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Decolorization of Olive Mill Waste-waters by Free and Immobilized *Phanerochaete chrysosporium* Cultures

Effect of the High-Molecular-Weight Polyphenols

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ABSTRACT

This paper describes the decolorization and chemical oxygen demand (COD) removal of olive mill waste-waters (OMW) by *Phanerochaete chrysosporium* grown in agitated submerged cultures. When *P. chrysosporium* was cultivated in the form of pellet, no decolorization of crude OMW was observed. Decolorization occured only after removing by ultrafiltration, the high-mol-wt (HM) polyphenolic fraction (>60 kDa). The use of high lignin peroxidase (LiP) producing medium yielded the highest levels of OMW decolorization and COD removal. In this case, extensive depolymerization and subsequent accumulation of phenolics with intermediates molecular weight were observed. Furthermore, increasing the concentration of the HM fraction decreased the color and COD removals. The decolorizing activity was lost when the concentration of the HM fraction reached 25% (v/v). Consequently, LiP activity was found to be completely inhibited in the presence of HM fraction, but not with the low-mol-wt (LM) polyphenolic fraction (<8 kDa). The use of *P. chrysosporium* immobilized on polyurethane foam resulted in efficient decolorization of crude OMW. Moreover, the addition of an induction medium was shown to perform several repeated batch cultures for OMW decolorization and COD removal.

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Index Entries: Olive mill waste-waters; decolorization; chemical oxygen demand; immobilization; *Phanerochaete chrysosporium.*

Abbreviations: LiP: lignin peroxidase; MnP: manganese peroxidase; COD: chemical oxygen demand; HM: high-mol-wt; MM: medium-mol-wt; LM: low-mol-wt.

INTRODUCTION

Environmental pollution owing to the release of phenolic compounds from industrial operations has become widespread in the world. Major efforts in many industrialized countries are being made to seek remedial action of this pollution problem. Pollution by olive mill waste-waters (OMW) is becoming a crucial problem in the Mediterranean area, particularly with the main producers of oil, Italy, Spain, Greece, and Tunisia. Indeed more than 3×10^7 m^3- of OMW are produced per year. This effluent is black and highly toxic owing to its high concentration of aromatic compounds. The maximum biological oxygen demand (BOD) and chemical oxygen demand (COD) concentration can be 100 and 200 g/L, respectively. The chemical composition of this waste includes polyphenols such as tannins, anthocyanins, and catechin (*1*). The phytotoxic and antibacterial effects of the OMW has been attributed to their phenolic content (*2*). Conventional bacterial water treatment processes are relatively ineffective for removing these pollutants. OMW must be diluted more than 10-fold prior to treatment by aerobic activated sludge or anaerobic digestion. In relation to this major pollution problem, the search for more effective treatments is crucial (*1,3*).

Phanerochaete chrysosporium is one of the organisms whose ligninolytic enzyme system has been studied extensively (*4*). Its high ligninolytic activity, rapid growth, and ability to produce asexual spores led to many attempts for a biotechnological application. Indeed, recent work has demonstrated that *P. chrysosporium* is capable of degrading a wide variety of phenolic compounds (*5*) and environmentally persistent xenobiotics and chlorinated hydrocarbons including DDT, TNT, alkyl halide insecticides, benzo(a)pyrene, chloroanilines, pentachlorophenols, and polychlorinated biphenyls (*6–11*). *P. chrysosporium* is also able to decolorize bleach plant effluent (*12,13*); humic acids (*14*); dyes such as Poly Blue 411, Poly R, Crystal violet, and methylene blue (*15*); and to decompose lignosulfonates (*16*).

In a previous paper (*17*), we demonstrated the ability of *P. chrysosporium* to decolorize OMW in static cultures and the possible involvement of the lignin degrading system. The decolorization of OMW corresponds to depolymerization of high-mol-wt (HM) aromatics combined with mineralization of a wide range of monoaromatics. In this paper, we report the decolorization of OMW by agitated submerged cultures of free and

immobilized *P. chrysosporium*. The inhibitory effect of the HM polyphenolics (>60 kDa) on the LiP activity and OMW decolorization was discussed.

MATERIALS AND METHODS

Strain

P. chrysosporium HD, a monoconidiosporous isolate from strain BKM-F-1767 (ATCC 24725), was used in this study. This strain was maintained at 4°C on 2% malt extract browth (Fluka) slants. Subcultures were routinely made every 2 mo.

Culture Conditions

Two media were used for the cultivation of *P. chrysosporium* with or without OMW. Medium A (*18*), which produces particularly MnP, contained (per liter: glucose, 10 g; KH_2PO_4, 2 g; $CaCl_2$, $2H_2O$, 0.132 g; $MgSO_4$, $7H_2O$, 1.45 g; thiamine hydrochloride, 1 mg; Tween-80, 0.5 g; D-diammonium tartrate, 1.2 m*M*; di-sodium tartrate pH 6.5, 20 m*M*; veratryl alcohol, 0.4 m*M*; and 70 mL of trace element solution without $MnSO_4$. This trace element solution contained (per liter): trinitroacetic acid, 1.5 g; $MgSO_4$, $7H_2O$, 3 g; NaCl, 1 g; $FeSO_4$, $7H_2O$, 0.1 g; $CoSO_4$, 0.1g; $CaCl_2$, $2H_2O$, 0.1 g; $ZnSO_4$, $7H_2O$, 0.1 g; $CuSO_4$, $5H_2O$, 0.01 g; $AlK(SO_4)_2$, $12H_2O$, 0.01 g; H_3BO_3, 0.01 g; and Na_2MoO_4, $2H_2O$, 0.01 g. High concentration of MnII was added to this medium as $MnSO_4$, H_2O (100 ppm) to increase the production of manganese peroxidases.

Medium B (*17*), which produces particularly LiP, contained (per liter): KH_2PO_4, 1 g; $CaCl_2$, $2H_2O$, 0.07 g; $MgSO_4$, $7H_2O$, 0.35 g; $FeSO_4$,$7H_2O$, 0.035 g; $ZnSO_4$, $7H_2O$, 0.023 g; and $CuSO_4$, $5H_2O$, 0.0035 g (*40*). This culture medium was buffered to pH 6.5 with di-sodium-tartrate (20 m*M*). Veratryl alcohol was added to 0.4 m*M*. The carbon sources were glycerol (10 g/L). The nitrogen source was diammonium tartrate at 20 m*M* of nitrogen. $MnSO_4$, H_2O was not added in this medium for increasing the production of LiP.

P. chrysosporium Free Pellets

Sterilized OMW (with or without the high-mol wt polyphenolic fraction) was added in the basal media A and B. Cultures were inoculated with 5×10^7 conidiospores / 50 mL of medium.

Immobilized Cultures

P. chrysosporium was cultivated in the synthetic medium B. Polyurethane foam (Filtren T45, from Recticel, Wetteren, Belgium) was used as the support. Seven polyurethane foam cubes, each 2 cm × 2 cm × 1 cm, were placed in 250-mL Erlenmeyers flasks and autoclaved at 120°C at 20 min.

After inoculation of the synthetic medium with 10^7 conidiospores/50 mL, conidiospores attached easily to the polyurethane foam and growth took place throughout the support. At d 4, the extracellular medium was removed and 20 mL of crude and sterilized OMW/cycle of decolorization were added. An activation medium was added to the crude OMW when mentioned in the text. This medium contained: glycerol, 2.5 g/L; diammonium tartrate, 0.45 g/L; yeast extract, 0.1 g/L; veratryl alcohol, 2.5 m*M*; and Tween-80, 0.25 g/L.

Cultures were grown in a rotary shaker (120 rpm, 2.5-cm-diameter cycle) and flushed with pure O_2 for 2 min at the time of inoculation and every day after the third day. All cultures were grown in triplicate.

OMW Fractionation

Crude OMW was divided into different molecular mass fractions by ultrafiltration (Gamma Filtration, France) through polysulfonate organic membranes (PCI, Laverstoke Mill, Whitchurch, England). These membranes have a multitubular configuration with a cut-off of 8 and 60 kDa. The molecular mass fractions chosen were low molecular mass (LM < 8 kDa), medium molecular mass (8 kDa < MM < 60 kDa) and high molecular mass (HM > 60 kDa).

Decolorization Assay

Each day, two cultures were harvested and the mycelium washed by filtration on Whatman Glass Microfiber Filters GF/D. Thirty-fold diluted supernatants were employed for the measurement of absorbance at 395 nm (Spectrophotometer UV-Visible Shimazu). Results were expressed in comparison to noninoculated and filtered cultures incubated in the same conditions. In the comparison of media A and B, decolorization was given per gram of biomass. The final pH of the cultures ranged between 5.3 and 5.5.

LiP and MnP Activities

Lignin peroxidase activity was determined using the veratryl alcohol oxidation assay (*19*). Manganese-dependent peroxidase was assayed according to Paszczynski et al. (*20*), using vanillylacetone as substrate. Enzymatic activities were expressed in nanokatals (nKat) (1 nKat/mL MnP and LiP activities give, respectively, 0.018 and 0.042 μM ($10^{-6}M$) of enzyme).

Molecular Weight Distribution of Polyphenolics

Gel filtration on Sephadex G-50 was used in the analysis of the polyphenolic compounds present in raw OMW and *P. chrysosporium* treated OMW. Samples (2 mL) were filtered and placed on a Sephadex coarse G-50 column (3 × 50 cm) previously equilibrated with NaOH 0.05*M*, LiCl 0.025*M*. The flowrate was adjusted to 0.33 mL/min, and 3 mL fractions

were collected. These fractions were measured spectrophotometrically at 280 nm.

COD Determination

COD was estimated as described by Knechtel (*21*).

RESULTS

Decolorization of OMW by *P. chrysosporium* Grown in Agitated Submerged Cultures in the Form of Pellets

Recently, manganese levels in the medium were shown to have a dramatic effect on the levels of production of MnP and LiP (*18*). We first examined the decolorization of unmodified OMW by *P. chrysosporium* grown in two different media. Medium A, which contains 100 ppm MnII, produces high titers of MnP (15–20 nKat/mL) and no detected LiP, whereas medium B, which did not contain MnII (0 ppm), exhibited relatively high titers of LiP (4–5 nKat/mL) and only traces of MnP. Figure 1 shows that *P. chrysosporium* is not able to decolorize unmodified OMW although a normal growth was observed (data not shown).

In order to determine the cause of this phenomenon, OMW was fractionated by ultrafiltration technique into different mol-wt polyphenols. Therefore, decolorization was obtained only after discarding the HM fraction (>60 kDa). Figure 1 shows that in the high MnP producing medium, decolorization of the modified OMW started after the third day but gave only 70% of OMW decolorization after 10 d of incubation. However, low MnII cultures that exhibited LiP decolorized these modified OMW at a high rate. In this case, decolorization started after 4 d but reached more than 85% at 9 d.

The medium expressing MnP decolorized OMW before that of LiP. This could be correlated to the fact that MnP is secreted before LiP. These results suggest also that polyphenols of HM (>60 kDa) could represent the limiting step of the decolorization of OMW by *P. chrysosporium* in agitated cultures.

COD removal was relatively appreciable (50%) only in the case where *P. chrysosporium* exhibited LiP. However, high MnII cultures give only a slight COD removal (30%) (Fig. 1B). In fact, the darkly colored fraction (8 kDa < MM < 60 kDa) contained in the fractionated OMW is present at low concentration (TS or COD) as shown in Fig. 2A. This suggests that the treatment of fractionated OMW, which is composed by the mixture (LM + MM), with *P. chrysosporium* gives a rapid depolymerization/decolorization without an important COD decrease.

The elution patterns of the fractionated OMW untreated and treated with *P. chrysosporium* were examined by gel filtration in order to clarify the expected change of OMW phenolic distribution (Fig. 2B). Untreated

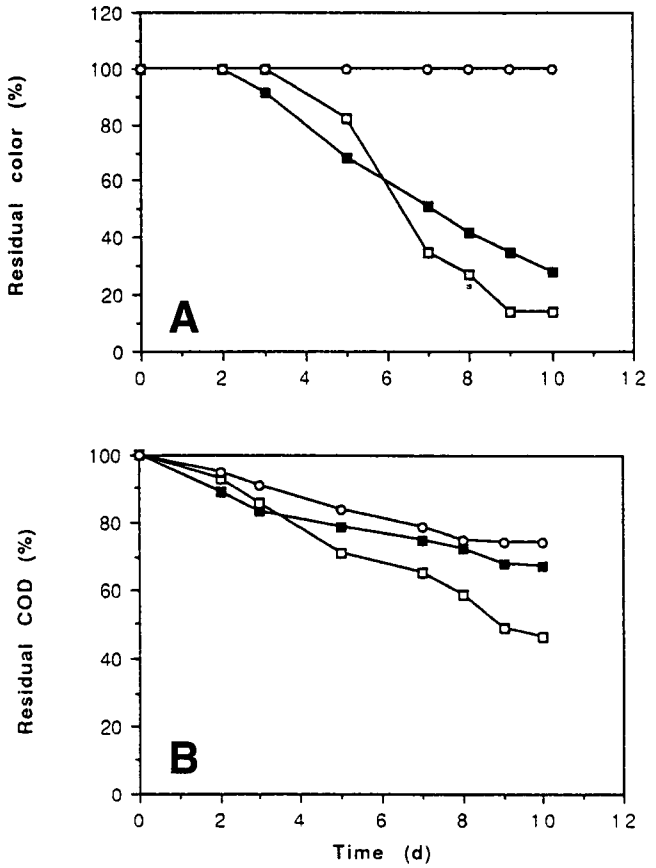

Fig. 1. Decolorization (**A**) and COD removal (**B**) of crude OMW (○) or OMW without the HM fraction (■,□) by *P. chrysosporium* HD grown in the form of pellets. (■), medium A, MnP production; (□), medium B, LiP production.

fractionated OMW showed three families of aromatic compounds. *P. chrysosporium* treated OMW showed extensive depolymerization/degradation with the LiP production medium. However, when MnP was the major enzyme, a depolymerization of the HM polyphenolics and a corresponding higher content of molecules with smaller hydrodynamic volumes were observed. This accumulation of aromatics having an intermediate molecular weight could correlate with the low COD removal.

Effect of the Addition of Different Concentrations of the High-Molecular-Weight Fraction (>60 kDa)

In order to determine the critical concentration of HM polyphenolics up to which *P. chrysosporium* is able to decolorize OMW, increased concentrations of this fractionated HM polyphenolics were supplemented into the mixture LM + MM). As shown in Fig. 3A, increasing the concentration of the HM fraction in the medium decreased the decolorization activities by *P. chrysosporium* in agitated cultures. However, using an LiP

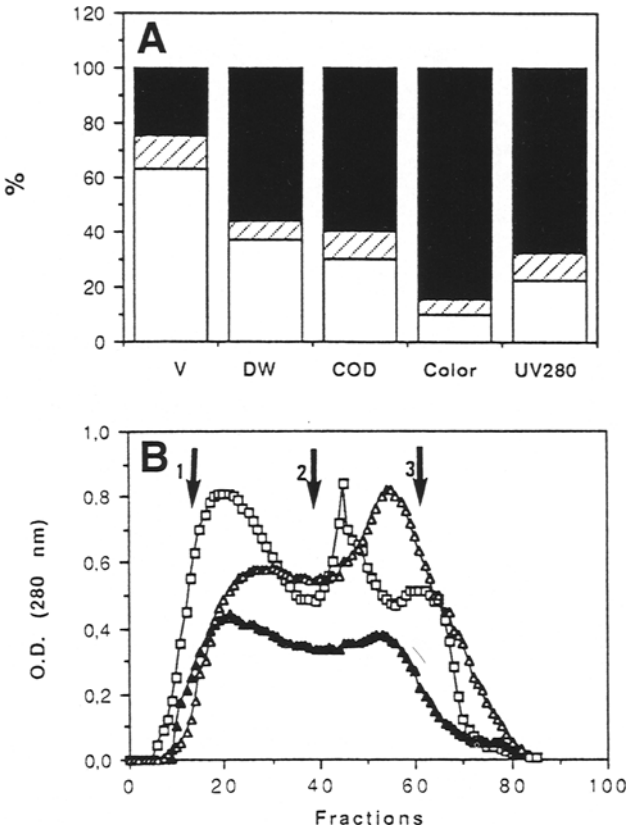

Fig. 2. (**A**) Characteristics of the 3 ultrafiltrated OMW polyphenolic fractions. V, volume; DW, dry weight. (□), LM < 8 kDa; (▨), 8 kDa < MM < 60 kDa; (■), HM > 60 kDa. (**B**) Molecular weight distribution of "phenolics" from untreated OMW (□) and after treatment with *P. chrysosporium* HD grown in agitated free cultures, in medium A (MnP production) (△) and in medium B (LiP production) (▲). 1, Blue dextran (mol wt = 200 kDa); 2, Lysozyme (mol wt = 15 kDa) 3, syringic acid (mol wt = 198 D).

producing medium, this fungus was able to achieve high decolorization levels when the concentration of the HM fraction was up to 10% (v/v). No decolorization was observed when the concentration of this black fraction reached 25% (v/v).

Increasing the concentration of the HM fraction leads also to the decrease of the COD removal as shown in Fig. 3B.

Inhibition of LiP Activity by OMW Phenolic Compounds

Extracellular LiP activity was compared in the basal medium B (as control), in the same medium containing the LM fraction (<8 kDa) at 10 g/L COD, and in the basal medium containing the HM fraction (>60 kDa) at the same COD concentration.

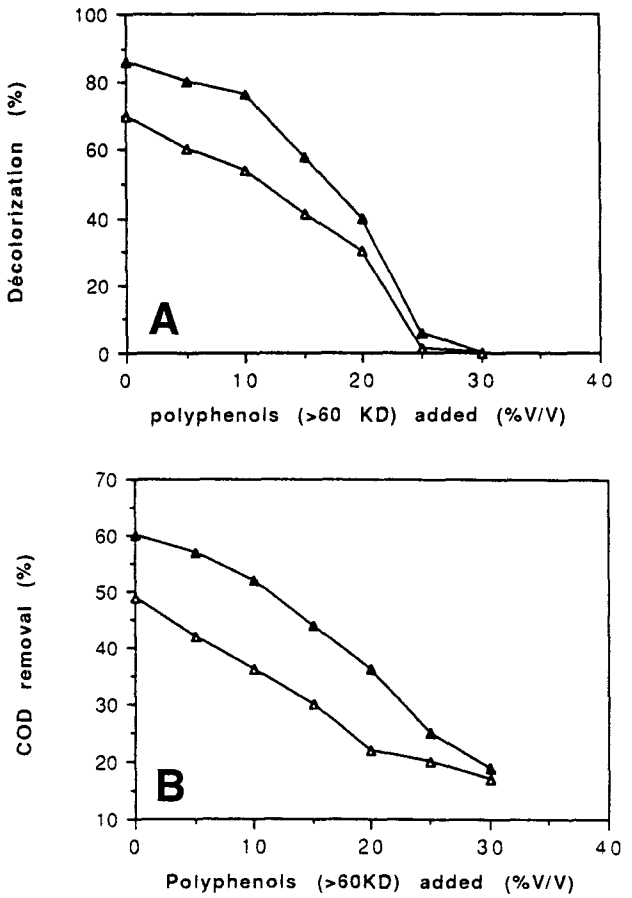

Fig. 3. Effect of the HM polyphenolic fraction on the decolorization (**A**) and COD removal (**B**) of OMW by *P. chrysosporium* HD grown in the form of pellets. (△), medium A, MnP production; (▲), medium B, LiP production.

Extracellular MnP was detected in the control but at a very low concentration (less than 0.5 nKat/mL). Figure 4 shows that, in the absence of OMW phenolics, *P. chrysosporium* produced 4.5 nKat/mL at 4 d. In the presence of LM aromatic fraction, this titre decreased to 2.5 nKat/mL, but LiP activity was still detected until 7 d. However, no LiP activity was detected in the presence of HM fraction, suggesting the high inhibitory effect of these compounds on LiP activity.

Taking into account that *P. chrysosporium* is able to decolorize in static cultures the OMW with its HM polyphenolic fraction, we can postulate that the low level of LiP activity (5 nKat/mL) produced in agitated cultures (compared to 14 nKat/mL in static cultures) was not sufficient for the depolymerization of the HM fraction. Hence, the decolorization of crude (unfractionated) OMW by *P. chrysosporium* in the form of a pellet could be

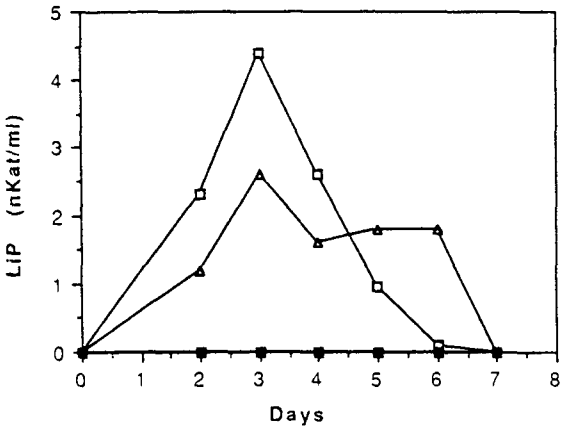

Fig. 4. Time frame of extracellular LiP activity produced by *P. chrysosporium* HD grown in agitated free cultures, without addition of OMW (□), in the presence of the LM polyphenolic fraction (△), or in the presence of the HM fraction (■).

hindered severely by the combined inhibition of LiP activity caused by the agitation and the concentrated high-mol-wt polyphenolics (>60 kDa).

Decolorization of Crude OMW by Repeated Batch of *P. chrysosporium* Immobilized on Polyurethane Foam

In the synthetic medium used in this study (medium B), *P. chrysosporium* HD produced a maximum LiP activity (20–23 nKat/mL) at 5 d. For OMW decolorization assays, extracellular medium was removed and crude OMW was introduced at 4 d. OMW decolorization by active mycelium of *P. chrysosporium* was found to be high with rotary agitation as well as with "swinging" agitation. Figure 5A,B illustrates two cycles of crude OMW decolorization. In the first cycle, decolorization appeared after only 16 h of incubation and more than 70% of the color and COD removals were achieved after 85 h, suggesting the high degradative ability of *P. chrysosporium* grown in an immobilized state. However, only 20% of OMW decolorization was obtained in the second cycle owing to abundant sporulation, which could suggest that the mycelium is in a severe starvation condition (OMW is nitrogen carenced effluent). An activation medium (described in the Methods section) was added to the crude OMW for repeated batch decolorization by *P. chrysosporium* immobilized mycelium. Figure (5C,D) showed that three cycles of OMW decolorization and COD removal could be achieved by reusing *P. chrysosporium* HD mycelium immobilized on polyurethane foam without sporulation or lysis.

P. chrysosporium imobilized cultures appeared to be the most adequate system for the efficient decolorization of OMW with its HM polyphenolic fraction.

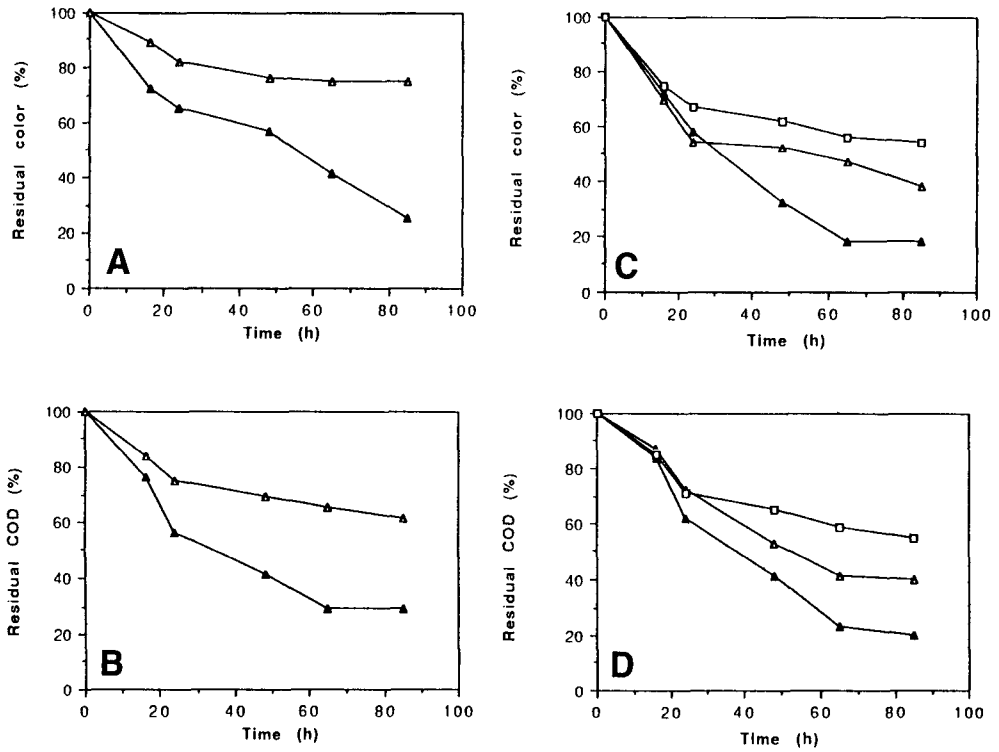

Fig. 5. Decolorization and COD removal of crude OMW by repeated batch of *P. chrysosporium* immobilized on polyurethane foam with (**C,D**) or without (**A,B**) addition of the induction medium. (▲), 1st cycle; (△), 2nd cycle; (□, 3rd cycle.

DISCUSSION

In the first report describing the decolorization of the phenolic rich effluent OMW by static cultures of *P. chrysosporium* (*17*), we proposed that the lignin degrading system was responsible for OMW decolorization. This conclusion was indirect and based on the use of several physiological factors known to enhance or repress lignin mineralization (i.e., high nitrogen and carbon concentrations, use of glutamate as nitrogen source, aeration, nature of carbon source) (*22*). This report confirms these results and extends them to include the relative predominant role of LiP in OMW decolorization and the deleterious effect of the HM polyphenolic fraction (>60 kDa) on its activity.

The absence of decolorization of unmodified OMW by *P. chrysosporium* grown in agitated free submerged cultures could be related first to the relative low LiP production levels by agitation. Indeed, agitation is known to have an inhibitory effect on both ligninolytic activity and secretion of LiP (*22*). Janshekar and Fieshter (*23*) have demonstrated ligninase pro-

duction in a 42-L stirred-tank reactor only when polypropylene glycol was present in the culture medium. Venkatadri and Irvine (*24*) reported that mechanical inactivation of ligninase (lignin peroxidase) is possibly the reason why ligninase is low or absent in agitated shake flask cultures. Agitation seems to affect the catalytic activity of lignin peroxidase and has no apparent effect either on the rate of liginase production or on the *P. chrysosporium* physiology.

Ultrafiltration technique has permitted the isolation of the LM and HM polyphenolic fractions. Our results showed that the HM fraction inhibited OMW decolorization as well as LiP activity. In the presence of the LM fraction, LiP activity was low at the first 3 d of incubation but remained constant up to the sixth day. This LM fraction contained simple phenolics, hydrolyzable tannins, some condensed tannins, and anthocyanins (*1,17*). Oligomeric tannins could be inhibitory to LiP activity but simple phenolics (syringate, veratrol, coumarate, and vanillate) would induce or enhance LiP activity when present at a moderate concentration. The HM polyphenolic fraction contained "humic acid like" compounds not studied or characterized for this effluent. Our results showed that this fraction completely inhibits the LiP activity. The study described by Blondeau (*14*) also reported that synthetic humic acids and melanoidins strongly inhibit the LiP activity, which was at least partly responsible for the humus decolorization. Natural or synthetic humic acids could inhibit peroxidase activity by competitive as well as noncompetitive interactions. More extensive study of the enzymatic specificities can provide useful information on the chemical structure of the HM polyphenolics contained in OMW.

In order to overcome the sensitivity of lignin peroxidase to agitation, *P. chrysosporium* was immobilized on polyurethane foam. Three repeated batches of OMW decolorization were achieved when a regeneration/activation medium was added to crude OMW, suggesting the possible use of the RBC (Rotating Biological Contactor) system (*13*), which would be the convenient system for the continuous decolorization of this effluent (the RBC allowed *P. chrysosporium* to be in alternating contact with the substrate and oxygen). Immobilization of *P. chrysosporium* was used as an efficient method in producing extracellular LiP as well as in depollution purposes. Numerous carriers have been tried for the immobilization of this fungus, such us nylon web, polyurethane foam, silicon tubing, sintered glass, porous ceramic, poly(styrene-divinylbezene), polypropylene stainless-steel, agarose, and agar gel beads (for detail *see* ref. *24*).

In conclusion, the use of *P. chrysosporium* for depollution of OMW by its conversion of phenolic compounds was evaluated. At present, agitation and HM polyphenolics (>60 kDa) showed to have negative effects on LiP activity and OMW decolorization. The immobilization procedure appears as a particularly attractive alternative for the efficient decolorization of such an effluent.

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REFERENCES

1. Hamdi, M., Kadir, A., and Garcia, J. L. (1991), *Appl. Microbiol. Biotechnol.* **34,** 829–831.
2. Moreno, E., Perez, J., Ramos-Cormenzanaand, A., and Martinez, J. (1987), *Microbios,* **51,** 169–174.
3. Boari, G., Brunetti, A., Passino, R., and Rozzi, A. (1984), *Agric. Wastes* **10,** 161–175.
4. Kirk, T. K. and Farrell, R. L. (1987), *Ann. Rev. Microbiol.* **41,** 465–505.
5. Leatham, G. F., Crawford, R. L., and Kirk, T. K. (1983), *Appl. Environ. Microbiol.* **46,** 191–197.
6. Hammel, K. E., Kalyanaraman, B., and Kirk, T. K. (1986), *J. Biol. Chem.* **261,** 16,948–16,952.
7. Bumpus, J. A. (1989), *Appl. Environ. Microbiol.* **55,** 154–158.
8. Kennedy, D. W., Aust, S. D., and Bumpus, J. A. (1990), *Appl. Environ. Microbiol.* **56,** 2347–2353.
9. Haemmerli, S. D., Leisola, M. S. A., Sanglard, D., and Fiechter, A. (1986), *Biol. Chem.* **261,** 6900–6903.
10. Lamar, R. T. and Dietrich, D. M. (1990), *Appl. Environ. Microbiol.* **56,** 3093–3100.
11. Thomas, D. R., Carswell, K. S., and Georgiou G. (1992), *Biotech. Bioeng.* **40,** 1395–1402.
12. Fukui, H., Presnell, T. L., Joyce, T. W., and Chang, H. M. (1992), *J. Biotechnol.* **24,** 267–275.
13. Joyce, T. W., Chang, H., Campbell, A. G., Gerrard, E. D., and Kirk, T. K. (1984), *Biotechnol. Adv.* **2,** 301–308.
14. Blondeau, R. (1989), *Appl. Environ. Microbiol.* **55,** 1282–1285.
15. Glenn, J. K. and Gold, M. H. (1983), *Appl. Environ. Microbiol.* **45,** 1741–1747.
16. Ritter, D., Jaklin-Farcher, S., Messner, K., and Stachelberger, H. (1990), *J. Biotechnol.* **13,** 229–241.
17. Sayadi, S. and Ellouz, R. (1992), *App. Microbiol. Biotechnol.* **37,** 813–817.
18. Bonnarme, P. and Jeffries, T. W. (1990), *Appl. Environ. Microbiol.* **56,** 210–217.
19. Tien, T. and Kirk, T. K. (1984), *Proc. Natl. Acad. Sci. USA* **81,** 2280–2284.
20. Paszcynski, A., Huynh, V. B., and Crawford, R. (1985), *FEMS Microbiol. Lett.* **29,** 37–41.
21. Knechtel, R. J. (1978), *Water Pollut. Control* **May/June,** 25–29.
22. Faison, B. D. and Kirk, T. K. (1985), *Appl. Environ. Microbiol.* **49,** 299–304.
23. Janshekar, H. and Fiechter, A. (1988), *J. Biotechnol.* **8,** 97–112.
24. Venkatadri, R. and Irvine, R. L. (1990), *Appl. Environ. Microbiol.* **56,** 2684–2691.
25. Ruckenstein, E. and Wang, X. B. (1994), *Biotech. Bioeng.* **44,** 79–86.