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MINI-REVIEW

Laccase catalysis for the synthesis of bioactive compounds

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Abstract The demand for compounds of therapeutic value is increasing mainly because of new applications of bioactive compounds in medicine, pharmaceutical, agricultural, and food industries. This has necessitated the search for cost-effective methods for producing bioactive compounds and therefore the intensification of the search for enzymatic approaches in organic synthesis. Laccase is one of the enzymes that have shown encouraging potential as biocatalysts in the synthesis of bioactive compounds. Laccases are multicopper oxidases with a diverse range of catalytic activities revolving around synthesis and degradative reactions. They have attracted much attention as potential industrial catalysts in organic synthesis mainly because they are essentially green catalysts with a diverse substrate range. Their reaction only requires molecular oxygen and releases water as the only by-product. Laccase catalysis involves the abstraction of a single electron from their substrates to produce reactive radicals. The free radicals subsequently undergo homo- and hetero-coupling to form dimeric, oligomeric, polymeric, or cross-coupling products which have practical implications in organic synthesis. Consequently, there is a growing body of research focused on the synthetic applications of laccases such as organic synthesis, hair and textile dyeing, polymer synthesis, and grafting processes. This paper reviews the major advances in laccase-mediated synthesis of bioactive compounds, the mechanisms of enzymatic coupling, structure-activity relationships of synthesized compounds, and the challenges that might guide future research directions.

Keywords Laccase · Bioactive compounds · Oxidative coupling · Structure-activity relationship

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Introduction

The search for cost-effective methods for producing bioactive compounds is a rapidly widening research niche with their market value predicted to rise by 4.71% between 2013 and 2018 (Infiniti Research Limited 2014). Bioactive compounds are compounds with nutritional benefits and are usually found in small quantities in plants (Kris-Etherton et al. 2002), sponges (Muller et al. 2004), bacteria, and fungi (Debbab et al. 2010). They are mainly secondary metabolites and can be broadly categorized into phenolic compounds, antibiotics, alkaloids, mycotoxins, food grade pigments, and growth factors (Martins et al. 2011). Industrial applications of these bioactive compounds are increasing. Apart from their application in pharmaceutical industries, bioactive compounds are now being employed in the food industry for the production of functional foods (nutraceuticals) (Gil-Chávez et al. 2013), in agrochemicals, cosmetics, geo-medicine, nano-bioscience, and in chemical industries (Guaadaoui et al. 2014).

Some of the presently used methods for extraction and production of bioactive compounds include the heat reflux extraction method, accelerated solvent method, supercritical fluid extraction, employing high-pressure protocols, use of microwave and ultrasound extraction processes, and chemical synthesis (Martins et al. 2011). Conventional physico-chemical processes employed in the production of bioactive

compounds are generally long, energy intensive, low yielding, and associated with excessive amounts of wastes which have a negative impact on the environment. Metrics such as the E factor have highlighted the inefficiencies of chemical synthesis; the amount of waste generated per kilogram of any fine chemical or pharmaceutical product manufactured was 5–100 times higher than the product (Li and Trost 2008). Such concerns have prompted the formation of bodies such as the American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable (ACS GCIPR) to promote the adoption of green technologies in pharmaceutical industries (Constable et al. 2007). Thus, newer, economically feasible, and environmentally benign processes have become a priority in a bid to meet the rising demand for bioactive compounds.

Biocatalysis is gaining notable attention in organic synthesis. This is because biocatalysts are environmentally benign and involve less process steps for the synthesis of valuable compounds. Unlike conventional means, enzymes are characteristically selective, a trait which is of importance when producing compounds of therapeutic value (Maugh 1984). However, laccases are an exception in this respect. Their catalytic mechanism leads to the formation of organic radicals as primary products, which frequently pose a challenge for biosynthesis purposes. While the highest possible yield of just one enantiomerically pure product would ideally be desired, radical processes typically lead to a range of different (and sometimes many) products appearing at rather low concentrations and as racemic mixtures. Laccases are one group of enzymes that have shown encouraging potential as biocatalysts in organic synthesis. Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belong to the multicopper oxidase family of enzymes, and their role in nature involves both synthetic and degradative reactions (Riva 2006). Laccases are generally regarded as "green catalysts" because of their ability to oxidize a diverse range of compounds (including phenols, diphenols, methoxy-substituted phenols, phenolic, and alkyl amines) to corresponding radicals in the presence of molecular oxygen, concomitantly producing water as the only by-product (Kudanga et al. 2011a). Their catalytic mechanism generally involves the abstraction of a single electron from substrates to produce reactive free radicals (Kudanga and Le Roes-Hill 2014). These free radicals are vital intermediates which undergo coupling reactions to produce dimeric, oligomeric, polymeric, or cross-coupling products (Fig. 1). Therefore, the ability of laccases to catalyze oxidative coupling reactions makes them relevant in organic synthesis. Coupling of naturally existing bioactive compounds can result in novel products with enhanced bioefficacy. As a result, in the past two decades, there has been an increase in research activity exploiting laccases in the synthesis of bioactive compounds. Although extensive reviews on the enzymology of laccases (Claus 2004; Madhavi and Lele 2009; Mayer and Staples 2002; Morozova et al. 2007a) and their industrial application potential (Cañas and Camarero 2010; Jeon et al. 2012; Kudanga and Le Roes-Hill 2014; Kudanga et al. 2011a,b; Mikolasch and Schauer 2009; Riva 2006; Rodríguez Couto and Toca Herrera 2006; Witayakran and Ragauskas 2009) have already been published, their application in the synthesis of compounds of therapeutic value has not been comprehensively reviewed in recent articles. This paper provides a consolidated review of the work that has been covered so far in the laccase-catalyzed production of bioactive compounds mainly in the synthesis or modification of phenolic anti-oxidants, antibiotics, and alkaloids. In addition, the reaction mechanisms, structure-activity relationships, and directions for future research are also provided.

Laccase-catalyzed production of bioactive compounds

Laccase applications in organic synthesis have been increasing in recent years mainly because the enzyme has a broad substrate specificity. Phenolic compounds, amino-phenols, polyamines, anilines, aromatic and alkyl amines, and benzenethiols all fall under the laccase substrate range (Kunamneni et al. 2008a; Madhavi and Lele 2009). Compounds carrying these functional groups have therefore become targets for biocatalytic reactions using laccases. The product range is further widened by coupling reactions involving a laccase substrate and a non-laccase substrate (variable reaction partner) to create new heteromolecular hybrid molecules (Mikolasch and Schauer 2009). The most frequently investigated compounds are phenolic anti-oxidants, alkaloids, and antibiotics.

Phenolic compounds

Phenolic compounds are widely distributed in the plant kingdom as secondary metabolites. They have been described as the "*first line in plant defense against infection*" (Matern and Kneusel 1988) because of their physiological role in the protection of plants from infections, harsh environments, and as a response to stress (Bhattacharya et al. 2010). Because of their bioactivity, phenolic compounds present a wide range of nutritional and therapeutic benefits ranging from anti-inflammatory, anti-allergenic, anti-artherogenic, anti-microbial, anti-oxidant, and anti-thrombotic activities and protection against several cardiovascular diseases (Balasundram et al. 2006; Pasha et al. 2013). Therefore, they have been obvious targets for researchers interested in bioactive compounds. Consequently, extensive research has focused on the application of laccase in the synthesis of phenolic compounds.

Laccase oxidation of substrates to their respective radicals is a pre-requisite for the production of dimeric, oligomeric, or polymeric compounds (through homomolecular coupling reactions) or cross-coupling products (through heteromolecular coupling of the radicals) (Kudanga et al. 2011b). Phenolic compounds have been modified mainly through homomolecular coupling (Table 1). Several studies have

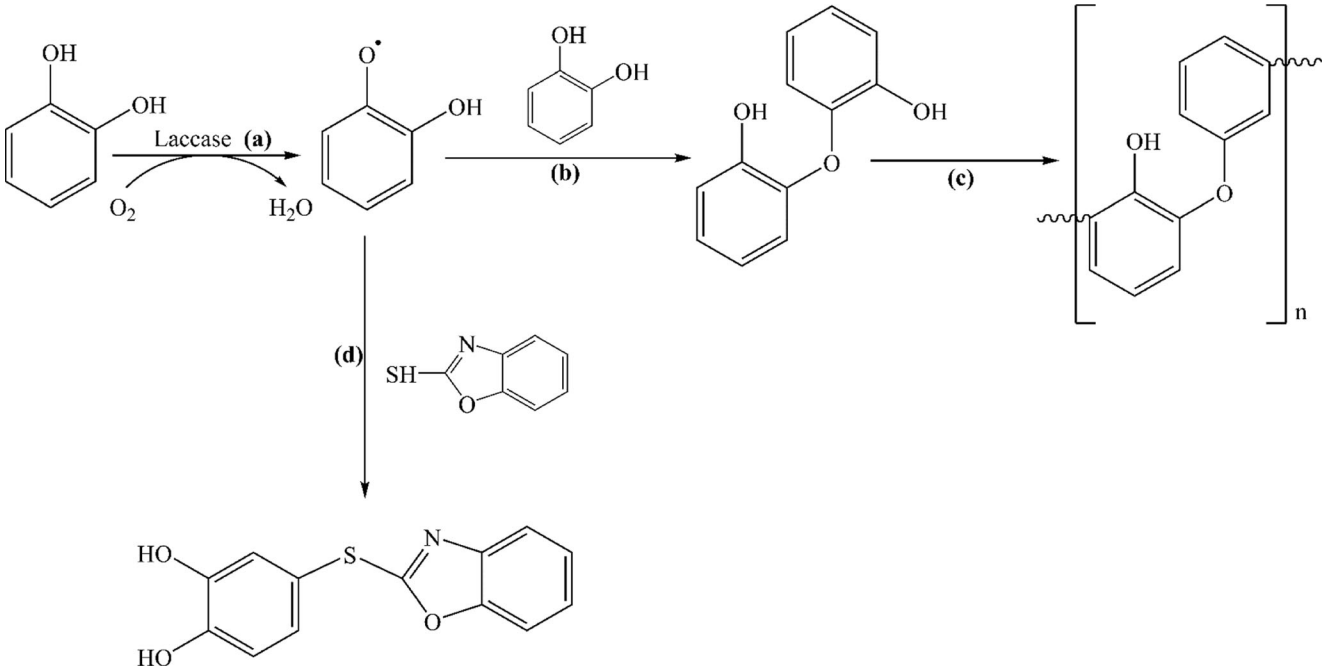


Fig. 1 Laccase synthetic mechanism of action which involves **a** laccase-catalyzed oxidation of substrate to form radicals, **b** radicals undergo oxidative coupling to produce dimers, **c** further coupling results in the formation of polymers through polymerization, and **d** coupling with a non-laccase substrate to form cross-coupling products. Adapted from Abdel-Mohsen et al. (2014) and De Regil and Sandoval (2013), with permission from Royal Society of Chemistry and MDPI

focused on producing novel anti-oxidant compounds through laccase-mediated dimerization of phenolic compounds. Adelakun et al. (2012a,b) used monomeric natural phenolic compounds as laccase substrates for the production of new anti-oxidants. Using ferulic acid as starting material, two derivatives, β-5 and β-β dimers, were successfully produced (Adelakun et al. 2012a). The β-5 dimers showed enhanced anti-oxidant activity, while β-β dimers had lower activity compared to ferulic acid. The enhanced activity of the β-5 dimer was attributed to the increase in electron-donating groups on the compound and the carboxylic acid group with an adjacent unsaturated C–C double bond, which can provide additional sites of attack for free radicals (Srinivasan et al. 2007). In related studies, 2,6-dimethoxyphenol (2,6-DMP) was also used in a laccase-oxidized reaction that resulted in the formation of a symmetrical C–C-linked 2,6-DMP dimer, 3,3′,5,5′-tetramethoxy biphenyl-4,4-diol, with approximately twice the anti-oxidant activity of 2,6-DMP (Adelakun et al. 2012b). During laccase catalysis, 2,6-DMP is oxidized to phenoxy radical species which form para-radical species through resonance stabilization; the dimer is subsequently formed through radical coupling of two para-radical species (Fig. 2). The superior anti-oxidant activity of the dimer was attributed to the increased functional groups with electron-donating capacity (Matsuura and Ohkatsu 2000), the reduction in the O-H bond dissociation energy, and increased stability of radical due to resonance delocalization (Sánchez-Moreno et al. 1998).

Laccase has been successfully used as catalyst for improving the properties of natural phenolic compound rutin. Rutin is naturally a hardly water-soluble flavonoid glycoside. *Myceliophthora* laccase was used as the catalyst to synthesize polymerized rutin (poly(rutin)), which showed significantly improved solubility and radical scavenging properties (Kurisawa et al. 2003a). Rutin is commonly found on the market as a dietary supplement with remarkable anti-oxidant activity. Recent research has revealed rutin as an effective anti-thrombotic agent (Jasuja et al. 2012). Rutin act as an excellent inhibitor of protein disulfide isomerase (PDI), the enzyme which, when secreted rapidly from platelets and endothelial cells, is responsible for thrombosis (blood clotting). The production of poly(rutin), which has already proved to have enhanced properties such as improved solubility, may potentially enhance its biological properties.

Lignans are dimeric forms of phenylpropanoid units that have been identified as one of the primary active groups of *Eucommia ulmoides*, a Chinese traditional medicine that is recognized for its anti-cancer activities (Li and Zhang 2008), anti-oxidant activity (Zhang et al. 2013), antibiotic properties (JI and SU 2008), blood pressure reduction (Greenway et al. 2011), and anti-hypertensive activity (Luo et al. 2004). Wan et al. (2007) used crude *Rhus* laccases (CRL) and purified *Rhus* laccases (PRL) derived from the *Rhus vernicifera* plant in a domino oxidation of phenylpropanoids to produce bioactive compounds. Even though *Rhus* laccases are often marginalized for their low activity, the investigation resulted in the

Table 1 Laccase-catalyzed production of bioactive compounds through homomolecular coupling reactions

Substrate	Substrate category	Source of laccase	Positive effects observed	Potential applications	References
17β-estradiol	Hormone	*T. pubescens* and *Myceliophthora* sp.	C–O and C–C dimers produced were more polar than 17β-estradiol[a]	Pharmaceutical drugs	Nicotra et al. (2004b)
2,6-Dimethoxyphenol (2,6-DMP)	Phenolic compound	*T. pubescens*	A 2,6-DMP dimer produced (20.91% yield) showed 100% increase in anti-oxidant activity compared to 2,6-DMP	Potential use in the development of nutraceuticals and as components for cosmetic products	Adelakun et al. (2012b)
3-Hydroxyanthranilic acid (3-HAA)	Phenolic compound (aminophenol)	*Pycnoporus cinnabarinus* and *Cytisus lirsutus*	Cinnabarinic acid was produced and expressed anti-bacterial activity against all bacterial strains it was tested against	Anti-bacterial compounds	Eggert (1997) and Eggert et al. (1995)
4-Methyl-3-hydroxyanthranilic acid	Phenolic compound (aminophenol)	*T. versicolor*	Actinocin was produced (74% yield). Actinocin has anti-microbial properties	Antibiotics	Osiadacz et al. (1999)
Catechin	Phenolic compound	*T. versicolor*	Hydrophilic linear oligomers with high anti-oxidant activity were produced	Useful as pharmaceutical drugs with anti-oxidant, anti-mutagenic, anti-carcinogenic, anti-viral, and anti-inflammatory properties	Jadhav and Singhal (2014)
(+)-catechin	Phenolic compound	*Myceliophthora* sp.	Poly(catechin) was produced, which showed improved radical scavenging ability and no pro-oxidant properties. It also showed xanthine oxidase inhibitory activity, which was hardly measurable in catechin	Therapeutic agent against oxidative stress	Kurisawa et al. (2003b)
Esculin	Phenolic compound	*T. versicolor*	Oligomeric esculin compounds which registered a 189-times increase in solubility compared to the natural esculin	Anti-oxidant additives for cosmetics, food, and beverages	Anthoni et al. (2010)
Ferulic acid	Phenolic compounds	*T. pubescens*	β-5 dimer produced displayed higher anti-oxidant activity than ferulic acid	Anti-oxidant additives for cosmetic and pharmaceutical industries	Adelakun et al. (2012a)
Ferulic acid	Phenolic compound	*T. versicolor*	Ferulic acid dilactones were produced. However, Adelakun et al. (2012a) reported lower activities compared to substrate	Anti-oxidant additives for food and pharmaceutical products	Constantin et al. (2012a)
Hydroxytyrosol	Phenolic compound	*T. pubescens*	Dimer, oligomers, and polymers of hydroxytyrosol with superior anti-oxidant properties. Dimer had 87.6% radical scavenging ability compared to hydroxytyrosol with 33%	Anti-oxidant additives, also a potential ingredient in skin care products and nutraceuticals	Burton and Davids (2012) and Zwane et al. (2012)
Lysergol/trans-dihydrolysergol	Alkaloids	*T. versicolor*	C-4 hydroxylated derivative of trans-dihydrolysergol and other egort alkaloids were produced. This is the first synthetic functionalization of egort alkaloids at the C-4 position to be reported[a]	Therapeutic drugs	Chirivì et al. (2012)
Phenylpropanoids	Phenolic compound	*R. vernicifera*	Dehydrodiisoeugenol and pinoresinol were produced (yield 8–25%)[a]	Anti-cancer and anti-oxidant drugs	Wan et al. (2007)
Penicillin X	Antibiotics	*T. versicolor*	No positive effect. Dimers of penicillin X produced had lower activity than penicillin X	Antibiotics	Agematu et al. (1993)

Table 1 (continued)

Substrate	Substrate category	Source of laccase	Positive effects observed	Potential applications	References
Rutin (quercetin-3-rutinoside)	Phenolic compound	*M. thermophila*	Poly(rutin) with improved water solubility and improved radical scavenging ability	Anti-thrombotic agent and anti-oxidant	Kurisawa et al. (2003a)
Sesamol	Phenolic compound	*T. versicolor*	A novel sesamol trimer (yield 61%)[a]	Anti-oxidant additive for food products	Constantin et al. (2012b)
Silybin A	Phenolic compound	*T. versicolor*	Symmetric dimer of silybin A produced (yield 87%) had significantly improved radical scavenging activity compared to monomeric silybin A	Anti-oxidant additives	Gavezzotti et al. (2014)
Silybin	Phenolic compound	*T. pubescens*	C-21-C-21′ and C-20-O-C-21′ dimers produced showed anti-radical activity	Anti-oxidant additive	Gažák et al. (2008)
Trans-resveratrol	Phenolic compound	*M. thermophila*	A trans-dehydrodimer produced had improved polarity properties as well as anti-oxidant activity	Anti-oxidant additive	Nicotra et al. (2004a)
Tyrosol	Phenolic compound	*Trametes trogii*	Tyrosol dimer produced showed higher anti-microbial activities compared to tyrosol. The dimer also expressed insecticide activity against *Tuta absoluta*	Antibiotic or insecticide	Chakroun et al. (2013)

[a] Product activity tests were not reported

formation of several compounds of therapeutic importance. Two compounds that were identifiable include pinoresinol (8 and 23.5% yield using CRL and PRL, respectively) and dehydrodiisoeugenol (24.5 and 25% yield using CRL and PRL, respectively) (Wan et al. 2007). Pinoresinol has proven to be an effective anti-inflammatory drug (During et al. 2012; Jung et al. 2010). Research also showed that pinoresinol-rich olive oil had chemopreventive properties (Fini et al. 2008). Dehydrodiisoeugenol is popularly used in treating gastrointestinal disorders (Li and Yang 2012) and can be applied as an anti-oxidant or anti-inflammatory agent (Murakami et al. 2005b).

Myceliophthora thermophila laccase was used as an oxidant in the synthesis of aminonaphthoquinones (Wellington and Kolesnikova 2012). The enzyme catalyzed the amination of 1,4-dihydroxy-2-naphthoic acid with primary aromatic amines by facilitating C–N bond formation. Aminonaphthoquinones are a class of phenolic compounds that are known to have anti-cancer activity. The process resulted in the synthesis of 11 compounds with varying physiological properties. Some of the compounds exhibited high potency when tested against TK10 (renal), UACC62 (melanoma), and MCF7 (breast) cancer cell lines. The compounds also recorded a weak cytotoxicity on HeLa cell lines, highlighting their importance as potential anti-cancer drugs (Wellington and Kolesnikova 2012).

Catechol thioethers have been produced by reacting laccase-oxidized catechol with thiols. Laccase oxidation of catechol produces o-benzoquinone, which subsequently reacts with a thiol by nucleophilic conjugate addition to produce a catechol thioether (Fig. 3) (Abdel-Mohsen et al. 2014). Using 2-mercaptobenzoxazole and 2-mercaptobenzothiazole as thiols, thioester yields in the range of 74–96% were produced at room temperature, atmospheric pressure, and a pH of 6.0 (Abdel-Mohsen et al. 2014). Catechol thioethers have potential application as anti-microbial and anti-oxidant agents (Adibi et al. 2011).

Laccase has also been used in the synthesis of 2,3-ethylenedithio-1,4-quinones by cross-coupling 1,2-ethanedithiol with substituted hydroquinones (Cannatelli and Ragauskas 2015a). The reaction proceeds via sequential oxidation and addition reactions initiated by laccase-catalyzed oxidation of a hydroquinone into the corresponding 1,4-quinone derivative. The highly reactive 1,4-quinones then undergo nucleophilic addition by 1,2-ethanedithiol followed by further oxidation and addition steps to produce the respective 2,3-ethylenedithio-1,4-quinone products (Fig. 4). It was argued that the products are similar to several quinone-containing derivatives of natural compounds which have exhibited anti-tumor and anti-microbial activities (Abraham et al. 2011; Bozic et al. 2010). In related studies, *Trametes villosa* laccase was employed in the α-arylation of benzoylacetonitrile by hydroquinones to produce benzylic nitriles (Cannatelli and Ragauskas 2015b). Benzylic nitriles are

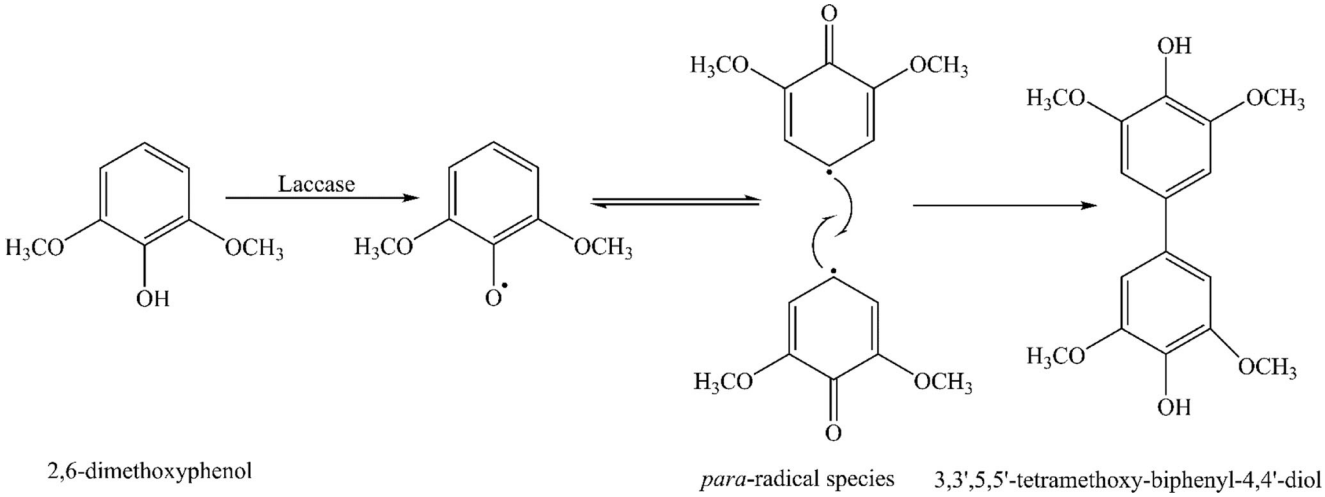


Fig. 2 Proposed reaction mechanism for the homomolecular coupling of 2,6-DMP to produce the C–C dimer (3,3′,5,5′-tetramethoxy biphenyl-4,4′-diol) (Adelakun et al. 2012b). Reprinted with permission from Elsevier

primary ingredients in the production of several pharmaceutical products such as anti-helmintic drugs and analgesics (Kermanshai et al. 2001; Vardanyan and Hruby 2006).

The synthetic reactions of phenolic bioactive compounds as with the ones described below for alkaloids and antibiotics are carried out in appropriate buffers usually in combination with miscible or immiscible organic cosolvents in monophasic or biphasic systems, respectively. Solvents are required to keep the substrates in solution (most are insoluble in aqueous environments), as well as minimize formation of polymeric products which are difficult to characterize. Ethyl acetate is frequently used in biphasic systems (Adelakun et al. 2012a,b; Gažák et al. 2008), while chloroform has also been used in a few studies (Agematu et al. 1993). In monophasic systems, methanol appears to be the most frequently used solvent (Abdel-Mohsen et al. 2014; Mikolasch et al. 2008a; Kurisawa et al. 2003a,b; Burton and Davids 2012; Zwane et al. 2012; Anthoni et al. 2010), while other miscible solvents such as acetone, methanol, dioxane, ethanol, 2-propanol, and n-butanol have also been used in some synthetic reactions (Nicotra et al. 2004a; Kurisawa et al. 2003b). Dimethyl formamide (DMF) can also be used for substrates that are difficult to dissolve but usually at low concentration (due to its high boiling point) in combination with other solvents (Gavezzotti et al. 2014).

Alkaloids

Although laccases have mostly been employed in the development of bioactive compounds of phenolic origin, inroads are being made in other areas such as in alkaloid synthesis. Alkaloids are organic compounds consisting of a nitrogenous moiety and are usually heteocyclic in nature (Pelletier 1983). They are naturally found in organisms as secondary metabolites and are essential for diverse physiological functions such as analgesic, anti-hypertensive, and anti-cancer activities (Roberts and Wink 1998). The ability of laccase to oxidize amines has been exploited in the modification of alkaloids to products with high bioactivities. A *Trametes pubescens* laccase has been used in the coupling of catharanthine and

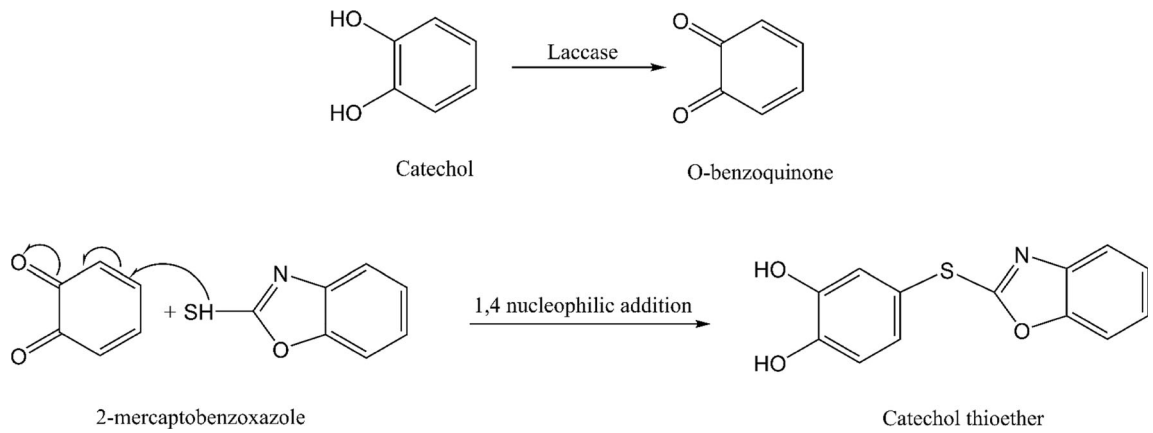


Fig. 3 Proposed reaction mechanism for the heteromolecular coupling of catechol and 2-mercaptobenzoxazole to produce catechol thioethers. Adapted from Abdel-Mohsen et al. (2014), with permission from Royal Society of Chemistry

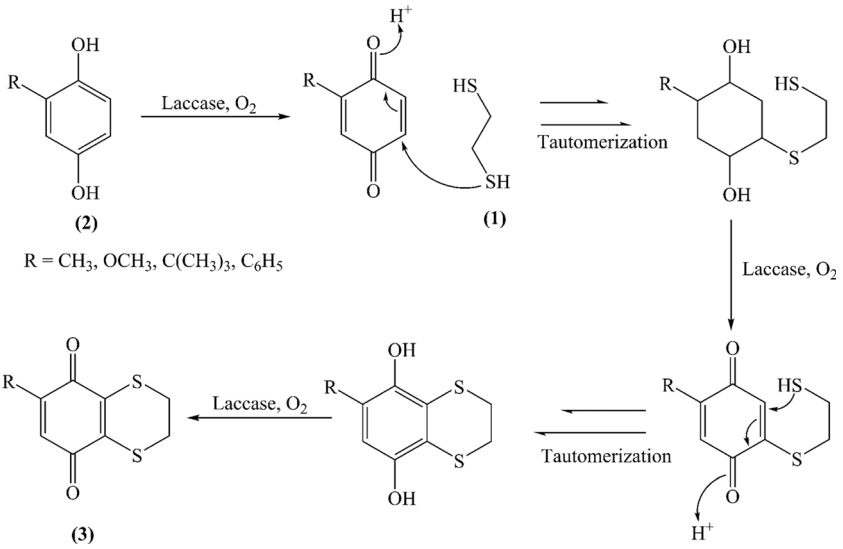


Fig. 4 Proposed reaction mechanism for the laccase-catalyzed reaction of 1,2-ethanedithiol (1) with substituted hydroquinones (2) to produce 2,3-ethylenedithio-1,4-quinones (3) (Cannatelli and Ragauskas 2015a). Reprinted with permission from Elsevier

vindoline to produce anhydrovinblastine (Sagui et al. 2009), an anti-neoplastic bisindole alkaloid which is reportedly useful in production of anti-tumor and anti-cancer drugs (van der Heijden et al. 2004). To date, the 56% yield obtained is the highest, compared to chemical synthesis methods and enzyme cocktail biocatalysis protocols previously employed. The low yields and costly production of bisindole alkaloids have hindered their commercial production, which has resulted in their replacement by semisynthetic analogs. The utilization of laccase thus comes as a welcome alternative that could provide a cost-efficient process that can potentially be scaled-up for industrial production.

Ergot alkaloid (EA) is a class of bioactive alkaloids of therapeutic value and find application as anti-Parkinson drugs, anti-hypertensive agents, cerebral dysfunction therapy, migraine treatment, and anti-prolactin drugs, among other uses (Gerhards et al. 2014). At the turn of the millennium, it was considered rather impossible to engineer a biocatalytic means of producing natural EA derivatives. However, recently, Chirivì et al. (2012) have, for the first time, reported the addition of a hydroxyl group at the C-4 position of the tetracyclic ergoline ring using a laccase obtained from *Trametes versicolor*. Because of the relatively low redox potential of the laccase, a mediator compound would be required for the oxidation of clavine EA with hydroxyl moieties. Surprisingly, the reaction also proceeded in the absence of the 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) mediator. This is because instead of oxidation to occur at the expected terminal CH_2OH site of trans-dihydrolysergol, a mild hydroxylation reaction occurred at the C-4 site, resulting in a 34% yield of the monohydroxylated derivative. The functionalization of EA at this position has not been achieved before even by chemical means (Chirivì et al. 2012). This is of particular importance, considering that many researchers developing EA-derived drugs have been striving to produce EA derivatives with narrowed biospecificity and therefore predictable bioactivity (Mantegani et al. 1999).

Antibiotics

Although there was already a general awareness of the presence of anti-microbial compounds among the scientific community, much interest emanated from the success of penicillin in treating various infectious diseases such as gangrene during the Second World War (Jones and Ricke 2003). Massive bioprospecting for new anti-microbials then led to the discovery of many antibiotics that helped treat diseases that were deemed incurable at the time. One challenge faced in the use of antibiotics is the development of resistance mechanisms by microorganisms, which results in the antibiotic losing its potency. This has become a global concern, with strains such as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (commonly referred to as the ESKAPE pathogens) notorious for devising mechanisms to "escape" the potency of antibiotics (Lewis 2013). Cases involving multidrug-resistant tuberculosis (MDR-TB) have become increasingly recurrent, and according to the World Health Organization, 480,000 cases were reported in the year 2013. Such cases of resistance, coupled with a decrease in discovery of new antibiotics, have caused scientists to consider the option of modifying existing antibiotics to their bioactive derivatives (Aminov 2010; France et al. 2004). Apparently, antibiotic modification dates as far back as the 1970s (Aminov 2010).

Laccase-catalyzed modification of antibiotics was first reported by Agematu et al. (1993). They reported the laccase-catalyzed dimerization of penicillin X. Penicillin X is generally oxidizable by laccase because of a hydroxyl group it possesses. An initial attempt to dimerize penicillin X was

unsuccessful because the products were not stable. Subsequently, the acetylation of the antibiotic resulted in the formation of stable dimers. Although the resulting dimers had no significant improvement in anti-microbial activity and stability, the work opened new avenues of antibiotic research (Agematu et al. 1993).

Subsequent work on laccase-mediated modification of antibiotics has focused mainly on heterocoupling as a way of improving efficacy of antibiotics (Table 2). Mikolasch and coworkers have carried out extensive work on the application of laccase in the production of novel antibiotics (Hahn et al. 2009a,b; Manda et al. 2006; Mikolasch et al. 2012, 2008a,b, 2007, 2006). Unlike the conventional modifications which generally explore the reactivity of moieties to form antibiotic derivatives with improved activity and lower cytotoxicity, they adopted the approach of coupling the existing antibiotics with other bioactive compounds to produce novel compounds with potentially improved therapeutic properties. Using derivatives of gentisic acid to cross-couple amoxicillin or ampicillin, eight novel penicillins were synthesized (Mikolasch et al. 2006). This approach appeared to be highly efficient, with yields of around 98% achieved within 3 h. The produced derivatives showed interesting bioactivity, particularly in vivo efficacy; they were able to protect mice infected with *S. aureus* (ATCC 6538 and 3841) without any signs of intoxication. Although the derivatives did not show a significant improvement in activity compared to amoxicillin or ampicillin, some coupling products were stable against β-lactamases that reduce activity of amoxicillin and ampicillin.

The presence of catechol groups on β-lactam-based antibiotics has been demonstrated to improve antibiotic activity by enhancing antibiotic penetration through the bacterial cell wall (Erwin et al. 1991). Using a laccase-catalyzed amination process, novel antibiotics were obtained by cross-coupling catechols and amino β-lactams such as cefadroxil, amoxicillin, and ampicillin (Mikolasch et al. 2008b). Several novel derivatives of N-analogous corollosporine (Mikolasch et al. 2008a), morpholines (Hahn et al. 2009b), and cephalosporins (Mikolasch et al. 2007) have also been reported (Table 2).

Synthesis of bioactive polymers

Laccase has been used for the functionalization of polymers through grafting reactions. For example, extensive work has been performed on the functionalization of lignocellulose material (Kudanga et al. 2011b, 2010a,b, 2009, 2008; Widsten et al. 2010). Recently, research activities have also focused on functionalization of polymers for the production of bioactive polymers (Table 3). Natural polymers, mainly chitosan, have been extensively investigated for possible grafting with several phenolics as bioactive compounds.

Chitosan is a readily available biopolymer that is usually produced from the deacetylation of crustacean shells such as shrimp and crab shells. It has recently received much attention as a potentially useful bioactive polymer. Although chitosan has been applied in food industry (Shahidi et al. 1999), medical industries (as antibiotics) (Raafat et al. 2008), and in wine industries (to prevent spoilage) (Bagder Elmaci et al. 2015), its application has been limited because of its poor solubility and poor anti-oxidant capacity (Aljawish et al. 2014b; Božič et al. 2013). The poor anti-oxidant property of chitosan is a result of limited number of hydroxyl groups on the biopolymer (Božič et al. 2012b). Grafting of phenolic compounds has been used to enhance the bioactive properties of chitosan. For example, grafting of laccase-oxidized ferulic acid (FA) and ethyl ferulate (EF) onto a chitosan backbone resulted in chitosan derivatives with superior anti-oxidant activity compared to natural chitosan (Aljawish et al. 2014b). Bozic and coworkers also performed a series of studies on chitosan functionalization, using several phenolic compounds such as tannic acid and quercetin (Božič et al. 2012a), gallic acid, and caffeic acid (Božič et al. 2013, 2012b). The resulting chitosan derivatives exhibited enhanced anti-oxidant activity. The derivatives also showed improved anti-microbial activity against *Escherichia coli* and *Listeria monocytogenes* (in the case of gallic and caffeic acid-functionalized chitosan derivatives) compared to the natural biopolymer.

Structure-activity relationships of enzymatically synthesized bioactive compounds

In laccase-catalyzed synthesis of bioactive compounds, the main aim is to produce coupling products exhibiting improved bioactive properties compared to the starting materials. Depending on the intended purpose of the bioactive compound, several structural factors determine the efficacy of the coupling products. In this section, some of these factors are discussed, with reference to the type of bioactive compounds produced.

Anti-oxidants

The bioefficacy of anti-oxidants is usually determined by the structure and stability of the synthesized compound (Table 4). Several researchers have analyzed the structure-activity relationship (SAR) of anti-oxidants (Bendary et al. 2013; Rice-Evans et al. 1996). Firstly, the anti-oxidant must have active groups (e.g., hydroxyl, alkyl, or aniline) (Bendary et al. 2013) attached to the aromatic ring, and the more active groups are present, the more bioactive the anti-oxidant can be (Bendary et al. 2013; Lien et al. 1999). For example, hydroxytyrosol consists of two hydroxyl groups attached to its aromatic ring; however, after a laccase-catalyzed oxidation process, a hydroxytyrosol dimer with four hydroxyl groups is produced (Zwane et al. 2012) (Table 4). This dimer showed a threefold increase in anti-

Table 2 Laccase-catalyzed production of bioactive compounds through heteromolecular coupling reactions

Substrates	Source of laccase	Positive effects observed	Potential applications	References
β-dicarbonyls + catechols	*Pleurotus ostreatus*	1,2-Ddihydroxylated aryl compounds were produced[a]	Potential ingredients in anti-hypertensive and anti-tumor drugs	Pietruszka and Wang (2012)
β-lactam antibiotics + catechols	*Trametes* sp.	New β-lactam-based antibiotics produced showed anti-microbial activity against gram-positive bacteria and drug-resistant *Staphylococcus* and *Enterococcus* strains	Antibiotic drugs	Mikolasch et al. (2008b)
1,4-Hydroquinone + L-phenylalanine	*P. cinnabarinus* and *M. thermophila*	A quinonoid compound was produced by heterocoupling[a]	Pharmacologically active amino acids potentially useful for production of antibiotic and chemotherapeutic drugs	Hahn et al. (2009a)
3-Tert-butyl-1H-pyrazol-5(4H)-1 + catechols	*Agaricus bisporus*	3-Tert-butyl-1H-pyrazol-5-ol derivatives were produced (yield 77–99%)[a]	Bioactive compounds with anti-tumor, hypoglycemic analgesic, anti-pyretic, and anti-inflammatory properties	Emirdağ-Öztürk et al. (2013)
Aminocephalosporins + 2,5-dihydrobenzoic acid derivatives	*Trametes* sp. and *M. thermophila*	Sixteen novel cephalosporins produced showed potency against gram-positive bacteria, including drug-resistant strains of *Staphylococcus* and *Enterococcus*	Antibiotic drugs	Mikolasch et al. (2007)
Benzoylacetonitrile + hydroquinones	*T. villosa*	Benzylic nitrile products were produced[a]	Important final ingredients in bioactive pharmaceutical products	Cannatelli and Ragauskas (2015b)
Catechol + 1,3-dicarbonyls	*M. thermophila*	5,6-Dihydroxylatedbenzo[*b*]furan derivatives produced showed cytostatic effects against renal (TK10), melanoma (UACC62), breast (MCF7), and cervical (HeLa) cancer cell lines	Anti-cancer agents	Wellington et al. (2013)
Catechol + thiols	*A. bisporus*	Catechol thioethers produced (yield 74–96%)[a]	Anti-microbial and anti-oxidant agents	Abdel-Mohsen et al. (2014)
Catharanthin e + vindoline	*T. pubescens*	A 56% yield of anhydrovinblastine was achieved (patented invention)[a]	Anti-tumor and anti-cancer drug	Baldelli et al. (2009) and Sagui et al. (2009)
Homogentisic acid + amino-β-lactams[b]	*M. thermophila* and *Trametes* sp.	Seven novel β-lactam antibiotics produced showed potency against gram-positive bacteria including *Staphylococcus aureus* and enterococci	Antibiotic drugs	Mikolasch et al. (2012)
Hydroquinones + 1,2-ethanedithiol	*T. villosa*	Compounds with the 2,3-ethylenedithio-1,4-quinones substructure similar to naturally derived bioactive quinones were produced[a]	Anti-cancer drugs and anti-microbial compounds	Cannatelli and Ragauskas (2015a)
Hyroquinone + mithramycin	*Polyporous anceps*	A 25% yield of the hydroquinone-mithramycin adduct was obtained[a]	Anti-tumor antibiotics	Anyanwutaku et al. (1994)
L-tryptophan + 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide	*P. cinnabarinus*	A quinonoid identified as 2-[2-(2-hydroxy-ethylcarbamoyl)-3,6-dioxo-cyclohexa-1,4 dienylamino]-3-(1H-indol-3-yl)-propionic acid was produced[a]	Potentially active ingredient for pharmaceutical products	Manda et al. (2006)
Morpholine + para-dihydroxylated aromatic compounds	*M. thermophila*	Six heteromolecular dimers and trimers produced showed moderate anti-microbial activity against some gram-negative and gram-positive bacteria as well as *Candida maltosa* yeast	Broad spectrum antibiotic drug	Hahn et al. (2009b)
N-analogous corollosporines + 2.5 dihydrobenzoic acid derivatives	*Trametes* sp.	Products were structurally similar to the ganomycin antibiotic class and active against gram-positive bacteria	Antibiotic drugs	Mikolasch et al. (2008a)

Table 2 (continued)

Substrates	Source of laccase	Positive effects observed	Potential applications	References
O-phenylenediamine + benzaldehydes	*A. bisporus*	2-Aryl-1H-benzimidazoles were produced (yield 50–99%)[a]	Therapeutic agents with potency against tumors, hypertension, and ulcers	Leutbecher et al. (2011)
Penicillins + 2,5-dihydrobenzoic acid derivatives	*Trametes* sp.	Hybrid dimers were produced[a]	Antibiotic drugs	Mikolasch et al. (2006)

[a] Product activity tests were not reported

[b] Amino-β-lactams used included cefadroxil, cefalexin, cefradin, cefaclor, loracarbef, amoxicillin, and ampicillin

oxidant activity when tested using the ferric-reducing anti-oxidant power (FRAP) assay (Zwane et al. 2012). Adelakun et al. (2012a) attributed the enhanced activity of the dimeric form of ferulic acid (β-5) to increased electron-donating groups. Functional groups such as alkyl, aniline, or hydroxyl groups enhance anti-oxidant activity (Bendary et al. 2013), while bulky alkyl groups contribute towards the stability of phenoxyl radicals (Decker 2008; Eskin and Przybylski 2000). On the other hand, compounds containing moieties such as nitro group or halogens, which are electron withdrawing groups, have poor anti-oxidant activity (Rakesh et al. 2015). The position of the active groups on the aromatic ring also determines the activity of the product. Enhanced activity of a phenolic anti-oxidant can be achieved when active groups occupy the *ortho* or *para* position to the hydroxyl group (Decker 2008). Recently, Najafi (2014) investigated the relationship between the position of active substituents on the daidzein aromatic ring and the compound's anti-oxidant activity. It was concluded that the *ortho* position can result in production of useful bioactive compounds (Najafi 2014).

The bond dissociation enthalpies (BDEs) of the active groups will also determine the inertia of the anti-oxidant in releasing the electron. The anti-oxidants containing active groups with lower BDE are better anti-oxidants because they readily release electrons to radical species (Szymusiak and Zielinski 2003). Adelakun et al. (2012a) showed that the β-β dimers of ferulic acid had a lower anti-oxidant activity than the monomeric ferulic acid. This is consistent with earlier findings, which showed that bis-ferulic acid (β-β dimers) had a higher BDE (85.76 kcal/mol) than ferulic acid (84.70 kcal/mol) (Murakami et al. 2005a). The determination of BDE varies with compounds and also experimental conditions; thus, many researchers focusing on the BDE of phenolic compounds have published contrasting results (Chandra and Uchimaru 2002; dos Santos and Simoes 1998; Klein and Lukeš 2006; Szymusiak and Zielinski 2003). However, in general, hydroxyl moieties have lower BDE than other active groups such as alkyl and aniline groups (Bendary et al. 2013), which probably explains why phenolics are frequently used as anti-oxidants.

An ideal anti-oxidant must also produce a stable radical, which will not facilitate the propagation of the oxidation chain bubble (Alov et al. 2015). The stability results from the resonance delocalization of lone electrons into the aromatic ring and absence of groups prone to attack by oxygen (Flora 2009; Shahidi and Naczk 2004). Although bulky groups on the *ortho* positions of the aromatic ring help stabilize anti-oxidant radicals (Shahidi and Naczk 2004), the bulky groups may also reduce anti-oxidant activity by steric masking of the phenolic hydroxyl group (Murakami et al. 2005a).

Hydrophobicity is also another attribute which affects anti-oxidant activity especially in a multicellular environment.

Table 3 Laccase-catalyzed production of bioactive natural polymers

Substrate	Source of laccase	Positive effects observed	Potential applications	References
Catechin + gelatin	Not provided	Catechin-gelatin conjugates produced exhibited improved water solubility as well as enhanced inhibition of low-density lipoprotein (LDL) oxidation	Soluble therapeutic drugs with anti-oxidant, anti-cancer, and anti-inflammatory properties	Chung et al. (2003)
Chitosan + caffeic acid	*T. versicolor*	Caffeic acid-functionalized chitosan with improved ABTS radical scavenging capacity	Pharmaceutical and cosmetic products	Božič et al. (2013, 2012b)
Chitosan + ethyl ferulate or ferulic acid	*M. thermophila*	Ethyl ferulate-functionalized chitosan produced preserved chitosan's initial anti-bacterial activity and improved ABTS radical scavenging activity	Anti-oxidant additives	Aljawish et al. (2014a,b)
Chitosan + ferulic acid	*M. thermophila*	Ferulic acid-functionalized chitosan derivatives produced had improved anti-oxidant activity	Anti-oxidant additives	Aljawish et al. (2014a,b)
Chitosan + gallic acid	*T. versicolor*	Gallic acid-functionalized chitosan produced had improved ABTS radical scavenging capacity	Pharmaceutical and cosmetic products	Božič et al. (2013, 2012b)
Chitosan/gelatin hydrogel + plant phenolic extracts	*Trametes* sp.	Chitosan/gelatin hydrogels cross-linked with phenolic compounds were stable under physiological conditions and resistant to degradation by wound enzymes such as lysozyme. The hydrogels also inhibited bacterial growth, thus promoting wound healing	Bioactive hydrogels for chronic wound healing	Rocasalbas et al. (2013)
Chitosan + quercetin	*T. versicolor*	Quercetin-chitosan derivatives had ABTS scavenging ability twofold higher than native chitosan	Bioactive materials for use in food and medical industries	Božič et al. (2012a)
Chitosan + tannic acid	*T. versicolor*	Improved anti-oxidant activity; more than double the activity of native chitosan	Bioactive materials for use in food and medical industries	Božič et al. (2012a)

Hydrophobicity increases the bioavailability of the anti-oxidant at sites where free radicals are generated (Ishige et al. 2001). For instance, hydrophobic anti-oxidants would be effective scavengers of free radicals generated from lipid peroxidation on the lipid bilayer because of their lipophilic properties (Lu et al. 2006). An ideal anti-oxidant would thus consist of a balance of electron-donating groups such as hydroxyl groups, which will ensure the free radical scavenging ability of the anti-oxidant, and also hydrophobic moieties which will enable the bioavailability of the anti-oxidant in multicellular systems. Research that focused on improving the hydrophilicity of silybin resulted in its compromised anti-oxidant activity in lipophilic environments (Gažák et al. 2010; Gažák et al. 2004), highlighting hydrophobicity as an important factor in the function of anti-oxidants in cell medium.

Antibiotics

Generally, microbial resistance to antibiotics is through three mechanisms: (i) enzymatic inactivation of the antibiotic, for example, hydrolysis of β-lactam-based antibiotics by β-lactamase enzymes; (ii) alteration of the targets; and (iii) reduced penetration of the antibiotic into the microorganism (Watanabe et al. 1987). Laccases have been used in developing antibiotics with enhanced penetration into host cell. These antibiotics have been produced by coupling catechols and β-lactam-based antibiotics (Mikolasch et al. 2008b). The produced β-lactam derivatives expressed significant activity against gram-positive bacteria, including drug-resistant *S. aureus* and enterococci. It has been demonstrated that the availability of a catechol moiety on the antibiotic improves its penetration into the bacterial cell through the iron transport system (Fung-Tomc et al. 1997; Silley et al. 1990). The incorporation of catechol groups onto antibiotic compounds thus enhances antibiotic activity of the antibiotic through effective drug delivery towards the targeted site. The coupling of monomeric antibiotic units to dimeric forms can result in enhanced efficacy. Some reports have highlighted the efficacy of dimeric vancomycin in inhibiting bacteria resistant to monomeric vancomycin units (Yoshida et al. 2011). Dimerization of monomeric antibiotics can also prevent enzymatic hydrolysis of the antibiotic since the dimerization alters the compound in such a way that hydrolyzing enzymes fail to recognize it.

Directions for future research

The use of laccases as biocatalysts offer economically viable domino processes for the synthesis of bioactive compounds. However, the translation of this green technology into a feasible industrial process requires several factors to be considered. For example, there is a need to develop a robust enzyme with properties that are ideal for industrial application. Specific research areas could include heterologous expression so as to produce enough enzyme with improved activity, thermostability, and

Table 4 Structure-activity relationships of laccase-catalyzed phenolic coupling products

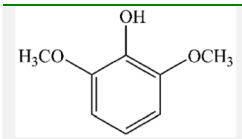
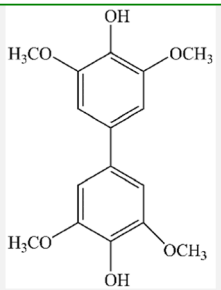
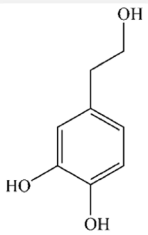
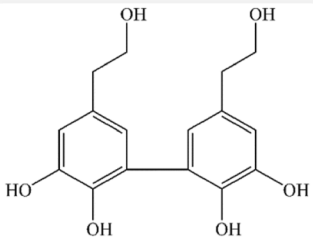
Substrate	Product	Effect	Structure – activity relationship	References
 2,6-DMP	 3,3′,5,5′-tetramethoxy biphenyl-4,4′-diol	Increased antioxidant activity	Increased electron donating groups on the dimer reduces the bond dissociation enthalpies (BDE) of the hydroxyl moieties and facilitated the subsequent formation of a stable radical due to the ease of electron delocalisation into the benzene ring.	Adelakun et al. (2012b); Wan et al. (2008a,b)
 3-hydroxytyrosol	 3-hydroxytyrosol dimer	Improved antioxidant activity	The increased number of hydroxyl groups on the dimer offers more attacking sites for free radicals, thus improving the free radical scavenging ability of the dimer.	Burton and Davids (2012); Zwane et al. (2012)

Table 4 (continued)

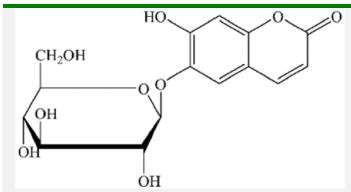
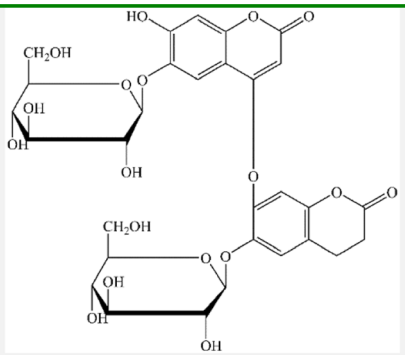
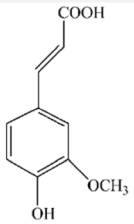
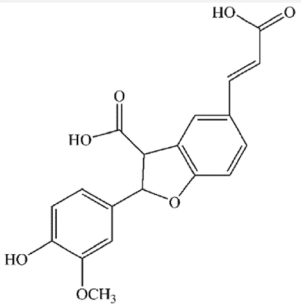
 Esculin	 Di-esculin	Improved solubility	Molecular modelling studies showed that the esculin monomer could form only 9 hydrogen bond interactions with water, while after dimerization, the number of hydrogen bonds increased to 22, thus the dimer had improved solubility.	Anthoni et al. (2010)
 Ferulic acid	 β–5 ferulic acid dimer	Improved antioxidant activity	Coupling of ferulic acid resulted in an increase in number of electron donating groups on the product. The unsaturated C=C bond along with its adjacent carboxylic acid group were preserved during oxidation process and offers extra attacking sites to	Adelakun et al. (2012a); Constantin et al. (2012a)

Table 4 (continued)

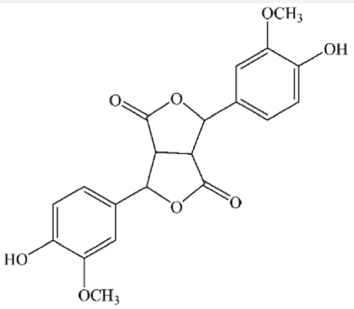
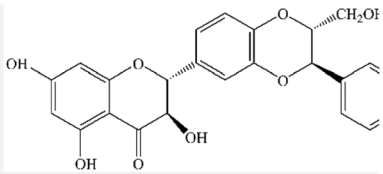
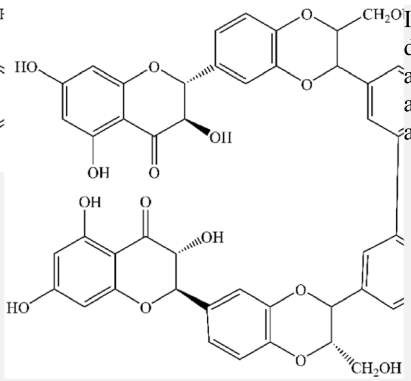
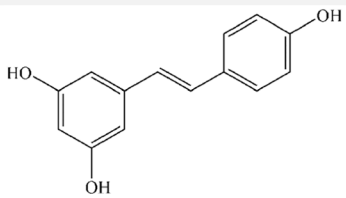
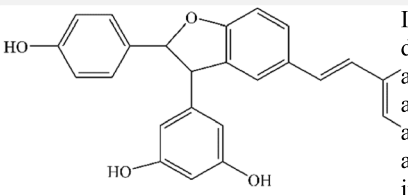
			free radicals.	
	 β–β ferulic acid dimer	Lower antioxidant activity compared to ferulic acid	The β–β dimer has lost the unsaturated C=C bond as well as the carboxylic acid group during the coupling process, which may explain its low antioxidant potential.	
 Silybin A	 Silybin A dimer	Increased antioxidant activity	The coupling of two silybin A monomers leads to higher resonance. High resonance allows the antioxidant to form stable radicals when it has been oxidised by free radicals.	Gavezzotti et al. (2014)
 Trans-resveratrol	 Trans-resveratrol dehydrodimer	Increased antioxidant activity and increased solubilit	Increased number of hydroxyl groups increases the antioxidant activity.	Nicotra et al. (2004a)

Table 4 (continued)

		y	The increased hydroxyl groups also enables the formation of more hydrogen bond interactions with water molecules thus increasing solubility.

ability to withstand organic solvents and inhibitors which are frequently encountered in industrial applications (Mate and Alcalde 2015; Kudanga and Le Roes-Hill 2014; Kunamneni et al. 2008b). Laccase-catalyzed reactions generally result in low product yield (see for example, Adelakun et al. 2012b; Wan et al. 2007). While the creation of radicals is a prerequisite for laccase synthesis, there are also some negative implications for biosynthesis. With radical processes, there is a possibility of many different radical forms of the oxidized molecule mainly due to resonance stabilization and non-specific radical-mediated reactions. Therefore, such processes usually result in a wide range of different racemic mixtures of products appearing at rather low concentrations. Frequently high concentrations of organic solvents are used to minimize radical proliferation, reduce polymerization reactions, and therefore increase yield, but the same solvents also inactivate enzymes. Therefore, reaction engineering to increase product yield remains a major challenge in laccase-mediated synthesis of bioactive compounds. However, other key research areas that need particular attention could include (i) the search for cheap substrate sources coupled with the bioprospection of natural laccase mediator systems (LMSs) and (ii) production of enantiomericaly pure products.

Biopolymers as substrate sources

The potential of laccases can be extended beyond the oxidation of its natural substrates through the LMS (Riva 2006). This involves the generation of radicals from small compounds within laccase's redox potential range (viz. 0.5–0.8 mV against a standard hydrogen electrode) (Witayakran and Ragauskas 2009). The generated radicals can then act as redox shuttles, oxidizing substrates with higher redox potentials and those too large to fit the enzyme active site (Zhu et al. 2014). The LMS technology has been extensively used in the textile industry, pulp and paper industry, alcohol oxidation, and lignin degradation (D'Alfonso et al. 2014; Morozova et al. 2007b; Fabbrini et al. 2002). Some researchers are of the opinion that the use of LMS presents an opportunity to mine the plethora of low-molecular-weight phenolics and other bioactive compounds entrapped within biopolymers such as lignin (Christopher et al. 2014; Rich et al. 2016). The degradation of lignin, which is the second most abundant biopolymer, and is laden with bioactive functional groups such as phenolic hydroxyls, benzyl alcohols, carbonyls, and methoxyls (Boeriu et al. 2004; El Mansouri and Salvadó 2007), can present a wealthy source of substrates for the synthesis of valuable bioactive compounds (Barclay et al. 1997; Božič et al. 2012a). The widely available artificial LMSs such as ABTS and 1-hydroxybenzotriazole (HBT) remain expensive and are potential contaminants when applied in the synthesis of compounds of therapeutic value (Cañas and Camarero 2010). Therefore, bioprospecting for more efficient natural mediator systems for the degradation of biopolymers also remains a key research area.

Towards the production of enantiomerically pure compounds

To date, much of the research on the exploitation of laccase for organic synthesis has only produced racemic mixtures of oligomeric and cross-coupling products. This is a limitation especially in the synthesis of therapeutic drugs, which in most cases requires enantiomerically pure compounds. It has also been observed that enantiomers can have significantly different bioactivities (Davis-Searles et al. 2005; Plíšková et al. 2005). Therefore, research is now also focusing on synthesizing enantiomerically pure compounds (Girol et al. 2012; Kim et al. 2012). Strikingly, in vivo laccase-catalyzed coupling

reactions are highly stereospecific, leading to the formation of compounds such as lignans, lignins, and suberins (Orlandi et al. 2001; Zoia et al. 2008). Development of a protocol that can mimic the same specificity in vitro will be valuable in industrial processes. Research towards production of pure final products thus represents a primary focus area for future research. A number of studies have laid a foundation for future studies in this respect as explained below.

Davin and colleagues demonstrated the role played by a 78-kDa protein (dubbed "dirigent" protein) isolated from *Forsythia intermedia* in the in vivo synthesis of stereospecific dimers of E-coniferyl alcohol, which are building units for lignin polymers in plants (Davin et al. 1997). Coupling reactions carried out in the absence of the dirigent protein resulted in the racemic dimers (±)-dehydrodiconiferyl alcohols, (±)-pinoresinols, and (±)-guaiacylglycerol 8-O-4-(coniferyl alcohol) ethers (Davin and Lewis 2005). However, in the presence of the dirigent protein, stereospecific coupling reaction occurred, resulting in (+)-pinoresinol as the only product (Fig. 5) (Davin et al. 1997; Davin and Lewis 2000; Halls et al. 2004). This trend was reproducible when either laccase, flavin mononucleotide (FMN), flavin adenine dinucleotide (FDN), ammonium peroxydisulfate, or an oxidase native to *F. intermedia* was used as oxidant, proving that stereoselectivity in the reaction was not promoted by the oxidant employed. The substrate specificity of the dirigent protein from *F. intermedia* restricts it only to the production of (+)-pinoresinol. This knowledge has already opened fresh avenues of enquiry, allowing scientists to bioprospect for their homologous proteins in nature (Präg et al. 2014; Pickel and Schaller 2013; Girol et al. 2012; Umezawa 2003) as well as taking advantage of the modern day tools such as molecular technology (Kazenwadel et al. 2013; Kim et al. 2012) to design modified proteins of such ilk that can control directed coupling to produce desired bioactive products.

In related studies, stereospecific bioactive lignans were synthesized by attaching chiral auxiliary compounds to the substrates (Orlandi et al. 2001). Riva and coworkers have also carried out extensive research on protecting functional groups of laccase substrates as a strategy for reducing the diversity of products formed in the oxidation reactions. A benzyl group was added to protect the OH group on the C′7 of silybin A, resulting in the 87% yield of its symmetric dimer (Gavezzotti et al. 2014) (Fig. 6). As shown by earlier studies, benzylation of functional groups seemed to be better than adding methyl groups, which made deprotection impossible (Gažák et al. 2008).

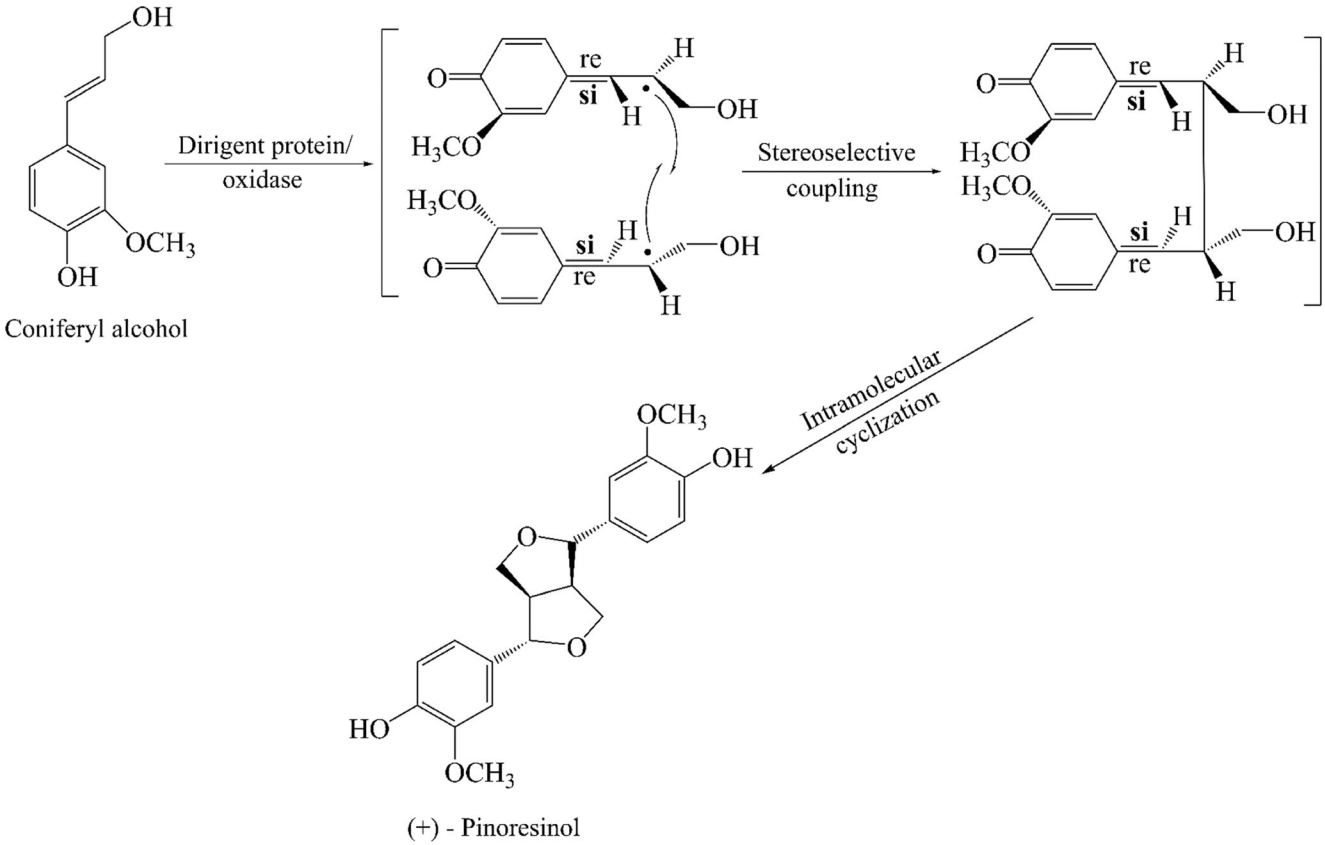


Fig. 5 The proposed in vivo stereoselective synthesis of (+)-pinoresinol in *Forsythia intermedia* involving dirigent protein-facilitated binding and orientation of coniferyl alcohol radicals. Adapted from Davin et al. (1997), with permission from the American Association for the Advancement of Science

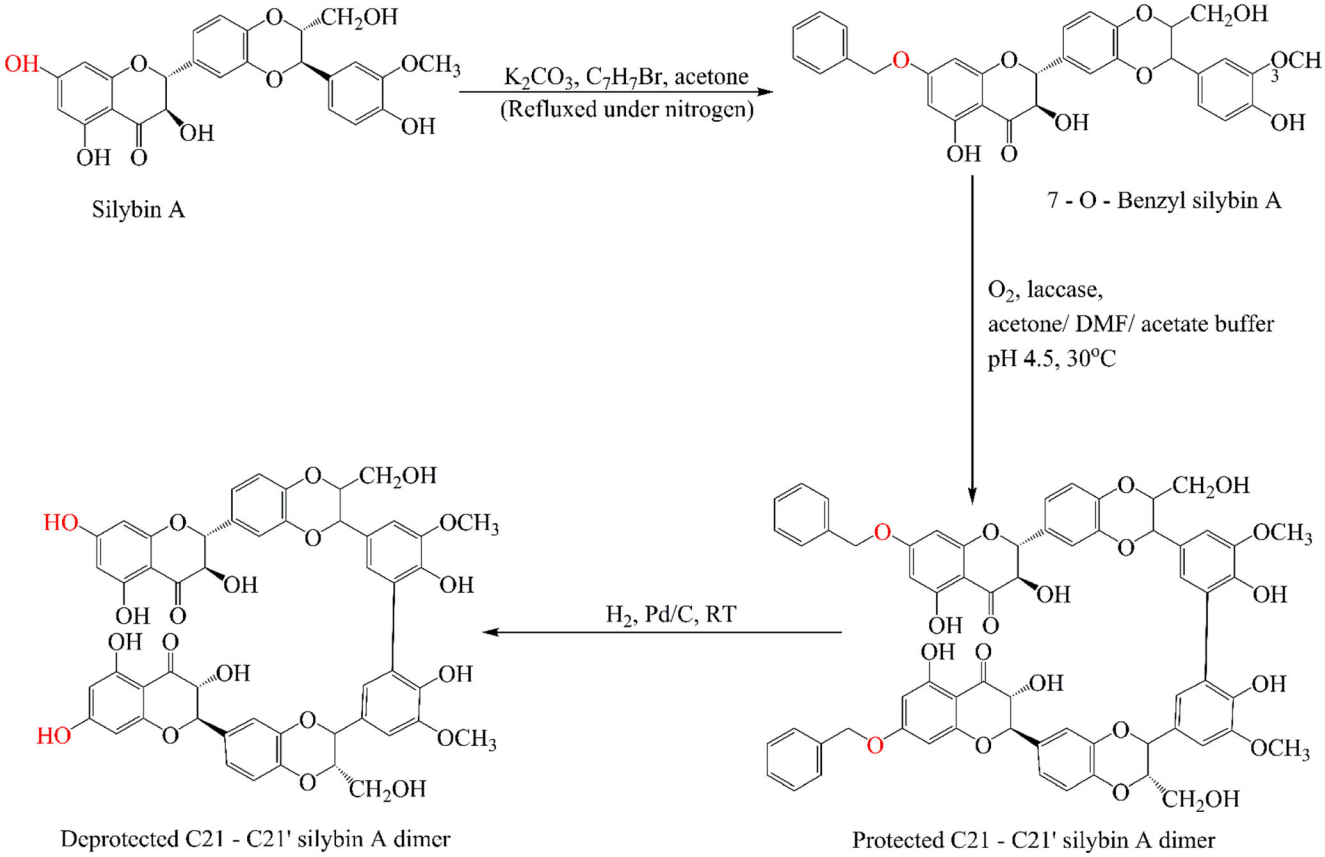


Fig. 6 Protection of the hydroxyl group on the C′7 of silybin A by benzylation to reduce the product range of the reaction. Adapted from Gavezzotti et al. (2014), with permission from Elsevier

It has also been reported that regioselectivity can be influenced by the reaction conditions such as pH and solvents (Chioccara et al. 1993; Orlandi et al. 2001). Horseradish peroxidase-catalyzed coupling of lignans (isoeugenol, methyl ferulate, or coniferyl alcohol) under acidic pH resulted in dimer formation; neutral pH promoted the formation of oligomers, while a racemic β-O-4 product was formed in the presence of methanol solvent (Chioccara et al. 1993).

The emergence and subsequent advances in the field of molecular biology have opened a host of opportunities in developing biocatalysts better equipped for industrial application. Besides the improved expression of proteins in heterologous hosts, molecular techniques also allow bioprospecting in unculturable microorganisms as well as database mining. Using bioinformatic databases, it is now possible to profile the sequence of the polypeptide chain. With this information, predictions can be made on how alteration of the amino acid sequence can affect the characteristics of the enzyme. Usually, these alterations are performed at or near the enzyme's catalytic core (Mate and Alcalde 2015; Prins et al. 2015; Turner 2009). However, in addition to carrying out these modifications with the goal of improving enzyme activity and/or robustness, genetic manipulation could also focus on improving stereoselectivity and facilitating the production of pure compounds (Robert et al. 2011).

Concluding remarks

The potential of laccase as a green biocatalyst in the synthesis of bioactive compounds is vast. Many studies have increased our understanding of reaction mechanisms involved, desired reaction conditions, and structure-activity relationships. Future research should highlight not only synthetic properties of the enzyme but also reaction engineering to optimize synthesis of specifically desired products of economic value. This could possibly facilitate transfer of the technology from bench scale to industrial application processes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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