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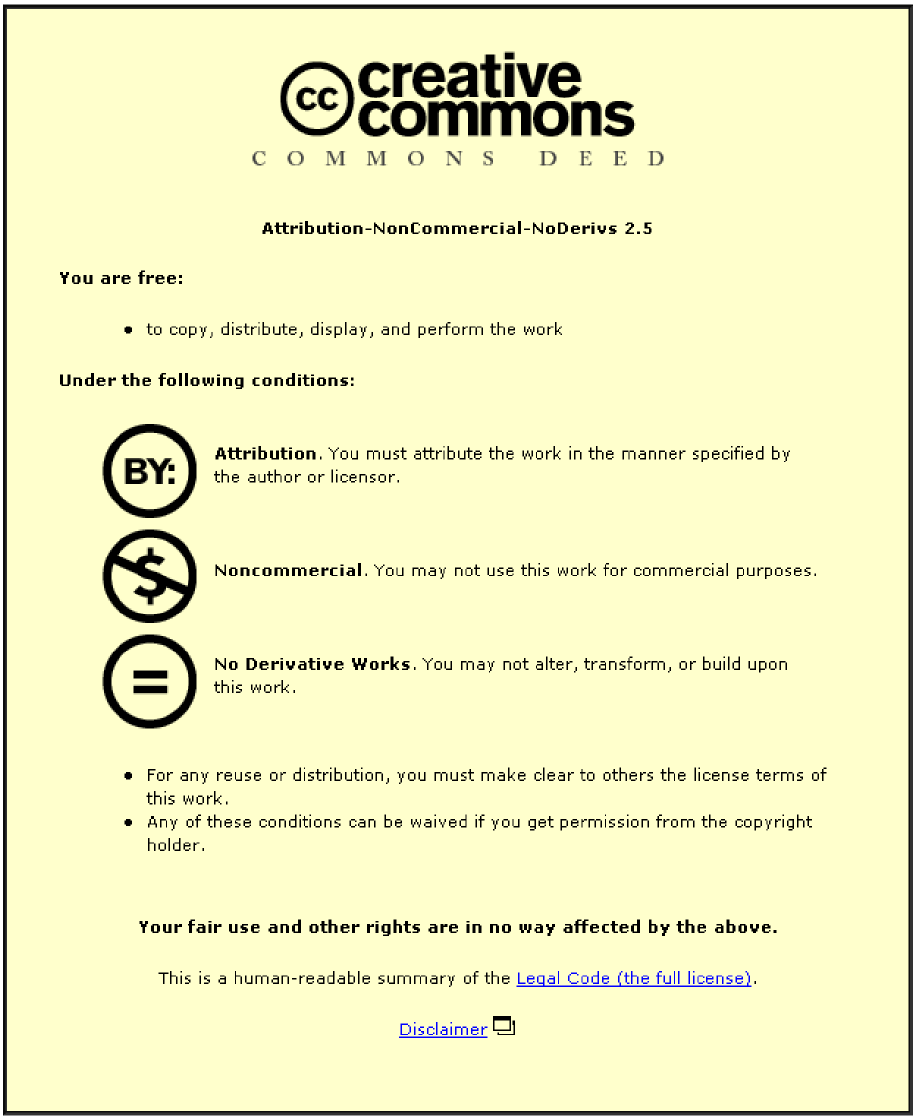

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Microbial Degradation Of Lignin And Lignin Related Aromatic Compounds

W. B. Betts 1987

MICROBIAL DEGRADATION OF LIGNIN AND LIGNIN RELATED AROMATIC COMPOUNDS

by

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M.Sc. (Loughborough University Of Technology)

A DOCTORAL THESIS SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE AWARD OF

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OF THE LOUGHBOROUGH UNIVERSITY OF TECHNOLOGY

JUNE 1987

SUPERVISOR : R. K. DART, B.Sc., Ph.D. M.I.Biol.

DEPARTMENT OF CHEMISTRY
LOUGHBOROUGH UNIVERSITY OF TECHNOLOGY

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THIS THESIS IS DEDICATED TO

My wife, Miriam

My two sons, Matthew and Jonathan

And to my Parents

SUMMARY

Eleven dimeric, trimeric and tetrameric lignin model compounds containing combinations of α-aryl ether, β-aryl ether and biphenyl linkages were synthesized. Those containing the biphenyl linkage were novel structures. Two ^{14}C-labelled lignin model compounds were synthesized.

Nine species of fungi were grown on solid media to assess their ability to degrade autohydrolysis aspen lignin and to establish the role of a secondary carbon source and nitrogen in lignin degradation. Some fungi previously unreported as lignin degraders were shown to degrade under certain conditions. The validity of the plate test as a screening method to demonstrate lignin degradation was established using known degraders.

Bacteria and fungi were screened, using a simple spray plate method, for their ability to degrade insoluble lignin-related aromatics with or without a secondary carbon source. Degradation was common but no regular pattern was found.

Physical associations between mycelia of _Aspergillus flavus_ and insoluble lignin model compounds were investigated using light and scanning electron microscopy. Strong and intimate associations were observed and reduction in crystal length of compounds during growth occurred.

The dimerization of 2,6-dimethoxyphenol to an insoluble product by _A. flavus_ was investigated. The dimer was identified and observed to form crystals attached to mycelia.

Quantitative growth measurements were made during growth of _A. flavus_ on model compounds. Products of degradation of dimeric, trimeric and tetrameric model compounds by _A. flavus_ were identified using TLC, GLC, ir, H-nmr and mass spectra. ^{14}C-labelled compounds were used to establish metabolic routes.

Degradation of larch wood by A. flavus was investigated. Changes in dry weight were measured and the lignin and carbohydrate content were assayed before and after degradation. The degraded wood samples were examined by electron microscopy. A reduction in dry weight, lignin and carbohydrate occurred and physical deterioration of the wood was observed after a lag period.

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INTRODUCTION

Introduction

Lignin is a major structural component of vascular plants and after cellulose is the most abundant naturally occurring organic polymer. The bulk of organic matter in terrestrial environments is in the form of the higher plants such as angiosperms, gymnosperms and monocotyledons. These have stems containing 18-25%, 25-35% and 10-30% of lignin respectively, on a dry weight basis, with cellulosic material making up much of the remainder (Cowling & Kirk, 1976). The degradation of lignin is probably a major rate-limiting step in the biospheric carbon-oxygen cycle (Evans, 1977).

Lignin fills the spaces between cellulose fibrils in wood cell tissues together with hemicellulose and pectin. The highest concentrations of lignin occur between wood cells in the middle lamella (Amer & Drew, 1980) but the majority of lignin (on a weight basis) is found in cell walls (Fergus et al., 1969). True lignin, although distributed widely, is not found universally in the plant kingdom. Non-lignified plant groups include the green algae and the Bryophyta (Erickson and Miksche, 1974 ; Gunnison & Alexander, 1975).

Lignin has many functions in plants. It acts as a bonding agent between cells providing resistance to mechanical stress. It helps plants to resist biochemical stresses such as microbial attack by acting as an impediment to penetration by destructive agents. It also has antioxidant, flame retardant properties and acts as a stabilizer against ultraviolet light. It reduces physio-chemical stresses and aids in responses to humidity, water balance and the transport of water and nutrients because of its waterproofing properties.

Native lignocellulose from sources such as wood, straw and vegetable fibre contains the largest store of lignin. However, related compounds are found in the macromolecular structures of lignite and bituminous coals (Hayatsu et al.,1979). Lignin also occurs in the wastes from many industries such as paper-making, forestry, lumbering, agriculture and textiles. Because these industries operate at very large capacity, the quantities of wastes produced are very large. A recent estimate of the annual production of lignin wastes in the United States is between 900 to 3000 million tons (Janshekar & Fiechter, 1983).

Lignin has great potential as a renewable resource of vast quantities. It is also important because, as a common waste material it can cause problems of disposal and pollution.

Lignin Structure

Lignins are highly branched, aromatic polymers composed of phenylpropane units linked by several non-hydrolysable bond types (Sarkanen & Ludwig, 1971; Adler, 1977; Higuchi, 1982). Natural lignins typically have molecular weights in excess of 100,000 daltons (Sarkanen & Ludwig, 1971). Figure 1 is an example of the structure of a softwood lignin (Sakakibara, 1983). The major linkages are arylglycerol-α-aryl ethers, arylglycerol-β-aryl ethers, biphenyls, 1,2-diarylpropanes, phenylcoumarans and diphenyl ethers. The proportions of these bonds vary depending on the source of the lignin but the arylglycerol-β-aryl ethers always predominate as shown in table 1.

In plant cell walls lignin is closely associated with

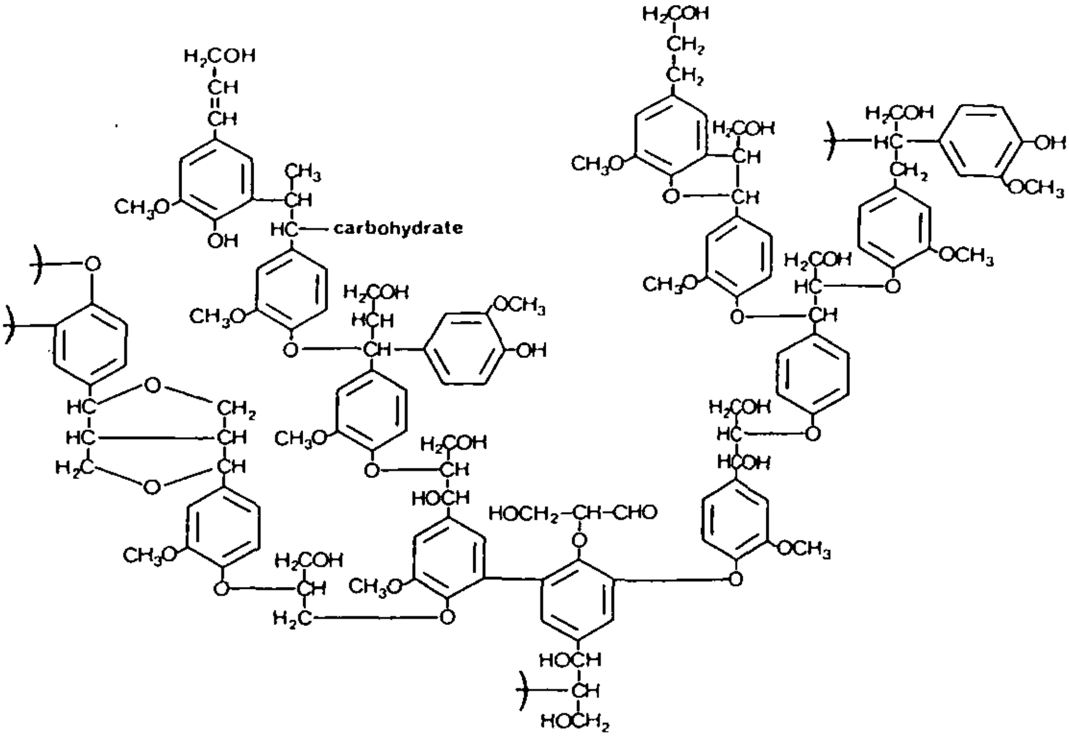


Figure 1. Schematic Structural Formula Of A Softwood Lignin

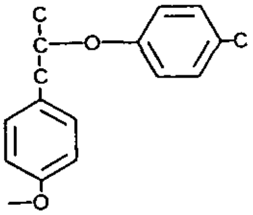
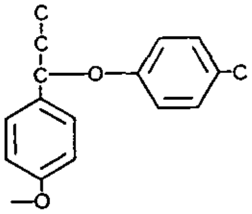
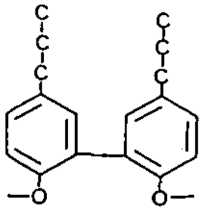
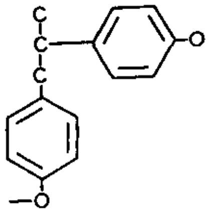
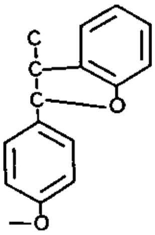
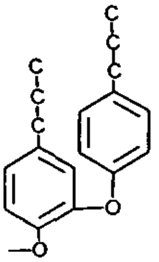
arylglycerol-β-aryl ether	arylglycerol-α-aryl ether	biphenyl
48%	6-8%	9.5-12%
		
1,2-diaryl-propane	phenyl-coumaran	diphenyl ether
7%	9-12%	3.5-4%
		

Table 1. Proportions Of Major Bond Types In Spruce Lignin

cellulose and hemicellulose. The separation of lignin from carbohydrates in lignocelluloses cannot be achieved by conventional methods such as gel filtration, electrophoresis, ultracentrifugation and hydrophobic-interaction chromatography (Janshekar & Fiechter, 1983). To explain this three theories have been considered: (1) hydrogen bonding between constituents; (2) covalent chemical bonds; and (3) encrustation and encasing of the carbohydrates by lignin (Fan et al., 1982).

Accumulating evidence points to both physical associations (Pew & Weyna, 1962; Wardrop, 1971; Kirk, 1975) and chemical linkages of various kinds (Freudenberg, 1964; Adler, 1977; Eriksson & Lindgren, 1977; Eriksson et al., 1980; Neilson & Richards, 1982). The frequency of occurrence of ether and ester linkages between lignin and hemicelluloses has been estimated at one linkage per 36 phenylpropane units (Obst, 1982). Investigations have suggested that the bonds are glycosidic linkages of lignin to D-galactose, L-arabinose and D-xylose (Koshijima et al., 1976).

The Biosynthesis Of Lignin

Lignin is synthesized by plants from carbon dioxide through the shikimate pathway (Higuchi et al., 1977; Grisebach, 1977) as shown in figure 2. Shikimic acid is converted to L-phenylalanine which is the primary precursor of the three cinnamyl alcohols, ρ-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol.

Removal of an electron from the phenoxy anions of these alcohols generates the corresponding phenoxy radicals. This is

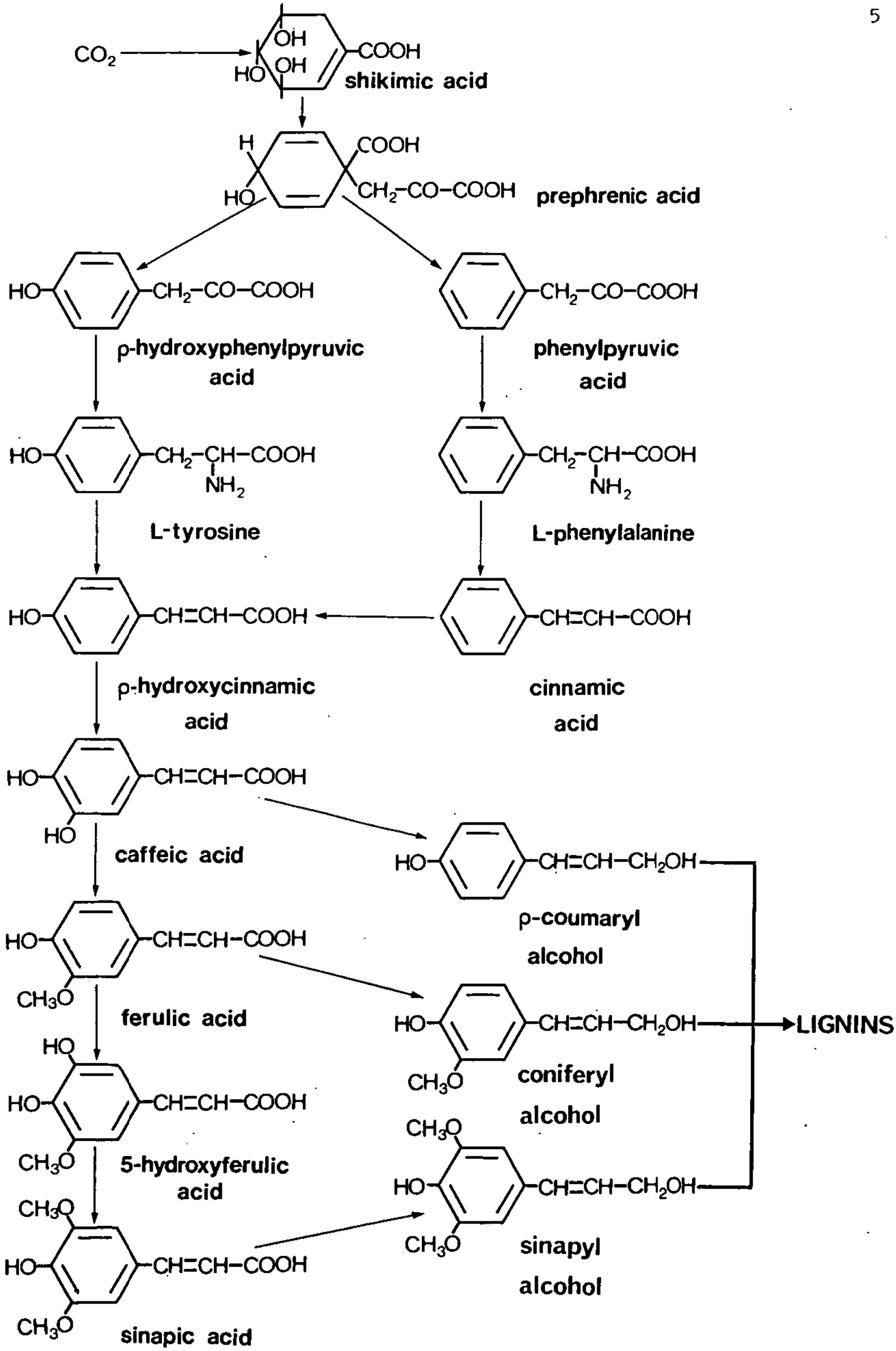

CO2
shikimic acid
prephrenic acid
p-hydroxyphenylpyruvic acid
phenylpyruvic acid
L-tyrosine
L-phenylalanine
p-hydroxycinnamic acid
cinnamic acid
caffeic acid
p-coumaryl alcohol
ferulic acid
coniferyl alcohol
5-hydroxyferulic acid
sinapyl alcohol
sinapic acid
LIGNINS

Figure 2. The Biosynthesis Of Lignins From CO_2

catalysed by phenoloxidases and is followed by spontaneous and irreversible polymerization (Harkin, 1967). The phenoloxidase initiating lignin biosynthesis is probably a peroxidase (Harkin & Obst, 1973).

The conjugated double bonds of the ring and side chain of *ρ*-hydroxycinnamyl derivatives allows delocalization of the electron and formation of a number of mesomeric structures. The specific radicals involved in the polymerization process will depend upon the relative concentrations and half-lives of each radical. The process can be repeated on the growing polymer to produce a very large, heterogenous structure. Examples of the initial stages of this process are shown in figure 3.

The proportions of each cinnamyl alcohol in a particular plant are determined by its phylogenetic origins (Sarkanen & Hergent, 1971; Higuchi, 1980; Vance et al., 1980). Lignins are often placed into one of three main groups depending on these proportions (Higuchi et al., 1977; Higuchi, 1980) :

(1) guaiacyl lignins (found in most conifers, lycopods, ferns and horsetails) which are composed mainly of coniferyl alcohol with small amounts of coumaryl and sinapyl alcohols. For example the relative proportions in spruce lignin are 80:14:6, respectively (Freudenberg et al., 1962);

(2) guaiacyl-syringyl lignins (found mainly in dicotyledonous angiosperms and some gymnosperms) which are composed of approximately equal amounts of coniferyl and sinapyl alcohols with minor amounts of coumaryl alcohol. In beech wood for example the relative proportions are 46:49:5, respectively (Freudenberg & Neish, 1968); and

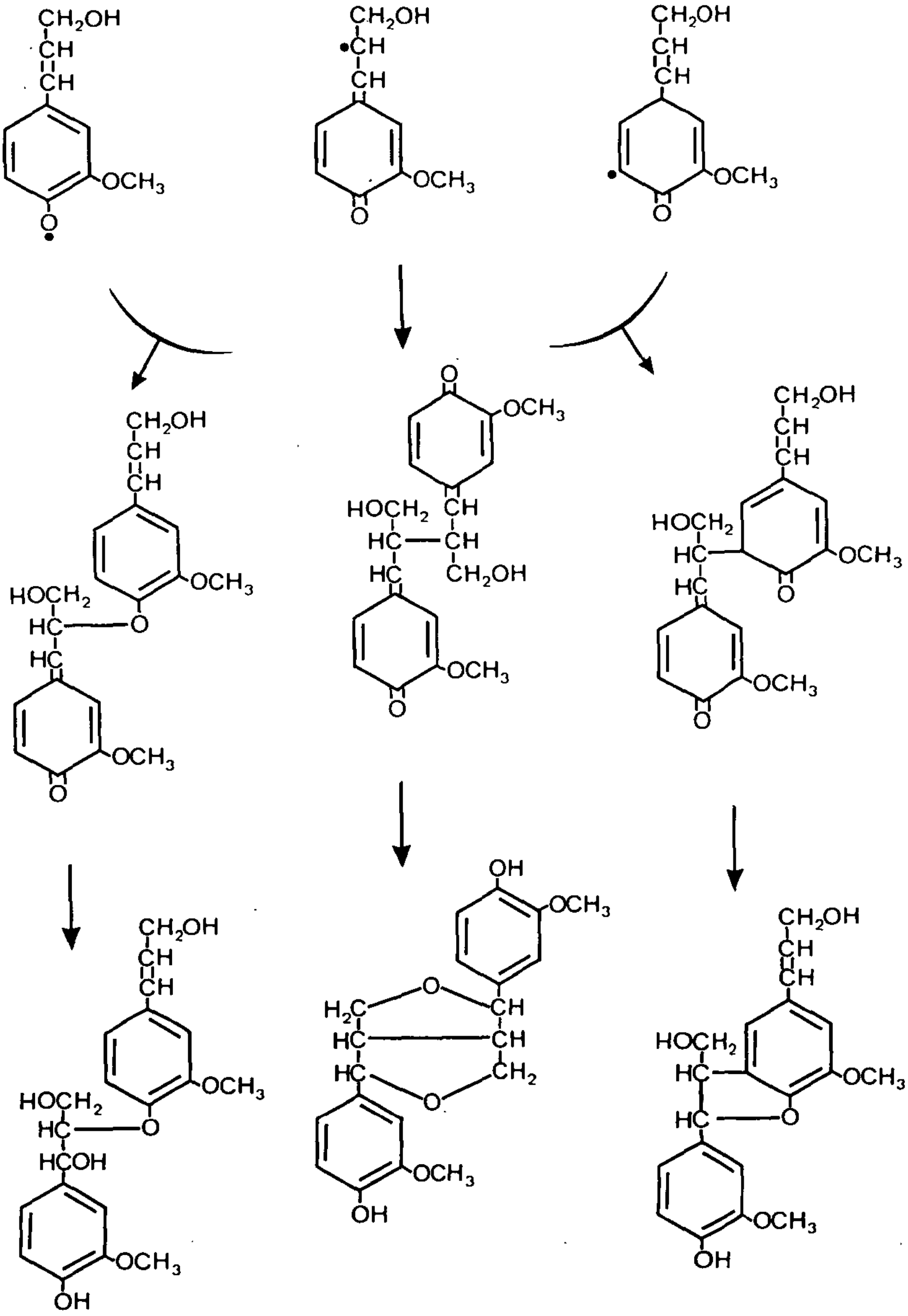

CH2OH
CH
CH
OCH3
O
OCH3
HOCH2
HC
HCOH
OH
H2C
CH2

Figure 3. Radical Coupling Of Coniferyl Alcohol During The Biosynthesis Of Lignin

(3) guaiacyl-syringyl-ρ-hydroxyphenyl lignins (found mainly in the highly evolved grasses and in the compression wood of conifers) which are composed of approximately equal amounts of all three alcohols (Higuchi et al., 1977).

The above classification of lignins has come under some criticism. The most important of these is that grass lignins contain considerable amounts of ρ-coumaric acid bound as esters (Shimada et al., 1971; Nakamura & Higuchi, 1976; Scalbert et al., 1985) and not actually incorporated into the polymer. It has been suggested that grass lignins are better classified as modified guaiacyl-syringyl lignins (Adler, 1977).

Lignin Degrading Micro-organisms

Lignin, although very resistant to chemical and biological degradation can be totally degraded by all three of the generally recognized groups of saprophytic micro-organisms. These are fungi, actinomycetes (filamentous bacteria) and eubacteria. However caution is needed in the interpretation of degradation as many more micro-organisms can cause simple modifications to the intact polmer rather than depolymerization and total conversion to carbon dioxide and water.

The wood-rotting fungi are of three major types: the 'white-rot' (Basidiomycete), the 'brown-rot' (Basidiomycete) and the 'soft-rot' (Ascomycete) fungi (Kirk, 1971; Kirk et al., 1977). A fourth group are generally considered which do not fall into these categories.

White-rot fungi can degrade lignin completely to carbon dioxide and water and are by far the most efficient degraders of lignin found to date. They can cleave $C\alpha$-$C\beta$, β-aryl ether, C1-$C\alpha$ bonds and aromatic rings and are able to oxidize α-carbons and $C\alpha{=}C\beta$ bonds as well as hydroxylating aromatic rings and demethylating methoxy groups (Ander et al., 1980; Ander et al., 1983 ; Ander et al., 1985; Buswell et al., 1979; Buswell et al., 1982 ; Buswell & Eriksson, 1979; Gupta et al., 1981; Eriksson et al., 1984; Kirk, 1983; Kuwahara et al., 1984; Tien & Kirk, 1984). Much of the research on white-rot fungi has centered around the use of Phanerochaete chrysosporium.

The brown-rot fungi can only cause a limited degradation. Brown-rot decay leads to humification (conversion of lignin into humic and fulvic acids) and demethylations, hydroxylations and some side-chain oxidations can occur. Aromatic rings are thought to remain intact and generally the carbohydrate portion of wood is removed to leave a residue of dark brown modified lignin (Kirk, 1971; Ander & Eriksson, 1978).

Soft-rot decay proceeds under moist conditions and causes a softening of the surface layer of wood with a very slow modification of lignin. This type of decay is more common in hardwood than in softwood (Ander & Eriksson, 1978). The extent of lignin degradation as measured by carbon dioxide evolution has been measured at 1 to 8% in soft-rot fungi as compared to 50 to 60% in some white-rot fungi (Ander & Eriksson, 1978; Hatakka & Uusi-Rauva, 1983).

Several bacteria have been reported to decompose lignin. These include species of Bacillus, Nocardia, Pseudomonas, Streptomyces and Xanthomonas (Crawford, 1981). However, bacteria probably play a more important role as secondary degraders which metabolise lignin substructures released by an initial fungal attack on the macromolecule. This is supported by the wealth of data showing the ability of bacteria to degrade low molecular weight aromatic compounds including lignin related monomers, dimers and other polymers (Kawakami, 1975 a ; Kawakami, 1975 b ; Fukuzumi & Katayama, 1977; Dagley, 1978; Katayama & Fukuzumi, 1978; Katayama & Fukuzumi, 1979(a); Katayama & Fukuzumi, 1979 b ; Chen et al., 1985; Dugan & Golovlev, 1985; Jokele et al., 1985; Odier & Rolando, 1985; Otuk, 1985; Pelmont et al., 1985; Sutherland, 1986).

Decomposition of the lignin macromolecule has been reported for relatively few strains of bacteria, most of these being the actinomycetes such as Streptomycete and Nocardia species (Schmidt & Bauch, 1980; Crawford, 1981; Crawford et al., 1982; Crawford et al., 1983; McCarthy & Broda, 1984). Lignin depletion rates of up to 98% in five days have been reported (Deschamps et al., 1981) but this is probably not typical.

There is evidence to show that a range of other fungi can degrade lignin but are not readily classifiable into the typical decay groups (Crawford & Crawford, 1980). This is because no other fungi of this type occur in a particular decay group or related fungi in a group do not degrade lignin in the same way. Examples of these include several Fusarium species (Higuchi, 1980; Iwahara, 1980; Sutherland et al., 1983) and Aspergillus fumigatus (Drew & Kadam, 1979; Kadam & Drew, 1986). A.fumigatus was found to be a very efficient degrader. Aspergillus and Fusarium species have been shown to degrade a range of lignin model compounds (Iyayi & Dart, 1982; Milstein et al., 1983; Nazareth & Mavinkurve, 1986).

The Use Of Model Compounds In The Study Of Lignin Biodegradation

The structure of lignin was shown earlier to be very complex. Attack on the numerous types of linkages during degradation can lead to the formation of a vast array of lower molecular weight compounds and repolymerization products. The interpretation of the many kinds of reactions which could occur to produce these intermediates is extremely difficult.

To simplify the understanding of lignin degradation, model compounds have been used by many researchers. These are based on substructures found in the macromolecule, on precursors of lignin or on degradation products.

The majority of substructure models have been monomers, dimers or deydrogenation polymers (DHP's). These are relatively low molecular weight compounds which contain aromatic ring(s) with typical lignin substituents such as methoxy, hydroxy and substituted propyl side-chains. Dimers and the less often used trimers and tetramers also contain examples of the major bond types found in lignin such as those found in table 1. DHP's are higher molecular weight polymers produced by enzymic polymerization of monomers such as coniferyl alcohol. They contain similar bonds and substituents to the monomers and dimers.

Examples of lignin biosynthesis precursors are coniferyl, sinapyl and ρ-coumaryl alcohols, cinnamic acid and ferulic acid (figure 2). The basic structures of these compounds are often still seen in the lignin macromolecule.

Products isolated from the degradation of lignin can be used as model compounds. These may be similar to the other model types and can include all the substructure and linkage features of lignin.

The examples of types of model compounds above can also be synthesized with incorporation of radiolabelled moeities. These are particularly useful in biodegradation studies to demonstrate complete metabolism to radiolabelled carbon dioxide, to help determine which bonds have been cleaved and to show aromatic ring cleavage etc.

The use of model compounds has attracted a certain amount of criticism because not all organisms isolated for their ability to degrade lignin models can degrade the macromolecule (Crawford, 1981). Conversely, known lignin degraders are generally able to decompose lignin models. A further problem is that many model compounds are low molecular weight and often water-soluble, and therefore may not allow for decomposition mechanisms involved in lignin dissimilation as lignin is a macromolecular, water-insoluble substance. Additionally, enzymes used to degrade model compounds are typically intracellular and often highly specific and these may not attack the complex extracellular lignin molecule. Because of these factors caution is needed in the interpretaton of results gained from the degradation of model compounds. However it is the use of these that has enabled lignin research to proceed so effectively over the past few years.

The General Biochemistry Of Aromatic Degradation

Molecules containing aromatic ring systems constitute a large part of the many complex organic compounds which are synthesized by biological systems (Dagley, 1975 ; Blumer, 1976; Callely, 1978). The degradation of these by micro-organisms follows many diverse routes but almost always involves the cleavage of an ortho- or para-substituted dihydric phenol (Dagley, 1978; Cain, 1980). Therefore these compounds provide foci for peripheral pathways of metabolism from a wide range of aromatic molecules.

Although evidence exists to show that dearomatization of the intact lignin polymer occurs, (Crawford, 1981) other work has shown that lignin degradation can procede via low molecular weight aromatic compounds (Chen et al., 1982). It is useful, especially when considering model compounds, to view aromatic degradations as : (i) initial reactions on the intact rings; and (ii) aromatic ring cleavage.

Initial Reactions

In lignin related compounds the ring substituents are generally methoxyl or hydroxyl groups with phenylpropanoid chains intact or oxidized to a carbonyl group. An essential step in the preparation of a monomeric intermediate for aromatic ring cleavage is removal of the methoxy group. As aryl-methoxyl ethers are resistant to hydrolytic attack most fungi and bacteria oxidize them, using a mono-oxygenase enzyme, to generate the corresponding phenol and formaldehyde or quinone and methanol (Frick & Crawford, 1983). Methyl groups substituted directly on the ring tend to remain intact and appear in the products of ring cleavage, or are oxidized to carboxyl to be lost as carbon dioxide (Cain, 1980).

Three further stages are involved in the production of dihydroxyphenols, which are susceptible to ring fission:

(1) oxidation of the C3 side-chain when phenylpropanoids from or analogous to lignin subunits are the substrates (Henderson, 1963);

(2) oxidation of alcohol and aldehyde groups, at C1 of the ring, to the carbonyl group; and

(3) Hydroxylation of monophenols to the appropriate diphenol (Ishikawa *et al.*, 1963 ; Kirk & Lorenz, 1973).

Overall, these transformations lead different lignin related aromatic compounds through converging pathways to common intermediates, as shown in figure 4.

Aromatic Ring Cleavage

With two hydroxyl groups in the correct relative positions the aromatic ring is sufficiently labile for cleavage and the variety of substrates possible at this stage means that various products can be formed, depending on the site and type of attack (Stanier & Ornston, 1973; Dagley, 1971; Chapman, 1972). Cleavage is usually initiated by a dioxygenase enzyme, a wide variety of which have been characterized (Chapman, 1972). Generally, monosubstituted aromatics are eventually catabolized via catechol and *para*-disubstituted aromatics are degraded via protocatechuic acid (Crawford, 1981; Dagley, 1975).

In 1,2-diphenols two mechanisms are possible for ring cleavage. These are the *ortho* (intradiol) cleavage or the *meta*

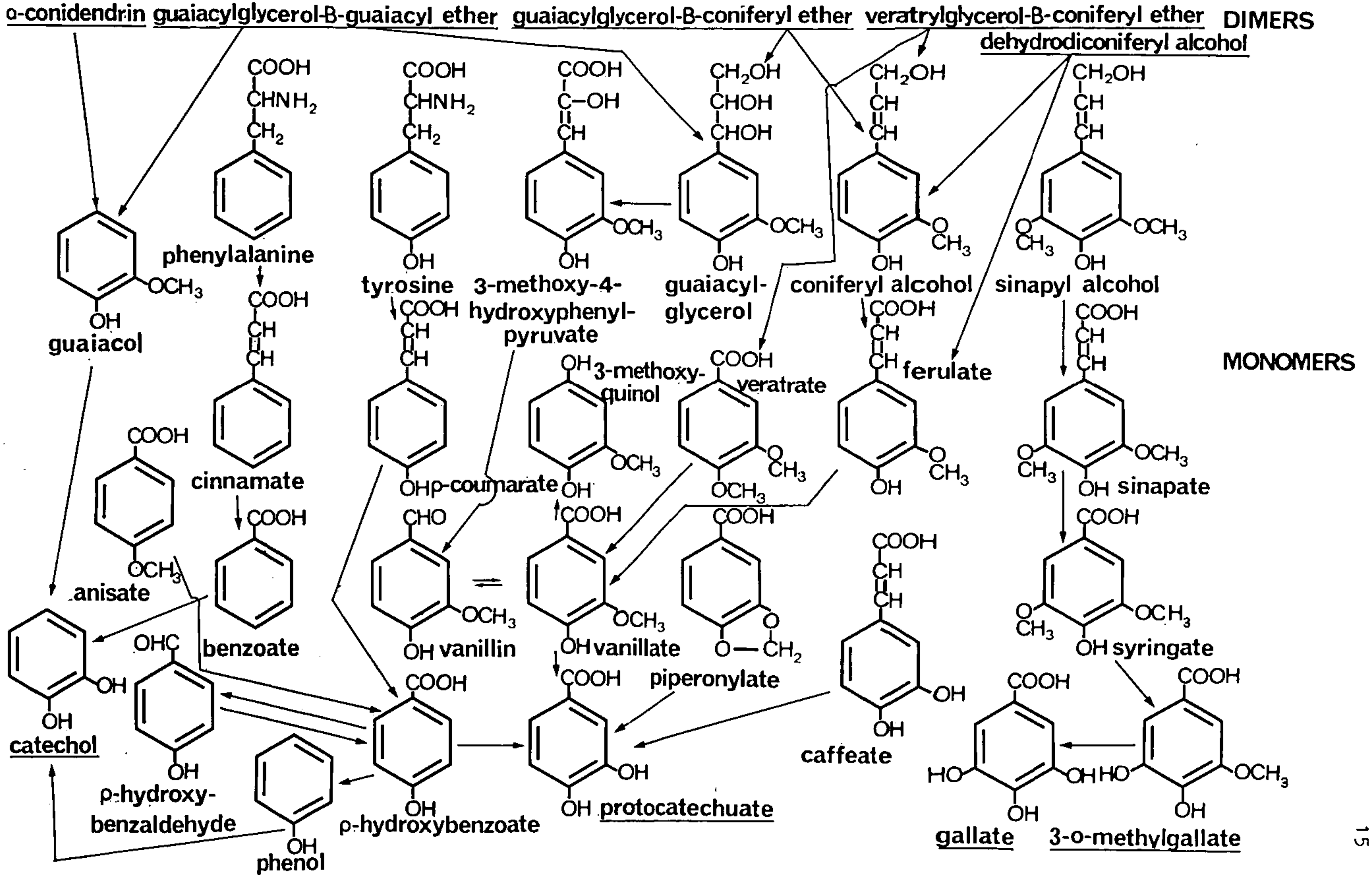

α-conidendrin
guaiacylglycerol-β-guaiacyl ether
guaiacylglycerol-β-coniferyl ether
veratrylglycerol-β-coniferyl ether
DIMERS
dehydrodiconiferyl alcohol
MONOMERS
phenylalanine
tyrosine
3-methoxy-4-hydroxyphenylpyruvate
guaiacylglycerol
coniferyl alcohol
sinapyl alcohol
guaiacol
cinnamate
p-coumarate
3-methoxyquinol
veratrate
ferulate
sinapate
anisate
benzoate
vanillin
vanillate
piperonylate
caffeate
syringate
catechol
p-hydroxybenzaldehyde
p-hydroxybenzoate
phenol
protocatechuate
gallate
3-o-methylgallate

Figure 4. Transformations Leading Different Lignin-related Aromatics Through Converging Pathways To Common Phenolic Intermediates

(extradiol) cleavage (Sugumaran & Vaidyanathan, 1978). See figure 6.

The ortho fission pathways of catechol and protocatechuic acid dissimilation are collectively known as the β-ketoadipate pathway (Stanier & Ornston, 1973). Although involving parallel chemistry, the two sequences are catalysed by physically distinct enzymes and have different intermediate compounds. Both converge on a common metabolite, β-ketoadipate enol-lactone to subsequently yield acetyl-CoA and succinate. See figure 5. It is highly probable that all bacteria and fungi use the same pathway for ortho cleavage in the conversion of catechol to β-ketoadipate (Cain, 1980).

Meta fission of catechol and protocatechuic acid in bacteria occurs via the α-keto acid pathway (Dagley, 1971; Bayley & Barbour, 1984). This results in characteristically different products to those of the β-ketoadipate pathway as shown in figure 7. Protocatechuic acid yields two molecules of pyruvate whereas catechol yields one molecule each of pyruvate and acetaldehyde. The only report of the occurrence of a meta pathway in eukaryotic micro-organisms is that of protocatechuic acid metabolism by a Penicillium species (Cain et al., 1968) but the evidence was tentative.

In the meta cleavage of 1,4-diphenols by bacteria a different cleavage mechanism is involved (Lack, 1959; Bayly & Barbour, 1984). For example in Pseudomonas ovalis, gentisate is oxidatively cleaved to form maleylpyruvate which isomerizes to fumarylpyruvate. This undergoes a hydrolytic fission to release

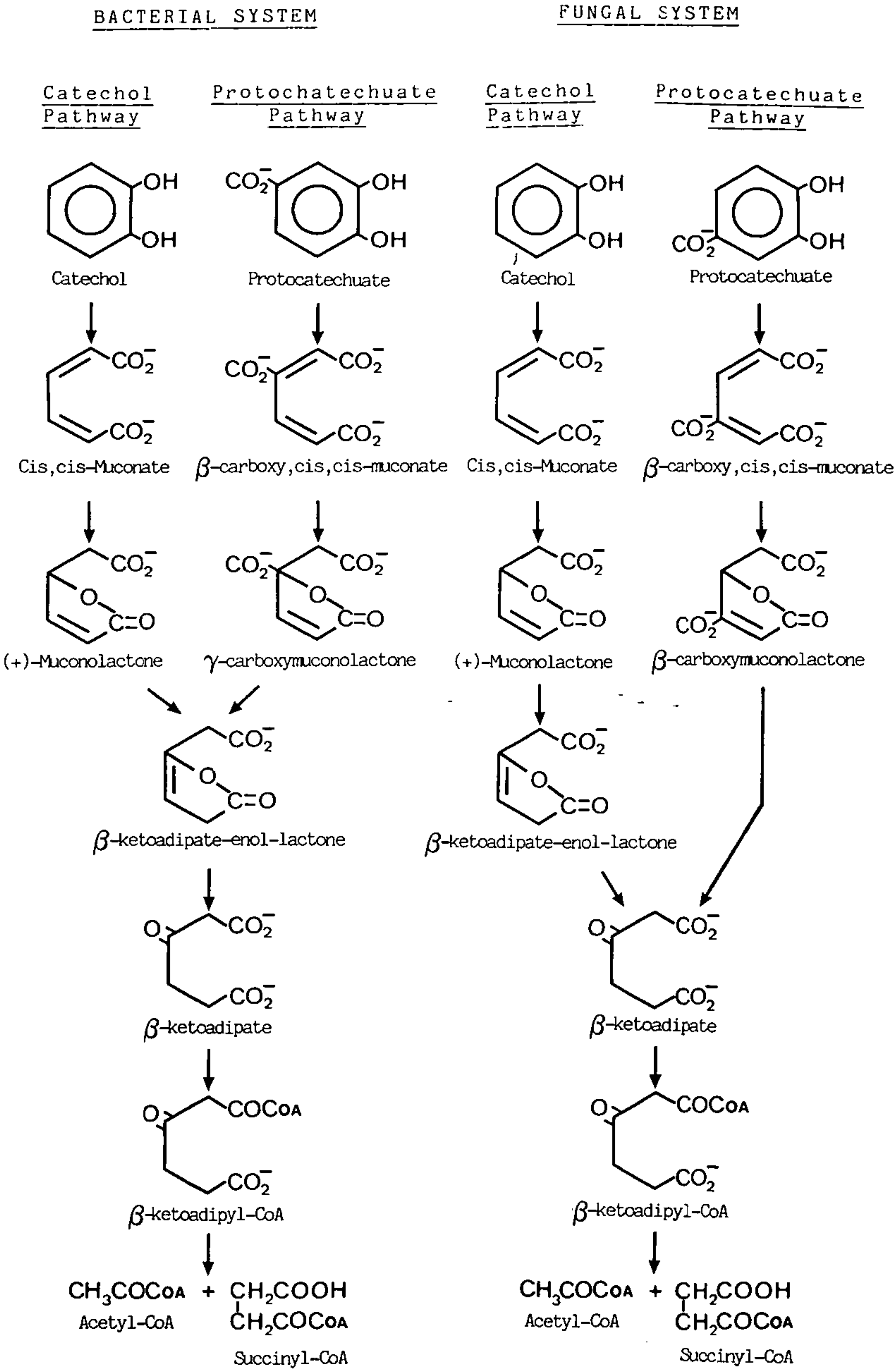

BACTERIAL SYSTEM
FUNGAL SYSTEM
Catechol Pathway
Protochatechuate Pathway
Catechol Pathway
Protocatechuate Pathway
OH
OH
Catechol
CO_2^-
Protocatechuate
Catechol
Protocatechuate
Cis,cis-Muconate
β-carboxy,cis,cis-muconate
Cis,cis-Muconate
β-carboxy,cis,cis-muconate
O
C=O
(+)-Muconolactone
γ-carboxymuconolactone
(+)-Muconolactone
β-carboxymuconolactone
β-ketoadipate-enol-lactone
β-ketoadipate-enol-lactone
β-ketoadipate
β-ketoadipate
COCoA
β-ketoadipyl-CoA
β-ketoadipyl-CoA
CH_3COCoA + CH_2COOH
CH_2COCoA
Acetyl-CoA
Succinyl-CoA
CH_3COCoA + CH_2COOH
CH_2COCoA
Acetyl-CoA
Succinyl-CoA

Figure 5. The *ortho* Pathway

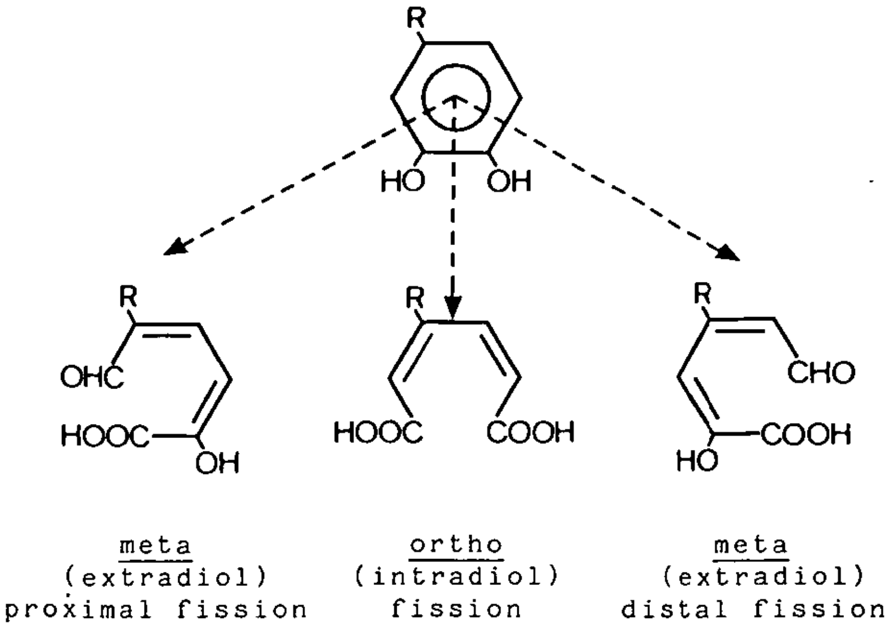

R
HO
OH
R
OHC
HOOC
OH
R
HOOC
COOH
R
CHO
HO
COOH
meta
(extradiol)
proximal fission
ortho
(intradiol)
fission
meta
(extradiol)
distal fission

Figure 6. Aromatic Ring Fission

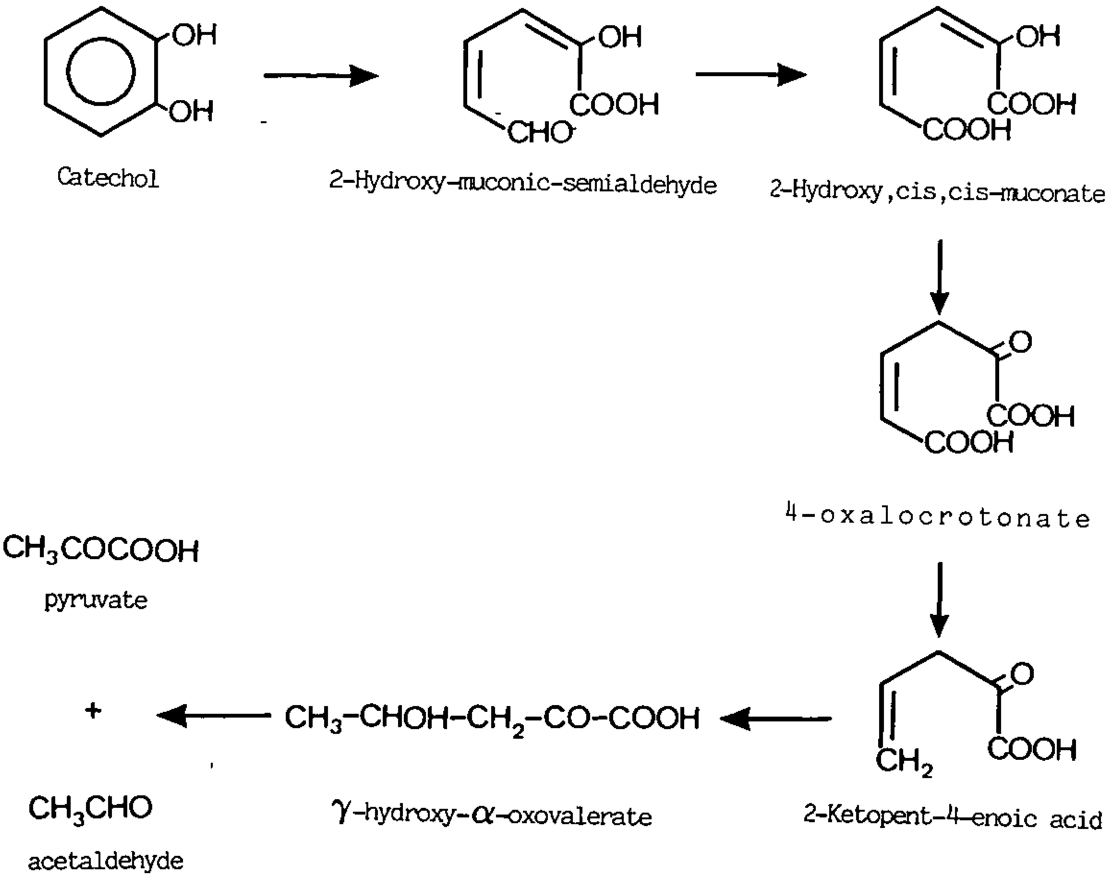

OH
OH
Catechol
OH
COOH
CHO
2-Hydroxy-muconic-semialdehyde
OH
COOH
COOH
2-Hydroxy,cis,cis-muconate
O
COOH
COOH
4-oxalocrotonate
O
COOH
CH2
2-Ketopent-4-enoic acid
CH3-CHOH-CH2-CO-COOH
γ-hydroxy-α-oxovalerate
CH3COCOOH
pyruvate
+
CH3CHO
acetaldehyde

Figure 7. The *meta* Pathway

fumarate and pyruvate.

After the stepwise degradation of aromatic compounds the final aliphatic fission products are funneled into the TCA cycle and are oxidized to carbon dioxide and water (Davis, 1961).

The Biochemistry Of Lignin Degradation

The elucidation of a 'two stage' attack on aromatic compounds has given much insight into the mechanisms underlying their degradation. However, to apply these principles to lignin degradation may not be entirely appropriate as lignin is not a simple, single ring molecule. Therefore reactions on the intact polymer must reflect the complexity of its subunit structure and internal bonding. It could be expected that many products of initial reactions would be formed, each having followed a different route from the macromolecule to the ring fission product. Some indication of this diversity was given in figure 4.

Additionally, two other important points must be considered. Firstly, different organisms may degrade lignin (or its subunits) by different mechanisms and so expand further the number of biochemical pathways. Secondly, as mentioned earlier, ring cleavage may occur on the intact polymer so that the initial reactions may be determined and limited by substituents on the ring undergoing cleavage. A speculative reaction sequence of this type devised by Crawford (1981) is shown in figure 8. In this scheme, the ring under attack is part of the macromolecule but still undergoes hydroxylation to labilize it for cleavage.

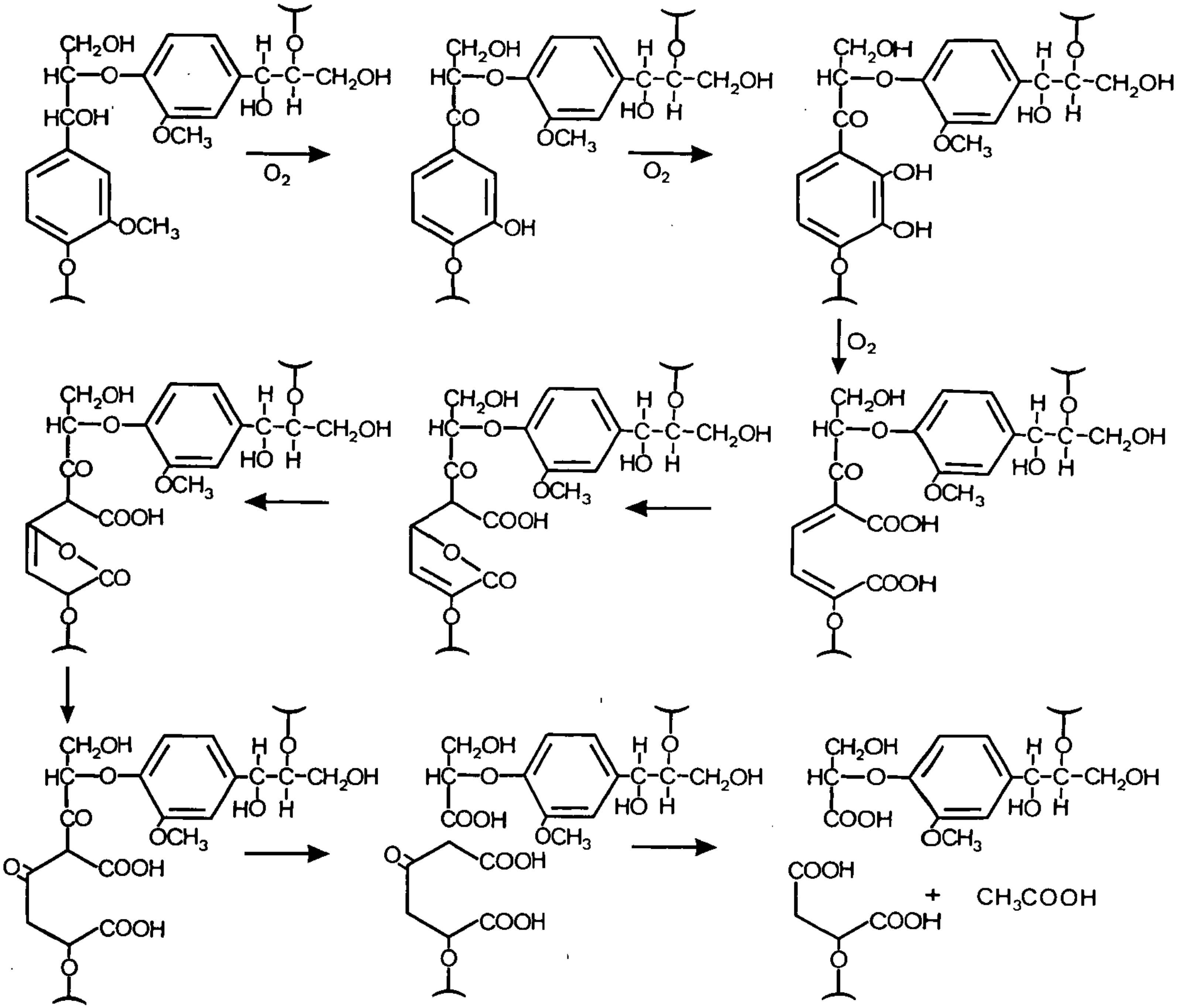


Figure 8. A Hypothetical Catabolic Sequence For The Microbial Degradation Of Lignin

Other reactions, analogous to those of the β-ketoadipate pathway result in ring fission and release of aliphatic molecules.

The elucidation of dissimilation pathways for lignin model compounds includes examples from a wide range of fungi and bacteria. Most work has been undertaken using Phanerochaete chrysosporium but the number of other micro-organisms capable of degrading lignin related compounds demonstrates two inportant points. In natural environments lignin degradation is achieved by a consortium of micro-organisms (Janshekar & Fiechter, 1983) and screening of a wide range of species is necessary to find the most efficient degraders for possible industrial applications.

Degradation Of Model Compounds Containing The Arylglycerol-β-aryl Ether Bond

Pathways for the degradation of veratrylglycerol-β-guaiacyl ether by Ph.chrysosporium and Pseudomonas acidovorans are shown in figures 9 & 10 respectively.

Veratryl alcohol and 4-ethoxy-3-methoxybenzyl alcohol have been identified as intermediates of degradation by Ph.chrysosporium (Enoki et al., 1980) suggesting cleavage of the Cα-Cβ bond. The additional identification of 2-(o-methoxyphenoxy)-1,3-propanediol as a metabolite indicates that the alkyl-phenyl bond is also cleaved. The degradative pathway of 4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether also gave results implying that Cα-Cβ cleavage is prefered to cleavage of the β-aryl ether linkage (Umezawa & Higuchi, 1985). However,

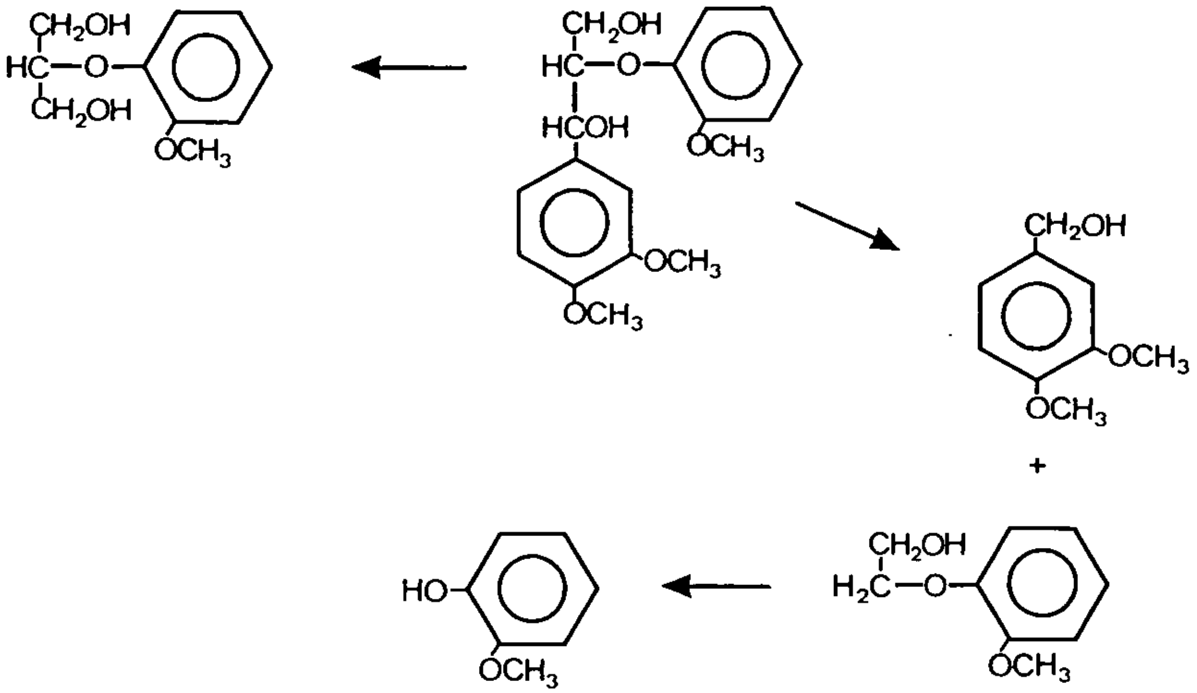


Figure 9. The Degradation Of Veratrylglycerol-β-guaiacyl Ether By The White-rot Fungus Phanerochaete chrysosporium

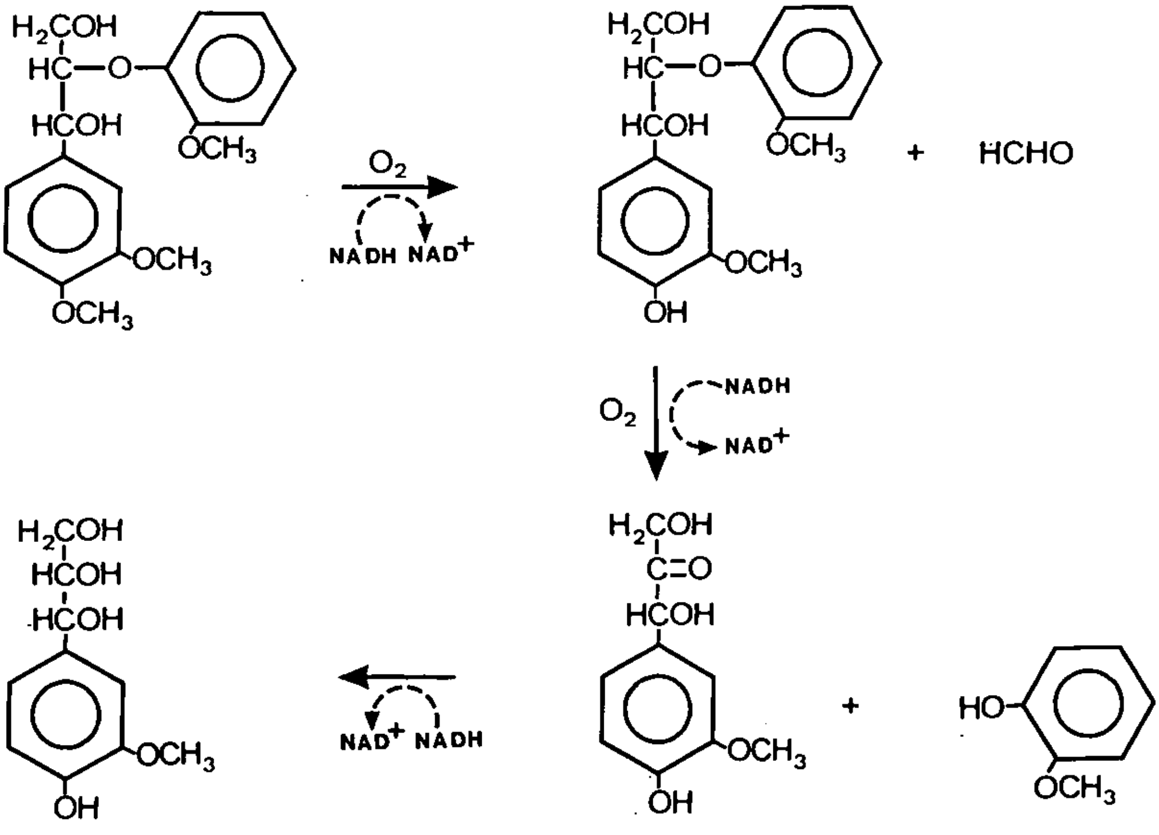

O2
NADH
NAD+
HCHO
O2
NADH
NAD+
NAD+
NADH

Figure 10. The Oxidative Cleavage Of Veratryl-glycerol-β-guaiacyl Ether By An Enzyme Preparation From The White-rot Fungus Poria subacida

Fukuzumi et al.(1969) observed oxidative cleavage of the β-aryl ether bond in the fungus Poria subacida when an enzyme preparation was used.

The proposed degradation of veratrylglycerol-β-guaiacyl ether by P.acidovorans showed a different position of cleavage to that in Ph.chrysosporium (Crawford et al., 1975). The β-aryl ether bond was cleaved and together with other examples showed that this type of cleavage may be prefered in bacteria (Fukuzumi, 1980). A recent exception to this was a Cα-Cβ cleavage by Pseudomonas cepacia (Odier & Rolando, 1985).

Degradation of Model Compounds Containing The 1,2-Diarylpropane Substructure

Ph.chrysosporium can cleave The Cα-Cβ linkage of 1-(3',-4'-diethoxyphenyl-1,3-dihydroxy-2-(4''-methoxyphenyl)-propane (Enoki & Gold, 1982) as shown in figure 11. A related compound, dimethoxyhydrobenzoin, was cleaved in a similar way by a ligninase preparation from this organism (Hammel et al., 1985).

Figure 12 shows that a different type of cleavage occurs when α-veratryl-β-guaiacylpropionic acid is degraded by Pseudomonas putida (Katayama & Fukuzumi, 1979a). This compound was converted to vanillic acid, veratraldehyde and α,β-di-guaiacylpropionic acid indicating an oxidative cleavage not involving the Cα-Cβ bond.

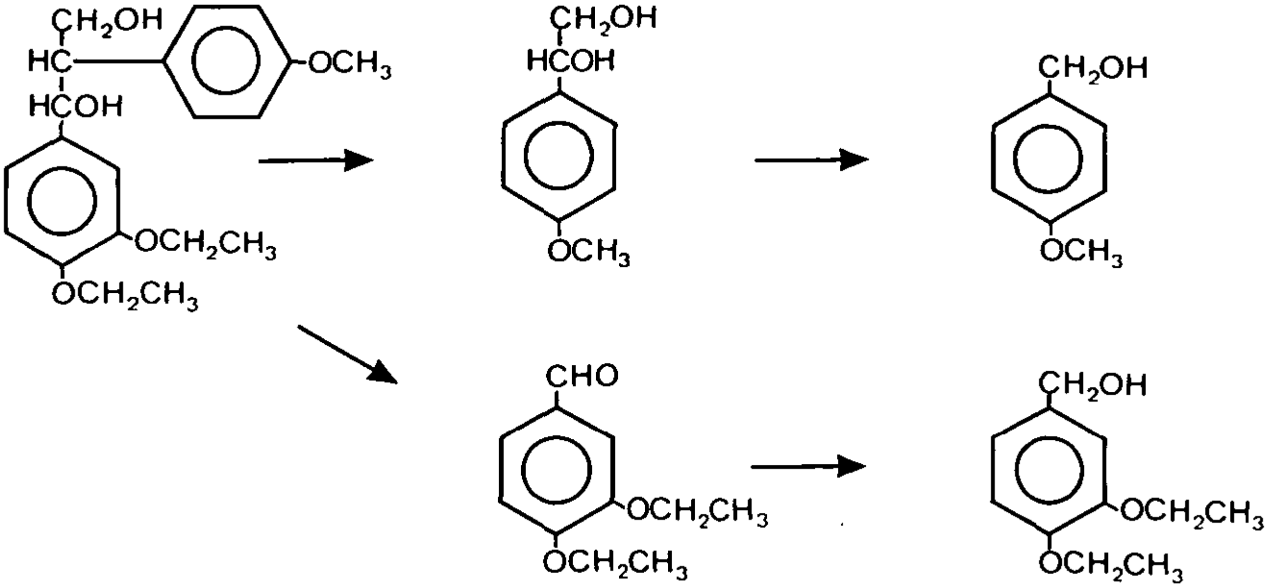


Figure 11. Degradation Of 1-(3',4'-Diethoxyphenyl)-1,3-dihydroxy-2-(4''-methoxyphenyl)-propane By Ph. chrysosporium

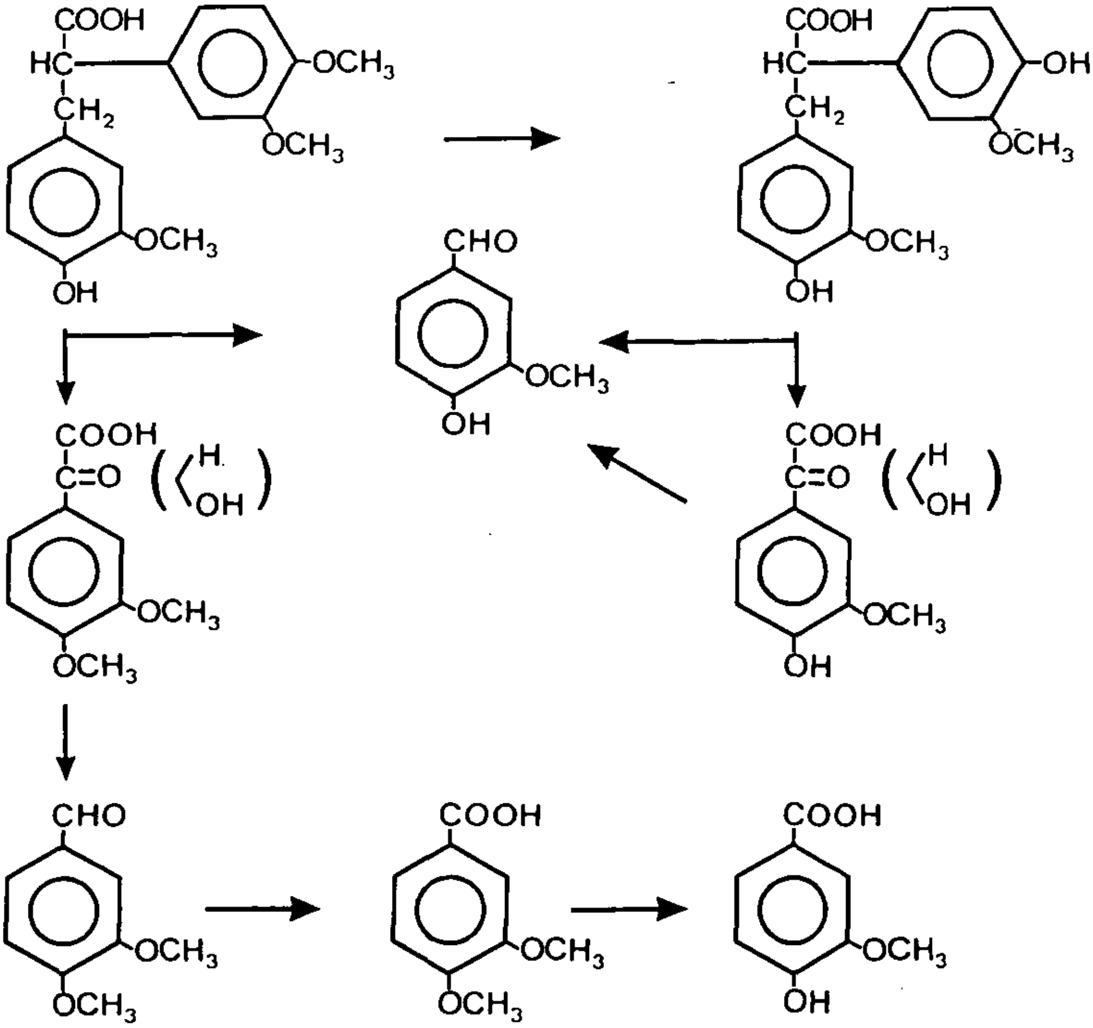


Figure 12. The Degradation Of α-veratryl-β-guaiacylpropionic acid By Pseudomonas putida

Degradation of Model Compounds Containing The Phenylcoumaran Substructure

Metabolism of these type of compounds has been demonstrated and cleavage of the coumaran ring was apparent in both fungi and bacteria. *Fusarium solani*, a fungus not readily classifiable into a specific decay group (Ohta *et al.*, 1979) and *Pseudomonas putida* (Katayama & Fukuzumi, 1978) have been shown to degrade dehydrodiconiferyl alcohol (figures 13 & 14).

Degradation Of Biphenyl Substructure Models

Pseudomonas putida rapidly assimilates dehydrodivanillic acid to produce a mixture of 5-carboxy vanillic acid and vanillic acid with a di-keto butyric acid group attached at the *meta* position (Fukuzumi, 1980) (Figure 15). Certain Streptomyces strains have been found to degrade dehydrodivanillin (Crawford, 1981) and an alkalophilic bacterium (strain KS-104) was also able to metabolize this compound using a similar route to the degradation of dehydrodivanillic acid by *Ps.putida* (Kawakami & Shumiya, 1983).

Degradation Of Monomeric Lignin Model Compounds

Although a very wide range of monomeric lignin models have been used the simple methoxylated aromatic acids, veratric acid and vanillic acid have probably been used the most frequently. These are usually demethylated by micro-organisms to yield protocatechuic acid which undergoes dioxygenase catalysed ring

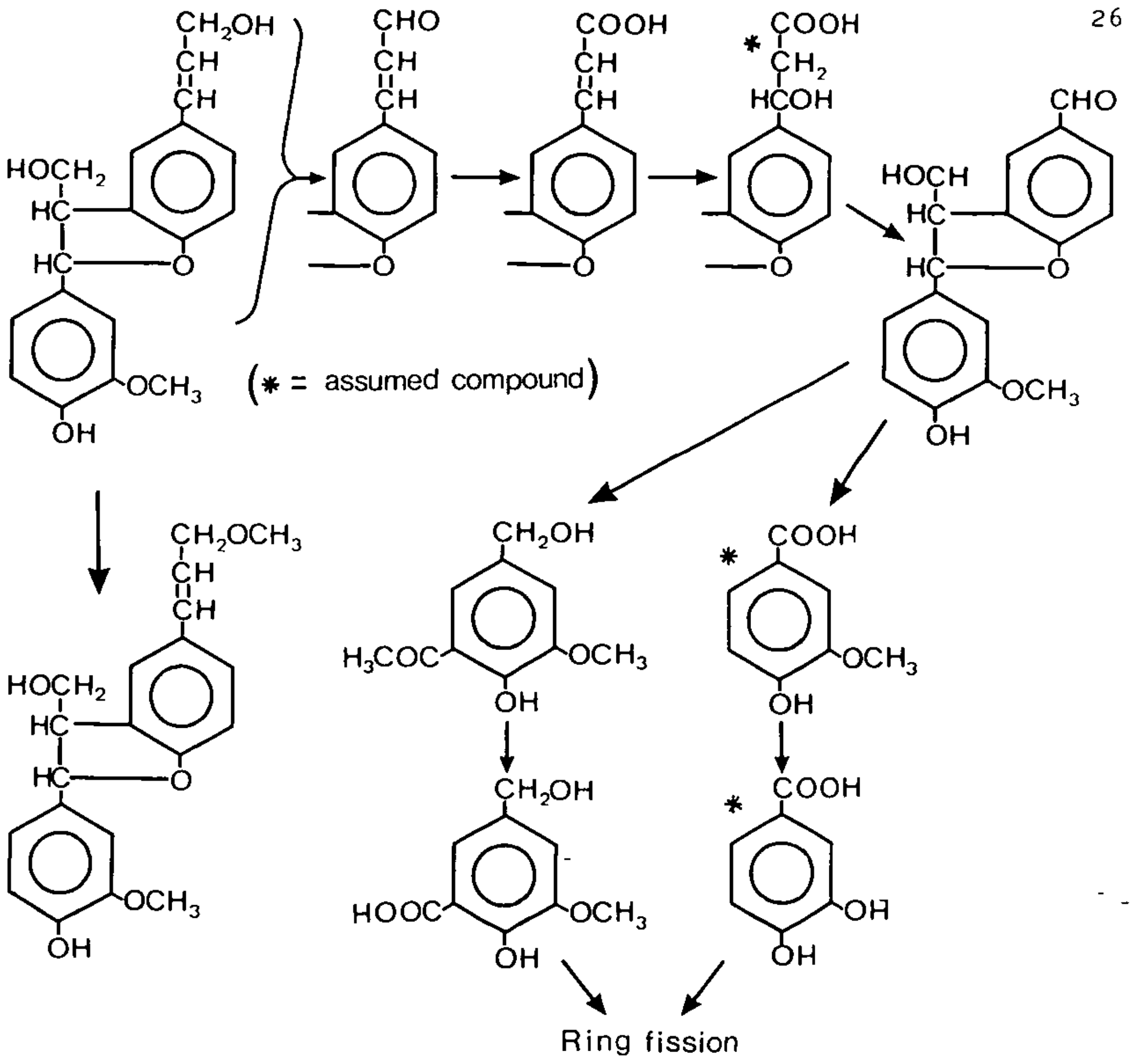

(* = assumed compound)
Ring fission

Figure 13. Catabolism Of Dehydrodiconiferyl Alcohol By F. solani

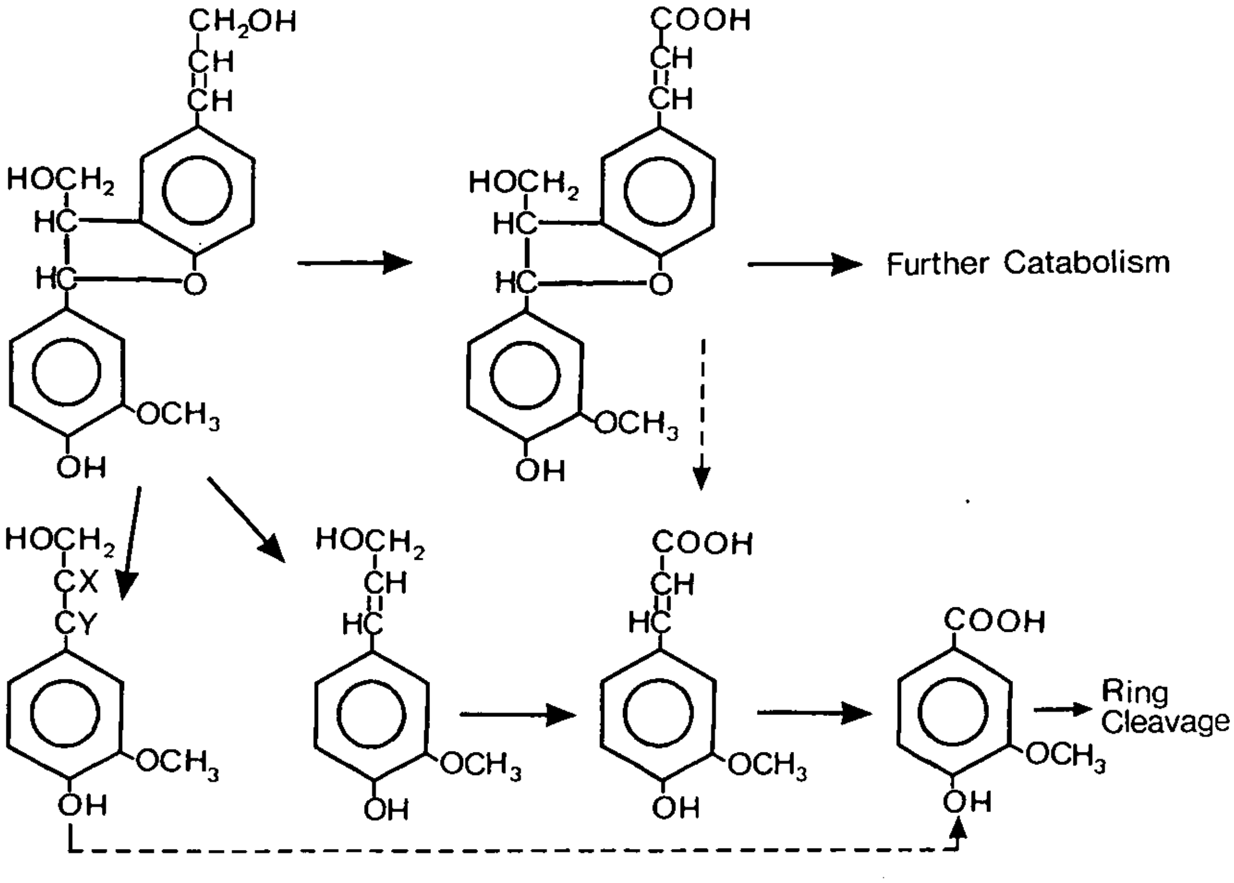

Further Catabolism
Ring Cleavage

Figure 14. Catabolism Of Dehydrodiconiferyl Alcohol By Ps. putida

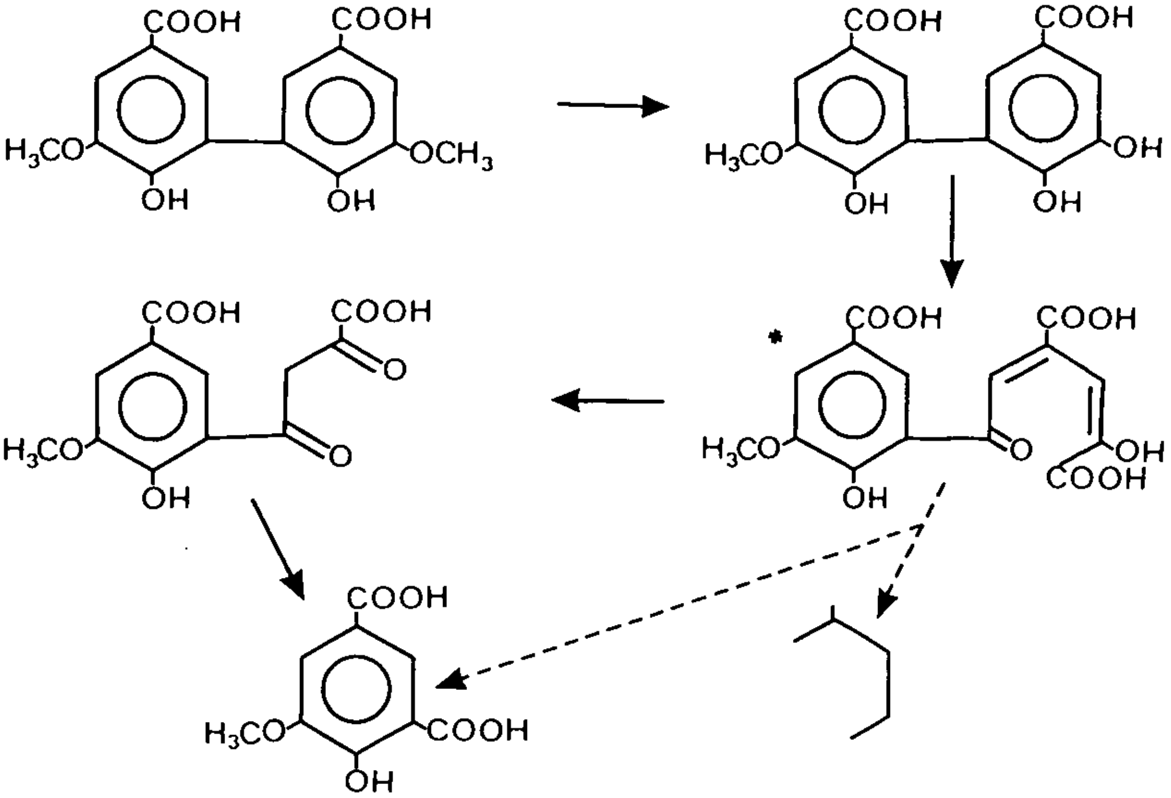
fission (Flaig & Haider, 1961; Crawford et al., 1973; Crawford et al., 1975; Kawakami, 1976) although an unusual oxidative decarboxylation of vanillate has been shown (Kirk & Lorenz, 1973). Some of the known transformations of vanillate and veratrate are summarized in figure 16.

The Enzymology Of Lignin Degradation

Relatively few enzymes have been implicated in lignin degradation (Paterson et al., 1984). The major enzyme groups thought to play a role in dissimilation of the macromolecule are laccases, aromatic alcohol oxidases or dehydrogenases, cellobiose:quinone oxidoreductase, and the recently characterized ligninases and peroxidases.

Laccases

These enzymes are copper-containing phenol oxidases that require molecular oxygen but no coenzyme and most fungal laccases exhibit maximal activity in the pH range 4 to 5.5 (Sjoblad & Bollag, 1981). Laccases generally have a wide specificity for the reducing substrate but this must contain a free phenolic group (Ishihara, 1980). They are members of groups of enzymes which can cause oxidative coupling reactions such as those involved in the synthesis of soil humic substances, tannins, melanins and alkaloids. In coupling reactions of laccases the enzymes catalyse the oxidation of phenols by removing an electron and a proton to form a free radical. The


COOH
COOH
H_3CO
OCH_3
OH
OH
COOH
COOH
H_3CO
OH
OH
OH
*
COOH
COOH
H_3CO
O
OH
OH
COOH
COOH
COOH
O
H_3CO
O
OH
COOH
H_3CO
COOH
OH

* = Hypothetical Structure

Figure 15. A Scheme For The Degradation Of Dehydrodivanillic Acid By *Ps. putida*

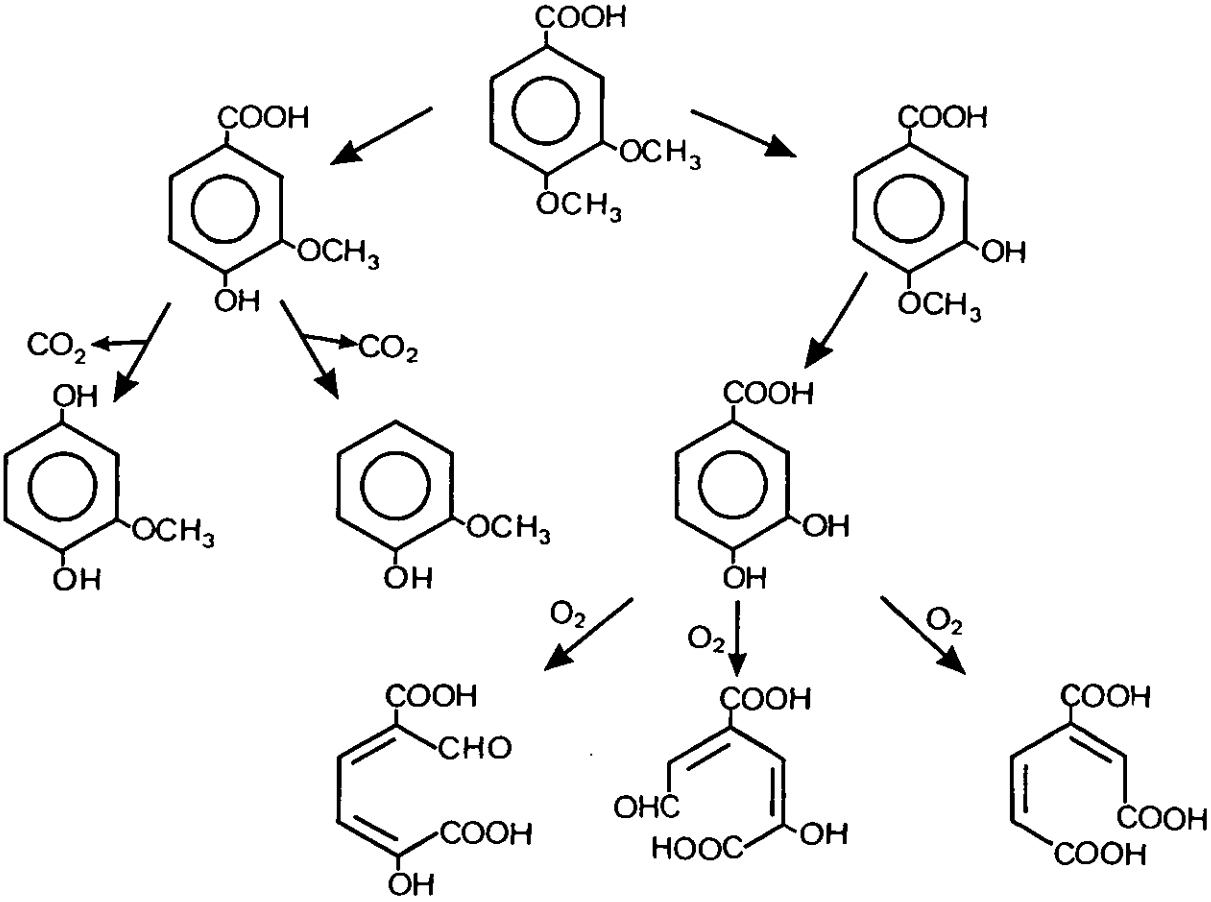

COOH
OCH_3
OCH_3
COOH
OCH_3
OH
COOH
OH
OCH_3
CO_2
CO_2
OH
OCH_3
OH
OCH_3
OH
COOH
OH
OH
O_2
O_2
O_2
COOH
CHO
COOH
OH
COOH
OHC
HOOC
OH
COOH
COOH
COOH

Figure 16. Microbial Transformations Of Veratric & Vanillic Acids

radicals spontaneously polymerize. (Haars & Huttermann, 1980; Sjoblad & Bollag, 1981; Evans, 1985).

The role of phenol oxidases in lignin degradation is controversial. It is known that levels of the enzyme can be elevated when fungi are grown on lignin (Arora & Sandhu, 1985) and lignin degrading micro-organisms are generally phenol oxidase positive (Sundman & Nase, 1971). Ander & Eriksson (1976), using mutants of Sporotrichum pulverulentum lacking phenol oxidase, showed that in this species they are needed for degradation. Evidence has indicated that cleavage of alkylphenyl C-C bonds and α,β C-C bonds can be catalysed by these enzymes (Goldsby et al., 1980; Kamaya et al., 1981; Katayama et al., 1981). A laccase preparation from Coriolus versicolor was able to remove hydrogen from the α and β positions of DHP side-chains (Noguchi et al., 1980). The cleavage of the ether bond of a lignin model compound has also been demonstrated (Kirk et al., 1968). Additionally, phenol oxidases have been shown to achieve demethylation of ring methoxy groups. This results in the formation of phenolic functions which are important in the labilizing of aromatic rings and can be accompanied by liberation of methanol (Ander et al., 1983; Leonowicz & Bollag, 1984; Lundquist & Kristersson, 1985). It has recently been suggested that a phenoloxidase was involved in the metabolism of lignin model compounds by Pseudomonas cepacia as the organism was unable to catabolize non-phenolic dimers but no radical species were found (Odier & Rolando, 1985). Phenoloxidase activity is generally thought to be absent from bacteria (Fukuzumi & Katayama, 1977).

Results which do not support the role of phenoloxidases in the degradation of lignin are also available.Leisola & Fiechter (1985) found mutants lacking the enzyme could not degrade lignin. Evans (1985) showed that although depolymerization of milled wood lignin by laccase could occur in the presence of H_2O_2 in vitro, this reaction was not the function of the enzyme in vivo. Many workers have shown that polymerization may or may not occur coupled to the depolymerization of lignin and lignin degradation products (Huettermann et al., 1980; Janshekar & Fiechter, 1983; Leonowicz et al., 1985). This would support the suggestion of laccase as a coupling mediator. Such coupling reactions may be of major importance in the formation of humic acids. Polymerization may also serve as a means of detoxification of poisonous compounds (Janshekar & Fiechter, 1983; Shuttleworth & Bollag, 1986).

Cellobiose:quinone Oxidoreductase

Cellobiose:quinone oxidoreductase is an enzyme produced by many white-rot fungi (Westermark & Eriksson, 1974). It catalyses the oxidation of cellobiose to cellobiono-δ-lactone with concomitant transfer of the two electrons to various quinones or phenoxy radicals, in particular, those produced in lignin by phenol oxidases. This mechanism could provide a cycle involving a phenol oxidase and cellobiose:quinone oxidoreductase to transfer electrons from cellobiose to oxygen or H_2O_2 with phenols (including lignin) acting as carriers (Ander & Eriksson, 1978). Cellobiose is the major extracellular product from white-rot mediated cellulose degradation (figure 17).

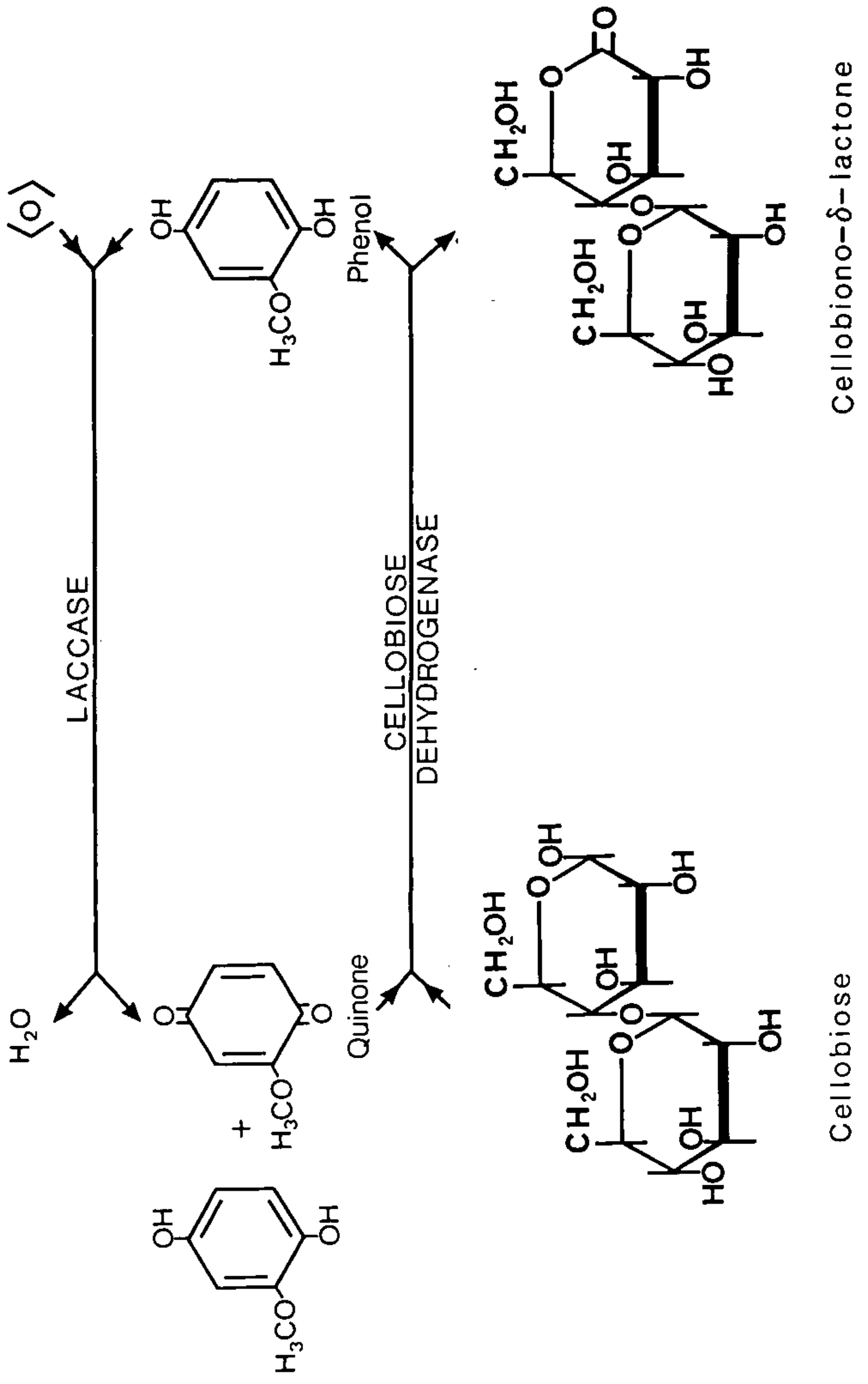

LACCASE
CELLOBIOSE DEHYDROGENASE
⟨O⟩
H_2O
Phenol
Quinone
+
Cellobiose
Cellobiono-δ-lactone

Figure 17. The Cellobiose:Quinone Oxidoreductase/Laccase Cycle

Aromatic Alcohol Oxidases or Dehydrogenases

The oxidation state at the α-carbon of aromatic rings is thought to influence the cleavage of the β-linkage between monomeric units of lignin (Fenn & Kirk, 1984). Aromatic alcohol oxidases or hydrogenases acting upon the side-chains of lignin-related compounds have been described in species of Fusarium, Polystictus, Rhodococcus and Pseudomonas (Farmer et al., 1959; Eggeling & Sahm, 1980; Iwahara et al., 1980; Pelmont et al., 1985). The fungal enzymes oxidize aromatic alcohols to aldehydes whilst reducing molecular oxygen to H_2O_2. The enzyme isolated from the Fusarium species shows oxygen uptake when using milled-wood lignin as substrate (Paterson et al., 1984).

Ligninases And Peroxidases

It has been shown recently that aerobic culture of Ph.chrysosporium produces two groups of extracellular H_2O_2-dependent, haem enzymes (Kuwahara et al., 1984; Glenn & Gold, 1985; Paszczynski et al., 1986). These are lignin peroxidases (ligninase, diarylpropane oxygenase) and Mn(II) peroxidases.

Three different molecular forms of ligninase have been identified (Renganathan et al., 1985). The main isozymic form has a MW of 41000 and all forms have a single iron protoporphyrin IX prosthetic group (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985). Ligninase is able to cleave bonds in lignin and can cause other oxidative transformations of lignin and lignin model compounds (Gold et al., 1984; Tien & Kirk, 1984; Kersten et al., 1985; Renganathan et al., 1985; Schoemaker et al., 1985).

Ligninase is dependent on H_2O_2 and is able to incorporate dioxygen into the products. It was therefore originally considered to be a unique peroxide dependent oxygenase (Tien & Kirk, 1984; Gold et al., 1984). However, more recent studies indicate that oxygenation proceeds even under anaerobic conditions with incorporation of ^{18}O from H_2O_2 into the products (Renganathan et al., 1986).

The enzyme is now thought to function as a peroxidase with a redox potential high enough to oxidize a methoxylated aromatic derivative to the corresponding radical cation (Tien et al., 1986). The radical cations can degrade without further enzyme involvement to yield the products typical of the enzymic process (Schoemaker et al., 1985; Harvey et al., 1985a). Hammel et al. (1985) found that carbon-centered and peroxyl radicals at $C\beta$ are major products of $C\alpha$-$C\beta$ cleavage by ligninase. Stable radical cations have been produced which can act as good oxidants and extract electrons from insoluble lignin at some distance from the active site of the enzyme (Harvey et al., 1986).

Ligninase can oxidize many substrates directly but it can also oxidize compounds indirectly. This indirect oxidation is mediated by veratryl alcohol. This may mean the surface of fungal hyphae could be the location of the ligninases, in the vicinity of sources of H_2O_2, and veratryl alcohol could be the agent causing oxidation of lignin at a distance from the active site of the enzyme (Harvey et al., 1986) (figure 18). Charge transfer might also occur between aromatic rings of the same lignin molecule and it may be this which could assist in depolymerization at a site distant to that of the initial

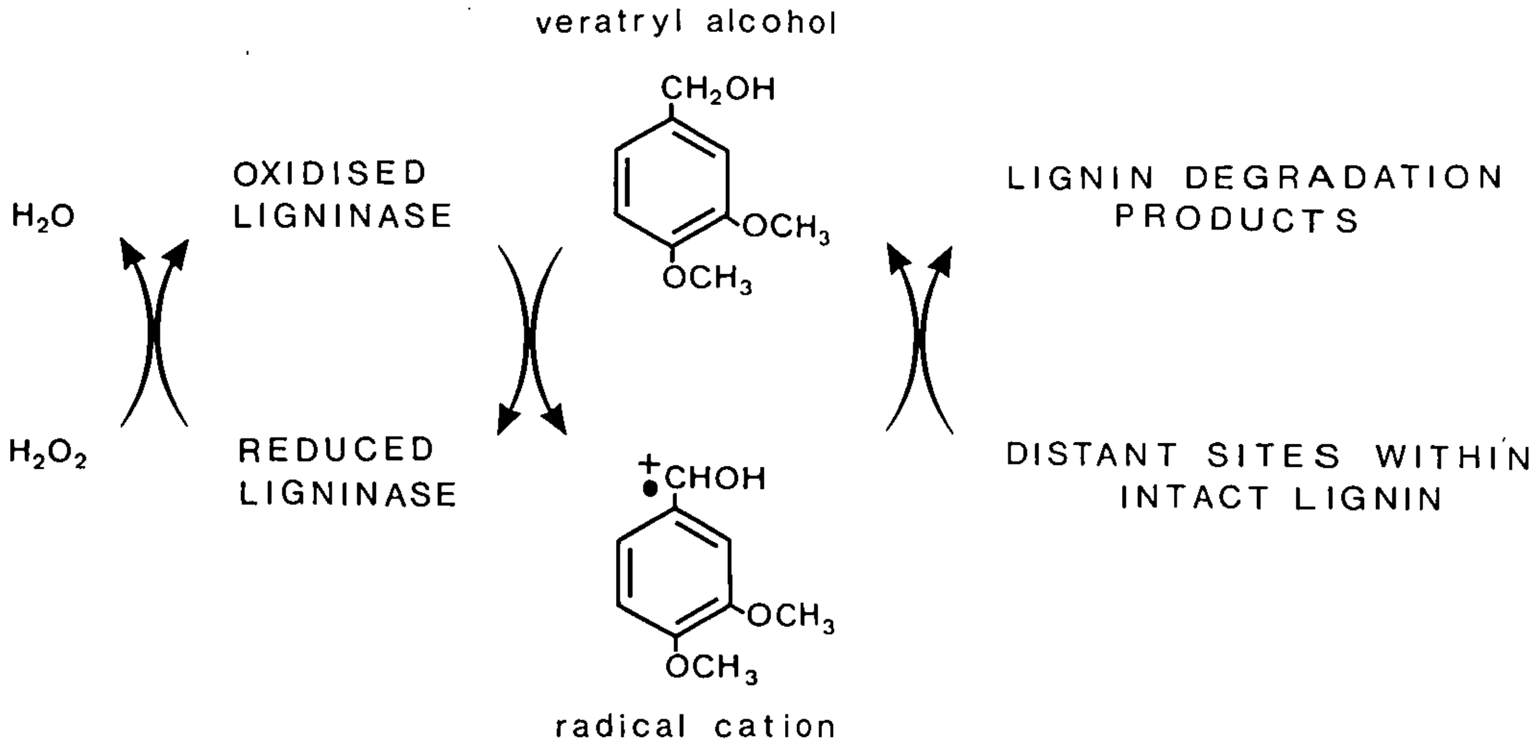

veratryl alcohol
CH₂OH
OCH₃
OCH₃
H₂O
OXIDISED LIGNINASE
LIGNIN DEGRADATION PRODUCTS
H₂O₂
REDUCED LIGNINASE
DISTANT SITES WITHIN INTACT LIGNIN
+•CHOH
OCH₃
OCH₃
radical cation

Figure 18. The Role Of Veratryl Alcohol As A Mediator Of Lignin Biodegradation

oxidation.

The second group of extracellular enzymes includes two or more Mn-dependent peroxidases whose role in lignin degradation is less clear (Kuwahara et al., 1984; Huynh & Crawford, 1985; Paszczynski et al., 1985). These act like classical peroxidases and can oxidize low-molecular weight phenols to form phenoxy radicals. They can also catalyse reactions such as H_2O_2-independent oxidations of compounds like NADPH and glutathione (Paszczynski et al., 1985). The peroxidase-M2 of Paszczynski et al. (1986) can also oxidize GSH (reduced glutathione) to GSSG (oxidized glutathione), DTT (dithiothreitol) to its disulphide derivative and dihydroxymaleic acid to its diketo derivative. The transformations required Mn^{2+} and the protons and electrons removed from the oxidized substrate were accepted by oxygen to yield H_2O_2 (figure 19). The H_2O_2 produced may become available to ligninase enzymes.

A glucose-dependent H_2O_2 production has been found in ligninolytic cultures of Ph. chrysosporium (Kelley & Reddy, 1986) and indications of glucose oxidase activity providing the H_2O_2 were found. The ultrastructural localization of H_2O_2 production in this organism was shown to be in the periplasmic space. Forney et al. (1982) suggested that by this arrangement the organism is able to avoid high levels of H_2O_2 in the cytoplasm. However the enzymes responsible for the production of H_2O_2 can be retained by the cells. The H_2O_2 could readily diffuse from the cells to undergo reductive cleavage producing •OH which is known to be involved in lignin degradation (Crawford & Crawford, 1984). A schematic representation of a

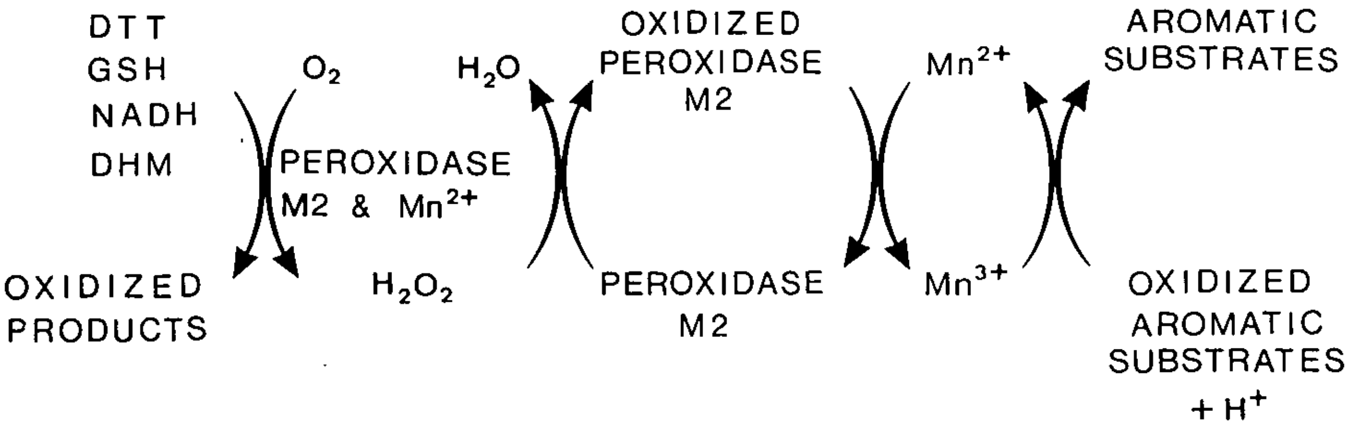

DTT
GSH
NADH
DHM
O2
PEROXIDASE M2 & Mn2+
OXIDIZED PRODUCTS
H2O2
H2O
OXIDIZED PEROXIDASE M2
PEROXIDASE M2
Mn2+
Mn3+
AROMATIC SUBSTRATES
OXIDIZED AROMATIC SUBSTRATES + H+

Figure 19. Transformations Mediated By Peroxidase-M2

DTT = Dithiothreitol

GSH = Reduced Glutathone

DHM = Dihydroxymaleic Acid

possible ligninolytic system is shown in figure 20.

It is interesting to compare the limited information on bacterial lignin and lignin model degraders with the above data for fungi. For example •OH scavenging agents do not inhibit the degradation of lignin by Streptomyces viridosporus but they do cause inhibition in Ph.chrysosporium indicating that actinomycetes may have different mechanisms for lignin degradation (Leisola & Fiechter, 1985). The ligniolytic bacterium (X.sp.99) found by Kern *et al.* (1984) however, produces H_2O_2 and this may indicate similarities between some bacterial and fungal ligninolytic systems.

Physiological Aspects Of Lignin Degradation

Secondary Carbon Source

It is thought to be a general rule that the white-rot fungi require an additional easily metabolizable carbon source to degrade lignin (Keyser *et al.*, 1978; Kirk *et al.*, 1978; Eriksson, 1981; Leatham, 1986). This requirement is unusual because lignin is potentially one of the most abundant sources of microbial carbon and energy (Kirk *et al.*, 1978). Possible explanations are that the energy obtained from lignin metabolism is too small to support growth or the level of ligninolytic activity is too low to support growth. The cost in terms of energy to degrade lignin may be higher than that recovered.

The role of a co-substrate is not universal. Fusarium solani has been shown to degrade lignin without a secondary carbon source (Norris, 1980) and Aspergillus japonicus degraded

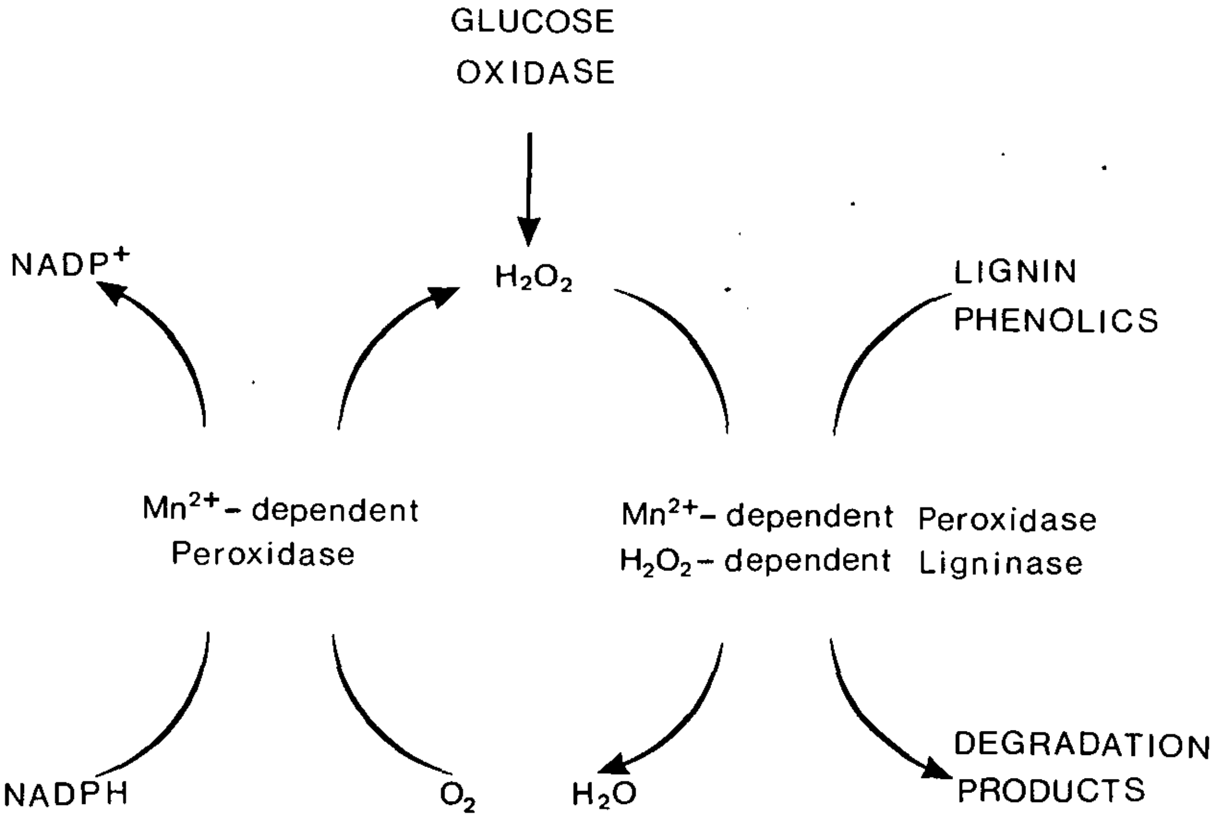

GLUCOSE OXIDASE
H_2O_2
$NADP^+$
LIGNIN PHENOLICS
Mn^{2+}- dependent Peroxidase
Mn^{2+}- dependent Peroxidase
H_2O_2- dependent Ligninase
NADPH
O_2
H_2O
DEGRADATION PRODUCTS

Figure 20. Relationship Between Enzymes Of The Ligniolytic System

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a lignocarbohydrate complex in the absence of additional carbon sources (Milstein et al., 1981). A strain of Streptomyces degraded milled wood lignin without a carbon supplement (Barder & Crawford, 1981) and gram-negative bacteria have been reported to degrade poplar lignin extracted with dioxane (Odier et al., 1981).

Nitrogen

Ph. chrysosporium develops ligninolytic activity as a response to nitrogen limitation (Keyser et al., 1978). Kirk & Fenn (1982) have concluded that this organism probably has a nitrogen-based regulatory system to control ligninolytic activity. The mechanism of this regulation seems to involve the level of intracellular cyclic AMP (Broda et al., 1983). Addition of glutamate to ligninolytic cultures suppressed lignin degradation and a rapid decrease in intracellular cAMP was observed. High protein turnover rates were measured by Fenn & Kirk (1981) at the onset of ligninolytic activity, reflecting the change from primary to secondary metabolism after depletion of nutrient nitrogen.

The increase in ligninolytic activity in response to nitrogen limitation is not universal. In other fungi there is no correlation between these two factors and the bacterium Streptomyces bodius shows increased ligninolytic ability with high levels of nitrogen (Janshekar & Fiechter, 1983).

Other Regulatory Factors

Ligninase activity is influenced by incubating cultures with enzyme substrates (Faison & Kirk, 1985). Veratryl alcohol was found to give the largest increase in activity of the enzyme. Leisola *et al.* (1984) and Ulmer *et al.* (1984) have reported that veratryl alcohol stimulates lignin degradation to CO_2 by *Ph. chrysosporium*. It is possible that veratryl alcohol protects and stabilizes the enzyme against inactivation or proteolytic decay (Haemmerli *et al.*, 1986). However Faison *et al.* (1986) suggested that this was not the case, rather the veratryl alcohol functions via an induction type of mechanism affecting only certain ligninase enzymes. Veratryl alcohol is a secondary metabolite of *Ph. chrysosporium* being synthesized from glucose (Lundquist & Kirk, 1978). It is then slowly degraded. Shimada *et al.* (1981) suggested that 3,4-dimethoxycinnamyl alcohol is the biosynthetic product and that this is degraded to veratryl alcohol. The relationship between the enhancement of glucose oxidation, H_2O_2 production and ligninase activity in the presence of veratryl alcohol is shown in figure 21.

Summary

To summarize, lignin is a high molecular weight polymer of variable structure. Its degradation, which is highly complex, is carried out *in vivo* by a consortium of micro-organisms of different types, using a plethora of different reactions many of which have not yet been adequately identified and categorized.

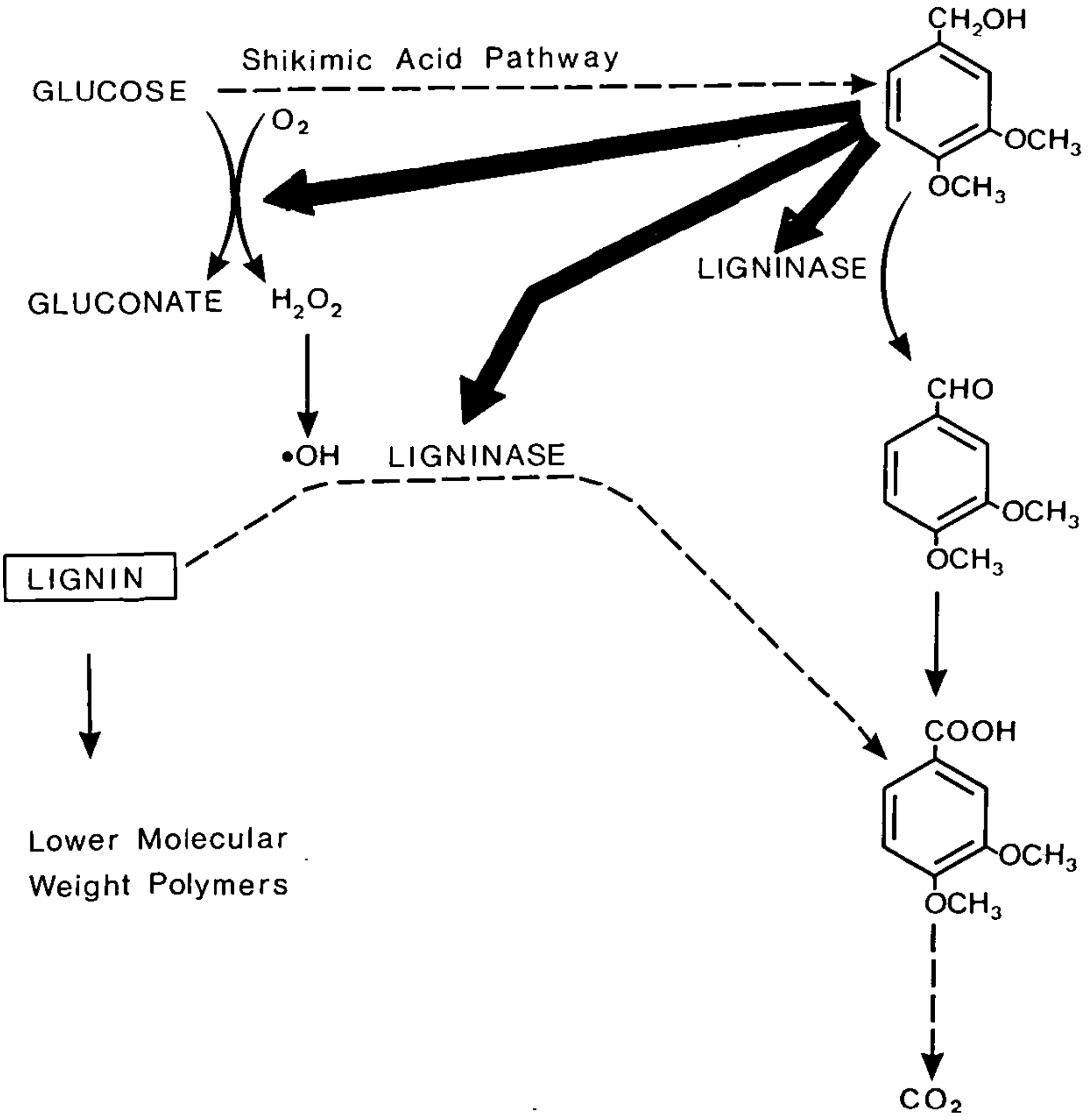

Shikimic Acid Pathway
GLUCOSE
O_2
GLUCONATE
H_2O_2
•OH
LIGNINASE
LIGNINASE
LIGNIN
Lower Molecular Weight Polymers
CH_2OH
OCH_3
OCH_3
CHO
OCH_3
OCH_3
COOH
OCH_3
OCH_3
CO_2

Figure 21. The Role Of Veratryl Alcohol In Lignin Degradation

(Large Arrows Show Points Of Activation)

MATERIALS

AND

METHODS

CHEMICALS

Unless specified in the text, chemicals were standard laboratory grade and supplied from the following companies.

Aldrich Chemical Company Limited, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4JL, U.K.

Sigma Chemical Company Limited, Fancy Road, Poole, Dorset, BH17 7NH, U.K.

Lancaster Synthesis Limited, Eastgate, White Lund, Morecambe, Lancs., LA3 3DY, U.K.

Fisons Limited, Bishop Meadow Road, Loughborough, Leics., U.K.

^{14}C-Sodium acetate solution (250 μCi) labelled in either the C1 or C2 position was obtained from:

The Radiochemical Centre Limited, Amersham International, Amersham, U.K.

Autohydrolysis aspen lignin was a gift from:

Professor M. Wayman, Department of Chemical Engineering & Applied Chemistry, University of Toronto, Canada.

SYNTHESIS OF MODEL COMPOUNDS

General Experimental Procedures

Melting points were uncorrected and were obtained using a Gallenkamp melting point apparatus.

Infra-red spectra were recorded on a Perkin-Elmer SP200 spectrophotometer. Proton-nmr spectra were measured on a Perkin-Elmer R32 nmr spectrometer at 90MHz and ^{13}C-nmr spectra were recorded on a Bruker WP-80 nmr spectrometer at 20,1 MHz. High resolution mass spectra were recorded on a Kratos MS 50/DS-55 mass spectrometer.

Analytical thin layer chromatography (TLC) was performed on SiO_2 plates (Merk, Kieselgel ,600, 0.25 mm thick). The spots were visualized by spraying the plates with 50% aqueous H_2SO_4 followed by heating at 110°C for 5 minutes. Column chromatography was carried out on SiO_2 (Merk, Kieselgel 40, 70-230 mesh ASTM). The solvent system for TLC and column chromatography was Benzene: Ethyl acetate, 9:1.

α-Bromoacetophenone.(1).

This compound was produced using a modification to the method of Landucci et al. (1981). Nitrogen was bubbled slowly through a solution of acetoveratrone (1.89 g) in CCl_4 (100 ml) in a separating funnel. The nitrogen flow was temporarily disconnected and bromine (1.23 g) was added to the solution. This was then shaken until a precipitate formed (approximately 60 seconds). The nitrogen flow was reconnected and the sides of the reaction vessel were washed with $CHCl_3$ (12 ml). The solution became a clear, yellow/red colour after approximately 30 minutes, at which time the nitrogen flow was stopped. The solution was neutralized with 50-60 ml of saturated $NaHCO_3$ solution. The organic phase was then separated, dried over anhydrous $MgSO_4$ and filtered. Evaporation of the solvents under reduced pressure left a pink solid which was washed several times with cold methanol to give yellow crystals of α-bromoacetoveratrone. These were dried under nitrogen. Yield 65%; mp 72-77°C (lit mp 73-77°C).

Synthesis of 3,4-Dimethoxy-ω-(2-methoxyphenoxy)-acetophenone.(2).

This was synthesized using a modification to the method of Adler et al. (1952). A mixture of α-bromoacetoveratrone (1) (3.6 g), guaiacol (3.6 g) and anhydrous potassium carbonate (3.6 g) in dry acetone (20 ml) was refluxed for 45 minutes with vigorous stirring. The acetone fraction was then collected by vacuum filtration. This was diluted with an equal volume of distilled water and extracted three times with 100 ml volumes of $CHCl_3$. The chloroform fraction was collected using a separating funnel and was then shaken with 100 ml of 2N NaOH solution. This was repeated until all excess guaiacol was removed as shown by TLC. The chloroform fraction was washed several times with water, separated and dried over anhydrous sodium sulphate. Subsequent filtration, removal of the chloroform under reduced pressure and recrystallization from methanol gave white crystals. Yield 76%; mp 88-90°C [lit. mp 90-92°C]; ir γ max 3090, 2915, 1685, 1590 cm^{-1}; H-nmr [$CDCl_3$] δ 7.65 (m, 3H, aromatic), 6.90 (m, 4H, aromatic), 5.30 (s, 2H, CH_2-O), 3.95 (s, 3H, OMe), 3.94 (s, 3H, OMe), 3.88 (s, 3H, OMe); ms m/z 302.1167.

Synthesis of 1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)-ethanol.(3).

Sodium borohydride (0.15 g) was added to a suspension of the ketone (2) (3 g) in absolute ethanol (20 ml). This was shaken at room temperature. The ketone dissolved after a short time and after a further 20 minutes a yellow precipitate formed. Shaking was continued until TLC showed that none of compound (2) remained. The resulting suspension was diluted with distilled water, extracted three times with $CHCl_3$ and dried over anhydrous Na_2SO_4. Chloroform was removed under vacuum to leave a pale yellow solid which was recrystallized from methanol to form white crystals. Yield 93%; mp 126-127°C ; ir max 3500, 2940, 1590 cm^{-1}; H-nmr [$CDCl_3$] 7.00 (m, 7H, aromatic), 5.15 (d, 1H, H-C-), 4.25 (o, 2H, CH_2-O), 4.02 (s, 3H, OMe), 4.00 (s, 3H, OMe), 3.98 (s, 3H, OMe), 2.45 (s, 1H, OH); ms m/z 304.1316

Synthesis of 4-Benzyloxy-3-methoxyacetophenone.(4).

A modification to the method of Leopold, (1950) was used to protect acetovanillone in the 4-position. A mixture of acetovanillone (1.2 g), benzyl bromide (1.26 g) and potassium carbonate (1.0 g) in acetone (30 ml) were refluxed, with vigorous stirring, for 3 hours. The reaction mixture was filtered under vacuum to remove the potassium carbonate. Evaporation of the acetone under vacuum and recrystallization from ethanol gave bright yellow crystals of (4). Yield 96%; mp 85-88°C [lit. mp 85-87°C].

Synthesis of 4-Benzyloxy-3-methoxy-ω-bromoacetophenone.(5).

This was prepared from 1.79 g of 4-benzyloxy-3-methoxyacetophenone dissolved in CCl_4 (100 ml) with subsequent addition of bromine (1.2 g) and $CHCl_3$ (12 ml) according to the procedure used to obtain (1). The white crystals formed were washed several times with cold methanol. Yield 70%; mp 100-103°C [lit. mp 98-102°C].

Synthesis of 4-Benzyloxy-3-methoxy-ω-(2-methoxyphenoxy)-acetophenone.(6).

This was prepared by refluxing for 4 hours, with vigorous stirring, a mixture of 4-benzyloxy-3-methoxy-ω-bromoacetophenone (1.62 g), guaiacol (0.67 g) and potassium carbonate (0.6 g) in dry acetone (8 ml) according to the procedure of Kratzl *et al.* (1959). The reaction mixture was subjected to the same treatment to remove excess guaiacol as described in the synthesis of (2). The brown oil remaining was recrystallized from ethanol to leave light yellow crystals. These were dried under nitrogen in a dessicator. Yield 91%; mp 96-101°C [lit. mp 96-101°C]; ir γ max 3070, 2930, 1685, 1590 cm^{-1}; H-nmr [$CDCl_3$] δ 7.62 (m, 3H, aromatic), 7.40 (m, 5H, aromatic), 6.98 (m, 4H, aromatic), 5.25 (s, 2H, CH_2-O-), 5.15 (s, 2H, CH_2-O-), 3.85 (s, 3H, OMe), 3.80 (s, 3H, OMe); ms m/z 378.1474.

Synthesis of 1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-methoxy-phenoxy)ethanol.(7).

Compound (7) was prepared from compound (6) (3 g) using a similar procedure to that used to produce (3). A golden oil was produced. This was purified by column chromatography to give a

bright yellow oil. After recrystallization from ethanol white crystals remained. Yield 91%; mp 67-68°C; ir γ max 3530, 2920, 1585 cm^{-1}; H-nmr [$CDCl_3$] δ 6.82 (m, 12H, aromatic), 5.10 (s, 2H, Ph-CH_2-O), 5.05 (q, 1H, H-C-), 4.02 (o, 2H, CH_2-O), 3.85 (s, 3H, OMe), 3.81 (s, 3H, OMe), 3.60 (s, 1H, C-OH); ms m/z 380.1620.

Synthesis of 4-Hydroxy-3-methoxyphenyl-ω-(2-methoxyphenoxy)-acetophenone.(8).

A mixture of the ketone (3.42 g) and 10% palladium on charcoal (0.6 g) in aqueous tetrahydrofuran (7.5 ml H_2O in 110 ml THF) was shaken vigorously under hydrogen (30 lb/in^2) at room temperature until a pressure drop to 15 lb/in^2 was recorded (approximately 10 minutes). The mixture was filtered to remove the catalyst and dried twice over anhydrous magnesium sulphate. After filtering, the THF was removed by evaporating under vacuum to leave a yellow oil which showed 2 spots on TLC. This was purified by column chromatography to give a light yellow oil showing one spot on TLC. Recrystallization from diethyl ether produced white crystals. Yield 87%; mp 88-89°C; ir γ' max 3010, 2940, 3150, 1660, 1585 cm^{-1}; H-nmr [$CDCl_3$] δ 7.65 (m, 3H, aromatic), 6.90 (m, 4H, aromatic), 6.22 (s, 1H, phenolic OH), 5.30 (s, 2H, CH_2-O-), 3.96 (s, 3H, OMe), 3.90 (s, 3H, OMe); ms m/z 288.1002:

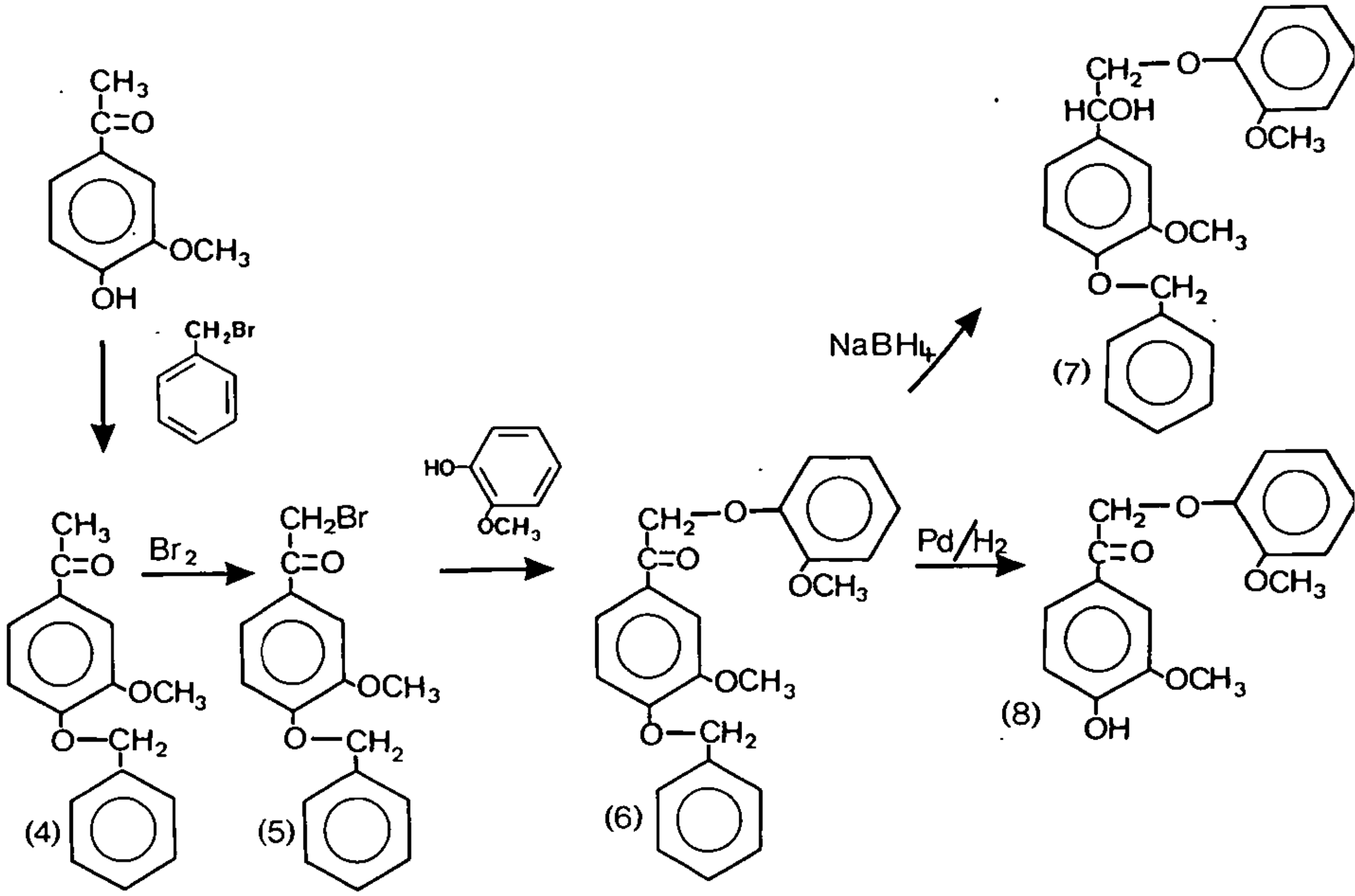

CH3
C=O
OCH3
OH
CH2Br
Br2
HO
OCH3
NaBH4
Pd/H2
(4)
(5)
(6)
(7)
(8)
CH2—O
HCOH
O—CH2

Figure 22. The Synthesis Of Lignin Model Compounds 6, 7 & 8 Containing The Guaiacyl Moiety

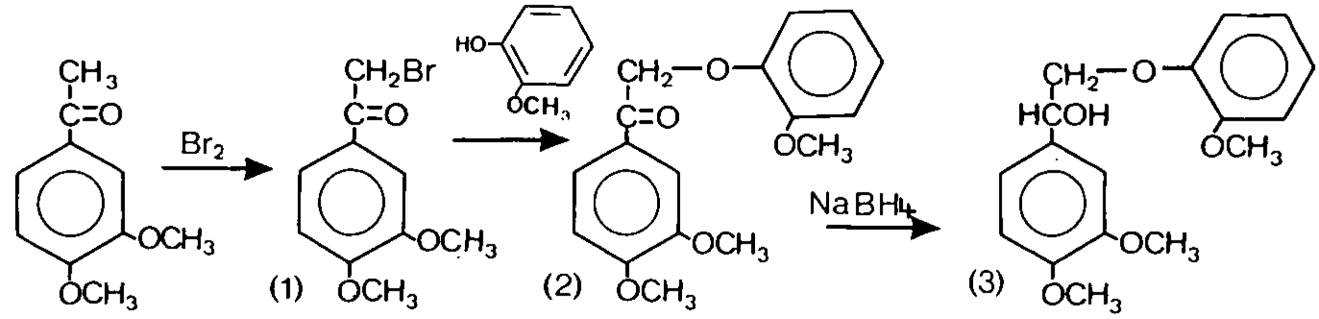

CH3
C=O
OCH3
Br2
CH2Br
HO
NaBH4
(1)
(2)
(3)
CH2—O
HCOH

Figure 23. The Synthesis Of Lignin Model Compounds 2 & 3 Containing The Guaiacyl Moiety

Synthesis of 3,4-Dimethoxy-ω-(2-phenylphenoxy)acetophenone.(9).

This was prepared using a similar method to that for the preparation of (2). A mixture of α-bromoacetoveratrone (3 g), 2-phenylphenol (1.97 g) and potassium carbonate (1.11 g) in dry acetone (15 ml) were refluxed for 4 hours. The acetone fraction was then collected by vacuum filtration and the acetone evaporated under reduced presure. The remaining solid was recrystallized twice from methanol and washed with cold methanol to leave white flakes of (9). Yield 87%; analytical TLC gave one spot Rf 0.43; mp 82-83°C; ir γ max 3100, 2980, 1685, 1590 cm^{-1}; H-nmr [$CDCl_3$] δ 7.54 (m, 12H, aromatic), 5.2 (s, 2H, CH_2-O), 3.95 (s, 3H, OMe), 3.85 (s, 3H, OMe); ms m/z 348.1358.

Synthesis of 1-(3,4-Dimethoxyphenyl)-2-(2-phenylphenoxy)ethanol.(10).

This was synthesized according to the procedure used to produce (3) and (7). Sodium borohydride (0.15 g) was added to a suspension of the ketone (9) (3 g) in absolute ethanol (20 ml). The previously described extraction left a light brown oil which was purified by column chromatography to leave (10) as a very pale green oil. Yield 97%; analytical TLC gave one spot Rf 0.24; decomposition at 200°C (1 mm Hg); ir γ max 3500, 3010, 2930, 1590 cm^{-1}; H-nmr [$CDCl_3$] δ 7.43 (m, 12H, aromatic), 4.82 (q, 1H, H-C-), 4.00 (o, 2H, CH_2-O), 3.80 (s, 3H, OMe), 3.74 (s, 3H, OMe), 2.64 (d, 1H, C-OH); ms m/z 350.1512.

Synthesis of 4-Benzyloxy-3-methoxy-ω-(2-phenylphenoxy)-acetophenone.(11).

Compound (11) was prepared from a mixture of 4-benzyloxy-3-methoxy-ω-bromoacetophenone (3 g), 2-phenylphenol (1.526 g) and potassium carbonate (1.11 g) in dry acetone (15 ml) according to the method used to produce (9). The yellow solid formed was recrystallized twice from ethanol and dried under nitrogen to give light yellow needles of (11). Yield 84%; analytical TLC gave one spot Rf 0.62; mp 82-83°C; ir γ max 3040, 2980, 1680, 1585 cm^{-1}; H-nmr [$CDCl_3$] δ 7.45 (m, 17H, aromatic), 5.28 (s, 2H, CH_2-O), 5.2 (s, 2H, CH_2-O), 3.90 (s, 3H, OMe); ms m/z 424.1678.

Synthesis of 1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-phenyl-phenoxy)ethanol.(12).

This was prepared from (11) (3 g) using the same procedure as the synthesis of (10) from (9). A light golden oil resulted. Yield 95%; analytical TLC gave one spot Rf 0.31; decomposition at 245°C (1.5 mm Hg); ir γ max 3515, 3020, 2912, 1590 cm^{-1}; H-nmr [$CDCl_3$] δ 7.25 (m, 17H, aromatic), 5.50 (s, 2H, Ph-CH_2-O), 4.76 (q, 1H, H-C-), 4.00 (o, 2H, CH_2-O), 3.76 (s, 3H, OMe), 2.54 (d, 1H, C-OH); ms m/z 426.1828.

Synthesis of 4-Hydroxy-3-methoxy-ω-(2-phenylphenoxy)-acetophenone.(13).

A similar procedure was used as for the production of (8) from (6). A mixture of the ketone (11) (3.86 g) and 10% palladium on charcoal (0.6 g) in aqueous tetrahydrofuran (3 ml H_2O in 110 ml THF) was shaken under hydrogen as described previously. The product was a pale yellow solid which was recrystallized to leave white crystals of (13). Yield 82%; analytical TLC gave one spot Rf 0.38; mp 135-137°C; ir γ max 3140, 3120, 2912, 1655, 1578 cm^{-1}; H-nmr [$CDCl_3$] δ 7.45 (m, 12H, aromatic), 6.05 (s, 1H, phenolic-OH), 5.11 (s, 2H, CH_2-O), 3.82 (s, 3H, OMe); ms m/z 334.1207.

Synthesis of 1-(4-Hydroxy-3-methoxyphenyl)-2-(2-phenylphenoxy)ethanol.(14).

Compound (14) was prepared from (13) according to the procedure used to obtain (10) from (9). A colourless oil resulted. Yield 93%; analytical TLC gave one spot Rf 0.21; decomposition at 190°C (1 mm Hg); ir γ max 3500, 3010, 2912, 1595 cm^{-1}; H-nmr [$CDCl_3$] δ 7.38 (m, 12H, aromatic), 5.55 (s, 1H, phenolic-OH), 4.76 (q, 1H, H-C-), 4.00 (o, 2H, CH_2-O), 3.76 (s, 3H, OMe), 2.51 (s, 1H, C-OH); ms m/z 336.1351.

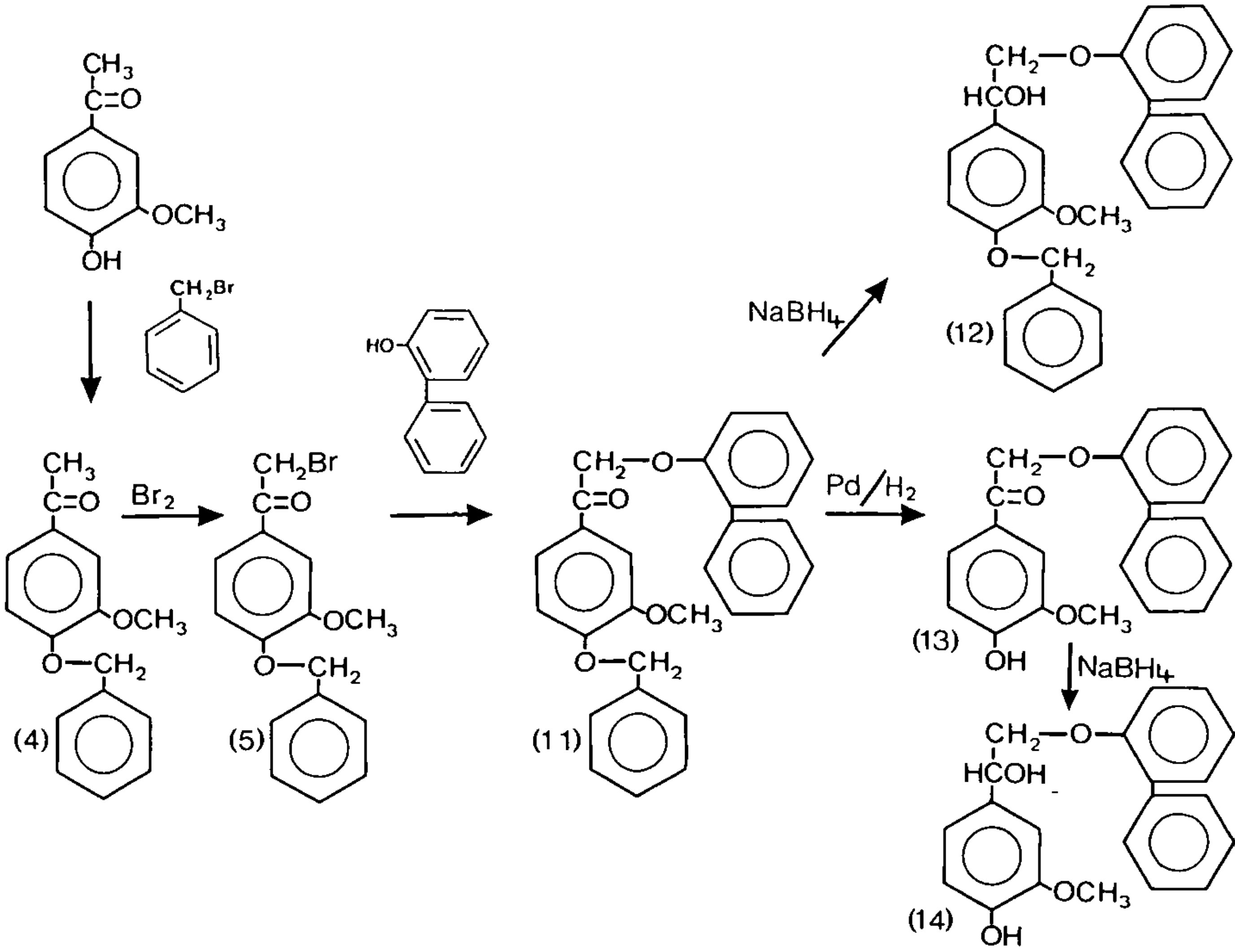

CH_3
C=O
OCH_3
OH
CH_2Br
Br_2
CH_2Br
HO
NaBH4
Pd/H2
NaBH4
$O-CH_2$
CH_2-O
HCOH
(4)
(5)
(11)
(12)
(13)
(14)

Figure 24. The Synthesis Of Lignin Model Compounds 11, 12, 13 & 14 Containing The Biphenyl Moiety

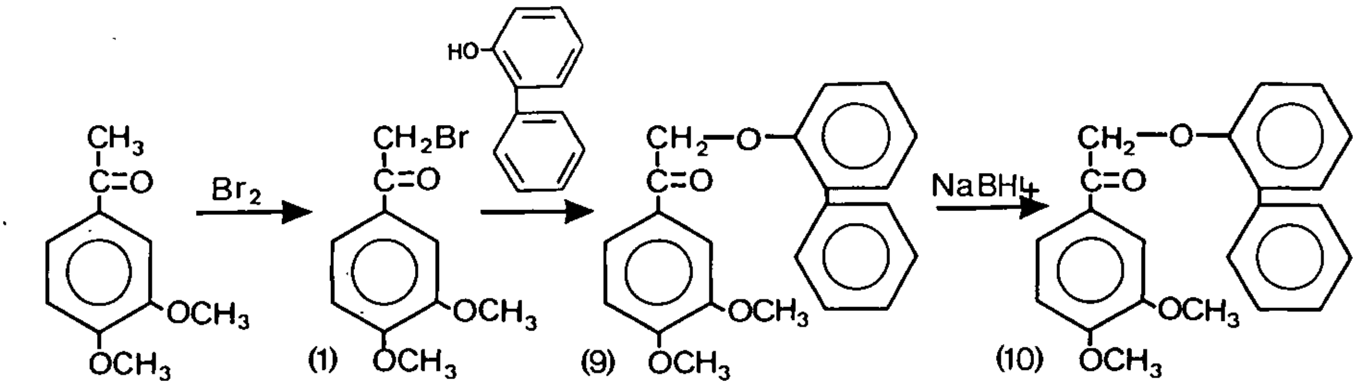

CH_3
C=O
OCH_3
Br_2
CH_2Br
HO
NaBH4
CH_2-O
(1)
(9)
(10)

Figure 25. The Synthesis Of Lignin Model Compounds 9 & 10 Containing The Biphenyl Moiety

SYNTHESIS OF RADIOLABELLED MODEL COMPOUNDS

3,4-Dimethoxy-ω-(2-methoxyphenoxy)acetophenone was synthesized with either the C1 or C2 of the molecule radiolabelled with ^{14}C. The syntheses were identical except for the use of ^{14}C-sodium acetate which was radiolabelled in either the C1 or C2 position.

Synthesis of [^{14}C]-Acetovanillone.

[^{14}C]-Sodium acetate solution (250 μCi) was freeze dried prior to the addition of glacial acetic acid (1.5 ml). This provided a source of [^{14}C]-glacial acetic acid.

A modification of the method of Landucci et al. (1981) was used to produce [^{14}C]-acetovanillone. [^{14}C]-glacial acetic acid (1.5 ml) and guaiacol (800 mg) were transfered to a three-necked flask. The flask was fitted with a condensor, an inlet port for BF_3 gas and a drying tube with an outlet connected to a series of traps containing water to absorb excess BF_3. The solution was stirred magnetically in an ice bath. A very slow stream of BF_3 gas was then introduced into the reaction vessel. The solution turned red after approximately 15 minutes and after 2 hours the BF_3 supply was disconnected and a 10% solution of sodium acetate was poured into the flask. A yellow precipitate was formed and the mixture was transfered into a separating funnel and extracted three times with 100ml portions of chloroform. The chloroform extract was washed with a saturated solution of sodium bicarbonate and water and was then evaporated under vacuum. The crude product was examined by TLC against an authentic sample of acetovanillone and was recrystallized from carbon tetrachloride to give pale yellow crystals of [^{14}C]-acetovanillone. Yield 43% from [^{14}C]-sodium acetate. The purity of the preparation was checked by analytical TLC and mp against an authentic sample.

Synthesis of [^{14}C]-Acetoveratrone.

[^{14}C]-Acetovanillone (1 g), dimethyl sulphate (2 g) and potassium carbonate (1.1 g) in dry acetone (30 ml) were refluxed for 5 hours. The reaction mixture was then cooled and filtered. Excess dimethyl sulphate and acetone were evaporated under a stream of nitrogen. The red/brown solid remaining was extracted with hot cyclohexane until TLC showed that no product remained in the residue. The light brown product was then recrystallized from cyclohexane to leave a light brown powder. Yield 55%. The purity of the product was checked by analytical TLC and mp against an authentic sample.

Synthesis of [^{14}C]-3,4-Dimethoxy-ω-(methoxyphenoxy) acetophenone.

This was produced from [^{14}C]-acetoveratrone in an identical way to the synthesis of the unlabelled compound as described earlier. The purity of the intermediates and the final product was checked by analytical TLC and mp against authentic samples.

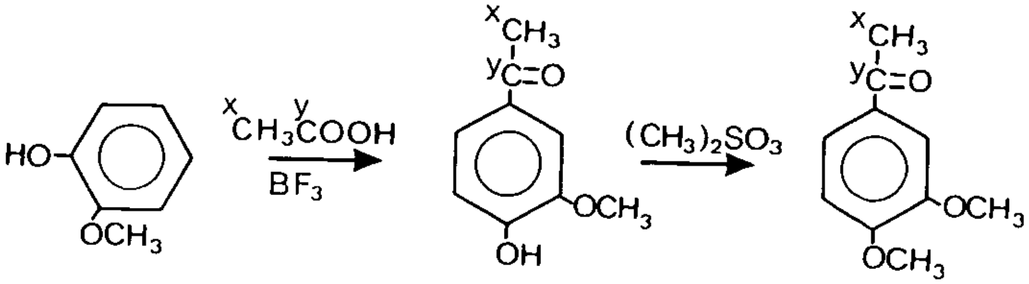


Figure 26. The Synthesis Of ^{14}C-Acetovanillone & ^{14}C-Acetoveratrone

MICRO-ORGANISMS

The following bacteria and fungi were used for investigations into the degradation of lignins and lignin model compounds and for phenoloxidase screening. A number of these organisms are known degraders of certain model compounds and lignins and were used as controls for the applicability of media and techniques.

FUNGI	MAINTENANCE MEDIUM	INCUBATION TEMP.(°C)
Sporotrichum pulverulentum CMI 174727	2	39
Polyporus dichrous NCWRF/FPRC 225	2	30
Polyporus versicolor CMI 79126	2	27
Aspergillus fumigatus CMI16153	3	30
Aspergillus flavus CMI 15959	1	30
Aspergillus nidulans CMI 16643	3	30
Schizophyllum commune FPRL 9	4	27
Byssochlamys fulva CMI 40021	3	30
Neurospora crassa CMI 75723	4	30
Saccharomyces cerevisiae NCTC 10716	3	30
Candida albicans NCYC 597	5	30
BACTERIA		
Pseudomonas aeruginosa NCIB 6749	7	37
Proteus vulgaris NCIB 8067	7	30
Nocardia opaca NCIB 9409	7	27
Nocardia petrophila NCIB 9438	7	27
Nocardia madurae NCTC 5654	7	27
Streptomyces species NCIB 8592	6	30

BACTERIA	MAINTENANCE MEDIUM	INCUBATION TEMP(°C)
Streptomyces albogriseolus NCIB 9604	6	30
Bacillus cereus LUT1 11755	4	30
Bacillus subtilis NCTC 10073	4	30
Bacillus megaterium NCIB 8291	4	30
Bacillus licheniformis NCTC 1097	7	30
Klebsiella aerogenes NCIB 8267	7	37
Staphylococcus aureus NCIB 8625	4	37
Escherichia coli NCTC 9001	4	37

SOURCES OF MICRO-ORGANISMS

NCIB - The National Collection Of Industrial Bacteria,
Torry Research Station, P.O. Box 31,
Aberdeen, AB9 8DG, U.K.

NCTC - The National Collection Of Type Cultures,
Central Public Health Laboratory,
Colindale Avenue, London, NW9 5HT, U.K.

CMI - The Commonwealth Mycological Institute,
Ferry Lane, Kew, Surrey, U.K.

NCYC - The National Collection Of Yeast Cultures,
Colney Lane, Norwich, NR4 7VA, U.K.

FPRL - The Forest Products Research Laboratory,
Aylesbury, Buckinghamshire, U.K.

MICROBIOLOGICAL MEDIA

Maintenance Media

All micro-organisms were subcultured at 2 week intervals to maintain pure cultures.

1. Malt Extract Agar

Malt extract	50 g
Agar	20 g
Distilled water	1000 ml

The ingredients were dissolved and made up to 1l with distilled water. The pH was adjusted to 5.2 before sterilization by autoclaving.

2. Modified Malt Extract Agar

Malt extract	50 g
Malic acid	5 g
Agar	20 g
Distilled water	1000 ml

The ingredients were dissolved in hot distilled water and made up to 1l. The pH was adjusted to 6.5 before sterilizing by autoclaving.

3. Maltose Based Agar

Maltose	38 g
Neutralized soya peptone	8 g
Yeast extract	2.5 g
Malt extract	2 g
Agar	20 g
Distilled water	1000 ml

The ingredients were dissolved and made up to 1l with distilled water. The pH was adjusted to 5.2 before sterilizing by autoclaving.

4. Neurospora Medium Extract

Ammonium tartrate	5 g	Sucrose	20 g
Ammonium nitrate	1 g	Biotin	5×10^{-6} g
KH_2PO_4	1 g	Trace elements soln.	1 ml
$MgSO_4.7H_2O$	0.5 g	Agar	20 g
NaCl	0.1 g	Distilled water	1000 ml
$CaCl_2$	0.1 g		

Trace Elements Soln. -

1. Boric acid soln. (60 mg/250 ml).
2. Ammonium molybdate soln. (260 mg/100 ml).
3. Ferric chloride soln. (1 g/100 ml).
4. Copper sulphate soln. (400 mg/100 ml).
5. Magnesium chloride soln. (80 mg/250 ml).
6. Zinc chloride soln. (2 g/100 ml).

The following solutions were mixed together - 2.5 ml of 1 and 5, and 1.0 ml of 2,3,4 and 6. The components were dissolved and made up to 1l with distilled water. The pH was adjusted to 5.2 (fungi) or 7.0 (bacteria) prior to autoclaving.

5. Yeast Glucose Agar

Yeast extract	3 g
Glucose	10 g
Neutralized soya peptone	5 g
Agar	20 g
Distilled water	1000 ml

The components were dissolved and the pH adjusted to 5.2 prior to autoclaving.

6. Rimosus Agar

Glucose	4 g
Yeast extract	4 g
Malt extract	10 g
Agar	20 g
Distilled water	1000 ml

The components were dissolved and the pH adjusted to 7.2 prior to autoclaving.

7. Nutrient Agar

Beef extract	10 g
Bacteriological peptone	10 g
NaCl	5 g
Agar	20 g
Distilled water	1000 ml

The components were dissolved. The pH was adjusted to 7.2 before sterilizing by autoclaving.

Fungal Sporulation Medium

$K_3PO_4 \cdot H_2O$	2 g
KNO_3	2 g
$CaCl_2$	0.25 g
Yeast extract	5 g
Glucose	10 g
Bacto Casamino acids	5 g
Metals soln.	0.1 ml
Distilled water to	1 l

The metals solution consisted of :

$MgSO_4.7H_2O$ - 0.5 mg/10 ml
$ZnSO_4$ - 0.02 mg/10 ml
$FeSO_4$ - 0.02 mg/10 ml
$MnSO_4$ - 0.01 mg/10 ml
$CuSO_4$ - 0.05 mg/10 ml

The pH of the medium was adjusted to 5.2 before autoclaving.

Fungal Growth Medium

When fungi were grown in liquid medium for mycelial pellet production, growth curve data or degradation analyses the following medium was used :

$NaNO_3$	5 g
KH_2PO_4	5 g
$MgSO_4.7H_2O$	0.5 g
Yeast extract	0.1 g
Carbon source	5 g or 1 g as indicated
Distilled water	1000 ml

The pH of the medium was adjusted to 5.2 and the medium was sterilized by autoclaving or by membrane filtration using 0.45 μm Millipore filters.

When insoluble model compounds were used, these were first dissolved in the minimum quantity of acetone and then added aseptically to the medium.

Insoluble lignin preparations were added in the minimum quantity of 0.2N KOH solution and the pH of the medium was then re-adjusted to 5.2 after addition of the lignin.

MICROBIOLOGICAL METHODS

Preparation And Harvesting Of Fungal Spores

The sporulation medium was dispensed as 250 ml quantities into 1 l Roux bottles. These were autoclaved, cooled, inoculated and incubated at 30°C for 20 days.

The mycelium was covered in spores after this time which were harvested and prepared using a modification of the method of Dart (1975).

The spores were dislodged from the mycelial mats by adding 100 ml volumes of sterile distilled water to the Roux bottles and agitating them for 5 minutes. The contents were then filtered through loose sterile cotton wool plugs and the filtrate was centrifuged for 15 minutes at 3500 X g. The supernatant was decanted off, sterile distilled water was added and centrifuged a second time to wash the spores. This was repeated until examination under the microscope showed that the spores were not contaminated with mycelia.

The spores were stored at 0°C as stock supensions diluted with sterile distilled water to approximately 10^5 spores/ml.

Fungal Growth

100 ml portions of the sterilized growth medium were dispensed into 250 ml Erlenmeyer flasks. The carbon source concentration was 1 g/l and water insoluble substrates were added as described earlier. When the carbon source was added in acetone the flasks were shaken at 40°C for 4 hours to allow the solvent to evaporate. Control flasks containing no carbon source

were treated similarly to take account of any acetone which might remain.

The flasks were inoculated with 1 ml of spore suspension containing 10^5 spores/ml and were incubated at 30°C in a Gallenkamp orbital incubator at 120 r.p.m. The dry weight of the mycelium was determined at intervals of 24 hrs by a modification of the method of Bruner et al. (1968). The contents of a flask were filtered through a previously dried (110°C) and weighed Whatman glass microfibre filter. The residue was washed with 5 volumes of distilled water and 2 volumes of acetone (or dioxane when lignin was the carbon source) and the filter and contents were dried to constant weight at 100°C. Dry weights were determined from the mean of six replicates for each time interval.

Plate Test For Fungal Degradation Of Lignin

Media

Fungi were grown on media of various types as shown in table 2. The media were labelled 1, 2, 3, 4, 5 and 6. Media 2 to 6 were modified versions of medium 1 which was described by Sundman & Nase (1971).

Autohydrolysis Aspen Lignin is water insoluble and prior to pouring the plates the lignin particles were uniformly dispersed in the media using a sterile homogenizer.

The pH of the media was 5.8. Incubation temperatures for the micro-organisms were given earlier and the incubation periods varied as shown in the results.

Lignin Degradation Plate Test

A modification to the method of Sundman & Nase (1971) was used to determine lignin degradation. After incubation for the period described in the results section, the diameter of the mycelial growth was measured. The mycelium was then scraped off the agar using a razor blade and 10 ml of the test reagent was added to the plate. This was left for 5-10 minutes after which time the reagent was poured off to allow viewing. The test agar was coloured green by the reagent and lignin degradation was shown by a disappearance of the green colour around or under the growth area.

Table 2

Media For Lignin Degradation Plate Test

COMPONENT (g/500ml)	MEDIA					
	1	2	3	4	5	6
Glucose	2.5	2.5	-	-	-	2.5
Ammonium tartrate	2.5	2.5	2.5	2.5	2.5	-
Malt extract	0.5	0.5	-	0.5	-	-
$MgSO_4.7H_2O$	0.25	0.25	0.25	0.25	0.25	0.25
$CaCl_2.2H_2O$	0.005	0.005	0.005	0.005	0.005	0.005
NaCl	0.05	0.05	0.05	0.05	0.05	0.05
$FeCl_3$	0.005	0.005	0.005	0.005	0.005	0.005
Vit.soln.(ml)	2.5	2.5	2.5	2.5	2.5	2.5
Lignin	0.5	-	0.5	-	-	0.5
Agar	20.0	20.0	20.0	20.0	20.0	20.0
Water (ml)	500	500	500	500	500	500

Table 3

Vitamin Solution For Lignin Degradation Plate Test

1. Thiamine	-	0.01 g	in 180ml
2. Pyridoxine	-	0.01 g	
3. Calcium pantothenate	-	0.01 g	
4. Nicotinic acid	-	0.01 g	
5. ρ-Aminobenzoic acid	-	0.01 g	
6. Folic acid	-	0.002 g	
7. Vitamin B12	-	0.016 g	in 1000ml
8. Biotin	-	0.008 g	in 1000ml

1 - 6 (in 180 ml) were combined with 10 ml each of 7 and 8

Test Reagent

The reagent was composed of a fresh mixture containing equal volumes of two solutions:

1. $FeCl_3$ - 10 g/l
2. $K_3(Fe(CN)_6)$ - 10 g/l

Screening Of Fungi And Bacteria For Their Ability To Degrade Insoluble, Lignin-Related Aromatic Compounds

The ability of a number of fungi and bacteria to degrade several insoluble lignin-related aromatic compounds was assessed using a rapid screening procedure.

Lignin-Related Aromatic Compounds

Ferulic acid, cinnamic acid, 3,4,5-trimethoxyacetophenone, 3,4,5-trimethoxycinnamic acid, 3,4,5-trimethoxybenzaldehyde and 3,4,5-trimethoxybenzoic acid.

Growth media

Four types of basal media were used: B1 (bacterial) and F1 (fungal) growth media included a carbon source whereas B2 (bacterial) and F2 (fungal) growth media contained no additional carbon source.

B1 consisted of: tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g; distilled water to 1 l; pH 7.2. This medium was essentially the same as the LB medium of Kiyohara *et.al.* (1982).

F1 was based on the medium described by Sundman & Nase (1971) and contained: glucose, 5.0 g; ammonium tartrate, 5.0 g; malt extract, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2.2H_2O$, 0.01 g; NaCl, 0.1 g; $FeCl_3$, 0.01 g; vitamin solution, 5 ml; agar, 20 g; distilled water to 1 l; pH 5.2. The vitamin solution consisted of: thiamine, 25 mg; calcium pantothenate, 25 mg; nicotinic acid, 25 mg; pyridoxine, 25 mg; ρ-aminobenzoic acid, 25 mg; folic acid, 5 mg; biotin, 50 g; vitamin B12, 100 g; distilled water to 1 l.

B2, as previously described by Kiyohara _et.al._ (1982), consisted of: $(NH_4)_2SO_4$, 2.376 g; $FeSO_4.7H_2O$, 0.0003 g; $CaCl_2.2H_2O$, 0.0131 g; $MgSO_4.7H_2O$, 0.2463 g; NaCl, 0.4973 g; Na_2HPO_4 /KH_2PO_4 buffer pH 7.0 to 1 l.

F2 contained: ammonium tartrate, 5.0 g; malt extract, 0.1 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2.2H_2O$, 0.01 g; NaCl, 0.1 g; $FeCl_3$, 0.01 g; vitamin solution, 5.0 ml; agar, 20 g; distilled water to 1 l; pH 5.2. The vitamin solution was as described for F1.

Inoculation of plates and application of model compounds

Plates were inoculated centrally with either a small piece of mycelium or a loopful of organism taken directly from a maintenance slope. A 24 h incubation period was allowed before application of the model compound.

The lignin models were dissolved in acetone (1 g in 10 ml) and were sprayed uniformly over the surface of the agar and the inoculation site. The acetone solutions were heated for a few seconds before spraying to effect an immediate vaporization upon contact with the agar. The use of very small quantities of model compounds was achieved by placing the acetone solutions into small glass sample tubes. These were then placed onto a support inside the reservoir of an aerosol spray unit.

Plates were incubated at 30°C for 1 to 5 days after spraying.

Degradation was indicated by the formation of clear areas in the opaque layer of the test compound.

Physical Aspects Of The Degradation Of Lignin And Lignin Model Compounds By *Aspergillus flavus*

The physical association between *A. flavus* and lignin/lignin model compounds was investigated during growth of the fungus on these substrates. The effect on the crystal size of compound (2) during its metabolism by *A. flavus* was also observed.

Growth Of Organism

The fungal growth medium was described earlier. Lignin model compounds were added to the basal medium as acetone solutions to provide finely dispersed suspensions. Autohydrolysis Aspen Lignin was added as a fine powder directly to the growth medium. The concentration of all carbon sources was 1 g/l.

Flasks were shaken at 30°C for 24 h before inoculation to allow evaporation of acetone as described earlier.

Spore suspension (1 ml containing approximately 10^5 spores) was used to inoculate 250 ml Erlenmeyer flasks containing 100 ml of medium. Cultures were incubated at 30°C on an orbital incubator at 120 r.p.m. For quantitative growth and crystal length measurements, samples were taken at 24 h intervals over a 5 day period. Otherwise samples were taken after 4 days incubation.

Light Microscopy

For light microscopy, the mycelial contents of flasks were collected and washed with distilled water before examination.

Scanning Electron Microscopy

The contents of flasks containing the various model compounds or lignin were filtered after the required inoculation period and washed five times with distilled water. Mycelial samples were placed on circular coverslips and excess moisture was removed with paper tissues. The coverslips were fixed to metal stubs and these were desiccated under vacuum for 2 days. The samples were then sputter coated with gold to a thickness of 10 nm prior to mounting in the electron microscope (ISI SS40).

It was occasionally necessary to tease open mycelial spheres to expose the interior before desiccation. Exposure of the interiors of spheres also occurred during desiccation as the mycelia split. This usually aided observations but it was sometimes necessary to recoat the samples with gold if they flared in the electron microscope.

Measurment Of Changes In Crystal Length Of Compound (2) During Incubation With A. flavus

Electron micrographs were used to measure the crystal size of compound (2). Low magnification fields were used to calculate the length of at least fifty crystals for each 24 h sample. The mean crystal lengths and the standard errors of the means were calculated.

Dimerization Of 2,6-dimethoxyphenol By *A. flavus*

The coupling of 2,6-dimethoxyphenol, a lignin-related monomer, by *A. flavus* was investigated. The coupling product was isolated and characterized and scanning electron microscopy was used to demonstrate its formation within mycelial spheres during their growth.

Growth Of Organism

The same growth medium and incubation conditions were used as for experiments on the physical aspects of the degradation of lignin model compounds. The concentration of 2,6-dimethoxyphenol was 1 g/l.

Light Microscopy

The contents of flasks taken at intervals during a five day incubation period were filtered and washed with distilled water. Whole mycelial spheres were carefully transfered to glass slides. The specimens were covered with cover slips and viewed directly under the light microscope. It was found that the mycelia could be seen more easily if the spheres were immersed for 5 minutes in phenol red followed by washing three times with distilled water.

Scanning Electron Microscopy

Preparation techniques for mycelial samples to be used for scanning electron microscopy were described earlier.

Isolation Of Product

The product could not be isolated by simple extraction procedures possibly because it was heavily enmeshed in fungal mycelia. A Soxhlet extraction technique was therefore used.

The combined mycelial contents of four 1 l flasks containing 500 ml of medium were filtered and washed with distilled water. Excess water was removed by pressing the mycelia between filter papers. The mycelia were placed in a paper thimble and then extracted in a Soxhlet extractor for 10 h using redistilled acetone. The acetone was then removed on a rotary evaporator to leave dark purple crystals. These were washed well with distilled water and dried in a desiccator over anhydrous calcium chloride.

Purification And Identification Of Isolate

The isolated product was subjected to preparative TLC. This was carried out on 1 mm thick Kieselgel 60 GF254 glass plates using a solvent system of benzene:ethyl acetate, 9:1. Bands on the TLC plates were visualized using UV light and after scraping a band from the plate it was extracted five times with acetone. The extract was filtered through a sintered disc funnel and the solvent was removed on a rotary evaporator.

The product was characterized by H-nmr and mass spectra.

BIOCHEMICAL METHODS

Degradation Of Lignin Model Compounds

Lignin model compounds were used as sole carbon source (1 g/l) in the basal fungal growth medium described earlier. Inoculation with spores of A. flavus was followed by incubation at 30°C in a orbital incubator (120 r.p.m.) for 3-4 days.

Isolation Of Degradation Intermediates

Mycelia were separated from the medium by filtration and washed well with distilled water. The medium and washings were combined and adjusted to pH 2 with 2N H_2SO_4. This was then extracted three times with ethyl acetate (3 X 250 ml per litre of medium) using a separating funnel. The extracts were combined and dried over anhydrous Na_2SO_4. The drying agent was removed by filtration and the extract was evaporated to dryness on a rotary evaporator. The typically dark red/brown residue was dissolved in a small volume of acetone for application to TLC plates.

Analysis Of Degradation Intermediates

Preparative TLC plates were prepared by coating glass plates (20 cm^2) to a thickness of 1 mm with a mixture of Keiselgel 60 GF_{254} and water (2 g : 4.8 ml). The plates were left to dry at room temperature for 2 days before activating for 2 h at 110°C in an oven. Analytical TLC was achieved using Whatman LK6DF 5 X 20 cm analytical TLC plates.

A 2% solution of the extract in acetone was applied to analytical TLC plates as a small spot using 5 μl pipettes. The solution was applied to preparative TLC plates using a syringe spray system to produce a thin line across the plate.

Various phenolic standards and their derivatives were used during analytical work and these were spotted alongside the samples for comparison of Rf values. These standards are indicated in the results section where appropriate.

TLC plates were developed using two solvent systems:

1. Benzene:ethyl acetate (9:1) (Crawford et al., 1975); and
2. Benzene:dioxane:acetic acid (90:25:4) (Randerath, 1968).

The TLC plates were developed in tanks lined with solvent soaked filter paper. Analytical plates were developed over a standard distance of 10 cm. Preparative plates were developed until the solvent front was 2 cm from the top of the plate.

The spots produced on analytical plates were visualized using UV light , 50% aqueous H_2SO_4 or the following reagents:

Phenols	-	diazotized sulphanilic acid followed by 1N NaOH;
Aldehydes & ketones	-	2,4-dinitrophenylhydrazine (0.5% solution in 2M HCl);
Carboxylic acids	-	aqueous $KMnO_4$(2%).

The bands were located under UV light and for preparative purposes the silica gel of the band under investigation was scraped from 2-5 plates into a flask. The silica gel was extracted by shaking vigorously with acetone which was then filtered through a sintered disc funnel. The procedure was repeated three times, the extracts were combined, filtered through a filter paper and the solvent removed on a rotary evaporator.

The extracted sample was dried under nitrogen in a desiccator before analysis using i.r., n.m.r. and mass spectroscopy. For i.r. spectra, the isolates were used as oil films or were mulled in Nujol. The n.m.r. and mass spectra were obtained as described in the section on synthesis of model compounds. Samples were also co-chromatographed with authentic standards when these were available.

Determination Of Methanol

Methanol produced from compounds (2) and (9) by *A. flavus* was determined by GLC.

The system used was a Phase-Sep Chromatograph with a Spectra-Physics SP4290 integrator. An F.I.D. detector was fitted to the instrument and the glass column was packed with 10% PPG on Chromosorb W, 80-100 mesh. The oven temperature was 70°C and the carrier gas was N_2 (8 lb/in^2). Butanol was used as the internal standard.

Samples of the growth medium (1 ml) were taken at 24 h intervals during growth of *A. flavus* on compounds (2) and (9). These aqueous samples were subjected directly to GLC analysis.

Methanol was identified by comparison of the retention times of peaks from samples with the retention time of an aqueous methanol standard. A control lacking substrate and an uninoculated control were used.

Determination Of Benzyl Alcohol

Benzyl alcohol produced from compounds (7) and (12) by *A. flavus* was determined by GLC.

The system used was a Pye series 4 chromatograph with a Spectra-Physics SP4290 integrator. An F.I.D. detector was fitted to the instrument and the glass column was packed with 10% Carbowax 20M on Chromosorb W, 80-100 mesh. The oven temperature was 160°C and the carrier gas was N_2 (17 lb/in^2). Hexadecanol was used as internal standard.

A sample of the aqueous medium after 4 days incubation was injected directly into the gas chromatograph. The retention time was compared with that of an aqueous solution of authentic benzyl alcohol. The two were also co-chromatographed.

Controls were used as for the determination of methanol.

Measurement Of $^{14}CO_2$ Released During Metabolism Of Compound (2) By *A. flavus*

$^{14}CO_2$ released from metabolism of Compound (2) was trapped and measured using modifications to the methods of Crawford & Crawford (1976) and Kadam & Drew (1986).

Experiments using radiolabelled compounds were carried out in 250 ml Erlenmeyer flasks equiped with a CO_2-free air supply and an arrangement to collect evolved $^{14}CO_2$ as shown in figure 27.

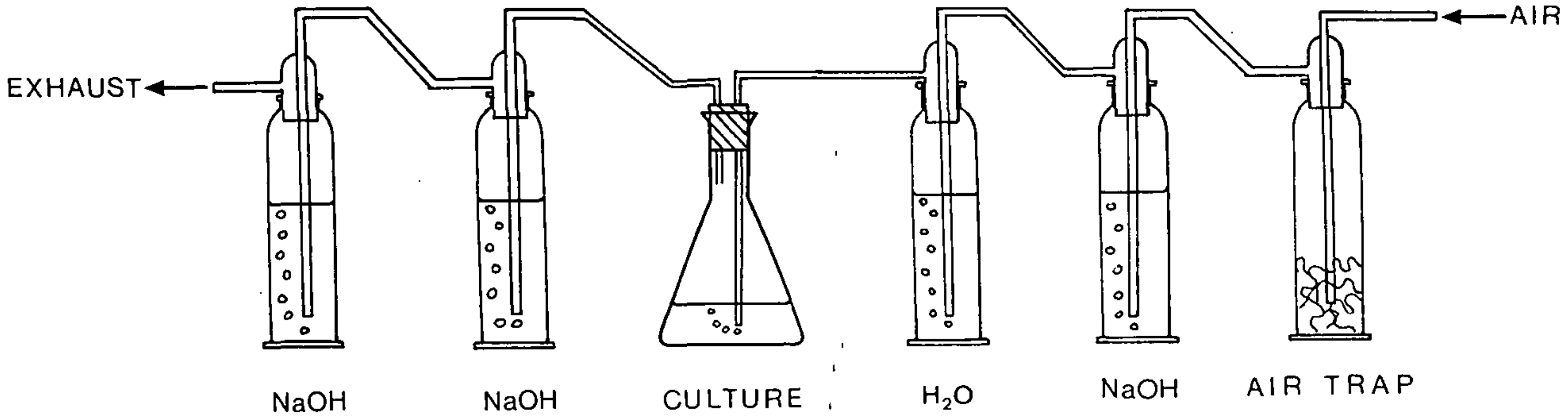

EXHAUST
AIR
NaOH
NaOH
CULTURE
H_2O
NaOH
AIR TRAP

Figure 27. The Apparatus Used For The Collecton Of $^{14}CO_2$ During The Metabolism Of Radiolabelled Compounds

100 ml volumes of the same growth medium (pH 5.2) used for unlabelled experiments was dispensed into 250 ml flasks. These were sterilized by autoclaving prior to addition of 5-10 μCi of compound (2) labelled with ^{14}C in the α-position (position 2). This was applied as an acetone solution. The acetone was allowed to evaporate as described earlier.

The flasks were inoculated with 1 ml of A.flavus spore suspension containing 10^5 spores and were incubated in an orbital incubator at 150 r.p.m. and 30°C.

The air supply to the flasks was passed through 2N KOH to trap ambient CO_2 and through glass wool to maintain sterility. The flow rate was adjusted to 1 ml per minute.

Metabolic $^{14}CO_2$ was collected from each Erlenmeyer flask by two traps connected in series and containing 20 ml of 1N KOH.

Experimental flasks were in triplicate and an uninoculated control and a no-substrate control were used.

Flasks were incubated for 5 days at 30°C and 150 r.p.m in an orbital incubator, after which time 1 ml samples of the KOH solution from the $^{14}CO_2$ trapping flasks was pipetted into 10 ml of scintillation fluid (Cocktail T). Radioactivity was determined using an LKB (Wallace) Display Unit 1215 Rack Beta liquid scintillation counter.

Counting efficiencies were calculated automatically from a previously constructed quench curve of external standards against counting efficiency. In all radioactivity measurements corrections were made for background counts.

Identification Of Radiolabelled Intermediates From The Metabolism Of 1-^{14}C- And 2-^{14}C-Compound (2)

A. flavus was grown on compound (2) labelled with ^{14}C in either the C1 or the C2 position of the sidechain for a three day period under identical conditions to those used for collection of $^{14}CO_2$.

The contents of flasks were filtered through a membrane filter apparatus to remove fungal growth and insoluble material. The pH of the filtrate was adjusted to 2 with 4N HCl and following transfer to a separating funnel the filtrate was extracted exhaustively with redistilled ethyl acetate. All extracts were combined and dried over anhydrous sodium sulphate prior to evaporation of the solvent using a stream of nitrogen.

The concentrated extract was diluted with exactly 1 ml of redistilled acetone to allow application to TLC plates for quantitative radio-analysis. Measured volumes of the acetone soluton were applied to TLC plates (as for unlabelled analyses) together with a range of authentic standards. The TLC plates were developed in two solvent systems :

1. benzene : ethyl acetate (9:1); and
2. benzene : dioxane : acetic acid (90:25:4).

The TLC plates were visualized under uv light and spots corresponding to standards were outlined. The silica gel marked in this way was scraped from the TLC plates and was placed into a scintered glass funnel. The silica gel was then washed five times with warm, redistilled acetone to extract the compound. Scintillation fluid (Cocktail T) (5 ml) was added to the residue after evaporation of the acetone. The radioactivity was measured using a scintillation counter as described earlier after shaking to dissolve the compound.

A quench curve was used for estimation of counting efficiency, background counts were deducted from measurements and no-substrate and uninoculated control flasks were used, as described earlier.

The identity of compounds found at each spot on the TLC plates was established by comparing the Rf values with authentic unlabelled standards and by co-chromatography.

DEGRADATION OF LARCH WOOD BY *Aspergillus flavus*

The degradation of native larch wood by *A. flavus* was investigated. Chemical analysis of wood components and scanning electron microscopy of wood samples during a five months incubation period were used to show any changes in the physical and chemical nature of the wood samples. A modified method of Otjen & Blanchette (1985) was used.

A. flavus was grown as mycelial mats on the surface of 10 ml quantities of malt extract broth in universal bottles.

Strips of larch wood were cut perpendicular to the grain to produce small blocks of dimensions 0.5 X 1.5 X 1.5 cm. These were dried at 105°C for 48 hours for dry weight determinations. The blocks were then moistened with distilled water and were placed into universal bottles containing 10 ml of vermiculite and 5 ml of distilled water. The bottles were then sterilized by autoclaving.

Using sterile forceps the previously grown mycelial mats were removed from the surface of the malt extract broth and placed onto the surface of the wood blocks. The bottles were then placed into a propagator containing a layer of water to provide an atmosphere of high humidity. The propagator was placed in a 30°C incubator.

Wood samples were taken for analysis at zero time and then at four week intervals. Uninoculated control wood samples were incubated under identical conditions.

The mycelial growth was scraped from all surfaces of the wood samples using a razor blade. Samples for chemical analysis were dried in an oven at 105°C for 48 hours prior to analysis.

This also allowed a dry weight determination. Samples for SEM were plunged into liquid nitrogen and then freeze dried before preparation.

Chemical Analysis Of Wood Samples

A modification to the method of Moore & Johnson (1967) was used to estimate extractives, lignin and total carbohydrate. Measurements were made on three replicate sets of samples and mean values determined.

Wood blocks (1.5-2.0 g) were disintegrated into fine particles before grinding in a vibratory mill to pass a 40-mesh sieve.

Extractives

Exactly 1.5 g of ground wood was placed into a thimble and extracted using a Soxhlet apparatus for 4 hours. A 95% ethanol : benzene (1:2) solvent system was used. The sample was then washed thoroughly with ethanol followed by a thorough washing with distilled water to remove the alcohol. The extractive-free wood was then dried at 105°C for 48 hours and the dry weight determined.

Lignin

15 ml of cold 72% H_2SO_4 (previously standardized) was added slowly with stirring to exactly 1.0 g of extractive-free wood. All lumps in the sample were broken up and the mixture was stirred constantly for 1 minute. The mixture was then left to stand for 2 hours at 20°C after which time it was washed into a

1 l Erlenmeyer flask. Distilled water (560 ml) was added and the mixture was refluxed for 4 hours.

The mixture was filtered through a previously weighed Gooch crucible and the residue washed with 500 ml of hot water. The crucible and contents were dried at 105°C for 2 hours and were then weighed. This was repeated until a constant weight for lignin was obtained.

Total Carbohydrate

Total carbohydrate was found by subtracting the weight of lignin and the weight of extractives from the dry weight of the whole wood.

Scanning Electron Microscopy Of Wood Samples

Freeze-dried wood samples were cleaved in a variety of planes and were placed onto metal stubs, sputter coated with gold to a thickness of 10 nm and viewed in the scanning electron microscope.

RESULTS

NOVEL MODEL COMPOUNDS

All of the model compounds containing the phenylphenol moiety were novel structures and contained bond types analogous to the β-aryl ether and biphenyl linkages found in native lignins. Some of these lignin models also contained a representation of the α-aryl ether linkage.

Acetovanillone was protected in the 4-position by benzylation with benzyl bromide using a modification to the method of Leopold (1950). This benzylation in the 4-position served a dual purpose, both to protect the 4-hydroxyl group and to act as analogue of the α-aryl ether linkage frequently represented in the lignin model (Adler, 1977). The α-aryl ether occurs to the extent of 6-8 % in natural lignin.

Bromination of acetoveratrone and 4-benzyloxy-3-methoxyacetophenone was accomplished using a modification of the procedure of Landucci et al. (1981).

The nucleophilic substitution of bromine by the 2-phenylphenate anion was achieved by a modification of the method of Kratzl et al. (1959).

Compound (13) was produced by catalytic hydrogenation of (11). The yield was considerably higher than the yield for the similar reaction by Landucci et al. (1981) as the α-carbonyl group of (11) was not appreciably reduced.

Subsequent reductions with sodium borohydride of (9), (11) and (13) gave rise to (10), (12) and (14) all in very high yields.

The syntheses of these novel compounds has been reported (Betts et al., in press).

TABLE 4

Summary Of Results From Lignin Degradation Plate Test

The media 1 to 6 and the test reagent were described in the methods section.

The plate test was conducted after incubation of the micro-organism on the medium for 5 days (3 days for *Ph. chrysosporium*).

+ = a positive result showed by the formation of a clear area under or surrounding the fungal growth.

- = a negative result i.e. no clear area was formed.

N.B.

1. *A. flavus* grown on Medium 6 - the result was repeatedly inconclusive.
2. *A. nidulans* grown on all media - the growth area became darker upon addition of the test reagent.
3. *N. crassa* grown on Medium 6 - this medium showed the most degradation.

TABLE 4

Summary Of Results From Lignin Degradation Plate Test

ORGANISM	MEDIUM					
	1	2	3	4	5	6
P.versicolor	+	-	+	-	-	+
P.dichrous	+	-	-	-	-	-
S.pulverulentum	-	-	-	-	-	+
A.fumigatus	+	-	+	-	-	-
A.flavus	+	-	-	-	-	?
A.nidulans	-	-	-	-	-	-
S.commune	+	-	+	-	-	+
B.fulva	+	-	+	-	-	+
N.crassa	+	-	+	-	-	+

PLATE 1

A Typical Positive (Above) And Negative (Below) Result Of The Plate Test For The Degradation Of Aspen Lignin

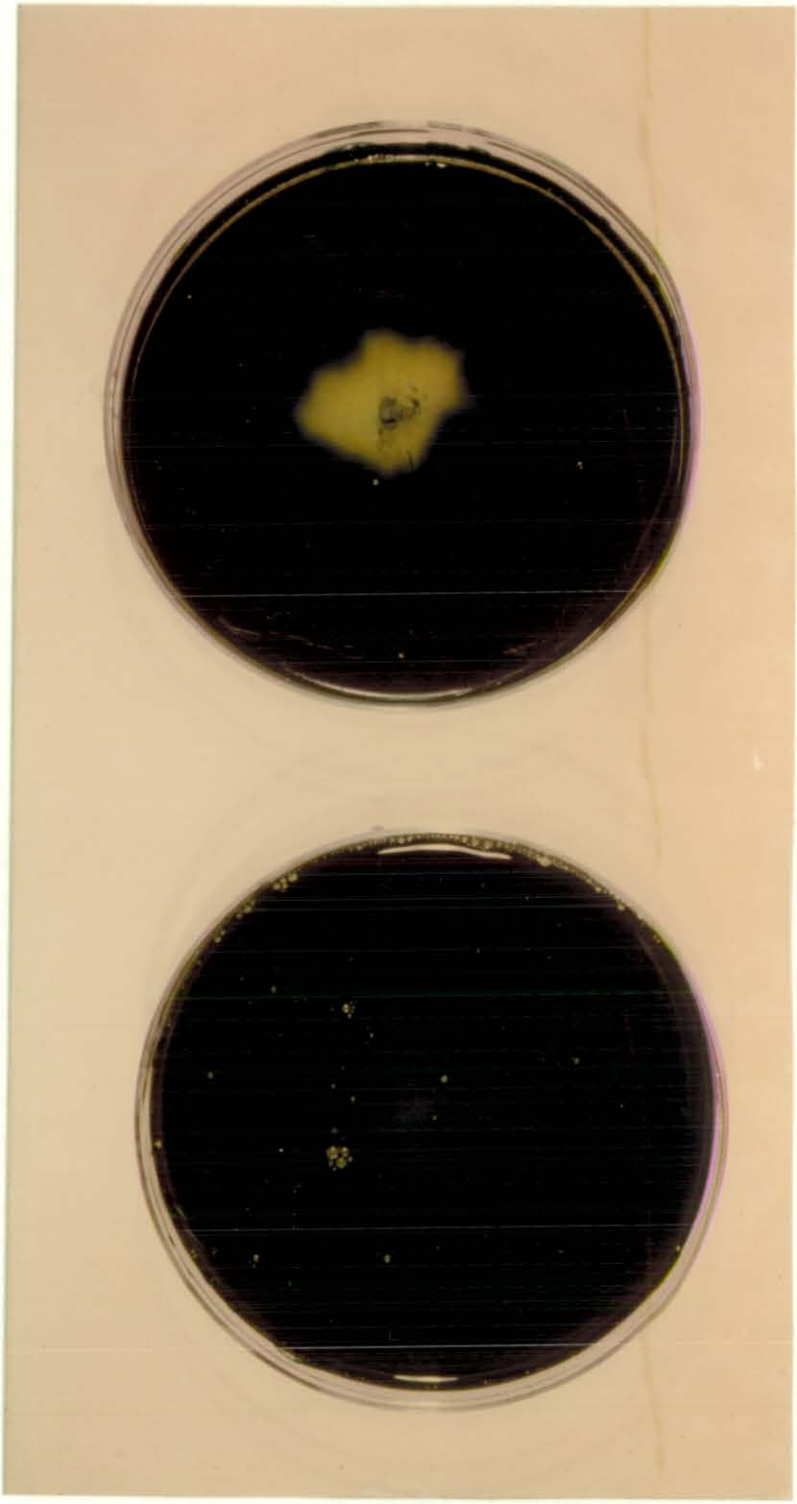


FIGURE 28

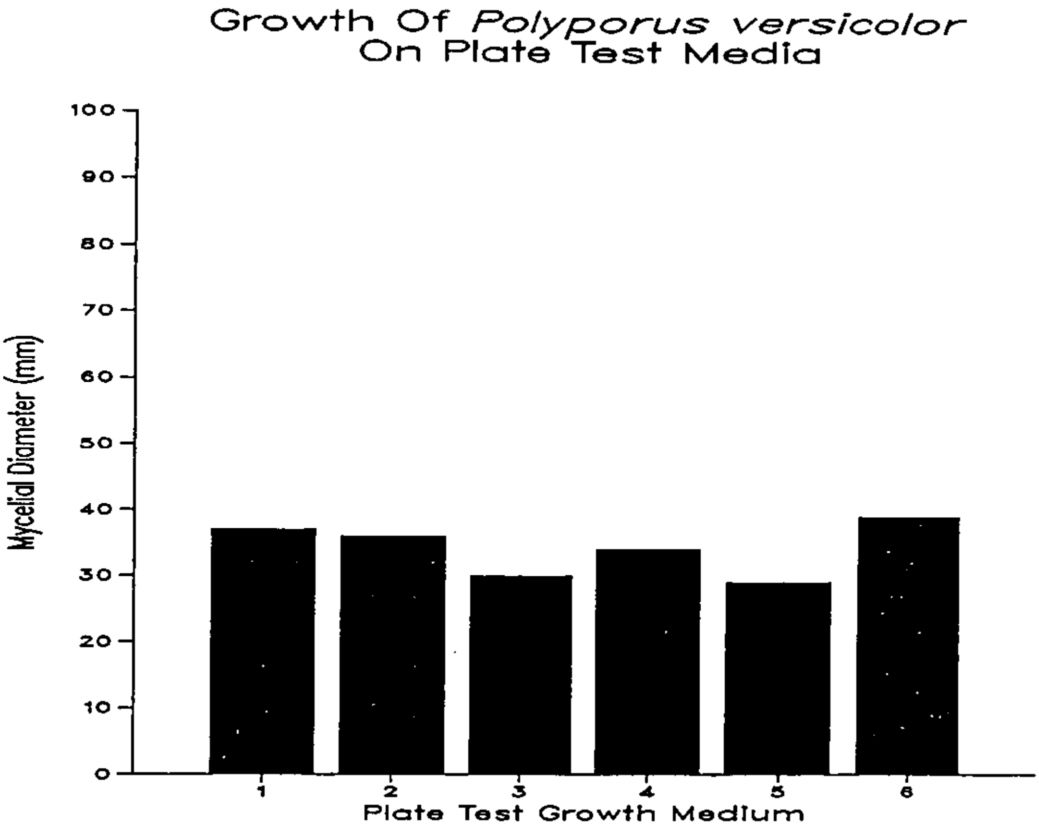


FIGURE 29

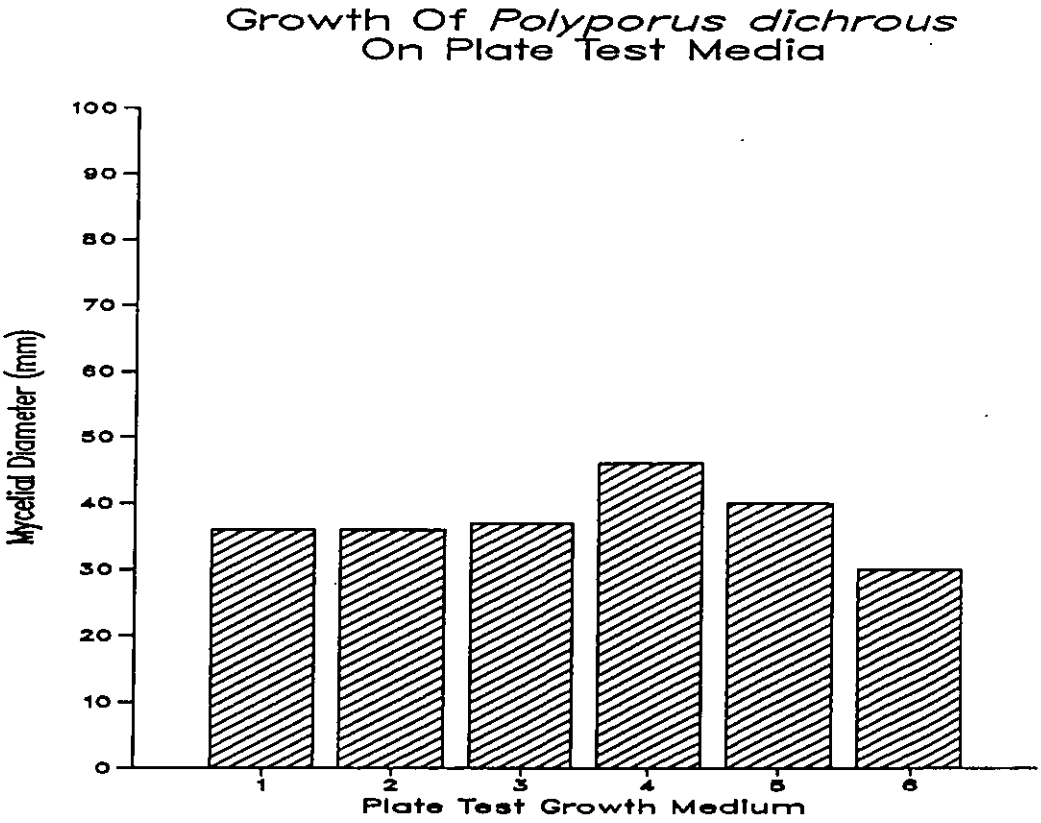


FIGURE 30

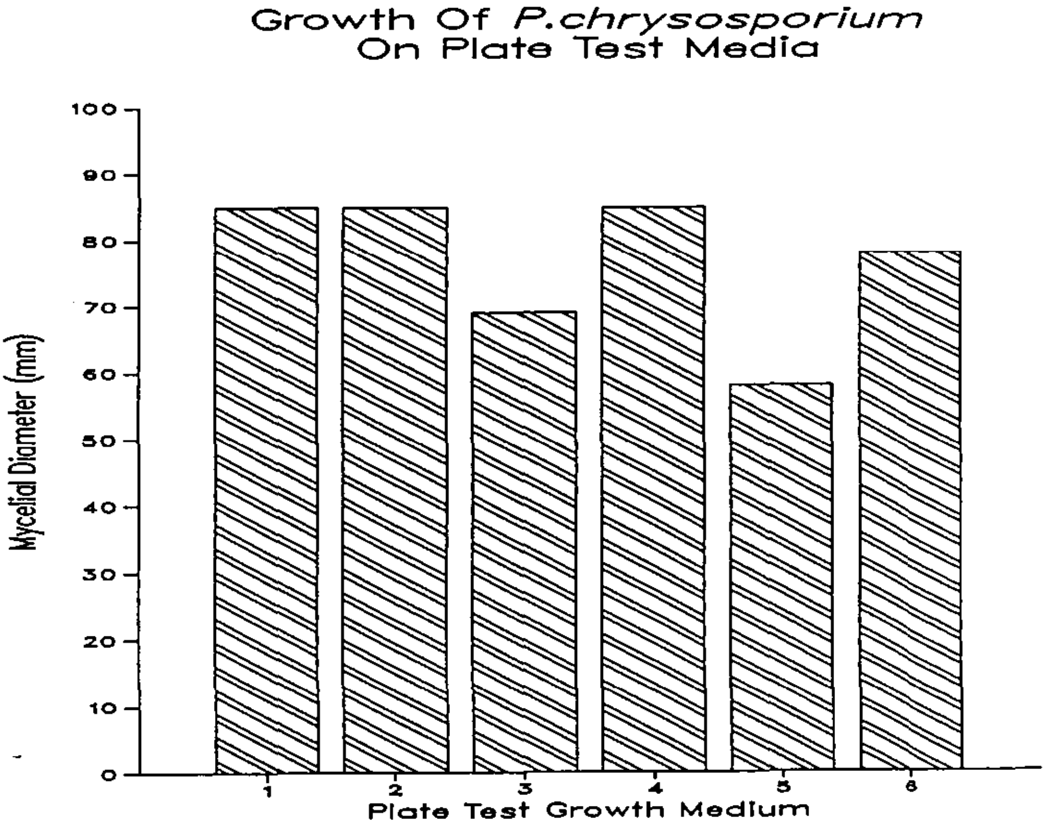


FIGURE 31

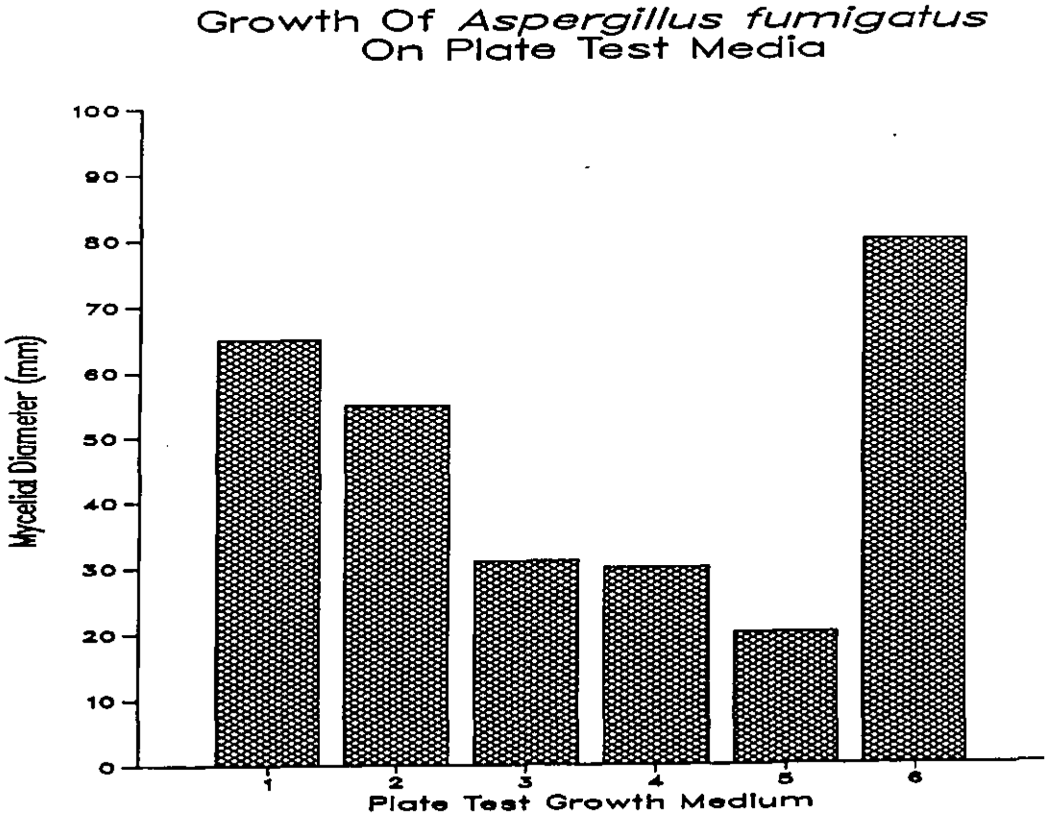


FIGURE 32

Growth Of *Aspergillus flavus*
On Plate Test Media

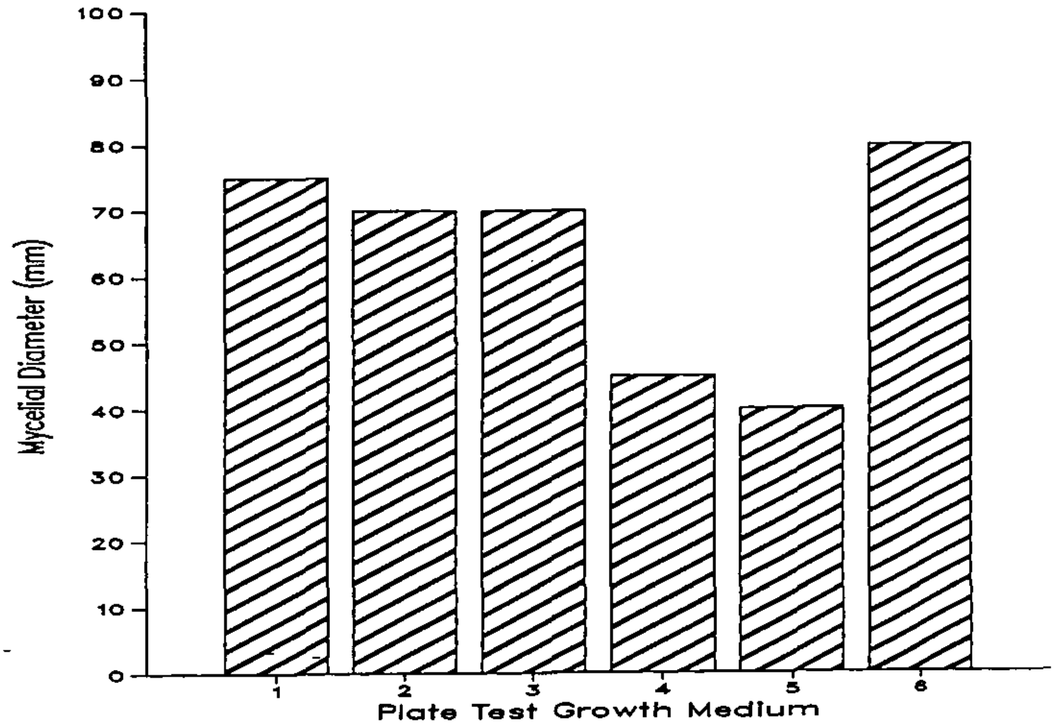


FIGURE 33

Growth Of *Aspergillus nidulans*
On Plate Test Media

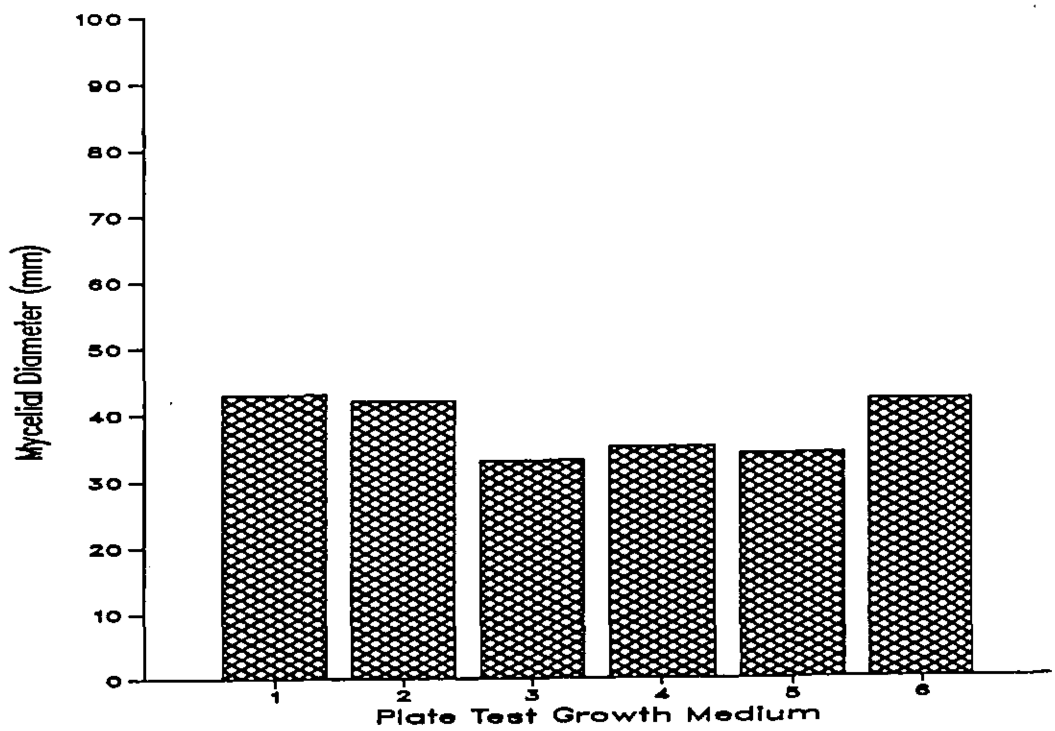


FIGURE 34

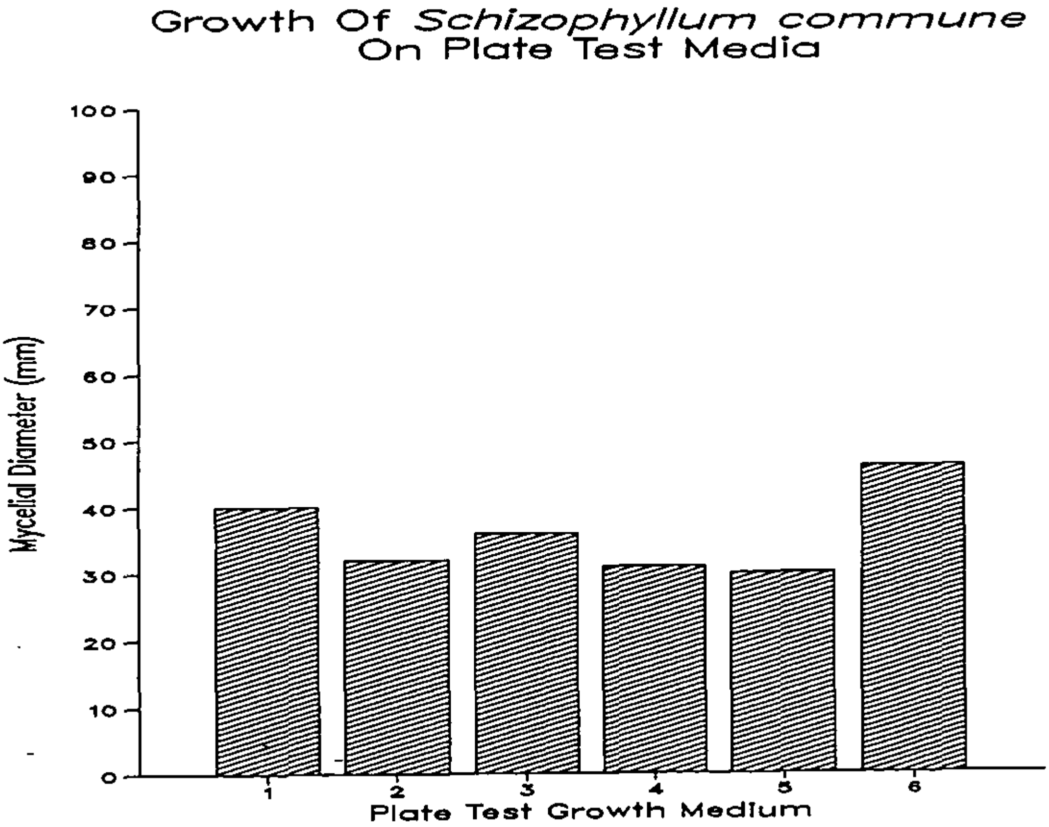


FIGURE 35

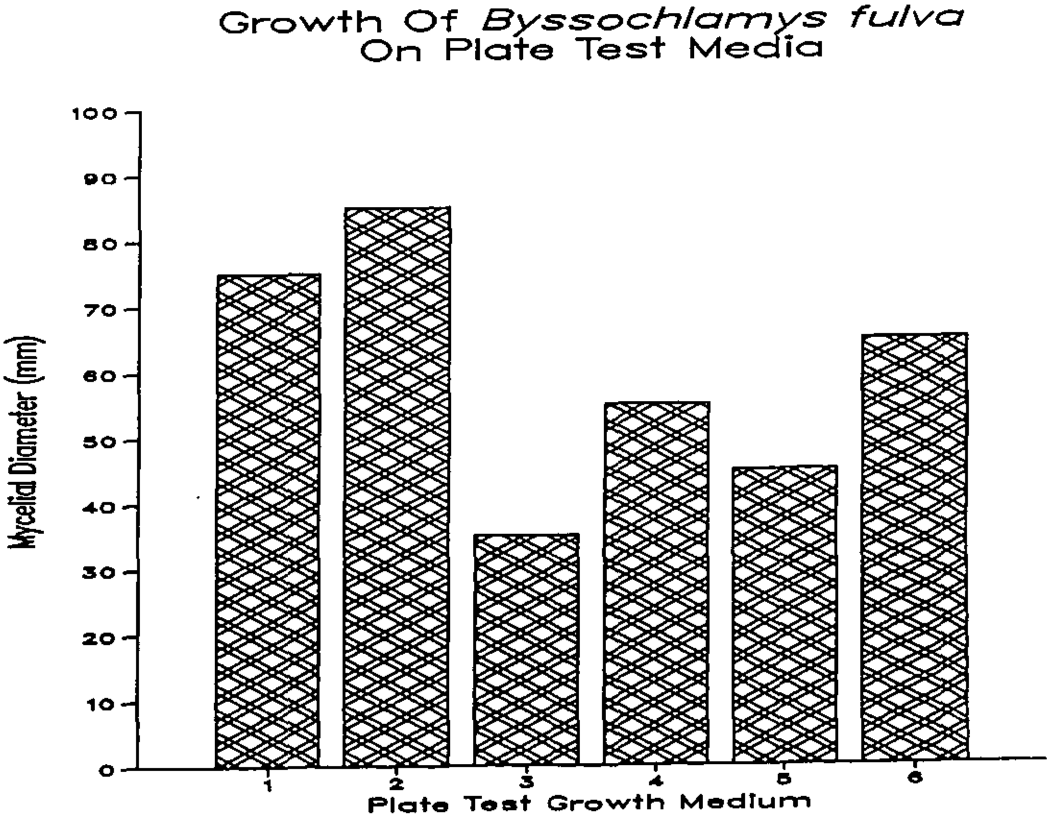
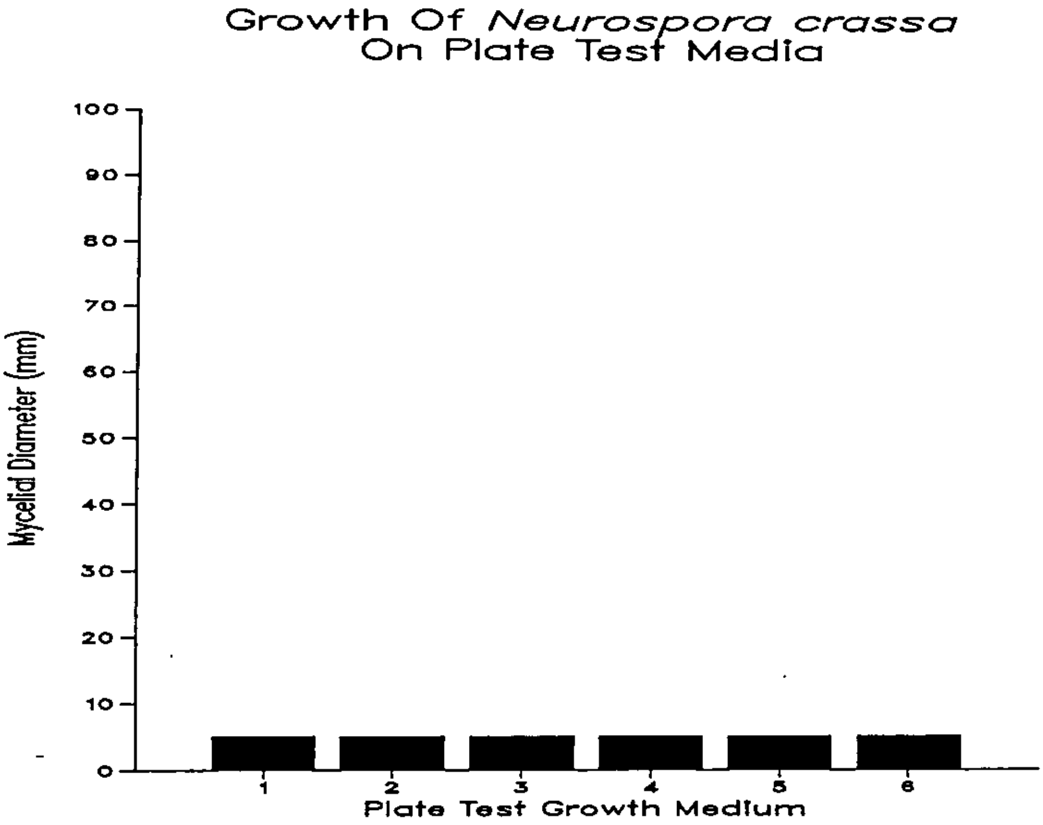


FIGURE 36


Growth Of *Neurospora crassa*
On Plate Test Media
Mycelial Diameter (mm)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
Plate Test Growth Medium

The Screening Of Fungi And Bacteria For Their Ability To Degrade Insoluble Lignin-Related Aromatic Compounds

Screening Of Bacteria

Table 5 shows the results obtained from plates inoculated with bacteria and incubated for between 1 and 3 days after spraying with one of five lignin-related aromatic compounds. Positive results are indicated by the formation of clear areas around the inoculation sites. Typical positive results are shown in Plates 2 to 5.

Screening Of Fungi

The results of experiments with plates inoculated with fungi and subsequently sprayed with six lignin-related aromatic compounds are shown in Table 6. Inoculation periods were between 1 and 4 days. Clear zones around mycelial growth were clearly seen in many cases. Plates 6 to 9 show examples of typical positive results. On occasions new inoculation sites, caused by distribution of spores over the plates during sporulation, were also observed to be surrounded by clear zones (Plates 6 & 7). Two organisms, Sacc. cerevisiae and C. albicans failed to grow on both media.

TABLE 5

Screening Of Bacteria For Ability To Degrade Lignin-Related Aromatic Compounds

+ indicates the presence of a clear zone around the inoculation site after incubation for 1 to 3 days.

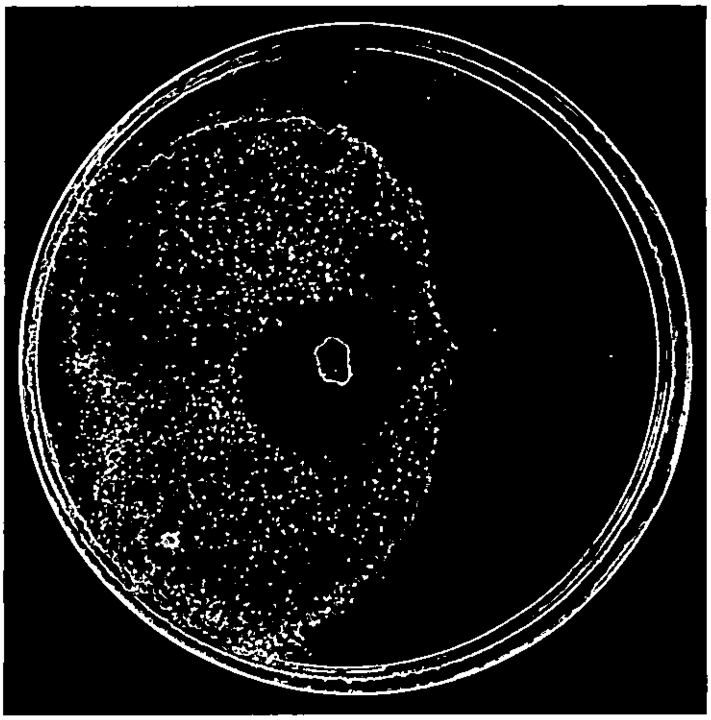
ORGANISM	Aromatic Compound & Growth Medium									
	i		ii		iii		iv		v	
	B1	B2	B1	B2	B1	B2	B1	B2	B1	B2
N.madurae	+	-	+	-	-	-	-	-	+	-
B.licheniformis	+	-	-	-	-	-	+	-	+	-
Ps.aeruginosa	+	-	+	+	-	-	+	+	+	+
Pr.vulgaris	+	+	+	-	-	-	+	+	-	-
N.petrophila	-	-	+	-	-	-	-	-	+	+
Strep. sp 8592	+	+	+	+	-	-	-	-	+	+
Strep.albogriseolus	+	+	+	+	-	-	-	-	+	-

i - ferulic acid
ii - cinnamic acid
iii - 3,4,5-trimethoxyacetophenone
iv - 3,4,5-trimethoxycinnamic acid
v - 3,4,5-trimethoxybenzaldehyde
vi - 3,4,5-trimethoxybenzoic acid

The media B1 & B2 are described in the methods

PLATE 2. Typical Positive Result Showing A Clear Region Around Streptomyces species 8592 (Medium B1; 3,4,5-Trimethoxybenzaldehyde)

PLATE 3. Typical Positive Result. Streptomyces albogriseolus (Medium B1; 3,4,5-Trimethoxybenzaldehyde)



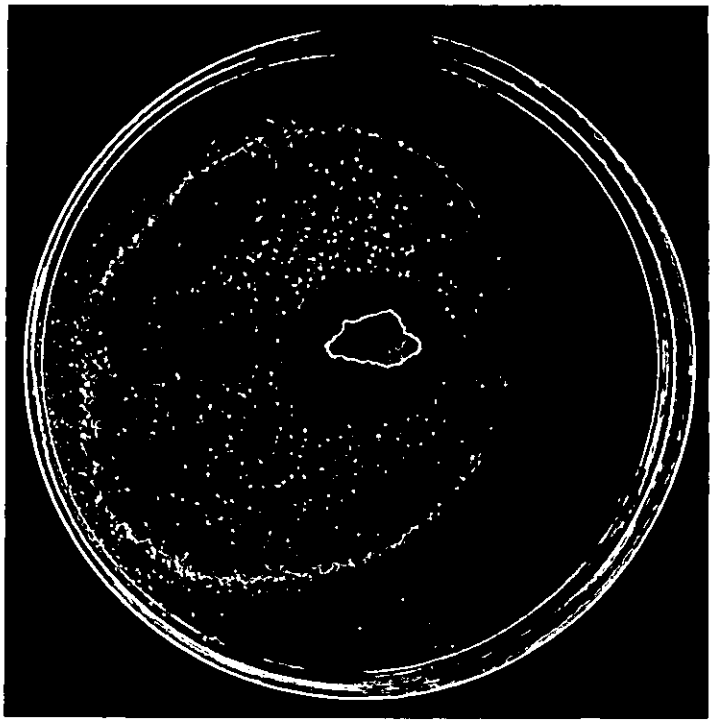
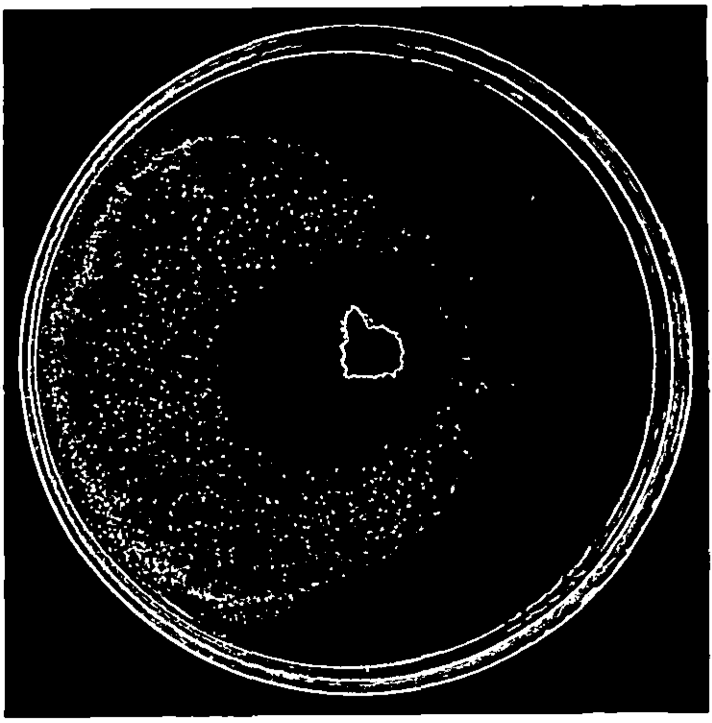


PLATE 4. Typical Positive Result. Bacillus licheniformis (Medium B1; 3,4,5-Trimethoxybenzaldehyde)

PLATE 5. Typical Positive Result. Proteus vulgaris (Medium B1; Ferulic Acid)



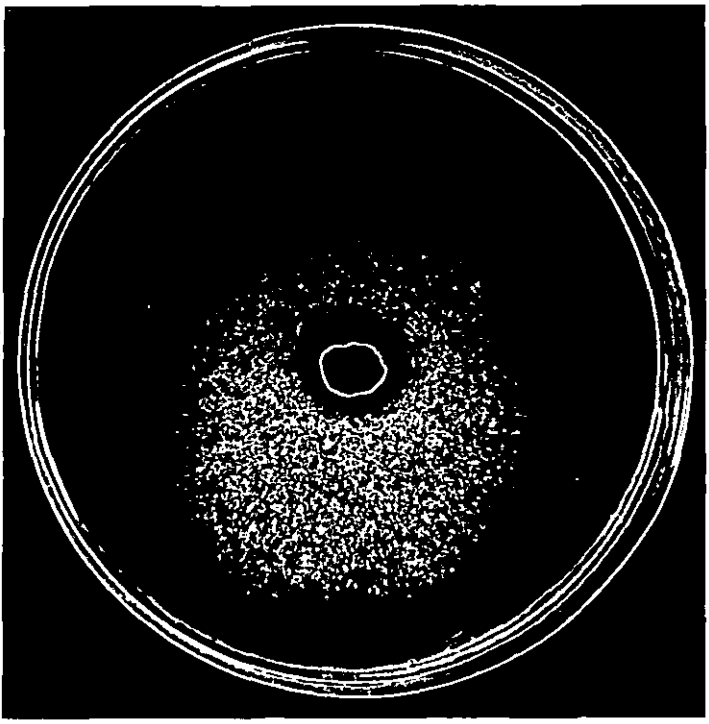


TABLE 6

Screening Of Fungi For Ability To Degrade Lignin-Related Aromatic Compounds

+ indicates the presence of a clear zone around the inoculation site after incubation for 1 to 3 days.

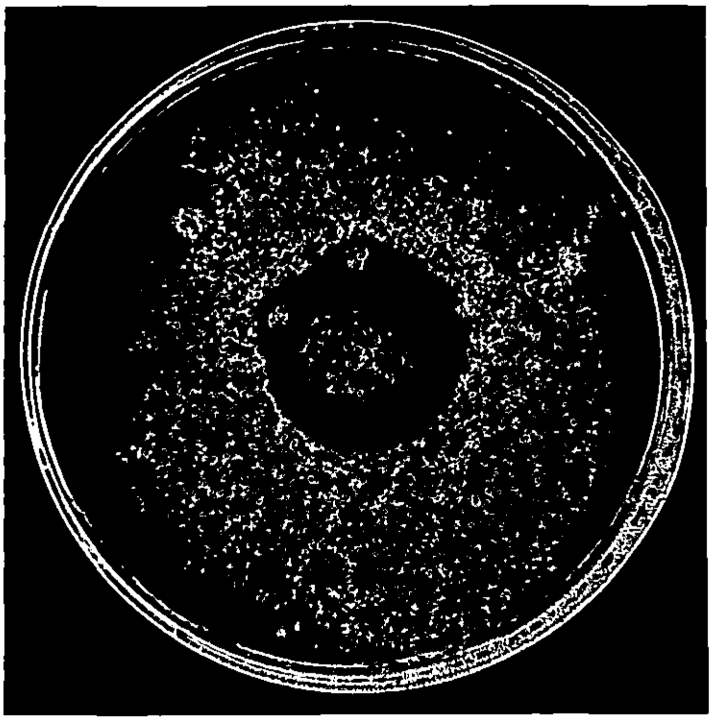
ORGANISM	Aromatic Compound & Growth Medium											
	i		ii		iii		iv		v		vi	
	F1	F2	F1	F2	F1	F2	F1	F2	F1	F2	F1	F2
Sacc.cerevisiae	-	-	-	-	-	-	-	-	-	-	-	-
Pol.versicolor	+	-	+	+	-	-	-	-	-	-	+	+
Pol.dichrous	+	+	+	+	-	-	-	-	-	-	+	-
A.fumigatus	+	+	+	+	-	-	+	+	-	+	+	-
A.flavus	+	+	+	+	-	-	+	+	+	+	+	+
A.nidulans	-	+	-	+	-	-	+	-	+	-	+	-
Schizo.commune	+	+	-	-	-	-	-	-	+	+	+	-
Byss.fulva	+	-	+	-	-	-	-	-	-	-	-	-
C.albicans	-	-	-	-	-	-	-	-	-	-	-	-

i - ferulic acid
ii - cinnamic acid
iii - 3,4,5-trimethoxyacetophenone
iv - 3,4,5-trimethoxycinnamic acid
v - 3,4,5-trimethoxybenzaldehyde
vi - 3,4,5-trimethoxybenzoic acid

The media F1 & F2 are described in the methods

PLATE 6. Positive Result Showing Clear Regions Around Colonies Of Aspergillus flavus (Medium F2; Ferulic Acid). The central colony was the inoculation site. Other colonies caused by distribution of spores are also surrounded by clear zones. This is typical of results found with many fungi forming aerial spores

PLATE 7. Positive Result. Aspergillus nidulans (Medium F2; Cinnamic Acid). Typically, other colonies caused by sporulation are also surrounded by clear zones.



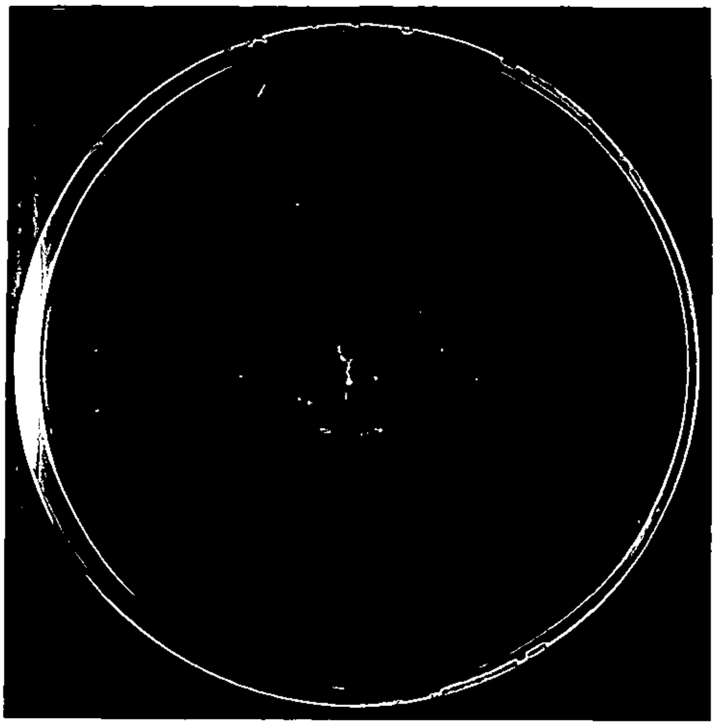
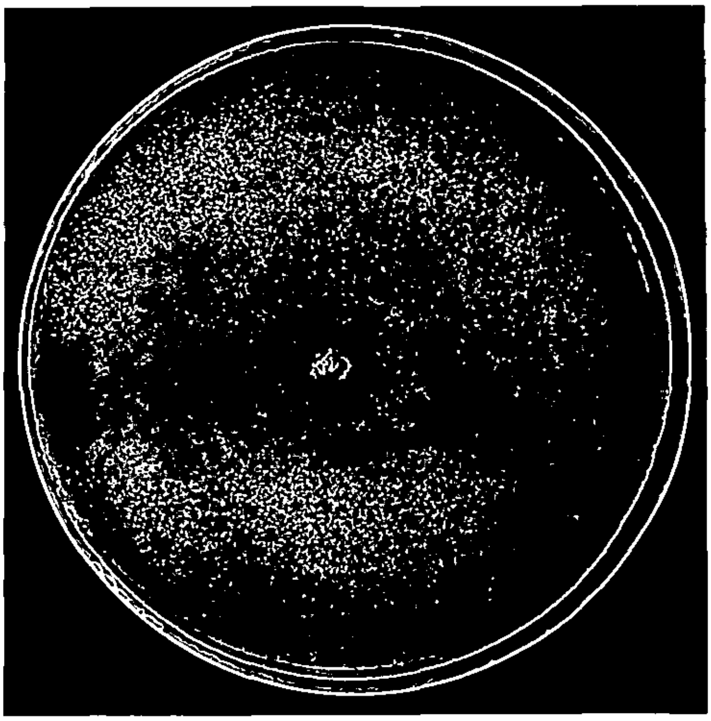
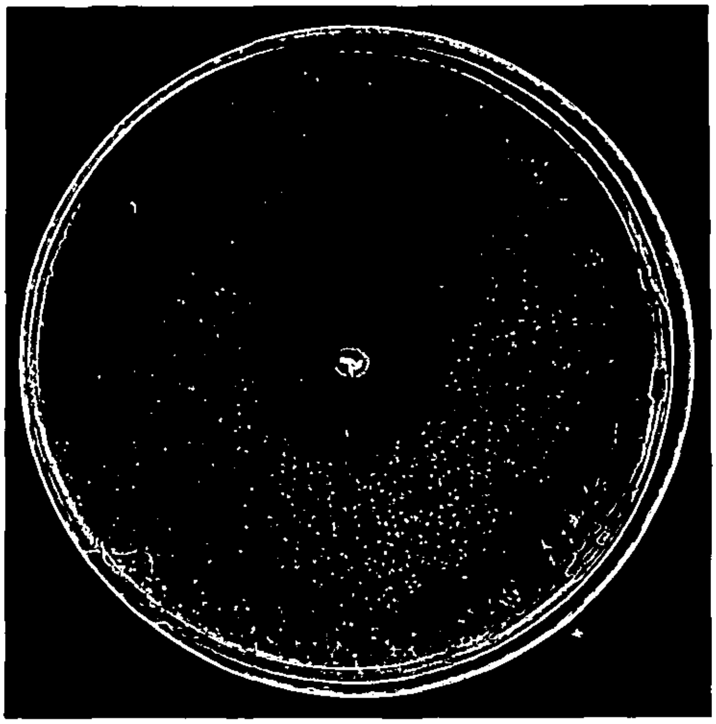


PLATE 8. Typical Positive Result. Byssochlamys fulva (Medium F1; 3,4,5-Trimethoxybenzaldehyde

PLATE 9. Typical Positive Result. Schizophyllum commune (Medium F2; Ferulic Acid)





FIGURES 37 to 44

Growth Of Aspergillus flavus On Lignin Model Compounds

The concentration of carbon sources was 1 g/l. The growth medium is described in the methods.

Shake cultures (120 r.p.m.) were inoculated with a standard inoculum of 10^5 spores/ml and incubated at 30°C. Samples for dry weight determination were taken at 24 hr intervals or less, over a 5 day period.

Each point is the mean of 6 determinations.

FIGURE 37

Growth Of *Aspergillus flavus* On Glucose

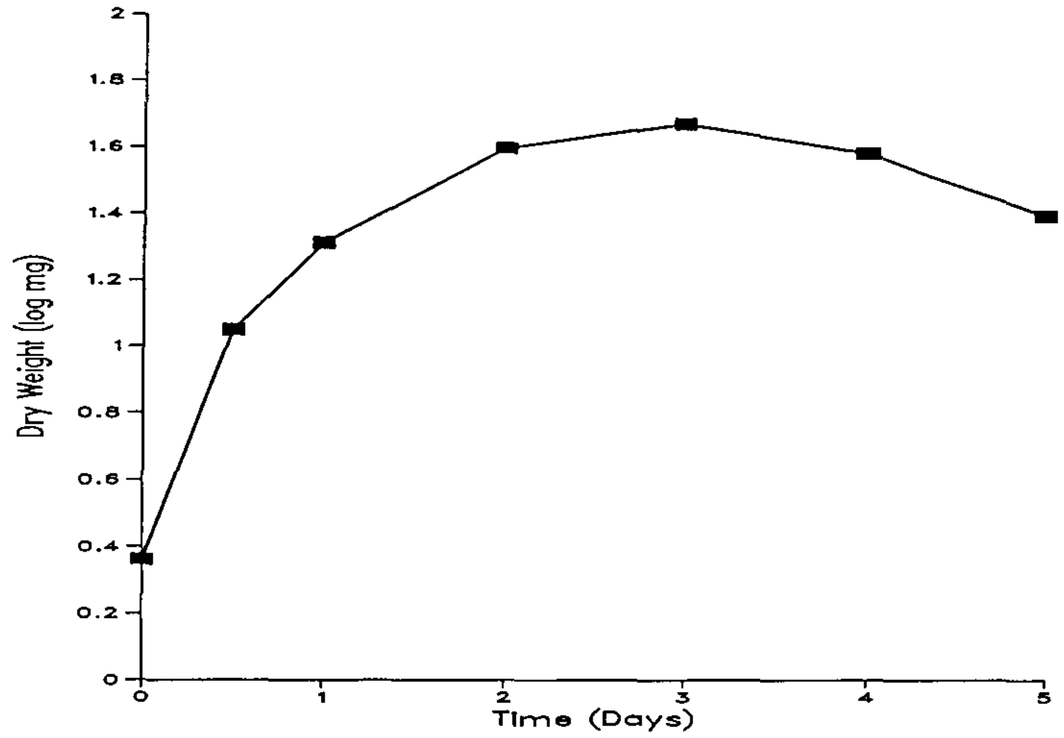


FIGURE 38

Growth Of *Aspergillus flavus* On Compound (2)

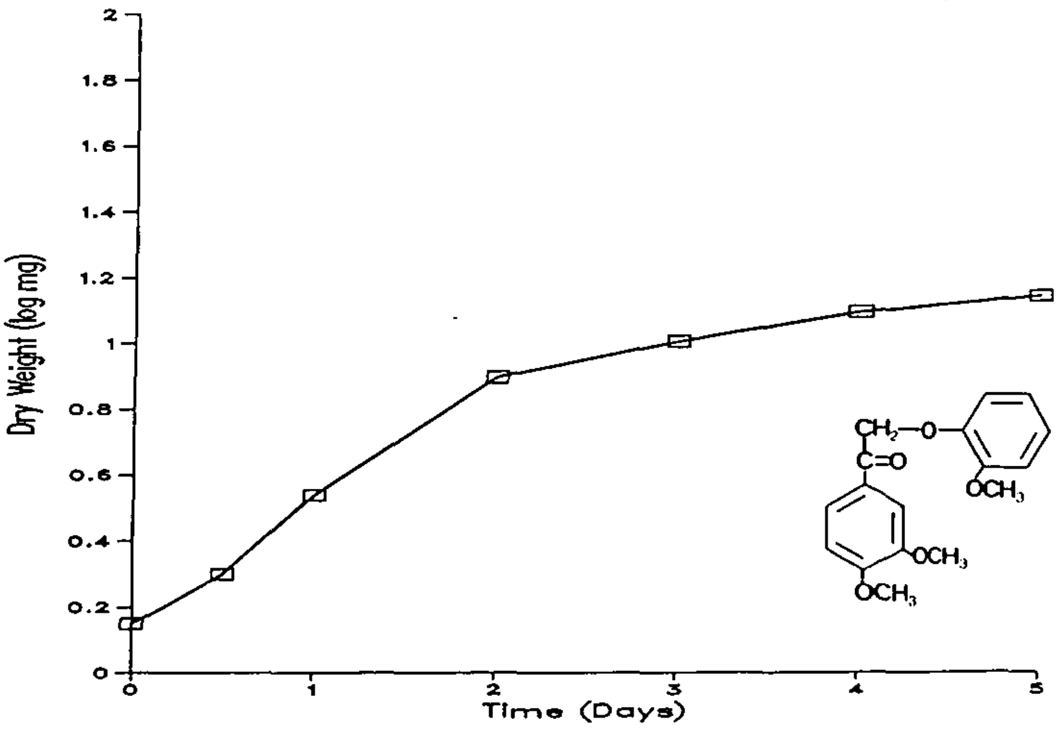
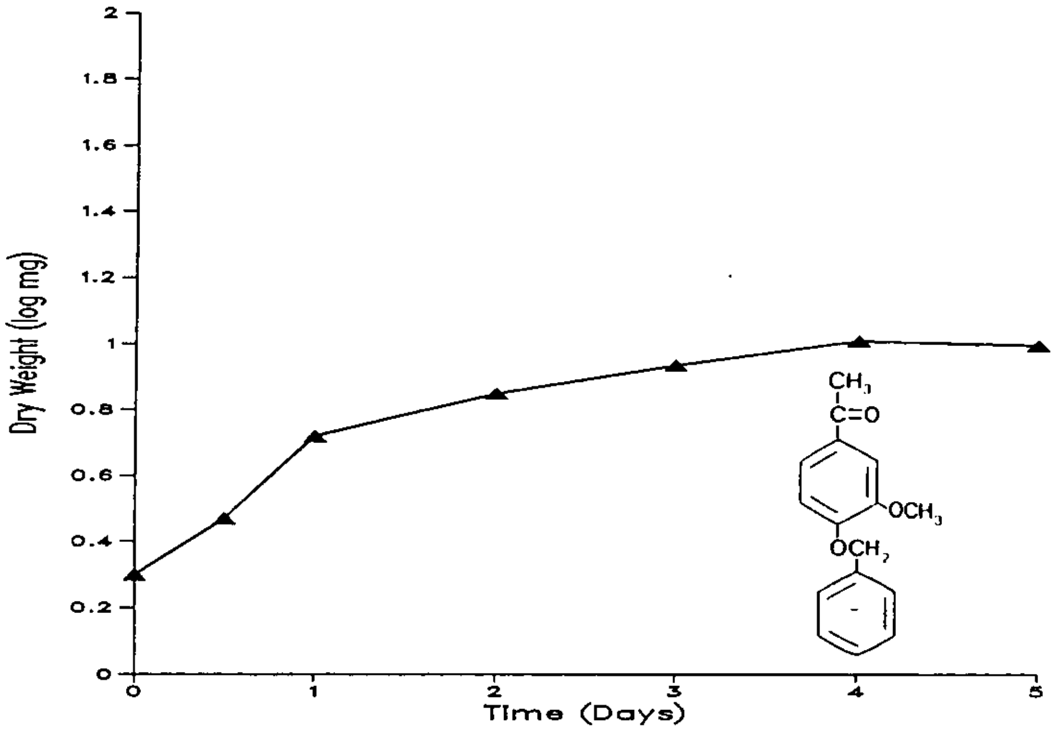
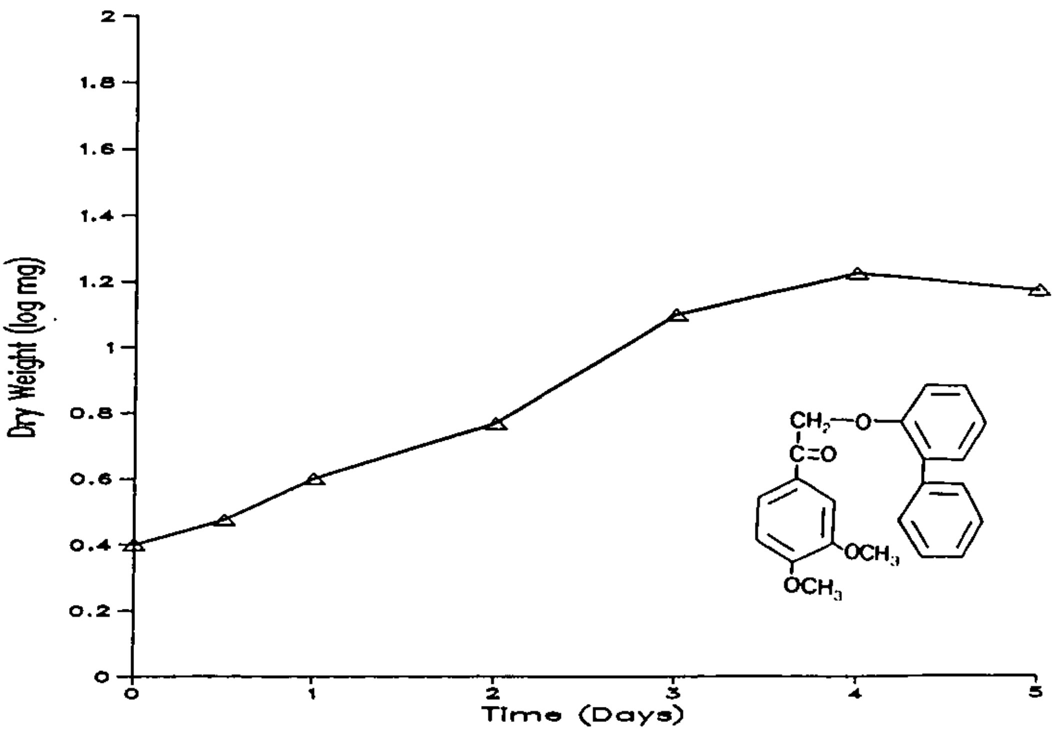


FIGURE 39



Growth Of *Aspergillus flavus* On Compound (4)

FIGURE 40



Growth Of *Aspergillus flavus* On Compound (9)

FIGURE 41

Growth Of *Aspergillus flavus* On Compound (6)

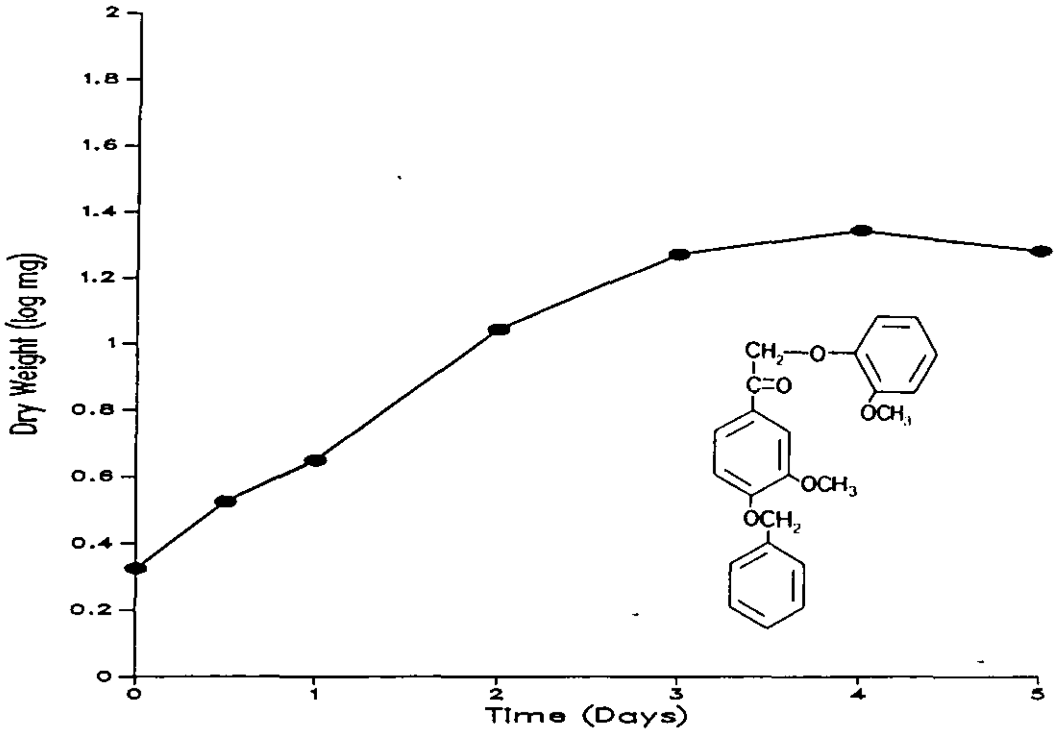


FIGURE 42

Growth Of *Aspergillus flavus* On Compound (7)

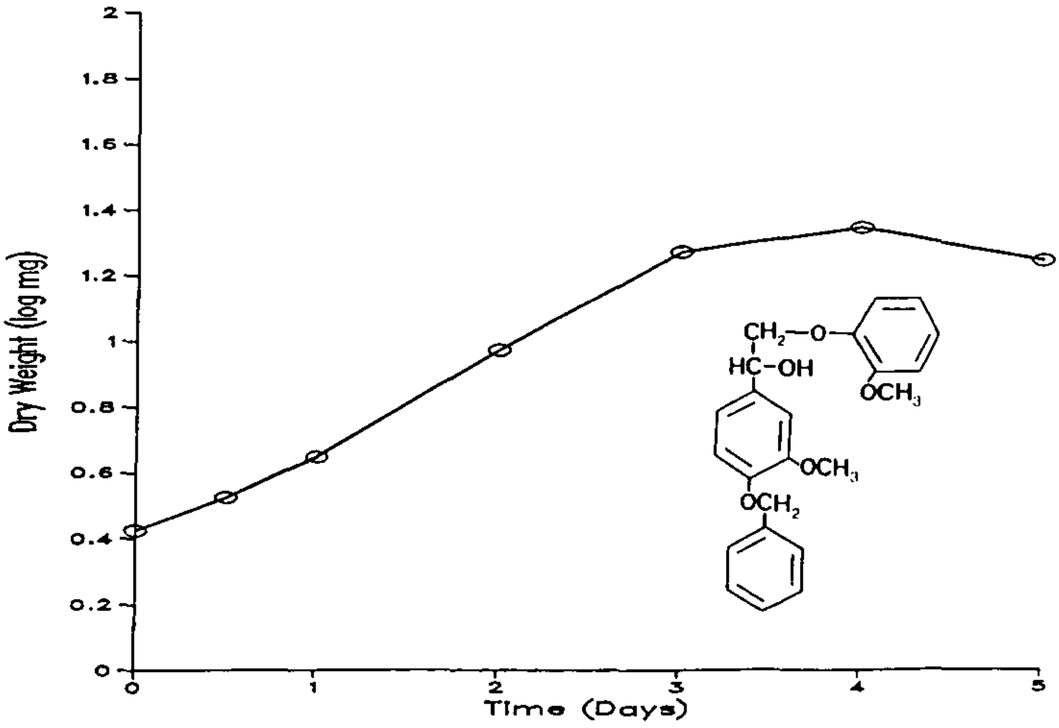
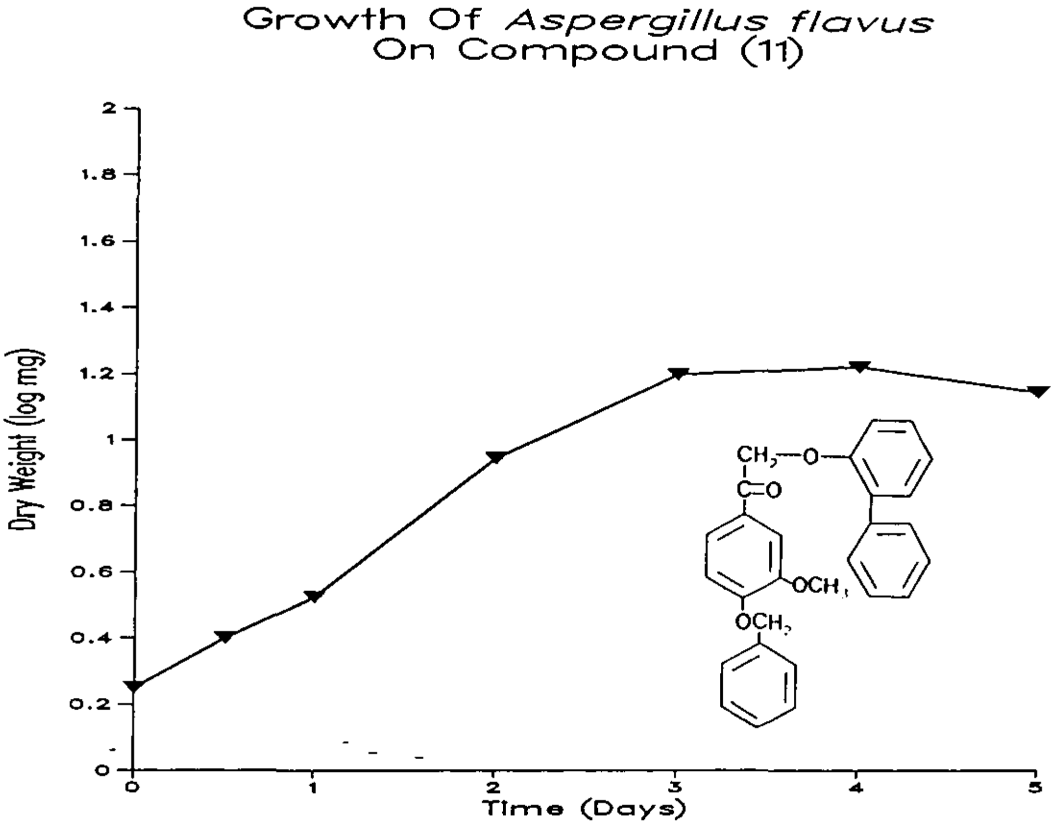
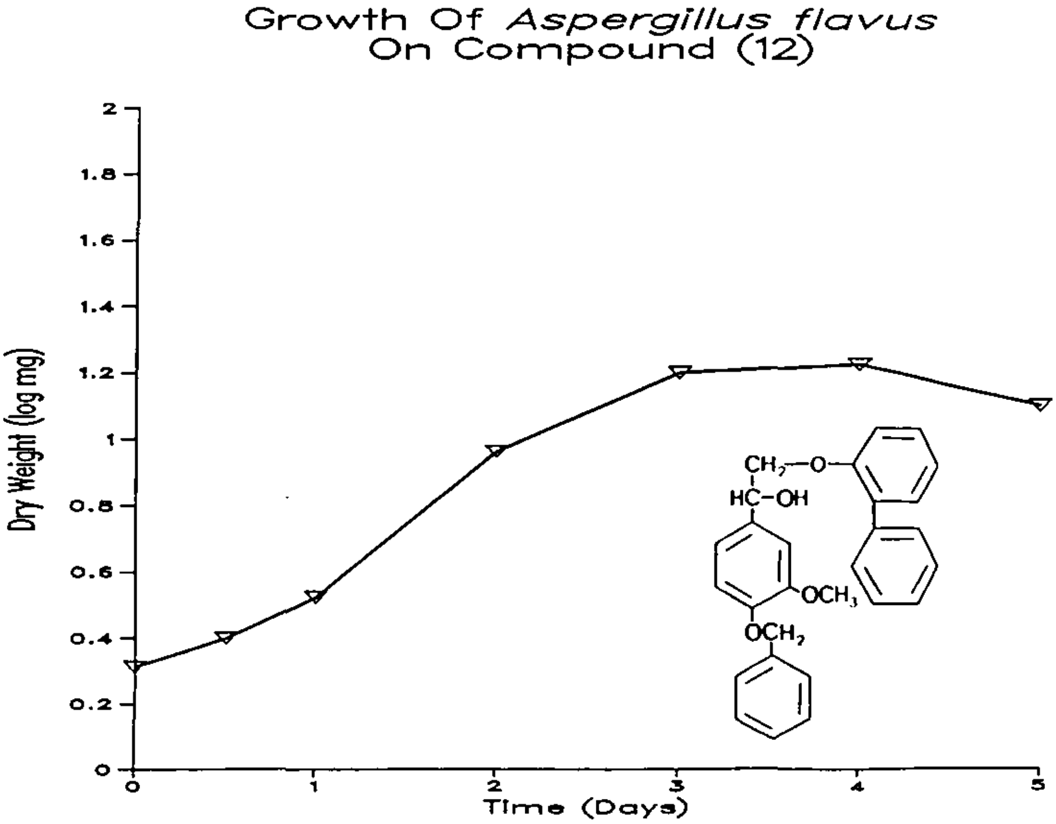


FIGURE 43



Growth Of *Aspergillus flavus* On Compound (11)

FIGURE 44



Growth Of *Aspergillus flavus* On Compound (12)

PHYSICAL ASPECTS OF THE DEGRADATION OF LIGNIN AND LIGNIN MODEL COMPOUNDS BY *Aspergillus flavus*

SEM Observations On Mycelial Samples After Growth On Lignin Model Compounds

3,4-Dimethoxy-ω-(2-methoxyphenoxy)-acetophenone (Compound 2)

The flasks containing Compound (2) had a cloudy appearance due to the compound in fine suspension at the time of inoculation. The flasks became clear over the following 3 to 4 days and electron microscopy showed that the mycelial pellets had become associated with crystals (Plates 10 & 11). The mycelial-crystal associations could also be seen clearly under the light microscope (Plate 12). Samples of these mycelial pellets were washed with water and then extracted with acetone to remove the attached crystals. Examination of the acetone extract by TLC showed the crystals to be Compound (2).

Closer examination of the mycelium-crystal associations showed two main effects. The mycelia were in intimate contact with the crystals. A typical hyphal strand (Plate 13) is shown which has collapsed under the vacuum either during dessication of the sample or during evacuation of the SEM. Flattened points can be seen along this strand suggesting firm attachment to the crystal. Mycelia were also observed on several occasions to be broken in preference to being pulled from the surface of the crystals (Plate 11, arrowed). The second effect is shown in Plates 14 and 15 (arrows), where erosion of the crystal face is apparent beneath the growing hyphal tip. This type of erosion was not observed in uninoculated control samples. Close examination of Plate 14 suggested that many hyphae lie in grooves (arrows) in the crystals and not on the flat surfaces of the crystals.

1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)ethanol (Compound 3)

The same effects were seen in samples taken from flasks containing Compound (3) as for those with Compound (2). The initial cloudiness of the medium disappeared over the growth period and containment of crystals of Compound (3) within mycelial pellets was seen in electron micrographs (Plate 16). TLC showed the associated crystals to be Compound (3). Intimate contact between mycelia and crystals was also observed in this sample (Plate 17).

4-Hydroxy-3-methoxy-ω-(2-methoxyphenoxy)acetophenone (Compound 8)

Similar effects were seen in samples grown on Compound (8) as those shown when organisms were grown on Compounds (2) and (3). Compound (8) however, appeared as flattened plates rather than obloid crystals. Aggregation and binding together of these plates by mycelia was frequently seen (Plate 18). Intimate attachment of a mycelial growing tip to a crystal plate was observed at higher magnifications (X 2145) (Plate 19).

1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-ethanol (Compound 7)

The addition of the acetone solution of Compound (7) to the growth medium produced a cloudy suspension at the time of inoculation. The medium became clear and mycelial pellets formed during the 4 day growth period. Electron micrographs showed that crystals had become associated with the mycelial pellets. Plate 20 shows splitting of a mycelial outer layer and exposure of crystalline material beneath. The crystals were shown to be Compound (7) using the same extraction and TLC procedure as described above.

4-Benzyloxy-3-methoxy-ω-(2-phenylphenoxy)acetophenone (Compound 11)

An aggregation effect similar to that described previously occurred when A. flavus was grown on Compound (11). Mycelial spheres became associated with a solid material and the initially cloudy growth medium became clear. The associated material was shown to be Compound (11) by the usual extraction and TLC method. The compound did not have a regular crystalline form but appeared as clumps of solid material (Plate 21). Intimate associations were observed and Plates 22 & 23 show typical observations of the binding together of the solid material by mycelia.

4-Hydroxy-3-methoxy-ω-(2-phenylphenoxy)acetophenone (Compound 13)

Plates and needles of crystalline material became associated with mycelia during the 4 days growth of A. flavus on Compound (13). The initial cloudy medium became clear. Close contact between mycelia and crystals could be seen at higher magnification (X 2500) (Plate 24). The crystals were shown to be Compound (13) by the method used previously.

Autohydrolysis Aspen Lignin

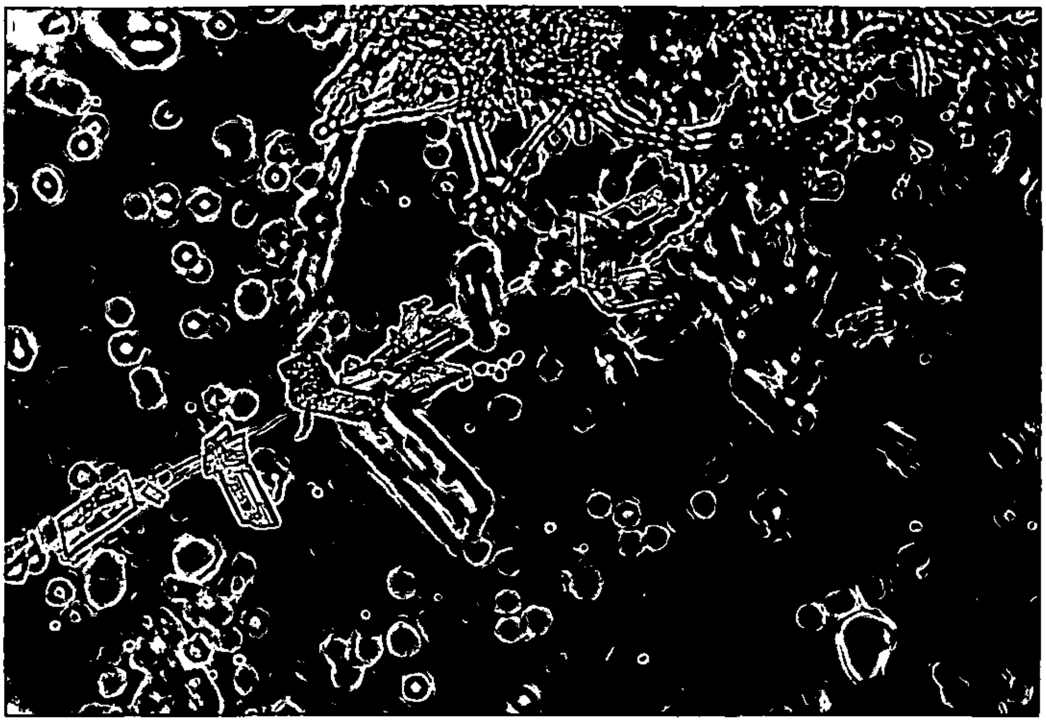
The lignin appeared as spherical particles in the electron micrographs. Aggregation of the lignin and associations with mycelial spheres occurred during growth and very little lignin remained as free solid in the medium after incubation for 4 days. Examples of mycelium-lignin contact are shown in Plates 25 & 26, and the strength of the associations is demonstrated by the strained hyphal strand (arrowed) which still remained attached to a lignin particle. A higher magnification (X 5200) of the intimate contact of a growing hyphal tip with a lignin particle is seen in Plate 27.

PLATE 10. Photomicrograph Showing Association Between Mycelial Pellets Of A. flavus And Crystals Of Compound (2). (X 790)

PLATE 11. Photomicrograph Showing Close Association Of Hyphae Of A.flavus With Large Crystals Of Compound (2). (X 1350)

PLATE 12. Light Microscope Photograph Of Mycelia Of A. flavus Showing Attachment Of Compound (2).

PLATE 13. Photomicrograph Of A Hyphal Strand Of A. flavus In Intimate Contact With A Crystal Of Compound (2). The strand has collapsed under the vacuum during dessication and points of very strong attachment are shown (arrowed). (X 4000)



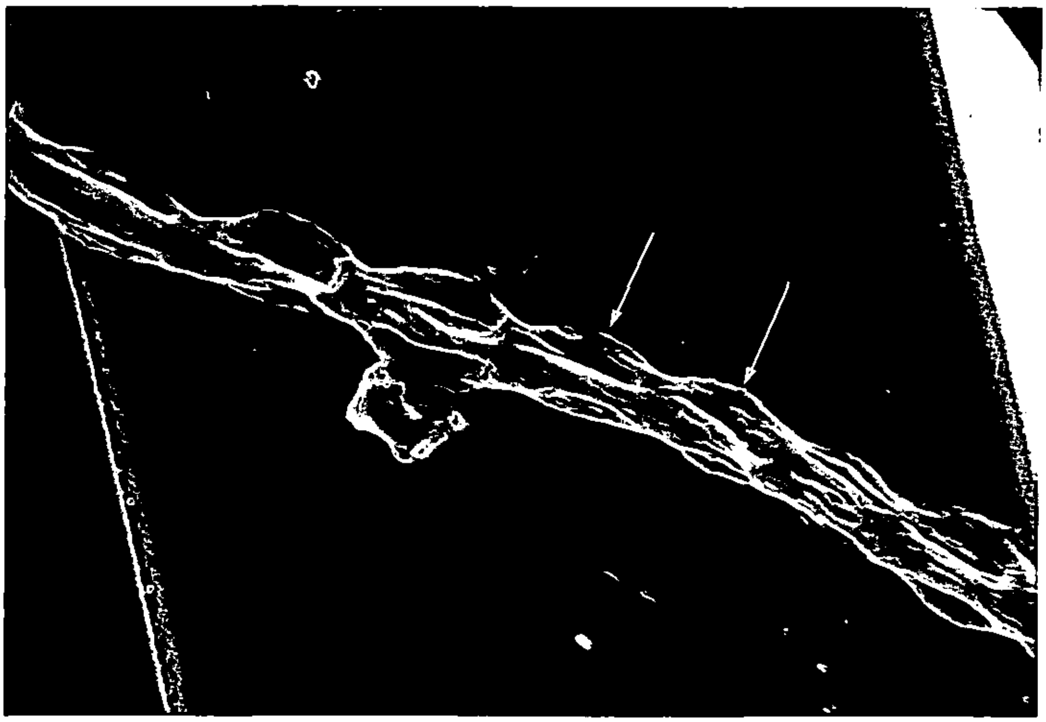


PLATE 14. Photomicrograph Of A Growing Hyphal Tip Of A. flavus Abov A Region Of Erosion Of A Crystal Of Compound (2). The photomicrograph also shows hyphae lying in grooves (arrowed) (X 4500)

PLATE 15. Photomicrograph Of Cultures Of A. flavus Incubated With Compound (2) For 72 h Showing Intimate Contact. Areas of crystal erosion can be seen (arrows). (X 2500)

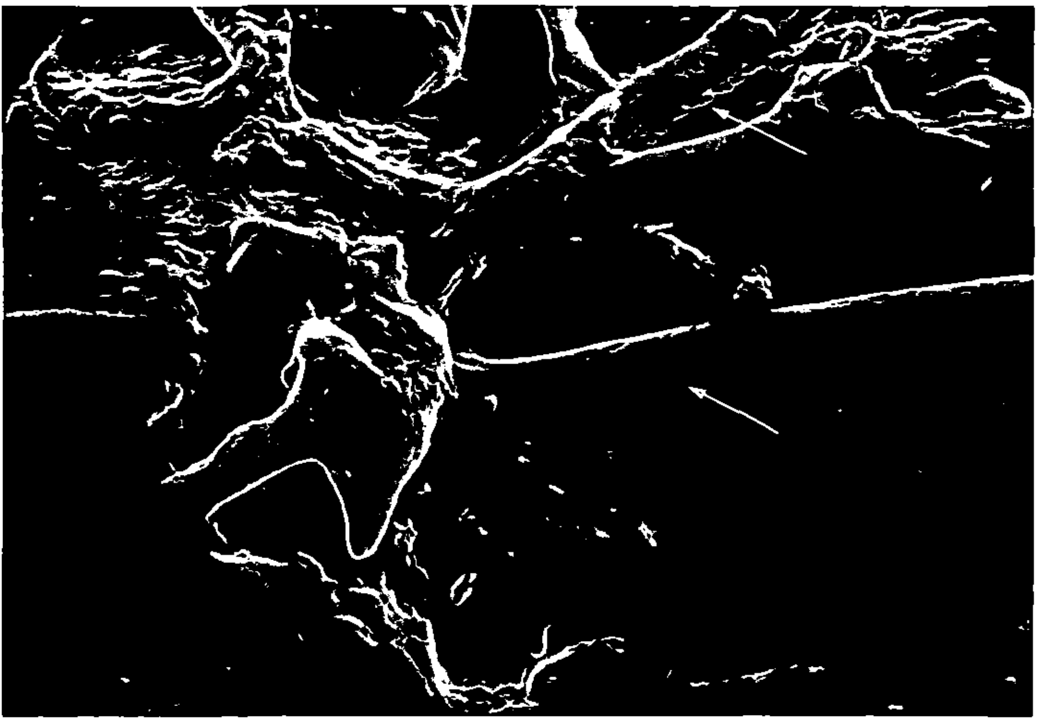
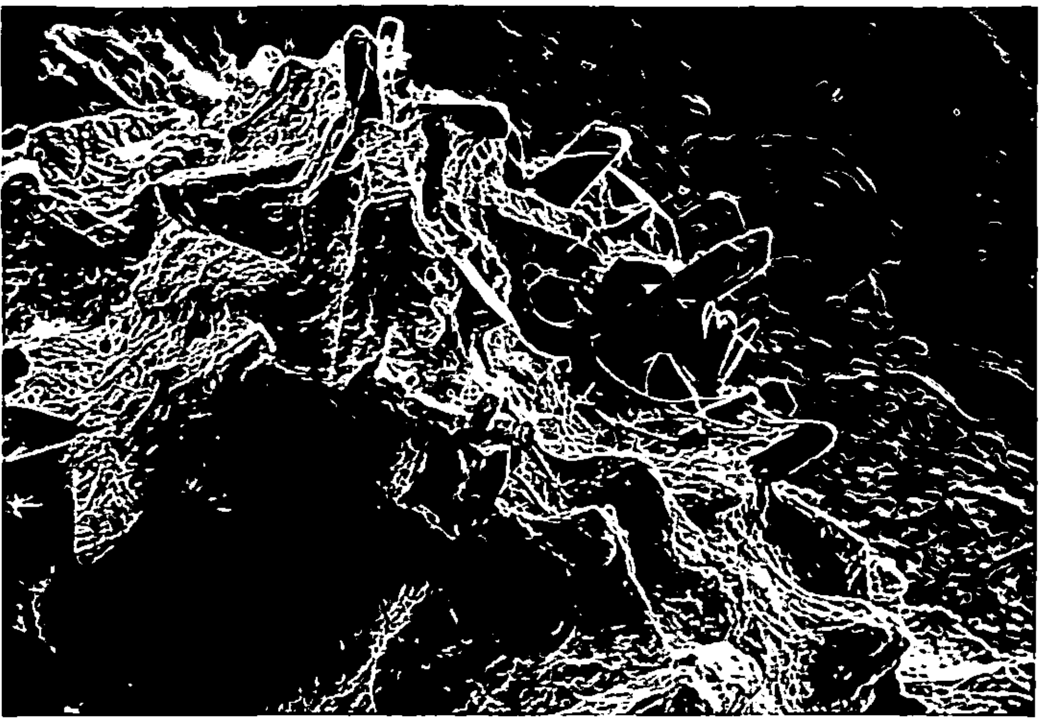


PLATE 16. Photomicrograph Showing Containment Of Crystals Of Compound (3) Within A Mycelial Pellet Of A. flavus. (X 200)

PLATE 17. Photomicrograph Showing Intimate Contact Between Mycelia Of A. flavus And Compound (3). (X 1000)



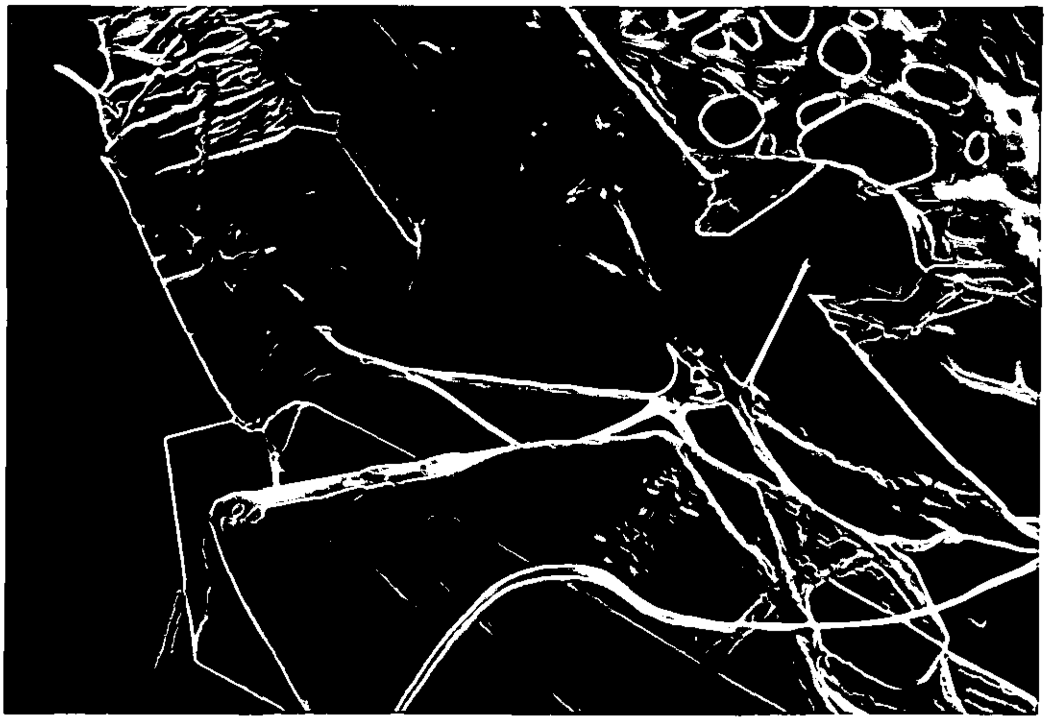


PLATE 18. Photomicrograph Showing Aggregation And Binding Together Of Plates Of Compound (8) By A. flavus. (X 600)

PLATE 19. Enlargement Of A Section Of Plate 18 To Show The Intimate And Strong Contact Between A Hyphal Tip Of A. flavus And A Crystal Of Compound (8). (X 3900)

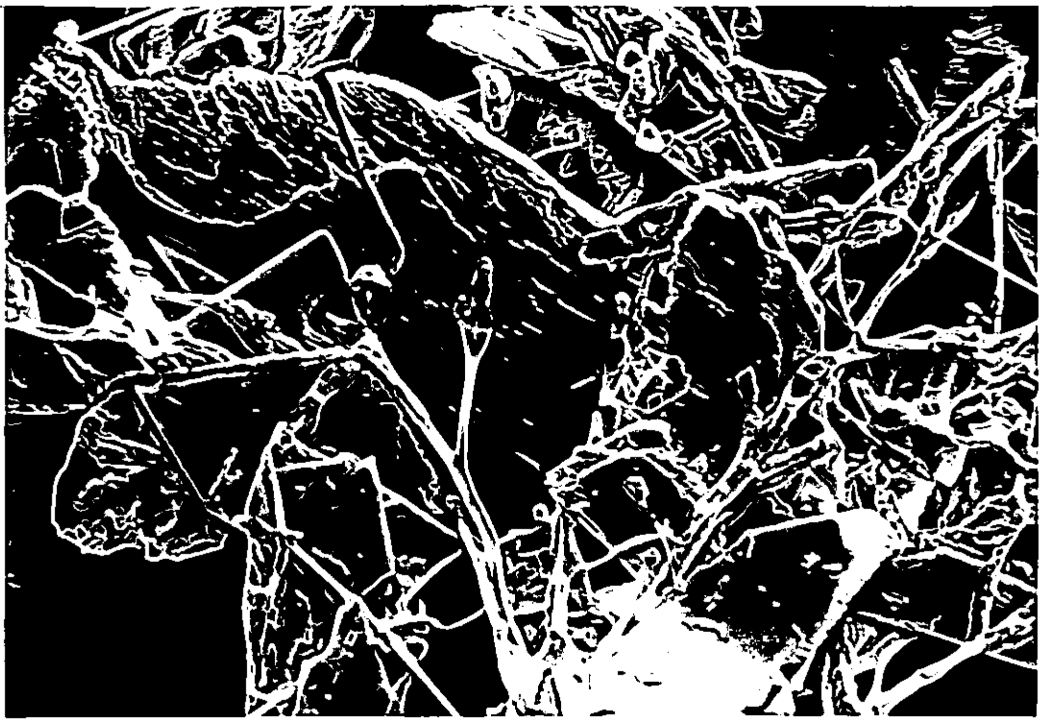
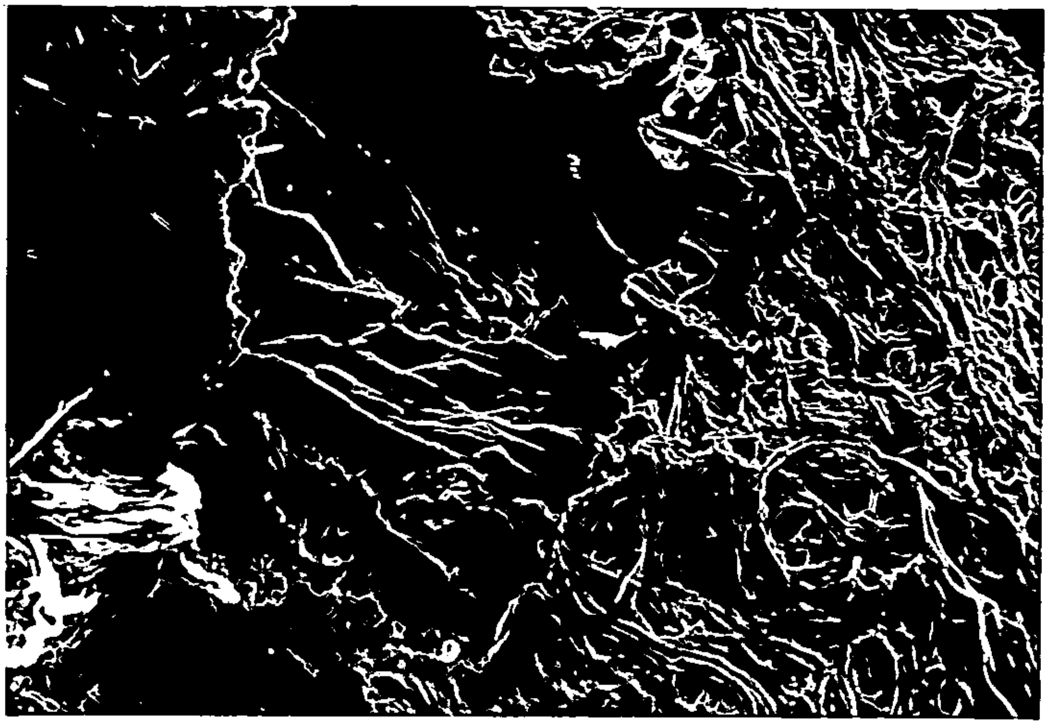


PLATE 20. Photomicrograph Showing Splitting Of The Outer Layer Of A Mycelial Pellet Of A. flavus Exposing The Crystalline Compound (7) Beneath. (X 810)

PLATE 21. Photomicrograph Of The Aggregations Of A. flavus And Compound (11). (X 44)



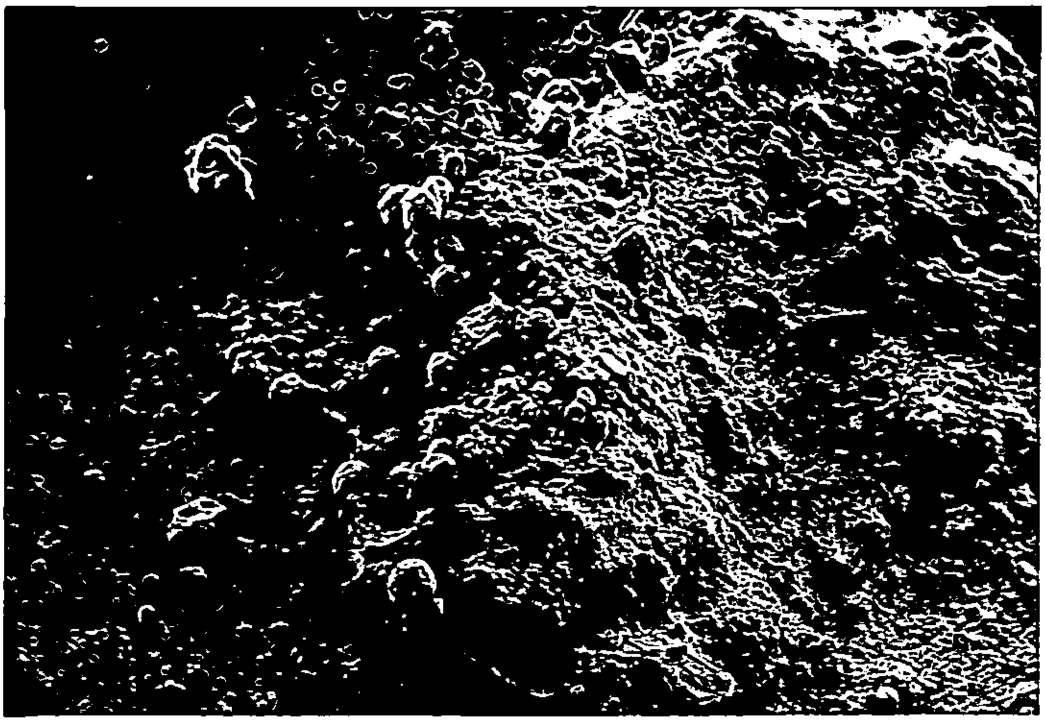
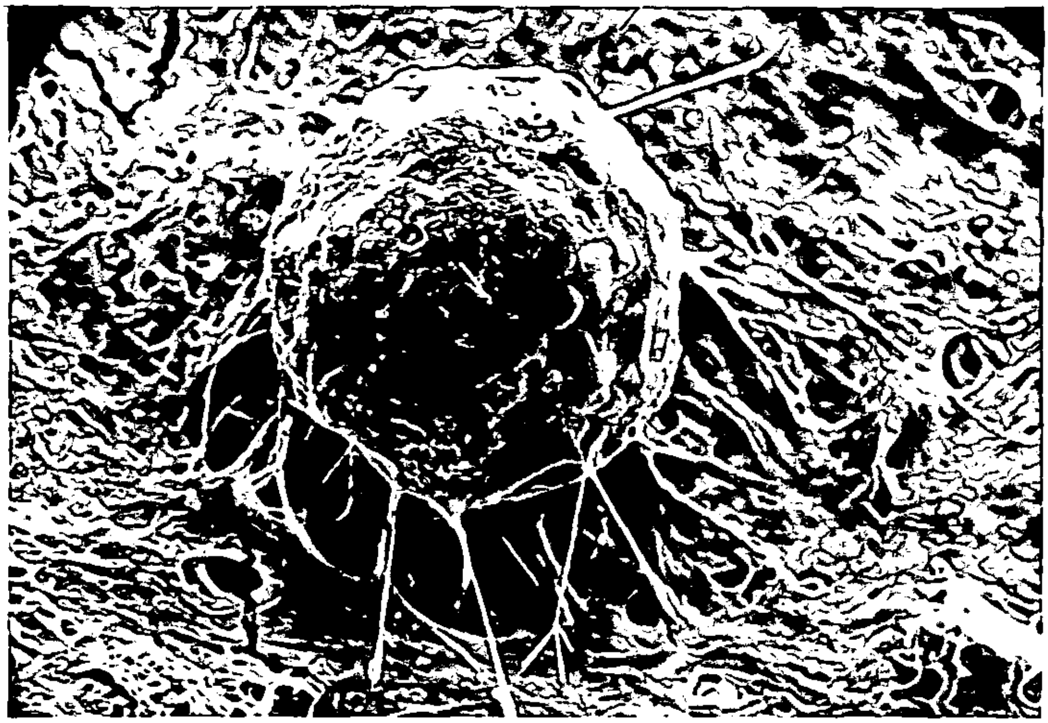


PLATE 22. Photomicrograph Showing The Very Strong And Intimate Contacts Between A. flavus And Compound (11). (X 630)

PLATE 23. Photomicrograph Showing Associations Between A. flavus And Compound (11) . The contacts remain even at points of great stress (arrowed) thus demonstrating their great strength. (X 460)



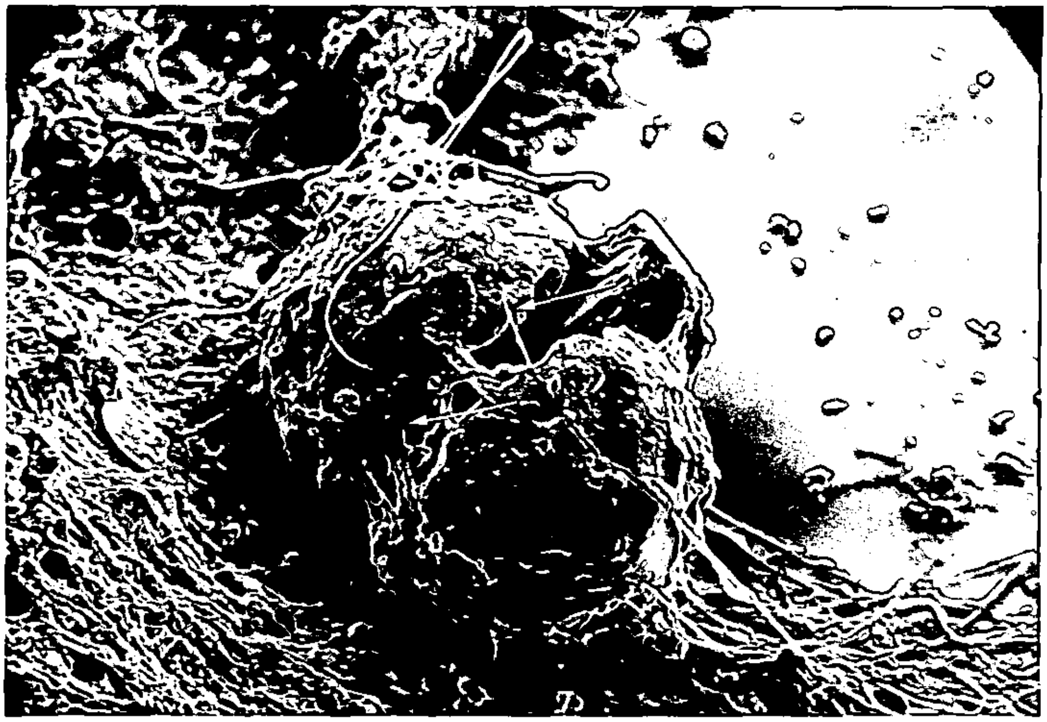


PLATE 24. Photomicrograph Showing Close Contact Between Mycelia Of A. flavus And Crystals Of Compound (13). Very close contact can be seen across the central crystal (arrowed). (X 2500)

PLATE 25. Photomicrograph Of A Mycelial Sphere Of A. flavus Associated With Autohydrolysis Aspen Lignin. A hyphal strand attached to a lignin particle (arrowed) demonstrates the strong contact. (X 1130)

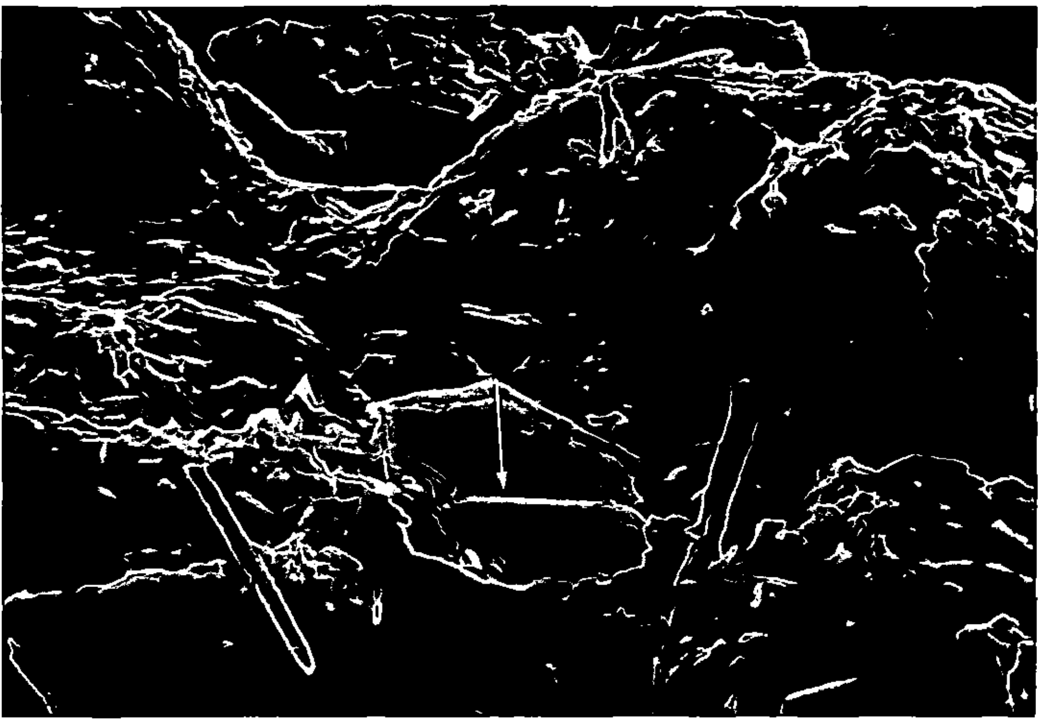
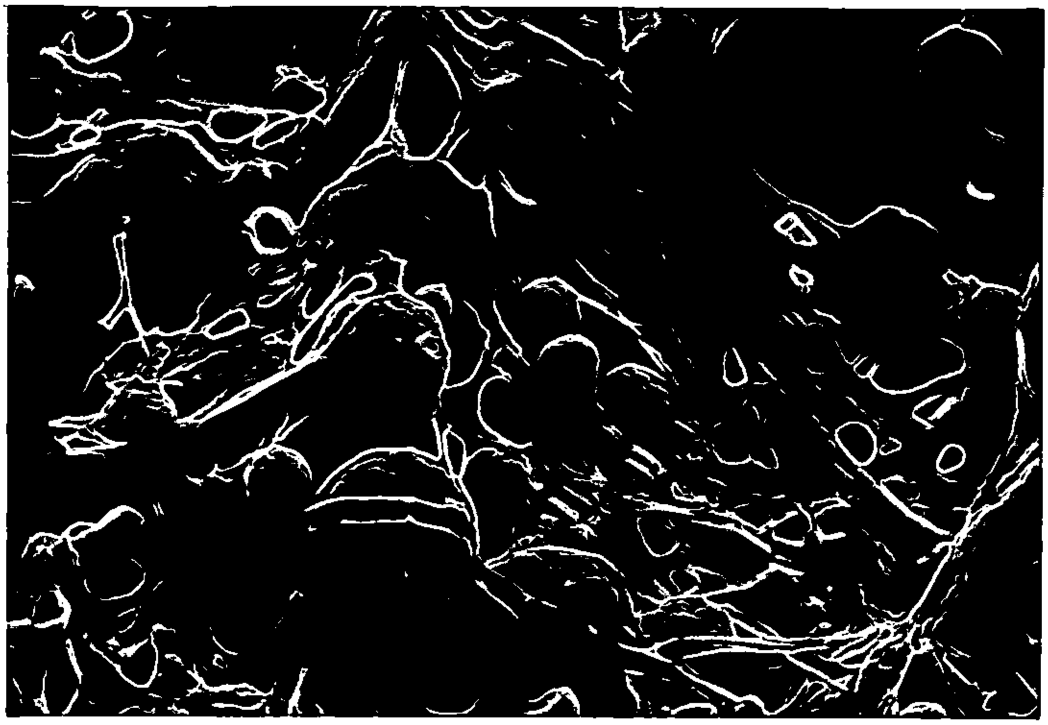


PLATE 26. Photomicrograph Showing Binding Together Of Lignin Particles By Mycelia Of A. flavus. (X 1130)

PLATE 27. Photomicrograph Of A Growing Hyphal Tip Of A. flavus Attached To A Particle Of Aspen Lignin. (X 5200)



SEM Observations And Measurement Of Changes In Crystal Length Of Compound (2) During Incubation With A. flavus

Dry weight measurements of A. flavus grown on Compound (2) over a 5 day period showed substantial growth (Figure 45) and measurements of crystal lengths of Compound (2) taken at various time intervals showed a reduction in mean length (Figure 46).

Identification of residual crystals by TLC (as previously described) after 5 days growth showed that they were Compound (2) and not a metabolite. Filtration of the medium to remove the mycelia and subsequent extraction as described in the methods section showed very little of Compound (2) to be present.

Long sharp crystals were seen in suspension in the medium at the time of inoculation (Plate 28).

Mycelial pellets had formed in the shaken flasks after 24 hours and electron micrographs showed some association between hyphae and crystals (Plate 29). Close association of mycelia and crystals could be seen after 48 hours and hyphae could be seen with crystals attached to them (Plate 30).

Growth for 72 hours produced dense pellets with a reduction in the amount of observable crystalline substrate (Plate 31). Crystals were generally smaller although a few large ones could still be seen.

Samples examined at 96 hours showed a few small crystals and fungal spores were present (Plate 31).

Higher magnification electron micrographs (X 2500) of cultures grown for 72 hours showed intimate contact of mycelia and crystals and erosion of crystal faces close to hyphae. This was shown in Plate 15.

Examination of mycelial pellets over the 5 day time course of experiments showed a concentration of crystals within the mycelia.

FIGURE 45

Growth Of *Aspergillus flavus* On Compound (2)

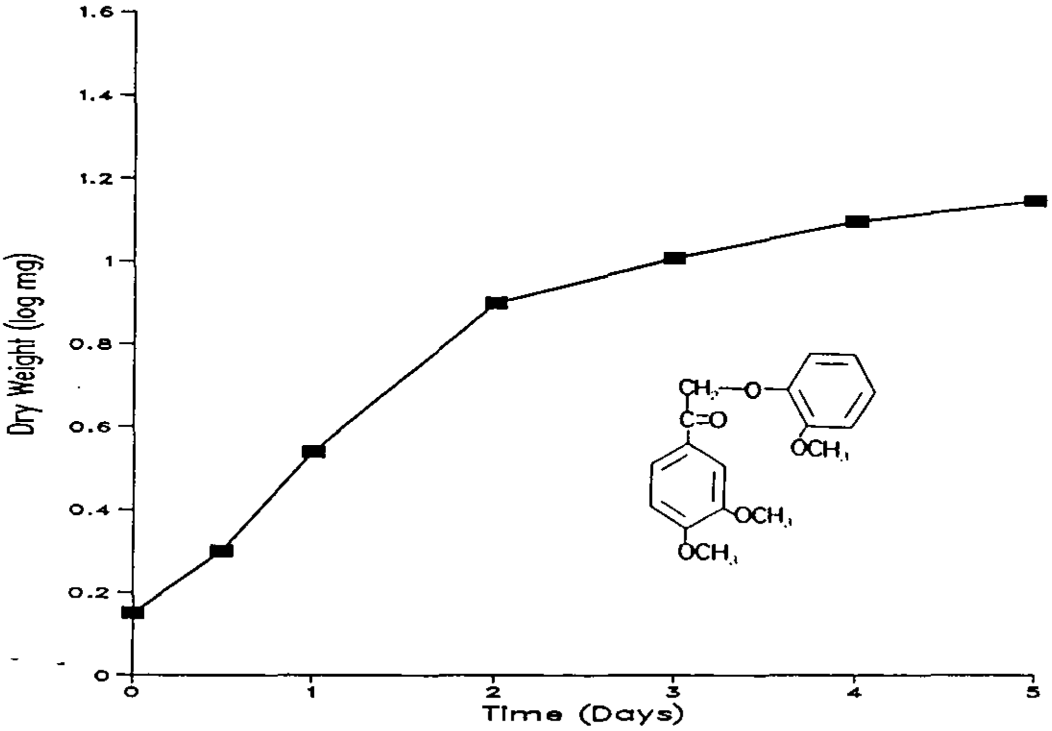


FIGURE 46

Decrease In Crystal Length Of Compound (2)

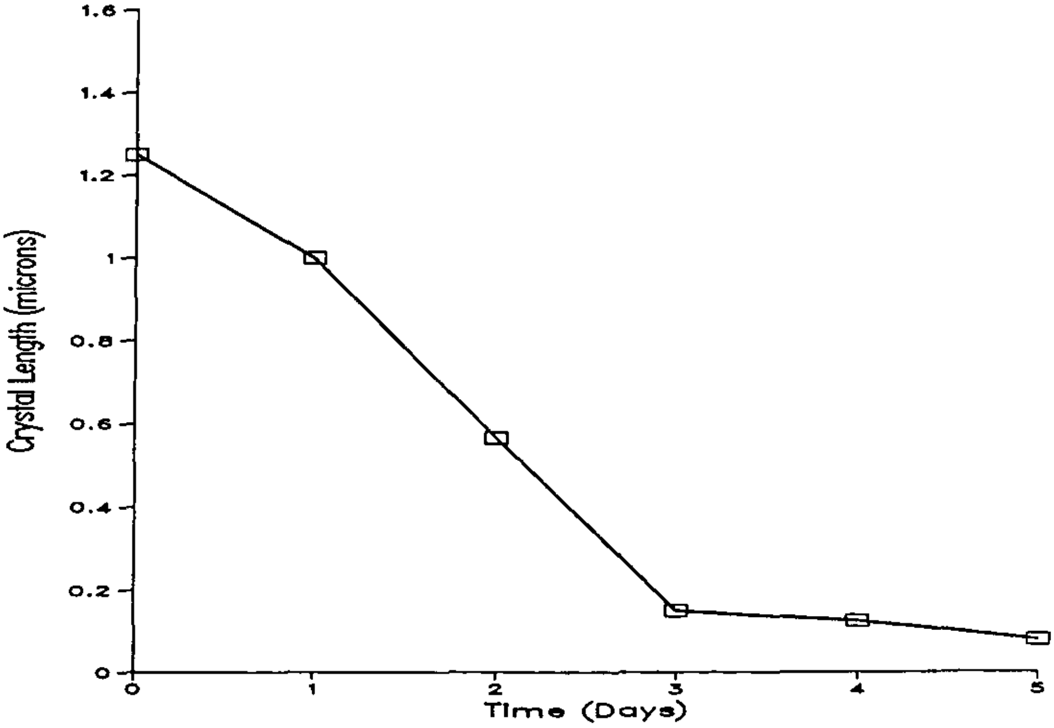


PLATE 28. Photomicrograph Showing Long Sharp Crystals Of Compound (2) At The Time Of Inoculation With Spores Of A. flavus. (X 1500)

PLATE 29. Photomicrograph Showing Some Associations Between A. flavus And Compound (2) 24 h After Inoculation. (X 1500)

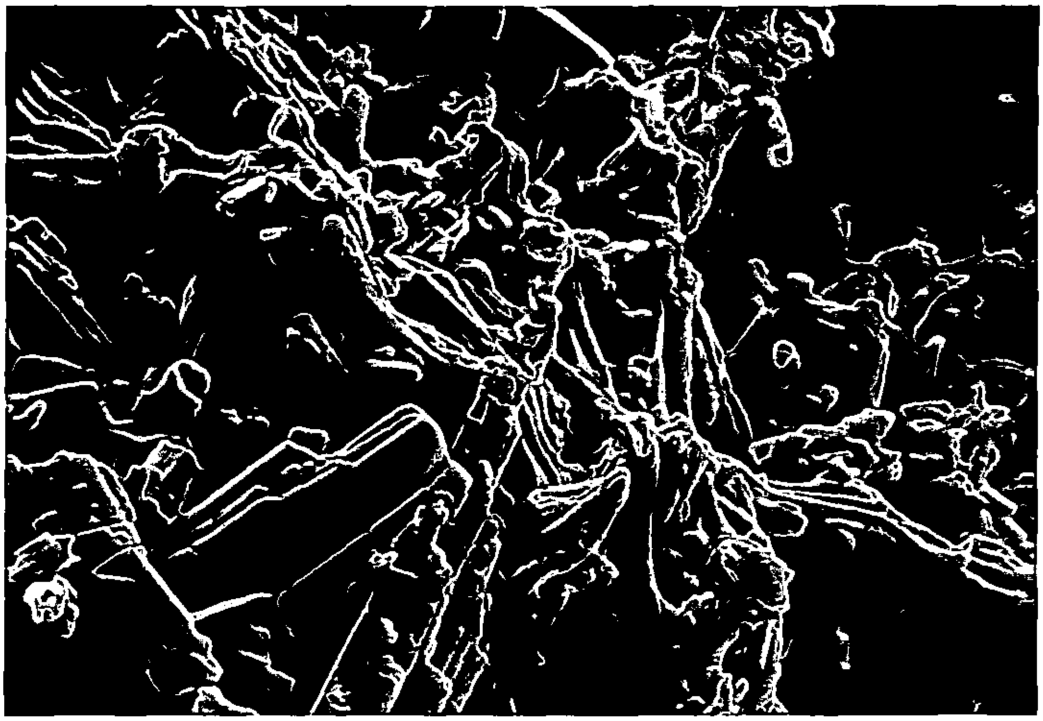
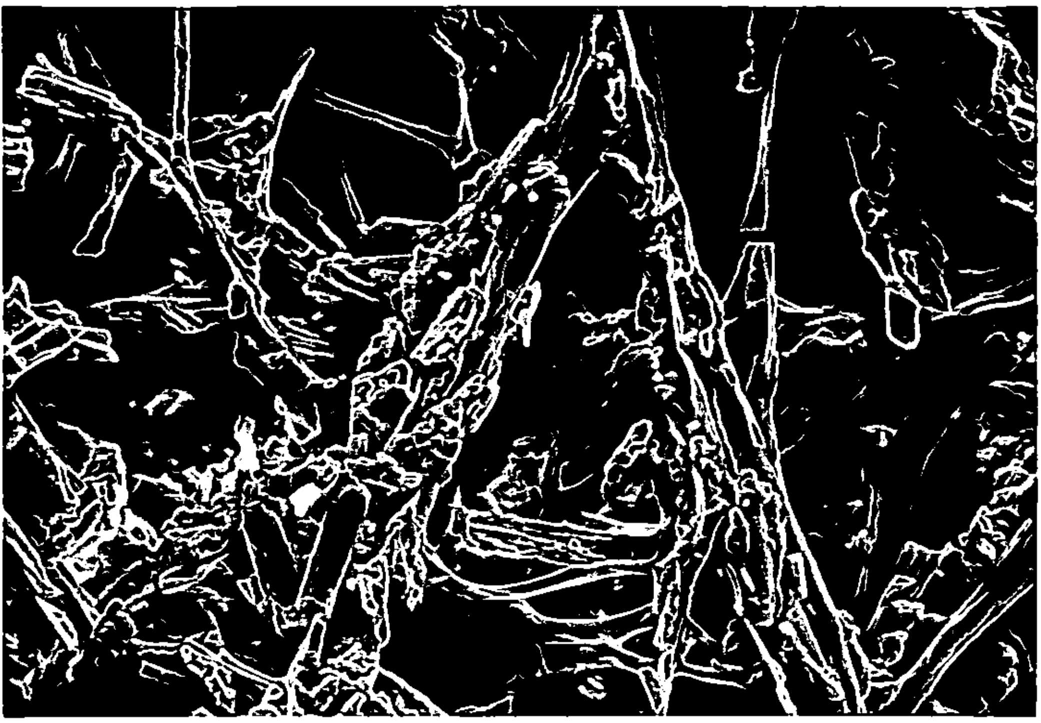
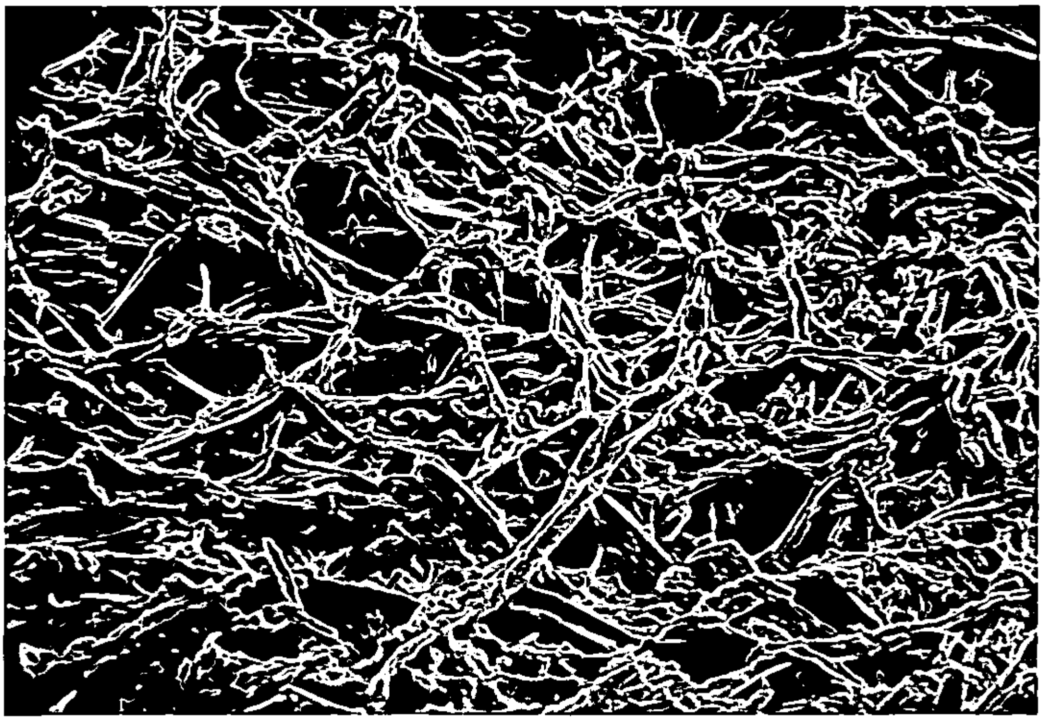


PLATE 30. Photomicrograph Taken 48 h After Incubation Of A. flavus With Compound (2). (X 1500). Crystals of compound can be seen attached to hyphae.

PLATE 31. Photomicrograph Taken After 72 h Incubation Of A. flavus With Compound (2) Showing Dense Mycelial Growth And Fewer Smaller Crystals. (X 1500)





Dimerization Of 2,6-dimethoxyphenol By *Aspergillus flavus*

Identification Of Isolate

The coupling product of 2,6-dimethoxyphenol was isolated as dark purple crystals. These were identified as tetramethoxy-ρ-dibenzoquinone.

Mp 291-293°C [lit. 294°C]; ms m/z 304.4218.

After acetylation - mp 238-240°C [lit. 239°C]; H-nmr [$CDCl_3$] δ 6.69 (s, 4H, aromatic), 3.90 (s, 12H, 4 X OCH_3), 2.41 (s, 4H, 2 X CH_3).

Microscopy

Long, dark needles of tetramethoxy-ρ-dibenzoquinone became visible within the mycelial spheres of *A. flavus* after approximately 2 days incubation of the organism with 2,6-dimethoxyphenol. The mycelial spheres appeared as dense, dark bodies after 5 days incubation (Plates 32 & 33). No crystals were found in random samples of the liquid growth medium and only a slight colour change could be observed in the medium. Many of the crystals associated with mycelia appeared attached to hyphae and it was seen that many crystals were aligned parallel to the hyphae (Plate 34).

No crystals were observed neither was there any colour change in the medium when it contained 0.05 g of glucose per 100 ml in addition to 2,6-dimethoxyphenol.

Scanning electron microscopy showed that mycelia were in very close contact with the crystals of dimer (Plates 35 & 36).

Plate 35 shows a mycelial strand which is attached to two crystals. The points of contact are intimate and the strength of the binding is demonstrated by the firm attachment of the mycelium to the crystals even with straining caused by drying and stretching.

PLATE 32. Light micrograph Showing Aggregation Of Crystals Of Tetramethoxy-ρ-dibenzoquinone Within A Whole Mycelial Sphere Of A. flavus. (X 40)

PLATE 33. Light micrograph Of Part Of A Mycelial Sphere Of A. flavus Showing Individual Crystals Of Tetramethoxy-ρ-dibenzoquinone. (X 156)

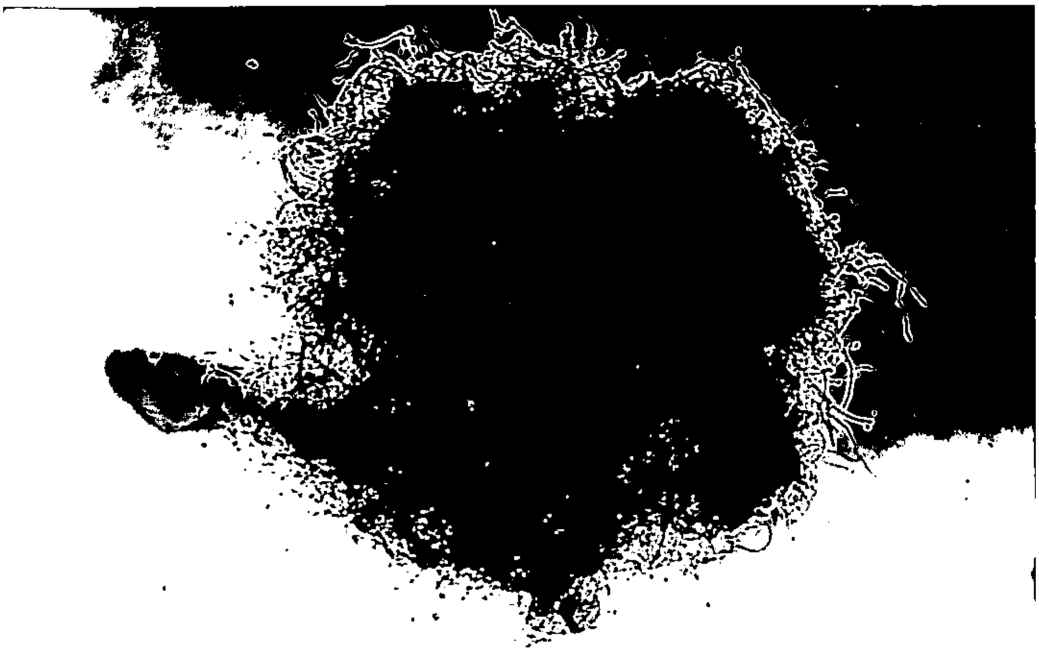


PLATE 34. Light micrograph Of The Edge Of A Mycelial Sphere Of A. flavus. Crystals of tetramethoxy-ρ-dibenzoquinone can be seen attached to mycelia. Typically, a crystal is aligned parallel to a hyphal strand (arrow). (X 156).

PLATE 35. Electron Micrograph Showing Attachment Of A Mycelial Strand Of A. flavus To Two Large Crystals Of Tetramethoxy-ρ-dibenzoquinone. (X 7000)

PLATE 36. Electron Micrograph Showing Associations Between Crystals Of Tetramethoxy-ρ-dibenzoquinone And Mycelia Of *A. flavus*. (X 3500)

THE DEGRADATION OF LIGNIN MODEL COMPOUNDS BY *A. flavus*

Intermediates of the metabolism of lignin model compounds by *A. flavus* were isolated from the growth medium after 3 days growth by the procedure described in the methods.

Analytical TLC was used to establish the Rf values of the intermediates in two solvent systems (see the methods) and these were compared with authentic standards. Preparative TLC was used to obtain sufficient quantities of intermediates for spectral analysis. Analysis by GLC was used to detect volatile intermediates such as methanol and benzyl alcohol in growth media.

The sequences of the degradation pathways of lignin model compounds were established as follows. Compounds previously found to be degradation intermediates of model compounds were used as the sole carbon source for growth of *A. flavus*. The degradation intermediates of these were then isolated and identified. In this way a pathway for the degradation of each model compound was found.

Degradation Of 1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)ethanol (Compound 7) By *A. flavus*

Detection And Isolation Of Intermediates

Extracts from media in which *A. flavus* was grown on Compound (7) showed several bands on TLC. Three of these corresponded to:

1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)ethanol
(Compound 7) (unused substrate);

4-Benzyloxy-3-methoxy-ω-(2-methoxyphenoxy)acetophenone
(Compound 6);and

4-Hydroxy-3-methoxy-ω-(2-methoxyphenoxy)acetophenone
(Compound 8).

The R_f values are shown in Table 7.

Identification Of Intermediates

Preparative TLC, isolation of the compounds and spectral analysis confirmed the identities of Compounds (6), (7) and (8) as shown in Table 8.

A positive result was obtained when the aqueous medium was assayed for benzyl alcohol by GLC. Its identity was confirmed by co-chromatography with an authentic sample on the column described in the methods. The quantity of benzyl alcohol found after incubation with 0.26 mM of Compound (7) for 4 days was 1.55×10^{-5} mM.

Sequence Of Degradation

The order in which the compounds were formed was determined by a replacement experiment using Compound (6) as the sole carbon source. Growth on Compound (6) produced several bands on TLC and two of these corresponded to Compound (6) (unused substrate) and Compound (8). Benzyl alcohol was detected using GLC. Compound (7) was not found. These results are shown in Table 9.

The above results suggest that Compound (7) is degraded by the sequence shown in Figure 49 of the discussion.

TABLE 7

Thin Layer Chromatography Of Extracts Obtained From Media Containing Compound (7) As Carbon Source

	Rf Values Extract	
Isolate	Solvent System 1	Solvent System 2
a	0.11	0.68
b	0.16	0.63
c	0.27	0.80

	Rf Values Authentic Compounds	
Compound	Solvent System 1	Solvent System 2
6	0.26	0.79
7	0.10	0.69
8	0.18	0.61

Solvent System 1 - Benzene:ethyl acetate 9:1

Solvent System 2 - Benzene:dioxane:acetic acid 90:25:4

TABLE 8

Spectral Analysis Of Isolates Derived From The Degradation Of Compound (7) By *A. flavus*

Isolate a

R_fs Solvent System 1 - 0.11, Solvent System 2 - 0.68

Identification 1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)ethanol (Compound 7)

ir γ max 3532, 2925, 1584 cm^{-1}

H-nmr [$CDCl_3$] δ 6.80 (m, 12H, aromatic), 5.12 (s, 2H, Ph-CH_2-O), 5.05 (q, 1H, H-C-), 4.00 (m, 2H, CH_2-O), 3.86 (s, 3H, OMe), 3.80 (s, 3H, OMe), 3.65 (s, 1H, C-OH)

ms m/z 380

o

Isolate b

R_fs Solvent System 1 - 0.16, Solvent System 2 - 0.63

Identification 4-Hyroxy-3-methoxy-ω-(2-methoxyphenoxy)acetophenone (Compound 8)

ir γ max 3011, 2939, 3148, 1659, 1584 cm^{-1}

H-nmr [$CDCl_3$] δ 7.66 (m, 3H, aromatic), 6.92 (m, 4H, aromatic), 6.24 (s, 1H, phenolic OH), 5.31 (s, 2H, CH_2-O-), 3.98 (s, 3H, OMe), 3.92 (s, 3H, OMe)

ms m/z 288

o

Isolate c

R_fs Solvent System 1 - 0.27, Solvent System 1 - 0.80

Identification 4-(4-Benzyloxy-3-methoxy-ω-(2-methoxyphenoxy)acetophenone (Compound 6)

ir γ max 3072, 2930, 1685, 1590 cm^{-1}

H-nmr [$CDCl_3$] δ 7.64 (m, 3H, aromatic), 7.42 (m, 5H, aromatic), 6.96 (m, 4H, aromatic), 5.25 (s, 2H, CH_2-O-), 5.14 (s, 2H, CH_2-O-), 3.90 (s, 3H, OMe), 3.85 (s, 3H, OMe)

ms m/z 378

1 Solvent System Benzene:ethyl acetate 9:1

2 Solvent System Benzene:dioxane:acetic acid 90:25:4

TABLE 9

Replacement Experiments To Establish The Sequence Of The Degradation Pathway Of Compound (7) By Aspergillus flavus

Carbon Source Added To Medium	Compounds Isolated After Incubation For 3 Days			
	Compound (6)	Compound (7)	Compound (8)	Benzyl Alcohol
Compound (6)	+	-	+	+
Compound (7)	+	+	+	+
Compound (8)	-	-	+	-

Degradation Of 1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-phenylphenoxy)ethanol (Compound 12) By *A. flavus*

Detection And Isolation Of Intermediates

Extracts from media in which *A. flavus* was grown on Compound (12) showed several bands on TLC. Three of these corresponded to:

1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-phenylphenoxy)ethanol
(Compound 12) (unused substrate);

4-Benzyloxy-3-methoxy-ω-(2-phenylphenoxy)acetophenone
(Compound 11);and

4-Hydroxy-3-methoxy-ω-(2-phenylphenoxy)acetophenone
(Compound 13).

The R_f values are shown in Table 10.

Identification Of Intermediates

Preparative TLC, isolation of the compounds and spectral analysis confirmed the identities of Compounds (11), (12) and (13) as shown in Table 11.

When the aqueous medium was assayed for benzyl alcohol by GLC a positive result was obtained. Its identity was confirmed by co-chromatography with an authentic sample. The quantity of benzyl alcohol found after incubation with 0.235 mM of Compound (12) for 4 days was 1.5 X 10^{-6} mM.

Sequence Of Degradation

The order in which the compounds were formed was determined by a replacement experiment using Compound (11) as the sole carbon source. Growth on Compound (11) produced several bands on

TLC and two of these corresponded to Compound (11) (unused substrate) and Compound (13). Benzyl alcohol was detected using GLC. Compound (12) was not found. These results are shown in Table 12.

The above results suggest that Compound (12) is degraded by the sequence shown in Figure 50 of the discussion.

TABLE 10

Thin Layer Chromatography Of Extracts Obtained From Media Containing Compound (12) As Carbon Source

Rf Values Extract

Isolate	Solvent System 1	Solvent System 2
d	0.33	0.80
e	0.37	0.80
f	0.63	0.88

Rf Values Authentic Compounds

Compound	Solvent System 1	Solvent System 2
11	0.63	0.89
12	0.36	0.79
13	0.33	0.78

Solvent System 1 - Benzene:ethyl acetate 9:1

Solvent System 2 - Benzene:dioxane:acetic acid 90:25:4

TABLE 11

Spectral Analysis Of Isolates Derived From The Degradation Of Compound (12) By A. flavus

Isolate f

R_fs Solvent System 1 - 0.63, Solvent System 2 - 0.88

Identification 4-(4-Benzyloxy-3-methoxy-ω-(2-phenylphenoxy)acetophenone (Compound 11)

ir γ max 3041, 2979, 1680, 1586 cm^{-1}

H-nmr [$CDCl_3$] δ 7.47 (m, 17H, aromatic), 5.29 (s, 2H, CH_2-O-), 5.22 (s, 2H, CH_2-O-), 3.94 (s, 3H, OMe)

ms m/z 424

——— o ———

Isolate e

R_fs Solvent System 1 - 0.37, Solvent System 2 - 0.80

Identification 1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-phenylphenoxy)ethanol (Compound 12)

ir γ max 3512, 3020, 2910, 1590 cm^{-1}

H-nmr [$CDCl_3$] δ 7.25 (m, 17H, aromatic), 5.50 (s, 2H, Ph-CH_2-O), 4.75 (q, 1H, H-C-), 4.00 (m, 2H, CH_2-O), 3.75 (s, 3H, OMe), 2.55 (d, 1H, C-OH)

ms m/z 426

——— o ———

Isolate d

R_fs Solvent System 1 - 0.33, Solvent System 2 - 0.80

Identification 4-Hyroxy-3-methoxy-ω-(2-methoxyphenoxy)acetophenone (Compound 8)

ir γ max 3139, 3121, 2914, 1657, 1577 cm^{-1}

H-nmr [$CDCl_3$] δ 7.47 (m, 12H, aromatic), 6.07 (s, 1H, phenolic OH), 5.09 (s, 2H, CH_2-O-), 3.83 (s, 3H, OMe)

ms m/z 334

Solvent System 1 - Benzene:ethyl acetate 9:1

Solvent System 2 - Benzene:dioxane:acetic acid 90:25:4

TABLE 12

Replacement Experiments To Establish The Sequence Of The Degradation Pathway Of Compound (12) By *Aspergillus flavus*

Carbon Source Added To Medium	Compounds Isolated After Incubation For 3 Days			
	Compound (11)	Compound (12)	Compound (13)	Benzyl Alcohol
Compound (11)	+	-	+	+
Compound (12)	+	+	+	+
Compound (13)	-	-	+	-

Degradation Of 1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)ethanol (Compound 3) By *Aspergillus flavus*

Detection And Isolation Of Intermediates

Extracts from media in which *A. flavus* was grown on Compound (3) as sole carbon source showed six major bands on TLC. These corresponded to:

1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)-
ethanol (Compound 3)(unused substrate);

3,4-Dimethoxy-ω-(2-methoxyphenoxy)-
acetophenone (Compound 2);

4-Hydroxy-3-methoxy-ω-(2-methoxyphenoxy)
acetophenone(Compound 8).

4-Hydroxy-3-methoxybenzene (Guaiacol)

4-Hydroxy-3-methoxybenzoic acid (Vanillic acid)

3,4-Dihydroxybenzoic acid (Protocatechuic acid)

The R_f values are shown in Table 13.

Identification Of Intermediates

Preparative TLC, isolation of the compounds and spectral analysis confirmed the identities of Compounds (2), (3), (8), Guaiacol, Vanillic Acid and Protocatechuic Acid as shown in Table 14.

Sequence Of Degradation

The order in which the compounds were formed was determined by a replacement experiment using metabolites of Compound (3) as the sole carbon source. Growth on Compound (2) produced several bands on TLC. These corresponded to Compound (2) (unused substrate), Compound (8), Guaiacol, Vanillic acid and Protocatechuic acid but not Compound (3). Growth on Compound (8) produced bands corresponding to Compound (8) (unused substrate), Guaiacol, Vanillic acid and Protocatechuic acid but not Compounds (2) or (3). These results are shown in Table 15.

When the aqueous medium which contained Compound (2) as sole carbon source was assayed for methanol by GLC a positive result was obtained. Its identity was confirmed by co-chromatography with an authentic sample. Methanol production over a 7 day growth period is shown in Figure 47.

The above results suggest that Compound (3) is degraded in the sequence shown in Figure 51 of the discussion.

TABLE 13

Thin Layer Chromatography Of Extracts Obtained From Media Containing Compound (3) As Carbon Source

	Rf Values Extract	
Isolate	Solvent System 1	Solvent System 2
g	0.00	0.40
h	0.05	0.66
i	0.13	0.68
j	0.15	0.64
k	0.19	0.72
l	0.50	0.78

	Rf Values Authentic Compounds	
Compound	Solvent System 1	Solvent System 2
2	0.18	0.73
3	0.04	0.66
8	0.11	0.67
Guaiacol	0.48	0.76
Vanillate	0.15	0.63
Protocatechuate	0.00	0.40

Solvent System 1 - Benzene:Ethyl acetate 9:1

Solvent System 2 - Benzene:dioxane:acetic acid 90:25:4

TABLE 14

Spectral Analysis Of Isolates Derived From The Degradation Of Compound (3) By A. flavus

Isolate g

R_fs Solvent System 1 - 0.00, Solvent System 2 - 0.40

Identification Protocatechuic acid

ir γ max 3505, 3300-2500, 1660, 1600 cm^{-1}

H-nmr [$CDCl_3$] δ 10.05 (s, 1H, O=C-OH), 7.60 (d, 2H, aromatic), 7.50 (d, 1H, aromatic), 6.95 (s, 1H, Phenolic OH), 6.87 (s, 1H, Phenolic OH)

ms m/z 154

Isolate h

R_fs Solvent System 1 - 0.05, Solvent System 2 - 0.66

Identification 1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)ethanol (Compound 3)

ir γ max 3505, 2945, 1590 cm^{-1}

H-nmr [$CDCl_3$] δ 7.05 (m, 7H, aromatic), 5.15 (d, 1H, H-C), 4.25 (m, 2H, CH_2-O), 4.00 (s, 3H, OMe), 3.95 (s, 3H, OMe), 3.80 (s, 3H, OMe), 2.40 (s, 1H, C-OH)

ms m/z 304

Isolate i

R_fs Solvent System 1 - 0.13, Solvent System 2 - 0.68

Identification 4-Hyroxy-3-methoxy-ω-(2-methoxyphenoxy)acetophenone (Compound 8)

ir γ max 3011, 2939, 3148, 1659, 1584 cm^{-1}

H-nmr [$CDCl_3$] δ 7.66 (m, 3H, aromatic), 6.92 (m, 4H, aromatic), 6.24 (s, 1H, phenolic OH), 5.31 (s, 2H, CH_2-O-), 3.98 (s, 3H, OMe), 3.92 (s, 3H, OMe)

ms m/z 288

TABLE 14 continued

Spectral Analysis Of Isolates Derived From The Degradation Of Compound (3) By *A. flavus*

Isolate j

R_fs Solvent System 1 - 0.15, Solvent System 2 - 0.64

Identification Vanillic acid

ir γ max 3500, 3200-2500, 1670, 1600 cm^{-1}

H-nmr [$CDCl_3$] δ 11.10 (s, 1H, O=C-OH), 8.15 (m, 1H, Phenolic OH), 7.55 (m, 2H, aromatic), 6.90 (d, 1H, aromatic, 3.95 (s, 3H, OMe)

ms m/z 168

* * *

Isolate k

R_fs Solvent System 1 - 0.19, Solvent System 2 - 0.72

Identification 3,4-Dimethoxy-ω-(2-methoxyphenoxy-acetophenone (Compound 2)

ir γ max 3091, 2917, 1685, 1590 cm^{-1}

H-nmr [$CDCl_3$] δ 7.64 (m, 3H, aromatic), 6.90 (m, 4H, aromatic), 5.35 (s, 2H, CH_2-O), 3.95 (s, 6H, OMe), 3.89 (s, 3H, OMe)

ms m/z 302

* * *

Isolate l

R_fs Solvent System 1 - 0.50, Solvent System 2 - 0.78

Identification Guaiacol

ir γ max 3500, 2990, 2880, 1601 cm^{-1}

H-nmr [$CDCl_3$] δ 6.95 (m, 4H, aromatic), 5.74 (s, Phenolic OH), 4.05 (s, 3H, OMe)

ms m/z 138

Solvent System 1 - Benzene:ethyl acetate 9:1

Solvent System 2 - Benzene:dioxane:acetic acid 90:25:4

TABLE 15

Replacement Experiments To Establish The Sequence Of The Degradation Pathway Of Compound (3) By *Aspergillus flavus*

Carbon Source Added To Medium	Compounds Isolated After Incubation For 3 Days					
	Compound (2)	Compound (3)	Compound (8)	Guaiacol	Vanillate	Protocatechuic Acid
Compound (2)	+	-	+	+	+	+
Compound (3)	+	+	+	+	+	+
Compound (8)	-	-	+	+	+	+

FIGURE 47

METHANOL PRODUCTION BY Aspergillus flavus

A. flavus was grown in 100ml volumes of medium in an orbital incubator at 30°C and 120 r.p.m.

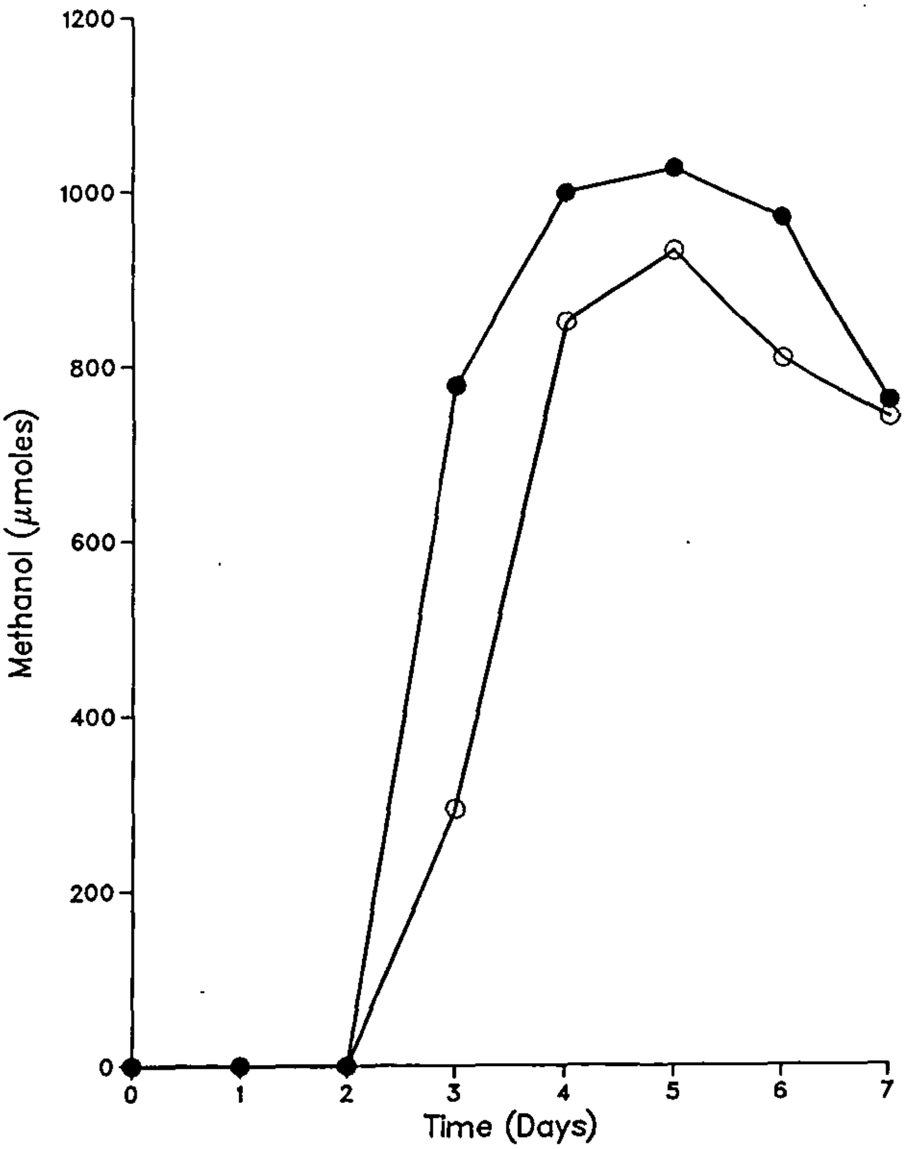
The growth medium was described in the methods.

1 ml samples of the medium were taken at 24 h intervals and assayed for methanol by GLC.

Each point is the mean of three determinations.

● - Grown On Compound (2) (332 μmoles)

○ - Grown On Compound (9) (387 μmoles)


1200
1000
800
600
400
200
0
Methanol (μmoles)
0
1
2
3
4
5
6
7
Time (Days)

Degradation Of 1-(3,4-Dimethoxyphenyl)-2-(2-phenylphenoxy)ethanol (Compound 10) By *Aspergillus flavus*

Detection And Isolation Of Intermediates

Extracts from media in which *A. flavus* was grown on Compound (10) as sole carbon source showed six major bands on TLC. These corresponded to:

1-(3,4-Dimethoxyphenyl)-2-(2-phenylphenoxy)-
ethanol (Compound 10)(unused substrate);

3,4-Dimethoxy-ω-(2-phenylphenoxy)-
acetophenone (Compound 9);

4-Hydroxy-3-methoxy-ω-(2-phenyl-phenoxy)
acetophenone(Compound 13).

2-Phenylphenol

4-Hydroxy-3-methoxybenzoic acid (Vanillic acid)

3,4-Dihydroxybenzoic acid (Protocatechuic acid)

The R_f values are shown in Table 16.

Identification Of Intermediates

Preparative TLC, isolation of the compounds and spectral analysis confirmed the identities of Compounds (9), (10), (13), 2-Phenylphenol, Vanillic Acid and Protocatechuic Acid as shown in Table 17.

Sequence Of Degradation

The order in which the compounds were formed was determined by a replacement experiment using metabolites of Compound (10) as the sole carbon source. Growth on Compound (9) produced five major bands on TLC. These corresponded to Compound (9) (unused substrate), Compound (13), 2-Phenylphenol, Vanillic acid and Protocatechuic acid but not Compound (10). Growth on Compound (13) produced bands corresponding to Compound (13) (unused substrate), Guaiacol, Vanillic acid and Protocatechuic acid but not Compounds (9) or (10). These results are shown in Table 18.

When the aqueous medium which contained Compound (9) as sole carbon source was assayed for methanol by GLC a positive result was obtained. Its identity was confirmed by co-chromatography with an authentic sample. Methanol production over a 7 day growth period is shown in Figure 47.

The above results suggest that Compound (10) is degraded in the sequence shown in Figure 52 of the discussion.

TABLE 16

Thin Layer Chromatography Of Extracts Obtained From Media Containing Compound (10) As Carbon Source

	Rf Values Extract	
Isolate	Solvent System 1	Solvent System 2
m	0.00	0.42
n	0.16	0.62
o	0.22	0.74
p	0.34	0.77
q	0.45	0.81
r	0.55	0.79

	Rf Values Authentic Compounds	
Compound	Solvent System 1	Solvent System 2
9	0.44	0.81
10	0.22	0.76
13	0.34	0.78
2-Phenylphenol	0.57	0.79
Vanillate	0.15	0.63
Protocatechuate	0.00	0.40

Solvent System 1 - Benzene:Ethyl acetate 9:1

Solvent System 2 - Benzene:dioxane:acetic acid 90:25:4

TABLE 17

Spectral Analysis Of Isolates Derived From The Degradation Of Compound (10) By A. flavus

Isolate m

R_fs Solvent System 1 - 0.00, Solvent System 2 - 0.42

Identification Protocatechuic acid

ir γ max 3500, 3300-2500, 1665, 1600 cm^{-1}

H-nmr [$CDCl_3$] δ 10.00 (s, 1H, O=C-OH), 7.65 (d, 2H, aromatic), 7.54 (d, 1H, aromatic), 6.95 (s, 1H, Phenolic OH), 6.90 (s, 1H, Phenolic OH)

ms m/z 154

Isolate n

R_fs Solvent System 1 - 0.16, Solvent System 2 - 0.62

Identification Vanillic acid

ir γ max 3505, 3200-2500, 1670, 1595 cm^{-1}

H-nmr [$CDCl_3$] δ 11.05 (s, 1H, O=C-OH), 8.15 (m, 1H, Phenolic OH), 7.55 (m, 2H, aromatic), 6.89 (d, 1H, aromatic, 3.95 (s, 3H, OMe)

ms m/z 168

Isolate o

R_fs Solvent System 1 - 0.22, Solvent System 2 - 0.74

Identification 1-(3,4-Dimethoxyphenyl)-2-(2-phenylphenoxy)ethanol (Compound 10)

ir γ max 3500, 3015, 1595 cm^{-1}

H-nmr [$CDCl_3$] δ 7.40 (m, 12H, aromatic), 4.76 (q, 1H, H-C), 4.05 (m, 2H, CH_2-O), 3.84 (s, 3H, OMe), 3.95 (s, 3H, OMe), 2.60 (s, 1H, C-OH)

ms m/z 350

TABLE 17 continued

Spectral Analysis Of Isolates Derived From The Degradation Of Compound (10) By A. flavus

Isolate p

R_fs Solvent System 1 - 0.34, Solvent System 1 - 0.77

Identification 4-Hyroxy-3-methoxy-ω-(2-phenylphenoxy)acetophenone (Compound 13)

ir γ max 3150, 2910, 1655, 1580 cm^{-1}

H-nmr [$CDCl_3$] δ 7.45 (m, 12H, aromatic), 6.00 (s, 1H, Phenolic OH), 5.10 (s, 2H, CH_2-O-), 3.85 (s, 3H, OMe)

ms m/z 334

o

Isolate q

R_fs Solvent System 1 - 0.45, Solvent System 2 - 0.81

Identification 3,4-Dimethoxy-ω-(2-phenylphenoxy-acetophenone (Compound 9)

ir γ max 3100, 2985, 1685, 1590 cm^{-1}

H-nmr [$CDCl_3$] δ 7.60 (m, 12H, aromatic), 5.24 (s, 2H, CH_2-O), 3.95 (s, 6H, OMe)

ms m/z 348

o

Isolate r

R_fs Solvent System 1 - 0.55, Solvent System 2 - 0.79

Identification 2-Phenylphenol

ir γ max 3570, 2985, 2885, 1595 cm^{-1}

H-nmr [$CDCl_3$] δ 7.37 (m, 9H, aromatic), 5.17 (s, Phenolic OH)

ms m/z 170

Solvent System 1 - Benzene:ethyl acetate 9:1

Solvent System 2 - Benzene:dioxane:acetic acid 90:25:4

TABLE 18

Replacement Experiments To Establish The Sequence Of The Degradation Pathway Of Compound (10) By Aspergillus flavus

Carbon Source Added To Medium	Compounds Isolated After Incubation For 3 Days					
	Compound (9)	Compound (10)	Compound (13)	Guaiacol	Vanillate	Protocatechuic Acid
Compound (9)	+	-	+	+	+	+
Compound (10)	+	+	+	+	+	+
Compound (13)	-	-	+	+	+	+

Degradation Of 1-(4-Hydroxy-3-methoxy-2-(2-phenylphenoxy)ethanol (Compound 14) By Aspergillus flavus

Detection And Isolation Of Intermediates

Extracts from media in which A. flavus was grown on Compound (14) as sole carbon source showed five major bands on TLC. These corresponded to:

1-(4-Hydroxy-3-methoxy)-2-(2-phenylphenoxy)-ethanol (Compound 14)(unused substrate);

4-Hydroxy-3-methoxy-ω-(2-phenylyphenoxy) acetophenone(Compound 13).

2-Phenylphenol

4-Hydroxy-3-methoxybenzoic acid (Vanillic acid)

3,4-Dihydroxybenzoic acid (Protocatechuic acid)

The R_f values are shown in Table 19.

Identification Of Intermediates

Preparative TLC, isolation of the compounds and spectral analysis confirmed the identities of Compounds (14), (13), 2-Phenylphenol, Vanillic Acid and Protocatechuic Acid as shown in Table 20.

Sequence Of Degradation

These results suggest that Compound (14) is degraded in the sequence shown in Figure 53 of the discussion.

TABLE 19

Thin Layer Chromatography Of Extracts Obtained From Media Containing Compound (14) As Carbon Source

Rf Values Extract

Isolate	Solvent System 1	Solvent System 2
s	0.00	0.40
t	0.15	0.65
u	0.20	0.73
v	0.35	0.79
w	0.58	0.80

Rf Values Authentic Compounds

Compound	Solvent System 1	Solvent System 2
13	0.34	0.78
14	0.21	0.73
2-Phenylphenol	0.57	0.79
Vanillate	0.15	0.63
Protocatechuate	0.00	0.40

Solvent System 1 - Benzene:Ethyl acetate 9:1

Solvent System 2 - Benzene:dioxane:acetic acid 90:25:4

TABLE 20

Spectral Analysis Of Isolates Derived From The Degradation Of Compound (14) By *A. flavus*

Isolate s

R_fs Solvent System 1 - 0.00, Solvent System 2 - 0.40

Identification Protocatechuic acid

ir γ max 3505, 3300-2500, 1665, 1605 cm^{-1}

H-nmr [CDCl$_3$] δ 10.02 (s, 1H, O=C-OH), 7.64 (d, 2H, aromatic), 7.54 (d, 1H, aromatic), 6.95 (s, 1H, Phenolic OH), 6.90 (s, 1H, Phenolic OH)

ms m/z 154

* * *

Isolate t

R_fs Solvent System 1 - 0.15, Solvent System 2 - 0.65

Identification Vanillic acid

ir γ max 3505, 3200-2500, 1670, 1595 cm^{-1}

H-nmr [CDCl$_3$] δ 11.04 (s, 1H, O=C-OH), 8.15 (m, 1H, Phenolic OH), 7.55 (m, 2H, aromatic), 6.90 (d, 1H, aromatic, 3.95 (s, 3H, OMe)

ms m/z 168

* * *

Isolate u

R_fs Solvent System 1 - 0.20, Solvent System 2 - 0.75

Identification 1-(4-Hydroxy-3-methoxyphenyl)-2-(2-phenylphenoxy)ethanol (Compound 14)

ir γ max 3500, 3015, 2910, 1595 cm^{-1}

H-nmr [CDCl$_3$] δ 7.40 (m, 12H, aromatic), 5.50 (s, 1H, Phenolic OH), 4.75 (q, 1H, H-C-), 4.00 (m, 2H, CH$_2$), 3.75 (s, 3H, OMe), 2.50 (s, 1H, C-OH)

ms m/z 336

TABLE 20 continued

Spectral Analysis Of Isolates Derived From The Degradation Of Compound (14) By A. flavus

Isolate v

R_fs Solvent System 1 - 0.35, Solvent System 2 - 0.79

Identification 4-Hyroxy-3-methoxy-ω-(2-phenylphenoxy)acetophenone (Compound 13)

ir γ max 3145, 2905, 1650, 1585 cm^{-1}

H-nmr [$CDCl_3$] δ 7.46 (m, 12H, aromatic), 6.05 (s, 1H, Phenolic OH), 5.12 (s, 2H, CH_2-O-), 3.85 (s, 3H, OMe)

ms m/z 334

Isolate w

R_fs Solvent System 1 - 0.58, Solvent System 2 - 0.80

Identification 2-Phenylphenol

ir γ max 3565, 2985, 2885, 1595 cm^{-1}

H-nmr [$CDCl_3$] δ 7.35 (m, 9H, aromatic), 5.16 (s, Phenolic OH)

ms m/z 170

Solvent System 1 - Benzene:ethyl acetate 9:1

Solvent System 2 - Benzene:dioxane:acetic acid 90:25:4

Metabolism Of 2-^{14}C-Compound (2)

Identification Of Radiolabelled Intermediates

The first monomeric units produced during metabolism of Compound (2) by A. flavus were previously shown to be Vanillic Acid and Guaiacol. 2-^{14}C-Compound (2) (unused substrate), 2-^{14}C-Compound (8) and ^{14}C-Guaiacol were detected after degradation of 2-^{14}C-Compound (2) by A. flavus for 3 days (Table 21). This indicates that cleavage of the dimer occurred between the C1-C2 (Cα-Cβ) bond of the side-chain.

A small proportion of the original radioactivity of 2-^{14}C-Compound (2) was detected as $^{14}CO_2$.

Growth of A. flavus for 3 days on 1-^{14}C-Compound (2) produced 1-^{14}C-Compound (2) (unused substrate), 1-^{14}C-Compound (8) and ^{14}C-Vanillic Acid (Table 22).

Radiolabelled isolates were identified by analytical TLC. Rf values were compared with those of authentic compounds and isolates were co-chromatographed with authentic standards.

TABLE 21

Radioactivity Remaining In Some Degradation Products Of 2-^{14}C-Compound (2)

Rf Values Extract

Isolate	Solvent System 1	Solvent System 2	% Original Activity
r1	0.18	0.75	63.76
r2	0.12	0.66	5.08
r3	0.50	0.75	3.86

Rf Values Authentic Compounds

Compound	Solvent System 1	Solvent System 2
2	0.18	0.74
8	0.11	0.65
Guaiacol	0.48	0.76

Solvent System 1 - Benzene:Ethyl acetate 9:1

Solvent System 2 - Benzene:dioxane:acetic acid 90:25:4

TABLE 22

Radioactivity Remaining In Some Degradation Products Of 1-^{14}C-Compound (2)

Rf Values Extract

Isolate	Solvent System 1	Solvent System 2	% Original Activity
r4	0.18	0.76	59.64
r5	0.11	0.66	4.35
r6	0.13	0.61	6.53

Rf Values Authentic Compounds

Compound	Solvent System 1	Solvent System 2
2	0.18	0.74
8	0.11	0.65
Vanillate	0.15	0.63

Solvent System 1 - Benzene:Ethyl acetate 9:1

Solvent System 2 - Benzene:dioxane:acetic acid 90:25:4

Degradation Of Larch Wood By *A. flavus*

Chemical Analysis Of Wood Samples

The proportions of extractives, lignin and total carbohydrate in undegraded larch wood were 5%, 26% and 68%, respectively.

Three months after inoculation with *A. flavus*, the dry weight of larch wood blocks had been reduced to 79% of the original value. Lignin and total carbohydrate values after this time were 72% and 81% of their original values respectively (Figure 48). All values were the mean of at least six determinations.

The reduction in dry weight was greatest after the first month of incubation with only small reduction occurring over the remainder of the incubation period. The rate of lignin and carbohydrate removal was also greatest during the first month of incubation. However, further substantial removal of both components occurred during the next two months.

The fall in the level of extractives was greatest during the second month of incubation when the value decreased to 44% of the original amount in undegraded wood.

Scanning Electron Microscopy

Inoculated wood samples were taken at monthly intervals for observation by scanning electron microscopy. The samples were compared with uninoculated samples.

Uninoculated wood blocks separated cleanly when sectioned and microscopy showed the cells to be intact and relatively free of particulate material (Plate 37). Vessel elements remained attached together.

One month after inoculation the wood samples had become covered with extensive growth of the fungus not only on the surface at the site of inoculation but over all surfaces. The colour of the wood had turned from light to dark brown. Electron microscopy showed that vessel elements had become detached from each other and particulate material was present. No infiltration of fungal mycelia was observed (Plate 38).

Much more extensive separation of vessel elements was observed by electron microscopy after two months of incubation with A. flavus (Plate 39). Cells of the decayed wood were separated at the middle lamella regions and disintegrated cells were common. Fungal mycelia were absent in deeper areas of the samples but some infiltration through the separated cells was observed.

Three months after inoculation wood samples showed a similar appearance to samples taken after two months with only slightly more particulate material present and with much disarrangement of the cell structure (Plate 40). These effects were not seen when deeper sections of the wood were viewed but a similar appearance was observed at all surfaces of the samples.

Samples observed after five months incubation showed similar but more extensive disintegration of cells and more particulate material. Large numbers of spores of A. flavus were found deeper in samples and on occasions hyphae were seen (Plate 41).

FIGURE 48

CHANGE IN COMPOSITION OF WOOD DURING INCUBATION WITH A. flavus

Aspergillus flavus was grown on small blocks of larch wood as described in the methods section.

Sample wood blocks were taken at 4 week intervals and their dry weight, extractives, lignin and total carbohydrate were determined.

Each point is the mean of three determinations.

■ - Dry Weight

□ - Extractives

● - Lignin

○ - Carbohydrate

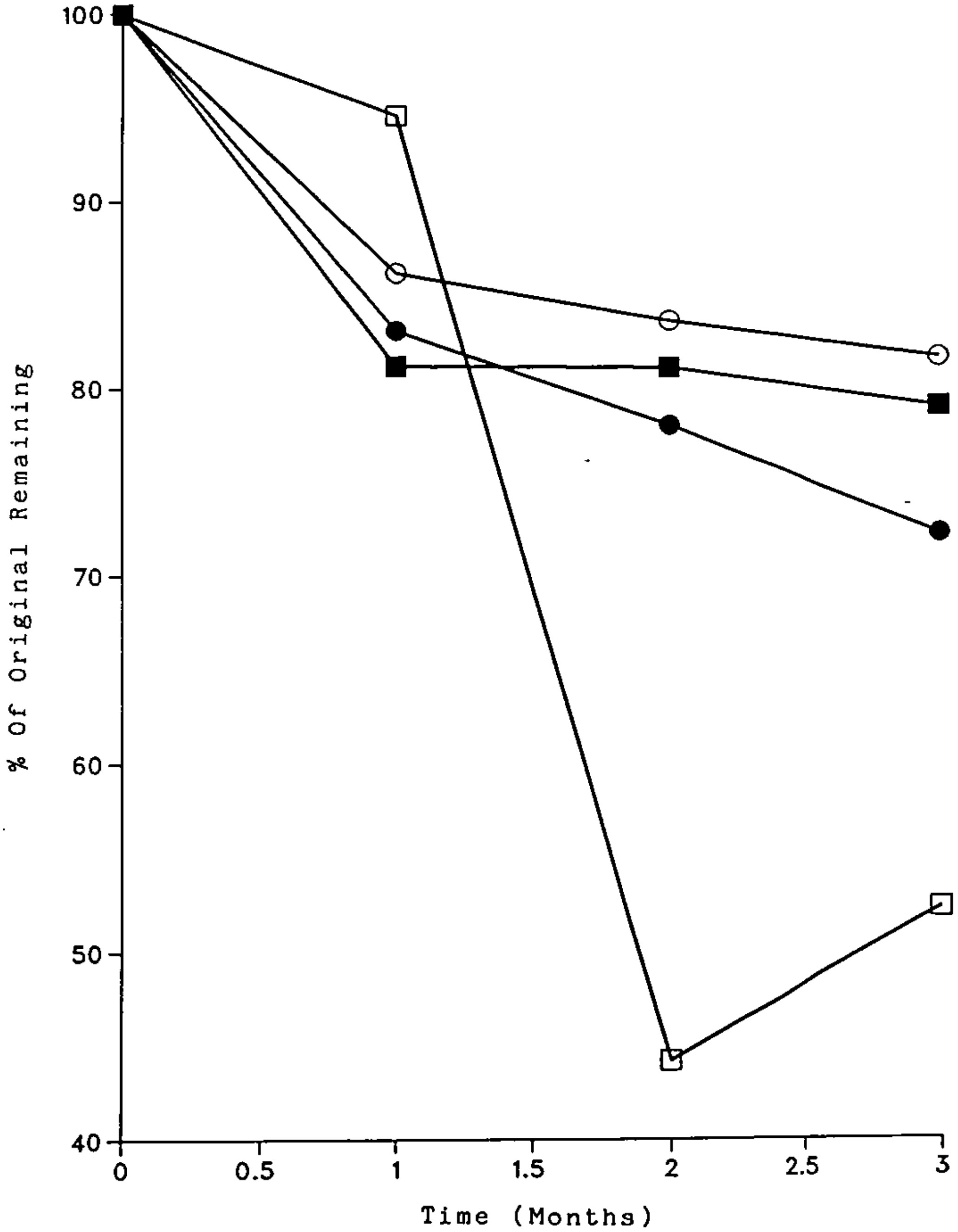
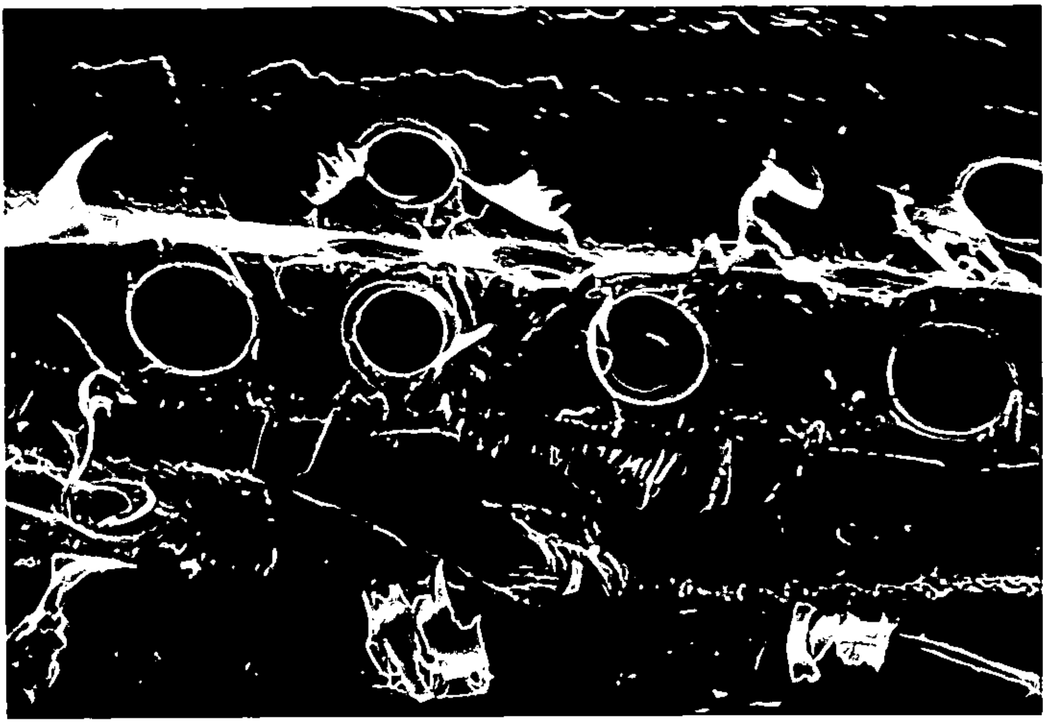

100
90
80
70
60
50
40
% Of Original Remaining
0
0.5
1
1.5
2
2.5
3
Time (Months)

Plate 37. Electron Micrograph Of Uninoculated Wood Showing Intact Cells Relatively Free Of Particulate Material (X 550)

Plate 38. Electron Micrograph Of Wood Inoculated With A. flavus For One Month Showing Detached Vessel Elements (Arrows) And Particulate Material (X 550)



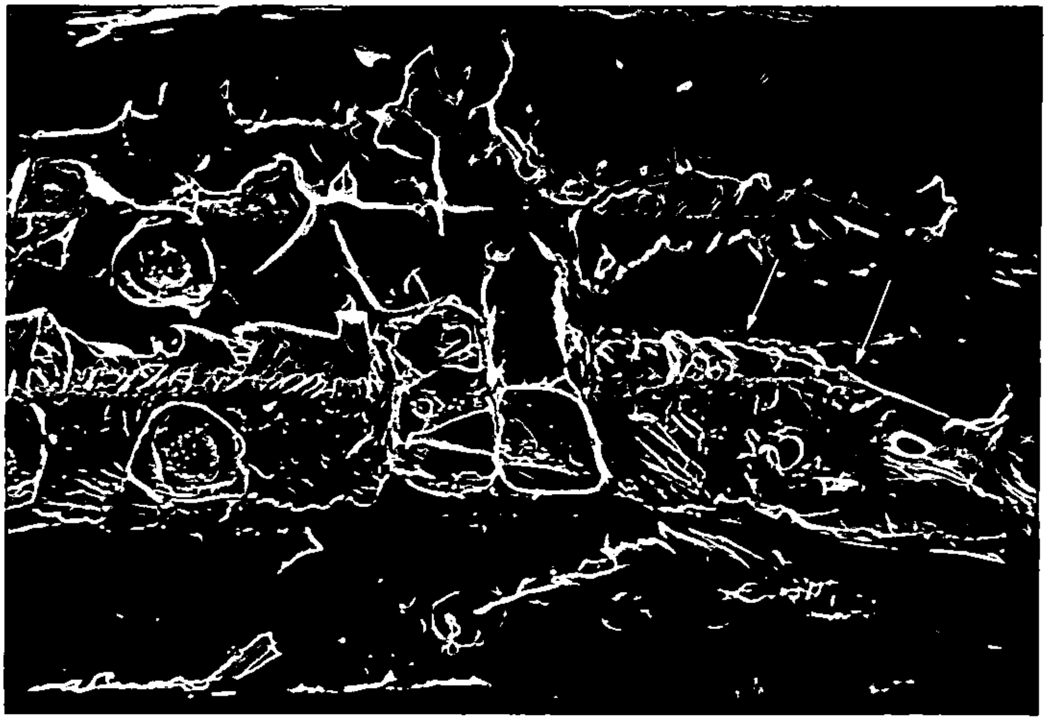
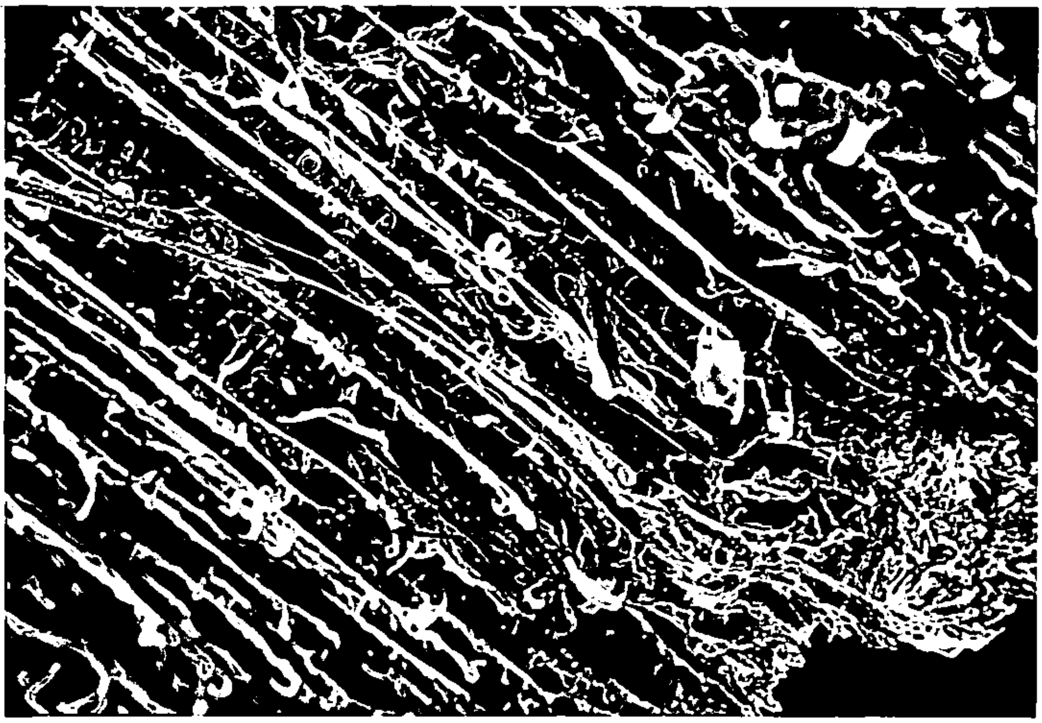


Plate 39. Electron Micrograph Of Wood Degraded By A. flavus For Two Months Showing Disintegrated Cells And Separation At Middle Lamellae (Arrows) (X 119)

Plate 40. Electron Micrograph Of Wood Degraded By A. flavus For Three Months Showing Cell Separation (Arrows) And Disruption (X 120)



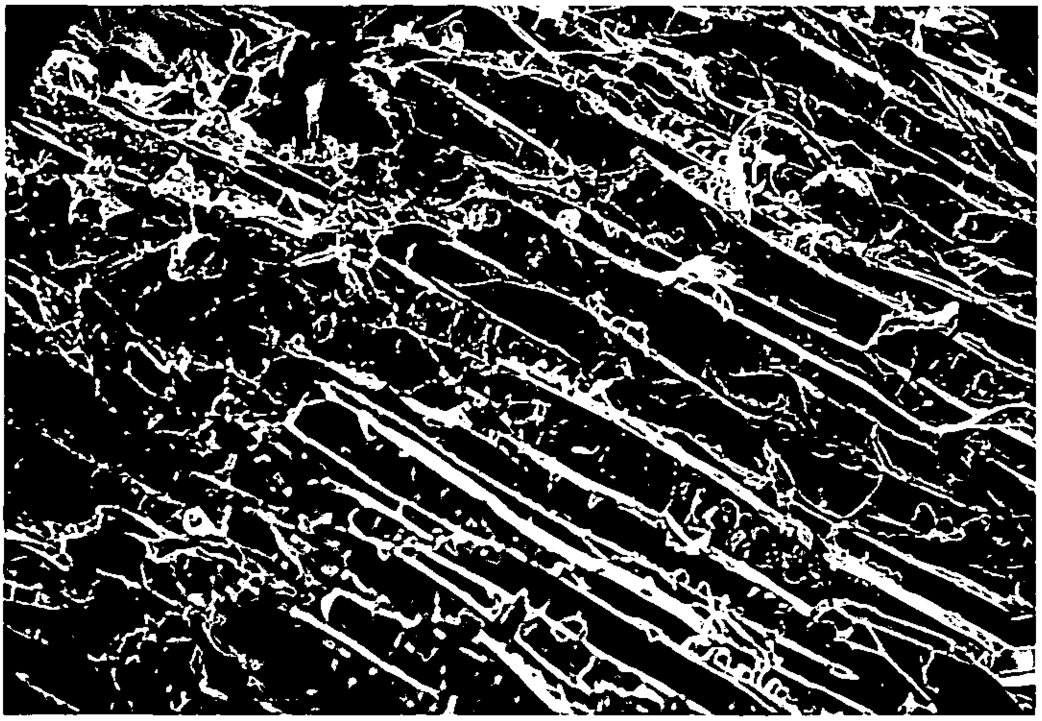


Plate 41. Electron Micrograph Of Wood Incubated With A. flavus For Five Months Showing Spore Masses (Arrow) (X 550)

DISCUSSION

DISCUSSION

Screening Procedures

Lignocellulose is the most abundant natural material present on the earth comprising 50% of all biomass (Wood, 1985). Lignin cannot be degraded hydrolytically by micro-organisms due to the nature of its structure. The protection of cellulose and hemicelluloses by lignin in lignocellulosic materials means that their utilization is limited. Therefore vast renewable resources are, at the moment, largely untapped.

The number of micro-organisms known to be capable of modifying lignin and thereby allowing access to the protected carbohydrate is relatively small. However, continuing efforts are being made to develope screening methods to increase this number and to find more efficient degraders of lignin.

It is widely recognized that the white-rot fungi e.g. Ph. chrysosporium are generally very efficient degraders but even though much work has been carried out on certain species within this group there is scope for further screening. Recently Trojanowski and Hutterman (1987) reported the high ligninolytic activities of wood-inhabiting fungi during screening procedures and found that two species of Polyporus previously unknown as degraders were able to solubilize up to 65% of a lignin preparation. This demonstrates the need for further screening with particular emphasis on finding micro-organisms with less demanding physiological conditions for degradation.

The recent findings that the ligninolytic systems of actinomycetes may be very different to that of the white-rot fungi (Leisola & Fiechter, 1985) probably indicates that there is considerable scope for finding degraders with less strict physiological requirements for lignin utilization.

Tien et al. (1987) have also proposed a method for the detection of lignin-degrading micro-organisms by a qualitative assay of ligninase. This could be especially useful for finding micro-organisms which produce quantities of these enzymes.

The screening techniques used in this study were of two main types. The first consisted of assessing the ability of species not previously reported to degrade lignin and to determine whether these and known degraders could carry out degradation with certain modifications to the growth medium. The most important modifications were the removal of the secondary carbon source and also the nitrogen source.

This screening gave the results shown in Table 4. Previously unreported degraders such as A. flavus and B. fulva gave positive results and these occurred under specific media conditions. Ph. chrysosporium, a known degrader, only degraded when a secondary carbon source was present and added nitrogen was absent. The results for Ph. chrysosporium are in agreement with results of other workers (Keyser et al., 1978; Kirk et al., 1978; Broda et al., 1983). This demonstrates the validity of the screening method, which is a modification of the method of Sundman & Nase (1971). Overall the results indicated that no predictable pattern of requirements for degradation was apparent. This emphasizes the need for continued screening to discover the most appropriate micro-organisms to use in bioconversion processes.

During these lignin degradation plate tests it was found that the extent of growth on any of the media did not correlate with the ability of the organism to degrade lignin (Figures 28 - 36). This was particularly true in the case of N. crassa where virtually no growth was detected over the incubation period, but substantial degradation was determined by the test. The opposite effect was seen with other organisms. For example Ph. chrysosporium showed extensive growth on plate test media (1) and (3) but did not degrade lignin.

The second screening procedure used was to indicate the ability of a range of fungi and bacteria to degrade insoluble lignin-related aromatic compounds with or without a secondary carbon source. The results, as shown in Tables 5 and 6, showed that many of the micro-organisms could degrade these compounds. However no definite pattern could be established for the requirement of a secondary carbon source. This is consistent with previous work done on the physiological aspects of aromatic degradation.

The value of this second screening procedure is twofold:

Firstly, the ability of a micro-organism to degrade lignin-related compounds may indicate its ability to degrade or modify lignin. Although this is not always true it is likely that the lack of this ability probably indicates the organism is not a lignin degrader.

Secondly, to understand fully the biodegradation of lignin in natural environments it is essential to realize that the process is undertaken by a consortium of micro-organisms. Within this consortium there are probably not only micro-organisms which initiate the degradation by attacking the intact polymer but also secondary degraders which are likely to be involved in the metabolism of low molecular weight compounds released during the initial stages of attack. Additionally, it is known, for example, that brown-rot fungi, although unable to metabolize lignin, can leave it in a considerably modified form (Harvey et al., 1985). Only by understanding the involvement of all members of the lignin degrading and modifying community can the process be elucidated.

The use of water-insoluble compounds in this study served two purposes. They may be better models of natural lignins which are insoluble molecules and this may be particularly important when considering the physical aspects of degradation. Secondly, the insolubility of the compounds used allowed a thin, visible film of solid to be deposited over agar layers. Metabolism of the compounds produced soluble intermediates which dissolved in the agar, producing the clear zones. Formation of soluble intermediates may have occurred due to side-chain oxidations, demethylation of methoxyl substituents and/or aromatic ring hydroxylations followed by ring cleavage. These types of reaction are well documented (Henderson, 1963; Ishikawa et al., 1963b; Chapman, 1972; Dagley, 1975; Kirk & Lorenz, 1973; Stanier & Ornston, 1973; Cain, 1980; Kirk et al., 1980; Crawford, 1981).

The possible dissolution of substrates due to factors other than degradation was considered. Release of alkaline metabolic products from the bacterial tryptone medium (B1) may have produced soluble salts of the acid substrates. However medium B2 contained no tryptone and yet gave many positive results. Additionally, the substrate 3,4,5-trimethoxybenzaldehyde could not form soluble salts but it gave positive results even in tryptone medium. This also applies to the fungal media F1 and F2. At both pH values used any other effect causing solubilization of the substrates would have caused clearing uniformly over the petri dishes. When negative or positive results were obtained uniform clearing was not observed. The conclusion was that clear areas around the microbial colonies were caused by solubilization due to degradative processes.

There are several important considerations when choosing compounds for the spray test. One is that compounds should be relatively insoluble and should not be volatile, as earlier trials showed that some compounds evaporated during the incubation period. However, these compounds are not precluded from use as thicker layers sprayed onto the agar plates and shorter incubation times still gave acceptable results. A further consideration is that an early intermediate of metabolism must be sufficiently soluble to dissolve in the agar.

Many insoluble lignin-related aromatic compounds are available commercially or can be synthesized. However, the high cost of purchase or the lack of facilities for synthesis means that the majority are not available for screening purposes. The compounds chosen in this study are freely available and inexpensive. Ferulic acid and cinnamic acid are well established as lignin model compounds (Crawford, 1981; Fukuzumi, 1980; Iwahara, 1980; Kawakami, 1980). The trimethoxy-substituted compounds relate well to the structure of certain lignin substructures. The 4-position methoxyl group replaces the substituted benzyloxy groups of the natural polymer. This type of compound has frequently been used as a lignin substructure model (Fukuzumi, 1980; Kirk & Tien, 1983). The techniques used in this screening technique are simple and applicable to a wide range of micro-organisms and aromatic compounds.

Growth Of A. flavus On Lignin Model Compounds

Growth of A. flavus was supported by the lignin model compounds synthesized during this study. The growth on compounds which were synthesized in sufficient quantity for dry weight measurements to be made are shown in Figures 38 to 44. Growth on model compounds was always less than that on glucose (Figure 37) although based on theoretical ATP production it would have been predicted to be higher in all cases. Reasons for this may be the production of toxic intermediates during degradation, lack of transport systems for certain intermediates or a deterioration of cell membranes caused by the models or their intermediates. This latter explanation would mean that constant repair to membranes may have utilized carbon and energy which would normally have been used to increase cell mass. The effect of phenolics on the membranes of micro-organisms is widely exploited in anti-microbial agents. Similar findings have been reported by other authors (Iyayi & Dart, 1982) but no explanation has been postulated.

The vigorous growth on the model compounds synthesized in this work is contrary to early observations into the degradation of lignin and insoluble model compounds by Ph. chrysosporium in agitated culture. This earlier work found that degradation and metabolism were inhibited (Kirk et al., 1978; Weinstein et al., 1980; Goldsby et al., 1980; Shimada et al., 1981) and this was explained by general physiological effects on secondary metabolism. The abrasive effect of solid particles on the delicate mycelial growth was also considered to be inhibitory.

More recent investigations have shown that the ligninolytic system may be very effective in shaken culture (Eaton et al., 1980). The degradation of aspen wood lignin and DHP has also been found to be as effective in shaken as in static culture (Reid et al., 1985) although it is probable that the limited availability of oxygen to the centre of mycelial spheres (Phillips, 1966; Kirk et al., 1978) causes some reduction in degradative capacity.

Fungal Associations With Insoluble Substrates

During growth of *A. flavus* on lignin and lignin model compounds it was noticed that the insoluble crystals of some compounds became incorporated into the mycelial pellets of the organism. Electron microscopy of the pellets showed strong and intimate contact between the crystals and the mycelia.

The degradation of Compound (2), which became associated with mycelia, was observed using electron microscopy over a five day period. This showed a gradual reduction in crystal size over the incubation period (Figure 46) and evidence was presented for erosion of crystals at the site of growing mycelial tips.

The recent discoveries on the enzymology of the ligninolytic system have suggested that certain enzymes, notably one of the peroxidase enzymes may reside on the surface of mycelia. This may explain the requirement for a close association between mycelium and substrate. However, the association does not necessarily mean that degradation took place on the mycelia. A recent paper by Stucki & Alexander (1987) indicates that so called 'insoluble substrates' are generally very slightly soluble and a process of gradual dissolution and metabolism of these small amounts from solution may occur. The solubility and rate of dissolution, which will differ for different substrates, may determine and limit the rate of its biodegradation. The substantial growth of fungus on lignin model compounds over a relatively short incubation time in this project would suggest that a dissolution rate is not a limiting factor.

Degradation Of Larch Wood By *A. flavus*

Cells frequently make close contact with the substrates during microbial attack of lignin and cellulose. It has been shown that the *Ph. chrysosporium* can bind synthetic lignins tenaciously (Chua *et al.*, 1983). The bacteria *Clostridium thermocellum*, *Cellvibrio fulvus*, *Sporocytophaga myxococcoides* and a *Cellulomonas* species have been reported to become attached to cellulose fibres (Berg *et al.*, 1972a; Berg *et al.*, 1972b; Weigel & Dijkstra, 1984).

However, the role of contact in degradation of both cellulose and lignin is controversial. In wood decayed by white and brown-rot fungi, hyphae can be seen in virtually every cell (Kirk, 1983). Electron microscopy has shown that the dissolution of the plant cell wall by white-rot fungi can occur along the lateral cell wall as well as near the growing tips of the mycelium (Schmid & Liese, 1964; Eriksson *et al.*, 1980).

In contrast, Blanchette & Reid (1986) have reported a white-rot depolymerization of lignin throughout cell walls and not only immediately adjacent to the hyphae. Work on the degradation of larch wood by *A. flavus* in this study supports this view. During the first three months of incubation, larch wood decreased in dry weight and lost quantities of both lignin and carbohydrate with disintegration of cells shown by electron microscopy. However, there was no sign of infiltration by fungal hyphae at this stage. Only after five months incubation was there any indication that hyphae were invading the wood tissues.

Lysis of cell walls which are not in the vicinity of hyphae is common with brown-rot fungi and Kauri & Kushner (1985) have shown that bacterial degradation of cellulose similarly does not necessitate contact. The evidence presented in this work indicates that _A. flavus_ degrades larch wood in a similar way to the brown-rot fungi.

Dimerization Of 2,6-Dimethoxyphenol

Oxidative coupling of phenolic compounds and aromatic amines is an important process in the synthesis of soil humic substances, lignins, tannins, alkaloids, melanins and antibiotics (Haider _et al._, 1975; Harkin, 1967; Sjoblad & Bollag, 1981). Reactions during coupling can give rise to a variety of C-C, C-O, C-N and N-N bonds.

The coupling mechanism in the case of phenols involves production of a phenoxy radical by removal of a proton and an electron from the hydroxyl group. Two free radical intermediates are then able to couple at _ortho_ or _para_ positions relative to the hydroxyl group, yielding dimeric compounds. Further oxidation can yield higher polymers. Oxidative coupling at _meta_ positions does not occur in phenols (Sjoblad & Bollag, 1981).

Various enzymes can catalyse the coupling processes. These include laccases and peroxidases. Members of both groups have also been suggested as likely to be involved in the depolymerization reactions during lignin biodegradation (Ander & Eriksson, 1976; Arora & Sandhu, 1985; Huynh & Crawford, 1985;

Ishihara, 1980; Kuwahara et al., 1984; Paszczynski et al., 1985; Paszczynski et al., 1986).

Both intracellular and extracellular laccases have been reported to be produced by white-rot fungi (Blaich & Esser, 1975). It is considered by some workers that the peroxidase of ligninolytic systems is extracellular (Glen & Gold, 1985; Trojanowski et al., 1985). However, most higher plants contain intracellular peroxidases. Three mycelium-bound peroxidase isoenzymes have been found in Trametes versicolor and Trametes hirsuta (Schanel et al., 1971).

A. flavus readily formed the dimer tetramethoxy-ρ-dibenzoquinone from 2,6-dimethoxyphenol. The dark purple, insoluble crystals of the dimer were only produced after mycelial spheres had formed and they were always produced within the spheres. Microscopy showed close associations between crystals of the dimer and mycelia of A. flavus (Betts et al., 1987).

This information suggests that the coupling of 2,6-dimethoxyphenol may occur at the surface of mycelia. Production of the same dimer from 2,6-dimethoxyphenol by Ph. chrysosporium has also been reported to occur on the mycelial surface, although this was in static culture (Paszczynski et al., 1986).

In a similar reaction, the oxidation of vanillyl glycol to produce methanol and polymeric quinoids (Lundquist & Kristersson, 1985) was reported to have been catalysed by laccase whereas the coupling reaction observed by Paszscynski et al. (1986) was suggested to have been catalysed by a peroxidase enzyme.

Some workers strongly support the role of laccase in lignin degradation (Ander & Eriksson, 1976; Ishihara, 1980) but others have suggested that laccase is not involved (Gierer & Opara, 1973; Haars & Hutterman, 1980). Various enzymes could cause these polymerization and depolymerization reactions but in lignin degradation evidence is accumulating which suggests that specific enzymes produce active oxygen species which then act in a non-specific way in the disassembly of the polymer.

Polymerization of phenols is a common occurrence with soil micro-organisms and it has been suggested that laccase is produced to detoxify the phenols (including lignin degradation products). When micro-organisms which can polymerize phenols are also found to degrade lignin or lignin model compounds then the complexity of the enzyme systems and the pathways involved becomes apparent. The involvement of both types of reaction in the degradation of lignin may explain the fact that low molecular weight degradation products have only been found in very small quantities (Janshekar & Fiechter, 1983).

Formation of tetramethoxy-ρ-dibenzoquinone did not occur when glucose was present in the growth medium. This would suggest glucose repression of the enzyme(s) involved.

Metabolism Of The Tri- And Tetrameric Lignin Model Compounds (7) And (12)

The pathways proposed for the initial degradation of compounds (7) and (12) are shown in Figures 49 and 50.

The initial degradation step involves oxidation of the α-hydroxyl group to a carbonyl group with both compounds (7) and (12). This may be analogous to the process by which white-rot fungi attack lignin as they tend to produce a modified lignin

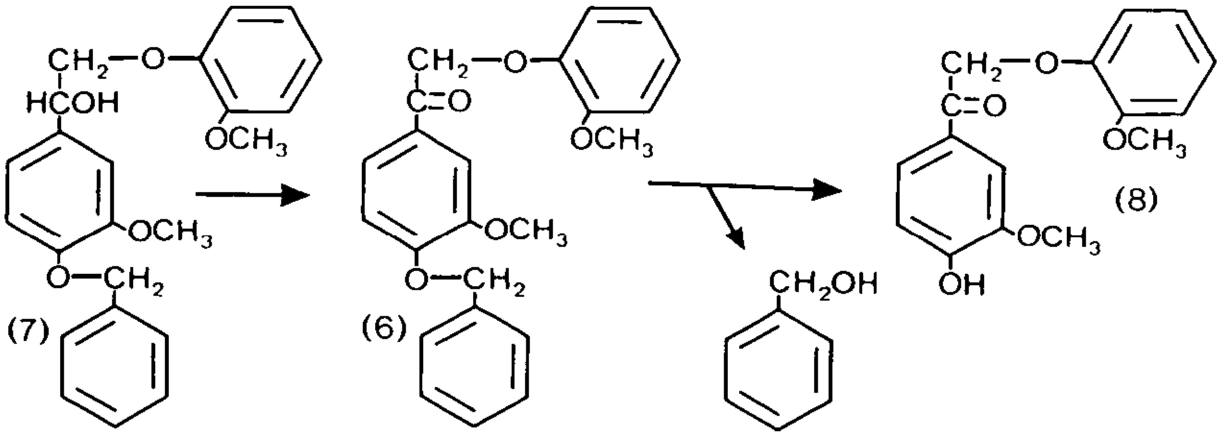

CH2—O
HCOH
OCH3
OCH3
O—CH2
(7)
CH2—O
C=O
OCH3
OCH3
O—CH2
(6)
CH2OH
CH2—O
C=O
OCH3
(8)
OCH3
OH

Figure 49. Proposed Sequence Of Degradation Of 1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)ethanol (Compound 7) By A. flavus

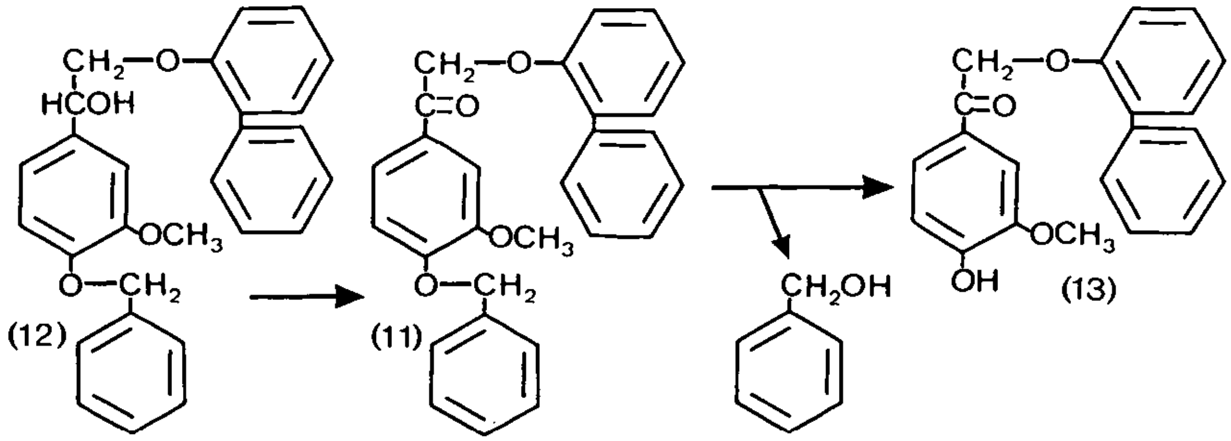

CH2—O
HCOH
OCH3
O—CH2
(12)
CH2—O
C=O
OCH3
O—CH2
(11)
CH2OH
CH2—O
C=O
OCH3
OH
(13)

Figure 50. Proposed Sequence Of Degradation Of 1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-phenylphenoxy)ethanol (Compound 12) By A. flavus

with increased amounts of benzoyl groups as compared to undecayed lignin (Kirk and Chang, 1974). Examples of this type of reaction are also found in non white-rot fungi and bacteria (Crawford et al., 1973; Higuchi, 1980; Pelmont et al., 1985). However, the enzyme systems involved in this oxidation may differ considerably with diffferent organisms. The reaction is catalysed by intracellular enzymes in Pseudomonas acidovorans and would almost certainly not attack an intact lignin molecule which would be extracellular (Crawford and Crawford, 1980). The range of enzymes capable of achieving this type of reaction is wide and includes a NAD^+ dependent dehydrogenase (Pelmont et al., 1985), a hydrogen peroxide dependent extracellular enzyme (Renganathan et al., 1986), and a phenol oxidase (Shimada, 1980). Several lignin degrading Fusarium species have been reported to excrete an enzyme which catalyses the oxidation of the primary alcohol group in the α,β-unsaturated side chain of guaiacylglycerol-β-coniferyl ether to the corresponding aldehyde. The enzyme seemed to be an aromatic alcohol oxidase (Janshekar and Fiechter, 1983). The oxidation state at the α-carbon is thought to be important to the mechanisms by which the α-β carbon bond between monomers is broken (Fenn and Kirk, 1984).

The second reaction to occur in the degradation of compounds (7) and (12) is the debenzylation of the 4-benzyloxy group to yield benzyl alcohol and the corresponding phenol. This is analogous to the very common demethylation reactions which occur in a very wide range of organisms removing methoxy groups both in the lignin macromolecule and in lignin model compounds. In this case the benzyloxy group can also be considered as an analogue of an α-aryl ether bond and this may have implications for the mechanism used to cleave this type of ether bond. No benzaldehyde was detected and the first product found was benzyl alcohol. The parent molecule remaining was the phenol. The very small quantities of benzyl alcohol detected may be due to certain properties of this compound. Benzyl alcohol is very insoluble in water and is volatile, evaporating readily from flasks of media incubated with shaking at 30°C. It would also be metabolized rapidly and therefore the levels detected may not have been representative of the true levels produced.

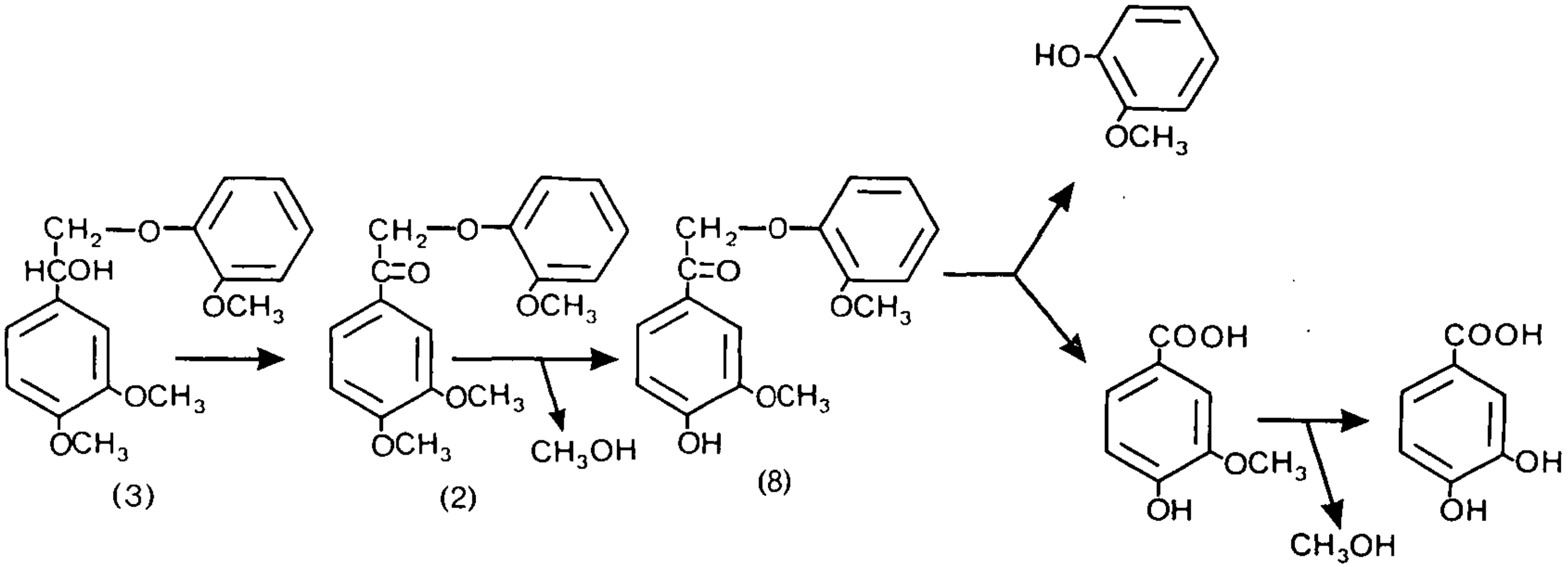
The results seem to indicate that after an initial oxidation of the α-hydroxyl group to a ketone, the next step is a cleavage of the benzyl ether bond in preference to attack of the β-aryl bond. The mechanism of this cleavage may be a debenzylation reaction similar to the demethylation reactions previously encountered with this organism.

Metabolism Of The Lignin Model Compounds (3), (10) and (14)

The sequences of degradation of Compounds (3), (10) and (14) are shown in Figures (51), (52) and (53), respectively.

The first step in the degradation of Compounds (3), (10) and (14) by A. flavus is the oxidation of the α-hydroxyl group to a carbonyl group. This is analogous to the initial reaction in the metabolism of Compounds (7) and (12) and it is likely that the same enzyme catalyses these similar steps.

The second degradation step for Compounds (3) and (10) is the demethylation of the 4-position methoxyl group. This is again analogous to the debenzylation of Compounds (7) and (12) and is one of the most common types of reactions by which many



(3)
(2)
CH_3OH
(8)
CH_3OH

Figure 51. Proposed Sequence Of Degradation Of 1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)ethanol (Compound 3) By A. flavus

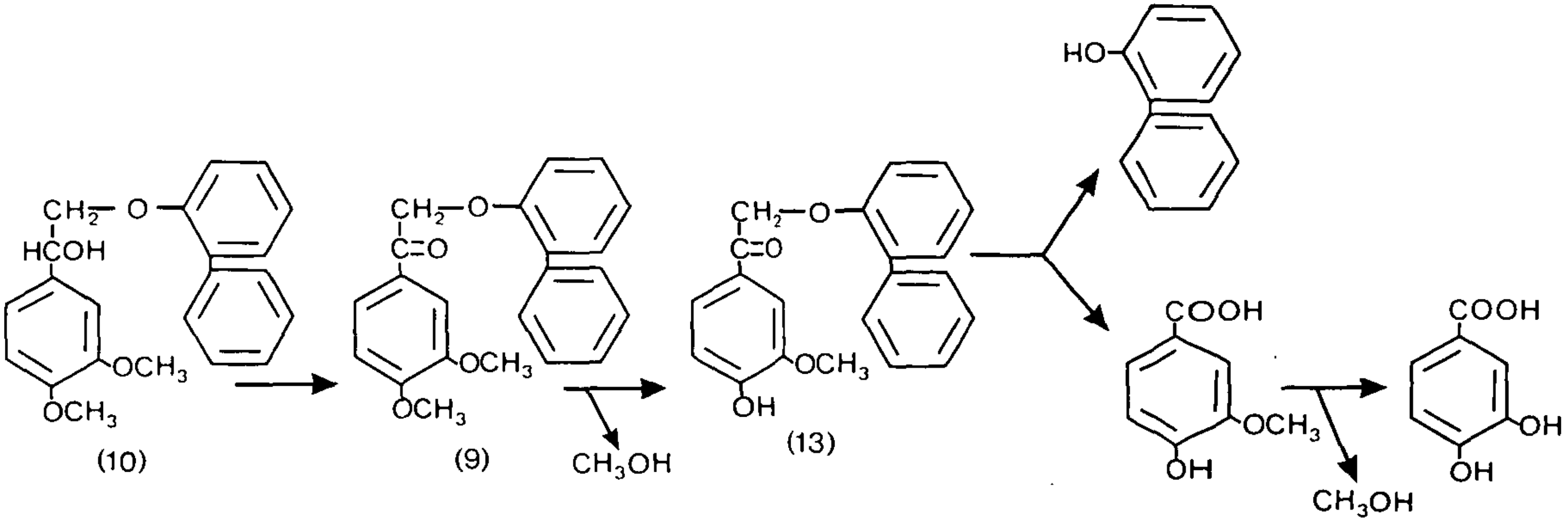

(10)
(9)
CH3OH
(13)
CH3OH

Figure 52. Proposed Sequence Of Degradation Of 1-(3,4-Dimethoxyphenyl)-2-(2-phenylphenoxy)ethanol (Compound 10) By A. flavus

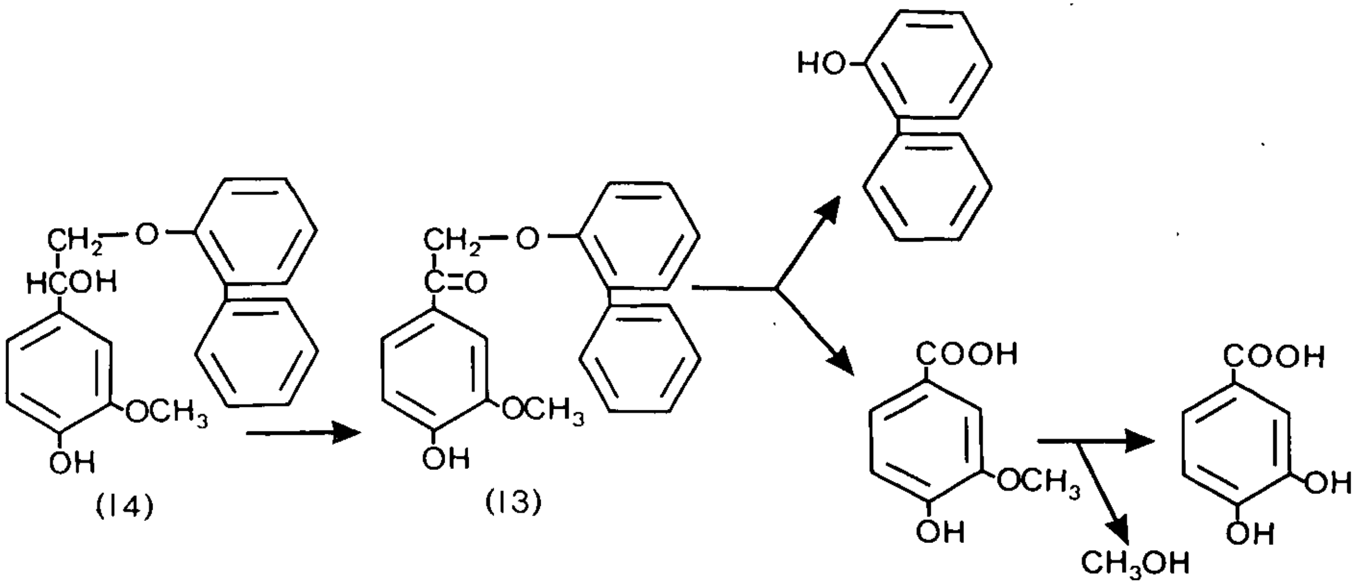

CH2—O
HCOH
OCH3
OH
(14)
CH2—O
C=O
OCH3
OH
(13)
HO
COOH
OCH3
OH
CH3OH
COOH
OH
OH

Figure 53. Proposed Sequence Of Degradation Of 1-(4-Hydroxy,-3-methoxy)-2-(2-phenylphenoxy)ethanol (Compound 14) By *A. flavus*

groups of micro-organisms attack the lignin polymer.

Demethylation mechanisms have not received as much attention as those for cleavage of the β-aryl ether in lignin research but there are several mechanisms proposed in the literature. Dagley (1978) proposed a hydroxylation of the methyl ether to form a hemiacetal. This is thought to undergo spontaneous aldol cleavage to give formaldehyde and the corresponding phenol. Donnelly and Dagley (1981) and Kersten et al. (1982) devised a mechanism in which the ether linkage was converted via a series of reactions to an ester which was hydrolysed to release methanol and give various intermediates of aromatic ring degradation. More recently, Kersten et al. (1985), has proposed a demethylation by the ligninase enzyme. This involves a one electron oxidation of the methoxybenzene by the oxidized enzyme and subsequent addition of water to the cation radical followed by or simultaneously with methoxy elimination as methanol. Previous work on the demethylation of coniferyl alcohol by A.flavus showed detection of methanol but not formaldehyde (Iyayi and Dart, 1982).

In the present study no formaldehyde was detected. Methanol was detected in each case and the remaining parent molecules were the phenols (8) and (13).

The metabolism was continued by cleavage of the dimer in each case to yield guaiacol and vanillic acid from Compound (8), and 2-phenylphenol and vanillic acid from Compound (13). Cleavage of either the aryl ether bond or the $C\alpha$-$C\beta$ could have resulted in these products. The explanation is as follows. If the methoxyl group of the guaiacyl moiety of Compound (8) had been demethylated prior to the cleavage of the $C\alpha$-$C\beta$ bond (as shown in Figure 54) then vanillic acid and guaiacol could have been produced. Alternatively, direct cleavage of the Cα-Cβ bond could have resulted in the production of veratrole, with subsequent demethylation to guaiacol, and vanillic acid (Figure 54). Conversely, cleavage of the aryl ether bond of Compound (8) may have resulted in guaiacol and a C6-C2 compound rapidly converted to vanillic acid (Figure 55).

In a similar way the cleavage of the Cα-Cβbond of Compound (13) could have resulted in the production of 1-phenyl-2-methoxybenzene (with subsequent demethylation to 2-phenylphenol) and vanillic acid. The cleavage of the aryl ether linkage of Compound (13) could have produced 2-phenylphenol and a C6-C2 compound converted to vanillic acid.

To establish which of the bonds had been cleaved radiolabelled compounds were synthesized and used in metabolic studies. When ^{14}C2-labelled Compound (2) was degraded by A. flavus radiolabelled guaiacol was detected. This indicated that the Cα-Cβ bond had been cleaved (and not the aryl ether bond) with a demethylation reaction occurring either before or after the cleavage (Figure 54).

The quantity of methanol detected during growth of A. flavus on Compound (2) also indicated that cleavage of the Cα-Cβ bond occurred. The maximum amount of methanol detected (Figure 47) was almost equivalent to the theoretically calculated amount of methanol derived from four methoxyl groups. Three of these are present in Compound (2) itself. A further methoxyl group could have arisen from cleavage of the Cα-Cβ bond. This could have appeared in Compound (15) (veratrole) if the Cα-Cβ bond of Compound (8) had been cleaved or in guaiacol if Compound (8) had been converted to Compound (16) prior to Cα-Cβ cleavage (Figure 54).

The maximum amount of methanol produced during metabolism of Compound (9) (Figure 47) was also found to almost equal the theoretically calculated amount. This supports a Cα-Cβ cleavge of Compound (9).

No further degradation products of guaiacol or 2-phenylphenol were detected but protocatechuic acid was found suggesting that vanillic acid had been demethylated. Conversion of vanillic acid to protocatechuic acid and its subsequent cleavage in A. flavus has been previously established (Iyayi & Dart, 1981).

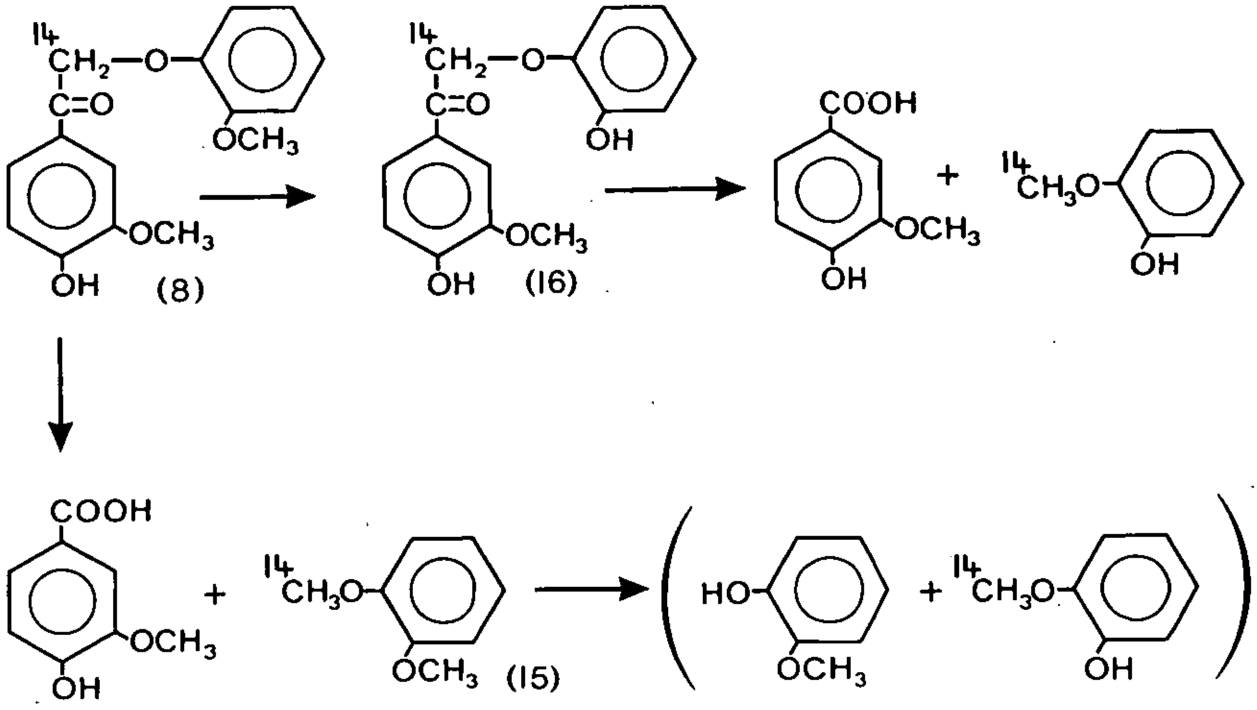


Figure 54. Alternative Sequences For

Cleavage Of The Cα-Cβ Bond Of Compound (8)

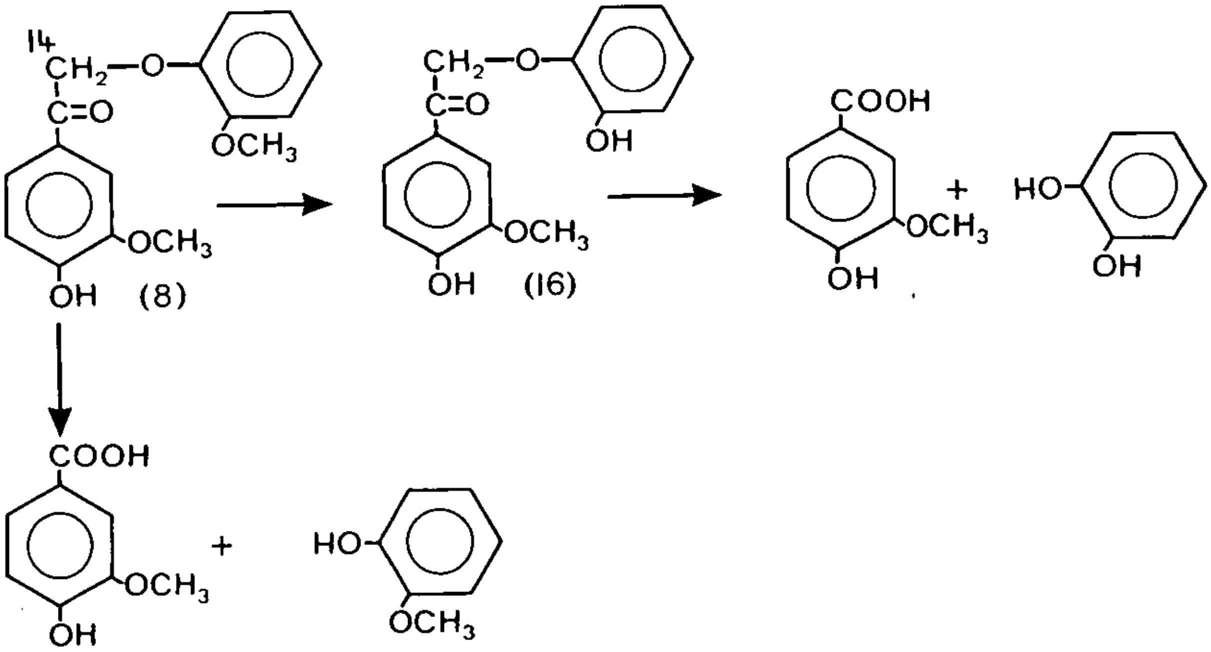


Figure 55. Alternative Sequences For

Cleavage Of The β-aryl Ether Bond Of Compound (8)

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APPENDICES

Dimerization of 2,6-dimethoxyphenol by *Aspergillus flavus*: evidence for the reaction occurring close to mycelia

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Abstract

Aspergillus flavus grown on 2,6-dimethoxyphenol as sole carbon source produced tetramethoxy-*p*-dibenzoquinone by a free radical mechanism. The product was identified by H-nmr and ms. Scanning electron microscopy and light microscopy were used to follow the growth of mycelia and the attachment of crystals to the mycelial surfaces. Formation of dimer was inhibited by the presence of glucose in the medium.

Introduction

Oxidative coupling of phenolic compounds and aromatic amines is an important process in the synthesis of soil humic substances, lignins, tannins, alkaloids, melanins and antibiotics (Haider *et al.*, 1975; Harkin, 1967; Sjoblad and Bollag, 1981). Reactions during coupling can give rise to a variety of C-C, C-O, C-N and N-N bonds.

The coupling mechanism in the case of phenols involves production of a phenoxy radical by removal of a proton and an electron from the hydroxyl group. Two free radical intermediates are then able to couple at *ortho* or *para* positions relative to the hydroxyl group, yielding dimeric compounds. Further oxidation can yield higher polymers. Oxidative coupling at *meta* positions does not occur in phenols (Sjoblad and Bollag, 1981).

Various enzymes can catalyse the coupling processes. These include laccases and peroxidases. Members of both groups have also been suggested as likely to be involved in the depolymerization reactions during lignin biodegradation (Ander and Eriksson, 1976; Arora and Sandhu, 1985; Huynh and Crawford, 1985; Ishihara, 1980; Kuwahara *et al.*, 1984; Paszczynski *et al.*, 1985; Paszczynski *et al.*, 1986).

Both intracellular and extracellular laccases have been reported to be produced by white-rot fungi, which are the most efficient lignin degraders (Blaich and Esser, 1975). It is considered by some workers that the peroxidase of ligninolytic systems is extracellular (Glen and Gold, 1985; Trojanowski *et al.*, 1985). However, most higher plants contain intracellular peroxidases. Three mycelium-bound peroxidase isoenzymes have been found in *Trametes versicolor* and *Trametes hirsuta* (Schanel *et al.*, 1971).

In this paper we describe the coupling of 2,6-dimethoxyphenol, a lignin-related monomer, by *Aspergillus flavus* which is found in wood-rotting systems. The dimeric product was identified and its formation within growing mycelial spheres was investigated using light and scanning electron microscopy.

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Materials and methods

Maintenance and growth of organism

The organism used was *Aspergillus flavus* CMI 15959. This was obtained from the Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, England. The maintenance, sporulation and growth media used have been described previously (Betts *et al.*, 1987).

Microscopy

The contents of flasks taken at intervals during the 5 day incubation period were filtered and washed with distilled water. Whole mycelial spheres were carefully transferred to glass slides. The specimens were covered with cover slips and viewed directly under the light microscope. It was found that the mycelia could be seen more easily if the spheres were immersed for 5 min in phenol red then washed three times with distilled water.

Mycelial samples to be used for electron microscopy were placed on circular coverslips and excess moisture was removed with paper tissues. The coverslips were fixed to metal stubs and were then desiccated under vacuum for 2 days. Following desiccation, the samples were sputter coated to a thickness of 10 nm with gold and were mounted in the scanning electron microscope (ISI SS40).

Isolation of product

It was difficult to isolate the product by simple extraction procedures possibly because the product was heavily enmeshed in fungal mycelia. A Soxhlet extraction technique was therefore used. The combined mycelial contents of four 1 litre flasks containing 500 ml of medium were washed with distilled water.

Excess water was removed by pressing the mycelia between filter papers. The mycelia were placed in a paper thimble and then extracted in a Soxhlet extractor for 10 h using redistilled acetone. The acetone was then removed on a rotary evaporator to leave dark purple crystals. These were washed well with distilled water and dried in a desiccator over anhydrous calcium chloride.

Thin layer chromatography

Preparative TLC was carried out on 1 mm thick Kieselgel 60 GF254 glass plates. The solvent system was benzene:ethyl acetate, 9:1 (Crawford *et al.*, 1973). Bands on the TLC plates were visualized using UV light and after scraping a band from the plate it was extracted five times with acetone. The extract was filtered through a sintered disc funnel and the solvent was removed on a rotary evaporator.

Identification of isolate

The product was characterized by its melting point, H-nmr spectrum (obtained on a Perkin-Elmer R32 spectrometer) and mass spectrum (recorded on a Kratos MS 50/DS-55 instrument).

Chemicals

2,6-Dimethoxyphenol was obtained from the Aldrich Chemical Company, Gillingham, Dorset, England.

Results

Identification of isolate

The coupling product of 2,6-dimethoxyphenol was isolated as dark purple crystals. These were identified as tetramethoxy-*p*-dibenzoquinone (MP 291—293°C) by H-nmr and ms.

Microscopy

Long dark needles of tetramethoxy-*p*-dibenzoquinone became visible within the mycelial spheres of *A. flavus* after approximately 2 days incubation of the organism with 2,6-dimethoxyphenol. The mycelial spheres appeared as dense, dark bodies after 5 days incubation (Figures 1 and 2). No crystals were found in random samples of the liquid growth medium and only a slight colour change could be observed in the medium. Many of the crystals associated with mycelia appeared attached to hyphae, and it was seen that many crystals were aligned parallel to the hyphae (Figure 3).

No crystals were observed neither was there any colour change in the medium when it contained 0.05 g of glucose per 100 ml in addition to 2,6-dimethoxyphenol.

Scanning electron microscopy showed that mycelia were in very close contact with the crystals of dimer (Figures 4 and 5). Figure 4 shows a mycelial strand which is attached to two crystals. The points of contact are intimate and the strength of the binding is demonstrated by the firm attachment of the mycelium to the crystals even with straining caused by drying and stretching.

Figure 1 Light micrograph showing aggregation of crystals of tetramethoxy-*p*-dibenzoquinone within a whole mycelial sphere of *A. flavus* x 40.

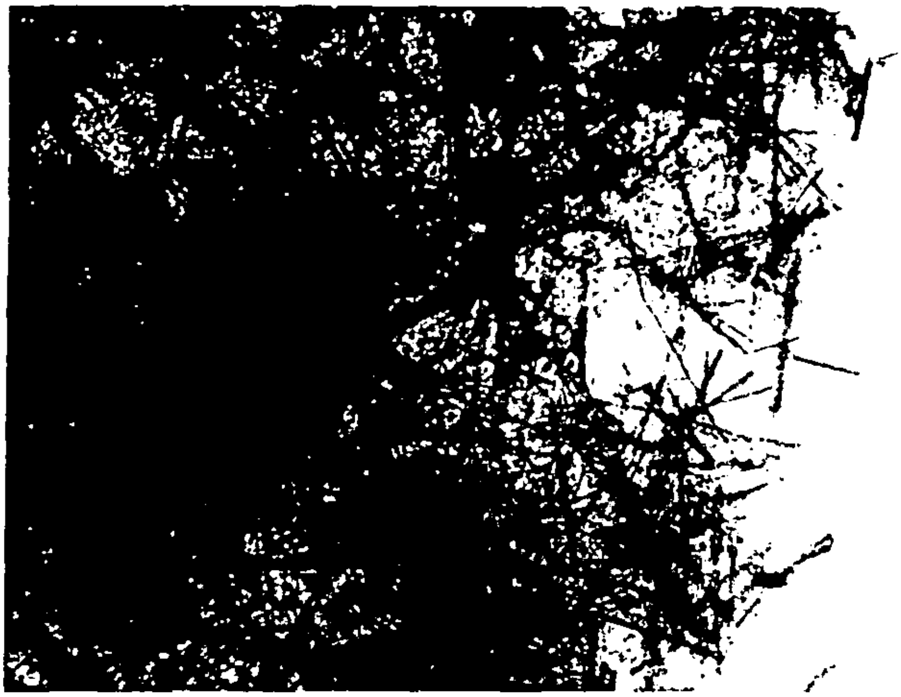

Figure 2 Light micrograph of part of a mycelial sphere of *A. flavus* showing individual crystals of tetramethoxy-*p*-dibenzoquinone. x 156.

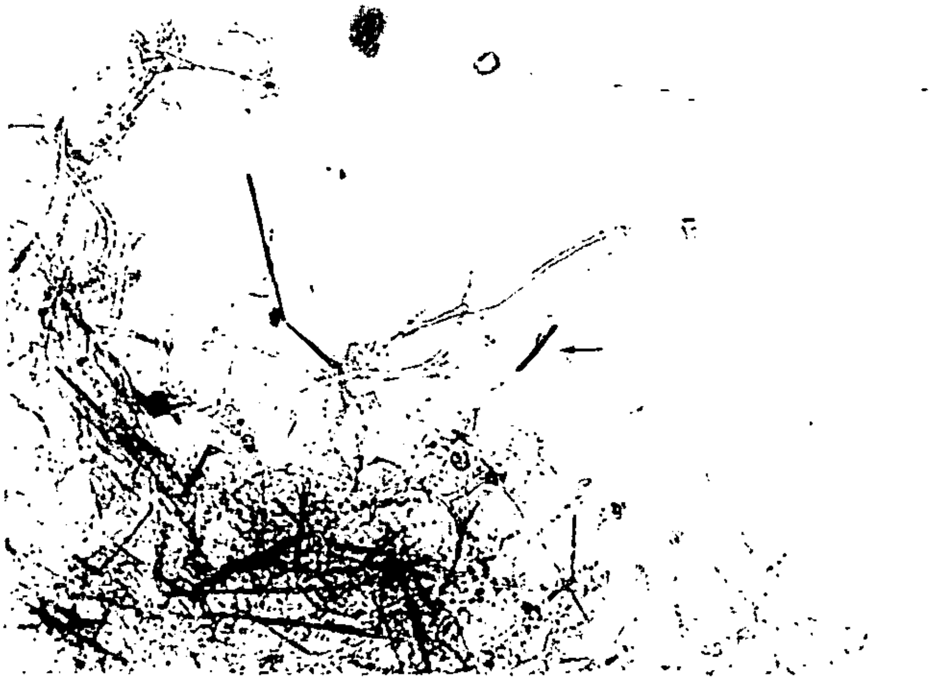

Figure 3 Light micrograph of the edge of a mycelial sphere of *A. flavus*. Crystals of tetramethoxy-*p*-dibenzoquinone can be seen attached to mycelia. Typically, a crystal is aligned parallel to a hyphal strand (arrow). x 156.

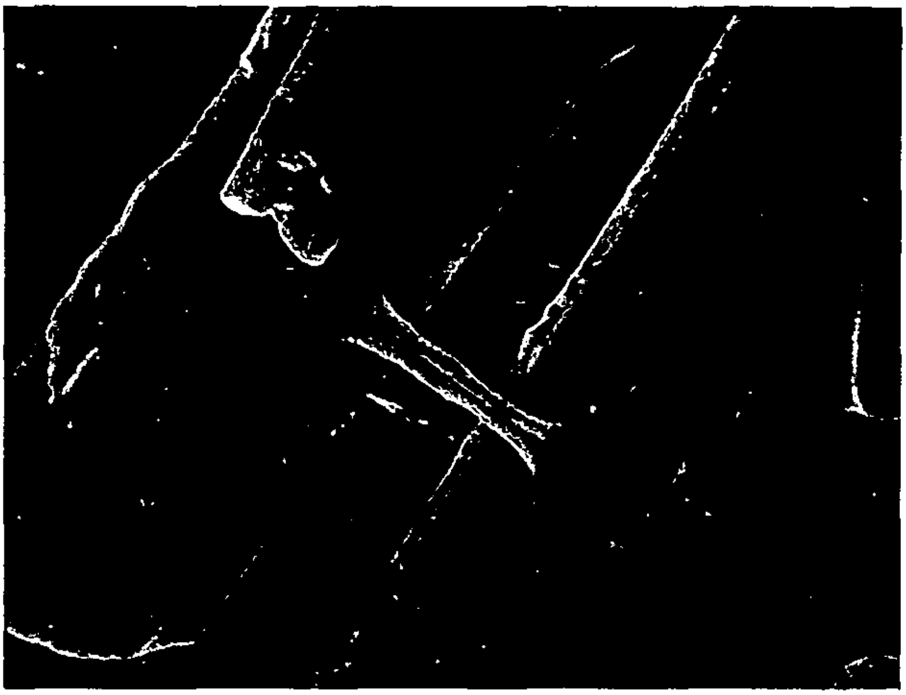

Figure 4 Electron micrograph showing attachemnt of a mycelial strand of *A. flavus* to two large crystals of tetramethoxy-*p*-dibenzoquinone. x 7,000.

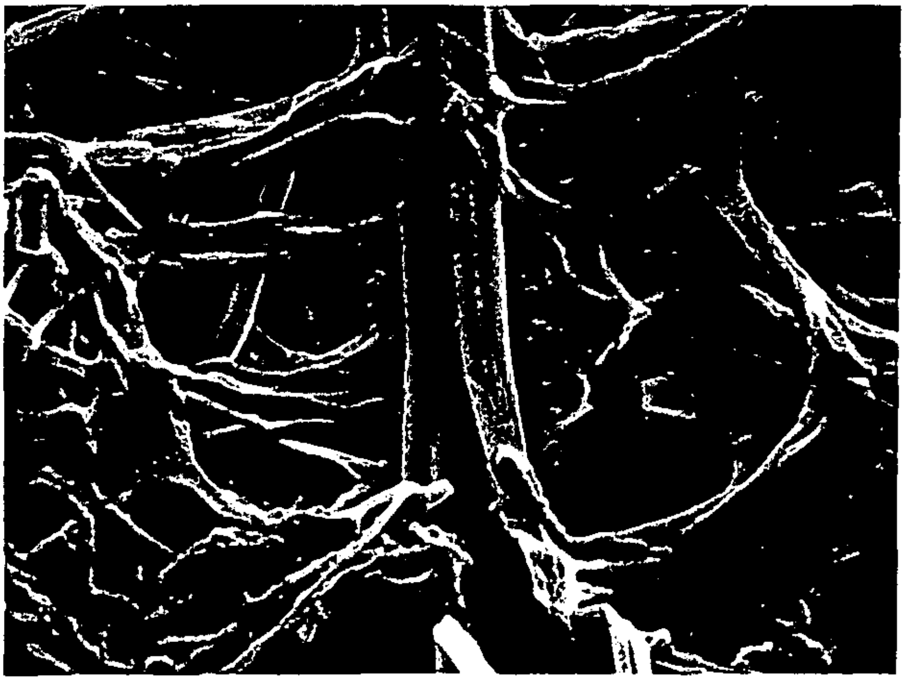

Figure 5 Electron micrograph showing associations between crystals of tetramethoxy-*p*-dibenzoquinone and mycelia of *A. flavus*. x 3,500.

Discussion

A. flavus readily formed the dimer tetramethoxy-*p*-dibenzoquinone from 2,6-dimethoxyphenol. The dark purple crystals of the dimer were only produced after mycelial spheres had formed and they were always produced within the mycelial spheres. Microscopy showed close associations between crystals of the dimer and mycelia of *A. flavus*.

The above information suggests that the coupling of 2,6-dimethoxyphenol may occur at the surface of mycelia. Close contact of the mycelia of *A. flavus* with insoluble lignin and lignin model compounds during shake culture has been observed previously (Betts *et al.*, 1987). Production of tetramethoxy-*p*-dibenzoquinone from 2,6-dimethoxyphenol by *Phanerochaete chrysosporium* has also been reported to occur on the mycelial surface, although this was in static culture (Paszczynski *et al.*, 1986).

In a similar reaction, the oxidation of vanillyl glycol to produce methanol and polymeric quinoids (Lundquist and Kristersson, 1985) was reported to have been catalysed by laccase whereas the coupling reaction observed by Paszczynski *et al.* (1986) was suggested to have been catalysed by a peroxidase enzyme. Some workers strongly support the role of laccase in lignin degradation (Ander and Eriksson, 1976; Ishihara, 1980), but others have suggested that laccase is not involved (Gierer and Opara, 1973; Haars and Huttermann, 1980). Various enzymes could cause these polymerization and depolymerization reactions but in lignin degradation evidence is accumulating which suggests that specific enzymes produce active oxygen species which can then act in a non-specific way in the disassembly of the polymer.

Polymerization of phenols is a common occurrence with soil micro-organisms and it has been suggested that laccase is produced to detoxify the phenols (including lignin degradation products). When micro-organisms which can polymerize phenols are also found to degrade lignin or lignin model compounds then the complexity of the enzyme systems and the pathways involved becomes apparent. The involvement of both types of reaction in the degradation of lignin may explain the fact that low molecular weight degradation products have only been found in very small quantities (Janshekar and Fiechter, 1983).

Formation of tetramethoxy-*p*-dibenzoquinone did not occur when glucose was present in the growth medium. This would suggest glucose repression of the enzyme(s) involved.

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Physical aspects of the degradation of insoluble tri- and tetrameric lignin model compounds by *Aspergillus flavus*

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Abstract

The three insoluble lignin model compounds 1-(4-benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-ethanol, 4-benzyloxy-3-methoxy-ω-(2-phenylphenoxy)-acetophenone and 4-hydroxy-3-methoxy-ω-(2-phenylphenoxy)-acetophenone each supported growth of *Aspergillus flavus* when used as sole carbon source. Scanning electron microscopy was used to show the concentration of the compounds within mycelial pellets over a 4 day period and to observe the intimate contact between mycelia and compounds.

Introduction

Lignin is a complex polymer containing a variety of non-hydrolysable bonds linking phenylpropane units (Sarkanen and Ludwig, 1971; Adler, 1977; Higuchi, 1982). Low molecular weight model compounds based on substructure units are often used in investigations into lignin biodegradation. These are frequently dimeric compounds with a β-aryl ether linkage which is the most common type of bond in the macromolecule (Sarkanen and Ludwig, 1971; Crawford, 1981).

The micro-organisms most often used in lignin biodegradation studies are white-rot fungi, particularly *Phanerchaete chrysosporium* which can degrade lignin completely (Kirk, 1980, 1981; Leisola and Fiechter, 1985). There have been reports of other types of ligninolytic fungi (Drew and Kadam, 1979; Haider and Trojanowski, 1980) and ligninolytic bacteria (Trojanowski *et al.*, 1977; Crawford and Sutherland, 1979; Kern *et al.*, 1984) but these have received less attention. In natural environments lignin is degraded by a consortium of micro-organisms (Janshekar and Fiechter, 1983).

The requirement for physical contact between lignin or related isoluble compounds and cells during degradation is inconclusive. An extracellular ligninase has attracted great attention recently but it is also proposed that a peroxidase associated with the mycelium plays a role in the degradation of lignin and model compounds (Paszczynski *et al.*, 1986; Asada *et al.*, 1986; Renganethan *et al.*, 1986). Forney *et al.* (1982), demonstrated that H_2O_2 production and catalase were located in the periplasmic structure of *P. chrysosporium* although no direct evidence has yet been provided to show the presence of a peroxidase in this location. Samples of wood decomposed by white- and brown-rot fungi contain hyphae in almost every cell (Kirk, 1983) and electron micrographs have shown dissolution of plant cell walls along the lateral surfaces of hyphae and also near the growing tips (Schmid and Liese, 1964; Eriksson *et al.*, 1980). However, the lysis of cell walls which are more distant from fungal hyphae has been reported by Ruel and Barnoud (1984).

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The bacterial degradation of cellulose which is also insoluble, frequently involves the attachment of cells to the substrate and very close contact has been observed (Berg *et al.*, 1972a, b; Weigel and Dijkstra, 1984). Kauri and Kushner (1985) have shown that although cell to fibre contact can occur, if it is prevented degradation of cellulose can still take place.

Recently, the metabolism of 2,6-dimethoxyphenol by *P. chrysosporium* was reported to result in the formation of the dimer tetramethoxy-*p*-dibenzoquinone attached to the mycelial surface (Paszczynski *et al.*, 1986). This suggests that the reaction occurred at the mycelial surface.

This study involves the growth of *Aspergillus flavus* on a number of insoluble tri- and tetrameric lignin model compounds which contained combinations of β-aryl ether, α-aryl ether and biphenyl linkages. The physical aspects of degradation were examined using the scanning electron microscope.

Materials and methods

Organism

Aspergillus flavus CMI 15959 was obtained from the Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, England.

Maintenance medium

A. flavus was maintained on slopes as previously described (Iyayi and Dart, 1982) and was subcultured at 2 week intervals.

Sporulation medium

The organism was induced to sporulate by growing it on a medium consisting of (per litre): $K_3PO_4.H_2O$, 2.0 g; KNO_3, 2.0 g; $CaCl_2$, 0.25 g; yeast extract, 5.0 g; glucose, 10.0 g; Bacto casamino acids (Difco), 5.0 g; metals solution, 0.1 ml; and agar, 20.0 g. The metals solution contained: $MgSO_4$, 50 mg; $ZnSO_4$, 2.0 mg; $FeSO_4$, 2.0 mg; $MnSO_4$, 1.0 mg; $CuSO_4$, 5.0 mg; and distilled water to 1 litre. The pH was adjusted to 5.2 with 0.1 M NaOH. The medium was dispensed into Roux bottles in 200 ml portions and these were then sterilised by autoclaving.

Growth medium

The basal growth medium contained (per litre): $NaNO_3$, 5.0 g; KH_2PO_4, 5.0 g; $MgSO_4.7H_2O$, 0.5 g; and yeast extract, 0.1 g. The pH was adjusted to 5.2 using 0.1 M NaOH and the medium was dispensed in 100 ml portions into 250 ml Erlenmeyer flasks which were then sterilised by autoclaving. The lignin model compounds were added to flasks as acetone solutions (0.1 g of compound in 2 ml of acetone per 100 ml of medium) to provide a finely dispersed suspension. The flasks were shaken at 30°C for 24 h before inoculation to allow evaporation of the acetone. Control flasks containing basal growth medium with the addition of acetone (2 ml per 100 ml of medium) with no model compound were used to check that the acetone had evaporated before inoculation.

Growth of organism

Fungal spores were prepared and harvested using the method of Dart (1975). These were diluted with sterile distilled water to a standard inoculum concentration of 10^5 spores per ml. Flasks were inoculated with 1 ml of spore suspension per 100 ml of medium.

Cultures were incubated at 30°C on an orbital incubator at 120 rpm. Uninoculated and no-substrate controls were used. The flasks were incubated for 4 days at which time samples were taken for electron microscopy.

Electron microscopy

Four days after inoculation the contents of flasks containing the various model compounds were filtered and washed five times with distilled water. Mycelial samples were placed on circular coverslips and excess moisture was removed with paper tissues. The coverslips were fixed to metal stubs and these were desiccated under vacuum for 2 days. The samples were then sputter coated with gold to a thickness of 10 nm prior to mounting in the electron microscope (ISI SS40).

It was occasionally necessary to tease mycelial spheres to expose the interior before desiccation. Exposure of the interiors of spheres also occurred during desiccation as the mycelia split. This usually aided observations but it was sometimes necessary to recoat the samples with gold if they flared in the electron microscope.

Chemicals

$NaBH_4$ reduction of an alcoholic suspension of 4-benzyloxy-3-methoxy-ω-(2-methoxyphenoxy)-acetophenone which was previously prepared by the method of Landucci *et al.* (1981) produced 1-(4-benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-ethanol (BMPMPE). Pure BMPMPE was obtained after extraction of the reaction mixture with $CHCl_3$ and subsequent column chromatography. The method of Betts *et al.* (1986) was used to prepare 4-benzyloxy-3-methoxy-ω-(2-phenylphenoxy)-acetophenone (BMPPA) and 4-hydroxy-3-methoxy-ω-(2-phenylphenoxy)-acetophenone (HMPPA). The purity of the compounds was checked by thin layer chromatography (solvent system, benzene:ethyl acetate, 9:1; visualizing agent, 50% H_2SO_4) and spectral analysis before use (ir, ^{13}Cnmr, Hnmr, ms).

Results

SEM observations

1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-ethanol (BMPMPE)

The addition of the acetone solution of BMPMPE to the growth medium produced a cloudy suspension of BMPMPE at the time of inoculation. During the 4 days growth period the growth medium became clear and mycelial pellets formed. Electron micrographs showed that crystals had become associated with the mycelial pellets. Figure 1 shows splitting of a mycelial outer layer and exposure of crystalline material beneath. Samples of mycelial pellets were washed carefully with distilled water and then extracted with acetone to remove

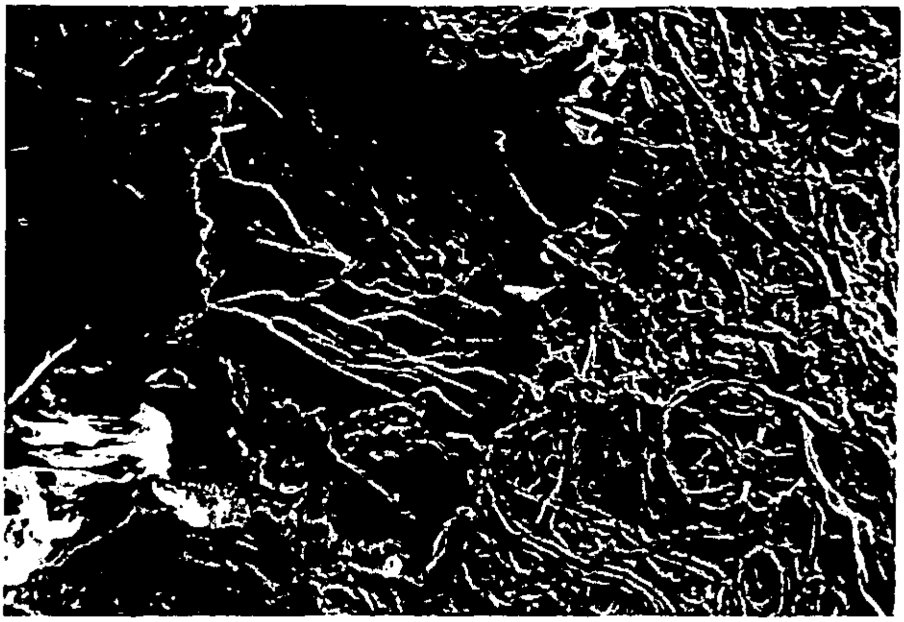

Figure 1 Photomicrograph showing splitting of the outer layer of a mycelial pellet of *A. flavus* exposing the crystalline BMPMPE beneath. x 696.

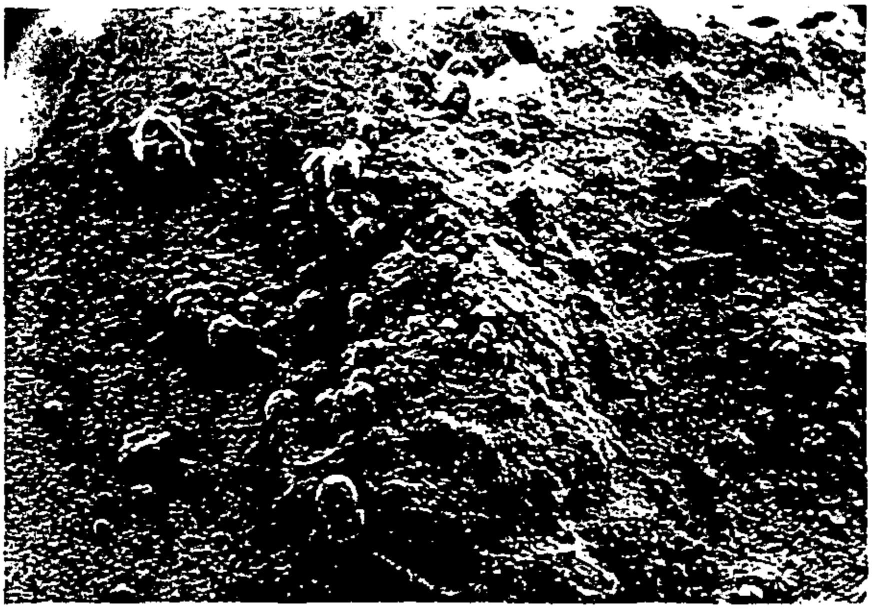

Figure 2 Photomicrograph of the aggregations of *A. flavus* and BMPPA . x 38.

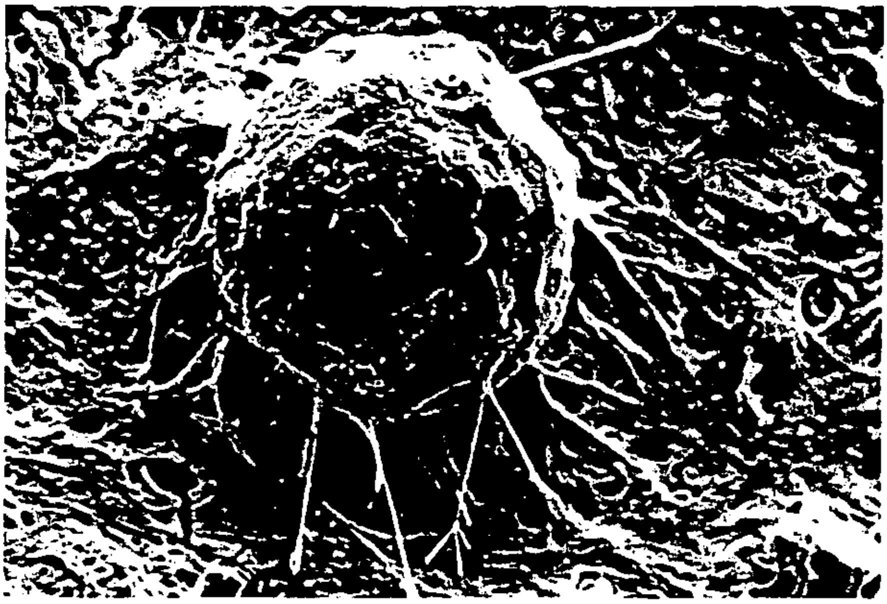

Figure 3 Photomicrograph showing the very strong and intimate contacts between *A. flavus* and BMPPA. x 542.

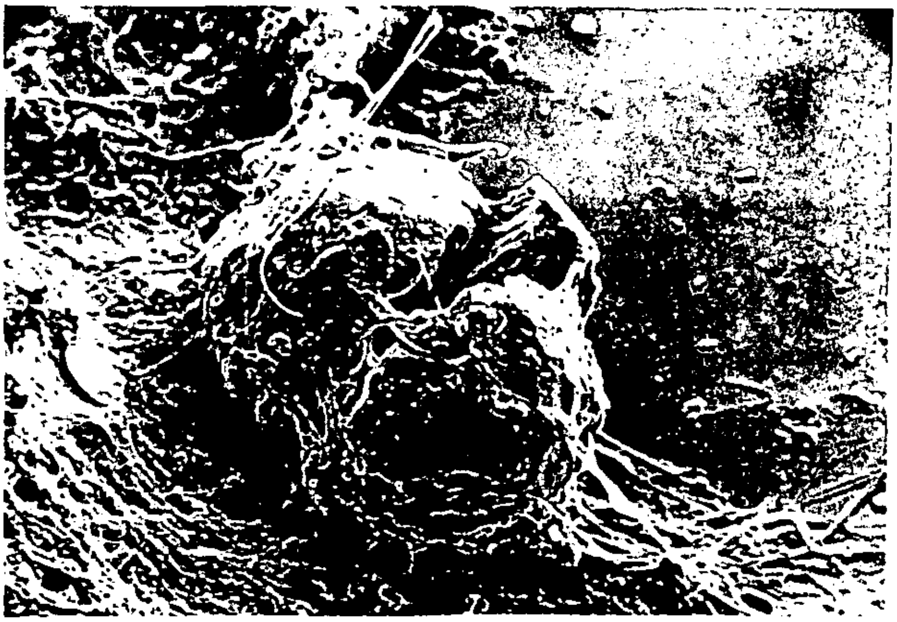

Figure 4 Photomicrograph showing associations between *A. flavus* and BMPPA. The contacts remain even at points of great stress (arrowed) thus demonstrating their strength. x 542.

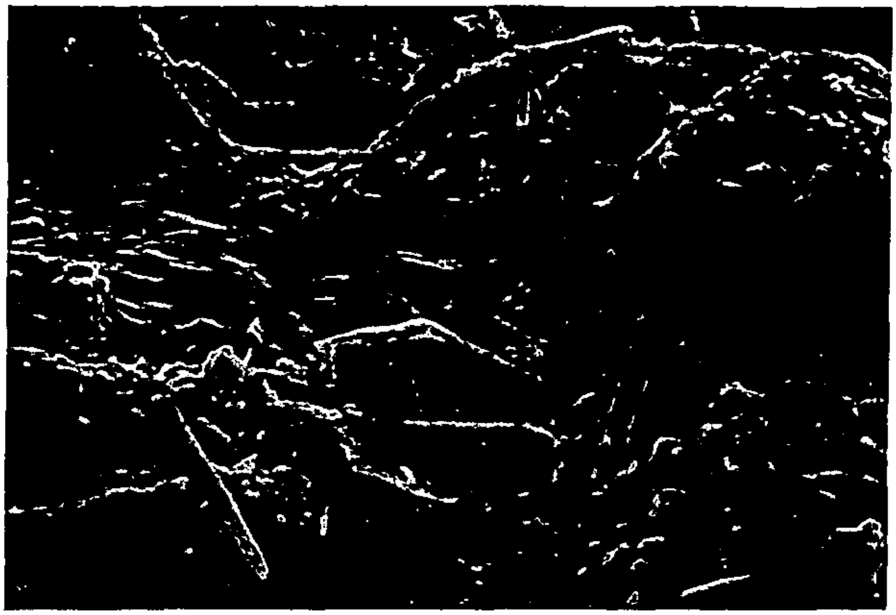

Figure 5 Photomicrograph showing close contact between mycelia of *A. flavus* and crystals of HMPPA. Very close contact can be seen across the central crystal (arrowed). x 2,150.

the crystals. Examination of the acetone extract by TLC (benzene:ethyl acetate, 9:1) showed that the crystals were BMPMPE.

4-Benzyloxy-3-methoxy-ω-(2-phenylphenoxy)-acetophenone (BMPPA)

An aggregation effect similar to that described above occurred when *A. flavus* was grown on BMPPA. Mycelial spheres became associated with a solid material and the cloudy growth medium cleared. Extraction of the spheres with acetone and subsequent TLC (benzene-ethyl acetate, 9:1) as described above showed the associated material to be BMPPA. The compound did not have a regular crystalline form but appeared as clumps of solid material (Figure 2). Intimate associations were observed and Figures 3 and 4 show typical observations of the binding together of the solid material by mycelia.

4-Hydroxy-3-methoxy-ω-(2-phenylphenoxy)-acetophenone (HMPPA)

Plates and needles of crystalline material became associated with mycelia during the 4 days growth of *A. flavus* on HMPPA. The initial cloudy medium became clear. At higher magnification (x 2,500) close contact between mycelia and crystals could be seen (Figure 5). The crystals were shown to be HMPPA by using the extraction and TLC procedures described above.

Discussion

The growth of *A. flavus* in shaken culture on the tri- and tetrameric lignin model compounds was vigorous and during growth the compounds became associated with and eventually concentrated within the mycelial pellets. Previous investigations on the growth of *P. chrysosporium* in agitated cultures with lignin and insoluble lignin model compounds have shown that degradation and metabolism were inhibited (Kirk *et al.*, 1978; Weinstein *et al.*, 1980; Goldsby *et al.*, 1980; Shimada *et al.*, 1981). An abrasive effect on the delicate mycelia may have been caused by the movement of the solid growth substrates in the shaking flasks and this could have disrupted and damaged cells (Reid *et al.*, 1985).

Shimada *et al.* (1981) suggested that the inhibition was due to physiological effects, perhaps affecting secondary metabolism generally. However Eaton *et al.* (1980) showed that the ligninolytic system could be very effective in shaken culture and recently the degradation of DHP and aspen lignin has been found to be as efficient in shaken culture as in static culture (Reid *et al.*, 1985). It is probable that because there is a limited availability of oxygen in the centre of mycelial pellets (Phillips, 1966) there will be some reduction in degradative capacity.

The results of this investigation have shown strong, intimate associations between mycelia of *A. flavus* and insoluble tri- and tetrameric lignin model compounds. Recent investigations on the ligninolytic system have shown the possible involvement of a mycelium-bound peroxidase and this may explain the requirement for a close mycelium-substrate association.

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159 Degradation of insoluble lignin model compounds by *A. flavus*

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NOTES AND BRIEF ARTICLES

GROWTH OF *ASPERGILUS FLAVUS* ON AN INSOLUBLE LIGNIN MODEL COMPOUND

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Aspergillus flavus was grown on 3,4-dimethoxy-ω-(2-methoxy-phenoxy)acetophenone, an insoluble lignin model compound. Electron microscopy of cultures over a period of 5 d showed close mycelial attachment to the insoluble crystals and erosion of crystal faces. Growth was correlated with reduction in mean crystal size and concentration of crystals within mycelial pellets was noted.

Lignin is a high molecular weight, water insoluble polymer consisting of phenylpropane units connected by a variety of carbon–carbon and carbon–oxygen bonds (Sarkanen & Ludwig, 1971).

Degradation of lignin has been investigated extensively using model compounds. These are usually of low molecular weight with one or two aromatic rings containing one of the major bond types found in lignin. Many of these model compounds are non-crystalline or are water soluble. Relatively few insoluble crystalline model compounds have been used, although these are probably a better representation of lignin for biodegradation studies (Betts, Dart & Ball, ~~in press (a))~~. (1987a)

The role of contact in degradation of both lignin and cellulose is controversial. In wood decayed by white and brown-rot fungi, hyphae can be seen in virtually every cell (Kirk, 1983). Electron microscopy has shown that the dissolution of the plant cell wall by white-rot fungi can occur along the lateral hyphal surface in contact with the cell wall as well as near the growing tips of the mycelium (Schmid & Liese, 1964; Eriksson, Grunewald & Vallander, 1980).

In contrast, Blanchette & Reid (1986) have reported a white-rot depolymerization of lignin throughout cell walls and not only immediately adjacent to the hyphae. Lysis of cell walls which are not in the vicinity of hyphae is common with brown-rot fungi, and Kauri & Kushner (1985) have shown that bacterial degradation of cellulose similarly does not necessitate contact.

Recently, Paszczynski, Huynh & Crawford (1986) found that degradation of 2,6-dimethoxyphenol by *Phanerochaete chrysosporium* resulted in the formation of the dimer, tetramethoxy-*p*-dibenzoquinone and showed that this dimer was attached to the mycelial surface. It was suggested that contact may be necessary for the reaction to occur and this may be explained by the presence of a peroxidase enzyme attached to the hyphae. This enzyme, together with an extracellular ligninase, is considered to play a role in the degradation of lignin and lignin model compounds (Paszczynski *et al.*, 1986; Renganethan, Miki & Gold, 1986).

A similar dimerization of 2,6-dimethoxyphenol has been shown in *Aspergillus flavus* (Betts, Dart & Ball, 1987 ~~in press~~ (b)).

It is proposed that two distinct ligninases exist in white-rot fungi, one hypha-bound and another diffusible (Otjen & Blanchette, 1985).

In this study we report the growth of *Aspergillus flavus* on the crystalline lignin model compound 3,4-dimethoxy-ω-(2-methoxyphenoxy)acetophenone (3,4-DMMPA) using this substrate as sole carbon source. This compound was chosen firstly because it contains a *β*-aryl ether linkage which accounts for approximately 50% of the intermonomeric linkages of lignin and secondly because like the natural polymer it is relatively insoluble. It was therefore considered that it would represent a better lignin model for studying the physical aspects of attachment of hyphae to substrate.

The model compound 3,4-dimethoxy-ω-(2-methoxyphenoxy)acetophenone (Fig. 1) was synthesized by the method of Landucci, Geddes & Kirk (1981). Its purity was checked by thin-layer chromatography (TLC) (solvent system, benzene : ethyl acetate, 9 : 1; visualizing agent, 50% aqueous H_2SO_4) and spectral analysis before use (i.r., H-n.m.r., ^{13}C-n.m.r., m.s.).

Aspergillus flavus IMI 15959 was obtained from the CAB International, Ferry Lane, Kew, Surrey, U.K. and maintained as previously described (Iyayi & Dart, 1982).

The experimental growth medium contained (l^{-1}) : $NaNO_3$, 5·0 g; KH_2PO_4, 5·0 g;

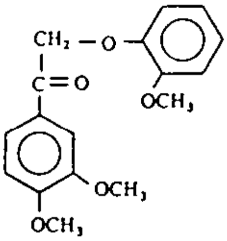


Fig. 1. 3,4-Dimethoxy-ω-(2-methoxyphenoxy) acetophenone (3,4-DMMPA).

$MgSO_4 . 7H_2O$, 0·5 g; yeast extract, 0·1 g. The pH was adjusted to 5·2. An acetone solution of 3,4-DMMPA was added to the culture medium in flasks to provide a finely dispersed suspension (1·0 g l^{-1}). Flasks were shaken at 30 °C for 24 h before inoculation to evaporate the acetone and then inoculated with 1 ml of spore suspension (approximately 10^6 spores ml^{-1}) 100 ml^{-1} of medium for quantitative growth experiments.

Cultures were incubated at 30° on an orbital incubator at 120 rev. min^{-1}. Quantitative growth measurements were made by dry weight determinations using the method of Bruner, Rohr & Silfen-Teifer (1968) and each determination was replicated six times. Uninoculated and no-substrate controls were used.

For light microscopy, the mycelial contents of flasks were collected at 24 h intervals after inoculation and washed with distilled water before examination.

Mycelial samples for scanning electron microscopy (SEM) were placed on circular coverslips and excess moisture removed with paper tissues. Samples were desiccated under vacuum for 2 d after fixing to metal stubs and were then sputter coated with gold (thickness 10 nm) prior to mounting in the scanning electron microscope (ISI SS40).

Electron micrographs were used to measure crystal size of 3,4-DMMPA. Low magnification fields were used to calculate the length of at least fifty crystals for each 24 h sample. Mean crystal lengths and the standard errors of the means were calculated.

Dry weight measurements of *A. flavus* grown on 3,4-DMMPA over a 5 d period showed substantial growth and measurements of crystal lengths of 3,4-DMMPA taken at various time intervals during growth showed a reduction in mean length (Fig. 2).

Identification of residual crystals by tlc (as previously described) after 5 d growth showed that

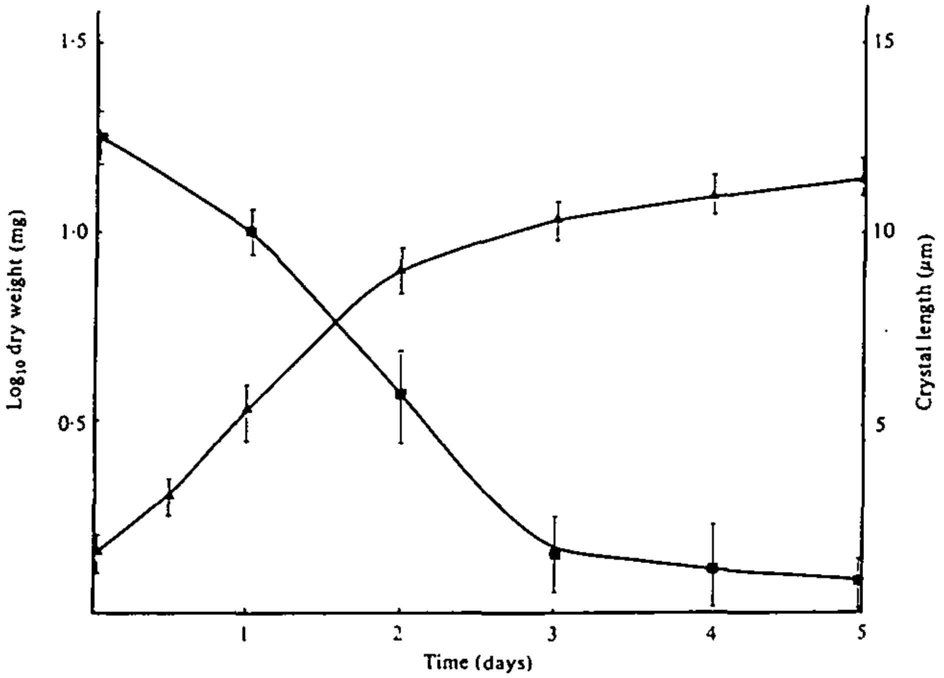
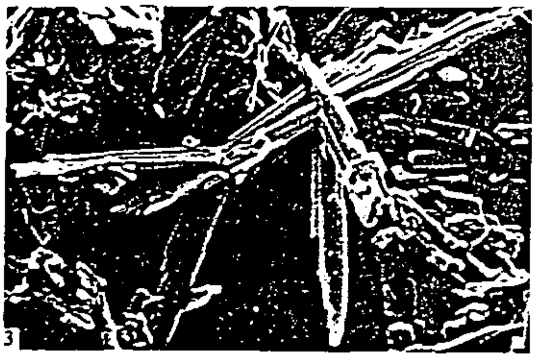
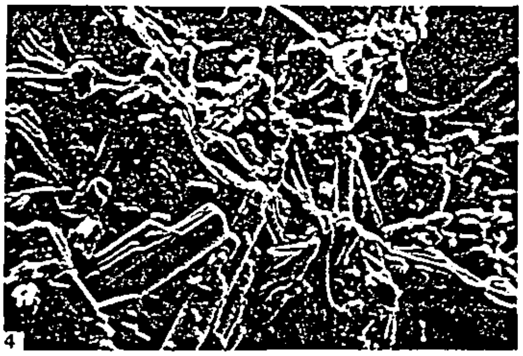
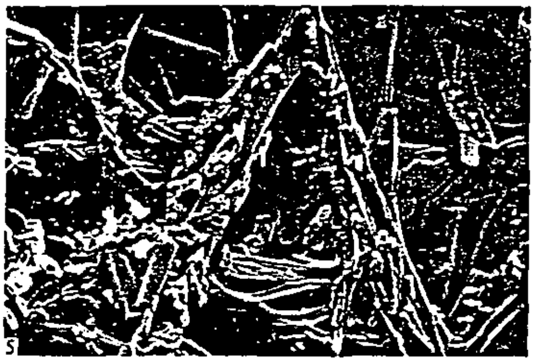
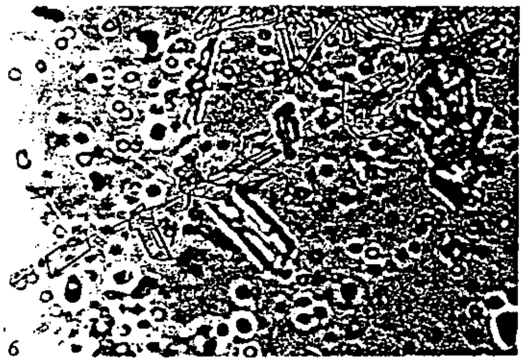


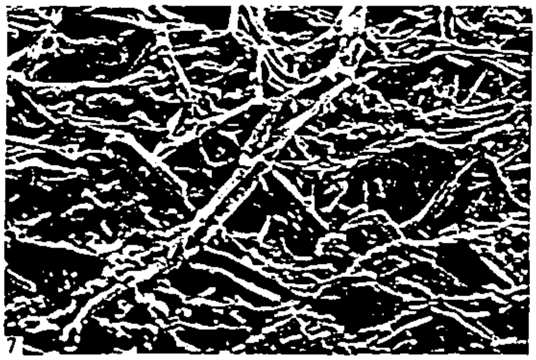
Fig. 2. Growth of *A. flavus* on 3,4-DMMPA. (▲), growth of *A. flavus*; (■), mean crystal length.

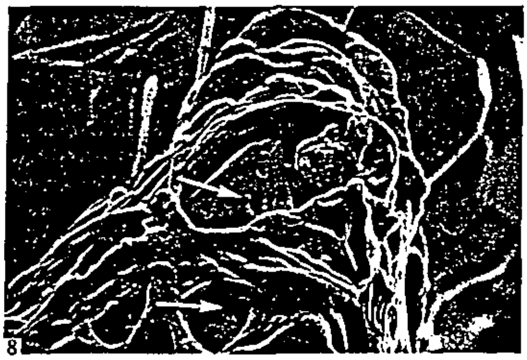












Figs. 3–8.

Fig. 3. Photomicrograph showing long sharp crystals of 3,4-DMMPA at the time of inoculation with spores of *A. flavus*. × 1500.

Fig. 4. Photomicrograph showing some association between *A. flavus* and 3,4-DMMPA 24 h after inoculation. × 1500.

Fig. 5. Photomicrograph taken after 48 h incubation of *A. flavus* with 3,4-DMMPA. × 1500. Crystals of 3,4-DMMPA can be seen attached to hyphae.

Fig. 6. Light microscope photograph of mycelia of *A. flavus* showing attachment of 3,4-DMMPA (arrows). Incubation time 48 h.

Fig. 7. Photomicrograph taken after 72 h incubation of *A. flavus* with 3,4-DMMPA showing dense mycelial growth and fewer, smaller crystals. × 1500.

Fig. 8. Photomicrograph of cultures of *A. flavus* incubated with 3,4-DMMPA for 72 h showing intimate contact. Areas of crystal erosion can be seen (arrows). × 2500.

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they were 3,4-DMMPA and not a metabolite. Filtration of the medium to remove the mycelium and extraction with three volumes of ethyl acetate followed by concentration and examination by tlc showed very little 3,4-DMMPA to be present.

Long sharp crystals were seen in suspension in the medium by SEM examination at the time of inoculation (Fig. 3).

Mycelial pellets had formed in the shaken flasks after 24 h and electron micrographs showed some association between hyphae and crystals (Fig. 4). Close association of mycelia and crystals could be seen after 48 h and hyphae could be seen which had crystals attached to them (Fig. 5). This effect was more obvious under the light microscope (Fig. 6).

Growth for 72 h produced dense pellets with a reduction in the amount of observable crystalline substrate (Fig. 7). Crystals were generally smaller, although a few large ones could still be seen.

Samples examined at 96 h showed a few small crystals and fungal spores were present.

Examination at higher magnification (× 2500) of cultures grown for 72 h showed intimate contact of mycelia and crystals and erosion of crystal faces close to hyphae (Fig. 8).

Examination of mycelial pellets over the 5 day time course of experiments showed a concentration of crystals with the mycelia.

The results presented in this paper show a close and intimate contact between mycelia and the insoluble substrate. Evidence is presented for erosion of the crystals within the vicinity of the mycelia and the almost total disappearance of the crystals. It is possible that the presence of residual amounts of crystals are due to a limitation of the availability of oxygen, an effect widely reported to occur in the centre of mycelial masses (Phillips, 1966; Kirk *et al.*, 1978). In spite of earlier doubts (Shimada *et al.*, 1981) more recent studies have shown that the ligninolytic system may be very effective in shake culture (Eaton, Chang & Kirk, 1980) and degradation of dehydrogenation polymers (DHP) and aspen wood lignin can be as efficient in shaken culture as in stationary cultures (Reid, Chao & Danson, 1985).

We wish to suggest that insoluble lignin model compounds such as the one used in this study provide a much better model for the physical aspects of lignin degradation than some of the soluble compounds used in previous studies.

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Scanning Electron Microscope observations of Fungal Associations with Lignin and insoluble Dimeric Lignin Model Compounds

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SEM
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Aspen lignin
Aspergillus flavus

Scanning Electron Microscope Observations of Fungal Associations with Lignin and Insoluble Dimeric Lignin Model Compounds

Summary

Aspergillus flavus was grown on autohydrolysis aspen lignin and the isoluble lignin model compounds 3,4-dimethoxy-ω-(2-methoxyphenoxy)acetophenone, 1-(3,4-dimethoxyphenyl)-2-(methoxyphenoxy)-ethanol and 4-hydroxy-3-methoxy-ω-(2-methoxypenoxy)acetophenone. Scanning electron microscopy of mycelial pellets after 4 days incubation with the compounds showed concentration of crystals within mycelial spheres and intimate contact between substrate and mycelia. Erosion of crystals in the vicinity of growing hyphal tips was also observed.

Introduction

The published work on the biodegradation of lignin contains frequent references to the use of dimeric model compounds (Reddy 1978; Cain 1980; Crawford and Crawford 1980; Kirk *et al.* 1980; Crawford 1981; Eggeling 1983; Janshekar and Fiechter 1983; Kirk 1984; Leisola and Fiechter 1985). Many of these compounds are water soluble or oils whereas natural lignin is a water insoluble polymer of phenylpropane ring (Sarkanen and Ludwig 1971; Adler 1977. It is therefore considered that insoluble compounds would probably be a better representation for work on the physical aspects of lignin biodegradation.

Cells frequently make close contact with the substrates during microbial attack of lignin and cellulose. It has been shown that the fungus *Phanerochaete chrysosporium* can bind synthetic lignins tenaciously (Chua *et al.* 1983). The bacteria *Clostridium thermocellum, Cellvibrio fulvus, Sporocytophaga myxococcoides* and a *Cellulomoas* species have been reported to become attached to cellulose fibres (Berg *et al.* 1972a; Berg *et al.* 1972b; Weigel and Dijkstra 1984).

However, the role of contact in the biodegradation of both cellulose and lignin is cotroversial. In wood decomposed by white-rot and brown-rot fungi virtually every wood cell contains fungal hyphae (Kirk 1983). Electron microscope examination of plant cell walls has shown dissolution alongside the lateral hyphal surfaces and near the growing tips (Schmid and Liese 1964; Eriksson *et al.* 1980). Recently it has been found that when *Ph. chrysosporium* degrades 2,6−dimethoxyphenol, the dimer tetramethoxy-*p*-dibenzoquinone is formed attached to the mycelial surface (Paszczynski *et al.* 1986).

In contrast, evidence has recently been presented to show that bacterial degradation of cellulose does not necessitate contact (Kauri and Kushner 1985). Ruel *et al.* (1984) have also roported cell wall lysis which is not in the vincinity of fungal hyphae.

Although lignin is degraded in natural environments by a consortium of micro-organisms (Leisola and Fiechter 1985), most investigations into lignin and lignin model degradation have been carried ot on the white-rot fungi, particularly *Ph. chrysosporium*. In this study we report the growth of *Aspergillus flavus* which is not a white-rot fungus on an insoluble lignin preparation and a number of insoluble dimeric lignin model compounds. Associations between the fungus and these substrates were examined using the scanning electron microscope.

Experimental

Organism

Aspergillus flavus CMI 15959 was obtained from the Commonwealth Mycolgical Institute, Ferry Lane, Kew, Surrey, U.K.

Media

A. flavus was maintained on slopes as previously described (Iyayi and Dart 1982) and was subcultured at two week intervals.

The Organism was induced to sporulate by growing it on a medium consisting of (per litre): KH_2PO_4, 2.0 g; KNO_3, 2.0 g; $CaCl_2$, 0.25 g; yeast extract, 5.0 g; glucose, 10.0 g; Bacto Casamino Acids (Difco), 5.0 g; metals solution, 0.1 ml; agar, 20.0 g. The metals solution contained: $MgSO_4$, 50 mg; $ZnSO_4$, 2.0 mg; $FeSO_4$, 2.0 mg;

$MnSO_4$, 1.0 mg; $CuSO_4$, 5.0 mg; distilled water to 1 l. The pH was adjusted to 5.2 with 0.1 M NaOH and the medium was dispensed into Roux bottles in 200 ml portions. These wre the sterilised by autoclaving.

The basal growth medium contained (per litre): $NaNO_3$, 5.0 g; KH_2PO_4, 5.0 g; $MgSO_4.7H_2O$, 0.5 g; yeast extract, 0.1 g. The pH was adjusted to 5.2 using 0.1 M NaOH and the mediu was dispensed in 100 ml portions into 250 ml Erlenmeyer flasks which were then sterilised by autoclaving. The lignin model compounds were added to flasks as acetone solutions (0.1 g of compound in 2 ml of acetone per 100 ml of medium). This provided a finely dispersed suspension. The flasks were shaken at 30°C for 24 hr befor inoculation to allow evaporation of the acetone. Control flasks containing basal grwth medium with the addition of 2 ml of acetone per 100 ml of medium with no model compound were used to ensure that the acetone had evaporated before inoculation.

Aspen lignin was added to the basal mdium as a separately sterilised solid (0.1 g per 100 ml of medium).

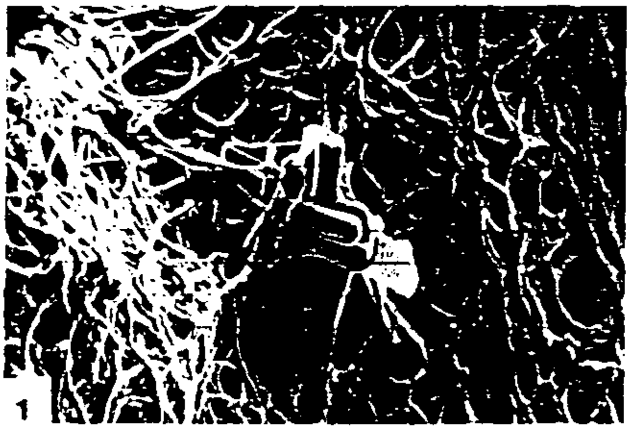
Growth of Organism

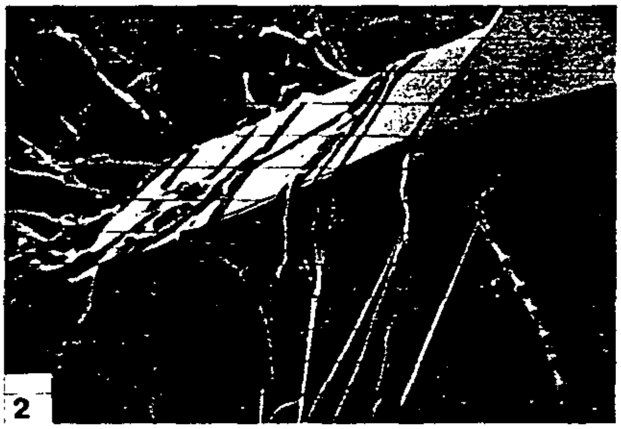
Fungal spores were prepared and harvested using the method of Dart (1975). These were diluted with sterile distilled water to a standard inoculum concentration of 10^5 spores per ml. Flasks were inoculated with 1 ml of spore suspension per 100 ml of medium.

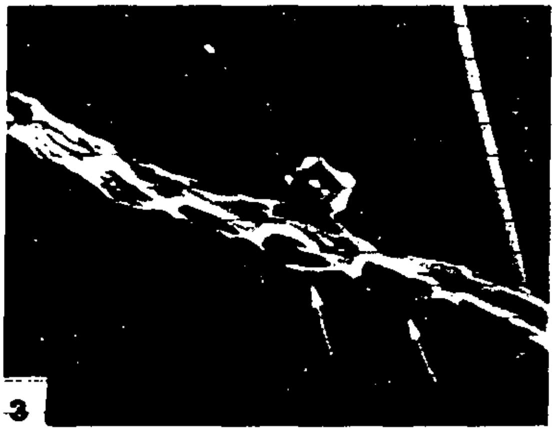
Cultures were incubated at 30°C on an orbital incubator at 120 r.p.m. Uninoculated and no-substrate controls were used. The flasks were incubated for 3 or 4 days at which time samples were taken for electron microscopy.

Electron Microscopy

At 3 or 4 days afert inoculation the contents of flasks containing lignin or the carious model compounds were carefully filtered and washed by pouring the contents into a fluted filter paper and allowing the medium to drain through. Befor the liquid had drained completely, distilled water was added to wash the contents and to prevent them from flattening onto the filter paper. This resuspending and washing was repeated 5 times, after which mycelial samples were placed on circular coverslips and excess moisture was removed with paper tissues. The coverslips were fixed to metal







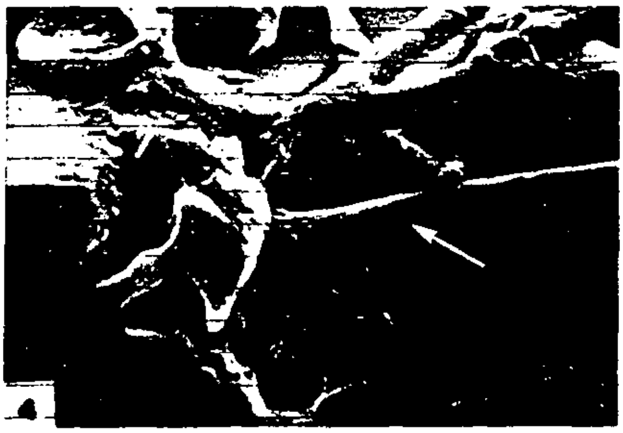


Fig. 1. Photomicrograph showing association between mycelial pellets of *Aspergillus flavus* and crystals of DMMPA. (× 435).

Fig. 2. Photomicrograph showing close association of hyphae of *A. flavus* with large crystals of DMMPA. (× 740).

Fig. 3. Photomicrograph of a hyphal strand of *A. flavus* in intimate contact with a crystal of DMMPA. The strand has collapsed under the vacuum during dessication and points of very strong attachment are shown (arrowed). (× 2200).

Fig. 4. Photomicrograph of a growing hyphal tip of *A. flavus* above a region of erosion of a crystal of DMMPA. The photomicrograph also shows hyphae lying in grooves (arrowed). (× 2475).

stubs and these were dessicated under vacuum for 2 days. The samples were then sputter coated with gold to a thickness of 10 nm prior to mounting in the electron microscope (ISI SS40). On occasions before preparation it was necessary to tease open mycelial spheres to expose the interior. Exposure of the interiors of spheres also occurred during dessication as the mycelia split. This usually aided observations, but it was occasionally necessary to recoat the samples with gold if they flared in the electron microscope.

Cemicals

A modification of the procedure of Landucci *et al.* (1981) was used to prepare 3,4-dimethoxy-ω-(2-methoxyphenoxy)acetophenone (DMMPA). Recduction of an alcoholic suspenion of DMMPA with $NABH_4$ was used to prepare 1-(3,4-dimethoxyphenoxyl)-2-(methoxyphenoxy)ethanol (DMPMPE). Extraction of the resulting solution with $CHCl_3$ followed by column chromatography (benzene: ethyl acetate, 9:1) gave pure DMPME. Catalytic hydrogenation of 4-benzyloxy-3-methoxy-ω-(2-methoxyphenoxy)acetophenone (BMMPA) prepared by the method of Landucci *et al.* (1981) gave 4-hydroxy-3-methoxy-ω-(2-methoxyphenoxy)-acetophenone (HMMPA). A solution of BMMPA in tetrahydrofuran/water (107:3) was shaken vigorously under hydrogen with a 10% Pd−C catalyst until TLC (benzene:ethyl acetate, 9:1) showed mainly the product HMMPA. This was subsequently filtered and recrystallized from ethanol to give pure crystals.

The purity of all the compounds was checked by thin layer chromatography (solvent system as above; visialisting agent, 50% H_2SO_4) and spectral analysis (ir, ^1H nmr, ^{13}C nmr, ms) before use.

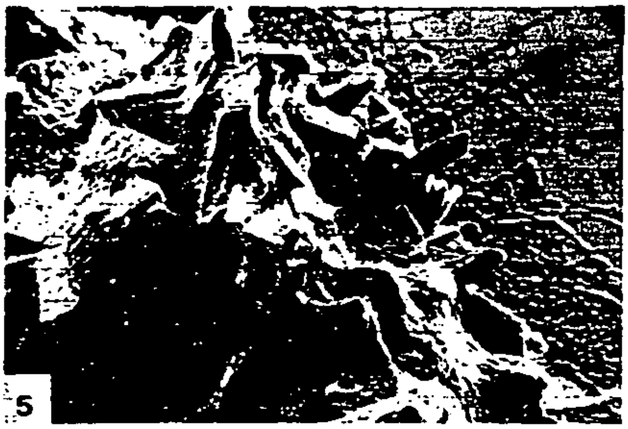
The sample of autohydrolysis aspen lignin was a gift of Professor M. Wayman, Department of Chemical Engineering and Applied Chemistry, University of Toronto, Canada.

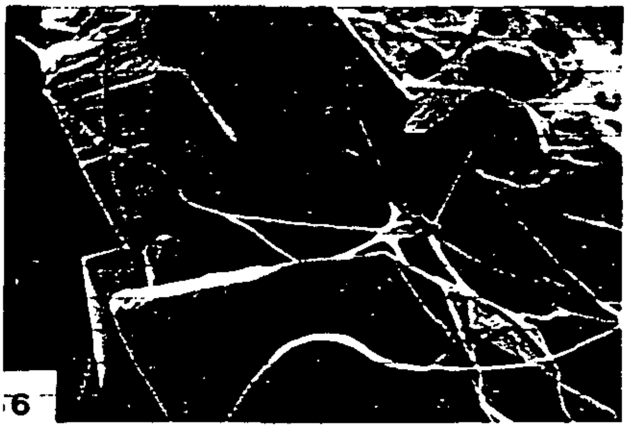
Results

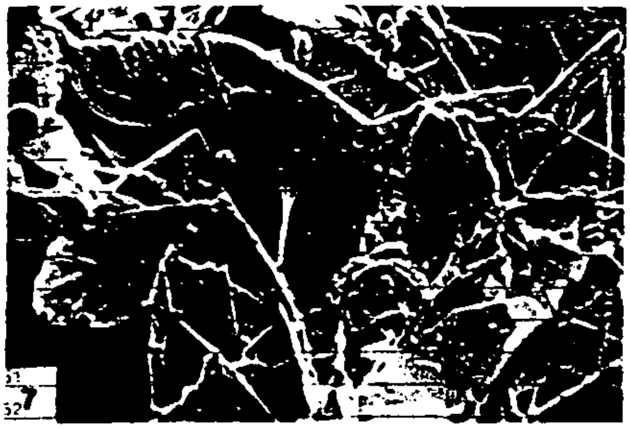
3,4-Dimethoxy-ω-(2-methoxyphenoxy)acetophenone (DMMPA)

The flasks containing DMMPA had a cloudly appearance due to DMMPA in fine suspension at the time of inoculation. During the following 3 to 4 days the flasks became clear and the electron micrographs showed that the mycelial pellets had become associated with crystals (Fig. 1 and 2). Samples of these mycelial pellets were washed with water and then extracted with acetone to remove the attached crystals. Examination of the acetone extract by TLC showed the crystals to be DMMPA.

Closer examinaton of the mycelium-crystals associations showed two main effects. The mycelia were in intimate contact with the crystals. A typical hyphal strand (Fig. 3) is shown which has collapsed under the vacuum either during dessication of the sample or during evacuation of the SEM. Along this strand are







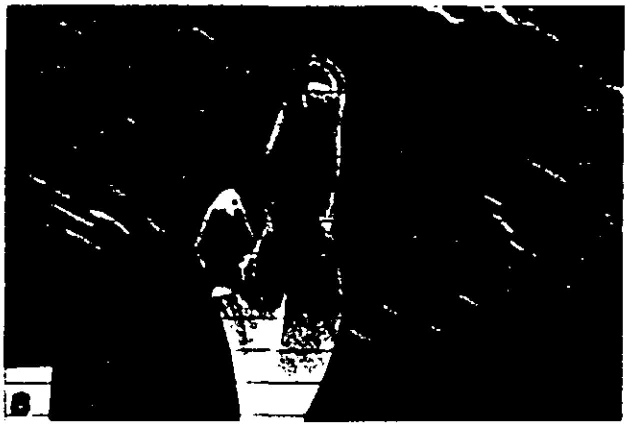


Fig. 5. Photomicrograph showing containment of crystals of DMPME within a mycelial pellet of *A. flavus*. (× 1100).

Fig. 6. Photomicrograph showing intimate contact between mycelia of *A. flavus* and crystals of DMPME. (× 730).

Fig. 7. Photomicrograph showing aggregation and binding together of plates of HMMPA by *A. flavus*. (× 375).

Fig. 8. Enlargement of a section of plate VII to show the intimate and strong contact between a hyphal tip of *A. flavus* and a crystal of HMMPA. (× 2145).

flattened points suggesting firm attachment to the crystal. On occasions mycelia were observed to be broken in preference to being pulled from the surface of the crystals. The second effect is shown in Fig. 4, where erosion of the crystal face is apparent beneath the growing hyphal tip. This type of erosion was not observed in uninoculated control samples. Close examination of Fig. 4 suggested that any hyphae lie in grooves in the vrystals and not the flat surfaces of the crystals.

1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)ethanol (DMPMPE)

The same effects were seen in samples taken from flasks containing DMPMPE as for those with DMMPA. The inital cloudiness of the medium disappeared over the growth period and containment of crystals of DMPMPE within the mycelial pellets was seen in electron micrographs (Fig. 5). TLC showed the associated crystals to be DMPMPE. Again, intimate conatact between mycelia and crystals was observed (Fig. 6).

4-Hydroxy-3-methoxy-ω-(2-methoxyphenoxy)acetophenone (HMMPA)

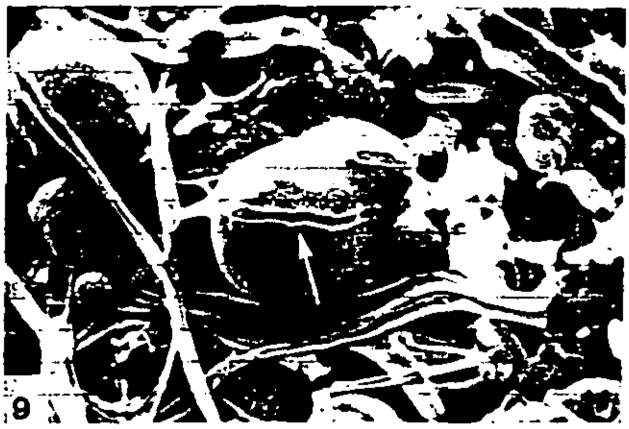
Similar effects were seen in samples grown on HMMPA as thōse grown on D̃MMPA and DMPMPE. However, HMMPA appeared as flattened plates rather than obloid crystals. Aggregation and binding together of these plates by mycelia was often seen (Fig. 7). At higher magnification (× 2145) intimate attachment of a mycelial growing tip to a crystal plate was observed (Fig. 8).

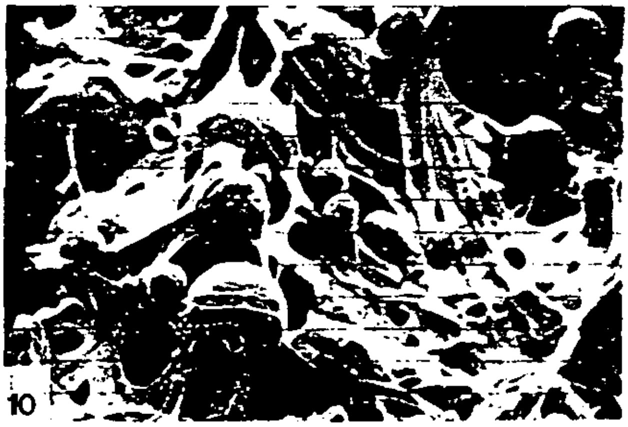
Autohydrolysis Aspen Lignin

The lignin appeared as spherical particles in the electron micrographs. Aggregation of the lignin and associations with mycelial spheres occurred during growth, and very little lignin remained as free solid in the medium after incubation for 4 days. Examples of mycelium-lignin contact are shown in Fig. 9 and 10, and the strength of the assosciations is demonstrated by the strained hyphal strand (arrowed) which still remained attached to a lignin particle. A higher magnification (× 2860) of the intimate contact of a growing hyphal tip with a lignin particle is seen in Fig. 11.

Discussion

Examination of mycelial spheres of *A. flavus* grown in shaken culture and utilising either autohydrolysis aspen lignin or several insoluble lignin model compounds showed a concentration effect of the solid materials within the spheres. Growth was vigorous in all cases. These effects are contrary to early observations into the degradation of lignin and isoluble model compounds by *Ph. chrysosporium* in agitated cul-





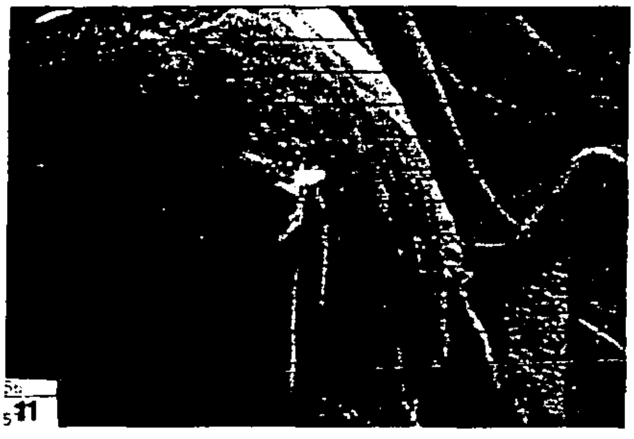


Fig. 9. Photomicrograph of a mycelial sphere of *A. flavus* associated with autohydrolysis aspen lignin. A hyphal strand attached to a lignin particle (arrowed) demonstrates the strong contract. (× 620).

Fig. 10. Photomicrograph showing binding together of lignin particles by mycelia of *A. flavus*. (× 620).

Fig. 11. Photomicrograph of a growing hyphal tip of *A. flavus* attached to a particle of autohydrolysis aspen lignin (× 2860).

tures, when Kirk *et al.* (1978), Weinstein *et al.* (1980), Goldsby *et al.* (1980) and Shimada *et al.* (1981) found that degradation and metabolism were inhibited. This was explained by general physilogical effects on secondary metabolism. The abrasive effect of solid paricles on the delicate mycelial growth was also considered to be inhibitory. However, more recent investigations have shown that the ligninolytic system may be very effective in shaken culture (Eaton *et al.* 1980). The degradation of aspen wood lignin and DHP has also been found to be as effective in shaken as in stationary culture (Reid *et al.* 1985), although it is probable that the limited availability of oxygen to the centre of mycelial slpheres (Phillips 1966; Kirk *et al.* 1978) causes some reduction in degradative capacity.

The contacts made between mycelia of *A. flavus* and the compounds used in this investigation have been shown to be intimate and very strong. These aspects of substrate – micro-organism associations have received little attention. The recent discoveries on the enzymology of the ligninolytic system have suggested that certain enzymes, notably a peroxidase, may reside on the surface of mycelia (Paszczynski *et al.* 1986, Renganathan *et al.* 1986) which could account for the need for a substrate-mycelium contact.

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SYNTHESIS OF SOME NOVEL TRI- AND TETRAMERIC LIGNIN MODEL COMPOUNDS

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Keywords
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Tetrameric
Synthesis
α-aryl Ether
Biphenyl Ether

Synthesis Of Some Novel Tri- and Tetrameric Lignin Model Compounds

A simple synthesis of tri- and tetrameric lignin model compounds containing the β-aryl ether, biphenyl linkage and the α-aryl ether is described. Initial bromination of Acetoveratrone or Acetovanillone benzyl ether was followed by a reaction with 2-Phenylphenol to yield respectively 3,4-Dimethoxy-ω-(2-phenylphenoxy)acetophenone (1) and 4-Benzyloxy-3-methoxy-ω-(2-phenylphenoxy)acetophenone (3). Catalytic hydrogenation of (3) formed 4-Hydroxy-3-methoxy-ω-(2-phenylphenoxy)acetophenone (5). Reduction of (5) with $NaBH_4$ yielded 1-(4-Hydroxy-3-methoxyphenyl)-2-(2-phenylphenoxy)-ethanol (6). Reduction of (1) and (3) with $NaBH_4$ gave rise to 1-(3,4-Dimethoxyphenyl)-2-(2phenylphenoxy)ethanol (2) and 1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-phenylphenoxy)ethanol (4), respectively.

Lignin is a high molecular weight polymer consisting of phenylpropane units linked by a variety of bonds Sarkanen and Ludwig 1971). Micro-organisms degrade lignin slowly (Eggeling 1983) and the use of low molecular weight model compounds in lignin degradation studies is common. Most published work only reports the use of monomeric and dimeric compounds. Several books and reviews have been published which describe and reference this work thoroughly (Reddy, 1978; Cain, 1980; Crawford and Crawford, 1980; Kirk, Higuchi and Chang, 1980; Crawford, 1981; Eggeling, 1983; Janshekar and Fiechter, 1983; Kirk, 1984; Leisola and Fiechter, 1985).

We report the synthesis of a series of novel trimeric and tetrameric model compounds containing the β-aryl ether linkage and also a biphenyl linkage both of which are represented in the typical lignin structures proposed by Nimz (1974) and Adler (1977).

The use of dimeric lignin model compounds containing the β-aryl ether linkage, which accounts for approximately 50% of the intermonomeric linkages of lignin (Crawford, 1981) has been reported by several authors. However, little work has been done on the biphenyl linkage which represents about 10% of the intermonomeric bonds in natural lignin. Relatively little work has been carried out on tri- and tetrameric lignin model compounds. This may be due to the more difficult syntheses necessary to produce these.

Acetovanillone was protected in the 4-position by benzylation with benzyl bromide using a modification to the method of Leopold (1950). This benzylation in the 4-position serves a dual function,either to protect the 4-hydroxyl group or to act as an analogue of the α-aryl ether linkage frequently represented in the lignin model (Nimz, 1974; Adler, 1977). The α-aryl ether linkage occurs to the extent of 6-8% in natural lignin. Bromination of acetoveratrone and 4-benzyloxy-3-methoxyacetophenone was accomplished using a modification of the procedure of Landucci _et al._ (1981).

The nucleophilic substitution of bromine by the 2-phenylphenate anion was achieved by a modification of the method of Kratzl _et al._ (1959).

Compound (5) was produced by catalytic hydrogenation of (3). The yield was considerably higher than the yield for the similar reaction by Landucci _et al._ (1981) as the α-carbonyl group of (3) was not appreciably reduced.

Subsequent reductions with sodium borohydride of (1), (3) and (5) gave rise to (2), (4) and (6).

Experimental

Melting points are uncorrected. Ir spectra were recorded on a Perkin-Elmer SP200 spectrophotometer. ^{1}H-nmr were measured on a Perkin-Elmer R32 nmr spectrometer at 90 MHz. High resolution mass spectra were recorded on a Kratos MS 50/DS-55 mass spectrometer. Analytical thin layer chromatography (tlc) was performed on SiO_2 plates (Merk, Kieselgel 60G, 0.25mm); the spots were visualized by spraying the plates with 50% aqueous H_2SO_4 followed by heating at 110°C for 5 min. Column chromatography was carried out on SiO_2 (Merk, Keiselgel 40, 70-230 mesh ASTM).

α-Bromoacetoveratrone

Nitrogen was bubbled slowly through a solution of acetoveratrone (1.89 g) in CCl_4 (100 ml) in a separating funnel. The nitrogen flow was temporarily disconnected and bromine (1.23 g) was added to the solution. This was then shaken until a precipitate formed (approximately 60 seconds). The nitrogen flow was reconnected and the sides of the reaction vessel were washed with $CHCl_3$ (12 ml). The solution became a clear, yellow/red colour after approximately 30 minutes, at which time the nitrogen flow was stopped. The solution was neutralized with 50-60 ml of saturated $NaHCO_3$ solution. The organic phase was then separated, dried over anhydrous $MgSO_4$ and filtered. Evaporation of the solvents under reduced pressure left a pink solid which was washed several times with cold methanol to give yellow crystals of α-bromoacetoveratrone. These were dried under nitrogen(1.23 g,65%); mp 72-77°C (lit mp 73-77°C).

3,4-Dimethoxy-ω-(2-phenylphenoxy)acetophenone.(1).

A mixture of α-bromoacetoveratrone (3 g), 2-phenylphenol (1.97 g) and potassium carbonate (1.11 g) in dry acetone (15 ml) were refluxed for 4 hours. The acetone fraction was then collected by vacuum filtration and the acetone evaporated under reduced presure. The remaining solid was recrystallized twice from methanol and washed with cold methanol to leave white flakes of (1) (2.67 g, 87%); analytical TLC gave one spot Rf 0.43; mp 82-83°C; ir γ max 3100, 2980, 1685, 1590 cm^{-1}; H-nmr [$CDCl_3$] δ 7.54 (m, 12H, aromatic), 5.2 (s, 2H, CH_2-O), 3.95 (s, 3H, OMe), 3.85 (s, 3H, OMe); ms m/z 348.1358.

1-(3,4-Dimethoxyphenyl)-2-(2-phenylphenoxy)-ethanol (2)

Sodium borohydride (0.15 g) was added to a suspension of the ketone (1) (3 g) in absolute ethanol (20 ml). This was shaken at room temperature. The ketone dissolved after a short time and after a further 20 minutes a yellow precipitate formed. Shaking was continued until TLC showed that none of compound (2) remained. The resulting suspension was diluted with distilled water, extracted three times with $CHCl_3$ and dried over anhydrous Na_2SO_4. Chloroform was removed under vacuum to leaving a light brown oil. This was purified by column chromatography (C_6H_6/EtOAc, 9:1) to leave (2) as a very pale green oil (2.91 g, 97%); analytical tlc (C_6H_6/EtOAc, 9:1) gave one spot R.f. 0.24; decomposition at 200°C (1 mm Hg); ir γ max 3500, 3010, 2930, 1590 cm^{-1}; H-nmr [$CDCl_3$] δ 7.43 (m, 12H, aromatic), 4.82 (q, 1H, H-C-), 4.00 (o, 2H, CH_2-O), 3.80 (s, 3H, OMe), 3.74 (s, 3H, OMe), 2.64 (d, 1H, C-OH); ms m/z 350.1512.

4-Benzyloxy-3-methoxyacetophenone

A mixture of acetovanillone (1.2 g), benzyl bromide (1.26 g) and potassium carbonate (1.0 g) in acetone (30 ml) were refluxed, with vigorous stirring, for 3 hours. The reaction mixture was filtered under vacuum to remove the potassium carbonate. Evaporation of the acetone under vacuum and re-crystallization from ethanol gave bright yellow crystals (1.15 g, 96%); mp 85-88°C [lit. mp 85-87°C].

4-Benzyloxy-3-methoxy-ω-bromoacetophenone

This was prepared from 1.79 g of 4-benzyloxy-3-methoxyacetophenone dissolved in CCl_4 (100 ml) with subsequent addition of bromine (1.2 g) and $CHCl_3$ (12 ml) according to the procedure used to obtain (2). The white crystals formed were washed several times with cold methanol (1.25 g, 70%; mp 100-103°C [lit. mp 98-102°C].

4-Benzyloxy-3-methoxy-ω-(2-phenylphenoxy)-acetophenone (3)

Compound (3) was prepared from a mixture of 4-benzyloxy-3-methoxy-ω-bromoacetophenone (3 g), 2-phenylphenol (1.526 g) and potassium carbonate (1.11 g) in dry acetone (15 ml) according to the method used to produce (1). The yellow solid formed was recrystallized twice from ethanol and dried under nitrogen to give light yellow needles of (3) (2.52 g, 84%); analytical tlc gave one spot Rf 0.62; mp 82-83°C; ir γ max 3040, 2980, 1680, 1585 cm^{-1}; H-nmr [$CDCl_3$] δ 7.45 (m, 17H, aromatic), 5.28 (s, 2H, CH_2-O), 5.2 (s, 2H, CH_2-O), 3.90 (s, 3H, OMe); ms m/z 424.1678.

1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-phenyl-phenoxy)ethanol (4)

Compound (4) was prepared from (3) (3 g) using the same procedure as the synthesis of (2) from (1). A light golden oil resulted (2.85 g, 95%); analytical TLC gave one spot Rf 0.31; decomposition at 245°C (1.5 mm Hg); ir γ max 3515, 3020, 2912, 1590 cm^{-1}; H-nmr [$CDCl_3$] δ 7.25 (m, 17H, aromatic), 5.50 (s, 2H, Ph-CH_2-O), 4.76 (q, 1H, H-C-), 4.00 (o, 2H, CH_2-O), 3.76 (s, 3H, OMe), 2.54 (d, 1H, C-OH); ms m/z 426.1828.

4-Hydroxy-3-methoxy-ω-(2-phenylphenoxy)-acetophenone.(5)

A mixture of the ketone (3) (3.86 g) and 10% Palladium on charcoal (0.6 g) in aqueous tetrahydrofuran (3 ml$\cdot H_2O$ in 110 ml THF) was shaken under hydrogen at room temperature until the pressure dropped to 15 lb/in^2. The mixture was filtered to remove the catalyst and dried twice over anhydrous magnesium sulphate. After filtering, the THF was removed by evaporating under vacuum to leave a pale yellow solid. This was recrystallized from ethanol to leave (5) as white crystals (3.16 g, 82%); analytical TLC gave one spot Rf 0.38; mp 135-137°C; irγ max 3140, 3120, 2912, 1655, 1578 cm^{-1}; H-nmr [$CDCl_3$] δ 7.45 (m, 12H, aromatic), 6.05 (s, 1H, phenolic-OH), 5.11 (s, 2H, CH_2-O), 3.82 (s, 3H, OMe); ms m/z 334.1207.

1-(4-Hydroxy-3-methoxyphenyl)-2-(2-phenylphenoxy)ethanol.(6)

Compound (6) was prepared from (5) according to the procedure used to obtain (2) from (1). A colourless oil resulted(2.79 g, 93%); analytical TLC gave one spot Rf 0.21; decomposition at 190°C (1 mm Hg); ir γ max 3500, 3010, 2912, 1595 cm^{-1}; H-nmr [$CDCl_3$] δ 7.38 (m, 12H, aromatic), 5.55 (s, 1H, phenolic-OH), 4.76 (q, 1H, H-C-), 4.00 (o, 2H, CH_2-O), 3.76 (s, 3H, OMe), 2.51 (s, 1H, C-OH); ms m/z 336.1351.

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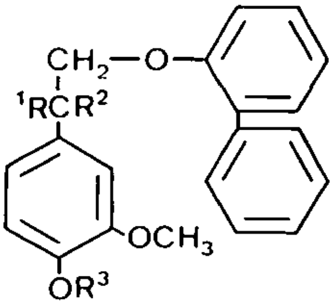
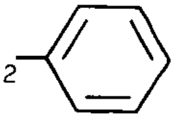
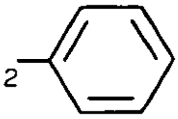


CH_2
O
$^1RCR^2$
OCH_3
OR^3

1 R_1R_2 = O; R_3 = CH_3

2 R_1 = H; R_2 = OH; R_3 = CH_3

3 R_1R_2 = O; R_3 = CH_2 

4 R_1 = H; R_2 = OH; R_3 = CH_2 

5 R_1R_2 = O; R_3 = H

6 R_1 = H; R_2 = OH; R_3 = H

INITIAL REACTIONS IN THE DEGRADATION OF TRI- AND TETRAMERIC LIGNIN MODEL COMPOUNDS BY *Aspergillus flavus*

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Initial Reactions in the Degradation of Tri- and Tetrameric Lignin Model Compounds by *Aspergillus flavus*

Keywords

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A. flavus
Degradation

Summary

Aspergillus flavus utilised 4-benzyloxy-3-methoxy-ω-(2-methoxyphenoxy)acetophenone and 4-benzyloxy-3-methoxy-ω-(2-phenylphenoxy)acetophenone as sole carbon source. Initial reactions in the degradation of these two compounds were oxidation of the α-hydroxyl group to the corresponding ketone and debenzylation of the benzyloxy group to yield benzyl alcohol and the corresponding phenol. Identification of the intermediates was accomplished using TLC, GLC and IR, NMR and mass spectroscopy.

INTRODUCTION

Micro-organisms which can decay lignin include the white-rot fungi and certain *Ascomycetes*, *Fungi Imperfecti*, *Eubacteria* and *Actinomycetes* (Ander and Erikson, 1978; Crawford *et al*., 1983; Drew and Kadam, 1979; Haider and Trojanowski, 1975; Hall *et al*., 1979; Higuchi, 1971; Kern, 1984; Kirk, 1971; Levi and Preston, 1965; Trojanowski *et al*., 1977). The most efficient degraders are the white-rot fungi and most research has been associated with this group. In particular, *Phanerochaete chrysosporium* has received great attention.

In order to simplify the understanding of lignin degradation by micro-organisms model compounds are often used (Ander and Eriksson, 1978; Crawford, 1981). These tend to be low molecular weight aromatic compounds many of which are soluble or oils. The majority contain either a single aromatic ring or two rings joined by one of the more common bonds found in lignin.

Degradation of dimeric lignin model compounds of the β-aryl ether type generally occurs by oxidation and splitting of either the Cα-Cβ bond or the β-aryl ether bond (Pelmont *et al*., 1985). Most fungi preferentially cleave the Cα-Cβ bond (Enoki *et al*., 1981; Fukuzumi *et al*., 1969; Katayama *et al*., 1980; Kirk *et al*., 1968; Tien and Kirk, 1983; Umezawa *et al*., 1983). The most common type of attack by bacteria involves cleavage of the β-aryl ether linkage (Crawford *et al*., 1973; Crawford *et al*., 1975; Fukuzumi, 1980; Kawakami, 1980; Borgmeyer and Crawford, 1985).

Most of the current theories on the mechanisms and enzymology of lignin degradation are based on research using monomeric or dimeric lignin models (Glenn and Gold, 1985; Hammel *et al*., 1985; Harvey *et al*., 1986; Huynh and Crawford, 1985; Kersten *et al*., 1985; Kirk, 1986; Renganathan *et al*., 1986).

In this paper we describe the initial reactions in the degradation of tri- and tetrameric model compounds containing the β-aryl ether and biphenyl linkages. Also present is a benzyloxy group which may be considered as an analogue to an α-aryl ether.

The organism used was *Aspergillus flavus* which has been isolated from wood-rotting systems and has previously been shown to degrade monomeric lignin model compounds (Iyayi and Dart, 1982). *A. flavus* is not a white-rot fungus and does not belong to any of the generally accepted groups of wood degraders.

METHODS

Maintenance and Growth of Organism.

Aspergillus flavus CMI 15959 was obtained from The Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, U.K. The maintenance and sporulation media used were as described previously (Betts *et al.*, 1987 [a]). The growth medium consisted of (l^{-1}): $NaNO_3$, 5 g; KH_2PO_4, 5 g; $MgSO_4.7H_2O$, 0.5 g; yeast extract, 0.1 g; lignin model compound, 1 g. Quantitative growth measurements were made by dry weight determinations using the method of Bruner *et al.*, (1968) and each determination was repeated six times. To account for any growth due to small amounts of carbon source in the yeast extract, the extent of growth in the medium without added lignin model compound was determined. This was deducted from subsequent growth measurements.

Chemicals

1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)ethanol (I) was produced by $NaBH_4$ reduction of an alcoholic suspension of 4-benzyloxy-3-

methoxy-ω-(2-methoxyphenoxy)acetophenone (II) which was previously prepared by the method of Landucci *et al.*, (1981). Catalytic hydrogenation of (II) gave 4-hydroxy-3-methoxy-ω-(2-methoxyphenoxy)acetophenone (III). A solution of (II) in tetrahydrofuran/water (107:3) was shaken vigorously under hydrogen with a 10% Pd-C catalyst until TLC using a solvent system of benzene:ethyl acetate, 9:1 showed mainly product (III). Subsequent filtering and recrystallization from ethanol gave (III) as pure crystals.

1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-phenylphenoxy)ethanol (IV), 4-benzyloxy-3-methoxy-ω-(2-phenylphenoxy)acetophenone (V) and 4-hydroxy-3-methoxy-ω-(2-phenylphenoxy) acetophenone (VI) were synthesised as described by Betts *et al.*, (1987 [b]). Benzyl alcohol was obtained from Aldrich Chemical Company, Gillingham, Dorset, U.K. The purity of the compounds was checked by TLC (solvent system, benzene:ethyl acetate, 9:1: visualising agent, 50% aqueous H_2SO_4) and spectral analysis (IR, ^{13}C-NMR, H-NMR, MS) before use in degradation studies.

Isolation of Intermediates

Aspergillus flavus was grown in 1 l. Erlenmeyer flasks containing 500 ml of growth medium with 0.5 g of either compound (I) or (IV). The flasks were incubated at 30°C in an orbital incubator (120 r.p.m.) for 4 days. The contents of 4 replicate flasks were then pooled and filtered. The mycelial material residue was washed well with distilled water, combined with the filtrate, acidified to pH 2.0 with 4 M HCl and extracted exhaustively with ethyl acetate. The extracts were combined and dried over Na_2SO_4. The organic phase was evaporated to dryness and the residue was dissolved in acetone. This concentrated extract was fractioned by TLC.

A control lacking substrate and an uninoculated control were also subjected to the above procedure.

All solvents used were redistilled.

Thin Layer Chromatography

Analytical TLC was carried out on Whatman LK6 DF silica gel plates. Preparative TLC was carried out on 1 mm thick Kieselgel 60 GF_{254} 20 x 20 cm glass plates. Solvent systems used were benzene:ethyl acetate, 9:1 (Crawford *et al.*, 1973) and benzene:dioxane:acetic acid, 90:25:4 (Randerath, 1968). Analytical plates were visualised using UV light and 50% aqueous H_2SO_4. Preparative plates were visualised under UV light and the band under investigation was scraped from the plate into a flask and the silica gel extracted five times with acetone. The extract was filtered using a sintered disc funnel and the volume of the acetone extract was reduced on a rotary evaporator.

Gas-liquid Chromatography

The system used was a Pye series 4 Chromatograph with a Spectra-Physics SP4290 integrator. An F.I.D. detector was fitted to the instrument and the glass column was packed with 10% Carbowax 20M on Chromosorb W80/100. The oven temperature was 160° and the carrier gas was N_2 (17 lb/in^2). Hexadecanol was used as internal standard.

Identification of Isolates

The Rf values of isolates were compared with authentic samples and isolates and authentic samples were co-chromatographed. Spectra (IR) were recorded on a Perkin-Elmer SP200. Spectra (H-NMR) were obtained on a Perkin-Elmer R32 using samples dissolved in $CDCl_3$. Mass spectra were recorded on a Kratos MS 50/DS-55 mass spectrometer. All spectra were compared with authentic samples. Benzyl alcohol was determined by GLC as described above. A sample of the aqueous medium after 4 days incubation was injected directly into the gas chromatograph. The retention time was compared to that of an aqueous solution of authentic benzyl alcohol. The two were also co-chromatographed.

RESULTS

Growth of *A. flavus* on Compounds (I) and (IV)

Dry weight measurements of *A. flavus* grown on both (I) and (IV) over a 5 day period showed substantial growth (Figs. 1 and 2).

Identification of Isolates

Extracts from media in which *A. flavus* was grown on compound (I) showed several major bands on TLC. Three of these corresponded to unused substrates and compounds (II) and (III). The Rf values are shown in Table 1. Preparative TLC, isolation of the compounds and spectral analysis confirmed the identities of (II) and (III).

Compound (II): IR ν max 3072, 2930, 1685, 1590 cm^{-1}; H-NMR (CDCl$_3$) δ 7.64 (m, 3H, aromatic), 7.42 (m, 5H, aromatic), 6.96 (m, 4H, aromatic), 5.25 (s, 2H, CH$_2$-O-), 5.14 (s, 2H, CH$_2$-O-), 3.90 (s, 3H, OMe), 3.85 (s, 3H, OMe); MS m/z 378.1478.

Compound (III): IR ν max 3011, 2939, 3148, 1659, 1584 cm^{-1}; H-NMR (CDCl$_3$) δ 7.66 (m, 3H, aromatic), 6.92 (m, 4H, aromatic), 6.24 (s, 1H, phenolic OH), 5.31 (s, 2H, CH$_2$-O-), 3.98 (s, 3H, OMe), 3.92 (s, 3H, OMe); MS m/z 288.1005.

Similar experiments using compound (IV) as substrate gave compounds (V) and (VI) which were identified in an identical manner. The Rf values are shown in Table 2.

Compound (V): IR ν max 3041, 2979, 1680, 1586 cm^{-1}; H-NMR (CDCl$_3$) δ 7.47 (m, 17H, aromatic), 5.29 (s, 2H, CH$_2$-O), 5.22 (s, 2H, CH$_2$-O), 3.94 (s, 3H, OMe); MS m/z 424.1672.

Compound (VI): IR ν max 3139, 3121, 2914, 1657, 1577 cm^{-1}; H-NMR (CDCl$_3$) δ 7.47 (m, 12H, aromatic), 6.07 (s, 1H, phenolic-OH), 5.09 (s, 2H, CH$_2$-O), 3.83 (s, 3H, OMe); MS m/z 334.1204.

The order in which compounds were formed was determined by growing *A. flavus* on compound (II). Growth on (II) produced several bands on TLC and two of these corresponded to unused (II) and compound (III). Both were isolated and their identities were confirmed as above.

When the aqueous medium was assayed for benzyl alcohol by GLC a positive result was obtained. Its identity was confirmed by co-chromatography with an authentic sample. The quantities of benzyl alcohol found after incubation with 0.1 g of compounds (I) and (IV) for four days were 0.0155 µmoles and 0.0015 µmoles, respectively.

The above results suggest degradation sequences as shown in Figures 3 and 4.

DISCUSSION

The initial degradation step involves oxidation of the α-hydroxyl group to a carbonyl group with both compounds (I) and (IV). This may be analogous to the process by which white-rot fungi attack lignin as they tend to produce a modified lignin with increased amounts of benzoyl groups as compared to undecayed lignin (Kirk and Chang, 1974). Examples of this type of reaction are also found in non white-rot fungi and bacteria (Crawford *et al.*, 1973; Higuchi, 1980; Pelmont *et al.*, 1985). However, the enzyme systems involved in this oxidation may differ considerably with different organisms. The reaction is catalysed by intracellular enzymes in *Pseudomonas acidovorans* and would almost certainly not attack an intact lignin molecule which would be extracellular (Crawford and Crawford, 1980). The range of enzymes capable of achieving this type of reaction is wide and includes a NAD^+ dependent dehydrogenase (Pelmont *et al.*, 1985), a hydrogen peroxide dependent extracellular enzyme (Renganathan *et al.*, 1986), and a phenol oxidase (Shimada, 1980). Several lignin degrading *Fusarium* species have been reported to excrete an enzyme which catalyses the oxidation of the primary alcohol group in the α,β-unsaturated side chain of guaiacylglycerol-β-coniferyl ether to the corresponding aldehyde. The enzyme seemed to be an aromatic alcohol oxidase (Janshekar and Fiechter, 1983). The oxidation state at the α-carbon is thought to be important to the mechanism by which the α-β carbon bond between monomers is broken (Fenn and Kirk, 1984).

The second reaction to occur in the degradation of compounds (I) and (IV) is the debenzylation of the 4-benzyloxy group to yield benzyl alcohol and the corresponding phenol. This is analogous to the very common demethylation reactions which occur in a very wide range of organisms removing methoxy groups both in the lignin macromolecules and in lignin model compounds. In this case the benzyloxy group can also be considered as an analogue of an α-aryl ether bond and this may have implications for the mechanisms used to cleave this type of ether bond. Demethylation mechanisms have not received as much attention as those for cleavage of the ß-aryl ether in lignin research, but there are several mechanisms proposed in the literature. Dagley (1978) proposed a hydroxylation of the methyl ether to form a hemiacetal. This is thought to undergo spontaneous aldol cleavage to give formaldehyde and the corresponding phenol. Donnelly and Dagley (1981) and Kersten *et al*. (1982) devised a mechanism in which the ether linkage was converted via a series of reactions to an ester which was hydrolysed to release methanol and give various intermediates of aromatic ring degradation. More recently, Kersten *et al*. (1985), has proposed a demethylation by the ligninase enzyme. This involves a one electron oxidation of the methoxybenzene by the oxidised enzyme and subsequent addition of water to the cation radical followed by or simultaneously with methoxy elimination as methanol. Previous work on the demethylation of coniferyl alcohol by *A. flavus* showed detection of methanol but not formaldehyde (Iyayi and Dart, 1982).

In this study, no benzaldehyde was detected and the first product found was benzyl alcohol. The parent molecule remaining was the alcohol. The very small quantities of benzyl alcohol detected may be due to certain properties of this compound. Benzyl alcohol is very insoluble in water and is volatile, evaporating readily from flasks of media incubated with shaking at 30°C. It would also be metabolised rapidly. Therefore the levels detected may not have been representative of the true levels produced.

The pathways proposed for the initial degradation of compounds (I) and (IV) are shown in Figures 3 and 4. The results seem to indicate that after an initial oxidation of the α-hydroxyl group to a ketone, the next step is a cleavage of the benzyl ether bond in preference to attack of the ß-aryl bond. The mechanism of this cleavage may be a debenzylation reaction similar to the demethylation reactions previously encountered with this organism.

Further work is proceeding to determine other intermediates of the dissimilation of compounds (I) and (IV). Vanillic acid has been tentatively identified suggesting that either the Cα-Cβ bond or the β-aryl ether bond has been cleaved. This would account for the substantial growth on (I) and (IV) which is not possible by only utilising the benzyl alcohol produced. It is hoped that the synthesis of compounds (I) and (IV) with the β-carbon labelled with ^{14}C will indicate which of the bonds is cleaved in the next stage of the degradation sequence.

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Table I

Thin Layer Chromatography of Extracts Obtained From Media Containing Compound I as Carbon Source

		r_F Values[1]			r_F Values[2]		
	Extract	0.11	0.16	0.27	0.63	0.68	0.80
Authentic Compounds	I	0.10	-	-	-	0.69	-
	II	-	-	0.26	-	-	0.79
	III	-	0.18	-	0.61	-	-

1 Solvent System Benzene:ethyl acetate 9:1

2 Solvent System Benzene:dioxane:acetic acid 90:25:4

Table II

Thin Layer Chromatography of Extracts Obtained from Media Containing Compound II as Carbon Source

		r_F Values[1]			r_F Values[2]		
	Extract	0.33	0.37	0.63	0.80	0.80	0.88
Authentic Compounds	IV	-	0.36	-	-	0.79	-
	V	-	-	0.63	-	-	0.89
	VI	0.33	-	-	0.78	-	-

1 Solvent System Benzene:ethyl acetate 9:1

2 Solvent System Benzene:dioxane:acetic acid 90:25:4

Figure 1.

Growth of *A. flavus* on 4-benzyloxy-3-methoxy-2-(2-methoxyphenoxy)ethanol (I). The organism was grown on 100 ml of medium in 250 ml Ehrlenmeyer flasks on a rotary shaker (120 r.p.m.). Each reading is the mean of six replicates.

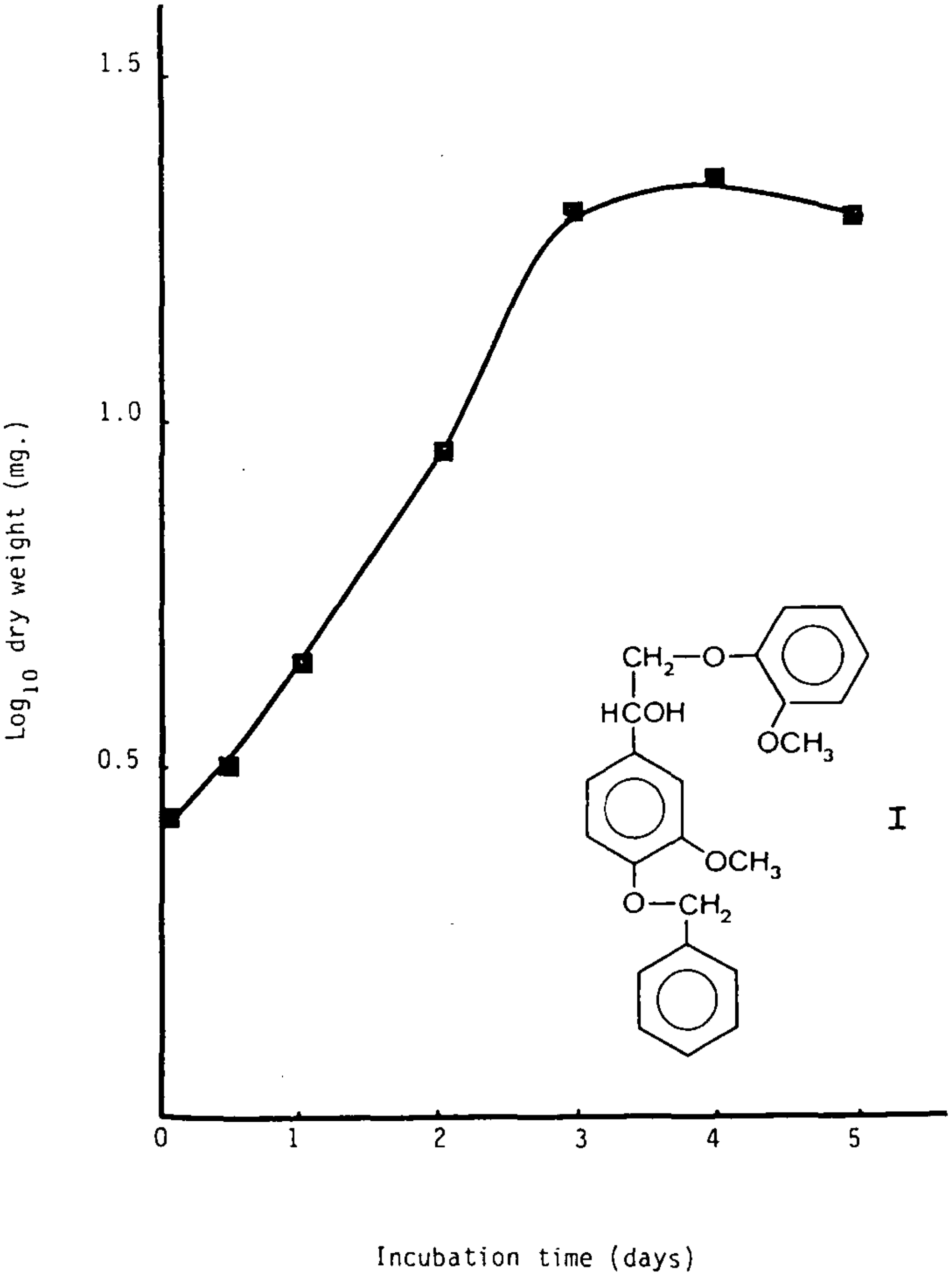

1.5
1.0
0.5
Log$_{10}$ dry weight (mg.)
0
1
2
3
4
5
Incubation time (days)
CH$_2$—O
HCOH
OCH$_3$
OCH$_3$
O—CH$_2$
I

Figure 2.

Growth of *A. flavus* on 4-benzyloxy-3-methoxy-2-(phenylphenoxy)ethanol (IV). The organism was grown on 100 ml of medium in 250 ml Ehrlenmeyer flasks on a rotary shaker (120 r.p.m.). Each reading is the mean of six replicates.

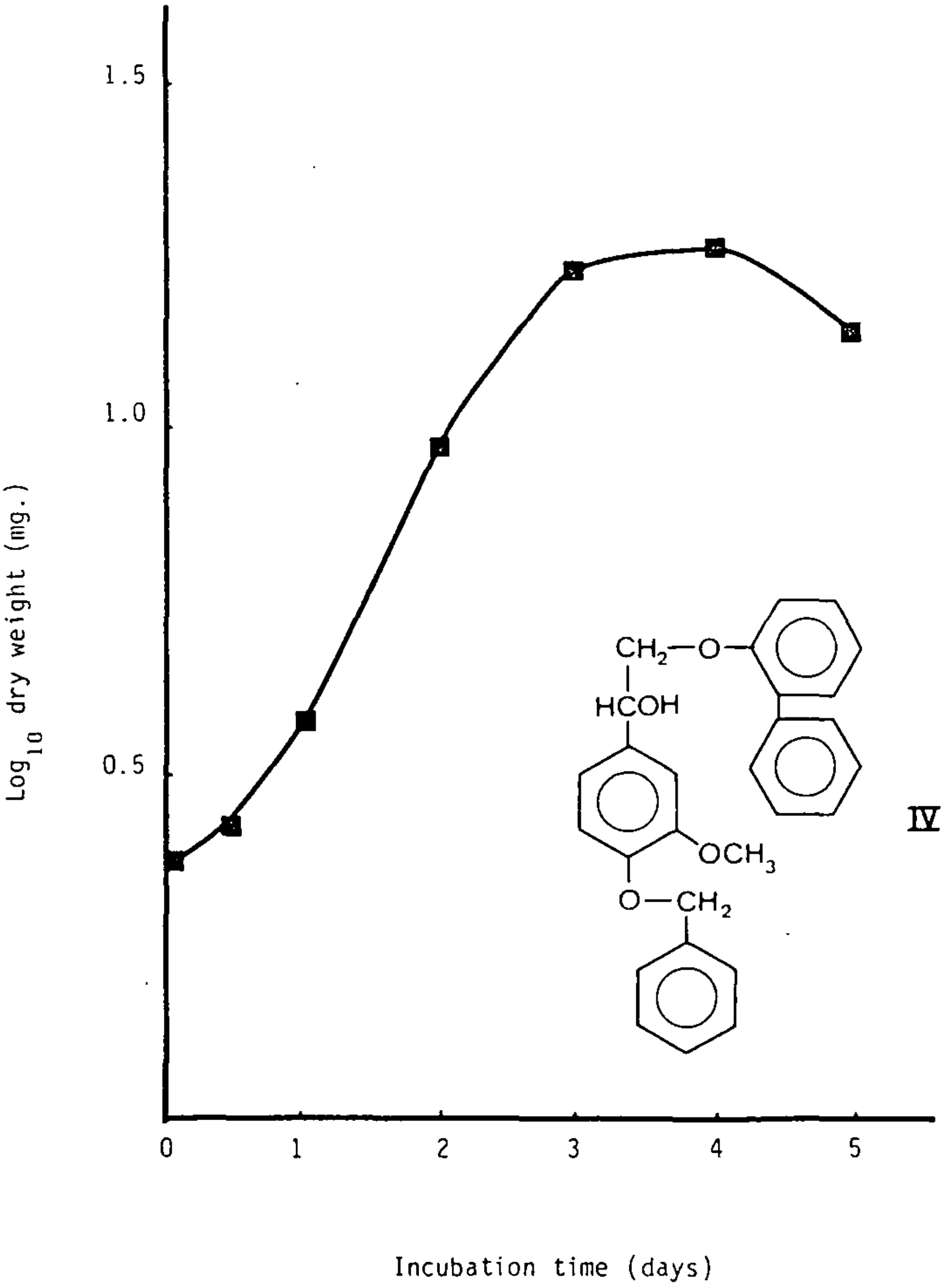
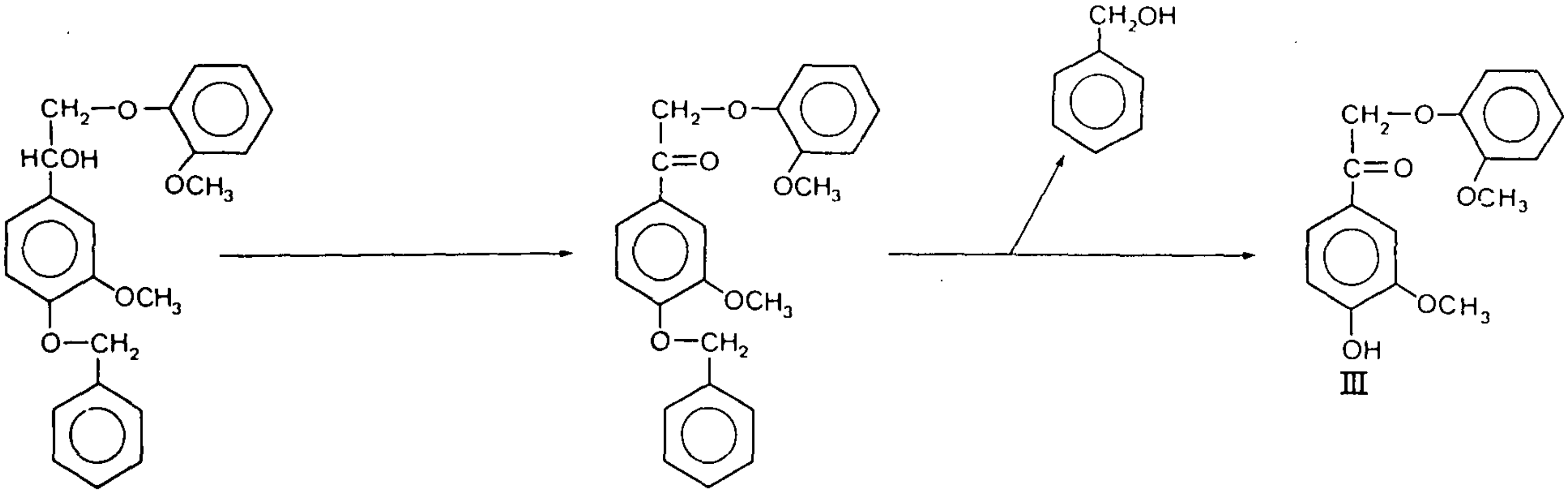

1.5
1.0
0.5
$\log_{10}$ dry weight (mg.)
0
1
2
3
4
5
Incubation time (days)
CH_2—O
HCOH
OCH_3
O—CH_2
IV

Figure 3.

Proposed Pathway for the Degradation of the Trimer 4-Benzyloxy-3-Methoxy-2-(2-Methoxyphenoxy)Ethanol (I) to a Dimer by *A. flavus*



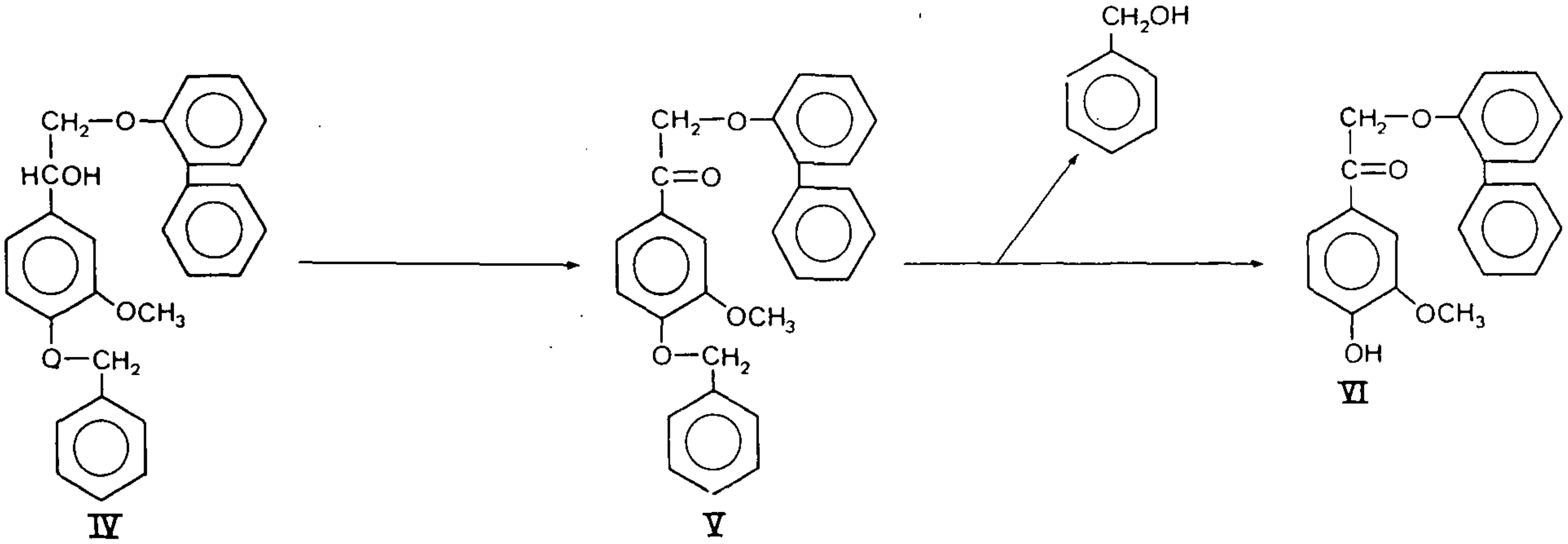
I

II

III

Figure 4.

Proposed Pathway for the Degradation of the Tetramer 4-Benzyloxy-3-Methoxy-2-(2-Phenylphenoxy)Ethanol (IV) to a Trimer by *A. flavus*


CH₂OH
IV
V
VI