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Original Article

# An *In Vitro* Comparative Approaches of Pahs Degrading Basidiomycetes Fungi from *Kolli* Hill, Tamil Nadu India

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ABSTRACT

Twenty one different wood decaying wild basidiomycetes fungi were collected from Kolli Hill, out of which five were identified for its ligninolytic potential. In this investigation basidiomycetous fungi were screened the ability to grow on various lignocellulosic substrates (80% moisture) including paddy straw, wood chips, wood powder and wood bark as well grown in solid state fermentation, the maximum fungal growth was noticed in paddy straw. In liquid state fermentation, the maximum growth was observed in basal salt media followed by potato dextrose broth (PDB) and molasses media. The selected basidiomycetes fungi were identified by using nucleotide sequences of the Internal Transcribed Spacer (ITS) region. The phylogenetic tree using maximum likelihood analysis revealed and the same was deposited in Gen bank and accession has been assigned for the same (Accession No. KM596809, KM596810, KM596813, KM596814 and KM596815) and these identified wood decaying fungus were subjected to assayed ligninolytic enzymes like laccase, manganese peroxidase and lignin peroxidase activities in both qualitative and quantitative respectively.

**Key words:** Kolli Hills, ITS, Ligninolytic activity and PAHs.

Polycyclic Aromatic Hydrocarbons (PAHs) are one of the important recalcitrant pollutants that are present in our environment. The environmental occurrence of PAHs has been associated with adverse effects on public health[1]. There is an ever increasing interest in the use of fungi in the remediation of creosote and PAHs polluted soil. High degradation of PAHs was obtained with white rot fungi *Phanaerochaete crysosporium*[2], *Pleurotus ostreatus*[3] and *Trametes versicolor*[4].

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The destruction and combustion of the World Trade Centre (WTC) towers which released complex mixture of toxicants including PAHs into New York City environment on and after 11th September, 2001 and caused several illness to the people who lived in that area[5, 6, 7].

Bioremediation industry has developed many novel approaches for biostimulation, bioaugmentation, combined processes and for monitoring and quantifying intrinsic bioremediation[8].

Microbial degradation of PAHs in soil is restricted by various factors that often result in lower than the expected bioremediation efficiency. One of these factors is the low bioavailability of the compounds[9]. A better basic understanding of the *in situ* competitive interactions between bacteria and fungi will probably result in much more straight forward approaches than which currently exist to screen for biodegradation potential[10].

White rot fungi are characterized by their ability to degrade lignin, which is a high molecular weight complex polymer in wood. Many species of white rot fungi such as *Phanerochaete chrysosporium*, *Coriolus versicolor* (*Trametes versicolor*), *Pleurotus ostreatus*, *Bjerkandera adusta*, and *Irpex lacteus* are able to oxidise a wide variety of xenobiotic organic pollutants including PAHs[11,12,13,14,15,16,17].

As a result of human ignorance in the safe use of chemicals, carelessness in the manufacture of synthetic compounds, occasional accidents, and improper disposal of chemical wastes, toxic anthropogenic chemicals have become ubiquitous contaminants of soils and waters worldwide[8].

Current research on biodegradation alert on the increasing environmental pollution due to petroleum products and on the significance of ligninolytic mushroom fungi in PAHs degradation states that there is a gap in awareness applied white rot fungi and the native micro flora in the PAHs contaminated sites.

Hence, the present investigation aimed to isolate wild basidiomycetes mushroom fungi with high ligninolytic activity of both qualitatively and quantitatively assayed from wild mushrooms collected from *Kolli* Hills of the Eestern Ghats, Tamil Nadu, India and liquid state fermentation (LSF) and solid state fermentation (SSF) of isolated basidiomycetes fungi was also analyzed. The main goal of the investigation was to evaluate the effects of ligninolytic potential of white rot fungus for the treatment of PAHs contaminated soil.

## 2. MATERIALS AND METHODS

### Methods

### Basidiomycetes fungi

The basidiomycetes fungal sporocarps were collected from two different sites of *Kolli* Hill (110 30' to 210 0' N Latitudes and 770 22' to 850 20' E Longitudes) a part of Eestern Ghats, Tamil Nadu, is located in Namakkal district, Tamil Nadu, India. The fungal sporocarps were carefully collected[18] and stored to study the characteristics, microscopical observation and necessary biochemical analysis for their identification[19]. Individual basidiomes of the preserved samples were cultured by the procedure recommended by[20] on Potato Dextrose Agar (PDA) medium for 7 days. The cultures were stored in a refrigerator and they were sub cultured once a month.

### Molecular identification of indigenous PAHs degrading wild basidomycetes fungus ITS primers

### Sequence Analysis

The sequences of ITS where compared against the sequences available from GenBank using the BLASTN program and were aligned using CLUSTAL W software. Distances were calculated and phylogenetic trees were constructed using the neighbour-joining method. Bootstrap analysis was done based on 1000 replications. The MEGA3 package was used for all analyses[21].

### Solid State Fermentation of different isolated basidomycetes fungus

Four different lignocellulosic waste materials from agricultural land such as air dried chopped paddy straw (80% moisture), chopped bark (2 cm of 80% moisture), timber wood chips (80% moisture) and wood powder (80% moisture) was saturated with water and allowed to drain off. Five grams of the wetted above mentioned material was taken in a 250 ml Erlenmeyer flask and autoclaved for 20 min at $121^0$C (Steffen *et al.*, 2007). After sterilization, 1 cm diameter fungal discs were taken from the 7 day old cultures grown on potato dextrose agar plates. The fungal discs were inoculated into the flask and it was incubated at 30°C in the dark and fungal growth was observed visually with respect to mycelial density as well as hyphae distribution in the solid substrate.

### Liquid State Fermentation of different isolated basidomycetes fungus

Sterile 100ml of BSM broth, molasses broth and PDB were prepared. Each flask was inoculated with 3 agar plugs (10mm diameter) of active mycelium of the selected basidiomycetes fungus inoculated and incubated at room temperature.

### Screening of basidiomycetes fungi for ligninolytic enzyme producing potential

### Qualitative screening for ligninolytic enzyme activities

Potato Dextrose agar medium was employed for the screening of ligninolytic enzymes activities. 0.0025% azure B (w/v) or 0.0025% phenol red (w/v) were used to detect lignin peroxidase[22] manganese peroxidase[23] correspondingly. Laccase was tested by adding 0.05% (w/v) gualacol, modified from[24]. Fungal discs (1 cm in diameter) were taken from 7day old cultures grown on PDA plates. Discs were inoculated onto triplicate. Plates were incubated at 30°C for 7 days. The diameter of the colored zone was measured.

### Quantitative estimation of ligninolytic enzyme activities

Flasks containing Potato Dextrose broth was inoculated with 1 cm diameter fungal discs were taken from 7 day old

cultures. The flasks were incubated at 30°C for 7 days. The culture filtrate was used for measuring the extracellular ligninolytic enzyme activities. The estimation of ligninolytic enzymes such as Lip[25], Mnp[26] and laccase[27].

**Estimation of Laccase enzyme activities**

Laccase activity was assayed with guaiacolas substrate. The reaction mixture contained 3.9 ml acetate buffer (10 mmol/l, pH 5.0), 1 ml guaiacol(2 mmol/L) and 0.1ml properly diluted enzyme solution was incubated at 50°C for 30 min. and the absorbance was read at 470 nm. In the blank, guaiacolwas replaced with acetate buffer[28].

**Estimation of lignin peroxidase enzyme activities**

The methodology of lignin peroxidase activity was determined by monitoring the oxidation of veratryl alcohol to veratraldehyde at 37°C as indicated by an increase in absorbance at 310 nm. The reaction mixture (2.5 ml) contained 500 µl enzyme extract, 500 µl (2 mmol/l) $H_2O_2$, 500 µl veratryl alcohol solution (10 mmol/l) and 1.0 ml sodium tartarate buffer pH 3.0 (10 mmol/l). One unit of enzyme activity is defined as the amount of enzyme oxidizing 1 µmol of substrate per minute[29].

**Estimation of Manganese peroxidase enzyme activities**

Manganese peroxidase activity was determined by measuring the increase of $Mn^{+3}$malonate formation at 270 nm at 25°C. The assay mixture (1 ml) contained sodium malonate buffer (70 mM, pH 4.5), $MnSO_4$ (2 mM), $H_2O_2$ (6 mM), and 25 µl of enzyme extract. One unit of MnP was defined as 1 µmol of product formed per milliliter per minute under the assay conditions[30].

**Statistical analysis**

Triplicate was maintained for each experiment and two way ANOVA was used to analyze the significant difference between the experiments. Results were analyzed statistically with computer software SPSS version 16.0.

## 3. RESULT AND DISCUSSION

**Collection of basideomycetes fungi:**

There is a general interest in studying the diversity of indigenous microorganisms capable of degrading different pollutants because of their varied effects on the environment[31].

Twenty one samples of basidiomycetes fungal sporocarps were collected at two different sites of *Kolli* Hill. The maximum rainfall (209 mm) and humidity (97.7%) during September and November, 2012 and minimum rainfall and humidity were recorded in during April and June 2013 was 5 mm and 21.7% respectively.

Among the twenty one collected samples, sixteen samples belonged to wood rot fungi while 5 samples belonged to soil inhabiting mushroom fungi. From the collected samples, 5 wood decaying basidiomycetes fungi were identified. Amid the identified samples grown on the culture media under lab conditions.

**Isolation and Identification of poly cyclic aromatic hydrocarbon (PAH) degrading basidiomycetes fungi**

The PAHs research in recent years has been on the degradation of high molecular weight PAHs which has resulted in the isolation of number of microorganisms that can mineralize and grow on four ringed PAHs as a sole carbon and energy source[32, 33].

In the present study, an indigenous PAH degrading basideomycetes fungi enrichment culture technique (Figure. 1) the sequence of the partial ITS gene isolate was compared against those available in the public databases. The ITS sequence of the isolate closely matched with those of *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pycnoporus sanguineus*, *Alternaria alternate* and *Trichoderma harzianum* (99% homology) in the database. Based on this study and on the morphological, cultural characteristics they were identified as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pycnoporus sanguineus*, *Alternaria alternate* and *Trichoderma harzianum*. The ITS fungal sequence has been deposited in Gen Bank database under accession number *KM596810, KM596809, KM596813, KM596814* and *KM596815*.

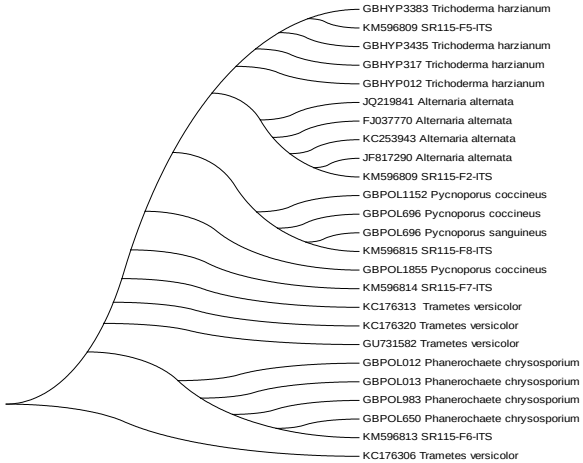


**Fig 1: ITS fungal genome identification**

**Evolutionary relationships of taxa**

The evolutionary history was inferred using the Neighbor-Joining method[34].The optimal tree with the sum of branch length = 2.48478745 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method[35] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 32 positions in the final dataset. Evolutionary analyses were conducted in MEGA6[36].

**Solid State cultivation of different isolated basidiomycetes fungus**

In solid state fermentation, the excellent growth was occurred in *Trametes versicolor* (wood chips (+++)) and wood powder (*Trametes versicolor* (+++)) good growth (++) was observed in *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pycnoporus sanguineus*, *Alternaria alternata* and *Trichoderma harzianum* (Paddy straw, wood chips, wood

powder and wood bark), moderate growth was exhibited in *Phanerochaete chrysosporium Trichoderma harzianum* and *Alternaria alternata* (Paddy straw, wood chips, wood powder and wood bark (+)) and no growth were showed in wood bark (*Phanerochaete chrysosporium*, *Trametes versicolor* (-)) (Table 1. and Figure.2). For the ligninolytic fungi, organic materials containing cellulose and hemicelluloses are the natural choice[37]. With an optimal growth substrate, soil invasion and consequently pollutant degradation can be enhanced. Most wood decayers utilize straw or wood shavings as preferred substrates[23].

**Fig 2: Various basidiomycetes fungus grown on SSF**

**Table 1: Growth of Fungal isolates on different lignocellulosic materials (SSF)**

| S.No. | Strains | Fungal growth on different lignocellulosic materials | | | |
|---|---|---|---|---|---|
| | | Paddy straw | Wood chips | Wood powder | Wood bark |
| 1 | *Phanerochaete chrysosporium* | + | ++ | ++ | - |
| 2 | *Trametes versicolor* | ++ | +++ | +++ | - |
| 3 | *Pycnoporus sanguineus* | ++ | ++ | ++ | ++ |
| 4 | *Alternaria alternata* | ++ | +++ | + | ++ |
| 5 | *Trichoderma harzianum* | ++ | + | + | + |

Excellent growth (+++), Moderate growth (+), Good (++), No growth (-).

## Liquid State cultivation of different isolated basidomycetes fungus

In Liquid state fermentation, the maximum growth was occured in *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pycnoporus sanguineus*, *Alternaria alternata* and *Trichoderma harzianum* (basal salt media and molasses media (+++)), good growth (++) was observed in *Phanerochaete chrysosporium*, *Pycnoporus sanguineus*, and *Trichoderma harzianum* (basal salt media, molasses media and PDB), moderate growth was exhibited in *Alternaria alternata* (Molasses media (+)) and no growth were showed in Molasses media and potato dextrose broth (*Trametes versicolor* and *Alternaria alternata* (-)) (Table 2. and Figure. 3).

**Fig 3: Various basidiomycetes fungus grown on LSF.**

**Table 2: Liquid state fermentation (LSF) of different isolated fungus.**

| S.No. | Strains | Fungal growth on different liquid media | | |
|---|---|---|---|---|
| | | Basal Salt Media | Molasses Media | Potato Dextrose Broth |
| 1 | *Phanerochaete chrysosporium* | ++ | ++ | ++ |
| 2 | *Trametes versicolor* | +++ | - | - |
| 3 | *Pycnoporus sanguineus* | ++ | +++ | ++ |
| 4 | *Alternaria alternata* | +++ | + | - |
| 5 | *Trichoderma harzianum* | +++ | +++ | ++ |

Excellent growth (+++), Moderate growth (+), Good (++), No growth (-).

## Screening of basidiomycetes fungi for ligninolytic enzyme producing potential

### Plate assay for ligninolytic enzymes of the isolated fungi.

PDA medium was employed for the screening of ligninolytic enzyme activities. All the basidiomycetes fungus produced significant colored zone (Table. 3 & 5 Fig.4.). The chromogen guaiacol is a very sensitive substrate that allows rapid screening of fungal strains producing extracellular guaiacol oxidizing enzymes by means of a color reaction[38]. The selected three strains produced the laccase, which catalyzes the oxidative polymerization of guaiacolto form reddish brown zones in the medium (Figure 1.1). The obtained results have good agreement with the report[39] for the isolation of laccase producing fungus.

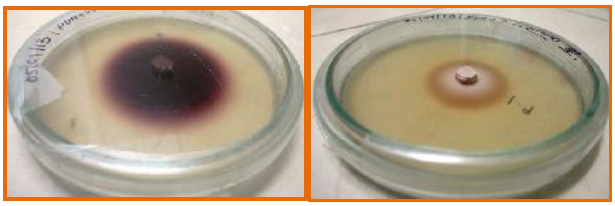

**Fig 4: Basidiomycetes plate assay for ligninolytic activity.**

**Table 3: Qualitative plate assay for ligninolytic activity of basideomycetes fungi**

| S.No. | Different Wood decaying fungi | Laccase activity | Manganese Peroxidase | Lignin peroxidase activity |
|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD |
| 1. | *Phanerochaete chrysosporium* | 1.2 ± 0.05 | 0.9 ± 0.05 | 2.1 ± 0.00 |
| 2. | *Trametes versicolor* | 1.8 ± 0.05 | 1.3 ± 0.01 | 1.7 ± 0.05 |
| 3. | *Pycnoporus sanguineus* | 0.7 ± 0.05 | 3.6 ± 0.05 | 3.1 ± 0.05 |
| 4. | *Alternaria alternata* | 2.5 ± 0.05 | 1.2 ± 0.00 | 1.2 ± 0.05 |
| 5. | *Trichoderma harzianum* | 0.6 ± 0.05 | 0.4 ± 0.05 | 0.4 ± 0.05 |

**Data are expressed as diameter of colored zone (mm) after 7 days of incubation at 30°C.**

## Screening the basidiomycetes fungi for quantitative ligninolytic enzyme production potential

Basal salt medium was employed for the quantitative screening of ligninolytic enzymes activities. All the basidiomycetes fungus produced significant results (Table 4). At the higher concentration of substrate, the fungus did not follow the Michaelis Menten enzyme kinetic rate model[40] for enzyme kinetics. Therefore, for further

optimization studies on bioprocess parameters, 2% (w/v) bagasse dose was taken as optimum for maximum ligninolytic enzymes activity, [41]reported that cotton stalk improved the maximum ligninolytic enzymes of *Pleurotus ostreatus* in 8 days incubation time. [42]showed lignin and manganese peroxidase activity in extracts from straw solid substrate fermentation in maximum at 6 days of inoculation. [43]reported the increased lignin biodegradation of straw by *P. ostreatus* with the increased activity of ligninolytic enzymes.

**Table 4: Quantitative ligninolytic enzyme production potential (U/ml) of basidiomycetes fungi**

| S.No. | Different Wood decaying fungi | Laccase activity | Manganese Peroxidase | Lignin peroxidase activity |
|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD |
| 1. | *Phanerochaete chrysosporium* | 2.2 ± 0.05 | 0.8 ± 0.05 | 2.1 ± 0.05 |
| 2. | *Trametes versicolor* | 3.1 ± 0.05 | 3.1 ± 0.05 | 3.7 ± 0.05 |
| 3. | *Pycnoporus sanguineus* | 3.6 ± 0.05 | 3.8 ± 0.05 | 3.1 ± 0.05 |
| 4. | *Alternaria alternata* | 1.8 ± 0.05 | 1.4 ± 0.05 | 1.7 ± 0.05 |
| 5. | *Trichoderma harzianum* | 0.1 ± 0.05 | 2.1 ± 0.05 | 2.1 ± 0.05 |

**Table 5: Two way ANOVA of both qualitative and quantitative enzyme assay for basidiomycetes fungi**

| Source | Dependent Variable | Type III Sum of Squares | df | Mean Square | F | Significant |
|---|---|---|---|---|---|---|
| Microorganisms | Laccase | 33.043 | 5 | 6.609 | 2.379E3 | 0.00 |
| | Manganese Peroxidase | 49.036 | 5 | 9.807 | 3.210E3 | 0.00 |
| | Lignin Peroxidase | 38.996 | 5 | 7.799 | 3.120E3 | 0.00 |
| Assay of both Qualitative & Quantitative | Laccase | 4.000 | 1 | 4.000 | 1.440E3 | 0.00 |
| | Manganese Peroxidase | 3.803 | 1 | 3.803 | 1.244E3 | 0.00 |
| | Lignin Peroxidase | 4.410 | 1 | 4.410 | 1.764E3 | 0.00 |

P< 0.05 Significant

## 4. CONCLUSION

This study focused on the isolation and characterization of white rot fungi from decaying wood sample, and isolated basidiomycetes fungus were subjected to analze ITS – gene identification , and those fungus were *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pycnoporus sanguineus*, *Alternaria alternata* and *Trichoderma harzianum*. In solid state fermentation *Trametes versicolor* and *Alternaria alternata* and liquid state fermentation basal salt media showed better results qualitative plate assay for ligninolytic activity all the basideomycetes fungi and quantitative ligninolytic enzyme production potential of basideomycetes fungi produced significant potential activity. This wood decaying fungus may able to degrade PAHs compounds and to protect polluted site or environment.

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