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Fungal laccases as tools for biodegradation of industrial dyes

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Abstract: Laccases are blue copper oxidases, found in some plants and secreted by a wide range of ligninolytic fungi. These enzymes are well known for their ability in oxidizing several organic compounds, mainly phenolics and aromatic amines, at the expenses of molecular oxygen. Therefore, they could find application in the field of enzymatic bioremediation of many industrial wastewaters, and in particular to bleach and/or detoxify dye-containing effluents. Not all industrial dyes behave as laccase substrates, but this limitation is often overcome by the judicious use of redox mediators. These could substantially widen the application range of laccases as bioremediation tools. The present study encompasses the main properties of the most used industrial dyes as related to their chemical classification, fungal laccases and their molecular and catalytic features, the use of redox mediators, limitations and perspectives of the use of fungal laccases for industrial dye bleaching.

Keywords: Laccase, textile dyes, bleaching, bioremediation

1 Introduction

1.1 Laccases: a brief overview

Since its discovery at the end of the 19th century in the latex of the Japanese lacquer tree [1], the enzyme laccase has drawn much attention for its outstanding features. Laccases are found in some higher plants [2,3], but also in certain bacteria [4,5]. However, ligninolytic basidiomycetes [6] are the sources of the most studied laccases, usually employed in technological applications. Ligninolytic basidiomycetes, the white rot fungi, secrete laccase isozymes, involved in lignin oxidative depolymerization/solubilization [7-17].

Laccases belong to the multicopper oxidases (along with other enzymes such as tyrosinase and ascorbate oxidase [18-22]), and usually contain four cupric ions, belonging to three distinct spectroscopic types (T1, T2, and T3 [23]), arranged within the enzyme active site so that two targets could be achieved: i) the one-electron oxidation of a reducing substrate by the T1 cupric ion, and ii) the regeneration of the resting state of the enzyme by means of molecular oxygen reduction to water, by the intervention of a tricupric cluster, formed by one T2 cupric ion and two T3 cupric ions, the latter anti-ferromagnetically coupled .

The T1 cupric ion in bacterial laccases presents a distorted tetrahedral geometry built up from a strong trigonal set of aminoacidic residues (His-Cys-His) and a relatively weaker ligand in the axial position, generally a Met. In multicopper oxidases such as fungal laccases the Met ligand is replaced by a non-coordinating hydrophobic Leu or Phe residue, leading to a trigonal planar geometry [24]. In any case, the peculiar ligation geometry of the T1 cupric ion and the nature of coordinating aminoacidic residues are responsible for the typical strong absorption at about 600 nm, corresponding to an intense blue color. By contrast, the contribution of T2 and T3 ions to the protein color is negligible. The polar versus non-polar nature of the axial aminoacidic residues correlates with the redox potential of the T1 cupric ion, and hydrophobic residues such as Phe or Leu have a positive effect, whereas a polar, coordinating residue such as that of Gln (present in the blue, single copper, low-potential protein stellacyanin) shows the opposite effect. Such effects have been studied in detail in the bacterial blue copper, high-redox-potential protein rusticyanin from *Thiobacillus ferrooxidans* [25]. In laccases, as a general rule, a more hydrophobic axial ligand corresponds to a higher redox potential. Bacterial and plant laccases usually have a Met as the axial ligand for the T1 cupric ion, and share a relatively low redox potential ($E^{o} < +500$ mV). Ascomycete and a few basidiomycete

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laccases have a non-coordinating Leu residue at the axial position and show intermediate redox potentials, ranging from about +500 mV to about +700 mV. Finally, high-potential (Eº > +700 mV) laccases (from basidiomycetes) almost invariably bear a non-coordinating Phe residue at the axial position [23]. Site-directed mutagenesis on some fungal laccases resulted in contrasting effects. Leu/Phe interchange could be without significant electrochemical and kinetic effects (*Rhizoctonia solani* and *Myceliophthora thermophila* laccases [26]). In another fungal laccase [27], the changing of Met for Phe resulted in substantial alterations of pH optimum and kinetic constants. In bacterial enzymes, such substitutions could be sometimes favorable [28], whereas in other cases the increase of Eº causes an excessive stabilization of the cuprous state of the T1 copper, thus substantially worsening the enzyme performances [24,29].

Although the crucial role of the aminoacidic residue in the axial position towards the redox potential and reactivity of the T1 cupric ion is firmly established, some other structural factors could exert to a certain extent a small influence towards redox potentials of the T1 cupric ions [30,31].

Once the one-electron oxidation of the substrate is accomplished by means of the one-electron reduction of the T1 cupric ion to its cuprous counterpart, the oxidized state of T1 copper is quickly restored by one-electron transfer to the tricopper cluster [32]. Such a transfer has to be repeated four times to reduce molecular oxygen to two water molecules. In fact, the transfers take place in two steps, each of two electrons, giving rise to a peroxide intermediate, which in turn is reduced to water in the second step, thus regenerating the resting state of the enzyme. Theoretical calculations favor a μ - η^2: η^2 arrangement for peroxide anion $^{-}O–O^{-}$ positioning between the two cupric ions of the T3 dicupric cluster, such as it was found in hemocyanins, tyrosinases, and catecholases. However, this forecast is contradicted by spectroscopic evidence, and the peroxide-tricopper cluster adduct is presumably arranged in a μ_3 – 1,1,2 geometry therefore involving the T2 cupric ion in peroxide ligation. The electron transfer takes place through the highly conserved His-Cys-His bridge between T1 and the tricopper cluster [33]. The dioxygen and peroxide ligation to the reduced tricopper cluster is irreversible; the ligation geometry when the first two-electron reduction (to peroxide dianion O_2^{2-}) takes place does not change substantially.

So, two distinct - although close to each other - sites exist within the laccase catalytic center: the T1 cupric ion, devoted to reducing substrate one-electron oxidation, and the tricupric cluster which, upon reduction to its cuprous state, binds dioxygen.

The Eº values deeply influence laccase efficiency in oxidizing substrates, being obviously comparatively more efficient those with high redox potentials, secreted by ligninolytic basidiomycetes, that also show a wider substrate specificity. Heterologous expression of non-secreted but interesting laccases is usually prevented by their glycoprotein nature [34]. Therefore, high-potential, secreted fungal laccases are the best candidates for biotechnological applications.

Fungal laccases usually show activity optima within the acidic pH range; however, pH increase favors deprotonation of substrates such as phenols (to the corresponding phenoxide anions) and aromatic amines (from ammonium salts to the corresponding free bases) that are more easily oxidized. However, the overall efficiency of laccase-catalyzed oxidations is lowered by increasing pH owing to the OH^- ligation to the tricopper T2/T3 cluster, therefore hindering the internal electron transfer form the T1 copper to the tricopper cluster [35]. In general, the hydroxide-depending disturbance towards the enzyme activity prevails over the facilitated oxidation of substrates upon deprotonation [34]. In principle, two distinct mechanisms could operate along laccase catalysis: i) the enzyme abstracts one electron from a lone pair pertaining to a heteroatom (usually oxygen, nitrogen, or sulfur), a cation radical arises, which in turn undergoes a nucleophilic attack by H_2O, OH^-, or so on (Electron Transfer mechanism, ET). In such cases, a second electron could hardly be abstracted from the cation radical, mainly owing to electrostatic reasons, and therefore no quinonoid product could be formed directly. However, at higher pH phenolics exist - at least in part - as their phenoxide counterparts. In such cases, no cation radicals arise, and neutral delocalized phenoxy radicals are easily produced, which can in turn give rise to oxidative coupling, or disproportionation, or also direct quinonization by means of a second electron abstraction by the enzyme; ii) the enzyme abstracts a hydrogen atom (in fact, one proton and one electron, Hydrogen Atom Transfer mechanism, HAT), from a non-dissociated phenol (at lower pH) or a non-protonated primary or secondary aromatic amine. In any case a neutral delocalized radical arises, which is further transformed as described above.

Fungal laccases are typically inducible enzymes, whose production could be dramatically increased by a variety of small aromatic molecules, sometimes resembling monolignols in chemical structure. A number of different isoenzymes are secreted in response to different inducers [36-41].

Being that laccases are cupro-proteins, copper salts under suitable conditions also lead to enhanced

enzyme secretion [42-45]. According to their official name, laccases (EC 1.10.3.2 Benzenediol: Oxygen Oxidoreductases, water forming) usually oxidize *ortho*- and *para*-diphenols (catechols and quinols, respectively) to the corresponding quinones [46-51]. However, generally speaking, also monophenols could behave as laccase substrates, provided that they bear at least one electron-donating substituent at the *ortho* or *para* positions. So, methoxyphenols such as guaiacol (2-methoxyphenol) and hydroquinone monomethylether (4-methoxyphenol) are good laccase substrates. The same considerations could be extended to aromatic amines as well as to aminophenols [52,53]. As noted above, laccases work through a one-electron abstraction (and also one proton could be extracted, depending on the particular substrates and the experimental conditions such as pH, *vide supra*) from an electron-rich aromatic substrate, provided that the arising radical could delocalize the unpaired electron, giving rise to a relatively stable entity. Such a radical usually evolves further to a more or less complex mixture of coupling, oligomerization, polymerization products, or could disproportionate to the starting substrate and a quinonoid product. The latter could in turn evolve to other products, such as Michael-type addition products with the remaining substrate [50,54]. All these possible pathways are summarized on Fig. 1.

Obviously, this pattern depends on a number of factors (the particular substrate, the pH, and the presence of excess molecular oxygen, among others). And the presence of molecular oxygen is of the greatest importance, since it could perform the dual role of laccase substrate and non-enzymatic reagent towards the free radicals arising from the action of the laccase or otherwise generated [55]. A very complex mixture of reaction products arises, comprising hydrogen peroxide, peroxyquinones, oxiranes (epoxides), and quinones.

In conclusion, laccase specificity is much wider than that suggested by its systematic name, as it can very often attack non-phenolic dyes, provided that organic dyes in general are typical for an extended electronic delocalization, in turn allowing the formation of delocalized radicals upon one-electron abstraction by the enzyme. Such radicals could evolve towards degradation, by following the general criteria outlined above, and according to their particular chemical structure.

1.2 Widening laccase specificity and potential use: redox mediators

Laccase-catalyzed oxidation of potential substrates is limited by a number of conditions, such as steric hindrance (for macromolecular compounds), very low affinity between the compound and the enzyme active site, and too high redox potential of the putative substrates when compared with the oxidizing power of the enzyme. Although a noticeable range of redox potential have been calculated for different laccases, and in many cases found to be comparatively high [14,56], still a huge number and variety of compounds remain out of the reach of these enzymes. This obstacle can be overcome in many cases by means of particular substances, usually small, water-soluble molecules, capable of behaving as one-electron shuttles between the enzyme and the to-be-oxidized compounds [57,58] (Fig. 1). In more detail, these compounds are laccase substrates, whose oxidation proceeds as usual with the one electron abstraction. The arising free radicals are long-lived enough to diffuse away from the enzyme active site, and are therefore capable of restoring their stable electronic configurations at the expenses of other substrates [59]. Conceptually, no great difference exists between 'conventional' substrates and

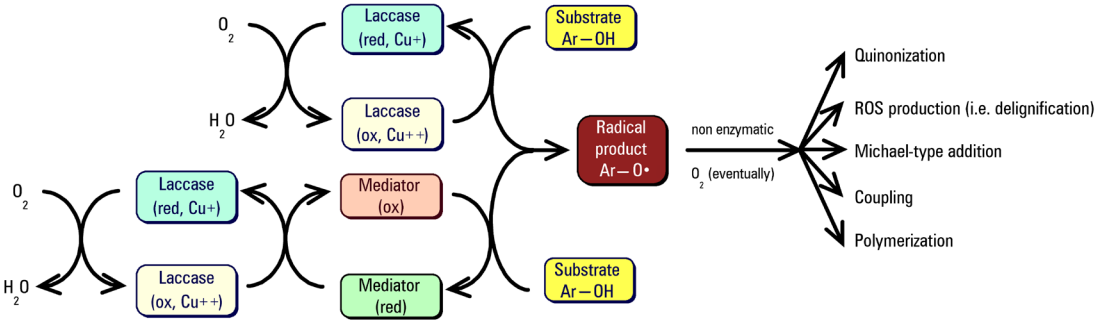


Figure 1: Simplified mechanism of laccase action in the presence and in the absence of redox mediators [54]. Some of the products (i.e. polymerized polyphenols) could be in turn laccase substrates.

mediators. However, a mediator *stricto sensu* is a substrate, whose radical is capable of restoring the original electronic configuration at the expenses of another compound, which is usually resistant against the direct action of laccase. These redox mediators (RMs) are well known in Nature, and many others have been synthesized. Most of them have been studied in detail, and some have been proposed in technological applications. The behavior of RMs is not purely catalytic: the intermediate radicals arising from their enzymatic oxidation can well behave as those arising from 'conventional' substrates, therefore disproportionating and affording more or less slowly the (relatively) stable quinonoid product(s). So, in a technological application the chosen RM has to be periodically replenished to obviate the unavoidable oxidation.

Among natural mediators [60], some are certainly present in decaying wood, and others are presumably produced by the same fungus that operates the rotting process. Most are phenolics, already well known as laccase substrates, and therefore they are rapidly quinonized by the enzyme. So, they undergo fast consumption and have to be continually replaced, unless they are naturally present or also formed in suitable amounts along laccase action. Some simple phenolics have been studied and described in detail as laccase RMs: *p*-hydroxybenzyl alcohol, *p*-hydroxybenzoic acid, vanillin, acetovanillone, syringaldehyde, acetosyringone, syringic acid, *p*-coumaric, ferulic, and sinapic acids (Fig. 2) [61,62].

In particular, phenolics combining the presence of a *p*-substitution (such as –CHO, –COOH, –CH=CH–COOH) with two *o*,*o*' methoxyls, are good laccase substrates but cannot dimerize or polymerize (unless decarboxylation, demethoxylation, or oxidative coupling – in the case of cinnamic derivatives – takes place), and moreover their quinonization is comparatively difficult, for the same reasons. Therefore, such compounds are among the most efficient phenolic-type RMs [63-65]. A particular case is that of *o*-hydroxyanthranilic (2-amino-3-hydroxybenzoic) acid, a simple *o*-aminophenol secreted by the white-rot fungus *Pycnoporus cinnabarinus*, which mediates lignin degradation by the fungal laccase, being at the same time slowly and irreversibly converted to its phenoxazone derivative, cinnabarinic acid [66]. However, hydroxyanthranilic acid, although effective in lignin degradation by *P. cinnabarinus*, is comparatively weak with respect to other RMs.

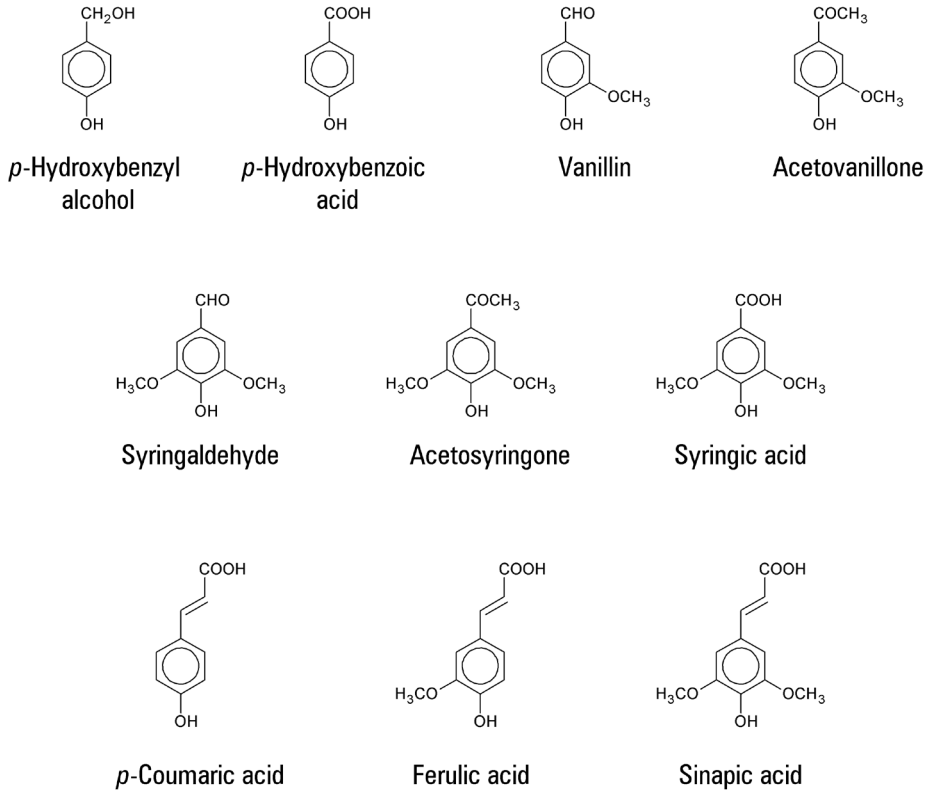
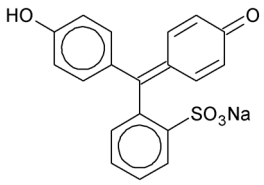


Figure 2: Natural redox mediators for laccase catalysis are mainly p-substituted phenolics.



Phenol red

Figure 3: Phenol red is a synthetic phenolic compound, effectively used as laccase redox mediator.

Many artificial compounds have been tested as potential RMs, and for many of them a noticeable effectiveness has been assessed. In particular, ABTS has been the first synthetic molecule for which a RM role has been shown [58]. Among synthetic phenolics, Phenol Red (a sulfophthalein, Fig. 3) has proved to be effective as a RM [67]. Hydroxylamine derivatives, all sharing the presence of a >N–OH moiety, have become highly popular as RMs for laccase industrial applications, as they are readily available at reasonable prices. Their main drawbacks are the well-known toxicity and their tendency to act as laccase inhibitors [60]. Fig. 4 presents the most used NOH-type RMs.

TEMPO (and analogues) is a quite particular case: it is a stable >N–O• radical, which is oxidized by laccase to the corresponding oxoimmonium cation. This acts by following an ionic mechanism [68-71]. These RMs share with the phenolic ones the HAT action mechanism [72,73] (Fig. 5), contrary to what is observed for ABTS which typically acts through an ET mechanism [74] (Fig. 6). The two mechanisms could sometimes be differentiated by using proper substrates that yield different products [75,76]. Interestingly, synergistic effects have been observed when two RMs with different (HAT or ET) mechanisms are used together [77,78].

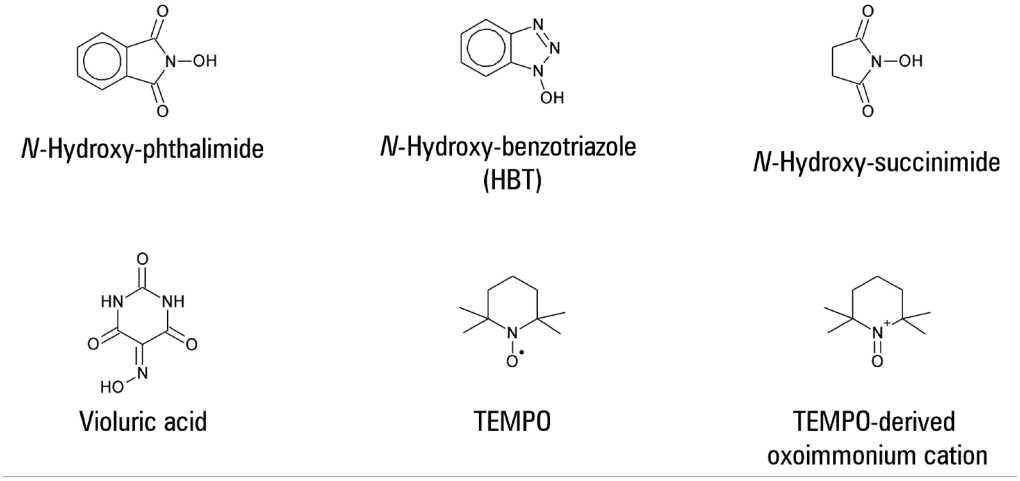


Figure 4: Hydroxylamine derivatives, bearing the typical N-OH moiety, are highly popular synthetic redox mediators for laccase catalysis.

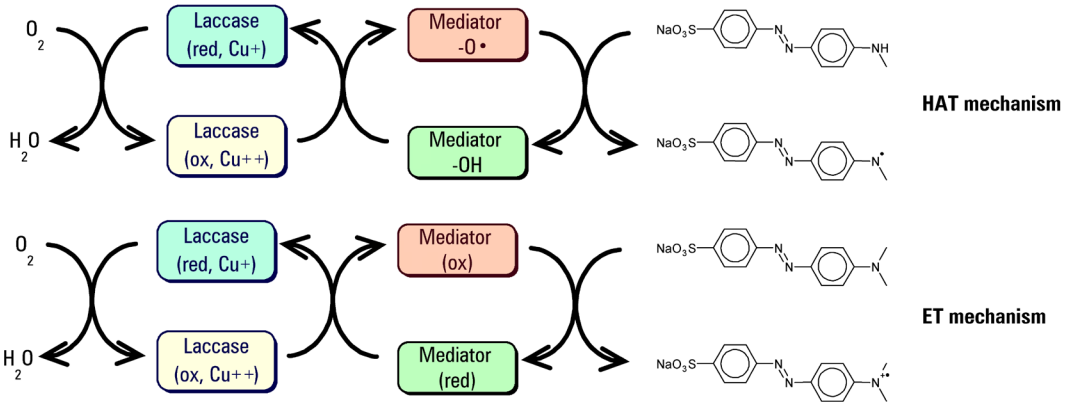


Figure 5: Laccase redox mediators can act both through HAT (hydrogen atom transfer) or ET (electron transfer) mechanism.

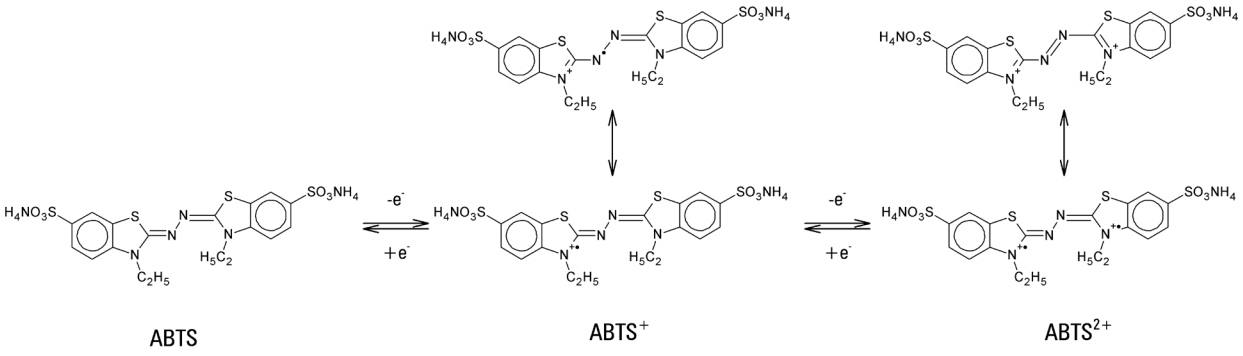


Figure 6: ABTS is a well-known laccase redox mediator, acting through ET mechanism, yielding cationic radicals stabilized by resonance.

1.3 Organic industrial dyes: molecular motifs, general reactivity

Industrial dyes constitute an enormous class of compounds, mainly organic, whose sole common feature is the absorption of visible light. Obviously, this feature is not enough, taken alone, to classify an organic, colored compound as a dye. In fact, a dye must be capable of conferring its color to a substrate to be dyed, such as fabrics, or plastics, or also foodstuff. Ideally, the obtained coloration should be stable in time, resistant against washings, and unaltered upon prolonged exposure to solar (or also artificial) light. Organic dyes are almost invariably aromatic compounds, showing extended electronic delocalization along π orbitals. Electronic delocalization of the chromophoric moiety of the dye molecule is reinforced by the presence, in suitable positions, of auxochromic/bathochromic groups such as hydroxy-, methoxy-, amino-, dimethylamino-groups and so on. Industrially relevant dyes should be inert enough against physical, chemical, and biological degradation, to show a proper durability under the application conditions.

A detailed discussion about structures and syntheses of industrial dyes is far beyond the scope of this paper; however, a brief description of the main classes of the relevant structures and main properties of these compounds could be useful for the readership and is therefore presented in the next chapter, which is mainly focused on the textile dyes.

1.3.1 Textile dyes in wastewaters

A huge amount of dyes are unavoidably released in wastewaters as a result of dyeing processes, adopted in textile plants [79]. Over 10,000 tons of dyes are produced every year [80] with quite low yields of textile processes (the percentage of the lost dye in the effluents can reach up to 50% [81]). Even greater amounts of water are consumed along the dyeing and washing processes (up to 200 L per Kg of textile fabricated, making the dye industry one of the most water-consuming sectors [82,83]). So a serious problem arises, of relatively low concentrations of dyes, contained in large volumes of water. Usually, no re-use of wastewater is performed [83], posing a serious issue about the economical and environmental sustainability of the processes (in fact water supplies are dropping, increasing its market price (up to almost 6 €/m^3 in the European Community [83]). Furthermore, the visual impact of the presence of dyes in wastewaters is high towards the public, so a high pressure towards textile plants and authorities involved in environmental defense has led to the development of an exceptionally high number of different methods, aimed to solve the problem of water bodies pollution by textile dyes [79,84-86].

These include biological, physical, and chemical approaches [82,84,87-89]. However, all of the methods suffer from significant drawbacks still affecting the economical feasibility of the processes. For instance, the common biological activated sludge treatment does not provide the flexibility required by the continuous differences in textile wastes composition [82,90]. On the other hand, the displacement of secondary waste produced by coagulation and adsorption techniques is sometimes less sustainable than the original wastewater [91]. Whereas, the chemical approaches usually suffer from high costs and low efficiency.

Textile dyes are usually water-soluble, or at least could be transiently changed into soluble derivatives that are in turn re-insolubilized and precipitated onto or within the fibers to be dyed, usually by means of a reduction (with sodium dithionite) followed by a re-oxidation (with air).

Therefore, the dyeing baths as well as the washing waters contain more or less elevated concentrations of dyes that should be removed before the wastes are funneled into receiving water bodies.

Given the extreme structural heterogeneity of the chemical structures of the hundreds of commonly used textile dyes, several methods should be kept under consideration when planning a treatment protocol for a particular textile wastewater, owing to the different reactivity of the different compounds.

In the present context, a very concise review of the main chemical classes of important dyes could be useful to the readership, which is directed to comprehensive reviews if necessary [84,92,93]. A reasonable knowledge of the fundamental structural motifs present in the different classes of the examined dyes could help with forecasting the pros and cons of the existing treatment protocols, or envisaging new ones.

Azo dyes are among the most used, as they are effective, stable, and inexpensive. The general structure of an azo dye R_1–N=N–R_2 gives evidence to the azo chromophore –N=N–, which is responsible for the main properties of the class [94]. R_1 and R_2 are aromatic (or heteroaromatic) moieties [95], where R_1 brings electron-withdrawing substituents whereas R_2 bears electron-releasing substituents. Electronic delocalization of an extended π system, also possibly involving the peripheral substituents, causes the compounds to strongly absorb visible light. The azo chromophore is *per se* an electron-withdrawing moiety (–I effect), although it possesses two lone pairs suitable for interactions with the adjacent aromatic systems (+M effect). When the substituents on the R_2 ring are –OH and/or $–NH_2$, the corresponding dye can exist in a protonated (–OH and/or $–NH_3^+$) or an unprotonated form ($–O^-$ and/or $–NH_2$), depending on pH. The alternative forms usually show different colors, and therefore could work as pH indicators. In many azo dyes, R_2 also contains electron-withdrawing substituents such as $–SO_3^-$ besides the electron-donating ones. The purpose of the sulfonic substituent is to confer solubility in water to the compound. Some prominent examples of azo dyes are encompassed in Fig. 7.

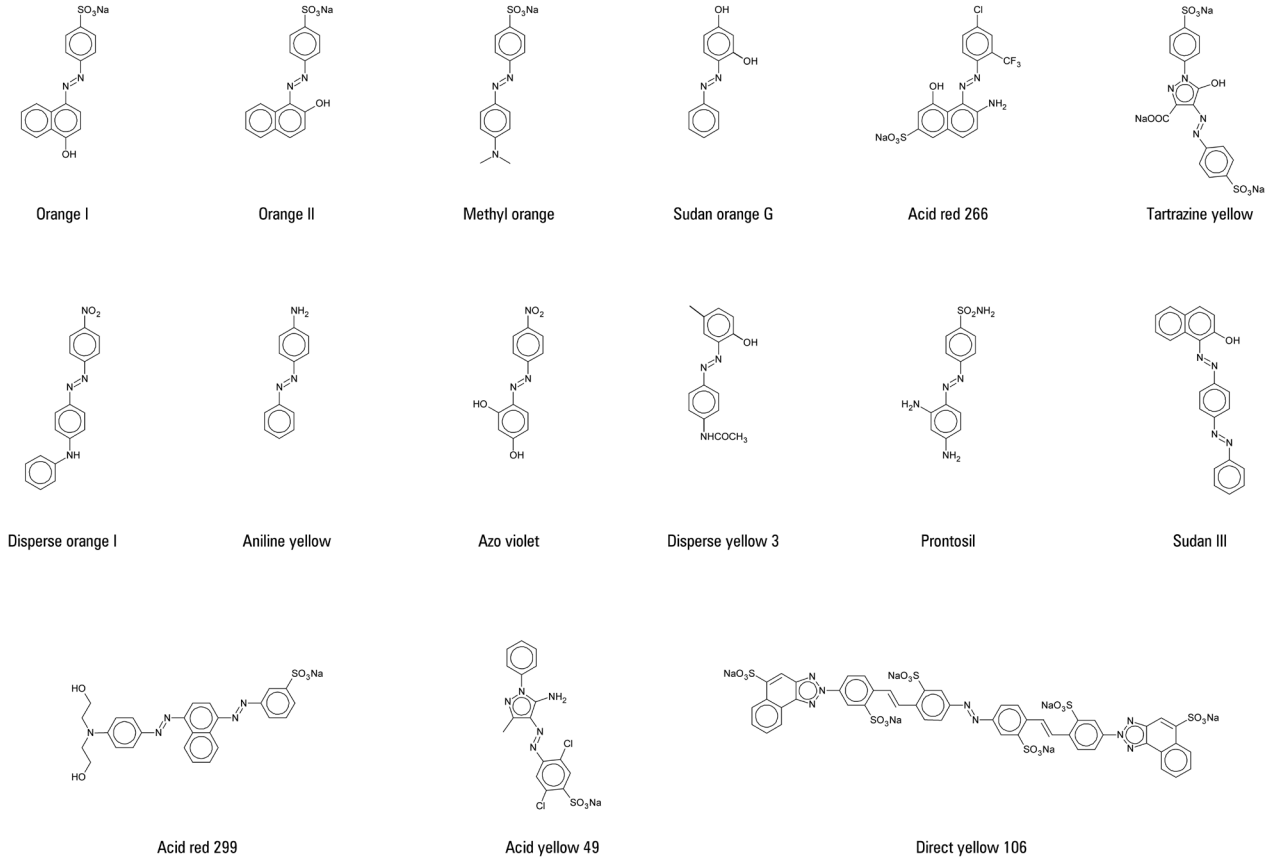


Figure 7: Chemical structures of the most diffused azo dyes.

9,10-Anthraquinone is the parent compound of a wide range of natural dyes and also of synthetic dyes, usually containing electron-donating substituents [96]. The iconic compound of this class is alizarin (1,2-dihydroxyanthraquinone) found in madder (*Rubia tinctorum*). A hydroxy and/or amino substituent should occupy the α position with respect to the carbonyl groups, to ensure the maximum overlap of the π orbitals and therefore the best tinctorial properties. On the whole, although typical for their light fastness and brightness, anthraquinone dyes are usually more costly and therefore cannot compete with the azo dyes. A selection of well-known anthraquinone dyes is presented in Fig. 8.

Indigoid dyes owe their name to indigo, obtained from the plant *Indigofera tinctoria*, which is the main source of 'natural' indigo [97]. Another famous dye is the imperial or Tyrian purple, which is 6,6'-dibromoindigo [98]. These dyes are in fact insoluble pigments, which need to be reduced to soluble leuco-compounds, and re-oxidized in turn by air directly on the fibers (vat dyes).

Simple indigoids are exceptionally inert and durable upon exposure to heating, sunlight, and extreme pH, although they can undergo both reversible reduction and oxidation under proper conditions. Synthetic indigo is largely used to dye denim fabric. Some outstanding indigoids are summarized in Fig. 9.

Cationic dyes are molecules, whose positive charge is an essential part of their chromophores; very different cationic dyes also exist, whose positive charge is not involved in the chromophoric delocalized electron system, so their cationic nature is only relevant to impart solubility in water and preferential adsorption on negatively charged fibers. Obviously, cationic dyes are a rather heterogeneous class of substances, owing to the very different chromophores they show.

Cyanine dyes are cationic substances containing polymethine bridges between two nitrogen atoms with a delocalized positive charge. The number of carbon atoms forming the bridge may vary within a wide range, thus extending the charge delocalization. Also, one or both nitrogen atoms can be part of cyclic or polycyclic structures, further expanding the structural variety of this dye class. Consequently, a huge number of cyanine dyes exist, with shades ranging from deep blue to

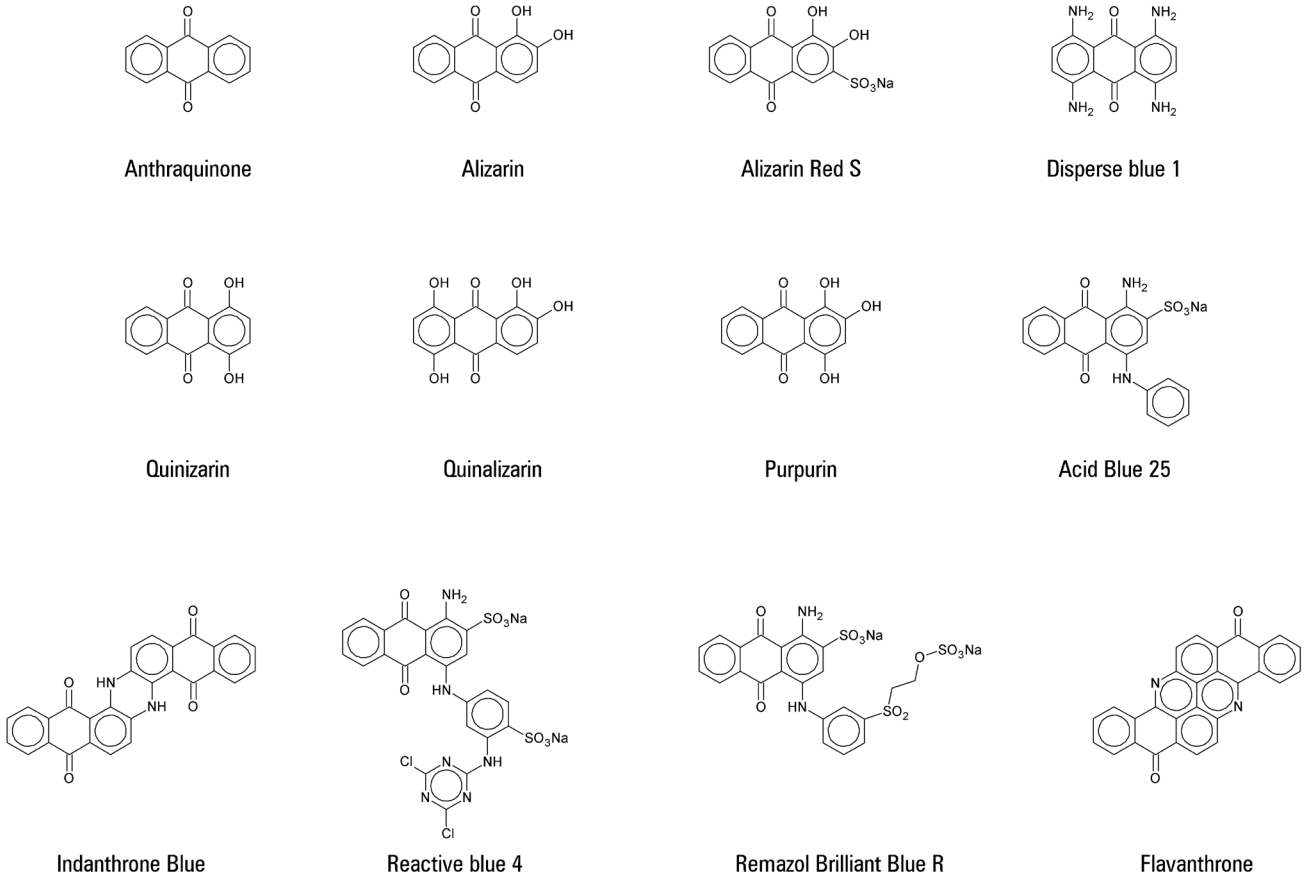


Figure 8: Chemical structures of the most diffused anthraquinone dyes.

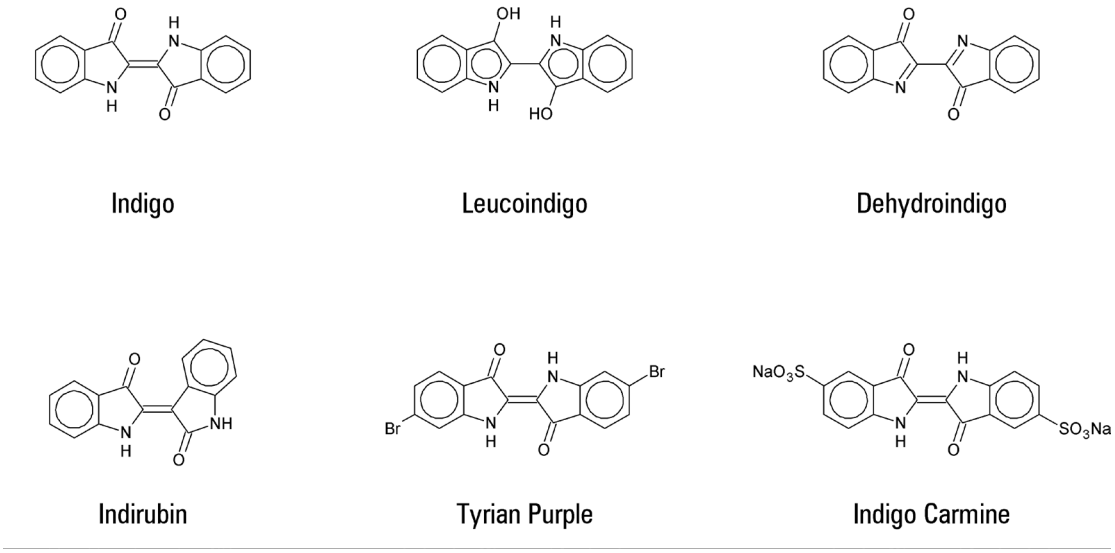


Figure 9: Chemical structure of indigo and the most diffused indigoid dyes.

green to yellow to red and purple. The fluorescence of many cyanine dyes widens their fields of application to photography, biotechnology, and lasers [99,100]. Some cyanine-based structures are depicted in Fig. 10.

Triphenylmethane cationic dyes, once referred to as fuchsoneimine derivatives, are more properly regarded as cyclic cyanine dyes where the methine bridge is incorporated within an aromatic ring. They could also be considered as delocalized triphenylmethyl cations. Some important triphenylmethane-based dyes are illustrated in Fig. 11.

Similarly, diphenylmethane-based cationic dyes have been considered as benzophenoneimine derivatives. When a nitrogen atom bridges the two aromatic rings, acridines are obtained; when the bridging atom is oxygen or sulfur, xanthenes or thiaxanthenes, respectively, are formed as is shown in Fig. 12. Other cationic dyes, also belonging to the cyanine family, are derived from phenazine, phenoxazine, and phenothiazine. Some outstanding examples of these are shown in Fig. 13. Some comprehensive discussions about the above mentioned cationic dyes are available [94,101,102].

Phthalocyanine dyes are macrocycles [103] usually obtained starting from *o*-phthalonitrile. Despite a feeble resemblance with porphyrins, these dyes sharply differ from the latter for being exceptionally stable compounds. Phthalocyanines are usually applied as the corresponding metal complexes, usually with cupric ions.

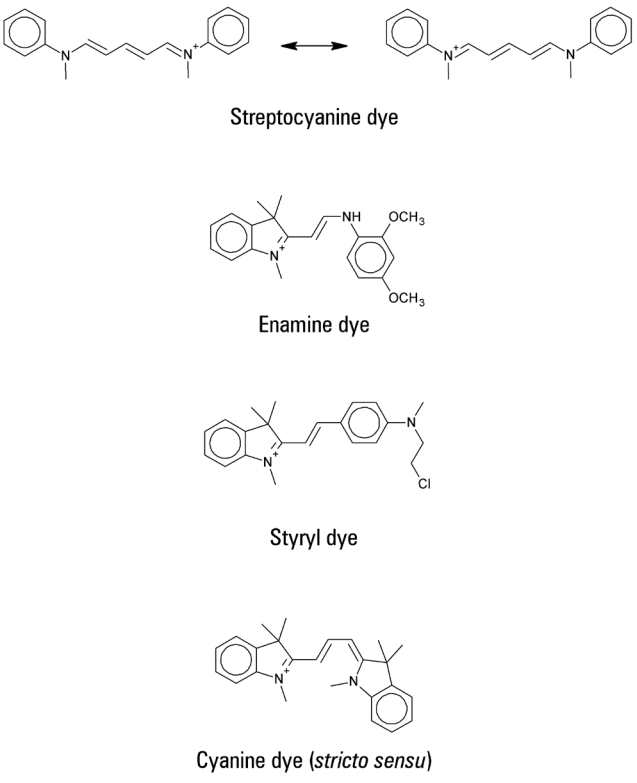


Figure 10: Chemical structures of some cyanine-based dyes.

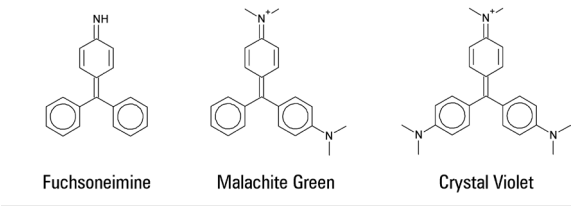


Figure 11: Chemical structures of some important triphenylmethane-based dyes.

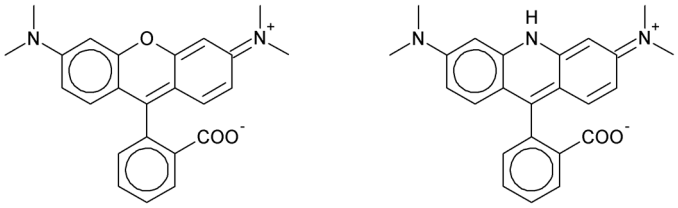


Figure 12: Chemical structure of acridine- and xanthene-based dyes.

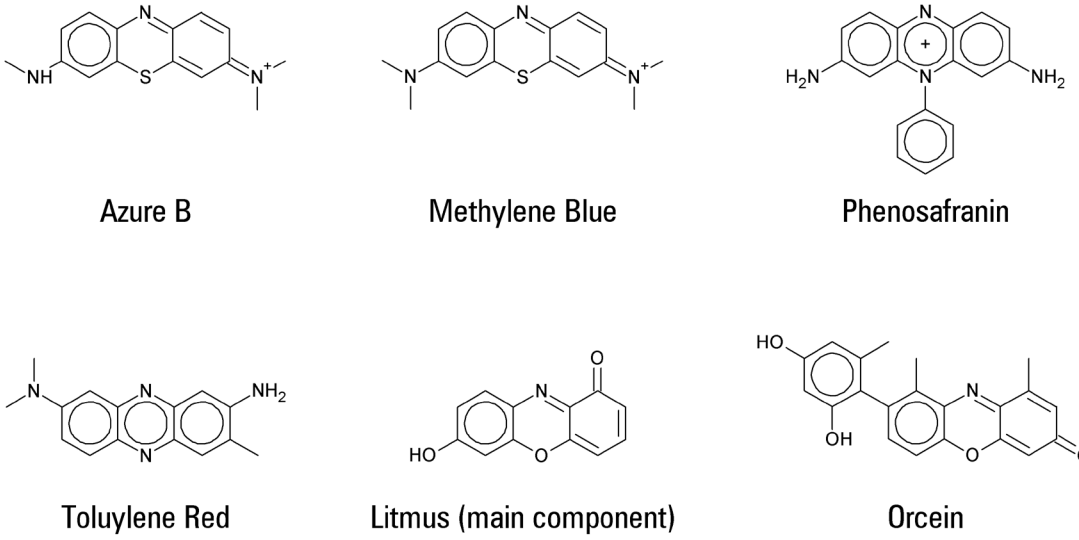


Figure 13: Several cationic dyes derive from phenazine, phenoxazine, and phenothiazine.

Copper phthalocyanine is a quite useful pigment, very stable against heating, sunlight, and harsh washings. Phthalocyanines (and their metal complexes) are completely water insoluble, and should be regarded as pigments rather than dyes *stricto sensu*. However, water-soluble phthalocyanine dyes are well known, bearing solubilizing moieties such as the sulfonate anion $-SO_3^-$. Relevant phthalocyanines are depicted in Fig. 14.

Sulfur dyes are obtained by heating suitable aromatics or heterocycles with sulfur or compounds capable of releasing sulfur upon heating. From the obtained melts the dyes can be extracted, showing non-definite structures. Sulfur atoms could be part of newly formed heterocyclic structures, and/or also constitute thiol, sulfide, or polysulfide moieties [104]. Very often sulfur dyes must be solubilized by reduction under alkaline conditions, to afford the corresponding thiolates; these are reoxidized by air thus giving the insoluble dye. Differently from other dye classes, univocal identification of a definite chromophore in sulfur dyes is usually impossible. Some sulfur dyes are shown in Fig. 15.

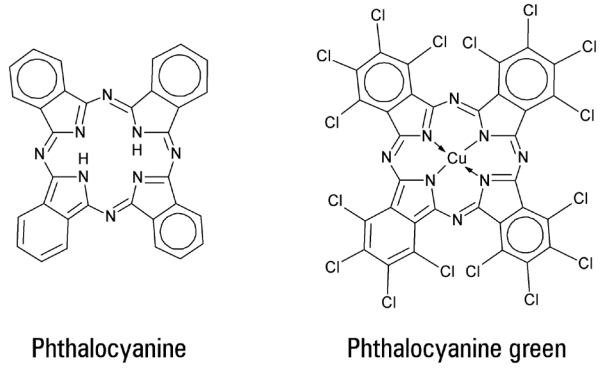


Figure 14: Chemical structures of some phthalocyanine dyes.

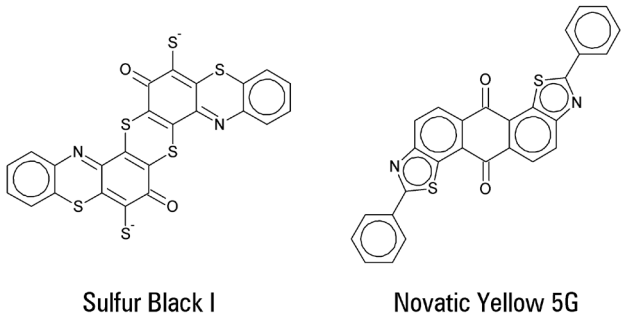


Figure 15: Chemical structures of some sulfur dyes. Univocal identification of a definite chromophore is seldom possible. For instance, Novatic Yellow 5G could be also regarded as an anthraquinone dye.

1.4 Fungal laccases within the frame of white rot fungi metabolism

Although laccases have been found in many fungi and also in bacteria, higher plants, and insects [34], they are typical for white-rot fungi [105-107]. These are the sole family of fungi capable of efficiently degrading lignin [108]. Lignin biosynthesis, structure, and function have been encompassed in some reviews [109-113]. Lignin is a very complex heteropolymer obtained by oxidative polymerization of the so-called monolignols (Fig. 16). A 3D lattice, very compact and rather hydrophobic, is obtained, where the monomeric units are mainly linked by means of C–C bonds and aryl ether functions (Fig. 17). Therefore, lignins are completely resistant towards hydrolysis, but can be gradually fragmented and solubilized by means of an enzymatic/non-enzymatic pathway, requiring some fungal enzymes, such as high-potential heme peroxidases (Lignin peroxidase EC 1.11.1.14, Manganese peroxidase EC 1.11.1.13, Versatile peroxidase EC 1.11.1.16) and laccase. White-rot fungi have been classified on the basis of the ligninolytic enzymatic patterns they express when growing on lignocellulosics [114], and laccase is generally believed to exert a key role in oxidative ligninolysis [5,16,108,115-120].

Among the known ligninolytic enzymes in white-rot fungi, laccases are by far the most widespread and the most manageable [121], contrary to peroxidases, which suffer for an exaggerated sensitivity to their substrate hydrogen peroxide, easily leading to irreversible enzyme inactivation [17,122-124], suggesting the use of biomimetic catalysts in their stead [125-127]. Therefore, the fact that a relatively low number of studies are devoted to the use

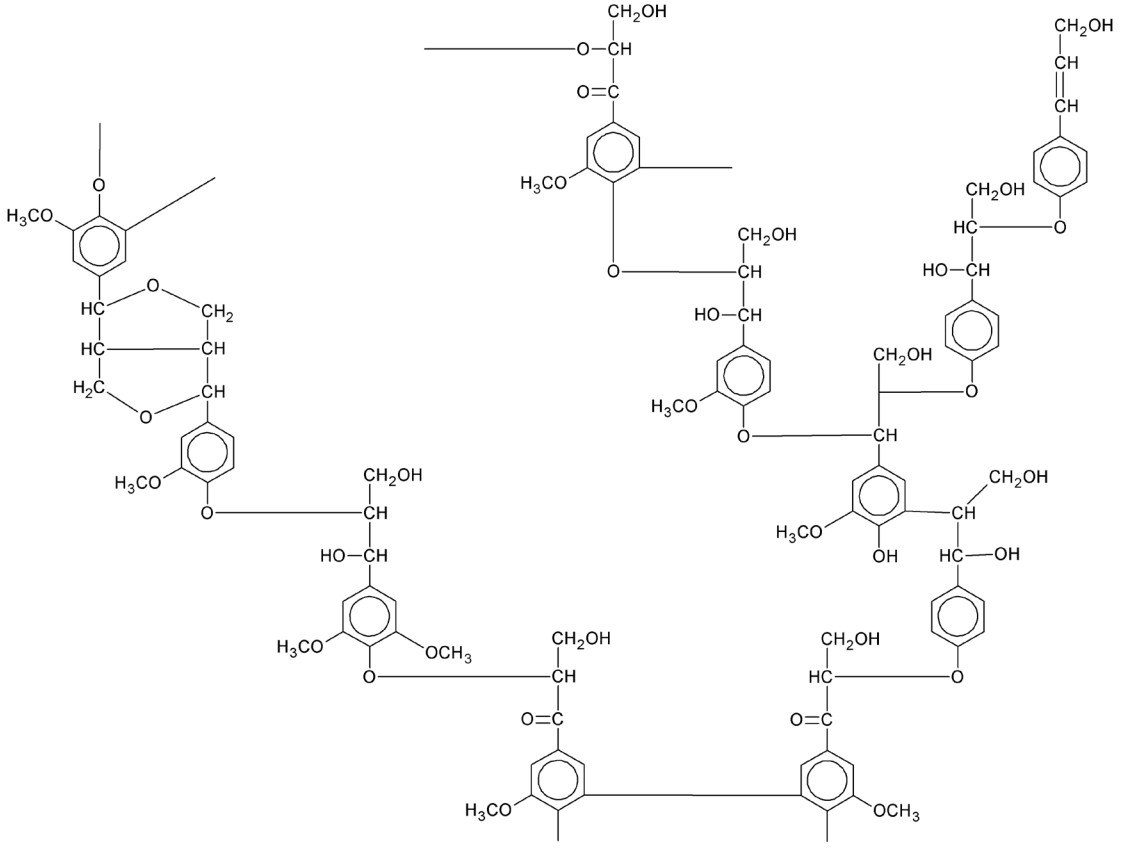


Figure 16: Hypothetical structure of a portion of a lignin molecule.

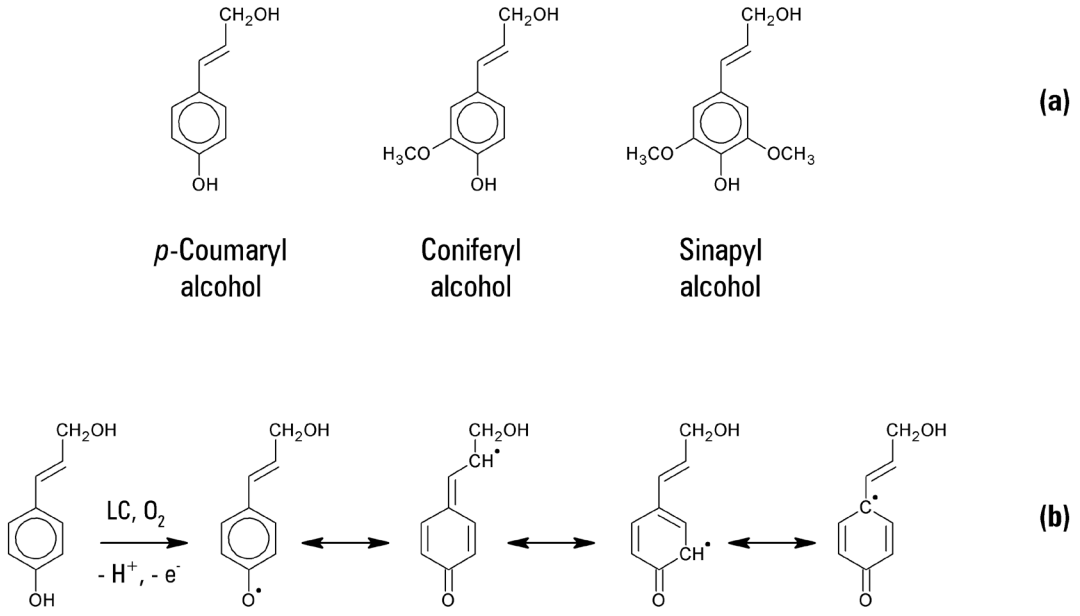


Figure 17: The three monolignols (a) are the starting units for the synthesis of lignin, by radical non-enzymatic addiction of the corresponding radicals (b) arising from laccase catalysis [17].

of fungal peroxidases in delignification technology and bioremediation processes is not surprising [128-131]. Also the need for more expensive hydrogen peroxide as the oxidizing agent, whereas laccases use the inexpensive oxygen from air, has to be taken into account to explain the sharp prevalence of laccase-based processes.

Laccase alone *in vitro* appears on the whole as incapable of promoting lignin oxidative breakdown, and on the contrary it seems capable of triggering a further polymerization amongst native lignin molecules [132]. Laccase attacks the peripheral regions of lignin macromolecules, where it specifically oxidizes phenolic units to the corresponding phenoxy radicals (Hydrogen Atom Transfer mechanism, HAT, also extensible to primary $-NH_2$ and secondary $-NHR$ amines, *vide infra*); these are most probably responsible for the observed polymerization reactions. In fact, laccase action towards lignin is rather complex [133,134] and a partial depolymerization also takes place (Fig. 18). It is commonly believed that laccase is particularly effective in oxidative degradation of relatively simple phenolics, arising from oxidative breakdown of the native lignin molecules, in turn caused by other enzymes such as high-potential fungal peroxidases, and/or by the intervention of ROS (Reactive Oxygen Species) formed as the result of direct, non-enzymic interactions between phenoxy radicals and excess molecular oxygen.

As an iconic example, vanillic (4-hydroxy-3-methoxybenzoic) acid gives rise to a number of oxidation products [135] such as methoxy-1,4-benzoquinone, and degradation products of 1,2-benzoquinone-4-carboxylic acid (Fig. 19). In other words, quinonization goes together with oxidative demethoxylation and/or decarboxylation.

Then, the crucial question is: why is laccase capable of delignification *in vivo*, whereas its action is rather disappointing *in vitro*? The answer most probably resides in the existence of some quinone-recycling fungal enzymes, localized within the periplasmic membranes, and NAD(P)H-dependent [136-138]. The arising quinols are quickly re-oxidized by laccase, giving rise to phenoxy radicals that are in turn efficient sources of ROS (the true responsible species for lignin oxidative fragmentation).

In conclusion, laccase is able to perform several different reactions (compare also Fig. 1) that can find useful technological application. Laccase versatility is often further increased by non-enzymatic action of molecular oxygen and its reactive derivatives, and very notably by the judicious use of RMs. Among the large range of laccase substrates undoubtedly are industrial dyes, as demonstrated by the huge number of theoretical and applicative studies dealing with dye oxidative bleaching upon laccase catalysis. The next section introduces dyes as substrates for laccases.

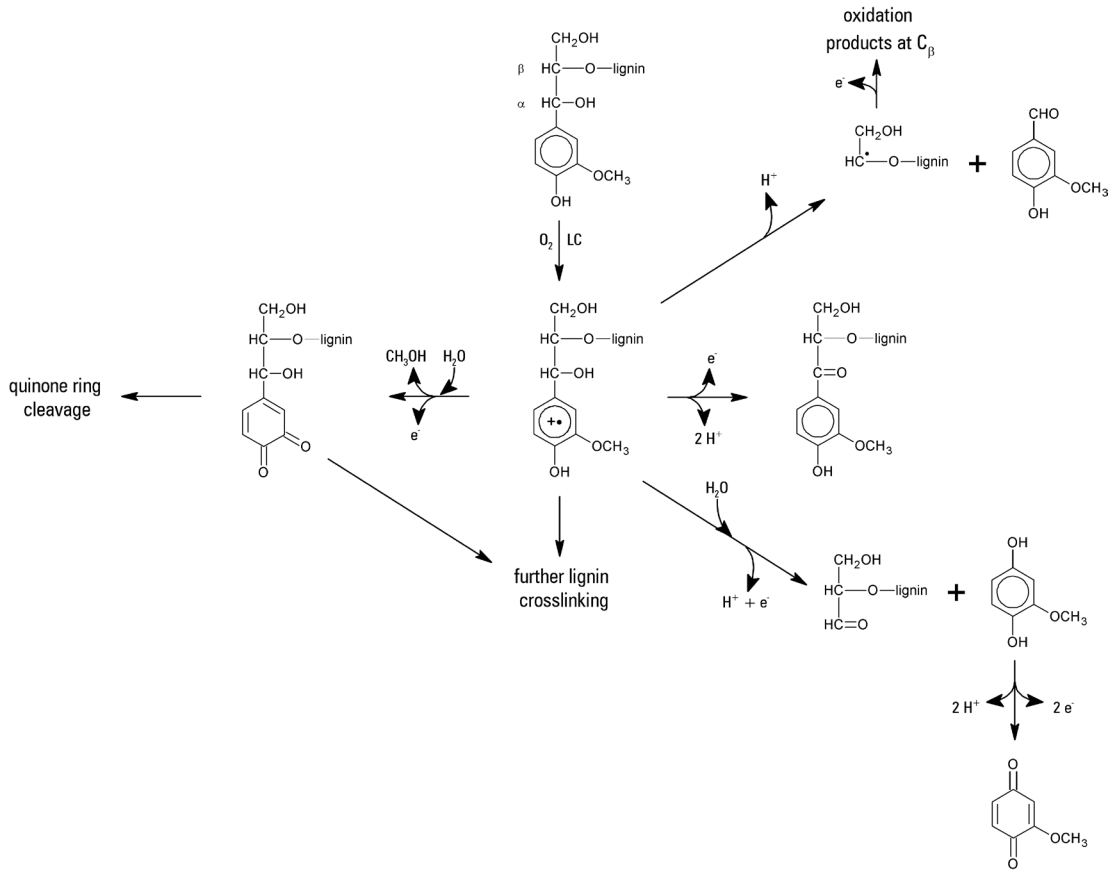


Figure 18: Laccase action towards lignin is quite complex, leading at least to a partial depolymerization whereas several side reactions occur [133,134].

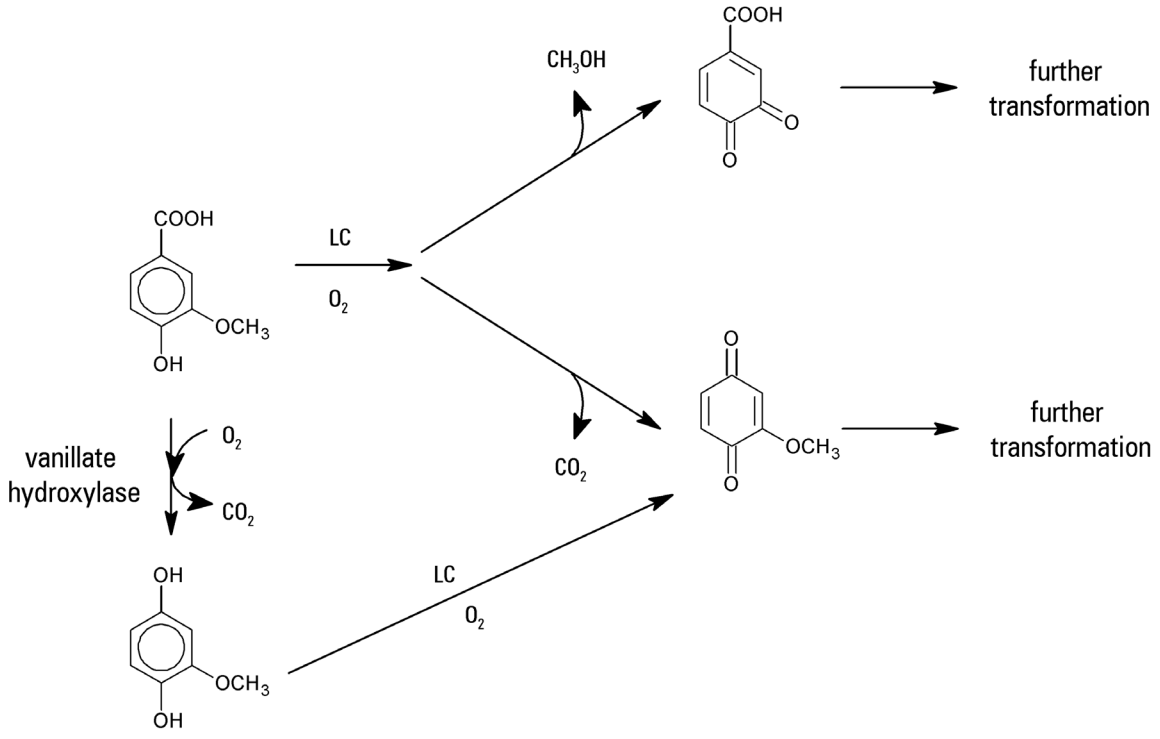


Figure 19: Laccase catalysis gives rise to a number of oxidation products starting from vanillic (4-hydroxy-3-methoxybenzoic) acid.

2 Industrial dyes as 'direct' laccase substrates

As noted above, dye molecules generally bear one or more auxochromic/bathochromic groups, namely –OH, $–OCH_3$, $–NH_2$, $–N(CH_3)_2$, $–NH–C_6H_5$, and so on, directly bound to an aromatic ring, with the aim of increasing the ε values and at the same time of shifting the λ_{max} well within the visible range. As both oxygen and nitrogen are more electronegative than carbon, these substituents show a feeble –I action, that is largely overcome by a +M effect, arising from the presence of lone pairs on the heteroatoms. So, when the dye also shows a net positive charge, usually an electron-rich region exists in the molecule, which is capable, under proper conditions, of losing one electron (eventually also one proton), giving rise to a radical. The further fate of such a radical depends on the particular dye considered, on the pH, on the presence and concentration of molecular oxygen. The agent, capable of performing such an electron abstraction could well be a fungal laccase, as demonstrated by a huge number of studies, dealing with industrial dye bleaching by fungal laccases [79,80,85,139-159].

2.1 Degradation of azo dyes

As noted in § 1.1.1, azo dyes almost invariably contain at least one phenolic or amine substituent, bound to one of the two aromatic systems linked by the –N=N– group. This electron-rich portion of the dye molecule would be the target of the laccase action. The main aim of dye-containing wastewater treatment with laccase (or any other method to decolorize such wastewater) is just decolorization, so not many studies are dedicated to the intimate mechanism(s) of dye oxidative degradation. However, some detailed degradation pathways have been proposed for selected azo dyes, that could well be extended to this enormous dye family. Both bacterial and fungal laccases were tested, and the obtained results speak strongly in favor of a common mechanism, starting with one-electron abstraction by laccase from the electron-rich aromatic ring of the dye [160-162].

In particular, a series of dyes, sharing the sulfonate anion as the electron-withdrawing substituent in the *para* position, and the phenolic nature of the electron-rich aromatic ring, were tested as substrates of a laccase from the plant pathogenic fungus *Pyricularia (Magnaporthe) oryzae*. As a general result, the cleavage of the dye molecules was observed, at the bond linking the azo chromophore and the phenolic ring. In the presence of chloro- or nitro- substituents on the phenolic ring, no degradation at all was observed. After oxidation of the dye substrates, the electron-rich aromatic (phenolic) ring was found as the corresponding *para*-quinone (and/or its degradation products) whereas elemental nitrogen was evolved and the sulfobenzene ring was released as the *para*-hydroperoxy-benzenesulfonic acid and its degradation products (Fig. 20).

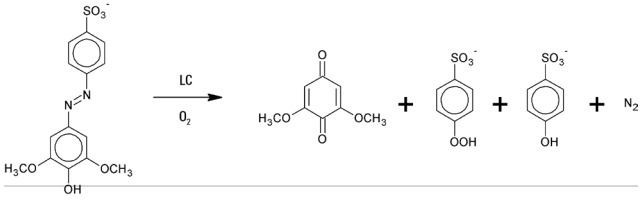


Figure 20: The reported reaction of the best substrate (2,6-dimethoxy-4-(4'-sulfophenylazo)-phenol) among the 4-sulfophenylazo dye family studied by Chivukula and coworkers [160].

Noticeably, oxidative cleavage of the studied dyes largely prevailed over oxidative coupling/polymerization [160]. In a somewhat similar study, two other azo dyes were studied [162], sharing the presence of the *meta*-benzenesulfonic moiety as the electron-poor portion of the dye molecules. The electron-rich portions were 4-dimethylaminobenzene or 2-hydroxynaphthalene, respectively. The laccase used came from the ligninolytic fungus *Trametes villosa*. The degradation pathway mainly paralleled that described above, but with the noticeable difference that a substantial fraction of the degradation products polymerize, also retaining the azo group (Fig. 21).

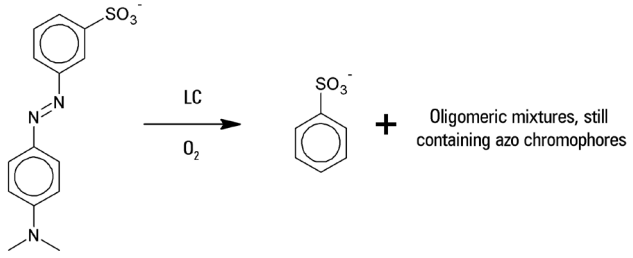


Figure 21: The degradation pathway of a laccase from ligninolytic fungus *Trametes villosa*, using a sulfophenylazo dye. In this case a substantial fraction of the degradation products polymerizes, also retaining the azo group [162].

The Authors therefore concluded that laccases are not suitable on the whole for the bioremediation of azo dyes. Another study on 19 azo dyes [161] showed that a recombinant bacterial laccase (CotA-Laccase, *Bacillus subtilis*) was able to efficiently degrade the majority of

the tested dyes, with the exception of reactive Yellow 81. This azo dye is however rather far from the 'conventional' azo dye structure, as it lacks the electron-rich moieties that are the preferred attack point for laccases. The fate of Sudan Orange G, which on the contrary has a very simple and 'conventional' structure, has been studied in detail. The degradation mechanism is still the same, leading to hydroxy-*p*-benzoquinone, benzene, phenol, and a polymer mixture of compounds, still retaining the azo moiety (Fig. 22). So, in this case the bioremediating power of laccase seems at least debatable.

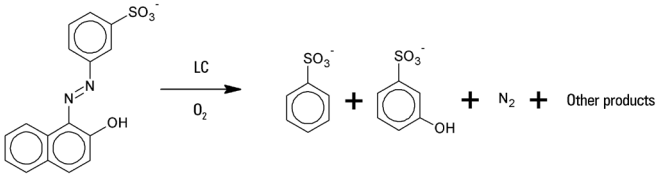


Figure 22: A recombinant bacterial laccase (CotA-Laccase, *Bacillus subtilis*) was able to efficiently degrade the majority of the tested azo dyes. The degradation mechanism is still the same, leading to a mixture of other products deriving from the transient o-naphthoquinone through oxidative coupling with both the starting dye and 3-hydroxy-benzenesulfonic acid [161].

A comprehensive, simplified scheme of the general degradation pathway is presented in Fig. 23. It is worth noting that amino azo dyes should follow an asymmetrical cleavage of the molecule, paralleling that observed in the case of phenolic azo dyes, and leaving the azo chromophore linked to the electron-poor aromatic ring, as a quite transient phenylazene derivative. The amine-bearing ring should afford a quinoneimine, which upon hydrolysis should afford ammonia and the corresponding benzoquinone.

Somewhat surprisingly, an asymmetrical cleavage opposite to that commonly observed, and leaving the azo chromophore linked to the dimethylaminophenyl moiety (under the form of the quite elusive 4-dimethylaminophenyl diazene [163]) has been reported as the cleavage product upon laccase action, starting from methyl orange [164,165].

Unlike laccase, ligninolytic peroxidases are able to achieve both symmetrical and asymmetrical cleavage paths (Fig. 24) [166]. This (apparent) difference is due to the sharply acidic pH values needed for peroxidase action. Under such conditions, a radical cation is formed, whose positive charge could be delocalized involving also the azo chromophore. Therefore this undergoes nucleophilic attack by water and subsequent hydrolysis, affording a nitroso derivative and an iminoquinone (and their further reaction products). Anyway, such a symmetrical cleavage mechanism could not be definitely ruled out in the case of laccase action, at least when working at low pH values.

It is worth noting that hydroxy- and amino- azo dyes could be oxidatively degraded upon bioinspired catalysis with redox-active metalloporphines and suitable oxidants. Also in this case, only asymmetrical mechanisms have been described [167]. However, in the case of the amino compounds, additional reactions (oxidative dealkylation of secondary and tertiary amines, followed by further oxidation giving the corresponding nitroso derivatives) take place, leading to condensation and/or oligomerization reactions that finally afford polyazo dyes, more recalcitrant than the parent dyes [167]. Therefore, although redox-active metalloporphines could oxidize a wider range of azo dyes than laccases can, such bioinspired catalysts often lead to worsening rather that solving of the problem.

Azo dyes can undergo the action of the rather non-specific enzymes azo reductases [94,168]. As a result of the action of azo reductases, the azo bridge is broken down and two arylamines arise. These are often carcinogenic, and usually more toxic than the dye that precedes them. Fortunately, such amines are good laccase substrates. Therefore, they are transiently changed into the corresponding quinoneimines, affording - upon hydrolysis - in turn quinones and/or their degradation/oxidation products.

2.2 Degradation of anthraquinone dyes

Anthraquinone itself is a rather unreactive molecule. Conversely, its hydroxy- and/or amino-derivatives could undergo a number of reactions, including oxidation, as is common knowledge. As expected, hydroxyanthraquinones can undergo oxidation by peroxidases [169] although sometimes oxidative coupling rather than cleavage takes place [170]. Hydroxy- and amino-anthraquinones basically behave as phenols and aromatic amines, although their reactivity is somewhat weakened by the electron-withdrawing power of the two carbonyl moieties. Nevertheless, in several cases they have proven to be good laccase substrates [171-174]. A comparative study about different fungi (*Pleurotus ostreatus* vs. *Phanerochaete chrysosporium*) showed that *Pleurotus* was sharply more efficient [175] in bleaching synthetic dyes dissolved in the culture broth, most probably owing to its higher laccase secretion than *Phanerochaete*. However, such efficiency dropped down when the culture filtrate was separated from the hyphae, therefore confirming the intervention of membrane-bound enzyme(s) helping laccase in its bleaching action. Such enzymes have been identified as

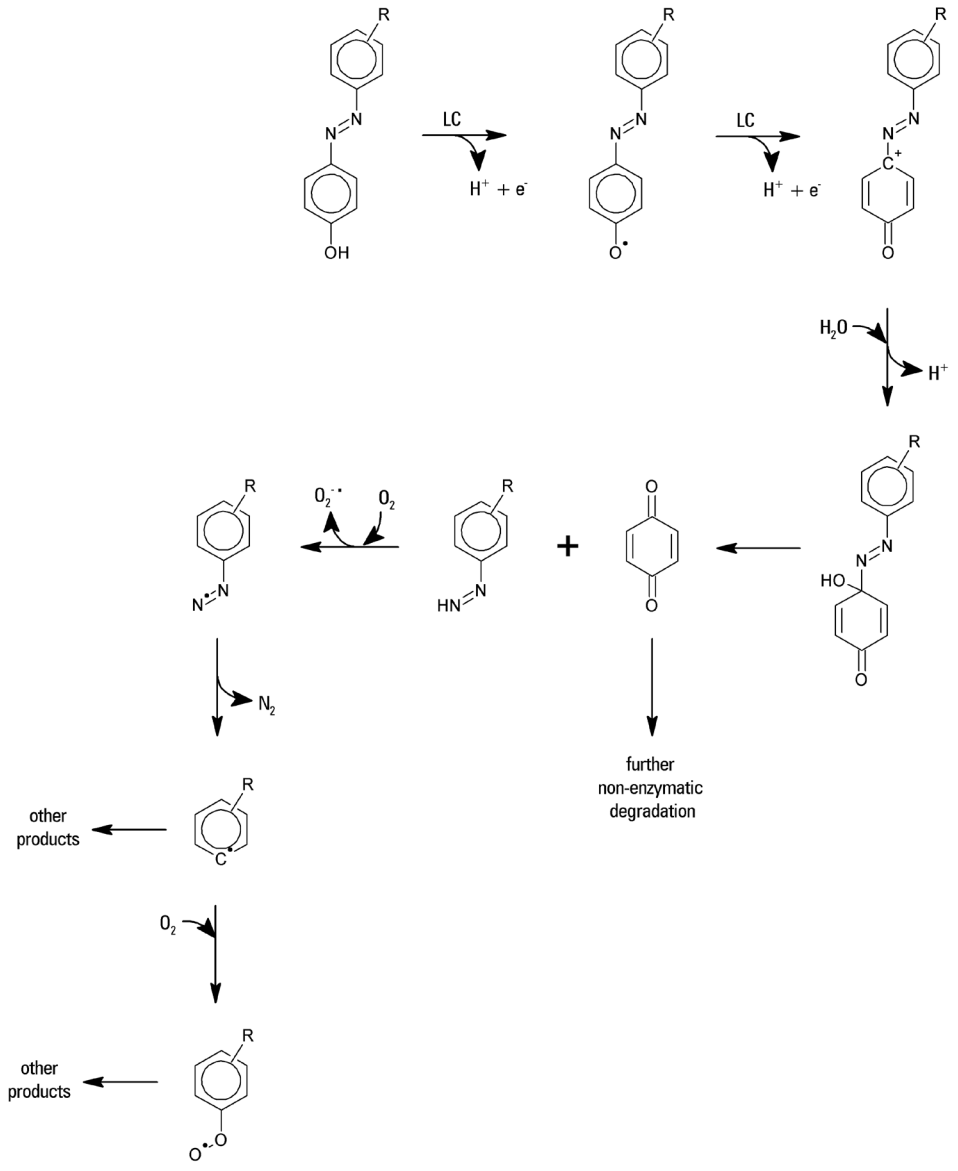


Figure 23: Comprehensive scheme of the general degradation pathway of azo dyes in the presence of laccase catalysis and molecular oxygen.

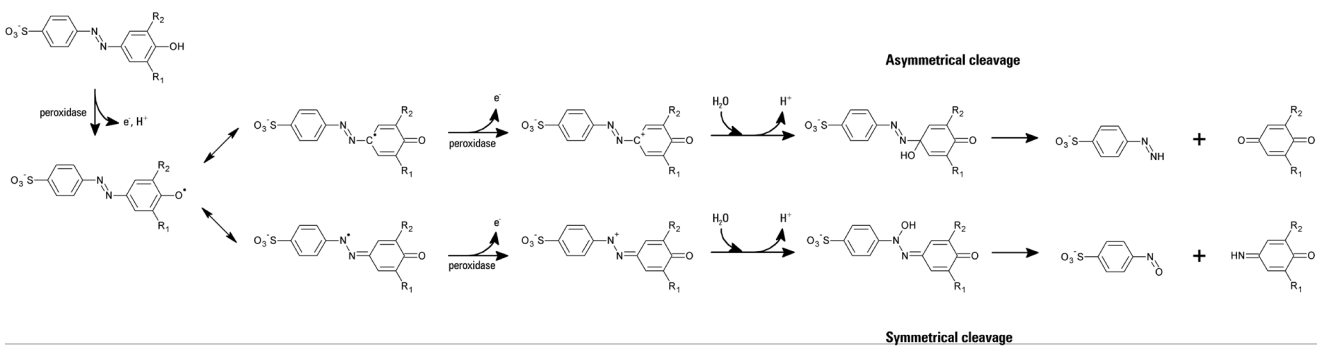


Figure 24: Proposed mechanism for asymmetrical and symmetrical cleavage of azo dyes by ligninolytic peroxidases (adapted from [166]).

NAD(P)H-dependent quinone reductases [137,138,176]. Quinones arising from laccase action are reduced to the corresponding quinols. These could be re-oxidized by laccase, or undergo direct (auto)oxidation by molecular oxygen, thus generating Reactive Oxygen Species (ROS) that are the true agents of dye bleaching (Fig. 25). This latter rapid (auto)oxidation is a typical feature of anthraquinols (as a point of fact, the industrial preparation of hydrogen peroxide is just based on re-oxidation of 3-methyl-anthraquinol to the corresponding anthraquinone by molecular oxygen).

Also aminoanthraquinones and aminohydroxyanthraquinones behave as substrates for laccase(s) and are therefore bleached with high efficiency [153,177]. A study on a laccase from *Lentinus* sp. showed that the enzyme could efficiently bleach both azo and aminoanthraquinone dyes. In this study, modeling calculations assessed the enzyme-substrate interactions at the active site [178].

2.3 Degradation of cyanine dyes

As underlined above (§ 1.1.1.) cyanine dyes are a very heterogeneous dye class, sharing a delocalized positive charge that confers them a cationic character, so they could well be defined as basic dyes. However, the dye net charge could be annihilated or also reverted by sulfonation that introduces one or more $-SO_3^-$ groups. These have a marginal impact on the dye hue, but increase solubility in water and change the tinctorial features, so sulfonated cyanine dyes are not uncommon at all. As a general rule, basic dyes should pose a challenge to bleaching by laccases: the enzyme should extract a negatively charged entity (one electron) from a positively charged molecule. Despite such a conjecture, many basic dyes are substrates for laccases, and are therefore bleached. Among the thionines (Fig. 13), methylene blue, the fully methylated member of the series, is not an ideal substrate for all the tested laccases [143,179,180]. These observations are not surprising: the dye has no –OH or $-NH_2$ groups, so no hydrogen atom could be abstracted from the molecule (HAT mechanism). Only a non-bonding electron could be drawn from a $-N(CH_3)_2$ group or also from the nitrogen and sulfur atoms incorporated in the thiazine ring (ET mechanism), therefore adding a supplementary positive charge to the dye cation.

Sharply different is the general behavior of the very similar dye, Azure B (identical to Methylene Blue, except for having three methyl groups only and therefore a $-NHCH_3$ instead of a $-N(CH_3)_2$). In this case, a more efficient bleaching has been described in the presence of different laccases. Most probably, Azure B degradation starts with the abstraction of the hydrogen of the $-NHCH_3$ group of the dye, producing a radical species, where the positive charge of the molecule was not increased by the laccase(s) [181-183] (Fig. 26): at least in this case, HAT seems to be more favorable than ET. A similar mechanism could occur in the presence of lignin peroxidases and their biomimetic counterparts [184,185].

Conversely, the cationic dye Phenosafranine (Fig. 13) seems not to be a substrate for laccase from *Pleurotus* at all [86,186], whereas it is bleached by lignin peroxidase.

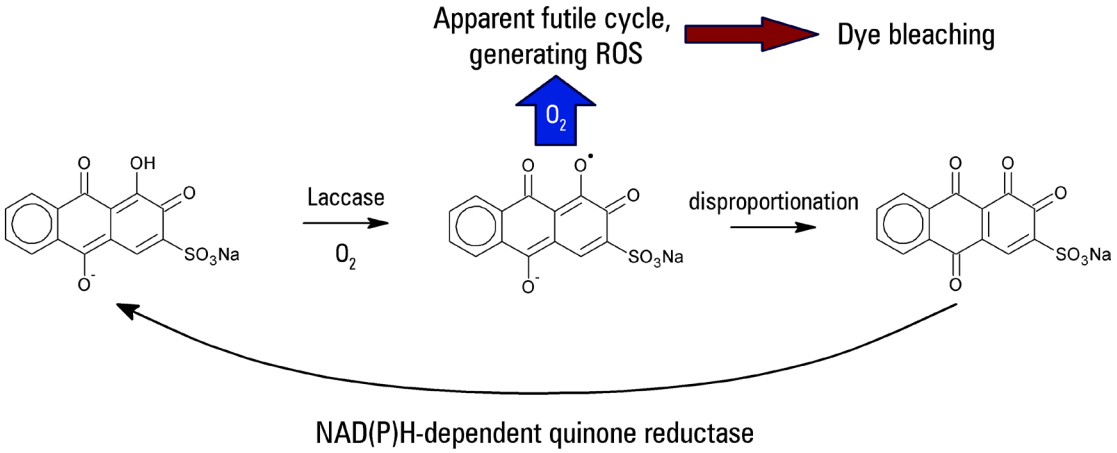


Figure 25: The mechanism for anthraquinone dyes involves an apparently futile cycle with ROS production, which seem to be the true agent of the bleaching. The presence of fungal membrane-bound NAD(P)H-reductases in combination with laccase is mandatory for the completion of the cycle. In the absence of reductase activity, the oxidative action of laccase drops, probably stopping at a quinone intermediate.

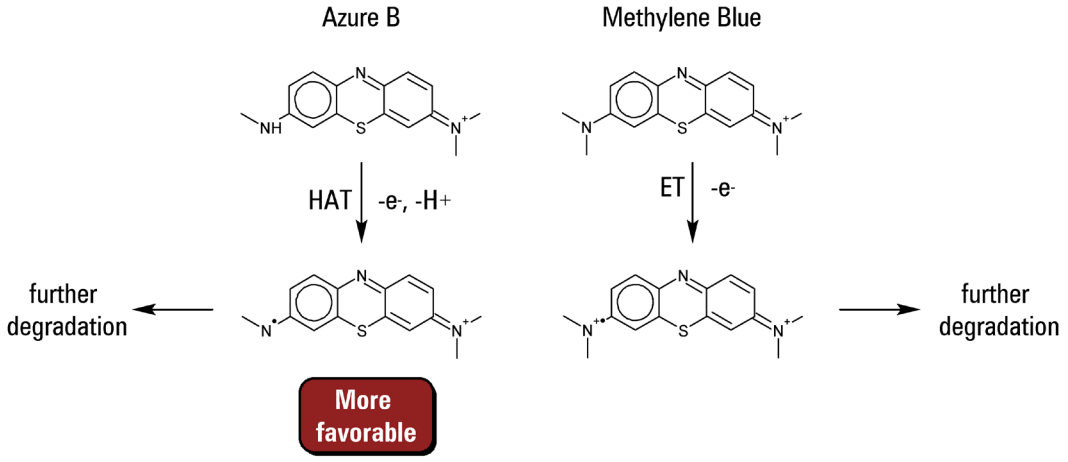


Figure 26: Thionine dyes can be bleached by both ET and HAT mechanism. The presence of a secondary amine group in Azure B allows for HAT mechanism. This is indeed more favorable, not increasing the charge of the molecule.

2.4 Degradation of triphenylmethane dyes

Among triphenylmethane dyes (Fig. 11) phthaleins, although poorly significant as industrial dyes, have been tested as laccase substrates. An outstanding example is that of the well-known pH indicator and electrophoresis marker and stain, bromophenol blue, which is bleached [187]. On the other hand, nitrogen-containing triphenylmethane dyes (Fig. 11) could be well regarded as a particular class of cyanines. They could be considered as virtually based on the fundamental structure of parafuchsine (pararosaniline) hydrochloride, to which methyl groups are bound directly to the free positions on the phenyl rings, or on the amine nitrogen atoms, or also on both, therefore forming a number of dyes, whose colors range from magenta to purple to blue till dark violet. In some cases, sulfonate groups are also introduced by sulfonation, giving rise to 'acid' triphenylmethane dyes. However, sulfonation does not abolish the typical positive net charge, delocalized along the three amine substituents. Basic triphenylmethane dyes are widely used owing to their brilliant hues and relatively low cost; many of them have been described as laccase substrates [179,188-192].

Some general conclusions can be drawn by the cited studies: among the triphenylmethane dyes, bromophenol blue is degraded rather easily, most probably because of the lack of any positive charge, which opposes electron abstraction by the enzyme; crystal violet, with its quaternized ammonium substituent, is the most recalcitrant because of the difficult abstraction of an electron from a cation. A deeper insight in the oxidative bleaching mechanism [193] revealed that almost invariably, the triphenylmethane moiety is cleaved at the central carbon atom. First of all, two series of experiments were conducted, one with the dyes dissolved in the culture media of actively growing *Trametes versicolor*, and the other on the same dyes with the purified laccase coming from the same fungus. *T. versicolor* was able to adsorb and beach all the tested dyes, with sharp preferences for the acid ones, which were quickly decolorized, without any adverse effect towards the fungus. The basic dyes on the contrary proved to be toxic to the fungus, thus inhibiting biomass production and mycelium development almost completely. Nevertheless, if the concentrations of the basic, toxic dyes were not too high, the fungus was able to degrade and bleach them. Obviously, bleaching of the triphenylmethane dyes by *T. versicolor* cannot be attributed to laccase alone, due to the presence of a rich enzyme arsenal within the fungal cells. All the studied dyes were also treated with the purified *T. versicolor* laccase. Quite different results were obtained: for example, acid fuchsine was resistant against enzymic bleaching, and was only slowly and partly degraded. The strong –I effect of the sulfonate substituents most probably prevents any electron abstraction from the dye molecule, thus suggesting the intervention of another enzymatic activity (i.e. high-potential ligninolytic peroxidase(s)) when decolorization was performed in the presence of actively growing mycelium. As a general rule, all the tested dyes caused a drastic deactivation of the enzyme within 24 hours of incubation, therefore suggesting the intermediacy of very reactive species along the degradation process, leading to irreversible enzyme inhibition.

2.5 Degradation of phthalocyanine dyes

Phthalocyanine dyes (mainly Cu-phthalocyanines) are particularly recalcitrant substances; however, some industrially relevant, water-soluble derivatives could be bleached by fungal laccases. This is the case of Reactive Blue 25, containing a Cu-phthalocyanine macrocycle (the chromophore) and a monochlorotriazine moiety (the reactive tether), which is at first adsorbed and then bleached by the mycelium of *Aspergillus ochraceus* NCIM-1146. It is worth noting that in this case laccase is accompanied by lignin peroxidase, so uncertainty exists about the role of the former in the degradation [194].

In another study some soluble Cu-phthalocyanines were at first adsorbed and then degraded by the iconic ligninolytic fungus, *Phanerochaete chrysosporium*. The dyes were demetallated by the intervention of manganese peroxidase, whereas laccase broke down the phthalocyanine macrocycle, leading to intermediates, that were in turn changed into simpler compounds, also comprising phthalimide [195].

It is also worth noting that in several cases no purified laccase was used, and bleaching experiments were carried out by adding the chosen dye to the culture media of the ligninolytic fungi. However, in all cited experiments, the bleaching effect of the studied fungi was clearly related to laccase production and secretion. The possible intervention of enzymes other than laccases should also be considered when working with cultured fungi and/or culture filtrates. Manganese peroxidase appears as a quite effective potential ancillary tool for laccase-assisted oxidative degradation [158].

3 Redox mediators allow laccases to bleach recalcitrant dyes

As noted above (§ 1.2) many industrial dyes are out of the reach of laccases, due to different factors such as too high redox potential, steric hindrance, lack of molecular motifs recognizable by the enzymes. In several cases, the problem of dye inertness when treated with laccases could be solved by the use of RMs. Moreover, RMs could also substantially enhance the bleaching efficiency for dyes that are *per se* (poor) laccase substrates.

The use of ABTS as a RM has resulted in several studies assessing its efficiency in dye bleaching and detoxification, as found in a combined treatment scheme adopted to remediate a real dye mill effluent [196]. In another study [197] the suitability of a bacterial laccase as a tool for azo dye decolorization was assessed in the presence of the RMs ABTS, acetosyringone, syringaldehyde, and *N*-hydroxybenzotriazole. A copper phthalocyanine dye was efficiently bleached, and the degradation products characterized, by a recombinant fungal laccase, in the presence of ABTS [198].

Another recombinant laccase (CotA laccase from *Bacillus pumilus*), remarkable for its high stability and activity at alkaline pH, proved to be much more efficient in the presence of the RM methyl syringate [172]. The enzyme was capable of bleaching Reactive Red 11, Reactive Blue 171 (azo dyes) and Reactive Blue 4, Reactive Brilliant Blue (anthraquinone dyes). A laccase secreted by *Pycnoporus sanguineus* decolorized some dyes in the presence of violuric acid and, better, methyl syringate [199]. The general properties of methyl syringate as laccase RM have been recently reviewed [200].

Among the >N–OH RMs, hydroxybenzotriazole (HBT) has a prominent role; the influence of benzene ring substituents towards efficiency in different HBTs has been recently reviewed [201]. HBT is very popular among >N–OH RMs due to its high efficiency [202-207].

In conclusion, the use of properly chosen RMs decidedly widens the application field of laccases in dye degradation/bleaching, and this is crucial when managing dyes, that are not 'direct' laccase substrates. However, the use of RMs represents an additional cost, in particular when poorly stable RMs are used, leading to inactive oxidation products. Finally, when using very toxic RMs, their persistence in the bleached wastewaters should be considered.

4 Open problems and perspectives

The problem of laccase and/or RMs waste when treating industrial wastewaters has noticeable economical concerns, as enzymes are generally costly and moreover are more or less quickly inactivated. Therefore, immobilization procedures could help solve these drawbacks, allowing enzyme and/or mediator recovery and recycling [208-214]. Adsorption is the most immediate approach for enzymatic immobilization, not involving the use of toxic reagents or complex chemical reactions [215]. However, adsorbing materials in principle are not the first choice for laccase immobilization: non-specific adsorptive properties would allow also dye adsorption, thus nullifying the bleaching power of the enzyme. On the other hand, laccases are usually immobilized with high yields on solid supports, provided that covalent linkages are formed between the enzyme and the chosen support, and many of such preparations have been described

as capable of efficiently degrading industrial dyes [216-223]. The most useful and popular methods for covalent immobilization of enzymes have been recently described and reviewed [208].

Immobilization of RMs could in principle help with recovering these compounds, but the drawback of mass transfer issues makes this approach almost useless. However, a very recent study [224] deals with substituted anthraquinones as RMs, covalently bound to carbon-based materials. In that study, 4-nitrophenol was reduced by sodium sulfide, thanks to the anthraquinone intermediacy. The study has nothing to do with enzymes, but could represent a starting point for future developments in that direction.

In conclusion, a judicious choice of both the particular laccase and the support for immobilization could afford tailor-made preparations, suitable for particular dyes to be bleached. Addition of RMs to the reaction mixtures could substantially enhance the bleaching rates and efficiencies.

Another novel strategy to improve the efficiency of the laccase-based treatments of textile dyes (especially in the perspective of decreasing economical impact of the whole process) is the heterologous production of recombinant laccases. Several attempts have been recently accomplished with promising results [150,161,188,198,225]. However, the presence of post-translation modifications (particularly, glycosylation) should be taken in due care, to obtain a fully functional enzyme.

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