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Fungal laccase, cellobiose dehydrogenase, and chemical mediators: Combined actions for the decolorization of different classes of textile dyes

Ilaria Ciullini, Silvia Tilli, Andrea Scozzafava, Fabrizio Briganti *

Laboratorio di Chimica Bioinorganica, Dipartimento di Chimica, Università degli Studi di Firenze, Via Della Lastruccia 3, 50019 Firenze, Italy

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Abstract

Dyes belonging to the mono-, di-, tri- and poly-azo as well as anthraquinonic and mono-azo Cr-complexed classes, chosen among the most utilized in textile applications, were employed for a comparative enzymatic decolorization study using the extracellular crude culture extracts from the white rot fungus *Funalia* (*Trametes*) *trogii* grown on different culture media and activators able to trigger different levels of expression of oxidizing enzymes: laccase and cellobiose dehydrogenase. Laccase containing extracts were capable to decolorize some dyes from all the different classes analyzed, whereas the recalcitrant dyes were subjected to the combined action of laccase and the chemical mediator HBT, or laccase plus cellobiose dehydrogenase. Correlations among the decolorization degree of the various dyes and their electronic and structural diversities were rationalized and discussed. The utilization of cellobiose dehydrogenase in support to the activity of laccase for the decolorization of azo textile dyes resulted in substantial increases in decolorization for all the refractory dyes proving to be a valid alternative to more expensive and less environmentally friendly chemical treatments of textile dyes wastes.

Keywords: *Trametes trogii*; Laccase; Cellobiose dehydrogenase; Textile dyes; Decolorization

1. Introduction

White rot fungi, a heterogeneous group of organisms are capable of degrading lignin and the other main wood components, fundamental for carbon flux in ecosystems. Their biodegradation capacities are due to highly non-specific, free-radical-mediated processes resulting from the activities of several enzymes secreted by these fungi such as laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) (Fu and Viraraghavan, 2001). These enzymatic systems enable white rot fungi to degrade a wide range of pollutants, including polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), pesticides, explosives, synthetic polymers and synthetic dyes (Pointing, 2001).

Synthetic dyes are being increasingly used in the textile, paper, pharmaceutical, cosmetics and food industries. Over 7×10^5 tonnes of approximately 10,000 different dyes and pigments are produced annually worldwide, of which about 50,000 tonnes are discharged into the environment (Lewis, 1999). The discharge of very small amounts of dyes (less than 1 ppm for some dyes) is aesthetically displeasing, impedes light penetration, affects gas solubility damaging the quality of the receiving streams and may be toxic to treatment processes, to food chain organisms and to aquatic life. For these reasons several countries are adopting stringent regulations for the release of colored industrial effluents. Azo, anthraquinone and indigo are the major chromophores found in commercial dyes. Decolorization of these dyes by physical or chemical methods is financially and often also methodologically demanding, time-consuming and mostly not very effective. Because of the range of chemical structures and properties, the degradation of mol-

* Corresponding author. Tel.: +39 0554573343; fax: +39 0554573333.
E-mail address: fbriganti@unifi.it (F. Briganti).

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ecules of dyes in the environment by microorganisms is very slow (Pierce, 1994). Moreover, the industrially important azo dyes are cleaved under anaerobic conditions by bacterial azo-reductases to the corresponding amines, many of which are mutagenic and/or carcinogenic generating potential health hazards (Banat et al., 1996).

At present, a number of studies have focused on the utilization of fungi since their mechanisms of dyes decolorization involve oxidative reactions which therefore do not produce toxic amines. However, using fungal biomass or single enzymes to remove color in a dye wastewater is still in the research stage. Recent studies have also shown that cellobiose dehydrogenase (CDH hereafter), an extracellular haemo-flavo-enzyme, produced by a number of wood-degrading and phytopathogenic fungi, has a role in the early events of lignocelluloses degradation and wood colonization (Henriksson et al., 2000), due to its ability to facilitate the formation of free hydroxyl radicals. CDH has been reported to display *in vitro* a synergism with laccases in the decolorization of an anthraquinonic dye, and directly in the oxidation of several chemicals (Vanhulle et al., 2007).

Funalia (*Trametes*) *trogii* is a widely distributed white rot basidiomycete, good producer of laccases and other ligninolytic enzymes (Levin et al., 2005). A few studies on its capabilities in the decolorization of only some dyes have also been reported (Colao et al., 2006; Levin et al., 2001). The *F. trogii* strain 201, subject of this research, secretes laccases and no peroxidases under the different growth conditions utilized in this study and we here report for the first time that in the presence of cellulose besides laccases also CDH is secreted in abundance.

In this paper, we report the investigation of the activities of the redox enzymes secreted by *F. trogii* 201 under different growth conditions on the decolorization of several classes of textile dyes among the most diffused in textile applications. The combined utilization of CDH or redox mediators with laccase was also investigated for the decolorization of the most recalcitrant dyes.

2. Methods

2.1. Chemicals

All the chemicals were purchased from Sigma Chemical Co. Agar and Yeast Extract were from Oxoid Ltd. Textile dyes utilized were from Eurocolor S.p.A.; International Color S.p.A.; Ciba Specialty Chemicals S.p.A.; Kem. Color S.p.A; Novacolor s.r.l; or from AlphaColor S.p.A.

2.2. Organism and culturing conditions for laccase production

The white rot fungus *F. trogii* 201 (DSM 11919) was maintained on basidiomycete rich medium (BRM) (Bezalel et al., 1997) agar plates at 4 °C and periodically transferred onto fresh BRM agar plates and grown at 28 °C. After 4–6 days of growth on agar plates, 500 ml shaken flask cultures containing 150 ml liquid BRM were prepared and inoculated with 10 plugs of fungal mycelia (about 25 mm^2) and grown in the dark at 28 °C under continuous stirring at 130 rpm. After 4 days the grown mycelia were transferred in baffled 2000-ml Erlenmeyer flasks, closed with sterile air permeable silicon corks, containing 1000 ml of fresh BRM liquid medium and grown under the same conditions. The laccase expression was induced by the addition of 150 μM $CuSO_4$ to the starting medium. Other inducers (veratryl alcohol 0.25 mM or Cu(II) 0.15, 0.5 or 1.0 mM) were added after 2 days of incubation. When the extracellular laccase activity reached a maximum about on day 7, the culture supernatant was collected by filtration through Whatman No. 1 paper and concentrated using an ultrafiltration Vivaflow 200 module (Sartorius group) with a 30,000 Da cut-off membrane.

2.3. Culturing conditions for simultaneous cellobiose dehydrogenase (CDH) and laccase production

The fungus *F. trogii* 201 was cultivated in the above described conditions but on modified BRM where 10 g l^{-1} of microcrystalline cellulose powder was added as carbon source instead of glucose to obtain the simultaneous production of CDH and laccase. When the extracellular CDH activity reached a maximum, about on days 8–9, the culture supernatant was collected by filtration and concentrated as reported above.

2.4. Enzyme assays

Laccase activity was determined spectrophotometrically based on the capacity of this enzyme to oxidize the non-phenolic compound 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) ($\varepsilon_{420} = 36{,}000\ M^{-1}\ cm^{-1}$), pH 3 at 25 °C; 1 U of laccase activity was defined as the amount of enzyme oxidizing 1 μmol substrate/min.

CDH activity was assayed by following the decrease in absorbance of the electron acceptor, i.e. 2,6-dichlorophenol-indophenol (DCIP), at 520 nm ($\varepsilon_{520} = 6.8 \times 10^3\ M^{-1}\ cm^{-1}$), pH 4.0 and 37 °C. One unit of enzyme activity is defined as the amount of enzyme reducing 1 μmol DCIP/min under the above reaction conditions. The combined determination of laccase and CDH activities was performed using 0.1 mM DCPIP following the method by Vasil'chenko et al. (2005). To understand if the DCPIP reducing activity observed was really due to CDH and not to a sugar oxidase usually present in the cellulolytic systems of fungi, i.e. glucose oxidase, in this assay we substituted cellobiose with D-glucose; no glucose oxidase activity was observed.

MnP activity was estimated by the formation of Mn^{3+}–tartrate complex (ε_{238}: 6500 $M^{-1}\ cm^{-1}$) at pH 5, 25 °C. LiP activity was determined by the H_2O_2-dependent veratraldehyde (3,4-dimethoxybenzaldehyde) formation ($\varepsilon_{310} = 9300\ M^{-1}\ cm^{-1}$), pH 3, 25 °C.