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Review

Fungal laccases

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ABSTRACT

Laccases are enzymes widely distributed in plants, fungi, bacteria, and insects. They are multicopper oxidases that catalyze the transformation of aromatic and non-aromatic compounds with reduction of molecular oxygen to water. These enzymes participate in processes such as biosynthesis and lignin degradation, morphogenesis, and pigment biosynthesis, among others. In this review we discuss relevant aspects of fungal laccases regarding the existence of fungal laccases gene families, the growing interest in investigating mechanisms of their molecular regulation, and factors that influence the production of laccases, due to their potential biotechnological applications. In addition we comparatively analyzed some structural similarities and differences depicting general features of laccases' active site, demonstrating their frequency as monomeric proteins with highly conserved cupredoxine type domains. Although inter- and intra-specific differences have been determined, structural differences encountered between fungal laccases remain unclear based on Crystallography and X-ray diffraction.

1. Introduction

Yoshida (1883) was the first to describe laccase in latex obtained from the tree *Rhus vernicifera*. A decade later, the enzyme was isolated and purified by Bertrand, who reported its mechanism of action (Bertrand, 1894; Yoshida, 1883). This type of activity was attributed exclusively to higher plants and fungi. However, it is now recognized that laccases are almost

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ubiquitous enzymes, since they have been isolated from plants, fungi (*Ascomycetes*, *Basidiomycetes* and *Deuteromycetes*), prokaryotes, and arthropods (Giardina *et al.*, 2010).

Laccases have been isolated from insects, where their main function is during sclerotization processing for epidermal cuticle synthesis (Nakamura and Goa, 2005; Sakurai and Kataoka, 2007). In bacteria laccases have several functions such as morphogenesis processes, copper homeostasis, pigment biosynthesis (such as melanin and brown spore pigment), and spore protection against UV light and hydrogen peroxide (Santhanam *et al.*, 2011; Strong and Claus, 2011). Furthermore, fungal laccases are involved in sporulation, pigment production, fruit body formation, and plant pathogenesis (Alcalde, 2007). The white rot *Basidiomycetes* are known for its efficient lignin, cellulose, and hemicellulose decomposition and transformation into carbon dioxide (Baldrian, 2006). Consequently, *Basidiomycetes* are a widely studied fungus.

At present, more than 100 laccases from *Basidiomycetes* and *Ascomycetes* fungi have been purified and characterized. Laccase purification from plant crude extracts is complex, and for this reason it has not been studied extensively (Strong and Claus, 2011).

Laccases (EC 1.10.3.2), also named p-diphenol: dioxide oxidoreductases are blue multicopper oxidases (MCOs) that have the ability to catalyze the oxidation of a wide variety of organic aromatic compounds, concomitantly with the reduction of molecular oxygen to water (Ruiz-Dueñas and Martínez, 2009; Sakurai and Kataoka, 2007). Although most laccase substrates are phenolic compounds (ortho and para-diphenols, methoxy-substituted phenols, polyphenols, aromatic amines, benzenethiols, hydroxindols, 1-naphthol, syringaldazine) enzyme activity can be extended to non-phenolic compounds by use of mediators like ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) and HBT (1-hydroxybenzotriazole) (Solís-Oba *et al.*, 2005).

Addition of mediators extend the use of laccases for industrial processes related to bioremediation; including delignification of lignocellulosics, color removal and detoxification of industrial dyes, bioremediation of xenobiotic compounds, pesticides, explosives, wastewater treatment, and treatment of other pollutants such as polycyclic aromatic hydrocarbons (PAHs), (Desai and Nityanand, 2011). Other substrates that can also be reduced by laccases include inorganic/organic metal compounds, such as Mn^{2+} and Fe $(EDTA)^{2-}$ (Thurston, 1994).

Oxidases and oxidoreductases are classified in more than 200 types, but only six classes (cytochrome-c oxidase, laccases, L-ascorbate oxidase, ceruloplasmin, bilirubin oxidase, and phenoxazinone synthase) have the ability to catalyze this type of oxygen reaction (Nakamura and Goa, 2005). It is difficult to categorize laccases based on the substrate reduced, due to the wide range of compounds that can be catabolized. Laccases have the property to change their redox potential (E^0), thus substrate type can change from one laccase to another (Giardina *et al.*, 2010; Strong and Claus, 2011).

General enzymology of laccases has been discussed in numerous reviews and several of them describe the broad range of biotechnological applications (Desai and Nityanand, 2011; Kunamneni *et al.*, 2008a, 2008b; Majeau *et al.*, 2010; Rodríguez Couto and Toca Herrera, 2006; Shraddha *et al.*, 2011; Singh Arora and Kumar Sharma, 2010; Widsten and Kandelbauer, 2008). In particular fungal laccases use has increased in recent years for certain biotechnological processes, since they do not use hydrogen peroxide in their catalytic process. In addition, they present high stability, and can be used in an immobilized form (Loera Corral *et al.*, 2006).

This review offers an overview of what is known about fungal laccase's molecular characteristics, as well as some details of the structural diversity of emzyme's crystallography, their heterologous expression and biotechnological applications.

2. Main reactions of laccases

During laccase's catalytic process, different free radical reactions result, depending on structure and reaction conditions. The most frequent reactions are coupling of free radicals that generate dimeric products or polymeric compounds and oxidative carboxylations. The oxidation of substrates is coupled to reduction of molecular oxygen; generating two water molecules. For each oxygen reduced, four molecules of substrate are oxidized without hydrogen peroxide production: $4H^+ + 4\ \text{substrate} + O_2 \rightarrow 2H_2O + 4\ \text{substrate}^+$ (Solomon *et al.*, 2008). Consequently, laccases are considered "ideal green" catalysts because they employ O_2 as a co-substrate and generate H_2O as a byproduct (Fig 1).

3. Characteristics of laccases

One of the fundamental characteristics of these enzymes is the direct relation of their redox potential (E^0) with the energy required to remove an electron from the reducer substrate. In fact, the catalytic behavior of laccases on most reducer substrates depends on the electron acceptor: E^0 Cu T1 (Wong, 2009; Xu *et al.*, 1998). Thus, laccases with greater E^0 T1 display a special interest for biotechnology, due to their greater potential to oxidize substrates with greater E^0. This is the case for

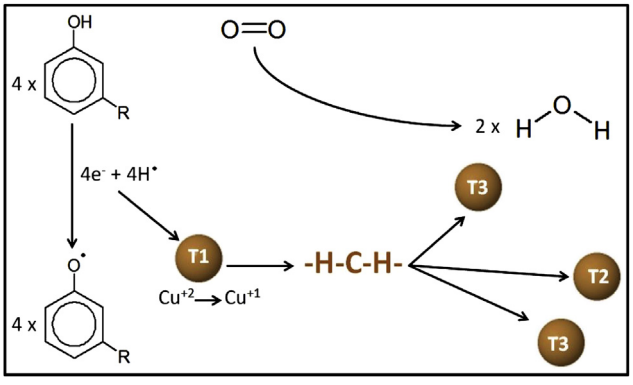

Fig 1 – Laccase catalytic cycle. Substrates are oxidized by the Cu-T1 center and electrons are transferred by a highly conserved motif: His-Cys-His (HCH) to the T2 and T3 copper centers. This is where reduction of molecular oxygen to water takes place. Figure modified from (Baldrian, 2006). In the scheme, copper atoms appear in brown, and were based on in the 1GYC structure (laccase-2 of *Trametes versicolor*).

certain polycyclic aromatic hydrocarbons or various organic synthetic dyes. The E^0 of several laccases has been studied utilizing spectrum-electrochemical techniques, mainly cyclic voltammetry and redox valuations (Kosman, 2010; Piontek *et al.*, 2002; Zhukhlistova *et al.*, 2008). The E^0 Cu T1 of some fungal laccases is greater (E^0 T1 near +800 mV) than isoenzymes from plant or bacteria (for example the laccase of *R. vernicifera* has an E^0 T1 = +400 mV), and other blue multicopper oxidases (as ascorbate oxidase, EC 1.10.3.3, have an E^0 T1 = +340 mV), (Alcalde, 2007; Rodgers *et al.*, 2010). Moreover, it is important to note that there are marked differences in the E^0 T1 of different fungal laccases. For example, E^0 T1 values can range from +465 mV in Ascomycete *Myceliophthora thermophila* to +790 mV in Basidiomycete *Pycnoporus cinnabarinus* (Kumar *et al.*, 2003; Wong, 2009). Therefore, researchers have attempted to elucidate the factors associated with E^0 Cu T1 differences; in particular because there is a high conservation degree in the Cu sites coordination geometries (Piontek *et al.*, 2002).

Factors affecting metalloprotein redox potential are varied and of great complexity. Many aspects such as solvation, metal–ligand interactions, intramolecular electrostatic interactions, and/or protein folding restrictions (governing the position and orientation of the ligands) can modulate the E^0 values of these enzymes (Li *et al.*, 2004).

4. Laccase gene families

Fungal laccases characterization studies, agree with gene multiplicity and numerous isoenzyme production in different species. Examples of fungal laccase gene families include the description of two different laccases (codified by *lcc*1 and *lcc*2 genes) in *Trametes villosa* (Yaver *et al.*, 1996) and five (*lcc*1-5) in *Trametes sanguinea* (Hoshida *et al.*, 2001), four (*lcc*1-4) in *Rhizoctonia solani* (Wahleithner *et al.*, 1996), three (*lcc*1-3) in *Trametes* sp. I62 (Mansur *et al.*, 1997), three (*lcc*A-C) in *Trametes* sp. AH28-2 (Xiao *et al.*, 2006), and two (*LAC1-2*) in *Gaeumannomyces graminis* (Litvintseva and Henson, 2002). Furthermore, laccase gene families have also been described in Pleurotus, with four genes (*lac*1-4) isolated from *Pleurotus sajor-caju* (Soden *et al.*, 2002), two (*pel*3-4) from *Pleurotus eryngii* (Rodríguez *et al.*, 2008), and six (*poxa*1a, *poxa*1b, *poxC*, *poxa*2, *poxa*3a and *poxa*3b) from *Pleurotus ostreatus* (Palmieri *et al.*, 2003).

In recent years study of laccase gene expression regulatory mechanisms has gained interest due to the need to understand the physiological role of different isoforms produced in distinct organisms. Furthermore, this knowledge can be translated into an improvement for industrial processes. Synthesis and secretion of laccases are highly influenced by nutrient levels (N/C ratio), culture conditions, fungal developmental state, as well as the addition of inducing agents to culture medium (Piscitelli *et al.*, 2011). Influencing factors act synergistically or antagonistically on laccase's expression, and include metal ion regulation (Collins and Dobson, 1997; Galhaup *et al.*, 2002), lignin or derivates related aromatic compounds (Terrón *et al.*, 2004), nitrogen (Collins and Dobson, 1997) and carbon sources (Soden and Dobson, 2001). Although, laccase's gene regulatory mechanisms have not been studied extensively, few reports suggest a complex system of *cis* regulatory elements (Piscitelli *et al.*, 2011). Currently, the general mechanisms for the transcriptional regulation of the laccases are not clear and the presence of multigenic families for this type of enzyme production raises the question: why does a fungus require more than one laccase enzyme?

Some authors argue the presence of numerous genes generating variants of the same enzyme; a physiological function diversity developed by laccases during their life cycle (Giardina *et al.*, 2010; Kües and Rühl, 2011). Evidence obtained from phylogenetic trees obtained by Neighbor-joining in *Basidiomycete's* laccases supports this argument by the presence of functional clusters (Hoegger *et al.*, 2006; Kües and Rühl, 2011).

The picture of laccase gene family has become even more complicated since the recent release of *P. ostreatus* genome (http://genome.jgi-psf.org), whose analysis disclosed the existence of new laccase genes, putatively coding for previously uncharacterized laccases (*lacc*3, *lacc*5, *lacc*7 and *lacc*8), thus enriching this panel up to 11 members in this organism and strengthening existing questions about the physiological role played by different laccase isoforms present in *Ascomycete* and *Basidiomycete* fungus.

5. Factors involved in laccase production

Although a large number of studies report intracellular laccases, most are extracellular glycoproteins (Kunamneni *et al.*, 2007), and their production usually occurs during the secondary metabolism of different fungi, and influenced by different aspects, such as fungal species, culture type (stationary or agitated), aeration and culture time (Brijwani *et al.*, 2010; Elisashvili and Kachlishvili, 2009; Kunamneni *et al.*, 2007). Furthermore, among the most critical factors are carbon, nitrogen, and inducer agent concentration (Majeau *et al.*, 2010). These requirements are considered limiting for fungal laccase large-scale production. Low enzyme yield can be obtained when fungi are grown on wood or in submerged culture (Piscitelli *et al.*, 2010). Microbial metabolism may be "controlled" by environmental conditions and culture medium composition. Multiple DNA regulatory sites, such as Metal Responsive Elements (MREs), Xenobiotic Responsive Elements (XREs) and Heat Shock Elements (HSEs) located 400 bp upstream of laccase gene promoter regions can be activated by certain xenobiotic compounds, heavy metals or thermal shock; influencing laccase's metabolism (Faraco *et al.*, 2002; Monteiro and De Carvalh, 1998).

High biomass culture concentration is not directly proportional to an increase in laccase production or laccase activity (Kunamneni *et al.*, 2007). Contrary, higher concentrations can be obtained by the addition of aromatic compounds such as 2,5-xylidine; one of the most efficient inducers (Kunamneni *et al.*, 2007; Tong *et al.*, 2007). It has been reported for *P. cinnabarinus* and *Trametes versicolor* that after 24 h of culture, addition of 2,5-xylidine at a concentration ranging from 0.01 mM to 1.25 mM produces a maximum laccase activity, compared to culture without an inducing agent (from 10^3 U L^{-1} to 9 × 10^3 U L^{-1}), (Eggert *et al.*, 1996; Kollmann *et al.*, 2005). Increase in laccase production is the result of a similarity of the inducer with the molecular structure of lignin

and other chemical agents of phenolic nature (Loera Corral et al., 2006). However, when these inducers are added in concentrations higher than 1.65 mM, a contrary effect might be observed with reduction of laccase activity (Eggert et al., 1996). Another important inducer for laccase production is copper. It has been shown that the addition of low copper concentrations to culture media stimulates laccases production in white rot fungi (Neifar et al., 2009; Shutova et al., 2008). Palmieri et al. (2000) found that the addition of 150 μM of copper sulfate, increased laccase activity from 6×10^2 to 30×10^3 U L^{-1} compared to control culture.

Although many investigations have focused on assaying different inducing agents to increase laccase production, combined induction has not been extensively explored. Few studies have been conducted that combine multiple inducers to evaluate laccase production and activity changes. Tong et al. (2007) induced laccase production in *Trametes* sp., 420, adding 0.5 mM Cu^{+2} and 6 mM o-toluidine. In this study laccase activity values ranged approximately between 68.1×10^2 U L^{-1} and 78.8×10^2 U L^{-1}. Tinoco et al. (2001) incorporated copper and lignin simultaneously and observed an increase in growth and volumetric activity of laccase from 10^3 up to 12×10^3 U L^{-1}; these results open new paths towards more efficient processes in the production of laccases from white rot fungi.

Regarding carbon source influence, it has been observed that rapidly degraded substrates such as glucose, mannitol and cellobiose, usually produce high laccase activities in comparison to other substrates that are degraded more slowly, such as cellulose or lactose (Mikiashvili et al., 2006). In some cases an increment in laccase activity occurs with a concomitant increase in fungal growth. Some studies have shown that specific activities of laccases can augment according to carbon source selection. For example, Mansur et al. (1998) reported a laccase specific activity increase when substituting fructose for glucose. In addition, Rodríguez Couto and Toca Herrera (2006) demonstrated in *Trametes hirsuta* that sequential addition of different carbon sources, such as glucose followed by glycerol, resulted in a higher laccase production rate, compared to cultures supplemented only with glucose or cellulose. For many species, glucose is a typical repressor of laccases production. An optimal concentration of inducer agents and carbon sources depends on fungal species and strain. This suggests that carbon source or inducer agent should not be assumed based on prior published experiments and the existence of different mechanisms for laccase gene regulation.

In regard to the influence of nitrogen, the majority of authors report laccase production by nitrogen source exhaustion (Majeau et al., 2010). Still others have found that for some strains nitrogen does not have an effect on enzyme yield and activity (Kunamneni et al., 2007). Furthermore, some authors have reported early laccase production in nitrogen rich culture media, compared to limited nitrogen source cultures (Elisashvili et al., 2008a, 2008b). These data display certain ambiguity regarding the selection of nitrogen's optimum concentration for laccase production. Some studies report high laccase activity with low carbon-nitrogen ratio (Hou et al., 2004). In contrast, other authors have reported higher laccase productions with high carbon-nitrogen ratio (Dong et al., 2005).

Additionally, metal contribution to regulation of laccase's expression is widespread in fungi. For *T. versicolor* 290, the highest laccase activity value ($\sim 23 \times 10^2$ U L^{-1}) was achieved in cultures grown in the presence of 400 μM Cu^{2+}, corresponding to an ~18-fold increase compared to cultures without copper (~ 128 U L^{-1}), (Collins and Dobson, 1997). Even in *Pleurotus* spp., the induction of laccase by copper addition has been widely documented. Giardina et al. (1999) obtained a laccase production of 30 U L^{-1} growing *P. ostreatus* (ATCC MYA-2306) in nutrient-rich medium with addition of 150 μM $CuSO_4$, whereas laccase production in the presence of copper traces resulted in values between 0.5 and 4 U L^{-1}. This regulation of laccase expression by copper has been demonstrated at the transcriptional level (Piscitelli et al., 2011).

Ions, including Ag^{+}, and Mn^{+2} have also been reported as modulators of laccase transcription (Manubens et al., 2007; Soden and Dobson, 2001). It is worth noting that the same metal can exert opposite effects in different fungal species (Manubens et al., 2007). Even though, Mn^{+2} can act as transcription inducer of laccase in *P. sajor-caju* (Soden and Dobson, 2001), *Clitocybula dusenii* and *Nematoloma frowardii* (Scheel et al., 2000), it has been shown to inhibit laccase expression in *Ceriporiopsis subvermispora* (Manubens et al., 2007).

Recently, Pezzella et al. (2013) performed the transcriptional analysis by quantitative RT-PCR of *P. ostreatus* laccase genes in different grown conditions and in presence of different inducers. In their work, they concluded that few laccase genes are strongly expressed in response to tested inducers, and some others were specifically unregulated during fructification. Moreover, most of the analyzed genes were poorly expressed in the majority of the tested samples, suggesting either limited involvement under the physiological conditions tested, or other as yet undefined functions. Finally, they proposed that it cannot be overruled that for some members of such a numerous laccase gene family redundant gene copies, produced less active or low redox potential laccases, not preferentially produced by the fungus.

6. Structural diversity of fungal laccases (bioinformatic analysis)

Usually laccases are monomeric extracellular glycoproteins with variable molecular weight, ranging between 50 and 140 kDa, with a great diversity both in size (10–45 % of the total weight) and glycosylation patterns (Claus, 2004). Most fungal laccases contain a total of 520–550 amino acids, not including the signaling peptide sequence (~20 residues) (Thurston, 1994). Fig 2, displays the typical folding in a *Basidiomycete*'s laccase. The three cupredoxin-like domains (a characteristic folding in blue multicopper oxidases and other small copper proteins such as plastocianine or bacterial azurine) (Nakamura and Go, 2005), organized in a sequential manner (domains 1, 2, and 3 is depicted in green, yellow, and red, respectively). Each one of them, have a β-barrel topology; common to all members of the MCOs family (Giardina et al., 2010). Copper type 1 (Cu T1) is located in domain 3, while the trinuclear center is integrated between domains 1 and 3; both domains provide residues for copper coordination. The structure is stabilized by two disulfide bonds; the first bond located between domains one and three and the other one between domains one and two (Zhukova et al., 2010).

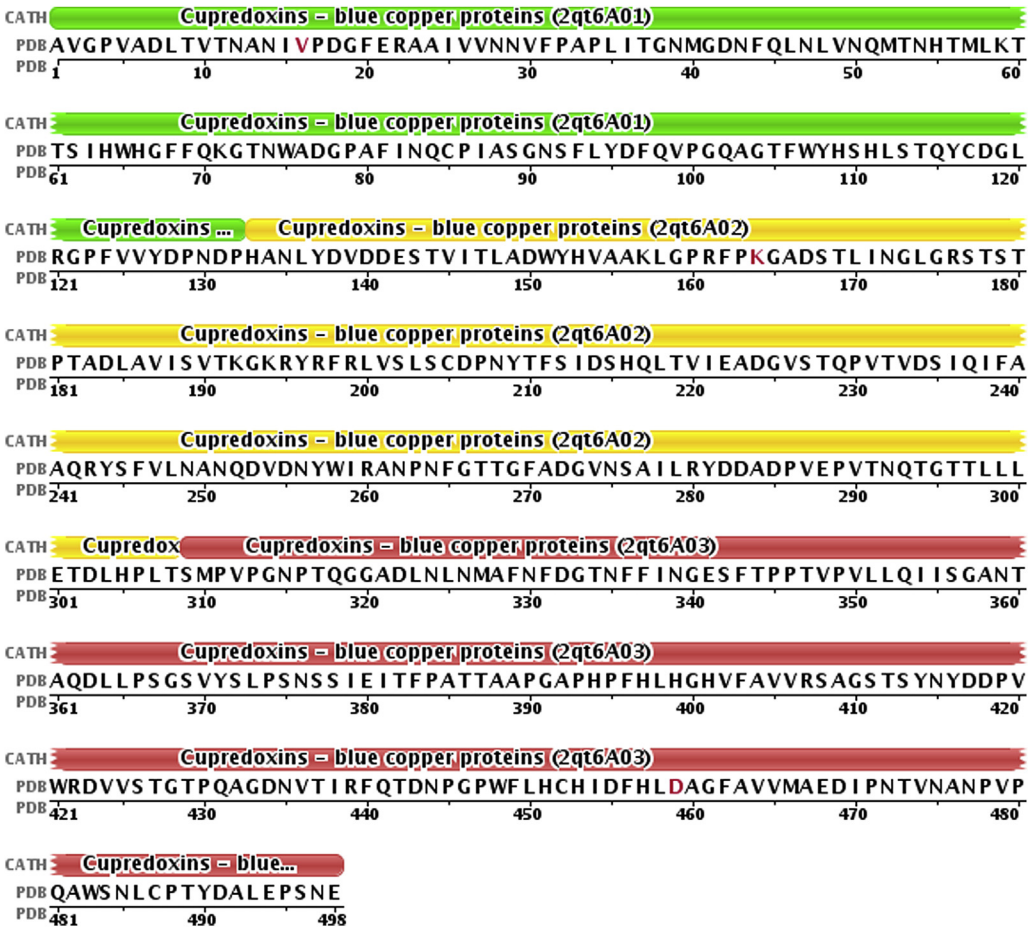

Fig 2 – Laccase's structure cupredoxin-like domains. The sequence shown corresponds to the 2QT6 laccase structure in *Lentinus tigrinus*. Domain one is represented in green, yellow for domain two, and red for domain three. The sequences used to generate the figure were retrieved from the Protein Data Bank (www.pdb.org) and Classification of Protein Structure Database CATH (www.cathdb.info).

Different multiple alignment studies, over 100 laccase sequences, have revealed their tertiary structure. Their redox sites and copper coordination are highly conserved (Kumar *et al.*, 2003; Valderrama *et al.*, 2003). The eight ligands of His in the trinuclear cluster T2/T3 show a highly conserved pattern with four His-X-His motifs. The X motif is a Cys that binds Cu T1, while the adjacent His bind each a Cu T3 site. At 35–75 amino acid distance there is another His-X-His motif, and close to the amino terminal there are two other motifs, separated by 35–60 amino acid residues (Solomon *et al.*, 1996). Twelve amino acids serve as ligands for four Cu atoms, and are located within four segments of separate sequences with an approximate length of 8 and 24 residues (L1–L4). This feature distinguishes laccase from other blue multicopper oxidases. Furthermore, laccases have an intra-protein homology between L1 and L3, as well as L2 and L4. This suggests that in laccase's evolution a duplication event occurred. The formidable active site conservation in different copper oxidases suggests that the activity related to the three different Cu sites was a very early evolutionary event (Zumárraga *et al.*, 2007). In order to show the highly conserved 3D structure, we conducted an alignment with 14 out of 38 sequences of fungal laccase structures from *Basidiomycetes* and *Ascomycetes* (Table 1). All sequences were obtained from Protein Data Bank (PDB) with previously reported crystallography structures. Alignment data does not include duplicate sequences, sequences with induced mutations, and sequences with no available citation (unpublished manuscript), (Fig 3).

Since the first report conducted in fungal laccase (Ducros *et al.*, 1998), only few crystallography laccase structures have been determined. Up to now, 66 structures of laccases can be found in PDB. Thirty-eight are of fungal origin, 26 of them come from 12 species of *Basidiomycetes*, *Coprinopsis cinerea*, *Cerrena maxima*, *Coriolopsis gallica*, *Coriolus zonatus*, *Lentinus tigrinus*, *P. cinnabarinus*, *Rigidoporus lignosus*, *Steccherinum ochraceum*, *T. hirsuta*, *Trametes* sp., *Trametes trogii* and *T. versicolor*, and the remaining 12 come from 3 species of *Ascomycetes*, *Botrytis aclada*, *Melanocarpus albomyces* and *Thielavia arenaria*.

Several crystallography resolutions ranging from 1.20 Å to 3.34 Å have been used to determinate laccase 3D structures (Andberg *et al.*, 2009; Bertrand *et al.*, 2002; De la Mora *et al.*, 2012; Ducros *et al.*, 1998, 2001; Ferraroni *et al.*, 2012; Ferraroni *et al.*, 2007; Garavaglia *et al.*, 2004; Ge *et al.*, 2010; Hakulinen

Table 1 – Laccase structures in *Basidiomycetes* and *Ascomycetes* fungi. These structures were reported in Protein Data Bank (PDB) obtained by Crystallography and X-ray diffraction, resolution units are Å, weights are in Da and lengths are in amino acid (AA). Proteins with two or more chains are homooligomeric

Fungi	Organism	Molecule	PDB	Resolution	Length	Chains	Structure weight	References
Basidiomycetes	*Coprinopsis cinerea*	Laccase	1A65	2.23	504	A	55080.08	(Ducros *et al.*, 1998)
		Laccase-1	1HFU	1.68	503		55367.24	(Ducros *et al.*, 2001)
	Trametes versicolor	Laccase	1KYA	2.40	499	A–B–C–D	221719.74	(Bertrand *et al.*, 2002)
		Laccase-2	1GYC	1.90		A	55989.82	(Piontek *et al.*, 2002)
	Rigidoporus lignosus	Laccase	1V10	1.70	521		55858.68	(Garavaglia *et al.*, 2004)
	Cerrena maxima		2H5U	1.90	499		55670.18	(Lyashenko *et al.*, 2006a; Lyashenko *et al.*, 2006b)
	Coriolus zonatus		2HZH	2.60			54122.88	(Lyashenko *et al.*, 2006c)
	Trametes trogii		2HRH		496		54247.49	*Not available*
			2HRG	1.58			55104.45	
	Lentinus tigrinus		2QT6	1.50	498	A–B	112749.96	(Ferraroni *et al.*, 2007)
	Trametes hirsuta		3FPX	1.80	499	A	56465.33	(Polyakov *et al.*, 2009)
	Cerrena maxima		3DIV	1.76			56220.02	(Zhukova *et al.*, 2010)
	Trametes sp. AH28-2	Laccase B	3KW7	3.44	502	A–B	110887.03	(Ge *et al.*, 2010)
	Trametes hirsuta	Laccase	3PXL	1.20	499	A	56354.35	*Not available*
	Coriolopsis gallica		4A2H	2.30	496		54464.58	(De la Mora *et al.*, 2012)
			4A2E	1.80			53906.09	
			4A2D	2.30			54383.02	
	Pycnoporus cinnabarinus		2XYB	1.75	497		57999.61	*Not available*
	Trametes hirsuta		3V9C	2.00	499		56402.35	
	Steccherinum ochraceum		3T6W	2.15	495	A–B–C	163303.82	(Ferraroni *et al.*, 2012)
			3T6V	2.00			163207.82	
			3T6X	2.15			163303.82	
			3T6Z				162875.51	
			3T71				163207.82	
	Coriolopsis gallica	Laccase	4A2G	1.80	496	A	53779.00	(De la Mora *et al.*, 2012)
			4A2F	1.90			53927.65	
Ascomycetes	*Melanocarpus albomyces*	Laccase-1	1GW0	2.40	559	A–B	132161.81	(Hakulinen *et al.*, 2002)
			2IH9	2.00			129934.94	
			2IH8				130864.68	
			2Q9O	1.30			131889.56	(Hakulinen *et al.*, 2008)
			3DKH	2.40			130716.04	(Andberg *et al.*, 2009)
			3FU9	2.00			128050.20	(Kallio *et al.*, 2009)
			3FU8	1.80			130976.90	
			3FU7	1.67			131164.01	
			3QPK	1.90			129328.47	(Kallio *et al.*, 2011b)
	Thielavia arenaria	Laccase	3PPS	2.50	604	A–B–C–D	276674.96	(Kallio *et al.*, 2011a)
	Botrytis aclada		3SQR	1.67	580	A	66902.22	*Not available*
			3V9E	1.70			66429.93	

et al., 2008, 2002, 2006; Kallio *et al.*, 2009; Kallio *et al.*, 2011a; Kallio *et al.*, 2011b; Lyashenko *et al.*, 2006a, 2006b, 2006c; Piontek *et al.*, 2002; Polyakov *et al.*, 2009; Zhukova *et al.*, 2010), (Table 1) and several differences have been observed as consequence of the natural differences in molecules, protein extraction conditions, crystallography techniques resolution or depending of X-rays doses.

For example, 12 different crystallographic structures of laccases have been obtained from *Ascomycetes*. Two correspond to laccase from *B. aclada*, nine to laccase-1 from *M. albomyces*, and only one corresponds to a laccase from *T. arenaria* (Kallio *et al.*, 2011a). They accomplished structure 3PPS with a 2.5Å resolution. For this structure, 3PPS Asn is the amino acid residue responsible for catalytic proton transfer, in contrast to Gln in laccase-1 from *M. albomyces*. In addition, the loops in the vicinity of copper type 1 site forming the pocket where the substrate binds, also differs to some extent (Kallio *et al.*, 2011a).

Ferraroni *et al.* (2007) obtained structure 2QT6 from a laccase in *L. tigrinus;* solved at 1.5 Å of resolution. This structure revealed an asymmetric unit (quaternary structure) containing two molecules of laccase A and B.

De la Mora *et al.* (2012), reported five different structures for the same laccase obtained from *C. gallica*: 4A2D, solved at 2.0 Å resolution; 4A2E, solved at 1.8 Å resolution; 4A2F, solved at 1.9 Å resolution; 4A2G, solved at 1.8 Å resolution, and 4A2H, solved at 2.3 Å resolution. Their differences rely on the fact they were extracted at different pH (4.5, 5.5 and 7.0 respectively) during fungi culture process. Structural changes were caused by crystallography resolution variations and radiation-induced reduction and radiolysis.

On the other hand, the crystal structure of a blue laccase from *S. ochraceum* has been solved at 2.0 Å resolution, using classic data acquisition from a single crystal (3T6V). The overall structural features are typical of this class of enzymes; however, distances inside the trinuclear copper cluster are indicative of a reduction of the metal centers induced by free electrons produced during X-ray data collection. Besides, for this work, authors obtained four additional structures with

PDB	L1			L2			L3			L4		
	*	****			*****			** ***** *			* **** *	
2H5U	60	TSVHWHGFF	69	105	FWYHSHLST	114	388	GAPHPFHLHGHTFA	402	448	WFLHCHIDFHLEG	461
3DIV	60	TSVHWHGFF	69	105	FWYHSHLST	114	388	GAPHPFHLHGHTFA	402	448	WFLHCHIDFHLEG	461
3FPX	60	TSIHWHGFF	69	105	FWYHSHLST	114	388	GAPHPFHLHGHTFA	402	448	WFLHCHIDFHLEG	461
1KYA	60	TSIHWHGFF	69	105	FWYHSHLST	114	388	GAPHPFHLHGHAFA	402	448	WFLHCHIDFHLEA	461
2HZH	60	TSVHWHGFF	69	105	FWYHSHLST	114	388	GGPHPFHLHGHAFA	402	448	WFLHCHIDFHLEA	461
1GYC	60	TSIHWHGFF	69	105	FWYHSHLST	114	388	GAPHPFHLHGHAFA	402	448	WFLHCHIDFHLEA	461
2QT6	60	TSIHWHGFF	69	105	FWYHSHLST	114	387	GAPHPFHLHGHVFA	401	447	WFLHCHIDFHLDA	460
4A2D	60	TSIHWHGFF	69	105	FWYHSHLST	114	387	GFPHPFHLHGHVFA	401	445	WFLHCHIDFHLEA	458
3KW7	60	TTIHWHGLF	69	105	YWYHSHLST	114	394	GAPHPFHLHGHAFS	408	451	WFLHCHIDFHLEA	464
3T6V	61	TSIHWHGEF	70	106	YWYHSHLTT	115	393	GGPHPFHLHGHDFA	407	448	WFLHCHIDWHLDA	461
1V10	81	TSIHWHGFF	90	126	FWYHSHLST	135	416	---HPFHLHGHNFD	427	468	WFLHCHIDWHLEA	481
1A65	60	TSIHWHGLF	69	105	FWYHSHFGT	114	392	GGPHPFHLHGHAFS	406	447	WFFHCHIEFHLMN	460
1GW0	89	TSIHWHGIH	98	134	SWYHSHFSA	143	427	SLPHPMHLHGHDFL	441	498	WLFHCHIAWHVSG	511
3PPS	130	TSMHWHGLR	139	175	SWYHSHFSA	184	468	SLPHPMHLHGHDFL	482	539	WLFHCHIAWHVSG	552

Fig 3 – Laccase ClustalW2 alignment (Larkin *et al.*, 2007). All sequences had previously determined crystallography structures. Identical positions are marked with an asterisk and joining sites between ligands (amino acids) with copper atoms indicated in black. L1–L4 represents four conserved regions of fungal laccases. The numbers refer to the amino acid sequence. The dashes represent gaps in the alignment.

different X-rays doses (3T6W, 3T6X, 3T6Z, and 3T71; all of them solved at 2.15 Å resolution). Observations made at progressively increased X-rays doses are consistent with reduction of copper centers which allows the binding of the oxygen molecule; the dioxygen is successively reduced to peroxide, and thus an additional reduction is observed resulting in its splitting in two oxide/hydroxide ions (Ferraroni *et al.*, 2012).

Structural information obtained from crystallography data is essential to understand the protein's general properties, in particular the catalytic site. To date the number of laccase structures obtained by crystallography represents only a small fraction when compared to the vast variety of laccase isoforms reported. The scarcity of data can be explained by difficulties, mainly related to isoform separation during laccase's crystallization process (Lyashenko *et al.*, 2006a, 2006c; Piontek *et al.*, 2002). These difficulties result in data from structural conformation that might not be consistent with reality. Even though, it is important to highlight the efforts addressed to understand laccase's structures and, despite the fact the rest of the molecule is variable among different laccases, a high degree of conservation is evident in domains related to copper. Resulting in what some authors have called "signature sequence" (Alcalde, 2007), and have characterized laccases in a unique way.

7. Mechanisms of action and biological activity

Studies carried out by spectroscopy and density functional methods (DTF) to clarify the coordination nature of the trinuclear copper center in laccases, revealed that T1 exhibits a planar triangular coordination with the sulfur atom of a cysteine and with the Nδ1 nitrogen of two histidines. The three T2/T3 ions are arranged in a triangular fashion and coordinated to a strongly conserved pattern of four His-X-His motifs. Six of such histidine residues coordinates the T3 copper pair, whereas the T2 copper is coordinated by the remaining two histidine residues (Giardina *et al.*, 2010; Solomon *et al.*, 2008), (Fig 4).

Three main steps are involved in the mechanism of laccase action. Initially, Cu type 1 is reduced by the action of a reducer substrate, which is subsequently oxidized. Then the electron is internally transferred from Cu T1 over >13 Å through a Cys–His pathway to the trinuclear center, formed by the atoms of coppers T2 and T3. The oxygen molecule binds to the trinuclear center for an asymmetric activation through a substrate binding site located near the His ligands of Cu T1 center. It has been proposed that the joining pocket for O_2 seems to restrict the access of oxidizing agents different than O_2. During the steady state of the process H_2O_2 is not detected outside the enzyme, indicating that reduction of four electrons from the O_2 to H_2O is occurring (Desai and Nityanand, 2011; Gianfreda *et al.*, 1999; Kunamneni *et al.*, 2008a, 2008b). Since oxidation of an electron in the phenolic substrate is linked to reduction of four oxygen electrons, it cannot be assumed the reaction's mechanism is simple. Therefore, it is assumed that laccase acts as a battery, storing electrons from individual oxidation reactions in order to reduce molecular oxygen. Therefore, oxidation of four substrate molecules is required to produce complete reduction of molecular oxygen to water (Desai and Nityanand, 2011; Solomon *et al.*, 2008).

In general terms, laccase substrate oxidation is a one-electron reaction generating a free radical. The initial product is typically unstable and may undergo a second oxidation by enzyme catalysis or from a non-enzymatic reaction as a hydration, polymerization or disproportionation (Kunamneni *et al.*, 2007). Natural substrate bonds, lignin, which can be separated, include oxidation of Cα-bonds, court of Cα–Cβ bonds, and court aryl–alkyl bonds (Kunamneni *et al.*, 2007; Martínez *et al.*, 2005), (Fig 5).

8. Laccases and chemical mediators: mimicking nature

Lignin is a structural component of plant cell walls. It is a complex and amorphous polymer of aromatic nature, which comprises approximately 20–32% of the dry weight of wood (Ralph *et al.*, 2007). Monomers forming lignin are p-coumaril,

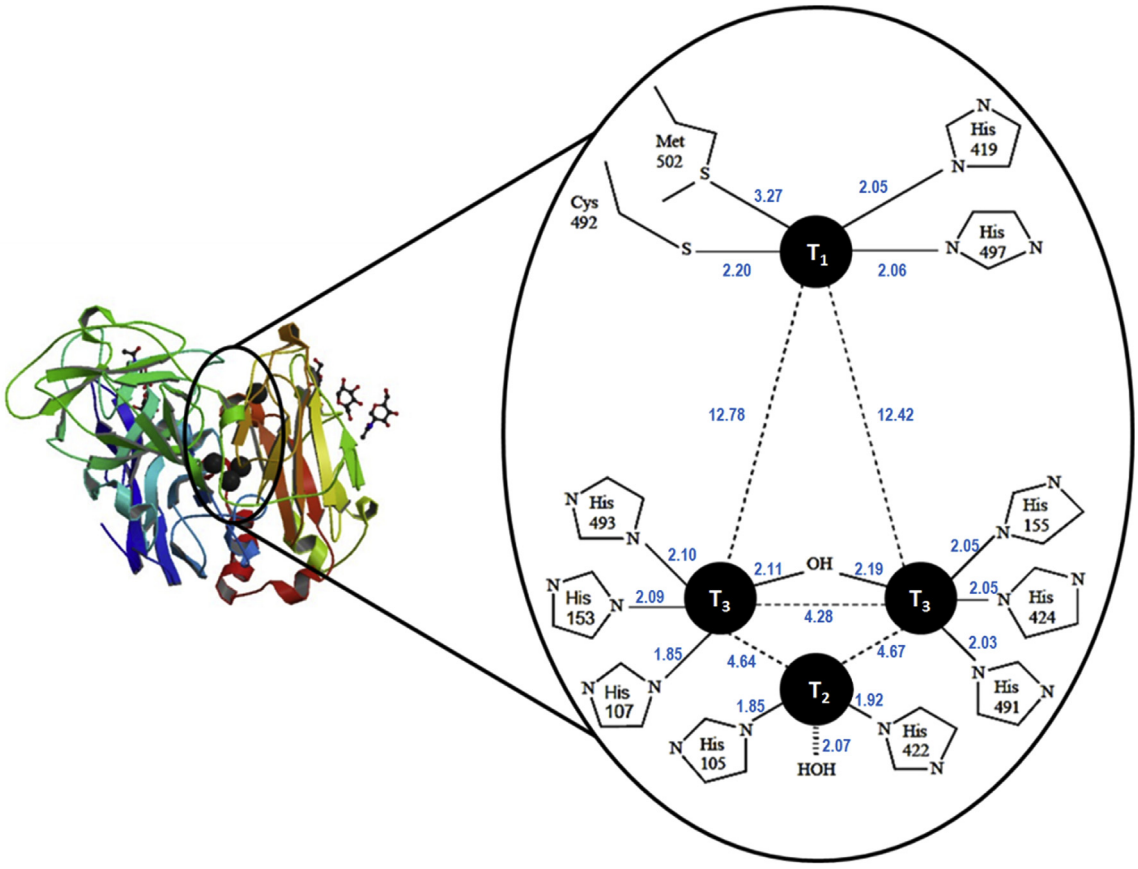

Fig 4 – Laccase active site. Illustration depicting the orientation regarding copper atoms and ligand distance (blue, distances in Å). The three-dimensional structure used for laccase 2HZH in *Coriolus zonatus* obtained from Protein Data Bank (PDB). With slight modifications from (Dwivedi *et al.*, 2011; Enguita *et al.*, 2003).

conyferil, and sinapyl alcohols; they differ from each other by the methoxylation degree. These monomers produce p-hydroxyphenyl, guaiacyl, and syringyl phenylpropanoid units, which are capable of generating electron delocalized radicals that couple at various sites (Ralph *et al.*, 2007; Widsten and Kandelbauer, 2008).

While in plant wood-tissues laccases are part of the lignin synthesizing system, the role of laccases in white rot fungi is to depolymerize and mineralize lignin. Laccase is a large molecule (Rodgers *et al.*, 2010) that cannot penetrate deep into wood and because it has a low redox potential (≤0.8 V) compared to ligninolytic peroxidases (>1 V), laccase can only oxidize lignin phenolic fragments. However, the number and type of substrates oxidized by laccase can be extended by a mechanism involving the participation of redox mediators. These mediators are low molecular weight compounds that

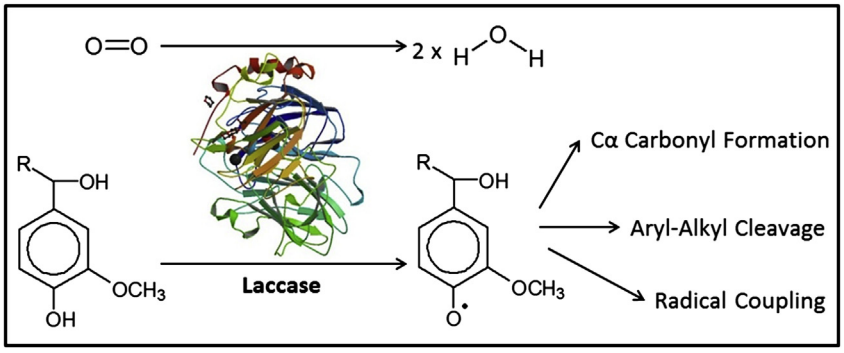

Fig 5 – Oxidation of lignin phenolic subunits by a laccase. The oxidation of the substrate is a reaction, which produces a free radical which is unstable and may form carbonyl Cα, aryl–alkyl breaking or radicals docking. Modified from (Kunamneni *et al.*, 2007). The structure of laccase used was obtained from *Coprinopsis cinerea* (1A65).

can easily be oxidized by laccase, producing very reactive and unstable cationic radicals. However, at the same time these cationic radicals can oxidize complex compounds (not including phenolic substrates) before returning to their original state (Torres *et al.*, 2003). By this mechanism mediators act as diffusible electron transporters, allowing indirect oxidation of polymeric substrates such as lignin, penetrating even to less accessible areas of its structure. Additionally, because of mediator use, laccases are able to oxidize compounds with greater redox potential than their own; an example of this is the oxidation mediated by polycyclic aromatic hydrocarbons or PAHs (Riva, 2006).

Since Bourbonnais demonstrated that mediator inclusion expanded laccase's catalytic activity toward non-phenolic substrates (Bourbonnais and Paice, 1990), more than 100 different mediators have been described with ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) and the more commonly used HBT (1-hydroxybenzotriazole) (Solís-Oba *et al.*, 2005). This laccase-mediator system can be applied to pulp and paper bleaching industry (Bourbonnais *et al.*, 1997; Call and Mucke, 1997), as well as bioremediation of xenobiotic-compounds such as PAHs (Alcalde *et al.*, 2002; Bourbonnais *et al.*, 1997; Call and Mucke, 1997; Collins *et al.*, 1996; Johannes *et al.*, 1996; Majcherczyk and Johannes, 2000; Majcherczyk *et al.*, 1998). In addition, it has been established that the combination of two or more mediators (e.g., ABTS and HBT) can generate a synergistic effect on oxidative activity (Pickard *et al.*, 1999). However, the elevated chemical mediator commercial costs, their high toxicity, and lack of studies on derivative effects, in addition to inactivation caused by their cationic radical exertion on laccases, makes the laccase-mediator system implementation still limited.

For these reasons, employment of natural mediators could have environmental and economic advantages. Many compounds involved in natural degradation of lignin can act as mediators in an effective manner. Such is the case for lignin degradation compounds derived from oxidized lignin units, or those secreted by white rot fungi. In a similar way the natural mediators 4-hydroxybenzoic acid, 4-hydroxybenzilic alcohol, veratrilic alcohol, syringaldehyde, acetosyringone, vanillin, and p-coumaric acid, among others, have been tested with different laccases with similar results to those obtained with artificial mediators. A clear example is the recently PAHs described degradation of benzo[a]pyrene, pyrene and anthracene using a laccase-mediator system from *P. cinnabarinus* and p-coumaric acid (Cañas and Camarero, 2010).

9. Atypical laccases

Usually laccases contain three domains in their structure (Fig 2), and a molecular mass of approximately 50–70 kDa or greater. Only some multicopper oxidases contain two domains in their structure and a molecular mass of approximately 30–40 kDa (Nakamura and Goa, 2005). However, it is not common to find laccases with molecular weights below 50 kDa. Only few reports describe laccases with low molecular weight from *Botrytis cinerea* (Pezet, 1998) and the fruit bodies of *Tricholoma giganteum* (Wang and Ng, 2004). Due to their small molecular mass, laccases with two domains may require to be assembled in order to acquire a quaternary structure. Data obtained by some authors suggest that these enzymes may also act as monomers (Giardina *et al.*, 2010).

Some laccases that exhibit a homodimeric structure have been isolated. Such is the case for those obtained from Basidiomycetes *T. villosa* (Yaver *et al.*, 1996), *Phellinus ribis* (Min *et al.*, 2001), phytopathogenic Ascomycetes, *R. solani* (Wahleithner *et al.*, 1996), *G. graminis* (Edens *et al.*, 1999), and for the aquatic Ascomycete *Phoma* sp. UHH 5-1-03. It is known that some of these enzymes present a dimerization depending on pH, as found in *Phoma* sp. UHH 5-1-03, where the predominant dimeric state occurred at a pH range between 5.0 and 8.0 (Junghanns *et al.*, 2009). Dimer formation is necessary to ensure proper enzyme functioning, as observed in homodimeric laccases with two subunit domains isolated from *Pleurotus pulmonarius* (De Souza and Peralta, 2003), *P. eryngii* (Wang and Ng, 2006), and the mycorrhizal fungus *Cantharellus cibarius* (Ng and Wang, 2004).

As previously mentioned laccases contain four copper atoms and present an absorption wavelength peak close to 600 nm. However, some laccases have unusual spectral properties, since they do not present absorption spectrum characteristic of Cu T1. These enzymes are referred to as "yellow" or "white" laccases, and some authors do not recognize them as true laccases. Others argue that considering laccases are enzymes capable of oxidize polyphenols, methoxy-substituted phenols, aromatic diamines, and a wide range of other compounds not including tyrosine, "yellow" and "white" laccases should be acknowledged as real laccases (Giardina *et al.*, 2010).

10. Recombinant laccases and heterologous production

Obtaining laccases from native sources does not provide sufficient yield to meet industrial processes requirements. Furthermore, high cultivation and purification increase costs. For this reason, heterologous laccase expression has become a promising alternative. With readily available commercial hosts that are simple to manipulate genetically and cultivate; productivity can become more efficient, reducing production costs (Piscitelli *et al.*, 2010).

Until now results obtained from laccase's heterologous production are promising, however many obstacles remain to be solved. Among the difficulties encountered are high expression levels in combination with an optimal biological activity. Such is the case for heterologous fungal laccase production, where *Ascomycetes* are considered friendlier for genetic manipulations, and industrial scaled-up processes. However, the much-desired laccase high redox potential is primarily obtained when expressed in *Basidiomycetes*; which are more difficult to manipulate genetically (Table 2), (Piscitelli *et al.*, 2010; Rodgers *et al.*, 2010; Salony *et al.*, 2008).

The ability to produce laccases in heterologous systems in an efficient manner depends largely on changes made to the original DNA sequence. For example, an increase in secretion of recombinant laccases has been obtained replacing the native signaling peptide by signal sequences present in proteins with a high degree of secretion by the host (Salony *et al.*, 2008). Another strategy employed includes inducing

Table 2 – Laccases heterologously expressed. List of some fungal laccases heterologously expressed in yeasts and filamentous fungi

Laccase	Origin	Host	Laccase activity (~U L^{-1})	References
LCC1	*Trametes versicolor*	*Pichia pastoris*	14×10^4	(Hong *et al.*, 2002)
			50×10^2	(Jönsson *et al.*, 1997)
			25	(O'Callaghan *et al.*, 2002)
LCC2		*Saccharomyces cerevisiae*	12×10^{-2}	(Larsson *et al.*, 2001)
LCCI		*Pichia pastoris*	39	(Gelo-Pujic *et al.*, 1999)
LCCIV			150	(Brown *et al.*, 2002)
LCC1		*Pichia methanolica*	98×10^2	(Guo *et al.*, 2006)
LACIIIb		*Yarrowia lipolytica*	230	(Jolivalt *et al.*, 2005)
LCCα		*Saccharomyces cerevisiae*	35×10^{-3}	(Necochea *et al.*, 2005)
LCC1,		*Pichia pastoris*	2.8	(Bohlin *et al.*, 2006)
LCC2		*Aspergillus niger*	27×10^2	
Gene IV		*Aspergillus niger*	592	(Téllez-Jurado *et al.*, 2006)
LAC	*Schizophyllum commune*	*Aspergillus sojae*	77×10^4	(Hatamoto *et al.*, 1999)
LAC1	*Pycnoporus cinnabarinus*	*Aspergillus niger*	70×10^5	(Record *et al.*, 2002)
POXA3	*Pleurotus ostreatus*	*Kluyveromyces lactis*	80	(Faraco *et al.*, 2008)
LCC	*Trametes trogii*	*Pichia pastoris*	25×10^2	(Colao *et al.*, 2006)
LCC1		*Kluyveromyces lactis*	6.6	(Camattari *et al.*, 2007)
LACB	*Trametes* sp.	*Pichia pastoris*	32×10^6	(Li *et al.*, 2007)
LACD	*Trametes* sp. 420	*Pichia pastoris*	83×10^3	(Hong *et al.*, 2007)
Pel3	*Pleurotus eryngii*	*Saccharomyces cerevisiae*	14×10^7	(Bleve *et al.*, 2008)

random mutagenesis and/or recombination to modify the protein's characteristics (Collins *et al.*, 1996; Majcherczyk *et al.*, 1998).

Results regarding the production of heterologous laccases imply that "the best host" or the "more promising" laccase still remains unknown. Currently, as a result of great advances in molecular biology and recombinant DNA technology, it is possible to explore new strategies aimed at producing laccases to industrial levels. Starting with synthetic genes coding for laccases to modify nucleotide sequences specifically to improve enzyme expression; or even attain new features representing an advantage for certain industrial processes.

Subsequently, based on our experience in *Pichia pastoris* heterologous proteins expression (Córdoba-Ruiz *et al.*, 2009; Landázuri *et al.*, 2009; Poutou-Piñales *et al.*, 2010), we proposed to implement the expression of synthetic and nucleotide-optimized laccase genes from *P. ostreatus* and *Ganoderma lucidum*. With the final objective to employ recombinant laccases for the degradation process of cellulose pulp and in the medium-term; we anticipate broadening our results to other types of industries, such as textile, for dye removal and pollutants from effluents.

11. Biotechnological applications

Laccases are of great interest to industry, and have been used in many processes such as delignification of lignocellulosic compounds, biopulping and biobleaching, transformation of colorants in the textile industry, wastewater treatment and degradation of explosives and pesticides (Rodríguez Couto and Toca Herrera, 2006; Shraddha *et al.*, 2011; Singh Arora and Kumar Sharma, 2010).

Laccases from white rot *Basidiomycetes* possess a great biotechnological potential because of the characteristics previously mentioned in this review. Laccases with high redox potential can be employed in almost the entire paper product production chain: paper pulp development, pulp chlorine-free washing, or for effluent treatment. Two other emerging areas of research for forest product industry are: (i) Design of lignocellulosic materials with new resistance and stability properties by means of phenolic compound grafts catalyzed by laccase, in the so-called "functionalization of cellulose fibers". (ii) Laccase use for improving compression degree in wood-based panels (through "*in situ*" enzyme lignin coupling), without the use of toxic adhesives containing formaldehyde.

Laccase's application in the textile industry range from cotton fiber washing, textile dye and bleaching as well as precursor of coloring matter production to elimination of the former in waste-waters (Fernández *et al.*, 2009). In fact, laccase from *M. thermophila* has been recently used for indigo dye oxidation in denim cloth (Riva, 2006). Furthermore, in nanobiotechnology, laccases have the necessary properties to function as phenol detectors.

In addition, they can be used in the development of biosensors for clinical and environmental analysis of oxygen, azides, morphine, codeine, catecholamines or flavonoids. For the pharmaceutical industry antitumor agents, benefiting from laccase's properties can develop new derivates for antibiotic use and cosmetics. Laccases can also be employed in the advancement of biofuel for clean electrical energy (without fossil fuel use) through laccase immobilization in the cathode. Lastly, the chemical industry can profit from laccases in chemical synthesis in the production of complex polymers (polycatechol for chromatography resins), (Kunamneni *et al.*, 2008a, 2008b; Shraddha *et al.*, 2011).

One of the most common applications of laccases is in delignification. Bourbonnais *et al.* (1995) demonstrated that laccase from *T. versicolor* was capable of paper pulp delignification in the presence of mediators, without the need of traditional chlorinated toxic reagent techniques. Many studies have been developed to optimize the entire process (Alcalde

et al., 2002; Bertrand *et al.*, 2002; Ferraroni *et al.*, 2012), and their utility in the degradation of lignocellulosic waste in general (Sarria-Alfonso *et al.*, 2013).

Enzymatic bioremediation through laccases is another area of great interest. For example, laccase can be utilized for bioremediation in compounds such as PAHs, chlorophenols, dimethoxyphenols, nitrophenols, and pesticides, askarels, among others (Alcalde *et al.*, 2002; Gayosso-Canales *et al.*, 2012; Hakulinen *et al.*, 2008). It is of common knowledge that pollution load in waste-water resulting from the washing process during paper production, is characterized by high levels of chemical oxygen demand (COD), color, and more than 500 different absorbable organic halide compounds (AOX), (Savant *et al.*, 2006).

Our research, to reduce pollutant load taking advantage of laccase's redox potential from *Basidiomycetes* has used an innovative strategy. The sequential use of *T. versicolor* fungus, followed by photocatalysis with UV/TiO_2/Ru_xSe_y, reduced bacterial and fungal populations by five logarithmic units with respect to control without treatment in industrial wastewater from paper pulping (Pedroza *et al.*, 2007). In addition, global treatment, resulted in 92% color removal (5800 CU), COD (59 g L^{-1}) reduction, and pentachlorophenol elimination of 99% after 96 h of treatment. On the other hand, with *T. versicolor*-UV/TiO_2 a 98% color removal of Reactive Black 5 (RB5) was achieved in 96 h of treatment (Henao-Jaramillo *et al.*, 2011).

Degradation of PAHs is considered of particular interest, since polycyclic aromatic hydrocarbons are a group of very dangerous xenobiotics (mutagenic, carcinogenic and/or teratogenic) widely distributed in terrestrial and aquatic environments. The main emission sources come from marine dumping, vehicle engines, industrial processes and forest fires. Recent trends for PAHs elimination aim to combine chemical and biological methods, such as shock treatment for oxidation (Kojima *et al.*, 1990; Riva, 2006). Thus, a greater effort is being made to design effective PAHs oxidation by laccases. These procedures require demanding process conditions, such as the presence of organic solvents and extreme environmental conditions, amongst others (Collins *et al.*, 1996; Johannes *et al.*, 1996; Kudangaa *et al.*, 2011).

Laccase's enzymatic activity coupled to physical transducers can be useful in biosensor design to detect O_2 and a wide variety of substrate reducers as phenols, anilines and glucose. In addition, indirect activity of other enzymes (for example, amylases, aminopeptidases, alkaline phosphatase, cellobiose oxidase, chymotrypsin or glucosidase) can be determined (Mayera and Staples, 2002; Minussi *et al.*, 2002; Xu, 2005). Finally, since laccases are related to an exclusive group of enzymes able to accept electrons, directly from an electrode (Call and Mucke, 1997), and can catalyze the reduction of oxygen to water, a promising laccase application is aimed at biofuel design and cleaning-up of certain explosives in soil, such as trinitrotoluene (TNT), (Shraddha *et al.*, 2011).

12. Conclusions

Laccases are part of the broad blue multicopper oxidases (MCOs) and have the ability to catalyze the oxidation of a great variety of organic aromatic compounds concomitantly with the reduction of molecular oxygen to water. This catalytic behavior depends on the E^0 of Cu T1, which is greater in some fungal laccases compared to those from plants or bacteria. Despite the fact that several factors such as solvation degree, metal–ligand interactions, intramolecular electrostatic interactions and/or restrictions of protein folding affect laccase's redox potential; the geometry for Cu site coordination is highly conserved. It is important to emphasize the multiplicity of genes and the existence of a complex system of regulatory *cis* elements, which promote the production of several isoforms; whose expression depends on nutrient levels, growing conditions, state of fungal development, and presence of inducing agents. The existence of numerous genes that produce isoforms is a consequence of the diversity in physiological functions performed by laccases during the fungal life cycle (delignification, development of the fruitful body, pathogenesis, and pigment formation during asexual development, and interactions of competition). Nonetheless, laccase's transcriptional regulation is not fully understood. 3-D structures of 38 fungal laccases have been determined, 26 from *Basidiomycetes*, and 12 from *Ascomycetes*. These structures have been obtained by Crystallography and X-ray diffraction with different resolutions. It is not clear whether the resolution has an effect on the structural differences encountered. Yet, it is clear that there are inter-specific differences, among laccases from different genera and species, and intra-specific differences, among laccases of the same fungus.

Although some laccases obtained by non-recombinant systems are successfully used at the industrial level, not all of them combine the attributes needed to promote production processes (stability and activity in broad ranges of temperature and pH, high redox potential and halide/hydroxide tolerance). Thus, the development of robust systems for heterologous expression could offer numerous benefits in comparison to direct enzyme purification from its native producer. It is clear that several aspects still require further studies. Forthcoming investigations might shed some light on gene expression regulation mechanisms, influence of glycosylation patterns on biological activity, and system designed for heterologous expression that meet industrial scale requirements, among others.

In conclusion, laccases are enzymes known for a long time with a great future. They remain relevant as a structure/function relationship model. In addition, they are promising as "green tools" in industrial processes, thus making laccase's characterization and more relevant knowledge.

Declaration of interest

Authors declare that there are no conflicts of interests in this work.

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