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Diclofenac biotransformation and toxicity assessment of laccase from *Pleurotus floridanus*

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ABSTRACT

Laccase producing fungus *Pleurotus floridanus* was isolated from Siruvani forest, Tamil Nadu, India. The potential of *P. floridanus* to produce laccase by using various lignocellulosic substrates was screened under submerged fermentation. Laccase production in the presence of lignocellulosic substrates such as rice, wheat and maize bran as a sole source of carbon as well as an additional supplement was examined. Laccase activity of *P. floridanus* using varied substrates was observed in the order of rice bran > wheat bran > maize bran. The isolate showed maximum laccase activity of 13.29±0.01 U/mL using rice bran as a carbon source within 11 days. This was 18 fold higher than the control media that lacks lignocellulosic substrates. The diclofenac tolerance was assessed in solid media at various concentrations and the results showed that the mycelia growth is not significantly affected by the drug. Finally, the laccase mediated degradation of diclofenac at a concentration of 10 mg/L showed 98% degradation in 2 h. The phytotoxicity of the crude laccase treated diclofenac was lower than the untreated diclofenac. In conclusion, findings suggested direct application of crude laccase produced from *P. floridanus* using agro-residues as ideal substrate for environmental applications.

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Introduction

The Western Ghats is one of 34 global biodiversity hotspots that span 1600 kilometers. Millions of plants, animals, and microorganisms, including a diverse range of fungi, reside in the Western Ghats. There are around 1600-1700 white-rot fungal species in the earthy surroundings. Due to the presence of ligninolytic enzymes such as laccase, lignin peroxidase, and manganese peroxidase, white-rot fungi are capable of decomposing a wide range of hazardous contaminants, including phenolic and aromatic chemicals (1). White rot fungal screening is a significant area of research in microbial biotechnology. Laccase (EC 1.10.3.2, benzenediol: oxygen oxidoreductase) is one of the most significant ligninolytic enzymes which degrades a wide range of contaminants, including phenolic and aromatic compounds. Fungi, bacteria, plants, lichens, and insects have this multi-copper oxidase. Laccase from white-rot fungi is notable among these sources because it can produce extracellular laccase with a high redox potential (2). The versatility of laccase is related to its broad substrate specificity, and it catalyzes oxidation without the use of any cofactor since it consumes oxygen and is reduced to water (3). Laccase can be found in a variety of industries, including paper and pulp, textiles, food, pharmaceuticals, cosmetics, and baking. It's also utilized to make biosensors and fuels, as well as

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organic synthesis and bioremediation of a wide range of substances (4).

One of the most important challenges in the industrial application of laccase is that the requirement of an enzyme is more while the enzyme produced is in low quantity. From an industrial point of view, higher productivity and lower production costs are recommended during the optimization of fermentation. Generally, laccase is produced either by solid-state or submerged fermentation (5). The enzyme activity of laccase produced by solid-state fermentation is more than submerged fermentation (6, 7). But the main limitation of solid-state fermentation is its difficulty in scaling up (8). One of the main reasons for the production of an enzyme with a high titer in solid-state fermentation is that the substrates used for the growth of the fungi are natural substrates. They are rich in soluble carbohydrates and proteins and minerals that will stimulate growth as well as enzyme production (9). Most of the studies in submerged fermentation focus on the usage of lignocellulosic components as co-substrates along with organic carbon sources. The laccase activity produced by natural substrates as co-substrates such as wheat bran (10), rice bran (11), and oak sawdust (12) was reported to be higher. The reports on the usage of these lignocellulosic components as a sole source of carbon for laccase production were also reported (13-15,6). In addition, the usage of agricultural residues as a substrate for laccase production is an effective way to reduce the cost associated with the production.

Pharmaceuticals and personal care products (PPCPs) are a unique group of emerging environmental contaminants due to their inherent ability to induce physiological effects in humans and animals at low doses (16). Pharmaceutical contamination is caused by the disposal of unwanted medications, pharmaceutical production facilities, hospital wastes, human excretions, and veterinary applications. Diclofenac, also known as 2-[(2,6-dichlorophenyl) amino] benzeneacetic acid, is a non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic effects. Diclofenac is also referred to as the "world's most popular pain killer" and the most often prescribed NSAID, with a market share that rivals that of the next three most popular medicines combined (ibuprofen, mefenamic acid, and naproxen). Diclofenac is used in around 1000 tonnes per year across the world. Diclofenac has primarily been found in freshwater basins across the world. After treatment, water containing diclofenac is released to surface water from wastewater treatment plants (17). The concentrations range from a few hundred to thousands of nanograms per liter. Diclofenac residues were shown to be the cause of vulture deaths in Pakistan and India and were also reported toxic to aquatic life (18). The commonly employed methods for the removal of diclofenac such as advanced oxidation, photolysis, ozonation, ultrasonic irradiation etc have been reported. Biological techniques are more reliable and cost-effective than oxidation procedures. Bioremediation is often 60–70% less expensive than competing technologies (19). White-rot fungi are well reported for bioremediation application, specifically due to their laccase-producing abilities. In view of the above-mentioned facts, the objective of the study was not only to identify the potential laccase producing fungus from the Western Ghats and enhance laccase activity using certain lignocellulosic substrates but also to assess the role of crude laccase in the removal of diclofenac. Further, the toxicity of the transformed products was also evaluated via phytotoxicity and algae inhibition studies.

Materials and methods

Chemicals

Diclofenac sodium, 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) [ABTS], guaiacol, Remazol brilliant blue R (RBBR), and Poly R-478 were obtained from Sigma-Aldrich. All other chemicals required for the study were also supplied by Sigma-Aldrich.

Collections of wood rot fungi

The fruiting bodies of fungal samples were collected during the northeast monsoon (November) from the Siruvani forest (part of the Western Ghats region; Latitude: 10.969758, Longitude: 76.647999), Tamil Nadu, India. A piece of wood containing fungi was collected, transferred to a sterile plastic bag, transported to the laboratory, and stored at 4ºC until culturing.

Table 1. Screening of laccase producing fungi by ABTS and guaiacol oxidation method.

<1.0 cm :- (very low/ambiguous); 1-2.5 cm:- + (low); 2.5-5.0 cm:++ (medium); >5.0 cm: +++ (high) >6 :- ++++ (very high)

S.No	Strain name	Intensity of oxidation			
		ABTS		Guaiacol	
		Zone diameter (cm)	Oxidation	Zone diameter (cm)	Oxidation
1	BPL F1	2	+	1.5	+
2	BPL F2	4	++	3.6	++
3	BPL F3	2.2	+	2.1	+
4	BPL F 4	3.1	++	2.8	++
5	BPL F 5	0	0	0	0
6	BPL F 6	1.5	+	0	0
7	BPL F 7	3.0	++	2.8	++
8	BPL F 8	4.5	++	4.0	++
9	BPL F 9	5.3	+++	4.5	++
10	BPL F 10	2.3	++	2.0	+
11	BPL F 11	0	0	0	0
12	BPL F 12	3.7	++	3.5	++
13	BPL F 13	4.8	++	4.0	++
14	BPL F 14	4.0	++	4.5	++
15	BPL F 15	7.3	++++	5.0	+++
16	BPL F 16	5.9	+++	4.4	++
17	BPL F 17	4.3	++	4.6	++
18	BPL F 18	1.1	+	1	0
19	BPL F 19	2.0	+	1.5	+
20	BPL F 20	3.4	++	3.0	++
21	BPL F 21	3.0	++	2.5	++
22	BPL F 22	0	0	0	0
23	BPL F 23	1.2	+	0	0
24	BPL F 24	0.5	-	0	0
25	BPL F 25	1.6	+	1.2	+
26	BPL F 26	0.7	-	0	0
27	BPL F 27	0	0	0	0
28	BPL F 28	1.5	+	1.3	+
29	BPL F 29	2.6	++	2.4	+
30	BPL F 30	3.1	++	2.6	++
31	BPL F 31	5.5	+++	4.7	++
32	BPL F 32	4.4	++	4.1	++
33	BPL F 33	0	0	0	0
34	BPL F 34	1.9	+	2.0	+
35	BPL F 35	2.5	++	2.6	++
36	BPL F 36	3.0	++	3.3	++
37	BPL F 37	1.4	+	1.6	+
38	BPL F 38	1.9	+	2.1	+
39	BPL F 39	2.8	++	3.0	++
40	BPL F 40	0	0	0	0
41	BPL F 41	3.4	++	3.2	++
42	BPL F 42	4.8	++	4.4	++
43	BPL F 43	2.6	++	0	0
44	BPL F 44	3.8	++	3.5	+
45	BPL F 45	2.2	+	2.0	+
46	BPL F 46	4.3	++	4.5	++
47	BPL F 47	1.0	+	0	0
48	BPL F 48	0	0	0	0
49	BPL F 49	2.5	++	2.0	++
50	BPL F 50	0	0	0	0
51	BPL F 51	1.6	+	1	+
52	BPL F 52	4.4	++	3.8	++
53	BPL F 53	6.3	++++	4.8	+++
54	BPL F 54	5.4	++	4.3	++
55	BPL F 55	3.9	++	3.0	++

Isolation of wood rot fungi

A small piece of fruiting bodies/wood segment containing fungi was cut using sterile tweezers/forceps and washed in distilled water to remove dirt and then washed in 0.1% (w/v) mercuric chloride solution, followed by repeated washing with distilled water and inoculation onto potato dextrose agar (PDA) medium plates. Plates were incubated at 27ºC for 7 days.

Screening of laccase activity

Laccase activity of the fungi was confirmed using ABTS and guaiacol as an indicator. Actively growing mycelial plug (0.5 cm) was inoculated into modified Kirk medium containing ABTS, and 2% malt extract agar with 0.01% guaiacol (20). ABTS was added after autoclaving the medium, while guaiacol was added before autoclaving. After inoculation, the plates were incubated for 3-10 days at 28ºC. The appearance of green/purple color and brownish color confirmed the laccase activity in ABTS and guaiacol-containing medium, respectively.

Dye decolorization in liquid media

The selected isolates (BPLF9, BPLF15, BPLF16, BPLF31, and BPLF53) based on higher laccase activities were further screened for their ability to degrade dyes such as RBBR and Poly-R-478 (21). The experiments were conducted in a 250 mL Erlenmeyer flask containing 50 mL of carbon-limited medium. The selected strains were grown in potato dextrose agar medium. An agar plug (0.5 cm) was taken from the growing end of mycelium and inoculated into 50 mL of the carbon-limited medium. The flasks were incubated on a rotary shaker at 27ºC at 115 rpm. After 2 days, RBBR (30 mg/L) and poly-R-478 (20 mg/L) were aseptically added to the flasks. The intensity of the color was measured at 600 nm (RBBR) and 513 nm (poly-R-478) at regular intervals using a UV-Vis spectrophotometer (Shimadzu-1800). The medium without dye served as a control. The dye reduction was calculated based on the equation given below:

$$\text{Dye reduction}(\%) = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100 \quad \text{(I)}$$

(I)

Identification of isolate BPLF15

The identification of potent isolate i.e. BPLF15 was carried out by molecular approaches using ITS (Internal Transcribed spacer) segment. DNA was extracted from the culture using Norgen's Plant/Fungi DNA Isolation Kit (Sigma) as per the manufacturer's instructions. The isolated DNA was checked for its purity and run on 1% agarose gel electrophoresis, which was later visualized under UV trans-illuminator. PCR amplification was performed for the isolated DNA using universal barcode primers, i.e., ITS1-F (5'-CTTGGTCATTTTAGAGGAAGTAA-3') and ITS4-R (5'-CAGGAGACTTGTACACGGTCCA G-3'). Polymerase chain reaction (PCR) was performed under the condition: initial denaturation (95˚C for 5 min) and then denaturation (35 amplification cycles at 95˚C for 30 s), annealing (54˚C for 30 s) primer extension (72˚C for 90 s), and final extension at 72˚C for 10 min (22). The amplified product was checked for its appropriate size by agarose gel electrophoresis and purified using a commercial column-based purification kit (Invitrogen, USA) and sequencing was performed with desired primers in ABI 3730 XL cycle Sequencer. Based on BLAST analysis, the first five hits were chosen considering the identity percentage and taken further for multiple sequence alignment (MSA) using ClustalW software and also for the dendrogram construction.

Effect of lignocellulosic wastes on laccase production

The laccase production was estimated in a basal medium (glucose-2%, yeast extract-0.5%, peptone-0.5%, $MgSO_4.7H_2O$-0.1%, and pH-5.0) (23) constituting different lignocellulosic wastes (2% w/v) such as rice bran, wheat bran, and maize bran. Two mycelia plugs (0.5 cm) of strain *P. floridanus* were individually inoculated into 100 mL of medium and the flasks were incubated under shaking conditions at 115 rpm at 27ºC.

Laccase assay

Laccase activity was estimated based on ABTS oxidation according to the methodology of Schubert et al. (24) with slight modification. Briefly, 100 µL of culture filtrate (obtained after centrifugation at 10,000 rpm for 10 min) and 900 µL of 1mM ABTS

containing 0.1M sodium acetate buffer (pH-4.5) were mixed. ABTS oxidation was monitored in a UV-Visible spectrophotometer at 436 nm. The laccase activity was calculated using the formula as mentioned below:

$$\text{Laccase activity (U/mL)} = \frac{\Delta A \times V \times df}{\Delta t \times \epsilon \times v} \quad \text{(II)}$$

where ΔA is the change in absorbance at 436 nm, V is the total reaction volume, df is the dilution factor of the sample, Δt is time, ε is the molar extinction coefficient of ABTS (29,300 M^{-1} cm^{-1}), and v is sample volume.

Radial growth studies in fungus

Diclofenac (0.1, 1, and 10 mg/L) were aseptically added into the sterile malt extract medium. Inoculum consisting of 0.5 mm diameter plug was taken from the growing end of mycelia growth (5-7 days old culture) and plates were incubated up to 10 days in dark conditions. The radial mycelium extension was measured every 24 h until the maximum extension occurs. The hyphal extension was measured from the center of the inocula to the edge of the plate and the maximum growth which was equal to the size of plates was assigned to be 9.0 cm. Non-inoculated plates were served as abiotic control while biotic controls were maintained without supplementing diclofenac.

Partial purification of laccase

Laccase was partially purified as per the method of Shanmugham et al. (25) with slight modifications. Laccase was precipitated using acetone in the ratio of 1:1. Pre-chilled acetone was added to culture filtrate and incubated for 4 h at cold condition. The precipitated proteins were collected by centrifugation of the filtrate at 8000 rpm 4ºC for 10 min. The obtained pellet was dialyzed against 0.1M sodium acetate buffer (pH-4.5) to remove acetone and the pellet was stored at -20ºC till further use.

Degradation of diclofenac by partially purified laccase

Diclofenac degradation studies were carried out using partially purified laccase. The reaction mixture contained a total volume of 5 mL with 0.1M sodium acetate buffer (pH-4.5), diclofenac at a concentration of 10 mg/L, and crude laccase with an activity of 100 U/L. Abiotic control was maintained which consisted of the heat-inactivated enzyme (110ºC for 10 min). A separate control test without the addition of enzymes to the reaction mixture was also maintained. Diclofenac transformations were assessed in UV-Visible spectrophotometer (JASCO V-750), changes in the absorption spectra were measured (200-600 nm), and compared with the control.

Phytotoxicity analysis of laccase treated diclofenac

The toxicity of the untreated and laccase-treated diclofenac was assessed by phytotoxicity analysis in *Vigna unguiculata* seeds. In the presence of drugs and metabolites, the seeds were allowed to germinate. The germination percentage of treated and untreated seeds was assessed and compared with the control. In addition, the shoot and root length of the plants were also measured after 5 days of development. Initially, the seeds were washed in distilled water and 5 seeds of *Vigna unguiculata* were placed on petri plates containing sterile filter paper, followed by various treatments. The plates were incubated at room temperature and watered using the respective solutions.

Algae growth inhibition test

Microalgae *Tetradesmus obliquus* BPL16 was obtained from microbial culture collection center, Bharathiar University, Coimbatore, India (26) and cultivated as described by Esakkimuthu et al. (27) on Bold Basal medium (BBM). The diclofenac solution before and after laccase treatment was added into the 100 mL of the medium at a final concentration of 10 mg/L and the flasks were incubated at 25 ºC at 130 rpm with the illumination of 100 mmol m^2s^{-1}. The growth of the algae was assessed by measuring optical density at 750 nm in a UV-Visible spectrometer (Shimadzu UV-1800) after 10 days. The algal growth in BBM after 10 days was assigned to be 100%. The growth inhibition was evaluated by comparing the growth rate of algae exposed to the diclofenac treated/untreated to that of algae growing in pure mineral media (controls) (28).

Statistical analysis

All the experiments were carried out in triplicate and results were expressed as mean ± standard deviation.

Results and discussion

Isolation and screening of laccase producing fungi

About 55 fungi were isolated (Figure 1) and tested for laccase production. Fungal cultures were tested for zone formation in ABTS or guaiacol amended solid media which are considered a standard substrate for laccase. The results of the laccase-producing ability of various isolated fungi are represented in Table 1. Among 55 isolates, 5 isolates named BPLF9, BPLF15, BPLF16, BPLF31, and BPLF53 were identified as maximum laccase producing fungi as they showed higher ABTS oxidation zone (>5cm). The morphology of BPLF15 in the PDA medium is represented in Figure 2a. The mycelium showed a cottony texture with abundant growth. BPLF15 showed a higher halo diameter of about 7.3 cm (ABTS) followed by BPLF53 which showed zone formation of about 6.3 cm. The green halo was more than the colony growth and represented in Figure 2b. Results showed that 82% of the strains were able to oxidize ABTS while only 71% of the strains oxidized the guaiacol and formed colored red products. Six isolates that showed positive in ABTS appeared as negative in guaiacol media. Five isolates (as described earlier) that showed higher laccase production with more than 5 cm zones were selected for further studies.

Figure 1. Collected fungal strains.

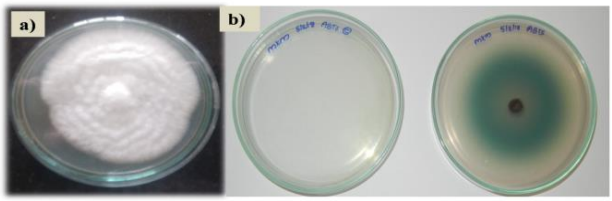

Figure 2. Morphological appearance of BPLF15 in **a)** Potato dextrose agar media and **b)** Modified kirk media showing ABTS oxidation.

Dye decolorization in liquid media

The percentage of dye decolorization increased with the incubation time and 99.69±0.124 and 97.45±0.165 % of the RBBR and poly-R-478 dye have been removed by isolate BPLF15 in 3 and 4 days, respectively (Table 2). BPLF53 removed 85.74±0.012 and 83.68±0.154% of the RBBR and poly-R-478 dye in 6 days. Isolate BPLF9, BPLF16, and BPLF31 showed a moderate level of dye decolorization which was greater than 50% in 6 days. Further studies were performed with the isolate BPLF15 as it showed higher dye decolorization potential.

Table 2. Dye decolorizing potential of selected laccase producing strains.

S. No	Strain name	Dye decolorization (%)	
		RBBR	Poly-R478
1	BPLF9	62.3±0.142	58.63±0.142
2	BPLF15	99.69±0.124	97.45±0.165
3	BPLF16	67.89±0.112	69.69±0.241
4	BPLF31	51.68±0.214	63.41±0.156
5	BPLF53	85.74±0.012	83.68±0.154

Identification of prominent laccase producing isolate

BPLF15 was identified based on morphological and molecular characterization methods. The BLAST annotation showed 100% identity, 0 E-value, and 99% query coverage with 5 *Pleurotus* reference strains. To identify the closely related sequence, multiple sequence alignment was performed in CLUSTALW software. The results of multiple sequence analysis show that the isolate BPLF15 was closely associated with the reference sequence of *Pleurotus floridanus*. The multiple sequence alignment and dendrogram is represented in Figure 3.

Effect of lignocellulosic wastes on laccase production

Laccase activity of the media supplemented with lignocellulosic compounds was higher than the organic substrates containing glucose. The enzyme activity appeared to increase after 6th day while in the case of simple sugar glucose, the increment was observed after 3rd day onwards. The maximum laccase activity using rice bran, wheat bran, and maize bran supplemented media were estimated as 7.98, 6.38, and 4.31 U/mL by *P. floridanus* (Table 3). It was 11, 9,

and 6 times higher than the control medium. The laccase activity of rice bran, wheat bran, and maize bran which served as a carbon source was higher than supplemental media. Laccase activity of about 13.29±0.19 (rice bran) and 7.89±0.26 U/mL (wheat bran) was reported on the 11th and 13th days, respectively. It was 18 and 11 times higher than control media.

S.No	Media	Laccase production (U/mL) on various Days						
		3	5	7	9	11	13	15
1	Basal gyp (Control)	0.147±0.078	0.32±0.02	0.54±0.02	0.710±0.07	0.615±0.03	0.412±0.01	0.31±0.01
2	G+ WB	0.145±0.034	1.24±0.036	2.42±0.014	4.89±0.036	6.38±0.03	5.79±0.024	3.56±0.021
3	G + RB	0.158±0.053	1.65±0.04	3.15±0.024	6.78±0.014	7.98±0.01	6.94±0.034	4.41±0.025
4	G + MB	0.124±0.032	1.12±0.06	2.25±0.07	3.75±0.045	4.31±0.02	3.97±0.04	2.12±0.024
5	WB	0.056±0.047	0.096±0.07	2.12±0.045	3.64±0.014	4.98±0.07	7.89±0.07	6.24±0.025
6	RB	0.045±0.066	0.072±0.04	4.11±0.053	5.87±0.021	13.29±0.01	11.67±0.045	10.97±0.027
7	MB	0.031±0.074	1.13±0.024	1.96±0.032	3.19±0.014	3.64±0.06	4.41±0.039	3.21±0.036

Table 3. Comparison of laccase production by *P. floridanus* on different natural substrates.

G-glucose, WB-wheat bran, RB-rice bran, MB-maize bran. Values are represented as mean±standard deviation

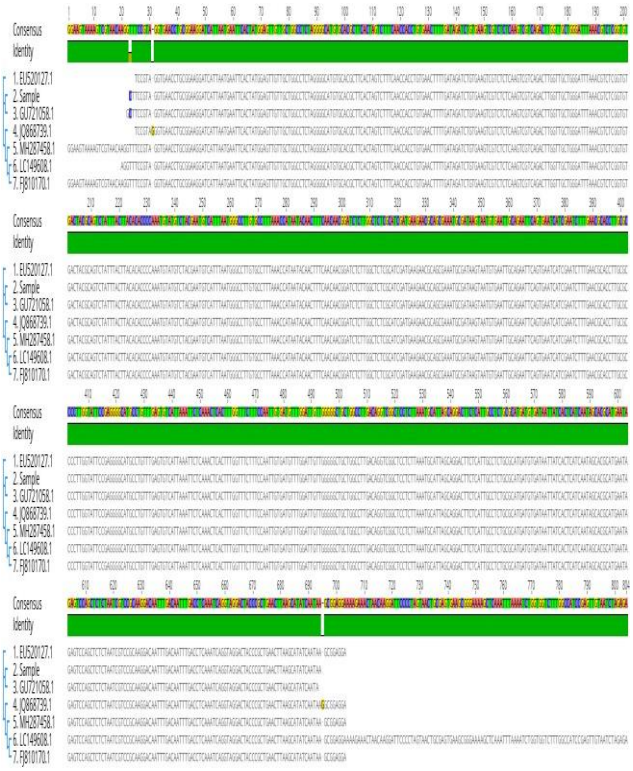

Figure 3. Multiple sequence alignment and identification of BPLF15 regarding other known species derived from BLAST result.

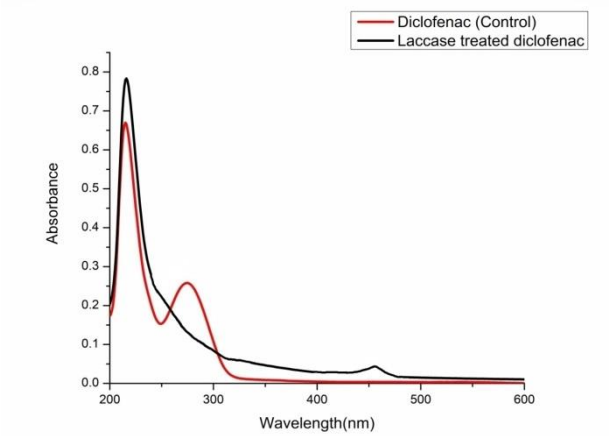

Figure 4 .UV-Visible absorbance spectra before and after treating with crude laccase.

Radial growth studies

In the present study, the radial growth studies of *P. floridanus* were assessed under various diclofenac concentrations. The mycelia growth in various concentrations was assessed after every 24 h and was represented in Table 4. The results showed that the diclofenac at a concentration of 0.1 and 1 mg/L did not have much effect on the growth of fungi, and maximum growth was attained within 10 days which was quite comparable with control. Diclofenac at a concentration of 10 mg/L showed milder inhibition

with a maximum mycelia growth of about 8.5 cm which was 0.4 cm lower than that of control. This showed that as the diclofenac concentration increased, the fungal growth was mildly inhibited.

Table 4. Effect of different concentration of diclofenac on the radial growth of *P. floridanus;* Incubation Days (A)

A	Mycelial Measurement (cm)			
	Concentration of diclofenac (mg/L)			
	0 (control)	0.1	1	10
3	1.75±0.099	1.46±0.024	1.24±0.045	1.09±0.99
4	3.4±0.110	3.1±0.021	2.84±0.135	2.75±0.055
5	5.1±0.115	4.5±0.025	4.39±0.066	4.1±0.022
6	5.7±0.031	5.3±0.034	5.1±0.086	4.9±0.048
7	6.96±0.076	6.1±0.056	5.96±0.156	5.6±0.054
8	7.9±0.084	7.1±0.075	6.98±0.034	6.6±0.021
9	8.3±0.014	8.1±0.063	7.8±0.065	7.5±0.065
10	8.94±0.021	8.82±0.064	8.6±0.074	8.5±0.046

Values are represented as mean±standard deviation.

Diclofenac degradation by crude laccase

UV spectral studies were conducted to find the catalytic potential of *P. floridanus* laccase for diclofenac transformation. The results of diclofenac absorbance spectra before and after laccase treatment are represented in Figure 4.

The diclofenac spectra showed a major absorption peak at 275 nm and correspond to the aromatic ring of diclofenac. After laccase treatment, the intensity of the peak at 275 nm was not observed, while a new peak was formed at 460 nm within 2 h. The shift of the peak was visible in the presence of laccase while the control without the addition of laccase displayed no changes in the peaks. The control with heat-inactivated laccase showed no changes in the diclofenac spectrum. This confirms the catalytic potential of laccase to oxidatively transform diclofenac.

Phytoxicity analysis

To find out the toxicity of metabolites, phytotoxicity analysis of the treated and untreated diclofenac solution was performed in *Vignaun guiculata* seeds and represented in Table 5. The seeds treated with distilled water served as a control and their maximum growth was assumed to be 100%. About 35% of the seeds failed to germinate in the presence of diclofenac. While after laccase treatment, the germination percentage considerably improved up to 92.14±1.04%. In the presence of the parent molecule, the root length and shoot length were considerably affected and showed lower root lengths of about 1.5±0.336 cm and 3.16±0.214 cm, respectively.

Table 5. Phytotoxicity analysis of laccase treated and untreated diclofenac on the growth of *Vignaun guiculata*.

Treatments	Germination (%)	Root length (cm)	Shoot length (cm)
Control	99.11±1.02	7.18±2.14	5.31±0.645
Untreated	65.18±1.24	3.16±0.214	1.5±0.336
Treated	92.14±1.04	6.38±0.798	4.78±0.098

Algal growth inhibition studies

A growth inhibition test in microalgae *Tetradesmus obliquus* BPLF16 was performed to assess the toxicity of diclofenac before and after laccase treatment. Untreated diclofenac showed 75% of growth inhibition, while after laccase treatment the growth inhibition reduced to 36% (Figure 5). This suggested that the transformation products formed by laccase treatment were of less toxic nature than diclofenac.

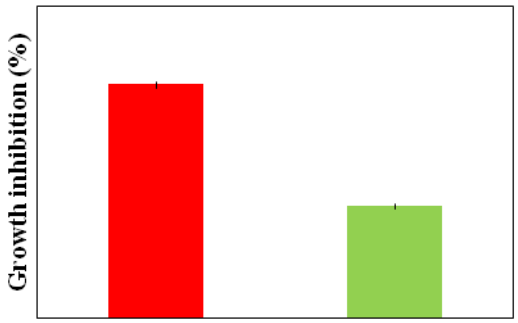

Figure 5. Growth inhibition studies in *T. obliquus* BPL16 before and after laccase treatment.

In this study, we evaluated the laccase-producing ability of various fungi isolated from the Western Ghats. The use of colored indicator compounds in solid media allows easy visualization of laccase-producing microorganisms (29). Among the various strains, isolated BPLF15 showed higher ABTS and guaiacol oxidation. A larger green halo than colony development showed a high yield and excellent laccase activity, which was consistent with the results described by Wang et al. (30). The ABTS and guaiacol oxidation abilities vary among the isolated strains. The utility of substrates may vary depending

on the fungus and the catalytic activity is not specific for a single substrate (31).

Polymeric dyes such as RBBR and poly R-478 have been widely used as model compounds for studying ligninolytic activities. All of the strains examined demonstrated moderate to high dye decolorization capacity, with BPLF15 showing faster dye decolorization than the other strains. A new white-rot fungus isolated from East Kalimantan, Indonesia named KRUS-G showed RBBR dye decolorization of about 84% (100 ppm) after 6 days (32). 95% of RBBR dye decolorization was also reported in *P. ostreatus* within 14 days. 50.16% and 75.47% of reactive black dye decolorization at a concentration of 0.01 and 0.05% was reported by *Alternaria alternata* in 19 and 30 days, respectively (33). 95% of reactive blue 19 decolorization was reported by *Ganoderma* sp. after 5 days. The potential of white-rot fungi to decolorize dyes vary among fungi. The major factors influencing dye decolorization by white-rot fungi are the growth condition and the characteristic of the dyes (34). The high dye decolorization percentage of BPLF15 in a shorter period indicates the biotechnological potentiality of the isolated strains for industrial applications**.**

The laccase-producing ability in the presence of various substrates was assessed by submerged fermentation. Higher laccase production was obtained in the presence of agriculture residues. The higher inductive capability of bran residues may be because of its lignocellulosic components. In addition, it also contains flavonoids and phenolic compounds that can activate laccase production (35). The lignocellulosic substrate contains a significant concentration of soluble carbohydrates and inducers that may also enhance laccase production (36). White rot fungi were mainly associated with the wood-decaying process and are responsible for the complete decomposition of wood including lignin. It might be the reason for the enhancement of laccase production in the presence of natural complex substrates. Similar results were obtained by Chawachart et al. (15). The author reported the usage and enhancement of laccase activity by 22 folds in rice bran media where it acted as a sole carbon source by *Coriolus versicolor*. The laccase activity obtained after 15 days in RB, WB, and rice straw media was 0.22, 0.09, and 0.01 U/mL, respectively (15). The laccase production obtained by *Aspergillus flavus* PUF 5 using rice bran and wheat bran as a carbon source in olga medium was 1.5 and 1.25 U/mL, respectively (13). Findings of this study suggested the innate potential of *P. floridanus* for laccase production in the presence of lignocellulosic substrates.

In the present study, the influence of diclofenac on *P. floridanus* was assessed at various concentrations. The radial growth of *P. floridanus* appears to be unaffected by diclofenac concentration at lower concentrations (0.1 and 1mg/L). A similar study in which clofibric acid at a concentration of 1 mg/L showed less inhibition was also reported earlier (37). On the contrary to the present results, the enhancement of radial growth after the addition of antibiotic ciprofloxacin was also reported in *P. ostreatus*. The radial growth experiments on PDA at various doses of ciprofloxacin (100-500 ppm) indicated that it had no negative impact on the fungus growth (19). At a higher concentration of diclofenac (10mg/L) there showed a mild reduction in the mycelia growth and the results were found in agreement with the report of Sadañoski et al.(38). Poly chlorinated biphenyl tolerance assay in solid media by various fungal isolates demonstrated that among ligninolytic enzymes, laccase plays a major role than other ligninolytic enzymes (38). The results showed the potential of *P. floridanus* to remain active in the presence of diclofenac at a concentration more than the environmentally relevant concentration (500 µg L^{-1}). The degradation potential of *P. ostreatus* to remove diclofenac from hospital wastewater was also reported (39). These studies suggest that the isolated *P. floridanus* strains are capable of tolerating a higher concentration of diclofenac and might be due to its laccase producing abililty.

A shift in λ max from 275 nm to 460 nm was also reported recently after treating diclofenac with *Scytalidium thermophilum* laccase. The formation of a peak at 460 nm suggested the transformation of diclofenac (40). Complete degradation of diclofenac (500µg/L) in the initial 5 h by *Trametes versicolor* crude laccase was reported and suggested hydroxylation followed by ring-opening as the major mechanism for laccase mediated degradation of diclofenac (17). 93% of diclofenac removal was reported in 6 h by *P. djamor* pellets (41). The present study reports a lower time duration

for the removal of diclofenac without any mediators than the reported studies. The findings of this investigation confirmed the application of *P. floridanus* laccase for diclofenac removal.

The inhibition of seed germination by diclofenac (10-50 mg/L) was also reported in black gram, green gram, horse gram, Bengal gram, pearl millet, and wheat seeds (42). The improved seed germination following laccase treatment showed that the degraded product was less hazardous. In contrary to present results, the enhancement of toxicity of diclofenac metabolites after photocatalytic degradation was also reported (43). The laccase-treated plants showed a better growth pattern in the presence of degraded products. This confirms the low toxic nature of the metabolites formed after laccase-mediated transformation of diclofenac. A reduction in toxicity after laccase mediator system treatment of sulfomethoxazole was also reported in the previous study (38). The reduction in toxicity after *S. thermophilum* laccase treatment of diclofenac which facilitated the enhancement of the growth of algae *Dunaliella salina*, was also reported (40). These results suggested that the crude *P. floridanus* laccase could be employed for treating effluents containing diclofenac.

In conclusion, the present study disclosed the potential of *P. floridanus* isolated from world's biodiversity hotspot (Western Ghats) to produce laccase that was further enhanced in the presence of lignocellulosic components. The maximum laccase activity which was 18 fold higher than glucose media was observed in the rice bran media. Laccase produced in natural substrates supplemented medium was also higher than the organic carbon. This suggested the possibility to utilize agricultural residues as ideal substrates for laccase production. The utilization of these substrates will reduce the cost of production and will mitigate the environmental pollutants. In addition, the potential of *P. floridanus* for mycoremediation purposes was assessed by radial growth studies in the presence of diclofenac that suggested the possibility of fungus for such environmental application. The detoxifying ability of diclofenac by crude laccase showed 98% degradation within two hours. The toxicity of the degraded products was evaluated by phytotoxicity analysis and the toxicity was observed lower than the parent molecule. Further, the algal growth inhibition studies also confirmed the less toxic nature of laccase treated diclofenac. Thus, findings of the present investigation suggested that *P. floridanus* laccase can be employed for the degradation of diclofenac.

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Interest conflict

The authors declare no conflict of interest.

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