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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.