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Efficient azoic dye degradation by *Trametes trogii* and a novel strategy to evaluate products released

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ABSTRACT

Culture filtrates from the white rot fungus *Trametes trogii* were used for the degradation of two azo dyes: Xylidine and Methyl Orange. These culture filtrates showed a great potential for decolorizing even high concentrations of both azo dyes, without the addition of redox mediators, possibly due to the synergistic action of their high contents of laccase (104 U ml^{-1}), accompanied by Mn-peroxidase (0.54 U ml^{-1}), cellobiose dehydrogenase (0.45 U ml^{-1}) and glioxal oxidase (0.38 U ml^{-1}) activities. 75% of Methyl Orange (980 ppm) and 96% of Xylidine (480 ppm) were degraded after 24 h, degradation occurred after 6 days with a 94% of Xylidine removed (1440 ppm). Biodegradation products from Xylidine were determined by capillary electrophoresis followed by MALDI-TOF mass spectra. Neither aromatic amines nor colored quinones were detected, major metabolites being naphtalene sulphonate and xylene.

1. Introduction

Azo dyes are the largest group of colorants used in industry for color printing, textile dyeing, etc. This chemical class of dyes, which is characterized by the presence of at least one azo bond (−N═N−) bearing aromatic rings, dominates the worldwide market of dyestuffs with a share of about 70% (Rodríguez Couto, 2007). However, the production and use of azo dyes result in environmental pollution due to the color visibility and the toxicity of certain dyes. They are designed to convey high photolytic stability and resistance towards major oxidizing agents (Zhao et al., 2011). The recalcitrance of azo dyes has been attributed to the presence of sulfonate groups and azo bonds, two features generally considered as xenobiotic (Rodríguez Couto, 2007). Various methods such as adsorption, biodegradation, photocatalytic and Fenton degradation can be used for the treatment of dyes (Zhao et al., 2011). The main disadvantages of these methods include high cost, secondary pollution or low efficiency, etc. Several combined anaerobic and aerobic microbial treatments have been suggested to enhance the degradation of azo dyes. However, under anaerobic conditions, azo reductases usually cleave azo dyes into the corresponding amines, many of which are mutagenic and/or carcinogenic. Furthermore, azo reductases have been shown to be very specific enzymes, thus cleaving only azo bonds of selected dyes (Rodríguez Couto, 2007). Therefore, the need of unspecific processes for the effective treatment of wastewater containing such dyes arises.

By far, the most efficient single class microorganism in breaking down synthetic dyes is white rot fungi (Wesenberg et al., 2003). The nonspecific nature of the lignin-degrading systems of white rot fungi composed mainly by laccase, Mn-peroxidase and lignin-peroxidase, is an advantage for the biotreatment of textile effluents, since a mixture of dyes, surfactants and other compounds exist in wastewater. Another important advantage for degradation of azo dyes using white rot fungi is that ligninolytic enzymes degrade azo dyes by oxidation, in contrast to the reduction pathway in bacterial decolorization. Thus, the hazardous aromatic amines, which usually generate from reduction of azo dyes, are not produced during fungal degradation (Zhao et al., 2007). Since Cripps et al. (1990) showed that three azo dyes were extensively degraded by the white rot fungus *Phanerochaete chrysosporium* under aerobic conditions, the possibility of using white rot fungi in the decolorization of different azo dyes in the wastewater has been intensively studied (Bumpus, 2004). Considering the high volume of effluents generated from dyeing units, the use of purified and/or

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immobilized enzymes increases the cost of detoxification processes. As the use of whole fungal cultures for decolorization is not easily applicable in a large scale, the studies of *in vitro* decolorization of synthetic dyes by crude enzymes have currently become more important (Kokol et al., 2007). Crude culture filtrates offer several advantages, their production process is inexpensive, they may include natural laccase-mediators secreted by the fungus (Johannes and Majcherczyk, 2000) and in addition, proteins, residual macronutrients, soluble extracellular metabolites or other factors present in the medium may stabilize crude enzymes (Papinutti et al., 2008). Moreover, to target a wider range of chemical structures in waste effluents, the use of crude culture filtrates containing more than one ligninolytic enzyme would be advantageous, since each one may attack different chemical structures.

The degradation of azo dyes has been extensively studied using a wide range of fungi and bacteria whereas work carried out using enzymes is more limited (Rodríguez Couto, 2007). Furthermore, knowledge of mechanisms and metabolites of fungal degradation of dyes is lacking (Zhao et al., 2007). Thus, effective analytical techniques are needed to identify the metabolites of azo dyes and understand their biodegradation mechanism. The combination of capillary electrophoresis (CE) and MALDI-TOF mass spectra, which employs small sample quantities, represents a good alternative to identify the metabolic degradation pathway of these azoic dyes.

In the present study, the ability to decolorize two azo dyes by crude culture filtrates from *Trametes trogii* with outstanding laccase (*p*-diphenol:dioxygen oxidoreductases; EC 1.10.3.2) activity, was assessed. Laccase from *T. trogii* was reported to have one of the highest redox potential among laccases (E° 0.79 at pH 5.0) (Garzillo et al., 2001), which makes this laccase particularly interesting since high redox potentials correlate with high laccase activity (Li et al., 1999). *T. trogii* is a worldwide distributed white-rot basidiomycete. It demonstrated to be a good laccases and other ligninolytic enzymes producer including LiP and MnP (Garzillo et al., 2001; Levin and Forchiassin, 2001; Levin et al., 2005; Zouari-Mechichi et al., 2006; Ciullini et al., 2008). Strain BAFC 463 of this fungus also proved to be an efficient tool for the degradation of several organic pollutants including nitrobenzene and anthracene, a combination of polychlorinated biphenyls (PCBs) (Aroclor 1150) and an industrial mixture of polycyclic aromatic hydrocarbons (PAHs) (Haglund et al., 2002; Levin et al., 2003). The ability of *T. trogii* to decolorize industrial dyes was confirmed by several studies (Yesilada et al., 2002; Deveci et al., 2004; Levin et al., 2005, 2010; Zouari-Mechichi et al., 2006; Ciullini et al., 2008; Grassi et al., 2011; Zeng et al., 2011). The potential of synthetic dye decolorization by purified laccases has been described in several species. However, most dyes are only transformed in the presence of redox mediators (Baldrian, 2004). Recently, *T. trogii* BAFC 463 culture fluids, or its purified laccase proved to be capable of decolorizing three azoic dyes in the absence of redox mediators: Congo Red, Xylidine and Fast Blue RR (40, 12.5 and 8% at half hour respectively; representing decolorization rates of 46.9, 5.6 and 12.4 mg l^{-1} h^{-1}). More than 90% of the Xylidine was degraded by the purified laccase after 1 day. Culture fluids even showed efficient decolorization at 70 °C and pH 7.0. Thus, *T. trogii* culture fluids could be effectively used to decolorize azo dyes (Grassi et al., 2011).

The main objectives of our work were: (i) to assess the ability of *T. trogii* culture fluids to decolorize high concentrations of two azo dyes: Xylidine (Ponceau 2R, Acid Red 26 C.I. 16150) and Methyl Orange (Acid Orange 52, C.I. 13025) (ii) to evaluate Xylidine degradation products by a novel strategy that involves small sample quantities.

2. Materials and methods

2.1. Microorganism

Strain BAFC 463 (MYA 28-11) of *T. trogii* (*Funalia trogii*) (Berk. in Trog.) (Polyporaceae, Aphyllophorales, Basidiomycetes) -an Argentinean strain isolated in Entre Rios province from decayed willow (*Salix humboldtiana*) wood – was used in these experiments. This strain was obtained from the Mycological Herbarium at the University of Buenos Aires (BAFC), and has been the subject of previous published papers (Levin and Forchiassin, 2001; Haglund et al., 2002; Levin et al., 2003, 2005, 2010; Grassi et al., 2011). Stock cultures were maintained on malt extract agar slants at 4 °C.

2.2. Culture media

Basal culture medium (GA) contained glucose, 20 g; L-asparagine monohydrate, 3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.6 g; $MnCl_2 \cdot 4H_2O$, 0.09 mg; H_3BO_3, 0.07 mg; $Na_2MoO_4 \cdot H_2O$, 0.02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 3.5 mg; thiamine hydrochloride, 0.1 mg; distilled water up to 1 l, supplemented with 1 mM copper sulfate. The initial pH of the medium was adjusted to 6.5 with NaOH 1 N.

2.3. Culture conditions

Incubation was carried out statically at 28 ± 1 °C in 500 ml Erlenmeyer flasks with 50 ml of medium, which were inoculated with four 25-mm2 surface agar plugs from a 7-d-old colony grown on Bacto-agar 2%. Cultures were harvested at day 22, filtered through a filter paper using a Büchner funnel, and dried overnight at 70 °C. Culture supernatants were used as enzyme sources. All chemicals were of analytical grade and were used without further purification.

2.4. Enzymatic determinations

Laccase activity was measured with 2,2-azinobis (3-ethyl benzthiazoline-6-sulphonic acid) (ABTS) in 0.1 M sodium acetate buffer (pH 3.5) at 30 °C. Oxidation of ABTS was determined by the increase in A_{420} (ε_{420} = 36,000 M^{-1} cm^{-1}) (Bourbonnais et al., 1995). MnP activity was determined using phenol red as the substrate in 0.05 M sodium succinate buffer (pH 4.5) at 30 °C (ε_{610} = 22000 M^{-1} cm^{-1}) (Glenn et al., 1986). Glyoxal oxidase (GLOX) activity was determined by using a peroxidase-coupled assay with methylglyoxal as GLOX substrate and phenol red as the peroxidase substrate (Kersten, 1990). International enzymatic units (U) were used (μmol product min^{-1}). Enzyme activity was expressed as U ml^{-1} of culture filtrate. Cellobiose dehydrogenase activity (CDH) was assayed by following the decrease in absorbance of an electron acceptor, 2,6-dichlorophenolindophenol (DCPIP) at 600 nm (ε_{600} = 18,500 M^{-1} cm^{-1}) according to a modified method based on Sadana and Patil (1988) and Baminger et al. (1999). The reaction mixture (in a total volume of 1 ml) consisted of DCPIP (0.05 ml, 2 mM in 10 mM phosphate buffer, pH 6.0), cellobiose (0.85 ml, 2.5 mM in the same buffer), sodium fluoride (0.05 ml, 4 mM in the same buffer as an inhibitor of laccases) and 0.05 ml of the culture filtrates. Heat-denatured culture filtrates served as controls. The reaction was started by addition of the enzyme and the decrease in 600 nm absorbance was monitored during 1 min at 37 °C. One unit of CDH activity is defined as the amount of enzyme reducing 1 μmol DCPIP min^{-1} ml^{-1} under the assay conditions.

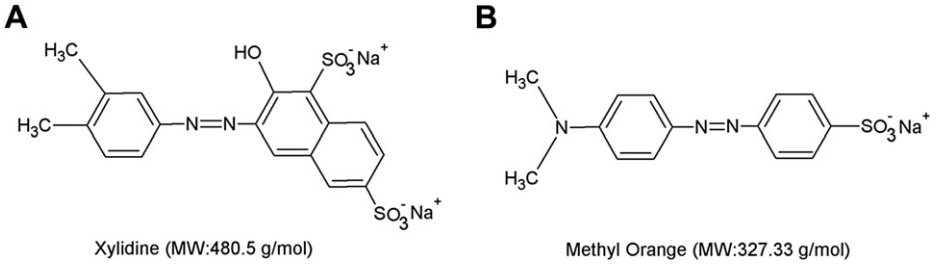


Fig. 1. Xylidine (a) Methyl Orange (b).

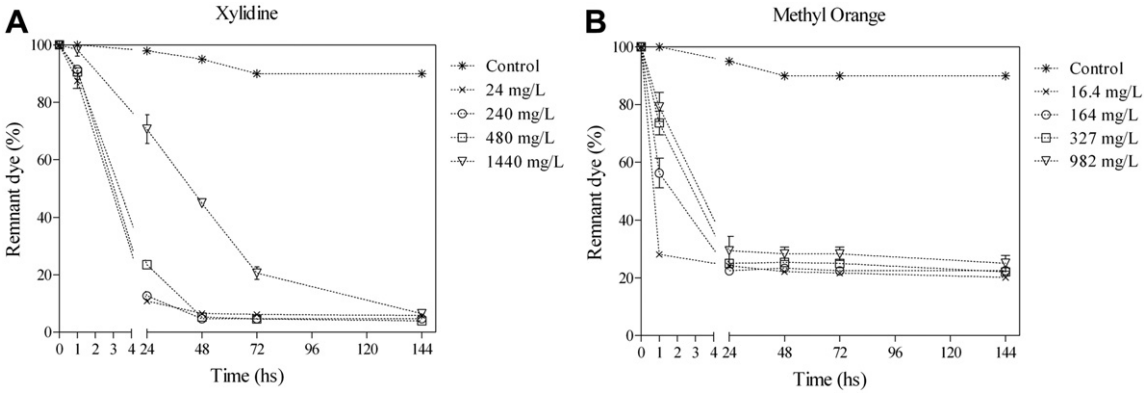


Fig. 2. A, **B**. Effect of dye concentration on decolorization attained with *T. trogii* culture filtrates. (**A**) Xylidine 24 up to 1440 mg l^{-1}; (**B**) Methyl Orange 16.4 up to 982 mg l^{-1}. Control: azo dyes treated with heat-inactivated culture filtrates. The data represent averages and error bars indicate SD.

2.5. In vitro decolorization of dyes by culture fluids

Decolorization activity was determined by measuring the decrease of dye absorbance at its maximum visible wavelength. A HP8452 diode array spectrophotometer and a quartz crystal cell were used to obtain the UV spectra. The reaction was carried out in test tubes at 30 °C, the reaction mixture contained sodium acetate buffer (10 mM, pH 4.5), 500 μl of crude filtrate (laccase activity 104 U ml^{-1}; MnP activity 0.54 U ml^{-1}) and an aliquot of the dye (Xylidine or Methyl Orange), in a total volume of 3 ml. Different concentrations of both dyes up to 3 mM were assayed (Xylidine: 24, 240, 480 and 1440 mg l^{-1}; and Methyl Orange: 16.4, 164, 327 and 982 mg l^{-1}). Samples were diluted in order to obtain 1.0 absorbance units at their maximum wavelength in the visible spectrum, respectively 505 and 460 nm. Controls where culture fluids were replaced by distilled water or heat-denatured supernatants were conducted in parallel. Decolorization was calculated by measuring the decrease in absorbance maximum according to the following expression: remnant dye (%) = $(A/A_0) \times 100$, where A_0 was the initial absorbance and A was the absorbance measured at 0, 1, 24, 48, 72 and 144 h. Results are expressed in terms of percentage (Levin et al., 2010). For laccase stability assays, samples of the decolorization reaction mixtures of both dyes were collected and residual activities were determined during 144 h. The data presented are mean values of triplicate assays with a SD within 10% of the mean.

2.6. Capillary electrophoresis-MALDI-TOF mass spectra

Separations were performed in a P/ACE MDQ (Beckman Coulter, Brea, CA, USA), equipped with a UV–vis photodiode array detector. Data were processed by 32 Karat™ software (Beckman Coulter). For all experiments, the CE system temperature was held at 25 °C. In all cases, UV detection at 254 nm was performed. An untreated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm I.D. × 375 μm O.D. was used. Its total length was 62 cm and the effective length was 50 cm to the detector. The mobile phase consisted of 50 mM ammonium carbonate buffer (pH 9.5). When the running solution was changed, the capillary was rinsed with 0.1 M NaOH for 5 min, followed subsequent rinses with distilled water for 3 min and running buffer for 4 min. The concentrations of both dyes were respectively 128 mg l^{-1} and 102 mg l^{-1} for Xylidine and Methyl Orange. Aliquots were taken at different times and injected into CE. Sample solutions were introduced by 5 s at 3.5 kPa and the separation voltage was 20 kV. After each run, the capillaries were

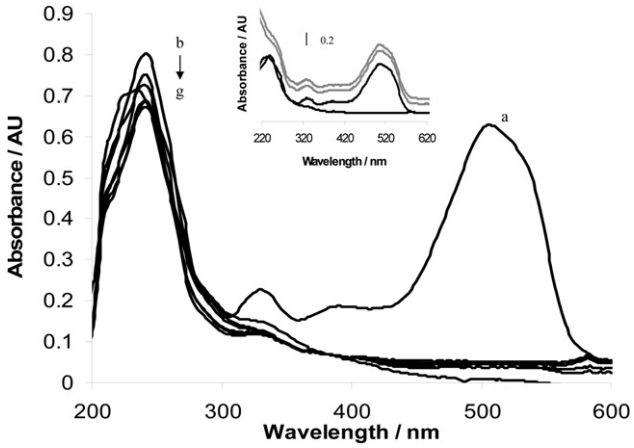


Fig. 3. UV-visible spectra of Xylidine (34.12 mg l^{-1}) treated with culture filtrates of *T. trogii* after different incubation periods (a: $t = 0$; b: $t = 5$ h; c: $t = 24$ h; d: $t = 48$ h; e: $t = 72$ h; f: $t = 168$ h; g: $t = 336$ h). **Insert**: UV–visible spectra of: non-treated Xylidine (grey) at $t = 0$ and $t = 14$ days; Xylidine treated with *T. trogii* culture filtrates (black) at $t = 0$ and $t = 14$ days.

Table 1
Decolorization of azo dyes by white rot fungi.

Strain	Decolorization mode	Dye	Initial concentration (mg l^{-1})	Percentage of decolorization/Time	Rate of decolorization (mg l^{-1} h^{-1})	References
Phanerochaete chrysosporium	Whole cultures	Methyl Orange	150	92% 15 days	0.38	(Pasti-Grigsby et al., 1992)
			300	88% 15 days	0.73	
Trametes versicolor	Whole cultures	Reactive Black 5	100	97–99% 14 cycles 200 days	0.4-0.6	(Borchert and Libra, 2001)
			500	92–99.5% 14 cycles 200 days	2–3	
Trametes versicolor	Whole cultures	Amaranth	33	100% 3.5 h	9.43	(Ramsay and Nguyen, 2002)
		Tropaeolin O	53	100% 24.8 h	2.14	
		Congo Red	30.5	100% 22 h	1.39	
		Reactive Black 5	23.5	100% 48 h	0.49	
		Cibacron Brilliant Red	48	84% 32.5 h	1.24	
		Cibacron Brilliant Yellow	34.5	69% 97 h	0.25	
Ganoderma sp. En3	Whole cultures	Methyl Orange	50	96% 72 h	0.67	(Zhuo et al., 2011)
			200	48.7% 72 h	1.35	
Trametes pubescens	Whole cultures	Reactive Red 243	200	64% 48 h	2.67	(Casieri et al., 2008)
			2000	61% 10 days	5.08	
Pleurotus ostreatus	Whole cultures	Reactive Red 243	200	50% 48 h	2.08	(Casieri et al., 2008)
			2000	44% 10 days	3.67	
Trametes versicolor	Purified laccase	Direct Red 28	20	33.4% 16 h	0.42	(Claus et al., 2002)
		Acid Orange	20	0% 16 h		
		Reactive Black 5	20	0% 16 h		
		Reactive Blue 15	20	0% 16 h		
Trametes villlosa	Purified laccase	Methyl Orange	32.73	76% 1 h	24.87	(Zille et al., 2004)
Trametes hirsuta	Crude laccase	Direct Black 168	133	67% 4 h	22.28	(Rodríguez Couto, 2007)
		Direct Blue 78	67	73% 4 h	12.23	
		DP 56L	83	24% 4 h	4.98	
		SSY 46L6	100	12% 4 h	3	
Dichomitus squalens	Purified laccase	RO 16	50	13% 2 h	3.25	(Susla et al., 2007)
Trametes sp. SQ01	Purified laccase	Orange G	100	70% 4 h	17.5	(Yang et al., 2009)
		Fast Blue RR	100	85% 4 h	21.25	
		Amido Black 10B	100	85% 4 h	21.25	
		Congo Red	100	47% 12 h	3.92	
		Acid Red	100	21% 12 h	1.75	
Ganoderna lucidum	Crude laccase plus HBT 1 mM (Without HBT no decolorization)	Remazol Black 5	50	62% 1 h	31	(Murugesan et al., 2007)
			300	2.5% 1 h	20.88	
				83.5% 12 h		
Ischnoderma resinosum	Culture filtrates	Remazol Black 5	100	15% 24 h	0.63	(Kokol et al., 2007)
		Reactive Red 22	100	10% 24 h	0.42	
		Reactive Yellow	100	8% 24 h	0.33	
Funalia trogii ATCC 200800	Whole cultures	Reactive Black 5	150	99% 21 days	0.29	(Deveci et al., 2004)
Trametes trogii SYBCLZ	Culture filtrates plus HBT 1 mM (Without HBT no decolorization)	Acid Red 1	10	90.2% 0.5 h	18.04	(Zeng et al., 2011)
		Reactive Black 5	18.3	65.4% 0.5 h	23.94	
Funalia trogii 201	Culture filtrates	Acid Yellow 49	500	23.3% 24 h	4.85	(Ciullini et al., 2008)
		Acid Red 42	500	93.4% 24 h	19.46	
		Reactive Yellow 39	500	21% 24 h	4.38	
		Reactive Red 272	500	21.4% 24 h	4.46	
		Acid Blue 158	500	99.3% 24 h	20.69	
		Acid Black 194	500	99% 24 h	20.62	
		Acid Yellow 129	80	27.5% 24 h	0.92	
		Acid Red 186	500	96.5% 24 h	20.10	
		Acid Black 1	500	95% 24 h	19.79	
		Direct Red 243	500	90.4% 24 h	18.83	
		Acid Red 374	40	100% 24 h	1.67	
		Direct Blue 71	500	21.5% 24 h	4.48	
		Direct Blue 22	500	31.3% 24 h	6.52	
Trametes trogii B6J	Purified laccase	Neolane Blue	50	91.5% 24 h	1.91	(Zouari-Mechichi et al., 2006)
		Neolane Pink	50	63.6% 24 h	1.33	
		Neolane Yellow	50	26.6% 24 h	0.55	
		Maxilon Blue	50	0% 24 h		
				15.1% with HBT in 24 h	0.31	
Trametes trogii BAFC 463	Purified laccase Laccase plus HBT 0.5 mM	Xylidine	20.5	20% 1 h	4.1	(Grassi et al., 2011)
				90% 1 h	18.09	
Trametes trogii BAFC 463	Culture filtrates	Congo Red	58.1	40% 0.5 h	46.9	(Levin et al., 2010)
		Xylidine	12.8	23% 0.5 h	5.9	
		Janus Green	76.7	69% 24 h	2.2	
Trametes trogii BAFC 463	Culture filtrates	Xylidine	480.4	76% 24 h	15.21	This work
			1441.2	28% 24 h	16.81	
		Methyl Orange	327.3	75% 24 h	10.23	
			982	74% 24 h	30.28	

washed with buffer for 5 min, and finally flushed with water at the end of the day (Takeda et al., 1999).

Positive MALDI-TOF mass spectra were recorded using an Omniflex Bruker Daltonics mass spectrometer operated in the linear mode at an accelerating voltage of 19 keV. The photomatrix solution was prepared by dissolving 10 mg ml^{-1} of 2,5 dihydroxybenzoic acid in water/acetonitrile (1:1) with 0.1% trifluoroacetic acid solution. The samples were made mixing the problem solutions with the photomatrix solution in a 1:10 ratio. One μl of the sample solution was applied to the target and dried down. Desorption/ionization was accomplished with a nitrogen UV laser (337 nm) (Calafell et al., 2007; Scodeller et al., 2010).

2.7. Results

The data presented are mean values of triplicate assays with a SD within 10% of the mean.

Results were analyzed using the STATISTICA 5.1 software (StatSoft, Tuls, Okla.).

3. Results and discussion

3.1. In vitro decolorization of dyes by culture fluids of T. trogii. Effect of increasing dye concentrations

In this paper, the decolorization of two mono-azo dyes with different structural patterns (Xylidine and Methyl Orange, Fig. 1) was investigated using crude extracellular culture extract of *T. trogii* BAFC 463. In order to examine the effect of dye concentration, the decolorization of both dyes was performed at various increasing concentrations of dye during different time intervals (Fig. 2A,B). Methyl Orange (Fig. 2B) was more easily decolorized than Xylidine (Fig. 2A). Methyl Orange decolorization increased from 0 to 24 h, when up to 75% of the dye was degraded, and from then onwards decolorization did not improve. Results were similar with all Methyl Orange concentrations evaluated (from 0.1 up to 3 mM). The efficiency of Xylidine decolorization was not significantly affected by increasing the dye up to 1 mM. Increase of Xylidine concentration over 1 mM up to 3 mM, decreased the decolorization level at the initial hours. However, after further incubation almost complete decolorization was observed. As the initial Xylidine concentration increased from 480 to 1440 mg l^{-1} the decolorization decreased from 76 to 28% after 24 h, but almost 100% of the Xylidine (up to initial concentration 1 mM) was removed after 48 h, while Xylidine 3 mM (1440 mg l^{-1}) was removed after 140 h. This suggests that the tested dye concentrations up to 1440 mg l^{-1} are not completely inhibitory to the enzyme. The dye did not affect the activity of laccase in up to 140 h of decolorization, maintaining 100% of the activity measured in the control without dye. Dye concentration affected dye degradation by white rot fungi and their ligninolytic enzymes in previous studies (Yesilada et al., 2002; Murugesan et al., 2007; Casieri et al., 2008). The difference of dye structure may result in the difference of efficiency of decolorization. It has been reported that the chemical structures of the dyes largely influence their decolorization rates with laccase and that its decolorization efficiency was limited to several azo dyes (Pasti-Grigsby et al., 1992; Chivuluka et al., 1995). A linear relationship exists between the redox potential of the azo dyes and the decolorization efficiency of laccase (Zille et al., 2004).

No significant decolorization (Fig. 2A,B) was observed after 140 h, in the experiments with heat-denatured culture filtrates and they did not show any changes on their visible spectrum along incubation time (Fig. 3 insert). Although decolorization rates increased with the addition of laccase redox mediators such as 1-hydroxybenzotriazole (HBT) (Grassi et al., 2011), these chemicals should probably be avoided as they may prove to be another pollutant in the dye wastewaters (Champagne and Ramsay, 2010).

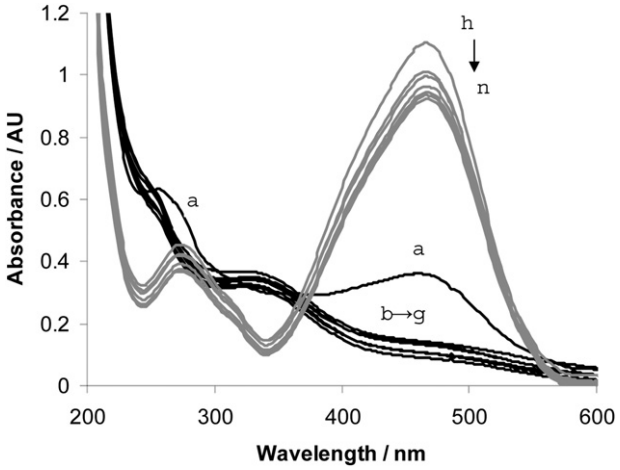


Fig. 4. UV–visible spectra of non-treated Methyl Orange (h–n, grey) and treated with culture filtrates of *T. trogii* (a-g, black) after different incubation periods (*t*: 0, 5, 24, 48, 72, 168, 336 h).

We further compared the decolorization capability of *T. trogii* culture filtrates with that of other strains reported previously (Table 1). As shown in this table *T. trogii* capacity to decolorize both azo dyes even at high concentration exceeds the previously described for many white rot fungi and their ligninolytic enzymes. Moreover the increase in dye concentrations did not affect the reaction rates. Results obtained with *T. trogii* may be attributed not only to the high titers of laccase detected in the culture fluids (104 U ml^{-1}) but also to the combined action of MnP (0.54 U ml^{-1}) and CDH (0.45 U ml^{-1}) detected in *T. trogii* BAFC 463 culture filtrates. Recently, the utilization of CDH in support of the decolorization activity of laccase for azo textile dyes, resulted in substantial increases in decolorization for all the refractory dyes (Ciullini et al., 2008).

3.2. Proposed pathway for Xylidine biodegradation

Fig. 3 illustrates the UV–visible absorbance spectrum for Xylidine treated with culture filtrates of *T. trogii* after different

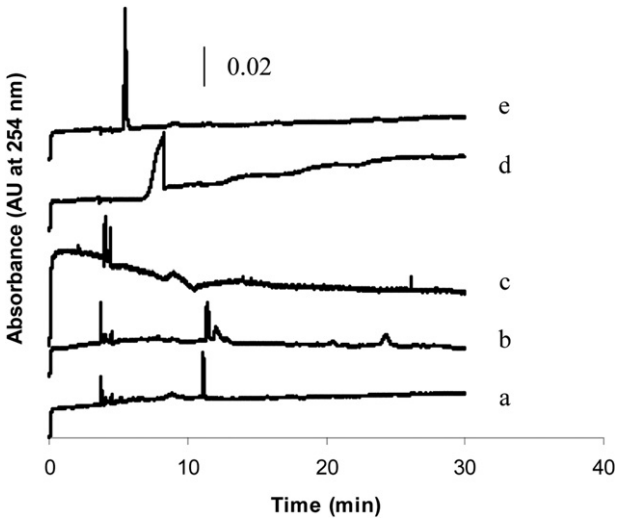


Fig. 5. Electropherograms of Methyl Orange (a: treated with *T. trogii*; e: non-treated sample) and Xylidine (b: treated with *T. trogii*; d: non-treated sample), Blank of culture filtrate of *T. trogii* (c).

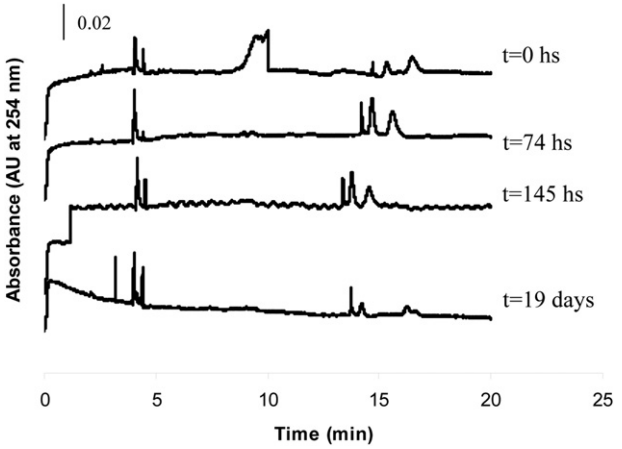


Fig. 6. Electropherograms of Xylidine treated with culture filtrates of *T. trogii* during different periods (0 up to 19 days).

incubation periods. Different structure units and groups in dye molecules have different absorbance peaks. The main conjugated structure includes an azo linkage, a benzene ring and a naphthalene ring. The chromophore containing the azo linkage has absorption in the visible region, while the benzene ring and the naphthalene ring absorb in the UV region, showing absorbance peaks at 230 and 330 nm respectively. The disappearance of the absorbance peak in the visible region (508 nm) after 5 h treatment with the culture filtrates of *T. trogii*, was associated with decolorization. A new absorbance peak appeared at about 245 nm, indicative of a new product. The active hydrogen atoms may attack the azo bonds and reduce them to hydrogenated azo bond structures, which may absorb UV-light at 245 nm (Feng et al., 2000). Nevertheless, this absorbance peak due to the reduced azo bond decreased after longer incubation periods. Thus, apparently, the role of *T. trogii* culture filtrates on Xylidine degradation does not limit to a reduction of the azo bond with loss of conjugation and consequently decolorization, but also other degradation pathways may be involved, as discussed later. The absorbance peak at 330 nm associated with the naphthalene ring showed only a very small decrease in height, thus apparently during dye degradation the naphthalene ring can not be destroyed effectively.

Fig. 4 illustrates the UV–visible absorbance spectrum for Methyl Orange treated with culture filtrates of *T. trogii* after different incubation periods. Methyl Orange showed two absorbance peaks at 464 and 278 nm, respectively. The fact that the decolorized sample did not show the corresponding absorbance peak at the visible spectra suggests decolorization. The peak at 278 nm appears after treatment at 262-254 nm. This hypsochromic shift of the spectral band accounts for the loss of conjugation in the double bonds. The Methyl orange degradation pathway has already been described (Telke et al., 2010; Mishra et al., 2011), thus it is not the subject of our work. Recently Telke et al. (2010) demonstrated that the first step in the decolorization of azo dyes using laccase of

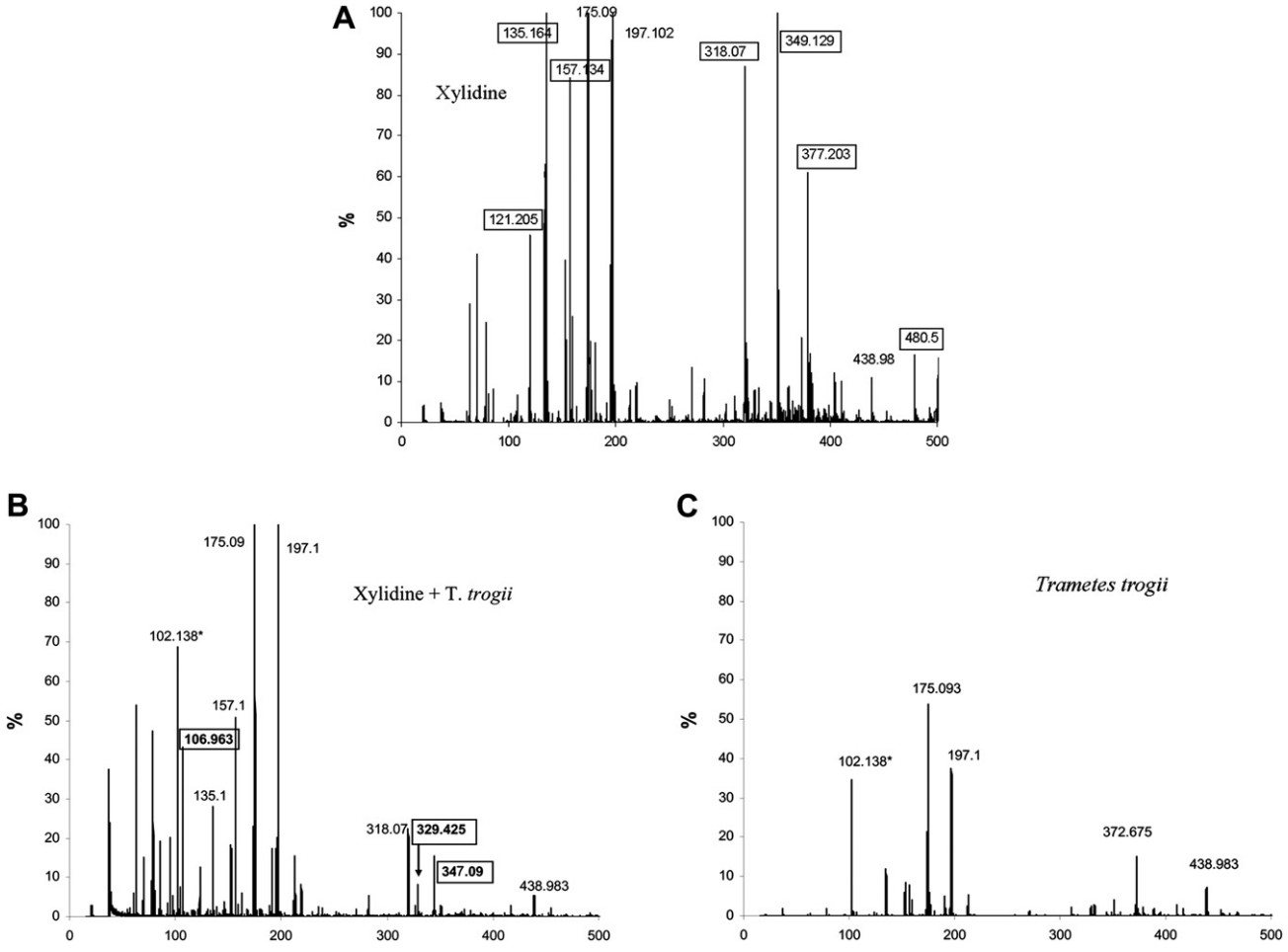
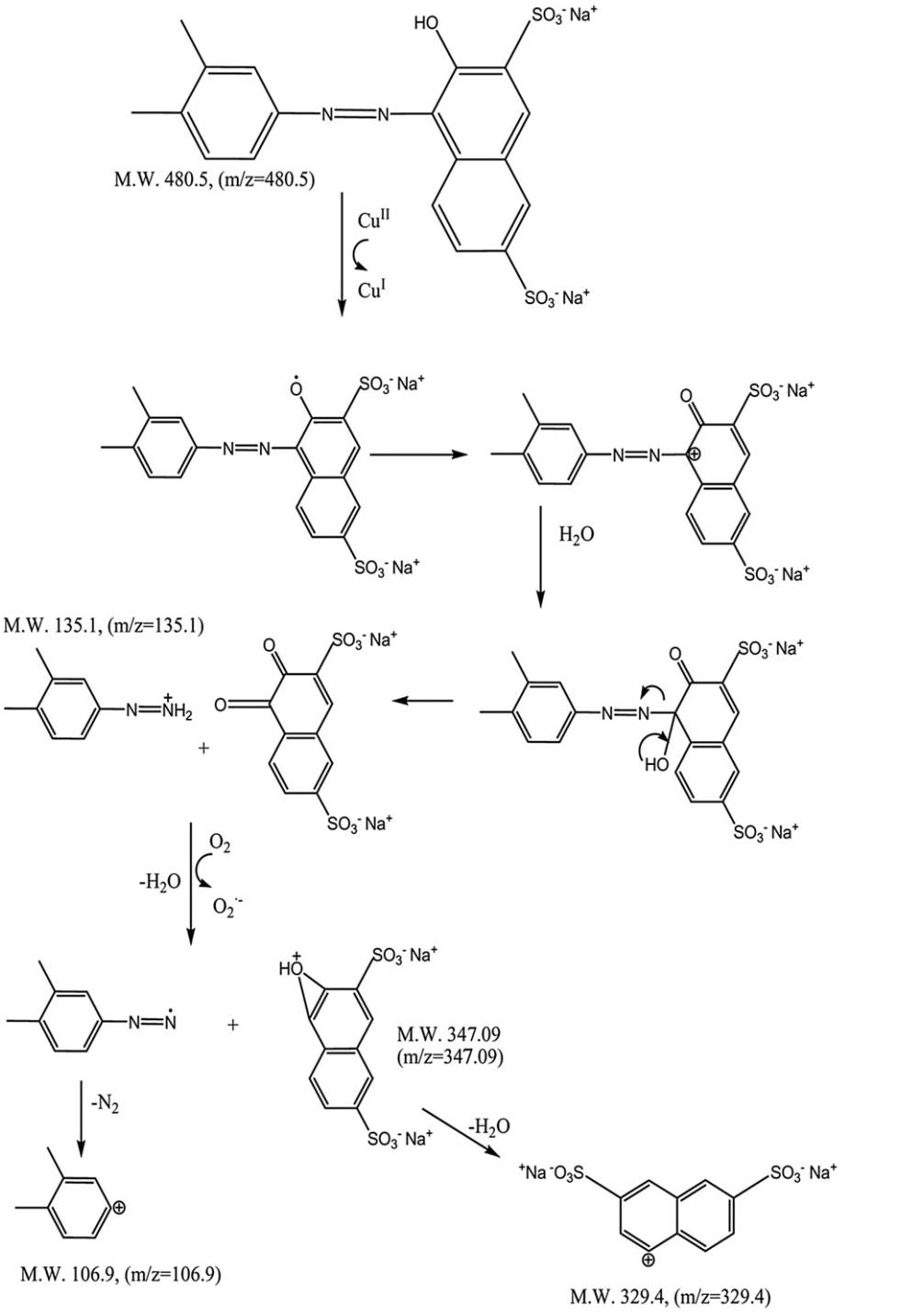


Fig. 7. A–C. MALDI-TOF Mass spectra of non-treated Xylidine (a), Xylidine treated with culture filtrates of. *T. trogii* (120 h) (b), culture filtrates of *T. trogii* (c).



Another products in the xylidine at time=0 hs

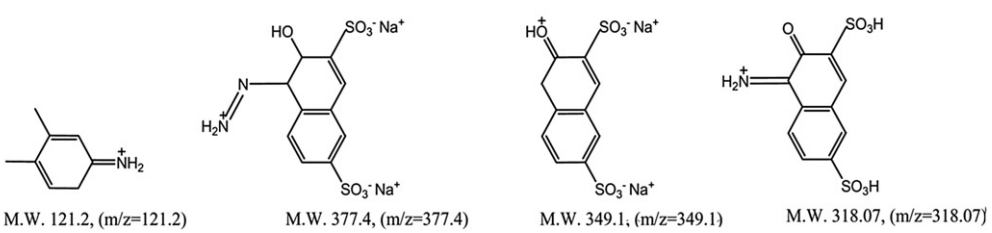


Fig. 8. Proposed degradation pathway for Xylidine.

Aspergillus ochraceus was the formation of an electron–deficient reaction center (carbocation). Carbocation created a highly reactive intermediate, which was often subject to nucleophilic attack by nucleophiles such as $-OH$, $-SO_3$ or halogen ions, resulting into asymmetric cleavage of the azo bond. The biodegradation of Methyl Orange involved asymmetric cleavage of the azo bond, resulting in the formation of p-N-N-dimethylamine phenyldiazine and p-hydroxybenzene sulfonic acid intermediates which were identified by Liquid chromatography-mass spectrometry studies. Similar findings were also reported by Mishra et al. (2011) when studying Methyl Orange biodegradation by whole cultures of a white rot fungus.

Fig. 5 shows the electropherograms of both dyes treated with *T. trogii*. When comparing the degradation profiles of both azo dyes [(Methyl Orange (a) and Xylidine (b)] it can be observed that one of the metabolites produced after *T. trogii* degradation shows the same retention time (11.2 min) in both electropherograms, suggesting that both degradation paths rendered similar products.

Fig. 6 shows the kinetic profile by CE–UV/DAD for Xylidine treated with culture filtrates of *T. trogii* during different periods (0 up to 19 days). Peaks that appeared around 3.5 min correspond to *T. trogii* culture filtrates and did not show significant changes along the incubation period. Xylidine profile (only at $t = 0$ h) appeared between 8 and 9.5 min, which almost completely disappeared by $t = 74$ h. The peaks shown at 14 min correspond to dye degradation products. After 19 days new smaller dye degradation products became visible.

On the basis of the analysis by MALDI-TOF mass spectra of the Xylidine prior to treatment (Fig. 7A), and the dye treated during 120 h with *T. trogii* culture filtrates (Fig. 7B), we propose the degradation pathway shown in Fig. 8. In the MALDI mass spectra of Xylidine potentially toxic structures appeared (m/z. 121.2, 377.4, 349.1, 318.07), which diminished significantly after the treatment with *T. trogii* culture filtrates, giving rise to the formation of other compounds that are in correspondence with the proposed degradation pathway (m/z: 106.9, 329.4, 347.09). The structure that corresponds to 3,4 dimethyl phenyldiazine ($m/z = 135.1$) decreased in intensity after the treatment with the culture filtrate, showing that the biodegradation process does not stop in the aromatic amine. On the contrary this phenyldiazine could be oxidized by molecular oxygen to the corresponding radical, which finally splits off molecular nitrogen under formation of the phenyl radical, being stabilized by the abstraction of a hydrogen radical from its surroundings. Similar results were described by Goszczynski et al. (1994), Spadaro and Renganathan (1994) and Chivuluka et al., 1995 for the degradation of two azo dyes by the lignin peroxidase of *P. chrysosporium*. Despite the fact that azo dye degradation in many works led to final products in the form of coloured quinones (Goszczynski et al., 1994; Spadaro and Renganathan, 1994; Zille et al., 2004; Lu et al., 2008), the final products in the degradation pathway proposed in this work are naphthalene sulfonate and xylene. CDH is able to reduce quinones (Roy et al., 1996; Wingate et al., 2005) and has been detected in culture filtrates of this strain as well as in another strain of *T. trogii* (Ciullini et al., 2008). The results obtained in this work with culture filtrates of *T. trogii* may be due to the synergistic action of laccase, MnP and CDH activities detected in *T. trogii* culture filtrates. On the other hand *T. trogii* is also an outstanding producer of the hydrogen peroxide-producing enzyme GLOX which may contribute to dye degradation. High levels of GLOX activity were produced by *T. trogii* (0.38 U ml^{-1}). Only a few of the 67 strains analyzed by de Jong et al. (1992) tested up to 0.003 U ml^{-1} of GLOX activity. Kersten (1990) using an optimized liquid medium, obtained 0.032 U ml^{-1} of GLOX activity in *P. chrysosporium*. All these enzymes may be responsible for generating highly reactive free radicals that cause a series of spontaneous cleavage reactions that may destroy organic structures with conjugated double bonds.

4. Conclusions

In view of the obtained results, it can be concluded that culture filtrates from *T. trogii* - an outstanding producer of laccase - have a great potential for decolorization of high concentrations of azo dyes, maybe due to the synergistic action of laccase, MnP, CDH and GLOX activities detected in these culture filtrates. They almost completely decolorized the Xylidine (480 and 1440 mg l^{-1}) after 48 h and 140 h respectively. Biodegradation products from Xylidine were determined by capillary electrophoresis followed by MALDI-TOF mass spectra. A new degradation pathway is proposed for Xylidine decolorization by *T. trogii* which involves colored quinones reduction by CDH, rendering naphthalene sulfonate and xylene as final products.

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