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In silico and *in vitro* assays suggests Congo red dye degradation by a *Lentinus sp.* laccase enzyme

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ABSTRACT

Laccase is a superfamily of ligninolytic enzymes known to degrade a wide variety of xenobiotics, including synthetic dyes. Congo Red (CR) has a diazo dye function, carcinogenic and mutagenic potential, and is currently applied in clinical analysis. The objective of this work was to produce and characterize the crude extract of *Lentinus sp.* in semi-solid fermentation (FSS) and perform *in vitro* and *in silico* studies to assess the potential of the crude extract to discolor the CR dye. Laccase activity was determined using ABTS as substrate and characterized. The *in vitro* discoloration was carried out using experimental design 2^2 at room temperature and monitored at 340 nm for 24h. Molecular docking and molecular dynamics simulations were performed between laccase and CR. The maximum laccase activity production was 29.63 U L^{-1} with six days of FSS. The optimal temperature and pH were 50 °C and 3.0, respectively. Discoloration of the CR dye was obtained only in tests containing $CuSO_4$. Laccase formed stable complexes with the dye, presenting negative binding energy values ranging from −70.94 to −63.16 kcal mol^{-1} and the occurrence of seven hydrogen bonds. Molecular dynamics results showed the stability of the system (RMSD ranging from 1.0 to 2.5 Ä) and protein-ligand interaction along simulation. RMSF values pointed residues at the end of chains A (residues 300 to 305, 480 to 500) and B (residues 650 to 655 and 950 to 1000) as the most flexible regions of the laccase. This study highlighted the enzymatic action in the bioremediation of CR *in vitro* in agreement with the *in silico* simulations that demonstrate the enzyme potential.

Abbreviations: 2D: two-dimensional; 3D: three-dimensional; ABTS: 2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonate); ALA: alanine; ASN: asparagine; ASP: aspartic acid; atm: atmospheric pressure; CCIBt: Culture Collection of Algae, Cyanobacteria and Fungi; CR: Congo Red; $CuSO_4$: Copper Sulfate; GLY: glycine; Hbond: hydrogen-bond interactions; IBt: Institute of Botany; K: Kelvin; kcal: kilocalorie; LEU: leucine; MAN: mannose; mb: moisture base; MEA: malt extract agar; min: minute; mg: milligram; mL: milliliter; mM: millimolar; nm: nanometer; ns: nanoseconds; °C: degrees Celsius; PBC: Periodic boundary conditions; PDB: Protein Data Bank; pH: Potential of Hydrogen; PRO: proline; RG: Radius of Gyration; RMSD: Root Mean Square deviation; RMSF: Root Mean Square Fluctuation; rpm: revolutions per minute; SASA: Solvent Accessible Surface Area; SER: serine; SSF: Semi-solid Fermentation; $T_{1/2}$: half-life; THR: threonine; U: international enzyme unit; VMD: Visual Molecular Dynamics; w/v: weight/volume; µL: microliter; µMol: micromole.

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Introduction

Effluent discharges containing synthetic dyes represent a source of visual pollution and changes in biological cycles, causing health problems to humans and damage to the environment (Khouni et al., 2011). Thus, prior treatment of effluents is essential to improve the conditions of life on the planet since synthetic dyes is harmful to various forms of organisms. Different physical and chemical methods have been developed for the treatment of effluents containing dyes, which use Congo red as a standard (Magdalane et al., 2021; Zheng et al., 2021; Ozola-Davidane et al., 2021). The CI Direct Red 28 dye, also called Congo Red (CR) is a dye commonly used in clinical analyzes to identify different diseases (Yakupova et al., 2019). Generally, treatment chemical methods are expensive when compared to biological treatments and in general promote more toxic and reactive molecules than the original such as benzidine and carcinogenic amines (Iark et al., 2019). In this context, biological treatments using basidiomycete fungi, and the ligninolytic enzymes produced

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by them, gain space due to the low cost combined with the production of less toxic effluents (Hsu et al., 2012; Iark et al., 2019; Moreira-Neto et al, 2022; Wang et al., 2017).

The use of ligninolytic enzymes has advantages such as high efficiency, selectivity, low energy requirement and reduced environmental impact, and the use of native enzymes in industrial processes has been shown to be effective for the removal of refractory compounds (Bilal et al., 2019).

In the international scenario, Brazil is the 11th largest producer of peanuts (*Arachis hypogaea* L.), with a production of 746.7 thousand tons in the 2021/2022 cycle (Conab, 2022) The peanut shell corresponds to 22 to 32% of the mass of the vegetal and may vary according to the variety (Sales, 2022). If improperly disposed of agricultural wastes, such as peanut shells, can become an environmental problem. But on the other hand, agricultural wastes can receive a more noble destination by application in fermentative processes to produce enzymes from basidiomycete fungi (Wang et al., 2019; Peralta et al., 2017).

Basidiomycete fungi produces a ligninolytic complex whose main enzymes are Lignin Peroxidase (LiP, EC 1.11.1.14), Manganese-dependent Peroxidase (MnP, EC 1.11.1.13), Laccase (EC 1.10.3.2), Peroxidase 1.11.1.16), in addition to accessory enzymes such as glyoxal oxidase (EC 1.2.3.5), aryl alcohol oxidase (EC 1.1.3.7), oxalate decarboxylase (EC 4.1.1.2) and cytochrome P-450 monooxygenase (EC 1.14.14.1) (Bilal et al., 2017) and low molecular weight compounds (Moreira et al., 2014). Among the enzymes of the ligninolytic complex, the laccase stands out, which is a multi-copper enzyme capable of degrading lignin and xenobiotic compounds by electron abstraction with concomitant reduction of oxygen to water (Bilal et al., 2019; Noman et al., 2019; Singh & Neeraj, 2020; Silva et al., 2022). The fungus *Lentinus sp.*, a white-rot basidiomycete fungus, which causes white wood to be lost, is recognized for its ability to produce laccase and to degrade organochlorine pollutants (Ballaminut & Dácio, 2007) and synthetic dyes (Bosco et al., 2017; Moreira-Neto et al., 2013).

Comprehension of laccase and recalcitrant compounds interactions is necessary to determine their effectiveness in wastewater bioremediation, and computational biology techniques can predict and elucidate the degradation possibility of substrates, such as dyes, by enzymes (Hsu et al., 2012; Reena et al., 2014). The application of these techniques favors a reduction in the number of laboratory tests, with a consequent reduction in the time spent and financial investments to identify dyes, and other molecules, which can be degraded by enzymatic treatments (Hadibarata et al., 2013; Prabhavathi et al. 2016). In addition, the molecular docking technique has the advantage of a fast-computational response, as well as an understanding of the enzyme's functions (Awasthi et al., 2015; Singh et al., 2014).

This work aimed to evaluate the *Lentinus sp.* potential to produce and characterize a crude enzymatic extract containing laccase, in Semi-solid Fermentation (SSF) with peanut pod as a substrate, and to evaluate the use of this crude extract, rich in laccase, in the CR discoloration *in vitro* using experimental design 2^2, in addition, the study of molecular simulations the interaction of *Lentinus* sp. with CR.

Methodology

Inoculum

Lentinus sp. CCIBt 2611 was provided by Culture Collection of Algae, Cyanobacteria and Fungi (CCIBt) from the Institute of Botany, IBt, in the Secretary of Infrastructure and Environment of the State of São Paulo. The fungus was grown in 2% MEA (Malt Extract Agar) medium at 28 °C for 15 days. Five disks of mycelial growth (Ø = 0,5 cm) were used as inoculum.

Laccase production kinetics by Lentinus sp

The SSF was conducted in Erlenmeyer flasks and consisted of 3 g of peanut pods, 2% soy oil (w/v) with humidity of 70% (mb). The Erlenmeyer flasks were sterilized in an autoclave for 40 min at 1 atm. After cooling and inoculated was incubated in an oven at $28\,^{\circ}C \pm 2\,^{\circ}C$ for 30 days.

Crude extract enzyme obtaining

50 mL of 50 mM sodium acetate buffer, pH 4.8 was added to the culture systems. The contents of each Erlenmeyer flasks were manually homogenized for 5 min, stirred at 120 rpm for 1 h at 25 °C and vacuum filtered using ordinary filter paper.

Enzyme activity

Laccase activity was determined by oxidation of 2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) at 420 nm ($\grave{\varepsilon}$ = 36 mM- 1 cm-1) for 600 s, in UV spectrophotometer - 1000th, according to Ballaminut and Dácio (2007). An enzyme unit corresponded to the amount of enzyme capable of oxidizing 1 μMol of substrate per minute. The tests were performed in triplicate.

Determination of pH

The determination of the pH in the crude extract enzyme was carried out on a digital pH meter (Instrumental model pH3600).

Characterization of laccase activity of crude extract enzyme

The tests were performed in duplicate, and the residual activity was calculated. a. **Determination of the optimal reaction temperature and pH:** it was carried out by the crude extract enzyme (1 mL) incubation in the presence of McIlvaine buffer solutions (1 mL) in the pH range of 2.2 to 6.6. Optimum temperature was determined by incubating the extract at temperatures of 30 to 80 °C, at the optimum pH; **b. pH stability:** was determined by incubating the extract (1 mL) and McIlvaine buffer solution (1 mL) at

Table 1. Experimental design levels for the potential evaluation of de *Lentinus sp.* crude extract enzyme to discolor CR dye *in vitro*.

Variables	−1	0	+1
CR (mg L^{-1})	20	40	60
$CuSO_4$ (mM)	0	0.5	1

Table 2. Experimental design matrix for the study of discoloration of the CR dye *in vitro*.

Tests	CR (mg L^{-1})	$CuSO_4$ (mM)
1	20 (−1)	0 (−1)
2	60 (+1)	0 (−1)
3	20 (−1)	1 (+1)
4	60 (+1)	1 (+1)
5	40 (0)	0.5 (0)

optimum pH at 25 °C for 72 h; c. **Thermal stability:** crude extract enzyme was incubated in a water bath in the temperature range of 30 °C to 80 °C at the optimum pH.

In vitro discoloration of the CR dye by crude extract enzyme

It was evaluated by the experimental design 2^2, with triplicate at the central point. The reactive medium consisting of extract (1 mL), copper sulfate solution (250 µL), sodium acetate buffer solution pH 4.8 (250 µL), CR dye solution (150 µL) was maintained at 25 °C, in the dark for 24 h. As a control, the crude extract heated for 5 min at 80° C was used. The tests were performed in triplicate. The levels of the independent variables and the planning matrix are presented in Tables 1 and 2, respectively.

The degradation of the CR dye was determined by reading the absorbance on a spectrophotometer. The percentage of discoloration in culture followed according to Equation 1, where A is the absorbance at 340 nm:

$$\%\ discoloration = \frac{A_{control} - A_{test}}{A_{control}}\ x\ 100$$

Molecular docking simulation

The three-dimensional structure of the *Lentinus sp.* laccase was obtained from the Protein Data Bank database (PDB ID 3X1B). Water molecules were removed from the file. The structure of the CR dye was obtained from the Zinc DataBase (ZINC34801951). The molecules were submitted to the PatchDock server (https://bioinfo3d.cs.tau.ac.il/PatchDock/), in *Protein—small ligand* mode option, with a Clustering RMSD of 1.5 angstroms. The ten best-ranked complexes obtained were refined with the Firedock tool (http://bioinfo3d.cs.tau.ac.il/FireDock/php.php), downloaded and analyzed (Schneidman-Duhovny et al., 2005). The enzyme-ligand complex was submitted to Discovery Studio (http://3dsbiovia.com/products/collaborative-science/biovia-discovery-studio/) for the identification of molecular bonds. Ghecom server (http://strcomp.protein.osaka-u.ac.jp/ghecom/) (Kawabata, 2010) analyzed laccase the structure to predict the enzyme binding sites.

Molecular dynamics simulation

All simulations and analyzes were performed using the GROMACS software package, version 4.6.7 (Spoel et al., 2005) in the GROMOS 43A1 parameter set (Van Gunsteren et al., 1996). The distances and angles restrictions between the atoms of the protein and ligand were established using the P-LINCS algorithm (Hess et al., 1997), and the geometry constraints of water molecules were made by the SETTLE algorithm (Miyamoto & Kollman, 1992). The SPC water model (BERENDSEN et al, 1987) were chosen to describe the interatomic interactions of the system in solvation. Counter ions were added to neutralize the system.

Computational calculations were performed in 3 steps: minimization, equilibration, and simulation. Initially, it was performed in a balance procedure using two protocols. In the first protocol, a simulation was performed in the NPT ensemble. The temperature was kept constant around 300 K using the Berendsen thermostat (Berendsen et al., 1984). The pressure was maintained constant through the Berendsen barostat, with no value of 1 bar. In the second protocol, a 100 ps relaxation was performed in the NVT set combined with the Berendsen thermostat used for 300 K. In a second step, before simulations, the systems were submitted to the energy minimization step in order to find a set of coordinates that minimize the potential energy of the system and avoid bad contact between atoms. For this purpose, the steepest descent algorithm was used, which uses the first derivative to determine the direction to the minimum (Arfken, 1985; Morse & Feshbach, 2001).

Finally, during a molecular dynamics step, the temperature was kept constant at 300 K by means of a V-rescale thermostat with a relaxation time of 0.1 ps coupled combined for solvent and solute. Pressure was maintained at 1 bar using the Berendsen barostat in a semi-isotropic scheme with a coupling constant of 1.0 ps. Periodic boundary conditions (PBC) were applied, in addition to the SPC water model. Total simulation time was 20 nanoseconds (ns). Trajectories analysis included calculations of Root-Mean Square Deviation (RMSD), Root-Mean Square Fluctuation (RMSF), Radius of Gyration (RG), Solvent Accessible Surface Area (SASA), and Hydrogen-bond interactions (H-bond).

Analysis of results

The results were evaluated by analysis of variance (ANOVA) and graphs generated by the computer programs Statistica TM (Version 8.0, from Statsoft, Inc.), Origin 2020. Computational simulations (molecular docking and molecular dynamics) were evaluated by the Visual Molecular Dynamics (VMD) (Version 1.9.3), PatchDock (Version 1.3), Firedock (Version 1.4), Ghecom (Version 1.0), GROMACS (Version 4.6.7) and Discovery Studio (Version 19.1) tools.

Results and discussion

The peak of laccase activity production in crude extract occurred at 6 days of FSS with an activity of 29.63 U L^{-1}

(Figure 1). The pH varied from 5 to 3, with the most acidic values occurring between the 18th and the 21st day of FSS. Many efforts have been made to increase laccase productivity with cost reduction, using agro-industrial waste (Almeida et al., 2018; Ballaminut & Dácio, 2007; Iark et al., 2019; Ozcirak & Ozturk, 2017). The reduction of pH during the kinetics of laccase production can occur because ring cleavage of alicyclic and aromatic compounds, leads to the formation of organic acids from fungal metabolism (Unuofin et al., 2019).

In crude extract, laccase optimal pH and temperature were determined at 3.0 (Figure 2a) and 50 °C (Figure 2b), respectively. These values are in accordance with values for basidiomycete laccases (Singh & Neeraj, 2020). Considering that *Lentinus sp.* laccases are present in the dimeric form (Maestre-Reyna et al., 2015), the activity reduction in values above the optimum temperature may be associated with the separation of monomers of this enzyme.

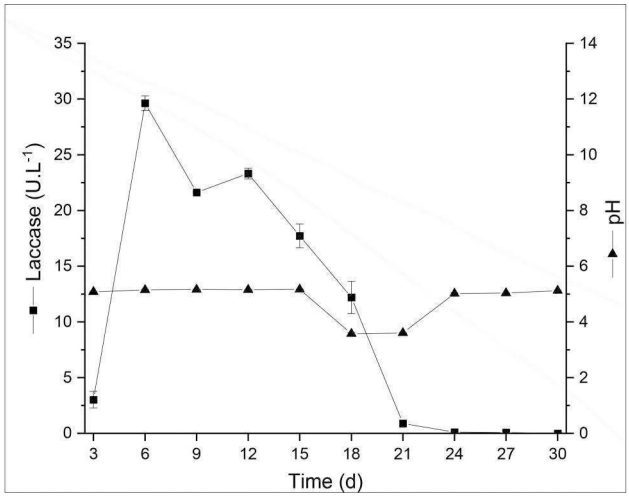

Figure 1. Kinetics profile of laccase production. Laccase activity with ABTS as substrate. The tests occurred in triplicate.

The half-life ($T_{1/2}$) of laccase activity in crude extract was 33 h at the optimum pH at 25 °C (Figure 3a) and $T_{1/2}$ at 30 min and 6 min at temperatures of 50 °C and 60 °C, respectively (Figure 3b). At temperatures of 30 °C and 40 °C, the enzyme showed high stability. The *Lentinus sp.* laccase activity proved to be more stable at the optimum pH than the *Pleurotus ostreatus* laccase, with $T_{1/2}$ of around 0.5 h (Das et al., 2016). The thermostability of laccases produced by fungi varies according to species (Singh & Neeraj, 2020).

In vitro tests showed that the discoloration of the CR dye by the crude extract enzyme occurred only in tests using the mediator, copper sulfate, with discoloration of 2.5, 2.52, and 10.4% in tests 3, 5, and 4, respectively, in 24 h. These results were higher to that found for the discoloration of red dyes by other basidiomycete fungi. The laccase of *Pycnoporus sanguineus* discolored 3.8% of the solution of the red azo dye 195 in 24 h, at 28 °C in the dark (Fabrini et al., 2016).

Diazo dyes are resistant to enzymatic treatment and require a long incubation period. Despite the low discoloration value obtained in this research, the crude extract of *Lentinus sp. is* relevant in the application in environmental treatment since this organism can reduce the 30% phytotoxicity of this dye, as reported by a study conducted *in vivo* by our research group (Coelho et al., 2020).

In factorial design, the copper sulfate and the initial concentration of CR dye showed positive effects on the discoloration dye (Table 3). In addition, increasing concentrations of $CuSO_4$ and the CR influenced the discoloration of the dye, with is confirmed by Pareto Diagram (Figure 4). In tests without copper sulfate, there was no discoloration, demonstrating the relevance of copper sulfate, which is recognized as an important mediator of laccase activity (Singh & Neeraj, 2020; Mejía-Otálvaro et al., 2021). The use of the laccase mediator system allows for the oxidation of non-phenolic compounds and substrates too large to bind to the active site (Kameshwar et al., 2018), resulting in an enhancement of their capability to oxidize compounds that the enzyme could not naturally transform (Cañas & Camarero, 2010).

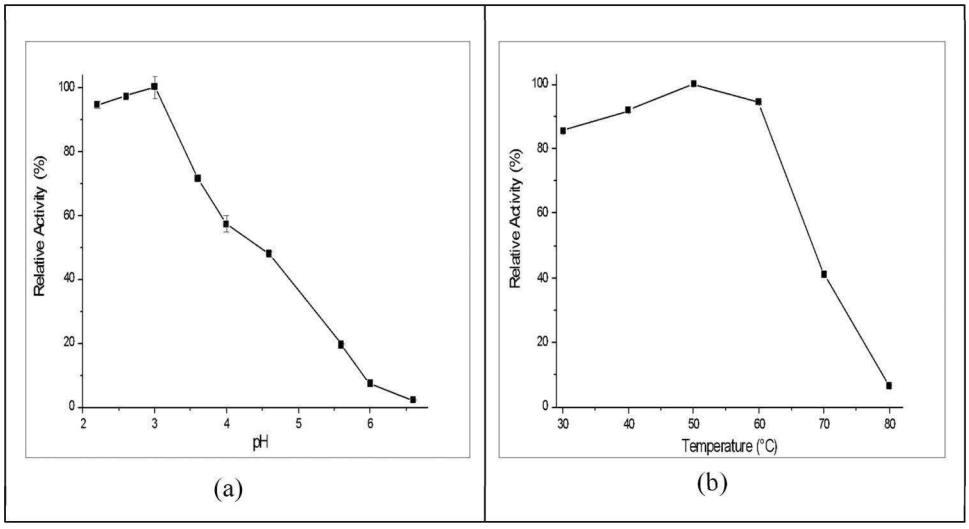

Figure 2. Effect of pH and temperature on the laccase activity of *Lentinus sp.* (a) The optimum pH was determined in McIlvaine buffer from 2.2 to 6.6 at 25 °C. (b) The optimum temperature was determined at the optimum pH. The bars indicate standard deviation of duplicates.

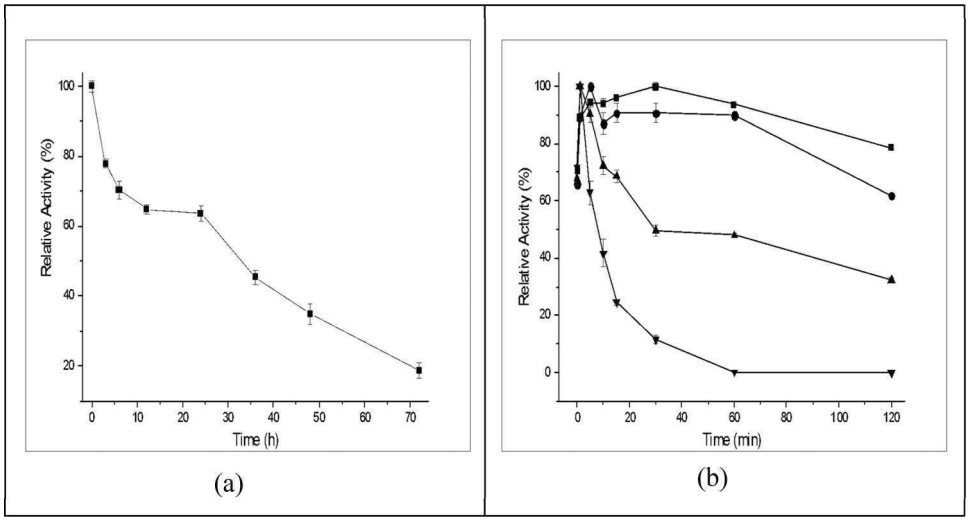

Figure 3. (a) *Lentinus sp.* laccase stability profile at optimal pH at 25 °C. (b) Thermal stability profile; thermostability at 30 °C (— ■ —), 40 °C (— ● —), 50 °C (— ▲ —), 60 °C (— ▼ —). the tests had ABTS as a substrate. The bars indicate standard deviation.

Table 3. Effect of variables obtained from factorial design 2^2 for discoloration of CR by *Lentinus sp.* (24h at 25 °C).

Factor	Effect of variables	*p*-value ($p < 0.1$)
Media	3.295268	0.013589
(1) Congo Red	3.950803	0.053299
(2) $CuSO_4$	6.450803	0.021030
1 by 2	3.950803	0.053299

The factors showed significance (*p*-value < 0.1).

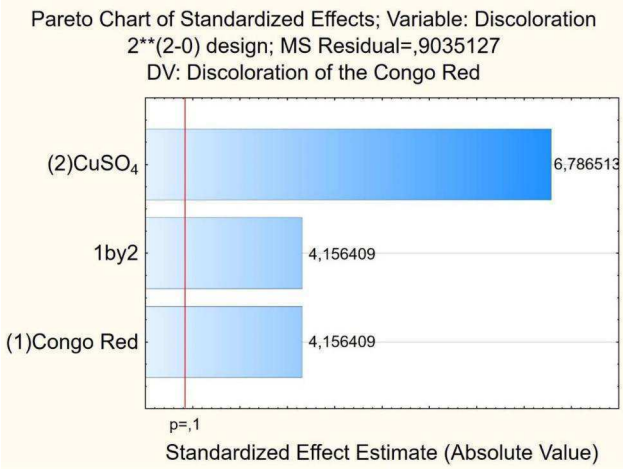

Figure 4. Pareto Diagram for evaluating the effect of initial concentrations of CR and $CuSO_4$ on the discoloration of the CR dye. The factors with significance (*p*-value < 0.1).

This discoloration of the CR dye showed statistically significant results ($p < 0.1$) in the effects of the variables for the model that predict the initial concentration of CR and $CuSO_4$ and the interaction between these variables. The coefficients obtained according to the significant variables confirmed by the Pareto Diagram led to the proposition of a mathematical model: y (discoloration of the dye) = 3.295268 + 1.975402 (CR dye) + 3.225402 (copper sulfate) + 1.975402 (CR dye × copper sulfate).

Statistical significance of the mathematical model was tested by ANOVA (Test F). The model could be considered to be predictive due the F value was significant ($F_{calculated}$ (22.7) > $F_{tabulated}$ (9.16)) with a high percentage of correlation coefficient (R^2 = 90.35). Therefore, it was possible to generate the response surface and contour plot (Figure 5), which suggests the optimization of the experiment to the experiment to explore the discoloration of the CR in higher concentrations of copper sulfate.

The laccase-CR complexes (Figure 6) obtained from the molecular docking simulation showed negative values of binding energy (-70.94 to −63.16 kcal mol^{-1}), this indicates affinity between these molecules (Maia & Amador, 2018). This result corroborates with the versatility and no specificity of laccases to substrates (Martínez-Sotres et al., 2015; Singh et al., 2016).

The CR showed interaction with the two enzyme monomers (A and B), identified each residue in the interaction with dye (Figure 7). Six interactions were identified of hydrogen between residues THR-A:175 and THR-B:175 of the laccase with two sulfonate groups (SO_3) of the CR and the residues GLY-A:186, SER-A:187, THR-A:200 and SER-B:187; and one interaction with the glycan MAN-A:604. All these interactions are important for the establishment of the laccase compound - CR and consequent degradation of the CR molecule. Additionally, these interactions show the importance of the SO_3 and amine groups in the establishment of the laccase-CR complex and the role of protein glycosylation in the laccase functionality (Maestre-Reyna et al., 2015). Although there are unfavorable interactions with aromatic structure (ASP-A:188 and ASN-A:288), the hydrogen bonds remain in a favorable interaction. When analyzed by comparing with Ghecom's output, it was possible to verify that all residues recognized are in pocket 1 (Supplementary Material).

The molecular docking results, associated with the *in vitro* results obtained here, represent evidence for laccase capacity to metabolize CR dye. Synthetic effluent CR contends degradation was identified by crude extract enzyme from *Lentinus sp.* (Coelho et al., 2020) and for a *Oudemansiella canarii* partially purified laccase (lark et al., 2019) of, different

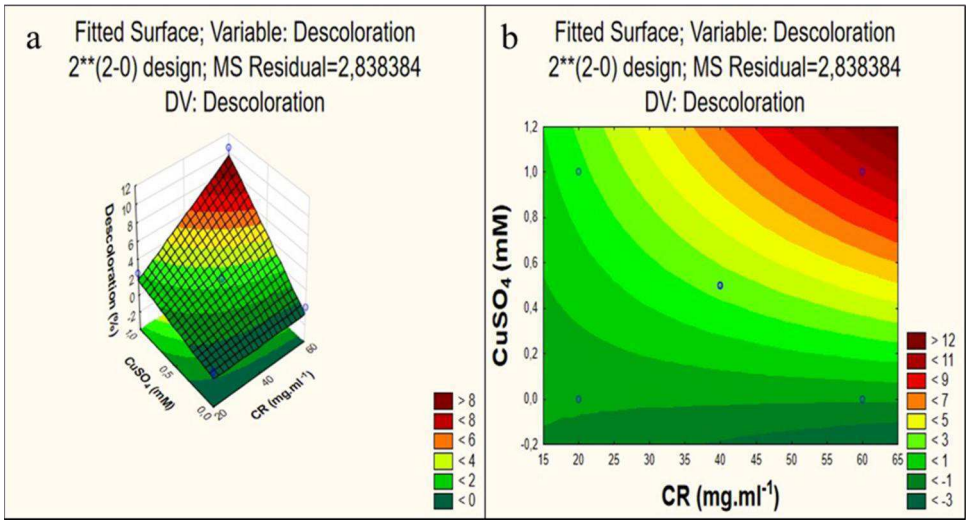

Figure 5. Response surface graph (a) and contour graph (b) for evaluating the effect of initial concentrations of CR and $CuSO_4$ on the discoloration of the CR dye. The red band indicates the significant effect on the 90% confidence level.

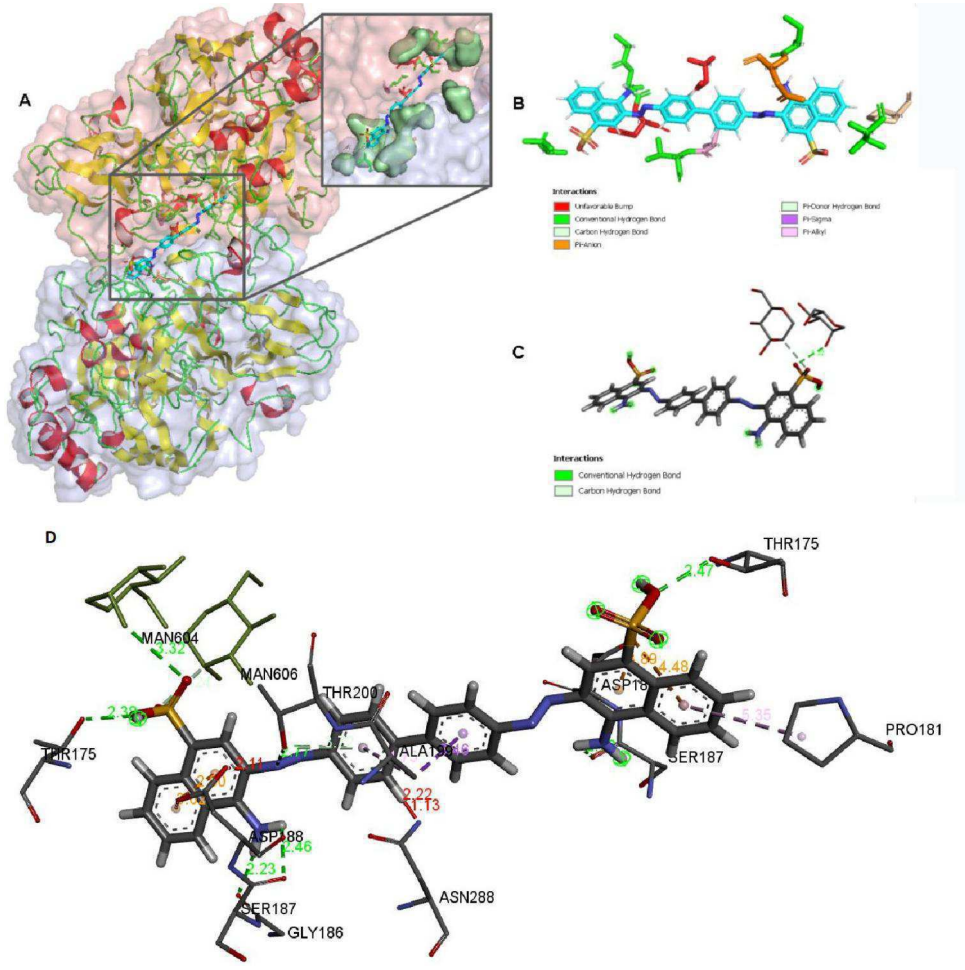

Figure 6. Interactions residues from laccase catalytic site and CR dye. A: laccase enzyme showing chain a on pink surface and chain B on light green surface. B: colored amino acids according to the type of interaction with CR. C: Hydrogen bonds between CR. and mannoses. D: epresentation of residues and mannoses that interact with CR.

from that observed for a *Ceriporia lacerada* laccase, in which there was an increase in toxicity due to the generation of toxic intermediates (Wang et al., 2017).

Torsion angle (dihedral) values for CR were measured before and after molecular docking. However, the geometry of the ligand did not show expressive changes, as can be observed in the Figure 8.

RMSD (*Root-mean-square deviation*) values from molecular dynamics trajectories are plotted in Figure 9. Ligand and enzyme RMSD initiated with low values (∼ 2.5 Ä and 1.5 Ä

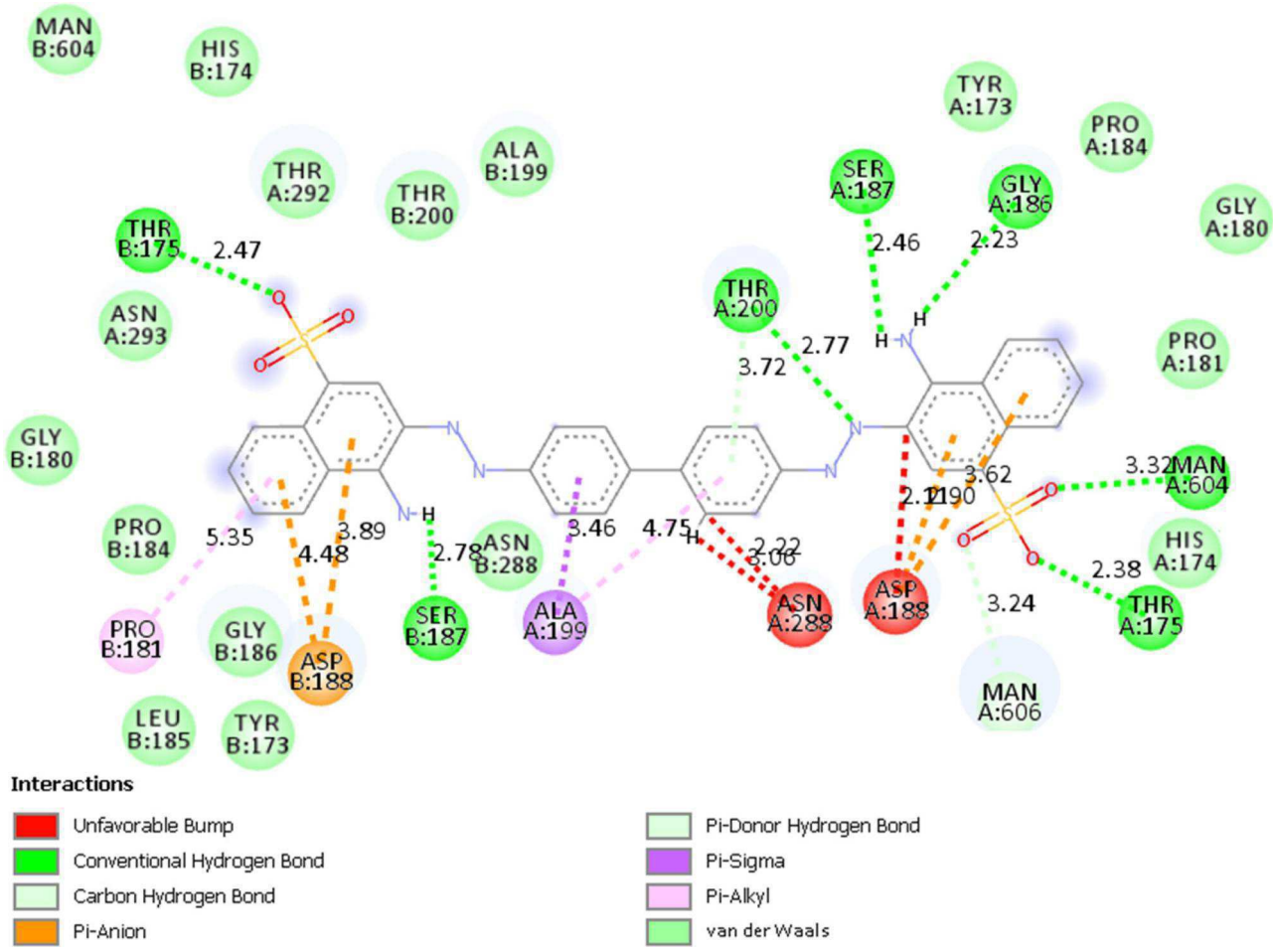

Figure 7. Representation of molecular interactions in the organic residue of CR dye from the enzyme-ligand complex. Legend: ASN288, ASP188 - unfavorable collision; GLY186, SER187, THR200, SER187, MAN604 - conventional hydrogen bonding; MAN606 - carbon-hydrogen bond; ASP188 - Pi-anion; ALA199 - Pi-Sigma; PRO181, ALA199 - Pi-Alkyl.

respectively) which indicates the stability of the system. Throughout the simulation, it is possible to observe that the ligand shows a slight increase in its RMSD 40 nanoseconds and 70 nanoseconds at ∼1.3 Ä, and then shows a slight decrease and keeps stable until 100 ns. Laccase, from approximately 10 ns reaches an RMSD of 4 Ä. At 40 nanoseconds, RMSD increases to the range of 5 Ä suggesting a new sub-conformational state, probably in response to the interaction with the ligand.

The low RMSD values among all simulation reinforces the enzyme-ligand complex stability. Many molecular dynamics studies of protein-ligand systems pointed low RMSD values as an indicator of the enzyme (Knapp et al., 2011; Pontes et al., 2016). The RMSD value from the simulation converged to under 1.0 Ä, a very similar value obtained in an *in silico* study with *Trametes versicolor* laccase (average RMSD ∼0.92 Å) (Christensen & Kasper, 2013).

Figure 10 shows the radius of gyration (RG) values. This parameter is useful to infer the expansion and compaction processes of globular proteins.

When analyzing the RG along with the graph, it is possible to notice that laccase alternate between processes of compaction and expansion with a subtle tendency to an increase in values. The final RG was in the range of 3.2 nm. This result suggests that the enzyme tends to compact itself slightly to accommodate the ligand. Radius of gyration is an important measurement of protein compactness and size associated with structure stability (Arnittali et al., 2019). In laccase enzymes, RG values showed lower values (Christensen & Kasper, 2013) which leads us to conclude that the interaction with CR induces an increase of protein size. An interesting behavior of laccase is that its radius of gyration increases from 40 ns onwards and reaches a stable pattern compatible with a conformational state, indicating an enzyme expansion process. This corroborates the conformational change observed in the RMSD graph, reinforcing the possible accommodation of the protein structure when interacting with the ligand.

SASA values for laccase are available in the graph in Figure 11, which is a relevant measurement of the solvent-accessible surface area of a molecule.

The SASA values increased slightly throughout the simulation. This indicates a decisive factor in the structural stability of the protein since the values remain little variable throughout the simulation. This result reinforces the stability suggested by the RMSD and RG values.

Figure 12 displays the occurrence of hydrogen bonds between laccase and CR dye of the entire course of the simulation.

The number of hydrogen bonds between protein-ligand ranged between 0 and 7 with an average of 3–4 bonds.

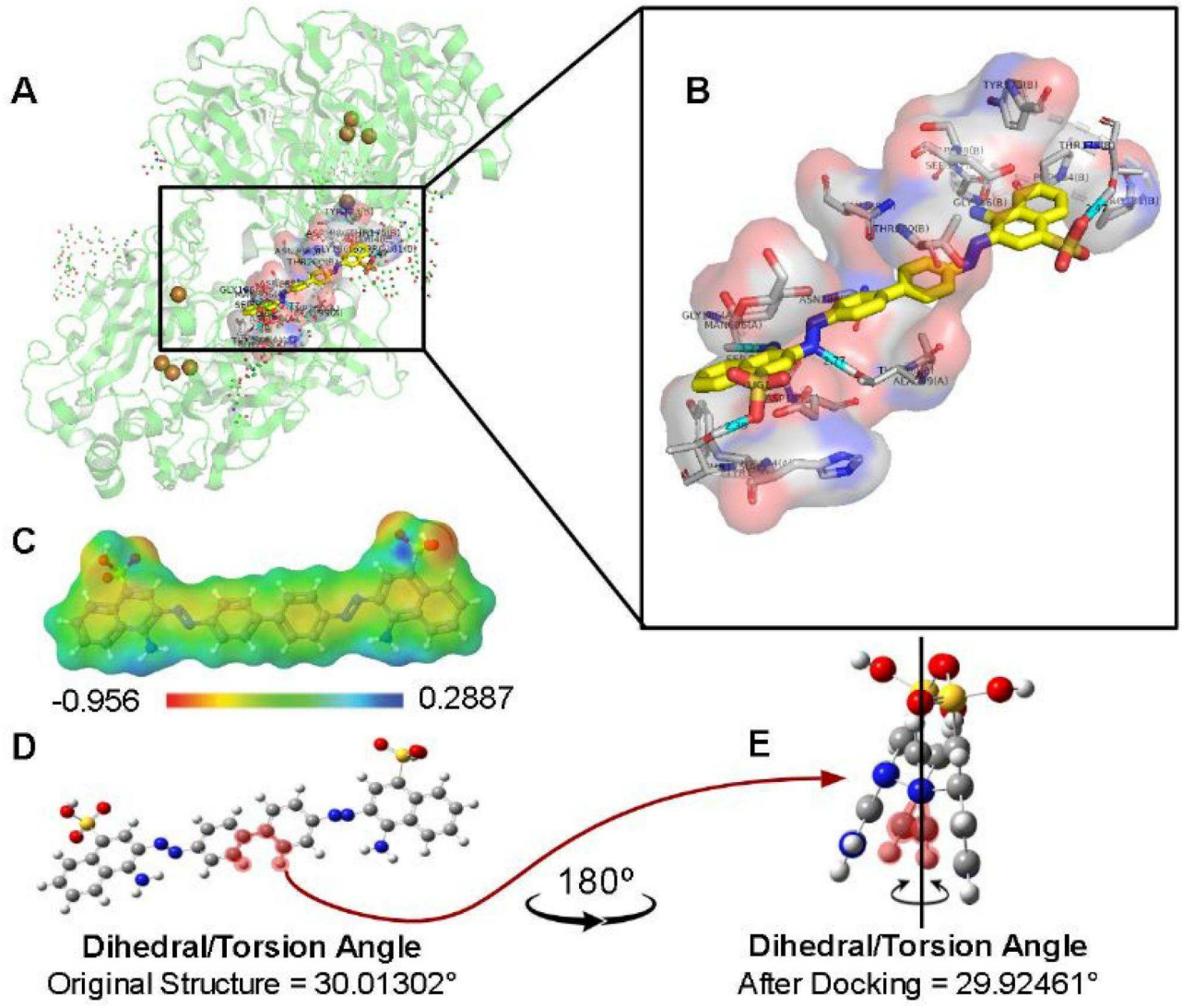

Figure 8. (A) Enzyme-ligand complex (laccase-CR) with copper ions. (B, C) Congo red electrostatic surface. (D) CR torsion angle before molecular docking. (E) CR torsion angle after molecular docking.

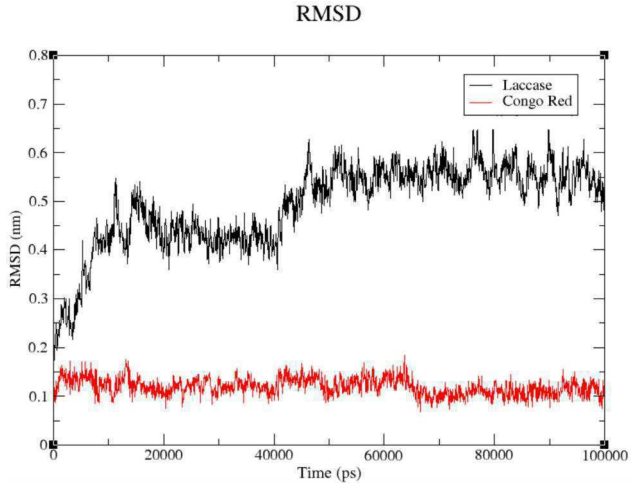

Figure 9. RMSD values of laccase enzyme and CR ligand.

H-bonds between molecules are present in entire simulation, with just some sparse peaks. It is evidence that the ligand interacts directly and continuously with the enzyme.

In order to investigate the most flexible regions of laccase, we performed RMSF (Figure 13).

In Figure 14, it is possible to see that the regions of high fluctuation occurred more in the chain A. This is a very interesting result because *Lentinus sp.* laccase is a homodimeric enzyme (Liu, 2014). The regions of greatest fluctuation are located in different domains of the chains, and it is possible to observe that some copper atoms are near to residues located in these regions of flexibility. This asymmetric behavior between the two subunits can be explained by the interaction of Congo red with this protein. This dye interacts in the interchain region of the enzyme, having anchor residues on both strands. However, it is possible to see in the 2D diagram (Figure 7) that the dye interacts in greater number with residues from the chain A. This greater interaction seems to be the reason why there is a major interference in the atomic fluctuations in chain B than in chain A. After the simulation, it was possible to verify a very interesting interaction involving the LEU185 residue (Figure 15).

Fu et al. (2018) recommends small distances of molecular bonds in molecular docking experiments (<3.5 Å). In

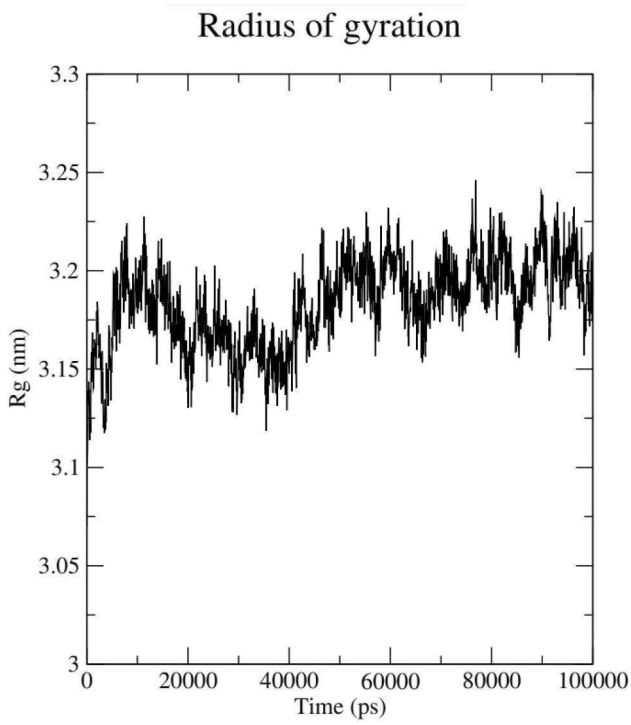

Figure 10. Radius of gyration for laccase enzyme.

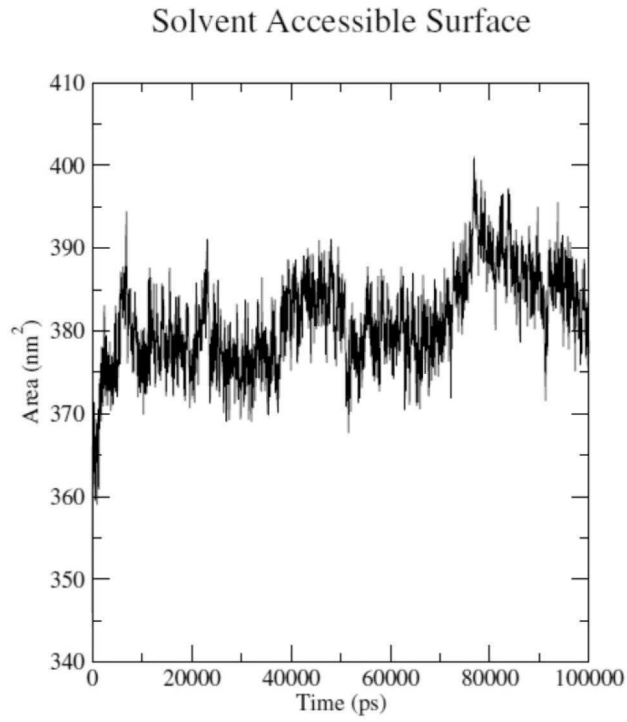

Figure 11. SASA values for laccase enzyme.

addition, the 6.76 Ð distance between the LEU185 residue and the copper T1 of laccase reinforces the need for the action of mediators of laccase activity and explains the discoloration *in vitro* only in presence of copper sulfate. In the literature, the action of low molecular weight compounds has been reported to be responsible for dye discoloration (Awasthi et al., 2015; Moreira-Neto et al., 2013).

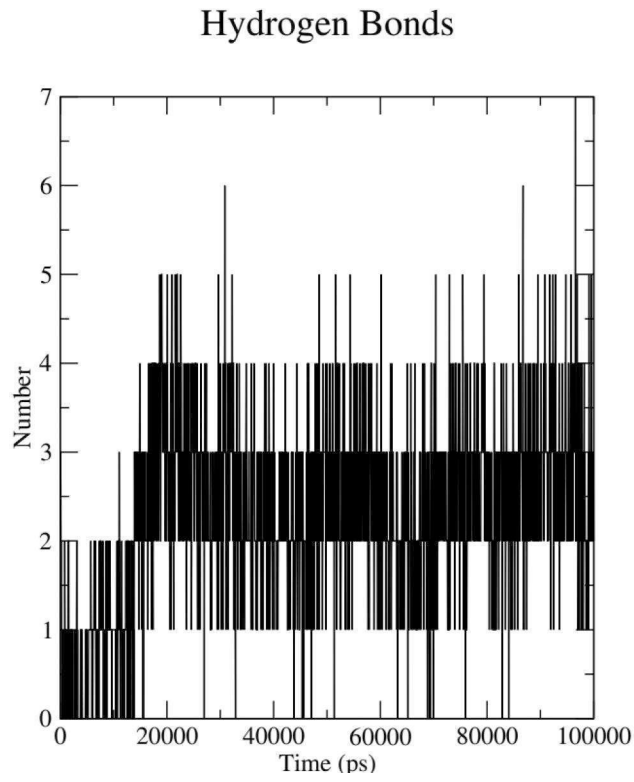

Figure 12. Number of hydrogen bonds formed between laccase and CR dye.

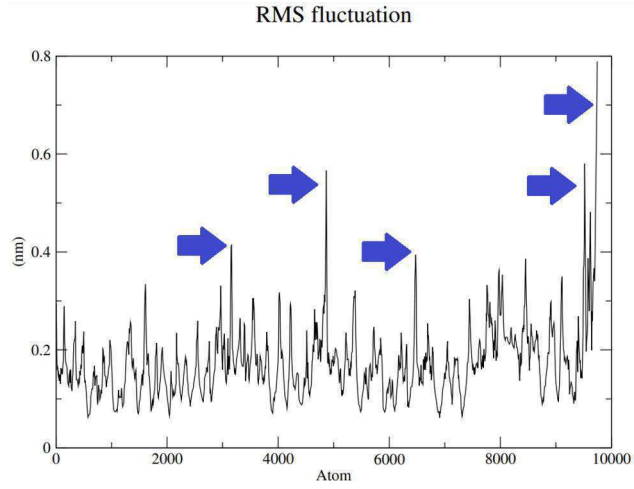

Figure 13. Root-Mean square fluctuation of laccase atoms. Arrows indicates the most flexible regions. The regions of greatest atomic fluctuation occurred at the end of chains A (residues 300 to 305, 480 to 500) and B (residues 650 to 655 and 950 to 1000). in chain B a high fluctuation peak (residues 970 to 1042) located in the C-terminal region is observed (Figure 14).

The LEU185 from chain A performs H-bond interaction with an aromatic ring of the CR dye. This residue also seems to interact directly with a copper atom. Maybe this residue performs a key interaction in dye catalyzing, possibly being an anchor residue.

Is it possible to use *Lentinus* laccase in the bioremediation of Congo red effluents? Potentially, yes. According to the results obtained here, in both experimental and *in silico* assays, laccase from *Lentinus* sp. shows a promising degradation activity of this polluting dye. However, this bioremediation potential must be test *in situ* in the future to ensure the efficiency and safety of laccase as a CR bioremediatory.

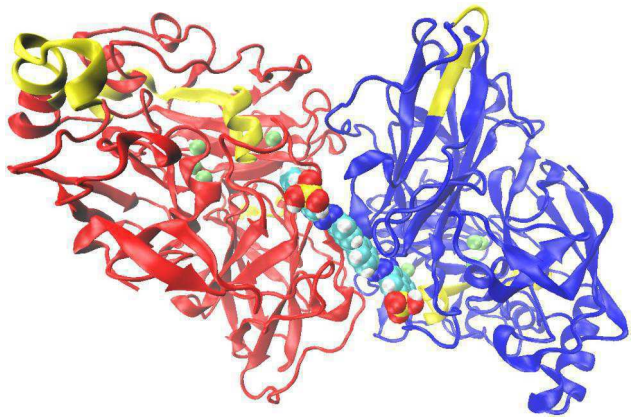

Figure 14. 3D Representation of laccase-CR complex highlighting the regions of greatest fluctuation. In blue, the chain A; in red, the chain B; in yellow, the regions with higher RMSF peaks. The CR dye (VDW representation) binds in an inter-chain region. Silver beads represent the copper atoms. Source: research data edited by VMD software.

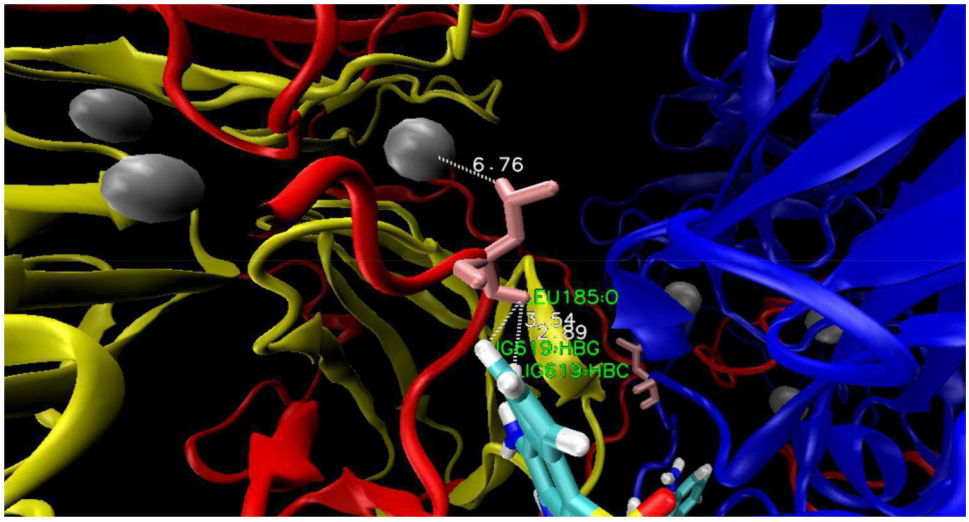

Figure 15. Deep view of the protein-ligand site. Silver beads are copper atoms; in licorice (pink), representation is the LEU185 residue; the CR representation is in atom type coloring.

The discoloration of dyes *in vivo* by laccase-producing fungi of the genus *Lentinus* is well documented in the literature (Almeida et al., 2018; Moreira-Neto et al., 2013), but studies *in vitro* favor the application of enzymatic technologies *in vitro* enable of treating the effluent without an alien microorganism being introduced into a new ecosystem, which represents an important advance in environmental protection.

Conclusion

The *Lentinus sp.* fungus was able to produce a relatively stable crude extract with potential industry use, using as substrate the peanut pod. In addition, this study evidenced the action of laccases in the bioremediation of the dye through the action of copper sulfate as an important mediator of CR discoloration. Screening with other redox mediators in the process of *in vitro* discoloration of dyes needs to be investigated.

Following the *in vitro* responses, the *in silico* analyses demonstrate the enzymatic potential of *Lentinus sp.* laccase in the remediation of diazo dyes by the occurrence of hydrogen bonds and the indication of the change of conformational state of the enzyme due to laccase-CR interaction, stimulating the optimization of technologies focused on the treatment of synthetic dyes.

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