Scale-up of a bioprocess for textile wastewater treatment using Bjerkandera adusta

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Abstract

Twelve basidiomycetes were investigated for their ability to degrade 13 industrial dyes and to treat four model wastewaters from textile and tannery industry, defined on the basis of discharged amounts, economic relevance and representativeness of chemical structures of the contained dyes. The best degradation yields were recorded for one strain of *Bjerkandera adusta* able to completely decolourise most of the dyes and to decolourise and detoxify three simulated wastewaters, showing a significant physiological versatility which is very useful for application purposes. The effects of different nutrient sources were investigated in order to optimize the yields of decolourisation and detoxification. Manganese-peroxidase and manganese-independent peroxidase were the only recorded enzymatic activities. In order to evaluate its true bioremediation potential, this strain was packed in a fixed-bed bioreactor, for treatment of large volumes of a real wastewater. The fungus resulted effective during 10 cycles of decolourisation, remaining active for a very long period, in non-sterile conditions.

1. Introduction

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 have proved to be the microorganisms most efficient in degrading synthetic dyes (Kaushik and Malik, 2009). 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 with a manganese-independent activity (MiP) and named versatile peroxidase (VP), sharing the catalytic properties of LiP and MnP, was described in several species of the genera *Pleurotus* and *Bjerkandera* (Mohorcic et al., 2009) and was demonstrated effective against a wide range of industrial dyes (Tinoco et al., 2007).

The literature is replete with reports demonstrating the excellent capacity of fungi to degrade dyes. Their potential so far, however, has not yet found a real application, mainly because of the difficulty in selecting organisms capable of growing and degrading in the very variable and restrictive conditions of the textile industry wastewaters. Actually, textile effluents are one of the most difficult-to-treat wastewaters on account of the considerable amount of suspended solids and of the massive presence of dyes, salts, additives, detergents and surfactants. They exhibit large fluctuations in terms of quantities and pollution load, pH and temperature depending upon customer orders, types of manufactured textile materials and production schedules (Hai et al., 2007 and Vanhulle et al., 2008).

Most of the screenings have been performed 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. Moreover, model dyes biotransformation cannot always be extrapolated to industrial dyes (Lucas et al., 2008).

In addition, most of the research studies on dyes degradation are conducted at Erlenmeyer scale. Nevertheless, before an industrial application can be implemented, fungal bioreactors which can be operated under industrial conditions, using real wastewaters must be developed (Blanquez et al., 2008). Some works have been published about dyes biodegradation by white-rot fungi in bioreactor, under conditions similar to those used at laboratory scale using Erlenmeyer flasks, without optimization for further application at full-scale (Mielgo et al., 2002, Rodriguez Couto et al., 2004, Romero et al., 2006 and Zhang et al., 1999). Very few papers related to the continuous treatment, under non-sterile conditions, of real industrial textile wastewaters in bioreactor by white-rot fungi were found (Blanquez et al., 2008).

Another critical point is the evaluation of the detoxification after fungal treatment, a parameter that is often not taken into account, even if it is a prerequisite to hypothesize a real application in the treatment of coloured wastewaters. Actually, decolourisation does not imply that the resulting molecules 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 (Oliveira et al., 2007).

The purpose of this study was the selection of fungal strains useful in the treatment of coloured wastewaters. The decolourisation efficiency of 12 basidiomycetes against 13 commercially important industrial dyes was investigated in solid conditions. The seven best strains were studied in greater detail, in liquid conditions, investigating the decolourisation activity towards simulated effluents that mimicked the real ones for pH, concentration of dyes, additives and salts. A very promising strain of *Bjerkandera adusta*was then cultured under specific conditions in order to optimize its degradation yield and two ecotoxicity tests were performed in order to estimate the evolution of toxicity due to the fungal treatment. Finally, this strain was packed in a fixed-bed bioreactor, for treatment of large volumes of a real wastewater, starting a first scale-up of a bioremediation process.

2. Methods

2.1. Dyes and effluents

Thirteen industrial dyes (Table 1) kindly provided by Town End (Leeds, UK) plc. were selected because representative of different structural dye types (comprising mono-, di-, polyazoic, stilbene and anthraquinonic chromophores) and commercially important, with a wide range of applications across the textile and tannery industries. They are of technical grade and dye molecules constitute the 30–90% of the total weight. The dyes were tested individually in solid conditions and mixed together in four simulated wastewaters, designed to mime effluents produced during cotton, wool or leather dyeing processes (Table 1) and already used to study the applicability of fungi in wastewater treatments (Prigione et al., 2008 and Faraco et al., 2009). In addition to the dyes, these simulated wastewaters mimic the industrial ones also for the presence of different salts, often in high concentrations, and for the pH values. The simulated wastewaters, previously developed by the industrial partners of the EC FP6 Project SOPHIED (NMP2-CT-2004-505899), were used under the permission of the SOPHIED Consortium. All the simulated wastewaters were sterilised by tyndallization (three 1 h cycles at 60 °C with 24 h interval between cycles at room temperature).

Table 1.

Dyes and wastewaters composition (EC FP6 Project SOPHIED, NMP2-CT-2004-505899).

Wastewater	Dyes and salts	Concentration (g l^{-1})	pH
Acid dye bath for wool (W1)	Acid blue 62 (ABu62)	0.10	5
	Acid yellow 49 (AY49)	0.10	
	Acid red 266 (AR266)	0.10	
	Na_2SO_4	2.00	
Reactive dye bath for cotton (W2)	Reactive blue 222 (RBu222)	1.25	10
	Reactive red 195 (RR195)	1.25	
	Reactive yellow 145 (RY145)	1.25	
	Reactive black 5 (RBk5)	1.25	
	Na_2SO_4	70.00	
Direct dye bath for cotton (W3)	Direct blue 71 (DrBu71)	1.00	9
	Direct red 80 (DrR80)	1.00	
	Direct yellow 106 (DrY106)	1.00	
	NaCl	5.00	
Acid dye bath for leather (W4)	Acid black 194 (ABk194)	0.10	5
	Acid black 210 (ABk210)	0.10	
	Acid yellow 194 (AY194)	0.10	

The real effluent (OMO) was kindly provided by Tintoria Pollone, Biella (Italy). It was sampled by the homogenisation tank in which were collected the 3 days discharges of processing and dyeing of wool, cotton, acrylic and polyester (containing acid, direct, reactive and disperse dyes in different concentrations). The initial pH was 9.3.

2.2. Organisms

Ten white-rot fungi, one brown-rot fungus (*Gloeophyllum odoratum*) and an unidentified basidiomycetes have been selected from previous experiments for their degradation capability towards industrial dyes (Casieri, 2005 and Anastasi et al., 2009b). The strains, listed in Table 2, belong to eight species ascribable to different families (*Meruliaceae*, *Phanerochaetaceae*, *Pleurotaceae*, *Polyporaceae*); they are lignicolous saprotrophs isolated both from carpophores and compost. The fungi are preserved at the *Mycotheca Universitatis Taurinensis* Collection (MUT, University of Turin, Department of Plant Biology) on malt extract agar at 4 °C.

Table 2
Decolourisation activity on two solid substrates (GHY, GLY) by 12 basidiomycetes towards 13 industrial dyes. Numbers represent degradation classes in function of an increasing decolourisation (0 = no decolourisation; 1 = light decolourisation; 2 = medium decolourisation; 3 = complete decolourisation); in brackets the day in which a complete decolourisation was obtained.

		Bjerkandera adusta MUT 3060	*Bjerkandera adusta* MUT 2843	*Bjerkandera adusta* MUT 2295	*Trametes versicolor* MUT 2473	*Gloeophyllum odoratum* MUT 2294	*Phlebia radiata* MUT 2300	*Pleurotus ostreatus* MUT 2976	*Pleurotus ostreatus* MUT 2150	*Trametes pubescens* MUT 2400	*Lenzites betulina* MUT 2451	*Phanerochaete chrysosporium* MUT 3190	*Unidentified basidiomycetes* MUT 3062
ABu62	GHY	3 (7)	3 (14)	3 (14)	3 (21)	1	1	2	2	1	1	0	1
	GLY	3 (21)	3 (21)	3 (21)	1	3 (21)	2	3 (21)	2	3 (21)	2	0	1
AY49	GHY	1	1	1	1	1	1	1	1	1	1	0	0
	GLY	1	1	1	0	2	2	3 (21)	3 (21)	0	0	1	0
AR266	GHY	3 (14)	3 (14)	3 (14)	3 (21)	2	2	2	2	2	2	1	0
	GLY	1	1	1	2	2	3 (21)	3 (21)	2	2	2	2	0
RBu222	GHY	3 (14)	3 (14)	3 (14)	3 (21)	2	0	0	0	1	0	0	0
	GLY	3 (14)	3 (21)	3 (14)	1	3 (21)	3 (14)	1	1	2	2	3 (21)	1
RR195	GHY	3 (14)	3 (14)	3 (14)	3 (21)	2	0	0	0	0	0	0	0
	GLY	3 (14)	3 (21)	3 (14)	2	1	3 (14)	2	2	3 (21)	1	2	0
RY145	GHY	3 (14)	3 (14)	3 (14)	3 (14)	1	0	0	0	0	0	0	0
	GLY	3 (14)	3 (14)	3 (14)	2	3 (14)	3 (14)	2	3 (14)	2	1	3 (14)	1
RBk5	GHY	3 (14)	3 (14)	3 (14)	3 (14)	1	1	1	1	3 (21)	1	0	0
	GLY	3 (14)	3 (14)	3 (14)	3 (21)	2	3 (21)	2	3 (14)	1	2	3 (14)	1
DrBu71	GHY	3 (14)	3 (14)	2	3 (14)	1	1	1	2	2	2	0	0
	GLY	3 (14)	3 (14)	3 (21)	3 (14)	3 (14)	3 (14)	3 (14)	3 (14)	3 (14)	3 (14)	3 (14)	0
DrR80	GHY	2	1	2	1	0	0	0	0	0	0	0	0
	GLY	3 (21)	3 (21)	3 (14)	3 (21)	2	2	3 (21)	2	2	2	3 (14)	0
DrY106	GHY	0	0	0	0	0	0	0	0	0	0	0	0
	GLY	0	0	0	0	0	0	0	0	0	0	0	0
ABk210	GHY	3 (14)	3 (14)	2	3 (21)	2	0	1	1	1	1	0	0
	GLY	3 (14)	3 (14)	3 (14)	3 (21)	2	3 (14)	3 (14)	3 (21)	1	2	3 (14)	0
ABk194	GHY	3 (7)	3 (7)	3 (7)	1	2	2	3 (14)	3 (14)	3 (14)	3 (14)	1	0
	GLY	2	2	2	1	2	3 (21)	2	1	2	2	0	1
AY194	GHY	3 (21)	3 (14)	3 (14)	3 (14)	3 (14)	1	2	2	3 (14)	2	1	0
	GLY	3 (14)	3 (14)	3 (21)	1	0	1	0	0	1	1	1	0
Total		64	63	62	51	43	40	40	39	39	33	27	6

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 two solid media (GLY and GHY), with different C:N ratios (250 and 10, respectively), supplemented with dyes at a final concentration of 500 ppm. GLY and GHY contained 0.152 and 3.18 g l^{-1} respectively of yeast extract, 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} 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, 0.001 g $NaMoO_4{\cdot}2H_2O$ (Leung and Pointing, 2002).

The experiment was performed in triplicate. Plates were incubated at 25 °C, and after 3, 7 and 14 days, decolourisation was measured. Since the fungal colonies determined a widespread loss of dye over the whole surface of the plate, it was not possible to measure the extent of a halo of discolouration. Four degradation classes were assigned in function of an increasing decolourisation (0 = no decolourisation; 1 = light decolourisation; 2 = medium decolourisation; 3 = complete decolourisation).

2.4. Wastewater decolourisation in liquid systems

The best seven strains selected from the previous experiment were inoculated as described above in 100 ml flasks containing 30 ml of GHY. The flasks were incubated in agitated conditions (130 rpm) at 28 °C. After 7 days, the culture broths were replaced with 30 ml of the four wastewaters. Each trial was performed in triplicate. The visible absorbance spectrum (from 360 to 790 nm) of the effluent was acquired daily for one week using a spectrophotometer (Amersham Biosciences Ultrospec 3300 Pro). The decolourisation percentage (DP) was calculated as the

extent of decrease of the spectrum area (the integral of the absorbance spectrum), with respect to that of the abiotic control. Significance of differences ($p \leqslant 0.05$) among DP values at the end of the experiment was calculated by the Mann–Whitney test (SYSTAT 10, SPSS Inc. 2000).

2.5. Optimization of wastewater decolourisation in liquid systems

In order to improve its decolourisation yield, *B. adusta* MUT 2295, the best strain from the previous experiment, was inoculated as described above in the wastewaters in the presence and in the absence of glucose and yeast extract in the proportion of GHY and GLY. The decolourisation percentage (DP) was monitored daily for one week together with the enzyme activities according to the following protocols. At the end of the experiments the evolution of toxicity was measured by means of two ecotoxicity tests described below.

2.6. Fixed-bed bioreactor configuration and operation conditions

Six stainless steel sponges were used as immobilisation supports. The sponges were autoclaved at 121 °C for 20 min, embedded with a GHY solution mixed with agar at a concentration of 7 g l^{-1} and colonized by *B. adusta* MUT 2295 for 10 days. The bioreactor consisted of a glass column (30 cm in height and 4 cm in diameter) packed with the colonized sponges. The column was connected to a bottle containing the wastewater kept circulating at a constant 10 ml min^{-1} by a peristaltic pump (Model SP311 VELP, Milan). Air was supplied in a continuous way. The bioreactor was kept at room temperature (about 20 °C). Periodically, 1 ml of wastewater was withdrawn from the system for DP and enzyme activities monitoring. The system was active for more than 70 days during which 6 cycles of decolourisation of W1 (300 ml each) and 4 cycles of OMO (1 cycle of 300 ml and 2 of 1 l) were conducted. Every 2 cycles of W1 a phase of conditioning was conducted replacing the wastewater with GHY. No conditioning was carried out between the cycles of the wastewater OMO.

2.7. Enzymes assays

Lac activity was assayed at 25 °C using 2.2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate (Niku-Paavola et al., 1988). MiP and MnP activities were measured at 25 °C using DMAB/MBTH (3-dimethylaminobenzoic acid/3-methyl-2-benzothiazolinone hydrazone hydrochloride) as substrates (Vyas et al., 1994). LiP activity was assayed at 35 °C using veratryl alcohol as substrate (Tien and Kirk, 1983). All the enzyme activities were expressed in International Units (IU), where one unit is defined as the amount of enzyme that oxidize 1 μmole of substrate in 1 min.

2.8. Ecotoxicity tests and COD determination

Evolution of toxicity before and after treatment (IV cycle) of the real OMO effluent was evaluated by means of the test on *Vibrio fischeri* according to the method UNI EN ISO 11348-3:2001. The luminescence of the bacteria was measured after 5, 15 and 30 min exposure to samples at 15 °C, utilizing Microtox® (Microtox Model 500; Microbics Corp., USA). Correction for colour, which might have an impact on the luminescent measurements, was performed on all samples according to the MicrotoxOmni™ manual. Toxicity was expressed as toxicological units (TU) calculated as $100/EC_{50}$, where EC_{50} is the pollutant effective concentration that provokes a response corresponding to the half of the maximum toxic effect.

Due to the strong colour interference the *V. fischeri* test wasn't applicable on the simulated wastewaters (W1–W4); hence we used two other ecotoxicity tests described below that, although very time consuming and with more complex operating conditions and elaborations, do not suffer the colour interference.

The *Lemna minor* L. (duckweed) ecotoxicity test was applied according to the standard ISO/WD 20079 to measure the toxicity of the simulated wastewaters before and after the treatment in batch with *B. adusta*MUT 2295. Due to the limited sample volume available the method was miniaturized after statistical control. The test was performed in 25 ml glass beakers in a working volume of 15 ml and with a sample dilution of 1:15 (ISO 2001). Toxicity was expressed as growth inhibition ($I_{\mu i}$) on frond number or dry weight calculated as follows: $I_{\mu i} = [(\mu_c - \mu_i)/\mu_c]$. 100, where μ (average growth rate) = $(\ln N_t - N_0)/T_n$; N_t is the value of the observed parameter (frond number or dry weight) at time t; N_0 is the value of the same parameter at time 0; T_n is the period of time between time 0 and t; μ_c is the average growth rate of the control; μ_i is the average growth rate of the sample.

Toxicity of the simulated wastewaters was also evaluated, before and after the fungal treatment, by means of a phytotoxicity test on *Cucumis sativus* L. seeds according to the method UNICHIM N. 1651 (2003). The germination index (GI) was calculated as a percentage of number of germinated seeds and root length values in the samples respect to the control (set up with distilled water instead of samples) according to the formula: GI = (Gs × Ls)/(Gc × Lc) × 100, where Gs is the mean number of germinated seeds in the sample, Ls is the mean root length of the sample, Gc is the mean number of germinated seeds in the control, Lc is the mean root length of the control.

Significance of the increase or decrease of toxicity values before and after fungal treatment ($p \leqslant 0.05$) was calculated by the Mann–Whitney test (SYSTAT 10, SPSS Inc. 2000).

Determination of the chemical oxygen demand (COD) with the dichromate method was performed before and after treatment of OMO effluent (IV cycle) 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 l^{-1} of O_2). Appropriate dilutions of each sample were assayed.

3. Results and discussion

3.1. Dyes decolourisation on agar plate

The screening on solid substrate made it possible to identify seven fungi (*B. adusta* MUT 2295, MUT 3060, MUT 2843, *Trametes versicolor* MUT 2473, *G. odoratum* MUT 2294, *Phlebia radiata* MUT 2300, *Pleurotus ostreatus* MUT 2976) with promising degradation capacity against different industrial dyes, at high concentrations (Table 2). The best degradation yields were recorded for the three strains of *B. adusta*, able to completely decolourise (class 3) most of the dyes and in both culture conditions. The physiological versatility of these strains, already demonstrated in a previous study on other industrial dyes (Casieri, 2005 and Anastasi et al., 2009b), is a very useful feature for application, since industrial effluents usually contain a range of different dyes and have a very complex and variable composition (Lucas et al., 2008). On the contrary, the need for specific cultivation conditions has so far represented a major limitation to the industrial application of fungi such as for example *P. chrysosporium*, for the treatment of coloured wastewater (Faraco et al., 2009 and Yu et al., 2006). In our study, not only *P. chrysosporium* but in general most of the other fungi showed a higher decolourisation activity on GLY, the medium

with the lowest content of nitrogen (Table 2). Actually, from the earliest studies on *P. chrysosporium*, it is known that the depletion of nitrogen triggers the production of the ligninolytic system (Kaal et al., 1995) and, hence, the degrading potential of fungi.

Another point of interest of this study is the use of industrial dyes. As recently highlighted by Lucas and colleagues (2008), verification of degradation against industrial dyes is essential. In fact the industrial dyes, although apparently similar to the model ones, differ from them in terms of purity of the chromophore, presence of auxiliary compounds and recalcitrance. In our study, the yellow dyes (AY49 and DrY106) were the most recalcitrant as also demonstrated by Faraco and colleagues (2009) for a yellow disperse dye (Disperse yellow 3).

3.2. Wastewater decolourisation in liquid systems

The screening in liquid allowed to verify the potential application of the seven fungi for wastewater treatments. As can be seen in Table 3, in the absence of any additional nutritive source, the two acid baths for the dyeing of wool (W1) and leather (W4) were extensively decolourised within few days, in particular by*B. adusta* MUT 2295 and MUT 3060. The absorption spectra of W1 and W4 at the end of the test were completely knocked down (Fig. 1), indicating the effectiveness of the two strains towards all the dyes (including AY49 that was only partially degraded during the screening in solid). Biomasses at the end of the process were colourless, indicating the absence of adsorption phenomena.

Table 3
Maximum DP values obtained by seven strains against four simulated wastewaters (in brackets the day of measure).

	W1	W2	W3	W4
B. adusta 2295	90 (3)	16 (1)	57 (5)	88 (2)
B. adusta 3060	91 (2)	34 (1)	41 (7)	83 (1)
P. ostreatus 2976	80 (7)	25 (1)	42 (7)	81 (7)
P. radiata 2300	75 (7)	19 (1)	22 (1)	75 (7)
T. versicolor 2473	59 (1)	31 (1)	20 (1)	73 (2)
B. adusta 2843	56 (7)	17 (1)	16 (1)	72 (5)
G. odoratum 2294	52 (1)	22 (1)	34 (5)	35 (1)

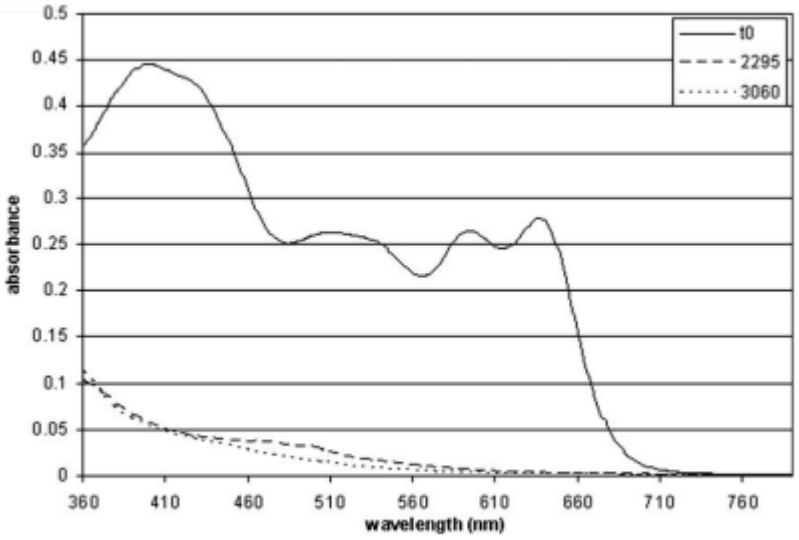

Fig. 1. Absorbance spectra of W1 before (t0) and after treatment with *Bjerkandera adusta* MUT 2295 and 3060.

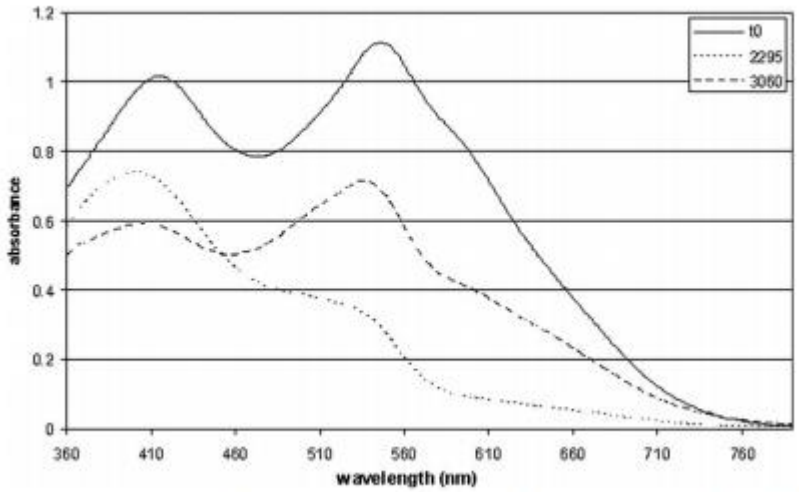

Fig. 2. Absorbance spectra of W3 before (t0) and after treatment with *Bjerkandera adusta* MUT 2295 and 3060.

Against the reactive wastewater (W2) all fungi determined, within the first day, only a 16–34% decrease of the absorbance spectrum, mainly due to biosorption phenomena (the absorbance spectra of the dyes did not present differences in shape and the fungal mycelia appeared strongly dyed). This can be ascribed to the very restrictive conditions of this wastewater (5000 ppm of dyes, pH 10 and 70 g l^{-1} of salt) that have hindered the growth and activity of fungi.

The direct wastewater (W3) was significantly decolourised by the two strains of *B. adusta* MUT 3060 and MUT 2295, the latter being able to determine a DP of 57%, corresponding to the removal of more than 1700 ppm of dye in 3 days. It is interesting to note that the two strains were able to preferentially remove different areas of the spectrum, and this is an important prerequisite for a

possible use in co-culture of the two strains in order to exploit their complementary effects (Fig. 2). In particular, the strain MUT 3060 was able to remove a significant portion of the spectrum corresponding to the dye DrY106 which was not affected on solid substrate.

On the whole the best results were obtained by the strain *B. adusta* MUT 2295, that displayed the highest decolourisation of three wastewaters in a very short time (1–2 days), in the absence of any additional nutrient source. Excellent degradation was also recorded for *B. adusta* MUT 3060 and *P. ostreatus* MUT 2976 which also worked extensively with W1, W3 and W4.

3.3. Optimization of wastewater decolourisation in liquid systems by *B. adusta* MUT 2295

According to the results of the previous experiment, where the fungus caused an almost complete degradation of the wastewater (W1 and W4), the addition of a nutrient source did not lead to a substantial improvement in the decolourisation yields (Table 4). On the contrary, for the other wastewaters, the addition of nutrients and, in the case of W2, the dilution to 1/3 and 1/5, substantially improved the degradation process, indicating that the higher the recalcitrance, more stringent the conditions for the growth and activity of the fungus become. In particular W2, despite the still high concentration of salts and dyes, was almost completely decolourised (up to 91% for W2 1/5). This finding is of significant impact considering that previous experiments on the same wastewater using high concentrations of purified enzymes (Faraco et al., 2009) had never led to a substantial degradation.

Table 4
Maximum values of decolourisation percentage (DP - in brackets the day of measure) of the simulated wastewaters (W1–4) by *Bjerkandera adusta* MUT 2295 in the presence and in the absence of GLY/GHY, peroxidase (MiP and MnP) activities (U l^{-1}) and pH values at the end of the experiment.

	DP	MnP	MiP	pH
W1	95 (1)	210	62	6.0
W1 GLY	95 (4)	164	47	6.9
W1 GHY	95 (2)	451	149	6.1
W2 1/3	45 (2)	55	19	7.4
W2 1/3 GLY	77 (3)	67	52	6.0
W2 1/3 GHY	70 (3)	207	67	5.4
W2 1/5	72 (2)	216	32	5.6
W2 1/5 GLY	91 (3)	142	66	5.6
W2 1/5 GHY	75 (4)	345	175	6.5
W3	68 (3)	269	96	5.9
W3 GLY	82 (7)	221	95	5.2
W3 GHY	71 (4)	587	201	7.5
W4	96 (3)	152	40	6.1
W4 GLY	93 (2)	283	173	6.4
W4 GHY	95 (2)	359	89	7.0
GLY	–	107	28	5.7
GHY	–	720	195	6.1

In the case of W2 and W3 it is also important to stress that the best yields of degradation were obtained in the presence of the lowest content of *N* (GLY), which was the only culture condition in which the fungus determined an almost complete degradation of even the most recalcitrant dyes, such as the reactive RY145 and RR195 and the direct DrY106. On the other hand, the higher content of *N*, both in presence and absence of the wastewaters, resulted in general in an increased

peroxidase activity (MnP) according to what has already been reported for *B. adusta* (Kaal et al., 1995 and Seker et al., 2008). MnP and MiP were the only recorded enzymatic activities during the decolourisation processes; Lac and LiP were never registered.

The higher enzyme production was never coupled with a more efficient decolourisation. The absence of a clear correlation between the decolourisation and the enzyme activities is particularly evident in W2 1/5 and W3, where in the presence of GLY there was a better decolourisation with significantly lower values of enzyme activity, in particular MnP. This finding may be partly explained by the pH values during the process of decolourisation. The decolourisation, in fact, was higher when the fungus metabolism resulted in a lowering of pH values around 5, regardless of the values of enzyme activity. This pH value is close to the *optimum* for peroxidases, that would therefore be more active and effective.

Other explanations of this apparent lack of correlation between decolourisation and enzyme production, already reported by other authors, could be: (1) the production by the fungus, in the different culture conditions, of enzyme isoforms with different affinity to the dyes and the substrates used in the reaction assays (Rabinovich et al., 2004), (2) the limited distribution of enzymes in the growth medium (Blánquez et al., 2004; Kuhad et al., 2004), (3) the rapid inactivation by proteolytic activity (Rabinovich et al., 2004). Moreover, in addition or alternative to a direct involvement, peroxidases may play an indirect role in the degradation process, starting a chain reaction that leads to the degradation of pollutants by generating free radicals which, in turn, can attack very recalcitrant molecules normally not directly attacked by enzymes (Anastasi et al., 2009a, Gavril et al., 2007 and Rabinovich et al., 2004). Finally, other enzymes (such as cytochrome P450 monooxygenase) or other degradative mechanisms not tested in this study may have taken part in the degradation process (Rabinovich et al., 2004).

The ecotoxicity tests also emphasized that the use of different culture conditions is important, not only to optimize the yields of decolourisation, but also and above all, to get a real wastewater detoxification. In the case of W1, W2 and W4 a significant reduction of toxicity (increase of GI and decrease of I_i) was almost exclusively observed in presence of GLY vs. both organisms (Table 5). It is important to note that the fungal treatment led to a detoxification of the wastewaters within the threshold limit values of the Italian law (GI > 50 and I_i < 50 − Legislative Decree 152/2006) in three cases against *C. sativus* (W1, W1 GLY, W4 GLY) and in three cases against *L. minor* dry weight (W1 GLY, W2 1/3 GLY, W2 1/5 GLY). In contrast, the treatment of W3 never resulted in a reduction of toxicity and, in several cases, an increase in toxicity was even observed. The verification that the process of decolourisation corresponds to detoxification is a crucial step in order to indicate a real application. However, this aspect until now has been evaluated only in a limited number of studies (Casieri et al., 2008, Gavril and Hodson, 2007, Hai et al., 2007, Husain, 2006, Vandevivere et al., 1998 and Vanhulle et al., 2008) that, in some cases, showed an ineffective detoxification even in presence of an extensive decolourisation (Hai et al., 2007, Husain, 2006 and Vandevivere et al., 1998). The fact that the ecotoxicological tests are rarely carried out in wastewater treatment studies may be partly due to the absence of a specific legislation stating the most appropriate test to be applied in different situations and providing tools for unequivocal interpretation of data. Actually, as also shown by our own data, different organisms (or even several parameters within the same test, i.e. frond number and dry weight of *L. minor* against W2 1/3 GLY before fungal treatment) showed a different sensitivity to the same effluent.

Table 5
Cucumis sativus germination index (GI, method UNICHIM N. 1651 2003) and *Lemna minor* growth inhibition ($I_{\mu i}$, method ISO/WD 20079) on frond number (f) and dry weight (dw) of the simulated wastewaters (W1–W4) before and after treatment by *Bjerkandera adusta* MUT 2295 in the presence and in the absence of GLY/GHY. Different letters indicate significant differences ($p \leq 0.05$) of values before and after fungal treatment. * indicates the cases in which the fungal treatment led to a detoxification of the wastewater with values of GI (>50) and I_i (<50) within the threshold limit values of the Italian law (Legislative Decree 152/2006).

	GI before treatment	GI after treatment		$I_{\mu i}$ before treatment	$I_{\mu i}$ after treatment
W1	44 a	65 b*	f	−13 a	15 b
			dw	−26 a	96 b
W1 GLY	41 a	93 b*	f	4 a	−10 b
			dw	56 a	18 b*
W1 GHY	29 a	45 b	f	9 a	8 a
			dw	56 a	68 a
W2 1/3	2 a	4 a	f	−3 a	−1 a
			dw	11 a	36 a
W2 1/3 GLY	2 a	10 b	f	11 a	−13 b
			dw	87 a	1 b*
W2 1/3 GHY	1 a	1 a	f	38 a	20 b
			dw	11 a	12 a
W2 1/5	29 a	34 a	f	−10 a	−10 a
			dw	−10 a	26 a
W2 1/5 GLY	18 a	29 b	f	−2 a	−10 a
			dw	53 a	11 b*
W2 1/5 GHY	27 a	9 b	f	4 a	9 a
			dw	9 a	13 b
W3	45 a	2 b	f	−10 a	16 b
			dw	13 a	34 b
W3 GLY	38 a	0 b	f	−5 a	3 a
			dw	21 a	17 a
W3 GHY	35 a	5 b	f	10 a	5 a
			dw	46 a	51 a
W4	67 a	78 a*	f	−9 a	10 b
			dw	11 a	36 a
W4 GLY	50 a	102 b*	f	−1 a	−12 b
			dw	43 a	−8 b
W4 GHY	56 a	35 b	f	−3 a	5 a
			dw	11 a	12 a

3.4. Textile effluent treatment in fixed-bed bioreactor

The experiment with the fixed-bed bioreactor is a first scale-up for a bioremediation process. The fungus in fact, resulted effective during 10 cycles of decolourisation (up to 84% DP) of large volumes (5.1 l total) of a simulated and a real wastewater, remaining active for a very long period (70 days), in non-sterile conditions (Fig. 3). Furthermore, after treatment of OMO effluent (IV cycle) the COD (from 330 to 230 mg O_2 l^{-1}) and the *V. fischeri* toxicity (from 4.6 to 1 TU) were effectively reduced, the latter parameter falling below the threshold limit values of the Italian law (Legislative Decree 152/2006).

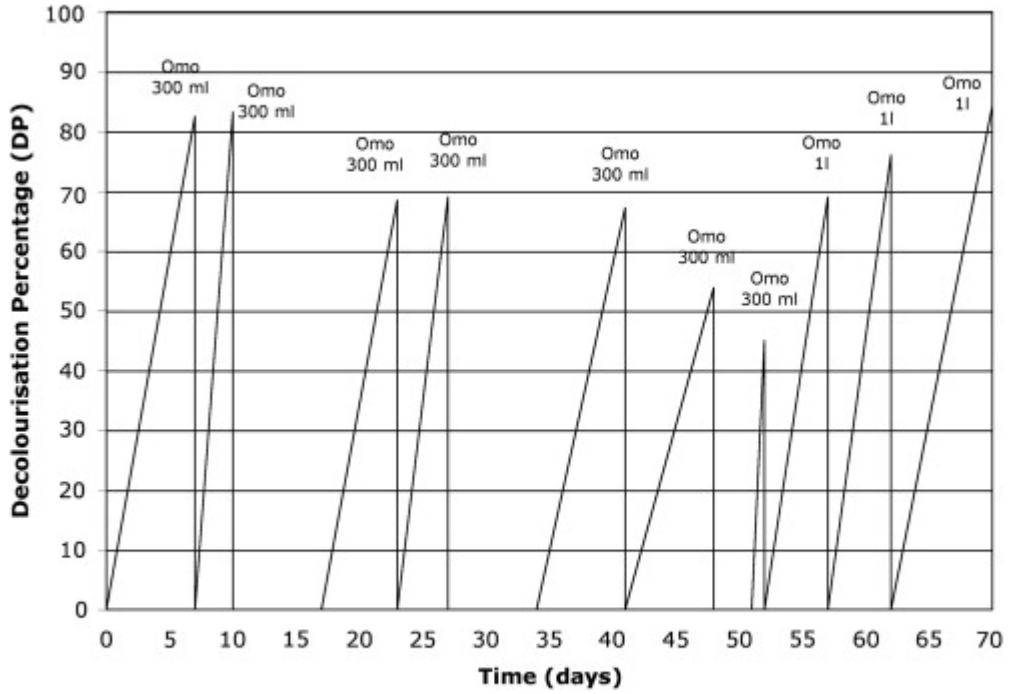

Fig. 3.

Decolourisation percentage (DP) of the simulated (W1) and the real (OMO) wastewater by *Bjerkandera adusta* MUT 2295 packed in a fixed-bed bioreactor.

A very important aspect to consider is the fact that generally the metabolic capacity of fungi decreases in the absence of sterile conditions, as demonstrated by Borchert and Libra (2001). In our case, the immobilized fungus confirmed its robustness remaining active during several cycles even in the presence of the microflora associated with the real dyeing wastewater. Certainly in these conditions is established a consortium responsible for the degradation in which it is no longer possible to define the actual contribution of the different organisms. Recently, some authors have emphasized that the very establishment of such consortia can determine overall good yields of decolourisation (Cetin and Donmez, 2006 and Saratale et al., 2009).

As far as we know, this is one of the few studies in which large volumes of a real wastewater were treated in repeated batches. In fact, most of the studies, even using bioreactors, were conducted in conditions very different from the actual characteristics of industrial wastes, with single dyes at low concentration. Thus, for example, a fixed-bed bioreactor packed with *Trametes pubescens* was able to decolourise, for four successive cycles, 200 ml of a solution of the dye Reactive Black 5 at a concentration of 60 ppm (Enayatzamir et al., 2009) and Casieri and colleagues (2008) have shown the efficiency of degradation of our own strain of *B. adusta* (previously identified as *T. pubescens*) and a strain of *P. ostreatus* against successive cycles of solutions containing 200, 1000 and 2000 ppm of one model and two industrial dyes.

Another important aspect is that our system did not require any addition of nutrients during the four cycles of treatment of OMO, in which a growing trend of degradation was observed (from 45% to 84%). In this regard it is particularly interesting a comparison with one of the few works on the treatment of a real wastewater, in a sterile bioreactor (Blanquez et al., 2008). In this study, *T. versicolor* was able to degrade 60% of a real wastewater in 15 days in a bioreactor continuously fed with glucose. The fact that in our case it was not necessary to intervene in the process by any dilution of the wastewater, the addition of nutrient sources or the control of chemical parameters,

such as pH, increases the chance of application of our system and confirms the robustness of this fungus. Indeed, a minor process control results in a significant cost reduction and a greater applicability.

4. Conclusions

This study opens new perspectives for the bioremediation of industrial effluents using white-rot fungi. In particular, *B. adusta* MUT 2295 was found able to survive and grow over a long period, in toxic and restrictive conditions and proved to be effective in the degradation and detoxification of a wide variety of wastewaters. The biotechnological potential of this strain was confirmed setting up a bioreactor which resulted effective in the treatment of a real industrial effluent, being active for a very long period, in absence of axenic conditions. No operational problems were detected, confirming the robustness and applicability of this system.

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References

Anastasi et al., 2009a A. Anastasi, T. Coppola, V. Prigione, G.C. Varese **Pyrene degradation and detoxification in soil by a consortium of basidiomycetes isolated from compost: role of laccases and peroxidases** J. Hazard. Mater., 165 (2009), pp. 1229–1233

Anastasi et al., 2009b A. Anastasi, V. Prigione, G.C. Varese **Industrial dye degradation and detoxification by basidiomycetes belonging to different eco-physiological groups** J. Hazard. Mater. (2009) http://dx.doi.org/10.1016/j.jhazmat.2009.12.027

Blanquez et al., 2004 P. Blanquez, N. Casas, X. Font, X. Gabarrell, M. Sarrà, G. Caminal, T. Vicent **Mechanism of textile metal dye biotransformation by *Trametes versicolor*** Water Res., 38 (2004), pp. 2166–2172

Blanquez et al., 2008 P. Blanquez, A. Sarrà, T. Vicent **Development of a continuous process to adapt the textile wastewater treatment by fungi to industrial conditions** Process Biochem., 43 (2008), pp. 1–7

Borchert and Libra, 2001 M. Borchert, J.A. Libra **Decolorization of reactive dyes by the white rot fungus *Trametes versicolor* in sequencing batch reactors** Biotechnol. Bioeng., 75 (2001), pp. 313–321

Casieri, 2005 L. Casieri **Biodegradation and biosorption of synthetic dyes by fungi** University of Turin, Italy (2005) Ph.D. Thesis

Casieri et al., 2008 L. Casieri, G.C. Varese, A. Anastasi, V. Prigione, K. Svobodova, V. Filipello Marchisio, C. Novotny **Decolorization and detoxification of reactive industrial dyes by immobilized fungi *Trametes pubescens* and *Pleurotus ostreatus*** Folia Microbiol., 53 (2008), pp. 44–52

Cetin and Donmez, 2006 D. Cetin, G. Donmez **Decolorization of reactive dyes by mixed cultures isolated from textile effluent under anaerobic conditions** Enzyme Microb. Technol., 38 (2006), pp. 926–930

Enayatzamir et al., 2009 K. Enayatzamir, H.A. Alikhani, S. Rodríguez Couto **Simultaneous production of laccase and decolouration of the diazo dye Reactive Black 5 in a fixed-bed bioreactor** J. Hazard. Mater., 164 (2009), pp. 296–300

Faraco et al., 2009 V. Faraco, C. Pezzella, A. Miele, P. Giardina, G. Sannia **Bioremediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete hrysosporium* and *Pleurotus ostreatus* and their enzymes** Biodegradation, 20 (2009), pp. 209–220

Gavril and Hodson, 2007 M. Gavril, P.V. Hodson **Investigation of the toxicity of the products of decoloration of amaranth by *Trametes versicolor*** J. Environ. Qual., 36 (2007), pp. 1591–1598

Gavril et al., 2007 M. Gavril, P.V. Hodson, J. McLellan **Decoloration of Amaranth by the white-rot fungus *Trametes versicolor*. Part I. Statistical Analysis** Can. J. Microbiol., 53 (2007), pp. 327–336

Hai et al., 2007 F.I. Hai, K. Yamamoto, K. Fukushi **Hybrid treatment system for dye wastewater** Crit. Rev. Environ. Sci. Technol., 37 (2007), pp. 315–377

Husain, 2006 Q. Husain **Potential applications of the oxidoreductive enzymes in the decolorization and detoxification of textile and other synthetic dyes from polluted water: a review** Crit. Rev. Biotechnol., 26 (2006), pp. 201–221

Kaal et al., 1995 E.J. Kaal, J.A. Field, T.W. Joyce **Increasing ligninolytic enzyme activities in several white-rot basidiomycetes by nitrogen sufficient media** Bioresour. Technol., 53 (1995), pp. 133–139

Kaushik and Malik, 2009 P. Kaushik, A. Malik **Fungal dye decolorization, recent advances and future potential** Environ. Int., 35 (2009), pp. 127–141

Kuhad et al., 2004 R.C. Kuhad, N. Sood, K.K. Tripathi, A. Singh, O.P. Ward **Developments in microbial methods for the treatment of dye effluents** Adv. Appl. Microbiol., 56 (2004), pp. 185–213

Leung and Pointing, 2002 P.C. Leung, S.B. Pointing **Effect of different carbon and nitrogen regimes on Poly R decolorization by white-rot fungi** Mycol. Res., 106 (2002), pp. 86–92

Lucas et al., 2008 M. Lucas, V. Mertens, A.M. Corbisier, S. Vanhulle **Synthetic dyes decolorization by white-rot fungi. Development of original microtitre plate method and screening** Enzyme Microb. Technol., 42 (2008), pp. 97–106

Mielgo et al., 2002 I. Mielgo, M.T. Moreira, G. Feijoo, J.M. Lema **Biodegradation of a polymeric dye in a pulsed bed bioreactor by immobilised *Phanerochaete chrysosporium*** Water Res., 36 (2002), pp. 1896–1901

Mohorcic et al., 2009 M. Mohorcic, M. Bencina, J. Friedrich, R. Jerala **Expression of soluble versatile peroxidase of *Bjerkandera adusta* in *Escherichia coli*** Bioresour. Technol., 100 (2009), pp. 851–858

Niku-Paavola et al., 1988 M.L. Niku-Paavola, E. Karhunen, P. Salola, V. Raunio **Ligninolytic enzymes of the white-rot fungus *Phlebia radiate*** Biochem. J., 254 (1988), pp. 877–884

Oliveira et al., 2007 D.P. Oliveira, P.A. Carneiro, M.K. Sakagami, M.V.B. Zanoni, G.A. Umbuzeiro **Chemical characterization of a dye processing plant effluent—identification of the mutagenic components** Mutat. Res., Rev. Mutat. Res., 626 (2007), pp. 135–142

Prigione et al., 2008 V. Prigione, V. Tigini, C. Pezzella, A. Anastasi, G. Sannia, G.C. Varese **Decolourisation and detoxification of textile effluents by fungal biosorption** Water Res., 42 (2008), pp. 2911–2920

Rabinovich et al., 2004 M.L. Rabinovich, A.V. Bolobova, L.G. Vasil'chenko **Fungal decomposition of natural aromatic structures and xenobiotics: A review** Appl. Biochem. Microbiol., 40 (2004), pp. 1–17

Rodriguez Couto et al., 2004 S. Rodriguez Couto, M.A. Sanroman, D. Hofer, G.M. Guëbitz **Stainless steel sponge, a novel carrier for the immobilisation of white-rot fungus *Trametes hirsuta*for decolourization of textile dyes** Bioresour. Technol., 95 (2004), pp. 67–72

Romero et al., 2006 S. Romero, P. Blanquez, G. Caminal, X. Font, M. Sarra, X. Gabarrell, T. Vicent **Different approaches to improving the textile dye degradation capacity of *Trametes versicolor*** Biochem. Eng. J., 31 (2006), pp. 42–47

Saratale et al., 2009 R.G. Saratale, G.D. Saratale, D.C. Kalyania, J.S. Chang, S.P. Govindwar **Enhanced decolorization and biodegradation of textile azo dye Scarlet R by using developed microbial consortium-GR** Bioresour. Technol., 100 (2009), pp. 2493–2500

Seker et al., 2008 U.O.S. Seker, T. Catal, Y. Taptik, C. Tamerler, H. Bermek **Enhanced production of manganese-peroxidase by the white-rot fungus *Bjerkandera adusta*using media engineering** Biotechnol. and Biotechnol. Eq., 22 (2008), pp. 844–848

Tien and Kirk, 1983 M. Tien, T.K. Kirk **Lignin-degrading enzyme from the Hymenomycete *Phanerochaete chrysosporium* Burds** Science, 221 (1983), pp. 661–663Tinoco et al., 2007 R. Tinoco, J. Verdin, R. Vazquez-Duhalt

Role of oxidizing and tryptophan 172 in the decoloration of industrial dyes by the versatile peroxidase from *Bjerkandera adusta* J. Mol. Catal. B: Enzym., 46 (2007), pp. 1–7 Vandevivere et al., 1998

P.C. Vandevivere, R. Bianchi, W. Verstraete **Treatment and reuse of wastewater from the textile wet-processing industry: review of emerging technologies** J. Chem. Technol. Biotechnol., 72 (1998), pp. 289–302

Vanhulle et al., 2008 S. Vanhulle, M. Trovaslet, E. Enaud, M. Lucas, S. Taghavi, D. van der Lelie, B. van Aken, M. Foret, R.C.A. Onderwater, D. Wesemberg, S.N. Agathos, Y. Schneider, A. Corbisier **Decolorization, cytotoxicity and genotoxicity reduction during a combined ozonation/fungal treatment of dye-contaminated wastewater** Environ. Sci. Technol., 42 (2008), pp. 584–589

Vyas et al., 1994 B.R.M. Vyas, J. Volc, V. Sasek **Effects of temperature on the production of manganese peroxidase and lignin peroxidase by*Phanerochaete chrysosporium*** Folia Microbiol., 39 (1994), pp. 19–22

Yu et al., 2006 G. Yu, X.H. Wen, R. Li, Y. Qian ***In vitro* degradation of a reactive azo dye by crude ligninolytic enzymes from nonimmersed liquid culture of *Phanerochaete chrysosporium*** Process Biochem., 41 (2006), pp. 1987–1993

Zhang et al., 1999 F.M. Zhang, J.S. Knapp, N.T. Kelvin **Development of bioreactor systems for decolorization of Orange II using white rot fungus** Enzyme Microb. Technol., 24 (1999), pp. 48–53