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Laccase-mediated oxidation of small organics: bifunctional roles for versatile applications

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Laccases have been widely used in several biotechnological areas, including organic synthesis, bioremediation, and pulp/textile bleaching. In most applications, the enzymatic actions start with single-electron oxidation of small organics followed by formation of the corresponding radicals. These radicals are subsequently involved in either oxidative coupling (i.e., bond formation) or bond cleavage of target organics. These bifunctional actions – catabolic versus anabolic – are readily identifiable in *in vivo* metabolic processes involving laccases. Here, we characterize the bifunctionality of laccase-mediated oxidation of small organics and present the view that knowledge of the biological functions of these metabolic processes *in vivo* can illuminate potential biotechnological applications of this bifunctionality.

Laccases and their substrates

Laccases are copper-containing oxidases that perform the single-electron oxidation of substrates, such as phenols and aliphatic or aromatic amines, to the corresponding radicals at the expense of molecular oxygen. Redox actions of these enzymes are readily found in several biological groups, including prokaryotes, fungi, insects, and plants [1,2]. Depending on the species, laccases are known to be naturally involved in either synthetic or degradation processes. For instance, fungal laccases play a critical role in lignin and humus degradation [3,4]. Plants and insects also use laccase anabolic actions in fiber synthesis and cuticle hardening, respectively [5,6].

Of the laccases available from nature, fungal laccases are of particular commercial interest because such enzymes are secreted extracellularly in response to simple inducers, making their production and purification relatively simple [1]. Genetic manipulation for enhancing yields and redox potentials of laccases is also popular due to diverse industrial needs [2,7]. Laccases use molecular oxygen as an electron acceptor, therefore, they create a less destructive environment than other similar oxidases, such as lignin and manganese-dependent peroxidases, which require enzyme-destabilizing hydrogen peroxide for their catalytic actions [8]. Accordingly, the use of laccases has been highlighted as an environmentally benign approach for several biotechnological oxidative applications, including organic synthesis [9–11], fiber modification/bleaching [12], and bioremediation [4,13,14].

What kinds of organics are potential substrates of laccase enzymes? The copper-containing cavity of laccases that accommodates substrates is wide. Thus, these enzymes are active toward a wide range of compounds, including phenols, polyphenols, anilines, hydroxyindoles, and benzenethiols, allowing several types of organics to be oxidized [1,4,9,10]. Laccases are able to perform their oxidative processes on the surfaces of polymers that display laccase-reactive groups, such as hydroxyphenyl groups [15], but in most cases the enzymatic actions start with single-electron oxidation of low-molecular-weight organics. In nature, small phenolics, such as monolignols and mono- or polyphenolics, are the main substrates for *in vivo* laccase-catalyzed reactions [6,16]. The potential substrates for *in vitro* biotechnological applications of laccases are more extensive. For example, nitroxyl compounds, such as 1-hydroxybenzotriazole (HBT), (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) and violuric acid (VA), and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), in addition to low-molecular-weight phenolics, have been reported as laccase substrates for *in vitro* oxidation reactions [4,9,10,12,14]. Chemical structures of the small organics discussed in this opinion are described in Figure 1.

Several small organics are efficiently transformed into the corresponding radicals by laccases. The radicals *per se* or in conjunction with their target organics subsequently lead to diverse outcomes that can be classified as either synthetic or degradative processes [9,10,12,17,18]. Bifunctional actions (i.e., catabolic or anabolic) involving laccase-catalyzed oxidation of small organics are ubiquitous in laccase-driven *in vivo* metabolic pathways and contribute to different biological functions [3–6,19,20]. This fact indicates that the *in vivo* functions of metabolic pathways can provide new insights into the potential biotechnological applications of the bifunctionality of laccase-catalyzed reaction of small organics.

In this opinion, we argue that the potential biotechnological applications of laccases can be revealed by viewing the oxidations catalyzed by these enzymes through the

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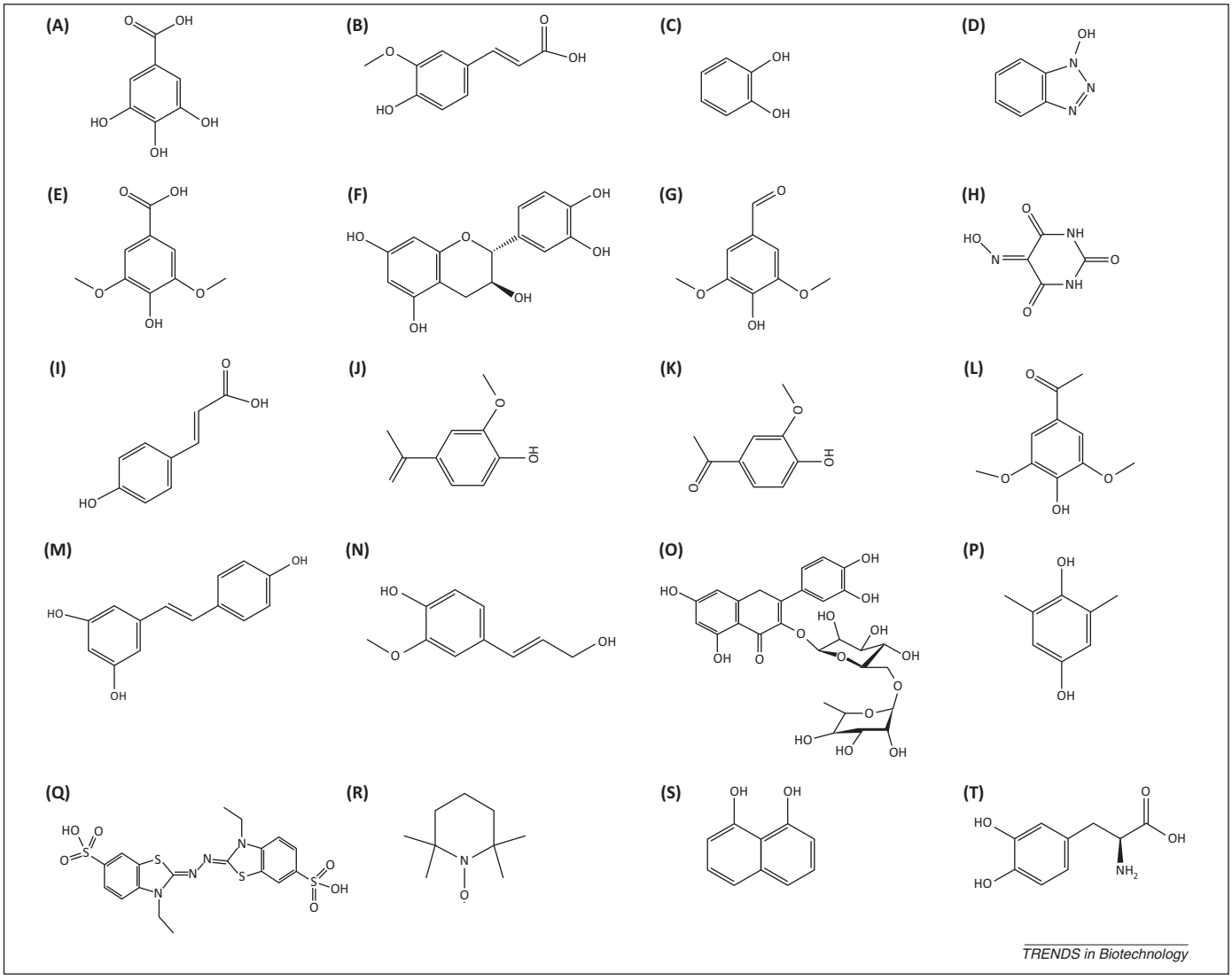

Figure 1. Chemical structures of small organics used for laccase bifunctionality. **(A)** Gallic acid, **(B)** ferulic acid, **(C)** catechol, **(D)** 1-hydroxybenzotriazole, **(E)** syringic acid, **(F)** catechin, **(G)** syringaldehyde, **(H)** violuric acid, **(I)** *p*-coumaric acid, **(J)** vanillin, **(K)** acetovanillone, **(L)** acetosyringone, **(M)** resveratrol, **(N)** coniferyl alcohol, **(O)** rutin, **(P)** 2,6-dimethoxy-1,4-benzohydroquinone, **(Q)** 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), **(R)** (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO), **(S)** 1,8-dihydroxynaphthalene, **(T)** L-3,4-dihydroxyphenylalanine (L-DOPA).

prism of their bifunctional properties *in vivo*. In support of this view, we provide the first characterization of the bifunctionality of laccase-catalyzed oxidation of small organics in a variety of *in vivo* contexts and discuss how *in vivo* metabolic processes and *in vitro* applications involving laccase biochemistry are mutually informative.

Dual roles of low-molecular-weight natural phenolics in metabolic processes involving laccases

Small phenolics are known to be key substrates for *in vivo* metabolic processes involving laccases. Depending on the biological species, phenoxyl radicals produced from laccase-catalyzed oxidations contribute to either morphogenesis via polymerization [5,6,16,20,21] or carbon recycling via depolymerization (Figure 2) [3,4,19,22].

In vivo, laccase-catalyzed anabolic processes generally use low-molecular-weight phenolics as building blocks. Single-electron oxidation by laccases allows the phenolics to be involved in oxidative-coupling reactions, producing homo- or heterodimers. Application of repeated coupling reactions to preformed oligomers leads to highly cross-linked macromolecules, thus contributing to several morphogenetic phenomena [21]. Direct evidence for the involvement of laccase-mediated natural phenolic reactions in anabolic processes in some species has been obtained from studies of genetic mutants. For example, a decrease in laccase-like oxidase expression in seed coats results in an increased concentration of flavonoid monomers, indicating that a deficiency of laccase activity delays oxidative polymerization of natural phenolics [16]. The extent of lignification in plants is also affected by laccase activity. Disruption of laccase genes in *Arabidopsis thaliana* is linked to a significant deficiency of lignification in stems. This observation supports the idea that laccase catalytic actions on natural phenolics such as monolignols contribute to plant lignification [6].

Other examples of anabolic processes involving laccases include cuticle sclerotization and melanogenesis in insects and fungi, respectively. Knockdown of laccase activity in beetles using RNAi affects the morphogenesis of cuticle

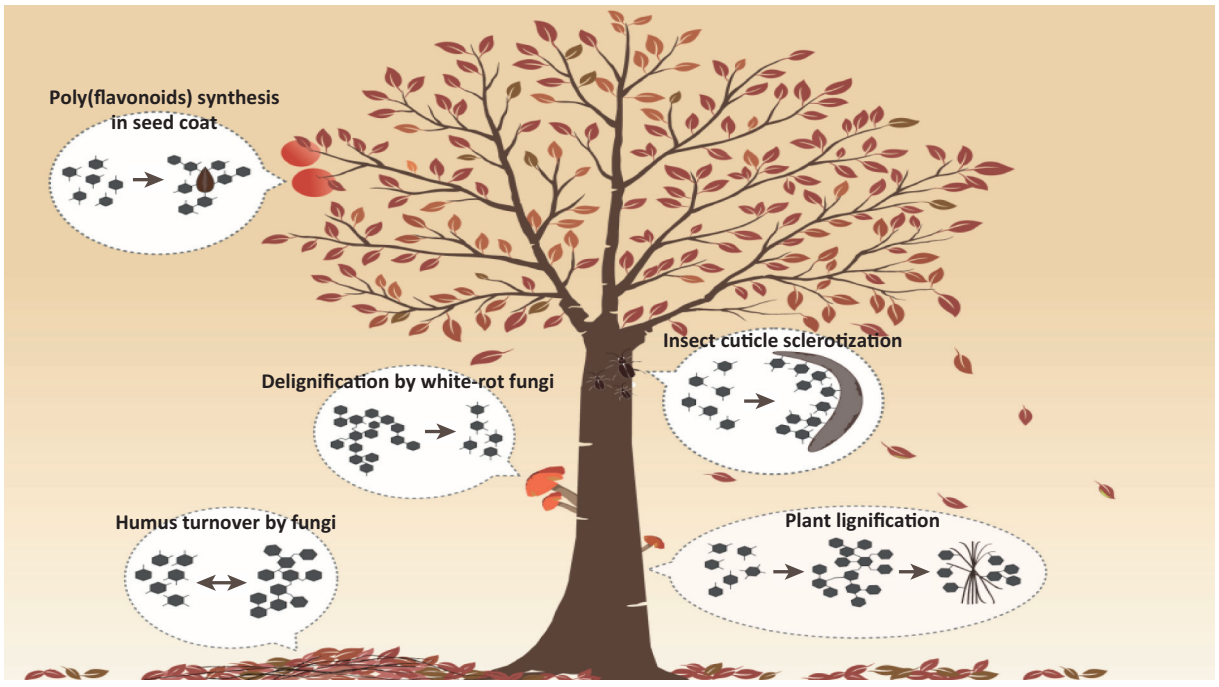

Figure 2. Laccase-catalyzed *in vivo* metabolisms of small phenolics present several biological phenomena regarding morphogenesis and carbon recycling. Plant lignification; small phenolics transported into apoplast during cell wall morphogenesis are polymerized with laccase anabolic actions followed by nonspecific crosslinking to plant fiber components. Insect cuticle sclerotization; small phenolics are cross-coupled with chitin and proteins, thus allowing the cuticle layers to be tightly filled. Poly(flavonoids) synthesis in seed coat; flavonols and condensed tannins are oxidatively polymerized in seed coat, thus contributing to pigmentation and protection against UV irradiation. Delignification by white-rot fungi; nonphenolic portion of lignin might be degraded through laccase-catalyzed redox cycling between lignin and small phenolics. Humus turnover by fungi; laccase-catalyzed oxidative-coupling reactions with biomass-derived small phenolics lead to humification, reversely delignification-like processes result in humus mineralization.

layers, giving rise to beetles with soft, colorless bodies [5]. Biochemical studies also provide critical clues supporting the idea that laccase-catalyzed cross-coupling of natural phenolics, such as catechol, with protein-based matrices is a key step in cuticle sclerotization [20]. Fungi are known to use laccase-catalyzed oxidation of natural phenolics for melanogenesis. Exogenously or endogenously supplied phenolics, such as dihydroxynaphthalene and dihydroxyphenylalanine, are oxidized by laccase activity, followed by subsequent coupling-based polymerization [23]. Beyond the physical barrier characteristics of the polymeric products derived from laccase-catalyzed natural phenolics during morphogenesis, several physicochemical properties, including reactive oxygen species (ROS) scavenging, and chromophore, adhesion, and antimicrobial activity [16,21,24], have been observed. Such properties are closely linked to the biological functions of *in vivo* anabolic processes, such as cell wall integrity, immunity, virulence, pigmentation, and humification [21].

Unlike anabolic processes, catabolic processes involving laccases are apparently restricted to some white- or brown-rotting fungi. Laccase-deficient mutants of these organisms are unable to induce wood decay, supporting the direct involvement of *in vivo* laccase activity in natural depolymerization processes [22]. The need for natural phenolics in laccase-driven wood degradation is highlighted by the fact that the redox potentials of these enzymes are insufficient to oxidize nonphenolic moieties that cover major portions of wood surfaces [1,4]. It has been suggested that two kinds of pathways involving natural phenolics allow laccases to oxidize nonphenolic compounds. First, as demonstrated in *Postia placenta*, laccase-catalyzed oxidation of 2,5-dimethoxyhydroquinone in the presence of ferrous iron (Fe^{2+}) acts through Fenton chemistry to induce the generation of ROS for wood decay [19]. Second, small phenolics capable of diffusing through the 3D architecture of woody matrices act as laccase redox mediators. Laccase-catalyzed redox cycling between phenoxyl radicals and natural polymers gives rise to the oxidized polymers that perform subsequent bond cleavage. The natural phenolics are either secreted from fungi (e.g., 2,5-dimethoxyhydroquinone for brown-rotting fungi) [19] or derived from natural macromolecules (e.g., mono- or polyphenols from lignin for white-rotting fungi) [4,25]. The biological functions of laccase-catalyzed catabolic processes involving small phenolics are closely associated with wood and humus mineralization, and thus contribute to carbon recycling in terrestrial ecosystems.

Which factors are involved in determining whether catabolic or anabolic pathways prevail during laccase-catalyzed natural phenolic reactions? Although scientific evidence is lacking, two factors might be involved. The first is redox potential. For example, extracellularly secreted fungal laccases responsible for catabolic processes exhibit relatively high redox potentials compared with laccases from other species that are mainly involved in anabolic processes [1,9]. The second factor is the stability of the phenoxyl radicals involved in mediating laccase-catalyzed

catabolic processes. For instance, lignin-derived S-type phenols, such as syringaldehyde and acetosyringone, show an enhanced ability to mediate catabolic processes owing to the structural stability and long half-life of their corresponding radicals [26].

Lessons from nature offer insights into the bifunctionality of laccase-mediated oxidation of small organics

Laccases perform both anabolic and catabolic functions using natural phenolics as substrates. These bifunctional actions can be readily reproduced *in vitro* for biotechnological applications. Substrates for *in vitro* reactions can be extended to include non-natural phenolics and nitroxyl compounds in addition to natural phenolics [4,9–12]. Laccase-catalyzed oxidation of small organics gives rise to the corresponding radicals, which are subsequently linked to synthetic or degradative processes.

The main mechanism of action of synthetic processes is oxidative coupling. Homo-coupling of small organics or cross-coupling with different organics results in homo- or heterodimers, respectively [9,10]. Structurally diverse products can be obtained through electron delocalization of organic radicals prior to coupling actions [27]. When polymeric surfaces are provided, laccase-catalyzed coupling of small organics is also useful for grafting reactions that lead to surface functionalization [12,28]. Combining repeated coupling processes with a long incubation time induces the production of homo- or heteropolymeric products. Enzymatic coupling can also induce cross-linking reactions among polymers. Homo/hetero-coupling and cross-linking are key mechanisms in anabolic processes involving laccase, such as lignification, poly(flavonoids) synthesis, and melanogenesis [5,6,16,20,21,23]. More interestingly, key structural features, such as multiple hydroxyphenyl groups, double-bond conjugations, and aromaticities attributed to several *in vivo* functionalities, including chromophore activity, radical scavenging, and adhesion, are similarly synthesized through laccase-catalyzed oxidation of small organics *in vitro* [9,10,21], thus extending the applicability of laccase-driven synthetic processes.

Degradative processes are primarily based on laccase-mediator systems. Two different mechanisms may underlie the mediator ability of small organics: electron transfer and hydrogen atom transfer. The radicals formed from laccase-catalyzed single-electron oxidations restore either hydrogen atoms or electrons from target organics. Mediating ability of ABTS is derived from electron transfer, whereas nitroxyl compounds such as HBT perform redox cycling with target organics through hydrogen atom transfer because of laccase-catalyzed removal of an electron, followed by spontaneous release of a proton from the N-OH type organics [4,29]. An unusual nonradical ionic mechanism has been also reported in the case of the stable nitroxyl radical TEMPO. Laccase-catalyzed oxidation of TEMPO results in the formation of the corresponding oxoammonium ion that is further involved in oxidation of targets [29–31].

Such redox actions leave the target organics oxidized, and are linked to either oxidative bond cleavage or oxidation of nonphenolic functional groups. Fiber bleaching [12,18] and degradation of recalcitrant organics [4,14,26] are feasible through laccase-mediator systems. In the early stage of laccase-mediator system development, synthetic mediators such as ABTS and HBT were highlighted, but insight into laccase-driven, *in vivo* lignin biodegradation has led to the use of lignin-derived small phenolics as mediators [4]. Several synthetic and natural organics are known to act as laccase mediators, but a detailed analysis has shown that bleaching and grafting occur

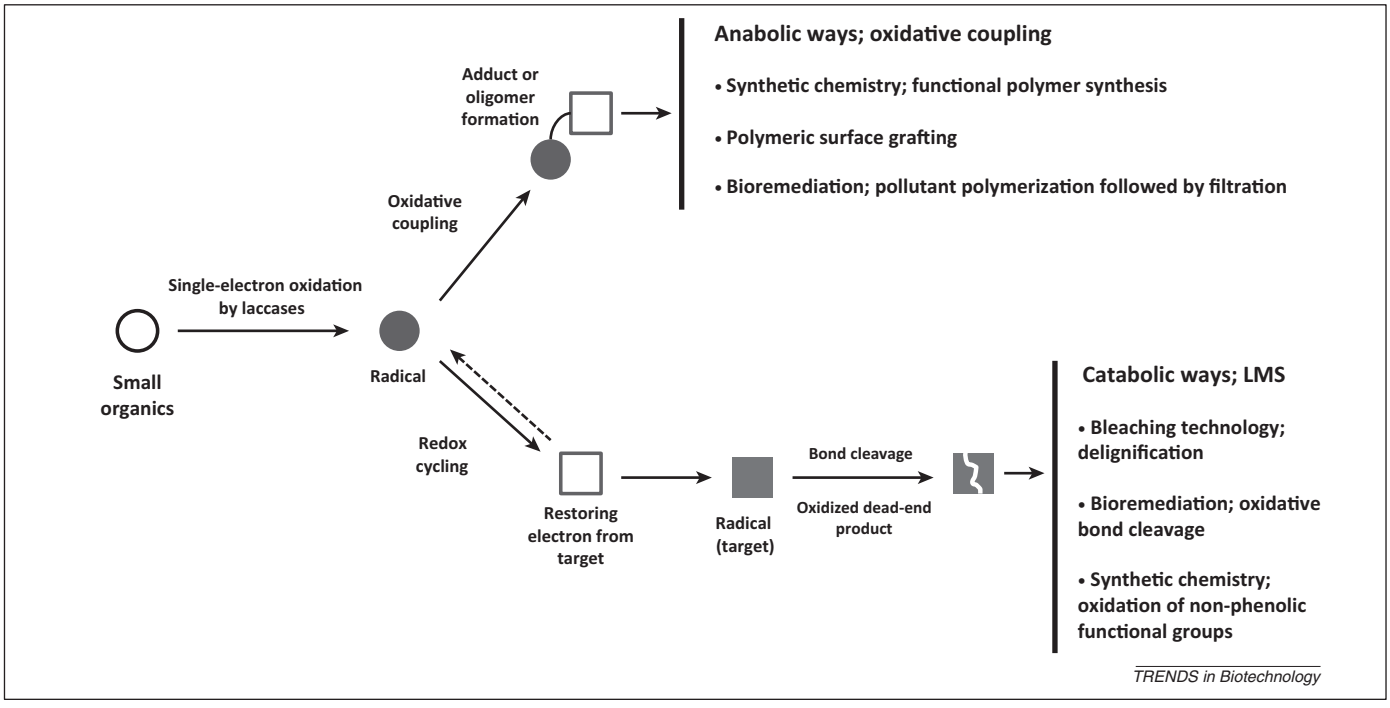

Figure 3. Bifunctional actions of laccase-small organics reactions pave the way for versatile applicability of laccases. Abbreviation: LMS, laccase-mediator system.

simultaneously during laccase-catalyzed oxidation of small organics with polymeric surfaces [12,32]. This indicates that mediating and coupling processes can occur competitively with a single type of laccase substrate. Redox potentials and the structural stability of radicals might govern the dominance of mediating or coupling processes.

Biotechnological applications of laccase-catalyzed oxidation of small organics

The bifunctionality of laccase-catalyzed *in vitro* oxidation of small organics manifests as catabolic and anabolic pathways. It has been demonstrated that the bifunctionality of laccase-catalyzed small organics reactions allows for diverse applications (Figure 3). In this section, we provide specific examples showing how catabolic or anabolic reactions pave the way for laccase applications (Table 1).

Oxidative coupling through laccase-catalyzed reactions of small organics is mainly applicable to organic synthesis and surface functionalization. Some antibiotics, such as actinocin and phenoxazinone, are efficiently synthesized through laccase-catalyzed dimerization of aminophenols involving homo-coupling and cyclocondensation [33]. Naturally occurring bioactive organics are also homo-coupled with laccases. For instance, dimers of *trans*-resveratrol showing ROS scavenging activity are synthesized with laccases [34]. Cross-coupling of different organics is an effective method for making functional heterodimers, as exemplified by Tinuvin, a UV-absorber, which is synthesized with 3-(3-tert-butyl-4-hydroxyphenyl) propionic acid methylester and HBT [35]. Functional polymers are synthesized through oxidative coupling of laccase-catalyzed small organics reactions. The synthesized polymers derived from these reactions have been shown to exhibit ROS scavenging [36], adhesion [37], conductivity [38], and chromophore [10] properties. A focus on individual physicochemical functionalities suggests that the development of certain products in cosmetic, pharmaceutical, and paper mill industries is feasible. For example, using laccase-catalyzed chromophore formation has been suggested as a novel product concept for eco-friendly hair-dyeing agents [10]. As is the case for the synthesis of other synthetic polymers, the molecular weights of the products are controllable by varying the kinds of organic solvents used during enzymatic polymerization [39]. Grafting or conjugation reactions with polymeric materials are also

Table 1. Selected references showing *in vivo* metabolism-inspired applications of laccase-small organics reactions

Small organics	Applicability	Comments on related *in vivo* metabolism	Refs
Gallic acid, syringic acid, ferulic acid, catechin, catechol	Hair dyeing for cosmetic product developments	Laccase-catalyzed oxidative coupling of small phenolics is associated with several pigmentation phenomena including fungal melanogenesis, poly(flavonoids) synthesis of plant seed coats, and tanning processes of beetles. Repeated double-bond conjugation through the polymerizations of small aromatics results in visible light-absorbing chromophore formation.	[10]
Syringaldehyde, 1-hydroxybenzotriazole, violuric acid	Delignification for pulp mill industries	It has been widely accepted that nonphenolic portion of lignin can be degraded with mediating organics of laccase redox actions produced as the byproducts during degradation of phenolic portion of lignin.	[12,18]
Syringaldehyde, coumaric acid, vanillin, acetovanillone, acetosyringone	Degradation of hazardous organics for bioremediation processes	Insight from laccase-involved delignification suggests that laccase catalytic actions can be extended toward nonphenolic compounds with small mediators, indicating that laccase-driven oxidation of small organics leads to degradation and detoxification of a lot of recalcitrant pollutants.	[14,26,43]
Rutin, resveratrol	ROS scavenging for cosmetic or pharmaceutical applications	Enhanced antioxidant capability of *in vitro* laccase-catalyzed oxidative coupling of small phenolics are consistent with the fact that high-molecular-weight phenolics in *in vivo* plant tissues are stronger antioxidants than that of the low-molecular-weight ones, although detailed biological roles of such high-molecular-weight phenolics remain to be determined.	[34,36]
Acetosyringone, gallic acid, coniferyl alcohol, syringaldehyde	Cross-coupling of small organics with hazardous organics for bioremediation processes	Laccase-catalyzed *in vivo* oxidative coupling of small organics gives rise to cross-linked biomacromolecules such as lignin and poly(flavonoids). Decreased solubility with increased molecular weight of co-polymeric products can be employed to separate soluble pollutants from water-containing matrices.	[14,45,46]
2,6-dimethoxy-1,4-benzohydroquinone	ROS generation for bioremediation processes	ROS generation through fungal laccase-catalyzed quinone cycling contributes to wood decay (i.e., delignification and cellulose destruction). Strong oxidative capability of ROS produced from fungi can also destroy recalcitrant anthropogenic organics, indicating that wood-rotting phenomena via ROS oxidation can be applied in bioremediation.	[19,47]

possible through laccase-catalyzed oxidation of small organics [17]. Depending on the small organic used, novel functionalities, such as antibiotics and antioxidants [28,40], are readily introduced into target polymeric products.

Laccase-mediator systems enable enzymes to recruit nonphenolic substrates. Several nonphenolic functional groups, such as alcohols, alkenes, and ethers, are oxidized by laccase-mediator systems, indicating that these systems are valuable tools with applications in organic synthesis [41]. The main application areas of these systems include natural polymer bleaching processes. As suggested by the *in vivo* roles of laccase mediators in lignin degradation, redox cycling of small organics with woody surfaces by laccase-mediator systems allows for delignification processes in biorefinery and pulp milling industries [12,18,32]. In addition to lignin, laccase-mediator systems also contribute to the degradation of free or conjugated pulp sitosterols, thus increasing the brightness of the fibers [42]. Synthetic mediators such as HBT and violuric acid exhibit relatively high bleaching efficiency [12,18], but some natural mediators have proven to be as effective as these synthetic organics [4,42].

One example involving both catabolic and anabolic applications of laccase is bioremediation and detoxification of recalcitrant organic pollutants. Laccase-mediator systems extend the bioremediation potential of laccases to nonphenolic pollutants. Several kinds of pollutants, including synthetic dyes, triclosan, pesticides, and polycyclic aromatic hydrocarbons, are degraded efficiently by these systems [4,14,26,43]. First, laccase-mediator systems dramatically accelerate the kinetics of pollutant removal compared with laccases alone. Second, these systems are able to induce oxidative-bond cleavage of pollutants. For example, the ether bond of triclosan can be cleaved using both synthetic and natural mediators, resulting in the formation of chlorophenols [43]. Transformations of organic pollutants by laccase-mediator systems also contribute to detoxification of hazardous organics, indicating that these enzymatic systems are effective tools for environmental applications [43].

Oxidative coupling is also involved in the cleanup of organic pollutants. Unlike laccase-mediator systems, these coupling processes give rise to adducts or copolymeric products of small organics. This approach is particularly useful for water treatment because polymerization makes the pollutants insoluble in water matrices [44], allowing subsequent filtration or sedimentation steps to readily remove pollutant-containing copolymeric components. The formation of adducts from laccase-catalyzed reactions of small organics with pollutants also leads to bioremediation. As demonstrated in pesticide transformation through laccase-catalyzed small organics reactions [14], mediation and adduct formation can depend on the kinds of target organics. Although mediating ability of syringaldehyde has been demonstrated with constant molar concentration of the mediator during dichlorophen removal, other types of pesticides, such as bromoxynil, mainly induce adduct formation with syringaldehyde, highlighting the complexity of the factors that determine mediating and coupling reactions [14,45,46].

Concluding remarks

The diverse laccase-catalyzed oxidation reactions with small organics are classified into two types: catabolic and anabolic. In catabolic pathways, the small organics act as laccase mediators, facilitating redox cycling between the organics and target compounds. Such laccase-mediator systems can be efficiently applied to fiber bleaching and recalcitrant pollutant removal. By contrast, anabolic pathways are based on oxidative coupling of small organics and give rise to adduct and polymeric products. These coupling processes readily lend themselves to functional organic synthesis and grafting for surface modification. Interestingly, similar bifunctional actions of laccase-catalyzed small organics reactions are observed in small phenolics metabolic pathways involving laccase *in vivo*, indicating that the biological functions of natural processes can inspire novel biotechnological applications of this bifunctionality. As illustrated by the development of natural mediators and new functional polymers through exploitation of laccase catabolic and anabolic pathways, respectively, the most productive avenue for future research into applications of laccase bifunctionality lies in developing a thorough understanding of *in vivo* metabolic pathways involving laccase.

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