LIGNIN BIODEGRADATION: REDUCED OXYGEN SPECIES

by

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I. INTRODUCTION

A. Sources, function, nature and structure of lignin

Lignocellulosic material comprises about 95% of the earth's land-based biomass. It is composed of approximately 50% cellulose, 25% hemicellulose and 25% lignin, the exact figures varying from species to species. Lignin forms an essential component of the woody stems of arborescent gymnosperms and angiosperms. It is not, however, restricted to arborescent plants, but is found as an integral cell wall constituent of stems, foliage and roots in all vascular plants including herbaceous varieties (1).

In addition to imparting rigidity to plants, lignin acts as a permanent bonding agent between plant cells (2). The fact that lignin is a cell wall constituent allows it to perform multiple functions that are essential to the life of the plant. Lignin decreases water permeation across cell walls in the conducting xylem tissues, and this plays an important role in the internal transport of water, nutrients and metabolities in the plant (1). Lignin also has the important function of protecting plant tissues from invasion by pathogenic microorganisms (1,3). Lignin is most concentrated in tissues where functions such as conduction of solutes (tracheids) and mechanical support (fibers) are specialized.

Plant cell structure and function of tracheids and fibers depend on the intimate association of three main features of

cellular morphology. Each cell is composed of cellulosic fibrils set in a largely amorphous carbohydrate matrix with a polyphenolic binder. The fibrils are largely microcrystalline with paracrystalline regions surrounding those of higher molecular order. The carbohydrate matrix is not a homopolymer of a hexose sugar, but rather, is typified by linear and branched pentose sugar polymers (hemicellulose). The major component of the adhesive or encrusting, amorphous, polyphenolic binder is lignin (4).

The three primary features of cellular morphology are combined in different ways to give overall structure to the plant cell. The two most obvious features of overall cellular structure are the primary wall, which contains 20-28% cellulose, and the thicker secondary wall, which contains 45-55% cellulose (4). Lignin is most concentrated in and between the primary walls (middle lamella) of adjacent cells (Figure 1).

Lignin varies in chemical structure from species to species and can be divided into three catagories (5): a) gymnosperms or softwood lignins, b) angiosperms or hardwood lignins and c) grass lignins. Homologies in the chemical structure of these lignins are best identified on the basis of nitrobenzene oxidation products (5). Whereas gymnosperm lignins yield mainly vanillin with some p-hydroxybenzaldehyde, both syringealdehyde and vanillin are obtained from angiosperm lignins, and significant amounts of all three aldehydes are obtained from grass lignin. In addition, different lignins occur within the same plant (1).

Within the bounds of species and locations dependent variability in chemical structure, lignin is a highly polydisperse, polyphenolic macromolecule of nine-carbon phenylpropane units linked in a random fashion by non-labile C-C and C-O-C interunit bonds (6). Polymeric

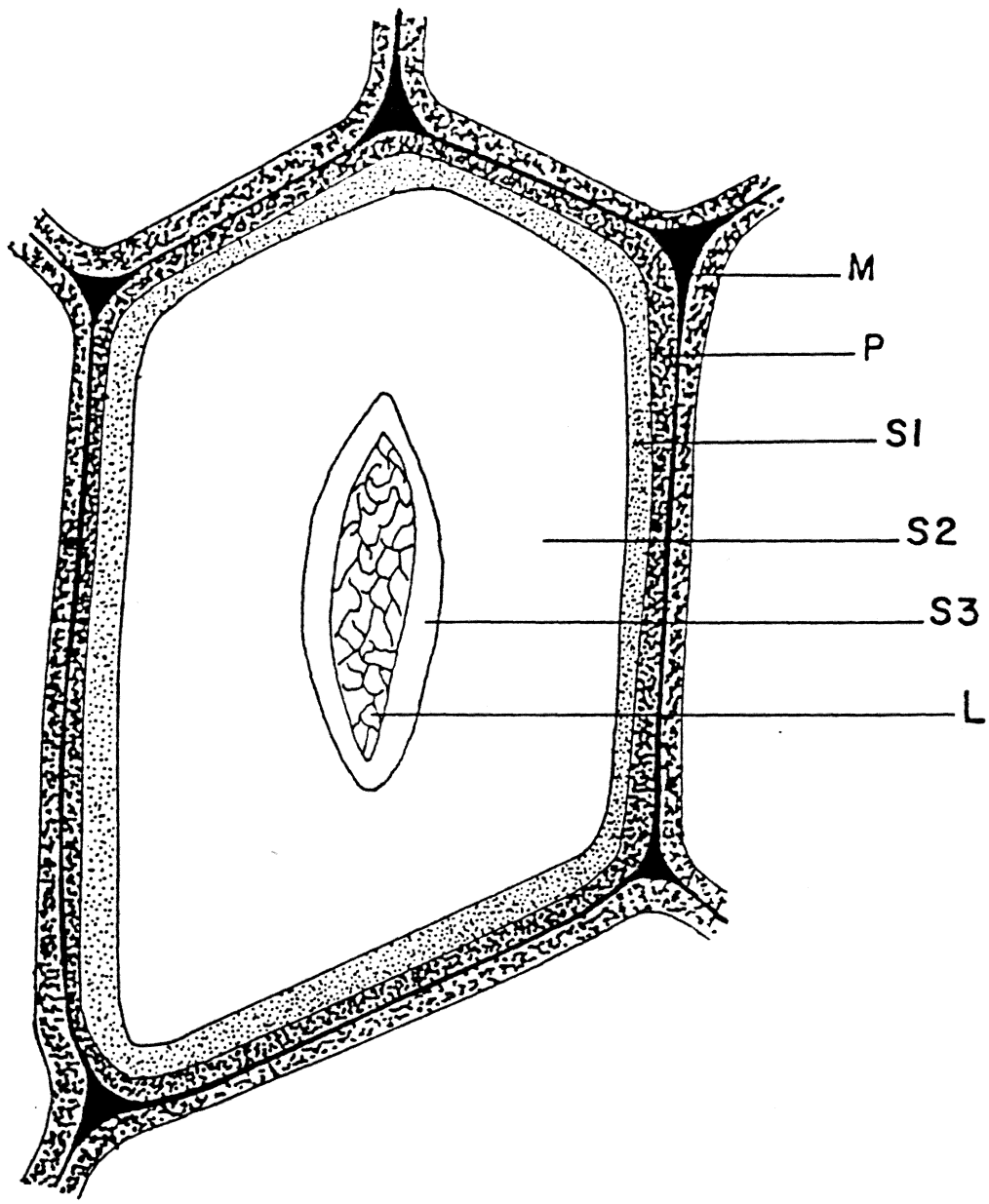
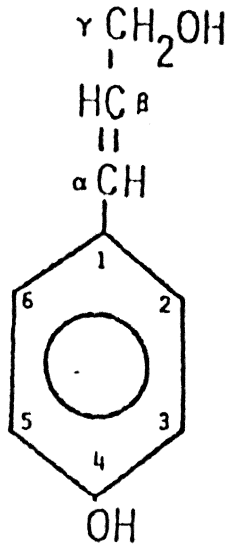


Figure 1. A schematic representing typical plant cell morphology; middle lamela (M), primary wall (P), first layer of secondary wall (S1), second layer of secondary wall (S2), third layer of secondary wall (S3), lumen (L).

natural lignins arise by enzyme - initiated dehydrogenative polymerization of these three primary precursors: trans-coniferyl, trans-sinapyl and trans-p-coumaryl alcohols (1) [Figure 2]. Softwood lignin typically contains 80% coniferyl alcohol, 14% p-coumaryl alcohol and 6% sinapyl alcohol. Hardwood lingin, on the other hand, contains similar amounts of coniferyl and sinapyl alcohols but a minor amount of p-coumaryl alcohol. Bamboo and grass lignin contain a larger amount of p-coumaryl alcohol than either softwood or hardwood lignin(3).

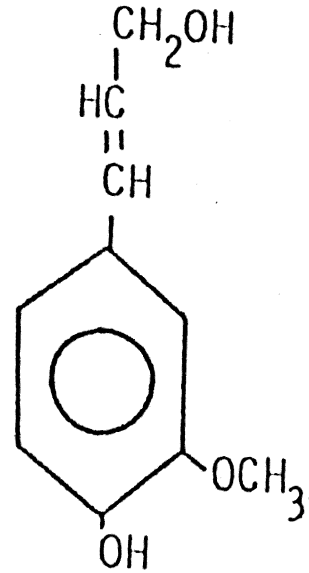
Softwood lignin characteristically contains cinnamyl alcohol and cinnamaldehyde end groups (7). Phenylpropanoid units containing free phenolic hydroxyl groups, common in conifer lignin, may possess an unsubstituted 5-C or aryl-, aryloxy, or alkyl-substituted 5-carbon and are often referred to as "uncondensed" and "condensed" phenolic units respectively. Lignin structures with side-chains connected to the next unit through a β-O-4 linkage are commonly called "arylglycerol-β-ether" structures. These linkages are the most abundant in lignin (7). Other types of structures found in lignin are β-1-lignols, 4-O-1 and 5-1 linkages, β-5 lignols, β-β lignols, etc. A computer-simulated structure for softwood lignin [Figure 3] has recently been proposed (6,8).

In addition to dependence on species and location within the plant, the chemical structure of isolated lignin is very much a function of the method of isolation. Isolation techniques lead



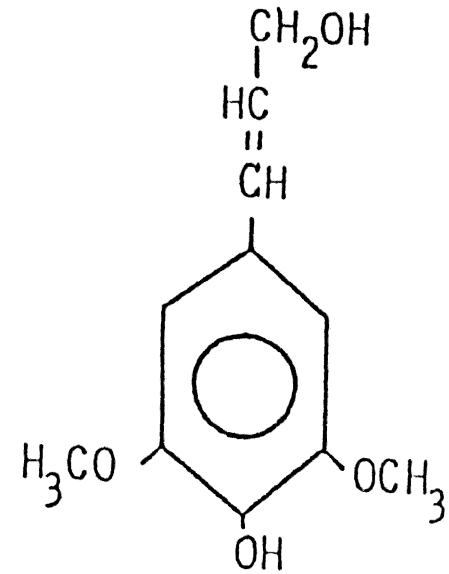
p-COUMARYL ALCOHOL

(14 %)



CONIFERYL ALCOHOL

(80 %)



SINAPYL ALCOHOL

(6 %)

Figure 2. Lignin building blocks and their relative abundance. Figures in parentheses represent approximate proportions of these monomers in softwood lignin.

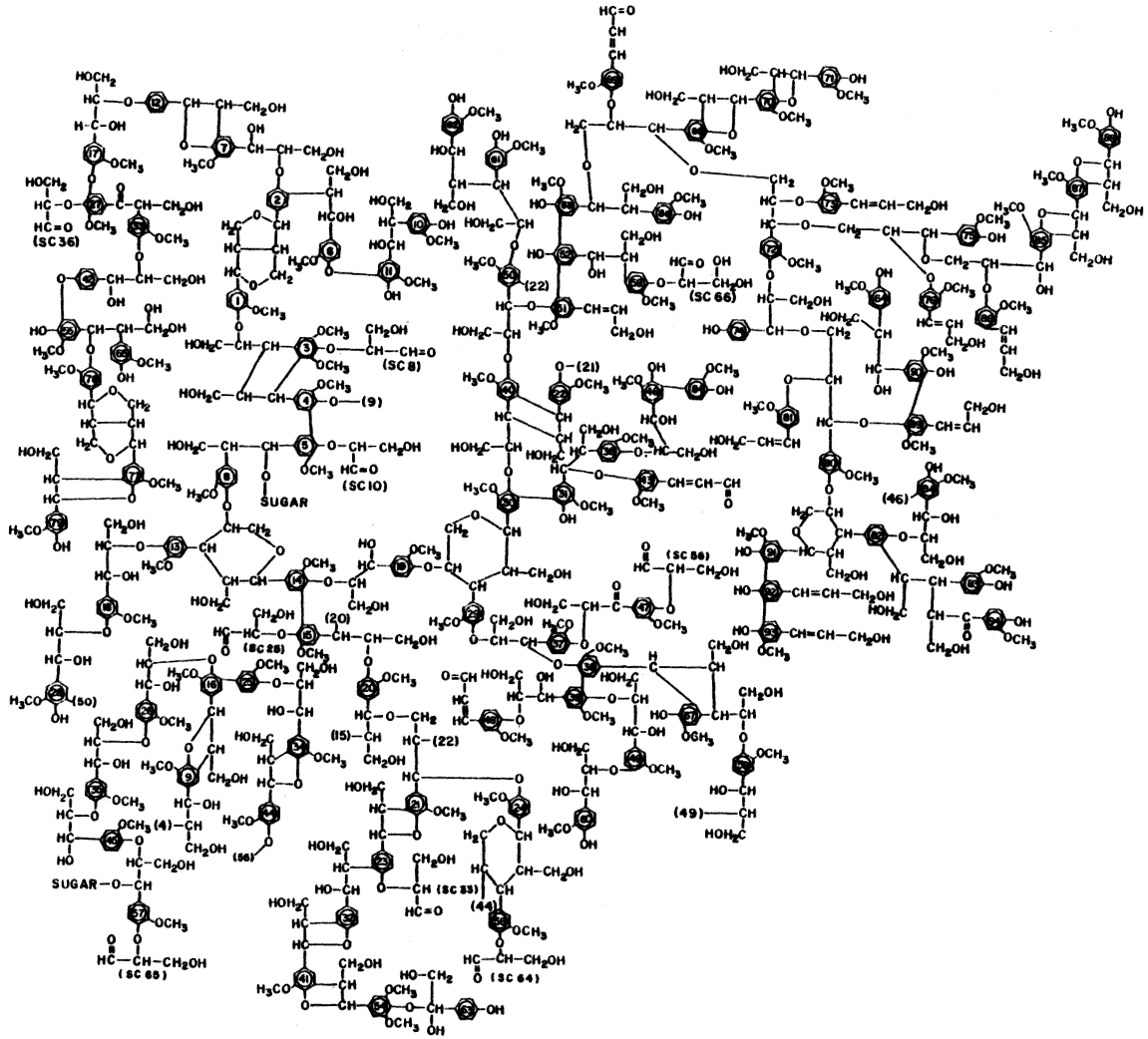


Figure 3. Computer-simulated structure of soft-wood lignin (Glasser and Glasser (8))

to the cleavage of certain bonds (e.g. C-C, C-O, arylalkyl ether), and alter the extent of lignin condensation, lignin molecular weight distribution, the content of certain functional groups (e.g. OCH_3, COOH, carbonyl), the distribution of phenolic and aliphatic moieties, the elemental analysis of the isolated lignin and the solubility of the resulting lignin in organic solvents and mineral acids. Table 1 summarizes some of the differences in isolated lignins as a function of the method of isolation.

B. Relevance of lignin utilization via biodegradation

Lignocellulosic biomass, an abundant renewable resource, represents a potentially enormous supply of chemicals and fuel material. Mineral acid or specific enzyme hydrolysis of the insoluble cellulose and hemicellulose components of lignocellulosic material has long attracted the attention of researchers devoted to the development of new production methods for chemicals, foods, and fuels (11-13). On the other hand, the utilization of the lignin fraction of such material has not yet been fully investigated, despite the fact that the lignin fraction of wood may represent an important source of aromatic chemicals (14).

Hydrolysis of cellulose and hemicellulose results in their conversion to soluble sugars. The sugars can then be microbially fermented to a large number of end products including organic acids, ethanol, and methane, which have industrial significance. Moreover, cellulose can be used as a carbohydrate substrate for

Table 1: Characteristics of differently isolated lignins

Method of Isolation	Lignin Recovery (unpurified) % of total lignin	Carbohydrate Content, % of recovered material	Phenolic units in recovered lignin moles/100 C9 units	Aromatic hydroxyl moles/100 C9 units	Elementary composition C%	H%	MeO%	Number average molecular weight
(1) Milled wood lignin								
(a) soft wood	40[7] - 50[10]	2 - 8[7]	25 - 35[a]	2.0 - 2.5[a]	64	6	15.7[7]	< 6,000[7]
(b) hard wood			20 - 30[a]		60	6	20	
(2) Brauns lignin								
(a) soft wood	8 - 10[7]	> 2[7]	25 - 35[7]	2.0 - 3.0[7]	64	6	15[7]	850 - 1000[7]
(b) hard wood	< 10[a]		20 - 30 [7]		62	5.8	15-18	
(3) Enzymatically liberated lignin								
(a) soft wood	23[10]	present[c]	25 - 35[a]	2.0 - 2.5[a]	64	6	15[7]	850 - 1000[7]
(b) hard wood			20 - 30[a]		62	5.8	15-18	
(4) Kraft Lignin								
(a) soft wood	∿ 80[a,b]	0.2[a]			66	6	14[9]	1600[9]
(b) hard wood	∿ 80[a,b]		> 35[9]	> 3[9]	65.5	∿6	19.2	1050[9]
(5) Iotech (Steam Chip explosion) lignin								
(a) soft wood	n.a.	n.a.	n.a.	n.a.		n.a.		n.a.
(b) hard wood	66[a]	< 5 [a]	Ca.50[a]	Ca.5[a]	65	7	20[a]	< 1000[a]

n.a.; data not available

a; personal communications with Dr. W. Glasser, Forest Products Department, VPI & SU

b; obtained by adding UV adsorbing and acid soluble fractions

c; the amount of carbohydrates depends on the period of incubation

d; numbers in brackets indicate reference quoted

the production of single cell protein, particularly yeast, for use as an animal feed protein supplement (13).

Degradation of the lignin fraction of wood should, theoretically, allow the production of important aromatic monomers and polymers and organic compounds of low molecular weight that can be used as fuels. Table II is a partial list of potential lignin-derived products and their applications. Lignin can be degraded chemically by a variety of methods (15), but these are generally too expensive for commercial applications. In addition, degradation of lignin by chemical or thermal means results in residual polymeric products of reduced chemical activity and isomeric mixtures of low molecular weight products. This led Rosenberg (14) to state that, "Chemically degraded lignin has a low commercial value.". In view of the disappointing results from efforts to chemically degrade lignin, the potential for selective degradation of lignin using biological systems seems to be an attractive alternative. Specific modification of lignin through the action of enzymes might result in a more reactive residual polymer and the resolution of isomeric mixtures of low molecular weight degradation products. In addition, production of energy and chemicals from lignocellulosic materials via biodegradation will require the integrated use of all the lignocellulosic components not just the cellulosic fraction. Moreover, biological conversion of lignin, in addition to being important from the standpoint of lignin utilization, is also important in the context of cellulose bioconversion technology. This is because the presence of lignin severely hampers the bioconversion of cellulose. The removal or

Table II. Potential lignin-derived products and industrial uses.

Compounds	Uses
Vanillin and vanillic acid	Food industry, cosmetics
Aromatic acids and aldehydes	Amino acids, aromatic alcohols
Modified lignin polymers	Adhesives, polymers
Phenols, phenylpropanoid derivatives, and quinones	Polymers, specialty chemicals
Acetaldehyde and other open chain aldehydes	Amino acids, alcohols, fuels

partial degradation of lignin in lignocellulosic materials appreciably improves the digestion of cellulose by microorganisms (15,16). For these reasons I believe a promising method of lignin utilization is biodegradation or biotransformation.

Unfortunately, the diversity and complexity of lignin chemistry has provided an effective roadblock to developing an understanding of biodegradation of lignin. Rapid, adequate chemical analysis of lignin and lignin degradation products is the single most important limiting factor in this quest for knowledge. In most cases only the most gross estimates of lignolytic activity are available. Although the precise mechanism(s) of lignin biodegradation is still unknown, white-rot and soft-rot fungi are capable of substantially degrading lignin (17-19). On the other hand, brown-rot fungi, which are usually associated with decaying wood (10,20), decompose the cellulose and hemicellulose in wood and cause very little change in the lignin (21). Despite the fact that bacteria have been implicated in the degradation of purified lignin (22,23), there still is much confusion about their role in lignin biodegradation in nature.

II. LITERATURE REVIEW

A. Lignin-degrading microorganisms

1. White-rot fungi

White-rot fungi, wood-decaying Hymenomycetes, are among the most active lignolytic microorganisms know (24) and are capable of degrading all the components of wood including lignin (24,25). Their name arises from the fact that they remove, at least in part, tannin materials from sound wood leaving bleached wood (3). Cowling (26) showed that Polyporus versicolor (Coriolus versicolor) can degrade over 97% of the lignin in sweet gum wood. Early studies (27) found that cultures of P. versicolor could be grown on isolated native aspen lignin as the sole carbon source. Van Vilet (28) also found that P. versicolor could be cultivated on a medium containing Brauns' lignin as sole organic material. These results are contradictory to more recent results by Eriksson et al. (25) and by Drew and Kadam (19) who have concluded that lignin cannot be degraded by white-rot fungi unless a more easily metabolized carbon source is used simultaneously. Since lignin isolation normally results in the presence of some carbohydrate impurities in the lignin, it is conceivable that the amount of carbohydrate present in the isolated lignin in earlier studies was enough to fulfill the requirement for cosubstrate.

That lignin degradation is oxidative is evidenced by the fact that lignin attacked by white-rot fungi contains higher amounts of

oxygen than the corresponding sound lignin (6,29). Hydroxyl group content has been reported by some to increase (30,31) and reported by others to decrease (17). Other observations (6) indicate that hydroxyl content changes very little if at all. Kirk and Chang (17,18) conclude from their extensive data on lignin obtained from white-rotted spruce that substantial side-chain oxidation occurs. They also conclude that, during lignin degradation in their system, oxidative ring-opening occurs while the lignin is still polymerized. This has been confirmed by Chen and Chang (32).

The content of total carbonyl and carboxyl is somewhat higher in degraded lignin than in sound lignin (30,31). The molecular weight of residual biodegraded lignin is usually higher than that of sound lignin (6). This polymerization may be attributed to the presence of laccase activity found extracellularly in lignin degrading systems (6).

Though it was reported that phenylpropane (C_6-C_3) compounds are intermediates of lignin degradation by white-rot fungi (30,33), only small amounts of such compounds were detected (30). Moreover, the amounts of vanillin and vanillic acid in the fermentation medium were between 0.002% and 0.006% of the lignin (30). Kirk (34) suggests that these compounds are formed from "side groups" or minor structural elements and not from the lignin polymer itself (exo-degradation). A recent study by Haars and Hüttermann (35) supports this suggestion. Based on the degradation of lignosulfonates by Fomes annosus (a Basidiomycete), Haars and Hüttermann conclude that lignin biodegradation in nature follows the mechanism of an exo-degradation. They further conclude that the fungus is unable to split the lignin molecule into intermediate-size pieces,

and that the fungus is most likely attacking side groups during the early stages of the fermentation while leaving the lignin polymer intact.

The fact that lignin is an extensively cross-linked and polydisperse polymeric structure may preclude direct uptake of lignin by microbial cells; thus extracellular enzymes which facilitate the initial attack on the polymer are plausible. Aromatic ring-opening enzymes are reportedly present in the soluble extracellular proteins in P. versicolor culture broth (36). This report on extracellular enzymes and others (37,38) treated later in this section lack confirmation (29). Hall et al. (6) have isolated only very small amounts of extracellular proteins from lignin degradation broths. The only potentially lignolytic extracellular enzymes found in lignin fermentation broths are laccases (39), phenoloxidase and quinone-oxidoreductase (6). However, laccase and peroxidase alone are not capable of depolymerizing lignin or even releasing low molecular weight fragments in detectable amount (40,41). Futhermore, incubation of lignin with cell free broth from a lignin degrading fermentation does not result in net reduction of lignin molecular weight (6).

In summary, white-rot fungi are able to completely degrade lignin under the proper conditions. The lignin undergoes oxidative changes during the early stages of degradation. Although very small amounts of low molecular weight intermediates have been found extracellularly, no extracellular enzymes with lignolytic activity have been isolated from lignin fermentation media. The mechanism

of initial lignin breakdown is still a mystery. I will speculate on such a mechanism later in this section.

2. Soft-rot fungi

Soft-rot fungi are Ascomycetes or Fungi Imperfecti and have been shown to degrade lignin and lignocellulosic materials (19,42,40,43). Hardwoods are more susceptible to soft-rot attack than are softwoods (3,41). Soft-rot fungi, many of which are thermophilic and degrade lignin best when incubated at high temperature (19,44), seem to have an absolute requirement for an easily metabolized carbon source as cosubstrate in lignin degradation (19,42). Eslyn et al. (42) have shown that carbohydrates in soft-rotted wood are depleted faster than lignin by four out of six species studied while the two remaining species depleted lignin faster than the carbohydrate. Rosenberg (44) concludes that the moisture content of the solid lignocellulosic substrate influences its degradation by some soft-rot fungi. Soft-rot fungi degrade lignosulfonate (45) as well as whole wood and Kraft lignin (19).

Haider and Trojanowski (46) conclude that soft-rot fungi can attack methoxyl groups, β-ether linkage of the side chain and also the aromatic ring structure of DHP* and lignin. Levi and Preston (40) presented evidence that the lignin remaining in soft-rotted beechwood is partially degraded, has a lower methoxyl

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*DHP's are made by dehydrogenative polymerization of coniferyl alcohol with peroxidase and are commonly used in lignin research (46,47).

content and is more acid soluble than the corresponding lignin in sound wood.

It has been suggested (3,47,48) that soft-rot fungi cause limited lignin degradation and that they are less efficient lignin degraders than white-rot fungi. This, however is not always true. Drew and Kadam (19) have found a strain of Asperigllus fumigatus (a soft-rot fungus) superior to C. versicolor in metabolizing Kraft lignin to CO_2 under identical conditions. From comparison studies of soft-rot and white-rot fungi, Takahashi (43) concludes that soft-rot fungi degrade lignin but that delingification varies not only with the fungal species but also the the type of wood.

3. Bacteria

Examination of early reports pertaining to bacterial degradation of lignin reveals that in many instances weaknesses in experimental technique have led to erroneous or questionable conclusions. Kirk (34) has enumerated these problems. However, reports suggest that bacteria from Flavobacterium, Micrococcus, Mycobacterium, Pseudomonas and Xanthomonas species are capable of lignin degradation. Ferm and Nilsson (49) reported that both Acetobacter and Pseudomonas species are capable of degrading the lignin in lignosulfonates. Kleinert and Joyce (50) previously reported this capability in Pseudomonas. Pandila (51) concluded that certain soil bacteria can degrade lignin.

Conflicting reports confuse the role of bacteria in lignin degradation. For example, in 1973 Crawford et al. (52) claimed that Nocardia carollina utilize aromatic compounds but were incapable of

degrading lignin. In 1977 Trojanowski et al. (53) provided concrete evidence for the ability of Nocardia sp. to degrade lignin; and in 1978 Gradziel et al. (54) claimed that Nocardia sp. was more active in producing $^{14}CO_2$ from ^{14}C-labeled plant lignin and DHP than soft-rot and white-rot fungi tested by Haider and Trojanowski (55).

It has been shown (22) that Pseudomonas ovalis degrades a large portion of high molecular weight milled wood lignin (MWL) while increasing the nitrogen content of residual lignin. Because no accumulation of low molecular weight intermediates was observed in the medium, Kawakami (22) concluded that the degradation of lignin by P. ovalis leads to successive breakdown from the terminal ends of the MWL molecule (exo-degradation). Haider et al. (23) used ^{14}C-labeled lignin to screen several species of Nocardia and Pseudomonas and concluded that Nocardia sp. were better lignin degraders than Pseudomonas. The release of p-hydroxybenzoic acid from aspen wood meal by Pseudomonas sp., isolated from the intestinal content of a larva living in whole aspen wood, has also been reported (56).

Cartwright and Holdom (57) reported that Arthrobacter sp. are capable of utilizing enzymatically released lignin as sole carbon source. (The fact that Arthrobacter sp. utilized lignin released by the action of brown-rot fungi might indicate that the lignin was partially modified due to the fungal attack, making it more susceptible to bacterial degradation, and contained enough carbohydrate to act as a cosubstrate.) The claim that the lignin

(impling native lignin) was the sole carbon source in this study would not be appropriate.

Odier and Monties (58) reported that a Xanthomonas strain, isolated from decomposing wheat straw, was able to utilize dioxane lignin as the sole carbon source. They observed that 77% of the initial lignin, in a mineral medium, was degraded in 15 days. They also reported that in the presence of glucose only 23% of the lignin was degraded. Futhermore, they reported that this strain of Xanthomonas was capable of degrading wheat dioxane lignin under anaerobic conditions in the presence of NO_3^- and glucose. Under anaerobic conditions small quantities of two phenolic acids were produced. Recently Deschamps et al. (59) observed that a strain of Aeromonas sp. was also capable of utilizing lignin as the sole carbon source. Their results indicate that the Aeromonas strain degraded 98% of the lignin present in their fermentation medium (1gm/L) in 5 days of incubation. Moreover, Crawford (60) provided proof that Streptomyces sp. can degrade lignin. Bacillus megaterium has also been shown to have lignolytic activity (61).

Reports of the effect of mixed bacterial cultures on the biodegradation of lignin are scarce. Crawford (62) reported that an Acinetobacter sp. grew on a lignin model compound and metabolized it to guaiacol, Nocardia corallina was then able to utilize the guaiacol. He also found that in the absence of N. corallina the Acinetobacter sp. died while N. corallina did not grow alone on the model compound. Kleinert and Joyce (50) showed that a mixed culture containing two fungal species, Penicillium and Aspergillus,

and Pseudomonas was capable of utilizing lignosulfonate as the sole source of carbon. Ban and Soljan (63) showed that a mixed culture consisting of two yeasts, two Arthrcbacter sp., Pseudomonas and Chromobacterium genera were able to degrade 50% of the initial lignosulfonate in the medium in 24 hours.

Many of the studies mentioned above indicate the importance of adaptation of the bacterial culture to the substrate prior to observation of lignin degradation (51,57,58). Close contact between the bacteria and the lignin is also reported to be important for extensive degradation (58). Most of the bacterial cultures apparently require the presence of a cosubstrate to initiate lignin degradation; Xanthomonas 23 (58) and Aeromonas L17 (59) are possible exceptions.

Changes occurring in the residual lignin, due to the action of P. ovalis, include a decrease in interunit β-aryl ether bonds, a decrease in molecular weight of MWL and a 70% and 80% decrease in coniferyl aldehyde content of pine and beech MWL (22). On the other hand, Nocardia sp. apparently is capable of removing methoxyl groups and cleaving the side chains and ring structures of DHP (53).

B. Microbial Ecology and Physiology of Lignin Degradation

The diversity of intermonomer linkages and monomer structures in lignin, as well as the abundance of various functional groups, provides an opportunity for a given microorganism to cause a variety of limited changes in lignin without necessarily effecting significant degradation. Research to date indicates that the capacity for complete degradation of lignin by a single microorganism may be limited to

white-rot fungi and related litter decomposing Basidiomycetes (3,34). It is possible that other fungi (e.g. Aspergillus fumigatus (6,19)) and bacteria (e.g. Xanthomonas sp. (58) and Aeromonas sp. (59)) can also completely degrade lignin to CO_2 and water.

Sutherland et al. (64) has established that Streptomyces flavovirens is capable of attacking and destroying the intergrity of the cell wall of Douglas-fir phloem. S. flavovirens degraded insoluble carbohydrates extensively but did not appreciably degrade the lignin. Their observations over an incubation period of 12 weeks indicated that the nonlignified wall was decomposed first, then the thick, heavily lignified portions of the wall were attacked. Scanning electron micrographs of decayed phloem taken at different time intervals during the incubation period showed the presence of cavities in the cell wall with the actinomycete hyphae penetrating these cavities causing loss of cell wall integrity. It also appears that part of the disruption was caused by the removal of carbohydrates. Crawford and Crawford (65) claimed preliminary evidence indicating that the lignin had been structurally altered as a consequence of the attack on the cell wall by S. flavovirens in the experiments of Sutherland et al. They (65) also claimed that this was the first report implicating a bacterium in the attack on lignin within intact woody tissues, thus indicating a wood decay role for actinomycetes. The observations of Sutherland et al. led Crawford and Crawford (65) to the conclusion that streptomycetes' attack on lignin may follow a pattern similar to white-rot attack on lignin.

More recently Phelan et al. (66) examined 6 streptomycete strains to determine their ability to degrade ^{14}C-labeled lignin from Douglas-fir. The substrates were ^{14}C-labeled in side-chains or ring structures of the lignin. Their results clearly indicate that aromatic ring components were cleaved after 1008 hours of incubation and that a substantial amount of the labeled ring carbon was released as $^{14}CO_2$. They also noticed that side-chains were only attacked to a small degree. Phelan et al. (66) concluded that streptomycetes preferentially attack the aromatic rings of the lignin polymer. In contrast Nocardia sp. (23) and Bacillus megaterium (61) preferentially degrade side-chain components. This may be an indication of the importance of hyphal penetration and disruption of the polymer structure facilitating extensive microbial degradation of lignin.

1. Succession of microorganisms in decaying wood

The biodegradation of lignocellulosic materials is undoubtedly an important step in the process of carbon cycling in nature. Decayed lignified plant tissues are normally attacked on the surface or the upper layers of the soil. The first step of microbial attack on lignocellulosic material, in nature, appears to be mediated by fungi which instigate the decomposition of this intractable substrate. Upon studying the decomposition of lignocellulosic materials by the successive action of two wood destroying fungi, Tichy (67) speculated that fungi provide the primary attack on lignocellulosic materials and cause alterations in the original substrate, other members of the soil microflora will normally amplify the fungal action.

Tichy further speculated that in normal succession the organism that will, at a given time, occupy the lignocellulosic material is the organism that can benefit the most from the changes brought about in the substrate by the foregoing partner.

Decomposition of lignocellulosic materials in nature is generally caused by the action of several fungal species and other microorganisms inhabiting the decaying wood. These microorganisms colonize the decaying wood in conjunction and/or in succession. It is reasonable to assume that each organism will, on its own, contribute to the final state of the decomposed substrate. The organism might, for example, suppress other organisms that have attacked the substrate earlier or be influenced by their activity. Tichy (68), who studied fungal succession in the primary attack on lignocellulosic materials, concluded that one important factor determining the colonization of decaying wood by a given organism is substrate availability. He stated that substrate availability is not only determined by the duration and character of the foregoing decomposition, but also by the type of wood being decomposed. Tichy's study (68) indicated a requirement for partial removal of cellulose by cellulose metabolizing fungi in order to make the lignin available for metabolism.

Smith and Ofosu-Asiedu (69) studied the distribution of fungi colonizing wood chips in storage piles. Their observations indicate that the majority of fungi isolated from the wood chip pile were Fungi Imperfecti and Ascomycetes. Futhermore, they observed that certain fungal species suppress other species at different time intervals during the decay of wood.

Recently, Schmidt and Walter (70) studied the succession and activity of microorganisms in stored bagasse. After 6.5 days of storage they were able to isolate 400 different organisms colonizing the bagasse. The flora consisted of bacteria, yeast, fungi and actinomycetes. They observed that microbial succession on the bagasse started with yeast species, followed by bacteria and then by actinomycetes and fungi. They concluded that this succession coincided with the enzymatic activities of the organism during fermentation, noting that residual sugars were consumed at first by the yeasts, followed by bacterial degradation of the pectin, hemicellulose and, in parts, the cellulosic fraction of the substrate. In the later stages the actinomycetes and Fungi Imperfecti attacked the hemicellulose, cellulose and, partially, the lignin fraction of the bagasse fiber. Similar observations concerning microflora colonizing hay during its storage have been reported (71).

These results indicate that wood degradation in nature is the result of the attack of a succession of microorganisms. Brown-rot fungi and bacteria colonize the wood during the early stages of decay, primarily attacking the carbohydrate fractions. Brown-rot fungi cause the removal of cell wall substance beginning in the S2 layer of the secondary wall and continuing through the S1 layer of the secondary wall. The primary wall and the middle lamella are very resistant to degradation by brown-rot fungi (72), presumably due to the high lignin content (4). White-rot fungi and actinomycetes amplify the effect of previous microorganisms and initiate significant delignification. In the advanced stages

of decay, after most of the polysaccharides are consumed, the cell wall collapses (3). At this point the lignin fraction has undergone physical and limited chemical changes that increase the availability of the lignin fraction and may promote colonization of the decaying wood by other lignin degrading fungi and bacteria. The succession of microbial attack on lignin in nature suggests that mixed or sequential culture techniques may be required for effective bioprocessing of lignin as a means towards its improved utilization.

2. Cosubstrate Requirement in Lignin Biodegradation

The role of bacteria in the degradation of lignocellulosic materials in nature is probably limited, in part, by the lack of mobility of bacteria through woody tissue as compared to the penetration of fungal hyphae. Aerobic degradation of lignin as a sole carbon source by Xanthomonas has been documented (58). It is interesting to note, however, that anerobic degradation of lignin by Xanthomonas 23 requires a cosubstrate and NO_3^- as a terminal electron acceptor (58). This specific case may help explain the role of cosubstrates in lignin degradation.

It may be that most lignin degrading organisms require a cosubstrate for lignin metabolism because the energy produced from lignin metabolism is inadequate to support microbial growth. An efficient aerobic electron transfer system, utilizing oxygen as a terminal electron accepter, can convert the energy inherent in the electrons to high energy phosphate compounds better than a less efficient system utilizing NO_3^- as a terminal electron acceptor. Thus in aerobic degradation of lignin by Xanthomonas 23, no cosubstrate

is required to give enough energy to support microbial growth. In the case of anaerobic degradation of lignin by this culture, less efficient energy conservation may make it impossible for the organism to grow unless an easily metabolizable, high energy cosubstrate is present. Although it seems likely that energy metabolism is closely linked to the ability to degrade lignin, it would not be justified to infer that anaerobic metabolism of lignin will occur in nutritionally rich media. The observations on Xanthomonas 23 (58) are singular, and the conclusions regarding metabolism in the absence of oxygen are in conflict with other studies (Drew, S. W. and J. Ferry, unpublished data, 73). Nevertheless, the studies with Xanthomonas 23 suggest an energy dependent requirement for cosubstrate that may transcend microbial classification.

The importance of energy efficiency in aerobic degradation of lignin may also be indicated by phenomenological observations on the effect of oxygen tension. Increased oxygen concentration allows a higher rate of turnover of the terminal electron acceptor, which results in more efficient energy conservation by the electron transport system. This could explain the observation by many researchers (74,75) that increased oxygen concentrations during lignin fermentations result in lignin decomposition to a greater extent and at a higher rate.

Odier and Monties' (58) observations concerning the ability of Xanthomonas 23 to utilize lignin as a sole carbon source suggest that non-cosubstrate requiring microoganisms could be used to selectively remove the lignin fraction of lignocellulosic material

while leaving the carbohydrates for other uses. Moreover, since the pulp and paper industry produces industrial wastes containing large amounts of lignin and small concentrations of carbohydrates, such non-cosubstrates requiring organisms might be utilized for treating these wastes without any requirement for additional carbohydrate.

Kadam (76) has shown, based on the evolution of $^{14}CO_2$ from radio-labeled lignin, that the ability of Coriolus versicolor to degrade lignin is enhanced by utilizing more slowly metabolized carbohydrates as cosubstrate. In contrast, the presence of an easily metabolized sugar (e.g. glucose) in small concentrations has been reported to increase the extent of lignin degradation (25). Another study (24), however, found no effect on the extent of lignin degradation due to the addition of small amounts of glucose. A more recent study (77) suggests that low concentrations of glucose (1.3%), markedly reduces the extent of lignin degradation, by Fusarium solani, as defined by the evolution of $^{14}CO_2$ from ^{14}C-labeled lignin under unbuffered conditions. Addition of high concentrations of glucose (35%), however, reduced the extent of lignin degradation by P. chrysosporium (24). This observation has been confirmed in our laboratory for C. versicolor as well.

Normally carbohydrates used as cosubstrates are metabolized more rapidly and degraded to a higher extent than lignin (78). Santra and Nandi (21), however, claim that Tremetes scabrosa, Phellinus badius and Daedalea flavida (all of which are white-rot fungi) utilize much of the lignin in rotted mahagony and sapwood and little of the cellulose.

Drew and Kadam (19), in their experiments utilizing A. fumigatus and C. veriscolor to degrade ^{14}C-labelled lignin, found that the ratio of lignin derived carbon dioxide to total carbon dioxide was variable. Based on this observation, they concluded that the lignolytic capacity of their cultures (as defined by metabolism of lignin to CO_2) rises and falls during the fermentation irrespective of total metabolism (as determined by total CO_2 evaluation). Moreover, they concluded that the metabolic characteristics of A. fumigatus, a soft-rot fungus, appear to be very different from those of C. versicolor, a white-rot fungus, suggesting a diversity of approaches to cosubstrate and lignin metabolism.

The desirability of noncosubstrate requiring, lignin degrading organisms has lately become the focus of much research (25,79,80). Ander and Eriksson (79) demonstrated that treating wood chips with cellulaseless mutants of white-rot fungi decreases the energy demand for the production of thermomechanical pulp due to lowered lignin content. In another paper (80), they concluded that some of the xylans and low molecular weight sugars in the wood were used as a cosubstrate. In a more recent paper (25), Eriksson, et al. studied the optimal conditions and the factors influencing the growth of cellulaseless mutants of white-rot fungi during wood rotting. More research is still needed in this field.

3. Nitrogen limitation and lignin biodegradation

A very interesting aspect of lignin degradation is the effect of nitrogen limitation on the breakdown of lignin. Many researchers (25,74,81,82) have concluded that lignin degradation

occurs only after the depletion of nutrient nitrogen and the elapse of a subsequent lag phase. Keyser et al. (82) showed that adding nutrient nitrogen to the fermentation system delayed the appearance of lignin degrading system. They also showed that such additions depress existing lignolytic activity. Reid (81) and Kirk and Yang (74) conlude that low nitrogen concentration enhances the rate of lignin biodegradation by P. chrysosporium. High nitrogen content, on the other hand, has an inhibitory effect which is relieved upon depletion of excess nitrogen (82,83). Eriksson et al. (25) determined the optimal C/N ratio for growth in fermentations containing lignin, for three different white-rot fungi and their cellulase mutants, to be between 160:1 and 400:1, depending on the organism. However, Drew and Kadam (19) concluded from their studies of lignin biodegradation by white-rot fungi, that fungal growth and lignolytic activity in the presence of carbohydrates are poorly correlated.

Although the response of lignolytic systems to nitrogen limitation is somewhat variable (19), this response is common in white-rot fungi. To date, no satisfactory explanation has been offered for such a phenomenon. I offer the following possible explanation for the effect of nitrogen limitation on lignin degradation. In the early stages of lignin fermentation the microbial metabolism favors cosubstrate utilization (19). A consequence of carbohydrate metabolization is the production of reducing equivalents (e.g. NADH and NADPH). Nitrogen in the medium will be utilized by the microorganism in conjunction with part of these reducing

equivalents for biosynthesis. Once the nutrient nitrogen is depleted, the biosynthesis process will slow or stop. As a result, excess reducing equivalents will accumulate within the cell. Mediation of the electron transport system could alleviate this intolerable condition by increasing electron transfer to the terminal electron acceptor (i.e., oxygen). This would result in the production of an increased concentration of reduced oxygen species (e.g., superoxide radical, hydrogen peroxide...etc.). Such species are very reactive and could conceivably attack the lignin causing its degradation. The capability of such species to degrade lignin will be discussed in detail later in this section.

4. Cultivation parameters and lignin degradation

Considerable controversy exists concerning optimum conditions of agitation and aeration. Careful evaluation of the literature has shown that many of the comparisons between static and shaken cultures were performed with conditions that were suboptimal for adequate oxygen transfer. However, the data indicate that conditions which allow morphological differentiation may favor fungal degradation of lignin. Yang et al. (24) have shown that degradation of lignin by white-rot fungi in shallow, liquid phase, stationary cultures was ten times as rapid as in agitated cultures. Wilke and co-workers (78) have demonstrated that the extent of lignin biodegradation in damp cultures is higher than in static or shaking submerged cultures. Moreover, lignin degradation by Phanerochaete chrysosporium was found to be 50% more rapid in submerged, shaken cultures under 100% oxygen than in those under normal air exchange (24). Other studies (75,78)

with P. chrysosporium indicate a similar increase in the extent of lignin degradation in submerged static cultures under 100% oxygen compared with normal air exchange. In contrast Wilke and colleagues (78) found no difference in the extent of lignin degradation in either submerged shake culture or damp cultures in response to a gas atmosphere of 100% oxygen or normal air. However, a slight increase in the rate of lignin degradation was observed in the case of damp cultures under a 100% oxygen atmosphere as compared with normal air exchange (78).

Another important factor, that influences lignin degradation by white-rot fungi, is the requirement for close proximity between the fungal hyphae and lignin (84). It has been suggested (75,78) that disrupted contact between lignin and the growing hyphae might be the reason that lignin degradation is inhibited in shaking cultures. My own observations, as well as those of Yang et al. (24), indicate that pellets of substrate and mycelia are formed and that the proximity of lignin to cell mass is quite close. Yang and coworkers speculate that interference with reactions requiring oxygen within the macrostructure of the pellets causes the adverse effect on lignin degradation by agitation.

The effect of illumination on wood decay was studied by Duncan (85). Her observations indicate that light from the visible spectrum stimulates the rate of wood decay of three wood-destroying fungi. She found a close correlation between the extent of decay inside blocks of wood and the intensity of light measured in foot-candles. She attributed some of the inconsistencies in

results obtained from "presumably well-controlled fermentations" to uncontrolled illumination of such fermentations.

Finally, Fenn and Kirk (86) have demonstrated that o-phthalate (often used as a buffer in lignin fermentations) inhibits lignin degradation. They reported that replacement of o-phthalate with 2,2-dimethyl succinate in P. chrysosporium fermentations doubled the rate of lignin degradation. Their observations suggest that lignin metabolism may be particularly sensitive to the presence of non-lignin mononuclear aromatic compounds

C. Analytical Techniques for the Study of Lignin Biodegradation

Analysis of residual lignin is an important step in the study of microbial degradation of lignin. Determination of the extent of lignin degradation, as well as the structural changes in the degraded lignin, are important factors in assessing the ability of a given microorganism to degrade lignin. Residual lignin analysis, however, is often plagued by interference from other wood components and constituents of the microbial biomass. Phytochemicals of high molecular weight which may be covalently linked to lignin and have phenolic character may be considered, from the analytical point of view, as being part of the lignin (87). However, results from residual lignin analysis will vary from sample to sample and from one method of analysis to another. This is an important first step in the interpretation of patterns of lignin biodegradation. Often the idiosyncrasies of analytical methodology compound errors in interpretation by the uninitiated. Unfortunately no single assay

is sufficient to characterize the nature or extent of lignin biodegradation. As a consequence, a thorough understanding of the strength and potential pitfalls of each method of lignin analysis is an essential second step in interpretation of the chemical history of lignin samples.

In the following I describe some of the more common methods used to quantify lignin content in biodegraded samples. Please note that these are not the only methods available, nor is the discussion of each method complete. For more complete discussion of analytical techniques the reader is encouraged to consult references 7 and 88.

1. Sulfuric acid (Klason's) method

In this method dried samples (usually 1 gm) of the residual lignin (with or without mycelium) are mixed with 15 ml of 72% sulfuric acid (88). The acid is added slowly while stirring. The mixture is then incubated in a water bath at 18-20°C for 2 to 3 hours with frequent stirring. The mixture is then diluted with distilled water to a sulfuric acid concentration of 3% (88). The weak sulfuric acid solution is boiled for 4 hours under reflux and the insoluble lignin allowed to settle prior to vacuum filtration. The solids are washed free of acid with hot water, dried and weighed.

This technique may underestimate the lignin content of samples which have undergone significant solubilization of lignin or overestimate the lignin content of sample containing large amounts of complexing inorganic salts. Therefore, this assay should be

accompanied by others to determine the soluble lignin content and purity of the residual lignin fraction.

2. Spectrophotometric methods

Lignin determination by ultraviolet absorption is essentially a measure of soluble lignin. In this method the lignin is dissolved in a suitable solvent, such as acetylbromide-acetic acid (89), 80% phosphoric acid (90) or 76% sulfuric acid (91), and the absorbance of the solution measured at 280 nm. Takahashi (43), after determining the insoluble lignin fraction by the Klason method, estimated the acid soluble fraction resulting from the hydrolyzate by measuring its ultraviolet absorption at 205 nm. This method was described first by Schöning and Johansson (92) in 1965.

Dissolved lignosulfonate can be determined by reacting the lignolsulfonate with nitrates in an acidic solution (Pearl-Benson test) (87). Goldschmidt et al. (93) describe a modified method in which the reaction between nitrite and lignosulfonate is carried out in an acetic acid solution. The resulting nitrosolignin derivative is characterized by its intense absorption at 430 nm.

Some of the problems encountered in these methods include the interference of undissolved particles (88) and, in the case of the Pearl-Benson test, interference from other polyphenolic compounds such as humic acids (87). Absorbance measured at 280 nm will not adequately estimate the non-aromatic fraction of lignin samples.

3. Chlorine number

The chlorine number method is an indirect method of determining the amount of lignin in pulp samples (88). It is based on the fact that lignin is easily oxidized by cholorine. Roe (94), who developed

a method for measuring the volume of chlorine gas consumed by pulp samples, defined the cholorine number as the weight in grams of chlorine consumed per gram of pulp under set conditions of time and temperature. Since the chlorine number is proportional to the lignin content in the specific woody material being analyzed, it can be related to lignin content only by an empirically determined factor. TAPPI Standard T202os is the current standard method for chlorine number determination. Other methods for determining the chlorine number utilize sodium chlorite instead of chlorine gas (87).

Hiroi and Eriksson (95) used the chlorine number method to assay for lignin disappearance during the degradation of milled wood lignin by Pleurotus ostreatus (a white-rot fungus). After analyzing their data they concluded that the chlorine number is influenced by the structural changes that occur in the lignin due to enzymatic alterations by the fungus. Futhermore, they calculated that lignin losses measured by this method are 10-15% higher than the true losses after 35 days incubation.

4. Permanganate (Kappa) number

In unbleached pulps, lignin is more easily oxidized by potassium permanganate than is cellulose (88). The amounts of potassium permanganate consumed by wood materials under standardized conditions give an estimate of lignin content. The Kappa number is usually expressed as the number of ml of 0.1N potassium permanganate soultion consumed by 1 gm of woody material. Factors affecting the permanganate number assay include the amount and concentration of permanganate and temperature and reaction time (88) although these

later conditions can be easily controlled.

A sample (1 gm) is prepared for Kappa number determination by vacuum drying and subsequent disintegration of the weighed woody material in water (500 mL) until it is free of fiber bundles. The disintegrated material is then reacted with 100 ml of 0.1N potassium permanganate and 100 mL of 4N sulfuric acid while maintaining the temperature of the reaction mixture at 25°C and constantly stirring for exactly 10 minutes. After 10 minutes the reaction is terminated by adding potassium iodide solution (20 mL of 1N). The liberated iodine is then titrated with 0.2N sodium thiosulfate using a few drops of starch indicator (88). Wu and Glasser (96) evaluated the applicability of Kappa number to the determination of lignin in biodegraded lignocellulose and found that the method is affected by the structural changes in the lignin caused by the action of the microorganism. They concluded that the degree of oxygenation of the lignin structure can increase the factor correlating lignin content with Kappa number by 25% or more. Moreover, they suggested that without simultaneous lignin structure analysis, Kappa number alone can lead to overestimation of the ability of microorganisms to delignify lignocellulosic materials.

The methods discussed above are able to quantify polymeric lignin but in some cases these methods are unacceptable for even that task. None of the above methods allow determination of the type or extent of structural change in residual lignin. Table III is a summary of the highlights of these methods.

Table III. Summary of methods for residual lignin analysis

Method of Analysis	Basis of the method	Advantages	Disadvantages	Detectability of lignin derived residuals	Method used in ref.
1. Sulfuric acid (Klason)	Insolubility of lignin in 72% sulfuric acid	Works well with soft wood lignin	•Does not work well with hardwood lignin that contain large fractions of acid soluble lignin •Can introduce positive error due to the condensation of carbohydrate degradation products which become acid insoluble	Quantification possible if acid insoluble Characterization not possible	14,24,25 84
2. Spectrophotometric	UV adsorbing properties of solubilized lignin	•Good precision •Simple •Fast	•Interference from insoluble particles	Quantification possible if soluble. Some characterization possible	43,45,97
3. Chlorine number	Selective response of lignin to Chlorination	Good for non-microbially degraded lignin	•Affected by structural changes in lignin caused by enzymatic attack	Quantification as well as characterization not possible	95, 98
4. Permanganate (Kappa) number	Selective response of lignin to oxidation by potassium permanganate	Good for non-degraded pulps	•Affected by structural changes in lignin caused by enzymatic attack	Quantification as well as characterization not possible	74,96

5. Computer assisted data reduction method

Glasser and Glasser (8,99) have developed an elegant computer assisted data reduction method for elucidating the type and extent of chemical changes in reacted lignins. The method is based on the correlation of several analytical procedures applied to polymeric lignin: elemental composition, OCH_3 content, total OH content, proton NMR spectroscopy of acetylated lignin, ash content and oxidative degradation by both alkaline permanganate and hydrogen peroxide, before and after oxidative depolymerization with alkaline cupric oxide. The products of this last analysis step are separated by gas and gel permeation chromatography. The results obtained from these analyses are used as input for a simulation computer program (SIMREL Analysis Program) which interprets the data and predicts the chemical behavior and properties of the lignin. Although this approach was developed to simulate the synthesis of natural lignins, its application to analysis of degraded lignin is highly promising.

Understanding the mechanism of lignin biosynthesis and the chemical and analytical behavior of lignin are the basis of SIMREL system (Simulation of Reactions with Lignin by computer). SIMREL can develop a lignin formula of 70 to 100 phenylpropane units by using random coupling reactions between mono- and oligomeric lignin precursors. Data obtained from the literature or from analytical observations can be used as an input for the simulation program. The program itself simulates the expected analytical behavior of the lignin, these predictions can normally be

correlated with analytical data obtained experimentally (100, 101).

This computer assisted data reduction method is by no means complete. The technique is still in an evolutionary state and is currently incapable of accurately determining the phenolic OH content, carboxyl group content, and the structural side chain features of the lignin polymer. Dr. W. G. Glasser of the Department of Forestry and Forest Products at Virginia Polytechnic Institute and State University and his group are working continously at complementing and filling the gaps in this promising technique for lignin analysis. An example of data generated by this analytical approach, applied to microbially degraded lignin, can be found in Reference 6.

6. Radioiosotopic tracer studies

Rapid, sensitive analysis of subtle changes in residual lignin structure and functionality still elude the research community. Although great strides have been made in sensitivity and breadth of analysis, virtually all of these procedures are tedious or require sophisticated instrumentation that may not always be available. On the other hand, a rapid and sensitive technique for assessing the overall extent of lignin metabolism and, in some cases, for pinpointing the functionality involved in sequential breakdown has been developed. This technique is based on the degradation of ^{14}C-labeled natural lignin (46,47) and extractive free lignocelluloses which contain ^{14}C only in their lignin component (46,102,103).

The evolution of $^{14}CO_2$ by cultures growing on ^{14}C-labeled lignin has given important clues to the mode of action and extent

of lignin degradation. The presence of low molecular weight intermediates in lignin degradation can be detected by monitoring water soluble ^{14}C-labeled compounds in the fermentation broth. Moreover, the study of sequential attack on the lignin molecule has been facilitated with lignins that are ^{14}C-labeled only in side-chains, methoxyl groups, or ring structures (46,53).

The methods for ^{14}C-labeling of the lignin component in lignocellulosic materials have been described by many researchers (46,102,103). An aqueous solution of L-[U^{14}C] phenylalanine or other suitable precursor is fed to live saplings through their cut stems (104). It has been shown that the resulting lignocellulosic materials contain at least 90% of their ^{14}C in non-acid-hydrolyzable components (102). In the preparation of specifically ^{14}C-labeled DHP's, specifically labeled coniferyl alcohols (^{14}C in aromatic rings, in propyl side-chains, or in methoxyls) are oxidatively polymerized *in vitro* using a peroxidase/H_2O_2 system as a catalyst (47). Radioisotopic methods for the study of lignin biodegradation have recently been reviewed by Crawford *et al*. (104,105).

A cautionary note about the utilization of DHP in lignin studies has been sounded by Glasser in his recently published, excellent review article (87). Glasser has enumerated the main shortcomings of utilizing DHP in lignin research. These short comings are: (1) the preparation of the polymer ignores the role of carbohydrates in the lignification mechanism, (2) polymer structure is greatly affected by polymerization conditions and (3) the polymer structure usually deviates from the structure of

isolated lignin. These observations could explain the differences between the character of ^{14}C-labeled DHP and ^{14}C-labeled lignin degradation observed by Trojanowski et al. (53) and Gradziel et al. (54).

D. The Chemistry of Lignin Biodegradation

It is perhaps presumptuous to speak of the chemistry of a phenomenon that is so poorly understood. Nevertheless, it is possible to glean, at least, a feeling for the sequence of events involved in the metabolism of lignin.

1. Low molecular weight intermediates and lignin biodegradation

The question of whether low molecular weight intermediates are formed as a consequence of lignin biodegradation is an important one. The ability to produce low molecular weight compounds from lignin by microbial degradation might allow commercial utilization of lignin, and their analysis could lead to a better understanding of the initial steps in lignin breakdown. Many researchers have addressed this subject (6,30,33,56,58,65,76,97) but no general consensus regarding the extent or type of low molecular weight species involved in lignin breakdown has been reached.

Phenylpropanoid (C_6-C_3) compounds were implicated in early studies (30,33) as being intermediates in lignin degradation by white-rot fungi. However, only small amounts of such compounds have been reported to accumulate (30,33). Kirk (34) suggests that such small amounts could have been formed from minor structural elements instead of being formed as a consequence of the breakdown

of the bulk of the polymer itself.

In a recent study Kawakami (22), who examined the degradation of MWL by P. ovalis, found no accumulation of low molecular weight intermediates in the culture medium. Meanwhile, Odier and Monties (97) were also unable to detect low molecular weight intermediates in the fermentation media during the degradation of dioxane lignin from wheat straw by Pseudomonas, Flavobacterium and Aeromonas species. However, in a later study (58) they reported detecting small quantities of two phenolic acids that appeared rapidly after innoculation; they did not observe significant accumulation of those compounds.

Kadam (76) utilized a dialysis fermentation in an effort to detect the presence of low molecular weight intermediates during biodegradation of ^{14}C-labeled Kraft lignin by a variety of white- and soft-rot fungi but was unable to detect accumulation of dialyzable intermediates. His results indicate that no more than 2% of the original radioactivity in the lignin was initially dialyzable and that biodegradation rapidly lowers their concentration. The organism probably competes with autorepolymerization for available dialyzable species. Perhaps low molecular weight lignin breakdown products not only fail to accumulate but also gradually disappear in lignin fermentations because of an imbalance in reaction rates. For example, the rate of microbial uptake of low molecular weight intermediates may be more rapid than their generation, or autorepolymerization may be so rapid that low molecular weight breakdown products are removed as fast as they

are released. Quite probably a combination of these possibilities occurs.

Low molecular weigh intermediates undergo rapid repolymerization, mediated by phenoloxidases which are normally found extracellularly during lignin fermentations. Haars and Hüttermann (35) used a homogeneous lignosulfonate preparation and thioglycolic acid, an inhibitor of polyphenoloxidase activity, in the study of lignosufonate degradation by the Basidiomycete Fomes annosus. Their data, over a 20-day incubation period, indicate the accumulation of phenols and phenolic ether compounds in the fermentation medium.

2. Sequential attack on the lignin molecule

The literature indicated that residual lignins have been biologically attacked in a sequential manner with the order and sites of attack dependent on the microbial species. Haider and Trojanowski (46) studied the biodegradation of specifically labeled ^{14}C- DHP's and found that soft-rot fungi attack the side-chains and aromatic rings in the early stages of degradation. Ultimately, radioactive carbon dioxide derived from methoxyl groups was released at a higher rate than radioactive carbon dioxide derived from side-chains or aromatic rings.

In studying the degradation of ^{14}C-labeled lignin from maize straw by a Nocardia sp., Trojanowski et al. (53) showed that $^{14}CO_2$ was released first from methoxyl groups followed immediately by the release of $^{14}CO_2$ from the side-chains. Twenty-four hours after inoculation, $^{14}CO_2$ from the aromatic rings was released; their data show that the highest rate of $^{14}CO_2$ released came from side-chains

followed by $^{14}CO_2$ released from methoxyl groups.

Kirk and Lundquist (106) found that P. veriscolor degraded sweetgum lignin by sequential attack starting with demethylation and proceeding to more extensive breakdown of the phenolic structure. Kirk et al. (73) offered the following scenario to describe the sequential degradation of lignin by white-rot fungi: first, the degradation proceeds by a demethylation of phenolic units in different parts of the lignin polymer to produce diphenolic units. This first step is thought by these workers to be the rate limiting step. Diphenolic units are then cleaved to produce aliphatic and carboxyl-rich residues. The residues formed are then further degraded by both hydrolytic and oxidative reactions. They speculated that while the attack on aromatic regions is progressing, some terminal side-chains are being oxidized to aromatic carboxyl residues. Presumably the low molecular weight aliphatic products are readily taken up by the fungal hyphae.

These studies suggest that the degradation of lignin follows an exodegradation pattern. That is, microbial attack proceeds from the outer macromolecular limits of the lignin and progresses towards the inside of the lignin molecule. It also appears that lignin degrading microorganisms are unable to split the lignin molecule into polyphenolic moieties of intermediate size during the early stages of degradation. This suggestion appears to have been partially confirmed by the recent work of Haars and Hüttermann (35).

E. Mechanism of Initial Attack on Lignin by Microorganisms

Complete microbial degradation of lignocellulosic material requires coordinated breakdown of both the carbohydrate fraction and the encasing lignin fraction. Delignification not only facilitates microbial penetration but also allows higher rates of enzymatic attack on the cellulose fraction. *In situ* depolymerization of cellulose by the action of extracellular microbial enzymes is well established, but the microbial breakdown of lignin is not well understood. Although lignolytic cultures do elaborate enzymes that modify lignin structure, none of these have the independent ability to depolymerize lignin, nor are whole culture filtrates able to reduce the number averaged molecular weight of residual lignin (6).

Although the plant synthesizes the precursors of lignin via enzymatic pathways, the ultimate formation of lignin is a random free-radical, one-electron oxidative polymerization of intermediates catalyzed by the action of plant peroxidases (107). The resultant polymer is randomly linked and polydisperse with no identifiable repeating subunits. In other words, nature has provided an extremely efficient mechanism for the synthesis of a nearly ideal multifunctional structural material. It follows that nature would also allow the development of highly efficient means for the breakdown of this important carbon source. Although there is some risk in second-guessing "Mother Nature", one might expect that such a system for lignin breakdown and carbon recycling might not have a plethora of extracellular enzymes, each specific for a different inter-and intra-unit bond. Rather, it seems likely that activities of broad specificity would develop that could lead to simpler substrates

for classical catabolic pathways. Such a system for the initial stage of lignin breakdown might involve cell-bound activities and require intimate contact between cell membrane and macromolecular lignin. Alternatively, there may be, unidentified, diffusible agents, enzymatic or non-enzymatic, that could catalyze the initial step(s) of lignin breakdown. Support for these possibilities exists in the literature, but in each case the data still leave inconsistencies.

Crawford et al. (108,109) studied the degradation of veratryl-glycerol-β-(o-methoxyphenyl) ether, a lignin model compound, by Pseudomonas acidovorans. This model compound contains the arylglcerol-β-aryl ether linkage which is quantitatively the most important inter-monomeric linkage in lignin (7). Their results indicate that P. acid-ovorans was capable of degrading the ring and propanoid side-chain of this model compound. Futhermore, they observed that an early step in its degradation was demethylation (108). This led them to speculate that the enzyme effecting such a demethylation is a mixed-function oxidase requiring $Fe^{2}+$. They also speculated, that further degradation of such a compound was effected by a dehydrogenase requiring NAD^{+} as a cofactor. It is interesting to note that this strain of P. acidovorans was found to be incapable of effectively degrading ^{14}C-labeled lignin from spruce. This led Crawford and Crawford (65) to conclude that the correlation between the degradation of model compounds and the degradation of lignin is that lignin degrading microorganisms can degrade lignin models while model-degrading microorganisms are not necessarily able to degrade lignin.

Kirk and Chang (17,18) have concluded from their analytical data of lignin obtained from rotted spruce that oxidative ring-opening degradation of lignin has occurred while the aromatic rings are still part of the lignin polymer. This observation has been supported in the recent literature (32). These results as well as others (73,110) seem to indicate that enzymes responsible for initial breakdown of lignin are of the mono- and dioxygenases. It is important to note that most of these enzymes require redox cofactors (e.g. NADH and NADPH) (29), thus making the possibility of extracellular attack by mono- and dioxygenases somewhat unlikely. Similarly, the suggestion that membrane bound mono- and dioxygenases cause the initial breakdown of lignin (18,65), requires that the lignin penetrate the cell wall to reach the membrane activities, a mechanically and chemically arduous task. The fact that lignin is a large cross-linked polymer appears to preclude such a penetration.

The early claim (36) that aromatic ring-opening enzymes were found extracellularly in P. veriscolor culture broths has not been confirmed (29). Other findings (111,112) which claim the presence of hydrolytic enzymes that can cleave β-O-4 linkages in lignin in clutures of several white-rot fungi still lack confirmation.

Higuchi and his coworkers (37,38) have proposed that oxidative cleavage between C_α and C_β of the phenylpropanoid side-chains of dehydrodiconiferyl alcohol suggests a mechanism for C=C double bond cleavage in lignin. Enzymes which can effect such a bond cleavage in lignin or lignin model compounds have not yet been found (29).

Kirk et al. (113) and Ishihara (39) have suggested that phenol oxidizing enzymes (e.g. laccase and peroxidase) play an important role in lignin degradation. However, the studies by Gierer and Opara (114), as well as other studies (115,116), indicate that neither peroxidase nor laccase alone can cause the depolymerization of lignin or the release of low molecular weight fractions.

To date, no enzymes capable of carrying out depolymerization of lignin have been shown to exist in lignin degrading systems. Hall (29), upon comparing the chemistry of biologically degraded lignin with the chemistry of reactions between reduced oxygen species and lignin model compounds has suggested that reduced oxygen species such as superoxide radical, hydroxyl radical, and singlet oxygen may be the attacking agents that cause the initial step in depolymerization of lignin. The possibility that reduced oxygen species excreted by microbial cells could initiate the breakdown of lignin can be supported by the following facts:

(1) The diversity of intermonomer linkages and monomer structures make it highly unlikely that one or several extracellular enzymes can cause lignin polymer cleavage.

(2) The nature of lignin biodegradation is oxidative as indicated by residual fermentation lignin which contains significantly more oxygen than the corresponding sound lignin (6,34).

(3) The extent of lignin biodegradation is increased by increasing the oxygen tension in fermentation media (24,78,117).

(4) Close proximity between microbial cell and lignin is required to achieve degradation of the lignin (6,78). Wilke et al. (78)

suggest that attacking agents needed for the initial breakdown of the lignin polymer by white-rot fungi are unstable or inactive at all but very small distances from the growing hyphae. The property of instability applies to reduced oxygen species. The presence of extracellular hydrogen peroxide formed by wood rotting fungi has been observed by Koenings (118,119). Based on his observations, Koenings (119) proposes that brown-rot fungi cause the partial decay of wood via a hydrogen peroxide - Fe^{2+} system.

Another aspect of lignin biodegradation that could indicate that extracellular enzymes have a limited role in lignin degradation is the fact that lignin adsorbs proteins to its surface. Recent observations indicate that enzymes added to fermentations containing lignin are readily adsorbed to the lignin with considerable loss of activity (P.L. Hall, personal communications). Moreover, the fact that reduced oxygen species cannot be produced microbially in the absence of oxygen during fermentation might explain why anerobic biodegradation of lignin is limited.

Finally an aspect of lignin degradation that has not yet been extensively studied, but is worth mentioning, is the antibiotic effect of lignin components on microorganisms. Zemek et al. (120), in a recent paper, have studied the effect of lignin model compounds with guaiacyl and suringyl structures on a variety of yeasts, bacteria and fungi. Their observations indicate that such compounds exhibit an inhibitory effect on the growth of the studied microorganisms.

III. OBJECTIVES

In this study I elected to use the fungus Coriolus versicolor, a representative member of the white-rot fungi, in order to study the role of reduced oxygen species in lignin degradation. The choice of C. versicolor was based on pervious literature (3,76) which indicate that C. versicolor is one of the most active lignin degrading fungi.

The objectives of this study are:

(1) To develop assays aimed at detecting the presence of extracellular superoxide radical and hydrogen peroxide (as representatives of reduced oxygen species) in lignolytic cultures.

(2) To study the effect of fermentation parameters, known to increase the extent of lignin degradation, on the concentration of extracellular superoxide radical in lignolytic cultures.

(3) To test the involvement of reduced oxygen species (namely superoxide radical) in lignin degradation.

(4) To study the effect of different aeration methods on the concentration of extracellular superoxide radical and on the extent of lignin degradation.

(5) To develop novel fermentation systems which could lead to improved lignin degradation in whole wood.

(6) To identify the possible site of production of such species within the microbial cell.

IV. MATERIALS AND METHODS

A. Materials

1. Microorganism; Coriolus versicolor (ATCC No. 12679) originally obtained from Dr. T. K. Kirk of the Forest Product Laboratory, USDA, Madison, Wisconsin. Inocula for the experiments were prepared by inoculating mycelia from working slants of malt extract agar (Difco Laboratories, Detriot, MI) into 500 ml of malt extract broth (BBL, Cockeysville, MD) in a 2800 ml fernbach flask. After 6-7 days incubation at room temperature (≈25°C) and agitation at high speed on an Eberbach table top rotary shacker (Eberbach Corp., Ann Arbor MI), the mycelia were harvested by centrifugation at 20,000xg for 20 minutes and washed twice with sterile distilled, deionized water. Mycelial pellets from the seed culture were homogenized in 30 ml of sterile distilled, deionized water in a Waring blender at full speed for 20 seconds. Five ml of the resulting uniform suspension were added to each 250 ml erlenmyer flask containing 45 ml defined fermentation medium, thus bringing the final volume of medium in each flask to 50 ml.

2. Fermentation medium: The defined fermentation medium contained per liter of the final volume Avicel (FMC Corp., Marcus Hook, PA), 10 gm; reprecipitated Kraft lignin Indulin ATR-Cl (Westvaco, North Charleston, SC), 1 gm; $NH_4H_2PO_4$, 2 gm; KH_2PO_4, 0.6 gm; K_2HPO_4, 0.4 gm; $MgSO_4.7H_2O$, 0.5 gm; $CaCl_2.2H_2O$, 75 mg; Thiamine.HCl, 1 mg; Ferric citrate, 12 mg; $MnSO_4.H_2O$, 4 mg; $ZnSO_4.7H_2O$, 7 mg; $CoCl_2.6H_2O$, 1 mg; $CuSo_4.5H_2O$, 1 mg; 100 ml of 0.1 M potassium

hydrogen phthalate solution. Kraft lignin was purified by acid reprecipitation from alkaline aqueous solution. The purified lignin was dissolved in 50 ml of 0.2 N KOH and filter sterilized (0.22 μm Millipore membrane filter) prior to its addition to the autoclaved medium. Calcium chloride and magnesium sulfate were autoclaved separately as concentrated solutions and added to the other presterilized medium constituents. Thiamine hydrochloride solution was filter sterilized (0.22 μm Millipore membrane filter) prior to addition to the sterile medium.

3. Reagents: All reagents were prepared with distilled, deionized water.

a) Phosphate buffer (0.1M; pH 7.8) prepared by dissolving 26.8 gm $Na_2HPO_4.7H_2O$, reagent grade (Fisher SCientific Co., Fairlawn, NJ) in 800 ml distilled water. The pH of the solution was adjusted to 7.8 using 0.1 N HCl and diluted to a final volume of 1000 ml with distilled water.

b) Ethylenediaminetetra-acetate, EDTA (10 mM) prepared by dissolving 3.8 gm of EDTA-$Na_4.H_2O$, reagent grade (Fisher Scientific Co., Fairlawn, NJ) in 800 ml distilled water. The pH of the solution was adjusted to 7.8 using o.1N HCl and then diulted to a final volume of 1000 ml with distilled water.

c) Gelatine (1% w/v) prepared by dissolving 1 gm granular gelatine, 270 Bloom (Fisher Scientific Co., Fairlawn, NJ) in 100 ml boiling phosphate buffer and allowed to cool before use.

d) Nitrobluetetrazolium (NBT) solution, prepared by dissolving 200 mg crystalline Nitrobluetetrazolium, grade III (Sigma Chemical Co., St. Louis, MO) in 50 ml phosphate buffer. The crystalline NBT was weighed out in subdued light and the solution stored in a dark bottle.

e) Nitrobluetetrazolium assay mixture for superoxid radical was prepared by mixing 18 ml phosphate buffer (pH 7.8), 15 ml EDTA, 15 ml gelatine solution and 9 ml NBT solution together. The solution was stored refrigerated in a dark bottle.

f) Cytochrome c solution for assay of superoxide radical (1.86 mg per ml) was prepared by dissolving 0.372 gm of solid cytochrome c type VI (Sigma Chemical Co., St. Louis MO) in 200 ml of phosphate buffer (0.1M $Na_2HPO_4.7H_2O$, final pH 7.8).

g) Chromic acid solution for assay of hydrogen peroxide was prepared by dissolving 5 gm CrO_3 in 200 ml distilled deionized water; the solution was then acidified by adding 5 ml of concentrated H_2SO_4.

h) Catalase solution containing 1 mg protein per ml of 0.1M phosphate buffer (pH 7.8), had 2000 units of catalase activity per ml.

i) Superoxide dismutase (SOD) solution containing 0.1 mg solid per ml of 0.1M phosphate buffer (pH 7.8) had 290 units of superoxide dismutase activity per ml of solution. Both catalase and superoxide dismutase were obtained in solid form from Sigma Chemical Co. (St. Louis, MO).

j) $^{14}CO_2$ trapping solutions: 1 N KOH and phenethylamine (Fisher Scientific Co., Fairlawn, NJ) were used, to trap $^{14}CO_2$ in

experiments where ^{14}C-labeled lignin was used to follow the degradation of lignin.

k) Scintilation cocktails: Commercially available scintillation cocktails, Readysolv HP (Beckman, Fullerton, CA) and Scintiverse (Fisher Scientific Co., Fairlawn, NJ) were used in these studies. Quench curves were used in calibrating CPM and dpm.

l) Acetylbromide - acetic acid (25% by volume Acetylbromide), used for spectrophotometric analysis of lignin, was prepared by mixing 25 ml of acetylbromide reagent grade (Matheson Coleman and Bell, East Rutherford, NJ) with 75 ml of glacial acetic acid (Fisher Scientific Co., Fairlawn, NJ).

m) Hydroxylamine hydrochloride (7.5M), used for spectrophotometric analysis of lignin, was prepared by dissolving 52.125 gm of hydroxylamine hydrochloride reagent grade (Fisher Scientific Co., Fairlawn, NJ) in a 100 ml distilled water.

n) Paraquat (= methyl viologen hydrate) was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI.

All other chemicals whether used to prepare the media or other reagents were of reagent grade and were purchased from Fisher Scientific Co., Fairlawn, NJ.

B. Methods

All phases of the experimental work involving microorganisms were carried out with sterile equipment and materials using aseptic technique. The sterilization of equipment and materials was done in a steam autoclave at 121°C for 20-120 minutes depending on the

size and contents. The only exception was the sterilization of the tapered column, which was used to carry out fluidization fermentations. The sterilization of the tapered column was accomplished by circulating low pressure steam, in the column assembly, for 4-6 hours, prior to loading it with the sterile wood chips and sterile mineral salts medium.

1. Shake flask fermentations: In all cases where experiments were carried out in 250 ml erlenmyer flasks, the final volume of medium and inoculum was 50 ml which guaranteed proper mixing and aeration (76). Flask containing the fermentation medium were incubated in a Psychrotherm Incubator Shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) with a two inch throw platform. All experiments were run at a temperature of 28°C and a shaking speed of 250 rpm.

In these experiments the flasks were either plugged with cotton plugs or covered using plastic caps (Kaputs, Bellco, Vineland, NJ). Both methods allow good sterile air transfer to the flask's contents. In the cases where ^{14}C-labeled lignin was used, the flasks were completely sealed using rubber stoppers, thus making the system air-tight. These flasks were flushed with air every 8-10 hours.

2. Hydrogen peroxide assay: Hydrogen peroxide reacts with chromic acid solution quantitatively yielding chromphoric chromium pentoxide (121,122). The blue colored chromium pentoxide was assayed by adding 10 ml samples, containing 9 ml of the fermentation broth and 1 ml distilled water, to 10 ml of the chromic acid solution

and 10 ml of anhydrous diethyl ether. The mixture was thoroughly shaken then allowed to separate, at ambient temperature, in a separatory funnel. The chromium pentoxide in the ether layer was determined spectrophotometrically at 450 nm capped cuvettes.

The blank for the spectrophotometric assay was prepared in a similar way except that the inoculated fermentation broth was heated at 60°C for 30 minutes to destroy any hydrogen peroxide present. Three standards with different hydrogen peroxide concentrations were prepared by adding 1 ml of preprepared hydrogen peroxide solutions (1.276×10^{-5}, 2.553×10^{-5} and 3.824×10^{-5} mole per ml) to 9 ml of the heated medium and assayed.

3. Superoxide radical assay: In the early stages of this study I developed a qualitative assay, based on the work of Beauchamp and Fridovich (146), to detect the presence of superoxide radical using the NBT assay mixture described earlier. Superoxide radical reduces nitrobluetetrazolium, such a reduction is accompanied by the formation of an intensely blue dye (Formazan) (123). The formazan dye is insoluble in aqueous solution and quickly precipitates. This precipitation of the dye makes the use of a spectrophotometer to detect NBT reduction by superoxide radical rather limited. However, Fried (124) suggested that the inclusion of gelatine in the assay mixture keeps the insoluble formazan in solution, and hence I included gelatine in the NBT assay mixture.

The qualitative assay was performed by adding 0.4 ml of the fermentation broth to 3.6 ml of NBT assay mixture in a test tube. In the case where superoxide radical was present in the broth I

visually observed the formation of the blue dye. On the other hand, when superoxide was not present in the broth or when I added 3 ml of SOD solution to the assay mixture in the test tube, addition of 0.4 ml of the fermentation broth did not produce the blue dye.

Since the formazan dye can be followed spectrophotometrically at 530-580 nm (125) I attempted to develop a method aimed at separating the microbial cells from the dye. Once the microbial cells could be separated from the dye in the assay mixture, quantitative determination of superoxide radical could be achieved spectrophotometrically by following the dye formation. My efforts in this aspect were fruitless, so the method was dropped in favor of another method utilizing ferricytochrome c to detect the presence of superoxide.

Superoxide radical reduces cytochrome c; this reduction is accompanied by an increase in absorbance at 550 nm with a molar extinction coefficient of $\Delta E_{550}=2x10^{4}$ liter.mole^{-1}.cm^{-1} (126). Superoxide radical was assayed by adding 2.5 ml of fermentation suspension to 1 ml of phosphate buffer in the body of a syringe fitted with a glass fiber filter and a final 0.22 μm membrane filter. The mixture was shaken by hand for 45 seconds and filtered directly into 1 ml of cytochrome c solution. The final working concentration of cytrochrome c in the assay mixture was $5x10^{-5}$ M. The mixture was then assayed spectrophotometrically at 550 nm after 6 minutes. Blanks for the spectrophotometric assay were prepared in a similar manner except that the inoculated medium was preheated at 60°C for 30 minutes prior to its incubation with the buffer in the

body of the syringe. Samples incubated with 1 ml of superoxide dimutase solution, instead of buffer, in the body of the syringe provided negative controls on superoxide radical-dependent reduction of cytochrome c.

The assay developed here relies on the continuing production of superoxide radical by the cell suspension in the body of the syringe. A schematic of the apparatus used for superoxide radical assay is shown in Figure 4. Spontaneous dismutation of superoxide radical occurs at an extremely rapid rate. Removal of cells by filtration introduces a finite time lag between generation of superoxide radical and its reaction with cytochrome c. Therefore the concentration of superoxide radical that I detect underestimates the actual concentration expected at the microbial cell wall in the fermentation medium itself.

Since I am measuring small quantities of superoxide radical it is necessary to assay replicates of the fermentation broth to allow statistical analysis of the results. It has been my experience over more than 40 assays in triplicate that the variance in superoxide radical concentration is ±16%. Moreover, since the culture conditions allow the production of variable amounts of pigments and chromophoric materials it is essential, in order to accuratley evaluate the concentration of superoxide dismutase sensitive reduction in optical density, to consider the change in optical density as a measure of the concentration of the radical.

4. Preparation of ^{14}C-labeled lignin: The procedure used to prepare ^{14}C-labeled Kraft lignin was based on the procedure described

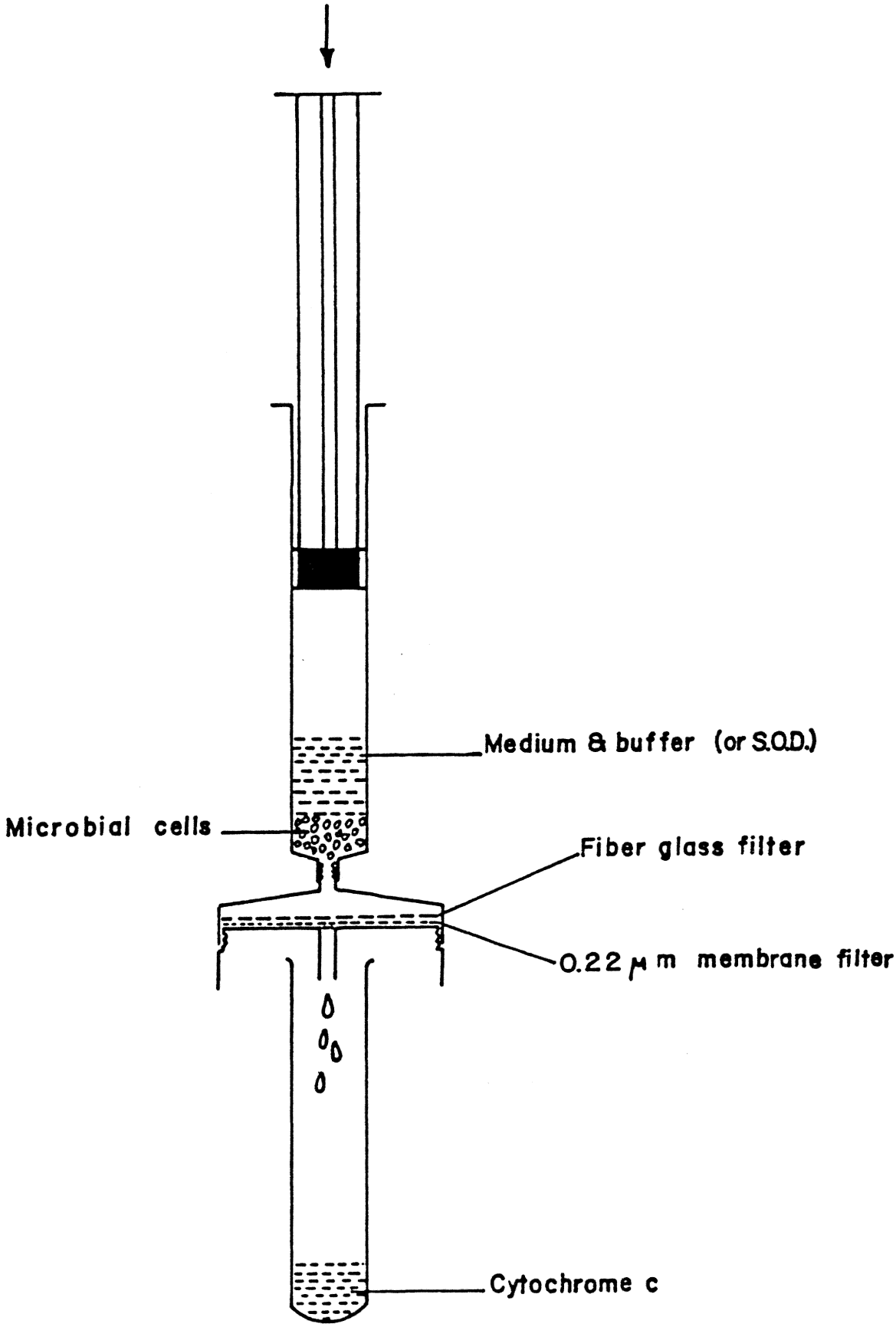

Figure 4. Schematic representing the set-up used for superoxide radical assay.

by Larsson and Miksche (127). Loblolly pine seedlings were grown in a greenhouse and allowed to take up L-(U-^{14}C) phenylalanine for 7-10 days.* Once the isotope-fed seedlings were at an average height of one foot they were stripped of needles and bark. The resulting radioactive wood was cut into one inch pieces and extracted with acetone: water (1:9) for two days. The wood was then air-dried and cooked, in cooking liquor, for three hours at 180°C in a sealed container. Five ml of cooking liquor (0.425 gm Na_2S + 1.274 gm NaOH in 38 ml H_2O) were used per gram of wood. The resulting pulp was then extracted using 2N NaOH and washed with water until the filtrate was clear. Chloroform was then added to the combined liquor and wash solutions to yield a 1% cholorform concentration. Lignin was then precipitated from the solution by adding excess 5N HCl. The precipitated lignin was then filtered out of the solution and washed with water to remove the acid. Finally, the lignin was air-dried yielding ^{14}C-labeled Kraft lignin.**

5. Measurement of $^{14}CO_2$: Experiments using ^{14}C-labeled lignin as substrate were carried out in 250 ml Erlenmeyer flasks equipped with side ports (Figure 5). The flasks were then incubated in the Psychrotherm Incubator-Shaker at 28°C and a shaking speed of 250 rpm.

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*Performed by Mr. Robert Kelly at Virginia Tech.

**The ^{14}C-labeled Kraft lignin was prepared by Mrs. Charlotte Barnett of the Forest Products Department at Virgina Tech.

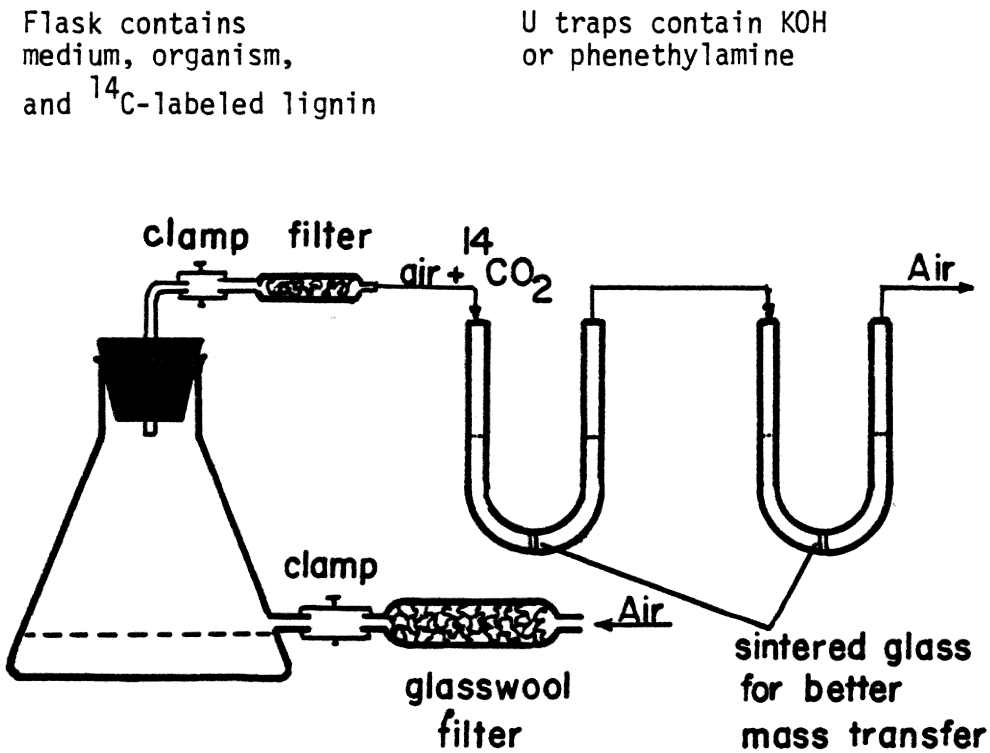

Figure 5. Schematic diagram of the set-up for ^{14}C-labeled lignin experiments.

The flasks were flushed every 8-10 hours with CO_2-free air and the displaced $^{14}CO_2$ was trapped for measurement. Glasswool filters at both ends of the flask were used to assure no contamination of the flask's contents during flushing.

The radioactive CO_2 was trapped in two traps (Figure 5), the first containing 5 ml of 1N KOH and the second containing 5 ml of phenethylamine. One ml of 1N KOH trapping solution was then transferred into Poly-Q vials (Beckman Instruments Inc., Irvine, CA) and 10 ml of Readysolv HP cocktail were added to the vial. In another Poly-Q vial, the five ml of phenethylamine from the second trap were transferred, and 10 ml of Scintiverse cocktail was added to the vial. The radioactivity in each vial was measured in a Beckman Liquid Scintillation counter (model LS 3155T, Beckman Instruments, Inc., Irvine, CA). $^{14}CO_2$ data reported are the total radiactivity of a sample, and represent the sum of five times the radioactivity observed in 1 ml KOH and in 5 ml phenethylamine.

Counting efficiencies were determined by establishing a plot of "external standard ratio" vs efficiency of counting. This plot, called a quench curve, was arrived at by using standard ^{14}C-labeled fructose of known radioactivity. The radioactivity of a particular sample was calculated by using the formula

DPM = (CPM - background reading)/efficiency of counting

where DPM = disintegration per minute or actual radioactivity

CPM = counts per minute or radioactivity measured by the counter

I counted several vials containing 1 ml KOH mixed with 10 ml of Readysolv cocktail to obtain the value of background reading for

KOH. No radioactive material was added to those vials. The same procedure was done to obtain the background reading for phenethylamine. Also, the quench curve for both trapping solutions was arrived at by adding the radioactivity to the appropriate amount of trapping solution (1 ml KOH or 5 ml phenethylamine) and adding 10 ml of the appropriate cocktail (Readysolv in the case of KOH or Scintiverse in the case of phenethylamine) and counting. This was done to take into consideration the chemiluminescence emitted by the aqueous trapping solutions which was counted as radioactivity by the Scintillation Counter.

6. Spectrophotometric lignin analysis: The method described by Johnson et al. (89) was used to determine the extent of lignin degradation in shake flask experiments, in fluidized bed experiments and solid state fermentations.

a)sample preparation

A known volume of shake flask fermentation broth was aseptically withdrawn from the flask into a test tube. The sample was then dried in a vacuum oven maintained at 65± 1°C and 13 psi vacuum.

Wood chips samples were withdrawn from fluidization experiments and solid state fermentations aseptically and dried in an oven at 65± 1°C for 4-6 days. Once the chips were dry they were ground in a rotary blade grinder to a fineness passing an 80-mesh screen. An acurately weighed sample from the fines was stored desiccated in a test tube until lignin analysis was performed.

b) Procedure for analysis

Ten ml of the 25% acetylbromide were added to the sample in the test tube. The tube was stoppered with a glass marble, to allow escape of evolved gasses, and heated in a water bath maintained at

70 ± 2°C for a period long enough to allow complete dissolution of the sample (30-45 minutes), during which the test tube was swirled at 8-10 minute intervals to enhance dissolution. Once the sample was completely dissolved the test tube was removed from the heating bath and placed in a beaker of water at 15-18°C and allowed to cool. The cooled sample was then transferred to a 200 ml volumetric flask already containing 9 ml of 2M NaOH and 50 ml of glacial acetic acid. A minimum amount of acetic acid was used to complete the transfer. Once this was accomplished, 1 ml of 7.5M hydroxylamine hydrochloride was added to the volumetric flask. The contents of the flask were then mixed, allowed to cool, and diluted to 200 ml using acitic acid. The absorbance of this solution at 280 nm is a measure of the lignin concentration in the sample.

To obtain the extinction coefficient of indulin, an accurately weighed sample was treated as described above and the extinction coefficient was calculated from the following formula

$$\text{Extinction coefficient} = \frac{\text{Absorbence of sample - Absorbence of blank}}{\text{lignin concentration (gm/l) in the final assay solution}}$$

The blank, not containing lignin, and the samples, were treated similarly. The extinction coefficient for lignin in loblolly pine was considered to be 23.7 liter $gm^{-1}cm^{-1}$ according to Johnson et al. (89).

7. Pretreatment of wood chips: The wood chips (loblolly pine) utilized in both fluidization experiments and solid state fermentations were pretreated, prior to their utilization, in the following manner; One hundred thirty-two gm of loblolly pine sawdust, passing through a 4-mesh screen but not through a 5-mesh screen, were added to 2000 ml

of distilled, deionized water in a 2800 ml fernbach flask. The flask and its contents were heated to 65-70°C for two hours while stirring frequently. After two hours a sample of the wash solution was withdrawn from the flask, allowed to cool and then assayed spectrophotometrically at 280 nm. Meanwhile, the wood was separated from the wash solution by pouring the contents of the flask on a 20-mesh screen. Once the wash solution was drained, cold distilled, deionized water was used to rinse the wood. The above procedure was repeated 19 times until the absorbance at 280 nm of the resulting wash solution reached a constant value of 0.7. The rinsed wood was then placed in a drying oven maintained at 65°C for 4 days until it was completely dryed. This procedure allowed the extraction of most water soluble phenolic compounds present in the wood.

Finally, a cumulative screen analysis was done on the dried wood, as described in reference 128, and a mean mass diameter of 0.288 cm was obtained for the resulting wood chips. The resulting chips were then stored desiccated until they were used.

8. Solid state fermentation: The desirability to work with fermentation systems that could be scaled to large industrial sizes, to facilitate utilization of lignocellulosic materials, and the fact that scale up of static or shake flask cultures is impractical, prompted me to develop novel fermentation systems for biological treatment of lignocellulosic materials. Moreover, reports by many researchers (58,78,84) indicating that close cell-lignin proximity, long residence time, and efficient oxygen transfer are prerequisites for efficient lignin degradation, requirements not easily met by stirred tank

fermentors, led me to study the possibility of utilizing solid state fermentation and fluidized bed fermentation to efficiently degrade lignocellulosic materials. Both solid state fermentations and fluidized bed fermentors can provide the above mentioned requirements.

With an eye on industrial applications, I reasoned that utilization of whole wood in these systems will be advantageous. Utilizing whole wood could eliminate the requirement of delignification which could find wide applications in the pulp and paper industry. Microbial treatment of whole wood could lead to the modification and/or partial degradation of the lignin fraction in wood, this could result in lower energy requirements for the production of thermo-mechanical pulp (79).

Solid state fermentation has many advantages when compared to submerged cultures. These advantages are;

a) The cultivation of fungi on solid lignocellulosic materials generally favors mycelial penetration of the substrate (129), an important requirement for lignin degradation in lignocellulosic materials (refer to the literature review for a discussion of the importance of mycelial penetration).

b) Solid state fermentations, in general, require a smaller working volume than submerged cultures (129).

c) In general, solid state fermentations require infrequent mixing thus resulting in significant savings of energy (130).

d) Solid state fermentations have lower technology requirements than submerged cultures (129).

e) Solid state fermentations produce much smaller amounts of waste water when compared to submerged cultures (129).

Another factor which weighted heavily in the decision to study solid state fermentors as a mean to biologically modify lignocellulosic materials, was the results presented by Wilke and Rosenberg (78) which indicate that the largest extent of lignin degradation was obtained in damp cultures (solid state fermentation) of lignocellulosic materials when compared with static and shaking submerged cultures.

Finally, I reasoned that cultivation of fungi on solid surfaces would allow for considerably more morphological differentiation than would submerged cultures. This, I further reasoned, could prove to be an important factor in lignin degradation by fungi. Moreover, cultivation of white-rot fungi on solid nutrient surfaces models the natural environmental conditions for lignocellulosic degradation.

The procedure used to prepare the solid state fermentation was as follows;

In a 2800 ml fernbach flask 20 gm of dry, pretreated wood chips were mixed with 3 gm $NH_4H_2PO_4$, 0.9 gm KH_2PO_4, 0.6 gm K_2HPO_4, 0.75 gm $MgSO_4.7H_2O$, 0.1125 gm $CaCl_2.2H_2O$, 1.5 mg thiamine-HCl, 18 mg ferric citrate, 6 mg $MnSO_4.H_2O$, 10 mg $ZnSO_4.7H_2O$, 1.5 mg $CoCl_2.6H_2O$, 1 mg $CuSO_4.5H_2O$ and 3.06 gm potassium hydrogen phthalate. Fifty-five ml distilled, deionized water were then added to the mixture. The flask was closed with a cotton plug and autoclaved for 20 minutes. The autoclaved material was wet but no excess liquid was observed.

Twenty ml of the homogenized cells were added aseptically to the flask. The flasks containing the inoculated solid state fermentations were then incubated in a constant humidity chamber (92% relative humidity) at 28°C.

9. Fluidized bed fermentation: Another approach studied was the utilization of a tapered fluidized bed as a bioreactor which could lead to efficient biodegradation of lingocellulosic materials. Although the use of fluidized beds as bioreactors has been studied by many researchers (131-133), no reports about their use for the degradation of lignocellulosic materials are available in the literature. In general, a fluidized bed bioreactor offers many advantages over more conventional high-solids bioreactors. These advantages include;

a) Enormous surface area provided by the solid particles allowing for a higher specific reaction rate (131,133).

b) Fluidized beds allow easy periodic removal of some of the particles and the addition of fresh ones which helps in maintaining the bed's high reactivity (132).

c) When operated in the boiling bed mode, fluidized beds provide excellent mixing, thus improving mass and heat transfer rates (134). As a consequence more efficient oxygen transfer is obtained (a prerequisite for efficient lignin degradation).

d) The control of fluidized beds is more easily accomplished than is the case in stirred-tank reactors (132).
However, in conventional (i.e., constant cross-sectional columns) fluidized beds the pressure drop maintains a constant value as the

velocity of fluidization increases (131), on the other hand, in tapered beds it decreases with increasing velocities. Moreover, conventional fluidized beds have a relatively narrow range of optimum operating conditions at high bed expansions making it rather difficult to maintain nonfluctuating operation (132). This is not the case in tapered fluidized beds which allow maximum flexibility in fluidization flow rate while minimizing washout of solids from the bed.

Figure 6 is a schematic diagram of the tapered fluidized bed used in this study. The column was constructed from Pyrex glass using a carbon mandrel. Its diameter at the bottom is 2.5 cm and increases to 7.6 cm at the top. The height of the column is 107 cm and the angle of taper is 1.37°.

The air-liquid contactor consists of an air sparger in the center of a 5 cm diameter stainless steel cylindrical casing tapered at both ends. The air sparger was made of a rubber pipet bulb (2 ml) pierced with holes. Air coming from the line, passes through a glass fiber filter, then through the sparger into the central part of the casing where it contacted the recycled liquid stream. A centrifugal pump was used to recycle the liquid.

In this study the column was operated in the batch mode. After sterilizing the column and the lines by passing steam through it, six liters of sterile salt medium (the same medium described previously except it did not contain Indulin and Avicel) were pumped into the column, through the drain line, using a peristaltic pump. Once this was done, 20 gm of inoculated wood chips were loaded into

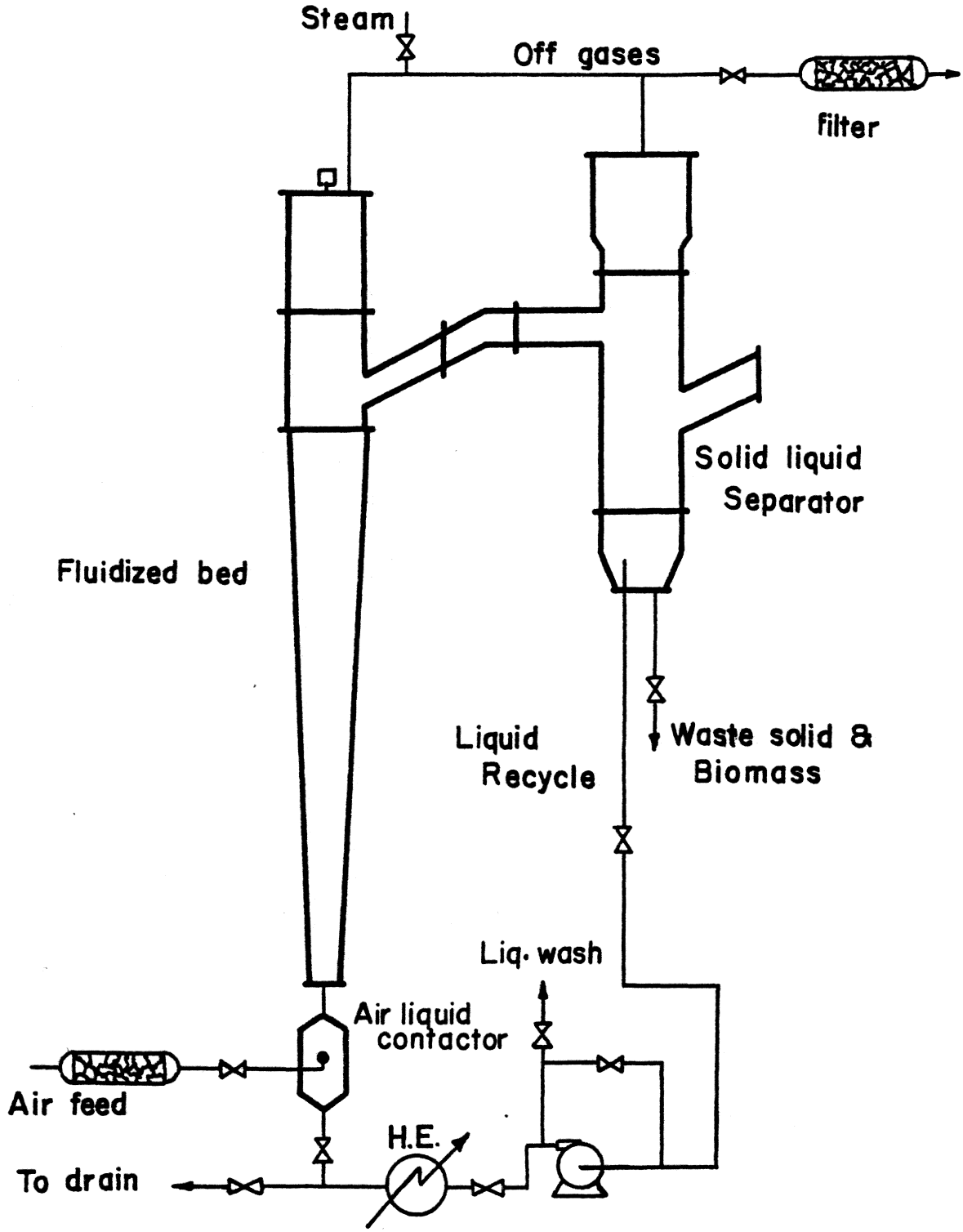

Figure 6. Fluidized bed bioreactor for batch wood degradation.

the column through a hole in the top flange.

The inoculated wood was prepared by mixing 4 gm of wood obtained from the solid state fermentation, after 12 days of incubation, to 16 gm of pretreated wood. The 16 gm of pretreated wood were autoclaved with 200 ml distilled deionized water prior to the addition of the 4 gm inoculum. Autoclaving the wood chips with excess water resulted in its saturation with water, thus allowing it to be fluidized (the wood did not float after this treatment).

The air flowrate was maintained at 0.75 liters.min^{-1} during the operation. Liquid circulation in the system was maintained at a rate of 2.8 l/min. Using the heat exchanger, with cold water circulating in the shell side, the temperature of the system was maintained at 28 ± 1°C. Daily samples of wood were withdrawn through the opening in the column's top flange using suction.

V. RESULTS AND DISCUSSION

(1) The presence of extracellular superoxide radical and hydrogen peroxide in lignolytic cultures of C. versicolor

C. Versicolor was grown in a simple defined fermentation medium with and without lignin. With lignin present during the fermentation, hydrogen peroxide in excess of 1.8 mM was detected after 4 days of incubation. After 6 days of incubation superoxide radical in excess of 0.004 mM was also detected in the extracellular broth. However, when lignin was absent from the medium, hydrogen peroxide was present at 1.5 mM concentration but no superoxide radical could be detected in the extracellular broth. Tables IV and V summarize my results for hydrogen peroxide and superoxide radical respectively. Results reported in Table V are averages of three replicate cultures with ±16% variation.

Observations on superoxide radical were qualitatively confirmed using the nitrobluetetrazolium assay mixture described previously. My observations with this assay system were consistant with the data presented in Table V. Again, the blue dye was formed when lignin was present in the system and was not formed when lignin was absent. Although quantitative determination of superoxide radical was not possible using this technique, the positive color reaction might provide a means of screening cultures for superoxide radical export.

The data clearly indicate that reduced oxygen species, namely superoxide radical and hydrogen peroxide, are present in the extracellular broth during lignin fermentation by C. versicolor.

Table IV: Extracellular Hydrogen Peroxide in Fermentation Broths of Coriolus versicolor

Sample description	Concentration of H_2O_2 mM	
	Lignin present	Lignin not present
(1) Sample containing water	1.87	1.45
(2) Sample containing catalase	2.9×10^{-4}	2.9×10^{-4}
(3) Control with dead cells	2.9×10^{-4}	2.9×10^{-4}

Table V: Extracellular Superoxide Radical in Fermentation Broths of Coriolus versicolor

Sample description	Concentration of O_2^- mM	
	Lignin present	Lignin not present
(1) Sample + water (final pH 4.2)	4.25×10^{-3}	5×10^{-5}
(2) Sample + catalase (final pH 7.1)	7.9×10^{-3}	5×10^{-5}
(3) Sample + 1/2 ml catalase + 1/2 ml SOD (pH 7.1)	8.7×10^{-4}	$<1 \times 10^{-6}$
(4) Sample + SOD	$<1 \times 10^{-6}$	$<1 \times 10^{-6}$
(5) Sample from flask inoculated with dead cells	$<1 \times 10^{-6}$	$<1 \times 10^{-6}$
(6) Sample from uninoculated flask	$<1 \times 10^{-6}$	$<1 \times 10^{-6}$

Futhermore, extracellular concentrations of superoxide radical are significantly higher in the presence of lignin than in its absence. The buffered samples from medium containing lignin (i.e., those to which catalase was added) showed somewhat higher concentrations of superoxide radical. In retrospect this observation was predicable. Fee and Valentine (135) indicate that the second order rate constant for the spontaneous dismutation of superoxide radical is $4x10^5$ M^{-1} sec^{-1} at pH 7.1 while it is $1x10^7$ M^{-1} sec^{-1} at pH 4.2. Adjustment of the pH of the cell suspension in the syringe established an environment which allowed a longer half-life for the highly unstable superoxide radical.

The biological production of superoxide radical is known to occur intracellulary in many systems. Superoxide radical is produced intracellularly in eucaryotic organisms during respiration via univallent reduction of oxygen in the mitochondria (136,137). Bioluminescent organisms are also thought to produce superoxide radical intracellularly (138), while phagocytic cells produce superoxide radical during phagocytosis (139). In phagocytosis, however, the vacuole membrane, which was once part of the outer membrane, is thought to be the site of superoxide radical production.

The observation of extracellular superoxide radical in microbial fermentations is unprecedented. On the other hand, phagocytic polymorphonuclear leukocytes, effector cells of acute inflammatory response, release large amounts of superoxide radical into the medium in which the stimulated cells are suspended (140) supporting my observation that certain biological systems can export superoxide radical into the extracellular environment.

A mathematical model for the prediction of superoxide radical concentration as a function of the distance from the source was developed. The model takes into consideration superoxide radical diffusion through the cytoplasm and second order dismutation of the radical in the presence of superoxide dismutase. Assuming superoxide dismutase concentration is constant within the cell at a value of 2×10^{-5}M and neglecting counter diffusion in the opposite direction to superoxide radical diffusion, the one dimensional differential equation describing this situation at steady state is

$$-D_{O_2^-,H_2O}\frac{d^2C_{O_2^-}}{dZ^2} + KC_E C_{O_2^-} = 0 \qquad (A)$$

where: D = Diffusion coefficient of O_2^- in water
= $10^{-5}cm^2.sec^{-1}$ (Based on data for diffusion of gases in water (141))

K = Second order rate constant for the dismutation reaction
= $2\times10^9\ M^{-1}\ sec^{-1}$ (135)

C_E = Superoxide dismutase concentration in the cell
= 2×10^{-5} M (Based on data for liver cells (123))

$C_{O_2^-}$ = Concentration of superoxide radical

Z = distance from the source of O_2^- production

The solution of equation A subject to the following boundary conditions:

at $Z = 0$ $\quad C_{O_2^-} = C_{O_{2_0}^-}$ (Concentration of O_2^- at the source is defined)

at $Z = \infty$ $\quad C_{O_2^-} = 0$

is $$\frac{C_{O_2^-}}{C_{O_{2_0}^-}} = \exp -\left(\frac{KC_E}{D_{O_2^-,H_2O}}\right)^{1/2} Z \qquad (B)$$

Substitution of the above mentioned values for K, C_E and D in equation B indicate that at a distance of 3μm from the point source of superoxide radical generation $C_{O_2^-}/C_{O_2^-_0} = 5.8 \times 10^{-9}$, i.e., if we assume superoxide radical is produced by respiration in the mitochandria, located at the center of the microbial cell, the concentration of superoxide radical just outside the cell could not be as large as we have detected unless about 0.7 moles per ml of the radical are produced by respiration. On the other hand, if superoxide radical is produced at the cell plasma membrane or close to it, and assuming that the cell wall thickness is 200°A, $C_{O_2^-}/C_{O_2^-} = 0.84$ or 84% of superoxide radical initially produced will still be present just outside the microbial cell. In such a case the presence of a hydrodynamic boundary layer that does not contain superoxide dismutase will allow an appreciable amount of superoxide radical to diffuse to the bulk of the fermentation medium. Recent experiments indicate that C. versicolor has a higher level of superoxide dismutase intracellularly when grown in the absence of lignin than when grown in its presence (P. L. Hall, personal communcations). Higher superoxide dismutase activities in the absence of lignin in the growth medium may explain our failure to detect superoxide radical in this system.

(2) Relation between extracellular superoxide radical and lignin degradation

To identify the role superoxide radical may play in lignin degradation, I decided to use a chemical capable of stimulating the microbial cells to produce excess superoxide radical. My first step

was to show that lignolytic cultures, to which such a chemical had been added, produce more superoxide radical when compared to lignolytic cultures not containing such a chemical (control). I could then monitor lignin degradation and determine whether the increase in extracellular superoxide concentration correlates with an increase in the extent of lignin degradation.

Recent literature (142,143) indicate that the treatment of certain biological systems with paraquat (=methyl viologen) results in the system generating superoxide radical. I studied the effect of different concentrations of paraquat, added to a simple growth medium (Malt extract broth), on the production of extracellular superoxide radical by C. versicolor. Figure 7 indicates that concentrations of 0.5 mM and 1.0 mM of paraquat induced the micro-organism to produce extracellular superoxide radical. Figure 8 illustrates the effect of different concentrations of paraquat on the extent of growth of C. versicolor incubated for seven days in the simple medium.

Once I established that paraquat stimulated the production of extracellular superoxide radical in cultures of C. versicolor, the second step was to grow the organism in the lignin containing medium. Two sets of 250 ml erlenmyer flasks, containing ^{14}C-labeled Kraft lignin, were inoculated with C. versicolor. One set contained 0.5 mM paraquat, while the control set did not contain paraquat. The flasks were incubated for seven days, and the time-course of the extracellular concentration of superoxide radical was followed. Figure 9 shows that in the presence of paraquat, higher concentrations of extracellular

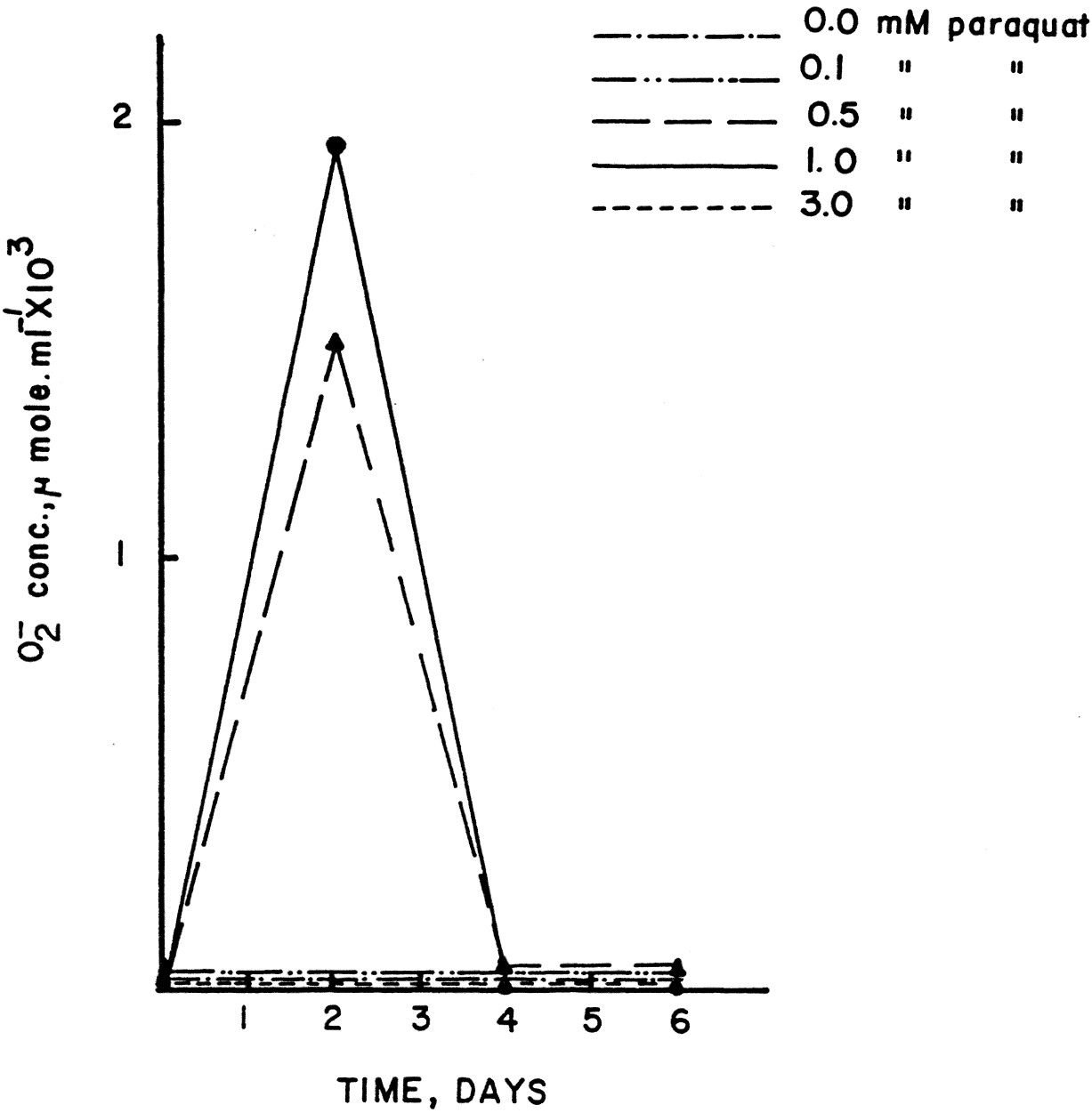

Figure 7. Effect of paraquat concentration on the concentration of O_2^- in fermentation broth of C. versicolor (malt extract broth).

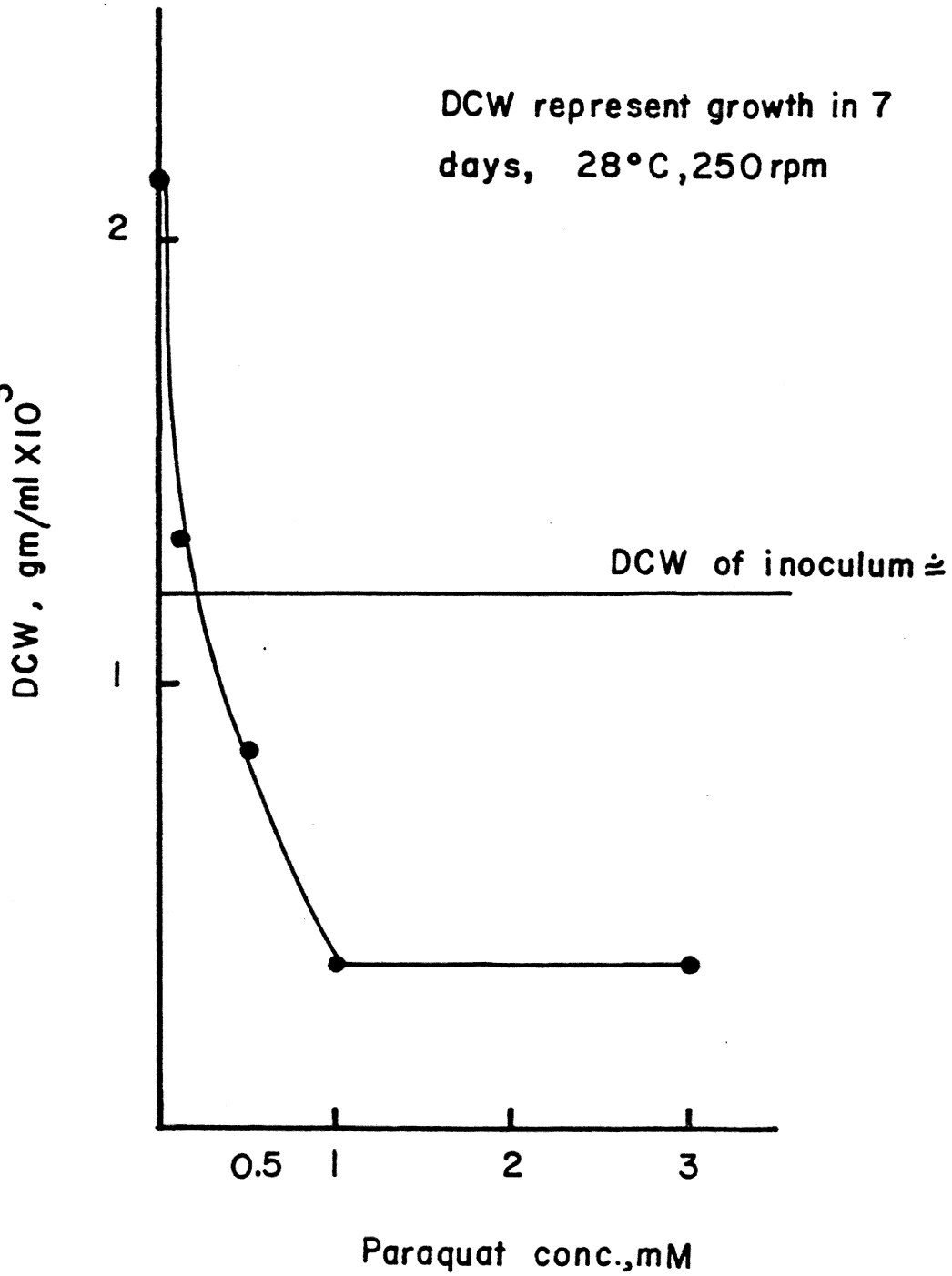

Figure 8. Effect of paraquat concentration on the growth of C. versicolor in malt extract broth.

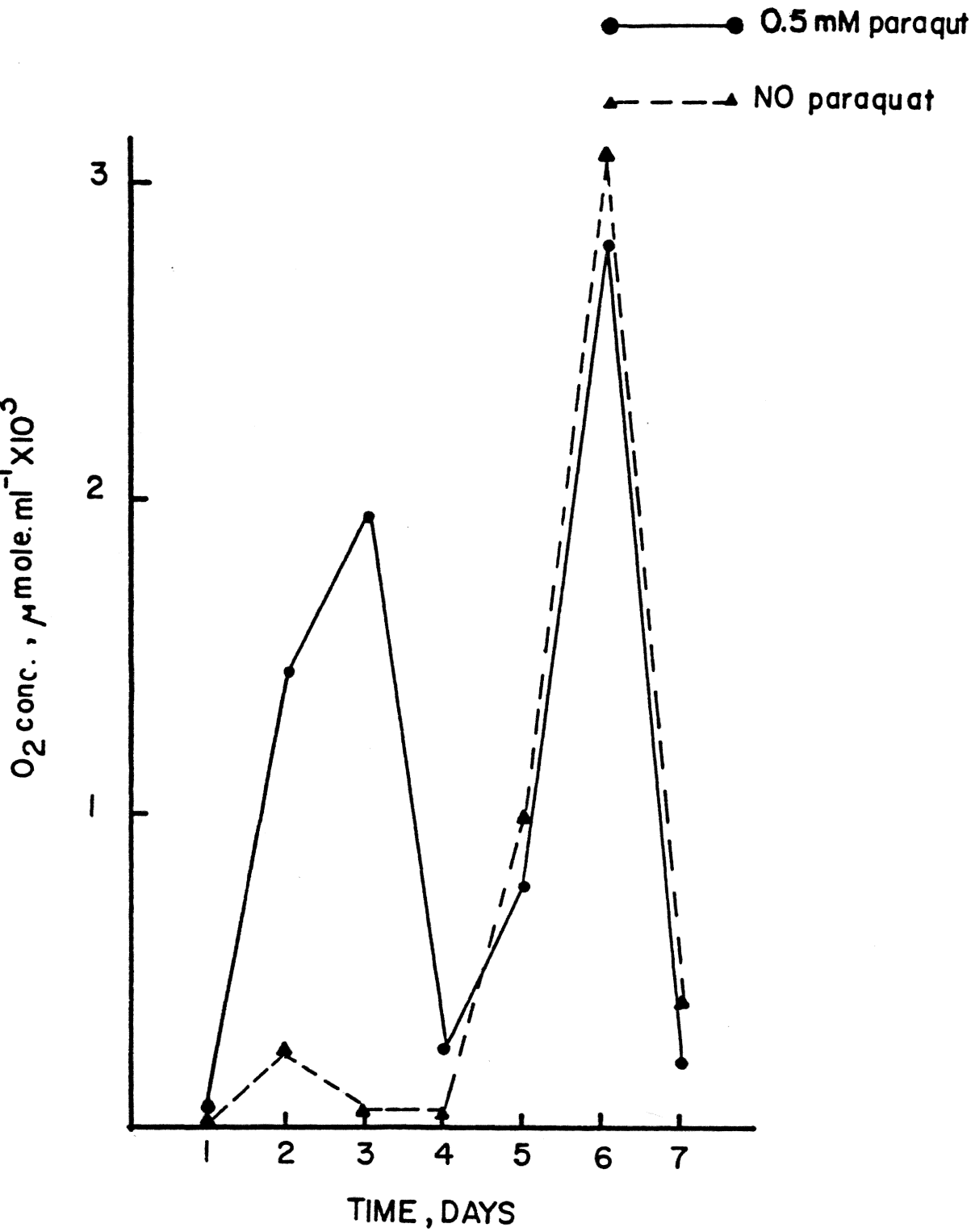

Figure 9. Effect of paraquat on the production of O_2^- by C. versicolor in lignin medium as a function of time.

superoxide radical were observed during the first four days of incubation.

The extent of lignin degradation in this experiment was also followed, by measuring the amount of $^{14}CO_2$ evolved due to the degradation of ^{14}C-labeled lignin. Figure 10 indicates that in the presence of paraquat, lignin is degraded to a larger extent than in the control. The error bars shown in Figure 10 are for a confidence limit of 80% as determined by the "student" t test as described in reference 144. An interesting observation concerning the results presented in Figure 10 is the fact that no $^{14}CO_2$ was evolved in the period around day 4. This could indicate a shift in metabolism. It is interesting to note that Kadam (76) showed that for the same organism grown in a similar medium the concentration of nutrient nitrogen falls to negligable levels at this point in time (at the end of three days of incubation). This could be an indication that once nutrient nitrogen is depleted from the medium a new metabolic machinery, capable of better lignin degradation, is developed in the microorganism. The development of such metabolic machinery appears to occur after the depletion of nutrient nitrogen in the medium and the elapse of a subsequent lag phase.

Also interesting, are the results presented in Figure 11. These indicate that during the first four days of incubation, the rate of $^{14}CO_2$ evolution is higher in lignolytic cultures containing paraquat as compared to cultures without paraquat. This is in good agreement with the results presented in Figure 9, which showed larger concentration of superoxide radical in paraquat containing cultures,

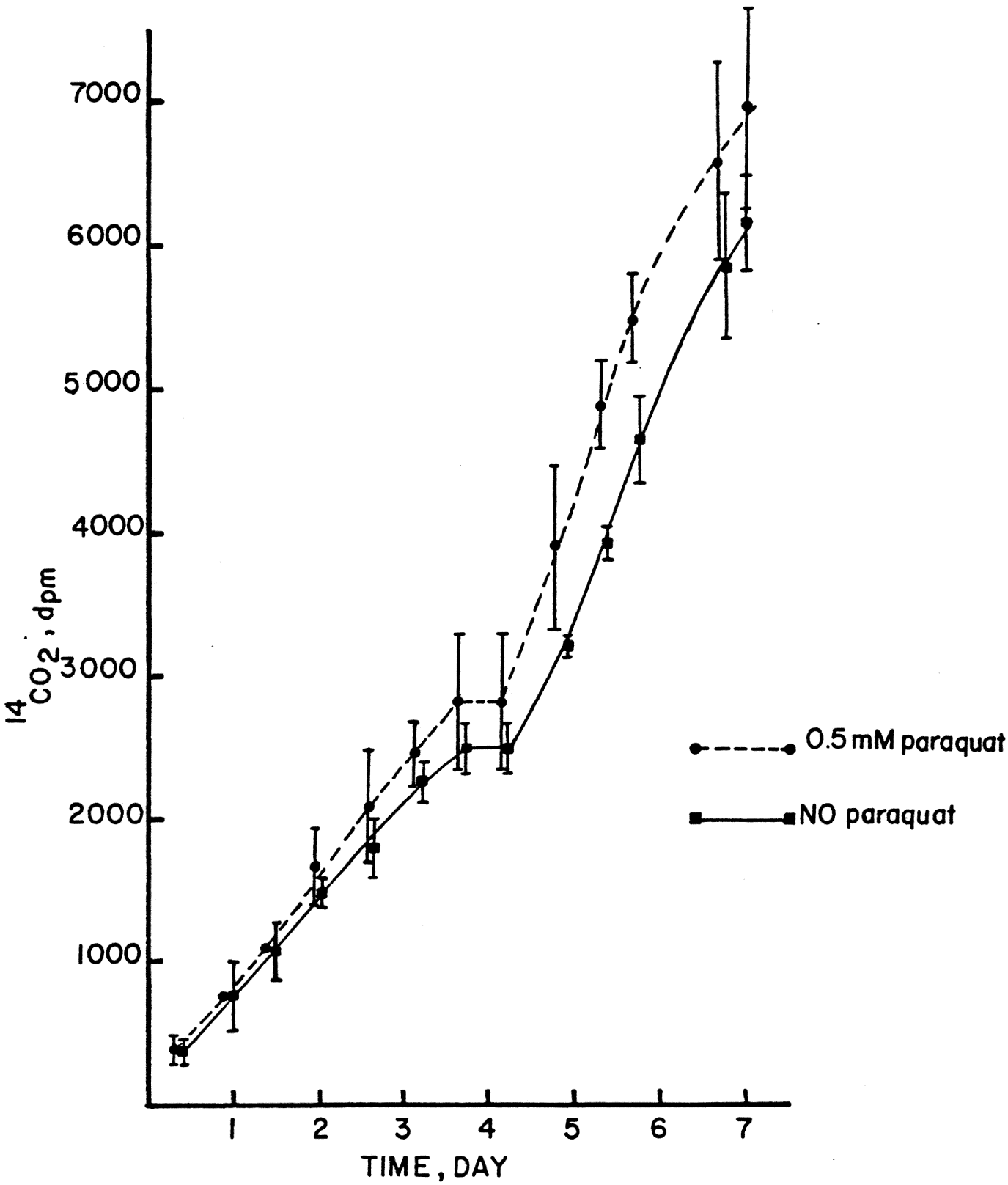

Figure 10. Effect of paraquat on the evolution of $^{14}CO_2$ from ^{14}C-labeled lignin by C. versicolor.

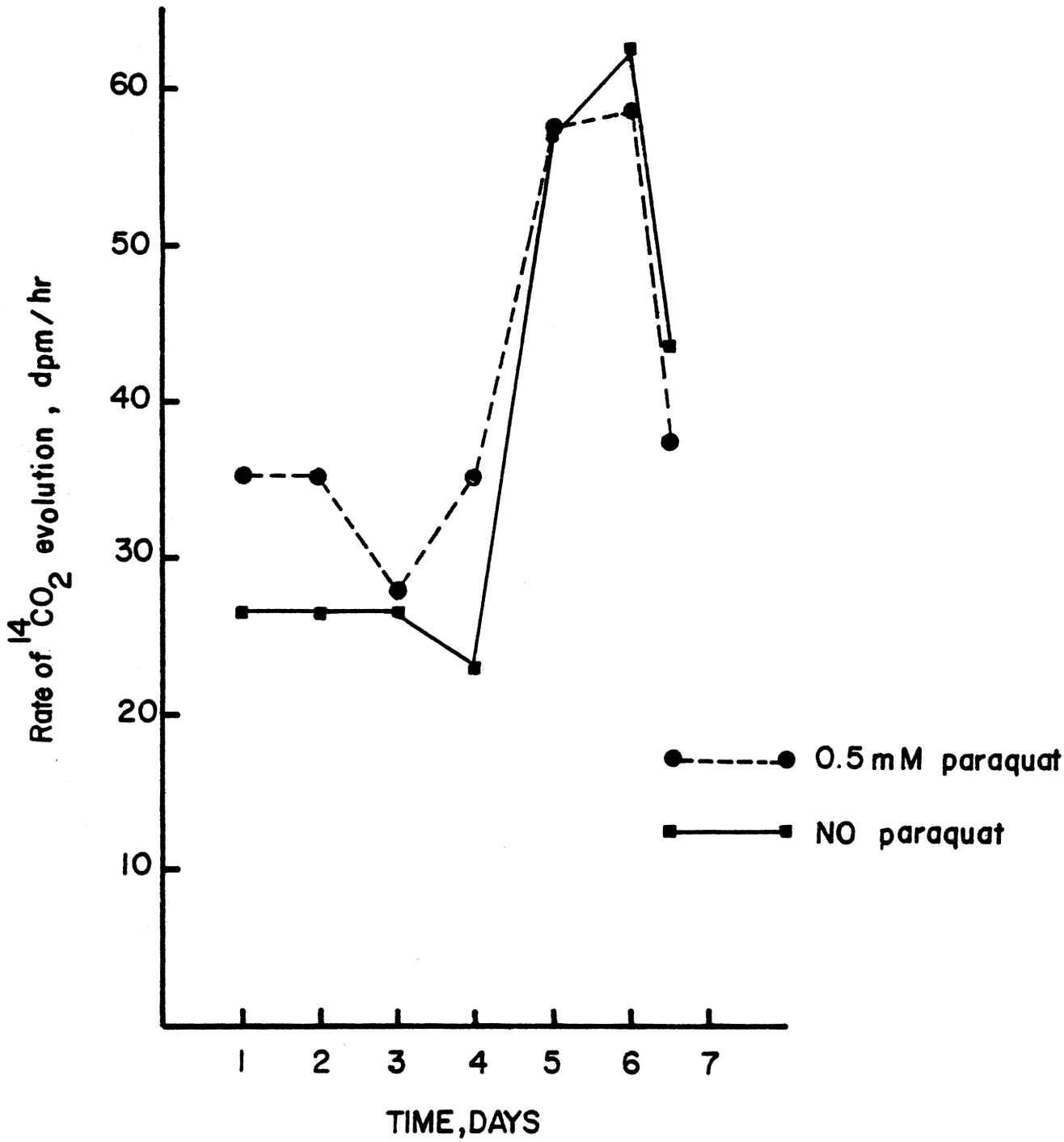

Figure 11. Effect of paraquat on the rate of $^{14}CO_2$ evolution, in lignolytic cultures of C. versicolor, as a function of time.

during the first four days of incubation. Moreover, the point of maximum extracellular superoxide concentration at six days (Figure 9) corresponds with the most rapid rate of $^{14}CO_2$ evolution from radioactive lignin (for both paraquat containing and control cultures, Figure 11).

The facts that higher rates of lignin degradation correspond to higher concentrations of extracellular superoxide radical, and that maximum rates of lignin degradation (as defined by the maximum rate of $^{14}CO_2$ evolution) coincide with maximum concentrations of extracellular superoxide radical, appear to support my contention that superoxide radical is actively involved in lignin degradation by the white-rot fungus C. versicolor. Admittedly, the initial breakdown of the lignin macromolecule (if instigated by superoxide radical and/or other related reduced oxygen species) is far removed from the evolution of CO_2 which should be the last step in lignin degradation, however, the similarity in timing of the two events is very suggestive. The initial breakdown of the lignin macromolecule to low molecular weight moieties may be the rate limiting step in lignin metabolism. The failure, by many researchers, to detect accumulation of low molecular weight lignin degradation products suggests that the rate of metabolism of such low molecular weight products to CO_2 is quite rapid compared to cleavage of the lignin macromolecule.

The results I have just presented strongly suggest a direct relationship between the rate of lignin conversion to CO_2 and the concentration of extracellular superoxide radical. It is probably true that the rate of CO_2 evolution from lignin during its degradation is also dependent on the concentrations of other related reduced oxygen species. However, a preliminary kinetic expression relating

the extracellular concentration of superoxide radical to the rate of CO_2 evolution from lignin, could be written in the following manner:

$$\frac{d[CO_2]}{dt} \propto [O_2^-]^n$$

where $\frac{d[CO_2]}{dt}$ is the rate of CO_2 evolution from lignin due to its degradation and n is a reaction order to be determined experimentally.

Because of the fact that I believe that my superoxide radical assay does not accurately predict its concentration in the medium, no attempt was made to determine a proportionality constant or n. However, I suggest that the utilization of ESR (Electron Spin Resonance) technique could lead to more accurate determination of extracellular superoxide radical concentration, and hence good estimates of the proportionality constant and n. Such a method has been successfully used to detect the presence of O_2^- in biological systems (135), but its usefulness might be limited by the interference of other reduced oxygen species present in the system.

(3) Effect of nitrogen deficiency in the medium on the concentration of extracellular superoxide radical

An important aspect of lignin degradation by white-rot fungi is the effect of nitrogen deficiency in the fermentation medium on lignin degradation. Reports in the literature (25,74,81,82) indicate that nitrogen deficiency in lignolytic cultures leads to increased lignin degradation. Figure 12 illustrates the time-course of the concentration of extracellular superoxide radical in the lignolytic culture of C. versicolor as a function of the initial concentration of nutrient

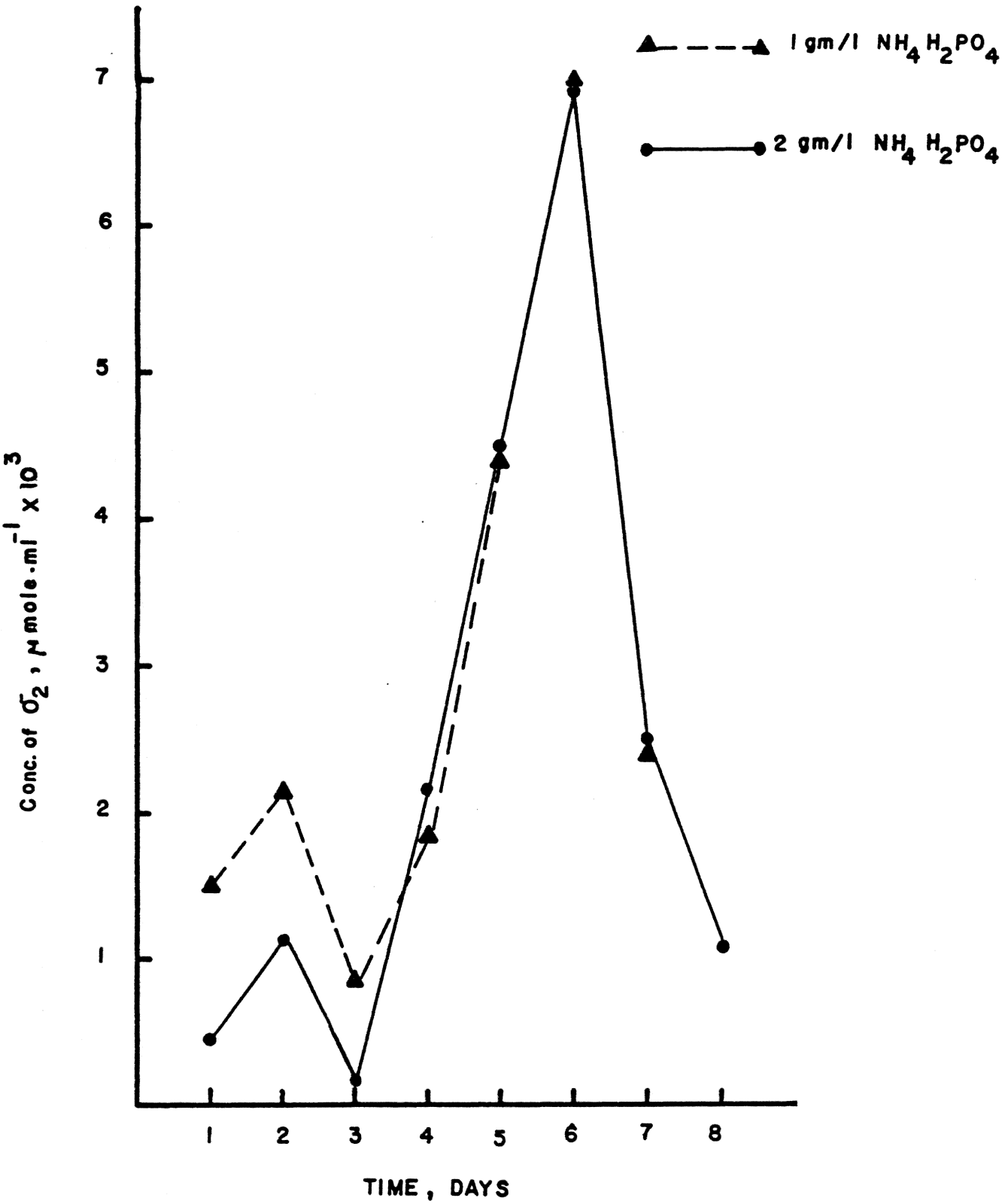

Figure 12. Effect of initial nitrogen concentration on the concentration of O_2^- as a function of time.

nitrogen in the medium. It is obvious that in the case where a lower initial concentration of nutrient nitrogen was used, higher concentrations of extracellular superoxide were observed during the first three days of incubation. On the other hand, starting at the fourth day superoxide radical concentrations for both cultures become similar. This observation could be explained by referring to Kadam's work (76) in which he found, using the same organism and medium composition, that nutrient nitrogen is depleted after three days of incubation. Based on Kadam's observations, I concluded that after three days of growth, both cultures presented in Figure 12 had depleted the nutrient nitrogen, and hence they behaved similarly.

The results presented in Figure 12 clearly indicate that at lower nutrient nitrogen concentrations, in the lignolytic cultures of C. versicolor, the organism produces higher levels of extracellular superoxide radical. This observation is consistant with my contention that superoxide radical is directly involved in lignin breakdown since it correlates with previous reports (74,81) which indicate that low nitrogen concentrations enhance the rate of lignin degradation. The higher concentrations of superoxide observed at low nutrient nitrogen concentration could be the cause for the enhanced lignin degradation in this case.

Moreover, the fact that once nutrient nitrogen is depleted (after three days incubation (76)) higher concentrations of extracellular superoxide radical are observed (starting at day four), correlates well with previous reports (81,82) which indicate that significant lignin degradation occurs only after the depletion of nutrient nitrogen, and the elapse of a subsequent lag phase. My results

in general indicate that high concentrations of superoxide radical occur after four days incubation which coincides with the higher rates of lignin conversion to CO_2 (refer to Figure 11). All these observations suggest that high nitrogen content in lignolytic cultures has an inhibitory effect on lignin degradation which is relieved once excess nitrogen is depleted.

(4) Effect of agitation and increased oxygen tension on lignin degradation

Since the biological production of superoxide radical is known to occur via the univalent reduction of molecular oxygen (145), it is reasonable to assume that the production of superoxide radical in lignolytic cultures requires the presence of oxygen. These observations may explain the fact that white-rot fungi cannot degrade lignin anaerobically (74,82). Moreover, since superoxide radical production requires the presence of oxygen in the lignolytic cultures, I reasoned that the partial pressure of oxygen over the medium could affect the extent of lignin degradation.

Utilizing the simple defined fermentation medium described previously, four sets of submerged lignolytic cultures were inoculated with C. versicolor and incubated for a period of seven days. In this experiment, I followed the time-course of extracellular concentration of superoxide radical, and the extent of lignin degradation using the spectrophotometric method described previously. Of the four similar sets of flasks, two were incubated on the shaking platform of the shaker incubator (referred to as shaking cultures) and the other two

sets were incubated on a shelf in the shaker incubator (referred to as static cultures). One set of the shaking cultures was flushed with pure oxygen every eight hours for 60 seconds while the other set was covered using Kaputs allowing continuous air exchange. The same was done to the static cultures.

Figure 13 indicates that in submerged shaking cultures, there is no noticeable difference in the concentration of extracellular superoxide radical, as a function of time, whether the culture was incubated under a pure oxygen atmosphere or under normal aeration. As a consequence, Figure 14 indicates that there also is no difference in the extent of lignin degradation, whether the shaking culture is incubated under an atmosphere of pure oxygen or under normal aeration conditions.

On the other hand, static cultures incubated under an atmosphere of pure oxygen, elaborated more superoxide radical than in the case of static cultures under normal aeration (Figure 15). Figure 16 indicates that this higher superoxide concentration observed in the case of static cultures under pure oxygen, corresponds with a higher level of lignin degradation. This is in comparison with static cultures under normal aeration, which elaborated a lower level of superoxide.

Results presented in Figures 14 and 16 are consistent with the results obtained previously by Rosenberg (14). His results clearly indicate that in the case of submerged shaking lignolytic cultures of C. pruinosum (imperfect forms of the basidiomycete P. chrysosporium), the partial pressure of oxygen over the fermentation medium does not

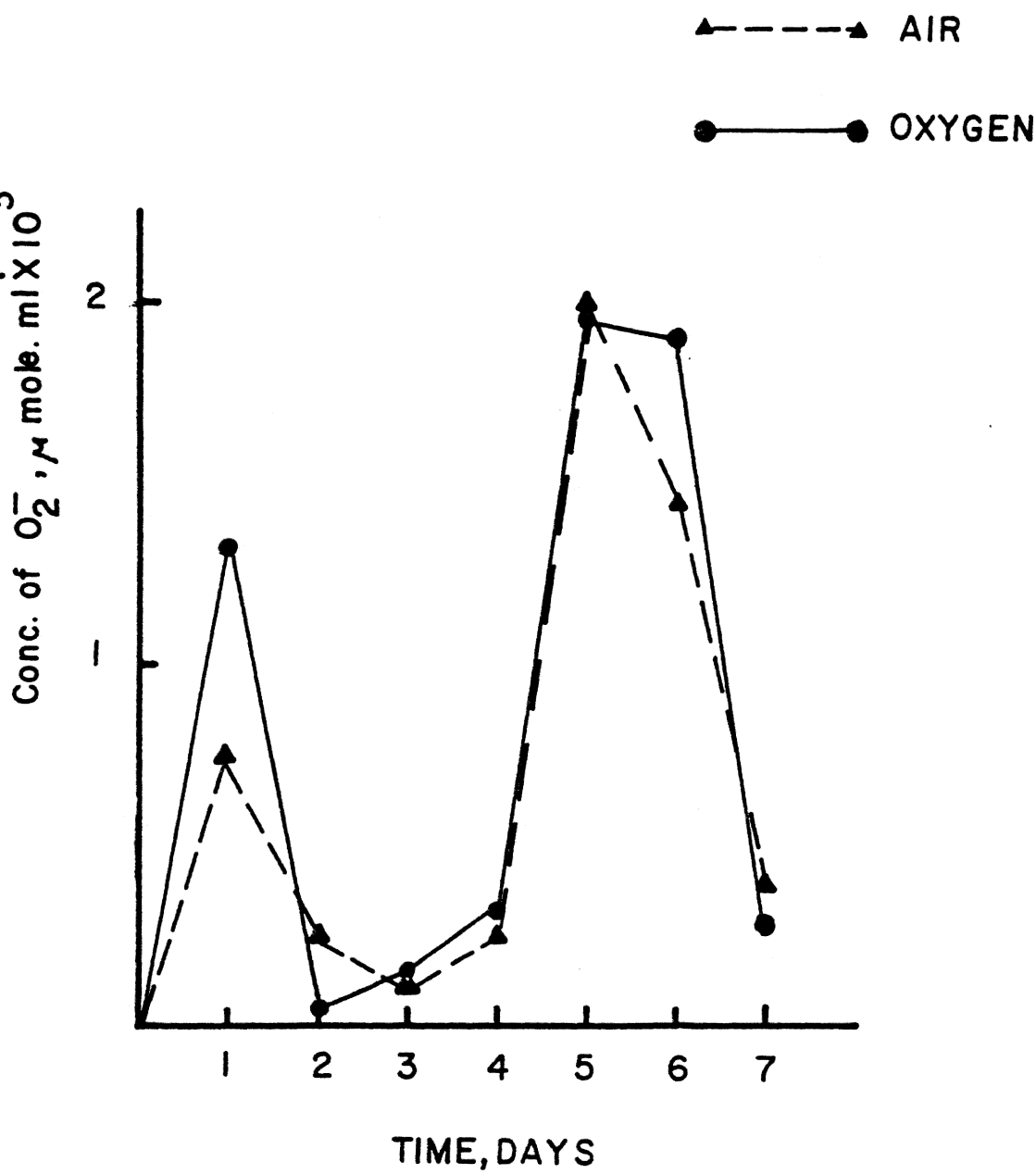

Figure 13. O_2^- conc. vs. time in submerged shaking lignolytic cultures of C. versicolor.

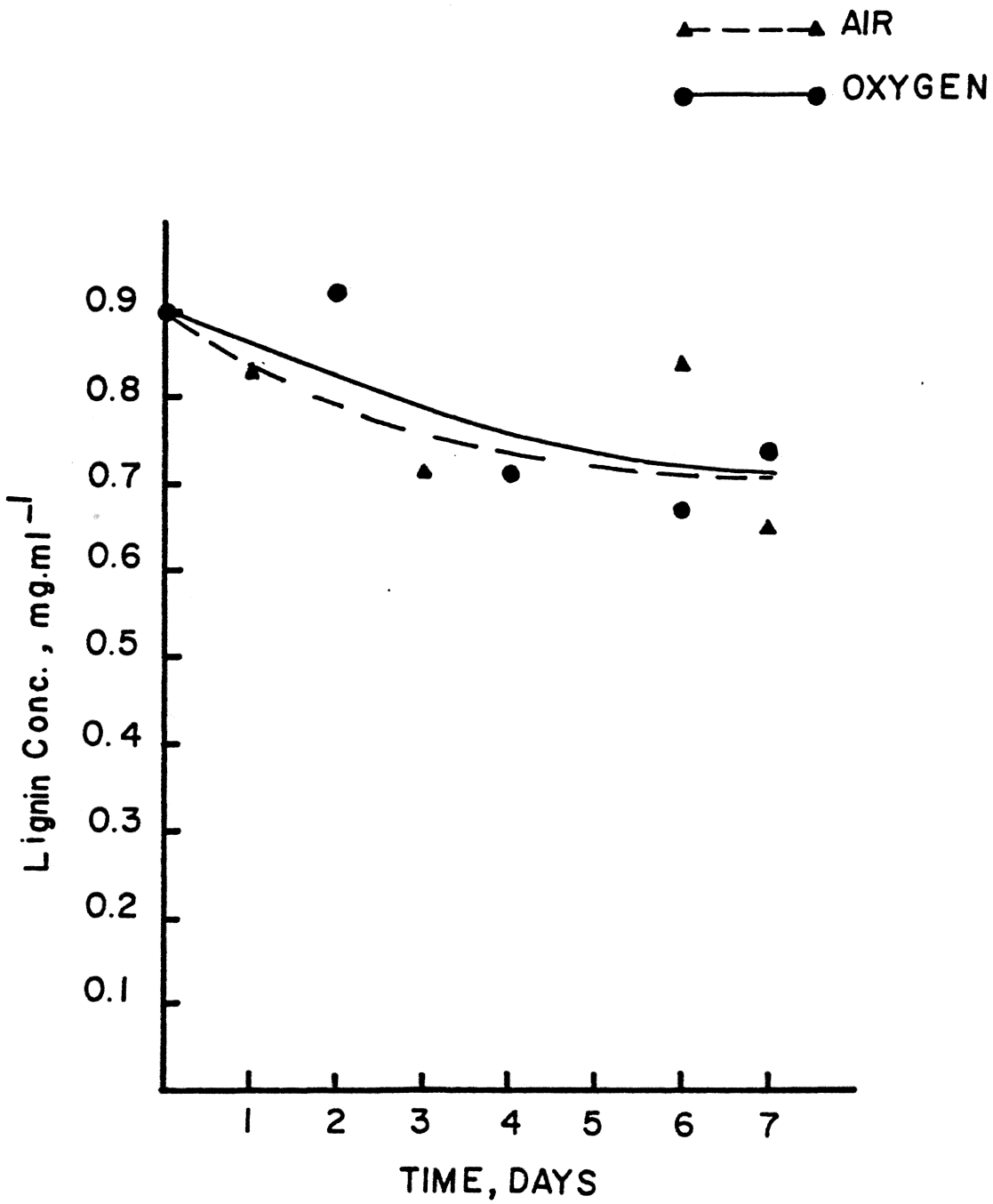

Figure 14. Lignin conc. vs. time in submerged shaking lignolytic cultures of C. versicolor.

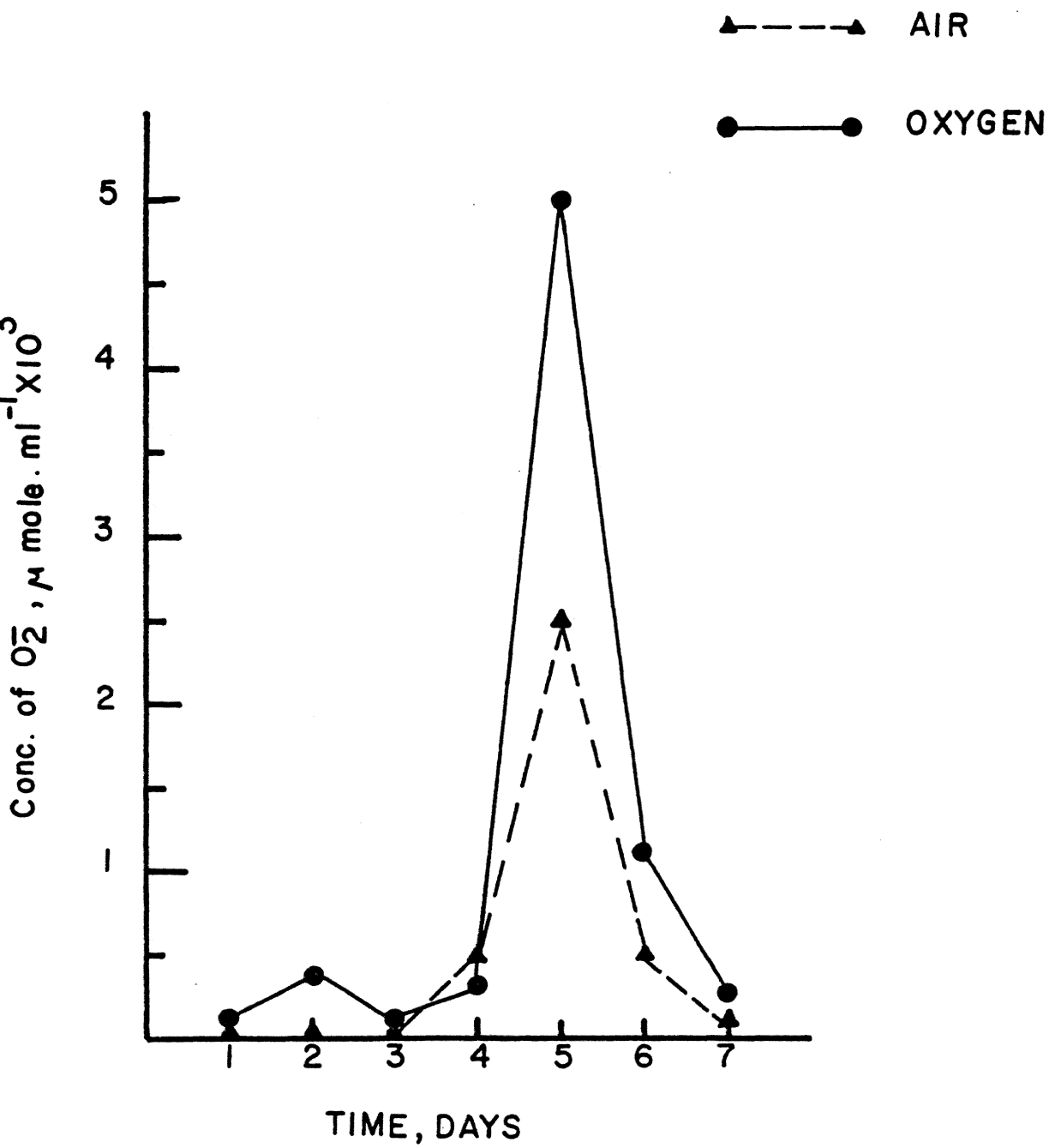

Figure 15. O_2^- conc. vs. time in submerged static lignolytic cultures of C. versicolor.

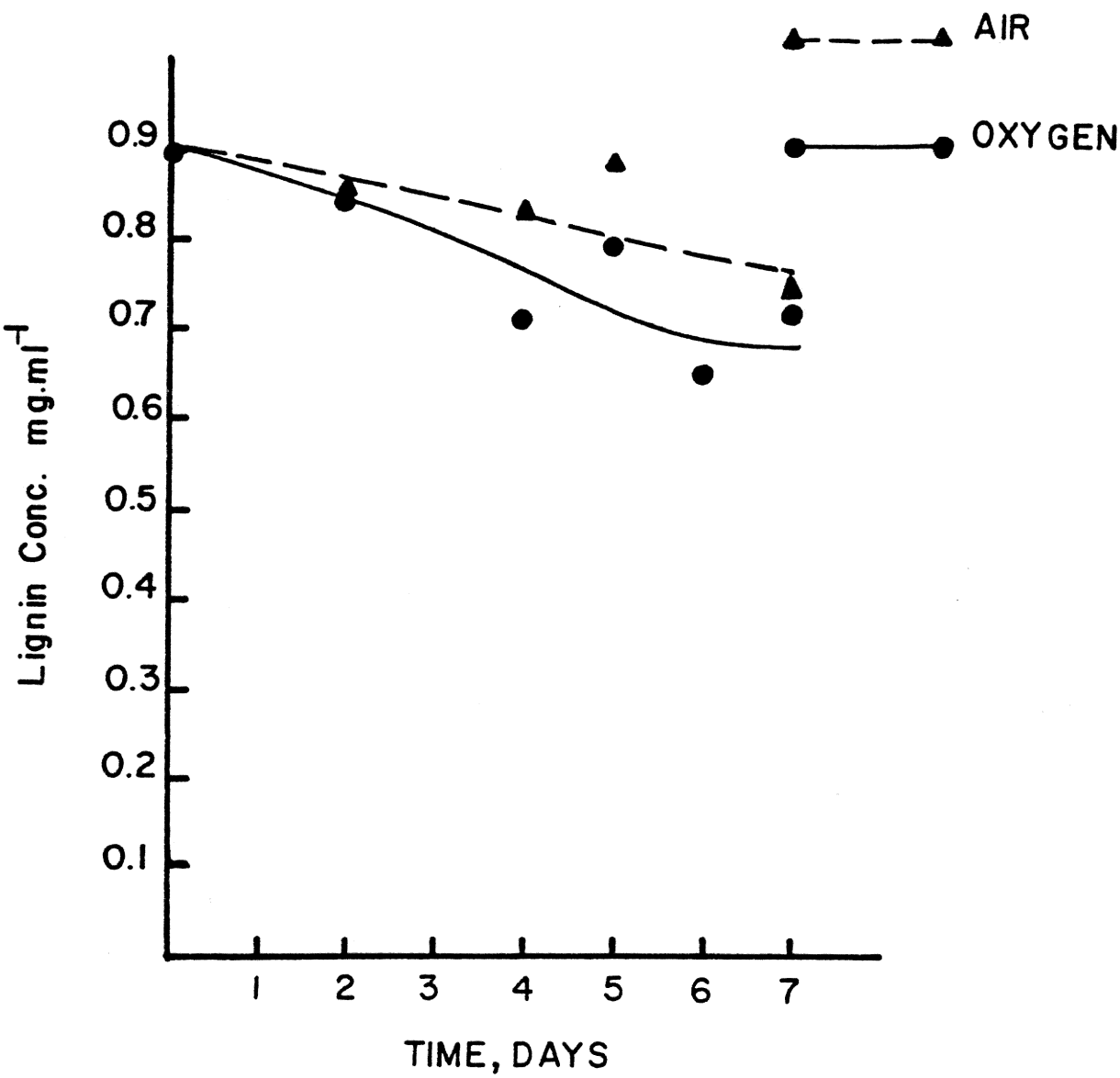

Figure 16. Lignin conc. vs. time in submerged static lignolytic cultures of C. versicolor.

affect the extent of lignin degradation. This is probably due to the fact that in both cases, the medium was saturated with oxygen. Moreover, Rosenberg's results also indicated that in the case of static cultures, the partial pressure of oxygen over the fermentation medium of C. pruinosum did influence the extent of lignin degradation.

Through visual observation I noted that, in the case of submerged shaking cultures pellets of substrate and mycelia were formed. On the other hand, in the case of static cultures a mycelial mat, which encompassed all of the substrate, was formed on the bottom of the flask. The morphological appearance of the mycelia in the mat differed markedly from the mycelia in the pellets. This observation combined with the fact that the extent of lignin degradation in static cultures under a pure oxygen atmosphere appear to be slightly greater than in shaking cultures, (Figure 17) seem to indicate that conditions which allow morphological differentiation may favor fungal degradation of lignin.

(5) Assessment of novel fermentation systems for lignin degradation

a) Solid State Fermentation (SSF)

The solid state fermentation flasks were incubated for a period of 30 days at 28°C and a constant humidity of 92%. Throughout the incubation period the wood chips were wet in appearance but no excess liquid was observed in the flasks. The analysis of the lignin fraction in the degraded wood was done using the spectrophotometric method described previously. Figure 18 illustrates the time-course of lignin losses in the solid state fermentations. The results

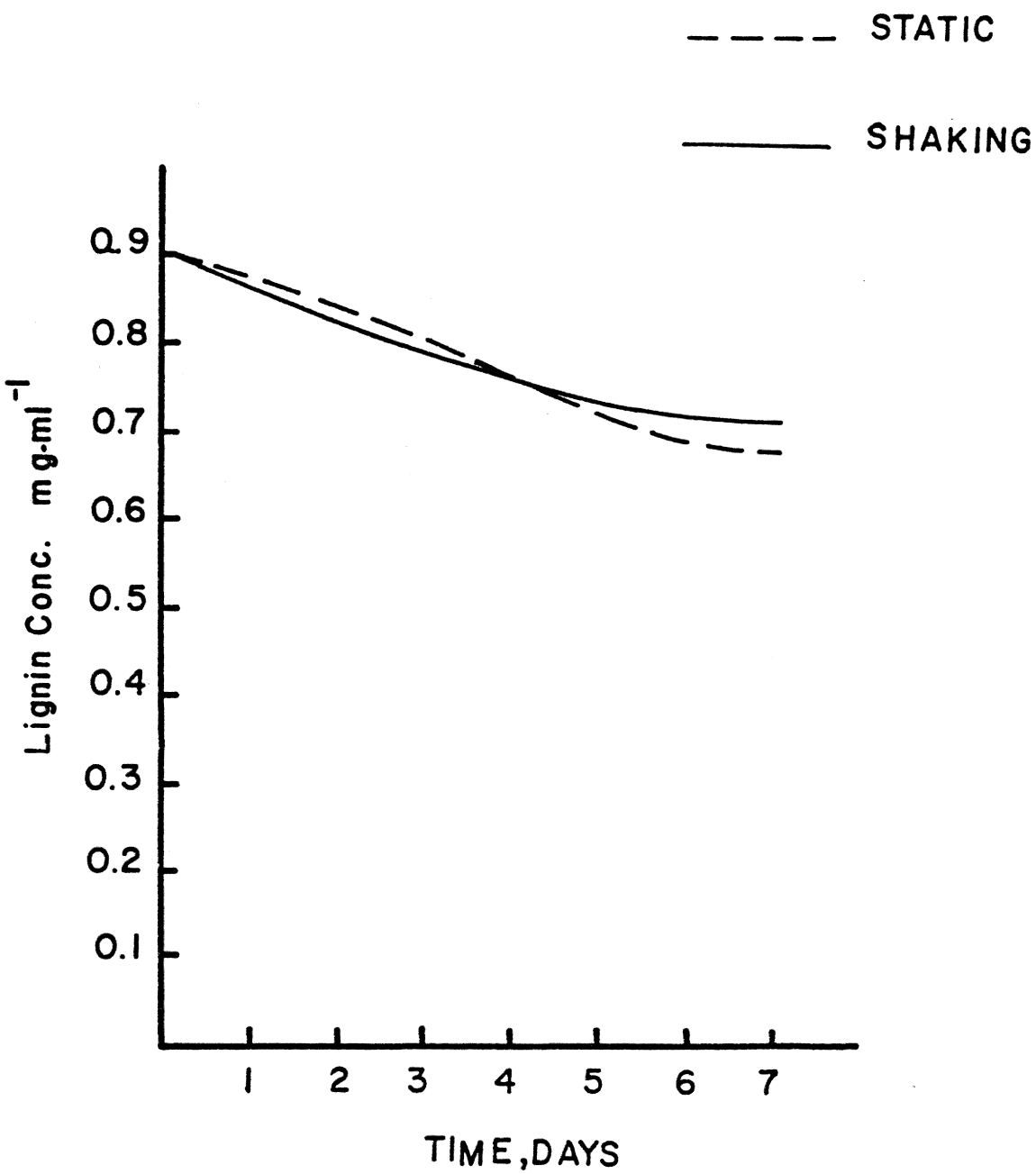

Figure 17. Comparison of lignin concentration in shaking and static lignolytic cultures of C. versicolor under pure oxygen. (curves obtained from figures 14 and 16)

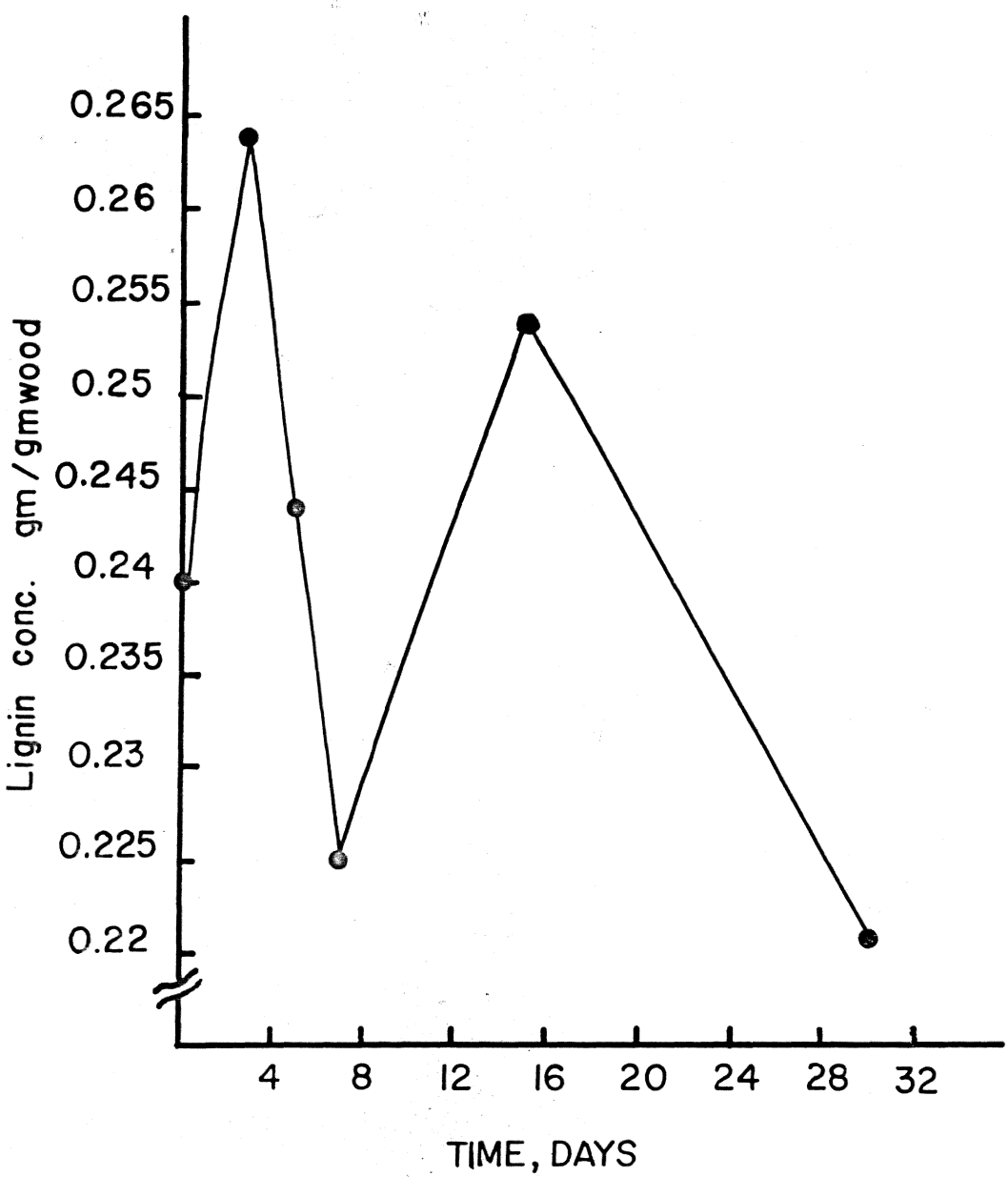

Figure 18. Lignin conc. vs. time in solid state fermentation of lignocellulosic material by C. versicolor.

presented in Figure 18 are averages of duplicate cultures with ±3 to ±5% variation.

Figure 18 indicates that 6.25% and 7.92% of the lignin originally present in the wood was degraded after 7 and 30 days incubation respectively. However, C. versicolor is also capable of degrading the cellulosic and hemicellulosic fractions of the wood (3), thus since the lignin concentrations were measured as grams of lignin per gram of wood my results obviously underestimate the extent of lignin degradation in the solid state fermentation.

Moreover, if the degradation of the carbohydrates fraction (cellulose and hemicellulose) in the wood proceeded at the same rate as the degradation of the lignin fraction I should have observed no decrease in the lignin concentration as a function of time. However, my results indicate a substantial decrease in lignin concentration as a function of time. This decrease in lignin concentration suggests that the rate of lignin degradation in solid state fermentation is higher than the rate of carbohydrates degradation. Based on the above arguments, it is my opinion that solid state fermentation of wood, favors the degradation of the lignin fraction over the degradation of the carbohydrate fraction.

b) Fluidized bed fermentation

Figure 19 illustrates the time-course of lignin losses in degraded loblolly pine chips during seven days incubation with C. versicolor, in the tapered fluidized bed. These results show that the concentration of the lignin fraction (grams lignin per

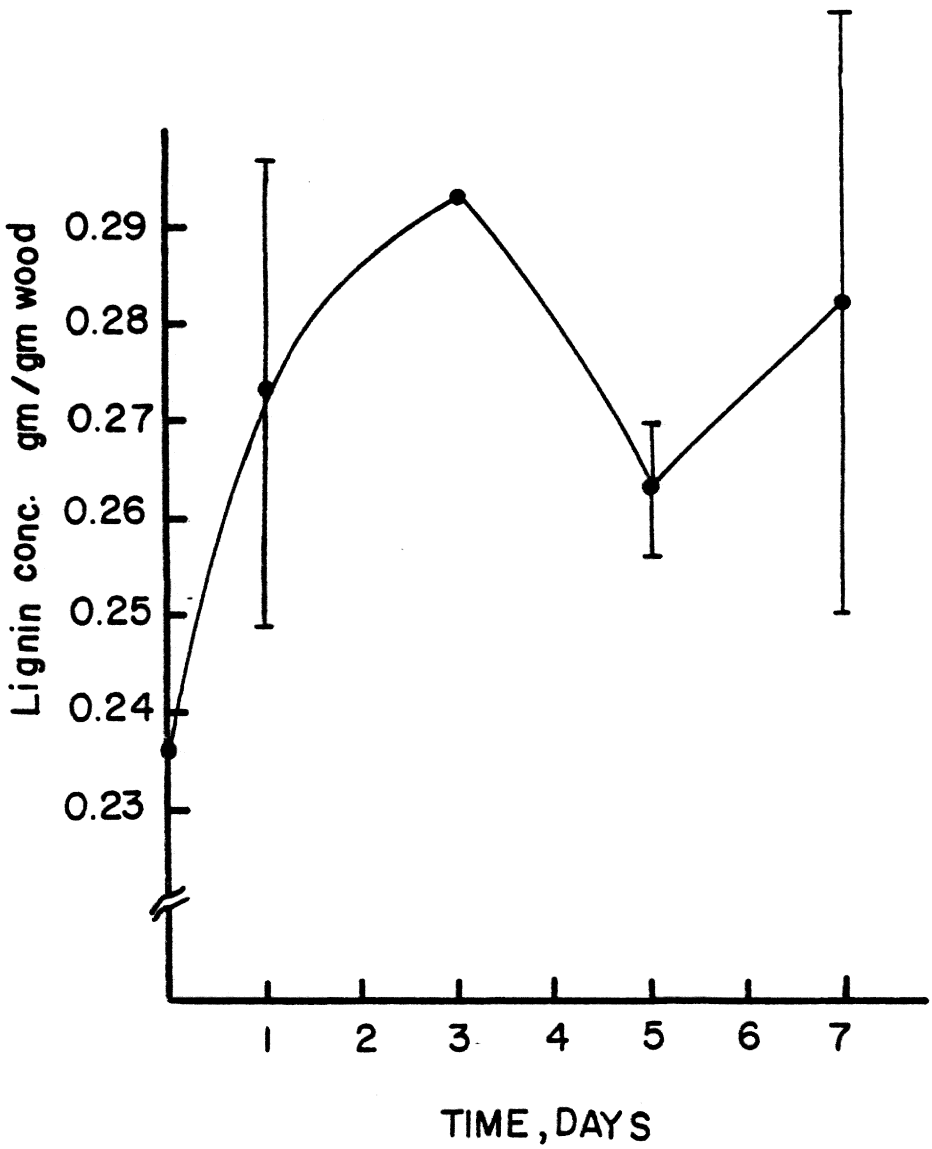

Figure 19. Lignin conc. vs. time in fluidized bed fermentation of lignocellulosic material by C. versicolor.

gram wood) increased during incubation. Since a substantial amount of microbial growth was observed, it is reasonable to conclude that the organism mainly utilized the carbohydrates fraction of the wood as its growth substrate, leaving the lignin behind. Thus the increase in lignin concentration was observed. The results indicate that the rate of lignin degradation is slower than the rate of carbohydrates degradation in the fluidized bed fermentor. In other words, the degradation of the carbohydrates fraction is favored over the degradation of the lignin fraction of the wood. This is opposite to the observations concerning the solid state fermentation of wood.

Another interesting observation, is the fact that the morphology of the culture grown in the fluidized bed fermentor differed markedly from the morphology of the culture in the solid state fermentation. In the solid state fermentation I observed the formation of reproductive fruits, however such fruits were not observed in cultures grown in the fluidized bed. This observation supports my conclusion that conditions which allow morphological differentiation may favor fungal degradation of lignin.

(6) Relevance of results to white-rot fungi in general

Despite the fact that no reports are available in the literature, concerning the elaboration of extracellular superoxide radical by other white-rot fungi during lignin degradation, it is my opinion that such a phenomenon is not limited to C. versicolor. Kayser et al. (82) have presented results indicating that the white-rot fungus Phanerochaete chrysosporium degrades lignin to CO_2 in a

manner very similar to the one observed for C. versicolor. In their results they observed that the rate of $^{14}CO_2$ evolution from radiolabeled lignin reaches a maximum after six days of incubation with P. chrysosporium. They also observed low rates of $^{14}CO_2$ evolution after seven days of incubation. Moreover, in their discussion section they mentioned observing a similar behavior for C. versicolor. These observations are consistant with the results presented in Figure 11 suggesting that both C. versicolor and P. chrysosporium behave similarly in lignolytic cultures.

In addition, the results presented in Figures 14 and 16 are consistant with results presented by Rosenberg (14) concerning the effect of aeration and agitation on lignolytic cultures of Chrysosporium pruinosum. Rosenberg (14) showed that solid state fermentation of lignocellulosic materials by C. pruinosum favored lignin degradation, while agitation in submerged cultures favored the degradation of carbohydrates in lignocellulosic materials. Again, these results are consistant with my observations concerning C. versicolor.

Based on the above discussion it is my opinion that white-rot fungi in general, degrade lignin in a similar manner. This leads me to conclude that elaboration of extracellular superoxide radical during lignin degradation is a general characteristic of white-rot fungi and not limited to C. versicolor. The observations by Keyser and his coworkers leads me to the conclusion that white-rot fungi elaborate superoxide in a manner similar to the one presented for C. versicolor in Figures 9, 12, 13 and 15.

(7) Addendum

Superoxide radical is known to be formed during aerobic respiration in microorganisms by the univalent reduction of dioxygen. Superoxide radical is the conjugate base of the weak acid $HO_2^{\cdot}$. It is highly unstable and undergoes the fast dimutation reaction; $O_2^- + O_2^- = H_2O_2 + O_2$. The rate constant for this autodismutation reaction, at physiological pH, is $1x10^5$ M^{-1} sec^{-1} (123). However, in the presence of superoxide dismutase the rate constant of the catalyzed dismutation reaction is $2x10^9$ M^{-1} sec^{-1} under the same conditions (123).

Superoxide radical is not considered a strong oxidant by itself, none the less, it is capable of acting as both an oxidant and a reductant (135). The fact that superoxide radical is a weak oxidant suggests that its capability to degrade lignin by itself is limited. However, the presence of extracellular hydrogen peroxide with superoxide radical, in the lignolytic cultures of C. versicolor, could allow the Haber-Weiss reaction to take place (137). Such a reaction; $O_2^- + H_2O_2 = O_2 + OH^- + OH^{\cdot}$, leads to the formation of hydroxyl ion (OH^-) and hydroxyl radical ($OH^{\cdot}$). Hydroxyl radical is considered to be the strongest oxidant known to mankind (137), thus capable of causing the initial breakdown of the lignin macromolecule.

Based on the preceding disucssion, it appears that although O_2^- may have a minimal role as a causative agent in the biological degradation of lignin by white-rot fungi, its presence, in lignolytic cultures, indicate that other reduced oxygen species which are strong oxidants capable of causing lignin breakdown are also present. The

presence of such species (e.g. hydroxyl radical) will be closely correlated to the presence of O_2^-. Such a correlation could explain the correlation between the concentration of superoxide radical and the rate of CO_2 evolution from degraded lignin discussed earlier.

Admittedly, the data presented in this study does not unequivocally prove the hypthesis that reduced oxygen species cause the initial breakdown of the lignin macromolecule during its biodegradation. However, the data do not refute such a hypothesis. The strong similarity between the shape of the curve representing the extracellular concentration of O_2^- vs. time and the one representing the rate of CO_2 evolution from degraded lignin vs. time are very suggestive. Such similarity does suggest that reduced oxygen species, present in lignolytic cultures of the white-rot fungus C. versicolor, may indeed be responsible for the initial breakdown of the lignin macromolecule during its biodegradation.

VI. CONCLUSIONS

Based on my study, my conculsions concerning certain aspects of lignin degradation by white-rot fungi are summarized in the following:

1. Initial lignin breakdown during its biodegradation by white-rot rungi is a nonenzymatic process. This initial step in lignin degradation may be caused by nonspecific, nonenzymatic attacking agents elaborated by the microorganism into the lignolytic medium. These attacking agents may be reduced oxygen species which are derived from ordinary dioxygen such as superoxide radical and hydrogen peroxide.

2. The site of superoxide radical production appears to be in the cell's plasma membrane. This conclusion precludes the possibility that reduced oxygen species responsible for lignin degradation are produced by normal respiration in the microbial cell.

3. High levels of superoxide radical (and probably other reduced oxygen species that may also be responsible for lignin degradation) are not elaborated by the microbial cell until nutrient nitrogen is exhausted in the lignolytic medium.

4. The concentration of oxygen in lignolytic cultures appears to have no effect on lignin degradation in the cases where the cultures were agitated.

5. In static submerged cultures, increased oxygen concentration, in lignolytic cultures, leads to a higher extent of lignin degradation.

6. Redox active compounds such as paraquat could be used to stimulate white-rot fungi to produce excess superoxide radical. This could be used as a means of increasing the capability of white-rot fungi to degrade lignin.

7. Conditions leading to the formation of reproductive fruits in lignin degrading fungi appear to cause increased lignin degradation.

8. The degradation of the lignin fraction of lignocellulosic materials is favored when such materials are degraded in the solid state. On the other hand, the carbohydrates are degraded preferentially in the case of submerged fermentations of lignocellulosic materials.

9. Shaking or vigorous mixing of lignolytic cultures of white-rot fungi have a detrimental effect on the extent of lignin degradation.

VII. RECOMMENDATIONS

Based upon my work with the biodegradation of lignin, I would like to make the following recommendations:

1. The most important obstacle towards gaining a complete understanding of the events occurring during lignin degradation is the analysis of residual degraded lignin. It is imperative to develop a quick and simple method for residual lignin analysis. Such a method should be capable of yielding accurate and reproducible results which would allow the elucidation of structural changes in residual, biodegraded lignin.

2. The development of an assay capable of more accurately estimating the extracellular concentration of superoxide radical in lignolytic cultures, could lead to the development of a kinetic model which relates lignin degradation to superoxide concentration. Electron Spin Resonance (ESR) could prove to be a desirable approach for developing such an assay.

3. The presence of extracellular superoxide radical and hydrogen peroxide in lignolytic cultures of C. versicolor suggest that other reduced oxygen species (e.g. hydroxyl radical, singlet oxygen...etc.) are also present. Development of assays capable of detecting the presence of such oxygen species will undoubtedly lead to a better understanding of the events occurring during lignin degradation by C. versicolor.

4. The possible involvement of reduced oxygen species in lignin degradation by white-rot fungi, other than C. versicolor, and by other wood degrading microorganisms, should be investigated.

5. It is possible that the biodegradation of aromatic polymers, other than lignin, is instigated by reduced oxygen species produced by microorganisms. Such a possibility should be investigated.

6. Since no low molecular weight products appear to accumulate during lignin biodegradation, applications other than the production of chemicals from lignin biodegradation should be investigated. Such novel applications include biochemical pulping, waste treatment of pulp and paper mill effluents, and single cell protein production. Another interesting application is the utilization of lignin degradation to produce modified polymers that can be used in the production of adhesives and polymeric insulating materials.

VIII. SUMMARY

Lignin degradation, is quite common in nature and is an important link in the natural carbon cycle. A large variety of microorganisms are know to degrade lignin in nature as well as in contrived fermentation systems. White-rot and soft-rot fungi, as well as Actinomycetes, are apparently the most active lignin degraders in nature.

The large, cross-linked, polymeric structure of the lignin macromolecule makes its direct uptake, during the initial stages of its degradation, by microbial cells improbable. Moreover, the fact that the lignin macromolecule is composed of different monomeric units linked by a large variety of non-hydrolyzable intermonomeric bonds precludes hydrolytic cleavage of the biopolymer. Despite the fact that many extracellular and membrane-bound enzymes have been suspected in the initial breakdown of lignin, such activities have not yet been found. A close review of the literature indicates that the initial breakdown of the lignin macromolecule may be nonenzymatic. In addition, the degradation of the lignin polymer appears to follow an exo-degradation mechanism. That is, many lignin degrading microorganisms are apparently incapable of splitting the lignin molecule into intermediate molecular weight polyphenolic moieties which are further degraded; instead, they attack the periphery of the macromolecule.

The possible involvement of reduced oxygen species produced by white-rot fungi in the initial breakdown of the lignin macromolecule, during its biodegradation, was investigated. Using C. versicolor as

a representative of white-rot fungi, I demonstrated that C. versicolor exports superoxide radical and hydrogen peroxide during lignin degradation, into the lignolytic medium. Results presented in this study indicate that a correlation between the concentration of extracellular superoxide radical in the medium and the extent of lignin degradation may exist. Moreover, I have shown that superoxide radical is produced in the cell membrane, and not the organism's mitochondria. This precludes the possibility that such reduced oxygen species are produced as a result of normal respiration by the organism.

An investigation of the effects of aeration and agitation indicated that agitation has a detrimental effect on the extent of lignin degradation. On the other hand, increased oxygen tension in lignolytic cultures appeared to enhance the extent of lignin degradation. Another interesting finding was the fact that conditions leading to the formation of reproductive fruits in the lignolytic microorganism favored the degradation of the lignin fraction in lignocellulosic materials.

A comparative study of two different fermentation schemes, designed to degrade lignin in lignocellulosic materials on a large scale, indicated that solid state fermentation of such materials led to greater lignin degradation. Fluidized bed fermentations, on the other hand, appeared to favor the degradation of the carbohydrates rather than the lignin fraction of lignocellulosic materials.

Studies of the biodegradation of monomeric lignin model compounds do not shed light on the initial step(s) involved in the breakdown of the lignin polymer. Such studies assume that microbial

breakdown of lignin model compounds is similar to microbial breakdown of lignin an assumption that may not be correct. It is true that degradation of monomeric lignin model compounds can conceivably elucidate the mode of degradation of low molecular weight moieties resulting from initial breakdown of the lignin macromolecule. However, the chemical identities of these low molecular weight intermediates are not yet known. The efficacy of studies using aromatic, monomeric lignin model compounds in attempts to identify intracellular pathways for metabolism of lignin depends on the assumption that lignin breakdown products are indeed mononuclear phenolic materials.

Careful analysis of soluble and insoluble residual lignin resulting from lignin fermentations is a critical step in assessing the lignolytic ability of microorganisms. Futhermore, such analyses are essential in understanding the steps involved in lignin metabolism by microorganisms. To date the methods for residual lignin analyses are complex, time consuming and error prone. There is an urgent need to develop a quick simple method for residual lignin analysis that will yield accurate and reproducible results capable of elucidating structural changes in residual, biodegraded lignin. The development of such an analysis technique will undoubtedly lead to a better understanding of the complex problem of lignin biodegradation.

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LIGNIN BIODEGRADATION: REDUCED OXYGEN SPECIES

by

Gamal I. Amer

(ABSTRACT)

Lignin degradation, is quite common in nature and is an important link in the natural carbon cycle. A large variety of microorganisms are know to degrade lignin in nature as well as in contrived fermentation systems. White-rot and soft-rot fungi, as well as Actinomycetes, are apparently the most active lignin degraders in nature.

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