Journal of Basic Microbiology
Environment · Health · Techniques

Research Paper

Water recycle as a must: decolorization of textile wastewaters by plant-associated fungi

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Textile dye effluents are among the most problematic pollutants because of their toxicity on several organisms and ecosystems. Low cost and ecocompatible bioremediation processes offer a promising alternative to the conventional and aspecific physico-chemical procedures adopted so far. Here, microorganisms resident on three real textile dyeing effluent were isolated, characterized, and tested for their decolorizing performances. Although able to survive on these real textile-dyeing wastewaters, they always showed a very low decolorizing activity. On the contrary, several plant-associated fungi (*Bjerkandera adusta*, *Funalia trogii*, *Irpex lacteus*, *Pleurotus ostreatus*, *Trametes hirsuta*, *Trichoderma viride*, and *Aspergillus nidulans*) were also assayed and demonstrated to be able both to survive and to decolorize to various extents the three effluents, used as such in liquid cultures. The decolorizing potential of these fungi was demonstrated to be influenced by nutrient availability and pH. Best performances were constantly obtained using *B. adusta* and *A. nidulans*, relying on two strongly different mechanisms for their decolorizing activities: degradation for *B. adusta* and biosorption for *A. nidulans*. Acute toxicity tests using *Daphnia magna* showed a substantial reduction in toxicity of the three textile dyeing effluents when treated with *B. adusta* and *A. nidulans*, as suggested by mass spectrometric analysis as well.

Abbreviations: PDA – potato dextrose agar; **LB** – Luria Bertani; **ARDRA** – amplified ribosomal DNA restriction analysis; **ITS** – internal transcribed spacers; **CR** – color removal; **ESI** – electron spray injection; **CFU** – colony forming unit; **DCW** – dry cell weight

Keywords: Plant-associated fungi / Bioremediation / Biodegradation / Biosorption / Textile dyeing decolorization

Received: July 12, 2012; accepted: October 12, 2012

DOI 10.1002/jobm.201200401

Introduction

In the last decades, the availability of large quantities of fresh, clean water has become a growing issue for the economic growth of textile companies [1, 2]. A significant portion of the costs associated to water management is related to effluent discharge [3]. Textile companies are the primary sources of contaminated effluents, whose major pollutants are residual dyes and derivatives thereof [4, 5]. The global production of dyes is about 2.3 million metric tons p.a., 80% of which is dedicated to the textile industry [6]. Typically, dyeing wastewaters may contain from 10–15% up to 50% of the original dye bath concentration [7–9], along with many other chemicals, from dispersing agents and mordants, to heavy metal ions and inorganic salts [10–13]. Thus, the overall toxicity of textile effluents is the result of the synergistic effects of all these chemicals, raising a substantial environmental issue: therefore, their reduction to acceptable levels is a must.

Routine strategies for the purification of dyeing wastewaters include chemical and electrochemical oxidation, filtration on micro-membranes, precipitation of dyes in the form of insoluble salts, photocatalytic degradation, and adsorption or electrosorption [14–20]. All these methods are highly costly and thus commercially unattractive. In addition, those based on chemical transformation may also be the source of carcinogenic

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substances (e.g. aniline derivatives) for humans and animals [21–25]. The urgency to invest in environmentally friendly and cost savings technologies to sustain the textile processing industry would drive the development of efficient treatments of wastewaters to be implemented conveniently on site. This approach would progress in the direction of specific and tailored technologies, allowing the recycling of water and eventually the generation of margins for industrial growth [26–28]. Saving water would also mean reduction of the overall environmental impact of the finished textile goods, from their manufacture to their disposal [29].

In recent years, bioremediation and more generally biotechnologies have been emerging competitive to traditional purification technologies, due to their cost-effective and eco-friendly profile [7, 30]. Some algae [31, 32], bacteria [33, 34], fungi [35–39], and yeasts [40, 41] were proved able to achieve dye removal. Concerning fungi, Basidiomycetes have been the most investigated for their dye degrading abilities, mainly based on the production of peroxidases and laccases [42–47], but several Ascomycetes have also been examined [48–50]. Studies have been carried out both on simulated and real industrial effluents, highlighting the prospective view for an industrial development [49, 51–54].

This paper explores the potential of several microorganisms as bioreactors, to decolorize different real textile effluents under aerobic conditions. The trials were carried out with fungal and bacterial isolates characterized as resident microflora in these wastewaters and with a selection of plant-associated fungi belonging to Basidiomycetes and Ascomycetes. The effect of nutritional sources and pH of effluents on the activity of these microorganisms were investigated. Furthermore, the acute toxicity of the treated and untreated effluents was also assessed.

Materials and methods

Dyeing effluents source and analysis of resident microflora

Three highly colored effluents were collected at the end of dyeing processes (Table 1) from Gruppo Tintoriale S.p.A. (Vaiano, Prato, Italy). Chemical structures were reported for those dyes commercially available as unmixed (Table 2). These effluents were filtered through sterilized filter paper (ULTRAFAST2, Whatman International Ltd, England) to remove any suspended impurity, and the filtrates were stored in sterile airtight plastic bottles at 4 °C until use. The initial absorbance of each effluent was measured using a UV-1650 spectrophotometer (Shimadzu Scientific Instruments, Colombia, MD, USA) over the range 200–700 nm. The microflora resident in these effluents was isolated and characterized using the plate-dilution method. Serial decimal dilutions were prepared from 5 ml aliquots of dye wastewaters previously diluted with 45 ml sterilized distilled water, spread onto Potato Dextrose Agar (PDA, Sigma, St. Louis, MO, USA) and Luria Bertani (LB) [55] agar plates, and incubated for 3 days at 28 °C. The results from plate counts were expressed as colony forming units (CFU) for 1 ml effluent. Colonies having different morphologies were separately streaked on PDA or LB, keeping record of their source. Duplicates were stored in 40% v/v aqueous glycerol at −80 °C until needed. The isolates were analyzed and taxonomically assigned by amplified

Table 1. Composition of the dyeing textile effluents used in this study.

Dyeing effluent	pH	λ_{max}[a] (nm)	Color	Dyes[b]	Additives (g L^{-1})	Textile substrates
A	3.70	252	Violet	Acid Yellow 29 Mix Acid Red Acid Blue 324	Hydrogen peroxide (1) Formic acid (2.5) Sodium chloride (10) Ethoxy amines (nd)	Wool
B	6.27	573	Yellowish	Mix Disperse (Yellow, Crimson, Navy)	Ethoxylated alcohol (1) Ammonium polyphosphate (1) Sodium naphthalene sulphate (3) Acetic acid (1) Monosodic phosphate (2)	Polyester
C	9.46	476	Dark black	Black Direct 22	Sodium phosphate (30) Sodium hydroxide (2) Sodium polyphosphate (3) Fatty acids (2)	Cotton

[a]Absorbance at λ_{max} at time zero.
[b]Names of dyes are according to the color index.

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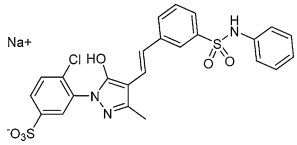
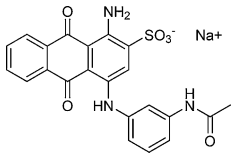
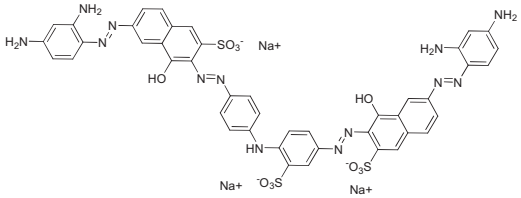
Table 2. Chemical structures of the dyes present in the textile effluents.

Dye (CI)[a]	Molecular formula	Molecular weight	CAS number	Chemical structure
Acid Yellow 29	$C_{22}H_{17}ClN_5NaO_6S_2$	569.97	6359-91-7	
Acid Blue 324	$C_{22}H_{16}N_3NaO_6S$	473.43	88264-80-6	
Black Direct 22	$C_{44}H_{32}N_{13}Na_3O11S_3$	1083.97	6473-13-8	

[a]CI, color index.

ribosomal DNA restriction analysis (ARDRA) [56], and direct double-strand sequencing of internal transcribed spacers (ITS) [57] and 16S rDNA [58, 59] (Eurofins MWG Operon Ltd, Ebersberg, Germany). Homologous sequences were obtained by interrogating the National Center for Biotechnology Information (NCBI) (http://ncbi.nlm.nih.gov), using BLASTN algorithm [60].

Fungal strains and culture conditions

Several plant-associated fungal strains were collected from different forestry Italian sites and deposited in the Fungal Collection of the Laboratorio di Patologia Vegetale Molecolare (LPVM) of the University of Florence. Identification numbers were listed in Table 3. These fungi were confirmed in their identity by sequencing of their ITS regions [57], prior to decolorizing experiments. The fungal strains were routinely grown and subcultured on PDA plates at 25 °C in the dark, and kept at 4 °C on slants on long term. Transfers were made at 1-month intervals.

Microbial decolorization analysis of real dyeing effluents

The three exhausted dyeing effluents were filter sterilized on 0.22 µm pore membranes (Millipore Inc., Bedford, MA, USA) prior to decolorization trials. Aliquots (30 ml) were then transferred into 100 ml Erlenmeyer flasks. According to the experimental purposes, the pH values of the samples were either kept at their original values or corrected to 6.0 by the addition of 0.1 N NaOH or 0.1 N HCl. Decolorizing experiments were carried out using the plant-associated fungi reported in Table 3 and

Table 3. Plant-associated fungi used in decolorizing trials.

Species	Code[a]	Phylum	Plant	Geographical origin
Bjerkandera adusta	BA10171	Basidiomycota	*Juglans regia*	Benevento
Funalia trogii	FT233	Basidiomycota	*Populus nigra*	Roma
Irpex lacteus	IL1036	Basidiomycota	*Populus nigra*	Arezzo
Pleurotus ostreatus	PE66	Basidiomycota	*Morus alba*	Monza
Trametes hirsuta	TH7489	Basidiomycota	*Sorbus spp.*	Trento
Aspergillus nidulans	AN8	Ascomycota	*Zea mays*	Codroipo
Trichoderma viride	TV4545	Ascomycota	*Solanum lycopersicum*	Portici

[a]Codes used at Laboratorio di Patologia Vegetale Molecolare (LPVM) of the University of Florence.

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Table 4. Predominant culturable fungi and bacteria isolated from dyeing effluents.

Effluent	CFU ml^{-1} ± SD on PDA	CFU ml^{-1} ± SD on LB	Fungal genus	Bacterial genus
A	10^2 ± 3.3	10^4 ± 7.0	*Pichia*	*Methanobrevibacter* *Enterobacter* *Escherichia*
B	10^1 ± 1.9	10^2 ± 0.6	*Pichia*	*Pseudomonas* *Desulfobacterium* *Enterobacter* *Escherichia*
C	0	0	nd	nd

nd, not determined.

the microorganisms isolated from the real textile effluents (Table 4). As far as bacteria are concerned, each Erlenmeyer flask was inoculated with a fresh bacterial suspension, grown for 16 h on LB, to an OD = 1.0 at 600 nm. Concerning fungi, a mycelial disc was taken from a 1-week culture grown on PDA, using a disc molder (1 cm diameter), and then inoculated. Decolorizing activity of plant-associated fungi was evaluated either by removing or not the PDA disc under the mycelial layer, before proceeding to its inoculation into the dyeing effluents. The inoculated effluents were then incubated at 25 °C, for 7 days under shaking (100 rpm) in the dark. All assays were conducted in triplicates and referred to un-inoculated controls. Time intervals of 2, 5, and 7 days after inoculation were chosen to assay the decolorization progress. At the same time intervals, the pH of the effluents was also monitored, and the fungal biomass estimated by means of dry cell weight (DCW) measurement, after 24 h drying at 105 °C or to constant weight in an oven. For each effluent, 2 ml aliquots were centrifuged at 14 000 rpm for 5 min, to remove any mycelial fragment or bacterial cell. On the supernatant, decolorization was evaluated by UV–Visible spectroscopic analysis, carried out on a Cary–4000 Varian spectrophotometer (Varian Inc., Palo Alto, CA, USA), using 1 cm quartz cuvettes over the range 200–700 nm. The decolorization activity was estimated by subtracting the control spectrum of the effluent from that of the treated effluent at the maximum absorption wavelength (λ_{max}) of each dye in the visible region (Table 1). Microbial decolorization was expressed as percentage of color removal (CR%), according to the following equation:

$$CR\% = \frac{(A_0 - A_t)}{A_0} \times 100$$

where A_0 and A_t are the absorbances of each effluent before inoculation and after time t, respectively. CR% refers to the percentage mean of decolorization assayed in three independent experiments with three replicates each. Data thus obtained were subjected to statistical analysis. Significant differences were determined by analysis of variance (ANOVA), and a significance level of 0.05 was chosen.

Mass analysis on discolored dyeing effluents

A 1.0 µl aliquot of each fungal treated and untreated dyeing effluent was diluted with 2 ml of HPLC grade methanol (Fisher Scientific, Leicestershire, UK), and then filtered on Titan2® Syringe Filter (ThermoFisher Scientific, Austin TX, USA). The filtered sample was analyzed using a ThermoScientific LCQ-Fleet mass spectrometer under electrospray ionization (ESI, $+c$ or $-c$ technique) (Thermo Scientific, Austin TX, USA) by direct infusion with a 500 KL Hamilton micro-syringe and using a 15 µl min^{-1} flow. The mass spectrometer parameters used were: sheet gas flow = 10, auxiliary gas flow = 0, sweep gas flow = 0, spray voltage = 5.00 kV, capillary tube temperature = 220 °C, capillary voltage = 10.0 V and tube lens = 75.0 V.

Acute toxicity determination

The short-term tests to determine acute toxicity were carried out using the water flea *Daphnia magna* [61]. This cladoceran microcrustacean is a standardized test organism widely used for toxicity assays for over 40 years [62, 63], and proposed as a representative invertebrate in the EU Guidance Document on Aquatic Ecotoxicology [64], in Commission Regulation No. 544 [65], and among EU criteria for classification of dangerous substances in Commission Directive 84/449/EEC [66]. Young organisms (6-24 h life) were used for toxicity determination and were not fed during the test period. For each sample and each experimental condition, 25 organisms were tested in triplicates, along with negative (water) and positive (untreated effluent) controls. After 48 h treatment at 21 °C and with a light–dark cycle 16 h + 8 h, the loss of mobility of the organisms was registered. The number of immobile individuals was

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evaluated, where *D. magna* organisms are considered immobile if they do not show any mobility during 30 s of observation, and this condition is officially accepted as associated to death.

Results

Characterization and decolorization performances of resident microflora from real dyeing effluents

The highest amount of viable and in vitro cultivable microorganisms was shown in effluent A, whilst the development of fungal and bacterial colonies was never observed on PDA and KB plates spread with aliquots of effluent C. A total of 812 isolates was recorded, then used in PCR for 16S rDNA or ITS amplification. On the deriving amplicons ARDRA analysis was carried out with *Alu*I as restriction enzyme. According to the comparative analysis of their restriction patterns, the 812 microbial isolates were assigned to 16 different ARDRA profiles (data not shown). Three isolates were then selected for each ARDRA group, and their 16S rDNA or ITS amplicons sequenced. According to nucleotide sequence data and BLASTN analysis, the microorganisms present in the textile dyeing effluents were thus assigned to the genera reported in Table 4. In effluent A and B, the predominant microorganisms were represented by bacteria, mainly belonging to the family of *Enterobacteriaceae* (*Enterobacter* and *Escherichia* spp.). Other Gram negative isolates were found, belonging to the genera *Pseudomonas*. As far as Gram positive bacteria are concerned, isolates of the genera *Methanobrevibacter* and *Desulfobacterium* were also found. Fungi were poorly represented in the effluent A and B, and restricted to yeasts belonging to the genera *Pichia*. An isolate for each of the 16 ARDRA groups was investigated to ascertain its decolorizing capabilities on the three real textile effluents. The results obtained are reported in Table 5. After 2 days of incubation, no decolorization was exhibited by any bacterial isolate on any of the three effluents. Moreover, at 5 and 7 days no bacterial decolorization was ever observed on effluents A and C, while on effluent B a very low activity (approximately 4%) was noted. Low decolorizing percentages were also found on effluent A after 7 days for resident *Pichia* spp. isolates. On the contrary, fungal activity started on effluents B and C just after 2 days of incubation. The best decolorizing performances (up to 7%) were reached by *Pichia* spp. on effluent B after 5 or 7 days.

Decolorizing activity of plant-associated fungi on real dyeing effluents

Five Basidomycetes (*B. adusta*, *F. trogii*, *I. lacteus*, *P. ostreatus* and *T. hirsuta*) and two Ascomycetes/Deuteromycetes (*T. viride* and *A. nidulans*) were isolated from several Italian forestry sites (Table 3) and selected according to the considerable attention already received for their decolorizing activity. Here, they were used in decolorization trials carried out on the three exhausted dyeing baths, in order to increase the availability of data on their

Table 5. Decolorizing activity of the resident microflora.

	Decolorizing activity (%)								
	Effluent A			Effluent B			Effluent C		
Isolates[a]	Day 2	Day 5	Day 7	Day 2	Day 5	Day 7	Day 2	Day 5	Day 7
P 0121	0	0	2 ± 1.2	3 ± 2.2	7 ± 4.5	7 ± 4.5	1 ± 0.6	1 ± 0.2	2 ± 0.9
P 0135	0	0	3 ± 2.2	4 ± 1.2	6 ± 2.6	6 ± 2.6	1 ± 0.5	1 ± 0.3	1 ± 0.4
P 0184	0	0	2 ± 0.9	1 ± 0.7	2 ± 1.1	2 ± 1.1	1 ± 0.5	1 ± 0.9	1 ± 0.8
P 0198	0	0	3 ± 2.2	4 ± 1.2	6 ± 2.6	6 ± 2.6	1 ± 0.5	1 ± 0.3	1 ± 0.4
P 0201	0	0	2 ± 0.9	1 ± 0.7	2 ± 1.1	2 ± 1.1	1 ± 0.5	1 ± 0.9	1 ± 0.8
L 0260	0	0	0	0	0	3 ± 2.8	0	0	0
L 0301	0	0	0	0	2 ± 1.8	2 ± 1.8	0	0	0
L 0365	0	0	0	0	0	0	0	0	0
L 0412	0	0	0	0	0	2 ± 1.8	0	0	0
L 0466	0	0	0	0	0	3 ± 2.8	0	0	0
L 0494	0	0	0	0	0	2 ± 1.7	0	0	0
L 0516	0	0	0	0	3 ± 2.2	3 ± 1.4	0	0	0
L 0552	0	0	0	0	0	0	0	0	0
L 0656	0	0	0	0	4 ± 3.5	4 ± 3.5	0	0	0
L 0663	0	0	0	0	0	3 ± 2.7	0	0	0
L 0754	0	0	0	0	2 ± 1.5	3 ± 2.3	0	0	0

Codes beginning with P and L refer to fungal and bacterial isolates, respectively.
[a]An isolate for each ARDRA group was tested.

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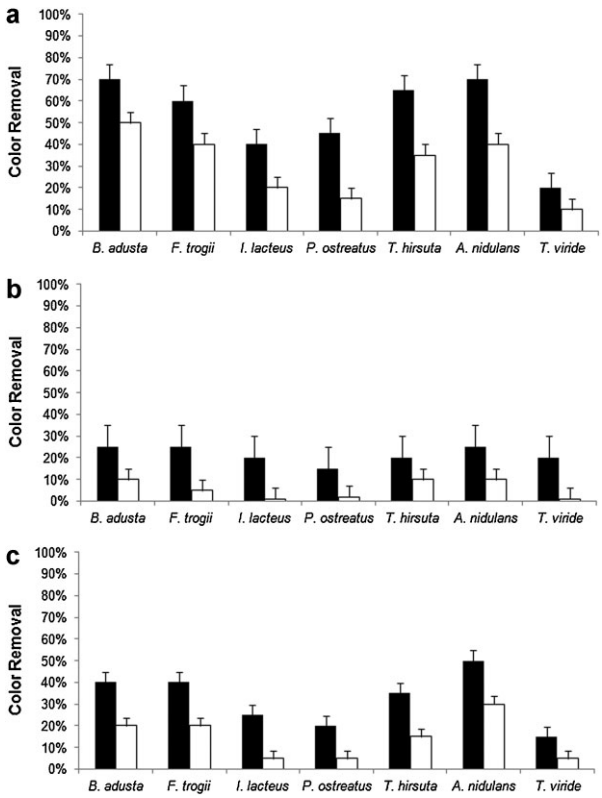

Figure 1. Decolorizing activity of plant-associated fungi on dyeing effluents A, B, and C. Fungal plugs were inoculated in presence (■) or not (□) of PDA layer under the plug. Decolorization was estimated after 7 days of incubation and expressed as percentage of color removal (+ standard deviation).

performances on industrial effluents, in the presence of several chemical additives commonly used in dyeing processes. Data were collected after 2, 5, and 7 days after fungal inoculation. The data obtained after 7 days were reported in Fig. 1, showing that the plant-associated fungal strains used in this study were able to decolorize the different effluents to various extents. The general finding was that all microorganisms achieved the highest percentages of decolorization in the presence of PDA as a source of nutrients. Specifically, in the case of effluent A good decolorization of the samples was observed in all cases, except for *T. viride* which performed modestly (5–20%). On the contrary, decolorization efficacy dropped below 30% of the initial color when effluent B was subjected to fungal treatment. *B. adusta*, *F. trogii*, *T. hirsuta*, and *A. nidulans* gained credit to decolorize effluent C up to 35–50% of the original color, showing higher efficacy than *I. lacteus*, *P. ostreatus*, and *T. viride*. Further to the higher activity of these fungi on effluent A and C, comparative studies were performed to assess the effect of pH on the decolorization power on these two effluents, in presence or not of a source of nutrients. Although a decrease in decolorizing performances was expected in the absence of PDA, a very strong reduction was observed on both effluent A and C with all the tested fungi if the initial pH values of these effluents were also adjusted to 6.0 (Table 6). Surprisingly, an increment of about 10% was observed in the decolorizing activity of *F. trogii* and *A. nidulans* on both effluents at pH 6.0 when the mycelial plugs were used keeping PDA. On the contrary decolorizing performances of *B. adusta*, *T. hirsuta*, and *T. viride* appeared not to be affected by the initial pH values of effluents A and C in the presence of PDA. In the same conditions, *I. lacteus* and *P. ostreatus* showed a decreased activity in color removal abilities on effluent A at pH 6.0, while decolorization data obtained on effluent C were shown to be pH independent (Table 6). On the basis of their excellent decolorizing performances and of their opposite behaviors with respect to pH variation, *B. adusta* and *A. nidulans* were selected for further experiments, aiming to ascertain if the pH values of effluents could be modified by the color removal activity of these fungi. Accordingly, *B. adusta* and *A. nidulans* were inoculated on effluents A and C, without any initial pH adjustment and maintaining the PDA layer under the mycelial plug. The

Table 6. Nutrients and pH influence on decolorizing activity of plant-associated fungi on effluents A and C.

	Decolorizing activity (%)							
	Effluent A				Effluent C			
	+ PDA		− PDA		+ PDA		− PDA	
Fungi	Not adjusted	pH 6.0	Not adjusted	pH 6.0	Not adjusted	pH 6.0	Not adjusted	pH 6.0
B. adusta	70 ± 4.7	70 ± 4.6	50 ± 3.7	15 ± 1.3	40 ± 3.6	40 ± 2.0	20 ± 1.8	5 ± 0.9
F. trogii	60 ± 4.5	70 ± 7.6	40 ± 4.6	5 ± 2.9	40 ± 2.9	45 ± 2.3	20 ± 2.6	15 ± 1.1
I. lacteus	40 ± 4.3	35 ± 3.6	20 ± 3.8	0	25 ± 2.9	25 ± 3.5	5 ± 1.1	0
P. ostreatus	45 ± 4.9	35 ± 4.7	15 ± 3.1	0	20 ± 2.4	20 ± 1.7	5 ± 1.3	0
T. hirsuta	65 ± 5.6	65 ± 4.1	35 ± 3.6	5 ± 3.5	35 ± 3.3	35 ± 2.3	15 ± 2.3	0
A. nidulans	70 ± 5.7	80 ± 6.5	40 ± 4.3	10 ± 3.7	50 ± 2.6	55 ± 4.3	30 ± 3.1	15 ± 1.6
T. viride	20 ± 5.2	20 ± 4.3	10 ± 3.5	0	15 ± 1.8	15 ± 1.8	5 ± 2.6	0

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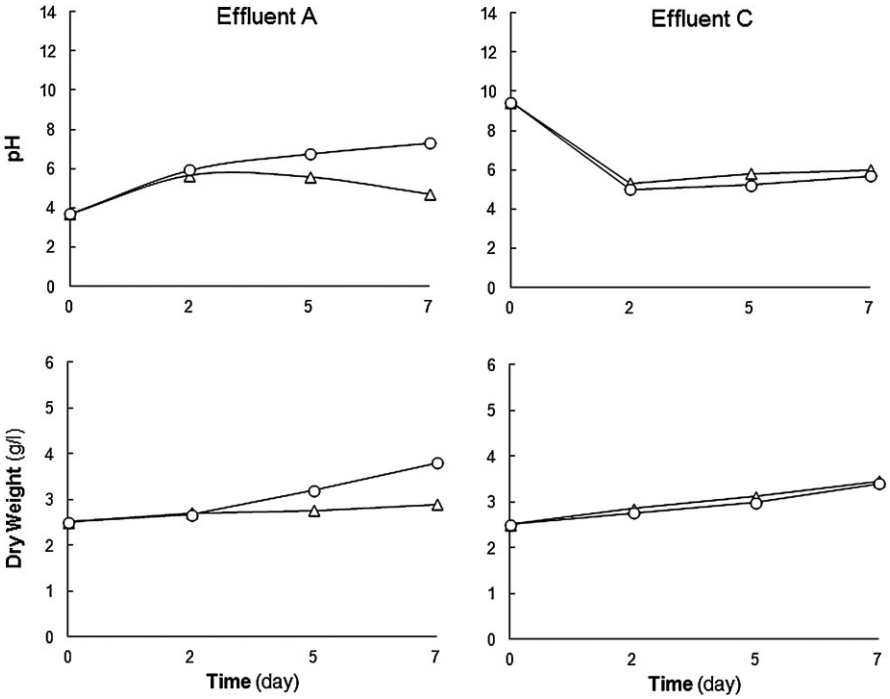

Figure 2. Fungal growth and pH variation on effluents A and C. *A. nidulans* (◯) and *B. adusta* (Δ) were inoculated on effluents A and C in presence of PDA. Fungal growth is expressed as DCW (g L^{-1}).

results shown in Fig. 2 indicated that *A. nidulans* shifted the pH towards 6.0 for both effluents, with higher rate in the case of effluent C and within 7 days of incubation. *B. adusta* behaved similarly to *A. nidulans* when inoculated into effluent C. This trend was not kept for effluent A, whose pH raised to 5.9 after 2 days, to decrease to 4.7 at the seventh day. Concerning fungal growth, after 7 days of incubation a significant increase in biomass (*ca*. 40%) was observed for *A. nidulans* both on effluent A and C, whereas *B. adusta* was shown able to grow on effluent C only and similarly to *A. nidulans*.

Analysis of fungal decolorization products by Ultra-Violet spectroscopy and mass spectrometry

The full electronic spectra of the three effluents treated with the plant-associated fungi reported in Table 3 were registered at the time intervals set for the monitoring of decolorization, and compared with untreated negative controls. The most significant spectrophotometric sets are reported in Fig. 3, exhibited by *B. adusta* and *A. nidulans* inoculated on effluents A and C respectively, without any initial pH adjustment and keeping PDA under the mycelial plug. Within the first 2 days of incubation on effluent A, *B. adusta* determined an appreciable reduction of the absorbance at 220 nm, with a depletion trend until the seventh day of culture (Fig. 3a). Furthermore, at the same time interval a peak at 271 nm appeared, then decreasing in the following 5 days. This evidence strongly suggested that *B. adusta* might have operated a metabolic process on the dyes present in the effluent A, presumably generating aromatic species which displayed the UV absorption in the region of the simplest benzene ring [67]. The addition of *A. nidulans* to effluent C promoted a remarkable depletion of the full absorption spectra in the first 2 days after fungal inoculation (Fig. 3b). Interestingly, the UV absorption plots for *A. nidulans* were superimposable across the whole wavelength range of investigation, in which the untreated effluent C displayed a maximum absorption at 476 nm. These data suggested that *A. nidulans* may have degraded the dye of effluent C by a biosorption mechanism, which may well be the primary mechanism of action for this fungus.

Mass spectrometric analysis is a useful technique to investigate the residual presence either of the dyes employed in the dyeing process or possibly, their derivatives arising from chemical or biochemical degradation. Analysis of effluent A and B could not support any rational interpretation of the data observed, due to the unknown and heterogeneous composition of the dyes (data not shown). Differently, the analysis of untreated effluent C showed the mass ion of CI Direct Black 22 in positive current at 1085.05 [M + 1] (Fig. 4) along with other peaks from unknown species. These could be either

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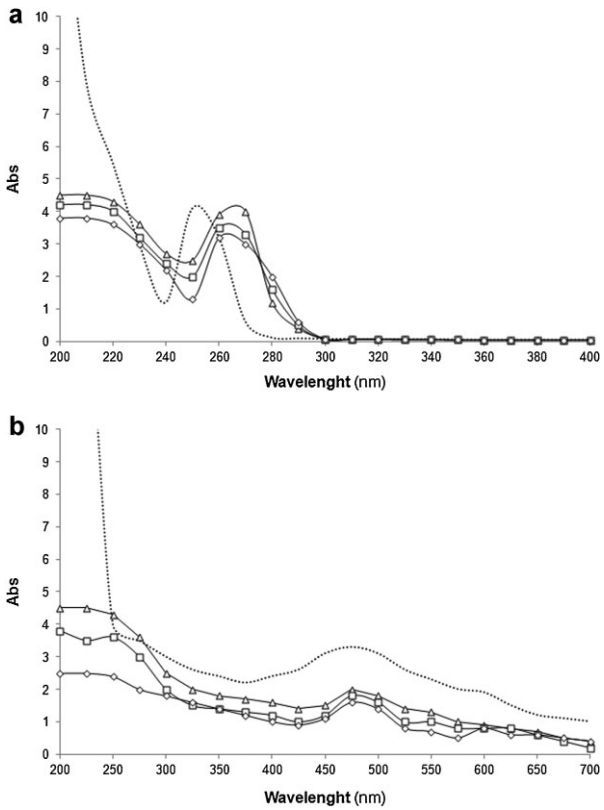

Figure 3. UV/visible spectra of effluent A and C treated with *B. adusta* and *A. nidulans*. Decolorization operated on effluent A and C by *B. adusta* (a) and *A. nidulans* (b), respectively, was estimated after 2, 5, and 7 days of incubation (Δ, □, and ◊, respectively). Untreated A and C effluents were used as negative controls (dashed line).

present in the CI Direct Black 22 original mixture, or added during the dyeing process for the adsorption and fixation of the dye to textiles. The structures reported in Fig. 5 could be originated photochemically or biologically by reductive cleavage of the azo group of CI Direct Black 22. It is noteworthy to observe that after fungal treatment of effluent C, the ion mass of CI Direct Black 22 disappeared. It is also truly evident that none of the detected peaks could be associated to a fragment directly derived from the dye structure, although it is quite tricky to state whether CI Direct Black 22 had been metabolized or simply biosorpted by *A. nidulans*. Moreover, the peak at 717 could not be assigned to any fragmented species of the dye either. Although CI Direct Black 22 is a not-banned azo dye according to the REACH regulation [68], compounds reported in Fig. 5 and derivatives thereof might represent a potential toxic threat. Their absence and the observed peaks were of particular encouragement to confirm the reliability of the method, which releases clear dyeing effluent potentially free from toxic compounds.

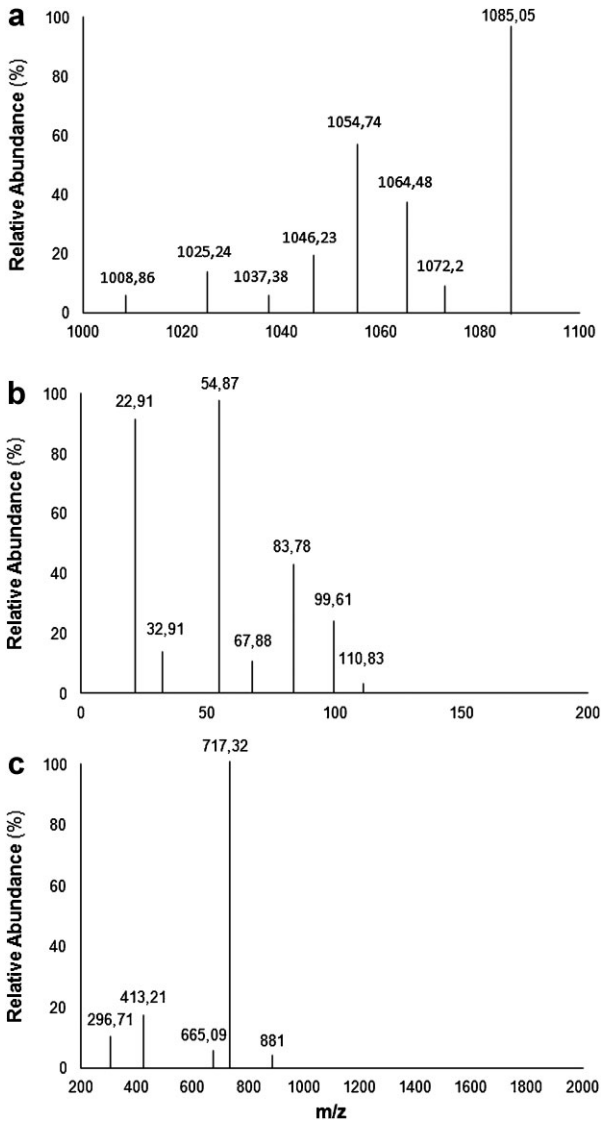

Figure 4. Spectrometric mass analysis of effluent C. Mass analysis (ESI, +c) of effluent C was carried out on effluent C before (a) and after treatment with *A. nidulans* (b and c).

Toxicity test

The assessment of the toxicity of textile wastewaters after decolorization is an important aspect for bioremediation studies, to demonstrate that the treated effluents are not toxic although visually clear. According to the results of decolorizing activity and to the UV–visible spectra, acute toxicity tests using *D. magna* were carried out on dyeing effluents A and C treated with *B. adusta* and *A. nidulans*. As shown in Table 7, the toxicity rates of effluent A and C dropped to 0 after 2 and 5 days treatment with *A. nidulans*. The same situation was found for effluent A, but after a 7-day treatment with *B. adusta*. On the other hand, a strong reduction of the zooplankton mortality could also be

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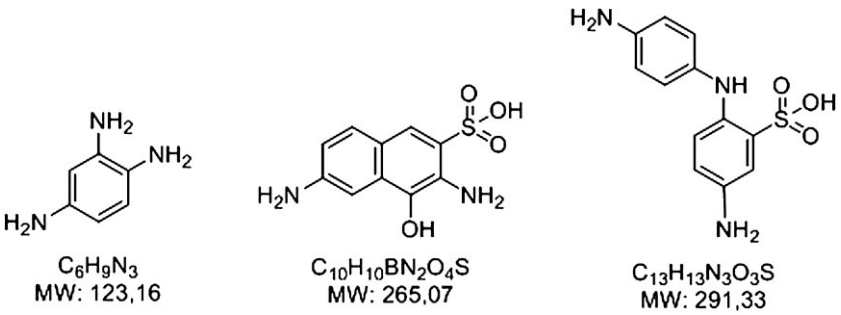

Figure 5. Potential toxic fragments derived from Black Direct 22 degradation by reductive cleavage of azo group.

observed on effluent A treated with *B. adusta* for 2 and 5 days (*ca*. 82 and 91%, respectively). A substantial reduction in acute toxicity was also found on effluent C treated with *B. adusta*, with values ranging from 74 to 86% for 2 and 7 days treatment, although the mortality never dropped to 0.

According to these data, it is reasonable to hypothesize that the differences here observed in the acute toxicity of the same effluent when treated with *B. adusta* or *A. nidulans* might be mainly related to putatively different mechanisms adopted by these fungi for decolorization.

Discussion

We have isolated and characterized the indigenous microflora associated to three different real textile-dyeing effluents. As it could be expected according to stressful conditions of these effluents, very few microorganisms were demonstrated to adapt and to stably survive in these wastewaters. A very low variability was also found, and the microbial species were mainly represented by mainly bacteria, although yeasts belonging to the genus *Pichia* were isolated. The decolorizing potentials of these isolates were evaluated on the three real exhausted effluents. Unfortunately, in the experimental conditions adopted here, no isolates were found having a substantial activity of color removal, which was never higher than 7%. In a very recent study on the decolorization of the monoazo dye Orange II, the native microflora was able to achieve 32% decolorization of unsterilized effluents under optimized conditions [69]. A rationale about our finding may be centered on the nature of the resident microorganisms, mainly represented by bacteria, and on the ordered sequence of metabolic events during bacterial degradation of dyes. It could be speculated that in specific and optimized conditions, a microbial consortium (e.g. the native microflora) may be more efficient than each of its component. Among the main reasons for that there could be the opportunity for a consortium to have complemented the different metabolic properties of each of its individuals. To this concern, it is worth to remind the steps occurring during the bacterial degradation of azo dyes. This process usually begins under anaerobic and static conditions, and gives origin to colorless aromatic amines, which can be then further oxidized only under aerobic conditions [70–72]. Moreover, the rate of dye removal by bacteria was proved to be linked with the exponential growth phase [73], whereas the bacterial concentration in these effluents was always very low. These entire hypotheses deserve in the next future an in depth investigation, to provide useful information for the industrial feasibility of the use of the native microflora to treat exhausted dyeing effluents. Still in this frame, decolorization efforts were directed towards the use of Basidiomycetes, Ascomycetes, and Deuteromycetes

Table 7. Acute toxicity tests using *Daphnia magna*, on effluents A and C treated with *B. adusta* and *A. nidulans*.

Fungi	Effluent	Time[a]	Mortality[b] (%)
B. adusta	A	0	100 ± 0
		Day 2	18.66 ± 6.11
		Day 5	9.33 ± 6.11
		Day 7	0 ± 0
	C	0	100 ± 0
		Day 2	26.66 ± 6.11
		Day 5	17.33 ± 4.62
		Day 7	14.66 ± 4.61
A. nidulans	A	0	100 ± 0
		Day 2	0 ± 0
		Day 5	0 ± 0
		Day 7	0 ± 0
	C	0	100 ± 0
		Day 2	2.67 ± 2.31
		Day 5	0 ± 0
		Day 7	0 ± 0

[a]Effluents were fungal-treated for 2, 5, and 7 days. Untreated effluents ($t = 0$) were used as negative controls.
[b]*D. magna* mortality was assessed after 48 h treatment and tests were carried out in triplicate, $n = 25$.

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plant-associated fungi on three different effluents from a real dyeing company. No acceptable decolorization was ever obtained on effluent B, consisting of a mixture of disperse dyes for polyester substrates. This could be related to the presence of large quantities of surfactants and other additives, hampering the metabolic activities of these plant-associated fungi. Conversely, *B. adusta*, *A. nidulans*, and *F. trogii* led to promising results on effluent A, consisting of a mixture of direct dyes and a limited set of additives used for wool dyeing. Similar decolorization was obtained for effluent C, which contained just a single direct dye for cotton. Examining the experimental parameters and their combination, the nutrients were demonstrated to be able to increase the rate of color removal for all the fungi tested. At the moment this finding cannot be assumed to depend from a direct relationship between fungal growth and color removal activity, as demonstrated for example by comparing the decolorizing performances and the growth of *B. adusta* on effluent A. To this regard, the effectiveness of the addition of organic nitrogen sources in promoting color removal is supposed to be related to the generation of NADH, which acts as an electron donor in the dyes reduction [74, 75]. Moreover, it was demonstrated that the decolorization operated by fungi depends not only on the amount of the fungal biomass, but on its chemical composition as well [49, 76]. A decrease in the efficiency of color reduction was recorded in the absence of nutrients, that further dropped when the pH of the effluents was adjusted to 6.0, although acidic or neutral pH were reported to give better decolorization and biodegradation activities for fungi and yeast [77]. Nevertheless, both *B. adusta* and *A. nidulans* were proved to be able to spontaneously shift the native pH of the effluent in presence of nutrients, with a general trend towards neutrality. Recent studies demonstrated that substantial variations of the pH of textile dyestuff can occur during decolorization, depending on the changes in the microbial metabolism related to nutrient availability [78]. Currently, it is purely speculative to associate the effects of pH variations on color removal efficacy to different mechanisms, such as changes occurring in fungal enzymatic activity or in the distribution of the charges on the dyes, or in the transport of dyes across the cell membrane as well [78]. More investigations are needed to provide information, which are essential for the industrial optimization of the parameters for fungal decolorization.

The UV–visible analyses of the treated effluents suggested that two different strategies might have been adopted by *B. adusta* and *A. nidulans* during the decolorization process. In the case of *B. adusta*, biodegradation of effluent A found an evidence with the enzyme promoted breakdown of the dye structures, resulting in different UV–visible absorption patterns between the spectra of treated and un-treated effluents. These findings would be in accordance with previous reports in the literature documenting the decolorizing abilities of white-rot fungi by means of extracellular peroxidases and laccases [43–47, 79, 80]. On the other hand, *A. nidulans* gave indication of a biosorption process for effluents C, triggering a rapid depletion of the dye here contained. Biosorption is associated to the adsorption of the dye on the fungal cell wall and the resulting UV–visible spectra were characterized by a proportional decrease of all the peaks that were initially present in the untreated samples [81]. Negligible variation of the qualitative absorption in the plot led to superimposable spectra, which may also support the absence of fungal metabolites. Moreover, the results coming from the toxicity studies carried out on *D. magna* were very supportive for the future industrial application of *A. nidulans*, which was able to bring the toxicity of treated effluent A and C to naught from the fifth and the second day onwards, respectively.

Some recent decolorization studies carried out on model and industrial dyes have shown the biosorption activity of living and dead biomass of several Ascomycetes and Deuteromycetes, among which some specie belonging to the genus *Aspergillus*, with *A. ochraceus* and *A. niger* among the most effective [25, 49, 76, 82–84]. To the best of our knowledge, until now *A. nidulans* was never reported to be able to efficiently decolorize textile dyes, although its properties as biosorbent have already been shown for the removal of very dangerous pollutants from wastewaters, such as endosulfan [85] and arsenic [86]. Thus, overall these data pose the basis for a practical bioprocess of dyeing wastewater decolorization privileging *A. nidulans*, for its versatile activity on several real very different textile-dyeing effluents even if used as such.

Conclusions

Real textile effluents were used in decolorizing experiments carried out by several microorganisms. Biodegradation and biosorption have found evidence in the decolorization of these wastewaters. The most promising results for future industrial applications were shown by the Ascomycete *A. nidulans*, whose decolorizing activity was strongly suggested to be based on a supposed biosorption mechanism. This mode of action by *A. nidulans* may well be exploited to devise wastewater treatment protocols to allow the recycle of water, after a

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simple filtration to remove mycelial residues. The reduction of the dyes promoted by *A. nidulans* without the generation of toxic fungal metabolites was supported by a dramatic decrease of the toxicity of treated wastewaters, as highlighted with the experiments carried out on *D. magna*. We believe that biosorption operated by *A. nidulans* may represent a novel opportunity for fungal treatment of dye wastewaters, encompassing a sustainable answer to the bioremediation of industrial dyeing effluents. Further studies about this topic will be progressed.

Acknowledgments

We gratefully acknowledge Ilaria Marsili Libelli and Massimo Rolla for their technical support, and Margaret Gordon and Marta Bencini for English revision. We do also acknowledge Dr. Paolo Capretti for providing us some of the fungi here used. We gratefully acknowledge Regione Toscana for financially supporting this research in the frame of project COLBIOTECH – POR CREO FSE 2007-2013. We also gratefully acknowledge "Ente Cassa di Risparmio di Firenze" (grant number 2007.1005-2010.0972).

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