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ORIGINAL PAPER

Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes

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Abstract The effect of *Phanerochaete chrysosporium* and *Pleurotus ostreatus* whole cells and their lignino-lytic enzymes on models of colored industrial wastewaters was evaluated. Models of acid, direct and reactive dye wastewaters from textile industry have been defined on the basis of discharged amounts, economic relevance and representativeness of chemical structures of the contained dyes. *Phanerochaete chrysosporium* provided an effective decolourization of direct dye wastewater model, reaching about 45% decolourization in only 1 day of treatment, and about 90% decolourization within 7 days, whilst *P. ostreatus* was able to decolorize and detoxify acid dye wastewater model providing 40% decolourization in only 1 day, and 60% in 7 days. *P. ostreatus* growth conditions that induce laccase production (up to 130,000 U/l) were identified, and extra-cellular enzyme mixtures, with known laccase isoenzyme composition, were produced and used in wastewater models decolourization. The mixtures decolorized and detoxified the acid dye wastewater model, suggesting laccases as the main agents of wastewater decolourization by *P. ostreatus*. A laccase mixture was immobilized by entrapment in Cu-alginate beads, and the immobilized enzymes were shown to be effective in batch decolourization, even after 15 stepwise additions of dye for a total exposure of about 1 month.

Keywords Textile dyes decolourization · Industrial effluent treatment · Ligninolytic fungi · Laccase · Manganese-peroxidase · Color industry

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Introduction

Dyes are widely used within the food, pharmaceutical, cosmetic, textile and leather industries. During industrial processing, up to 40% of the used dyestuff are released into the process water (Vaidya and Datye 1982), producing highly colored wastewaters that affect aesthetics, water transparency, and gas solubility in water bodies. Moreover and most importantly, there is a general concern regarding toxicity of some of these dyes. Because of both the high discharged volumes and the effluent composition, wastewaters from the textile industry can be considered as the most polluting among all industrial sectors, thus greatly requiring appropriate treatment technologies (O'Neill et al. 1999). All the dyes used in the textile industry are designed to resist fading even upon exposure to many chemicals including oxidizing agents. Although some a-biotic methods

for the reduction of several dyes exist, these require highly expensive catalysts and reagents (Robinson et al. 2001b). Biotechnological approaches were proven to be potentially effective in treatment of this pollution source in an eco-efficient manner (Willmott et al. 1998; McMullan et al. 2001; Robinson et al. 2001b; Borchert and Libra 2001; Beydilli et al. 1998; Zissi and Lyberatos 2001). The white rot fungi (WRF) are, so far, the microorganisms most efficient in degrading synthetic dyes, with basidiomycetous fungi that are able to depolymerize and mineralize lignin. This WRF's property is due to the production of extracellular lignin-modifying enzymes (LMEs), which, because of their low substrate specificity, are also able of degrading a wide range of xenobiotic compounds (Barr and Aust 1994; Pointing 2001; Scheibner et al. 1997) including dyes (Glenn and Gold 1983; Pasti-Grigsby et al. 1992; Paszczynski et al. 1992; Spadaro et al. 1992). The main LMEs are manganese peroxidases (MnP), E.C. 1.11.1.13, (Glenn et al. 1986), lignin peroxidases (LiP), E.C. 1.11.1.14 and laccases (Lac), E.C. 1.10.3.2, (Edens et al. 1999). LiP, MnP, and laccase play significant roles in dye metabolism by WRF (McMullan et al. 2001), due to the structural similarity of the most commercially relevant dyes to lignin (sub)structures amenable to be transformed by LMEs. However, the profiles of LME production during dye decolourization can be different in different fungi. For instance, Lac is the main enzyme involved in dye decolourization by cultures of *Phlebia tremellosa* (Kirby et al. 2000; Robinson et al. 2001a), *Pleurotus sajor-caju* (Chagas and Durrant 2001), and *Pleurotus ostreatus* (Palmieri et al. 2005a). Whereas, MnP, with or without LiP cooperation, was reported as the main enzyme involved in dye decolourization by *Phanerochaete chrysosporium* (Chagas and Durrant 2001; Kirby et al. 1995), and LiP was considered as the principal decolorizing enzyme of *Bjerkandera adusta* (Robinson et al. 2001b).

There is a gap in current knowledge of decolourization and, even more, of mineralization mechanisms, that limits our capacity to evaluate the true technical potential of WRF and their LMEs. Moreover, these difficulties are even greater if one considers that industrial effluents show a complex composition that is extremely variable even within the same factory, as the case of textile industry well demonstrates. Thus, decolourization of real effluents requires an appropriate choice of fungal strains as well as of operative conditions. Real textile dye effluents contain not only dyes but also salts, sometimes at very high ionic strength and extreme pH values, chelating agents, precursors, by-products, surfactants, etc. . Thus, in spite of the high efficiency in dye decolourization by some strains, decolorizing a real industrial effluent is quite troublesome.

In this report, models of acid, direct and reactive dye wastewaters from textile industry -defined on the basis of discharged amounts, economic relevance and representativeness of chemical structures of the contained dyes- were used to evaluate bioremediation capabilities of the WRF *Pleurotus ostreatus* and *Phanerochaete chrysosporium* and their LMEs.

Materials and methods

Microorganisms

Strains of the WRF *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) and *P. chrysosporium* Burdsall M1 (DSM 13583), were maintained through periodic transfer at 4°C on agar (1.5% w/v) plates containing undiluted and 10-fold diluted PDY medium [24 g/l potato dextrose (Difco, Detroit, Michigan, USA) and 5 g/l yeast extract (Difco)], respectively. The fungus *P. chrysosporium* shows a higher growth rate than *P. ostreatus* in PDY medium, and a comparable growth of both the fungal strains was obtained using a 10-fold diluted PDY medium for *P. chrysosporium*.

Dye containing wastewater models

Composition of the model wastewaters containing dyes that were used in this work is reported in Table 1. The wastewaters were sterilized by tindallyzation, consisting of three 1 h cycles of incubation at 60°C with 24 h interval between two cycles.

Treatment of dye containing wastewater models by *P. chrysosporium* and *P. ostreatus*

The inocula for liquid cultures were prepared by preinoculating 300 ml of ME broth [20 g/l malt extract (Difco)] in 1 l Erlenmeyer flask with 6 agar plugs of *P. ostreatus* (11 mm diameter) or *P. chrysosporium* (14 mm diameter) mycelia, from the edge of a

Table 1 Composition of colored wastewater models

Wastewater model	Dye				[Dye] (g/l)	Salt	[Salt] (g/l)	pH	Base or Acid	Concentration of base or acid (g/l)
	Name	Abbreviation	Chemical structure class	λmax						
Direct	Direct Blu 71	DrBu 71	Trisazo	582	1.00	NaCl	5	9	Na_2CO_3	2
	Direct Red 80	DrR 80	Polyazo	542						
	Direct Yellow 106	DrY 106	Stilbene	419						
Reactive	Reactive Blue 222	RBu 222	Disazo	280, 609	1.25	Na_2SO_4	70	10	NaOH	2
	Reactive Red 195	RR 195	Monoazo	291, 543						
	Reactive Yellow 145	RY 145	Monoazo	292, 419				12	Na_2CO_3	20
	Reactive Black 5	RBk 5	Disazo	593						
Acid	Acid Blue 62	Abu 62	Anthraquinonic	595, 637	0.10	Na_2SO_4	2	5	Acetic Acid	2
	Acid Yellow 49	AY 49	Monoazo	402						
	Acid Red 266	AR 266	Monoazo	307, 500						

7-days-old agar culture, in a temperature-controlled incubator at 28°C on rotary shaker (at 125 rpm). Fifty milliliters of a 5-day-old pre-culture were transferred in 1 l flasks containing 450 ml of model wastewater in the presence or in the absence of 2% malt extract. Cultures were incubated in the dark at 28°C on a rotary shaker at 125 rpm.

Performances of fungal cells in model wastewater decolourization were evaluated by recording light absorption spectra between 280 and 800 nm at different times (every 24 h), compared with the corresponding spectra of un-inoculated controls. Decolourization was measured as the extent of decrease of spectrum area recorded between 380 and 740 nm with respect to a control sample. All spectra were recorded after 1:100 dilution of the sample in water.

Samples of fungal cultures of wastewater treatment were daily withdrawn and used to perform enzyme activity assays.

Enzyme assays

Laccase activity was assayed using 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate (Giardina et al. 1996). The assay mixture contained 2 mM ABTS and 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$).

Dye-decolourizing peroxidase (DYP) activity was assayed using Remazol Brilliant Blue R (RBBR) as substrate (Shin et al. 1997). The assay mixture contained 50 μM RBBR and 0.1 mM H_2O_2 in 20 mM sodium acetate buffer, pH 4.0. RBBR degradation was followed by absorbance decrease at 592 nm ($\varepsilon = 9{,}000\ M^{-1}\ cm^{-1}$).

Aryl alcohol oxidase (AAO) activity was determined using veratryl alcohol as substrate (Sannia et al. 1991). The reaction mixture contained 4 mM veratryl alcohol in 50 mM sodium phosphate buffer, pH 6.0. Oxidation of veratryl alcohol to veratrylaldehyde was followed by absorbance increase at 310 nm ($\varepsilon = 9{,}300\ M^{-1}\ cm^{-1}$).

Manganese peroxidase (MnP) activity was determined using manganese sulfate as substrate (Giardina et al. 2000). The reaction mixture contained 0.5 mM manganese sulfate and 0.1 mM H_2O_2 in 50 mM sodium malonate buffer, pH 4.5. Oxidation of Mn^{2+} to Mn^{3+} was followed by absorbance increase at 270 nm ($\varepsilon_{270} = 11{,}590\ M^{-1}\ cm^{-1}$) due to the formation of malonate—Mn^{3+} complex.

Lignin peroxidase (LiP) activity was determined using veratryl alcohol as substrate (Tien and Kirk 1984). The reaction mixture contained 2 mM veratryl alcohol and 0.5 mM H_2O_2 in 50 mM sodium tartrate buffer, pH 2.5. Oxidation of veratryl alcohol was followed by measuring the absorbance increase at 310 nm ($\varepsilon_{310} = 9{,}300\ M^{-1}\ cm^{-1}$).

All the enzyme activities were measured at 25°C and expressed in International Units (IU), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes one μmole of substrate in 1 min.

Production and characterization of laccase mixtures from *P. ostreatus*

A total of 50 ml of a pre-culture of *P. ostreatus* were inoculated in 1 l Erlenmeyer flasks containing 450 ml of PDY [24 g/l potato dextrose (Difco), 5 g/l yeast extract (Difco)], containing 0.15 mM $CuSO_4$ (Sigma) and supplemented with 2 mM ferulic acid (Sigma) after 2 days culture. After 8 days of fungal growth, the broth was filtered, and proteins were precipitated by the addition of 80% $(NH_4)_2SO_4$ at 4°C and centrifuged at 10,000*g* for 30 min. Protein precipitate was resuspended in 50 mM sodium phosphate buffer pH 6.5 and extensively dialyzed against the same buffer. The sample was centrifuged, and the supernatant was concentrated on an Amicon PM-10 membrane (Millipore). Enzyme samples were treated with 1 mM PMSF (Sigma) and stored at −80°C to be used in the treatment of dye containing wastewater models or for analyses of laccase isoenzyme composition.

Laccase isoenzyme fractionation

Ultra-filtrated protein samples were loaded on a Resource Q (GE Healthcare) column equilibrated with 50 mM Tris–HCl buffer pH 7. The column was washed at a flow rate of 1 ml/min with 3 ml of buffer, and a 0–0.5 M NaCl linear gradient (20 ml) was applied. Fractions containing laccase activity were pooled and concentrated.

Non-denaturing polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed at alkaline pH under nondenaturing conditions. The separating and stacking gels were, respectively, at 9 and 4% acrylamide, buffer solutions were 50 mM Tris–HCl (pH 9.5) for separating gel and 18 mM Tris–HCl (pH 7.5) for stacking gel, and the electrode reservoir solution was 25 mM Tris, 190 mM glycine, pH 8.4. Gels were stained for laccase activity using ABTS as substrate.

Treatment of dye containing wastewater models by laccase mixtures from *P. ostreatus*

Batch decolourization experiments using laccase mixtures as free enzymes were performed incubating crude preparation of laccase mixture containing different enzyme amounts (0.1, 1, 10 or 100 U/ml) with wastewater models, at room temperatures. Decolourization was evaluated recording UV-VIS absorption spectra at different times (10 min, 30 min, 1 h, 4 h, 24 h) of enzyme incubation and calculated as the extent of decrease of spectrum area recorded between 380 and 740 nm in comparison with the corresponding area of the spectrum of the untreated wastewater model.

Immobilization of laccase mixtures from *P. ostreatus*

The immobilization was carried out according to the procedure reported by Palmieri et al. (2005b). 2000 U of crude laccase preparation of laccase mixture were mixed with 35 ml of 3% sodium alginate solution (low viscosity, Sigma, St. Louis, MO), centrifuged at 4,000 rev min^{-1} for 5 min to remove air bubbles, and extruded drop by drop through a needle (0.4 mm internal diameter) into a 0.15 M $CuSO_4$ aqueous solution (pH 4.0) under continuous stirring. The resulting spherical blue beads were left to solidify for at least 30 min in the copper solution and then washed exhaustively with distilled water until pH 5.0–5.5 was reached. The total wet weight of beads obtained from 1 ml of sodium alginate solution was about 0.7 g. The beads were stored wet at 4°C.

Batch-mode decolourization by immobilized *P. ostreatus* laccase mixtures

In a typical experiment, 1 g of beads (with a mean diameter of 2.7 mm) containing about 50 U laccase activity was added to 2 ml of acid dye wastewater model solution. The reaction was incubated at room temperature under vigorous stirring. Each experiment was performed in a repeated sequencing batch decolourization. Each cycle consisted of the addition of the acid dye wastewater model solution to the laccase alginate beads. A new cycle was initiated when no further change in the dye (Abu 62) concentration was observed by monitoring the absorbance decrease at 637 nm. Control samples, consisting of alginate beads without any entrapped enzyme, were run in parallel under identical conditions. Each experiment has been repeated at least three times, and standard deviations were determined.

Production of manganese peroxidase and lignin peroxidase containing mixtures from *P. chrysosporium*

Different *P. chrysosporium* growth conditions in liquid culture have been tested for MnP and LiP activity production. 50 ml of a pre-culture of *P. chrysosporium* (6 agar plugs with 14 mm diameter from the edge of 7-days-old agar culture in 1 l flask containing 300 ml of 2% malt extract) were inoculated in 1 l Erlenmeyer flasks containing 450 ml of 10-fold diluted [2.4 g/l potato dextrose (Difco), 0.5 g/l yeast extract (Difco)] PDY broth or 2% malt extract broth supplemented with 0.1 or 1 mM $MnSO_4$. Culture broth was concentrated by ultra-filtration by using a 30 kDa cartridge in the Quix Stand Benchtop system (GE Healthcare).

Analysis of detoxification abilities

Toxicity of wastewater models before and after fungal treatment or incubation with enzyme mixture was measured with Lumistox 300 system (HACH LANGE, S.r.l. Milan—Italy), using freeze-dried *Vibrio fischeri* bioluminescent bacteria as test micro-organism. The inhibition of the natural light emission of these microorganisms, caused by toxic substances, was measured.

All the samples to be tested were prepared according to the manufacture's instructions. Conductivity, pH and optical density (OD) were measured. The pH was set to 7.0 ± 0.2 with HCl or NaOH and their salt content was adjusted to 2% NaCl when the measured conductivity was lower than 35 mS/cm.

Both acid and direct dye model wastewaters (with and without malt extract addition) showed O.D. values >1,800 mE, that required preparation of wastewaters dilutions to have final O.D. values <1,800 mE. All the measures were performed in color correction mode in order to reduce the interferences caused by physical absorption or light scattering. Toxicity values (defined as percent of inhibition of bacterial bioluminescence) of these wastewaters at the new defined concentrations were measured.

Analysis of COD

Determination of COD (Chemical Oxygen Demand) with the dichromate method was performed using HACH (COD High range vials) apparatus (HACH LANGE, S.r.l. Milan—Italy) according to the manufacture's instructions. A calibration curve was obtained using HACH COD standard solution (800 mg O_2/l). Appropriate dilutions of each sample were assayed.

Results and discussion

Treatment of dye containing wastewater models by *P. chrysosporium* and *P. ostreatus* cultures

The effect of *P. chrysosporium* and *P. ostreatus* liquid cultures on colored acid, direct and reactive dye industrial wastewater models was evaluated both in the presence and in the absence of nutrients. The wastewater models were defined in the frame of European Union Sixth Framework Program funded project SOPHIED (contract NMP2-CT2004-505899) on the basis of discharged amounts by textile industry, economic relevance and representativeness of chemical structures of the contained dyes (Table 1). Values of pH and salt concentration were chosen as those reproducing the characteristics of the real industrial effluents.

P. chrysosporium caused an effective decolourization of direct dye wastewater models when supplemented with nutrients, reaching 46% decolourization in only 1 day of treatment, and 87% decolourization within 7 days. Spectra analysis revealed that fungal treatment is effective on all the dyes present in the wastewater model, giving a complete depletion of the peaks at 582, 541 and 419 nm corresponding to the dyes Direct Blue 71 (Trisazo), Direct Red 80 (Polyazo) and Direct Yellow 106 (Stilbene), respectively (Fig. 1a). However, a new absorbance peak centered at 357 nm was observed in the spectrum of the wastewater model after fungal treatment, thus suggesting the formation of a new product inducing a significant shift of wastewater colour from dark to yellow. The complete removal of the major visible light absorbance peaks and the significant spectral change observed after *P. chrysosporium* treatment of direct wastewater suggest a degradation of the dyes by the fungus (Knapp et al. 1995). Fungal treatment of the wastewater also determined a pH shift of the growth medium from 8.4 to 4.5 within 7 days. Spectra of samples of direct

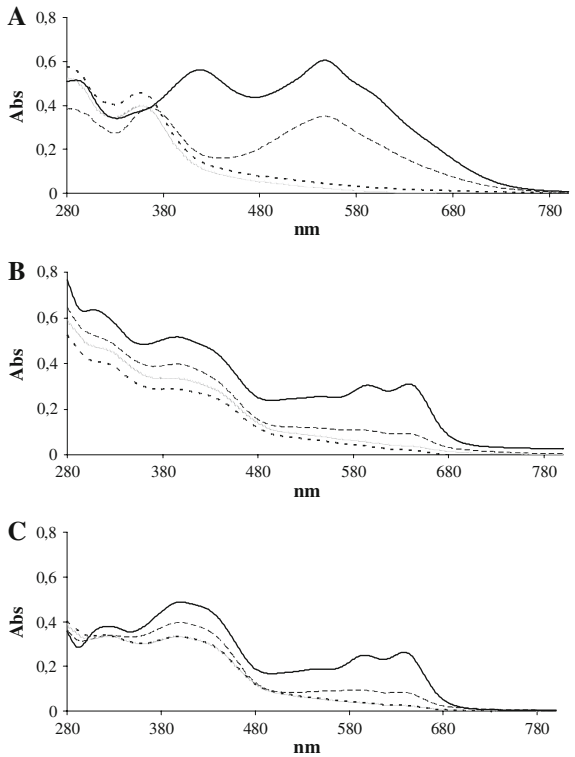

Fig. 1 Light absorption spectra between 280 and 800 nm of: direct dye wastewater treated by *P. chrysosporium* (**a**), acid dye wastewater treated by *P. ostreatus* in the presence (**b**) and in the absence (**c**) of malt extract, after 0 (——), 1 (– – –), 7 (·····) and 14 days (·····) of treatment

dye wastewater model at different pH values in the range 8.4–4.5 were recorded allowing us to rule out that the variations observed in the spectrum after fungal treatment were due to a pH change. We could not directly correlate the decolourization of the wastewater by *P. chrysosporium* to the production of oxidative enzyme activities potentially involved in the process, since residual color interfered with assays of MnP and LiP activities, the enzymes reported as the mainly responsible for decolourization by *P. chrysosporium* (Champagne 2005).

P. ostreatus proved to be capable to decolorize acid dye wastewater model with and without addition of malt extract, providing 40% decolourization after only 1 day of treatment in the absence of nutrient, and reaching up to 60 and 66% of decolourization after 7 and 14 days, respectively, in the presence of nutrients. Spectra analysis revealed that fungal treatment is more effective on the dyes Acid Blue 62 (anthraquinonic dye) and Acid Red 266 (azo dye), with a dramatic reduction of the maximum absorbance peaks at 637 and 595 nm, due to Abu62, and of maximum absorbance peak at 500 nm due to AR266. On the other hand, Acid Yellow 149 (azo dye) seems to be more recalcitrant to biodegradation, its maximum absorbance peak at 402 nm being only slightly decreased (Fig. 1b). These results indicated a different efficiency of *P. ostreatus* in degrading the tested acid dyes.

Oxidative enzyme activities [laccase, Mn Peroxidase (Mnp), Lignin peroxidase (LiP) dye decolourizing peroxidase (DyP, Johjima et al. 2003), Veratryl Alchol Oxidase (VAO)] were assayed in decolorized samples treated by *P. ostreatus*. Only laccase activity was revealed since the first day of incubation, and a constant level of enzyme production (10^{-3} U/ml) during fungal treatment of the wastewater model was observed. Therefore, laccase activity seems to be the oxidative activity mainly involved in dye decolourization by *P. ostreatus*.

No decolourization of reactive wastewater model was obtained, neither by *P. chrysosporium* nor by *P. ostreatus*, both in the presence and in the absence of added nutrients. This can be ascribed to the loss of fungi vitality, due to the extreme pH (10–12) and salt concentration (70 g/l) conditions of the waste. As a matter of fact, it has been demonstrated that both the fungi loose their vitality after treatment of reactive dye wastewater, whilst vitality was retained after treatment of the acid and direct dye wastewater models. The observation that *P. chrysosporium* was able to decolourize the direct wastewater, characterized by a high pH value (9), suggested that the loss of the fungus vitality during the treatment of reactive dye wastewater can be ascribed to the higher salt concentration (70 g/l) of this wastewater in comparison with that of direct wastewater (5 g/l).

Hence the fungal strains *P. ostreatus* and *P. chrysosporium* showed different specificities in decolorizing the dye wastewater models. *Phanerochaete chrysosporium* proved to be able to decolourize direct dyes having complex -Trisazo, Polyazo and Stilbene-structures, whilst *P. ostreatus* did not decolorize these dyes but was showed to be able to decolourize anthraquinonic and aniline azo dyes. That is consistent with some previously reported studies (Knapp et al. 1995; Swamy and Ramsay 1999; Balan and Monteiro 2001; Martins et al. 2003; Eichlerova et al. 2005; Nozaki et al. 2008), showing different specificities of WRF in dye decolorization.

The different decolourization specificities of *P. ostreatus* and *P. chrysosporium* can be due to the different profiles of produced LMEs, mainly laccases in *P. ostreatus* and MnP in *P. chrysosporium*.

Treatment of dye containing wastewater by *P. ostreatus* laccases

In order to test the ability of *P. ostreatus* laccases to decolorize dye wastewater models and to verify the hypothesis that laccase activity is the oxidative activity mainly involved in decolourization of acid dye wastewater by *P. ostreatus*, an extracellular enzyme mixture endowed with high laccase activity levels was produced from *P. ostreatus* and incubations of the wastewater models with the laccase mixture were performed.

High levels of laccase activity production (up to 130×10^3 U/l) were obtained in a potato-dextrose (24 g/l) broth supplemented with yeast extract (5 g/l), 0.15 mM $CuSO_4$ and 2 mM ferulic acid, a low cost growth medium (4.4 euro/l). In this growth condition a quite constant production level of laccase activity was also detected throughout the 8–11 days period. Laccase isoenzyme composition was determined in culture supernatants, withdrawn after 8 days of fungal growth, and fractionated by ammonium sulphate precipitation and anionic exchange chromatography fractioning. Two laccase activity peaks were obtained. The corresponding fractions were pooled, concentrated, and analyzed by electrophoretic separation on native PAGE gels stained for laccase activity. The identity of the laccase isoenzymes in the laccase activity peaks was ascertained on the basis of the different electophoretic mobility of the laccase isoenzymes. The extracellular enzyme mixture was showed to contain POXA3 (Palmieri et al. 2003) and POXC (Giardina et al. 1996) isoenzymes, the latter one being the most abundant (99%).

When the wastewater models (Table 1) were prepared for incubations with the enzyme mixture, they were not tindallysed, since antiseptic conditions are not strictly required. Reactive dye wastewater model was used at pH 10.

The laccase mixture decolorized acid dye wastewater model up to 35% decolourization after incubation with only 0.1 U/ml of laccase in 24 h, and up to 30% after only 1 h incubation with 1 U/ml. An increase in enzyme concentration does not correspond to a proportional increase in the extent of decolourization (Fig. 2). The modifications of the absorption spectra following the enzymatic treatment of the wastewater model are similar to those produced by *P. ostreatus*, thus suggesting that laccases are the main agents of the wastewater model decolourization by the fungus. A quick depletion of the maximum absorbance peaks at 637 and 595 nm—due to Acid Blue 62—was observed just after an incubation of 10 min. This behaviour was confirmed by treating the single dyes with the laccase mixtures, that provided a fast decolourization of Acid Blue 62 (anthraquinone dye), a slower decolourization—taking 24 h—of Acid Red 266 (azo dye), whilst Acid Yellow 49 (azo dye) was resistant to laccase decolourization. The differences in decolourization efficiency can be ascribed to structural differences of the dyes and to substrate specificity of the laccase isoenzymes. As reported in literature, anthraquinonic dyes are good substrates for laccases, while degradability of azo dyes depends on their different aromatic substitution patterns (Chivukula and Renganathan 1995), and among them phenol and aniline azo dyes can be degraded by laccases (Kandelbauer et al. 2004). This could allow us to explain the more efficient degradation of the anthraquinone-type dye Acid Blue 62, compared with that of the aniline azo dye Acid Red 266. Lack of a hydroxyl or an amine group on the aromatic ring of the azo dye Acid Yellow 49 should explain the inability of *P. ostreatus* laccases to degrade this dye.

The laccase mixture proved to be poorly active in the decolourization of direct dye wastewater, even when up to 100 U/ml of enzyme were used. The extreme conditions of pH and salt concentration of this wastewater model could prevent efficient

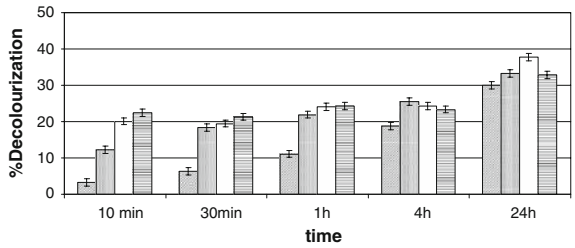

Fig. 2 Percent of decolourization of acid dye wastewater model after incubation with various amounts (0.1 U/ml, ▨; 1 U/ml, ▩; 2U/ml, □; 10 U/ml, ▤) of laccase mixture, at room temperature, and recording UV-VIS absorption spectra at different times (10 min, 30 min, 1 h, 4 h, 24 h)

functioning of laccases in decolourization. In fact a slight increase in decolourization is achieved by lowering the pH of direct dye wastewater from 9 to 5. Spectra analysis revealed that the enzymatic treatment is effective only on Direct Blue 71 (Trisazo), as indicated by the decrease of absorbance intensity at the wavelength of maximum absorption of this dye (582 nm), while the other two dyes Direct Red 80 (Polyazo) and Direct Yellow 106 (Stilbene) showed to be resistant to the enzymatic degradation.

No decolourization was observed for reactive dye wastewater after incubation with laccase mixture, even if the enzyme concentration was increased up to 100 U/ml. In order to verify that the absence of any decolourization effect is due to the extreme pH value (10) of the reactive wastewater, decolourization experiments were performed lowering the pH of the wastewater model to 6. No decolourization was observed for reactive dye wastewater although the pH lowering, thus ruling out the hypothesis that the extreme pH value of this wastewater model had inhibited enzyme decolourization activity.

Treatment of wastewater models by laccase mixtures from *P. ostreatus* entrapped in Cu-alginate beads

Effectiveness of *P. ostreatus* laccase mixture entrapped in Cu-alginate beads in decolourizing RBBR (Remazol Brilliant Blue R) had been previously established (Palmieri et al. 2005a, b). Although the reported data suggested the possibility to use this system in wastewater treatment, it was necessary to verify the stability of the support in the wastewater models. No alteration of beads consistency was observed after 24 h incubation in acid dye wastewater model, whereas a big stress was given by direct dye wastewater, and a complete dissolution of beads was produced by reactive dye wastewater model.

The enzymatic extracellular mixture was immobilized by entrapment in Cu-alginate beads and the immobilized enzymes used in batch decolourization experiments on acid dye wastewater model.

The dye decolourization process was efficient even after 15 stepwise dye additions taking about 1 month, providing up to 80% decrease of absorbance at 637 nm (Fig. 3a). Even if the time needed to achieve 50% decolourization exponentially increased (Fig. 3b), the $t_{1/2}$ of the 15th cycle was as low as 7 h.

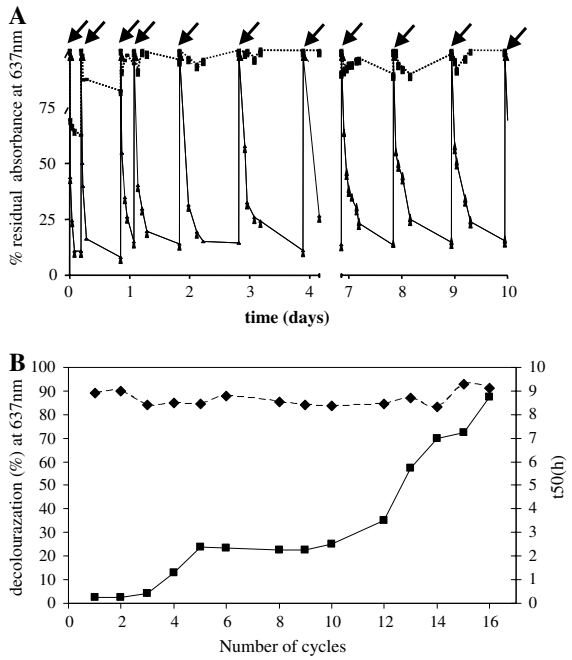

Fig. 3 Decolourization of acid dye wastewater model obtained by crude laccase mixture entrapped in copper alginate beads. (**a**) Decolourization percentage (residual absorbance at 637 nm) during 10 cycles (▲) compared to that obtained in the presence of alginate beads with no enzyme entrapped (■). Arrows indicate new dye additions. (**b**) Decolourization percentage (◆) during 16 cycles and time needed to obtain 50% decolourization (t_{50}) for each cycle (■)

Production of manganese peroxidase mixtures from *P. chrysosporium* for treatment of dye containing wastewater models

To test the performance of *P. chrysosporium* extracellular enzymes in the decolourization process, conditions to optimize enzyme (MnP and LiP) production and to prepare enriched enzyme mixtures have been investigated. Among the different *P. chrysosporium* growth conditions tested, the best selected growth medium for MnP production contained potato-dextrose broth (2.4 g/l) added with yeast extract (0.5 g/l) and 0.1 mM $MnSO_4$. Further increase of $MnSO_4$ concentration resulted in a decrease of MnP activity production. However, LiP activity was not detected in any tested condition.

The enriched enzyme mixture containing MnP activity was used in batch decolourization experiments performed incubating 0.1 or 0.01 U/ml enzyme with direct dye wastewater model and recording UV-VIS absorption spectra at different times (1, 24 and

48 h). Incubations were carried out in H_2O or 50 mM Sodium Malonate pH 4.5 with or without addition of 0.5 mM $MnSO_4$ and of 0.1 mM H_2O_2, by using the wastewater at pH 9 or lowering pH to 4.5. The best performance of the enzyme mixture was observed in decolorizing the 10-fold diluted wastewater model, in 50 mM Sodium Malonate pH 4.5 in the presence of $MnSO_4$. The mixture was able to decolorize undiluted wastewater giving about 40% decrease of absorbance at 600 nm after only 1 h, whilst to observe a complete depletion of the 600 nm absorbance peak, a 10-fold dilution of the wastewater was required. When decolourization was evaluated as decrease of spectrum area between 380 and 740 nm, only 15% decolourization was revealed after 48 h, whilst about 50% of decolourization after 24 h was observed on 10-fold diluted direct dye wastewater model. The addition of 0.1 mM H_2O_2 did not affect the decolourization efficiency. When tested on each of the dyes present in the direct dye wastewater model, the MnP containing samples were shown to be more effective in decolorizing Direct Blue 71, reaching 50% of decolourization, evaluated as the decrease of spectrum area between 380 and 740 nm of the undiluted dye, after 24 h.

Only some of the spectral modifications produced by *P. chrysosporium* treatment were observed after enzyme incubations, thus suggesting involvement of other fungal metabolites or enzymes during in vivo decolourization process.

Variability of MnP production by fungus and low stability of the enzymes hindered further scale up of the system. The low stability is reported as a common property of MnP enzymes (Sutherland and Aust 1996; Timofeevski and Aust 1997).

Analyses of detoxification and COD reduction abilities

Toxicity of the acid dye wastewater model after 24 h treatment with 0.1U/ml of the laccase mixture and at the end of treatment with *P. ostreatus* was evaluated by using Lumistox 300. EC50 could be only measured for acid dye wastewaters with malt extract added, and the values determined for untreated and fungal treated were 43.3 and 10.4% respectively. For the acid dye wastewater without malt extract, toxicity of a 50% dilution with a final O.D. values <1,800 mE was tested. Reduction of the toxicity of the wastewater was provided by fungal treatment both in the presence and in the absence of added nutrients, whilst a less extent of toxicity reduction was obtained by the enzyme incubation (Table 2), thus suggesting the involvement of other fungal components in detoxification during in vivo process.

Toxicity of direct dye wastewater model treated by *P. chrysosporium* could not be evaluated because of the low reproducibility of toxicity measurements obtained with these samples, due to too low sample concentration (required by the color correction mode).

Reduction of COD of acid and direct dye wastewaters after fungal treatment was not detectable in the presence of added malt extract because of the high contribute of the nutrient to overall COD value. On the other hand, 33% COD reduction of acid dye

Table 2 Detoxification abilities of *P. ostreatus* and its laccases evaluated by using Lumistox 300 (reduction of % Inhibition of *Vibrio fisheri* growth by dye after fungal or laccase treatment)

Wastewater	Bioremediation system	Toxicity
Acid with malt extract added	Control (tyndalized)	EC50:10.4%
	P. ostreatus	EC50: 43.3%
Acid	Control (tyndalized)	% Inhibition (at 50% diluition): 37.8% ± 1.1%
	P. ostreatus	% Inhibition (at 50%diluition): 6.1%
Acid	Control (Not tyndalized)	% Inhibition (at 50%diluition) = 28.6 ± 6.0%
	Laccase mixture	% Inhibition (at 50%diluition) = 11.5 ± 3.2%
Direct with malt extract added	Control (tyndalized)	Not detectable
	P. chrysosporium	Not detectable

wastewater without malt extract was produced by *P. ostreatus* treatment. 31% COD reduction of the wastewater was measured after enzyme treatment, whilst no COD reduction of model wastewater was provided by *P. chrysosporium*.

Conclusions

In conclusion, in this report decolourization capabilities of the fungi *P. chrysosporium* and *P. ostreatus* and of free and immobilized laccase mixtures from *P. ostreatus* on industrial dye wastewaters have been demonstrated. A different decolourization specificity was exhibited by the fungi, *P. chrysosporium* being active in decolorizing the direct wastewater model containing dyes with complex—Trisazo, Polyazo, Stilbene- structures, whilst *P. ostreatus* provided decolourization of the acid wastewater model. Laccases were identified as the main agents of wastewater decolourization by *P. ostreatus* and their preference in decolourizing anthraquinonic type dye and aniline mono-azo dye allowed us to explain ability of *P. ostreatus* to decolourize acid wastewater model. On the other hand, *P. ostreatus* is unable to decolourize the direct wastewater model, possibly because of the presence of complex poly-azo and stilbene structures, not degraded by laccases. A system based on immobilized laccase mixtures was also shown active in decolurization and can be further improved for large scale applications. On the other hand, manganese peroxidases are involved in direct wastewater model decolourization by *P. chrysosporium*, and the different profiles of LMEs produced by the fungi can explain their different decolorization specificities.

It's worth noting the ability of *P. chrysosporium* to decolourize the direct wastewater that is characterized by a high (9) pH value, whilst for majority of the fungi the optimum pH for dye decolourization lies in the acidic range (Asgher et al. 2008; Kapdan et al. 2000; Parshetti et al. 2007). However, such low pHs are not suitable for the wastewater treatment and hence, fungal strains able to decolourize dyes efficiently at wider pH ranges are desirable for industrial applications.

While many studies were devoted to biodecolourisation of the textile dyes, few manuscripts have been reported on decolourization of dye effluents in which the presence of salts and high dye concentration may be inhibitory to biological agents. Decolorization of reactive dye industry effluents was demonstrated by the fungi *Aspergillus fumigatus* (Jin et al. 2007) and *Phanerochaete sordida* (Harazono and Nakamura 2005), and by fungal oxidative enzymes (Zille et al. 2003; Mohorcic et al. 2006). Knapp and Newby (1999) reported decolourization of a diluted chemical industry effluent containing a diazo-linked chromophore by white-rot fungi. Taking also into account the high dye concentrations and the presence of salts in the tested wastewaters, *P. chrysosporium* and *P. ostreatus* exhibited good decolourization performances.

The potential of *P. ostreatus* to be used as an effective detoxifying microorganism for acid dye wastewaters have also been demonstrated. To the best of our knowledge, this is the first report on this detoxifying potential.

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