RESEARCH ARTICLE

Isolation and Characterization of Newly Laccase-Producing Endophytic Fungi in Submerged Cultures from *Calotropis gigantean* Plant Leaves

Sabiha Naz[1*], Shailendra Gupta[2], Tanushree Chatterjee[1]

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Abstract

The aim of the study to isolate and identify new laccase sources from an endophytic fungal source that could be used as a weapon for eliminating and detoxifying contaminants found in wastewater and aquatic habitats. Isolation was done from *Calotropis gigantean* plant leaves from different locations of paper mill effluents from the "Raipur" region of Chhattisgarh, India. Positive isolates were obtained with a dark brown color below and surrounding the fungal colony due to guaiacol oxidation on potato dextrose agar. One potent endophytic fungal isolate that produces laccase is identified as *Aspergillus turcosus* by using the internal transcribed spacer (ITS) and BLAST analysis. After optimization, maximum laccase production was obtained at the following conditions: medium (Czapeck Dox Broth), carbon source (sucrose), nitrogen source (sodium nitrate), pH (6), activator tannic acid (20 mM), incubation period (35°C) and duration (8 days) with 3 (8 mM fermentation) disc inoculums. The maximum laccase activity was obtained at 65 U mL^{-1} in submerged optimized conditions, which was more than two fold compared to the unoptimized conditions. As estimated by SDS-PAGE, The molecular mass of the monomer of pure laccase was determined to be 66 kDa. After five days of treatment with the laccase of *A. turcosus*, the synthetic dyes phenol red, bromophenol blue, methyl orange, and Congo red lose their colour. Clearance rates for chemical oxygen demand were 59.46 and 48.57%, and phenolic contaminants were 80 and 22.3% in coal and textile effluents during the required treatment periods, respectively. One novel and potent laccase-producing endophytic fungus was successfully isolated, which can be utilized as a laccase-producing source for various industrial applications.

Keywords: *Aspergillus turcosus,* Endophytic fungus, Industrial effluents, Internal transcribed spacer, Laccase, Optimization.

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Introduction

Several factors, including post-ingestion metabolic excretion, industrial waste, and incorrect disposal, contribute to pharmaceuticals' environmental contamination (Huber *et al*., 2018). The health of people and animals is significantly impacted by the pollutants and toxins that accumulate in water bodies. However, varieties of diverse pollutants are challenging and are discharged into water bodies (Daughton, 2004). Biotechnologists are creating innovative tools and environmentally friendly procedures all over the world to mitigate the consequences of environmental contamination. Because of the fact that remarkable selectivity high productivity while maintaining a low impact on the ecosystem, microorganisms and microbial enzymes are being used to eliminate industrial and environmental contaminants (Klaper and Welch, 2011).

Recently, the potential application of multi-copper enzymes made of glycoproteins termed fungal laccases which EC 1.10.3.2, and also comes under class of p-diphenol: dioxygen oxidoreductase, in the detoxification of resistant pollutants and the bioremediation of phenolic and non-phenolic chemicals, have heightened interest in these enzymes. The protein fungus laccase of 60–70 kDa with an amino acid sequence between 220–800 and an isoelectric point of 4.0 for acid. Many species of ascomycetes and basidiomycete, such as *Melanocarpus albomyces*, *M. unicolour*, *Magnoporthe grisea*, *Trametes versicolour*, *Trichoderma resei,* and *Xylaria polymorpha* have provided the majority of the laccase enzyme for purification. Various soil-borne and freshwater species of ascomycetes from the genera *Aspergillus, Curvularia*, and *Penicillium* and pathogenic plant species have been observed to produce laccase. Their property is to catalyze a diverse array of substrates and change various contaminants into nontoxic compounds. Because their co-substrate, oxygen, is often available in their surroundings, they don't need any other co-factors. Laccase production from fungi was done during secondary metabolism, which was commonly impacted by a variety of cultivation methods like carbon and nitrogen, pH, temperature, media, inducers, incubation periods, etc. (Garcia-Morvales *et al.* 2015) The majority of laccases are extracellular, making purifying processes simple. The majority of fungal species often show a high degree of stability in the extracellular environment and the induced production of laccases. Endophytic fungi identified in

[1]Department of Biotechnology, Raipur Institute of Technology, Swami Vivekanand Technical University, Raipur, Chhattisgarh, India.

[2]Department of System Biology and Bioinformatics, University of Rostock, Germany.

***Corresponding author:** Sabiha Naz, Department of Biotechnology, Raipur Institute of Technology, Swami Vivekanand Technical University, Raipur, Chhattisgarh, India, Email: sabihanaz4@gmail.com

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various plant habitats may be beneficial to mankind in various ways. Endophytes bioactive secondary metabolites serve as a chemical reservoir for substances that are antibacterial, antioxidant, immune-stimulating, anticancer, and anti-parasitic (Gopi and Jayaprakashvel, 2017). Laccase can also be used as a bioreporter or biosensor. It can be applied as a biological fuel cell, which is of special importance for medical purposes (Kudanga and Rose-Hill, 2014) and in nanobiotechnology (Rodriguez-Delgado and Aleman Nava, 2015). Discoveries of endophytic fungi are significant because they have the biological potential to produce novel chemicals for use in industrial, medicinal, and agricultural applications, in addition to their abundance and diversity (Meenambiga *et al.* 2020). Depending on the organism, laccases play a variety of roles in nature and take part in both anabolic and catabolic processes (Eisenman *et al.* 2007). Low molecular weight phenolics are oxidized in normal anabolic reactions (Sun *et al.* 2021) and can potentially create dimeric radicals that can then either self-couple or cross-couple to form trimers or polymers. Lignin/lignocellulose depolymerization (Kundanga *et al.* 2017) and humus degradation are two examples of laccase-mediated catabolic processes, which may be an effective biotechnological tool in a wide range of industrial uses (Rico *et al.* 2014). The activity of microorganisms in the fermentation process led to the reduction of the complexity of the polymeric lignocellulose materials into simple monomers (Preethi *et al.* 2018).

The utilization of various dyes is widespread throughout many different industries, like the textile industry, paper manufacturing, printing, cosmetics, and the medical field. It is estimated that 10–15% of the pigments used during the dying process are lost in the effluent. Due to their intricate structural makeup, most of these dyes are resistant to decolorization. There are chemical ways for cosmetics to get rid of these colors; however, these processes are both expensive and harmful to the environment (Vantamuri and Kaliwal, 2016). Because laccase drastically decreases the biological oxygen demand, chemical oxygen demand and amount of particulates present in grey water, it has become an essential enzyme in the mycoremediation process used to remediate grey water (Al Gheethi *et al.* 2019). The innovative technology of forward osmosis, which uses laccase to help remove micropollutants from sewage and enhance water potability, was developed to accomplish these two goals (Liu *et al.* 2019). Because of its excellent catalytic activity and reusability, laccase plays an important role in the breakdown of organic substances, which is an essential step in reducing the contamination of water (Gu *et al.* 2020). Due to the unavailability of laccases, their utilization has been ignored in many commercial fields. Therefore, this research aimed to examine the effectiveness of the endophytic isolates for laccase production, their optimization in fermentation conditions, and their application to protect the environment.

Materials and Methods

Samples Collection

Samples were taken from the *Calotropis gigantean* plant's leaves from different locations of industrial area effluents in the Raipur region (41°24'12.2"N) of Chhattisgarh State, India. The samples were cut, labelled, and placed separately in polythene bags after the removal of excess moisture and kept in a refrigerator at 4 °C till further use.

Isolation and Screening

The endophytic fungus was isolated by Arnold *et al.* (2000) method. 5–6 segments of sterile samples were incubated on potato dextrose agar (PDA) for 72 hours at 25 ± 2°C. Potato dextrose agar plates were spread with 100 µL of the final and second-final distilled water washes, used as a sterility control. The fungi that came out the leaf tissue were transferred on fresh PDA media with no antibiotics. All the isolated organisms were subcultured and maintained for further use. Fungal isolates obtained were used for the preliminary screening step, and each isolate was then spot-inoculated on petri plates containing PDA medium, amended with 0.01% Guaiacol by Kiiskinen *et al.* (2014) method. The emergence of a reddish-brown oxidation zone surrounding the colonies showed the fungus was able to produce extracellular laccase. A strain with the greatest activity of laccase was chosen for further research.

Inoculum Preparation and Laccase Production

The laccase-production inoculum was produced by incubating two 8 mm-diameter discs of a 5-day-old culture for three days at 25 ± 2°C using 50 mL of the laccase-producing liquid medium in a 250 mL Erlenmeyer flask. As part of quantitative screening, the chosen isolates were exposed to submerged fermentation to ascertain their capacity for maximum laccase production by growing in a liquid medium that produces laccase using the techniques described by Monnat *et al.* (2012). The production of laccase by fermentations were performed in Erlenmeyer flasks (250 mL) which contained 100 mL of medium formulated up of compoents (g/L) with pH (4.5) : sucrose-2.0; urea-0.14 ; yeast extract-0.34; $CaCl_2{\cdot}2H_2O$-0.004 ; KH_2PO_4-0.1; $MgSO_4{\cdot}7H_2O$-0.07; and Na_2HPO_4-0.3). Two discs with a diameter of 8 mm each were taken from a culture that was five days old. These discs were then inoculated with fermentation media, put in a rotary shaker (Rivotek, India), and incubated at a temperature of 25 ± 2°C for seven days. Following the completion of the time of incubation, the medium used was processed for the extraction of the laccase enzyme.

Laccase Extraction

After an incubation period of 7 days, the transparent supernatant was extracted by centrifugation (using a REMI C-24BL cooling centrifuge from India) at 5000 x g for 5 minutes at 4°C. This procedure was carried out in accordance with Sidhu *et al.* (2014).

Laccase Assay and Protein Estimation

The activity of laccase was evaluated using a technique that was explained by Jhadav *et al.* (2009). This involved observing the oxidation of a buffer containing 10 mM guaiacol with (pH 5.4) 100 mM sodium acetate buffer at 530 nm for 10 minutes while 470 nm was used for the measured blank. The reaction mixture (5 mL) consisted of 1 mL of culture filtrate, 1-mL of 10 mM Guaiacol, and 3 mL of buffer, whereas culture filtrate was excluded in the blank tube. The amount of laccase activity was measured and expressed in enzyme units per millilitre (U/mL). Laccase activity was calculated and expressed in enzyme units

(U) per mL or ($U.mL^{-1}$). The Bradford (1976) test was used to estimate the sample's protein content.

Calculation

$$\text{Volume Activity (U/mL)} = \frac{\Delta \text{ A420 nm/Min x 4 x Vt x dilutions factor}}{\in \text{ x Vs}}$$

Where,

Vt = reaction mixture final valume (ml) = 5.00

Vs = volume of sample (mL) = 1

€ = guaiacol extinction co-efficient = 6,740 M/cm

4 = derived from unit principle and definition

Identification of Fungal Isolates

Initially, the fungi was identified by microscopic analysis according to the cultural characteristics identified on PDA media, as well as microscopic investigation carried out by lactophenol cotton blue slides at the research center of Raipur Institute of Technology, Raipur. Further identification of the fungi, up to the species level, was carried out by Molecular characterization Internal Transcribed Spacer (ITS) at Bioreserve Pvt. Ltd., in Hyderabad (India).

Optimization Studies

Three different media (PDB, Czapek Broth, and Carboxymethyl Cellulose Broth) were screened for laccase production. Temperature (25–45°C with 10°C increments), pH (4–8), number of discs (8 mm in diameter) varied from 1 to 5, number of days (5–10), carbon source concentration range of 10 g/L (glucose, sucrose, fructose, galactose), and nitrogen source concentration range of 10 g/L (urea, peptone, ammonium nitrate, sodium nitrate). This sterilized medium was inoculated with two discs of 8mm diameter, a culture that was five days old. The culture was then incubated for 7 days at 25 ± 2°C and a pH of 4.5. All of the optimization studies were carried out in triplicates using the traditional one-factor-at-a-time constant. The culture is harvested, filtrated, and used to determine enzyme activity.

Laccase Production on Optimized Parameters and Purification

In order to evaluate the influence that all of the parameters listed above have combined and to achieve the maximum amount of laccase production, a medium for laccase production was produced with all the optimal parameters. The enzyme activity was calculated after the period of incubation and compared to the medium that unoptimized medium after purification in triplicates. The culture filtrate was purified using ammonium sulphate between 0 and 75% saturation and dialysis, as described by Patel *et al.* (2009). The dialyzed protein was subjected to gel filtration chromatography, and the dialyzed enzyme extract (3.0 mL) was loaded onto a Sephadex G-100 column.

Characterization of Isolated Laccase

Molecular weight determination by SDS-PAGE

Standard protein markers ranging in size from 25–170 kDa (Ge-Nei, Bangaluru, India) were used in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), with a stacking gel (10%) and resolving gel (12%) to ascertain the purity and molecular weight of the purified protein fraction (Laemmli, 1970). Following the electrophoresis, the separated bands using Coomassie Brilliant Blue R-250 as a stain.

Effect of Various Factors (Temperature, pH, Inducers, and Inhibitors) on Laccase Activity

Enzymes were pre-incubated for 10 minutes at various temperatures (from 20–60°C) and a range of pH (from 3 to 8) to determine the impact of these variables on laccase activity. The effect of different inducers and inhibitors in different concentrations (5–20 mM) of $CuSO_4$, EDTA, sodium azide, and tannic acid on enzyme activity was measured by pre-incubating with inducers and inhibitors for 10 minutes prior to adding the substrate, allowing them 10 minutes of reaction time at 30°C. The laccase activity was quantified as per the priorly mentioned laccase assay method in triplicates.

Kinetic properties

Pure laccase was used in spectrophotometric measurements of substrate oxidation at the wavelength of interest. In a pH 5.5 buffer composed of 100 mM sodium acetate, the rise at the A470 for Guaiacol was measured. The rate of reaction at guaiacol was measured at concentrations ranging from 0.18 to 10 mM. The Km and Vmax of enzyme kinetic constants were obtained by employing a Lineweaver-Burk plot with guaiacol as the substrate.

Dye Decolourization by Endophytic Fungal Laccase

Specific absorption peak of each dye solution at 1% dye solution was determined with the help of a UV-vis spectrophotometer, which scanned the wavelength. 1% of the synthetic dye solutions were prepared by dissolving the dyes bromophenol blue, phenol red, methyl orange, and congo red in water. On the basis of this information, the calibration curve for each dye was obtained in order to conduct additional dye concentration measurements across a variety of treatments. The procedure for decolorization was performed in triplicates inside a 20 mL test tube with 10 mL of 1% dye solution, a buffer with a pH of 6, and laccase in a volume of 500 µL at a temperature of 35°C for 0 to 9 days. For the purpose of all color measurements, the day on which effluents were first added to the pre-grown cultures is referred to as zero day and was regarded as 100%. According to Schmitt *et al.* (2012), the actual concentration of the dye in the medium can be determined by basing the calculation on the decolorization percentage.

Phenol Deduction by Endophytic Fungal Laccase

In 20 L of waste effluent samples were collected from the coke oven section of the Bhilai steel plant, Bhilai, and the dye and clothing industries, Bhilai, and stored at 4°C. The method of sampling that is used is known as composite sampling. The procedure involved incubating 500 mL of enzyme culture supernatant in triplicate with 10 mL of a 10% final concentration of effluent solution for 6 to 12 hours at 35°C. A control consisting of 10% untreated effluent solution was also included in the experiment. Per the Rice *et al.* (2012) method, the concentrations of phenolic content were analyzed with the Folin-Ciocalteu reagent using a spectrophotometer set at 765 nm. The reaction mixture consisted of 1000 µL of effluent, 250 µL of sodium carbonate solution 12 g/L as a concentration, and 25 µL of Folin-Ciocalteu at a concentration of 2 µL. Every sample was held at 20°C for a period of half an hour. In order to establish the levels in the samples, a calibration curve was

developed with the standards using pure phenol. This curve was used as a calibration curve for the standards.

Chemical Oxygen Demand Deduction by Endophytic Fungal Laccase

This was accomplished by incubating (20 U/mL) enzyme culture supernatant with 50 mL of a 10% final concentration of effluents to eliminate COD for 0–9 days at 35°C in triplicates. The incubation time was different for each day. For the purpose of all colour measurements, the day on which effluents were first added to the pre-grown cultures is referred to as day zero and was regarded as 100%. Throughout the process of treatment, the effluent was kept in the dark and stirred very slowly. The Rice *et al.* technique (2012), which was used to measure COD, was followed.

Statistical Analysis

The results of all the observations were the mean of three experiments. The description of statistics of the observations were shown as Mean Standard ± error in this representation. The results of the statistical analysis can be observed in the form of tables below. The data were statistically analyzed using a one-way ANOVA followed by Tukey's multiple comparison tests with a statistical significance of $p < 0.05$. The Origin Pro 2018 (version 10.0) statistical programme was used for the analysis.

Results

Isolation of laccase-producing endophytic fungi from various plant leaves from different locations of paper mill effluents from the "Durg-Bhilai-Raipur" region of Chhattisgarh, India, was collected. In total, eight were isolated from *C. gigantean* plant leaves. The result of qualitative screening showed that out of eight isolated endophytes from *C. gigantean* plant leaves; only one laccase-producing species produced a maximum zone (brown colour) under and around the fungal colonies from the leaves of the *C. gigantean* plant (Fig. 1). The morphological characteristics of the isolation of one novel maximum laccase producing endophytic fungal strain show velvety, grey-turquoise colonies, loosely columnar conidial heads were seen after lactophenol cotton blue stain, showing similarity to *Aspergillus sp.* The endophyte 2Ca fungal strain isolated is identified as *Aspergillus turcosus* based on 18S rRNA molecular identification done by Bio Reserve Pvt. Ltd. in Hyderabad, India. The Gene Bank accession numbers of strains of the endophytic fungus *A. turcosus MK966400.1* show similarity (E-value =0.0, pair similarity =100% identical) with the sequenced laccase-producing endophytic fungi.

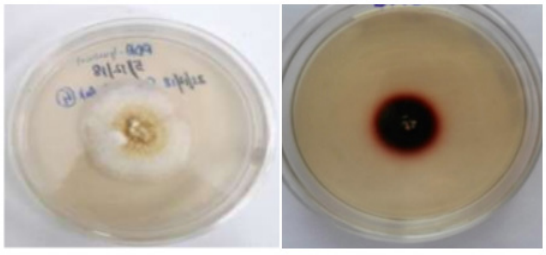


Fig. 1: Screening of laccase producing endophytic fungi by plate tests using the indicator Guaiacol.

The effects of three different media (Potato Dextrose Broth, Czapek Dox Broth, and Carboxymethyl Cellulose Broth) were screened for laccase sythesis, and the results revealed that the Czapek Dox Broth media was best for laccase production (39 ± 1.95 $U.mL^{-1}$) (Table 1a, and Fig. 2a). The medium formulation, which must include the appropriate carbon and nitrogen sources, is thought to be of the greatest significance in the early stages of the formation of any process. The effect of adding 1% glucose, sucrose, fructose, and galactose to the Czapek Dox Broth was studied.

Carbon is the most important source of energy for an organism's maturation and growth. The presence of 1.0 g/L of sucrose produced in highest laccase production of 16.5 ± 0.825 ($U.mL^{-1}$), and it was significant ($p < 0.05$) (Table 1b and Fig. 2b) for *A. turcosus*. The effect of both inorganic and organic nitrogen sources was investigated to find out which of these sources influenced laccase production the most. According to the conclusions of the study on nitrogen source optimization, inorganic sources of nitrogen are preferable, like 1.0 g/L of sodium nitrate, which resulted in a maximum laccase synthesis of 20.8 ± 1.04 ($U.mL^{-1}$), and it was significant ($p < 0.05$) (Table 1c, Fig. 2c).

The result of the influence of the pH range from 4 to 8 of the initial medium on the laccase production by *A. turcosus* was reported to be the maximum titer of laccase (25.2 ± 1.26 $U.mL^{-1}$)

Table 1a: Effect of various media on laccase production from *A. turcosus*

S.No.	*Effect of Media*	*Laccase activity ($U.mL^{-1}$)*
1.	Potato Dextrose Broth	38 ± 1.9
2.	Czapeck Dox Broth	43 ± 2.15
3.	Carboxymethyl Cellulose Broth	29 ± 1.45

Table 1b: Effect of various carbon sources on laccase *laccase* production from *A. turcosus*

S.No.	*Carbon Sources*	*Laccase activity ($U.mL^{-1}$)*
1.	Glucose	5.9 ± 0.295
2.	Sucrose	16.5 ± 0.825
3.	Fructose	9.4 ± 0.47
4.	Galactose	1.9 ± 0.095

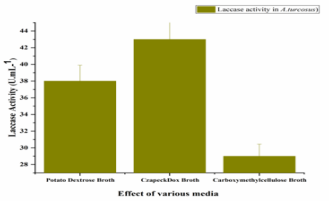


Fig. 2a: Effect of various media on laccase production

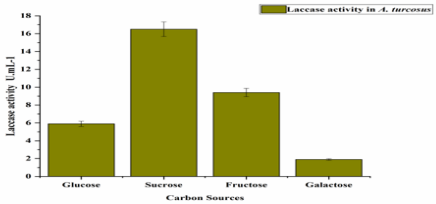


Fig. 2b : Effect of various carbon sources on laccase production

at pH 6.0 after 7 days of the incubation period, respectively **(**Table 1d, **Fig. 2d),** and it gradually decreased at higher medium pH (6–8). The statistical evaluation of the collected data showed that pH 6 was significant ($p < 0.05$) for the greatest laccase synthesis by *A. turcosus* compared to the other media pH levels. The ideal temperature for the laccase production by *A. turcosus* was identified at many temperatures ranging from 25–45°C with a 10°C increment. Maximum laccase synthesis was observed in *A. turcosus* at 35°C (58 ± 2.9 $U.mL^{-1}$), and with the increase in temperature, laccase production was recorded at its lowest at 45 °C**.** As shown in (Table 1e, Fig. 2e), statistical analysis of the data revealed that the temperature of 35°C was significantly ($p < 0.05$) higher than the other temperature levels for laccase production by *A. turcosus*.

The influence of inoculum number on laccase synthesis by *A. turcosus* was evaluated by altering the quantities of inoculum introduced to the fermentation medium. Laccase production was only significant (59 ± 2.95 $U.mL^{-1}$) at the three discs (8 mm diameter) inoculum number (Table 1f, and Fig. 2f), and enzyme activity dropped dramatically at the 4 and 5-disc (8 mm diameter) inoculum numbers, which could be influenced by the presence of a favorable growth environment, such as adequate nutrients and oxygen, which would boost laccase synthesis, and the implication was significant ($p < 0.05$). The influence of the incubation period was investigated on the laccase production of *A. turcosus* during 5–11 days of incubation at different experimental conditions, with the maximum output observed on the 8th day of incubation. (49 ± 2.45 $U.mL^{-1}$) and steadily declined until the 11th day of incubation (Table 1g). As there was a decrease in nutrient food for laccase from the 8th day onwards, there was a decrease in enzyme production of a significant magnitude. The statistical evaluation of the data revealed that the 8th day was significant ($p < 0.05$) for the production of laccase by *A. turcosus* compared with the laccase production at the other temperature ranges, as shown in Fig. 2g.

Laccase production was done at optimized parameters on liquid fermentation media by *A. turcosus*. The result demonstrated that *A. turcosus* produced a maximum total protein concentration of 35.299 (µg/mL) whereas laccase produced 65 ± 3.25 $U.mL^{-1}$.Production in optimized parameters was more than 2 fold higher than in unoptimized conditions (Fig. 3). The test of significant data was performed for all optimization parameters using one-way ANOVA, in which the value of F was found and the $p < 0.05$. The analyzed optimum parameters are best for maximum laccase production and activity. Under the optimum parameters obtained, the highest laccase activity of 65 ± 3.25 $U.mL^{-1}$ represents more than twice the increase from 26.8 ±1.34 $U.mL^{-1}$ in the basic medium and was further procured in an optimized medium containing (g/L): (10 g sucrose, 10 g sodium nitrate, and 10 g urea instead of 2.0 g sucrose, 0.34 g yeast extract, and 0.14 g urea in the fermentation medium, pH 6).

The extracellular laccase that was extracted from *A. turcosus* was purified to 292.88 total purity and had a yield of 79.98% after going through a variety of purification stages (Table 2), including ammonium sulfate precipitation, dialyzes, and gel filtering utilizing Sephadex G-100 column chromatography. On SDS-PAGE, the molecular mass of the isolated laccase enzyme was shown to be a separate band at 66.0 kDa (Fig. 4). SDS–PAGE analysis showed that the protein was monomeric and had a molecular mass of 66 kDa.

The influence of temperature on laccase activity was examined by pre-incubating the enzyme for 10 minutes at different temperatures (25–65°C) and pH (3–8) for the partially

Table 1c: Effect of various nitrogen sources on laccase production from *A. turcosus*

S.No.	*Nitrogen Sources*	*Laccase activity ($U.mL^{-1}$)*
1.	Ammonium Nitrate	14.65 ± 0.7325
2.	Peptone	10.92 ± 0.546
3.	Sodium Nitrate	20.8 ± 1.04
4.	Urea	15.64 ± 0.782

Table 1d : Effect of various pH on laccase production from *A. turcosus*

S.No.	*Different pH of Media*	*Laccase activity ($U.mL^{-1}$)*
1.	4	7.5 ± 0.375
2.	5	14.5 ± 0.725
3.	6	25.2 ± 1.26
4.	7	5.1 ± 0.395
5.	8	4.9 ± 0.345

Table 1e : Effect of various temperatures on laccase production from *A. turcosus*

S.No.	*Temperatures (°C)*	*Laccase activity ($U.mL^{-1}$)*
1.	25	45 ± 2.25
2.	35	58 ± 2.9
3.	45	34 ± 1.7

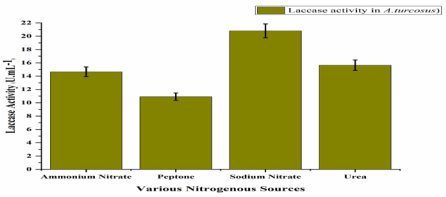


Fig. 2c : Effect of various nitrogen sourcess on laccase production

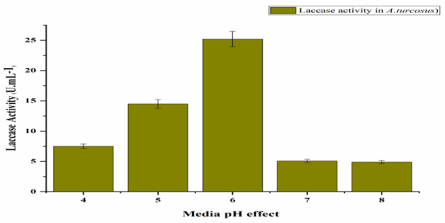


Fig. 2d : Effect of various pH on laccase production

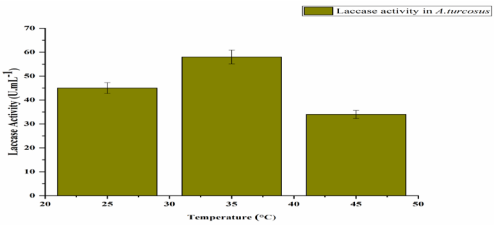


Fig. 2e: Effect of various tempearture on laccase production

Table 1f : Effect of no. of inoculum disc (8 mm in dia) on laccase production

S.No.	*Inoculums number of disc in Dia. (8 mm)*	*Laccase activity (U.mL^{-1})*
1.	1	53 ± 2.65
2.	2	54 ± 2.7
3.	3	59 ± 2.95
4.	4	43 ± 2.15
5.	5	40 ± 2.0

Table 1g : Effect of incubation period (no. of days) on laccase production

S.No.	*Effect of incubation period (Days)*	*Laccase activity U.mL^{-1}*
1.	5th	28 ± 1.4
2.	6th	31 ± 1.55
3.	7th	34 ± 1.7
4.	8th	49 ± 2.45
5.	9th	17 ± 0.85
6.	10th	5 ± 0.25
7.	11th	2 ± 0.1

Table. 2: Purification of laccase from *A. turcosus*

Purification steps	*Activity (U.mL^{-1})*	*Protein Con centration (mg.mL^{-1})*	*Specific Activity (U. mg^{-1})*	*Yeild (%)*	*Fold purity (%)*
Crude	26.2	35.98	0.745	100	1
Dialysis	65.0	33.48	1.941	93.05	262.16
Gel filtration chroma tography	62.8	28.78	2.182	79.98	292.88

Table 3a: Effect of temperature on laccase activity of *A. turcosus*

S. No.	*Effect of Temperatures (°C)*	*Laccase activity (U.mL^{-1})*
1.	25	45.2 ± 2.26
2.	35	58.6 ± 2.93
3.	45	34.7 ± 1.735
4.	55	10.3 ± 0.515
5.	65	5.2 ± 0.26

purified laccase activity. The result showed that the partially purified laccase enzyme has the highest activity at pH 6.0 and 35°C. The enzyme activity decreases above and below the optimal pH and temperature (Tables 3a and 3b, Figs 5a and b). The effect of different inducers and inhibitors was measured by the addition of (5–20 mM) concentrations of $CuSO_4$, tannic Acid, EDTA, and sodium azide to the enzyme solution. Adding $CuSO_4$ and tannic acid activators significantly increased the partially purified laccase enzyme activity. Tannic acid promotes maximum laccase activity (65 ± 3.25 U.mL^{-1}) at a concentration of 20 mM, whereas $CuSO_4$ achieves an optimal level with a gradual increase of 10 mM concentration and after an increase in concentration, it inhibits laccase activity (Table 3c, and Fig. 5c). The results reveal that sodium azide and EDTA absolutely

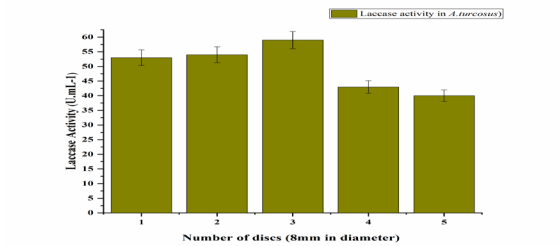


Fig. 2f : Effect of no. of inoculum disc (8 mm in dia) on laccase production

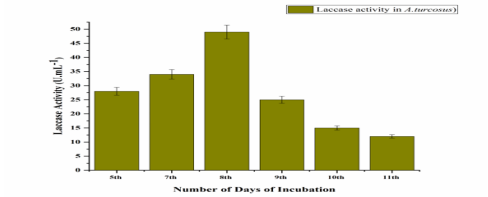


Fig. 2g: Effect of incubation period (no. of days) on laccase production

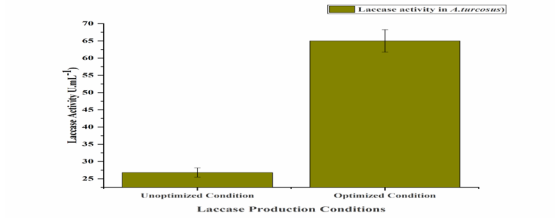
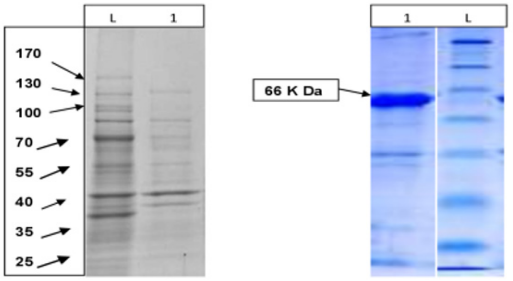


Fig. 3: Endophytic fungal laccase production from *A. turcosus* at optimized conditions



Lane1, is filtrate from *A. turcosus* respectively. Lane L: High molecular weight protein size ladder (size 25–170 kd)

Fig. 4: SDS PAGE (12%) of unpurified and purified laccase from *A. turcosus* endophytic fungi

inhibit the enzyme activity at all concentrations (Table 3d, and Fig. 5d). The kinetic parameters Km and V_{max} of purified laccase produced from *A. turcosus* have been estimated to be 2.4 mM and 1.6 mol min^{-1}, respectively (Fig. 5e).

The application of *A. turcosus* laccase (500 µL) to degrade several colors over a period that extended from 0 to 9 days revealed that laccases were capable of positive degradation and decolorization of 1% of synthetic colors phenol red, bromophenol blue, methyl orange, and congo red. Decolorization was done up to 26.96, 19.08, 40.52, and 14.48% within 5 days as a change in the original color and apparent disappearance of color. The decolorization of the dyes improved until the 5th day, after which there was no visible change (Table 4, Fig. 6). This observation demonstrated that *A. turcosus* laccase was most applicable to decolorized methyl orange.

Table 3b: Effect of pH on laccase activity of *A. turcosus*

S.No.	*Effect of pH*	*Laccase activity (U.mL^{-1})*
1.	3	43.2 ± 2.16
2.	4	45.9 ± 2.29
3.	5	49.8 ± 2.49
4.	6	60.5 ± 3.02
5.	7	49.4 ± 2.47
6.	8	29.5 ± 1.47

Table 3c: Effect of activators on laccase activity isolated from *A. turcosus* endophytic fungi

Activators	*Different concentration of activators (mM)*	*Enzyme activity (U.mL^{-1}) A. turcosus*
$CuSO_4$	0	53 ± 2.65
	5	55.9 ± 2.795
	10	56.8 ± 2.84
	20	56.1 ± 2.805
Tannic Acid	0	53 ± 2.65
	5	57.8 ± 2.89
	10	57.3 ± 2.865
	20	65 ± 3.25

Table 3d: Effect of inhibitors on laccase activity isolated from *A. turcosus* endophytic fungi

Inhibitors	*Different concentration of inhibitors (mM)*	*Enzyme activity (U.mL^{-1}) A. turcosus*
EDTA	0	53 ± 2.65
	5	52.6 ± 2.63
	10	51.9 ± 2.595
	20	52 ± 2.6
Sodium Azide	0	53 ± 2.65
	5	52.9 ± 2.645
	10	52.2 ± 2.61
	20	52.0 ± 2.6

Table 4 : % of various dyes degradation by *A. turcosus* laccase treatment.

Synthetic dye	*% of Dye degradation by A. turcosus laccase treatment*
Congo red	19.08 ± 0.954
Bromophenol Blue	26.96 ± 1.348
Methyl orange	40.52 ± 2.026
Phenol red	14.48 ± 0.724

According to the time course of phenolic pollutants, elimination curves (Table 5, Fig. 7), and purified *A. turcosus* laccases eliminated 80% of phenolic pollutants from coal industry effluents and 23% of phenolic pollutants from textile industry effluents after 12 hours, respectively. In the negative controls, there was no sign of phenol removal.

The effect of isolated laccase from endophytic fungal *A. turcosus* sources on the elimination of the chemical oxygen

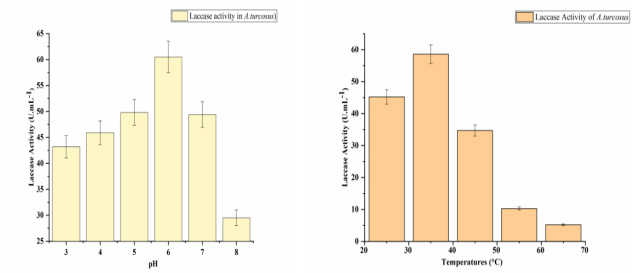


Fig. 5a-b: Effect of pH and Temperature on laccase activity from *A. turcosus* endophytic fungi

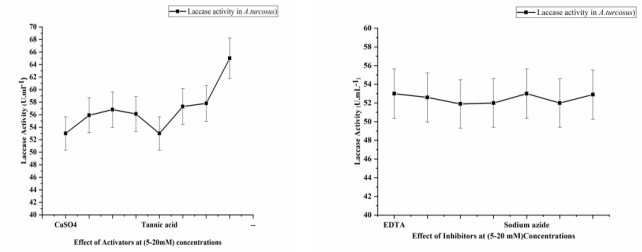


Fig. 5c-d: Effect of activators and inhibitors on laccase activity of *A. turcosus* endophytic fungi

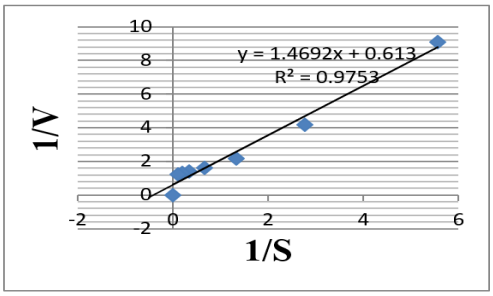


Fig. 5e: Lineweaver- Burk plot with guaiacol as substrate

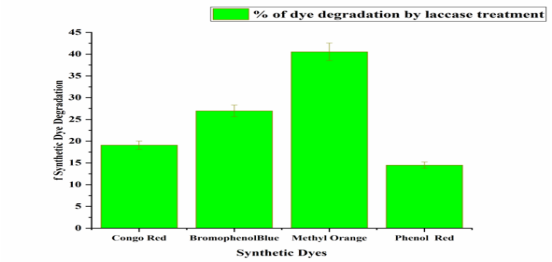


Fig. 6: % of various synthetic dyes degradation by *A. turcosus* laccases treatment

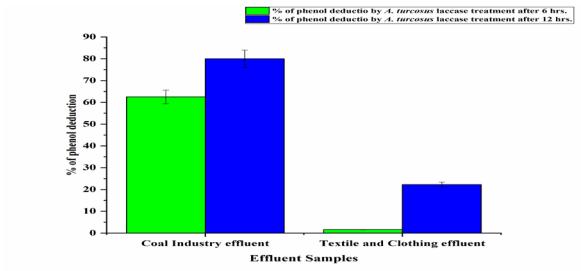


Fig. 7: % of deduction of phenolic pollutant from coal and textile effluents by *A. turcosus* laccase treatment

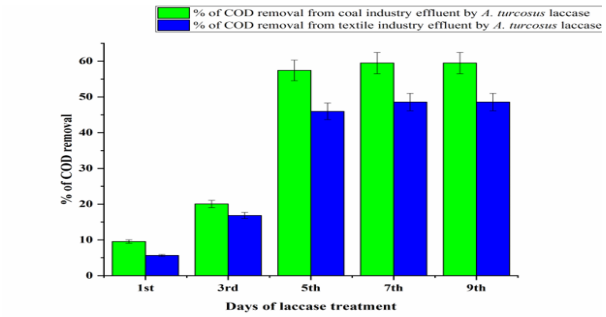


Fig. 8: % of COD removal from coal and textile effluents effluent by *A. turcosus* laccase treatment.

Table 5 : Percentage of deduction of phenolic pollutant from coal effluents by *A. turcosus* laccase enzyme treatment.

Industrial Effluent	*A. turcosus laccase % of deduction of phenolic pollutant*	
	6 hours	*12 hours*
Coal Industry effluent	62.5	80.0
Textile and Clothing effluent	1.6	22.3

Table 6 : Percentage of COD removal of coal and textile industry effluent by *A. turcosus* laccase laccase enzyme treatment

S No.	*No. of days of incubation after laccase enzyme (20 UmL^{-1}) treatment*	*% of COD removal from effluents*	
		Coal Industry effluent	*Textile effluent*
1.	1st	9.56 ± 0.478	5.64 ± 0.282
2.	3rd	20.05 ± 1.0025	16.82 ± 0.841
3.	5th	57.40 ± 2.87	45.95 ± 2.2975
4.	7th	59.46 ± 2.973	48.57 ± 2.4285
5.	9th	59.46 ± 2.973	48.57 ± 2.4285

demand from effluents from the coal and textile industries was examined over a period of 0–9 days. The findings indicated that the laccases produced by *A. turcosus* could positively remove COD from the effluents of the coal and textile industries. As shown in the time duration of COD removal elimination curves of coal and textile industry effluents from the first to the seventh days of treatment by *A. turcosus* laccase, removal was 60 and 50%, respectively, and remained the same on the ninth day (Table 6, Fig. 8).

Discussion

Laccase-producing endophytic fungi were isolated from the *C. gigantean* plant leaves from different locations of paper mill effluents from the "Durg-Raipur-Bhilai" region of Chhattisgarh, India. Eight endophytic fungi were isolated and screened for laccase production. After seven days in dark conditions, the results of screening show that out of eight isolated endophytes, only one has the capability to produce laccase (a brown color) under and around the fungal colonies from guaiacol oxidation. In previous studies, similarly, *Scytalidium lignicola* pesante (Sidhu *et al.* 2017) and *Myrothecium verrucaria* (Sun *et al.* 2017), *P. eryngii, P. florida* and *P. sajor caju* (Mathur *et al.* 2021), *Beauveria pseudobassiana* (Sharma *et al.* 2023), *Beltraniopsis* sp. ET-17and *Phlebia floridensis* (Laura *et al.* 2023) were described as novel laccase-producing fungi in a media containing 0.02% Guaiacol.

One of the essential components in the creation of any procedure is the medium formulation, which must have the proper carbon and nitrogen sources. Carbon is the most essential source of energy for an organism's growth and development. Of the wide variety of carbon sources that were examined, sucrose was the most effective carbon source for *A. turcosus,* probably because it is rich in carbon, which can be suitably used as an energy source. Among the different carbon sources, 1.0 g/L of sucrose addition gave the maximum laccase production. Several research findings also demonstrated that sucrose is one of the greatest carbon sources for fungal laccase formation (Eugenia *et al.*, 2009), *Pseudomonas aeruginosa* ADN04 (Kumar, 2017), and *Agaricus* species (Jose and Joel, 2014). In this study, the impacts of a wide variety of inorganic and organic nitrogen sources were analyzed to determine which of these sources had the most impact on laccase production. The optimization of nitrogen sources suggests that inorganic nitrogen sources like 1.0 g/L of sodium nitrate result in the maximum laccase production. Leatham and Krik (1983) state nitrogen depletion frequently stimulates laccase production. Similarly, for *Pichia* sp. Dw^2 and *Kluyveromyces* sp. The best nitrogen source is dw1, sodium nitrate (3 g/L) (Wakil *et al.* 2017).

The investigations concerned with the optimization of medium pH indicated that *A. turcosus* produced the highest laccase production at pH 6.0. According to several studies, the optimum pH for laccase production for most fungi is 5.0 to 6.0 (Minussi *et al.* 2007). The optimum temperature for the production of laccase by *A. turcosus* was measured at several temperatures ranging from 25–45°C with an increment of 10°C. Maximum laccase productivity was obtained in *A. turcosus* at 35°C, and with the increase in temperature, laccase production was recorded at its lowest at 45°C**.** Different studies have shown similarity: *Pichia sp. Dw2 and Kluyveromyces sp. Dw1 have* ideal temperatures of *30–35°C* (Wakil *et al.* 2017), *P. ostreatus* prefers 25–30°C, *T. versicolour prefers* 35°C (Snajdr and Baldrian, 2007), *Neurospora crassa AJAS1* at 35°C (Nair *et al*. 2021), and *T. harzianum* at 35°C and pH 5 (Abd *et al*. 2016) for maximum laccase production. The incubation period is an important consideration for obtaining optimum enzyme productivity on a large scale in the least amount of time. Laccase enzyme production decreased significantly after the eighth day due to decreased nutrient intake. Laccase enzyme production decreased significantly after the eighth day due to decreased nutrient intake. As there was a decrease in nutrient food for laccase from the 8th day onwards, there was a decrease in enzyme production of a significant magnitude. Previous research indicates that a progressive increase in the incubation period, which resulted in a corresponding rise in laccase amount beyond the optimal, led to a decrease in enzyme production in *A. flavus* NG85 (8.422 U.mL^{-1}) on the eighth day of incubation (Ali *et al.* 2015). The initial fungal count significantly impacts the intake rate of medium nutrients; hence, optimizing inoculum size is vital to maintaining optimum enzyme production. The optimal inoculum size for maximum production of laccase by *A. turcosus* was obtained to be 3 discs (8 mm in diameter), which could be influenced by the presence of a favorable growth environment, such as adequate nutrients and oxygen, which

would boost laccase synthesis. Among the various inoculum sizes, the addition of 3 discs (8 mm in diameter) resulted in the optimum for maximum laccase production. A further increase in inoculum numbers causes a decrease in laccase production. A lower inoculant concentration is insufficient to induce proliferation, while a larger concentration inhibits growth due to rapid food depletion (Jadhav *et al.* 2009). Five discs were the optimal inoculum number for laccase production from *P. martensii* NRC 345 (Elshaefei *et al.* 2012). Similarly, the optimal conditions for *Beauveria pseudobassiana* laccase synthesis were a pH of 6.0, a temperature of 25°C, and a time period of 8 days (Sharma *et al.* 2023).

It was determined that *A. turcosus* was a high laccase producer; hence, the optimal parameters that were evaluated the best ones for achieving maximum laccase production from the fungus. Under the optimal parameters, the maximum laccase activity represented more than twice the increase in laccase production and was further procured in an optimized medium. SDS-PAGE analysis revealed a unique band with a molecular mass of 66.0 kDa for the pure laccase enzyme. According to earlier research, the molecular weight of *Agaricus bisporus* was 66 kDa (Perry *et al.* 1993). In most cases, the molecular weight varies between 60 and 90 kDa, and it encodes for 500–600 distinct amino acids found in laccase protein (Vishwanath *et al.* 2014), and variation might be related to glycosylation, which is prevalent in laccases (Ike *et al.* 2015). The temperature effect on enzyme activity was observed at various temperatures from (25–65°C) and pH (3–8) for the purified laccase activity, which showed the highest activity at pH 6.0 and 35°C. The effect of different inducers and inhibitors was measured by the addition of 5–20 mM concentrations of $CuSO_4$, tannic acid, EDTA, and sodium azide to the enzyme solution. The addition of $CuSO_4$ and tannic acid significantly increased the purified laccase enzyme activity. Tannic acid promotes maximum laccase activity at a concentration of 20 mM, whereas $CuSO_4$ achieves an optimal level with a gradual increase of 10 mM concentration, and after an increase in concentration, it inhibits laccase activity. The results reveal that EDTA and sodium acetate completely inhibit enzyme activity at all concentrations. Previous studies revealed that laccase production is increased in the growth medium with the addition of tannic acid (Rothschild *et al.*, 1995), copper, and $CuSO_4$ (Palmieri *et al.*, 2000), and that *T. pubescens* (Apkinar and Urek, 2017) laccase gene expression rises. According to the findings that Mainak and Rintu (2015) presented, Km value of the pure yellow laccase was 0.0714 mM, and its V_{max} value was 0.0091 mM min^{-1}, respectively. Tannic acid at a concentration of 2 mM and copper sulfate at three different concentrations were observed to boost laccase activity to around 45% in *P. eryngii, P. florida,* and *P. sajor caju* (Mathur *et al.*, 2021). Laccase synthesis in *Beauveria pseudobassiana* was significantly increased by the addition of 0.003% (w/v) copper sulphate (Sharma *et al.* 2023). According to the findings of Moon-Jeong and colleagues (2015), the Km value of the enzyme for the substrate ABTS is 12.8 M, and its corresponding Vmax value is 8125.4 U mg^{-1}. According to the research done by Xiao and colleagues in 2003, the Km value for ABTS by the laccase produced by *Trametes* sp. strain AH28-2 was 25 M. The research conducted by Shujing *et al.* (2013) find that the Km and V_{max} values of the pure laccase were 0.296 mM and 0.0645 mM min^{-1} when using ABTS as the substrate. The Km values for the pure laccases of *Fusarium solani* MAS2 (Wu *et al.* 2010) and *Trichoderma harzianum* WL1 (Sadhasivam *et al.* 2008) were found to be 10.23 and 2.66 mM.

Previous studies demonstrated that *Aspergillus* sp. have the capacity to decolourize red dye maximum by 70% at day 15 and orange dye up to a maximum of 35% at day 20 when the original dye concentration was 0.15 g/250 mL (Rao *et al.* 2014). After 15 days of incubation, in-vitro testing validated the effectiveness of *P. floridensis* by achieving a decolorization rate of 53.61% of a dye combo (brilliant blue-Congo red. proportion 1:1) (Laura *et al.*, 2023). Synthetic colors are discouraged since they are both carcinogenic and toxic. In a study with similar findings, researchers found that incubating bisphenol A at 0.015% with 1.5 U/mL of Lac^{-1} from *Grifola frondos* for six hours resulted in the degradation of bisphenol A at a 30% concentration without the intervention of a mediator (Nitheranant *et al.* 2011). *T. versicolour*, a white-rot fungus, was found to be possess efficient species, with a reduction in total phenol content of 93% under ideal conditions in just seven days (Bernats and Junha, 2007). This current result can effectively be used for the degradation of phenol from industrial sources. After 14 days of screening, similar results in *T. pubescens* MB 89 were attained in flask cultures. They achieved 79% COD removal, 80% total phenol removal, and a 71% color decrease (Strong and Burgess, 2015). This demonstrates that COD removal may be increased with an increased period and enzyme concentration. Laccase-producing fungi could be used for bioremediation of phenolic and non-phenolic waste contamination from various industrial effluents, including pharmaceuticals, as lignin degraders, as well as for other biotechnological uses.

Conclusion

It can be concluded that *A. turcosus, which* has been isolated from the *C. gigantean* plant leaves of the paper mill effluents of Raipur, Chhattisgarh, India, is potent enough to produce laccase from endophytic sources, which is applicable in the industrial production of various value-added products. After optimization, the following conditions were found to produce the most laccase: medium (Czapeck Dox Broth), carbon source (Sucrose-10 g/L), nitrogen source (Sodium Nitrate-10 g/L), pH (6), activator Tannic Acid (20 mM), and incubation time (35°C), and duration (8 days) with three (8 mM) disc inoculums. In submerged fermentation, the maximum laccase activity was found at 65 $U.mL^{-1}$, which was more than two times higher than the unoptimized conditions (26.8 $U.mL^{-1}$). SDS-PAGE revealed that the isolated laccase was a monomer with a molecular mass of 66 kDa. The study also emphasizes the significance of the traditional approach to laccase production optimization, which would serve as a baseline for subsequent optimization using statistical tools. The findings also show that nutritional and physical factors have an impact on enzyme production. Moreover, the ability of catalyzed phenolic and nonphenolic compounds to stimulate laccase production can be exploited for biodegradation to remove phenolic and nonphenolic waste from many industrial effluents. The laccase-mediated synthesis of physiologically active natural products and their analogs, including lignans and neolignans, dimeric stilbenoids, biflavonoids, biaryls, and other substances that may be of

interest to the pharmaceutical sector. Laccase is also used in natural polymer modification. This partially purified enzyme has a prospective application in biotechnological operations.

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Author Contribution

Sabiha Naz Conceived and designed the analysis, Collected the data, Contributed data or analysis tools, performed the analysis, I wrote the paper. Shailendra Gupta contributed suggestions and guidance in paper writing. Tanushree Chatterjee contributed for suggestions and guidance in paper writing.

Conflict of Interest

An author that they do not have any conflicts of interest.

Ethics Statement

None. Studies involving humans and animals must have been performed with the approval of an appropriate ethics committee and provide the reference number.

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