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# Roles of Lignin Peroxidase and Manganese Peroxidase from *Phanerochaete chrysosporium* in the Decolorization of Olive Mill Wastewaters

SAMI SAYADI* AND RADHOUANE ELLOUZ

*Centre de Biotechnologie de Sfax, 3038 Sfax, Tunisia*

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**The relative contributions of lignin peroxidase (LiP) and manganese peroxidase (MnP) to the decolorization of olive mill wastewaters (OMW) by *Phanerochaete chrysosporium* were investigated. A relatively low level (25%) of OMW decolorization was found with *P. chrysosporium* which was grown in a medium with a high Mn(II) concentration and in which a high level of MnP (0.65 μM) was produced. In contrast, a high degree of OMW decolorization (more than 70%) was observed with *P. chrysosporium* which was grown in a medium with a low Mn(II) concentration but which resulted in a high level of LiP activity (0.3 μM). In this culture medium, increasing the Mn(II) concentration resulted in decreased levels of OMW decolorization and LiP activity. Decolorization by reconstituted cultures of *P. chrysosporium* was found to be more enhanced by the addition of isolated LiP than by the addition of isolated MnP. The highest OMW decolorization levels were obtained at low initial chemical oxygen demands combined with high levels of extracellular LiP. These data, plus the positive effect of veratryl alcohol on OMW decolorization and LiP activity, indicate that culture conditions which yield high levels of LiP activity lead to high levels of OMW decolorization.**

Environmental pollution due to the release of phenolic compounds from industrial operations has become widespread in the world. Major efforts are being made in many industrialized countries to seek remedial action against this pollution problem. Pollution by olive mill wastewaters (OMW) is becoming a crucial problem in the Mediterranean area, particularly for the main producers of olive oil, Italy, Spain, Greece, and Tunisia, which produce more than $3 \times 10^7$ $m^3$ of OMW per year. This effluent is black and highly toxic because of its high concentration of aromatic compounds. The maximum biological oxygen demand and chemical oxygen demand (COD) can be 100 and 200 g/liter, respectively (39). The chemical composition of this waste includes the black high-molecular-weight polyphenols such as tannins, anthocyanins, and catechins (16). The phytotoxic and antibacterial effects of the OMW have been attributed to the phenolic content of OMW (33). Conventional bacterial water treatment processes are relatively ineffective at removing these pollutants. OMW must be diluted more than 10-fold prior to treatment with aerobic activated sludge or by anaerobic digestion. The search for more effective treatments is crucial (2, 16).

*Phanerochaete chrysosporium* produces two classes of extracellular peroxidases involved in lignin biodegradation (24). Lignin peroxidase (LiP) catalyzes the one-electron oxidation of various aromatic compounds, with subsequent formation of aryl cation radicals which are decomposed spontaneously by various pathways. Manganese peroxidase (MnP) catalyzes the oxidation of Mn(II) to Mn(III), which in turn can oxidize several phenolic substrates (14). Both LiP and MnP, with $H_2O_2$-generating enzymes, are produced during secondary metabolism in response to nitrogen or carbon starvation (14, 24).

The irregular and recalcitrant nature of lignin and the fact that it contains substructures found in primary pollutants such as phenols, anisoles, biphenyls, and diarylethers led researchers to postulate that the nonspecific ligninolytic system produced by white rot fungi might be able to oxidatively degrade such persistent aromatic pollutants. Indeed, recent work has demonstrated that *P. chrysosporium* is capable of degrading a wide variety of phenolic compounds (30) and environmentally persistent xenobiotics and chlorinated hydrocarbons, including DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane], TNT (2,4,6-trinitrotoluene), alkyl halide insecticides, chloroanilines, polychlorinated phenols, and polychlorinated biphenyls (6, 7, 10, 17–19, 23, 29, 31, 42). *P. chrysosporium* is also able to decolorize humic acids (1), bleach plant effluent from paper mills (11, 22, 32), and dyes such as Poly Blue 411, Poly R, crystal violet, and methylene blue (8, 13, 26) and to decompose lignosulfonates (37).

In a previous paper (38) we demonstrated the ability of *P. chrysosporium* to decolorize OMW and the possible involvement of the lignin-degrading system in this process. The decolorization of OMW corresponds to depolymerization of high-molecular-weight aromatics combined with mineralization of a wide range of monoaromatics. In this paper, we report the roles of LiP and MnP in the decolorization of OMW. Mn(II) regulation was used for varying the levels of extracellular LiP and MnP. The influence of veratryl alcohol and initial COD on the level of extracellular LiP and the rate of OMW decolorization was examined.

## MATERIALS AND METHODS

**Strains and culture conditions.** *P. chrysosporium* HD, a monoconidiosporous isolate from *P. chrysosporium* BKM-F-1767 (ATCC 24725) (38), and BKM-F-1767 were the principal strains used in this study. These strains were maintained at 4°C on 2% malt extract (Fluka) slants. Subcultures were routinely made every 2 months.

Two media were used for the cultivation of *P. chrysosporium* with or without OMW. Medium A contained (per liter) (21) glucose, 10 g; $KH_2PO_4$, 2 g; $CaCl_2 \cdot 2H_2O$, 0.132 g; $MgSO_4 \cdot 7H_2O$, 1.45 g; thiamine hydrochloride, 1 mg; Tween 80, 0.5 g; D-diammonium tartrate, 1.2 mM; sodium tartrate (pH 6.5), 20 mM; veratryl alcohol, 0.4 mM; and trace-element solution without $MnSO_4$, 70 ml. This trace-element solution contained (per liter) trinitroacetic acid, 1.5 g; $MgSO_4 \cdot 7H_2O$, 3 g; NaCl, 1 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; $CoSO_4$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $ZnSO_4 \cdot 7H_2O$, 0.1 g; $CuSO_4 \cdot 5H_2O$, 0.01 g; $AlK(SO_4)_2 \cdot 12H_2O$, 0.01 g; $H_3BO_3$, 0.01 g; and $Na_2MoO_4 \cdot 2H_2O$, 0.01 g. In the presence of 100 ppm of Mn(II), this medium (high-glucose–low-nitrogen medium) allows *P. chrysosporium* to selectively produce high titers of MnP (3).

* Corresponding author. Mailing address: Centre de Biotechnologie de Sfax, B.P. "W," 3038 Sfax, Tunisia.

Medium B contained (per liter) $KH_2PO_4$, 1 g; $CaCl_2 \cdot 2H_2O$, 0.07 g; $MgSO_4 \cdot 7H_2O$, 0.35 g; $FeSO_4 \cdot 7H_2O$, 0.035 g; $ZnSO_4 \cdot 7H_2O$, 0.023 g; and $CuSO_4 \cdot 5H_2O$, 0.0035 g (38). This culture medium was buffered to pH 6.5 with disodium tartrate (20 mM). Veratryl alcohol was added to 0.4 mM. The carbon source was glycerol (10 g/liter). The nitrogen source was diammonium tartrate at 20 mM nitrogen. This high-glycerol–high-nitrogen medium exhibited increased levels of LiP activity due in part to the poorer growth rate of the fungus on glycerol, since glycerol can be used to mimic the nutritional status of carbon limitation (34).

Mn(II) was added separately as $MnSO_4 \cdot H_2O$ to obtain the specified concentrations of Mn(II) as free ions (low, 0 ppm; medium, 10 ppm; or high, 100 ppm). Centrifuged and sterilized OMW was diluted to 50 g of COD/liter (in all experiments except for the study of the influence of the initial COD) in the basal media A and B. Cultures were grown in 150- and 250-ml flasks containing 10 and 25 ml, respectively. Cultures were flushed with pure $O_2$ only at day 0. Medium B was used in the studies of veratryl alcohol and initial-COD effects. All cultures were grown in triplicate.

**OMW oxidation with purified LiP or MnP.** For LiP and MnP reactions, crude OMW was separated by ultrafiltration into low-molecular-mass (L) (<8-kDa) and high-molecular-mass (H) (>60-kDa) fractions.

LiP and MnP were a gift from the Laboratoire de Microbiologie (Institut National de la Recherche Agronomique, Grignon, France) (34). LiP isozymes with pIs of 4.6 and 4.8 and MnP isozymes with a pI of 5.15 were used. In vitro OMW depolymerization was carried out in 4-ml total volumes at 28°C for 1 h in 50 mM L-tartrate buffer (pH 5) containing enzymes (LiP or MnP, 0.06 μM), 2 ml of OMW (fraction L or H), and 66 μM $H_2O_2$. Veratryl alcohol (1.6 mM) was added for LiP reactions, and $MnSO_4$ (0.2 mM) was added for MnP reactions. Enzymes (LiP or MnP) and $H_2O_2$ were added every 15 min.

**Decolorization of OMW in reconstituted cultures with added purified LiP or MnP.** Reconstituted cultures (28, 31) were used to study the decolorization of OMW with added LiP and MnP. For this, *P. chrysosporium* HD was cultivated on medium B [10 ppm of Mn(II)]. Pellets and extracellular medium from several cultures were separated by decantation. The pellets were collected on cheesecloth in a funnel, gently washed with distilled water, and allowed to drain. Drained pellets were weighed, and a sample was taken for dry-weight determination. Reconstituted cultures were obtained by mixing 3 g of pellets (fresh weight) with 5 ml of OMW diluted in tartrate buffer (pH 5) to give 30 g of COD per liter. LiP and MnP were used at 0.06 μM. Veratryl alcohol (0.4 mM) was added for LiP reactions. $MnSO_4$ (0.2 mM) was added for MnP reactions. Glucose (13.3 mM) and glucose oxidase (1.25 nkat/ml) were used as the $H_2O_2$-generating system. Enzymes and veratryl alcohol were added every 8 h.

**Decolorization assay.** Each day, two cultures were harvested and the mycelia were washed by filtration on Whatman glass microfiber filters (GF/D). Filtrates diluted 30-fold were employed for measurement of $A_{395}$ (Shimadzu UV-visible spectrophotometer). Results were expressed as comparisons between uninoculated and filtered cultures incubated under the same conditions. For the comparison of media A and B, decolorization was expressed per gram of biomass. The final pHs of the cultures ranged between 5.3 and 5.5.

**LiP and MnP activities.** LiP activity levels were determined by the veratryl alcohol oxidation assay (41). MnP was assayed according to the method of Paszczynski et al. (35) with vanillylacetone as a substrate. Enzymatic activities were expressed in nanokatals or micromolar units.

**Molecular mass distribution of the polyphenolics.** Gel filtration on Sephadex G-50 was used in the analysis of the polyphenolic compounds present in raw OMW, *P. chrysosporium*-treated OMW, and enzyme (LiP or MnP)-treated OMW. Samples were diluted (volume per volume) in 0.05 M NaOH–0.025 M LiCl and filtered, and 2 ml of each sample was placed on a Sephadex coarse G-50 column (3 by 50 cm) which had been previously equilibrated with 0.05 M NaOH–0.025 M LiCl. Otherwise-insoluble compounds and polymerized materials are completely soluble in this buffer. The flow rate was adjusted to 0.33 ml/min, and 3-ml fractions were collected. The $A_{280}$s of the fractions were measured spectrophotometrically.

**COD determination.** The COD was estimated as described by Knechtel (27).

## RESULTS

**Contributions of LiP and MnP to OMW decolorization by intact cultures.** The time courses of LiP and MnP activities were determined in parallel with that of OMW decolorization activity (Fig. 1).

MnP activity was first detected in the extracellular medium A culture fluid between days 2 and 3 of incubation. The levels of this activity increased to a maximum on days 3 to 4 and declined to low levels by day 6 (Fig. 1). LiP activity, on the other hand, was produced at high levels in medium B. It first appeared between days 3 and 4; its level reached a maximum between days 5 and 6 and then rapidly declined.

For medium A, high-Mn(II) cultures exhibited a high level of MnP activity which reached a maximum (0.65 μM) on day 3 to 4, whereas no LiP activity was detected. In these conditions, decolorization started after the sixth day but resulted in decolorization of only 25% of OMW after 10 days of incubation. However, low-Mn(II) cultures exhibited more than 0.1 μM LiP activity, whereas either the level of MnP activity had declined or no MnP activity had been detected. In this case OMW decolorization started on day 5 and reached its relative maximum rate on day 6, when the level of LiP activity was maximal.

In contrast to medium A, low-Mn(II) cultures of medium B exhibited high levels of LiP activity which reached more than 0.3 μM at day 4 whereas no MnP activity was detected. The rate of OMW decolorization was greatest with these cultures (70%) (Fig. 1). The decolorization started also at day 2, concomitantly with LiP production. Analogously, the highest rate of OMW decolorization (day 4) correlated with the maxima of LiP activity (day 4). LiP appears to yield a higher OMW level of decolorization by *P. chrysoporium* (75%) than does MnP (25%).

*P. chrysosporium* HD cultures on medium B were used for monitoring LiP activity as a function of Mn(II) concentration. The effect of Mn(II) concentration (0 ppm [low] to 50 ppm [high]) on OMW decolorization and LiP activity in the decolorized OMW culture fluid is shown in Fig. 2. An increase in the Mn(II) concentration was accompanied by decreases in levels of OMW decolorization and LiP activity. The level of LiP activity fell to an undetectable level at an Mn(II) concentration of 40 ppm. For lower Mn(II) concentrations, high OMW decolorization levels were obtained after only 5 days, and the prolongation of incubation to 17 days only slightly enhanced the OMW decolorization.

**Biodegradation of polyphenolics.** The culture fluids from medium A [high Mn(II) concentration, high titers of MnP] and medium B [low Mn(II) concentration, high titers of LiP] were analyzed for OMW aromatic compounds (Fig. 3). Untreated OMW showed two "families" of aromatics: the first family eluted at the exclusion peak and consisted of material in excess of 30 kDa, and the second family corresponded to low-molecular-mass fractions of less than 2 kDa. Extensive depolymerization was found with the medium giving high levels of LiP production (Fig. 3). In contrast, when MnP was the major enzyme in the medium, high-molecular-mass aromatics persisted and minimal depolymerization was obtained. The OMW decolorization correlates with the extent of depolymerization. However, the gel filtration method used is not able to show if polymerization or polymerization followed by depolymerization occurred, particularly in the case of high levels of LiP production. Nevertheless, aromatics with intermediate molecular masses appeared in both the systems, and the low-molecular-mass aromatics were degraded efficiently in either the LiP or the MnP production medium.

**Utilization of purified enzymes.** The reactions of LiP and MnP in vitro were carried out under the optimized experimental conditions described in Materials and Methods by using two OMW fractions. The high-molecular-mass fraction is composed of polyphenols with molecular masses higher than 60 kDa. The second fraction contains polyphenolics with molecular masses of less than 8 kDa.

During the reaction of LiP or MnP with these fractions, an increase in color was observed, particularly with LiP ($\partial$ $OD_{395}$/min = 0.076 and 0.085 for low- and high-molecular-mass fractions, respectively, where $\partial$ $OD_{395}$ is optical density at 395 nm). To examine whether the dark color is caused by polymerization of OMW, the molecular mass distributions of the reaction mixtures were measured and compared with that of the control. No significant change in the elution patterns of the two

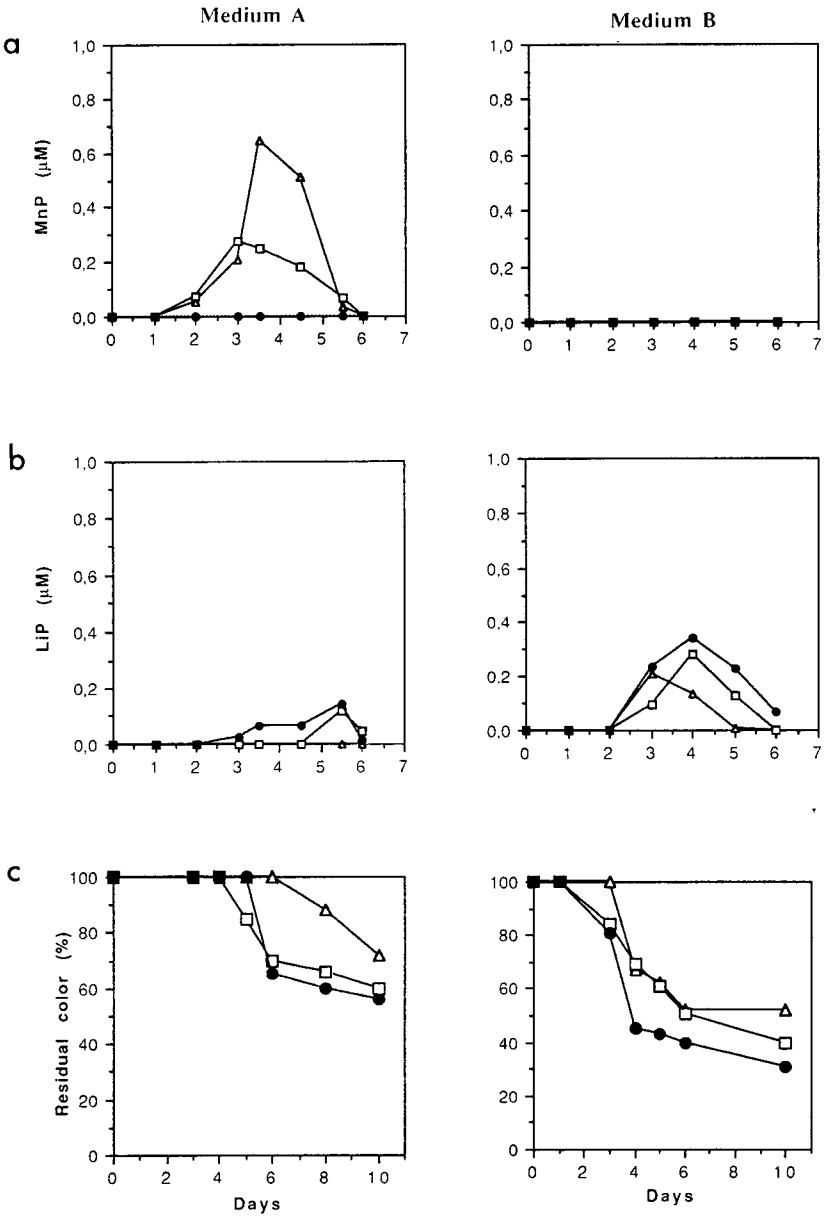


FIG. 1. Effects of culture medium and Mn(II) concentration on MnP and LiP activities and OMW decolorization by *P. chrysosporium* BKM-F-1767. MnP and LiP activities and OMW decolorization were measured in parallel in the synthetic media and the OMW culture media, respectively. The Mn(II) concentrations were 0 ppm (●), 10 ppm (□), and 100 ppm (△). (a) MnP activity; (b) LiP activity; (c) OMW decolorization. See Materials and Methods for further details.

OMW fractions after treatment with MnP was observed. However, a polyphenol fraction with high hydrodynamic volumes was formed after OMW oxidation by LiP, suggesting that polymerization had taken place (data not shown). Thus, in a cell-free system, polymerization of the OMW phenolics by LiP is shown to be the main reaction. For this reason, the effect of LiP or MnP supplementation on the decolorization of OMW was examined in reconstituted cultures (see Materials and Methods). Figure 4 shows that the addition of purified LiP provokes more decolorization than does the addition of MnP. LiP appears to be the limiting factor for OMW decolorization.

**Influence of veratryl alcohol: decolorization of OMW and its relation to extracellular LiP.** OMW decolorization experiments with *P. chrysosporium* cultures were conducted in basal medium with or without veratryl alcohol (Fig. 5). The OMW decolorization rate and the extracellular LiP level reached 69% and 3.5 nkat/ml, respectively, in the presence of veratryl alcohol, whereas only 51% of OMW color was removed and 1.8 nkat of extracellular LiP per ml was present in the absence of this secondary metabolite. Thus, the high level of OMW decolorization appears directly related to the production of extracellular LiP. *P. chrysosporium* HD produced 14 to 16 nkat of LiP per ml in the basal medium supplemented with veratryl alcohol. In the presence of OMW, this titer drops to 1.8 nkat/ml without veratryl alcohol but rises to 3.5 nkat/ml when this secondary metabolite is added. This suggests that the inhibitory effect of the OMW phenolic compounds is on LiP activity.

**Effect of initial COD. (i) OMW decolorization, COD removal, and LiP activity.** When *P. chrysosporium* was first incu-

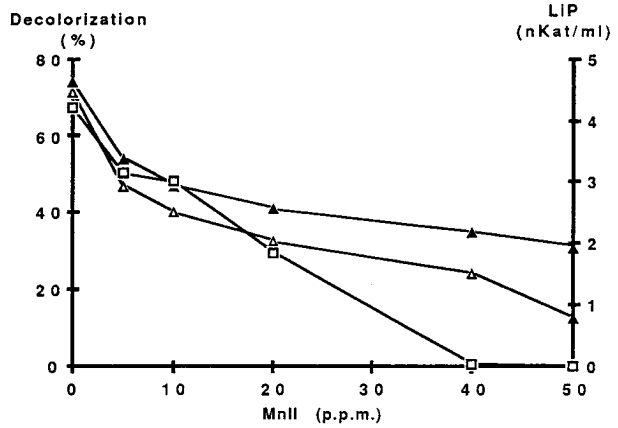


FIG. 2. Effect of Mn(II) concentration on LiP activities and decolorization of OMW by *P. chrysosporium* HD. LiP activities were determined in the OMW culture fluid (□). OMW decolorization was measured after the 5th (△) and 17th (▲) days of incubation with *P. chrysosporium* HD.

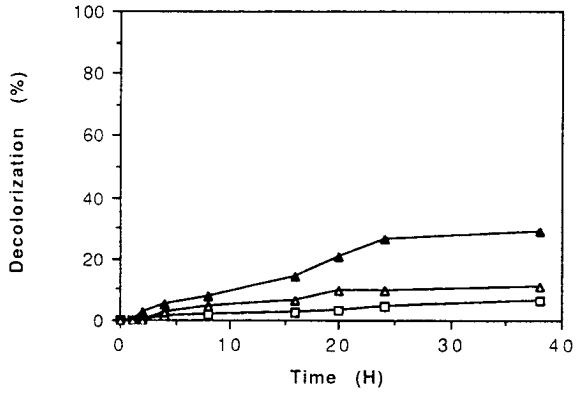


FIG. 4. Decolorization of OMW in reconstituted cultures of *P. chrysosporium* without addition of enzymes (□) or with addition of purified LiP (▲) or MnP (△).

bated with OMW as the sole carbon source, no growth was observed, presumably because of the high phenolic content of the OMW and perhaps the presence of other inhibitory compounds (140 to 160 g of COD/liter). Hence, OMW was diluted to give CODs of 28, 48, 63, 80, 105, and 145 g/liter (undiluted) (Fig. 6a). The OMW decolorization, COD removal, and extracellular LiP activity were monitored with respect to the initial COD. Figure 6a shows that OMW decolorization was detectable from an initial COD of 80 g/liter ($d$ = 0.5). The decolorization increased and reached a relative stationary phase when the initial COD was adjusted to less than 63 g/liter ($d$ = 0.66). Indeed, at a COD of 28 g/liter ($d$ = 0.87), the decolorization did not exceed 76%.

The correlation of COD removal to decolorization shows strong correlation with the colored and high-molecular-mass aromatics (Fig. 6a).

LiP activity was detected in the extracellular medium (3.11 nkat/ml) when the initial COD did not exceed 63 g/liter. This LiP activity level increased to 11.5 nkat/ml when media with a COD of 28 g/liter were used. Although the decolorization was optimal at this LiP concentration, the efficiency of the OMW decolorization was not enhanced.

**(ii) Biodegradation of high-molecular-mass aromatics.** Figure 6b shows the results of a gel filtration analysis of untreated OMW and treated OMW at different initial CODs. Low-molecular-mass aromatics were efficiently degraded at the different CODs used. However, for high-molecular-mass aromatics, the rate of depolymerization was severely affected by the initial COD; e.g., OMW at an initial COD of 80 g/liter was fairly well depolymerized. However, the reduction of the molecular masses of the aromatic compounds was more enhanced in OMW with a COD of less than 63 g/liter. For CODs of 48 and 28 g/liter, the G50 profiles are broadly similar and the depolymerization was not enhanced. These results correlate with OMW decolorization, in which the percentages of color removal for CODs of 48 and 28 g/liter are similar.

OMW depolymerization reached a stationary phase even at low initial CODs. Aromatics with intermediate molecular masses accumulated, and the yellow color remained even after 1 month of incubation.

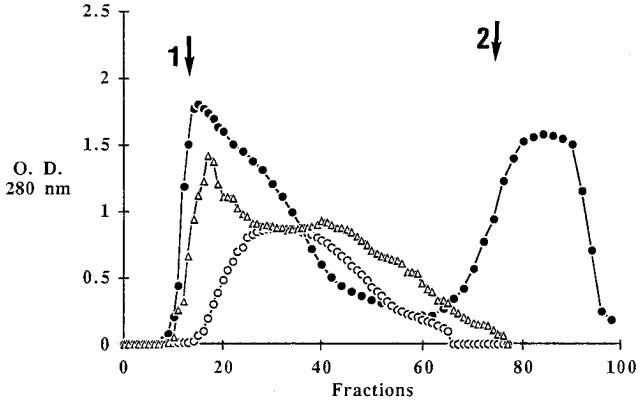


FIG. 3. Molecular mass distribution of phenolics from untreated OMW (●) and after treatment with *P. chrysosporium* BKM-F-1767 grown in medium A (MnP production) (△) and in medium B (LiP production) (○). Blue dextran (molecular mass, 200 kDa) (arrow 1) and syringic acid (molecular mass, 198 Da) (arrow 2) are indicated.

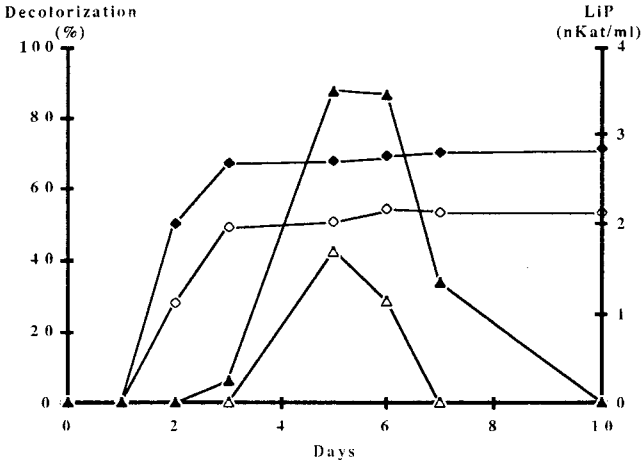


FIG. 5. Effect of veratryl alcohol on OMW decolorization by *P. chrysosporium* HD (● and ○) and on synthesis of extracellular LiP (▲ and △). Cultures were grown on basal medium B either supplemented with veratryl alcohol (● and ▲) or unsupplemented (○ and △).

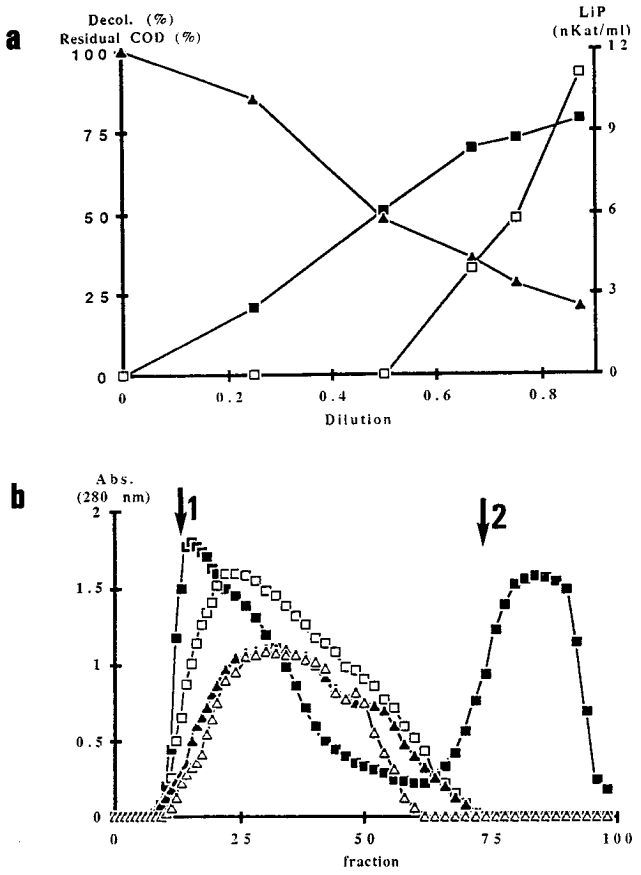


FIG. 6. (a) Effect of dilution of OMW on OMW decolorization (■), residual COD (▲), and extracellular LiP (□). (b) Changes in the molecular mass distribution of untreated OMW (■) and OMW treated for 20 days at different initial CODs: 80 g/liter (□), 48 g/liter (▲), and 28 g/liter (△). Blue dextran (molecular mass, 200 kDa) (arrow 1) and syringic acid (molecular mass, 198 Da) (arrow 2) are indicated.

## DISCUSSION

In the first report describing the decolorization of the phenolic-rich effluent OMW by *P. chrysosporium* (38), we proposed that the lignin-degrading system was responsible for OMW decolorization. This conclusion was indirect and was based on the use of several physiological factors which are known to enhance or repress lignin mineralization (i.e., high nitrogen and carbon concentrations, the use of glutamate as the nitrogen source, aeration, and the nature of the carbon source) (9, 24). We have also demonstrated in a previous study (39) that strains producing low levels of LiP did not efficiently decolorize OMW, in contrast to the strains producing high levels of LiP (HD and BKM-F-1747), which rapidly decolorized OMW. This report confirms these results and extends them to include the relative contributions of LiP and MnP to OMW decolorization. Our results suggest that culture conditions that yield high levels of LiP activity lead to high levels of OMW decolorization.

The appearance of LiP in the extracellular medium is delayed with the onset of OMW decolorization (Fig. 1 and 5). This phenomenon was also observed for $^{14}$C-lignin biodegradation (25). This could be explained by the fact that the veratryl alcohol oxidation assay was not sensitive enough to detect LiP which nevertheless was present in amounts sufficient to achieve the observed OMW decolorization. A fraction of LiP could also be associated with the fungal cell wall. Kurek and Odier (28) found that some fraction of LiP was strongly associated with the mycelium, since washed pellets retain some lignin-degrading ability. Moreover, the existence of LiP in association with the fungal cell wall has been detected by immunochemical methods (12). In the same way, the presence of cell envelope-bound MnP activity cannot be ruled out even at low Mn(II) concentrations. This type of enzyme is capable of generating freely diffusible Mn(III) which in turn oxidizes the terminal phenolic substrate. Polyphenolics may undergo degradation-dependent binding to the fungal mycelium, and such bound enzymes could be more active than extracellular ones. Phenolic degradation fragments could serve as substrates of other enzyme systems or be sequestered as osmiophilic granules within the fungal sheath. Arylalcohol oxidase and other intracellular or extracellular enzymes could be involved in the OMW decolorization process.

Veratryl alcohol has a positive effect on OMW decolorization, and a higher degree of OMW decolorization paralleled the high level of LiP activity obtained in the basal medium supplemented with this secondary metabolite (Fig. 5). Veratryl alcohol has been shown to enhance LiP activity and lignin mineralization (9). The inhibitory effect of OMW phenolic compounds on LiP activity was observed. Indeed, phenolic compounds are able to serve as reductants of LiP in its oxidized (compound I) state, with subsequent polymerization. For such cases LiP did not complete its catalytic cycle, and its functionality was lost. Veratryl alcohol prevents this inhibition by phenolic compounds, and thus its addition resulted in greater LiP activity and better decolorization. Veratryl alcohol could act as a mediator of oxidation of phenolic compounds as suggested by Harvey et al. (20). Alternatively, Wariishi and Gold (43) have shown that veratryl alcohol can convert LiP compound III (inactive form) to the native state.

Purified LiP or MnP was not able to decolorize even the low-molecular-mass polyphenolic fraction of OMW (<8 kDa). Instead, LiP polymerizes this fraction to yield aromatics with higher molecular masses. However, the relative increase in the percentage of OMW decolorization after the addition of purified LiP to reconstituted cultures (Fig. 4) could suggest the important role of LiP in the presence of mycelia of *P. chrysosporium*. LiP can degrade OMW polyphenolics into small fragments which can then be further absorbed by fungal cells and subsequently mineralized. Several authors have hypothesized that this way of binding lignin to the fungal cell wall in ligninolytic cultures is necessary for effective lignin degradation rather than polymerization (24) to occur. Until now, an OMW treatment process based on whole fungus grown under conditions for high-level LiP production has been preferable.

In somewhat analogous studies, the important roles of LiP in the dechlorination of polychlorinated phenols (19), the oxidation of several recalcitrant compounds such as 2,4-dichlorophenol (42), polycyclic aromatic hydrocarbons, dibenzo[*p*]dioxins, benzo[*a*]pyrene, and methylene blue (15, 18, 26), and the decolorization of humic acids (1) have been described. In the same way, Perez and Jeffries (36) reported that the mineralization of a $^{14}$C-ring-labeled dehydrogenative polymerisate of coniferyl alcohol by *P. chrysosporium* correlates with the appearance of LiP but not MnP and confirmed the results of Boominathan et al. (4), who showed that the *lip* mutant of *P. chrysosporium*, which lacks the ability to produce LiP but can produce a full component of MnP, exhibits only 16% of the ligninolytic activity of the wild-type strain. However, Michel et al. (32) noted that MnP of *P. chrysosporium* plays an important role in the decolorization of kraft bleach plant effluent.

The higher level of OMW decolorization obtained by LiP

production media than by MnP production media is in accordance to that of lignin degradation but is in contrast to the decolorization of bleach plant effluent from paper mills. With regard to substrate characteristics, OMW contains a wide range of aromatics with low to high molecular masses. The latter are more responsible for the black color of the effluent. Decolorization amounts to the depolymerization of these high-molecular-mass aromatics, which appear to require attack by LiP rather than MnP (LiP is rate limiting), as for lignin polymer. Nevertheless, cultures which exhibited exclusively high titers of MnP bring about only minimal depolymerization of the high-molecular-mass aromatics but efficiently degrade the low-molecular-mass aromatics, which are also predominantly phenolics. Wariishi et al. (44) reported that in a cell-free system, manganese peroxidase was able to depolymerize different $^{14}C$-labeled dehydrogenative polymerisates but did not achieve degradation to $^{14}CO_2$.

In conclusion, the use of *P. chrysosporium* for depollution of OMW by its conversion of phenolic compounds appears to be a particularly attractive approach. At present LiP appears to be one of the key enzymes in this process.

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