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ENZYMATIC "COMBUSTION": THE MICROBIAL DEGRADATION OF LIGNIN[1,2]

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INTRODUCTION

Lignin is the most abundant renewable aromatic material on earth. Growing evidence indicates that the complex plant polymer is biodegraded by a unique

[1]Dedicated to the memory of Professor Erich Adler.

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enzymatic "combustion," i.e. a nonspecific enzyme-catalyzed burning. Lignin is degraded by a narrower array of microbes than the other major biopolymers. Lignin biodegradation is central to the earth's carbon cycle because lignin is second only to cellulose in abundance and, perhaps more significantly, because lignin physically protects most of the world's cellulose and hemicelluloses from enzymatic hydrolysis.

Research on lignin biodegradation has accelerated greatly during the past 10 years, mainly because of the substantial potential applications of bioligninolytic systems in pulping, bleaching, converting lignins to useful products, and treating wastes (52, 58, 59, 103, 126, 130, 158, 270). Rapid progress in lignin biodegradation research is reflected in the number of reviews published in the last 5–6 years on the subject in general (25a, 34, 37, 110, 111, 126, 154, 179) or selected aspects of it (26, 103, 113, 114, 146, 155, 162, 168, 274). In addition, two international meetings have focused on lignin biodegradation (115, 163).

Our review encapsulates the major findings of the past 5–6 years, emphasizing the research that immediately preceded and followed the discovery in 1982 of the first lignin-degrading enzyme. That discovery projected the field into the realm of biochemistry and molecular biology and opened up new prospects for application.

LIGNIN AS A SUBSTRATE

Lignin is found in higher plants, including ferns, but not in liverworts, mosses, or plants of lower taxonomic ranking. Wood and other vascular tissues generally are 20–30% lignin. Most lignin is found within the cell walls, where it is intimately interspersed with the hemicelluloses, forming a matrix that surrounds the orderly cellulose microfibrils. In wood, lignin in high concentration is the glue that binds contiguous cells, forming the middle lamella.

Biosynthetically, lignin arises from three precursor alcohols: *p*-hydroxycinnamyl (coumaryl) alcohol, which gives rise to *p*-hydroxyphenyl units in the polymer; 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, the guaiacyl units; and 3,5-dimethoxy-4-hydroxycinnamyl (sinapyl) alcohol, the syringyl units. Free radical copolymerization of these alcohols produces the heterogeneous, optically inactive, cross-linked, and highly polydisperse polymer. Most gymnosperm lignins contain primarily guaiacyl units. Angiosperm lignins contain approximately equal amounts of guaiacyl and syringyl units. Both types of lignin generally contain only small amounts of *p*-hydroxyphenyl units. For convenience we use the term "lignin" for the family of related polymers.

Figure 1 is a schematic formula for an angiosperm lignin, depicting several

important features; note the nomenclature of the carbons (Unit 1). Over 10 interphenylpropane linkage types occur, including four that predominate. The schematic illustrates only the major linkages; the dominant linkage (>50%) is the β-O-4 type, seen between units 1 and 2, 2 and 3, 4 and 5, 6 and 7, 7 and 8, and 13 and 14. In the polymerization process, secondary reactions lead to cross-linking between lignin and hemicelluloses (Unit 5 in Figure 1). In addition, the lignins of grasses and certain woods contain aromatic or cinnamic acids esterified through side-chain hydroxyl groups of lignin (Unit 12 in Figure 1). The major features of lignin structure are now well understood (2, 61, 70, 240); although additional details are still being clarified, it is not likely that they will be of consequence to biodegradation research.

The structural features of lignin dictate unusual constraints on biodegradative systems responsible for initial attack: They must be extracellular, nonspecific, and nonhydrolytic. Analogies with other biopolymer-degrading systems, which are hydrolytic and specific, and with the intracellular systems

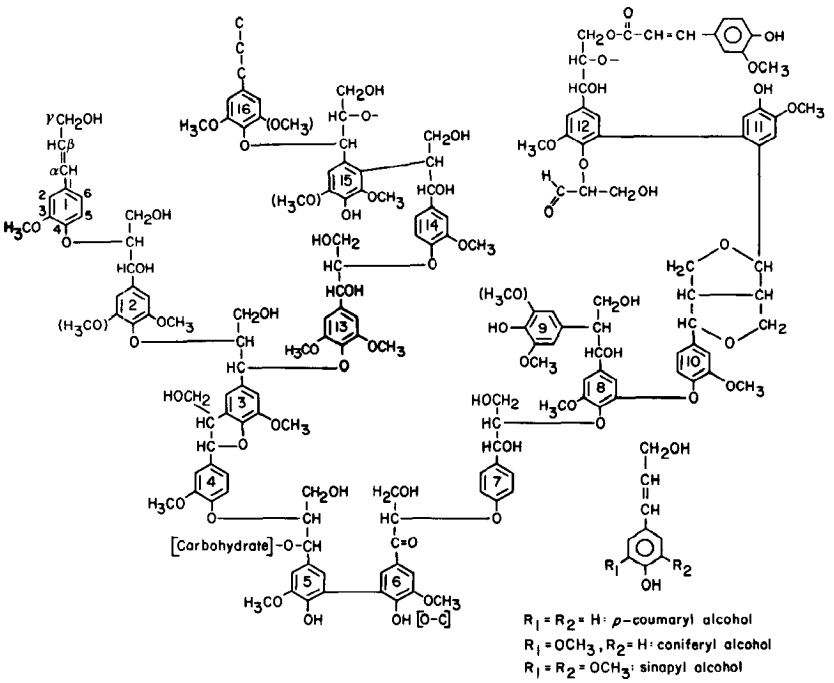

Figure 1 Schematic structural formula for lignin, adapted from Adler (2). The structure illustrates major interunit linkages and other features described in the text; it is not a quantitatively accurate depiction of the various substructures. The three precursor alcohols are shown at the *lower right;* their polymerization, following one-electron oxidation, produces lignin.

that degrade low–molecular weight aromatics cannot readily be drawn. We define lignin as a 600–1000-kd molecule, i.e. too big to enter cells. We have chosen not to treat the biodegradation of low–molecular weight lignin-related compounds, except where these compounds have been used as models to elucidate specific reactions in the polymer.

The structural features of lignin also cause problems in methodology, mainly in quantitative determination. Research has been greatly aided by the introduction of ^{14}C-labeled lignins, produced either in vivo (36, 38, 95) or in vitro (95, 157, 159). Conversion of labeled lignins to $^{14}CO_2$ has served as a useful assay for biodegradation, even though it does not indicate the full extent of degradation because lignin is biodegraded in part to water-soluble intermediates (e.g. 187, 231). Care must be taken in preparing labeled lignins. Incorporation of precursors by plants into nonlignin components (proteins, aromatic acids esterified to hemicelluloses or to lignin, and other aromatics such as lignans) must be avoided or circumvented (15, 34). One must also be sure that the lignin is polymeric. This is a potential problem with in vitro lignins [often termed "dehydrogenative polymerizates" (DHPs)] and possibly also with lignins labeled in vivo.

Another important point for microbiologists is that some of the linkages of lignin are unstable, particularly those involving C_α (as between Units 3 and 13, and 15 and 16 in Figure 1). Slow abiotic degradation, favored by high-temperature, acidic, or alkaline environments, releases small fragments. We suspect that the facile biodegradation of such fragments has led to erroneous conclusions concerning polymer biodegradation.

Crawford (34), Janshekar & Fiechter (126), and Buswell & Odier (25a) have discussed the merits of various isolated lignins used in biodegradation studies. In most recent work synthetic lignins or lignin-labeled plant tissues have been used. Some studies, however, have been done with kraft lignins and lignosulfonates, both of which are structurally modified by-products of commercial pulping operations (see 25a, 34, 126, 210, 240).

MICROBIOLOGY OF LIGNIN BIODEGRADATION

Despite numerous studies, it is not entirely clear which microbes, other than certain fungi, degrade the lignin polymer. The uncertainty reflects the experimental difficulties mentioned above and insufficient comprehensive study with selected species. Moreover, the apparent inability of microorganisms to use lignin as sole carbon/energy source for growth precludes the isolation of lignin-degraders by standard enrichment procedures and the use of growth on lignin as a criterion for degradative ability.

Anaerobic Conditions

Lignin is apparently not biodegraded anaerobically. Zeikus et al (275) studied the decomposition in anaerobic lake sediments of synthetic lignins, an alkali-degraded synthetic lignin, a dimeric lignin model compound, and lignin-related phenols, all labeled with ^{14}C. Both $^{14}CO_2$ and $^{14}CH_4$ were monitored. Degradation during 110 days was limited to the low–molecular weight materials (<600 daltons). Colberg & Young (32) obtained similar results in studies with Douglas fir wood labeled by [^{14}C]phenylalanine feeding. In a study by Holt & Jones (117), beech wood buried in anaerobic seawater, freshwater, or brackish muds was only slightly degraded after 18 mo. Similarly, [^{14}C]lignin-labeled aspen wood was not degraded significantly during a 6-mo anaerobic incubation in seven soils (212). We suggest that very limited anaerobic metabolism of lignin-labeled plant tissues by various microflora during extensive incubations (14, 16) can be attributed to nonlignin components or to low–molecular weight materials freed abiotically.

Aerobic Conditions

Neither rapid nor extensive bacterial degradation, even under highly aerobic conditions, has been reported. The most rapid and extensive degradation described to date is caused by certain fungi, particularly the white-rot fungi, in highly aerobic environments.

BACTERIA Bacterial lignin degradation has been most extensively studied in actinomycetes, particularly *Streptomyces* spp. *Streptomyces viridosporus* and *Streptomyces setonii* caused losses of 32–44% of the lignin in spruce, maple, and *Agropyron* lignocelluloses, as determined by chemical analyses of the insoluble residues (9). Characterization of lignin isolated from *S. viridosporus*–degraded spruce phloem indicated oxidative alterations similar to those reported for white-rot fungi (35). Nevertheless, in all studies of degradation of [^{14}C]lignin-labeled lignocelluloses by the streptomycetes, a maximum of about 20% of the ^{14}C has been converted to $^{14}CO_2$, and attack on fully lignified xylem tissues has been minimal. In recent studies, maximum degradation of [^{14}C]lignin in wheat straw to $^{14}CO_2$ was 8% in 14 days (199, 200).

Degradation of grass tissues by the actinomycetes produces a water-soluble residue termed "acid-precipitable polymeric lignin," or APPL (39, 201, 221, 222), which contains varying amounts of carbohydrate. APPLs, therefore, might result from polysaccharide rather than lignin degradation (201). The lignin component is similar to sound lignin (39, 201) and is resistant to further degradation by the actinomycetes that produced it (201, 222).

Recent studies with other bacteria have failed to demonstrate extensive

degradation. Experiments that indicated high rates and extents of degradation of a kraft lignin by various bacteria did not employ ^{14}C-labeling, and cell adsorption was not ruled out (42). Janshekar & Fiechter (125) isolated strains of *Nocardia, Pseudomonas,* and *Corynebacterium* for ability to grow on lignin-related phenols, but none was able to degrade any of four different lignins. A bacterium reported to degrade lignin released only 3% of the ^{14}C from [^{14}C]lignin-labeled *Spartina* lignocellulose, whereas pepsin solubilized 19% of the label (150). Although a *Xanthomonas* strain mineralized over 30% of synthetic [^{14}C]lignin in 20 days, the lignin also underwent abiotic depolymerization (149); a very recent study indicated that the low–molecular weight components are mineralized (H. Kern & T. K. Kirk, unpublished). Benner et al (17), using ^{14}C-labeling, found that bacteria were primarily responsible for the degradation of plant material in certain environments, but mineralization of the lignin was minimal.

Nilsson & Holt (208) described a new type of wood decay caused by bacterial consortia that appear to belong to the Myxobacteriales or Cytophagales (G. Daniel & T. Nilsson, personal communication). Analyses of woods after 3-mo exposure to such a consortium indicated extensive lignin loss from birch and pine woods.

To date, however, no study has shown that lignin is mineralized rapidly or extensively by aerobic bacteria. As in the case of the anaerobes, a limiting factor might be the size of the lignin polymer. Lignin-related dimeric compounds (72, 85, 86, 142, 220, 238) and even tetrameric (638- and 666-dalton) compounds (129) are metabolized by numerous species of bacteria, presumably intracellularly, and apparently with little specificity.

FUNGI Soft-rot wood decay caused by various species of ascomycetes and fungi imperfecti involves lignin degradation, although wood polysaccharides are preferentially degraded (see 154, 160). *Chaetomium piluliferum* converted 20–30% of ^{14}C-labeled synthetic lignins to $^{14}CO_2$ in 50 days (96). Unfortunately, the soft-rot fungi have received very little attention; virtually nothing is known about the enzymology of their degradation of lignin.

Several ascomycetes, fungi imperfecti, and phycomycetes that are not associated with soft rot of wood, including 12 marine fungi (245), 18 *Trichoderma* strains (65), and *Trichoderma harzianum* (147), failed to degrade lignin significantly. A supposed kraft lignin degradation by a *Candida* sp. (31) could have been cell adsorption. A report of degradation of ^{14}C-labeled kraft lignin by a strain of *Aspergillus fumigatus* (131) did not include the molecular size of the lignin components, so it is not clear that high–molecular weight material was degraded. Several recent studies have treated *Fusarium* species, and especially their degradation of phenolic-lignin model compounds. These species do not, however, appear to degrade lignin. Thus

Norris (209) reported that an isolate of *Fusarium solani* released only 4–5% of the ^{14}C in labeled synthetic lignins as $^{14}CO_2$ in 30 days, and Sutherland et al (246), using 18 *Fusarium* strains, found a maximum of 5% conversion of ^{14}C-labeled lignin in spruce wood to $^{14}CO_2$ in 60 days.

A few species of ascomycetes (e.g. species of *Xylaria, Libertella,* and *Hypoxylon*) cause white-rot wood decay accompanied by substantial lignin loss. None of these fungi have been studied extensively, but recent work has shown that they fail to degrade gymnosperm wood (guaiacyl lignin) and that they preferentially attack the syringyl units of angiosperm lignin (T. Nilsson, J. R. Obst & T. K. Kirk, unpublished). This finding has interesting implications for the ligninolytic enzymes of these fungi, which remain to be studied.

Lignin degradation by non–white-rotting basidiomycetes has been reported. The gasteromycete *Cyathus stercoreus,* which is associated with litter decomposition, degrades lignin in wheat straw (269); the degradation is as extensive as that by various white-rot fungi (3). Several other *Cyathus* species degrade grass lignin, but members of the related genera *Nidula* and *Crucibulum* do not (1). Ectomycorrhizal fungi (*Cenococcum, Amanita, Tricholoma,* and *Rhizopogon*) only slowly mineralized ^{14}C-labeled synthetic lignins and corn lignin (253); however, poor degradation by two white-rot fungi included for comparison suggests that further studies with different culture conditions are needed.

Basidiomycetes that cause the important brown-rot wood decay also partially decompose lignin. They are closely related to the white-rot fungi; many were formerly assigned to the same genera. They invade the lumens of wood cells, where they secrete enzymes that decompose and remove the polysaccharides, leaving behind a brown, modified lignin residue. Studies show that the lignin undergoes limited aromatic hydroxylation and ring cleavage, but that the major effect is demethylation of aromatic methoxyl groups (154, 156). In accord, brown-rot fungi converted substantial percentages of $[O^{14}CH_3]$lignins to $^{14}CO_2$, whereas conversion of side-chain carbon to CO_2 was significantly lower, and conversion of aromatic carbons lower still (96, 159). Ability of the brown-rot fungi to mineralize the backbone lignin polymer thus seems to be limited. Some brown-rot fungi degrade dimeric model compounds, but to different, as yet unidentified, products from those produced by white-rot fungi (48, 49). Overall, however, lignin degradation by this group of fungi has been studied very little.

LIGNIN DEGRADATION BY WHITE-ROT FUNGI

The white-rot basidiomycetes degrade lignin more rapidly and extensively than other studied microbial groups. Like the brown-rot fungi, they invade the

lumens of wood cells, where they secrete enzymes that degrade lignin and the other wood components. The electron-microscopic features of wood decay by white-rot fungi, including species that selectively degrade the lignin plus hemicelluloses, have been studied recently (19–21, 215, 216, 237). These studies reveal, among other things, that lignin is degraded at some distance from the hyphae and is removed progressively from the lumens toward the middle lamella.

During its mineralization by white-rot fungi, lignin undergoes a number of oxidative changes, including aromatic ring cleavage (see 26). Recent studies have shown that a progressive depolymerization occurs and releases a wide array of low–molecular weight fragments (27, 28, 230, 231, 247, 248). Fragments of <1 kd seem to predominate (57, 187).

One species of white-rot fungus, *Phanerochaete chrysosporium* [=*Sporotrichum pulverulentum* (23, 223)] has been studied widely. Culture conditions for lignin degradation have been optimized, and *P. chrysosporium* exhibits the highest reported rates of lignin degradation. Yang et al (272) reported degradation of 2.9 g lignin per gram of fungal cell protein per day in a wood pulp; assuming 15% protein in the mycelium (63), this rate is about 200 mg lignin per gram of mycelium per day. Ulmer et al (255) reported rates three times higher for a lignin from wheat straw.

In the following sections, we briefly review recent progress in the study of the physiology, biochemistry, genetics, and molecular biology of degradation of lignin by *P. chrysosporium*. Where available, data for other white-rot fungi are included.

Physiology

Research during the late 1970s demonstrated that several nutritional and cultural parameters are important for lignin degradation by *P. chrysosporium:* (*a*) presence of a cometabolizable substrate, (*b*) high oxygen tension, (*c*) growth as mycelial mats rather than as submerged pellets in agitated cultures, (*d*) correct choice of buffer, (*e*) correct levels of certain minerals and trace elements, and (*f*) growth-limiting amounts of nutrient nitrogen (reviewed in 25a, 154, 168). Through subsequent research this list has been refined and physiological features have been elucidated.

Recent studies have added to the evidence that lignin is not a growth substrate for white-rot fungi. *P. chrysosporium* and *Lentinula edodes* metabolize various lignin preparations only when an alternate carbon/energy source is present (176, 255). Several studies have confirmed earlier observations that hemicelluloses and cellulose, or added carbohydrates, are always metabolized with the lignin in lignocelluloses (e.g. 4, 19, 186, 189, 215, 230, 269, 272). Nevertheless, the balance between energy-producing and energy-yielding reactions in lignin mineralization is not known.

Earlier observations (167, 185) that molecular oxygen can be crucial in determining the rate of lignin degradation by *P. chrysosporium* as well as by certain other white-rot fungi have been confirmed (13, 108, 232, 273). Increasing the O_2 level in the medium has a multiple effect (13); it leads to an increase in the titer of the ligninolytic system, including ligninase (see section on biochemistry, below), and the H_2O_2-producing system(s) (54, 55), and it also increases the activity of the existing lignin-degrading system, evidently by increasing the supply of O_2 for degradative reactions and for H_2O_2 production. Extracellular H_2O_2 production can be rate limiting in *P. chrysosporium* (170).

Culture agitation, usually used to increase oxygen tension, almost completely suppressed lignin degradation (167) as well as metabolism of dimeric models (45), synthesis of veratryl alcohol (244), and formation of ligninase (55). More recently, however, degradation of lignin by submerged pellets in agitated cultures has been achieved by using a mutant strain (82) or by adding detergent (12, 123), veratryl alcohol (180), or benzyl alcohol (T. K. Kirk & S. C. Croan, unpublished) to cultures of wild-type strains. The agitation-induced suppression and its alleviation have not been explained.

The choice of buffer in the culture medium can affect lignin degradation significantly. Kern (148), for example, showed that cultures buffered with a polymer, polyacrylic acid, mineralize lignosulfonate more rapidly than those buffered with 2,2-dimethylsuccinate, which is widely used.

A medium formulated in initial studies (167) was later found to contain about the correct amounts and balance of several inorganic nutrients (127). Some stimulation was noted when Zn, Fe, and Mo concentrations were decreased 10-fold over the basal level (127). In addition, Ca concentration is important; 1 mM is more favorable than either 0.1 or 10 mM (176). Ligninase production was increased by increasing either Cu or Mn (161). Growing evidence implies that Mn is important in lignin degradation: Not only does increased Mn lead to increased ligninase production, but this element accumulates as MnO_2 deposits during degradation of lignin in wood by several white-rot fungi (18). Also, Mn concentration has a marked influence on lignin degradation by *L. edodes* (176). Finally, a Mn-dependent peroxidase has recently been discovered in *P. chrysosporium* (see section on biochemistry, below).

In *P. chrysosporium,* lignin is degraded only during secondary (idiophasic) metabolism, which is triggered by limiting cultures for nutrient N (127, 153, 167, 228, 229), C, or S (127). Lignin degradation by several other species (105, 106, 177, 213), but not by all white-rot fungi (69, 176, 177), is stimulated by N-limitation. N-limited conditions are natural for the white-rot fungi because wood is N-poor (33). For practical purposes N, rather than C or S, is usually limited in experiments with *P. chrysosporium;* triggering of

lignin degradation by S-limitation is not easily demonstrated, and C-limitation leads to autolysis and only transient lignin degradation (127).

In addition to lignin degradation, other features of secondary metabolism triggered by N-limitation in *P. chrysosporium* have been studied. In glucose-grown cultures, appearance of new hyphal outgrowths (124, 187), formation of an extracellular glucan (162, 178, 191), and de novo synthesis of the secondary metabolite veratryl (3,4-dimethoxybenzyl) alcohol (191, 193, 244) are manifestations of secondary metabolism. The N-regulated transitions from primary to secondary metabolism and vice versa are associated with increased and decreased levels of cyclic AMP, respectively; interestingly, cyclic AMP levels are controlled in part by secretion (194, 195).

The transition from primary to secondary metabolism is associated with a transient increase in intracellular glutamate (63); addition of exogenous glutamate or other nitrogen sources sharply suppresses secondary metabolism, including activity of enzymes involved in lignin degradation (62). The titer of ligninase (see section on biochemistry) is lowered sharply by adding glutamate to cultures (55). Variation in glutamate content is associated with changes in the levels of glutamate-synthesizing and glutamate-degrading enzymes (24, 62; reviewed in 211).

The association of veratryl alcohol with lignin degradation has been studied in N-limited cultures of *P. chrysosporium*. Veratryl alcohol is synthesized from phenylalanine via 3,4-dimethoxycinnamyl alcohol, which is oxidized to 1-(3,4-dimethoxyphenyl)glycerol; this is then oxidized to veratraldehyde (244). Interestingly, these oxidations are catalyzed by ligninase in vitro, but their catalyst in vivo is not yet known. In vivo, the veratraldehyde is reduced to the alcohol (244). Veratryl alcohol is a substrate for ligninase; it is oxidized to veratraldehyde and other products (see section on biochemistry). Another possible contribution of veratryl alcohol is that it stimulates the oxidation of other compounds by ligninase (93, 104). In addition, exogenous veratryl alcohol hastens the appearance of the ligninolytic system ([^{14}C]lignin $\rightarrow$ $^{14}CO_2$) as a part of secondary metabolism; in the absence of added veratryl alcohol, the ligninolytic system and the biosynthesized metabolite appear simultaneously (188). Added veratryl alcohol also increases the titer of ligninase (55, 161, 184), probably via induction (56). Leisola et al (188) suggested that veratryl alcohol is the normal inducer of the ligninolytic system. Recent studies have shown that veratryl alcohol is also synthesized de novo by the white-rot fungi *Coriolus versicolor* (141), *Pycnoporus cinnabarinus, Phlebia radiata* (105), and a *Trametes* species and by four other unidentified white-rot fungi (H. Silva & E. Agosin, personal communication). However, whether it is synthesized by all white-rot species is not yet known. In any case, the association of veratryl alcohol with lignin degradation and secondary metabolism deserves further study. Studies with mutants

might help elucidate its importance, but have so far provided only inconclusive data (191).

Recent studies have shown that added lignin also increases the titers of ligninase and the complete lignin-degrading system (55, 254). This probably explains the failure to saturate the lignin-degrading system of *P. chrysosporium* with lignin; the more lignin is added to cultures, the more is degraded (30, 176). The ligninolytic system of *L. edodes,* by contrast, is easily saturated (176).

The role of the extracellular glucan in lignin degradation by *P. chrysosporium,* if any, remains speculative. The glucan, which is of undetermined structure, seems to occur both as a hyphal sheath (161) and free in culture fluid (251). Reportedly it is remetabolized when carbon becomes limiting (53). Leisola et al (178) presented evidence that the glucan inhibits lignin degradation. Recent studies, however, have linked degradation of wood by white-rot fungi with the presence of mucopolysaccharide hyphal sheaths and extracellular tripartite membranes (66, 217). Palmer et al (217) suggested that wood-degrading enzymes are embedded in the sheaths. The tight, transient binding of lignin to mycelia during degradation (30, 124) might involve the glucan. Although extracellular ligninase is easily separated from the glucan (251), recently reported particulate bodies with ligninase (?) activity (88) might have been formed by sedimenting the activity with the glucan at high centrifuge speeds.

Biochemistry

The recent discovery of several enzymes that are thought to have roles has projected lignin biodegradation research into the realm of biochemistry. These enzymes include ligninases, Mn peroxidases, phenol-oxidizing enzymes, and H_2O_2-producing enzymes.

LIGNINASE (LIGNIN PEROXIDASE) In 1983, two groups announced discovery in *P. chrysosporium* of an extracellular H_2O_2-requiring enzyme activity that catalyzes several of the reactions formerly seen with intact cultures (81, 169). A third group (243) reported an extracellular membrane-bound enzyme activity which in retrospect might have been the same. Papers published by the first two groups later in 1983 described reactions catalyzed by the crude enzyme (75) and by the isolated enzyme, which, importantly, partially depolymerized methylated lignins (250).

Ligninase activity has recently been detected in other white-rot fungi, including *Phlebia radiata* (107; A. Hatakka, M.-L. Niku-Paavola, personal communication), *Panus tigrinus* (196), *Coriolus versicolor, Pleurotus ostreatus,* and *Bjerkandera adusta* (R. Waldner, M. Leisola & A. Fiechter, personal communication). Failure to detect the enzyme in *Fomes lignosus,*

Trametes cingulata (R. Waldner, M. Leisola & A. Fiechter, personal communication), and *L. edodes* (176) might reflect assay insensitivity or failure to solubilize the enzyme. Oki et al (214) recently detected an enzymatic activity in *L. edodes* that cleaves model compounds in the same manner as ligninase.

Nature and properties Ligninase from *P. chrysosporium* was initially isolated by various chromatographic procedures and was shown to contain one mole of protoheme IX per mole of enzyme, to have molecular mass of 41–42 kd, and to be glycosylated (82, 174, 251). In reactions of ligninase with $H_2{}^{16}O_2$, $^{18}O_2$, and lignin model compounds, ^{18}O was incorporated into C_α–C_β cleavage products; therefore the enzyme was referred to as an H_2O_2-requiring oxygenase (82, 251).

Subsequent spectroscopic studies have shown that the ligninase is distinct from P_{450} oxygenases, shares some properties with oxygen-carrying heme proteins, and is a true peroxidase. ESR spectral studies (8, 219) showed that the iron is high-spin Fe(III). Detailed ESR and electronic spectral studies indicated that the heme environment resembles those of other peroxidases (8, 172). Raman resonance spectroscopy confirmed the high-spin ferric nature of the heme iron and indicated that the fifth ligand in the pentacoordinate heme is histidine (8, 172). From their resonance Raman studies, Andersson et al (8) concluded that the active site in ligninase is hexacoordinate at low temperatures. Kuila et al (172) showed that ligninase is pentacoordinate at room temperature. Recent work has confirmed the temperature dependence of axial ligation (M. H. Gold, personal communication).

Ligninases from other fungi are only beginning to be characterized. Two ligninases from *Phlebia radiata* are apparently very similar in size to those from *P. chrysosporium* (M.-L. Niku-Paavola, personal communication). A somewhat larger ligninase (50 kd) has also been isolated from *C. versicolor* (J. Palmer & C. Evans, personal communication).

Enzyme mechanism Renganathan & Gold (234) characterized the H_2O_2-oxidized forms of ligninase by electronic absorption spectroscopy. They demonstrated formation of compound I (the two-electron oxidized form) and compound II (the one-electron oxidized form). Compound I was converted to compound II by one equivalent of a one-electron substrate such as a phenol, or by 0.5 equivalent of the two-electron substrate, veratryl alcohol. Ligninase therefore resembles HRP in many of its properties, although it has a higher oxidation potential than HRP (98; P. J. Kersten, K. E. Hammel, B. Kalyanaraman & T. K. Kirk, unpublished).

Hammel et al (99) showed that O_2 uptake during cleavage of a hydrobenzoin lignin model by ligninase results from addition of O_2 to carbon-centered radicals. These authors, as well as Renganathan et al (236), also

showed that ligninase cleaves β-1 model substrates under anaerobic conditions using peroxide alone as the oxidant. These studies proved that the essential mechanism of ligninase is peroxidative.

Tien et al (252) made a detailed study of the oxidation of veratryl alcohol by ligninase. With excess veratryl alcohol they observed a stoichiometry of one mole of veratraldehyde per H_2O_2 and estimated a maximum turnover number of 7.8 sec^{-1}. Steady-state kinetic studies indicated a ping-pong mechanism (K_m = 29 μM for H_2O_2 and 72 μM for veratryl alcohol) in which H_2O_2 first reacts with the enzyme, and the oxidized enzyme then reacts with veratryl alcohol. Attempts to detect intermediate substrate-free radicals were not successful. The results indicated that veratryl alcohol is oxidized at the active site by direct oxygenation or via two rapid one-electron oxidations.

The oxidation of the normal secondary metabolite veratryl alcohol by ligninase might have special significance. Harvey et al (104) showed that veratryl alcohol increases the rate of oxidation of anisyl substrates by ligninase/H_2O_2; in its absence these substrates are only incompletely oxidized. Haemmerli et al (93) similarly reported stimulation by veratryl alcohol of ligninase oxidation of benzo(*a*)pyrene. Harvey et al (104) suggested that veratryl alcohol is oxidized to a cation radical (see below), which acts as a diffusible one-electron oxidant to interact with other substrates. However, the results of Tien et al (252) seem to discount this postulate. It seems more likely that the veratryl alcohol simply protects the enzyme from inactivation by anisyl substrates. Alternatively, it might act as an electron relay at the enzyme active site, or it might alter enzyme conformation.

The reactions of aromatic substrates on ligninase oxidation were puzzling at first in their diversity and complexity, but can now be understood. Investigations by Kersten et al (152) and Hammel et al (97, 99) established the basic simplicity of ligninase oxidation: Susceptible aromatic nuclei are oxidized by one electron, and this produces unstable cation radicals, which undergo a variety of nonenzymatic reactions. Based on direct ESR spectroscopic observations of the cation radicals produced by purified ligninase, Kersten et al (152) proposed that this is the basic mechanism that accounts for the various reactions. At about the same time, Schoemaker et al (241) suggested a cation-radical mechanism based on some of the reactions catalyzed, and Harvey et al (102) showed that chemical one-electron oxidation of certain compounds gives the same products as ligninase. Hammel et al (97) proved the one-electron mechanism of ligninase action by showing that radical coupling dimers are produced stoichiometrically on anaerobic ligninase cleavage of special model compounds, that carbon-centered radical products can be trapped under anaerobic cleavage, and that peroxyl radicals are produced by addition of O_2 to carbon-centered radicals in aerobic ligninase reactions.

The key reaction of ligninase with lignin model compounds therefore is one-electron oxidation. With certain substrates, such as with veratryl alcohol, a second electron apparently can be removed from the substrate before it leaves the enzyme active site (252). Phenolic substrates are oxidized to phenoxy radicals, as evidenced by dimerization of 4-*tert*-butylguaiacol (251) and by direct ESR detection (E. Odier, M. D. Mozuch, B. Kalyanaraman & T. K. Kirk, unpublished). Reactions of phenoxy radicals are discussed below in connection with phenol-oxidizing enzymes. The following discussion deals with nonphenolic substrates.

Whether an aromatic nucleus is a substrate for ligninase depends in part on its oxidation potential (98). Strong electron-withdrawing substituents such as C_α-carbonyl groups tend to inactivate aromatic nuclei to oxidation by ligninase, whereas alkoxyl groups activate it. The positions of the latter groups also affect oxidizability by ligninase. In lignin, the positions of the alkoxyl groups are set, but the number varies from one to three (Figure 1); the oxidation rates are expected, therefore, to be in the order syringyl > guaiacyl > *p*-hydroxyphenyl. The nature and pattern of the substituents also affect the subsequent reactions of the cation (171), including nucleophilic attack by water or an internal hydroxyl group, loss of the acidic proton at C_α, and C_α–C_β cleavage.

A variety of sequential reactions can follow these initial reactions. Included are addition of molecular oxygen to carbon-centered radicals, one-electron oxidation or reduction, and (in the absence of O_2, which scavenges radicals) radical-radical coupling. The many reactions that the cation radicals undergo lead to many different products; this explains the surprisingly large number of degradation intermediates formed from lignin as it is degraded by white-rot fungi (26–28, 247, 248).

It is this nonspecific oxidation of lignin, which leads to a variety of subsequent reactions and products determined only by the kinetics of reaction intermediates, that leads us to conclude that the initial process is essentially enzymatic "combustion" (see final section, below).

In the following we illustrate the diversity of reactions and products with model compounds of the β-O-4 type, which is the dominant lignin substructure. Figure 2 is based on studies from four different laboratories with isolated ligninase and many model compounds. Higuchi (114) has summarized the reactions leading to most of these products and the mechanistic information gained with specially synthesized models and various isotopes. Ligninase can oxidize ring *A* and ring *B*. In lignin the two rings might have the same substitution and be equally susceptible; the more accessible ring would be expected to be oxidized first. In Figure 2, we have labeled the arrows *A* or *B* to denote which ring was oxidized.

Products *1–3* in Figure 2 are formed on C_α–C_β cleavage (75, 82, 91, 171,

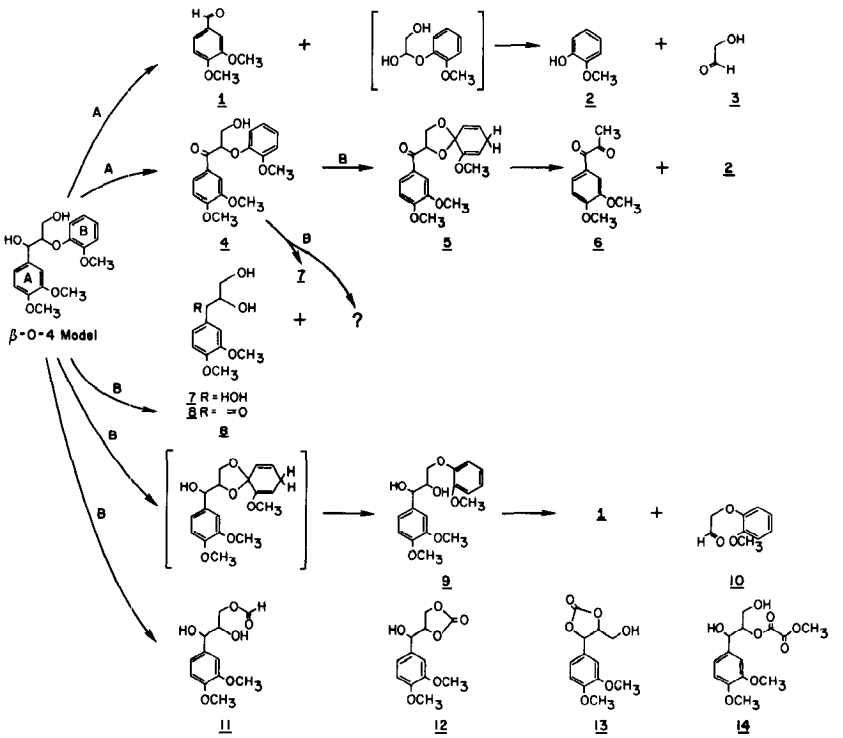

Figure 2 Products of oxidation of β-O-4 model compounds by ligninase/H_2O_2. Oxidation can be in ring *A* or ring *B*, as indicated (see text).

202, 203, 236, 251, 252). By analogy with other types of models, it is clear that the new hydroxyl oxygen atom at C_β comes from O_2 (97, 99, 251). Studies have shown that C_α–C_β cleavage is the major reaction in intact cultures of *P. chrysosporium* (45, 47, 64, 116). Importantly, studies of polymeric lignin that had been partially degraded by white-rot fungi also showed that C_α–C_β cleavage is prominent (26).

Product *4* is formed by C_α oxidation; this is analogous to veratryl alcohol oxidation to veratraldehyde (75, 82, 91, 171, 236, 250, 251). C_α oxidation in model compounds is also seen in intact cultures (64), and polymeric lignin contains substantial numbers of C_α-carbonyl groups after partial degradation by white-rot fungi (26, 29).

Product *5* formation involves an intramolecular nucleophilic attack by the C_γ-hydroxyl group at C_4 of the ring *B* cation and subsequent reduction (171). Spontaneous decomposition of product *5* yields *6* and *2* (171).

Products *7* and *8* (75, 82, 91, 171, 202, 203) result from nucleophilic attack

on the ring *B* cation, again at C_4, but this time by water. Product *8* arises from C_α-carbonyl structures such as that in product *4* (171), or perhaps by C_α-oxidation of product *7*. However, product *7* is more likely to undergo C_α–C_β cleavage to products *1* and *3*. Formation of product *7* in intact cultures has been studied extensively (46, 47, 256, 260, 262).

Demethoxylation products (not shown), reflecting attack by water at C_3 of the ring *B* cation, are prominent in anaerobic ligninase reactions (203). Their importance in aerobic reactions is unclear, but small amounts of methanol are formed from β-O-4 models (171). Ligninase-initiated demethoxylation could be responsible for the formation of methanol from lignin and related low–molecular weight aromatics by *P. chrysosporium* (7). It is unclear, however, whether ligninase oxidation alone accounts for the substantial methoxyl deficiency in lignin after partial degradation by white-rot fungi (26).

Product *9* is presumably formed via an intermediate analogous to *5*, (shown in brackets), with subsequent opening of the heterocyclic ring. C_α–C_β cleavage produces *10* and *1* (121, 202, 258).

Products *11–14* (114, 259, 261, 263) involve cleavage of ring *B* following its oxidation. Umezawa et al (261) suggested that the initial ring cleavage product might be a methylmuconate formed by oxygenative cleavage between C_3 and C_4; this has now been proven (T. Higuchi, personal communication). Demethoxylation is not involved (114). Products *11–14* are closely related to each other. They are substrates for further ligninase oxidation, and have only been found in trace amounts. Even so, all have been identified in intact cultures (114). Ligninase oxidation probably accounts for ring cleavage fragments found in the lignin polymer following partial degradation by white-rot fungi (26, 29, 94) and for various low–molecular weight ring cleavage products formed during polymer degradation (26–28, 247, 248).

The first ring cleavage product identified in cultures of *P. chrysosporium* was a cyclic carbonate formed from a β-O-4 model compound (257). Shortly thereafter, Leisola et al (183) identified two isomeric lactones formed from veratryl alcohol via oxygenative cleavage between the methoxyl-bearing carbons. It was demonstrated that ligninase is responsible for their formation (114, 183).

Model β-1 compounds (Units 9 and 10, Figure 1) undergo reactions analogous to those involved in the formation of products *1–4* from the β-O-4 models (Figure 3); the relative simplicity of their reactions has provided much insight into the mechanism of ligninase action (82, 91, 97, 99, 236, 250, 251). The reactions shown in Figure 3 were first found in intact cultures (44, 80, 165, 207), and arise from ring *A* oxidation. Reactions initiated by ligninase oxidation of ring *B* have not been studied per se; it is possible that the products would be the same as those from oxidation of ring *A*. Also, ring cleavage products from ligninase oxidation of β-1 models have not yet been

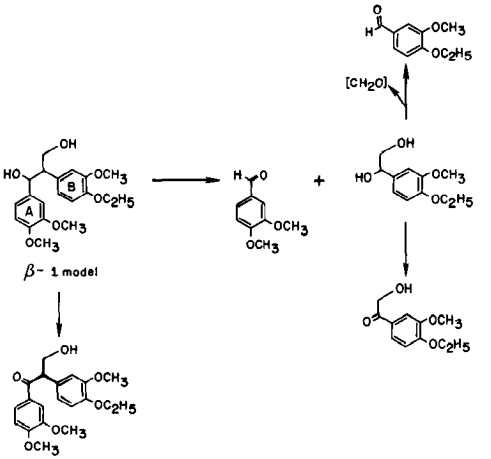

Figure 3 Oxidation of β-1 model compounds by ligninase/H_2O_2.

observed, but such products were tentatively identified among low–molecular weight compounds formed during white-rot of wood (28).

Interestingly, one-electron oxidants, including isolated hemes with appropriate oxidants, convert β-1 models to the same products as ligninase (90, 118, 242), and should have utility in basic and applied studies.

Most of the products in Figures 2 and 3 have been identified in intact cultures of *C. versicolor* (135, 138–140) as well as *P. chrysosporium*. Initial studies with a number of other white-rot fungi indicate degradation of β-O-4 and β-1 models via the same reactions (48, 49). Thus it seems clear that ligninase is primarily responsible for the initial degradation of nonphenolic β-O-4 and β-1 model compounds and presumably also for the initial degradation of the same structures in lignin.

Ligninase oxidation of lignin model compounds representing other substructures has not yet been examined. However, some (not all) of the reactions in the degradation of a nonphenolic β-5 model compound (substructure 3–4, Figure 1) in intact cultures of *P. chrysosporium* (206) can probably be attributed to ligninase. Other spontaneous reactions that follow ligninase oxidation have been seen with single-ring compounds; these include dihydroxylation of styryl structures and hydroxylation of C_α-methylene groups (75, 82, 236, 251).

In addition to lignin-related aromatics, polycyclic aromatics and certain dibenzodioxins with appropriate oxidation potentials are oxidized by ligninase to quinones and unidentified products (93, 98, 239). The reactions are consistent with a cation-radical mechanism: The cation radical from dibenzodioxin oxidation was directly demonstrated, and $H_2{}^{18}O$-labeling

studies were consistent with a cation-radical intermediate from ligninase oxidation of pyrene to pyrenediones (98).

Intact cultures of *P. chrysosporium* degrade a variety of chlorinated aromatics (10, 22, 43, 119); this indicates that the degrading system is nonspecific. The role of ligninase is not yet known, but it oxidizes several chlorophenols (K. E. Hammel, personal communication).

Multiplicity In one of the early studies electrophoresis indicated that ligninase activity in *P. chrysosporium* might be associated with more than one protein (250). Later work verified this multiplicity (59, 123, 161, 181, 182, 219, 235). The number of isoenzymes reported (from 2 to 15) reflects not only separation efficiency, but probably postsynthesis modifications, and perhaps differences in strains, culture conditions, and ages. The number of structural genes involved is not yet known. All of the isoenzymes oxidize veratryl alcohol. Those examined further (59, 181, 219, 235; R. L. Farrell, K. Murtagh, M. Tien & T. K. Kirk, unpublished) have molecular mass of 39–43 kd and similar spectral and catalytic properties, and they are glycoproteins. Polyclonal antibody reactions and protease digestion patterns indicate considerable homology. The isoenzymes differ in isoelectric points, in specific activity by at least fivefold, and slightly in K_m for veratryl alcohol oxidation and for H_2O_2 reduction. The important question of whether they differ in oxidation potential has not yet been answered.

Tien et al (252) provided evidence that each preparation of ligninase isoenzyme H-8 (161) consists of a mixture of active and inactive enzyme. The two could not be separated. Once isolated, ligninase is stable (219). Paszczyński et al (219) found that the specific activity of the ligninases varied with culture age, but this might reflect differences in isoenzyme mixtures.

Production Ligninase production by *P. chrysosporium* has been increased substantially through improvement of strains and culture parameters. Originally the activities, measured in N-starved stationary cultures, were low (~5 units per liter) based on veratryl alcohol oxidation (251). Buswell et al (25) later measured over 400 U liter^{-1} in N-sufficient stationary cultures of a new strain, INA-12, grown on glycerol. Faison & Kirk (55) showed that the various culture parameters that favor complete degradation of lignin similarly influence ligninase titer in cultures. Ligninase titer is increased by adding lignins or related low–molecular weight aromatics (55), including veratryl alcohol (55, 184), to cultures.

Volume scale-up was achieved with a mutant strain, SC-26, in rotating disc fermenters (161) and with a wild-type strain immobilized on the interior wall of a rotating plastic carboy (219). Linko et al (190) achieved continuous and repeated batch production of up to 245 U liter^{-1} of ligninase with wild-type mycelium immobilized in agar or agarose gels.

Further improvements in ligninase production have been obtained with agitated submerged cultures. Gold et al (82) first reported ligninase production in such cultures by a mutant strain, and Leisola et al (184) later demonstrated production of 60 U liter^{-1} in wild-type cultures to which veratryl alcohol had been added. Activities of up to 670 U liter^{-1} (specific activity = 36 U mg^{-1}) were obtained in carbon-limited cultures with concentrated mycelial pellets. Jäger et al (123) found that detergent in the medium permits wild type strains to produce ligninase, and to degrade lignin to CO_2, in agitated submerged cultures, and Asther et al (12) measured activity of over 1250 U liter^{-1} (specific activity = 29 U mg^{-1}) with strain INA-12 growing in a glycerol medium to which oleic acid emulsified with Tween 80 had been added. This is the highest activity reported to date, but it represents production of less than 50 mg liter^{-1} of ligninase protein, which suggests that considerable improvements might still be made. We have obtained activity of over 1000 U liter^{-1} with strain SC-26 in 2-liter stirred tank fermentors, and have found that culture additives, pellet size, stirring rate, and aeration are critical parameters (T. K. Kirk & S. Croan, unpublished).

MANGANESE PEROXIDASE Kuwahara et al (174) discovered a peroxidase activity different from ligninase in extracellular growth fluid of ligninolytic cultures of *P. chrysosporium*. The isolated 46-kd enzyme exhibited a requirement for H_2O_2, Mn(II), and lactate. Like horseradish peroxidase, it oxidized phenol red, *o*-dianisidine, and polymeric dyes. A similar enzyme of 45–47 kd isolated from ligninolytic cultures (120) also required Mn and H_2O_2, but did not require lactate (possibly because tartrate was present as buffer) or oxidize phenol red. It contained an easily dissociable heme.

Both Mn peroxidases were later purified and characterized (74, 218). The enzymes now seem to be either identical or isoenzymic. Both contain a single protoheme IX, with high-spin ferric iron. The enzyme oxidizes Mn(II) to Mn(III), which in turn oxidizes the organic substrates (73, 74, 218, 219). Glenn & Gold (74) reported 3–20× stimulation of oxidation rates by lactate or related compounds, which probably reflects stabilization of the Mn(III). The enzyme described by Paszczyński et al (219) contained 17% neutral carbohydrate and a high proportion of acidic amino acids. Leisola et al (181) separated six Mn peroxidases from the extracellular fluid of *P. chrysosporium*, and we separated four (T. K. Kirk & R. L. Farrell, unpublished). Mn peroxidases appear to function as phenol-oxidizing enzymes and perhaps participate in H_2O_2 production.

LACCASE AND OTHER PHENOL-OXIDIZING ENZYMES Most white-rot fungi produce extracellular laccase (EC 1.10.3.2). This blue copper oxidase catalyzes the one-electron oxidation of phenols to phenoxy radicals, eventually transferring four electrons to O_2 (233). The effect on the substrate phenols is

the same as that of horseradish peroxidase, despite fundamental differences in enzyme mechanism.

Laccase apparently has a role in sexual fruiting (271) and in lignin degradation. Work with various phenolic model compounds and isolated laccase or HRP shows that certain degradative reactions occur, particularly with syringyl models (114, 168). Among the consequences of the one-electron oxidation in lignin-related phenols are C_α-oxidation, limited demethoxylation, and aryl-C_α cleavage (168). Phenol-oxidizing enzymes account for many of the degradative reactions in phenolic models seen in intact cultures of lignin-degrading fungi (84, 114, 132, 133) and *Fusarium solani* (113, 122, 136, 137).

Recent work shows that laccase can also cause C_α–C_β cleavage in phenolic syringyl structures (T. Higuchi, personal communication). Earlier work (134) had shown that HRP as well as whole cultures of *P. chrysosporium* caused the same cleavage in a phenolic syringyl dimer. Presumably all of the enzymes that catalyze one-electron oxidation of phenols catalyze similar reactions in lignin.

Coupling/polymerization is a major consequence of one-electron oxidation of lignin-related phenols and isolated lignins (e.g. 132, 192; see 168). Polymerization of phenols is also a consequence of ligninase oxidation (92); as discussed below, polymerization by ligninase and the other phenol-oxidizing enzymes must be limited in vivo by mechanism(s) yet to be elucidated.

Phanerochaete chrysosporium belongs to a minority of white-rot fungi that produce no detectable laccase. But apparently all white-rot fungi secrete enzymes capable of oxidizing phenols. Simple color tests are used to determine whether these are of the laccase type or the peroxidase type (which includes ligninase and Mn peroxidase) (100).

H_2O_2-PRODUCING ENZYMES The discovery that H_2O_2 is required for ligninase activity prompted several investigations into its origin. Crude cell extracts from ligninolytic cultures produce H_2O_2 in the presence of added glucose (68). Peroxisomelike structures just beneath the cell walls in hyphae that stain for catalase/H_2O_2 might be the in vivo site of the activity (67). Glucose oxidase activity in ligninolytic mycelia (226, 227) was characterized as a glucose-1-oxidase (gox) (144). Because the activity is produced during growth on various sugars and is associated only with ligninolytic cultures, Kelley & Reddy (145, 227) concluded that gox is the primary source of H_2O_2. Gox mutants had lost their ability to degrade [^{14}C]lignin to $^{14}CO_2$ while retaining other idiophasic functions, and Gox$^+$ revertants regained their ligninolytic capability (143); exogenous gox, however, did not restore ligninolytic activity to a Gox mutant. In related work, Eriksson et al (53)

purified and characterized an intracellular glucose-2-oxidase that was also idiophasic. None of these studies with glucose oxidases demonstrated that the enzyme's action results in production of extracellular H_2O_2. Maltseva et al (196), however, reported extracellular idiophasic glucose oxidase activity in *Panus tigrinus*.

Other work has focused on other possible sources of extracellular H_2O_2. Greene & Gould (87) observed its production by washed, starved mycelia in the absence of added substrates, and attributed H_2O_2 production to fatty acyl CoA oxidase. Paszczyński et al (218) showed that the above-described Mn peroxidase oxidizes various reduced substrates, including glutathione, NADPH, and dihydroxymaleic acid, with the coupled reduction of O_2 to H_2O_2 (see also 11). Surprisingly, extracellular NAD(H) and NADP(H) are present in N-starved ligninolytic cultures (175). Very recently, Kersten & Kirk (151) demonstrated a new extracellular, idiophasic enzyme, glyoxal oxidase, in ligninolytic cultures. Glyoxal, methyl glyoxal, and several other α-hydroxy carbonyl and dicarbonyl compounds serve as substrates; their oxidation is coupled to the reduction of O_2 to H_2O_2. Both glyoxal and methyl glyoxal were identified in the extracellular culture fluid of idiophasic cultures (151). In summary, it appears that the H_2O_2 required for ligninase activity may be supplied by several different oxidases; supply by intracellular enzymes, however, has not been shown directly.

OTHER ENZYMES ACTING ON THE LIGNIN POLYMER As wood is degraded by white-rot fungi, the lignin exposed on the interior microsurfaces within the wood is oxidized. The partially degraded lignin polymer has been produced under controlled laboratory conditions, isolated by solvent extraction, and purified. It has been characterized chemically and physically, most recently by ^{13}C-NMR spectroscopy (26). These studies revealed that fungal attack decreases the methoxyl, phenolic, and aliphatic hydroxyl contents, cleaves aromatic nuclei to aliphatic carboxyl-containing residues, creates new C_α-carbonyl and carboxyl groups, and forms alkoxyacetic acid, phenoxyacetic acid, and phenoxyethanol structures (reviewed in 26). Many of the same effects were observed in specifically ^{13}C -labeled synthetic lignins following incubation with white-rot fungi (94). Surprisingly, ^{13}C spectroscopic studies also revealed significant amounts of aliphatic hydrocarbon structures (26, 29, 95).

It is now apparent that many of these degradative changes in the lignin polymer can be attributed to ligninase; however, some cannot, which indicates that other enzymes participate in polymer breakdown. For example, formation of aromatic carboxyl groups has not been observed in any of the studies with ligninase and model compounds. This suggests that there is an undiscovered extracellular aromatic aldehyde oxidase. Similarly, enzymes

other than ligninase are evidently involved in producing the alkoxyacetic acid, phenoxyacetic acid, and phenoxyethanol structures. The aliphatic hydrocarbon structures are perhaps best attributed to covalently bound lipids. As mentioned earlier, it is not known whether the demethoxylation caused by ligninase and phenol-oxidizing enzymes accounts for the methoxyl loss in lignin (7, 26). Brown-rot fungi demethylate methoxyl groups in the polymer (156) by an unknown biochemical mechanism; it is possible that the white-rot fungi possess the same system. Huynh & Crawford (120) reported an extracellular H_2O_2-dependent enzyme activity in *P. chrysosporium* that demethylates 2-hydroxy-3-phenylbenzoic acid.

One of the puzzles of lignin degradation is the mechanism that prevents polymerization of lignin and phenolic products by ligninase and phenol-oxidizing enzymes. Polymerization of lignin is not prominent in vivo (30, 57, 231). This suggests that phenols are rapidly oxidized past the phenoxy radical step or that the radicals are reduced back to the phenols by an undiscovered enzyme and/or mechanism that prevents polymerization.

Westermark & Eriksson (268) suggested some time ago that phenoxy radicals might be reduced back to phenols by the enzyme cellobiose:quinone oxidoreductase (CBQase). CBQase, discovered in *P. chrysosporium* (268), oxidizes cellobiose, transferring the electrons to various quinones. We recently found that CBQase apparently does not prevent polymerization of phenols by ligninase or horseradish peroxidase (E. Odier, M. Mozuch, B. Kalyanaraman & T. K. Kirk, unpublished).

ACTIVATED OXYGEN SPECIES The requirement of H_2O_2 for lignin degradation was discovered through investigations into the possible involvement of activated oxygen species: H_2O_2, superoxide radical ($O_2\cdot^-$), hydroxyl radical ($\cdot OH$), and singlet oxygen ($^1O_2$). All of these species have been reported in biological systems. Only $^1O_2$ and $\cdot OH$ are reactive enough to be considered lignin oxidants. Initial studies by several groups suggested involvement of both of these species (reviewed in 168), but subsequent investigations have discounted involvement of both $^1O_2$ (166, 173) and $\cdot OH$ (164).

Genetics

Genetic approaches have been applied to the study of bioligninolytic organisms, particularly *P. chrysosporium*, to fully elucidate fundamental principles and manipulate the system. The methods of classical genetics such as the use of good selectable markers and the formation of recombinant strains (in the classic sense) are paramount to the basic studies. Comparisons with better-understood prokaryotic secondary metabolic systems cannot be readily drawn, unfortunately, since the enzymes produced in lignin degradation are unlike those in most metabolic pathways (see section on enzymatic "combustion").

Phanerochaete chrysosporium apparently has a classical three-stage life cycle (homokaryotic haploid, multikaryotic haploid, and homozygotic diploid), and it is probably homothallic or self-fertile (H. H. Burdsall & K. Nakasone, personal communication; M. Alic, C. Letzring & M. H. Gold, personal communication). Prolific conidiation, which is unusual among white-rot fungi, gives *P. chrysosporium* distinct advantages over other species for mutant production. Ultraviolet irradiation of asexual spores (conidia) was originally used to induce mutations in *Polyporus adustus* (50). Gold & Cheng (76) facilitated mutant analysis by inducing colonial growth on solid-agar plates and developing replica-plating techniques. They also elucidated the physiological conditions for fruit body formation, which permit genetic recombination (77). They showed that fruiting is controlled by glucose and nitrogen metabolite repression and that adenosine-3',5'-cyclic monophosphate reverses the effects of glucose repression (77).

Table 1 lists major described genetic mutants of *P. chrysosporium*. Various auxotrophic mutants of *P. chrysosporium*, of which most are auxotrophic for amino acids or cofactors (Table 1), have been isolated and used in complementation studies. Gold et al (79, 83) isolated UV and X-ray mutants; some phenotypically similar mutations apparently occurred at different loci. Protoplasts were fused and mycelia were regenerated in various strains (78).

Table 1 Some mutants of *Phanerochaete chrysosporium*

Organism (ATCC number)	Strain or mutation	Phenotype[a]	Reference
32629	44	C^-; X^+; POx^-	6
32629	44-2	C^-; X^-; POx^+	51
32629	63-2	C^-; X^-; POx^+	51
32629	31	C^+; L^+	128
32629	3113	C^-; L^+	128
32629	85118	C^-; L^+	128
24725	SC26	L-enhanced	161
34571	*leu3,1,2*	Leucine auxotroph	204
34571	*ade1,2*	Adenine auxotroph	204
34571	*rib1*	Riboflavin auxotroph	204
34571	LMT 320	Riboflavin auxotroph	191
34571	*nic1*	Nicotinamide auxotroph	204
34571	*arg1,3,4*	Arginine auxotroph	204
34571	*his1,2*	Histidine auxotroph	204
34571	LMT 31	Histidine auxotroph	191
34571	*met1*	Methionine auxotroph	204
34571	*leu1*	Leucine auxotroph	204
34571	LMT 30	Leucine auxotroph	191
34571	LMT 26	POx^-; L-enhanced	191

[a] C = cellulase; X = xylan degradation; POx = phenol oxidase activity; L = lignin degradation; + = positive.

Marker strains carrying multiple mutations were recovered by fruiting heterokaryons of *P. chrysosporium* (5). In further complementation studies certain gene mutations and enzyme deficiencies were identified (204).

Eriksson and coworkers (51) developed various Cel^- as well as phenol oxidase positive (POx^+) strains in attempts to improve selective lignin degradation (Table 1). Cross-breeding of homokaryotic Cel^- strains has improved lignin-degrading ability (128). Unfortunately, highly lignin-degrading strains derived from cross-breeding may not be intercrossable, since 75% of the strains did not fruit. Cel^- strains 3113 and 85118 degraded lignin in wood (128) and synthetic lignins (170). In a study with several strains, there was no correlation between lignin degradation and ligninase production (170). SC-26, a mutant selected for enhanced decolorization of a lignin by-product, had about six times the amount of accumulated ligninase as the parent (161, 170).

Gold et al (83) isolated a POx mutant that was pleiotropically lacking in several secondary metabolic functions; a revertant regained all of the functions. Recently, Liwicki et al (191) isolated a number of POx mutants and found that they shared the following idiophasic traits: increased intracellular cAMP levels, sporulation, extracellular glucan, veratryl alcohol synthesis, and lignin-degrading ability. The authors concluded that mutations that result in the loss of lignin-degrading ability are not necessarily pleiotropic with other idiophasic functions.

As pointed out above in the section on physiology, idiophasic metabolism is initiated by N-limitation. Marzluf (198) has extensively studied nitrogen metabolism and its control in *Neurospora crassa, Aspergillus nidulans,* and *Saccharomyces cerevisiae*. Analogous studies with *P. chrysosporium* should contribute to an understanding of N-regulation of lignin degradation. A new selection procedure that uses lignin model compound–amino acid adducts has been developed: ligninase cleaves the compounds, releasing the amino acids, which can serve as growth nitrogen (249). The compounds are now being used to select N-deregulated mutants for lignin degradation (M. Tien, personal communication).

Genetic study of *P. chrysosporium* can be used on the one hand for acquiring more complete basic understanding of the organism, its primary and secondary metabolism, and regulation of ligninolytic activity, and on the other for manipulation in industrial applications. As described in the previous section, a single enzyme, even ligninase, does not completely degrade lignin; therefore, although molecular approaches are warranted for isolation and expression of pertinent ligninolytic enzyme gene(s), fundamental knowledge of the total system is still required. The genetic approach, including manipulation, selection for desired metabolic characteristics, and development of new recombinant strains, is probably the most accessible and clearest approach available for elucidation of bioligninolytic systems.

Molecular Biology

Following the isolation and characterization of ligninase, several laboratories began to apply recombinant DNA research to lignin degradation. The large-scale production of recombinant ligninolytic enzyme(s) in genetically engineered organisms is on the horizon.

CLONING Two similar, simple methods for isolating high-quality DNA and/or RNA that appear general for filamentous fungi have been developed with basidiomycetes, including *P. chrysosporium* (224, 225). Translatable mRNA from *P. chrysosporium* was first isolated (109) by a procedure adapted from that of Garber & Yoder (71).

Comparison of ligninolytic and nonligninolytic mycelial in vitro–translated mRNAs showed several differences in 40–50 species of polypeptides resolved in one-dimensional gels (109). Differences were also observed between in vitro–translated products from 3-day-old and 6-day-old nonligninolytic cultures; this points out the difficulty in interpreting these types of results.

In vitro–translated mRNAs isolated from ligninolytic (N-limited) cultures of *P. chrysosporium* strain BKM (ATCC 24725) contained several translation products in the 25- and 40-kd regions that were not found in mRNAs isolated from nonlignolytic cultures, including a unique 40-kd protein that reacted with affinity-purified polyclonal ligninase antibodies (Y. Devash & A. Anilionis, personal communication).

Ligninase gene(s) encoding isoenzymes of *P. chrysosporium* have been cloned by two groups (60, 252a). Tien & Tu (252a) very recently reported the cloning (in *E. coli*) and sequencing of a cDNA for a ligninase isoenzyme. They showed that synthesis of ligninase is regulated by N-limitation at the mRNA level. Sequence analysis revealed that mature ligninase is preceded by a 28-residue leader, and that the mature protein contains 345 amino acids. Sequence homology around histidine residues of ligninase and turnip, cytochrome *c*, and horseradish peroxidase suggested a similarity in catalytic mechanism (252a). Lignin model compound assays were used to characterize the activities of recombinant ligninase H8, cloned by Farrell (60). With tetramethoxybenzene, veratryl alcohol, and a β-O-4 model (see Figure 2) as substrates, recombinant ligninase catalyzed aromatic demethoxylation, C_α-oxidation, and C_α–C_β cleavage, respectively. The specific activities of recombinant ligninase (rH8) in these assays were essentially equivalent to those of *P. chrysosporium* H8. Oxidation of veratryl alcohol (VA) to veratraldehyde occurred at a specific activity of 25 μmol VA oxidized min^{-1} mg^{-1} enzyme with rH8 compared to 22 μmol VA oxidized min^{-1} mg^{-1} with *P. chrysosporium* H8.

In the presence of kraft lignin (Indulin AT from Westvaco) and milled wood (loblolly pine) lignin the recombinant ligninase exhibited substrate-

dependent peroxide uptake. The extent and rate of this reaction were comparable to those of the reaction catalyzed by *P. chrysosporium* ligninase. Analysis of lignin treated with recombinant ligninase by ionization difference spectroscopy indicates that the recombinant enzyme probably catalyzes C_α-oxidation of these polymers, as well as other functional group changes (T. Maione & R. Farrell, unpublished).

Besides the ligninase H8 gene, other genes have been cloned from *P. chrysosporium*. Zhang et al (276) used synthetic oligonucleotide probes to identify putative ligninase cDNAs. P. Broda and coworkers (personal communication) isolated a *trpC* gene by complementation of the corresponding *Escherichia coli* auxotroph. Further, they isolated *P. chrysosporium* acetyl CoA mutants by selecting fluoracetate-resistant strains. This work is aimed at developing a transformation system in *P. chrysosporium*. Rao & Reddy (225) reported several DNA sequences capable of promoting autonomous replication of plasmid Ylp5 in *S. cerevisiae;* these sequences may be useful for vector construction for such a transformation system.

EXPRESSION AND PRODUCTION OF RECOMBINANT ENZYMES The utility of any clone lies in the expression of its gene product. To achieve expression one must consider basic properties of the host organism and their relevance to the gene product of interest. Such properties include fermentability, expression level (percentage of recombinant gene product in the total protein produced), ability to secrete and/or glycosylate, and presence of proteases, which decrease recombinant protein stability.

The major hosts used for expression of recombinant proteins are *Escherichia coli, Bacillus subtilis,* and *Saccharomyces cerevisiae*. Other host organisms that are currently being studied and developed include *Pseudomonas* sp., other yeasts, *Aspergillus nidulans, Aspergillus niger,* mammalian systems, and to a lesser extent plant and insect systems and *Trichoderma reesei*. The highest expression levels have been demonstrated in *E. coli,* which has the disadvantage of inability to glycosylate. In addition, proteins that are normally soluble are often expressed in *E. coli* in an insoluble form in inclusion bodies (101). Solubilization requires denaturation and renaturation, and has been achieved for some proteins (89, 197). Vectors and hosts have been developed for *S. cerevisiae,* and fermentation is probably superior to that by strains of any other species, in large part owing to brewing-industry research (265). The drawbacks of yeast are relatively low expression level and inappropriate glycosylation patterns. The development of transformation systems for *A. nidulans* has permitted investigation for heterologous gene expression in this organism (reviewed in 40, 41, 266). Basic studies will probably require different host organisms (e.g. *A. nidulans*) from those used as industrial expression systems (e.g. *A. niger*). Owing to physiological similarities to fungi such as *P. chrysosporium,* the ability to recognize heterologous

promoters and secretion signals, and a high capacity for accumulating protein in the extracellular medium, *Aspergillus* is an excellent choice as a host organism for expression of recombinant ligninolytic organisms.

For recombinant ligninase production, heme incorporation is required in vivo and in vitro. Heme-containing proteins have been expressed in prokaryotic hosts, but usually as the apoprotein. Human myoglobin cDNA expressed as a gene fusion product made up about 10% of *E. coli* total cellular protein. The insoluble recombinant Mb was solubilized from inclusion bodies by denaturation, and apoMb was reconstituted in vitro with heme (267). Electronic absorption spectra were identical to those of native Mb. Nagai et al (205) demonstrated that the oxygen-binding properties of reconstituted recombinant hemoglobin were essentially the same as those of human native hemoglobin. Both of these studies indicate that in vitro incorporation of heme in the recombinant protein resulted in physical characteristics comparable to those of the native protein. P-450, the most similar to ligninase of all cloned proteins, has been successfully expressed in prokaryotic hosts (264). A small percentage of P-450 molecules expressed in *E. coli* appeared to incorporate heme appropriately, but the majority of *E. coli* recombinant P-450 was apoprotein. A greater percentage of P-450 expressed in *S. cerevisiae* had appropriate in vivo incorporation of heme.

Future molecular work will include the cloning of more ligninolytic genes and comparison of their expression and gene products in a variety of hosts. It is likely that ligninase is expressed by a multigenic family, and the structure of the DNAs may give important clues to how they arose (e.g. by gene duplication) and how they are regulated (R. Farrell, unpublished). For eventual application we must develop the most efficient expression systems and maximize the ease of processing active material.

CONCLUSIONS AND RECOMMENDATIONS

Our review leads us to certain general conclusions which in turn point to needs for further research.

1. The literature does not contain convincing evidence that polymeric lignin is biodegraded under anaerobic conditions. We suggest that the slight degradation that has been reported can be attributed to low–molecular weight fragments already present in the samples, freed through the action of hemicellulases or esterases or released abiotically during long incubations.

2. Many aerobic bacteria, including actinomycetes, can degrade certain lignocellulosic materials, and they probably degrade lignin fragments produced abiotically and by other microbes. Some bacteria can apparently degrade a wide range of lignin-related models, which suggests that they possess nonspecific intracellular systems. Further research is needed to determine whether bacteria produce extracellular, polymer-degrading enzymes, i.e. to

determine the ability to attack the polymeric lignin backbone. We suggest that comprehensive studies aimed at maximizing rates and extents of lignin degradation and at identifying the responsible enzymes be undertaken with selected species, with carefully characterized ^{14}C-labeled synthetic lignins, and with dimeric model compounds.

3. Certain ascomycetes and fungi imperfecti, particularly those that cause soft rot and the few that cause a white rot of wood, can degrade lignin. Because of their abundance in soils and plant debris, many of these fungi would seem to be of prime importance in lignin mineralization. As with aerobic bacteria, we suggest that selected species be studied in detail to maximize their rates and extents of lignin degradation and to identify the responsible enzymes.

4. Basidiomycetes other than white-rot fungi have been studied little, but members of other groups clearly degrade lignin. Selected species of litter-degraders, mycorrhizal fungi, brown-rot fungi, and other groups should be investigated. Of particular interest from a biochemical standpoint is the extracellular methoxyl-demethylating system of the brown-rot fungi.

5. Good progress is being made in describing the lignin-degrading enzyme system of white-rot fungi. We suggest that forthcoming biochemical and molecular biological investigations continue to concentrate on *P. chrysosporium;* recent evidence suggests that other white-rot fungi possess a similar enzyme system.

Growing evidence indicates that ligninase (lignin peroxidase) is the key lignin-degrading enzyme of white-rot fungi. Its basic mechanism, involving cation-radical intermediates, has been established, and can explain almost all of the degradation intermediates of model compounds seen in intact whole cultures. Further studies are needed to clarify the mechanism and the relative importance of aromatic ring cleavage by ligninase. Also, the possible role of veratryl alcohol in ligninase catalysis needs to be clarified.

Evidence indicates that enzymes other than ligninase are also involved. What are the roles of the multiple Mn peroxidases and other phenol-oxidizing enzymes? How is repolymerization of reacted lignin and phenolic degradation products controlled in vivo? We suggest that new assays be developed, some based on phenols and polymeric lignin, to facilitate identification of remaining enzymes.

6. Certain physiological questions need to be answered for the *P. chrysosporium* system. What are the roles of the extracellular glucan, Mn, and glutamate? What are the details of induction and regulation of the lignin-degrading enzymes? What is the relationship between energy-consuming and energy-yielding reactions during lignin degradation?

7. We suggest that major emphasis be placed on molecular biology to answer important basic questions about ligninase and other enzymes involved in lignin degradation: (*a*) What is the molecular basis for multiple isoen-

zymes? What purpose does the multiplicity serve? (*b*) What is the role of glycosylation in activity and stability? (*c*) What is the maximal expression of the recombinant ligninolytic enzymes in appropriate vehicles? (*d*) Will heme incorporation into recombinant protein be facilitated in vivo or in vitro?

ENZYMATIC "COMBUSTION"

The size, nonhydrolyzability, heterogeneity, and molecular complexity of lignin dictate that its initial biodegradation be oxidative and nonspecific, and that it be mediated by an extracellular system. It is clear from observations of burning lignin that its conversion to CO_2 and H_2O is thermodynamically favored. In biological systems the oxidative mineralization of organics, unlike combustion, normally takes a channeled route (i.e. metabolic pathway) to thermodynamically stable end products. The reactions are catalyzed by enzymes with a fidelity that is rarely matched even by the best organic chemist. The initial steps in the depolymerization of lignin by white-rot fungi are also catalyzed by enzymes, but with a nonspecificity that leads to a potpourri of diverging reactions that is probably unsurpassed by any other enzyme system. Depolymerization is kinetically favored because ligninases oxidize their substrates by one electron; the diversity of subsequent reactions of the unstable intermediates is a function of their structures. It is this nonspecific subsequent oxidation of lignin that leads us to refer to the process as enzymatic combustion. It is likely that similar types of systems are responsible for the initial reactions in lignin polymer degradation by other groups of microbes. We speculate that enzymatic combustion, as opposed to oxidation catalyzed by specific oxygenases and dehydrogenases, could be important in the microbial degradation of other complex aromatics as well as compounds produced wholly or partly abiotically.

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