

## Free hydroxyl radical is not involved in an important reaction of lignin degradation by *Phanerochaete chrysosporium* Burds

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Hydroxyl radical ( $\text{HO}\cdot$ ) has been implicated in the degradation of lignin by *Phanerochaete chrysosporium*. This study assessed the possible involvement of  $\text{HO}\cdot$  in degradation of lignin substructural models by intact cultures and by an extracellular ligninase isolated from the cultures. Two non-phenolic lignin model compounds [aryl- $\text{C}(\alpha)\text{HOH}-\text{C}(\beta)\text{HR}-\text{C}(\gamma)\text{H}_2\text{OH}$ , in which R = aryl ( $\beta$ -1) or R = *O*-aryl ( $\beta$ -*O*-4)] were degraded by cultures, by the purified ligninase, and by Fenton's reagent ( $\text{H}_2\text{O}_2 + \text{Fe}^{2+}$ ), which generates  $\text{HO}\cdot$ . The ligninase and the cultures formed similar products, derived via an initial cleavage between  $\text{C}(\alpha)$  and  $\text{C}(\beta)$  (known to be an important biodegradative reaction), indicating that the ligninase is responsible for model degradation in cultures. Products from the Fenton degradation were mainly polar phenolics that exhibited little similarity to those from the biological systems. Mass-spectral analysis, however, revealed traces of the same products in the Fenton reaction as seen in the biological reactions; even so, an  $^{18}\text{O}_2$ -incorporation study showed that the mechanism of formation differed. E.s.r. spectroscopy with a spin-trapping agent readily detected  $\text{HO}\cdot$  in the Fenton system, but indicated that no  $\text{HO}\cdot$  is formed during ligninase catalysis. We conclude, therefore that  $\text{HO}\cdot$  is not involved in fungal  $\text{C}(\alpha)-\text{C}(\beta)$  cleavage in the  $\beta$ -1 and  $\beta$ -*O*-4 models and, by extension, in the same reaction in lignin.

Hall (1980) speculated that diffusible reactive species (activated forms of oxygen), rather than enzymes directly, are responsible for the initial degradation of lignin by white-rot wood-decay fungi. Two years later Forney *et al.* (1982) discovered that ligninolytic activity ( $^{14}\text{C}$ -lignin  $\rightarrow$   $^{14}\text{CO}_2$ ) is correlated with production of  $\text{H}_2\text{O}_2$  by cell extracts of *Phanerochaete chrysosporium*. The involvement of  $\text{H}_2\text{O}_2$  in ligninolytic activity was also supported by the work of Kutsuki & Gold (1982) and of Faison & Kirk (1983), who showed that added catalase inhibits lignin degradation. Forney *et al.* (1982) and Kutsuki & Gold (1982) concluded that hydroxyl radical ( $\text{HO}\cdot$ ), generated by the one-electron reduction of  $\text{H}_2\text{O}_2$  by metal ions (Fenton's reagent), is involved in lignin degradation by this organism. This conclusion was based on the observations that ligninolytic cultures oxidize 4-methylthio-2-oxobutyric acid and methional (Kutsuki & Gold, 1982), and that  $\text{HO}\cdot$  scavengers inhibit these oxidations as well as the oxidation of lignin itself.

The involvement of  $\text{HO}\cdot$  in lignin degradation is further supported by the work of Bes *et al.* (1983).

These workers demonstrated that Fenton's reagent readily oxidizes lignin and that  $\text{HO}\cdot$  scavengers such as mannitol and benzoate inhibit degradation of lignin in ligninolytic cultures of *P. chrysosporium*. Further evidence in support of  $\text{HO}\cdot$  involvement in lignin degradation was provided by Kirk & Nakatsubo (1983), who showed that an  $\text{HO}\cdot$ -generating system (glucose/glucose oxidase/ $\text{Fe}^{2+}$ ) partially mimics ligninolytic cultures of *P. chrysosporium* in degrading a dimeric lignin model compound.

Since the above investigations, an extracellular enzyme has been discovered in *P. chrysosporium* that catalyses lignin oxidation (Tien & Kirk, 1983); the enzyme has now been purified and partially characterized (Tien & Kirk, 1984). It catalyses several different oxidative reactions, exhibits no stereoselectivity in the cleavage reactions, is an iron (haem)-containing protein, and has an absolute requirement for  $\text{H}_2\text{O}_2$ . Glenn *et al.* (1983) and Kuwahara *et al.* (1984) have reported an enzyme that is apparently the same. The characteristics of the enzyme, together with a report by Evans *et al.* (1984), prompted us to

examine the possibility that HO· plays a role in the enzyme's action.

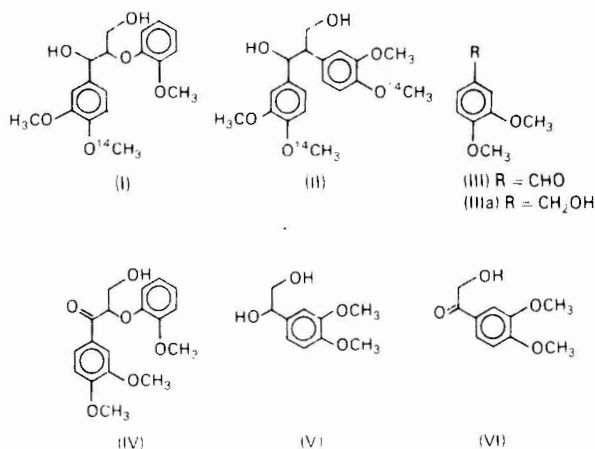
Evans *et al.* (1984) isolated an extracellular haemoprotein from a ligninolytic fungus, *Corioliolus versicolor*. They found that in the presence of H<sub>2</sub>O<sub>2</sub> this haemoprotein oxidizes methional and that this activity is inhibited by HO· scavengers. Although these workers stated that it is unclear whether the HO· is generated by the catalytic action of the enzyme (or by Fe released from destruction of the haem), they did propose that the radical (HO·) is involved in lignin degradation.

To assess this possibility we performed the following experiments: (1) compared the enzymic degradation of two lignin model compounds with degradation by chemically generated HO· (Fenton's reagent); (2) determined whether HO· can be detected during enzymic cleavage; (3) determined whether the purified ligninase and intact cultures degrade lignin models in the same way (i.e. whether the ligninase is the actual agent *in vivo*). We used 'dimeric' model compounds (I) and (II), which represent the arylglycerol β-aryl ether (β-O-4) and 1,2-diarylpropane-1,3-diol (β-1) substructures respectively. Together, the β-O-4 and β-1 substructures represent over 60% of the interunit linkages in lignin (Adler, 1977). The models were labelled with <sup>14</sup>C to facilitate quantification.

## Materials and methods

### Origin of models and other chemicals

Model compound (I) (27mCi/mmol) was prepared from unlabelled 4-hydroxy-3-methoxy-α-(2-methoxyphenoxy)-β-hydroxypropiophenone (Lan-ducci *et al.*, 1981) by methylation with <sup>14</sup>CH<sub>3</sub>I (Kirk & Nakatsubo, 1983), followed by reduction with NaBH<sub>4</sub>. Compound (IV) was prepared by methylation with CH<sub>3</sub>I of the propiophenone derivative used to prepare model compound (I).



Compounds (III) and (IIIa) were from Aldrich (Milwaukee, WI, U.S.A.). Model compound (II) (6mCi/mmol) and unlabelled compounds (V) and (VI) were synthesized by methods used previously to prepare close analogues (Kirk & Nakatsubo, 1983). Unlabelled model compounds (I) and (II) were also prepared, and were used in two experiments to dilute the labelled model compounds to equal specific radioactivities. <sup>18</sup>O<sub>2</sub> (98%) was from KOR Isotopes (Cambridge, MA, U.S.A.). 5,5-Dimethyl-1-pyrroline *N*-oxide was obtained from Aldrich and vacuum-distilled. All other chemicals were reagent grade, and were used without further purification.

### Degradation of model compounds (I) and (II) by intact cultures

Cultures (10ml) in 125ml Erlenmeyer flasks were set up as described previously (Kirk & Nakatsubo, 1983), except that strain BKM-1767 (A.T.C.C. 24725) was used. The cultures were maintained under O<sub>2</sub> at 39°C. Model compound (I) or (II) (0.45 μmol, 1 × 10<sup>5</sup> d.p.m. in 0.5ml) was added to cultures and incubated under O<sub>2</sub> at 39°C for 16h (model compound I) and 4h (model compound II). <sup>14</sup>O<sub>2</sub> was determined quantitatively (Kirk *et al.*, 1979, and the pooled triplicate cultures were extracted with 1 vol. of chloroform/acetone (1 : 1, v/v) three times, followed by 1 vol. of chloroform (Nakatsubo *et al.*, 1982). Radioactivity in the aqueous and organic layers was determined (Chua *et al.*, 1983), and the aqueous fraction was discarded. Principal products from the model compounds had been identified previously; they and residual compounds (I) and (II) were isolated from the organic fraction by t.l.c. (Kirk & Nakatsubo, 1983), and quantified by scintillation counting (Fenn & Kirk, 1984). Phenolic products were made visible on replicate t.l.c. plates by spraying with *p*-nitrobenzenediazonium tetrafluoroborate (Kodak, Rochester, NY, U.S.A.).

### Degradation of model compounds (I) and (II) by the ligninase

The ligninase was purified from culture filtrates of 6-day-old cultures of *P. chrysosporium* as described previously (Tien & Kirk, 1984). Reaction mixtures, open to the air, contained enzyme (16 μg), H<sub>2</sub>O<sub>2</sub> (0.3 μmol), Tween 80 (0.1%) and substrate [model compound (I), 2.0 × 10<sup>5</sup> d.p.m., 3.4 nmol; or model compound (II), 1.7 × 10<sup>5</sup> d.p.m., 13 nmol], in a final volume of 1 ml of 0.1 M-sodium tartrate buffer, pH 3.0, at 39°C. Addition of H<sub>2</sub>O<sub>2</sub> started the reactions. Reactions were terminated by adding acetone/chloroform. Extraction and work-up was done as with cultures.

*Degradation of model compounds (I) and (II) by Fenton's reagent*

HO· was generated by the Fenton reagent (Fe<sup>2+</sup> + H<sub>2</sub>O<sub>2</sub>) (Walling, 1975). Model compound (I) or (II) (8.8 μmol, 5 x 10<sup>4</sup> d.p.m.; in 17 μl of *NN*-dimethylformamide) was added to 5ml of an aqueous solution of FeCl<sub>2</sub> (4.6 mM). With the vessel capped and the solution stirring at room temperature, 570 μl of 0.3% H<sub>2</sub>O<sub>2</sub> solution (0.5 mmol) was added over 1 min. The reaction mixture was stirred 20min; <sup>14</sup>CO<sub>2</sub> was determined quantitatively and other products were examined as in intact cultures. Reaction of model compound (II) under <sup>18</sup>O<sub>2</sub> was done after purging the reaction solution with N<sub>2</sub> and then <sup>18</sup>O<sub>2</sub> before the addition of H<sub>2</sub>O<sub>2</sub>. Products were analysed as the trimethylsilyl derivatives by gas chromatography/mass spectrometry (Tien & Kirk, 1984).

*E.s.r. spectroscopy*

E.s.r. measurements were performed with a Varian E-109 spectrometer operating at X-band (9.5GHz) employing 100kHz field modulation. Reaction mixtures were in quartz flat cells.

**Results**

*Degradation of model compounds by the ligninase and Fenton's reagent*

*β-O-4 Model compound (I)*. The ligninase and the Fenton system both degraded model compound (I) in part to products (III) and (IV), but the yields differed markedly in the two reactions (Table 1). Products (III) and (IV) accounted for over 70% of the original <sup>14</sup>C in the enzyme reaction, but for much less than 1% in the chemical reaction. Unidentified water-soluble compounds were the

major products from the Fenton degradation, accounting for over 70% of the <sup>14</sup>C of the model compound that had reacted; the ligninase produced 7.4% water-soluble compounds. The Fenton reagent produced 4.5% and the ligninase 0% <sup>14</sup>CO<sub>2</sub> from the model. Unidentified organic-soluble compounds accounted for about 20% of the <sup>14</sup>C in both systems, but these products differed in the two reactions. As indicated by t.l.c. examination, those from the Fenton degradation were primarily polar phenolics, whereas no phenolic products were detected among the enzyme products.

*b-1 Model compound (II)*. Like model compound (I), model compound (II) was degraded in part to the same products (III, V and VI) by the ligninase and the Fenton system. Again, product yields differed substantially (Table 2). Products (III), (V) and (VI) together accounted for over 90% of the <sup>14</sup>C of the model compound (II) that had reacted in the enzyme reaction, but for less than 10% in the Fenton reaction. The Fenton system converted over 90% of the reacted model into unidentified water-soluble and organic-soluble compounds. These same fractions contained 7.5% of the <sup>14</sup>C from the enzyme reactions. Less <sup>14</sup>CO<sub>2</sub> was produced from model compound (II) than from model compound (I) by the Fenton reagent; again, none was formed in the enzyme reaction.

Further experiments compared the Fenton and enzyme reactions as to the origin of the oxygen atom in the new (benzylic) hydroxy group in product (V). By using <sup>18</sup>O<sub>2</sub>, we have already shown that this oxygen atom comes from O<sub>2</sub> and not from H<sub>2</sub>O<sub>2</sub> in the ligninase reaction. Here we examined the product (V) that was formed in the Fenton degradation conducted under <sup>18</sup>O<sub>2</sub> in the presence of H<sub>2</sub><sup>16</sup>O<sub>2</sub>. Mass spectrometry showed that 30% of the new hydroxy-group oxygen was derived from

Table 1. Products formed from *β-O-4* model compound (I) on degradation by purified ligninase, by Fenton reagent and by intact cultures of *P. chrysosporium*

For experimental details see the text. Yield was calculated, on a <sup>14</sup>C basis, from the amount of model compound (I) that had reacted: Fenton reagent, 83.1%; cultures, 97%; ligninase, 92%.

Products	Yield (% of total products)		
	Ligninase	Fenton reagent	Cultures
Identified organic-soluble compounds			
(III)	55.3	<0.01	—
(IIIa)*	—	—	55.5
(IV)	15.7	<0.1	7.6
Unidentified organic-soluble compounds	21.3	23.5	16.4
Unidentified water-soluble compounds†	7.4	70.5	13.0‡
CO <sub>2</sub>	0	4.5	7.5

\* Product (III) is rapidly reduced to product (IIIa) in cultures.

† <sup>14</sup>C not extracted into organic solvent.

‡ Includes <sup>14</sup>C associated with mycelium.

Table 2. Products formed from  $\beta$ -1 model compound (II) on degradation by purified ligninase, by Fenton reagent and by intact cultures of *P. chrysosporium*

For experimental details see the text. Yield was calculated, on a  $^{14}\text{C}$  basis, from the amount of model compound (II) that had reacted: Fenton reagent, 60.6%; cultures, 99%; ligninase, 98%.

Products	Yield (% of total products)		
	Ligninase	Fenton reagent	Cultures
Identified organic-soluble compounds			
(III)	68.2	4	—
(IIIa)*	—	—	63.0
(V)	8.2	0.5	6.5
(VI)	16.1	4.4	6.3
Unidentified organic-soluble compounds	5.5	49.2	9.2
Unidentified water-soluble compounds†	2.0	39.6	11.0‡
$\text{CO}_2$	0.0	1.8	3.0

\* Product (III) is rapidly reduced to product (IIIa) in cultures.

†  $^{14}\text{C}$  not extracted into organic solvent.

‡ Includes  $^{14}\text{C}$  associated with mycelium.

$\text{O}_2$  (Fig. 1); in contrast, over 95% incorporation was observed with the enzyme (Tien & Kirk, 1984).

#### E.s.r. detection of $\text{HO}\cdot$

The 5,5-dimethyl-1-pyrroline *N*-oxide- $\text{HO}\cdot$  adduct formed by the reaction of 5,5-dimethyl-1-pyrroline *N*-oxide with  $\text{HO}\cdot$  produced by the Fenton reagent was detected by e.s.r. spectroscopy. The *g*-value of 2.006, symmetrical quartet with a 1:2:2:1 signal intensity, and the hyperfine splitting constant of  $A^{\text{N}} = A_2^{\text{H}} = 1.49\text{mT}$  confirmed the generation of  $\text{HO}\cdot$  (Finkelstein *et al.*, 1980) (Fig. 2a). Addition of ethanol to the incubation mixture resulted in trapping of the ethanol radical (not shown), diagnostic of  $\text{HO}\cdot$  formation (Finkelstein *et al.*, 1980).

No  $\text{HO}\cdot$  was detected in incubations containing ligninase and  $\text{H}_2\text{O}_2$  (Fig. 26). The addition of model compound (I) or (II) to the reaction mixture did not result in detection of  $\text{HO}\cdot$ .

#### Degradation of model compounds by ligninase and cultures

Degradation of model compounds (I) and (II) by intact cultures was very similar to degradation by the ligninase. The cultures reduced product aldehyde (III) to the corresponding alcohol (IIIa), but the yields of products (III) and (IIIa) were quite similar. As expected, the intact cultures degraded the products further than the ligninase, as indicated by the formation of  $^{14}\text{CO}_2$  and of more unidentified water-soluble compounds. Nevertheless, the major identified products were the same. Phenolic products were not detected among the

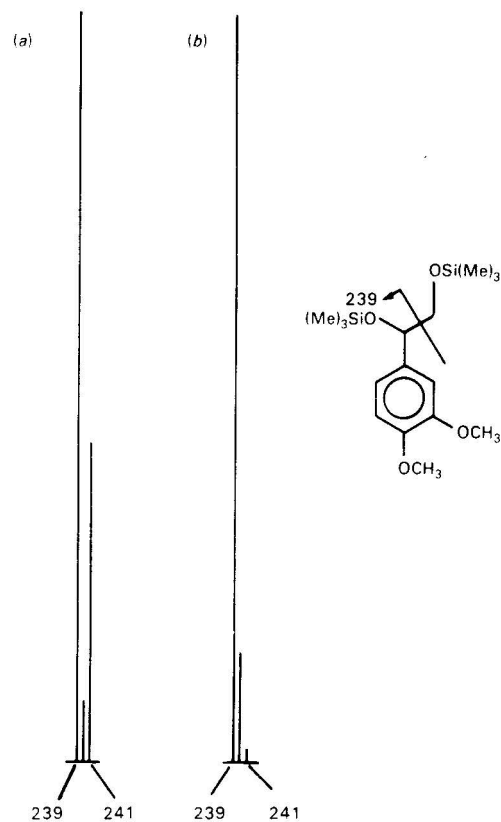


Fig. 1. Diagnostic portions of the mass spectra from degradation of model compound (II) with Fenton's reagent.

The spectra of the major fragment from the trimethylsilyl derivative of product (V) is shown. The reaction was with  $\text{H}_2^{16}\text{O}_2$  and either  $^{18}\text{O}_2$  (a) or  $^{16}\text{O}_2$  (b). Regions shown contain the base peak.

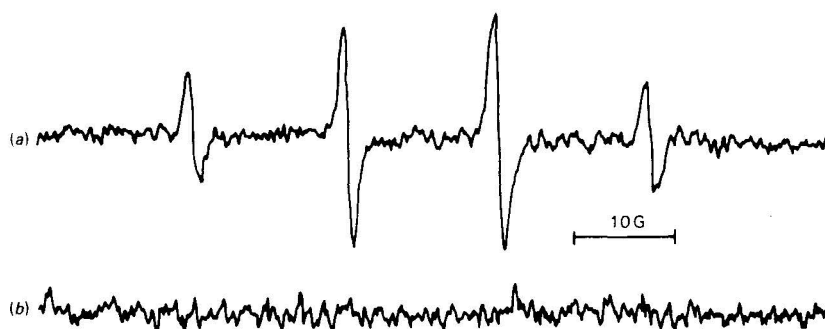


Fig. 2. (a) *E.s.r. spectrum of the 5,5-dimethyl-1-pyrroline N-oxide-HO-adduct* and (b) *e.s.r. spectrum with ligninase* (a) The reaction mixture contained 0.3mM-H<sub>2</sub>O<sub>2</sub>, 0.3mM-FeCl<sub>2</sub> and 10mM-5,5'-dimethyl-1-pyrroline N-oxide in 100mM-sodium tartrate buffer, pH 3.0. (b) The reaction mixture contained ligninase (16 µg/ml), 0.3mM-H<sub>2</sub>O<sub>2</sub> and 10mM-5,5'-dimethyl-1-pyrroline N-oxide in 100mM-tartrate buffer, pH 3.0. Instrument conditions; scan range, 10mT; modulation amplitude, 0.1 mT; scan time, 8 min; time constant, 0.5s; microwave power, 20mW; gain,  $1.25 \times 10^4$ .

organic-soluble compounds from the cultures, in accord with the ligninase reaction, but in contrast with the Fenton reaction.

### Discussion

Our results indicate that the ligninase does not function simply by generating free HO· and releasing it in the vicinity of substrate. This conclusion is based on the following: (1) the substantial differences in product distribution from the oxidation of the lignin models with the Fenton's reagent and the enzyme or the cultures; (2) the differences in the origin of the benzylic hydroxy-group oxygen atom in product (V) from model compound (II); (3) the absence of detectable HO' from the ligninase reaction. These results do not preclude enzyme-bound HO· or other oxidant of similar potential as a transient intermediate in catalysis.

The predominant reactions with the Fenton's reagent appear to be demethylation of aromatic methoxy groups and ring hydroxylation. Both of these reactions are known to occur with the Fenton system (Steenken & O'Neill, 1977). The phenolic products detected by t.l.c. in the Fenton degradation of the model compounds here presumably arose via both reactions. The fact that much of the <sup>14</sup>C from both models was found in the non-extractable water-soluble compounds after Fenton degradation probably reflects formation of [<sup>14</sup>C]methanol via demethylation of the [<sup>14</sup>C]-methoxy groups.

With the ligninase and cultures, the predominant reaction is cleavage of the C(α)-C(β) bond. On the basis of similarities in product identities, our results indicate that the ligninase is responsible for

degrading model compounds (I) and (II) in intact cultures.

Although the Fenton's reagent and ligninase degradation differ substantially, the former does have slight biomimetic action, confirming earlier preliminary results (Kirk & Nakatsubo, 1983). This slight ability suggests that the Fenton's reagent generates intermediates that behave similarly to the intermediates generated by the enzyme (i.e. the enzyme may generate radical transients during catalysis). Nevertheless, our results indicate that Fenton chemistry is of little importance in the fungal degradation of β-1 and β-O-4 substructure models and, by extension, these same structures in lignin.

Several reports have suggested high rates of HO· generation in cultures on the basis of ethylene formation via 4-methylthio-2-oxobutyrate oxidation (Kutsuki & Gold, 1982; Kelley & Reddy, 1982). We have observed that the purified ligninase can also oxidize 4-methylthio-2-oxobutyrate. This is in accord with the observation by Glenn *et al.* (1983) with the crude culture filtrate of *P. chrysosporium*. Thus this enzymic (ligninase) activity may account, in part, for the considerable amount of ethylene generated from 4-methylthio-2-oxobutyrate in cultures, which was previously attributed to HO· generation (Kutsuki & Gold, 1982; Kelley & Reddy, 1982).

Even so, the interaction of H<sub>2</sub>O<sub>2</sub> with Fe<sup>3+</sup> or Fe<sup>2+</sup>, which are present in the milieu of ligninolytic cultures (Faison & Kirk, 1983), will certainly result in some HO· formation (Barb *et al.*, 1950). Indeed, assays other than 4-methylthio-2-oxobutyrate oxidation also indicate its presence (Forney *et al.*, 1982; Bes *et al.*, 1983; Faison & Kirk, 1983). The partial inhibition of lignin

degradation by HO· scavengers would suggest a significant role for such HO·, although we question the specificity of these scavengers, especially in a biological system. Bes *et al.* (1983) have shown that chemically generated HO· can oxidize lignin, but this fact clearly does not establish that it plays a role in lignin biodegradation. The HO· can oxidize virtually any carbon-hydrogen bond, and also add to double bonds (Walling, 1975).

In conclusion, our data indicate that free HO· is not involved in one major reaction of lignin degradation (C(a)-C(b) cleavage) by *P. chrysosporium*. Its possible involvement in other reactions is not ruled out, but evidence for any role of HO· is presently indirect and inconclusive.

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## References

- Adler, E. (1977) *Wood Sci. Technol.* **11**, 169-218
- Barb, W. G., Baxendale, J. H., George, P. & Hargrave, K. R. (1950) *Trans Faraday Soc.* **47**, 462-500
- Bes, B., Ranjera, R. & Bondet, A. M. (1983) *Biochimie* **65**, 283-289
- Chua, M. G. S., Choi, S. & Kirk, T. K. (1983) *Holzforschung* **37**, 55-61
- Evans, C. S., Farmer, J. Y. & Palmer, J. M. (1984) *Phytochemistry* **23**, 1247-1250
- Faison, B. D. & Kirk, T. K. (1983) *Appl. Environ. Microbiol.* **46**, 1140-1145
- Fenn, P. & Kirk, T. K. (1984) *J. Wood Chem. Technol.* **4**, 131-148
- Finkelstein, E., Rosen, G. M. & Rauckman, E. J. (1980) *Arch. Biochem. Biophys.* **200**, 1-16
- Forney, L. J., Reddy, C. A., Tien, M. & Aust, S. D. (1982) *J. Biol. Chem.* **257**, 11455-11462
- Glenn, J. K., Morgan, M. A., Mayfield, M. B., Kuwahara, M. & Gold, M. H. (1983) *Biochem. Biophys. Res. Commun.* **114**, 1072-1083
- Hall, P. L. (1980) *Enzyme Microb. Technol.* **2**, 170-176
- Kelley, R. L. & Reddy, C. A. (1982) *Biochem. J.* **206**, 423-425
- Kirk, T. K. & Nakatsubo, F. (1983) *Biochim. Biophys. Acta* **756**, 376-384
- Kirk, T. K., Connors, W. J., Bleam, R. D., Hackett, W. F. & Zeikus, J. G. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2515-2519.
- Kutsuki, H. & Gold, M. H. (1982) *Biochem. Biophys. Res. Commun.* **109**, 320-327
- Kuwahara, M., Glenn, J. K., Morgan, M. A. & Gold, M. H. (1984) *FEBS Lett.* **169**, 247-250
- Landucci, L. L., Geddes, S. A. & Kirk, T. K. (1981) *Holzforschung* **35**, 67-70
- Nakatsubo, F., Reid, I. D. & Kirk, T. K. (1982) *Biochim. Biophys. Acta* **719**, 284-291
- Steenken, S. & O'Neill, P. (1977) *J. Phys. Chem.* **81**, 505-508
- Tien, M. & Kirk, T. K. (1983) *Science* **221**, 661-663
- Tien, M. & Kirk, T. K. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2280-2284
- Walling, C. (1975) *Acc. Chem. Res.* **8**, 125-131