Bacterial laccases: Some Recent Advances and Applications

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ABSTRACT

Laccases belong to the large family of multicopper oxidases (MCOs) that couple the one-electron oxidation of substrates with the four-electron reduction of molecular oxygen to water. Because of their high relative nonspecific oxidation capacity particularly on phenols and aromatic amines and the lack of requirement for expensive organic cofactors, they have found application in a large number of biotechnological and industrial fields. The vast majority of studies and applications were performed using fungal laccases but bacterial laccases show interesting properties for diverse biotechnological applications such as optimal temperature above 50°C, optimal pH at the neutral to alkaline range, thermal and chemical stability and increased salt tolerance. Additionally, bacterial systems benefit from a wide range of molecular biology tools that facilitates their engineering and achievement of high yields of protein production and the set-up of cost effective bioprocesses. In this review we will provide up to date information on the distribution and putative physiological role of bacterial laccases, will highlight their distinctive structural and biochemical properties, discuss the key role of copper in the biophysical and biochemical properties, unveil thermostability determinants and finally, will review biotechnological applications with a focus on the putative catalytic mechanisms of phenolics and aromatic amines.

DISTRIBUTION AND PHYSIOLOGICAL ROLE OF LACCASES IN THE PROKARYOTIC WORLD

Laccases act on a surprisingly wide range of substrates, including diphenols, polyphenols, differently substituted phenols, diamines, aromatic amines, benzenethiols, and even some inorganic compounds such as metal ions such as Mn(II), Cu(I) and Fe(II). Laccases are claimed to be one of the oldest enzymes ever isolated that for almost have been mostly characterized from plan and fungal sources (Mayer and Staples 2002), however in the last two decades a wide number of laccases of prokaryotic origin have been identified and characterised; for recent reviews see (Martins et al. 2015; Chandra and Chowdhary 2015; Tonin et al. 2016; Chauhan et al. 2017; Guan et al. 2018). In **Fig. 1** a phylogenetic analysis is shown with laccases which genes were cloned and enzymes were characterized (sequences retrieved from NCBI (**Table 1**)). In the next sub-sections we will discuss the physiological relevance of proteins within the different groups.

Proteins involved in copper resistance. The distribution of laccases involved in copper resistance in the phylogenetic tree suggests that this is a physiological role widely conserved in

bacteria. Copper is a redox active metal with two different oxidation states, Cu(I) and Cu(II), that is a micronutrient required by living organisms that in excessive amounts exerts cellular toxicity. The microorganisms have developed different mechanisms of copper resistance that includes the action of MCOs playing a role in oxidising Cu(I) to the less toxic Cu(II) in the periplasm (Pérez et al. 2018). **A.1. CueO.** *Escherichia coli* CueO, one of the best characterized MCO, involved in copper resistance (Grass and Rensing 2001) is included in **a** phylogenetic branch including different enzymes from proteobacteria. CueO, in addition of oxidizing Cu(I) in the periplasmic space, also oxidizes catecholate siderophores, avoiding that Cu(II) is reduced back to Cu(I) (Grass et al. 2004). *cueO* is regulated at the transcriptional level by CueR that also regulates the copper ATPase efflux CopA, which works in coordination with CueO in copper detoxification (Outten et al. 2000; Rademacher and Masepohl 2012). **A.2. PcoA.** In addition to CueO which is chromosomally encoded (Outten et al. 2001), some *E. coli* strains possess another plasmid encoded MCO involved in copper resistance mechanisms (Brown et al. 1995). This MCO was named PcoA and similarly to CueO shows cuprous oxidase activity in the periplasm (Huffman et al. 2002). The operon contains 7 genes and its expression from two different promoters is controlled by a two-components regulatory system encoded within the same operon (Rouch and Brown 1997). Proteins with similarity to PcoA have been detected in other gammaproteobacteria, as in *Pseudomonas syringae* (Cha and Cooksey 1991) or *Stenotrophomonas maltophilia* (Galai et al. 2011), and named CopA, the same name used for the copper efflux ATPase associated to CueO. The presence of these genes in microorganisms able to degrade lignin and their enzymatic activity on lignin model compounds has been considered as an indication of a possible role in lignin degradation (Granja-Travez and Bugg 2018). **A.3. MCOs in *Myxococcus xanthus*.** *Myxococcus xanthus*, a deltaproteobacterium with a complex life cycle encodes three different MCO (CuoA, CuoB and CuoC) involved in copper resistance (Muñoz-Dorado et al. 2016). CuoB and CuoC cluster with an enzyme from the alphaproteobacterium *Pedomicrobium* and the CuoA one is in a different branch (**Fig. 2**). CuoB is involved in an immediate response reaching a maximum after 2 hours of copper adition (Sanchez-Sutil et al. 2007); the expression of *cuoB* is regulated by the extracytoplasmic sigma factor (ECF) CorE which activity is activated by Cu(II) and inactived in the presence of Cu(I) (Marcos-Torres et al. 2016). CuoA and CuoC, participate in the copper response maintenance and are slowly induced reaching a maximum at 24 h. This response is regulated by the two-component regulatory system CosSR, which periplasmic domain is responsible for Cu sensing (Sánchez-Sutil et al. 2016). **A.4. MCOs in Firmicutes.** The involvement of MCOs in copper resistance has been also described in some Firmicutes as it is the case of *Staphyloccus aureus* that synthesizes a MCO whose gene expression is induced by copper and which deletion resulted in an increased copper sensitivity (Sitthisak et al. 2005). The genes coding for the *S. aureus* MCO have been detected in mobile genetic elements present in many clinical isolates and proposed to play a role in virulence (Zapotoczna et al. 2018). *Pediococcus* and *Lactobacillus* synthesize MCOs that are very close in the phylogenetic tree to the *S. aureus* enzyme. These enzymes have been studied after recombinant production showing enzymatic activity and the capacity to degrade biogenic amines (Callejon et al. 2016, 2017)
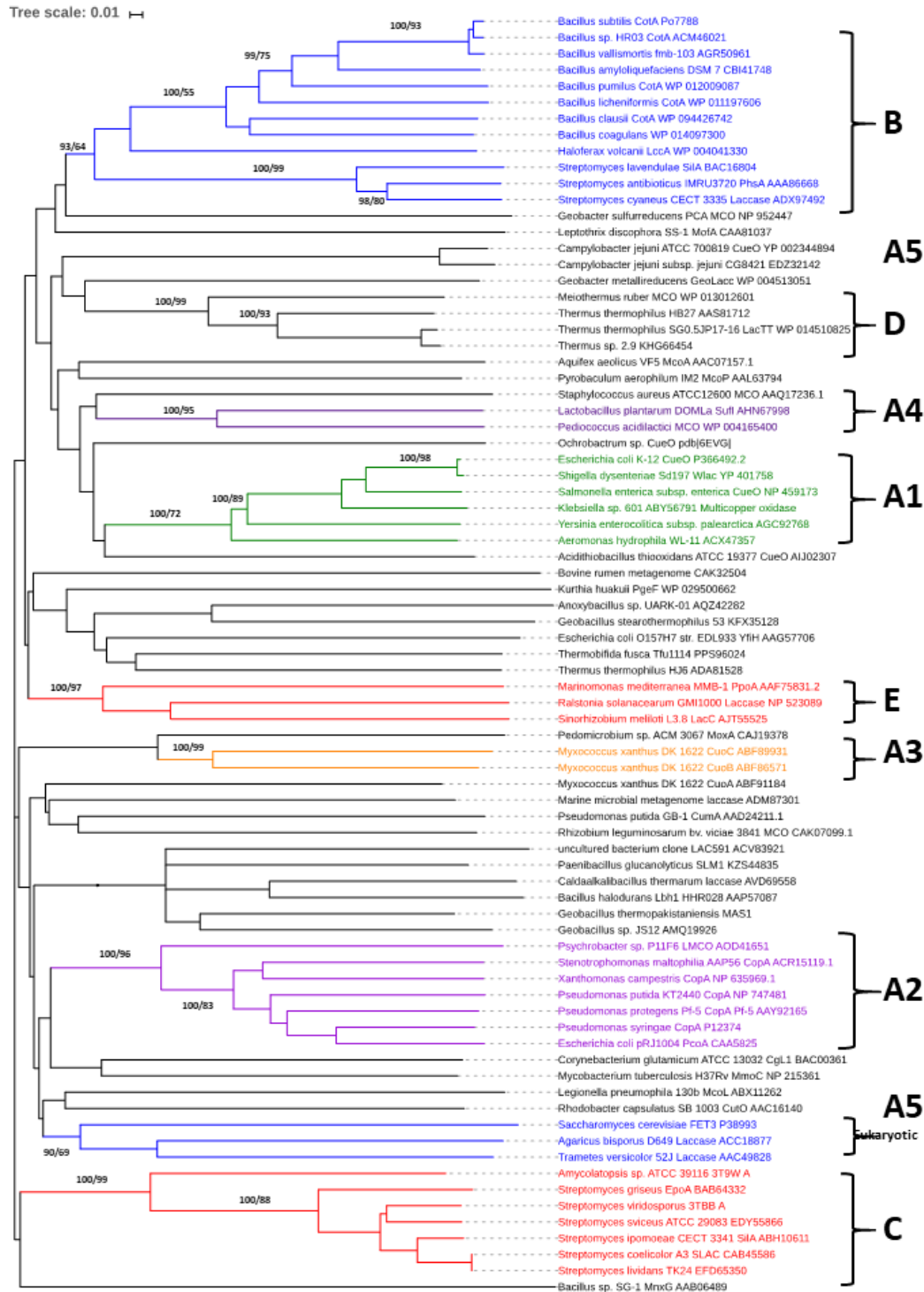
Figure 1. Phylogenetic relationship of bacterial multicopper oxidases with reported laccase activity. The phylogenetic analysis was performed using the program MEGA X with sequences aligned by the program MUSCLE built into MEGA (Kumar et al. 2018). The tree using the Neighbor-Joining (NJ) method is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. The final display of the tree was obtained by using iTOL (Letunic and Bork 2019). A tree was also constructed by the Maximum Likehood (ML) method. Numbers at branches indicate bootstrap values higher than 80% for the NJ method and the boostrap vaule obtained with the ML method in those branches. The enzymes in the same group are marked with the same color and are annotated as explained in the text. Phylogenetic relationship of bacterial multicopper oxidases with reported laccase activity.

A5. Other MCOs involved in copper resistance. The expression of gene coding for CutO from *Rhodobacter capsulatus* was shown to be induced at high copper concentrations through a regulatory mechanism operating at the post-transcriptional level (Rademacher et al. 2012). Similarly the gene coding for the MCO of *Campylobacter jejuni* is induced with high copper concentrations and the mutation of the gene determines an increase in copper sensitivity (Hall et al. 2008)

B. Proteins involved in spore pigmentation. This group comprises the most well studied bacterial laccase, CotA, from *Bacillus subtilis* (Hullo et al. 2001; Martins et al. 2002). Most of the laccases identified in *Bacillus* are part of the outer coat that protects spores from a diverse range of stresses, playing roles in the biosynthesis of a brown melanin-like spore pigment and protection from UV-light and hydrogen peroxide and cluster in the same group as shown in the tree (Table 1 and references in there). Very related to those proteins is a laccase synthesized by the archaeum *Haloferax volcanii* that had been most likely acquired by horizontal gene transfer from bacteria of the phyla *Firmicutes* (Uthandi et al. 2010). Some proteins of actinobacteria are found closely associated to the *Bacillus* CotA (**Figure 2**), for example, phenoxazinone synthase Phs from *Streptomyces antibioticus* that catalyses the oxidation of aminophenol required for the synthesis of secondary metabolites, such as the antibiotic actinomycin. As an alternative physiological role, it was proposed that Phs could participate in spore pigment synthesis similarly to CotA-laccases (Jones 2000).

C. Small laccases in *Streptomyces*. Some laccases from from actinobacteria of the Order *Actinomycetales* are in a clearly differentiated branch (Figure 1). This group contains the secreted enzymes denominated "Small laccases" (SLACS) characterized by having only two structural domains and the *Streptomyces coelicolor* enzymes id the most extensively characterized laccase (Machczynski et al. 2004). These are secreted enzymes that have been implicated in morphogenesis, sporulation, pigmentation, ligninocellulose degradation, bacteria-bacteria interactions or antibiotic production (Table 1 and references in there).

D. Laccases in plant associated proteobacteria. The *Marinomonas mediterranea* laccase PpoA was one of the first bacterial laccases described (Solano et al. 1997). This protein is a membrane associated protein showing an N-terminal His rich region which could be related to its capacity to oxidize not only the substrates characteristic of laccases, but also the monophenol L-tyrosine (Solano et al. 2001; Sanchez-Amat et al. 2001). The physiological role of PpoA is unknown; mutant strains lacking this protein are not affected in pigment synthesis and no evidences for involvement in Cu resistance were found (Fernandez et al. 1999). The laccase *Ralstonia solanacearum* laccase was proposed to participate in defence against phenolic compounds (Hernandez-Romero et al. 2005). This could relate to the association of the producing bacteria with plants: *M. mediterranea* was isolated from the microbiota of the marine plant *Posidonia oceanica* (Espinosa et al. 2010), *R. solanecarum* is a plant pathogen and *Rhizobium* is a symbiont of plants (Pawlik et al. 2016).

E. Laccases in thermophilic bacteria (and archaea). Several MCOs from thermophilic and hyperthermophilic bacteria that cluster in the same group (Miyazaki 2005; Fernandes et al. 2007; Kalyani et al. 2016; Navas et al. 2019). Notably, the MCO from *Thermus thermophilus* shows extreme stability at high temperatures with a half-life at 80°C of around 14 h (Miyazaki 2005). This group also comprises the only hyperthermophilic archaeal-type MCO, designated McoP

from *Pyrobaculum aerophilum* (Fernandes et al. 2010). A MCO was also characterized from *Aquifex aeolicus* that grows optimally at 89°C and occupies the deepest branch of the bacterial phylogenetic tree (Fernandes et al. 2007). The gene coding for McoA is part of a putative copper-resistance determinant and the enzyme was shown to exhibit higher efficiency for Cu(I) and Fe(II) metal ions than for aromatic substrates.

F and G. Other groups. There are other clusters revealed by the phylogenetic analyses for which no physiological role has been yet proposed. For example, the MCO from *Thermus thermophilus* HJA shows a small molecular mass (Kim et al. 2015) and is not included in the same group that other enzymes from the same species which are in group E. Genes encoding proteins similar to those in group F can be detected in other bacteria, including some *E. coli* strains (Kim et al. 2015) but their physiological role remains unknown.

STRUCTURE, MECHANISMS AND BIOCHEMICAL PROPERTIES

These enzymes have three distinct copper sites, Cu types 1, 2 and 3; the oxidation of the reducing substrate occurs at the type 1 (T1) Cu site while the reduction of O_2 occurs at the T2/T3 trinuclear cluster (Jones and Solomon 2015). The coordination of the copper centres is largely conserved among laccases. The oxidation of the reducing substrates occur at the T1-Cu site while the reduction of O_2 occurs at the T2/T3 trinuclear cluster (**Figure 2**) (Solomon et al. 1996; Jones and Solomon 2015; Kosman 2010, 2017). The T1 Cu is coordinated by two histidine nitrogen atoms and a cysteine sulphur and it is characterised by an intense $S(\pi) \rightarrow Cu(d_{x^2-y^2})$ charge transfer absorption band at around 600 nm, $\varepsilon_{600nm} > 3000$ $M^{-1} cm^{-1}$ responsible for the intense blue colour of the enzymes. The T2 copper site, strategically positioned close to the T3 binuclear copper centre, is usually coordinated by two histidine residues and a water (or hydroxyl) molecule, while each T3 copper is coordinated by three histidines and a bridging ligand such as a hydroxyl moiety, displaying an absorption in the near-UV, with $\lambda_{max} = 330$ nm. The mononuclear T1 Cu site interacts with the trinuclear cluster T2/T3 through the highly conserved HCH motif, where the cysteine binding to the T1 Cu shuttles electrons to each of the adjacent histidines binding to one of the T3 copper ions. The routes of electron transfer, the oxidation states of the Cu centres and the mechanism of oxygen reduction in laccases were recently reviewed; please see (Jones and Solomon 2015).

The first three dimensional structures of prokaryotic laccases were reported for *E. coli* CueO (Roberts et al. 2002) and *Bacilus subtilis* CotA-laccase (Enguita et al. 2003), followed by *Streptomyces coelicolor* SLAC (Skalova et al. 2009), *Pyrobaculum aerophilum* McoP (Sakuraba et al. 2011) *Campylobacter jejuni* McoC (Silva et al. 2012), *Streptomyces sviceus* Ssl1 (Gunne et al. 2014) and more recently *Ochrobactrum* sp. CueO (Granja-Travez et al. 2018). Structural information is also available for the Tht *Thermus thermophila* HB27 laccase in the Protein Database Bank (Serrano-Posada et al. 2011). The overall bacterial laccase structural fold comprises the typical three cupredoxin-type domains, characterised by a Greek key β-barrel topology (Hakulinen and Rouvinen 2015) (**Fig 2**). Laccases from *Streptomyces*, as well as *Amycolatopsis* sp. 75iv2, designed SLACs (<u>s</u>mall <u>lac</u>cases)) lack the second of the three domains and their crystal structures reveal a trimeric quaternary arrangement of the two-domain protein chains (Skalova et al. 2009; Komori et al. 2009; Gunne et al. 2014; Majumdar et al. 2014). Evolutionary theories proposed two-domain proteins as the intermediate step in the evolution

between one domain proteins with a cupredoxin fold, and three domain laccases (Nakamura and Go 2005; Komori et al. 2009).
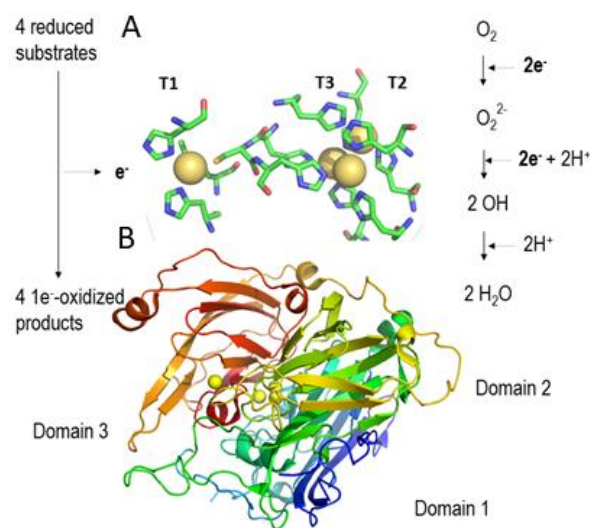

Figure 2. Three-dimensional representation of the copper centers arrangement (A) and the overall structure in the *Bacillus subtilis* CotA-laccase (Enguita et al. 2003). In picture (A) the mononuclear T1 center is on the left and the trinuclear center is on the right. The distance between the T1 and T2/T3 centre is ≈13Å. In picture (B) the cupredoxin domains are coloured differently (residues 1-173, domain 1: blue to green; residues 182-340, domain 2: green to yellow; residues 369-501, domain 3: yellow to red). The four copper atoms are shown as yellow spheres. Pictures drawn in PyMol software using the deposited structure PDB1w6L.

In bacterial laccases the T1 Cu is weakly coordinated by an axial ligand, a methionine, in tetrahedral geometry. Fungal laccases have non-coordinating phenylalanine or leucine at this position, favouring a trigonal planar geometry for the site, that may contribute to the higher redox potential (~ 800 mV) observed in these enzymes as compared to of bacterial ones (~ 500 mV) (Xu et al. 1999; Durao et al. 2006; Hong et al. 2011). However, variations in redox potential of the T1 center observed among laccases cannot be assigned to this single structural feature but to a sum of factors including the nature of the second sphere residues influencing solvent accessibility, hydrogen bonding, and dielectric anisotropy around the site (Durao et al. 2008b; Matera et al. 2008; Marshall et al. 2009).

In general, laccases show a broad binding pocket buried between domain 1 and 3 and close to the T1-Cu centre primarily stabilised through hydrophobic interactions (Hakulinen and Rouvinen 2015). In *B. subtilis* CotA-laccase the T1 Cu centre is sited at the bottom of the substrate binding region, relatively exposed to the solvent and interacting with the substrate molecules through the imidazole ring of one of its His ligands (Enguita et al. 2004) similarly to what was observed in fungal structures complexed with phenolics or related substrates (Bertrand et al. 2002; Hakulinen et al. 2002; Matera et al. 2008). However, in the remainder of 3-domain bacterial laccases whose structures have been reported the T1 site is occluded by several secondary structure elements, with varied length, composition and structure (**Figure 3**). The small substrate binding pocket of these enzymes seems to be in accordance to their higher specificity to small metal ions Cu(I) and Fe(II) (Singh et al. 2004; Fernandes et al. 2010; Silva et al. 2012), indicating that these MCOs should behave predominantly as metallo-oxidases in their native microorganisms with a role in copper resistance (please see above). However, even

at reduced efficiency these enzymes are also able to oxidise phenolic or non-phenolic substrates and it is plausible that they possess additional binding pockets to accommodate bulkier substrates (Zaitsev et al. 1999; Liu et al. 2011; Bello et al. 2014).
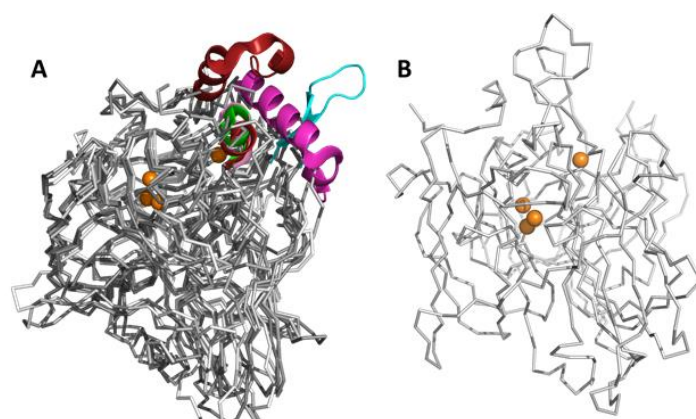

Figure 3. (A) Ribbon superimposition highlighting the major secondary structure elements interfering with the access to the T1-Cu center in *E. coli* CueO (magenta, PDB 1KV7), *C. jejuni* McoC (red, PDB 3ZX1), *P. aerophilum* McOP (green, PDB 3AW5) and *T. thermophilus* Tth (cyan, 2XU9). (B) *B. subtilis* CotA-laccase (PDB 1w6L) shows a widely exposed T1-Cu centre. Cu ions are represented as dark orange spheres.
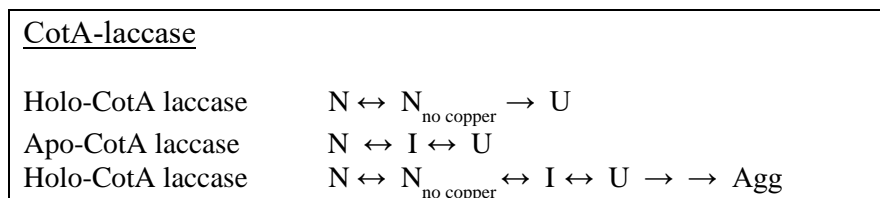
One important structural distinctive feature of bacterial laccases as compared to those of fungal origin is the lack of negatively charged residues in the substrate binding pocket with consequences in the optimal pH for substrates with phenolic or aromatic amine functional groups. Fungal laccases contain a conserved acidic residue (either an Asp or Glu) close to the T1 Cu centre that in their carboxylate form, i.e. in acidic conditions, are able to hydrogen bond with OH or NH_2 groups of substrates aiding in their deprotonation by stabilising the radicals formed during the catalytic reaction (Bertrand et al. 2002; Kallio et al. 2009; Madzak et al. 2006). In the case of bacterial laccases such as *B. subtilis* CotA-laccase, in the absence of negatively charge residues close to the T1 Cu centre, the oxidation relies mainly in the substrates structures, in particular, in their protonation/deprotonation equilibria, which is dependent of the pKa values (d'Acunzo and Galli 2003). Considering that oxidative enzymatic reactions are more prone to occur after deprotonation of OH or NH_2 groups, fungal laccases show an optimal pH at the acidic range and bacterial laccases at the neutral to alkaline range, above the pKa value of the respective phenolic and aromatic amines substrates (Pereira et al. 2009a; Pereira et al. 2009b; Rosado et al. 2012).

COPPER ROLE AND STABILITY DETERMINANTS OF BACTERIAL MCOs

Copper incorporation in MCOs is still a poorly understood process and remains an important issue of discussion in the current literature (Kosman 2017) . Understanding this mechanism is important both at the biochemical, spectroscopic and structural viewpoints but also from a biotechnological perspective since copper depletion is a limiting factor to achieve full catalytic activity and stability with consequences in the implementation of cost-effective industrial applications. Bacterial laccases are typically heterologously overproduced in the cytoplasm of *E. coli* that contains limiting concentrations of copper (10 µM) in aerobic conditions due to the operation of copper homeostasis mechanisms (Changela et al. 2003). In order to surpass this limitation *E. coli* cells needs to be cultivated under microaerobic growth conditions (in copper-

supplemented media) where significantly higher amounts of copper accumulates allowing the production of fully copper loaded recombinant laccases (Durao et al. 2008a).

The key role of copper to the stability and folding of CotA laccase. The CotA laccase from *Bacillus subtilis* has been used as one model to dissect stability features and determinants of multi-copper oxidases. The long-term stability of CotA revealed that the enzyme denatures irreversibly according to a simple pathway of unfolding and deactivation (first-order process) with a half-life of inactivation of 50-172 min (at 80°C) depending on the pH and copper content (Durao et al. 2008a; Fernandes et al. 2011; Brander et al. 2014). This value is larger than the half-life of *B. clausii* CotA (20 min) but smaller than the 350, 540 and 868 min measured for the thermophilic laccases from *T. terrenum*, *C. thermarum* and *T. thermophiles*, respectively (Miyazaki 2005; Brander et al. 2014, 2015; Ghatge et al. 2018). The thermodynamic stability of *B. subtilis* CotA was further characterised showing that at 25°C the native state is very stable with a free energy change of 10 kcal/mol and a mid-point of 4.6 M guanidinium hydrochloride upon unfolding of the tertiary structure (Durao et al. 2006). These studies showed that copper depletion from the T1 site is the key event in the inactivation of CotA laccase preceding the unfolding of the secondary and tertiary structures similarly to the reported in plant *Rhus vernicifera* laccase (Agostinelli et al. 1995) and *Coriolus hirsutus* and *Coriolus zonatus* fungal laccases (Koroleva et al. 2001), and an unfolding pathway was proposed as shown in **Box 1** (Durao et al. 2006; Fernandes et al. 2012). Copper depletion affects the k_{cat} values of the enzymes and the T1 Cu site redox potential E^{o}_{T1} of the holoprotein is 525 nm as compared to 455 mV of the partially depleted enzyme (2.5 moles of copper per mole of protein) (Durao et al. 2006; Durao et al. 2008a).

CotA-laccase	
Holo-CotA laccase	$N \leftrightarrow N_{no\ copper} \rightarrow U$
Apo-CotA laccase	$N \leftrightarrow I \leftrightarrow U$
Holo-CotA laccase	$N \leftrightarrow N_{no\ copper} \leftrightarrow I \leftrightarrow U \rightarrow \rightarrow Agg$

Box 1 - Chemically-induced unfolding pathways of CotA laccase where N, I and U are the native, intermediate and unfolded states, respectively, and Agg is an aggregated state.

The role of copper in CotA laccase was addressed in more detail to reveal a subtle balance between copper loading and enzyme folding; please see a recent review on MCOs metallation (Kosman 2017). Copper incorporation was shown to be critical in the fine tuning of CotA-laccas folding in the cytoplasm of *E. coli* (Durao et al. 2008a). Firstly, the production of fully copper loaded CotA preparations in *E. coli* cells was shown to be achieved after a switch from aerobic to microaerobic growth conditions that favour the presence of Cu(I) ion in *E. coli* cytoplasm. Secondly, *in vitro* copper incorporation into apo-CotA preparations result in a fully copper loaded enzyme only if Cu(I) and not Cu(II) is used. It was shown that the small structural rearrangement that occurs *in vivo* when copper is incorporated seems to occur in the vicinity of the T2 copper site and persists after copper depletion *in vitro*. The results clearly indicated that Cu(I) is the most efficient Cu redox state and its incorporation during enzyme folding *in vivo* is key to achieve a fully copper loaded and fully functional and stable enzyme.

A sequential copper incorporation was proposed, with the T1 site being the first to be reconstituted *in vitro*, followed by the T2 and T3 centers (Durao et al. 2008a) similarly to what

was described in *E. coli* CueO (Galli et al. 2004). It is the T1 site that provides a template for the assembly of the native protein indicating that the ancestral cupredoxin is the starting point for assembly of a fully metallated protein (Kosman 2017). The mutant G304K of CueO laccase may well be a good example of structural tuning imparted by copper binding taking place in this case *in vitro* (Wang et al. 2018). Addition of copper induced conformational changes in a methionine-rich helix and in a regulatory loop resulting in enhanced activity. Molecular dynamics simulations of the holo- and apo-forms of the multicopper oxidase from *Thermus thermophilus* HB27 also revealed a different conformation for the linker connecting the β-strands 21 and 24 of the cupredoxin fold with possible implications in the process of electron transfer (Bello et al. 2012). Timely copper incorporation into the multicopper oxidase ceruloplasmin is also crucial to prevent misfolding and assure successful biosynthesis *in vivo* (Sedlak and Wittung-Stafshede 2007).

Deeper insight into the unfolding pathway and copper incorporation in CotA was gathered through acid-induced unfolding and double-jump stopped-flow experiments (Fernandes et al. 2012). In these double-jump experiments, CotA was first unfolded by a decrease in pH and after fixed time intervals when the enzyme unfolds in a delay loop (20-1000 ms) refolding was promoted by mixing the unfolded enzyme preparation with a buffer at higher pH. If CotA unfolded according to a two-state process where only the native and the unfolded states accumulate, a simple refolding phase would be observed. On the contrary, if an intermediate accumulated between the native and the unfolded state two refolding phases would be observed especially for longer delay times where the accumulation of the unfolded state becomes more significant. Apo-CotA display two refolding phases at delay times over 200 ms indicating that a three-state process describes the unfolding of the enzyme. Even in the absence of copper there is an intermediate in between the native and the unfolded only revealed through kinetic measurements as shown in **Box 1**. The apo-form of human ceruloplasmin also unfolds with at least one intermediate (Palm-Espling et al. 2012). Double-jump experiments for holo-CotA also revealed the presence of an intermediate in between the native and the unfolded state, indicating that the presence of copper in the unfolded holo-protein prevents its refolding back to the native state due to protein aggregation as summarised in **Box 1**. In summary, copper has to be incorporated at later stages of *in vitro* folding (Fernandes et al. 2012) but this incorporation is important to adjust the structure to obtain a fully functional and stable enzyme (Durao et al. 2008a).

The removal of the single intradomain disulfide bridge of the CotA laccase showed no significant effects at the structural or functional levels (Fernandes et al. 2011). Conformational dynamics are known to be affected by copper binding such as in the case of the multicopper oxidase Fet3p and the copper chaperones Atox1 and CopZ (Sedlak and Wittung-Stafshede 2007; Palm-Espling et al. 2012) and the *Thermus thermophilus* HB27 laccase (Bello et al. 2012). Interestingly not only the unfolding kinetics of CotA measured by stopped-flow, which report on the free energy change between the native and the transition state are faster for the mutant protein, indicating that the native state is less stable in the absence of the disulfide bridge, but additionally, the kinetics of copper release is increased in the mutant. Overall the results indicate that the CotA disulfide-bond has a role in tuning copper binding a the key parameter in the folding and stability of CotA.

The hyperthermophilic nature of the *A. aeolicus* McoA and *P. aerophilum* McoP. The thermal stability of the hyperthermostable metallo-oxidases from *A. aeolicus* (McoA) (Fernandes et al. 2009) and *P. aerophilum* (McoP) (Fernandes et al. 2010) was studied by DSC revealing the complex process characterised by three independent thermal transitions showing Tm values of 105, 110 and 114°C for McoA and 97, 102 and 112°C for McoP. Three independent thermal transitions were also observed for ascorbate oxidase (Savini et al. 1990), ceruloplasmin (Bonaccorsi di Patti et al. 1990) and CotA laccase (Durao et al. 2008a) and seem to correlate well with the structural organisation of three cupredoxin-like domains for the respective enzymes. The high thermal stability of both McoA and McoP is in agreement with a significant long-term stability; McoP display a half-life time of 5.5 h at 80°C (Fernandes et al. 2010) while McoA shows a more complex deactivation pathway due to protein aggregation but the first-order deactivation step is characterised by a half-life time of 5.7 h at 80°C (Fernandes et al. 2007). Despite the hyperthermophilic nature of both enzymes, the chemical-induced unfolding reveals a relatively low thermodynamic stability especially for McoA with a mid-point of 2.7 M guanidinium hydrochloride and a free energy change in buffer at 25°C of 2.8 kcal/mol (Fernandes et al. 2009). Copper depletion from McoA precedes largely the unfolding of the tertiary structure and is the key event for inactivation in the presence of chemical denaturants as mentioned previously for CotA. The low equilibrium thermodynamic stability measured for McoA refers thus to the apo form (Fernandes et al. 2009; Pozdnyakova et al. 2001). A fast unfolding rate constant for the apo form compared to the holo form was measured; this was assigned to the stabilising effect of copper on the native state and quantified as 1.5 kcal/mol, which is very similar to the value of 1.4 kcal/mol measured for CotA using a different approach (Durao et al. 2006). Copper loading increases the stability of McoA and the dependence of stability on temperature becomes less flat (**Fig 4A**). Interestingly, a flat dependence of the free energy change on temperature is one of the mechanisms that leads to enhanced thermal stability in proteins from thermophiles (Fitter and Heberle 2000; Robic et al. 2003). The comparison between the stability curves of McoA and CotA clearly shows that the flat dependence of stability on temperature observed for McoA allows the protein to remain folded over a wider range of temperatures as compared with CotA (**Fig 4B**). For McoA, the flat dependence of stability on temperature imposed by a low heat capacity change upon unfolding (0.5 compared to 2.6 kcal mol^{-1} K^{-1} for CotA) results from the aggregation of the enzyme as proved by gel filtration chromatography and light scattering measurements. From a mechanistically point of view, the aggregation process confers residual structure to the final state resulting in a low heat capacity change and a flat dependence of stability on temperature. One hypothesis is that McoA suffers kinetic partitioning between aggregation and first-order deactivation which is an uncommon feature seldom observed for other enzymes (Baptista et al. 2003) that can explain the low heat capacity change. This hypothesis is in accordance with the low cooperativeness of the transition from the folded to further states under equilibrium conditions as evaluated by the thermodynamic parameter *m* (-1.0 ± 0.1 kcal.mol^{-1} M^{-1}); both the heat capacity change and *m* correlate to the amount of surface area exposed upon unfolding. This atypical behaviour of McoA explains the low thermodynamic stability observed upon chemical-induced destabilisation but simultaneously contributes to an increased stability over a wider range of temperatures and thus to the hyperthermophilic nature of McoA.
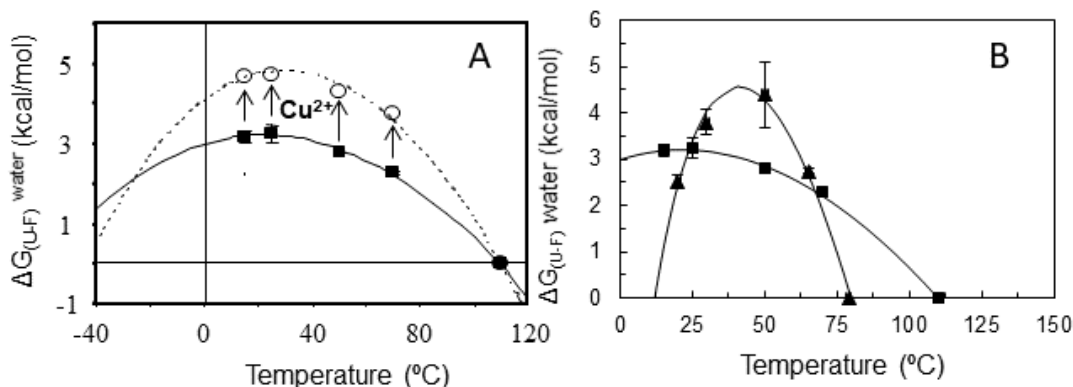
Figure 4. (A) Temperature dependence of McoA stability built with an average Tm measured by DSC and chemical unfolding induced by guanidinium hydrochloride at different temperatures. Experimental data was fitted with the Gibbs-Helmholtz equation and the dotted line is the fit using the same equation after adding 1.5 kcal/mol due to the stabilizing effect of copper (adapted from (Fernandes et al. 2009)). (B) Protein stability curves of McoA (squares) and CotA (triangles) at pH 3, fitted according to the Gibbs-Helmholtz equation (partly adapted from (Fernandes et al. 2009)).

APPLICATION OF BACTERIAL LACCASES IN OXIDATIVE BIOPROCESSES

Oxidation of lignin-related phenolic and non-phenolic compounds using bacterial laccases.
Laccases are promising ligninolytic enzymes and therefore it is expected that the number of laccase-based industrial oxidation processes will increase significantly in the next years in particular in the lignocellulose biorefinery field. Enzymatic depolymerisation of lignin into phenolic platform chemicals is envisaged as one of the potential environmentally friendly breakthrough applications for the successful valorisation of lignin bio-wastes (Sun et al. 2018). The fragmentation of lignin into lower molecular weight fractions with increased reactivity, bioequivalents, for example, to oil-based compounds used as resins, adhesives, composites, and foams is crucial for its valorisation. Bacterial laccases have been successfully tested in the oxidation of eucalyptus kraft pulp (Arias et al. 2003; Eugenio et al. 2011), wheat straw pulps (Zheng et al. 2012), kraft lignin (Huang et al. 2013), diverse hardwood and softwood lignocellulose samples (Sondhi et al. 2015; Singh et al. 2017), woody materials (Goacher et al. 2018; Navas et al. 2019), lignin samples and a broad range of low molecular weight lignin model compounds (Moya et al. 2011; Huang et al. 2013; Reiss et al. 2013; Majumdar et al. 2014; Hamalainen et al. 2018) confirming their potential in lignin degradation and valorisation.

The enzymatic oxidation of phenols is based in one electron abstraction which generates resonance-stabilised phenoxy radicals. These may undergo a second enzyme-catalysed oxidation into quinonic structures, or react further in non-enzymatic reactions (hydration, disproportionation or homo and heteromolecular coupling reactions giving to oligo- or polymeric products) (Mogharabi and Faramarzi 2014; Kudanga et al. 2017; Romero-Guido et al. 2018). Most of reports using bacterial laccases for the oxidation of lignin-related phenolic acids, including sinapic, ferulic, caffeic and coumaric acids leads to the formation of dimeric structures (Koschorreck et al. 2008; Ricklefs et al. 2014; Xie et al. 2015). The phenolics oxidation was shown to be affected by substituents in the *ortho* positions, with a preference in the oxidation of syringyl-type over the guaiacyl- or hydroxyphenyl-subunits and the carbon chain length at the *para* position of the phenyl group. Phenolic and non-phenolic dimeric β-O-4 compounds, for

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example guaiacyl glycerol-β-guacyl ether (GGE) and adlerol, models to study the Cα–Cβ bond cleavage, that represent more than 50% of lignin structure, were used as substrates by several bacterial laccases; SLCAS from *S. coelicolor* A3(2), *S. lividans* TK24, *S. viridosporus* T7A and *Amycolatopsis* sp. 75iv2 (Majumdar et al. 2014), CopA from *P. putida* KT2440 and *P. fluorescens* Pf-5 (Granja-Travez and Bugg 2018), CueO from *Ochrobactrum* sp. (Granja-Travez et al. 2018), CtLac from *C. thermarum* (Ghatge et al. 2018) and the *Thermus* sp. 2.9 laccase (Navas et al. 2019). Overall, the tested bacterial laccases catalyses the oxidative coupling of GGE units, but not the cleavage of the Cα–Cβ bond with the exception of *B. amyloliquefaciens* CotA that degraded GGE in a set of products identified as guaiacol, 4-vinylguaiacol, vanillin and other related compounds (Yang et al. 2018). The presence of these compounds indicate that the main degradation pathway of GGE follows the oxidation of Cα and the cleavage of β-O-4 and Cβ-Cγ bonds, highlighting the potential of the *B. amyloliquefaciens* CotA laccase for lignin degradation.

A mechanism of lignin-related phenolics oxidation and their role in mediating non-phenolics by bacterial laccases was thoroughly described (**Fig. 5**) (Rosado et al. 2012). The oxidation rates for three syringyl-type phenolic models (syringaldehyde (SA), acetosyringone (AS)) and methyl syringate (MS) were measured showing that the enzymatic rates (SA > AS >> MS) are in line with the electron withdrawing capability of the para-substituting groups (aldehyde (SA) > ketone (AS) > ester (MS)), and concordant with the pKa values (and oxidation potential) of compounds
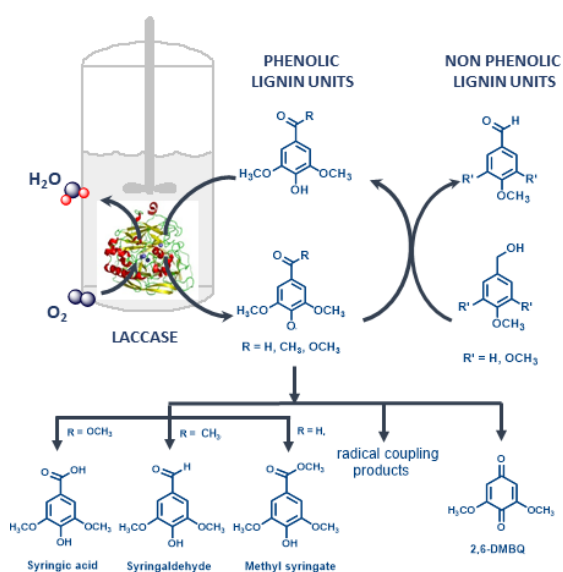

Figure 5. The catalytic cycle of laccase-mediator systems showing the formation of intermediate radicals upon oxidation of the phenolic substrates and the routes leading to the formation of different oxidation products (adapted from Rosado et al 2012). The phenoxy radicals are not only involved in the oxidation of the non-phenolics but additionally are involved in the formation of 2,6-dimethoxy-*p*-benzoquinone (DMBQ), other phenolic compounds and in coupling reactions, which can include radical recombination, cross-coupling and self-coupling proceeding with or without release of substituent groups.

(SA < AS < MS). The positive effect of electron-withdrawn substituents in the rates of enzymatic oxidation suggest that these improve the stabilisation of the phenolate anions. The results indicate that TvL oxidises the three substrates in the phenolic form (maximal rates at pH 4) while CotA oxidises them optimally in the phenolate form (maximal rates at 8-9), i.e. after deprotonation of the phenolic group. The action of these phenolics were tested as "natural" laccase mediators (present in lignocellulose, cheap and environment-friendly avoiding the latent toxicity and high cost of synthetic mediators (Camarero et al. 2005; Canas and Camarero 2010; Fillat et al. 2010)) for the oxidation of the non-phenolics, veratraldehyde, 4-methoxybenzyl alcohol and 3,4,5-trimethoxybenzyl alcohol. Interestingly, the mediator activity of the syringyl-type phenolic reflected a balance between reactivity and stability of the radicals formed as the

measured conversion yields of non-phenolics was higher for MS > AS > SA, showing that the stability of radicals is improved by the presence of electron donor groups at the para-position. Moreover, maximal conversion yields (up to 80%) were achieved at large excess of mediators (10:1) implying an interplay of competitive routes (**Fig. 5**). Phenoxy radicals intermediates are involved in (i) the oxidation of the non-phenolic monomers, forming hydroxybenzyl radicals that are converted to the correspondent aldehydes, (ii) the conversion to 2,6-dimethoxy-*p*-benzoquinone and, finally (iii) coupling reactions including radical recombination, cross-coupling and self-coupling, with or without release of substituent groups. This catalytic cycle points out the need to control the reaction conditions in order to guide the depolymerisation/re-polymerisation balance of lignin samples (Rosado et al. 2012).

Although laccases can play a major role in lignin depolymerization, the achievement of a correct balance between the depolymerisation and re-polymerisation processes is though as the great challenge (Roth and Spiess 2015; Hamalainen et al. 2018). This balance depends on several reaction conditions including the substrate structures, the difference between the redox potentials of enzyme and substrate, pH of reaction, temperature and solvent. Recent advances highlighted the potential of bacterial laccases as a promising tool to tackle the lignin depolymerisation/re-polymerisation bottleneck. MetZyme® LIGNO^{TM}, a laccase of bacterial origin, was successfully used for lignin depolymerisation at 50°C at pH 10.5 where lignin is soluble in water (Hamalainen et al. 2018). After the enzymatic treatment, extensive lignin depolymerisation was observed accompanied by chemical activation *via* demethylation and benzylic oxidation as well as increased solubility in neutral and acidic pH. Importantly, organic solvent-free soluble lignin fractions with defined molecular weights were obtained using membrane separation technologies with increased dispersibility and solubility in water. The reaction mechanism proposed for the laccase-mediated oxidation of lignin at alkaline pH is a benzylic oxidation at the Cα position of the lignin units (**Fig. 6**). This modification is expected to lead to changes in the electronic nature of the substituent leading to a drop in the *pKa* of the phenolic group from 9-11 to 7-8. For the phenolic units, this change is responsible for increased lignin solubility at neutral to acidic pH.
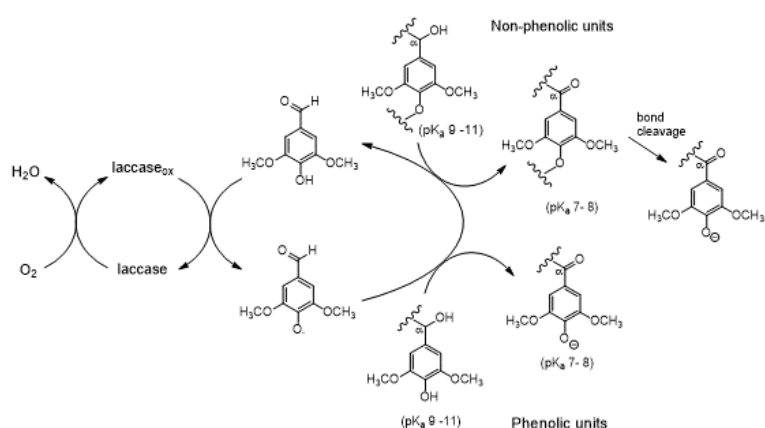

Figure 6. Scheme of phenolic and non-phenolic lignin units oxidation using a MetZyme® LIGNO^{TM} at alkaline conditions (pH 10.5) (adapted from (Hamalainen et al. 2018)).

For the non-phenolic units, the electron withdrawing nature of the *para*-substituent group promote depolymerisation or demethylation reactions in the lignin polymer. Therefore the authors demonstrate that depolymerisation of lignin prevailed over polymerisation pathways at strong alkaline and aeration conditions (Hamalainen et al. 2018). This enzymatic-based solution

open up new opportunities for lignin valorisation crucial to make economically feasible present and future lignocellulose biorefineries.

Oxidation of aromatic amines using bacterial laccases. Substituted aromatic amines including The bacterial CotA laccase oxidises at the neutral to alkaline range of pH values a wide range of aromatic amines with different substitution patterns (*ortho*-phenyldiamines, substituted *para*-diphenylamines, *ortho*-amino-phenols, among other) that are precursors of dimeric and trimeric dyes (Sousa et al. 2013; Sousa et al. 2016; Sousa et al. 2019) as well as of substituted heterocyclic frameworks (phenazine, phenoxazinone, carbazole derivatives) (Sousa et al. 2014; Sousa et al. 2015; Sousa et al. 2018). Phenazine and phenoxazine cores are multifunctional and versatile building blocks widely distributed in a vast array of biologically active compounds, such as anti-tumour agents, (Nakaike et al. 1992; Bolognese et al. 2002; Corona et al. 2009) antibiotics and antibacterial agents, (McDonald et al. 1999; Zeis et al. 1987; Borrero et al. 2014) agrochemicals, (Starke et al. 2004) biosensors (Pauliukaite et al. 2010) and dyes and polymers. (Dailey et al. 2001; Yamamoto et al. 1996; Yamamoto et al. 2003) Due to the importance and broad field of applications of the aforementioned molecules, the development of new enzymatic, greener methodologies, is crucial as alternative (bio)synthetic routes for the formation of these aromatic frameworks.

Homocoupling reactions of *para*-substituted aromatic amines using CotA-laccase result in trimers with diaminated quinone-diimine structures (**Fig 7**) (Sousa et al. 2013) at very good to excellent overall conversion yields. Additionally, the involvement of some of these amines as primary bases in cross-coupling reactions with different couplers mediated by CotA-laccase (Sousa et al. 2016) resulted in the production of indo dyes, widely used in the permanent hair and leather coloration. (Corbett 1999; Morel and Christie 2011) The substrate´s reactivity is dependent on the nature and position of the substituents in the aniline ring (Sousa et al. 2013; Sousa et al. 2016) and in general, laccase´s activity is enhanced by the presence of electron-donating groups. Additionally, the efficiency of the CotA-laccase enzymatic system is strictly dependent on the difference of the redox potential between the enzyme (0.55 V) (Durao et al. 2008a) and the substrates. The pH of reaction is a paramount parameter since it affects both the catalytic activity of laccase and the redox potentials of the substrates i.e. their susceptibility for oxidation is a critical operational parameter (Sousa et al. 2015). In the pathway proposed for the oxidation of substituted aromatic amines (**Fig 7),** the initial step is the abstraction of an electron from aromatic amines, followed by deprotonation of the primary intermediate (oxidation base) and formation of two short-lived intermediates (an aminium cation radical ($A^{·+}$), a neutral radical species ($A^{·}$), the aminyl radical) or the formation of the benzoquinonediimine intermediate (**A**) and the corresponding conjugated acid (AH^+). Therefore, starting from the intermediate species, by sequential self-conjugation (**path a**) or cross-coupling with different couplers (**paths b and c**), the reaction proceeds through N-C coupling in the ring activated positions. Path **a**) forms a homomolecular dimeric structure which is subsequently transformed in the final 1,4-substituted-2,5-benzoquinonediimine trimer, while paths **b**) and **c**) yield dinuclear leuco dyes (**B**) and result in the final indoaniline or aminoindamine dyes (**C**) or the trinuclear indo dyes (**D**). According to the proposed pathway the stability of the radicals, enhanced by the presence of electron donor substituents, seems to be of major importance for the laccase catalytic efficiency (Sousa et al. 2013; Sousa et al. 2016).
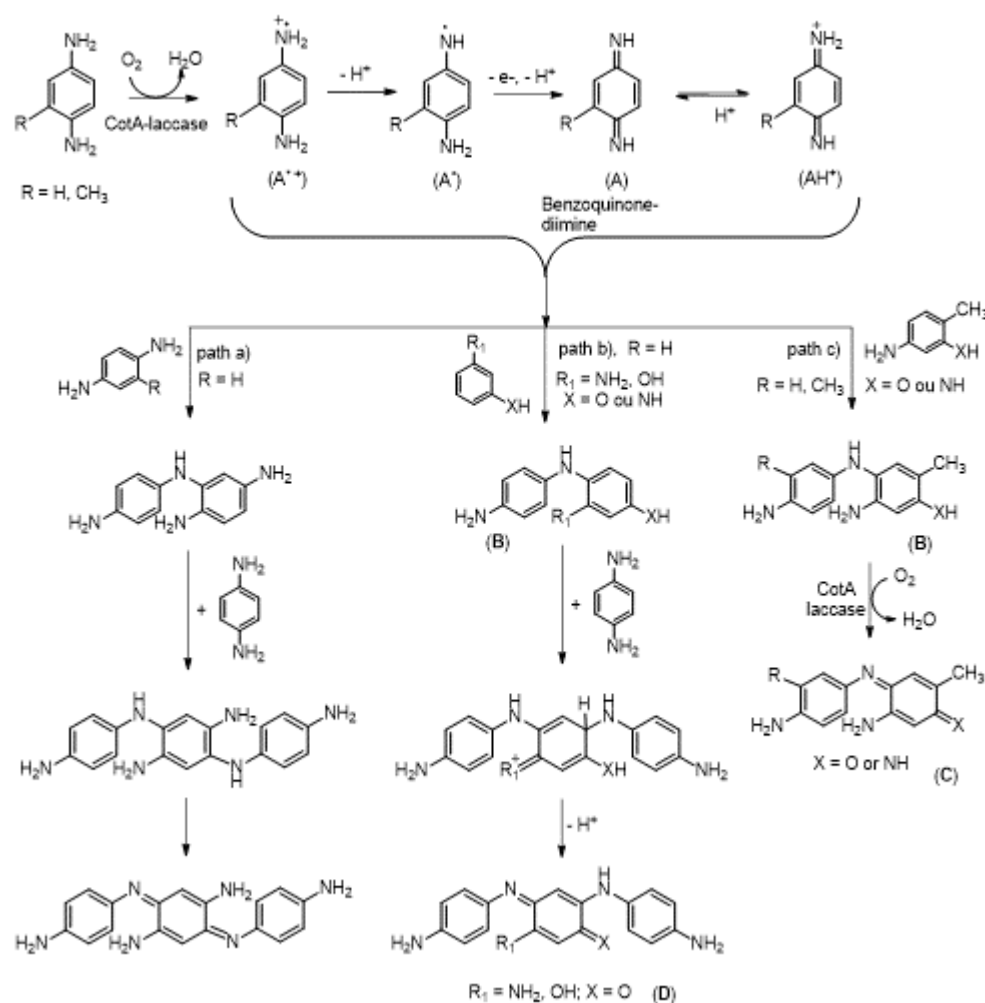
Figure 7. Proposed pathways for CotA-laccase mediated homocoupling reactions involved in the formation of 1,4-substituted-2,5-benzoquinonediimine trimers and heterocoupling reactions involving the primary intermediates 1,4–PDA or 2,5-DAT and *meta-* or *meta,para*-substituted couplers (adapted from (Sousa et al. 2013; Sousa et al. 2016)).

The formation of different heterocyclic scaffolds, *e.g.* symmetric and asymmetric phenazines, phenoxazinones and carbazoles by oxidation of structurally different aromatic substrates assisted by CotA-laccase was also reported (Sousa et al. 2014; Sousa et al. 2015; Sousa et al. 2018) (see **Fig. 8**). The efficiency of the reactions leading to the formation of heterocoupled dinuclear or trinuclear dyes was found strongly dependent on the presence of electron donor groups on the *meta*-substituted couplers. A mechanistic pathway for the synthesis of substituted symmetric and asymmetric heterocycles with phenazine and phenoxazine frameworks was proposed (**Fig. 8**) where an *ortho*-diamine or *ortho*-aminophenol is enzymatically oxidized through two successive one-electron oxidations, generating *ortho*-quinone-diimine or *ortho*-quinone-imine intermediates (**A**). Under the reaction conditions, these species suffer rapid nucleophilic addition by other substrate molecules in its most electrophilic carbon atom, followed by a proton shift, yielding the first coupling intermediate (**B**). The second 2e⁻ oxidation is enzymatic and an intramolecular Michael addition of an amino group (or phenol) to the C5 atom, with the displacement of a R group, leads to an aminophenazine or fully reduced aminophenoxazine, which are spontaneously oxidised in air to produce the final heterocycle products. (Sousa et al. 2014; Bruyneel et al. 2012) For the *meta,para*-disubstituted aromatic amines, the first step is the

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in-situ generation of an *para*-benzoquinonediimine intermediate (**A'**) in a similar way as described above. This intermediate further reacts with the nucleophilic amino group of another molecule at the *ortho*-position, adjacent to the R_1 group leading to the formation of dimeric structures. This second step, followed by a proton loss, yields the first coupling intermediate (**B**). This non-isolable product underwent a subsequent oxidation, probably mediated by laccase, followed by an intramolecular Michael addition to form the symmetric substituted phenazines.
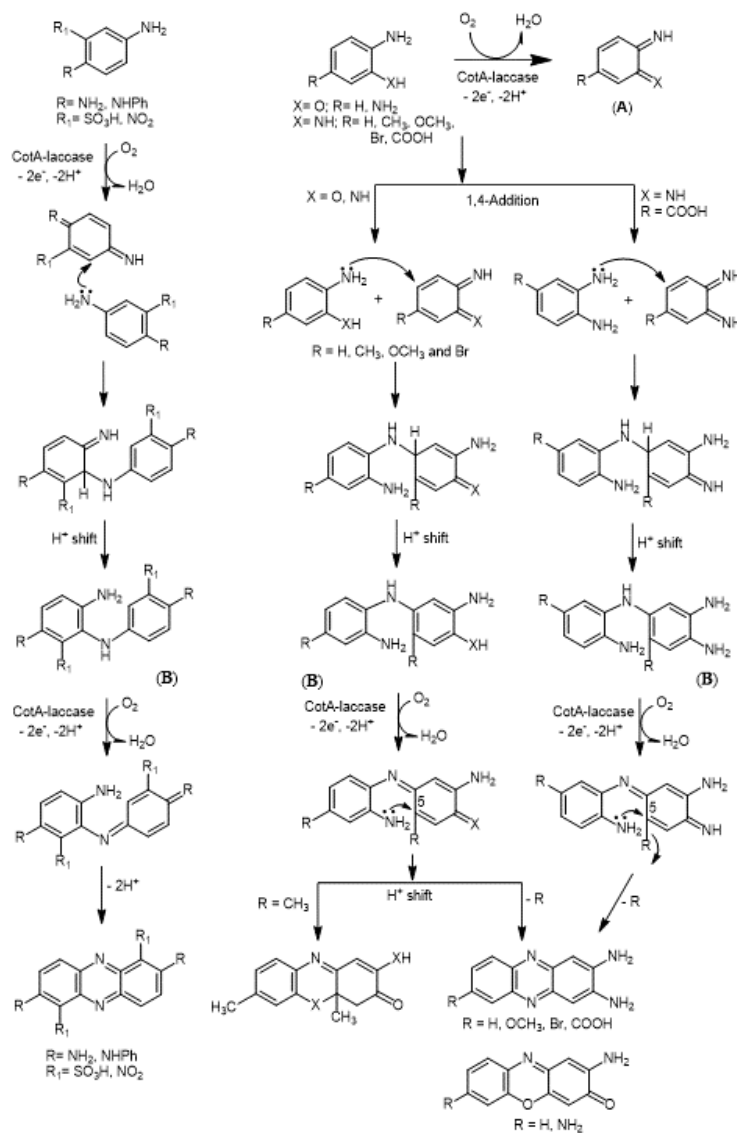

Figure 8. Proposed pathways involved in the formation of symmetric and asymmetric phenazines and phenoxazines from substituted aromatic amines by CotA-laccase (adapted from (Sousa et al. 2014, 2018)).

The (bio)synthesis of azo dyes biotransformation was also reported as minoritary products of laccase´s aromatic amines oxidation (Sousa et al. 2013; Sousa et al. 2014, 2018) and as a product of degradation of the antraquinonic Acid Blue 62 dye (Pereira et al. 2009a; Enaud et al. 2010). Recently, the enzymatic oxidation of aromatic amines was directed to the formation of azo dyes, by the choice of appropriate substrates and the use of ABTS as mediator and several *ortho* and *para*-substituted azo dyes were formed and the correspondent mechanistic pathway proposed (Sousa et al, 2019). The variety of different aromatic scaffolds obtained by this enzymatic

approach clearly show that laccases are promising tools for aromatic amines oxidation, boosting new eco-friendly alternatives to the production of value-added aromatic compounds.

Acknowledgments

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