

Influence of Molecular Size and Ligninase Pretreatment on Degradation of Lignins by *Xanthomonas* sp. Strain 99

HARTMUT W. KERN¹ AND T. KENT KIRK^{2*}

Institut für Biotechnologie, Kernforschungsanlage Jülich, D-5170 Jülich, Federal Republic of Germany,¹ and Forest Products Laboratory, Forest Service, U.S. Department of Agriculture, Madison, Wisconsin 53705-2398²

Received 16 March 1987/Accepted 22 June 1987

The purpose of this study was to examine the relationship between the molecular size of lignin in several preparations and extent of degradation (mineralization) by *Xanthomonas* sp. strain 99. The influence of ligninase pretreatment was also examined. Five synthetic lignins and one ¹⁴C-methylated spruce lignin were used. The extent of mineralization to ¹⁴CO₂ was greatest for the samples containing the most low-molecular-weight material, and the low-molecular-weight portions were preferentially (or perhaps solely) degraded. Pretreatment of the five synthetic lignins with crude ligninase increased their molecular size and decreased their degradability by the xanthomonad. Pretreatment of the methylated spruce lignin with crude ligninase caused both polymerization and depolymerization but resulted in a net decrease in bacterial degradability. Our results suggest that the xanthomonad can degrade lignins only up to a molecular weight of 600 to 1,000.

The ability of bacteria from several taxa to degrade lignin model compounds, chemically depolymerized lignins, and at least to some extent, natural and synthetic lignins, is well established (1, 3, 4, 9, 13, 17-19; J. Buswell and E. Odier, in G. G. Stewart and I. Russell, ed., *Critical Reviews in Biotechnology*, in press). Their ability to degrade (mineralize) high-molecular-weight lignin, i.e., the lignin backbone, has not been rigorously established however (17; Buswell and Odier, in press). Thus, although it seems apparent that bacteria participate in lignin degradation by mineralizing low-molecular-weight fragments present in lignocellulose samples, released by abiotic means, or by fungal degradation, their role as primary lignin degraders is not clear. One of the most active bacteria in degrading synthetic lignins is *Xanthomonas* sp. strain 99 isolated from soil (13, 14).

Our aim here was to examine the relationship between the molecular size of lignin in several ¹⁴C-lignin preparations and degradation by this xanthomonad. We characterized the lignins by molecular size and molecular size distribution, then incubated them with the bacterium, and measured the released ¹⁴CO₂; with selected lignins, we also measured the cell-associated ¹⁴C and the changes in molecular size distribution. We also examined the question whether pretreating the lignin preparations with ligninase enhances their degradability by *Xanthomonas* sp. strain 99. Ligninase (lignin peroxidase), recently discovered in the basidiomycete *Phanerochaete chrysosporium*, has been reported to depolymerize lignin (7, 20). The properties and mode of action of ligninase have been studied in several laboratories, and the findings were reviewed recently (17; Buswell and Odier, in press).

MATERIALS AND METHODS

Abbreviations. DHP, dehydrogenative polymerizate (synthetic lignin); DMF, *N,N*-dimethylformamide; GPC, gel permeation chromatography; HPLC, high-performance liquid chromatography; THF, tetrahydrofuran.

Organism and culture conditions. The bacterial strain *Xanthomonas* sp. strain 99 was grown under conditions

previously described (13, 14). Briefly, the bacteria were cultivated in a complete medium (glucose-yeast extract) in shaking cultures under air at 30°C. After centrifugation and several washings, the cells were suspended in a mineral medium (13) and supplied with ¹⁴C-labeled lignin preparations which were introduced as DMF solutions. Incubations at 30°C were in 200-ml baffled Erlenmeyer flasks, each containing 22 ml of the suspended bacteria (ca. 30 × 10⁹ bacteria per flask). The flasks were closed with rubber stoppers carrying small glass cups containing 0.5 ml of 10% NaOH (wt/vol) to trap ¹⁴CO₂ released during incubation (9) and were shaken at 100 rpm (2.5-cm diameter). In some experiments (as noted), the supply of the medium with trace elements was incomplete and in others the medium was further supplemented with Tween 80 at a final concentration of 0.005% (vol/vol).

Origins and characterizations of the ¹⁴C-labeled lignin preparations. The origins and specific activities of the six lignins are shown in Table 1. Also given are the molecular weights of the preparations as determined by HPLC or GPC. For HPLC determination only, portions of the dry samples were acetylated with pyridine-acetic anhydride (1:1 [vol/vol]) for 24 h at 50°C, precipitated in distilled water, collected on 0.22-μm-pore-size membrane filters, and dried. These acetylated samples, dissolved in THF, were analyzed by HPLC with polystyrene columns (5, 13). Because of the high specific radioactivity of lignin preparation VI (Table 1), acetylation and subsequent procedures were carried out in the presence of an unlabeled DHP as a carrier. In all cases, radioactivity in the effluents from the columns was determined by liquid scintillation spectrometry (model 4530; Packard Instrument Co.) and correlated with the respective molecular weights deduced from a calibration curve constructed with polystyrene standards. The molecular weight of lignin V was estimated by GPC on a Sephadex LH-20 column (2); a sufficient quantity of the sample for the other procedure was not available.

Fractionation of ¹⁴C-labeled products from cultures. The ¹⁴CO₂ evolved during incubation with the bacteria, trapped with NaOH, was measured every second day (9). After termination of the experiments (14 days), the uninoculated controls, as well as the inoculated samples, were mixed with

* Corresponding author.

TABLE 1. Characteristics of the ^{14}C -labeled lignin preparations and their degradation to $^{14}\text{CO}_2$ by *Xanthomonas* sp. strain 99

Lignin prepn	Lignin type (sp act (dpm · mg ⁻¹))	Reference	Mol wt ^a	% Release of $^{14}\text{CO}_2$ ^b		
				Incomplete	Standard	Optimal
I	Guaiacyl [^{14}C -ring]DHP (1.27×10^5)	13	1,850 (1,100)	9.0	20.7	23.7
II	Guaiacyl [^{14}C -ring]DHP (9.2×10^5)	15	2,600 (1,050)	4.2	ND	5.3
III	Guaiacyl [β - ^{14}C]DHP (1.3×10^5)	13	1,100 (850)	ND	26.0	28.4
IV	Guaiacyl [β - ^{14}C]DHP (2.15×10^5)	6	5,500 (2,400)	1.0	1.2	1.3
V	Syringyl/guaiacyl [β - ^{14}C]DHP (13.4×10^4)	6	900	13.0	ND	25.1
VI	^{14}C -methylated spruce lignin (1.9×10^8)	20	1,200	8.5	ND	14.7

^a Values given are the weight average molecular weights; numerical averages are given in parentheses (5). The molecular weight of lignin V was estimated from GPC on a Sephadex LH-20 column (2).

^b Values for different culture media: one with an incomplete supply of trace elements, standard (13), and standard plus added Tween 80 (0.005% [wt/vol]) (optimal). ND, Not determined. Incubations were for 14 days.

equal volumes of dioxane and stored for 24 h at room temperature. After this, the suspensions were centrifuged ($10,000 \times g$) and the residual bacteria were thoroughly washed with dioxane and dried. The ^{14}C content of the bacteria was determined by combustion (Tri-Carb sample oxidizer 306; Packard Instrument Co.), followed by liquid scintillation spectrometry. The filtrates of three pooled replicate cultures were quantitatively collected. Aliquots were withdrawn for the determination of ^{14}C . The residual extract was supplied with 1 ml of DMF, and the volume of this extract was reduced in a rotary evaporator to ca. 1 ml. The centrifuged solution was analyzed by GPC on a Sephadex LH-20 column (1.2 by 40 cm), with DMF as the solvent (2) at a flow rate of $0.5 \text{ ml} \cdot \text{min}^{-1}$. Radioactivity in each 0.5-ml fraction was measured by liquid scintillation spectrometry. For better demonstration of changes in the distribution of the molecular sizes, the total radioactivity from each run was adjusted to 1.5×10^5 dpm (2.5 kBq). Radioactivity administered to each culture was at least 5×10^4 dpm (833 Bq).

Preparation of extracellular enzymes from *P. chrysosporium*. Mutant strain SC26 (16) was grown in 1-liter un baffled Erlenmeyer flasks (300 ml of medium) with shaking at 39°C (11). The basal medium BIII (16) was supplemented with 0.1% Tween 80 (wt/vol), 0.4 mM veratryl alcohol, 1.1 mM ammonium tartrate, 1% glucose, and sevenfold-higher concentrations of trace elements (11, 16). Buffer was omitted because the pH remained at 4.5 during the 4-day incubation. For inoculum, cultures were grown in Fernbach flasks as stationary cultures for 3 days, blended, and added to the medium according to Jäger et al. (11). Inoculated cultures were flushed with O_2 , shaken at 39°C and 130 rpm (2.5-cm diameter) for 3 days, reflushed with O_2 , and harvested after one more day. At that time, ligninase activity (16) was $250 \text{ U} \cdot \text{liter}^{-1}$. The filtered fluid was concentrated by ultrafiltration (Amicon YM10), dialyzed against distilled water, and lyophilized (crude ligninase). Purified ligninase H8 (16) was also used in one experiment.

Treatment of lignin preparation with ligninase. Before some incubations with the bacteria, selected lignin preparations were treated with crude ligninase or with ligninase H8 (16). Aliquots of the lignin, dissolved in DMF, were suspended in a 2-ml reaction mixture consisting of sodium tartrate, pH 3 (50 mM), Tween 80 (0.1%), Mn^{2+} (0.1 mM), veratryl alcohol (1 mM), and an enzyme preparation corresponding to 1.5 U (16). Veratryl alcohol was included because it reportedly enhances ligninase oxidation (10). The reaction, started by the addition of $100 \mu\text{l}$ of 8 mM H_2O_2 , was at room temperature for 80 min. During this period, six further additions of $50 \mu\text{l}$ of 8 mM H_2O_2 were made at 10-min intervals. After termination, about 0.5 ml of DMF was added and the volume of the mixture was reduced by vacuum

evaporation. The resulting solution was centrifuged and added to the cultures as described above or was analyzed on a Sephadex LH-20 column.

RESULTS AND DISCUSSION

The six lignin preparations, which included five synthetic lignins (I to V) and one methylated Brauns lignin of spruce (VI), exhibited a range of molecular weights (Table 1) and molecular size distributions (Fig. 1). One of the synthetic lignins (V) was of the angiosperm (guaiacyl/syringyl) type; the other preparations were of the gymnosperm (guaiacyl) type.

All six lignins were incubated with *Xanthomonas* sp. strain 99 for 14 days in a simple medium with or without added trace elements and detergent. The amount of evolved $^{14}\text{CO}_2$ varied from 1.0 to over 28% of added label (Table 1). In all cases, the rate of evolution of $^{14}\text{CO}_2$ had become negligible by 14 days, indicating that the remaining lignin was resistant. Added trace elements and detergent seemed to enhance degradation, but these additions were not tested with all lignins, and conclusions are not definite. Notably,

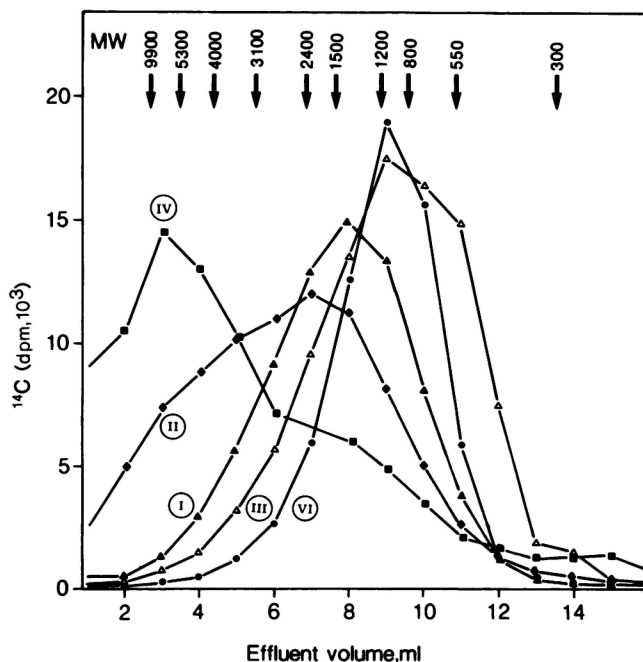


FIG. 1. Molecular size distributions of acetylated lignin preparations I to IV and preparation VI, determined by HPLC and GPC with THF as the solvent (5). Molecular weight (MW) standards are shown at the top.

TABLE 2. Distribution of ^{14}C of lignin preparations before and after pretreatment with crude ligninase^a

Lignin prepn	Pretreatment	% $^{14}\text{CO}_2$	% ^{14}C in aqueous dioxane extracts	% ^{14}C after aqueous dioxane extraction (cell-associated ^{14}C)	% Recovery
I	None	23.7	64.1	12.0	99.8
	Crude ligninase	15.8	67.0	9.5	92.3
III	None	28.4	55.6	14.0	98.0
	Crude ligninase	17.2	64.0	10.6	91.8
VI	None	14.7	75.5	5.9	96.1
	Crude ligninase	8.2	78.9	4.0	91.1

^a The lignin preparations (Table 1) were pretreated with crude ligninase as described in Materials and Methods. Incubation for 14 days with *Xanthomonas* sp. strain 99 was under optimal conditions corresponding to the last column in Table 1. Average values from triplicate assays are presented.

however, there was a clear relationship between the molecular size distribution and the extent of degradation to $^{14}\text{CO}_2$; those lignins containing the greatest proportions of low-molecular-weight material (I, III, and V) were degraded to the greatest extent. Even though we did not determine the molecular size distribution of lignin V by the same technique used with the others, it was clear from LH-20 chromatography that it contained a high proportion of low-molecular-weight material; this was also shown previously (6). Lignin VI was exceptional; although it contained a high proportion of low-molecular-weight material, it was degraded to a lesser extent than lignins I, III, and V were. Lignin VI, however, was also exceptional chemically; not only had it been 4-O-methylated, but the label was in the 4-O-methyl carbon atom. The significance of $^{14}\text{CO}_2$ production from that carbon atom to lignin degradation, therefore, is questionable. This lignin was included for reasons discussed below.

Earlier studies with lignins IV and V (6) showed that their degradation by the lignin-degrading fungus *P. chrysosporium* was faster and more extensive than by *Xanthomonas* sp. strain 99. Over 40% of the ^{14}C in both lignins was converted to $^{14}\text{CO}_2$ in 72 h, and this mineralization had not slowed when the experiment was terminated. That same study showed that the high- and low-molecular-weight portions of lignin IV were degraded at the same rate, in contrast to the findings here with the *Xanthomonas* system.

We analyzed the fates of lignins I, III, and VI in greater detail. This was done by measuring, in addition to $^{14}\text{CO}_2$, the cell-associated ^{14}C (aqueous dioxane insoluble), as well as the aqueous dioxane-soluble ^{14}C and its molecular size distribution, at the end of 14-day incubations with the xanthomonad. The amount of cell-associated ^{14}C in each case was equal to about half of the evolved $^{14}\text{CO}_2$ (Table 2). The remaining ^{14}C , in the aqueous dioxane solutions, was transferred to DMF, and these samples were analyzed by Sephadex LH-20-DMF chromatography (Fig. 2). Results showed clearly that the low-molecular-weight materials in lignins III and VI were preferentially (or solely) degraded. The Sephadex LH-20-DMF system did not resolve lignin I sufficiently to determine whether the low-molecular-weight portion of that sample, too, was preferentially degraded. The chromatographic system did, however, reveal production of small amounts of low-molecular-weight materials from all three lignins, indicating that some depolymerization occurred.

The earlier study with *P. chrysosporium* showed that the fungus rapidly and extensively depolymerized lignin IV and especially lignin V (6). As mentioned above, a study with

lignin IV revealed no preference for the low-molecular-weight portion.

Using lignins I, III, and VI, we examined the influence on bacterial degradation of pretreatment with a crude ligninase preparation from *P. chrysosporium*. The crude ligninase was reported by Glenn et al. (7) to depolymerize a synthetic lignin, and a ligninase isolated by gel electrophoresis was shown in an earlier work to partially depolymerize a high-molecular-weight fraction of lignin VI, which as pointed out, is 4-O-methylated (21). In contrast, Haemmerli et al. (8) recently reported that a purified ligninase actually polymerized three different lignins. Because lignin VI was known to be depolymerized by ligninase, it was included in the study here.

With all three lignins (I, III, and VI), we found decreased bacterial degradation after ligninase pretreatment. $^{14}\text{CO}_2$ production after 14 days was 15, 17, and 8% for these lignins,

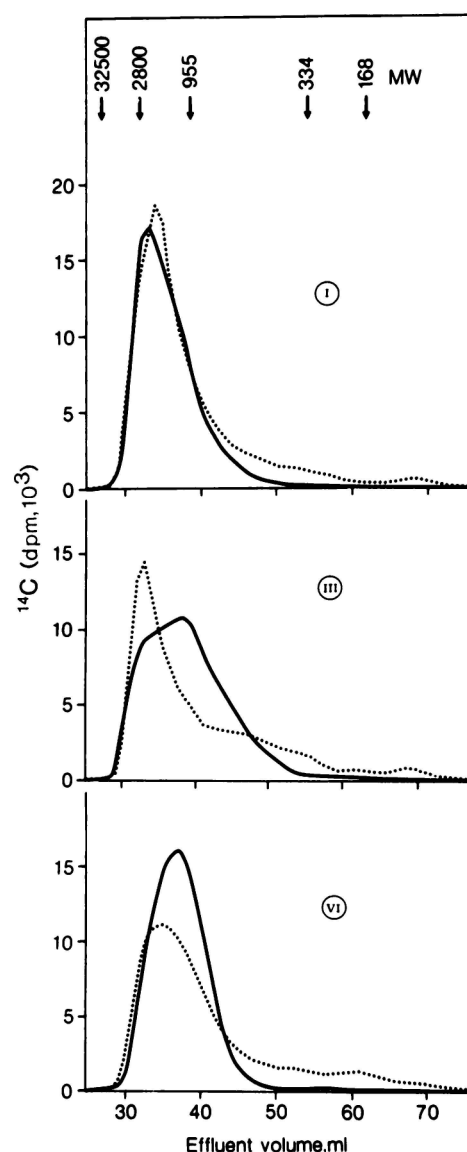


FIG. 2. Molecular size distribution of lignins I, III, and VI incubated 14 days without (—) and with (···) *Xanthomonas* sp. strain 99. Fractionation was with a Sephadex LH-20-DMF column (2). Molecular weight (MW) standards are shown at the top.

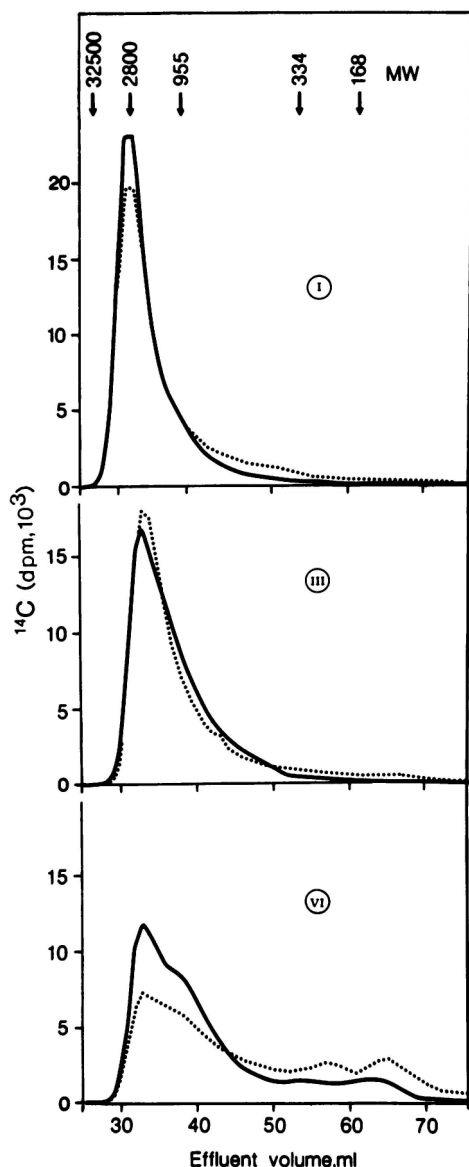


FIG. 3. Molecular size distribution of lignin preparations I, III, and VI pretreated with crude ligninase and then incubated 14 days without (—) or with (···) *Xanthomonas* sp. strain 99. Fractionation was with a Sephadex LH-20-DMF column (2). Molecular weight (MW) standards are shown at the top.

i.e., only about 50 to 60% of the values for the untreated lignins (Table 1).

The explanation for this decreased degradability apparently lies in the fact that the ligninase increased the molecular size of the lignins, in agreement with the results of Haemmerli et al. (8). Sephadex LH-20-DMF chromatography showed clearly that lignins I and III were polymerized by the ligninase treatment; this was most noticeable with lignin III because the chromatographic system resolved this preparation better (cf. Fig. 2 and 3). Lignin VI was partially polymerized, and in agreement with previous work (20), was partially depolymerized (cf. Fig. 2 and 3). The elution profiles of the aqueous dioxane-soluble extracts from cultures with ligninase-treated lignins I and III were essentially the same as the profiles of the extracts from the control

incubations; controls were ligninase treated but incubated without bacteria (Fig. 3). Incubation of ligninase-treated lignin VI resulted in further depolymerization (Fig. 3). The fact that this was not reflected in increased degradation of lignin VI to $^{14}\text{CO}_2$ evidently reflects a resistance of the released methylated structures. This supposition is supported by failure of the xanthomonad to degrade the veratryl alcohol present in the ligninase-treated samples (data not shown). Separate experiments demonstrated that neither the veratryl alcohol nor the crude ligninase affected the degradation of susceptible lignins by the xanthomonad. Using lignin III, we showed in preliminary studies that purified ligninase H8 had the same polymerizing effect on it as the crude ligninase did.

We interpret our results to indicate that *Xanthomonas* sp. strain 99 degrades the low-molecular-weight portions of lignins but probably is unable to depolymerize the high-molecular-weight backbone lignin polymer. This suggests that the cells do not secrete lignin-depolymerizing enzymes as *P. chrysosporium* and other white rot basidiomycetes do. Our results suggest further that the bacterial cells are able to take up lignin molecules of up to molecular weight 600 to 1,000 (perhaps oligomers of 15 units) and then to metabolize them to CO_2 . The same interpretation might be applicable to findings with *Nocardia* sp. (9), and various actinomycetes (3, 4, 18). Jokela et al. (12) showed recently that mixed bacterial cultures are able to metabolize a synthetic tetrameric lignin model ($M_w = 650$). In any event, these results imply the presence of a relatively nonspecific enzyme system within these various bacteria, because steric and structural heterogeneity, even in the low-molecular-weight portions of lignin, is quite large. Further research should focus on the enzymology of degradation of defined structures, model compounds, by selected species such as the *Xanthomonas* sp. studied here.

ACKNOWLEDGMENTS

The excellent technical assistance of M. Mozuch, U.S. Forest Products Laboratory, and of T. Boddecker and M. Bouzgarrou, Kernforschungsanlage, is gratefully acknowledged.

LITERATURE CITED

1. Benner, R., M. A. Moran, and R. E. Hodson. 1986. Biogeochemical cycling of lignocellulosic carbon in marine and freshwater ecosystems: relative contributions of prokaryotes and eucaryotes. *Limnol. Oceanogr.* **31**:89-100.
2. Connors W. J., L. F. Lorenz, and T. K. Kirk. 1978. Chromatographic separation of lignin models using Sephadex LH-20. *Holzforschung* **32**:106-108.
3. Crawford, R. L. 1981. Lignin biodegradation and transformation, p. 154. John Wiley & Sons, Inc., New York.
4. Crawford, R. L., and D. L. Crawford. 1984. Recent advances in studies of the mechanisms of microbial degradation of lignins. *Enzyme Microb. Technol.* **6**:434-442.
5. Faix, O., W. Lange, and G. Besold. 1981. Molecular weight determinations of DHP's from mixture of precursors by steric exclusion chromatography (HPLC). *Holzforschung* **35**:137-140.
6. Faix, O., M. D. Mozuch, and T. K. Kirk. 1985. Degradation of gymnosperm (guaiaicyl) vs. angiosperm (syringyl/guaiaicyl) lignins by *Phanerochaete chrysosporium*. *Holzforschung* **39**:203-208.
7. Glenn, J. K., M. A. Morgan, M. B. Mayfield, M. Kuwahara, and M. H. Gold. 1983. An extracellular H_2O_2 -requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* **114**:1077-1083.
8. Haemmerli, S. D., M. S. A. Leisola, and A. Fiechter. 1986.

- Polymerisation of lignins by ligninases from *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **35**:33-36.
9. Haider, K., J. Trojanowski, and V. Sundman. 1978. Screening for lignin degrading bacteria by means of ¹⁴C-labeled lignins. Arch. Microbiol. **119**:103-106.
 10. Harvey, P. J., H. E. Schoemaker, and J. M. Palmer. 1986. Veratryl alcohol as a mediator and the role of radical cations in lignin biodegradation by *Phanerochaete chrysosporium*. FEBS Lett. **195**:242-246.
 11. Jäger, A., S. Croan, and T. K. Kirk. 1985. Production of ligninases and degradation of lignin in agitated submerged cultures of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **50**:1274-1278.
 12. Jokela, J., J. Pellinen, M. Salkinoja-Salonen, and G. Brunow. 1985. Biodegradation of two tetrameric lignin model compounds by a mixed bacterial culture. Appl. Microbiol. Biotechnol. **23**:38-46.
 13. Kern, H. W. 1984. Bacterial degradation of dehydropolymers of coniferyl alcohol. Arch. Microbiol. **138**:18-25.
 14. Kern, H. W., L. E. Webb, and L. Eggeling. 1984. Characterization of a ligninolytic bacterial isolate: taxonomic relatedness and oxidation of some lignin related compounds. Syst. Appl. Microbiol. **5**:433-447.
 15. Keyser, P., T. K. Kirk, and J. G. Zeikus. 1978. Ligninolytic enzyme system of *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. J. Bacteriol. **135**:790-797.
 16. Kirk, T. K., S. Croan, M. Tien, K. E. Murtagh, and R. L. Farrell. 1986. Production of multiple ligninases by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of a mutant strain. Enzyme Microb. Technol. **8**:27-32.
 17. Kirk, T. K., and R. L. Farrell. 1987. Enzymatic combustion: the microbial degradation of lignin. Annu. Rev. Microbiol. **41**:465-505.
 18. McCarthy, A. L., A. Paterson, and P. Broda. 1986. Lignin solubilisation by *Thermomonospora mesophila*. Appl. Microbiol. Biotechnol. **24**:347-352.
 19. Nilsson, T., and E. Holt. 1983. Bacterial attack occurring in the S₂ layer of wood fibres. Holzforschung **37**:107-108.
 20. Tien, M., and T. K. Kirk. 1983. Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. Science **221**:661-663.