Degradation of [^{14}C]Lignin-labelled Wheat Lignocellulose by White-rot Fungi

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[lignin- ^{14}C]Lignocellulose was prepared from wheat seedlings, injected with L-[U- ^{14}C]-phenylalanine, by a procedure that selectively removed radiolabelled low molecular weight compounds and protein. ^{14}C was mainly localized in the acid-insoluble fraction ('Klason lignin') of the lignocellulose and the polymeric nature of the radiolabelled material was confirmed by gel permeation chromatography. Ferulic and *p*-coumaric acids were found to be esterified to both carbohydrate and lignin fractions of the wheat lignocellulose, but contained only a small proportion of the total ^{14}C . The white-rot fungi *Phanerochaete chrysosporium*, *Sporotrichum pulverulentum* and *Coriolus versicolor* degraded the radiolabelled substrate; the *S. pulverulentum* culture was the most active, releasing almost 50% of the ^{14}C as $^{14}\text{CO}_2$ in 15 d at 37 °C. In all three species $^{14}\text{CO}_2$ evolution commenced after a lag period during which growth occurred, and was greatly enhanced by reducing the nitrogen concentration in the medium. This indicated that grass lignin degradation by these fungi is subject to the same nutrient nitrogen regulation as the degradation of wood and synthetic lignins. In a culture of *P. chrysosporium* grown on the radiolabelled substrate for 50 d at 37 °C, $^{14}\text{CO}_2$ was the main product. Low molecular weight degradation products present in the culture medium were acid-soluble and accounted for a much smaller proportion of the original [^{14}C]lignin.

INTRODUCTION

The study of lignin biodegradation has progressed rapidly since the development of assays with ^{14}C -labelled natural or synthetic lignins. The latter, radiolabelled dehydrogenative polymers of coniferyl alcohol, have been central to the study of the physiology of lignin degradation by white-rot fungi, while studies using [^{14}C]lignocellulose or milled wood lignin have provided evidence that a range of other micro-organisms can attack the lignin polymer (for a review see Crawford, R. L., 1981). The use of $^{14}\text{CO}_2$ evolution as an indicator of lignin degradation has demonstrated that, in the best-studied white-rot fungus *Phanerochaete chrysosporium*, lignin degradation is a secondary metabolic activity (Fenn & Kirk, 1981), is enhanced under conditions of high O_2 tension (Kirk *et al.*, 1978) and possibly involves hydroxyl radical formation (Forney *et al.*, 1982; Kutsuki & Gold, 1982).

In the field of lignin research, emphasis has traditionally been placed on the woody plants, both angiosperms and gymnosperms, whereas grass lignins, which are structurally different, have been relatively neglected. In the plant cell wall, lignin is synthesized by polymerization of coniferyl, sinapyl and *p*-coumaryl alcohols to produce large molecules of indefinite size in which aromatic monomers are linked by a number of different chemical bonds. Grass lignins are polymers of all three monomer units, rather than of mainly coniferyl and sinapyl alcohols (angiosperms) or coniferyl alcohol (gymnosperms) (Higuchi, 1980). In addition, grass lignins

Abbreviation: GPC, gel permeation chromatography.

contain ester-linked ferulic and *p*-coumaric acids (Higuchi *et al.*, 1967) which also occur linked to polysaccharide (Hartley, 1973) and may act as cross-links between lignin and carbohydrate in the plant cell wall (Morrison, 1974; Himmelsbach & Barton, 1980; Hartley, 1981). These structural features may have important implications for biodegradation, since Crawford, D. L. (1981) has demonstrated that grass lignins are more susceptible to actinomycete attack than wood lignins. The higher lignin content of wood lignocelluloses also results in much lower values for total biodegradation (e.g. Zadrazil & Brunnert, 1982). Waste grass lignocellulose, widely available in the form of cereal straw, is therefore a preferable feedstock for bioconversion processes.

In this paper, we describe a procedure for the preparation of [*lignin*- ^{14}C]lignocellulose by direct injection of wheat seedlings with L-[U- ^{14}C]phenylalanine, the primary precursor of the three substituted cinnamyl alcohols. The distribution of ^{14}C in the radiolabelled lignocellulose is analysed and the substrate used to examine grass lignin degradation by white-rot fungi.

METHODS

In vivo ^{14}C -labelling of wheat seedlings. Wheat seedlings were cultivated from seed in trays of compost exposed to a natural diurnal light cycle. After approximately 2 weeks growth at room temperature, when the second leaf had emerged, the seedlings were injected with 5 μl (0.5 μCi) L-[U- ^{14}C]phenylalanine (450 mCi mmol $^{-1}$, 16.7 GBq mmol $^{-1}$) (Amersham) by means of a glass needle inserted approximately 0.5 cm above soil level. The needle was removed after the solution had been taken up (within 24 h) and the plants allowed to grow for a further 3–4 weeks prior to harvesting. Harvested stems and leaves were ground in liquid nitrogen, dried and weighed.

Removal of low molecular weight compounds and protein. The procedure described by Crawford & Crawford (1978) was used to prepare extractive-free lignocellulose from the ^{14}C -labelled plant material. Release of ^{14}C -labelled compounds was monitored throughout by the addition of samples of extractant to 3.5 ml of a scintillation cocktail which contained: 1,4-di-2-(5-phenyloxazolyl)benzene (POPOP), 0.032 g; 2,5-diphenyloxazole (PPO), 0.5 g; toluene, 100 ml; ethanolamine, 30 ml; methanol, 30 ml. Radioactivity was counted in a liquid scintillation counter (1216 Rackbeta II, LKB Wallac) with quench correction by the channels ratio method.

^{14}C -labelled protein was removed from the extractive-free lignocellulose by treatment with protease. The weighed material was suspended in 150 ml 0.2 M-sodium phosphate buffer (pH 7.6) containing 0.03% (w/v) sodium azide and 0.05 g protease (P5130 type 6 ex *Streptomyces griseus*; Sigma) in a 250 ml conical flask. The flask was shaken with glass beads at 37 °C for 24 h and the solubilized radioactivity determined as described. The protease treatment was repeated twice and the insoluble lignocellulose preparations were freeze-dried after each treatment. The specific activity of the [^{14}C]lignocellulose was estimated by trapping the $^{14}\text{CO}_2$ produced by complete combustion at 800 °C. Experiments demonstrated that ethanolamine was a more efficient trapping agent than either 1 M-NaOH or phenylethylamine. Filter paper strips impregnated with D-[U- ^{14}C]glucose (Amersham) were used to optimize conditions for recovery of 96–98% of the ^{14}C as $^{14}\text{CO}_2$.

Acid hydrolysis. A modified Klason procedure (Effland, 1977) was used to determine the partitioning of ^{14}C between the lignin and polysaccharide components. The specific activity of ^{14}C in the acid-insoluble 'Klason lignin' was determined by combustion. Radioactivity in the acid-soluble fraction was counted and the fraction neutralized with saturated Ba(OH) $_2$. The sugars released from [^{14}C]lignocellulose by acid hydrolysis were separated by ascending thin layer chromatography (TLC) on cellulose plates (Merck: layer thickness 0.1 mm) in the solvent system formic acid/butanone/tert. butanol/water (15:30:40:15, by vol.). Chromatograms, developed twice, were sprayed with a solution of phthalic acid (3.25 g) in 100 ml water-saturated butanol plus 2 ml aniline and heated at 100 °C for 5 min to locate sugars. Portions of the cellulose layer corresponding to R_f values of reference sugars were removed and radioactivity counted.

Alkali treatment. Samples (20–25 mg) of [^{14}C]lignocellulose were suspended in 10 ml 1 M-NaOH and agitated at room temperature for 48 h. Solubilized radioactivity was determined and [^{14}C]phenylalanine estimated using the procedure described by Crawford, R. L. (1981). High performance liquid chromatography (HPLC) was used to estimate esterified [^{14}C]phenolic acids and determine the molecular weight range of the [^{14}C]lignin solubilized by 1 M-NaOH. The alkali extract was neutralized with conc. HCl, freeze-dried and resuspended in 1.5 ml distilled water. The pH was adjusted to 1.0–2.0 and the acid-insoluble material removed by centrifugation and freeze-dried. Phenolic acids in the supernatant were separated by reverse-phase chromatography (Hartley & Buchan, 1979) and the radioactivity in eluted fractions was counted. A range of monomeric aromatic compounds were used as reference standards. Freeze-dried samples of both the acid-insoluble residue and the supernatant were resuspended in dimethylformamide + 0.5% (v/v) acetic acid and analysed by gel permeation chromatography (GPC) in a Zorbax PSM60s column. The column was developed with dimethylformamide + 0.5% (v/v) acetic acid (Kristersson *et al.*, 1983) at 1.0 ml min $^{-1}$ flow rate and radioactivity counted in fractions of the eluted ^{14}C -labelled samples. Polystyrene standards (Polymer Laboratories, Church Stretton, UK) in the molecular weight

range $0.5\text{--}34.5 \times 10^3$ in addition to monomer (anisic acid) and dimer (α -conidendrin; supplied by Dr M. Salkinoja-Salonen, University of Helsinki) standards were used to calibrate the GPC column.

Cellulase treatment. Samples (20 mg) of [^{14}C]lignocellulose were placed in 25 ml conical flasks each containing 10 ml 0.1 M-sodium acetate buffer (pH 4.5), 15 mg CT cellulase (ex *Trichoderma reesei*: 10000 carboxymethyl cellulase units g^{-1} , Sturge Enzymes, Selby, N. Yorks, UK) and 0.03% (w/v) sodium azide. Flasks, including a control without cellulase, were incubated with shaking at 60 °C for 18 h and the amount of released radioactivity was determined. UV absorption spectra (250–400 nm) were compared before and after treatment with 0.1 vol. 1 M-NaOH. The alkali-treated cellulase supernatant of [^{14}C]lignocellulose was further analysed for phenolic acids as described. For comparison, a 0.1 M-NaOH extract of unlabelled wheat lignocellulose, prepared by the method described for [^{14}C]lignocellulose but not cellulase treated, was analysed similarly.

Biodegradation of [^{14}C]lignocellulose. *Phanerochaete chrysosporium* Burdsall ME-446 (ATCC 34541), *Sporotrichum pulverulentum* Novobranova (ATCC 24725) and *Coriolus versicolor* [(Linnaeus) Fries] Quélet (FPRL 28A) were maintained on slopes of 2% (w/v) malt extract agar, incubated at 37 °C (*P. chrysosporium* and *S. pulverulentum*) or 25 °C (*C. versicolor*) and subsequently stored at 4 °C with subculture every 4 weeks. Inocula for the lignin degradation assay were prepared by growing cultures to approximately 25 mg wet weight in 25 ml of a high nitrogen growth medium which contained: 56 mM-D-glucose; 1.47 mM- KH_2PO_4 ; 0.2 mM- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.09 mM- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 10 mM-sodium 2,2-dimethyl succinate; 1 ml l^{-1} vitamin solution (Kirk *et al.*, 1978); 1 ml l^{-1} of mineral salts solution (Jeffries *et al.*, 1981); 24 mM (2.4 mM for low nitrogen medium) $(\text{NH}_4)_2\text{HPO}_4$, pH 4.6. The harvested mycelia were homogenized in 20 ml fresh growth medium and 0.5 ml samples used as inocula. In the lignin degradation assay, cultures in 250 ml conical flasks were flushed periodically with 100% O_2 and the evolved CO_2 trapped in the ethanalamine scintillation cocktail. To test this system, a streptomycete was grown on D-[U- ^{14}C]glucose (Amersham) for 10 d with O_2 flushing at 2 d intervals. The ^{14}C recovered in the biomass, spent culture medium and evolved $^{14}\text{CO}_2$ together accounted for 90% (approx.) of the added radioactivity. Flasks containing 2.0–3.0 mg [^{14}C]lignocellulose ($6.0\text{--}9.0 \times 10^4$ d.p.m.) plus 30 mg vibratory ball-milled cereal straw (mean particle size $< 5 \mu\text{m}$ diameter) were autoclaved at 121 °C for 15 min, and 25 ml medium was added. Flasks were inoculated and incubated at 37 °C or 25 °C. For each strain, three flasks containing low nitrogen and two containing high nitrogen medium were flushed with 100% O_2 at 3 d intervals and evolved $^{14}\text{CO}_2$ was trapped and counted. Experiments were terminated after 15 d when the solubilized radioactivity in membrane-filtered culture supernatants was determined.

In an additional *P. chrysosporium* culture in low nitrogen medium, $^{14}\text{CO}_2$ evolution was assayed up to 35 d at 37 °C. After addition of sterile D-glucose and $(\text{NH}_4)_2\text{HPO}_4$ to give final concentrations of 18 mM and 1.4 mM, respectively, $^{14}\text{CO}_2$ evolution was measured for a further 15 d when the solubilized radioactivity in the culture medium was determined. The method described for HPLC analysis using GPC was applied to estimate the molecular weight range of the soluble ^{14}C -labelled compounds.

RESULTS

A total of 113 wheat seedlings yielded 3.79 g dry weight of ^{14}C -labelled plant material, of which approximately 50% remained after successive extractions with hot water, benzene/ethanol and ethanol. Exhaustive protease treatment released 27% of the radioactivity from this extractive-free preparation to provide ^{14}C -labelled wheat lignocellulose with a specific activity of 3.04×10^4 ($\pm 0.02 \times 10^4$, SEM; $n = 3$) d.p.m. mg^{-1} .

Distribution of radioactivity in the [^{14}C]lignocellulose

Analysis demonstrated that 86% of the radioactivity was located in the acid-insoluble fraction of the lignocellulose, defined as 'Klason lignin'. No radioactivity was detected in the sugars derived from cellulose and hemicellulose, indicating that the remainder was in the form of acid-soluble lignin components (see Crawford, R. L., 1981). Alkali treatment solubilized approximately 80% of the radioactivity, but TLC showed that only approximately 2% of the total radiolabel was present as [^{14}C]phenylalanine. These values (Table 1) are comparable to those reported for other ^{14}C -labelled natural lignins (Crawford, R. L., 1981).

Ferulic and *p*-coumaric acids were the only ^{14}C -labelled phenolic acids detected in the alkali-soluble fraction of [^{14}C]lignocellulose from wheat; together they accounted for approximately 5% of the total radioactivity. Analysis of the ^{14}C -labelled material released by cellulase treatment demonstrated that phenolic acids were not exclusively bound to lignin. A UV absorption maximum was detected at 324 nm, shifted to 340 nm by 0.1 M-NaOH treatment to break the ester bond, and this suggested that the carbohydrate ester of ferulic acid had been released by

Table 1. *Distribution of ^{14}C -labelled compounds in protein-free radiolabelled wheat lignocellulose*

Treatment	Component	Percentage of ^{14}C in labelled lignocellulose*
Acid hydrolysis†	Insoluble fraction	86.0
	Soluble fraction	13.2
Alkali extraction‡	Soluble fraction	81.5
	[^{14}C]Phenolic acids	4.7
	[^{14}C]Phenylalanine	2.1
Cellulase treatment§	Soluble fraction	9.9
	[^{14}C]Ferulic acid	2.2
	<i>p</i> -[^{14}C]Coumaric acid	0.5

* Specific activity = $3.04 \pm 0.02 \times 10^4$ d.p.m. mg^{-1} .

† 72% (v/v) H_2SO_4 for 1 h at 30 °C. Diluted sample autoclaved for 1 h at 121 °C.

‡ 1 M-NaOH for 48 h at 25 °C. Three replicates.

§ Samples incubated at 60 °C for 18 h with *Trichoderma reesei* cellulase. Two replicates.

Fig. 1

Fig. 2

Fig. 1. Separation of phenolic acids by reverse-phase HPLC. (a) 0.1 M-NaOH extract of unlabelled wheat lignocellulose. (b) 0.1 M-NaOH extract of the cellulase-soluble fraction of ^{14}C -labelled wheat lignocellulose. (c) Location of ^{14}C in components of (b). *p*-C, *p*-coumaric acid; F, ferulic acid.

Fig. 2. Molecular weight range of the products of 1 M-NaOH extraction and *P. chrysosporium* degradation of [*lignin*- ^{14}C]lignocellulose. The samples were analysed using a GPC column calibrated with polystyrene molecular weight standards. The dashed lines represent, from left to right, the positions of α -conidendrin (dimer) and anisic acid (monomer) markers. (a) 1 M-NaOH extract: acid-insoluble fraction. (b) 1 M-NaOH extract: acid-soluble fraction. (c) Water-soluble ^{14}C -labelled products in a *P. chrysosporium* culture grown for 50 d at 37 °C.

Fig. 3

Fig. 3. Degradation of wheat [*lignin-¹⁴C*]lignocellulose to ¹⁴CO₂ by white-rot fungi. Filled symbols, low nitrogen medium; open symbols, high nitrogen medium. ○, ●, *C. versicolor* (25 °C); □, ■, *P. chrysosporium* (37 °C); △, ▲, *S. pulverulentum* (37 °C). The bars represent SEM values. Three replicates were done using low nitrogen medium, and two using high nitrogen medium.

Fig. 4

Fig. 4. Degradation of wheat [*lignin-¹⁴C*]lignocellulose to ¹⁴CO₂ by *P. chrysosporium* grown in low nitrogen medium at 37 °C. After 35 d, D-glucose (18 mM) and (NH₄)H₂PO₄ (1.4 mM) were added to the culture.

cellulase (Hartley, 1973; Hartley *et al.*, 1974). This was confirmed by HPLC analysis which showed that both [¹⁴C]ferulic acid and, to a lesser extent, *p*-[¹⁴C]coumaric acid were linked to carbohydrate (Fig. 1). Together these accounted for approximately 30% of the radioactivity solubilized by cellulase (Table 1). Ferulic and *p*-coumaric acids were also released from the lignocellulose by mild alkali treatment, but in this case *p*-coumaric acid was the major component (Fig. 1), suggesting that while ferulic acid is primarily linked to carbohydrate, *p*-coumaric acid is mainly bound to the lignin of wheat lignocellulose.

GPC of the alkali-extracted fraction of [¹⁴C]lignocellulose confirmed that most of the radioactivity was located in lignin itself (Fig. 2), rather than in associated low molecular weight phenolic compounds. The ¹⁴C-labelled material which precipitated when the alkali-soluble fraction was acidified had a molecular weight range of 1–30 × 10³. The supernatant contained a smaller amount of high molecular weight lignin but in both cases the major radiolabelled components were of similar molecular weight (2.5–4.0 × 10³). However, these are unlikely to be absolute values since the molecular weights of the α-conidendrin and anisic acid standards were overestimated by the polystyrene calibration (Fig. 2) and some acid-mediated polymerization of the extracted lignins could have occurred.

Biodegradation of [¹⁴C]lignocellulose

Phanerochaete chrysosporium, *S. pulverulentum* and *C. versicolor* degraded [¹⁴C]lignocellulose to ¹⁴CO₂ when the cultures were grown in low nitrogen medium (Fig. 3). The *S. pulverulentum* culture consistently gave the highest values, converting almost 50% of the radiolabelled substrate to ¹⁴CO₂ in 15 d at 37 °C. In all three species, the onset of ¹⁴CO₂ evolution was delayed, none being detected after 3 d during which a significant amount of primary growth was observed. A negligible amount of ¹⁴CO₂ was produced by cultures grown on [¹⁴C]lignocellulose in high nitrogen medium (Fig. 3). The overall pattern of ¹⁴CO₂ evolution described here for *P. chrysosporium* is an established feature of wood and synthetic lignin degradation by this organism.

[^{14}C]Lignin was also solubilized to a greater extent by all three species during growth with limited nitrogen. However, soluble ^{14}C was also detected in high nitrogen cultures, especially *P. chrysosporium*, which solubilized up to 20% of the original ^{14}C in 15 d at 37 °C. In uninoculated controls, only 1–2% of the radioactivity was detected in the culture medium and degradation of radiolabelled material other than [^{14}C]lignin could not totally account for the solubilization under high nitrogen conditions, which may represent limited attack on lignin. In another experiment with *P. chrysosporium* there was little further $^{14}\text{CO}_2$ evolved after 12 d incubation in low nitrogen medium until the addition of glucose and $(\text{NH}_4)_2\text{PO}_4$ after 35 d stimulated a second phase of rapid lignin degradation (Fig. 4). After 50 d total incubation time, 52% of the [^{14}C]lignin had been converted to $^{14}\text{CO}_2$ and a further 25% was solubilized in the culture medium. The latter was totally acid-soluble and although compounds were not identified specifically the culture filtrate was analysed by GPC. The molecular weight range ($0.5\text{--}2 \times 10^3$) was significantly lower than that determined for undegraded [^{14}C]lignins extracted with 1 M-NaOH (Fig. 2). Comparison with the α -conidendrin and anisic acid standards shows that an appreciable amount of radioactivity was located in monomeric and dimeric material derived from the [^{14}C]lignin.

DISCUSSION

Direct injection of radiolabelled phenylalanine into the growing plant followed by a period of further growth in a diurnal light cycle resulted in the incorporation of ^{14}C into the lignin component of wheat lignocellulose. This method minimizes interference with the natural biosynthesis of lignin and has been used previously to prepare [*lignin*- ^{14}C]lignocellulose from maize seedlings, with [^{14}C]ferulic acid as the precursor (Haider & Trojanowski, 1975). Radiolabelled grass lignocelluloses have also been prepared by the method routinely applied to woody plants (Crawford & Crawford, 1976; Maccubbin & Hodson, 1980) in which a small limb or stem is incubated in a solution of the radiolabel for 24–48 h. While this method may be advantageous in that larger amounts of mature radiolabelled material can be conveniently prepared, possible disruption of lignin biosynthesis as a result of injury casts some doubt on the validity of the lignocellulose as a substrate for biodegradation studies. A number of indirect methods have been used to provide evidence that the radioactivity is largely localized in lignin. Klason fractionation has been used routinely to demonstrate that the greater proportion is present in the acid-insoluble fraction and that the cellulose and hemicellulose are not ^{14}C -labelled. Haider & Trojanowski (1975) also included nitrobenzene oxidation to show that ^{14}C was present in the products derived from lignin but the use of GPC/HPLC analysis of radiolabelled lignocellulose has not been reported previously. Our results clearly demonstrate that in both the acid-soluble and insoluble fractions of alkali-extracted [^{14}C]lignocellulose from wheat, the radiolabel is concentrated in high molecular weight polymeric lignin. While these analyses are sufficient to show that the substrate is primarily [^{14}C]lignin labelled, they provide no information on the complexity of grass lignocellulose structure. Phenolic acids esterified to lignocellulose have been detected previously in members of the Gramineae (Hartley, 1981) where they may be involved in cross-linking between lignin and carbohydrate (Morrison, 1974; Himmelsbach & Barton, 1980) or act as precursors of lignification (Hartley, 1981). There is also some evidence that these phenolic acids may limit accessibility of cellulose and hemicellulose to enzymic attack (Hartley, 1972) and could therefore be a rate-limiting factor in lignocellulose bioconversion. We have found that wheat lignocellulose, in common with that from other grasses, contains more ferulic acid than *p*-coumaric acid ester-linked to carbohydrate, and we have confirmed the observation of Higuchi *et al.* (1967) that the *p*-hydroxyphenylpropane content of grass lignin is mainly due to esterified *p*-coumaric acid. However, [^{14}C]phenolic acids accounted for only 5% of the total radiolabel, and those esterified to carbohydrate, less than 3%. A further 6% (approx.) of the ^{14}C released from wheat lignocellulose by cellulase was not identified and it is possible that this fraction comprised other aromatic monomers and low molecular weight polymers, e.g. diferulic acid (Hartley & Jones, 1976), which are also an integral part of the grass lignocellulose complex.

The degradation rates by white-rot fungi reported here for wheat [*lignin*- ^{14}C]lignocellulose are

of the same order of magnitude as those published for other ^{14}C -labelled natural and synthetic lignins (Leatham & Kirk, 1983; Reid, 1983a). It is now well established that degradation of lignin to CO_2 by *P. chrysosporium* ($\equiv$ *S. pulverulentum*) occurs during secondary metabolism and that nitrogen starvation is the most potent initiator of ligninolytic activity (Kirk & Fenn, 1982). These regulatory features also apply to the degradation of grass lignin, as demonstrated in this paper by data on the kinetics of $^{14}\text{CO}_2$ evolution and the effect of nitrogen concentration. *Coriolus versicolor* showed a similar $^{14}\text{CO}_2$ evolution pattern but, although this species produced less $^{14}\text{CO}_2$ than *P. chrysosporium* and *S. pulverulentum* strains under low nitrogen conditions, the basal level of $^{14}\text{CO}_2$ production in high nitrogen cultures was slightly higher. While this may not be significant, it is interesting to note that Leatham & Kirk (1983) observed that *C. versicolor* was more active against ^{14}C -labelled synthetic lignin than *P. chrysosporium* when nitrogen was not a limiting factor.

It has been postulated that lignin degradation ceases in *P. chrysosporium* cultures when carbohydrate is exhausted (Kirk *et al.*, 1976; Reid, 1983b; Ulmer *et al.*, 1983). We also found that an increase in the rate of $^{14}\text{CO}_2$ evolution from [^{14}C]lignin could be effected by adding carbon and nitrogen sources to a *P. chrysosporium* culture which was no longer rapidly degrading lignin. This could be due to the provision of an energy source (glucose), required for lignin degradation or the stimulation of further primary growth leading to another phase of secondary metabolic activity. Although the reproducibility of this effect was not evaluated, it does indicate that ligninolytic activity could be maximized by monitoring and adjusting the nutrient balance in *P. chrysosporium* cultures. Depolymerization of lignin was also demonstrated by analysis of the molecular weight range of water-soluble ^{14}C -labelled compounds in a *P. chrysosporium* culture. Reid *et al.* (1982) showed that the water-soluble products of aspen lignin degradation by *P. chrysosporium* were mostly acidic and Chen *et al.* (1982) identified a range of aromatic acids in extracts of spruce lignin degraded by *P. chrysosporium*. It has also been suggested that recalcitrant lignin fragments accumulate in the culture medium as degradation proceeds (Reid *et al.*, 1982). The products of wheat lignin degradation by *P. chrysosporium* remained in solution when the culture filtrate was acidified. This, together with the molecular weight range of the products, suggests that both aromatic acids and small lignin-derived fragments were produced in addition to large amounts of CO_2 .

The structural difference between grass and wood lignins had no effect on the regulation of ligninolytic activity by nutrient nitrogen in the white-rot fungi studied. The efficiency of these organisms as lignin degraders is indicated by the fact that $^{14}\text{CO}_2$ is the main end-product of attack on radiolabelled lignin. However, grass lignins are more readily attacked by actinomycetes than wood lignins (Antai & Crawford, 1981; Crawford, D. L., 1981), these organisms being capable of only limited degradation of lignin to CO_2 (McCarthy *et al.*, 1984). In both groups of micro-organisms, the molecular basis of lignin degradation has not been elucidated. Our objective is to obtain an improved understanding of the mechanisms involved by applying genetical methods to organisms whose ability to attack lignin has been well documented.

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