

Versatility of oxidoreductases in the remediation of environmental pollutants

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Brief review of oxidative enzymes and reaction mechanisms
 - 3.1. Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2)
 - 3.2. Tyrosinase (Tyros; E.C. 1.14.18.1, monophenol monooxygenase)
 - 3.3. Lignin peroxidase (LiP; EC: 1.11.1.14)
 - 3.4. Manganese peroxidase (MnP; EC 1.11.1.13)
 - 3.5. *Coprinopsis cinerea* peroxidase (CiP; EC 1.11.1.7)
 - 3.6. Versatile peroxidase (VP; EC 1.11.1.16)
 - 3.7. Heme-thiolate peroxidases
 - 3.8. Dye-decolorizing peroxidases (/DyP; EC 1.11.1.X)
 - 3.9. Secreted plant peroxidases (horseradish peroxidase (HRP, EC 1.11.1.7) and soybean peroxidase (SBP, EC 1.11.1.7)
4. Applications of oxidoreductases for environmental purposes
 - 4.1. Soil remediation
 - 4.2. Waste water treatment
 - 4.3. Decoloration of dyes
 - 4.3.1. Peroxidases in dye decoloration/degradation
 - 4.3.2. Laccases in dye decoloration/degradation
5. Perspective
6. Acknowledgements
7. References

1. ABSTRACT

Enzymatic transformation of recalcitrant and other pollutants is a promising eco-friendly alternative to physico-chemical methods in environmental remediation. This review summarizes some of the significant advances in applications of oxidative enzymes for treatment of xenobiotics. The review also discusses some of the underlying principles and enzyme reaction mechanisms in the detoxification or removal of xenobiotic compounds such as dyes, phenolic compounds, nitroaromatic compounds and polyaromatic hydrocarbons, as a way of remediating contaminated soils or wastewaters.

2. INTRODUCTION

Life on this planet depends entirely on the continuous recycling of elements among living organisms and their interaction with non-living things. Unfortunately, over the years, the massive mobilization of resources and the industrial synthesis of various chemicals have resulted in waste treatment problems leading to environmental pollution and limited incorporation of most of the synthesized molecules in the biological cycles. This is particularly true for many xenobiotic compounds which exhibit structural elements or substituents that are rarely found in nature. Thus environmental pollution is now a

major concern worldwide and has been linked to global warming, diseases and unbalanced ecosystem.

Nevertheless, advances in molecular biology, applied microbiology and enzyme technology over the past two decades have renewed hopes for the development of effective environmentally friendly processes for treatment of wastes at production sites as well as bioremediation of contaminated sites. This is driven by the desire to develop environmentally friendly waste processing technologies as alternative strategy to conventional chemical and physical methods. Enzyme-based methods are believed to have a minimal impact on ecosystems as compared to physicochemical methods. They also present some other interesting properties such as low energy requirements, easy process control and operation over a wide range of pH, temperature and ionic strength (1). For example, advances in metagenomics, a new emerging field that enables us to study microorganisms which were, until recently, unknown to us, is greatly helping scientists in identifying novel enzymes, since present estimations suggests that more than 99 % of the microorganisms in most ecosystems cannot be cultured in the laboratory (2,3) and thus very little is known about their enzymes.

Among enzymes being actively pursued for environmental purposes are oxidative enzymes. Oxidative enzymes are a large group of enzymes catalyzing oxidation/reduction reactions. The potential application of oxidative enzymes from bacteria, fungi and plants is increasingly being demonstrated and among these enzymes are peroxidases, laccases and phenoloxidases (tyrosinases) which are reviewed in this paper. Strategies being employed include activation of notorious xenobiotics and their subsequent coupling onto large polymers or polymerization thereby either immobilizing or detoxifying the xenobiotics or ring opening reactions (complete degradation of aromatic compounds) leading to less toxic or non toxic metabolites.

Given the tremendous effort over the past decade, this review summarizes some of the significant advances and state of the art applications of oxidative enzymes for treatment of xenobiotics. The review also discusses some of the underlying principles and enzyme reaction mechanisms in environmental detoxification of xenobiotic compounds such as dyes, nitroaromatic compounds, halogenated phenols and polyaromatic hydrocarbons.

3. BRIEF REVIEW OF OXIDATIVE ENZYMES AND REACTION MECHANISMS

3.1. Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2)

Laccase is a blue multicopper oxidoreductase capable of mono-electron oxidation of various substrates such as ortho- and para-diphenols, methoxy-substituted monophenols and aromatic and aliphatic amines while simultaneously reducing molecular oxygen to water. Structurally, laccases are monomeric, dimeric or tetrameric glycoproteins with four copper atoms (type 1 copper; type

2 copper and two type 3 copper atoms) at the active site of each monomeric unit. They are widely distributed in plants, prokaryotes and fungi (4,5,6,7,8) and have also been isolated in insects (9).

Laccase catalysis is accomplished by the cluster of the copper atoms in the active site. Type I copper, which has a higher oxidation potential, is the site where substrate oxidation occurs by removal of electrons to form radicals. Type 2 and Type 3 copper atoms form a trinuclear center to which the electrons are transferred and where reduction of molecular oxygen to water takes place. A full cycle of reaction involves oxidation of four substrate molecules to four radicals and concomitant reduction of one molecule of oxygen (by four removed electrons) to two molecules of water. The radicals can then undergo several reactions which include radical coupling to form dimers, oligomers or polymers, or radical mediated degradation of complex compounds or polymers by cleavage of covalent bonds.

3.2. Tyrosinase (Tyros; E.C. 1.14.18.1, monophenol monooxygenase)

Tyrosinase is a glycoprotein which contains a type 3 dinuclear copper in its active site. It catalyzes the *o*-hydroxylation of monophenols to yield *o*-diphenols (cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase activity). Like laccases, tyrosinases are ubiquitous in nature; they have been isolated in bacteria, fungi, plants and animals.

The catalytic mechanism of tyrosinase has been comprehensively reviewed (10). The model involves the three copper states of the active site and is based on an associative ligand substitution at the active site (10). Both the cresolase and catecholase activity use molecular oxygen. In a full cycle of the reaction, molecular oxygen is incorporated during hydroxylation to give an *o*-diphenol while the catecholase activity oxidizes two *o*-diphenols to two *o*-quinones with concomitant four electron reduction of molecular oxygen to two molecules of water.

3.3. Lignin peroxidase (LiP; EC: 1.11.1.14)

Lignin peroxidase (LiP) is a heme-containing glycoprotein oxidoreductase capable of catalyzing the oxidation of phenols and aromatic amines, aromatic ethers and polycyclic aromatic hydrocarbons. The bulk of LiPs in use today in processes such as bioremediation (11,12) and lignin degradation (13,14,15) are produced from the fungus *Phanerochaete chrysosporium* where it is secreted during secondary metabolism as a response to nitrogen limitation. However, many other fungi are also known to produce the enzyme (16).

The catalytic cycle involves initial oxidation of the enzyme by H₂O₂ to form a two-electron deficient intermediate termed compound I which comprises an Fe (IV) oxoferryl centre and a porphyrin-based cation radical. Compound I then oxidizes substrates by one electron and forms compound II, a more reduced Fe (IV) oxoferryl intermediate which can in turn oxidize substrates by one electron and return the enzyme to its resting state (17).

3.4. Manganese peroxidase (MnP; EC 1.11.1.13)

Manganese peroxidase (MnP) is a heme-containing glycoprotein oxidoreductase which preferentially uses Mn^{2+} as the electron donor (reductant). A comprehensive review of sources of this enzyme shows that MnP production appears to be limited to certain basidiomycetous fungi (18) with no records of isolation in bacterium, yeast mold or mycorrhiza-forming basidiomycete (19). The first phase of the catalytic cycle involving 2 electron oxidation of enzyme by H_2O_2 to compound I and subsequent reduction to compound II by oxidation of suitable substrate (various monomeric and dimeric phenols, including phenolic lignin model compounds) or Mn^{2+} , is similar to that of other peroxidases. However, unlike other peroxidases, compound II strictly requires Mn^{2+} as electron donor (20).

3.5. *Coprinopsis cinerea* peroxidase (CiP; EC 1.11.1.7)

Coprinopsis cinerea peroxidase is a haem-containing extracellular peroxidase isolated from the ink cap fungus *Coprinopsis cinerea* (*Coprinus cinereus*). It readily oxidizes phenols and smaller dye molecules but unlike LiP it is unable to oxidize veratryl alcohol (21) and lacks the Mn^{2+} binding site specific for MnP and VP (22,23). The purification, crystallization and structural characterization of CiP were first reported by Morita *et al.* (24). Since then several studies have been carried out to further characterize and classify the enzyme and details of its structure, function and classification have been recently reviewed (12).

3.6. Versatile peroxidase (VP; EC 1.11.1.16)

Versatile peroxidase is a hybrid-type haem-containing enzyme that combines the catalytic functions of MnP and LiP in that they can oxidize Mn^{2+} as well as high redox-potential compounds such as Reactive Black 5. Catalytic and physical properties have shown that the multiple catalytic properties are due to the co-existence of different catalytic sites on the same enzyme. The catalytic cycle of the enzyme combines activities reminiscent of both MnP and LiP. At the lignin-peroxidase site, the enzyme is capable of Mn^{2+} -independent oxidation of various organic compounds including high redox potential non-phenolic aromatics and dyes following the catalytic cycle similar to that of LiP as described earlier. Due to steric hindrance, long range electron transfer (LRET) from a protein radical at the surface of the enzyme (which would act as the substrate oxidizer) to the haem cofactor, has been suggested to explain oxidation of large compounds such as the lignin polymer, large aromatic substrates or complex redox mediators (25). At the manganese oxidation site Mn^{2+} is oxidized to Mn^{3+} using the MnP reaction mechanism. The generated Mn^{3+} acts as a diffusible oxidizer of phenolic lignin and free phenols and has also been reported to initiate lipid peroxidation reactions which may be implicated in the biodegradation of recalcitrant materials (25,26).

3.7. Heme-thiolate peroxidases

The most relevant enzymes in this group include *Caldariomyces fumago* chloroperoxidase (CfuCPO; EC 1.11.1.10) and aromatic peroxigenases (APOs). The most

significant activity of these enzymes in environmental pollution control is their ability to transfer peroxide-borne oxygen to diverse substrates. For example, Hofrichter and co-workers have reported the transfer of peroxide oxygen to recalcitrant aromatics, heterocycles, and ethers (27,28). The main activity of *C. fumago* chloroperoxidase (CfuCPO) is the oxidation of chloride (Cl^-) into hypochlorous acid which in turn acts as a strong oxidant and can chlorinate organic compounds (12). However, in the absence of halides, CfuCPO directly oxidizes phenols and anilines through a peroxidative mechanism (29). Of huge environmental importance is the oxidative dehalogenation of highly chlorinated derivatives (e.g., pentachlorophenol, PCP) into insoluble polymeric materials which occurs at about one to two magnitudes faster than the reactions of HRP, LiP, or VP (30). The oxidation reactions of CfuCPO follow the typical cycle of heme peroxidases involving compound I and II formation as explained previously (31,32). In addition, CfuCPO and APOs can catalyze epoxidation reactions by adding oxygen to double bonds of unsaturated compounds such as alkenes and cycloalkenes while APOs are also able to epoxidize and hydroxylate aromatic rings (12). These reactions are important in conversion of recalcitrant materials to epoxides which are important substrates in organic synthesis.

3.8. Dye-decolorizing peroxidases (EC 1.11.1.X)

Dye-decolorizing peroxidases (DyP-type peroxidases) comprise a new superfamily of heme-containing glycoproteins that oxidize anthraquinone-type dyes and their derivatives in addition to typical peroxidase substrates (33,34). In addition, some DyPs are able to oxidize aromatic sulphides (35), nonphenolic β -O-4 lignin model compound (36) as well as cleave β -carotene and other carotenoids (37). The enzyme was first identified in the fungus *Geotrichum candidum* Dec 1 (later assigned to the phytopathogenic basidiomycete *Thanatephorus cucumeris*) and has also been isolated in bacteria (12,38,39).

The typical characteristic of DyP-type peroxidases is their ability to oxidize the high-redox potential anthraquinone-type dyes which are hardly oxidized by other peroxidases (39). This has led to the suggestion that they incorporate hydrolytic fission capability to normal peroxidase function (33,34), making them extremely important in the bioremediation of high redox potential anthraquinone-type dyes. They are also structurally different from all other peroxidases - show little sequence similarity (0.5–5%) to classic fungal peroxidases and lack the typical heme-binding region, which is conserved in the plant peroxidase superfamily (one proximal Histidine, one distal Histidine, and one essential Arginine) (33,34,40).

3.9. Secreted plant peroxidases (horseradish peroxidase (HRP, EC 1.11.1.7) and soybean peroxidase (SBP, EC 1.11.1.7))

The most important plant peroxidases in bioremediation are horseradish peroxidase (HRP, EC

Oxidoreductases for environmental purposes

Table 1. Application of oxidative enzymes in soil remediation

Chemical	Mode of remediation	Enzyme: source of enzyme	Reference
TNT	Oxidative coupling to guaiacol	Laccase: <i>Pyricularia oryzae</i> , <i>Trametes versicolor</i>	(51)
	Oxidative coupling to catechol	Laccase: <i>Trametes villosa</i> , <i>Trametes modesta</i>	(52,53,54,55)
	Oxidative coupling to Syringaldehyde, syringic acid, protocatechuic acid, phenol, or caffeic acid	Laccase: <i>T. villosa</i> , <i>T. modesta</i>	(53,54,55)
	Coupling to ferulic acid, guaiacol	Laccase: <i>T. modesta</i>	(54,55)
Halogenated phenols	Coupling to soil organic matter; oxidative coupling accompanied by dehalogenation	Laccase: <i>T. versicolor</i> , <i>Pycnoporus cinnabarinus</i> , <i>Pyricularia oryzae</i> , <i>T. villosa</i> , <i>Rhizoctonia praticola</i>	(56,57,58,59,60,61,62,63,64,65,66)
		Manganese peroxidase: shepherd's purse root	(67)
		Horseradish peroxidase: horseradish roots	(65,68)
		Tyrosinase: mushroom	(65)
Chlorinated phenols	Oxidative coupling accompanied by dehalogenation	Horseradish peroxidase: horseradish	(63)
		Tyrosinase: mushroom	
4 Chloroaniline	Coupling to syringic acid, ferulic acid, vanillic acid, or guaiacol	Laccase: <i>R. praticola</i>	(62)
Halogen-, alkyl-, and alkoxy-substituted anilines	Coupling to guaiacol	Laccase: <i>Ceriporiopsis Subvermispora</i> , <i>Phanerochaete chrysosporium</i>	(69)
	Oligomerisation	Laccase: <i>R. praticola</i> and <i>T. versicolor</i>	(70)
Dichlorodiphenyltrichloroethane (DDT)	Degradation of DDT	Laccase: <i>T. versicolor</i> , <i>R. praticola</i> (only active with p-methoxyaniline),	(71)
		Laccase: white rot (polyporous)	(72)
Bisphenol A, nonylphenol	Polymerization and precipitation to water insoluble products	Laccase: fungus (family <i>Chaetomiaceae</i>)	(73,74)
1-Naphthol, 2-naphthol	Polymerization	Laccase: <i>Polyporus pinsitus</i> , <i>Myceliophthora thermophila</i> , <i>Coprinus cinereus</i> , <i>Rhizoctonia solani</i>	(75)
Herbicide Dymron; postharvest fungicide imazalil	Mediator assisted degradation	Laccase: <i>Trametes</i> sp.	(76,77)
Herbicide Bentazon	Coupling to humic monomers guaiacol and ferulic acid	Laccase: <i>Polyporus pinsitus</i>	(78)
2,5-Diaminobenzene sulfonic acid	Oxidative coupling to catechol	Horseradish peroxidase: Horseradish	(79)
Polycyclic Aromatic Hydrocarbons (PAHs): acenaphthene, acenaphthylene, anthracene, and fluorene Nitrobenzene anthracene	Mediator assisted oxidative degradation	Laccase: <i>T. villosa</i>	(80,81,82,83,84)
		Laccase: <i>Trametes trogii</i> , <i>T. versicolor</i> , <i>Nematoloma frowardii</i>	
		Lignin peroxidase, Manganese peroxidase: <i>Nematoloma frowardii</i>	(84)
		Horseradish peroxidase: horseradish	
Catechol, methyl catechol, tyrosol, hydroxytyrosol	Oxidative degradation	Tyrosinase: mushroom	
		Laccase: <i>Cladosporium sphaerospermum</i>	(85)
		Manganese peroxidase, Lignin peroxidase: <i>Phanerochaete laevis</i> <i>P. chrysosporium</i>	(86)
Halogenated pesticides (pentachlorophenol, dichlorophen, bromoxynil)	ABTS mediated oxidation/ Simple oxidation	Laccase: <i>Cerrena unicolor</i>	(87)
	Oxidative transformation	Versatile peroxidase: <i>Bjerkandera adusta</i>	(88)

1.11.1.7) and soybean peroxidase (SBP, EC 1.11.1.7). They are both heme-containing enzymes that utilize hydrogen peroxide to oxidize a wide variety of organic and inorganic compounds. HRP is secreted from horseradish (*Armoracia rusticana*) roots and is now produced on a large scale for commercial purposes. It is the most widely used of the plant peroxidases mainly because of its high stability in aqueous solution (41). SBP is obtained from soybean seed coats. SBP has, until recently, been neglected despite being a viable alternative to HRP; it shows an unusually high thermal stability and is less susceptible to haem loss (42) and permanent inactivation by hydrogen peroxide (43) than

HRP. SBP and HRP are homologous - very similar in their amino acid sequence and overall three-dimensional structure (including active sites) (44). However, SBP has higher haem affinity than HRP (42) which explains its higher thermal stability. They also have the same catalytic mechanism, which is similar to that of other peroxidases. The first step in the catalytic cycle is the reaction between H_2O_2 and the Fe (III) resting state of the enzyme to generate compound I, a compound which is two oxidizing equivalents above the resting state. In the next step, a reducing substrate reduces compound I by one electron reduction to compound II which is one oxidizing equivalent

Oxidoreductases for environmental purposes

Table 2. Application of oxidative enzymes in waste water treatment¹

Pollutant removed	Mode of remediation	Enzyme: source of enzyme	References
Phenol	Polymerisation and precipitation of simple phenols	Laccase: <i>Panus tigrinus</i> , <i>Coriolus versicolor</i> , <i>Trametes pubescens</i> , Basidiomycete Euc-1	(89,90,91,92,93)
		Coprinus cinereus peroxidase: <i>Coprinus cinereus</i>	(94,95)
	Precipitation and degradation	Laccase: <i>Streptomyces psammoticus</i>	(96)
	Degradation in presence of mediator (HBT)	Laccase: <i>Coriolopsis rigida</i>	(97)
	Precipitation	Manganese peroxidase: <i>Panus tigrinus</i>	(89)
		Tyrosinase: <i>Agaricus bisporus</i>	(98)
Phenol and aromatic amine	Enzymatic treatment and precipitation by a coagulant (chitosan)	Tyrosinase: mushroom	(99,100,101,102)
Polycyclic Aromatic Hydrocarbons (PAHs): acenaphthene, acenaphthylene, anthracene, and fluorene Nitrobenzene anthracene	Mediator assisted oxidative degradation	Laccase: <i>Trametes trogii</i> , <i>T. versicolor</i> , <i>Nematoloma frowardii</i>	(80,81,82,83,84)
		lignin peroxidase, manganese peroxidase: <i>N. frowardii</i> Horseradish peroxidase: horseradish Tyrosinase: mushroom	(84)
PAHs: anthracene, phenanthrene, fluorine, pyrene, fluoranthene and perylene	Oligomerisation through oxidative coupling	Laccase: <i>Pleurotus ostreatus</i>	(103)
	Oxidative degradation	Manganese peroxidase, Lignin peroxidase: <i>P. laevis</i> , <i>P. chrysosporium</i>	(86,104)
Bisphenol A, triclosan nonylphenol, octylphenol, ethynylestradiol	Mediator assisted Oxidative transformation	Laccase: <i>Clavariopsis aquatica</i> , fungal strain coded as UHH 1-6-18-4; <i>Coriolopsis polyzona</i> , <i>T. villosa</i> , <i>Pycnoporus coccineus</i> , <i>T. versicolor</i> , <i>P. ostreatus</i>	(59,74,105,106,107,108, 109,110,111,112,113,114, 115,116,117)
Bisphenol A	Oxidative polymerization	Coprinus cinereus peroxidase: <i>Coprinus cinereus</i>	(118)
1-Naphthol, 2-naphthol	Polymerization	Laccase: <i>T. versicolor</i> , <i>Pyricularia oryzae</i> , <i>R. praticola</i> ; <i>Polyporus pinsitus</i> , <i>Myceliophthora thermophila</i> , <i>Coprinus cinereus</i> , <i>Rhizoctonia solani</i>	(64,75,119,120)
Herbicide Dymron	ABTS-mediated degradation	Laccase: <i>Trametes</i> sp.	(76)
Fungicide Imazalil	Mediator-assisted degradation		(77)
Phenolic effluent	Oxidative coupling to form insoluble precipitates/ mediator assisted degradation	Laccase: <i>T. versicolor</i> , <i>T. villosa</i> , <i>Lentinula edodes</i> <i>Botrytis cinerea</i> , <i>Lentinula edodes</i>	(121,122,123,124,125, 126,127,128)
Alkylated, hydroxylated, halogenated and amino phenols	Oxidative coupling to form insoluble precipitates	Coprinus cinereus peroxidase: <i>C. cinereus</i>	(94)
Benzyl alcohol and 1-phenylethyl alcohol	TEMPO-mediated oxidation	Laccase: <i>T. versicolor</i>	(129)
Halogenated phenols	Oxidative dehalogenation	Laccase: <i>C. versicolor</i> , <i>T. villosa</i>	(66,87,130)
Chlorinated phenols	Coupling to coniferyl alcohol/phenol	Laccase: <i>R. praticola</i> and <i>C. unicolor</i>	(131,132)
2,5-Diaminobenzene sulfonic acid	Oxidative coupling to catechol	Laccase: <i>T. villosa</i>	(79)
Syringaldazine, o-dianisidine, and 2,6-dimethoxyphenol	Oxidative polymerization	Laccase: <i>P. ostreatus</i>	(133)
Acetaminophen	Oxidative oligomerisation	Laccase: commercial (Sigma-Aldrich)	(134)
Guaiacol, catechol and <i>m</i> -cresol, xlenol	Oxidative precipitation	Laccase: Novozym 51003 from genetically modified <i>Aspergillus</i> spp; <i>Trametes</i> sp.; <i>T. versicolor</i>	(135,136,137)
<i>N,N'</i> - (Dimethyl)- <i>N</i> - (2-hydroxyphenyl)urea	Oxidative precipitation	Laccase: <i>T. versicolor</i>	(138)
Catechol, methyl catechol, tyrosol, hydroxytyrosol	ABTS mediated oxidation	Laccase: <i>C. unicolor</i>	(87)
Polychlorinated biphenyls (PCBs)	Oxidative degradation	Laccase, Lignin peroxidase, manganese peroxidase: <i>P. chrysosporium</i> , <i>T. versicolor</i> , <i>Coriolopsis polyzona</i> , and <i>P. ostreatus</i>	(139)

¹Treatment of dye effluents not included

Oxidoreductases for environmental purposes

above the resting state. A second one-electron reduction step, involving oxidation of another substrate, returns compound II to the resting state of the enzyme (45).

4. APPLICATIONS OF OXIDOREDUCTASES FOR ENVIRONMENTAL PURPOSES

The ability of oxidoreductases to catalyze coupling reactions through C-C, C-O- and C-N-coupling (sometimes coupled with polymerization reactions, demethylations and dehalogenations) or to create reactive radicals which can cleave covalent bonds forms the basis for their application in environmental pollution control. These reactions allow the enzymes to act on specific recalcitrant pollutants by precipitation and transforming to other products which are either non-toxic, less-toxic or can easily be removed in subsequent treatment procedures. Several reports have therefore been written on potential application mainly in soil bioremediation and industrial waste water remediation. Although intracellular systems that are generally present in most fungi, such as cytochrome P-450 monooxygenase, may also be involved in organopollutant degradation (46), ligninolytic enzymes produced by white rot fungi are active extracellularly, making them better candidates for the bioremediation of highly apolar pollutants than non-ligninolytic microorganisms (47). Therefore the main enzymes that have been utilized are laccases and secreted peroxidases while limited applications have been carried out with tyrosinases and catechol oxidases. The main applications of oxidoreductases reviewed here are in the areas of soil remediation, waste water treatment and dye decoloration using mainly peroxidases and laccases.

4.1. Soil remediation

Anthropogenic land pollution has become a serious issue especially in this 21st century, mainly due to an increase in environmental consciousness and the manifestations of its effects. Industrial activities, munitions waste and agricultural practices have been identified as the three main sources of pollution (48). Although various industrial processes have improved human life, many of the products and by-products contain toxic materials which accumulate in soils – many of which are carcinogenic and/or mutagenic (49). This has necessitated research into enzymatic modification of toxic materials to less or non-toxic materials or their coupling to other molecules rendering them less toxic or easier to degrade. Following suggestions by Bollag and Liu (50) that laccase can mediate xenobiotic binding and incorporation into the humus, several studies have reported successful coupling of xenobiotics to humic substances using oxidative enzymes as a way of remediating soils contaminated with toxic compounds. Table 1 summarizes some of the reports on the potential application of oxidative enzymes in soil remediation.

Research findings have demonstrated that both chlorinated phenols and anilines can be dechlorinated by oxidative coupling mediated by laccase or HRP (63). However, in tyrosinase-mediated polymerization of chlorophenols, chloride ions were removed from the

formed o-quinones during nucleophilic attack by phenoxide ions (63). Ahn *et al.* (60) demonstrated the oxidative dehalogenation of 2,4-dichlorophenol using immobilized laccase. Although much less enzyme was required than with free laccase, there was a 23% loss in enzyme activity during the immobilization process (60).

4.2. Waste water treatment

Waste water treatment has recently become an area of major scientific interest as indicated by the large quantity of related research output. Although a number of technologies have been developed, only a few have been accepted by industry mainly because they are either energy intensive and therefore expensive, unsustainable or detrimental to the environmental. Therefore biotechnological applications in particular oxidative enzymes have been attracting a lot of interest as alternative technologies in water treatment or as pretreatment agents. The bulk of recalcitrant compounds in wastewaters are phenolic compounds many of which originate from industrial processes such as chemical plants, pulp and paper plants, food industries, petroleum refining, coking and coal conversion, and foundries. Successful bioremediation of phenolic wastewater by oxidative enzymes relies on many factors but mainly oxidative oligomerisation or precipitation to water insoluble products which are removed by sedimentation or filtration while in mediator assisted systems xenobiotics are removed mainly by oxidative degradation. Research reports in this regard have continued to increase over the years. Table 2 summarizes research output over the last two decades. Remediation of dye effluents has been deliberately excluded as it is covered separately in section 3.3.

4.3. Decoloration of dyes

Dyes are now an integral part of industrial wastewaters due to extensive usage mainly in the textile, paper, printing, and ceramic industries. It is estimated that between 2 and 15% (140,141,142) of the 7×10^5 tonnes of 100 000 different dyestuffs produced annually (143) may be found in wastewater. Many of the dyes have a synthetic origin and complex aromatic molecular structures which make them inert and difficult to biodegrade when discharged into waste streams (144). The removal of these potentially carcinogenic (145,146,147,148) dyes from industrial effluents is, therefore, one of the most significant environmental problems. Of the biotechnological methods currently being investigated for dye removal, ligninolytic enzymes such as laccases, MnPs and LiPs are being extensively investigated because of the structural similarity of most of the commercially available dyes to lignin constituents. Consequently much research has been published reporting dye removal using these enzymes.

4.3.1. Peroxidases in dye decoloration/degradation

It has been suggested that the oxidation of a dye such as azo dye by peroxidases such as LiP at the phenolic group to produce a radical at the carbon bearing the azo linkage, followed by attack by a water molecule at this phenolic carbon and subsequent cleavage of the molecule, is the basis of dye degradation by peroxidases. A number of

Oxidoreductases for environmental purposes

Table 3. Application of peroxidases in dye degradation

Dye removed	Enzyme	Source of enzyme	Reference
Polymeric dyes	Phenol oxidase Lignolytic enzymes	<i>P. chrysosporium</i>	(149,150)
Crystal Violet triphenylmethane dyes (Pararosaniline, Cresol Red, Bromphenol Blue, Ethyl Violet, Malachite Green, and Brilliant Green)	Lignin peroxidase Horseradish peroxidase	<i>P. chrysosporium</i> Horseradish	(150,151,152)
Azo dyes (Orange II, Tropacolin O, Crocein Orange G and Congo Red), and the heterocyclic dyes e.g. Azure B	Lignin peroxidase Manganese peroxidase	<i>P. chrysosporium</i>	(142,150,153,154,155, 156,157)
Sulfonated azo dyes	Lignin peroxidase, Manganese peroxidase,	<i>P. chrysosporium</i> <i>Streptomyces chromofuscus</i>	(158,159)
Anthraquinone-type dyes, metal complex and Indigo	Lignin peroxidase	<i>P. chrysosporium</i>	(142)
Anthraquinone-type dye (Remazol Brilliant Blue R)	Lignin peroxidase Horseradish peroxidase Manganese peroxidase	<i>P. ostreatus</i> <i>T. versicolor</i> Horseradish <i>Irpex lacteus</i>	(160,161,162,163)
Azo, diazo and anthraquinone -type dyes	Unique dye-decolorizing peroxidase, DyP	<i>Thanatephorus cucumeris</i> Dec 1	(33,39)
	Horseradish peroxidase	Horseradish	(39)
	Versatile peroxidase	<i>T. cucumeris</i> Dec 1	(164)
	Manganese peroxidase	white rot fungus, strain L-25	(165)
	Manganese peroxidase, lignin peroxidase	<i>Bjerkandera adusta</i> , <i>Pleurotus eryngii</i>	(166)
Methylene Blue	Lignin peroxidase, Horseradish peroxidase (less efficiently)	<i>P. chrysosporium</i> , Horseradish	(167,168)
Azo, anthraquinone-type, triphenyl methane (Crystal Violet, Bromophenol Blue, Brilliant Green), and Phthalocyanine dyes (Procion Brilliant Blue H-7G)	Plant peroxidase (unidentified)	Leaves of <i>Ipomea palmate</i> and <i>Saccharum spontaneum</i>	(169)
Sulfonphthalein dyes (Phenol Red, <i>ortho</i> -Cresol Red, <i>meta</i> -Cresol Purple, Bromophenol Red, Bromocresol Purple, Bromophenol Blue, Bromocresol)	Manganese peroxidase manganese-independent peroxidase	<i>P. chrysosporium</i> , <i>P. ostreatus</i>	(170,171)
Azo dyes (Amaranth, Reactive Black 5 (RB5) and Cibacron Brilliant Yellow)	Manganese peroxidase	<i>T. versicolor</i>	(172,173)
Reactive Black 5 (RB5)	Versatile peroxidase	<i>Pleurotus eryngii</i>	(25,174)
Azo reactive dye (Reactive Red 120)	Manganese peroxidase	<i>Phanerochaete sordid</i>	(175,176)
Azo dyes, Direct Red-80 and Mordant Blue-9	Lignin peroxidase and manganese peroxidase	<i>P. chrysosporium</i>	(177,178,179)
Ponceau 2R, Malachite Green and Anthraquinone Blue	Manganese peroxidase	<i>T. trogii</i>	(180)
Stilbene dye Direct Yellow 11, methine dye Basazol 46L	Soybean peroxidase Horseradish peroxidase	Soybean Horseradish	(181)

researchers have, therefore, investigated the potential of peroxidases in dye degradation (Table 3).

The involvement of LiP in dye decoloration has been clearly demonstrated (142,150,151,153,154) while dyes such as Azure B (182) and Poly R (183) have been proposed as standard substrates for determination of LiP activity. However, the failure of LiP to decolorize Congo Red which was decolorized by whole cells suggested the involvement of other enzymes (153) while substantial degradation of Crystal Violet by non-ligninolytic (nitrogen-sufficient) cultures of *P. chrysosporium*, suggests the existence of another mechanism complementary to the lignin-degrading system (151). Although supplementation with optimum veratryl alcohol and H₂O₂ increased dye decoloration by LiP, the dyes investigated were not decolorized by MnP (142). However, a number of studies have reported dye decoloration mediated by MnP (156,170,171). In the oxidation of sulfonphthalein dyes, the MnP was shown to prefer the position of methyl group at *ortho* than at *meta* on the chromophore while rate of oxidation and hence decoloration was reduced by halogenations (171). Similarly, in azo dye decoloration, Amaranth and Reactive Black 5 were decolorized the most rapidly by MnP since they have a hydroxyl group in an *ortho* position and a sulfonate group in the *meta* position relative to the azo bond (172).

The mechanism for the degradation of dyes carrying a phenolic group has been proposed (158,159). Generally the phenolic hydroxyl group is oxidized by the peroxidase through abstraction of a hydrogen atom to create radicals which are either degraded or polymerized to other molecules; in the case of azo dyes, the azo linkages are cleaved in the process. Using LC-MS and GC-MS, Goszczynski *et al.* (159) elucidated the mechanism of

Oxidoreductases for environmental purposes

Table 4. Application of laccases in dye degradation

Dye removed	Source of laccase	Mediator	References
Phenolic azo dye (Methyl-, methoxy-, chloro-, and nitro-substituted derivatives of 4- (4 (prml)-sulfophenylazo)-phenol)	<i>Pyricularia oryzae</i>	None	(187)
Azo dyes (amaranth, Reactive Black 5 (RB5) and Cibacron Brilliant Yellow), anthraquinone-type dye (Remazol Brilliant Blue R)	<i>T. versicolor</i> <i>T. villosa</i>	None	(172,173,188)
Reactive Black 5, Acid Blue 25, Methyl Orange, Remazol Brilliant Blue B, Methyl Green and Acid Green 27	<i>Myceliophthora thermophila</i>	1-hydroxybenzotriazole (HBT)	(189)
Azo dye Reactive Black 5	<i>Pleurotus sajor-caju</i>	HBT	(190)
	<i>T. pubescens</i>	None	(191)
Anthraquinone-type dye (Remazol Brilliant Blue R)	Genetically modified <i>Aspergillus</i>	HBT, violuric acid (VA), phenothiazine-10-propionic acid	(192,193)
	<i>P. ostreatus</i>	None	(194)
Disazo dyes	<i>Aspergillus niger</i>	HBT or VA	(195)
	<i>Pycnoporus sanguineus</i> , <i>Pycnoporus cinnabarinus</i>	None	(196,197)
	<i>T. troglitii</i>	With or without HBT	(198)
	<i>P. ostreatus</i>	Ferulic acid	(199)
Acid Blue 74 (indigoid dye), Reactive Blue 19 (anthraquinoid dye), and Aniline Blue (triarylmethane-type dye) Reactive Black 5 (diazo dye) and Azure B (heterocyclic dye)	<i>P. cinnabarinus</i> , <i>T. villosa</i>	Lignin-Derived Compounds (2,6-dimethylphenol, 2,4,6-trimethoxyphenol, ethyl vanillin, vanillyl alcohol, <i>p</i> -coumaric acid, vanillin, acetovanillone, methyl vanillate, syringaldehyde and acetosyringone), synthetic mediators (ABTS, HBT, VIO, TEMPO, HNNS, NNDS, PZ, HAA).	(200)
Lignin	<i>P. eryngii</i> , <i>P. cinnabarinus</i> , <i>T. villosa</i> ; <i>Streptomyces cyaneus</i> <i>T. versicolor</i>	HBT and ABTS	(201,202,203)
		None	(204)
Azo dye Direct Red 28, the indigoid Acid Blue 74 and anthraquinonic dyes (Reactive Blue 19, Acid Blue 25)	<i>T. versicolor</i> , <i>Polyporus pinisitus</i> and the ascomycete <i>Myceliophthora thermophila</i>	HBT (a few without HBT)	(205)
Reactive Red 239, Reactive Yellow 15 and Reactive Blue 114; Reactive Red 180	genetically modified <i>Aspergillus</i>	None	(206,207,208)
Reactive Black 5, Reactive Yellow 176, Reactive Yellow 15, Reactive Red 239, Reactive Red 180, Reactive Blue 114	genetically modified <i>Aspergillus</i>	ABTS, HBT), <i>N</i> -hydroxyacetanilide (NHA), polioxometalates, VA, TEMPO	(209,210)
Reactive Blue 19	<i>Ganoderma</i> sp.	None	(211)
Reactive Blue 198 and Reactive Red 35	<i>Pleurotus florida</i>	None	(212)
Remazol Brilliant Blue R (RBBR, anthraquinoid dye) and Coomassie Brilliant Blue G-250 (CBB, triphenylmethane dyes), Acid Red (diazo dye), Indigo Carmine (indigoid dye) Azure A (heterocyclic dye).	<i>Pichia pastoris</i> (genetically modified)	Thymol, and VA	(213)
Acid Blue 62, Reactive Blue 81 Acid Blue 40 C.I. Direct Black 22 and Acid Red 27	<i>C. unicolor</i>	None	(214,215)
Diamond Black	<i>Sclerotium rolfsii</i>	None	(216)
Reactive Blue 198	<i>Coriolopsis gallica</i>	HBT	(217)
Indigo Carmine, Methyl Orange and Methyl Green	<i>Coriolopsis rigida</i> <i>T. villosa</i>	None HBT	(218) (79)
Drimarene Blue X3LR	<i>Funalia troglitii</i>	None	(219)
Remazol Brilliant Blue R (RBBR) and Drimaren Blue CL-BR	<i>P. ostreatus</i> , <i>C. versicolor</i> <i>F. troglitii</i> and <i>Irpex lacteus</i>	None	(163,220)
Reactive Orange 16, Naphtol Blue Black, Remazol Brilliant Blue R, copper (II)phthalocyanine Bromophenol Blue	<i>I. lacteus</i>	None	(221)
Anthraquinone-type dye SN4R	<i>P. ostreatus</i>	With or without ABTS	(222)
Indigo Carmine	<i>Streptomyces coelicolor</i>	Syringaldehyde	(223)

Oxidoreductases for environmental purposes

	<i>Sclerotium rolfsii</i> , <i>Trametes hirsuta</i> <i>T. villosa</i>		(224,225)
Azo dyes (Acid Orange 52, Acid Orange 5 and Direct Blue 71, Reactive Black 5 and Reactive Orange 16 and 107)	<i>T. modesta</i>	None	(226,227,228)
Chicago Sky Blue, poly B-411, Remazol Brilliant Blue R, Trypan Blue and Reactive Blue 2	<i>Daedalea quercina</i>	None	(229)
Luganil dye, Sella Solid Red	<i>T. hirsuta</i>	None	(230)
		ABTS, HBT	(231)
Azo-dye Diamond Black PV 20	<i>T. modesta</i>	None	(232)
Ranomill Yellow, Texacid Fast Red A	<i>Cyathus bulleri</i>	With or without ABTS	(233)
Triarylmethane, indigoid, azo, and anthraquinonic dyes	<i>T. hirsuta</i>	None	(234)
	<i>T. modesta</i>	HBT and 2-methoxyphenothiazine	(235)
Anthraquinone –type dyes (Reactive Blue 19 and Dispersed Blue 3), indigoid (Acid Blue 74), azo (Acid Red 27 and Reactive Black 5) dyes	<i>T. versicolor</i>	None	(236)
Remazol Brilliant Blue R, Remazol Black B, Reactive Orange 122 and Reactive Red 251	<i>T. versicolor</i>	None	(237)
Indigo Carmine, Phenol Red, Bromophenol Blue, Lanaset Marine, Malachite Green, Methyl Orange, Methyl Green	<i>T. hirsuta</i> , <i>T. versicolor</i>	None	(238,239,240,241,242,243,244)
Levafix Blue CA and Cibacron Blue FN-R	<i>Trametes</i> sp.	None	(245)
Lissamine Green B, Nickel (II) phthalocyanine, Lissamine Green B and Acid Black 48	<i>T. hirsuta</i>	None	(246,247)
Ponceau 2R, Malachite Green and Anthraquinone Blue	<i>T. trogii</i>	None	(180)
Blue Tubantin GLL 300, Black Tubantin VSF 600, Blue Solophenyl	<i>T. trogii</i>	None	(248)
Poly R-478	<i>T. versicolor</i>	None	(249)
Acid Fuchsin, Congo Red, Indigo Carmine	<i>T. versicolor</i>	None	(250)
Triphenylmethane dyes (Acid Fuchsin, Brilliant Green 1, Basic Fuchsin, Methyl Green, Acid Green 16)	<i>T. versicolor</i>	None	(251)
3- (4-Dimethylamino-1-phenylazo) benzenesulfonic acid, 3- (2-hydroxy-1-naphthylazo) benzenesulfonic acid	<i>T. villosa</i>	None	(252)
Stilbene dye Direct Yellow 11, methine dye Basazol 46L	<i>T. villosa</i>	ABTS, HBT, VA	(181,253)
Blue Paper Color	<i>T. villosa</i>	TEMPO	(254)
Derma Pardo 5 GL, Direct Black 168, Acid Red 119, Direct Blue 78, Acid Yellow 166	<i>T. hirsuta</i>	None	(255)
Grey Lanaset G	<i>T. versicolor</i>	None	(256)

peroxidase-catalyzed sulfonated azo dye degradation by peroxidases. Their findings showed that azo linkage is split both asymmetrically and symmetrically (Figure 1) to produce unstable intermediates which underwent further redox, oxidation, and hydrolytic transformation to more stable organic compounds and ammonia (Figure 2). Recently a mechanism for the degradation pathway of a hydroxyl-free anthraquinone-type dye by a unique peroxidase, DyP, was shown (33). Using LC-MS and nuclear magnetic resonance (NMR), it was shown that an anthraquinone-type dye, Reactive Blue 5 was oxidized to red-brown compounds, one of which was generated by peroxidase action, and phthalic acid which was produced through a hydrolase- or oxygenase-catalyzed reaction (33).

4.3.2. Laccases in dye decoloration/degradation

One of the most promising applications of laccase is in the decoloration of dye effluent. The wide substrate range of laccase which can be further widened by use of mediator systems makes them particularly useful in the remediation of wastewater contaminated with many different dye compounds. Consequently several studies have reported the involvement of laccase in dye decoloration (184,185,186). Some of the research output within the last two decades is summarized in Table 4.

Usually hydroxyl-free anthraquinone-type dye Remazol Brilliant Blue R require redox mediator for decoloration to occur (189,192,193), although some

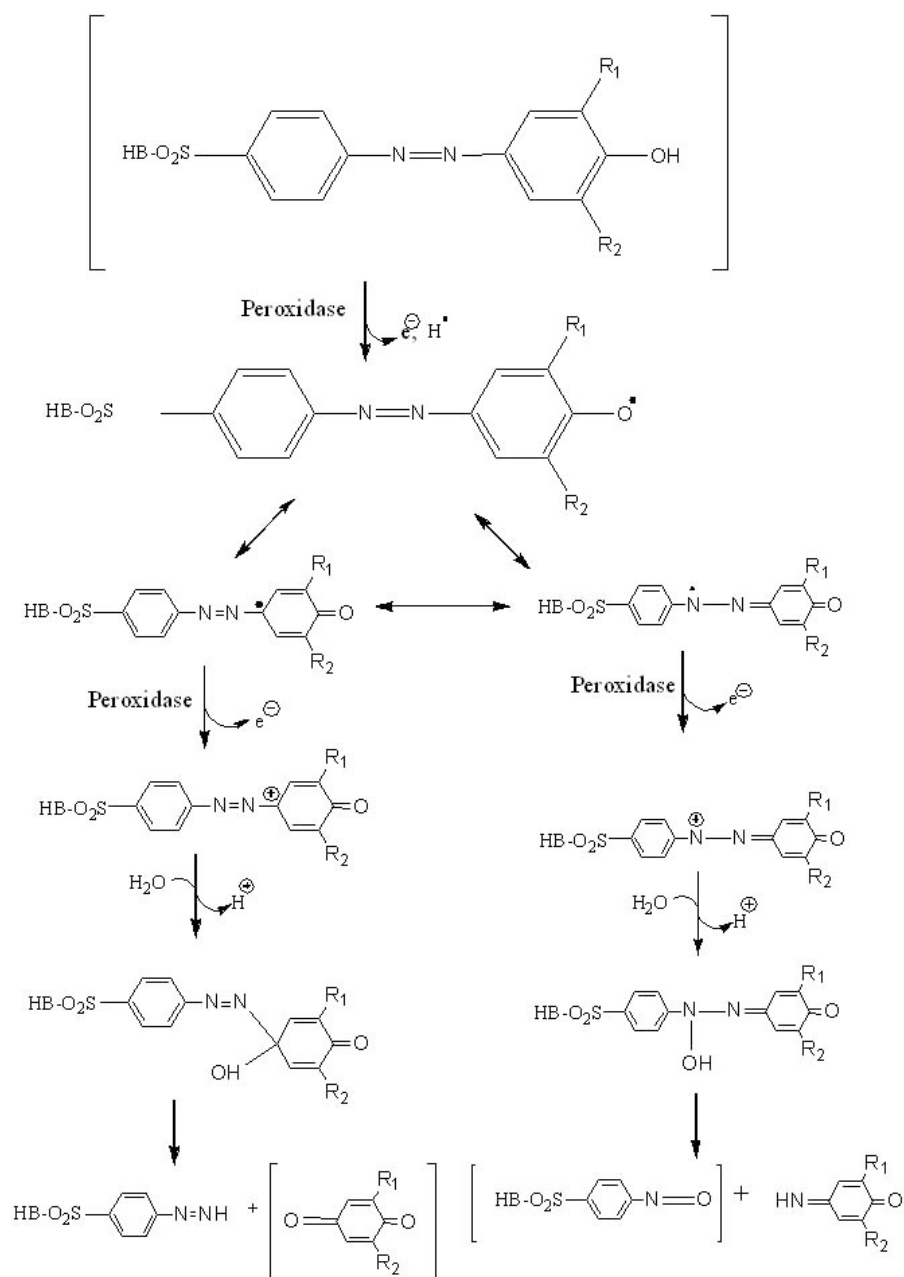
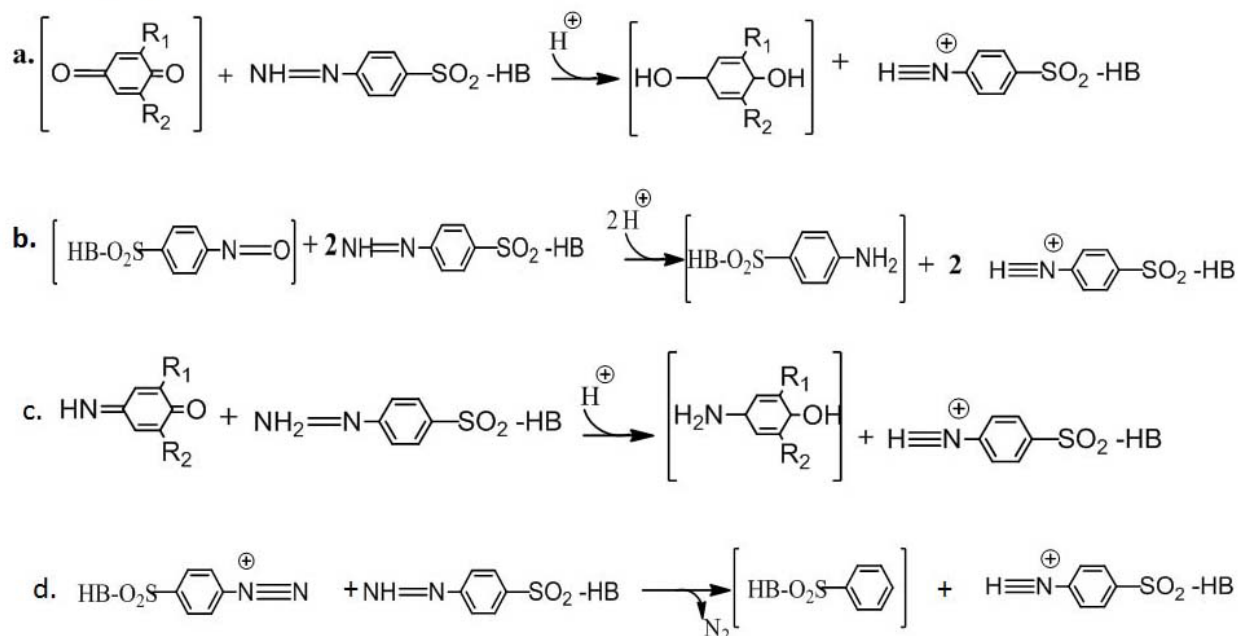


Figure 1. Proposed mechanism for degradation of sulfonated azo dyes by *P. chrysosporium* and *S. chromofuscus* peroxidases (ligninases). The compounds represented by structures in brackets were found in the reaction mixture. Azo dye 1, R= R₂ = CH₃ and B = O; azo dye 2, R = H, R₂ = OCH₃, and B = NH (159). Reproduced with permission from the American Society for Microbiology.

efficient laccases can work without mediators (196,198). Similarly a diazo Reactive Black 5 was not decolorized without mediator (189,190). It was suggested that the high-redox potential of the dye (1.4 V), together with steric hindrances which reduced the accessibility of the -NH₂ and -OH, were the reasons for failure to decolorize the dye (189). In contrast other anthraquinone-type dyes such as Acid Blue 25 and Acid Green 27 can be directly decolorized without mediators by high redox potential laccases (189,205).

Apart from mediator systems, ultrasound has been used successfully to complement laccase oxidation systems in azo dye degradation with volumetric scale-up showing a positive correlation between the energy input and the absolute amount of dye degraded (225,226 227,228). Although higher energy tend to inactivate the enzyme which must be compensated by higher doses of enzyme, it was shown that a combination of laccase and ultrasound treatments can have synergistic effects in dye degradation (225,228).

Redox processes



Hydrolysis

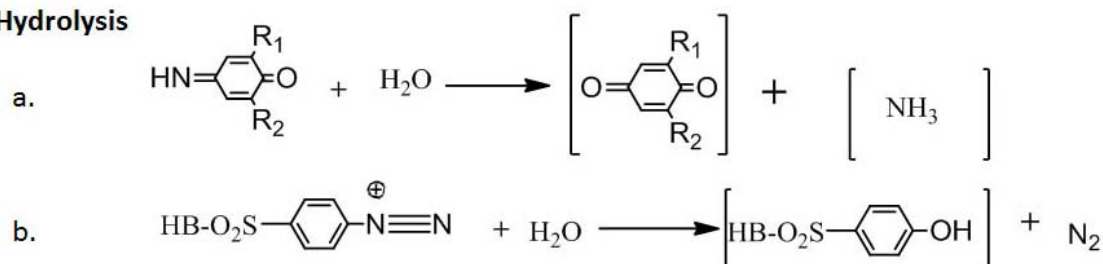


Figure 2. Transformations of the intermediate products of the initial sulfonated azo dye degradation. The compounds represented by structures in brackets were found in the azo dye reaction mixtures. Original azo dye 1, RI = R2 = CH₃ and B = O; azo dye 2, RI = H, R2 = OCH₃, and B = NH (159). Reproduced with permission from the American Society for Microbiology.

The mechanism of laccase-catalyzed azo dye oxidation is similar to that proposed for peroxidases (187,195,252). The azo dye undergoes one electron oxidation to form a phenoxy radical which is further oxidized to a carbonium ion in which the charge is localized on the phenolic ring carbon with the azo linkage (195,252). This is followed by a nucleophilic attack by water on the phenolic ring carbon bearing the azo linkage and subsequent cleavage of the molecule as shown in Figure 3 for a disazo dye (195).

5. PERSPECTIVE

Enzymatic transformation using oxidative enzymes is a promising environmentally friendly, energy efficient and potentially cost-effective way of environmental remediation. However, limited quantities of

commercially available robust and inexpensive enzymes and cheap environmentally friendly mediators are major barriers to the widespread application of oxidative enzymes in various industrial sectors. Many of the recalcitrant xenobiotics are also effective enzyme inhibitors while increasing doses of mediators beyond certain levels can also inactivate enzymes. Although remarkable progress has been made to solve these problems through for example, genetic engineering to increase enzyme quantities and biochemical and chemical modification to enhance stability, there are still challenges related to optimization of the processes since the enzymes will need to work in highly variable environments. More research especially focused on simulating contaminated environments is required so that enzymes which are active and stable over wide environmental conditions can be selected and produced in large quantities.

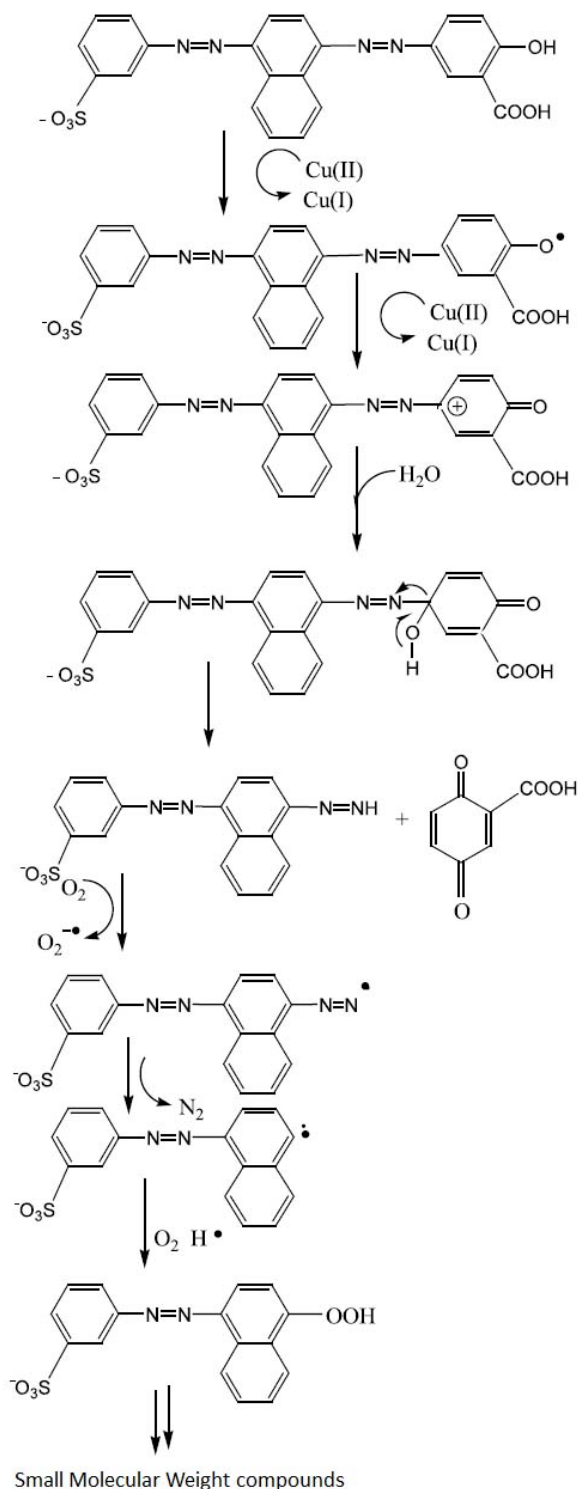


Figure 3. Proposed mechanism of degradation of disazo dyes by laccase (195). Reproduced with permission from Elsevier.

There has also been evidence that some products of biotransformation are actually more toxic than the starting material and hence the need to monitor toxicity of intermediates and final products of a bioremediation process. A recent research showed a reduction in toxicity of

azo and indigo-type dyes accompanied by an increase in toxicity of anthraquinone-type dyes during a laccase mediated decoloration process (236), emphasizing the importance of tailoring these processes to specific dyes. In addition, during prolonged azo dye decoloration, the

polymerization reactions of laccase can result in the formation of polymeric phenolics which retain azo group integrity and consequently unacceptable color levels, limiting the application of laccases as bioremediation agents (252). Thus research efforts need to be also directed at synergistic processes such as the recent efforts to complement laccase oxidation of dyes with use of ultrasound (227) or a use of a cocktail of different enzyme systems. For example a laccase from *Streptomyces psammoticus* which is stable at alkaline pH range of 6.5–9.5 (optimum 8.5) and capable of degrading phenolic compounds (96) can be combined with fungal laccases (active in acidic conditions) for treatment of wastes in highly variable conditions. A recent observation that the organism itself was more effective than laccase in degrading triphenylmethane dyes, with no aromatic residues detected after fungal treatment (251), emphasizes the potential of enzyme mixtures to mineralize dyes and other xenobiotics and also opens an opportunity to widen substrate range.

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Oxidoreductases for environmental purposes

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