Maja Vaukner Gabrič, Franc Pohleven[1]

Laccase Application for Upgrading of Lignocellulose Fibers

Primjena lakaze za dogradnju lignoceluloznih vlakanaca

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ABSTRACT • *Laccases have the ability to oxidize both phenolic and trough mediators non-phenolic lignin related compounds. When reacting on lignin, they can display both ligninolytic and polymerizing (cross-linking) abilities, which makes them very useful for their application in industries based on lignocellulose material. Most of the published papers and applications of laccase and laccase-mediator systems on lignocellulose material relate to the pulp, paper and textile industry. Recent research has been done in terms of laccase assisted biografting of phenols and other compounds on wood surface and use of laccase for adhesion enhancement in fiberboard production. They can be introduced to wood technology as environmentally friendly enzymes. The paper reviews the application of laccases in industries based on lignocellulose material and discusses the future outlook and development in the above mentioned fields.*

Key words: *laccase, laccases mediators, lignocellulose material, lignin functionalization*

SAŽETAK • *Enzimi lakaze imaju sposobnost oksidacije fenolnih spojeva uz pomoć medijatora i nefenolnih spojeva lignina. Kada reagiraju na lignin, mogu pokazati i ligninolitičke i polimerizacijske (unakrsno povezujuće) sposobnosti, što ih čini vrlo korisnima za primjenu u industriji utemeljenoj na lignoceluloznim materijalima. Većina objavljenih radova i primjena enzima lakaze i posredničkih lakaza-sustava na lignoceluloznim materijalima odnose se na celulozu, papir i proizvode tekstilne industrije. Nedavno su provedena istraživanja o kalemljenju fenola i drugih spojeva uz pomoć lakaza na površinu drva i o primjeni lakaza za poboljšanje adhezije u proizvodnji ploča vlaknatica. Lakaze se mogu upotrijebiti u drvnoj tehnologiji kao ekološki prihvatljivi enzimi. U radu se analizira primjena enzima lakaze u industriji utemeljenoj na lignoceluloznim materijalima i razmatraju se buduće perspektive i razvoj na spomenutim područjima.*

Ključne riječi: *lakaze, posrednici lakaze, lignocelulozni materijal, primjena lignina*

1 INTRODUCTION
1. UVOD

Laccases (EC 1.10.3.2., p-diphenol:dioxygen oxidoreductase) belong to the multicopper oxidases family and are also called blue enzymes. They are widely distributed in many plant and fungal species (Riva, 2006). An enzyme of this group was first described by Yoshida (1883) at the end of the 19th century as a component of the resin ducts of the lacquer tree *Rhus vernicifera* (Yoshida, 1883). The physiological function of these biocatalysts is different in various or-

[1] Authors are assistant and professor at University of Ljubljana, Biotechnical Faculty, Department for Wood science and technology, Ljubljana, Slovenia.
[1] Autori su asistentica i profesor Sveučilišta u Ljubljani, Biotehnički fakultet, Odsjek za znanost o drvu i drvnu tehnologiju, Ljubljana, Slovenija.

ganisms but they all catalyze polymerization or depolymerization processes. It has been suggested that laccases are involved in cuticle sclerotization in insects and in the assembly of UV-resistant spores in *Bacillus* species. In plants, they are involved in cell wall formation and, together with peroxidases, in lignification (Mayer and Staples, 2002). In white rot fungi, the laccases are among the main enzymes involved in delignification process (Tavzes *et al*., 2009).

Meeting the challenges of reducing formaldehyde emissions from the adhesives and improving product recyclability, calls for innovative approaches to minimize the amount of binder while ensuring product quality. Enzymatic treatment of wood fibers or other wood particles before their pressing into composite boards, such as medium density fibreboards (MDF), has been investigated by Felby, Kharazipour and coworkers since 1997. For example, laccase has been shown to greatly improve the internal bond (IB) of fiber boards (Felby *et al*., 1997; Kharazipour *et al*., 1997; Hüttermann *et al.,* 2001; Felby *et al*., 2002; Felby *et al*., 2004). This effect may be explained by the fact that laccase oxidizes the phenolic structures in lignin, which leads to relatively stable free radicals that may participate in fiber bonding by subsequent radical coupling (Fackler *et al.*, 2008).

Others, like Widsten (2008b), made research for combining boards without petroleum-derived wood adhesives. Furthermore, an important topic related to wood products is the chemical modification of their surface properties to improve their resistance. Enzyme technology offers an environmentally friendly method for modifying solid wood, pulp, or other lignocellulosics by biografting of phenols and other molecules on their surfaces. Properties such as antimicrobial, antifungal, UV- and weathering stability, and fire retardancy have been or can potentially be imparted to lignocellulosic substrates (Widsten *et al*., 2008a).

2 DISTRIBUTION, STRUCTURE AND MECHANISMS OF LACCASE
2. DISTRIBUCIJA, STRUKTURA I MEHANIZMI ENZIMA LAKAZE

Laccases are common enzymes in nature. The first laccase was reported in *Rhus vernicifera*, the Japanese lacquer tree (Reinhammar, 1984). Subsequently laccases have been discovered in numerous plants. The majority of laccases characterized so far have been from wood decay fungi especially from white-rot basidiomycetes (Fig. 1) that are efficient lignin degraders. Well-known laccase producers also include fungi belonging to the ascomycetes, deuteromycetes and basidiomycetes (Sharma *et al*., 2007).

The first report of prokaryotic laccase is from the rhizospheric bacterium *Azospirillum lipoferum* (Givaudan *et al.,* 1993). The best-studied bacterial laccase is the CotA, the endospore coat component of *Bacillus subtilis*. The CotA gene codes for a 65-kDa protein belonging to the outer spore coat. CotA participates in the biosynthesis of the brown spore pigment, which mutants in the gene encoding CotA lost the ability to produce. The protein, which was over expressed in E. coli, has a molecular mass of 65 kDa, an isoelectric point of 7.7 and is highly thermo stable (Martins *et al*., 2002). Fungal laccases molecular mass ranges from 50 to 100 kDa with a 10 to 45 % covalently linked carbohydrate molecules. For the catalytic activity, a minimum of four copper atoms per protein unit are needed, and they are divided in three Types (T1, T2, and T3). T1 is a paramagnetic copper with absorbance at 610 nm and confers the blue color to the multicopper proteins, which results from the intense electronic absorption caused by the covalent copper-cysteine bond. It is also the site where substrate monoelectronic oxidation takes place. It has a high redox potential of +790 mV. On the other hand, T2 copper shows no absorption in the visible spectrum but reveals paramagnetic properties in Electron Paramagnet Resonance (EPR) studies. It is positioned close to the T3, a binuclear center spectroscopically characterized by an electron adsorption at 330 nm. T2 and T3 copper form a trinuclear cluster, where reduction of molecular oxygen and release of water take place. The oxidation of substrates (p-diphenols) creates reactive radicals that can undergo non-enzymatic reactions like cross-linking of monomers, degradation of polymers and ring cleavage of aromatic compounds (Claus, 2003; 2004) (Fig. 2).

Figure 1 White-rot caused by basidiomycetes *Oudemansiella mucida* (photo by Franc Pohleven, 2011)
Slika 1. Bijela trulež uzrokovana basidio gljivicama *Oudemansiella mucida* (foto: Franc Pohleven, 2011)

Because of their redox potential of around ≤0.8 V, their action is restricted to the oxidation of the phenolic lignin moiety, whereas non-phenolic substrates having redox potential above 1.3 V cannot be oxidized by laccases directly (Cañas and Camarero, 2010).

For catalyzing the oxidation of non-phenolic substrates, laccase requires the presence of a mediator. A mediator is a small molecule in the medium that behaves like an "electron shuttle" between laccase and substrate. They act as redox mediators and oxidize other compounds that are not substrates of laccase (Sharma *et al*., 2007). In 1990, the first artificial mediator 2,2'-azino bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was used in the laccase mediator systems (LMS) for pulp delignification (Bourbonais *et al*.,

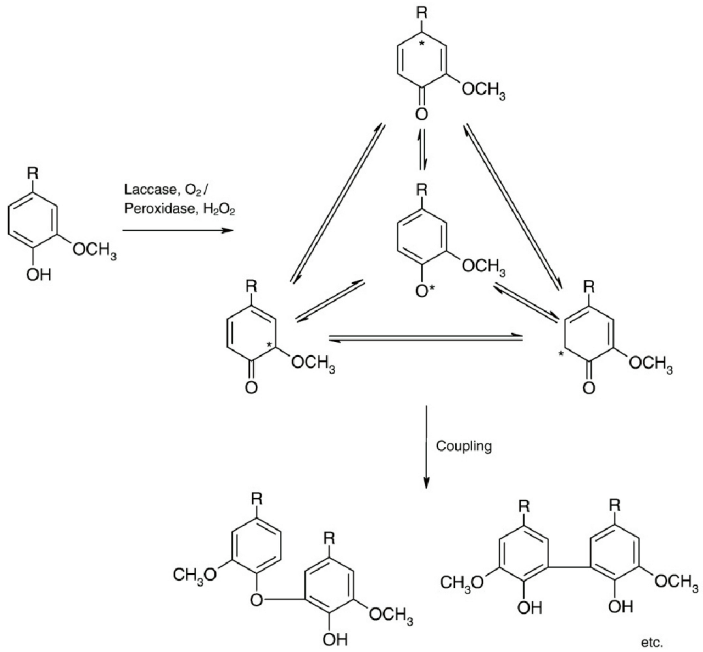

Figure 2 Coupling reactions of phenoxy radicals on lignocellulosic substrates treated with phenol-oxidizing enzymes (Widsten and Kandelbauer, 2008b)
Slika 2. Reakcija fenoksi radikala na lignoceluloznim podlogama tretiranim fenol-oksidacijskim enzimima (Widsten i Kandelbauer, 2008b)

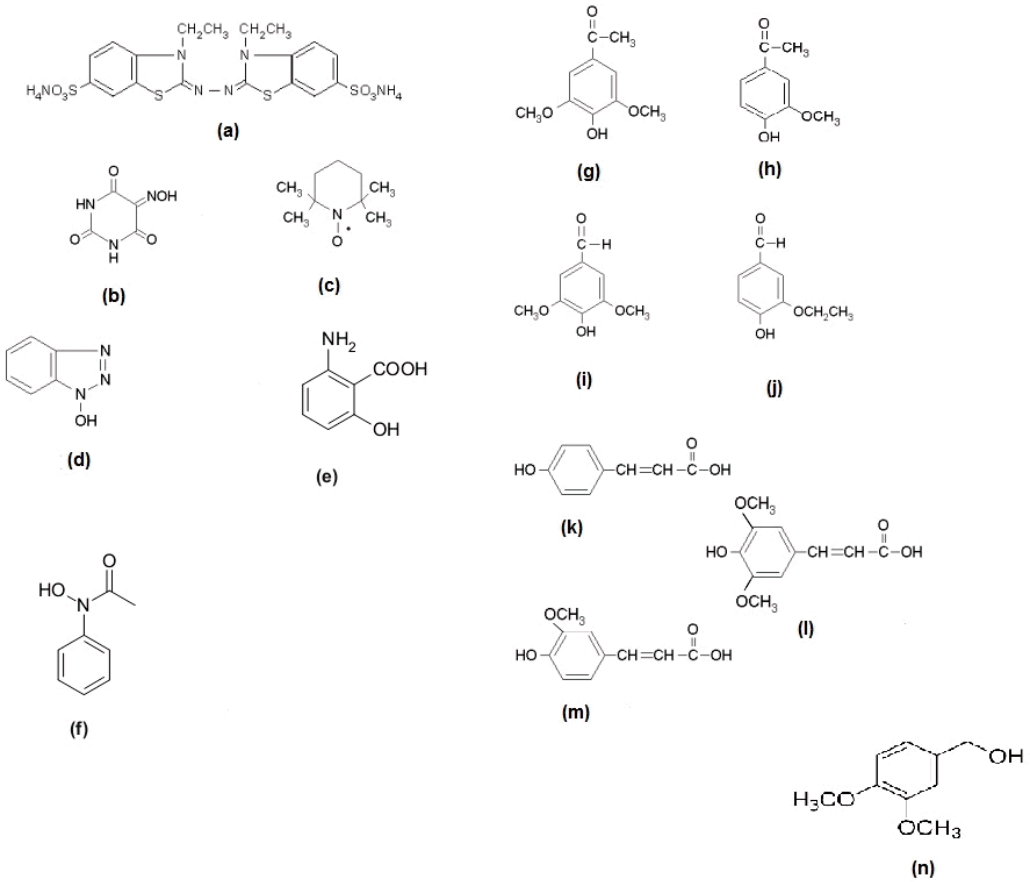

Figure 3 Examples of laccases artificial (a-f) and natural (g-m) mediators and veratryl alcohol. (a) 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS); (b) violuric acid; (c) 2,2,6,6-tetramethylpiperidine-1-yloxy (TEMPO) (VLA); (d) N-hydroxybenzo-triazole (HBT); (e) 3-Hydroxyanthranilic acid (HAA); (f) N-hydroxyacetanilide (NHA); (g) acetosyringone; (h) acetovanillon; (i) syringaldehyde; (j) vanillin; (k) *p*-coumaric acid; (l) sinapinic acid; (m) ferulic acid; (n) 3,4-dimethoxybenzyl alcohol (VA) (Riva, 2006; Kunamneni et al., 2008)
Slika 3. Primjeri umjetnih (a-f) i prirodnih (g-m) posrednika lakaze i veratril alkohola (n): (a) 2,2'-azino-bis (3-etilbenzotiazolin-6-sulfonska kiselina) (ABTS); (b) violurična kiselina; (c) 2,2,6,6-tetrametilpiperidin-1-iloksi (TEMPO) (VLA); (d) N-hidroksibenzo-triazol (HBT); (e) 3-hidroksiantranilna kiselina (HAA); (f) N-hidroksiacetanilid (NHA); (g) acetosiringon; (h) acetovanilon; (i) siringaldehid; (j) vanilin; (k) p-kumarinska kiselina; (l) sinapinska kiselina; (m), ferulična kiselina; (n) 3,4-dimetoksibenzil alkohol (VA) (Riva, 2006; Kunamneni *et al.*, 2008.)

1995). Since then, more than hundred other compounds have been tested for their ability to oxidize lignin or lignin models (Fig. 3). Riva and coworkers (2006) proved the most effective mediators for lignin degradation to be the N – heterocycles bearing N – OH groups (Fig. 3 b, d, f, i).

The cost and toxicity of synthetic mediators tend to be prohibitive for implementation in industry. This has generated interest in natural mediators obtainable from plants, fungi or as industrial by-products. Potentially cost-effective lignin derived natural mediators like p-coumaric acid, syringaldehyde and acetosyringone have been investigated by Camarero et al. (2007). Not only organic compounds have been investigated as laccase mediators, but also inorganic polyoxometalate and other transition metal complexes (Widsten and Kandelbauer, 2008a).

3 LIGNOCELLULOSE MATERIAL AS LACCASE SUBSTRATE
3. LIGNOCELULOZNI MATERIJAL KAO PODLOGA LAKAZE

Lignin is an amorphous polymer; it comprises approximately 20–32 % of dry wood mass and functions as a cementing material in wood cells. Lignin consists of p-hydroxyphenyl, guaiacyl and syringyl-type phenylpropane units in which the aromatic units bear 1, 2 or 3 free or etherified hydroxyl groups. The phenylpropane units are linked together by alkyl aryl ether (α-O-4, β-O-4), aromatic ether (4-O-5') bonds and carbon-carbon bonds (5-5' or β-5) in condensed structures (Fig. 4). A good understanding of lignin structure and chemistry is helpful in the development of laccase based treatment technology for wood (Widsten and Kandelbauer, 2008a).

Adhesion improvement of lignocellulosic products, such as medium-density fiberboard and particleboard, by enzymatic bonding methods are well summarized in a paper by Widsten and Kandelbauer (2008b). They mention two approaches; one is to improve the self-bonding properties of the particles by oxidation of their surface lignin before they are fabricated into boards. Another method involves using enzymatically pre-treated lignins as adhesives for boards and laminates.

Fackler *et al*. (2008) functionalized spruce wood particles by fungal laccase combined with 4-hydroxy-3-methoxybenzylamine (HMBA) or 4-hydroxy-3 methoxybenzylurea (HMBU). The expectation was cross-linked with urea-formaldehyde (UF) resins in subsequent bonding processes, which should improve strength properties of particle boards (Fig 5). Mechanical testing and multivariate data analysis revealed an

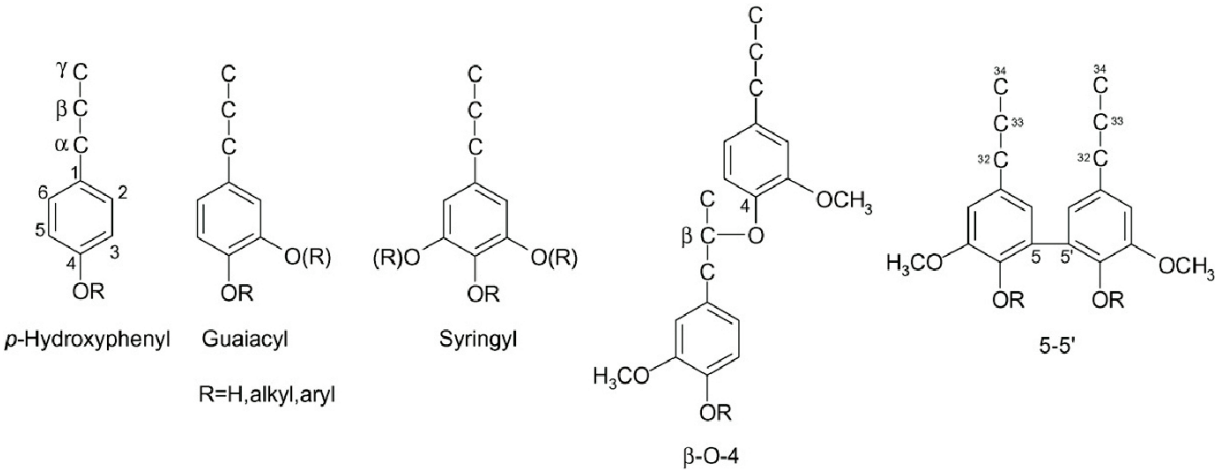

Figure 4 Lignin model compounds (p-Hydroxyphenyl, Guaiacyl, Syringyl);a β-O-4 ether linkage; an example of condensed lignin structures, a biphenyl 5-5' lignin substructure (Widsten and Kandelbauer, 2008a)
Slika 4. Modeli spojeva lignina (p-hidroksifenil, Guajacil, Siringil); β-O-4 eter veza; primjer kondenziranih ligninskih struktura, bifenil 5-5' ligninska podstruktura (Widsten i Kandelbauer, 2008a)

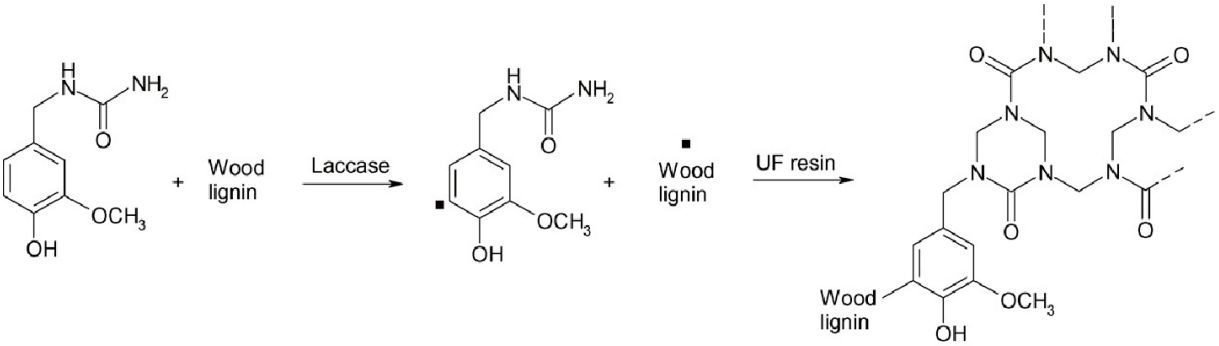

Figure 5 Functionalization of lignin with laccase and 4-hydroxy-3-methoxybenzylurea (HMBU), and cross-linking of functionalized lignin and urea-formaldehyde (UF) resin in particleboards (Fackler *et al.*, 2008)
Slika 5. Funkcionalizacija lignina primjenom enzima lakaze i 4-hidroksi-3-metoksibenzilurea (HMBU) te umreženje funkcionaliziranog lignina i urea-formaldehidne (UF) smole u pločama ivericama (Fackler *et al*., 2008.)

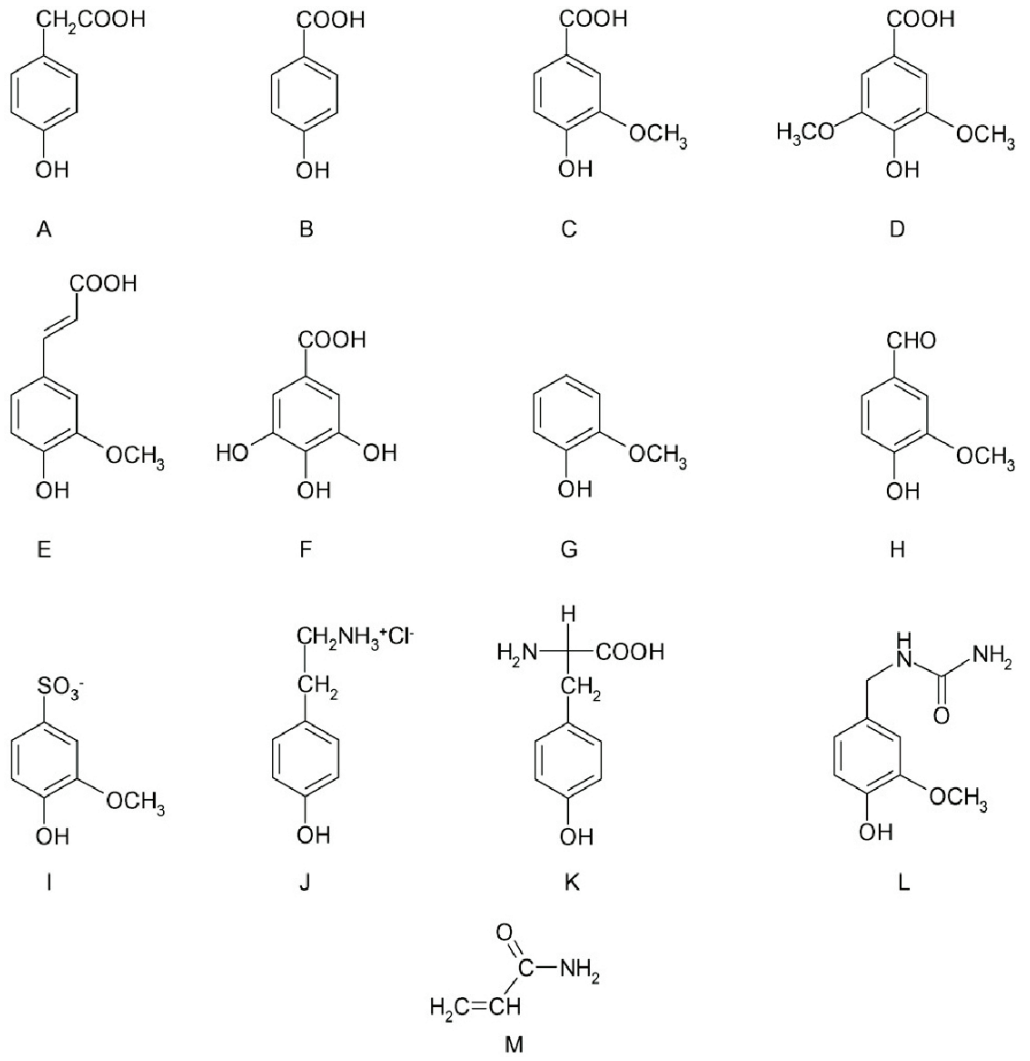

Figure 6 Examples of low-molecular weight compounds biografted to lignocellulosic materials with the aid of laccase: (A)4-hydroxyphenylacetic acid; (B)4-hydroxybenzoic acid; (C) vanillic acid; (D) syringic acid; (E) ferulic acid; (F) gallic acid; (G) guaiacol; (H) vanillin; (I) guaiacol sulfonate; (J) 3-hydroxytyramine hydrochloride; (K) tyrosine; (L) 4-hydroxy-3 methoxybenzylurea; (M) acrylamide (Widsten in Kandelbauer, 2008a)

Slika 6. Primjeri spojeva male molekularne težine kalemljeni na lignocelulozne materijale uz pomoć enzima lakaze: (a) 4-hidroksifeniloctena kiselina; (B) 4-hidroksibenzojeva kiselina; (C) vanilična kiselina; (D) siringijska kiselina; (E) ferulična kiselinu; (F) galna kiselina; (G) gvajakol; (H) vanilin; (I) gvajakol sulfonat; (J) 3-hidroksitiramin hidroklorid; (K) tirozin; (L) 4-hidroksi-3 metoksibenzilurea; (M) akrilamid (Widsten u Kandelbauer, 2008a)

increase of internal bond (IB) as a result of functionalization with HMBU. HMBA showed no significant increase of IB.

Biografting is a method for tailoring the surface of lignocellulose material (LM) under mild conditions and usually without harmful solvents. Series of reactions result in the introduction of new functional groups that may alter the physicochemical properties of LM in a desired way. Laccase may act as a catalyst for the covalent binding of compounds with low-molecular weight to lignin in wood and pulp fibers (Chandra *et al.,* 2002, 2004a,b; Grönqvist *et al.,* 2006). Examples of grafted molecules are shown in Figure 6.

Kudanga *et al*. (2010b) showed for the first time the mechanistic evidence of a laccase-catalyzed method of covalently grafting hydrophobicity enhancing fluorophenols onto *Fagus sylvatica* veneers. Coupling was made of fluorophenols (4-fluoro-2-methylphenol, 4-[4-(trifluoromethyl)phenoxy] phenol and 4-(trifluoromethoxy)phenol) onto complex lignin model compounds guaiacylglycerol β-guaiacyl ether and syringylglycerol β-guaiacyl ether (Fig 7). The covalent bonding was demonstrated by LC–MS, NMR and XPS analysis.

Furthermore, they report of laccase-mediated grafting of long chain alkylamines onto LMs and how it can be potentially exploited for improving their hydrophobicity (Kudanga *et al*. (2010a). Concomitantly the grafting of dihexylamine (DHA) or dodecylamine (DA) onto beech veneers resulted in a 53.8 % and 84.2 % increase in hydrophobicity, respectively when compared to simple adsorption (Kudanga *et. al.*, 2010a). The advantage of laccase-mediated covalent binding of molecules onto wood surface is that the grafted molecules are not easily released into the environment.

Schubert (2013) represented laccase-catalysed iodination of wood as an efficient method for wood protection. The enzymatic oxidation of iodide (I-) to

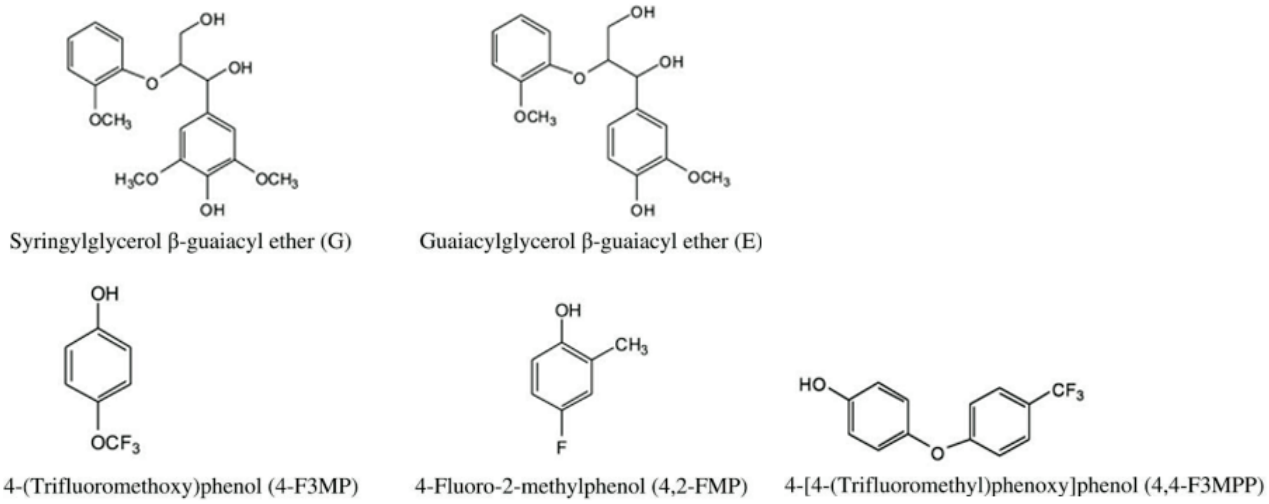

Figure 7 Chemical structures of the lignin model compounds and fluorophenols used in the coupling reactions in an attempt to achieve higher surface hydrophobicity. (Kudanga *et. al.*, 2010b)
Slika 7. Kemijske strukture spojeva lignina i fluorofenola koji se upotrebljavaju u kondenzacijskim reakcijama kako bi se postigla veća hidrofobnost (Kudanga *et al*., 2010b)

iodine (I_2) in the presence of wood led to an enhanced resistance of the wood surface against all microorganisms, even after exposure to leaching.

4 SUCCESSFUL APPLICATION OF LACCASE ON OTHER LM
4. USPJEŠNA PRIMJENA LAKAZE NA DRUGIM LIGNOCELULOZNIM MATERIJALIMA

In textile industry, laccase is used to bleach textiles and even to synthesize dyes. In 1996 Novozyme from Denmark launched a new industrial application of laccase enzyme in denim finishing called DeniLite®. It is the first industrial bleaching enzyme acting with the help of mediator molecule. In 2001, the company Zytex from India developed the formulation based on a laccase mediator system (LMS) Zylite® capable of degrading indigo. Due to laccases potential to degrade dyes of diverse chemical structure, it seems an attractive solution for removal of dyes from industrial effluents (Couto *et al.*, 2006).

Production of chemical pulp paper requires separation and degradation of lignin in wood pulp, pretreatments of wood pulp with ligninolytic enzymes provides milder and cleaner strategies than polluting chlorine-based procedures. In pulp and paper industry, laccase and LMS are used for a variety of processes like biopulping, biobleaching, deinking, pitch control by pulp treatment, enhancing paper strength properties, mill process water and effluent treatment (Widsten and Kandelbauer, 2008a). LMS have also found commercial applications in paper industry such as Lignozym® - process (Couto *et al.*, 2006).

Tavzes et al. (2009) tested the chemical changes induced in melanin as a result of treatment with laccase and 1-hydroxybenzotriazole (HBT). Since melanin is a black pigment produced by moulds and various blue-staining fungi which also infest many art objects, the implication of these findings can be applied in art conservation science (Tavzes *et al.*, 2009).

5 FUTURE OUTLOOK
5. POGLED U BUDUĆNOST

Mayer and Staples (2002) titled their paper "Laccases: New Functions for an Old Enzyme" and almost ten years later we are discovering new functions and possible applications for this "old" enzyme. Not only can we use it for new wood technological processes, but we can also use it for bioremediation of old wood preservatives that were used for wood protection in the past, such as lindan (gamma-hexachlorocyclohexane) and PCP (pentachlorophenol). Ulčnik (2012) demonstrated that white rot fungi can degrade lindan. With the use of enzymes, wood industry has the potential to become part of many fields in biotechnology and modern environmentally friendly technologies. Who knows what else the future holds for these "old eco-friendly" enzymes.

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Corresponding address:

MAJA VAUKNER GABRIČ, uni. dipl. ing. chem. and chem. teh.

University of Ljubljana, Biotechnical Faculty
Department of Wood Science and Technology
Rožna dolina c. VIII/34
1000 Ljubljana, SLOVENIA
e-mail: maja.vaukner@bf.uni-lj.si