the bacterium *C. thermocellum* and one of the thermophilic ethanol producing bacteria, ethanol can be produced directly from cellulose. Mixed cultures have given rise to 1.4 moles ethanol/mole glucose[18].

Other biotechnical processes based on cellulosic materials

The utilization of cellulosic materials in biotechnical processes naturally depends upon the development of economically feasible processes. 2 fermentation processes for the production of fodder protein based on cellulosic waste materials from forest industries are already in use and may serve as examples. The first process is founded on the yeast *Candida utilis*, and the other on the fungus *Paecilomyces varioti*. Both of these processes have in common that the substrate is mainly monosaccharides in spent sulfite liquor. Disaccharides and higher oligosaccharides are utilized only to a very limited extent. However, on the basis of the knowledge gained in the studies of the enzymatic degradation of cellulose a fermentation process based on the white-rot fungus *S. pulverulentum*[19] has been developed. The process allows fermentation of solid as well as dissolved lignocellulosic waste and gives, as products, purified water and protein. It seems possible to use the developed technique for the total closure of the white-water system in a newsprint paper mill or in a fiberboard mill. The process has recently been studied in pilot plant scale. The results show no build-up of organic matter taking place and, thus, that all dissolved substances of lignocellulosic origin dissolved from wood in mechanical grinding can be utilized by the white-rot fungus. The fungal mycelium produced in the process can be used either as animal feed[20] or be added to the paper. Both possibilites have been tested and found feasible.

With the present escalation of oil prices it can be foreseen that renewable resources, mainly cellulosic materials, will substitute petroleum as raw material although the extent to which, cannot be surveyed at present. Knowledge of the metabolic pathways of microbial cellulose degradation will, with certainty, play an important role in this evolution.

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Biodegradation of lignin: Biochemistry and potential applications

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Introduction

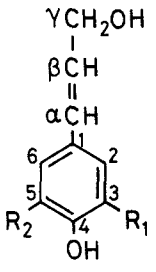
An estimated 65% of our biomass is produced on land. Of that biomass, lignin is the most abundant natural polymer next to cellulose and is an important renewable source of aromatic carbon on earth. Since lignin and cellulose, together with the hemicelluloses, are the structural components of the vascular tissues of higher land plants, biodegradation of the vascular tissues is the key process in the recycling of terrestrial biosynthetic carbon.

However, lignin, which is a heterogeneous aromatic polymer containing various biologically stable carbon-to-carbon and ether linkages, is interspersed with hemicelluloses surrounding cellulose microfibrils, resulting in an organic composite material protected from the degradative enzymes of microorganisms. Therefore, elucidation of the lignin biodegradation process is essential for understanding the circumstances of the recycling of carbon on earth, for establishing technology for bioconversion of plant

residues and waste lignins to useful materials, and for protecting the environment from lignin-related pollutants.

Biochemistry of lignin biodegradation

Lignins have been shown to be dehydrogenative polymers of p-hydroxycinnamyl alcohols such as p-coumaryl (**1**), coniferyl (**2**) and sinapyl (**3**) alcohols:



(1) $R_1 = R_2 = H$
(2) $R_1 = OCH_3$, $R_2 = H$
(3) $R_1 = R_2 = OCH_3$

gymnosperm lignin is formed from coniferyl alcohol; angiosperm lignin from mixtures of coniferyl and sinapyl alcohol; and grass lignin from mixtures of coniferyl, sinapyl and p-coumaryl alcohols. These p-hydroxycinnamyl alcohols are dehydrogenatively polymerized by the mediation of peroxidases via radical coupling and subsequent nucleophilic reactions of various nucleophiles to quinonemethide intermediates, which results in β-β', β-5', β-1', β-O-4', 5-5' and 3-O-4' linkages between phenyl-propanoid units[1] (fig. 1).

Investigations have indicated that white-rot fungi (Basidiomycetes) and closely related litter-decomposing fungi are dominant lignin-degraders; the brown-rot fungi (Basidiomycetes) cause limited degradation of lignin[2,3]; and soft-rot fungi (Fungi imperfecti), various soil Ascomycetes and Fungi imperfecti *(Fusarium, Aspergillus)* as well as some bacteria *(Nocardia, Streptomyces)* partly degrade lignin. Kirk et al.[4] recently found that the addition of cellulose or glucose is necessary for the decomposition of synthetic [ring-^{14}C]-lignin (DHP) to $^{14}CO_2$ by *Phanerochaete chrysosporium* (a white-rot fungus). Lignin cannot serve as a growth substrate and has no influence on synthesis of the ligninolytic system, which simply appears as part of secondary metabolism in the fungus incubated in a medium of excess carbon source (glucose or cellulose) with a limiting amount of nitrogen source (ammonium salts and some amino acids). The oxygen concentration strongly influences the rate and extent of lignin degradation: stationary cultures (10 ml/125-ml Erlenmeyer flask) at 37–39 °C, pH 4.5 in an atmosphere of approximately 60% O_2 in N_2 gave maximum rates of lignin degradation (ligninolytic culture). Culture agitation resulting in pellet formation greatly suppressed lignin degradation at all O_2 levels. These investigations of the physiological parameters of lignin catabolism have greatly contributed to establishing a sensitive and reproducible assay method in the study of lignin biodegradation.

Chemistry of degraded lignin by white-rot fungi

Analytical comparisons[5] of sound and white-rotted lignins extracted with various solvents have shown that the degradation process is heavily oxidative:

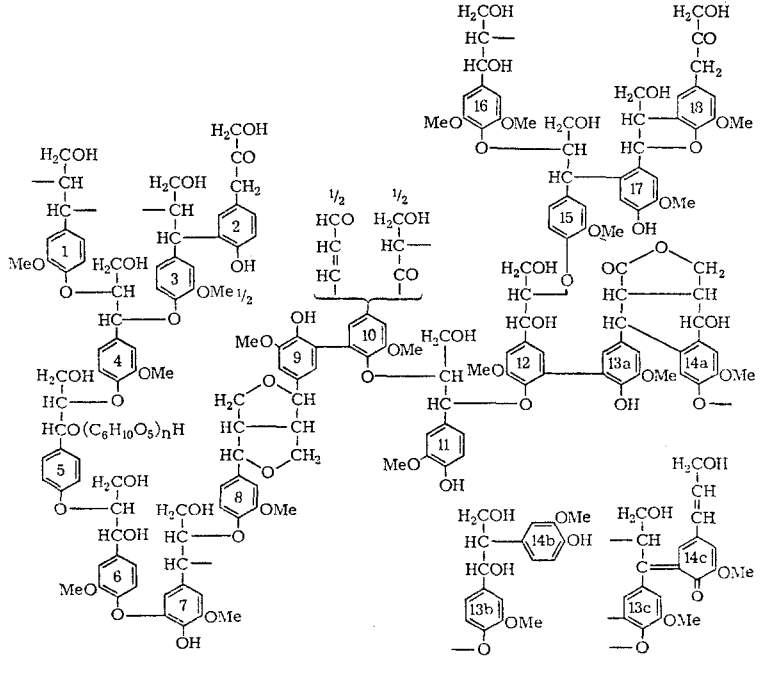


Figure 1. Schematic constitution of spruce lignin (Freudenberg)[1].

degradation of lignin polymer occurs in the side chains, which are oxidized with the formation of α-carbonyl and α-carboxyl groups, and in the aromatic nuclei, which are oxidatively cleaved following demethylation and the introduction of hydroxyl groups in phenolic units to give 2,3- and/or 3,4-dihydroxyphenyl moieties (table). The degraded lignin contained α,β-unsaturated carboxyl groups which were not derived from side chains but presumably from aromatic rings: relative intensity of the 1515 cm^{-1} band due to the aromatic ring in the IR-spectrum was considerably low in decayed lignins in accord with ring cleavage in the lignin polymer. The degradation study of ^{13}C-DHP by various lignin degraders[6] supports the findings on the degradation of aromatic nuclei in the lignin polymer. The low molecular weight fractions extracted from the decayed spruce wood by *P. chrysosporium* contained several aromatic acids (**4–10**), among which vanillic acid (**4**) was by far the most abundant. All of these acids obviously involved Cα-Cβ cleavage, which resulted either in the direct formation of the aromatic acids, or was followed by oxidation to then yield the acids. Traces of several compounds, each containing an intact ring attached to an aliphatic residue which clearly was formed via oxidative cleavage of a 2nd aromatic ring, have been tentatively identified by GC-MS (fig. 2 (**9**), (**10**))[7].

These results seem to suggest that lignin is degraded by white-rot fungi primarily via a few key reactions; a) demethylation of methoxyl groups, b) hydroxylation at C-2 in aromatic rings as found in the decayed lignin by brown-rotted lignin, c) aromatic ring cleavages in lignin polymers (intradiol 2,3- and 3,4-), with the subsequent release of aliphatic products, and d) Cα-Cβ cleavage, which is perhaps a major reaction releasing both aliphatic and aromatic products. This picture suggests that various lignin structural units are degraded by the mediation of extracellular enzymes which attack both low molecular weight and polymeric substrates via many intermediate products along several different pathways.

Degradation of dilignols

The use of low molecular weight compounds of a specific substructure (2-unit segments containing specific types of interunit linkage) in lignin is indispensable in elucidating the details of the reactions involved in the degradation of various linkages in lignin macromolecules. Thus, investigations have been done on the degradation of dilignols by *Fusarium solani* M-13-1 which was isolated by an enrichment technique using DHP as the sole carbon source and by *P. chrysosporium*. On being added to the shaking culture of *Fusarium*, guaiacylglycerol-β-coniferyl ether (β-O-4 substructure model, 40–65% in lignins) (**11**) was degraded to (**15**) via compounds (**12**), (**13**) and (**14**). The compound (**15**) was then degraded to (**16**) which was converted to (**17**) and/or (**18**). 2,6-Dimethoxy-p-benzoquinone (**19'**) was isolated when syringylglycerol-β-vanillic acid ether (**15'**) was used as a substrate, which indicates that the β-vanillic acid ethers are degraded via alkylphenyl cleavage as shown in figure 3[8–10]. However, cleavage of the β-O-4 dilignol linkage to β-hydroxypropiovanillone and

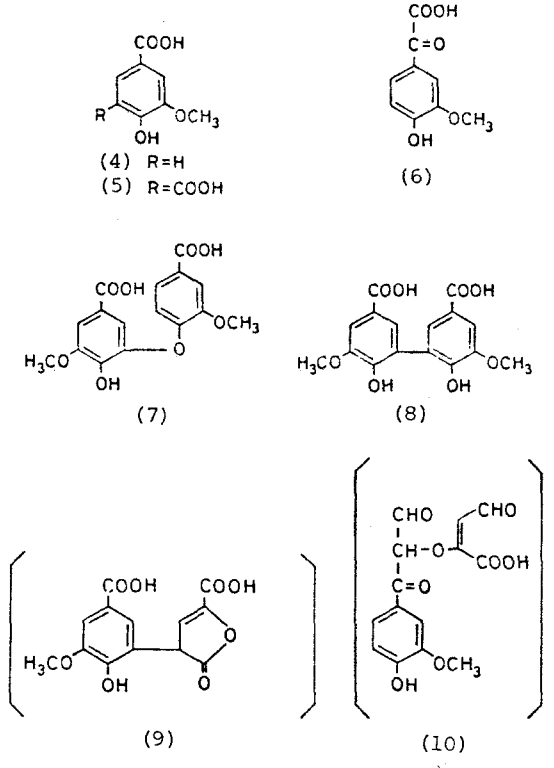


Figure 2. Aromatic acids identified in extracts of white-rotted spruce wood.

Analytical properties of lignin isolated and purified from spruce wood before and after decay by white-rot fungi (Chang et al.)[5]

Lignin	Formula for average C_9-unit	Functional groups (moles/C_9-unit) Conjugated carbonyl	Total carboxyl	Hydroxyl Phenolic	Aliphatic	Total
Sound	$C_9 H_{8.66} O_{2.75} [OCH_3]_{0.92}$	0.07	0.10	0.24	0.92	1.16
Decayed by						
Polyporus anceps	$C_9 H_{7.70} O_{3.80} [OCH_3]_{0.72}$	0.16	0.59 (0.17)*	0.10	0.77	0.87
Coriolus versicolor	$C_9 H_{7.26} O_{3.95} [OCH_3]_{0.74}$	0.17	0.55	0.11	-	-

* Aromatic acid as estimated by ^1H-NMR.

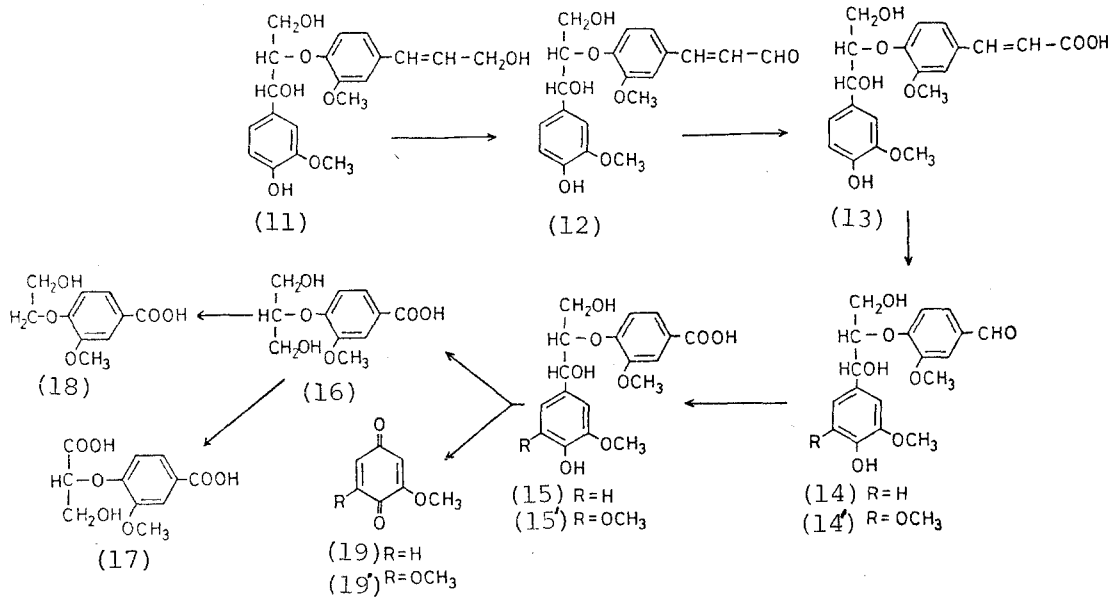


Figure 3. Degradation pathway of guaiacylglycerol-β-coniferyl ether by *Fusarium solani* M-13-1.

coniferyl alcohol was found in *Pseudomonas putida*[11,12].

On investigating *P. chrysosporium*[13] the non-phenolic β-O-4 compound (**20**) was converted to (**21**) which was then converted to (**22**) and (**23**). The vanillic acid ether (**23'**) was converted to (**24**) via Cα-Cβ cleavage instead of alkyl-phenyl cleavage in phenolic β-O-4 model (fig. 4).

Recently, cleavage of the Cα-Cβ and/or alkyl-phenyl linkages in veratrylglycerol-β-guaiacyl ether, which is a β-O-4 model but not a true degrading intermediate, was also reported in white-rot fungus *(Phanerochaete chrysosporium)*[14] and in bacteria[15].

The β-5' (phenylcoumaran) substructure model, dehydrodiconiferyl alcohol (**26**) was degraded by *F. solani* M-13-1 to (**29**) via (**27**) and (**28**). The compound (**29**) was further oxidized to the compounds (**30**) and (**31**)[16]: phenylcoumaran-α'-aldehyde with syringyl group (**29'**) was converted to phenylcoumarone (**32**)[17]. The results suggest the participation of a dioxygenase in the cleavage of the coumarone ring to 5-acetylvanillyl alcohol (**30**) and vanillic or syringic acids (fig. 5). Enzymes which oxidize allyl alcohol groups in side chains of β-O-4 and β-5' dilignols as well as of the lignin polymer have been found in the culture filtrate of *Fusarium*[18] and *Nocardia*[19], respectively.

On the other hand, in ligninolytic cultures of *P. chrysosporium*[20] the cinnamyl alcohol side chain of 4-O-methyl dehydrodiconiferyl alcohol (**33**) was degraded to (**35**) via the compound with the glycerol side chain

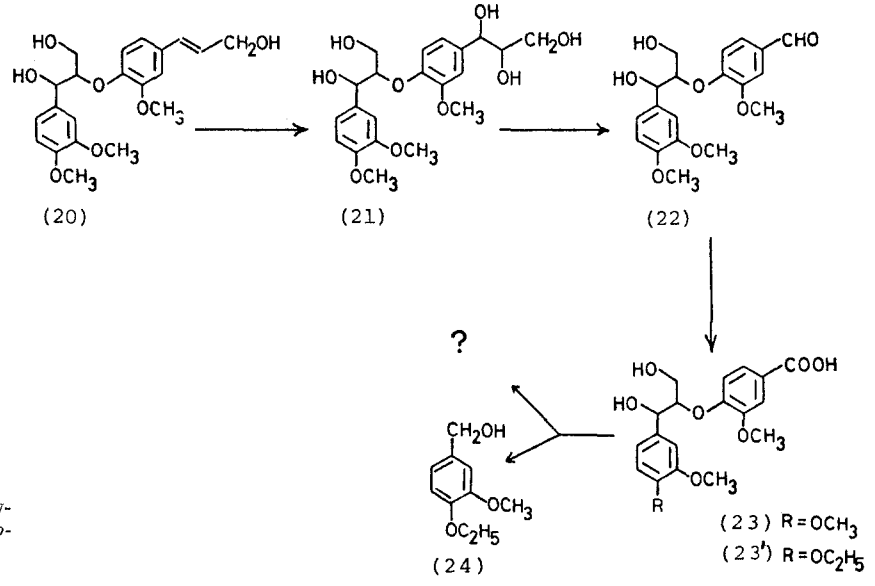


Figure 4. Degradation pathway of arylglycerol-β-aryl ether by *Phanerochaete chrysosporium*.

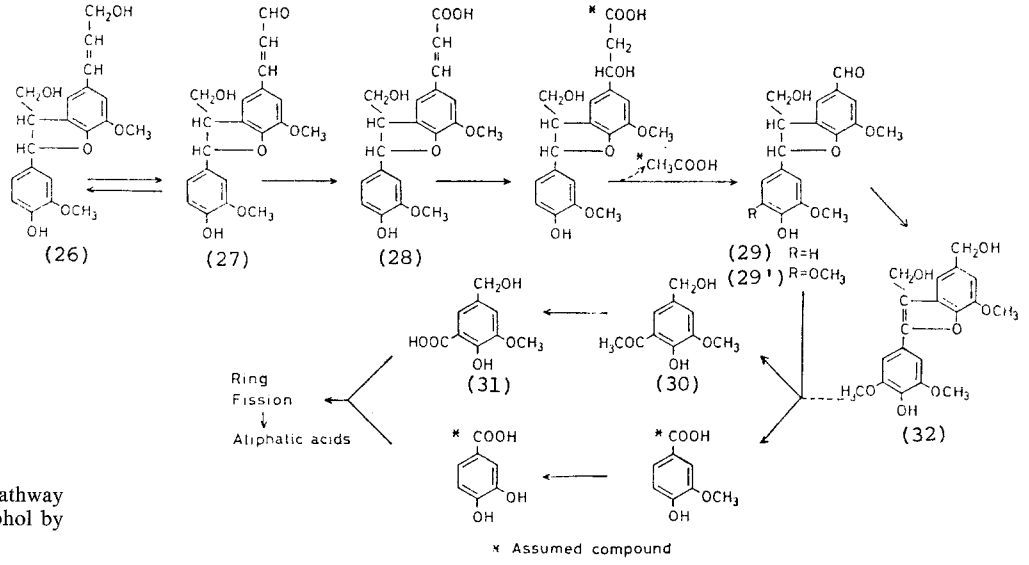


Figure 5. Degradation pathway of dehydrodiconiferyl alcohol by *Fusarium solani* M-13-1.

(**34**) as in the degradation of β-O-4 dilignol, and then (**35**) was degraded to 3-methoxy-4-ethoxybenzoic acid via phenylcoumarone (**36**) (fig. 6). Phenylcoumaran-α'-aldehyde with the syringyl group (**35'**) was converted to phenylcoumarones (**36'**) and α-hydroxyphenylcoumaran (**37**) which was degraded to 2,6-dimethoxy-p-benzoquinone (**19'**) and/or syringaldehyde[21]. Oxygenases seem to be involved in the formation of the glycerol side chain by this fungus.

The β-β' substructure model, syringaresinol monobenzyl ether (**38**) was converted by *F. solani* M-13-1[22] to (**39**), (**40**), (**41**) and 2,6-dimethoxy-p-benzoquinone via alkyl-phenyl cleavage (fig. 7). Dibenzyl and dimethyl ethers of syringaresinol were not degraded, which suggests that both phenolic hydroxyl groups are indispensable for complete degradation of the resinol by this fungus. Diguaiacylpropane-1,3-diol (β-1') substructure model (**42**) was degraded to (**43**), and (**44**), and from 1,2-disyringylpropane-1,3-diol (**42'**), (**43'**), 2,6-dimethoxy-p-benzoquinone (**19'**), (**45**) and syringaldehyde (**46**) were obtained, which again indicates that the compounds were degraded via alkyl-phenyl cleavage as illustrated in figure 8[23].

The degradation products of the main dilignols by *F. solani* M-13-1 and *Phanerochaete* showed the occurrence of the following degradation reactions[24]: 1. cinnamyl alcohol groups are oxidized to the corresponding cinnamic acids via cinnamaldehyde by *Fusarium* but are converted to glycerol groups by *Phanerochaete,* 2. both cinnamic and glycerol side chains are converted to the C6-C1 acid side chains via C6-Cl aldehydes (fig. 9), 3. α-hydroxydilignols such as arylglycerol-β-aryl ethers and diarylpropanes are degraded via cyclohexadienone radical derivatives to the corresponding hydroquinones and glyceraldehydes by alkyl-phenyl cleavage, 4. α-ether (alkoxy and phenoxy) dilignols such as pinoresinol and phenylcoumaran are first converted to α-hydroxy

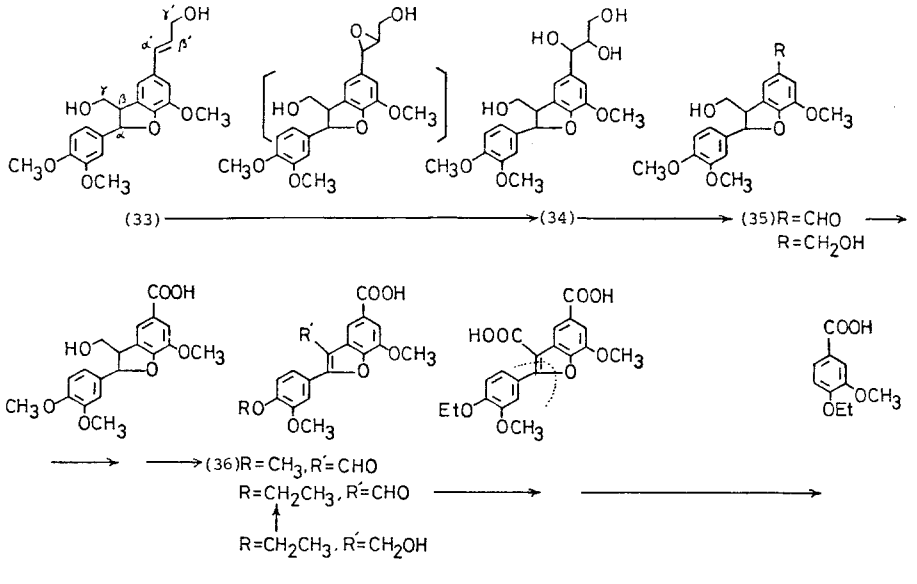


Figure 6. Degradation pathway of 4-O-methyl ether of dehydrodiconiferyl alcohol by *Phanerochaete chrysosporium.*

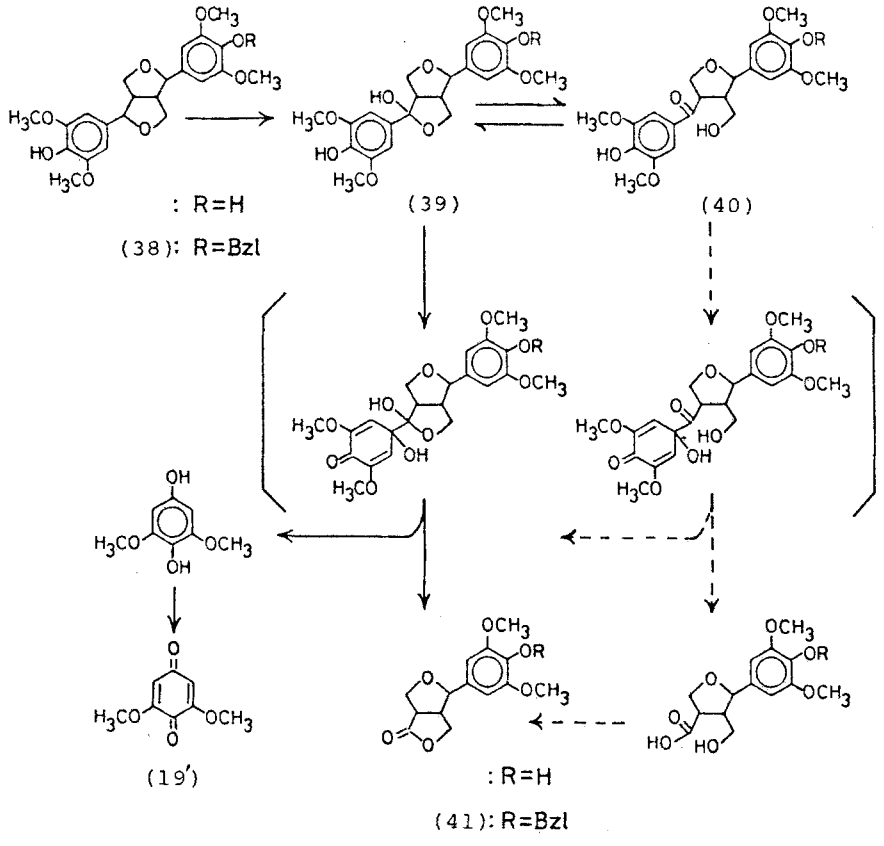


Figure 7. Degradation of syringaresinol by *Fusarium solani* M-13-1.

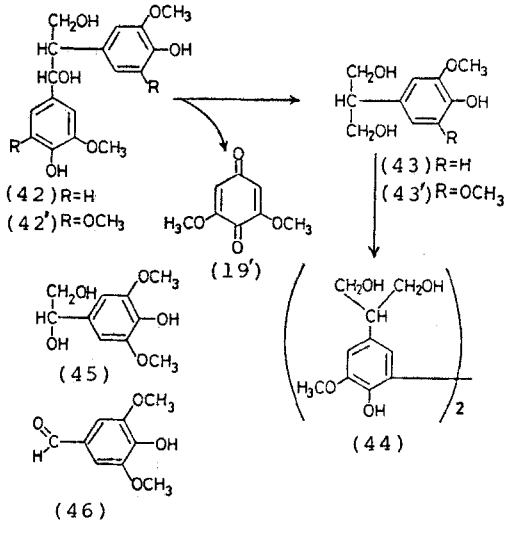


Figure 8. Degradation of 1,2-diarylpropane-1,3-diols by *Fusarium solani* M-13-1.

ethers via quinonemethide intermediates and then α-hydroxy ethers are degraded to the corresponding hydroquinones and lactone or ester derivatives by both fungi (fig. 10), 5. non-phenolic dilignols are not degraded by *Fusarium* but cleaved mainly between Cα and Cβ by *Phanerochaete*. Degradation processes of the phenolic dilignols with *Fusarium* and other white-rot fungi seem to be similar. Even in *Phanerochaete* phenolic dilignols are degraded much faster than non-phenolic dilignols.

Concluding remarks

Chemical analyses of degraded lignins and the degradation of dilignols lead to the conclusion that lignin undergoes simultaneous oxidative degradation of both aliphatic side chains and the aromatic nuclei from the surface of the lignin macromolecule. Aromatic moieties with free phenolic hydroxyl groups

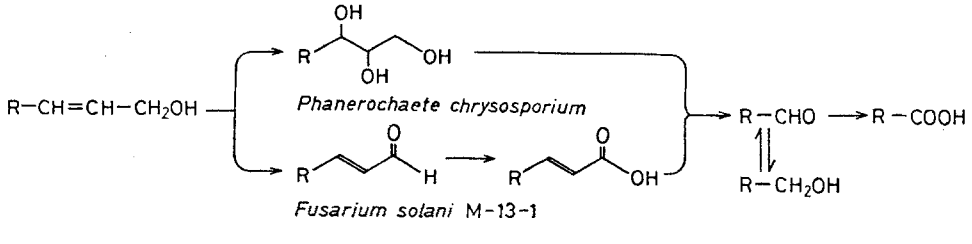


Figure 9. Oxidative degradation of allyl alcohol side chains of dilignols by *F. solani* M-13-1 and *P. chrysosporium*.

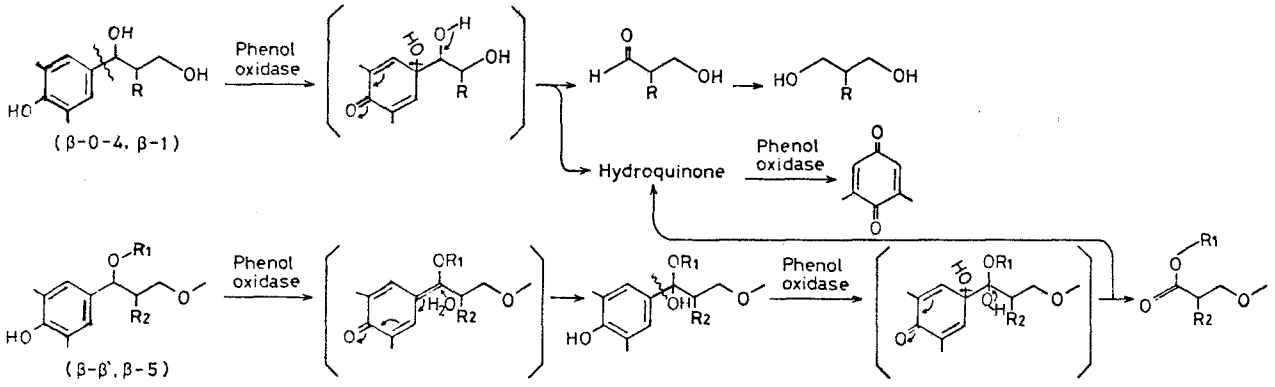


Figure 10. Alkyl-phenyl cleavage of phenolic dilignols by *F. solani* M-13-1 and *P. chrysosporium*.

(20–30% in lignin) would be preferentially attacked via alkyl-phenyl cleavage by the phenol oxidizing enzymes, giving moieties with newly formed phenolic groups which could again be oxidized by the enzymes. Non-phenolic aromatic moieties are mainly cleaved between *Cα* and *Cβ* and the aromatic products formed are ring cleaved in polymers. Aromatic alcohol dehydrogenases and monooxygenases are involved as key enzymes in the oxidative degradation of side chains, and dioxygenases are indispensable for cleavage of aromatic rings of lignin. Vanillic and syringic acids and hydroquinones usually found as degradation products of lignin could be metabolized following the decarboxylation of the acids, hydroxylations, and ring cleavage processes as found by Ander et al.[25] (fig. 11), and via humus polymers in soil[26]. We expect that the use of trilignols and tetralignols as a substrate will provide missing links in the degradation processes between dilignols and lignin polymers[24].

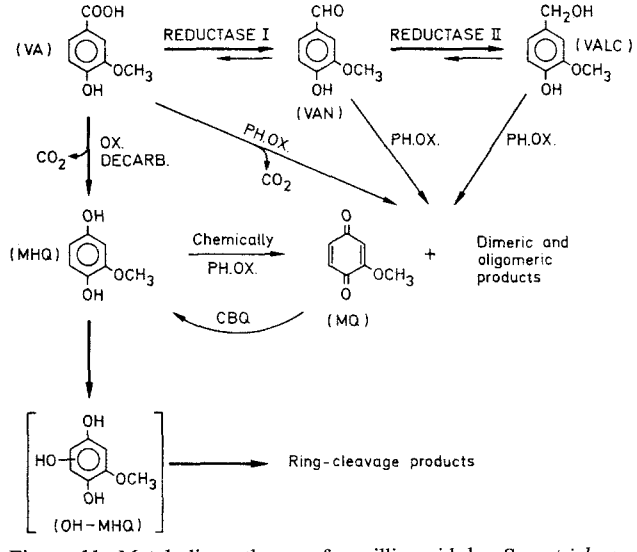


Figure 11. Metabolic pathway of vanillic acid by *Sporotrichum pulverlentum* (Ander et al.)[25].

Applications

Lignin biodegradation research is still at a basic level. However, a considerable potential is expected for the future development of lignin bioconversion processes. Kraft lignins are mostly utilized as fuel to recover chemicals for pulp digestion, whereas lignosulfonates are mainly used as dispersants, stabilizers, binders and for vanillin production. However, a large amount of waste lignin remains still unutilized. Lignin, an aromatic polymer would be the predominant source of low molecular weight chemicals and of high polymers in place of those derived from petroleum. In these fields, lignin bioconversion could be successfully applied with several advantages: conversions with less energy consumption; selected and specific alterations mediated by specific enzymes and/or microorganisms etc. If genetic manipulation is applied to strains of lignin degrading microbes these possibilities could be greatly enhanced[27,28]. Demethylation, hydroxylation, side chain shortening and ring cleavage of lignin could satisfactorily alter polymeric lignin for chemical modifications. Preferential removal of lignin from wood by lignin degraders is useful for biological pulping, and for pretreatment in the production of ethanol and livestock feed from wood polysaccharides. Eriksson and Vallander[29] obtained a cellulase-less mutant (Cel 44) of *Sporotoricum pulverlentum* (= Phanerochaete chrysosporium) by irradiation of a spore suspension with UV-light. 10 days' pretreatment of wood chips by Cel 44 significantly decreased the energy consumption in the production of mechanical pulp. Upon treating thermomechanical pulp[30] and unbleached Kraft pulp[31] with ligninolytic fungi, the maximum rate of lignin degradation was 3% per day over a 2-week incubation; the unbleached Kraft pulp was partially delignified on incubation with ligninolytic fungi, reducing the necessity for bleaching chemicals. Another approach is fungal decolorization of Kraft bleach plant effluents. About 60–70% color reduction was achieved by fungal decolorization of

bleach plant effluent with *P. chrysosporium*[32] and *Tinctoporia borbonica*[33] within 2–4 days.

These kinds of potential applications obviously require an improved understanding of the chemistry and biochemistry of biodegradation, of the nature and properties of the products, and of the microorganisms which could be used. Although such applications seem to be a long way off in the future, research is rapidly providing the fundamental knowledge needed to realize the applications. Recent reviews[2,34–36] and two recent books[3,37] should be consulted for more detailed information on lignin biodegradation.

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