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REVIEW ARTICLE

Recent Developments and Challenges in the Application of Fungal Laccase for the Biodegradation of Textile Dye Pollutants

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Abstract: According to the European Environment Agency, the textile industry is responsible for 20% of global water pollution due to dyeing and finishing products, thus facing severe environmental challenges. It is essential to design more biocompatible and sustainable treatment processes capable of removing dyes from industrial wastewater to fight this environmental hazard. Chemical industries must change traditional chemical-based concepts to more environmentally friendly and greener processes to remove pollutants, including dyes. Enzymatic bioremediation is a smart tool and a promising alternative for environmental pollutant degradation. The use of enzymes in dye decolourization makes the process a green and clean alternative to conventional chemical treatments. Moreover, enzyme-mediated biocatalysis decreases the formation of toxic by-products compared to chemical reactions. The most used enzyme for the decolourization of dyes is laccase. Laccase is a multicopper oxidase found in diverse organisms such as fungi. It promotes the oxidation of phenolic compounds and has a wide range of substrate specificity, making it a promising enzyme for removing different dyes used by the textile industry, including recalcitrant aromatic dyes. The present article gives a comprehensive revision of textile dye decolourization, its types, recent developments in laccase-mediated dye bioremediation technologies, the mechanism of biocatalysis, and their limitations and challenges. Emphasis on the chemical pathways of laccase reaction mechanisms for dye bioremediation processes is also provided. In addition, a brief overview of textile industries and the respective traditional treatment processes for textile wastewater is also presented.

Keywords: Decolourization, enzymes, textile dyes, biocatalysis, fungal laccases, textile wastewater, greener processes.

1. INTRODUCTION

The textile industry is one of the most water-intensive and polluting sectors. The dye or mixture of dyes are used to add colour to textiles; however, since not all dyes bind to the textiles, the remaining dye is released through the effluent from the textile industry. According to the European Environment Agency, the textile industry is responsible for 20% of global water pollution due to its dyeing and finishing processes [1]. However, regulations set a colour restriction but do not specify the precise dye limits, occasionally just demand colour measurement rather than coloured water limits, and do not require biotoxicity analysis [2]. Even at low concentrations, dyes are harming the environment [3]. More biocompatible and sustainable treatment methods capable of removing and degrading dyes from industrial textile wastewater must be developed to combat this environmental threat. Various types of treatments, such as physical, chemical, or biological, have been investigated to assess the long-term removal of dyes from the textile effluent [2,3]. Biotechnological approaches are a greener and cleaner alternative to traditional chemical treatments [4]. More specifically, enzymatic bioremediation is an effective and potential alternative for removing dyes from the environment. Compared to chemical reactions, enzyme-mediated biocatalysis produces fewer hazardous by-products [5]. Laccase is the most commonly utilized enzyme for colour bioremediation [2]. Laccase is a multicopper oxidase found in fungi and other organisms that enhances the oxidation of phenolic chemicals and has a broad substrate specificity, making it promising to remove many textile dyes, including resistant aromatic dyes. The laccase application in wastewater treatment and, more specifically, dye degradation has been recently reviewed in the [2,6–8]. However, degradation routes and degradation products are often overlooked.

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Therefore, this review focuses on textile dyes, dye wastewater treatments, breakthroughs in laccase-mediated dye decolourization technologies, and presents chemical biodegradation mechanisms for each dye family.

2. TEXTILE INDUSTRIES

The textile industry represents one of the oldest and most significant industrial sectors contributing to the global economy, either in developed or developing countries [9,10]. It is one of the most labour-intensive industries responsible for generating a significant number of jobs worldwide [11,12]. Due to the world population growth along with increasingly consumerist behaviour, the textile industry is expected to reach a larger development [11]. However, although this sector displays a remarkable role in the economy of many countries, it is also a major source of environmental pollution [9]. The textile industry uses a wide range of chemicals at the different stages of the manufacturing process, mainly in the dyeing and printing steps, while large amounts of fresh water are consumed [13]. As a result, a huge amount of coloured wastewater is released, generally without appropriate treatments, which represents one of the major challenges that the whole world is facing [14]. Globally, the wastewater released from textile industries contains various highly toxic and recalcitrant compounds, highlighted in the dyes [13,15,16]. Other pollutants include organic surfactants (carboxylates, sulphonates and alkyl ether phosphates), alkalis (NaOH, Na_2CO_3), salts, reducing agents, phenols, heavy metals such as lead, cadmium, arsenic, chromium, zinc and nickel, as well as hazardous chemical solvents (L-chlorinated hydrocarbons, dichloromethane, trichloroethane) [9,17]. The dyestuff industry is responsible for up to 20% of industrial water pollution [18].

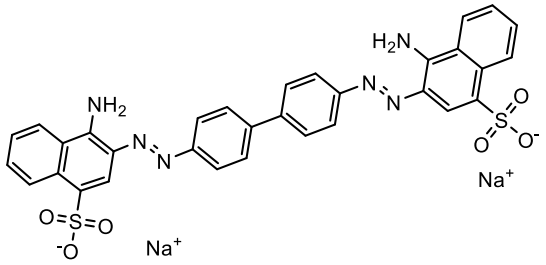
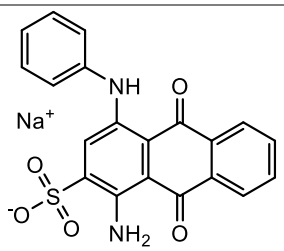
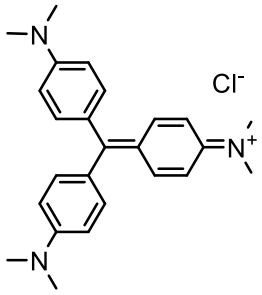
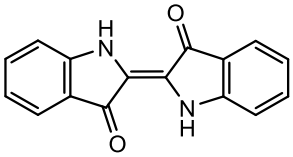
2.1. Textile dyes: classification and properties

Dyes are used to adding colour to a variety of substrates, such as cloth, paper, and leather, among others, and are generally applied in aqueous solutions due to their affinity to water [17]. Textile dyes are aromatic and heterocyclic compounds, characterized by their capacity to absorb light radiation in the visible spectrum and have at least one chromophore group (colour-bearing group), such as azo, (–N=N–), carbonyl (–C=O), nitro (–N=O) and quinoid groups [17,19,20]. Besides chromophores, most dyes contain auxochrome groups, being highlighted the hydroxyl (–OH), amine ($–NH_2$), carboxyl (–COOH) and sulphonate ($–SO_3H$) [17]. Although these are not responsible for colour, their presence is essential to influencing dye solubility [21]. Amongst the multiple types of dyes applied, these are mainly categorized depending on their origin into two main groups: natural and synthetic dyes [17,22,23]. Natural dyes are derived mainly from plants and are getting more attention as they are environmentally friendly and generate less hazardous effluents during the dyeing process [22]. Nevertheless, they are little used, and their industrial-scale application is restricted due to limitations associated with the low affinity to the textile fibres, which represent the main drawback of their use [22]. In addition, the use of mordant and chemicals for fixation onto the textile fibres is required and is associated with lower yield and colour fastness [22,24]. Specific extraction techniques are also needed to eliminate the non-colourant components from their original sources [25]. On the other hand, synthetic dyes are synthesized from chemical compounds and are extensively used due to their chemical stability and remarkable colour fastness [17]. Globally, more than 10,000 types of synthetic dyes are used in the textile industries during the dyeing process [26,27].

Dyes can be classified according to the application method in different substrates, dyeing mechanism, or based on the chemical structure, particularly the nature of the chromophore group [28,29]. Regarding the chemical structure, they can be classified as azo dyes (e.g. reactive yellow 4, reactive black 5 and direct blue 71), anthraquinone dyes (e.g. alizarin, reactive blue 4 and acid blue 25), triarylmethane dyes (e.g. basic red 9 and crystal violet), nitro and nitroso dyes (e.g. naphthol yellow S and mordant green 4), indigo dyes (e.g. indigo blue and blue acid 74), xanthene dyes (e.g. fluorescein, eosin Y and erythrosine B) and phthalocyanine dyes (e.g. pigment blue 15/3 and nickel (II) tetrasulfonic acid), among others [17]. Based on their applicability and affinity for several fibres, dyes can be divided into two distinct families: soluble and water-insoluble dyes [28]. Among the soluble dyes are the acid or anionic, basic or cationic, reactive and direct dyes, while water-insoluble ones are the disperse dyes, vat dyes, sulphur dyes and pigments [28]. Several synthetic dyes are extensively used in the textile industry, including azo, vat, reactive, acidic and basic dyes [17]. Azo dyes belong to the most prominent family of synthetic dyes, constituting 60-70% of all dyes used in the textile industry during the dyeing process [20,30]. That is due to their versatility, cost-effectiveness and high intensity of colour, as well as to their reactive units that allow a quick attachment to fibres [31]. They are highly water-soluble and consist of one or more azo groups (-N=N-) linked to the -OH or $-NH_2$ type auxochrome groups, generally containing one, two, three, or more azo linkages [32,33]. Reactive dyes are the most popular dyes used in this sector since they provide a broad colour spectrum and a higher colour fastness due to their capacity to create a covalent bond with the hydroxyl groups within the fibres under alkaline conditions [11]. Moreover, they have relatively low costs and high applicability [11].

Regarding the acid dyes, the correspondent dyeing process is carried out in an aqueous solution at acidic pH ranging from 2.0 to 6.0, comprising around 30-40% of the total usage of dyes [30]. These are applied to hydrophilic fibres that contain an amino group (NH_2), such as wool and silk [30]. The azo, anthraquinone or triarylmethanes constitute most of these dyes [28,30]. Vat dyes are reported for their outstanding brightness properties and better colour fastness (e.g. blue indanthrene MS and vat green 1) [28,30]. They are mainly soluble in hot water, and some are soluble in sodium carbonate [20]. Regarding the basic dyes, these are reported to be cheaper and soluble in alcohol but not easily soluble in water, and therefore they have limited applicability [20]. Table 1 presents the chemical structures of some common synthetic dyes used in the textile industries.

Table 1 – Chemical structures of some synthetic dyes used in the textile industries.

Dye Classification	Examples of dyes	Dye chemical structure	Ref.
Azo	Congo red		[11]
Anthraquinone	Acid blue 25		[30]
Triarylmethane	Crystal violet		[2]
Indigo	Indigo blue		[28]
Phthalocyanine	Pigment blue 15:3		[28]

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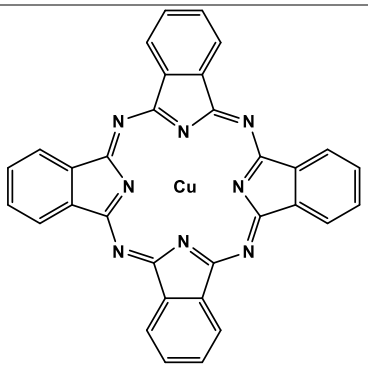
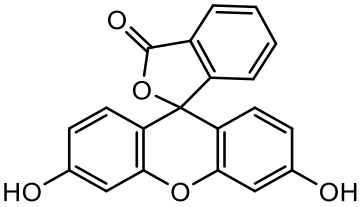
			
Xanthene	Fluorescein		[28]

2.2. Toxicity of textile dyes and their intermediates on the environment and human health

Since dyes are not totally attached to the target fibres during the dyeing process, they get discharged as effluents along with the wastewater into the aquatic resources such as rivers, lakes and streams and persist in nature due to their aromatic and complex structure, causing serious environmental threats [14]. In addition, they are resistant to light, chemicals, biological activity and other environmental conditions [34]. Some of the negative contributions of dyes to the environment include reducing the dissolved oxygen level in the water and, on the other hand, an increase in the chemical oxygen demand (COD) and biochemical oxygen demand (BOD) levels of aquatic resources due to the alteration of the pH level [26,34]. Dyes can block sunlight from the aquatic system, reduce gas solubility, and therefore interfere with photosynthetic activity, leading to eutrophication problems while disturbing the dynamic of the aquatic environment [13], [34]. Although all dyes are naturally toxic, a dye can be differentiated from the others regarding its toxicity level, which depends on dye structures and functional groups, as well as their sizes [9].

Additionally, most of the dyes and their converted products have a toxic effect on aquatic bodies, and some of them may present toxicity and carcinogenic and/or mutagenic effects on humans and other animals [35–37]. For example, aromatic amines are mentioned to cause allergies and consequently affect human health [34]. More particularly, according to the World Health Organization (WHO), benzidine is reported to be a carcinogen for humans [26]. Several benzidine-derived dyes, such as direct black 38, direct blue 6 and direct brown 95, cause cancer in human beings [26]. Azo dyes are considered among the most dangerous synthetic dyes produced in textile manufacturing, constituting a major environmental concern due to their stable chemical structures containing azoic linkages, aromatic rings and amino groups [38]. They display stability to water, light, bleach, heat and detergents [34]. Thus, their complex and almost non-biodegradable structure make them resistant to biological and chemical degradation [39]. Moreover, some of these dyes are mentioned to be toxic and mutagenic for aquatic organisms, as well as their reductively cleaved products and chemically related aromatic amines, which affect human health [9]. Another example of an azo dye is the *p*-phenylenediamine, a contact allergen [9,40].

In summary, with the growing industrialization implying the use of more dyes, it is of extreme importance a proper treatment of textile wastewater since the presence of dyes has a negative impact on the environment and, more particularly, on aquatic systems and human health, due to their toxic, carcinogenic and mutagenic effects.

3. TRADITIONAL TREATMENT PROCESSES FOR TEXTILE WASTEWATER

Various strategies of treatment have been investigated over the last two decades to evaluate the sustainability and removal of dyes from textile wastewater. However, no ideal treatment technology applies to all types of textile effluents. As a result, a combination of approaches is the ideal solution for treating all types of textile effluents, which can be classified as physical, chemical, or biological processes [41].

3.1. Physical Treatments

Physical, often also classified as physicochemical methods, are typically procedures based on mass transport principles. These are the most used methods and are frequently chosen for their simplicity and effectiveness. Adsorption, coagulation/flocculation, ion exchange, and membrane-based filtrations are the most used physical procedures for dye removal from textile wastewater.

3.1.1. Adsorption

Adsorption became one of the preferred physical methods for the treatment of textile effluents due to its exceptional ability to remove practically all types of dye [42–46]. Most recent research on adsorbent development has focused on using carbon-based materials, specifically activated carbon [47–49]. However, its high cost and regeneration restrict its applicability. As a result, there is a growing interest in adopting low-cost and natural adsorbents, such as agricultural and industrial residues [50–53]. Rafatullah *et al.* [42] collected 185 research publications on low-cost adsorbents to demonstrate the presence of both inexpensive and effective adsorbents.

3.1.2. Coagulation and Flocculation

This approach is a low-cost, easy, and energy-efficient method for removing colours from textile effluents [54–56]. The main disadvantage of this treatment is the production of massive volumes of concentrated sludge, as well as the pH dependence [57]. Furthermore, this treatment method is incompatible with soluble dyes such as reactive, azo, acid, and basic dyes [58]. Synthetic polymer coagulants have also been linked to plenty of environmental issues, as well as neurotoxic and carcinogenic consequences to humans [59].

3.1.3. Ion exchange

This procedure involves the formation of strong bonds between the solutes and the resin, resulting in the separation and generation of high-quality water [60]. The main disadvantages of ion exchange systems are their inability to handle extremely concentrated wastewater, the ease with which the matrix gel fouls, and their extraordinary sensitivity to the pH of the effluent [50].

3.1.4. Membrane-based filtration

Membrane filtration procedures pass wastewater through tiny pores and treat textile dyeing effluent using the membrane's selective permeability. Membrane-based filtrations are classified according to the membrane´s pore size, namely microfiltration, ultrafiltration, nanofiltration, and reverse osmosis, ranging from largest to smallest, respectively [9,53]. These techniques apply to any type of dye, have a high separation efficiency, are simple to use, and are ecologically safe. However, this technique is still not appropriate for large-scale use since it needs specialized equipment, a significant investment, and a limited lifespan owing to membrane fouling [61,62].

3.2. Chemical Treatments

Chemical treatments for textile wastewater are mostly based on oxidation processes and can be classified as chemical oxidation and advanced oxidation processes (AOPs). Under ambient conditions, these mechanisms can be employed independently or in synergy with other processes and break down the harmful original compounds, dyes, pesticides, etc. [63]. However, the bulk of these oxidation systems fail to completely decompose the complex chemicals, particularly in the case of real wastewater, and hence cannot be employed to handle the vast volumes of waste formed [64]. Chemical colour removal procedures are also commercially unappealing since they require specialized equipment and a lot of energy [65].

3.2.1. Chemical oxidation

Chemical oxidation technologies use oxidizing agents such as ozone (O_3) and hydrogen peroxide (H_2O_2) to form highly reactive radicals at high pH values [63]. Because of their high oxidation potential, these radicals can effectively break the conjugated double bonds of dye chromophores as well as other functional groups, such as dyes' complex aromatic rings [66]. One significant advantage of ozonation is that ozone may be employed in its gaseous form, which does not increase the volume of the wastewater and does not result in sludge production. However, it may form toxic by-products even from biodegradable dyes in wastewater [67]. Furthermore, these oxidizing agents degrade at a slower pace than the ones used in AOPs [68].

3.2.2. Advanced Oxidation Processes (AOPs)

AOPs comprise the formation and subsequent reaction of hydroxyl radicals, which are extremely powerful oxidizing species capable of reacting with complex organic and inorganic compounds in textile effluent water that cannot be oxidized by traditional oxidizing agents [64,68] The most common AOPs are photocatalytic oxidation and Fenton-based reactions, both of which have been shown to produce excellent outcomes for either the total mineralization of reactive dyes or their transformation into less complicated compounds that are more easily biodegradable [69].

The UV-TiO_2 treatment method is an efficient heterogeneous photocatalytic process that employs UV light energy to break down different compounds in wastewater [70]. In this process, an electron is excited from the valence band of TiO2 to the conduction band (Equation 1). Then, the charge carrier of the valance band hole (h_{VB}^+) and conduction band electron (e_{CB}^-) can react with oxidizing species such as OH^-, H_2O, organic compounds and reducing species such as O_2 present in the solution (Eqs. 2–4). Mainly these combinations lead to the formation of OH• and superoxide radical anions ($O_2•^-$) on the surface of TiO_2 that are capable of destroying a large variety of organic compounds (Eqs. 5–7) [71]. The photocatalytic oxidation of organic compounds by the UV-TiO_2 system is represented in Figure 1.

$TiO_2 + h\nu \rightarrow TiO_2 (e_{CB}^- + h_{VB}^+)$ (1)

$TiO_2 (h_{VB}^+) + H_2O \rightarrow TiO_2 + H^+ + OH•$ (2)

$TiO_2 (h_{VB}^+) + OH^- \rightarrow TiO_2 + OH•$ (3)

$TiO_2 (e_{CB}^-) + O_2 \rightarrow TiO_2 + O_2•^-$ (4)

Organic compound + HO• → Degradation products (5)

Organic compound + $TiO_2 (h_{VB}^+)$ → Oxidation products (6)

Organic compound + $TiO_2 (e_{CB}^-)$ → Reduction products (7)

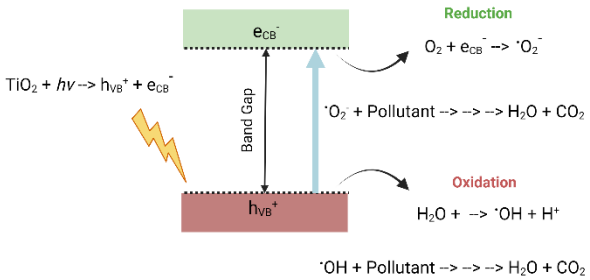


Figure 1 – Photocatalytic oxidation of organic compounds from textile wastewater under UV-TiO_2. Adapted from Almamun *et al.* [72].

Meanwhile, Fenton-based processes use hydrogen peroxide and ferrous ions (Fe^{2+}) to generate highly oxidant species like hydroxyl radicals [73,74] Fenton reaction comprises a simple redox reaction in which Fe^{2+} ions are oxidized to Fe^{3+}, and H_2O_2 is reduced to hydroxyl ion and hydroxyl radical (steps are presented in Eqs. 8–12) [75].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + HO^- \quad (8)$$
$$HO\bullet + Fe^{2+} \rightarrow Fe^{3+} + HO^- \quad (9)$$
$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2\bullet + H^+ \quad (10)$$
$$Fe^{2+} + HO_2\bullet \rightarrow Fe^{3+} + O_2 + H^+ \quad (11)$$
$$H_2O_2 + \bullet OH \rightarrow HO_2\bullet + H_2O \quad (12)$$

Still, several issues limit the performance of these oxidation processes, including reaction parameters [76], the expensive cost of hydrogen peroxide, and excessive chemical consumption [77]. Furthermore, the risks connected with transporting, processing, and storing large volumes of H_2O_2 have made the process dangerous and economically challenging [78].

3.3. Biological Treatments

Comparing to physical/oxidation methods, biological treatment procedures for removing contaminants from wastewater are considered very helpful owing to their eco-friendly nature, low chemical usage, low energy consumption, economic viability, and low sludge creation [79]. The biological treatment techniques work on the premise of converting biodegradable wastes into simpler and harmless species involving various microorganisms (bacteria, fungi, algae, plants). However, the biological process only eliminates dissolved matter from textile wastewater, and its effectiveness is affected by the ratio of organic load/dye and microbe load, as well as the system temperature and oxygen content. Biological techniques can be characterized as aerobic, anaerobic, and anoxic, or facultative, or a mix of these based on their oxygen requirements [53]. In reality, a mix of anaerobic and aerobic methods is commonly used, with the anaerobic treatment of COD textile wastewater followed by aerobic polishing treatment of the resulting low COD textile wastewater [63]. Although microorganisms can express enzymes that break down even the most challenging contaminants, they are filled with complications. Furthermore, microorganisms degrade contaminants at a relatively slow rate, limiting their total practical use for this purpose.

Considering the limitations of the mentioned physical and chemical methods, namely requiring high assets and involving high operational costs, biological treatment processes are considered the cheapest and safest alternative for the eco-friendly removal of dyes and other pollutants from textile effluents. Within the branches of biological processes, the use of enzymes is becoming increasingly popular [80], and will be extensively explored in the following sections of this review.

4. ENZYME ACTION IN THE DEGRADATION OF DYES

Researchers have been increasingly interested in using enzymes to decolourize and degrade dyes present in textile industry wastewaters as an alternative to existing physicochemical approaches [81,82]. Enzymes are particularly effective for dye degradation since they present high specificity and catalytic activity, producing a reduced amount of by-products, operate in mild conditions and are biodegradable [83,84]. The entire procedure is considered ecologically friendly and minimally invasive. Nevertheless, several important parameters and conditions influence the effectiveness of the enzymatic degradation of dyes, including dye structure and concentration, oxygen transfer, presence of redox mediators, pH and temperature [85]. Enzymes of the oxidoreductases family, namely peroxidases, azo reductases and laccases, are able to oxidize a large number of different compounds [86]. The products of degradation are often precipitants or simple chemical compounds that can be easily handled or removed [14].

4.1. Peroxidases

Peroxidases, like lignin and manganese peroxidases (LiP and MnP, respectively), are heme glycoproteins produced mainly by *Phanerochaete chrysosporium* fungus that catalyses the oxidation of phenolic, non-phenolic and polycyclic aromatic hydrocarbons compounds as well as a variety of recalcitrant aromatic compounds. Several researchers have reported other microbiological origins of different peroxidase isoforms, such as various fungi and bacteria [87–90]. These enzymes require H_2O_2 as a cofactor, and the reaction occurs through a mechanism of electron oxidation followed by a series of non-enzymatic reactions [91]. The reaction mechanism of both LiP and MnP begins with the oxidation of the enzyme by H_2O_2. In the case of phenolic-azo dyes decolourization (Figure 2), after two consecutive one-electron oxidations of the phenolic by the enzyme, a carbonium ion is produced. This phenolic carbon bearing the azo bond is then attacked by a water molecule, generating an unstable hydroxyl intermediate, which then cleaves into a quinone and an amidophenyldiazine. The latter product is subsequently oxidized by oxygen into the equivalent phenyldiazene radical, which, upon nitrogen removal, yields a phenyl radical that is lastly oxidized by

oxygen. Since no aromatic amines are produced, this method results in azo dyes detoxification [92].

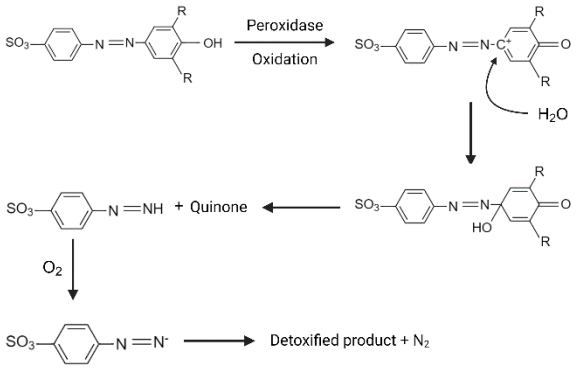


Figure 2 – Proposed mechanism for azo dyes degradation by peroxidases. Adapted from Torres-Duarte and Rafael Vazquez-Duhalt [93].

In the specific case of MnP, it requires free manganese ion for its activity. Initially, it catalyses the oxidation of Mn^{2+} to Mn^{3+} oxidizing, subsequently, several phenolic compounds. As the MnP enzyme varies from species to species, the ability to dye decolourization also changes with the type of MnP used and with the reaction conditions [94]. Several studies report the peroxidases decolourization and degradation of dyes [95–98]. Dye degradation has also been accomplished using peroxidases from plants, such as purified peroxidase from *Saccharum spontaneum* leaf [99] and *Senna angustifolia* [100], reaching degradation efficiencies of 100% and 97% for certain dyes, respectively.

4.2. Azoreductases

Azoreductases are the largest group of enzymes involved in azo dyes' degradation. They are extracellular or intracellular enzymes found in the intestinal microbiota that catalyze the azo bonds reductive cleavage to generate predominantly colourless aromatic amine products [101]. To perform the degradation reaction, azoreductases require the existence of reducing agents like $FADH_2$, NADH and NADPH. Most azo dyes are large molecules of high molecular weight with sulphonate substituent groups, making them unable to cross the cell membrane [102]. As a result, the enzyme reducing action is not determined by the dye's intracellular uptake [103]. According to Russ *et al.* [104], cell membranes also limit the reducing agents' transference from the cytoplasm to the azo dyes. Therefore, the effective mechanism of dye reduction by azoreductases depends not only on reducing agents but also on other compounds. Gingell and Walker [105] reported the use of low molecular weight redox mediators as electron vehicles between the azo dye and NADH-dependent enzyme in the external membrane. Additionally, cytoplasmic reducing enzymes will deliver electrons to the redox mediators [106]. The proposed mechanism for azo dyes reduction by azo reductase is presented in Figure 3. This mechanism of action refers to the anaerobic degradation of dyes. Under aerobic circumstances, oxygen will block this reduction pathway because it will preferentially oxidize the reduced redox mediator rather than the azo dye. A particular aerobic azoreductase structure is required for the aerobic degradation of azo dyes [107].

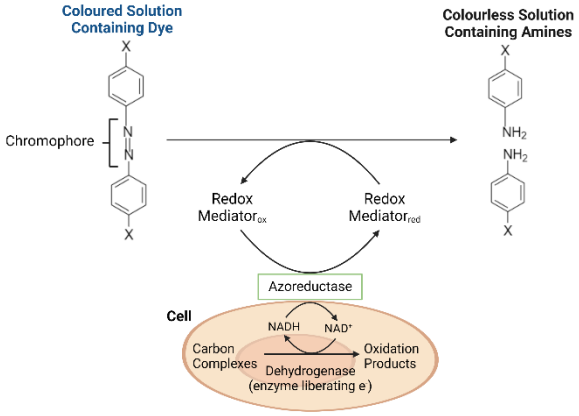


Figure 3 – Mechanism for azo dyes reduction by azo reductase. Adapted from Ajaz *et al.* [108].

This group of enzymes is highly diverse depending on the source they come from (organism or even species). The potential of azoreductases for the degradation of dyes was already described by many authors, who have cloned and characterized the enzymes from diverse bacteria [109–112]. Numerous investigations were conducted to determine the physiological activities of azoreductases, including functional and structural studies [113,114], which have indicated their association with responses to oxidative stress, quinones and heavy metal stress [113,115]. *Escherichia coli* azoreductase showed to be upregulated under thiol-specific stress, which could explain its ability to destroy azo dyes and the requirement for several enzymes in a single strain [113]. In addition to its considerable sequence differences, azoreductases also differ in flavin cofactor (FMN, FAD or flavin free), electron donor preference (NADH, NADPH or both) and azo dye substrate range [116]. It is worth noting that certain microorganisms have numerous azoreductases, which adds to the enigma surrounding their physiological function. For example, three unique azoreductases with markedly varying substrate specificity were reported from *Pseudomonas aeruginosa* [117].

4.3. Tyrosinases

Tyrosinases are oxidoreductases, also called polyphenol oxidases, which catalyse the oxidation of phenolic and other aromatic molecules without cofactors' use. These enzymes are found in a wide range of sources, from bacteria to mammals and they can even be found in several organs of the same organism [118]. Tyrosinases are tetramer enzymes with four copper atoms per molecule and binding sites for two oxygen and aromatic compounds [119]. The degradation of dyes has been reported by tyrosinases from several bacteria [120]. This enzyme is widely known for catalysing two distinct and consequent oxygen-dependent reaction steps: the first one includes the monophenol o-hydroxylation, resulting in the generation of o-diphenols known as mycophenolate (cresolase activity); the second one comprises the o-diphenols oxidation to o-quinone, known as o-diphenolase (catecholase activity) [121]. The efficiency of tyrosinases extracted from plants for the degradation of textile dyes has also been demonstrated [122].

Peroxidases, azoreductases and tyrosinases proved to be potential to remove dyes from aqueous solutions and wastewaters. However, one of the enzymes that has a high

potential to promote the degradation of dyes is laccase. Thus, due to the effective catalytic properties of laccase and the well-established technical applications, the comprehensive information of all laccases namely characteristics of the enzyme and their capacity in dye removal is presented in next Section 5.

5. LACCASES FOR DYE REMOVAL

Laccases (benzenodiol:oxygen oxidoreductase; EC 1.10.3.2) are multicopper polyphenol oxidases originally present in bacteria, fungi, plants and animals. Laccase belongs to the oxidoreductases enzymes class, also known as a blue copper oxidase. This enzyme plays a variety of biological functions in each different organism, being the detoxification a common role of laccases in all organisms [123]. Laccase was discovered by Yoshida in 1883 in the lacustrine tree (*Rhus vernicifera*), and characterized by Bertrand in 1985 after its isolation and purification [124]. Fungal laccases were discovered during the XIX century [125] and actually, more than one hundred laccases from Basidiomycetes and Ascomycetes have been characterized [2]. Laccase can be produced by many plants [126] and fungi [127], as well as by bacteria [128]. Since bacterial laccases have low redox potential, fungal laccases are more studied and applied due to their high redox potential [129]. Among fungal laccase producers, white-rot fungi are the main producers, being fungal laccase extracellular proteins. [130,131]. Laccase oxidizes a wide range of aromatic and nonaromatic compounds, using molecular oxygen as the final electron acceptor [123,132]. The broad substrate specificity and no requirement for cofactors make laccase a great candidate to be used in bioremediation by transforming and degrading a variety of toxic compounds present in the environment [132].

5.1. Molecular and structural properties of laccase

Usually, the fungal laccase active site is composed of four copper atoms with different oxidation states, one type-1 (Cu1), one type-2 (Cu2) and two type-3 (Cu3) each one with an important role in the laccase catalytic mechanism [133]. Copper Cu1 is involved in the capture and transfer of the electron to the trinuclear copper centre Cu2/Cu3, which is involved in bonding with oxygen [125]. Laccases usually comprise 520-550 amino acids, with a molecular weight ranging from 43 to 383 kDa depending on the fungal species [134]. These enzymes are known to have high stability due to the covalently linked carbohydrate moiety (10-45 %) [135].

The four copper atoms can be distinguished using UV/visible and electroparamagnetic resonance spectroscopy (EPR). One of the copper atoms belongs to the paramagnetic site T1, is EPR detectable and presents a strong absorption at 610 nm, which gives rise to the typical blue colour of the copper oxidases. This is the place where substrate oxidation starts. Another atom belongs to the copper paramagnetic site Cu2, which does not confer colour but is EPR detectable. The other pair of copper atoms are strongly coupled by a hydroxyl bridge and belong to the diamagnetic site Cu3. This pair gives a weak absorbance in the near UV at 330 nm but is not detected by EPR. The Cu1 copper is usually coordinated to two nitrogens from two histidines and to sulphur from cysteine. It is the bond of copper Cu1 to sulphur that is responsible for the characteristic blue colour of typical laccase enzymes. The reduction of molecular oxygen and the release of water occur in the trinuclear cluster formed by Cu2 and Cu3 coppers. Cu2 copper is coordinated by two histidines and Cu3 copper by six histidines [135]. Figure 4 shows the three-dimensional structure of laccase produced by *Trametes versicolor* from protein data bank (PDB) [136,137].

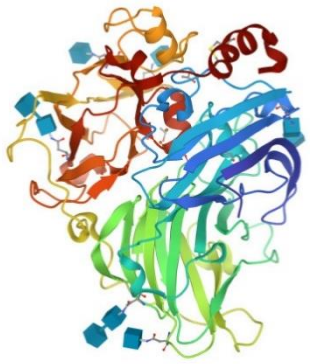


Figure 4 **–** Diagram of laccase from *Trametes versicolor*. [136,137]**.**

The redox potential was determined for several laccases using different mediators and ranges from 430 mV to 800 mV. It is important to remark that the redox potential of fungal laccases is independent of their origin species [134,138]. Table 2 summarizes the important properties of fungal laccases.

Table **2** – Main properties of fungal laccases [125,134].

Property	Fungal Laccases
pH-Optimum	2.0 – 7.5
Temperature-Optimum (ºC)	25 – 80
Molecular weight (kDa)	43 – 390
Copper content (atoms per molecule)	2 – 16
Redox potential (mV)	430 – 800
Isoelectric point	2.6 – 7.6

Beyond the interest in investigating laccase structure, catalytic mechanism and electrochemical properties, recently there has also been a great interest in developing new laccases through molecular evolution techniques [139–141].

5.2. Catalytic cycle of laccases

Despite not knowing the exact catalytic mechanism of laccases, some mechanisms have been proposed, which are consistent with the available properties. As explained above, the active site of the laccase structure comprises four copper atoms which offers a complex reaction pathway and where each atom plays its role in the direct oxidation reaction (Figure 5).

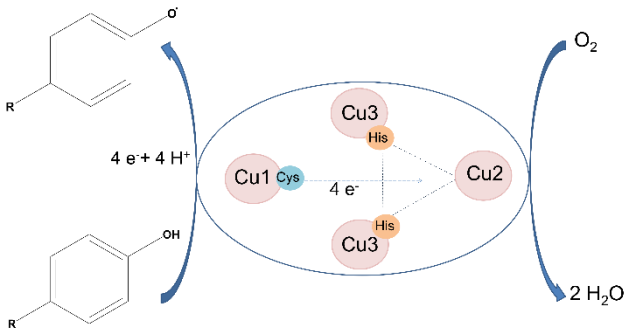


Figure 5 – Scheme of the direct catalytic cycle of laccases. Adapted from Baldrian [134].

The laccase substrate oxidation occurs at the Cu1 atom site, where the cooper atom gets an electron from the substrate [121,133]. Then, the electron is transferred through functional groups from the protein amino acids to the trinuclear copper cluster formed by Cu2/Cu3. In this site, after four electrons are collected, the Cu3 atom transfers the electrons to the Cu2 atom, which causes the reduction of oxygen. Subsequently, the reduced oxygen atoms are transformed into the water with the help of two carboxylate groups from protein amino acids, which transfer the necessary hydrogen atoms [133].

After the enzymatic reaction, a free radical is formed which leads to non-enzymatic radical reactions, such as polymerization and degradation [132,133]. In the environmental field, laccase is a promising tool for detoxication, once it promotes the oxidation of the compounds to free radicals or quinones, then, polymerization and/or partial precipitation take place [132]. In their insoluble form, the contaminants became less hazardous and can be removed using physical techniques. However, the complete understanding and knowledge of the detoxication process are still to discover.

5.3. Free laccase for dye degradation

Laccase has been reported to oxidize many recalcitrant compounds, like halogenated phenols, hydroxy-indols, aromatic amines, organophosphorus compounds, polycyclic aromatic hydrocarbons, bisphenol A, pesticides, insecticides, and dyes.

Firstly, fungal systems producing laccases were studied as an option for the detoxification of wastewaters containing dyes [132,142]. To understand the mechanism of decolourization when using fungal systems, Rodriguez *et al.* [143] evaluated the decolourization of 23 industrial dyes using 16 different white-rot fungal strains. In crude extracts from solid-state cultures, despite laccase, manganese peroxidase, lignin peroxidase, and aryl alcohol oxidase activities being detected, being only the laccase activity were determined to be related to the colour removal. Then, two laccase isoenzymes from *Trametes hispida* were purified and their performance for dye degradation was evaluated. The purified enzymes were able to decolourize *in vitro* 11 of the 23 industrial dyes studied. When comparing fungal systems, species secreting higher laccase concentrations were frequently found to be more efficient in dye removal [132]. These findings open the door and recognized laccases as potential biocatalysts for the bioremediation of dyes from wastewaters.

The use of laccase for the degradation of different dyes has been widely investigated during the last decades. The studies revealed that multiple factors play a role in the success of dye degradation. For example, the presence and position of different functional groups in dye structures influence dye degradation [144]. The presence of substituents in ortho- and meta- positions in dye structure is advantageous for laccase oxidation, instead of para- substituents. Another important aspect is the presence of electron-donating group aid biodegradability, whereas substituents from the electron-withdrawing group make a ring more resistant to biological oxidation [144].

To better understand and improve the biodegradation of dyes, firstly, it is important to study the chemical reactions that lead to the degradation and the role that the laccase plays in the degradation pathway. Laccase is capable to degrade a multitude of pollutants via direct oxidation, depending on the enzyme source and dye structure [145]. However, to extend the range of molecules that laccase can oxidase, it is common the use of redox mediators. The use of mediators could significantly improve the catalytic oxidation efficiency of laccase to the target compound. Thus, for the degradation of some dyes, laccase needs the presence of redox mediators, a compound to act as an intermediate substrate for laccases, known as laccase mediator system (LMS), an indirect oxidation route [145,146]. The mediator serves as an electron carrier that transfers electrons to the dye molecule. The redox mediators are usually low molecular weight compounds that are oxidized by laccase and diffuse away from the enzyme to oxidize the dye, thus, improving the performance of laccases [147]. Some compounds can be used as laccase mediators such as ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), 3-hydroxyanthraniliec acid, 1-hydroxybenzotraiazole, acetosyringone, syringaldehyde, methyl syringate and hydroxybenzotriazole [148]. The reaction mechanism using LMS was investigated by Fabbrini *et al.* [149] and Tavares *et al.* [146] and several routes were proposed depending on the mediator used. Laccase catalysed the mediator oxidation, for further degradation of the nonphenolic substrate (Figure 6). Thus, the use of LMS improves the degradation efficiency of different compounds including dyes.

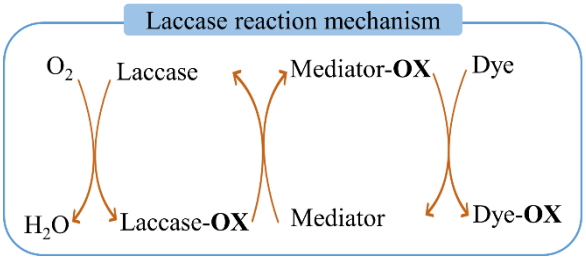


Figure 6 – Scheme of indirect laccase oxidation reaction in the presence of a mediator. Adapted from Fabbrini *et al.* and Tavares *et al.* [146,149].

5.3.1. Azo dyes degradation by laccase

Azo dyes represent a large part of the dyes used in the textile industry, being imperative to have a sustainable process for the treatment of effluents rich in this dye. Azo dye degradation using fungal laccases has been explored by several authors over the years. For example, using *Pichia pastoris*, Xu *et al.* [150] created a recombinant laccase from *Coprinopsis cinerea*. Using the recombinant laccase, the

researchers investigated the dye degradation of five distinct azo dyes, namely reactive blue M-2GE, reactive brilliant red KD-8, reactive brilliant red KM-8B, reactive brilliant orange K-7R, and acid red 6B. The decolourization rate was investigated using this enzyme in the absence and presence of five distinct laccase mediators (including 3,5-Dimethoxy-4-hydroxybenzaldehyde, methyl syringate, syringic acid, 1-hydroxybenzotriazole, and ABTS). Among them, methyl syringate produced the best results for all dyes evaluated. The recombinant laccase using this mediator attained a decolourization rate of reactive blue M-2GE, reactive brilliant red KD-8, reactive brilliant red KM-8B, reactive brilliant orange K-7R, and acid red 6B of 71.9, 89.2, 82.5, 94.9 and 98.6, respectively, in 140 min or less, at 30 ºC [150]. Using commercial laccase from a genetically modified *Aspergillus* sp. provided in a formulation with laccase, a mediator and a non-ionic surfactant, Tavares *et al.* [146] study the degradation of 3 different azo dyes: Remazol Black B, Remazol Yellow GR and remazol brilliant red 3BS using 4 distinct redox mediators, namely ABTS, violuric acid, N-hydroxyacetanilide; 1-hydroxybenzotriazole and 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO). The most effective mediator was ABTS, leading to a decolourization rate of 73%, 59% and 76% for Remazol Black B, Remazol Brilliant Red 3BS and Remazol Yellow GR, respectively [146]. Using the same enzyme, Cristóvão *et al.* [151] achieved a 93% dye removal with Remazol Brilliant Red F3B.

5.3.1.1 Mechanism of azo dye degradation by laccase

In order to understand the biocatalytic process, several researchers have studied and proposed dye degradation mechanisms [152–155]. Telke *et al.* [152] studied the mechanism of degradation of methyl orange using laccase from *Aspergillus ochraceus.* In this case, the mono azo bond suffers an asymmetric cleavage, forming p-N,N'-dimethylamine phenyldiazine and p-hydroxybenzene sulfonic acid intermediates. More recently, Navas *et al.* [156] studied azo dye degradation using a recombinant laccase from *Thermus* sp. produced in *E. coli* using 1-hydroxybenzotriazole as a mediator. The proposed biochemical pathway for the degradation of methyl orange is represented in Figure 7.

Laccase is able to degrade azo dyes by forming a carbocation (a positively-charged carbon atom), a highly reactive molecule, frequently attacked by nucleophiles [152], provoking an asymmetric cleavage of the azo bond [152–155]. Then, the intermediate molecules will be subject different chemical reactions depending on dye structure, such as oxidative cleavage, desulfonation, deamination, demethylation and dihydroxylation [145].

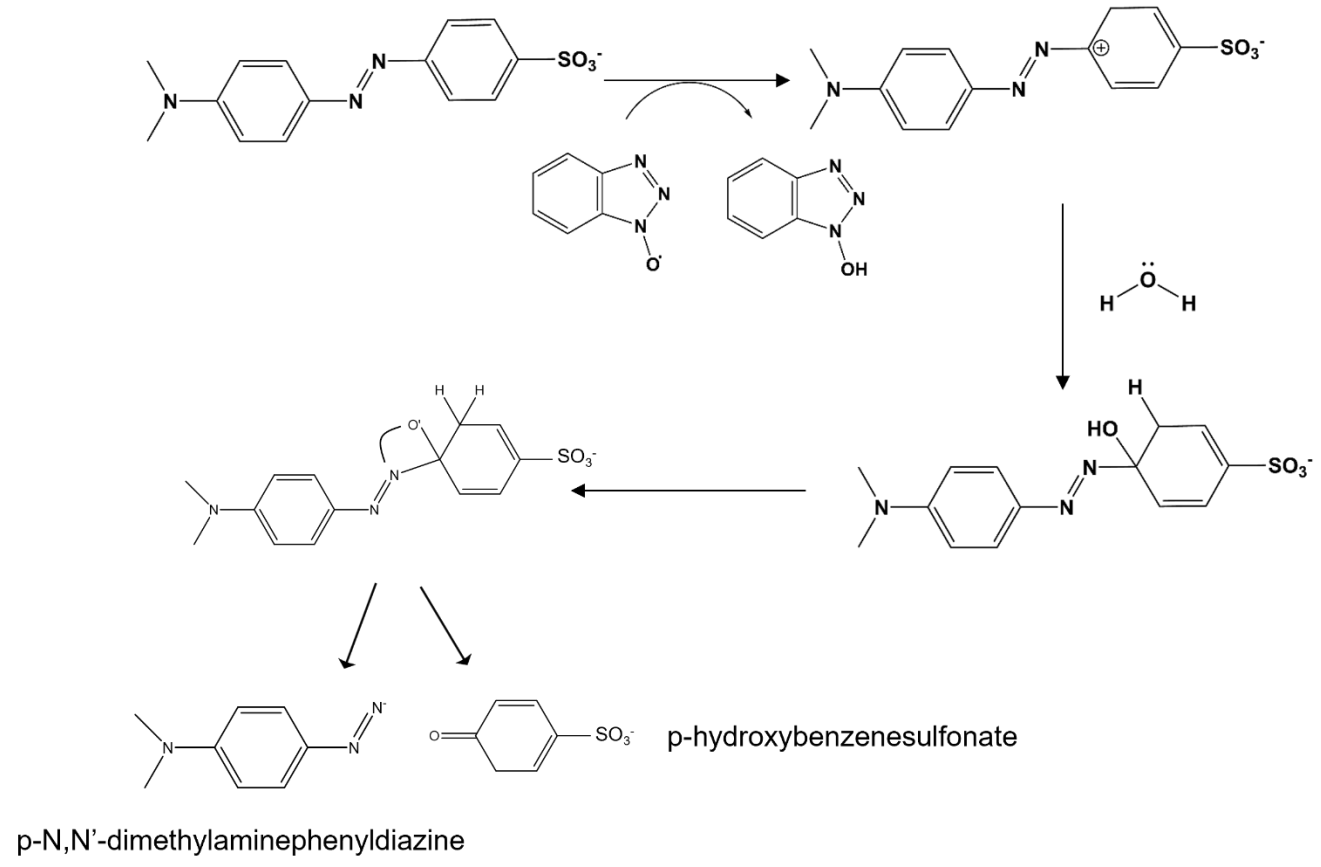


Figure 7 – Proposed route for the degradation of methyl orange (azo dye) by laccase. Adapted from Navas *et al.* [156].

However, azo dyes can have more than one azo bond, making the degradation mechanism more complex and with more reaction products. Using laccase from *Peroneutypa scoparia*, Pandi *et al.* [157] explored different leather dyes degradation, namely acid red 97, acid black 210, acid brown 282, acid green 16, acid yellow 42, acid blue 7, acid blue 193 and acid violet 54. The best result achieved was for acid red 97, with a decolourization rate of 75% without the use of mediators. The degradation occurs in three steps: first, laccase oxidizes the dye's phenolic group with one electron in the first phase, forming a phenoxy radical, which is followed by carbonium ion oxidation in the second phase. In the third phase, naphthalene 1,2-dione and 3-(2-hydroxy-1-naphthylazo) benzenesulfonic acid are produced by a nucleophilic attack by water on the phenolic ring carbon-containing the azo bond. [157]. Si *et al.* [158] studied the degradation of congo red with laccase from *Trametes pubescens*. The enzyme achieved a decolourization rate of 80.5 % within 72 h. The initial step of dye degradation was to reduce the azo bond, which resulted in the synthesis of two reactive intermediates, which then led to a series of reactions (deamination and desulphonation) that produced a stable intermediate (Figure 8), until the reaction products naphthalene amine, biphenyl amine, biphenyl and naphthalene diazonium be detected. Iark *et al.* [155] also explored the degradation of congo red with a laccase from *Oudemansiella canarii*. The enzyme is capable to decrease by 80 % the dye present in 24 at 30 ºC. During the degradation process, an asymmetric cleavage of the azo link occurred, resulting in the formation of multiple degradation products [155]. The authors propose that the dye molecule was oxidized, resulting in species with the NH_2 group nitrified and the SO_3 group lost. The oxidation of the amine groups present in congo red and the other intermediates can promote the release of naphthalene derivatives like $C_{10}H_6N_3O_7S^-$, $C_{10}H_5N_2O_7S^-$, and $C_{10}H_7N_2O_6S^-$, which are nitrified and/or hydroxylated species [155]. Finally, the benzene ring-opened in the final step of degradation, forming $C_8H_3N_2O_8^-$, a fully oxygenated molecule. Toxicity tests indicate that the degradation products are far less hazardous than the parent molecule (congo red) [155].

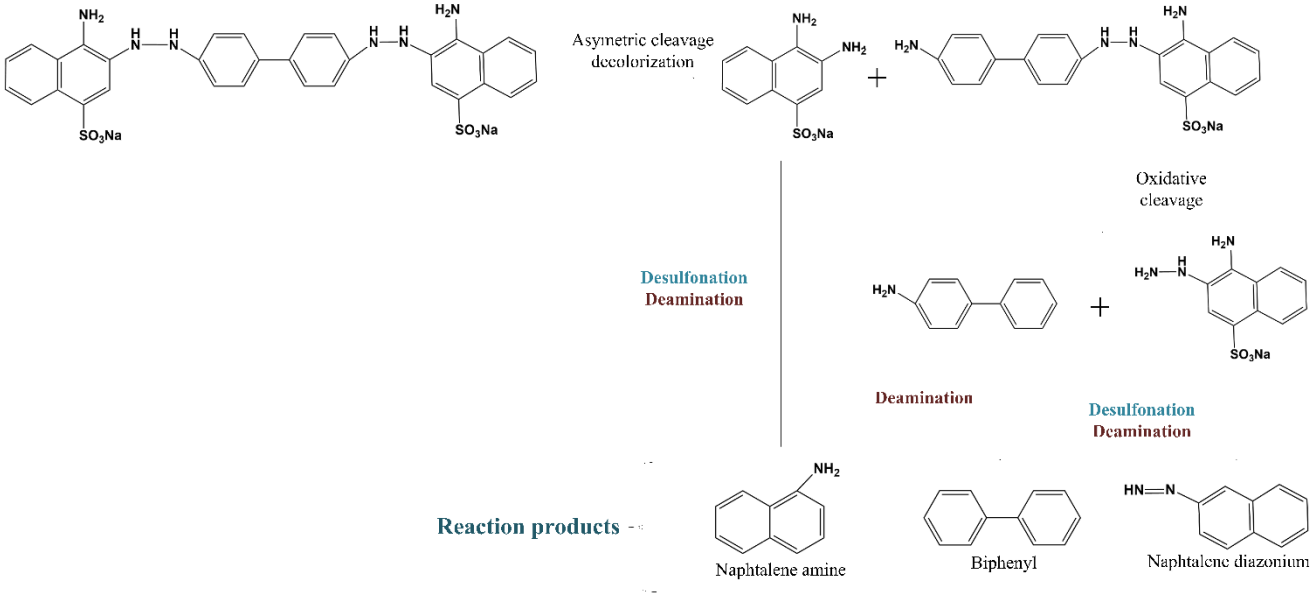


Figure 8 – Proposed route for the degradation of congo red (azo dye) by laccase. Adapted from Si *et al.* [158].

5.3.2. Triphenylmethane dyes degradation by laccase

Triphenylmethane dyes are used in many industrial dyeing processes causing environmental issues [159]. Triphenylmethane degradation using fungal laccase has been explored by several authors over the years and its degradation by laccase usually requires the presence of a mediator. Yang *et al.* [160] used a thermostable laccase from *Trametes trogii* S0301 to degrade triphenylmethane dyes (malachite and crystal violet dyes) at 60 °C using LMS and hydroxybenzotriazole as the mediator. The LMS revealed efficient decolourization ability towards malachite and crystal violet, with decolourization rates of 100 and 99.1%, respectively, in 5 h [160]. Without the presence of the mediator, the decolourization rate decreased to 16 and 8.6% for malachite and crystal violet, respectively, in 20h. However, in another work, a higher efficient decolourization

using the same enzyme was achieved without the use of mediators. Yan *et al.* [161] used a thermostable laccase from *T. trogii* S0301 to degrade triphenylmethane dyes (bromophenol blue, malachite green, crystal violet and acid red) at 30 °C. The degradation of bromophenol blue with laccase was achieved at a decolourization rate of 96.4% in 4 h. In addition, the enzyme revealed an efficient decolourization ability toward malachite green, crystal violet and acid red, with decolourization rates of 83.6, 95.7 and 81% respectively, in 11 h [161].

5.3.2.1. Mechanism of triphenylmethane dye degradation by laccase

To better understand the malachite green degradation, the reaction mechanism was studied, and two pathways were proposed (Figure 9) [162,163]. The first one, proposed by Yang *et al.* [162] explored malachite green degradation by laccase from *Cerrena* sp. and achieved a 91.6% decolourization rate, in less than 3h. The second one, proposed by Zhuo *et al.* [163], used *Pleurotus ostreatus* HAUCC laccase to degrade malachite green. It was found that the degradation reached 91.5%, within 24 h. Both works proposed the degradation mechanism via two pathways (Figure 9). The laccase-mediated breakdown of malachite green consists of two concurrent transformation steps: demethylation (Pathway I) and hydroxylation (Pathway II). Pathway I resulted in a consecutive N-demethylation of malachite green with a lower molecular weight. Demethylation does not eliminate the chromophore, therefore, additional degradation to open the ring is crucial for the complete decolourization. In Pathway II, malachite green was initially hydroxylated, followed by ring removal and demethylation. Both degradation pathways were found during the analysis of the transformations [162,163] and laccase was able to degrade the dye quickly and efficiently when both pathways are present [145]. However, Zhuo *et al.* [163] argue that endogenous mediators present in the extracellular fluid could be degradation facilitators in Pathway II [163]. In another work, Nejad *et al.* [164] also explored the degradation of malachite green, however, using purified laccase from *P. chrysosporium*, though, in the proposed mechanism only one pathway was presented, different from the Yang *et al.* and Zhou *et al.* [162,163]. The degradation was achieved with a 99% decolourization rate within 24 h at 30 ºC. The dye degradation mechanism proposed starts with malachite green suffering an oxidation/reduction reaction to produce leucomalachite green [164]. This intermediate was then demethylated and deaminated before being oxidized to generate (amino-phenyl)-phenyl-methanone. The final products obtained were di-benzyl methane and 4-(dimethylamino) benzaldehyde [164].

In summary, according to the type of triphenylmethane dyes and source of laccase, the degradation reaction mechanisms change [145].

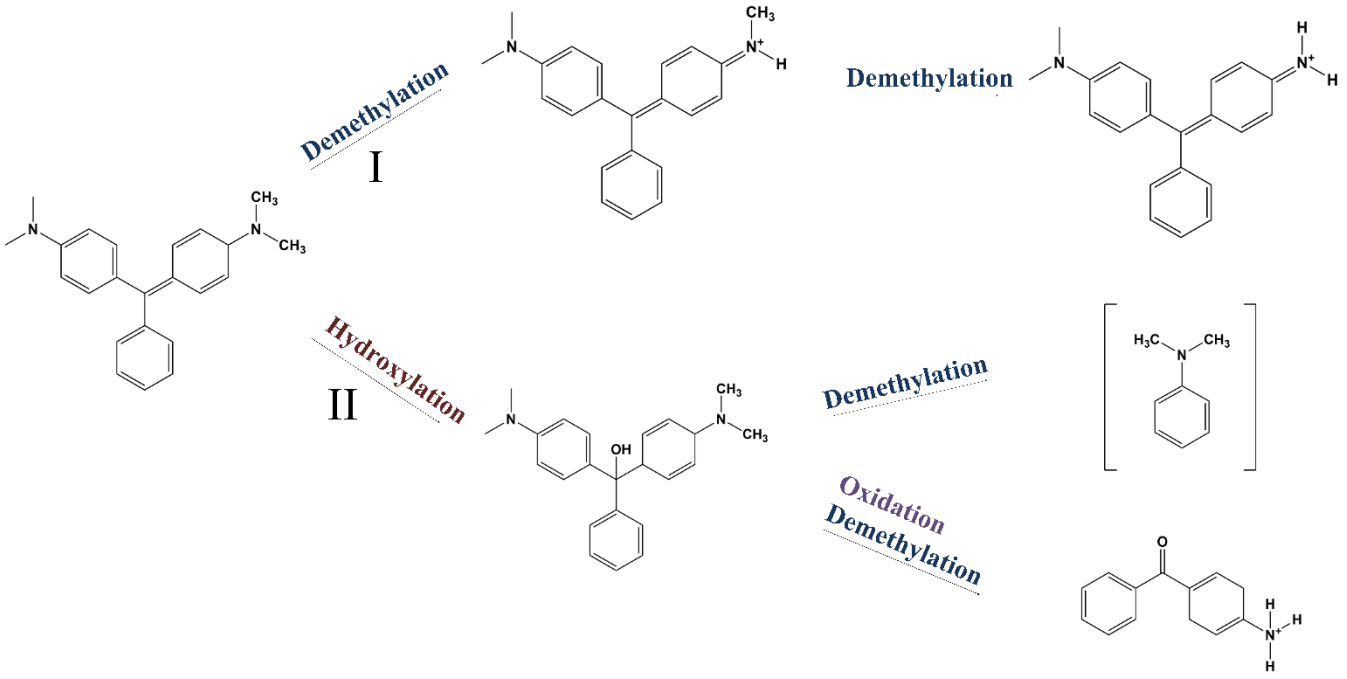


Figure 9 – Routes of degradation of malachite green (Triphenylmethane dye) by laccase. Adapted from Yang *et al.* and Zhou *et al.* [162,163].

5.3.3. Anthraquinone dyes degradation by laccase

Anthraquinone dyes are considered one of the most difficult to work with because of their fused aromatic structure [165]. Among anthraquinone dyes, RBBR has been one of the most extensively studied dyes. In the absence of redox mediators, RBBR is difficult to decolourize, however, different laccases can highly impact the degradation success. For example, RBBR decolourization by laccase from *Arthrospira maxima* was achieved, without the use of mediators [166]. This enzyme was capable to degrade RBBR at a 49% decolourization within 96 h (and 84% decolourization of Reactive Blue 4), without the use of

mediators [166]. In another work, Mechichia and co-works [167] used laccase from *Thielavia* sp. to degrade RBBR without the use of mediators [167]. The results proved that laccase was capable of efficiently degrading RBBR with a decolourization of 90% within 24 h [167]. Another anthraquinone dye, reactive blue 114 was degraded by laccase from *Trametes villosa* using 2 mediators: violuric acid and 1-hydroxybenzotriazole [168]. The decolourization rate was 92.3% with violuric acid and 81.1% with 1-hydroxybenzotriazole as a mediator, in only 10 min and 99.2% and 99.6%, respectively, within 1 h.

5.3.3.1. Mechanism of anthraquinone dye degradation by laccase

The anthraquinone degradation mechanism was investigated by several authors [163,165]. In 2010, Osma *et al.* [165] studied the transformation pathway of RBBR by immobilized laccase from *T. pubescens.* In 42 h, 44% of decolourization was achieved. Using *P. ostreatus* laccase to degrade RBBR, Zhou *et al.* [163] found that the degradation reached 84.9%, within 24 h. In both works, RBBR is broken down into two sub-products, which would lead to two routes. The last ring opened after successive deamination, hydroxylation, and oxidation [163,165] and the mechanism proposed is present in Figure 10. In addition, Osma *et al.* [165] studied the phytotoxicity with *Lolium perenne*, as a representative of grasslands species and found that RBBR degradation products are still harmful to *L. perenne*, however, not as much as the original dye.

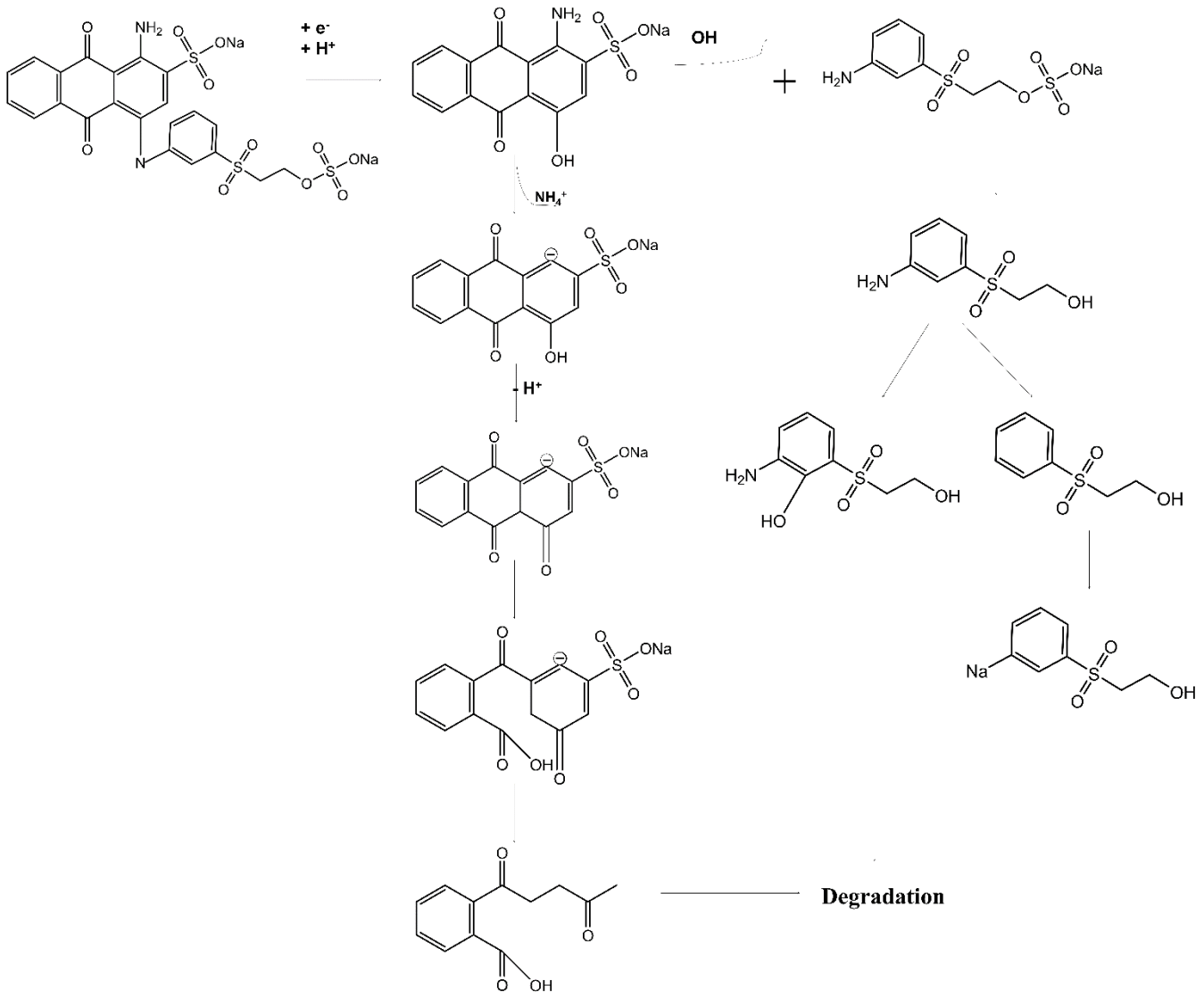


Figure 10 – Route of degradation of RBBR (anthraquinone dye) by laccase. Adapted from Zhou *et al.* and Osma *et al.* [163,165].

5.3.4. Indigo dyes degradation by laccase

Through the years, the degradation of indigo dyes by laccase has been extensively studied and has been reviewed by Choi *et al.* [169]. The degradation of indigo carmine dye by laccase alone is challenging, so the use of LMS is required. Xu *et al.* [150] produced a recombinant laccase from *Coprinopsis cinerea* using *P. pastoris.* The dye degradation of indigo carmine using the recombinant laccase was studied. The decolourization rate was studied without and with 5 different mediators, namely ABTS, 3,5-Dimethoxy-4-hydroxybenzaldehyde, methyl syringate, syringic acid and 1-hydroxybenzotriazole. The best result for the degradation of all dyes was obtained for methyl syringate as a mediator. At optimal conditions, the LMS with methyl syringate achieved a decolourization of indigo carmine of 99.1% [150]. Using a different approach, Bento *et al.* [170] studied the degradation of indigo carmine dye using laccase without the use of mediators. To improve the process, the team used ionic liquids (IL)-based surfactants, specifically decyltrimethylammonium bromide ($[N_{10111}]Br$) and 1-decyl-3-methylimidazolium chloride ($[C_{10}mim]Cl$). The ILs formed micelles to encapsulate laccase and Indigo Carmine in aqueous solutions, this process creates a microenvironment that allows the *in situ* degradation of the dye inside the micelles, improving the enzyme performance and dye degradation [170]. $[N_{10111}]Br$ present the best results achieving a degradation efficiency of

the dye of 82% within 30 min, compared to a 6% degradation without the IL, and 93% after 24 h [170].

5.3.4.1. Mechanism of indigo dye degradation by laccase

Indigo dye's degradation pathway by laccase from *T. hirsuta* was proposed by Campos *et al.* [171] (Figure 11). Laccases oxidize indigo dyes by removing four electrons from the molecule of indigo in a sequential process [171]. The first phase in the degradation of indigo dyes is the electrochemical oxidation of indigo into dehydroindigo, which is followed by a nucleophile attack (e.g. water) that results in the integration of O-atoms into the degradation products. More specifically, laccase can degrade indigo by converting it into isatine (indol-2,3-dion). Isatine is then spontaneously converted to anthranilic acid (2-aminobenzoic acid) via decarboxylation of isatic acid, a hydrolytic intermediate generated during isatine breakdown. The mechanism proposed for indigo degradation by laccase from *T. hirsuta* is presented in Figure 11.

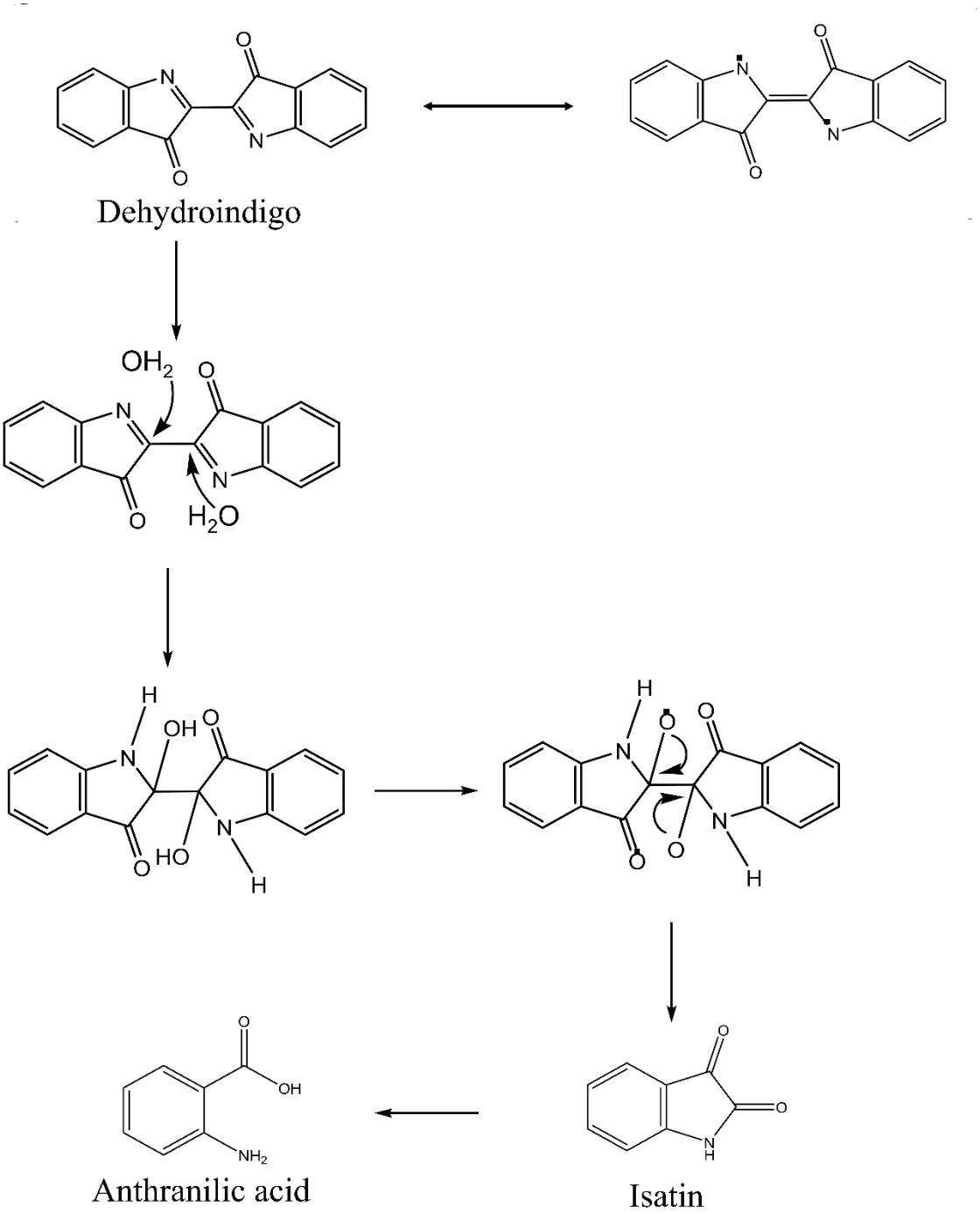


Figure 11 – Proposed route of indigo carmine (indigo dye) degradation by laccase. Adapted from Campos *et al.* [171].

All works presented and described prove the outstanding potential of laccases to remove and degrade different types of dyes with different chemical structures. However, more research is needed to better understand their properties and mechanisms in order to apply the enzymatic technology in diverse wastewaters containing dyes. To recognize and solve the difficulties at an industrial scale, it is necessary to evaluate several paraments such as the redox potential of laccases, the use of mediators, the reaction pH and temperature, the enzyme stability, the by-products generated and their toxicity and the reuse of the enzyme. Several authors are exploring the use of immobilized laccases to allow the reuse of the biocatalysts.

5.4. Immobilized laccase

Due to the limitations of free enzymes such as sensitivity to adverse environmental conditions and loss of activity and stability, enzyme immobilization methodologies are usually applied. It is known that free laccase has a high solubility in water and, thus, it is not easy to be separated from the reaction products. Moreover, laccase is an expensive enzyme leading to high bioprocesses costs [172]. These difficulties limit the practical application of laccase at the industrial level. Immobilized laccases raise catalytic activity in addition to offering stronger stability, improved durability against harsh environmental conditions, longer half-lives, resistance to proteases, and the capacity for reuse [8]. The use of immobilized laccase for bioremediation and more specifically, dye degradation, has been explored through the years and the literature was recently reviewed by Morsy *et al.* [8] and Zhou *et al.* [7]. Using this strategy, laccase is attached to a solid support by different immobilization methods, namely adsorption, covalent binding, entrapment, and encapsulation. Numerous materials have been proposed in recent years to immobilized laccase and are used in dye degradation.

Immobilized laccase has been demonstrated to be very effective for dye decolourization and researchers have been exploring laccase immobilization and comparing decolourization with the free form. Arica *et al.* [173] immobilized laccase from *T. versicolor* on fibrous polymer-grafted polypropylene chloride film for the decolourization of three dyes: Procion Green H4G, Brilliant Blue G, and Crystal Violet. According to the results, the immobilized laccase degraded dyes more effectively than the free laccase. After 120 min of enzymatic treatments, in a continuous enzyme reactor containing the immobilized enzyme, the decolourization for Crystal Violet was 99% and for Brilliant Blue G was 100%, after 60 min, and 90% for Procion Green H4G after 120 min. The chemical structure of the dyes had a significant impact on the degradation time. After eight cycles of Procion Green H4G decolourization, the immobilized laccase retained 53% of its initial activity [173]. Using the cross-linked enzyme aggregate (CLEA) approach, laccase from *Oudemansiella canarii* was immobilized [174] and used to degrade RBBR. Both free and immobilized laccase were effective in decolorizing RBBR. The enzyme's operational and storage stabilities were both improved by laccase immobilization. The dye was decolourized by 80% in the presence of both free and immobilized laccase after 24 h at 30 ºC. Moreover, after 6 cycles of reaction, the immobilized laccase was still effective in decolourising the dye, while the free enzyme was not reused. In addition, the toxicity was evaluated and, after laccase treatment, the toxicity of the RBBR solution was reduced by 90% (Microtox assay) [174]. Shan *et al.* [175] designed a novel material, a geopolymer microspheres with amino acids (histidine and cysteine) to immobilized both laccase and ABTS, with the aim of improving the degrading capacity of dyes, since the enzyme and the mediator will be close. The immobilized laccase was used to degrade congo red, reaching a degradation of 94.78%, superior to 79.23% and 58.13% attained using free enzyme and ABTS and only free enzyme, respectively. The immobilized laccase showed excellent pH resistance, storage stability, and reusability up to 10 cycles of reaction (decolourization efficiency of 80.3% at the fifth cycle, and 50.7% at the tenth cycle) [175]. Using a different immobilization approach and creating a more eco-friendly and biocompatible option, Gao *et al.* [176] encapsulated recombinant laccase from *T. versicolor* expressed in *Spodoptera frugiperda* in protein nanocages called major vault proteins (MVP). Laccase performance was significantly increased by encapsulation at industrially relevant conditions, such as a wider pH range and relatively high temperatures. Reactive Blue 19 and Acid Orange 7 were swiftly decolourized, 72% and 80% in 24 h, respectively by immobilized laccase, while free laccase (laccase from *T. versicolor*) achieved 40% and 32% decolourization, respectively [176]. On model bacterial, algal, and insect cells, the toxicity of the dyes was investigated, and treated samples with encapsulated laccase presented a toxicity reduction, compared to the original dyes [176].

Recently, Escarpa and co-authors [177] designed a very interesting material for the immobilization of laccase from *T. versicolor*. Laccase was immobilized on micromotors composed of poly(3,4-ethylenedioxythiophene)-polypyrrole and platinum. The micromotor's platinum layer created O_2 by breaking down H_2O_2 and allowing it to move. The micromotors were used to investigate Procion Red, Reactive Green 5, Reactive Brown 10, Reactive Green 19, Cibacron Blue F3GA, Alkali Blue 6B, and Brilliant Blue 6 decolourization. According to the authors, in comparison to the free enzyme, the self-propelling behaviour of laccase modified micromotor improved the decolourization ability of the laccase enzyme due to continuous movement, in terms of efficiency, reaction time and convenience of use. The decolourization rate using this material varies between 76 and 94% (depending on the dye) within 10 min, while the use of free enzyme presents a decolourization rate of 34-55% [177]. The authors also suggested the incorporation of a magnetic component in the micromotor structure to facilitate immobilized enzyme recovery.

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The use of magnetic materials is very interesting since it offers easy and fast recovery of the immobilized laccase by the simple use of a magnet [178]. Ulu *et al.* [178] used a magnetic hybrid material (composed of magnetite and thiolated chitosan) to immobilized laccase from *T. trogii*. The immobilized laccase was used in the decolourization of Reactive Blue 171 and Acid Blue 74, with a decolourization of 79% after 10 cycles and 56% after 8 cycles, respectively [178]. Amir *et al.* [179] create a metal-organic material with magnetic properties and immobilized laccase from *T. versicolor* and used it to degrade Reactive Black 5 and Alizarin Red S. The results showed a total decolourization of Alizarin Red S and 81% of Reactive Black 5 within 24 h. Furthermore, the reusability of the immobilized laccase for the removal of target dyes was tested for up to five cycles of reaction, with small efficiency declines, with Alizarin Red S and Reactive Black 5 removal rates of 92% and 73%, respectively, after the fifth cycle [179]. Alsaiari *et al.* [180] immobilized laccase from *T. versicolor* in nanoparticles of magnetite and copper ferrite and used it to remove the dye Direct Red 23. The immobilized laccase reuse for the decolourization was evaluated for six cycles of reaction, with a decolourization rate of over 70%, indicating good reusability and outstanding stability. Immobilized laccases, not only had stronger resistance to a wider pH and temperature range, but also had significantly improved thermal stability, storage stability, and reuse [178–180].

Laccase immobilized on pine needle biochar was used to investigate the decolourization and degradation of malachite green dye [181]. Within 5 h, more than 85% of the malachite green dye was removed. The degradation products obtained were leuco malachite green, methanone, [4-(dimethyl amino)pheny]phenyl, and 3-dimethyl-phenyl amine. The enzymatic breakdown produced fewer hazardous metabolites than the original dye, according to phytotoxicity studies, realized with *Vigna radiata*. Peng *et al.* [182] immobilized laccase purified from A*spergillus* sp. in a metal-organic framework constituted by aluminium and polyvinyl alcohol cryogel and applied it in the removal of alizarin green. The immobilized enzyme reached 95.86% of removal efficiency in 12 h. After six reaction cycles, the enzyme maintained more than 60% of its initial activity. The authors also explored the degradation products and identified the degradation process' end products: p-toluidine, 2-(2,3,6-trihydroxybenzyl acyl) benzoic acid and phthalic acid. Besides, the degradation mechanism was proposed and C- α oxidation was discovered to be the major mechanism [182]. In Table 3 is summarized the decolourization of dyes using immobilized laccase on different solid materials.

Table 3 – Dye decolorization using immobilized laccase on different solid materials.

Material	**Laccase source**	**Target dye**	**Degradation**	**Ref**
Fibrous polymer-grafted polypropylene chloride film	*Trametes versicolor*	Procion Green H4G, Brilliant Blue G, and Crystal Violet	90%, 100% and 99%, respectively.	[173]
Cross-linked enzyme aggregate (CLEA)	*Oudemansiella canarii*	RBBR	80	[174]
Geopolymer microspheres with amino acids	Not specified	Congo red	94.78%. 80.3% at the fifth cycle	[175]
Major vault proteins (MVP)	*T. versicolor* (recombinant laccase expressed in *Spodoptera frugiperda*)	Reactive Blue 19 and Acid Orange 7	72% and 80%, respectively	[176]
Poly(3,4-ethylenedioxythiophene)-polypyrrole and platinum micromotors	*T. versicolor*	Procion Red, Reactive Green 5, Reactive Brown 10, Reactive Green 19, Cibacron Blue F3GA, Alkali Blue 6B, and Brilliant Blue 6	76-94%	[177]
Magnetite and thiolated chitosan hybrid materail	*Trametes trogii*	Reactive Blue 171 and Acid Blue 74	79% after 10 cycles and 56% after 8 cycles, respectively	[178]
Amino-Functionalized Magnetic Metal–Organic Framework	*T. versicolor*	Reactive Black 5 and Alizarin Red S	81% and 100%, respectively	[179]
Nanoparticles of magnetite and copper ferrite	*T. versicolor*	Direct Red 23	70 after 6 cycles	[180]
Pine needle biochar	Not specified	Malachite green	85%	[181]
Metal–organic frameworks (with aluminium and polyvinyl alcohol cryogel)	A*spergillus* sp.	Alizarin green	95.86%. 60% after 6 cycles	[182]
Modified rice husks	*Boletus edulis*	Reactive Blue-19	91%	[183]

Copper alginate beads	*Ganoderma* sp.	Indigo Carmine, RBBR and Malachite green	100%, 100% and 82%, respectively	[85]
Polydopamaine nanoparticle/bacterial cellulose composite	*Myceliophthora thermophile*	RBBR	91%. 65% after 5 cycles	[184]
Chitosan-polyacrylic acid microspheres	*T. versicolor*	Naphthol green B and Indigo Carmine	81% and 72% of initial activity after 5 cycles, respectively	[185]
Nanocellulose from quinoa husk	Not specified	Malachite green and congo red	98% and 60%, respectively. And 83% dye removal after 18th runs for MG.	[186]
Green coconut fibre	*Aspergillus* sp.	Reactive Black 5, Reactive Blue 114, Reactive Yellow 15, Reactive Yellow 176 and Reactive Red 239	90%, 90%, 80%, 30% and 30%, respectively.	[187]

In addition to solid supports for enzyme immobilization, the use of liquid supports for enzyme reuse is an interesting alternative. In this context, the use of biphasic systems can be applied for enzyme retention in one phase, allowing enzyme recovery and reuse. For example, Ferreira *et al.* [188] evaluated the use of aqueous biphasic systems (ABS) as liquid support to immobilize and reuse laccase for cyclically degrading RBBR (Figure 12). The ABS was composed of polypropylene glycol (PPG) 400 and other compounds, namely, 3 different cholinium-based ILs (cholinium dihydrogen citrate ([Ch][DHC]), cholinium dihydrogen phosphate ([Ch][DHP]), cholinium acetate ([Ch][Acet])), polyethylene glycol (PEG) 400 and K_2HPO_4. The best results were achieved with the ABS constituted by 46 wt% PPG 400 +16 wt% [Ch][DHC], with complete degradation of the dye. Laccase was recovered from the IL rich phase and reused for 6 cycles of RBBR decolourization, with a dye degradation yield of 96% in the final cycle.

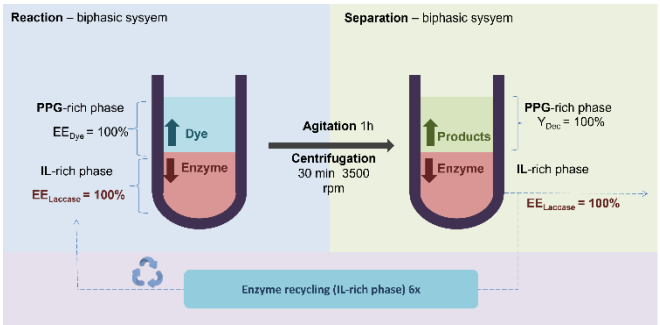

Figure 12 – Process developed using ABS as liquid supports for enzymatic degradation of dyes and enzyme reuse. Adapted from Ferreira *et al.* [188].

The liquid enzyme immobilization approaches present a high potential to be applied as novel techniques in dye decolourization and detoxification of wastewaters. Current immobilization methods using solid supports have limitations due to enzyme leakage from the carrier, unwanted interactions between the carrier and the enzyme, or even the enzyme's inability to react with its substrate [8]. Researchers are working on new enzyme immobilization methods, as it is a promising subject with better outcomes and lower costs for industrial application. However, the focus has been on finding novel materials, rather of the in-depth study of its application in the treatment of wastewater with dyes, including the study with real effluents.

6. ENZYMATIC DECOLORIZATION OF REAL TEXTILE WASTEWATER WITH LACCASES

The experimental results previously presented from the literature suggest that laccases have been extensively studied regarding the decolourization of diverse types of dyes. Nevertheless, relatively few experiments have been proposed with laccases for the treatment of real textile wastewaters containing dyes. Industrial wastewaters can be complex, comprising not only dyes but also detergents, and salts, sometimes with extremely high ionic strength and pH values, as well as chelating agents, precursors, by-products and surfactants [189]. In addition, the treatment of textile wastewaters is even more challenging considering that their complex composition is tremendously variable within a single textile factory. The first studies on the degradation of real textile wastewater by laccase describe its use in its free form, as reported by Khlifi *et al.* [190] in 2010. These authors tested the capacity of a crude laccase from *T. troggi* to decolourize and detoxify industrial wastewater from a textile factory from Tunisia containing several dyes and other chemical compounds. The ability of laccase to decolourize the real wastewater proved to be limited. However, in the presence of some redox mediators, the laccase oxidative reaction was improved with a wastewater decolourization above 50%. Nevertheless, it was concluded that wastewater decolourization is not equivalent to wastewater detoxification, as the enzymatic reaction products were still toxic [190]. In another work, the decolourization and detoxification of two textile industry wastewaters (A and B) from the same textile factory in Tunisia were further studied by Benzina *et al.* [191] using a LMS with 1-hydroxybenzotriazole as mediator. The wastewaters were treated at 30 ºC for 20 h to replicate industrial conditions. After optimizing the conditions, a maximum decolourization of 68% and 88% were attained, for wastewater A and B, respectively. Although some toxicity was still found in the treated wastewaters, the authors reported reductions in growth inhibition of yeast and bacterium, used as ecotoxicity parameters. Due to the high salt concentrations of textile industry wastewaters, a LMS of salt-tolerant laccase from *Peniophora cinerea* was also tested for the treatment of

a real wastewater from a textile industry in São Paulo, Brazil, promoting 54.6% of decolourization after 72 h [192]. A novel laccase from *Cerrena* sp. HYB07 showed a complete decolorization in 3 days of a textile wastewater from Septwolves Group Co. in Fujian, China. [193]. Motamedi *et al*. [194] studied the use of a thermostable/halotolerant metagenome-derived laccase in the treatment of a real wastewater from a textile factory in Iran. The metagenome-derived laccase proved to be very fast and effective, achieving decolourization efficiencies above 84% of the wastewater sample in less than 2 min.

Recently, reports of real textile wastewaters decolourization using LMS with immobilized enzyme show that, in fact, the immobilization is very important for the enzymes at an industrial scale. Immobilized enzymes have improved stability in extreme conditions, increasing their shelf life. Furthermore, the use of mediators increases the treatment performance and process costs, so enzyme immobilization and reuse are also great advantages for industrial applications. Sondhi *et al.* [195] reported the use of a laccase from *Bacillus* sp. MSK-01 immobilized onto Cu-alginate beds with ABTS as mediator for the construction of a continuous flow packed bed bioreactor for the treatment of textile dye wastewater from a textile mill in Punjab, India. According to the results of the treated sample, the LMS resulted in the generation of some dye degradation products, nevertheless, the colour of the real wastewater was reduced by 66% [195]. In another work, a new polyacrylamide-alginate cryogel was showed also to be an effective support to immobilize laccase for dye removal from real textile wastewater from a plant in Turkey, attaining a maximum decolourization of 56% [196].

6.1. Dye mineralization using laccase

Dye decolourization is clearly different from dye mineralization. There is a significant gap in current information about the mechanisms of decolourization and, even more so, mineralization. The ability to assess the full potential of laccase for textile industrial wastewater treatment remains limited due to a lack of knowledge about potentially hazardous although colourless accumulating intermediates. In this sense, lately, some authors have decided to deepen their studies not only in terms of the decolourization of textile wastewater but also regarding their mineralization. Navada and Kulal [197] used laccase from gamma-irradiated endophytic fungus in the treatment of wastewater from a textile washing factory in Karnataka, India, to study its decolourization and also to determine the laccase's potential role in the mineralization of the wastewater. The treatment with laccase led to a 99% decolourization in 2.5 h, and the pollution parameters were below the allowed limits, with 67% and 47% reductions in COD and BOD, respectively, validating the mineralization of the textile wastewater [197]. A bacterial laccase from *Alcaligenes faecalis* XF1 was co-immobilized with a natural mediator acetosyringone (ACS) onto chitosan-clay composite beads and studied in terms of its ability to decolourize and detoxify real textile wastewater from the textile industry in India, using a packed bed reactor system [198]. The use of this system revealed a marked reduction of the prominent peaks in the spectrum of the treated wastewater compared to the original sample, reaching 78% of decolourization. In addition, the treated sample presented a reduction of 91% and 87% of BOD and COD values, respectively, as well as a significant decrease in the respective microtoxicity.

Nevertheless, for the introduction of the immobilized laccase mediated systems in the treatment of textile industries wastewaters, more research is needed on the cost-effectiveness of the system and the enzymatic dye decolourization mechanisms. The elucidation of the decolourization mechanism is a difficult mission due to the inherent complexity of both the dyes' structures and the enzyme degradation mechanisms.

7. CONCLUSION AND FUTURE PERSPECTIVES

The release of textile dyes into the environment has serious negative effects on the aquatic systems causing high pollution levels. Textile dyes are poorly biodegradable and cause mutations and metabolic alterations, besides the potential carcinogenic effects. Depending on the type and concentration of the textile dyes and the volume of wastewater to treat, the traditional chemical, physical and biological wastewater treatments are not recommended due to the generation of secondary by-products and low efficacy. Thus, the enzymatic degradation of textile dyes using fungal laccases has gained interest due to their outstanding potential to discourse this problem and to their eco-friendlier nature and exceptional decolourization. Among microbial laccases, fungal laccases are extensively used to degrade textile dyes because of their high redox potential and consequently improved decolourization rates of diverse types of dyes. Laccases are able to attack the chemical structure of dyes in different ways to rapidly decolourize the complex chemical structure of textile dyes. Current laccase immobilization methods are highly beneficial and successful in the problem at hand, nevertheless, it is important to address activity and stability loss issues due to enzyme leakage from support, undesired enzyme-support reactions or even the enzyme failure to react with the substrate. Future opportunities and improvements may relate to the development of new immobilization strategies to ensure a stable, sustainable and cost-effective solution to achieve commercial success. It is also important to note that, although fungal laccases have the ability to degrade textile, lack of knowledge of possibly toxic, although colourless accumulating intermediates limits the evaluation of laccase's full potential for treating textile industry effluents. The intrinsic complexity of both the dye and enzyme structures, as well as the complex and variable composition of real textile wastewaters, also makes the understanding of the degradation mechanisms challenging. Future directions in this domain include more studies to better understand their characteristics and properties and to optimize the performance of the existing methodologies not only in terms of textile wastewater decolourization but also regarding their mineralization in order to expand this enzymatic technology. Moreover, life cycle impact analysis and economic cost-benefit evaluation should be also performed to demonstrate the effectiveness of enzymatic technologies based on fungal laccases at an industrial scale.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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