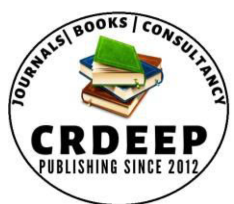

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International Journal of Environmental Sciences
(ISSN: 2277-1948) (Scientific Journal Impact Factor: 6.043)

UGC Approved-A Peer Reviewed Quarterly Journal

Full Length Research Paper

A Comprehensive Assessment of the Environmental Impact of the Laccase-Mediated Degradation of Azo dyes by *Phanerochaete chrysosporium*

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ARTICLE DETAILS

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Key words:
P. chrysosporium, Azo dyes, Corazol Violet SR, Guaiacol, Laccase and Solid and liquid state degradation

ABSTRACT

P. chrysosporium is a white rot fungus known for its ability to produce laccase, an enzyme that plays a crucial role in the degradation of azo dyes, particularly reactive dyes. In this study, experimental evidence has clearly shown the effectiveness of laccase in degrading textile dyes, including Red color, Blue CA, and Corazol Violet SR dye. The current study emphasizes the *P. chrysosporium* laccase production, optimization with various parameters and solid and liquid state degradation of azo dyes. Laccase production was significantly enhanced by the addition of guaiacol at a concentration of 100mM, resulting in a laccase high yield of 42.82 U/ml observed on the tenth day of incubation. Additionally, a process of optimization was conducted to stabilize enzyme production under various conditions to improve its efficacy in the dye degradation process. The study also evaluated laccase production with various carbon sources. Among these, sucrose yielded the highest laccase production at 41.93 U/ml. Optimal temperature and pH conditions were identified, with 30 °C producing 29.59 U/ml and pH 4 resulting in 28.70 U/ml. For nitrogen sources, urea achieved a production of 24.28 U/ml. additionally, the presence of $CuSO_4$ at a concentration of 2.5 mM contributed to a laccase production of 24.90 U/ml. further, the study focused on the solid-state degradation of the reactive dye, degradation commenced on the fourth day of incubation, with complete degradation achieved by the tenth day on Corazol Violet SR only. In contrast, other dyes were not fully degraded by *P. chrysosporium* under similar conditions. During the liquid-state degradation process, the reactive red dye showed complete discoloration by the tenth day, whereas the other dyes remained undegraded by *P. chrysosporium*. In this study, we observed that using the same medium and similar organisms resulted in different outcomes in the degradation process. Specifically, the same organism exhibited different dye degradation properties in solid-state and liquid-state methods. This discrepancy highlights an intriguing aspect that warrants further investigation into the underlying mechanisms of degradation. Understanding these differences could provide valuable insights into optimizing dye degradation techniques.

1. Introduction

1.1 Biocatalyst Laccase

Indeed, biocatalyst has gained significant attention as a promising alternative for environmental remediation due to its potential to address many of the challenges associated with traditional chemical-based methods. Biocatalyst involves the use of natural catalysts, such as enzymes, to facilitate chemical reactions. These enzymes are typically derived from living organisms and have the ability to accelerate specific chemical transformations (Givaudan et al. 1993). The use of enzymes, particularly oxidoreductases like laccase, for the removal of dyes from natural water bodies has indeed emerged as a

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Received: 12-Nov-2024; Sent for Review on: 15-Nov--2024; Draft sent to Author for corrections: 22-Nov -2024; Accepted on: 30-Nov--2024; Online Available from 03-Dec-2024

DOI: 10.13140/RG.2.2.33981.37606

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significant application of biocatalysts. Laccase have gained attention due to their broad substrate specificity and ability to catalyze the degradation of various dye molecules (Abdullah et al. 2000). To enhance the catalytic activity of laccase and improve their overall performance, researchers have explored different methods, with immobilization being a widely adopted approach. Immobilization involves attaching or confining the enzyme onto a solid support material, which can be in the form of particles, fibers, or membranes. This immobilization process offers several advantages for laccase in the context of dye removal (Parshetti et al. 2007). The utilization of immobilized laccase for the detoxification and decolorization of dye effluents has shown great potential as an eco-friendly and efficient approach. Immobilization methods include physical adsorption, entrapment within matrices, covalent binding, and cross linking. Each method offers unique advantages and can be optimized based on the specific requirements of the application. To further enhance immobilized laccase stability, reusability, and general performance, more research and development is being done in this area. By combining the advantages of laccase with the benefits of immobilization, this biocatalytic solution can play a revolutionary role in addressing the environmental challenges posed by dye effluents (Antonio Ballesteros; Miguel Alcalde et al. 2005*).* Laccase, a member of the polyphenol oxidase enzyme family, is notable for its multicopper-containing structure. Classified among the blue oxidases, this enzyme is essential for catalyzing the oxidation of various organic and inorganic compounds, such as phenols, polyphenols, diamines, and aromatic amines *(*Angeles Sanroman *et al.* 2006*)*. The enzyme facilitates the removal of one electron at a time from the substrate while utilizing molecular oxygen as the electron acceptor. This process results in the formation of a free radical as the substrate loses a single electron but the resulting unstable radical proceeds through a number of non-enzymatic processes, including disproportionate, polymerization, and hydration (Gianfreda *et al.* 1999). One of the notable features of laccase is its ability to reduce molecular oxygen to water while simultaneously oxidizing substrates. This dual functionality makes laccase a versatile enzyme with significant applications in various industries (Buddolla Biswanath *et al.* 2014).

1.2 Fungal laccase

Laccase are widely distributed in nature, being found in higher plants, bacteria, fungi, and even insects. In the plant kingdom, laccase are present in vegetables such as cabbages, turnips, potatoes, pears, and apples. Moreover, laccase have been successfully isolated from various types of fungi including Ascomycetes, Deuteromycetes, and Basidiomycetes. Among these, the white-rot Basidiomycetes fungi stand out as efficient lignin degraders, making them crucial players in biodegradation processes. In comparison, Ascomycetes and Deuteromycetes fungi tend to focus more on the oxidation of phenolic compounds (Robinson et al. 2001). The diverse distribution and functional versatility of laccase highlight their importance in various biological processes and industrial applications. From environmental roles in lignin breakdown to their potential use in biotechnology, laccase continue to captivate researchers and industries alike due to their unique enzymatic capabilities (Kersten et al., 2007). Fungal laccase play a significant role in the degradation of lignin and the protection against toxic phenolic monomers derived from polyphenols (Kiiskinen et al. 2004) These enzymes have demonstrated their utility in various industrial applications, ranging from preventing wine discoloration to aiding in paper processing, dye oxidation, detoxification of environmental pollutants, and chemical production from lignin. In particular, laccases are effective in decomposing azo dyes through oxidative methods (Michael et al. 2005). Compared to ligninolytic peroxidase, laccase possess a lower redox potential, typically within the range of 450 to 800 mV.

1.3 Phanerochaete chrysosporium laccase

One of the hallmarks of this intricate engagement is the appearance of ethereal white patches, akin to cellulose canvases, etched upon the remnants of plant structure. These patches are the result of a mesmerizing vanishing act – lignin, a structural component that once fortified the plant, dissolves into the shadows (Roca albas et al. 2013). This vanishing act, orchestrated by P*. chrysosporium*, illuminates the path for other organisms to join the feast of transformation, paving the way for new life forms to emerge from the ashes of the old. An intriguing facet of *P. chrysosporium* is its benign nature towards humans and animals. Unlike some of its fungal counterparts, this enigmatic entity refrains from inflicting harm upon living creatures. It remains a silent, yet pivotal, contributor to the orchestration of life's cyclical journey. The current study is focused on the efficient production of laccase using *P. chrysosporium*, an organism isolated from the Tamarind tree. This research aims to explore the diverse aspects of its ability to degrade azo dyes, holding great promise for environmental and industrial applications. This study centers on optimizing the production of laccase from the *P. chrysosporium* strain, recognized for its unique enzymatic capabilities. The Tamarind tree, from which this strain was isolated, adds an interesting ecological dimension to the research. Understanding the intricate mechanisms underlying the enzyme's interaction with azo dyes is crucial. The study may involve spectroscopic techniques and kinetic analyses to elucidate the enzymatic pathways and reaction intermediates involved. The findings of this research hold immense potential for practical applications. The laccase enzyme from *P. chrysosporium* could be employed in wastewater treatment plants, textile industries, and other sectors requiring dye removal, contributing to sustainable practices. This involves evaluating the toxicity of the degradation products and their potential effects on the ecosystem.

2. Methodology

2.1 Collection of sample

The fungi used in the study were collected from the tree of Tamarind bark in the area of Potheri, Chengalpattu District. The fungal sample was obtained in sterile and sealed, plastic covers, and transported to the lab under aseptic conditions for further analysis.

2.1.1 Isolation of laccase producing fungi

The gathered fungal samples were finely diced using aseptic blades. Potato Dextrose Agar (PDA) was prepared and subjected to sterilization. After sterilization, the media was enriched with guaiacol and meticulously mixed. Subsequently, it was poured into sterile petri plates and left to solidify. The isolated fungal specimens were then sectioned into small fragments utilizing sterile blades and positioned onto the Potato Dextrose Agar plates. These plates were subjected to aerobic incubation at a temperature range of 27°C to 30°C for duration of 8-10 days, facilitating the growth of fungi.

2.1.2 Identification of laccase producing fungi

2.1.3 Macroscopic and Microscopic Examination

Fungal identification using macroscopic methods involves observing and analyzing visible characteristics of fungal colonies grown on agar plates. After incubation, observed the fungal colonies' macroscopic characteristics such as color, texture, elevation, margin, Size and Surface features and all results noted and tabulated. Macroscopic characteristics provide valuable information, but isolated fungi require microscopic examination for accurate identification. Prepared slide with a small piece of the colony and added LBCP then covered with a cover slip. The fungal sample was observed under a compound microscope to examine the fungal morphology and results were noted.

2.2 Fungal Genotype identification

2.2.1 Fungal DNA Extraction Procedure

Two ml eppendorf tubes should be sterilized. Add 0.5 ml of extraction buffer to one set of tubes and 0.3 ml of isopropanol to the other set. Using a toothpick, remove 10–30 mg of fungal mycelia from the PDA plate and transfer it to an extraction buffer. Homogenize the fungal mass with a pestle (previously dipped in 70% ethanol). The fungal cell lysate was centrifuged at 5000 rpm for ten minutes. The supernatant should be disposed of with isopropanol. After carefully inverting the tubes to combine the lysate and isopropanol, centrifuge for ten minutes at 12,000 rpm. Discard the supernatant. Wash the pellet with 0.8ml of 70% ethanol, then centrifuge at 12000 rpm for 10 minutes. Discard the supernatant, and dissolve the pellet in distilled water. Mix extracted DNA with gel loading dye and loads it onto a 1% agarose gel for visualization (Houhala *et al.* 2018).

2.2.2 PCR Amplification

The following PCR reagents sequentially to PCR tubes: Sterile water: 15.3µl,10X buffer: 2.0µl,10mM dNTPs: 0.2µl, the following primers were used for amplication, 0.5µl of forward primer, 0.5µl of reverse primer, 1µl of template DNA, and 0.5µl of Taq polymerase. 40 cycles of denaturation and activation at 95°C for 15 minutes, denaturation at 94°C for 20 seconds, annealing at 60°C for 70 seconds, and extension at 72°C for 1 minute are the conditions under which PCR runs. Mix the PCR product with gel loading dye and load onto a 1.5% agarose gel. Run the gel at 70V for 1 hour until the tracking dye reaches 3/4 of the gel length. Visualize the gel using a gel imager.

2.2.3 DNA Sequencing (Sanger's Method)

2.2.3.1Template Preparation:

Prepare template strands with a short known sequence at the 3' end. Prepare a DNA primer with a known sequence at the 3' end. Attach the template strand to a single-stranded cloning vector (M13) at the 3' end for primer binding.

2.2.3.2 Generation of Nested Fragments:

Divide each template into four branches for different replication reactions. Use standard primer and DNA polymerase-1 in all branches. Add ddATP to terminate the reaction in batch one, and similarly ddCTP, ddGTP, and ddTTP to other batches.

2.2.3.3 Electrophoresis and Gel Reading:

In separate wells on a polyacrylamide gel, load reaction mixtures from each branch. To ascertain the base order of the complementary strand, conduct electrophoresis and examine the gel's autoradiogram. The autoradiogram can be read from bottom to top in sequence because the bands of the shortest fragments are located at the bottom.

2.3 Laccase Production from production media

The *P. chrysosporium* had grown for seven days on Potato Dextrose Agar plates, which were used to produce laccase; it was sliced into tiny discs (5 mm in size). A total of ten Potato Dextrose Agar discs with fungal mycelia were put into 250 ml Erlenmeyer flasks holding fifty milliliters of liquid culture medium. The media compositions used for laccase production were (g/l) glucose-20, peptone-5, ammonium tartrate-10, yeast extract-1.0, KCl-0.5, KH2PO4-1, MgSo4.7H2O-0.5, CuSo4.5H2O0.25, and pH adjusted to 5.5. 100 mM guaiacol (substrate) was used to induce the fungal culture in order to produce laccase in the medium and the medium without guaiacol was kept as a control. Each culture flask was incubated on a rotary shaker set at 120 rpm for 7–10 days at a temperature of 28–30°C. Every 24 hours, samples were taken out of culture flasks, filtered, and centrifuged for 10 minutes at 10,000 rpm. The supernatant was then tested for enzyme activity. A triplicate assay was performed.

2.3.1 Laccase activity measurement
Guaiacol was used as the substrate in a UV-VIS Spectrophotometer to measure laccase activity. Three milliliters of a 100 mM guaiacol solution diluted in a sodium acetate buffer containing 10% acetone and one milliliter of the culture filtrate made up the reaction blend. After incubating for 15 minutes, the absorbance was measured at 470 nm. When the enzyme helped convert the substrate (guaiacol) and produced one milliliter of colored product per minute that was the measurement of one unit of laccase activity. All of the components had been included in the blank, with the exception of the functional enzyme.

2.4 Optimization of laccase production
Continuous optimizing investigations were carried out with the traditional method of one element at a time. Laccase activity was evaluated every 24 hours while the fungal culture was cultured for 7–12 days to find the ideal duration for laccase formation. Flasks were incubated under both natural and induced circumstances at a range of temperatures, from 25°C to 60°C, in order to assess the impact of temperature on enzyme synthesis. Furthermore, the effects of various pH values (pH 2, 3, 4, 5, 6, 7, and 8) on the synthesis of enzymes were examined. Investigations were also conducted on the effects of other carbon sources on laccase yield, including glucose, fructose, and sucrose at 100 mM each. Additionally, the impact of 100 mM sodium nitrate, ammonium chloride, and urea as nitrogen sources on laccase synthesis was investigated. After the inoculation, the culture medium was supplemented with copper sulfate at concentrations of 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM after 24 hours. The impact of this addition on laccase production was assessed after the fourth day of incubation.

2.5 Decolorization of Textile Dyes
Reactive dyes play a pivotal role in the textile industry. This study focuses on three specific reactive dyes: i) Blue CA, ii) Corazol Violet SR, and iii) Red color dye.

2.5.1 Solid State Decolorization Studies of the dyes
A screening test was conducted following the methodology outlined by (*Cenek Novotny et al.* 2001). The experiment began with carefully preparing Potato Dextrose Agar. For every dye that was taken into consideration, a dye solution was created with a concentration of 75 mg/l. Following the sterilization process carried out under aseptic conditions, the media was readied. With the sterilized media at hand, a precise volume of 1 ml of the 75 mg/l dye solution was added to every 50 ml of the prepared media. This addition was facilitated using a nylon syringe filter to ensure accuracy. For each individual agar plate, a small disc containing the inoculum was strategically placed at the center. Additionally, plates with uninoculated media serve as a control and triplicates were maintained for the assessment. These assembled plates were then subjected to incubation at ambient room temperature. Throughout this incubation period, diligent observations were made to monitor any potential decolorization phenomena. This served as a key indicator of dye degradation or transformation. By employing this systematic approach, we aimed to assess the capability of the *P.chrysosporium* to decolorize the dyes effectively.

2.5.2 Aqueous state Dye Decolorization Studies
Studies conducted and (Srinivasan and Murthy et al. 2000) demonstrated that the use of a certain concentration of glucose improved the color removal process by the white rot fungus through decolorization. This enhancement was based on the results obtained from a screening assay that evaluated the efficiency of decolorization for various dyes. Notably, dyes like Red, Corazol Blue CA, and violet SR were chosen for this assessment. To carry out the experiment, a solution containing 0.5% glucose and 50 ml of distilled water was prepared in conical flasks and subsequently sterilized. Following the sterilization process under aseptic conditions, dye solutions were introduced into each conical flask. In a separate flask serving as a control for the dye, fungal spore suspension ($9x10^{6/}$ ml) was introduced. The progress of decolorization was monitored using a UV-VIS spectrophotometer (Beckman DU-40). This enabled the observation and quantification of the rate at which decolorization occurred over time.

3. Results and Discussion
White rot fungi and their laccase enzymes have garnered attention for their ability to bioremediate pollutants, notably dyes. Laccase, multicopper oxidase plays a key role in breaking down lignin and aromatic compounds. They effectively degrade challenging synthetic dyes, often recalcitrant to conventional methods. Laccase take apart dye chromophores, diminishing color and complexity. This occurs through radical-driven oxidation, facilitated by laccase, disintegrating chemical bonds and converting dyes into smaller, less chromatic forms. The process involves direct dye oxidation by laccase and mediation via electron-transferring molecules.

3.1 Isolation and identification of laccase producing fungi
A collection of white rot fungi sample obtained from tree bark was subjected to cultivation on Potato Dextrose Agar (PDA) infused with guaiacol. For the purpose of obtaining pure colonies, distinct colonies were individually inoculated onto separate PDA plates. The ensuing pure colonies underwent both macroscopic and microscopic examinations, with the outcomes noted in Table 1.Through a combination of macroscopic and microscopic observations, a distinct white rot fungus *Phanerochaete sp* were isolated and identified from the *T. indica* specimen. In a prior investigation by

(Vaidyanathan Vinoth Kumar et al. 2011), a range of fungal species such as *Agaricus bisporus, Aspergillus niger, Aspergillus terreus, Aspergillus flavus, Fusarium solani, Metarhizium anisopliae, Trichoderma harzianum, and Trichoderma viride* were isolated. Similarly, (Ram Kumar Pundir et al. 2016) isolated Apophysomyces sp in their study. Another study (Buddolla et al. 2010) results revealed that *P. chrysosporium, Theliophora terristrus, Stereum ostreatus, Lenzites betulina, Cunninghamella echinulata, A.flavus, A.niger, and P.rubrum.* It's important to note that the outcomes of the current investigation may vary from those of other researchers. Such discrepancies can be attributed to variations in the sources of isolation, geographic locations, and environmental conditions under which the white rot fungal species were cultivated.

Table: 1 showed a growth on PDA medium and small, white, circular colonies rough colony structures such as cotton and sticky colony was observed. The PDA medium supplemented with guaiacol for enhanced growth.

S.NO	Macroscopic observation Identification of fungi		
1	Sample from the source	Sample inoculated on PDA	White Buffy colony growth on PDA with Guaiacol supplementation
2			

Table: 2 Microscopic identification of *Phanerochaete sp.* Under microscopic view the fungal species showed a Septate and unbranched hyphae.

S.NO	**Microscopic observation**		**Identification of fungi**
1	Septate, unbranched hyphae and solitary, thick-walled globular sporangia were observed under microscopic observation.		*Phanerochaete sp*

Upon conducting microscopic and macroscopic observations of the fungal species, further identification was carried out through 18S rRNA analysis (ITS sequence). The fungal species was conclusively identified as Phaeonochaete chrysosporium through comprehensive analysis. The gene sequence was then uploaded to the GenBank database of the NCBI in order to receive a Gene Bank accession number. The accession number assigned to the submitted gene sequence was PP469578 (Fig.1).

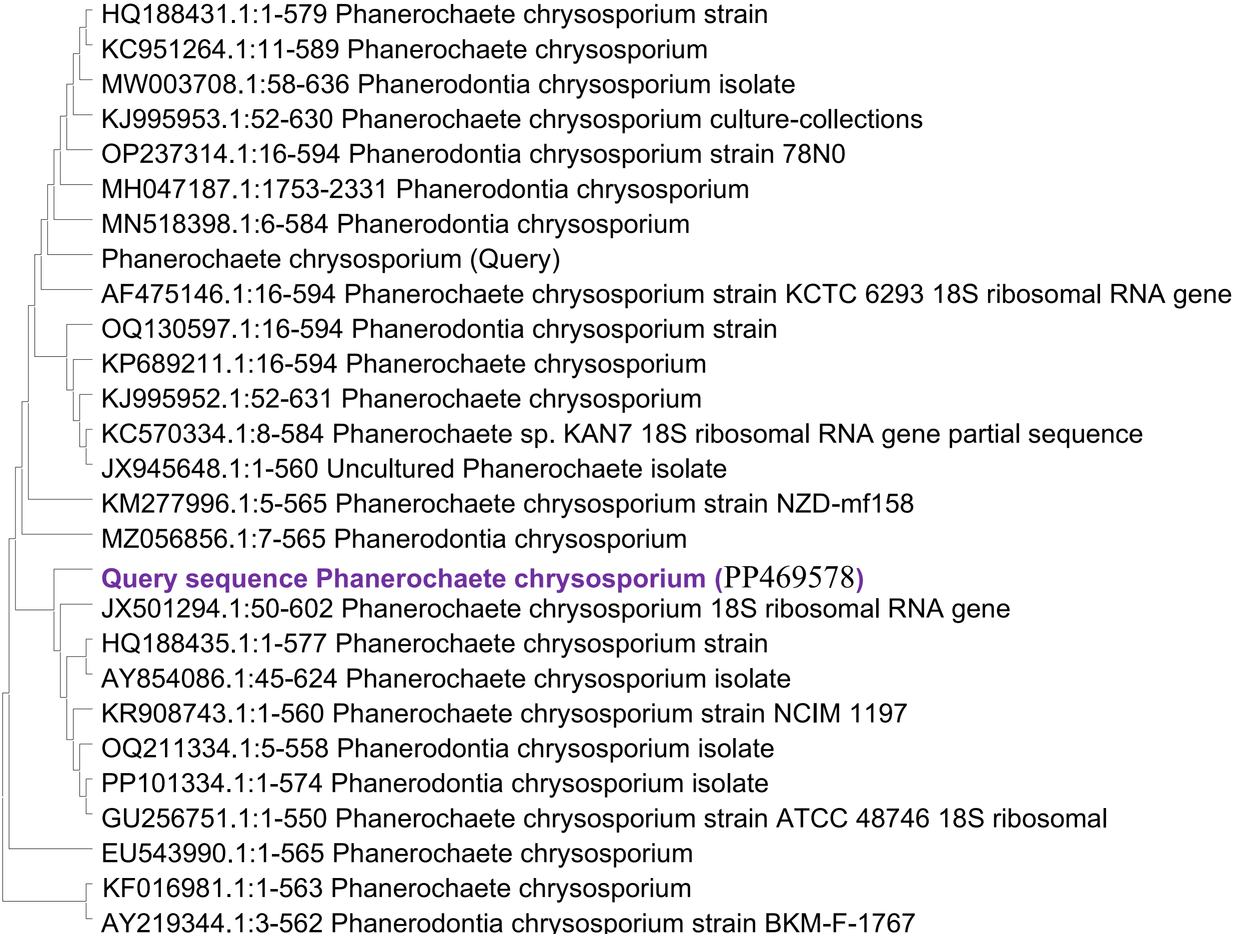

Fig: 1 Phylogenetic tree constructed using Mega 11 software's neighbor joining method for isolated species. The Phanerochaete species that were selected for analysis showed a strong relationship with *P. chrysosporium*, in comparison to all other species.

3.2 Laccase Production in media

The fungi capable of producing laccase were initially screened, and particular white rot fungus with predominant laccase production was chosen. This selected fungus was cultivated on a Potato Dextrose Agar plate for 12 days at a temperature of 30°C. After this incubation period, the laccase activity of the fungus was assessed using guaiacol as a substrate, and measurements were taken using a UV-Spectrophotometer. The extracellular nature of the laccase was confirmed by the presence of initial activity in the culture's supernatant. The *P. chrysosporium* growth analysis of revealed that initially, during initial period i.e., after 4th day the biomass measured 20.03 U/ml. Subsequently, it increased throughout the other phase, reaching of 23.24 U/ml on the 48hours incubated sample. This phase indicated a proportional increase in total laccase activity along with biomass production. In further investigation, induced conditions led to a shorter average laccase production time, with the highest activity observed on the 10th day incubated sample 42.82 U/ml and then laccase production declined (as depicted in Graph.1). Additionally, compared to the control condition, laccase activity significantly doubled when the inducer guaiacol was present.

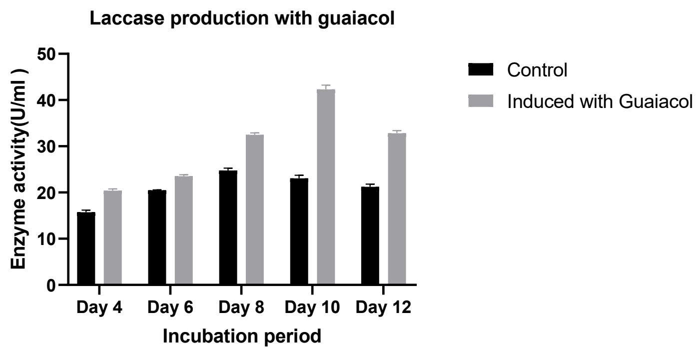

Fig. 2 shows laccase production with the induction of guaiacol. The significant level of enzyme production was noted at 10th day of incubation. On all the incubation days, laccase production was compared with the control.

Over a span of 16 days, cultures treated with 100 mM guaiacol and untreated normal cultures were cultivated, and their laccase activity was evaluated every 24 hours.
Laccase secretion peaked early on the second day of incubation under normal conditions. Notably, by the tenth day, the highest recorded activity of 54.671 U/ml was observed, indicating a consistent and ongoing production, which aligns with findings reported by other authors (Scheel et al. 2021). In a study conducted (Adiveppa bheemappa et al. 2015), Marasmius sp, Pleurotus sp, Aspergillus sp, Penicillium sp, Rhizoctonia sp, and Fusarium sp demonstrated the highest laccase activity. Additionally, the culture supernatant containing the enzyme showed an initial laccase activity of 3.2 U/ml, as reported and (Senthivelan et al. 2019), supporting the laccase's extracellular nature.

3.3 Optimization of laccase synthesis

3.3.1 Optimization of carbon sources

Synthesis of Laccase in *P. chrysosporium* increased in response to the external addition of different carbon sources, including fructose, glucose, and sucrose, as noted in Fig. 3(a). Laccase started to produce in the medium on the fourth day. It slowly increased up to the tenth day (maximum production) and started to decline during the subsequent incubation period. The medium supplemented with sucrose showed a higher laccase production (41.93 U/ml) than other sources. Fructose and glucose sources laccase production was observed to be 29.49 and 26.48 U/mL, respectively. According to (Gawande et al. and Kamat et al. 2000) research, the type and concentration of the sources of carbon available in the medium significantly influenced the synthesis of enzymes. During the carbon supplementation experiment, (sucrose 23.89 U/ml) and fructose (19.49 U/ml) had significantly higher maximal activity (18.96 U/ml) than glucose. (Mansur et al. 1997) reported that the use of fructose boosted the specific laccase activity in Basidiomycete fungi by an average of 100. But by the second day, laccase production from glucose supplementation was higher than that from sucrose and fructose. The organism's rapid and effective use of glucose may be the cause of this early increase in enzyme synthesis (Figs.3 (a).

3.3.2 Optimization of temperature

Temperature influences each of the organism's metabolic processes, notably the accessibility and absorption of nutrients, and it also impacts the characteristics of the watery environment. It has been demonstrated that temperature significantly affects enzyme activity while trying to optimize culture conditions to produce the maximum amount of laccase from P. chrysosporium. Day-by-day activity increased significantly, with maximum activity occurring on the 10th at 30ºC and produced 29.59 U/ml and results were noted in fig: 3(b). The results also showed that laccase production steadily decreased when the incubation temperature was raised above 35ºC. After the tenth day, the fungal culture began to depreciate and color change was observed at 50ºC. The amount of enzyme production was significantly reduced when temperature increased at 50ºC (17.69U/ml).

3.3.3 Optimization of laccase production with PH

This made it obvious that the pH had a substantial impact on *P. chrysosporium* laccase enzyme production. The tenth day of enzyme level was determined and has researched 28.70 U/ml at pH 4. At pH 5, enzyme production 24.37 U/ml was recorded. Subsequently, the enzyme activity had a sharp decline, reaching 19.14 U/ml at pH 7 (Fig. 3(c)). Most laccase in other fungi have an optimal pH of about 3 when using ABTS as a substrate, while pH optimality may also depend on the substrate utilized (Fukushima et al. 1995; Palmieri et al. 1997; Garzillo *et al.*1998; Perie et al*.* 1998; Shin *et al.* 2000; and Jung *et al.* 2002). The enzyme remained steady and increased at pH levels of 4 and decreased at 7, with pH 4 being its highest enzyme production point. As a result, the laccase under investigation was mesophilic by nature and enzyme production at the ideal pH and temperature.

3.3.4 Optimization of laccase production nitrogen sources

Nitrogen is a necessary ingredient for the synthesis of amino acids, which is needed for the production of proteins and other compounds with added value. Considering the effect of various nitrogen sources on the production of laccase by different organisms, there seems to be a great deal of discrepancy (Collins et al.1997 and Domınguez et al. 2007). Notably, it has been observed that many fungi exhibit increased production of ligninolytic enzymes when cultivated in nitrogen-rich media. The purpose of the current study was to ascertain whether different sources of nitrogen, such as ammonium chloride, urea and sodium nitrate, had an effect on laccase production. The nitrogen source evaluated at 100 mM increased laccase synthesis on the tenth day, urea produced the highest amount of enzymes (24.28 U/ml) among the nitrogen sources that were examined, compared to the control (12.83 U/ml).

The optimal nitrogen source for laccase production was similarly discovered and its source (Shraddha et al. 2011) to be sodium nitrate. Additionally, on the fourth day of incubation, ammonium chloride noticeably increased laccase activity. According to (Stajic et al. 2006), *P. ostreatus* fungus exhibited the maximum laccase activity when exposed to ammonium chloride. Even while the laccase activity in the urea-supplemented culture was the lowest i.e. 34.971 U/ml, it was still significantly higher than that of the control i.e. 13.485 U/ml. Conversely, (Lee et al. 2006) study state that *P. sanguineus* showed that elevated Laccase synthesis with urea than sodium nitrate, when comparing the effects of various nitrogen sources on laccase synthesis. In this study, the highest laccase production was achieved using urea as the nitrogen source, outperforming other nitrogen sources tested. These results suggest that urea plays a crucial role for the production of laccase production.

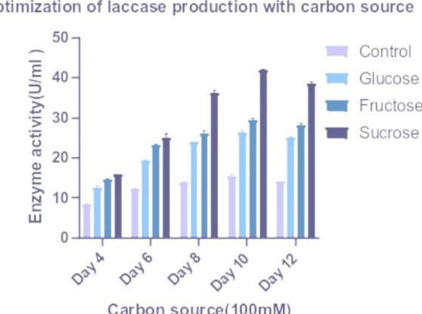

Fig.3 (a): Optimization of laccase production with carbon sources like glucose, fructose, and sucrose. Significant laccase production was recorded in sucrose on the 10th day of incubation.

Fig.3(b): Optimization of laccase production with various temperatures and laccase production was recorded at 30ºC on the fourth day of incubation.

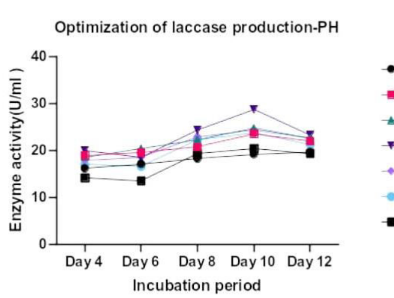

Fig.3 (c): Depicts laccase production with different PH. Enhanced laccase production was noted at PH 4 than other PH condition.

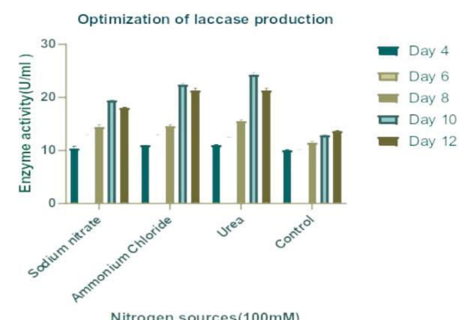

Fig.3 (d): showed laccase production with three different sources among the sources, urea has produced more laccase than other nitrogen sources.

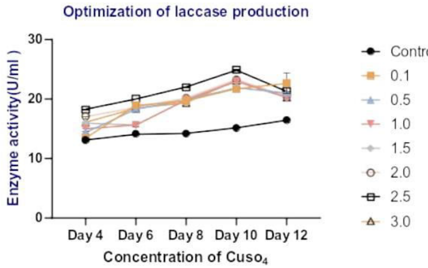

Fig.3 (d): showed laccase production with three different sources among the sources, urea has produced more laccase than other nitrogen sources.

Fig. 3 denotes analysis of various optimal conditions for laccase production. Laccase production with different carbon sources, temperature, PH, nitrogen source and copper sulphate.

3.3.5 Optimization of laccase production with Cuso₄

Laccase, multicopper oxidase, require copper as a micronutrient for optimal activity and stability. Copper ions activate enzymes, catalyzing substrate oxidation, essential for biological and industrial processes. Maintaining adequate copper levels ensures effective laccase production. The results of the study indicated that at 2.5 mM, a high concentration of laccase (24.90 U/ml) synthesis occurred on the tenth day of incubation. Copper has been identified as a potent inducer of laccase in the fungi *P.chrysosporium, Trametes versicolor* and reported by (Dominguez *et al.* 2007; Collins and Dobson, 1997. While (Palmieri *et al*. 2000**)** discovered that the ideal copper sulfate concentration for laccase activity was 150 µM, followed by Hao et al. (2007) investigated different concentration of CuSO4 on Pestalotiopsis sp and reported that 2.0 mM CuSO4 was the most optimal concentration of copper for laccase.

3.4 Dye degradation by Solid State Assay [SSA]

During the dye decolorization study, the fungal mycelia growth was initiates from day one but actual decolorization activity become more visible and evident on fourth day onwards. On Day 4, the dye decolorization is in its early stages. By Day 6, it is observed that *P.chrysosporium* exhibit a more rapid degradation rate compared to control. Moving to Day 8,

P.chrysosporium displays an increased degradation performance and by Day 10, continues to exhibit complete degradation than the control. The degree of degradation ability of the *P.chrysosporium* has been noted in figure no.4. *P. chrysosporium* more effectively decolorize Corazol Violet SR than any of the other two dyes.

Number of Days	Solid state discoloration		Degree of discoloration
	Control plate	*P.chrysosporium inoculated plate*	
Day:1			No discoloration
Day:2			+
Day:4			+ +
Day:8			+ ++
Day:10			+ +++

Fig. 4 showed a solid state degree of discoloration. Discoloration was observed from day one to tenth day. Discoloration starts from fourth day of incubation and degree of dye degradation increasing when days of incubation period increased.

Sathiya Moorthi et al. (2007) noticed that *P. florida* decolorized the dye with greater efficiency than Trametes hirsute. Specifically, *P. florida* exhibited a remarkable 96.4% decolorization on the 10th day of incubation, whereas T. hirsute achieved 91.1% decolorization. Similarly, Vaidyanathan Vinoth Kumar et al., (2011) found that *P. ostreatus* demonstrated significant degradation rates, achieving 66% decolorization for reactive blue, 49% for reactive red 243, and 55% for other reactive red dyes. In contrast to the other dyes tested, Corazol Violet SR decolorized more quickly, according to the results of the current investigation. During the optimization study, laccase production gradually increased as the study extended for further incubation period. According to the optimization study results, they would be paired with the decolorization study; hence, laccase is a major direct evident and responsible enzyme involved in the decolorization process.

3.5 Aqueous state decolorization Day -5 and Day-10

The decolorization of various textile dyes was assessed on the 5th and 10th days of the incubation period, with absorbance measured at 580 nm. The findings are represented in Figure 5. *In an aqueous state, P. chrysosporium* exhibited a higher degree of decolorization on red dye than other dyes. Blue and Corsalviolet-SR dyes showed minimal degradation by *P. chrysosporium.* No degradation was observed in the control. Murugesan et al. (2007) studied decolorization on *P. florida* and, following T. hirsute, used a low concentration of blue dye (CA), i.e., 25 mg/l. The significant decolorization was observed as 93.54% produced by *P. florida* on the tenth day. In a similar condition, *T. hirsuta* showed 92.17% discoloration. In addition, When compared to other dyes, Black B133 shown reduced decolorization. *P. florida* and T. hirsuta achieved maximum decolorization at 25 mg/l concentrations of 64.67% and 57.21%, accordingly. *P. florida* and T. hirsuta reported efficient decolorization of Corazol violet SR. P. florida showed 83.70% decolorization at 25mg/l, while T. hirsute showed 62.02%. However *P. chrysosporium* showed more degradation, further its degradation potential was analyzed with different concentration of red dye.

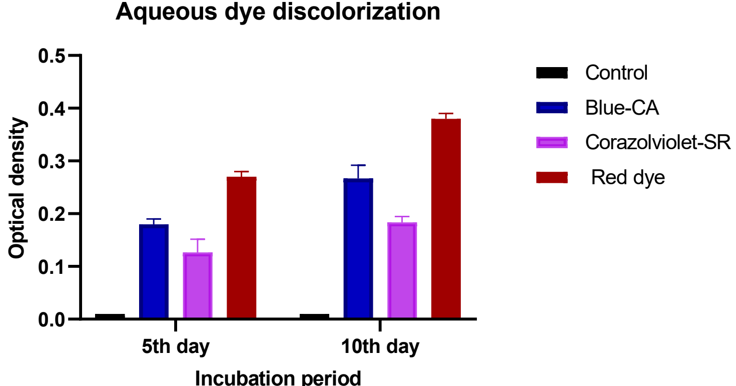

Fig.5: showed a red dye decolorization by *P. chrysosporium*. The degradation level was observed in different incubation periods (Days 5 and 10). Complete decolorization was noted on the tenth day of incubation.

3.5 Different concentration of textile red dyes Day-5and Day -10

P. chrysosporium has the ability to degrade dye at 25%, 50%, 75%, and 100%. At 120% and 150% no degradation was observed. Different dye concentration was taken for these analyses. Among these entire concentrations *Phanerochaete sp* showed complete decolorization in 25% of the dye at 10th day of incubation. Kalaichelvan *et al.,* (2007) found that *P. florida* and *T. hirsuta* showed varying decolorization rates at different concentrations. *P. florida* discolored at a concentration of 75 mg/l, while T. hirsuta discolored at a concentration of 28.57% and 24.04%. Blue CA-decolorization rate was low at 75 mg/l, while Corazol violet SR-decolorization was highest at 64.67% and 57.21%.

4. Conclusion

The *P. chrysosporium* exhibiting a remarkable rate of laccase production underwent comprehensive analysis, delving into both genotype characterization and optimization studies to enhance the efficiency of laccase production. This multifaceted approach aimed to unravel the genetic makeup of the fungal strain and fine-tune environmental and nutritional parameters for optimal enzyme expression, especially in dye degradation. The current study emphasizes the preliminary part of the dye discoloration process, indicating that extensive studies are still required to understand the complete mechanism of dye degradation carried out by P. chrysosporium.

5. Declaration of Competing Interest

The authors declare no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

6. Data Availability

The data supporting this study will be made available upon request.

7. Acknowledgements

We sincerely thank all individuals and institutions who contributed to this research.

8. Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Monica R Vinodhini E and Sriram V. The draft of the manuscript was written by Abirami Arasu.

9. References

Abdulla E, Tzanov T, Costa S, Robra K-H, Cavaco-Paulo A, Gübitz GM (2000). Decolourization and detoxification of textile dyes with laccase from *Trametes hirsute*. *Appl. Environ. Microb. 66: 3357-3362*

Adeline Seek May Chua (2010). A Quantitative Method for Fungal Ligninolytic Enzyme Screening Studies. Asia-Pacific Journal of Chemical Engineering, *6: 589-595.*

Adiveppa Bheemappa Basawanneppa Kaliwal. (2015). Production and optimization of laccase by submerged fermentation. Current Research Vol. 7, Issue, 07, pp.18308.
Ala-Houhala M, Koukila-Kähkölä P, Antikainen J, Valve J, Kirveskari J, Anttila VJ. (2018) Clinical use of fungal PCR from deep tissue samples in the diagnosis of invasive fungal diseases: a retrospective observational study. Clin Microbiol Infect. (3):301-305. doi: 10.1016/j.cmi.
Colao, M.C., Caporale, C., Silvestri, F. *et al.* (2009). Modeling the 3-D Structure of a Recombinant Laccase from *Trametes trogii* Active at a pH Close to Neutrality. *Protein J* **28**, 375–383 https://doi.org/10.1007/s10930-009-9204-1.
Collins, P.J., Dobson, A.D.W., (1997). Regulation of laccase gene transcription in Trametes versicolor. Appl. Environ. Microbiol. 63, 3444–3450.
Collins, P.J., Dobson, A.D.W., (1997). Regulation of laccase gene transcription in Trametes versicolor. Appl. Environ. Microbiol. 63,3444–3450.
Domınguez, A., Gomez, J., Lorenzo, M., Sanroman, A., (2007). Enhanced production of laccase activity by Trametes versicolor immobilized into alginate beads by the addition of different inducers. World J. Microbiol. Biotechnol. 23, 367–373.
Domınguez, A., Gomez, J., Lorenzo, M., Sanroman, A., (2007).Enhanced production of laccase activity by Trametes versicolor immobilized into alginate beads by the addition of different inducers. World J. Microbiol. Biotechnol. 23, 367–373.
Fukushima Y, Kirk TK. Laccase component of the Ceriporiopsis subvermispora lignin-degrading system.(1995) Appl Environ Microbiol. Mar;61(3):872-6. doi: 10.1128/aem.61.3.872-876.1995.
Gawande, P.V., Kamat, M.Y., (2000). Production of xylanases by immobilized Aspergillus sp using lignocellulosic waste. World J.Microbiol. Biotechnol. 16, 111–112.
Gianfreda, L., Xu, F., & Bollag, J. M. (1999). Laccases: A Useful Group of Oxidoreductive Enzymes. *Bioremediation Journal*, *3*(1), 1–26. https://doi.org/10.1080/10889869991219163.
Gianna Palmieri, Paola Giardina, Carmen Bianco, Andrea Scaloni, Antonio Capasso, Giovanni Sannia, A Novel White Laccase from Pleurotus ostreatus(1997) Journal of Biological Chemistry,Volume 272, Issue 50, Pages 31301-31307,ISSN 0021-9258,https://doi.org/10.1074/jbc.272.50.31301.
Givaudan, A., Effosse, A., Faure, D., Potier, P., Bouillant, M.L., Bally, R., (1993). Polyphenol oxidase in Azospirillum lipoferum isolated from rice rhizosphere: evidence for laccase activity in non-motile strains of Azospirillum lipoferum. FEMS Microbiol. Lett. 108 (2), 205–210.
Hao, J., Song, F., Huang, F., Yang, C., Zhang, Z., Zheng, Y., Tian,X., (2007). Production of laccase by a newly isolated deuteromycetes fungus Pestalotiopsis sp. and its decolorization of azo dye. J. Ind.Microbiol. Biotechnol. 34, 233–240.
Jung H., Xu F., Li K., (2002) Enzyme Microb. Technol. 30 161–168
Kalaichelvan Thangavelu, Malarvizhi Kaliyaperumal, Pudupalayam, Ganoderma australe from southern India, (2008) Microbiological Research, Volume 163, Issue 3, , Pages 286-292, https://doi.org/10.1016/j.micres.2007.01.003.
Kersten, P., Cullen, D., & Kersten, P. (2007). Laccase - a Multi-purpose Biocatalyst at the Interface of Biology, Chemistry, and Physics. Current Opinion in Biotechnology, 18(5), 1-8.
Kiiskinen LL, Rättö M, Kruus K. (2004)Screening for novel laccase-producing microbes. J.Appl.Microbiol.;97(3):640-6. doi: 10.1111/j.1365-2672.2004.02348.x.
Lee, Y., Park, C., Lee, B., Han, E., Kim, T., (2006). Effect of nutrients on the production of extracellular enzymes for decolorization of reactive blue 19 and reactive black 5. J. Microbiol. Biotechnol. 16, 226–231.
Mansur, M., Sua´ rez, T., Ferna´ ndez-Larrea, J.B., Brizuela, M.A.,Gonza´ lez, A.E., (1997).Identification of a laccase gene family in the new lignin-degrading basidiomycetes CECT 20197. Appl. Environ.Microbiol. 63, 2637–2646.
Michael, M.T., M.G. Georg and R. Astrid (2005). Degradation of Azo dyes by laccase and ultrasound treatment. Appl. Environ. Microbiol. 71: 2600–2607.
Miguel Alcalde, Thomas Bulter, Miren Zumárraga, Humberto García-Arellano, Mario Mencía, Francisco J. Plou, Antonio Ballesteros. (2005) Screening Mutant Libraries of Fungal Laccases in the Presence of Organic Solvents,SLAS Discovery,Volume 10, Issue 6,2005,Pages 624-631,ISSN 2472-5552,https://doi.org/10.1177/1087057105277058.
Murugesan, Kumarasamy, Nam, In-Hyun,Kim, Young-MoChang, Yoon-Seok.(2007). Decolorization of Reactive Dyes by a Thermostable Laccase Produced by Ganoderma lucidum in Solid State Culture. Enzyme and Microbial Technology. DO 10.1016/j.enzmictec.2006.08.028.
Palmieri, G., Giardiana, P., Bianco, C., Fontanella, B., Sannia, G.,(2000). Copper induction of laccase isoenzymes in the ligninolytic fungus Pleurotus ostreatus. Appl. Environ. Microbiol. 66, 920–924.
Parshetti, Kalme G.K. S.D, Gomare S.S, Govindwar S.P. (2007) Biodegradation of reactive blue-25 by Aspergillus ochraceus NCIM-1146 Bioresour. Technol.23:124-132.
Perie F, Reddy G., N. Blackburn, M. Gold, Arch. Biochem. Biophys. 353 (1998) 349–355.
Pundir RamKumar, Mishra VK, Rana S, et al. Screening of laccase producing fungi from soil samples: An In Vitro Study. (2016). Electronic Journal of Biology, , Vol.12(3): 254-257.
Robinson T, Chandran B, Nigam P. (2001). Studies on the production of enzymes by white-rot fungi for the decolorization of textile dyes. Enzyme. Microb. Tec hnol. 29: 575-579.
Sathiya M Periyar S Sasikalaveni A Murugesan K Kalaichelvan PT. (2007) Decolorization of textile dyes and their effluents using white rot fungi Journal / African journal of Biotechnology / Vol. 6 No. 4 .

Scheel AM, Schijen MRMJ, Lakens D. An Excess of Positive Results: Comparing the Standard Psychology Literature With Registered Reports. Advances in Methods and Practices in Psychological Science. 2021;4(2). doi:10.1177/25152459211007467.

Senthivelan T., Kanagaraja J, Rames, Pandab C,NarayanT (2019).Screening and production of a potential extracellular fungal laccase from Penicillium chrysogenum: Media optimization by response surface methodology (RSM) and central composite rotatable design (CCRD). Biotechnology Reports,*23: 00344.*

Shin K.S., Lee Y.J., (2000) Arch. Biochem. Biophys. 384 109–115.

Shraddha, R.S., Sehgal, S., Kamthania, M., Kumar, A., (2011). Laccase: microbial sources, production, purification and potential biotechnological applications. Enzyme Res. http://dx.doi.org/10.4061/2011/217861.

Srinivasn SV and Murthy DVS (2000).Potential for bioremediation of xenobiotic compounds by the white rot fungus Phanerochaete chrysosporium. Biotechnol. prog. 11: 368-379.

Stajic, M., Persky, L., Friesem, D., Hadar, Y., Wasser, S.P., Nevo, E.,(2006). Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected Pleurotus species. Enyzme Microbiol. Technol. 38, 65–73.

Vaidyanathan Vinoth Kumar, Selvaraj Dinesh Kirupha, Premkumar Periyaraman and Subramanian Sivanesan Screening and induction of laccase activity in fungal species and its application in dye decolorization.(2011) African Journal of Microbiology Research Vol. 5(11), pp. 1261-1267, DOI: 10.5897/AJMR10.894.

Viswanath Buddolla, Rajesh B, Janardhan A, Kumar AP, Narasimha G. Fungal laccases and their applications in bioremediation.(2014) Enzyme Res. 2014;2014:163242. doi: 10.1155/2014/163242.