In Silico Generation of Laccase Mutants from Lacc 6 of *Pleurotus ostreatus* and Bacterial Enzymes

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In this research, 63 complete amino acid sequences of laccase enzymes from fungi (40), bacteria (5), insects (8), and plants (10) were used for a phylogenetic analysis. A common ancestor that diverged in the laccases in prokaryote and eukaryote was observed. Additionally, it was determined that there was a difference in the type of amino acids bound to the histidines linked to the copper atoms of the active site, and that a fungal laccase of approximately 279 Ma was an ancestor of the laccases in the basidiomycetes considered in this study. In contrast, Lacc 6 of *Pleurotus ostreatus* was used as a template to generate mutants. Through modeling, the changes in the secondary and tertiary structures of this enzyme were observed with the substitution of amino acids adjacent to histidines in the conserved region of the active site, *via* the presence of amino acids in a similar place in the laccases of bacteria.

Keywords: Laccase; Phylogenetic analysis; Mutants; Pleurotus ostreatus

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INTRODUCTION

The laccases (EC 1.10.3.2) belong to the Multi-Copper Oxidase (MCO) family of enzymes that are produced by different organisms and have gained great importance since the twentieth century in different industries such as paper, textile, and cosmetics to name a few. The enzymes have great potential in degradation applications of toxic compounds and xenobiotics, such as polyphenols and aromatic and non-aromatic amines. The laccases are glycoproteins that catalyze the oxidation of phenolic compounds through the transfer of an electron with the final reduction of oxygen to water (Claus 2004). Laccases have a molecular weight range between 25 to 80 kDa (Díaz *et al*. 2013), the isoelectric point (pI) of these enzymes is between 2.5 to 6.5, the optimal pH values are between 2.0 and 7.0 (Kirk-Kent and Cullen 1998), and the optimal glycosylation level is between 10% and 25% (Mayer and Staples 2002; Morosova *et al*. 2007). The first study reported on this type of enzyme was from the Japanese lacquer tree *Rhus vernicifera* (Yoshida 1883), and a few years later, the existence of laccases in fungi was reported (Bertrand 1896; Laborde 1896). This enzyme type has been found and studied in bacteria, fungi, plants, and insects. More than 100 different proteins have been identified (Shuresh-Kumar *et al*. 2003).

Laccase's physiological function in plants is in the lignification processes; while in fungi, its function is in the morphogenesis process (formation of spores, pigments of fruiting bodies), pathogenesis, virulence, and the degradation of lignin. Likewise, it has also been reported that in some insects the analyzed enzyme acts in the formation of

cuticles in the process of sclerotization (Kramer *et al*. 2001), and in bacteria it has the function of monomer cross-linking, polymer degradation, and the breakdown of aromatic rings (Sharma *et al*. 2007). In contrast, bioinformatic studies of these enzymes have been performed, analyzing the alignment between amino acid sequences in different bacterial laccases as reported by Sharma *et al*. (2007), in which the presence of conserved regions between the amino acid sequences was observed. Suresh-Kumar *et al*. (2003) reported the presence of conserved regions in laccases from fungi and plants related to the Cu1 or Cu2 in copper sites. Diez-Fernandez (2015) carried out a bioinformatic analysis to determine the similarities between prokaryotic and eukaryotic laccase genes, observing that there is a phylogenetic relationship between different kingdoms. Hoegger *et al*. (2006) performed a phylogenetic analysis that supports the classification of multi-copper oxidase enzymes, dependent on their physico-chemical characteristics and the substrates that they are able to degrade.

However, there is no information on the variations of the structure of the laccase and specifically of its active site, due to a series of natural mutations that were carried out over millions of years and that allowed it to have its current activity. Therefore, in this study silico laccase mutants were generated by substituting amino acids adjacent to the active site of Lacc 6 for those present in laccases of bacteria that tolerate high temperatures and low humidity. In addition, the changes in the secondary and tertiary structure of the models of mutants were observed.

EXPERIMENTAL

Materials

Selection of complete sequences of known laccase enzymes

The search for complete amino acid sequences of laccase enzymes from bacteria, fungi, insects, and plants was performed using the public databases of NCBI and UniProt.

Methods

Analysis of the sequences

To determine the family of each enzyme through the obtained sequences, the conserved regions of the active sites associated with copper atoms contained in the laccases were searched, using the Hidden Markov Models (HMMs) corresponding to an algorithm of the Pfam program (version 31.0, European Molecular Biology Laboratory and European Bioinformatics Institute (EMBL-EBI), Hinxton, Cambridge, England). This program compares the similarity of the protein domains to define the family or families to which it belongs (Finn *et al*. 2014, 2016). From this analysis, the regions where the copper atoms are located and the amino acids in the vicinity of the active site were identified.

Once the sequences with conserved regions of each protein were selected, multiple alignments were performed using the MAFFT program (version 7.0, Immunology Frontier Research Center, Osaka, Japan) (Katoh *et al*. 2005, 2013). This program was based on the fast Fourier transform (FFT), which correlates the physicochemical properties of the amino acids of each protein and improves the alignment; after which a manual editing was performed based on the histidine residues present in the active site of the multicopper oxidases. These alignments were used for phylogenetic analysis.

Phylogenetic analysis

Phylogenetic analysis was performed with the BEAST program (held by the Centre for Computational Evolution and Department of Computer Science, University of Auckland, v1.8.4, Auckland, New Zealand) (Drummond and Rambaut 2007; Drummond *et al*. 2012), which was built based on the model reported by Whelan and Goldman (2001); the algorithm estimates empirical amino acid replacement matrices from large databases of aligned protein sequence families. A maximum likelihood procedure is used, and the evolutionary relationships within the families are recorded through the use of phylogenies for each family according to the matrix of Whelan and Goldman (WAG-matrix), as well as, a model of gamma heterogeneity sites and invariant sites with a number of Gamma 4 categories. Later, to estimate the divergence times between the sequences, a Lognormal model with a relaxed clock (Uncorrelated) was used (Drummond *et al*. 2006). Ten million generations were run using a Markov Chain Monte Carlo algorithm (MCMC). For the second phylogenetic analysis, where the approximate age of onset of fungal laccase enzymes was determined, the estimated time of 290 Ma for the taxonomic group of Agaricomycetes was used (Floudas *et al*. 2012). Ten million generations were executed using the MCMC algorithm.

The consensus of the phylogenetic tree was realized with the program TreeAnnotator (v1.8.4 supported by the Department of Computer Science, University of Auckland, Auckland, New Zealand), using the parameters preset in the program (Bouckaert *et al*. 2014; Álvarez-Cervantes *et al*. 2016). Later, the consensus of the phylogenetic tree was visualized with the program FigTree (v1.4.3 8, Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, Scotland), and finally the image of the tree was edited with the program Inkscape Project (Free Software Foundation, v0.91, Boston, MA, USA).

Protein modeling

Based on the results obtained from the phylogenetic analysis, the sequences corresponding to the bacteria laccases were selected as a model. Due to the geographical region where they were isolated, extreme temperature conditions could have led to the evolution of bacteria causing mutations that resulted in stable and functional laccases under these conditions. In contrast, laccase enzymes called Lacc 6 from *Pleurotus ostreatus* PC15 with access number KDQ26322.1 was proposed as a mold, because this enzyme showed greater expression and stability to the different pH levels of development of the producer organism (Díaz *et al*. 2013). The modeling of laccases from bacteria and Lacc 6 was performed using the Swiss-Model program accessible in The Swiss Institute of Bioinformatics (University of Basel Klingelbergstrasse, v8.05, Basel, Switzerland) to observe possible differences in the trinuclear center and their structures, with respect to the alpha helices and beta sheets domains (Biasini *et al*. 2014; Waterhouse *et al*. 2018). The obtained models were visualized with the program Python Molecular Viewer (PMV) available in The Molecular Graphics Laboratory (The Scripps Research Institute, v1.4.5, La Jolla, CA, USA) (Sanner 1999; Dallakyan 2010).

Generating mutant

The Lacc 6 enzyme was used as a model. The design of mutants was focused on the active site and the histidine channel where the electron transfer was performed. The bacterial laccase sequences were taken, observing the type and physicochemical characteristics of the adjacent amino acids to the channel of histidines that are united to the

atoms of copper, to observe hypothetical changes that have occurred during the past millions of years. The bacterial laccase sequences were of bacteria that grow in environments of high temperature and low humidity.

Structural comparison

For the structural comparison, 46 laccases were studied and those enzymes are of the group that can found as First citation of Laccase on PDB, and are from different kingdoms. It was determined that the following items are included: 37 laccases with structures of 3 domains of Cu-oxidase (L-3MCO) (Zhukhlistova *et al*. 2008) and 9 with structures of 2 domains of Cu-oxidase (L-2MCO) (Komori *et al*. 2009; Skálová *et al*. 2009). A structural study of the domains was also carried out with the Pfam database (Finn *et al*. 2016). The crystallographic structure of the 46 laccases was superimposed on the crystallographic structure of the enzyme mgLAC L-2MCO (Lawton *et al*. 2009) (PDB ID: 4E9X), due to its conjecture of "missing link" between the families of oxidoreductases from two to three domains (Komori *et al*. 2009). The above was done through the Secondary Structure Matching algorithm (SSMA) using the Chimera software and Root Mead Squarer Deviation (RMSD) (Goddard *et al*. 2017). To compare the architecture of domains through evolution, a phylogenetic tree containing 256 non-redundant laccase structures was performed through the server Simple Modular Architecture Research Tool (SMART) and InterProScan (Letunic *et al*. 2015).

RESULTS AND DISCUSSION

Selection of Laccase Sequences

Based on the results of the BLAST analysis, 63 protein sequences corresponding to laccase enzymes from different organisms were obtained; 40 of basidiomycete fungi, five of bacteria, 10 of plants, and eight of insects, all above sequences showed regions conserved in the amino acids binding to the copper atoms. The name of the organisms and their laccase access number are described below.

For basidiomycetes, one of *Phanerochaete chrysosporium* (AAO42609.1), six of *Trametes versicolor* FP-101664 SS1 (EIW62366.1, EIW63935.1, EIW64151.1, EIW60660.1, EIW58134.1, and EIW58136.1), 12 of *Pleurotus ostreatus* (AAR21094.1, BAA85185.1, CAA84357.1, CAR48257.1, CAC69853.1, KDQ29908.1, KDQ26322.1, KDQ24506.1, KDQ26265.1, KDQ27218.1, KDQ27220.1, and KDQ26299.1), two of *Pleurotus eryngii* var. *Ferulae* (AKE48164.1 and ACI62810.1), 2 of *Pleurotus eryngii* (ADG01835.1 and CAO79914.1), four of *Pleurotus salmoneostramineus* (BAI66147.1, BAI66142.1, BAI66139.1, and BAI66145.1), one of *Pleurotus* sp. *Florida* (CAA80305.1), two of *Pleurotus pulmonarius* (AAX40733.1 and AAX40732.1), one of *Pleurotus sapidus* (CAJ00406.1), one of *Coprinus comatus* (AFD97049.1), three of *Lentinus sajor-caju* (CAD45379.1, CAD45380.1, and CAD45381.1), one of *Stereum hirsutum* FP-91666 SS1 (EIM79648.1), two of *Heterobasidion irregulare* TC 32-1 (XP_009545278.1 and ETW82985.1), one of *Hypsizygus marmoreus* (KYQ44884.1), and one of *Leucoagaricus* sp. *SymC.cos* (KXN81384.1).

For bacteria, two of *Halovivax ruber* (WP_015301801.1 and WP_015300460.1), one of *Haloferax sp.* ATCC BAA-644 (WP_008572767.1), one of *Haloferax volcanii* (WP_013034979.1), and one of *Halorubrum saccharovorum* (WP_004045604.1).

For plants, three of *Malus domestica* (XP_008357173.1, XP_008363777.1, and XP_008362821.1) and seven of *Solanum tuberosum* (XP_006363100.1, XP_006367545.1, XP_015163413.1, XP_015163415.1, XP_015159950.1, XP_006360308.1, and XP_006363101.1). For insects, one of *Bactrocera cucurbitae* (XP_011179084.1), one of *Microplitis demolitor* (XP_014297903.1), one of *Musca domestica* (XP_011290564.1), one of *Pimpla hypochondriaca* (CAD20461.1), one of *Plutella xylostella* (XP_011555576.1), one of *Polistes canadensis* (XP_014599609.1), and two of *Zootermopsis nevadensis* (KDR15437.1 and KDR11754.1).

Analysis of the Sequences

The Pfam database suggests that the laccase enzymes of all organisms used in this study belong to the large family of multicopper oxidases; in addition, it showed the position of the active site and the domains of each of the sequences. Figure 1 shows the alignment of the sequences in the regions conserved in the histidine channels, which are the amino acids bound to the copper atoms and catalytic site of the enzymes. This analysis also allowed for the observation of changes in the amino acids adjacent to the histidine channels.

Phylogenetic Analysis

The phylogenetic analysis allowed for the construction of a tree with the 63 sequences of laccases. In phylogenetic construction, two well-defined groups, one belonging to prokaryotes (bacteria) and the other to eukaryotic cells (fungi, plants, and insects), were observed. Additionally, in eukaryotes, a grouping corresponding to the phylum was observed; on the one hand the laccases of the fungi, on the other side of the plants and one more of insects. An evolutionary selection was observed in the laccase enzymes of insects and fungi before that of the plants; in contrast, the tree made it possible to observe that there was a common ancestor that gave rise to insect laccases and diverged in the laccases of plants and fungi. It was also possible to observe that the Lacc 6 enzyme of *Pleurotus ostreatus* appeared approximately 5.57 million years ago (MYA) and has a phylogenetic relationship with the enzymes AKE48164 and ACI62810 of *Pleurotus eryngii* var. Ferulae and with the isoenzyme KDQ26265 of *Pleurotus ostreatus*, as shown in Fig. 2. Figure 3 shows the phylogenetic analysis constructed with 40 laccase sequences of basidiomycete fungi, showing the estimated time of appearance of the common ancestor of all fungal laccases used in this study. The time that was used to make the calculation was proposed by Floudas *et al*. (2012), suggesting that the appearance of agarycomicetes was 290 MYA. It can be observed that there was a common ancestor of laccase in all the fungi and that approximately 278.48 MYA was divided to give place to a laccase of *Phanerochaete chrysosporium* and to a group of the rest of fungal laccases used in this study. It can be seen that the laccase of *Phanerochaete chrysosporium* has not changed over time, and the other group showed a separation into two groups 148.87 MYA. The divergence of laccase enzymes over time suggests that the fungi evolved, obliged by the changing conditions of their environment, becoming more efficient for the degradation of lignocellulosic compounds present in their natural substrates (Heckman *et al*. 2001; Honrubia 2009). Jones and Dangl (2006) suggested a co-evolution between plants and host pathogens that affects their growth and reproduction. Plants respond to infection using a two-branched innate immune system, the first branch recognizes and responds to molecules common to many kinds of microorganisms, even non-pathogens. The second branch responds to the virulence factors of the pathogens, either directly or through their effects on the host targets.

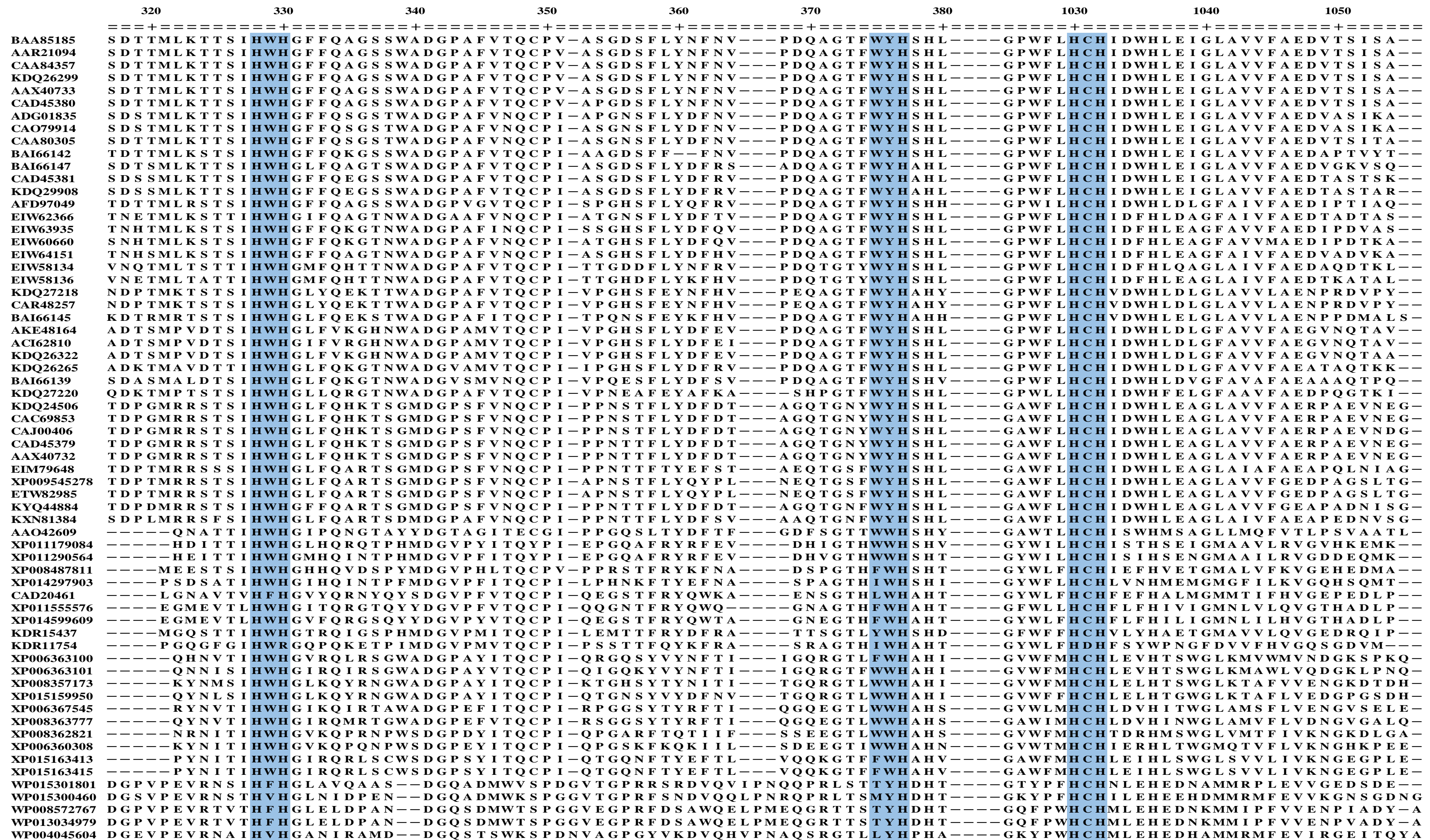

Fig. 1. Alignment of the amino acid sequences of the active site region of laccase enzymes. The three conserved regions are between amino acids 328 to 330, 377 to 379, and 1030 to 1032. The left column shows the laccases access numbers that were mentioned above. The differences in the sequences are indicated with the sign (-). The conserved regions of the histidine channel bound to the copper atoms and active sites are highlighted in blue.

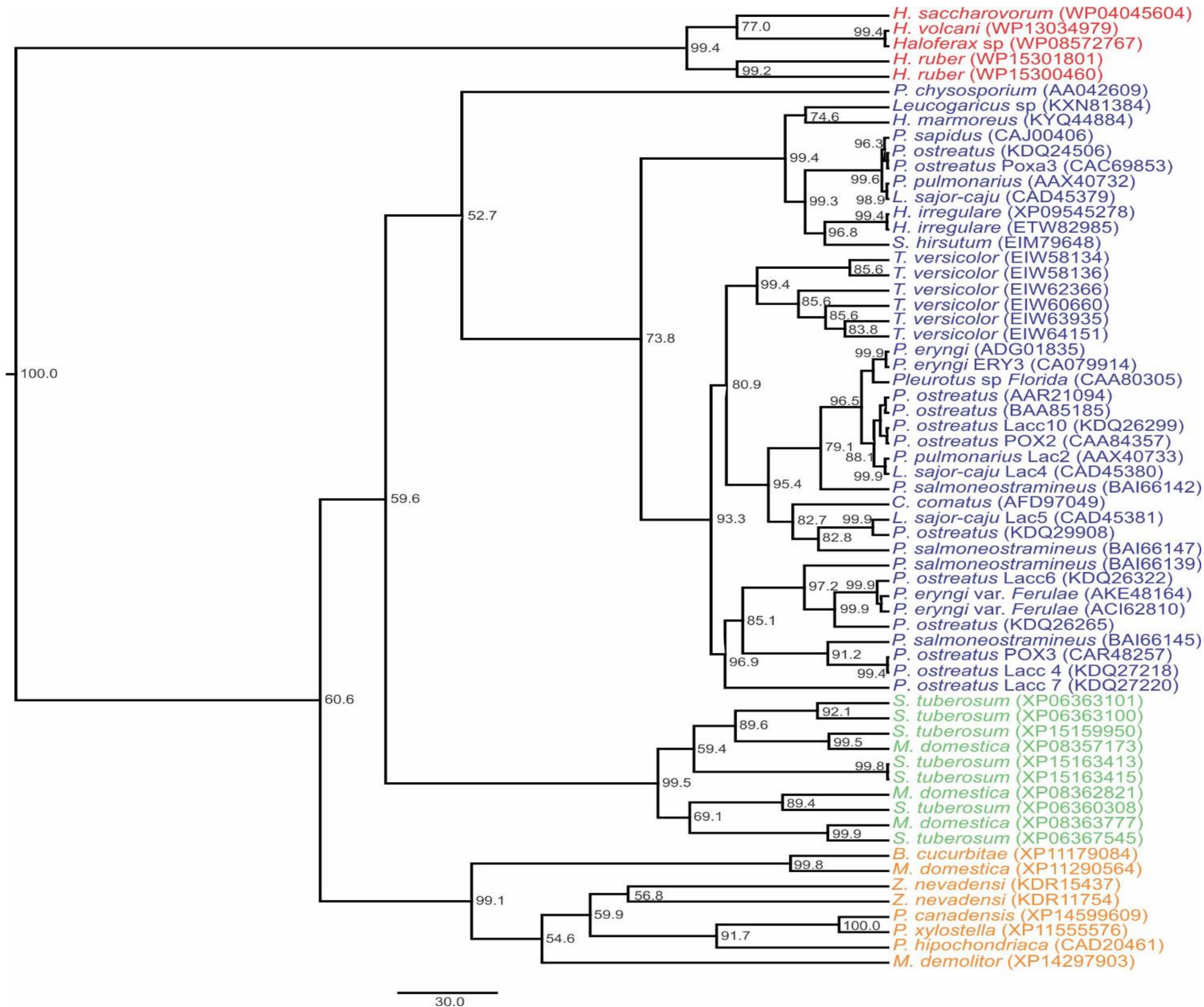

Fig. 2. Phylogenetic tree of the amino acid sequences of laccase enzymes of bacteria (fonts in red), basidiomycetes (fonts in blue), plants (fonts in green), and insects (fonts in orange). The values in the nodes represent the Bayesian posterior probabilities in percentage.

These two scenarios predict different results with respect to the timing and amplitude of the responses that are triggered and also causes a rise in multiple evolutionary pressures in both the host and the pathogen. In agreement with this study, it was suggested that the fungi co-evolved with respect to their carbon source, promoting an adaptation and greater catalytic effectiveness of the enzymes involved in their degradation, including laccases.

Hoegger *et al*. (2006) performed a phylogenetic comparison of the laccase enzymes of fungi, insects, plants, and bacteria to develop a classification and relation between the sequences of these type of enzymes. The comparison resulted in an observation that these multicobre oxidases appear grouped according to the type of organism and also that some species encode for proteins of the ferroxidase type, while other proteins have different oxidative characteristics. As such, this observation differed from this study, which only worked with proteins classified as multicobre oxidase. Given the importance of these enzymes and the wide range of organisms capable of producing them, it is interesting to know the phylogenetic relationship with a common ancestor that can correlate their pathogenic, saprophyte, or synthesis functions of producing organisms. The phylogeny described in this research showed the clustering of the protein sequences according to the order of each phylum. A common ancestor was observed for prokaryotic and eukaryotic cells. From this ancestral protein, a divergence occurred to give origin to the proteins of bacteria, where it was observed that during millions of years they have not undergone considerable changes in structure, and to a protein from which the laccase enzymes emerge from the other three kingdoms (fungi, plants, and insects). From this second group, it was observed that a protein gave rise to fungi and plant laccases and also to insect enzymes. From these enzymes, it was observed that insect and fungal proteins were formed earlier than those of plants, possibly because they are used in the degradation of substrates in insects and fungi; whereas in plants, they are mainly used for structure synthesis (Hoegger *et al*. 2006; Floudas *et al*. 2012).

Floudas *et al*. (2012) conducted a phylogenetic analysis of the paleozoic origin of ligninolytic enzymes from 31 fungal genomes, including the basidiomycete and ascomycheta families. The study predicted an approximate time of 290 Ma for the agaricomycetes, which was used to determine the approximate time of appearance of the different laccases through the use of the phylogenetic analysis of the fungi sequences. The results observed coincided with those reported by Floudas *et al*. (2012).

Relationship of the structure of laccase enzymes with respect to phylogeny

The structures of all laccase enzymes were analyze and showed conserved regions related to the active site that did not have changes in the type of amino acid bound to the copper atoms. However, there were changes in the sequence of the rest of the peptide chain, causing differences in the domains of proteins, and thus, in the tertiary structure. Through the Swiss-Model program (Biasini *et al*. 2014; Waterhouse *et al*. 2018), it was observed that some enzymes contained a different number of copper atoms in the active site, and in some cases, contained molecules of oxygen, hydroxyl ion, or zinc. In the structures of the laccases of plants, the hydroxyl ion was preferentially found; in those of bacteria and insects, the oxygen molecule existed, and only in some cases of fungal enzymes zinc was present. However, the presence of these molecules could not be correlated evolutionarily with respect to the phylogeny.

The conserved regions of the active site and the amino acids where the copper atoms are attached were observed in all the laccase structures of this study.

For the design of the mutants, the laccase sequence Lacc 6 of *Pleurotus ostreatus* was used, which has shown a high and efficient enzymatic activity for the degradation of lignocellulosic compounds (Díaz *et al*. 2013), as well as the sequences of laccases of the bacteria *Halorubrum saccharovorum*, *Haloferax volcanii*, *Haloferax* sp. ATCC BAA-644, and *Halovivax ruber* (with two sequences) because they grow in environments of high temperature and low humidity.

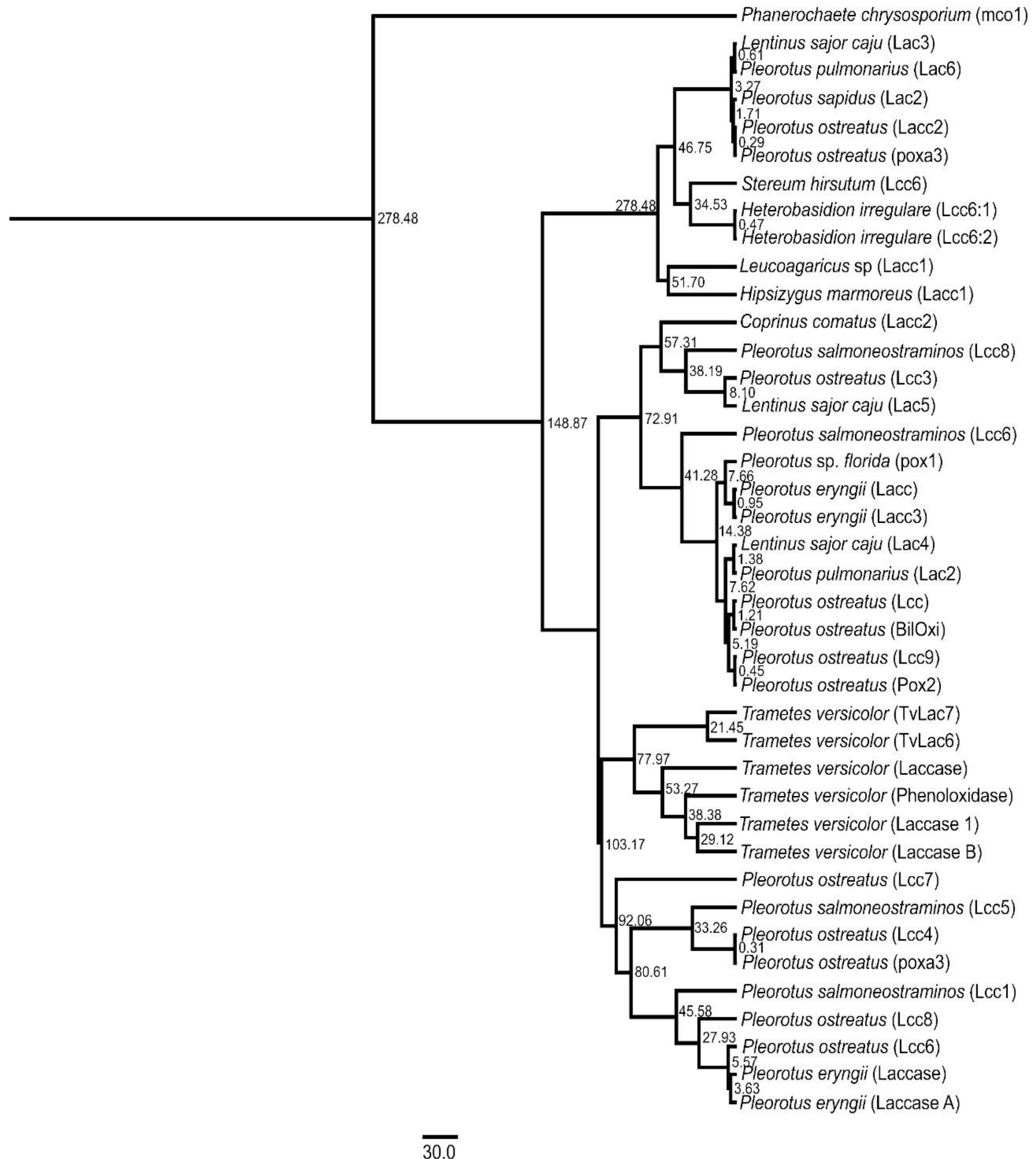

Fig. 3. Phylogenetic analysis of the amino acid sequences of laccase enzymes of basidiomycetes; numbers indicate the estimated time of divergence for enzymes (MYA)

Additionally, the signal peptide of each of the enzymes of this study was determined. The length of the signal peptides of the enzymes used for the modeling ranged from 20 to 31 amino acids. The conserved regions binding to the copper atoms for all cases of bacteria were between 140 to 200 amino acids and 440 to 570 amino acids. For the case of Lacc 6, the conserved regions were found between 180 to 230 amino acids and between 410 to 480 amino acids. In these regions there is a channel of histidines that bind to the copper atoms and transfer the electrons to the oxygen to form water at the end of the catalysis of the substrates (Fig. 4).

Fig. 4. Elements that make up the primary structure of laccase enzymes of bacteria *Halovivax ruber*, *Haloferax* sp. ATCC BAA-644, *Haloferax volcanii*, *Halorubrum saccharovorum*, and the sequence of Lacc 6 of *Pleurotus ostreatus* (KDQ26322.1). The numbers indicate the amount of amino acids or position of the signal peptide (SP). For conserved copper binding regions, the amino acid number attached to the copper atom is shown: the first copper bond (blue), the second copper bond (green), the third copper bond (red), and the total number of amino acids in the chain of proteins is shown in black.

The comparison and structural overlap of the active site (Fig. 5) show the high evolutionary conservation of the copper ligation site, and independently of the amino acid composition that surrounds this (Zhukhlistova *et al*. 2008; Skálová *et al*. 2009), an invariable characteristic of the laccase enzyme group is that the copper atoms of the active site are constituted by the same number of histidine and cysteine residues, besides being structured purely by domains 1 and 2 Cu-oxidase type. The superposition of the domains (D1, D2 and D3), with an RMSD of approximately 0.7 Å, 1.7 Å and 1.0 Å between nuclei for each domain reveals surprising structural similarities, demonstrating that the secondary structure, architecture and general orientation of the Cu-oxidases domains in laccases are highly conserved, despite having low homology in sequence (0-30%) (Fig. 6). Using a phylogenetic map (Fig. 7), constructed from 256 non-redundant sequences of laccases and made with SMART (Skálová *et al*. 2009), the evolutionary positions for some laccase structures deposited in the PDB were located. The phylogenetic map in conjunction with

the structural superposition confirms the conserved evolutionary nature of the laccase protein (Finn *et al.* 2016; Goddard *et al*. 2017), thus corroborating our hypothesis of a common ancestor.

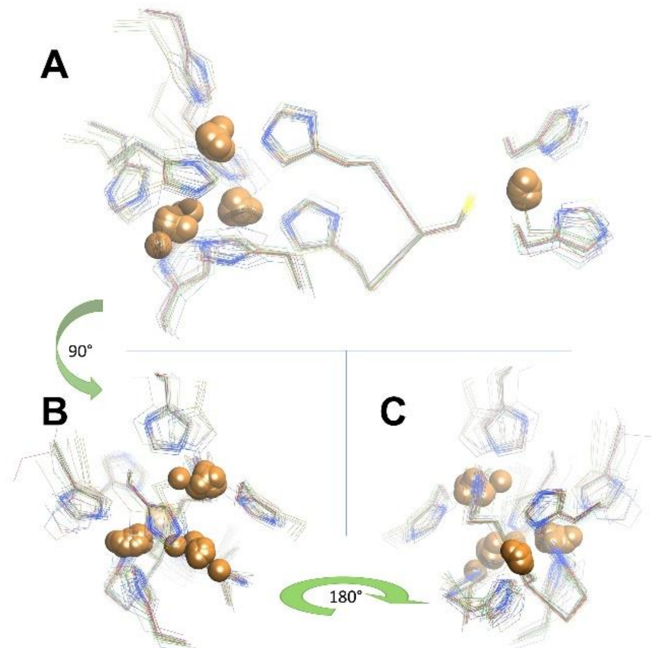

Fig. 5. Schematic and structural representation of the catalytic motif for the laccase enzyme shows an evolutionarily conserved structure with an RMSD less than 1.0 Å. A) Tetranuclear center of copper atoms, B) Site T2/T3 or trinuclear and C) Site T1 or mononuclear. Within the color code the copper atoms are ocher and the set of HIS/CYS in gray-blue-yellow lines.

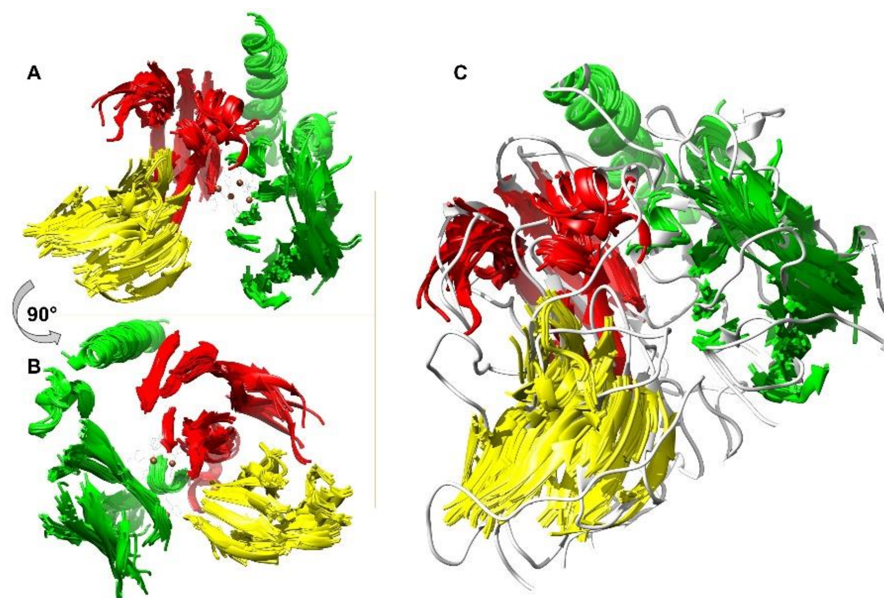

Fig. 6. General architecture of the laccase enzyme and superposition of the structures deposited in the PDB. The three subunits (architecture type Cu-oxidase) are shown in red (D1), green (D2) and yellow (D3), as well as the disordered regions are exposed in white. A) and B) show a high degree of overlap between nuclei of the Cu-oxidase architecture. C) Structure and connectivity of architectural domains.

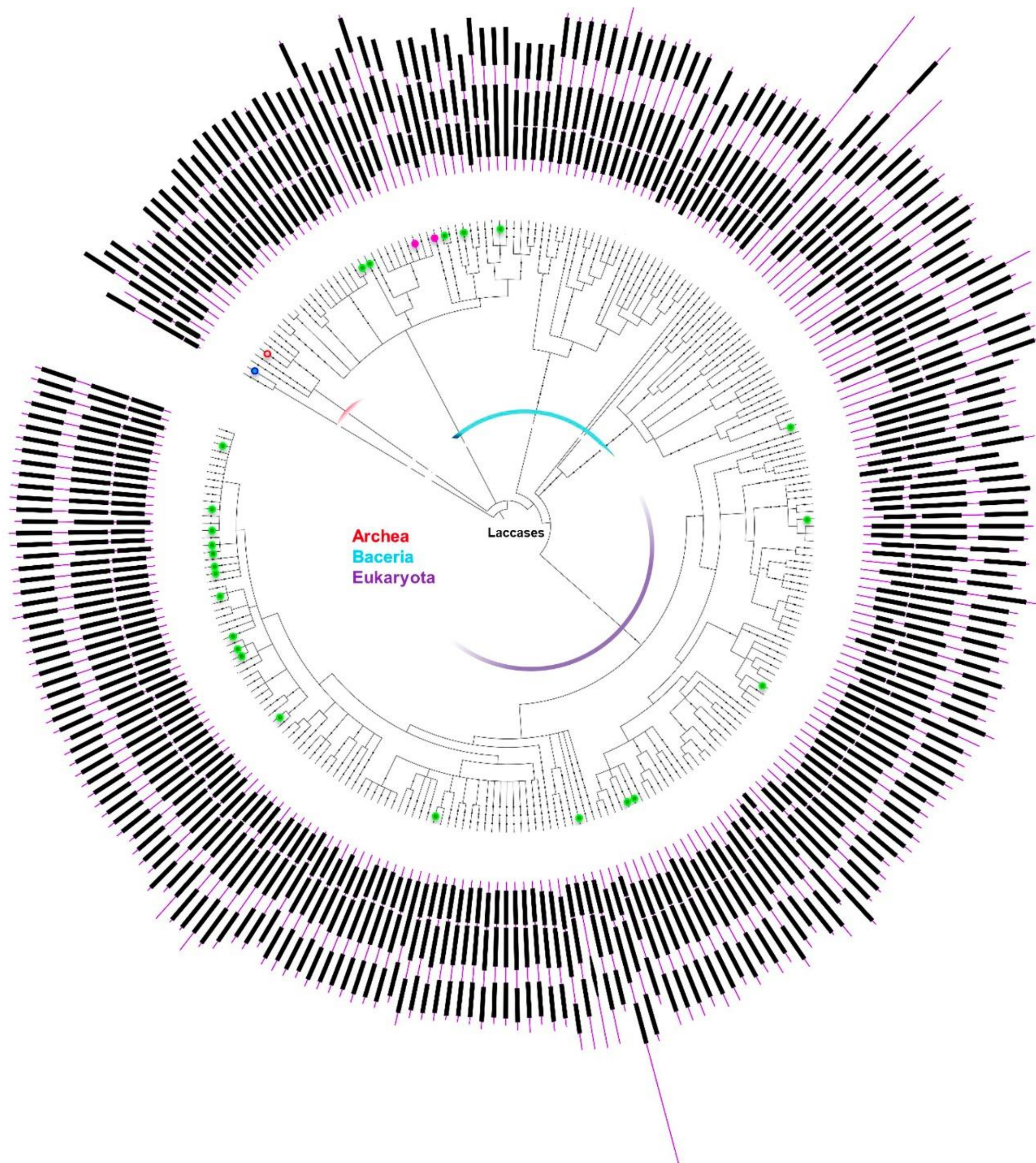

Fig. 7. Phylogenetic tree. The evolutionary displacement of a common orthologous ancestor is appreciated, diverging through the three characteristic kingdoms. On the phylogenetic circle, the location of mgLac is seen in blue. In pink and green the location a fraction of the 2-LMCO and 3-LMCO used in the structural superposition of the laccases, in red color *H. volcanii*. Out of the phylogenetic circle in black bars the number and type of Cu-oxidase domain corresponding to each of the 256 structures.

Laccase enzymes are classified as blue-copper oxidases that contain at least four copper atoms, one of type 1 (Cu1), one of type 2 (Cu2), and two of binuclear coupled type 3 (Cu3). A trinuclear junction of copper between the Cu2 and Cu3 atoms is formed, stabilized by magnetic forces generated by these same copper atoms (Campos *et al*. 2001; Sang-Kyu *et al*. 2002; Claus 2004; Pazarlioglu *et al*. 2005). The copper atoms are bound to histidines at the active site and are responsible for the transfer of electrons during the oxidation of phenolic compounds such as lignin, the natural substrate of these enzymes.

The active site was identified in this study by modeling each of the enzymes and observing the interaction with the protein structure forming the gap where the substrate is coupled for its transformation into product. The active site of the laccases was studied with respect to the amino acids attached to the copper atoms and the amino acids adjacent to the histidines channel, in an effort to replace these amino acids by others with similar chemical characteristics and found in the protein sequence of the laccases of bacteria. Highly conserved regions were observed with respect to the amino acid sequences that make up the catalytic site of these enzymes, and in some cases, a hydroxide molecule was shown that possibly helped regulate the pH changes during their catalytic action (Quintanar *et al*. 2005; Solomon *et al*. 2014).

Modeling of laccase enzymes

Figure 8 shows the laccase enzyme structures of Lacc 6 and the bacteria that were analyzed in this study. It was observed that the selected proteins showed similar numbers of copper atoms except for an isoenzyme of *Halovivax ruber*. From the orientation of the isoenzyme and the domain of the amino acid sequences, there was a predominance of beta sheets for all the cases, with little presence of alpha helices. The position of the laccase enzyme models show the gap of the active site and the copper atoms.

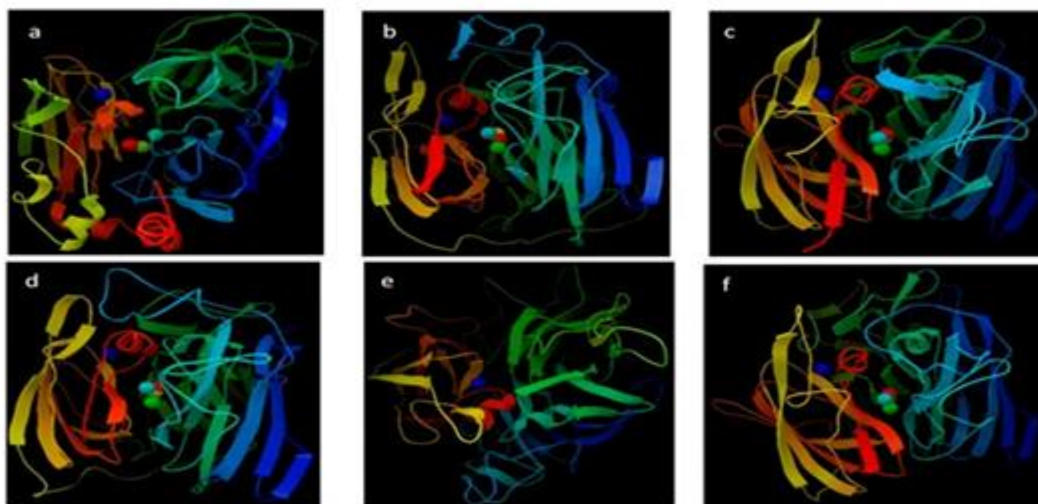

Fig. 8. Laccase enzymes models of (a) Lacc 6, (b) *Haloferax* sp., (c) *Haloferax volcanii,* (d) *Halorubrum saccharovorum*, (e) *Halovivax ruber*, and (f) *Halovivax ruber*

For the case of bacterial enzymes the oxygen molecule that is next to the type 2 and 3 coppers is observed and are Cu2 and Cu3 diamagnetic to the center of the enzyme. In the case of the enzyme Lacc 6 (Fig. 8a), the structure is laxer with respect to the enzymes of the bacteria and was not present in the model of the oxygen molecule. Additionally, in the enzymes of bacteria, it was observed that the domains of the secondary structures were more compact and a difference was observed in the distance between the copper atoms. An isoenzyme from *Halovivax ruber* (Fig. 8e) showed only one type 3 copper atom in the center of the active site and the structure of the protein was more dispersed with respect to the other bacterial enzymes. Regardless of the origin of the laccase, the histidine channel was formed through the hollow of the active site to perform the catalysis. The domains of the secondary structures were similar in all samples.

Generating mutant

Using the Lacc 6 enzyme as a model and looking for the greatest similarity in the active site with respect to the laccase enzymes of bacteria (because of their origin of isolation in areas with high temperatures and low humidity), the amino acids united to the copper atoms and the amino acids adjacent to the histidines channel were identified. Given the analysis of these amino acids, it was proposed that replacing the amino acids adjacent to the histidines channel in the Lacc 6 enzyme (Table 2) suggests possible mutations that have occurred over time; however, silent mutations may have occurred that did not affect their structure or catalytic capacity. Table 2 indicates the amino acids and their position in the protein sequence as well as its substituent amino acid for obtaining *in silico* the models of possible mutants from Lacc 6.

Table 2. Substitution of Amino Acids Adjacent to Histidines Channel of Lacc 6

Mutant	Amino Acid and its Position	Substitute Amino Acid
1	W, 85; S, 130; L, 132; L, 416; G, 420; F, 468; L, 469; F, 481	F, D, T, I, L, P, F, M
2	W, 85; S, 130; L, 132	V, D, T
3	L, 416; G, 420	I, L
4	F, 468; L, 469; F, 481	P, F, M
5	W, 85; S, 130; L, 132; L, 416; G, 420	F, D, T, I, L
6	W, 85; S, 130; L, 132; F, 468; L, 469; F, 481	F, D, T, P, F, M
7	L, 416; G, 420; F, 468; L, 469; F, 481	I, L, P, F, M

In most cases, the original amino acids had physicochemical characteristics similar to substitute amino acids. After replacing the respective amino acids in the original Lacc 6 sequence, modeling was performed and the mutants that could possibly exist millions of years ago were obtained to give rise to the current enzyme (Fig. 9).

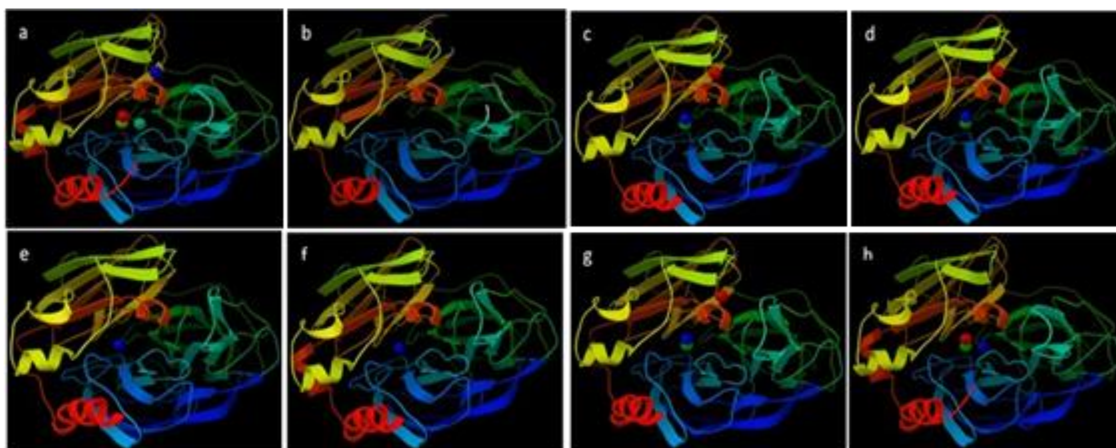

Fig. 9. Models of (a) Lacc 6 and their mutants; (b) to (h) correspond at mutants 1 to 7. The colored spheres in the structures indicate the copper atoms in the active site

In all cases, the mutants showed noticeable changes in their secondary structure and number of copper atoms. Mutant 1 showed the greatest change, because no copper atoms were observed in the protein and its secondary structure changed. Mutants 2, 3, and 6 showed two copper atoms in the central part of the protein of types Cu2 and Cu3 and a copper atom in the initial part of the histidines channel of type Cu1. Mutants 4 and 5 showed only one copper atom in the gap of active site of type Cu2. Mutant 7 showed three

copper atoms at the active site of the enzyme (Cu2, Cu3, and Cu4). The copper atom in mutant 7 was not found at the beginning of the electron transfer channel. Mutants 1, 2, 3, 4, 5, and 6 had changes in residues at the end of the protein, which caused a change in their alpha helix structure. Mutant 7 did not have this effect on its structure. Several studies have been conducted on the generation of *Pleurotus ostreatus* laccase enzyme mutants as an alternative for their application in the biodegradation of xenobiotic and/or recalcitrant compounds. Miele *et al*. (2010) randomly performed the generation of 2300 mutants of the POXA1b (Lacc 6) isoenzyme of *Pleurotus ostreatus* to develop biocatalysts from this enzyme, but no proposal was made to know how the possible mutations occurred on the active site of this enzyme; also no proposal was made to obtain models that would show the effect on the trinuclear copper region by modifying amino acids adjacent to the histidines bound to these metals as is done in this research. In contrast, Prins *et al*. (2015) observed the effect of mutations near the Cu1 copper site of *Streptomyces coelicolor* A3(2). To evaluate their biochemical characteristics against the native enzyme, three mutations (M298F, V290N, and V290A) were obtained, with the V290N mutant showing approximately double the activity. Likewise, the activity was four to five times greater of the mutant M298F; however, the thermal stability of this mutant was decreased.

CONCLUSIONS

1. It is suggested that there was an ancestral protein that gave rise to all laccases. Consequently, over time there were mutations and differences between the laccases, which gave rise to the multiple proteins of laccase and their biological function.
2. The change in the adjacent amino acids caused noticeable changes in the type of ligand to copper, in the number of copper atoms, and in the domains of the secondary structure of the mutated proteins proposed in this study.
3. The mutants generated were made through structural changes in the vicinity of the active site; however, the production of the mutant enzymes in a biological system will make it possible to determine the certainty of this study.

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