APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1994, p. 459–466
0099-2240/94/$04.00+0

Vol. 60, No. 2

Lignin Peroxidase Oxidation of Aromatic Compounds in Systems Containing Organic Solvents

RAFAEL VAZQUEZ-DUHALT,† DONALD W. S. WESTLAKE, AND PHILLIP M. FEDORAK*

Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

Received 29 July 1993/Accepted 15 November 1993

Lignin peroxidase from *Phanerochaete chrysosporium* was used to study the oxidation of aromatic compounds, including polycyclic aromatic hydrocarbons and heterocyclic compounds, that are models of moieties of asphaltene molecules. The oxidations were done in systems containing water-miscible organic solvents, including methanol, isopropanol, *N,N*-dimethylformamide, acetonitrile, and tetrahydrofuran. Of the 20 aromatic compounds tested, 9 were oxidized by lignin peroxidase in the presence of hydrogen peroxide. These included anthracene, 1-, 2-, and 9-methylanthracenes, acenaphthene, fluoranthene, pyrene, carbazole, and dibenzothiophene. Of the compounds studied, lignin peroxidase was able to oxidize those with ionization potentials of <8 eV (measured by electron impact). The reaction products contain hydroxyl and keto groups. In one case, carbon-carbon bond cleavage, yielding anthraquinone from 9-methylanthracene, was detected. Kinetic constants and stability characteristics of lignin peroxidase were determined by using pyrene as the substrate in systems containing different amounts of organic solvent. Benzyl alkylation of lignin peroxidase improved its activity in a system containing water-miscible organic solvent but did not increase its resistance to inactivation at high solvent concentrations.

The use of fossil fuels for energy and as raw materials during the last century has been the origin of some widespread environmental pollution. Among these pollutants are the polycyclic aromatic hydrocarbons (PAHs) that are considered to be potential health risks because of their possible carcinogenic and mutagenic activities.

The ability of ligninolytic fungi to attack organic pollutants and xenobiotics has been studied (14, 18, 29), and the capacity to biotransform and biodegrade PAHs has been investigated. Among the PAHs studied are acenaphthene (33), anthracene (20), benzo[*a*]pyrene (2, 36), biphenyl (41), fluoranthrene (32), fluorene (16), methylanthracenes (6), phenanthrene (19, 29, 38), pyrene (22, 25), some monoaromatic compounds (47), and the PAH components of the anthracene oil from coal tar distillation (1).

The white-rot fungus *Phanerochaete chrysosporium* is capable of degrading the lignin present in woody plant tissue by means of the extracellular enzymes (9, 21), and lignin and manganese peroxidases are thought to be major components of the degradation system in vivo. Recently, Hammel et al. (21) clearly demonstrated the role of lignin peroxidase in the breakdown of synthetic lignins (average molecular weight, 4,200) in vitro.

P. chrysosporium can also metabolize several PAHs, but the role of lignin peroxidases in the oxidations of these compounds appears to vary (19, 36, 38, 47). For example, anthracene was oxidized by cultures of *P. chrysosporium* and by purified lignin peroxidase (20), whereas phenanthrene was metabolized by cultures of *P. chrysosporium*, but neither the culture supernatant, containing extracellular enzymes, nor purified lignin peroxidase was able to modify phenanthrene (19, 38). An intracellular cytochrome P-450 system has been proposed to be involved in phenanthrene degradation by *P. chrysosporium* (38). However, the intermediates formed could not be attributed to mono-oxygenases (19). Other unidentified enzymes are probably involved. In contrast, lignin peroxidase oxidized benzo[*a*]anthracene (22), benzo[*a*]pyrene (14, 22, 36), and pyrene (22, 25) in vitro. The ability of purified lignin peroxidase to oxidize some PAHs was determined by measuring changes in their UV-visible spectra (22). This was correlated with the ionization potentials of the PAHs, i.e., with the energy required to remove an electron and form the PAH cation radical. Those PAHs with an ionization potential lower than 7.55 eV were modified (22). The values of ionization potentials used were obtained from the charge-transfer absorption spectra and from polarographic data and are sometimes as much as 0.5 eV lower than those obtained by the electron-impact method (27, 35).

Our recent work has focused on the biocatalytic modifications of asphaltenes (12), with the long-term goal of "biocracking" this material as a means of upgrading heavy oils and bitumens that are abundant in Alberta, Canada. Strausz et al. (37) have proposed a structure for a hypothetical asphaltene molecule (molecular mass, approximately 6,100 Da) that contains a variety of ring structures connected by methylene bridges of various lengths (2 to 24 carbons). The ring structures are not highly condensed and include alicyclic and aromatic moieties, some of which contain heteroatoms, with sulfur being most abundant.

To hasten the biocatalytic activities on asphaltenes, these large, highly hydrophobic molecules must be dissolved in organic solvents to minimize mass transfer limitations. Therefore, we have studied the effect of organic solvents on the enzymatic activity of peroxidases (12, 42) and cytochrome *c* (43, 44). In a previous study, the oxidation of a variety of model sulfur-containing compounds was demonstrated (44), but there was no evidence of bond breakage, which is essential for a biocracking process. Recently, it has been reported that lignin peroxidase is able to degrade synthetic lignins in systems containing 30 to 40% water-miscible organic solvent (21). In the present study, we examined the activity of lignin peroxidase on 20 aromatic compounds that are models of moieties found

* Corresponding author. Mailing address: Department of Microbiology, University of Alberta, Edmonton Alberta, Canada T6G 2E9. Phone: (403) 492-3670. Fax: (403) 492-2216.

† Present address: Instituto de Biotecnologia-UNAM, Cuernavaca, Morelos, 62271 México.

in asphaltene molecules. This investigation addresses the effects of organic solvents on the activity of lignin peroxidase. The effects of chemical modifications on the reaction kinetics and enzyme stability of lignin peroxidase were estimated.

MATERIALS AND METHODS

Chemicals. Lignin peroxidase, manganese peroxidase, and a crude extract from *P. chrysosporium* were purchased from Tienzyme, Inc. (State College, Pa.). All of the PAHs, heterocyclic compounds, and the oxidizing agents *t*-butyl hydroperoxide, 3-chloroperoxybenzoic acid, and peroxyacetic acid were obtained from Aldrich (Milwaukee, Wis.). Cumene hydroperoxide and hydrogen peroxide were obtained from Sigma Chemical Co. (St. Louis, Mo.). The high-performance liquid chromatography (HPLC)-grade solvents methanol, isopropanol, acetonitrile, *N,N*-dimethylformamide, and tetrahydrofuran were obtained from Fisher Scientific (Fairlawn, N.J.). Tetrahydrofuran was distilled in the presence of ferrous sulfate to eliminate peroxides.

Reaction conditions. To initially test the specific activities of lignin peroxidase, manganese peroxidase, and crude extracts from *P. chrysosporium*, reaction mixtures contained 20 μM anthracene and 100 μM 3-chloroperoxybenzoic acid in 10% (vol/vol) tetrahydrofuran in 40 mM sodium succinate buffer (pH 4.5). This mixture was supplemented with 100 μM Mn^{2+} for the test with manganese peroxidase.

For routine use, the enzymatic reaction mixture (1 ml) contained 20 μM polycyclic aromatic compound and from 37 to 370 nM lignin peroxidase in 40 mM sodium succinate buffer (pH 4.0) containing different concentrations of organic solvent. The reactions were carried out at room temperature (20 to 22°C) and started by adding 30 nmol of hydrogen peroxide. The reaction progress was monitored by HPLC. Decreases in the amounts of aromatic substrates were determined by measuring the decreases in their peak areas at A_{255} or A_{225} with an integrator (Hewlett-Packard 3390A) after calibration with standards. Pyrene oxidation was monitored spectrophotometrically as a decrease in A_{335} and by using 32,600 M^{-1} cm^{-1} as the extinction coefficient, which was experimentally determined when no cosubstrate was present. All reactions were done in triplicate, and the mean and standard deviations are reported.

To obtain enough products for their identification, 10-ml reaction mixtures containing 20 μM aromatic compounds were treated with enzyme and hydrogen peroxide. Because the enzyme is inhibited by hydrogen peroxide (39), three additions of 740 pmol of lignin peroxidase and 300 nmol of hydrogen peroxide were made at 1-h intervals to ensure that nearly all of the aromatic compounds reacted. The reaction mixtures were then saturated with NaCl and extracted five times with 2 ml of toluene. The extracts were combined, dried over Na_2SO_4, and concentrated under N_2 prior to analysis by gas chromatography (GC). The products from pyrene were fractionated on a silica gel column (1 by 25 cm) eluted with a gradient of 0 to 100% methanol in methylene chloride, and the fractions were monitored at A_{255}. The fractions containing the isolated products were dried under N_2 and analyzed by high-resolution mass spectrometry (Mass Spectrometry Laboratory, Department of Chemistry, University of Alberta).

The stability of lignin peroxidase was measured by incubating the reaction mixture, containing 15% tetrahydrofuran and 30 μM hydrogen peroxide, for different times. The reaction was started by adding 20 μM pyrene, and the activity remaining was reported as a percentage of initial activity.

Lignin peroxidase modifications. Reductive alkylation with benzaldehyde was done by using sodium cyanoborohydrate (42). Polyethylene glycol-modified lignin peroxidase was prepared at pH 4.0 by using polyethylene glycol dialdehyde (43).

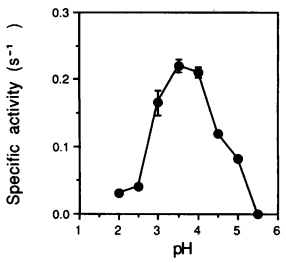

FIG. 1. Effect of pH on the specific activity of lignin peroxidase for pyrene oxidation. The reaction mixture contained 20 μM pyrene, 30 μM hydrogen peroxide, and 74 nM lignin peroxidase.

The concentrations of unmodified and modified lignin peroxidase were estimated by protein measurement with the Bio-Rad procedure and by spectrophotometry using an extinction coefficient of 168,000 M^{-1} cm^{-1} at 409 nm (9).

Analytical methods. HPLC was done with a Waters-Millipore HPLC system with a UV detector. The substrates and products were detected at 255 or 225 nm and eluted at 1 ml min^{-1} with acetonitrile-water (70:30 [vol/vol]) from a Resolve 5-μm (particle size) C_{18} column (Waters-Millipore).

The GC analyses were performed with a Hewlett-Packard (model 5730) gas chromatograph equipped with a flame ionization detector. The oven temperature was held at 150°C for 2 min and then programmed at 4°C per min to 250°C and held for 16 min. The proportions of products formed by the enzymatic oxidations were calculated for the peak areas from GC analyses, assuming an equal response factor for each product. The method used for electron impact ionization GC-mass spectrometry was described previously (13). Some samples were analyzed by GC-Fourier transform infrared spectroscopy to help identify the products (11).

The ionization potentials of the aromatic compounds studied were estimated by using the charge-transfer absorption spectra of a complex with chloranil (15). For this determination, equal volumes of chloroform solutions of each aromatic compound (0.1 M) and chloranil (saturated solution) were used.

RESULTS

The activities of lignin and manganese peroxidases and the crude extract from *P. chrysosporium* were tested on anthracene in a system containing 10% tetrahydrofuran and 100 μM 3-chloroperoxybenzoic acid. The specific activity for the oxidation of anthracene by lignin peroxidase was 550 (±15) μmol min^{-1} g of $protein^{-1}$, and that by the crude extract was 300 (±20) μmol min^{-1} g of $protein^{-1}$. Manganese peroxidase did not oxidize anthracene in the presence of 100 μM Mn^{2+} under the experimental conditions used, even though it has a more positive oxidation-reduction potential than lignin peroxidase (−88 mV and −137 mV, respectively) (28). As expected, the partially purified lignin peroxidase showed higher specific activity than the crude extract.

The effect of pH on lignin peroxidase activity was determined by using pyrene as the substrate in a reaction mixture containing 10% tetrahydrofuran (Fig. 1). The highest values of specific activity found were in systems with pHs between 3.5 and 4.0. At pH 4.0, lignin peroxidase showed better stability

TABLE 1. Effect of water-miscible organic solvents on the kinetic constants of pyrene oxidation by lignin peroxidase[a]

Solvent	Concn (%)	k_{cat} (s^{-1})	$K_{m,app}$ (μM)	$k_{cat}/K_{m,app}$ (s^{-1} mM^{-1})
Isopropanol	20	4.4	96	46
	25	0.8	30	25
	30	0.2	11	17
	35	NR[b]		
Acetonitrile	10	1.6	9	188
	15	2.6	31	84
	20	0.8	11	69
	25	0.2	5	28
	30	NR		
N,N-dimethylformamide	10	1.2	28	43
	15	0.4	43	8
	20	NR		
Tetrahydrofuran	10	1.3	9	149
	15	1.1	8	148
	20	0.4	9	45
	25	NR		

[a] The reaction mixture contained 20 μM pyrene and from 47 to 190 nM lignin peroxidase.
[b] NR, no reaction detected.

TABLE 2. Specific activity for lignin peroxidase oxidation and ionization potentials of polycyclic aromatic compounds that are models of moieties of asphaltene molecules[a]

Compound	Sp act (s^{-1})	Ionization potential (eV) measured by:	
		Electron impact[b]	Charge transfer
Pentacene	NEO[c]	6.55	8.69
9-Methylanthracene	3.01 (±0.35)[d]	7.23	7.26
Carbazole	1.69 (±0.39)	7.20	7.92
1-Methylanthracene	1.62 (±0.33)	NA[e]	7.36
Anthracene	0.76 (±0.09)	7.55	7.41
Pyrene	0.33 (±0.04)	7.72	7.45
Acenaphthene	0.28 (±0.01)	7.70	7.70
2-Methylanthracene	0.25 (±0.01)	7.42	7.35
Fluoranthene	0.15 (±0.02)	7.76	8.15
Dibenzothiophene	0.06 (±0.01)	7.93	8.14
Chrysene	NR[f]	8.01	8.12
Phenanthrene	NR	8.03	8.18
Triphenylene	NR	8.19	8.10
Naphthalene	NR	8.25	8.19
1-Methylnaphthalene	NR	8.50	7.97
Fluorene	NR	8.52	8.03
1,3-Diphenylpropane	NR	8.60	8.53
Dibenzofuran	NR	8.77	8.42
Biphenyl	NR	8.80	8.46
Diphenylmethane	NR	9.00	8.60

[a] The reaction mixture contained 20 μM aromatic compound and 30 μM hydrogen peroxide in 10% tetrahydrofuran-sodium succinate buffer.
[b] Literature values from references 26, 27, and 35.
[c] NEO, nonenzymatic oxidation.
[d] Values in parentheses are standard deviations.
[e] NA, not available.
[f] NR, no reaction detected.

than it did a pH 3.5. Thus, a sodium succinate buffer with a pH of 4.0 was used for the enzymatic reactions.

The effect of five water-miscible organic solvents on pyrene oxidation by lignin peroxidase was determined. Pyrene was not soluble in the reaction mixtures that contained 15% isopropanol or 25% methanol. No reaction was observed in a mixture containing 30% methanol, the concentration required to dissolve 20 μM pyrene. The kinetic constants for pyrene oxidation by lignin peroxidase in four other water-miscible solvents are shown in Table 1. The maximum values of k_{cat} in different solvents were in the following order: isopropanol > acetonitrile > tetrahydrofuran > *N,N*-dimethylformamide. In this study, we focused on solvent systems containing tetrahydrofuran because it is the only water-miscible organic solvent able to dissolve an appreciable amount of asphaltenes (43).

Twenty aromatic compounds, including PAHs, methyl-substituted PAHs, fused-ring hydrocarbons, polyphenylalkanes, and heterocycles (Table 2), were tested for oxidation by lignin peroxidase in 10% tetrahydrofuran. Nine of these were oxidized by the enzyme in the presence of hydrogen peroxide. Each of the compounds oxidized by lignin peroxidase had an ionization potential of <8.0 eV, as determined by electron impact (Table 2). Pentacene, which has the lowest ionization potential (6.55 eV by electron impact) was oxidized by the hydrogen peroxide (30 μM) alone. In the presence of both hydrogen peroxide and lignin peroxidase, the extent of this oxidation was reduced, probably because of a competitive reaction between lignin peroxidase and hydrogen peroxide.

In the presence of hydrogen peroxide and lignin peroxidase, the rate of this oxidation was decreased. In general, the lower the ionization potential of the aromatic compound, the higher the specific activity of the lignin peroxidase reaction (Fig. 2). The ionization potential of 8.0 eV (by electron impact) appeared to be a threshold, and none of the compounds tested that had ionization potentials of >8.0 eV was transformed by lignin peroxidase.

The ionization potentials determined from the charge-transfer absorption spectra of the aromatic compounds by using chloranil are also shown in Table 2. The values that were determined are in good agreement with those found in the literature (27, 35). The aromatic compounds that were transformed by the lignin peroxidase had ionization potentials between 7.26 and 8.15 eV (by charge transfer). However, several of the compounds that did not react had ionization potentials in this range (Table 2). Thus, a clear threshold value,

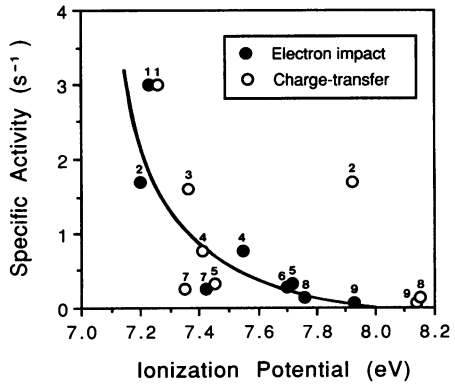

FIG. 2. The influence of ionization potential on the specific activity of the lignin peroxidase oxidation of the following polycyclic aromatic compounds: 9-methylanthracene (symbols 1), carbazole (symbols 2), 1-methylanthracene (symbol 3), anthracene (symbols 4), pyrene (symbols 5), acenaphthene (symbols 6), 2-methylanthracene (symbols 7), fluoranthene (symbols 8), and dibenzothiophene (symbols 9). Ionization potential was determined by electron impact or charge transfer.

TABLE 3. Mass spectral data of products formed from polycyclic aromatic compounds by lignin peroxidase and hydrogen peroxide

Substrate	Products	Mass spectral ions (*m/z*)[a]
Anthracene	Anthraquinone	209 (16), 208 (100), [M$^+$], 207 (15), 181 (14), 180 (98), 153 (10), 152 (76), 151 (35), 150 (18), 126 (11), 76 (42), 75 (15)
1-Methylanthracene	1-Methylanthraquinone	223 (16), 222 (100) [M$^+$], 221 (26), 194 (19), 166 (13), 165 (50)
2-Methylanthracene	2-Methylanthraquinone	223 (16), 222 (100), [M$^+$], 221 (11), 207 (14), 194 (30), 193 (10), 166 (23), 165 (64), 164 (10), 163 (10)
9-Methylanthracene	Anthraquinone	209 (16), 208 (100) [M$^+$], 207 (15), 181 (14), 180 (98), 153 (10), 152 (76), 151 (35), 150 (18), 126 (11), 76 (42), 75 (15)
	9-Methyleneanthranone	207 (23), 206 (100) [M$^+$], 179 (22), 178 (100), 177 (29), 176 (53), 152 (28), 151 (24), 150 (15), 89 (20), 88 (28), 75 (11)
	9-Methanol-9,10-dihydroanthracene	210 (26) [M$^+$], 209 (100), 206 (21), 178 (26), 176 (10), 152 (25), 151 (11), 77 (13), 76 (12)
Acenaphthene	1-Acenaphthenol	170 (100) [M$^+$], 169 (98), 168 (14), 167 (13), 153 (35), 152 (49), 151 (15), 141 (37), 140 (13), 139 (41), 115 (22), 76 (11), 63 (12)
	1-Acenaphthenone	169 (18), 168 (100) [M$^+$], 141 (15), 140 (100), 139 (89), 113 (11), 89 (10), 70 (17), 69 (14), 65 (15)
Pyrene	1,8-Pyrenedione	232 (100) [M$^+$], 204 (41), 176 (91), 175 (39), 174 (29), 144 (54), 97 (22), 83 (31), 69 (38), 57 (64), 55 (40)
	Unknown	238 (33), 237 (18), 236 (100), 201 (27), 200 (34), 199 (9), 118 (14), 100 (8)
Dibenzothiophene	Dibenzothiophene sulfoxide	201 (11), 200 (80) [M$^+$], 188 (12), 185 (14), 184 (100), 172 (43), 171 (64), 167 (12), 152 (13), 139 (35)

[a] Values in parentheses are relative intensities (in percent). [M$^+$], molecular ion.

based on these ionization potentials, was not observed. Figure 2 also includes the specific activities for lignin peroxidase oxidation of the nine reactive compounds plotted versus the ionization potential determined by charge transfer. Again, there is a general trend of higher specific activity with lower ionization potential.

Many of the products resulting from the lignin peroxidase reactions with the aromatic compounds were identified by mass spectrometry or GC-mass spectrometry. The major ions of the products are listed in Table 3. The product of anthracene oxidation was thought to be anthraquinone. Indeed, it had the same GC retention time and mass spectrum as an authentic standard of 9,10-anthraquinone. This mass spectrum (Table 3) showed the characteristic biphenylene ion (*m/z* = 152) which forms by the ejection of two molecules of carbon monoxide from the two keto groups of a quinone (34). By using mass spectrometry, Hammel et al. (20) also identified 9,10-anthraquinone as the oxidation product from anthracene.

The products of lignin peroxidase oxidation of 1- and 2-methylanthracene had molecular ions at *m/z* 222, which is 14 mass units greater than that of 9,10-anthraquinone and consistent with the products being 1- and 2-methylanthraquinone, respectively. Ejection of two molecules of carbon monoxide from the keto groups would yield a methylbiphenylene ion (*m/z* = 166). This ion was observed in the mass spectra of these products (Table 3).

Three products were detected from the oxidation of 9-methylanthracene. The mass spectrum of one product matched that of authentic anthraquinone. The molecular ion of the second oxidation product was at *m/z* 206, and this compound is believed to be 9-methyleneanthranone. The intense ion at *m/z* 178 would result from the loss of CO, and the biphenylene ion (*m/z* = 152) that was observed would result from the subsequent loss of C_2H_2. The mass spectrum of the third oxidation product showed a weak molecular ion at *m/z* 210. The base peak was at *m/z* 209, indicating the presence of a labile hydrogen atom. The product reacted with *N,O*-bis(trimethylsilyl)acetamide, in a silylation reaction, giving a derivative with a molecular weight of 282. The results were consistent with this product being 9-methanol-9,10-dihydroanthracene.

The mass spectra of the two products from acenaphthene matched those of 1-acenaphthenone and 1-acenaphthenol given by Pothuluri et al. (33). The product of dibenzothiophene oxidation by lignin peroxidase was its sulfoxide, as determined by comparing the GC-mass spectrometry analysis with a sample of authentic dibenzothiophene sulfoxide.

One product from pyrene oxidation had a molecular ion at *m/z* 232, which indicated the addition of two oxygen atoms to pyrene. The mass spectrum shows one ion at *m/z* 204 [(M-28)$^+$], which would be produced by the loss of carbon monoxide, and an intense ion at *m/z* 176 [(M-56)$^+$], which would result from the ejection of a second molecule of carbon monoxide. The UV spectrum of this product most closely matched that of 1,8-pyrenedione given by Fatiadi (10). A minor second oxidation product (≈10% of total products) was also detected. The mass spectral data of this second product from the pyrene oxidation are given in Table 3. Upon irradiation with visible light, pyrenediones are reduced to the corresponding dihydroxypyrenes (40); thus, this unidentified compound could be a product from the subsequent reduction of 1,8-pyrenedione.

Figure 3 summarizes the products identified from the lignin peroxidase reactions with seven of the nine compounds tested. Although fluoranthene and carbazole were oxidized, their products were not identified. The amounts of substrates oxidized (expressed as nanomoles) after three additions of enzyme and hydrogen peroxide are shown on the left side of Fig. 3, and these amounts were 60 to 100% of the original 200 nmol of substrate in the reaction mixture, as determined by HPLC. The products were detected by GC, and because there was the possibility that some products may have formed that were not amenable to GC analysis, the results shown on the right side of Fig. 3 are the relative amounts of each product observed by the GC analysis.

The rates of pyrene oxidation by lignin peroxidase with different oxidizing agents and cosubstrates were determined, and the specific activities are shown in Table 4. Hydrogen peroxide was the best oxidizing agent when no cosubstrate was present or when veratryl alcohol was added. The presence of phenol and guaiacol decreased the specific activity of pyrene oxidation because these compounds act as competitive sub-

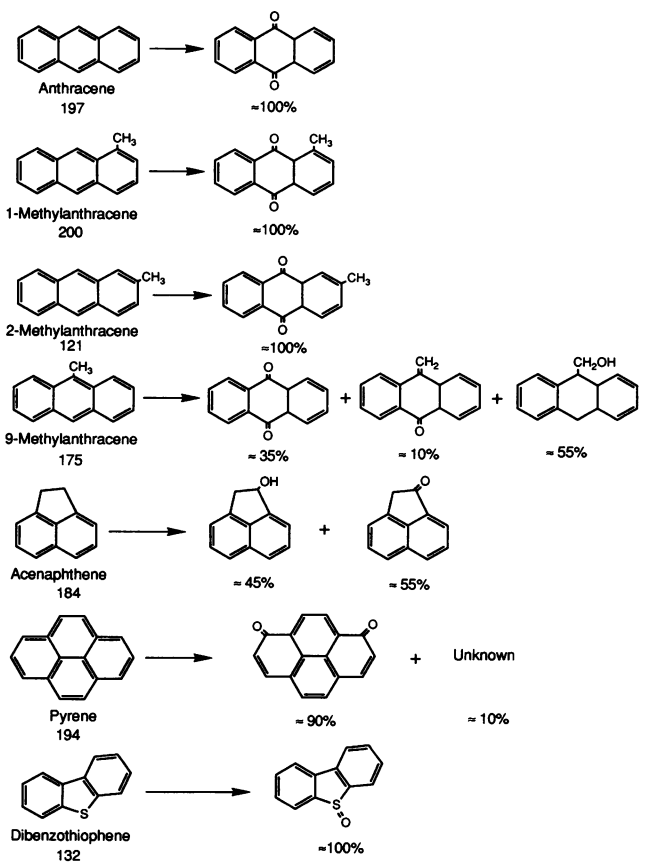

FIG. 3. Oxidation products from the reaction of lignin peroxidase and hydrogen peroxide with 200 nmol each of seven polycyclic aromatic compounds. Under the name of each substrate is the number of nanomoles that reacted after three additions of enzyme and hydrogen peroxide. Under each product is its proportion (percent) detected by GC analysis.

strates. The presence of polymeric guaiacol was detected after the reaction by changes in color, with a maximum absorbance at 470 nm.

The kinetic constants for the enzymatic oxidation of pyrene by using hydrogen peroxide or 3-chloroperoxybenzoic acid, with or without veratryl alcohol, and in different concentrations of tetrahydrofuran are shown in Table 5. The presence of veratryl alcohol slightly enhanced the activity (k_{cat}) when hydrogen peroxide was used (Table 5), but it offered no protective effect against the inhibitory action of an increase in the tetrahydrofuran concentration, as determined by measuring the reaction velocity over time in the presence and absence of veratryl alcohol (data not shown). This is supported by the lower values of the second-order constants, $k_{cat}/K_{m,\ app}$, in the reaction containing veratryl alcohol (Table 5; $K_{m\ app}$ is the apparent K_m). At an increased concentration of organic solvent, the value of $K_{m,\ app}$ increased significantly, provoking a decrease of the catalytic efficiency ($k_{cat}/K_{m,\ app}$). 3-Chloroperoxybenzoic acid showed lower values of k_{cat} and catalytic efficiency than hydrogen peroxide, and no pyrene oxidation could be detected in 20% tetrahydrofuran systems.

With the objective of improving the activity and the stability of lignin peroxidase in the tetrahydrofuran-water systems, two chemical modifications of lignin peroxidase were done. The kinetic constants of pyrene oxidation by the two modified lignin peroxidase preparations are compared with those for the unmodified enzyme in Table 6. The benzyl modification increased the value of k_{cat} by more than twofold, whereas the polyethylene glycol modification lowered the k_{cat} value below that of the unmodified lignin peroxidase. The chemical modifications did not significantly preserve the enzyme activity in solutions with higher concentrations of tetrahydrofuran (Fig. 4).

TABLE 4. Specific activity of lignin peroxidase on pyrene with different oxidizing agents and different cosubstrates[a]

Oxidizing agent	Cosubstrate	Sp act (s^{-1})
Hydrogen peroxide	None	0.60 (±0.01)[b]
	Veratryl alcohol	0.52 (±0.02)
	Phenol	0.11 (±0.01)
	Guaiacol	0.10 (±0.02)
3-Chloroperoxybenzoic acid	None	0.32 (±0.01)
	Veratryl alcohol	0.29 (±0.02)
	Phenol	0.14 (±0.06)
	Guaiacol	0.10 (±0.02)
Peracetic acid	None	0.42 (±0.01)
Cumene hydroperoxide	None	0.13 (±0.05)
t-Butylhydroperoxide	None	0.07 (±0.00)

[a] The reaction mixture contained 20 μM pyrene, 50 μM oxidizing agent, and 50 μM cosubstrate in 10% tetrahydrofuran-succinate buffer (pH 4.0).
[b] Values in parentheses are standard deviations.

DISCUSSION

Peroxidases from *P. chrysosporium* are characterized by their ability to oxidize substrates with high redox potentials, and these enzymes have active sites that are more electron deficient than horseradish peroxidases (28). Lignin peroxidase activity is affected by the increase in organic solvent concentration (Table 1). The sequence of the maximum k_{cat} values (isopropanol > acetonitrile > tetrahydrofuran > *N,N*-dimethylformamide) is similar to that found for cytochrome *c* activity (43). No activity was found in systems containing more than 30% organic solvent (Table 1).

Lignin peroxidase oxidation of veratryl alcohol has an apparent optimum near pH 2, with activity rapidly decreasing at lower pH and no appreciable activity at pH values above 5 (39). With pyrene as the substrate, the greatest lignin peroxidase activity was found at pH 3.5 (Fig. 1). This difference could be a product of two factors. First, free radical cations derived from veratryl alcohol do not appear to be released from the enzyme during catalysis, suggesting that the alcohol may react with compound I either directly, to form resting enzyme and aldehyde, an apparent oxygenation reaction, or to form compound II and cation-free radicals (39). In contrast, other substrates such as PAHs are known to be converted to cation radicals (17, 25). Second, unlike other peroxidases, no pH dependence was observed for the reaction of ferric lignin peroxidase with hydrogen peroxide to form compound I (3). Thus, the oxidations of veratryl alcohol and pyrene appear to involve different mechanisms.

The capacity of polycyclic aromatic compounds to form radical cations is related to their ionization potential, which depends on the π-electron charge distribution of the compound. The removal of one electron from the π-system generates a cation radical. The positions of the highest charge density are the most susceptible to nucleophilic substitution (4). Ionization potential seems to be important in other

TABLE 5. Effect of tetrahydrofuran concentration and the presence of veratryl alcohol on the kinetic constants of lignin peroxidase oxidation of 20 μM pyrene

Oxidizing agent	Tetrahydrofuran concn (%)	Veratryl alcohol (100 μM)	k_{cat} (s^{-1})	$K_{m,app}$ (μM)	$k_{cat}/K_{m,app}$ (s^{-1} mM^{-1})
Hydrogen peroxide	10	−	1.3	9	144
	15	−	1.1	8	138
	20	−	0.4	9	44
	10	+	1.5	5	300
	15	+	1.6	53	30
	20	+	1.0	42	24
3-Chloroperoxybenzoic acid	10	−	0.3	6	50
	15	−	0.4	64	6
	10	+	0.4	20	20
	15	+	0.2	24	8

biological reactions, such as the binding of PAHs with DNA by a horseradish peroxidase-catalyzed reaction (5).

From our in vitro survey of the oxidation of aromatic compounds by lignin peroxidase in 10% tetrahydrofuran, the ionization potential of the aromatic compound appears to have an important role in the ability of the enzyme to oxidize the compound. Hammel et al. (22) reported that PAHs having an ionization potential lower than 7.55 eV are modified by lignin peroxidase. The ionization potential values used by those authors were obtained from the charge-transfer absorption spectra and polarographic data, which differ from the values obtained by the electron impact method (Table 2). The ionization potentials of PAHs obtained by other methods, such as photoelectron spectroscopy, show significant variations for a given compound (27, 35), whereas the values obtained from the electron impact technique commonly vary by <0.1 eV (26).

The ionization potential of a molecule is defined as the energy required to completely remove an electron from the neutral particle in its ground state. When the ionization potential is obtained by extrapolation of vibrational bands, as in the case of a charge-transfer spectrum, the value is affected by the solvent in the system. This effect could be more important when heteroatom substituents, which affect the electronegativity of the molecule, are present. To the extent that such ions in solution resemble the gaseous ions formed by electron impact, the energies of gaseous ions will be useful in interpreting the mechanism of lignin peroxidase in solvents. However, the electron impact values depend only on molecular characteristics and are not affected by any solvent interaction.

Lignin peroxidase oxidation introduced keto and hydroxyl groups onto many of the aromatic molecules tested (Fig. 3). Anthracenes were oxidized in positions 9 and 10 to form quinones. 9,10-Anthraquinone was the only product detected in the anthracene oxidation, in agreement with Hammel et al. (20), whereas 9-methylanthracene oxidation yielded three products, 9-methanol-9-hydroanthracene, 9,10-anthraquinone, and 9-methyleneanthranone. The last product has been found as an oxidation product of 9-methylanthracene in a sodium hypochlorite solution (30). Of the three isomers of methylanthracene tested, only the methyl carbon from 9-methylanthracene was removed (Fig. 3).

1-Acenaphthenol and 1-acenaphthenone were the oxidation products from acenaphthene treatment with lignin peroxidase and hydrogen peroxide (Fig. 3), and these products were detected as metabolites of acenaphthene in a *Cunninghamella elegans* culture (33). Pyrenediones were also found by Hammel et al. (22) as major products of pyrene oxidation by lignin peroxidase, and 3,4-pyrene-dihydrodiols were produced during the degradation of pyrene by a *Mycobacterium* sp. (23). The sulfur atom was the site of the lignin peroxidase-mediated oxidation of dibenzothiophene, yielding the corresponding sulfoxide, which was also observed as the product of the cytochrome *c*-mediated oxidation of dibenzothiophene in the presence of hydrogen peroxide (44).

Lignin peroxidase is inhibited by modest concentrations of hydrogen peroxide, showing a behavior of competitive inhibition (39). Of the peroxides tested, hydrogen peroxide showed higher activities than the others (Tables 4 and 5). The same result was obtained with manganese peroxidase (45) but not with other peroxidases (31) or cytochrome *c* (44). Veratryl alcohol improves lignin peroxidase production in cultures (8, 24), benzo[*a*]pyrene mineralization in vivo (29), and the oxidation of benzo[*a*]pyrene by lignin peroxidase in vitro (17). However, the presence of veratryl alcohol had no effect on fluorene oxidation in situ (16). The formation of a veratryl

TABLE 6. Kinetic constants in pyrene oxidation with lignin peroxidase and hydrogen peroxide in 10% tetrahydrofuran-succinate buffer (pH 4.0)

Enzyme prepn	k_{cat} (s^{-1})	$K_{m,app}$ (μM)	$k_{cat}/k_{m,app}$ (s^{-1} mM^{-1})
Unmodified	1.3	8.7	150
Polyethylene glycol modified	0.8	14	57
Benzyl modified	2.9	30	97

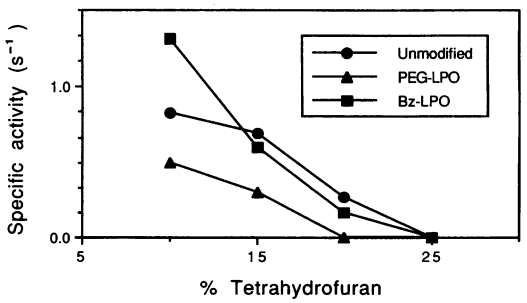

FIG. 4. Effect of tetrahydrofuran concentration on the specific activity of three preparations of lignin peroxidase, namely, unmodified enzyme, benzyl-modified lignin peroxidase (Bz-LPO), and polyethylene glycol-modified lignin peroxidase (PEG-LPO). The reaction mixture contained 20 μM pyrene, 30 μM hydrogen peroxide, and from 20 to 114 nM lignin peroxidase preparation.

alcohol-lignin peroxidase complex (46) and/or the production of veratryl alcohol radicals (39) may play a role in the enhanced activity of lignin peroxidase in a high concentration of organic solvent when veratryl alcohol is present (Table 5).

Phenol and guaiacol are competitive substrates (Table 3). In the presence of guaiacol, the reaction mixture became brown and had an absorbance spectrum similar to those of polymeric guaiacol formed by horseradish peroxidases. Phenol is a well-known competitive substrate in peroxidase reactions forming *o,o'*-biphenol (7).

The value of k_{cat} obtained with pyrene as the substrate (1.3 s^{-1}; Table 5) is in the same range as that found for veratryl alcohol (2.9 s^{-1} at pH 3.5 and 0.8 s^{-1} at pH 4.5) (39). The catalytic efficiency ($k_{cat}/K_{m,\ app}$) of pyrene oxidation with hydrogen peroxide (150 s^{-1} mM^{-1}) is nearly the same as that of veratryl alcohol oxidation at pH 2.5 (147 s^{-1} mM^{-1}) (39).

Benzyl modification of horseradish peroxidase improved its catalytic activity in water-immiscible solvents by increasing the superficial hydrophobicity of the enzyme (42). The introduction of benzyl groups onto lignin peroxidase improved the enzyme activity in a system containing tetrahydrofuran (Table 6). This modification may increase the affinity of the substrate and the enzyme. However, the chemical modification did not protect the enzyme from inactivation by higher concentrations of tetrahydrofuran in the reaction mixture (Fig. 4).

In conclusion, lignin peroxidase oxidizes several aromatic compounds in reaction mixtures containing tetrahydrofuran. In general, compounds with an ionization potential of <8.0 eV (measured by the electron-impact technique) were oxidized. The products were mainly aromatic ketones and hydroxycompounds. A carbon-carbon bond cleavage was observed leading to the removal of the methyl carbon from 9-methylanthracene. The activity and stability of the enzyme were decreased when the concentration of organic solvent was increased. Finally, reductive alkylation of the enzyme with benzyladehyde improved the activity on pyrene in a reaction mixture containing 10% tetrahydrofuran.

ACKNOWLEDGMENTS

This work was funded by a contract from the Alberta Oil Sands Technology and Research Authority.

We thank Karen Dodge for technical assistance.

REFERENCES

1. **Bumpus, J. A.** 1985. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **55:**154–158.
2. **Bumpus, J. A., M. Tien, D. Wright, and S. D. Aust.** 1985. Oxidation of persistent environmental pollutants by white rot fungus. Science **228:**1434–1436.
3. **Cai, D., and M. Tien.** 1991. Lignin peroxidase of *Phanerochaete chrysosporium*. Evidence for an acidic ionization controlling activity. J. Biol. Chem. **266:**14464–14469.
4. **Cavalieri, E. L., and E. G. Rogan.** 1984. One-electron and two-electron oxidation in aromatic hydrocarbon carcinogenesis, p. 323–396. *In* W. A. Pryor (ed.), Free radicals in biology, vol. IV. Academic Press, Inc., New York.
5. **Cavalieri, E. L., E. G. Rogan, R. W. Roth, R. K. Saugier, and A. Hakam.** 1983. The relationship between ionization potential and horseradish/hydrogen peroxide-catalyzed binding of aromatic hydrocarbons to DNA. Chem. Biol. Interactions. **47:**87–109.
6. **Cerniglia, C. E., W. L. Campbell, P. P. Fu, J. P. Freeman, and F. E. Evans.** 1990. Stereoselective fungal metabolism of methylated anthracenes. Appl. Environ. Microbiol. **56:**661–668.
7. **Danner, D. J., P. J. Brignac, D. Arceneaux, and V. Patel.** 1973. The oxidation of phenol and its reaction product by horseradish peroxidase and hydrogen peroxide. Arch. Biochem. Biophys. **156:**759–763.
8. **Faison, B. D., T. K. Kirk, and R. L. Farrell.** 1986. Role of veratryl alcohol in regulating ligninase activity in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **52:**251–254.
9. **Farrell, R. L., K. E. Murtagh, M. Tien, M. D. Mozuch, and T. K. Kirk.** 1989. Physical and enzymatic properties of lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **11:**322–328.
10. **Fatiadi, A. J.** 1965. Separation of pyrenediones by column chromatography. J. Chromatogr. **20:**319–324.
11. **Fedorak, P. M., and D. Grbić-Galić.** 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. Appl. Environ. Microbiol. **57:**932–940.
12. **Fedorak, P. M., K. M. Semple, R. Vazquez-Duhalt, and D. W. S. Westlake.** 1993. Chloroperoxidase-mediated modification of petroporphyrins and asphaltenes. Enzyme Microb. Technol. **15:** 429–437.
13. **Fedorak, P. M., and D. W. S. Westlake.** 1986. Fungal metabolism of *n*-alkylbenzenes. Appl. Environ. Microbiol. **51:**435–437.
14. **Field, J. A., E. de Jong, G. Feijoo-Costa, and J. A. M. de Bont.** 1993. Screening for ligninolytic fungi applicable to biodegradation of xenobiotics. Trends Biotechnol. **11:**44–49.
15. **Foster, R.** 1960. The absorption spectra of molecular complexes. Tetrahedron **10:**96–101.
16. **George, E. J., and R. D. Neufeld.** 1989. Degradation of fluorene in soil by the fungus *Phanerochaete chrysosporium*. Biotechnol. Bioeng. **33:**1306–1310.
17. **Haemmerli, S. D., M. S. A. Leisola, D. Sanglard, and A. Fiechter.** 1986. Oxidation of benzo(a)pyrene by extracellular ligninases of *Phanerochaete chrysosporium*. J. Biol. Chem. **261:**6900–6903.
18. **Hammel, K. E.** 1989. Organopollutant degradation by ligninolytic fungi. Enzyme Microb. Technol. **11:**776–777.
19. **Hammel, K. E., W. Z. Gai, B. Green, and M. A. Moen.** 1992. Oxidative degradation of phenenthrene by the ligninolytic fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58:**1832–1838.
20. **Hammel, K. E., B. Green, and W. Z. Gai.** 1991. Ring fission of anthracene by a eukaryote. Proc. Natl. Acad. Sci. USA **88:**10605–10608.
21. **Hammel, K. E., K. A. Jensen, M. D. Mozuch, L. L. Landucci, M. Tien, and E. A. Pease.** 1993. Ligninolysis by a purified lignin peroxidase. J. Biol. Chem. **268:**12274–12281.
22. **Hammel, K. E., B. Kalyanaraman, and T. K. Kirk.** 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]dioxins by *Phanerochaete chrysosporium* ligninase. J. Biol. Chem. **261:**16948–16952.
23. **Heitkamp, M. A., J. P. Freeman, D. W. Miller, and C. E. Cerniglia.** 1988. Pyrene degradation by a *Mycobacterium* sp.: identification of ring oxidation and ring fission products. Appl. Environ. Microbiol. **54:**2556–2565.
24. **Kirk, T. K., S. Croan, and M. Tien.** 1986. Production of multiple ligninase by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of a mutant strain. Enzyme Microb. Technol. **8:**27–32.
25. **Kirk, T. K., M. Tien, P. J. Kersten, B. Kalyanaraman, K. E. Hammel, and R. L. Farrell.** 1990. Lignin peroxidase from fungi: *Phanerochaete chrysosporium*. Methods Enzymol. **188:**159–171.
26. **Lee, M. L., and R. A. Hites.** 1977. Mixed charge exchange-chemical ionization mass spectrometry of polycyclic aromatic hydrocarbons. J. Am. Chem. Soc. **99:**2008–2009.
27. **Levin, R. D., and S. G. Lias.** 1982. Ionization potentials and appearance potentials measurements, 1971–1981. U.S. National Standard Reference Data Series No. 71. U.S. National Bureau of Standards, Washington, D.C.
28. **Millis, C. D., D. Cai, M. T. Stankovich, and M. Tien.** 1989. Oxidation-reduction potentials and ionization states of extracellular peroxidases from the lignin-degrading fungus *Phanerochaete chrysosporium*. Biochemistry **28:**8484–8489.
29. **Morgan, P., S. T. Lewis, and R. J. Watkinson.** 1991. Comparison of abilities of white-rot fungi to mineralize selected xenobiotic compounds. Appl. Microbiol. Biotechnol. **34:**693–696.
30. **Onodera, S., K. Igarashi, A. Fukada, J. Ouchi, and S. Suzuki.**

1989. Chemical changes of organic compounds in chlorinated water. XVI. Gas chromatographic-mass spectrometric studies of reactions of tricyclic aromatic hydrocarbons with hypochlorite in dilute aqueous solutions. J. Chromatogr. **466:**233–249.

31. **Paul, K. G., P. I. Ohlsson, and S. Wold.** 1986. The formation of peroxidase compound I with alkylhydro-peroxides. Multiple regression or pattern recognition analysis?, p. 25–30. *In* H. Greppin, C. Penel, and T. Gaspar (ed.), Molecular and physiological aspects of plant peroxidases. University of Geneva, Geneva, Switzerland.
32. **Pothuluri, J. V., J. P. Freeman, F. E. Evans, and C. E. Cerniglia.** 1990. Fungal transformation of fluoranthene. Appl. Environ. Microbiol. **56:**2974–2983.
33. **Pothuluri, J. V., J. P. Freeman, F. E. Evans, and C. E. Cerniglia.** 1992. Fungal metabolism of acenaphthene by *Cunninghamella elegans*. Appl. Environ. Microbiol. **58:**3654–3659.
34. **Proctor, C. J., B. Kralj, E. A. Larka, C. J. Porter, A. Maquestiau, and J. H. Beynon.** 1981. Studies of constitutive reactions of quinones in a reversed geometry mass spectrometer. Org. Mass Spectrom. **16:**312–323.
35. **Rosenstock, H. M., K. Draxl, B. W. Steiner, and J. T. Herron.** 1977. Energetics of gaseous ions. J. Phys. Chem. Ref. Data **6**(Suppl. 1)**:**I-34–I-690.
36. **Sanglard, D., M. S. A. Leisola, and A. Fiechter.** 1986. Role of extracellular ligninases in biodegradation of benzo(*a*)pyrene by *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **8:**209–211.
37. **Strausz, O. P., T. W. Mojelsky, and E. M. Lown.** 1992. The molecular structure of asphaltene: an unfolding story. Fuel **71:** 1355–1363.
38. **Sutherland, J. B., A. L. Selby, J. P. Freeman, F. E. Evans, and C. E. Cerniglia.** 1991. Metabolism of phenanthrene by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **57:**3310–3316.
39. **Tien, M., T. K. Kirk, C. Bull, and J. A. Fee.** 1986. Steady-state and transient-state kinetic studies on the oxidation of 3,4-dimethoxybenzyl alcohol catalyzed by ligninase of *Phanerochaete chrysosporium*. J. Biol. Chem. **261:**1687–1693.
40. **Tintel, C., J. Terheijden, J. Lugtenburg, and J. Cornelisse.** 1987. Photoreduction and photoaddition reactions of pyrenediones. Tetrahedron Lett. **28:**2057–2060.
41. **Thomas, D. R., S. Carswell, and G. Georgiou.** 1992. Mineralization of biphenyl and PCBs by the white rot fungus *Phanerochaete chrysosporium*. Biotechnol. Bioeng. **40:**1395–1402.
42. **Vazquez-Duhalt, R., P. M. Fedorak, and D. W. S. Westlake.** 1992. Role of enzyme hydrophobicity in biocatalysis in organic solvents. Enzyme Microb. Technol. **14:**837–841.
43. **Vazquez-Duhalt, R., K. M. Semple, D. W. S. Westlake, and P. M. Fedorak.** 1993. Effect of water-miscible organic solvents on the catalytic activity of cytochrome c. Enzyme Microb. Technol. **15:**936–943.
44. **Vazquez-Duhalt, R., D. W. S. Westlake, and P. M. Fedorak.** 1993. Cytochrome c as a biocatalyst for the oxidation of thiophenes and organosulfides. Enzyme Microb. Technol. **15:**494–499.
45. **Wariishi, H., H. B. Dunford, D. MacDonald, and M. H. Gold.** 1989. Manganese peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. J. Biol. Chem. **264:**3335–3340.
46. **Wariishi, H., J. Huang, H. B. Dunford, and M. H. Gold.** 1991. Reactions of lignin peroxidase compounds I and II with veratryl alcohol. J. Biol. Chem. **266:**20694–20699.
47. **Yadav, J. S., and C. A. Reddy.** 1993. Degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **59:**756–762.