

Mineralization of the methoxyl carbon of isolated lignin by brown-rot fungi under solid substrate conditions

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Summary. The objectives of this work were to begin developing an experimental system for studying the demethylation of lignin by brown-rot fungi and to examine the influence of selected culture parameters. As substrate for demethylation, we used partially 3-O-demethylated lignin that had been isolated earlier from brown-rotted spruce wood; we remethylated with $^{14}\text{CH}_3\text{I}$, giving a lignin with both [3- ^{14}C]methoxyl and [4- ^{14}C]methoxyl groups. This lignin was added to pine wood flakes, which were incubated with selected brown-rot fungi, and the evolved $^{14}\text{CO}_2$ was trapped and measured. Of eight fungi examined, *Gloeophyllum trabeum* and *Wolfporia cocos* gave the highest rates of mineralization of the ^{14}C -methoxyl carbons. With the former but not the latter fungus, methoxyl mineralization was over twice as fast in an atmosphere of O_2 than in air. Amending the cultures with ammonium tartrate suppressed mineralization to some extent. Further studies with *G. trabeum* showed that glutamate lowered the rate of mineralization and that glucose and glycerol sharply suppressed it. Addition of Fe^{2+} and Mn^{2+} slightly increased the rate of mineralization. Our results suggest that in unsupplemented cultures the rate-limiting step in methoxyl mineralization is the initial demethylation. Thus the two likely initial C_1 products, methanol and formaldehyde (as ^{14}C compounds), were mineralized much more rapidly than the methoxyl carbon of the lignin (as was formic acid), and no low molecular weight labeled intermediates from the [^{14}C]-methoxyl lignin accumulated in the cultures. Our results also provide evidence that the spruce lignin was partially polymerized by *G. trabeum*. Mineralization of the methoxyl carbon of a synthetic [3- ^{14}C]-methoxyl lignin was slower than that of the spruce lignin, suggesting either that the synthetic lignin was more recalcitrant or that the [4- ^{14}C]methoxyl group in the [3,4- ^{14}C]-methoxyl spruce lignin was attacked more readily.

Introduction

Brown-rot fungi decompose and remove the cellulose and hemicelluloses in wood, leaving a modified brown lignin residue. Early chemical studies of brown-rotted lignin showed that the methoxyl content is decreased (Apenitis et al. 1951; Grohn, Deters 1959; Pew, Weyna 1962; Brown et al. 1968). Later studies showed that new phenolic

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hydroxyl groups are present and that they arise both by demethylation of methoxyl groups (Kirk, Adler 1970; Kirk 1975) and by regiospecific hydroxylation of aromatic nuclei (Kirk et al. 1970). Results indicated that both of these modifications occur without depolymerization of the lignin to low molecular weight products. Both types of modification in the polymer are of interest, not only from the standpoint of the biochemistry involved but also from a practical standpoint. The increased phenolic hydroxyl content should greatly enhance the reactivity of lignin, which should make it more attractive industrially. Understanding the biochemistry could lead to biotechnological or chemical means for harnessing the reactions.

Very few studies have been made of lignin degradation by brown-rot fungi since the early chemical investigations. In the only major study since the work cited above, Haider and Trojanowski (1980) evaluated the ability of several brown-rot fungi to mineralize specifically labeled lignins and related aromatics in liquid cultures, finding that most of the fungi preferentially mineralized (i.e. converted to CO₂) the methoxyl carbon. Some mineralization of side chain and aromatic ring carbons was observed, in agreement with previous chemical studies, which had also indicated that demethylation and hydroxylation were not the only changes caused by the fungi (Kirk 1975). The physiology and biochemistry of the demethylating (and the hydroxylating) reactions remain virtually unstudied.

The paucity of knowledge about the brown-rot systems is in contrast to the increasingly sophisticated understanding of lignin decomposition by white-rot fungi (reviews by Buswell, Odier 1987; Kirk, Farrell 1987; Tien 1987). The white-rot and brown-rot fungi are closely related taxonomically; several species of the two groups belong to the same genera of hymenomycetous basidiomycetes. Thus, some known principles of the white-rot system might be applicable to the brown-rot system, although evidence that white-rot fungi are able selectively to demethylate lignin is lacking. In contrast to the brown-rot fungi, white-rot fungi mineralize all the carbons of lignin.

Our objective in the work reported here was to begin developing an experimental system for studying brown-rot demethylation of lignin. We first screened several fungi for their ability to mineralize the methoxyl carbon of lignin (i.e., to convert [methoxyl]-¹⁴C-lignin to ¹⁴CO₂). Using experience gained with white-rot fungi as a guide (Buswell, Odier 1987; Kirk, Farrell 1987), we then evaluated the influences of selected culture parameters on this mineralization. We employed a solid wood substrate to which we added methoxyl-labeled lignin.

Materials and methods

Fungi

The studied strains and their origins are as follows: *Gloeophyllum trabeum* (Pers. ex Fries.) Murr, Mad. 617-R; *Neolentinus lepideus* Fr. QKM-2414-S; *Phaeolus schweinitzii* Fries 14854-S; *Postia placenta* (Fries) Cooke, Mad. 698-R; and *Wolfiporia cocos* (Schw.) FP-90850-R, all from the Center for Forest Mycology Research, Forest Products Laboratory, USDA Forest Service, Madison, Wisconsin; and *Lentinus lepideus* Fr., Mad. 534; *Polyporus balsameus* Peck, Mad. 74; *Poria incrassata*

(Berk. & Curt.) Burt, Mad. 563, from the Forest Products Laboratory, Mississippi State University. The fungi were maintained on 2% malt extract agar at room temperature.

Chemicals

Methoxyl-labeled lignin was prepared from brown-rotted spruce lignin from a previous study (Kirk 1975). The lignin was first treated with NaBH₄ to reduce any carbonyl groups, including quinonoid structures if present (Fenn, Kirk 1984), and then methylated with ¹⁴CH₃I (ICN¹, Irvine, CA) (Tien, Kirk 1983), followed by methylation with unlabeled CH₃I. The methylated lignin was fractionated with a column of Sephadex LH 20/60 (1:1) (Pharmacia, Piscataway, New Jersey) in N, N-dimethylformamide (DMF), using procedures similar to those used by Tien and Kirk (1983) with Sephadex LH 20. A center-cut fraction was used in this study. The specific activity of this preparation was 8.15 KBq · mg⁻¹.

The brown-rotted lignin used here was shown earlier (Kirk 1975) to have a phenolic hydroxyl content of 0.58 per C₉ unit and a methoxyl content of 0.61 per C₉ unit. Two sound spruce lignins analyzed for comparison contained 0.92 and 0.96 (0.94 average) methoxyls per C₉ unit. Assuming that the methoxyl-deficient units were still present in the lignin as 3-OH groups, which is likely (Kirk, Adler 1970), approximately $0.94 - 0.61 = 0.33$ C₉ units contained phenolic groups at C₃. (Hydroxyl groups at C₂, formed via hydroxylation, were negligible in this sample (Kirk 1975)). Thus 57% ($0.33 - 0.58 \times 100$) of the phenolic hydroxyls were at C₃; in accord, the methylated sample would be expected to have 57% of its ¹⁴C-methoxyl groups at C₃.

A synthetic [3-O-¹⁴CH₃]lignin was prepared earlier (Kirk et al. 1975); its specific activity was 1.52 KBq · mg⁻¹. Both lignins were stored at -20°C in DMF solution. For use in cultures, these solutions were added with stirring to sterile water to give sterile suspensions (Kirk et al. 1978).

¹⁴C-Labeled methanol (specific activity of 7.2×10^5 KBq · mmol⁻¹), formaldehyde (specific activity of 4.1×10^5 KBq · mmol⁻¹), and formic acid (specific activity of 2.6×10^5 KBq · mmol⁻¹) were obtained from Sigma Chemical Company, St. Louis, MO.

All other chemicals were reagent grade and were used as purchased.

Culture conditions and assay

The initial experiment compared the methoxyl-mineralizing activities of the fungi. Cultures were grown in 125-ml Erlenmeyer flasks, each containing 10 ml of 1% malt extract agar. The agar was seeded with a 5-mm-diameter mycelium-covered plug cut from a fresh petri dish culture on malt agar. Sterile (autoclaved) flakes of loblolly pine (*Pinus taeda*) sapwood (1500 mg on a dry weight basis) were added to each flask. The labeled brown-rotted lignin (1.67 KBq) in 350 µl of sterile water was dispersed over

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the flakes. The final moisture content of the flakes was 65% (dry weight basis). The flasks were then closed with sterile rubber stoppers fitted with ports to allow periodic flushing and trapping of evolved $^{14}\text{CO}_2$ (Kirk et al. 1975). Cultures were incubated at 28°C in the dark. The fungi grew over the flakes slowly. Cultures were first flushed with air after 14 days. Thereafter, cultures were flushed with air every 7 days, and the $^{14}\text{CO}_2$ was trapped and counted,

The second experiment evaluated the influence on methoxyl mineralization of (1) two levels of nutrient nitrogen, (2) two carbon sources, and (3) two oxygen concentrations. The experimental setup was similar to that in the first experiment, except that the malt extract was omitted from the agar. The nitrogen and carbon additions were ammonium tartrate (5 mmol and 20 mmol based on the total water in the flasks), and D-glucose and glycerol (1% based on total water in the flasks). These nutrients were added, together with 1.67 KBq of the labeled brown-rotted lignin, in a total of 350 μl of sterile water, with care being taken to dispense the additives evenly over the flakes. Control cultures received only the lignin in water. Cultures were flushed at 7-day intervals with sterile O_2 or with sterile air. The experiment had three replicates of each treatment combination. Data were evaluated by two-way analysis of variance and Duncan's multiple range test with $p = 0.95$.

Additional experiments were performed using experimental setups similar to that in the second experiment, except that the cultures were allowed to grow before being amended with lignin and additives. These additional experiments examined (1) the effect of adding L-glutamate (26 mmol), glycerol (1%), or glucose (1%) alone to cultures actively evolving $^{14}\text{CO}_2$, (2) the effect of adding Fe^{2+} (25.1×10^{-3} mmol based on total water) and Mn^{2+} (20.3×10^{-2} mmol), (3) mineralization of the methoxyl carbon in the synthetic lignin, and (4) mineralization of labeled methanol, formaldehyde, and formic acid. With the methanol and formaldehyde, it was necessary to circumvent flushing out the labeled compounds themselves instead of, or with, the $^{14}\text{CO}_2$. The $^{14}\text{CO}_2$ was therefore trapped directly in the culture flask in 1.5 ml of ethanolamine in a small cup suspended over the culture. We found that the formic acid did not volatilize from the cultures, so the normal flushing and trapping procedure was satisfactory.

Molecular weight distribution of labeled residual lignin

A special experiment was set up to determine changes in molecular weight distribution of the residual labeled lignin as a function of incubation time. The labeled lignin was added to pregrown flake cultures (4 weeks old) in 125-ml Erlenmeyer flasks. Cultures were flushed with O_2 to remove evolved $^{14}\text{CO}_2$ at 3-day intervals, and immediately after each flushing three cultures were terminated by adding 30 ml of dioxane-water (1:1) to each culture. The stoppered cultures were incubated overnight at room temperature on a shaker, and insoluble materials were then removed by centrifugation. The dioxane-water solubles from three cultures were pooled (approx. 90 ml), 1 ml of DMF was added to a 10-ml sample of the pooled extracts, and the volume was reduced on a rotary evaporator to approx. 1 ml, mostly DMF. One-milliliter samples of each DMF solution were analyzed on a 1×40 -cm column of Sephadex LH 20/60 (1:1) in DMF (Connors et al., 1978). The flow rate was approx. 0.5 ml/min, and 55

to 60 fractions (approx. 0.5 ml each) were collected for each run. Fractions were collected directly in 8 ml of Ecolume scintillation fluid (ICN, Irvine, CA) for radioactivity determination. Molecular weight markers were veratryl alcohol, 1-(4-benzyl-oxy-3-methoxy)-2-(4-formyl-2-methoxyphenoxy)-ethane-1-one, and spruce milled wood lignin (MWL, largely excluded from the gel).

Results

The fungi screened were chosen because they had been studied previously, and/or because they grow relatively rapidly. They were compared for their ability to mineralize the ^{14}C -methylated brown-rotted lignin. Following this first experiment, two of the most promising fungi, *G. trabeum* and *W. cocos*, were further evaluated to determine the effect of selected culture parameters on methoxyl mineralization. Further studies were then done with *G. trabeum*.

Methoxyl mineralization by selected brown-rot fungi

In the screening, *G. trabeum* and *W. cocos* mineralized the methoxyl carbon of the lignin at significantly higher rates than did the other fungi, although total conversion to $^{14}\text{CO}_2$ in this experiment was low for all, probably due to the presence of malt extract (s. results in next section). After 42 days, the total conversions, as a percentage of the initial label, were as follows:

<i>G. trabeum</i>	6.0
<i>W. cocos</i>	5.5
<i>N. lepideus</i>	2.9
<i>Poria placenta</i>	2.8
<i>Phaeolus schweinitzii</i>	2.4
<i>Polyporus balsameus</i>	1.1
<i>L. lepideus</i>	1.1
<i>Poria incrassata</i>	1.0

On the basis of these results, *G. trabeum* and *W. cocos* were chosen for further study.

Effect of culture parameters

Mineralization by *G. trabeum* in an atmosphere of O_2 was over twice that in air, but it was the same with *W. cocos* in both atmospheres (Fig. 1). Total mineralization by both fungi was greater than in the screening experiment, since the malt extract, which contains sugars, was eliminated from the medium in this experiment. After 41 days, conversion of labeled methoxyl to $^{14}\text{CO}_2$ was 26% for *G. trabeum* under O_2 , approximately 11% for *G. trabeum* in air, and 11% for *W. cocos* in both atmospheres; at termination after 52 days, these values had increased to 30%, 16%, and 16%, and the cultures were still active.

The effect on methoxyl mineralization of supplementing the cultures at the outset with glucose or glycerol, and with two concentrations of ammonium tartrate, was

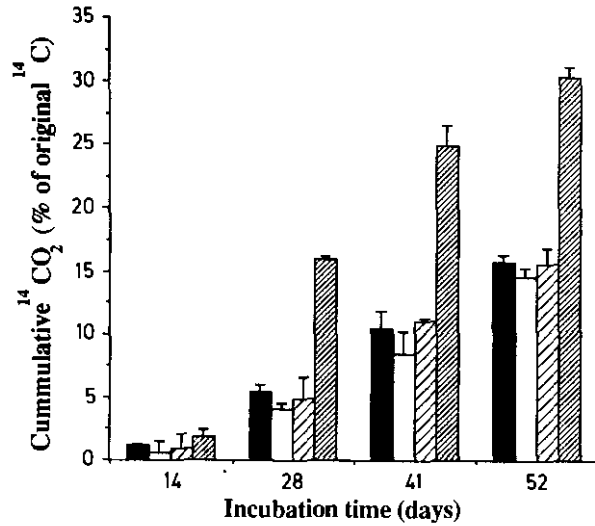


Fig. 1. Mineralization of methoxyl carbon in ¹⁴C-methylated lignin by *Gloeophyllum trabeum* and *Wolfiporia cocos* under atmospheres of oxygen and air. ¹⁴CO₂ production was monitored for a 52-day period at 7-day intervals. □, *W. cocos* flushed with air; ◻, *W. cocos* flushed with O₂; ▨, *G. trabeum* flushed with air; and ▩, *G. trabeum* flushed with O₂.

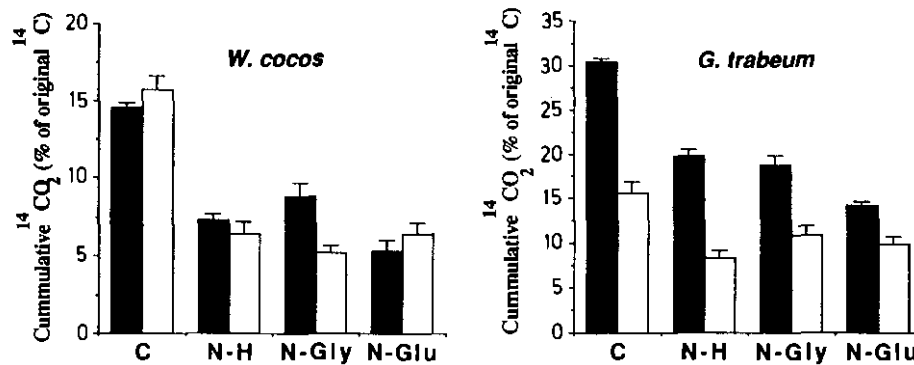


Fig. 2. Influence of ammonium tartrate, with and without added glucose or glycerol, on mineralization of the methoxyl carbon in ¹⁴C-methylated lignin. The compounds were added at the time of inoculation and were in the following initial concentrations, based on total water in the cultures: ammonium tartrate, 20 mM; glucose or glycerol, 1%. Bars show total ¹⁴CO₂ after 52 days. C, control; H, water; N, ammonium tartrate; Glu, glucose; and Gly, glycerol. □, flushed with O₂; ◻, flushed with air. Vertical bars denote standard deviation.

studied in both air and O₂ atmospheres with both fungi. The rates of ¹⁴CO₂ evolution by the two fungi (data not shown) were similar to those in the unsupplemented cultures (Fig. 1). Thus, total percentages of degradation after 52 days (Fig. 2) accurately mirror the nutrient effects throughout the incubation. Degradation by the two fungi was suppressed at both concentrations of nutrient nitrogen (the data for 20 mM are shown in Fig. 2). The added carbon sources with the presence of nutrient nitrogen were without substantial effect, except that glucose was slightly suppressive at the higher level of ammonium tartrate.

Additional experiments were conducted with *G. trabeum*. We found that L-glutamate, which strongly suppresses lignin mineralization by *P. chrysosporium* (Fenn et al. 1981), also suppresses mineralization of the methoxyl-labeled lignin by *G. trabeum*

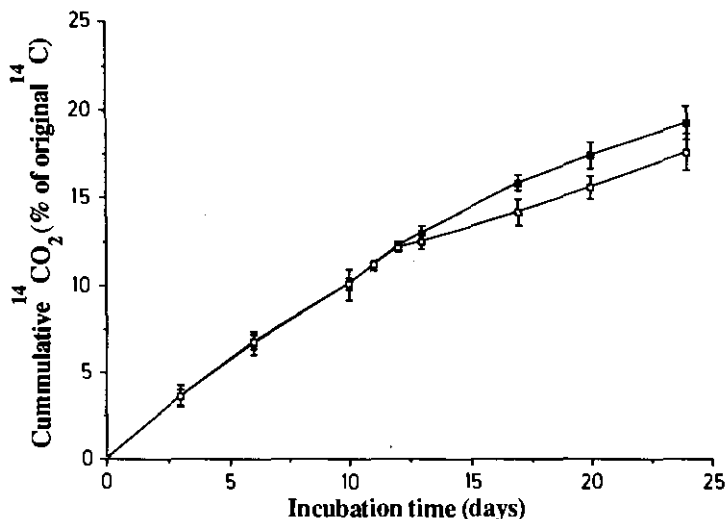


Fig. 3. Influence of L-glutamate on mineralization of the methoxyl carbon in ¹⁴C-methylated lignin by *Gloeophyllum trabeum*. The ¹⁴C-lignin, in 300 μ l of water, was added after 5 weeks of growth and the glutamate, in 300 μ l of water, was added ten days later. Initial glutamate concentration was 26 mM based on total water in the flask. Control cultures received water only. ○, glutamate addition, □, control. Vertical bars denote standard deviation

(Fig. 3). Addition of glucose or glycerol alone to the cultures sharply suppressed mineralization (Fig. 4). Addition of Fe²⁺ (25.1×10^{-3} mmol) plus Mn²⁺ (20.3×10^{-2} mmol) enhanced the mineralization rate by approximately 20%.

Mineralization of methoxyl-labeled synthetic lignin

Methylation of the brown-rotted lignin with ¹⁴CH₃I used in these experiments generated ¹⁴CH₃-methoxyl groups at C₃ and C₄. Determining whether the C₃-methoxyl group (which is the only natural one in spruce lignin) was mineralized was therefore of interest. For this work, synthetic [3-O-¹⁴CH₃]lignin was used. Incubation of this lignin with competent cultures of *G. trabeum* resulted in production of ¹⁴CO₂, although at a substantially lower rate than from the methylated spruce lignin (Fig. 5).

Mineralization of C₁ compounds

The two fungi rapidly mineralized methanol, formaldehyde, and formic acid in unsupplemented wood flake cultures (Fig. 6). Because the mineralization of ¹⁴C-formaldehyde was lower than that of the other two compounds, it was reexamined, and the results were the same as in the first experiment. Other experiments also showed that oxidation to ¹⁴CO₂, and not simple volatilization of these compounds, was being measured (s. Materials and methods).

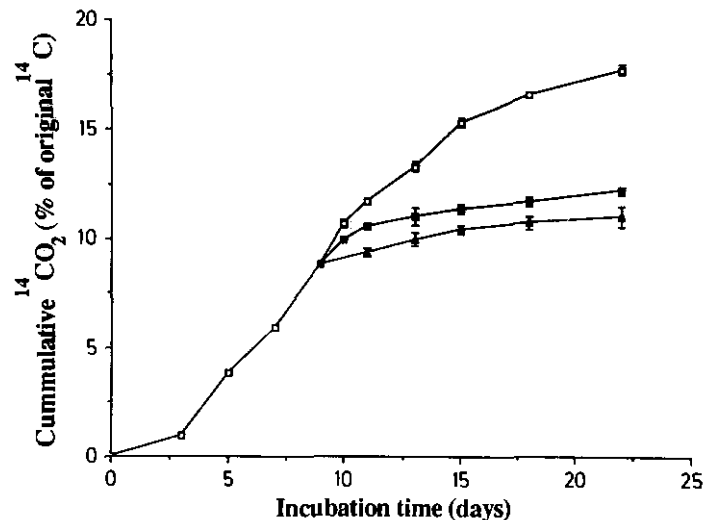


Fig. 4. Influence of glucose and glycerol on mineralization of the methoxyl carbon in ^{14}C -methylated lignin by *Gloeophyllum trabeum*. The ^{14}C -lignin, in 300 μl of water, was added after 4 weeks of growth, and the glycerol and glucose, in 300 μl of water, were added after an additional 9 days. Control cultures received water only. □, control; ■, glucose added; and ▲, glycerol added. Vertical bars denote standard deviation

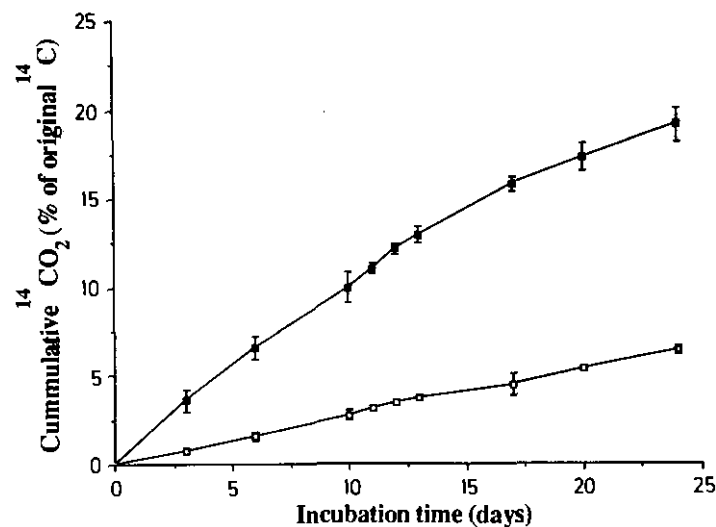


Fig. 5. Mineralization of the methoxyl carbon of synthetic $[3\text{-O-}^{14}\text{CH}_3]$ -lignin and $[3,4\text{-O-}^{14}\text{CH}_3]$ -lignin (methylated brown-rotted lignin) by *Gloeophyllum trabeum*. The ^{14}C -lignins, in 300 μl of water, were added after 5 weeks of growth. ■, methylated brown-rotted lignin; □, methylated synthetic lignin. Vertical bars denote standard deviation

Fig. 7. Molecular size distribution of ^{14}C -material extracted from cultures of *Gloeophyllum trabeum*. Methoxyl-labeled lignin was added after 4 weeks of growth. The column was Sephadex LH 20/60 in DMF. Molecular weight markers were veratryl alcohol; 1-(4-benzyloxy-3-methoxy)-2-(4-formyl-2-methoxyphenoxy)-ethane-1-one; and spruce milled wood lignin (MWL, largely excluded from the gel)

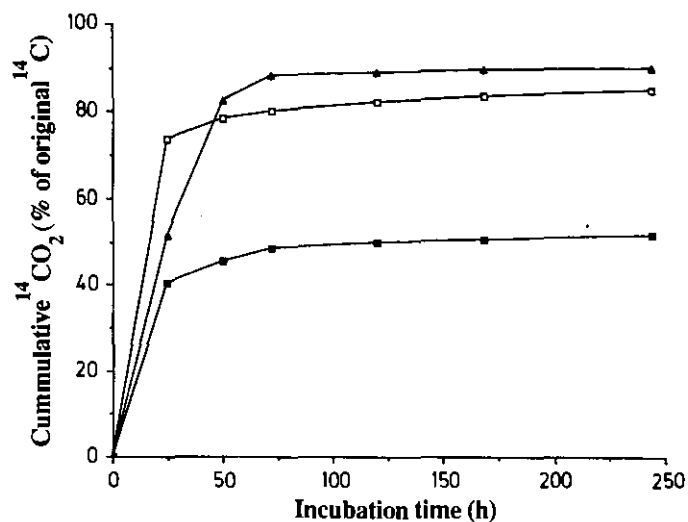
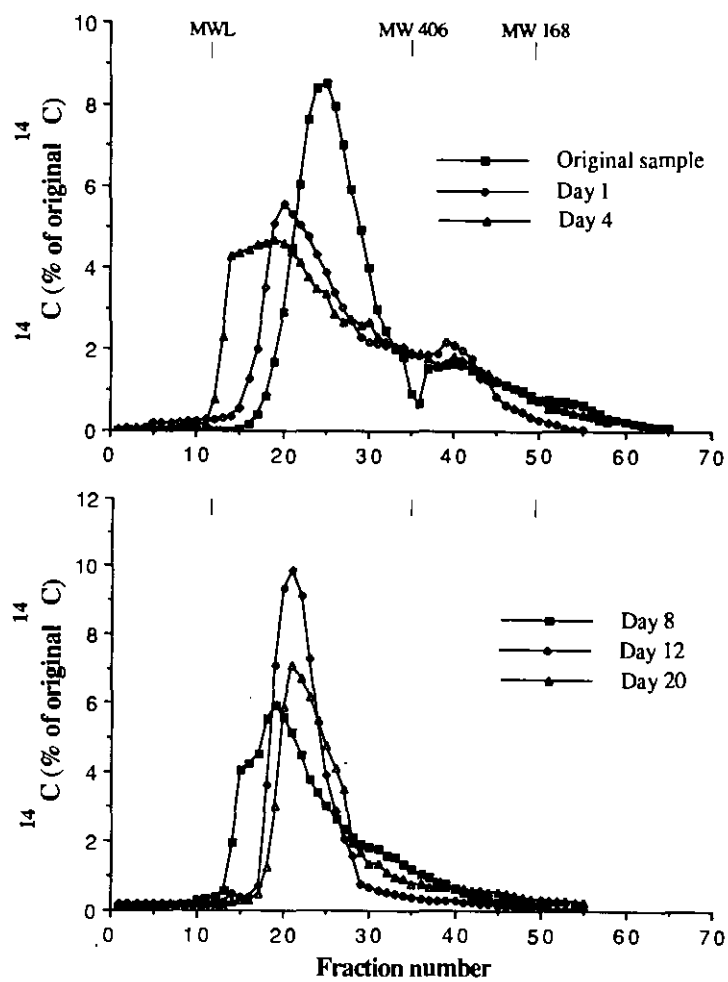


Fig. 6. Mineralization of methanol, formaldehyde, and formic acid by *Gloeophyllum trabeum*. The ¹⁴C₁-compounds were added after 5 days of growth. □, methanol; △, formic acid; ○, formaldehyde



Molecular size distribution of the residual lignin

The ^{14}C recovered in the extracts of cultures of *G. trabeum* varied from 70.19% to 93.25% of the original ^{14}C added. The total ^{14}C (including $^{14}\text{CO}_2$) and $^{14}\text{CO}_2$ recovered are given in the following (percentage recovered as $^{14}\text{CO}_2$ in parentheses: day 0, 92.1 (0); day 1, 93.3 (0.9); day 4, 88.0 (3.8); day 8, 79.6 (8.9); day 12, 77.2 (11.5); day 16, 72.4 (15.5); and day 20, 70.2 (19.5)).

Molecular size distribution of the original ^{14}C -lignin (brown-rotted lignin sample) and residual lignin at five different incubation times are shown in Fig. 7. The amount of ^{14}C applied to the column used for determining molecular size distribution was proportional to the ^{14}C extracted from the cultures, so the quantitative changes in elution profiles reflect losses due to $^{14}\text{CO}_2$ evolved and losses due to binding to the wood or mycelium, as well as changes in molecular weight. Incubation of the labeled lignin with the fungus altered its elution profile. During the 20-day incubation, the lignin first underwent an apparent partial polymerization, which was followed by a loss of the highest molecular weight portion. No low molecular weight labeled material was observed during the 20-day incubation, except that present in the original sample, which had disappeared by the end of the incubation.

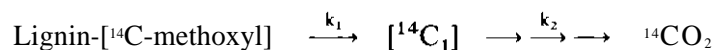
Discussion

We made the assumption at the outset that the fungal system being measured is extracellular and attacks the lignin polymer rather than acting on low molecular weight compounds intracellularly. The assumption seems valid: our methylated lignin had only a minor low molecular weight component, so most of it probably did not enter the cells. Furthermore, the evidence indicates that the fungi created no methoxylated low molecular weight products that might have entered the cells and been demethylated. Finally, and most importantly, demethylation in the polymer by *G. trabeum* (formerly *Lenzites trabea*) was demonstrated earlier in straightforward chemical studies (Kirk, Adler 1970).

In this initial work, our assay for demethylation was conversion to $^{14}\text{CO}_2$ of the labeled methoxyl groups of $^{14}\text{CH}_3$ -methylated brown-rotted lignin. The $^{14}\text{CO}_2$ is obviously not the first demethylation product, so it was necessary to determine whether labeled intermediates were accumulating or being lost due to volatilization. Probable initial products are the (labeled) C_1 compounds methanol and formaldehyde, or alternatively, some $^{14}\text{C}_1$ derivative of a metabolite. We found no low molecular weight labeled material in the culture extracts, indicating that no intermediate was accumulating. This rules out the accumulation of a derivative of the initial demethylation product, although such an intermediate could still be involved. Also, methanol and formaldehyde (and formic acid) were oxidized to CO_2 much more rapidly than was the methoxyl carbon of lignin.

Mineralization of the methoxyl groups can be viewed as in Scheme 1, in which k_1 is the net forward rate constant for the demethylation reaction (i.e., conversion of methoxyl carbon to a C_1 intermediate) and k_2 is the net forward rate constant for further conversion of the intermediate to $^{14}\text{CO}_2$. Our results indicate that $k_2 \gg k_1$. Thus the quantitative assay for the rate of demethylation based on $^{14}\text{CO}_2$ production is valid.

Scheme 1:



In the study with the three C₁ compounds, we do not know why the rate and extent of mineralization of formaldehyde was lower than that for methanol and formic acid. Similar results were obtained by Ander et al. (1988). One possibility is that the formaldehyde reacted with culture components, rendering it less degradable. The acidic conditions might be expected to favor formaldehyde condensation reactions. In this regard, we have shown in other experiments that formaldehyde reacts readily with brown-rotted lignin.

Our results do not predict what the immediate C₁ product of cleavage is. In the monooxygenase-catalyzed demethylation of aryl methoxyl groups by bacteria, formaldehyde is the initial product (Ribbons 1970). On the other hand, methanol is the product from the action of white-rot fungi on lignin (Ander et al. 1985), and methanol is the product of the action of *G. trabeum* and other brown-rot fungi on vanillic and ferulic acids (Ander et al. 1988).

The brown-rotted lignin studied here provided a natural substrate, except that in remethylating it, we also methylated the free phenolic hydroxyl groups at C₄ (which are found in sound lignin), as well as those at C₃ (which arose via fungal demethylation). Our assay for demethylation, therefore, did not distinguish between ¹⁴CO₂ formed from the C₃- and C₄-methoxyls. The fact that the [3-O-¹⁴CH₃]-group in the synthetic lignin was mineralized shows that the 3-methoxyl group is attacked under our conditions, indicating that in the case of the methylated brown-rotted lignin at least some of the ¹⁴CO₂ came from the C₃-methoxyl group. The lower rate of mineralization of the methoxyl groups in the synthetic lignin than in spruce lignin suggests that the C₄-methoxyl was more readily mineralized than the C₃-methoxyl, or that the synthetic lignin was relatively recalcitrant. Interestingly, Haider and Trojanowski (1980) found that the 4-methoxyl group of veratric acid was mineralized much faster than the 3-methoxyl group by brown-rot fungi. The relevance of that result with a small molecule to the lignin polymer is not clear, however. In any event, because of the heterogeneity of lignin, it would not be surprising if the biochemical system that demethylates it is nonspecific, as is the case with the lignin-degrading system of the white-rot fungi, so that the same system that attacks the C₃-methoxyl also attacks the C₄.

Our study of the influence of culture parameters was patterned after previous studies of lignin degradation by the white-rot fungus *P. chrysosporium*. The results suggest that the ligninolytic system of *P. chrysosporium* and the methoxyl-degrading system of the brown-rot fungi share some regulatory properties.

The strong enhancement by molecular oxygen of methoxyl mineralization by *G. trabeum* is also seen with *P. chrysosporium* in its mineralization of all of the carbons of lignin (Kirk et al. 1978). The effect in the white-rot fungus is a dual one: the O₂ increases the amount of the degrading system, and it enhances its action (Bar-Lev, Kirk 1981). Increasing the O₂ level in the medium increases the total activity of lignin peroxidase in cultures (Faison, Kirk 1985) and of the hydrogen peroxide-producing system (Faison, Kirk 1983). Further work with *G. trabeum* will attempt to determine the basis for enhancement by O₂, and whether H₂O₂ is involved. Surprisingly, high O₂ had no effect on methoxyl mineralization by *W. cocos*. This might reflect the fact that

W. cocos has a much sparser mycelium than *G. trabeum*, facilitating O₂ accessibility. In *P. chrysosporium*, the effect of O₂ is not observed in very shallow cultures (Leisola et al. 1983), presumably because of increased accessibility of O₂ to the hyphae.

The effect of nutrient nitrogen on methoxyl mineralization by *G. trabeum* and *W. cocos* resembles that seen in lignin mineralization by *P. chrysosporium*. Maximum lignin degradation is seen under nutrient limitation, and lignin degradation can be markedly suppressed by adding nutrient nitrogen to cultures (Keyser et al. 1978; Fenn, Kirk 1981). The most suppressive source of nitrogen among those studied was L-glutamate (Fenn et al. 1981), which also had a clear but only partial suppressive effect in the present study with *G. trabeum*. This suppression could have affected the demethylation reaction (k_1 in Scheme 1) or further oxidation (k_2).

The slight enhancing effect of added manganese and iron on methoxyl mineralization suggests that these elements might have been limiting in the pine wood used here. Their influence presumably was on the demethylation reaction (k_1 in Scheme 1), because further oxidation of the C₁ intermediate was not rate limiting. In previous studies, Jeffries et al. (1981) found that these and other trace elements have strong effects on lignin mineralization by *P. chrysosporium*. Manganese also enhances the production of lignin peroxidase in *P. chrysosporium* (Kirk et al. 1986).

The very strong suppressive effect of added glucose or glycerol on methoxyl mineralization by *G. trabeum* could have been on the initial demethylation reaction rate (k_1 in Scheme 1) or on further oxidation (k_2). Yang et al. (1980) found that at low concentrations, added glucose was without significant effect on *P. chrysosporium* degradation of lignin in a lignocellulose substrate; however, at high concentrations it was suppressive.

The polymerization of the lignin by *G. trabeum* suggests that phenolic groups liberated by the demethylating system were converted to phenoxy radicals, which coupled, Rösch (1962) reported that certain brown-rot fungi have intracellular laccase activity, which could be responsible for phenoxy radical generation if it were released by the hyphae - as, for example, by autolysis. More puzzling than the polymerization here was the subsequent apparent partial depolymerization. However, the high molecular weight material may have simply become bound to the wood rather than having been depolymerized. The absence of low molecular weight ¹⁴C-materials argues against depolymerization.

Based on the assay used in this preliminary work, *G. trabeum* clearly is the best of the examined fungi for further study. Haider and Trojanowski (1980) also found *G. trabeum* to be the best among nine brown-rot fungi in mineralizing methoxyl-labeled lignins. Further research should be directed at obtaining expression of the demethylating system in defined liquid cultures, for which the present results and the earlier work of Haider and Trojanowski (1980) provide useful guidelines.

References

- Ander, P.; Eriksson, K.-E. 1985: Methanol formation during lignin degradation by *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. 21: 96–102
- Ander, P.; Stoytschev, I.; Eriksson, K.-E. 1988: Cleavage and metabolism of methoxyl groups from vanillic and ferulic by brown-rot and soft-rot fungi. Cellulose Chem. Technol. 22: 255–266

- Apenitis, A.; Erdtman, H.; Leopold, B. 1951: Studies on lignin V. The decay of spruce wood by brown-rotting fungi, Sv. Kem. Tidskr. 63: 195–207
- Bar-Lev, S. S.; Kirk, T. K. 1981: Effects of molecular oxygen on lignin degradation by *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. 99: 373–378
- Brown, W.; Cowling, E. B.; Falkehag, S. I. 1968: Molecular size distribution of lignins liberated enzymatically from wood. Sv. Papperstidn. 71: 811–821
- Buswell, J. A.; Odier, E. 1987: Lignin biodegradation. In: Stewart, G. G.; Russell, I (eds.): Critical Reviews in Biotechnology. pp 1–60. Boca Raton, FL: CRC Press
- Connors, W. J.; Lorenz, L. F.; Kirk, T. K. 1978: Chromatographic separation of lignin models by molecular weight using Sephadex LH-20. Holzforschung 32: 106–108
- Faison, B. D.; Kirk, T. K. 1983: Relationship between lignin degradation and production of reduced oxygen species by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 46: 1140–1145
- Faison, B. D.; Kirk, T. K. 1985: Factors involved in the regulation of a ligninase activity in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 42: 299–304
- Fenn, P.; Kirk, T. K. 1981: Relationship of nitrogen to the onset and suppression of ligninolytic activity and secondary metabolism in *Phanerochaete chrysosporium*. Arch. Microbiol. 130: 59–65
- Fenn, P.; Kirk, T. K. 1984: Effects of C_a-oxidation in the fungal metabolism of lignin. J. Wood Chem. Technol. 4: 131–148
- Fenn, P.; Choi, S.; Kirk, T. K. 1981: Ligninolytic activity of *Phanerochaete chrysosporium*: physiology of suppression by NH₄ and L-glutamate. Arch. Microbiol. 130: 66–71
- Grohn, H.; Deters, W. 1959: Über den Abbau von Fichtenholz durch *Lenzites saepiaria*. Holzforschung 13: 8–12
- Haider, K.; Trojanowski, T. 1980: A comparison of the degradation of ¹⁴C-labeled DHP and corn stalk lignins by micro- and macrofungi and bacteria. In: Kirk, T. K. Higuchi, T.; Chang, H.-M. (eds.): Lignin Biodegradation: Microbiology, Chemistry and Potential Applications. Vol 1, pp. 111–134. Boca Raton, FL: CRC Press
- Jeffries, T. W.; Choi, S.; Kirk, T. K. 1981: Nutritional regulation of lignin degradation by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 42: 290–296
- Keyser, P.; Kirk, T. K.; Zeikus, J. G. 1978: Ligninolytic enzyme system of *Phanerochaete chrysosporium*: Synthesized in the absence of lignin in response to nitrogen starvation. J. Bacteriol. 135: 790–797
- Kirk, T. K. 1975: Effects of a brown-rot fungus, *Lenzites trabea* on lignin in spruce wood. Holzforschung 29: 99–107
- Kirk, T. K.; Adler, E. 1970: Methoxyl-deficient structural elements in lignin of sweetgum decayed by a brown-rot fungus. Acta. Chem. Acad. 24: 3379–3390
- Kirk, T. K.; Farrell, R. L. 1987: Enzymatic “combustion:” the microbial degradation of lignin. Ann. Rev. Microbiol. 41: 465–505
- Kirk, T. K.; Larsson, S.; Miksche, G. E. 1970: Aromatic hydroxylation resulting from attack of lignin by a brown-rot fungus. Acta. Chem. Scand. 24: 1470–1472
- Kirk, T. K.; Connors, W. J.; Bleam, R. D.; Hackett, W. F.; Zeikus, J. G. 1975: Preparation and microbial decomposition of synthetic ¹⁴C-lignins. Proc. Natl. Acad. Sci. USA. 72: 2515–2519
- Kirk, T. K.; Schultz, E.; Connors, W. J.; Lorenz, L. F.; Zeikus, J. G. 1978: Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. 117: 277–285
- Kirk, T. K.; Croan, S.; Tien, M.; Murtagh, K. E.; Farrell, R. L. 1986: Production of multiple ligninases by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of a mutant strain. Enz. Microb. Technol. 8: 27–32
- Leisola, M. S. A.; Ulmer, D.; Fiechter, A. 1983: Problem of oxygen transfer during degradation of lignin by *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. 17: 113–116
- Pew, J. C.; Weyna, P. 1962: Fine grinding, enzyme digestion, and the lignin-cellulose bond in wood. Tappi 45: 247–256
- Ribbons, D. W. 1970: Stoichiometry of *o*-demethylase activity in *Pseufoomonas aeruginosa*. FEBS Lett. 8: 101–104
- Rösch, R. 1962: Über die intracellulären Polyphenoloxidasen der Braunfäulepilze. II. Arch. Mikrobiol. 43: 392–401

- Tien, M. 1987: Properties of ligninase from *Phanerochaete chrysosporium* and their possible application. *CRC Critical Reviews Journals in Microbiology* 15: 141-168
- Tien, M.; Kirk, T. K. 1983: Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. *Science* 221: 661-663
- Yang, H.-H.; Effland, M. J.; Kirk, T. K. 1980 Factors influencing fungal decomposition of lignin in a representative lignocellulosic, thermomechanical pulp. *Biotechnol. Bioeng.* 22: 65-77

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