Prospects for Inhibition of Lignin Degrading Enzymes to Control *Ganoderma* White Rot of Oil Palm

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Abstract: Oil palm (OP) is prone to a rot by the fungus *Ganoderma* which may be capable of being controlled by enzyme inhibitors. Palm oil is used in the production of vegetable oil for foods, cosmetics, pharmaceuticals and, most recently, biodiesel. However, the fundamental process of the disease as “white rot” has been ignored by researchers. White rot fungi are capable of degrading lignin ultimately to carbon dioxide and water: Celluloses become available as nutrients for the fungus. One potential control method is to inhibit the ligninolytic enzymes. There are few data on the lignin of OP and none on how it is degraded by OP *Ganoderma* and so specific examples on how to inhibit the enzymes of the fungus is impossible. Fortunately, there is more information on lignin and lignin model compounds degraded by other fungi. The taxonomy of *Ganoderma* is confused; hence drawing direct comparisons between other taxa within the genus in terms of ligninolysis is of limited utility. In general, ligninolytic enzymes can be inhibited by (a) temperature, pH and aeration, (b) high carbon and high nitrogen and (c) halides, metal chelators, heavy metals, and reducing agents. These factors require to be tested against the enzymes from *Ganoderma* from OPs *in vitro* with a view to developing control methods in the field, and this is how the area requires to be progressed. Furthermore, the procedures may be useful to control other rots of trees and wood products. In the case of OP, such compounds could be (a) injected into (b) sprayed onto and/or (c) added to the soil of the OP. In conclusion, the control of *Ganoderma* of OP would benefit from investigating the effect of inhibitors on the lignin degrading enzymes *in vitro* to enable this to be applied in plantations.

Keywords: Oil palm, palm oil, *Ganoderma*, white rot, lignin, laccase, manganese peroxidase.

INTRODUCTION

Palm oil is a very important commodity the current market value of which has trebled recently. The palm is subjected to a white rot by the fungus *Ganoderma* [1], which may be controlled by enzyme inhibitors. *Ganoderma* disease is the major constraint in the traditional growing countries, Malaysia and Indonesia [2]. Oil palms (OP) are grown increasingly in other countries with tropical climates (e.g. Latin America). The oil is employed in approximately 10% of food and cosmetics and it is also has pharmaceutical properties for which there is a market. The economic value of the product has increased significantly by it becoming a recognised biofuel. A recent workshop confirmed that the palm oil industry takes the disease seriously indeed [3]. However, major collected works on the disease [4-6] have failed to focus on the fact that *Ganoderma* is a white rot fungus that degrades lignin by employing an arsenal of extracellular enzymes.

This is the topic that was addressed by Paterson [2] to redress the balance. By implication, *Ganoderma* attacks lignin to obtain energy from cellulose. In addition, Paterson *et al.* [7] indicated how altering the lignin in OP may be possible and what the current constraints may be to employ this as a control measure to inhibit *Ganoderma*. Another review addresses the secondary metabolite production of *Ganoderma*,

some of which may be derived from the lignin of host trees [8]. These metabolites may be involved in the disease process and could act as diagnostic aids for the disease. Indeed, lignin biodegradation is effectively a secondary metabolic process undertaken by the fungi.

Lignin biodegradation is crucial for carbon recycling in land ecosystems. White rot fungi degrade (a) many living trees and (b) wood used in manufacturing causing billion-dollar losses, and so methods to reduce decay of these are also required. In addition, white rot fungi are being investigated to treat industrial pollutants because these can act as substrates for the unspecific ligninolytic enzymes produced [9]. Basidiomycete fungi are the predominant organisms responsible because they possess the complete lignin degradation capabilities – an ancient evolutionary trait. They are loosely classified as white-rot and brown-rot fungi based mainly on macroscopic characteristics of the rotted wood. These fungi overcome limitations to decay including the low nitrogen content of wood (OP stem is 0.9% protein), by translocation and the presence of toxic and antibiotic compounds probably by detoxification [10]. Incidentally, low nitrogen is a factor that increases lignin degradation (see later).

LIGNOCELLULOSIC STRUCTURE

An in-depth review of lignin structure and degradation is required if an understanding of how to inhibit the ligninolytic enzymes is to be obtained, because of the complexity of the fields. Lignocellulosic materials (e.g. wood) are

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formed by three predominant polymeric constituents, cellulose, lignin, and hemicelluloses. Furthermore, OP stem has a high concentration of starch [11] which may influence decay. Obviously, a sound knowledge of the structure of lignin is beneficial if the intention is to inhibit the enzymes involved in degrading the substrate. Lignin is three-dimensional and built of dimethoxylated, monomethoxylated and non-methoxylated phenylpropanoid units (syringyl, guaiacyl, and *p*-hydroxyphenyl) (S, G and H) respectively. These are derived from the corresponding *p*-hydroxycinnamyl alcohols, giving a variety of subunits including various ether and C—C bonds. Acetylated lignin units are present in non-woody plants. However, it is becoming clear increasingly that lignins are derived from several more precursors than simply the three traditional monolignols [12].

There are two factors that affect the cell wall degradation rate, the (a) lignin concentration and (b) monomer composition. Lignin is impervious exquisitely to chemical and biological degradation, and confers mechanical resistance to wood (Martínez *et al.* [13]). However, it is more useful to consider the role of lignin as to protect cellulose - the component of real mechanical strength. The highest concentration of lignin is in the middle lamella, cementing wood fibres, but it is also present in the cell wall (especially the secondary cell-wall), forming an amorphous matrix in which the cellulose fibrils are embedded and protected against biodegradation together with hemicelluloses. Vascular bundles in the stem are scattered in monocots. The situation for OP requires being determined.

It is imperative that the components of OP lignin are known (see later). The H:G:S ratio of lignin varies between different vascular plant groups: Woody gymnosperms (softwoods) possess the highest lignin concentration which consists of predominantly G units. That of woody angiosperms (hardwoods) (e.g. palms) consists of S and G, and non-woody angiosperms also contain H. However, the lignin of Ceibo, Ulin wood, Bangkirai, Koki, Malas, are primarily constituted of guaiacyl units, notwithstanding the fact the woods are "typical" hardwoods. The major structural differences between guaiacyl and syringyl/guaiacyl lignins are probably associated with the degree of condensation - involving C—C and C—O—C linkages, primarily at C5 [14]. Composition between wood tissues and cell-wall layers varies: Middle-lamella lignin typically has a lower S/G ratio than that from the secondary wall (making it liable to degradation). In complete contrast, cellulose is a linear and ordered polymer of cellobiose (D-glucopyranosyl- α -1,4-D-glucopyranose) that represents over 50% of wood weight. The macromolecule is often present in crystalline form which is more difficult to degrade.

Fibres, vessels, and parenchyma cells form predominantly the wood tissue elements. In gymnosperms wood tissues consist of 90–95% tracheid cells (softwood fibres) and low amounts of parenchyma, which includes the specialized resin channels in conifers. They have a simpler structure than in angiosperms. Parenchymatic rays have a radial arrangement in wood and contain phenolic and lipophilic extractives and water-soluble compounds as storage material. Vessels are large cells with a longitudinal arrangement and transport water and nutrients along the plant stem. Finally, fibres rep-

resent most of the wood volume and are characterized by their thick cell walls, which are also longitudinally arranged and provide support for the tree [13]. This information on the architectural arrangements is important when considering how fungi degrade plant materials

CHEMICAL ANALYSIS OF LIGNIN

The complex chemical structure of lignin is required to be understood if lignin degradation and its inhibition, are to be comprehended. Lignin is estimated traditionally by the Klason method, based on (a) total acid hydrolysis of polysaccharides, (b) determining ash and protein and (c) gravimetric estimation of the lignin concentration. The method is time-consuming, and requires a considerable sample volume. Klason lignin is often combined with analysis of wood polysaccharide composition by GC of monosaccharides in the acid hydrolysate. In one of the rare works on OP, trunk fibre had the following chemical composition (% dry wt, w/w): cellulose 41.2%, hemicelluloses 34.4%, lignin 17.1%, ash 3.4%, extractives 0.5%, and ethanol solubles 2.3% [15]. Syringaldehyde was the predominant phenolic component (important because lignin is a polyphenol), which comprised 65.6–68.5% of the total phenolic monomers in the oxidation mixtures. This basic result may explain the high degree of biodegradability of OP as this unit is more susceptible compared to guaiacyl containing wood lignin [16]. Vanillin was the second major phenolic component. The presence of syringaldehyde and vanillin results from the degradation of non-condensed syringyl and guaiacyl units respectively. The lower yields of alkaline nitrobenzene oxidation of these lignin fractions indicated a higher degree of condensation of the isolated lignins compared to the corresponding yields of hardwood lignins. The authors found that the lignin in OP trunk contained a high proportion of aryl ether-linked syringyl units. The presence of (a) a large proportion of non-condensed syringyl, (b) a small amount of guaiacyl and (c) fewer *p*-hydroxyphenyl units indicated that the fractions can be considered as straw or grass type lignin. This is helpful, as more information is available on these lignins compared to that for OP. In addition, lignin of OP frond was characterised by the presence of significant amounts of esterified *p*-hydroxybenzoic acid together with small amounts of etherified *p*-hydroxybenzoic acid. Vanillic and syringic acids were esterified or etherified to lignin. Wall polysaccharides of OP frond are composed of cellulose and significantly high concentration of arabinoxylan [17].

FUNGAL ROTS

The enzymes involved in the process include laccases, high redox potential ligninolytic peroxidases (lignin peroxidase, manganese peroxidase, and versatile peroxidase), and oxidases. It is important to appreciate the externally-produced nature of the enzymes, especially in relation to the extraordinary extracellular splitting of the aromatic ring of lignin degradation products. It is this step which is most important to target for inhibition on the face of it, although it is perhaps more valid to focus on the steps (i.e. enzymes) before degradation has progressed to this stage. The reactions catalyzed by the enzymes are considered herein, including

the synergistic action on lignin, and the structural bases for their unique catalytic properties. This will contribute fundamentally to better control of OP decay by *Ganoderma* by widening our knowledge of lignocelluloses biodegradation.

The state of knowledge concerning the enzymes involved in lignin biodegradation was described by Martínez *et al.* [13] (these authors supply a useful Figure in their paper which can be referred to). Laccases or ligninolytic peroxidases produced by white-rot fungi oxidize the lignin polymer, and generate aromatic radicals. These take part in non-enzymatic reactions: (1) C4-ether breakdown, (2) aromatic ring cleavage, (3) C α -C β breakdown, and (4) demethoxylation. The substrates for H₂O₂ generation by AAO in cyclic redox reactions involving also AAD are the aromatic aldehydes released from C α -C β breakdown of lignin, or synthesized de novo by fungi. Phenoxy radicals from C4-ether breakdown can repolymerize on the lignin polymer if they are not first reduced by oxidases to phenolic compounds, as reported for AAO. The phenolic compounds formed can be again reoxidized by laccases or peroxidases. Phenoxy radicals can also be subjected to C α -C β breakdown, yielding *p*-quinones. Quinones contribute to oxygen activation in redox cycling reactions involving QR, laccases, and peroxidases. This results in reduction of the ferric iron present in wood by superoxide cation radicals or directly by the semiquinone radicals, and its reoxidation with concomitant reduction of H₂O₂ to the hydroxyl free radical (OH $\cdot$). The OH $\cdot$ is a very strong oxidizer that can initiate the attack on lignin in the initial stages of wood decay, when the small size of pores in the still-intact cell wall prevents the penetration of ligninolytic enzymes. Then, lignin degradation proceeds by oxidative attack of the enzymes described above. Finally, simple products from lignin degradation enter the fungal hyphae and are metabolised *via* intracellular catabolic routes.

The most frequently-encountered wood-rotting organisms are the white-rot basidiomycetes which are characterized by the ability to degrade lignin, hemicelluloses and cellulose, sometimes resulting in cellulose-enriched white material. However, it is perhaps not too obvious to mention that wood subjected to white rot do not always become this colour. White rots can be further subdivided into the ability to degrade lignin selectively or simultaneously with cellulose. The two patterns are associated with different types of wood, i.e. sequential decay (or selective delignification), and simultaneous rot. Clearly, the situation requires being determined for *Ganoderma* on OP to confirm the white rot mode. Currently, it is assumed rather than demonstrated, although it has been referred to as a dry rot (or even "dry stem rot" [6], which is surely a misunderstanding of the basic definitions of fungal rots [2]. The most convincing evidence of these fungi as white rots is from an unedited extended conference abstract by Ali *et al.* [18], which clearly indicates the pathogenic *Ganoderma* of OP (a) produce lignin degrading enzymes, and (b) reduce lignin in dead OP samples *in vitro*. However, it would be advantageous for this information to be published in an international journal to ensure wider acceptance. To continue, brown-rot fungi represent only 7% of wood-rotting basidiomycetes and which grow mainly on softwoods (OPs are hardwoods). The fungi degrade wood polysaccharides after only a partial modification of lignin resulting in a brown material consisting of oxi-

dized lignin which represents a potential source of aromatic compounds for the stable organic matter fraction in forest soils. Wood degradation patterns can be identified macroscopically and microscopically. Certain changes in the chemical composition of wood also can be observed: Precise analysis of the degradation type requires chemical determinations of the concentration and modifications of cellulose and lignin. However, this work has not been carried out with OP affected by *Ganoderma*.

Of direct relevance to *Ganoderma* rot of OP, modern techniques are used to analyse wood decay, such as spectroscopic and degradative methods. Pyrolysis coupled to gas chromatography-mass spectrometry (Py-GC/MS) has advantages by allowing rapid analysis of small samples, providing identification of the proportion of H, G, and S lignin units. For example, Py-GC/MS analysis of the fungal decay of a hardwood (*Eucalyptus globulus*) has been undertaken (OP is hardwood). Consequently, Py-GC/MS analyses can be used to compare the changes in the relative proportion of lignin and polysaccharides in decaying wood. Moreover, the amounts of S and G lignin can be estimated by this analysis. For examples, the white-rot basidiomycete *Ceriporiopsis subvermispora*, gives a decrease in the lignin peaks and an increase in the carbohydrate peaks. In contrast, the soft-rot deuteromycete *Paecilomyces* sp. decreased cellulose concentration to a larger extent than lignin.

The (hard)wood degradation patterns by (a) the basidiomycetes *Bjerkandera adusta*, *Ceriporiopsis subvermispora*, *Coniophora puteana*, *Crepidotus variabilis*, *Funalia trogii*, *Melanotus hepatochrous*, *Phanerochaete chrysosporium*, *Phlebia radiata*, and *Pleurotus pulmonarius*, (b) the ascomycetes *Mollisia* sp, *Ophiostoma piliferum* ("Cartapip") and *Ophiostoma valdivianum*, and (c) the deuteromycetes *Fusarium oxysporum*, *Kirramyces eucalypti* and *Paecilomyces* sp. can be differentiated based on their lignin/carbohydrate and S/G Py-GC/MS ratios. The highest cellulose enrichments were produced by *C. subvermispora* and *C. variabilis*, the former also caused the highest modification of lignin, as revealed by the S/G ratio. In addition, *Paecilomyces* sp. and other deuteromycetes produced an increase in the relative lignin content in wood because of preferential removal of polysaccharides, whereas the ascomycetes only slightly modified the wood composition [19]. Clearly, this work must be repeated with OP and *Ganoderma* to determine conclusively which type of rot occurs if progress is to be made.

It is apparent that lignin degradation by basidiomycetes is the key step in lignocellulose decay (including OP). Laccases have many roles in insect, plants, and fungi including synthesis of pigments, detoxification, and fruit-body morphogenesis. Their production in fungal plate cultures was considered to be a characteristic unique to white-rot basidiomycetes. However, some brown-rot fungi produce laccase in liquid cultures. They are perhaps not the most important as these phenoloxidases have a low redox potential that allows direct oxidation only of phenolic lignin units, which often comprise less than 10% of the total polymer. Consequently these may be less of a priority to inhibit. Some lignin-derived phenols could act as efficient laccase mediators in degradation.

However, lignin peroxidase (LiP) and manganese peroxidase (MnP) as discovered from *P. chrysosporium* are true ligninases because of the high redox potential. These may be more important to focus on as a target for inhibition within the present context. LiP degrades non-phenolic lignin units (up to 90% of the polymer), whereas MnP generates Mn^{3+} , which acts as a diffusible oxidizer on phenolic or non-phenolic lignin units *via* lipid peroxidation reactions. Versatile peroxidase (VP) has been described in *Pleurotus* and other fungi as a third type of ligninolytic peroxidase that combines the catalytic properties of LiP, MnP, and plant/microbial peroxidases oxidizing phenolic compounds [20]. Other relevant extracellular enzymes are oxidases generating H_2O_2 , and mycelium associated dehydrogenases that reduce lignin-derived compounds. The former include the aryl-alcohol oxidase (AAO) from *Pleurotus eryngii* and other fungi, and *P. chrysosporium* glyoxal oxidase. Aryl-alcohol dehydrogenases (AAD) and quinone reductases (QR) are also involved in lignin degradation. Finally, the lignin degrading capabilities of four *Ganoderma* strains isolated from ornamental arboreal plants were variable between the strains [21].

MOLECULAR BIOLOGY

Southern blot screening for lignin peroxidase and aryl-alcohol oxidase genes was undertaken for 30 fungal species. Hybridization signals were undetected in *T. versicolor*, *P. chrysosporium* and *G. applanatum*. AAO activity was reported previously in *T. versicolor*, however, the authors failed to detect AAO in this fungus. This activity has been reported also in *G. applanatum* and *P. chrysosporium*. AAO is associated with the mycelium and shows physico-chemical and kinetic properties different to those for extracellular AAO. Most of the basidiomycetes involved in lignin degradation demonstrated evidence of the presence of either *lpo*-type genes (nine species) or *aao*-type genes (five *Pleurotus* species) or both (*B. adusta*). This suggests that the presence of genes closely related to *aao* and *lpo* is not a required feature of white-rot fungi, according to the different patterns of ligninolytic enzymes reported. Other peroxidases, such as MnP and the versatile peroxidase reported in *Pleurotus* and *Bjerkandera* species, and H_2O_2 -producing extracellular oxidases must be involved in lignin biodegradation [22].

Two aspects of molecular structure provide ligninolytic peroxidases unique catalytic properties: (a) a heme environment which confers high redox potential to the oxo-ferryl complex; and (b) the existence of specific binding sites and mechanisms for oxidation of their characteristic substrates, including non-phenolic aromatics in the cases of LiP, manganese iron in the case of MnP, and both types of compounds in the case of the recently described VP. Recent studies have contributed to the identification of the substrate binding sites in ligninolytic peroxidases. The aromatic substrate and the manganese binding sites were first identified in LiP and MnP, and then confirmed in the crystal structure of VP. These studies revealed that the novel catalytic properties of VP are due to its hybrid molecular architecture. Mn^{2+} oxidation is produced at a binding site near the cofactor, where this cation is bound by the carboxylates of three acidic residues, which enables direct electron transfer to one of the

heme propionates. In contrast, veratryl alcohol, and other lignin model substrates, is oxidized at the surface of the protein by a long-range electron transfer mechanism that initiates at an exposed tryptophan residue. The rationale of the existence of this electron transfer mechanism is related to the fact that many LiP/VP aromatic substrates, including the lignin polymer, cannot penetrate inside the protein to transfer electrons directly to the cofactor. Therefore, these substrates are oxidized at the enzyme surface. The H_2O_2 responsible for oxidative degradation of lignin is generated by extracellular fungal oxidases, which can reduce dioxygen to peroxide in a catalytic reaction. Obviously, inhibition of these enzymes requires to be considered in relation to the OP disease. Flavin cofactors are generally involved in this reaction, as in the *Pleurotus* flavoenzyme AAO, although glyoxal oxidase from *P. chrysosporium* is a copper-containing oxidase and is involved. Among flavoenzyme oxidases, fungal glucose oxidase crystal structure has been used as a template to predict the molecular structure of AAO. AAO is a unique oxidase as determined by spectroscopic characteristics and to the wide range of aromatic and aliphatic polyunsaturated primary alcohols and aldehydes that it is able to oxidize [23].

The crystal structures of the laccases from the basidiomycete *T. versicolor*, and the ascomycete *Melanocarpus albomyces* were reported. The active site of laccases includes four copper ions. Type-I copper acts as electron acceptor from substituted phenols or amines (the typical laccase substrates); and type-II copper, which transfers the electrons to the final acceptor, dioxygen, which is reduced to water. The two type-III coppers act as intermediates in the electron transfer pathway that also includes one cysteine and two histidine protein residues. The molecular environment of laccase type-I copper apparently regulates the redox potential of the enzyme. The fact that laccase can use atmospheric oxygen as the final electron acceptor represents a considerable evolutionary advantage favouring lignin biodegradation compared to peroxidases, which require a continuous supply of H_2O_2 . However, this somewhat contradicts the evidence that suggests laccase can degrade only the limited amounts of lignin with phenolic components. More work is required to prioritise enzymes in terms of the most effective to inhibit.

In summary, most of the multienzymatic processes involved in wood lignin degradation (peroxidases, oxidases, and laccases acting synergistically) have been identified, and the mechanisms of action of several of them have been established precisely. It needs to be appreciated that these enzymes cannot penetrate sound wood tissues due to their comparatively large molecular size. Therefore, small chemical oxidizers, including activated oxygen species and enzyme, are probably involved in the initial steps of wood decay. These factors only add to the astonishingly complex process of lignin biodegradation. Nevertheless, the situation as it occurs in OP with *Ganoderma* requires being determined urgently if progress in understanding the disease is desired.

ENZYME INHIBITION

Laccases can be inhibited by heavy metals (Hg^{2+} , Sn^{2+} , Ca^{2+} , Zn^{2+} , K^{2+} , Cd^{2+} , Cu^{2+} , Fe^{2+} , Ag^{2+} , Mn^{2+} , Fe^{3+} , Pb^{2+}) and humic substances [24-27]. In addition, metals can activate

Table 1. Effect of Metals on Laccase of Lignin Model Compounds [9]

Lignin Model Compound	Metal	Effect
Sella solid Red decolourisation	Hg ⁺²	Decreases laccase stability. Irreversible laccase inactivation
Bisphenol	Cu ⁺² , Fe ⁺² at <1.0 mM	Inhibit electron transport systems of laccase, hence inhibit substrate conversion
Poly R-478 decolourisation	Cd ⁺² , Cu ⁺² , Zn ⁺²	Increases

proteolytic enzymes which may degrade laccase; hence activity can be lost in this manner [28]. Ions such as iron and copper may interrupt the electron transport system of laccase and substrate conversion [29, 30]. Hg²⁺ decreases strongly the stability of laccases: Whereas mercury acts by binding to thiol groups (SH) in the active or regulatory sites of the enzyme, causing irreversible inactivation [31]. Of course, the toxicity of this compound may limit its utility. Furthermore, laccase contain copper ions that accept and transfer electrons to reduce molecular oxygen, and the copper is susceptible to chelating or reducing agents. Diethyldithiocarbamate, cysteine, and sodium cyanide inhibit laccase in these manners [32, 33]. The practical use of such compounds would need to be assessed for toxicity. The Type 2/3 trinuclear site of laccases is inhibited by halides. Oxygen cannot be reduced to water stopping terminal electron acceptance and a decrease in redox potential between the two copper sites. F⁻>Cl⁻>Br⁻ in inhibition potency can be attributed to limited accessibility of the T2/T3 copper site by larger halides (Table 2) [34, 35]. Smaller size and higher electronegativity are the important factors here. Furthermore, ionic strength of the enzymatic solutions is one of the most important factors in inhibition [29]. The chlorine ion was the cause of high levels of reduced activity (e.g. from NaCl) [36]. Organic solvents affect activity and DMSO is a strong inhibitor: Laccase was inactivated by greater than 10% acetone, acetonitrile, or DMSO. Anionic surfactants inhibited laccase: Sodium dodecylsulphate (SDS) caused complete inhibition at only 0.1%. Anionic surfactants seem to act with the positively charged side chain of an amino acid after penetration of the 3-dimensional protein structure of the hydrophobic tail [9, 37]. Heavy metals also inhibit laccase as determined by lignin model compounds (Table 1).

Table 2. Inhibition of Laccase by Halides [9]

Halide	Concentration Range (mM)	Max % Inhibition
F ⁻	0.02- 2.00	100
Cl ⁻	0.4 – 200	100
Br ⁻	5-1600	I ₅₀
NaF	0.1	100

The laccase activity of, *inter alia*, *Ganoderma* sp and lignin degradation of olive pomace was determined by Haddadin *et al.* [38] under a variety of conditions. Lignin degradation and laccase activity decreased with increased ammonium tartrate (0, 1.2 and 2.4 mM): Laccase was undetectable at 2.4 mM. pH was tested from 4.0 to 6.0 and the lowest activity was at 6.0 at which value lignin degradation or laccase were undetected. Highest activity was at 4.5. Temperature

was also tested in these regards from 25 to 37° C and the higher temperature was least effective for ligninolytic activity. Haddadin *et al.* [38] discuss that aeration or oxygen transfer is important for lignin degradation with high degradation of lignin being associated with good oxygen availability. These data are useful initially to determine some parameters to control the rot of OPs. For example, OPs could be (a) protected from wind to reduce oxygen transfer, (b) maintained at pH 6.0, and (c) grown at temperatures approximating 37° C by avoiding shade.

Furthermore, the fungi *P. chrysosporium* and *G. lucidum* were used to investigate efficient methods for reducing dyes which may cause environmental problems. The biosynthetic pathways involved are related to those for lignin biodegradation and hence are relevant to the present discussion. Blocking lignin peroxidase (LiP) and manganese peroxidase (MnP) production did not substantially affect Victoria Blue B (VB) decolorization. Particular growth media were suitable for blocking this activity. Such enzymes may be more suitable targets for inhibition than laccase as discussed previously. Nevertheless, laccase production was inhibited by various compounds again as determined by VB analysis (Table 3).

Oxygenase and/or oxidases contribute to lignin degradation. A ferroxidase assay showed higher activity in malt-extract-grown cultures in terms of decolorizing VB but was minimal in Sabaroud broth medium. When sodium azide was used to inhibit endogenous catalase and cytochrome P-450 oxygenase activities, there was 100% reduction in VB decolorization and again these enzymes are related to lignin biodegradation. Sodium azide inhibits catalase, resulting in the accumulation of hydrogen peroxide, which is believed to be the primary source of OH by a Fenton-type reaction. However, addition of sodium azide to the culture did not increase the decolorization activity, suggesting that hydrogen peroxide is not the limiting factor in VB decolorization and by extension, lignin degradation [39] (Table 3). Such factors may be important in controlling lignin breakdown in OP. The addition of 1-aminotriazole, a known cytochrome P-450 oxygenase inhibitor resulted in nearly 70% decrease in VB decolorization, suggesting that this may inhibit lignin degradation. These results are consistent with the involvement of oxygenases in lignin degradation. Hydrogen peroxide is known to be produced in cultures of wood-rotting fungi. Hence, another possible mechanism of lignin degradation is the involvement of hydrogen peroxide-derived OH•. As mentioned, such dye decoloration studies could usefully be employed for studying *Ganoderma* from OP. However, the impact of these compounds on the environment needs to be considered.

Benzoate has the ability to trap hydrogen peroxide-derived hydroxyl radicals (OH•) and may be useful for inhib-

Table 3. Effect of Different Compounds on Decolorization (%) of VB in Culture by *Phanerochaete chrysosporium* which was Grown for 4 Days in Kirk Medium [39]

Addition	Final Concentration (mM)	Decolorization
NaN ₃	0.1	0
ABTS	2	19
Triazole	50	32
ZnCl ₂	60	40
Benzoate	5	46
EDTA	60	57
CaCl ₂	60	59
NaCl	60	60
Control culture	-	79
CuSO ₄	150	83

iting lignin peroxidase enzymes e.g. manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase (collectively known as LME) and other oxygenases which contribute to the degradation process. Benzoate addition to cultures of *P. chrysosporium* decreased the decolourization efficiency by 50% suggesting hydroxyl radical involvement in the ligninolytic process. However, other mechanisms of inhibition by benzoate may be possible. LMEs are commonly perceived as the primary lignin-degrading enzymes and their actions appear to be mediated by hydroxyl radicals. Aromatic cation radicals also may be involved in reactions catalyzed by LME. In summary, (a) sodium azide, (b) 3-amino-1,2,4-triazole, and (c) benzoate inhibit endogenous (a) catalase, (b) cytochrome P-450 oxygenase and (c) hydroxyl radical concentration respectively. Hence, these may be useful for reducing lignin breakdown in OP by *Ganoderma*, although they have to be tested *in vitro* before any field tests.

Copper is a laccase inducer for fungi and, for example, increased the decolourization of dye by *P. chrysosporium*. However, 2, 20-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) is known to enhance the effects of laccase. Halides such as ZnCl₂, NaCl and CaCl₂ are laccase inhibitors (Table 2) by binding to the trinuclear copper site. EDTA chelates metals such as copper and so may be useful for decreasing laccase activity in OP in accordance with the main thrust of the present review. CuSO₄ enhanced laccase activity. Obviously, these, or similar compounds, should not be available to OP where *Ganoderma* may grow or at least only be present at non stimulatory levels. Ethylene diamine tetracetate (EDTA) and sodium azide inhibited laccase activity and decolourization. Mediators, small molecules that act as electron shuttles, increase enzymatic oxidation of phenolic and non-phenolic compounds. The presence of high glucose and nitrogen levels (HCHN) as in Saboraud dextrose broth and malt extract broth medium block the production of MnP and LiP. However, a well known medium referred to as Kirk's broth (high glucose, low nitrogen) [40] induce laccase. So maintaining low nitrogen in OP will stimulate degradation and nitrogen levels in wood in general are low. Laccase is insensitive to high N and C levels. However, nitrogen-limiting conditions supported higher laccase production

and limited MnP and LiP production. In addition, *G. lucidum* produced much higher laccase activity than *P. chrysosporium* [39].

P. chrysosporium produces LiP and MnP under ligninolytic conditions. Furthermore, *P. chrysosporium* metabolizes several (a) organopollutants under conditions which are unfavourable for the expression of extracellular ligninolytic enzymes and (b) non-phenolic aromatics under conditions where LiP production was suppressed [41]. Biphenyl (BP), biphenylene, dibenzofuran (DF), dibenzo-*p*-dioxin, and diphenyl ether (DE) utilisation by *P. chrysosporium* was investigated. The fate of BP, DE, and DF cultures was of great interest which were not oxidized by LiP. Nevertheless, the compounds disappeared in cultures, indicating that they were effectively metabolized. Furthermore, degradation was shown to occur more effectively under ligninolytic high carbon and low nitrogen (HCLN) conditions, where the dry weight of mycelium was three fold less than that under high carbon and high nitrogen (HCHN) conditions. This indicated that the metabolic reactions proceeded more effectively under ligninolytic (HCLN) conditions, and that the initial reaction was found to be the hydroxylation reaction catalyzed by cytochrome P450. To clarify whether the hydroxylated intermediates found were formed *via* the action of P450s, the effect of piperonyl butoxide, a P450 inhibitor, on hydroxyl intermediate formation was examined. It is suggested that fungal cytochrome P450s may play a central role in the metabolism of highly recalcitrant aromatic compounds. LiP-mediated degradation of dibenzo-*p*-dioxin has been described to be initiated by the formation of an aryl cation radical, which is followed by the nucleophilic attack of water to form quinone products. *P. chrysosporium* was also reported to reduce quinones to the corresponding hydroquinones by quinone reductase. Thus, the apparent hydroxylation reaction was initiated by the activation of aromatic rings by LiP. The hydroxylation of aromatic ring results in lowering the ionization potential of recalcitrant aromatic substrates. These resulted in forming the chemical structure more susceptible for a further metabolism such as oxidation by peroxidases and laccase, and in this way may be significant for lignin biodegradation. The medium (pH 4.5) used in

this study was as previously described, with 1% glucose and either 1.2 or 12mM ammonium tartrate as the carbon and nitrogen sources, respectively. Some reducing and chelating agents which inhibit laccase are provided in Table 4. Coumaric acid and kojic acid are interesting as they are well-known fungal secondary metabolites. Paterson [42] provides details of the numerous fungal compounds with known enzyme inhibitory effect. Indeed some of the compounds reported in that publication may be useful for inhibiting ligninolytic enzymes. For example, p-coumaric acid is considered to be a precursor of ubiquinones [43] and so ubiquinones may be useful compound to inhibit ligninolytic enzymes.

Table 4. Inhibiting Effect of Reducing Agents and Metal Chelating Agents on Laccase Production [9]

Compound	Concentration (mM)
NaN ₃	0.0021 – 100; ns
Diethyldithiocarbamate	0.6-10; ns
Thioglycolic acid	0.5-2
EDTA	0.5-25
KCN	1-10
L-cysteine	0.1-1.0; ns
Dithiothreitol (DDT)	0.1-1; ns
Tropolone	0.08-10; ns
Kojic acid	0.1-3.0; ns
p-coumaric acid	1; ns
Thiourea	1-5
2-mercaptoethanol	10
Salicylhydroxamic acid	1
N-hydroxyglycine	0.05
H ₂ O ₂	1
Acetylacetone	10
Glutathione	0.1-1.0
β-mercaptoethanol	0.1; ns

ns actual concentration not specified in some reports.

ENZYMES SPECIFICALLY FROM *GANODERMA* AND WHAT IS KNOWN ABOUT OIL PALM LIGNIN BIODEGRADATION

A valid exercise would be to include a section under these headings. However, to do so would place too much reliance on the accuracy of species delineations within the genus. There is not a great deal to be gained from listing the data from *G. lucidum* for example, and implying that this represents the situation on the fungus from OP. This is even more the case now that four species are associated with the OP disease (see [2]). The equivalent situation exists for specifically OP lignin degradation – there are no data, which is the reason that this field needs urgent experimentation.

CONCLUSIONS

The effect of various compounds which are known to affect ligninolytic enzymes needs to be investigated *in vitro* as

possible control chemicals of *Ganoderma* on OP. These may become soil or spray treatments in the field in the future. Maintaining high temperatures could be beneficial in the plantation perhaps by planting away from shade. Aeration could be affected by growing palm in protected areas of the plantation from winds such as beside hills or other plants which may also tend to increase temperature. The effects of pH, temperature and oxygen transfer need to be determined on *Ganoderma* attack of OP. Ammonium tartrate, halides, and the other compounds require to be tested *in vitro* before selecting appropriate conditions for field trials. The various observations regarding soil type, location of planting, moisture, etc. affecting disease may ultimately relate to how this affects the growth of the fungus and hence lignin biodegradation. Obviously, the effects of these treatments on other aspects of producing healthy OP need to be considered. The fact that the basic physiology of the fungus has not been studied surprises the current authors. What are the optimal conditions for producing and activity of ligninases? These basic data do not appear in the literature. As mentioned, the bulk of the data relates to spread of the fungus by roots, and variation in DNA of *Ganoderma* [2]. Only by a consideration of the disease as primarily one involving lignin degradation will a logical approach emerge to control which is likely to involve enzyme inhibition. However, specific work on pathogenic *Ganoderma* from OP and as it attacks OP lignin requires to be undertaken in the laboratory with a view to field application.

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