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Industrial dye degradation and detoxification by basidiomycetes belonging to different eco-physiological groups

Antonella Anastasi, Valeria Prigione, Giovanna Cristina Varese*

Department of Plant Biology, University of Turin, Viale Mattioli 25, 10125 Turin, Italy

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ABSTRACT

Twenty-five basidiomycetes belonging to 17 species and ascribable to different eco-physiological groups were screened for their ability to decolorize 9 commercially important industrial dyes comprising a variety of anthraquinonic, azoic and phtalocyanin chromophores. The influence of the culture medium, particularly its C:N ratio, on decolourisation capacity was considered on solid substrate. Three strains of *Bjerkandera adusta* performed the highest decolourisation yields being able to degrade all dyes on all media and to produce a wide spectrum of oxidative enzyme activities. Hence, *B. adusta* strains were selected for further experiments in liquid cultures together with other 6 fungi that resulted effective in the decolourisation of the largest number of molecules in the broadest spectrum of cultural conditions. Particularly *B. adusta* MUT 3060 was found very effective (decolourisation percentage over 90%) in the treatment of simulated effluents composed of single and mixed dyes at high concentration (1000 mg/l). Peroxidase activity dependent (up to 362 U/l) and independent from manganese (up to 57 U/l) were detected during the decolourisation process. The *Lemna minor* toxicity test showed a significant reduction of toxicity after the fungal treatment indicating that decolourisation corresponded to an actual detoxification of the wastewater.

1. Introduction

Textile effluents are one of the most difficult-to-treat wastewaters on account of the massive presence of weakly biodegradable and often toxic substances such as additives, detergents, surfactants and dyes [1]. Traditional technologies have proven to be markedly ineffective for treating textile effluents because most of the synthetic dyes are highly resistant to biological, physical or chemical treatments and therefore dyes and their derivates accumulate in the environment [2]. Actually, the release of coloured wastewater in the ecosystem is a remarkable source of eutrophication and perturbations in aquatic life. The presence of dyes or their degradation products in water even at very low concentrations can be toxic, and sometimes carcinogenic, mutagenic or teratogenic to various organisms, man included [3–5].

The implementation of strict legislation in many countries, combined with the awareness of the negative environmental impact of dyestuffs, has resulted in recent years in an increasing number of environmental researches for the development of processes that can effectively treat textile effluents. White-rot fungi (WRF) have proved to be the microorganisms most efficient in degrading synthetic dyes [6]. Their extracellular enzymatic system, which is involved in lignin degradation, consists mainly of oxidative enzymes like laccases (Lac), lignin peroxidases (LiP) and manganese peroxidases (MnP). Recently, another type of peroxidase named versatile peroxidase (VP), sharing the catalytic properties of LiP and MnP, was described in several species from the genera *Pleurotus* and *Bjerkandera* [7] and was demonstrated effective against a wide range of industrial dyes [8]. In recent years, some authors have highlighted the degradation potentialities of other eco-physiological groups of basidiomycetes (i.e. brown-rot fungi—BRF, compost fungi), underlining the importance to investigate other fungal species till now neglected [9,10].

However, most of the screenings have been performed so far on single model dyes at low concentrations, but these conditions are poorly predictive of the actual decolourisation efficiency of real effluents in which dyes are usually present as a mix and often in quite high concentrations. Therefore, the selection of representative compounds to carry out a reliable and significant screening is a key point since model dyes biotrasformations cannot always be extrapolated to industrial dyes with apparent similar structure [11].

Another critical point is the evaluation of the detoxification after fungal treatment, a parameter that is often not taken into account, even if required by recent Italian legislation (Legislative Decree 152/2006), and that is the prerequisite to hypothesize a real application in the treatment of coloured wastewaters. Actually, decolourisation does not imply that the resulting molecules

* Corresponding author. Tel.: +39 011 6705984; fax: +39 011 6705962.
E-mail address: cristina.varese@unito.it (G.C. Varese).

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Table 1
Industrial dyes used in the experiment, their commercial and C.I. name, chromophore, chemical group, wavelength of maximum visible absorbance (λ_{max}), biodegradability and molecular concentration.

Common name	Commercial name	C.I. name	Chromophore	Chemical group	λ_{max} (nm)	Biodegradability[a]	Dye molecule concentration
B113	Nylosan bleu marine N-RBL P 187	Acid blue 113	Azoic	Acid	541	Unknown	55–75%
B214	Drimaren bleu marine X-GN CDG	Reactive blue 214	Azoic	Reactive	607	~0%	50–60%
B225	Nylosan bleu F-2RFL P 160	Acid blue 225	Anthraquinonic	Acid	590–626	20–30%	55–65%
B41	Drimaren turquoise X-B CDG	Reactive blue 41	Phtalocyanin	Reactive	616–666	~80%	50–60%
B49	Drimaren bleu P-3RLN GR	Reactive blue 49	Anthraquinonic	Reactive	586–625	10–20%	Unknown
B81	Solar bleu G P 280	Direct blue 81	Azoic	Direct	577	Unknown	Unknown
R111	Scarlet nylosan F-3GL 130	Acid red 111	Azoic	Acid	499	60%	85–90%
R243	Drimaren red X-6BN CDG	Reactive red 243	Azoic	Reactive	517	25%	Unknown
R80	Solar red BA P 150	Direct red 80	Azoic	Direct	540	46%	30–35%

[a] Method OECD 302B.

are less toxic than the parent ones. On the contrary, it has been shown that anaerobic degradation leads to reduction and cleavage of the azo-bonds of dyes derived from benzidine and the formation of potentially carcinogenic aromatic amines [5].

In the present work the decolourisation efficiency of 25 basidiomycetes ascribable to different species and eco-physiological groups against 9 commercially important industrial dyes was investigated in solid conditions. Several culture conditions were tested, in order to select fungi endowed with high degradation capabilities over a wide range of conditions and, hence, more suitable to be exploited in the treatment of different wastewaters. The 9 best strains were studied in greater detail, in liquid conditions, investigating the decolourisation activity towards simulated effluents containing single or mixed dyes and the enzymatic mechanisms involved. A very promising strain of *Bjerkandera adusta* was then cultured under specific conditions in order to optimize its degradation yield and the *Lemna minor* ecotoxicity test was performed in order to estimate the evolution of toxicity due to the fungal treatment.

2. Materials and methods

2.1. Dyes

Nine industrial dyes kindly provided by Clariant Italia S.p.A. were selected for decolourisation assays because of their recalcitrance to biodegradation by conventional wastewater treatment plants. They are all commercially important dyes, with a wide range of applications across the textile industries and are representative of the most used structural dye types, comprising a variety of anthraquinonic, mono-, di-, polyazoic and phtalocyanin chromophores. Their acronyms, chemical groups, maximum absorbance peak wavelengths, dye molecule concentration and biodegradability are listed in Table 1. Since reactive dyes are lost in effluents due to the hydrolysation of reactive groups occurring in dyeing bath conditions, the reactive dyes were hydrolysed by a 2 h heat treatment (80 °C) in alkaline solution (Na_2CO_3 0.1 M), and then neutralized, before use.

2.2. Organisms

The 25 strains, listed in Table 2, belong to 17 species ascribable to 8 families (Bolbitiaceae, Corticiaceae, Ganodermataceae, Meruliaceae, Nidulariaceae, Pleurotaceae, Polyporaceae, Strophariaceae) and represent different eco-physiological groups (wood saprotroph, WS, plant pathogen, PP, and litter saprotroph, LS, Table 2). In most cases, the fungi were isolated in pure culture from carpophores and identified on the basis of basidiome morphological features; in two cases (*B. adusta* MUT 2843, MUT 3060) fungi were isolated as sterile mycelia from compost and were identified on the basis of rDNA sequencing (Internal Transcribed Spacer—ITS 1, 2- and 18S) and comparison with sequences in gene databases (Genbank, NCBI). These strains have been selected out of 300 isolates of basidiomycetes from a screening experiment for their degradation capability against 3 model dyes (the antraquinonic RBBR and Poly R 478 and the azoic Poly S 119) [12] and are preserved at the *Mycotheca Universitatis Taurinensis* (MUT, University of Turin, Department of Plant Biology) on Malt Extract Agar at 4 °C.

2.3. Dye decolourisation on agar plate

Fungi were inoculated as an agar plug (5 mm of diameter), taken from the edge of an actively growing colony, in the middle of Petri dishes (5 cm of diameter) containing 10 ml of 3 solid media (GN0.1, GN1 and GN4), with different C:N ratios (91, 10 and 2.5 respectively), supplemented with dyes at a final concentration of 200 mg/l. GN0.1, GN1 and GN4 contained 2, 1 and 4 g l^{-1} respectively of di-ammonium tartrate, 18 g l^{-1} agar, 10 g l^{-1} glucose, 2 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g l^{-1} $CaCl_2 \cdot 2H_2O$, 0.01 g l^{-1} of biotin, 0.01 g l^{-1} thiamine, 10 ml l^{-1} mineral stock solution. The mineral stock solution contained for 100 ml of distilled water, 0.05 g $MnSO_4 \cdot 5H_2O$, 0.1 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $CoCl_2 \cdot 6H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$, 0.001 g $CuSO_4 \cdot 5H_2O$, 0.001 g $AlK(SO_4)_2$, 0.001 g H_3BO_3 and 0.001 g $NaMoO_4 \cdot 2H_2O$.

The experiment was performed in triplicate. Dishes were incubated at 25 °C, and after 3, 7 and 14 days, colony diameter and decolourisation halos were measured. Four degradation classes were assigned in function of the diameter (mm) of the decolourisation halo (a = 40–50 mm; b = 25–40 mm; c = 1–25 mm; d = no halo).

2.4. Dye decolourisation in liquid systems

The 9 best strains selected from the previous experiment were inoculated as described above in deep Petri dishes containing 20 ml of liquid GN0.1 added with dyes at the final concentration of 200 mg l^{-1}. Abiotic controls were set up in order to check any decrease in absorbance not due to the fungal presence (i.e. photobleaching). Cultures were incubated in static conditions at 25 °C, in the dark. Each trial was performed in triplicate. After 7 days, the decolourisation and the enzymatic activities (according to the protocol illustrated in the following paragraph) were monitored using a spectrophotometer (Amersham Biosciences Ultrospec 3300 Pro). The decolourisation percentage (DP) was calculated measuring the absorbance of the medium at the maximum absorption wavelength for each dye and expressed as decrease of absorbance as follows: $DP = [100 \times (Abs_0 - Abs_t)/Abs_0]$ where Abs_0 is the absorbance value at time 0 and Abs_t the absorbance value at time t.

Since industrial effluents usually contain a mixture of different dyes deriving from dye baths of different dyeing machines, decolourisation abilities of fungi were also investigated in 150 ml flasks containing 50 ml of GN0.1 broth added with equal amounts of each of the 9 dyes, to a final concentration of 1000 mg l^{-1} (MIX).

Table 2
Degradation capability of the 25 tested fungi belonging to different eco-physiological groups: wood saprotroph (WS), plant pathogen (PP), litter saprotroph (LS). Small letters represent degradation classes in function of the diameter of the decolourisation halo (*a*, 40–50 mm; *b*, 25–40 mm; *c*, 0–25 mm; *d*, no halo); numbers represent days necessary to reach the maximum decolourisation; in brackets the medium on which decolourisation occurred (A = GN0.1, B = GN1, C = GN4).

	Anthraquinonic dyes		Azo dyes						Phtalocyanin	Number of dyes efficiently decolourised (class a) on the 3 media		
	B 225	B 49	B 113	R 111	B 81	B 214	R 80	R 243	B 41	GN0.1	GN1	GN4
Bjerkandera adusta MUT2843 (WS)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (A, C), a 14 (B)	a 7 (all)	9	9	9
Bjerkandera adusta MUT 3060 (WS)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	9	9	9
Bjerkandera adusta MUT 2295 (WS)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (all)	a 7 (A, B), a 14 (C)	a 7 (B, C), a 14 (A)	a 7 (B), a 14 (A, C)	9	9	9
Gloeophyllum odoratum MUT 2294 (WS)	a 7 (all)	a 7 (all)	a 7 (all)	a 14 (all)	a 14 (all)	a 14 (all)	a 14 (all)	a 14 (all)	a 14 (A), b 14 (B), c 14 (C)	9	8	8
Lenzites betulina MUT 2451 (WS)	a 7 (all)	a 7 (B, C), a 14 (A)	a 7 (all)	a 14 (all)	a 14 (all)	a 14 (all)	a 14 (A), c 14 (B, C)	a 14 (A), c 14 (B, C)	a 14 (all)	9	7	7
Trametes pubescens MUT 2400 (WS)	a 7 (all)	a 7 (all)	a 7 (all)	a 14 (all)	a 7 (C), a 14 (A, B)	a 7 (A), a 14 (B, C)	a 14 (A, B), c 14 (C)	a 14 (A), c 14 (B), d (C)	b 14 (A, B), c 14 (C)	8	7	6
Pleurotus ostreatus MUT 2976 (WS)	a 7 (A, C), a 14 (B)	a 7 (C), a 14 (A, B)	a 7 (all)	a 14 (all)	a 14 (all)	a 14 (A, B) c 14 (C)	a 14 (A, B) d (C)	a 14 (A), b 14 (C), d (B)	b 14 (A), d (B, C)	8	7	5
Trametes versicolor MUT 2473 (WS)	a 14 (all)	a 14 (all)	a 14 (all)	a 14 (all)	a 14 (all)	a 14 (C), c 14 (A, B)	a 14 (A), d (B, C)	a 14 (all)	a 14 (A), b 14 (C), c 14 (B)	8	6	7
Phlebia radiata MUT 2300 (WS)	a 14 (all)	a 14 (all)	a 14 (A, B), c 14 (C)	a 14 (all)	a 14 (A, B) b 14 (C)	a 14 (A) c 14 (B), d (C)	a 14 (all)	a 14 (A, B), c 14 (C)	b 14 (A, B), c 14 (C)	8	7	4
Cyathus stercoreus MUT 2171 (WS)	a 14 (all)	a 14 (all)	a 14 (all)	a 14 (A), b 14 (B), d (C)	a 14 (A), b 14 (B, C)	a 14 (A), d (B, C)	d (all)	d (all)	a 14 (A), d (B, C)	7	3	3
Chondrostereum purpureum MUT 3075 (PP)	a 14 (all)	a 14 (all)	a 14 (all)	a 14 (all)	b 14 (all)	b 14 (B), c 14 (C), d (A)	a 14 (all)	a 14 (B, C), b 14 (A)	b 14 (all)	6	7	7
Pleurotus ostreatus MUT 2979 (WS)	a 7 (A, C), a 14 (B)	a 7 (all)	a 7 (all)	a 14 (all)	a 14 (A, C), b 14 (B)	c 14 (B, C), d (A)	a 14 (A), c 14 (B, C)	d (all)	b 14 (A), d (B, C)	6	4	5
Panus conchatus MUT 1885 (WS)	a 14 (all)	a 14 (all)	a 14 (A), b 14 (B, C)	b 14 (B), b 14 (A, C)	b 14 (all)	b 14 (A), d (B, C)	a 14 (A), b 14 (B, C)	b 14 (A), c 14 (B, C)	a 14 (A), d (B, C)	6	2	2
Porostereum spadiceum MUT 1585 (WS)	a 7 (all)	a 7 (all)	a 7 (B, C), a 14 (A)	a 14 (all)	a 7 (C), a 14 (A, B)	a 14 (B, C), b 14 (A)	a 14 (B), b 14 (C), d (A)	c 14 (C), d (A, B)	a 14 (C), c 14 (B), d (GN0, 11)	5	7	7
Pleurotus ostreatus MUT 2977 (WS)	a 14 (all)	a 7 (A, B), a 14 (C)	a 7 (A, B), a 14 (C)	a 14 (all)	a 14 (all)	d (all)	d (all)	d (all)	d (all)	5	5	5
Pleurotus ostreatus MUT 2978 (WS)	a 7 (A), a 14 (B, C)	a 7 (A, C), a 14 (B)	a 7 (A, C), a 14 (B)	a 14 (all)	a 14 (A, B), b 14 (C)	d (all)	d (all)	d (all)	d (all)	5	5	4
Cyathus stercoreus MUT 1122 (WS)	a 14 (all)	a 7 (all)	a 7 (all)	a 7 (A), b 14 (B, C)	a 14 (all)	d (all)	d (all)	d (all)	b 14 (A), d (B, C)	5	4	4
Hypholoma sublateritium MUT 385 (WS)	a 14 (A, B), b 14 (C)	a 14 (A), b 14 (B), c 14 (C)	a 14 (A), b 14 (B), c 14 (C)	a 14 (A), d (B, C)	a 14 (A), b 14 (B), c 14 (C)	d (all)	d (all)	d (all)	c 14 (A), d (B, C)	5	1	0
Polyporus ciliatus MUT 3082 (WS)	a 14 (all)	a 14 (all)	a 7 (A), a 14 (B, C)	a 14 (A), c 14 (B), d (C)	b 14 (A), c 14 (B, C)	a 14 (C), c 14 (B), d (A)	b 14 (A), d (B, C)	b 14 (A), d (B, C)	b 14 (A), d (B, C)	4	3	4
Agrocybe farinacea MUT 2755 (LS)	a 14 (all)	a 14 (all)	a 14 (all)	b 14 (all)	a 14 (A), b 14 (C), c 14 (B)	c 14 (all)	c 14 (A), d (B, C)	b 14 (B), c 14 (C), d (A)	b 14 (A), d (B, C)	4	3	3
Agrocybe praecox MUT 2968 (LS)	a 14 (all)	a 14 (A), c 14 (B), d (C)	a 14 (A), c 14 (B, C)	b 14 (A, B), c 14 (C)	a 14 (A), b 14 (B), d (C)	d (all)	d (all)	d (all)	d (all)	4	1	1
Ganoderma applanatum MUT 3044 (PP)	a 14 (A), b 14 (B, C)	a 14 (A), b 14 (B, C)	a 14 (A, C), b 14 (B)	d (all)	a 14 (A), d (B, C)	c 14 (A), d (B, C)	d (all)	d (all)	d (all)	4	0	1
Agrocybe farinacea MUT 2754 (LS)	a 14 (A, C), b 14 (B)	a 14 (A, C), b 14 (B)	a 14 (all)	c 14 (all)	b 14 (A), c 14 (B, C)	b 14 (A, B), c 14 (C)	d (all)	d (all)	b 14 (A), d (B, C)	3	2	2
Polyporus squamosus MUT 2234 (WS)	d (all)	a 14 (all)	b 14 (all)	d (all)	d (all)	b 14 (all)	d (all)	d (all)	d (all)	1	1	1
Total										147	117	113

The flasks were incubated in agitated conditions (130 rpm) at 25 °C. Each trial was performed in triplicate. The DP was monitored approximately every 3 days for 1 month acquiring the complete absorbance spectrum of the effluent and calculated as the extent of decrease of the spectrum area from 360 to 790 nm, with respect to that of the abiotic control.

Significance of differences ($P \leq 0.05$) among DP values was calculated by the Mann–Whitney test (SYSTAT 10, SPSS Inc., 2000).

2.5. Enzymes assays

Lac activity was assayed at 25 °C using 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate [13]. MnP and manganese-independent peroxidase (MiP) activities were measured at 25 °C using DMAB/MBTH [3-dimethylaminobenzoic acid/3-methyl-2-benzothiazolinone hydrazone hydrochloride] as substrates [14]. Aryl alcohol oxidase (AAO) activity was assayed at 45 °C using veratryl alcohol as substrate [15]. LiP activity was assayed at 35 °C using veratryl alcohol as substrate [16]. All the enzyme activities were monitored on 0.1 ml of extracellular culture medium and were expressed in International Units (U), where 1 U is defined as the amount of enzyme that oxidize 1 μmole of substrate in 1 min.

2.6. Batch-mode decolourisation by B. adusta MUT 3060

In order to improve its decolourisation yield, *B. adusta* MUT 3060, the best strain from the previous experiment, was pre-grown before putting it in contact with the MIX and the experiment was performed in agitated conditions. The fungus was inoculated as mycelial plugs in 150 ml Erlenmeyer flasks containing 50 ml of GN0.1 and incubated at 130 rpm at 25 °C. After 7 days, the mycelium was statically sedimented for few minutes, and the culture supernatant was withdrawn and replaced with the same amount of fresh GN0.1 added with the MIX at 1000 mg l^{-1}. Every 2 days and for 2 weeks, 1 ml of the culture medium was taken from each sample for decolourisation and enzyme activities monitoring. The experiment was performed in triplicate.

2.7. Ecotoxicity test

The *L. minor* L. (duckweed) ecotoxicity test was applied to measure the toxicity of the MIX before and after the fungal treatment. The test was performed in 250 ml glass beakers according to the standard ISO/WD 20079 in a working volume of 150 ml and with a sample dilution of 1:10 (ISO 2001). Distilled water was used as the control in the test. Ten fronds of *L. minor* (2 or 3 fronds per colony) of similar size were used as the inoculum. The test was carried out in a climatic exposure test cabinet, calibrated at 24 ± 2 °C, with fluorescent tubes on top that provided continuous lighting (light intensity, 100 μE s^{-1} m^{-2}) for 7 days. At the end of the experiment, fronds number and plant dry weight were used to calculate the growth inhibition ($I_{\mu i}$) by $I_{\mu i} = [(\mu_c - \mu_i)/\mu_c] \times 100$ where μ (average growth rate) = $(\ln N_t - N_0)/T_n$; N_t, value of the observed parameter (fronds number or dry weight) at time t; N_0, value of the same parameter at time 0; T_n, period of time between time 0 and t; μ_c, average growth rate of the control; μ_i, average growth rate of the treatment sample. The test was performed in triplicate.

3. Results and discussion

3.1. Dye decolourisation on agar plate

The strains of *B. adusta* MUT 2295, MUT 2843 and MUT 3060 were able to decolourise all dyes on all media, showing a significant physiological versatility (Table 2). These results are in agreement with Nordstrom et al. [17] who found in plate experiments that a *Bjerkandera* sp. strain had the potential to decolourise several dyes regardless of a different content of nitrogen. The finding of strains efficient against a wide range of structurally different dyes and not strictly dependent on culture conditions is a very crucial aspect with a view to application, since industrial effluents usually contain a range of different dyes and have a very complex and variable composition [11]. Another point of interest is the fact that two of these strains, MUT 2843 and MUT 3060, were isolated from compost that, being a substrate rich in lignocellulosic materials, can be an important source of microorganisms, including ligninolytic fungi, able to degrade pollutants [18]. Hence, *B. adusta* strains were selected for further experiments in liquid cultures together with other 6 fungi that resulted effective in the decolourisation of the largest number of molecules in the broadest spectrum of cultural conditions: *Gloeophyllum odoratum* and *Lenzites betulinus*, able to decolourise all dyes at least on one medium, and *Trametes pubescens*, *Pleurotus ostreatus* MUT 2976, *Trametes versicolor* and *Phlebia radiata*, able to decolourise 8 of the 9 dyes at least on one medium (Table 2).

With the exception of the *Bjerkandera* strains, the medium composition strongly affected the decolourisation capability of many strains; in particular GN0.1, the medium with the lowest content in ammonium tartrate (C:N ratio = 91), showed to be almost always the best medium for decolourisation (Table 2). Therefore this medium was selected for the next phase of the work. Actually, from the earliest studies on *Phanerochaete chrysosporium*, it is known that the depletion of nutrients, including the utilizable nitrogen source, triggers the production of ligninolytic system [19] and, hence, the degrading potential of fungi. The results obtained in cultures of *P. chrysosporium* have been widely accepted as a paradigm for the physiology of ligninolysis among WRF. However, more recently, several white-rot fungi were shown to have a distinct physiology with respect to the regulation of ligninolytic enzymes by nitrogen [19]. For example, *Bjerkandera* spp., *Lentinula edodes* and *P. ostreatus* were shown to have a higher production of ligninolytic enzyme activities in presence of high levels of NH_4^+ [19,20]. However, Moreira et al. [20] observed that the MnP produced by a *Bjerkandera* strain under nitrogen excess conditions was unstable, probably depending on proteolytic activity; N-sufficient conditions were therefore recommended for larger scale production of the enzyme.

Most isolates showed the capability to decolourise a broad spectrum of different molecules, whereas some strains had a specific decolourising activity, being able to decolourise extensively only one or few dyes (Table 2). For example *Polyporus squamosus*, although effective against 3 anthraquinonic and azo model dyes [12], was found able to decolourise completely (class a) only B49 among the anthraquinonic industrial dyes. Therefore, the degradation of model dyes can be considered only predictive of the applicability of white-rot fungi for wastewaters treatment and, as already stressed by Lucas et al. [11], it cannot be separated from the verification of the effectiveness in industrial dyes degradation. Indeed, industrial dyes, even with apparent similar structure, differ from the model ones in terms of purity of the chromophore, presence of auxiliary compounds and recalcitrance.

Considering the number of strains able to extensively decolourise the dyes (class a), it was possible to establish a dye recalcitrance order, namely: R243 > B41 > R80 > B214 > B81 > R111 > B113 > B49 = B225. In general, the azo and the phtalocyanin dyes resulted more recalcitrant than the anthraquinonic ones. On the contrary, no correlation seemed to occur between recalcitrance and chemical class of the dyes (acid, reactive or direct).

Table 3
Decolourisation percentage (DP) and enzyme activity ($U\,l^{-1}$) obtained by 9 basidiomycetes on different industrial dyes.

		B214	B49	R80	B225	B113	R111	B41	B81	R243	Average DP for each isolate
Bjerkandera adusta MUT 3060	DP	98	99	100	97	80	76	96	88	67	90
	Lac	3	2	3	1	1	1	1	2	–	
	MiP	278	2	42	39	24	1	31	76	10	
	MnP	3	12	19	25	6	3	18	9	39	
	LiP	25	2	5	8	8	3	2	18	–	
	AAO	6	–	8	22	–	1	–	4	7	
Bjerkandera adusta MUT 2295	DP	94	98	99	96	81	81	93	63	55	85
	Lac	2	–	2	–	1	–	1	92	1	
	MiP	133	8	23	2	55	1	89	4	17	
	MnP	4	4	5	2	1	–	25	–	29	
	LiP	1	5	2	2	6	–	6	4	–	
	AAO	5	3	9	32	4	60	31	–	16	
Trametes pubescens MUT 2400	DP	100	97	99	99	81	65	73	45	57	81
	Lac	40	50	95	112	387	146	391	8	281	
	MiP	2	2	2	–	–	–	–	63	–	
	MnP	12	9	–	5	9	–	–	1	–	
	LiP	–	–	–	–	4	–	1	1	–	
	AAO	1	–	6	–	–	–	2	9	4.9	
Lenzites betulinus MUT 2451	DP	83	94	97	92	80	57	69	36	50	75
	Lac	58	8	5	89	76	65	98	22	20	
	MiP	–	3	–	4	–	–	2	22	–	
	MnP	8	6	6	5	–	–	1	–	12	
	LiP	4	4	–	–	1	17	–	6	13	
	AAO	–		1	–	–	–	–	–	5	
Gloeophyllum odoratum MUT 2294	DP	75	91	97	89	76	78 17	84	43	33	74
	Lac	154	48	63	89	145	–	104	31	51	
	MiP	–	–	–	–	–	–	–	–	–	
	MnP	–	–	–	–	–	10	–	6	–	
	LiP	–	6	4	2	8	13	7	4	4	
	AAO	10	8	3	1	4	–	2	3	9	
Trametes versicolor MUT 2473	DP	90	90	98	83	80	70	30	54	53	73
	Lac	84	34	9	106	33	53	330	36	55	
	MiP	8	4	2	3	3	1	12	2	3	
	MnP	10	13	6	–	28	–	–	–	3	
	LiP	4	9	1	1	3	5	14	2	13	
	AAO	1	–	13	10	2	–	–	14	4	
Bjerkandera adusta MUT 2843	DP	99	95	44	75	79	76	39	74	63	73
	Lac	1	–	–	–	–	–	–	1	–	
	MiP	4	13	5	–	2	–	2	2	–	
	MnP	2	–	5	–	–	–	1	–	1	
	LiP	1	9	2	1	–	1	4	2	2	
	AAO	–	1	–	–	–	–	–	24	–	
Pleurotus ostreatus MUT 2976	DP	91	95	98	72	80	17	4	19	28	56
	Lac	1	23	34	–	55	2	3	28	101	
	MiP	2	1	–	–	1	–	1	–	–	
	MnP	–	2	–	–	15	–	–	3	–	
	LiP	10	–	–	12	11	4	44	7	5	
	AAO	–	2	–	3	–	3	2	–	6	
Phlebia radiata MUT 2300	DP	81	39	65	76	73	26	18	22	14	51
	Lac	2	1	3	18	1	2	13	2	1	
	MiP	6	1	–	–	1	–	3	–	1	
	MnP	13	1	11	3	4	5	2	8	1	
	LiP	1	28	10	6	6	7	18	42	7	
	AAO	–	1	–	–	2	2	–	–	–	
Average DP for each dye		90	89	89	87	79	61	56	49	47	

3.2. Dye decolourisation in liquid systems

Liquid culture conditions allowed a more precise evaluation of the degradation capabilities of the 9 selected strains and the monitoring of ligninolytic enzymes. No dye sorption to mycelia was visually observed in the biotic lines at the end of the experiment and no decolourisation occurred in the abiotic controls. Decolourisation efficiency varied according to the isolates. Comparing the average of the DPs obtained on the 9 different dyes by each isolate, *B. adusta* MUT 3060 and 2295 presented, as in solid conditions, the highest efficiency, followed by *T. pubescens*, *L. betulinus*, *G. odoratum*, *T. versicolor*, *B. adusta* MUT 2843 and finally by *P. ostreatus* and *P. radiata* (Table 3).

The recalcitrance of the dyes was evaluated calculating the average DP by the different isolates (Table 3). As observed in solid conditions, the azoic and the phthalocyanin dyes were in general more recalcitrant than the anthraquinonic dyes. However, some differences in the recalcitrance order occurred and may be attributed to the different culture conditions. The lower recalcitrance in liquid compared to solid conditions, as in the case of the azo dyes B214 and R80, may be attributed to the fact that in liquid cultures degradation is favoured by a more direct contact between

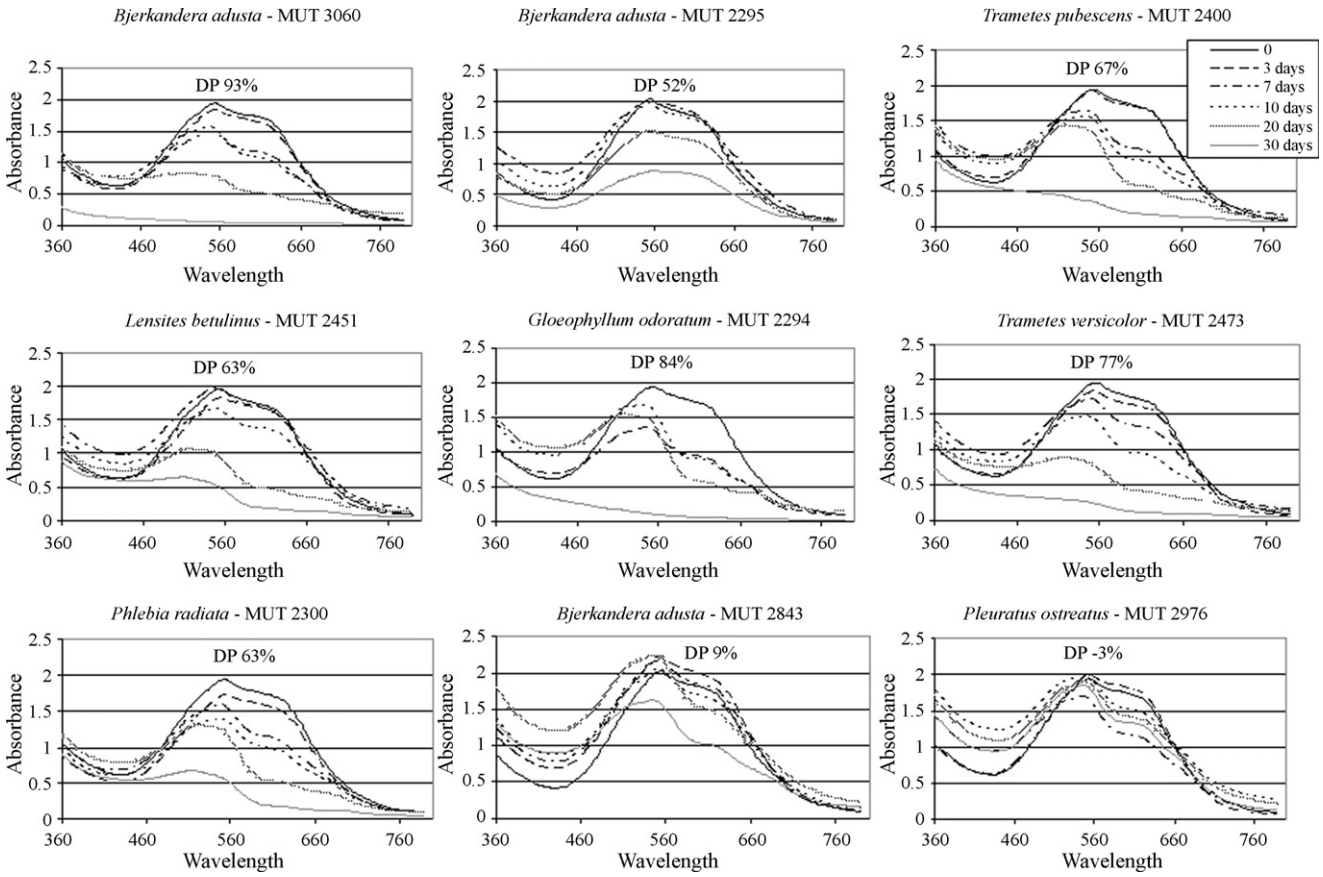

Fig. 1. Absorbance spectra of MIX effluent at day 3, 7, 10, 20, 30 and final decolourisation percentage (DP) after treatment by 9 fungal strains.

the dye molecule and the organism and its enzymes. Differences in the decolourisation of dyes also belonging to a same class may be attributed to steric factors, electron distribution, and charge density [21]. The recalcitrance of several azo dyes may be due to the fact that these compounds do not occur naturally, thus specific adaptive mechanisms of biodegradation by the microorganisms have not been developed [10]. Phthalocyanine dyes are widely used in the textile and dyestuff industries, but little is known about the decolourisation and biodegradation of these compounds [21]. An important consideration concerns the data on the biodegradability of the tested molecules provided by the manufacturer (Table 1). Our fungal strains were found effective also in the degradation of dyes with a very low biodegradability (0–30%) as B214, B225, B49. Only the dye R243, whose reported biodegradability is of 25%, confirmed its recalcitrance being the less degraded molecule by the tested fungi, both in liquid and in solid conditions.

Many papers have documented the involvement of ligninolytic enzymes like Lac and different types of peroxidases in dye degradation [9,10,22], and some authors have already suggested the involvement of other enzymes recently found in the lignocellulose degradation pathway, i.e. glucose oxidases and AAO [23]. However, the participation of these enzymes in the dye degradation is not obvious and very little is known about their combined role and their interactions during the degradation process [24]. In our experiment, each fungus produced a different pattern of oxidative enzyme activities during dye decolourisation (Table 3). MiP/MnP and AAO were the prevailing activities during decolourisation by *B. adusta* MUT 3060 and 2295. On the contrary, very low activities were expressed by the less efficient strain of *B. adusta* MUT 2843, indicating that MiP/MnP have a crucial role in dye degradation by *B. adusta* and that different strains, even when belonging to the same species, can have a different enzymatic behaviour and degradation activity. Lac was the main secreted enzyme by the WRF *T. pubescens*, *L. betulinus*, *T. versicolor* and *P. ostreatus* and also by the BRF *G. odoratum*, while *P. radiata* produced mainly LiP. Each dye strongly affected the enzymatic activities of the different isolates. Noteworthy is the induction of MiP by B214 in *B. adusta* MUT 3060, of Lac by B113 and B41 in *T. pubescens* and by B41 in *T. versicolor* (Table 3). These data confirm the observations of Martins et al. [25] about the change of the enzymatic pattern of *P. chrysosporium* cultured in the presence of different dyes.

The 9 isolates displayed different DPs against the MIX of dyes at 1000 mg l^{-1}: after 30 days the DP ranged from 0% (*P. ostreatus*) to 93% (*B. adusta* MUT 3060) (Fig. 1). The incubation time affected the decolourisation intensity. The DP was negligible within 3 days with the only exception of *P. radiata* (12.2%); after the 7th day most of fungi started an effective decolourisation which continued till the end of the experiment and a great increase in decolourisation was observed also after 20 days of incubation. Only two fungi, *B. adusta* MUT 2843 and *P. ostreatus*, were ineffective in the decolourisation of the MIX and were almost inactive until the 14th day. Obviously these fungi, although active on individual dyes, are not able to act when they are mixed together. Changes in visible absorbance spectra were observed for all the isolates (Fig. 1). In particular the shape of the spectrum was always affected over the 600 nm wavelength (corresponding to absorbance peaks of blue dyes). On the contrary, at the wavelengths corresponding to the maximum absorbance of the red dyes (e.g. R80 and R243), among the most recalcitrant when used individually (singly), a weaker absorbance decrease was observed during the experiment. A complete knock down of the visible spectrum was obtained exclusively for *B. adusta* MUT 3060.

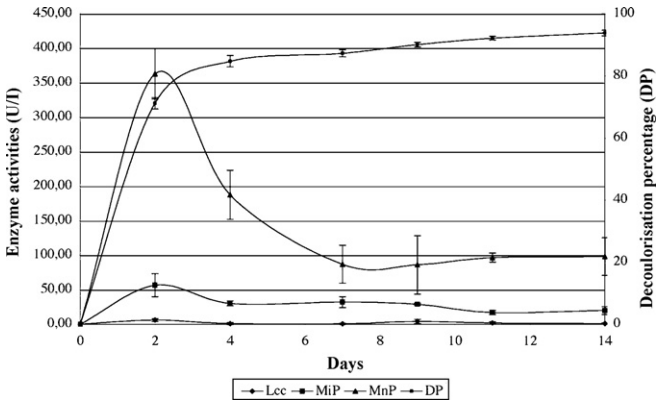

Fig. 2. Decolourisation of the MIX of 9 industrial dyes by *B. adusta* MUT 3060 and laccase (Lac), manganese peroxidase (MnP), manganese-independent peroxidase (MiP) activities (average of 3 replicates ± SE).

These results are very promising from an applicative point of view, since until now very few fungi were tested against a mixture of dyes and in these experiments often lower dye concentrations were used respect to our experiments. Using *B. adusta* Mohorcic et al. [26] succeeded in decreasing the colour of an artificial effluent containing 170 mg l^{-1} of dyes. Martins et al. [27] reported that pre-cultured biomasses of *P. chrysosporium* and *T. versicolor* degraded respectively about 40% and 80% of a 50 mg l^{-1} mixture of 8 syringol derivates of azo dyes and Blanquez et al. [28] found that *T. versicolor* efficiently decolourised (90%) a 150 mg l^{-1} of a mixture of metal complexed dyes named Grey Lanaset Faraco et al. [22] reported a 60% decolourisation of a 300 mg l^{-1} mixture of acid dyes by *P. ostreatus* and a 87% decolourisation of a 3000 mg l^{-1} mixture of direct dyes by *P. chrysosporium*.

3.3. Batch-mode decolourisation and detoxification by B. adusta MUT 3060

In order to optimize the decolourisation process, *B. adusta* MUT 3060 was pre-activated and cultured in agitated conditions. These culture conditions really accelerated the process: in 14 days the fungus almost completely decolourised (DP = 94%) the MIX of 9 dyes at 1000 mg l^{-1} (Fig. 2). The sharpest fall in dyes concentration (DP = 71%) was evident after 2 days and a significant decrease in decolourisation occurred till the end of the experiment. The MIX can be regarded as representative of a real wastewater, both for number and concentration of dyes; hence, such high yields of decolourisation are very promising from an applicative point of view and confirm the data recently obtained by several authors with *Bjerkandera* spp. strains in the decolourisation of textile dyes [17,26,29].

Extracellular ligninolytic enzyme activities were detected throughout the experiment, but varied greatly from one enzyme to another and in function of the sampling time, displaying always the maximum activity on day 2, when the maximum decolourisation occurred (Fig. 2): MnP activity was the highest (maximum 363 U l^{-1}), followed by MiP activity (maximum 57 U l^{-1}) and Lac (maximum 6 U l^{-1}). LiP and AAO activities were never detected. Hence, in agreement with other studies on the decolourisation activity of *B. adusta*, MnP is the main class of enzymes involved [20,30]. Mohorcic et al. [26] provided indirect evidence for the involvement of this enzyme in the decolourisation process of artificial textile dye baths demonstrating an effective absorbance reduction by purified MnP produced by *B. adusta*. Moreover, another interesting feature of this enzyme from a biotechnological point of view is the quite simple improvement in the production using media engineering as demonstrated by Seker et al. [31]. The presence of MiP activity suggests that also versatile peroxidase (VP) may be involved in the dye degradation process by *B. adusta* MUT 3060. VPs are a new family of ligninolytic peroxidases independent of manganese and with characteristics intermediate between MnP and LiP. They are generally found in *Pleurotus*, *Bjerkandera*, *Lepista*, *Trametes* and are known to degrade dyes and several other recalcitrant pollutants [32]. Among the different basidiomycetes peroxidases, VP presents particular interest due to its catalytic versatility including the degradation of compounds that other peroxidases are not able to oxidize directly [33]. VP versatility permits its application in Mn-mediated or Mn-independent reactions on both low and high redox-potential aromatic substrates and dyes, among others. Moreover, by contrast with LiP, VP does not require mediators to oxidize phenolic and non-phenolic compounds and this is a characteristic of the highest biotechnological interest [33].

After the fungal treatment the growth inhibition of *L. minor*, measured both as fronds number and dry weight, significantly decreased indicating a reduction of the toxicity (Fig. 3). The *L. minor* ecotoxicity test presents high sensitivity for dye-containing samples [34] and the results of this study give us a first indication that the decolourising activity of *B. adusta* is coupled to detoxification events. Detoxification of true or simulated industrial effluents after fungal treatment has been verified in only very few studies [34–36]. The observation that decolourisation is not always followed by detoxification, as already demonstrated by several papers dealing with chemical–physical treatments (i.e. ultrasound and photochemical treatment) and biological degradation mainly of azo dyes [4,37], indicates that the effluent toxicity before and after the treatment should be always monitored.

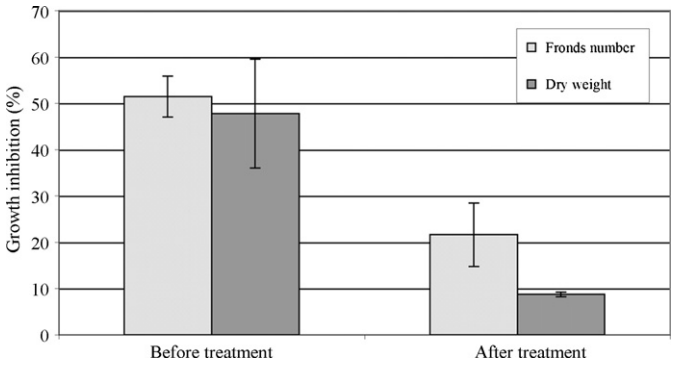

Fig. 3. Growth inhibition ($I_{\mu i}$, average of 3 replicates ± SE) measured by frond number and plant dry weight before and after the decolourisation of the MIX of 9 dyes by *Bjerkandera adusta* MUT 3060.

4. Conclusions

The results obtained from this wide screening on the degrading potentialities of basidiomycetes belonging to different eco-physiological groups against industrial dyes, allow to draw several conclusions:

- Most of the tested isolates showed the capability to decolourise a broad spectrum of structurally different molecules and some strains were found effective in the decolourisation of all dyes on all media, displaying a physiological versatility which is a very crucial aspect with a view to application.
- The capability of our selected isolate of *B. adusta* MUT 3060 to survive and grow in presence of very high concentration of toxic dyes is an adaptive feature that, together with its degradation capability and physiological versatility (high degradation capa-

bility over a wide range of different C:N ratio conditions), make it a very promising candidate for the treatment of real wastewaters.
- The decolourisation after fungal treatment corresponds to a substantial detoxification of the wastewater, a parameter that is often not taken into account, but that is the prerequisite to hypothesize a real application in the treatment of coloured wastewaters.

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