Enzyme and Microbial Technology 49 (2011) 465–471

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Differential decolorization of textile dyes in mixtures and the joint effect of laccase and cellobiose dehydrogenase activities present in extracellular extracts from *Funalia trogii*

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ARTICLE INFO

Article history:
Received 6 April 2011
Received in revised form 1 June 2011
Accepted 10 August 2011

Keywords:
Textile dyes mixtures
Funalia trogii
Laccase
Cellobiose dehydrogenase
Decolorization
Bioremediation

ABSTRACT

The largest part of the bio-decolorization investigations have been performed to date on a single dye without exploring the behavior in complex mixtures as the real dyeing baths. Therefore, mixtures of dyes belonging to azo and anthraquinonic classes, chosen among the most utilized in textile wool dyeing, were employed for comparative enzymatic decolorization studies using the extracellular extracts from the white rot fungus *Funalia trogii*, to understand how the concomitant presence of more than one dye could influence their degradation course and yield.

Fungal extracts containing laccase activity only were capable to partially decolorize dyes mixtures from the different classes analyzed. The deconvolution of the decolorization with time allowed to monitor the degradation of the single dyes in the mixtures evidencing a time dependent differential decolorization not observed for the singles alone. Some dyes in the blend were in fact decolorized only when the most easily converted dyes were largely transformed. These experiments would allow to help the dyeing factories in the selection of the most readily degraded dyes.

Since *F. trogii* grown on different media and activators shows diverse levels of expression of the redox enzymes laccase and cellobiose dehydrogenase (CDH), the dyes mixtures recalcitrant to decolorization by laccase activity alone, were subjected to the combined action of extracts containing laccase and CDH. The use of CDH, in support to the activity of laccase, resulted in substantial decolorization increases (>84%) for all the refractory dyes mixtures.

1. Introduction

Synthetic dyes are extensively used in the textile, paper, tanning, pharmaceutical, cosmetics and food industries. Over 50,000 t of approximately 10,000 different dyes and pigments produced annually worldwide are discharged into the environment [1]. Color is usually the first contaminant to be recognized in wastewater. The discharge of 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 microorganisms utilized in treatment processes, to food chain organisms and to aquatic life [2]. For these reasons several countries adopted demanding regulations for the release of colored industrial effluents.

Azo, anthraquinone and indigo are the major chromophores found in commercial dyes [3]. Decolorization of these dyes by physical or chemical methods (adsorption and precipitation methods, chemical degradation or photodegradation) is financially and often also methodologically demanding, time-consuming and mostly not very effective [4]. The degradation of synthetic dyes in the environment by microorganisms is generally very slow due to the variety of their chemical structures and properties. It is known that 90% of reactive textile dyes entering the conventional bio-treatments with activated sludge sewage treatment plants will be discharged to rivers unchanged [5]. Moreover the industrially important azo dyes, under anaerobic conditions are transformed by bacterial azoreductases into the corresponding mutagenic and/or carcinogenic amines generating the expected health hazards [4].

As a consequence several studies have focused on the utilization of fungi since their mechanisms of dyes decolorization involve oxidative reactions which therefore do not produce toxic amines [2]. Their biodegradation capacities are generally 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) [6]. The

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doi:10.1016/j.enzmictec.2011.08.002

peroxidases have the highest redox potentials (>1.0 V), being able to catalyze directly the oxidation of non-phenolic compounds, however, they need hydrogen peroxide as a cosubstrate for functioning. Laccases, although showing a lower redox potential (0.5–0.8 V), only use molecular oxygen as the final electron acceptor, therefore being more adequate for industrial and environmental applications. Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are copper-containing enzymes that catalyze the one electron oxidation of substituted phenols, anilines, and aromatic thiols to their corresponding radicals with the concomitant reduction of molecular oxygen to water. Their substrate range can be extended to non-phenolic compounds in the presence of low molecular mass compounds acting as redox mediators [7,8]. The ability of fungi to degrade dyes appears as a promising alternative to replace or supplement present treatment processes. However, using fungal biomass or single enzymes to remove color in a dye wastewater is still in the research stage.

Funalia trogii is a widely distributed white-rot basidiomycete, good producer of laccases and other ligninolytic enzymes [9]. The *F. trogii* subject of this research secretes one major laccase (>90% of the total laccase activity) and low levels of four additional laccases but no peroxidases under the growth conditions utilized in this study [10]. The structure of the major laccase secreted by this strain has been recently solved and analyzed [11]. We also demonstrated that in the presence of cellulose besides laccases also cellobiose dehydrogenase (CDH hereafter) is secreted in abundance [12].

Recent work has shown that CDH is an extracellular haemoflavo-enzyme that is produced by a number of wood-degrading and phytopathogenic fungi and it has a proposed role in the early events of lignocelluloses degradation and wood colonization [13]. Due to its ability to facilitate the formation of free hydroxyl radicals, potent oxidants with a standard reduction potential of +2.3 V, CDH has been reported to display *in vitro* a synergism with laccases in the decolorization of many dyes [12].

The majority of the enzymatic decolorization studies have been performed on a single dye [14–18]. On the contrary the real textile dyeing processes primarily utilize mixtures of different dyes. Only a limited number of studies explored the biodegradation of composite dyes blends without investigating the distinct degradation of the individual dyes in the mix and if the decolorization of each dye would influence the course of the degradation of the others [19–24]. We report here a study on the decolorization induced by the redox enzymes laccases and CDH secreted by *F. trogii* on assorted dyes mixtures with the aim of understanding if the presence of more than one dye influences their mutual degradation course and final yields.

2. Materials and methods

2.1. Chemicals

All the chemicals were purchased from Sigma Chemical Co. Agar and Yeast Extract were from Oxoid Ltd. Textile dyes utilized are: Acid Red 42 (Are 42), Acid Black 1 (Aba 1), from Eurocolor S.p.A.; Acid Black 194 (Aba 194) from International Color S.p.A.; Reactive Blue 69 (Rbu 69), Reactive Red 272 (Rre 272), Lanasol Black R®, from Ciba Specialty Chemicals 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) [25] and grown on liquid media as previously reported [12]. The laccase expression was further induced by the addition of 150 μM $CuSO_4$. When the extracellular laccase activity reached a maximum on days 7–9 (7–8 U mL^{-1}), 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 CDH and laccase production

The fungus *F. trogii* 201 showed detectable CDH production only in media containing cellulose powder as carbon source. Therefore we modified the above mentioned medium substituting the glucose, which inhibits CDH expression, with 10 g L^{-1} cellulose [26]. When the extracellular CDH activity reached a maximum, about on days 8–9 (up to 0.6 U mL^{-1}), the culture supernatant was collected and concentrated as reported above.

Manganese peroxidase and lignin peroxidase activities were not detected in any of the different conditions utilized for fungal growth.

2.4. Enzyme assays

The activities of three ligninolytic enzyme families: peroxidases, laccases and CDH, that can be involved in textile dyes decolorization have been determined by using a variety of methods as reported by Ciullini et al. [12].

2.5. Enzymatic dyes decolorization

The reaction mixtures for dyes-decolorizing activity were prepared in 50–500 mL shaken flasks and consisted of an aqueous solution of dyes the concentrations of which are reported in Table 2, in a total volume of 20–200 mL, respectively. The reactions were initiated adding fungal crude extract (to reach final concentrations from 0.14 to 15.4 U mL^{-1} laccase and CDH from 0.3 to 1.5 U mL^{-1} when requested) and incubated at 30 °C with shaking (300 rpm) for the appropriate times. Samples of dyes solutions were taken at regular times, centrifuged at 13,000 rpm for 5 min to eventually remove suspended particles and the decolorization extent was measured spectrophotometrically after appropriate dilution (1:10). Control samples were tested under identical conditions. UV–visible measurements were carried out on double beam Perkin Elmer EZ 301 spectrophotometer using 1 cm path length Hellma 110 quartz suprasil cells thermostated with a Lauda Ecoline low temperature thermostat RE112. The absorption spectrum was recorded (400–800 nm) for each dyes mixture or individual dye and decolorization was followed monitoring the % decreasing absorption areas utilizing the formula: $(Area_{initial} - Area_{final}) \times 100/Area_{initial}$. The spectral deconvolution was performed as previously reported [27].

The partially or totally recalcitrant dyes were also tested for decolorization with the combined action of laccase and CDH activities, in a total volume of 20–200 mL dye solution.

All the dyes that were totally or partially decolorized by the catalytic action of extracts in the course of the present study did produce extensive precipitation of polymerized products, within 24 h, easily eliminated by decanting, filtering or through low speed centrifugation.

To exclude that the incomplete decolorization of some dyes would not be the result of enzymes inactivation with time, a supplementary addition of fresh extract, equal to that initially added, was always made after 48 h but this did not result in any further decolorization.

3. Results and discussion

3.1. Dyes mixtures decolorization with crude extracellular laccase extract

Several mixtures of textile dyes, selected on the basis of their extensive utilization in dyeing factories, were tested for enzymatic decolorization. As previously observed, a laccase activity of 1.5 U mL^{-1} afforded optimal results for single dyes and it was also utilized for these mixtures [12].

3.1.1. Mixture I

Mixture I is composed by four dyes of different classes chosen among those readily decolorized by laccase extracts as singles: Are 42 (monoazo dye), Aba 1 (disazo), Aba 194 (mono-azo chromo complexed), and Rbu 69 (anthraquinonic).

The dyes which constitute mixture I were individually tested for laccase decolorization at pH 7.0, the results (see Table 1) confirmed that these dyes are mostly degraded by the laccase action alone since Are 42, Aba 1, Rbu 69, and Aba 194 exhibit overall decolorizations in the range 84–90%.

The decolorization of the anthraquinonic Rbu 69 dye by laccase was expected since high potential laccases have been shown to decolorize anthraquinonic dyes more efficiently than azo and other classes of dyes [28]. Laccases are known to modify azo dye structures by destroying their chromophoric assemblies. In a first step, one electron is abstracted from the phenolic/naphtholic rings, yielding a phenoxy radical. After abstraction of a second