Chapter 2: Screening of potential lignin degrading fungus from natural habitat

2.1 Introduction

Lignocellulosic (LC) biomass is the most abundant naturally available renewable organic raw material on the Earth. It has been estimated to account for approximately 50% of the biomass in the world, and to have a yearly production of about 200 billion tons per year (Teghammar 2013). It is composed of carbohydrate polymers (cellulose, hemicellulose) and a complex aromatic polymer (lignin).

Lignin physically surrounds and protects the carbohydrate polymers from enzymatic hydrolysis and is also the most recalcitrant component of plant cell wall (Sanchez 2009). Only a very small amount of the total LC biomass produced is utilized for useful applications, while most are disposed causing environmental problems. However, LC biomass actually has a great potential as feedstock for production of more value added products (Sanchez 2009; Isroi et al. 2011). Therefore accessibility and availability of the carbohydrate polymers and thereby effective use of LC depend on delignification of plant material.

Many microorganisms are capable of degrading and utilizing cellulose and hemicelluloses as carbon and energy sources. However, only a smaller group of filamentous fungi known as white-rot fungi (WRF) possess the unique ability to efficiently degrade lignin to CO₂. The most rapid degraders in this group are basidiomycetes (Sanchez 2009). Lignin biodegradation by WRF is an oxidative process mediated by a unique set of extracellular enzyme system known as ligninolytic system. It is comprised of three major classes of enzymes designated as lignin peroxidases (EC 1.11.1.14) (LiP), manganese peroxidases (EC 1.11.1.13)
(MnP) and laccases (EC 1.10.3.2) (Ryu et al. 2003; Couto 2009; Patrick et al., 2010).

The enzymes involved in ligninolytic system are nonspecific, non stereoselective and effective against a broad spectrum of aromatic compounds. These enzymes have high biotechnological interest, as demonstrated by their role in wood pulp delignification, dye decolorization, ethanol production, wine processing, degradation of a variety of persistent environmental pollutants, bioremediation and for the realization of biosensors (Couto 2009; Sanchez 2009; Patrick et al. 2010). Recently, lignin degrading enzymes are also used for degradation of pharmaceuticals and their metabolites from surface, ground and drinking water (Wen et al. 2009) and as a component of enzyme cocktail in deinking of paper fiber.

Although over the past 2 to 3 decades, enormous research and/or progress have been made in lignocellulose technology, the industrial exploitation of these enzymes is limited. The main challenges facing the fungal lignocellulolytic enzymes research are summarized by Mtui (2012). He suggested to improve technologies to maximize productivities and vis-à-vis to reduce production costs. He further stressed on (i) stability of enzymes at adverse conditions (ii) sustainable production and (iii) availability of enzymes in large amountshas to be consider.

In recent years in order to maximize enzyme productivity and reduce cost, recombinant DNA technology has been frequently used. In addition, DNA shuffling methods and protein engineering has been employed to improve the stability, activity and/or specificity of an enzyme, thus tailor made enzymes can be produced.
to suit the individual processes (Piscitelli et al. 2010; More et al. 2011). However, there could be a number of predictable and unpredictable severe risks/ potential hazards related to use and release of Genetically Modified Organism in the environment including damage and destruction of ecosystem, biodiversity as well as human health. The various aspects of risks are reviewed in detail by Prakash et al. (2011). Moreover, the issues related to stability of modified organism and/or enzyme has to be addressed. Further development and application of this technology is, therefore, hindered as issues began to rise on how ecological upset would be prevented.

In comparison to above mentioned methods a classical strategy comprising sampling, cultivation followed by identification may yield promising wild strains with desired characteristics. Moreover, the application of which is not subject to any of the above mentioned risks. To meet the current biotechnological challenges, one of the solutions is to intensify bioprospecting initiatives, focusing on the vast habitats of fungi to look for novel strains that produce robust enzymes that are stable and have wide pH and temperature ranges (Jordaan 2005; Mtui 2012). Even though for more than 50 years the main focus of lignocellulose research is isolation of hyper producer, till today only an average of less than one per cent of the potential microbes in the biosphere have been identified (Howard et al. 2003). Moreover, amongst 10,000 species of white-rot fungi reported in the world only not more than 3 dozen have been more properly studied. The majority of studies have focused on Phanerochaete chrysosporium, followed by Trametes versicolor, Bjerkandera adusta and Pleurotus sp. (Coelho-Moreira et al. 2013). Thus
discoveries of more robust enzyme-producing fungi can set a new stage in this area of study.

Screening of the environment for ligninolytic microorganism is, therefore, necessary for two reasons: a) it may provide an opportunity for discovering unique genes which can be used for future applications and b) the number of isoforms of the enzymes present varies between species and also within species and the fact that search for fungal laccases with different properties is still on-going since enzyme with unique characteristics is required for individual application.

The relatively neglected fungal biodiversity of Jalgaon district, a region of North Maharashtra (India), provides an opportunity to test the hypothesis that potentially valuable source of ligninolytic enzymes could be obtained from rotten wood samples available in the forest area.

During bioprospecting studies a large number of samples are collected from diverse habitat; selecting potential strains efficiently from a large number depends on intelligent selection of screening methodology. Nevertheless, the screening methods preferably, an inexpensive, rapid and sensitive. The screening strategy assumes to minimize the possibility of losing the potent strains.

The aim of this study is to isolate efficient white rot fungi producing lignin degrading enzyme, from biodiversity of Jalgaon district, useful for industrial application. During this study, therefore, traditional and recent screening strategies were applied and a combination of both was used in order to facilitate efficient screening.
2.2 Objective

To isolate efficient white rot fungi producing lignin degrading enzyme from biodiversity of Jalgaon district, North Maharashtra region that may help realize industrial applications of ligninolytic enzyme technology.

2.3 Materials and Methods

2.3.1 Chemicals

Remazol Brilliant Blue R was purchased from Sigma Aldrich (USA). 2,2’-azino-bis(3-ethylbenzthiazoline- 6-sulphonic acid) (ABTS) was purchased from Fluka Analyticals (USA). O-phenyl phenol was purchased from Lancaster. Gallic acid, tannic acid and K₃Fe(CN)₆ was purchased from Sigma chemicals. All the other reagents and media were purchased from HiMedia, Mumbai, India. All chemicals used in this work were of analytical grade.

2.3.2 Sample collection

All samples were collected from the forest area of the Jalgaon district, North Maharashtra, India. Prior to sampling, information related to climate, geology and vegetation of the region was gathered. A majority of samples were collected from three forest areas namely Raver forest, Manudevi-Yawal forest and Patnadevi forest (Chalisgaon) while a few from Jalgaon city. The collected samples were brought to the laboratory in clean plastic bags marked with information such as number, procurement location.
To design an effective sampling regimen, GIS approach was used. Geo-referenced sampling frame suitable for spatial analysis was used to prepare the grid of 10 x 10 km using ArcGIS 9.2 desktop software. Random sampling points were generated in the forest area of the Jalgaon district using the software. The non-probability sampling technique is used while collecting the samples with subjective judgment of road accessibility to the sampling location using GPS instrument and Remote Sense images. Use of GIS and GPS in sampling design was implemented through the help of experts at JalaSRI, Watershed Surveillance Institute, M. J. College, Jalgaon.

2.3.3 Isolation and culturing techniques

Media: Different types of media were used in this study.

a) Isolation media

Two different media were used for isolation: malt extract agar (MEA) and O-phenyl phenol (OPP) agar. Malt extract agar (MEA) contained malt extract 1.5% and agar 2% and was amended with 250 µg ml⁻¹ streptomycin sulphate. O-phenyl phenol (OPP) agar was prepared as follows: Weighed quantity of O-phenyl phenol was dissolved in a small volume of Sodium hydroxide solution (1 M) with gentle heating. It was then neutralized with Hydrochloric acid solution (0.1 M) till the appearance of precipitate which would not re-dissolved on shaking. It was then sterilized by membrane filtration (Hi-media, 0.45µ). The filtrate was added to 2% malt agar in such a way that the final concentration of OPP in the medium is 0.3%.

Wood chips measuring 5 mm × 5 mm × 1 mm were removed from the rotten wood and aseptically transferred to Petri plates containing two different cultural
media: 1.5% MEA and 0.3% OPP agar. The first medium was intended to isolate total fungi and the second medium to specifically isolate phenol tolerant fungi.

The plates were incubated at 30 °C for 7–10 days to ensure growth of culturable fungi. Two plugs were removed from these plates and subcultured at opposite ends of a Petri-plate containing the selective medium. This subculturing was performed until a monoculture had been attained for all culturable fungi. The fungal isolates were maintained on potato dextrose agar (PDA) and stored at 4°C. These were then subjected to different plate assays for screening of ligninolytic enzymes.

b) Screening media

Different types of media were used for qualitative and quantitative assay of lignin degrading enzymes (LDE) production.

i) Qualitative media

Four different media were used for screening of fungal isolates for LDEs production, namely Tannic acid agar (TA), Gallic acid agar (GA), Guaiacol agar (GuA) and Lignin agar (LA). Tannic acid agar (TA), also known as Bavendamm’s medium (Kiiskinen et al. 2004), contained 0.5% tannic acid, 2% malt extract and 2% agar agar, adjusted with sodium hydroxide to pH 4.5. Similarly Bavendamm’s medium containing 0.5% gallic acid and 0.01% guaiacol instead of tannic acid was also used.

Gallic acid and guaiacol were added to the media before autoclaving; however tannic acid was autoclaved separately before addition to the media. The fungal strains were inoculated as plugs (One cm diameter) cut with a cork borer.
from the growing edge of PDA cultures. The plates were incubated at 30 °C for 7 day.

**Sundman and Nase test (Test for dephenolization of lignin)**

Ligninolytic activity was determined by using Sundman and Nase plate assay as described by Hedger (1982). Lignin agar contained (g/l): Sucrose-30, NaNO₃-2, KCl-0.5, KH₂PO₄-1, MgSO₄ (Hydrated)-0.5, FeSO₄-0.5, Yeast extract-0.5, lignin-0.15 % and agar agar -15, pH- 6.0. The lignin paste was separately autoclaved and then added to sterile base medium.

One cm diameter plug cut from the growing edge of PDA cultures of phenol tolerant isolates, was centrally inoculated on the surface of lignin agar. The plates were incubated at 30 °C for 7 day. The un-inoculated plate served as a control. After 7 day incubation, the aerial mycelium was scrapped off and the plates were flooded with freshly prepared ferricyanide reagent (1% FeCl₃ solution was mixed with 1% K₃Fe(CN)₆ solution in 1:1 ratio just prior to use). After 10 min incubation in dark, the excess reagent was decanted and colour developed was compared with control.

**ii) Quantitative media**

The selected fungal strains were cultivated in liquid fermentation media for quantitative estimation of enzyme activities and to study the type of ligninolytic enzymes produced. A general medium (2% malt extract broth) was used to study the constitutive production of ligninases. In addition, to study the production of LiP and MnP, the basal medium described by **Tien and Krik (1988)** was used. All vitamins were filter sterilized and glucose was autoclaved separately from other
media components. All media contained basal III and trace element solutions as described by Tien and Kirk (1988).

To study MnP production 100 mg/L MnSO$_4$·H$_2$O was added to basal medium. The medium described by Eggert et al. (1996) was used for laccase production. Cultivation was carried out in 100-ml Erlenmeyer flasks (triplicate) with 20 ml of medium. The medium was inoculated with two plugs of one cm diameter and incubated stationary at 30°C for 7 day. Contents of the replicate flasks were then pooled, and centrifuged at 4°C, 10000 rpm for 10 minutes to remove the mycelial mat. The supernatant was used for the enzyme assays as described in methods section.

2.3.4 Qualitative tests to assess lignin degrading enzymes (Pointing 1999)

Azure-B agar

One cm diameter plug cut from the growing edge of PDA cultures of selected isolates, was centrally inoculated on the surface of azure B agar. The medium containing (g/L) Glucose-0.2%, KH$_2$PO$_4$-1, Yeast Extract-0.01, Diammonium tartarate-0.5, CuSO$_4$.5H$_2$O-0.001, MgSO$_4$.7H$_2$O-0.5, FeSO$_4$.0.001, CaCl$_2$.2H$_2$O-0.01, MnSO$_4$.H$_2$O-0.001 and agar agar-20; was supplemented with 0.01 % w/v Azure B. The plates were incubated at 30 °C for 7 day. The uninoculated plate served as a control. The production of lignin peroxidase and Mn peroxidase was recorded as clearance of blue colored medium.

ABTS agar

This test is performed in a similar way using medium mentioned in Azure B agar except that Azure B was substituted with 0.1 % w/v ABTS. The formation of a
green color in colorless growth medium was used as an indication of production of laccase.

**α-napthol agar**

This test is performed in a similar way using medium mentioned in Azure B agar except that Azure B was substituted with 0.005 % w/v α-napthol. The formation of a blue color in colorless growth medium was used as an indication of production of laccase.

**2.3.5 Dye decolorization**

Decolorization of the anthraquinone dye Remazol Brilliant Blue R (RBBR) was assayed by the following methods.

**Solid state decolorization**

Decolorization experiments were carried out in medium containing glucose-1%, peptone-0.1% malt extract 1% and agar agar 2%. After autoclaving the medium was supplemented with sterile RBBR at a concentration of 500 mg l\(^{-1}\). The medium was inoculated centrally with a plug of one cm diameter cut from the growing edge of PDA culture and incubated at 30°C for 7 day. Cultivations were done in triplicate with un-inoculated dye containing plate as control. The decolorization of RBBR was recorded daily by visual observation in comparison to control.

**Liquid state decolorization**

Decolorization experiments were carried out in 100 ml Erlenmeyer flasks with 20 ml of medium containing glucose-1%, peptone-0.1% and malt extract 1%. The medium was supplemented with RBBR at a concentration of 500 mg l\(^{-1}\) and the pH was adjusted to 5.0. The medium was inoculated with two plugs of one cm
diameter cut from the growing edge of PDA cultures and incubated stationary at 30°C for 7 day. Cultivations were done in triplicate with biotic (without dye) and abiotic (without fungus) controls. The mycelium was separated by centrifugation (10 min, 10,000 rpm at 4°C) and color reduction of the supernatant was recorded at 595 nm using Shimadzu UV-1800 spectrophotometer. The absorption of the dye to mycelium was quantified by extraction with methanol (Bumpus 1988). The decolorization efficiency of the different isolates was expressed as percentage RBBR decolorization (Lopez et al., 2006) as per the following equation:

\[
\text{Decolorization (\%)} = \frac{(A_{\lambda, \text{initial}} - A_{\lambda, \text{final}})}{A_{\lambda, \text{initial}}} \times 100
\]

Where \(A_{\lambda, \text{initial}}\) = initial absorbance and \(A_{\lambda, \text{final}}\) = absorbance after 7 day culture.

**In vitro decolorization of RBBR**

The decolorization experiments with the extracellular fluid were also performed. Fungal mycelium and extracellular fluid were separated by centrifugation (10,000 rpm for 10 min at 4°C). The reaction mixture contained 1.0 mL of the enzymatic extract, 20 µl distilled water and 10 µl 2% RBBR solution. After 1 h, *in vitro* decolorization of RBBR was assayed by the decrease in absorbance at 595 nm (Neto et al. 2009). One unit of decolorization was defined as the amount able to catalyze a reduction of 0.01 in absorbance compared to a control prepared with previously heat-inactivated (10 min) enzymatic extract.

**2.3.6 Enzyme Assays**

After the mycelium was removed by centrifugation (10 min, 10,000 rpm at 4°C), LDEs activity in the culture supernatant was determined. All spectrophotometric measurements were carried out at 25°C using a Shimadzu UV-
1800 spectrophotometer with UV probe software. The substrates used to assay LDEs are presented in Table 2.1. Some stock solutions of the substrate had to be prepared in ethanol or methanol before they could be mixed with buffer. For each assay the absorbance was also measured without addition of laccase to determine the stability on auto-oxidation of the substrate.

**TABLE 2.1: Evaluation of Substrate preference for LDEs**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme to be assayed</th>
<th>Concentration of substrate</th>
<th>Molar weight [g/mol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>Lignin peroxidase</td>
<td>1 mM</td>
<td>548.0</td>
</tr>
<tr>
<td>ABTS</td>
<td>Laccase</td>
<td>1 mM</td>
<td>548.0</td>
</tr>
<tr>
<td>Azure B</td>
<td>Peroxidases</td>
<td>0.160 mM</td>
<td>305.83</td>
</tr>
<tr>
<td>Veratryl alcohol</td>
<td>Lignin peroxidase</td>
<td>0.092 M</td>
<td>168.19*</td>
</tr>
<tr>
<td>Phenol red</td>
<td>Manganese peroxidase</td>
<td>0.1 mM</td>
<td>354.38</td>
</tr>
</tbody>
</table>

*Solvent used for preparation of substrate was ethanol otherwise, it was water.

**ABTS assay method for laccase**

The nonphenolic dye 2,2′-azino-bis-(3-ethylbenzthiazolinesulphonate)(ABTS) is oxidized by laccase to the more stable state of the cation radical (Figure 2.1). The concentration of the cation radical responsible for the intense blue-green colour can be correlated to enzyme activity. ABTS assay is, therefore, commonly used by several researchers (Bourbonnais and Paice 1990; Pavko and Novotný et al. 2008; Garcidueñas et al. 2012; Ilyas and Rehman 2013) and is most often read between 415 nm and 420 nm.
FIGURE 2.1: The laccase-catalyzed oxidation of ABTS to a cation radical (ABTS\(^{+}\))(Bar 2001).

The oxidation of 1 mmol l\(^{-1}\) ABTS buffered with 50 mM phosphate buffer (pH 4.0) was monitored at 420 nm (\(\varepsilon_{\text{mM}} = 36 \text{ mM}^{-1}\text{cm}^{-1}\)) using a Shimadzu UV-1800 spectrophotometer with UV probe software. Enzyme activity was expressed as international units (IU) where 1 IU is defined as the amount of enzyme forming 1 \(\mu\text{mole}\) of product per minute (Neto et al. 2009). The reaction mixture contained 2 ml phosphate buffer (pH 4.0; 30 °C), 50 \(\mu\text{l}\) of substrate and 10 \(\mu\text{l}\) of enzyme. The laccase activity in U/ml (\(\mu\text{mole cation radical released} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}\)) was calculated as follows:

\[
U/\text{ml} = \frac{\Delta A \text{ min}^{-1} \times V}{v \times \varepsilon \times d}
\]

Where:
- \(V\) = Total reaction volume (ml)
- \(v\) = Enzyme volume (ml)
- \(\varepsilon\) = Extinction coefficient of ABTS at 420 nm (\(\varepsilon_{\text{mM}} = 36 \text{ mM}^{-1}\text{cm}^{-1}\)).
- \(d\) = Light path of cuvette (cm)
- \(\Delta A \cdot \text{min}^{-1}\) = Absorbance change per minute at 420 nm

**ABTS assay method for Peroxidase**

Peroxidase activity was determined by adding to the laccase assay solution \(\text{H}_2\text{O}_2\) to 100 mmol l\(^{-1}\) final concentration and subtracting the increase in absorbance caused by laccase activity (Eggert et al. 1996).

**Phenol red assay for manganese peroxidase**
The oxidation of phenol red is most commonly used to measure the Mn peroxidase activity, \((\text{Minussi et al. 2001; Arora et al. 2002; Cruzet al.2004})\). The phenol red assay was performed as follows:

Five milliliter of reaction mixture contained 1.0 ml sodium succinate buffer (50 mM, pH 4.5), 1.0 ml sodium lactate (50 mM, pH 5.0), 0.4 ml manganese sulphate (0.1 mM), 0.7 ml phenol red (0.1 mM), 0.4 ml \(\text{H}_2\text{O}_2\) (50 \(\mu\)M), gelatin 1 mg ml\(^{-1}\) and 0.5 ml of enzyme extract. The reaction was initiated by adding \(\text{H}_2\text{O}_2\) and conducted at 30 \(^\circ\)C. One milliliter of reaction mixture was taken and 40 \(\mu\)l of 5 N NaOH was added to it. Absorbance was taken at 610 nm. After every minute the same steps were repeated with 1 ml of the reaction mixture up to 4 min. One unit of enzyme activity is equivalent to an absorbance increase of 0.1 units \(\text{min}^{-1} \text{ml}^{-1}\).

**Veratryl alcohol assay for lignin peroxidase**

The VA assay for LiP used 50 mM Natartrate buffer (pH 2.5 or 4.5)-0.1 mM \(\text{H}_2\text{O}_2\)-2 mM VA in a 1.0-ml reaction volume, and activity was monitored at 310 nm.

**Azure B assay for lignin peroxidase**

Azure B as a substrate to assay lignin peroxidase is commonly used by various researchers \((\text{Archibald 1992; Arora and Gill 2001; Cruz 2004})\). Reactions were carried out in 3 ml cuvettes containing 1 ml of 125 mM sodium tartrate buffer (pH 4.0), 500 \(\mu\)l of 0.160 mM Azure B, 500 \(\mu\)l of enzyme extract and 500 \(\mu\)l of 2 mM \(\text{H}_2\text{O}_2\). The optical density (OD) decrease was read at exactly 651 nm.

**2.3.7 Study of growth curve**
The method developed by Langvad (1999) was used to measure mycelial growth using 96 well microtitre plates, which were read on a ELISA plate reader (Thermo Fisher scientific) at 630 nm, where 1 absorbance unit is equivalent to 4.2 mg/ml dry fungal biomass concentration. Data is represented as means of octaplate samples ± standard deviation.

The malt extract broth was used in this experiment. The inoculum was prepared from 5 mycelial circles of diam. 6 mm cut from the mycelial mat using a cork borer. The circles were transferred in 30 ml sterile distilled water and homogenized for 30 s. The microplate wells were inoculated with 20 µl of this homogenate.

Column 1 of amicrotitre plate containing 180 µl of growth medium and 20 µl of sterile distilled water served as blank. Columns 2 to 12 containing 180 µl of growth medium was inoculated with 20 µl homogenate described before. The plates were then incubated at 30°C and the absorbance was read daily on a ELISA plate reader at 630 nm. The result is expressed as a mean of the 8 wells in each column.

2.3.8 Measurement of biomass (dry weight)

The biomass concentration was determined by fungal mycelium dry weight. The mycelium was washed by filtration on filter paper (Whatman No.1) and dried overnight at 100 °C to a constant weight together with pre-weighed filter paper. The mycelium weight was calculated by subtracting the weight of pre-weighed filter paper. Growth yield was expressed as mg of mycelium dry weight per ml of culture.
2.3.9 Calculation of growth parameters

The specific growth rate was determined as the slope of the natural logarithm of the exponential portion of the plotted growth curve. The best four points were used as determined by analyzing the best fit for the linear regression in Microsoft Excel. Lag phase was determined as the X intercept of two linear plots, the exponential portion of the curve (the four points exhibiting the best fit) and the linear regression for the initial lag phase (Jordaan 2005).

2.3.10 Determination of apical extension rate

The fungal growth on solid medium is more advantageous than submerged cultures in studying the mechanisms involved in the control and regulation of mycelial growth, as fungi are adapted to growth on solid substrates. In the present study, apical extension rate was determined as described by Reeslev and Kjoller (1995). The PDA agar plates were centrally inoculated with a plug of mycelia (1 cm diameter) taken from an actively growing mycelial mat. During incubation at 30±2°C, the diameter of the widest part of colony was measured every 24 h. The radius vs. day plot was analyzed with linear regression and the slope of the line was recorded in µm/h which gave the apical extension rate, Kr of the colony (Biswas 2001). The experiment was repeated twice with three replicates in each experiment. Furthermore, the finding that the Kr of a fungal colony was related to the specific growth rate obtained in submerged culture by the equation Kr=μ*w, where w is the peripheral growth zone and μ is the specific growth rate.

2.3.11 Identification of the isolate
The morphological identification of the isolates was attempted by microscopic observations using illustrated Atlas of Common Plant Pathogenic Fungi from Department of Botany, Moolji Jaitha College, Jalgaon. Also morphological identification was attempted at Agarkar Research Institute, Pune. The molecular analysis was conducted at National Centre for Cell Science (NCCS), Pune (India); the details of which are as follows:

**DNA isolation, PCR amplification and Sequencing**

DNA was isolated from strains L-168 and L-169 using a DNeasy™ Tissue Mini Kit (Qiagen), following the manufacturers protocol for fungi. The extracted DNA was quantified by NanoDrop spectrophotometer (ND–1000, Thermo scientific, USA). Universal primer pairs ITS1F (5’- TCC GTA GGT GAA CCT GCG-3’) and ITS4 (5’-TCC TCC GCT TAT TGA TAT GC-3’) (White *et al*. 1990) were used to amplify desired region using ABI2720 thermocycler (Applied Biosystems®). PCR reaction mixture (25 µl) comprised of 200 µM of dNTP,10 picomole each primer, 50ng DNA template and 1U Taq DNA polymerase (Bangalore Genei, Bangalore, India) with reaction buffer containing 1.5 mM MgCl₂, supplied by the manufacturer. Polymerase chain reaction was performed using the following thermocycling pattern: 94°C for 5 min (1 cycle); 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min (30 cycles); and 72°C for 10 min (1 cycle). PCR products were run on 1% agarose gel which was stained with SYBR® Safe DNA Gel Stain (Invitrogen™) before casting. After completion of run gel was photographed using Gel doc system (Syngene G:BOXChemi XR5 system) using GeneSys software. The successfully amplified PCR products were purified using
PEG-NaCl method (Sambrook et al. 1989). The products so obtained were sequenced from both the ends using BigDye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems) by following manufacturer’s protocol. Sequences were obtained using an automatic DNA sequencer (3730xl DNA analyzer, ABI).

**Construction of similarity matrix**

A percent similarity matrix was constructed to compare both the sequences obtained in this study using dnadist from Phylip (version 3.69) package as described in Salunke et al., 2012. Sequences obtained in this study were considered for pairwise comparisons.

**Sequences for alignment, their editing and Phylogenetic analysis**

Twenty six sequences from GenBank were retrieved. Sequences obtained under this study were aligned with these database sequences using ClustalX (version 2.0.9) (Larkin et al. 2007). All sequences were manually edited and unrooted phylogenetic tree was constructed using MEGA 5 with 1000 bootstrap replication (Tamura et al. 2011). For Bayesian inference of phylogeny, the program MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) was used. The analysis for each gene consisted of 30,00,000 generations with sampling every 100 generations. First 12000 trees (40%) were discarded as burn in. Before carrying out the probabilistic phylogenetic analyses, appropriate models of sequence evolution for each dataset were chosen via Akaike Information Criterion (AIC) using program MrModeltest2.2 (Nylander 2002). The selected model of nucleotide substitution was ‘GTR+I+G’ and the final alignments consisted of 556bp. Three independent runs were performed for each dataset. In phylogenetic trees, levels of confidence for
each node are shown in the form of Bayesian posterior probabilities (BPP). BPP below 0.50 are not shown. Accession numbers for each sequence was shown after each species name in parenthesis.
2.4 Results

Sample collection

As the occurrence of white rot fungi depends on substratum, host availability and climate, appropriate timing for sampling was initially determined. The month of August and September (the end of rainy season for Jalgaon district) was found to be most appropriate. The geo-climatic conditions of sampling sites are presented in Table 2.2. The total reserved forest area of Jalgaon district is 801.58 sq. miles. The Figure 2.2 shows forest map of Jalgaon district with random sampling points generated with software.

**TABLE 2.2: Geo-climatic conditions of sampling sites and sampling profile**

<table>
<thead>
<tr>
<th>Habitat /Source</th>
<th>Average rainfall (mm)*</th>
<th>Temperature (°C)* Aug - Sept.</th>
<th>Number of samples collected</th>
<th>Sample code</th>
<th>Number of phenol tolerant isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raver forest</td>
<td>683</td>
<td>30-32</td>
<td>80</td>
<td>L 1 to L 80</td>
<td>12</td>
</tr>
<tr>
<td>Manudevi - Yawal forest</td>
<td>698.4</td>
<td>30-32</td>
<td>50</td>
<td>L 81 to L 130</td>
<td>20</td>
</tr>
<tr>
<td>Patnadevi forest, Chalisgaon</td>
<td>664</td>
<td>30-32</td>
<td>51</td>
<td>L131 to L 181</td>
<td>17</td>
</tr>
<tr>
<td>Jalgaon city</td>
<td>718</td>
<td>30-35</td>
<td>14</td>
<td>L182 to L195</td>
<td>2</td>
</tr>
</tbody>
</table>

*The data on average rainfall and temperature of the Jalgaon District is kindly provided by “JalShri”, Watershed Surveillance Research Institute, Moolji Jaitha College, Jalgaon.

A total of 195 white rot samples were collected (Plate 2.1). The screening profile of samples collected is summarized in Table 2.3. A total of 51 isolates showing phenol tolerance were obtained from all the sources explored. Out of the four habitats explored, Manudevi forest proved to be the best source since maximum of 40% phenol tolerant isolates were obtained from the site. It is followed by Patnadevi forest from where 33% isolates were obtained. All the fungal isolates were maintained on Potato destrose agar (PDA) and stored at 4°C.
Qualitative assay for lignin degrading enzymes (LDEs) production

The ligninolytic enzyme production ability of 51 phenol tolerant fungal isolates was qualitatively determined using agar plate assay (Plate 2.2). When screened for guaiacol oxidation (Plate 2.3), among 51 phenol tolerant isolates, 17 isolates showed a halo of intense brown color under and around the fungal colonies, indicating the presence of ligninolytic enzymes. Similarly ability of these isolates to oxidize gallic acid (Plate 2.4) and tannic acid (Plate 2.5) was evaluated using a four point grading system (Table 2.3). The oxidation ability of isolates was repeatedly checked up to seven subcultures (Plate 2.6). Interestingly, amongst 51, the ability of 17 isolates to form brown color on Gallic and tannic acid weakened during sub culturing. Such weakening was not observed with guaiacol. In view of the results obtained, altogether 34 isolates were selected for further study. The screening profile of 34 fungal isolates by plate assay using different indicators is shown in Table 2.3.
**TABLE 2.3: Screening profile using different indicators**

<table>
<thead>
<tr>
<th>Sr.no</th>
<th>Sample No.</th>
<th>Oxidase production on</th>
<th>Growth on Malt agar with Guaiacol (0.01%)</th>
<th>% RBBR decolorization</th>
<th>Decolorization Units/ml</th>
<th>Mycelial extension rate cm/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gallic acid agar (0.5%)</td>
<td>Tannic acid agar (0.5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>L40</td>
<td>4+</td>
<td>-</td>
<td>0.19</td>
<td>0.2</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>L47</td>
<td>2+</td>
<td>1+</td>
<td>2+</td>
<td>61.12</td>
<td>8.01</td>
</tr>
<tr>
<td>3</td>
<td>L48</td>
<td>4+</td>
<td>3+</td>
<td>44.5</td>
<td>ND</td>
<td>0.64±0.05</td>
</tr>
<tr>
<td>4</td>
<td>L56</td>
<td>4+</td>
<td>-</td>
<td>1+</td>
<td>1.78</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>L58</td>
<td>-</td>
<td>4+</td>
<td>-</td>
<td>0.19</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>L62</td>
<td>2+</td>
<td>1+</td>
<td>0.30</td>
<td>ND</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>7</td>
<td>L63</td>
<td>4+</td>
<td>3+</td>
<td>0.90</td>
<td>ND</td>
<td>1.7±0.06</td>
</tr>
<tr>
<td>8</td>
<td>L84</td>
<td>3+</td>
<td>-</td>
<td>-</td>
<td>0.32</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>L91</td>
<td>-</td>
<td>4+</td>
<td>-</td>
<td>0.00</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>L96</td>
<td>4+</td>
<td>3+</td>
<td>1+</td>
<td>62.00</td>
<td>8.2</td>
</tr>
<tr>
<td>11</td>
<td>L109</td>
<td>4+</td>
<td>2+</td>
<td>0.80</td>
<td>ND</td>
<td>0.80±0.1</td>
</tr>
<tr>
<td>12</td>
<td>L114</td>
<td>4+</td>
<td>2+</td>
<td>0.14</td>
<td>ND</td>
<td>1.2±0.04</td>
</tr>
<tr>
<td>13</td>
<td>L115</td>
<td>-</td>
<td>3+</td>
<td>0.11</td>
<td>ND</td>
<td>1.0±0.16</td>
</tr>
<tr>
<td>14</td>
<td>L116</td>
<td>4+</td>
<td>3+</td>
<td>0.15</td>
<td>ND</td>
<td>0.49±0.15</td>
</tr>
<tr>
<td>15</td>
<td>L126</td>
<td>1+</td>
<td>-</td>
<td>0.19</td>
<td>ND</td>
<td>0.55±0.02</td>
</tr>
<tr>
<td>16</td>
<td>L129</td>
<td>3+</td>
<td>1+</td>
<td>0.19</td>
<td>ND</td>
<td>1.01±0.06</td>
</tr>
<tr>
<td>17</td>
<td>L137</td>
<td>-</td>
<td>4+</td>
<td>-</td>
<td>12.74</td>
<td>1.42</td>
</tr>
<tr>
<td>18</td>
<td>L138</td>
<td>3+</td>
<td>2+</td>
<td>1+</td>
<td>54.28</td>
<td>7.00</td>
</tr>
<tr>
<td>19</td>
<td>L139</td>
<td>4+</td>
<td>2+</td>
<td>1+</td>
<td>59.3</td>
<td>7.89</td>
</tr>
<tr>
<td>20</td>
<td>L140</td>
<td>4+</td>
<td>2+</td>
<td>1+</td>
<td>70.1</td>
<td>9.23</td>
</tr>
<tr>
<td>21</td>
<td>L141</td>
<td>4+</td>
<td>2+</td>
<td>1+</td>
<td>66.00</td>
<td>8.74</td>
</tr>
<tr>
<td>22</td>
<td>L143</td>
<td>4+</td>
<td>1+</td>
<td>2+</td>
<td>79.6</td>
<td>10.02</td>
</tr>
<tr>
<td>23</td>
<td>L149</td>
<td>4+</td>
<td>2+</td>
<td>-</td>
<td>80.18</td>
<td>10.5</td>
</tr>
<tr>
<td>24</td>
<td>L151</td>
<td>4+</td>
<td>2+</td>
<td>1+</td>
<td>74.3</td>
<td>9.96</td>
</tr>
<tr>
<td>25</td>
<td>L154</td>
<td>1+</td>
<td>-</td>
<td>1+</td>
<td>0.57</td>
<td>0.1</td>
</tr>
<tr>
<td>26</td>
<td>L155</td>
<td>4+</td>
<td>4+</td>
<td>3+</td>
<td>96.60</td>
<td>11.1</td>
</tr>
<tr>
<td>27</td>
<td>L159</td>
<td>1+</td>
<td>-</td>
<td>1+</td>
<td>0.19</td>
<td>0.25</td>
</tr>
<tr>
<td>28</td>
<td>L160</td>
<td>4+</td>
<td>4+</td>
<td>3+</td>
<td>95.00</td>
<td>12.0</td>
</tr>
<tr>
<td>29</td>
<td>L162</td>
<td>2+</td>
<td>1+</td>
<td>-</td>
<td>0.10</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>L168</td>
<td>4+</td>
<td>-</td>
<td>3+</td>
<td>98.53</td>
<td>13.00</td>
</tr>
<tr>
<td>31</td>
<td>L169</td>
<td>4+</td>
<td>-</td>
<td>3+</td>
<td>98.00</td>
<td>12.8</td>
</tr>
<tr>
<td>32</td>
<td>L185</td>
<td>3+</td>
<td>4+</td>
<td>-</td>
<td>0.21</td>
<td>0.18</td>
</tr>
<tr>
<td>33</td>
<td>L181</td>
<td>2+</td>
<td>-</td>
<td>-</td>
<td>10.20</td>
<td>0.98</td>
</tr>
<tr>
<td>34</td>
<td>L182</td>
<td>3+</td>
<td>4+</td>
<td>1+</td>
<td>72.00</td>
<td>9.01</td>
</tr>
</tbody>
</table>

All values reported as mean ± SD for three replica cultures; ND = Not determined

"-" Negative test

Zone diameter (mm) of colored reaction measured after 7 day incubation at 30°C on:

1. gallic acid agar and tannic acid agar: 1+ → 20-30mm; 2+ → 30-40mm; 3+ → 40-50mm; 4+ → above 50mm

2. Guaiacol agar - 1+ → 20-30mm; 2+ → 30-40mm; 3+ → above 40mm

Ph.D Thesis: Minal K. Narkhede, North Maharashtra University, Jalgaon (January 2014)
Response to Gallic acid, tannic acid and guaiacol was graded as good \( (4^+/3^+) \), moderate \( (2^+/1^+) \) and no response.

FIGURE 2.3: Response by the isolates to different indicators during qualitative screening for LDEs.

It is evident from Table 2.3 and Figure 2.3 that, the response shown by the isolates to different indicators is variable. 67.6% isolates showed good response \( (4^+/3^+) \) to gallic acid whereas only 11.8% to guaiacol. On the contrary, only 20.6% isolates showed moderate response \( (2^+/1^+) \) to gallic acid but 41.2% to guaiacol. However, in case of tannic acid the percentage isolates showing good and moderate response was equal i.e. 35.3%.

When ferricyanide reagent was used to check dephenolization of lignin on lignin agar plates (Plate 2.7), development of yellow green coloration indicated lignin degradation ability against the blue green colour of the control plate. Although the ability of oxidation of gallic acid, tannic acid and guaiacol varied amongst the 34 isolates, all exhibited lignin dephenolization ability.

**Dye decolorization**

RBBR decolorization was studied for all the selected isolates (Plate 2.8, Plate 2.9). It was observed that 14 isolates showed more than 50% decolorization of the dye. However only 4 isolates (L-155, L-160, L-168 and L-169) showed more
than 90% decolorization. Of the thirty four isolates, L-168 and L-169 were most effective in the decolorization, which showed 98.53% and 98.00% decolorization, respectively. The percentage decolorization of RBBR by the isolates is presented in Figure 2.3 and the response shown by the phenol tolerant isolates to RBBR decolorization test is depicted in Figure 2.4.

FIGURE 2.4: Response by the phenol tolerant isolates to RBBR decolorization.

It was observed that the mycelial mats in the flasks remained clear throughout the experiment. To confirm this, the separated mycelia of cultures incubated with dye were suspended in methanol, extracted and centrifuged. The absence of color in the extracted supernatant proved that decolorization is due to degradation and not due to adsorption onto the mycelium.

Ligninolytic enzyme production using liquid and solid media

In view of the results obtained, four strains, namely L-168, L-169, L-155 and L-160 which gave more than 90% dye decolorization were prioritized for a better understanding of ligninolytic enzyme system. Numerous substrates have been used to study activity of LDEs; many of which are attacked by both laccase and
peroxidase (LiP/MnP). Considerable effort, therefore, was subsequently expended to determine the type of enzyme in selected four strains.

When cultivated in liquid medium, laccase was the only ligninolytic enzyme detected in all four isolates. No peroxidase activity was detected by ABTS oxidation in the presence of H₂O₂. The LiP assay carried out with azure B as well as veratryl alcohol was also negative. Moreover, MnP-type peroxidase was also absent since it could not oxidize phenol red, a standard substrate for MnP’s. The laccase activity of four isolates by cultivation in liquid medium is shown in Table 2.4

**TABLE 2.4: Laccase activity exhibited by four isolates**

<table>
<thead>
<tr>
<th>Fungal isolates</th>
<th>Laccase activity (UL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-168</td>
<td>507.92 ±18.2</td>
</tr>
<tr>
<td>L-169</td>
<td>458.22± 21.6</td>
</tr>
<tr>
<td>L-155</td>
<td>278.19± 11.8</td>
</tr>
<tr>
<td>L-160</td>
<td>285.06± 12.5</td>
</tr>
</tbody>
</table>

The presence of laccase and absence of peroxidase was qualitatively confirmed by cultivation of the isolates on Azure-B, ABTS and α-napthol agar. Decolorization of the dye Azure-B by fungi is positively correlated with production of lignin peroxidase and Mn dependent peroxidase, however, this dye is not a substrate for laccase. No decolorization of azure B was found for all four isolates; however, green coloration on ABTS agar due to the oxidation of ABTS and blue coloration on α-napthol agar confirmed laccase activity (Plate 2.10).

Although the laccase activities observed in our screening trial were much lower, it is important to note that the conditions for cultivation were not optimized for the strains. Different inducers, media components and cultivation conditions could interact with different strains, which could enhance the laccase production.
Study of growth curve and Growth Kinetic Parameters

It would also be important to determine the growth characteristic and growth rate of hypersecretary strains along with ability to produce high titer of laccase. The growth curve as well as the method used for calculation of the growth kinetic parameters is shown in Figure 2.5a and 2.5b.

**FIGURE 2.5:** Growth curve for BspL-168 measured as: a) microplate technique described in methods section and b) biomass dry weight.
The growth profile shown in Figure 2.5 a and b were used for the determination of the lag phase. The x intercept of the two linear plots of the lag phase and exponential phase was used as the lag time for a sample.

![Image](image_url)  
**Figure 2.6 a** and b: Measurement of specific growth rate with natural log of the biomass yield

The graph shown in Figure 2.6 (a and b) were used to calculate specific growth rate a) measured with the microplate technique and b) measured by biomass dry weight. The specific growth rate is represented by the slope of the linear plot, of the natural logarithm of biomass yield at each specific time measurement.
The apical extension rate (Kr) as recorded in µm/h from linear regression of the radius vs. day plot is 246 µm/h (Figure 2.7).

**FIGURE 2.7: Determination of radial extension rate of isolate BspL-168.**

**Identification of fungal isolates**

The microscopic observations of the isolates (L-168 and L-169) revealed that the isolates were non sporulating. However, the isolates were not identifiable based on biological and morphological characteristics. Further, morphological characterization at ARI, Pune was also inconclusive. Molecular characterization was therefore, preferred for species identification with the help of experts at National Center for Cell Science, Pune.

**Comparison of sequences obtained in this study**

Sequences obtained in this study were compared with each other. Similarity matrix constructed using the sequences obtained for L-168 and L-169 showed 99.8% similarity with each other. A BLAST sequence homology search was done to compare the sequences with available database sequences in GenBank. These results suggest that the L-168 was 99% similar with
Enzymological and Biotechnological Prospects in Lignolytic system of White Rot Fungi.

*Perenniporiattephropora*(JN048763), *Polyporales* sp. (JQ312162) and *Rigidoporus* sp. (AJ537410). Same way strain L-169 *Polyporales* sp. (JQ312162), *Basidiomycete* sp. (AY605709) and *Pleurotusnebrodensis*(FJ572269). These results showed negligible differences in total score and maximum score. To avoid this confusion, phylogenetic tree was constructed.

The phylogenetic tree (Figure 2.8) was constructed to determine evolutionary relationship among isolates L-168, L-169 and already reported fungi. It consists of sequences from this study along with 26 database sequences representing 7 genera and 4 species which are either having name as *Basidiomycete* sp. or Fungal sp.

**ITS sequence of Isolate L-168**

| CCTGATTGGAGTGTCAGAGTCAATGTGTTGTCCTCANAAGAGACGATTAGAAGCTCGCCA  |
| AACGCTTTACGTCGGTCCGGCGTAGACAAATTATCTACACCAGAGGCAGCCGATCCCAGAAGTT |
| CAAGCTAATGCTTTAAGAGGAGGCCAGCCGGTTAGAGCCGACTCCAGCTTCAAGGATCTGC |
| CTGGGAATACCAAGGAGGCACAGACGTTCGTTCAAGAAGATCGATTTCTTGAGAAGTA |
| AATTCAACATTACCTTTCGCTGTTTCCTCATCGATGCAGAGGCGAGGCTAGTCAGGCA |
| CTGAAACCCACACATAGTGCAAGTTGGAAGTGTAGAGTGAGTAGAGAGGGCAGGGTCACATGCT |
| CGGAGGCAGTCACACCCAGTCGAAAATCGCATATAATGCCTTCCAGTACGACC |

**ITS sequence of Isolate L-169**

| CCTCCGTATATGATATGCTTAAAGTGTCAGAGGTTAGTCTCCTACCATGATTGATGTCAGAGGTC |
| AATGTGTGTGTCATGTAAGAGACGATTAGAAGCTCCGCAAAGCCGTTACGTCGGTCCGGGT |
| AGACAAATTAATTTACACCAGAGGCCAGCGATCCCAGAAGATAAGCTAATGCACTTTTAAAGAGG |
| AGCCGACCCTTACGCCGACAGCGCTGCATCAAGGTGAAACCCAGGAGGAGAATTTA |
| TAGGTTGGAAGATTTTCATGACACTCAAGGAGGCTGCTCCGGAATACACCAGGAGAGGCA |
| AGGTGGCTCAAGAAGGTTGATGTTCACTGCAAATTTCGCTACCATTTAATTTCGCAATTT |
| TCCGGTTATCTCATGTTGGAGGCCAGAAAAGACTGCTTGGTGTTAAGATTTGATAATAG |
| ATGCTGTTATATGCTTACACATTATTCTTATTCTTGTGAAAAGGTTTATGATATAAAGCCGGGC |
| AGACGCTTTCNCGAACCCTGAAGGCTCGCTACCGCCTGCTGCAAACCACAGTAAATGTCGA |
| CAGGTTGAGATGGAGTGCCAGGGCGTGCCACATGCTCCGGAAGGCCAGGTCAACACCCA |
| GTCAAAACTGATATGATGTTCTTCAGTCAGGGTTTGATGTCGACTTACGGAAG |

*Ph.D Thesis: Minal K. Narkhede, North Maharashtra University, Jalgaon (January 2014)*
FIGURE 2.8: Phylogenetic tree for ITS using the neighbor joining algorithm of ClustalX, indicating the placement of organisms of interest in bold. (Names of species are followed by the relative GenBank accession numbers for the retrieval of sequences used.)
Phylogenetic Analysis

Phylogenetic analysis revealed that alike to Similarity matrix isolate L-168 and L-169 are highly similar to each other and also cluster together. These two isolates with 6 other fungal sequences form a major cluster which compromises of two sister clades. One clade consists of L-168 (JF412304) and L-169 (JF412303) along with the Basidiomycotasp. (HQ018817). While other clade was formed by isolate Basidiomycetesp. (AY605709), Pleurotusnebrodensis(FJ572269), Rigidoporussp. (AJ537500) and Rigidoporussp. (AJ537410) which is supported by good Bayesian posterior probabilities value (0.78).

Thus, based on molecular data the isolated fungal strains L-168 and L-169 are Basidiomycotasppecies. This isolates are very close to genus Pleurotus and Rigidopus. However for exact genus and species level identification, there is further need to carry out morphological or biochemical characterization. The isolates Basidiomycota sp. L-168 and L-169 are newly isolated species. The full lineage of the isolates is as follows: Cellular organisms; Eukaryota; Opisthokonta; Fungi; Dikarya; Basidiomycota; unclassified Basidiomycota

The fungal isolates obtained could be potential candidates for use in bioremediation or other biotechnological application. The approach used to isolate one potential strain from a total of 195 rotten wood samples is depicted in Figure2.9.
FIGURE 2.9: Screening profile for potent fungal isolate from wood samples
2.5 Discussion

In the present investigation, various advanced tools such as satellite imagery, GIS software and GPS were used to design the sampling regimen. In our knowledge, this is the first report on use of these tools for the sampling of ligninolytic fungi. This methodology has the added benefit of providing a geo-referenced sample, and helps to correlate with spatial characteristics of the physical environment. Remote Sense images and GPS served as a guide for subjective judgment of road accessibility to the sampling location. The non-probability sampling technique was more effective in this regard.

The importance of phenol tolerance in screening of ligninolytic fungi is emphasized since long period. As the growth requirements of different white rot fungal species are quite different, it is difficult to screen these fungi from natural source. To overcome this difficulty, in addition to general medium (2% malt extract agar), a medium amended with OPP was also used. The isolation of phenol tolerant fungi facilitated the isolation of ligninolytic fungi. The use of OPP for selective isolation of white-rot fungi is reported by Wilcox (1964) (0.006%) and Hedger (1982) (0.003%). However, in the present study 0.3 % OPP was used to isolate high phenol tolerant fungi. From 195 rotted wood samples, altogether 51 phenol tolerant fungi were isolated, which were further screened for LDEs using different indicators.

The traditional screening of fungi for LDEs production is routinely carried out by qualitative assays (Pointing 1999; Kiiskinen et al. 2004). They are particularly useful in screening large number of fungal isolates for several classes of
enzymes. Several different substances have been used as indicator for ligninolytic enzymes production e.g. gallic acid and tannic acid (Cookson 1995; Kamada et al. 2002; Kiiskinen et al. 2004), guaiacol (Lopez et al. 2006; Kim et al. 2010; Bodke et al. 2012) and α naphthol (Pointing 1999; More et al. 2011).

Most of the earlier researchers have used single indicator compound during screening. However, it is evident from the present study that, all fungi that oxidize gallic acid, do not necessarily oxidize tannic acid and vice versa. Therefore, an isolate that do not oxidize gallic acid cannot be considered negative for presence of LDEs. The same is true for tannic acid oxidation. Secondly, since individual species of white-rot fungi differ in the composition of their sets of ligninolytic enzymes (Hatakka 1994; Palaez et al. 1995), using single indicator during screening seems to be insufficient. This could be explained by the observation during the present study that: some of the isolates (L-40, L-56, L-84, L-129, L-143, L-168 and L-169) gave strong positive reaction (4+ or 3+) with gallic acid but showed very poor (1+) or no response with tannic acid. In contrast to this, some isolates (L-58, L-91, L-115, and L-137) showed strong positive reaction only with tannic acid. Interestingly, some isolates (L-48, L-63, L-96, L-116, L-155, L-160, L-185 and L-182) showed strong positive reaction with both gallic acid and tannic acid.

Amongst 51 phenol tolerant, the ability of 17 isolates to form brown color on Gallic and tannic acid weakened during sub culturing. Kiiskinen et al. (2004) also reported such weakening of ability to form brown color on tannic acid. However, during present study, we found this phenomenon on both gallic and tannic acid. Such weakening was not observed with guaiacol.
As compared to gallic acid and tannic acid, guaiacol is more closely related to lignin monomer structure. Therefore, study of guaiacol oxidation is considered to be more important in screening ligninolytic fungi. In the present study, out of 34 phenol tolerant isolates, seventeen fungal isolates were positive for guaiacol oxidation. However, it was observed that those strains that were positive only with tannic acid, all were found negative for guaiacol oxidation. Only some those were positive with gallic acid gave guaiacol oxidation positive and some gave negative. Similar findings are reported by D’Souza et al. (2006), where they reported only 7.5% isolates positive for guaiacol. Hence, it was very difficult to rely on use of single indicator, moreover use of only plate assay was found to be inadequate for screening of large number of samples. Therefore, these isolates were further screened by RBBR oxidation and enzyme assay.

RBBR decolorization has been widely used for study of ligninolytic system. The advantages of use of polymeric dyes as model compounds in studies of ligninolytic fungi compared to conventional substrates are discussed by Machado et al. (2005) and Neto et al. (2009). The correlation between decolorizing and ligninolytic abilities of white-rot fungi has been established by several authors (Mohorčič et al. 2004; Babic and Pavko 2007; Sarnthima et al. 2009; Singh et al. 2010). It was observed that fourteen isolates (L-47, L-96, L-138, L-139, L-140, L-141, L-143, L-149, L-151, L-155, L-160, L-168, L-169 and L-182) showed RBBR decolorization more than 50%. Among these fourteen isolates, ten isolates showed positive reaction for all indicators tested i.e. gallic acid, tannic acid and guaiacol. However, the isolates, L-168 and L-169 oxidized gallic and guaiacol but
did not oxidize tannic acid whereas L-149 oxidized gallic and tannic acid but could not oxidize guaiacol. Among four isolates (L-155, L-160, L-168 and L-169) that showed more than 90% decolorization, only two were positive for tannic acid.

Furthermore, all isolates showing good response to gallic acid and / or tannic acid were not efficient in RBBR decolorization where as all showing good response to guaiacol were found efficient in RBBR decolorization. It clearly indicates that among the three indicators guaiacol seems to be more applicable during screening of LDEs. But surprisingly, L-149 even though is negative for guaiacol oxidation, showed 80% RBBR decolorization.

The observations in this study are parallel with those reported by Kiiskinen et al. (2004). An excellent guideline for laccase activity screening based on comparison of the reactions with different indicators is suggested by them. Further, they reported excellent correlation of presence of laccase activity with polymeric dyes (RBBR and Poly R-478) as well as with guaiacol while no such correlation was found with tannic acid. Moreover, tannic acid seemed to be less specific, as 10 strains positive with other indicators failed to oxidize tannic acid.

Although the ability of oxidation of gallic acid, tannic acid and guaiacol varied amongst the 34 isolates, all exhibited lignin dephenolization ability. Since lignin dephenolization ability clearly indicates presence of LDEs, the variation in response to oxidation of gallic acid, tannic acid, guaiacol as well as RBBR decolorization was due to the difference in substrate specificity of the enzymes. Nevertheless, one of the primary goals of screening tests is to establish a correlation of presence of LDEs with the oxidation ability as well as with dye
decolorizing ability. Overall, it may, therefore, be concluded that during screening of LDEs, if the purpose is collection of isolates with ability to produce LDEs, use of any single indicator is applicable. However, if the purpose is to screen a single efficient isolates with potential for biotechnological application, then a combination of traditional and recent strategies is more useful.

**D’Souza et al. (2006)** studied decolorization of RBBR (0.04%) by incubating the culture supernatant (without the fungal biomass) containing laccase enzyme. They reported 67% and 46% decolorization within 6 and 12 h incubation respectively. RBBR decolorization to about 57.6% within 1 h by the crude enzyme from *Lentinus polychrous* was reported by **Sarnthima et al. (2009)**. However, in the present study, 98% decolorization of RBBR (0.05%) was achieved within 5 day of incubation of culture of the isolate L-168. The same percentage of decolorization was obtained within 1 h by incubating the culture supernatant (without the fungal biomass) containing laccase enzyme in our isolate.

The selection of fungi with high growth rates is one of the desirable characteristics in the application of bioremediation processes since this factor can make the strains more competitive with the native biota (**Machado et al. 2005**). The time taken (5 day) for the four strains, L-168, L-169, L-155 and L-160, to decolorize the dye RBBR compares favorably with reports on other white-rot fungi which required periods of between 7 and 20 day to achieve 90% decolourisation in a diverse range of synthetic dyes (**Toh et al. 2003; Kirby et al. 2000**). It also compares favorably against Ganoderma strains that typically required 14 day to achieve substantial dye decolourisation (**De Souza Silva et al. 2005**). These results
showed the biotechnological potential of ligninolytic enzymes from our isolates in bioremediation.

All ligninases (LiP, MnP and Laccase) are reported to be involved in guaiacol oxidation as well as RBBR decolorization (Ryu et al. 2003; Wesenberg et al. 2003; Kiiskinen et al. 2004). Therefore, in the present study, to know the type of ligninases produced by the isolates, quantitative estimation of ligninolytic enzymes was carried out by cultivation of isolates in liquid medium. The only ligninolytic enzyme detected in four isolates (L-155, L-160, L-168 and L-169) was laccase. The presence of laccase as a sole ligninolytic enzyme was also confirmed by oxidation of ABTS and α-napthol.

The presence of laccase as the sole lignin-degrading enzyme is reported in Pycnoporus cinnabarinus (Eggert et al. 1996), Phlebia tremmellosa (Robinson et al. 2001) and Pleurotus sojarcaju (Chagas and Durrant 2001). The isolate L-168 expressed a high titer (507.92 U/L) of laccase. To compare this value with data from the literature, the laccase activity of the isolated strain exceeds that of Pleurotus ostreatus (ranging 1.6–5.9 of U/L), which has been successfully used in bioremediation applications under various culture conditions (Kaal et al. 1995). Hou et al. (2003) reported production of laccase up to 0.4 U/L by a strain of Pl. ostreatus. Although the higher laccase titer is reported by some investigators, the laccase titer obtained with L-168 is significant since no specific laccase inducer (e.g. 2,5 xylidine) was used in the medium. Therefore, further increase in the titer of laccase by L-168 could be obtained by induction studies. Also, 98% decolorization
within 5 day with the laccase titer of 507 U/L, indicates the high catalytic efficiency of the enzyme.

Species identification based primarily on morphological characteristics or breeding similarities is time-consuming and an experience-based science (Taylor et al. 2000; Jordaan 2005) and therefore, not readily available to many researchers. Currently, use of molecular biology techniques based on DNA sequence analysis is the preferred method of species identification and study of the phylogenetic relationships within various organisms. More specifically, the non-coding internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal DNA are the most frequently employed analytical tools (Jordaan 2005; Coelho-Moreira et al. 2013). In the present study, therefore, molecular analysis was used to identify the isolated fungal strains.
2.6 Conclusion

In an attempt to find potential ligninolytic enzyme producing fungus, various rotten wood samples were collected from the relatively neglected biodiversity of Jalgaon district, Maharashtra (India). The application of various advanced geo-informatics tools was found to be effective in sampling. A combination of traditional and recent screening strategies resulted in isolation of potent laccase producer. The present study reports two new fungal isolates with bioremediation potential. Furthermore, molecular and bioinformatics techniques helped to establish the phylogenetic relationship and identified as *Basidiomycota* sp. L-168 and *Basidiomycota* sp. L-169. The Genbank Accession number of both isolate is JF 412304 and JF 412303, respectively. The laccase was the only ligninolytic enzyme detected in both the isolates.

From industrial point of view, to enhance enzyme productivity, there is need to optimize production of laccase from the above said organism i.e. *Basidiomycota* sp. L-168 and for simplicity it is abbreviated as BspL-168 in the following chapters. In next chapter, therefore, production and optimization of laccase from BspL-168 is described with respect to nutrients, inducer, pH, temperature etc.