peroxidase genes in anthracene transforming soil cultures of *P. chrysosporium* revealed that the anthracene transformation occurred throughout and likely to involve mechanisms distinct from those involved in oxidation of non LiP substrate PAH (Boga *et al.* 1996). The other xenobiotics effectively degraded are the pesticides (DDT), chlorinated phenoxyacetates (2,4 D and 2,4,5, T), chlorinated anilines, polychlorinated phenols, chlorinated alicyclic insecticides, polychlorinated biphenyls and 2,4 TNT (Higson, 1991).

When bleach plant effluents were treated with the white rot fungi, high rates of decolorisation, dechlorination and a decrease in COD were observed. In long-term experiments with bleaching effluent of the alkaline stage of a sulfite mill, a maximum of 80 per cent decolorisation was accompanied by 50 per cent COD reduction and 80 per cent toxicity reduction against *Daphnia magna* after one passage through a trickling filter of 1 m length.

Another area of application ligninolytic system is in the treatment of lignosulfonates, produced as a by product in sulfite pulping. Ritter *et al.* (1990) employed *Phanerochaete chrysosporium* cultivated on foam cubes according to MYCOPOR process. They employed whole cells of *P. chrysosporium* to polymerise the lignosulfonates as dark brown compounds which are depolymerised and degraded later. Diluting the sulfite liquor decreased the lignosulfonate degradation by 30 per cent when the undiluted liquor can be degraded to an extent of 70-80 per cent on the 16th day. The degradation rates might be too slow for industrial level needs but useful chemicals can be produced if metabolic mutants are developed and a part of the liquor can be productively used.

Substrate for crude fungal and edible protein

Conversion of wood, bark, and lignocellulosic wastes into crude fungal protein has also received attention, although the most effective systems developed so far involve the utilisation of water-soluble carbohydrates. White-rot fungi growing on lignified materials are comparatively poor candidates for this type of technology due to their relatively slow growth rates. However, a continuous process which uses *P. chrysosporium* for protein production from mixed lignocellulosic wastes accompanied with water purification has been proposed. Considerably more attractive commercially is the direct cultiavtion of edible mushrooms on wood or agricultural residues (Table 5). This approach already represents a major industry in Japan and other part of Asia where the forest mushroom *L.edodes*, or shiitake is particularly important (Kirk, Higuchi and Chang, 1980).

Conversion of lignocellulosics

Agricultural and forest industry byproducts unsuited for pulping are potential sources of dietry

Table 5. Commercial mushroom cultivation on untreated lignocellulosic substrates (Kirk *et al.*, 1980)

Species	Substrates
Auricularia spp.	Hardwood logs
Coprinus fimetarius	Straws
Flamulina velutipes	Sawdust
Lentinus edodes	Hardwood logs, Sawdust
Pholiota nameko	Hardwood logs
Pleurotus spp.	Hardwood logs, Sawdust, Straw
Tremella fuciformis	Hardwood logs
Volvariella volvacea	Straws

energy for ruminants. However, the feed value is limited by the low digestion in the rumen, mainly due to lignin. Zadrazil and Brunnert (1980) have examined the ability of several white rot fungi to convert the straw and other plant wastes into feed through processes like the solid state fermentations. Relatively higher lignin degradation was achieved with straw compared to wood. The solid state fermentation of aspen wood with *Merulinus tremellosus* removed 52 per cent of the lignin and increased the digestability from 18 - 53 per cent.

The other applications include the production of chemicals. A variety of valuable low molecular weight compounds formed as intermediates during lignin degradation could be used. *P. chrysosporium* produces vanillic acid, isovanillic acid, veratric acid, m-hemipimic acid and dehydrodevanillic acid from soft rot lignin. A water soluble polymeric lignin carbohydrate complex is produced during degradation of graminaceous lignocellulose by actinomycetes. This high molecular weight material is enriched in phenolic hydroxy groups and its use as chemical feed stock for industrial applications. The lignocellulosics like the coir waste could be efficiently composted by the ligninolytic systems of *Pleurotus sojar caju* which is commercially used for on lignin rich waste to hasten the process (Ramasamy, Sesha Reddy and Chitra, 1989 of composting).

Anaerobic degradation of lignin

So far, much work has been done on the degradation of aromatic monomers released by

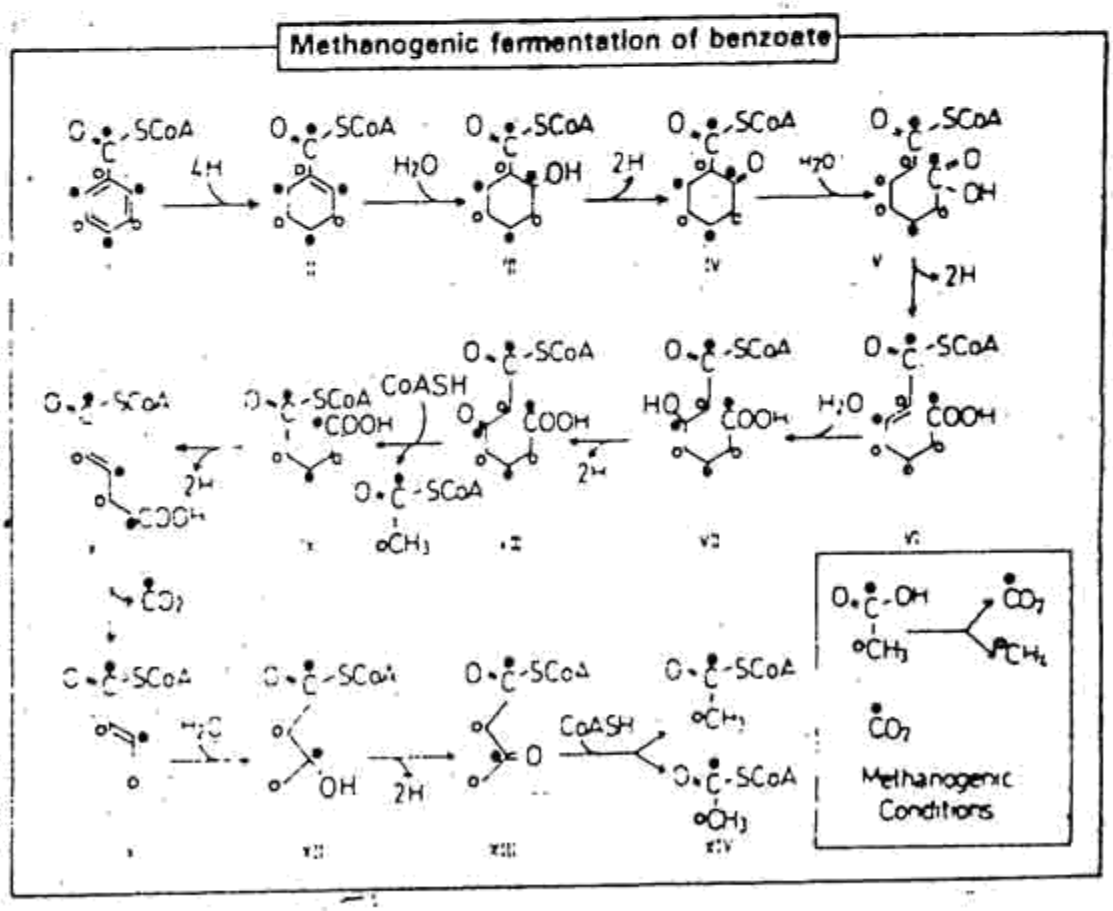

Fig.1. Proposed pathway of benzoate fermentation to CH_4 and CO_2 under methanogenic conditions (Fuchs *et al.*, 1989) (I) Benzoyl CoA, (II) cyclo-1-ene carboxyl CoA, (III) trans -2-hydroxy cyclo- hexane carboxyl CoA, (IV) 2-oxocyclohexane carboxyl CoA, (V) pimelyl-CoA, (VI) 2,3 dihydropimelyl CoA, (VII) 3-hydroxy pimelyl CoA, (VIII) 3-oxopimelyl CoA, (X) glutaconyl CoA, (XI) crotonyl CoA, (XII) 3-hydroxybutyryl CoA, (XIII) 3-oxobutyryl CoA, (XIV) acetyl CoA.

aerobic ligninolytic organisms. Their fate in natural systems has yet to be determined. However, it has been postulated that if further biodegradation does not occur in aerobic environment, such compound may eventually enter anaerobic sediments where they would be subjected to anaerobic transformations. The first evidence on anaerobic degradation of corn stalks was reported by Boruff and Buswell (1934). About 54 per cent of the lignin was converted to CO_2 and CH_4 after 600 days of incubation. Although elucidation of pathways is still lacking, anaerobic metabolism of aromatic monomers is now known to occur during anaerobic photometabolism, under nitrate and sulphate reducing conditions. Four major aromatic compounds i.e., benzoic, phenylacetic phenylpropionic and cinnamic acids widely studied were completely mineralised to CO_2 and CH_4 by an inoculum of sewage sludge.

In the absence of oxygen, the degradation of aromatics necessarily require a novel means of ring fission. The aromatic ring is activated in such a manner that it can be reduced by specific enzyme. Benzoate was used as model substrate and in common involve benzoyl CoA. in which the aromatic ring is activated by the adjacent thioesterified carboxyl group. The products of benzoyl CoA degradation by the methanogenic

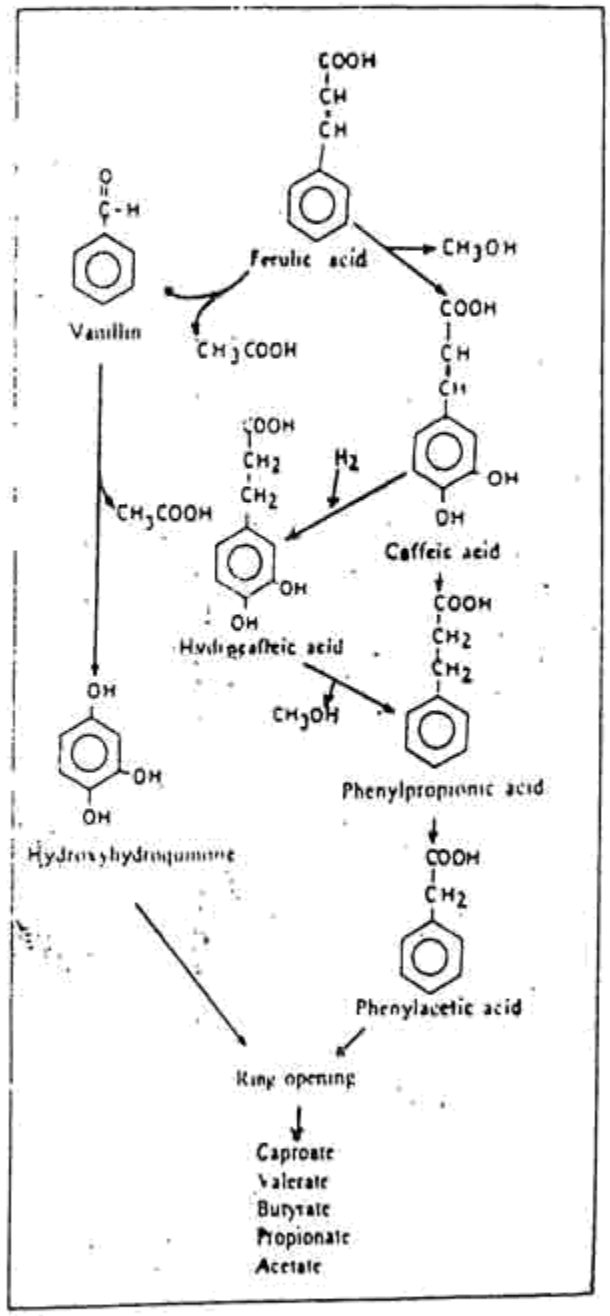

Fig.2. Ferulic acid degradation under anaerobic conditions (Doraisamy, 1994)

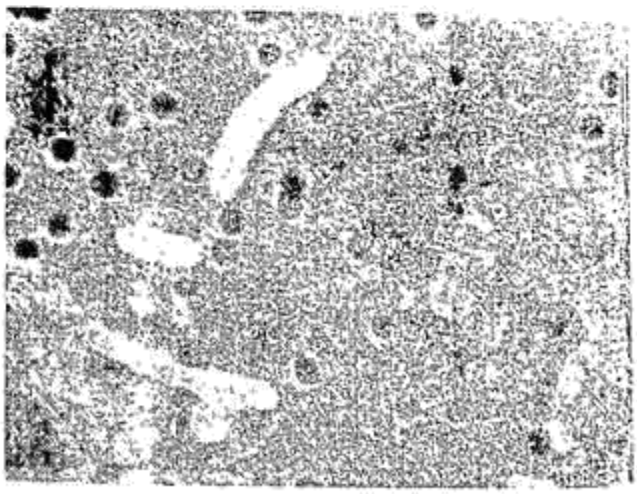

Fig.3. Scanning electron micrograph of the ferulic acid degrading anaerobic strain (x 6000) Doraisamy, 1994)

consortia are acetyl CoA, CO_2 from C2/C6 of the ring carbon and six reducing equivalents (Fig.1) (Fuchs *et al.*, 1994). Ferulic acid gets catabolised in many steps to vanillin, caffeic acid, phenyl propionic acid and hydroquinone (Fig.2) performed by anaerobic rod shaped bacteria isolated from the enrichments (Fig.3) (Doraisamy, 1994). The phenol degradation by a stable methanogenic consortia was also demonstrated whereby a 50 per cent reduction was achieved (Doraisamy and Ramasamy, 1996).

SUMMARY

Lignin, an important cell wall constituent is abundant in nature. It is highly complex in its structure which involves a variety of microorganisms and their enzymes to perform degradation. The basidiomycetes fungi are the major group involved and studied in detail in view of its efficient ligninolytic system. These organisms release its ligninases during the secondary metabolism of growth under nitrogen, carbon or sulphur limited conditions. The major enzymes involved are the lignin peroxidases, Mn peroxidases and laccases and their release is highly specific to the substrate. Upon chemical/biological degradation the lignin yields many intermediary products which find numerous applications, major being the biopulping, biobleaching and bioremediation of pollutants. Also, recently, it was reported that the commercially viable hydroxy styrenes could be obtained on degradation of the lignin rich maize seedcoats. Though many applications are available, the utilisation is not a commercially viable competitive method owing to the same innate complexity. This remains a further task to the scientific community besides elucidating the anaerobic metabolism of lignin.

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