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Bioalteration of Kraft Pine Lignin by *Phanerochaete chrysosporium*

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Abstract. Bioalteration of the organic-soluble ether-insoluble fraction of Kraft pine lignin (KL-O) was studied. Various types of inocula of *Phanerochaeta chrysosporium* were compared using a standing mode of cultivation under nitrogen limitation. Pellet inoculated and mycelial cultures required a period of about 10 days to become ligninolytically active. When spores were used as inoculum the bioalteration of lignin commenced earlier but the rate was considerably less. The total decrease in absorbance measured amounted to 61% within 20 days after addition of lignin to active mycelial cultures. This corresponded to a reduction of 50% in Klason lignin. Further reduction was possible only when the free lignin was extracted from the culture, purified and mixed with new active cells. Elemental analysis, Klason lignin content, absorptivity coefficient, molecular weight distribution and the presence of saccharides and proteins for free and "cell recovered" lignin were compared with KL-O. Microscopic observation showed the formation of new outgrowths in the form of short hyphae appearing concurrently with ligninolytic activity.

Key words: Kraft lignin – *Phanerochaete chrysosporium* – Inocula – Biodegradation – Polymerization

Kraft lignin, a waste product of the major pulping processes, is the main contributor to the colour and toxicity of plant effluent. A biological approach to the utilization of industrial waste lignin is rapidly gaining more attention (Sundman et al. 1981).

The white rot, wood decomposing basidiomycete, *Phanerochaete chrysosporium* has been shown to be able to oxidize ^{14}C-lignin and various lignin-related model compounds (Shimada et al. 1981; Enoki et al. 1980; Martin and Haider 1979; Kirk et al. 1978). The ligninolytic activity of this fungus can be induced using standing cultures (Yang et al. 1980; Kirk et al. 1978), nitrogen starvation (Fenn and Kirk 1981; Fenn et al. 1981) and an atmosphere of oxygen (Bar-Lev and Kirk 1981; Reid and Seifert 1980. Limitation of carbohydrate or sulphur but not that of phosphorus can also trigger ligninolytic activity. The balance of trace elements such as Mg^{2+} and Ca^{2+} may also be important for lignin degradation (Jeffries et al. 1981). It has recently been suggested that glutamate metabolism may play a role in suppressing ligninolytic activity (Fenn and Kirk 1981). Lignin itself does not seem to serve as a growth substrate for this organism and it does not influence the appearance of the ligninolytic system.

In this study, standing cultures of *P. chrysosporium* under conditions of nitrogen starvation and an atmosphere of oxygen were investigated as to their ability to alter commercial Kraft lignin. The effects of different types of inocula and culture age on the time required before onset of lignin alteration, the extent of alteration and various culture parameters were compared. In addition the morphology of the fungus was examined in an attempt to correlate any changes with ligninolytic activity.

This study was undertaken to try and clarify observations made with respect to the extent and mechanism of lignin alteration. The lignin remaining after bioalteration consists of two separate entities which were named free lignin and "cell recovered" lignin. Further study of these could be of great value.

Materials and Methods

Lignin Preparation. Kraft pine lignin polymer (Indulin AT, Westvaco Co. Charleston, SC.) was fractionated and purified according to Lundquist and Kirk (1980). The organic-soluble ether-insoluble fraction, KL-O, of this lignin was used for alteration studies.

Organism and Inoculum. The white rot fungus *Phanerochaete chrysosporium (Sporotrichum pulverulentum)*, ATCC 24725, was maintained at room temperature on 2% malt agar slants. 1.5–3.5 week – old slants provided spore inoculum. The inoculum build up scheme is shown in Fig. 1.

Media and Culture Conditions. The constituents per litre of the medium used were those of Solution A described by Janshekar et al. (1982). The nitrogen sources were replaced by 0.22 g ammonium tartrate (Goldsby et al. 1980).

This medium was autoclaved at 121°C for 20 min. In continuous culture experiments the medium was filter sterilized using the 0.2 µm Pall ultipor disposable filter assembly. The bioreactor used for continuous cultivation had an effective volume of 4 l, agitation, aeration and dilution rates were 600 rpm, 0.5 vvm and 0.01 h^{-1} respectively.

Lignin degradation was studied in standing cultures in 200-ml Erlenmeyer flasks containing 50 ml culture media under an atmosphere of oxygen. Gas-flushing was performed as described by Kirk et al. (1978). The media was inoculated with pellets or spores, or was mycelial culture taken directly

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Abbreviations: GPC, Gel Permeation Chromatography; TLC, Thin Layer Chromatography; rpm, revolutions per minute; vvm, volume per volume per minute

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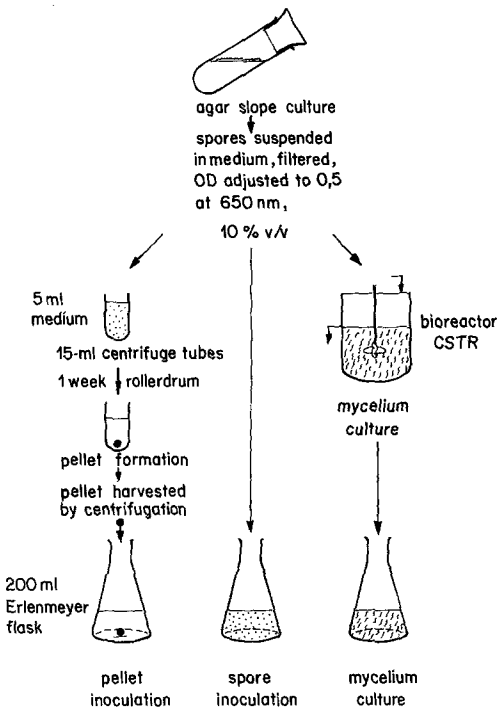

Fig. 1. Inoculum build-up scheme

from the chemostat (Fig. 1). Lignin was ether-sterilized and added as a solution in 2 ml of sterile KOH (0.2 M) to each flask to give a final concentration of 1 g l^{-1}. The pH of the medium was adjusted to 4.5 with KOH or H_3PO_4. Stationary cultures were buffered with dimethylsuccinate (DMS, Fluka: 14150) at a concentration of 30 mM (Fenn and Kirk 1979). Cultivation and lignin alteration were carried out at 38 – 39° C.

Analytical Methods. At the indicated intervals the entire contents of flasks were blended by a kinetic high-frequency homogenizer, 10 ml of homogenized culture was suction filtered (Sartorius type 13400, glass fiber filter). The filtrate was used for *glucose* (glucose analyser 23A, Yellow Springs Instrument Co.), *total carbon* (Rapid C, Heraeus), *nitrogen* (ammonia electrode, Philips, coupled to a pH-meter 632, Metrohm) and *protein* (Lowry et al. 1951) analyses. The vacuum filtered material was washed several times with dioxane-H_2O 1:1 (v/v) and was dried overnight for *mycelium dry weight* values.

Lignin Assays. The lignin was measured spectrophotometrically (Janshekar et al. 1981). The schedule for separation, purification, and determination of free and "cell-recovered" lignin is presented in Fig. 2.

Total Hydrolysis of Carbohydrate and Klason Extraction

About 150 mg of the vacuum dried samples were placed in 10 ml test tubes and digested with 1.5 ml cold (5° C) 75 % (w/v) H_2SO_4 at 30° C for 1 h with continuous shaking. Contents of the tubes were quantitatively transferred to 200-ml Erlenmeyer flasks and diluted with 42 ml of water. The flasks were covered with aluminium foil and placed in an autoclave (120° C, 1 h). The residual acid insoluble Klason lignin was collected on a tared filter (Sartorius type 11311, 0.01 µ), vacuum dried, and weighed. The filtrate

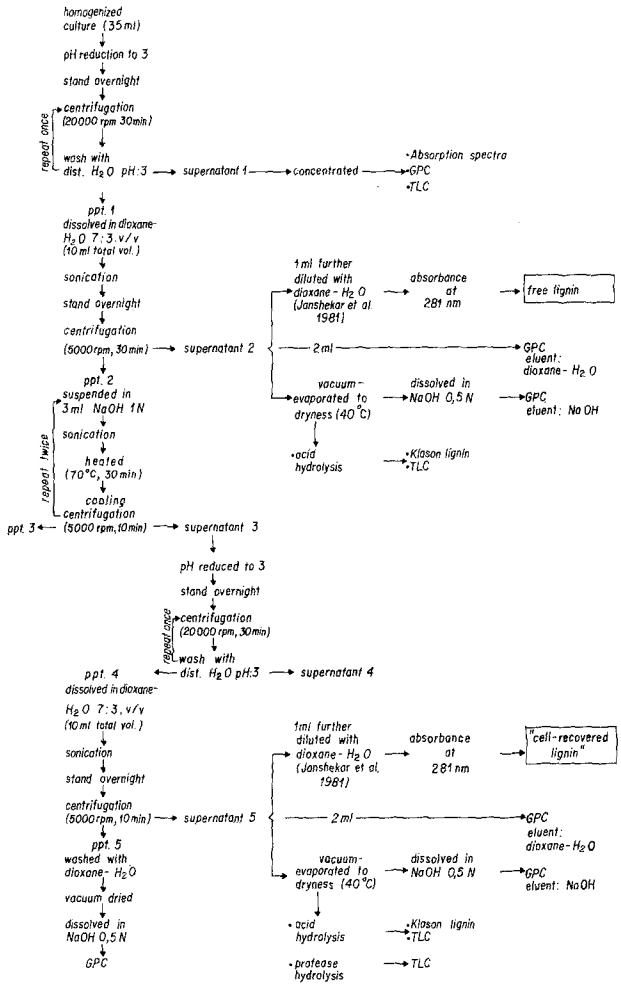

Fig. 2. Schedule for separation, purification and analysis of lignins. *GPC* Gel permeation chromatography; *ppt* precipitate; *TLC* thin layer chromatography

was cooled and diluted to 100 ml with water, then neutralized with anion exchange resin (Dowex 2, 20 – 50 mesh) which had been regenerated with a 1 M solution of Na_2CO_3. The resin was filtered off with a glass filter and washed with about 50 ml water. The combined solution of sample and wash water was concentrated under reduced pressure to ~ 0.5 ml (see also Saeman et al. 1963). The sugars in the solution were then analyzed with thin layer chromatography (TLC).

Enzyme Hydrolysis of Proteins

Proteins were hydrolyzed with two different commercial protease preparations. Protease type VI from *Streptomyces griseus* (Sigma P-5130) and protease type VIII from *Bacillus subtilis* (Sigma P-5380) were used at a concentration of 0.1 g l^{-1} in 100 mM phosphate buffer, pH 7.4, at 37° C. Cytochrome *c*, bovine serum albumin and alcohol dehydrogenase were used to test the efficiency of hydrolysis. Amino acids formed were detected with TLC.

TLC of Monosaccharides

Various monosaccharides were separated with TLC on silica gel (Kieselgel 60 F_{254}, Merck) according to Poller and Unger (1974).

TLC of Phenolic Compounds

The presence of small molecular weight phenolic compounds were checked with TLC on silica gels (Kieselgel 60 F_{254}, Merck) according to Martin et al. (1967).

TLC of Amino Acids

Amino acids were detected with TLC on cellulose plates according to a Merck information pamphlet (Mitteilungen zur Dünnschicht-Chromatographie X).

Gel Permeation Chromatography

GPC was performed by the descending method described previously by Janshekar et al. (1982). Additionally Sephadex G-75 packed in a column of 1.6 × 87 cm eluted with 0.5 NaOH at a flow rate of 0.28 ml min^{-1} was utilized. The standards used were blue dextran (MW = 2×10^6), albumin from hen eggs (MW = 45000), chymotrypsinogen A (MW = 25000), cytochrome *c* (MW = 12500), glucagon (MW = 3483) and phenol (MW = 94) (Forss and Fuhrmann 1978).

Results

Pellet Inoculum

The results obtained using pellet inoculation are presented in Fig. 3. The level of free lignin in the culture remained almost constant for the first 9–10 days. This was followed by a period during which a sharp decrease in the free lignin absorbance was observed. The absorbance decreased to about 55% of its starting value within 5 days. The greatest reduction, 22%, occurred between days 11 and 12. During the 3rd week of cultivation the rate of decrease in absorbance was retarded, only 5% of the total reduction in free lignin absorbance occurred in the last 5 days. There was a steady drop in the glucose concentration throughout the experiment. During the first, non-active 9–10 days 0.7–1.0 g l^{-1} glucose was consumed. Glucose utilization per litre of culture increased with culture age, in 20 days 3.8 g l^{-1} glucose was consumed of which 44% was used in the last 5 days although the dry wt. increase in this period was not significant (0.03 g l^{-1}, day 15–20). The pH of the culture varied between 3.9 and 4.5. During the active phase it was about 4 and it tended to increase towards day 20.

Spore Inoculum

The previous experiment was repeated using spores as inocula. Samples were taken every 5 days and the experiment was carried out for 50 days. A decrease in the level of free lignin was observed from the beginning. However, it should be noted that the first sample was taken 5 days after inoculation. Therefore a non-ligninolytic phase similar to that observed with pellet inoculation if occurring in the first 5 days could not be detected. Although the decrease in free lignin absorbance began earlier, the rate of decrease was considerably less. In the first 5 days of cultivation the free lignin absorbance was only reduced to 95% of its starting value. The free lignin level which was reached by the pellet inoculated culture within 10 days (10 days after the start of the ligninolytic active phase) was achieved here but after 30 days. The retardation of ligninolytic activity began when the culture

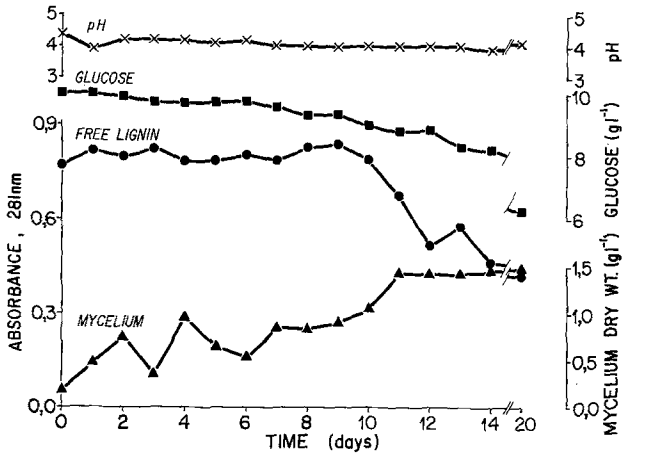

Fig. 3. Pellet inoculated, standing cultures of *Phanerochaete chrysosporium*. Lignin was added at the time of inoculation. ■ Glucose; ▲ mycelium dry weight; ● free lignin; × pH

was 25–30 days old, although a decrease in free lignin absorbance was observed after day 30.

Glucose consumed by the culture in the first 5 days was 1.3–1.8 g l^{-1}. This was higher than that of the pellet inoculated cultures (0.3 g l^{-1}). No correlation could be found between the amount of glucose used by the culture, and the culture age, as had been observed in the previous experiment. The pH of the culture dropped to 3.4 after 5 days in spite of the presence of buffer. Therefore the pH was corrected to 4.5 at 5 day intervals. Without this correction the decrease in free lignin absorbance ceased, although the increase in mycelium dry weight was not affected.

Mycelial Culture

Lignin bioalteration studies using mycelial cultures gave similar results to those observed with the experiment using pellet inocula. The cultures used were obtained from a chemostat without making any change in its content. An addition of nitrogen not sufficient to remove the limitation (2.4 mM) had been shown in previous experiments (unpublished data) to cause a greater reduction in glucose concentration without favouring the extent of decrease in free lignin absorbance or shortening the period of inactivity. The concentration of nitrogen dropped to 0.1 mM, and less, within 5 days.

The effect of the age of the standing culture on the extent and rate of absorbance decrease was investigated by adding lignin to cultures of increasing age at 5 day intervals. The decrease in free lignin absorbance was determined in each case after 5 days. Mycelial cultures that were standing for less than 10 days before lignin addition were not able to effect a significant decrease in the free lignin absorbance. Older cultures, standing for 10–20 days before lignin addition all reduced the free lignin absorbance to almost the same extent (50–60%). Glucose consumption by the cultures increased with increasing culture age. After observing the effect of culture age on reduction of free lignin absorbance, lignin was added only to mycelial cultures which had been standing for 10 days.

Figure 4 compares the behaviour of cultures with and without lignin addition. The glucose concentration and mycelium dry weight in the chemostat were 6.5 and 0.84 g l^{-1} respectively. In standing cultures without lignin addition, the

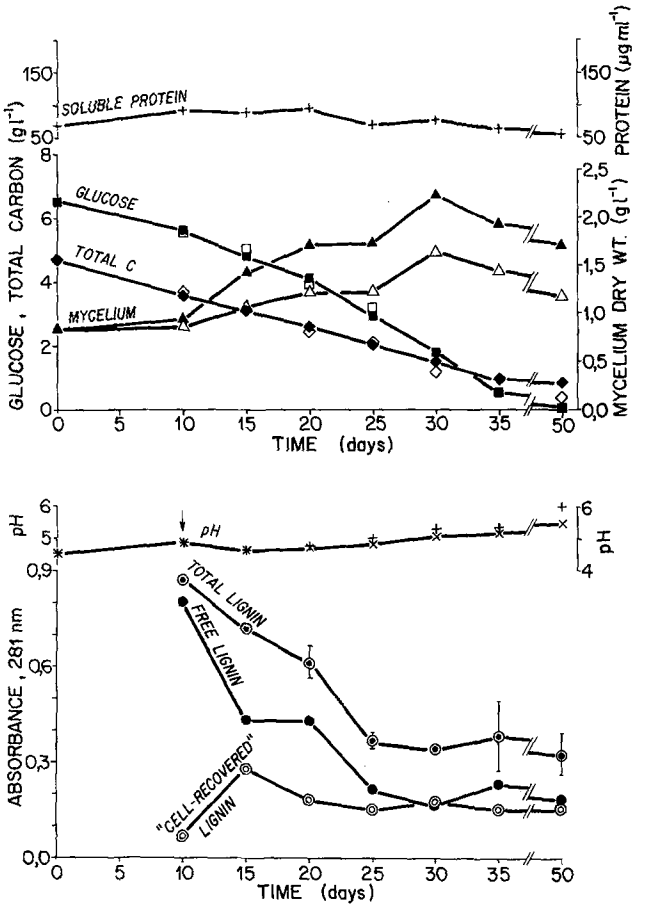

Fig. 4. Standing cultivation of *Phanerochaete chrysosporium* with and without lignin addition. Cultures were obtained from a chemostat operating at a dilution rate of $0.02\,h^{-1}$ as described in Materials and Methods. Lignin was added after 10 days (*arrow*). For cultures without lignin: □ Glucose; ◇ total soluble carbon; △ mycelium dry weight; + soluble protein and pH. For cultures with lignin addition: ■ Glucose; ◆ total soluble carbon; ▲ mycelium dry weight; × pH

mycelium dry weight increased only slightly up to day 30 and with a lower yield on glucose (16%) than that obtained in the chemostat (24%). After day 30, a decrease was observed, perhaps due to cell lysis. This decrease in mycelium dry weight occurred even though the glucose content of the culture was not depleted. The glucose consumption rate of the cultures increased only up until day 30 but total depletion of glucose took place after 50 days of standing cultivation.

After lignin was added to cultures, dry wt. values increased when compared to cultures without lignin addition, but the difference between the values became constant 10–15 days after addition of lignin. In those cultures to which lignin was added, no attempt was made to correct the dry weight values for lignin remaining after dioxane-H_2O washing (see Materials and Methods). It seems possible that lignin absorbance by the cells is the cause of the observed difference rather than increased biomass formation due to lignin degradation. This explanation is supported by the fact than when the level of "cell recovered" lignin becomes constant, the difference in mycelium dry weight between cultures with and without lignin also becomes constant.

Assuming that only glucose-C can be assimilated by the fungus the sum of glucose (measured by glucose analyzer) and non-glucose carbon (based on starting values) should be equal to the total carbon measured. In fact the measured total carbon was found to be less than the sum of glucose and non-glucose carbon. The concentration of non-glucose carbon in the medium was $2.2\,g\,l^{-1}$ of which 97% was contributed by the dimethylsuccinate buffer. Thus it would appear that the buffer can be assimilated by the fungus although at a lower rate than glucose.

The pH of the cultures in the lignin degrading phase was about 4.8 and increased to over 5 towards the end of the experiment. As shown in Fig. 4 no significant difference was observed in glucose concentration, total carbon or pH of cultures with and without lignin.

The soluble protein level did not vary to any great extent. It rose slightly up to day 20, then tended to decline. Data is not reported for cultures to which lignin was added because of interference with the assay due to the presence of soluble phenolic compounds.

The maximum ammoniacal nitrogen measured in the supernatant during standing cultivation was $0.2\,mg\,l^{-1}$ (0.01 mM).

The free lignin absorbance decreased after the addition of lignin, but the "cell recovered" lignin absorbance first increased and then decreased. However, the sum of these two, the total lignin, decreased. The sharp decrease in the absorbance due to free lignin within the first 5 days was followed by a retardation period of 5 days after which time the decrease in absorbance continued. The free lignin absorbance decreased up to 15–20 days after lignin addition. In spite of the presence of a carbon and energy source, no further change was seen in the following 20 days. The respective decreases in the free and total lignin absorbances amounted to 79 and 61% of their starting values within 20 days.

The absorbance spectra of free and "cell recovered" lignins were compared. The characteristic absorption band of the KL-O fraction in dioxane-H_2O with a maximum at 281 and a minimum at 271 nm was maintained. Furthermore no new peak or shoulder which could be evidence for the formation of new groups or compounds was observed. The free lignin seemed to be little different from the KL-O fraction with respect to its molecular size distribution (Fig. 5). "Cell recovered" lignin, on the other hand was polymerized when compared with KL-O. This was verified by all three different gels used. Similar gel permeation chromatography, using lignin which together with non-ligninolytic cells (cultures standing for less than 10 days) had undergone the same treatment presented in Fig. 2, ruled out the possibility of such polymerization happening during the extraction process. The elution profile of free lignin did not change with culture age whereas that of cell recovered lignin did exhibit changes (Fig. 6). Five days after addition of lignin to the cultures, the distribution curve of "cell recovered" lignin showed a higher proportion of molecules having a molecular weight greater than 45,000. The elution profile moved back towards its starting pattern in the next 5 days and after that did not change.

Elemental analysis showed that free and "cell recovered" lignin are different from KL-O with respect to their composition (Table 1). Differences were also observed in the Klason lignin content and absorptivity coefficients of these lignins which were lower in both cases than the values for KL-O. The analyses were made for cultures to which lignin had been added 20 days previously. Whether the values may change with culture age is not, however, clear. The lowest

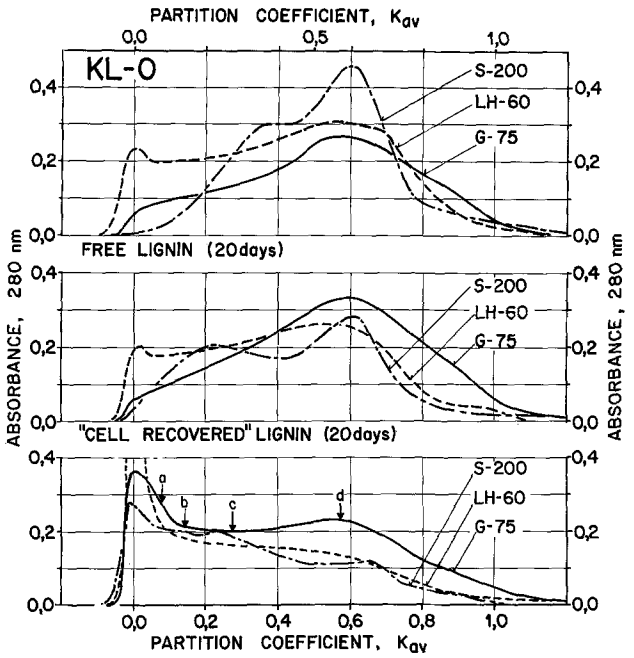

Fig. 5. Elution patterns of the organic-soluble ether insoluble fraction of Kraft pine lignin (KL-O) before and after attack by *Phanerochaete chrysosporium*. Lignin was added to 10 day old standing cultures (see Fig. 4). Chromatographic separation was performed on free and "cell recovered" lignin (see Fig. 2) after 20 days. Standards used in Sephadex G-75 column: *a*, albumin (MW = 45,000); *b*, chymotrypsinogen (MW = 25,000); *c*, cytochrome *c* (MW = 12,500); *d* glucagon (MW = 3,483)

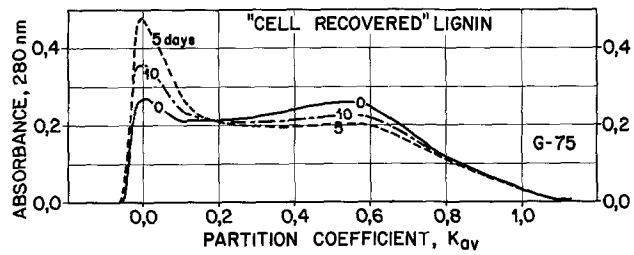

Fig. 6. Changes in the elution pattern of the "cell recovered" lignin. Lignin was added to 10 day standing cultures. The times shown refer to the number of days after lignin addition. The elution curves for the lignin extracted at days 15 and 20 are similar to that of day 10. For standards see Fig. 5

carbon and highest nitrogen contents were found in the "cell recovered" lignin.

In terms of Klason lignin, 50% of the original KL-O was degraded within 20 days. "Cell recovered" lignin which had been vacuum dried was partly soluble in phosphate buffer (100 mM, pH 7.4). GPC of the soluble part showed that the molecular weight distribution took the form of a normal distribution curve with the maximum at a molecular weight of about 1,500.

TLC of the acid hydrolyzate of dried "cell recovered" lignin showed the presence of 2.5% glucose which was verified using a glucose analyzer. The acid hydrolyzate showed traces of amino acids. Glucose was also found in the acid hydrolyzates of KL-O and free lignin but at lower concentrations (< 0.5%). This was in spite of the fact that the KL-O had been treated with a pyridine, acetic acid, chloroform and water system. The dried "cell recovered" lignin was additionally subjected to enzymatic hydrolysis with bacterial proteases. TLC of the hydrolyzate showed no detectable amino acids. Probably the proteins associated with the lignin are so denatured that they can no longer be hydrolyzed under the conditions used to an extent detectable with TLC.

Supernatant 1., the aqueous part of the culture (Fig. 2) was subjected to further analyses. The absorbance spectra obtained of this fraction from cultures with lignin against that from cultures without lignin had a significant absorbance in the range 260–360 nm. The absorbance increased with culture age during the 15 days after lignin addition. At 280 nm for example, the absorbance increased by a factor of 10 during this period. This accords with the period in which the decrease in free lignin absorbance took place (Fig. 4). GPC using Sephadex LH-20 gel which has a resolving power in the low molecular weight region (MW < 1700, Connors et al. 1978), showed that after 5 days of cultivation with lignin, the molecular weight distribution curve of Supernatant 1. from cultures with lignin was approximately the sum of the respective profiles of similarly treated Supernatant 1. from cultures without lignin and lignin containing medium. After 15 days of cultivation with lignin this relationship no longer applied. The peak eluted at a partition coefficient of 0.75 showed a greater increase for lignin containing cultures when compared to cultures to which lignin had not been added. This was also seen for those molecules that eluted at K_{av} values less than 0.6. According to the correlation found by Connors et al. (1978), this means compounds with a molecular weight greater than 300. TLC tests of Supernatant 1. for phenolic monomers showed little difference between cultures with and without lignin addition.

Can Lignin be Altered Only to a Limited Extent?

As shown in Fig. 4 and already mentioned, the decrease in absorbance of lignin stops about 15–20 days after lignin has been added to the culture. This indicates that lignin alteration could only have taken place to a limited extent. To investigate whether this is the case, cultures to which lignin had been added 20 days previously were divided into two parts (A and B). From one part (A), free lignin was extracted, concentrated and vacuum dried. This was then added to 10 day old mycelial cultures as previously described. To cultures of the second part (B), an equal volume of 10 day old standing cultures without lignin was added. Half of these culture mixtures were then blended with a homogenizer, while the other half were mixed only by manual shaking. All the flasks described above were then cultivated for a further 25 days. The absorbance of the lignin was found to be decreased only in those cultures which had received extracted free lignin, however, at a lower rate and to a lesser extent than observed in previous experiments. The percentage decrease in free lignin absorbance which had been achieved in 5 days (Fig. 4) occurred here after 25 days. A resumption in the decrease of free lignin absorbance was not observed in the two-culture mixtures regardless of whether they were blended or unblended.

Microscopic Observations

During the course of cultivation and lignin alteration studies, cells were regularly subjected to microscopic examination.

Table 1. Elemental analysis, Klason lignin content and absorptivity coefficients of the organic-soluble ether insoluble fraction of Kraft pine lignin (KL-O) before and after 20 days cultivation with *Phanerochaete chrysosporium*. Absorptivity coefficients were measured in dioxane-H_2O 1:1 (v/v)

Lignins	% w/w							Absorptivity at 281 nm $1\,g^{-1}\,cm^{-1}$
	C	H	N	S	O	Ash	Klason lignin	
KL-O	64.06	5.37	1.51	1.08	27.28	0.70	89.0	22.1–25.5
Free lignin (after 20 days)	60.53	6.56	0.72	0.82	31.37	0.00	83.1	15.0–16.0
"Cell recovered" lignin (after 20 days)	53.70	5.41	2.58	0.53	35.58	2.20	76.3	17.0–17.4

Differences in the morphology of the fungus were observed according to the age of standing cultures. Cells taken directly from the chemostat exhibited long mycelial hyphae, some having a spiral configuration, little side branching was seen. Formation of chlamydospores was also observed in such cultures. This morphology remained unchanged even after continuous cultivation periods of 1 month or more. When cultures were transferred from an agitated to a standing mode of cultivation, mat formation occurred during the first 10 days, but no significant change in morphology was observed. After this period, formation of new outgrowths could be seen (Fig. 7). Development of the many new hyphae which emerged directly from the walls of older mycelia appeared to differ from new growth normally observed at hyphal tips. The extension of the growing zone seemed to be limited and newly formed hyphae remained short. This mode of growth and the structures formed were not dependant on the presence or absence of lignin in the culture. Formation of such structures was not observed in standing cultures with an adequate nitrogen content i.e. a nitrogen concentration 10 times greater than that of nitrogen-limited cultures.

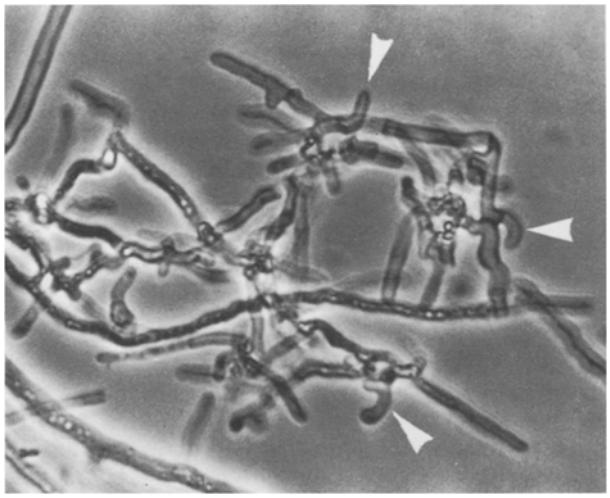

Fig. 7. *Phanerochaete chrysosporium* mycelia from a nitrogen-limited standing culture older than 10 days. The initial culture was taken from the chemostat. The structures indicated by arrows appeared after 10 days standing cultivation at 38–39° C under an atmosphere of oxygen, and continued to be observed for the remainder of the experiment (50 days); ×1,000

Discussion

Data to enable comparison of the different experiments is presented in Table 2. From the table it can be seen that the stage of growth of the culture is important with regard to the onset, rate and extent of decrease of free lignin absorbance and the glucose consumed. Mycelial cultures or cultures inoculated with pellets needed about 10 days after transferring to a standing mode of growth to become ligninolytically active. This period of inactivity was reduced to less than 5 days when spore inocula were used. The work of other authors (Fenn et al. 1981; Keyser et al. 1978) using spore inocula, and the ability of cultures to release $^{14}CO_2$ from ^{14}C-labelled synthetic lignin as a criterion of lignin alteration showed similar results with respect to the length of the ligninolytically inactive period. In their cultures nitrogen supplied in the medium was depleted between day 1 and 1.5 and activity appeared 2–3 days after near depletion of nitrogen. With spore inocula it can be seen that although the onset of ligninolytic activity is earlier, the decrease in free lignin absorbance proceeds more slowly than with mycelial or pellet inoculated cultures. The most considerable decrease in free lignin absorbance occurred between days 10 and 15 in all cultures. It seems possible from this data that the initial stages of decrease in free lignin absorbance may be rapid because certain moieties of the lignin molecule are more susceptible to bioalteration and that after this initial alteration the molecule remaining is resistant to further alteration. This hypothesis is supported by data from the experiment where free lignin was extracted and dried, then added to 10 day old cells in the normal manner. The rate and extent of degradation in these cultures was less than that observed with KL-O.

Kirk et al. (1978) have reported a degradation to CO_2 of 5 mg of [ring-^{14}C]-lignin concomitantly with the utilization of approximately 100 mg of glucose within 21 days. On the basis of Klason lignin in mycelial culture, we obtained 13.5 mg decrease in Klason content of 1 g KL-O accompanied by utilization of 100 mg glucose. The glucose consumed by cultures per unit time seems to increase with culture age up until day 30 at which time the biomass begins to decrease. Possibly the glucose required by the culture for maintenance increases with increasing culture age or is additionally used for new growth. From the dry weight values it seems possible that cells may take of part of the glucose and use it to build storage polysaccharides. More study is being done to clarify this. Since pH, glucose and total carbon values are very similar for cultures both with and without lignin this indicates that glucose is unlikely to be directly related to ligninolytic activity. Jeffries et al. (1981) report a maintenance requirement of about $0.3\,g\,g^{-1}\,day^{-1}$ for *Phanerochaete chrysosporium* under conditions of nitrogen limita-

Table 2. Reduction in free lignin absorbance with culture age as a percentage of the starting value and as a percent decrease in absorbance per gram of glucose consumed concurrently in 5 days. Comparison is made of cultures inoculated with pellets or spores, and mycelial cultures from the chemostat, to which KL-O was added at the beginning of standing cultivation. Data on "redegradation" is from an experiment in which the free lignin remaining after 20 days incubation with *Phanerochaete chrysosporium* was extracted and added to an "active" culture (10 days old)

Culture age (day)	Absorbance of free lignin (% of starting value)				% Decrease in absorbance of free lignin per g glucose consumed concurrently in 5 days			
	pellet	mycelia	spore	"redegradation"	pellet	mycelia	spore	"redegradation"
0	100	100	100	α	0	0	0	α
5	100	101	95	α	0	0	4	α
10	100	100	91	100	0	0	7	0
15	55	55	79	83	40	65	28	34
20	50	54	76	60	3	1	3	47
25	–	27	55	65	–	18	52	0
30	–	21	51	49	–	7	2	8

α Lignin added after 10 days standing cultivation

tion. Using the dry weight values of the samples without lignin addition (Fig. 4) a specific glucose uptake rate of 0.25 g g^{-1} day^{-1} between days 10 and 30 can be calculated.

Although cultures were nitrogen starved a slow increase in biomass formation was observed. It has been reported that wood-destroying basidiomycetes are remarkable well adapted to nitrogen deficient environments and are capable of recycling nitrogen (Merrill and Cowling 1966). The maximum ammoniacal nitrogen measured in mycelial cultures from the chemostat was 0.2 mg l^{-1} (0.01 mM), suppression due to NH_4^+ has been measured and found to occur at concentrations $\geqslant 0.7$ mM (Fenn et al. 1981). Thus the 10 day lag before onset of ligninolytic activity cannot be attributed to NH_4^+ suppression. This points towards the possibility that ligninolytic activity may not be a direct consequence of nitrogen starvation. It is possible that nitrogen limitation and standing conditions are prerequisites for formation of the structures observed. These may be involved in lignin alteration since they are found only in cultures under conditions suitable for lignin alteration. Effect of N-limitation on hyphal growth and wall properties are discussed by Gooday and Trinci (1980). To show that these structures are involved in lignin alteration, biochemical and physiological studies of the cell wall structure, morphogenesis and tip growth as related to bioalteration would be necessary.

The polymerization of "cell recovered" lignin observed would seem to be very rapid, because cultures to which lignin had been added and which were immediately assayed or frozen for subsequent analysis already showed some degree of polymerization. This phenomenon was found to be specific to ligninolytic cells, e.g. it did not take place with cells grown under sufficient nitrogen supply. Polymerization of Kraft and Spruce lignin has also been observed in cultures of *Fomes annosus*. This has been postulated to be a result of laccase activity (Hütterman et al. 1980). Elemental analysis of this polymer indicates that it is not a pure lignin. It has a higher concentration of nitrogen, lower concentrations of carbon and Klason lignin and a lower value for its absorptivity coefficient when compared to KL-O. Results obtained from TLC and elemental analysis indicate that in this polymer lignin is associated with carbohydrates and proteins. Our observations of this polymer lead us to speculate that lignin may be attached by polymerization, and subsequently depolymerized in the vicinity of new hyphal growth.

The absorbance spectrum and molecular weight distribution of free lignin have the same general appearance as those of the original lignin but both the Klason lignin content and the absorptivity coefficient are less than those of KL-O. This decrease in the absorptivity coefficient without a parallel change in the molecular weight distribution could be caused by intrapolymeric cleavage, i.e. ring cleavage within the intact polymer (Crawford and Crawford 1980).

Several possible explanations for the termination of changes in lignin can be postulated. Firstly that the free lignin has been changed and cannot be further altered. Secondly that only during a certain period of culture age are the cells ligninolytically active, and thirdly that ligninolytic activity has been inhibited or repressed by something that is related either to the cells or lignin. Our experiments have shown free lignin can be further altered, but that the addition of new active cultures of cells to cultures of old, no longer active cells gave no additional change in free lignin absorbance. Therefore it would seem that lignin alteration comes to a halt due to inhibition or repression, or because the lignin is inacessible to new active cells.

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