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Laccase as Tools for the Synthesis of Bioactive Compounds and Biomaterials: A Review

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ABSTRACT: Filamentous fungi is used as a source of enzyme for biotechnological applications are preferred over bacteria due to their excellent yield and total production volume. Fungal laccase is potent enzyme due to its high redox potential and low substrate specificity to xenobiotics. The biodegradation of polycyclic aromatic hydrocarbons (PAHs) such as pyrene (Pyr) and benzo[a]pyrene (BaP) in soils by laccase are also been reported. Laccase can be effectively used in the bioremediation of environmental effluents with the application of immobilization of enzyme using carriers such as chitosan. Apart from its action in catabolic, depolymerizing and polymerizing processes, laccases have also been shown to be powerful enzymes for coupling two different molecules to create new low-molecular-weight products in high yield. Thus, laccases are increasingly finding applications in biotechnology in the fields of environment-friendly synthesis of fine chemicals and for the gentle derivatization of biologically active compounds e.g., antibiotics, amino acids and antioxidants. Finally, oligomerization and polymerization reactions can lead to new homo- or heteropolymers and biomaterials. These may be useful in a wide range of applications including the production of polymers with antioxidant properties, the copolymerizing of lignin components with low-molecular mass compounds, the coating of cellulosic cotton fibers or wool, the coloring of hair and leathers, or the cross-linking and oligomerization of peptides.

KEYWORDS - biodegradation, biomaterials, biological applications, laccase, oligomerization

I. INTRODUCTION

Laccases of fungal origin are more useful in industrial and biotechnological applications because of their catalytic properties, high redox potentials and low substrate specificity for synthetic dyes, phenols, polyphenols, aryl diamines, anilines, hydroxyindols, methoxy substituted phenols, benzenethiols, inorganic/ organic metal compounds and many other xenobiotics (Kunamneni *et al.*, 2008).

Studies have shown that some Wood Rot Fungi (WRF) contain all three classes of ligninolytic enzymes, whereas others may contain only one or two of these enzymes (Poojary *et al.*, 2012). *Trametes versicolor*, *Pleurotus ostreatus*, *Pleurotus eryngii*, *Agaricus blazei*, and *Clitocybe maxima* are suitable for large-scale production of laccases while *Ganoderma lucidum* is an efficient producer of laccases, Mn oxidizing peroxidases and lignin peroxidases (Aftab and Ahmad, 2015). Martins *et al.* (2011) also reported that fungi such as *Phanerochaete chrysosporium* and *Trametes versicolor*, *Trametes hirsuta*, *Bjerkandera adusta*, *Irpex lacteus*, *Dichomitus squalens*, *Funalia trogii*, *Pycnoporus sanguineus*, *Phlebia radiata* and *Daedalea flavida* are of biotechnological importance, because they carry out enzymatic degradation of a wide variety of recalcitrant compounds including synthetic dyes resulting in detoxification and mineralization.

Globally, more than 100,000 dyes are commercially available with an annual production of over 7×10⁵ tonnes of the dyestuffs (Itodo *et al.*, 2017). Extensive utilization of these occur in diverse industrial processes such as textile and leather dyeing, paper printing and coloured photography, cosmetics, food, pharmaceuticals as well as additives to petroleum products (Gonawala *et al.*, 2014). As a result, approximately 10 - 15% of used dyes are released into the environment as wastewater which contaminates surface water, soils and underground water by infiltration (Hassaan and Nerm, 2017). Among the many classes of synthetic dyes used in the textile and dyeing industries, triphenyl

methane group of dyes such as crystal violet and malachite green are the largest and most commonly used in almost every type of application. Most of the synthetic dyes are characterized by high stability in presence of light and high temperatures, and are fast to detergents and attacks of microorganisms. In addition, they exist in wide arrays of colours along with the ease and low cost of synthesis compared to natural dyes (**Roushdy and Abdel-Shakour, 2011**).

However, many synthetic dyes are toxic, carcinogenic or prepared from known carcinogens or other aromatic compounds that may cause harm to humans, animals or other forms of aquatic resources (**Songserm et al., 2012**). For instance, Malachite green which is employ in dyeing of jute, leather, silk, cotton, wool and ceramics is also a potent antiprotozoan and antifungal. Nevertheless, it is highly poisonous to cells of mammals and has been implicated in promoting the formation of hepatic tumors in rodents as well as reproductive abnormalities in rabbits and fish. When reduced to leucomalachite green, it may persist in tissues of edible fish for extended periods through bioaccumulation, and therefore it is of public health concern in terrestrial and aquatic habitats (**Fallah and Barani, 2014**). The structural similarity of malachite green to other carcinogenic triphenyl methane dyes also raises suspicion of carcinogenicity. Crystal violet on the other hand has been identified as a thyroid and liver carcinogen in rodents while pararosanine is a bladder carcinogen in humans (**Suwannawong et al., 2010**)/

Filamentous fungi as a source of bio proteins for biotechnological applications are preferred over bacteria and yeasts due to their excellent yield and total volume of production. Fungal laccases are useful because of high redox potentials and low substrate specificity to xenobiotics. However, challenges for large-scale utilizations are low enzyme yield and high cost of production. Saw-dust of *Terminalia superba* abounds locally and was utilized as alternative substrate for production using various optimization processes. *Trametes* sp. isolate B7 was isolated and identified using molecular techniques. Optimum pH and temperature for total soluble protein (TSP) and laccase activity were pH 5.0 (3.6 mg/mL, 2356 U/mL) and 25°C (2.3 mg/mL, 2395 U/mL), respectively. Enhanced production of TSA occurred in Cu²⁺ (2.70 mg/L) and Ca²⁺ (2.50 mg/L) at 3 mM. Laccase activity was recorded at 1 - 2 mM Cu²⁺ (2379 U/mL) and 3 - 4 mM Ca²⁺ (2385 U/mL). Productions of TSP and laccase activity were higher using ammonium sulphate and ammonium chloride respectively. Glucose induced the best TSP and laccase activity of 3.6 mg/mL and 2395 U/mL respectively. TSP and laccase activity were best on day 14 (3.6 mg/mL) and day 18 (2395 U/mL) respectively. Percentage degradation of synthetic dyes using crude laccase 1000 U/mL (and 2000 U/mL) were:

Remazol Brilliant Blue Royal 100% (77%), Phenol red 28% (36%), Congo red 75% (64%), and Malachite green 62% (28%) respectively. The ability of laccase to degrade Phenol red and other synthetic dyes without using enzyme mediators made it a valuable tool for biotechnological applications.

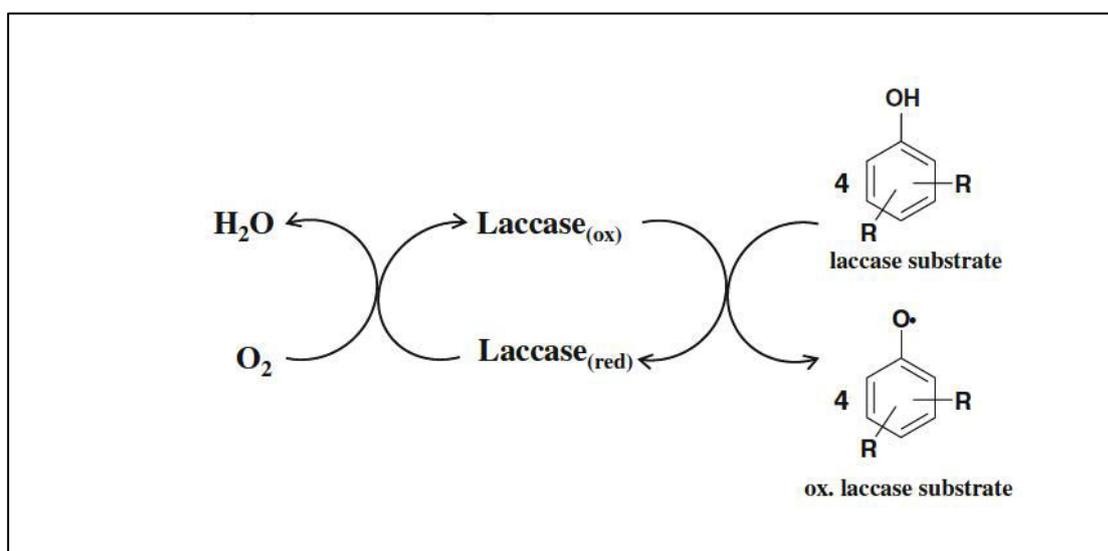
Aromatic compounds and their derivatives are pollutants of high priority because they impact negatively on aquatic organisms even at low concentrations. Treatments of these pollutants can be achieved using conventional methods. However, these methods are expensive, time consuming and often lead to formation of toxic residues (**Villegas et al., 2016**). Several studies have reported the decolorization of synthetic dyes by fungal species using laccase as the main enzyme during the process (**Songserm et al., 2012**). The use of laccase an oxido-reductive enzyme in biological treatment effectively oxidises pollutants to less soluble compounds that are easily removed by sedimentation and filtration (**Moturi and Singara-Charya, 2009**). In addition, biological treatments are less expensive and environmentally-friendly.

II. BIOLOGICAL ROLE OF LACCASE

The biological roles of laccase are diverse (Mayer and Staples 2002). Together with lignin peroxidase, manganese peroxidase, and manganese independent peroxidases, it is aligninolytic enzyme (Leonowicz et al. 2001; Martinez et al.2005). As a result of laccase oxidation, radicals (cationic)can be generated in lignin, and these can cause subsequentaliphatic or aromatic C–C bond cleavage and lignindepolymerization (Hatakka 1994; Bourbonnais et al. 1995;Eggert et al. 1995; Leonowicz et al. 1999; Barreca et al.2003). Laccase seems to be involved also in the depolymerization of coal (Willmann and Fakoussa 1997; Temp etal. 1999; Hölker et al. 2002), humus, and black-colored solutions of humic acids (Cohen and Gabriele 1982;Fakoussa and Hofrichter 1999) depolymerization of plant cell wall material in mycorrhizalsymbiosis (Burke and Cairney 2002). Additionally, di- orpolyhydroxylated monoaromatic, methoxyphenolic, ormonohydroxylated biarylic compounds (laccase sub-strates) such as hydroquinone, catechol, vanillic acid,syringic acid, ferulic acid, or hydroxylated biphenyls canalso be oxidized by laccase (Fig. 1).The one-electronoxidation of hydroxylated aromatic substrates is accompa-nied by the reduction of molecular oxygen to water by thetransfer of four electrons. The oxidation of a reducing substrate by laccase typically involves the loss of a singleelectron and the generation of a free radical (Solomon et al.1996, 2001; Xu 1999). The laccase catalysis is

mediated by the presence of different highly conserved copper binding regions in the enzyme molecule. Many laccases are characterized by the presence of one type-1, one type-2, and two type-3 copper ions. The substrates are oxidized by the type-1 copper, and the extracted electrons are transferred to the type-2/type-3 copper site, where molecular oxygen is reduced to water (Ong et al. 1997; Baldrian 2006). Laccases are divided into “low-redox potential” and “high-redox potential” laccases depending on the structure and properties of the copper center. The high-redox potential laccases occur mainly in basidiomycetes, especially white-rot fungi (Gutierrez et al. 2006; Rebrikov et al. 2006; Quarantino et al. 2007; Cherkashin et al. 2007; Hernandez-Luna et al. 2008), the low-redox potential laccases seem to be widely distributed in molds (Jung et al. 2002), bacteria, insects, and plants. However, detailed studies by Klonowska et al. (2002, 2005) have shown that the white-rot fungus *Trametes* sp. C30 possesses not only the high-redox potential laccase (LAC1) but also two additional low-redox potential laccases (LAC2 and LAC3) with minor activity.

Fig- Catalytic Cycle of Laccase Catalysed Substrate Oxidation



Given their versatility and broad substrate specificity, laccases as a family of copper-containing oxidases catalyzing a variety of oxidations could become among the most important biocatalysts in fungal biotechnology. Because of this, their biochemical properties and molecular evolution are of considerable interest and have been summarized in several reviews (Xu 1999; Claus 2004; Nakamura and Go 2005; Baldrian 2006; Zhuklistova et al. 2008). Their biotechnological applications range from delignification of lignocellulosics (Elegir et al. 2005; Widsten and Kandelbauer 2008), purification of colored waste waters (Duran and Esposito 2000; Ikehata et al. 2004), textile dye decolorization (Shah and Nerud 2002; Wesenberg et al. 2003; Husain 2006), beverage and food treatment (Minussi et al. 2002), the desulfurization and solubilization of coal to their use in enzyme-based biosensors (Marco and Barcelo 1996), and the transformation and inactivation of toxic environmental pollutants (Gianfreda et al. 2006; Hwang et al. 2007). These diverse applications have been extended by the use of laccase-mediator systems applied to refractory substrates which have been reviewed in recent years (Burton 2003; Couto and Toca-Herrera 2006; Riva 2006; Wells et al. 2006; Morozova et al. 2007a, b).

III. LACCASE AS TOOL FOR ORGANIC SYNTHESIS

The laccase reactions presumably proceed by formation of a radical cation, with subsequent deprotonation of the hydroxyl group to give a radical (Fig. 1). The radical may then undergo formation of a quinonoid derivative (Manda et al. 2007) or intermolecular nucleophilic attack by itself to produce homomolecular dimers (Table 1). These dimers can be C–O coupled (defined as type I dimers) or C–C coupled (defined as types II a and II b dimers; Fig. 2 left part). These dimer formation reactions are referred to as “oxidative coupling,” “oxidative condensation,” and “phenolic oxidative coupling,” respectively. After longer reaction times, oligomers and polymers can be generated from the dimers. In addition to these processes, other reaction types which involve transformations without coupling have been described.

These include phenolic oxidation of the substrate resulting in oxidized laccase substrates (Agematu et al. 1993a), oxidative cleavage resulting in lower molecularweight products (Fukuda et al. 2004), and oxidative decarboxylation resulting in decarboxylated laccase substrates (Agematu et al. 1992, 1993b). To use the reactions listed in Table 1, it is necessary to scale up the process and identify possible bottlenecks. Filamentous fungi are able to secrete large amounts of the enzyme laccase, but an efficient production system at bioreactor scale is still lacking (Couto and Toca-Herrera 2007). This has, so far, been one of the main limitations to the use of laccase as a biocatalyst. However, some of the laccases have been cloned and overexpressed and are now commercially available (Berka et al. 1997; Koschorreck et al. 2008). Immobilization of the laccase can reduce the amount of enzyme required for use in the synthesis process, and several methods have been shown to be applicable for this purpose (Palmieri et al. 1994; Crecchio et al. 1995; Duran et al. 2002). However, in some cases, the products synthesized may interact with the immobilization matrix, resulting in decreased yields. Because of this, suitable and efficient production systems at the bioreactor scale are necessary.

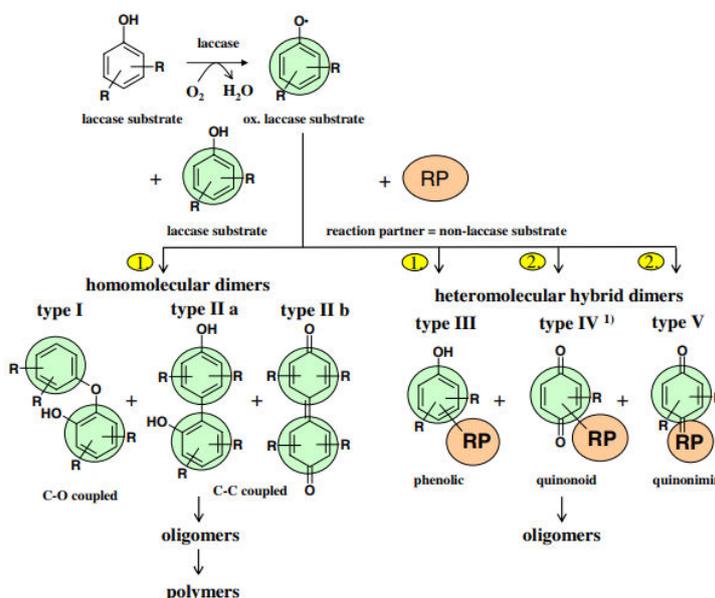


Fig 2 - Formation of homo molecular and hetero molecular dimers (simplified structure).

Several substituents or ring systems. Reaction type =oxidative coupling, oxidative condensation or phenolic oxidative coupling. 2. Reaction type =oxidation coupled with nuclear amination. 1) The formation of type IV dimers requires dihydroxylated laccase substrates; para-dihydroxylated substrates result in para-quinonoid products; ortho-dihydroxylated substrates result in ortho-quinonoid products

IV. APPLICATIONS OF LACCASE FOR THE PRODUCTION OF BIOLOGICALLY ACTIVE COMPOUNDS

Enzymatic transformation of antibiotics Laccase is of great interest in the enzyme-catalyzed production of new antibiotics via phenolic oxidation (Agematu et al. 1993a), phenolic oxidative coupling (Agematu et al. 1993c; Anyanwutaku et al. 1994), and oxidation coupled with nuclear amination (Mikolasch et al. 2006, 2007, 2008c; Table 3). The resulting products were produced for the first time. Whereas the phenolic oxidative couplings of penicillin X derivatives (Fig. 6A) combine two molecules of one antibiotic containing a phenolic moiety to form ortho-ortho C-C, ortho-para C-C/C-O, and ortho C-O coupling products, the phenolic oxidative couplings of hydroquinone and mitramycin (Anyanwutaku et al. 1994) result in the formation of heteromolecular hybrid dimers (Fig. 6B). The antibiotic activity of the formed products was lower than the activity of the substrates. Furthermore, several novel penicillin and cephalosporin derivatives were synthesized by oxidation followed by nuclear amination forming C-N coupled aminoquinonoid heterodimers type IV (Fig. 4C, Mikolasch et al. 2006, 2007, 2008c). The amino quinonoid heterodimers consist of one molecule of the oxidized laccase substrate and one molecule of the reaction partner. All of these products inhibited the growth of several gram positive bacterial strains including methicillin-resistant

Staphylococcus aureus and vancomycin-resistant Enterococci in an agar diffusion assay, and they protected mice against a lethal *S. aureus* infection. Cytotoxicity and acute toxicity of the new compounds were negligible. Another possible use of laccase-catalyzed amination in the field of antibiotic synthesis is the derivatization of new bioactive compounds isolated from biological resources. In this regard, we reported the straightforward laccase-catalyzed amination of dihydroxylated arenes with N-analogous coumarins (Fig. 6C, Mikolasch et al. 2008a). In biological assays, the amination products obtained are more active than the parent compounds. This technology for derivatization of potentially active lead compounds has many advantages over other classical synthetic technologies both because a variety of new substances can be synthesized smoothly within a short time and because sensitive natural substances can be functionalized in this way. In this respect, it is worth noting that active compounds, which are not themselves substrates of laccase, can be linked with the appropriate substrates of the enzyme.

Table 1: Applications of laccase for enzymatic production of antibiotics

Laccase substrate	Reaction	Type of reaction partner	Reaction products/activity	Source of laccase	Reference
7-(4-Hydroxyphenyl-acetamido)-cephalosporanic acid	–	Phenolic	Oxidized substrate/antibiotic (lower than the substrate)	<i>Trametes versicolor</i>	Agematu et al. 1993a
Penicillin X methyl ester	–	Phenolic	Homomolecular dimers type I, type II, and similar to type VI	<i>Trametes versicolor</i>	Agematu et al. 1993c
pivaloyloxymethyl ester	Coupling		(Fig. 6A)/antibiotic (lower than the substrate)		
Hydroquinone	Mitramycin	Phenolic	Heteromolecular dimers type III	<i>Polyporus anceps</i>	Anyanwutaku et al. 1994
	Oxidative		(Fig. 6B)/antitumor antibiotic		
	Coupling		(no activity)		
2,5-Dihydroxybenzoic acid derivatives	Ampicillin or amoxicillin with nuclear Amination	Oxidation coupled	Heteromolecular dimers type IV/antibiotic (comparable to ampicillin and amoxicillin)	<i>Trametes spec.</i> from ASA Spezialenzyme	Mikolasch et al. 2006
2,5-Dihydroxybenzoic acid derivatives	Cefadroxil or cefalexin with nuclear	Oxidation coupled	Heteromolecular dimers type IV (Fig. 4C)/antibiotic (comparable to cefadroxil or cefalexin)	<i>Trametes spec.</i> from ASA Spezialenzyme, <i>Myceliophthora thermophila</i>	Mikolasch et al. 2007
	Cefaclor	amination	Heteromolecular dimers type IV (Fig. 4C)/antibiotic	from Novozymes	



(comparable)

Derivatization of amino acids The enzymatic derivatization of pure amino acids by use of laccase was achieved by oxidation coupled with nuclear amination of dihydroxylated laccase substrates with amino acids (Table 4, Fig. 4D). The main products were identified as heteromolecular dimer type IV and demonstrate that laccase-catalyzed C–N coupling occurred on the α -amino group. During the incubation of L-phenylalanine with para-hydroquinone using laccase as biocatalyst, one or two main products were formed. Dependent on the substitution grade of the hydroquinones, mono- and diaminated products were detected (Fig. 4D). Whereas para-hydroquinone and methyl-para-hydroquinone reacted to the mono- and diaminated products, only monoaminated products were detected in the laccase-catalyzed reaction from di- and trimethyl-para-hydroquinone with L-phenylalanine (Hahn et al. 2008). Both the α - and the ϵ -amino group of the amino acid lysine participates in the laccase-catalyzed C–N coupling (Manda 2006). Hence, these enzymatic reactions may be suitable to cross-link proteins which contain large amounts of lysine and may be important for developing new types of adhesives and biomaterials.

Table 2 Applications of laccase for derivatization of amino acids

Laccase substrate	Reaction partner	Type of reaction	Reaction products	Source of laccase	Reference
Hydroquinone	Glycine	Oxidation coupled with nuclear amination	Heteromolecular dimer type IV	<i>Boletus badius</i>	Michalek and Szarkowska 1959
2,5-Dihydroxy-N-(2-hydroxyethyl)-benzamide	L-Tryptophan	Oxidation coupled with nuclear amination	Heteromolecular dimer type IV	<i>Pycnoporus cinnabarinus</i>	Manda et al. 2006
2,5-Dihydroxy-N-(2-hydroxyethyl)-benzamide	L-Tyrosine	Oxidation coupled with nuclear amination	Heteromolecular dimers type IV	<i>Pycnoporus cinnabarinus</i> , <i>Myceliophthora thermophila</i> from Novozymes	Manda 2006
propionic acid	L-Phenylalanine	Oxidation coupled with nuclear amination	Heteromolecular dimers type IV (Fig. 4D)	<i>Pycnoporus cinnabarinus</i> , <i>Myceliophthora thermophila</i> from Novozymes	Hahn et al. 2008

This table is arranged in chronology of the references

Applications of laccase for the production of polymers and new biomaterials

Although polymerization reactions of phenolic compounds by oxidases and peroxidases have been known to be important for several biosynthetic steps in plants and microorganisms (O’Malley et al. 1993; Williamson et al. 1998; Tsai et al. 1999) and lignolytic enzymes from whiterot fungi are useful for polymerization, detoxification, and diminishing of bioavailability of environmental pollutants (Minard et al. 1981; Rüttimann-Johnson and Lamar 1996; Jonas et al. 2000; Schultz et al. 2001; Uchida et al. 2001), their application in organic and polymer chemistry remains a relative new field in fungal and white biotechnology.

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