Biochemical features of dye-decolorizing peroxidases: Current impact on lignin degradation

Biochemical features of Dye-decolorizing peroxidases: current impact on lignin degradation

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Abstract

Dye-decolorizing peroxidases (DyP) were originally discovered in fungi for their ability to decolorize several different industrial dyes. DyPs catalyze the oxidation of a variety of substrates such as phenolic and non-phenolic aromatic compounds. Catalysis occurs in the active site or on the surface of the enzyme depending on the size of the substrate and on the existence of radical transfer pathways available in the enzyme. DyPs show the typical features of heme containing enzymes with a Soret peak at 404-408 nm. They bind hydrogen peroxide that leads to the formation of the so-called Compound I, the key intermediate for catalysis. This then decays into Compound II yielding back Fe(III) at its resting state. Each catalytic cycle uses two electrons from suitable electron donors and generates two product molecules.

DyPs are classified as a separate class of peroxidases. As all peroxidases they encompass a conserved histidine that acts as the fifth heme ligand, however all primary DyP sequences contain a conserved GxxDG motif and a distal arginine that is their characteristic. Given their ability to attack monomeric and dimeric lignin model compounds as well as polymeric lignocellulose, DyPs are a promising class of biocatalysts for lignin degradation that not only represents a source of valuable fine-chemicals, but it also constitutes a fundamental step in biofuels production. Research efforts are envisioned for the improvement of the activity of DyPs against lignin, through directed evolution, ration protein design or one-pot combination with other enzymes to reach satisfactory conversion levels for industrial applications.

Introduction

Every year the global demand for energy increases. World energy consumption will rise 50% between 2018 and 2050 reaching a total consumption of 911 quadrillion British thermal units (Btu) in 2050. As a consequence, the use of renewable energy sources has become a key strategy to avoid increased greenhouse emissions. Projections for 2050 indicate that renewable energies will become the leading source of primary energy consumption accounting for 250 Btu [1] (Figure 1). Biomasses are the primary source of renewable energy that can be exploited for the production of chemicals and fuels [2, 3]. Plants are widely distributed and their biomass can be transformed to produce liquid biofuels such as bioethanol, biodiesel or biobutanol [4]. First generation biofuels typically derive from corn, palm oil or sugarcane that are extracted from plants to obtain raw materials [5]. These biomasses have been extensively used worldwide thanks to well established technologies. Nevertheless, first generation biofuels are limited by the fact that the amount of carbon that is recovered throughout the process does not exceed the amount that feedstocks release during the production. Moreover, global increasing demand of biomass-derived biofuels [6] can shift the production from food crops to biofuels crops posing both macroeconomics and ethics questions of “fuel versus food” [7]. For this reason, lignocellulosic material could be a better substrate for biofuels production. Production of lignocellulose is 200 x 10^9 tons per year [8] and its cost is approximately 50% lower than other feedstock. Lignocellulose is the most abundant and least used resource of fuels and raw materials [9]. Plant cell walls are a primary source of lignocellulosic material that is made of cellulose, hemicellulose, lignin and other components like organic acids, tannins and proteins [10]. The chemical structure of lignocellulosic material cannot be predicted accurately [2] and it can be deeply influenced by growth, storage and harvest conditions. While hemicellulose and cellulose have a more regular structure characterized by the repetitions of the sugar units lignin

is characterized by a complex aromatic structure that is harder to attack and has been considered a low-value product by most bio-rafineries [2]. The cross-linking of lignin is obtained via oxidative coupling of the various small units such as: synaptyl, coniferyl and *p*-coumaryl alcohols [11]. Given the structure of these compounds, lignin potentially is an excellent source of fine chemicals and its valorization can be obtained through a deconstruction process [12] leading to the breakage of carbon-oxygen and carbon-carbon bonds in its complex structure. This is a task that can be done by powerful tools such as enzymes that are commonly available in small microorganisms. A white rot fungus*, Phanerochaete chrysosporium* is the best studied organism for lignin degradation [13]. The microorganism synthetizes and secretes a set of lignin peroxidases isozymes that are able to attack lignin [14-16] through a radical transfer mechanism [17, 18]. Lignin peroxidases [19], can oxidize a variety of different phenolic aromatic compounds as well as non-phenolic lignin model compounds [20]. They are characterized by the presence of two Ca^{2+} binding sites, two glycosylation sites and four disulfide bridges that stabilize the structure of the enzyme [21]. Their molecular weight ranges between 35 and 48 kDa and the isoelectric point between 3.1 and 4.8 [21].

Peroxidases were also used as model systems to understand the interaction between proteins and electrodes using mediators to shuttle electrons between the working electrode and the enzyme [22, 23]. Ferrocene mediators (negatevely charged, positevely charged and neutral) were found to be able to interact with Cytochrome c Peroxidase (CcP) in different ways due to the presence of a cluster of negative amino acids on the surface of the enzyme that interact better with opposite (positive) charges [22]. Further characterization of the system performing site directed mutagenesis of a single amino acid on the surface of CcP, Asp34Lys, resulted in a faster electron transfer confirming the importance of optimizing surface chemistry interaction [23, 24].

Previous reports have highlighted a role of several different classes of enzymes in attacking lignin or lignin model compounds. Lignin peroxidases [25-28], manganese-dependent peroxidases [29-31], versatile peroxidases [32] and laccases [13, 33-36], have been extensively studied for their lignin degradation activity. These enzymes, except for laccases, all depend on H_2O_2 for their activity. Interestingly recent works highlight the importance of H_2O_2 to drive the catalytic cycles of other class of enzymes. Among them, cytochrome P450 peroxygenases are very promising candidates to be exploited for the production of biofuels as they are able to react with H_2O_2 to generate catalytically competent heme iron-oxo species [37-39]. Moreover cytochromes P450 were found to be a valuable catalysts for lignin bioconversion [40]. Cytochromes P450 are known to be extremely powerful catalysts with broad substrate specificity [41, 42] that can be exploited for pharmaceutical, environmental and bionsensing applications [43], but except for the peroxygenase subgroup, their activity relies in the proper shuttling, coupling and optimization of electrons from a reductase partner [44, 45]. This review focusses on the structure/function relationship of a new family of peroxidases, named dye-decolorizing peroxidases, that have demonstrated a role in lignin breakdown [46], through the use hydrogen peroxide as co-substrate and high potential for biotechnological applications thanks to their presence in bacterial genomes [47, 48] that facilitates heterologous production in *E.coli*.

Dye-decolorizing peroxidases

DyP peroxidases were first discovered in fungi in 1995 when the basidiomycete *Geotrichum candidum (G. candidum)* was found to be able to decolorize several different dyes [49]. A few years later, in 1999, the enzyme from *G. candidum* responsible for the decolorization reaction was purified [50] and upon characterization it showed the typical features of a heme containing

enzyme with a Soret peak at 406 nm [50]. Bioinformatics analysis revealed that DyP peroxidases belong to a separate class of enzymes as they do not share sequence homology or structure with other known peroxidases [51]. For an outline of the characteristics of DyP from *T.cucumeris* (formerly *G. candidum)* and an in-depth analysis of the DyP-type peroxidase family please refer to Sugano 2009 [52].

The range of industrial dyes attacked by DyPs is very wide [50], particularly those derived from anthraquinone. DyPs are classified as peroxidases (EC 1.11.1) with EC number 1.11.1.19. Initially they were thought to be only of fungal origins, but now they also have been found in the genome of bacteria and archea [48, 51]. Analysis of their role in these organisms highlighted how these enzymes can have both oxidative and hydrolytic activity [51]. Today it can be stated that DyP peroxidases constitute a microbial super family of heme peroxidase not present in plants or animals [51] and their physiological role in bacteria and fungi is not yet clear. Substrates tested include a variety of aromatic compounds classified as dyes [50, 53-56], beta-carotene [57], aromatic sulfides [48], some of which are poorly metabolized by other heme peroxidases. Numerous studies have shown that DyP peroxidases play a key role in the degradation of lignin [54, 55, 58-65]. Their catalytic properties make them very interesting because the bacterial enzymes can be easily engineered, heterologously expressed in *E.coli* and purified [65] avoiding the issues of using an eukaryotic hosts.

DyP peroxidases are classified in four subclasses: A, B, C, D [51]. All Dyps possess a Type b non covalently bound heme. Most bacterial DyPs, are cytoplasmic enzymes and belong to the subclasses B and C. The DyPs belonging to the subclass A, present a signal sequence called "Tat-signal", responsible for their export to the extracellular environment [66, 67]. Subclass D mainly contains enzymes of a fungal nature that are secreted outside the cell [51, 68].

Structural features and catalytic cycle

While the most widely used classification of DyP is purely sequence-based Yoshida and Sugano in 2015 have proposed a new classification that is based on the tertiary structure of the DyPs [68]. In the new classification Class P replaces former Class B; former Classes A becomes Class I, former Classes C and D are joined in Class V. P indicates “primitive”, I stands for “intermediate” and V represents “advanced”. The new division into 3 Classes highlights: 1) the common structural features shared by Class C and D in the same structure regions reflected also by their higher catalytic efficiencies of their members; 2) the fact that class A proteins have the extra sequences fewer than those of class V and 3) the uniqueness of Class P displaying a more overall compact structure.

In terms of the the primary sequence DyP peroxidases contain a conserved histidine that acts as the fifth heme ligand, a conserved GxxDG motif and a distal arginine [52]. As expected, in general mutation of the histidine to an alanine results in the loss of the heme and complete inactivation of the enzyme [48]. Within the GxxDG motif, aspartate plays an important role by performing deprotonation of H_2O_2 that is crucial for Compound I formation [69]. Nevertheless, it has been found that, in DypB from Rhodococcus jostii RHA1, the conserved aspartate is not required for peroxidase activity and replacement of this residue by alanine only marginally affects the reactivity towards H_2O_2 and the formation of compound I [70]. Furthermore, the dye-decolorizing peroxidase EfeB/YcdB from E.coli was found to maintain its activity even when Asp 235 is mutated to asparagine suggesting that EfeB is a unique DyP protein member [71]. It follows that the specific role of aspartate can show some variability in some specific enzymes and the matter remains under question.

The conserved arginine in the active site was also studied. The role of this residue seems to be structural as its mutation compromises the architecture of its distal heme cavity and access channel [72].

The molecular weight of monomeric DyP is between 35-60 kDa. However these enzymes have been shown to exist in different states: monomeric [50, 69, 73], to dimeric [57, 71, 74, 75], tetrameric [76] and hexameric [75]. The various enzymes belonging to the different classes of DyPs highlight how these enzymes do not have a high degree of sequence homology [47]. From the structural point of view, on the other hand, these enzymes show a highly conserved topology [47]. All DyPs have two alpha and beta domains that are organized in a ferrodoxin-type folding structure, consisting of four beta anti-parallel sheets surrounded by alpha helices [47]. The two domains, alpha and beta, form the active site cavity that hosts heme. To date, 39 crystal structures of DyPs are known (Table 1) [64, 70, 71, 72, 75, 77-90].

Detailed studies of the catalytic cycle show that hydrogen peroxide deprotonation is the first step (Figure 2). This step requires the presence of an amino acid that works as a base yielding a Fe(III)-OOH complex that is normally called Compound 0. This reaction is very fast and therefore it is hard to experimentally catch the reaction intermediate. At this point heterolytic cleavage of the O-O can occur leading to the formation of the Fe(IV)-O porphyrin radical Compound I. The formation of Compound II (FeIV-OH) from Compound I is achieved through a 1-electron reduction producing a first radical species [72]. Finally, the ferric Iron resting state (FeIII) is regenerated by a second 1-electron reduction with generation of a second radical [72]. An earlier shunt of the catalytic cycle is also possible through immediate 2-electron reduction of Compound I generating ferric Iron resting state (FeIII).

Long range electron transfer

DyPs are able to catalyze the oxidation of substrates that due to their large size are not able to enter the active site of the enzyme [80]. For this reason electron transfer must occur from the active site to the surface of the enzyme via a long range electron transfer pathway. The best candidates to perform catalysis at the surface of the enzyme are typically tryptophan and tyrosine residues [80]. These amino acids can form stable radicals that participate directly to charge transfer. Site directed mutagenesis followed by EPR spectroscopy gave evidenced different abilities of the enzyme to generate radical species. Cross-linking experiments, where enzymes are incubated with hydrogen peroxide at different pH to test the possibility of forming covalent dimers by SDS-Page, allowed the identification of the specific surface residues involved. Shrestha et al. reported the involvement of W263 in substrate oxidation in a class A DyP from *Thermomonospora curvata* [77]. Mutation of the tryptophan resulted in 50% loss of activity and sigmoidal kinetics for Reactive Blue 19 dye (RB19) as substrate. On the contrary, the same authors found also that Trp336 is an off-pathway electron transfer destination of the enzyme and that mutation of this residue improves the stability of compound I and avoids covalent cross-linking of enzymatic units in the absence of substrate [77].

A class B DyP peroxidase from *Vibro Cholerae* was characterized for its ability to perform substrate oxidation at the enzyme surface. Interestingly, in *Vibro cholerae* two tyrosine couples are responsible for enzyme covalent cross-linking (Y109 and Y133) and enzymatic activity respectively (Tyr129 and Tyr235 Figure 3A) [80]. Moreover, while radical formation occurs at every pH, substrate oxidation occurs at lower pH and cross-linking is observed mainly at higher pH. Therefore the authors have hypothesized the presence of a pH dependent switch in the radical transfer pathway controlled by H178 that forms a hydrogen bond with Thr277 and drives conformational change in a pH dependent manner [80]. In order to understand if this pathway

is conserved in other DyPs we have performed a three dimensional structural alignment of Vibrio Cholerae DyP with other members of the same class and the overlay shows that at least one of the two productive pathways is probably present exploiting the conserved His178 and Tyr235 (Figure 3B).

For class D DyP peroxidases Tyr337 and Leu357 were reported to be involved in the radical transfer patwhay of AauDyPI to the surface [91] [88].

Lignin degradation

Over the last 20 years, many different dye-decolorizing peroxidases have been biochemically characterized. This is usually achieved by first identifying a strain that shows activity against a lignocellulose matrix, then isolating the enzyme that is responsible for lignin degradation. Given the complex structure of lignin, once the enzymatic target is expressed and purified in a suitable host, different options are available to evaluate the activity of the enzyme. A bottom-up approach consists in testing a variety of monomer compounds that represent a specific class, for instance phenolic or non-phenolic compounds. A second level of complication entails the testing of the activity of the enzyme against one or more dimeric lignin model compounds. Further analysis requires the assessment of enzymatic activity against either wheat straw lignocellulose, nitrated lignin, kraft lignin or organosolv lignin (Figure 4A). Recently, a rapid and economic colorimetric assay, based on the reaction of 2,4-dinitrophenylhydrazine with the carbonyl product of the enzyme for lignin oxidation was reported. This allows a simple oxidative screening of enzymes involved in lignin degradation [92] accelerating the initial phase of the characterization process (Figure 4B). Indeed, dye-decolorizing peroxidases very often demonstrate activity against phenolic compounds, but they do not always accept non-phenolic compounds or more complicated lignin molecules. New sources of bacterial or fungal dye-

decolorizing peroxidases active on polymerized lignin have become available from a variety of organisms [46, 53, 64, 93-95]. The jelly fungus Auricularia auricular-judae Dyp peroxidases AjP I and AjP II were found to be active on aldlerol, a non phenolic beta-O-4 lignin model dimer exhibiting maximum activity at pH 1.4 [53]. AjP I resulted more stable than AjP II and upon incubation at at pH 2.5 did not lose activity over a time span of 4 hours [53]. DypB from Rhodococcus jostii RHA1 was tested against pinoresinol and three different beta-aryl ether model compounds: a *p*-hydroxy analogue, a guaiacyl analogue and a syringyl analogue. Guaiacyl beta-aryl ether was gradually consumed in time with specific activity for the erythro and threo isomers of 0.017 micromol/min and 0.005 micromol/min respectively [46]. DypB from Rhodococcus was also able to attack wheat straw lignocellulose or kraft lignin in the presence of $MnCl_2$ [46]. DypA and Dyp1B from Pseudomonas fluorescens exhibited Michaelis-Menten like kinetic behavior upon incubation with kraft Lignin whereas in the presence of Mn(II) only Dyp1B showed activity against wheat straw lignocellulose yielding a lignin fragment containing two aryl-C3 units [93]. Reactivity against kraft lignin was also reported for a Dyp peroxidase from *Thermobifida fusca* [64]. The enzyme showed the conversion of guaiacylglycerol-beta-guaiacyl ether to corresponding beta-aryl monomer as a consequence of the oxidative dimerization of the model compound [64].

Conclusion

DyP peroxidases are biocatalysts with great potential in second generation biofuel production. The data available in literature point towards a primary role of DyPs in the degradation of lignin, a complex matrix that contains highly polymerized molecules with variable structure, which cannot be easily attacked by a single enzyme. Nevertheless, it should be mentioned that lignin degradation activities of DyPs are low when compared with those of lignin peroxidases and

manganese peroxidases [96]. Future works should aim at directed evolution of DyPs to improve their catalytic activity and testing the combination of DyP peroxidases and other lignin degrading or modifying enzymes to perform a more radical and effective transformation of lignin matrix. The combination of a multi-enzyme approach will yield a fraction of low molecular mass aromatic compounds that can be easily adopted by the industry as starting raw materials. Furthermore, rational design efforts will also be beneficial for DyP peroxidases. Recent work showed how adjustment of bacterial growth combined with protein engineering allows protein overexpression at yields > 100 mg/L [65]. Future work should also be aimed at improving the activity of the enzymes at higher pH because the acidic conditions can limit their application [97].

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Two images were Created with BioRender.com.

Conflict of interest

None

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Figures

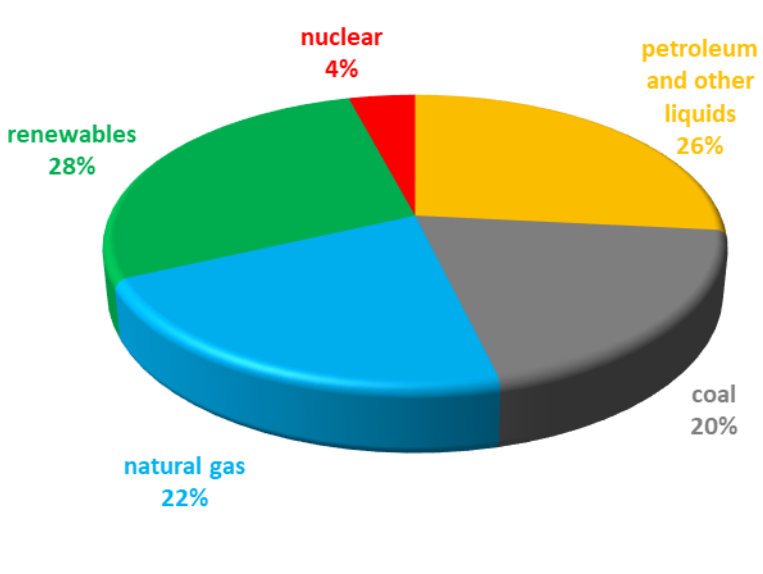

Figure 1 Projections of world energy consumption in 2050 classified by source

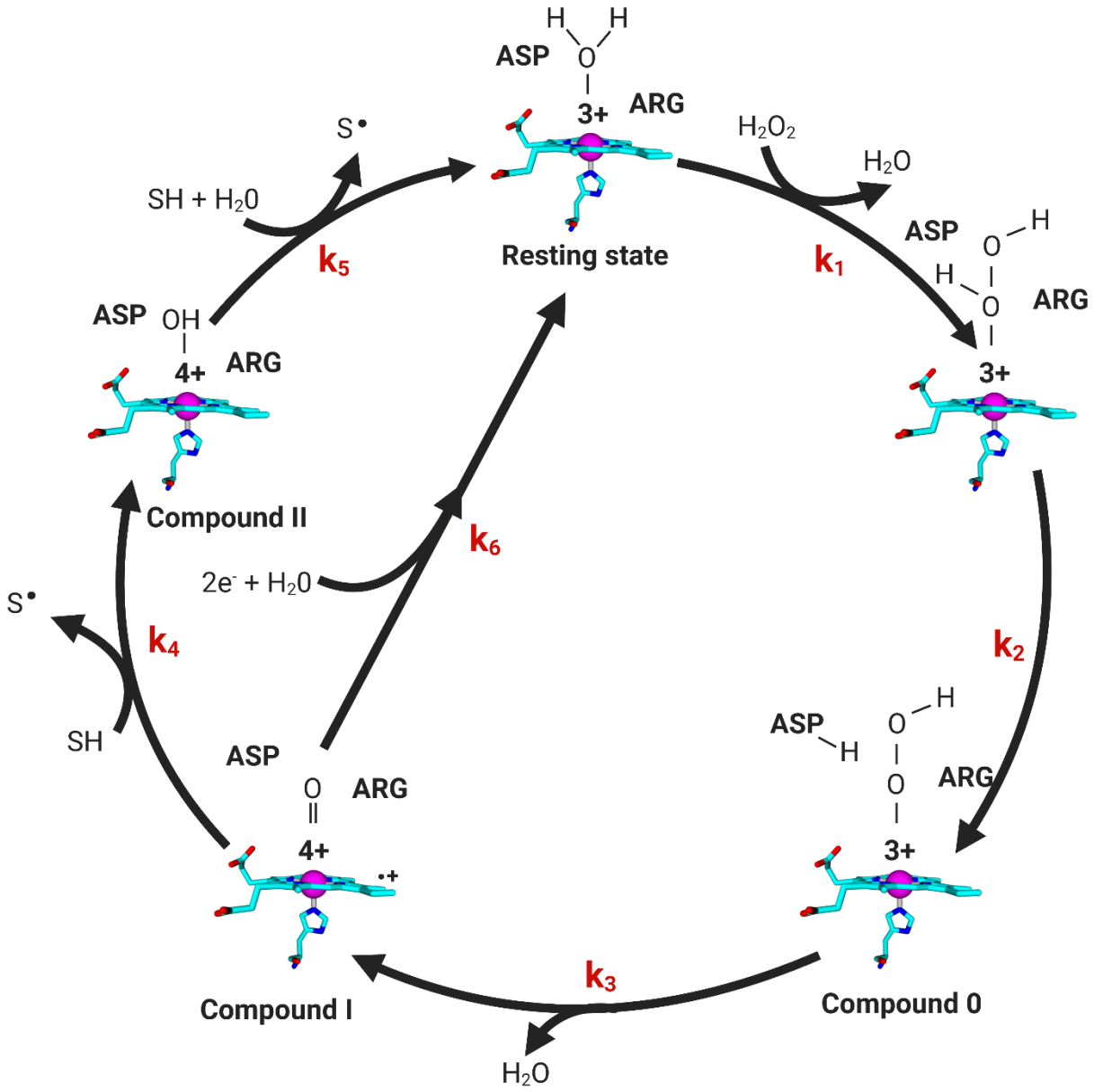

Figure 2 Detailed catalytic cycle of dye-decolorizing peroxidases. Resting state Fe(III) binds H_2O_2. Deprotonation of H_2O_2 by aspartic acid leads to the formation of Compound 0. Compound I is formed after heterolytic cleavage of dioxygen resulting in the highly reactive Fe(IV) species. Compound I can either decay into Compound II receiving one electron from a suitable substrate yielding one molecule of radical product or directly to resting state Fe(III)

when 2 electrons are provided by the substrate. Compound II decays into Fe(III) resting state yielding a second radical product molecule.

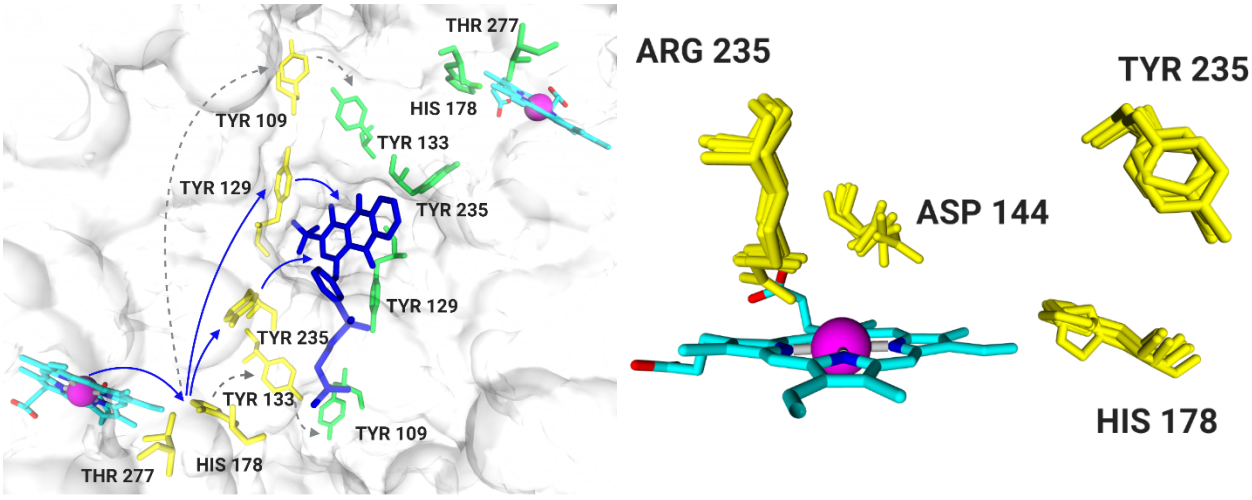

Figure 3. Radical transfer pathways. A) Postulated pathway for *Vibrio Cholerae* DypB protein dimer. The radical is generated by the heme iron and is transferred to His 178. In acidic conditions His 178 transfers the radical to Tyr 129 or Tyr 235 resulting in the attack of the substrate molecule. In alkaline conditions His 178 transfers the radical to Tyr 133 or Tyr 109 that by transferring the radical to the Tyr residues at the surface result in covalent dimerization. Substrate molecule and productive pathways are shown in blue, grey dashed arrows indicate unproductive pathways. B) Structural alignment showing conserved active site residues of *Vibrio Cholerae* DypB (PDB ID 5de0) overlayed on representative members of Class B DyPs. PDB IDs: 6fks, 5vj0, 3qhr, 2hag. All residue are conserved except for *Rhodococcus Jostii* (3qhr) where the tyrosine is replaced by a phenylalanine.

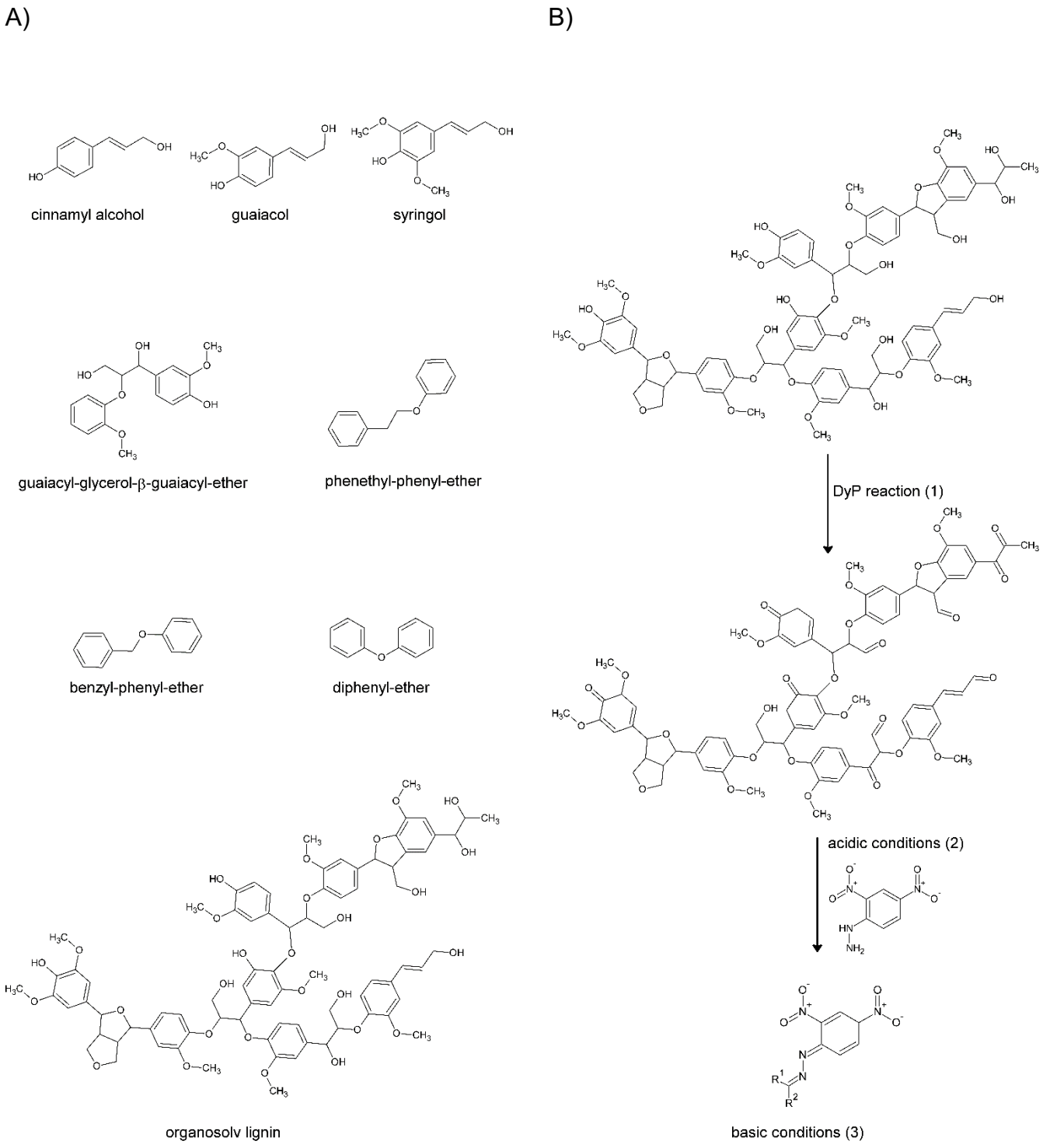

Figure 4. A) Constituents of lignin with increasing level of complexity. Monomeric structures: cinnamyl alcohol, guaiacol and syringol. Dimeric model compounds: guaiacyl-glycerol-β-guaiacyl-ether, phenethyl-phenyl-ether, benzyl-phenyl-ether; Polymeric structure: organosolv lignin. **B) Colorimetric screening assay for investigating lignin oxidative**

activity. [92] In the first step the enzymatic reaction in the presence of DyP peroxidase (or suitable enzymes) is carried out. In the second step 2,4-dinitrophenylhydrazine reacts with the carbonyl groups generated by enzymatic oxidation in acidic conditions yielding a dinitrophenylhydrazone derivative that in basic conditions (step 3) can be easily detected at 450 nm.

Tables

Table 1 Crystal structures of DyP peroxidases

CLASS	ORGANISM	PROTEIN NAME	PDB CODE	Reference
A	*Thermomonospora curvata*	TcDyP	5JXU	[77]
	Thermobifida fusca	TfuDyP	5FW4	[64]
	Escherichia coli O157	EfeB/YcdB	2Y4D, 2Y4E, 2Y4F	[71]
	Cellulomonas bogoriensis	CboDyP	6QZO	[78]
B	*Klebsiella pneumoniae*	Kp DyP	6FKS, 6FIY, 6FL2, 6FKT	[72]
	Enterobacter lignolyticus	El DyP	5VJ0	[79]
	Vibrio cholerae	VcDyP	5DE0	[80]
	Rhodococcus jostii RHA1	DyPB	4HOV, 3VEC, 3VED, 3VEE, 3VEF, 3VEG, 3QNR	[81] [70] [82]
	Shewanella oneidensis	TyrA	2HAG	[75]
	Bacteroides thetaiotaomicron	BtDyP	2GVK	[75]
C	*Anabaena sp.*	AnaPX	5C2I	[83]
D	*Pleurotus ostreatus*	PosDyP4	6FSK	[84]
	Auricularia auricula-judae	A. auricula-judae DyP	5IKD, 5IKG, 4W7J, 4W7K, 4W7L, 4W7M, 4W7N, 4W7O, 4UZI, 4AU9	[85] [86] [87] [88]
	Bjerkandera adusta	BadDyP	3VXI, 3VXJ, 2D3Q, 3MM1, 3MM2, 3MM3	[89] [90]