Decolorization of Azo Dyes by White Rot Fungi

Emrah Ahmet Erkurt, Hatice Atacag Erkurt, and Ali Unyayar

Abstract White rot fungi (WRF) produce various isoforms of extracellular peroxidases (lignin peroxidase-LiP and manganese peroxidase-MnP) and phenoloxidases (laccases), which are involved in the degradation of lignin in their natural lignocellulosic substrates. This ligninolytic system of WRF is directly involved in the degradation of various xenobiotic compounds and dyes. Liquid fermentation or solid-state fermentation techniques can be used for enzyme production. Crude enzymes or purified enzymes of WRF can be used for decolorization of azo dyes. Repeated-batch decolorization technique is a new approach that can be used for decolorization. There are different procedures to determine the enzyme(s) responsible for decolorization. Single step isolation and identification procedure (SSIIP) is a new and simple method that can be used for detection of the enzyme responsible for biodegradation of azo dyes.

Keywords Azo dye, Biodegradation, Decolorization, Laccase, Peroxidase, White rot fungus

Contents

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Abbreviations

LiP	Lignin peroxidase
LME	Lignin modifying enzyme
MnP	Manganese peroxidase
PAGE	Polyacrylamide gel electrophoresis
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSIIP	Single step isolation and identification procedure
WRF	White rot fungi

1 Introduction

Azo dyes represent the largest group of organic dyes synthesized and account for about 70% of all textile dyes produced. During the dying process most reactive dyes are hydrolysed and later released into waterways. Although these dyes are not toxic by themselves, after release into the aquatic environment, they may be converted into potentially carcinogenic amines [1, 2] that impacted the ecosystem downstream from the mill. The public demands for colour-free discharges to receiving waters have made decolourization of a variety of industrial wastewater a top priority [3]. Microbial decolourization has been claimed to be less expensive and less environmentally intrusive alternative [4]. Many bacteria and fungi are used for the development of biological processes for the treatment of textile effluents [5–7]. Containing various substituents such as nitro and sulfonyl groups, synthetic dyes are not uniformly susceptible to decomposition by activated sludge in a conventional aerobic process. Attempts to develop aerobic bacterial strains for dye decolourization often resulted in a specific strain, which showed a strict ability on a specific dye structure [8]. The use of lignin-degrading white rot fungi (WRF) has attracted increasing scientific attention, as these organisms are able to degrade a wide range of recalcitrant organic compounds. Their lignin modifying enzymes (LME), that is MnP, LiP and laccases, are directly involved in the degradation of not only lignin in their natural lignocellulosic substrates [9, 10] but also various xenobiotic compounds [11, 12] including dyes [13–18]. Peroxidases and laccases of WRF are oxidative enzymes, which do not need any other cellular components to work. They have broad substrate specificity and are able to transform a wide range of toxic compounds. These enzymes, which are widely distributed in nature, have been studied for many years because of their potential use as biocatalysts in pulp

and paper bleaching, wastewater treatment, soil remediation, on-site waste destruction and medical diagnostics [19–23].

2 White Rot Fungi Capable of Decolorizing Azo Dyes

List of selected white rot fungi are given in Table 1.

Table 1 Selected white rot fungi and their enzymes able to decolorize azo dyes

WRF	Enzyme	Dye	References
Phanerochaete chrysosporium	LiP	Diazo dyes	[52]
	LiP	Reactive Brilliant Red K-2BP	[53]
	LiP and MnP		
	MnP and β-glucosidase	Amaranth, new coccine, and Orange G	[54]
Trametes vesicolor	–	Reactive Red 2	[55]
	–	Remazol Black B	[56]
Coriolus versicolor	Laccase	Drimarene Blue	[16]
Funalia trogii	Laccase	Astrazone Blue	[34]
	Laccase	Drimarene Blue	[16]
Pleurotus ostreatus	Laccase	Drimarene Blue	[16]
	LiP	Disperse Orange 3	[57]
	–	Methyl Red and Congo Red	
	LiP	Disperse Orange 3 Disperse Yellow 3	[58]
Phanerochaete sordida	MnP	Reactive Red 120	[59]
Pleurotus sajorcaju	Laccase	Amaranth, new coccine, and Orange G	[14]
	Laccase	Reactive Black 5	[17]
Irpex lacteus	–	Methyl Red and Congo Red Reactive Orange 16, Congo Red, Reactive Black 5, Naphthol Blue Black, Chicago Sky Blue	[60]
	MnP		[61]
Ganoderma lucidum	Laccase	Reactive Black 5	[42]
Ganoderma sp. WR-1	LiP	Amaranth	[62]
Ischnoderma resinosum	Laccase	Orange G	[15]
Dichomitus squalens	Laccase and MnP	Orange G	[15]
Pleurotus calyptratus	Laccase	Orange G	[15]
Strain L-25 (newly isolated white rot fungus)	MnP	Direct-Orange 26, Direct Red 31, Direct Blue 71, Acid Orange 56, Acid Red 6, Mordant Yellow 3, Mordant Blue 13, Mordant Black 11, Reactive Orange 16, Reactive Black 5	[25]
Lentinula edodes	MnP	Congo Red, Trypan Blue, Amido Black	[13]

3 Enzymes of White Rot Fungi Involved in Azo Dye Decolorization

WRF are key regulators of the global C-cycle. Some WRF produce all three LME, while others produce only one or two of them [10]. The main LME are oxidoreductases, that is two types of peroxidases, LiP and MnP, and a phenoloxidase Laccase. Catalytic cycles of peroxidases and laccases are given in Figs. 1 and 2, respectively. LME are produced by WRF during their secondary metabolism.

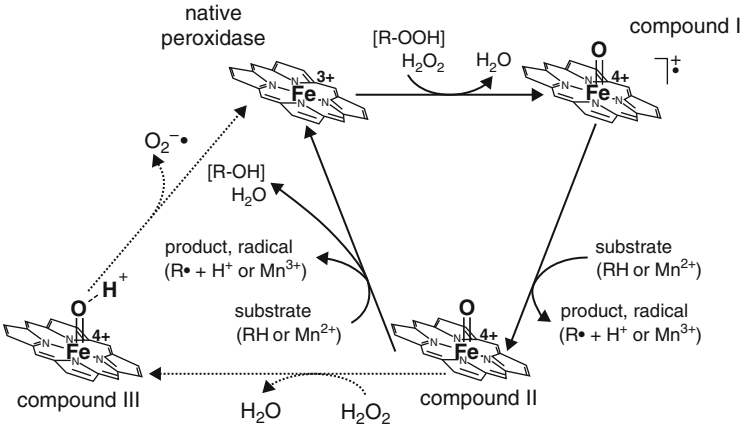

Fig. 1 Generic scheme of the catalytic cycle of peroxidases (taken from [24])

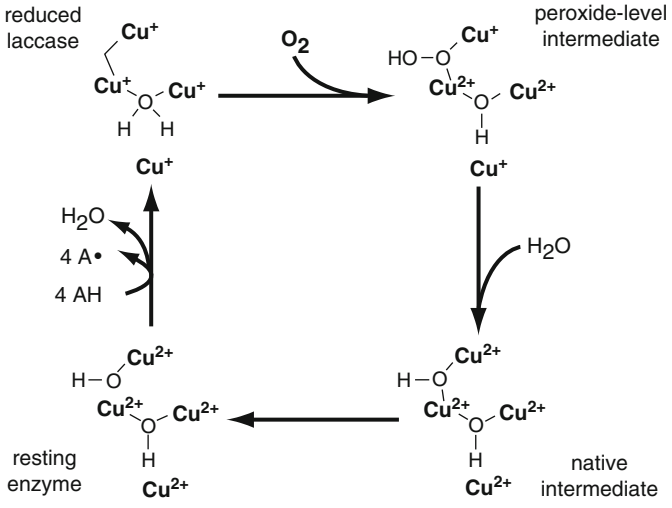

Fig. 2 The catalytic cycle of laccases (taken from [24])

Synthesis and secretion of these enzymes are often induced by limited nutrient (C or N) levels [24].

The proposed mechanism for the functionality of MnP involves the oxidation of manganous ions Mn^{2+} to Mn^{3+}, which is then chelated with organic acids. The chelated Mn^{3+} diffuses freely from the active site of the enzyme and can oxidize secondary substrates [25].

LiP catalyze several oxidations in the side chains of lignin and related compounds [26] by one-electron abstraction to form reactive radicals [27]. Also the cleavage of aromatic ring structures has been reported [28]. The role of LiP in ligninolysis could be the further transformation of lignin fragments, which are initially released by MnP.

Fungal laccases as part of the ligninolytic enzyme system are produced by almost all wood rotting basidiomycetes. This group of *N*-glycosylated extracellular blue oxidases with molecular masses of 60–390 kDa [29, 30] contain four copper atoms in the active site (as Cu^{2+} in the resting enzyme). Laccases catalyze the oxidation of a variety of aromatic hydrogen donors with the concomitant reduction of oxygen to water (Fig. 2). Laccase is an oxidase with a redox potential of 780 mV and can catalyse the oxidation of organic pollutants by reduction of molecular oxygen straightforwardly to water in the absence of hydrogen peroxide or even other secondary metabolites [31]. While anthraquinone was directly oxidized by the laccase, azoic and indigo dyes were not the substrates of laccase, and small molecule metabolites mediated the interaction between the dyes and the enzyme [32].

4 Enzyme Production and Decolorization Methods

Most studies on lignin biodegradation and dye decolourization have been carried out using liquid culture conditions [15]. Homogenized mycelium [16] or pellets [33, 34] of WRF can be used for biodegradation of azo dyes. In batch mode, at the beginning of the decolorization process, adsorption of dye by cells might be observed. However, this color sometimes disappeared when enzymes were released by fungal strains [25]. Liquid media including lignocellulosic substrates are also used for ligninolytic enzyme production [35].

In some researches, solid-state fermentation (SSF) is being used as the media for ligninolytic enzyme production [36]. SSF reflect the natural living conditions (i.e. in wood and other lignocellulosic substrates) of these fungi. SSF is defined as the growth of microorganisms on solid materials in the presence of a small amount of free water [37]. The list of different substrates used for the cultivation of microorganisms on SSF is long, including several agricultural materials, such as wheat bran, wheat straw, sugar cane bagasse and corn cob. The choice of corn cob was due to the low amounts of natural coloured pigments found in this material. The pigments found in other lignocellulosic substrates, such as wheat bran and wheat straw, could interfere in the dye decolorization experiments [13].

SSF containing wheat bran and soybean as a substrate was chosen for the production of ligninolytic enzymes for *Funalia trogii* ATCC200800 [18] as it mimics the natural environment of the WRF and permits the concentration of dyes by absorption process prior to biological treatment [4, 38, 39]. It is possible to stimulate the yield of laccase activity of *Trametes versicolor* by using several agricultural wastes [40].

Crude enzyme of *Earliella scabrosa* obtained in SSF showed higher decolourization percentage of Navy FNB and Red FN-3G dyes than *Trametes maxima* and *Ganoderma zonatum* (B-18). *T. maxima* exhibited the best decolourization percentage in submerged cultures supplemented with Navy FNB, Red FN-3G and yellow P-6GS dyes. Growing biomass of *T. maxima* could supply other enzymes and mediators for dye transformation. Peculiar behaviour was observed with *G. zonatum* (B-18); it had a similar dyes biodegradation in both liquid and solid bed fermentation and there was no positive correlation between ligninolytic enzymes production and decolourization pattern. The employment of crude enzymes produced in the solid bed of bagasse could be an attractive option for biological removal of textile dyes [41].

Forest residue wood chips contain a mixture of fungi and bacteria, which is an advantage when complex molecules should be degraded. The wood chips furthermore provide the microorganisms with carbon source, which make the addition of, for example, glucose unnecessary. The decolourization of a mixture of 200 mg/L each of Reactive Black 5 and Reactive Red 2 dye was studied in batch experiments using microorganisms growing on forest residue wood chips in combination with or without added WRF, *Bjerkandera* sp. BOL 13. The microorganisms growing on the forest residue wood chips decolourized the mixture of the two dyes; adding extra nutrients approximately doubled the decolourization rate [42].

Dye decolorizing potential of the WRF *Ganoderma lucidum* KMK2 was demonstrated for recalcitrant textile dyes. *G. lucidum* produced laccase as the dominant lignolytic enzyme during SSF of wheat bran, a natural lignocellulosic substrate. Crude enzyme shows excellent decolorization activity to anthraquinone dye Remazol Brilliant Blue R without redox mediator, whereas diazo dye Remazol Black-5 (RB-5) requires a redox mediator [43].

Funalia trogii ATTC 200800 pellets and enzymes were used wherein an efficient decolourization was observed within 24 h [16, 34]. The direct decolourization of textile dyes by crude enzymes of *F. trogii* ATCC200800 would provide a cost-effective solution for textile industry. On the other hand, using pellets would also provide a cost-effective solution as repeated addition of dyes is possible. Yesilada et al. reported a 86% decolorization efficiency at the end of tenth cyle [34]. Repeated-batch mode represents a potential alternative mode of fermentation, in which medium or some part of the medium is drawn and fresh medium is refilled periodically without changing the pellets [24]. This process allows the maintenance of long-term activity of the pellet for a long period of time and achieves better results compared with batch cultivation [44]. With this method, it also possible to store the pellets and reuse them. Thus, repeated-batch-type laccase production represents a process which may be applicable for industrial purposes [33].

Using purified enzymes of WRF is another method used for degradation of azo dyes [45]. Purified laccase from *Pleurotus sajorcaju* was reported to be used for decolorization of Reactive Black 5, and increased decolourization was observed with increase in enzyme concentration [43].

Both purified laccase as well as the crude enzyme from the WRF *Cerrena unicolor* were used to convert the dyes in aqueous solution. Biotransformation of the dyes was followed spectrophotometrically and confirmed by high performance liquid chromatography. The results indicate that the decolorization mechanism follows MichaeliseMenten kinetic and that the initial rate of decolorization depends both on the structure of the dye and on the concentration of the dye. Surprisingly, one recalcitrant azo dye (AR 27) was decolorized merely by purified laccase in the absence of any redox mediator [46].

5 Detection of Enzymes Responsible for Azo Dye Decolorization

Measuring Lignin peroxidase, Laccase and MnP activities in decolorization medium is a method to determine the enzyme responsible for decolorization [15, 17, 25].

Lentinula (*Lentinus*) *edodes* produced only Mn peroxidase, and the production of both laccase and lignin peroxidase was, apparently, negligible. Consider that a strict relation between the production of Mn peroxidase and the dye decolorization ability was observed in vivo [13].

Statistical analysis of enzyme amounts could be used to demonstrate which enzyme plays an important role in the decolourization process of azo dyes, and it was reported that the complete decolourization time and enzyme activity are negatively correlative [47].

Molecular masses of the same enzymes of different species are different. Molecular mass of the laccase of *Pleorotus ostreatus* was found to be 66.8 kDa by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [48]. Purified enzyme of *T. versicolor* having a single band with a molecular mass of ~68 kDa was in the same range with the molecular weights of laccase isoforms isolated from 2,5-xylidine-induced cultures of *T. versicolor* [49].

Using SDS-PAGE or native polyacrylamide gel electrophoresis (PAGE) methods is another method to determine enzyme(s) responsible for decolorization. The degradation of the disazo dye Chicago Sky Blue 6B by a purified laccase from *Pycnoporus cinnabarinus* showed a band having a molecular size of 63 kDa determined by SDS-PAGE [50]. Unyayar et al. had reported the Drimarene Blue X3LR decolourizing enzymatic activity in the culture filtrate of *F. trogii* by using SDS-PAGE [18]. In this method, two SDS-PAGEs were performed. One of them was used for determining molecular weight of protein bands (Lane A, Fig. 3). The other one was used for single step isolation and identification procedure. The staining activity was done with Drimarene Blue X3LR dye and guaicol after the gel was re-natured. After the gel was stained with Drimarene Blue X3LR dye and incubated

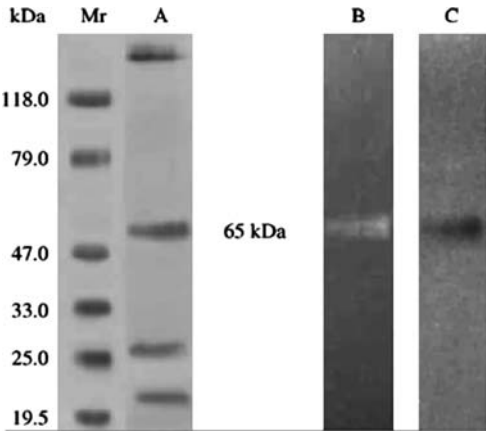

Fig. 3 SDS-PAGE Photograph: Separation (Lane Mr and A) and activity staining (Lane B and C) of the crude filtrate of *Funalia trogii*. Lane Mr standard molecular weight markers (β-galactosidase, 118.0 kDa; bovine serum albumin, 79.0 kDa; ovalbumin, 47.0 kDa; carbonic anhydrase, 33.0 kDa; β-lactoglobulin, 25.0 kDa; and lysozyme, 19.5 kDa). Relative mobilities of the standard markers vs. common logarithms of their molecular masses were plotted.With the linear regression output, the molecular masses of the proteins in the crude filtrate were estimated (taken from [18])

at 30°C for 30 min, a colourless zone was observed (Lane B, Fig. 3). This colourless zone was found to be equal to 65 kDa in Lane A (Fig. 3). Afterwards, the gel was treated with guaicol. This colourless zone turned into orange colour after incubation with guaicol, which is a classical indicator and substrate of laccase (Lane C, Fig. 3), and so it was concluded that this enzyme responsible for decolourization of Drimarene Blue X3LR was a laccase [18].

A similar method was used by Murugesan et al. PAGE of crude enzyme and oxidation of guaicol on gels confirm that the laccase enzyme was the major enzyme involved in the decolorization of RB5. Native and SDS-PAGE indicates the presence of single laccase with molecular weight of 43 kDa [43].

A microtitre plate-based method was developed for a fast screening of numerous fungal strains for their ability to decolourize textile dyes. In 3 days, this method allowed to estimate significant fungal decolourization capability by measuring the absorbance decrease on up to 10 dyes. WRF strains belonging to 76 fungal genera were compared with regards to their capability to decolourize five azo and two anthraquinone dyes as well as the dyes mixture. The most recalcitrant dyes belonged to the azo group. Several new species unstudied in the bioremediation field were found to be able to efficiently decolourize all the dyes tested [51].

Decolorization of azo dyes by WRF technology improvements will require integration of all major areas of industrial biotechnology: novel enzymes and microorganisms, functional genomics, protein engineering, biomaterial development, bioprocess design and applications.

The enzymes of WRF will play a significant role for the working of treatment processes. As a result, the mechanical equipments will be reduced and also pre-investment expenses will drop. The biotechnological methods presented in this work will be expected to reduce the operational cost.

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