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# ISOLATION, SCREENING AND IDENTIFICATION OF LACCASE PRODUCING FUNGI

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## ABSTRACT

The enzyme laccase (p-diphenol: oxygenoxidoreductase; EC 1.10.3.2) is known to degrade many phenolic aromatic compounds. This enzyme is found in many plant species and is widely distributed in fungi including wood-rotting fungi where it is often associated with lignin peroxidase or manganese dependent peroxidase, or both. The objective of the present study was to isolate the potential fungus and characterize the organism with reverence to laccase production. About 150 fungal strains were isolated from natural habitat such as decaying wood, soil and mushrooms in the area of Dharwad and Uttara Kannada district. Karnataka, India and screened for their ability to produce laccase on solid medium containing 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Guaiacol, Tannic acid and Syringaldazine. Among 150 fungi isolated only 8 isolates have ability to produce laccase. Out of the 8 isolates, one (FS6) was presumed to be potent, another (FS1) showed medium potency and six (FS2, FS3, FS4, FS5, FS7 and FS8) showed week laccase producing ability. The FS6 was identified by conventional and molecular methods. Fungal DNA was extracted, ITS-rDNA amplified by PCR, and PCR amplification of the 18S rDNA were performed and its nucleotide sequence was done at BLAST-n site at NCBI server. ITS regions were sequenced. Based on the molecular characterization, the laccase producing novel isolate was identified as *Marasmius sp.* BBKAV79 (Accession no. KP455496, KP455497).

**KEYWORDS: Laccase, ABTS, Guaiacol, Tannic acid, Syringaldazine, Molecular Identification.**

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# INTRODUCTION

Laccase (E.C. 1.10.3.2, p-benzenedial: oxidoreductases) is an oxidoreductase able to catalyze the oxidation of various aromatic compounds (particularly phenol) with the concomitant reduction of oxygen to water [1]. Laccase was first discovered in the sap of the Japanese lacquer tree *Rhus vernicifera* and its characteristic as a metal containing oxidase was discovered by Bertrand in 1985 [2]. Laccases are belonging to the group of oxidases also called as a blue copper oxidases or blue copper proteins. They are sometimes refers to as polyphenol oxidases (PPOs). They are extracellular enzymes. Laccases catalyze the oxidation of a variety of phenolic compounds diamines and aromatic amines pigment formation, lignin degradation and detoxification [3]. Laccases are widely distributed in nature in higher plants, fungi and bacteria [4]. The white-rot Basidiomycetes fungi efficiently degrade the lignin in comparison to Ascomycetes and Deuteromycetes which oxidize phenolic compounds to give phenoxy radicals and quinines [5]. Laccases have received much attention from researchers in last decades due to their ability to oxidise both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants, which makes them very useful for their application to several biotechnological processes. Laccases find wide commercial applications within food industry, pulp and paper industries, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutants and removal of endocrine disruptors [6]. As laccase act on various types of substrates, several different compounds have been used as indicators for laccase production. Some indicators used are Guaiacol, Tannic acid 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), Syringaldazine and polymeric dyes like remazolbrilliant blue-R (RBB-R) [7-14]. Therefore, the present investigation was undertaken on isolation, screening, identification of laccase producing fungi from various environmental samples using different indicator compounds in agar plates and methods.

# MATERIALS AND METHODS

Solid media was used in the screening, the reagent grade chemicals are potato dextrose agar (PDA), Glucose, Peptone, Yeast extract and Copper sulphate were procured from Hi-Media, Mumbai. Guaiacol, ABTS, Tannic acid and Syringdalzine were purchased commercially from SIGMA, USA and used for analytical study.

### *Collection of Samples*

The fungi used in this study was isolated from natural habitat such as decaying wood, soil and mushrooms in the area of Dharwad and Uttara Kannada District, Karnataka, India. The samples were collected in sterile plastic bags and were sealed and brought to the lab aseptically for further study.

### *Isolation of fungi*

1 gram of the collected sample was added to 10 ml of sterile water and mixed. The suspension was serially diluted from $10^{-1}$ to $10^{-7}$ dilution factors. Later, 1.0 ml of each dilution was spread on the surface of Potato Dextrose Agar (PDA) medium plate which is containing 0.01% Chloramphenicol and incubated at 30 $^0$C for 7 days. Fruiting bodies of mushroom used for the isolation. Mycelium was isolated by aseptically moving the upper unexposed part of the basidiocarp on PDA. The plates were incubated at 37 °C for 7 days. Distinct fungal colonies were isolated and repeatedly subcultured until pure cultures were obtained. The cultures were maintained on PDA slants at 5 °C [15].

### *Qualitative screening*

Laccase production were carried out by inoculation of mycelium from each strain onto PDA plates containing 0.02% Guaiacol, 1mM ABTS, 0.5% Tannic acid and 0.1% Syringaldazine as indicator compound and it was incubated at 30 $^0$C for 7 days. The formation of reddish brown halo in Guaiacol, Tannic acid and Syringaldazine supplemented plates, dark-purple halo in the ABTS supplemented plates indicated a positive laccase secretion.

***Quantitative screening***

Screening for laccase positive cultures was carried out in 250 mL Erlenmeyer flasks containing 100 mL of yeast extract peptone dextrose-copper sulphate (YPD-Cu) medium. Selected mycelia from PDA plates were used to inoculate the production. Flasks were sterilized at 121 ºC for 15 min and incubated with shaking at 120 rpm at room temperature. Sampling was done at regular intervals for laccase activity.

***Screening of culture media***

Two semi synthetic culture media with the following composition were used for the study: 1. Yeast extract peptone dextrose-Copper sulphate (YPD-Cu) medium Glucose 20 g/l, Peptone 5 g/l, Yeast extract 2 g/l, Copper sulphate 100mg/l and 2. Glucose Peptone Broth (GPB) media Glucose 10.0 g/l, Peptone 3.0, $KH_2PO_4$ 0.6, $ZnSO_4$ 0.001, $K_2HPO_4$ 0.4, $FeSO_4$ 0.0005, $MnSO_4$ 0.05, $MgSO_4$ 0.5, $CuSO_4$ 0.01.

***Extracellular enzyme activity***

The Laccase activity was assayed at room temperature by using 10mM Guaiacol in 100 mM sodium acetate buffer (pH 5.0).The reaction mixture contained 3ml acetate buffer, 1ml Guaiacol and 1ml enzyme source. The change in the absorbance of the reaction mixture containing guaiacol was monitored at 470 nm for 10 mins of incubation using UV Spectrophotometer. Enzyme activity is measured in U/ml which is defined as the amount of enzyme catalyzing the production of one micromole of colored product per min per ml [17].

$$\text{Volume activity (U/ml)} = \frac{\Delta A470nm/min \times 4 \times Vt \times \text{dilution factor}}{€ \times Vs}$$

***Calculation***

Where,

Vt = final volume of reaction mixture (ml) = 5.0

Vs = sample volume (ml) = 1

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

4 = derived from unit definition & principle

## RESULTS AND DISCUSSION

The present study was mainly targeted to laccase producing fungi from the different environment samples from Karnataka, India. A total of 150 fungi colonies were isolated from the wood decaying region, soil and mushrooms. Isolated fungal were maintained in PDA medium. They were screened for potential laccase producing ability using four indicators namely ABTS, Guaiacol, Syringadalzine and Tannic acid. Laccase is a very potent enzyme with ability to act on a number of substrates. Due to which it gains high industrial importance. All the isolates were inoculated in potato dextrose agar plate which is containing 0.02% Guaiacol, 1mM ABTS, 0.5% Tannic acid and 0.1% Syringaldazine as indicator compound and it was incubated at 30 $^0$C for 7 days. The results of the screening tests are given in the Table 1.

**Table 1**
***Response of fungal isolates to the indicators***

| Isolates | Indicators | | | |
|---|---|---|---|---|
| | Guaiacol (0.02%) | Tannic acid (0.5%) | ABTS (1mM) | Syringaldazine (0.1%) |
| FS 1 | **Positive** | Negative | Negative | Negative |
| FS 2 | Negative | **Positive** | Negative | Negative |
| FS 3 | **Positive** | Negative | Negative | Negative |
| FS 4 | Negative | **Positive** | Negative | Negative |
| FS 5 | **Positive** | Negative | Negative | Negative |
| FS 6 | **Positive** | **Positive** | **Positive** | **Positive** |
| FS 7 | Negative | Negative | Negative | **Positive** |
| FS 8 | Negative | Negative | **Positive** | Negative |

*Positive result means that the fungal isolate is able to produce colour halo with the indicator compound around its colony and negative result means the absence of colour halo. In the presence of Guaiacol, Tannic acid and Syringaldazine intense reddish brown colour was produced in the medium around the fungal colonies and was taken as the positive reaction for the production of laccase enzyme [7, 8, and 10]. In the presence of ABTS purple-colour in the presence of fungal colonies and was taken as the positive reaction for the production of laccase [10, 18].

The results revealed that out of 150 fungi only 8 isolates have ability to produce laccase. Out of 8 only FS6 was act on all four indicators (fig.1).The isolates which did not show any colour change, It's because of lack or absence of laccase activity and were not considered for further work. The isolated laccase producing fungi were assigned the labels from FS1 to FS8 is the abbreviation for Fungal Strain. They were quantitatively screened by Guaiacol assay method [17]and the best strain was further identified at the molecular level.

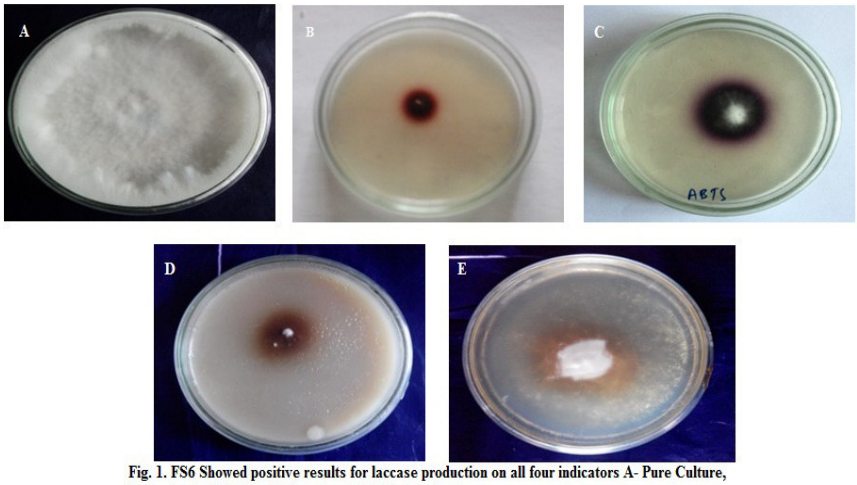



**Fig. 1. FS6 Showed positive results for laccase production on all four indicators A- Pure Culture, B-Guaiacol, C-ABTS, D- Tannic acid, E- Syringadalzine**

The morphological characteristics of promising laccase producing FS1 to FS8 were observed. As the morphological characteristics were not promising. 18S rDNA sequencing was used for the identification for excellent laccase producing Fungal Strain FS6. The ITS and LSU regions of isolate were amplified and sequenced at Agharkar Research Institute, Pune, India. The sequences were analysed by BLAST and submitted to GenBank and Accession number is KP455496, KP455497. FS6 18S rDNA showed 99% homology with *Marasmius sp.* CBB-361. (Accession no AY2164761.1). The phenograms reflecting the phylogenetic of FS6 constructed using data from the BLAST analysis of the rDNA region of FS6 are shown in Figure 2 and 3. The isolate FS6 was identified as *Marasmius sp.* BBKAV79 (KP455496, KP455497).

**Figure 2**
***Phylogenetic Tree obtained by analysis of LSU homologous sequences by MEGA6* software**

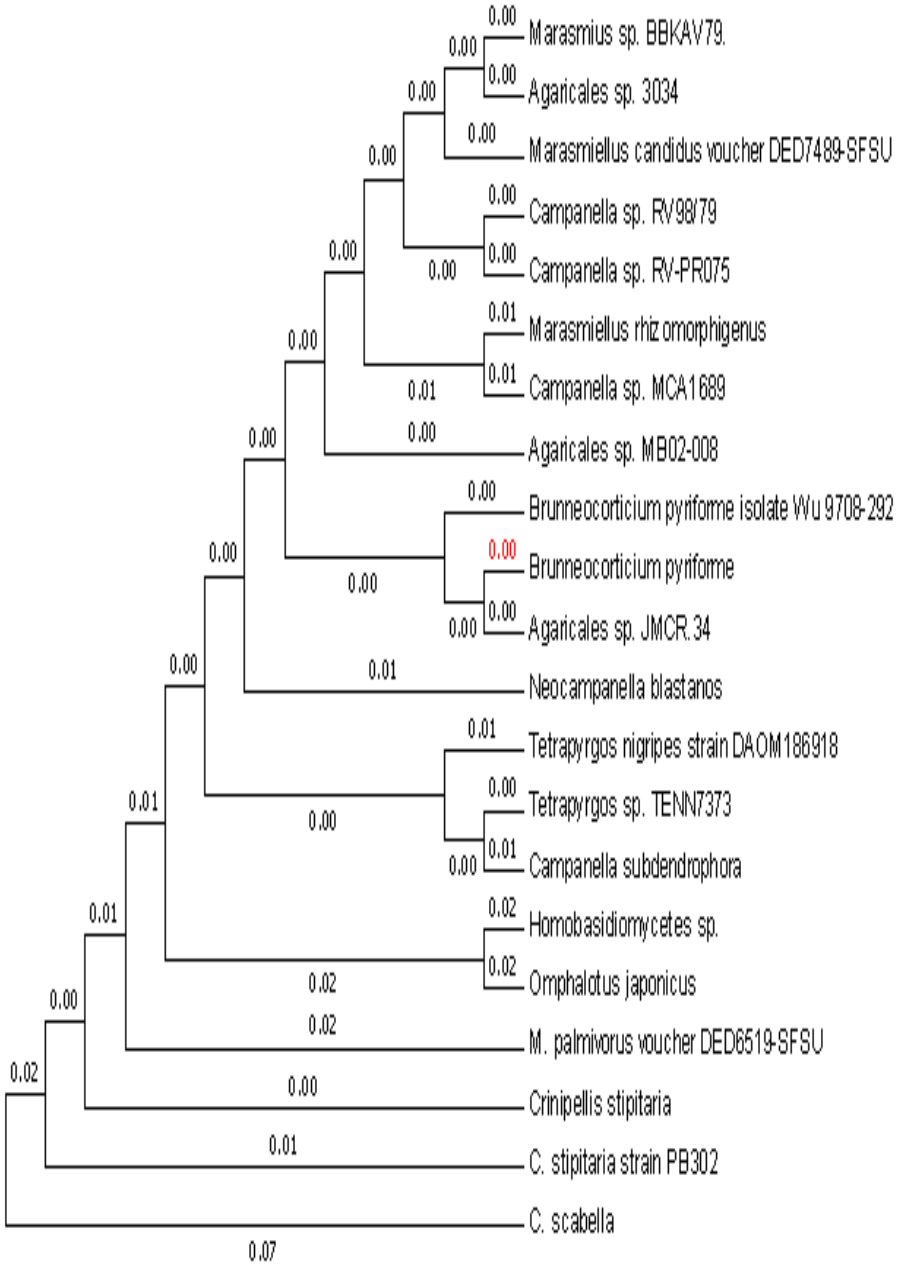
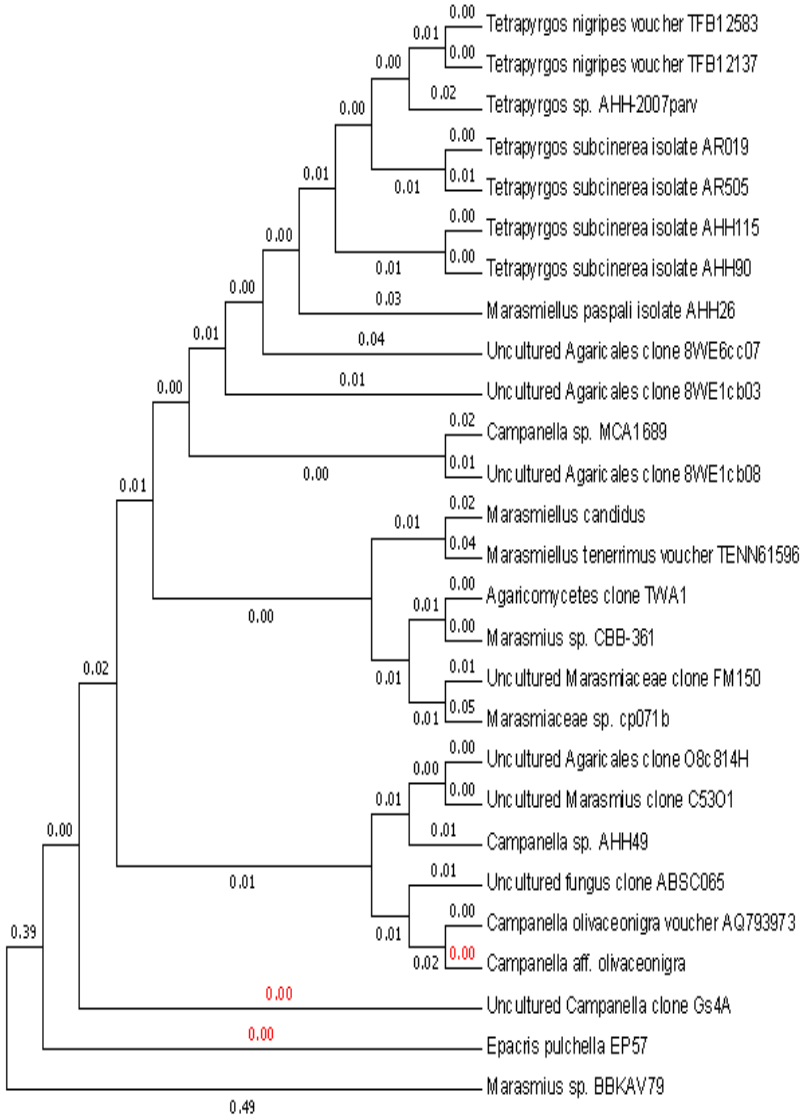
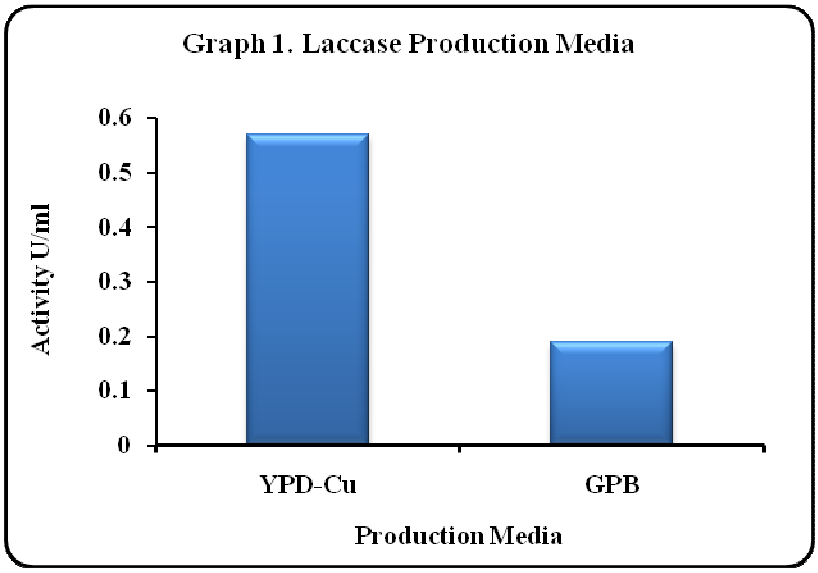


**Figure 3**
***Phylogenetic Tree obtained by analysis of ITS homologous sequences by MEGA6 software***




The laccase enzyme production in the YPD-Cu and GPB media showed marked variations (Graph1). FS6 produced highest quantity of laccase enzyme in YPD-Cu and lowest in GPB media viz., 0.570 U/ml and 0.190 U/ml respectively. Copper has been reported to be a strong laccase inducer in several species, among them, *T. versicolor*[19] and *P. chrysosporium*[20] the same results were obtained in this study. The isolated fungal strains were grown in production medium. After 5 days of incubation, the culture filtrate was taken from the flasks and analysed for the laccase activity. The FS6 showed maximum laccase activity on 8$^{th}$day 0.718 U/ml followed by FS5, FS1 and FS3 with 0.189 U/ml, 0.100 U/ml and 0.90 U/ml respectively (graph 2). The morphological and colonial identification of fungal isolates was carried out using by lactophenol cotton blue staining technique and morphological characterization as observed under light microscopy[21] (Table 2, Fig 4).



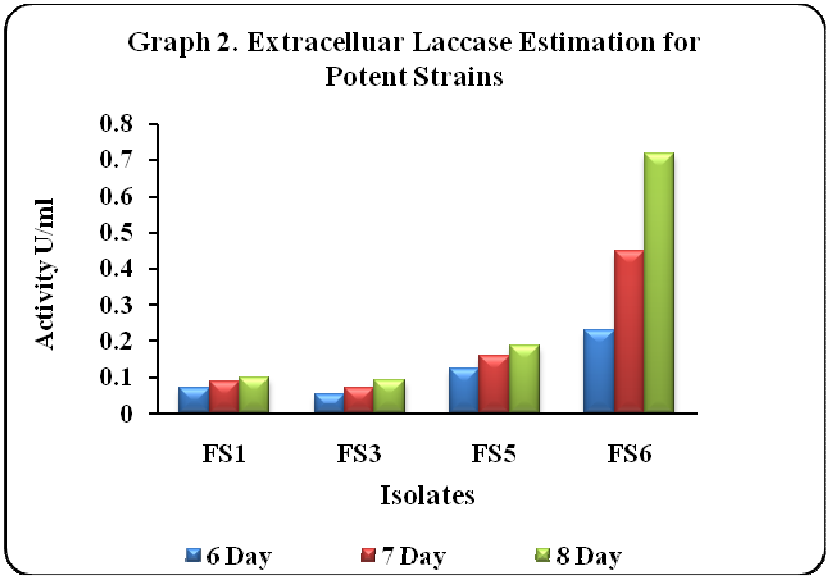
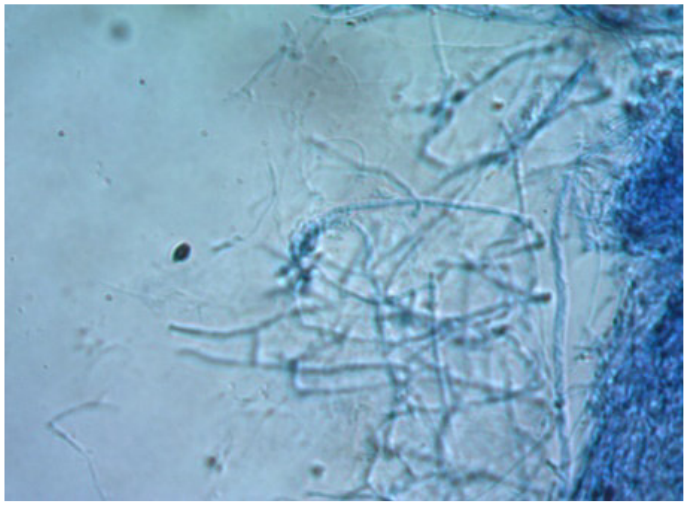




**Table 2**

***Identification of selected strains by Lactophenol cotton blue technique***

| Strain No. | Species |
|---|---|
| FS1 | *Pleurotus sp.* |
| FS2 | *Aspergillus sp.* |
| FS3 | *Pleurotus sp.* |
| FS4 | *Penicillium sp.* |
| FS5 | *Pleurotus sp.* |
| **FS6** | ***Marasmius sp.*** |
| FS7 | *Rhizoctonia sp.* |
| FS8 | *Fusserium sp.* |

**Figure 4**
***Microscopic Image of FS6***



## CONCLUSION

During this study, different cultivation techniques have been done in order to facilitate efficient screening. Guaiacol substrate showed a very strong ability to facilitate the growth and the isolation of the interested fungi with the laccase activity, 150 fungal strains were isolated from the wood decaying region, soil and mushrooms. The results revealed that out of 150 isolated fungi only 8 isolates have ability to produce laccase. Out of 8 laccase producing isolates only FS6 had significant laccase production. Only FS6 was act on all four indicators. Molecular identification of FS6 was done at Agharkar Research Institute, Pune, India. The identified novel strain is *Marasmius sp*. BBKAV79 (Accession no. KP455496, KP455497) which will be used for further research work.

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