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Four marine-derived fungi for bioremediation of raw textile mill effluents

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Abstract Textile dye effluents pose environmental hazards because of color and toxicity. Bioremediation of these has been widely attempted. However, their widely differing characteristics and high salt contents have required application of different microorganisms and high dilutions. We report here decolorization and detoxification of two raw textile effluents, with extreme variations in their pH and dye composition, used at 20–90% concentrations by each of the four marine-derived fungi. Textile effluent A (TEA) contained an azo dye and had a pH of 8.9 and textile effluent B (TEB) with a pH of 2.5 contained a mixture of eight reactive dyes. The fungi isolated from mangroves and identified by 18S and ITS sequencing corresponded to two ascomycetes and two basidiomycetes. Each of these fungi decolorized TEA by 30–60% and TEB by 33–80% used at 20–90% concentrations and salinity of 15 ppt within 6 days. This was accompanied by two to threefold reduction in toxicity as measured by LC_{50} values against Artemia larvae and 70–80% reduction in chemical oxygen demand and total phenolics. Mass spectrometric scan of effluents after fungal treatment revealed degradation of most of the components. The ascomycetes appeared to remove color primarily by adsorption, whereas laccase played a major role in decolorization by basidiomycetes. A process consisting of a combination of sorption by fungal biomass of an ascomycete and biodegradation by laccase from a basidiomycete was used in two separate steps or simultaneously for bioremediation of these two effluents.

Keywords Marine fungi · Raw dye-containing effluents · Laccase · Toxicity · Decolorization · Chemical oxygen demand

Introduction

Natural pigments used for coloring textiles have been replaced by "fast colors" which do not fade on exposure to light, heat and water. These features unfortunately go with the perils of harmful effluent quality. About 15% of the dyes used for textile dying are released into processing waters (Mishra and Tripathy 1993). Besides being unaesthetic, these effluents are mutagenic, carcinogenic and toxic (Chung et al. 1992). Chemical and physical methods

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for treatment of dye wastewater are not widely applied to textile industries because of exorbitant costs and disposal problems. Green technologies to deal with this problem include adsorption of dyestuffs on bacteria and fungal biomass (Dönmez 2002; Fu and Viraraghavan 2002; Prigione et al. 2008a, b) or low-cost non-conventional adsorbents (Crini 2006; Ferrero 2007). Treatment with basidiomycetous fungi or their lignin-degrading enzymes, lignin peroxidase, manganese-dependent peroxidase and laccases has been widely reported (Wesenberg et al. 2003; Raghukumar et al. 2008; Blánquez et al. 2008). These act on a broad range of substrates and hence are able to degrade several xenobiotics (Kim and Nicell 2006) including synthetic dyes (Wesenberg et al. 2003). Several ascomycetous and hyphomycetous fungi also produce laccase (Baldrian 2006). Laccases (EC 1.10.3.2) have been lately reported to be produced by several marine and marine-derived fungi (Raghukumar et al. 1994, 1999, 2008; Pointing et al. 1998; Pointing and Hyde 2000; D'souza-Ticlo et al. 2009).

A majority of previous studies have focused on treatment of simulated effluents (Prigione et al. 2008b) with one to several dyes added to defined media. The raw effluents also contain high concentrations of various inorganic chemicals such as sulfides, sulfates, chlorides and carbonates. Therefore, these are required to be diluted several fold (Wesenberg et al. 2002). Textile effluents differ widely in their chemical characteristics and pH (Hai et al. 2007). Therefore, no single organism can detoxify and decolorize them. Efficiency of marine-derived fungi in treatments of such effluents has largely remained unexplored. Marine-derived fungi grow and produce degradative enzymes in seawater media (Raghukumar et al. 2008) and thus may be useful in treating wastewaters with high salt content. A basidiomycete *Phlebia* sp., strain MG-60 isolated from mangrove stands was reported as a hypersaline-tolerant lignin-degrading fungus which participated in biobleaching of pulp and decolorization of dyes (Li et al. 2002) in the presence of different concentrations of sea salts. Purified laccase from the marine fungus NIOCC #2a was not inhibited in the presence of NaCl up to 0.3 M concentration and retained 75% of its activity in the presence of half strength sea water (D'souza-Ticlo et al. 2009). Besides, it decolorized several synthetic dyes in the presence of sea water.

The objective of the present study was to achieve decolorization and detoxification of two raw, dye-containing textile mill effluents varying in their pH, chemical and dye composition, added at high concentrations in media prepared with sea water. Two marine ascomycetes with rapid growth and high biomass-yield and two basidiomycetes with relatively high titer of laccase were used for bioremediation. Efficiency of the whole cultures (in situ) in removal of color and toxicity, reduction in COD and total phenolics from these two effluents was studied. The mechanism of color removal by these two different groups of fungi was evaluated. A further objective of this study was to develop an efficient bioprocess for industrial application involving use of these fungi and their enzymes in combination.

Materials and methods

Isolation of fungi

Detritus and decaying wood pieces were collected from mangrove swamps of Chorao and Sal in Goa, India (73°55′E and 15°30′N) in December 2006. Fungi from detritus were isolated by particle plating method (Damare et al. 2006) and from decaying wood material by moist chamber incubation method (D'souza-Ticlo et al. 2006). They were maintained in Boyd & Kohlmeyer (B & K) medium prepared with sea water of 15–17 ppt salinity and contained 2% glucose, 0.1% yeast extract and 0.2% peptone (Kohlmeyer and Kohlmeyer 1979). They were routinely checked for purity by light microscopy.

Screening of fungi for lignin-degrading enzymes

Preliminary screening of the isolates for the presence of lignin-degrading enzymes was carried out on B & K agar medium containing model compounds such as Poly-R 478 (Poly-R), guaiacol and ABTS (2,2′-azino-bis-3-ethylbenthiazoline-6-sulfonate). Decolorization of Poly-R from pink to yellow or colorless indicated presence of lignin peroxidase and/or manganese peroxidase. The production of an intense brown color under and around the fungal colony in guaiacol-supplemented medium and deep green color in

ABTS-supplemented medium indicated presence of laccase activity (D'souza-Ticlo et al. 2006).

Identification of fungi

Fungi with telomorphic and/or anamorphic stages (spores/conidia) produced in cultures were identified by morphology using the taxonomic keys (Kohlmeyer and Kohlmeyer 1979). Molecular identification of all the fungi was carried out by sequence analyses of partial 18S and complete ITS regions of rRNA gene. Partial region of 18S rDNA was PCR-amplified using a universal fungal specific primer sets (White et al. 1990), NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS4 (5′-CTTCCGTCAATTCCTTTAAG-3′). Full length of ITS region was amplified (White et al. 1990) using the primers ITS1 (5′-TCCGTA GGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGC TTATTGATATGC-3′).

Purified PCR products were sequenced at National Centre for Cell Sciences, Pune, India. Sequence data were edited using Chromas Pro version 1.34. For tentative identification, fungal 18S and ITS rDNA sequences were compared with NCBI (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov) database. The 18S and ITS fungal rDNA in this study and the identical/similar sequences from GenBank were edited and aligned using CLUSTAL-X, version 1.81 (Thompson et al. 1997). The aligned sequences were imported into DAMBE 4.5.47 (Xia and Xie 2001). Neighbor-joining (NJ) trees were created using pairwise genetic distances using MEGA 4.1 (Kumar et al. 2008). The quality of the branching patterns for NJ was assessed by bootstrap resampling of the data sets with 1,000 replications.

Culture conditions

Four laccase-producing fungi, two belonging to the class ascomycetes and two to basidiomycetes were short listed for this study. They were grown in B & K broth (20 ml in 100 ml Erlenmeyer flasks) for 7 days, homogenized in sterile seawater in Omni Macro-homogenizer (model No. 17505, Marietta, GA, USA) for 5 s. The mycelial suspension (10%, v/v) was used for inoculating 20 ml of B & K broth in 250 ml Erlenmeyer flasks. The fungi were incubated at room temperature (30°C) under static conditions.

Decolorization of textile mill effluents by whole cultures (in situ)

Textile effluent A (TEA) and textile effluent B (TEB) were each added separately at 20, 50 and 90% final concentration (equivalent to 5, 2 and 1.1-fold dilutions, respectively) to 4 day-old cultures raised in B & K broth as described above. The day of addition of effluents to the pre-grown cultures was considered as day zero for all the color measurements. Decolorization of these two effluents was monitored by changes in the absorbance scanned from 360 to 800 nm wavelengths. Percentage decolorization was calculated as the extent of decrease of the spectrum area with respect to that of the control (0 day sample). Triplicate cultures were maintained for each treatment.

Residual color from the fungal biomass was extracted in 10 ml of methanol:water (1:1) by homogenization for 1 min in a Macro-homogenizer. Mycelial fragments were removed by filtering the content over Whatman No. 1 filters. The filtrate was lyophilized and the residue was re-suspended in 1 ml of water and the percentage color adsorbed was calculated spectrophotometrically as described above.

As the two effluents differed in their pH, we normalized this parameter in one of the experiments by changing the pH of TEA from 8.9 to 5.0 with glacial acetic acid before adding it to the pre-grown cultures. Decolorization was measured as described above and compared with that obtained by using pH-unaltered TEA.

Estimation of lignin-degrading enzymes

Laccase activity was assayed in the culture supernatants by measuring oxidation of 1 mM ABTS substrate buffered with 0.2 M glycine–HCl buffer, pH 3, at 405 nm (Niku-Paavola et al. 1988). Laccase units (U) were calculated as micromole of the substrate transformed per minute per litre of culture supernatant and expressed as U l^{-1}. Lignin peroxidase (LiP) activity was determined by measuring the rate of oxidation of veratryl alcohol to veratraldehyde (Tien and Kirk 1988). Manganese-dependant peroxidase (MnP) activity was determined by measuring the rate of oxidation of veratryl alcohol to veratraldehyde in the presence of Mn (Paszczynski et al. 1988). Units were calculated as micromole of the substrate transformed per minute per litre of culture

supernatant and expressed as U l^{-1}. Glucose oxidase was estimated with peroxidase and chromogenic oxygen acceptor *O*-dianisidine which results in formation of a colored product which is measured at 460 nm (Tsuge et al. 1975). The enzyme units were expressed as U l^{-1}.

Toxicity test

Detoxification of culture supernatants of different fungi grown in the presence of effluents (at 20, 50 and 90%) was assayed on day 6 using nauplii of *Artemia salina* (Barahona-Gomariz et al. 1994). Fungal-treated and untreated effluents were diluted to different concentrations with 0.22 μm-filtered seawater and larval mortality was assayed in these. The nauplii (15–25 organisms) were incubated in the diluted effluents at room temperature and mortality was estimated after 24 h. Multiple dilutions were used to obtain linearity in concentration against mortality. Lethal concentration that resulted in 50% mortality (LC_{50} value) by 24 h was calculated by plotting dilutions of effluent versus number of dead organisms. Average values of triplicate treatments were recorded.

Estimation of total phenolics and chemical oxygen demand

Total phenolics were measured in the culture supernatants of fungi grown in the presence of effluents on day 0 and 6 using Folin–Ciocalteau method and calculated in g l^{-1} (Singleton and Rossi 1965). Catechol (Sigma Chemicals, Mo, USA) was used as the reference standard. The reduction in total phenolics from day 0 (obtained immediately after addition of effluents to the cultures) to day 6 was expressed in percentage.

Chemical oxygen demand (COD) was estimated by open reflux method (APHA 2005) in culture supernatants on day 0 and 6 and the COD values were calculated in mg O_2 l^{-1} and expressed as percentage reduction.

Mass spectrometric analyses of treated effluents

Electrospray ionization mass spectrometry (ESI-MS) analysis was performed on a quadrapole-time of flight mass spectrometer (Model Qstar XL, Applied Biosystems, Rotkrenz-Switzerland). Culture supernatants from fungi with TEA and TEB (added at 20%), respective control cultures without effluents and uninoculated B & K broth were diluted with methanol:water (1:1) and directly analyzed by ESI-MS. The samples were introduced at a constant flow rate into the electrospray source using an integrated syringe pump. The mass/charge (m/z) MS-survey range was 0–1,000 in positive mode. The spectra shown in the figures are after subtracting common peaks found in the uninoculated and inoculated B & K broth.

Decolorization of effluents by culture supernatants (*ex situ*)

As all the four fungi showed laccase activity in the culture supernatants, their efficiency in decolorization of the two effluents was tested *ex situ*. For this purpose the fungi were grown in low nitrogen medium because it is reported to support high laccase production (D'souza-Ticlo et al. 2006). Concentrated culture supernatants from 12-day old cultures (when maximum laccase activity was recorded) were used for decolorization of TEA and TEB. The effluents were diluted to 50% with sodium acetate buffer 0.1 M (pH 5.0) and incubated with culture supernatants for varying time period and reduction in color was monitored. The percentage decolorization was calculated as described above.

Mediators such as ABTS, vanillic acid, veratryl alcohol, *p*-coumaric acid, 1-hydroxy benzotriazole (HBT), and acetosyringone (Wong and Yu 1999) were added at 50 and 500 μM concentrations to enhance the decolorization of TEA and TEB obtained by laccase. Appropriately diluted effluents were incubated with culture supernatants of these fungi along with mediators. These were scanned from 360 to 800 nm wavelengths at 0, 6, 12, 24, 48 and 72 h. The reduction in color was calculated and was expressed in percentage as described above. The results were compared with control treatment that did not receive any mediators.

As NIOCC #2a produced highest laccase titer among the four test fungi, its efficiency was compared with a commercial laccase preparation from *Trametes versicolor* (Sigma Chemicals, USA) for decolorization of TEA and TEB. For this purpose multiple concentrations of these two laccases were

incubated with 20% TEA and TEB and reduction in color was measured at 12 and 36 h.

Reusing fungal biomass for decolorization

The fungi were tested for repeated use in decolorization of these effluents. Briefly, to 4-day old cultures, the effluents were added at 20% final concentration and the reduction in color was measured after 6 days in the culture supernatants. After draining the culture supernatants, fresh half-strength B & K broth with 20% effluent was added to the fungal biomass and the reduction in color was measured once again after 6 days. This procedure was repeated twice with each fungus.

Decolorization of bioadsorbed effluent

The possibility of decolorization of the adsorbed color from the fungal biomass by culture supernatant with high laccase activity was tested. Mycelial biomass of the ascomycete #C3 after adsorption was homogenized and incubated with 100 U of laccase from the basidiomycete #2a for 48 h at 120 rpm and the residual color in the fungal biomass was extracted and measured as described above. In the control treatment the mycelial biomass was incubated with distilled water for 48 h and the color removal was compared with laccase-treated samples.

Developing a process for enhanced decolorization

To enhance the decolorization process, the possibility of using a combination of fungal biomass and laccase from different fungi was tested. To achieve this, 4-day old ascomycetous fungal biomass from #C3 and laccase from #2a were incubated with 20% TEA or TEB for 48 h and the residual color, both in the supernatant and the mycelial biomass was measured.

Statistical analyses

All comparisons between treatments or cultures were analysed by student *T*-test and correlation coefficient in Excel (Microsoft, USA) program for statistical significance.

Results

Laccase producing fungi and their identification

About 40 fungi isolated from mangroves showed laccase activity in the qualitative assay with guaiacol and ABTS. Only NIOCC #2a decolorized Poly-R indicating the presence of manganese peroxidase.

The fungi could not be identified by classical morphological taxonomy since no reproductive structures were observed, hence were identified by molecular methods. Out of these fungi, two ascomycetes (NIOCC #16V and #C3) and two basidiomycetes (NIOCC #2a and #15V) showing high laccase activity in the culture medium and decolorization of the two textile effluents were chosen for further studies. The two ascomycetes were identified as (Table 1) *Diaporthe* sp. and *Pestalotiopsis* sp. (NIOCC #16V and #C3, respectively). Sequence analyses of 18S of NIOCC #15V identified it to be *Coriolopsis byrsina* and NIOCC #2a as *Cerrena unicolor* (Table 1). However, ITS sequence analyses of NIOCC #15V showed 88% homology to *Ganoderma* sp. and that of #2a showed 100% homology to an unknown basidiomycete, but the closest positively identified match was *C. unicolor*. The phylogenetic analyses of the four test fungi based on 18S and ITS sequences are shown in the supplementary Fig. 1a and b. The cultures #15V, #16V and #C3 were deposited at American Type Culture Collection, USA under the accession No. ATCC MYA-4557, ATCC MYA-4558 and ATCC MYA-4556, respectively. The fungus #2a was deposited at Microbial Type Culture Collection (MTCC), Chandigarh, India under the Accession No. MTCC 5159.

Laccase production and decolorization by whole cultures (in situ)

The two textile effluents differed in their dye composition, chemical constituents, pH, and salt content (Table 2). The structures of component dyes (wherever available), C.I. names and λ_{max} are listed in Table 3. Ascomycetes produced lower titer of laccase than basidiomycetes in the presence of both the effluents (Fig. 1a–d). The basidiomycetes showed 20 to 60-fold (Fig. 1a, c) and the ascomycetes two to tenfold (Fig. 1b, d) higher laccase production in the presence of TEB than in the presence of TEA.

Table 1 Sequences producing significant alignments with various rRNA gene sequences

Fungus # accession no. of 18S and ITS sequences	18S sequence		ITS sequence	
	Identity (max % identity) class	Accession no. Q-coverage (error value)[a]	Identity (max % identity) class	Accession no. Q-coverage (error value)[a]
NIOCC # 2a	*Cerrena unicolor*	EF059806.1	Basidiomycetes sp.	EF029817.1
18S: EE059806.1	(100%)	100	(99%)	98%
ITS: FJ010208.1	Basidiomycetes	(0)	Basidiomycetes	(0)
NIOCC # 15V	*Coriolopsis byrsina*	AY336773.1	*Ganoderma* sp.	AJ60811.1
18S: EU725822.1	(100%)	100	(88%)	97%
ITS: EU735845.1	Basidiomycetes	(0)	Basidiomycetes	(0)
NIOCC # 16V	*Diaporthe* sp.	EF211126.1	*Diaporthe phaseolorum*	AF001025.2
18S: EU725818.1	(99%)	100	(99%)	99%
ITS: EU735847.1	Ascomycetes	(0)	Ascomycetes	(0)
NIOCC # C3	*Pestalotiopsis maculans*	AB 220236.1	*Pestalotiopsis uvicola*	AY 687297.1
18S: EU725821.1	(100%)	100	(100%)	100%
ITS: EU735843.1	Ascomycetes	(0)	Ascomycetes	(0)

[a] Accession numbers of the closest match found in the BLASTn search

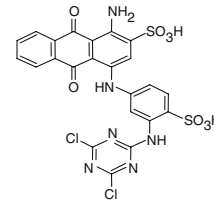
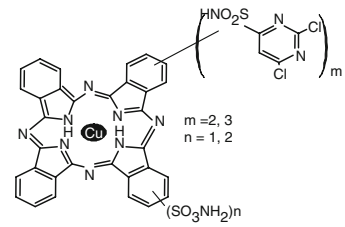
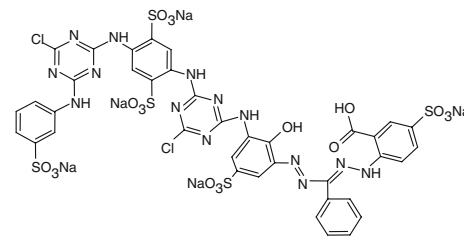
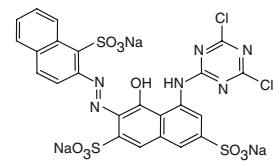
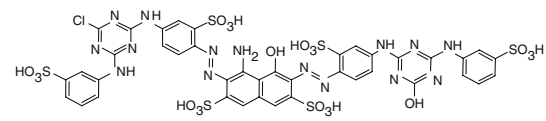
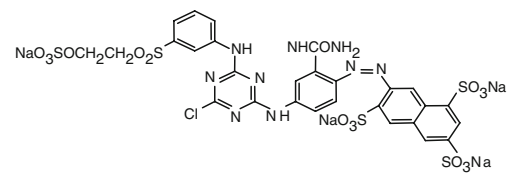
Table 2 Characteristics of textile effluent A (TEA) and textile effluent B (TEB)

Parameters	TEA	TEB
pH	8.9	2.5
Color (Pt–Co units)	1,44,180	52,500
Absorbance maxima (nm)	505	667
COD (mg l^{-1})	30,000	20,000
Total phenolics (g l^{-1})	0.1	0.02
Dye components	Azo dye-20	Reactive blue 4, reactive blue 140 base, reactive blue 140, reactive blue 160 base, reactive blue 163, reactive red 11, reactive yellow 145, reactive green 19
Total solids (g l^{-1})	0.254	0.51
Carbonates (g l^{-1})	30.0	36.0
Na^+ (g l^{-1})	0.043	0.013
Ca^+ (g l^{-1})	0.03	0.009
SO_4 (g l^{-1})	7.23	1.23
Cl (g l^{-1})	150.0	191.7
PO_4 (g l^{-1})	0.021	0.02

Decolorization of 20% TEA and TEB by the two ascomycetes reached plateau by day 3, irrespective of the laccase titer produced by them (Fig. 1b, d). Among the two effluents TEB was decolorized to the higher extent by all the four fungi than TEA (Fig. 1a–d). Most of the decolorization of TEB by ascomycetes was achieved by day 2 (Fig. 1d) whereas this was not the case with TEA. In general, about 40–60% color reduction of TEA and 60–80% color removal of TEB was obtained by day 9. No correlation was observed between decolorization of TEA or TEB with laccase production. Color removal of both the effluents by adsorption on the fungal biomass was greater in ascomycetes than in the basidiomycetes (Table 4).

The cultures were tested for the presence of other lignin-degrading enzymes. The culture # 2a alone produced a maximum of 70 U l^{-1} of MnP but not LiP

Table 3 Component dyes of the effluents, their C.I. name, maximum visible absorbance and chemical structure (where available)

C.I. name	Absorbance maxima (nm)	Chemical structure
Reactive blue 4	599	
Reactive blue 140	654	
Reactive blue 160	609	
Reactive red 11	545	
Reactive yellow 145	419	
Reactive green 19	634	

in the B & K medium. Addition of TEA to this medium did not inhibit MnP production but TEB inhibited its production by five to sixfold. The basidiomycetes produced about 200–300 U l^{-1} glucose oxidase in this medium supplemented with TEB, whereas the ascomycetes produced about 50–100 U l^{-1}. The production of this enzyme was reduced by four to fivefold in the presence of TEA in the medium. In the ascomycetes, its production was largely inhibited (data not shown).

In order to verify whether the low decolorization of TEA was due to alkaline pH, decolorization

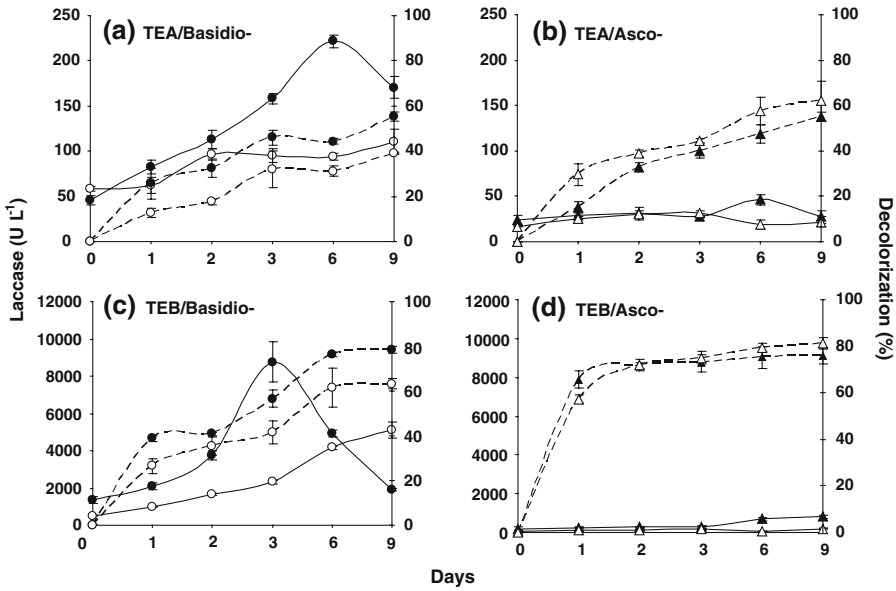


Fig. 1 Laccase production (*solid line*) and decolorization (*dashed line*) of TEA by (**a**) the basidiomycetes NIOCC #2a (*filled circle*) and # 15V (*open circle*) and (**b**) the ascomycetes #16V (*filled triangle*) and #C3 (*open triangle*). Laccase production (*solid line*) and decolorization (*dashed line*) of TEB by (**c**) the basidiomycetes NIOCC #2a (*filled circle*) and #15V (*open circle*) and (**d**) the ascomycetes #16V (*filled triangle*) and #C3 (*open triangle*). The effluent was added to 6-day old culture and this was considered day 0 for the decolorization measurements

Table 4 Percentage of color adsorbed on the live fungal biomass after growing them in the presence of 20% TEA and TEB for 6 days

Cultures (class)	TEA	TEB
# 2a (Basidiomycete)	32.3 ± 5.4	6.8 ± 1.9
# 15V (Basidiomycete)	27.7 ± 2.3	8.2 ± 0.6
# 16V (Ascomycete)	38.9 ± 4.8	41.3 ± 4.6
# C3 (Ascomycete)	43.0 ± 5.7	29.8 ± 2.3

Fungal biomass grown in the presence of TEA and TEB was collected by centrifugation, homogenized in methanol:water (1:1) and filtered. The filtrate was lyophilized and re-suspended in water and the color was determined spectrophotometrically as described under "Materials and methods"

experiment was also performed after lowering the pH of TEA from 8.9 to 5. No increase in percentage decolorization was noticed after altering the pH, ruling out the possibility that alkaline pH was the limiting factor for decolorization.

Spectral scans of culture supernatants from fungi grown in the presence of TEA and TEB showed a decrease in absorbance throughout the visible range (Supplementary Fig. 2a, b). Spectral scans of #C3, #2a and #16V-treated TEB showed total disappearance of absorbance maximum peaks in the region 560–700 nm. The absorbance between 400 and 450 nm reduced considerably without altering the pattern of the peak. The λ_{max} of reactive yellow 145 fell into this spectral range (Table 3).

As all the four fungi decolorized both TEA and TEB incorporated at 20% (fivefold diluted) in the medium, efficiency of these fungi to decolorize higher concentrations of these effluents was tested by adding them at 50 and 90% concentration (twofold and 1.1-fold diluted). All the four fungi showed biomass and laccase production in the presence of TEA and TEB at these concentrations (Tables 5, 6). The growth in the presence of TEA and TEB was better than the control in most of the fungi (Tables 5, 6). Decolorization of TEA in the range of 27–57% (Table 5) and 34–68% of TEB (Table 6) added at 50 and 90% concentrations was possible with these fungi.

Detoxification of textile mill effluents in whole cultures (in situ)

Detoxification of TEA as measured by a decrease in percentage mortality of Artemia larvae with reference to untreated control was best by NIOCC #15V followed by #16V, #C3 and #2a (Fig. 2a) whereas

Table 5 Response of the fungi in the presence of TEA added at 20, 50 and 90% to 6-day old cultures (in situ)

Parameters measured	Conc. of the effluent (%)	NIOCC #2a *Cerrena unicolor*	NIOCC #15V *Coriolopsis byrsina*	NIOCC #16V *Diaporthe* sp.	NIOCC # C3 *Pestalotiopsis* sp.	Control[b]
Biomass (g l^{-1})	Control[a]	3.6 ± 0.2	4.1 ± 0.5	3.7 ± 0.9	5.1 ± 0.3	–
	20	4.0 ± 0.3	4.6 ± 0.5	4.3 ± 0.8	6.3 ± 0.3	
	50	4.9 ± 0.7	5.3 ± 0.9	4.8 ± 0.6	6.2 ± 1.1	
	90	4.2 ± 1.7	4.4 ± 0.8	5.3 ± 1.3	5.9 ± 0.8	
Laccase (U l^{-1})	Control[a]	2,015 ± 615.8	1,951 ± 74.5	52 ± 12.9	39 ± 8.1	–
	20	141.9 ± 42.6	82.4 ± 16.8	32.8 ± 7.1	22.3 ± 4.1	
	50	46.3 ± 4.5	39.8 ± 11.6	41.5 ± 12.1	41.8 ± 28.6	
	90	38.4 ± 7.4	40.9 ± 5.9	47.8 ± 3.6	23.6 ± 2.4	
% Decolorization	20	44.4 ± 1.4	31.2 ± 2.1	48.0 ± 4.1	58.0 ± 6.0	–
	50	55.6 ± 1.5	29.9 ± 0.7	27.3 ± 0.5	56.6 ± 4.3	
	90	23.9 ± 5.8	26.7 ± 7.2	31.9 ± 4.3	30.7 ± 7.9	
24 h-LC_{50}	20	14.2 ± 1.4	28.0 ± 14.1	18.1 ± 1.8	16.7 ± 2.2	6.5 ± 1.4
	50	4.3 ± 0.1	5.0 ± 1.1	5.7 ± 1.3	4.7 ± 0.5	1.9 ± 0.0
% Reduction in total phenolics	20	19.3 ± 0.9	28.1 ± 1.1	21.1 ± 0.6	22.8 ± 2.4	–
	50	22.6 ± 2.5	24.9 ± 6.6	21.7 ± 5.4	34.2 ± 2.8	
% Reduction in COD	20	48.3 ± 3.0	62.9 ± 11.3	90.0 ± 0.6	69.1 ± 5.5	–
	50	20.6 ± 11.7	28.5 ± 4.4	24.1 ± 6.8	40.4 ± 1.2	

All the parameters were estimated on day 6 after addition of the effluent

[a] Without any effluent

[b] Only TEA in the medium without any culture

in TEB, lowest mortality was noticed in the presence of #2a followed by #C3, #16V and #15V (Fig. 2b). The four fungi brought about three to fivefold reduction in toxicity of TEA (Table 5) whereas toxicity of TEB was reduced two to threefold (Table 6). In general TEA was more toxic than TEB (Tables 5, 6). About 19–34% reduction in total phenolics and 50–90% reduction in COD of TEA were brought about by the four fungi (Table 5). Treatment of TEB with the four test fungi resulted in a reduction in total phenolics by 50–90% and 44–98% reduction in COD (Table 6). Toxicity of TEB was reduced better by basidiomycetes whereas ascomycetes proved better in COD reduction (Table 6).

Mass spectrometry analysis of the effluents

Mass spectrometric scans of culture supernatants of fungi grown in the presence of TEA and TEB showed distinct changes indicating fragmentation and degradation of the components of these effluents (Figs. 3, 4). These modifications appear to reflect in percentage decolorization. Chromatograms of TEA showed decrease in intensity and disappearance of most of the peaks by all the fungi (Fig. 3). Scan of #15V in the presence of TEB showed the maximum number of degradation products and the rest of the fungi showed maximum disappearance of peaks (Fig. 4).

Decolorization by culture supernatants (*ex situ*)

The efficiency of culture supernatants of the four fungi in decolorization of TEA and TEB at 50% concentration was tested. No color reduction of TEA was observed up to 72 h. Culture supernatants of NIOCC # 2a, #15V, #16V and #C3 with the laccase titer of 64, 29, 0.06 and 0.03 U ml^{-1}, respectively, brought about a color reduction of TEB by 23, 17, 9 and 5%, respectively within 72 h.

As TEA was not significantly decolorized by the culture supernatants alone of all the fungi, effect of low molecular weight mediators to enhance decolorization was tested. Although decolorization of TEA used at 10% concentration did occur, no clear effect of mediators was observed. Therefore, TEA was finally used at 1% in combination with various mediators.

Table 6 Response of fungi in the presence of TEB added at 20, 50 and 90% to 6-day old cultures (in situ)

Parameters measured	Conc. of the effluent (%)	NIOCC #2a *Cerrena unicolor*	NIOCC #15V *Coriolopsis byrsina*	NIOCC #16V *Diaporthe* sp.	NIOCC # C3 *Pestalotiopsis* sp.	Control[b]
Biomass (g l^{-1})	Control[a]	3.6 ± 0.2	4.1 ± 0.5	3.7 ± 0.9	5.1 ± 0.3	–
	20	3.9 ± 0.2	3.7 ± 0.1	6.1 ± 0.9	4.4 ± 0.1	
	50	6.9 ± 1.3	4.3 ± 0.5	9.0 ± 1.6	7.5 ± 0.8	
	90	3.1 ± 0.1	4.2 ± 0.2	9.4 ± 2.4	6.7 ± 1.6	
Laccase (U l^{-1})	Control[a]	2,015 ± 615.8	1,951 ± 74.5	52 ± 12.9	39 ± 8.1	–
	20	5,015 ± 519.7	4,165 ± 95.1	406 ± 69.8	105 ± 13.0	
	50	534 ± 24.9	145 ± 20.3	159 ± 24.7	30 ± 3.0	
	90	855 ± 61.9	284 ± 85.7	146 ± 20.5	191 ± 69.5	
% Decolorization	20	76.4 ± 0.9	61.5 ± 8.7	75.1 ± 4.6	79.3 ± 1.7	–
	50	42.8 ± 1.6	32.4 ± 3.7	53.5 ± 4.8	58.1 ± 1.3	
	90	38.6 ± 3.0	38.8 ± 4.7	59.7 ± 5.3	67.9 ± 2.5	
24 h-LC_{50}	20	57.9 ± 14.4	38.2 ± 9.9	39.6 ± 9.5	53.3 ± 3.7	19.8 ± 2.7
	50	13.5 ± 3.4	13.7 ± 2.9	8.4 ± 0.8	8.5 ± 1.0	6.5 ± 0.8
	90	7.9 ± 1.3	9.5 ± 2.7	10.6 ± 2.5	7.5 ± 0.9	3.8 ± 0.4
% Reduction in total phenolics	20	68.5 ± 5.3	52.9 ± 6.6	70.6 ± 4.8	76.5 ± 9.2	–
	50	82 ± 4.1	70 ± 15.3	87 ± 5.9	82 ± 2.3	
	90	83 ± 2.4	67 ± 11.2	79 ± 9.4	71 ± 5.9	
% Reduction in COD	20	43.7 ± 7.0	52.3 ± 7.6	73.3 ± 0.6	98 ± 2.1	–
	50	46 ± 1.6	37 ± 8.8	90 ± 1.3	84 ± 2.3	
	90	48 ± 1.2	55 ± 15.2	72 ± 6.9	86 ± 10.3	

All the parameters were measured on day 6 after addition of the effluent

[a] Without any effluent

[b] Only TEB in the medium without any culture

The mediators HBT, vanillic acid and acetosyringone were effective in enhancing the decolorization efficiency of the basidiomycetes #2a and #15V (Fig. 5). Decolorization by the ascomycete #16V was comparatively lesser than basidiomycetes. However, it was enhanced by all the three mediators whereas none of the mediators were effective in enhancing the decolorization efficiency of the ascomycete #C3. These studies indicated that the mediators were more efficient in decolorization of both the effluents in the presence of culture supernatants from basidiomycetes than the ascomycetes.

Culture supernatants from laccase-hyper-producing isolate #2a and commercial laccase preparation of *Tremetes versicolor*, at varying concentrations of laccase (0.5–20 U ml^{-1}) were effective in decolorization of 20% TEA and TEB. Decolorization correlated with laccase concentrations in these studies (Table 7). Polymerization, as indicated by reduction in percentage decolorization with longer incubation period was noticed to occur occasionally in TEA treated with both the laccase preparations.

Reusing fungal biomass in decolorization

Reuse of fungal biomass in decolorization of TEA was not effective whereas decolorization of TEB up to 3 cycles was effective with the basidiomycetes #2a and #15V. In the ascomycetes #16V and #C3, a 50% reduction in decolorization efficiency was observed in the second cycle itself (Fig. 6). However, color removal in the first cycle was comparable with basidiomycetes.

Decolorization of the residual color from the fungal biomass

The culture supernatants of #C3 grown in the presence of TEA or TEB were removed and in the second step the fungal biomass was incubated with

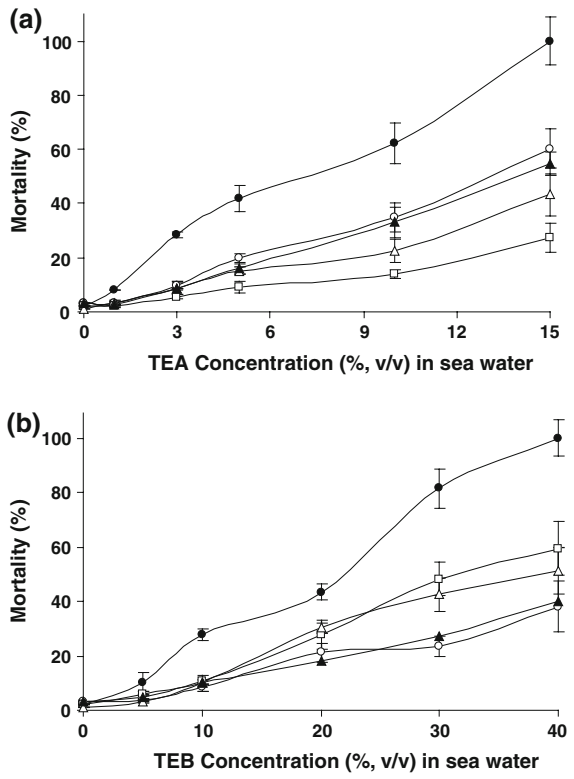


Fig. 2 (**a**) Mortality percentage of *Artemia salina* growing in the presence of untreated TEA (*filled circle*), and treated with #2a (*open circle*), #C3 (*filled triangle*), #16V (*open triangle*) and #15V (*open square*). (**b**) Untreated TEB (*filled circle*), and treated with #15V (*open square*), #16V (*open triangle*), #C3 (*filled triangle*) and #2a (*open circle*)

100 U of laccase from #2a for 48 h. The residual color of TEA and TEB from the fungal biomass was removed by 49 and 84%, respectively. The respective controls, incubated with distilled water did not show any decolorization. These results indicated that bioadsorbed effluent can also be decolorized using laccase from a basidiomycete.

A process for enhanced decolorization

Based on the above results, sorption capacity of the ascomycete #C3 and efficiency of laccase from the basidiomycete #2a were coupled together to enhance decolorization process. This was carried out by incubating pre-grown fungal biomass of #C3 with 100 U laccase from #2a and 20% TEA or TEB. No decolorization was noticed in the control treatment where the fungal biomass was incubated with distilled water. A total of 52% decolorization of TEA and 93% of TEB occurred from the biomass and the supernatant together within 48 h. It was noticed that by this process, decolorization of the effluent in the liquid phase and the solid phase occurred simultaneously.

Discussion

Generally each kind of effluent is decolorized by a specific fungus (Faraco et al. 2009) or a consortium (Senan and Abraham 2004). We are reporting decolorization of two raw textile effluents with differing dye composition, salt content and pH values by four of the marine fungi, two each belonging to ascomycetes and basidiomycetes.

Most of the industrial effluents contain various inorganic chemicals such as sulfides, sulfates, chlorides and carbonates (Bartlett 1971) and such effluents with high salt contents are required to be diluted several fold for any biotreatment. In the present study, high concentrations of effluents (20–90%) could be decolorized, thus minimizing dilutions. The fungi used in our studies showed growth, laccase production and decolorization in media prepared with seawater of 15–17 ppt salinity. Decolorization and detoxification in seawater medium indicate that they can be used for effluents containing high salt content and varying pH. A few fungi belonging to the class zygomycetes have been demonstrated to decolorize and detoxify simulated textile wastewaters of varying composition characterized by high concentrations of salts and dyes by bioadsorption (Prigione et al. 2008a, b). Bioremediation by dye sorption has been reported in *Aspergillus* spp. (Fu and Viraraghavan 2002; Corso and de Almeida 2009) and by laccase and other lignin-degrading enzymes in basidiomycetes (Wesenberg et al. 2002; Faraco et al. 2009).

Dye decolorization is dependent on their structure, pH, concentration of dyes and enzyme (Wong and Yu 1999). Anthraquinonic dyes that are substrates for laccase are easily degraded whereas non-substrates like azo and indigo dyes are removed to a lesser extent. In the present studies, decolorization of TEB which contained a mixture of reactive dyes was much higher than TEA which contained only one azo dye. Azo dyes are usually difficult to remove in wastewater (Riu et al. 1997). However, a number of white-rot fungi have been reported to breakdown individual azo

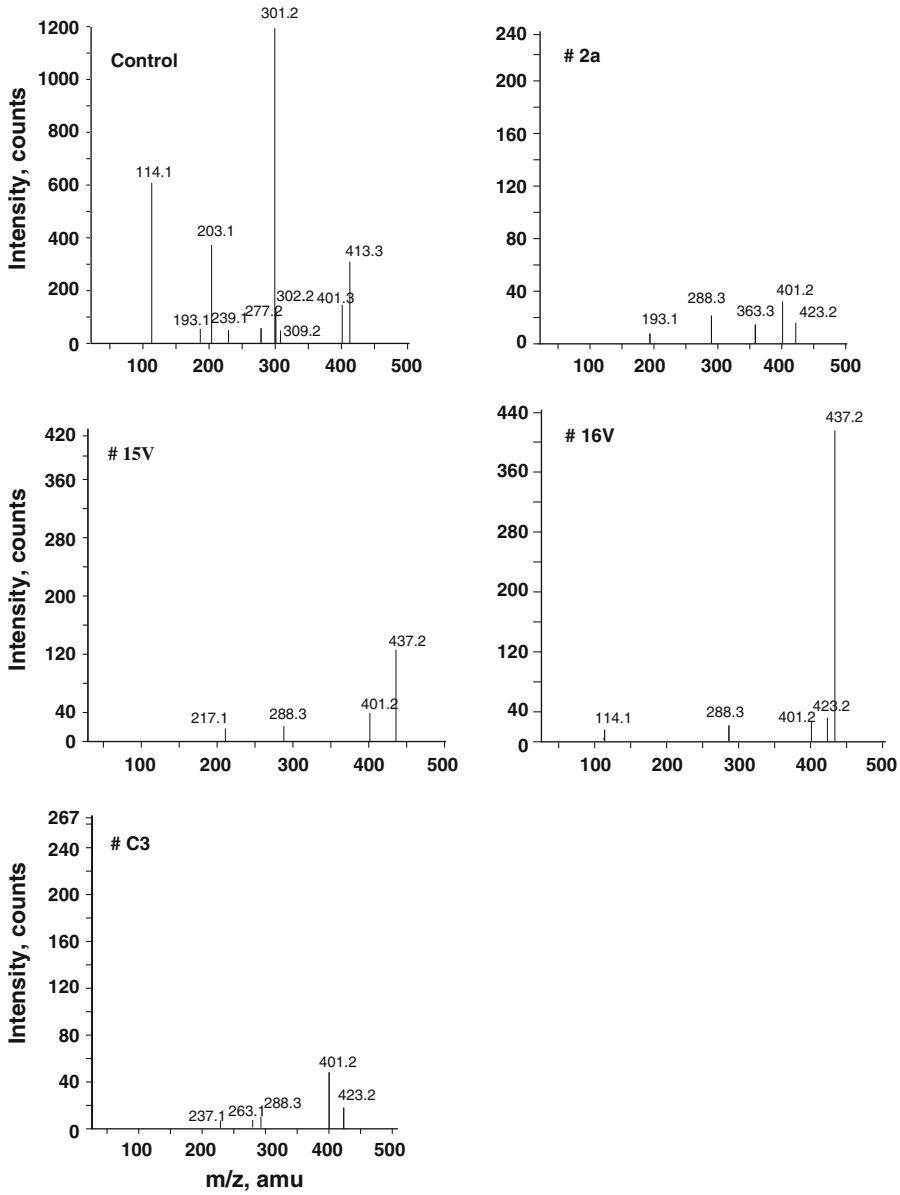


Fig. 3 Electronspray ionization mass spectra with characteristic ions related to specific low mass ions were recorded at 0–1,000 m/z in positive mode. Mass spectra of untreated (control) and fungal-treated (in situ) TEA. Note the difference in intensity counts (*Y* axis) in various cultures and the control

dyes (Nyanhongo et al. 2002). Wesenberg et al. (2002) have reported about 22% color removal of textile effluent (used at 25% concentration) containing azo dyes by day 9 with a white-rot fungus, *Clitocybula dusenii*. Our cultures removed 27–57% of color from 50% TEA containing azo dye-20 after 6 days. Altering the pH of TEA from alkaline to acidic did not increase decolorization indicating that pH alone was not the limiting factor.

Physical adsorption and enzymatic degradation are the mechanisms for color removal by fungi (Ali et al. 2008). In many cases, adsorption of dye to the fungal surface is the primary mechanism of decolorization (Zümriye and Karabayir 2008; Prigione et al. 2008a, b). In the present study, adsorption of color from TEA and TEB by fungal biomass was two to threefold higher in the ascomycetes than in the basidiomycetes. Initially higher color removal by fungal biomass was noticed in ascomycetes (NIOCC # 16V and # C3) than in basidiomycetes indicating adsorption as the primary mechanism (see Figs. 1d, 6).

Enzymatic degradation plays a primary role in biodegradation of colored effluents in basidiomycetes (Wesenberg et al. 2003; Faraco et al. 2009). The

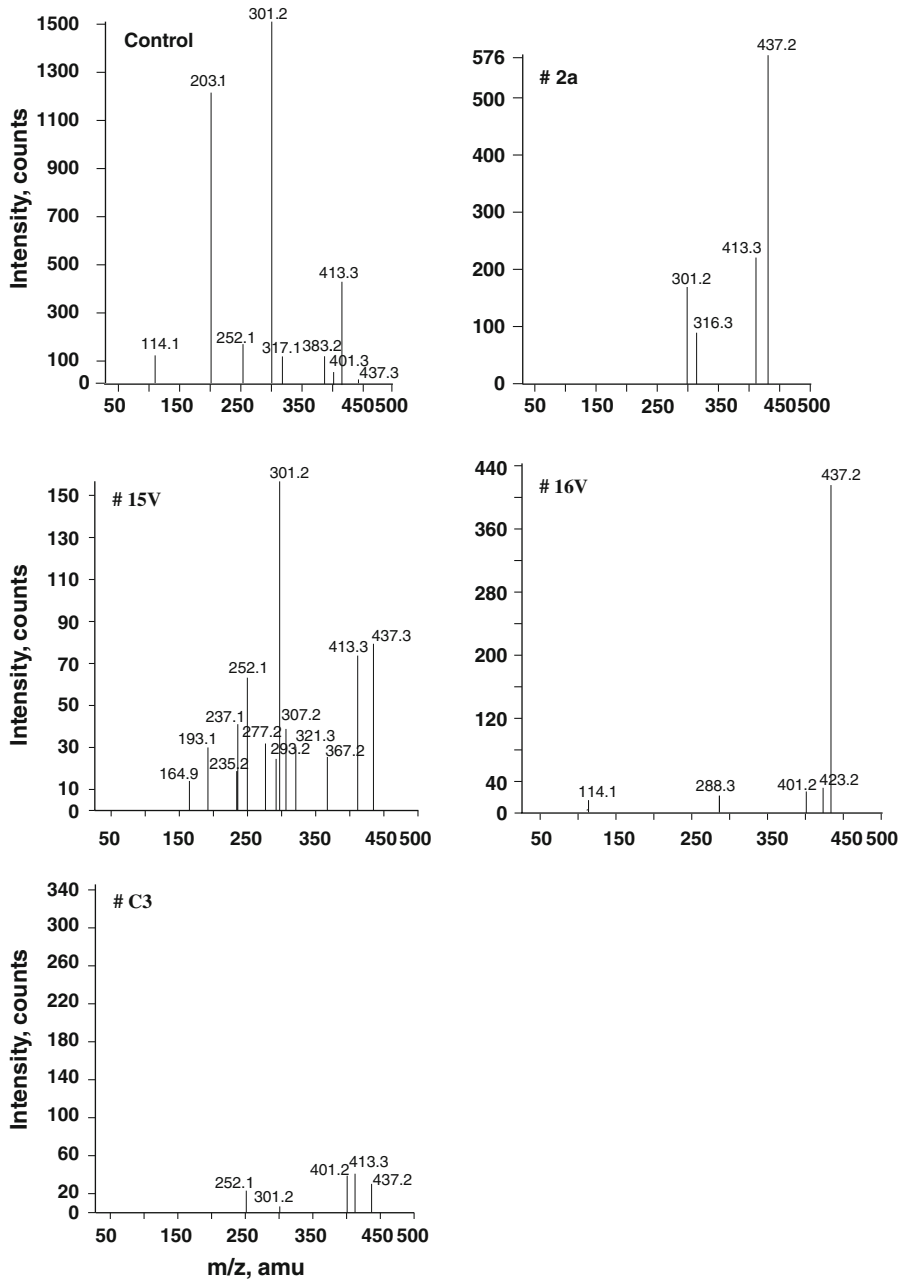


Fig. 4 Electronspray ionization mass spectra with characteristic ions related to specific low mass ions were recorded at 0–1,000 m/z in positive mode. Mass spectra of untreated (control) and fungal-treated (in situ) TEB. Note the difference in intensity counts (*Y* axis) in various cultures and the control

following points suggest that decolorization of dye-containing effluents in the basidiomycetes appeared to be primarily laccase-mediated. (1) Decolorization of TEA by the culture supernatants (*ex situ*) increased in the presence of laccase mediators in the basidiomycetes NIOCC #2a and #15V whereas in the ascomycetes #16V and #C3 with lower laccase activity, this effect was not evident (see Fig. 5). (2) *Ex situ* experiment further demonstrated that laccase from NIOCC #2a was as efficient as the commercial laccase from *Trametes versicolor* in decolorization of TEA and TEB (see Table 7). (3) Increased amounts of laccase from NIOCC #2a and commercial laccase from *Trametes versicolor,* showed corresponding increase in decolorization of both the effluents (see Table 7). (4) Reusing the fungal biomass from NIOCC #2a and #15V (basidiomycetes) showed decolorization in three subsequent cycles indicating

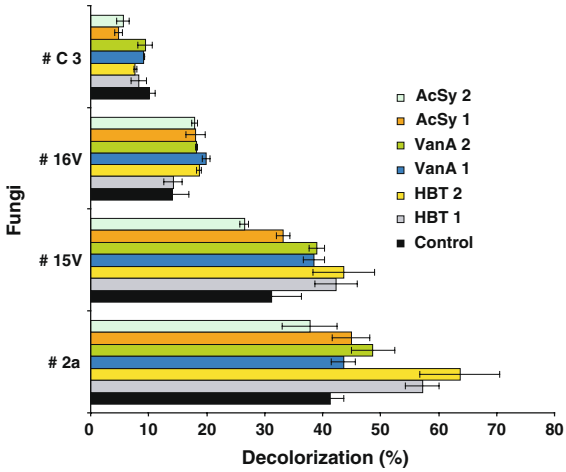


Fig. 5 Effect of mediators on decolorization of TEA (1% concentration) after 72 h incubation with culture supernatants of four fungi at 30°C. Control is without any mediators, 1 = 50 μM and 2 = 500 μM. *AcSy* acetosyringone, *VanA* vanillic acid, *HBT* 1-hydroxy benzotriazole

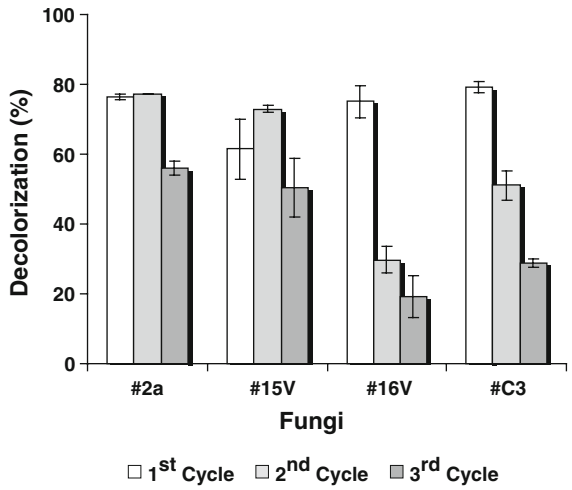


Fig. 6 Decolorization of TEB by reusing the fungal biomass in three cycles

involvement of laccase whereas with ascomycetes with much lower laccase titer showed a substantial reduction in decolorizing capacity in the second cycle itself (see Fig. 6). (5) Laccase production in the presence of TEB was high and it also got decolorized to a greater extent than TEA. Faraco et al. (2009) reported laccase to be solely responsible for decolorization of model dye-containing industrial wastewaters by *Pleurotus ostreatus*. Our studies also indicate that laccase appears to be involved in decolorization of textile effluents by basidiomycetes.

A direct correlation between lignin-degrading enzyme production and industrial effluent decolorization was reported by Wesenberg et al. (2002). By *ex situ* studies Wong and Yu (1999) demonstrated that increased decolorization capacity of laccase from *Trametes versicolor* involved decolorization of non-substrate dyes in effluents via substrate dyes that act as mediators. *Ex situ* experiments established a linear relationship (r value ≥ 0.8) between the laccase titer and decolorization in our studies. On the other hand, studies involving the whole cultures (in situ) did not show direct correlation between laccase titer and extent of decolorization consistently due to the

Table 7 Decolorization of TEA and TEB (20%) *ex situ* by laccase from NIOCC #2a and commercial laccase from *Trametes versicolor*

Laccase (U ml^{-1})	Decolorization (%)							
	TEA (20%)				TEB (20%)			
	NIOCC #2a (*C. unicolor*)		*T. versicolor*		NIOCC #2a (*C. unicolor*)		*T. versicolor*	
	12 (h)	36 (h)	12 (h)	36 (h)	12 (h)	36 (h)	12 (h)	36 (h)
0.5	0	0	3 ± 0.9	4 ± 1.1	10 ± 0.9	13 ± 1.4	3 ± 0.2	7 ± 2.1
2.5	14 ± 2.3	18 ± 3.8	13 ± 1.6	11 ± 0.8	12 ± 3.8	16 ± 3.3	13 ± 2.8	19 ± 0.7
5.0	26 ± 1.9	21 ± 4.9	12 ± 2.2	16 ± 2.9	15 ± 0.8	28 ± 2.7	15 ± 1.9	27 ± 0.8
10.0	33 ± 3.7	29 ± 5.6	30 ± 5.7	29 ± 2.8	39 ± 3.6	42 ± 2.7	17 ± 5.5	51 ± 2.2
20.0	39 ± 7.3	39 ± 4.7	25 ± 6.5	22 ± 6.6	43 ± 6.4	52 ± 5.9	29 ± 3.3	60 ± 9.7

Effluents were diluted with 0.1 M sodium acetate buffer (pH 5.0) to 20% final concentration and incubated with different concentrations of laccase from NIOCC #2a (*Cerrena unicolor*) or *Trametes versicolor* (Sigma Chemicals, USA). Absorbance spectra of TEA and TEB from 360 to 800 nm were acquired and percentage decolorization was calculated by the difference in spectral area from those of 0 h samples

involvement of several other factors such as nature of effluent, pH, adsorption capacity of the fungi, presence of natural mediators in the culture supernatants and presence of other oxidative enzymes which may trigger cascade reactions. Only NIOCC #2a produced MnP in low quantity. However, all the cultures showed glucose oxidase activity to a varying degree in the culture medium. Glucose oxidase may play role in generating H_2O_2 which by Fenton type of reaction can produce highly reactive hydroxyl radicals (Henriksson et al. 2000). These hydroxyl radicals can participate in methoxylation/hydroxylation of many aromatic compounds in converting nonphenolic compounds to phenolic ones. These in turn are easily oxidized by laccases or peroxidases (Hilden et al. 2000). In situ decolorization in our cultures appears to be a collective action of laccase and other cascading reactions besides adsorption.

Reports of decolorization of dye-containing textile effluents using ascomycetes or hyphomycetes are very few. *Aspergillus fumigatus* XC6 is one such fungus reported to decolorize dye industry effluent although laccase production in this fungus was not reported (Jin et al. 2007). A laccase-producing ascomycete, *Pestalotiopsis* sp. (Hao et al. 2007) was reported to decolorize an azo dye and another ascomycete *Myceliophthora thermophila* was reported to decolorize several synthetic dyes by the action of laccase (Kunamneni et al. 2008) but these were not tested for decolorization of dye-containing raw effluents. We are demonstrating decolorization and detoxification of dye-containing raw textile effluents by marine ascomycetes.

Reduction in toxicity is one of the important criteria to be considered while developing a process for decolorization of dye wastewaters. We observed a substantial reduction in toxicity as evidenced by LC_{50} dosage values, total phenolics and COD in treated effluents. There was a reduction in toxicity, COD and total phenolics by different fungi when grown in the presence of 90% TEB. Mass spectrometric analyses also indicated a distinct change in the spectra of untreated and fungus-treated effluents suggesting degradation of effluent components. Laccases were shown to be responsible for reduction in toxicity and COD of model textile effluents by *Pleurotus ostreatus* (Faraco et al. 2009). In the present study, both ascomycetes and basidiomycetes were able to reduce toxicity irrespective of laccase titer.

Although bioadsorption is an efficient method of color removal from effluents (Prigione et al. 2008a, b), as observed in our present study also, it only transfers color from liquid phase to the solid phase. Therefore, the problem of final color removal persists. Based on our studies we suggest a bioremediation process involving these two groups of fungi which include instant color removal by adsorption using ascomycetes followed by treatment with laccase from basidiomycetes to remove the adsorbed color from the fungal biomass. An added advantage in growing the ascomycetes in effluents is the reduction in COD, total phenolics and toxicity in contrast to use of inert material for adsorption (Rodríguez-Couto et al. 2009). We have also demonstrated that pre-grown ascomycetous biomass and laccase from a basidiomycete can be used simultaneously to enhance and speed up decolorization of raw textile effluents. The fungal biomass is able to decolorize and detoxify highly concentrated effluent (50–90%) and therefore the proposed method has high applicability at industrial scale. Although the cultures did not grow in plain effluents without added nitrogen and carbon source, they showed better growth in the medium containing 50 and 90% effluent than in the control medium. One of the laccases of the culture #2a has been characterized in detail and was found to be highly thermo- and alkaline-stable besides being halo- and metal tolerant (D'souza-Ticlo et al. 2009). These points further favor their use on an industrial scale.

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