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Review

Bacterial enzymes involved in lignin degradation

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ABSTRACT

Lignin forms a large part of plant biomass. It is a highly heterogeneous polymer of 4-hydroxyphenylpropanoid units and is embedded within polysaccharide polymers forming lignocellulose. Lignin provides strength and rigidity to plants and is rather resilient towards degradation. To improve the (bio)processing of lignocellulosic feedstocks, more effective degradation methods of lignin are in demand. Nature has found ways to fully degrade lignin through the production of dedicated ligninolytic enzyme systems. While such enzymes have been well thoroughly studied for ligninolytic fungi, only in recent years biochemical studies on bacterial enzymes capable of lignin modification have intensified. This has revealed several types of enzymes available to bacteria that enable them to act on lignin. Two major classes of bacterial lignin-modifying enzymes are DyP-type peroxidases and laccases. Yet, recently also several other bacterial enzymes have been discovered that seem to play a role in lignin modifications. In the present review, we provide an overview of recent advances in the identification and use of bacterial enzymes acting on lignin or lignin-derived products.

Contents

1. Introduction

Plant biomass is the most abundant renewable biomass on earth and is considered as an attractive source of bioenergy and biobased chemicals. It is mainly composed of lignin, cellulose and hemicellulose. The lignin percentage in lignocellulosic biomass is around 10–30% and is the second most abundant natural organic polymer. Lignin enables plants to generate rigid structures and provides protection against hydrolysis of cellulose and hemicellulose. The biotechnological conversion of lignocellulose into different carbohydrates, including glucose, is the basis for the production of ethanol, carbohydrates and aromatic products (Asgher et al., 2014; Ragauskas et al., 2014; Kawaguchi et al., 2016). Such plant biomass derived products can be used as fuel, polymer precursors, food and flavor compounds, and pharmaceutical building blocks. For optimizing the use of plant biomass through biorefining, lignin

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degradation has become a key target in the last few years. Efficient and cost-effective methods for selective lignin degradation are in high demand. It is worth noting that, while the recent intensified efforts in complete valorization of plant biomass, lignin was already considered as a major industrial by-product in the first half of the previous century (Gottlieb and Pelczar, 1951).

While cellulose and hemicellulose are built from carbohydrates, lignin is a highly cross-linked polymer formed by polymerization of 4-hydroxyphenylpropanoid monomers (monolignols) through various ether and carbon–carbon bonds. The phenolic moieties of the monomeric units are *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) groups and the percentage of each depends on the plant species and tissue. The formation of lignin is triggered by plant peroxidases and/or laccases. By oxidizing the phenolic monolignols into their respective phenolic radical, formation of dimers is catalyzed. Subsequent enzyme-catalyzed single electron oxidations promote polymerization. Monolignols can couple *via* various bonds with a preference of coupling through the β-carbon. The most occurring linkages involve β—β, β—*O*—4, and β—5 bonds (Vanholme et al., 2010), as shown in Fig. 1.

Due to its aromatic nature and highly branched polymer network, lignin is rather inert towards degradation (Abdel-Hamid et al., 2013). Yet, to complete global carbon cycling, nature has evolved catabolic pathways since the time that plants started to produce lignin (Nelsen et al., 2016). White-rot fungi have developed a rich collection of extracellular oxidative enzymes to attack and degrade lignin. They employ different types of heme-containing peroxidases, which include the so-called lignin peroxidases (LiP), manganese peroxidases (MnP), versatile peroxidases (VP), and dye-decolorizing peroxidases (DyP) (Lambertz et al., 2016). While some of these peroxidase are capable of attacking lignin or lignin fragments, peroxidases also attack lignin from a distance. By oxidizing mediators, small oxidizing agents are generated that can penetrate the branched lignin polymer to trigger depolymerization via radical chemistry (Nousiainen et al., 2014; Baciocchi et al., 2002; Glenn and Gold, 1999). Known mediators are lignin derived aromatic compounds (e.g. formation of veratryl alcohol cation radical) and manganese ions (Hunt et al., 2013). For effective peroxidase-based lignin degradation, also various fungal oxidases are secreted to produce the required hydrogen peroxide. Candidates for the extracellular production of hydrogen peroxide are aryl alcohol oxidases, glyoxal oxidases, and various carbohydrate oxidases. Except for peroxidases, fungi also secrete various copper-containing oxidative laccases that assist in lignin degradation. Intriguingly, it seems that the same types of enzymes used for lignin synthesis in plants (peroxidases and laccases) are used by fungi to recycle the aromatic polymer. Genome sequence analysis of ligninolytic fungi has revealed that there is not one defined set of enzymes for lignin degradation (Floudas et al., 2012). The composition of the set of oxidative enzymes being produced depends on the fungus.

While a wealth of biochemical knowledge has been obtained on fungal degradation of lignin, the ligninolytic capacity of bacteria has been less well studied. While it appears that white-rot fungi are very well equipped for lignin degradation, evidence is growing that also bacteria are capable of delignification. Already in 1930 Phillips et al. reported on a thorough study on lignin decomposition by "soil microorganisms", which presumable were bacteria (Phillips et al., 1930). While many claims of bacterial lignin degradation have been reported since then, only in the last few decades some bacterial enzymes involved in delignification have been identified. With this review we aim at providing an overview of the bacterial enzymes that have been implicated to be involved in degrading lignin or the oxidation of lignin derived degradation products.

2. Bacterial enzymes acting on lignin

2.1. DyP-type peroxidases

As described above, white-rot fungi produce several different kinds of heme-containing peroxidases to trigger lignin decomposition. However, homologs of the most common fungal ligninolytic peroxidases, LiPs MnPs and VPs, have not been encountered in biochemical studies on ligninolytic bacteria. Also when analysing sequenced genomes (Davis et al., 2013) or proteomes (Brown et al., 2011) of ligninolytic bacteria, no homologs emerge. It seems that these lignin-degrading peroxidases, belonging to the superfamily of plant peroxidase (Class II) (Welinder, 1992), are restricted to fungi. Yet, recently it has become clear that bacteria are relatively rich in another type of peroxidase, the so-called dye-decolorizing peroxidases (DyPs, EC 1.11.1.19) (Van Bloois et al., 2010). DyPs represent a newly discovered family of heme-containing peroxidases, which has recently received attention due their ability to degrade lignin and other compounds (Sugano, 2009; Colpa et al., 2014; Singh and Eltis, 2015; Yoshida and Sugano, 2015). The first discovered member of this enzyme family, DyP from *Bjerkandera adusta*, was isolated and characterized in 1999 (Kim and Shoda, 1999). Studies on the activity of this enzyme on synthetic anthraquinone and azo-dyes have served to name this family of peroxidases (Sugano et al., 2007). In recent years a large number of bacterial DyPs have been described in literature (Lambertz et al., 2016) which is in line with the observation that putative DyP-encoding genes are abundantly present in bacterial genomes (Table 1) (Van Bloois et al., 2010). In fact, already in 1988 a bacterial 'lignin peroxidase' was described from *Streptomyces viridosporus*. Unfortunately, no sequence has ever been deposited for this protein or the respective gene while several papers have appeared on cloning of the respective gene (Ramachandra et al., 1998; Wang et al., 1990; Thomas and Crawford, 1998). Yet, when analysing the recently sequenced genome of this *Streptomyces* isolate, a gene encoding a putative Tat-secreted DyP can be identified (Davis et al., 2013). This may well be the enzyme that was described long before the first fungal DyP was described.

DyPs have a protomer weight of around 40–60 kDa and various oligomeric states have been observed (Colpa et al., 2014). They belong to the peroxidase-chlorite dismutase superfamily of proteins and contain a non-covalently bound heme *b* cofactor (Zámocký et al., 2015). DyPs show a dimeric ferredoxin-like fold consisting of a four-stranded anti-parallel β-sheet surrounded by α-helices. DyP-type peroxidases contain a highly conserved GXXDG-motif and a conserved proximal histidine, which acts as the fifth ligand of the heme iron. Yet, while DyPs are structurally unrelated to the common fungal peroxidases, they exhibit similar catalytic properties with having similar redox potentials and reactivities (Liers et al., 2014). Furthermore, some of the bacterial DyPs are secreted via the Tat secretion machinery which adds to the analogy with the secreted fungal peroxidases.

Based on sequence characteristics, DyPs have been divided in four classes in the PeroxiBase database (Fawal et al., 2013). Proteins belonging to classes A–C are mainly found in bacteria, while class D DyPs are extracellular fungal representatives (Yoshida and Sugano, 2015). Class A DyPs typically have a Tat-signal sequence and are therefore secreted. In contrast, the DyP protein sequences of class B and C DyPs do not disclose any secretion signal peptides, suggesting that they are intracellular enzymes. The InterPro database currently contains 8318 DyP sequences. Approximately thirty of these enzymes have been isolated and characterized (Colpa et al., 2014; Yoshida and Sugano, 2015). DyPs are mainly active at acidic pH and show a very broad substrate profile, including several classes of synthetic dyes, monophenolic compounds, veratryl alcohol, β-carotenes, Mn^{+2} and lignin model compounds, but their physio-

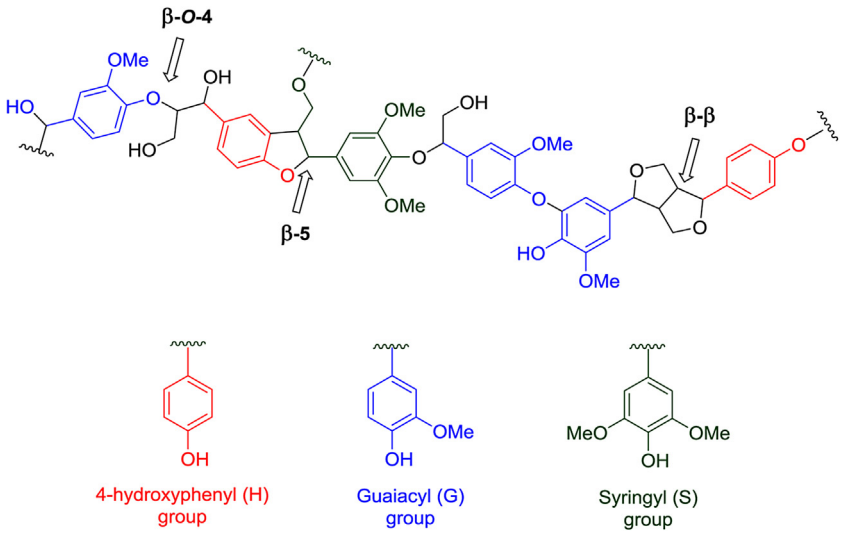

Fig. 1. Example of a lignin structure containing the most frequent bonds as well as the corresponding monomers that take part of its structure: 4-hydroxyphenyl (H), guaiacyl (G) and syringyl (S).

Table 1
Occurrence of DyPs in bacterial genomes. By performing a BLASTP analysis of the predicted proteomes, homologs of known DyPs were identified.

Organism	DyP type		
	A	B	C
Escherichia coli K-12	1	1	
Thermobifida fusca YX	1		
Rhodococcus jostii RHA1	1	1	
Streptomyces viridosporus strain T7A	1		
Streptomyces coelicolor A3(2)	2	1	
Amycolatopsis sp. 75iv2	1		2
Pseudomonas sp. strain YS-1p		2	

logical substrates still remain unknown. DyP-peroxidases can also catalyse interesting synthetic reactions such as enantioselective sulfoxidations (Van Bloois et al., 2010), heme deferrochelatations (Létoffé et al., 2009) and even carbonyl olefination processes in the absence of hydrogen peroxide (Weissenborn et al., 2016). A fungal DyP has recently been found to enhance lignocellulose degradation (Linde et al., 2015).

In the last years, several bacterial DyP-type peroxidases have been implicated in the degradation of lignin and lignin model compounds. DyP-mediated oxidation of veratryl alcohol and the lignin model dimers guaiacylglycerol-β-guaiacol ether and veratrylglycerol-β-guaiacol ether has been reported. The bacterial DyPs investigated to date appear to have a lower oxidizing power than the fungal counterparts, and seem to be limited to the oxidation of less recalcitrant phenolic lignin models. DyP-type peroxidases are generally active on monophenolic substrates, but several bacterial DyPs have shown significant activity towards the nonphenolic veratryl alcohol: *Bs*DyP from *Bacillus subtilis* KCTC2023 (Min et al., 2015), *Pp*DyP from *Pseudomonas putida* MET94 (Santos et al., 2014), *Svi*DyP from *Saccharomonospora viridis* DSM 43017 (Yu et al., 2014) and *Tfu*DyP from *Thermobifida fusca* (Van Bloois et al., 2010). Both *Svi*DyP and *Tfu*DyP are class A DyPs and are secreted via the Tat-system. This would be in line with extracellular degradation on lignin. The A-type *Tc*DyP from *Thermomonospora curvata*, although showing a relaxed substrate specificity, was inactive towards veratryl alcohol. Nonetheless, it was able to decarboxylate the nonphenolic lignin-related substrate 4-methoxymandelic acid, yielding *p*-anisaldehyde as final product (Chen et al., 2015). Interestingly, the C-type DyP2 from *Amycolatopsis sp.* 75iv2 was also able to decarboxylate 4-methoxymandelic acid in the presence of Mn^{2+} and O_2, with no need of H_2O_2 (Brown et al., 2012). This hints to the ability of DyPs to act as oxidases.

Several bacterial DyPs are able to oxidize the phenolic lignin dimer guaiacylglycerol-β-guaiacol ether. For instance, *Tfu*DyP has been tested for the oxidation of this lignin-model compound. It was found that *Tfu*DyP does not cleave the ether bond in the model compound but oxidizes the phenolic moiety resulting in oxidative coupling of the guaiacylglycerol-β-guaiacyl ether, mainly yielding in dimeric and trimeric products (Lončar et al., 2016; Rahmanpour et al., 2016). This is in line with the observation that *Tfu*DyP efficiently dimerizes several monophenolic compounds (e.g vanillin, vanillin alcohol and vanillin ketone) (Fig. 2a). This behaviour is different from *Tc*DyP and DyPB, a B-type DyP from *Rhodococcus jostii* RHA1. The use of the latter two enzymes resulted in a more diverse product profile, which could be explained by the degradation of the Cα-Cβ linkages of the model substrate and subsequent radical coupling of the products formed (Chen et al., 2015; Ahmad et al., 2011). The main oxidation products of DyPB-treated guaiacylglycerol-β-guaiacol ether were guaiacol, guaiacol trimers and vanillin (Ahmad et al., 2011) (Fig. 2b). Some of the compounds recovered after treatment of the lignin model substrate with *Tc*DyP could be identified as hydroxylated guaiacol pentamers and cresol dimers, as shown in Fig. 2c (Chen et al., 2015). DyP2 has also shown activity on this phenolic lignin dimer, but the products formed have not been characterized, so its degradation pathway remains unclear (Brown et al., 2012).

Veratrylglycerol-β-guaiacol ether has been also used as lignin model for investigating the oxidative potential of DyPs. This compound does not contain a phenolic moiety and is more recalcitrant to oxidation by DyP-type peroxidases. None of the enzymes mentioned above were able to oxidize this lignin model dimer. Remarkably, *Bs*DyP, which was inactive towards the phenolic lignin dimer mentioned above, showed activity towards both veratryl alcohol and the veratrylglycerol-β-guaiacol ether (Min et al., 2015), as indicated in Fig. 2d. The decomposition of the lignin dimer was measured based on the release of the product veratraldehyde, the same product formed when using LiP (Kirk et al., 1986). The degradation of the dimer occurred through the breakage of the Cα-Cβ bond. Thus, *Bs*DyP is the first bacterial DyP showing activity towards this compound. This activity was also described for a few fungal DyP (Liers et al., 2013).

Several bacterial DyPs, including DyPB, *Tfu*DyP and the two DyPs obtained from *Pseudomonas fluorescence* Pf-5: DyP1B and

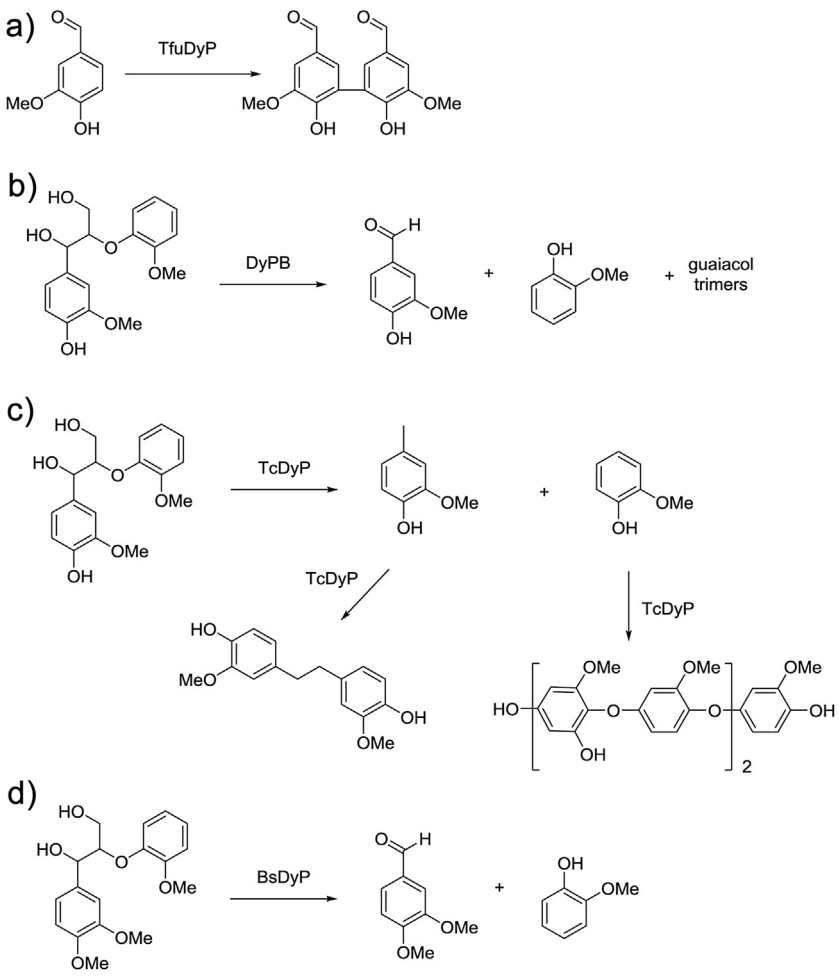

Fig. 2. Some of the degradation reactions catalyzed by DyP-type peroxidases: (a) *Tfu*DyP-catalyzed dimerization of vanillin; (b) oxidation of guaiacylglycerol-β-guaiacol ether by DyPB leading to guaiacol, guaiacol trimers and vanillin; (c) *Tc*DyP-catalyzed degradation of guaiacylglycerol-β-guaiacol to hydroxylated guaiacol pentamers and cresol dimers, and (d) *Bs*DyP-catalyzed degradation of veratrylglycerol-β-guaiacol ether.

DyPA, have been shown to act on alkali Kraft lignin, a by-product of the paper industry (Rahmanpour and Bugg, 2015). *Svi*DyP has shown interesting results in biobleaching processes, which makes it a promising candidate for further industrial applications. This thermostable bacterial peroxidase (60% remaining activity after incubating at 70 °C for 2 h) with a high alkali tolerance (>80% activity after incubation at pH 5–10 at 37 °C for 1 h) has been employed successfully as biocatalyst in the biobleaching of eucalyptus Kraft pulp (Yu et al., 2014).

Most DyP substrates are too big to enter the active site and are therefore unable to interact directly with the heme cofactor. Structural analysis of DyPs (DyP2, the N246A mutant of DyPB, and a fungal DyP) have revealed the presence of surface exposed substrate binding sites (Brown et al., 2012; Yoshida et al., 2012; Singh et al., 2013). Besides these sites, a long-range electron transfer (LRET) pathway between the heme cofactor and a surface exposed tyrosine or tryptophan has been suggested, as previously described for LiPs and VPs (Doyle et al., 1998; Pérez-Boada et al., 2005). In fact, similar to the typical fungal LiPs, MnPs and VPs, DyPs also seem to be able to promote lignin degradation by oxidizing redox mediators. Redox mediators as veratryl alcohol, monophenolic substrates and Mn^{+2} have been tested as DyP substrates. Some DyP-type peroxidases were shown to be active on veratryl alcohol, monophenolic substrates and Mn^{2+}. Many DyPs are active on monophenolic substrates. It is also worth noting that AnaPX (a C-type DyP) from *Anabaena* sp. strain PCC 7120 showed a significantly enhanced activity towards several azo-dyes in the presence of the natural mediator syringaldehyde (Ogola et al., 2009). The activity towards Mn^{2+} and/or the use of Mn^{2+} as mediator in DyP-catalysed degradation of lignin has been widely studied for several bacterial DyPs. DyP2, DyP1B, DyPB, *Bs*DyP and *Pp*DyP from *Pseudomonas putida* MET94 (Santos et al., 2014) have been tested for activity with Mn^{2+}. DyP2 from *Amycolatopsis* sp. 75iv2 showed the highest activity on this cation, with a k_{cat} of $24 \pm 1\ s^{-1}$ and a k_{cat}/K_M value only one to two orders of magnitude lower than the activities from respectively VP (*Pleurotus eryngii*) and LiP (*Phanerochaete chrysosporium*) (Brown et al., 2012).

From the three DyP peroxidases obtained from *Pseudomonas fluorescence* Pf-5 and overexpressed in *Escherichia coli*, only DyP1B showed activity for the oxidation of Mn^{2+} and for the degradation of powdered wheat straw lignocellulose. Using Mn^{2+}, formation of a lignin dimer from this lignin material could be boosted (Rahmanpour and Bugg, 2015). A more extensive study on the potential lignin degradation capacity by a bacterial DyP in the presence of Mn^{2+} was performed using DyPB from *Rhodococcus jostii* RHA1 (Ahmad et al., 2011). DyPB cleaves the Cα-Cβ linkage of the phenolic lignin dimer guaiacylglycerol-β-guaiacol ether (Fig. 2b) and is also able to act on Kraft lignin. These activities were enhanced by 23 and 6.2 times, respectively, through the addition of 1.0–1.5 mM $MnCl_2$. DyPB also showed activity towards wheat straw lignocellulose and wheat straw milled wood lignin when incubated in the presence of 1.0 mM $MnCl_2$ and in absence of

H_2O_2. The obtained products have not been characterized, but HPLC analysis has revealed various breakdown products. Lignin degradation did not occur in the absence of Mn^{+2}. Using purified DyPB it could be confirmed that it catalyses the peroxide-dependent oxidation of Mn^{2+}, albeit less efficiently than fungal manganese peroxidases. An engineered variant of DyPB, containing the N246A mutation, showed an 80-fold increased activity towards Mn^{2+} ($k_{cat} = 39 \pm 3\ s^{-1}$) (Singh et al., 2013). This mutant has been tested in the transformation of hard wood Kraft lignin and on its solvent extracted fractions. This resulted in recovery of syringaldehyde and 2,6-dimethoxybenzoquinone as major products. These results highlight the potential of bacterial enzymes as biocatalysts to transform lignin.

In contrast to A-type DyPs, B- and C-type DyPs typically lack a secretion signal. This may not exclude a role as extracellular enzyme. The extracellular fraction of the Δ*dypB* mutant of *Rhodococcus jostii* RHA1 showed a highly reduced activity towards nitrated lignin, suggesting that the location of DyPB is extracellular. Thus, it has been proposed that this enzyme might be exported through another mechanism, potentially through encapsulation and subsequent secretion of DyPB. Approximately 14% of the genes of B-type DyPs are located in an operon together with an encapsulin gene. Sutter et al. have shown that these DyPs often contain a 30–40 amino acid C-terminal extension. Enzymes containing this C-terminal extension, for instance DyPB, *Bl*DyP and *Mt*DyP, are targets for encapsulation by a protein-based cages, the so-called encapsulins (Sutter et al., 2008; Contreras et al., 2014). Interestingly, DyPB, when being encapsulated, showed an eight-fold enhanced activity towards nitrated lignin (Rahmanpour and Bugg, 2013), when compared with DyPB alone. This indicates that in some way encapsulation which enhances DyP-mediated lignin degradation.

2.2. Lignin-modifying bacterial laccases

Laccases (EC 1.10.3.2) are multi-copper oxidases able to perform the single electron oxidations of organic compounds to the corresponding radical species. Laccases employ a cluster of four copper ions for such oxidations which use dioxygen as electron acceptor, generating water as byproduct. The formed radical products can undergo further oxidation or undergo other reactions such as hydration, disproportionation or polymerization reactions. Laccases are ubiquitous in nature, being found in plants, fungi, bacteria and insects. They are often secreted as extracellular catalysts and typically perform polymerization or depolymerization reactions (Riva, 2006). Laccases vary largely in their molecular weight, oligomeric state and structure (Santhanam et al., 2011). While most laccases consist of three structural domains, also laccases that lack one of these three domains exist. Similar to fungal laccases, many bacterial laccases are secreted. For the export of bacterial laccases the Tat secretion system is used, which facilitates the export of folded and holoproteins across the cell membrane.

Laccases are industrially attractive biocatalysts, as unlike many oxidoreductases, they do not require the addition of cofactors. Additionally, different from most oxidases, they do not produce toxic hydrogen peroxide as byproduct. Laccases have been employed in bioremediation processes to decontaminate industrial wastewaters, in food industry for the stabilization of beverages and improvement of the organoleptic properties of food, in the synthesis of pharmaceuticals and other fine chemicals, in textile dye transformation, and in the delignification of wood, especially during the bleaching process (Shraddha et al., 2011). Laccases can be also employed in the pretreatment of softwood samples with the aim of improving the subsequent hydrolysis treatment (Palonen and Viikari, 2004).

Most of the laccases known, studied and applied to date are from fungal origin. From these studies it has become clear that laccases play a role in lignin degradation. Only in recent years bacterial laccases have gained considerable interest concerning their possible role in lignin degradation and other biotechnological applications. The first laccase was described in 1995 (Faure et al., 1995). Recent advances in genome analysis and other approaches have allowed the identification of numerous laccases in bacteria (Alexandre and Zhulin, 2000; Santhanam et al., 2011; Martins et al., 2015). The role and efficacy of bacterial laccases in lignin degradation is nowadays heavily studied (Chandra and Chowdhary, 2015).

In order to perform the degradation and depolymerization of lignin, laccases require the presence of small molecules, the so-called mediators, which act as redox shuttles between the laccase active site and the lignin structure (Fig. 3). Several compounds have been identified as effective mediators. The first one was ABTS (2,2′-azino-bis(3-ethylbenozthiazoline-6-sulfonic acid) which is used in pulp degradation (Bourbonnais and Paice, 1990). The most effective mediator in lignin transformation are *N*-heterocycles bearing N—OH groups, for instance, *N*-hydroxybenzotriazole (HBT).

Similar to DyPs, the most studied bacterial laccases in lignin degradation are from actinomycetes, particularly from *Streptomyces* species (Fernandes et al., 2014). Most of the laccases present in this genus belong to the group of two-domain laccases. The ability of these so-called small laccases to assist on lignin modification was confirmed by studying knock-out strains. Furthermore, crystal structures with lignin model compounds bound to laccases have been elucidated (Majumdar et al., 2014). Recently, a PCR primer was developed in order to identify the two domains (Lu et al., 2014) of *Streptomyces* genes during composting of agricultural waste. Gene libraries obtained were clustered with *S. coelicolor*, *S. violaceusniger* and *S. griseus*. The observed increase in the *Streptomyces* small laccase genes during the initial stages of composting was due to the presence of relatively high amounts of degradable organic compounds. A clear correlation was found between the abundance of laccase-encoding genes and the lignocellulose degradation rates.

In 2009, a halotolerant laccase (SilA) from *Streptomyces ipomoea* CECT 3341 was isolated and analyzed (Molina-Guijarro et al., 2009). Depending on the substrate, this enzyme showed an optimal activity at acid or basic conditions. SilA was highly active at alkaline pH for the oxidation of the phenolic compound 2,6-dimethoxyphenol, which has only been described for a few laccases. SilA was able to work at high saline concentrations (100% of activity retained at 1.0 M NaCl, pH 8.0), which makes the enzyme really attractive for industrial purposes. Thus, recombinant SilA obtained from cultures of *E. coli* BL21 has been applied in biobleaching processes on *Eucalyptus globulus* Kraft pulps using acetosyringone as mediator (Eugenio et al., 2011). In order to evaluate the enzymatic system, a further alkaline extraction of the Kraft pulp followed by hydrogen peroxide treatment was developed. The enzymatically treated biobleached pulps resulted in a significant reduction in the kappa number (41.5%) due to the degradation of both lignin and the hexenuronic acids present in the eucalyptus pulp. Addition of oxygen during the delignification processes was required in order to obtain slightly improved results. The laccase-mediator system has also an advantageous effect on the pulp treatment, as it was observed that less hydrogen peroxide was required for the treatment after the enzymatic procedure, which leads to lower amounts of pollutants effluents. Laccase-mediator degradation led to a decrease in the pulp viscosity as well as affording a high decrease in pulp brightness. SilA was recovered from the biobleaching treatments with only a slight deactivation (36%) when compared with the remaining activity of other laccases after this process.

Four small-laccases from *Streptomyces* (*S. coelicolor* A3(2), *S. lividans* TK24, *S. viridosporus* T7A) and *Amycolatopsis* sp. 75iv2 were expressed in *E. coli* and purified with high yields (15–20 mg/L culture). The enzymes were found to be very stable and active over a wide pH range (3–10), which makes them interesting candidates

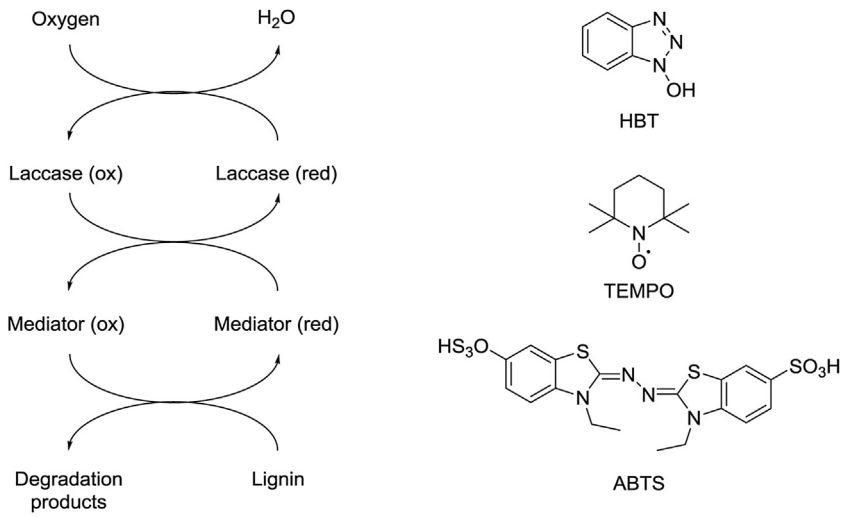

Fig. 3. Laccase-catalyzed redox cycle for lignin degradation in the presence of different chemical mediators (left) and the structures of the most employed mediators *N*-hydroxybenzotriazole (HBT), (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).

for industrial applications (Majumdar et al., 2014). The four laccases were able to degrade a phenolic model compound into a mixture of different products including vanillin, but most of the other compounds obtained were not fully characterized. Conversion of a nonphenolic lignin model compound was only observed in the presence of mediators such as ABTS and HBT while these were not needed for oxidizing a phenolic derivative. It was suggested that the observed activities can explain the role of bacterial laccases in lignocellulosic degradation by modifying the lignin properties in order to allow the access of other enzymatic systems to cellulose and hemicellulose.

Purified laccase from *S. coelicolor* A3(2) was tested in the *in vitro* degradation of ethanolsolv lignin. This lignin derivative is obtained from the treatment of lignin with ethanol and sulfuric acid, presenting a lower molecular weight and a higher water-solubility. After a 16 h treatment at 37 °C, chromatography studies showed a loss in material solubility, due to the polymerization of small lignin units. This process can be explained by the laccase oxidation of lignocellulosic substrates to produce aryl cation radicals that can rearrange and promote repolymerization, which will be in competition with the enzymatic depolymeration of the starting material. Under *in vivo* conditions, the degradation of lignin by depolymerization can be explained by the presence of cooperative enzymes that prevent the formation of radical species, thereby acting as quenchers.

In addition to the "small" two-domain laccases, some other laccases presenting three-domains have been used for the degradation of lignin derivatives. The endophytic bacterial strain *Pantoea ananatis* Sd-1 was isolated from rice seeds using a surface sterilization method (Xiong et al., 2013). This gram-negative bacterium was able to degrade lignin and rice straw. The sequenced *Pantoea ananatis* Sd-1 genome revealed that this microorganism has the potential to produce intra- and extracellular laccases (Shi et al., 2015). Four putative laccase-encoding genes were discovered and studied. From sequence analyses it was concluded that Lac4 had the highest homology to typical bacterial laccases. Thus, recombinant Lac4 was characterized as being a dimeric enzyme with high activity at low pH values and a moderate thermostability. *In vitro* lignin degradation by Lac4 in the presence of ABTS as mediator resulted in 38% conversion after 12 h. The formed products contained several low molecular weight aromatic compounds such as 1,4-benzedicarboxaldehyde, benzenepropanoic acid and phenol.

Laccases from *Bacillus* species are in general tolerant to high temperatures and alkaline conditions, which makes them suitable for lignin degradation. Unfortunately, most of these laccases are intracellular which complicates large scale production. Recently, an extracellular laccase was obtained from *Bacillus tequilensis* SN4 (Sondhi et al., 2015), an bacterium isolated from a paper mill effluent. The optimum temperature for this laccase was 80–90 °C, while it even retains part of its activity at 100 °C. The SN4 laccase showed an optimal activity at pH 8.0. Besides being an extracellular enzyme, its stability make SN4 laccase a useful biocatalyst to be exploited on industrial scale. Laccase production was optimized by using a medium containing Mn^{+2} and Fe^{+2} sulfates as well as 3.5% v v^{-1} ethanol. SN4 laccase was employed in the biobleaching of softwood pulp resulting in a reduction of 28% in the kappa number and a 7.6% increase in brightness. Addition of *N*-hydroxybenzotriazole (HBT) as mediator at low concentration (2.0 mM) led to a further improvement in its performance.

Several bacterial strains obtained from soils of a biodiversity-rich rainforest in Peru have been tested in the oxidation of ABTS (Huang et al., 2013). This resulted in the isolation of two *Bacillus* strains: *Bacillus atrophaeus* (strain B7) and *Bacillus pumilus* (strain C6). Both microorganisms exhibit intracellular and extracellular laccase activities. Furthermore, Kraft lignin and the lignin model compound guaiacylglycerol-β-guaiacyl ether were successfully degraded by both strains. This suggests that these strains harbor interesting laccases.

A laccase from *Bacillus pumilus* (CotA) discovered by genome mining has been successfully cloned and overexpressed in *E. coli* (Reiss et al., 2011). This enzyme showed a high thermostability with a maximum activity at 70 °C. CotA was capable of oxidizing several phenolic compounds showing optimal values at pH neutral to alkaline, which makes it an interesting biocatalyst for further biotechnological applications.

Thermus thermophilius HB27 is a thermophilic bacteria which produces an intracellular laccase: Tth-laccase (Miyazaki, 2005). This bacterial laccase was successfully expressed in *E. coli*. Treatment of wheat straw pulp with this laccase was studied (Zheng et al., 2012). Using optimized conditions, the pulp brightness was increased while the kappa number was reduced. These beneficial effects of laccase activity can be further improved when the enzymatic bleaching is combined with some other chlorine-free chemical bleaching process. The used of the laccase-treated wheat pulp affords a 25% save in the use of hydrogen peroxide in subse-

quent pulp treatments. The use of ABTS or guaiacol as Tth-laccase mediator (5 mM) improved the delignification process with an increase in the delignification degree of 9% and 5.6%, respectively. Opposite, HBT has no effect on this process, probably due to the differences between the redox potential of the mediators.

Wheat straw-rich soda pulp has recently been treated with an alkalophilic laccase isolated from γ-proteabacterium JB (Bains et al., 2003). This microorganism was only able to produce the laccase, but no other lignin degrading enzymes. Three parameters (laccase units, pH and ABTS concentration) were optimized using response surface methodology based on central composite design in order to achieve the best degradation process. This led to an increase in pulp brightness of around 6.0%, while the kappa number was reduced in a 21% extent (Singh et al., 2008). When a fresh chemically bleached pulp sample was subjected to laccase treatment, the final pulp properties were significantly improved in terms of chromophores, reducing sugars and hydrophobic compounds. The application of this laccase can reduce the use of hypochlorite by 10% in the pulp bleaching procedure.

Recently, a biosensor for the detection of aromatic lignin degradation products has been developed (Strachan et al., 2014). This biosensor identified a novel multicopper oxidase by screening the metagenome of coal bed bacteria. This enzyme has a high similarity to CopA from *Pseudomonas stutzeri* ATCC14405 and *Pseudomonas putida*, two strains that able to degrade a wide range of aromatic compounds. The discovered oxidases were employed in the degradation of a lignin substrate, leading to 2,6-dimethybenzene-1,4-diol as the major identified product after 3 h.

2.3. Glutathione-dependent β-etherases acting on lignin degradation products

In the '80 s the first glutathione-dependent β-etherases were discovered capable of catalyzing the reductive cleavage of β-ether bonds in lignin-related compounds (Masai et al., 1989). The first detailed studies were reported for the β—O—4 aryl-ether cleaving enzyme system from the α-proteobacterium *Sphingobium* sp. SYK-6. This system is composed of three separate proteins; LigD (aCα-dehydrogenase), LigF (a β-etherase) and LigG (a glutathione lyase), which have been successfully expressed in *E. coli*. The multienzyme system cleaves the β-aryl ethers of a model lignin dimer: guaiacylglycerol-β-guaiacyl. The role of each of the three enzymes in the lignin degradation is the following: (1) LigD catalyzed the NAD^+-dependent oxidation of the Cα of lignin substrate from the alcohol to the corresponding ketone; (2) LigF cleaves the intermediate with the attachment of glutathione at the Cβ position, and finally (3) LigG oxidizes glutathione and releases the final product (Fig. 4) (Sato et al., 2009). Two other glutathione reductases from *Sphingobium* sp. strain SYK-6 (LigE and LigP) are also able to catalyze the same reaction as LigF, but these enzymes are active on the opposite enantiomer of the carbonyl compound formed by the oxidation of guaiacylglycerol-β-guaiacyl with LigD (Tanamura et al., 2011). Recently, the stereopreference of LigG has been studied together with two novel glutathione lyases (LigG-NS from *Novosphingobium* sp. PP1Y and LigTD from *Thiobacillus denitrificans* ATC 25259) in the enzymatic degradation of a lignin model substrate employing an enzymatic cascade of β-etherases and glutathione lyases. The tested enzymes showed β-(*R*)-enantiopreference. This enantiopreference can be increased or even reversed by mutagenesis (Picart et al., 2015). As these enzyme systems are inherent intracellular (as NAD^+ is an intracellular metabolite), they are not involved in the initial degradation of lignin. Yet, they may play a role in the degradation of the small lignin oligomers formed in the first steps of lignin degradation and may develop as useful biocatalysts in the field of lignocellulose utilization.

2.4. The role of superoxide dismutases in bacterial lignin modification

Recently two bacterial manganese-dependent superoxide dismutases (MnSODs) were discovered to have lignin degrading activity (Rashid et al., 2015). Superoxide dismutases typically catalyze the disproportionation of superoxide anion radical into molecular oxygen and hydrogen peroxide and play a key role in cellular protection against oxidative stress. Superoxide dismutases are in general intracellular enzymes, but some examples of extracellular activity have been reported. Recently two extracellular MnSODs from *Sphingobacterium* sp. T2 (MnSOD1 and MnSOD2) were identified and partially purified (Rashid et al., 2015). Interestingly, both enzymes are able to perform the degradation of Organosolv and Kraft lignin, as well as different lignin model substrates, into several compounds. All the products formed resulted from aryl-Cα and Cα-Cβ bond oxidative cleavage reactions as well as from *O*-demethylation activity. The lignin oxidation reactivity of these MnSODs can be assigned to the formation of a hydroxyl radical, with a high oxidant efficiency. It remains to be established whether such superoxide dismutases are promising candidates for biotechnological application in the area of lignocellulose degradation and whether they are typical bacterial ligninolytic enzymes.

2.5. Catalase-peroxidases are associated with lignocellulose degradation

Recently, by a proteomic approach, a catalase-peroxidase (Amyco1) was identified as a heme-containing enzyme secreted by *Amycolatopsis* sp. 75iv2 when incubated with lignocellulosic material (Brown et al., 2011). To provide further evidence for its involvement in lignin degradation, the enzyme was recombinantly produced and purified. Amyco1 was found to be able to convert a phenolic lignin model compound, while a methylated derivative was not a substrate. With experimental confirmation that this catalase-peroxidase acts on a lignin-like compound, a role of Amyco1 in lignin modification seem feasible. Future research will tell whether such extracellular bacterial catalase-peroxidases are frequently used by bacteria to modify lignin.

2.6. Bacterial dioxygenases may play a role in lignin degradation

In 2013 a peculiar bacterial enzyme was described that is composed of a dioxygenase domain and a lignin-binding domain (Bianchetti et al., 2013). Again, this enzyme was isolated from a *Streptomyces* isolate and was found to be associated with a woodwasp which lays its eggs in wood. Therefore it was anticipated that the isolated bacterium would exhibit plant biomass degradation capabilities. Indeed, a detailed characterization revealed a suite of secreted enzymes involved in hydrolytic and oxidative attack of lignocellulose. As part of the identification of secreted proteins, the SACTE_2871 protein emerged. Based on the protein sequence, it appeared to be the result of a fusion of a intradiol dioxygenase and a carbohydrate binding module. The protein also contains an N-terminal Tat-translocation signal peptide which confirms its extracellular location. Recombinant expression of the enzyme in *E. coli* was successful and allowed the elucidation of the crystal structure of the dioxygenase domain. Furthermore, it could be confirmed that the enzyme was active as dioxygenase with several catechol derivatives. Intriguingly, it was discovered that the carbohydrate binding module displayed affinity towards synthetic lignin polymers.

The involvement of a dioxygenase in a bacterial degradation pathway of lignin-related compound is not unprecedented. One of the bacteria from which a β-etherase was identified, *Sphingomonas paucimobilis* SYK-6, was found to harbor another

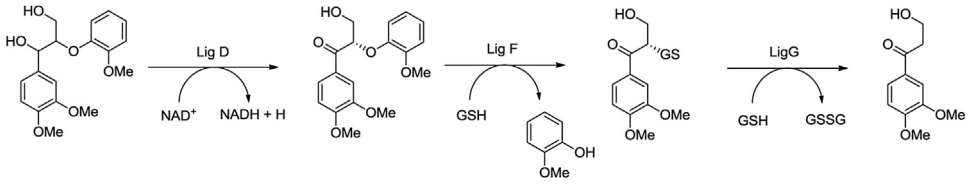

Fig. 4. Degradation of guaiacylglycerol-β-guaiacyl by *Sphingobium* sp. SYK-6: (1) LigD catalyzes the alcohol oxidation of the lignin substrate Cα; (2) LigF catalyzes the incorporation of glutathione at the Cβ position, and finally (3) LigG oxidizes glutathione, leading to the final product.

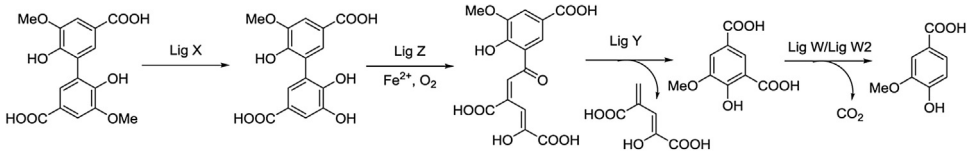

Fig. 5. Multienzymatic degradation of the biphenyl lignin derivative 2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxybiphenyl by *Sphingomonas paucimobilis* SYK-6. Four enzymes are involved: (1) LigX catalyzes the demethylation of the methoxy group; (2) ring fission is catalyzed by the dioxygenase LigZ; (3) C—C hydrolysis is catalyzed by LigY, and (4) conversion of 5-carboxyvanillic acid into vanillic acid is performed by two decarboxylases (LigW and LigW2).

interesting degradation pathway related to lignin degradation, which includes a dioxygenase. A biphenyl compound (2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxybiphenyl) was identified as a growth substrate for *Sphingomonas paucimobilis* SYK-6 (Sonoki et al., 2009). Four different types of enzymes are involved in the initial steps of degrading this biphenyl: LigW/LigW2 (decarboxylases), LigY (a C—C hydrolase), LigX (an iron-dependent demethylase) and LigZ (an extradiol dioxygenase) (Fig. 5). The demethylation of the methoxy group takes place by LigX. The product from this cleavage step is the substrate for the dioxygenase LigZ, which leads to a ring fission product yielding 5-carboxyvanillic acid and 4-carboxy-2-hydroxypentadienoic acid. The product 5-carboxyvanillic acid is converted into vanillic acid in a process catalyzed by two decarboxylase enzymes (LigW and LigW2). These findings suggest that dioxygenases represents another tool used by bacteria to assist in lignin degradation.

3. Outlook

Bacteria do not possess the regular peroxidases that fungi employ for lignin degradation. This may be due to intrinsic difficulties in expressing these rather complex proteins that are typically glycosylated, contain several disulphide bonds, and incorporate several calcium ions and a heme cofactor. Folding and processing may require special conditions that are not compatible with the bacterial machinery for protein production. In line with this, it is worth noting that most of the attempts to produce peroxidases from the plant peroxidase superfamily failed. Yet, the DyP-type peroxidases are somewhat less complex concerning their protein structure (no calcium binding sites, glycosylation or disulphide bonds needed) and are wide spread among bacteria. Recombinant expression of various DyPs in *E. coli* typically yielded high levels of expression which is in sharp contrast with the expression of fungal peroxidases (Lambertz et al., 2016). This makes these bacterial peroxidases interesting targets for enzyme development. The bacterial laccases also seem to be suited for large scale recombinant enzyme production. A recent detailed study revealed that by optimizing expression conditions, various bacterial laccases can be produced in *E. coli* (Ihssen et al., 2015).

While the number of known bacterial peroxidases and laccases that may be involved in lignin degradation has grown remarkably in the last two decades, there may still be undiscovered enzyme types around that play a role in bacterial lignin degradation. Several new candidates have surfaced in the last few years, vide supra. Furthermore, one class of enzymes that has been overlooked are the enzymes that need to provide the bacterial peroxidases the required hydrogen peroxide. Such oxidases have been identified for the fungal peroxidases, and similarly it is expected that bacteria secrete oxidases. Yet, what type of oxidases and their respective substrates have still to be established.

It is interesting to note that only recently, also for the degradation of the cellulose part of plant biomass oxidative enzymes are recognized as major players in the degradation process. The copper-containing so-called lytic polysaccharide monooxygenases (LPMOs) have been found to be essential in the attack of the rather inert parts of cellulose and other polysaccharides. The recent finding of the Eijsink group that the LPMOs sequestered the required electrons from the lignin part of plant biomass suggests a strong link between the degradation of the different biomass moieties (Westereng et al., 2015). It will be interesting to see whether bacterial LPMOs can be linked to other redox enzymes secreted by bacteria and whether bacterial lignin degradation is intertwined with plant polysaccharide degradation.

With the realization of the bacterial ability to modify the lignin part of plant biomass, biochemical studies on bacterial lignin degradation pathways and their respective enzymes has been revitalised. With new approaches to identify new lignin-degrading bacteria (Strachan et al., 2014; Picart et al., 2016) in combination with detailed genomic, proteomic and biochemical studies, the identities and roles of bacterial ligninolytic enzymes will be uncovered in the coming years.

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