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## Explorations and Applications of Enzyme-linked Bioremediation of Synthetic Dyes

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Additional information is available at the end of the chapter

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### Abstract

Extensive use of synthetic dyes and their subsequent release in industrial wastewater is a growing environmental problem. These dyes are recalcitrant in nature, and some dyes are also well established to be potentially carcinogenic and mutagenic as well as genotoxic. Research efforts have been devoted to develop new, low-cost, and eco-friendly treatments capable of reducing and even eliminating synthetic dye compounds from the environment. Enzymatic approach has attracted much interest recently in the decolorization of textile and other industrially important dyes from wastewater as an alternative strategy to conventional chemical, physical, and biological treatments, which pose serious limitations. In this chapter, the accumulated research data on the potential of the oxidoreductive enzymes—high redox potential peroxidases (lignin peroxidase [LiP], EC 1.11.1.14; manganese peroxidase [MnP], EC 1.11.1.13; dye decolorizing peroxidase [DyP], EC 1.11.1.19; and versatile peroxidases [VP], EC 1.11.1.16), laccases (benzenediol–oxygen oxidoreductase, EC 1.10.3.2), polyphenol oxidases (EC 1.14.18.1), and azoreductases (azobenzene reductases, EC 1.7.1.6)—that have been exploited in the decolorization and degradation of synthetic dyes are presented. An overview of enzyme technology, including the importance of redox mediators for enhanced range of substrates and efficiency of degradation, current biodegradation applications, and suggestions to overcome the limitations to these proteins' large scale and efficient use, is made. Different strategies currently being used and future prospects for the potential use of genetic engineering techniques to improve the performance of these oxidoreductases in terms of stability, selectivity, and catalytic activity in dye bioremediation technologies are also explored.

**Keywords:** Dye decolorization, oxidoreductases, enzyme immobilization, genetic engineering, bioremediation

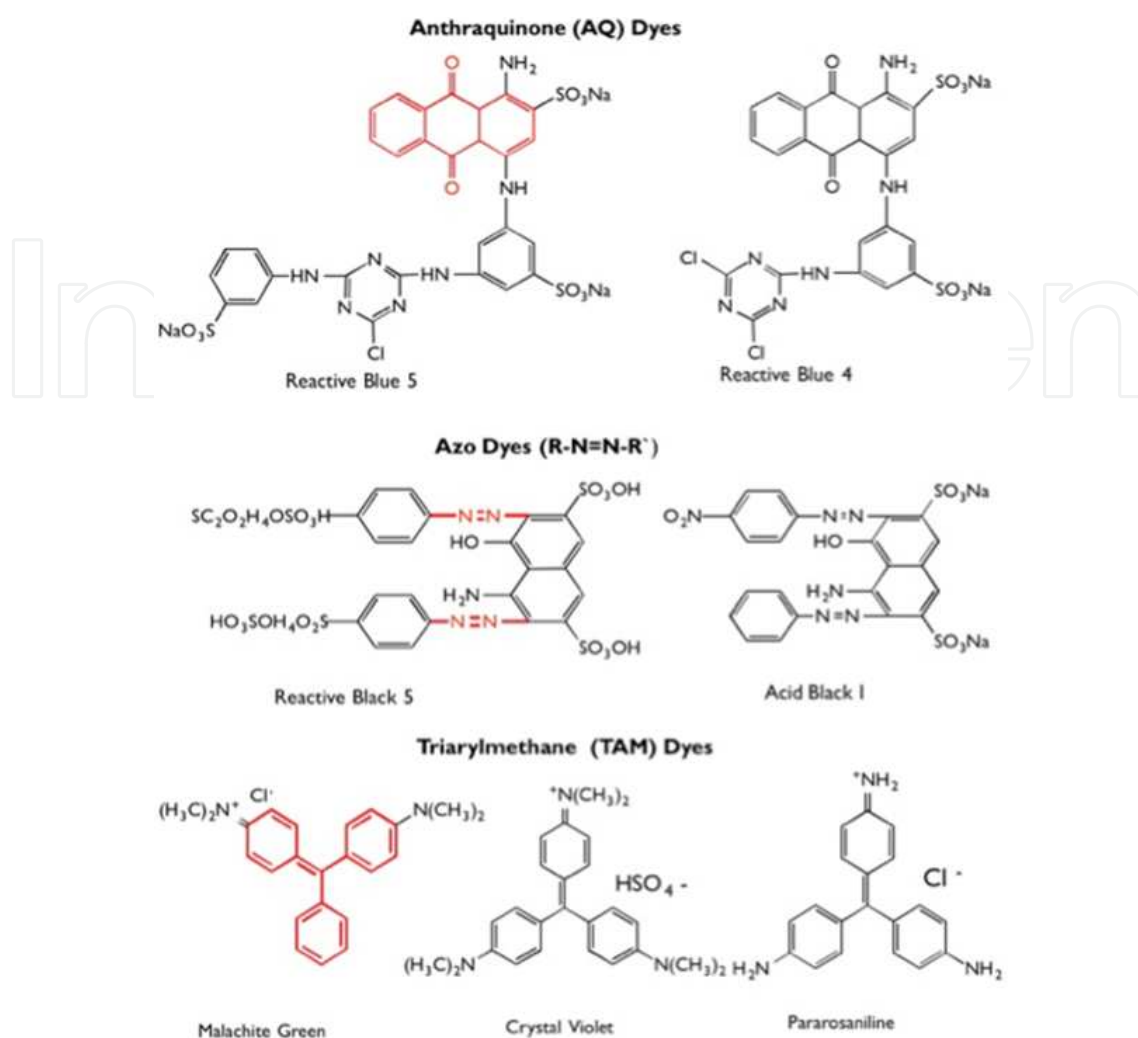
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## 1. Introduction

Dyes are compounds that absorb light with wavelengths in the visible range, i.e., 400 to 700 nm, thereby giving different colors [1]. Generally, dyes contain chromophores, delocalized electron systems with conjugated double bonds responsible for light absorption in dye molecules, and auxochromes, electron-withdrawing or electron-donating substituent that cause or intensify the color of the chromophore by altering the overall energy of the electron system. The chromophores include  $-C=C-$ ,  $-C=N-$ ,  $-C=O$ ,  $-N=N-$ ,  $-NO_2$ , and quinoid rings, whereas the auxochromes include  $-NH_2$ ,  $-COOH$ ,  $-SO_3H$ , and  $-OH$ . On the basis of chemical structure or chromophore, 20 to 30 different groups of dyes can be discerned [2]. Synthetic dyes are therefore named according to the chemical structure of the chromophoric group (azo dyes, anthraquinone dyes, indigoid dyes, xanthene dyes, triarylmethane dyes, etc.) [2, 3] or according to the dyeing method (their mode of binding to the fiber) as reactive dyes, direct dyes, and cationic dyes [4].

Azo ( $R-N=N-R'$ ), anthraquinone, and triphenylmethane dyes are quantitatively the largest classes of commercially produced colorants (Figure 1). Azo dyes make up approximately 70% of all dyes by weight and account for the majority (more than 3000 different varieties) of all textile dyes produced globally because their synthesis is easy and cost-effective, they are stable, and produce a wide variety of colors [2]. These dyes include at least one or more azo ( $R-N=N-R'$ ) double bond, with one or more aromatic systems, and classified into two subgroups according to number of their double bond as mono-azo and poly azo types [2]. However, these dyes are recalcitrant in the environment as the breakdown of azo bonds ( $R-N=N-R$ ) is quite difficult, and they can be stable in acidic and alkaline conditions. They are also resistant to high temperatures and light.

After azo dyes, anthraquinone compounds are the next most important textile dyes. These dyes are known for their good fastness and light fastness [2, 5] and a large range of colors, and they are commonly used to dye cellulosic fabric, wool, and polyamide fibers. Another group of dyes, the triphenylmethane dyes, e.g., malachite green, crystal violet, and pararosaniline, are characterized by the presence of chromogens, which contain three phenyl groups bound by a central carbon atom [2]. These dyes are extensively used for dyeing nylon, polyacrylonitrile-modified nylon, wool, silk, and cotton. They are also used by other dyestuff manufacturing industries as a biological stain and in printing paper [3]. Most of these dyes are stable against light, temperature, and biodegradation and therefore accumulates in the environment as recalcitrant compounds [1, 2, 4].



**Figure 1.** Chemical structure of major synthetic dye compounds showing their chromophoric groups (in red) and different auxochromes.

The increased demand for dyed products such as textiles coupled with the proportional increase in their production and the use of synthetic dyes have together contributed to dye wastewater becoming one of the substantial sources of severe pollution problems in current times [6]. Due to their synthetic origin and complex aromatic molecular structure, some of the dyes are thought to be toxic and mutagenic, resistant to biological degradation, and may accumulate in the food chain [2, 7]. In recent years, increased public concern and ecological awareness regarding the polluting properties of dyes have led to a stricter legislative control of wastewater discharge. This has led to increased interest in various methods of dye decolorization. Dye decolorization using physicochemical processes such as adsorption, adsorption on activated carbon, electrocoagulation, flocculation, froth flotation, ion exchange, membrane filtration, ozonation, and reverse osmosis and oxidation with ozone has proved to be effective [8, 9]. However, these processes are generally expensive, generate large volumes of sludge, and require the addition of environmentally hazardous chemical additives [5-7]. Research efforts have been devoted to develop new, low-cost, innovative, and eco-friendly treatments,

such as biological processes capable of reducing and even eliminating synthetic dye compounds from the environment.

## 2. Microbial bioremediation of synthetic dyes

Biological decolorization and degradation are an environmentally friendly, cost-competitive, and efficient alternative to physical/chemical decomposition [3, 6, 7, 10]. Decolorization by biological means may take place in either one of three ways: (1) adsorption (or biosorption) on the microbial biomass, (2) biodegradation by cells, and (3) biodegradation by enzymes. Biosorption involves the entrapment of dyes in the matrix of the adsorbent (microbial biomass) without destruction of the pollutant. In contrast, biodegradation involves the fragmentation of the original dye structure into smaller compounds, resulting in the decolorization of synthetic dyes. Several studies have described the use of microorganisms as biosorption agents in the removal of dye pollutants from wastewater [11, 12]. However, relative to the operational simplicity and adaptability of microorganisms to a given set of conditions, the biodegradation mechanism is considered efficacious and hence preferable to biosorption for treatment of dye wastewater [13].

There are numerous reports of microorganisms capable of decolorizing synthetic dyes: bacteria [14-16], fungi [17-19], yeasts [20, 21], actinomycetes [22, 23], and algae [24, 25]. Several fungi are capable of mineralizing pollutant compounds by action of their highly oxidative and nonspecific ligninolytic enzymes, which are also responsible for the decolorization and degradation of many different dyes. There are reports that white rot fungi (WRF), members of the basidiomycetes such as *Funalia trogii* [26], *Phanerochaete chrysosporium* [17, 18], *Trametes versicolor* [19], *Trametes hirsuta* [27], *Irpex lacteus* [28], and *Lentinula edodes* [29], can efficiently degrade xenobiotic textile dye compounds. Lignin-degrading fungi also degrades a wide range of aromatics owing to the relatively nonspecific activity of the extracellular ligninolytic enzymes, such as lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), and laccase.

Bacterial strains that can aerobically decolorize azo dyes have also been isolated during the past few years. However, there are only very few bacteria that are able to grow on azo compounds as the sole carbon source. The degradation of various azo dyes by mixed aerobic and facultative anaerobic microbial consortia under anoxic conditions has also been reported [30]. *Pseudomonas luteola*, *Aeromonas hydrophila*, *Bacillus subtilis*, *Pseudomonas* sp., and *Proteus mirabilis* can also decolorized azo dyes under anoxic conditions [30-32]. These bacteria are specific toward their substrate, cleaving R-N=N-R bonds reductively and using the resultant amines as a source of carbon and energy for their growth.

The efficiency of dye degradation is mainly dependent on the following: (1) type of microorganisms used; (2) process conditions such as temperature, oxygen level, pH, available carbon, and nitrogen sources that affects microbial growth; and (3) dye concentration and chemical structure [10, 33]. Dependence on microbial growth to metabolize the toxicants makes the bioremediation of dyes by these organisms a relatively slow process. Another drawback to the

microbial anaerobic reduction of synthetic dyes is its production of compounds such as carcinogenic aromatic amines [34, 35].

### 3. Major enzymes used in bioremediation of synthetic dyes

Over the last two decades, there has been increasing interest in the use of formulated enzymes rather than live bacteria as bioremediation agents owing to their specificity, and the ease with which their robustness can be enhanced with engineering [36]. Other advantages of using enzymes include increased enzyme production, enhanced stability and/or activity, and lower costs by using recombinant DNA technology. However, the high structural complexity of dye molecules means that only a few enzymes can degrade these compounds. These dye-degrading enzymes share one common mechanistic feature—they are all redox-active molecules that exhibit relatively wide substrate specificities. The most important dye-degrading enzymes are azoreductases, laccases, and peroxidases.

In this chapter, the accumulated research data on the potential of the oxidoreductive enzymes—high redox potential peroxidases, polyphenol oxidases, and azoreductases—that have been exploited in the decolorization and degradation of dyes are presented. The current initiatives and future prospects for the potential use of genetic engineering techniques to develop novel enzyme variants that are more durable and versatile biocatalyst, with respect to both the varieties of xenobiotics degraded and the operative conditions of dye bioremediation technologies are also discussed.

#### 3.1. Peroxidases

Peroxidases (oxidoreductases, EC 1.11.1.x) are a diverse group of versatile heme-containing enzymes that utilize hydrogen peroxide or organic hydroperoxides (R-OOH) electron acceptor to catalyze oxidation of numerous substrates. Due to their catalytic versatility and enzymatic stability, peroxidases are one of the most studied groups of enzymes, more so as potential industrial/environmental biocatalysts [37]. Several good reviews have summarized years of peroxidase research and described their potential applications [37-40].

Generally, peroxidases are ubiquitous in nature; found in fungi, plants, animals, and eubacteria; and are classified within different superfamilies on the basis of their sequence homologies; animal and nonanimal peroxidases (former plant peroxidases) form the largest groups. The plant superfamily is further grouped into three subclasses according to cellular localization: class I—intracellular, organelle-associated, and bacterial peroxidases (e.g., cytochrome *c* peroxidase [CCP]); class II—secretory fungal peroxidases, including ligninolytic peroxidases such as LiPs, MnPs, and VPs [41-43]; and class III—secreted plant peroxidases (horseradish peroxidase [HRP]) [37]. Among peroxidases, a new superfamily—“dye-decolorizing peroxidases”—has arisen. These enzymes are known to successfully oxidize a wide range of substrates, but most importantly, they effectively degrade high redox synthetic dyes such as anthraquinone and azo dyes. DyPs form a novel superfamily of peroxidases that are generally

characterized by “atypical” molecular architecture and divergent mechanistic behavior that is not fully understood yet but different from the classical peroxidases [37, 40, 41].

Structurally, peroxidases share several features, including the overall protein fold and the general architecture of the heme pocket with the high-spin ferric iron ( $\text{Fe}^{\text{III}}$ ) coordinated to the proximal histidine and the conserved distal histidine and arginine residues [38, 39]. These highly conserved residues in the heme catalytic pocket are critical for peroxidase activity, i.e., in the generation and stabilization of compound I and II. The functional and catalytic diversity of heme peroxidases is thereby attributable to specific structural differences around the heme-binding site, including the nature of the axial ligand, and the environment of the substrate-binding site.

Catalytically, peroxidases share a general reaction mechanism by using hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) as the final electron acceptor in the oxidation of a broad range of substrates (AH) to radicals ( $\text{AH}^\cdot$ ), which involves a three-step sequential mechanism via compound I and compound II intermediates [37, 38, 40, 42]. Initially, the native enzyme, which is in the ferric form  $\text{Fe}^{\text{III}}$ , undergoes two-electron oxidation by  $\text{H}_2\text{O}_2$  to produce compound I (oxoferryl porphyrinyl radical [ $\text{Fe}^{\text{IV}}=\text{O}^\cdot$ ]). One electron is removed from the ferric iron  $\text{Fe}^{\text{III}}$  to form the ferryl  $\text{Fe}^{\text{IV}}$ , whereas the second electron is withdrawn from the porphyrin ring to form a porphyrin cation radical [43]. During this reaction step,  $\text{H}_2\text{O}_2$  is reduced to water. Next, compound I oxidizes substrates by one electron and is reduced to compound II. In this step, the porphyrin ring gains an electron. Thus, compound I is able to oxidize substrates with a higher redox potential than compound II [38, 44]. The reduction of compound II, by a second electron of substrate, brings the enzyme back to the native state and completes the catalytic cycle.

In the absence of substrate and in the presence of excess  $\text{H}_2\text{O}_2$ , compound II is converted to compound III, a ferrous-oxy or ferric superoxide species. In this process,  $\text{H}_2\text{O}_2$  reduces compound II by one electron to produce a ferric enzyme and a superoxide radical. The latter readily combines with the ferric peroxidase to produce compound III. In the peroxidase catalytic cycle, the generation of highly reactive radicals that undergo a complex series of spontaneous cleavage reactions accounts for the degradation ability of peroxidase toward numerous substrates, e.g., phenolic and nonphenolic aromatics, metal ions, and complex dyestuff molecules [45].

### 3.1.1. Ligninolytic peroxidases

Lignin-modifying peroxidases (LMPs; LIP, MnP, and VP) refer to a group of glycosylated, heme-containing ligninolytic enzymes produced by the fungi during secondary metabolism in nutrient starved cultures [46]. These enzymes are produced in multiple isoforms and are affected by many external factors, such as nutrient level, mediator compounds, and metal ions. Phylogenetically, they belong to class II extracellular fungal peroxidases in the so-called “plant peroxidase superfamily.”

Lignin peroxidases (diarylpropane: oxygen, hydrogen peroxide oxidoreductase, EC 1.11.1.14) were first described in the basidiomycete *P. chrysosporium* Burdsall in 1983 [47] and have

become the most studied peroxidase since then. They have also been reported in several species of white rot basidiomycetes [48-51], actinomycetes [52, 53], and some bacteria, such as *Brevibacillus laterosporus* MTCC 2298 [54] and *Streptomyces viridosporus* T7A [55]. LiPs are generally dependent on H<sub>2</sub>O<sub>2</sub> and have very high redox potential and low optimum pH [56, 57]. Both these characteristics are important for their ability to oxidize a variety of reducing substrates, including polymeric substrates such as complex dye compounds. LiPs have a typical enzymatic cycle, characteristic of other peroxidases. LiP, MnP, and VP share an almost identical heme environment, which is responsible, among other factors for their high redox potential. However, they differ in the substrates that they can oxidize because of the presence of different catalytic sites in their molecular structures. LiP oxidizes nonphenolic lignin model compounds in direct contact with a tryptophan radical exposed to the solvent [58].

Biochemically, LiPs utilize veratryl alcohol (VA) to complete the catalytic cycle by reducing compound II to a resting enzyme to avoid inactivation. VA (3,4-dimethoxybenzyl alcohol) is a secondary metabolite, concomitantly produced by basidiomycetous fungi, along with LiPs usually synthesized from glucose in liquid cultures. Moreover, VA acts as a cation radical redox mediator of remote substrates [58, 59]. Similar to other peroxidases, LiP shows little substrate specificity, and, due to its high redox potential, reacts with a wide variety of lignin model compounds and even unrelated molecules. Interestingly, LiP has the distinction of being able to oxidize methoxylated aromatic rings without a free phenolic group, generating cation radicals that can react further by a variety of pathways, including ring opening, demethylation, and phenol dimerization [60], a typical mechanism also employed in the degradation of dye compounds. Numerous studies have reported the application of LiPs in the decolorization of various synthetic dyes and industrial dye wastewaters [20, 43, 48, 54, 61-63].

MnP (Mn<sup>II</sup>:hydrogen peroxide oxidoreductase, EC 1.11.1.13), like LiPs, is a heme-containing peroxidase and is the most common ligninolytic peroxidase produced by almost all white rot basidiomycetes [37]. MnP is a glycoprotein with a heme (ferric protoporphyrin) group that shares the mechanistic properties of other peroxidases and the formation of oxidized intermediates, compound I and compound II, in the presence of H<sub>2</sub>O<sub>2</sub> for aromatic and nonphenolic substrates oxidation [44]. Catalytically, MnP requires Mn<sup>II</sup> as an electron donor; Mn<sup>II</sup> is oxidized to Mn<sup>III</sup> [45], a deviation from other typical peroxidase reaction mechanisms. This specific oxidation of Mn<sup>II</sup> by MnP generally occurs in a small channel formed by three acidic residues located directly on the heme internal propionate [64]. MnP depends on Mn<sup>II</sup> as a substrate for compound I and II formation. In this reaction scheme, MnP oxidizes Mn<sup>II</sup> to Mn<sup>III</sup> in the presence of H<sub>2</sub>O<sub>2</sub>, and the Mn<sup>III</sup> formed oxidizes a variety of compounds [44]. The chelation of Mn<sup>II</sup> to Mn<sup>III</sup> by organic acids, such as oxalate, is necessary for MnP activity [53]. Its unique catalytic activity enables MnPs to biodegrade dyes, as well as decolorize various types of synthetic dyes, indicating their potential application in the environmental bioremediation of dye industry wastewater [65-67].

VPs (EC 1.11.1.16) are also known as hybrid peroxidases or lignin–manganese peroxidases because of their dual LiP and MnP catalytic properties. They are relatively new fungal peroxidases that were first thought to be MnPs but have since been isolated and thoroughly characterized in *Pleurotus* and *Bjerkandera* [68-72]. In contrast to other ligninolytic peroxidases,



VPs possess two catalytic sites, one for the direct oxidation of low- and high-redox potential compounds and the other for oxidation of  $Mn^{II}$  in a preferred manner [70-72]. *Pleurotus eryngii* VP (PeVP) possesses three acidic amino acid residues for  $Mn^{II}$  binding and a catalytic efficiency ( $k_{cat}/K_m$ ) for  $Mn^{II}$  oxidation that is typical of MnPs. In addition, PeVP has a tryptophan residue, Trp164, which is analogous to the PeLiP Trp171 that participates in electron transfer from aromatic donors and consequently enables the enzyme to oxidize nonphenolic lignin-related structures [64]. The dual catalytic mode of action observed accounts for their ability to catalyze the direct degradation/oxidation of a broad spectrum of persistent substrates (e.g., nonphenolic lignin compounds, dyes, such as RB5 and others) in the absence of mediators [71, 72], an important feature as a potential catalyst for a variety of biotechnological applications.

### 3.1.2. Dye decolorizing peroxidases

Dye decolorizing peroxidases (DyPs) comprise a novel group of heme-containing enzymes, named for their ability to efficiently oxidize high redox potential trichromatic anthraquinone (AQ) dyes. They were first reported in the extracellular secretions of a plant pathogenic fungus, *Bjerkandera adusta* Dec 1 (wrongly annotated previously as *Thanetophorus cucumeris*) [73]. The main features of DyP from *B. adusta* Dec1 include the following: (1) a monomeric 60-kDa glycosylated enzyme having higher specificity for AQ than for azo dyes, and different degradation spectra for phenolic compounds such as 2,6-dimethoxy-phenol, guaiacol, and VA; (2) a low pH optima (pH < 3.0); (3) lack of a conserved active site for distal histidine; and (4) structural divergence from classical plant and animal peroxidases (Figure 2) [73, 74]. To date, these enzymes, the physiological function of which is still unclear, have been identified from the genomes of fungi, bacteria, and archaea (<http://peroxidase.toulouse.inra.fr/index.php>). Interestingly, there is increasing evidence for the key role that microbial DyP peroxidases play in the degradation of lignin (see [37, 40, 41] up-to-date reviews on DyP-type peroxidases and their known biological, chemical, and structural features).

Similar to other peroxidases, DyPs are catalytically bifunctional enzymes displaying both oxidative and hydrolytic activity. They exhibit significant catalytic versatility arising from their ability to oxidize a variety of organic compounds, some of which, including dyes, phenols,  $\beta$ -carotene, lignin model compounds, and aromatic sulfides, are poorly degraded by conventional peroxidases [75-78]. Moreover, studies have demonstrated that DyPs are very robust enzymes in terms of pH [76], temperature, and pressure [78]. Several mediators can also improve DyPs substrate range. For example, we observed a drastic enhancement of azo dye oxidation in presence of a natural mediator syringaldehyde by AnaPX, a bacterial DyP [75], whereas  $Mn^{II}$  activates *Rhodococcus jostii* DyP2 activity [79]. The potential utility, as industrial/environmental biocatalysts in the bioremediation of wastewater contaminated with synthetic dyes specifically — the recalcitrant and xenobiotic AQ dyes that are generally not substrates of peroxidases such as HRP — has made DyPs the focus of significant interest.

Biochemically, the physicochemical properties of DyPs such as UV-vis spectral characteristics, molecular masses, or isoelectric points resemble those of classical heme proteins [76, 80, 81]. DyPs are structurally divergent from typical peroxidase; the basic architecture has a dominant  $\alpha$ - $\beta$ -helical secondary structure with extended loop regions (Figure 2). The N-terminal and C-

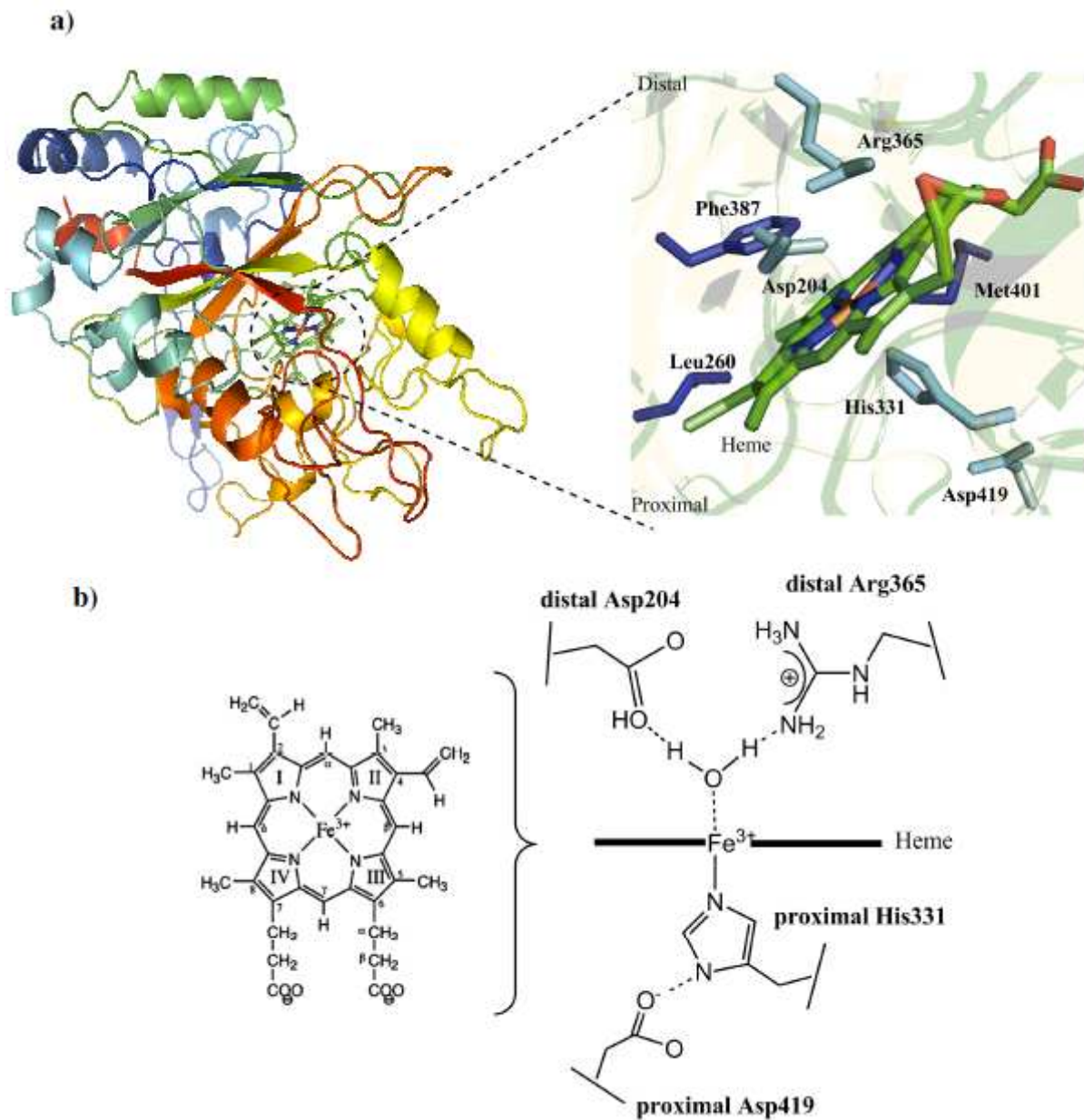
terminal domains contain an antiparallel  $\beta$ -sheet that is arranged into a characteristic ferredoxin-like motif on the distal side of the heme moiety [41, 82, 83]. Obviously, structural peculiarities, including the nature of the axial ligands, the environment of the substrate-binding site, and the involvement of intramolecular electron transfer, appear to account for the novel and the varied catalytic differences between DyPs and other peroxidases. Although DyPs possess a heme iron prosthetic group with a conserved proximal Fe–His–Asp triad found in most other peroxidases, the generally conserved distal His is absent in DyPs. Instead, an Asp residue forming the absolutely conserved novel GXXDG motif and an Arg in the distal position of DyPs are present (Figure 2). The distal Asp residue assumes the part of the catalytic base, mediating peroxide cleavage and thus the formation of compound I [84]. Moreover, the distal Asp's "swinging movement" determines heme cavity access for small organic molecules, making it function as the heme cavity's gatekeeper [75, 85]. The observed smaller heme-access channel for DyPs indicates that the oxidation of bulky substrates such as bulkier AQ dyes requires another substrate interactions sites. The existence of substrate interaction sites, involving long-range electron transport (LRET) via either surface-exposed tyrosyl or tryptophan residues, has been demonstrated in several DyPs [77, 82]. A Mn<sup>II</sup> binding site, which accounts for the oxidase activity of DyP2, in the absence of peroxide, has also been reported in *R. jostii* DyP2 [79].

### 3.2. Laccases

Laccases belong to the multicopper oxidase family of enzymes that catalyze the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water, through a radical-catalyzed reaction mechanism [86]. They are mainly of fungal or plant origin, although a few representatives have been identified and isolated in bacteria and insects [87, 88]. The most studied laccases are fungal in origin, mainly in phyla Ascomycota, Zygomycota, and Basidiomycota (see reviews [87, 88]). The most biotechnologically useful laccases are also of fungal origin. Physiologically, the functions of laccases are diverse, ranging from lignolysis, pigment formation, detoxification, to pathogenesis. All these functions are attributed to the enzymes' ability to oxidize a wide range of aromatic substrates such as polyphenols and diamines and even some inorganic compounds [87, 88].

Compared with fungal laccases, bacterial laccases are generally more stable at high pH and temperatures [89]. Although fungal laccases can be both intra- and extracellular, bacterial laccases are predominantly intracellular. Laccase have been isolated from the rhizospheric bacterium *Azospirillum lipoferum* [90], the melanogenic marine bacterium *Marinomonas mediterranea*, and the endospore coat component (CotA) of *B. subtilis* [89, 91]. In addition, the optimum pH of bacterial laccases is higher than that of fungal laccases [89, 91], whose optimum pH is acidic [89, 91]. The optimum temperature for most laccases is between 50°C and 70°C, with thermal stability dependent on the microbial source.

The structure of an active holoenzyme laccase molecule is a dimeric or tetrameric glycoprotein containing four copper atoms per monomer, bound to three redox sites (T<sub>1</sub>, T<sub>2</sub>, and T<sub>3</sub> Cu pair) [87, 88]. The four Cu atoms differ from each other in their specific properties, such as the characteristic electronic paramagnetic resonance (EPR) signals that allow them to play an



**Figure 2.** The structural model of DyP showing the heme active site architecture. (a) Typical  $\alpha\beta$  protein structure of DyP and the heme active site showing the conservation of key catalytic proximal Fe–His–Asp triad residues and substitution of His with Asp in the distal heme active. (b) Possible coordination of distal Asp and other active site residues with a water molecule involving hydrogen-bond network in the heme active site as implicated by the resolved crystal structures of ferric DyP enzymes [82–84].

important role in the catalytic mechanism of the enzyme. For catalytic activity, a minimum of four Cu atoms per active protein unit is needed. The  $T_2$  and  $T_3$  Cu atoms form a trinuclear cluster site, which is responsible for the binding and reduction of oxygen to water.  $T_2$  Cu is coordinated by two His and  $T_3$  Cu pair by six His. The strong antiferromagnetic coupling between the two  $T_3$  Cu atoms is maintained by a hydroxyl bridge [87, 88]. The function of the  $T_1$  site, in this type of enzyme, involves electron abstraction from reducing substrates (electron

donors), with a subsequent electron transfer to the  $T_2/T_3$  Cu cluster. Generally, laccase catalyzes the four-electron reduction of oxygen to water (at the  $T_2$ - $T_3$  trinuclear Cu centers) by the sequential one-electron uptake from a suitable reducing substrate (at the  $T_1$  mononuclear Cu center) [86]. Laccases are categorized into high potential laccases (HPLs) and low potential laccases (LPLs) on the basis of  $T_1$ - $T_3$  Cu having a redox potential of 0.6–0.8 V and 0.4–0.6 V, respectively,

The catalytic efficiency of laccases ( $k_{cat}/K_m$ ) depends on the redox potential of  $T_1$  site; consequently, laccases with high redox potential at the  $T_1$  site are of special interest in biotechnology for their potential application in bleaching and bioremediation processes [92]. Compared with LiP, MnP, and VP that exhibit higher redox potentials (1.15–1.25 V), most laccases typically have low redox potential (0.5–0.8 V). Consequently, most laccases lack the ability to degrade nonphenolic aromatic substrates due to their low redox potential. However, in the presence of mediators, particularly small chemical compounds with redox potential higher than 0.9 V, the substrate range of laccases can be expanded to include the oxidation of nonphenolic compounds such as lignin and complex dye compounds [27, 86, 87]. A mediator is a low molecular weight (LMW) chemical compound that is continuously oxidized by the laccase enzyme and subsequently reduced by the substrate. Due to its bulky size, high molecular weight (HMW) substrates cannot enter the laccase active site, and the mediator acts as a carrier of electrons between the enzyme and the substrate, thereby overcoming the steric hindrances that exist between them [86].

Laccases and laccase-mediator systems (LMSs) have been intensively studied with regard to their degradation of various recalcitrant compounds, such as chlorophenols, polyaromatic hydrocarbons (PAHs), lignin-related structures, organophosphorous compounds, phenols, and synthetic dyes [27, 87]. These enzymes have great potential in various biotechnological processes mainly because of their high nonspecific oxidation capacity, the lack of a requirement for cofactors, and the use of readily available oxygen as an electron acceptor. Laccases and LMSs have found various biotechnological and environmental applications, including as analytical tools/biosensors for phenols, and in the development of oxygen cathodes in biofuel cells, textile dye degradation, organic synthesis, immunoassay labeling, delignification, demethylation, and in bleaching of craft pulp [87, 88, 92].

### 3.3. Azoreductases

Azoreductases are a group of oxidoreductive enzymes that catalyze the NAD(P)H-dependent reduction of azo compounds to the corresponding amines, via cleavage of the azo linkages (R-N=N-R), resulting in azo dye degradation [93]. They are a varied family of enzymes that have been identified in almost all species except in viruses. Physiologically, these enzymes participate in enzymatic detoxification systems, involving the reduction of quinones, quinone imines, azo dyes, and nitro groups, and protect cells against the toxic effects of free radicals and reactive oxygen species arising from electron reductions [93].

Azoreductase activity has been characterized from a wide variety of bacteria, including *Pigmentiphaga kullae* K24, *Xenophilus azovorans* KF46F, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus* sp. strain OY1-2, *Pseudomonas aeruginosa*, and *Rhodobacter*

*sphaeroides* [93-101]. There are at least two different types of bacterial azoreductases: those that require flavin and those that do not [94]. Flavin-dependent azoreductases can be further classified into two families according to their amino acid sequences. Azoreductases from *E. coli* and *Bacillus* sp. strain OY1-2 are representative of the two flavin-dependent azoreductases, respectively. *Bacillus* sp. strain OY1-2 azoreductase is a 23-kDa protein with the ability to reduce the azo dyes Rocceline, Sumifix Red B, and Methyl red, producing dimethyl p-phenylenediamine and o-aminobenzoic acid in the presence of  $\beta$ -NADPH [102]. On the other hand, *Xenophilus azoovarians* KF46 and *P. kullae* K24 azoreductases are monomeric flavin-free enzymes that use NADPH as a cofactor to degrade the azo dyes carboxy-Orange II and I. These two enzymes exhibit different substrate specificities and sizes (21 and 30 kDa) and require the presence of hydroxyl groups in the aromatic ring of the substrate [95].

### 3.4. Other dye-degrading enzymes

In addition to peroxidases, laccases, and azoreductases, the bioremediation of synthetic dye compounds with other enzymes such as tyrosinases [103], aryl alcohol oxidases [104], and biosulfidogenic hydrogenases [105] have been demonstrated. Similar to laccases, tyrosinases (monophenol monooxygenase, EC1.10.3.1) are oxidoreductases that can catalyze the oxidation of phenolic and other aromatic compounds, without the use of a cofactor in presence of oxygen. Catalytically, these enzymes possess both cresolase activity (ortho-hydroxylation of monophenols to o-diphenols) and catecholase activity (the oxidation of o-diphenols to o-quinones) [103]. Tyrosinases also degrade aromatic amines and o-aminophenols via the similar ortho-hydroxylation and oxidation reactions. The resultant products undergo subsequent polymerization, giving rise to oligomeric products.

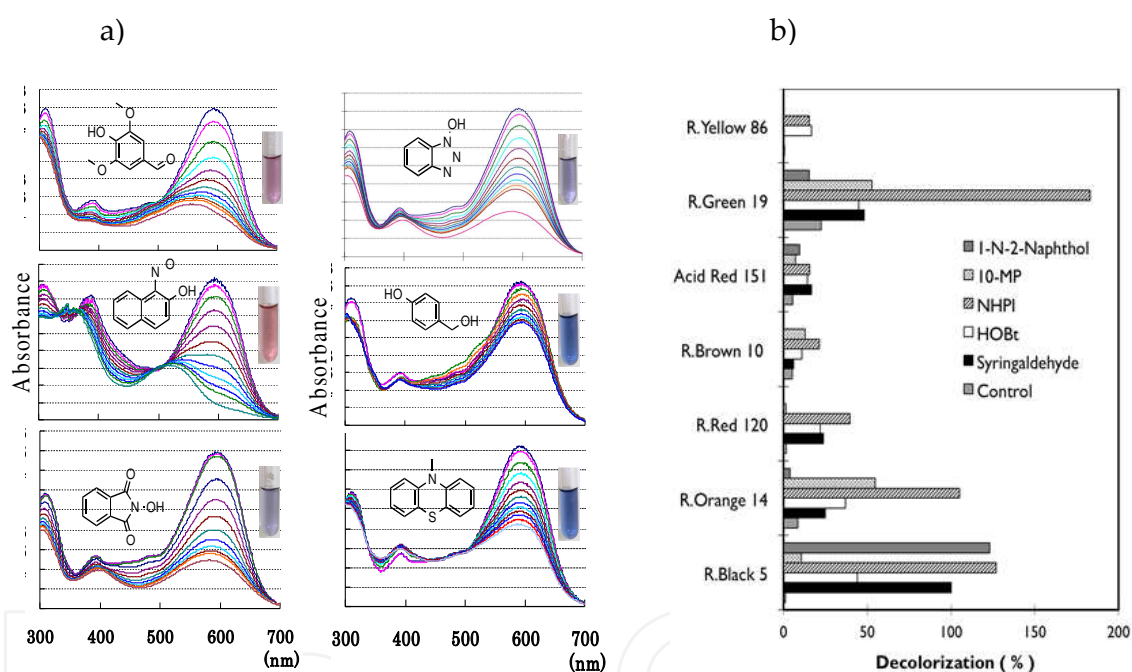
Biotechnologically, tyrosinases are used as markers of the oxidative enzymes involved in the degradation of azo dyes. For example, the involvement of tyrosinase in the degradation of Direct Blue-6 by *Pseudomonas desmolyticum* NCIM 2112 [106], disperse dye brown 3REL by a microbial consortium consisting of *Galactomyces geotrichum* MTCC 1360, and sulfonated azo dyes by *Brevibacterium* sp. strain VN-15 [103] and *Bacillus* sp. VUS [62] has been demonstrated. The activity of aryl alcohol oxidase (AAO) has been reported in *B. adusta* Dec 1; it oxidizes VA to veratraldehyde producing  $H_2O_2$ , which is important for supporting the *in vivo* dye-decolorizing activity of fungi [104]. The involvement of a similar enzyme in *Comamonas* sp. UVS during the decolorization of Red HE7B and Direct Blue GL has also been reported [107].

## 4. Enzyme-linked biotransformation of industrial dyes

### 4.1. Enzymatic degradation of azo dyes

A general mechanism for the peroxidase-catalyzed oxidation mechanism for azo dye degradation, via either the symmetrical and/or asymmetrical azo bond cleavage, has been proposed. Goszczynski et al. [108] proposed mechanisms in the course of which these enzymes convert dyes to cation radicals and become susceptible to nucleophilic attack by water or hydrogen peroxide. This results in the simultaneous split of the azo linkage, both symmetrically and

asymmetrically, to produce intermediates that subsequently undergo several redox reactions, such as depolymerization, repolymerization, and demethylation, finally leading to more stable intermediates. In this reaction scheme, two successive one-electron oxidations of the phenolic ring, by the hydrogen peroxide–oxidized forms of the enzyme, produce a carbonium ion. A water molecule then reacts with the phenolic carbon, bearing the azo linkage, and an unstable hydroxyl intermediate, which breaks down into a quinone, and an amidophenyldiazine, is formed. The latter compound is then oxidized, by oxygen, into the corresponding phenyldiazene radical, which, after elimination of nitrogen, gives a phenyl radical that is reoxidized by oxygen. This mechanism leads to the detoxification of azo dyes because no aromatic amines are formed. LIP, MnP, and VP from basidiomycetous fungi are able to oxidize several azo dyes by following this mechanism [20, 43, 48, 54, 61-63]. Although exhibiting a general preference toward trichromatic AQ dyes than azo dyes, it is possible that DyP-type peroxidases also use the same mechanism.



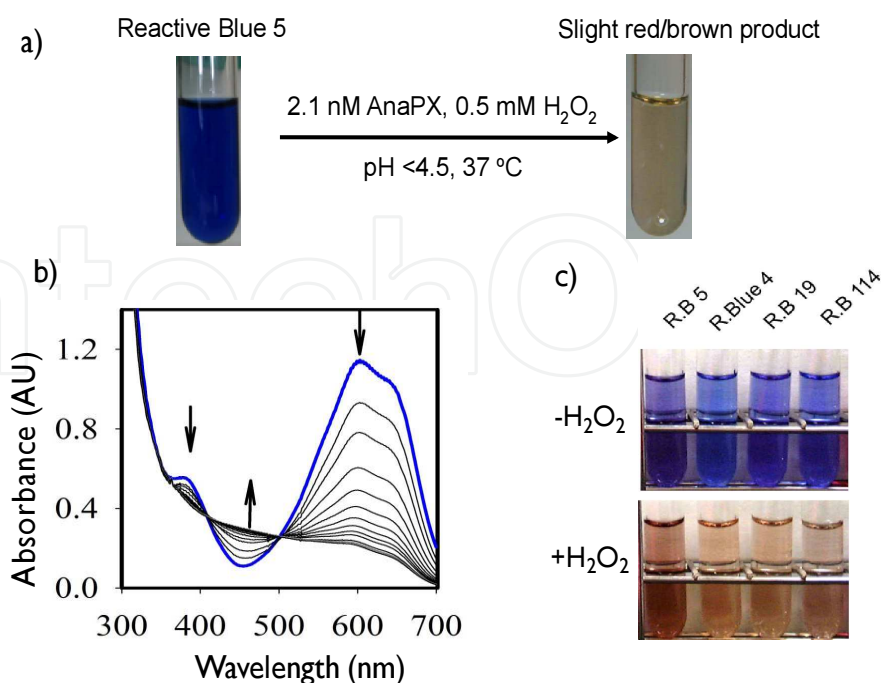
**Figure 3.** Decolorization of azo dyes in the presence of mediators by AnaPX. (a) Biotransformation of Reactive Black 5 by AnaPX in the presence of various mediators. Inset shows the structures of the mediators and the resultant product upon decolorization. (b) Decolorization profile of several azo dyes by AnaPX in presence of different mediator compounds.

Some azo dyes are oxidized effectively only in the presence of mediator compounds. In previous studies, we have shown that the azo degradation ability of AnaPX was significantly improved in the presence of redox mediators [75]. The decolorization range and oxidation rates of AnaPX, compared with HRP for azo dyes, increased markedly (2- to 5-fold) in the presence of a lignin-derived phenolic redox mediator, syringaldehyde (SA) (Figure 3). The degradation of Reactive Black 5, in presence of SA, was pH dependent, with the AnaPX-mediator reaction exhibiting maximal activity at pH 6.0, although the enzyme optimum pH is 4.5 [75]. Similar

results have been reported in the presence of NOH-type (1-hydroxy-1H-benzotriazole (HOBt), *N*-hydroxyphthalimide (NHPI), 1-nitroso-2-naphthol), and quinine-containing mediators such as 10-methylphenothiazine (10-MP); however, decolorization efficiencies were different, possibly due to differences in the type of dye structure (Figure 3). Differences in the position of the substituents (-CH<sub>3</sub>, -OCH<sub>3</sub>) and/or their substitution with -Cl or -NO<sub>2</sub> groups on the phenolic ring affect the electronic character of dye compounds and may render the azo dye more or less susceptible to oxidation by enzymes, resulting in the observed dye-structure-related effects on decolorization efficiencies [109]. Our earlier work also showed that the transformation of Reactive Black 5 resulted in a decrease in the intensity of the dye absorption band, at  $\lambda_{\max}$  = 600, 400, and 310 nm, indicating the degradation of the dye. The complete decolorization of the dye resulted in the formation of light brownish products within 1 min in the presence of SA and 1-nitroso-2-naphthol. In contrast, other mediators showed varied decolorization efficiency for Reactive Black 5, illustrating the importance of mediator specificity toward different functional groups. It has been postulated that phenoxy radicals from SA act similarly to the -NO- radicals from -NOH- compounds, i.e., they extract a hydrogen atom from the substrate [110, 111]. It is very likely that the varied oxidative ability observed for the different AnaPX mediators is governed by the dissociation energy of the corresponding bond, which consequently affects the azo dye degradation.

The laccase-catalyzed oxidation of azo dyes has also been postulated to follow a similar mechanism, albeit with slight modifications. In this reaction scheme, fungal laccases oxidize azo dyes through a highly nonspecific free radical mechanism to form phenolic type compounds without the cleavage of the azo bond [89, 109, 112]. A similar mechanism has been reported in the biotransformation of the azo dye, Sudan Orange G by bacterial Cot-laccase from *B. subtilis*, that exhibits an optimal pH of around 8–9 for dye decolorization [89]. According to this mechanism, laccases initially catalyze a one-electron transfer reaction of the dye to generate a phenoxyl radical that is sequentially oxidized to various unstable radical molecules, with the concomitant destruction of the chromophoric structure of the dye. The resultant biotransformation radical species undergo coupling reactions to form less toxic oligomeric or polymeric condensation products. This laccase-catalyzed oxidation of phenolic azo dyes is however dependent on the electronic character and position of the substituent on the phenolic ring [89, 109]. For example, azo dyes with electron-donating 2-methyl or 2-methoxy substituents are more easily oxidized than compounds containing a methyl group in position 3, or those unsubstituted or substituted with 2-chloro and 2-nitro groups. Generally, the laccase-catalyzed oxidation of azo dyes excludes the formation of toxic aromatic amines obtained under reductive conditions, making these enzymes important in azo dye bioremediation.

All azoreductases reduce azo compounds via a Ping Pong Bi Bi mechanism [94, 101]. In the proposed mechanism, azo compound reduction undergoes two cycles of NAD(P)H-dependent reduction; the azo substrate is reduced to a hydrazine in the first cycle, and the hydrazine is further reduced to two amines in the second cycle. In this reaction, FMN serves as a redox center in the electron-transferring system by mediating the electron transfer from NAD(P)H to the azo substrate [94, 113]. However, corresponding aromatic amines, formed during anaerobic azo reduction, are generally more toxic, mutagenic, and carcinogenic than azo



**Figure 4.** Biotransformation of AQ dye Reactive Blue 5 by AnaPX. The formation of characteristic light reddish brown product (2,2-disulfonyl azobenzene) upon enzymatic degradation of (a) Reactive Blue 5 and (c) other AQ dyes, Reactive Blue 4, Reactive Blue 19, and Reactive Blue 114. (b) UV absorption spectra of the degradation process showing the decrease in absorbance at 600 nm and an increase in absorbance at 400–500 nm. Each spectrum was taken after 4-s intervals.

substrates [32, 94, 113]. Moreover, the requirement for expensive cofactors is a barrier to the wider utilization of azoreductases in bioremediation.

#### 4.2. Enzymatic biotransformation of anthraquinone dyes

Although there are many reports on the involvement of peroxidases in the biodegradation of azo dyes as described above, very few studies have reported the degradation of anthraquinone (AQ) dyes by these peroxidases. Since the first report on the DyP peroxidases' high specificity to AQ dyes [73], several proteins have been isolated and characterized, and their ability to decolorize synthetic dyes was demonstrated. In our study, AnaPX decolorized over 90% of the AQ dyes—Reactive Blue 5 (262 U mg<sup>-1</sup>), Reactive Blue 4 (167 U mg<sup>-1</sup>), Reactive Blue 114 (491 U mg<sup>-1</sup>), and Reactive Blue 19 (401 U mg<sup>-1</sup>)—within 5 min [75]. These dyes have a vinyl sulfonic reactive moiety in their structure; their aromatic anthracene-9,10-dione structure is highly stabilized by resonance, accounting for their general resistance to both chemical and enzymatic oxidation. The enzyme also decolorized over 70% of Reactive Blue 4 and the triazine dyes, Procion Blue H-ERD and Procion Blue H-EXL, within 2 h. The kinetic parameters determined for AnaPX clearly revealed that it has a higher affinity and greater redox potential for H<sub>2</sub>O<sub>2</sub> and RB5 than HRP and other peroxidases. This may explain the higher decolorization activity of AnaPX toward RB5. The decolorization of RB5 and Acid Blue 45 by AnaPX results in a decrease in absorbance at 600 nm and an increase in absorbance at 400–500 nm, accompanied



by the formation of a light reddish-brown product (Figure 4). Similar results have been reported in other DyP peroxidases [75-78, 82, 83, 114-117].

The biotransformation of AB62 (an AQ), by *B. subtilis* Cot-laccase, also results in a decrease in the intensity of the dye absorption bands, at  $\lambda_{\max} = 600$  and 630 nm, along with an increase in absorption around 500 nm, due to the formation of reddish products [118]. Thus, it is probable that both laccase and peroxidase utilize similar mechanism for AQ degradation.

In the transformation of Reactive Blue 5 by *B. adusta* Dec 1 DyP, analysis of the final enzymatic reaction mixtures, by NMR and MS techniques, showed that dye degradation results in three reaction products: (1) phthalic acid, (2) product 2 ( $m/z = 472$ ) lacking the anthraquinone frame, and (3) product 3 ( $m/z = 305$ ), formed from the loss a 2,5-diaminobenzene sulfonic acid (ABS) molecule from product 2 [119]. In the proposed reaction mechanism, the anthraquinone frame undergoes initial oxidative ring opening due to attack of the carbonyl group by the H<sub>2</sub>O molecule. This oxygenase/hydrolase-like activity leads to the production of phthalic acid. In contrast, the formation of products 2 and 3 proceeds via cationic radical catalysis, typical of peroxidases, followed by the subsequent dimerization and polymerization of the intermediates to form final products with high molecular weights, such as 2,2-disulfonyl azobenzene. The formation of 2,2-disulfonyl azobenzene resulted in the characteristic reddish-brown product observed during the DyP-catalyzed degradation of Reactive Blue 5 (Figure 4) [75, 119]. Further treatment of the final product with TcVP1, a VP from *B. adusta* Dec 1, decolorized these colored intermediates to colorless by products [74]. The concerted action of these two enzymes, for the complete decolorization of Reactive Blue 5, illustrates the potential utility of DyPs in dual-enzyme systems as a novel strategy in the treatment of dye wastewater.

### 4.3. Biodegradation of other synthetic dyes by enzymes

Dye-degrading enzymes can also be applied in the degradation of other synthetic dyes such as indigoid, triarylmethane, and phthalocyanine dyes. Similar to the laccase-catalyzed degradation mechanism for azo dyes, the initial hydrolytic attack by the water molecule, coupled with laccase-catalyzed electron transfer, causes the cleavage of the indigoid frame, forming an intermediate isatin. The subsequent decarboxylation of isatin leads to the formation of anthranilic acid as a final stable oxidation product [120]. This process is used industrially to achieve the stonewashed effect of indigo-dyed denim fabric via mild enzymatic decolorization. This process can be used to treat textile wastewaters containing indigoid dyes. *P. chrysosporium* extracellular lignolytic enzymes such as MnP and LiP have been demonstrated to successfully decolorize indigoid dyes [121, 122]. In the peroxidase-catalyzed decolorization of indigo carmine, isatin sulfonic acid is formed as a final yellowish product when LiP is used. In MnP-catalyzed oxidation of indigo carmine, a stable reddish product, probably a dimeric condensation product, is formed instead. *P. chrysosporium* cultures, extracellular fluid, and purified peroxidases have been reported to degrade generally recalcitrant crystal violet and six other triphenylmethane dyes [123, 124]. The degradation of these dyes follows N-demethylation reactions. For example, the decolorization of crystal violet has been shown to form Michler's ketone, a metabolic dead-end product [125].

The removal of phthalocyanine dyes in aqueous solution by peroxidase has been widely reported, especially by white rot fungi [51, 67]. Phthalocyanine dyes are reactive dyes containing metallic complexes, mostly Cu, used to produce blue and green shades in textile dyes. The peroxidase-catalyzed degradation of these dyes involves cleavage of the nitrogen bonds in the inner ring of the phthalocyanine molecule and release of  $\text{Cu}^{2+}$  from the metal complex [126]. However, the resultant products tend to be more toxic in the environment [126].

## 5. Evolutionary issues and scope for improvement of dye-degrading enzymes

Enzymes are capable of carrying out a tremendous range of biochemical functions, including dye bioremediation. However, their efficiency, stability, and costs often do not correspond to the needs of industrial operation [127]. In dye bioremediation, the choice of enzymes also depends on the effluent characteristics, operational requirements, and costs. Although some peroxidases and laccases are being employed successfully in industry, there is still no enzyme that combines the desired attributes of being stable and active over a range of temperatures and pH values, with high reduction potential [128].

To overcome this limitation, tailor-made biocatalysts can be created from wild-type enzymes by protein engineering using either rational design via computer-aided molecular modeling and site-directed mutagenesis, or by directed evolution techniques. These techniques can be used to successfully modify protein activity, stability, enantioselectivity, soluble expression, and binding affinity. In this regard, the availability of the structure of the enzyme and knowledge about the relationships between structure and function is requisite to undertake rational design and is consequently very information intensive [129]. Rapid progress in solving protein structures, and the enormously increasing number of sequences stored in public data bases have significantly eased access to data and structures, making rational protein engineering possible.

To overcome challenges faced by rational design, directed evolution has emerged as a key technology for protein engineering, generating impressive results [129]. Direct evolution involves four key steps: (1) selecting a starting gene sequence, (2) creating a library of variants, (3) selecting variants by high-throughput screening with improved function, and (4) repeating the process until the improvement or function is achieved [130]. The most common mutagenesis method used includes error-prone PCR (ep-PCR), saturation mutagenesis, and DNA shuffling [131]. For both approaches to protein engineering, the gene(s) encoding the enzyme(s) of interest, a suitable expression system, and a sensitive detection system are prerequisites.

### 5.1. Engineering for specificity

There have been many attempts to use rational approaches to engineer laccases over the last couple of decades. Using site-directed mutagenesis, Xu et al. [132] developed a collection of variants with structural perturbations at the  $T_1$  Cu center to determine what parameters define the catalytic activity and the redox potentials of laccase. In the study, F463M mutation resulted

in a  $T_1$  Cu site with an EPR signal intermediate between that of the wild-type laccase and plastocyanin, an altered UV-visible spectrum, and a decreased redox potential (by 0.1 V). In oxidizing phenolic substrate, the mutation also led to a more basic optimal pH as well as an increase in  $k_{cat}$  and  $K_m$ . Similarly, triple mutations V509L/S510E/G511A and L466V/E467S/A468G near  $T_1$  Cu center of *Myceliophthora* and *Rhizoctonia* laccase, respectively, resulted in a phenol-oxidase activity with an altered  $K_m$ ,  $k_{cat}$  fluoride inhibition, and pH optimum shifted 1 unit lower and higher, respectively [133]. These observations were attributed to mutation-induced structural perturbations on the molecular recognition between the reducing substrate and laccase and on the electron transfer from the substrate to the  $T_1$  Cu center. Modifications in the amino acid composition in the enzyme active site of *Tinea versicolor* laccase also improved enzyme activity and affinity toward larger phenolic substrates [134].

Random mutagenesis experiments on *Pleurotus ostreatus* laccase POXC and POXA1B cDNAs, using ep-PCR, have been reported to result to variant library with altered enzyme properties [135]. In this study, two variants 2L4A and 3L7H showed a higher specific activity than the wild-type enzyme toward typical aromatic substrates and expanded dye degradation specificities [136]. Several directed evolution studies of bacterial laccase CotA have also been used to successfully improve enzyme substrate specificity and functional expression [137-140]. Gupta and Farinas [138] reported a variant of CotA having 120-fold more specificity for ABTS with unexpectedly enhanced thermal stability with the half-life for the heat inactivation ( $t_{1/2}$ ) at 80°C increased by 62 min. This newly generated laccase variant represents a helpful “evolved form” of the enzyme that is more durable and versatile as a biocatalyst, with respect to both the varieties of xenobiotics degraded and the operative conditions.

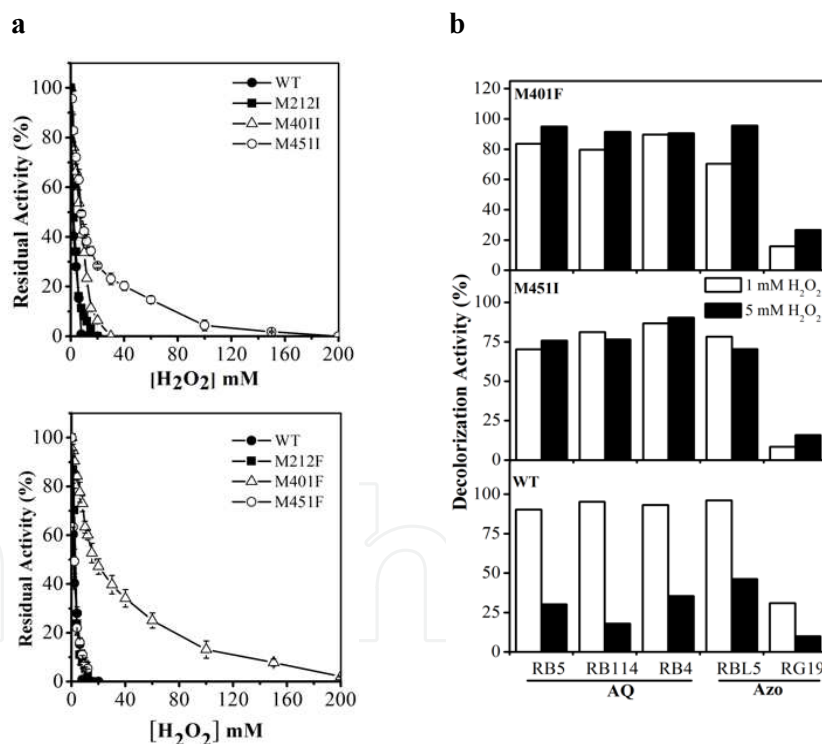
Similar to laccases, several attempts have been made to engineer peroxidases specificity using rational approaches [68, 141-143]. Using a combination of site-directed mutagenesis and *in vivo* shuffling, Garcia-Ruiz et al. [68] developed VPL2 variants of *P. eryngii* with enhanced VP activity (~129-fold) compared with the parental VPL2. Engineering of the cavity of cytochrome c peroxidase (CCP) via W191G mutation has been shown to alter the specificity of the enzyme toward substrates 2-aminothiazole [144]. Two mutations (A147M and A147Y) in CCP have also been reported to exhibit unique specificities toward oxidation of small substrates [142].

## 5.2. Engineering for properties of enzymes

Protein thermostability is a crucial issue in the practical application of enzymes in dye bioremediation applications. Several studies have reported the application of protein engineering techniques to improve thermal stabilities of peroxidases [68, 145, 146], azoreductase [147], and laccases [148, 149]. *In silico* design and site-directed mutagenesis of thermo-labile residues of *Coprinus cinereus* peroxidase (CiP) resulted in two variants (S323Y and E328D) with increased thermostability over the wild-type enzyme in addition to conserved catalytic activity [145]. Similarly, five rounds of mutagenesis/recombination followed by high-throughput screening yielded a variant 1B6, showing 300-fold higher half-life at 50°C than that exhibited by the homodimeric wild-type PpAzoR azoreductase from *Pseudomonas putida* [147]. In *P. ostreatus* VPL2, directed evolution involving six rounds of DNA shuffling cycle was used to improve enzyme secretion, activity, and stability [68]. The generated variant had a higher  $T_{50}$  of 8°C and increased enzyme stability at alkaline pH. In addition, the  $K_m$  for  $H_2O_2$  was enhanced

15-fold with the catalytic efficiency maintained, accompanied by an improvement in peroxide stability.

In our study, we have reported the stabilization of bacterial DyP AnaPX against H<sub>2</sub>O<sub>2</sub>-induced inactivation by replacing the Met residues in the heme pocket with high redox residues Ile, Leu, and Phe [80]. The heme cavity variants M401L, M401I, M401F, and M451I had significantly increased H<sub>2</sub>O<sub>2</sub> stabilities of 2.4-, 3.7-, 8.2-, and 5.2-fold, respectively. Surprisingly, M401F and M451I variants retained 16% and 5% activity at 100 mM H<sub>2</sub>O<sub>2</sub>, respectively. In addition, the two mutants maintained high dye decolorization activity toward AQ and azo dyes at 5 mM H<sub>2</sub>O<sub>2</sub> and exhibited a slower rate of heme degradation than the wild-type enzyme (Figure 5). The observed stabilization of AnaPX was attributed to (1) the replacement of potentially oxidizable Met residues, (2) the increased local stability of the heme pocket, or (3) the alteration of the self-inactivation electron transfer pathways due structural perturbations of the heme pocket by the above mutations. The observed increased stabilities and broad substrate specificity can be potentially useful for the further practical application of these AnaPX mutants in bioremediation of wastewater contaminated with recalcitrant AQ, under conditions of higher peroxide concentrations.



**Figure 5.** (a) H<sub>2</sub>O<sub>2</sub> stability of wild-type AnaPX and Met-substituted variants. (b) Dye decolorization activity of AnaPX and two improved variants (M401F and M451I) on AQ and azo dyes at two different H<sub>2</sub>O<sub>2</sub> concentrations (1 and 5 mM) [80].

Strategies for further improvements of laccase through genetic, metabolic, and protein engineering in suitable heterologous hosts for enzyme overproduction and enhanced enzyme kinetics and substrate binding, improving enzyme activity and stability, have been reported

[128]. The substitution of the aromatic amino acids residues with nonaromatic residues of *T. versicolor* laccase resulted in increased resistance to inactivation by free radicals [150]. Directed evolution has been used to increase laccase activity by 170- to 32,000-fold, pH, and temperature stability [151, 152]. The resultant mutants also exhibited increased tolerance to organic solvents such as ethanol and acetonitrile by 30% and 20%, respectively [153]. In addition, the directed evolution of high redox potential laccases has been used to overcome the obstacles associated with their functional expression in host suitable for *in vitro* evolution experiments [151, 153].

## 6. Current and potential synthetic dye biodegradation applications

### 6.1. Free enzyme biodegradation of industrial dyes

Enzymes, as potent biocatalysts, have been employed in numerous fields primarily for their immense catalytic potential [36]. In dye bioremediation, cell-free or isolated enzymes are preferred over the intact organism, especially when the effluent to be treated contains pollutants that cannot support growth. The key to successful application of enzymes for dye decolorization is the selection of appropriate enzyme cocktail that will exhibit versatility and efficiency, even under mild reaction conditions. The delivery system selected must be well suited to the purpose, simple, efficient, and cost-effective. Enzymes may be delivered to the target effluent in different ways in either cell-free or immobilized form.

Currently, there are commercial preparation of peroxidases and laccases used for different applications. DeniLite II S<sup>®</sup>, a commercial laccase formulation containing laccase, a mediator, and a nonionic surfactant, is used in the finishing process for indigo stained clothes [154]. A laccase from ascomycete fungus *Myceliophthora thermophila*, sold commercially by Novozymes as Suberase<sup>®</sup>, is used in the removal of astringency of cork stoppers for wine bottles. Ligno-Zym<sup>®</sup> system (GmbH), a mixture containing *T. versicolor* laccase and a group of mediators (N-OH-, N-oxide-, oxime-, or hydroxamic acid-functional groups), is used to remove 50% to 70% of lignin from pulp within 1 to 4 h [154]. However, the potential of these commercial laccase preparations for treatment of effluents containing dyes has yet to be demonstrated.

In the industrial scale of operation, the use of pure enzymes in effluent treatment is not economically feasible due to high start-up and running costs. The use of free enzymes as compared with their immobilized forms also show some significant drawbacks such as (1) thermal instability, (2) susceptibility to attack by proteases, (3) activity inhibition, (4) high sensitivity to several denaturing agents, and (5) difficulty of separating or reusing the free catalyst at the end of the reaction from the reaction mixture [155].

### 6.2. Immobilized enzyme bioremediation of synthetic dyes

In recent years, it has been shown that many industrial dyes can be decolorized by laccases and peroxidases immobilized on different supports. In contrast to soluble enzymes, immobilization offers higher enzyme stability, reusability, and capability to work in aqueous as well as in organic solvents due to protection against denaturants and proteolysis and reduced susceptibility to microbial contamination. This may be partly due to enzyme stabilization effect

on immobilization leading to restricting the protein unfolding process, as a result of the introduction of random intra- and intermolecular cross-links [155]. The development of immobilization methods has consequently caused a significant increase in the application of oxidoreductases in various technological processes [156].

Laccases have been immobilized on various supports such as glass-ceramic materials, imidazole-modified silica, montmorillonite, alginate-gelatin mixed gel, hydrophobic sol-gel, and green coconut fiber and applied in decolorization/degradation of various textile and nontextile dyes and phenolic compounds [27, 157-160]. For example, *Trametes modesta* laccase immobilized on alumina decolorized 41 commercial azo, triphenylmethane, indigoid, and heterocyclic dyes. The immobilization of laccase on alginate mixed gels or hydrophobic sol-gel also led to it improved pH stability, thermostability, and reusability of the enzymes, although a slight decrease in enzyme activity and dye affinity was observed. The entrapment of laccase in alginate-gelatin, alginate-chitosan mixed gels, or hydrophobic sol-gel matrix of trimethoxysilane and propyltetramethoxysilane has been reported to lead to significant laccase stability toward heat denaturation [161]. The reported improved decolorization of wastewaters by immobilized laccases is attributed to both enzymatic catalysis and support adsorption [112].

There are comparatively fewer investigations on dye decolorization by immobilized peroxidases, probably due to their requirement of  $H_2O_2$  for activity. For peroxidase catalysis,  $H_2O_2$  must be added or generated *in situ* to avoid enzyme deactivation and to achieve a stable decolorization process [158, 162]. For example, the half-life of *Saccharum spontaneum* peroxidase immobilized on polyethylene was favored by careful addition of  $H_2O_2$  to the reactor to decolorized 15 batches of Procion green HE-4BD [158]. Higher loading rates of  $H_2O_2$  resulted in 50% loss in decolorization activity of Orange II within 2 h by *Bjerkandera* sp MnP in a membrane reactor; however, the enzyme maintained 96% efficiency under optimized  $H_2O_2$  and enzyme feeding rates [163]. Similar to laccases, the immobilization of peroxidases into a sol-gel matrix of tetramethoxysilane and propyltrimethoxysilane or in alginate gel and mixed alginate-pectin gel improves their storage stability, pH stability, and thermostability, in addition increased enzymes reusability and decolorization efficiency. The above-mentioned examples illustrates importance of immobilization as a powerful technique in expanding the application of oxidoreductases in bioremediation, particularly in those circumstances where the enzyme can be reused in the application many times to reduce operation costs.

## 7. Future prospects and conclusion

In this chapter, we have discussed the descriptive information on the oxidoreductases from various microorganisms, including their discovery, biochemistry, current biodegradation applications, and limitations to their large scale and efficient use. An ideal enzyme for dye bioremediation application should have the following properties: (i) broad substrate specificity; (ii) high redox potential; (iii) high tolerance to inactivation by radicals, organic solvents, and shearing forces; (iv) ability to work with a large number of mediators; (v) broad pH and temperature optima; (vi) high enzyme activity and stability; and (vii) low production costs.

The current oxidoreductases such as peroxidases and laccases are not well suited for industrial applications that require particular substrate specificities and application conditions (pH and temperatures) in addition to high expression levels. Consequently, effluent treatment using enzymes on a large scale is still not economically viable.

Within the last decade, there is increased research interest in the application of genetic engineering techniques to develop “designer” enzyme cocktails for large-scale dye bioremediation applications in different industrial sectors. The availability of high-throughput screening assays and functional expression systems plus the crystal structures of the enzymes has helped these efforts enormously. Breakthroughs through protein engineering involving combination of directed evolution with both hybrid and rational approaches, including computational studies, will permit the conversion of the current array of oxidoreductases into versatile biotechnological products for dye bioremediation. In addition, an interdisciplinary approach to wastewater treatment involving nanotechnology and enzyme technology will enable the utilization of peroxidases, laccases, and azoreductases to their full potential. These studies need to be conducted in the context of dye mixtures simulating real dyeing baths, as real bioremediation process is affected by all the factors involved in the dyeing processes, such as components and auxiliaries present in the wastewater, that markedly affects dye decolorization and/or the enzyme.

In conclusion, the promise of the concerted research efforts evident thus far and the potential of modern microbial and enzyme technologies to make radical improvements in the oxidoreductases give confidence that the development of successful technologies for industrial synthetic dye bioremediation will be possible in the near future.

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