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In silico characterization of laccase gene isoforms of edible and medicinal basidiomycetes

Caracterização *in silico* de isoformas dos genes de lacase de Basidiomicetos comestíveis e medicinais

Caracterización *in silico* de isoformas del gen de lacasa de Basidiomycetes comestibles y medicinales

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Abstract

Laccases are part of the family of ligninolytic enzymes and have played essential roles in several biological filamentous fungi processes, including fruiting body formation and lignin degradation. This study aimed to identify and characterize laccase genes *in silico* of several basidiomycete strains. The applied guaiacol oxidation test allowed the selection of seven out of 11 strains with ligninase activity, which were used for DNA extraction and amplification of the copper-binding region. A single amplicon of approximately 450 bp, was produced by all selected strains and they were further sequenced. Sequence analysis has suggested the presence of a new subdivision of the laccase genes. Clustering analysis confirmed the existence of two groups: cluster A with six strains and singleton B with U8-11 strain. The structural predictions of the U8-11 protein were dissimilar compared to other proteins described in our study due to the absence of the ALAVIN motif and, therefore, the U8-11 amino acid sequence was separated in a different cluster.

Keywords: *Agaricus*; Isogenes; *Lentinus*; *Pleurotus*; White-rot fungi.

Resumo

As lacases fazem parte da família das enzimas ligninolíticas e desempenham papel essencial em vários processos biológicos de fungos filamentosos, incluindo a formação do corpo de frutificação e a degradação da lignina. Este estudo teve como objetivo identificar e caracterizar *in silico*, genes de lacase em diversas linhagens de Basidiomicetos. O teste de oxidação do guaiacol permitiu a seleção de sete de 11 linhagens com atividade ligninolítica, as quais foram usadas para extração de DNA e amplificação da região de ligação ao cobre. Todas as linhagens selecionadas produziram um único amplicon de aproximadamente 450 bp, que foi posteriormente sequenciado. A análise da sequência sugeriu a presença de uma nova subdivisão dos genes da lacase. A análise de agrupamento confirmou a existência de dois

grupos: agrupamento A com seis linhagens e o singleton B com a cepa U8-11. As previsões estruturais da proteína U8-11 foram diferentes em comparação com outras proteínas descritas em nosso estudo devido à ausência do motif ALAVIN e, portanto, a sequência de aminoácidos U8-11 ficou separada em um cluster diferente.

Palavras-chave: *Agaricus*; Isogenes; *Lentinus*; *Pleurotus*; Fungos da podridão-branca.

Resumen

Las lacasas son parte de la familia de enzimas ligninolíticas y desempeñan funciones esenciales en varios procesos biológicos de hongos filamentosos, incluso la formación de cuerpos fructíferos y la degradación de la lignina. El objetivo general fue identificar y caracterizar genes del lacasa *in silico* de varias cepas de Basidiomycetes. La prueba de oxidación con guayacol aplicada permitió la selección de siete de las 11 cepas con actividad ligninasa, que se utilizaron para la extracción del ADN y la amplificación de la región de conexão al cobre. Todas las cepas seleccionadas produjeron un único amplicón de aproximadamente 450 pb que fueron posteriormente secuenciados. El análisis de la secuencia sugirió la presencia de una nueva subdivisión de los genes de lacasa. El análisis de agrupamiento confirmó la existencia de dos grupos: el cluster A con seis cepas y el singleton B con la cepa U8-11. Las predicciones estructurales de la proteína U8-11 fueron diferentes en comparación con otras proteínas descritas en nuestro estudio debido a la ausencia del motif ALAVIN y, por lo tanto, la secuencia de aminoácidos U8-11 se separó en un cluster diferente.

Palabras clave: *Agaricus*; Isogenes; *Lentinus; Pleurotus*; Hongos de pudrición blanca.

1. Introduction

Laccases are widely distributed in fungi, plants, bacteria, and insects (Rivera-Hoyos et al., 2013; Shraddha et al., 2011) and are typically extracellular monomeric glycoproteins that belong to the multicopper oxidase family (Mate & Alcalde 2017; Rodgers et al., 2010; Baldrian 2006). These enzymes catalyze the oxidation of phenol and polyphenol substrates with concomitant reduction of molecular oxygen to water (Cázares-García et al., 2013). In different biotechnological applications such as biopulping, bioremediation, dye breakdown, enzymatic conversion of chemical intermediates, and synthesis of pharmaceutical products among others they have been used (Sirim et al., 2011; Sharma et al., 2018; Lira & Orlanda, 2020). However, most of the laccases that have been

used in biotechnological processes are from fungi (Cázares-García et al., 2013; Mate & Alcalde, 2017; Rodgers et al., 2010).

Laccases from fungi have been related to the degradation of lignocellulosic material, sporulation, pigment production, fruit body formation, and plant pathogenesis (Cázares-García et al., 2013). The multiplicity of laccase genes that are organized in gene families is common in fungi and can produce a great number of laccase isoforms (Piscitelli et al., 2011b). For different species such as *Pleurotus ostreatus* which has at least 12 laccase isoenzymes (Jiao et al., 2018) and *Lentinus edodes*, which has 14 laccase genes identified this has been reported (Yan et al., 2019).

Genome analyses have revealed the existence of multiple laccase genes in several basidiomycetes (Kües & Rühl, 2013) and the characterization of these genes could provide new perspectives in biotechnological applications. Thus, the objective of this study was to identify and characterize laccase genes *in silico* of several basidiomycete strains.

2. Metodology

Fungal strains from the culture collection of the Molecular Biology Laboratory of Paranaense University (Brazil) were used for experiments such as *Agaricus subrufescens* Peck (*Agaricus blazei* Murrill and *Agaricus brasiliensis* Wasser et al., synonyms) U2-4, U7-6, U7-7, and U6-5 strains, *Lentinula edodes* (Berk.) Pleger U6-11 strain, *Pleurotus eryngii* (DC.) Quél. U8-11 strain, *Pleurotus ostreatus* (Jacq.) P. Kumm. U6-10, U2-9, U2-11, and U6-9 strains, and *Lentinus crinitus* (L.) Fr. U9-1 strain (Table 1). The cultures were maintained on potato dextrose agar plates grown at 28 ºC and cryopreserved at -80 ºC (Mantovani et al., 2012; Zaghi Jr. et al., 2018).

Laccase activity

Petri dishes containing malt extract agar (35 g MEA l^{-1}, Himedia®) with 0.01% guaiacol (2-methoxyphenol) (mass: volume) were inoculated with a mycelial disk (6 mm diameter) and incubated at 28 ºC for seven days. A brown-reddish halo around the colony formed by guaiacol oxidation was verified as an indicator of ligninolytic enzyme production. Fungal cultures with the halo were considered positive for laccase activity (Kiiskinen et al., 2004) and were further analyzed.

DNA extraction, amplification, and sequencing

The strains with a positive reaction for laccase activity were selected to DNA extraction, amplification, and sequencing. The mycelia were grown in 100 mL liquid malt extract medium (2%, mass: volume) at 28 ± 1 °C for seven days and used for DNA extraction (Volpini et al., 2016). The mycelial biomass was filtered, washed with ultrapure water, macerated with liquid nitrogen, and mixed with 2.1 mL extraction buffer g^{-1} (1 M Tris-HCl, pH 8.0, 5 M NaCl, 0.5 M EDTA, pH 8.0, and 10% SDS) at 65 °C for 15 min (Volpini et al., 2016). For PCR reactions, Plac primers (Piscitelli et al., 2011a) were used to amplify partial laccase sequences such as forward (5'-CATTGGCATGGCTTCTTTCA-3') and reverse (5'-GCGCGAATCCAGTAATTGCGAC-3') Plac primers. The PCR reaction contained 0.2 pmol of each primer, dNTPs (0.15 mM in PCR 1x buffer, 20 mM tris-base, pH 8.4, and 50 mM KCl), $MgSO_4$ (1.5 mM), *Taq* DNA polymerase (2.0 U), DNA (25 ng), and ultrapure water to complete the final reaction volume to 50 µL. DNA was amplified in a thermocycler (Eppendorf Mastercycler Gradient®) at 94 ºC for 4 min initially, followed by 35 cycles of 94 ºC for 1 min; 50 ºC for 1 min, 72 ºC for 2 min, and a final cycle at 72 ºC for 7 min. The PCR products were submitted to electrophoresis in 0.8% agarose gel stained with ethidium bromide, visualized, and photographed under UV light using photographic documentation equipment (Loccus Biotecnologia®). Each amplicon was purified utilizing a PCR purelink purification kit (Life Technologies®) according to the manufacturer's recommendations and sequenced in an ABI-Prism 3100 genetic analyzer (Applied Biosystems®, Foster City, CA, USA) according to the manufacturer's recommendations.

The electropherograms obtained from DNA sequencing reactions were analyzed using Quality Checker Sanger (ThermoFisher Scientific®) and Bioedit (Hall 2011) programs. The resulting sequences from these PCR were deposited in the GenBank® of the National Center for Biotechnology Information (NCBI) (Table 2).

Sequence analysis

The DNA sequences of the basidiomycete strains were compared to DNA sequences obtained from the GenBank databank (NCBI). Sequences of laccase isoforms of *Pleurotus sajor-caju* genes *lac1*, *lac2*, *lac3*, and *lac4* (Soden & Dobson, 2001) were used as a reference. Sequences of laccase from *A. subrufescens*, *L. edodes*, *P. eryngii*, *P. ostreatus*, and *L. crinitus* were also included in the analysis. The inclusion criterion of sequences in the analysis

considered coverage above 90% and the 10 sequences with the greatest percentage of identity were chosen. Duplicates were excluded from the analysis. A phylogenetic tree was built by utilizing DNA sequences from a matrix of estimated pair distances using the maximum composite likelihood approach from the Kimura 2-parameter model (Kimura 1980), selected according to Bayesian information criterion score, and 1000 Bootstrap replicates in the Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6) (Tamura et al., 2013).

The DNA sequences were translated into amino acids by the European Molecular Biology Open Software Suite (EMBOSS) from the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) (Chojnacki et al., 2017) and validated by the Conserved Domains Database (CDD) of NCBI (Marchler-Bauer et al., 2017). The amino acid grouping analysis was performed by the Multiple Alignment using Fast Fourier Transform (MAFFT) software (Katoh et al., 2019) and the structural prediction was done by the structural bioinformatics web-server (https://swissmodel.expasy.org) (Waterhouse et al., 2018) using an automatic pipeline that identifies quaternary structures from the SWISS-MODEL Template Library (SMTL). For the analyses related to structural alignments, the Protein Data Bank (PDB) (https://www.rcsb.org/pages/analyze_features#Sequence) (Berman et al., 2000) and the Rhône-Alpes Bioinformatics Pole Gerland Center (PRABI-GERLAND French Institute of Bioinformatics) with the Garnier-Osguthorpe-Robson (GOR) IV method (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_ gor4.html) for prediction of secondary structures in proteins (Garnier et al., 1996) were used.

3. Results

Of 11 basidiomycete strains, seven were positive for laccase activity according to the guaiacol oxidation test and selected for the next experimental phase (Table 1).

Table 1. Laccase activity screening of basidiomycete strains by guaiacol oxidation test on malt extract agar.

Fungus	**Strain**	**Enzymatic activity***
Agaricus subrufescens	U2-4	+
Agaricus subrufescens	U7-6	-
Agaricus subrufescens	U7-7	-
Agaricus subrufescens	U6-5	-
Lentinula edodes	U6-11	-
Pleurotus eryngii	U8-11	+
Pleurotus ostreatus	U6-10	+
Pleurotus ostreatus	U2-9	+
Pleurotus ostreatus	U2-11	+
Pleurotus ostreatus	U6-9	+
Lentinus crinitus	U9-1	+

*: + = presence of laccase activity, - = absence of laccase activity.
Source: Authors (2020).

DNA fragments amplified with Plac primers corresponding to the copper-binding domain resulted in a single PCR product of approximately 430 bp from every investigated strain (data not shown) and the partial sequences ranged from 105 to 240 bp (231 bp median) were deposited in the NCBI GenBank (Table 2).

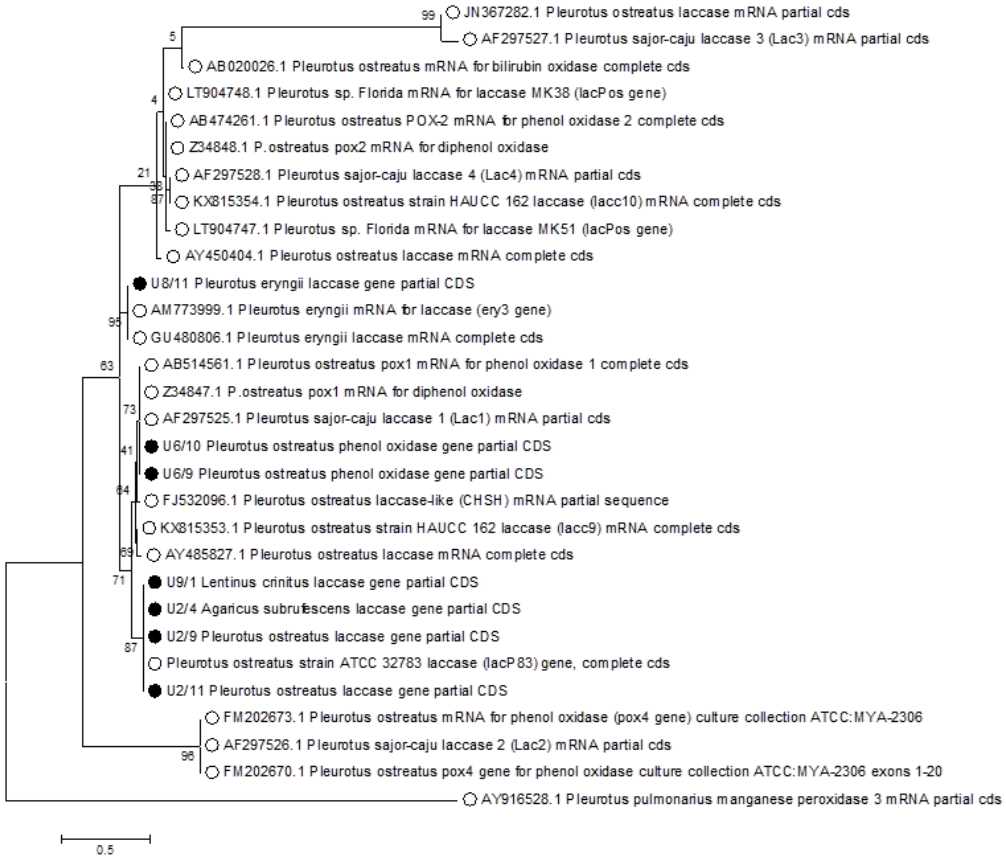
Table 2. Basidiomycete strain identification and sequencing access number deposited in the GenBank (NCBI).

Fungus	Strain	Accession number	Nucleotide sequence (bp)
Agaricus subrufescens	U2-4	KT447635	231
Pleurotus ostreatus	U2-9	KT447636	240
Pleurotus ostreatus	U2-11	KT447637	105
Pleurotus ostreatus	U6-9	KT447638	240
Pleurotus ostreatus	U6-10	KT447639	240
Pleurotus eryngii	U8-11	KT447640	225
Lentinus crinitus	U9-1	KT447641	204

Source: Authors (2020).

Phylogenetic analysis grouped strains U6-9 and U6-10 with *pox1* laccase gene of *P. ostreatus* (AB514651 and Z34847) and *lac1* laccase gene of *P. sajor-caju* (AF297525) in a cluster (Figure 1) and the strains U2-11, U9-1, U2-9, and U2-4 formed another cluster related to *lacP83* laccase gene of *P. ostreatus* (Figure 1), indicating a nucleotide sequence diversification. The *lac1* laccase gene of *P. sajor-caju* (AF297525) formed the first cluster and the strain U8-11 formed a cluster with two other *P. eryngii* gene (AM773999 and GU480806) sequences (Figure 1).

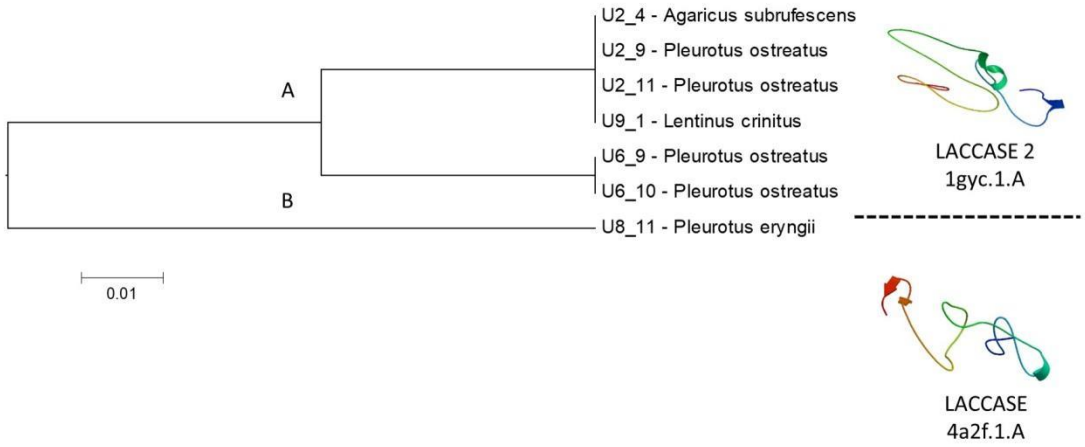
Figure 1. Molecular phylogenetic analysis by maximum likelihood method of the laccase nucleotide sequences from the study and the GenBank database. Outgroup = laccase gene sequence of *Pleurotus pulmonarius* (AY916528); ● = sequences from our study; ◯ = sequences from the GenBank



Source: Authors (2020).

All sequences of amino acids found in the nucleotide sequencing apparently belong to the multicopper oxidase domain, however, the clustering analysis of protein molecular characterization revealed the existence of two clusters (Figure 2). Cluster A formed by two subclusters with six strains (U2-4, U2-9, U2-11, U9-1, U6-9, and U6-10) and cluster B with the strain U8-11 of *P. eryngii* (Figure 2).

Figure 2. Dendrogram built from amino acid sequences and their structural predictions obtained by SWISS-MODEL. The LACCASE model (4a2f.1.A) can be associated with cluster A and LACCASE 2 model (1gyc.1.A) with the cluster B. The bar indicates the distance among strains.



Source: Authors (2020).

The analysis with the SWISS-MODEL structural predictor resulted in the coincidence of all structural predictions of the proteins belonging to cluster A, based on the LACCASE 2 model (1gyc.1.A). However, there was no coincidence with the prediction of singleton B, which was based on the LACCASE model (4a2f.1.A). The separation into two clusters was due to small changes in the U8-11 sequence of amino acids as the absence of the ALAVIN motif. These changes are responsible for the predicted proteins primary structure modifications (Figure 3). However, other aspects relative to the protein functionality may not have been affected.

Figure 3. Structural predictions obtained by GOR IV method of amino acid sequences of laccases, showing the predominance of the random coil (Cc), extended strand (Ee), and alpha helix (Hh) structures

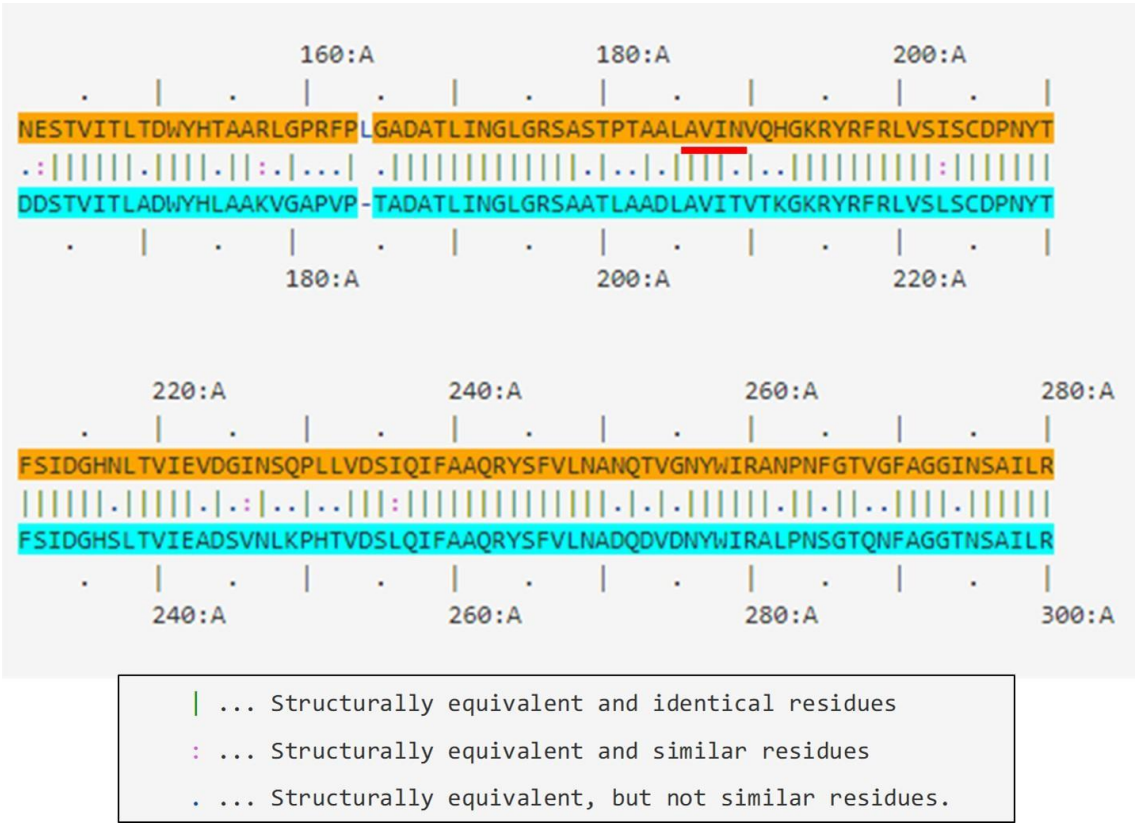
Source: Authors (2020).

The GOR IV method predicted on average 61.6% randomly entangled amino acids, 25.3% organized the extended strand and 13.2% in alpha helices (Figure 3). Besides the structural features (secondary structures), it is possible to verify that the amino acid sequences were different only at sites 8, 14, 29, 32, and 35 (Figure 3). The observed changes in sites 8,

14, and 29 may have not resulted in structural alterations because the entanglement was random for all amino acids in this position. However, in sites 32 and 35, the predicted alpha-helix in cluster A, was substituted for a simple stretched tape in singleton B and, therefore, the protein structure of the strain U8-11 was modified in relation to cluster A (Figure 2 and Figure 3). Thus, the strain U8-11 of *P. eryngii* presents a laccase isoform distinct to the ones previously described for fungi (Soden & Dobson, 2001).

The structural comparison of structural LACCASE 2 (1gyc.1.A) and LACCASE (4a2f.1.A) models in PDB (Protein Data Bank) indicates structural implications in the ALAVIN motif (red line, Figure 4) found in the cluster A and absent in the cluster B (Figure 4). However, the absence of the ALAVIN structural motif may not affect the functionality of the enzyme, but collaborates with the variability of structural isoforms that are able to perform the same function even when presenting distinct structural composition.

Figure 4. Partial structural alignment between LACCASE 2 (1gyc.1.A) model (orange amino acid sequence) and LACCASE (4a2f.1.A) model (blue amino acid sequence), pointing out the structural ALAVIN motif (red line).



Source: Authors (2020).

4. Discussion

Copper-binding sites in laccases structure are highly conserved among basidiomycetes (Cannatelli & Ragauskas, 2017). The results obtained in our study revealed that Plac primers, initially developed for highly conserved copper-binding regions of *P. sajor-caju* laccase genes (Soden & Dobson, 2001), amplified the same genetic region of *A. subrufescens* U2-4, *P. ostreatus* U2-9, U2-11, U6-9, and U6-10, *P. eryngii* U8-11, and *L. crinitus* U9-1 strains, confirming that these genetic regions are conserved among these basidiomycetes.

However, unlike the other isolates, the microorganism U8-11 showed high similarities with sequence AM773999.1 (Figure 1) being considered one another isoform for this amplified region. This indicates that although conserved, this region also has a small genetic variability.

It is important to emphasize that the laccase genes from strains U9-1, U2-4, U2-9, U2-11 were similar to *P. ostreatus* laccase gene *lacP83* (Téllez-Téllez et al., 2012) and U8-11 were similar to *ery3* from *P. eryngii* (Bleve et al., 2008). These genes show good catalytic characteristics and high stability at changes in pH and temperature (Grandes-Blanco et al., 2017), characteristics of interest in the food and agro-industrial industry. Knowing isoforms laccase it is possible to generate subsidies for the genetic improvement of these microorganisms and their biotechnological application as a result.

Structural enzymatic alterations in the metal-binding site are frequently observed in superfamilies with high functional differentiation (Valasatava et al., 2018) such as those of fungal laccases, also verified in fungal genomes with multiple isogenes for this enzyme (Sirim et al., 2011; Kües & Rühl, 2011). Regardless of any eventual mutations at the metal site, the functional role of the enzyme is generally preserved (Valasatava et al., 2018). However, even maintaining the functionality, these structural changes could affect the enzyme efficiency (Gupta et al., 2019; Kataoka et al., 2010). The hydrogen linking, Van der Waals, and ionic interactions are critical for the maintenance of structures and enzymatic functions.

Alterations in preserved sites can modify the protein conformation, affect ionic interactions, and alter enzyme activity (Gupta et al., 2019). In addition, an increase in the enzymatic activity was observed in a microorganism with two mutations in the copper-binding site (Kataoka et al., 2010), indicating that the investigation of small structural modifications is important for proper knowledge of the enzyme efficiency and, therefore, their biotechnological potential.

5. Conclusion

According to the results, the Plac primers were effective in identifying and *in silico* characterization of laccase isogene from basidiomycete strains. Also, was identified two different laccase sequences in *P. ostreatus* and a new isogenic group in *P. eryngii*. These results offer the possibility of new studies of functional roles, physiological functions, and biotechnological applications of those isogene.

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