Recent advances in laccase activity assays: A crucial challenge for applications on complex substrates

Elise Martin, Pascal Dubessay, Eric Record, Fabrice Audonnet, Philippe Michaud

Recent advances in laccase activity assays: a crucial challenge for applications on complex substrates

Elise Martin[a], Pascal Dubessay[a], Eric Record[b], Fabrice Audonnet[a] and Philippe Michaud[a,*]

[a] Université Clermont Auvergne, Clermont Auvergne INP, CNRS, Institut Pascal, F-63000 Clermont-Ferrand, France; fabrice.audonnet@uca.fr (F.A.), pascal.dubessay@uca.fr (P.D.), elise.martin@uca.fr (E.M.), philippe.michaud@uca.fr (P.M.)

[b] INRAE, Aix-Marseille Université, UMR1163, Biodiversité et Biotechnologie Fongiques, 13288 Marseille, France ; eric.record@inrae.fr (E.R.)

Abstract

Despite being one of the first enzymes discovered in 1883, the determination of laccase activity remains a scientific challenge, and a barrier to the full use of laccase as a biocatalyst. Indeed, laccase, an oxidase of the blue multi-copper oxidases family, has a wide range of substrates including substituted phenols, aromatic amines and lignin-related compounds. Its one-electron mechanism requires only oxygen and releases water as a reaction product. These characteristics make laccase a biocatalyst of interest in many fields of applications including pulp and paper industry, biorefineries, food, textile, and pharmaceutical industries. But to fully envisage the use of laccase at an industrial scale, its activity must be reliably quantifiable on complex substrates and in complex matrices. This review aims to describe current and emerging methods for laccase activity assays and place them in the context of a potential industrial use of the enzyme.

Keywords

Laccase; oxidase; enzyme; activity assay; lignin

1. Introduction

The use of enzymes has gained special attention in recent years, both in academic research and in the industrial sector. Many industries are looking for alternatives to physico-chemical methods as the environmental concerns grows in societal debates. To include more green chemistry in industrial processes, biocatalyst is an interesting approach as it allows to get rid of the limitations of chemical catalysis such as high energy requirements, poor efficiency, low selectivity, production of toxic side products, overall high cost and sometimes complicated operation conditions [1]. Among enzymes, laccases are being actively studied as they have a wide range of substrates and potential applications. As an indicator of this growing interest for laccases in the scientific community, some 630 patents have been published about laccases (according to the PATENTSCOPE database provided by the World Intellectual Property Organization [2]) and 46 537 articles (according to the Dimensions database [3]) over the past five years. Laccases (EC 1.10.3.2) are part of a larger group of enzymes known as multi-copper oxidases and were first discovered in 1883 by H. Yoshida [4], in the sap of a japanese tree called *Rhus vernicifera*. It is well known now that laccases can be found in a wide variety of organisms, i.e. 7300 cellular-organism sources listed in UniProtKB (https://www.uniprot.org/) [5], but mostly in higher plants, bacteria and fungi [6] in which they play diverse biological functions. They catalyse the one-electron oxidation of numerous substrates, basically any substrate with characteristics like *p*-diphenol, by the concomitant reduction of O_2 to water [7]. Their main natural substrate is lignin, an alkyl-aromatic heteropolymer which is constitutive of the pectocellulosic walls of plants. Lignin is a complicated polymer to break down because of its complex chemical structure and great heterogeneity [8]. Indeed, depending on the plant species, lignin structure can change considerably even if it always involves three building blocks called monolignols, i.e. p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol [9]. Due to this complex, variable and sometimes difficult-to-identify structure, lignin is currently

under-utilized and under-valued. The scientific community has therefore focused on the use of laccases to overcome this problem, as laccases are a key enzyme group in the biodegradation of lignin in wood by fungi and bacteria. As a result, 76 patents involving the activation and modification of lignin by laccase have been issued since 2019 (PATENSCOPE database [2]). A growing number of publications on the subject is also observed with a peak of 4800 new articles in 2022, especially in engineering, industrial biotechnology, and environmental journals (Dimensions database [3]).

The already huge range of substrates of laccases can be further extended by the use of small molecules acting as mediators in the laccase-mediator system (LMS) [10]. These enzymes seem therefore to still have the potential to expand its use in the coming years and find industrial applications in many fields. The current market size for laccases is of USD 3 million in 2021 and is set to increase in the coming years with a projection of USD 4 million by 2028 [11]. To do so, however, it is essential to be able to quantify its activity in different matrices and on a maximum of substrates. Currently, the most common method to determine laccase activity is spectrophotometry, by monitoring the appearance of a coloured oxidation product. The chemicals used to determine laccase activity are mostly monomers that do not reflect the complexity of lignin. This well-known method is not the only way of evaluating laccase activity though. The aim of this review is to present other techniques used for the determination of laccase activity and to investigate how these methods could be integrated in an industrial context.

2. Laccases: origin, mechanism, and laccase-mediator system

2.1. The origin and physiological functions of laccases

Laccases (EC 1.10.3.2) are oxidoreductases belonging to the blue multi-copper oxidases (MCOs) enzyme family with ascorbate oxidases, ceruloplasmins, bilirubin oxidases,

phenoxazinone synthases and metallo-oxidases [5]. They catalyse the oxidation of many phenolic and non-phenolic compounds by reducing O_2 to H_2O and without releasing any harmful products (**Figure 1**).

In plants, laccases are involved in lignification, wound healing and plant responses to the environmental stresses. Studies have shown evidence that laccases play a crucial role in lignin polymerisation by oxidising monolignols and thus enabling polymerisation [12]. In 2006, Liang *et al* [13] get the genetic proof of the importance of laccase in lignin biosynthesis. To do so, they identified two mutants for a laccase gene (AtLAC15) in *Arabidopsis* and showed that a mutation of this gene affected the content of extractable lignin in seeds, with a decrease of about 30% compared to a wild-type seed. Laccases can also participate in the cross-linking of cell wall structural proteins and thus promote tissue regeneration after wounding [14]. De Marco *et al* [14] also observed an immediate activation of laccase activity after leaf wounding, supporting the hypothesis that laccases play a regenerative role in plants. Indeed, laccase activity was detected in protoplasts culture and could be a substitute to peroxidases in the healing process when hydrogen peroxide is not yet available. Zhang *et al* [15] were interested in the involvement of laccase in plant defence mechanisms. Their study demonstrated that the cotton laccase gene (GhLAC15) was functionally induced in presence of the pathogen *Verticillium dahlia,* a fungus responsible for vascular wilt disease. In this work, laccase expression is proved to correspond to an immune response of plants that induced the lignification of cell walls as defence barrier against pathogen invasion.

Fungal laccases participate in lignin decomposition but also in sporulation, pathogenesis, virulence, defence/protection, iron metabolism, humic acid degradation, litter decomposition and metals oxidation [16]. As example, researchers have investigated the importance of laccase during sporulation showing that there was an accumulation of a natural substrate of the enzyme during the late stage of sporulation. This endogenous substrate was not identified in the study

but could inhibit competitively the enzyme activity and then impact the sporulation phenomenon [17].

Laccases found in insects are mostly involved in cuticle sclerotization. They catalyse the oxidation of catecholic compounds in insect cuticle [18]. This is the only example of the presence of laccases in animals.

In bacteria, laccases are used for lignin degradation, cell pigmentation, morphogenesis, detoxification and defence against antagonistic organisms [16].

2.2. Structure of laccases and their catalytic mechanism

To date, some 100 different laccases have been isolated and characterized. They are dimeric or trimeric glycoproteins with a molecular weight of 50-130 kDa [10]. The catalytic activity of laccases relies on the Cu atoms, distributed in three copper centers in the enzyme, that can be differentiated thanks to their characteristic electronic paramagnetic resonance (EPR) signals and spectroscopic behaviour. These centers are name Type-1 (T1), Type-2 (T2) and Type-3 (T3) copper centers [19]. T1 is a mononuclear site with one Cu atom, responsible for the characteristic blue color of the enzyme, and where takes place the substrate oxidation. It is also responsible of the redox potential of laccase. T2 and T3 form a trinuclear cluster with three Cu atoms (one type-2 and two type-3). The T2/T3 site recovers the electron extracted from the substrate in T1 and reduces oxygen to water [20].

The mechanism of action of laccase takes place in several steps, illustrated in **Figure 2**. First, the substrate is stripped of an electron at the T1 site. The Cu^{2+} atom is reduced to Cu^{+}. When four substrate molecules have been oxidized by laccase, all the enzyme's copper atoms are reduced to Cu^{+}. The enzyme is said to be in reduced form. In a second step, Cu^{+} ions at sites T2 and T3 reduce molecular oxygen to water. During this reduction step, a peroxide intermediate and an oxy radical intermediate are passed through. At the end of the reduction of O_2 to H_2O,

the four Cu^{+} ions have recovered their 4 electrons and the enzyme is "regenerated" in its native form, ready for another catalytic cycle [21].

Within the enzyme's active site, copper atoms are linked to amino acid residues by coordination bonds (a bond between a Lewis base, in this case an amino function of the amino acid, and a Lewis acid, the Cu^{2+} ion). The 2 copper atoms of T3 coordinate with a total of 6 histidines (**Figure 3**). Five of these share their Nε2 nitrogen and one shares its Nδ1 nitrogen in the bond. Site T2 is linked to 2 histidines by their Nε2 and to an oxygen atom, originating from a water molecule. Finally, T1 is linked to 2 histidines by their Nδ1 nitrogen and to a cysteine residue by its sulfur atom [22]. The laccase thus described has a redox potential generally between 400 and 800 mV. This redox potential, either high (>700 mV) or low (<700 mV), depends on the origin of the laccase [23]. Several studies have demonstrated that plant and bacterial laccases have lower redox potential than fungal ones [24].

Regarding 3D structure, laccase has around 500 amino acids in three domains, with a greek key β barrel topology circulated in a single molecule [25]. Crystal structures of different laccases have been elucidated over the past few years with, for example, fungal laccases from *Trametes versicolor* [22], *Trametes trogii* [25], *Pycnoporus sanguineus* [26], bacterial laccases from *Bacillus subtilis* [27], *Streptomyces coelicolor* [28], *Pediococcus acidilactici* [29] and plant laccase from *Zea mays* [30].

Fungal, bacterial and plants laccases have a similar molecular architecture despite their wide diversity of substrates and large taxonomic distribution. Sequence analysis have shown that copper binding motives are highly conserved in all three laccases. Few differences exist however, depending on the origin of the enzyme. The N-terminal region of bacterial laccase looks distorted compared to those of fungal and plant laccase. Bacterial laccases have also a coiled section to link Domain 1 and 2 of its structure, coiled section that is absent in fungi and plants, but the latter have instead α-helical regions to connect these two domains and the 2 and

3 ones. Finally, a protruding section making a lid-like structure over the substrate binding site, have been identified by Dwidedi *et al* [31] as a distinctive feature of bacterial laccases. They also observed, thanks to X-ray determination of crystal structures, that the bacterial laccase has a largest binding site cavity than fungal and plants laccases. All these observations suggest that, despite a rather similar 3D structure, some conformation differences exist between laccases from different organisms, giving them their physiological functions within the organism.

It is also important to differentiate between laccase and heme-peroxidases (EC 1.11.1.7) even if they can be found in similar organisms. The reactions they catalysed are very closed. Both the enzymes are able to oxidise phenolic compounds and aromatic amine thanks to a one electron oxidation [32]. Heme-peroxidases is a generic term for several enzymes: lignin peroxidases, manganese peroxidases, and manganese-independent peroxidases. Heme-peroxidases differ from laccases by their generally higher redox potentials. Laccases and heme-peroxidases have also different prosthetic groups in their protein structures: protoporphyrin IX in lignin and manganese peroxidase structure [33] versus pyrroloquinoline quinone in laccases [34]. Finally, a last but not least difference between laccases and heme-peroxidases, is the fact that laccases only require molecular oxygen O_2 for their catalysis mechanism while peroxidases require hydrogen peroxide H_2O_2 [35].

2.3. The laccase-mediator system

There is a system known as the laccase-mediator system (LMS), which enables the enzyme to oxidize compounds other than phenolic ones, with a mediator acting as an intermediate between the enzyme and the substrate (**Figure 4**). In this way, laccase oxidizes the mediator, which in turn oxidizes the substrate. Mediators have higher redox potentials than laccase and, once

oxidized, form stable compounds capable of oxidizing not only non-phenolic chemical groups but also groups inaccessible to the enzyme [21].

Indeed, laccase cannot penetrate some substrates, like the lignocellulosic matrix for example, due to their size and geometry in space. The mediator therefore overcomes this obstacle by acting as an electron "shuttle", bringing the oxidizing power into the matrix. The ideal mediator is a small-sized molecule, capable of being oxidized into a stable radical and then recycle. Moreover, the radical form of the mediator must not be an inhibitor of the enzyme. Finally, given the targeted applications for laccases and laccase-mediator system, mostly for environmental purposes in industries, the mediators should be environmental-friendly and cheap [36]. Laccase mediators can be divided into two groups: natural mediators (used by lignolytic organisms) and synthetic mediators. Natural laccase mediators are defined as molecules of natural origin which can be found in lignocellulosic biomasses and that are the usual mediators of white-rot fungi lignolytic laccases. Thus, during lignin biodegradation by fungi, depolymerization products such as phenoxyl radical fragments or phenolic monomers are generated and can oxidize non phenolic compounds, explaining the ability of fungal laccases to degrade lignin despite their rather low redox potential [36]. Natural mediators include for example syringaldehyde, vanillin, acetosyringone, acetovanillone, sinapic acid and ferulic acid, that are monomers derived from the lignin biodegradation, and 3-hydroxyanthranilic acid that is a fungal metabolite (**Figure 5**).

In contrast to natural mediators, synthetic mediators are those that are chemically synthesized. They include for example 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazole (HBT or HOBt), and (2,2,6,6-tetramethylpiperidin-1-yl)oxyl, (TEMPO) (**Figure 6**).

The mediator is first oxidized by laccase and then, there are two main mechanisms for the mediators to oxidize different compounds: electron transfer (ET) and hydrogen atom transfer

(HAT) (**Figure 7**) [37]. For example, HBT, once oxidised by the enzyme, can oxidise the substrate via a radical hydrogen atom transfer mechanism. This mechanism seems to be applicable to all the laccase mediators with a N-OH group that is oxidized into a nitroxyl radical capable of tearing an H atom from the substrate. HPI (N-hydroxyphtalimide), VLA (violuric acid) and NHA (N-hydroxyacetanilide) are also N-OH type compounds reacting using HAT mechanism. ABTS on the contrary, reacts with the substrate via an electron transfer mechanism [38].

3. Evaluation of laccase activity: a wide variety of methods

3.1. The spectrophotometric assay

The most used method to determine enzymes activity is probably the spectrophotometric assay. If this method is so widespread among biologists it is because of its low cost, rapidity, robustness, reproducibility, and ease to implement. This technique can be used when the product of an enzymatic reaction is an absorbing species that produces a measurable change in absorbance compared to the reaction substrate. If there isn't any contaminants in the sample, the amount of analyte in solution is then measured by simply taking absorbance measurements at the wavelength of interest using a spectrophotometer [39]. The wavelength range of a UV-Vis spectrophotometer is usually between 190-900 nm. If the reaction is followed in the visible range (400-800 nm), this assay is called a colorimetric assay and the change in colour can be seen by naked eye.

With the low concentrations of chromophores used in most biochemical experiments and if there are no complications linked to the reaction product (such as polymerization), absorbance is directly proportional to concentration and path-length, making it possible to simply use the Beer-Lambert law (**Equation 1**) to trace the product concentration [40].

$$A = \log\left(\frac{I_0}{I}\right) = \varepsilon\, l\, C$$ ***Equation 1***

where A is the absorbance, I_0 and I are the incident and transmitted light intensities, respectively, C is the concentration of the solution ($mol.L^{-1}$), ε is the molar extinction coefficient ($L.mol^{-1}.cm^{-1}$) and l is the length of the path cell (usually, but not always 1 cm).

As for a majority of enzyme assays, laccases activity can be determined by a spectrophotometric assay. Classical assay method for laccase involves ABTS, an electron-rich but non phenolic substrate [41]. Under laccase action, ABTS undergoes a one-electron oxidation to give the stable radical cation $ABTS^{+\bullet}$ (**Figure 8**), which absorption can be monitored at λ_{420nm}. The $ABTS^{+\bullet}$ radical cation then undergoes a slow oxidation into $ABTS^{2+}$ dication [42].

Other compounds can be used to test laccase activity such as syringaldazine [43], guaiacol [44], 2,6-dimethoxyphenol [45], o-tolidine [46], p-cresol, diaminobenzidine, dopa (3,4-dihydroxyphenylalanine), p-phenylenediamine, toluquinol [47] or 3-hydroxyantranilic acid (HAA) [48].

A variant of this assay method, allowing a direct spectrophotometric assay of laccase has been developed by Moshtaghioun *et al* [49]. For all the previous substrates cited, an increase of the absorbance is observed at their λ_{max} while being oxidised by the laccase, the absorption of the product being different from the substrate spectrum. Thus, the enzyme activity isn't measured directly by the depletion of its substrate but by the appearance of the product. This raises a problem: indeed, most of laccase oxidation products are radicals that can react with themselves, a molecule of substrate or even another existing species in the solution. All these phenomena change the absorbance and therefore the accuracy and precision of enzymatic activity measurements. Moshtaghioun *et al* worked on diazo derivatives of guaiacol and showed that they can be used as substrate for a direct spectrophotometric study of laccases by following the decrease in absorbance during laccase oxidation.

Although widely used for daily analysis [50–52] and with many advantages, the spectrophotometric assay also has some major disadvantages. Indeed, it is hard to find a relevant substrate for the determination of laccase activity by spectrophotometry. For example, the reaction product of syringaldazine with laccase is a quinone, insoluble in aqueous solution over time, and therefore complicated to manipulate for the laccase assay [53]. The reaction product of ABTS with laccase is a radical cation that can react with many other molecules in complex media, and which may therefore lead to an underestimation of laccase activity as it was demonstrated in a medium with tannic acid by Terrón *et al* [54]. Finally, spectrophotometric assays can't avoid the interference from turbidity and UV-vis light-absorbing substances [55]. That is why this review will introduce some other methods that may be relevant in other conditions.

3.2. Laccase assay using Fourier-transform infrared spectroscopy (FTIR)

FTIR is a technique used for measuring the infrared (IR) absorption and emission spectra of samples. IR region covers wavelengths from 780 nm to 1 mm. Infrared light is absorbed at specific frequencies directly related to the vibrational bonding energies of atom-to-atom within the molecule. When the vibrational bond energy and the average infrared light energy are equivalent, the bond can absorb this energy. Different bonds vibrate at different energies in a molecule and so they absorb different wavelengths of IR radiation. The frequency and intensity of these absorption bands contribute to the overall spectrum, creating a characteristic fingerprint of the molecule [56].

An FTIR spectrometer works by the principle of transmission with an interferometer. This interferometer is composed of an IR source that emits radiations. These radiations strike a beam splitter that splits the radiation into two beams. One beam passes towards the fixed mirror while the other travels towards the mobile mirror (a mirror moving with constant velocity). These two

beams are then reflected and, after recombination, the beam is transmitted through the sample and finally, reach the detector [57].

The FTIR method can be used to follow spectral changes of substrates and products during an enzymatic reaction and therefore, when combined with multivariate data analysis, evaluate the enzyme activity. This is what Perna *et al* [58] did to measure laccase activity using FTIR spectral fingerprinting. They used the fact that substrate and products have different spectral fingerprints in the mid infrared region and these spectral changes can be followed in real time during an enzymatic reaction. By using Parallel Factor Analysis (PARAFAC), the authors have been able to prove that this method can be used to quantitively assess laccase activity. This method is not only applicable to laccase, but to a wide range of enzymes [59].

One of the main disadvantages of the FTIR technique is the difficulties encountered when samples contain free water. Indeed, water has a very strong FTIR signal with OH stretching or bending bands that can mask the real bands of the analysed sample. As enzyme activity measurements are almost always carried out in aqueous solution, Perna *et al* [58] have conducted their FTIR experiments with deuterium oxide as solvent for both buffer and enzyme solutions to get rid of the water problem. Samples can nevertheless be measured using water as a solvent, if the bands of interest do not overlap with the water peak, or if the area of interest is in the fingerprint part of the infrared spectrum.

A variant of the technique, ATR (Attenuated Total Reflectance)-FTIR have also been used by Juárez-Hernández *et al* [60] to follow the decolourization process of a synthetic dye, Remazol Blue, thanks to laccase action. ATR is a technique which is fast, easy to use and non-destructive, particularly suitable for routine analysis of solids or liquids and therefore perfectly suited for enzyme activity measurements. ATR-FTIR allows Juárez-Hernández *et al* [60] to follow the activity of laccase on Remazol blue dye during an entire week thanks to an analyse by partial

least squares method of the variation of intensity in some main bands identified for this molecule such as C-N and S=O vibrations.

In ATR, infrared radiation passes through a crystal made in a material with a high refractive index (diamond, ZnSe or germanium), which is brought into direct contact with the sample, penetrating the first few microns of the solid or liquid. It is then reflected to a detector and computer-processed to obtain the infrared spectrum [57]. ATR crystals are chosen depending on several parameters: spectral range, refractive index, crystal geometry, hardness index, chemical compatibility (pH), pressure and temperature range, price [61]. Indeed, ATR crystals have different spectral ranges which must be carefully selected according to the sample to be analysed. Moreover, the crystal must have a higher refractive index than the sample and of course shouldn't react with it. One major advantage of using the ATR-FTIR technique rather than the FTIR classical one is that it can attenuate the effect of water in biological samples analyses. Indeed, ATR immersion and flow cells have short optical paths which enables species with high IR absorption capacities to be easily monitored, when in FTIR, these species would cause signal saturation.

3.3. Fluorimetric method for laccase activity determination

Fluorimetry is a measurement of fluorescence intensity of molecules. Fluorescence is caused by photons that can excite a molecule at specific wavelengths, raising the molecule to an electronic excited state. This excited state is highly unstable and is quickly followed by a relaxation phenomenon where electrons from excited singlet go down to singlet ground state (**Figure 9**). By doing so, the molecule emits a photon of lower energy, at a longer wavelength than the absorbed photon [62]. This technique has long been used in biology for DNA and RNA quantification [63,64], protein-ligand interaction study [65] and fluoroimmunoassays [66].

In 1997, Lonergan *et al* [67] have developed a novel fluorescence-based method for the detection and estimation of laccase activity in fungal cultures using homovanillic acid (HVA) as substrate. The authors expressed laccase activity against HVA with H_2O_2 equivalents, using the fluorescence of standard curves of H_2O_2 and compared their results with the activity obtained by a colorimetric assay with ABTS. They have thus proved that HVA is a suitable fluorometric substrate for laccase, especially in fixed time assays and allows direct observation of rate of change of fluorescence.

More recently, T. Wang *et al* [68] have also created a fluorescence-based method to evaluate laccase activity, but this time using Amplex Red (AR: 10-acety-3,7-dihydroxyphenoxazine), a colourless, non-fluorescent derivative of resorufin (RSF) as substrate for the laccase. After enzymatic reaction, Amplex Red turned into a highly fluorescent derivative and the fluorescence intensity can be correlated with laccase activity.

Arabi *et al* [69] have also chosen to develop a fluorescent assay to measure laccase activity. Their strategy is to conceive a dopamine-functionalized graphitic carbon nitride (g-C_3N_4) sensor. The dopamine part of the sensor can be oxidised in dopaquinone in presence of laccase and a photoinduced electron transfer (PET) phenomenon [70] (**Figure 10**) happens between the dopamine and the g-C_3N_4. The dopaquinone then becomes an electron acceptor and the g-C_3N_4 an electron donor which results in quenching the fluorescence spectrum of the g-C_3N_4-Dopa sensor. The signal reduction in fluorescence allows a quantitative evaluation of laccase activity with the fluorescence intensity cause by laccase measured at λ_{466nm}. Their study has led to the development of a wide-range, highly sensitive detector with a limit of detection of about $2U.L^{-1}$ and a laccase activity range detection of $5\text{-}400U.L^{-1}$.

Fluorescence spectroscopy for the study of enzymes activity has a bright future ahead of it. The advantages of such a method are the high sensitivity and specificity, allowing the detection of small quantities of products, a wide linear range, a good repeatability, and a minimum sample

preparation effort [71]. Moreover, it requires only a simple routine equipment available in most laboratories. However, the main disadvantage it that it requires to find a fluorescence compound among the enzyme substrates, or to design one through organic synthesis with all its limitations: yield, time and reagents needed.

3.4.The calorimetric method

Calorimetry is the process of measuring heat changes (released or absorbed) during a chemical reaction or a physical phenomenon. Those changes are measured by a calorimeter that consists of a container with walls kept at constant temperature and in which the thermal phenomenon is investigated. A variant of this method is microcalorimetry, where very sensitive devices are used to measure very small heat changes in small samples [72].

T. Z. Wang *et al* [73] were able, thanks to microcalorimetry, to determine laccase activity and kinetics parameters. Their methodology consists of a first step of determination of the molar reaction enthalpies for laccase-catalysed oxidation of different substrates. They then used **Equation 2** to evaluate the enzyme activity:

$$EA = \frac{L_m}{Ea} = -L_m \times \Delta_r H_m \times \Omega_{max}^{-1} \qquad \textit{Equation 2}$$

Where L_m is the mass of laccase (g), $\Delta_r H_m$ is the overall molar enthalpy of the reaction ($J.mol^{-1}$), Ω_{max} is the maximum exothermic rate ($J.s^{-1}$), EA is the amount of enzyme (g) which converts one mol of substrate per second, Ea (enzyme activity) is the number of moles per second per g of enzyme. The minus sign resents an exothermic reaction. According to the authors, this method has the advantages of being applicable not only to purified enzyme but also to heterogenous system like partially purified enzyme or tissue samples.

Islam *et al* [74] used isothermal titration calorimetric (ITC) method as a tool for evaluating lignin conversion by a fungal (*Trametes versicolor)* laccase. Indeed, many of the conventional

assay techniques for laccase activity are not suitable when working with more complex substrates such as lignin. Thanks to ITC, enzymatic reaction on complex substrates can be followed by using the variation of the molar enthalpy ΔH of the catalysed reaction. The total heat generated or consumed during laccase reaction is proportional to the total conversion that takes place during the enzymatic reaction and its molar enthalpy variation. The pH and the temperature are carefully controlled during measurements. To evaluate the temperature difference that may occur under laccase action, the temperature is measured continuously in the sample and in the reference cell. This new approach allows the author to determine kinetics parameters of the laccase on lignin and is then a powerful tool to investigate conversion rate of complex substrates by laccases.

Volkova *et al* [75] also investigated the oxidation of syringic acid by laccase using isothermal titration calorimetry and compared the kinetics parameters of two laccases of different origin. This method is applicable not only to laccase but also to a large range of enzymes [76–79].

Calorimetry is therefore a universal technique for enzymatic assays, offering real-time monitoring of the reaction, without post-reaction separation needed, and more importantly, the possibility to work with opaque samples of insoluble substrates that could not, or with great difficulty, be analysed by spectroscopy [78]. A disadvantage does exist, however, is that a large amount of sample is often needed, which can be problematic while working with enzymes as they are not always available in large quantities.

3.5. Electrochemical evaluation of laccase activity

As discussed in the 3.1 sub-section, colorimetric technique is the most used to determine laccase activity. What is interesting though, is that the most used laccase substrates, ABTS and syringaldazine, are also electrochemical active compounds. Constant potential amperometry and cyclic voltammetry can then be used to evaluate the enzyme activity.

Cyclic voltammetry is an electrochemical technique (potential sweep method) used to investigate reduction and oxidation processes of redox species and reactions near the electrode surface [80,81]. To do so, a three-electrodes set up is needed. The working electrode is made of redox-inert material and carries out the electrochemical reaction. A potentiostat is used to control the applied potential of the working electrode. The reference electrode is an electrode with a well-defined and stable equilibrium potential. It is against this reference electrode that the potential of other electrodes is going to be measured in an electrochemical cell. Finally, a counter electrode, that completes the electrical circuit, is needed [82]. Thanks to this experimental set-up, a voltage sweep with imposed sweep speed is conducted and the response is measured in current intensity. The results are then represented in the form of a voltammogram [83].

Amperometry is another electrochemical technique that consists of applying a constant reducing or oxidizing potential to a working electrode and to measure the resulting steady-state current. The resulting current can be linked to the concentration of electroactive species by the following equation, known as the Cottrell equation (**Equation 3**):

$$I = nFAC_b\sqrt{\frac{D}{\pi t}} \qquad \textit{Equation 3}$$

Where I is the diffusion current in A, t is the electrolysis time in second, n is the number of electrons involved in the reaction, F is the Faraday constant (96 485 $C.mol^{-1}$), A is the electrode area (m^2), D is the diffusion coefficient of the electroactive species ($m^2.s^{-1}$) and C_b is the concentration of the electroactive species ($mol.L^{-1}$). Cottrell's equation is established in the case of a reversible redox reaction (i.e. a reaction whose kinetics is very fast and whose current is controlled by diffusion), assuming semi-infinite linear diffusion perpendicular to the electrode surface.

Amperometry is therefore a simple technique based on the relationship between transient current and concentration for the quantification of redox compounds, in diffusion controlled redox measurements [84].

These two techniques have been used by Gáspár *et al* [85] to evaluate laccase activity in must. The authors used carbon fiber microelectrodes for the analyse of $ABTS^{+\bullet}$ by cyclic voltammetry and screen-printed gold electrodes for constant potential amperometry. The approach based on cyclic voltammetry allowed the authors to detect laccase activity, but they faced a major problem of underestimation of this activity, compared to a spectrophotometric assay using syringaldazine. The same problem was encountered with the constant potential amperometry technique. This underestimation was attributed by the authors to other phenolic compounds, resulting from sample pre-treatment and capable of being transformed by laccase but not detected by electrochemical method. An adsorption phenomenon of must components onto the carbon fiber microelectrodes could also explain this underestimation. The authors plan to develop gold microelectrodes in the near future to overcome this problem.

Voltammetry was also used by Klis *et al* [86] to evaluate the catalytic activity of laccase for two substrates : ABTS and hydroquinone. They compared their techniques with spectrophotometry and found a good agreement between them. Among the advantages, voltametric assay is an accurate, reproducible, and simple method that allows the analysis of a larger number of substrates than spectrophotometry, including compounds that do not absorb in the visible range. Moreover, it allows to follow both the consumption of substrate and the formation of product at the same time.

In the same way, Lulea *et al* [87] created a paper-assisted electrochemical assay for laccase activity in grapes using an optical method coupled with an electrochemical one. The current produced by the reduction of oxidized ABTS at 0V is quantitatively correlated with laccase

activity. Their method allowed them to determine enzymatic activity down to 1 $U.mL^{-1}$ in a repeatable, robust, and simple way.

As another example, one can mention the amperometric measurements conducted by J-X. Liu *et al* [55] to detect laccase activities in compost. They developed a sensor based on glassy carbon electrode modified with multi-wall carbon nanotubes as working electrode, a saturated calomel electrode as reference one and a Pt auxiliary electrode. This system allowed them to find a good correlation between the current and the laccase activities. Previous works [88] also reported a determination of laccase activity by oxygen consumption rate measures, using an amperometric oxygen electrode. They observed a decrease in the potential of the sample due to the laccase action on quinol and were able to prove that this decrease is proportional to laccase activity. The main advantages of these electrochemical techniques are the fact that, unlike optical devices, electrodes are easy to miniaturize, allowing in-fields measurements.

Polarography is another type of voltammetry in which redox active molecules undergo oxidation or reduction at the surface of a dropping mercury electrode at an applied potential [89]. Polarography has been used in past research to investigate laccase activity by following oxygen consumption of the enzyme reaction [90–92].

Direct electrochemistry is a powerful tool for understanding enzymes, and how they react and interact with their substrates. Thus, this technique must not be reduced solely to studies of reactional kinetics and enzymatic activity.

3.6. Electron paramagnetic resonance spectroscopy for laccase activity investigations

Electron paramagnetic resonance (EPR) or electron spin resonance (ESR) spectroscopy is a very sensitive technique for the detection of unpaired electrons, that can move between their two spins states if an external magnetic field is applied. It also allows the characterization of their chemical environment [93,94]. This method is based on the correlation that exists between

magnetic field and unpaired electrons. This correlation is described by the g-value that is unique for a given paramagnetic species [95]. It has been used by Munk *et al* [96] for quantitative real time measurements of radical formation induced by laccase on three different lignins. They observed an immediate time dependent increase in the isotropic signal intensity after laccase treatment and an accumulation of radicals linked to laccase dose in their lignin suspensions. They extracted kinetics parameters of laccase on lignin thanks to this technique and were also able to show that laccases acted on insoluble lignins in suspension. The same kind of experiments were conducted by Perna, Agger *et al* [95] who also used EPR to determine kinetics parameters of laccase on lignins. Thanks to EPR, they were able to directly assess lignin oxidation by laccase by following radical formation. It is interesting as it allows direct monitoring of the first product of the enzymatic reaction, and therefore the "true" action of the enzyme, avoiding quantifying recombinations and secondary reactions that take place at a later stage.

EPR technique is particularly well-suited for the study and quantification of complex macromolecules. It is also a very sensitive technique, making it an ideal technique for measuring laccase activity on substrates other than model substrates. Indeed, EPR detects only the paramagnetic material and is blind to the many paired electrons of a molecule and will therefore not be subject to interference, even in optically opaque and turbid solutions [97,98]. It would thus appear that EPR is a very powerful and specific technique for measuring laccase activity. Nevertheless, there are some major drawbacks to its development, the main one being its low resolution compared with other techniques such as NMR. A larger number of paramagnetic materials also require very low temperatures (as low as 20 K) to be detected which is an experimental constraint, but above all, a significant cost for labs and industries.

3.7. Laccase activity and kinetics quantification using chromatography

Chromatography is not commonly used for enzyme activity tests. However, some authors have looked into the possibility of determining laccase activity using it.

Badiani *et al* [99] have conducted laccase assay on guaiacol using high performance liquid chromatography (HPLC). They compared their results with a classic spectrophotometric assay. They used a RP-18 Hibar LiChrosorb RP- 18 (4 mm x 25 cm) column and a UV detector set at 275 nm. The elution was performed isocratically using 18 mM ammonium acetate in butanol/methanol/acetic acid/water (2/5/12.5/83, v/v) at a flow rate of 1 $mL.min^{-1}$ at room temperature. These conditions allowed them to measure laccase activity following the number of micromoles of guaiacol metabolized per minute.

Their HPLC method found higher kinetics parameters and activity for laccase than the spectrophotometric method, making them think that the spectrophotometric method is not so reliable because side reactions resulting from the primary oxidation of guaiacol can form molecules whose absorbance will affect the absorbance at 465 nm and thus distort the results obtained spectrophotometrically.

Another study was conducted by Anders *et al* [100] to investigate the laccase-mediator system using high performance anion exchange chromatography with a pulsed amperometric detection (HPAEC-PAD). This experimental set up allowed to quantify the LMS activity of a laccase from *Trametes versicolor* associated with the mediator HBT. They chose adlerol as substrate and determine an activity of 0.47 $U.mg^{-1}$ of protein for a laccase ratio of 1.75 U $laccase.g^{-1}$ HBT. This method has been automated to become a tool for the screening and quantification of mediators and lignin-related substrates that cannot be assayed by spectrophotometric ways.

Chromatography is a promising technique for the monitoring and further understanding of enzyme-catalyzed reactions and to determine catalytic properties of the enzymes. In the case of laccases, this technique can broaden the range of substrates that can be studied and those for industrial uses. However, one of the limitations that can be encountered when using

chromatography to measure enzymatic activity is the low solubility of the products of enzymatic reaction. For example, Kraft lignin is poorly soluble in water and in most solvents, making it very difficult to analyse by liquid chromatography. Among the few solvents possible for the dissolution of Kraft lignin, dimethyl sulfoxide (DMSO) [101] or tetrahydrofuran (THF) can be used. Sometimes a filtration step is also needed before injection into the column to remove insoluble part [102]. Finally derivatization with acetobromination or acetylation of lignin samples can be done to reach complete dissolution and prevent polar associative interactions within lignin [103]. Still, using chromatography for complex substrates remain challenging. Finally, the cost of such analyses would be considerable if they were to be carried out on a routine basis due to the cost and sensitivity of chromatographic equipment.

4. Practical applications of laccase activity determination methods: from lab scale to industrial applications

4.1. Industrial applications of laccases: an untapped potential

Laccases have a great potential in various industrial areas because of their wide range of substrates which includes both phenolic and non-phenolic compounds [104]. However, even though some laccases are commercially available, the industrial-scale use of laccase is slowed down by the high cost of the enzyme, the lack of sufficient enzyme stocks and the toxicity of some of the laccase mediators [105]. Research to increase the amount of laccase produced is therefore essential.

However, few laccases are already commercialised for industrial uses. For example, Novozymes Company (Denmark) has commercialized different preparations with laccases: suberase for cork modification, Novozym 51003 for paper pulp delignification and DeniLite for cold denim bleaching. Amano Enzyme (USA) sells a product named Laccase Y120 (LC-Y120) that aims to improve the quality of plant-based meats. Lignozym GmbH (Germany)

developed Lignozym-process for lignocellulosic material decomposition. As a final example, the company Genencor Inc. (USA) launches Indistar Coloradjust System for denim clean-up and bleaching [106].

Even if the production in native fungal strains reached the gram per liter at lab scale [107], laccase production from non-recombinant sources is not feasible for industrial-scale production due to yield problems. Heterologous production of laccase in recombinant system could play a key role in the future to initiate a green transition with the use of enzymes in the industrial world [108]. Heterologous production of laccases have been done so far in filamentous fungi, in Ascomycota [109] and Basidiomycota [110], bacteria [111], yeasts [112] and plants [113]. In future therefore, it will probably be possible to select a host according to the industrial application targeted for the laccase, and thus to take into account factors other than yield alone.

4.2. A green tool for the pulp and paper industry

Among the possible industrial applications for laccases, those in the pulp and paper industry seem to make sense. Indeed, pulp and paper industry is among the biggest industry of the world, with a huge economic and ecological impact. Pulp and paper mills still mostly rely on fossil fuels, with an energy consumption accounting for 6% of total industrial worldwide energy consumption in 2017 and emit a lot of fossil carbon dioxide [114]. The raw material of this industry is wood that contains lignocellulose, and one of the main challenges is pulping. Pulping is a step of paper production when cellulose fibers are separated and cleaned from the rest of the wood component. This can be performed either by mechanical or chemical mean. Mechanical pulping consists of grinding lignocellulosic fibres with an abrasive material without extracting lignin. As a result, the paper produced by this method often turns yellow and becomes brittle over the time. Chemical pulping involves different processes like alkaline (Kraft) or sulfite ones. These methods all involve solubilizing lignin under heat, in concentrated solutions

of acid or base [115]. Another step of bleaching is then needed to remove the residual lignin from the cellulose fibers and thus get rid of the brown colour. Here again, a chemical treatment is often used, involving large quantities of reagents including chlorine, chlorine dioxide, and sodium hydroxide. Moreover this processes produce highly persistent and bioaccumulative organochlorines, known to be endocrine disruptors [116].

Laccases could be of great help in pulping process as fungal laccases are implied in wood degradation. Using laccases for paper pulp delignification would save energy, eliminate the need for most chemical reagents and of course improve the quality of bleach pulp [117].

Literature lists many laccases that can be used in bleaching without chlorine compounds. For example, Sigoillot *et al* [118] studied the treatment of wheat straw Kraft pulp using recombinant and native laccases from *Pycnoporus cinnabarinus*. They associated this laccase with a redox mediator (HBT) to reach a delignification up to 75%.

Bourbonnais *et al* [119] associated a laccase from *Trametes versicolor* with two mediators (ABTS and HBT) in order to perform Kraft pulp delignification. They also tested other crude laccases from different white-rot fungi and deduced that none of them could efficiently delignified kraft pulp without mediator. Many other studies point in the same direction [120–123]. Indeed, laccases have, most of the time, a high molecular weight that prevents them for penetrating the pulp fibers. Moreover, their low-redox potential makes them unable to oxidize others groups than phenolic groups and thus without mediator, laccases can oxidize less than 20% of lignin on the pulp surface. Some other intrinsic factors of laccases still need to be improve to really consider using laccases for industrial biobleaching as for instance thermostability of the enzyme, optimum pH of laccases, grafting effects [124].

4.3. A phenol eliminator for the food industry

Thanks to its wide range of substrates, laccases also fit perfectly in the food industry. Some applications have already been imagined such as in beverage processing where they can prevent the change of colour and taste of juices by degrading the phenolic compounds responsible of the darkening of these products [125]. Wine stabilisation can also imply laccase to selectively remove the polyphenols. Wines are complex mixtures made of different chemical components and laccases can target specially polyphenols and thus preserve the organoleptic characteristics of wines [126]. In the same way, laccases also have a bright future in beer stabilisation for removing the unwanted oxygen in the finished product and increase the storage life of beer. Laccases are shown to prevent the formation of off-flavour compounds by removing oxygens and thereby prevent its reaction with fatty acids, amino acids, proteins and alcohol in beers [106].

Some applications can be considered for laccases in the baking industry [127] and a patent has been filed, specifying that the use of laccase allows an increase in the volume and an improvement if the crumb structure coupled with an increase in the softness of the baked product. The enzyme also promotes strength, stability and less stickiness of the dough for easier machinability [128].

It might also be possible to use laccases for agro-food wastes valorization, using them as a culture substrate for laccase-producing fungi. Some fungal cultures have already been performed using oil palm parenchyma tissue, sunflower seed hulls, rice residue, barley bran, chestnut shell waste, potato pulp, banana skins, mandarin peels, kiwifruit wastes, and grape seeds [125]. This alternative way of reusing food waste is a good example of circular economy, which involves reducing waste to a minimum. When a product reaches the end of its life, the resources it contains are kept in the economic cycle through recycling. They can then be used again to recreate value. Laccases fit perfectly into this system of “reduce-reuse-recycle” by being able to recycle and valorise food waste.

4.4. A great potential in the textile industry

Laccase is attracting growing interest in the textile industry because it can be used in two main ways: to optimize the bleaching of textile by dye decolourization and to treat wastewater and remove dye in effluents.

For this last application, it is important to know that textile wastewaters are heavily loaded with persistent by-products that can pollute the environment, and eventually food chain, representing both an ecological and a human health problem. These by-products are mainly irritants, toxins and hazardous chemicals and it is estimated that nearly 10-15% of dyes end up into wastewater after manufacturing process [129]. Many studies have proven that laccases could be good candidates for a cost-effective and eco-friendly way of treated the textile industry wastewaters. Motamedi *et al* [130] produced a recombinant thermostable and halotolerant laccase for decolorization and detoxification applications. Yadav *et al* [131] recently evaluated the biodegradation power of chemical synthetic dyes by a laccase from *Arthrographis kalrae*. They obtained an efficient decolourisation without using any mediator and the enzymatically treated dye solution was proven to be less phyto- and cytotoxic. This is also possible with immobilized laccases as done by Gao *et al* [132]. In their work, they immobilized the enzyme in vault nanoparticles and find that the decolorization and detoxification was improved compared to free laccase. This better performance may be explained by the microenvironment of the enzyme, with a higher concentration of dyes in the interior of vaults and a protection for the laccase against inhibiting compounds generated during the reaction.

4.5. A promising start in the pharmaceutical industry

As the synthesis of pharmaceutical molecules becomes increasingly complex, the use of biological catalysts appears to be a simple and eco-friendly alternative for certain chemical synthesis steps. Among enzymes, the oxidative activity of laccases is of particular interest for

the synthesis of organic molecules, especially in the pharmaceutical field. Laccases offer the advantage of being quite stable, easy to handle and able to work both in aqueous and organic media. Actinocin for example, was synthesized using laccase from 4-methyl-3-hydroxyanthranilic acid, and was identified as an anticancer agent that blocks transcription of tumor cell DNA [133]. Other anticancer drugs could be synthetized using laccase. Vinblastine which is use in leukaemia cases is a natural product extracted from plants but too small in quantities for medical use. As the precursors of this molecule, katarantine and vindoline are produced at higher concentrations in the plant, a hemi-synthesis strategy involving oxidative coupling by laccases could be considered to obtain the product in sufficient quantities [134]. Many other compounds of interest could be synthesized or modified by laccases such as antibiotics or alkaloids. However, there are still a number of challenges to be met before laccases can be widely used in the pharmaceutical industry like a strict stereospecificity as therapeutic drugs need to be enantiomerically pure [135].

4.6. Laccases biosensors: a green technique with many applications

A biosensor is an analytical device which uses cells or biological molecules to detect the presence of a specific chemical. Usually, a biosensor is made of two elements: a molecular recognition element (cells, enzymes, antibodies, nucleic acids), and a physico-chemical detector component or transducer, that can be electrochemical, optical, mass-based or piezoelectric [136]. Since the first enzymatic biosensor using glucose oxidase was created by Clark and Lyons in 1956, the market have grown considerably [137]. Indeed, enzymes have the great advantages of being highly specific for their substrate and having a high turnover rate, two essential requirements for a biosensor [136]. One of the key points of enzymatic biosensors is the enzyme immobilization to create a reusable, and more stable system than a free enzyme one.

Laccases have proved themselves to be interesting enzymes for biosensor applications, the most promising one being for phenol detection. Many researchers are currently working on this issue. Albayati *et al* [138] have, for example, developed a biosensor with a covalent immobilization of laccase on a glassy carbon electrode modified by conducting polymer, gold nanoparticles and carboxylated multiwalled carbon nanotubes. This biosensor function was to detect catechol, a model phenolic compound and happened to do so with a low detection limit and a high sensitivity. Another team [139] investigated the use of laccase for the detection and quantification of phenolic secondary metabolites in two *in vitro* cultivated plants. Their immobilization technique was based on the entrapment of the enzyme into a nanocomposite film during the electrodeposition of a multiwalled carbon nanotube-chitosan solution. The resulting biosensor had interesting properties with low detection limit, stability and reproducibility making it a future great tool for in field measurement. A different immobilization strategy was chosen by Shimomura *et al* [140] who immobilized their laccase on a mesoporous silica powder coated onto a glassy carbon electrode. This device allowed them to perform an amperometric detection of catechol with a linear response for substrate concentrations between 2.0-100 µM. Other researchers have chosen to use polydopamine-laccase-nickel nanoparticles loaded carbon nanofibers [141] or reduced graphene oxide supported palladium-copper alloyed nanocages for the laccase immobilization [142]. Some applications for wastewater detection of pollutants can be considered for those biosensors.

In the medical field, laccase biosensors have also been considered. For example, Bragazzi *et al* [143] tested it to do therapeutic drug monitoring, to tracking psychiatric drug level in blood of patients. In their study, they followed the concentration of clomipramine in blood sample using a Langmuir-Blodgett thin film of recombinant laccase from *Rigidoporus lignosus* and with a potentiometric and amperometric detection.

Others potential industrial applications for laccases are under development: biocatalysis, bioremediation, use in detergent formulation, functionalization and grafting of polymers and the synthesis of dyes for paints and coatings [105].

5. Conclusion

With green chemistry on the rise, the industrial use of enzymes is likely to become more widespread in the decades to come. Much research remains to be done, and a few locks to be lifted, before laccase is used to its full potential in industry. First, there is a need to reduce the costs production and purification of the enzyme by optimizing fermentation processes and producing organisms. It would be also useful to get thermotolerant and “pH-tolerant” laccases to extend the scope of application of laccases, and this can be done by investigating into biodiversity or enzyme engineering. The development of cheaper and less toxic mediators is also capital as most of the applications for laccase imply the use of these coumpounds. Finally, good encapsulation methods will also be needed to use and reuse laccases for industrial applications [144]. Other cutting-edge biotechnology and bioengineering research on laccases are underway to extend their applications and properties. Novel approach such a chimeragenesis, that consists of forming chimeras between proteins that are encoded by homologous cDNAs are developed for laccases, to create thermostable enzymes for example [145]. Enzyme reconstruction and resurrection, which is the action of recreating ancestral enzyme using phylogenetic analysis and ancestral interference algorithms [146], is also being studied for laccases [147]. Finally, KnowVolution, knowledge gaining directed evolution, is another strategy to consider, in the aim of producing enzymes for industrial uses, for example at alkaline pH [148]. All these studies are still very recent and will, with no doubt, deciding the industrial future of laccases.

It will then be vital to be able to reliably quantify laccase activity on complex substrates. Indeed, industrial applications involve substrates far more complex than those conventionally measured with spectrophotometry including lignin, effluents, textile dyes... It is also necessary to deal with more complex matrices and mixtures and thus, different analytical methods must be developed. This review listed and described several methods that could help solve this problem, like FTIR, fluorimetry, calorimetry, electrochemistry, or electron paramagnetic resonance, but an important work of optimization must be carried out in the coming years before considering these methods as routine analyses.

References

[1] Morsi R, Bilal M, Iqbal HMN, Ashraf SS. Laccases and peroxidases: The smart, greener and futuristic biocatalytic tools to mitigate recalcitrant emerging pollutants. *Science of The Total Environment* 2020;714:136572. https://doi.org/10.1016/j.scitotenv.2020.136572.

[2] PATENSCOPE - World Intellectual Property Organization (WIPO) https://www.wipo.int/patentscope/fr/ (accessed August 21, 2023).

[3] Digital Science & Research Solutions Inc. Dimensions https://www.dimensions.ai/ (accessed August 21, 2023).

[4] Yoshida H. LXIII.—Chemistry of lacquer (Urushi). Part I. Communication from the Chemical Society of Tokio. *J Chem Soc*, Trans 1883;43:472–86. https://doi.org/10.1039/CT8834300472.

[5] Arregui L, Ayala M, Gómez-Gil X, Gutiérrez-Soto G, Hernández-Luna CE, Herrera De Los Santos M, et al. Laccases: structure, function, and potential application in water bioremediation. *Microb Cell Fact* 2019;18:200. https://doi.org/10.1186/s12934-019-1248-0.

[6] Solomon EI, Sundaram UM, Machonkin TE. Multicopper Oxidases and Oxygenases. *Chem Rev* 1996;96:2563–606. https://doi.org/10.1021/cr950046o.

[7] Mehra R, Muschiol J, Meyer AS, Kepp KP. A structural-chemical explanation of fungal laccase activity. *Sci Rep* 2018;8:17285. https://doi.org/10.1038/s41598-018-35633-8.

[8] Ralph J, Lapierre C, Boerjan W. Lignin structure and its engineering. *Current Opinion in Biotechnology* 2019;56:240–9. https://doi.org/10.1016/j.copbio.2019.02.019.

[9] Katahira R, Elder TJ, Beckham GT. Chapter 1. A Brief Introduction to Lignin Structure. In: Beckham GT, editor. Energy and Environment Series, Cambridge: Royal Society of Chemistry; 2018, p. 1–20. https://doi.org/10.1039/9781788010351-00001.

[10] Morozova OV, Shumakovich GP, Shleev SV, Yaropolov YaI. Laccase-mediator systems and their applications: A review. *Appl Biochem Microbiol* 2007;43:523–35. https://doi.org/10.1134/S0003683807050055.

[11] Laccase Market Size, Share, Growth, and Industrial Analysis, by Type (Fungi Source, Plants Source), By Application (Textile Industry, Food Industry, Pharmaceutical Industry, and Others) Regional Forecast To 2028. Business Research Insight; 2022. https://www.businessresearchinsights.com/market-reports/laccase-market-101221 (accessed August 21, 2023)

[12] Wang J, Feng J, Jia W, Chang S, Li S, Li Y. Lignin engineering through laccase modification: a promising field for energy plant improvement. *Biotechnol Biofuels* 2015;8:145. https://doi.org/10.1186/s13068-015-0331-y.

[13] Liang M, Davis E, Gardner D, Cai X, Wu Y. Involvement of AtLAC15 in lignin synthesis in seeds and in root elongation of Arabidopsis. *Planta* 2006;224:1185–96. https://doi.org/10.1007/s00425-006-0300-6.

[14] De Marco A, Roubelakis-Angelakis KA. Laccase activity could contribute to cell-wall reconstitution in regenerating protoplasts. *Phytochemistry* 1997;46:421–5. https://doi.org/10.1016/S0031-9422(97)00301-4.

[15] Zhang Y, Wu L, Wang X, Chen B, Zhao J, Cui J, et al. The cotton laccase gene *GhLAC15* enhances Verticillium wilt resistance via an increase in defence-induced lignification and lignin components in the cell walls of plants: GhLAC15 enhanced Verticillium wilt resistance. *Molecular Plant Pathology* 2019;20:309–22. https://doi.org/10.1111/mpp.12755.

[16] Janusz G, Pawlik A, Świderska-Burek U, Polak J, Sulej J, Jarosz-Wilkołazka A, et al. Laccase Properties, Physiological Functions, and Evolution. *IJMS* 2020;21:966. https://doi.org/10.3390/ijms21030966.

[17] Chakraborty TK, Das N, Sengupta S, Mukherjee M. Accumulation of a Natural Substrate of Laccase in Gills of Pleurotus florida During Sporulation. *Current Microbiology* 2000;41:167–71. https://doi.org/10.1007/s002840010112.

[18] Asano T, Seto Y, Hashimoto K, Kurushima H. Mini-review an insect-specific system for terrestrialization: Laccase-mediated cuticle formation. *Insect Biochemistry and Molecular Biology* 2019;108:61–70. https://doi.org/10.1016/j.ibmb.2019.03.007.

[19] Deska M, Kończak B. Immobilized fungal laccase as “green catalyst” for the decolourization process – State of the art. *Process Biochemistry* 2019;84:112–23. https://doi.org/10.1016/j.procbio.2019.05.024.

[20] Debnath R, Saha T. An insight into the production strategies and applications of the ligninolytic enzyme laccase from bacteria and fungi. *Biocatalysis and Agricultural Biotechnology* 2020;26:101645. https://doi.org/10.1016/j.bcab.2020.101645.

[21] Rakotovelo A, Peruch F, Grelier S. Lignine : structure, production et valorisation chimique. *Techniques de l’ingénieur* 2019.

[22] Piontek K, Antorini M, Choinowski T. Crystal Structure of a Laccase from the FungusTrametes versicolor at 1.90-Å Resolution Containing a Full Complement of Coppers. *Journal of Biological Chemistry* 2002;277:37663–9. https://doi.org/10.1074/jbc.M204571200.

[23] Frasconi M, Favero G, Boer H, Koivula A, Mazzei F. Kinetic and biochemical properties of high and low redox potential laccases from fungal and plant origin. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 2010;1804:899–908. https://doi.org/10.1016/j.bbapap.2009.12.018.

[24] Xu F, Shin W, Brown SH, Wahleithner JA, Sundaram UM, Solomon EI. A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* 1996;1292:303–11. https://doi.org/10.1016/0167-4838(95)00210-3.

[25] Matera I, Gullotto A, Tilli S, Ferraroni M, Scozzafava A, Briganti F. Crystal structure of the blue multicopper oxidase from the white-rot fungus Trametes trogii complexed with p-toluate. *Inorganica Chimica Acta* 2008;361:4129–37. https://doi.org/10.1016/j.ica.2008.03.091.

[26] Orlikowska M, De J. Rostro-Alanis M, Bujacz A, Hernández-Luna C, Rubio R, Parra R, et al. Structural studies of two thermostable laccases from the white-rot fungus Pycnoporus

sanguineus. *International Journal of Biological Macromolecules* 2018;107:1629–40. https://doi.org/10.1016/j.ijbiomac.2017.10.024.

[27] Liu Z, Xie T, Zhong Q, Wang G. Crystal structure of CotA laccase complexed with 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) at a novel binding site. *Acta Crystallogr F Struct Biol Commun* 2016;72:328–35. https://doi.org/10.1107/S2053230X1600426X.

[28] Skálová T, Dušková J, Hašek J, Štěpánková A, Kovaľ T, Østergaard LH, et al. Structure of laccase from *Streptomyces coelicolor* after soaking with potassium hexacyanoferrate and at an improved resolution of 2.3 Å. *Acta Crystallogr F Struct Biol Cryst Commun* 2011;67:27–32. https://doi.org/10.1107/S1744309110046099.

[29] Olmeda I, Casino P, Collins RE, Sendra R, Callejón S, Huesa J, et al. Structural analysis and biochemical properties of laccase enzymes from two *Pediococcus* species. *Microb Biotechnol* 2021;14:1026–43. https://doi.org/10.1111/1751-7915.13751.

[30] Xie T, Liu Z, Wang G. Structural basis for monolignol oxidation by a maize laccase. *Nat Plants* 2020;6:231–7. https://doi.org/10.1038/s41477-020-0595-5.

[31] Dwivedi UN, Singh P, Pandey VP, Kumar A. Structure–function relationship among bacterial, fungal and plant laccases. *Journal of Molecular Catalysis B: Enzymatic* 2011;68:117–28. https://doi.org/10.1016/j.molcatb.2010.11.002.

[32] Gaur N, Narasimhulu K, Y P. Biochemical and kinetic characterization of laccase and manganese peroxidase from novel *Klebsiella pneumoniae* strains and their application in bioethanol production. *RSC Adv* 2018;8:15044–55. https://doi.org/10.1039/C8RA01204K.

[33] Heinzkill M, Bech L, Halkier T, Schneider P, Anke T. Characterization of Laccases and Peroxidases from Wood-Rotting Fungi (Family *Coprinaceae*). *Appl Environ Microbiol* 1998;64:1601–6. https://doi.org/10.1128/AEM.64.5.1601-1606.1998.

[34] Karhunen E, Niku-Paavola M-L, Viikari L, Haltia T, Van Der Meer RA, Duine JA. A novel combination of prosthetic groups in a fungal laccase; PQQ and two copper atoms. *FEBS Letters* 1990;267:6–8. https://doi.org/10.1016/0014-5793(90)80273-L.

[35] González-González P, Gómez-Manzo S, Tomasini A, Martínez Y Pérez JL, García Nieto E, Anaya-Hernández A, et al. Laccase Production from Agrocybe pediades: Purification and Functional Characterization of a Consistent Laccase Isoenzyme in Liquid Culture. *Microorganisms* 2023;11:568. https://doi.org/10.3390/microorganisms11030568.

[36] Cañas AI, Camarero S. Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. *Biotechnology Advances* 2010;28:694–705. https://doi.org/10.1016/j.biotechadv.2010.05.002.

[37] Christopher LP, Yao B, Ji Y. Lignin Biodegradation with Laccase-Mediator Systems. *Front Energy Res* 2014;2. https://doi.org/10.3389/fenrg.2014.00012.

[38] Cantarella G, Galli C, Gentili P. Free radical versus electron-transfer routes of oxidation of hydrocarbons by laccase/mediator systems. *Journal of Molecular Catalysis B: Enzymatic* 2003;22:135–44. https://doi.org/10.1016/S1381-1177(03)00014-6.

[39] Mantle TJ, Harris DA. Chapter 7 Spectrophotometric assays. *Spectrophotometry and Spectrofluorimetry: A Practical Approach, Oxford University Press*; 2000. https://doi.org/10.1093/oso/9780199638130.003.0011.

[40] Eisenthal R, Danson MJ, editors. Enzyme assays: a practical approach. 2. ed. Oxford: Oxford University Press; 2002.

[41] Childs RE, Bardsley WG. The steady-state kinetics of peroxidase with 2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. *Biochemical Journal* 1975;145:93–103. https://doi.org/10.1042/bj1450093.

[42] Chaurasia PK, Yadav RSS, Yadava S. A review on mechanism of laccase action. *Research & Reviews in BioSciences* 2013;7:66-71.

[43] Harkin JM, Obst JR. Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. *Experientia* 1973;29:381–7. https://doi.org/10.1007/BF01926734.

[44] Boidin J. Recherches de la tyrosinase et de la laccase chez les Basidiomycètes en culture pure. Milieux différentiels. Intérêt systématique. *Revue de Mycologie* 1951;16:173–97.

[45] Prillinger H, Esser K. The Phenoloxidases of the Ascomycete Podospora anserina. XIII. Action and interaction of genes controlling the formation of laccase. *Molecular & General Genetics: MGG* 1977;156:333–45. https://doi.org/10.1007/bf00267190.

[46] Miller R, Kuglin J, Gallagher S, Flurkey WH. A spectrophotometric assay for laccase using o-tolidine. *J Food Biochemistry* 1997;21:445–59. https://doi.org/10.1111/j.1745-4514.1997.tb00199.x.

[47] Flurkey WH, Kuglin J, Dawley R. Tyrosinase, Laccase, and Peroxidase in Mushrooms (Agaricus, Crimini, Oyster, and Shiitake). *J Food Science* 1994;59:824–7. https://doi.org/10.1111/j.1365-2621.1994.tb08137.x.

[48] d'Acunzo F, Barreca AM, Galli C. Determination of the activity of laccase, and mediated oxidation of a lignin model compound, in aqueous-organic mixed solvents. *Journal of Molecular Catalysis B: Enzymatic* 2004;31:25–30. https://doi.org/10.1016/j.molcatb.2004.07.001.

[49] Moshtaghioun SM, Haghbeen K, Lotfi Sahebghadam A, Legge RL, Khoshneviszadeh R, Farhadi S. Direct Spectrophotometric Assay of Laccase Using Diazo Derivatives of Guaiacol. *Anal Chem* 2011;83:4200–5. https://doi.org/10.1021/ac200501w.

[50] Elsaba YM, El-Hennawi HM, Ibrahim MM, Wehaidy HR. Production of a novel laccase from Ceratorhiza hydrophila and assessing its potential in natural dye fixation and cytotoxicity against tumor cells. *J Genet Eng Biotechnol* 2023;21:14. https://doi.org/10.1186/s43141-023-00473-1.

[51] Vázquez V, Giorgi V, Bonfiglio F, Menéndez P, Gioia L, Ovsejevi K. Lignocellulosic residues from bioethanol production: a novel source of biopolymers for laccase immobilization. *RSC Adv* 2023;13:13463–71. https://doi.org/10.1039/D3RA01520C.

[52] Yang Y, Ghatge S, Hur H-G. Improvement of thermoalkaliphilic laccase (CtLac) by a directed evolution and application to lignin degradation. *Appl Microbiol Biotechnol* 2023;107:273–86. https://doi.org/10.1007/s00253-022-12311-4.

[53] Farnet AM, Ferre E, Gil G, Gastaldi S. A new substrate to measure laccase activities in complex environments: Application to litters. *Soil Biology and Biochemistry* 2010;42:1001–5. https://doi.org/10.1016/j.soilbio.2010.02.005.

[54] Terrón MC, López-Fernández M, Carbajo JM, Junca H, Téllez A, Yagüe S, et al. Tannic acid interferes with the commonly used laccase-detection assay based on ABTS as the substrate. *Biochimie* 2004;86:519–22. https://doi.org/10.1016/j.biochi.2004.07.013.

[55] Liu J-X, Zhou W-J, Gong J-L, Tang L, Zhang Y, Yu H-Y, et al. An electrochemical sensor for detection of laccase activities from Penicillium simplicissimum in compost based on carbon nanotubes modified glassy carbon electrode. *Bioresource Technology* 2008;99:8748–51. https://doi.org/10.1016/j.biortech.2008.04.029.

[56] Skoog DA, Holler JF, Crouch SR. Principles of instrumental analysis. 7th ed. Cengage Learning; 2017.

[57] Blum M-M, John H. Historical perspective and modern applications of Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR): Modern applications of ATR-FTIR. *Drug Test Analysis* 2012;4:298–302. https://doi.org/10.1002/dta.374.

[58] Perna V, Baum A, Ernst HA, Agger JW, Meyer AS. Laccase activity measurement by FTIR spectral fingerprinting. *Enzyme and Microbial Technology* 2019;122:64–73. https://doi.org/10.1016/j.enzmictec.2018.12.009.

[59] Baum A, Meyer AS, Garcia JL, Egebo M, Hansen PW, Mikkelsen JD. Enzyme activity measurement via spectral evolution profiling and PARAFAC. *Analytica Chimica Acta* 2013;778:1–8. https://doi.org/10.1016/j.aca.2013.03.029.

[60] Juárez-Hernández J, Zavala-Soto ME, Bibbins-Martínez M, Delgado-Macuil R, Díaz-Godinez G, Rojas-López M, et al. FTIR Spectroscopy Applied in Remazol Blue Dye Oxidation by Laccases. *AIP Conference Proceedings*, vol. 992, Campinas, São Paulo (Brazil): AIP; 2008, p. 1253–7. https://doi.org/10.1063/1.2926829.

[61] Lewiner F, Klein JP, Puel F, Févotte G. On-line ATR FTIR measurement of supersaturation during solution crystallization processes. Calibration and applications on three solute/solvent systems. *Chemical Engineering Science* 2001;56:2069–84. https://doi.org/10.1016/S0009-2509(00)00508-X.

[62] Dong CY, So P.T. Fluorescence Spectrophotometry. ENCYCLOPEDIA OF LIFE SCIENCES 2002.

[63] Rengarajan K, Cristol SM, Mehta M, Nickerson JM. Quantifying DNA concentrations using fluorometry: A comparison of fluorophores. *Molecular Vision* 2002;8:416–21.

[64] Morozkin ES, Laktionov PP, Rykova EY, Vlassov VV. Fluorometric quantification of RNA and DNA in solutions containing both nucleic acids. *Analytical Biochemistry* 2003;322:48–50. https://doi.org/10.1016/j.ab.2003.07.009.

[65] Mocz G, Ross JA. Fluorescence Techniques in Analysis of Protein–Ligand Interactions. In: Williams MA, Daviter T, editors. *Protein-Ligand Interactions*, vol. 1008, Totowa, NJ: Humana Press; 2013, p. 169–210. https://doi.org/10.1007/978-1-62703-398-5_7.

[66] Smith DS, Al-Hakiem MHH, Landon J. A Review of Fluoroimmunoassay and Immunofluorometric Assay. *Ann Clin Biochem* 1981;18:253–74. https://doi.org/10.1177/000456328101800501.

[67] Lonergan G, Mew E, Schliephake K, Baker WL. Phenolic substrates for fluorometric detection of laccase activity. *FEMS Microbiology Letters* 1997;153:485–90. https://doi.org/10.1111/j.1574-6968.1997.tb12614.x.

[68] Wang T, Xiang Y, Liu X, Chen W, Hu Y. A novel fluorimetric method for laccase activities measurement using Amplex Red as substrate. *Talanta* 2017;162:143–50. https://doi.org/10.1016/j.talanta.2016.10.006.

[69] Arabi MS, Karami C, Taher MA, Ahmadi E. Fluorescence detection of laccases activity by the photoinduced electron transfer (PET) process. *J Biol Inorg Chem* 2020;25:151–9. https://doi.org/10.1007/s00775-019-01748-0.

[70] Escudero D. Revising Intramolecular Photoinduced Electron Transfer (PET) from First-Principles. *Acc Chem Res* 2016;49:1816–24. https://doi.org/10.1021/acs.accounts.6b00299.

[71] Nour IM, Mohamed AR, Hasan MA, Badrawy M. Eco-friendly-assessed micellar-fluorimetric platform for concurrent analysis of empagliflozin and prucalopride succinate in biological fluids: Docking simulation. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 2023;296:122715. https://doi.org/10.1016/j.saa.2023.122715.

[72] Calvet E, Prat H, Skinner HA. Recent Progress in Microcalorimetry. Pergamon Press. 1963.

[73] Wang T-Z, Li W-P, Wan H-W, Zhou P-J, Qu S-S. Assay for Laccase activity by microcalorimetry: Laccase was extracted from China lacquer of Rhus vernicifera. *Journal of Biochemical and Biophysical Methods* 2000;45:57–63. https://doi.org/10.1016/S0165-022X(00)00098-1.

[74] A. Islam ST, Zhang J, Tonin F, Hinderks R, Deurloo YN, Urlacher VB, et al. Isothermal titration calorimetric assessment of lignin conversion by laccases. *Biotech & Bioengineering* 2022;119:493–503. https://doi.org/10.1002/bit.27991.

[75] Volkova N, Ibrahim V, Hatti-Kaul R. Laccase catalysed oxidation of syringic acid: Calorimetric determination of kinetic parameters. *Enzyme and Microbial Technology* 2012;50:233–7. https://doi.org/10.1016/j.enzmictec.2012.01.005.

[76] Todd MJ, Gomez J. Enzyme Kinetics Determined Using Calorimetry: A General Assay for Enzyme Activity? *Analytical Biochemistry* 2001;296:179–87. https://doi.org/10.1006/abio.2001.5218.

[77] Olsen SN. Applications of isothermal titration calorimetry to measure enzyme kinetics and activity in complex solutions. *Thermochimica Acta* 2006;448:12–8. https://doi.org/10.1016/j.tca.2006.06.019.

[78] Wang Y, Wang G, Moitessier N, Mittermaier AK. Enzyme Kinetics by Isothermal Titration Calorimetry: Allostery, Inhibition, and Dynamics. *Front Mol Biosci* 2020;7:583826. https://doi.org/10.3389/fmolb.2020.583826.

[79] Maximova K, Wojtczak J, Trylska J. Enzyme kinetics in crowded solutions from isothermal titration calorimetry. *Analytical Biochemistry* 2019;567:96–105. https://doi.org/10.1016/j.ab.2018.11.006.

[80] Bard AJ, Faulkner LR, White HS. Electrochemical Methods: fundamentals and applications. John Wiley & Sons 2022.

[81] Yamada H, Yoshii K, Asahi M, Chiku M, Kitazumi Y. Cyclic Voltammetry Part 1: Fundamentals. *Electrochemistry* 2022;90:102005–102005. https://doi.org/10.5796/electrochemistry.22-66082.

[82] Elgrishi N, Rountree KJ, McCarthy BD, Rountree ES, Eisenhart TT, Dempsey JL. A Practical Beginner's Guide to Cyclic Voltammetry. *J Chem Educ* 2018;95:197–206. https://doi.org/10.1021/acs.jchemed.7b00361.

[83] Bogdan M, Brugger D, Rosenstiel W, Speiser B. Estimation of diffusion coefficients from voltammetric signals by support vector and gaussian process regression. *J Cheminform* 2014;6:30. https://doi.org/10.1186/1758-2946-6-30.

[84] Adeloju SB. AMPEROMETRY. *Encyclopedia of Analytical Science*, Elsevier; 2005, p. 70–9. https://doi.org/10.1016/B0-12-369397-7/00012-1.

[85] Gáspár S, Brinduse E, Vasilescu A. Electrochemical Evaluation of Laccase Activity in Must. *Chemosensors* 2020;8:126. https://doi.org/10.3390/chemosensors8040126.

[86] Klis M, Rogalski J, Bilewicz R. Voltammetric determination of catalytic reaction parameters of laccase based on electrooxidation of hydroquinone and ABTS. *Bioelectrochemistry* 2007;71:2–7. https://doi.org/10.1016/j.bioelechem.2006.09.008.

[87] Lulea AC, Ruginescu R, Banciu RM, Pantazi C, Brinduse E, Ion M, et al. Fast Electrochemical Measurement of Laccase Activity for Monitoring Grapes' Infection with *Botrytis cinerea*. *Processes* 2022;10:575. https://doi.org/10.3390/pr10030575.

[88] Zouari N, Romette J-L, Thomas D. Continuous-flow estimation of laccase activity in rotten grape juice by a computerized electrode. *J Chem Technol Biotechnol* 2007;40:195–201. https://doi.org/10.1002/jctb.280400305.

[89] Nimmo M. POLAROGRAPHY | Inorganic Applications. *Encyclopedia of Analytical Science*, Elsevier; 2005, p. 198–206. https://doi.org/10.1016/B0-12-369397-7/00470-2.

[90] Wood DA, Goodenough PW. Fruiting of Agaricus bisporus: Changes in extracellular enzyme activities during growth and fruiting. *Arch Microbiol* 1977;114:161–5. https://doi.org/10.1007/BF00410778.

[91] Wood DA. Production, Purification and Properties of Extracellular Laccase of Agaricus bisporus. *Microbiology* 1980;117:327–38. https://doi.org/10.1099/00221287-117-2-327.

[92] Blaich R, Esser K. Function of enzymes in wood destroying fungi: II. Multiple forms of laccase in white rot fungi. *Arch Microbiol* 1975;103:271–7. https://doi.org/10.1007/BF00436360.

[93] Wilson MJ, editor. Clay Mineralogy: Spectroscopic and Chemical Determinative Methods. Dordrecht: Springer Netherlands; 1994. https://doi.org/10.1007/978-94-011-0727-3.

[94] Khulbe KC, Ismail AF, Matsuura T. Electron Paramagnetic Resonance (EPR) Spectroscopy. Membrane Characterization, Elsevier; 2017, p. 47–68. https://doi.org/10.1016/B978-0-444-63776-5.00003-6.

[95] Perna V, Agger JW, Andersen ML, Holck J, Meyer AS. Laccase Induced Lignin Radical Formation Kinetics Evaluated by Electron Paramagnetic Resonance Spectroscopy. *ACS Sustainable Chem Eng* 2019;7:10425–34. https://doi.org/10.1021/acssuschemeng.9b00723.

[96] Munk L, Andersen ML, Meyer AS. Direct rate assessment of laccase catalysed radical formation in lignin by electron paramagnetic resonance spectroscopy. *Enzyme and Microbial Technology* 2017;106:88–96. https://doi.org/10.1016/j.enzmictec.2017.07.006.

[97] Hogg N. Detection of nitric oxide by electron paramagnetic resonance spectroscopy. *Free Radical Biology and Medicine* 2010;49:122–9. https://doi.org/10.1016/j.freeradbiomed.2010.03.009.

[98] Roessler MM, Salvadori E. Principles and applications of EPR spectroscopy in the chemical sciences. *Chem Soc Rev* 2018;47:2534–53. https://doi.org/10.1039/C6CS00565A.

[99] Badiani M, Felici M, Luna M, Artemi F. Laccase assay by means of high-performance liquid chromatography. *Analytical Biochemistry* 1983;133:275–6. https://doi.org/10.1016/0003-2697(83)90082-9.

[100] Anders N, Schelden M, Roth S, Spiess AC. Automated chromatographic laccase-mediator-system activity assay. *Anal Bioanal Chem* 2017;409:4801–9. https://doi.org/10.1007/s00216-017-0423-6.

[101] Alzagameem A, Khaldi-Hansen B, Büchner D, Larkins M, Kamm B, Witzleben S, et al. Lignocellulosic Biomass as Source for Lignin-Based Environmentally Benign Antioxidants. *Molecules* 2018;23:2664. https://doi.org/10.3390/molecules23102664.

[102] Ekeberg D, Gretland KS, Gustafsson J, Bråten SM, Fredheim GE. Characterisation of lignosulphonates and kraft lignin by hydrophobic interaction chromatography. *Analytica Chimica Acta* 2006;565:121–8. https://doi.org/10.1016/j.aca.2006.02.008.

[103] Asikkala J, Tamminen T, Argyropoulos DS. Accurate and Reproducible Determination of Lignin Molar Mass by Acetobromination. *J Agric Food Chem* 2012;60:8968–73. https://doi.org/10.1021/jf303003d.

[104] Imran M, Asad MJ, Hadri SH, Mehmood S. Production and industrial applications of laccase enzyme. *Journal of Cell and Molecular Biology* 2012;10:1.

[105] Zerva A, Simić S, Topakas E, Nikodinovic-Runic J. Applications of Microbial Laccases: Patent Review of the Past Decade (2009–2019). *Catalysts* 2019;9:1023. https://doi.org/10.3390/catal9121023.

[106] Osma JF, Toca-Herrera JL, Rodríguez-Couto S. Uses of Laccases in the Food Industry. *Enzyme Research* 2010;2010:1–8. https://doi.org/10.4061/2010/918761.

[107] Lomascolo A, Record E, Herpoel-Gimbert I, Delattre M, Robert JL, Georis J, et al. Overproduction of laccase by a monokaryotic strain of Pycnoporus cinnabarinus using ethanol as inducer. *J Appl Microbiol* 2003;94:618–24. https://doi.org/10.1046/j.1365-2672.2003.01879.x.

[108] Piscitelli A, Pezzella C, Giardina P, Faraco V, Sannia G. Heterologous laccase production and its role in industrial applications. *Bioengineered Bugs* 2010;1:254–64. https://doi.org/10.4161/bbug.1.4.11438.

[109] Record E, Punt PJ, Chamkha M, Labat M, Van Den Hondel CAMJJ, Asther M. Expression of the *Pycnoporus cinnabarinus* laccase gene in *Aspergillus niger* and characterization of the recombinant enzyme: *P. cinnabarinus* laccase gene expression in *A. niger*. *European Journal of Biochemistry* 2002;269:602–9. https://doi.org/10.1046/j.0014-2956.2001.02690.x.

[110] Alves AMCR, Record E, Lomascolo A, Scholtmeijer K, Asther M, Wessels JGH, et al. Highly Efficient Production of Laccase by the Basidiomycete *Pycnoporus cinnabarinus*. *Appl Environ Microbiol* 2004;70:6379–84. https://doi.org/10.1128/AEM.70.11.6379-6384.2004.

[111] Cheng C-M, Patel AK, Singhania RR, Tsai C-H, Chen S-Y, Chen C-W, et al. Heterologous expression of bacterial CotA-laccase, characterization and its application for biodegradation of malachite green. *Bioresource Technology* 2021;340:125708. https://doi.org/10.1016/j.biortech.2021.125708.

[112] Hong F, Meinander NQ, Jönsson LJ. Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*: Heterologous Expression of Laccase in *Pichia Pastoris*. *Biotechnol Bioeng* 2002;79:438–49. https://doi.org/10.1002/bit.10297.

[113] Wang B, Yan Y, Xu J, Fu X, Han H, Gao J, et al. Heterologous Expression and Characterization of a Laccase from Laccaria bicolor in Pichia pastoris and Arabidopsis thaliana. *J Microbiol Biotechnol* 2018;28. https://doi.org/10.4014/jmb.1807.08042

[114] Lipiäinen S, Kuparinen K, Sermyagina E, Vakkilainen E. Pulp and paper industry in energy transition: Towards energy-efficient and low carbon operation in Finland and Sweden. *Sustainable Production and Consumption* 2022;29:421–31. https://doi.org/10.1016/j.spc.2021.10.029.

[115] Singh G, Arya SK. Utility of laccase in pulp and paper industry: A progressive step towards the green technology. *International Journal of Biological Macromolecules* 2019;134:1070–84. https://doi.org/10.1016/j.ijbiomac.2019.05.168.

[116] Virk AP, Sharma P, Capalash N. Use of laccase in pulp and paper industry. *Biotechnol Progress* 2012;28:21–32. https://doi.org/10.1002/btpr.727.

[117] Singh D, Gupta N. Microbial Laccase: a robust enzyme and its industrial applications. *Biologia* 2020;75:1183–93. https://doi.org/10.2478/s11756-019-00414-9.

[118] Sigoillot C, Record E, Belle V, Robert JL, Levasseur A, Punt PJ, et al. Natural and recombinant fungal laccases for paper pulp bleaching. *Appl Microbiol Biotechnol* 2004;64:346–52. https://doi.org/10.1007/s00253-003-1468-3.

[119] Bourbonnais R, Paice MG, Freiermuth B, Bodie E, Borneman S. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. *Appl Environ Microbiol* 1997;63:4627–32. https://doi.org/10.1128/aem.63.12.4627-4632.1997.

[120] Moldes D, Díaz M, Tzanov T, Vidal T. Comparative study of the efficiency of synthetic and natural mediators in laccase-assisted bleaching of eucalyptus kraft pulp. *Bioresource Technology* 2008;99:7959–65. https://doi.org/10.1016/j.biortech.2008.04.002.

[121] Knutson K, Ragauskas A. Laccase-Mediator Biobleaching Applied to a Direct Yellow Dyed Paper. *Biotechnol Prog* 2004;20:1893–6. https://doi.org/10.1021/bp049833+.

[122] Vivekanand V, Dwivedi P, Sharma A, Sabharwal N, Singh RP. Enhanced delignification of mixed wood pulp by Aspergillus fumigatus laccase mediator system. *World J Microbiol Biotechnol* 2008;24:2799–804. https://doi.org/10.1007/s11274-008-9809-0.

[123] Iracheta-Cárdenas MM, Rocha-Peña MA, Galán-Wong LJ, Arévalo-Niño K, Tovar-Herrera OE. A *Pycnoporus sanguineus* laccase for denim bleaching and its comparison with an enzymatic commercial formulation. *Journal of Environmental Management* 2016;177:93–100. https://doi.org/10.1016/j.jenvman.2016.04.008.

[124] Singh G, Kaur K, Puri S, Sharma P. Critical factors affecting laccase-mediated biobleaching of pulp in paper industry. *Appl Microbiol Biotechnol* 2015;99:155–64. https://doi.org/10.1007/s00253-014-6219-0.

[125] Mayolo-Deloisa K, González-González M, Rito-Palomares M. Laccases in Food Industry: Bioprocessing, Potential Industrial and Biotechnological Applications. *Front Bioeng Biotechnol* 2020;8:222. https://doi.org/10.3389/fbioe.2020.00222.

[126] Minussi RC, Rossi M, Bologna L, Rotilio D, Pastore GM, Durán N. Phenols removal in musts: Strategy for wine stabilization by laccase. *Journal of Molecular Catalysis B: Enzymatic* 2007;45:102–7. https://doi.org/10.1016/j.molcatb.2006.12.004.

[127] Molina MA, Cazzaniga A, Milde LB, Sgroppo SC, Zapata PD, Fonseca MI. Purification and characterization of a fungal laccase expressed in *Kluyveromyces lactis* suitable for baking. *Journal of Food Science* 2023;88:1365–77. https://doi.org/10.1111/1750-3841.16497.

[128] Si JQ. USE OF LACCASE IN BAKING. WO 1994/028728, 1996.

[129] Dauda MY, Erkurt EA. Investigation of reactive Blue 19 biodegradation and byproducts toxicity assessment using crude laccase extract from Trametes versicolor. *Journal of Hazardous Materials* 2020;393:121555. https://doi.org/10.1016/j.jhazmat.2019.121555.

[130] Motamedi E, Kavousi K, Sadeghian Motahar SF, Reza Ghaffari M, Sheykh Abdollahzadeh Mamaghani A, Hosseini Salekdeh G, et al. Efficient removal of various textile dyes from wastewater by novel thermo-halotolerant laccase. *Bioresource Technology* 2021;337:125468. https://doi.org/10.1016/j.biortech.2021.125468.

[131] Yadav A, Yadav P, Kumar Singh A, Kumar V, Chintaman Sonawane V, Markandeya, et al. Decolourisation of textile dye by laccase: Process evaluation and assessment of its degradation bioproducts. *Bioresource Technology* 2021;340:125591. https://doi.org/10.1016/j.biortech.2021.125591.

[132] Gao Y, Wang M, Shah K, Singh Kalra S, Rome LH, Mahendra S. Decolorization and detoxification of synthetic dye compounds by laccase immobilized in vault nanoparticles. *Bioresource Technology* 2022;351:127040. https://doi.org/10.1016/j.biortech.2022.127040.

[133] Osiadacz J, Al-Adhami AJH, Bajraszewska D, Fischer P. On the use of *Trametes versicolor* laccase for the conversion of 4-methyl-3-hydroxyanthranilic acid to actinocin chromophore. *Journal of Biotechnology* 1999;72:141-149. https://doi.org/10.1016/S0168-1656(99)00100-5

[134] Kunamneni A, Camarero S, García-Burgos C, Plou FJ, Ballesteros A, Alcalde M. Engineering and Applications of fungal laccases for organic synthesis. *Microb Cell Fact* 2008;7:32. https://doi.org/10.1186/1475-2859-7-32.

[135] Kudanga T, Nemadziva B, Le Roes-Hill M. Laccase catalysis for the synthesis of bioactive compounds. *Appl Microbiol Biotechnol* 2017;101:13–33. https://doi.org/10.1007/s00253-016-7987-5.

[136] Nguyen HH, Lee SH, Lee UJ, Fermin CD, Kim M. Immobilized Enzymes in Biosensor Applications. *Materials* 2019;12:121. https://doi.org/10.3390/ma12010121.

[137] Newman JD, Setford SJ. Enzymatic Biosensors. *Molecular Biotechnology* 2006;32:249–68. https://doi.org/10.1385/MB:32:3:249.

[138] Albayati SAR, Kashanian S, Nazari M, Rezaei S. Novel fabrication of a laccase biosensor to detect phenolic compounds using a carboxylated multiwalled carbon nanotube on the electropolymerized support. *Bull Mater Sci* 2019;42:187. https://doi.org/10.1007/s12034-019-1850-0.

[139] Diaconu M, Litescu SC, Radu GL. Laccase–MWCNT–chitosan biosensor—A new tool for total polyphenolic content evaluation from in vitro cultivated plants. *Sensors and Actuators B: Chemical* 2010;145:800–6. https://doi.org/10.1016/j.snb.2010.01.064.

[140] Shimomura T, Itoh T, Sumiya T, Hanaoka T, Mizukami F, Ono M. Amperometric detection of phenolic compounds with enzyme immobilized in mesoporous silica prepared by electrophoretic deposition. *Sensors and Actuators B: Chemical* 2011;153:361–8. https://doi.org/10.1016/j.snb.2010.10.048.

[141] Li D, Luo L, Pang Z, Ding L, Wang Q, Ke H, et al. Novel Phenolic Biosensor Based on a Magnetic Polydopamine-Laccase-Nickel Nanoparticle Loaded Carbon Nanofiber Composite. *ACS Appl Mater Interfaces* 2014;6:5144–51. https://doi.org/10.1021/am500375n.

[142] Mei L-P, Feng J-J, Wu L, Zhou J-Y, Chen J-R, Wang A-J. Novel phenol biosensor based on laccase immobilized on reduced graphene oxide supported palladium–copper alloyed nanocages. *Biosensors and Bioelectronics* 2015;74:347–52. https://doi.org/10.1016/j.bios.2015.06.060.

[143] Bragazzi NL, Pechkova E, Scudieri D, Terencio TBC, Adami M, Nicolini C. Recombinant Laccase: II. Medical Biosensor. *CritRevEukarGeneExpr* 2012;22:197–203. https://doi.org/10.1615/CritRevEukarGeneExpr.v22.i3.30.

[144] Leynaud Kieffer Curran LMC, Pham LTM, Sale KL, Simmons BA. Review of advances in the development of laccases for the valorization of lignin to enable the production of lignocellulosic biofuels and bioproducts. *Biotechnology Advances* 2022;54:107809. https://doi.org/10.1016/j.biotechadv.2021.107809.

[145] Mateljak I, Rice A, Yang K, Tron T, Alcalde M. The Generation of Thermostable Fungal Laccase Chimeras by SCHEMA-RASPP Structure-Guided Recombination *in Vivo*. *ACS Synth Biol* 2019;8:833–43. https://doi.org/10.1021/acssynbio.8b00509.

[146] Alcalde M. When directed evolution met ancestral enzyme resurrection. *Microb Biotechnol* 2017;10:22–4. https://doi.org/10.1111/1751-7915.12452.

[147] Gomez-Fernandez BJ, Risso VA, Rueda A, Sanchez-Ruiz JM, Alcalde M. Ancestral Resurrection and Directed Evolution of Fungal Mesozoic Laccases. *Appl Environ Microbiol* 2020;86:e00778-20. https://doi.org/10.1128/AEM.00778-20.

[148] Novoa C, Dhoke GV, Mate DM, Martínez R, Haarmann T, Schreiter M, et al. KnowVolution of a Fungal Laccase toward Alkaline pH. *ChemBioChem* 2019;20:1458–66. https://doi.org/10.1002/cbic.201800807.

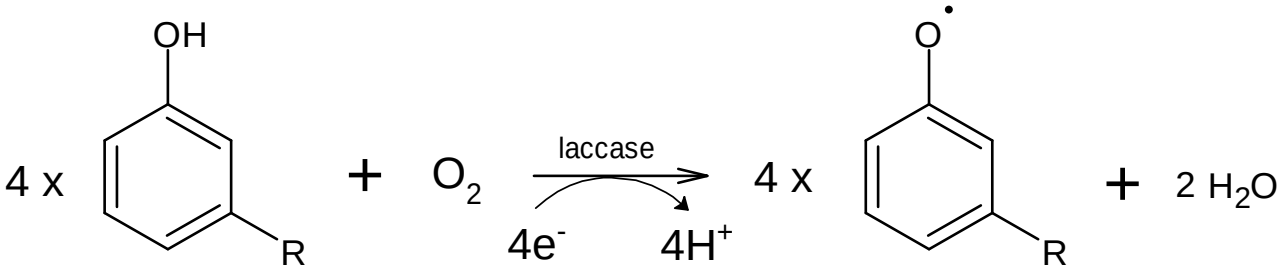


Figure 1 *: Laccase general catalytic reaction mechanism*

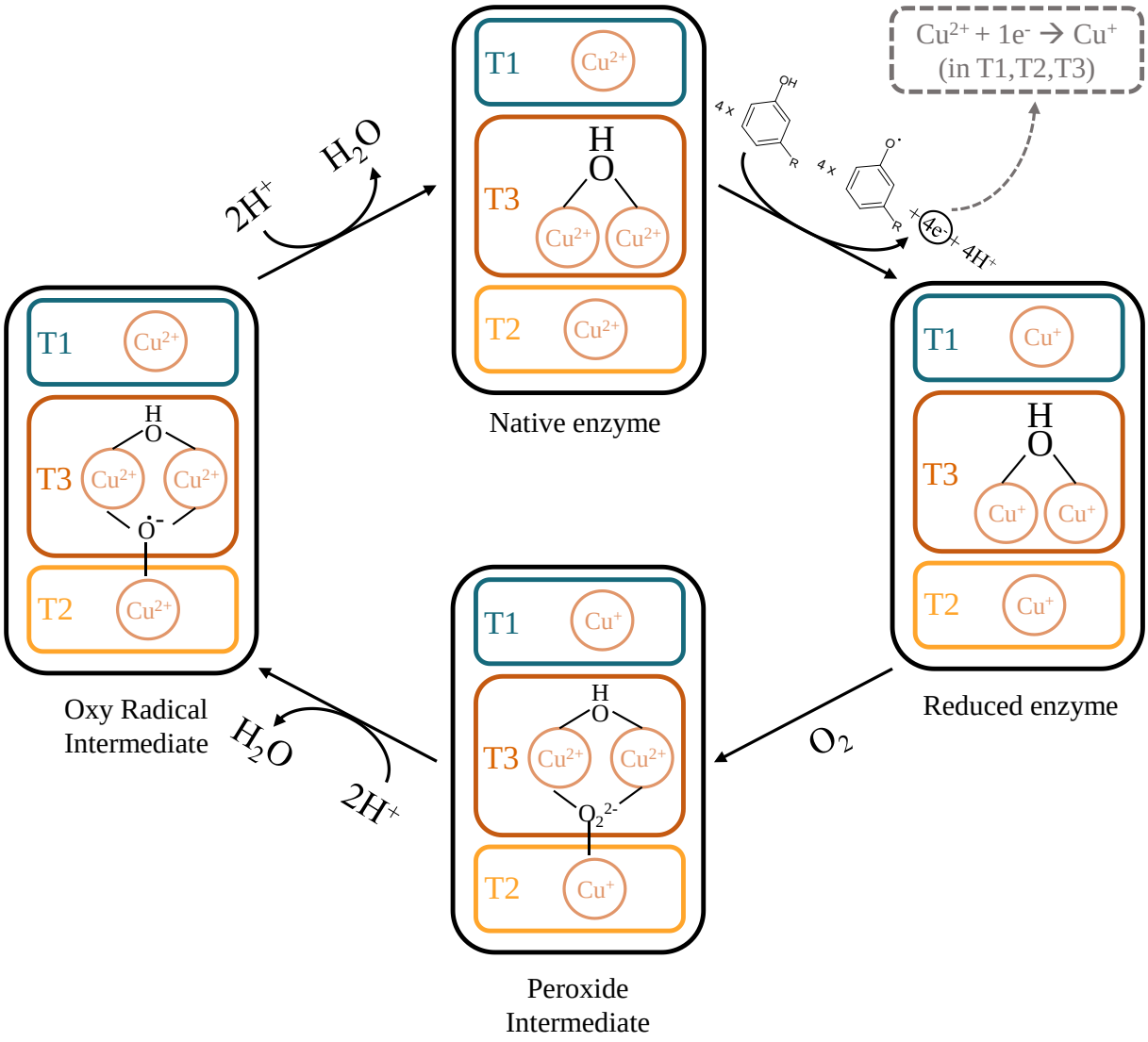


Figure 1 : *Schematic representation of the steps of laccase mechanism of action*

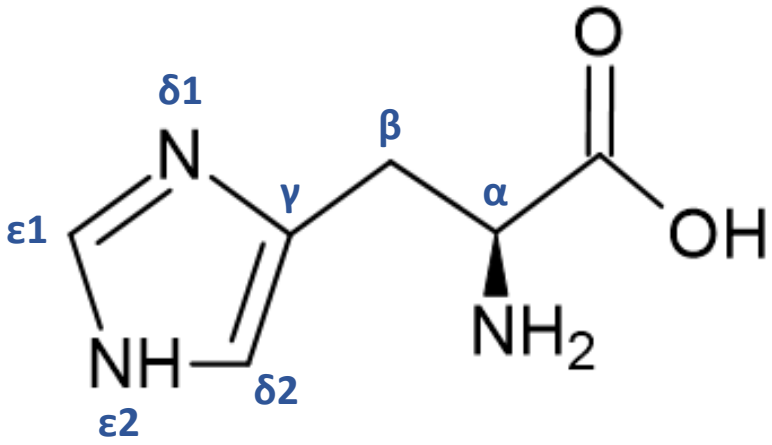


Figure 1 : *Histidin molecule and its atoms numbering*

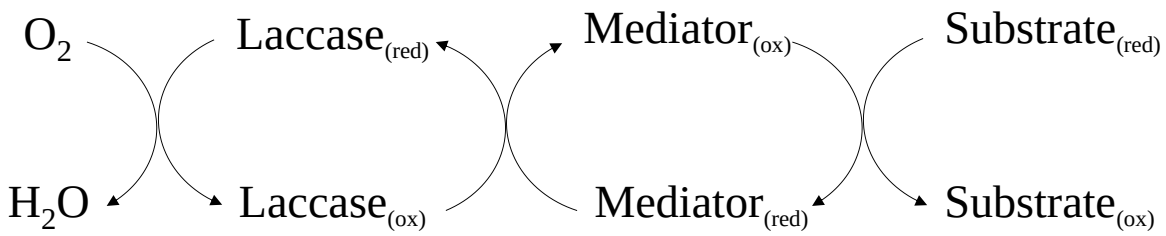
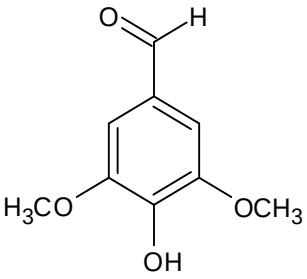
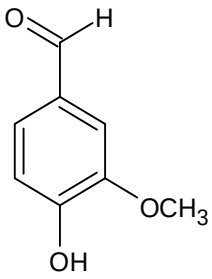


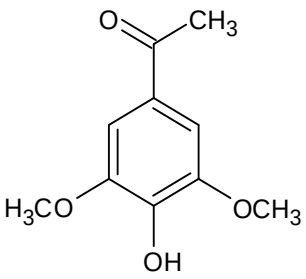
Figure 1: *Schematic diagram of how the laccase-mediator system works*



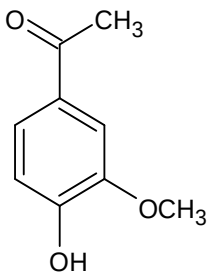
Syringaldehyde



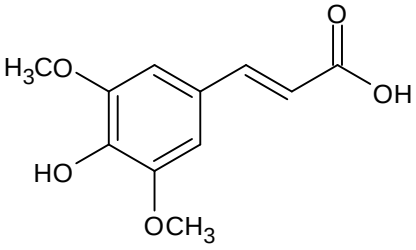
Vanillin



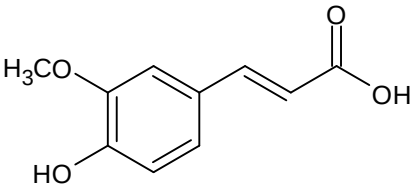
Acetosyringone



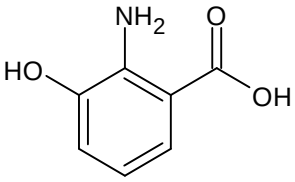
Acetovanillone



Sinapic acid

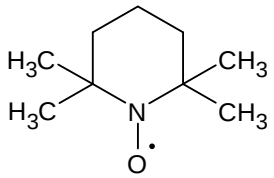


Ferulic acid

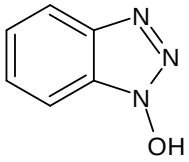


3-hydroxyanthranilic acid

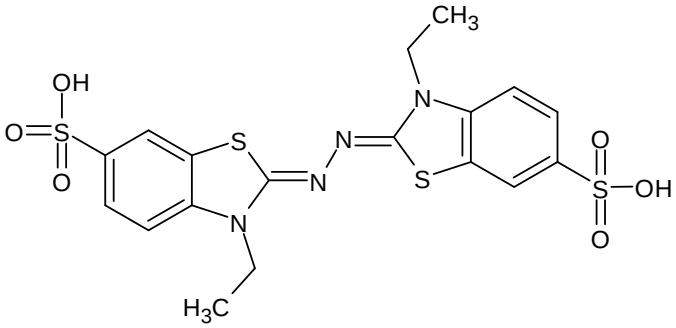
Figure 1: *Chemical structure of some natural laccase mediators*



(2,2,6,6-tetramethylpiperidin-1-yl)oxyl
TEMPO



1-hydroxybenzotriazole
HBT or HOBt



2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ABTS

Figure 6: *Chemical structure of some synthetic laccase mediators*

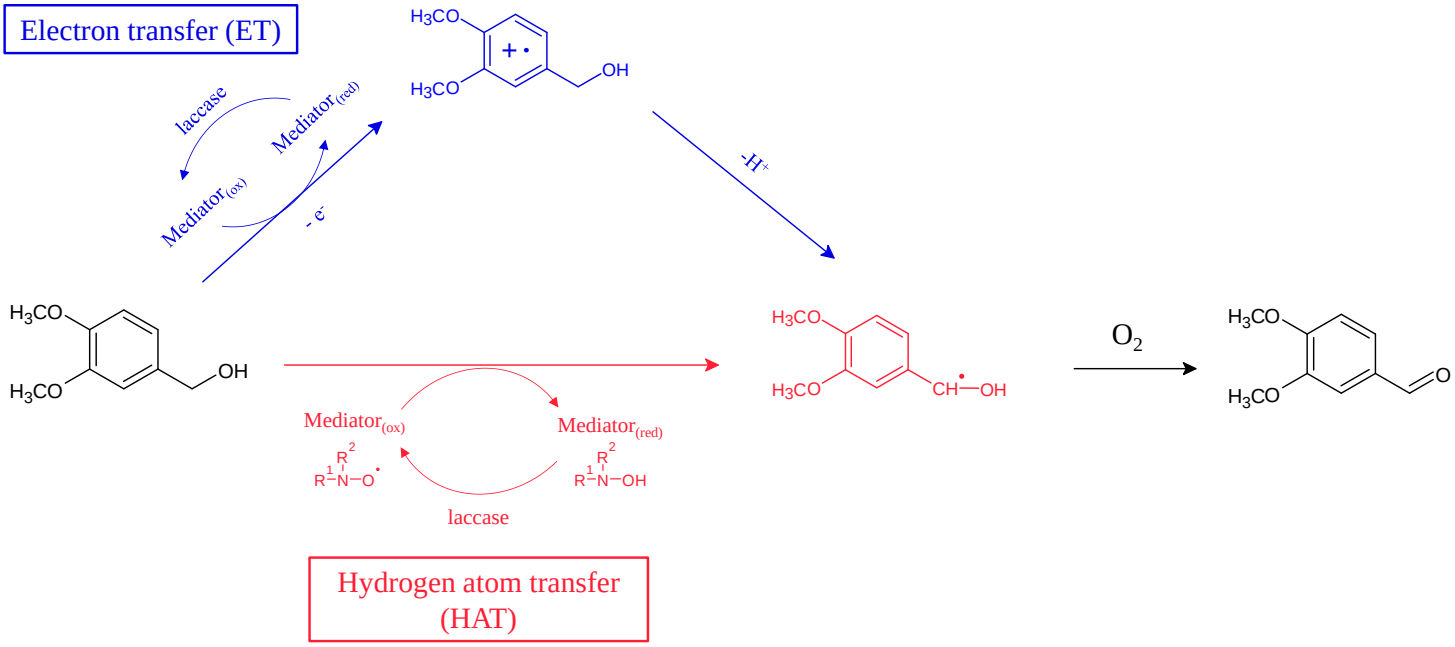


Figure 7: *Possible mechanism for the oxidation of a lignin model compound (veratryl alcohol) by the laccase-mediator system*

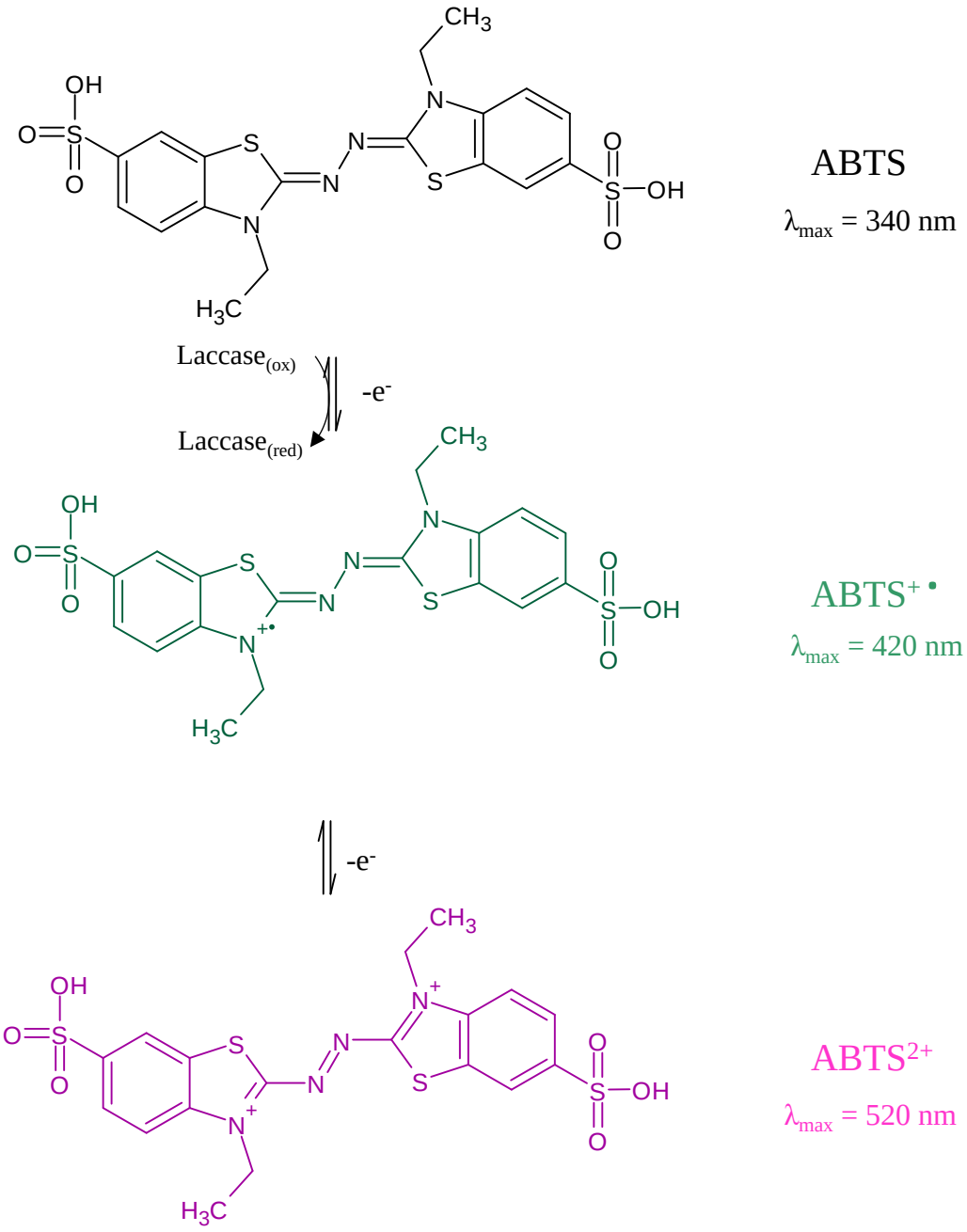


Figure 8: *Oxidation steps of ABTS after laccase action*

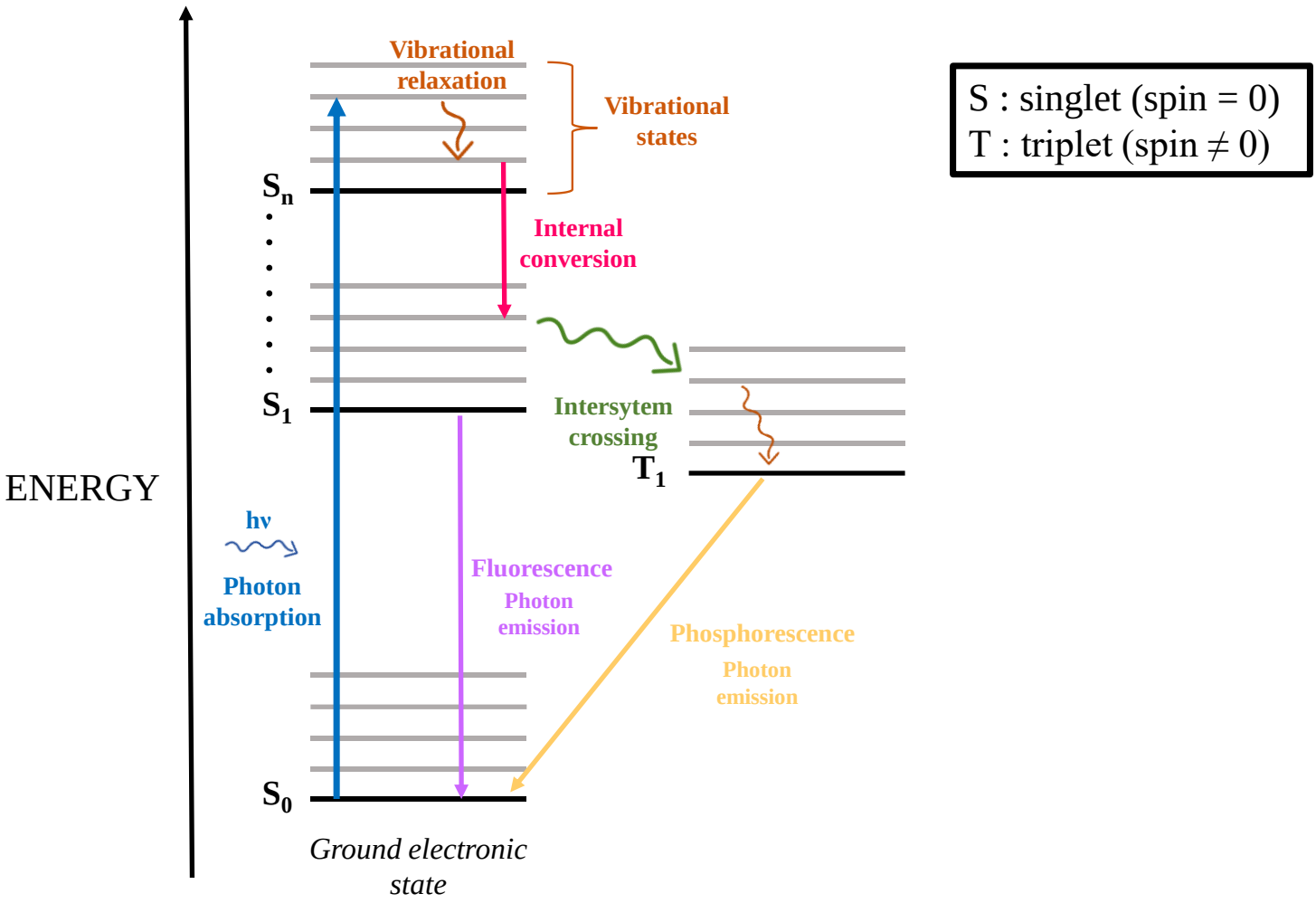
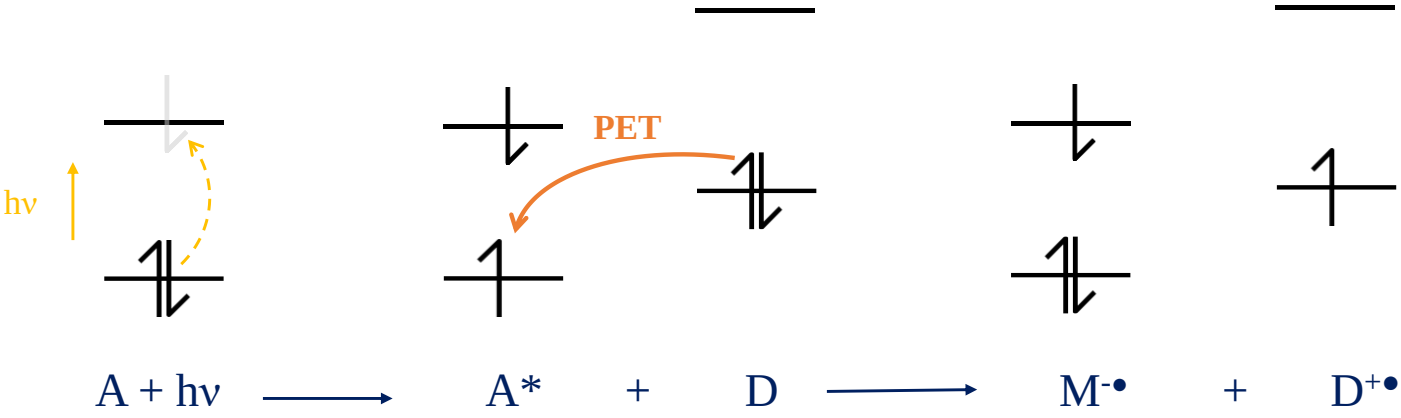


Figure 9: *Jablonski diagram, representation of electronic states of a molecule and the transitions between these states*



A : Acceptor
D : Donnor
LUMO: Lowest Unoccupied Molecular Orbital
HOMO: Highest Occupied Molecular Orbital

***Figure 10**: Principle of photoinduced electron transfer (PET)*