

***In vivo* and laccase-catalysed decolourization of xenobiotic azo dyes by a basidiomycetous fungus: characterization of its ligninolytic system**

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Received 12 February 2003; accepted 22 July 2003

Keywords: Azo dyes, bioremediation, decolourization, laccase, wastewater, white-rot fungi

Summary

Bioremediation is considered a promising eco-efficient alternative for industrial wastewater treatment. Particular attention is currently being given to biological degradation of synthetic dyes and more specifically to colour removal by fungi. This work looks at the extracellular enzymatic system of strain Euc-1. Its ability to decolourize 14 xenobiotic azo dyes was evaluated and compared with the well-known species *Phanerochaete chrysosporium*. Strain Euc-1 is a mesophilic white-rot basidiomycete, the main secreted ligninolytic enzyme being laccase (0.38 U ml⁻¹). Although low manganese-dependent peroxidase activity (0.05 U ml⁻¹) was also detected, neither lignin peroxidase nor aryl alcohol oxidase could be found in batch culture. Optimum pH values of 4.0 and 5.0 were obtained in the laccase-catalysed oxidation of guaiacol and syringaldazine, respectively. Laccase activity increased with the temperature rise up to 50–60 °C and remarkable thermal stability was observed at 50 °C with a half-life of 12 h and no deactivation within the first 2 h. Solid-plate decolourization studies showed that basidiomycete Euc-1 decolourized 11 azo dyes whereas *P. chrysosporium* only two. Moreover, it is shown that purified laccase from basidiomycete Euc-1 efficiently decolourizes the azo dye acid red 88.

Introduction

Dyes and pigments are extensively used for several industrial applications such as textiles, printing, and the manufacture of pharmaceuticals, foods, and toys. Dyes can be classified according to their chemical class (chromophore group) and application range. Azo dyes, the most widely used chemical class in the textile and food industries, are xenobiotic compounds characterized by the presence of one or more azo linkages (–N=N–) and aromatic rings. About 15% of annual synthetic dye production is released in industrial effluents. In traditional textile dyeing about 100 l of water are required to process 1 kg of dyed fabrics (Abadulla *et al.* 2000), thus generating vast quantities of coloured wastewater. Unfortunately, conventional wastewater treatment plants are unable to perform a complete dye removal. For example, 90% of reactive textile dye persists after activated sludge treatment and is discharged into the receiving water-bodies (Pierce 1994). Other physicochemical methods for wastewater decolourization are adsorption, membrane filtration, coagulation–flocculation, and oxidation: however, high-costs

and operational problems limit these applications (Stolz 2001).

Decolourization and dye removal using a biotechnological approach remains technically attractive and several biological processes have been suggested (McMullan *et al.* 2001; Stolz 2001). Although bacteria could decolourize textile wastewater, under anoxic environments azo dyes potentially form carcinogenic aromatic amines (McMullan *et al.* 2001) while aerobic bacteria usually tend to be specific towards a particular dye (Reddy 1995). However, the most efficient lignin-degrading organisms, the white-rot basidiomycetes (Kirk & Farrell 1987; Breen & Singleton 1999), have been reported to degrade a wide range of xenobiotic compounds and other pollutants (Reddy 1995; Pointing 2001) under aerobic conditions. Cripps *et al.* (1990) first reported the decolourization of azo dyes by a white-rot fungus. These authors demonstrated that *Phanerochaete chrysosporium* decolourized acid orange 6, orange II and Congo red. Since then, it has been found that strains of other genera such as *Bjerkandera*, *Phlebia*, *Pleurotus*, and *Trametes* have superior dye removal capabilities relative to *P. chrysosporium* (Rodríguez *et al.* 1999;

Swamy & Ramsay 1999; Pointing *et al.* 2000; Gill *et al.* 2002). This action is mainly ascribed to the non-specific nature of their extracellular ligninolytic enzymatic systems i.e. lignin peroxidase (LiP; EC 1.11.1.14), manganese-dependent peroxidase (MnP; EC 1.11.1.13), and laccase (EC 1.10.3.2) being the key enzymes (Hatakka 1994). Despite the fact that all of these enzymes have been implicated in colour removal, laccase, a copper-containing enzyme that catalyses non-specific oxidation of aromatic substrates with a concomitant four-electron reduction of molecular oxygen to water, has received increased attention due to its potential for pollution abatement (Gianfreda *et al.* 1999).

Nowadays, great attention is being directed towards other white-rot isolates (McMullan *et al.* 2001) and their ligninolytic systems. Recently, we reported extensive decolourization and dephenolization of olive mill wastewater (Dias *et al.* 2001), a darkly phenolic-rich effluent, by basidiomycetous strain Euc-1. In this work we investigate its physiological and ligninolytic characteristics and potential for bioremediation of xenobiotic compounds. Thus, *in vivo* and *in vitro* decolourization of several azo dyes was studied and for comparative purposes *P. chrysosporium* was also included. Taking into account our results, the role of laccase in this process is discussed.

Materials and methods

Reagents, dyes and microbiological media

Guaiacol, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS), syringaldazine (SGZ) and the azo dyes acid red 88 (C.I. 15620), amaranth (C.I. 16185), methyl orange (C.I. 13025), orange G (C.I. 16230) and orange II (C.I. 15510) were purchased from Aldrich. The remaining azo dyes acid blue 113 (C.I. 26360), acid red 337 (C.I. 17102), acid yellow 49 (C.I. 18640), reactive black 5 (C.I. 20505), reactive orange 16 (C.I. 17757) and reactive violet 5 (C.I. 18097) were kindly provided by DyStar (Portugal) and acid orange 67 (C.I. 14172), acid orange 127 (C.I. 26502) and acid red 57 (C.I. 17053) were a gift from Clariant (Portugal). Potato-dextrose agar (PDA) was obtained from Merck.

Fungal strains

Phanerochaete chrysosporium ATCC 24725 and strain Euc-1 were maintained on PDA plates at 4 °C and periodically subcultured. Strain Euc-1, from the UTAD-Colecção de Culturas de Microbiologia Ambiental, was isolated from decaying eucalyptus leaf litter submerged in the Olo river (Northern Portugal). In spite of unsuccessful fruiting bodies induction, this strain has been identified as a white-rot basidiomycetous fungus by DSMZ (Braunschweig, Germany). Light microscopic observations revealed the presence of septate hyphae

and clamp connections. When cultivated on PDA, the mycelium grows as a fairly flat white mat and the colony base was also white. Advancing hyphae were colourless and the plate (9 cm diameter) was fully covered after one week incubation at 28 °C. On the basis of phylogenetic analysis, strain Euc-1 resembles the genera *Trametes* and *Pycnoporus* (J.P. Sampaio, personal communication).

Effect of temperature on the growth of strain Euc-1

Using a sterile cork borer, 4-mm agar discs punched from the front of an actively growing Euc-1 fungus in PDA were used to centrally inoculate 3.9% PDA plates filled with 15 ml medium/plate. Triplicates were prepared for each of the following temperatures (°C): 10, 15, 20, 25, 28, 30, and 35. Mean growth rates were determined by measuring the mycelium diameter in two perpendicular directions.

Production of ligninolytic enzymes by strain Euc-1

Batch cultures adjusted to pH 4.5 with 2 M NaOH contained the following (per litre): 5.0 g glucose, 2.2 g ammonium tartrate, 1.0 g KH₂PO₄, 0.26 g NaH₂PO₄, 0.5 g MgSO₄·7H₂O, 2.9 g 2,2-dimethylsuccinic acid, 1.0 mg CuSO₄·5H₂O, 74 mg CaCl₂·2H₂O, 6.0 mg ZnSO₄·7H₂O, 5.0 mg FeSO₄·7H₂O, 5.0 mg MnSO₄·4H₂O, 1.0 mg CoCl₂·6H₂O, and 0.5 ml of vitamin solution prepared with (per litre): 5.0 g thiamine-HCl, 1.6 g nicotinic acid, 1.6 g pyridoxine-HCl, 0.8 g calcium pantothenate, and 0.01 g biotin. Two 10-mm agar plugs removed from the front of an actively growing Euc-1 fungus in PDA were used to inoculate 250-ml Erlenmeyer flasks containing 100 ml of medium. Incubations were carried out on an orbital shaker (Certomat H-S; B. Braun) set at 100 rev min⁻¹ and 28 °C. Flasks were periodically sampled for determination of enzymatic activities and glucose depletion using the dinitrosalicylic acid reagent (Miller 1959) for reducing sugars. Mycelial dry weight was determined after filtering the cultures through Whatman GF/A filters and drying at 80 °C until constant weight.

Determination of enzymatic activities

All assays were monitored by absorbance measurements at 25 °C using 0.4 ml of appropriately diluted enzyme in 1.5 ml total volume. Laccase activity was routinely determined by the oxidation of 2.0 mM ABTS in 100 mM phosphate-citrate buffer pH 4.0, followed at 420 nm ($\epsilon = 36 \text{ mM}^{-1} \text{ cm}^{-1}$) (Bourbonnais *et al.* 1995). Oxidation of SGZ (0.02 mM ethanolic solution) and guaiacol (4.0 mM) followed at 525 nm ($\epsilon = 65 \text{ mM}^{-1} \text{ cm}^{-1}$) and 470 nm ($\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$), respectively were also used. MnP, LiP, and aryl alcohol oxidase were determined by standard methods according to Heinfling *et al.* (1998), Tien & Kirk (1988), and Guillén *et al.* (1992), respectively. Enzyme units are defined as one μmol of