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Research review paper

Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes

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ABSTRACT

Laccases are oxidoreductases which oxidize a variety of aromatic compounds using oxygen as the electron acceptor and producing water as by-product. The interest for these old enzymes (first described in 19th century) has progressively increased due to their outstanding biotechnological applicability. The presence of redox mediators is required for a number of biotechnological applications, providing the oxidation of complex substrates not oxidized by the enzyme alone. The efficiency of laccase–mediator systems to degrade recalcitrant compounds has been demonstrated, but still the high cost and possible toxicity of artificial mediators hamper their application at the industrial scale. Here, we present a general outlook of how alternative mediators can change this tendency. We focus on phenolic compounds related to lignin polymer that promotes the *in vitro* transformation of recalcitrant non-phenolic structures by laccase and are seemingly the natural mediators of laccases. The use of eco-friendly mediators easily available from lignocellulose, could contribute to the industrial implementation of laccases and the development of the 21th century biorefineries.

Contents

1. Introduction

Laccases are widely distributed multicopper oxidases that have been subject of increasing research since their discovery in 19th century (Bertrand, 1896; Yoshida, 1883), due to their high biotechnological applicability. In fact, the biotechnological potential of laccases has been exploited since ancient China when *Rhus vernicifera* resin, rich in laccase and urushiol (a catechol derivate), was used to produce lacquer. Lacquer craft constitutes the first example of using an enzyme as biocatalyst for manufacturing plastic polymers.

Fungal laccases are secreted by most white-rot basidiomycetes during lignin biodegradation and have been widely investigated. However, for years lignin degradation studies focussed on ligninolytic peroxidases due to their higher redox potential, relegating laccase studies to a second position. Description of certain synthetic

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compounds that promote the oxidation capabilities of laccase towards more recalcitrant non-phenolic lignin compounds pushed forward the research on laccases and laccase-mediator systems.

Current research on oxidation of recalcitrant compounds by fungal laccases and alternative mediators of natural origin is described here. We especially focus on lignin-related phenolic compounds that act as efficient redox mediators of laccase (promoting the oxidation of non-phenolic substrates) and the mechanisms by which these phenolic mediators, once oxidized by the enzyme, oxidize the target substrate. Finally, we illustrate the possible application of these enzymatic systems on environmental and sustainable biotechnological processes, as plausible alternatives to the use of expensive, and perhaps unsafe, artificial mediators.

Description of these plant-derived compounds as the natural mediators of laccase, suggests a central role for fungal laccases in lignin biodegradation, and smoothes the progress for the industrial application of these green biocatalysts and their natural redox mediators, contributing to develop the future lignocellulose biorefineries.

2. Laccases and laccase-mediator systems

Laccases (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductases, Lignin Oxidases Family 1, http://foly.esil.univ-mrs.fr/index.htlm), are multicopper-containing oxidases with phenoloxidase activity, which catalyze the oxidation of substituted phenols, anilines and aromatic thiols, at the expense of molecular oxygen (Thurston, 1994).

Four copper ions at the active site are involved on the catalysis. "Blue" copper (T1 site) is implicated in the oxidation of the reducing substrate, capturing the electrons that are then transferred to the trinuclear copper cluster (T2/T3 site), in which O_2 is reduced. Electrochemical potential of Cu T1 site is one of the most significant features of laccases and might vary from 0.4 to 0.8 V, providing greater biotechnological interest for high redox potential fungal laccases (Morozova et al., 2007b; Yaropolov et al., 1994).

Laccases are widely distributed in nature and perform a multiplicity of functions linked to either synthetic or degradation processes (Mayer and Staples, 2002). So far, biological roles of laccases range from chitin or lignin synthesis (in insects and higher plants, respectively) to lignin and humic acids degradation carried out by fungi. Fungal laccases are also involved in the synthesis of melanin-like pigment synthesis (Nagai et al., 2003; Sharma and Kuhad, 2008; Tetsch et al., 2006). On the other hand, laccases have been also described in bacteria, where they would play roles related to pigmentation and resistance of spores and pathogenesis (Claus, 2003).

Laccases have been found in all types of higher fungi (Baldrian, 2006), being involved in the cycling of soil organic matter in nature. Secretion of extracellular oxidoreductases, namely laccases and peroxidases, confers ligninolytic basidiomycetes a unique capability to degrade the lignin polymer in decayed wood, as well as to detoxify a variety of recalcitrant aromatic compounds. White-rot basidiomycetes are the main laccase producers. However, litter-decomposing and mycorrhizal basidiomycetes (two other ecophysiological groups that live together in soil with wood-decayed fungi (Steffen et al., 2003) also secrete laccases. In these fungi laccases might play important detoxification and nutritional roles, participating in the humus turnover. In fact, forest soils contain a diversity of laccase genes. The saprophytic fungi appearing to display a higher diversity of laccase genes than the mycorrhizal ones, according to the variety of carbon sources they ought to make use of (Luis et al., 2004).

Fungal laccases possess huge biotechnological potential in the pulp and textile industry, beverage processing, organic synthesis, the manufacture of biodevices or the detoxification of pollutants (Husain and Husain, 2008; Kunamneni et al., 2008; Minussi et al., 2002; Riva, 2006; Rodríguez-Couto and Toca, 2006; Widsten and Kandelbauer, 2008b). The broad substrate specificity, the use of molecular oxygen as electron acceptor (compared to H_2O_2 requirement of fungal peroxidases which, besides, hold low operational stability, mostly due to their rapid deactivation by hydrogen peroxide) and the generation of water as the sole reaction by-product (Paice et al., 1995), are responsible for this high applicability of laccases. However, these green biocatalysts possess relative low redox potential ($\leq$0.8 V) compared to ligninolytic peroxidases (>1 V). Thus, their action would be restricted to the oxidation of the phenolic lignin moiety (less than 20% of lignin polymer) (Kawai et al., 1987) whereas non-phenolic substrates having redox potential above 1.3 V cannot be oxidized by laccases directly. In fact, the first attempts of wood pulp treatment with laccase did not lead to lignin degradation (Bourbonnais and Paice, 1992; Bourbonnais et al., 1995).

Nevertheless, this limitation has been overcome through mimicking nature, by using redox mediators in the so-called laccase-mediator systems. The presence of certain small-molecular weight compounds, that act as redox mediators, expand the catalytic activity of laccase towards more recalcitrant compounds such as non-phenolic lignin units (Barreca et al., 2003). Mediators act as electrons shuttles, providing the oxidation of complex substrates (such as lignin polymers) that do not enter the active site due to steric hindrances. Once oxidized by the enzyme and stabilized in more or less stable radicals, mediators diffuse far away from the enzymatic pocket and, by mechanisms different from the enzymatic one, enable the oxidation of target compounds that in principle are not substrates of laccase because of their high size or high redox potential (Bourbonnais and Paice, 1990; Bourbonnais et al., 1997b; Kawai et al., 1989).

The ideal redox mediator would be a small-size compound, able to generate stable radicals (in its oxidized form) that do not inactivate the enzyme, and which reactivity would allow its recycling without degeneration. In addition, from the point of view of their industrial and environmental application, laccase mediators should be environmental-friendly and available at low cost.

3. Artificial laccase mediators

The first synthetic compound to be demonstrated as laccase mediator for oxidation of non-phenolic lignin model compounds was ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) (Bourbonnais and Paice, 1990). The first stage in ABTS oxidation generates the cationic radical ($ABTS^{\bullet+}$) which is slowly oxidized to the dication ($ABTS^{2+}$). Oxidation of non-phenolic lignin model compounds (Bourbonnais et al., 1998) and organic dyes (Solis-Oba et al., 2005)

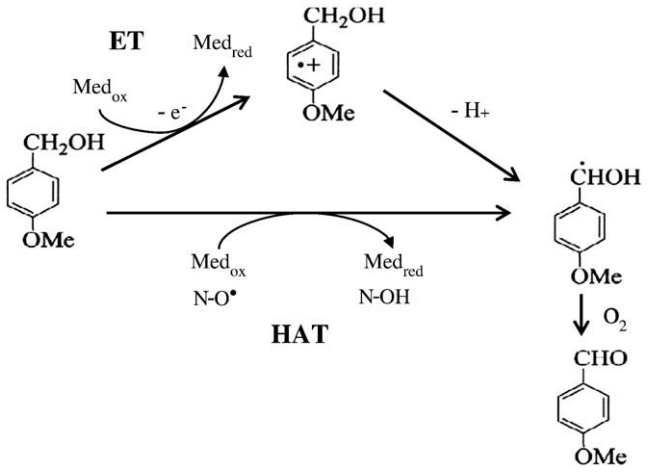

Fig. 1. Oxidation of a non-phenolic lignin model compound (p-anisilic alcohol) by laccase mediator following two different oxidation mechanisms: ET (electron transfer) and HAT (hydrogen atom transfer) (adapted from (Fabbrini et al., 2002)).

by laccase-ABTS occur via an electron transfer (ET) route (Baiocco et al., 2003) (Fig.1).

Since 1990, many efforts were directed towards the search for new synthetic mediators and elucidation of their oxidation mechanisms. The most efficient laccase mediators for oxidation of recalcitrant aromatic compounds, such as non-phenolic lignin, are the >N–OH mediators, such as 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI), violuric acid (VLA) or N-hydroxyacetanilide (NHA) (Call, 1994; Paice et al., 1997; Srebotnik and Hammel, 2000; Xu et al., 2000) (Fig. 2). The oxidation of this type of mediators by laccase generates a highly reactive nitroxyl radical (>N–O$^{\bullet}$), owing to the enzymatic removal of an electron followed by release of a proton. Nitroxyl radicals oxidize the target substrate by hydrogen atom transfer (HAT) mechanism (Bourbonnais et al., 1997b; Fabbrini et al., 2002; Xu et al., 2000) (Fig. 1). Therefore, the enthalpic balance between the dissociated bond (C–H) in the target substrate and the forming bond (NO–H) in the mediator, is the driving force of this mechanism (Barreca et al., 2003; Cantarella et al., 2003).

The case of the stable nitroxyl radical TEMPO is different because it is oxidized by laccase forming an oxoammonium ion (oxidation to >N=O^{+} occurs at a redox potential that matches that of a high redox fungal laccase) that follows a non-radical-ionic-mechanism (Fabbrini et al., 2002).

Other organic compounds, such as nitroso compounds or triphenylamine and phenothiazine derivates (i.e. promazine, phenothiazine-10-propionic acid or 2-nitro-1-naftol-4-sulfonic acid), have been tested as laccase mediators for the oxidation of lignin dimers, kraft paper pulp or textile dyes (Bourbonnais et al., 1997a; Camarero et al., 2005; Fabbrini et al., 2002; Paice et al., 1997). More recently, phenylpyrazolones have been demonstrated to mediate the oxidation of veratryl alcohol or xenobiotics by laccase (Shleev et al., 2003; Shleev et al., 2004). On the other hand, metal ions, such as Mn^{2+} could act as laccase mediators when they are oxidized by the enzyme (Muñoz et al., 1997). In addition, metallic complexes, the so-called polyoxometalates, have been described as laccase mediators for the degradation of lignin (Gamelas et al., 2005; Gamelas et al., 2007).

The versatility of laccase-mediator systems for oxidative transformation of aromatic compounds is evidenced by the variety of possible applications such as: delignification and bleaching of paper pulps, decolorization of industrial dyes or detoxification of pollutants (Bajpai, 2004; Barreca et al., 2004; Claus et al., 2002; Husain and Husain, 2008; Morozova et al., 2007a).

During the last two decades, extensive research has been carried out in order to reduce the use of toxic chlorinated chemicals in pulp bleaching and to optimize the conditions for enzymatic delignification and bleaching of paper pulps (up to pilot-scale in some cases), attaining better pulp qualities and chemical savings (Bajpai et al., 2006; Bourbonnais and Paice, 1992; Call, 1994; Paice et al., 1995). Laccase–HBT system is particularly effective in woody and non-woody pulp bleaching and delignification. Its integration in total chlorine free bleaching sequences has yielded pulp properties which are difficult to reach using only chemical reagents (Call and Mücke, 1997; Camarero et al., 2004; García et al., 2003; Ibarra et al., 2006a). Moreover, the laccase–HBT system is also very efficient in removing pitch deposits, which represent a major problem in the paper industry, whereby lowering the paper quality and increasing production costs (Gutiérrez et al., 2006; Gutiérrez et al., 2008; Speranza et al., 2007).

Research into dye decolorization by laccase and redox mediators has also provided excellent results (Claus et al., 2002; Couto and Sanroman, 2007; Hu et al., 2009; Rodriguez et al., 1999; Tavares et al., 2008; Wong and Yu, 1999). DeniLite®, the commercial laccase preparation (with phenothiazine–propionate as mediator) from Novozymes (Denmark), is used in textile industry for indigo decolorization in jeans fabric processing. In 2001 the company Zytex (Zytex Pvt. Ltd., Mumbai, India) also developed a formulation based on a laccase–mediator system capable of degrading indigo. The trade name of the product is Zylite.

Enzymatic bioremediation with laccases and redox mediators is another research field of increasing interest, due to the versatility of this enzymatic systems to transform and detoxify a variety of the most common organic pollutants released from industrial processes: polycyclic aromatic hydrocarbons, chlorophenols, polychlorinated biphenyls, phenols, organophosphorated pesticides, dyes, etc (Amitai et al., 1998; Cambria et al., 2008; Collins et al., 1996; Husain and Husain, 2008; Majcherczyk et al., 1998).

4. Phenolic compounds as laccase mediators

In spite of the proven efficiency of laccase–mediator systems to degrade recalcitrant aromatic compounds of biotechnological and environmental interest, the application of these enzymatic systems is in part hindered by the economic cost of the artificial mediators and the generation of possible toxic species (even though the lack of studies in this matter is noteworthy). For these reasons, many studies have focussed on searching alternative mediators which could present environmental and economic advantages. First attempts were directed towards fungal metabolites as natural laccase substrates (supposed to be involved in the mechanisms by which fungi naturally degrade lignin).

3-Hydroxyanthranilic acid (3-HAA) (see Fig. 3), a secondary metabolite synthesized by the ligninolytic fungus *Pycnoporus cinnabarinus*, was shown to mediate the oxidation of non-phenolic substrates and synthetic lignin catalyzed by laccase (Eggert et al., 1996a). Description of its mediating capability was one of the first evidence of the contribution of redox mediators of natural origin to lignin biodegradation (Eggert et al., 1996b; Eggert et al., 1997). However, the role of this phenolic metabolite has been questioned in subsequent studies because the oxidative coupling of 3-HAA generates cinnabarinic acid, that is unable to mediate the oxidation of non-phenolic compounds (it has no substituent group that can be further oxidized to phenoxyl radical by laccase) (Li et al., 2001). Moreover, this metabolite did not mediate oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase (Johannes and Majcherczyk, 2000).

Johannes and co-workers achieved the enzymatic oxidation of several polycyclic aromatic hydrocarbons mediated by two other fungal phenolic metabolites: 4-hydroxybenzoic acid and 4-hydroxybenzylic alcohol. The positive effect of amino acids and certain natural compounds bearing thiol groups (such as reduced glutathione) on PAH oxidation by laccase was also described (Johannes and Majcherczyk, 2000). 4-hydroxybenzoic acid has been also used as mediator for fungicide degradation and detoxification (Maruyama et al., 2007). Nevertheless, the high mediators' concentration used in these studies (mediator/PAH ratios between 40 and 200) might cause reaction

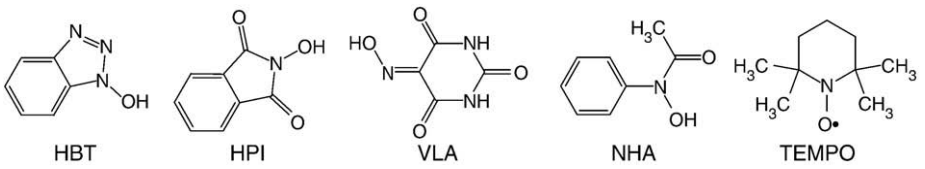

Fig. 2. Synthetic laccase mediators of >N–OH type: (HBT) 1-hydroxybenzotriazole, (HPI) N-hydroxyfthalimide, (VLA) violuric acid, (NHA) N-hydroxyacetanilide and TEMPO.

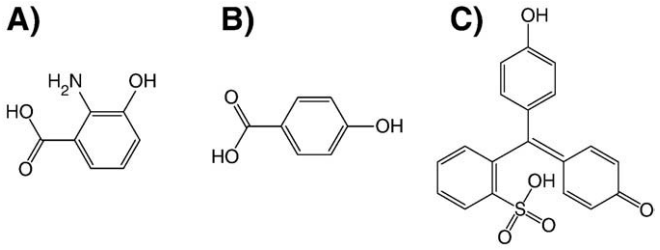

Fig. 3. Chemical structure of three phenolic compounds described as laccase mediators: A) 3-hydroxyantranilic acid, B) 4-hydroxybenzoic acid, and C) phenol red.

conditions different from those that occur in nature, being able to produce other oxidation routes that could give place to unknown metabolites (Branchi et al., 2005; Cantarella et al., 2003).

Another phenolic compound, in this case of synthetic origin, demonstrated to act as laccase mediator is phenol red. In spite of its slow oxidation rate by laccase, after prolonged exposure to the enzyme, phenol red is oxidized to its resonance-stabilized phenoxy radical, providing the oxidation of the non-phenolic substrates like p-anisilic alcohol (d'Acunzo and Galli, 2003).

5. Natural mediators of laccase related to lignin polymer

Lignin biodegradation is an oxidative process described as "enzymatic combustion" (Kirk and Farrell, 1987), wherein enzymatic and radical reactions result in degradation of non-phenolic aromatic structures (more than 80% of lignin polymer). In this process, free radicals of fungal metabolites or products derived from the own biodegradation process are supposed to act as redox mediators of ligninolytic oxidoreductases. The contribution of these low-molecular weight compounds would be especially noticeable in those white-rot fungi that secrete laccase as the main or sole ligninolytic oxidoreductase (Eggert et al., 1996b; Eggert et al., 1997; Geng and Li, 2002).

With this idea in mind and the aim of searching for new laccase mediators, we performed a colorimetric screening in which the efficiency of a series of naturally-occurring substituted phenols related to lignin polymer to act as laccase mediators, was demonstrated (Camarero et al., 2005). Syringaldehyde, acetosyringone, vanillin, acetovanillone, methyl vanillate and *p*-coumaric acid significantly promoted the oxidation of recalcitrant dyes by laccase (Fig. 4). These phenolic compounds are present in herbaceous plants (*p*-hydroxycinnamic acids) either as extractives or forming lignin-carbohydrate bridges; or they originate from lignin degradation (phenolic aldehydes, ketones and acids derived from the oxidation of the phenyl propane lignin units), and are subsequently incorporated into the soil organic matter (humus).

In this sense, the term "natural", not only would stand for the natural origin (lignocellulosic materials, fruits, seeds, etc) of these compounds, but also for their role in nature, being most likely the usual (true) mediators of laccase activities during the biodegradation of lignin polymer carried out by white-rot fungi. Thus, phenoxyl radical fragments generated from lignin oxidation by laccase (or else ligninolytic oxidoreductase), or from certain phenolic monomer residuals from the building up of lignin polymer, could oxidize non-phenolic residues of lignin thereby causing the breakdown of its alkyl network. This hypothesis is supported by the experimental data reviewed here, demonstrating the oxidation of recalcitrant aromatic compounds by laccase in the presence of its natural mediators. These data suggest a central role for laccases in lignin biodegradation, despite the seemingly lower oxidation power of these enzymes with respect to ligninolytic peroxidases.

Lignin degradation by fungal oxidoreductases has been a subject of research for years due to the importance of this process for the wood-pulp and paper industry. Many of these studies focused on the use of

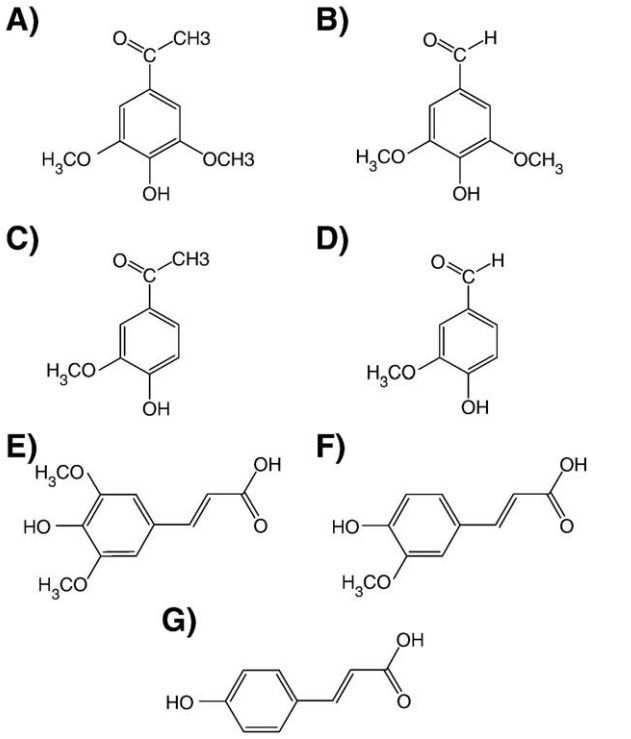

Fig. 4. Chemical structure of the phenolic compounds related to lignin polymer described as laccase mediators. A) Acetosyringone, B) syringaldehyde, C) vanillin, D) acetovanillone, E) sinapic acid, F) ferulic acid and G) *p*-coumaric acid.

laccases since the description of the first redox mediators, their use providing the means to oxidize non-phenolic lignin substructures by laccase. Syringaldehyde was described as a laccase mediator for the oxidation of the non-phenolic lignin model compound, veratryl alcohol (Kawai et al., 1989), even before than description of ABTS as a laccase mediator for the oxidation of this compound and non-phenolic lignin dimers (Bourbonnais and Paice, 1990). More recently, the oxidation of different benzylic alcohols (as models of non-phenolic structural moieties in lignin) by laccase and hydroquinone as a phenolic mediator has been successfully performed (Calcaterra et al., 2008).

The notable suitability of laccases and artificial redox mediators (especially HBT) to remove lignin from pulp has been demonstrated over the course of these years (Call and Mücke, 1997; Ibarra et al., 2006b; Bourbonnais and Paice, 1996). The suitability of natural phenolic mediators for enzymatic bleaching and delignification of kraft pulp has been also investigated. First evidence of natural phenols to mediate enzymatic delignification of paper pulps showed syringaldehyde and acetosyringone as promising laccase mediators, resulting in 25% lignin removal (Camarero et al., 2007). Four naturally-occurring *p*-hydroxycinnamic compounds have been also recently tested as laccase mediators for sisal pulp biobleaching. Although enzyme inactivation was markedly reduced in the presence of these natural mediators, pulp bleaching was less efficient than in the presence of HBT because these phenolic compounds tend to bind to pulp fibers (Aracri et al., 2009), according to previous results found with *p*-coumaric acid (Camarero et al., 2007). However, this effect could be used to functionalize fibers in order to improve the intrinsic properties of pulp (Aracri et al., 2009). Methyl syringate, has been also shown to act as a laccase mediator for lignin oxidation in kraft pulp and, simultaneously, as a grafting compound for fiber modification (Liu et al., 2009).

On the other hand, lipid compounds such as fatty and resin acids, sterols and triglycerides are responsible for pitch deposits during the manufacture of wood chemical pulps, reducing paper strength during

sheet formation or increasing energy consumption during paper production and reducing effluent toxicity (Back and Allen, 2000). Laccase–HBT system has been demonstrated to remove lipid extractives responsible for pitch deposits in pulp (Gutiérrez et al., 2006; Gutiérrez et al., 2008; Speranza et al., 2007). Alternatively, lignin-related phenols can be used as laccase mediators for combating pitch problem, as demonstrated by the efficient lipid-removal attained with laccase and syringaldehyde or acetosyringone (Gutiérrez et al., 2007). It is noteworthy to point out that these two phenolic compounds are among the main low-molecular-mass compounds extractable from black liquors of kraft pulping of eucalypt wood (Ibarra et al., 2006b). The possibility to obtain these mediators from natural resources wastes at low cost, makes the difference with the expensive synthesis of artificial mediators.

Naturally-occurring phenols related to lignin polymer have been also tested as laccase mediators for degradation of other recalcitrant aromatic compounds, such as synthetic organic dyes, PAH or diverse pesticides, attaining excellent results.

Several studies on dye decolorization with laccase in the presence of naturally-occurring phenolic mediators of natural origin have been published recently. Acetosyringone and syringaldehyde, both dimethoxy substituted phenols derived from syringyl lignin units, where described as the fastest and most efficient laccase mediators, providing dye decolorization rates higher than those obtained with the powerful HBT mediator or other synthetic and natural mediators (Camarero et al., 2005; Campos et al., 2001; Dube et al., 2008; Murugesan et al., 2009). Moreover, the strong mediating capability of acetosyringone and syringaldehyde provides maximal decolorization rates using low mediator concentrations (substrate/mediator ratios of 2) (Camarero et al., 2005). In a broader approach, the presence of a phenolic extract from wheat bran enhanced the decolorization of malachite green as effectively as using HBT (Murugesan et al., 2009).

The positive effect of the phenolic compounds derived from the lignin polymer and constituents of humic acids on the oxidative transformation of widespread pollutants such as chlorinated phenols, had already been demonstrated (Park et al., 1999). These naturally occurring phenols have been also used to mediate the oxidation of the fungicide cyprodinil. Syringaldehyde, vanillic acid, vanillin, and *p*-coumaric acid resulted as the best laccase mediators with cyprodinil transformation rates up to 70% (Kang et al., 2002). More recently, laccase, in the presence of different natural and synthetic mediators, has been tested for degradation of different halogenated pesticides and emerging pollutants such as antibacterial agents (i.e. triclosan) or drugs (i.e. naproxene) used in cosmetics or medicines (Marco-Urrea et al., 2010; Murugesan et al., 2010). The use of syringaldehyde and acetosyringone caused the highest transformation rates of dichlorophen or niclosamide by laccase. Laccase–syringaldehyde system was able to produce both oxidative dehalogenation of pesticides and formation of pesticide–mediator adducts, which could be related to the reduction of their environmental impact (Torres-Duarte et al., 2009).

We have also tested these lignin-related phenolic compounds as laccase mediators for the degradation of PAH with excellent results. The recalcitrant aromatic structures of these pollutants have similarities with lignin network. In this case, vanillin, acetovanillone, ferulic acid and especially *p*-coumaric acid significantly promoted anthracene and benzo[a]pyrene transformation by *Pycnoporus cinnabarinus* laccase (Cañas et al., 2007). The outstanding efficiency of *p*-coumaric acid (PCA) to promote the oxidation of anthracene and benzo[a]pyrene by laccase (up to 100% removal), was much better than those found for other natural mediators described elsewhere or ABTS, with close similarity to that of HBT. Even more recalcitrant PAH like pyrene or phenanthrene were transformed by laccase–PCA (Camarero et al., 2008; Cañas et al., 2007). Comparison of the mediating capabilities of *p*-hydroxycinnamic acids, *p*-coumaric, ferulic and sinapic acid, revealed the higher efficiency of sinapic acid to promote dye decolorization by laccase whereas, ferulic and specially *p*-coumaric acid, easily mediated PAH oxidation.

In the course of these studies important pieces of evidence about the oxidation mechanisms performed by the phenoxyl radicals of these natural mediators were established, as described below.

6. Oxidation mechanisms of natural phenolic mediators

Substituted phenols, having an electrochemical potential comparable to that of laccase, are good laccase substrates. Laccase activity towards phenols is enhanced by the presence of electron-donating substituents at the benzene ring that decrease their electrochemical potential. In this sense, acetosyringone, syringaldehyde or sinapic acid that presents two methoxy substituents in ortho positions to the phenol group, are rapidly oxidized by laccase. These natural dimethoxy phenols have been found to act as extraordinarily fast and efficient laccase mediators for dye decolorization (Camarero et al., 2005; Camarero et al., 2008). The electron-donating groups in *ortho* stabilize phenoxyl radicals and increase their lifetime by preventing 5–5′ coupling reactions. Thus, the speed and efficiency of these dimethoxy phenols as laccase mediators in different oxidation processes would be related to both, the high concentration of free radicals generated by their easy oxidation by laccase, and the stability of their phenoxyl radicals.

On the other hand, not only the formation of long-living radicals but also of secondary species that can act as mediators (due to the existence of additional phenolic groups), has been suggested as determining factors in the feasibility and efficiency of phenol red as laccase mediator (d'Acunzo and Galli, 2003). According to this premise, we found that the mediating capability of sinapic acid, providing fast and easy decolorization of different dyes by laccase, was related to generation of phenolic dimeric products capable to act as redox mediators (Camarero et al., 2008). Phenoxyl radicals of sinapic acid have high tendency for β–β′ coupling so that oxidation of sinapic acid by laccase immediately renders dehydrodisinapic acid lactones (Fig. 5) (Lacki and Duvnjak, 1998). The latter compounds would be in turn oxidized by laccase (this oxidation being favored by their high antioxidant activity) yielding stable phenoxyl radicals (since additional coupling reactions are not possible). These phenolic dimeric products would act as laccase mediators as demonstrated by the easy oxidation of the analogous dimer syringaresinol by *P. cinnabarinus* laccase, and the significant increase of dye decolorization rates in the presence of syringaresinol (Camarero et al., 2008).

But, what is the mechanism by which these phenolic mediators of laccase oxidize non-phenolic substrates? In the course of experimental studies with phenol red as laccase mediator, a mechanistic parallel between >N–OH and phenolic mediators were established. Phenoxyl radicals ($PhO^{\bullet}$) generated during phenol red oxidation by laccase, would act analogously to nitroxyl radicals (>N–$O^{\bullet}$), following an hydrogen atom transfer (HAT) oxidation mechanism (d'Acunzo and Galli, 2003), and different from the electron transfer (ET) mechanism followed by $ABTS^{\bullet+}$ radicals (Cantarella et al., 2003). The bond dissociation energy of phenol derivates appears certainly appropriate to a moderately exothermic H-abstraction route from methoxy benzylic moieties, in keeping with the case of >N–OH intermediates (Astolfi et al., 2005).

The analysis of the reaction products obtained during PAH oxidation by laccase in the presence of *p*-coumaric acid (PCA) as mediator, corroborated this assertion (Cañas et al., 2007). Indeed, differences on the anthracene and benzo[a]pyrene oxidation products obtained with laccase–ABTS versus those obtained with laccase–HBT or laccase–PCA uphold the dissimilarity of their oxidation mechanisms. Although benzo[a]pyrene 1,6-, 3,6-, and 6,12-quinones were found in all cases as minor products, benzo[a]pyrenyl acetate intermediate was only accumulated when using laccase–ABTS due to the oxidation of this PAH by electron transfer. The absence of this

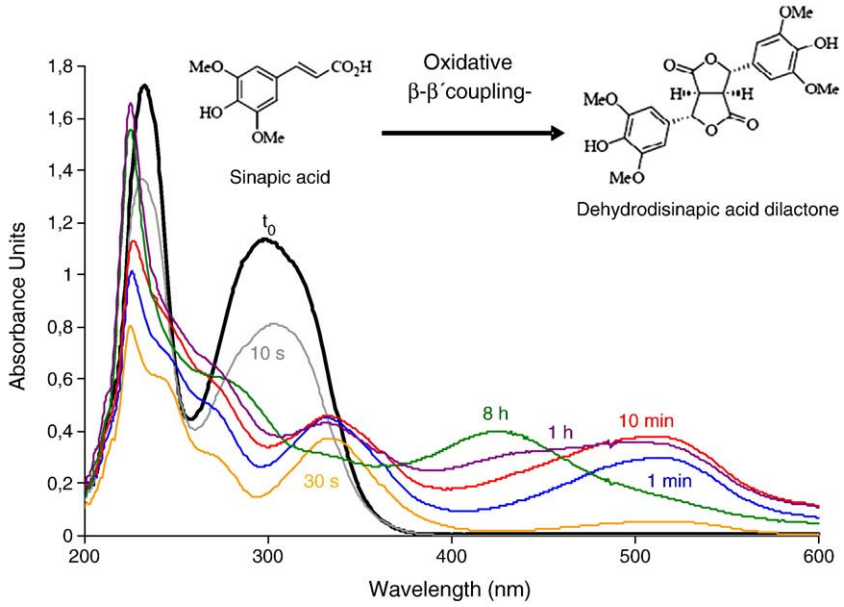

Fig. 5. Changes in the UV–visible spectrum of sinapic acid during its oxidation by laccase, reflecting the appearance of at least two different species. Increase of absorbance at 512 nm corresponds to formation of dehydrodisinapic acid dilactone by β–β´ oxidative coupling of sinapic acid (inset). (Figure adapted from Camarero et al., 2008).

intermediate when using laccase and PCA or HBT as mediators, sustains the performance of a radical oxidation route leaded by PhO• (and >N–O•) species. Thus, formation of benzo[a]pyrene free radicals, by H-abstraction with PCA phenoxyl radicals (and HBT nitroxyl radicals), impedes the formation of benzo[a]pyrene cation radicals and, therefore, the subsequent nucleophilic attack by the acetate ions of the buffered medium (see Fig. 6).

Slightly later, Calcaterra and co-workers confirmed that the oxidation of non-phenolic model substrates (bencylic alcohols) induced by laccase in the presence of two phenolic mediators (phenol red and hydroquinone) was attained by a radical (HAT) mechanism. By H-abstraction, the benzylic C–H bond was cleaved and PhO–H was restored (Calcaterra et al., 2008).

It has been found that a combination of two mediators in the same reaction catalyzed by laccase can increase the oxidation rates separately obtained with each laccase–mediator system. Synergic effect between ABTS and HBT had been described during PAH transformation by laccase (Pickard et al., 1999). Positive effect has also be found by joining the artificial mediator ABTS and vanillin or acetovanillone during pentachlorophenol transformation by laccase

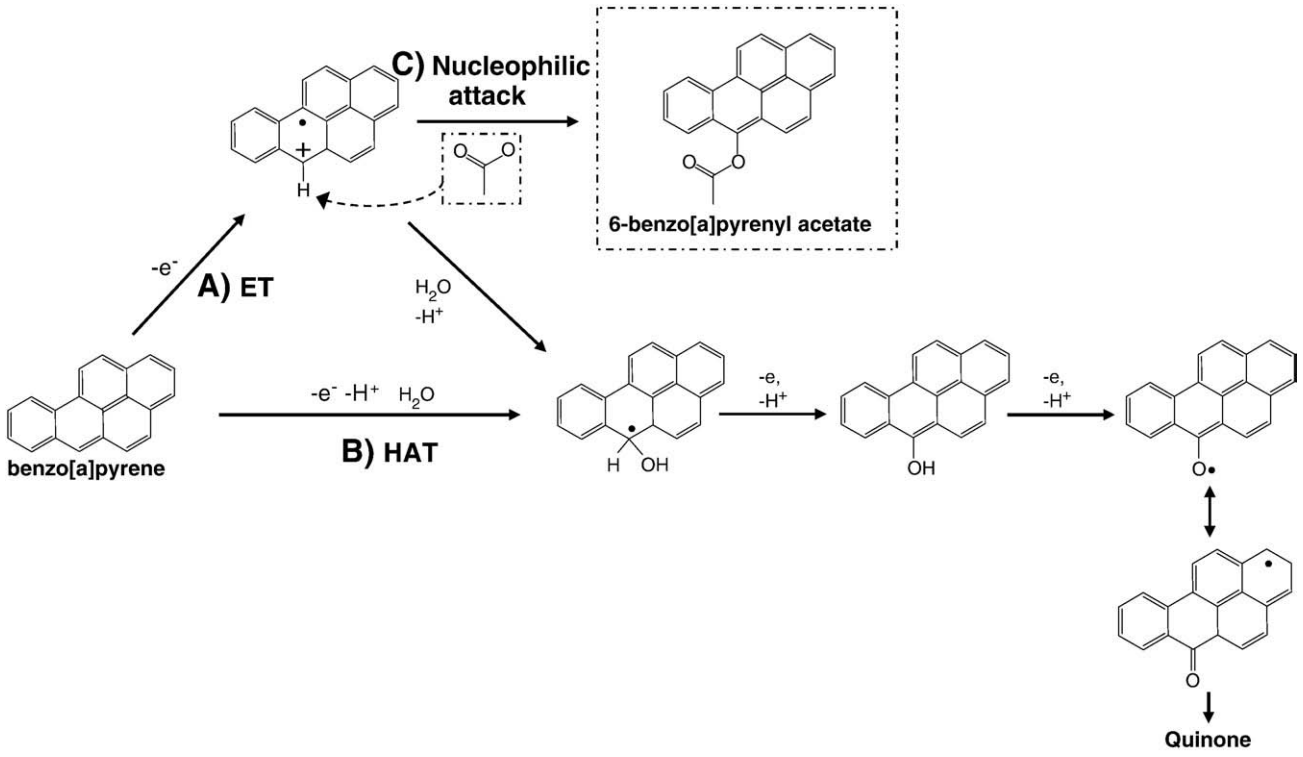

Fig. 6. Benzo[a]pyrene oxidation routes caused by different laccase–mediator systems: A) electron transfer route by laccase–ABTS; B) H atom transfer route by laccase–PCA and laccase–HBT; and C) nucleophilic attack of the acetate ions of the reaction mixture towards the benzo[a]pyrene cation radicals produced by the laccase–ABTS system. Figure adapted from (Cañas et al., 2007).

(Jeon et al., 2008). On the contrary, combination of both natural phenolic mediators produced no enhancement onto pentachlorophenol oxidation. The synergic effect between two laccase mediators seems to occur whenever their active species oxidize the target substrate by different mechanisms, confirming therefore the HAT mechanism for phenoxyl radicals versus ET of ABTS radicals.

Lipid peroxidation produced by laccase in the presence of *p*-coumaric acid as natural mediator (Fig. 7) corroborates the generation of free radicals via HAT by PCA phenoxyl radicals (Camarero et al., 2008). The addition of unsaturated lipids enables the transformation of very recalcitrant PAH such as phenanthrene by laccase using HBT as mediator, through the formation of peroxyl radicals that act as strong oxidizers (Bohmer et al., 1998). More-environmentally-friendly degradation of phenanthrene via lipid peroxidation reactions can be obtained using natural mediators. Thus, laccase in the presence of *p*-coumaric acid and unsaturated lipids rendered peroxyl radicals and phenanthrene transformation ratios similar to those obtained with HBT. The absence of lipid peroxidation with laccase alone or using ABTS as mediator (ET route) corroborates that HAT route is indispensable for lipid peroxidation to occur (Camarero et al., 2008).

As commented above, different factors determine the suitability of a certain compound as redox mediator. One of them is that radical mediator species should not inhibit laccase activity. In this sense, >N–O$^{\bullet}$ radicals inactivate laccase (Amann, 1997; Freudenreich et al., 1998) whereas certain phenolic compounds are capable to increase laccase stability (Mai et al., 2000) or protect its activity from extreme conditions in biotechnological applications (Aracri et al., 2009). Furthermore, the strong reactive HBT radicals decay to an inactive species (such as benzotriazole), in contrast to some reactive phenoxyl radicals which coupling produces phenolic dimers than act as laccase mediators (Camarero et al., 2008).

7. Laccases and natural mediators in bioremediation

Phenols are typical natural laccase substrates although laccase can directly oxidize non-phenolic substrates (i.e. anthracene) whenever their electrochemical potential is sufficiently low. On the other hand, the oxidation of specific phenolic substrates by laccase might be hindered by solubility or steric issues (i.e. Reactive Black 5 dye). In this event, small-size phenolic mediators can interact with complex substrates that cannot access enzymatic pocket directly. Moreover, substrates endowed with high electrochemical potential can be oxidized by radical mediators (PhO$^{\bullet}$) through the operation of H-abstraction mechanism.

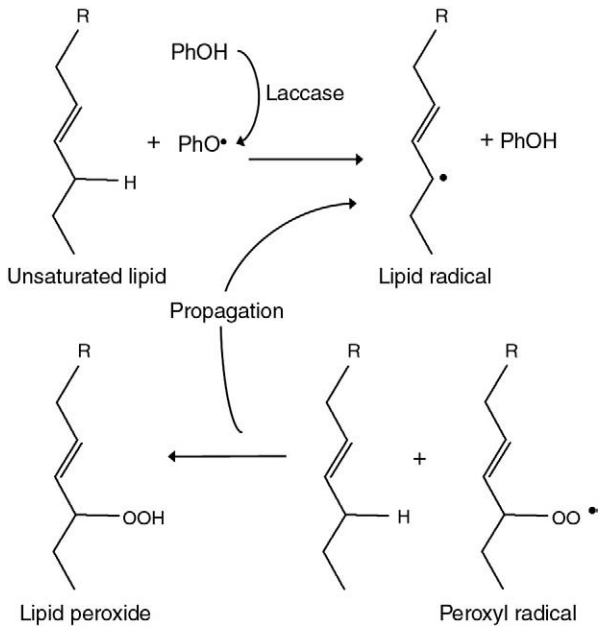

Fig. 7. Peroxidation of unsaturated lipids by the phenoxyl radicals (PhO$^{\bullet}$) generated during oxidation of natural phenolic mediators (PhOH) by laccase. (Figure adapted from http://search.com reference).

Likewise, successful *in vitro* transformation and detoxification of recalcitrant aromatic contaminants (dyes, PAHs, or chlorophenols) (Cañas et al., 2007; Camarero et al., 2005; Itoh et al., 2000; Cho et al., 1999; Chhabra et al., 2008) by laccase using phenolic compounds of natural origin as redox mediators, supports an alternative role of the above mentioned enzymatic systems in the *in vivo* degradation of aromatic pollutants by the endogenous microorganisms secreting this type of oxidoreductases in soils.

Laccases are ubiquitous enzymes in saprophytic fungi growing on soil and decayed plants (Claus, 2004). *p*-Hydroxycinnamic acids and lignin derived phenols, which are also present in soil, induce laccase expression by white-rot fungi (Terrón et al., 2004; Herpoël et al., 2000). Phenolic compounds, originated from tree litter (Kuiters and Denneman, 1987) and incorporated to humic substances, are also present in free form (Felbeck, 1971), being used as bioindicators of the ongoing process of humus formation (Djurdjevic et al., 2006). *p*-Coumaric acid and ferulic acid are abundant in forest litter (Suominen et al., 2003) and in soils containing high contents of non-woody plants (Fan and Deng, 2002), being also present in root exudates of trees (Munzenberger et al., 1990).

Taking the above information into account, decontamination of phenolic pollutants in soil could be directed towards their immobilization (humification) by oxidative coupling catalyzed by laccases (Tatsumi et al., 1992; Bollag, 1992), reducing their biodisponibility so far (Ahn et al., 2002; Bollag et al., 1980; Dawel et al., 1997; Dec and Bollag, 2000; Tatsumi et al., 1992). On the contrary, pollutants might undergo oxidative transformation (degradation) by laccases to easily up-taken products by soil microflora (Gianfreda and Rao, 2004) (Fig. 8). In this event, oxidation of recalcitrant pollutants would be promoted by the presence of lignin-related phenols acting as redox mediators. Moreover, the presence of unsaturated fatty acids in the mycelium hyphae (Lestan et al., 1990) could expanded the oxidative potential of these enzymatic systems onto more recalcitrant pollutants by formation of peroxyl lipid radicals, as described above.

Analogously, decontamination of industrial effluents by means of either oxidative coupling or oxidative transformation of pollutants by laccase (and natural mediators), can be achieved (Fig. 8). Oxidative coupling of chlorophenols or other contaminating phenols onto polymers can be easily removed by filtration. In addition, the oxidative transformation of aromatic pollutants by laccase implies their detoxification as revealed by production of dehalogenated products during oxidation of chlorophenols (Bollag et al., 2003) or generation of less toxic benzo[a]pyrene derivates by laccase and *p*-coumaric acid (Cañas et al., 2009).

8. Role of laccases and natural mediators in lignin biodegradation

As explained above, phenolic compounds related to recurring phenolic structures in lignin are capable of mediating the oxidation of non-phenolic compounds by a radical mechanism (once oxidized to phenoxyl radicals by laccase). This finding might be notably relevant to support the role of laccases in lignin biodegradation by means of no other mediators than the very phenolic groups of lignin. Hence, the first attack of laccases (or certain ligninolytic peroxidases) towards phenolic lignin units would bring the release of phenolic *sedox* mediators that could undertake the oxidation of the more recalcitrant non-phenolic lignin moiety.

The phenolic lignin moiety is the principal site of oxidative attack by white-rot fungi before acting on non-phenolic benzylic moieties (Camarero et al., 1994). Preferential oxidation of phenolic lignin units and side-chain oxidation would bring the release of phenolic residues with oxidized side chains. Phenolic aldehydes, ketones or acids, once

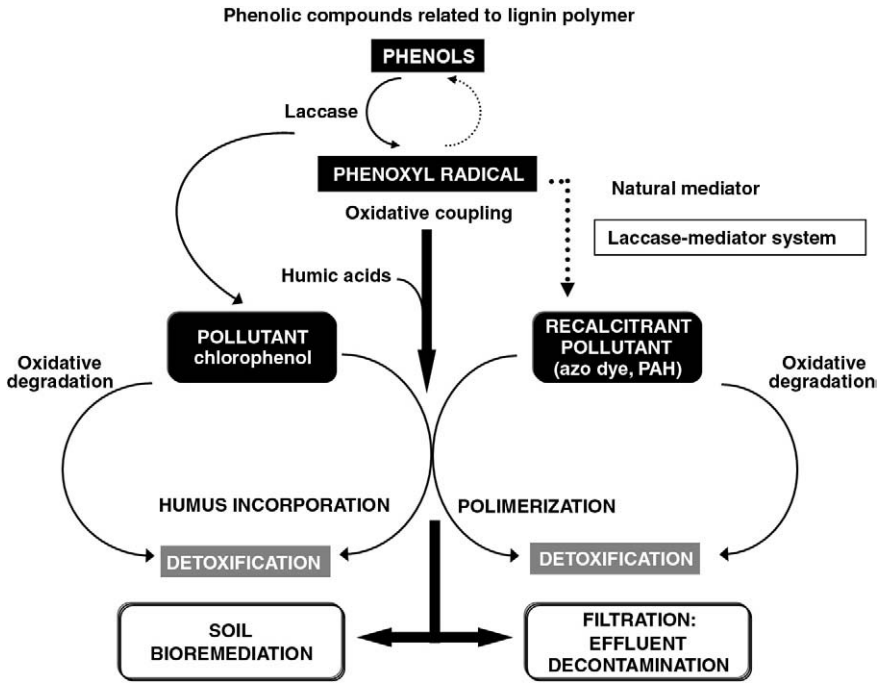

Fig. 8. Possible role of laccases and their natural phenolic mediators in soil bioremediation and detoxification of industrial effluents.

oxidized by laccase to form stable radicals, could move inside the lignin polymer and act as mediators oxidizing more recalcitrant (non-phenolic) lignin units by H-abstraction mechanism. On this topic, d'Acunzo and co-workers stressed the importance of phenoxyl radicals as true natural mediators of laccase. Bifunctional probes (with both phenolic and benzylic moieties together as substituents in the same substrate) have structural features comparable to portions of lignin. By using this type of probe, the initial formation of phenoxyl radical on one side of the aromatic bifunctional molecules might activate the benzylic substituent on the other side (d'Acunzo et al., 2006). Moreover, as the benzylic radical species is highly reactive with oxygen, easily generate side-chain oxidized products (Crestini et al., 2003). Profuse oxidation of lignin-side chains has also been found during pulp treatment with laccase–HBT (Ibarra et al., 2006b; Ibarra et al., in press).

Based on the feasible degradation of non-phenolic aromatic structures by laccases in the presence of redox mediator compounds related to lignin polymer, we suggest the potential biotechnological use of these green enzymes and their natural mediators to improve biomass conversion in the future lignocellulose biorefineries to produce renewable chemicals, materials, and biofuels.

The use of laccases to remove lignin from biomass might be achieved by different means, depending on the type of lignocellulosic material and its further use. Thus, natural phenolic mediators could be obtained as by-product or residue during the own industrial process of biomass conversion, i.e. from the water process of kraft pulping and used afterwards for pulp bleaching in enzymatic stages. Alternatively, the addition of natural mediators might not be necessary if laccase is being directly used for biomass pretreatment (i.e. in ethanol production). Under adequate conditions, the phenolic residues present in the lignocellulosic material (i.e. *p*-hydroxycinnamic acids highly abundant on herbaceous plants) could act as *in situ* laccase mediators promoting removal of the recalcitrant lignin moiety as already demonstrated with model compounds (Cho et al., 2008; Nousiainen et al., 2009).

9. Use of laccases and natural mediators in future lignocellulose biorefineries

The pulp and paper industry, together with the corn bioethanol industry, is an excellent starting point for launching the lignocellulose biorefineries ever since it possesses the best biomass conversion facilities coupled with practical industrial experience. Besides, the paper and pulp industry has been practicing certain aspects of the biorefinery philosophy since its beginnings, given that mill energy is supplied by combustion of wood residues and black liquors, the chemical reagents are recovered and kraft pulping byproducts (tall oils) are sold to produce high-value added products (adhesives, emulsifiers, detergents etc).

However, the adaptation of the forest-product industry (wood and paper) to integrated lignocellulose biorefineries requires biotechnological innovations to make available new high-value added products and bioenergy, together with traditional products. Among the innovations forecast for 2020 by the American Forest & Paper Association in partnership with the U.S. Department of Energy (Agenda 2020, 2007) are: 1) the introduction of new lignocellulosic resources (forest and herbaceous crops) rendering high industrial and energy yields; 2) the extraction of hemicelluloses previous to pulping to produce ethanol or chemicals; and 3) the gasification of biomass residues to produce energy power, liquid fuels or chemicals (Fig. 9). Gasification of biomass presents the advantage to convert lignocellulosic components, both fermentable (polysaccharides) and non-fermentable (lignin), in syngas. However, oxygen constitutes 40–45% of biomass that is converted to non-profitable CO_2 or water. Another drawback is the fibrous nature of plant biomass which hinders its pulverization.

On the other hand, biotechnology can contribute to the biorefinery concept providing higher plant integration, better use of vegetal resources, and reduction of residues. Laccase technology is applicable in every sector of forest-product industry, since laccases can be used either in those processes where lignin removal is the main objective (paper manufacture), or in those having lignin polymerization as the main goal (manufacture of fiberboards and others) (Widsten and Kandelbauer, 2008a). Actually, new and challenging applications of laccases, related to the synthesis of new materials and products from lignocellulosic resources, are currently being materialized. We have included in Fig. 9 the different points wherein biotechnological application of laccases (and natural mediators) could contribute more significantly to develop the Integrated Lignocellulose (Forest Products) Biorefineries.

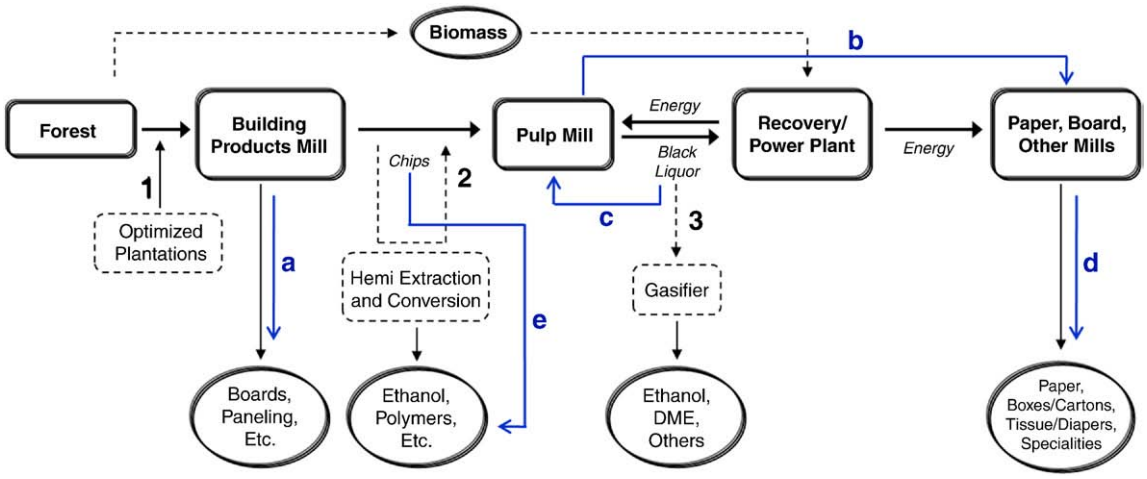

Fig. 9. Scheme of the Integrated Forest Products Biorefinery (IFPB) with the innovations forecast in Agenda 2020: 1) new lignocellulosic resources; 2) extraction of hemicelluloses previous to pulping; and 3) gasification of biomass residues. The points wherein laccase technology might contribute more significantly to this concept are also depicted: a) manufacture of new wood (or lignocellulose) products; b) develop of more efficient and eco-friendly industrial processes for paper pulp manufacture; c) recycling of black liquors and extraction of natural mediators; d) manufacture of cellulose products with new properties; or e) improved production of ethanol. (Figure adapted from Agenda 2020, 2007).

Wood products (boards and panels) employed in furniture construction are made with high cost adhesives derived from petroleum. Most of these adhesives are formaldehyde-based resins of which its highly hazardous emissions concern public opinion and limit the reuse of the fiberboards (Felby et al., 1997; Hüttermann et al., 1989). Laccases can contribute to reduce (or eliminate) the use of these toxic synthetic adhesives by catalyzing the cross-linking of phenolic residues in lignin-based materials to produce medium density fiberboards (MDF).

The use of laccases and their natural mediators can also contribute to develop new white industrial processes more efficient and clean. These enzymatic systems could be integrated at different stages of paper pulp manufacture: from pulp bleaching in enzyme-aided TCF bleaching sequences for removing the residual lignin responsible for pulp color (Camarero et al., 2004; Camarero et al., 2007), to control pitch deposits that reduce pulp quality and accumulates in circuits (Gutiérrez et al., 2007), or at the end of the process, for treating bleaching effluents rich in aromatic compounds. Generation of no organochlorinated compounds during the process and absence of pitch deposits in the mill circuits would allow the reuse of the process waters, contributing to the launch of zero-waste biorefineries. Of high relevance is the fact that these natural phenolic mediators can be obtained as by-product of the pulp manufacture process. As an example, black liquors from eucalypt kraft pulping are rich in some of these phenolic compounds (namely acetosyringone and syringaldehyde) which might be easily extracted at low cost from the recycled process waters (Camarero et al., 2005).

Another emerging research area, together with adhesion enhancement in binderless wood boards, is the tailoring of lignocellulosic materials by laccase-assisted biografting of phenols and other compounds. Laccase-assisted functionalization of wood and non wood fibers to modify different properties has been achieved (Chandra and Ragauskas, 2002; Lund and Ragauskas, 2001; Yamaguchi et al., 1992). Biografting of phenols onto different types of pulps can provide new physico-mechanical properties to paper such as improved strength due to promotion of new H-bonding and cross-linking between phenoxyl groups on pulp surface (Chandra and Ragauskas, 2002; Chandra et al., 2004). Laccase-assisted grafting of phenols can also modify optical properties like color. Even more, new antimicrobial resistance can be acquired using this enzymatic methodology to produce cellulose products for food packing or sanitary material (Elegir et al., 2008).

Finally, biotechnology has to face up several challenges to accomplish the complete conversion of biomass for ethanol production. One of them is the improvement of lignocellulose pretreatment and the use of ligninolytic oxidoreductases, that facilitate the further action of hydrolases on polysaccharides, is one of the biotechnological tools potentially available. Thermochemical pretreatment of lignocellulose generates furfural and phenols that inhibit the hydrolases used in the subsequent stage. Moreover, chemicals' recycling is difficult and expensive. Enzymatic pretreatment of biomass with laccases could diminish the impact of these phenolic compounds on the subsequent saccharification and fermentation stages. Indeed, enzymatic detoxification of steam-exploded wheat straw has been attained by using fungal laccases. Significant reduction of phenols by laccase-aided polymerization promoted yeast growth, glucose consumption and notably increased production of ethanol (Jurado et al., 2009). Furthermore, it is possible to develop yeast strains resistant to the phenols of lignocellulose hydrolyzates by laccase expression (Larsson et al., 2001), rendering higher growth and fermentation yields. This approach might be of much interest for simultaneous saccharification and fermentation of lignocellulose for ethanol production.

10. Conclusions and perspectives

Naturally-occurring phenols related to lignin polymer efficiently promote the *in vitro* oxidation of recalcitrant aromatic compounds by laccase. Degradation of non-phenolic structures, otherwise not oxidized by laccase, is provided in the presence of these phenolic mediators by means of an oxidation mechanism (H-abstraction) different from the enzymatic one. These phenolic compounds (released during lignin depolymerization or present as free acids in plants) appear to be the true mediators of fungal laccases in nature. Owing the feasible availability of these natural mediators and the ubiquity of laccase producers basidiomycetes in decayed wood and forest soils, a new and central role of laccase on the lignin biodegradation process, is conferred. Moreover, the application of these enzymes on sustainable industrial processes can be pushed forward by the presence of these environmentally-friendly mediators, easily available from lignocellulose biomass.

Acknowledgements

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