

## Influence of Culture Parameters on Lignin Metabolism by *Phanerochaete chrysosporium*

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**Abstract.** Culture parameters influencing metabolism of synthetic <sup>14</sup>C-lignins to <sup>14</sup>CO<sub>2</sub> in defined media have been studied in shallow batch cultures of the ligninolytic wood-destroying Hymenomycete *Phanerochaete chrysosporium* Burds. Study of the effect of O<sub>2</sub> concentration in the gas phase above non-agitated cultures indicated essentially complete absence of attack on the lignin polymer at 5% O<sub>2</sub> in N<sub>2</sub>, and a 2- to 3-fold enhancement by 100% O<sub>2</sub> as compared to air (21% O<sub>2</sub>). Agitation of the cultures resulting in the formation of mycelial pellets greatly suppressed lignin decomposition. The optimum culture pH for lignin decomposition was 4 to 4.5, with marked suppression above 5.5 and below 3.5. The source of nutrient nitrogen (NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, amino acids) had little influence on lignin decomposition, but the concentration of nitrogen was critical; decomposition at 24 mM was only 25 – 35% of that at 2.4 mM N. Thiamine was the only vitamin required for growth and lignin decomposition. Under the optimum conditions developed, decomposition of 5 mg of synthetic lignin was accompanied by utilization of approximately 100 mg of glucose. The influence of the various culture parameters was analogous for metabolism of synthetic lignin labeled in the ring-, side chain-, and methoxyl carbon atoms.

**Key words:** White-rot fungi – Nutrient nitrogen metabolism – Fungus physiology – Mycelial pellets – pH – Growth substrate – Wood decay.

Little is known about the nutritional, physiological, and environmental factors that influence the microbial degradation of lignin, the second most abundant biopolymer on earth. Two major reasons for this lack of knowledge are: (1) the structure of the complex, heterogeneous lignin polymer was not clear until the

1960's; and (2) until recently no unequivocal and sensitive quantitative assay for lignin biodegradation had been developed. Understanding the factors that influence lignin biodegradation is necessary to clarify wood decay, the formation of humic substances, coal and petroleum, and to explore realistically the practical potential for utilizing lignin-degrading organisms or enzymes in lignocellulose bioconversion processes.

A sensitive and definitive biodegradation assay based on the decomposition of synthetic <sup>14</sup>C-lignins to <sup>14</sup>CO<sub>2</sub> has been reported (Kirk et al., 1975) for lignins synthesized with <sup>14</sup>C in the ring, side chain, or methoxyl portions of the polymer. This assay procedure has been used to examine some of the environmental factors important in lignin degradation in various natural materials (Hackett et al., 1977), and to initiate detailed studies on the degradation of the complex aromatic polymer by white-rot fungi, which are wood-destroying Hymenomycetes. The white-rot fungi are by far the most active ligninolytic organisms described to date. We have already reported that *Phanerochaete chrysosporium* and *Coriolus versicolor* require a growth substrate such as glucose or cellulose in order to metabolize lignin to CO<sub>2</sub> (Kirk et al., 1976). We report here that several culture parameters have a pronounced effect on the ligninolytic activity of white-rot fungi.

### Materials and Methods

#### Lignins

Synthetic [*U*-ring-<sup>14</sup>C]-, [methoxyl-<sup>14</sup>C]-, and [side chain (β,γ)-<sup>14</sup>C]-lignins with specific activities of 9.3 × 10<sup>4</sup>, 9.1 × 10<sup>4</sup>, and 2.2 × 10<sup>5</sup> dpm/mg were prepared as described earlier (Kirk et al., 1975). Identically prepared unlabeled synthetic lignin was used in certain experiments to dilute the <sup>14</sup>C-lignins. All lignins were stored at -30°C in aqueous suspension. For use, these lignins were thawed, dried on a rotary film evaporator at <50°C, dissolved in *N,N*-dimethylformamide (DMF) to give approximately 10% solutions, and stored at -30°C. Lignin suspensions for addition to cultures were prepared by slowly adding the DMF solutions aseptically to

vigorously stirred sterile water (> 1 ml H<sub>2</sub>O/50 µl DMF solution). No contamination of cultures resulted from addition of lignin.

### Chemicals

All chemicals were reagent grade, and were used as purchased except *p*-dioxane, which was further purified according to Vogel (1956). Powdered cellulose (Solka Flok SW-40, Brown Co., Berlin, N.H.) was from wood pulp; this cellulose was analyzed (Saeman et al., 1954) and found to contain approximately 90% glucan, 8% xylan, and 2% mannan.

### Fungi and Inoculum

*Phanerochaete chrysosporium* Burds. (ME-446) was obtained from the Center for Forest Mycology Research, Forest Products Laboratory, U.S.D.A., Madison, Wisconsin, and was maintained at room temperature on 2% malt agar slants. Inoculum consisted of filtered (glass wool) conidial suspensions ( $A_{650\text{nm}} = 0.5/\text{cm}$ ,  $\approx 2.5 \times 10^6$  spores/ml); conidia were taken from 3–4-week-old slants, and exhibited > 90% viability.

### Experimental Cultures

Unless stated in the text, basal medium contained the following per liter of distilled H<sub>2</sub>O: KH<sub>2</sub>PO<sub>4</sub>, 0.2g; MgSO<sub>4</sub> · 7 H<sub>2</sub>O, 0.05 g; CaCl<sub>2</sub>, 0.01 g; mineral solution, 1 ml; and vitamin solution, 0.5 ml. Minerals (per 1 dist. H<sub>2</sub>O), consisted of nitrilotriacetate, 1.5 g; MgSO<sub>4</sub> · 7 H<sub>2</sub>O, 3.0g; MnSO<sub>4</sub> · H<sub>2</sub>O, 0.5g; NaCl, 1.0g; FeSO<sub>4</sub> · 7H<sub>2</sub>O, 100 mg; CoSO<sub>4</sub>, 100 mg; CaCl<sub>2</sub>, 82 mg; ZnSO<sub>4</sub>, 100 mg; CuSO<sub>4</sub> · 5H<sub>2</sub>O, 10 mg; AlK(SO<sub>4</sub>)<sub>2</sub>, 10 mg; H<sub>3</sub>BO<sub>3</sub>, 10 mg; NaMoO<sub>4</sub>, 10 mg. Vitamins (per 1 dist. H<sub>2</sub>O), consisted of biotin, 2 mg; folic acid, 2 mg; thiamine · HCl, 5 mg; riboflavin, 5 mg; pyridoxine · HCl, 10 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 5 mg; DL-calcium pantothenate, 5 mg; *p*-aminobenzoic acid, 5 mg; thioctic acid, 5 mg. The basal medium was prepared in 10–100-fold concentrations and diluted to the above concentration in the final medium.

Various nitrogen sources and concentrations, and buffers were used as stated in the text. D-Glucose in a final concentration of 1% (56 mM), unless noted otherwise, served as growth substrate.

Experiments were conducted with 10 ml cultures in 125-ml Erlenmeyer flasks. Filter-sterilized basal medium + nitrogen source, autoclaved buffer and glucose solution (15 min, 121°), lignin suspension in water, and spore suspension (1 ml for each 10 ml culture) were combined and dispensed (10 ml per culture flask) to give replicated cultures. Aliquots (1 ml) from the complete media were taken for radioactivity determinations; a modified Bray's scintillation fluid was used (Kirk et al., 1975). Final DMF concentration in the cultures was usually 0.25% by volume or less, although concentrations of DMF up to 1% v/v did not suppress growth or lignin decomposition.

Culture flasks were fitted with rubber stoppers through which two 6-mm-glass tubes were inserted. One of these tubes extended into the flask within 1 cm of the culture fluid; outside the flask it was connected via natural rubber tubing to a cotton-plugged gas-sterilizing filter. The second glass tube extended 1 cm or less into the flask, and outside was connected via rubber tubing to a 3-cm, 18-gauge, Luer-type needle. Screw clamps on both of the rubber tubings allowed the flasks to be closed. Flasks were flushed every 3–4 days by opening the clamps and forcing flushing gas through the cotton filters at 100–200 ml per minute for 15 min. This was found to remove all evolved <sup>14</sup>CO<sub>2</sub>, which was trapped by placing the exit needle in 10 ml of an ethanolamine-containing scintillation fluid (Kirk et al., 1975) in a 20-ml scintillation vial. Studies demonstrated that <sup>14</sup>CO<sub>2</sub> trapping was > 98% efficient. Flushing more frequently than every 3–4 days did not enhance growth or lignin decomposition.

Cultures were incubated at the optimum growth temperature of 39–40°C. In experiments with agitated cultures, flasks were incubated at 39–40°C on a rotary shaker (125 rpm, amplitude = 2.5 cm).

In studying the effect of gaseous O<sub>2</sub> concentration in the cultures, three gases were used to flush the flasks: 5% by volume O<sub>2</sub> in N<sub>2</sub> (obtained as a prepared mixture or mixed as needed through a pair of calibrated flow-meters); air (21% O<sub>2</sub> by volume); and 100% O<sub>2</sub>.

Most degradation data are presented as percent of original <sup>14</sup>C recovered as <sup>14</sup>CO<sub>2</sub> as a function of incubation time. Data are corrected for background radioactivity, and for counting efficiency. The latter was monitored routinely with an automatic external standard using a <sup>226</sup>Ra source (Packard Instruments Co., Inc., Downers Grove, Illinois), and periodically with an internal standard of <sup>14</sup>C-toluene. Counting efficiency was ≈65% and ≈78% for the <sup>14</sup>CO<sub>2</sub>-trapping and modified Bray's scintillation fluids, respectively. Counting of samples was continued at least for sufficient time to give values accurate within ±4% (95% confidence limits). All experiments were conducted at least in triplicate and all values are reported as means ± 1 standard deviation (s.d.).

Mycelium weights were determined after collection and drying on tared filter paper discs. Residual medium glucose was determined by the procedure of Nelson (1944).

Aqueous dioxane extracts were prepared from certain terminated cultures. Dioxane (10 ml) was added to each flask, and these were flushed with N<sub>2</sub>, stoppered, and held at room temperature overnight. Insoluble materials were removed by centrifugation and combusted to CO<sub>2</sub> (Lundquist et al., 1977) to determine total radioactivity. The dioxane-water solubles were counted in the modified Bray's scintillation fluid. Over 90% of the original radioactivity was recovered in all examined cultures.

### Gel Permeation Chromatography

To the pooled aqueous dioxane extracts of three cultures (≈ 55 ml), 5 ml of DMF was added and the volume reduced on a rotary evaporator to approximately 3 ml. The remaining liquid, mostly DMF, was freed of insolubles by centrifugation. Approximately 85% of the <sup>14</sup>C in the extracts was recovered in the DMF.

One-milliliter samples of the DMF solutions were analyzed by gel permeation chromatography by the descending method on a 1 × 77-cm column (bed size) of Sephadex LH-20 in DMF (flow rate ≈ 20 ml/h). Fractions (0.96 ml) were collected and added to 10 ml of the modified Bray's scintillation fluid for radioactivity determinations. The column was calibrated with a variety of aromatic compounds structurally related to lignin (Connors et al., 1978).

## Results

### Effect of Oxygen

Because lignin degradation has been shown to be an oxidative process (Hackett et al., 1977; Kirk and Chang, 1975), lignin metabolism as a function of O<sub>2</sub> concentration was examined. Figure 1 gives the relationship between growth, glucose depletion, and decomposition of [ring-<sup>14</sup>C]-lignin in cultures maintained under a one-atmosphere gas phase that contained 5%, 21%, or 100% O<sub>2</sub>. Growth during the first 3 days of incubation was similar at all three O<sub>2</sub> concentrations, and was accompanied by utilization of approximately 25% of the glucose. Lignin decomposition to CO<sub>2</sub> began after the initial growth period under 21% and 100% O<sub>2</sub>, but not under 5% O<sub>2</sub>.

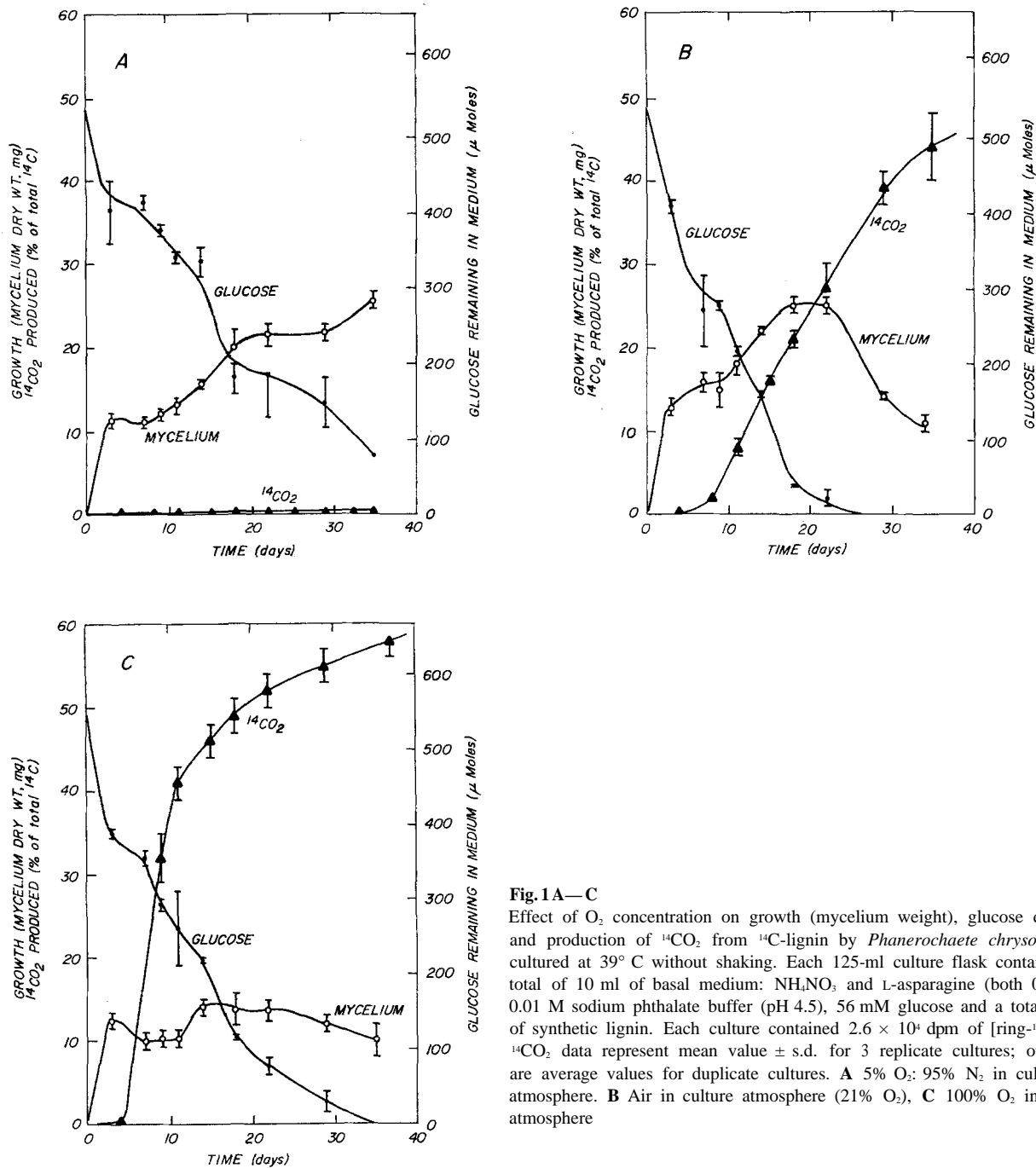


Fig. 1A—C

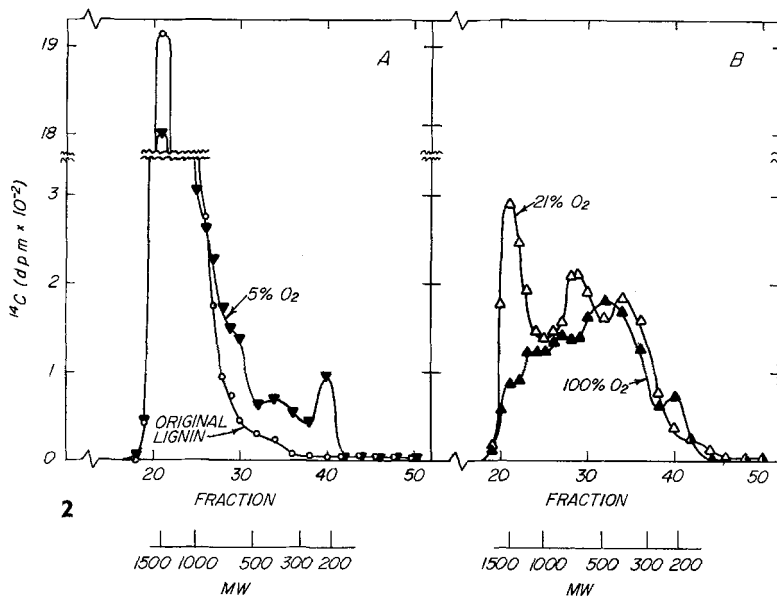
Effect of O<sub>2</sub> concentration on growth (mycelium weight), glucose depletion, and production of <sup>14</sup>CO<sub>2</sub> from <sup>14</sup>C-lignin by *Phanerochaete chrysosporium* cultured at 39° C without shaking. Each 125-ml culture flask contained, in a total of 10 ml of basal medium: NH<sub>4</sub>NO<sub>3</sub> and L-asparagine (both 0.6 mM), 0.01 M sodium phthalate buffer (pH 4.5), 56 mM glucose and a total of 1 mg of synthetic lignin. Each culture contained 2.6 × 10<sup>4</sup> dpm of [ring-<sup>14</sup>C]-lignin. <sup>14</sup>CO<sub>2</sub> data represent mean value ± s.d. for 3 replicate cultures; other data are average values for duplicate cultures. **A** 5% O<sub>2</sub>; 95% N<sub>2</sub> in culture atmosphere. **B** Air in culture atmosphere (21% O<sub>2</sub>), **C** 100% O<sub>2</sub> in culture atmosphere

After the initial 3 days of growth under 100% O<sub>2</sub> (Fig. 1C), only small changes in mycelium weight occurred for the next 37 days. Most of the conversion of lignin to CO<sub>2</sub> occurred between days 4 and 10, although evolution of <sup>14</sup>CO<sub>2</sub> was continuing even at 37 days. Glucose was depleted from the cultures before day 35.

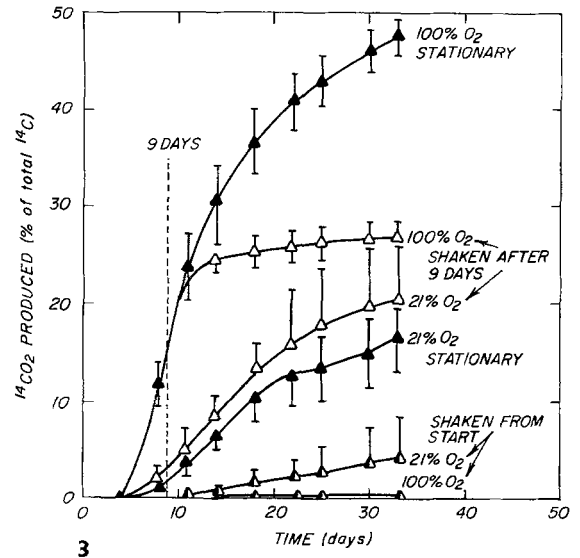
At 21% O<sub>2</sub>, the glucose was depleted from the cultures by day 25 (Fig. 1 B). Following the initial rapid growth phase (first 3 days), the mycelium continued to increase in weight gradually up to approximately

day 23, at which time mycelial weight was twice the 3-day weight. After day 23, mycelium weight decreased rapidly to less than half its maximum by day 35; the decrease coincided with depletion of glucose. The rate of <sup>14</sup>CO<sub>2</sub> production from the lignin was linear from day 8 to day 30, but was lower than that observed at 100% O<sub>2</sub>.

At 5% O<sub>2</sub>, the mycelium continued gradually to increase in weight after an initial period of rapid growth, as observed at 21% O<sub>2</sub>. At the time the cultures



**Fig. 2A and B.** Gel permeation chromatography of material extracted from 35-day cultures of *Phanerochaete chrysosporium* grown in the presence of [ring- $^{14}\text{C}$ ]-lignin under atmospheres of 5%, 21%, and 100%  $\text{O}_2$ . Radioactivity applied to the column, 6300, 3600, and 2900 dpm for cultures grown under 5%, 21%, and 100%  $\text{O}_2$ , respectively, was proportional to the amount extracted from the cultures; 5000 dpm of control lignin was applied



**Fig. 3.** Effect of agitation on decomposition of  $^{14}\text{C}$ -lignin by *Phanerochaete chrysosporium* cultured at  $39^\circ$  under gas atmospheres of 23%  $\text{O}_2$  (air) and 100%  $\text{O}_2$ . Each 125-ml culture flask contained, in a total of 10 ml of mineral salts medium:  $\text{NH}_4\text{NO}_3$  and L-asparagine (both 0.6 mM), 0.01 M sodium phthalate buffer (pH 4.5), 56 mM glucose, and  $3 \times 10^4$  dpm of [ring- $^{14}\text{C}$ ]-lignin. Cultures were incubated under three regiments. Values are means  $\pm$  s.d. for 4 replicate cultures

were terminated (day 35) about 90% of the glucose was depleted from the medium and decline in fungal weight was not observed. Lignin was not decomposed to  $\text{CO}_2$  during the 35-day incubation period.

As described in "Materials and Methods", the cultures were flushed at 3 - 4 day intervals in the above experiment. Separate experiments demonstrated that daily flushing gave similar results.

Experiments demonstrated that the effect of 0, was similar with glucose, cellulose, or xylose as growth substrate.

The  $^{14}\text{C}$  not recovered as  $^{14}\text{CO}_2$  from the above cultures by day 35 was recovered in the dioxane: water (1:1 by volume) solubles and in the mycelial residues left after aqueous dioxane extraction. Distributions of label in the cultures maintained under 5%, 21%, and 100%  $\text{O}_2$ , respectively, were:  $^{14}\text{CO}_2$ ,  $< 1$ ,  $47 \pm 4$ , and  $49 \pm 2\%$ ; aqueous dioxane extract, 71, 41, and 33%; and mycelial residue, 22, 9, and 9%. (Triplicate cultures at each  $\text{O}_2$  concentration were pooled for extraction with aqueous dioxane.)

The aqueous dioxane solubles from the cultures described above were transferred to DMF and analyzed by gel permeation chromatography. The radiolabeled

material from the cultures at 5%  $\text{O}_2$  differed only slightly in its chromatographic properties from the original lignin. Almost all of the radiolabel in both samples was excluded from the gel (Fig. 2A). In contrast, the extracts from the cultures maintained at 21% and 100%  $\text{O}_2$  contained approximately 35% and 10% labeled excluded material, respectively (Fig. 2B). Assuming that the  $^{14}\text{C}$ -labeled materials in the extracts of the 21% and 100%  $\text{O}_2$  cultures behaved chromatographically as lignin-related compounds, they were in the molecular weight range of 300 to 1000. No very low molecular weight material was present in either sample. Also, no radiolabel was detected beyond fraction 46 in any samples (i.e., the elution volume was always smaller than the total column volume), suggesting an absence of significant partitioning or adsorption effects. With each sample, all of the  $^{14}\text{C}$  applied to the column was recovered.

#### Effect of Culture Agitation

Experiments were conducted to test the effect of culture agitation on lignin decomposition to  $\text{CO}_2$ . Data are presented in Figure 3 for the degradation of the [ring- $^{14}\text{C}$ ]-lignin in cultures either maintained from the time

of inoculation on a rotary shaker (150 rpm; 2.5-cm amplitude), or maintained as non-shaken cultures for 9 days and then placed on the shaker. The 9-day still period was chosen after preliminary experiments revealed that lignin decomposition during incubation on the shaker increased with the length of the initial still period, up to 8 - 10 days. Constant shaking from the start suppressed lignin decomposition. At 21% O<sub>2</sub>, cultures that were shaken after 9 days of still incubation decomposed lignin much faster than cultures continuously shaken, and at a similar rate to that in non-shaken cultures. Identical effects of agitation on lignin decomposition were observed with side chain- and methoxyl-labeled lignins.

Cultures continuously shaken under 100% O<sub>2</sub> did not degrade lignin, although growth appeared equivalent to that at 21%. Also, at 100% O<sub>2</sub>, shaking after a 9-day stationary period greatly slowed lignin decomposition in comparison to stationary cultures (Fig. 3).

In parallel experiments, cultures under 21% O<sub>2</sub> that were initially shaken for 8 days formed 2 - 3 mm mycelial pellets, and began metabolizing lignin after remaining still for an additional 3 - 5 day period. During this still period, mycelial mats were formed as outgrowths from the pellets. Before these mats were formed, 48% of the radioactivity was found in the supernatants - i.e., not pellet-bound. The rate of degradation after mat formation was 30 - 50 % of that in stationary cultures. Thus, the rates observed were proportional to the amount of lignin not bound to the pellets.

### Effect of pH

The medium pH was found to be critical to lignin decomposition (Table 1). An optimum was observed at approximately pH 4.5, with substantial suppression of decomposition below pH 3.5 and above pH 5.5. The optimum pH for growth was somewhat higher than for lignin decomposition (Table 1).

### Effect of Medium Composition

Our initial studies (Kirk et al., 1976) on lignin metabolism by *P. chrysosporium* employed a defined culture solution that contained basal medium, 0.01 M phthalate buffer (pH 4.9, NH<sub>4</sub>NO<sub>3</sub> and L-asparagine (both 0.6 mM = 2.4 mM N) as N sources, <sup>14</sup>C-lignin, and 1% carbohydrate as growth substrate. Varying the ionic strength by increasing the basal medium concentration 10-fold did not affect the onset, rate, or extent of lignin decomposition. After 21 days, the percentages of total <sup>14</sup>C recovered as <sup>14</sup>CO<sub>2</sub> were 53 ± 2 and 51 ± 5 at the "low" and "high" ionic strengths, respectively. (Experiments were performed in tri-

**Table 1.** Effect of medium pH on growth and lignin decomposition by *Phanerochaete chrysosporium*<sup>a</sup>

pH		Growth <sup>b</sup> mg dry mycelium	Lignin decomposed to <sup>14</sup> CO <sub>2</sub> <sup>c</sup> % of total <sup>14</sup> C
Initial	Final		
3.0	2.9	7.8 ± 0.3	< 1
3.5	3.6	10.7 ± 0.2	8 ± 6
4.0	4.3	12.6 ± 0.4	37 ± 2
4.5	4.9	13.9 ± 0.4	38 ± 4
5.0	5.1	13.9 ± 0.5	20 ± 2
5.5	4.9	14.4 ± 0.8	8 ± 1
6.0	4.9	12.0 ± 0.1	3 ± 1

<sup>a</sup> Each 125-ml-culture flask contained, in a total of 10 ml of mineral salts medium: 0.6 mM L-asparagine, 0.3 mM ammonium tartrate, 0.01 M sodium aconitate (pH 4.5), 56 mM glucose, and 3 × 10<sup>4</sup> dpm of [ring-<sup>14</sup>C]-lignin. Cultures were grown without agitation, under 100% O<sub>2</sub> for 9 days

<sup>b</sup> Mean ± s.d. for 2 cultures

<sup>c</sup> Mean ± s.d. for 5 cultures

**Table 2.** Effectiveness of various compounds as growth substrates for degradation of synthetic <sup>14</sup>C-lignin by *Phanerochaete chrysosporium* in defined medium<sup>a</sup>

Compound <sup>b</sup>	Lignin decomposed to <sup>14</sup> CO <sub>2</sub> after 13 days <sup>c</sup> % of total <sup>14</sup> C
Glucose	38 ± 6
Glycerol	29 ± 3
Cellulose (Solka Floe)	41 ± 4
Sodium succinate (pH 4.5)	23 ± 3 <sup>d</sup>
Potassium gluconate (pH 4.5)	2 ± 2 <sup>d</sup>
Glucuronolactone	7 ± 2 <sup>d</sup>
Sodium vanillate (pH 4.5)	< 1

<sup>a</sup> Each culture flask contained in a total of 10 ml of basal medium: 0.6 mM NH<sub>4</sub>NO<sub>3</sub>, 0.6 mM L-asparagine, 300 μg of synthetic [ring-<sup>14</sup>C]-lignin, and 40 mg of growth substrate carbon [except vanillate, which was used at a non-toxic level of 6 mM (5.6 mg of C/culture)]. Cultures were grown without agitation, under 100% O<sub>2</sub>

<sup>b</sup> Similar growth occurred with all compounds except vanillate (no growth)

<sup>c</sup> Mean ± s.d. for 4 cultures with each compound

<sup>d</sup> Control of pH was inadequate

plicate; cultures initially contained 4.8 × 10<sup>4</sup> dpm of [ring-<sup>14</sup>C]-lignin, 2.4 mM N, 56 mM glucose, and were grown without shaking, under 100% O<sub>2</sub>.)

Previous studies (Kirk et al., 1976) demonstrated that any of several carbohydrates can serve as growth substrates supporting lignin metabolism. Analysis of other growth substrates has now shown that glycerol and sodium succinate can serve as carbon and energy sources and that both support lignin decomposition

**Table 3.** Effect of lignin concentration and label site on decomposition by *Phanerochaete chrysosporium*<sup>a</sup>

Original amount of lignin per culture µg	Lignin decomposed after 17 days µg <sup>b</sup>		
	[ring- <sup>14</sup> C]	[side chain- <sup>14</sup> C]	[methoxyl- <sup>14</sup> C]
300	50 ± 10	100 ± 10	130 ± 10
1300	350 ± 80	360 ± 50	520 ± 70
5300	1060 ± 180	1240 ± 100	1710 ± 320
10300	580 ± 260	1200 ± 390	2740 ± 470

<sup>a</sup> Each 125-ml culture contained, in a total of 10 ml of basal medium: NH<sub>4</sub>NO<sub>3</sub> and L-asparagine (both 0.6 mM), 0.01 M sodium phthalate buffer (pH 4.5), 56 mM glucose, and 3–4 × 10<sup>4</sup> dpm of <sup>14</sup>C-lignin. Unlabeled synthetic lignin was used to dilute the <sup>14</sup>C-lignins to obtain desired lignin concentrations. All cultures contained 90 µl of DMF. Cultures were grown without agitation, under 100% O<sub>2</sub>.

<sup>b</sup> (µg of lignin in original culture) · (% of total <sup>14</sup>C recovered as <sup>14</sup>CO<sub>2</sub>) ÷ 100. Values are means ± s.d. for 3 to 5 replicate cultures

(Table 2). Potassium gluconate and glucuronolactone also supported growth, but considerably less <sup>14</sup>CO<sub>2</sub> was produced from <sup>14</sup>C-lignin than with glycerol or succinate as growth substrate. (Under the conditions used, control of pH was inadequate with the gluconate, glucuronolactone, and succinate.) Vanillate did not support growth or lignin decomposition. Lignin decomposition was best with glucose and a wood pulp cellulose (Table 2).

The effect of concentration of lignin on its decomposition to CO<sub>2</sub> was investigated with glucose as growth substrate. The ring- and side chain-degrading activities became saturated at approximately 5 mg (0.005%) of lignin per 10 ml culture, but the methoxyl-degrading activity was not saturated even at 10 mg (Table 3).

Increasing the glucose concentration up to approximately 56 mM, in cultures containing 0.05% lignin, resulted in increasing rate and extent of lignin decomposition. Lignin decomposition after 21 days had essentially ceased in cultures with initial glucose concentrations of 28 mM or less (Table 4). These data suggest that *Phanerochaete chrysosporium* is able to degrade 5 mg of synthetic lignin concomitantly with the utilization of approximately 0.56 mMole (100 mg) of glucose.

The source of nutrient nitrogen did not significantly influence the rate or extent of lignin degradation. For example, degradation of ring-labeled lignin after 30 days with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, casamino acids (Difco), or NH<sub>4</sub>NO<sub>3</sub> + L-asparagine as nitrogen sources (N = 2.4 mM) was 55 ± 3%, 49 ± 8%, and 53 ± 4% CO<sub>2</sub>, respectively (mean ± s.d. for 3 cultures). In other experiments L-asparagine, ammonium tartrate, urea,

**Table 4.** Effect of glucose concentration on lignin decomposition by *Phanerochaete chrysosporium*<sup>a</sup>

Original concentration of glucose per culture mM	Lignin decomposed to <sup>14</sup> CO <sub>2</sub> % of total <sup>14</sup> C	
	(after 10 days)	(after 21 days)
0.00	0	0
0.14	2 ± 0	2 ± 0
0.21	5 ± 2	7 ± 2
0.28	10 ± 1	13 ± 1
0.42	15 ± 2	29 ± 3
0.56	21 ± 3	39 ± 3
1.11	22 ± 4	43 ± 6

<sup>a</sup> Culture conditions as described in Table 3. Each culture contained 5300 µg of lignin, including 2.3 × 10<sup>4</sup> dpm of [ring-<sup>14</sup>C]-lignin. Values are means ± s.d. for 5 replicate cultures

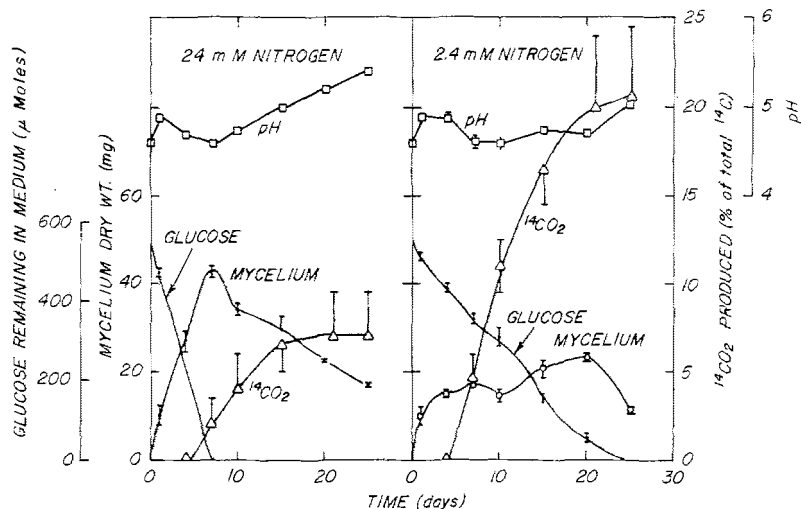
and sodium nitrate gave comparable results. The organism grew only very slowly from spores with nitrate as sole nitrogen source, but grew much more rapidly with nitrate-grown mycelia as inoculum. Degradation of lignin to CO<sub>2</sub> in nitrate medium inoculated with nitrate-grown mycelia began after 8–10 days, corresponding to establishment of a mycelial mat, and then proceeded at a rate and to an extent comparable to those on reduced nitrogen media. Conversions of [methoxyl-<sup>14</sup>C]-lignin to <sup>14</sup>CO<sub>2</sub> after 27 days were 62 ± 13% and 55 ± 3% in cultures grown with NaNO<sub>3</sub> and ammonium tartrate, respectively.

Experiments were conducted in quintuplicate; cultures initially contained basal medium, 0.01 M phthalate buffer at pH 4.5, 2.4 mM N, 56 mM glucose, and 3.4 × 10<sup>4</sup> dpm of lignin. They were grown without shaking, under 100% O<sub>2</sub>.

Although the source of N was not critical, the concentration of N greatly influenced lignin decomposition. Figure 4 depicts the relationship between growth, glucose utilization, lignin degradation, and pH changes at 24 and 2.4 mM nitrogen (12 and 1.2 mM ammonium tartrate). Lignin degradation began near the end of an initial growth phase. Both gain in mycelium weight and rate of glucose depletion during this growth period was over twice as much in the high-N medium as in the low-N medium. Growth appeared to be glucose-limited in the high-N medium, and mycelial weight declined immediately after glucose depletion. In the low-N medium, growth appeared to be limited by nitrogen at 2–3 days; glucose was not depleted until 20–25 days. Mycelium weight increased slowly between the 7th and 25th days, then declined, in the low-N medium. In both high- and low-N media, the pH fluctuated similarly for the first 7–10 days, and then rose in the high-N medium. The extent and rate of lignin decomposition was lower in the high-N medium

Fig. 4

Effect of nutrient nitrogen concentration on lignin decomposition by *Phanerochaete chrysosporium* cultures grown without agitation, under 100% O<sub>2</sub>. Each 125-ml Erlenmeyer culture flask contained, in a total of 10 ml of mineral salts medium: ammonium tartrate (1.2 or 12 mM), 0.05 M sodium aconitate buffer (pH 4.5), 56 mM glucose, and a total of 1 mg of synthetic lignin, including  $5 \times 10^4$  dpm of [ring-<sup>14</sup>C]-lignin. <sup>14</sup>CO<sub>2</sub> data represent mean value ( $\pm$  s.d.) for 5 replicate cultures; other data are average values for two cultures



than in the low-N medium. Lignin decomposition ceased in the former after 15 days.

Additional experiments examined the effect of nutrient nitrogen concentration at a constant pH of 4.5 in cultures buffered with 0.05 M aconitate, and containing either 0.6 or 6.0 mM NH<sub>4</sub>KO<sub>3</sub> and L-asparagine (total N = 2.4 or 24 mM). Decomposition of [ring-<sup>14</sup>C]-lignin to <sup>14</sup>CO<sub>2</sub> after 9 days was  $5 \pm 1\%$  and  $14 \pm 4\%$  in the high- and low-N cultures, respectively. After 15 days the conversions were  $7 \pm 2\%$  and  $27 \pm 4\%$ . (Cultures initially contained 56 mM glucose,  $3 \times 10^4$  dpm of <sup>14</sup>C-lignin, and were grown without agitation, under 100% O<sub>2</sub>.)

Experiments demonstrated that the strong influence of nutrient-N concentration was the same with cellulose and with xylose as with glucose as growth substrate.

Examination of the vitamin supplement requirements of *P. chrysosporium* showed that only thiamine is necessary for growth and lignin metabolism. Thiamine alone was as good as the complete vitamin mixture for lignin degradation, but the complete mixture was somewhat better for growth. Mycelial dry weights after 7 days, measured after the third serial transfer in homologous media, were  $9.1 \pm 0.9$  mg,  $19.6 \pm 0.5$  mg, and  $24.0 \pm 2.7$  mg for medium containing no vitamin addition, thiamine only, and complete vitamins, respectively. Decomposition of [ring-<sup>14</sup>C]-lignin during 15 days following the fourth serial transfer was  $< 1\%$ ,  $50 \pm 4\%$ , and  $49 \pm 3\%$ , respectively.

Experiments were performed in quadruplicate; cultures initially contained  $4 \times 10^4$  dpm of [ring-<sup>14</sup>C]-lignin, NH<sub>4</sub>NO<sub>3</sub> + L-asparagine (both 0.6 mM) as N-source, 56 mM glucose, and were grown under 100% O<sub>2</sub> without agitation.

Phthalate buffer was used in most experiments reported here, despite the fact that its buffering capacity (pK<sub>a</sub> = 5.5) at the optimum pH for lignin biodegradation ( $\approx$  pH 4.5) is not as great as other buffers such as acetate, succinate, tartrate, aconitate, and citrate, all of which have pK<sub>a</sub>'s between 4.2 and 4.8. Acetate could not

be used because it is toxic to *P. chrysosporium*, even at 0.01 M, and succinate was used as a carbon source. The others are not used as carbon source, nor are they toxic at 0.05 M or less. However, comparison of tartrate, aconitate, and phthalate (all at: pH 4.5, 0.05 M) revealed that best lignin decomposition occurred with phthalate buffer. Conversions of [ring-<sup>14</sup>C]-lignin to <sup>14</sup>CO<sub>2</sub> after 16 days was  $34 \pm 6\%$ ,  $18 \pm 2\%$ , and  $25 \pm 3\%$  in media buffered with aconitate, phthalate, and tartrate, respectively. (Experiments were performed in triplicate: cultures initially contained  $3 \times 10^4$  dpm of [ring-<sup>14</sup>C]-lignin, basal medium, NH<sub>4</sub>NO<sub>3</sub> + L-asparagine (both 0.6 mM) as N source, 0.01 M buffer at pH 4.5, and 56 mM glucose. They were grown without agitation, under 100% O<sub>2</sub>.)

The effects on lignin decomposition of the various culture parameters examined here were found to be the same in experiments conducted with side chain-, ring-, and methoxyl-labeled lignin. However, in all experiments, methoxyl-C was converted to CO<sub>2</sub> faster and to a greater extent than ring-C and side chain-C, which were similar (e.g., Table 3).

Studies not reported here, with *Coriolus versicolor*, a white-rot fungus taxonomically distinct from *P. chrysosporium*, indicated very similar effects of the above parameters on lignin metabolism.

## Discussion

These data demonstrate that unique culture parameters are critical for lignin decomposition by the white-rot fungus *Phanerochaete chrysosporium*. Limited studies with *C. versicolor* suggest that the findings for *P. chrysosporium* might be applicable to white-rot fungi in general.

Lignin metabolism by *P. chrysosporium*, even under the best conditions described here, is a relatively slow process. This is clearly evidenced by a comparison of the rates (mg/day) of glucose depletion and lignin depletion

The data presented here extend the range (Kirk et al., 1976) of growth substrates that support lignin

metabolism. The requirement for a growth substrate is a surprising one because lignin potentially is one of the most abundant sources of microbial carbon and energy. The basis for the requirement of a growth substrate is not known, but speculations include: (1) The energy recovered in the metabolism of lignin is simply too little to support growth; and (2) the level of the ligninolytic activity is too low to support growth. The relationship between ligninolytic activity, growth, and specific metabolic enzyme activities needs further investigation.

The lack of lignin decomposition at a low O<sub>2</sub> concentration (5%), at which growth still occurred (Fig. 1A), suggests that the lignin-decomposing system has a high O<sub>2</sub> requirement. A virtually complete lack of attack on the lignin polymer was indicated by the gel chromatography results. Previous studies, in which polymeric lignins heavily degraded by *C. versicolor* and *Polyporus anceps* Peck were characterized chemically and spectroscopically (Kirk and Chang, 1975), indicated that the degradative reactions are largely oxidative, and that cleavages of ether linkages and aromatic nuclei occur in the polymeric lignin. This led to speculation that extracellular oxygenases might play a central role. Perhaps ligninolytic oxygenases have a relatively low affinity for O<sub>2</sub>, and do not compete effectively with other O<sub>2</sub>-requiring enzymes.

Whatever the specific site of the O<sub>2</sub> effect, agitation—which is generally used to increase the rate of gas exchange between the atmosphere and culture media—was not beneficial. In fact, an initial period with no agitation was needed to avoid severe inhibition of lignin decomposition.

In both agitated and non-agitated cultures, a substantial portion of the initially suspended lignin became associated with the hyphae during the first few days of incubation (approximately half by day 9). Binding of lignin to fungal mycelia is a recognized phenomenon (Gottlieb and Pelczar, 1951; Tono et al., 1968; Konishi and Inoue, 1971). It is plausible that in the continuously shaken cultures most of the lignin bound by the hyphae became entrapped in the fungal pellets as they were formed. The O<sub>2</sub> concentration in the pellets might have been so low (Phillips, 1966) that lignin decomposition was prevented on all but the outer surfaces. This could account for the low rate of degradation. This explanation is supported by the fact that pelleted cultures, when removed from the shaker, formed mats and then decomposed the lignin at a rate and extent proportional to the amount of lignin not already associated with the pellets. Culture agitation gave different results under 100% O<sub>2</sub> than under air. Lignin decomposition under 100% O<sub>2</sub> was inhibited by agitation whether or not an initial period without agitation was allowed. The basis for this is not known and was not further examined.

A marked influence of culture pH on lignin decomposition was noted. Control of pH was problematic at high concentrations of nutrient nitrogen and when salts of carboxylic acids served as growth substrate. The results with the variety of culture media examined here indicated that the strong effect of pH was not related to the amount of growth (e.g., Table 1, Fig. 4).

The basis for the adverse effect of "high" nitrogen concentrations on lignin metabolism is not known, but three possibilities can be considered: (1) High N promotes rapid depletion of the growth substrate known to be necessary (Kirk et al., 1976) for lignin metabolism. (2) Nitrogen metabolism competes with lignin metabolism through requirements for the same cofactor(s). (3) Nitrogen regulates the synthesis of one or more components of the lignin-degrading system. The bikaverin synthetase system in *Gibberella fujikuroi* is not synthesized until nutrient nitrogen becomes limiting (Bu'Lock et al., 1974), and thus provides an example of this third possible basis for the nitrogen effect.

These various experiments demonstrate that lignin metabolism by *P. chrysosporium* occurs in a simple, defined medium, but that control of certain culture parameters is essential for optimum ligninolytic activity. Understanding the physiological bases of these unique culture requirements will facilitate investigation of the ligninolytic enzyme system—which is at present essentially undescribed—and will be of value for the potential exploitation of lignin-decomposing fungi and their enzymes.

*Acknowledgments.* This research was supported in part by NSF grants GB41861 and PCM76-11144.

## References

- Bu'Lock, J. D., Detroy, R. W., Hostalek, Z., Munum-Al-Shakarchi, A.: Regulation of secondary biosynthesis in *Gibberella fujikuroi*. *Trans. Brit. Mycol. Soc.* **62**, 377–389 (1974)
- Connors, W. J., Lorenz, L. F., Kirk, T. K.: Chromatographic separation of lignin models by molecular weight using Sephadex LH-20. *Holzforchung* **32** (in press, 1978)
- Gottlieb, S., Pelczar, M. J., Jr.: Microbiological aspects of lignin degradation. *Bacteriol. Rev.* **15**, 55–76 (1951)
- Hackett, W. F., Connors, W. J., Kirk, T. K., Zeikus, J. G.: Microbial decomposition of synthetic <sup>14</sup>C-labeled lignins in a variety of natural materials. *Appl. Environ. Microbiol.* **33**, 43–51 (1977)
- Kirk, T. K., Chang, H.-m.: Decomposition of lignin by white-rot fungi. II. Characterization of heavily degraded lignins from decayed spruce. *Holzforchung* **29**, 56–64 (1975)
- Kirk, T. K., Connors, W. J., Bleam, R. D., Hackett, W. F., Zeikus, J. G.: Preparation and microbial decomposition of synthetic [<sup>14</sup>C]-lignins. *Proc. Nat. Acad. Sci. (Wash.)* **72**, 2515–2519 (1975)
- Kirk, T. K., Connors, W. J., Zeikus, J. G.: Requirement for a growth substrate during lignin decomposition by two wood-rotting fungi. *Appl. Environ. Microbiol.* **32**, 192–194 (1976)

- Konishi, K., Inoue, Y.: Decomposition of lignin by *Coriolus versicolor*. III. Mode of action of laccase-type enzyme on lignin. J. Jap. Wood Res. Soc. **17**, 255–262(1971)
- Lundquist, K., Kirk, T. K., Connors, W. J.: Fungal degradation of kraft lignin and lignin sulfonates prepared from synthetic <sup>14</sup>C-lignin. Arch. Microbiol. **112**, 291–296(1977)
- Nelson, N. : A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. **153**, 375–380(1944)
- Phillips, D. H.: Oxygen transfer into mycelial pellets. Biotech. Bioeng. **8**, 456 - 460 (1966)
- Saeman, J. F., Moore, W. E., Mitchell, R. L., Millett, M. A.: Techniques for the determination of pulp constituents by quantitative paper chromatography. Tappi **37**, 336 – 343 (1954)
- Tono, T., Tani, Y., Ono, K.: Microbial treatment of agricultural wastes. I. Adsorption of lignin and clarification of lignin-containing liquor by moulds. J. Ferment. Technol. **46**, 569 - 576 (1968)
- Vogel, A. I.: A textbook of practical organic chemistry. New York: Wiley 1956

Received January 13, 1978