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RESEARCH ARTICLE

Laccase Production Under Optimized Parameters by *Aspergillus oryzae*, an Endophytic Fungus and their Application to Waste Water Treatment

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Abstract

Objective: To isolate an endophytic fungal laccase producer from the *Ziziphus mauritiana* plant leaves in the surroundings of paper mill effluents and to study its role in the decolorization of synthetic dyes, removal of COD and phenol from industrial effluents. **Methods:** Laccase-producing endophytic fungi were isolated and screened by using an agar plate method. The positive isolates were identified, and laccase activity was determined by using spectrophotometric methods to monitor the oxidation of guaiacol and optimizing various parameters affecting laccase production. The molecular mass of the purified laccase enzyme was determined by using 12% SDS-gel electrophoresis. Industrial effluents were treated with laccase to remove phenol, decolorize dyes, and reduce chemical oxygen demand. For the analysis, a spectrophotometric method was employed. **Findings**: One of the most effective endophytic fungal isolates, *Aspergillus oryzae*, was screened as a maximum laccase producer. The optimal pH of 6, temperature of 35 ^{o}C, inoculation period of 8 days, and the inoculum number of 3 discs/100 ml of Czapek Dox Broth in submerged culture were determined for the maximum laccase production. Sucrose and sodium nitrate, as carbon and nitrogen sources, considerably assisted laccase production. The molecular weight of the isolated laccase from *A. oryzae* was 66 kDa. The greatest activity was determined to be 64.2 U/mL, which is two times more than under unoptimized conditions. After the fifth day of exposure, the *A. oryzae* laccase decolorizes the synthetic dyes Bromophenol blue, Congo red, Methyl orange, and Phenol red.Chemical oxygen demand and phenolic pollutants' clearance rates were 38–43% and 60% from coal and textile effluents during their exposure times, respectively. **Novelty**: *A. oryzae* was discovered to be a potent natural laccase producer endophytic fungus from paper mill effluents, which may be used for decolorizing non-textile dyes, treatment of various industrial effluents, and other industrial purposes.

Keywords: Endophytic Fungi; Aspergillus oryzae; Laccase; Chemical oxygen demand; Synthetic dyes

1 Introduction

Phenols are ubiquitous pollutants that enter waterways through the effluents of industrial processes, including the production of phenol in petrochemicals, pulp, paper, paints, textiles, and coal refineries[(1)]. Huge volumes of industrial waste are produced by several industries, and its disposal poses a serious threat to the environment. Untreated phenol and phenolic chemical discharge may pose serious health risks to people, animals, and aquatic ecosystems. One of the basic tenants of industrial production efficiency is enzymatic catalysis. Utilizing enzymes reduces the need for harmful solvents while also allowing for the recycling of biowaste. They are also secure and effective in terms of energy. However, the cost, stability, and low rate of recycling and reuse continue to be barriers to their application in biotechnological processes. Laccases are glycosylated polyphenol oxidases (benzenediol: oxygen oxidoreductases; EC1.10.3.2)[(2)]. Fungal laccases (LCs), one of the numerous industrial enzymes, are excellent, adaptable catalytic oxidants that just need molecular oxygen to function, making them ideal choices to be used as a biotechnological tool. Laccase is capable of effectively reusing lignocellulosic biomass for the production of enzymes, the breakdown of phenolic and non-phenolic components, bioactive compounds, or clean energy while reducing the number of chemicals used. It is used in the chemical, textile, food, and wood processing industries, as well as the pharmaceutical and pharmaceutical industries[(3)].

The demand to identify promising laccases in large quantities has been sparked by their potential in biotechnological and environmental applications, and because of this, the manufacturing of this enzyme must be cost-effective. Due to the commercial unavailability of laccase, although there has been an increase in public awareness of endophytic fungi, their potential as enzyme producers has yet to be entirely realized. Because there has been no systematic research conducted, it is difficult to estimate the number of ascomycete species that produce laccases. Laccase was produced by some *Aspergillus* species, *A. flavus, A. nidulance, A. niger, and A. fumigates*, as well as some plant pathogenic species. Although *A. oryzae* produces a wide variety of enzymes related to the degradation of plant polysaccharides, there is still uncertainty regarding laccase production, optimization, etc. Native fungi that produced laccase may have coordinated their natural abilities with the environmental circumstances in the area. Endophytic fungi found in paper mill effluent may be able to produce laccase enzymes, which can be used for detoxification or bioremediation of industrial and agricultural waste as well as other harmful substances[(4)].

In this new era of dye oxidation, laccase is a crucial biological mediator and the greatest substitute for chemical mediators, making it the green enzyme of choice for dye breakdown[(5)]. In a broad range of industries, including textiles, paper, printing, cosmetics, and medicine, synthetic dyes are widely exploited 10-15% of the colours used during dyeing are lost in the effluent. Most of these dyes resist biodecolorization because of their complex structural makeup. Although there are physicochemical methods for getting rid of these dyes, they are expensive and unfriendly to the environment[(6)]. Since laccase significantly lowers the chemical oxygen demand (COD), biological oxygen demand (BOD), and solids present in grey water, it has become a key enzyme in the mycoremediation of grey water treatment[(7)]. With the help of laccase, the new method of forward osmosis is utilized to improve the potability of water and remove micropollutants from wastewater[(8)]. Due to its superior catalytic activity and reusability, laccase is also employed in the biodegradation of organics and is crucial in lowering water pollution[(9,10)].

However, laccase's limited applicability due to its high production cost and ineffectiveness has reinforced the need to create an economically viable technique[11]. Since the majority of naturally occurring strains are known to be poor laccase producers, the production yield of an enzyme depends on the type of producing strain. However, getting high and affordable laccase yields still depends on screening and choosing powerful laccase-producing fungi and optimizing production conditions. Additionally, it has been observed that improving medium composition and cultivation factors can boost laccase production[12]. For the commercial production of laccase, submerged fermentation is widely used. Hence, the goal of this study was to focus on the laccase production by the isolated endophytic fungus *Aspergillus oryzae* (Gene Bank accession number MG786521.1) from the paper mill effluents of *Ziziphus mauritiana* plant leaves and analyze various factors that influence laccase production in submerged fermentation for maximum production. The goal was expanded to include a study of isolated laccase applications in synthetic dye degradation, phenol removal, and COD removal from various industrial effluents.

2 Methodology

2.1 Isolation and screening of laccase-producing endophytic fungi

The endophytic fungi were isolated from the leaves of *Ziziphus mauritiana* collected from paper mill effluents in the Dongargaon and Raipur regions of Chhattisgarh state, India. Isolation of endophytes was done by Arnold et al.[13].For the preliminary screening step, fungal isolates were collected, and each isolate was subsequently spot inoculated on Petriplates containing PDA medium supplemented with 0.01% Guaiacol and incubated for 7 days at 25°C in the dark according to Kiiskinen et al.[14]. Guaiacol oxidation resulted in the creation of an intense brown colour under and around the fungal colony, which was regarded as a favorable reaction.

2.2 Preparation of Inoculum and Laccase Production

The inoculum preparation for laccase production was done by inoculating two discs (8 mM in diameter) of a 5-day-old culture in 50 mL of production medium in a 250 mL flask and incubating them for 3 days at 25 ± 2 °C. As part of the quantitative screening, the selected isolates were treated to submerged fermentation to test their capacity for maximum laccase production by growing in a laccase-producing liquid medium, as described by Monnat et al.[15]. The laccase fermentation was performed in Erlenmeyer (250 mL) flasks containing 100 mL of media with the following components (g/L): Urea-0.14; Sucrose-2.0; Yeast Extract-0.34; $MgSO_4 \cdot 7H_2O$-0.07; $CaCl_2 \cdot 2H_2O$-0.004; $NiSO_4 \cdot 7H_2O$-0.003; KH_2PO_4-0.1; and Na_2HPO_4-0.3). Two discs (8 mM in diameter) from a 5 day old culture were inoculated in this media and incubated for seven days at 25 ±2 °C at pH 4.5 on a Rotary Shaker (Rivotek, India) at 150 (x g). After the incubation period, the production medium was prepared for laccase enzyme extraction.

2.3 Laccase Extraction, Assay, and Protein Estimation

The clear supernatant was collected after 7 days of incubation by centrifugation (REMI C-24BL, Cooling centrifuge; India) at 5000 (x g) for 5 min at 4 °C, as described by Sidhu et al.[16]. The laccase activity was evaluated in triplicates by the method of Jadhav et al.[17], monitoring the oxidation of 10mM Guaiacol buffer with 100mM Sodium Acetate buffer (pH 5.4) for 10 minutes at 530nm while the blank was measured at 470nm. 1 mL of culture filtrate, 3 mL of buffer, and 1 mL of 10 mM Guaiacol were used to prepare the reaction mixture (5 mL), whereas the blank tube excluded culture filtrate. Laccase activity was determined and expressed in enzyme units per mL (U/mL). The Bradford method[18] was used to determine the protein concentration of the sample, with bovine serum albumin serving as the standard.

2.4 The Characterization of the Maximum producer

Microscopic examination based on cultural characters observed on PDA medium by Lactophenol Cotton Blue slide has been used to further characterize the maximum producer. Further fungal identification up to the species level was carried out by Bioreserve Pvt. Ltd. in Hyderabad, India.

2.5 Optimization Studies

Laccase production was examined in three distinct media: Potato Dextrose Broth, Czapek dox Broth, and Carboxy Methyl Cellulose Broth. The medium that produced the most enzymes was then employed in optimization trials. The method described by Mahmoud et al.[19] was used to optimize laccase production from endophytic fungus at various parameters; temperature

(25 to 45 °C with 10 °C increments), pH (4-8), number of discs (8mm in diameter) varied from 1 to 5, number of days (5-11), carbon source concentration range of 10 g/L (Glucose, Fructose, Galactose, Sucrose), nitrogenous source concentration range of 10 g/L (Ammonium Nitrate, Peptone, Sodium Nitrate, Urea). Two discs (8 mM in diameter) from a 5-day-old culture were injected into this sterilized media and incubated at pH-4.5 and 25 ±2 °C for 7 days. All of the optimization studies were carried out in triplicates using the traditional one-factor-at-a-constant. The culture is harvested, filtered, and the enzyme activity is measured.

2.6 Enzyme production under optimized conditions

A laccase production medium and supplement with 10 g/L sucrose and sodium nitrate as carbon and nitrogen sources and all of the optimal parameters were developed to investigate the combined effect of the above stated factors and to maximize laccase production. The media was sterilized before being infected with three discs (8mm in diameter) of a five-day-old culture and incubated for eight days at 35 ± 2 °C in triplicates. The enzyme activity of the purified extract was measured and compared to the unoptimized medium.

2.7 Purification of the Enzyme

The purification of the culture filtrate was carried out according to Patel et al. [20]. Total protein precipitation with Ammonium Sulphate in the range of 0–75% saturation was performed on the supernatant obtained from the culture filtrate. Dialysis was carried out with a Sodium Acetate solution of 100 mM (pH 4.5).

2.8 Molecular weight determination by SDS–PAGE

The method of Laemmli [21] was used to assess the homogeneity and molecular weight of the isolated protein fraction. Purified fractions and standard protein markers ranging from 25–170 kDa (Ge-Nei, Bangaluru, India) were electrophoresed on SDS-PAGE, comprising of a resolving gel (12%) and a stacking gel (10%). The separated bands were stained with Coomassie Brilliant Blue R-250 following electrophoresis.

2.9 The effect of various factors on laccase activity

2.9.1 Effect of temperature and pH on laccase activity

The effect of temperature was determined by pre-incubating laccase enzyme at various temperatures (20, 30, 40, 50, and 60 °C) for 10 minutes before measuring enzyme activity at a suitable pH with a substrate to explore the effect of temperature on enzyme activity in triplicates. The effect of pH on laccase activity was assayed in 50 mM acetate buffer (pH range 3-6) and phosphate buffer (pH range 7-8) at 35 °C.

2.9.2 Effect of inducers and inhibitors on enzyme activity

The effect of different inducers and inhibitors in the enzyme solution at different concentrations (5–20 mM) of $CuSO_4$, Tannic Acid, EDTA, and Sodium Azide. Before adding the enzyme solution to the substrate, it was pre-incubated for 10 minutes with inducers and inhibitors and allowed to react for 10 minutes at 30 °C in triplicates. Laccase activity was measured using the enzymatic test method previously mentioned.

2.10 Statistical Analysis

The results were calculated using the average of three experimental replicates. The descriptive statistics of the observations are represented by the Mean and Standard Deviation. The tables present the findings of the data statistical analysis. The data were analyzed using One-Way ANOVA and Tukey's multiple comparison tests, with $P < 0.05$ chosen as the statistical significance level. The statistics tool Origin Pro 2018 (version 10.0) was used for the analysis.

2.11 Applications of Laccase in Dyes Decolorization

A UV-Vis spectrophotometer was used to determine the specific absorption peak by wavelength scanning of each dye solution at 1% dye solution. 1% synthetic dye solutions of bromophenol blue, congo red, methyl orange, and phenol red were prepared in water. Based on this, the calibration curve of each dye was obtained for additional dye concentration assessment in various treatments. The decolorization process took place in a 20 mL test tube with 10 mL of dye solution (1%), buffer (pH 6), and

laccase (500 μL) at a temperature of 35 °C for 0 to 9 days in triplicates. For all colour measurements, the day of adding effluents to the pre-grown cultures is designated day zero and was considered to be 100%. The final concentration of the dye in the medium is calculated based on the percentage of decolorization according to Schmitt et al.[22].

2.12 Applications of Laccase in Phenol Deduction

A total of 20 L of effluent was collected and stored at 4° C from industries in the Bhilai region (Coke oven department of the Bhilai steel plant, Bhilai, and Dyes and clothing industries, Bhilai). Composite sampling is the sampling method used.

This was accomplished by incubating 500μL of enzyme culture supernatant in triplicate with a 10mL of 10% final concentration of effluent solution for 6 to 12 hours at 35°C, with 10% of untreated effluent solution serving as a control. The concentrations of phenolic content were determined using the Folin-Ciocalteu reagent through a spectrophotometer at 765nm according to the Rice et al. method[23]. The reaction mixture was: 1000 μL of effluent, 250 μL of sodium carbonate solution (12g/L), and 25μL of Folin-Ciocalteu 2 mol/L. All samples were kept at 20°C for 30 min. To establish the levels in the samples using a calibration curve created with the standards using pure phenol.

2.13 Applications of Laccase in Chemical Oxygen Demand Deduction

This was done by incubating (20 U/mL) enzyme culture supernatant with 50 mL of 10% final concentration of effluents to remove COD from 0-9 days at 35°C in triplicates. For all colour measurements, the day of adding effluents to the pre-grown cultures is designated day zero and was considered to be 100%. During the treatment, the effluent was kept in the dark with slow agitation. COD was measured according to the Rice et al. method[23].

3 Results and Discussion

3.1 Isolation and Screening

Laccase-producing endophytic fungi were isolated from *Ziziphus mauritiana* plant leaves from several locations of paper mill effluents in the ”Dongargaon and Raipur” region of Chhattisgarh, India. Twelve distinct endophytic fungi were isolated from *Ziziphus mauritiana* plant leaves. According to the findings, only three endophytic fungal isolates developed laccase in a qualitative agar plate method employing Guaiacol, while only one laccase-producing endophytic fungus gave a maximal zone (brown halos) under and around the fungal colonies, according to the findings (Figure 1). Previous reports of the guaiacol plate assay suggested that guaiacol was one of the best assays for screening of laccase producers[24].

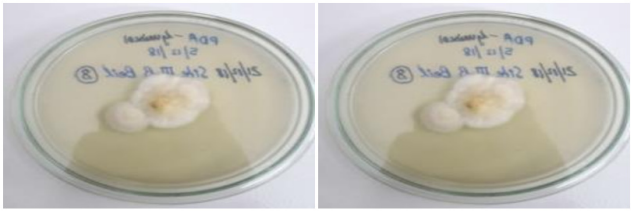

Fig 1. Screening of laccase-producing endophyte by plate tests using the indicator Guaiacol

3.2 Characterization and identification of highly efficient endophyte

The identification of one distinct endophytic fungal isolate with maximal laccase production potential is described as filamentous growth, hyphal branching, and multicellularity, which are comparable to *Aspergillus sp*. after lactophenol cotton blue staining. Based on of 18s RNA molecular identification done by Bioreserve Pvt. Ltd. in Hyderabad, India, the endophyte 2Ca fungal strain was identified as *Aspergillus oryzae*. The sequenced laccase-producing endophytic fungus is similar to *Aspergillus oryzae* MG786521.1 strain (E-value = 0.0, pair similarity = 100% identical).

3.3 Optimization of Various Parameters

The laccase production was examined using three different media (Potato Dextrose Broth, Czapek Dox Broth, and Carboxy Methyl Cellulose Broth), and the findings revealed that the Czapek Dox broth media produced the most laccase enzyme

(45±2.25 U/mL) (Figure 2). Different media were optimized for laccase production, and it was proved that Czapek Dox broth was the best medium [25]. The basic medium for laccase production was Czapek Dox broth media.

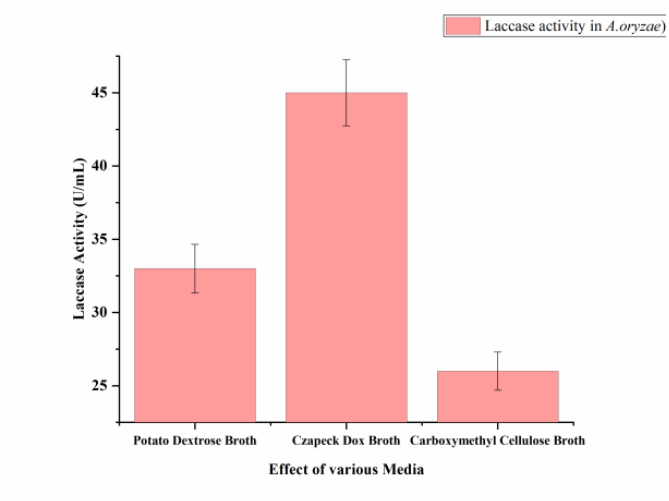

Fig 2. Effect of various media on laccase production from *Aspergillus oryzae*

Four different carbon sources were investigated for their capacity to stimulate laccase production at the ideal levels. The effect of 1% Glucose, Fructose, Galactose, and Sucrose added to the Czapek dox Broth. In *Aspergillus oryzae* (20.7± 1.035 U/mL), found that Sucrose promotes maximal laccase production which was significant (P ≤0.05) among the different carbon sources (Figure 3). The results of some studies also revealed that sucrose is one of the best carbon sources for fungal laccase production in the case of *Trametes versicolor* MTCC138 [26], *Pycnoporus sanguineus* [27] and *Agaricus sp.* [28].

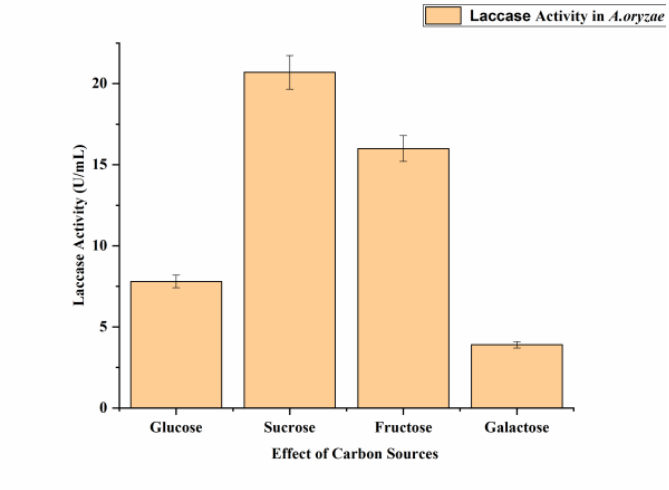

Fig 3. Effect of various carbon sources on laccase production from *Aspergillus oryzae*

The impacts of several organic and inorganic nitrogen sources were studied to see which ones had the greatest impact on laccase production. Sodium nitrate was the greatest nitrogenous source for laccase synthesis in *A. oryzae* (30.7±1.535 U/mL), which was significant (P < 0.05) among the different nitrogen sources (Figure 4). Inorganic sources of nitrogen are more effective at promoting laccase production and appropriate fungal development, so they are favoured [29]. Sodium nitrate appears to be the optimum nitrogen source for laccase production in *Scytalidium lignicola* [25], *Pichia sp.* Dw2 and *Kluyveromyces sp.* Dw1 [30], and *Dictyoarthrinium synnema* [31]. A significant impact of sodium nitrate on laccase production can be observed.

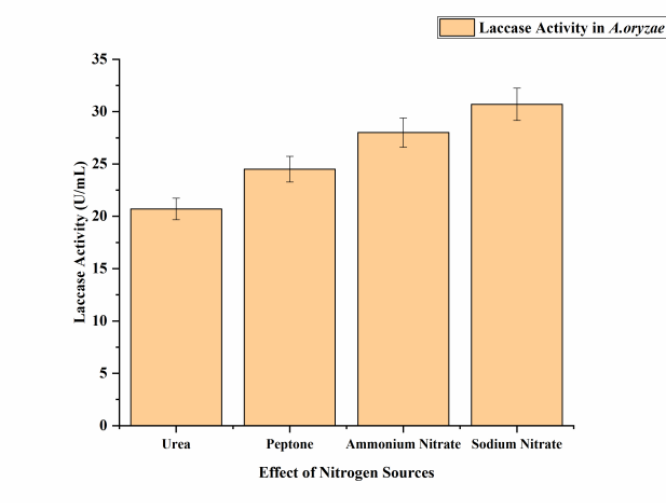

Fig 4. Effect of various nitrogen sources on laccase production from *Aspergillus oryzae*

The effect of the preliminary medium on *Aspergillus oryzae* laccase production was investigated in the pH range of 4–8. However, after 7 days of incubation, *Aspergillus oryzae* (23.0 ±1.15 U/mL) obtained the highest laccase production at pH 6.0 and the most constant, while it steadily declined at increasing medium pH (6 to 8). As indicated in Figure 5 , statistical analysis of the data revealed that pH 6 was significantly (P<0.05) associated with *A. oryzae* laccase production when compared to the other pH levels. According to previous studies, the ideal pH for laccase synthesis in most fungi is between 5.0 and 6.0. In *Scytalidium lignicola* 6 pH also shows the maximum activity of the laccase [25].

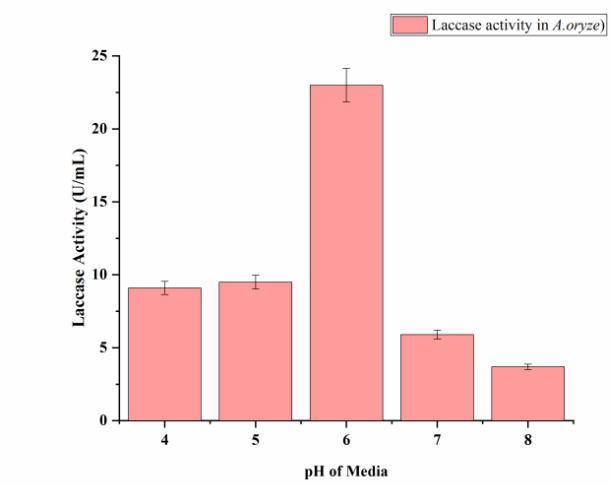

Fig 5. Effect of various pH on laccase production from *Aspergillus oryzae*

In organisms, the temperature has a significant impact on the formation and synthesis of extracellular laccase. Temperatures in the range of 25-45 °C with a 10°C increment were used to determine the optimum temperature for laccase production by *Aspergillus oryzae*, and the highest production was obtained at 35 °C (51± 2.55 U/mL) and the lowest at 45 °C as the temperature increased (Figure 6). Statistical analysis of the data revealed that a temperature of 35 °C was significantly ((P < 0.05) related to *A. oryzae* laccase production. Laccase synthesis was reduced as the temperature was raised above the optimal pH. *Kluyveromyces sp.* Dw1 and *Pichia sp*. Dw2 requires temperatures ranging from 30-35 °C for laccase production [21]. Similarly, *T. versicolor* and *T. harzianum* prefer temperatures of 35 °C for maximum laccase production [26,32].

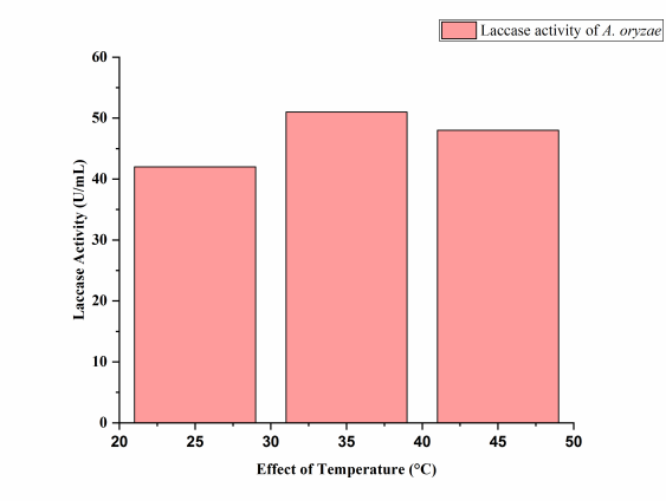

Fig 6. Effect of various temperatures on laccase production from *Aspergillus oryzae*

The effect of inoculum numbers on laccase production by *Aspergillus oryzae* was investigated by varying the amount of inoculum introduced to the production medium. Laccase production was only significant (53± 2.95 U/mL) at the 3disc (8mm diameter) inoculum numbers (Figure 7) and it was significant ($P < 0.05$). The production of laccase was reduced as the inoculum numbers increased even more. A lower level of inoculum may not be enough to stimulate development, whereas a larger quantity may result in competitive inhibition [20]. Due to rapid food depletion, increased inoculum numbers inhibits laccase synthesis, resulting in decreased metabolic activity [21].

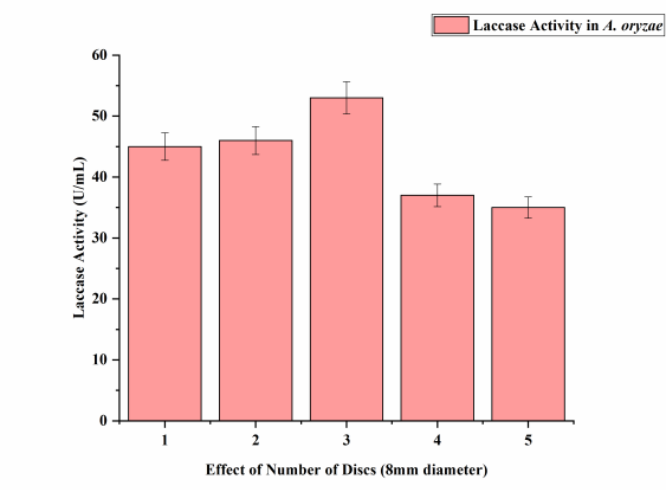

Fig 7. Effect of inoculums numbers on laccase production from *Aspergillus oryzae*

From 5 to 11 days of incubation under diverse cultural conditions, the influence of the incubation duration on *Aspergillus oryzae* laccase production was investigated, with maximum production observed on the 8th day (46 ± 2.45 U/mL) and rapidly declining until the 11th day of incubation (Figure 8). There was no food from day 8 onwards, a rise in enzyme activity of a significant magnitude. The statistical analysis of the data showed that the 8th day was significant ($P < 0.05$) for the laccase production by *A. oryzae* compared with the other temperature levels. Similarly, on the eighth day of incubation in *A. flavus* NG85, (8.422 U/mL) produced maximum laccase [33]. The maximum laccase production by *A. flavus* (17.39 IU/ml) was observed on the 12th day of culture [34], *Ganoderma sp.*, was recorded on the 10th day of incubation, *Pleurotus florida* produced (4.60 IU/ml) after 12 days under stationary conditions [35]. Appreciable levels of laccase in some fungal species required a longer

production time of 12–30 days. Previous results revealed that maximum cases required more periods for large-scale production by fungal species.

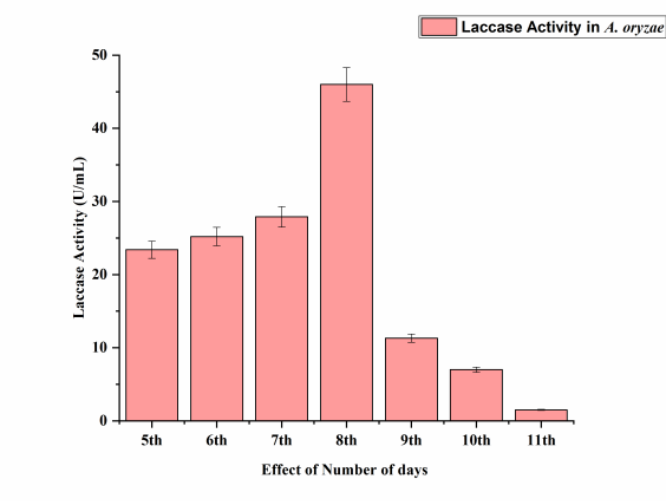

Fig 8. Effect of incubation periods on laccase production from *Aspergillus oryzae*

3.4 Production at optimized parameters

Aspergillus oryzae produces laccase under optimum conditions in liquid fermentation media. Total protein and laccase enzyme activity were calculated using the dialyzed sample. The test of significance of data was performed for all optimization parameters using one-way ANOVA in which the value of F was found and a P value less than (level of significance = 0.05%). This shows that the data is significant and the production of laccase at various parameters was done significantly at a 0.05% level of significance. The analyzed optimum parameters are best for maximum laccase production (64.2 ± 3.21 U/mL). Under optimized circumstances, laccase production was more than 2 times ($P < 0.05$) higher than in unoptimized parameters (20.0 ± 1.0 U/mL) (Table 1).

Table 1. Comparison of laccase production in various conditions byAspergillus oryzae.

S. No.	*Aspergillus oryzae* laccase production	Laccase activity (U/mL)
1.	Unoptimized condition	24.6±1.23
2.	Optimized condition	64.2±3.21

3.5 Molecular weight determination of purified laccase by SDS–PAGE

On SDS-PAGE, the pure laccase enzyme's molecular weight showed a clear band of 66.0 kDa under the purified laccase sample by using 12% SDS-gel electrophoresis. Similarly, a significant band with a molecular weight of 66 kDa was seen on SDS-PAGE of *Aspergillus nidulans* laccase (KF974331)[36] and also find that laccase molecular mass, which is generally in the range of 60 to 90 kDa and codes for 500–600 amino acids in the laccase protein[37].

3.6 Characterization of Laccase Activity

3.6.1 Effect of pH, temperature, activators, and inhibitors on laccase activity

Our investigation of purified *Aspergillus oryzae* laccase revealed that the maximum activity of laccase was obtained at 35°C and that the activity began to decrease proportionally as the temperature was raised above 35°C. At pH 6, the laccase enzyme had the greatest activity (Figure 9). Laccase activity appears to be hindered in an acidic environment. The optimal temperature for high laccase activity was determined in *Aspergillus niger* to be above 30 °C, confirming our findings[38].

$CuSO_4$, Tannic Acid, EDTA, and Sodium Azide (5–20 mM) amounts were added to the enzyme solution to determine the effect of different inducers and inhibitors. Both $CuSO_4$ and Tannic Acid considerably boosted the activity of the partly purified

laccase enzyme. Tannic Acid stimulates maximal laccase activity (59 ± 3.25 U/mL) at a concentration of 20 mM, whereas $CuSO_4$ obtains an optimal level with a gradual increase of 10 mM concentration and inhibits laccase activity following the increase in concentration (Figure 9). Before measuring laccase activity, the effect of EDTA and sodium azide as inhibitors were examined by adding the compounds at a concentration of (5-20 mM) (Figure 9). Previous findings explain that copper sulphate had the maximum laccase activity at a concentration of 10 mM. The addition of copper to the culture medium has been found to cause the laccase to fold properly due to the more efficient transport of copper to the Golgi components [39]. Previous studies also revealed that copper and $CuSO_4$ increase Trametes pubescens laccase production [40]. With the addition of inducer Tannic Acid, the laccase gene's expression rises, and laccase production increases in the growth medium [41]. It has previously been observed that binding of sodium azide to the types 2 and 3 copper sites affects internal electron transfer, decreasing laccase activity and that formaldehyde, as an organic solvent, alters the pH of an aqueous solution, resulting in lower enzyme output [42].

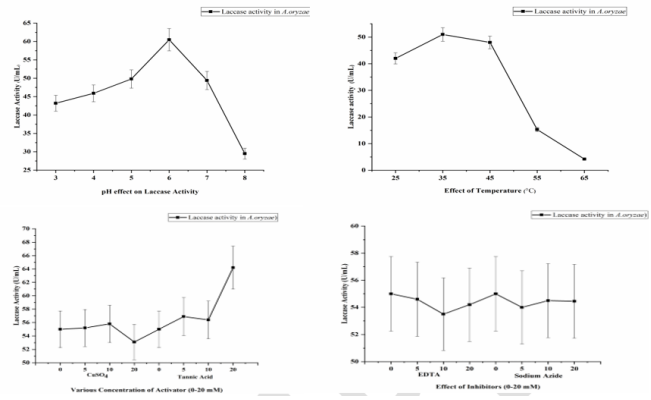

Fig 9. Effect of various factors on *Aspergillus oryzae* laccase activity

3.7 Application of Laccase

Laccase was isolated and used in industrial effluents to decolorize dyes, phenol, and phenolic compounds, and remove chemical oxygen demand.

3.7.1 Dyes Decolorization

The use of *Aspergillus oryzae* fungal laccase (500μl) to degrade different colours over a period ranging from 0 to 9 days revealed that laccases were capable of positive degradation and decolorization of 1% of synthetic dyes Bromophenol blue, Congo red, Methyl Orange, and Phenol Red. Decolorization was done up to 35.98%, 41.78%, 25.49%, and 20.62%, within 5 days as a change in the original colour and an apparent removal of colour. The decolorization of the dyes improved until the 5th day, after which there were no noticeable changes (Figure 10). Earlier studies found that even when the laccase mediator was present at the highest concentration, the *A. oryzae* laccases did not exceed the degraded Congo red dye by more than 28% [43], whereas in this work endophytic *A. oryzae* fungal laccases degraded 41.78%. *A. flavus* degrades Congo red and Bromophenol blue, two textile dyes, at 23.80% and 33.33%, respectively which was less than the present work [44]. It was previously also shown that with an initial dye concentration of 0.15g/250mL, *Aspergillus sp.* was able to decolorize red dye up to a maximum of around 70% at day 15 and orange dye up to a maximum of 35% at day 20 [45]. Synthetic dyes are carcinogenic and poisonous, and are efficiently broken down by the laccase enzyme from *A. oryzae*.

3.7.2 Phenol Removal by Laccase Treatment

Purified *Aspergillus oryzae* laccases eliminated 57.9% and 60% of phenolic pollutants from coal and textile industry effluents after 12 hours, respectively, as shown in the time course of phenolic pollutant elimination curves (Figure 11). In the case of negative controls, there was no evidence of elimination. Similar findings indicate that Bisphenol A (0.015%) was incubated with 1.5 U/mL of Lac 1 from *Grifola frondos* is capable of degrading Bisphenol A 30% without the use of a mediator in 6 hrs [46]. In just seven days, *T. versicolor*, a white-rot fungus, was found to be the most effective of the species, being reduced by 93% total phenol content under optimal conditions [47]. This present result is efficiently applicable to degrade phenol from industrial effluents.

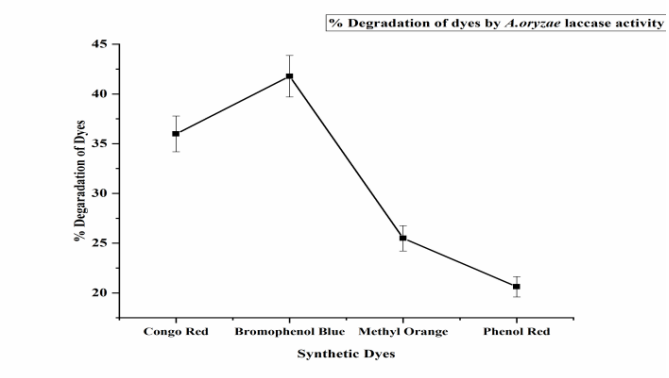

Fig 10. % of various dyes degradation by *Aspergillus oryzae* laccase activity

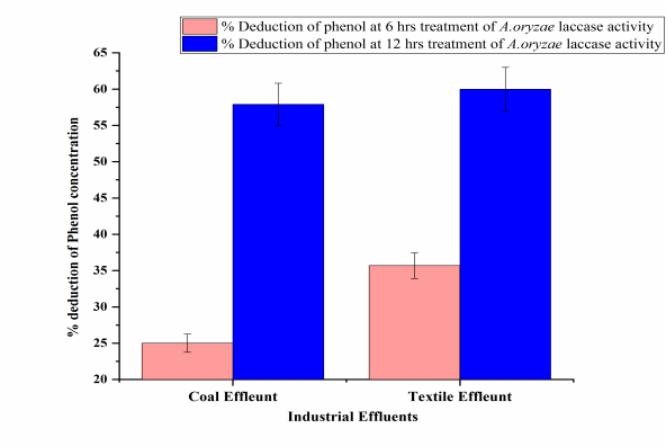

Fig 11. % deduction of phenolic pollutant from industrial effluents by *A. oryzae* laccase treatment

3.7.3 Chemical Oxygen Demand Removal by Laccase Treatment

For 0–9 days, the effect of isolated laccase from endophytic fungal *Aspergillus oryzae* sources on removing chemical oxygen demand from coal and textile industry effluents was studied. The results showed that *A. oryzae* laccases were capable of positive COD removal of both coal and textile industry effluents. As indicated in the time course of COD removal elimination curves of coal and textile industrial effluents from 1st to 7th days of treatment by *A. oryzae* laccase, 42.75%, and 35.98%, respectively, and were consistent on the ninth day (Figure 12). Similar results in *T. pubescens* MB 89 was achieved in flask cultures after 14 days of screening and achieved 79 % COD removal, 80 % total phenols removal, and 71 % colour decrease[40], which shows that with increased period and enzyme concentration, COD removal can be increased.

4 Conclusion

The present study shows an endophytic fungus, *Aspergillus oryzae*, isolated from *Ziziphus mauritiana* plant leaves from the surroundings of paper mill effluents in the ”Dongargaon and Raipur” region of Chhattisgarh, India, is capable of producing laccase appreciably (64.2 $\pm$ 3.21 U/mL). The enzyme production was shown to be enhanced by more than 2 fold through the optimization done in this investigation. This enzyme has the potential to decolorize various synthetic dyes and remove phenol and chemical oxygen demand from textile and coal industrial effluents. Thus, it can be a prospective organism for the source of laccase enzyme in various industrial applications, including further biotechnological exploitation. Enzymatic elimination of phenol and related hazardous substances by *Aspergillus oryzae* greatly enhanced the quality of wastewater that was known to be toxic to biological treatment systems. The host plant itself has naturally served as a selection system for microbes producing enzymes with reduced toxicity toward higher organisms. Therefore, the biotransformation reactions mediated by endophytic fungi and isolated enzyme systems are gaining tremendous attention among process researchers for sustainable

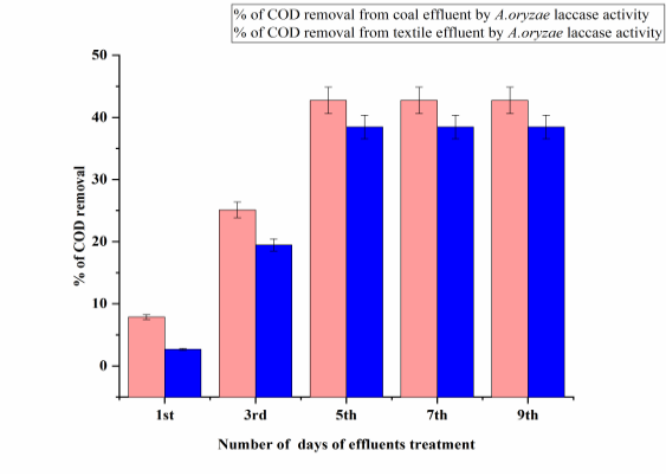

Fig 12. % of COD removal from Coal and Textile industry effluent by *A. oryzae* laccase treatment

development. The present research highlights the multifaceted potential of endophytic fungi assisted in the decolorization of dyes, bioremediation, and detoxification of pollutants.

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