LACCASES AS VERSATILE ENZYMES: FROM INDUSTRIAL USES TO NOVEL APPLICATIONS

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ABSTRACT

The application of enzymes offers an enormous potential in the improvement of existing industrial procedures and in the establishment of new processes for obtaining high-added value products. Enzymes provide cleaner and more efficient industrial processes and contribute to the sustainability concept. In this sense, laccases are very versatile biocatalysts currently used in food, textile and pulp and paper sectors among others. During the last years, scientific efforts have been diverted to the exploitation of such interesting enzymes in novel fields like lignocellulosic biorefineries, biosensors or enzymatic biofuel cells.

This review provides a general vision of the use of laccase enzymes describing their main characteristics and mode of action. Furthermore, their current uses in industrial processes are summarized and the most novel potential application of laccases are revealed. The increasing interest on laccases is also demonstrated by the research efforts on enzyme engineering as it is detailed in this review.

1. INTRODUCTION

Enzymes are used to provide clean and more efficient industrial processes, and to contribute to the sustainable development concept. Enzymatic processes have advantages over traditional chemical processes, including milder reaction conditions (in terms of temperature, pressure and pH) and superior specificity and selectivity, which results in a better use of resources and a lower generation of by-products and waste. Among various enzymes described, laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) represent an interesting group due to their great potential for biotechnological and environmental applications.[1-3] This is mainly due to the simple requirements of laccases (usually substrate and oxygen) together with their apparent stability and lack of inhibition when compared to other enzymes such as peroxidases (peroxidase reductases, EC 1.11.1.x), that can be inhibited by H_2O_2,[4] and due to the possibility of being reused.[5]

Laccases are multicopper-containing oxidases with phenoloxidase activity. They have been described for many years in plants, insects, bacteria and fungi[6] and depending on the source, their physiological functions may differ. Laccases catalyze the oxidation of wide range of substituted phenols, anilines and aromatic thiols, and other aromatic compounds coupled to the reduction of molecular oxygen to water.[7] The catalytic site of laccases involves four copper ions. On one hand, type-T1 copper, known as blue copper, is implicated in the oxidation of the reducing substrate, acting as the primary electron acceptor. On the other hand, type-T2 copper together with two type-T3 coppers form a tri-nuclear copper cluster where the transferred electrons reduce the molecular oxygen to water. Electrochemical potential of type-T1 copper is one of the most significant properties of laccases, varying between 0.4−0.8 V.[8] Plant and bacterial laccases have comparatively low redox potential, whereas the highest values are generally described for fungal laccases.[8] Nevertheless, substrates having redox potential

above 1.3 V, such as non-phenolic molecules, cannot be oxidized by laccases directly. In this sense, certain low molecular compounds, once previously oxidized by laccases, can act as redox mediators (laccase-mediator systems, LMS), aiding laccases to oxidize recalcitrant substrates.[9]

Due to their oxidative versatility, low catalytic requirements, and ability to catalyze degradation or polymerization reactions, laccases have shown huge applicability in different sectors (Figure 1). This review provides a brief overview of laccase enzymes, from its role in nature, including plants growth, cuticle formation in insects, and lignocellulose degradation by microorganisms, among others, until their potential industrial applications in lignocellulosic biorefinery, pulp and paper industry, food and textile sectors, bioremediation, and biosensor applications. Besides, innovative approaches such as the use of laccase in enzymatic fuel cells for wearable devices will be also commented.

The industrial use of laccase enzymes requires that enzyme properties are suitable to maximize their effectiveness. However, laccases are specifically designed for a different purpose than their industrial use. Therefore, different novel engineering approaches have been developed for the optimization and improvement of the catalytic efficiency of laccases. Here, novel biological engineering approaches combining directed evolution, rational design, and/or computational methods will also be discussed.

2. INTERESTING ROLES IN NATURE

As mentioned before, laccases are ubiquitous enzymes that have been found or isolated from higher plants, insects, fungi and bacteria.[6] In plants, laccase play a crucial biological role in lignification. Lignin is essential for plants because it confers integrity

and critical properties required for water transport and structural support.[10] Plants form lignin from monolignols (*p*-hydroxyphenil, H; guaiacyl, G; and syringyl, S*)* that are secreted from cells and activated by oxidation systems located in the cell wall during lignin synthesis (Figure 2). Oxidative enzymes such as O_2-dependent laccases and H_2O_2-dependent peroxidases initiate the assembling of monolignols in plant cell wall. In this context, the characterization of multiple knockout mutants has led to the identification of the role of laccases in the lignification of *Arabidopsis thaliana*.[11,12] Due to their important role in lignin formation, laccase and laccase-specific microRNA genes have been recently identified as interesting targets to modulate lignin content or composition in lignocellulosic biomass.[13-15] However, in spite of the increase number of scientific publications, the mechanisms regulating the spatio-temporal patterning of lignin polymerization still remain unclear.[10]

Laccase enzymes also have an important role in plants that produce seeds. The seed coat of many species contains hydrophobic lignin and laccase enzymes may contribute to release seeds from dormancy to stimulate germination.[16] In this sense, recent studies have proposed the application of fungal laccases to stimulate seed germination via enzymatic scarification. Enzymatic stimulation of germination implies less risk of damage to the embryo, which is of utmost importance for conservation purposes of rare plant or species difficult to cultivate.[16] Besides, laccase-like enzymes produced endogenously in plants have also been involved in browning reactions in seeds due to the formation of quinones and oxidation of phenolic compounds[17] as well as regulate seed size.[18]

Apart from plants, laccases also have interesting roles in animals, more concretely in the phylum Arthropoda. Arthropods body is covered by a cuticle, mainly composed of chitin and proteins, that works as exoskeleton serving as barrier to

maintain homeostasis. Laccase catalyze the oxidation of catecholamines that is a crucial reaction for hardening the insect cuticle.[19]

Multicopper oxidases, among which laccases form the largest subclass, have been also found to help pathogens to survive during an infection and are considered necessary determinants of virulence. The effect of laccases on virulence against mammals has been extensively studied using *Cryptoccocus neoformans*, which is highly infectious in immunocompromised patients, as model organism.[20,21] Melanin pigment that requires laccases to be synthetized has shown to be one of the important virulence factors in this opportunistic pathogen. By contrast, the exceptional versatility of laccases has also been demonstrated in a research work showing antimicrobial activity of *Pseudomonas putida* laccase against several fungal plant pathogens.[22] In this context, several antimicrobial effects have been identified in some laccase-catalyzed compounds (e.g. cinnabarinic acid, iodovanillin, iodoethylvanillin)[23-25].

Fungal laccases have also shown a significant role in stress management of fungi.[19] As a matter of fact, multiple fold increase in laccase activity was observed when subjecting "white-rot" fungi to different stress factors.[25] In addition these "white-rot" basidiomycetes are able to depolymerize and mineralize lignin by an extracellular and unspecific oxidative enzymatic system that include peroxidases and laccases.[8]

Apart from the antimicrobial activities previously mentioned, laccases have demonstrated great opportunities in the developments of anticancer drugs, antifungal drugs and in the synthesis of melanin, prostaglandin and sedatives.[26] For example, laccase from *Deinococcus* bacterium was applied for the oxidative coupling of katarantine and vindoline to yield vinblastine, which is especially useful in treating leukemia.[27] Actinocin is another anti-cancer drug that could be synthesized using this laccase.[27] Resveratrol, playing a role in the prevention of carcinogenesis has also been

selective oxidized on a preparative scale by laccase.[28] In this sense, gaining knowledge on laccase mode of action is a valuable opportunity for the development of novel antimicrobial therapies and formulation of new drugs in the pharmaceutical sector.

3. LACCASES AS VERSATILE ENZYMES: TRADITIONAL AND NEW INDUSTRIAL APPLICATIONS

3.1. LIGNOCELLULOSIC BIOREFINERY

A sustainable economy based on lignocellulosic biorefineries for the production of sugars-based biofuels and chemicals represents a priority to replace the current fossil-based industry. Lignocellulose, mainly composed by cellulose, hemicelluloses, and lignin, constitutes a compact architecture difficult to disrupt. Lignin acts as a physical barrier that hinders the accessibility to carbohydrates, in addition to promoting unspecific adsorption of hydrolytic enzymes during biomass conversion processes. Laccases (LMS included) are capable of modifying and/or partially eliminating lignin from pretreated biomass, improving the subsequent enzymatic hydrolysis of carbohydrates and reducing hydrolytic enzymes loadings. This property constitutes a powerful biotechnological tool for the complete utilization of lignocellulosic biomass. Furthermore, laccases can also be applied for the detoxification of pretreated biomass by the selective oxidation of phenolic compounds (considered as inhibitors of hydrolytic enzymes and fermentative microorganisms).

3.1.1. Delignification

Most of the biorefinery schemes for lignocellulosic biomass conversion comprise a pretreatment step followed by enzymatic hydrolysis of carbohydrates, resulting in simple sugars that will be the basis for producing biofuels (ethanol and higher alcohols) and chemicals (organic acids, alkenes, lipids and other chemicals) via fermentation.[29]

The effectiveness of pretreatment technologies to improve the enzymatic hydrolysis has been attributed, among others, to the elimination and redistribution of lignin.[30] However, most of the methods to overcome the lignin barrier (e.g. physical technologies as milling; chemical methods especially alkali- and acid-based pretreatments; and physicochemical pretreatments as steam explosion) are very energy-intensive and involve harsh conditions (high temperatures and pressures) or even toxic and hazardous chemicals. As alternative, biological delignification involves low energy demand, low environmental impact, and high product yield.[31] It comprises the selective degradation of lignin by the use mainly of "white-rot" basidiomycetes such as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Ceriporiopsis subvermispora*, *Pycnoporus cinnabarinus* and *Pleurotus ostreatus*.[31] Nevertheless, certain ascomycetes (the endophytes *Ulocladium* sp. and *Hormonema* sp.) and bacterial strains (*Bacillus macernas*, *Cellulomonas cartae* and *Zymomonas mobilis*) have also shown good delignification capabilities.[32,33]

Laccases are lignin specific and show high reaction rates, reducing significantly the delignification process time without any consumption of biomass sugars compared to microorganism populations.[1] The direct lignin oxidation by laccases, restricted to phenolic units (Figure 3), can lead to lignin elimination in lignocellulose. Different fungal laccases, including enzymes with low and high redox potential such as *Myceliophthora thermophila*[34] and *P. cinnabarinus*[35] laccases, respectively; or bacterial laccases from *Streptomyces ipomoea* have shown this ability.[36,37] In addition to lignin removal, laccases can also modify properties of lignin, producing a reduction of unspecific adsorption of hydrolytic enzymes.[1] An increase in the porosity and surface area of laccase-treated materials,[38] as well as a reduction in lignin hydrophobicity,[39]

have been described as the main effects that *Trametes hirsuta* laccase can produce on lignin.

Different pretreated lignocellulosic materials have been subjected to the LMS action (Figure 3), with the chemical mediators 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT) being two of the most widely applied.[1] As a result, a significant lignin degradation by decrease of both aromatic and aliphatic lignin units has been reported with *T. villosa*-HBT system,[40] increasing sugars yields after enzymatic hydrolysis. The improvement of saccharification due to lignin modification by LMS has also been described with *M. thermophila* laccase and HBT, triggering an increase in the amount of secondary OH groups and in the degree of lignin condensation.[41] Alternatively, low cost and environmentally natural mediators derived from lignin (methyl syringate) have also shown their potential in presence of *M. thermophila* laccase for improving delignification and saccharification of woody materials.[34]

3.1.2. Detoxification

Pretreatment of lignocellulosic biomass usually generates certain degradation compounds (furan derivatives, weak acids, and phenols included) that are inhibitors of the hydrolytic enzymes and fermentative microorganisms, compromising sugar production and conversion yields.[30] Although different physical (e.g. filtration and washing, vacuum evaporation, activated charcoal) and chemical (e.g. dithionite and sulfite, $Ca(OH)_2$) approaches have been proposed for minimizing the effect of such inhibitors,[42] biological methods (microorganisms and their enzymes) offer benefits such as lower energy necessities, milder reaction conditions, no chemical addition, and fewer side-reactions.[31]

Laccases have been largely used to detoxify different pretreated materials, avoiding sugar consumption and reducing treatment times.[1] These enzymes act selectively oxidizing phenolic compounds (syringaldehyde, acetosyringone, vanillin, *p*-cinnamic acids included) derived from lignin degradation without affecting furan derivatives and weak acids. Thus, the generated phenoxy-radicals polymerize between them leading to less toxic aromatic compounds (Figure 3). The most common laccases used for this purpose derive from "white-rot" fungi (*T. villosa*, *P. cinnabarinus*, *M. thermophile* and *P. ostreatus*).[31] Nonetheless, certain bacterial laccases from *S. ipomoea* have also shown this ability.[36]

The phenolic removal by laccases boosts the fermentative performance of different microorganisms involved in ethanol production.[31] Generally, the direct addition of laccase to pretreated materials improves the fermentative performance of *Saccharomyces cerevisiae*, xylose-fermenting *S. cerevisiae* strains and thermotolerant strains such as *Kluyveromyces marxianus* CECT 10875, reporting improved yeast growth together with higher sugar consumption rate, ethanol productivity and ethanol yields.[43-45] In addition to ethanol fermentative strains, other microorganisms such as *Clostridium acetobutilycum* and *Clostridium thermocellum*, involved in acetone-butanol-ethanol[46] (ABE) and biohydrogen[47] production, respectively, have also shown positive effects after laccase detoxification.

Laccase detoxification not only improves the fermentative performance of different microorganisms, but can also have a positive influence on other aspects such as the water economy and operation conditions.[1] After laccase detoxification, pretreated materials may not be filtered and washed, therefore saving freshwater and diminishing the amount of wastewater. Moreover, conversion processes can be performed at higher substrate loadings, increasing product concentrations and productivities.

3.2. PULP AND PAPER INDUSTRY

Pulp and paper industry has been dealing with lignocellulosic biorefinery aspects since its early stages, obtaining cellulosic pulps for paper production and generating other high-value added products (e.g. cellulose derivatives, nanocellulose) and energy for pulp mills. The interest for the application of biotechnology in this sector started at targeting energy and chemical reagents savings in mechanical and chemical pulp production, respectively, by the use of "white-rot" basidiomycetes such as *P. chrysosporium* and *C. subvermispora*.[48] Today, laccases and/or LMS are industrially applied in this sector with several purposes as depicted in Figure 1.

One of the most studied uses of biotechnology in the pulp and paper industry is the pulp bleaching process.[3,48] Bleaching focusses on removing and/or modifying compounds that give color to the pulps by means of chemical reagents such as chlorine derivatives, without degrading the cellulose fibers.[49] Lignin, major responsible for the color of the pulps, must be transformed to reduce their light absorption characteristics, or oxidized, reduced or hydrolyzed, to make it soluble. The need to reduce organochlorine compounds generated during conventional bleaching processes has led to the study of less polluting bleaching technologies. In this sense, laccase enzymes and LMS have been extensively applied for environmentally friendly production of high-quality bleached cellulosic pulps avoiding the use of polluting compounds.

Different fungal laccases (from *P. cinnabarinus*, *T. versicolor*, and *Pleurotus eryngii*) and synthetic mediators such as HBT and ABTS have been assayed by Camarero *et al.*,[50] achieving delignification values of up to 90% using a totally chlorine free approach consisting of laccase stage plus hydrogen peroxide bleaching. However, due to high cost of synthetic mediators and the possible generation of toxic species from laccase-mediator reactions, their use is one of the critical factors for the use of laccases

in the bleaching sequences.[51] Alternatively, natural compounds from lignin have also been proposed as mediators in laccase-aided bleaching sequences. As a matter of fact, syringaldehyde, acetosyringone and *p*-coumaric acid have been used as natural mediators in combination of *P. cinnabarinus, T. villosa* or *M. thermophila* laccases increasing the brightness of the pulps after peroxide bleaching.[52,53]

As it is known, xylanases have been used in biobleaching sequences even at industrial scale due to their capacity to enhance conventional bleaching. However, these enzymes do not act directly on the lignin as it is the case of laccases. In this context, not only a typical LMS (*M. thermophila* laccase and acetosyringone as mediator) but also xylanases have been used separately or in combination to bleach soda pulps from olive tree pruning and oil palm.[54,55] Saleem *et al.*[56] even bleached wheat straw pulp using xylanase and laccase together with peroxidase enzyme.

Generally, the low thermostability and the acidic working pH of fungal laccases are two other factors that compromise its use for bleaching processes.[51] Instead, bacterial laccases are able to fulfill the conditions of temperature and pH required by the pulp and paper industry during the bleaching process, so their applicability may be easier than the case of fungal laccases. As a matter of fact, both *Streptomyces cyaneus* CECT 3335 and *S. ipomoea* CECT 3341 laccases were successfully used for the biobleaching of eucalypt pulps.[57,58]

Pitch control and deinking processes are other laccase applications in the field of pulp and paper industry. In this context, *P. cinnabarinus* laccase in the presence of either synthetic or natural mediators has been used for controlling pitch deposits of different woody and non-woody pulps, increasing the quality of final pulps and reducing the pitch accumulation in circuits.[59,60] Moreover, *T. villosa* laccase and HBT as mediator have been also employed for deinking secondary fibers.[61]

In addition to improve the quality of final product, as well as reducing the environmental impact of pulp and paper industry, laccases could increase the sustainability and competitiveness of this industry by revalorizing lignin by-products and obtaining cellulosic fibers with improved or new properties. The depolymerization ability of laccases could transform the lignin polymer into several phenolic and aromatic compounds significantly demanded by food, pharmaceutical and cosmetic industries.[62] On the other hand, emerging materials such as engineering plastics, thickeners, fillers, and adsorbents have been developed by laccase grafting co-polymerization of lignin by-products and acrylic compounds.[63,64] Furthermore, wood composite boards have been produced by cross-linking of phenolic compounds in lignin-based materials via laccase;[65] and modification of cellulosic fibers properties including hydrophobicity[66] and strength and optical properties like color[67] has been achieved by laccase-assisted grafting of phenolic or other compounds. Strikingly, antimicrobial and antioxidant properties could be incorporated using this enzymatic strategy to produce cellulose-based materials for food packaging or sanitary use.[68]

As discussed previously, depending on where the enzymatic stage takes place in the pulp and paper industry, a selection of both appropriate laccases and mediators will be necessary.[51]

3.3. FOOD INDUSTRY

Laccase enzymes have also shown a high efficiency in multiple processes in the food industry (Table 1). They can be used as additives to prevent oxidation reactions in food and beverages during fruit juice processing or baking, and to improve food sensory parameters. In addition, laccases have also been tested for the determination of certain compounds in beverages as well as for the bioremediation of food industry wastewater.[69-72]

3.3.1 Wine and beer stabilization

The application of laccase enzymes for wine stabilization (usually made with physical-chemical adsorbents) implies the elimination of polyphenols avoiding decolorization and flavor alteration. As early as 1986, *Polyporus versicolor* laccase was utilized to eliminate 50% of the polyphenols in the black must, obtaining a stable wine with good flavor.[73] However, in order to not change the organoleptic properties of the wine the elimination of polyphenols by laccases must be highly selective and laccase must be stable at acid pH.[69,74]

The use of laccases to stabilize beer has also been described by some authors with successful results. Laccases from *T. villosa* and *T. versicolor* are able to eliminate polyphenols and reduce undesirable oxygen at the end of beer processing avoiding the formation of haze and facilitating beer conservation.[75,76]

3.3.2 Fruit juice processing

The use of enzymes for the clarification of fruit juices has been studied for years with the aim of eliminating the hazes produced by the interactions between proteins and polyphenols. However, contradictory results have been published regarding laccase. Sammartino *et al.*[77] observed that the laccase-treated apple juice was less stable than the one conventionally treated. Contrary, several authors assayed a filtration step after laccase treatment with good results. More concretely, the use of laccases (e.g. *Polyporus fomentarious* laccase) followed by a cross-flow filtration has been shown as a promising strategy for producing clear apple, pomegranate and sour cherry juice concentrates with high color and flavor stability.[69,71,78]

3.3.3 Baking

The ability of cross-linking polymers in laccases has been one of the reasons for their used in baking. The addition of laccase enzymes gives the dough strength and stability.

Furthermore, laccases increase dough volume and reduce stickiness improving its machinability.[71] In this sense, Renzetti *et al.*[79] reported the ability of a commercial laccase for improving the bread making performance of oat flour due to the increased softness, deformability and elasticity of oat batters. Moreover, the textural quality of oat bread was also enhanced. Manhivi *et al.*[80] found that the total phenolic content of amadumbe dough decreased up to 93% when activity of *T. versicolor* laccase was increased (0–3 U/g flour). Besides, rheological properties of laccase-treated dough were improved due to laccase-catalyzed cross-linking of proteins and polysaccharide esterified with phenolics, and an increase in the elastic character of the dough was obtained.

3.3.4 Food sensory parameters improvement

One of the main priorities identified in the food industry is the good preservation of the final products without physical and/or chemical deterioration. The use of laccase enzymes can help to control the odor, enhance the taste or reduce the generation of some undesired compounds in some food products. In this sense, several patents based on laccases (e.g. *Coriolus versicolor* laccase) have been developed to reduce bitterness and other undesirable tastes in cacao nibs and its products.[81] Reduction of tannin content in cocoa pod husk by *P. ostreatus* laccase has been described to improve its nutritive value when used as ingredient for animal feeds.[82] Moreover, oils, food products that contain oils and other products such as juices, soups, purées, can be deoxygenated by laccases (e.g. *T. villosa* and *T. versicolor* laccases) and lignin derivatives to avoid the formation of undesirable volatile compounds as a result of the reaction of such food products with oxygen.[83-85]

3.4. TEXTILE

Laccases have also been widely used in the textile industry. Their applications in this sector include bioremediation of effluents, cotton bleaching, denim finishing, textile dyeing and coating, and cleansing during cloth-washing to eliminate the odor on fabrics and the detergents.[86,87]

With a similar approach to the pulp and paper industry, laccases and LMS have been used in the textile finishing industry to bleach cotton and indigo-dyed denim fabric, yielding in the later its abrasion effect. With this purpose, Pazarlioğlu *et al.*[88] reported that *T. versicolor* laccase was more effective in denim finishing than other LMS, even in the absence of any mediator compound. In addition to bleaching processes, laccases offer an alternative bio-based catalyst to synthesize novel dye chemicals and simultaneously reduce the environmental footprint of the conventional dye synthetic processes. For instance, Kim *et al.*[89] used a LMS (*M. thermophila* laccase and the mediators K5[SiW11VVO40]·11H2O and H5[PMo10VV2O40]·13H2O) for the oxidative polymerization of catechol, resulting in relatively high molecular weight polycatechol for dyeing of flax fabrics. This dyeing polymer provided better color fixation and color resistance when compared to other LMS systems (e.g. laccase-HBT) or laccase alone. Pezzella *et al.*[90] obtained new mixtures of synthesized dyes with *P. ostreatus* POXA1b laccase by using resorcinol and 2,5-diaminobenzenesulfonic acid (DABSA) as substrates. The resulting mixtures were used for dyeing nylon and wool textiles with good and comparable end quality. Besides coloration, these enzyme-based polymers also confer additional properties including electrical conductivity, antimicrobial and antioxidant behavior in the applied textiles. Furthermore, *in situ* polymerization of phenolic compounds may also be applied to improve substantivity of these new polymers onto the fiber surface. Zhang *et al.*[91] developed an *in-situ* enzyme dyeing method (polymerization of DABSA with *T. versicolor* laccase) obtaining wool fabrics with special pH-responsive,

color-changing and conductive properties. Su *et al.*[92] coated textile fabrics (cotton, wool, and polyethylene terephthalate) with poly(catechol) and poly(p-phenylenediamine) using native and PEGylated *M. thermophila* laccase. The resulting colored fabrics presented high levels of coloration with additional conductive properties and good fastness behavior after washing. These fabrics also showed antimicrobial activity against gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) microorganisms, and exhibited antioxidant properties in terms of ABTS scavenging activity.[93]

3.5. BIOREMEDIATION

Due to the intrinsic characteristics of laccase enzymes, they show numerous potential applications in bioremediation and removal of environmental pollutants.

Among others, biologically active compounds from drugs, cosmetics or endocrine-disrupting chemicals are considered emerging contaminants that can severely affect the environment and living beings. Some of these compounds may provoke disorders of the nervous, hormonal and reproductive systems and even causing cancer. For this reason, their elimination from the environment is of paramount importance. Anti-inflammatory drugs, antiepileptic and antidepressants are present in high concentration in wastewater and are challenging to be removed completely due to their physicochemical properties and resistance towards biological attack.[94,95] Laccase enzymes can catalyze oxidation-reduction reactions for efficiently biodegrade of some of these contaminants in a very efficient way and have been widely studied to improve the biodegradation of pharmaceutical contaminants (Table 2).[96-99] The structure of the toxic compound will determine its toxicity degree and the appropriated laccase to be used for its degradation. Besides, different factors such as redox potential, reaction temperature, or pH, play a key role in the degradation of contaminants.[95,100,101]

Becker and co-workers studied the degradation of 38 antibiotics and concluded that the phenolic and amine group in amoxicillin could be the reason for the better removal of this antibiotic with laccase.[102] These authors also stated that the presence of methyl or carboxyl groups in enrofloxacin and pipemidic acid could facilitate their removal with laccase despite lacking phenol or amine group. Besides, in spite of the 5–6 phenolic groups present in tetracyclines used in the mentioned study, only a medium removal by laccase treatment was observed.[102]

The biodegradation of the organochlorine pesticide dichlorophen was successfully evaluated using the *Coriollopsis gallica* laccase immobilized on mesoporous nanostructures showing a significant reduction in the cytotoxic and genotoxic effects after laccase treatment.[103] Other chlorine-derived compounds (chlorolignins, chlorophenols, and chloroaliphatics included) contained in bleaching effluents from pulp and paper industry have also been removed by chemical and laccase combination, including fungal (e.g. *Pleurotus sajor* and *Rhizopus oryzae*) and bacterial (e.g. *Bacillus tequilensis* SN4) laccases.[104]

Dye-derived products from textile processing are often carcinogenic and affect the biological and photosynthesis processes in the aquatic environment.[105,106] Effluent treatment by laccases or LMS has been considered a green alternative for the degradation of dyes with diverse chemical structure, usually showing degradation yields of about 100% at mild reaction conditions (pH close to neutral and temperatures of 20-50 °C).[107] Wastewater from the brewing, distillery, and olive industries also have a high environmental impact due to their high phenolic content, high soluble organic matter content, and dark colour.[71] In this sense, Strong and Burgess[108] showed the capacity of a laccase from *Trametes pubescens* to remove color and the total phenolic compounds from different distillery wastewater. Similarly, Berrio *et al.*[109] showed that an

immobilized laccase from *P. coccineus* was able to produce both degradation and polymerization of the phenolic compounds present in olive mill wastewater.

3.6. BIOSENSING

Biosensors are analytical devices developed for the detection of compounds, and analysis of parameters of biological interest. The unique redox properties of laccases, especially those produced from fungi, make them interesting tools to be applied for bioelectrochemical purposes in biosensor devices.[110] The potential of prokaryotic laccases in electrochemistry has been not fully understood yet. However, bacterial laccases seem to exhibit better stability than fungal laccases in response to changes in some parameters such as temperature and pH.[111]

Laccase biosensors have been recently developed to monitor some toxic and/or harmful chemicals like phenolic compounds.[112-114] They have also been tested for immune assays, hormone monitoring in clinical diagnosis and the quantitative analysis of beverages.[115,116] Regarding the latter, several types of laccase biosensors have been developed to determine for instance the tannins in wine[117] or polyphenols in tea infusions and other beverages.[118,119]

Laccases have been also utilized as components in biosensors to detect pesticide and other harmful compounds released in nature or present in food. *T. versicolor* laccase has been used for in situ total phenol estimation in tap water.[120] Besides, an electrochemical sensor based on F,N-doped carbon dots was recently employed for detection of catechol in tap water and lake water.[121] Catechol was also detected using laccase immobilized on titania (TiO_2), TiO_2/Nafion and only Nafion.[122] In this case, the biosensor based on TiO_2/Nafion/laccase presented the best electro-chemical properties with regard to sensitivity, stability and detection limit. Different carbamates, some of

them acting as endocrine disruptor compounds, have been successfully detected in food samples by means of laccase biosensors.[123,124]

Recently, Coelho *et al.*[125] have developed a laccase biosensor for the determination of dopamine in pharmaceutical injection and synthetic biological samples, showing a good selectivity even in the presence of uric acid and ascorbic acid, as well as other phenolic compounds.

Another very innovative use of laccase is their utilization in bioelectrochemical devices such enzymatic biofuel cells (EBFCs). EBFCs convert biofuel into electrical energy, by utilizing the chemical energy of the biochemical pathways catalyzed by enzymes such as electro-catalysts, instead of metal catalysts.[126] EBFCs have been used in implantable devices in the healthcare industry for powering devices like pacemakers,[127] and laccase-based EBFCs have been even proposed as wearable electronic devices such as smart watches, fitness bands and wearable ECG detectors.[128]

The immobilization of laccase enzymes on the surfaces of different materials to constitute a biosensing platform is however, a critical factor in the development of bioelectrochemical sensing devices. In this sense, copper oxide nanoparticles[113], nanocomposites containing molybdenum disulphide[117], graphene quantum dots[115] and ionic liquid membranes[129] have become compatible matrices for laccase immobilization in biosensing devices.

4. NOVEL ENGINEERING APPROACHES ON LACCASE

As reviewed in the aforementioned sections, many industries have benefit from laccases and LMS in a broad variety of processes. To fulfill process requirements and increase their enzymatic performance it may be of utmost importance to subject these catalysts to protein engineering for making them active and stable under specific process

conditions.[130,131] Table 3 summarizes some of the engineering approaches leading to improved catalytic activity of laccases under certain specific conditions.

According to the oxidative versatility and low catalytic requirements, fungal high-redox potential laccases (HRPL) are the most interesting biocatalysts for the different industry sectors. Nevertheless, "white-rot" fungi are less prone to genetic manipulation. For this reason, heterologous expression of HRPLs has been mainly investigated in yeasts (e.g. *Kluyveromyces lactis, Pichia methanolica, Pichia pastoris, S. cerevisiae, Yarrowia lipolytica*) that grow as individual colonies, do not produce endogenous laccases, and secrete the recombinant products directly into the extracellular medium.[130] With the aim of boosting the expression levels of the corresponding recombinant enzymes, heterologous expression of laccase is usually combined with the use of strong promoters, multicopy vectors, and effective signal peptides. The optimization of codon usage, the simultaneous cloning of chaperones needed for protein folding, and the post-translational modifications (e.g. glycosylation, disulfide bonds) are also required approaches in some cases to obtain functional heterologous laccase. Heterologous expression of laccase can also be improved by directed evolution. For instance, Bulter *et al.*[131] subjected *M. thermophila* laccase to ten rounds of error-prone PCR and *in vivo* shuffling, improving both heterologous expression (8-fold) and total activity. Such enhancement was attributed to two mutations in the native signal propeptide and one mutation in the C-terminus.

Laccases have also been subjected to both rational and directed evolution engineering strategies targeting at modulating the redox potential, pH activity profile, thermostability, halide tolerance, and substrate specificity to obtain highly efficient enzymes under specific operational conditions. These engineering methods have been previously reviewed in detail by Pardo and Camarero[132] and Mate and Alcalde[130].

Rational methods involving site-directed mutagenesis allow obtaining new recombinant enzymes by replacing specific residues according to previous structural information of the enzyme. In this context, Wang *et al.*[133] investigated the D501G variant of *Bacillus amyloliquefaciens* laccase, exhibiting better stability and catalytic efficiency during decolorization of indigo carmine. Khodakarami *et al.*[134] constructed several mutants increasing the activity towards ABTS and substrate specificity to both ABTS and siringalzadine. Site-direct mutagenesis has also been combined with computational methods to predict substrate binding and electron transfer on each variant. With a computer-aided engineering method, Santiago *et al.*[135] introduced two point mutations (N207S/N263D) in the active site of a chimeric laccase, improving oxidation of aniline and N,N-dimethyl-p-phenylenediamine (DMPD) substrates. Semi-rational approaches such as saturation mutagenesis have also been applied to construct all potential mutant variants (or a representative selection of amino acids) from a single or multiple (combinatorial saturation) targeted codons.[136]

Both rational and semi-rational mutagenesis methods have been often applied to substitute amino acids located in the substrate binding pocket or in the proximity of the catalytic copper sites. However, directed evolution studies have identified alternative mutations in non-catalytic-related positions which have shown to be beneficial for the overall enzyme activity. By combining both semi-rational and directed evolution strategies, Scheiblbrandner *et al.*[137] screened new variants of *Botrytis aclada* laccase to increase its stability and activity at pH 6.5. Following this approach, 4 enzyme variants (3 variants with mutations around the T1 copper and the variant T383I) were obtained with increased specific activity at pH 7.5 and increased thermostability. Another interesting approach for enzyme engineering is the so-called KnowVolution strategy.[138] Having directed evolution, saturation mutagenesis, and computer assisted methods as

basis, KnowVolution identifies improved enzyme variants in only 4 steps and ensures the molecular understanding of improved enzyme properties. Using this strategy, Novoa *et al.*[139] have engineered *Melanocarpus albomyces* laccase (variant L365E/L513M) increasing its activity towards 2,6-dimethoxyphenol at pH 9.8.

Enzyme variants with the desired functions can also be obtained through chimeragenesis and/or enzyme resurrection. The latter allows the heterologous expression of non-specialized ancestral enzymes with promiscuous activities, which favors the subsequent enzyme evolution under desired process conditions.[140] In this case, genes are reconstructed from sequence databases by phylogenetic/inference methods based on bioinformatics. On the other hand, chimeragenesis combines DNA fragments with certain sequence identity (without considering the genetic background) to produce chimeric enzymes with new properties.[141] For instance, Pardo *et al.*[142] used chimeragenesis to obtain a chimeric laccase from the already evolved variants OB1 (obtained from *Coriolopsis* sp. PM1 laccase) and 3PO (obtained from *P. cinnabarinus* laccase), by exchanging D2 domain from OB1 for that of 3PO. The resulting laccase showed high stability to temperature, pH, and organic solvents, while retaining the capacity to oxidize substrates with high-redox potential. Mateljak *et al.*[143] used the SCHEMA-RASPP structure guided recombination *in vivo* to generate a family of thermostable chimeric laccases from three fungal laccase orthologs with about 70% protein sequence identity.

As an alternative to biological engineering methods, enzymes with improved activities and properties have also been attained by subjecting laccase enzymes to low-frequency rotating magnetic field (10–50 Hz) or by pre-incubation with organic solvents (including acetone, methanol, ethanol, dimethyl sulfoxide, and dimethyl formamide).[144,145]

5. CONCLUSIONS AND REMARKS

The versatility and interesting traits present in laccases discussed herein point at the huge potential of using laccases for industrial applications. Besides, the wide number of recent research publications on this topic is a clear probe of the increasing interest on these biocatalysts.

Laccase enzymes have a crucial role in nature and their application in some industrial sectors such as pulp and paper, food processing or textile industry is not new. Notwithstanding, they are progressively gaining attention in new niches such as lignocellulosic biorefineries that are crucial to gradually replace the present industry based on fossil fuels to promote a sustainable economy. Within the biorefinery context, laccases could constitute a powerful tool for the complete utilization of lignocellulosic biomass by means of delignification and detoxification strategies.

Laccases are also offering new opportunities to treat emerging contaminants that can severely affect the environment and consequently living beings and they can be explored as source of anticancer and antifungal drugs among others. Besides, new applications of laccase that were unexplored not long time ago are currently being investigated as it is the case of EBFCs for health-care applications in pacemakers or wearable electronic devices such as smart watches, fitness bands and wearable ECG detectors.

However, to implement laccases in novel industrial applications and increase their effectiveness in current industrial uses it may be of great importance to subject these enzymatic catalysts to protein engineering for making them active and stable under specific process conditions. In this context, cutting-edge research on laccase encloses

novel approaches for enzyme improvement such as chimeragenesis, enzyme resurrection or KnowVolution.

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Table 1. Role of laccases in food industry

Food sector	Enzyme source	Role	Reference
Wine and beer	*P. versicolor*	Wine stabilization by polyphenols elimination	[73]
	T. villosa	Phenol oxidation to avoid haze formation in beer	[75]
	T. versicolor	Clarification and flocculation of crude beer by reduction of undesirable oxygen	[75]
Fruit juice	*P. fomentarious*	Elimination of hazes for producing apple, pomegranate and sour cherry juice concentrates	[69, 71, 78]
Baking	Commercial laccase *(NS 26021, Novozymes)*	Improvement of the bread making performance of oat flour due to the increased softness, deformability and elasticity of oat batters	[79]
	T. versicolor	Phenolic content reduction of amadumbe dough, improving rheological properties of laccase-treated dough	[80]
Food sensory parameters	*C. versicolor*	Reduction of bitterness in cacao nibs	[81]
	P. ostreatus	Reduction of tannin in cocoa pod husk improving its nutritive value	[82]
	T. villosa and T. versicolor	Deoxygenation of oils, food products that contain oils and others such as juices, soups, and purées avoiding undesirable volatile compounds	[83, 84, 85]

Table 2. Biodegradation strategies of persistent pharmaceutical laccase or LMS

Enzyme source	Pharmaceutical compound	Strategy	Biodegradation efficiency	Reference
S. mutabilis	Sulfadiazine Sulfathiazole	50°C, pH 6.0, 1 h with 1mM HBT as mediator	73% 90%	[97]
T. pubescens	Triclosan Diclofenac Naproxec Salicilic acid	25 °C, pH 7	57% 52% 69% 94%	[146]
T. versicolor	Triclosan Diclofenac	26 °C, pH 7	90% 24%	[96]
T. versicolor	Amoxicillin Ampicillin Penicillin Pipemidic acid famethoxazole	25 °C, pH 6, 24 h 0.07m/s flow, in a membrane reactor with syringaldehyde as mediator	90% 90% 90% 60% 99%	[102]
T. versicolor	Sulfapyridine Sulfathiazole	25 °C, pH 4.5, 8 h 135 rpm	100 %	[147]
A. oryzae	Ciprofloxacin	60 °C, pH 6, 5 h, 200 rpm with ultrasound (75 W, 22 kHz, 50% duty cycle)	51%	[148]
P. sanguineus	Triclosan	25 °C, pH 5	92%	[149]
T. atroviride	4-Chlorophenol	30 °C, pH 4.5	30%	[150]

Table 3. Novel engineering approaches on laccase and improvements achieved

Engineering strategy	Enzyme source	Improvements	Reference
Rational mutagenesis	*B. amyloliquefaciens*	- Better stability and catalytic efficiency - 3.5 times higher decolorization of indigo carmine	130
	Bacillus HRO3	- T415I, 4-fold increased catalytic efficiency towards ABTS - T418I and T415G, 1.5-fold increased catalytic efficiency towards ABTS - T415I and T418I, increased substrate specificity to ABTS and syringaldazine	134
	Coriolopsis sp. PM1	- Improved oxidation of aniline and N,N-dimethyl-p-phenylenediamine	135
Combined directed evolution and rational mutagenesis	*B. aclada*	- T831, 2.6-fold increased half-life thermostability - D236E, I424G, L499F increased specific activity by 5-fold at pH 7.5	137
	M. albomyces	- Higher activity (3-fold) towards DMP at pH 9.8	139
Chimeragenesis	OB1 (from *Coriolopsis* sp. PM1) and 3PO (from *P. cinnabarinus*)	- Laccase chimera with higher activity in the presence of ethanol or methanol - Superior half-lives at 50–70 °C - Improved stability at acidic pH and similar catalytic efficiency for DMP - Capacity to solubilization of Kraft lignin	142
	OB1, Lac3 *Trametes* sp. (based on *Trametes* sp. AH28–2) and 3PO	- 5-fold half-life thermal inactivation at 70ºC - Several laccase chimeras with stability at acidic pH	143
Rotating magnetic field	*T. versicolor*	- 10% higher activity	144
Pre-incubation in organic solvents	*Cerrera* sp. RSD1, *T. versicolor, Agaricus bisporus, M. thermophila*	- 1.5- to 4-fold higher activity	145

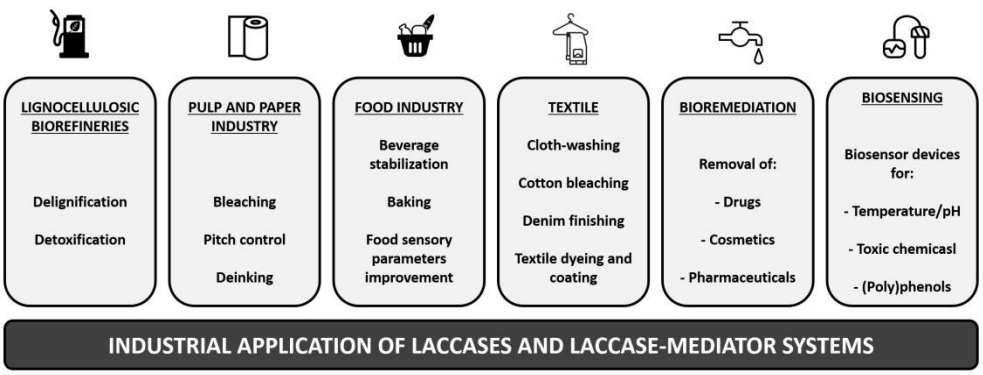

Figure 1. Areas of industrial application of laccase and laccase-mediator systems

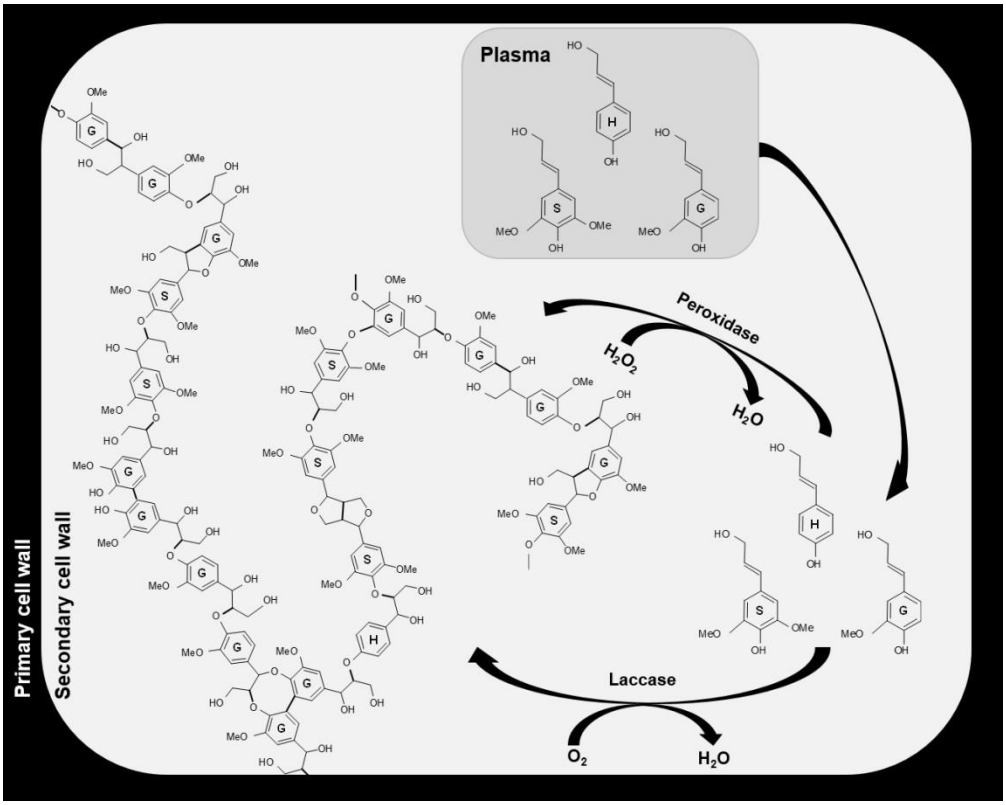

Figure 2. The lignification process in plant cells. After being transported to the cell wall, monolignols (p-hydroxyphenil, H; guaiacyl, G; and syringyl, S, phenylpropanoid units included) are oxidized to radicals by laccases or peroxidases after which they undergo purely chemical radical coupling reactions to polymerize to lignin polymer

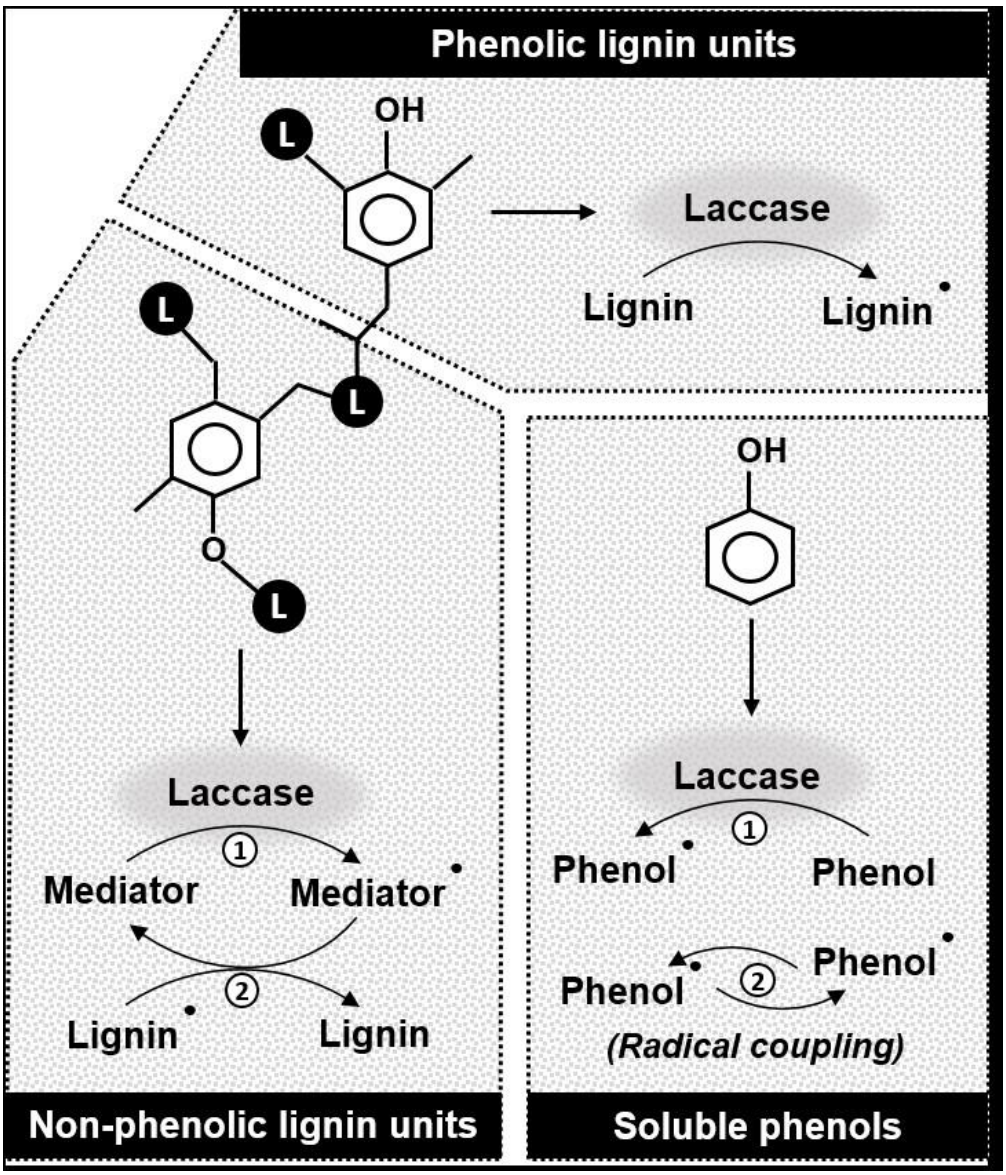

Figure 3. Role of laccase or laccase-mediator systems (LMS) towards phenolic and non-phenolic lignin units and soluble phenols. Catalytic reactions include ether bond degradation, C–C degradation and aromatic ring cleavage as main delignification reactions, while oxidative polymerization is the main detoxification reaction

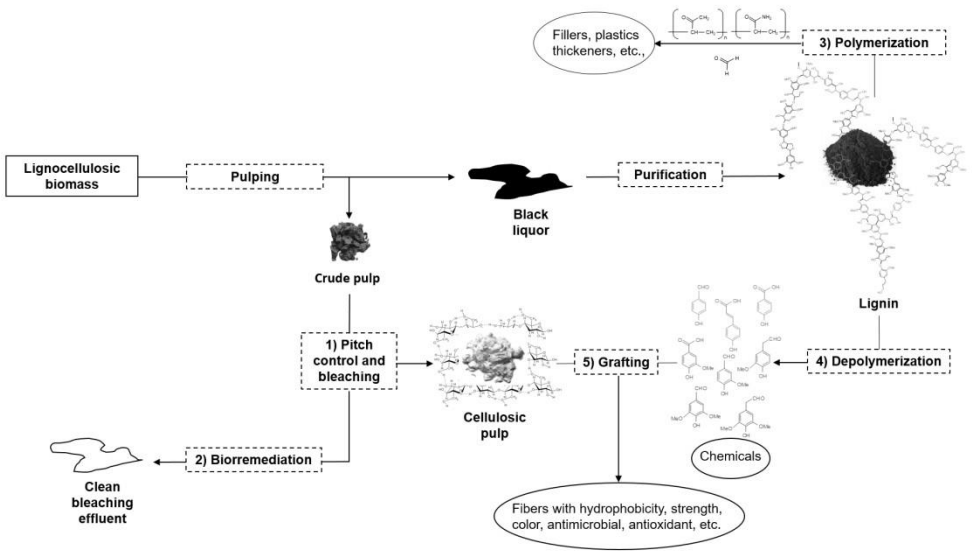

Figure 4. Scheme of laccase and LMS applications in pulp and paper industry: 1) pitch control and bleaching; 2) removal of chlorine-derived compounds contained in bleaching effluents; 3) co-polymerization of lignin waste with acrylic compounds or formaldehyde for production of fillers, plastics, thickeners, etc.; 4) depolymerization of lignin waste to chemicals; and 5) grafting of phenolic compounds or others on cellulosic pulps for new or improved properties such as hydrophobicity, strength color, antimicrobial, antioxidant