STUDIES ON THE PRODUCTION, PURIFICATION AND CHARACTERIZATION OF LACCASES BY THE NOVEL ISOLATES OF BASIDIOMYCETES

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DECLARATION

I, **Harisha Poojary**, hereby declare that the Research Thesis entitled **"Studies on the production, purification and characterization of laccases by the novel isolates of Basidiomycetes"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy** in **Chemical Engineering** is a bonafide report of the research work carried out by me. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

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This is to certify that the Research Thesis entitled **"Studies on the production, purification and characterization of laccases by the novel isolates of Basidiomycetes"** submitted by **Harisha Poojary** (Register Number: **CH08F02**) as the record of the research work carried out by him, is accepted as the Research Thesis submission in partial fulfillment of the requirements for the award of degree of **Doctor of Philosophy**.

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ABSTRACT

Lignin degradation is in a central position in the earth's carbon cycle. The most renewable carbon present in the nature is either present in lignin or in compounds, which are protected by lignin from enzymatic degradation (cellulose and hemicellulose). Lignin biodegradation is responsible for much of the natural destruction of wood in use, and it may have an important role in plant pathogenesis. Lignin degrading enzymes are becoming an effective tool in industrial processes, from crude applications such as bioremediation to fine processes such as biotransformation processes. Recently, there has been a growing interest in studying the lignin modifying enzymes of a wide array of white-rot fungi, not only from the standpoint of comparative biology but also with the expectation of finding better lignin-degrading system for use in various biotechnological applications. They have received considerable attention for industrial application due to both their broad substrate range and their ability to degrade the most recalcitrant natural polymer, lignin. They may provide environmentally friendly technologies for the pulp and paper industry and for the treatment of many xenobiotic compounds, stains, and dyes. This group of enzymes was therefore taken as target enzyme for the present study.

Lignin biodegradation was considered an unusual biological process involving extracellular oxidations. The organisms principally responsible for lignocellulose degradation are aerobic filamentous fungi, and the most rapid degraders in this group are basidiomycetes. These fungi are unique in their ability to degrade most components of wood due to their capability to synthesize the relevant hydrolytic and oxidative extracellular enzymes. Wood-rotting basidiomycetous fungi are usually divided into white-rot, brown-rot and litter decomposing fungi. White rot fungi and related litter degrading fungi produce various isoforms of extracellular Lignin Modifying Enzymes (LMEs): lignin peroxidase, manganese dependent peroxidase and laccase. Some wood degrading fungi contain all three classes of ligninolytic enzymes, while other contains only one or two of these enzymes.

Present research is undertaken with the aim of isolating and screening novel autochthonal fungal strains for ligninolytic enzymes production. The study also deals with the optimization and formulation of an inexpensive medium for acquiring high titre of the enzyme by submerged fermentation process. Latter use of cheap agro-industrial substrates for enzyme production by solid state fermentation is carried out. Further, purification of the enzyme is carried out by using simple and economical method such as three phase partitioning (TPP). The purified enzyme is characterized with respect to its activity and stability at various pH and temperature ranges, molecular weights determinations, kinetic constants and effects of compounds on the activity of the enzyme.

In the present study, forty five basidiomycetous fungi were isolated from a village forest of Mangalore, Karnataka, India. These fungi were screened using solid agar medium containing indicator compounds for ligninolytic enzymes production. For LiP's and MnP's activities, screening was performed based on the decolorization ability of the dye azure-B. For screening laccase activity, guaiacol oxidation was used. Further confirmation and selection of predominant ligninolytic enzymes producers was done by growing the isolates in liquid cultures in defined medium for 14 days. Among forty five isolates, 12 strains exhibited dye decolorization and 24 strains showed the oxidation of guaiacol on solid agar plates within 20 days of incubation period. However, 5 strains were found to be laccase hyperproducers. Screened strains were further cultivated in specific media for the production of laccase and peroxidases (LiPs and MnPs). Only 6 out of 17 strains were able to produce significant amounts of laccase, MnP and LiP. Moreover, two isolates such as *Peniophora sp.* hpF-04 and *Phellinus noxius* hpF-17 were obtained as efficient laccase producers. Hence, further work is focused on the production of laccase by these two novel isolates. The effect of different carbon, nitrogen and inducer sources on laccase production by these fungi was carried out to select suitable carbon, nitrogen and inducer sources, respectively.

Six different carbon sources such as glucose, carboxy methyl cellulose (CMC), sucrose, cellobiose, xylan, and starch were selected to study the effect of carbon on

laccase production. Similarly, seven different nitrogen sources such as KNO_3, NH_4Cl, NH_4NO_3, ammonium tartarate, aspargine, urea and yeast extract were used for studying the effect of nitrogen on laccase production. Compounds, such as copper sulphate (0.5, 1.0 and 2.0 g/l) veratryl alcohol (0.1, 0.3, and 1 ml/l), Tween 80 (0.1, 0.5, and 1.0 %), 2,5-xylidine (0.5, 1.0 and 2 mM) and guaiacol (0.1, 0.5 and 1.0 %) were selected to study their inducing effect on laccase production by both the isolates.

The time course production of laccase in basal medium showed similar enzyme production curves. Both fungi showed their maximum laccase activities and biomasses within 6-8 days of incubation period. *Peniophora* sp. hpF-04 and *P.noxius* hpF-17 respectively produced laccase of 1052±54 U/l and 545.5±14 U/l during 6 days of cultivations. Temperature of 30°C was observed to be optimum incubation temperature for laccase production in *Peniophora sp*. hpF-04 and *Phellinus noxius* hpF-17. Experiments on effects of different carbon and nitrogen proved that the presence of dissimilar carbon and nitrogen sources affected laccase production in both the fungi. Fungus *Peniophora sp.* hpF-04 showed maximum laccase activity in the medium containing sucrose (1249.0±12 U/l). Presence of CMC as a carbon source showed laccase activity of 1060.0±12.5 U/l in this culture. In *Phellinus noxius* hpF-17, maximum laccase activity (557±28 U/l) was found in the presence glucose supplemented media. *Peniophora sp.* hpF-04 showed maximum laccase production (1082.1±13.4 U/l) in case of ammonium chloride supplemented medium, whereas, *Phellinus noxius* hpF-17 produced maximum laccase in the presence of ammonium tartarate (557±23.1 U/l). Inducers like veratryl alcohol, guaiacol and 2,5-xylidine suppressed the production of laccase in both the fungi. On the other hand, increase in copper in the media, enhanced laccase in both *Peniophora sp* .hpF-04 and *Phellinus noxius* hpF-17.

The optimization of medium components for enhancement of laccase production was carried out by statistical methods by using Placket-Burman design (PBD) and central composite design of response surface methodology (CCD-RSM). Initial screening of medium components was performed using a PBD and the variables showing significant

effects on laccase production were identified. The interactions among the screened variables were studied by CCD. Variables such as CMC, ammonium chloride and copper sulphate were found to influence the laccase production in *Peniophora sp.* hpF-04. Statistical optimization process resulted in optimum concentration of variables for maximized laccase production. The values obtained were, CMC (15 g/l), ammonium chloride (1.35 g/l) and copper sulphate (807 µM) with laccase yield of 1890±12 U/l. There was an approximate of 1.81 fold improvement in laccase production over the previous yield with un-optimized medium was achieved. In case of *Phellinus noxius* hpF-17, variables such as glucose, ammonium tartarate and Tween 80 were found to influence the laccase production significantly. Optimum values of tested variables for maximum laccase production are glucose (20 g/l), ammonium tartarate (2.25 g/l) and Tween 80 (2.08 ml/l) were obtained by CCD-RSM. By using this optimal fermentation medium, the laccase yield was increased up to 780±7.9 U/l, an approximate 1.43 fold improvement as compared to the previous yield with un-optimized medium.

Present investigation was also aimed to exploit locally available, inexpensive agro-industrial wastes as a substrate for laccase production under SSF. Seven substrates *viz.*, sugar cane bagasse (SCB), wheat bran (WB), rice bran (RB), corn stover (CS), saw dust (SD), grass powder (GP) and Jatropa seed cake (JSC) were evaluated. Solid state fermentation was carried out with and without nutrient supplements. Respective optimized laccase media of *Peniophora sp*. hpF-04 and *Phellinus noxius* hpF-17 were used as nutrient supplements. In *Peniophora sp.* hpF-04, no laccase activities (very negligible in SCB and RB) were found during 5^{th} day of all the substrates, when the nutrient supplements were used in SSF. Among all the substrates, the fungus showed high level of laccase activity in case of Jatropa seed cake. Contrastingly, with the presence of nutrient supplements, detectable amounts of laccase activities were found in all the substrates during 5^{th} day of incubation. The similar results were obtained for SSF using *Phellinus noxius* hpF-17 also. The present study revealed that, the presence of specific nutrients during SSF altered the laccase production in both the fungi, either by enhancing or by reducing the laccase synthesis. Among the seven agro-industrial wastes

employed for SSF, only JSC was found more potential in producing laccase in both fungi, with and without nutrient supplements. Hence, a detailed study of SSF with JSC was carried out to study time course production profile, effect of pH, effect of moisture on laccase production. During this, *Phellinus noxius* hpF-17 produced laccase in higher titre than *Peniophora sp.* hpF-04. It produced laccase of 79.92±2.3 U/g of the substrate and *Peniophora sp*. hpF-04 produced low level of laccase of 14.32±2.4 U/g during 10^{th} day of incubation. *Phellinus noxius* hpF-17, produced maximum laccase at 70% moisture level and at pH.5.0, where as fungus *Peniophora sp*. hpF-04 showed maximum laccase at 80% of moisture level and at pH range between 5.0-6.0.

The purification was achieved by simple method with a combination of ultrafiltration and three phase partitioning (TPP). Ultrafiltration by tangential flow filtration (TFF) was used to concentrate the culture supernatant. Later the laccase from the concentrated crude sample was separated and purified by three phase partitioning (TPP). The purity of the separated laccase was analyzed by RP-HPLC method. The purified enzyme was further characterized with respect to its activity and stability at various pH and temperature ranges. SDS-PAGE profiling and determination of enzyme's K_m and V_{max} parameters were also carried out. The effect of metal ions and certain compounds such as EDTA, TEMED, SDS, β-mercaptoethanol and organic acids was investigated.

A one-step TPP was employed for the purification of laccase using organic solvent *tert*-butanol. Further, the effect of different TPP conditions such as salt saturation levels (w/v), aqueous phase to *tert*-butanol ratio (v/v), pH and temperature to optimize laccase separation was carried out. Concentration by ultrafiltration achieved 94.41% yield of laccase with a 2.28-fold purification in *Peniophora sp.* hpF-04. By single step TPP process, crude enzyme solution of pH 4.0 saturated to 55% (w/v) ammonium sulphate with a crude extract to *tert*-butanol ratio of 1:1.5(v/v) at 45^oC resulted in 96.4% recovery of laccase with 2.61fold purification of laccase produced by this fungus. On characterization, HPLC study resulted in a single peak in TPP purified sample which

revealed the purity of the separation. Optimum pH and temperature for maximum activity were determined as pH 4.0 and temperature range of 45-50^{o}C. This laccase was found to be stable at 50^{o}C and 60^{o}C for more than 15 h. The kinetic constants (V_{max} and K_m) measured using Lineweaver–Burk double reciprocal (1/ [v] vs 1/[S]) plots obtained were, 30.3 mM and 0.06 mM/min for ABTS, 8.77 mM and 0.27 mM/min for 2, 4-dimethoxy phenol and 1.49 mM and 0.28 mM/min for guaiacol, respectively. SDS-PAGE profiling revealed a single band of laccase with molecular weight of 67KDa. The laccase contained 3.12±0.2 Cu with an atypical spectrum lacking peak around 600 nm. According to these features, we classified *Peniophora sp.* hpF-04 with so-called yellow laccases, recently found in basidiomycetes. This laccase enzyme was completely inhibited by β-mercaptoethanol, oxalic acid and almost by sodium azide.

The laccase produced from *Phellinus noxius* hpF-17 also separated and purified by a combination of ultrafiltration and TPP. However, one step combination method of TPP resulted in poor laccase yield and purity in this fungal laccase. Hence, optimization of TPP variables had to be carried out for effective separation of laccase from the crude extract. The optimization was achieved by using Box-Behnken design (BBD) of statistical experiments. BBD was employed with experimental factors of ammonium sulphate saturation; 20-80% (w/v), ratio of crude extract to *tert*-butanol; 1.0:0.5 – 1.0:2.5 (v/v) and temperature; 20–60°C to optimize TPP process parameters. The second-order regression equation provided the levels of laccase yield and purity as a function of $(NH_4)_2SO_4$ saturation, ratio of crude extract to *tert*-butanol and temperature was generated. Ultrafiltration by TFF resulted in 64% laccase yield with purity fold of 1.58 during *Phellinus noxius* hpF-17 laccase concentration. The combination of 57% (w/v) of ammonium sulfate with 1:1.7 (v/v) ratio of crude extract to *tert*-butanol at pH 5.0 and incubation temperature of 44^{o}C obtained through optimization experiments was found to be suitable combination for maximum recovery of *Phellinus noxius* hpF-17 laccase. When compared to commercial purification procedure, which generally includes salt precipitation and chromatography techniques, *etc*., the extraction of laccase by this process was proved to be much easier and eco-friendly.

Isolated *Phellinus noxius* hpF-17 laccase did not show any blue coloration which was further confirmed by the lack of absorption peak at 610 nm under spectral scanning. This laccase found to be bigger in size than *Peniophora sp.* hpF-04 laccase with molecular weight of 75KDa as it determined by SDS-PAGE analysis. It showed high stability at acidic pH and elevated temperatures. The optimum pH of purified laccase produced by *Phellinus noxius* hpF-17 was found to be 3.0 for ABTS oxidation. When purified laccase was incubated for 20 h at room temperature at pH 3.0, it remained quiet stable. The laccase was found to be highly thermostable with broad temperature range of 65-85^{o}C with an optimum temperature being 75^{o}C. At 75^{o}C, the enzyme was very stable even after 72 h of incubation. But at 85^{o}C, the enzyme retained only 4% of its original activity after 24 h of incubation. The catalytic properties of this laccase were similar to the corresponding enzymes of other related group. Like *Peniophora sp.* hpF-04 laccase, laccase form this fungus also showed high affinity towards ABTS substrates. Compounds like β-mercaptoethanol, TEMED and sodium azide were very effective as inhibitor of *Phellinus noxius* hpF-04 laccase. However, In case of CuCl, 8-fold increase in laccase activity was observed.

Overall, the laccases produced by both isolates were found to be acid stable and thermostable, unlike most of the other fungal enzymes listed in the literature. The longer stability and higher temperature and acidic pH, lack of inhibition makes these enzyme suitable for industrial purpose/or bioremediation practices dealing with harsh process. However, further studies of structural and catalytic properties of these enzymes would be necessary for elucidation of novelty of these laccases.

Keywords: Ligninolytic enzymes; basidiomycetes; enzyme activity; statistical optimization; solid state fermentation; three phase partitioning; characterization.

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NOMENCLATURE

Description	Symbol
Absorbance unit	AU
Atmosphere	atm
Base pairs	bp
Centimeter	cm
Daltons	Da
Degree Centigrade	^{o}C
Diameter	d
Difference in absorbance	ΔA
Extinction coefficient	ε
Gram per litre	g/l
Grams	g
Gravitational force	g
Greater than	<
Hour	h
International unit	IU
Lesser than	>
Light path length	d
Litre	l
Logarithm	Log
Maximum specific growth rate	μ_{max}
Meter	m

Micro grams	µg
Micro litre	ml
Micromole per minute	µM/min
Milligrams	mg
Millimeter	mm
Millimolar	mM
Millimole per minute	mM/min
Minute	min
Moles	M
Nanometer	nm
Not determined	ND
Not reported	n.r.
Optical density	OD
Parts per billion	ppb
Parts per million	ppm
Per minute	min^{-1}
Percentage	%
Revolutions per minute	rpm
Specific growth rate	U
Substrate concentration	S
Substrate utilization constant	Ks
Time	t
Units per grams	U/g

Units per litre	U/l
Units per milligrams	U/mg
Units per milliliter	U/ml
Volume	v

ABBREVIATIONS

AAS	atomic absorption spectrophotometer
ANOVA	analysis of variances
ABTS	2,2´-azinobis(3-ethylbenzthiazoline-6-sulphonate)
BBD	box behnken design
BSA	bovine serum albumin
CCD	central composite design
Cu	copper
2,6-DMP	2,6-dimethoxyphenol
FPLC	fast protein liquid chromatography
GP	grass powder
IEF	isoelectric focusing
JSC	Jatropa seed cake
Lac	laccase
LBM	ligninolytic basal medium
NMWCO	normal molecular weight cut off
LiP	lignin peroxidase
LME's	lignin modifying enzymes
LSM	laccase screening medium
MEA	malt extract agar
MnP	manganese peroxidase
MW	molecular weight
PBD	placket burman design
pI	isoelectric point
RB	rice bran
RP-HPLC	reverse phase high performance liquid chromatography
RSM	response surface methodology

SCB	sugar cane bagasse
SD	saw dust
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SmF	submerged state fermentation
SSF	solid state fermentation
TFF	tangential flow filtration
TPP	three phase partition
Tween-80	polyoxyethylene (20) sorbitan monooleate
Uf	ultra filtration
VP	versatile peroxidase
WB	wheat bran
WP	wood powder
WRF	white rot fungi
YE	yeast extract

Chapter 1

GENERAL INTRODUCTION

Lignin is a complex natural polymer present in wood along with cellulose and hemicellulose. It is second only to cellulose in abundance on earth. It is the major component of the middle lamella region of wood. Most of the lignin is found within the secondary wall, where it is covalently bonded to hemicelluloses. The cellulose fibrils are embedded in the lignin-hemicellulose matrix. It is one of nature's most chemically heterogeneous and complex major polymers (Sanchez 2009). Because of its chemical and physical properties as well as its location in wood, lignin plays a central role in determining the properties of wood and its industrial processing. Degradation products of lignin are a major source of pollution in pulping and bleaching operations. The potentials of lignin degrading microbes and that of enzymes which degrade and modify lignin has been recognized by researchers since 1970's (Kirk et al. 1975; 1978). Utilization of lignin-degrading organisms and their enzymes in many potential applications have become more attractive, because they may provide environmentally friendly technologies for the pulp and paper industry and for the treatment of many xenobiotic compounds, stains, and dyes.

1.1 Lignin Biodegradation

Lignin degradation is central to earth's carbon cycle, because most renewable carbon is either in lignin or in compounds protected by lignin from enzymatic degradation (cellulose and hemicellulose) (Kirk 1983). Lignin biodegradation is also responsible for much of the natural destruction of wood in use, and it may have an important role in plant pathogenesis. Despite its significance, lignin biodegradation has only slowly been understood chemically and biochemically. One of the main reasons for that was the poor knowledge of the chemical structure of lignin until the late 1960s when it became better known (Kirk 1971; 1983; Adler 1977; Eriksson et al. 1990). Lignin biodegradation was considered an unusual biological process involving extracellular oxidations and reductions.

1.2 Historical Outline

Prior to the 1920s, little research was conducted on lignin biodegradation (Kirk 1983). Some findings, summarized in the 1930s (Kirk 1983), are still today valid:

1) Lignin is among the plant cell wall polymers the most resistant to biological degradation, although it is degraded,

2) White-rot fungi degrade lignin in wood, and

3) Completely selective removal of lignin (without concomitant removal of wood carbohydrates) had not been observed.

Waksman et al. (1939) had studied lignin degradation, e.g., in compost and soil environment (reviewed by Tuomela et al. 2000). Gottlieb and Pelczar (1951) in their review reported that the white-rot fungus *Polyporus* (syn. *Trametes*) *versicolor* used Brauns' native lignin as the growth substrate. Although the lignin structure is unaltered due to the mild procedure, the preparation has a relatively low molecular weight. This finding, indicating that lignin could be used as a sole carbon and energy source for white-rot fungi, has not been verified. In the1950s in addition to white-rot fungi, other groups of fungi were found to degrade lignin, at least partially, namely basidiomycetous litter-decomposing and brown-rot fungi as well as soft-rot fungi (reviewed by Kirk 1971; 1983).

In the late 1970s and in the beginning of the 1980s many important findings in the physiology of lignin degradation by *P. chrysosporium* were made (Kirk et al.1975; 1978). The most important discoveries can be listed as follows:

1) The effect of nutrient nitrogen, showing that low nitrogen was required for lignin degradation, and indicating that the mineralization of lignin occurred during secondary metabolism,

2) The effect of atmosphere, 100% oxygen giving the highest mineralization, thus demonstrating that lignin degradation is oxidative,

3) The detrimental effect of agitation in lignin mineralization, and

4) The concomitant production of veratryl alcohol during lignin degradation.

In the 1990s, in addition to detailed studies on catalytic and enzymatic properties of the lignin-modifying peroxidases as well as their molecular biology, major lines of research have dealt with the potential applications of white-rot fungi and their enzymes in biopulping (biomechanical pulping) and pulp bleaching (Eriksson et al. 1990).

1.3 Lignin-degrading enzymes

A set of enzymes that can extensively degrade lignin and reproduce the effect of a white-rot fungus has not yet been identified. However, three extracellular oxidative enzymes are commonly found in ligninolytic cultures of white-rot fungi. It is clear that different combinations of the known enzymes are produced by various lignin-degrading fungi, suggesting that there is more than one successful strategy for lignin biodegradation (Hatakka 1994; 2001).

1.3.1 Laccases (lac)

Laccases (Lac, benzenediol: oxygen oxidoreductase (EC 1.10.3.2) belongs to multicopper oxidase family (Hoegger et al. 2006; Alcalde 2007). These are copper containing enzymes, sometimes also referred as blue copper oxidases. Laccases catalyze the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water (Yaropolov et al. 1994). Most laccases were reported from fungal cultures and most biotechnologically useful laccases are also of fungal origin (Kalmiş et al. 2008). Probably the first report on the presence of laccase in fungi was from Laborde in 1897 (Mayer and Harel 1979). Over 60 fungal strains belonging to the phyla Ascomycota, Zygomycota and especially Basidiomycota have shown laccase activities (Kiiskinen et al. 2004; Baldrian 2006).

1.3.2 Lignin peroxidases (LiPs)

LiPs (EC 1.11.1.14) also belong to the family of oxidoreductases (Higuchi 2004; Martínez et al. 2005; Hammel and Cullen 2008). LiPs were first described in the basidiomycete *Phanerochaete chrysosporium* Burdsall (order Corticiales) in 1983 (Glenn

et al. 1983; Tien and Kirk 1988). This enzyme has been documented for several species of white-rot basidiomycetes (Buswell et al. 1987; Kirk and Farrell 1987; Pointing et al. 2005) and in actinomycetes (Perie and Gold 1991; Perie et al. 1996; Niladevi and Prema 2005). Unlike laccase, LiP is an extracellular hemeprotein, dependent of H_2O_2, with an unusually high redox potential and low optimum pH (Gold and Alic 1993; Haglund 1999; Piontek et al. 2001; Erden et al. 2009). LiP is capable of oxidizing a variety of reducing substrates including polymeric substrates (Oyadomari et al. 2003). Due to their high redox potentials and their enlarged substrate range, LiPs have great potential for application in various industrial processes (Erden et al. 2009).

LiP shows little substrate specificity, reacting with a wide variety of lignin model compounds and even unrelated molecules (Barr and Aust 1994). It has the distinction of being able to oxidize methoxylated aromatic rings without a free phenolic group, generating cation radicals that can react further by a variety of pathways, including ring opening, demethylation, and phenol dimerization (Haglund 1999). LiP in contrast with laccases does not require mediators to degrade high redox-potential compounds but it needs hydrogen peroxide to initiate the catalysis.

1.3.3 Manganese peroxidase (MnPs)

Manganese peroxidases (EC 1.11.1.13) belong to the family of oxidoreductases (Higuchi 2004; Martinez et al. 2005; Hammel and Cullen 2008). Following the discovery of LiP in *Phanerochaete chrysosporium*, Manganese peroxidase secreted from the same fungus was found to be another lignin degrading enzyme (Glenn and Gold 1985; Paszczynski et al. 1985). Subsequent investigations have shown that MnP is distributed in almost all white-rot fungi (Hofrichter 2002).

Manganese peroxidases (MnP) seem to be more widespread among white rot fungi than lignin peroxidases (Hammel and Cullen 2008). It oxidizes Mn^{2+} to $Mn^{3+,}$ which in turn oxidizes phenolic structures to phenoxyl radicals (Hofrichter 2002). The product Mn^{3+} is highly reactive and forms a complex with chelating organic acid, as

oxalate or malate, which is produced by the fungus (Kishi et al. 1994; Galkin et al. 1998; Makela et al. 2002). The redox potential of the Mn peroxidase system is lower than that of lignin peroxidase. On the other hand, studies indicates that contrary to LiP, MnP may oxidize Mn(II) without H_2O_2 with decomposition of acids and concomitant production of peroxyl radicals that may affect lignin structure (Hofrichter et al. 1999).

1.3.4 Versatile Peroxidase (VPs)

Versatile peroxidase (EC 1.11.1.16) oxidizes Mn^{2+} as MnP does and also has high redox potential in the presence of aromatic compounds, as LiP does. The interest in VP has increased during the last years, both as a model enzyme and as a source of industrial/environmental biocatalysts (Martinez et al. 2005; Martinez et al. 2009; Ruiz-Duenas et al. 2009).

1.4 Biological functions of ligninolytic enzymes

Lignin degrading enzymes have found their uses not only in the degradation of many compounds but also have different biological functions in the microorganisms. Laccases generally provides spore resistance, help in rhizomorph formation, pathogenesis, fruiting body formation in higher fungi and synthesis of pigments. The main role of lignin peroxidase is to furnish various defense mechanisms against pathogens in fungai. However, specific role of manganese peroxidase is unknown but has some inter-specific fungal interactions. Some of the biological functions of these enzymes in the fungal systems are given in Table 1.1.

Table 1.1 Biological functions of ligninolytic enzymes

Enzyme	Application	Reference
Laccase	Spore resistance, rhizomorph formation, pathogenesis, fruit bodies formation, pigment synthesis, lignin degradation	Mayer and Staples 2002 Claus 2004 Minussi et al. 2007
Lignin peroxidase	Biodegradation of lignin, defence of fungi against pathogens	Score et al. 1997 Piontek et al. 2001 Trejo-Hernandez et al. 2001
Manganese peroxidase	Degradation of lignin, inter-specific fungal interactions	Score et al. 1997 Trejo-Hernandez et al. 2001

1.5 Fungal degradation of lignin

The only microorganisms known to extensively degrade lignin are fungi (Kirk and Farrell 1987). Lignin degrading fungi are classified into three major categories based on the type of wood decay caused by these organisms: white-rot fungi, brown-rot fungi and soft-rot fungi (Table 1.2). Among these three groups, white-rot fungi are the most effective lignin degraders and have been the most extensively studied so far. In this group the most commonly used model organism in lignin biodegradation studies is strains of *Phanerochaete chrysosporium* (Boominathan and Reddy 1992).

Table 1.2 Lignin degrading fungi, their actions and distributions (Ward et al. 2004)

Organism	Example	Action	Distribution
White rot fungi	*Phanerochaete sp, Pleurotus sp., Bjerkandera sp., Trametes sp. and Phlebia sp*.	Mineralize lignin to CO_2 and H_2O; some preferentially remove lignin whereas others degrade bith lignin and cellulose.	Predominantly degrade wood from deciduous trees (angiosperms), containing hardwood

Brown rot fungi	*S. lacrymans, P. betulinus, G. trabeum and P. placenta*	Modify lignin by demethylation, limited aromatic hydroxylation, and ring cleavage	Preference for coniferous substrates (gymnosperms), which are softwoods
Soft rot fungi	*Chaetomium sp., Ceratocystis sp. and Phialophora sp.*	Some lignin modification	Active generally in wet environments as well as in plant litter; attack both hardwood and softwood

1.6 Applications of lignin modifying enzymes

1.6.1 Food Industry

Laccases can be applied to certain processes that enhance or modify the colour appearance of food or beverage. It can be used for the elimination of undesirable phenolics responsible for the browning, haze formation and turbidity in clear fruit juice, beer and wine (Rodriguez and Toca 2006). Laccase is also employed for ascorbic acid determination, sugar beet pectin gelation, baking and in the treatment of olive mill wastewater (Ghindilis 2000; Minussi et al. 2002; Rodríguez and Toca 2006; Selinheimo et al. 2006; Minussi et al. 2007).

Lignin peroxidase (LiP) and manganese peroxidase (MnP) have potential to produce natural aromatic flavors (Lesage-Meessen et al. 1996; Lomascolo et al. 1999; Zorn et al. 2003; Barbosa et al. 2008).

1.6.2 Pulp and paper industry

Laccases are able to depolymerize lignin and delignify wood pulps, kraft pulp fibers and chlorine-free in the biopulpation process (Bourbonnais et al. 1997; Lund and Ragauskas 2001; Chandra and Ragauskas 2002; Camarero et al. 2004; Rodriguez and

Toca 2006; Vikineswary et al. 2006). One of the most studied applications in the industry is the laccases-mediator bleaching of kraft pulp, the efficiency of which has been proven in mill-scale trials (Strebotnik and Hammel 2000). This ability could be used in the future to attach chemically versatile compounds in the fiber surfaces and let recycled pulp for new use (Rodriguez and Toca 2006; Mocchiutti et al. 2005; Saparrat et al. 2008; Widsten and Kandelbauer 2008).

Lignin peroxidases (LiP) compared to laccases, are the biocatalysts of choice for bleaching (Bajpai 2004; Sigoillot et al. 2005). LiP and MnP were reported to be effective in decolourizing kraft pulp mill effluents (Ferrer et al. 1991; Michel et al. 1991; Moreira et al. 2003). In laboratory scale, the consumption of refining energy in mechanical pulping was reduced with MnP pretreatment with a slight improvement in pulp properties (Kurek et al. 2001; Wasenberg et al. 2003; Maijala et al. 2007).

1.6.3 Textile industry

Laccases-mediator system finds potential application in enzymatic modification of dye bleaching in the textile and dyes industries (Abadulla et al. 2000; Kunamneni et al. 2008). Most currently existing processes to treat dye wastewater are ineffective and not economical (Mc Kay 1979; Cooper 1993; Riu et al. 1998; Rodriguez and Toca 2006). Therefore, the development of processes based on laccase utilization seems to be an attractive solution due their potential in degrading dyes of diverse chemical structures including synthetic dyes currently employed in the industry (Wong and Yu 1999; Abadulla et al. 2000; Blanquez et al. 2004; Hou et al. 2004; Rodriguez et al. 2005; Rodriguez and Toca 2006; Kunamneni et al. 2008).

The degradation performance of lignin peroxidases (LiP) was evaluated by decolourizing different synthetic dyes too (Cripps et al. 1990; Pointing 2001; Robles-Hernandez et al. 2008; Gomes et al. 2009). MnP can biodegrade dyes as well as decolourize various types of synthetic dyes in aqueous cultures and packed-bed bioreactors (Kasinath et al. 2003; Shin 2004; Champagne and Ramsay 2005).

1.6.4 Bioremediation

Laccases are involved in green biodegradation due its catalytic properties. The xenobiotic compound is a major source of contamination in soil and laccase degrades it more efficiently (Rodriguez and Toca 2006). Moreover, polycyclic aromatic hydrocarbons (PAHs), which arise from natural oil deposits and utilization of fossil fuels, are also degraded by laccases (Pointing 2001; Anastasi et al. 2009). Many PAHs have been found to exhibit cytotoxic, mutagenic and carcinogenic properties that cause serious risk to human health (Bamforth and Singleton 2005).

Lignin peroxidases (LiP) present a non-specific biocatalyst mechanism. Due to their ability to degrade azo, heterocyclic, reactive and polymeric dyes, it degrades 1,1,1-trichloro-2,2-bis-(4-chlorophenyl) ethane (DDT), 2,4,6-trinitrotoluene (TNT) and polycyclic aromatic hydrocarbons (PAH's) too (Koller et al. 2000; Abraham et al. 2002; Ohtsubo et al. 2004; Robles-Hernandez et al. 2008; Gomes et al. 2009; Wen et al. 2009). LiP from *P. chrysosporium* was one of the first enzymes of basidiomycetes capable for PAH degradation (Bumpus and Aust 1987).

1.6.5 Organic, medical, pharmaceutical, cosmetic and nanotechnology applications

Recently, increasing interest has been focused on the application of laccase as a new biocatalyst in organic synthesis (Milstein et al. 1989; Mayer and Staples 2002). Enzymatic polymerization using laccase has drawn considerable attention since laccase or laccase-mediator system (LMS) are capable of generating straight forwardly polymers that are impossible to produce through conventional chemical synthesis (Akta and Tanyolac 2003). Laccases have been employed for several applications in organic synthesis as the oxidation of functional groups, the coupling of phenols and steroids, medical agents (anesthetics, anti-inflammatory, antibiotics and sedatives), the construction of carbon-nitrogen bonds and in synthesis of complex natural products as well as cosmetic industries (Baminger et al. 2001; Fabbrini et al. 2001; D'Acunzo et al.

2002; Mikolasch et al. 2002; Baiocco et al. 2003; Barilli et al. 2004; Nicotra et al. 2004; Xu 2005; Rodríguez and Toca 2006; Ponzoni et al. 2007; Mikolasch and Schauer 2009).

Laccases also can be applied as biosensors or bioreporters (Bauer et al. 1999; Xu 1999; Durán and Esposito 2000; Ghindilis 2000; D'Souza 200; Kuznetsov et al. 200; Kunamneni et al. 2008; Szamocki et al. 2009). Laccases still could be immobilized on the cathode of biofuel cells that could provide for small transmitter systems (Ghindilis 2000). Laccase based miniature biological fuel cell is of particular interest for many medical applications calling for a power source implanted in a human body (Rodriguez and Toca 2006; Heller 2004).

Lignin peroxidase (LiP) exhibits high bioelectro-catalytic activity at atomic resolution and this property makes available for commercial development of biosensors for polymeric phenol or lignin (Christenson et al. 2004). Manganese peroxidase (MnP) produced by the basidiomycetae *Bjerkandera adusta* was used for acrylamide polymerization (Iwahara et al. 2000). MnP from *P. chrysosporium* can degrade styrene which was considered as water, air and soil pollutant (Soto et al. 1991; Lee et al. 2006). MnP is also a redox enzyme with efficient direct electron transfer (DET) properties with electrodes. Therefore, MnP is used for many applications such the development of biosensors based on DET, effective biofuel cells and selective bioorganic synthesis (Ferapontova et al. 2005).

1.7 Research hypothesis

1.7.1 Scopes and objectives of the present research

Based on the extensive literature survey on isolation, screening, medium formulation, purification and characterization of ligninolytic enzymes, it was found that

- Most of the studies on lignin modifying enzyme production were carried out with fungal cultures procured from the culture collection centers. There are many fungi that need to be explored with respect to their LME's complexes. In addition, there is

little information available with regard to the production of extracellular oxido-reductases by autochthonal fungal strains belonging to different eco-physiological and taxonomic groups. Hence, a study on LME's enzyme complex on isolated strains may add some novel strains to the list of already existing fungi producing LME's.

- It is known from the literature that the LME complex is species dependant. Some fungi produce all the three enzymes of LME's while other produces only one or two of them. Hence, the study may help in characterizing the isolated fungus with respect to its LME's which can further add its application in relevant areas.

- All ligninolytic fungi are able to grow and utilize various natural substrates containing lignocelluloses due to the ability of producing extracellular LME's. The utilization of agro-industrial residues as low cost substrates for the production of these enzymes may reduce the production cost of these enzymes.

- Most of the purification studies on LME's reported in the literature were found be tedious and economically undesirable which includes the methods like high salt saturation followed by dialysis, column chromatography including Sephadex, DEAE-cellulose and ion-exchange chromatography. Hence, there is a need to explore simple and economical methods for the purification of these enzymes.

- The LME's enzyme characteristics such as pH and temperature optima, stabilities, compositions, structural configurations, etc., are dependent on the microorganisms. Hence, the study on enzyme characterization might reveal the novel characteristics of the enzymes produced by new strains.

Therefore, the aim of the present research is to isolate a novel autochthonal fungal strains belonging to family Basidiomycetes, to optimize and formulate an inexpensive medium for obtaining higher titre of the enzyme, to use a simple and economical methods such as three phase partitioning (TPP) for the purification of enzyme and to characterize the purified enzyme with respect to its activity and stability at various pH and

temperature ranges, molecular weights determinations, kinetic constants and effects of compounds on the activity of the enzyme.

1.7.2 Objectives of the present research

Based on the above scopes, the following objectives were undertaken:

i. Isolation, screening and identification of LME's producing indigenous basidiomycetous fungi.

ii. Production of LME's in liquid cultures and selection of most promising producer of a ligninolytic enzyme.

Solid and liquid screening of fungi for LMEs revealed only efficient laccase producers. No strains producing significant LiPs and MnPs were discovered. Hence, the present investigation was continued with the aim of laccase enzyme studies, with the following objectives

iii. Production of laccase by the selected strains in non-optimized media under submerged fermentation.

iv. Influence of pH, temperature, carbon sources, nitrogen sources, and inducers on laccase production.

v. Optimization of medium components for enhanced laccase production by the isolates using statistical tools.

vi. Utilization of low cost substrates such as agro-industrial residues for the production of laccase under Solid State Fermentation (SSF).

vii. Partial purification and characterization of laccases produced by the novel isolates.

Chapter 2
LITERATURE REVIEW

2.1 Isolation and screening of fungi for ligninolytic enzyme production

Discovery of novel ligninolytic enzymes with different substrate specificities and improved stabilities is important for industrial applications. Screening of ligninolytic enzyme producing species and their variants is important for selecting suitable strains for ligninolytic enzyme production. The screening strategy must aim to identify fungal strains and enzymes that will work under industrial conditions which should rely on the use of inexpensive, rapid and sensitive testing methods (Grabner et al. 1997; Monteiro and De Carvalho 1998).

Qualitative assays are powerful tools for screening fungi for ligninolytic enzyme production. Such tests give a positive or negative indication of enzyme production. They are particularly useful in screening of large numbers of fungal isolates for several classes of enzymes, when definitive quantitative data are not required (Pointing 1999).

Microbes that produce lignin modifying enzymes (LMEs) have been screened either on solid media containing coloured indicator compounds that enable the visual detection of ligninase production or with liquid cultivations monitored with enzyme activity measurements. The use of coloured indicators is generally simpler as no sample handling and measurement is required. Several different compounds have been used as indicators for LMEs screening due their non-specificity to most of the compounds (Nishida et al. 1988; De Jong et al. 1992; Barbosa et al. 1996; Szklarz et al. 1989; Pelaez et al. 1995; Luterek et al. 1997).

The various screening procedures employed for the screening of the LME's producing fungi is described by Pointing et al. (1991). Some of the major staining methods are, staining after growth of fungi on lignin agar (lignin agar), tannic acid agar (Bavendamm test), Poly-R agar clearance (Poly-R agar), Azure-B agar clearance (azure B agar test), ABTS agar, well tests for LMEs (syringaldazine well test), p-cresol agar, etc.

Screening of LMEs by using tannic and gallic acid is one of the traditional screening methods (Harkin and Obst 1973). Nowadays, these reagents have been replaced with synthetic phenolic reagents, such as guaiacol and syringaldazine (Nishida et al. 1988, De Jong et al. 1992) or with the polymeric dyes Remazol Brilliant Blue R (RBBR) and Poly R-478 (Barbosa et al. 1996; D'Souza et al. 1999; Raghukumar et al. 1999). RBBR and Poly R-478 can be decolourized by lignin-degrading fungi (Gold et al. 1988; Barbosa et al. 1996). The production of ligninolytic enzymes will be observed as a colourless halo around microbial growth when these compounds are used as indicators. When guaiacol is used as an indicator, a positive reaction is indicated by the formation of a reddish-brown halo zones (Nishida et al. 1988). With tannic and gallic acid, the positive reaction of a dark-brown coloured zone will be seen (Harki and Obst 1973).

Kiiskinen et al. (2004) evaluated the efficiency of different screening methods for LMEs production by fungi. They used 0.04% (w/v) RBBR, 0.04% Poly R-478, 0.01% guaiacol and 0.5% tannic acid. Tannic acid gave relatively weak positive reaction with many samples. However, the ability of most positive microbes to form brown colour on tannic acid weakened during sub-culturing, whereas with other indicators, this effect was not observed. Comparison of the reactions with different indicators showed that the polymeric dyes RBBR and Poly R-478 gave very similar results. From these observations, it can be concluded that either one can be chosen as an indicator in future screening procedures. Reactions with guaiacol also well correlated with the reactions of polymeric dyes. Colour reactions with synthetic dyes and guaiacol are more easily detectable as more laccase-positives were detected and thus, these compounds can reliably be used for laccase activity screening.

Eriksson et al. (1990) used entirely different procedure to screen lignin degrading fungi. The lignin-degrading ability of a microorganism was evaluated by measuring $^{14}CO_2$ evolution from ^{14}C-labeled lignin preparations, such as ^{14}C-ring-labeled dehydrogenation polymerizate (DHP). The measurement of $^{14}CO_2$ evolution is the most sensitive and accurate method for testing ligninolytic activity.

It is also possible to simplify the procedure by using less defined basal growth medium such as peptone plus yeast extract, or malt extract during screening. Supplements of nutrients during solid agar screening not only support the growth of the fungi but also enhance the enzyme excretion. Such basal media have been used in several studies at varying concentrations (Gessner 1980; Egger 1986; Raaska and Itavaara 1990; Rohrmann and Molitoris 1992; Pointing 1992; Raghukumar et al.1994; Vrijmoed and lones 1999; Niku-Paavola 2008).

Some of the major isolation and screening work on ligninolytic enzyme production were summarized in Table 2.1

Table 2.1 Major investigations on isolation and screening of fungi for LMEs

Author	**Investigation**	**Inference**
Raqayyah et al. (2011)	Screened 313 strains of white rot fungi from Malaysian terrestrial habitat.	Found six strains producing all three classes of ligninolytic enzymes, one strain producing two classes and five strains producing only one class of ligninolytic enzymes.
Hamed et al. (2010)	Isolated 88 marine fungi.	Found only seven isolates which showed positive indication of LMEs production on solid medium containing guaiacol.
Sapparat et al. (2008)	Isolated *Mothele subargentea strain 436* from the trunk of the tree, Argentina.	The fungus was able to produce only laccase in SSF using saw dust.
Mtu (2008)	Isolated *Flavodon flavos*, a marine fungus.	Studied its LME's production.

Tortella et al. (2008)	Wood-rotting Basidiomycete were collected from indigenous hardwood forest of South of Chile	Twenty-eight strains were identified and qualitative enzymatic tests for peroxidases, laccase, tyrosinase, xylanase and cellulase production were performed in solid medium. Eleven selected strains were evaluated in liquid medium to quantify their ligninolytic enzyme production and their capacity to grow in solid medium supplemented with 2,4-dichlorophenol(2,4-DCF),2,4,6-trichlorophenol (2,4,6-TCF) and pentachlorophenol(PCP).
Papinutti et al. (2007)	Studied LMEs of WRF *Fomes scleroderma* in solid state fermentation.	Detected only MnP and laccase.
D'Souza et al. (2006)	Isolated 40 fungi from decayed wood pieces of mangrove swamps from Chorao Island in Goa, India.	Only 3 isolates showed positive reaction for laccase activity when grown in the presence of guaiacol.
Dhouib et al. (2005)	Isolated 315 strains of fungi from different Tunisian biotopes	Found 8 cultures producing significant amount of MnP, Lac and LiP in liquid cultures.
Levin et al. (2004)	Evaluated the ability of native Argentinean WRF for ligninolytic enzymes.	Found essentially Lac activity.
Boer et al. (2004)	Studied LME's of *Lentinula*	The fungus was a good

	edodes.	producer of Lac and MnP.
Takahashi (2002)	Isolated wild type strain of *Lenzites betulinus* from rotten wood and fruit bodies by screening on RBBR dye containing solid media.	It was found to be good producer of MnP.
Risna and Suhirman (2002)	Isolated 65 *polyporous* basidiomycetes from several forests in Lombok Island, Indonesia.	Found twelve isolates produced all three kinds of enzymes.
Muzariri et al. (2001)	Screened 224 fungal strains from Zimbabwe for ligninolytic and cellulolytic activities.	Studied the isolates for potential application in remediation of effluent water from the pulp and paper industry
Read et al. (2001)	Isolated 10 strains of WRF from decaying wood from Zimbabwe.	Identified based on their gross and microscopic characters as *Trematus Sp*, *Lentinus sp, Pycnoporous sp, Datrionia sp, Irpex sp*, and *Creptidotus sp,* producing no LiP in any of the tested culture conditions.
Okino et al. (2000)	Isolated 116 Brazilian tropical rainforest basidiomycetes.	Found laccase and peroxidase enzymes producing strains
Nerud et al. (2000)	Isolated several species belonging to the genus *Armillaria* such as *A. gallica*; *A ostoyae*, *A borealis* and *A*	No strains were able to produce LiP in any of the media tested.

	mellea.	
Okino et al. (2000)	The lignicolous fungus *T. villosa* CCB176 was isolated from basidioma collected in a seasonal forest located in the municipality of Assis, interior of the State of São Paulo, Brazil	Enzyme characteristics were performed.
Pelaez et al. (1995)	Screened 68 species isolated from fruit-bodies of basidiomycetes collected in the Central region of Spain for Ligninolytic enzymes.	Laccase and Mn^{2+}-oxidizing peroxidase were more common ligninolytic enzymes than LiP in the studied conditions.
Eriksson et al. (1995)	Studied ligninolytic system of WRF *Pycnoporous cinnabariaus.*	The fungus was found to produce an unusual set of extracellular phenolooxidases consisting of a single isoform of laccase and peroxidase that was neither LiP nor MnP.
Bont (1992)	Studied ligninolytic enzymes of *Bjerkandera sp.*	Detected novel enzyme named Mn-inhibited peroxidase(MIP) but no Lac and LiP.

2.2 Laccase: An important member of oxido-reductases

Among all ligninolytic enzymes, laccase has gained more importance and has been highly studied for many environmental applications. In contrast to most enzymes, which are generally substrate specific, laccase do act on wide range of substrates like diphenols, polyphenols, aromatic amines, benzenethiols and even some inorganic

compounds such as iodine (Viswanath et al. 2008). The enzymatic catalysis by laccases in different industrial applications such as textile dye bleaching, pulp bleaching and bioremediation could serve as a more environmentally benign alternative than the currently used chemical processes.

Laccases have a lower redox potential (450-800 mV) than those of ligninolytic peroxidases (>1V), so it was initially thought that laccases would only be able to oxidize phenolic substrates (Kersten et al. 1990). However, the range of substrates oxidized by laccases can be increased through a mediator-involved reaction mechanism. Mediators are low molecular weight compounds that are easily oxidized by laccase, producing very unstable and reactive cationic radicals. These further can oxidize more complex substrates before returning to their original state. The electrons taken by laccases are finally transferred back to oxygen to form water (McGuirl and Dooley 1999; Wong and Yu 1999). Thus, discovery of novel laccases with different substrate specificities and improved stabilities is very important for industrial applications.

2.3 Laccase sources

Laccases are ubiquitous in nature generally present in higher plants, bacteria, fungi, insects and lichens (Arakane et al. 2005; Riva 2006; Lisov et al. 2007). The first report of laccase existence dates from 1883, when Yoshida detected laccase-like activity in *Rhus vernicifera* (O´Malley et al 1993). However, it was not until 1962 that laccase was designated a p-diphenol oxidase (Benfield et al. 1964) and accepted as part of the lignification process in plants (O´Malley et al. 1993).

2.3.1 Plants

Structural analysis of plants together with the development of new techniques over the years have helped to confirm that plant laccases participate in the early steps of lignification by catalyzing the oxidation of monolignols. They are however not able to act on complex structures such as phenolic arrangements with multiple aromatic rings

(O´Malley et al. 1993). Besides lignification, plant laccases play an important role in wound healing and also in defense mechanism against external factors (Dwivedi et al. 2011).

Some examples of plant sources from which laccases have been isolated includes Sycamore maple (*Acer pseudoplatanus*), Loblolly pine (*Pinus taeda*) (O´Malley et al. 1993), *Rhus vernicifera* (Benfield et al. 1964) and *Populus euramericana* (Ranocha et al. 1999).

2.3.2 Bacteria

Findings of laccase activity in bacteria have been recently reported (Held et al. 2005; Sharma et al. 2007). Some of these laccases can function in the presence of high concentrations of chloride and copper ions at neutral pH values.

In bacteria, the first reported laccase was from *Azospirrullum lipoferum*, in which laccase was associated with the melanin production for cell pigmentation (Faure et al. 1994). In other bacterial species, it was related to morphogenesis (Endo et al. 2002) or the resistance of spores against hydrogen peroxide and UV (Held et al. 2005, Sharma et al. 2007). The enzyme isolated from *Sinorhizobium meliloti* is a dimeric protein with pI 6.2 consisting of two similar 45-kD subunits (Rosconi et al. 2005), whereas laccase from *Pseudomonas putida* is a monomeric 59-kD protein stable at pH 7.0 (McMahon et al. 2007). Both enzymes can oxidize syringaldazine. Characterization of bacterial laccases has revealed that they have a low redox potential (0.45-0.54 V) (Durao et al. 2006) but that they are active and stable at high temperatures (66 h at 60°C), pH (7-9) and salt concentrations (Held et al. 2005; Dwivedi et al. 2011). Recently some more bacteria such as *Streptomyces lavendulae*, *Streptomyces cyaneus*, and *Marinomonas mediterranea* were reported to produce laccases. Laccases activities were also found in *Bacillus sphaericus* (Claus and Filip 1997), *Escherichia coli* (Grass and Rensing 2001), *Serratia marcescens* (Verma and Madamwar 2003), *Bacillus halodurans* (Ruijssenaars and Hartmans 2004) and *Streptomyces psammoticus* (Niladevi and Prema 2008).

2.3.3 Fungi

Fungal laccases are involved in delignification of lignocellulosic material, protection against toxic compounds, formation of fruiting body, fungal morphogenesis, sporulation (Dwivedi et al. 2011) and synthesis of molecules with virulent activity (*i.e.,* melanin) that cause fungal diseases (Riva 2006).

The number of fungal laccase producers is immense (Couto and Toca-Herrera 2006; Morozova et al. 2007) and most of them belong to Basidiomycetes and Ascomycetes. Laccase activity has never been reported in the lower fungi belonging to the Zygomycete (Glomeromycete) and Chytridiomycete.

Among the basidiomycetes, *Trametes versicolor* (formerly known as *Coriolus versicolor* or *Polyporus versicolor*) is the most studied and characterized fungus for the production of laccase. It is one of the principal white-rot decaying fungi since it is able to produce other oxidoreductases besides laccase (Mikiashvili et al. 2005). Other laccase producers of *Trametes* include *T. pubescens* (Galhaup et al 2002), *T. hirsuta* (Couto et al. 2003) and *T. gallica* (Dong et al. 2005).

Basidiomycetes such as *Phanerochaete chrysosporium, Theiophora terrestris,* and *Lenzites betulina* and white-rot fungi such as *Phlebia radiate, Pleurotus ostreatus,* and *Trametes versicolor* produces laccase. Many *Trichoderma* species such as *T. atroviride, T. harzianum,* and *T. longibrachiatum* are the sources of laccases. Laccase from the *Monocillium indicum* was the first laccase to be characterized from Ascomycetes which shows peroxidase activity. *Pycnoporus cinnabarinus* produces laccase as ligninolytic enzyme while *Pycnoporus sanguineus* produces laccase as phenol oxidase (Shraddha et al. 2011).

Apart from the usual sources like soil, textile effluent, municipal waste, tree barks etc, some of the laccase producing fungi belonging to class Ascomycete and Basidiomycete have been also isolated from the marine samples (Verma et al. 2010).

Coriolopsis byrsina, Cerrena unicolour, Diaporthe phaseolorum, Pestalotiopsis uvicola are some of marine derived fungi.

2.3.4 Lichens

The laccase activity was recently discovered in lichens of different taxonomic and substrate groups (Laufer et al.2006; Zavarzina and Zavarzin 2006). It is well known that lichens are symbiotic organisms representing associations of fungi (most often ascomycetes) and green algae and/or Cyanobacteria. Depending on the host (higher plants, rocks, or soil), lichens can be divided into epiphytic, epilythic, or epigeic species. They are well known for tremendous abilities to adapt and survive under extreme conditions and for a rapid restoration of their metabolic activity (Purvis 2000). Lichens are able to develop even in an oligothroph environment (foreign for other organisms); they constitute more than 8% of the total terrestrial vegetation.

High- and low-('large' and 'small')-molecular-weight (MW) laccases were isolated and characterized from the thalli of lichens *Solorina crocea* and *Peltigera aphthosa* (the order Peltigerales) by Lisov et al. (2007).

2.4 Structure of laccases

Three-dimensional structural analysis of several fungal, bacterial and plant laccases reveals that all are composed of three sequentially arranged cuprodoxin-like domains. Each domain has a greek key β-barrel topology, highly related to small copper proteins such as azurin and plastocyanin (Dwivedi et al. 2010; Giardina et al. 2010). The multiple alignment of primary sequences of laccases shows that the copper binding motifs are highly preserved in all sequences, which reflects a common mechanism for copper oxidation and oxygen reduction. However, putative binding pocket analysis reveals that bacterial laccases have larger binding cavities when compared to those from plants and fungi (Dwivedi et al. 2010).

Generally, laccase contains four copper atoms which have been classified into three groups based on the absorption and electron paramagnetic resonance spectra (Fig. 2.1). Type 1 (T1) site is paramagnetic, referred to as “blue” copper. It has an intense absorption at 600-610 nm, which is caused by the covalent copper-cysteine bond and confers the typical blue colour to the multi copper proteins. The T1 copper has a trigonal coordination with two histidines and one cysteine. In bacterial laccases, the axial ligand is conformed by methionine and in fungal laccase; it is by leucine or phenylalanine (Witayakran and Ragauskas 2009). Type 2 (T2) site is also paramagnetic, “non-blue” copper and has no visible absorption spectrum. It is coordinated by two histidines. Type 3 (T3) is a diamagnetic, coupled binuclear copper center, with an absorption band at 330 nm. It is coordinated by six histidines (Claus 2004; Witayakran and Ragauskas 2009; Thurston 1994). Nevertheless, it is possible to find non-blue laccases in nature (Palmieri et al. 1999). Recently, the “White” laccases and “Yellow” laccases, as they are called, have been structurally characterized and atypically show the presence of one copper, one iron and two zinc atoms per molecule.

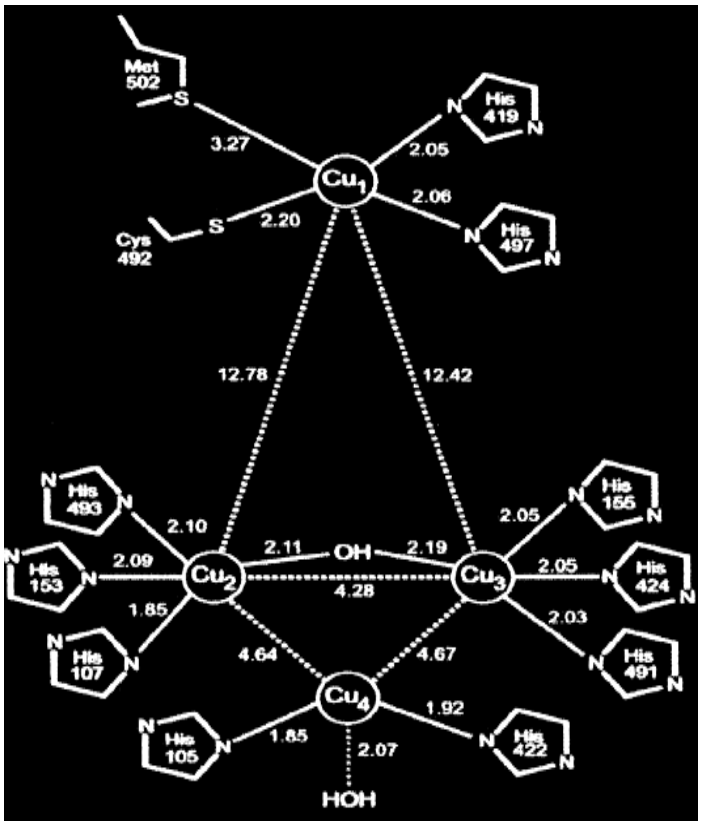

Fig. 2.1 Schematic representation of copper centers in fungal laccase. Modified from Claus 2004

Structural analysis of *Tramates versicolor* laccase and site-directed mutagenesis in *Bacillus sp*. laccase have revealed that the axial ligand in T1 copper is responsible for displaying the redox potential. T1 copper has no axial ligand in *T.versicolor* laccase and this has given rise to a modest elevation of its redox potential to 0.78 V (Piontek et al 2002). Moreover, mutations of *Bacillus sp.* laccase have been used to confirm that modifications in the axial ligand of T1(methionine was replaced by phenylalanine or leucine) which allowed changes in the redox potential (the change of amino acids led to an increase of 0.06-0.1 V of the redox potential as compared to the wild type) (Durao et al 2006). The redox potential is directly related to how good a laccase will catalyze oxido-reduction reactions.

2.5 Catalytic mechanism of laccase

Fig. 2.2 illustrates the catalytic mechanism of laccase where molecular oxygen undergoes a reduction reaction to form water. Starting from the native intermediate, the substrate reduces the T1 site, which transfers the electron to the trinuclear cluster T2/T3. Here, two possible mechanisms for reduction of the trinuclear cluster are possible: either T1 and T2 sites together reduce T3, or each copper on the cluster is sequentially reduced by electron transfer starting from T1. Once the enzyme is completely reduced, one oxygen atom is bound with the T2 and T3 copper ions, and the other oxygen atom is bound with the other copper ion of T3, forming the peroxide intermediate. Subsequently, the peroxide bond (O-O) is broken to produce a native intermediate (fully oxidized form), which will end the catalytic cycle with the reduction of oxygen to water. Sometimes the native intermediate is converted to a completely oxidized cluster called the "resting" form, where the T2 copper is isolated from the coupled T3 coppers. In this form, the T1 can still be reduced by the substrate, but the electron transfer is too slow to be significant (Duran et al 2002; Witayakran and Ragauskas 2009).

The use of molecular oxygen as the oxidant and the fact that water is the only by-product are very attractive catalytic features, rendering laccases as excellent 'green' catalysts (Riva 2006).

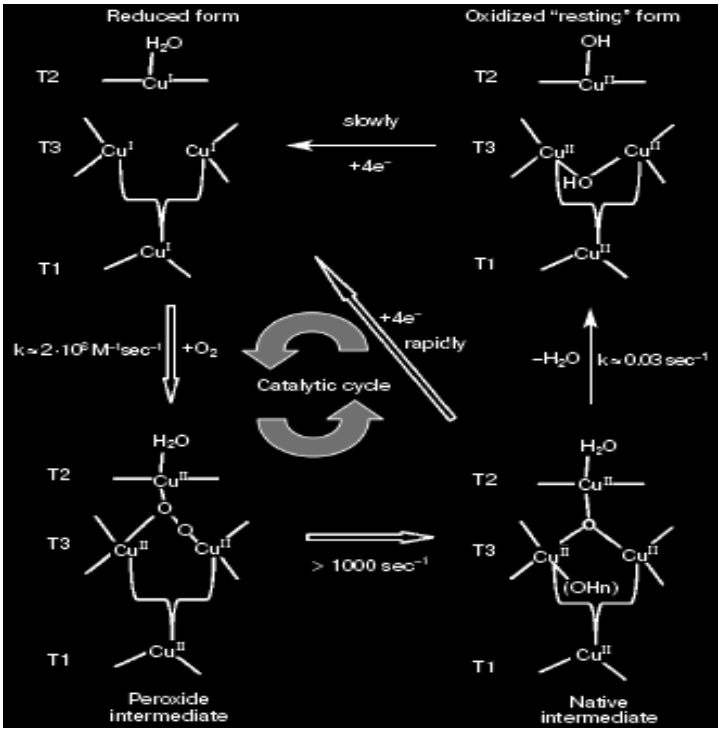

Fig. 2.2 Mechanism of four-electron reduction of molecular oxygen to water in the catalytic cycle of laccase (Shleev et al. 2006)

2.6 Determination assays for laccase measurements

Different assay methods are used for quantifying the laccase production from different sources. Some of the methods include HPLC assay, manometry, order spectrum method and spectrophotometry (Zhu et al. 2006). Among all the methods, laccase assay by spectrophotometry is widely used owing to its simplicity and sensitive characteristics. Usually, there are several compounds that have been used as substrates for spectrophotometry methods. The substrates like 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Fernandes et al. 2005), syringaldazine (Park et al. 2006), o-dianisidine (Silva et al. 2005), guaiacol (Arora et al. 2002) and its diazo derivatives (Farhadi et al. 2011), 2,6-dimethoxyphenol (2,6-DMOP) (Gold et at. 1992) and o-tolidine (Miller et al. 2007) were employed for the determination of laccase quantity. Assay sensitivity for enzyme is largely depended upon the efficiency of substrates. Thus,

sensitivity of substrates is vital in evaluating the enzyme activity. Though ABTS is one of the common substrates exploited for the laccase assay by many authors; but there is an interference caused by the certain medium components. The other common substrate largely used in the laccase assay is syringaldazine, in which the there is a lack of stability in the coloured compound (Melnig et al. 2008). Consequently, there is a large confusion regarding the choice of substrates for the laccase determination.

2.7 Fermentation techniques for the production of laccase

Fermentation techniques can be divided into two main groups: Solid-State Fermentation (SSF) and Submerged fermentation (SmF). The difference between these two techniques consists in the quantity of free flowing liquid present in the system. SSF involves the growth of microorganisms on solid materials in the absence or near-absence of free flowing water, whereas in SmF the microorganisms grow on a continuous liquid phase. There are many contributions to the field of laccase production under SmF using different microorganisms, at different scales and with the possible use of immobilization supports as well as the addition of inducers. Some of the most remarkable results in terms of laccase activity were obtained by the *Trametes* genus: *T. pubescens* (Galhaup et at. 2002), *T. versicolor* (Font et al. 2003; Tavares et at. 2005) and *T. hirsuta* (Roriz et at. 2009).

2.7.1 Submerged fermentation

The efficiency of the laccase producing organisms can only be exploited with the aid of media designed with optimum concentrations of every component which influences the production. Research conducted in this regard mainly aimed at picking up suitable carbon sources, nitrogen sources and inducers. Most of the submerged fermentation research works reported thus far, have been tried to design a optimum production medium by varying concentrations of media components, introducing natural (saw dust, corn cob, bagasse particles, etc.) and synthetic (veratryl alcohol, indulin,

guaiacol etc.) inducers in the media (Kiiskinen et al. 2004; Patel et al. 2008; Desai and Nityananda 2011).

2.7.1.1 Influence of carbon and nitrogen sources on laccase production

Glucose, mannose, maltose, fructose, and lactose are the commonly used carbon sources for laccase production by many fungi (Shraddha et al. 2010, Desai and Nityananda 2011). Among them, glucose is the easiest carbon source for fungi to metabolize but its effect on laccase production depends on the fungal strain. In *Trametes pubescens* (Galhaup et al. 2002), *Trametes versicolor* (Tavares et al. 2005) and *Phlebia sp*. (Arora and Rampal 2002), it repressed the production of laccase but it has been found to enhance the production in *Galerina sp.* HCl (Gulden et al. 2005). Fructose was shown to be a good carbon source for laccase production in *Pleurotus sajor-caju* (Bettin et al. 2009), cellobiose in *T. pubescens* (Galhaup et al 2002), and lactose or glycerol in *Pseudotrametes gibbosa*, *Coriolus versicolor* and *Fomes fomentarius* (Revankar and Lele 2006). However, in some cases it was found that the excess of glucose and sucrose reduce the production of laccase by obstructing the initiation (as reviewed by Desai and Nityananda 2011). This problem of production of enzyme can be improved by using polymeric substrates like cellulose, starch, carboxy methyl cellulose.

Yeast extract, peptone, urea, $(NH_4)_2SO_4$, and $NaNO_3$ are the commonly used nitrogen sources for the production of laccase. It was reported in some cases that the laccase production was triggered when nitrogen depletion occurs in the media. However, some nitrogen sources have showed no affect on the enzyme activity. It was reported in some studies that the elevated laccase activity was achieved by using low carbon-to-nitrogen ratio while others reported that it was achieved at high carbon-to-nitrogen ratio (as reviewed by Couto and Herrara 2007; Shraddha et al. 2010; Desai and Nityananda 2011). For e.g low nitrogen levels (as yeast extract) improved the laccase production in *Pleurotus ostreatus* (Prasad et al. 2005), *Coriolus versicolor* (Revankar and Lele 2006) and *Pycnoporus sanguineus* (Pointing et al. 2000) whereas high concentrations of

nitrogen were needed for *Trametes pubescens* (Galhaup et al. 2002), *Trametes gallica* (Dong et al. 2005) and *Galerina sp.* HC1(Gulden et al. 2005).

Casein, another nitrogen source was successfully used for the production of laccase in *Pleurotus sajor-caju* (Bettin et al. 2009). In *Trametes versicolor* and *Coriolopsis polyzona,* the laccase production was significantly improved when NH_4NO_3, $(NH_4)_2SO_4$, KNO_3 and peptone were used as supplementary nitrogen sources (Elisashvili et al. 2008). On the other hand, agricultural residues have been used as carbon and nitrogen sources for laccase production by some strains. They are cheap substrates and may have additional nutritional metabolites. Cultivation of *Galerina sp.* HC1 on bagasse and orange peels demonstrated the potential of the agro residues as substrates for laccase production: the production was increased 1 and 4 fold, respectively when compared to cultivation in glucose containing medium (Gulden et al. 2005).

2.7.1.2 Effect of inducers on enzyme production

Laccase are produced in low concentrations by many fungi but higher concentrations can be obtained with the addition of supplements to media. The compounds like veratryl alcohol (VA), lignin, 2,5-xylidine, Tween 80 or other xenobiotic compounds can induce laccase significantly. The addition of chemicals to the cultivation can enhance the laccase production based on their ability to induce the expression of the different isoforms (de'Souza et al. 2004).

Compounds like p-coumaric acid, α-benzoin oxime and 2,5-xylidine were highly improved laccase production in *Galerina* sp. HC1, whereas lignin or lignin-related structures slightly enhanced the laccase production (Gulden et al. 2005). Other lignin-related chemicals such as ferulic acid and/or vanillin proved as an inducer by increasing the laccase production up to 10 times in *Pleurotus pulmonarius* (de'Souza et al 2004). Vanillin also induced laccase production in *Phanerochaete flavido-alba* (de la Rubia et al. 2002) and caffeic acid in *Coprinus comatus* (Lu and Ding 2010).

Vitamins like biotin, riboflavin and pyridoxine hydrochloride as well as amino acids such as methionine, tryptophan, glycine and valine stimulated laccase production in *Cyathus bulleri*, whereas cysteine inhibited the production in this fungus (Dhawan and Kuhad 2002).

Antibiotics like apramycin sulfate stimulated laccase production in *Cyathus bulleri* and *Pycnoporus cinnabarinus* (Dhawan et al. 2005). Metals like Mn^{2+} led to a 4.5 fold increase in the laccase production by *Coprinus comatus* (Lu and Ding 2010).

Copper had both inducing and toxic effect in many organisms (Xing et al. 2006). Generally low concentration of copper is required for laccase production. The growth of *Galerina sp.* HC1 was highly favoured when 0.01g/l copper sulfate was added to the cultivation (Gulden et al. 2005). Interestingly, the fungus was able to tolerate and produce the enzyme at copper concentrations as high as 0.1g/l when yeast extract concentration was increased up to 35g/l.

Galai et al. (2009) observed enhanced laccase activity when Triton-X-100 (0.1% v/v) was used in reaction mixture. The enzymes had improved catalytic efficiency (5 to 10 fold) in a-pinene-rich environment, while optimal reaction rates were in high-water content systems (15.5% v/v). Laccase activity was increased 2.6 fold by the addition of copper sulfate (10mM). Valeriano et al. (2009) obtained high activity when inducers 2,5-xylidine or ethanol were used in specific concentration in case of laccase from *Pycnoporus sanguineus*. Among various inducers used like gallic acid (1mM), catechol (1mM), ammonium tartarate (55µM), hydroxybenzoic acid (1mM) and vanillin (1mM), it was observed that only ammonium tartarate increased the enzymatic activity reaching to days in case of laccase from *Lentinula edodes* (Cavallazzi et al. 2005).

2.7.1.3 Influence of temperature

It has been found that the optimal temperature for fruiting body formation and laccase production is 25°C in the presence of light, but 30°C for laccase production when the cultures are incubated in the dark (Thurston 1994). In general, the fungi were

cultivated at temperatures between 25°C and 30°C for optimal laccase production (Vasconcelos et al. 2000). When cultivated at temperatures higher than 30°C, the activity of ligninolytic enzymes was reduced (Nyanhongo et al. 2002).

2.7.1.4 Influence of pH

There is not much information available on the influence of initial medium pH on laccase production, but when fungi are grown in a medium of which the pH was optimal for growth (pH 5.0), the laccase was produced in excess (Thurston 1994). Most reports indicated initial pH levels set between pH 4.5 and pH 6.0 prior to inoculation, but the levels are not controlled during most cultivation (Vasconcelos et al. 2000). Nyanhongo et al. (2002) reported that an initial pH of 7.0 was the best for optimal growth and laccase production by a newly isolated strain of *Tramates modesta*.

2.7.1.5 Influence of agitator

Agitation is another factor that affects laccase production. Hess et al. (2007) found that mycelia are damaged when fungus was grown in the stirred tank reactor and laccase production in *Trametes multicolour* was considerably decreased. Cultivation of white-rot fungus *Bjerkandera adusta* in a stirred tank reactor yielded very low activities of laccase (Mohorcic et al. 2009). However, agitation did not play any role in the production of laccase by *T. versicolor* (Tavares et al. 2007).

Table 2.2 presents the major literatures on laccase sources, various media used for its production, inducers and substrate used for activity determination.

Table 2.2 Laccase sources, various media used for the production, inducers and substrate utilized for determining laccase activity in submerged fermentation

Organism	Media	Inducer	Substrate	Activity Definition	Reference
Pleurotus spodoleucas, Mycena purpurifusca, Pleurotus florida, Pleurotus abalonus, Tremella aurantialba, Pleurotus ostreatus	Minimal laccase media (MLM)		ABTS	Amount of enzyme catalyzing the activity of 1umol of ABTS/ minute	Zhang et al. 2012
Pleurotus sp.	Potato dextrose broth (PDB)		ABTS	Amount of enzyme catalyzing the activity of 1umol of ABTS/ minute	Saravanakumar et al. 2010
Pleurotus florida	Basal medium	Xylidine, Copper sulfate, VA	ABTS	Amount of enzyme catalyzing the activity of 1umol of ABTS/minute	Palavannan and Sathishkumar 2010
Pestalotigis sp. Diaporthe sp. Cyathus byriana Cerena unicolour	B and K broth		ABTS	Amount of enzyme required to oxidize 1micromole substrate per minute per litre of culture broth	Verma et al. 2010
Pleurotus ostreatus	Laccase media		ABTS	Amount of enzyme catalyzing the	Liu et al. 2009

	2 (LM2)			activity of 1umol of ABTS/minute	
Tramates hirsutae	Basal media	2,5-Xylidine	ABTS	Amount of enzyme producing 1millimole of product	Tzialla et al. 2009
Cerena unicolour	Tien and Kirck medium	Copper sulfate	ABTS	Amount of enzyme releasing 1 micro mol product per minute per litre of culture supernatant	D'Souza-Ticlo et al. 2009
Tramates versicolor *Aspergillus niger*	Potato dextrose broth medium		ABTS	Amount of enzyme oxidizing 1 micro mol substrate per min at 25^{o}C	Bajpai et al. 2009
Pycnoporus sanguineus	Malt extract medium	2,5-Xyldine Ethanol	ABTS	Amount of enzyme oxidizing 1 micro mol substrate at standard condition	Valeriano et al. 2009
Phanerochaete chrysosporium	Potato dextrose broth medium		Guaiacol	Amount of enzyme catalyzing the production of one micromole of coloured product per min per ml	Jhadav et al. 2009
Trichoderma harzanium	Basal medium	Copper sulfate	ABTS	Amount of enzyme catalyzing the activity of 1umol of ABTS/minute	Sadashivam et al. 2008
Streptomyces psammolicus	Basal media with	Gallic acid, Ferulic acid,	ABTS	1micro mol of ABTS oxidized per minute	Niladevi and Prema 2008

	agricultural residues	Guaiacol, VA, Pyrogallol, Vanillic acid, Copper sulfate			
Trametes sp.	Kirk's basal media		ABTS	Amount of enzyme producing 1millimole of product	Li et al. 2008
Funalia trogii	Basidiomycete rich medium	Copper sulfate Veratryl alcohol	ABTS	Amount of enzyme oxidizing 1 micromole substrate per min	Ciullini et al. 2008
Stereum ostrea P.chrysosporium	Medium of Olga et al.(1998), Munoz et al. (1997), Coll et al.(1993), PDB,		Guaiacol	Amount of enzyme causing 1 mole of substrate conversion per second	Visvanath et al. 2008

	Slomczyski				
Habeloma cylindrosporum	Melin's medium	Apple scraping, Pine nudles Copper sulfate	ABTS	Amount of enzyme required to oxidize 1 millimol substrate per min at 25^0C	Ramesh et al. 2008
Trichoderma harzanium	Basal media	CuSO4	ABTS	Quantity of enzyme oxidizing 1 micro mol substrate per minute	Sadhasivum et al. 2008
Pannus tigrinus	Basal medium		DMP	Amount of enzyme which produces 1umol product per minute under assay condition	Quarantero et al. 2007
Xylaria polymorpha	TMJ	2,5-Xyldine	ABTS	Amount of enzyme catalyzing the activity of 1umol of ABTS/ minute	Liers et al. 2007
Termitomyces clypeatus	Basal medium		ABTS	Amount of enzyme catalyzing the activity of 1umol of ABTS/ minute	Bose et al. 2007
Pycnoporous sanguineous, Coriolopsis polyzana, P.ostreatus	Peptone glucose yeast extract media (PGLY)	Ferulic acid, Mannan oligo-saccharide	ABTS	1 micro mol of product formed per minute at room temperature	Vanhulle et al. 2007

Trametes sp.	XH media	O-toluidine Copper	Guaiacol	Amount of enzyme catalyzing the activity of 1µmol of substrate/ minute	Tong et al. 2007
Minimidochium parvum	Basal media	Anthracene, ethanol, Guaiacol, humic acid, Kraft lignin, Tween 20, VA	DMP	Enzyme activity releasing 1 picomol of oxidized product per second	Saparrat et al. 2007
T.versicolor	VH medium	2,5-Xylidine	Syringaldazine	Amount of enzyme required to oxidize 1 micromole substrate per minute per litre of culture supernatant	Cordi et al. 2007
Trichoderma reesi *P.chrysosporium* *Polyporous sp.* *Armillaria sp.*	Avicel liquid		Catechol	Change in absorbance at 440nm in 1ml reaction mixture per min	Safari-Sinegani et al. 2006
Cerena versicolor	Basal medium	p-anisidne, gallic acid,	ABTS	Amount of enzyme catalyzing the activity of 1umol of ABTS/ minute	Madhavi et al. 2006

		ferulic acid, guaiacol, 2,5-Xylidine, Ethanol, Catechol, Vanillic acid			
Basidiomycete sp.	B and K media	p-anisidine	ABTS	Amount of enzyme oxidizing 1 micromole substrate per min	Bajpai et al. 2006
T.versicolor	TDM	Copper, Xylidine, Phenolic mixture	ABTS	Amount of enzyme catalyzing the activity of 1umol of ABTS/ minute	Tavares et al.2005
T.versicolor	Basal Synthetic media	Mandarin peel	Syringald azine	Amount of enzyme that transferred 1umol substrate per min	Mikiashvili et al. 2005
T.versicolor	CPDA medium		Syringald azine		Udaysoorian and Prabhu 2005
T.versicolor	TD media	Copper, xylidine Phenol	ABTS	Amount of enzyme oxidizing 1 micromole substrate per min	Tavares et al. 2005

		mixture			
Lentinus edodes		Catecol Ammonium tartarate Gallic acid Vanillin	ABTS	Amount of enzyme causing 1 micromole substrate per min	Gomez et al. 2005
Pycnoporus cinnabarinus	Minimal media		ABTS	Amount of enzyme catalyzing oxidation of 1 nanomol ABTS per second (Nanokatal)	Alves et al. 2004
P.ostreatus	N-limited medium		ABTS	Absorbance increase at 420nm at 30^{o}C	Hou et al. 2004
T.multicolor	Basal media	Copper sulfate	ABTS	Amount of enzyme catalyzing the activity of 1umol of ABTS/ minute	Hess et al. 2002
M.parvum	C-limited medium	Anthracene, Ethanol, Humic acid	2,4-DMP	1 picomol product per second	Sapparat et al. 2002
C.versicolor	Natural and synthetic media		Syringaldazine	Amount of enzyme that transferred 1unol substrate per min	Kahraman and Gurdal 2002

Phlebia fascicularia, P.brivispora, Paralepetopsis floridensis	MEM		Guaiacol	Colourimetric units per ml	Arora and Rampal 2002
T. versicolor	TDM and TaK medium	Lignin Lignosulphonate VA	ABTS	Amount of enzyme causing 1 micromole substrate per min	Xavier et al. 2002
T.versicolor, T.pocas T.cingulata, T.velutinus Pycnoporous sanguineus, Daldinia concentrica, Crataegus molliss,	Bonnarme (1990)	Cycloheximide	Guaiacol	-	Tekere et al. 2001
T.versicolor, Phylidorea squalens, Phlebia fascicularia, Pleurotus floridensis	MSB	Guaiacol, VA, Agro-residues	Guaiacol	Colourimetric units per milliliter of enzyme	Arora and Gill 2000

Botrytis cineria, Podospora anserina, Fomes annosus, entinusedodes, P.chrysosporium, Sporotrichum palverulentum, Phialophora mutabilis, Rhizoctonia practicola, T.versicolor	Sugar rich liquid media	2,5-Xylidine	Syringald -azine	Amount of enzyme which cause a change in optical density 0f 1.0unit per minute	Bollag and Leonowicz 1984

2.7.2 Solid state fermentation for the production of laccase

Solid state fermentation (SSF) holds tremendous potential for the production of various microbial enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source. In addition to the conventional applications in food and fermentation industries, microbial enzymes have attained significant role in biotransformations involving organic solvent media, mainly for bioactive compounds. This system offers numerous advantages over submerged fermentation (SmF) system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipments, etc.

Since the biotechnological applications require large amounts of low cost enzymes, one of the appropriate approaches for this purpose is to utilize the potential of lignocellulosic wastes as substrates for enzyme production. These substrates may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis ensuring efficient production of ligninolytic enzymes (Elisashvili et al. 2001; Reddy et al. 2003; Moldes et al. 2004). The selection of appropriate plant residue adequate for fungus growth and the production of target enzymes play an important role in the development of an efficient biotechnology process.

The selection of a substrate for enzyme production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate. Thus, it is necessary to screen the suitable substrate among the several agro-industrial residues. In SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as an anchorage for the mycelia. The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate. However, some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates. In such cases, it would become necessary to supplement the required nutrients externally. It has also been a practice to pre-treat

(chemically or mechanically) some of the substrates before using in SSF processes, thereby making them more easily accessible for microbial growth.

SSF processes are distinct from submerged fermentation (SmF) culturing, since microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents. The availability of water in lower or higher concentrations affects microbial activity adversely. Thus, it is crucial to provide optimized water content and control the water activity (a_w) of the fermenting substrate. Moreover, water has profound impact on the physico-chemical properties of the solids and this, in turn, affects the overall process productivity (Pandey 1992).

The use of lignocellulosic materials for enzyme production has, at least, two advantages with respect to the SmF processes: high production of enzymes using a low-cost media (Viniegra-Gonza´les et al. 2003) and the possible use of the bioconverted substrate due to its increased digestibility (Mukherjee and Nandi 2004). Recent works have shown that some lignocellulosic wastes stimulate enzyme production by basidiomycetes (Rosales et al. 2002; Reddy et al. 2003; Kapich et al. 2004; Elisashvili et al. 2006). SSF is suitable for the production of enzymes by using natural substrates such as agricultural residues because they mimic the conditions under which the fungi grow naturally. The lignin, cellulose and hemicelluloses are rich in sugar and promote fungal growth in fermentor and make the process more economical (Pandey 1992). The lignocellulosic agro-industrial residues have great potential to act as substrate for the production high titres of laccase. Many of the researches have already reported the efficiency of many such residues (Table 2.3).

Chawachart et al. (2003) conducted SSF using three substrtaes; Rubber wood meal (*Heavea sp*.), Hang nok young wood meal (*Delonix regia*) and rice bran. The highest laccase activity of 36 days of cultivated was obtained on rice bran (1.98U/g), those of rubber meal and Hang nok young wood meal were 0.06 and 0.07U/g respectively.

Octavio et al. (2006) reported that sago hampas, oil palm frond parenchyma tissue (OPFPt) and rubber wood sawdust supplemented with nutrient solution and nitrogen in the form of urea were utilized as substrates with *Pycnoporus sanguineus* for laccase production. Safari-Sinegani et al. (2006) observed that fungal laccase activities in extracts of solid media were higher when compared with those in extracts of liquid media. Different agro-industrial residues were employed to screen the potential substrate for laccase production by *Ganoderma sp*. by Revankar et al. (2007). Residues such as sugarcane bagasse, wheat bran, wood shavings, peanut hull and oat bran were used in their study. They observed that minimum laccase production seen in sugar cane bagasse and maximum production was observed in wheat bran. Ellen et al. (2008) exploited the potential of orange bagasse, major industrial food waste arising from processing orange for juice as solid support for laccase production from *B. rhodiana*. A good enzyme titre was seen in solid state fermentation without added nutrients, indicating nutrient sufficiency of orange bagasse at a solids concentration of 16% (w/v) to sustain growth and high enzyme titres.

Kapoor et al. (2009) reported the potential of wheat straw as a natural support for the production of laccase from *Lentinus edodes.* The study has also reported the efficiency of several organic compounds such as rice bran, corn steep meal, peanut meal, soya meal and wheat bran as supplements to wheat straw for laccase production. Laccase source when on cultivated on both supplemented wheat straw combinations and un-supplemented wheat straw (control), with 70% humidity, solid support with optimal concentration of supplement yielded a good growth and enzyme activity.

There are reports that describe increased production of extracellular laccases in many species of white rot fungi when grown on natural substrates, such as cotton stalk (Ardon et al. 1996), molasses waste water (Kahraman and Gurdal 2002), wheat bran (Souza et al. 2002) and barley bran (Couto et al. 2002). Many researchers supports that the presence of growth factors, vitamins and proteins may be the reason for enhancement of enzyme activities during SSF (Risna and Suhirman 2002).

F. sclerodermeus growing on wheat bran produced up to 270U g/1 of laccase (Papinutti et al. 2003). Chawachart et al. (2004) reported laccase production by *Coriolus versicolor* strain on rice bran with activity of 1.98 U/g after 36 days of cultivation. The results from the study carried out by Hatakka et al.(1995) shows that white rot fungus *Phlebia radiata 79 (ATCC 64658)* produced high titer of laccase during SSF of wheat straw when compared to the laccase produced in the commonly used glucose low-nitrogen liquid medium.

Wheat straw was used for cultivating several fungal strains to produce laccase. Wheat bran is an abundant source for hydroxycinnamiacids, particularly ferulic and p-coumaric acids, which are known to stimulate laccase production (Neifar et al. 2009). Indeed these hydroxycinnamic acids, covalently bound to cell wall polymers (pectins, arabinoxylans and xyloglucans) through ester linkages, could be released after feruloyl esterase action as described in several white-rot fungi (Dinis et al. 2009). Growth and lignocellulolytic enzymes production by two *Morchella esculenta* strains (BAFC 1728 and BEL 124) growing in solid state fermentation using different lignocellulosic materials was characterized by Papinutti and Lechner (2008). Both strains were able to grow on the three substrates: wheat bran, wheat bran plus corn starch and rolled oat. Maximum laccase production was observed in wheat bran by the strain BAFC.

Neifar et al. (2011) reported production of laccase by *Fomes fomentarius* on wheat bran with activity of 151.1 U/g of dry substrate on 13th day of cultivation. Highest records of laccase production were obtained in *Coriolopsis rigida* (108 U/g/1) (Gomez et al. 2005) and *Trametes hirsute* (68.4 U/g/1) (Couto et al. 2005), when both fungi grown on barley bran.

Rice bran contains total carbohydrate 82% (w/w) approximately and the main composition (31%) was hemicellulose (Claye et al. 1996). The main component of rice bran, hemicellulose was expected to be arabinoxylan as most sugars found are xylose and arabinose (Mod et al. 1978). When the levels of those carbon sources decrease, laccase synthesis was induced by phenolic compounds present in rice bran, leading to incresed

laccase production. This induction mechanism may help fungus to degrade lignin or aromatic compounds in rice bran so as to utilize nutrients such as carbon and nitrogen sources. Similar pattern in production of laccase and hemicellulytic enzymes was also found when several white- and brown rot fungi were cultivated on *Eucalyptus grandis* wood chips (Machuca and Ferraz 2001).

Lentinus edodes and *Pleurotus* species from various origins were compared for their ability to produce laccase enzyme in solid-state (SSF) and submerged state (SmF) fermentation methods using various plant raw materials. SSF of tree leaves was favorable for laccase secretion by the majority *L. edodes* and *Pleurotus* strains, whereas SmF enhanced the production of hydrolytic enzymes. Several food wastes, such as mandarin, apple, and banana peels, which are rich in easily metabilizable sugars, have been tested to evaluate basidiomycete's laccase enzyme activity (Elisashvili et al. 2006).

Pleurotus ostreatus showed atypical laccase production under submerged and solid-state fermentation. Cultures grown in submerged fermentation showed laccase activity of 13,000U/l, with a biomass production of 5.6 g/l and it showed the presence of four laccase isoforms. However, cultures grown in solid state fermentation had a much lower laccase activity of 2,430 U/l, biomass production of 4.5 g/l and three laccase isoforms were identified. The results indicated that *P. ostreatus* performed much better in submerged fermentation than in solid-state fermentation, unlike other fungi. The differences observed in the production of laccases might be mainly due to the presence of oxygen in the fermentation system. The fact that SmF showed higher laccase production than SSF might be a result of the stress caused by the little amount of oxygen available in SMF (Fernández et al. 2008).

Given the potential applications of laccases and the need for the development of economical methods for improving laccase production from fungi with an overall aim of reducing the cost of the industrial processes, the use of SSF, especially using agro-wastes as a support-substrate, is an appalling alternative. Table 2.3 represents various reports on

the laccase production by several white-rot fungi grown on different natural supports under solid-state conditions.

Table 2.3 Laccase production by different white-rot fungi grown on different natural supports under SSF conditions

Support	**Microorganism**	**Reference**
Orange peelings	*T.hirsuta*	Rosales et al. (2007)
Banana skin	*T.pubescens*	Osma et al. (2007)
Paper cuttings	*T.hirsuta*	Couto (2007)
Rubber wood sawdust, oil palm frond, parenchynia tissue, sago hampas	*Pvcnoporus sanguincus*	Vikineswary et al. (2006)
Wheat bran flakes	*T.pubescens*	Osma et al. (2006)
Groundnut seeds, groundnut shells, grape seeds	*T.hirsuta*	Couto et al. (2006)
Corncob	*P.pulmonaris*	Tychanowicz et al. (2004)
Kiwi fruit	*T.hirsuta*	Kapich et al. (2004)
Barley bran	*T. hirsuta, T.versicolor*	Couto et al. (2004)
Wheat straw	*Fomes sclerodermeus*	Papinutti et al. (2003)
Grape seeds, barley bran	*T.hirsuta*	Moldes et al. (2003)
Eucalyptus grandis	*Ceriporiopsis subvernispora*	Fenice et al. (2003)
Barley bran	*T. versicolor*	Couto et al. (2003)
Canola roots	*Cvathus olla*	Shinners-Carnelley (2002)

Barley bran, apple peelings, orange peelings, potato peelings	*T.hirsuta*	Rosales et al. (2002)
Neem hull, wheat bran, sugarcane bagasse	*P. ostrearus, P. chiyxosponurn*	Pradeep and Dutta (2002)
Wheat bran, wheat straw	*P. pubnonarius*	deSouza et al. (2002)
Wheat straw, barley straw, wood shavings, barley bran	*T. versicolor*	Couto et al. (2002)
Corncob	*P. chrysosporium P. radians*	Cabaleiro et al. (2002)
Wheat straw	*P. ostreatus*	Baldrian and Gabriel (2002)
Chestnut shell, barley bran Coconut flesh	*Coriolopsis rigida, T.hirsuta*	Baldrian and Gabriel (2002)
Sawdust, grapewine cuttings	*Coriolus hirsutus, Daedaleopsis confragosa, Marasmius albeaus, P. chrysosporium*	Elisashvili et al. (2001)
Banana waste	*P. ostreatus, Pleurotuss cajor-caju*	Elisashvili et al. (200)
Cotton stalks	*P. chrysosporium, Funalia trogu*	Sik and Unyayar (1998)
Cotton wastes	*Pleurotus ostreatus, Pleurotus custidiosus, Pleurotus pulmonarius, Pholiota naineko*	Jaszet et al. (1998)
Corncob	*P. chrysosporium*	Couto et al. (1998)

Barley bran	*P. chrysosporium*	Couto and Ratto (1998)
Straw	*Pleurotus sp.*	Lang et al. (1996)
Ballieo seed	*Botriosphaeria sp.*	Barbosa et al. (1996)
Corn stalks	*Lentinus edode* strain CS-495	D'Annibale et al. (1996)
Wheat straw	*Phlebia radiata*	Vares et al. (1995)
Sugar beet bagasse	*T. versicolor*	Pal et al. (1995)

2.8 Statistical approach of medium optimization for laccase production

Statistical experimental designs have been widely used for several decades (Plackett and Burman 1946; Box and Hunter 1957). Contour surfaces, central composite, Plackett-Burman and response surface technology (RSM) are examples of statistical experiments designs that increase efficiency, improve products and decrease costs, therefore, they have received increasing attention. Statistical experimental designs can be applied at different aspects in process optimization. The Plackett-Burman is very useful for the screening of the most important factors from a lot of candidates (Plackett and Burman 1946). This design does not consider the interaction effects between variables but the most important factors affecting the results. RSM is a factorial experiment design for examining the effect of test variables on measured responses. The RSM approach initially requires an experimental design followed by fitting experimental data into an empirical model equation to determine the optimum conditions.

In most instances, the microbiologist begins with some medium and set of conditions that allow for atleast modest production of metabolite or enzyme activity. The task is then to improve that production to a level sufficient for its isolation, characterization and for its industrial application. A production improvement program may begin by measuring a product yield as a response to factors like medium strength, incubation temperature and culture pH. It is also common practice in the early stages of development to replace the original carbon and nitrogen sources of the medium with

widely used ingredients using classical methods of experimentation. Traditional methods of optimization involve changing one independent variable while keeping the other variables fixed at certain levels. This one dimensional approach is laborious, time consuming, expensive and most importantly, incapable of providing the optimal conditions due to the lack of the interactions between different variables (Furuhashi and Takagi, 1984). On the other hand, experimental design techniques present a more balanced alternative to one-factor-at-a-time approach. Experimental design is a sequential process. First categorical factors are studied to determine which nutrients and physical conditions hold the most promise for optimizing the production. Then large number of continuous factors (usually 2-5) is screened and insignificants are eliminated in order to obtain a smaller, more manageable set of factors. The remaining factors are optimized by predicted response surface modeling. Finally after model building and optimization, the practical optimum is verified.

The Orthogonal and Plackett–Burman designs are important methodologies that can reduce the number of runs to an absolute minimum. The principal objectives of these designs are to screen main factors for further optimization processes from a large number of process variables (Castro et al. 1992; Parra et al. 2005). This enables a better picture of the possible effects of each component in the medium. However, the main disadvantage of these designs is that they consider only first order effects and ignore the interactions between variables. On the other hand, a full factorial design provides almost every possible combination, but it requires a large number of experiments, which is impractical to perform. Optimum performance has been determined using mathematical tools such as multiple regression of a partial or full factorial design to obtain a model of the production system, usually involving fitting of data to a polynomial equation, often using stepwise multiple regression. RSM has also been used to investigate the optimal regions of production of useful products (Prapulla et al. 1992).

RSM, firstly described by Box and Wilson (Box and Wilson 1951), is a collection of mathematical and statistical techniques (Myers et al. 2002), which is not only used to

evaluate the relationship between a set of experimental factors and observed results but also to seek the optimum conditions for multivariable system. RSM has been established as a convenient method for developing optimal conditions for processes with reduced cost and efficient screening of parameters (Vohra and Satyanarayana 2002). The models from RSM take into account the interactions of variables in generating a process response effectively. In many processes, the relationship between the response and the independent variables is usually unknown; therefore, the first step in RSM is to evaluate the function (response) in terms of analyzing variables (independent variables). Usually, this process employs a low-order polynomial equation in a pre-determined region of the independent variables, which is later analyzed to locate the optimum values of independent variables for the best response. RSM has been successfully employed for many bioprocesses, particularly in optimization of medium ingredients and operating parameters. With the development of genetic engineering, biomaterials and other bioprocess technologies like biodegradation and bioremediation, more scientists are interested in adopting statistical experimental design to improve their biological processes and production by shortening time and increasing efficiencies (Lee and Gilmore 2005).

Reducing the costs of enzyme production by optimizing the fermentation medium is the basic research for industrial application. Applications of laccase in biotechnological processes require its production in high amount at low cost and hence, current focus on laccase research is oriented towards the optimization of medium components by various statistical methods. Different statistical designs for medium optimization has been recently employed for lysozyme, xylanase, amylase and laccase production by fungal cultures (Thayer et al. 1987; Dey et al. 2001; Francis et al. 2003; Lee et al. 2003; Parra et al. 2005; Teerapatsakul et al. 2007). It is a well-known fact that extracellular laccase production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources and inducers. When many factors and their interactions regulate the desired response, RSM is an effective statistical tool for optimizing the process by solving the multivariate equations (Palavannan and Sathiskumar 2010).

Statistical methods were employed to optimize laccase production in *C.unicolour*. Five factors such as glucose, ammonium chloride, copper sulfate, sodium chloride and Tween 80 were screened using Placket Burman design (PBD) and central composite design (CCD) was employed to optimize the concentration of carbon (glucose) and nitrogen (ammonium chloride) (Abdul and Annuar 2009). Bar (2001) carried out a factorial experiment with a completely randomized design to optimize laccase production by *C. micaceus*. Three factors (carbon source, inducer and pH) were replicated three times. Molasses (4%) and malt extract (20 g/l) were evaluated for laccase production. Two inducers were evaluated: 500 mg/l of $CuSO_4$ and 5 mg/l of p-hydroxybenzohydrazine. Mishra et at. (2008) optimized laccase production media for *C. versicotor* MTCC 138 using a Box-Benhken statistical experimental design method. Parameters such as medium pH, temperature, moisture content, inducers, ground nut shell and cyanobacterial mass were considered for optimization. Highly influencing parameters were determined through standard Placket-Burman method and empirical model developed through Response Surface Method depicted the optimum concentrations of those factors.

The growth medium for *P. tigrinus* laccase production was studied through RSM. The impact of five crucial variables on laccase production, including glucose and nitrogen concentrations as well as three putative inducers: copper sulfate, 2,5-xylidine and olive-mill wastewater was investigated thoroughly (Quaratino et al. 2008). Box-Wilson central composite design was applied to optimize copper, veratryl alcohol and L-asparagine concentration for ligninolytic enzyme production under submerged fermentation of *T. trogii* (Trupkin et al. 2003). A seven-level Box-Behnken factorial design was employed to optimize the culture medium composition of *Ganoderma* sp. KU-Alk4 for improved laccase production (Teerapatsakul et al. 2007). Box-Behnken experiment design was also applied in biodegradation of the reactive dye by *P. chrysosporium*. Three variables that included dye concentration, incubation days, and nitrogen concentration, at three levels were studied to identify the correlation between those variables on biodegradation level (Nagarajan 1999).

The application of RSM requires the use of models which effectively describe the response quantitatively. The models generally are multinomial in nature and the most adequate degree for each factor and interaction can be chosen in the final form of relationship obtained. This technique finds most utility in optimization of different processes to get the best performance under given constraints.

2.9 Purification and Characterization of laccase

2.9.1. Purification of laccase

The cost of the downstream processing of proteins is an extremely important factor which constitutes up to 80% of the overall production costs of proteins/ enzymes. Purification of protein is usually an important post production step; it is generally difficult achieve, despite the development of a range of techniques. Laccase has been purified and characterized extensively from different species of white rot fungi. Most reported separation methods for laccase purification were developed for the purpose of enzyme characterization. These methods involve a combination of precipitation, membrane filtration, dialysis, ion exchange, hydrophobic interaction, gel permeation and affinity chromatography. Concentration is usually the initial step of downstream processing that is generally used to reduce liquid volume with minimal loss of activity. Although simple to perform, protein concentration by salting out has disadvantages of low process temperature, long time for protein aggregation and low product purity. Ultrafiltration, the other most commonly used concentration method, also has certain drawbacks such as extensive pre-filtration and membrane fouling. The other separation methods mentioned above involve chromatographic techniques that are time consuming, require pretreatment and used mainly for purification of therapeutic proteins.

A single-step purification procedure for *Neurospora crassa* laccase was reported by Judewicz et al. (1998) using celite chromatography and they obtained a specific activity of 333 U/mg with 54% fold purification. Kiiskinen et al. (2004) purified laccase from LLP13 and AH2 strains using DEAE Sepharose and Phenyl Sepharose fast flow

columns. AH2 laccase was further purified with gel filtration on a Sephacryl S-100 HR column. Han et al. (2005) purified laccase from *Trametes versicolor*, using ethanol precipitation, DEAE-Sepharose, Phenyl-Sepharose and Sephadex G-100 chromatography. *T. versicolor* 951022 secreted a single monomeric laccase showing a high specific activity of 91,443 U/mg for 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as the substrate.

Cordi et al. (2007) purified laccase from *T. versicolor* by applying the crude extract to ion exchange DEAE Sephadex A-50 column followed by gel filtration on Sephacryl S- 200-HR column in a FPLC System and obtained a specific activity value of 101 U/ml with 34.8 fold purification. Minussi et al. (2007) purified laccase from *T. versicolor* (CCT 4521) using ammonium sulphate precipitation followed by Sephacryl S-200 column and DEAE cellulose. They obtained a purification of 41.3 fold with 43.3 units/mg specific activity.

Khammuang and Rakrudee (2009) purified laccase from fruiting bodies of *Ganoderma sp*. MK05 by ammonium sulfate precipitation with 40-70% saturation and DEAE cellulose chromatography. They obtained 1.34 and 3.07 fold purification, respectively. Viswanath et al. (2008) purified extracellular laccase from *Stereum ostrea* and obtained up to 70-fold purification from the culture filtrate by a two-step protocol-ammonium sulphate (80% w/v) and Sephadex G-100 column chromatography. Yang et al. (2009) purified laccase from *Termetes sp*. using DEAE Cellulose and Superdex-75.

2.9.1.1 Three-phase partitioning of laccase purification

Purification difficulties led to the development of interfacial protein enrichment method, called three phase partitioning (TPP), which was first used by Tan and Lovrein in 1972. TPP has been reported as an efficient alternative method for concentration and purification of various industrially important enzymes (Dennison and Lovrein 1997). This technique uses a combination of ammonium sulfate and *tertert-butanol* to precipitate proteins from crude extracts. *tertert-butanol* binds to the precipitated proteins, thereby

increasing their buoyancy and causing the precipitates to float above the denser aqueous salt layer. Optimum pH, temperature, ammonium sulfate and *tert-butanol* concentrations can selectively precipitate proteins at the interface of the organic and aqueous phases. K osmotropy, salting out, co-solvent precipitation, isoionic precipitation, osmolytic electrostatic forces, conformation tightening and protein hydration shifts all contribute to protein precipitation at the interface (Lovrein et al.1987; Dennison and Lovrein 1997). In many cases, TPP enhances the activity of various enzymes, resulting in apparent higher yields of 100–1000% (Dennison and Lovrein 1997, Singh et al. 2001). This method has been successfully employed for the purification of various proteins mainly due to its simplicity, easy of handling, feasibility at room temperature. Morever, this method is inexpensive and environmental friendly. TPP has been successfully used in the purification of proteins like cellulase, amylase, pectinase, pullulanase, alkaline phosphatase, peroxidase, protease, phospholipase, aryl alcohol oxidase, carbonic anhydrase, catalase and superoxide dismutase, invertase, amylase and protease inhibitor. It has been used both for upstream and downstream protein purification processes and some time it has been used as a one-step purification protocol.

In addition to concentration, purification with TPP was found comparable to chromatographic techniques. For example, Saxena et al. (2007) obtained a 20.1-fold purification of a wheat germ protease/amylase bi-functional inhibitor using TPP. They further observed that a combination of fractional ammonium sulfate, affinity, ion-exchange and gel filtration yielded amaximum 22-fold purification.

Thus, the novelty of TPP lies in its ability to concentrate proteins from crude broths with higher purification than conventional concentration methods. Scalability, rapid recovery and a requirement for only minimal pretreatment are additional advantages of TPP. TPP system is able to precipitate and collect proteins as a separate layer formed between aqueous and organic phases with the aid of a dissolved inorganic salt (generally ammonium sulphate) and *tert*-butanol.

2.9.1.2. Optimization of TPP parameters

The ultimate objective of protein purification is to achieve both high yield and purity. Like other techniques, the efficiency of TPP purification can be affected by factors including the natural properties of the target protein (pH, isoelectric point, hydrophobicity, concentration, solubility and denaturation of the protein) and purification conditions (ammonium sulfate saturation, ratio of crude extract to *tert-butanol* and temperature). To obtain both high yield and purity, it is important to understand the relationship between these two goals and the purification factors and to optimize purification conditions accordingly. The partitioning of proteins in TPP purification is influenced by factors such as ammonium sulfate saturation, ratio of crude extract to *tert-butanol* and temperature. RSM is an efficient mathematical approach widely applied in the optimization of purification processes, including immobilized metal affinity chromatography (IMAC), dye ligand affinity membrane chromatography, reverse miceller extraction and aqueous two phase extraction (ATPE). It can give information about the interaction between variables, provide the information necessary for design, and process optimization (Vinoth Kumar 2011).

2.9.2 Characterization of purified laccase

The typical laccase is a 60-80 kDa molecule of which 15-20% is carbohydrate (although the sugar composition of the glycan moiety has only been analyzed in a few examples, such as *Podospora anserina* and *Botytis cinerea*). They are all extracellular glycoproteins. It is important to note that many of these enzymes show very considerable heterogeneity after purification. This is an inevitable consequence of the extracellular location of these proteins, as fungal media often contain substantial proteolytic and glycosidic activities (Wood 1985) such that a significant proportion of enzyme molecules become modified by trimming of carbohydrate or nicking of the polypeptide chain (Perry et al.1993).

2.9.2.1 Molecular weights of laccase enzymes

The molecular weight of laccase is predicted to be in the range of 50-97 kDa from the experimental reports. An important feature is that a covalently-linked carbohydrate moiety (10-45% of total molecular mass), which may contribute to the high stability of the enzyme.

In sedimentation analysis, the *Agaricus bisporous* laccase appears to be substantially larger (100 kDa), but this is not in accord with data from electrophoretic analysis under denaturing conditions (that gives a value of about 65 kDa) or gene sequence analysis. It may be beacause this laccase is a dimer of identical subunits (where the size computed from sedimentation analysis is an underestimate because of imperfect correction for the part protein/part carbohydrate composition of the molecule). Similarly, the molecular mass value for the *Aspergillus nidulans* conidial laccase obtained from gel filtration (110 kDa) indicates that this enzyme is a dimer. This method does not reliably measure molecular mass as Stokes' radius (which determines migration rate in a gel matrix) is not simply related to mass for non-spherical molecules.

The other obvious exception is laccase 1 of *Podospora anserine* which is a tetramer of identical subunits (from which the free 80 kDa laccase I11 is derived). It should be noted, in this context, that care must be taken to fully denature some laccases prior to estimation of molecular mass. The *Schiophyllzum commune* laccase migrates during SDS-PAGE with an apparent molecular mass of 36 kDa, but after boiling migrates as a 64 kDa species, Laccase I of *Armillaria mellea* behaves in the same way, but the heat required for complete denaturation is less (Rehman 1991). These are presumably very compact and/or non-spherical molecules prior to denaturation.

Judewicz et al. (1998) reported laccase of molecular weight 64.8 kDa. Lac IId (isozyme of laccase from *Cerrena unicolour*) purified by D'Souza-Ticlo et al. (2009) showed a molecular weight of 59 kDa and pI of 5.3 when analyzed by 2-D PAGE. Han et

al. (2005) purified laccase enzyme from *Trametes versicolor*, with a molecular mass of approximately 97 kDa as determined through SDS-PAGE, larger than those of other laccases reported so far. The molecular mass of purified laccases from *Tramates* was found to be approximately 66 kDa, as reported by Cordi et al. (2007) through calibrated gel filtration and SDS-PAGE.

2.9.2.2 Isozymes of laccase

Many laccase producing fungi secrete isoforms of the same enzyme. These isozymes have been found to originate from the same or different genes encoding for the laccase enzyme. The number of isoforms vary with species and also within species. The biochemical characteristics of isoenzymes vary depending upon the source and culture conditions.

Palmieri et al. (1997) observed two laccase isoenzymes (POXA1 and POXA2) produced by *Pleurotus ostreatus* with molecular weight of 61 and 67 kDa, pI of 6.7 and 4, respectively. Four laccase isozymes (LCC1, LCC2, LCC3 and LCC4) synthesized by *Pleurotus ostreatus* strain V-184 were purified and characterized (Mansur et al. 2003). LCC1 and LCC2 have showed molecular masses of about 60 and 65 kDa, respectively and exhibited the same pI value (3.0). Laccases LCC3 and LCC4 were characterized by SDS-PAGE, with their molecular masses around 80 and 82 kDa, respectively and pI of 4.7 and 4.5, respectively. When these isozymes were stained with ABTS and guaiacol in native polyacrylamide gels, different specificities were observed for LCC1/LCC2 and LCC3/LCC4 isozymes. Cordi et al. (2007) extracted two isoenzyme forms of laccase from *Trametes sp.* Three laccase isoenzymes Lac I, Lac II and Lac III from *C. unicolour* showed significantly varying biochemical characteristics (D'Souza-Ticlo et al. 2009).

2.9.2.3 Copper content of laccase enzyme

The measured copper content of the purified laccases varies between four and about two atoms per enzyme molecule (subunit). There are two reasons why the lower values must be regarded with caution. Purified preparations of some fungal laccases

include a fraction of molecules that are demonstrably damaged and therefore, unlikely to retain their full complement of copper. Secondly, as discussed by Reinhammar & Malmstrom (1981), although the copper ions in these proteins are regarded as firmly bound, selective depletion of one copper centre (type 2) can be achieved experimentally and may occur during purification. It has also been found that type 1 copper centres that lack a liganding methionine, as is true for all the fungal laccases, are relatively unstable (Karlsson et al. 1989). For the laccase of *Phlebia radiate,* a novel combination of prosthetic groups has been proposed (Karhunen et al. 1990; Saloheimo et al. 1991). This laccase is one in which about two atoms of copper per enzyme molecule are found and the claim is that these work in concert with a pyrroloquinoline quinine (PQQ) cofactor. This combination of copper and PQQ in fungal enzymes has since been questioned (Klinman et al. 1991; Maccarone et al. 1991). In addition, the laccases for which complete amino acid sequence has been deduced, including the *P. radiata* sequence, show conservation of the eleven residues involved in binding four copper atoms. The problem is accentuated by the presence of numerous compounds in some fungal culture fluids with similar spectroscopic properties to PQQ. At present, it seems that there is insufficient evidence to sustain any proposal other than those active laccase enzymes contain four copper atoms per molecule/subunit.

2.9.2.4 pH and temperature optima of laccase enzyme

The optimum pH value for laccases varies depending on the substrates employed, even though many reports have been reported a bell shaped profile for laccase activity. pH optima varies considerably due to reactions caused by substrate utilized, molecular oxygen or enzyme itself. The difference in redox potential between the phenolic substrate and the T1 copper could increase oxidation of the substrate at high pH values, but the hydroxide anion (OH^- binding to the T2/T3 centres. These two opposing effect can play an important role in determining the optimal pH of the biphasic laccase enzyme (Kunamneni et al. 2007). The optimal temperature of laccase can differ greatly from one strain to another.

Cordi et al. (2007) examined the effect of pH on activity and found to be in the range 3.0-8.0, using syringaldazine as substrate. L1 (isozyme of laccase) showed to have pH in acidic range, with an optima of pH 4.0, whereas the optimum pH for L2 was 5.0. Laccase enzyme extracted from *Trametes versicolor* by Han et al. (2005) exhibited high enzyme activity over broad pH and temperature ranges with optimum activity at pH 3.0 and a temperature of 50°C. Laccase purified from *Stereum ostrea* found to be active and stable at optimal pH 6.0 and temperature 40°C (Valeriano et al. 2009).

Stability experiments by Cordi et al. (2007) showed that L1 (isoenzyme of laccase) was stable at 60°C retaining 100% activity after 20 min of incubation while the amount of residual activity at 70°C amounted to 47%. On the other hand, the L2 isoenzyme was less stable retaining only 28.1% initial activity upon 20 min of incubation at 60°C. Two of the other isoenzymes exhibited an optimum temperature at 40°C and relative activities at 60 and 70°C were 65.0 and 37.0%, respectively.

Three laccases, Lac I, Lac II and Lac III enzyme extracted from mangrove (*Cerrena unicolour*) by D'Souza-Ticlo et al. (2009) of differing molecular masses were resolved by anion exchange chromatography. The optimum pH and temperature for Lac IId were 3.0 and 70°C respectively, the half-life at 70°C being 90 min. The enzyme was most stable at pH 9.0 and retained >60% of its activity up to 180 min at 50 and 60°C.

2.9.2.5 Substrate specificity and kinetic constants

Laccases can act on wide range of substrates. These enzymes catalyze one electron oxidation of a wide variety of organic and inorganic substrate, including poly-phenols, methoxy-substituted phenols, aromatic amines and ascorbate with the concomitant four electron reduction of oxygen to water (Kunamneni et al. 2007).

Li et al. (2008) compared three substrates; ABTS (2,2'-azinobis-(3-ethyl benzthiazoline-6-sulfonate), O-dianisidine and guaiacol to assay the laccase activity from *Trametes sp.* and obtained higher activity when ABTS was used compared to others. Tzialla et al. (2009) used various ternary systems consisting of monoterpenes (a-pinene

or D-limonene), *tert-butanol* and water as reaction media to enhance the catalytic performance of laccases from various fungi sources (*Trametes versicolor*, *T. hirsuta* and *Botrytis cinerea*).

The enzyme kinetic constants, Km and Vmax, vary from source to source, type of the substrate utilized and also other parameters used in experiment. Km values are in the range of 2-5000 μM. The Km values are different for laccases from different source organism having different substrate preference (Baldrian 2003).

The Km value of the enzyme from *Trametes versicolor* for substrate ABTS was 12.8 M and its corresponding Vmax value was 8125.4U/mg as reported by Han et al. (2005). Valeriano et al. (2009) reported that for laccase from *Stereum ostrea*, Km and Vmax values for the substrate guaiacol were found to be 13.25 mM and 255 nkat/ml of protein, respectively. In another report by D'Souza-Ticlo et al. (2009), for the isozyme Lac IId (isozyme of laccase form *C. unicolour*), specificity constant (Kcat/Km) of 120/min/μM was observed with ABTS at 70°C and pH 3.0.

Galai et al. (2009) isolated laccase from bacterium *Stenotrophomonas maltophilia* AAP56. The enzyme showed Km=53μM using syringaldazine, Km=700 μM using 2,2'-azino-bis(3-ethylbenzthiazoline-6- sulfonic acid) and Km=25 μM using pyrocatechol. The Lineweaver-Burk plot yielded a Km value of 28.6μM for L1 and 5 μM for L2 using syringaldazine as substrate for the enzyme purified from *T. versicolor* (Minussi et al. 2007).

2.9.2.6 Effect of inhibitors on enzyme activity

Different compounds have been reported as inhibitors of laccase. Among them, anions like azide, cyanide and fluoride inhibit laccase by binding to T2/T3, thus preventing electron transfer from T1. Other inhibitors like metal ions, fatty acids and quaternary ammonium detergents replace or chelate the copper centers and may also denature the protein (Witayakran and Ragauskas 2009).

Laccase respond to inhibitors. They may inhibit enzyme activity either by binding to Type 2 or 3 copper, resulting in the interruption of internal electron transfer (metal ions such as azide halides, cyanides), amino acid modification, conformational changes of Cu chelation (metal ions, fatty acids, kojic acids), selective removal of Cu by chelating agents (EDTA and dimethyl glyoxime). *Trametes versicolor* laccase was inhibited by copper-chelating agents like sulphamic acid, oxalic acid, hydroxylammonium chloride, malonic acid, citric acid and EDTA (Lorenzo et al. 2005), whereas *Fusarium sp*. BOL35 and *Galerina sp.* HC1 laccases were barely inhibited by EDTA (Terrazas 2005).

Among the various inhibitors tested by D'Souza-Ticlo et al. (2009) for Lac IId, isozyme (isozyme of laccase from *C. unicolor*), activity was inhibited in the presence of sodium azide, SDS and mercaptoethanol. Approximately 56 and 48% of Lac IId activity was inhibited in the presence of Cr and W, whereas in the presence of Sn, Ag and Hg, the inhibition was only 32-37%. The other metal ions did not show significant inhibition. Dube et al. (2008) observed that ethylene diamine tetraacetic acid (EDTA) (5mM) totally inhibited laccase activity. EDTA, SDS and arginine showed prominent inhibitory effect on laccase activity (Valeriano et al. 2009).

Table 2.4. enlists the biochemical characteristics of purified laccases from different fungal sources.

Table 2.4 Bio-characteristics of some major fungal laccases

Organism	Nature	M.W	pH Optima	Temperature Optima (^{o}C)	Glycosy -lation	Cu atoms	References
Basidiomycete PM1(CECT 2971)	Monomeric	64	4.5	80	6.5	4	Coll et al. (1993)
Fungi Chaetomiceae	Monomeric	73-80	7.0	42	n.r.	4	Saito et al. (2003)
A.occhraceus NCIM-1146	Monomeric	68	4.0	60	n.r.	n.r.	Telke et al. (2010)
P.ostreatus	Monomeric	40, 72	6.0	45	n.r.	3.66	Kumar et al. (2011)
Schyzophyllum commune IBL-06	Monomeric	63	6.0	40	n.r.	n.r.	Irshad et al. (2011)
Myrothecium verrucaria NF-05 (white laccase)	Monomeric	66	4.0	30	n.r.	3 (1Fe)	Zhao et al. (2012)
Phellinus ribis	Dimeric	76	5.0	37	28	1(1Mn,2Zn)	Min et al. (2000)
Coriolopsis rigida LacI	Monomeric	66	2.5	n.r	9	n.r.	Saparrat et al. (2002)
Coriolopsis rigida LacII	Monomeric	66	3.0	n.r.	9	n.r.	
Peniophora sp.UD4	Monomeric	62	6.5	70	n.r.	n.r.	Justin (2011)
Neurospora crassa	Monomeric	64.8	n		11	3.3	Frochner and Eriksson (1974)
Pleurotus sp.	Monomeric	40	4.5	5.0	n.r.	n.r.	More et al. (2010)
Cerrena Versicolor	Monomeric	57.61	n.r.	n.r.	n.r.	2.8	Kim et al. (2002)

P.cinnaba-rinus	LacI	Monomeric	81	n.r.	n.r.	n.r.	n.r.	Otterbein et al. (2000)
	Lac II	Monomeric	86	n.r.	n.r.	n.r.	n.r.	
Agaricus blazei		Monomeric	66	5.5	20	n.r.	n.r.	Ullrich et al. (2004)
Pleurotus Eryngii	Lac I	Monomeric	65	4.0	65	7	n.r.	Munoz et al. (1997)
	Lac II	Monomeric	61	3.5	55	1	n.r.	
Trametes trogii	Lac I	Monomeric	62	2.5	50	n.r.	n.r.	Zourari-machichi et al. (2006)
	Lac II	Monomeric	62	2.5	50	n.r.	n.r.	
T.trogii BATC463	Lac I	Monomeric	60	3.4	30	n.r.	n.r.	Levin et al. (2002)
	Lac II	Monomeric	38	3.4	30	n.r.	n.r.	
Botrytis cinerea 61-34		Monomeric	74	3.5	60	49		Slomczynski et al. (1995)
Ganoderma lucidum		Monomeric	40&60	n.r.	n.r.	n.r.	n.r.	D'souza et al. (1999)
T.versicolor		Monomeric		4.5	40	n.r.	n.r.	Stoilova et al. (2010)
P. sanguineus	LacI	Monomeric	80	4.8	n.r.	n.r.	n.r.	Garcia et al. (2006)
	LacII	Monomeric	68	4.2	n.r.	n.r.	n.r.	
T.hirsuta Bm-2		Monomeric	65	4-4.5	40-60	n.r.	n.r.	Zapata-Castillo et al. (2012)
Loweporus lividus MTCC-1178			64.8	5.0	60	n.r.	n.r.	Sahay et al. (2009)
Odontotermes formosanus		Monomeric	65	4.0	10-30	n.r.	n.r.	Zaou et al. (2010)
T.versicolor		Monomeric	97	3.0	50	n.r.	n.r.	Han et al. (2005)

951022								
P.ostreatus D1		Monomeric	64	4.0	n.r.	n.r.	n.r.	Pozdyakova et al. (2006)
Cerena Unicolour	Lac Ia1		45	5.5	50	3.5	3.88	Rogalski and Janusz (2010)
	Lac ia2		47	5.5	50	1.8	3.67	
	Lac Ib		54	5.5	50	1.84	4.21	
	Lac IIa1		62	5.5	50	1.65	3.71	
P.ostratus (White laccase)	POXA1	Monomeric	61	3.0	65	3	0.7(1Fe,1Zn)	Palmieri et al. (1997)
	POXA2	Monomeric	67	3.0	60	9	3.3	
	POXC	Monomeric		3.0	35	n.r.	3.7	
Physisporinus rivulosus	Lac 3.5	Monomeric	65	2.0	60	n.r.	n.r.	Hilden et al. (2007)
	Lac 4.8	Monomeric	67	2.0	60	n.r.	n.r.	
C.unicolour strain 137	Lac I	Monomeric	64	2.5	25	n.r.	n.r.	Michniewicz and Ullrich (2005)
	Lac II	Monomeric	57	3.0	50	n.r.	n.r.	

Chapter 3

ISOLATION, IDENTIFICATION AND SCREENING OF INDIGENOUS BASIDIOMYCETES FOR LMEs PRODUCTION

3.1 Introduction

The use of microbial enzymes in the diverse field of industrial applications has been of greater importance in recent years. Many of such potential enzymes are widely distributed in nature. Lignin modifying enzymes (LMEs) are one among them. LMEs are complex set of extracellular enzyme constituting laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP) (Hammel 1997). These enzyme systems exhibit differential characteristics depending on the species, strains and culture conditions (Kirk and Farrell 1987; Eggert et al. 1996).

For more than fifty years, one of the main areas of biotechnology research on lignocellulose has been driven with the need of isolating and identifying microorganisms which could either enzyme hyper-producers and/or sufficiently robust to withstand conditions of the intended application. The research also aimed to isolate and identify the organisms which are novel to produce lignocellulolytic enzymes. In terms of enzyme novelty from an applications perspective, interest is focused not only on finding enzymes which could break down lignocellulose much more rapidly but also on enzymes which could withstand pH, temperature and inhibitory agents more resiliently depending on the intended application. Production of such enzymes has already been reported in large number of micro-organisms, including bacteria (McCarthy 1987; Vicuna 1988; Zimmermann 1990), fungi (Kirk and Farrel 1987, Falcon et al.1995; Hatakka 2001; Baldrian and Gabriel 2003) and Actinomycetes (Vicuna 2000). In the fungal kingdom, a majority of LMEs producing organisms belongs to the group of white rot Basidiomycetes, many of which have been well studied and characterized (Ghosh et al.1993).

There is very little information available with regards to the production of extracellular oxidoreductases by autochthonal fungal strains belonging to different eco-physiological and taxonomic groups (Dhouib et al. 2005). The attention on the applications of lignin degrading enzymes has led to the discovery of many new enzymes with novel characteristics from various microorganisms. Hence, in this study we aimed

on i) isolating autochthonal fungal strains from a village forest of Mangalore district, Karnataka, India ii) screening of these fungal isolates for their ligninolytic activities and iii) selecting efficient enzyme producing strains for further study on their enzyme production and enzyme characteristics.

3.2 Materials and Methods

3.2.1 Collection of Basidiomycetes

Fungal samples were collected from a village forest of Mangalore, Karnataka, India. Fruiting bodies were collected from their respective habitats like decaying wood, plant litter, soil debris and also from live tree trunk. In some cases, wood logs showing the signs of mycelial growth were also collected for fungal isolation. The collected samples were placed in clean and sterile plastic bags and labeled. While collecting the fruiting bodies, various parameters like colour, length, shape, gills, texture etc., were noted down for possible fungal identification.

3.2.2 Isolation of the fungi

The collected fungal samples were taken to laboratory, washed with the sterile water to remove the adhered soil and spores of other lower fungi. The samples were cut into small pieces which were further surface sterilized with the 0.01% $HgCl_2$. These samples were again washed with sterile water and dried. 2% (w/v) MEA media was used for the isolation. Isolation was carried out by placing fungal samples on 2% (w/v) malt extract agar media supplemented with chloramphenicol (250 mg/l) (Appendix Ia). The plates were incubated at 25-28^{o}C for 7 to 10 days under dark conditions. Colonies of different morphotypes were isolated and sub-cultured on similar media. The purity of isolates was checked and confirmed by light microscopy observations.

3.2.3 Identification of the cultures

The collected fungi were identified based on their fruiting body morphology. The following features were considered during the identification process *viz*; texture (soft and

fleshy, gelatinous, cartilaginous, brittle, corky, leathery, or woody; dry, moist, or sticky; upper surface smooth, velvety, hairy or scaly), colour of internal tissues (context and spore bearing component); white, pale brown, dark brown or other (e.g. black, red), form (pileate - projecting out from the substrate surface, resupinate - forming a flat sheet or crust, or effused-reflexed-forming a shelf with the base extending down over the substrate as a flat sheet; if pileate: simple or compound; stalked (stipitate) or sessile; solitary or clustered - possibly imbricate, i.e. several shelves overlapping one above the other); if stalked, attached at the side or centrally), spore and basidia-bearing surface (smooth, folded or warty, usually forming the fruit body under surface (e.g. corticioid fungi); lining vertical gills or lamellae (e.g. agaric fungi; *Lenzites*, *Panus*, *Pleurotus* species); lining vertical, downward-directed pores (e.g. polypore fungi, boletes); on vertically hanging teeth or spines (hydnoid fungi); on erect branches (e.g. ramarioid fungi), etc. Dimensions (size of fruiting body, width of context, pores, lamellae) and microscopic features of the basidiocarps were considered.

3.2.4 Screening for Ligninolytic enzymes on solid media

Solid agar medium containing indicator compounds were used to detect the ability of the fungal strains to produce lignin-modifying enzymes. Different procedures were followed for screening laccases, lignin peroxidases and manganese peroxidases.

For the screening of lignin peroxidase (LiP) and manganese peroxidase (MnP) activities, dye decolourization method was followed. Screening was performed in Petri dishes (90 mm diameter) containing 15 ml of solid LBM (ligninolytic basal medium) supplemented with 0.01% of Azure-B (Appendix Ic). The composition of LBM medium was (g/l); KH_2PO_4(1.0), yeast Extract (0.01), $C_4H_{12}N_2O_6$(0.5), $CuSO_4.5H_2O$(0.001), $MgSO_4.7H_20$(0.5), $Fe_2(SO_4)_3$(0.001), $CaCl_2.2H_2O$ (0.01), $MnS0_4.H_2O$ (0.001). Plates were inoculated with agar disks (0.5 mm diameter) of active mycelia previously cultured in 2% malt extract agar and were incubated at 28-30°C for 10-20 days. The plates were observed periodically for mycelial growths and dye decolourization hallows. Decolourization of the dye Azure-B by fungi has been positively correlated with

production of lignin peroxidase and Mn dependent peroxidase; however this dye is not a substrate for laccase (Archibald 1992; Pointing 1999).

The ability of the fungal strains to produce extracellular laccase activity was screened by using an indicator compound. Guaiacol was used as an indicator compound, the oxidation of which by the enzyme results in the production of reddish coloured intermediate. Screening was performed in Petri dishes (90 mm diameter) with 15 ml of LSM (Laccase Screening media) (Appendix Ib) supplemented with 0.01% guaiacol (Sigma). The composition of LSM was (g/l): peptone (3.0), glucose(10.0), 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), agar (20.0) (pH-6) and guaiacol (0.2). Plates were inoculated with agar disks (0.5 mm diameter) of active mycelia previously cultured in 2% malt extract agar and were incubated at 28-30°C for 10-20 days. The plates were observed periodically for mycelial growths and reddish brown coloured hallow zones. Colonies that showed reddish brown zones in the media that exceeded the colony diameter were considered as laccase hyperproducers.

3.2.5 Production of ligninolytic enzymes in liquid cultures

Decolourizing Azure-B dye and guaiacol oxidizing strains were screened further on liquid cultures for LiP, Mn^{2+}-oxidizing peroxidases and laccase enzymes productions. For laccase activity determination, fungi were grown on modified LMM medium (Appendix Id). Composition of modified LMM medium was (g/l): glucose (10.0), ammonium tartarate (2.0), KH_2PO_4(1.0), $MgSO_4.7H_2O$(0.5), KCl(0.5), yeast extract (1.0), $CuSO_4.5H_2O$ (150µM), and 10 mg/l of trace elements containing per litre of distilled water: EDTA (0.5g), $FeSO_4$(0.2g), ZnSO4.$7H_2O$ (0.01g), $MnCl_2.4H_2O$ (0.003g), H_3BO_4(0.03g), $CoCl_2.6H_2O$ (0.02g), $CuCl_2.2H_2O$ (0.001 g) and $Na_2MoO_4.2H_2O$ (0.003g). Mycelial agar plugs (4 plugs of 7mm diameter) were cut along the edge of the actively growing colony and used as the inocula. The fungi were cultivated in 30 ml of medium in 100ml Erlenmeyer flasks at 30°C on a rotary shaker (150 rev/min) for 10

days. The contents of the flasks were centrifuged to separate the biomass. The filtrate was analyzed for laccase activity.

For LiP and MnP's determination, the isolated fungi were grown at 30°C for 12 days in static conditions in 250-ml Erlenmeyer flasks containing 30 ml of a (SMM) (Appendix Ie). The composition of the medium was: glycerol (10), ammonium tartarate (1.84), sodium tartarate (2.3), KH_2PO_4(2), $MgSO_4.7H_2O$ (0.7), $CaCl_2.2H_2O$ (0.14), $FeSO_4.7H_2O$ (0.07), $ZnSO_4.7H_2O$ (0.046), $MnSO_4.7H_2O$ (0.035), $CuSO_4.5H_2O$ (0.007), thiamine (0.0025), yeast extract (1.0), veratryl alcohol (0.067) and tween80 (0.5). Mycelial agar plugs (4 plugs of 7mm diameter) were cut along the edge of the actively growing colony and used as the inocula. After incubation, the contents of the flasks were centrifuged and mycelia- free filtrate was analyzed for LiP and MnP activities.

3.2.6 Assay for enzyme activities

3.2.6.1 Laccase assay

ABTS oxidation method was used for the laccase activity measurements. This method is commonly used for the determination of laccase activity. This method was developed by Bourbonnais and Paice (1990). The reaction mixture consisted of sodium acetate buffer (0.1M, pH 4.5), ABTS (50 mM) and 1.5 ml culture filtrate. The absorbance of the cation radical was monitored at 420nm ($€_{mM}$=36/mM/cm) at room temperature using UV-Vis spectrophotometer (GBC). Enzyme activity was expressed as international units (IU) where 1 IU is defined as the amount of enzyme forming 1umole of product per minute. The laccase activity in U/ml (µmol cation radical released/min/ml enzyme) was calculated.

3.2.6.2 Lignin Peroxidase assay

Lignin-peroxidase (LiP) activity measurement was based on the oxidation of veratryl alcohol (VA) to veratraldehyde in the presence of H_2O_2 (Bonnen et al.1994). The assay solution contained 2 mM VA in 25 mM tartarate buffer of pH 2.5 and was initiated

by the addition of 0.4 mM H_2O_2. The change in absorbance was monitored at 310 nm. One unit of enzyme oxidizes 1umol/min VA to veratraldehyde ($€_{310}$= 9,300/M/cm).

3.2.6.3 Manganese Peroxidase assay

MnP activity was estimated using phenol red as a substrate. The reaction mixture consisted of 0.01 % phenol-red (Sigma) in 100 mM sodium tartarate buffer of pH 5.0 (Michel et al. 1991), in presence of 0.1mM H_2O_2 and 1mM $MnSO_4$. One unit of enzymatic activity was defined as the amount of enzyme transforming 1μmol of substrate per minute.

3.3 Results and Discussion

3.3.1 Isolation and identification of Basidiomycetes

The Basidiomycota (colloquially basidiomycetes) are a large group of fungi with over 30000 species. They include many familiar mushrooms and toadstools, bracket fungi, puffballs, earth balls, earth stars, stinkhorns, false truffles, jelly fungi and some less familiar forms. Most basidiomycetes are terrestrial with wind-dispersed spores, but some grow in freshwater or marine habitats. Many are saprotrophic and are involved in litter and wood decay. Common woodland mushrooms such as species of *Amanita, Boletus* and their allies grow in a mutually symbiotic relationship with the roots of trees, forming ectotrophic (sheathing) mycorrhiza. As saprotrophs, basidiomycetes play a vital role in recycling nutrients by decaying wood components. In our study, we collected fruiting bodies and/or mycelia of different basidiomycetes from various habitats for their isolation. The fungi were found growing on the dead wood, leaf litter and other saprophytic conditions. Sometimes, vegetations showing the sign of mycelia growths such as twigs, branches and leaves were also collected for isolating fungi. During collection of basidiocarps, the sample characteristics such as colour and nature of the fruiting body, growth conditions, habitats of the fungi, dimensions of fruiting body, etc., were noted down for their possible identification process. Isolation of fungi were carried

out during rainy season as most of the fungi grows and fruits during this season due to the favorable conditions of moisture and temperature.

Total 45 fungi were successfully isolated on MEA media. The fungi were designated with the code words ranging from hpF-01 to hpF-45. Fig. 3.1 shows the photographs of the collected fungal samples. These strains were identified by comparing their physical characteristics like the form of the fruiting bodies, location of pileus, hair present on the pileus (Appendix III) (Krieger 1967; Zoberi 1972; Alexopoloulose et al. 1979; Hall et al. 2003; Kuo 2007). The characteristics of the fungal samples are listed in Table 3.1. Based on the physical characteristics, some mushrooms were identified to their generic levels (Table 3.1).

The fungi such as hpF-01, hpF-07, hpF-08, hpF-11, hpF-12, hpF-19 and hpF-21 were collected from soft woods which were almost degradred. In many of these fungi, no fruiting body was seen; instead thick mycelial mat on the surface of the wood was observed. These mycelial mats were cut into smaller pieces, washed and surface sterilized and used for their isolation. The fungi such as hpF-04, hpF-09, hpF-10, hpF-16, hpF-26, hpF-28, hpF-29, hpF-31, hpF-34, hpF-36, hpF-39, hpF-40, hpF-41, hpF-42, hpF-43, hpF-44 and hpF-45 were found growing on hard dead woods which were about to undergo decay process. Most of these fungi found with distinct fruiting bodies, with or without stipes, varying in sizes and colours, with tubular (poroid) gills, found growing laterally on their substrata. Many of these fungi were identified as *Polyporous sp*.

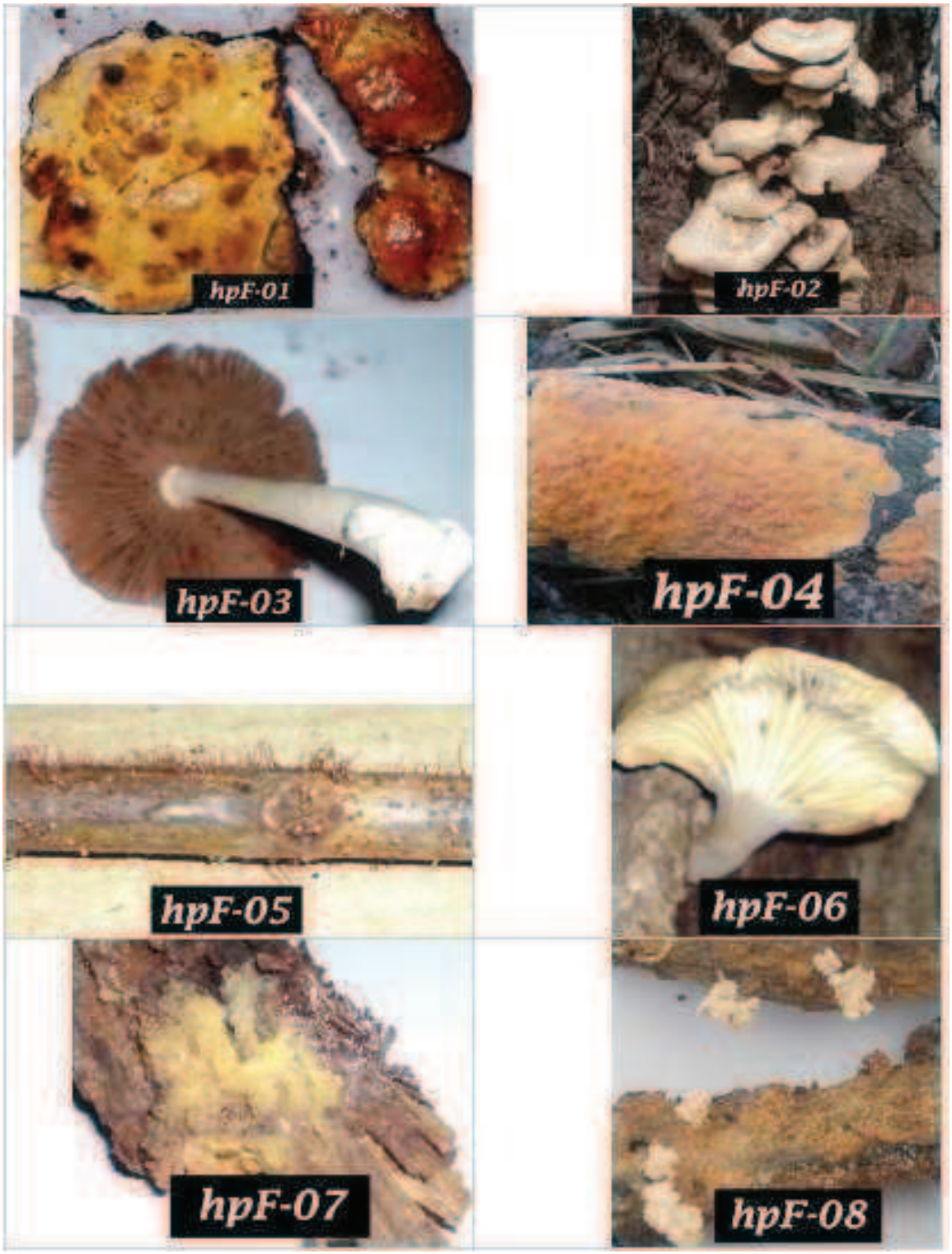

Fig. 3.1. Photographs of the fungal samples collected for isolation

Fig. 3.1. Photographs of the fungal samples collected for isolation (cont..)

Fig. 3.1. Photographs of the fungal samples collected for isolation (cont..)

Fig. 3.1. Photographs of the fungal samples collected for isolation (cont..)

Fig. 3.1. Photographs of the fungal samples collected for isolation (cont..)

Fig. 3.1. Photographs of the fungal samples collected for isolation (cont..)

Table 3.1 Characteristics of the isolated fungi, habitat and their possible identification				
Sl.No	**Code**	**Characteristics**	**Habitat**	**Identification**
1	hpF-01	No fruiting body was seen. In the stage of development. Only thick mycelial mat or mass was observed on decayed wood.	Soft wood	Unidentified
2	hpF-02	Found on decaying wood with funnel shaped fruiting body, pale tan to creamy yellowish, with brownish scales that are radially arranged, with tough lateral stem having pores underneath the cap.	Soft wood	*Polyporus craterellus*
3	hpF-03	Found in dry condition on soil, with predominant fruiting body or pileus, umbonate, with lammelloid and seceding gills underneath, supported by a long stipe (stalk), without hairs and annulus (ring).	Soil	Unidentified
4	hpF-04	Resupinute fruiting body, forming thin irregular patches found on sticks, dark brown in colour.	Wood sticks	Unidentified
5	hpF-05	Tiny morphology, hair like structures found on twig, with small minute round cap and narrow stem in groups.	Stick	Unidentified
6	hpF-06	Broadly convex fruiting body, purple brown in colour, gills running down the stem, tough and small stem with minute hairs.	Live tree	Likely *Panus sp.*
7	hpF-07	Brown coloured, powdery spores were found on hard wood.	Hard wood	Unidentified
8	hpF-08	Brownish fruiting bodies with repeatedly branched, flattened and cristate found on hard wood.	Hard wood	Likely *Clavulina sp.*

9	hpF-09	Ear shaped, jelly like fruiting body, irregular, gathered together, brown coloured, gelatinous found on twig.	Hard wood	*Auricularia sp.*
10	hpF-10	Fruiting bodies in clusters found on dead wood log, depressed or funnel shaped, pale brown with wavy margin (dried), gills lamellate, running down the laterally stem, hairy near the base.	Hard wood	*Pleurotus sp.*
11	hpF-11	No cap was found, mycelial mass was observed on wood debris.	debris	Unidentified
12	hpF-12	Concave fruiting body, yellowish in colour, curved margin, irregular, lamellate gills running down the small lateral stem.	debris	Unidentified
13	hpF-13	Found growing in between the rocks with convex cap, irregular margin and small fruiting body, lammelloid gills supported by small stipe, without a ring.	Soil	Unidentified
14	hpF-14	Brown in colour, both cap and stem, tiny cap but long thin stem.	Leaf	*Marasmius sp.*
15	hpF-15	Tiny brown cap, convex, black stem without ring found on leaf debris.	Leaf	*Marasmius sp.*
16	hpF-16	Tiny umbonate fruiting body, brown in colour, long stem, with ridges underneath, found on hard wood.	Stick	*Marasmius sp. likely*
17	hpF-17	Fruiting body found on trunks, single without, but was attached to substrate by a broad base like a shelf. Flattened half circled pileus with dark reddish brown upper surface. The margin is white and blunt or rounded at the apex.	Hard wood	*Phellinus sp.*
18	hpF-18	With smooth bay brown or chestnut coloured, umbillicate cap or	Soil	*Boleotus sp.*

		pileus, with tube terminating into pores, brown sufficiently lengthy stem or stipe without ring.		
19	hpF-19	Fruiting bodies in clusters found on wood debris, depressed or funnel shaped, pale brown with wavy margin (dried), gills lamellate, running down the laterally stem, hairy near the base.	Debris	*Pleurotus sp.*likely
20	hpF-20	White surface, with depressed /flat cap, brownish stem without a ring found in soil.	Soil	Unidentified
21	hpF-21	Funnel-shaped fruit bodies with dark lids on woody debris; the lid opens to reveal the egg-like peridioles. Each peridium or 'nest' contains typical four or five silvery flattened 'eggs.' The outside of each peridium is covered with fine grey-brown hairs; the inner surface, in contrast, is smooth and hairless.	Woody debris	Birds net fungi
22	hpF-22	Found with a gasteroid (enclosed), ovate fruiting body, reddish brown in colour, smooth surface with dark pink warts on surface, yellow elongated tubes opens into pores, with long brown stem, found on soil.	Soil	unidentified
23	hpF-23	Small, solitary fruiting body with central depression or vase shaped with gills running down the stem, close, tough and lateral stem with minute hairs found on a live tree.	Live tree trunk	Likely *Panus sp*.
24	hpF-24	White convex fruiting body or cap, found growing in clusters, usually 5-6 in numbers, smoothy texture with brown scales on the surface,	Soil	Unidentified

		brownish stem with an annulus.		
25	hpF-25	Ovate brown fruiting body, white stem without ring, covered with brown powdery spores.	Soil	Unidentified
26	hpF-26	Funnel shaped cap, brownish in colour, with a central depression, wavy margin (dry), with different shades of colouration, underneath the cap lamellate gills, running down the lateral stem.	Hard wood	Unidentified
27	hpF-27	Voilet coloured, deeply depresses, funnel-shaped fruting body, vevety, shining, with irregular margins lamellate gills underneath.	Soil	Unidentified
28	hpF-28	Small minute, white coloured fruiting body with lamellate gills running down the stem, found attached to a wood branch.	Wood branch	*Crepidotus sp.*
29	hpF-29	Orange yellow, jelly types, small cushion and slightly flattened fruit body found on hard wood.	Hard wood	*Calocera sp.*
30	hpF-30	Brownish fruiting bodies with repeatedly branched, flattened and cristate structure.	Live tree trunk	Unidentified
31	hpF-31	White coloured, small cushion and slightly flattened fruit body found on hard wood.	Hard wood	Unidentified
32	hpF-32	No fruiting body/cap developed, a thick mycelial smooth, pale flesh was observed on hard wood	Hard wood	Unidentified
33	hpF-33	The sample was dried, funnel shaped fruiting body, pale yellow in	Live tree	*Polyporous sp.*

		colour, wavy margin, pores underneath, with a lateral stem found attached on live tree branch	twig	
34	hpF-34	Concave, large fruiting body, brown coloured with different shades, hard flesh, with pores, without stem collected from hard wood	Hard wood	*Tramatus sp.*
35	hpF-35	Well developed broad cap, convex, tough with velvety surface, brown to violet colour, folded over edge, with zones of colouration without a stem found on hard wood	Hard wood	*Ischnoderma sp.*
36	hpF-36	Fruiting bodies in clusters found on dead wood log, depressed or funnel shaped, pale brown with wavy margin (dried), gills lamellate, running down the laterally stem, hairy near the base.	Hard wood	*Pleurotus sp.*
37	hpF-37	Deeply depressed, funnel shaped fruiting body, dark brown in colour during fresh, gills underneath, with long bent stem, hairy without ring or annulus, found in groups.	Debris.	Unidentified
38	hpF-38	Large fruiting body, reddish brown in colour, with irregular curved fruiting body, thin with different zones of colouration on the surface, with pores underneath, smooth texture. Found on hard wood.	Hard wood	*Trametes sp.*
39	hpF-39	Convex cap, reddish brown in colour, covered with scales, inrolled margin, gills attached to the stem, found on hard wood	Hard wood	*Gymnopillus sp.*
40	hpF-40	Fruiting bodies in clusters found on dead wood log, depressed or funnel shaped, pale brown with wavy margin (dried), gills lamellate,	Decaying wood	Unidentified

		running down the laterally stem.	debris	
41	hpF-41	Large compound fruiting bodies due to overlapping , brownish cap with concentric zones of colouration, found on hard wood	Hard wood	*Polyporous sp.*
42	hpF-42	Slightly large fruiting body, hard, white coloured fruiting body, without a stem, with pores underneath, found on a hard wood.	Hard wood	*Daedaleopsis sp.*
43	hpF-43	Funnel shaped fruiting body dark reddish colour (dried sample was collected) with enrolled and wavy margins, leathery flesh but was very hard due to dry weather with white tubes beneath the cap, found on fallen branch	Tree branch	*Microporus sp.*
44	hpF-44	Small tiny fruiting bodies, which are overlapped to form a compound structure, white in colour, without colouration patches, shrinked in some regions, smooth surface found on hard wood.	Hard wood	*Polyporous sp.*
45	hpF-45	Large fruiting body, reddish brown in colour, with irregular curved fruiting body, thin with different zones of colouration on the surface, with pores underneath, smooth texture. Found on hard wood.	Hard wood.	*Polyporous sp*.

3.3.2 Screening of isolates for LMEs Production

Screening of LMEs producing species and their variants is important for selecting suitable LMEs producing strains. Screening for oxidative enzymes or mediators involves the investigation of many samples, as there are many parameters that have to be considered. For this reason one usually relies on the use of inexpensive, rapid and sensitive testing methods. Microbes that produce LMEs have been screened either on solid media containing coloured indicator compounds that enable the visual detection of LMEs production (Nishida et al. 1988, Barbosa et al. 1996) or with liquid cultivations monitored by enzyme activity measurements (Kiiskinen et al. 2004; Vishwanath et al. 2008). The use of coloured indicators is generally simpler as no sample handling and measurement is required. Since LMEs oxidizes various types of substrates, several different compounds have been used as indicators for detecting LMEs production. Similar strategy was followed in our study for screening the isolates for the LMEs production. The isolates which were considered as predominant LMEs producers under solid screening tests were further employed in liquid cultivations for quantification of enzyme production.

In the present study, pure cultures of 45 indigenous fungal strains, isolated from a village forest of Mangalore, Karnataka, India were screened for azure-B decolourization and guaiacol oxidation using solid agar plates. Among them, 12 strains exhibited dye decolourization within 4-20 days of incubation in LBM plates and 24 strains showed the oxidation of guaiacol in LSM plates. Laccase when excreted by the fungus oxidizes the guaiacol supplemented through the medium (Fig. 3.2). The oxidized product of guaiacol is reddish brown in colour which indicates the presence of laccase. On the other hand, LiP and MnP acts on azure B dye and decolourize the dye to a non-colour substrate. As a results clear zones appears on the plate if the fungi excretes LiP and MnPs (Fig. 3.3) (Gold et al. 1988; Nishida et al. 1988; Barbosa et al. 1996).

Positive fungal cultures considered as most promising strains for the production of ligninolytic enzymes are shown in Table 3.2.

Table 3.2 Most promising strains observed for guaiacol oxidation and dye decolourization

Isolate	Substrate/habitat	Genus	Guaiacol oxidation	Dye decolou--rization
hpF-02	Dead wood	*Polyporous sp.*	3.5/3 (6)	+(15)
hpF-04	Hard wood	*Peniophora sp.*	4.75/4 (4)	
hpF-10	Tree trunk	*Polyporous sp.*	5/5 (4)	+ (17)
hpF-15	Leaf litter	*Marasmius sp.*		+ (15)
hpF-16	Decayed wood	*Pleurotus sp.*	5/5 (4)	+(10)
hpF-17	Living tree trunk	*Phellinus sp.*	2.5/2 (4)	+(15)
hpF-30	Tree trunk	*Perenniophora sp.*		+ (10)
hpF-31	Decayed wood	*Unidentified*	5/5 (8)	+ (15)
hpF-33	Living tree trunk	*Panus sp.*		+ (5)
hpF-34	Tree trunk	*Trametes sp.*		+ (6)
hpF-35	Tree trunk	*Trametes sp.*	5/5 (4)	+(10)
hpF-37	Decayed wood	*Unidentified*	4/4.5 (8)	+(10)
hpF-38	Living tree trunk	*Polyporous sp.*		+(15)
hpF-39	Hard wood	*Unidentified*	4/4 (8)	+(10)
hpF-42	Decayed wood	*Daedaleopsis sp.*		+(15)
hpF-43	Living tree twig	*Microporous sp.*	3.75/4 (6)	+(12)
hpF-44	Decayed wood	*Polyporous sp.*		+ (6)

Ratio in brackets: (diameter of the reddish brown zone/diameter of the colony); Values in parentheses: day on which ratio is maximum and complete decolourization of azure-B was observed

A strain was qualified as a high extracellular guaiacol oxidizing activity producer if the reddish brown colour appeared in the first look of incubation and the ratio

(diameter of the colour zone/diameter of the colony) was greater than 1. Among the 24 strains exhibiting guaiacol oxidation, only 5 were estimated as laccase hyper producers and were chosen for detailed investigations in liquid cultures.

In the present investigation, most of the fungi showing laccase activities rather than LiP and MnPs activities were isolated. This indicates that laccase production is relatively common in the wood-rotting fungi tested. The literature also supports our findings that laccase is produced by most white-rot fungi, but it depends on culturing conditions (Jong et al. 1994; Risna and Suhirman 2002). However, the varying intensity in colour production in the plates could be due to different laccase activity values of the isolates. This indicates that the ability of the isolates to produce laccases varies, i.e., it is dependent on either the isolates or strains. These differences between variations in laccase production probably may be due to the differences in their physiological functions as described by Eggert et al. (1996).

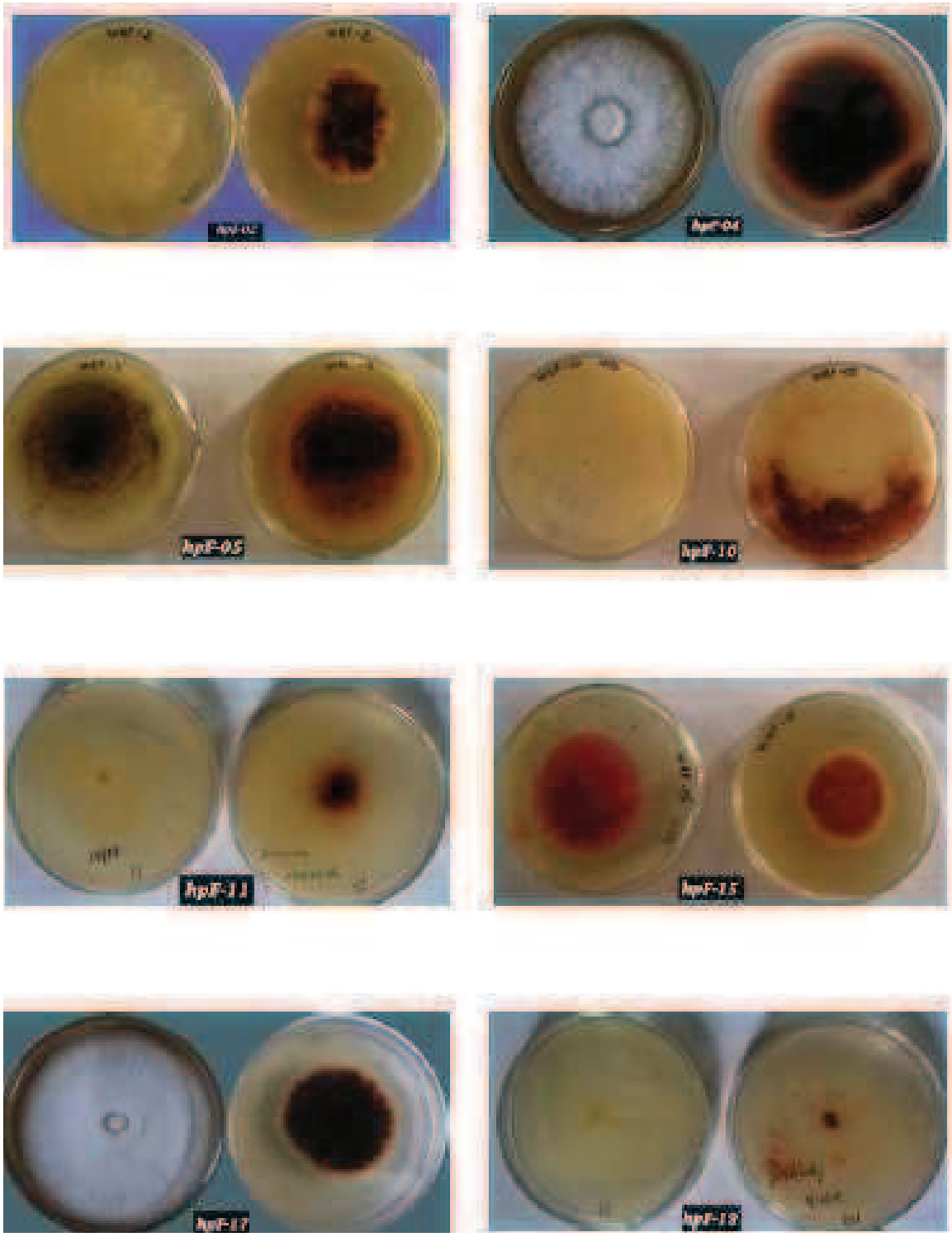

Fig. 3.2 Oxidative polymerization of guaiacol to form reddish brown zones due to laccase production by the isolates during screening experiments

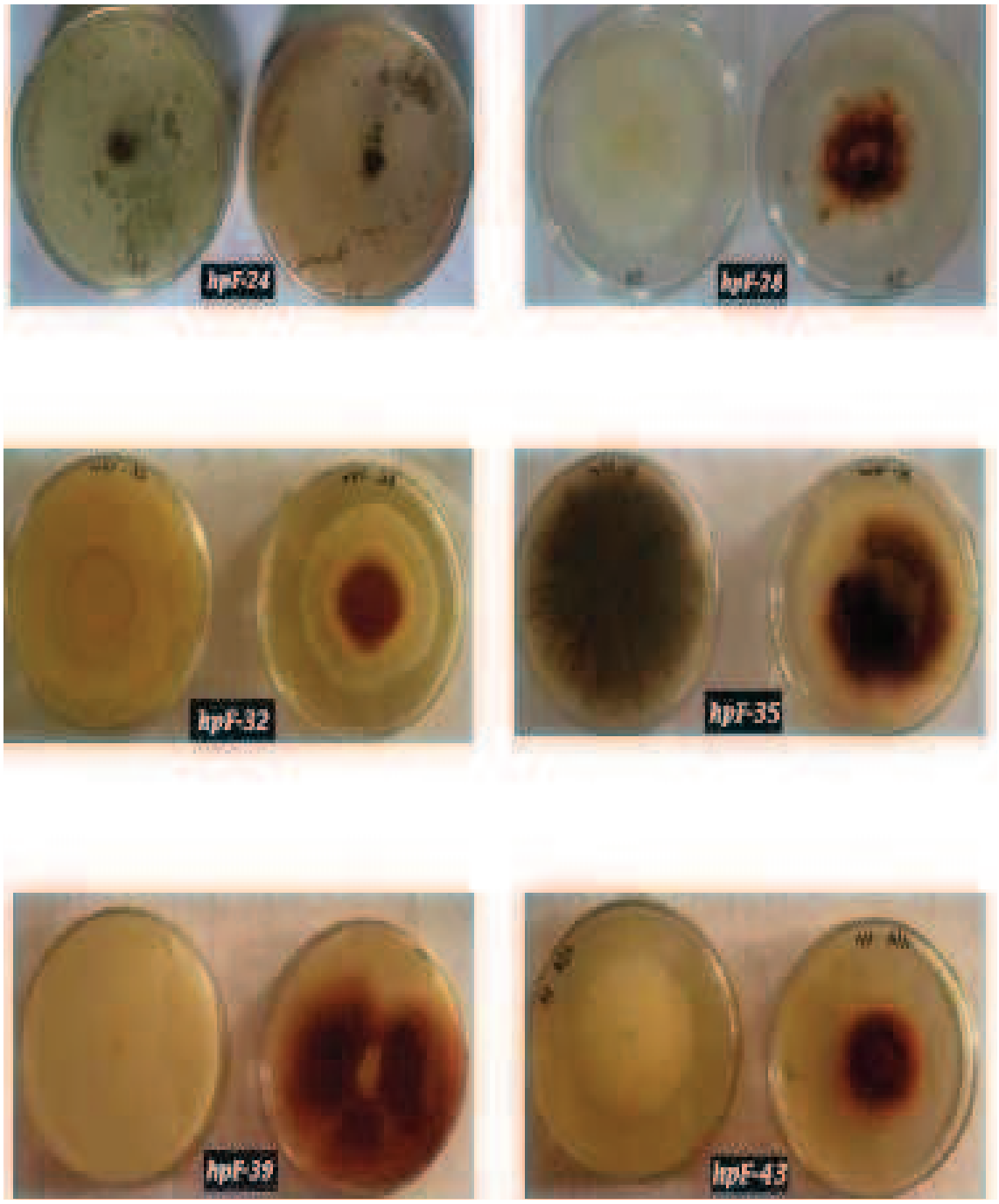

Fig. 3.2 Oxidative polymerization of guaiacol to form reddish brown zones due to laccase production by the isolates during screening experiments (cont..)

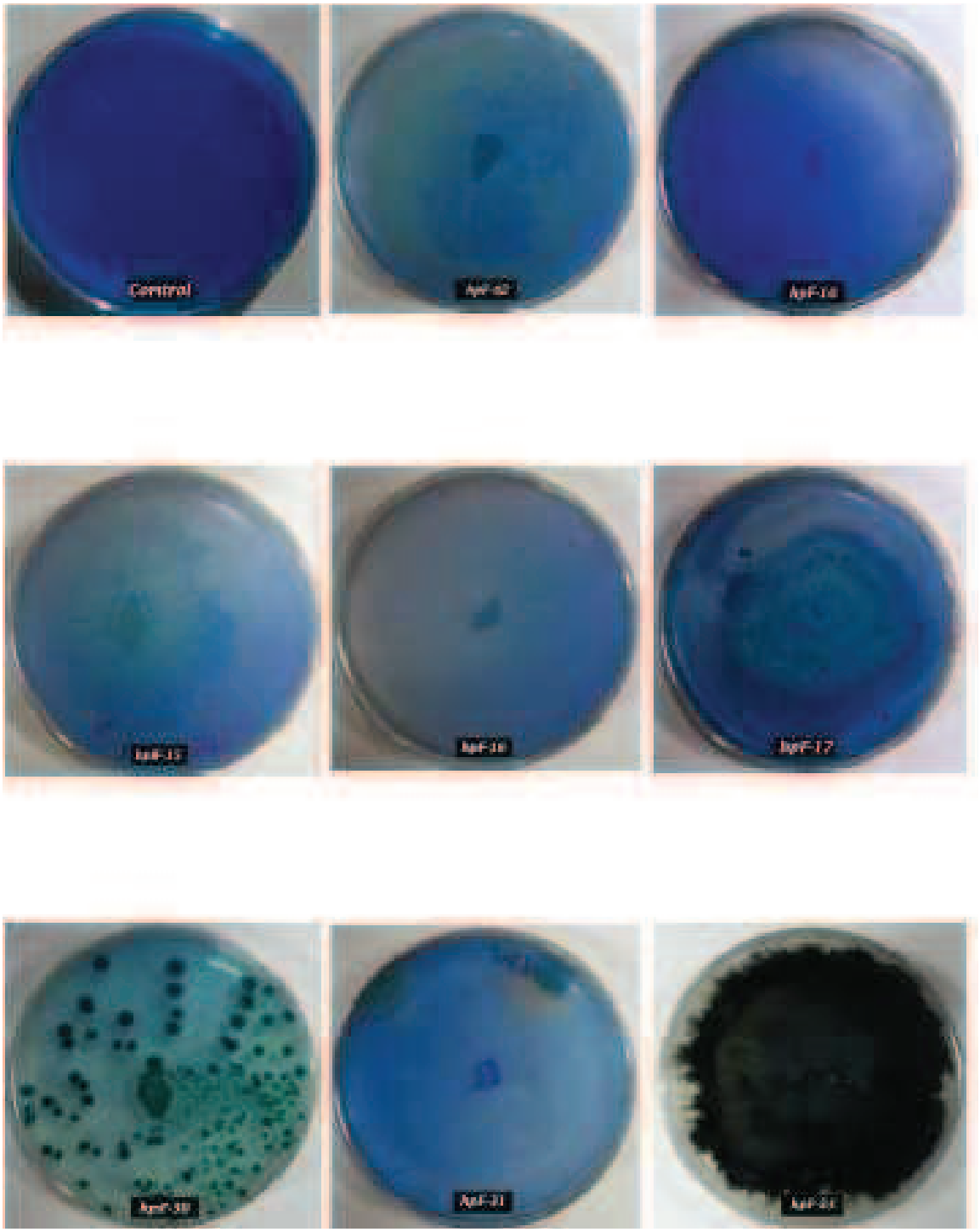

Fig. 3.3. Azure-B decolourization to form clear zones due to LMEs production by the isolates during screening experiments

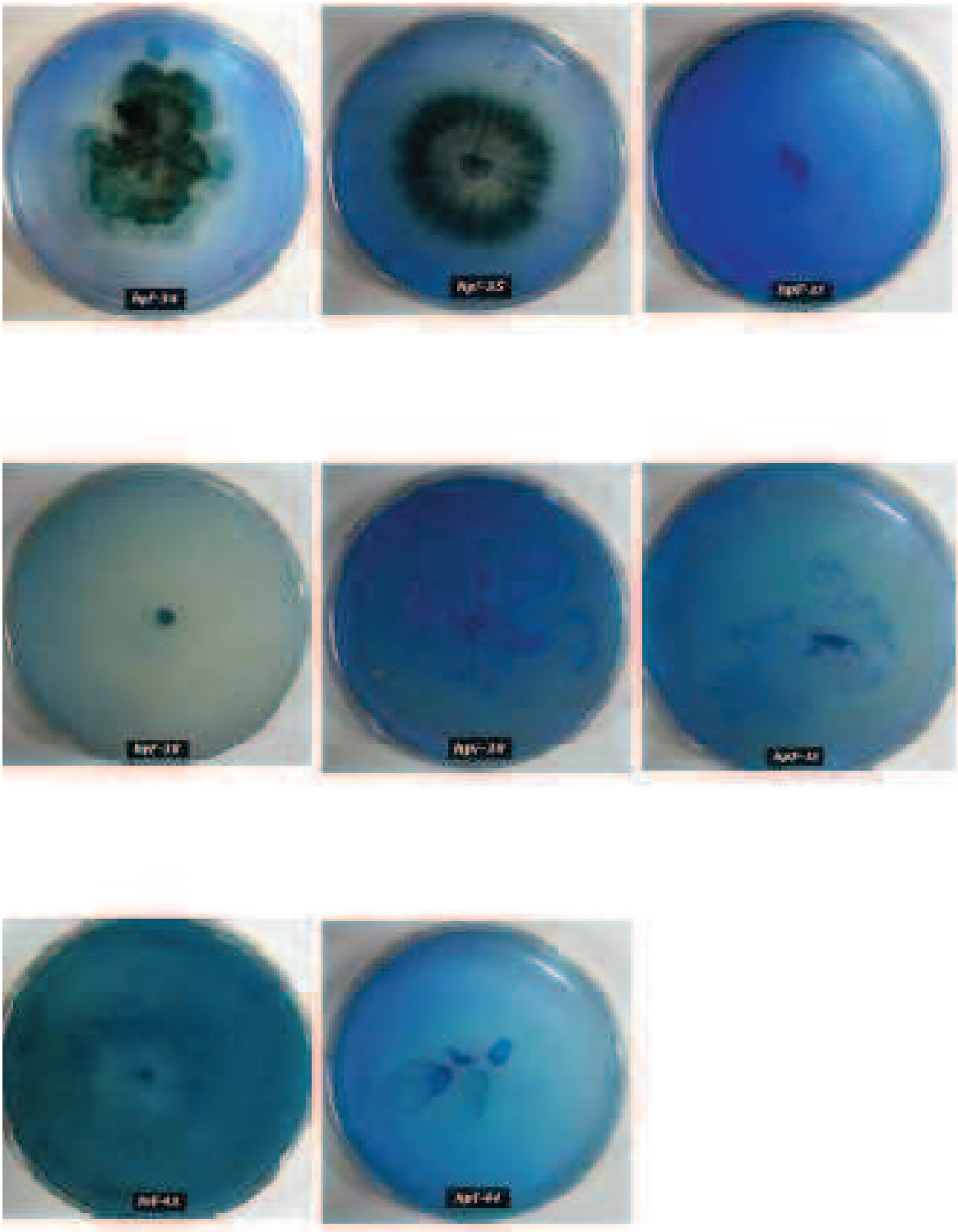

Fig. 3.3. Azure-B decolourization to form clear zones due to LMEs production by the isolates during screening experiments (cont..)

Use of several compounds for screening of ligninolytic enzymes were described in the scientific literatures. Kiiskinen et al. (2004) used four different indicator compounds in order to detect microbes that produced ligninolytic enzymes. The compounds which were added to solid media were, 0.04% (w/v) RBBR, 0.04% Poly R-478, 0.01% guaiacol and 0.5% tannic acid. Among all compounds, tannic acid gave relatively weak positive reaction with many isolates. However, the ability of most positive microbes to form brown colour with tannic acid was weakened during sub-culturing. This effect was not observed with other compounds. Comparison of the reactions with different indicators showed that the polymeric dyes RBBR and Poly R-478 resulted in very similar effects, thus either one can be chosen as an indicator in future screening procedures. Reactions with guaiacol also well correlated with reactions of polymeric dyes.

Eriksson et al. (1990) used entirely different procedure to screen lignin degrading fungi. The lignin-degrading ability of a microorganism was evaluated by measuring $14CO_2$ evolution from 14C-labeled lignin preparations, such as 14C-ring-labeled dehydrogenation polymerizate (DHP). The measurement of $14CO_2$ evolution is the most sensitive and accurate method for testing ligninolytic activity.

Supplementation of nutrients is also important during screening experiments, as it promotes growth and also induces enzyme secretions. Less defined basal growth medium such as peptone plus yeast extract, or malt extract have been used in several studies at varying concentrations (Gessner 1980, Egger 1986; Niku-Paavola et al.1990; Rohrmann and Molitoris 1992; Pointing 1992; Raghukumar et al.1994; Vrijmoed and lones 1999). However, it should be noted that LME production in fungi is generally repressed under conditions of nutrient sufficiency. A working strength of 0.01 % w/v peptone and 0.001 % w/v yeast extract in an undefined growth medium is acceptable (Reddy and D'Souza 1994, Eggert et al. 1996). In many fungi the presence of a redox mediator in the growth medium significantly enhances LMEs production (Reddy and D'Souza 1994, Eggert et

al.1996). This could be the reason for non indication of extracellular lignolytic enzymes in case of some basidiomycetes which were isolated from wood substrata.

In the present study, solid plate screening resulted in 12-azur B decolourizing strains and 5 strains of guaiacol oxidation hyperproducers. These strains were cultivated as agitated cultures on LMM and SMM media for the production of laccase and peroxidase enzymes, respectively. Only 6 out of 17 strains were able to produce significant amounts of laccase, MnP and LiP (Table 3.3).

Table 3.3 Production ligninolytic enzymes by the novel isolates

Isolate	Genus	LiP (U/l)	MnP (U/l)	Laccase (U/l)
hpF-04	*Peniophora sp.*	--	20±2(6)	1025.4±23 (4)
hpF-10	*Polyporous badius*	45±10 (3)	17±2 (3)	--
hpF-16	*Pleurotus sp.*	27±3 (4)	32±5(4)	246.5±12 (6)
hpF-17	*Phellinus sp.*	20±2 (6)	--	585.5±15(4)
hpF-33	*Panus sp.*	165±17 (7)	16±4 (7)	---
hpF-35	*Tramates sp.*	13.4±2(3)	68.5±15(6)	254.2±12 (4)

Values in parentheses: day on which maximum production of enzyme observed

As it is seen from the Table 3.3, the highest level of laccase production was observed in the strain *Peniophora sp.* hpF04, which also produced MnP but no LiP. It produced laccase activity of 1025.4±23 U/l on day 4. The strain *Phellinus sp.* hpF-17 also showed significant laccase activity of 585.5±15 U/l on day 4 and traces of lignin peroxidase activity (20±2 U/l) but no manganese peroxidase. The strain *Tramatus sp*. hpF-35 produced highest amount of MnP (68.5±15 U/l), in which production of significant amount of LiP (13.4±2 U/l) and laccase (254.2±12 U/l) was also observed. The strain hpF-33 *Panus sp.* produced maximum amount of LiP. Traces of MnP could be detected in this strain but no laccase.

The enzyme production profile shown in Table 3.3 is only an indicative sign of promising producers of ligninolytic enzymes. Negative LiP or MnP or laccase

production in some strains may suggest that these fungi either produce no significant levels of these enzymes or their production require different conditions other than those tested here. It has been observed that several strains of *Trametes versicolor* and *Bjerkandera adusta* which are known as LiP producers do not always produce lignin peroxidase as the production of enzymes is dependent on strain or culture conditions (Kirk and Farrell 1987; Waldner et al. 1988). Nerud and Misurcova (1996) considered that ligninolytic enzyme production is highly conditioned or strain-related.

In the present study, ligninolytic enzyme activity was detected in all the selected isolates, but only two isolates demonstrated simultaneous production of laccase, LiP and MnP (Table 3.3). This indicates that some fungi do not require all three enzyme types at one time during the lignin degradation process. In previous study by de Jong et al. (1994), it was described that most wood-rotting fungi produce three groups of enzymes, but the some fungi produce only one or two groups of these enzymes. Similar results were also reported by Risna and Suhirman (2002).

When compared to previous studies on production of LMEs by fungal isolates, the strains isolated in our study showed very low levels of LMEs in liquid cultivations. The low values of enzyme activities reported here may have resulted because the fungi were grown at unsuitable conditions or because of the presence of some unknown inhibitors in the growth media. As described in many previous studies, it may be necessary to use inducer compounds to enhance laccase activities. Several substances can induce 1accase production such as indulin AT, tannic acid (Arora and Sandhu 1985) and xylidine (Eggert et al.1996). The use of veratryl alcohol as a substrate for LiP activity appears to be suitable for the Lombok fungi. It is however, necessary to observe concentrations of the substrate in assay, nutrient limitations, as well as the incubation period that will give optimum values of LMEs activities.

Isolation and screening of autochthonal fungi was previously carried out by many investigators. Pelaez et al. (1995) reported 68 species of basidiomycetes from Central region of Spain producing laccase and Mn^{2+}-oxidizing peroxidase rather than LiP under

the studied conditions. Okino et al. (2000) isolated 116 Brazilian tropical rainforest basidiomycetes producing laccase and peroxidase enzymes. Muzariri et al. (2001) screened 224 fungal strains from Zimbabwe for ligninolytic and cellulolytic activities. Read et al. (2001) isolated 10 strains of WRF from decaying wood of Zimbabwe and identified based on their gross and microscopic characters as *Trematus sp.*, *Lentinus sp., Pycnoporous sp., Datrionia sp., Irpex sp.*, and *Creptidotus sp.* producing no lignin peroxidase in any of the tested culture conditions. Risna and Suhirman (2002) isolates 65 polyporous basidiomycetes from several forests in Lombok Island, Indonesia and found twelve isolates producing all three kinds of enzymes. Saparrat et al. (2002) and Levin et al. (2004) evaluated the ability of native Argentinean WRF for their ligninolytic enzymes and found significant Lac activities. Dhouib et al. (2005) isolated 315 strains of fungi from different Tunisian biotopes and found only 8 cultures producing significant amounts of MnP, Lac and LiP in liquid cultures. In an another study by Papinutti et al. (2007), they isolated and studied LMEs of WRF *Fomes scleroderma* in solid state fermentation and detected only MnP and laccase. Recently Raqayyah et al. (2011) screened 313 strains of white rot fungi from Malaysian terrestrial habitat and found 6 strains producing all three classes of ligninolytic enzymes, 1 strain producing two classes and 5 strains producing only one class of ligninolytic enzymes.

To conclude, the present work reports the isolation and screening of novel indigenous Basidiomycetous fungal species capable of producing ligninolytic enzymes. Production of efficient LiP or MnPs was not observed in any of the strains. Two fungal strains identified as *Peniophora sp. and Phellinus sp.* were found to be efficient laccase producers. Hence, further work will be focused on the production of laccase by these two novel isolates. As on today, no detailed work on production and characterization of laccase enzymes from these two genera is discussed in the literature.

Chapter 4

PRODUCTION OF LACCASE BY NOVEL ISOLATES OF BASIDIOMYCETES

4.1 Introduction

In recent years, several enzymes have gained great importance in industries; laccases are one among them which are widely distributed in the nature. Laccases are the oldest and most studied enzymatic systems (Williampson 1994). They are widely distributed in higher plants, bacteria, fungi, and insects. In plants, laccases are found in cabbages turnip, potatoes, pears, apples, and other vegetables. They have been isolated from Ascomyceteous, Deuteromycteous and Basidiomycetous fungi to which more than 60 fungal strains belongs (Gianfreda et al.1999). Basidiomycetes and saprotrophic fungi are the most widely known species that produce substantial amount of laccase in variable quantity (Hataka 2001). However, laccase producing capability of a brown-rot fungus is not known and no laccase has been purified.

During the of ligninolytic enzymes following isolation, we discovered two laccase hyper producers belonging to class Basidiomycetes. The strains were identified as *Peniophora* sp. hpF-04 and *Phellinus sp.*hpF-17 based on their fruiting body morphology. *Peniophora* belongs to the order Aphyllophorales and family Corticiaceae. This family contains some well known laccase producer-genera e.g. *Phlebia*. *Peniophora* strains are common decomposers of pine slash, pulpwood and other wood products. *Phellinus* is a member of the family Hymenochaetaceae, order Aphyllophorales. Laccase production by these two fungal genera, however, has not been described in detail previously in the literature.

Laccases from fungi have been implicated in lignin degradation, in differentiation and in protection from toxic phenolic monomers of polyphenols. They are used for many industrial purposes such as paper processing, prevention of wine discolouration and detoxification of environmental pollutants, oxidation of dye, production of chemicals from lignin. Laccases can degrade several dye structures (Aadulla et al. 2000), transform toxic compounds into safer metabolites and hence be useful for application in environmental pollution control measures (Gianfreda et al.1999). Laccases are also useful for the decomposition of azo dyes by oxidative methods (Michael et al. 2005). In view of

its importance in large scale applications, the present work the aimed on the production of laccases b *Peniophora sp*. hpF-04 and *Phellinus sp.* hpF-17 under submerged fermentation process.

In many studies, after successful screening program, optimization of the laccase enzyme production is extensively explored with the selected organisms. A large majority of the studies on the production of the enzymes have been carried out with defined media. This clearly indicates that basidiomycetes display wide diversity in their response to medium components and their concentrations (Elisashvili et al. 2002, Galhaup et al. 2002; Mikiashvili et al. 2005; Elisashvili et al. 2006; Wang et al. 2008). Laccases are generally produced during the secondary metabolism of white-rot fungi growing on natural substrate or in submerged culture (Gayazov and Rodakiewicz-Nowak 1996). Various cultivation parameters that influence laccase production and activity have been described. These factors include carbon limitation, nitrogen source and concentration, inducer compounds and microelements. Hence, study also aimed to evaluate the influence of alternate carbon, nitrogen and inducers to enhance the laccase activities. Different cultivation parameters like initial medium pH and incubation temperature were also checked in order to facilitate efficient production.

In this study, we also employed different enzyme assay methods for laccase activity measurements and compared the sensitivities of different substrates. The results can provide a reference for the substrate selection for similar kind of studies in future.

4.2 Materials and Methods

4.2.1 Organism maintenance and culture conditions

Fungi *Peniophora* sp. hpF-04 and *Phellinus* sp. hpF-17 were maintained in 2% MEA slants and refrigerated at 4°C. The fungi were sub-cultured in the same media before use.

4.2.2 Identification of the fungi

Based on their morphology, fungi were identified as *Peniophora* sp. hpF-04 and *Phellinus sp*. hpF-17. Further, rDNA sequencing method was used for further confirmation of their identification. The identification was carried out in Aghrakar Research Institute (ARI), Pune, India, with NCBI Accession number GU017513.1 and HQ400698.1 respectively, for hpF-04 and hpF-17.

4.2.3 Time course studies of laccase production in defined liquid medium

Fungi were grown on laccase modified medium (LMM) medium (Dhouib et al. 2005) to study their time course production of laccase. Composition of LMM medium was (g/l): glucose(10.0), ammonium tartarate(2.0), KH_2PO_4(1.0), $MgSO_4.7H_2O$(0.5), KCl(0.5), yeast extract(1.0), $CuSO_4.5H_2O$(150 μM), and 10 ml/l of trace elements containing per litre of distilled water (g/l): EDTA(0.5), $FeSO_4$(0.2), $ZnSO_4.7H_2O$(0.01), $MnCl_2.4H_2O$(0.003), H_3BO_4(0.03), $CoCl_2.6H_2O$(0.02), $CuCl_2.2H_2O$(0.001), $Na_2MoO_4.2H_2O$(0.003). Mycelial agar plugs (4 plugs of 7mm diameter) were cut along the edge of the actively growing colony and these plugs were used as inocula. The fungi were cultivated in 30 ml of medium in 100ml Erlenmeyer flasks at 30^oC on a rotary shaker (150 rev/min). The flasks were withdrawn in duplicates at an interval of every two days till fourteen days. The contents of the flasks were centrifuged at 5000rpm (REMI) to obtain mycelia-free culture extracts. The clear filtrate was used for the measurement of enzyme activities.

4.2.4 Effect of temperature and pH

Fungi were cultivated in chemically defined LMM medium according to the method described by Dhoubi et al. (2005). 25 ml of the chemically defined medium was dispensed into 100ml Erlenmeyer flask and sterilized, inoculated with three 7mm agar disc plugs of the white-rot fungus and incubated at 23°C to 37°C for 6 days. Thereafter, the culture mycelium was harvested and the mycelium-free filtrate was used to determine laccase activity.

To study the influence of initial medium pH, 25 ml of the LMM medium was dispensed into 100ml Erlenmeyer flask and pH was adjusted by using 0.1N HCl and 0.1N NaOH to 4.0, 5.0, 6.0, 7.0 and 8.0. The flasks were sterilized, then inoculated with three 7mm agar disc plug of the white-rot fungus and incubated at 30°C for 6 days. Thereafter, the culture mycelium was harvested and the mycelium-free filtrate was used to determine laccase activity.

4.2.5 Effect of carbon and nitrogen sources

The selection of the suitable carbon and nitrogen sources was based on the screening of different carbon and nitrogen sources in media preparations. The LMM medium was supplemented by replacing glucose with other carbon sources such as carboxy methyl cellulose (CMC), sucrose, cellobiose, xylan and starch. Different nitrogen sources such as inorganic (KNO_3, NH_4Cl, NH_4NO_3 and the N source of original medium), and organic (aspargine, urea and yeast extract) were employed to select the suitable nitrogen source. In these experiments, the initial yeast extract was eliminated from LMM medium. The organisms were grown at pH 6 and incubated for 6 days. Thereafter, the culture mycelium was harvested and the mycelium-free filtrate of 6^{th} day cultivation was used to determine laccase activity.

4.2.6 Effect of inducers

In the present study, five different inducers used were copper sulphate (0.5, 1 and 2 g/l) veratryl alcohol (0.1, 0.3 and 1 ml/l), Tween 80 (0.1, 0.5 and 1%), 2,5-xylidine (0.5, 1.0 and 2.0 mM) and guaiacol (0.1, 0.5 and 1%). The original LMM medium was supplemented with above said inducers and flasks were incubated in similar growth conditions. After six days of incubation period, the culture filtrate were collected and analyzed for laccase activity.

4.2.7 Biomass estimation

The biomass of the cultures was determined by filtering the mycelia contents through oven dried, pre weighed Whatman No.1 filter paper. The dry weight of the fungus was calculated by the difference in weight after drying the filter paper at 60^{o}C until a constant weight.

4.2.8 Enzyme assays

4.2.8.1 ABTS assay method for laccase

This method was first described by Bourbonnais and Paice (1990) and is common in use. This method is based on the oxidation of 2,2'-azinobis-bis-(3-ethylbenzthiazolinesulphonate)(ABTS) by laccase. The non phenolic dye ABTS gets oxidized by laccase to a more stable blue-green coloured and preferred state of cation radical (Fig. 4.1). The concentration of the cation radical is responsible for the intense blue-green colour which can be correlated to enzyme activity (Macherczyk et al.1995). The absorbance of the cation radical is most often read between 415nm and 420nm.

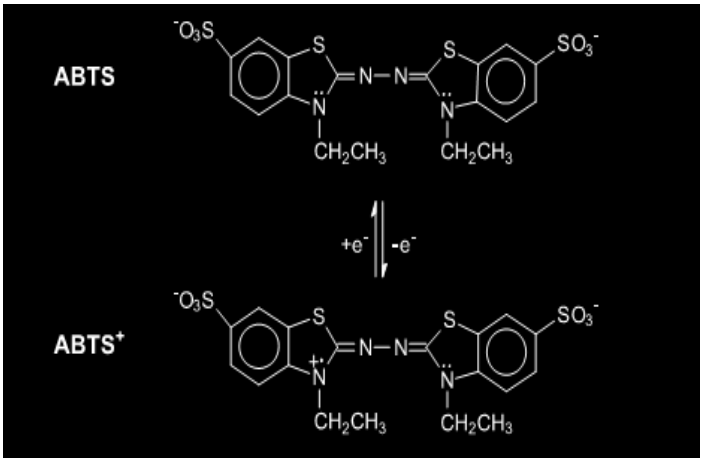

Fig. 4.1 The laccase-catalyzed oxidation of ABTS to a cation radical ($ABTS^+$) (Taken from Macherczyk et al. 1998)

The reaction mixture consisted of sodium acetate buffer (0.1M, pH 4.5), ABTS (50mM) and 1.5ml culture filtrate. The absorbance of the cation radical was monitored at 420nm(ϵ_{mM}=36$mM^{-1}cm^{-1}$) and at room temperature using UV-Vis spectrophotometer (GBC). Enzyme activity was expressed as international units (IU) where 1 IU is defined as the amount of enzyme forming 1µmole of product per minute. The laccase activity in U/ml (µmol cation radical released/min/ml enzyme) was calculated as follows;

$$U/ml = 2\left(\frac{\mathrm{V}}{\mathrm{v} \times \varepsilon \times \mathrm{d} \times \Delta\mathrm{A/min}}\right)$$

$$U/ml = 2\left(\frac{2.25}{0.75 \times 36 \times 1 \times \Delta\mathrm{A/min}}\right)$$

$$\text{ABTS Laccase } U/ml = 0.16 \times \Delta\mathrm{A/min}$$

where, V= Total reaction volume (ml)

v= Enzyme solution (ml)

ε= Extinction Coefficient of ABTS at 420nm=36/m/Mcm

d= Light path of curve (cm)

$\Delta\mathrm{A/min}$= Absorbance change per minute at 420 nm.

4.2.8.2 Syringaldazine assay method for laccase

This assay method was based on the oxidation of 4,4'-[azinobis(methanylylidene)]bis(2,6-dimethoxyphenol)(syringaldazine) to the corresponding quinine, 4,4'-[azinobis(methanylylidene)]bis(2,6-dimethoxycyclohexane-2,5-diene-1-one) (Fig. 4.2). An increase in absorbance at 530 nm is followed to determine laccase activity in international units (IU) where 1 IU is defined as the amount of enzyme forming 1µmol of product per minute.

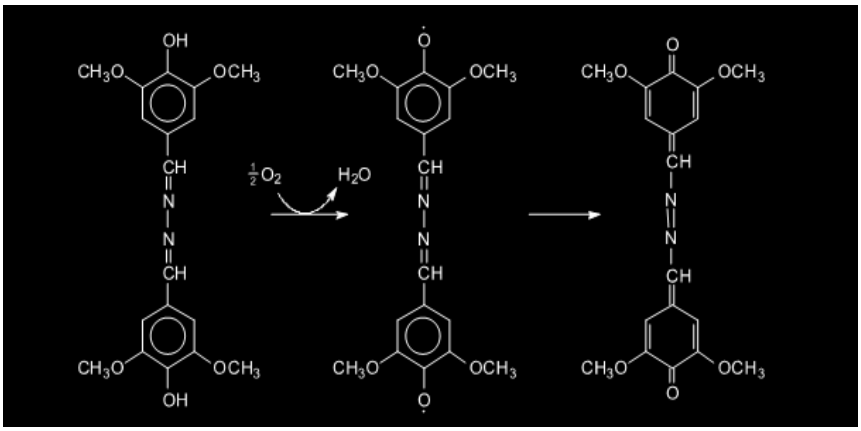

Fig. 4.2 The laccase-catalyzed oxidation of syringaldazine to its corresponding quinone. (Taken from Sanchez-Amat and Salan 1997)

The reaction mixture consisted of 2.20ml of acetate buffer (10mM, pH 4.0). 0.3 ml of syringaldazine (0.216 mM in absolute methanol) and 0.5 ml of culture filtrate. The laccase activity in U/ml (µmol cation radical released/min/ml enzyme) was calculated as follows;

$$U/ml = 2\left(\frac{\mathrm{V}}{\mathrm{v} \times \varepsilon \times \mathrm{d} \times \Delta\mathrm{A/min}}\right)$$

$$U/ml = 2\left(\frac{3.0}{0.5 \times 65 \times 1 \times \Delta\mathrm{A/min}}\right)$$

Syringaldazine Laccase $U/ml = 0.18 \times \Delta\mathrm{A/min}$

where,

V= Total reaction volume (ml)

v= Enzyme solution (ml)

ε= Extinction Coefficient of Syringaldazine at 530nm=65/m/Mcm

d= Light path of curve (cm)

$\Delta\mathrm{A/min}$ = Absorbance change per minute at 530 nm

4.2.8.3 DMP Assay method for laccase

2,6-Dimethoxyphenol (DMP) is oxidized to its quinine, 2,6-dimethoxycyclohexane-2,5-diene-1-one, by laccase catalyzed reaction (Fig. 4.3). The yellow orange colour formed by the quinine is measured spectrophotometrically and can be correlated to laccase activity.

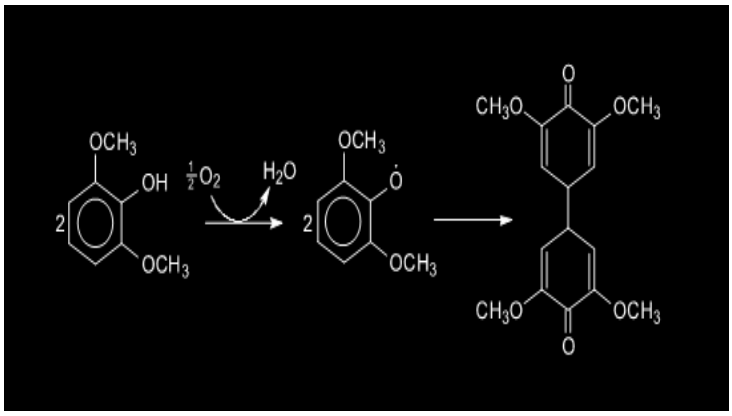

Fig. 4.3 The laccase-catalyzed oxidation of DMP to its corresponding quinine (taken from Sanchez-Amat and Salan 1997)

The laccase activity was determined at 468nm (€=49600/M/cm). The reaction mixture consisted of 3.0 ml malonate buffer (50 mM, pH 4.5), 1.0 ml DMP (1 mM) and 1.0 ml culture medium. The laccase activity in U/ml (umol cation radical released/min/ml enzyme) was calculated as follows;

$$U/ml = 2\left(\frac{\mathrm{V}}{\mathrm{v} \times \varepsilon \times \mathrm{d} \times \Delta\mathrm{A/min}}\right)$$

$$U/ml = 2\left(\frac{2.5}{0.5 \times 14.8 \times 1 \times \Delta\mathrm{A/min}}\right)$$

2,4-DMP Laccase $U/ml = 0.68 \times \Delta A/min$

where,

V= Total reaction volume (ml)

v= Enzyme solution (ml)

ε= Extinction Coefficient of 2,4-DMP at 468nm=14.8/m/Mcm

d= Light path of curve (cm)

$\Delta A/\min$ = Absorbance change per minute at 468 nm

4.2.8.4 O-dianisidine assay

Laccase assay was also carried out with o-dianisidine oxidation at 460 nm (€=11 300/M/cm). The 1.0 ml of reactive mixture consisting 50 mM acetate buffer (pH.5.0), 100 µl o-dianisidine (1.0 mM), 600 µl culture medium and 100 µl hydrogen peroxide (2mM) was used (silva et al.2005).

$$U/ml = 2\left(\frac{\mathrm{V}}{\mathrm{v} \times \varepsilon \times \mathrm{d} \times \Delta\mathrm{A}/\min}\right)$$

$$U/ml = 2\left(\frac{1.8}{0.6 \times 12.1 \times 1 \times \Delta\mathrm{A}/\min}\right)$$

O-dianisidine Laccase $U/ml = 0.5 \times \Delta\mathrm{A}/\min$

where,

V= Total reaction volume (ml)

v= Enzyme solution (ml)

ε= Extinction Coefficient of O-dianisidine at 450 nm=12.1/m/Mcm

d= Light path of curve (cm)

$\Delta A/min$ = Absorbance change per minute at 450 nm

4.2.8.5 Guaiacol oxidation assay

In this assay the oxidized product of guaiacol was measured at 470nm (€=12100/M/cm). The reactive mixture consisted of 3.0 ml acetate buffer (10 mM, pH 5.0), 1.0 ml guaiacol (2 mM) and 1.0 ml culture medium (Arora et al. 2002).

$$U/ml = 2\left(\frac{\mathrm{V}}{\mathrm{v} \times \varepsilon \times \mathrm{d} \times \Delta\mathrm{A/min}}\right)$$

$$U/ml = 2\left(\frac{5}{1 \times 12 \times 1 \times \Delta\mathrm{A/min}}\right)$$

Guaiacol Laccase $\mathrm{U/ml} = 0.84 \times \Delta\mathrm{A/min}$

where,

V= Total reaction volume (ml)

v= Enzyme solution (ml)

ε= Extinction Coefficient of guaiacol at 470 nm = 12/m/Mcm

d= Light path of curve (cm)

$\Delta\mathrm{A/min}$ = Absorbance change per minute at 470 nm

4.3 Results and Discussion

4.3.1 Growth characteristics of fungal isolates

The growth patterns of the isolates is shown in the Fig. 4.4 Both isolates showed radial symmetry of growth, the growth of the hyphae started from the centrally placed inoculum towards the periphery of the petriplates. Isolate hpF-04 hyphae system is monomitic, hypahe nodose-septate, fairly uniform, raised, composed of thin basal layer with loose texture. Hypahe was hyaline to brownish, mainly horizontal, usually glued together (Fig. 4.4 LHS). Reverse was colourless at the initial stage turning into reddish to brown after few days. On the other hand, hypahe of the isolate hpF-17 was white in colour, even and flat with dense texture (Fig. 4.4 RHS). Mycelium was loose-cottony to cottony-wooly in the beginning, becoming rough/elastic matt afterwards. Brown cluster areas formed within one week at some places. Reverse was colourless to light brownish.

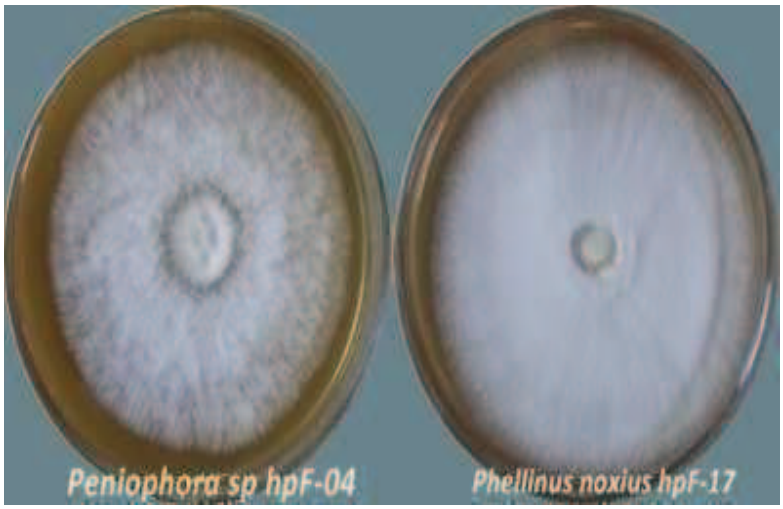

Fig. 4.4 Growth of the fungal isolates in 2% MEG plates

4.3.2 Identification of the fungal isolates

Identifications of the isolates, hpF-04 and hpF-17 were confirmed by rDNA sequencing. The rDNA sequence of the fungi was compared with the rDNA sequence of the other fungi present in the database. Strain hpF-04-NCBI- GU017513.1 showed close similarity (98%) with the genus *Peniophora sp*. and strain hp-F17-NCBI-HQ400698.1 showed maximum sequence similarity (98%) with genus *Phellinus* and species *noxius* (Fig. 4.5). Henceforth, the strain hpF-04 is designated as *Peniophora sp*. hpF-04 and the strain hpF-17 as *Phellinus noxius* hpF-17. The sequence of the isolates is shown in Fig. 4.5.The sequences of the hpF-04 and hp-F17 were deposited in DDBJ data bank with the Accession numbers AB639021 and AB639022, respectively.

Fig. 4.5 Sequence analysis 618 bases of hpF-04 (NCBI sequence Accession GU017513.1) (652 bases) of hpF-17 (NCBI sequence Accession HQ400698.1)

4.3.3 Time course production of laccase in the defined medium

Fig. 4.6 presents the results of time course study of biomass and laccase production by *Peniophora sp.* hpF-04. The amount of biomass produced by *Peniophora sp.* hpF-04 in cultures grown on LMM medium and its extracellular lac activity was estimated for 14 days. These cultures developed yellowish mycelial pellets, with no spores. The trophophase was restricted to first 6 days of culture growth, achieving a biomass level of 12.7±0.3 g/l of LMM medium. The culture subsequently entered into the idiophase under carbon limited conditions. Then, the level of biomass remained constant during 14 days of incubation. Extracellular Lac activity increased during the trophophase, reaching maximum levels during 6^{th} and 8^{th} days of cultivation. Laccase activities of

1052±54 and 1084±35 U/l was observed on 6th and 8th day, respectively. Hereafter, the enzyme activity reduced drastically.

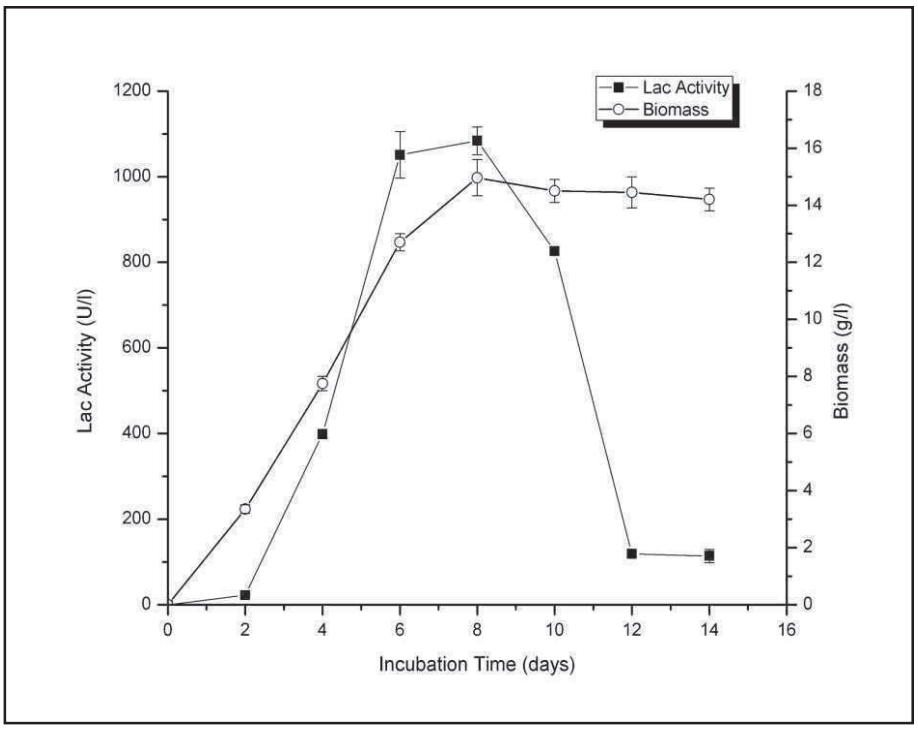

Fig. 4.6 Time course production of biomass (g/l) and laccase (U/l) by *Peniophora* sp. hpF-04

Fig. 4.7 presents the results of time course study of biomass and laccase production by *Phellinus noxius* hpF-04 in LMM medium. Similar trend of laccase and biomass production was observed in case of *P.noxius* hpF-17. However, the laccase and biomass production by *Phellinus noxius* hpF-17 grown in similar medium was considerably lesser compared to *Peniophora sp.* hpF-04. High laccase activities were estimated during 6th and 8th days of incubation, wherein respective laccase activities of 545.5±14 U/l and 535±17 U/l, respectively, were observed (Fig. 4.7). Mycelial biomass of 7.2±0.21 g/l was achieved during 6th day and there after the biomass remained almost constant and reached idiophase. On further incubation till 14 days, laccase activity drastically reduced to 16.05±7.7 U/l. These studies confirmed that both the isolates were good producers of extracellular laccase.

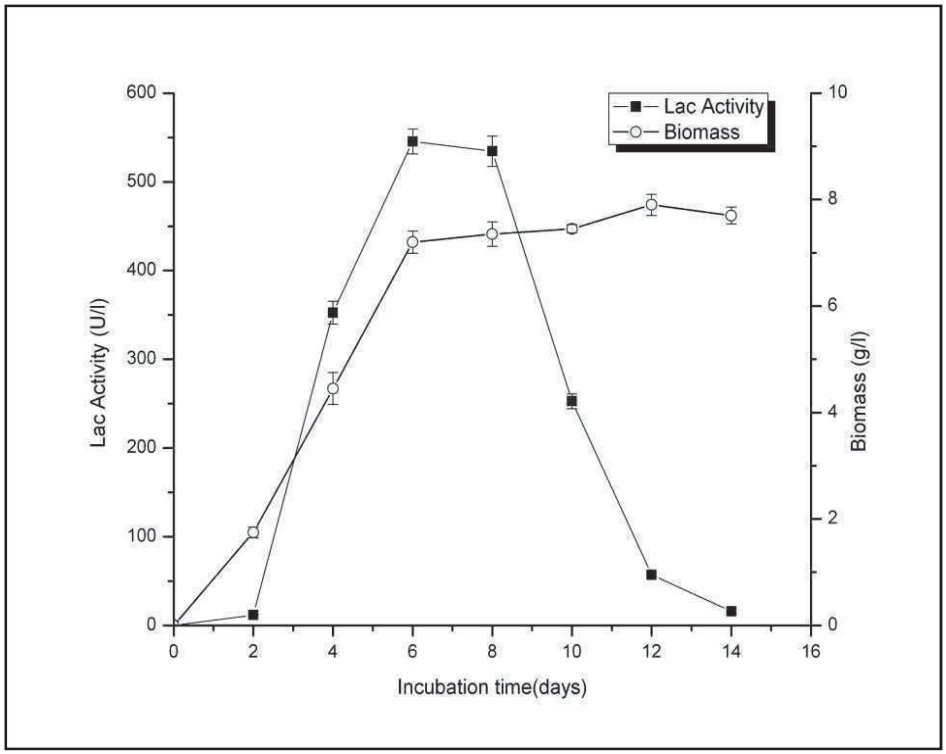

Fig. 4.7 Time course studies on biomass (g/l) and laccase (U/l) by *Phellinus noxius* hpF-17 in LMM medium

4.3.4 Sensitivity of substrates for laccase determination assay

The culture filtrate obtained after 6 days of incubation period was analyzed for laccase activity using different substrates. The laccase activity values measured using five different methods greatly differed with respect to their sensitivity towards enzyme reaction. Among all methods, laccase activity value in terms of ABTS oxidation method resulted in higher enzyme titre in both the fungi (Fig. 4.8), which probably may be due to its higher sensitivity towards enzyme reaction. Laccase assay by o-dianisidine and 2, 6 - DMP methods were also sensitive enough to quantify laccase measurements but were lesser to ABTS. It was observed that, assay with syringaldazine was dependent on reaction buffer pH. Assay mixture containing Britton Robinson buffer of pH 6.5 resulted in colour change in the case of *Peniophora sp.* hpF-04 culture filtrate and not with *Phellinus noxius* hpF-17 and the latter required acetate buffer of pH.4.5 for the laccase

activity measurements. It could be due to the laccase enzyme characteristics produced by these isolates. The laccase produced by *Phellinus noxius* might be active at lower pH range.

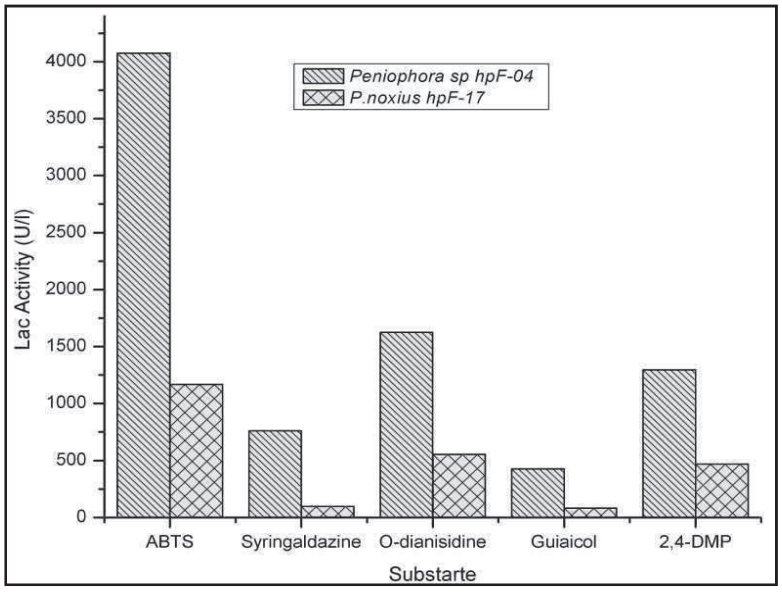

Fig. 4.8 Laccase determination assay using different substrates

Figure 4.9 shows the stability of the colour formation due to the oxidation of assay substrates by laccase enzyme. Colour formed in case of 2,4-DMP and syringaldazine was found to be unstable within an hour of incubation. However, the colour produced in the presence of other substrates was quiet stable for longer time. Because of the instability of colour produced by Syringaldzine and 2,4-DMP, there could be an inconsistency in laccase activity values during laccase assay. The colour produced in the presence of ABTS was stable for a longer time and hence, was highly accepted as a substrate for laccase assay by many investigators and was common in use (Li et al. 2008). ABTS is an electron rich non phenolic compound (Wilson et al. 1982), the oxidation potential of which is not pH-dependant within the range 2-11(Schott et al.1964) and proceeds in one step.

The great differences in the oxidation rates of the different substrates could be attributed to differences in redox potential between the enzyme and substrate

(Reinhammar and Malmström 1981). Guaiacol is a metonymy substituted monophenol, which can be oxidized to aldehydes. O-dianisidine is a substituted diamine and was oxidized to compounds containing disazo groups.

Fig. 4.9 Visual observation of colour formation in laccase determination assay. The experiments were carried out as per the method described in section 4.2.8. Different colour formation is due to the oxidation of individual substrates employed in the study by laccase enzyme to form coloured products

Gonzalez and coauthors (2004) demonstrated that certain medium compounds like tannic acid and other related organic compounds chemically reduce ABTS, thus it reduces laccase activity measurements. So it may be necessary to use most stable

components for the laccase measurements especially in the culture medium containing organic substances. In some study it was explained that catalytic efficiency of laccase from fungi tested was much lower for the substrates of o-dianisidine (Robles et al. 2000) and guaiacol (Liers et al. 2007) than that for ABTS. It was also reported that enzyme inactivation by reaction products had been responsible for the lower activity of laccase towards guaiacol (Robles et al. 2000). Melnig et al. (2008) observed the activity of laccase in terms of syringaldazine oxidation which was superior to that of hydroquinone, vanillic acid and tannic acid.

4.3.5 Effect of cultural conditions on biomass and laccase production

4.3.5.1 Effect of incubation temperature on biomass and laccase production

Temperature showed significant effect on mycelial biomass yield and extracellular laccase activity in *Peniophora* sp. hpF-04 and *P.noxius* hpF-17. With the increase in incubation temperature, fungal mycelial biomass yield and laccase activity found decreased in both fungi. Temperature of 30°C was observed to be optimal temperature for high laccase production and maximum biomass yield.

Fig. 4.10 presents the results of the effect of incubation temperature on biomass and laccase production by *Peniophora sp.*hpF-04. In this fungus, maximum lac activity of 1100.5±14.98 U/l, with respective biomass of 11.6±0.6 g/l was obtained at 30°C. At incubation temperature of 28°C, laccase activity of 1077.58±15.08 U/l with biomass of 12.33±0.5 g/l was estimated. The fungus also showed similar laccase activity of 1025.5±13.44 U/l with respective biomass of 9.64±0.21 g/l at incubation temperature of 32°C. At 37°C, it showed low level of laccase activity of 60.05±0.9 U/l. At this temperature, organism showed less growth of 1.45±0.23 g/l. Low level of laccase activity (139±4 U/l) and biomass (3.5±0.05 U/l) was also observed at low incubation temperature of 23°C.

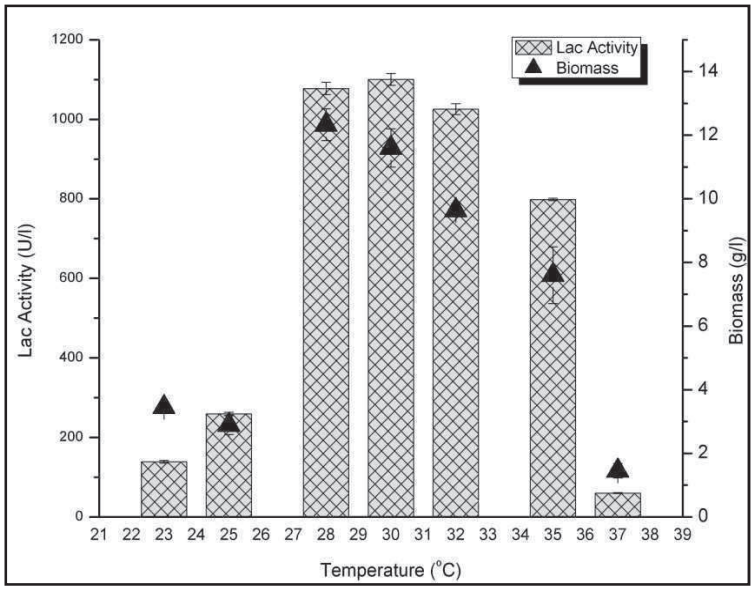

Fig. 4.10 Effect of incubation temperature on biomass and laccase production by *Peniophora sp.* hpF-04

Fig. 4.11 presents the results of the effect of incubation temperature on biomass and laccase production by *Phellinus noxius* hpF-17. In *Phellinus noxius* hpF-17, maximum laccase activity of 658.9±1.2 U/l was observed at 30^{o}C. At this temperature, fungus showed highest biomass of 5.98±0.32 g/l. At incubation temperatures of 28°C and 32°C, fungus produced laccase of 643.8±19.5 U/l and 589.6±3.05 U/l, respectively. Respective biomass of 12.3±0.5 g/l and 9.64±0.2 g/l was estimated at 28°C and 32°C. Similar to *Peniophora sp.* hpF-04, in this fungus also low level of laccase activity and biomass was observed at high and low temperatures. The fungus produced 77±9.2U/l of laccase, with biomass of 0.72±0.27 g/l at 37°C and 25.9±4.2 U/l of laccase, with biomass of 2.89±0.12 g/l at 23°C.

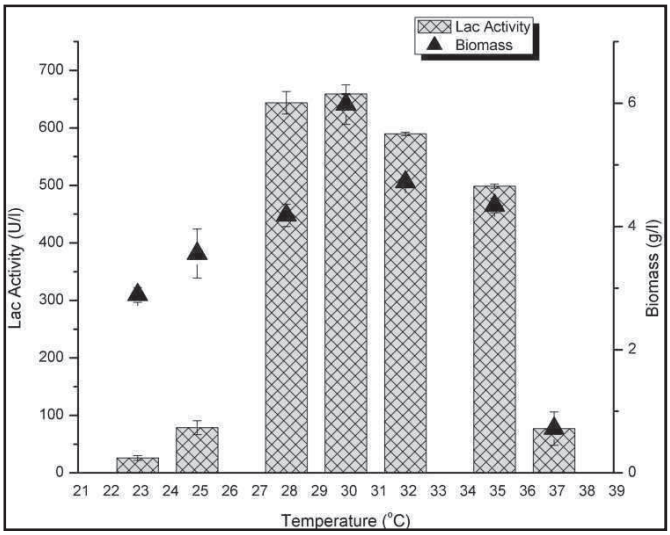

Fig. 4.11 Effect of incubation temperature on biomass and laccase production by *Phellinus noxius* hpF-17

4.3.5.2 Effect of medium pH

Fig. 4.12 presents the results of effect of initial medium pH on laccase production by *Peniophora sp.* hpF-04. Increase in fungal mycelial biomass yield and laccase activity was observed from pH 4.0 to 6.0. On further increase in pH, it reduced laccase activity as well as fungal growth. The highest fungal mycelial biomass production was recorded at pH 6.0 for *Peniophora sp.* hpF-04 (12.8±0.8 g/l). At this pH, maximum laccase activity of 1025.87±12.8 U/l of LMM medium was obtained. At alkaline pH*, i.e.* at pH 8.0, laccase activity of 24.2±1.1 with respective biomass of 0.9±0.03 was obtained. Low level of laccase (151.3±4.5 U/l) and biomass (3.0±0.2 g/l) was also observed at acidic medium pH 0f 4.0.

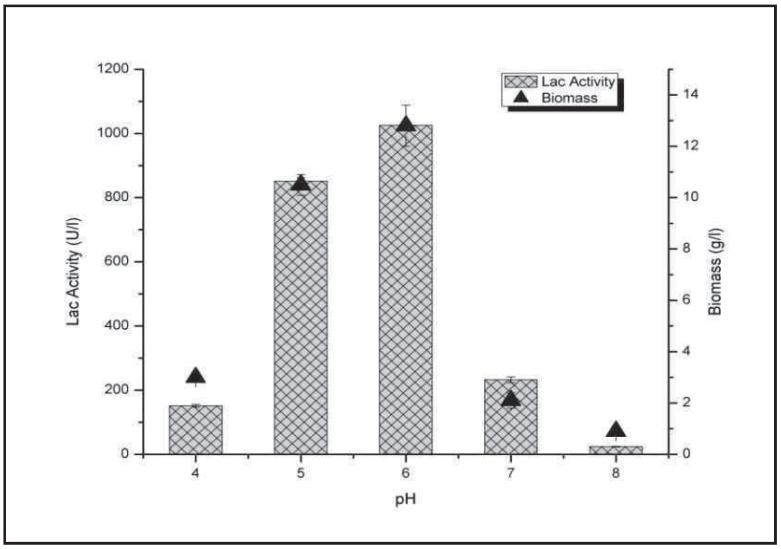

Fig. 4.12 Effect of medium pH on biomass and laccase production by *Peniophora sp.* hpF-04

The effect of initial medium pH on biomass and laccase production by *Phellinus noxius* hpF-17 is presented in Fig. 4.13. This fungus also followed similar trend of laccase and biomass production at different initial medium pH. It showed highest laccase production at medium pH of 6.0. At this pH, a laccase activity of 570.34±15.6 U/l with highest biomass of 7.66±0.34 g/l of LMM medium was recorded. At pH 8.0, laccase activity and biomass values were reduced to 12.65±0.25U/l and 1.95±0.25g/l, respectively.

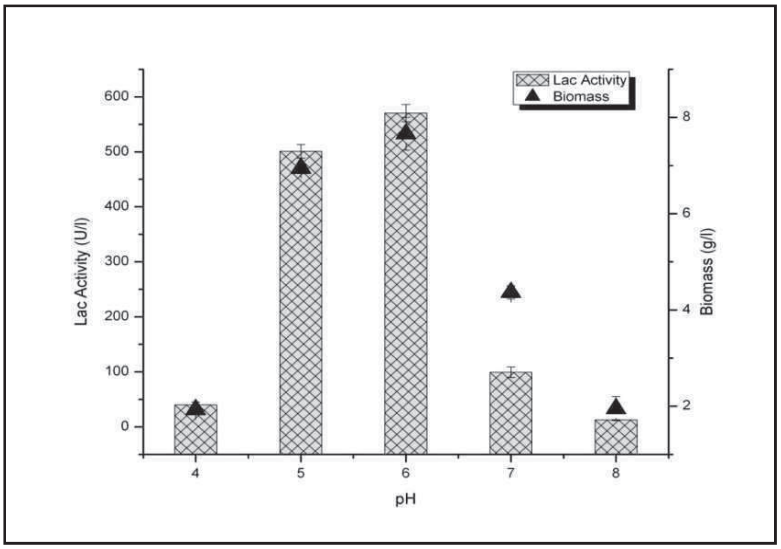

Fig. 4.13 Effect of medium pH on biomass and laccase production by *Phellinus noxius* hpF-17

Biomass production and laccase activity of the fungi were not favoured by low or high pH and temperatures. Zadrazil et al. (1999) reported that temperatures higher than 30°C reduce the activity of ligninolytic enzymes. Most of the enzymes which are capable for metabolic reactions get inactivated at elevated temperatures and hence could be the reason for decrease in fungal growth and activity. Change in pH may alter the three – dimensional structure of the enzymes (Pelzer et al. 2004). Similar observations were made by Gbolagade et al. (2006) during biomass production of *Pleurotus florida* and by Adejoy et al. (2009) in Nigerian edible mushroom, *Schizophyllum commune (fr.)*. Safari et al. (2005) also reported that culture pH is an index of fungi enzyme activity; whenever the pH was low, fungi activity was high. For these reasons, enzymes are known to be active over a certain pH range.

4.3.5.3 Effect of carbon source on biomass and laccase production

Laccase production by fungi has been previously shown to depend markedly on composition and concentration of medium components. Components like carbon source, nitrogen content and phenolic inducer have been reported to have significant effects on laccase production (Hatakka et al. 1991; Desai and Nityananda 2011). Hence, further

work was taken up to test the effect of various combinations of different nitrogen and carbon source as well as compounds that have been reported to induce laccase production by *Peniophora sp.* hpF-04 and *Phellinus noxius* hpF-17, respectively. We used different carbon sources such as glucose, sucrose, CMC, xylan and starch to study the effect on biomass and laccase production by *Peniophora sp.* hp-F04 and *Phellinus noxius* hpF-17.

Fig.4.14 presents the results of effect of carbon source on biomass and laccase production by *Peniophora sp.* hp-F04. This isolate showed maximum laccase activity in the medium containing sucrose (1249±12 U/l). Presence of CMC as a carbon source also showed laccase activity of 1060±12.5 U/l by this fungus. Glucose and cellobiose showed almost similar laccase activities of 735±6.0 U/l and 697±44.4 U/l, respectively. In starch and xylan supplemented media, the enzyme production was reduced to 32.5±1.05 U/l and 34.6±2.35 U/l, respectively. Among the carbon sources tested, glucose (a simple sugar) supported the maximum biomass yield followed by cellobiose (monosaccharide). Biomass yield was observed in the following descending order: glucose (12.65±0.35), cellobiose (10.85±0.05g/l), sucrose (9.5±0.1 g/l), CMC (9.23±0.17 g/l), starch (8.48±0.22 g/l) and xylan (3.85±0.05 g/l).

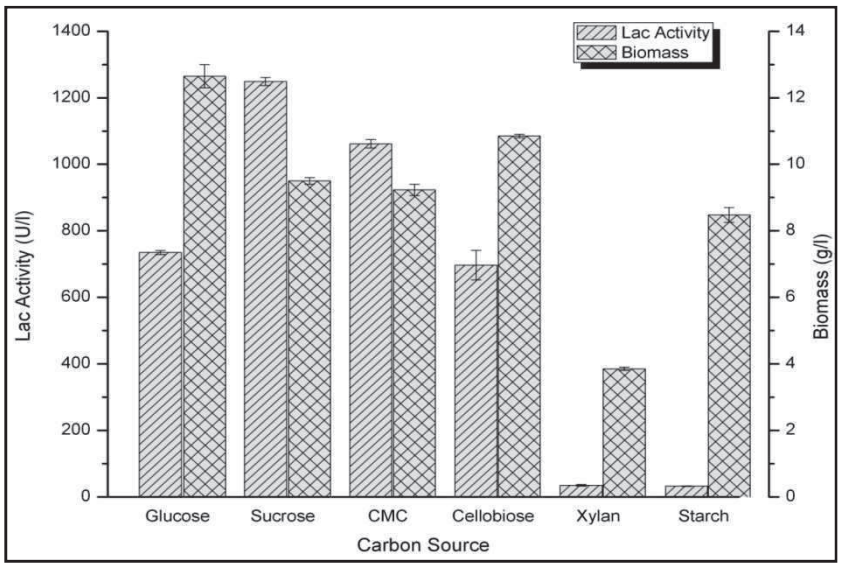

Fig. 4.14 Biomass and laccase production by *Peniophora sp.* hpF-04 cultivated in LMM medium supplemented with various carbon sources

The results of effect of carbon source on biomass and laccase production by *Phellinus noxius* hpF-17 is presented in Fig. 4.15. Maximum laccase activity was found in glucose (557±28 U/l) containing medium. Detectable levels of laccase activities were observed in sucrose (53.8±8.2 U/l) and cellobiose (36.90±3.3 U/l). In case of starch barely measureable laccase activity could be detected (1.95±2.1U/l). In this fungus, among the carbon sources tested, glucose (a simple sugar) supported the maximum biomass yield followed by starch (polysaccharide). Biomass yield (g/l) was observed in the following descending order: glucose (8.6±0.63), starch (8.3±1.59), xylan (6.4±1.48), sucrose (4.6±0.56), cellobiose (3.25±0.85), CMC (ND).

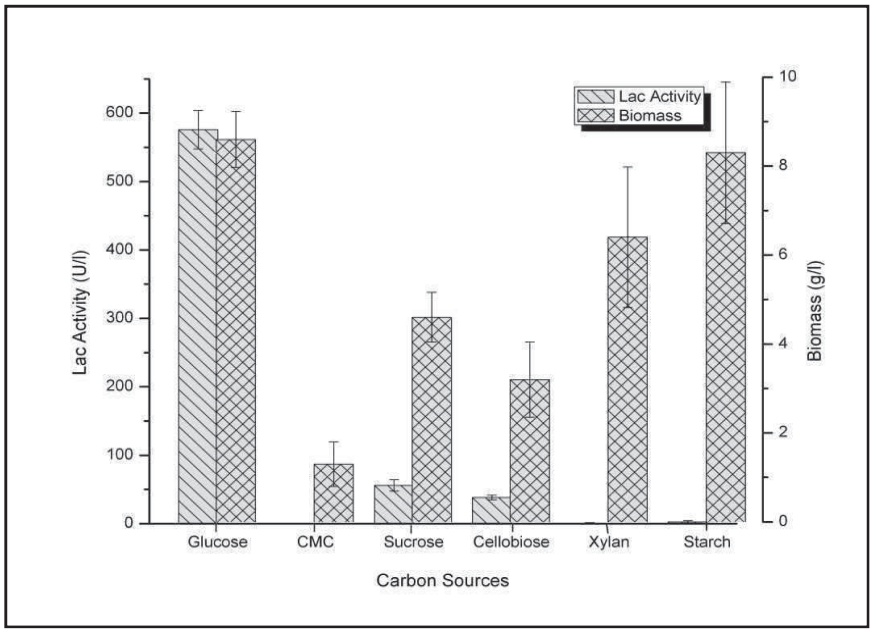

Fig. 4.15 Biomass and laccase production by *Phellinus noxius* hpF-17 cultivated in LMM medium supplemented with various carbon sources

In many fungi, maximum laccase production was observed during the stress conditions; *i.e* when nutrient depletion occurs (Baldrian 1994; Gianfreda et al. 1996; Couto and Herrera 2006; Shraddha et al. 2011). The simple sugars like glucose, sucrose and cellobiose were usually more rapidly assimilated and metabolized by cells. There is greater tendency for organisms to grow rapidly in the media containing simple sugars than that which contains complex sugars like starch and xylan. As a result, nutrient

depletion occurs at a faster rate in case of media containing simple sugars which in turns enhances the laccase activity. However, high levels of laccase activity and biomass in case of CMC could be due to the ability of the fungus to secret endo-gluconase which acts on CMC to release glucose and cellobiose. The simple sugars formed may increase the growth at higher rate, which in turn might have resulted in faster nutrient depletion. Due to the nutrient limitation, enhanced laccase production might have occured. The lack of ability to produce xylanase and amylase could be reason for the less growth in the media supplemented with xylan and starch as carbon sources and hence, low levels of lac activity were obtained.

In many studies on the effect of medium components on laccase production, it has been reported that laccase enzyme is inducible and it can be regulated by catabolic repression of laccase genes (Thurston 1994; Mayer and Staples 2002; Riva 2006). High levels of laccase production by the fungus employedthis study might be due to the carbon limitation as well as inducing effect of glucose. Though the fungi were able to grow well in the presence of complex sugars like starch and xylan, these sugars might have not induced laccase production which resulted in low or absence of laccase activity.

The use of excessive concentrations of glucose as carbon source in cultivation of laccase producing fungal strains has an inhibitory effect on laccase production (Egger et al.1996; Mayer and Staples 2002). It was found that increase in the amount of glucose in the media resulted in a delay of laccase production. An excess of sucrose or glucose can reduce the production of laccase, as these components allows constitutive production of the enzyme, but repress its induction. A simple but effective way to overcome this problem is by using cellulose as carbon source during fungal cultivation (Egger et al.1996).

Observations by many investigators on the effect of carbon sources on laccase production indicate that some carbohydrate appears to regulate the laccase expression in white rot fungi and fungus specific carbon source should be elucidated to maximally enhance the enzyme synthesis (Bettin et al. 2008; Revankar and Lele 2006; Elisashvili

and Kacchlishvili 2009). Lu et al. (1996) found a strong correlation between hyphal branching and the expression and secretion of laccase. The addition of cellobiose could induce profuse branching in certain *Pycnoporus* species and consequently increased laccase activity. The addition of cellobiose and lignin could increase the activity of extracellular laccases without an increase in total protein concentration (Garzillo et al. 1998; Lu et al. 1996). Mansur et al. (1998) reported inducing effect of fructose which enhanced laccase activity of 100-fold in *Basidiomycete sp*. I-62. Galhaup et al. (2002) reported that glucose and cellobiose were efficiently and rapidly utilized by *Trametes pubescens* resulting in high laccase activity. The replacement of crystalline cellulose or xylan by cellobiose increased lac activity of *Cerrena unicolor* by 21- and 70-fold, respectively (Elisashvili et al. 2002). Mikiashvili et al. (2005) also reported similar results in *T. versicolor* laccase production. In another study, Mikiashvili et al (2006) reported high laccase production in CMC supplemented medium by *Pleurotus ostreatus*. *F. fomentarius* also was found to produce high levels of laccase in presence of polysaccharides with especially high specific laccase activity in medium containing carboxymethyl cellulose (Elisashvili and Kacchlishvili 2009). Stajic et al. (2006) observed the highest laccase activity in the presence of mannitol, glucose and sodium gluconate in two strains of *P. ostreatus*.

4.3.5.4 Effect of nitrogen source on biomass and laccase production

Another factor reported to be essential for efficient laccase production is the nature and concentration of nitrogen sources in media. We examined the ability of four inorganic and three organic nitrogen sources on the production of laccase by the fungi.

Fig.4.16 presents the results of effect of nitrogen sources on biomass and laccase production by *Peniophora sp.* hpF-04. It showed efficient laccase activities in the presence of inorganic nitrogen sources such as ammonium chloride, ammonium tartarate and ammonium nitrate. Organic nitrogen sources were also able to produce laccase in suitable amounts. Among the inorgnic sources, highest laccase production was seen in case of ammonium chloride (1082.1±13.4 U/l), followed by ammonium tartarate

(941.95±11.6 U/l). In case of potassium nitrate and ammonium nitrate, laccase activity of 571.83±9.1U/l and 825.35±10.1U/l was observed, respectively. Among the organic nitrogen sources, yeast extract (YE) supplemented media produced highest laccase activity of 891.55±14.7U/l, followed by aspargine (505.18±4.3) and urea (392.7±12.3). Biomass yield (g/l) of the fungus was observed in the following descending order: yeast extract (11.65±0.2), aspargine (7.88±0.03), urea (7.50±0.2), ammonium tartarate (6.95±0.3), potassium nitrate (6.57±0.9), ammonium chloride (6.28±0.1) and ammonium nitrate (6.15±0.01).

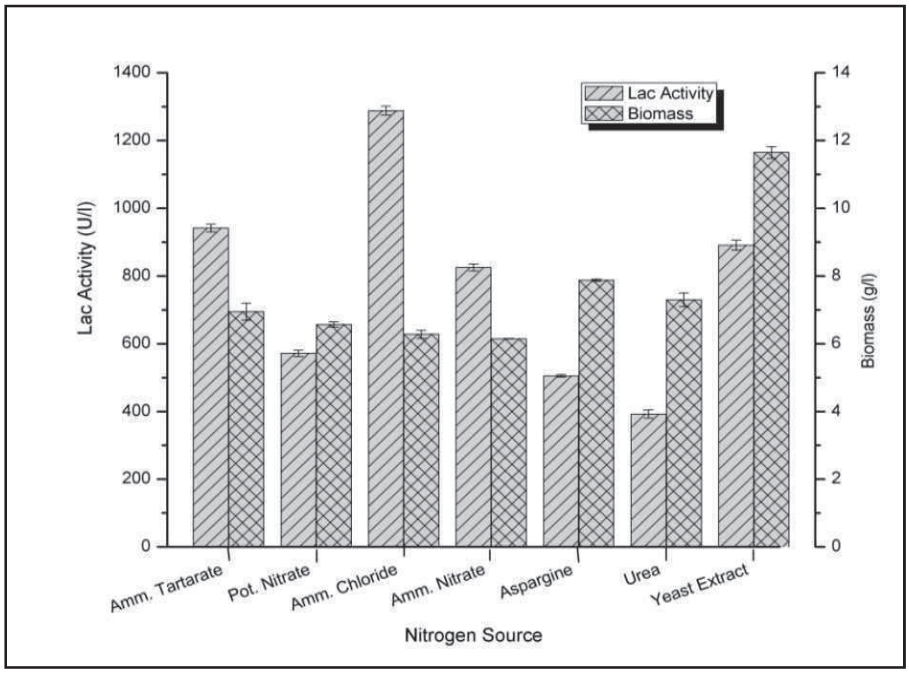

Fig. 4.16 Biomass and laccase production by *Peniophora sp.* hpF-04 cultivated in LMM medium containing different nitrogen source

The results of effect of nitrogen source on laccase production by *Phellinus noxius* hpF-17 is presented in Fig. 4.17. This fungus showed highest laccase activity in the presence of ammonium tartarate (557±23.1 U/l). This fungus also showed sufficient laccase production in all inorganic nitrogen sources employed in the study. In potassium nitrate and ammonium nitrate supplemented medium, fungus produced laccase of 272.55±12.2 U/l and 186.8±24 U/l, respectively. In the presence of ammonium chloride,

low level of laccase activity of 192.4±14.5U/l was observed. Among the organic nitrogen sources, urea showed considerable laccase activity of 392.75±9.1 U/l, in this fungus. Followed by urea, yeast extract produced laccase of 274.4±20.1 U/l. In the presence of aspargine as nitrogen source, laccase production of 125.95±8.0 U/l was observed. Biomass in grams per litre of the fungus was found in the following descending order: yeast extract (14.9±2.1), ammonium tartarate (9.4±1.2), ammonium chloride (5.9±1.0), urea (5.25±0.4), aspargine (2.55±0.4), potassium nitrtate (1.45±0.2), ammonium nitrate (0.6±0.1). In the study, it was observed that different nitrogen sources stimulated the growth of the fungi as well as laccase production in varying degrees.

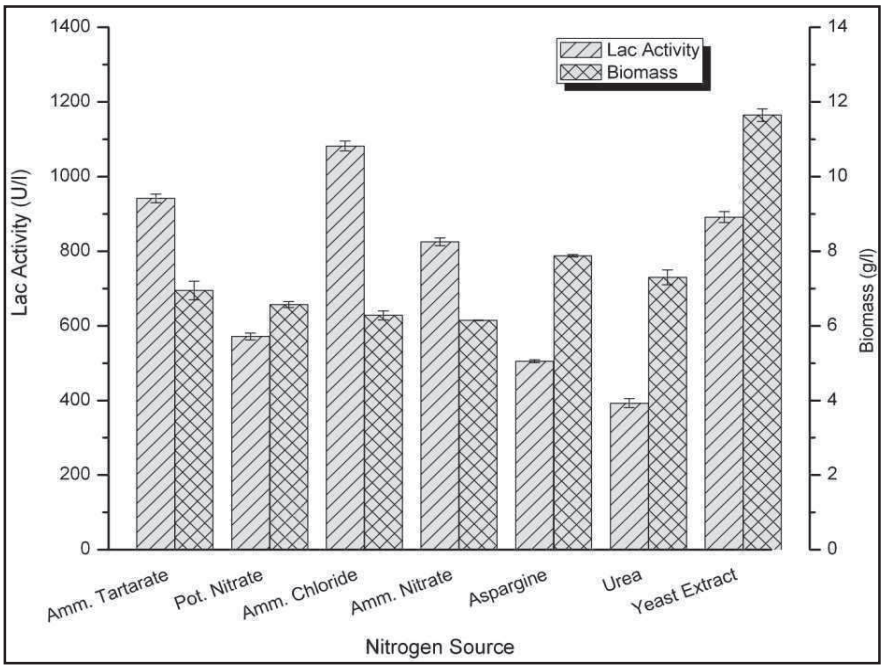

Fig. 4.17 Biomass and laccase production by *Phellinus noxius* hpF-17 cultivated in LMM medium containing different nitrogen source

In the literature, contradictory evidence exists for the effects of nature and concentration of the nitrogen sources on laccase production. Heinzkill et al. (1998) also reported a higher yield of laccase using nitrogen rich media rather than the nitrogen-limited media usually employed to induce oxidoreductases. While high nitrogen media gave the highest laccase activity in *L.edodes*, *Rhigidoporous lignonus* and *Trametes*

pubesccens, the nitrogen limited conditions enhances the production of the enzyme in *Pycnoporous cinnabarinus*, *P.sanguinens* and *Phlebia radiate* (Mester and Field 1997; Gianfreda et al. 1999; Galhaup et al. 2002). Elisashvili et al. (2001) observed highest laccase activity in *C. unicolour* IBB 62 in a medium with ammonium sulphate as the nitrogen source. D'Souza-Ticlo et al. (2006) showed that well defined organic nitrogen sources such as glutamic acid and glycine were better than beef extract and corn steep liquor for laccase production. The role of these compounds in the regulation of enzyme synthesis depends not only on the physiology of the tested fungi but also on the medium composition (Couto et al. 2004; Kapich et al. 2004).

The result of this investigation showed that the fungi *Peniophora sp.* hpF-04 and *Phellinus noxius* hpF-17 were able to utilize the various carbon and nitrogen sources as their nutrient substrates. Fungi being heterotrophs obtain their required nutrients from the organic matter in the environment through the presence of efficient and extensive systems of powerful enzymes.

4.3.5.5 Effect of inducer on biomass and laccase production

Laccase production has been found to be highly dependent on the cultivation conditions of the fungus (Heinzkill et al. 1998). The media supporting high biomass does not necessarily support high laccase yields (Xavier et al. 2001). However, one of the most effective approaches to increase the yield of laccase enzyme is the supplementation of the nutrient medium with an appropriate inducer. The addition of aromatic compounds such as 2,5-xylidine (Bollang and Leonowicz 1984; Elisashvili et al. 2002; Revankar and Lele 2006), lignin and veratryl alcohol (Lele et al. 2006), Guaiacol (Lele et al. 2006) are known to induce and increase laccase activity. Surfactants like Tween 80 enhanced the production of laccase in many fungi (Barbosa et al. 2007).

To study the inducing effect of different xenobiotic components on laccase production by *Peniophora sp*. hpF-04 and *Phellinus noxius* hpF-17, respectively, the cultivation was carried in LMM media containing glucose as a carbon source and

ammonium tartarate as a nitrogen source. The initial copper sulphate of LMM medium was omitted in these experiments as copper sulphate was also considered as an inducer. The fungi were grown at 30°C under shaking conditions and 6 day culture filtrates were used for measuring laccase activity. The inducers and their concentrations with their respective biomass and laccase activities obtained are presented in Table 4.1.

Table 4.1 Synergetic effect of inducer on laccase production by *Peniophora sp.* hpF-04 and *Phellinus noxius* hpF-17

Inducer	Concen-tration	Laccase activity (U/l)		Biomass (g/l)	
		***Peniophora sp.* hpF-04**	***P.noxius* hpF-17**	***Peniophora sp.* hpF-04**	***P.noxius* hpF-17**
Veratryl alcohol (ml/l)	0.1	147.7 ±2.1	303.2±10.2	8.7±0.9	10.4±0.2
	0.3	111.3±0.2	102.4±8.6	6.1±0.3	9.8±0.3
	1	100.8±3.2	144.5±5.5	5.8±0.5	6.7±0.1
Copper sulphate (g/l)	0.5	634.9±4.5	330.8±21	12.3±0.4	13.5±0.2
	1	1052.3±21	770.0±8	13.5±0.5	12.4±0.3
	2	1047.5±12	667.0±6	14.5±0.4	10.2±0.2
Guaiacol (ml/l)	0.1	568.8±9.4	65.9±2.8	9.9±0.2	12.4±1.0
	0.5	24.95±2.3	12.5±0.7	6.9±0.6	11.4±0.6
	1	1.45±0.2	8.2±2.1	5.7±0.6	8.7±0.2
2,5-xylidine (mM)	0.5	893.0±2.5	243.2±12	13.0±1.0	14.4±1.0
	1	1042.0±12.4	193.4±14	10.6±0.2	12.8±0.3
	2	673.0±2.2	183.5±1.8	10.9±0.3	10.6±0.4
Tween 80 (%)	0.1	338.5±3.5	470.1±10	14.5±0.2	13.5±0.5
	0.5	805.2±13.7	534.2±21	15.8±0.8	15.5±0.3
	1	1201.3±24.5	601.4±5.5	15.9±0.4	14.9±0.5

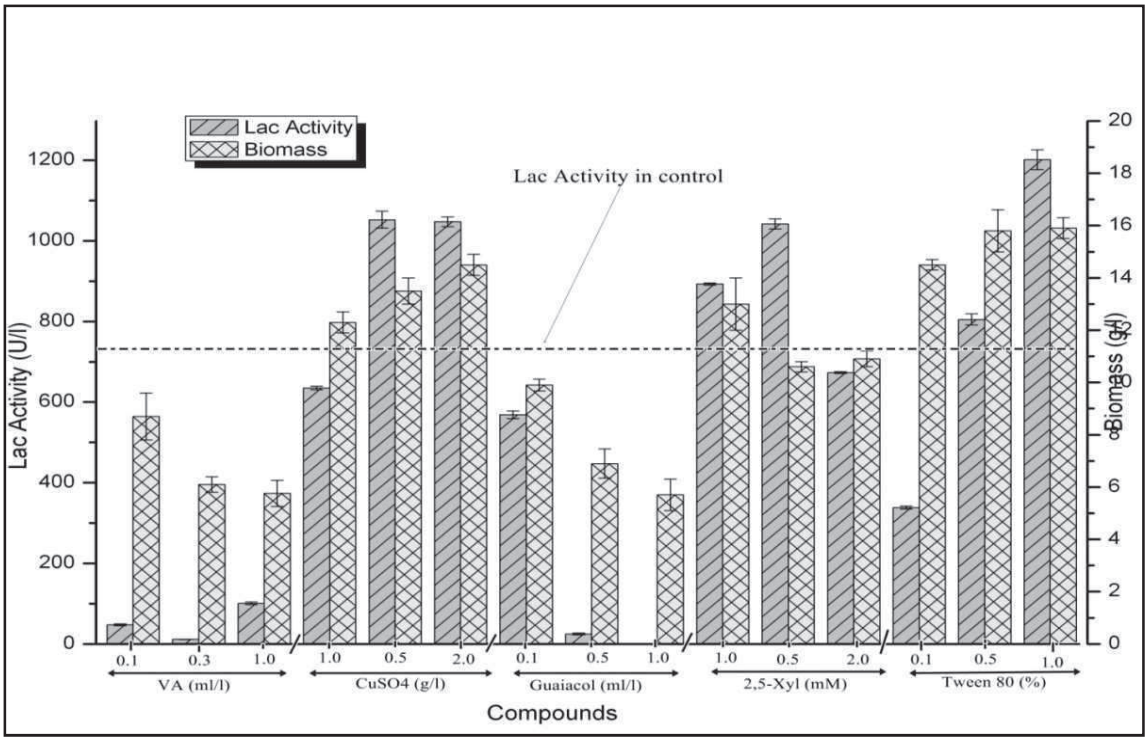

Fig. 4.18 Effect of different inducer on the biomass and laccase production by *Peniophora sp*. hpF-04. Dotted line represents the laccase production in control.

Fig. 4.18 presents the synergic effect of inducers on biomass and laccase production by *Peniophora sp*. hpF-04. The compounds like VA and guaiacol were insignificant in enhancing laccase activity at any of their concentrations. Instead, the presence of these compounds reduced laccase activity significantly. On the other hand, $CuSO_4$, 2,5-xylidine and Tween 80 significantly enhanced the laccase activity. In the case $CuSO_4$, laccase activity increased from 634.9±4.5 U/l to 1052.3±21 U/l when copper content of the media was increased from 0.5 to 1.0 g/l. Further increment of copper (2.0 g/l) slightly reduced the laccase activity (1047.5±12U/l). Tween 80 showed similar effect as that of copper sulphate. With the increase in Tween 80 concentration from 0.1 to 1.0 ml/l, the laccase activity increased from 338.5±3.5 to 1201.3±24.5 U/l. In the presence of 1.0 mM of 2,5-xylidine, laccase activity of 893.0±2.5 U/l was observed. However, laccase activity reduced to 673.0±2.2 U/l on further increment of 2,5-xylidine

concentration in the medium. In the presence of different concentrations of veratryl alcohol, laccase activity of 147.7 ±2.0 U/l, 111.3±0.2 U/l and 100.8±3.2U/l was observed at 0.1, 0.3 and 1.0ml/l of VA concentration in the medium. Similar trend of reduction in laccase activities were observed in the presence of guaiacol as shown in Table 4.1.

Presence of inducer in the medium also produced drastic changes in biomass production of the fungus. Presence of VA and guaiacol not only reduced laccase activity but also reduced dry mycelial biomass of *Peniophora sp*.hpF-04. Biomass of 8.7±0.9 g/l was obtained when medium is supplemented with 0.1ml/l of VA which was reduced to 5.8±0.5 U/l at VA concentration of 1ml/l. Similar trend in reduction in biomass value was observed during guaiacol supplementation in the medium. Biomass value of 9.9±0.2 g/l (at 0.1ml/l) was reduced to 5.7±0.6 g/l (at 1.0 ml/l) of guaiacol supplementation. However, presence of copper sulphate and Tween 80 enhanced the biomass production as shown in Table 4.1.

Fig. 4.19 presents the results of synergic effect of different inducers on biomass and laccase production by *Phellinus noxius* hpF-17. Compounds like VA, guaiacol and 2,5-xylidine did not show any increment in laccase activity but reduced the laccase activity significantly. The effect of copper sulphate and Tween 80 on laccase production was almost similar to that observed for *Peniophora sp*. hpF-04. In the presence of $CuSO_4$, laccase activity increased from 330.8±21U/l to 770.0±8 U/l when copper content was increased from 0.5 to 1.0 g/l but the enzyme activity was reduced (667.0±6 U/l) with further increment of copper (2.0 g/l). The laccase activity of *Phellinus noxius* hpF-17 increased from 470.1±10 U/l to 601.4±5.5 U/l with the increment of Tween 80 concentration from 0.1 to 1 U/l as shown in Fig.4.19. In this fungus, 2,5-xylidine also reduced the laccase activity. Laccase activity of 243.2±12 U/l, 193.4±14 U/l and 183.5±1.8 U/l was obtained at 2,5-xylidine concentration of 0.5, 1.0 and 2 mM, respectively. The values of respective biomass were shown in Table.4.1.

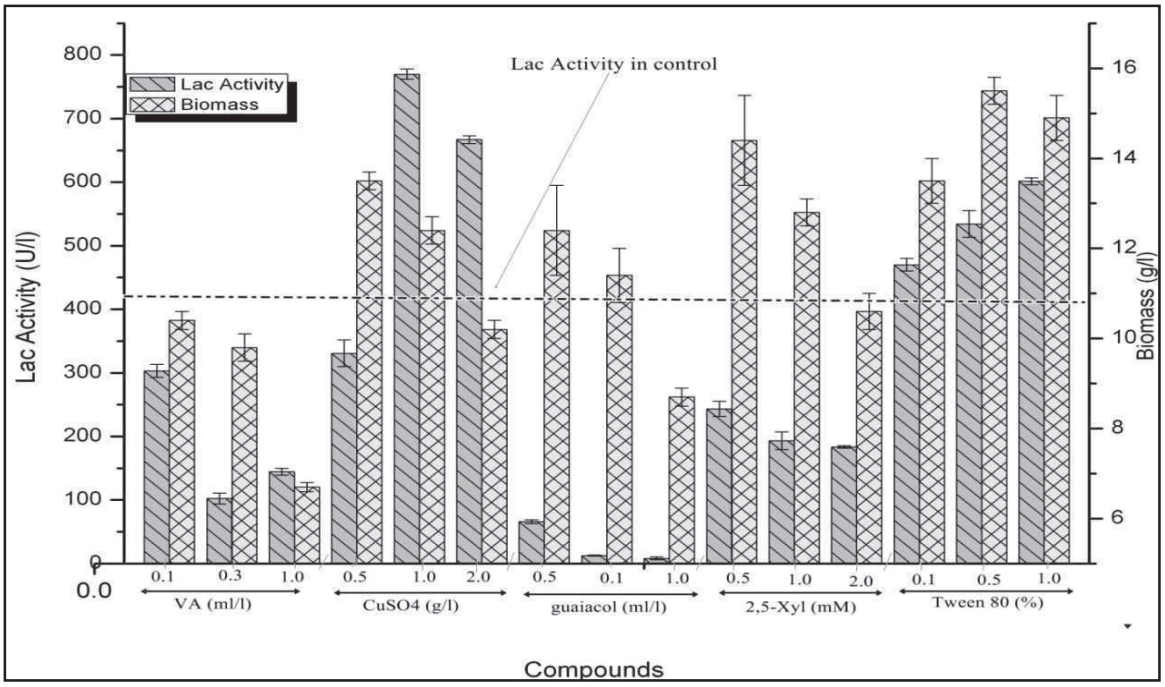

Fig. 4.19 Effect of different inducer on biomass and laccase production by *Phellinus noxius* hpF-17. Dotted line represents the laccase production in control.

Many of the compounds which resemble lignin molecules or other phenolic chemicals can act as inducers for laccase production (Marbach et al. 1985; Farnet et al. 1999). Veratryl (3, 4-Dimethoxybenzyl) alcohol is an aromatic compound known to play an important role in the synthesis and degradation of lignin. The addition of veratryl alcohol to cultivation media of many white-rot fungi has resulted in an increase in laccase production (Barbosa et al.1996). Sometimes these compounds affect the metabolism or growth rate (Froehner and Eriksson 1974). Eggert et al. (1996) reported that the addition of xylidene as inducer had the most pronounced effect on laccase production. The addition of 10 µM xylidine after 24 h of cultivation gave the highest induction of laccase activity and increased laccase activity to 9 fold (Eggert et al. 1996). They further observed that at higher concentrations, the xylidene had a reduction effect, probably due to its toxicity. Clear stimulation of the extracellular enzyme formation by xylidine was obtained in the cultures of *Fomes annosus*, *Pholiota mutabilis*, *Pleurotus ostreatus* and *Trametes versicolor*, whereas *Rhizoctonia praticola* and *Botrytis cinerea* were not

affected by the xylidine and in the case of *Podospora anserina* a decrease in laccase activity was observed (Bollag and Leonowicz 1984). Laccase offers protection for the fungus against toxic phenolic monomers of polyphenols (Eggert et al.1996; Assavanig et al.1992).

The addition of low concentrations of copper to the cultivation media of laccase producing fungi stimulates laccase production (Assavanig et al.1992). Palmieri et al. (2000) found that the addition of 150µM copper sulphate to the cultivation media can resulted in a 50-fold increase in laccase activity compared to the basal medium. According to the literature, the amount of copper required to enhance laccase production varies greatly among fungi, generally within the range 0.003–40µg Cu/ml. There are reports that show that higher concentrations of copper sulphate (5 mM $CuSO_4$ or 320 µg Cu/ml) effectively stimulate laccase synthesis (Galhaup and Haltrich 2001). Tavares et al. (2005) established the significance of induction by copper sulphate and 2,5-xylidine on *T.versicolor* culture for laccase production. Palvannan and Sathishkumar (2008) also reported the inducing effect of copper sulphate in *Pleurotus flourida* NCIM 1243. Similar copper-mediated increases in laccase synthesis were reported for *P. ostreatus* (Medeiros et al. 1999), *T. pubescens* (Galhaup and Haltrich 2001) and *B.rhodina* (Barbosa et al. 2007).

In our study, presence of Tween 80 not only increased the enzyme titer but also a larger biomass was obtained in the case of both the fungi. This increase in biomass might be attributable to the ability of fungi *Peniophora sp.* hpF-04 and *Phellinus noxius* hpF-17 to use Tween 80 as carbon source. Similar observation was made in *B.rhodina,* in which the presence of Tween 80 increased the laccase titer as well as biomass (Barbos et al. 2007). *B. rhodina* was able to grow in the presence of Tween 60 as sole carbon source and to produce laccase. *Acinetobacter radioresistens* has also been reported to use Tween 80 as sole carbon source, but it produce a lipase. The use of surfactants in enzyme production has been well-documented. Surfactants increase the permeability of the membrane's lipid bilayer, which facilitates the secretion of intracellular enzymes.

The promoter regions of the genes encoding for laccase contain various recognition sites that are specific for xenobiotics and heavy metals (Sannia et al. 2001). These can bind to the recognition sites when present in the substrate and induce laccase production. White-rot fungi were diverse in their responses to tested inducers for laccase. The addition of certain inducers can increase the concentration of a specific laccase or induce the production of new isoforms of the enzyme (Robene-Soustrade and Lung-Escarmant 1997). Some inducers interact variably with different fungal strains (Eggert et al.1996).

To conclude, in the present study, two isolates were found to be promising sources of laccase enzyme. The production of enzyme by these isolates was influenced by nutrient and cultivation conditions. Among the different assay used for the determination of laccase, ABTS assay was found to be most sensitive substrate which was further used for the laccase determination assay. The substrates such as 2,4-DMP and syringaldazine may not be considered as a substrate for laccase assay due to the instability of colour formation during the reaction. Isolate *Peniophora sp.* hpF-04 produced highest laccase in the presence of sucrose and ammonium chloride while the maximum laccase production in the case of *Phellinus noxius* hpF-17 was obtained in the presence of glucose and ammonium tartarate. The presence of copper sulphate and Tween 80 increased laccase production in both the fungi. The literature data and the results obtained in this work show that the effect of carbon, nitrogen and inducer sources depends on the fungal strain and nature of the compound tested. Factors like carbon, nitrogen and inducer sources and their concentrations have always been of great interest to the researchers in the enzyme industry for the low-cost media design. It is also known that 30–40% of the production cost of industrial enzymes is estimated to be the cost of growth medium. Therefore, it is of great significance to optimize the conditions for cost-efficient enzyme production. However, further optimization of important parameters by statistical methods will be focused in future experiments.

Chapter 5

STATISTICAL OPTIMIZATION OF PROCESS VARIABLES FOR ENHANCED LACCASE PRODUCTION

5.1 Introduction

Two isolated fungi, *Peniophora sp.* hpF-04 and *Phellinus noxius* hpF-17 were found to be the good producers of laccase. The production of laccase by these fungi was influenced by various factors like initial medium pH, incubation temperature and nutrient components. In our preliminary experiments, we evaluated the suitability of various carbon sources, nitrogen sources and inducers for the effective production of biomass and extracellular laccase by these two isolates. Both fungi produced high biomass and laccase production at a temperature of 30^{o}C and medium pH of 6.0. The influence of alternative sources of carbon, nitrogen and inducers were studied by traditional one-factor-at-a-time method. This method of optimization was carried out by changing one independent variable while fixing the other variables at a certain level. This single-dimensional search is laborious, time-consuming and incapable of reaching a true optimum due to interactions among the variables. Hence in this study, we exploited statistical methods to find out the true variables and its concentration to maximize laccase production in these fungi.

The preliminary data indicated that the major medium constituents that affected production of biomass and laccase in *Peniophora* sp. hpF-04 were the carbon sources (glucose, CMC and sucrose), nitrogen sources (ammonium tartarate, ammonium chloride, ammonium nitrate, aspargine and yeast extract) and inducers (copper sulphate, 2,5-xylidine and Tween 80) and those for *Phellinus noxius sp*. hpF-17 were carbon sources (glucose, sucrose and cellobiose), nitrogen sources (ammonium tartarate, potassium nitrate, ammonium nitrate, urea and yeast extract) and inducers (copper sulphate, 2,5-xylidine and Tween 80). Further identification of the significant variables was done by employing Placket-Burman design. Later, Central Composite Design (CCD) of Response Surface Methodology (RSM) was used for further optimization of medium variable concentrations to enhance the laccase production.

Response surface methodology (RSM) is a powerful and efficient mathematical approach widely applied in the optimization of various media components on enzyme

production (Adinarayana and Ellaiah 2002; Park et al. 2002; Pui et al. 2002), production of other metabolites (Zhang et al. 1996; Sunitha et al.1998; Sadhukhan et al.1999; Hujanen et al. 2001), spore production (Yu et al. 1997) and biomass production optimization (Lhomme and Roux 1991), etc. When many factors and their interactions regulate the desired response, RSM is an effective statistical tool for optimizing the process by solving the multivariate equations. RSM has been successfully applied in the optimization of the medium components for laccase production from different microorganisms (Arockiasamy et al. 2008; Niladevi et al. 2009; Bhattacharya et al. 2011).

5.2 Materials and Methods

5.2.1 Culture and maintenance

The fungal strains *Peniophora sp*. hpF-04 and *Phellinus noxius* hpF-17 were maintained in 2% malt extract agar plates and periodically sub-cultured on the same media before use.

5.2.2 Statistical optimization of laccase production by *Peniophora sp*. hpF-04

The optimization of medium components for laccase production was accomplished in two stages:-

5.2.2.1 Plackett-Burman Design (PBD)

Plackett-Burman design was used to find the significant nutritional factors influencing laccase production by *Peniophora sp. hpF-04***.** Total eleven variables (variable k=11) were selected based on the previous experiments. Each variable was kept at two levels, represented as -1 for low and +1 for high level. The selected variables for the present study were carbon sources (glucose, CMC and sucrose); nitrogen sources (ammonium tartarate, ammonium chloride, ammonium nitrate, yeast extract and aspargine); and inducers (copper sulphate, 2,5-xylidine and Tween 80) (Table 5.1). Effect of these variables was studied by PBD involving twelve experiments. However, the effect

of process parameters such as medium pH and incubation temperature were optimized initially and were not included in the design. All experiments were carried out in duplicate and average of the laccase activity was considered as response. From the Pareto chart, the variables showing the highest positive effect on laccase production were further selected for optimization using central composite design of response surface methodology.

Table 5.1 Experimental range and the levels of process variables used in PBD study

Variable	Code	Low Level (-1)	High Level (+1)	Effect
Glucose (g/l)	A	5	10	-1408.9
Sucrose (g/l)	B	5	10	1479.4
CMC (g/l)	C	2.5	5	2142.6
Ammonium tartarate (g/l)	D	0.2	2	340.1
Ammonium chloride (g/l)	E	0.2	2	843.1
Ammonium nitrate (g/l)	F	0.2	2	237.5
Yeast extract (g/l)	G	2.5	5	-1073.8
Aspargine (g/l)	H	2.5	5	-859.9
Copper Sulphate (uM)	J	300	600	2748.1
2,5-xylidine (mM)	K	0.2	2	-350.8
Tween 80 (%)	L	0.1	0.5	138.6

5.2.2.2 Optimization of screened process variables by RSM

CCD of RSM was used to optimize the concentration of significant variables that might enhance laccase production. The behavior of the system was explained by the following quadratic equation.

$$Y = \beta_0 + \Sigma \beta i\, Xi + \Sigma \beta ii\, Xi^2 + \Sigma \beta ij\, XiXj \qquad (5.1)$$

Where Y represents response variable, βo is the interception coefficient, βi is coefficient of the linear effect, βii is the coefficient of quadratic effect and βij is the coefficient of interaction effect.

A 2^3 factorial design augmented by 6 axial points (α = 1.682) was implemented in 17 experiments wherein the effect of each variable on laccase production was taken as a response. Design Expert Version 8.0 (Statease) was used for multiple regression analysis and to construct the plots of the obtained data. The coded and uncoded values of the variables at various levels are given in Table 5.2. The coded variables were Carboxy Methyl Cellulose (CMC) (A), ammonium chloride (B) and copper sulphate (C). These values were converted into their actual values to find out the optimum range of variables for the production of laccase as described by Palvannan and Sathishkumar (2010).

Table 5.2 Experimental range and levels of independent variables used in CCD-RSM study

Code	Factor	Range and level				
		Very low level (-1.68)	Low level (-1)	Mid level (0)	High level (+1)	Very igh level (+1.68)
A	CMC (g/l)	6.60	10	15	20	23.4
B	Ammonium chloride (g/l)	0.26	0.70	1.35	2.10	2.40
C	Copper sulphate (μM)	200	400	800	1200	1400

5.2.3 Statistical optimization of laccase production by *Phellinus noxius* hpF-17

5.2.3.1 Plackett-Burman Design (PBD)

Plackett-Burman design was used to find the significant nutrient variables influencing laccase production. Total eleven components (variable k=11) were selected for the study with each variable being represented at two levels: -1 for low and +1 for high level as before. The selected variables considered in the present study were carbon sources (glucose, sucrose and cellobiose); nitrogen sources (ammonium tartarate,

potassium nitrate, ammonium nitrate, yeast extract and urea); and inducers (copper sulphate, 2,5-xylidine and Tween 80) (Table1). Based on Pareto chart, the variables showing highest positive effect on laccase production were identified from each category. These significant variables were further optimized through CCD of RSM.

Table 5.3 Variables showing medium components used in Plackett-Burman design

Variable	Code	Low level (-1)	High level (+1)	Effect
Glucose (g/l)	A	5	10	818.4
Sucrose (g/l)	B	5	10	-313.9
Cellobiose (g/l)	C	5	10	44.0
Ammonium tartarate (g/l)	D	0.2	2	1142.6
Potassium nitrate (g/l)	E	0.2	2	-537.9
Ammonium nitrate (g/l)	F	0.2	2	-21.9
Yeast extract (g/l)	G	2.5	5	-537.9
Urea (g/l)	H	2.5	5	527.8
Copper sulphate (uM)	J	300	600	63.4
2,5-xylidine (mM)	K	0.2	2	220.6
Tween 80 (%)	L	0.1	0.5	647.8

5.2.3.2 Response surface methodology (RSM)

From PBD experiments, the factors such as glucose, ammonium tartarate and Tween 80 were found to have significant effect on laccase production in *P.noxius* hpF-17. These three variables were further optimized by CCD-RSM. The coded and uncoded values of the variables at various levels are given in Table 5.4. The coded variables were glucose (A), ammonium tartarate (B) and Tween 80(C). These values were converted into their actual values to find out the optimum range of variables for the production of laccase as described earlier.

Table 5.4 Experimental range and levels of independent variables

Code	Factor	Range and level				
		Very low level (-1.68)	Low level (-1)	Mid level (0)	High level (+1)	Very high level (+1.68)
A	Glucose(g/l)	3.18	10	20	30	36.82
B	Ammonium tartarate (g/l)	0.15	1	2.25	3.5	4.35
C	Tween 80 (ml/l)	0.32	1	2	3	3.68

5.2.4 Laccase assay

Laccase activities were measured spectrophotometrically (GBC) using 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) (Sigma) as a substrate with an absorbance coefficient value of $36000M^{-1}cm^{-1}$ at 420nm. The reaction mixture consisted of 1.5 ml acetate buffer (1 mM, pH 5.0), 1.5 ml ABTS (50 mM) and 1.5 ml culture filtrate. One unit (U) of laccase activity was defined as the amount of enzyme catalyzing the production of one micromole of coloured product per min per ml (Bourbonnais and Paice 1990).

5.3 Results and Discussion

5.3.1 Statistical optimization of process variables for laccase production by *Peniophora sp.* hpF-04

5.3.1.1 Plackett-Burman design

The influence of eleven medium factors namely, glucose, sucrose, CMC, ammonium tartarate, ammonium chloride, ammonium nitrate, yeast extract, aspargine, copper sulphate 2,5-xylidine and Tween 80, for laccase production was investigated in twelve runs using PBD. Laccase activity was observed in the range of 0.38 U/l to 7881.3 U/l in twelve runs (Table 5.5). This variation reflected the importance of medium optimization to attain higher yields.

Table 5.5 Experimental design matrix and the results of experimental runs of PBD for laccase production by *Peniophora* sp. hpF-04

Sl. No.	Coded variable											Laccase activity (U/l)
	A	B	C	D	E	F	G	H	I	J	K	
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	2567.40
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	761.50
3	- 1	1	1	-1	1	-1	-1	-1	1	1	1	1198.00
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	1498.00
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	6754.60
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	2876.50
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	1528.00
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	2842.00
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	7881.30
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	0.38
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	1876.00
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	456.30

The Pareto chart illustrates the order of significance of the variables affecting laccase production (Fig. 5.1). Among the 11 variables, copper sulphate showed highest significance on laccase production as indicated by the high positive effect. Followed by copper sulphate, CMC showed higher positive effect. Interestingly, glucose showed a very high negative effect. However, for further optimization using CCD, the factors which showed highest positive influence on laccase production in each of the category *viz*. carbon source, nitrogen source and inducers were selected. Among the carbon sources, CMC showed highest significance than glucose and sucrose. Among itrogen source, ammonium chloride showed larger significance. In the category of inducers, copper sulfate showed highest significance than Tween 80 and 2,5-xylidine. The variables, CMC, ammonium chloride and copper sulphate were screened based on their significance on laccase production and interactions among these variables were further

studied by central composite design. Hence, these variables were considered for optimization by CCD.

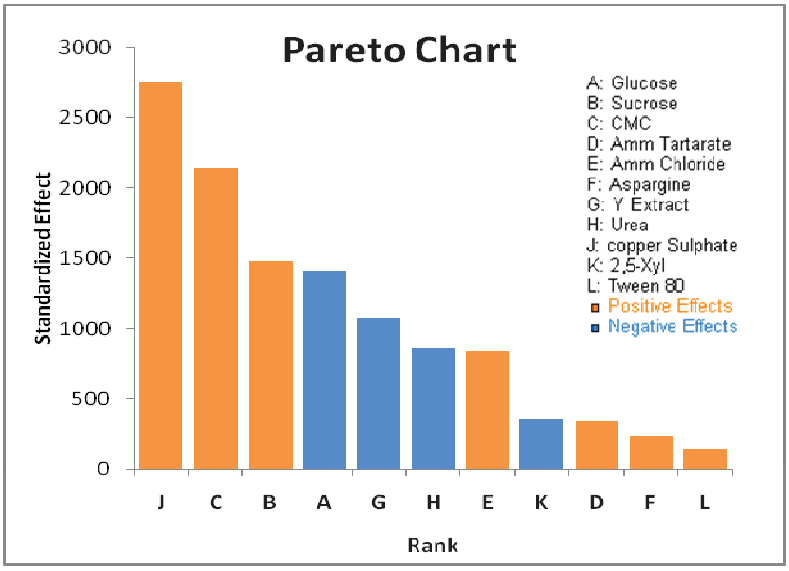

Fig. 5.1 Pareto chart of eleven-factor standard effects on laccase production by *Peniophora sp.* hpF-04

5.3.1.2 RSM-CCD for *Peniophora sp.* hpF-04

Central composite design was employed to study the combined effect of the variables, CMC (A), ammonium chloride (B) and copper sulphate(C). According to the design, 16 runs replicated twice at central points were performed. In each run experimental and predicted responses were recorded (Table 5.6). The relationship between variable was determined by fitting a second order polynomial equation to data obtained from the 16 runs.

Table 5.6 Full factorial central composite design matrix and their observed response

Run order	A (g/l)	B (g/l)	C (µM)	Laccase activity (U/l)		
				Actual response	Predicted response	Residual value
1	0	-1.68	0	403	693.533	290.533
2	0	0	-1.68	1645	1724.076	-79.076
3	-1	1	1	179	151.91	27.09
4	1	1	1	144	217.581	-73.581
5	1	-1	1	801	437.245	363.755
6	0	0	1.68	109	214.952	105.952
7	1.68	0	0	1229	1498.097	269.097
8	-1.68	0	0	505	420.931	84.069
9	-1	-1	-1	855	694.363	160.637
10	1	1	-1	1856	1691.369	164.631
11	1	-1	-1	2014	1911.033	102.967
12	0	1.68	0	430	324.496	105.504
13	-1	1	-1	285	474.698	-189.69
14	-1	-1	1	338	371.575	-33.575
15	0	0	0	1890	1908.569	-18.569
16	0	-1.68	0	1856	1908.569	51.431

The predicted and observed responses along with design matrix are presented in Table 5.6 and the results were analyzed by ANOVA. The second-order regression equation provided the levels of laccase activity as the function of CMC, ammonium chloride and copper sulphate which can be presented in terms of coded factors as in the following equation:

$$Y\ (response) = +1909.38+320.40A+(-109.73)B+(448.95)C+(-10.75)AB+(-287.75)AC+(-11.00)BC+(336.34)A^2+(-495.62)xB^2+(332.81)xC^2 \quad (5.1)$$

Where Y is the laccase activity (U/l), A, B, and C are CMC, ammonium chloride and copper sulphate, respectively.

The statistical significance of the Equation 5.1 was checked by F-test and the analysis of variance for response surface quadratic model is shown in Table 5.7. Analysis of Variance (ANOVA) of regression model demonstrates that the model is highly significant as it is evident from the Fischer test with very low probability value. The value of lack of fit, Model F and model P>F were found to be 34.95, 11.66 and less than 0.05 respectively, indicating that model was significant. Fisher F-test with a very low probability value (Pmodel >F = 0.001) and also insignificant lack of fit demonstrates a very high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient (R^2). The coefficient of determination (R^2) was calculated to be 0.945. This implies that 94.5% of experimental data of the laccase production was compatible with the data predicted by the model and only 5.5% of the total variations were not explained by the model. The value of the adjusted determination coefficient (Adj R^2 = 0.898) is also high to advocate for a high significance of the model. At the same time relatively low coefficient variation (CV=29.27%) confirm the precision and reliability of the experiment performed. From Table 5.7, it can be observed that the factors with higher significances were A, C, AC and squared terms of A^2, B^2 and C^2. The interaction terms AB and BC were found to be insignificant, which can be removed from the model without affecting the goodness of the model.

The three dimensional (3D) response surface graphs for laccase production based on the final model are depicted in Fig. 5.2. The graphs were generated for the pair-wise combination of the three factors while keeping the other one at its optimum levels. The response at the central point corresponds to a maximum achievable laccase activity for those factors. Almost all the interactions in the designed experiments produced a 'nearly spherical' variance function. This indicated that the effects of variables as individuals as well as interrelated allows for the prediction of optimum concentration levels for maximized laccase activity.

Table 5.7 Regression coefficients and their significance in the quadratic model for laccase production by *Peniophora sp.* hpF-04

Source	Sum of Squares	Df	Mean Squares	F Value	p-value Prob>F	
Model	7530000	9	837200	11.66	0.0037	Significant
A-CMC	140200	1	140200	19.53	0.0045	
B-Ammonium chloride	164400	1	164400	2.29	0.1809	
C-Copper Sulphate	275300	1	275300	38.35	0.0008	
AB	92450	1	924.5	0.013	0.9133	
AC	662400	1	662400	9.23	0.0229	
BC	96800	1	968.0	0.013	0.9113	
A2	104800	1	1048000	14.60	0.0087	
B2	227600	1	2276000	31.71	0.0013	
C2	102600	1	1026000	14.30	0.0092	
Residual	430600	6	71771.25			
Lack of Fit	428200	5	85635.5	34.95	0.1277	Not significant
Pure Error	2450	1	2450			
Cor Total	7965000	15				
Model fitting						
	C.V= 29.27%		R-Sq = 94.5%		R-Sq (adj) = 89.8%	

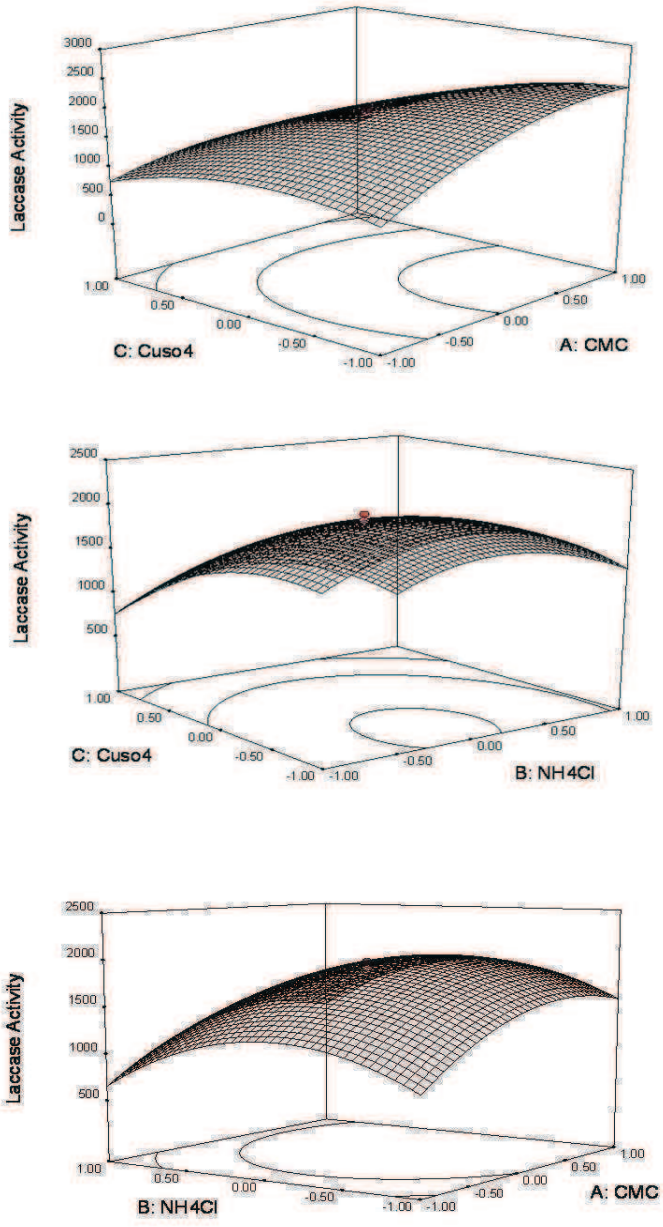

Fig. 5.2 Three dimensional (3D) response surface graphs of laccase production by *Peniophora sp*. hpF-04

The predicted optimum levels of the tested variables, namely, CMC, ammonium tartarate and copper sulphate were obtained by applying regression analysis on Equation 5.1. The optimal levels were as follows: A=0(15.0 g/l), B=0(1.35 g/l), C=0.018(807 µM)

with the corresponding laccase activity, Y=1900 U/l. Verification of the predicted values was conducted by using optimal medium in inoculation experiments and a response of 1890±12 U/l was obtained. This result corroborated the validity and the effectiveness of this model.

5.3.2 Statistical optimization of process variables for laccase production by *Phellinus noxius* hpF-17

5.3.2.1 PBD for *Phellinus noxius* hpF-17

Variations in laccase activity ranging from 24.9 U/l to 2876.5 U/l in twelve experiments of PBD was observed (Table 5.8). This variation reflected the importance of medium optimization to attain higher yields.

Table 5.8 Experimental design matrix and the results of experimental runs of PBD for laccase production by *Phellinus noxius* hpF-17

Coded Variable											Laccase
A	B	C	D	E	F	G	H	J	K	L	activity (U/l)
+	-	+	-	-	-	+	+	+	-	+	1589
+	+	-	+	+	-	+	-	-	-	+	1239.2
-	-	-	+	+	+	-	+	-	-	+	1783.4
-	-	+	+	+	-	+	+	-	+	-	884.7
-	+	+	+	-	+	+	-	+	-	-	396.3
+	+	+	-	+	+	-	+	-	-	-	536.1
+	-	+	+	-	+	-	-	-	+	+	2876.5
+	+	-	+	-	-	-	+	+	+	-	2478.5
-	+	+	-	+	-	-	-	+	+	+	143.6
-	+	-	-	-	+	+	+	-	+	+	542.5
+	-	-	-	+	+	+	-	+	+	-	30.06
-	-	-	-	-	-	-	-	-	-	-	24.9

The Pareto chart illustrates the order of significance of the variables affecting laccase production (Fig. 5.3). Among the variables, ammonium tartarate showed highest significance by showing higher positive effect. However, for further optimization using CCD, the factors which showed highest positive influence in each of the category *viz*. carbon source, nitrogen source and inducers were selected. Among the carbon sources, glucose showed higher significance as compared to sucrose and cellobiose and hence was included in CCD. Among nitrogen sources, ammonium tartarate showed larger significance. In the category of inducers, Tween 80 showed higher significance than copper sulfate and 2,5-xylidine, hence Tween 80 was selected as a variable for CCD.

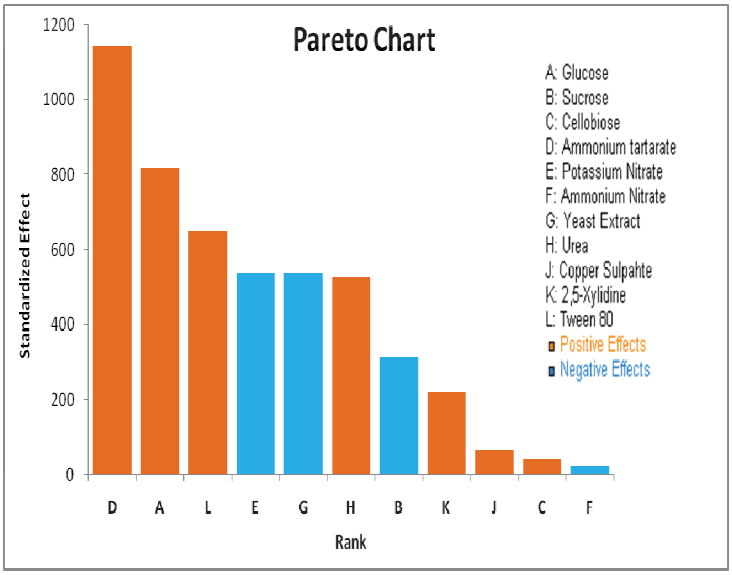

Fig. 5.3 Pareto chart of eleven-factor standard effects on laccase production by *Phellinus noxius* hpF-17

5.3.2.2 Response Surface Methodology (RSM)

From PBD, variables such as glucose, ammonium tartarate and Tween 80 were obtained as significant variables. To examine the combined effect of these factors, a central composite design (CCD) was employed within a range of –α (-1.68) and +α (+1.68) in relation to the production of laccase. According to the design, 17 runs replicated three times at central points were performed. The experimental and predicted

responses were recorded (Table 5.9). Relationship between the variables was determined by fitting a second order polynomial equation to the data obtained from these 17 runs.

Table 5.9 Full factorial central composite design matrix and their observed response in *Phellinus noxius* hpF-17

Run order	A (g/l)	B (g/l)	C (ml/l)	Laccase activity(U/l)		
				Actual response	Predicted response	Residual Value
1	-1	-1	-1	213.33	214.295	-0.965
2	+1	-1	-1	269.12	350.231	-81.111
3	-1	+1	-1	1	0.855	0.145
4	+1	+1	-1	10.96	31.251	-20.291
5	-1	-1	+1	242.5	212.9	29.642
6	+1	-1	+1	1.5	-7.705	9.205
7	-1	+1	+1	720	629.538	90.462
8	+1	+1	+1	313.75	303.434	10.316
9	-1.68	0	0	435.83	502.326	-66.496
10	+1.68	0	0	395.83	342.586	53.244
11	0	-1.68	0	197.08	166.842	30.238
12	0	+1.68	0	205.42	248.910	-43.490
13	0	0	-1.68	70.83	5.478	65.352
14	0	0	+1.68	154.3	232.904	-78.604
15	0	0	0	787.5	774.215	13.285
16	0	0	0	795.83	774.215	21.615
17	0	0	0	741.67	774.215	-32.545

The predicted and observed responses along with design matrix is presented in Table 5.9 and the results were analyzed by ANOVA. The second-order regression equation provided the levels of laccase activity as a function of glucose, ammonium

tartarate and Tween 80 which can be presented in terms of coded factors as in the following equation:

$$Y\ (response) = +774.32-47.51A+24.43B+67.64C-26.39AB-89.12AC+157.33BC-124.64A^2-200.51B^2-231.86C^2 \quad (5.2)$$

Where Y is the laccase activity (U/l), A, B, and C are glucose, ammonium tartarate and Tween 80, respectively.

Table 5.10 Analysis of Variance (ANOVA) for response surface quadratic model for the production of laccase by *Phellinus noxius* hpF-17

Source	Sum of Squares	Df	Mean Squares	F Value	p-value Prob>F	
Model	1199000	9	133200	24.27	0.0002	Significant
A-Glucose	30820.04	1	30820.04	5.61	0.0497	
B-Ammonium tartarate	8133.62	1	8133.62	1.48	0.2630	
C-Tween 80	62478.32	1	62478.32	11.38	0.0119	
AB	5569.35	1	5569.35	1.01	0.3474	
AC	63546.13	1	63546.13	11.57	0.0114	
BC	198500	1	198500	36.15	0.0005	
A2	175100	1	175100	31.89	0.0008	
B2	453200	1	453200	82.54	< 0.0001	
C2	6.06100	1	606100	110.37	< 0.0001	
Residual	38437.10	7	5491.01	38437.10		
Lack of Fit	36736.08	5	7347.22	8.64	0.1070	Not significant
Pure Error	1701.03	2	850.51			
Cor Total	1238000	16				
Model fitting						
	C.V= 22.67%		R-Sq = 96.9%		R-Sq(adj) = 92.9%	

The statistical significance of Equation 5.2 was checked by F-test and the analysis of variance for response surface quadratic model is shown in Table 5.10. ANOVA of regression model demonstrates that the model is highly significant as it is evident from the Fischer test with very low probability value. The value of lack of fit, Model F and model P>F were found to be 8.64, 24.27 and less than 0.05 respectively, indicating that model was significant. Fisher F-test with a low probability value (Pmodel >F = 0.0002) and also insignificant lack of fit demonstrates a very high significance of the regression model. The goodness of fit of the model was checked by the determination coefficient (R^2). The coefficient of regression (R^2) was calculated to be 0.969. The value of the adjusted regression coefficient (Adj R^2 = 0.929) was also high, which advocates for high significance of the model. At the same time relatively low coefficient variation (CV=22.67 %) confirms the precision and reliability of the experiment performed. From the table 5.10, it can be seen that the factors with higher significance were A, C, AC, BC and squared terms of A^2, B^2 and C^2. The interaction terms AB seems to be insignificant, which can be removed from the model without affecting the goodness of the model.

The relationship between the actual and predicted laccase activity (response) is shown in Fig. 5.4. The cluster of measurements near the diagonal line in the parity plot indicates a good fit of the model and demonstrates a satisfactory correlation between the actual and predicted values. The minimum response of 1U/l laccase activity was obtained with 10g/l of glucose 3.5 g/l of ammonium tartarate and 1% Tween 80. The maximum response of 795.83 U/l laccase activity was obtained with 20 g/l glucose, 2.25 g/l ammonium tartarate and 2% Tween 80.

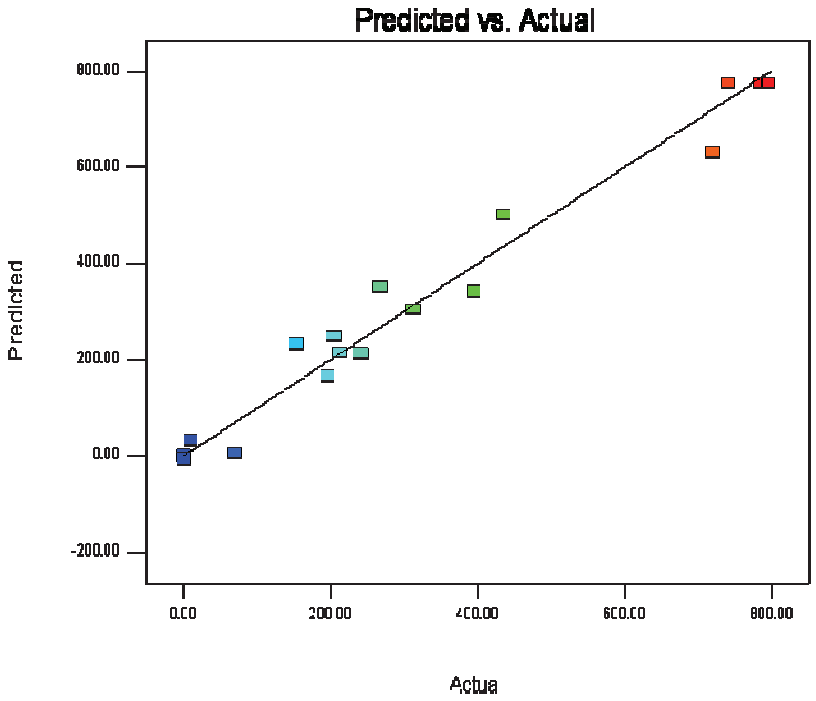

Fig. 5.4 Predicted v/s actual laccase production by *Phellinus noxius* hpF-17

The three dimensional (3D) response surface graphs of laccase production based on the final model are depicted in Fig. 5.5. The graphs were generated in pair-wise combination of the three factors while keeping the other one at its optimum level. The response at the central point corresponds to a maximum degree of achievable laccase activity for that factor. Almost all the interactions in the designed experiments produced a 'nearly spherical' variance function. This indicated that the variables as individuals as well as interrelated, allowed for the prediction of optimum concentration levels for maximized laccase activity.

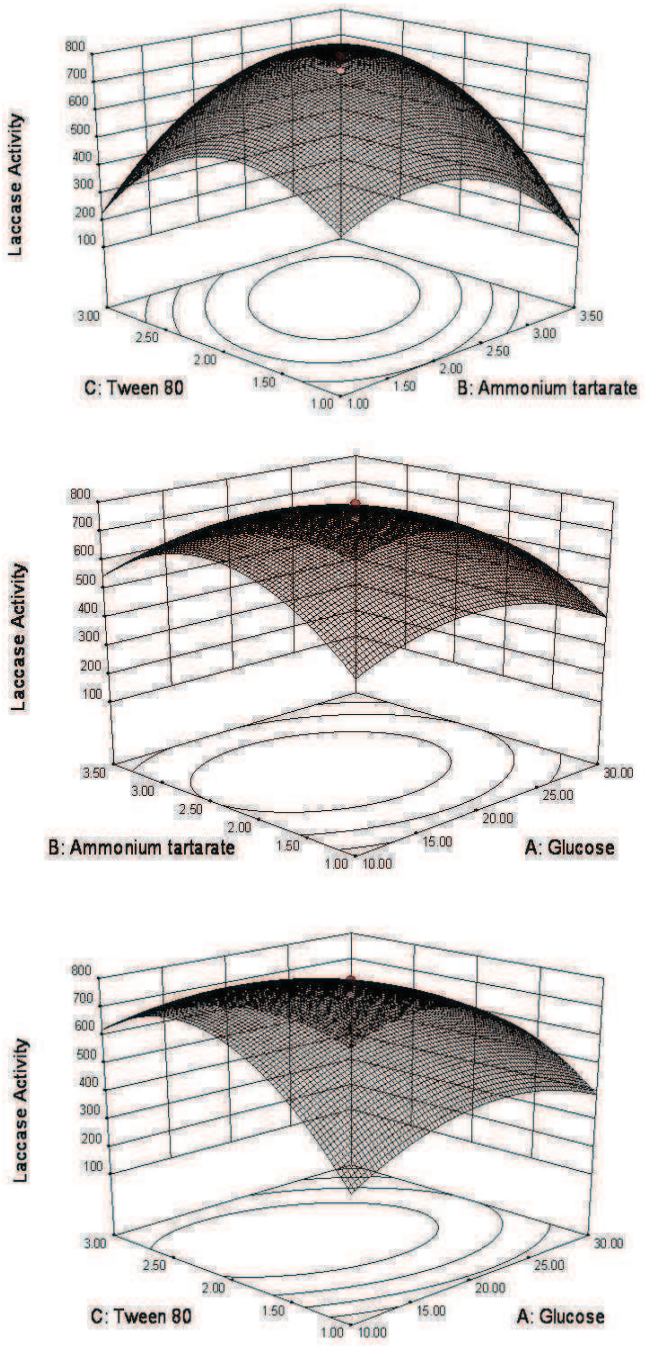

Fig. 5.5 3D response surface plot for the effect of a) Tween 80 and ammonium tartarate b) Ammonium tartarate and glucose and c) Tween 80 and glucose for the production of laccase by *Phellinus noxius* hpF-17

The predicted optimum levels of the tested variables, namely, glucose (A), ammonium tartarate (B) and Tween 80 (C) were obtained by applying regression analysis on Equation 5.2. The optimal levels were as follows: A=0 (20 g/l), B=0 (2.25 g/l), C=0.08 (2.08 ml/l) with the corresponding laccase activity, (Y) = 785 U/l. Verification of the predicted values was conducted by using optimal medium in inoculation experiments and the laccase activity of 780±7.9 U/l was achieved. This result corroborated the validity and the effectiveness of this model.

To conclude, the effect of process variables on the production of laccase by *Peniophora sp.* hpF-04 and *Phellinus noxius* hpF-17 was studied using PB design. Production of laccase in *Peniophora* sp. hpF-04 was found to be influenced by medium components such as CMC, ammonium chloride and copper sulphate. These variables were selected for further optimization studies using RSM. Optimum values of tested variables for maximum laccase production were; CMC (15 g/l), ammonium chloride (1.35 g/l) and copper sulfate (807 μM). Under this optimal condition, the laccase yield was increased to 1890 U/l. There was an approximate of 81% increase of laccase yield when compared to laccase activity in un-optimized medium was achieved.

On the other hand, medium components such as glucose, ammonium tartarate and Tween 80 were found to influence the laccase production in *Phellinus noxius* hpF-17. Optimum values of tested variables were glucose (20 g/l), ammonium tartarate (2.25 g/l) and Tween 80 (2.08 ml/l). With this optimal fermentation medium, the laccase yield of 780U/l was obtained. There was an approximate of 1.4 fold increase in laccase yield when compared to laccase activity in un-optimized medium.

Chapter 6

UTILIZATION OF AGRO-INDUSTRIAL RESIDUES FOR THE PRODUCTION OF LACCASE UNDER SOLID STATE FERMENTATION

6.1 Introduction

Solid state fermentation (SSF) holds tremendous potential for the production of various fungal enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source. This system offers numerous advantages over submerged fermentation (SmF) system in terms of productivity, relatively higher concentration of the products, cost-effectiveness in labour, time and medium components, requirement for simple fermentation equipments in addition to environmental advantages like less effluent production, waste minimization, etc. It has been generally claimed that product yields are mostly higher in SSF when compared to submerged fermentation (SmF) (Pandey 1992; Couto and Toca-Herrera 2007; Singhania et al. 2008). The use of SSF, especially using agro-wastes as a support-substrate, is an appalling alternative for industrial processes for laccase production at a lower cost.

Agro-industrial wastes are generated during the industrial processing of agricultural or animal products. Those derived from agricultural activities include materials such as straw, stem, stalk, leaves, husk, shell, peel, lint, seed/stones, pulp or stubble from fruits, legumes or cereals (rice, wheat, corn, sorghum, barley etc.), bagasses generated from sugarcane or sweet sorghum milling, spent coffee grounds, brewer's spent grains and many others. These wastes are generated in large amounts throughout the year, and are the most abundant renewable resources on earth. They are mainly composed of sugars, fibres, proteins and minerals, which are compounds of industrial interest. Due to the large availability and composition rich in compounds that could be used in other processes, there is a great interest on the reuse of these wastes, both from economical and environmental view points. The economical aspect is based on the fact that such wastes may be used as low-cost raw materials for the production of other value-added compounds, with the expectancy of reducing the production costs. The environmental concern is because most of the agro-industrial wastes contain phenolic

compounds and/or other compounds of toxic potential; which may cause deterioration of the environment when the waste is discharged to the nature (Singhania et al. 2008).

There are several reports describing use of agro-industrial residues for the production of laccase e.g. sugar cane bagasse by *Trametes versicolor* (Pal et al. 1995), Coconut flesh, groundnut shells and groundnut Seeds by *T. hirsuta* (Perez-Correa and Agosin 1999; Perez-Guerra et al. 2004), wheat straw and wheat bran by *Phlebia radiate* (Vares et al. 1995), *P. ostreatus* (Baldrian and Gabriel 2002), *P. pulmunaris* (de Souza et al. 2002), *T.versicolor* (Couto et al. 2002) and *Fomes sclerodermeus* (Papinutti et al. 2003), corn stalks by *Lentinus edodes* (D'Annibale et al. 1996), hampas, rubberwood sawdust by *Pycnoporus sanguineus* (Vikineswari et al. 2006), wheat bran flakes by *T. pubescens* (Osma et al. 2006), grape seeds by *T.hirsuta* (Couto et al. 2006), etc. Most of these wastes contain lignin or/and cellulose and hemicellulose, which act as inducers of the ligninolytic activities. Moreover, most of them are rich in sugars, which make the whole process much more economical. All these make them very suitable as raw materials for the production of secondary metabolites of industrial significance by microorganisms. However, individual microbial strain is unique in their molecular, biochemical, metabolic and enzyme production properties. The ability of the fungi to utilize the different substrates for growth and to produce laccase also varies among the strains.

In particular, the present investigation was aimed to exploit the locally available, inexpensive seven agro-industry wastes *viz*., sugar cane bagasse (SCB), wheat bran (WB), rice bran (RB), corn stover (CS), saw dust (SD), grass powder (GP), and Jatropa seed cake (JSC) as the potential solid state substrates for laccase production by *Peniophora sp*. *hpF-04* and *Phellinus noxius* hpF-17. Previously, the production of laccase by these isolates was carried out by submerged fermentation (SmF) using defined liquid medium and latter the medium was optimized to enhance the laccase titre in both the fungi. Here, in this study, the ability of the isolates to produce laccase under SSF was tested. Solid state fermentation was carried out with and without nutrient supplements.

The respective optimized laccase medium of *Peniophora sp*. hpF-04 and *Phellinus noxius* hpF-17 was used as nutrient supplements in case of SSF with nutrients supplements. The fermentation was carried out for 15 days and production of laccase was monitored at regular time interval of 5, 10 and 15 days. However, the other parameters like effect of pH, incubation temperature, moisture levels were studied only in the case of JSC, in which both the fungi showed maximum laccase production.

6.2. Materials and Methods

6.2.1. Cultures

Two fungal cultures *Peniophora sp*. hpF-04 and *Phellinus noxius* hpF-17 were maintained in 2% MEA plates and were subcultured before use.

6.2.2. Substrates for SSF

Solid state fermentation for the production of laccase was carried out with seven different agricultural wastes. The substrates used were sugarcane bagasse (SCB), wheat bran (WB), saw dust (SD), grass powder (GP), corn stover (CS), Jatropa seed cake (JSC) and rice bran (RB) (Fig. 6.1). No pretreatment was done for any of the substrates except for Jatropa seed cake kernel as it contains oil which could affect the fungal growth. De-oiling was carried out by using petroleum ether solution. All the substrates were finely powdered and sieved to get similar sized particles before employing them for SSF.

Fig. 6.1 Substrates employed in solid state fermentation for laccase production

6.2.3. Solid state fermentation (SSF)

SSF was carried out in 100 ml conical flasks containing 2-4g of solid substrates. The contents of the flasks were autoclaved at 121^{o}C at 15lbs pressure for 15 minutes. SSF was carried out in two different ways for both the fungi. One set of experiment was carried out with the addition of optimized nutrients solution of *Peniophora sp*. hpF04 and *Phellinus noxius* hpF-17, respectively. The second set of experiment was performed without the addition of nutrients. In this case, sterile distilled water was used as wetting agent. For the first set, inoculum was prepared by homogenizing 30 mycelial agar plugs of actively growing fungus (9mm in diameter) in 200ml of sterile nutrient medium. For the second set of experiment, the inoculum was prepared in similar way but with sterile water. The flasks were inoculated with 5-6ml of respective homogenized cultures to provide 80% of moisture level in the flasks. The flasks were incubated at 28-30^{o}C for 10-15 days under dark condition. The flasks were withdrawn at time intervals of 5, 10 and 15 days. The experiments were carried out in duplicates and average of the laccase activity was reported.

6.2.4 Extraction of enzymes for SSF flasks and enzyme assay

The enzyme extraction was done by adding 20 ml of acetate buffer (pH 4.5) to the contents of each flask. The flasks were rotated in rotary shaker for 2 hrs at 150 rpm to extract the crude laccase. The contents of the flasks were centrifuged at 5000 rpm for 10 minutes to separate the solid particles. The clear supernatant extract was collected and used for laccase determination assay.

6.2.5. Laccase assay

ABTS oxidation method was used to determine the laccase concentration. The reaction mixture consisted of sodium acetate buffer (0.1M, pH 4.5), ABTS (50mM) and 1.5ml enzyme extract. The absorbance was monitored at 420nm (ϵ_{mM}=36mM^{-1}cm^{-1}) at room temperature using GBC UV-Vis spectrophotometer. Enzyme activity was expressed as international units (IU) where 1IU is defined as the amount of enzyme forming 1umole

of product per minute. The laccase activity in U/g (µmol cation radical released/min/g of the substrate) was calculated.

6.2.6 SSF with Jatropa seed cake

Among the several SSF substrates used for laccase production by *Peniophora sp.* hpF-04 and *Phellinus noxius* hpF-17, only in the presence of JSC, both fungi showed significant laccase production. Moreover, the production of laccase in both fungi was high when no nutrient supplements were provided during SSF. Therefore, JSC was considered as a suitable SSF substrate for laccase production in these fungi. Further experiments of SSF were carried out by using JSC.

6.2.6.1 De-oiling of JSC

The seed kernels were finely powdered using motor and pestle. To a known amount of powdered kernel, sufficient amount of petroleum ether was added. The solution was mixed well and placed in an electrical oven maintained at 40-60^{o}C. After 30 minutes, the defatted cake was separated from the oil using a muslin cloth. This de-oiled cake was employed for the solid state fermentation.

6.2.6.2 Time course production of laccase in JSC

SSF was carried out in 100 ml conical flask containing 5g of de-oiled Jatropa seed cake kernel moistened with distilled water to an initial moisture level of 75%. All flasks were sterilized at 121°C for 30 min, inoculated with 2ml of inoculum, prepared by homogenizing 10 mycelial agar plugs (9mm) from the actively growing fungus in 100ml of sterile distilled water. Flasks were incubated at 28°C for 14days and samples were withdrawn at regular time intervals of 2 days to determine laccase activity.

6.2.6.3 Effect of moisture level

The effect of initial moisture content on laccase production by *Peniophora sp*. hpF-04 and *Phellinus noxius* hpF-17 was studied. The flasks were maintained at different

moisture levels using distilled water ranging from 40 to 80% (40%, 50%, 60%, 70% and 80%). The inoculum was prepared by homogenizing 30 agar plugs (9 mm in diameter) of respective fungi in 100 ml of sterile water. The flasks were inoculated with 1ml of the homogenized inoculum of the respective fungi and incubated at 28-30°C under dark condition. The contents were extracted on 10th day of incubation period for laccase assay using acetate buffer.

6.2.6.4 Effect of pH

To study the effect of pH on laccase production by SSF using JSC, different initial medium pH in the range of 4-7 was maintained for SSF. The effect of initial pH on enzyme production was assayed by adjusting pH of the substrate medium with 3N NaOH/HCl prior to its sterilization. SSF was carried out at 70% moisture level for *Phellinus noxius* hpF-17 and at 80% for *Peniophora sp.* hpF-04. The flasks were incubated at 28±2°C and the contents were extracted on 10th day for laccase assay.

6.3 Results and Discussion

6.3.1 Laccase production by *Peniophora sp.* hpF-04

Production of laccase under SSF using different agro-industrial residues without any nutrient supplements is shown in Fig. 6.2. No laccase activity was observed on 5th day (except negligible laccase activities of 0.08 U/g in SCB and 0.09 U/g in RB) during SSF in the presence of all the substrates. On 10th day, increase in laccase activities were observed with respect to all the substrates and on further incubation, *i.e.* on 15th day, the laccase production gradually reduced in most of the substrates. Among the substrates studied, a high level of laccase activity was found in Jatropa seed cake (JSC). In the case of JSC, laccase activity of 12.87± 0.7 U/g was found during 10th day of incubation period and later it reduced to 3.31±0.5 U/g on 15th day. Followed by JSC, wheat bran as a substrate showed laccase activity of 5.45±0.5 U/g during 10th day incubation period which was further reduced to 1.01±0.02 U/g after 15 days. In SCB and RB, similar

laccase activities of 3.58±0.3 U/g and 3.44±0.2 U/g was obtained, respectively, on 10th day of incubation period. In case of saw dust and corn stover, low levels of laccase activities were quantified on 10th day of incubation period. In these substartes, laccase activity of 0.02 U/g and 0.17 U/g was observed, respectively for saw dust and coern stover on 10th day incubation period.

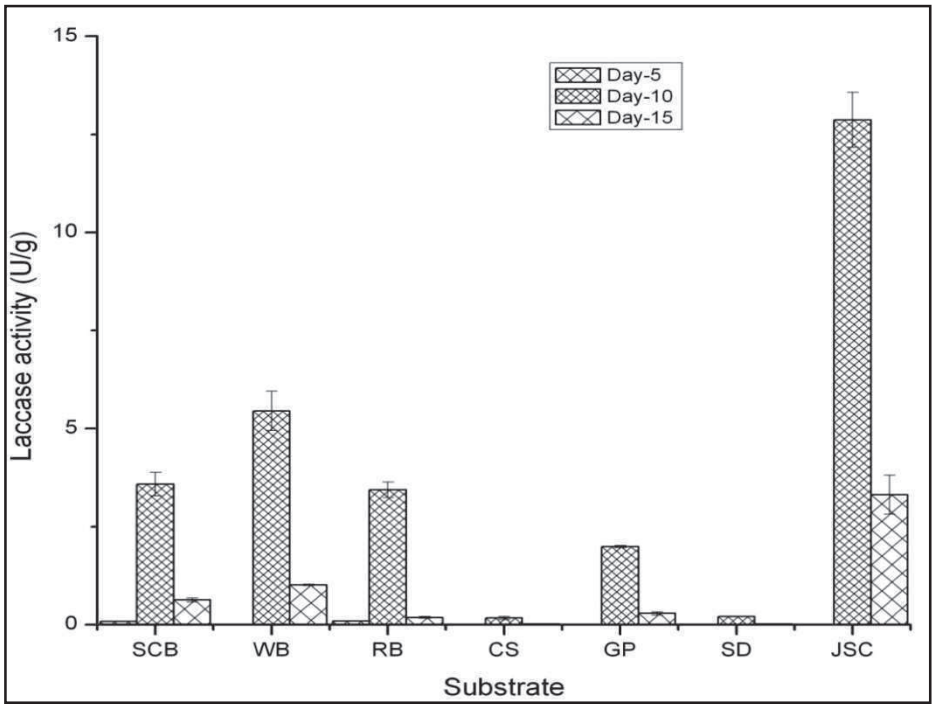

Fig. 6.2 Laccase production by *Peniophora sp.* hpF-04 in the absence of nutrients by SSF

Contrastingly, detectable amounts of laccase activities were found in all the substrates during 5th day of SSF when fermentation was carried out with nutrient supplements (Fig. 6.3). During 5th day, laccase activities (in U/g) of 0.18±0.02, 0.22±0.02, 0.14±0.01, 1.55±0.4, 0.13±0.05, 0.21±0.01, 0.009 observed, respectively, in SCB, WB, RB, CS, GP, SD and JSC. However, reduction in laccase production among

all the substrates, except JSC, was observed during the course of incubation time. On 10th day, respective laccase acticities in U/g observed were: SCB (0.17±0.02), WB (0.12±0.01), RB (0.65±0.09), CS (0.15), GP (0.09), SD (0.06) and JSC (15.8±1.4). In JSC, laccase activity reduced to 5.42±1.1 U/g, on 15th day. These findings are in contradicted with the results of previous trials where the fermentation was carried out in the presence of water alone. However, a high level of laccase activity was found in the presence of JSC as a SSF substrate similar to earlier case. With JSC as substrate, a laccase activity of 15.08 U/g was observed on 10th day of incubation.

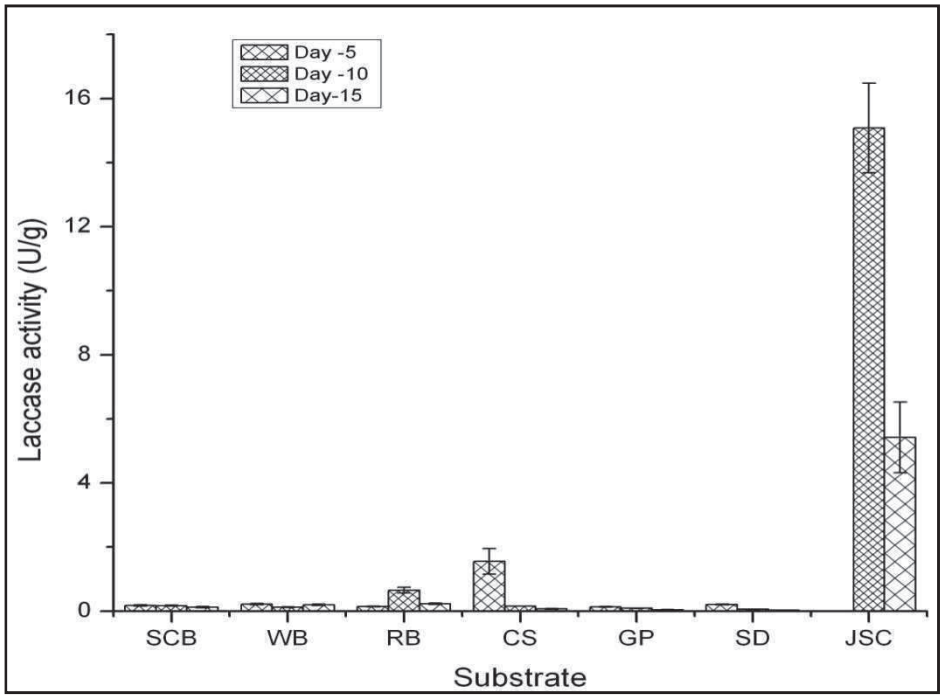

Fig. 6.3 Laccase production by *Peniophora sp.* hpF-04 in the presence of nutrients by SSF

6.3.2. Laccase production by *Phellinus noxius* hpF-17

Fig. 6.4 shows the production of laccase by *Phellinus noxius* hpF-17 under SSF of seven different agro-industrial residues without nutrients supplementation. However, the presence of nutrients during SSF showed positive effect on laccase production in case of

all the substrates by enhancing laccase activities (Fig. 6.5). Highest laccase production was observed in JSC in cases of both with and without nutrients. In the absence of nutrients, laccase productions of 19.6±4 U/g, 79.92±2.5 U/g and 67.82±5 U/g were observed respectively, during 5th, 10th and 15th day of incubation in JSC. The laccase activity in the presence of nutrients was 24.02±0.5 U/g, 128.5±12.4 U/g and 105.32±12.3 U/g respectively, during 5th, 10th and 15th day of incubation.

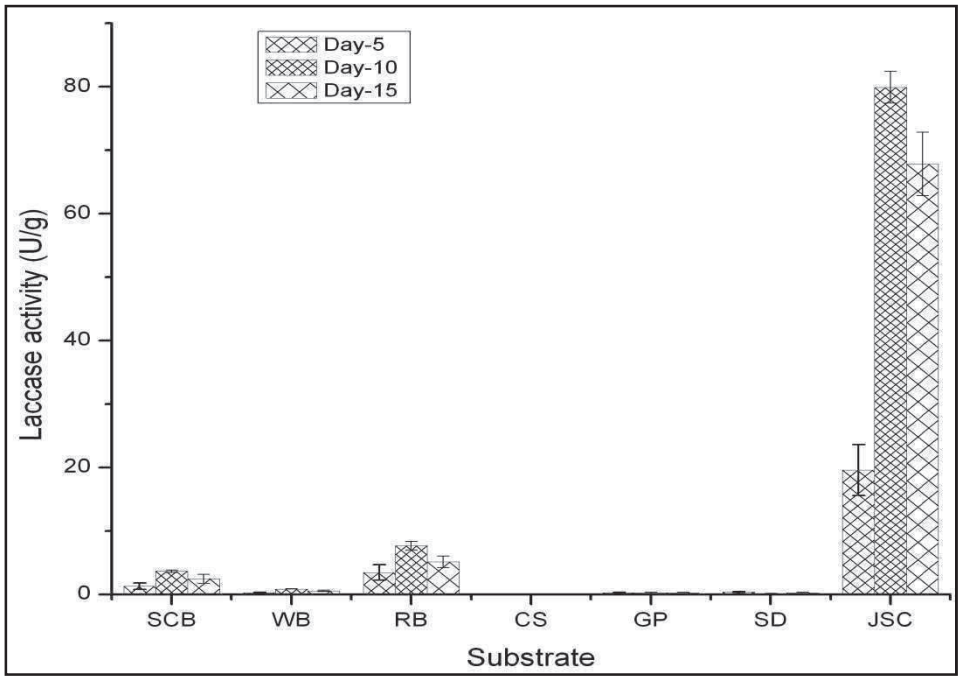

Fig. 6.4 Laccase production by *Phellinus noxius* hpF-17 in the absence of nutrients by SSF

In corn stover, no or fairly detectable laccase production was observed even with nutrient supplementation. Moreover, very less growth was seen in the flask containing CS. Similar to *Peniophora sp.* hpF-04, *Phellinus noxius* hpF-17 also produced high levels of laccase on 5th day fermented samples of all the substrates when nutrients were supplemented. The production of laccase gradually decreased in the course of time. In the presence of nutrients, on day 5, sugar cane bagasse showed laccase yield of 1.45±0.2 U/g; wheat bran showed laccase yield of 49.8±0.12 U/g, saw dust 75.53±2.4 U/g, grass

powder 94.58±11.3 U/g and rice bran showed laccase of 54.88±3.5 U/g of the substrate, respectively. Fig. 6.6 shows the fungal growth in the presence of different substrtaes during SSF.

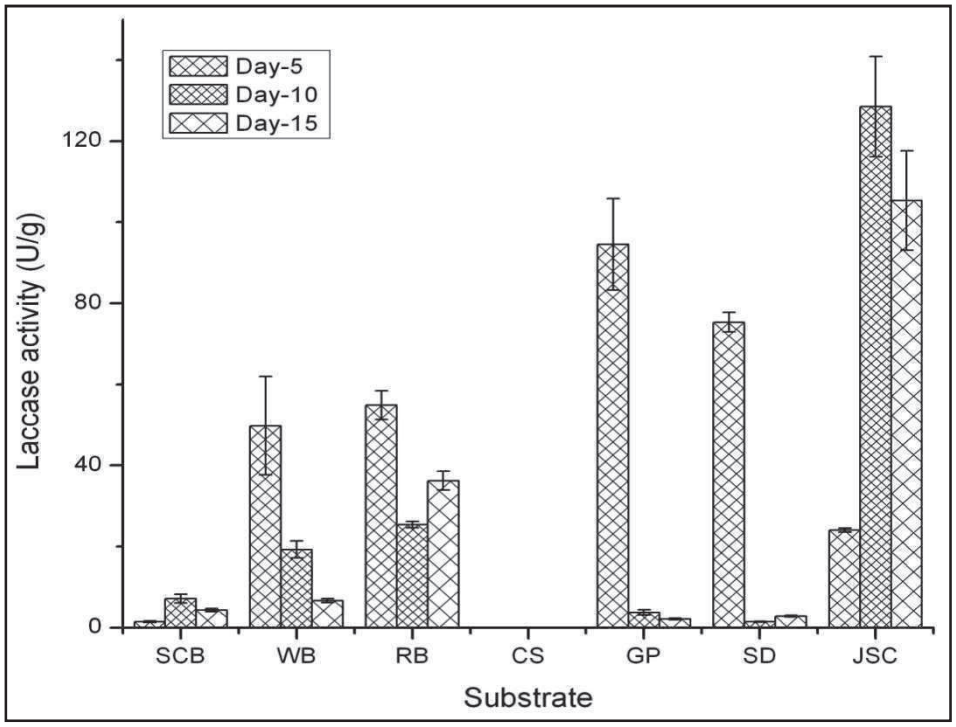

Fig. 6.5 Laccase production by *Phellinus noxius* hpF-17 in the presence of nutrients by SSF

The laccase production markedly differed among the different substrates used in SSF process by *Peniophora sp*. hpF-04 and *Phellinus noxius* hpF-17. The study also revealed that the presence of nutrients during SSF altered the laccase production. It could either enhance or reduce the laccase synthesis. The enhancement of laccase production in some substrates was possible since optimized nutrient solution was used in the study. On the other hand, the decrement in the production of laccase in the presence of nutrients may be due to its ready availability and thus prolonging the nutrient depletion effect. It was reported previously that fungal laccases are produced during secondary metabolism when there is a limitation for carbon or nitrogen sources (Baldrian 1994, Couto and Herrara 2007). Sugar cane bagasse, in general, contains easily hydrolysable simple sugars which

are ineffective in inducing laccase synthesis. Hence, low levels of laccase activities could be detected on day 5th in SSF using this substrate. However, most other substrates that are rich in lignocellulosic composition favoured in higher levels of laccase synthesis.

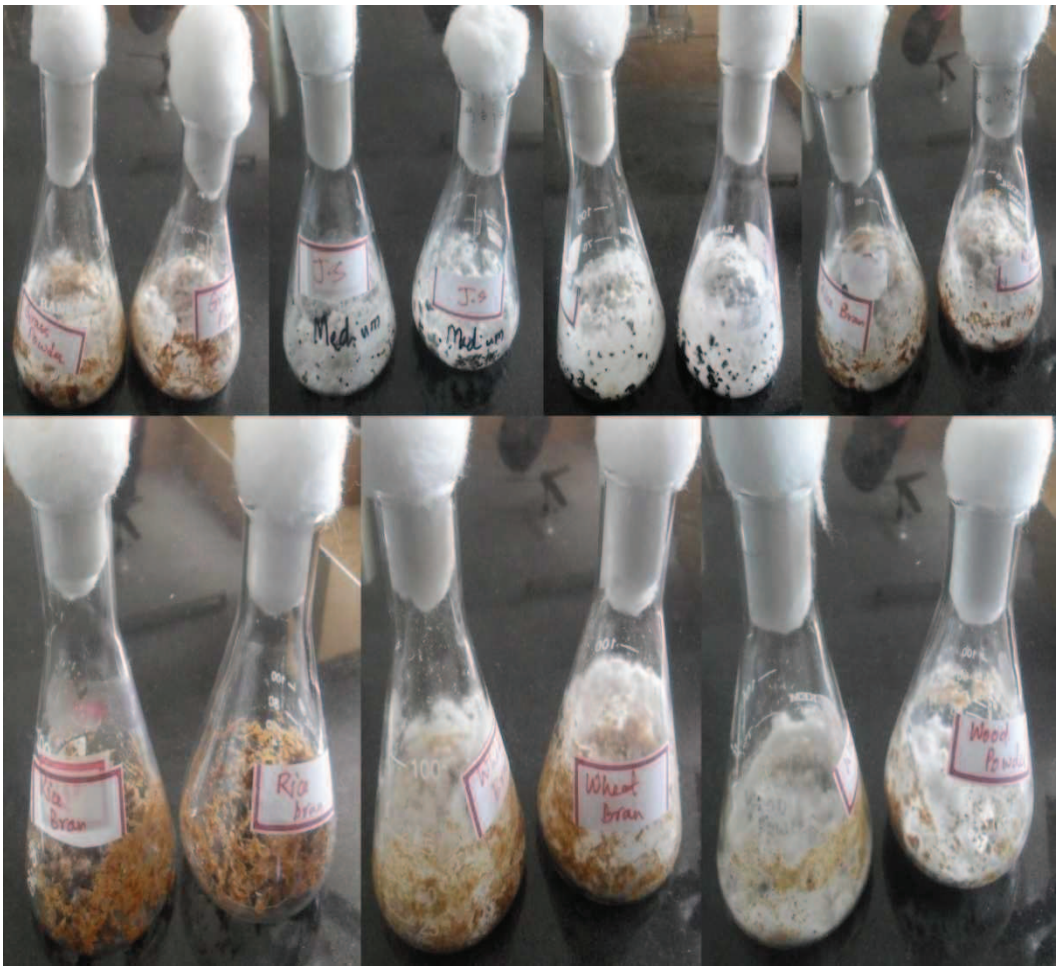

Fig. 6.6 Fungal growth observed in the presence of different substrates during SSF

Previous investigation of SSF using different agro-residues also proved that the presence lignocellulose induced laccase production in many fungi (Elisashvili et al. 2001; Reddy et al. 2003; Moldes et al. 2004). Since, the exact chemical composition of the agro-industrial residues used in our study for SSF was not known; it is not possible to depict the exact reason for variation in laccase production among the substrates. However, the variation found in enzyme production might be also due to the relative composition of polysaccharides, the size of the wastes used and the presence of natural inducers such as aromatic compounds in the substrates. Many aromatic compounds present in wood and other lignocellulosic materials are potent inducers of laccases, for example the ferulic acid, ubiquitously present in plant cell walls, induced laccase activity in several fungi (Leonowicz and Trojanowski 1975; Herpoel et al. 2000). The stimulating effect of lignin was observed for laccase production in white rot fungi, *T. hirsutus*. The rice bran inductive capability is based on the phenolic compounds such as ferulic acid, and vanillic acid which induce the laccase production (Munoz et al. 1997). According to the literature, the chemical composition of de-oiled Jatropa seed cake seems to be an appropriate nutrient to support good microbial growth and also the presence of phenolic antioxidants acts as inducers for various enzyme synthesis including laccase ().

SSF is a microbial process occurring mostly on the surface of solid materials, which can absorb and contain water in the absence / presence of soluble nutrients. The use of defined liquid medium and an inert support improved the laccase production in many fungi (Pandey 1992). However, the enzyme production also depends on the physical characterization of the support (particle size, shape, porosity, consistency), which favours gas and nutrient diffusion as well as the movement and attachment of the fungus (Pandey 1992). Generally smaller substrate particle provide a larger surface area for microbial colonization, but if they are too small, it may affect the substrate agglomeration which results in poor growth. In contrast, larger particle may provide better aeration but a limited surface for the microbial growth. Therefore, microbial growth and its enzyme production would be observed at its best if a compromised particle size is selected. Since in our study, SSF was carried out without maintaining a definite particle size for any of

the substrate, this could be one of the reasons for variation in level of laccase production among the different substrates.

Among the seven agro-industrial wastes employed for SSF, only JSC was found to be more potent substrate for SSF production of laccase by both fungi. Hence, further studies of SSF were carried using JSC to check the time course production, effect of moisture and effect of initial medium pH.

6.3.3 Solid state fermentation of JSC

Time course production of laccase by *Peniophora sp*. hpF-04 and *P.noxius* hpF-17 was shown in Fig. 6.6. Both fungi showed maximum laccase production on 10^{th} day of incubation period and laccase yield gradually decreased thereafter. Among the two fungi, *Phellinus noxius* hpF-17 showed laccase in higher titre than *Peniophora sp.* hpF-04. It showed laccase activity of 79.92±2.3 U/g on 10^{th} day while *Peniophora sp*.hpF-04 produced laccase of 14.32±2.4 U/g on the same day. In *Peniophora sp.* hpF-04, laccase activity of 13.2±2.6 U/g and 6.1±0.5 U/g of substrtae observed, respectively, on 12^{th} and 14^{th} of solid state fermentation. Moreover, the laccase production profile curve of *Phellinus noxius* hpF-17 lies above to that of *Peniophora sp*. hpF-04. This finding is completely dissimilar to laccase production observed in submerged fermentation using defined liquid medium, where *Peniophora sp.* hpF-04 showed laccase activity that was almost three times more than that of *Phellinus noxius* hpF-17. This indicates that, the components of JSC have induced laccase production in *Phellinus noxius* hpF-17. Therefore, high value of laccase activity was detected during solid state fermentation.

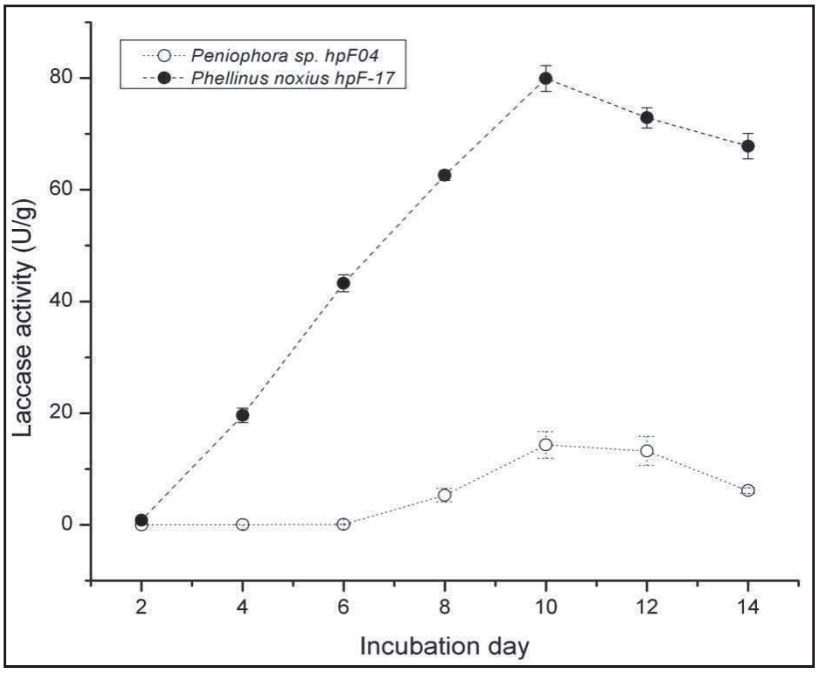

Fig. 6.7 Time course production of laccase by SSF using Jatropa seed cake (JSC) by the fungi

6.3.4 Effect of moisture level on SSF of JSC

Fig. 6.7 shows the effect of moisture level on laccase production. In both the fungi, variation in moisture levels in SSF showed varied laccase production. *Phellinus noxius* hpF-17 produced maximum laccase activity at 70% moisture level, whereas *Peniophora sp.* hpF-04 showed maximum laccase activity at 80% of moisture level. Fungus, *Peniophora sp.* hpF-04 produced laccase activity of 2.1±0.4 U/g, 4.4±0.8 U/g, 9.2±1.2 U/g, 13.4±1.5 U/g and 14.0±2 U/g, respectively at 40%, 50%, 60%, 70% and 80% of moisture level. On the other hand, *Phellinus noxius* hpF-17 produced laccase activity of 38.1±2.4 U/g, 48.9±3.2 U/g, 64.1±5.5 U/g, 98.5±6.8 U/g and 84.3±3.5 U/g, respectively, at 40%, 50%, 60%, 70% and 80% of moisture level. In general, *Peniophora sp.* being a brown rot fungus requires high moisture level for its growth and metabolic process as it is demonstrated in many litter degrading brown rots (Baldrian 1994; Hatakka et al. 2006). On the other hand, white rot fungi are able to grow on hard wood with less moisture level due to its complex enzyme mechanisms.

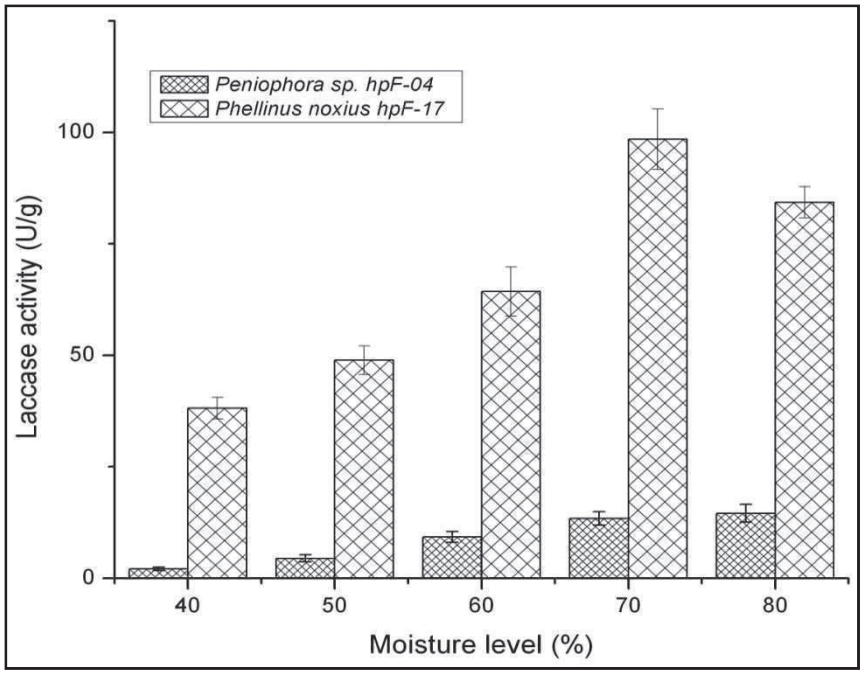

Fig. 6.8 Effect of moisture on laccase production by SSF using JSC kernel by the fungi

6.3.5 Effect of pH

In many fungi, it is reported that the production of laccase requires compromised pH level as it affect the enzyme structure which later has impact on fungal growth and mechanisms**.** Fig. 6.9 shows the effect of pH on SSF using JSC. Fungus *Phellinus noxius* hpF-17 produced maximum laccase of 83±8.1U/g, at pH 5.0. Similar laccase activity of 80.5±4.5 U/g was observed at pH 6.0. At pH 4.0 and pH 7.0, laccase activity was reduced and the observed values were 43.2±3.2 and 64.3±8.8 U/g, respectively. *Peniophora sp*. hpF-04 produced maximum laccase within pH range of 5-6. Laccase activity value of 11.3±0.8 U/g and 12.2±1.2 U/g was observed, respectively, at pH 5.0 and pH 6.0. At pH 4.0 this fungus produced laccase of 8.8±2.1 U/g and at pH 7.0, it showed laccase activity of 10.34±3.1 U/g.

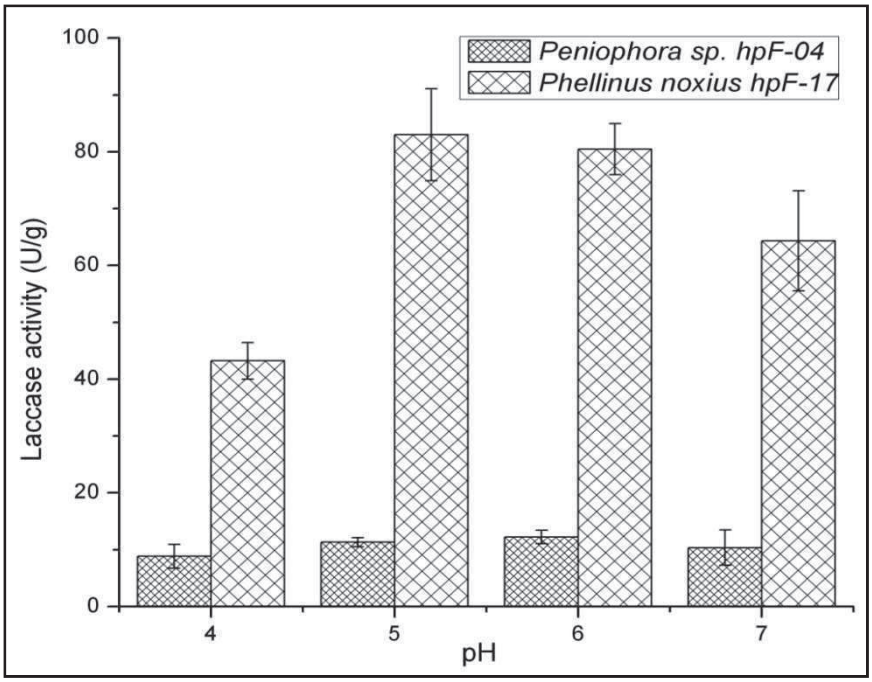

Fig. 6.9 Effect on pH of laccase production by SSF using JSC kernel

The use of lignocellulosic materials for enzyme production has, at least, two advantages with respect to SmF processes: high production of enzymes using a low-cost media (Viniegra-Gonzales et al. 2003) and the possible utilization of bio-converted substrate due to its increased digestibility (Mukherjee and Nandi 2004). When the substrate is based on high protein containing raw materials such as soy bran, its nutritional value is increased. Thus, because of its low cost, worldwide abundance and the resulting high levels of enzyme production, the SSF using soy and wheat bran as substrate could be used to scale up the production of lignocellulases (ligninases, pectinases, xylanases and cellulases). To conclude, the results of the present study of SSF using agro-industrial residues presents a new approach of utilizing the agro-industrial waste as fermentation feed stock for the production of laccase enzyme.

It is evident that after extraction of oil from the Jatropa seeds generate huge quantity of residual deoiled seed cake. Jatropa production is forecasted about 25000kg/seed/hectare under Indian conditions (Mahanta et al. 2008). Considering 40-50% oil in it, the extraction will generate approximately 1000kg seed cake per hectare

crop. The deoiled *J. curcas* seed cake cannot be used as a cattle feed, unlike other oilseeds, mainly due to the presence of toxic phorbol esters in it. Hence, its safe disposal and meaning full utilization is very important. Investigators tried detoxifying phorbol esters from Jatropa seed cake kernel by biological methods (Peace and Aladesanmi 2008, Belewu et al. 2010). Jatropa contains approximately 47% of crude fat, 25% of crude protein, 10% of crude fibre, 5% of moisture and 8% of carbohydrate (Akintayo 2004). This composition is suitable to support microbial growth and its enzyme production. Use of Jatropa seed cake as SSF substrate thus appears logical and worth exploring. SSF using sugar cane bagasse has been employed to grow microbes and to produce extracellular enzymes like protease (Germano et al. 2003; De Azerdo et al. 2006; Mahanta et al. 2008), xylanases (Yang et al. 2006), lipase (Mahadik et al. 2002; Diaz et al. 2006; Mahanta et al. 2008), etc. Although large number of lignocellulosic residues have been worked out as suitable substrates for SSF, there is no report so far on SSF using Jatropa seed cake for the production of laccase to the best of our knowledge.

To sum up, seven agricultural residues were successfully employed in the present investigation for the production of laccase by two novel isolates of Basidiomycetes. Both fungi were able to grow and produce laccase on all the substrates employed in the study under solid state fermentation condition. Moreover, high laccase production in case of JSC without the supplementation of nutrient was an important finding of our research, which could prove to be an alternative support for the production of other enzymes in future.

Chapter 7

PARTIAL PURIFICATION AND CHARACTERIZATION OF LACCASE PRODUCED BY *PENIOPHORA SP.* HPF-04

7.1 Introduction

The practical applications of laccases in various fields of biotechnology are increasing, it is very essential to produce and isolate a novel laccase with different physico-chemical and catalytic properties. Obtaining an enzyme in its pure form and enhancing its stability and maintaining the desired level of activity over a long period of time are two significant points which are taken into account for the selection of laccase in industrial applications. Laccase has been purified and characterized extensively from different species of basidiomycetes. Our initial studies of screening for LMEs producing fungi, a brown rot fungus *Peniophora sp.* hpF-04 showed remarkable laccase production in liquid medium. Although, some species from this genus were reported to produce laccase, there is no report on purification of laccases from this genera and no information available on laccase's kinetic properties and also the factors that influence the stability. Niku-Paavola et al. reported the production of laccase by *Peniophora* species for the first time in 2004 and studied enzyme characteristics in its crude form. Laccase from *Peniophora* species was found to be thermostable with temperature optima around 60°C (Niku-Paavola et al. 2004). The purpose of the present work was to separate and purify laccase from crude enzyme extracts of *Peniophora* sp. hpF-04 by advanced methods and to study the biochemical characteristics of laccase as well as the parameters that improve the activity and stability of the enzyme.

Most reported separation methods for laccase purification were developed for the purpose of characterization of laccase enzymes. Such methods involve a combination of precipitation, membrane filtration, dialysis, ion-exchange, hydrophobic interaction, gel permeation and affinity chromatography. Concentration is usually the initial step of down-stream processing to reduce liquid volume with minimal loss of activity. Although simple to perform, protein concentration by salting out has disadvantages of low process temperature, generally requires long time for protein aggregation and results in low product purity. Ultra-filtration, the other most commonly used concentration method, also has certain drawbacks such as extensive pre-filtration and membrane fouling. The other

separation methods mentioned above involve chromatographic techniques that are time consuming, require pretreatment and used mainly for therapeutic proteins (Rajeeva and Lele 2011).

In recent times, these traditional techniques were being replaced by advanced methods of protein purification such as Three-phase partitioning (TPP). TPP has been reported as an efficient alternative method for concentration and purification of various industrially important enzymes (Dennison and Lovrein 1997). This technique uses a combination of ammonium sulphate and *tert*-butanol to precipitate proteins from crude extracts. Organic solvent such as *tert*-butanol binds to the precipitated proteins, thereby increasing their buoyancy and causing the precipitates to float above the denser aqueous salt layer. Optimum pH, temperature, ammonium sulphate and *tert-butanol* concentrations can selectively precipitate proteins at the interface of the organic and aqueous phases. Scalability, rapid recovery and a requirement for only minimal pretreatment are additional advantages of TPP (Lovrein et al.1987, Dennison and Lovrein 1997). However, there are only few reports are available in the scientific literature on separation and purification of laccases by using TPP method.

In the present work, we report the recovery of laccase from culture broth of the fungus *Peniophora* sp. hpF-04 using TPP. A one-step TPP was employed for the purification of laccase using organic solvents. Though many organic solvents were used in TPP, it was reported in many studies that *tert*-butanol is a better organic solvent for separation of proteins by TPP. Hence, we employed *tert-butanol* as a solvent for TPP separation of *Peniophora sp.* laccase. Previous report indicates that significant precipitation can be achieved by varying the concentration of ammonium sulphate and the aqueous phase to *tert-butanol* ratio (Rajeeva and Lele 2011). Pike and Dennison (1989) reported that physical conditions such as temperature and pH also affect partitioning during TPP. Therefore, we exploited different combinations of salt saturation levels, aqueous phase to *tert*-butanol ratio, pH and temperature to optimize laccase separation. The partially purified laccase was then subjected for its characterization to

study its biochemical and catalytic properties. The laccase enzyme from *Peniophora sp*. was characterized for its temperature optimum, pH optimum, stability at different temperature and pH, copper and carbohydrate contents, specificity and kinetic properties for the first time.

7.2 Materials and Methods

7.2.1 Culture maintenance and production of laccase

The fungus *Peniophora sp.* hpF-04 was maintained in 2% MEA slants and subcultured in the same media before its use. Optimized medium which was formulated in our previous study was used for the production of laccase. The compositions of the medium was (g/l); carboxy methyl cellulose (15.0), ammonium chloride (1.35), copper sulfate (0.2), yeast extract (2.5), KH_2PO_4 (1), KCL (0.5), $MgSO_4$ (0.5) and 10mg/l of trace elements containing per litre of distilled water: EDTA (0.5g), $FeSO_4$ (0.2g), $ZnSO_4.7H_2O$ (0.01g), $MnCl_2.4H_2O$ (0.003g), H_3BO_4 (0.03g), $CoCl_2.6H_2O$(0.02g), $CuCl_2.2H_2O$ (0.001g), $Na_2MoO_4.2H_2O$ (0.003g). Mycelial agar plugs (4-5 plugs of 9mm diameter) were cut along the edge of the actively growing colony and used as the inocula. The fungi were cultivated at 30^oC on a rotary shaker (150 rev min^{-1}). After six days, the mycelium-free culture filtrate was collected and used for the experiments.

7.2.2 Concentration of culture filtrate using Tangential Flow Filtration (TFF)

TFF was used to concentrate the laccase enzyme from the culture filtrate. It was carried out using MILLIPORE XL 50 assembled with PELLICON XL 50 membrane device with normal molecular weight cut-off (NMWCO) value of 10,000 Dalton.

The general assembly of the filtration unit is shown in Fig. 7.1. The Pellicon XL device was first flushed with 500 ml distilled water to remove trace amounts of glycerin and preservatives. The device was pre-conditioned by flowing distilled water for 10-20 minutes at a flow rate of 30-50 ml/min.

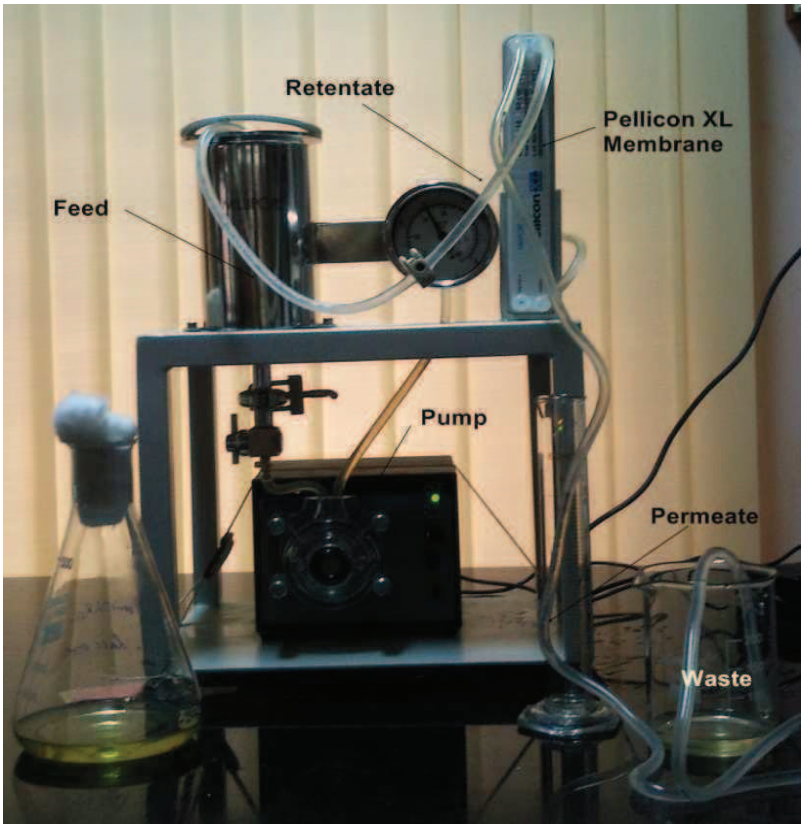

Fig. 7.1 TFF set up for the concentration of laccase

The tangential flow filtration was carried out as follows

1. Clean containers and tubing connections were arranged as shown in Fig. 7.1
2. The feed container was filled with 90ml of crude filtrate solution of *Peniophora sp.* hpF-04
3. The pump was turned on with a flow rate of 30-50ml/min
4. The solution was filtered till the volume reduced to 55ml and around 35 ml of waste was collected.
5. The pump was turned off. The tubing connections were removed. The retentate (concentrate) as well as permeate (waste) were collected in clean containers.
6. The membrane was cleaned by pumping 0.5N NaOH till 250ml of waste was collected in the waste container.

7. The samples were analyzed for laccase activity, protein content and specific activity.
8. The % yield, fold purification and concentration factor were calculated as follows

$$\% \text{ Yield} = \frac{\text{Total activity in purified sample x 100}}{\text{Total initial activity}}$$

$$\text{Fold purification} = \frac{\text{Specific activity of purified sample (U/mg protein)}}{\text{Specific activity of crude sample (U/mg protein)}}$$

$$\text{Concentration factor} = \frac{\text{Volume of the initial sample}}{\text{Volume after concentration}}$$

7.2.3 Precipitation of proteins by TPP

Purification of the laccase produced by *Peniophora sp.* hpF-04 was performed by one-step purification TPP based on modified procedure described by Lele and Rajeeva (2011).

Single-step TPP was carried out as follows

1. Crude laccase solution (15 ml) with an initial pH 5.2 was saturated with varying amounts of ammonium sulphate ranging from 30-70 % (w/v).
2. Then, *tert*-butano*l* (15 ml) was added and the tube was vortexed gently for 2min and incubated at room temperature.
3. After 1 h, the mixture formed three phases (upper organic phase, middle interfacial precipitate and lower aqueous phase).
4. The interfacial precipitate layer was removed and dialyzed in distilled water for 24h at 4°C.
5. The dialyzed interfacial precipitate and lower aqueous layer were analyzed for laccase activity and protein content.

7.2.3.1 Effect of temperature on protein separation

1. Crude enzyme solution was saturated with 55% (w/v) ammonium sulphate.
2. It was followed by addition of *tert*-butanol in volumetric aqueous phase-to-*tert*-butanol in the ratio 1:1 (v/v).
3. The mixtures were allowed to stand at different temperatures such as 30, 40, 45 50, 55 and 60°C for 1 h, after which the precipitate and organic layer were separated.
4. The resultant interfacial precipitate was analyzed for laccase activity and protein content.

7.2.3.2 Effect of saturated crude extract to *tert*-butanol ratio

1. Crude enzyme solution was saturated with 55% (w/v) ammonium sulphate.
2. It was followed by the addition of *tert*-butanol in different volumetric aqueous phase to *tert*-butanol ratios of 1:0.5, 1:1; 1:1.5 and 1:2 (v/v).
3. The mixtures were allowed to stand at 45°C for 1 h, after which precipitate and organic layer were separated.
4. The resultant interfacial precipitate was analyzed for laccase activity and protein content.

7.2.3.3 Effect of pH on TPP separation of laccase

1. Crude enzyme was buffered at different pH values (3–9). The buffered solutions were saturated with 55% (w/v) ammonium sulphate.
2. It was followed by the addition of *tert*-butanol in volumetric aqueous phase to *tert*-butanol in the ratio 1:1.5 (v/v).
3. The mixtures were allowed to stand at 45°C for 1h, after which the precipitate and organic layer were separated.
4. The resultant interfacial precipitate was analyzed for laccase activity and protein content.

7.2.4 Characterization of laccase

7.2.4.1 Effect of pH on activity and stability of the purified laccase

The effect of pH on the activity of the purified laccase was determined by assaying the enzyme activity at different pH values ranging from 2.5 to 9.0 using 0.1M of the following buffer systems: acetate (3.0, 4.0 and 5.0), phosphate (pH 6.0 and 7.0) and Tris–HCl (pH 8.0 and 9.0) buffer systems. The pH stability of *Peniophora sp.* hpF-04 laccase was investigated in the pH 4.0. Therefore, 1 ml of the enzyme was mixed with 1ml of the buffer solutions mentioned above and incubated at room temperature for 20h. Aliquots of the mixtures were then taken to measure the laccase activity (%) with respect to control, under standard assay conditions.

7.2.4.2 Effect of temperature on activity and stability of the purified laccase

The effect of temperature on the activity of the purified laccase was determined by performing the standard assay procedure at different temperatures ranging from 20 to 80^{o}C. Before the addition of enzymes, the substrate (50mM) ABTS and acetate buffer (pH 4.0) were pre-incubated at the respective temperatures for 10 min. Thermal stability studies were conducted without any additives. The enzyme solution was incubated at various temperatures ranging from 20–80^{o}C (20, 25, 30, 35, 40, 45, 50, 60, 70 and 80^{o}C) in a temperature-controlled water bath for 15-16h and the relative enzyme activity was measured at regular intervals of time. The relative activity was calculated as the percentage ratio of activity at a given temperature to the activity at optimum temperature.

7.2.4.3 Effect of metal ions, organic acids and various compounds on the laccase activity

Metal compounds (10mM) ($CoCl_2$, NaCl, $NiCl_2$, $MgCl_2$, $ZnSO_4$, $MnCl_2$, CuCl, $AgCl_2$), organic acids (10mM) (citric acid and oxalic acid) and various compounds (EDTA(10 mM), TEMED (0.1%), SDS (0.01%) and β-mercaptoethanol (0.1%), p-Chlorophenol (0.1%), sodium azide(0.005 mM)) were tested for their effect on purified

laccase activity in acetate buffer (pH 4.0). The enzyme was pre-incubated for 1hr at 45°C with listed components.

7.2.4.4 Protein determination and enzyme activity

Protein concentration was determined by Lowry's method using bovine serum albumin (BSA) as standard. The laccase activity was determined using ABTS as the substrate. Enzymatic activity was expressed as 1U=1 µmol of ABTS oxidized per min at 45°C (±1).

7.2.4.5 Determination of kinetics parameters

The kinetic constants (Vmax and Km) were determined using Lineweaver–Burk (LB) double reciprocal (1/v Vs 1/S) plot, where different concentrations of guaiacol (0.1 to 2.0 mM), ABTS (0.002 to 0.1 mM) and 2,4 dimethoxyphenol (2,4 DMOP) (0.2 to 1.0 mM) were used as substrate at pH 4.0 and 45°C.

7.2.4.6 Electrophoretic analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) or denaturing gel electrophoresis was used to monitor the development of the purification process, to determine the homogenecity and the relative molecular mass of the laccase enzyme. SDS-PAGE was carried out on a 10% resolving gel and 5% stacking gel according to a method adapted from Laemmli et al.(1970). SDS-PAGE was performed using vertical electrophoresis system and proteins were separated at 20 mA per gel.

Proteins were visualized by staining for 3-5 hours with Coomassie Brilliant Blue-R250. Gels were then distained using distilled water. The approximate molecular mass of the laccase was determined by calibration against broad range (3kDa to 205kDa) molecular weight markers (Genie), which contained the proteins myosin, rabbit muscle (205 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6.5 kDa) and insulin (α and β chain).

7.2.4.7 Laccase purity analysis using RP-HPLC studies

Purity of the enzyme was confirmed by RP-HPLC analysis using Dionex HPLC system. RP-HPLC was carried out according to the method of Divakar et al. (2010). The crude and TPP purified laccase was applied on to C-18 column (Zorbax C-18, 4.6mm×250mm i.d., 5µm particle size, Agilent technologies). For elution, buffer C consisted of 0.1% (v/v) trifluroacetic acid (TFA) in water and buffer D consisted of 0.1% (v/v) TFA in acetonitrile. After loading, the column was washed with 2% (v/v) buffer D for 2 minute, to elute any unbound protein. The bound proteins were eluted from column using a 25 minutes linear gradient from 2 to 100% (v/v) buffer D at the constant flow rate of 1ml/min. Column temperature was maintained at 25°C and column eluent was monitored at 280 nm.

7.2.4.8 Laccase spectroscopic studies and determination of copper and carbohydrate contents

The absorption spectrum of purified laccase was determined at room temperature (28°C) in the range of 300-700 nm (Kumar et al. 2010).

The copper content of the protein was determined by atomic absorption spectrometry (AAS) (GBC Avanta, Australia).

Amount of carbohydrate present in the protein was determined by using phenol sulphuric acid method as described by Sadashivam and Manikckam (2003).

7.3 Results and Discussion

7.3.1 Separation and partial purification of laccase

7.3.1.1 Ultra filtration by tangential flow filtration

Crude enzyme extract produced by *Peniophora sp.* hpF-04 was subjected to ultra filtration using Tangential Flow filtration (TFF). TFF is particularly well suited for concentrating and desalting large volumes of protein solution. The volume of the

retentate slowly drops as low molecular weight solutes and water in the filtrate pass through the membrane and collects as waste. Laccase concentration using ultrafiltration is summarized in Table 7.1. During the process, 90 ml crude extracellular culture filtrate having total enzyme activity of 181.13 U was fed into the system and operated for 20 min till the volume reduced to 55ml. The final total enzyme activity was increased to 279.81U. Hence, 94.41% yield of laccase with a 2.28-fold purification was obtained in the retentate after ultrafiltration (Table 7.1). No enzyme activity was found in the permeate ensuring that there was no laccase lost during filtration along with the waste.

Table 7.1 Ultrafiltration of laccase produced by *Peniophora sp.* hpF-04

	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	% yield
Feed	90.0	181.13	387	42.12	---	--
Retentate	55.0	279.81	160.05	96.16	2.28	94.41
Permeate	35.0	ND	--	--	--	--

7.3.1.2 Three phase partitioning (TPP)

Separation of laccase produced by *Peniophora sp.* hpF-04 was carried out by single step TPP process. Since separation by TPP depends on several factors, the conditions for efficient separation must be optimized. In TPP, selective precipitation of proteins requires a specific portion of ammonium sulphate and *tert*-butanol. Generally salt conditions lower than those used in the salting out procedures have been used to carry out TPP efficiently (Roy and Gupta 2002). However, similar salt concentration to precipitate amylase inhibitor by both salting out and TPP has been cited (Sharma and Gupta 2011). In our study, culture filtrate was initially treated with low concentrations of ammonium sulphate ranging from 10-20% to remove most of the unwanted proteins. Then single step TPP purification procedure was carried out by varying the amount of ammonium sulphate from 30-60% (w/v) with an aqueous phase to *tert*-butanol ratio of

1.0:1.0 at room temperature. Salt saturation of 55% resulted in 87.86% yield of laccase with 2.44 fold purification (Fig. 7.2). We also noticed that, at lower concentrations of 30 to 40% salt saturation, there was poor laccase recovery. This low recovery of laccase might be due to the presence of insufficient protein in the broth for aggregation at low salt levels. Similar findings were reported by Lele and Rajeeva (2011), while performing TPP for laccase separation produced by *Ganoderma* sp.WR-1.

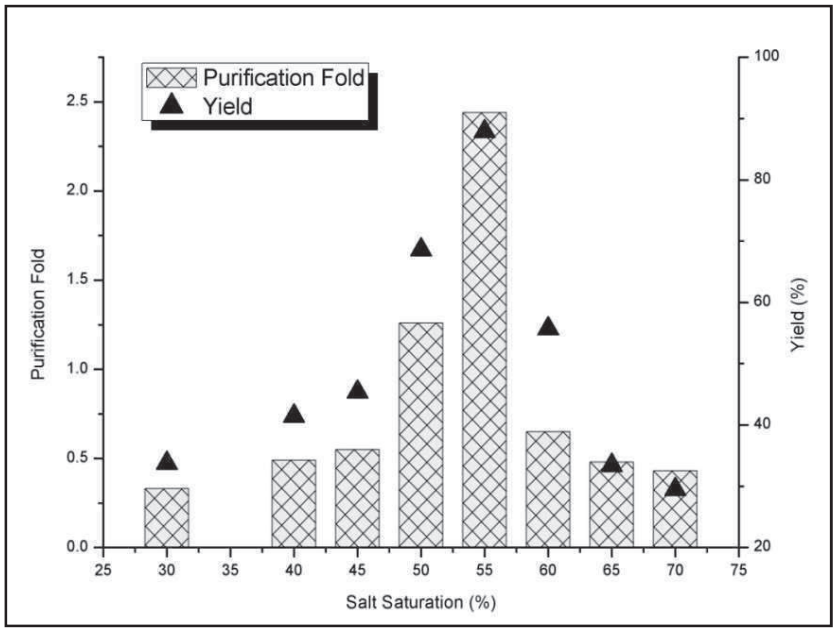

Fig. 7.2 Effect of salt saturation on laccase separation by TPP

Different ratios of crude extract to *tert*-butanol were performed to find the optimum ratio for efficient laccase separation. Fig. 7.3 presents the effect of different ratio of crude extract to *tert*-butanol on laccase recovery by TPP. Crude extract to *tert*-butano*l* ratio of 1.0: 1.5 resulted in 87.5 % laccase yield with 2.75 purification fold.

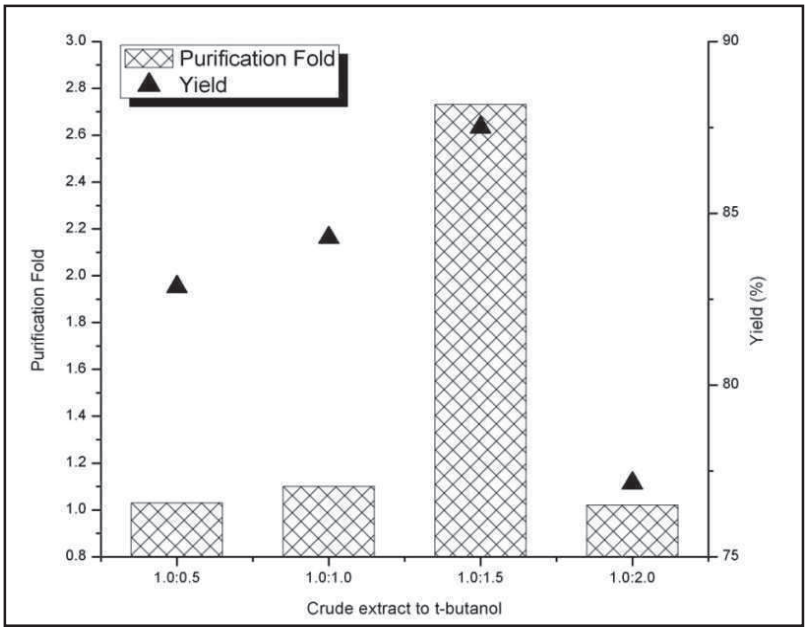

Fig. 7.3 Effect of crude extract to *tert-butanol* ratio on laccase recovery by TPP

Most concentration processes are usually carried out at low temperatures. The use of low temperature in solvent or salt precipitation dissipates the heat generated, ensuring minimal protein denaturation. However, there are several reports available on TPP process with higher incubation temperature of more than 40^{o}C also. Sivanesan et al. (2012) used 42±3°C for efficient separation of laccase from *P.ostreatus* using TPP. Lele and Rajeeva and Lele (2011), however reported that temperature was not a critical factor for laccase separation by TPP. Though we studied the effect of temperature on laccase separation by incubating separation mixture at a temperature ranging from 30-55°C, higher laccase yield was achieved at temperature ranging from 40-50^{o}C and there after it reduced. At incubation temperature of 45°C, laccase yield of 89.64% with 2.69 purity fold was obtained which was high among any of the other temperature conditions (Fig. 7.4).

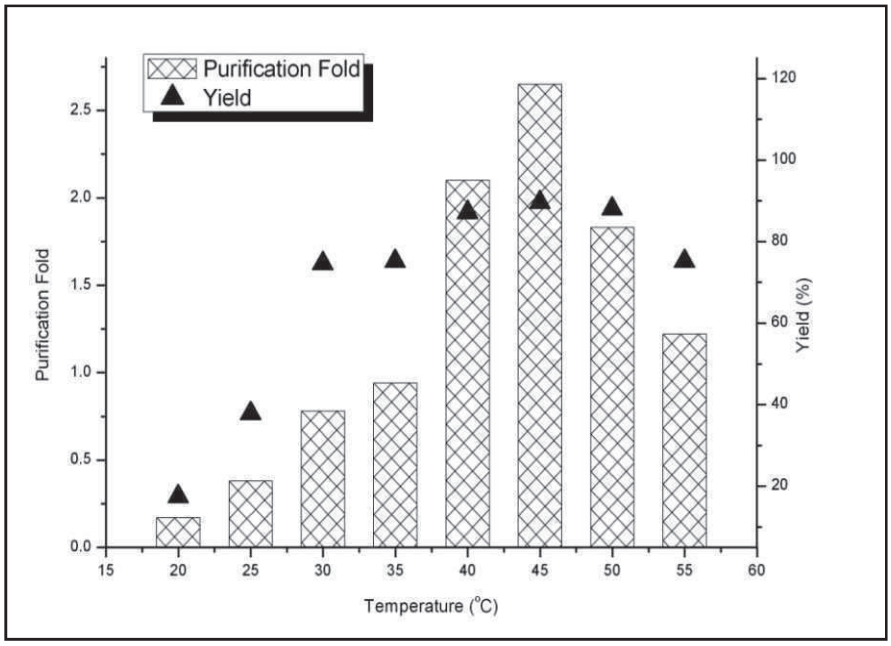

Fig.7.4 Effect of temperature on laccase separation by TPP

Another factor which influences the protein separation by TPP was pH of the crude extract. Protein concentration by salting out depends on the sulphate concentration and pH-dependent net charge of the proteins. Electrostatic forces and binding of sulfate anions to cationic protein molecules, which promote macromolecular contraction and conformational shrinkage, are the main causes of the strong sulphate pH dependency in salting out. Proteins tend to precipitate most readily at their pI (isoelectric point). Below the pI, proteins are positively charged and quantitatively precipitated out by TPP. On the other hand, negatively charged proteins are more soluble and not easily precipitated (Dennison and Lowry 1997). Reports of invertase precipitating selectively at its pI and leaving most of the contaminant proteins in the aqueous phase confirmed the relevance of pH as an important parameter (Ozer et al. 2010).

As a thumb rule, for broth containing proteins with varied pI values, a range of pHs between 3 and 7 is taken to study TPP of a desired protein. Fig. 7.5 depicts the effect of pH on the partition behavior of laccase during TPP.

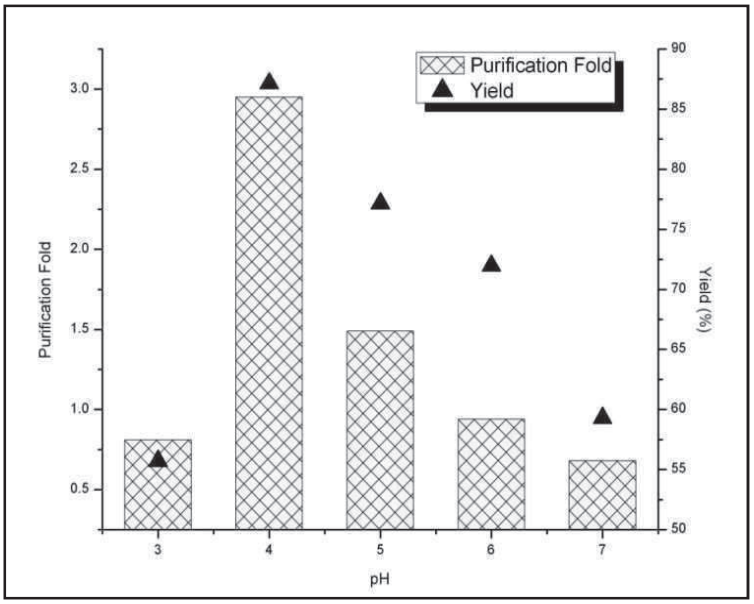

Fig. 7.5 Effect of culture filtrate pH on laccase recovery by TPP

Highest laccase recovery was found at pH 4 and it decreased thereafter (Fig. 7.5). The increased yield at pH 4.0 could be due to the better conformational stability of laccase towards *tert*-butanol. Various fungal laccases have been reported as relatively stable when stored at acidic to neutral pH (Baldrian et al. 2006, Morozoa et al. 2007).

The present study revealed the suitable conditions of three phase portioning (TPP) for the separation of *Peniophora* laccase. Maximum recovery was obtained with salt saturation level of 55%, incubation temperature of 45^{o}C, crude extract pH of 4.0 and *tert*-butanol ratio to 55% saturated crude extract was 1.0:1.5. The combinations of these conditions resulted in 96.42 % laccase yield with 2.61 fold purity protein. Although TPP produced a less favourable increase in the purity of laccase compared to chromatography process, this difference is negligible when the cost aspect is taken into account. Complete comparison of the TPP with other techniques has also been made (Table 7.2). It is seen in Table 7.2 that the present work is an excellent technique for purification of laccase which is cheap, simple and efficient.

Table 7.2 Comparison of the previously reported purification methods for laccase purification from various sources

Microorganism	Purification method	Yield (%)	Purity (Fold)	Reference
Pycnoporous sanguineus	Ultra filtration	87	2.14	Lu et al.2007
Ganoderma sp.	Diafiltration	90.4	3.7	Rajeeva and Lele 2010
Tramates sp.	Foam fractioning	72	11.7	Gerken et al.2006
Agaricus bisporus	Aqueous two phase system	95	2.48	Mayolo-Deloisa et al.2009
Ganoderma sp.	Three Phase Partitioning	60	13.1	Lele and Rajeeva 2011
P. ostreatus	Three Phase Partitioning	162	27.8	Sivanesan et al.2011
P.ostreatus	Ammonium Sulfate Precipitation-Immobilized metal affinity chromatography	57.4	46	Freixo et al.2011
Ganoderma sp. *WR-1*	Single-step TTP	65	4.9	Lele and Rajeeva 2011
Unidentified *Basidiomycete UD4*	ASP-Super Q anion exchange chromatography	57	7.1	Leukes et al.2004
Peniophora sp. hpF-04	Single step-TPP	96.42	2.61	Present study

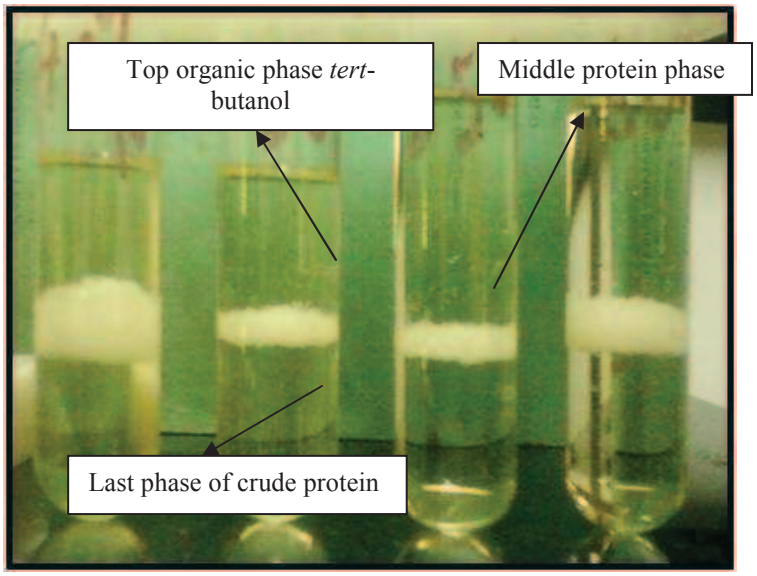

Fig. 7.6 Visible observations of three phase separation of proteins

7.3.2 Characterization of partially purified laccase

7.3.2.1 Effect of pH on laccase activity and its stability

Laccase activity was measured at varying pH (2.5 to 9.0). The optimum pH for purified laccase produced by *Peniophora sp*. hpF-04 was found to be 4.0 for ABTS oxidation (Fig.7.7). A further rise in pH was found to deactivate laccase protein. The purified laccase remained quiet stable within the pH range 3.5 to 4.5 after 1hr of incubation. The enzyme retained 31.5% of its original activity at this pH after 20hr of incubation at room temperature for the current purified laccase (Fig. 7.8). It is reported that the inactivation process is found to be faster at high alkaline pHs due to disulfide exchange, which usually occurs at near neutral and alkaline conditions. Therefore acid stable laccase characterized in this study can be used for the decolourization and degradation of acidic (pH 3.8-4.4) wool dyeing effluent.

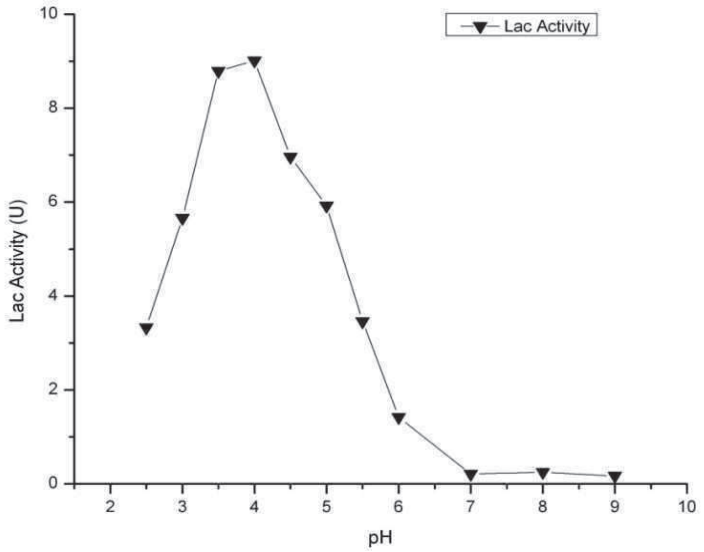

Fig.7.7 Effect of pH on laccase activity of *Peniophora sp.* hpF-04

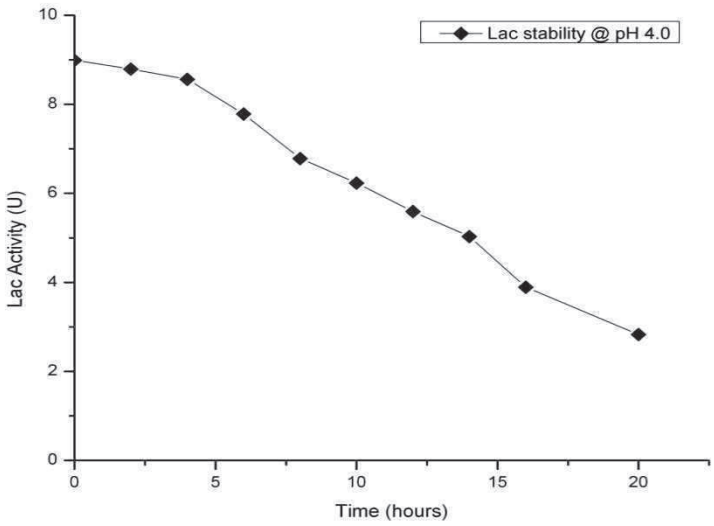

Fig.7.8 pH stability of purified *Peniophora sp*. hpF-04 laccase

These findings were in correlation with several fungal laccase pH optima of 3.0-3.6 for ABTS. Niku-Paavola et al. (2004) reported pH maxima for *Peniophora sp.* crude laccase as 4.0 for guaiacol and the enzyme retained its full activity at pH 6.0-7.0 for 22h at 20^{o}C. A similar result was obtained with pH optima of 4.0 for TPP purified laccase

from *Peniophora sp*.hpF-04. Contrastingly, the laccase activity from this species has drastically reduced at neutral pH range 0f 6.0-7.0. The majority of fungal laccases have been found to function as laccase under mild acidic conditions (pH 4–6). Many laccases with pH optima at acidic range have been reported from many fungi. A laccase from *Mauginiella* sp. had optimal activity at acidic pH of 2.4 and remained reasonably stable within pH range of 4-8 after 24-h incubation (Palonen et al. 2003). Crude laccase from *T. versicolor* showed its optimum pH at 4.5 (Stoilova et al. 2010). Boer et al. (2004) reported pH 4.5 as optimum pH for *Lentinula edodes* laccase. The optimum pH for *Coniothyrium minitans* laccase was reported to be 3.5 (Dahiya et al. 1998).

The pH optima of laccases are highly dependent on substrates. For phenols, the optimum pH can range from 3.0 to 7.0 for fungal laccases and up to 9.0 for plant laccases. When ABTS used as a substrate, the pH optima are in more acidic range usually in the range between 3 and 5 (Heinkill et al. 1998). In general laccase activity has bell shaped profile with an optimal pH that varies considerably. This variation may be due to the changes to the reaction caused by the substrate, O_2, or the enzyme itself (Xu 1997). The difference in redox potential between the phenolic substrate and the T1 copper could increase oxidation of substrate at high pH values, but the hydroxide ion (OH^-) binding to T2/T3 coppers results in an inhibition of the laccase activity due to the disruption of internal electron transfer between the T1 and T2/T3 sites. These two opposing effects can play an important role in determining the optimum pH of the biphasic laccase enzymes (Xu 1997). The role of T1 copper in the pH optima of the enzymes was confirmed by Palmieri et al. (1997), who was found that T1 copper was absent in laccases exhibiting more neutral pH optima.

7.3.2.2 Effect of temperature on laccase activity and its stability

The *Peniophora sp* .hpF-04 laccase was active over a broad temperature range of 30-70^{o}C with an optimum temperature of 45^{o}C (Fig. 7.9). Moreover, the enzyme was found to active with a broad range of temperature up to 60°C. Hence, the stability of the enzyme at higher temperatures was checked by incubating the enzyme at 50°C and 60°C.

The activity was completely lost after 40h at 50°C. The enzyme retained only 6% of its original activity at 50°C after 30h of incubation. At 60°C the enzyme retained only 8.9% of its original activity after 24h of incubation (Fig. 7.10). When compared to other fungal laccases, laccase from this strain has high temperature stability at 50°C and 60°C. These findings of temperature optima and temperature stability were similar to that of *Peniophora sp.* as reported by Niku-Paavola et al. (2004). Crude laccase from *Peniophora sp.* was thermostable with pH optima of 60-70°C. This confirms that the thermostability at higher temperatures is a specific characteristic of *Peniophora* species laccases.

The optimum temperature obtained in the present study is in agreement with the studies of laccase characterization of different strains. Laccase isolated from Ganoderma lucidium showed optimum temperature of 20-25°C and was found to be stable between 10-50°C for 4h (Ko et al. 2001). Xiao et al. (2004) found the optimum temperature of laccase from Trametes sp. as 45°C. Similarly, maximum activity for Pleurotus ostreatus laccase was determined at 45°C. Stoilova et al. (2010) reported T.versicolor laccase with similar temperature optimum of 45°C. Recently Irshad et al. (2011) reported a laccase from S. commune IBL-06 which was active at a temperature of 40°C and the enzyme retained only 60% of its original activity at 60°C when incubated for 1h.

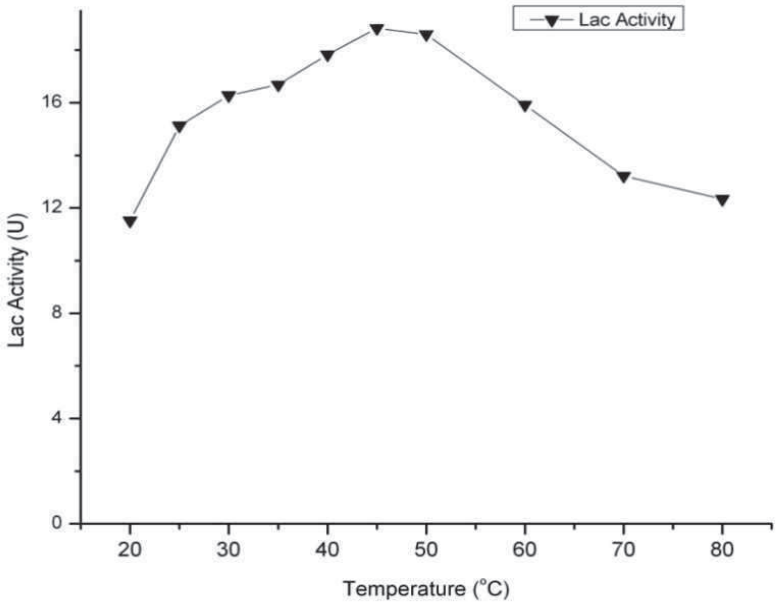

Fig.7.9 Effect of temperature on laccase activity of *Peniophora sp.* hpF-04

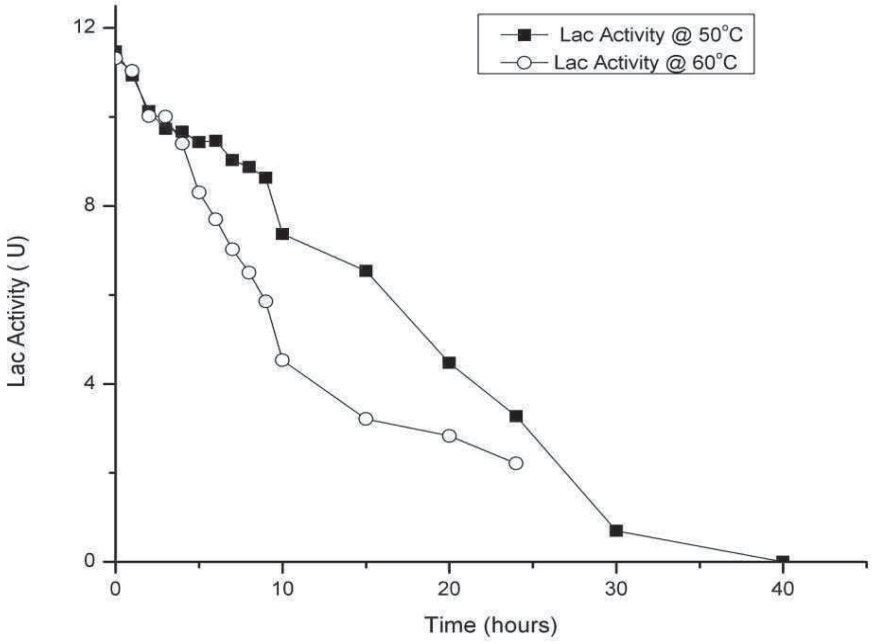

Fig. 7.10 Stability of *Peniophora sp.* hpF-04 laccase at 50°C and 60°C

7.3.2.3 Determination of kinetic parameters

The K_m and V_{max} values of purified laccase were determined by using different substrates such as ABTS, guaiacol and 2,4-DMP. Enzyme activities were measured under standard assay conditions and results were used to construct reciprocal plots using Line-Weaver and Burk equations (Fig. 7.11). The reciprocal of laccase activity (1/[V]) in IU/ml was plotted against the reciprocal of substrate concentrations (1/[S]) in mM.

The values of kinetic parameters V_{max} and K_m are shown in Table 7.3. For guaiacol, V_{max} and K_m values are 1.49 mM and 0.28 mM/min; for ABTS, 30.3 mM 0.06 mM/min and for 2,4-DMP, 8.77 mM and 0.27 mM/min, respectively. The relationship between rate of the reaction and concentration of the substrates depends on the affinity of the enzyme for its substrates expressed as K_m and high V_{max} value for ABTS reflects its higher affinity for the enzyme. The high specific activity towards ABTS is also reported in *Peniophora* sp. laccase (Niku-paavola et al. 2004).

Table 7.3 Kinetics of *Peniophora sp.* hpF-04 purified laccase

Substrate	V_{max} (mM)	K_m (mM/min)
Guaiacol	1.49	0.28
ABTS	30.30	0.06
2,4-DMP	8.77	0.27

The enzyme kinetic constants, K_m and V_{max} vary from source to source, type of the substrate utilized and also other parameters used in the experiments (Desai and Nityananda 2011). K_m value of *T. versicolor* laccase for ABTS was 12.8M and its corresponding V_{max} value was 8125.12U/mg (Han et al. 2005). Valeriano et al. (2009) reported K_m and V_{max} values of 13.25µM and 255nKat/mg for *Stereum ostrea* laccase for guaiacol, respectively. In the study by D'souza et al. (2009), for the isoenzyme Lac III (from *C.unicolour*), specificity constant (K_{cat}/K_m) of 120/min/µM was observed for ABTS at 70°C and pH 3.0. The Line-Weaver Burk plot yielded K_m value of 386µM for

L1 and 5µM for L2 using syringaldazine as substrate, for laccase purified from *T. versicolr* (Minusssi et al. 2007). Irshad et al. (2011) reported K_m value of 0.025 mM and V_{max} of 80mM/min for ABTS for laccase of *S. commune* IBL-06. Similar kinetic constant values were reported for laccases of *C. maxima* (Koroleva et al. 2002), *Pycnoporus sanguineus* (Litthaver et al. 2007) have reported to show higher K_m and lower V_{max}.

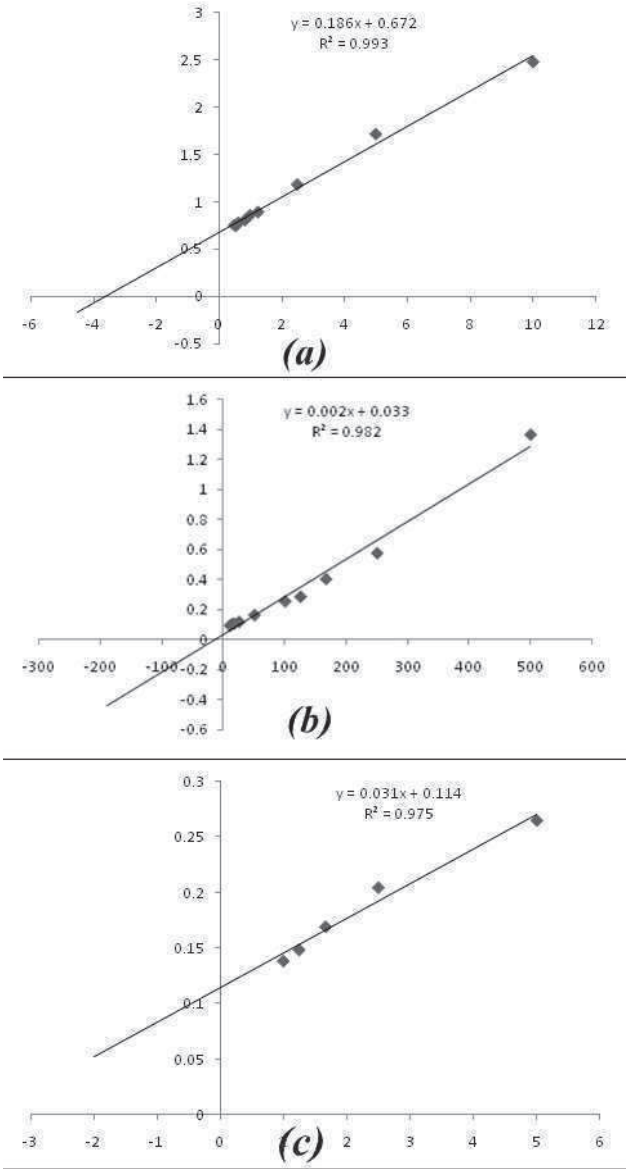

Fig. 7.11 Reciprocal plots for determination of K_m and V_{max} for laccase produced by *Peniophora sp.* hpF-04 using Guaiacol (a), ABTS (b) and 2, 4-DMP(c)

7.3.2.4 Effect of compounds and metal ions

Among various ions and organic compounds used in the study, β-mercaptoethanol and oxalic acid completely reduced laccase activity after 1hr of incubation at room temperature (Fig. 7.12). Sodium azide also showed around 98% of reduction in laccase activity. Interestingly, compounds like TEMED, EDTA and SDS, are proved to be inhibitors of enzymes of different types, did not show any inhibition of laccase activity in case the of *Peniophora sp.* hpF-04 laccase. Inhibition by other compounds used in the study was also insignificant. Such observation of high inhibition of azide compound and low inhibition of EDTA was also reported by Niku-paavola et al. (2004) for *Peniophora* laccase crude extracts. Similar results of inhibition on fungal laccases were also reported earlier in *Marasmius quercophilus* and *Sinorhizobioum melioloti* CE53G laccases, respectively, which were not affected by EDTA (1.0mM), mercaptoethanol (0.1mM), $MnCl_2$, while strongest inhibition was observed with sodium azide (Ryan et al. 2003; Fernet et al. 2004; Stajic et al. 2006). Inhibition by sodium azide was also reported in *P.ostreatus* laccase (Pradeep et al. 2010). In a study conducted by Bollag and Leonowiqz (1984), it was found that azide, thioglycolic acid inhibited laccase activity, whereas EDTA affected the enzyme activity to a lesser extent. Small ions such as halides, azide, cyanide and hydroxide bind to type 2 and type 3 copper, resulting in an interruption of the internal electron transfer and activity inhibition (Madhavi and Lele 2009).

Conversely, in the present study, the presence of cupric ions reduced *Peniophora sp.* hpF-04 laccase activity. This phenomenon was also reported in laccase from *P.ostreatus* K16-2. Laccase inhibition may occur through amino acid residue modification, copper chelation, or conformational changes of the enzyme (Morozova et al. 2007). Although laccase is a copper containing protein, excess supply of Cu^{+2} ions might cause change in the laccase structure.

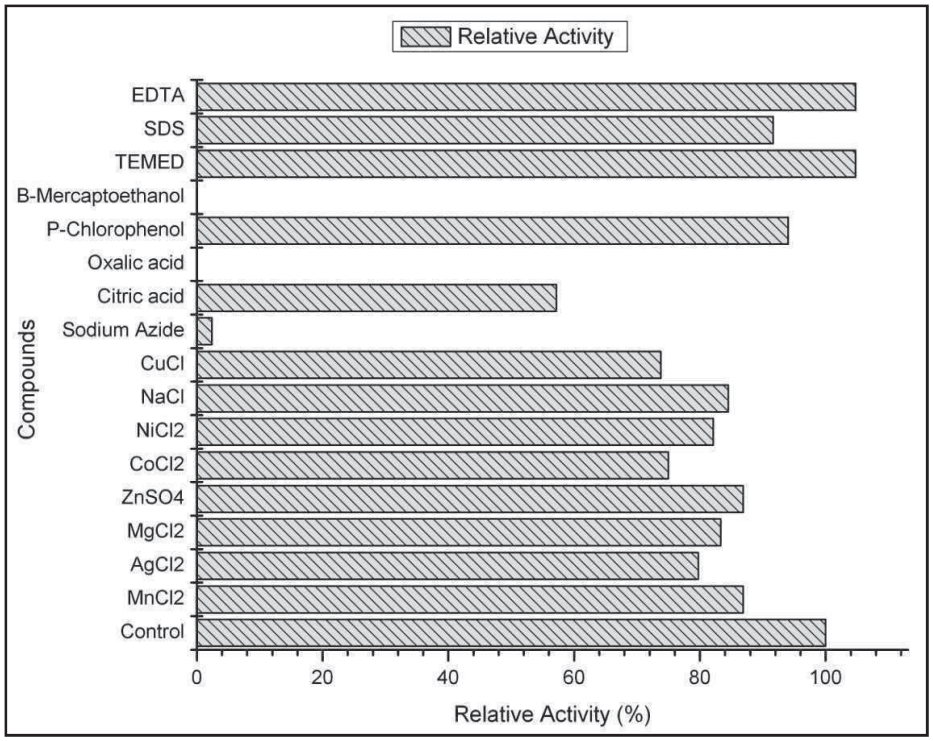

Fig. 7.12 Effect of compounds on laccase activity of *Peniophora sp.* hpF-04

7.3.2.5 SDS-PAGE analysis for molecular weight determination

The TPP purified laccase was subjected to denaturing PAGE on 10% acrylamide gel. SDS–PAGE was performed to determine sample purity and to determine the approximate mass of the laccase. SDS–PAGE revealed the presence of single protein band with molecular weight of 67kDa (Fig. 7.13).

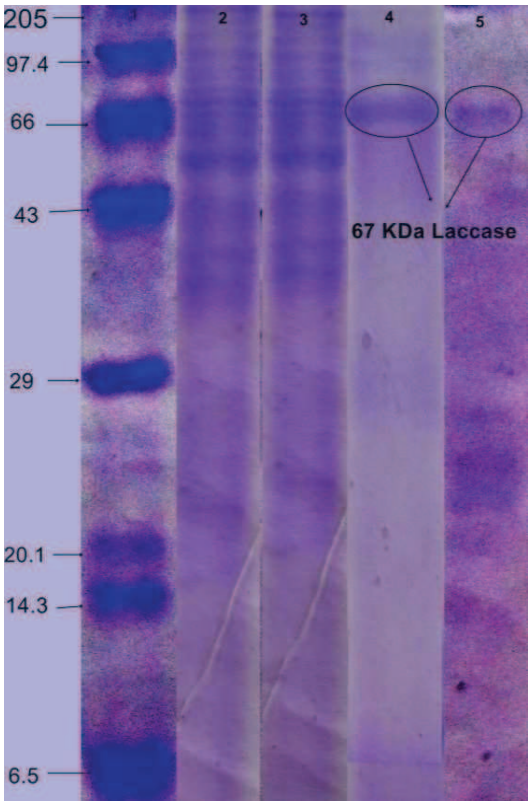

Fig. 7.13 SDS-PAGE of TPP purified laccase Lane 1: Molecular weight standard, lane 2 and 3: Crude enzyme; Lane 4 and 5: Interfacial precipitate. Molecular weight markers contained the proteins myosin, rabbit muscle (205 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa) and aprotinin (6.5 kDa)

7.3.2.6 Laccase purity analysis using RP-HPLC

During purification, especially after the last purification steps the purity of the final preparation has to be verified. The most commonly used method to check the purity of a protein/enzyme preparation is 1D or 2D gel electrophoresis. More rarely capillary electrophoresis is applied. An alternative method is RP-HPLC method. It separates according to the difference in their hydrophobicity and separation is based on interactions between hydrophobic patches on the surface of biomolecules and the hydrophobic

surfaces of a chromatography medium. Proteins are detected at 250nm or 280nm. RP-HPLC offers great flexibility in separation condition. Separations can be performed using isocratic elution, however, gradient elution is used in order to minimize the run time. In the present work, the analysis was carried out in multistep gradient using buffer A (0.1% TFA in water) and buffer B (0.1% TFA in ACN) for 30 min at 25°C with a flow rate of 1.0 ml/min. The absorption was monitored at 280nm.

Fig. 7.14 and Fig. 7.15 presents the HPLC chromatogram of crude filtrate and TPP purified laccase, respectively. A major peak at 23.57 min was confirmed as laccase peak in both the cases. Crude filtrate showed many small individual peaks of other unwanted proteins present in the culture filtrate along with the predominant laccase peak (Fig. 7.14), whereas, in TPP purified laccase sample (Fig. 7.15, Table 7.5), most of these peaks were eliminated which explains the purity of laccase separated by TPP.

Table 7.4 Properties of chromatographic peaks obtained for crude filtrate

No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	1.53	n.a.	14.919	1.468	16.46	n.a.	BMb
2	1.75	n.a.	3.730	0.405	4.55	n.a.	bMB
3	2.17	n.a.	0.563	0.034	0.38	n.a.	BMb
4	2.38	n.a.	10.453	1.776	19.92	n.a.	bMB
5	3.54	n.a.	7.040	2.044	22.93	n.a.	BMB
6	4.35	n.a.	0.206	0.022	0.25	n.a.	BMB
7	5.43	n.a.	0.262	0.115	1.29	n.a.	BMB
8	5.62	n.a.	0.712	0.088	0.99	n.a.	BMb
9	5.84	n.a.	0.336	0.062	0.69	n.a.	bMB
10	6.61	n.a.	1.163	0.306	3.43	n.a.	BMB
11	7.79	n.a.	0.311	0.069	0.78	n.a.	BMB
12	8.38	n.a.	0.432	0.099	1.11	n.a.	BMB
13	8.98	n.a.	0.705	0.152	1.71	n.a.	BMB
14	10.22	n.a.	1.167	0.245	2.74	n.a.	BMB
15	11.08	n.a.	0.211	0.059	0.66	n.a.	BMB
16	11.80	n.a.	0.320	0.096	1.08	n.a.	BMB
17	14.50	n.a.	0.574	0.181	2.04	n.a.	BMB
18	15.96	n.a.	0.684	0.161	1.81	n.a.	BMB
19	17.78	n.a.	0.100	0.022	0.24	n.a.	BMB
20	18.57	n.a.	1.064	0.277	3.10	n.a.	BMB
21	20.76	n.a.	0.162	0.029	0.32	n.a.	BMB
22	22.37	n.a.	0.078	0.010	0.11	n.a.	BMB
23	23.96	Laccase	3.139	1.130	12.68	1.529	BMB
24	28.89	n.a.	0.205	0.065	0.72	n.a.	BMB
Total:			48.535	8.915	100.00	1.529	

Table 7.5 Properties of chromatographic peaks obtained for TPP purified laccase

No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	14.26	n.a.	0.756	0.219	4.06	n.a.	BMB
2	17.44	n.a.	1.769	0.889	16.49	n.a.	BMB
3	23.57	Laccase	14.870	4.222	78.28	5.711	BMB
4	28.56	n.a.	0.173	0.064	1.18	n.a.	BMB
Total:			17.569	5.393	100.00	5.711	

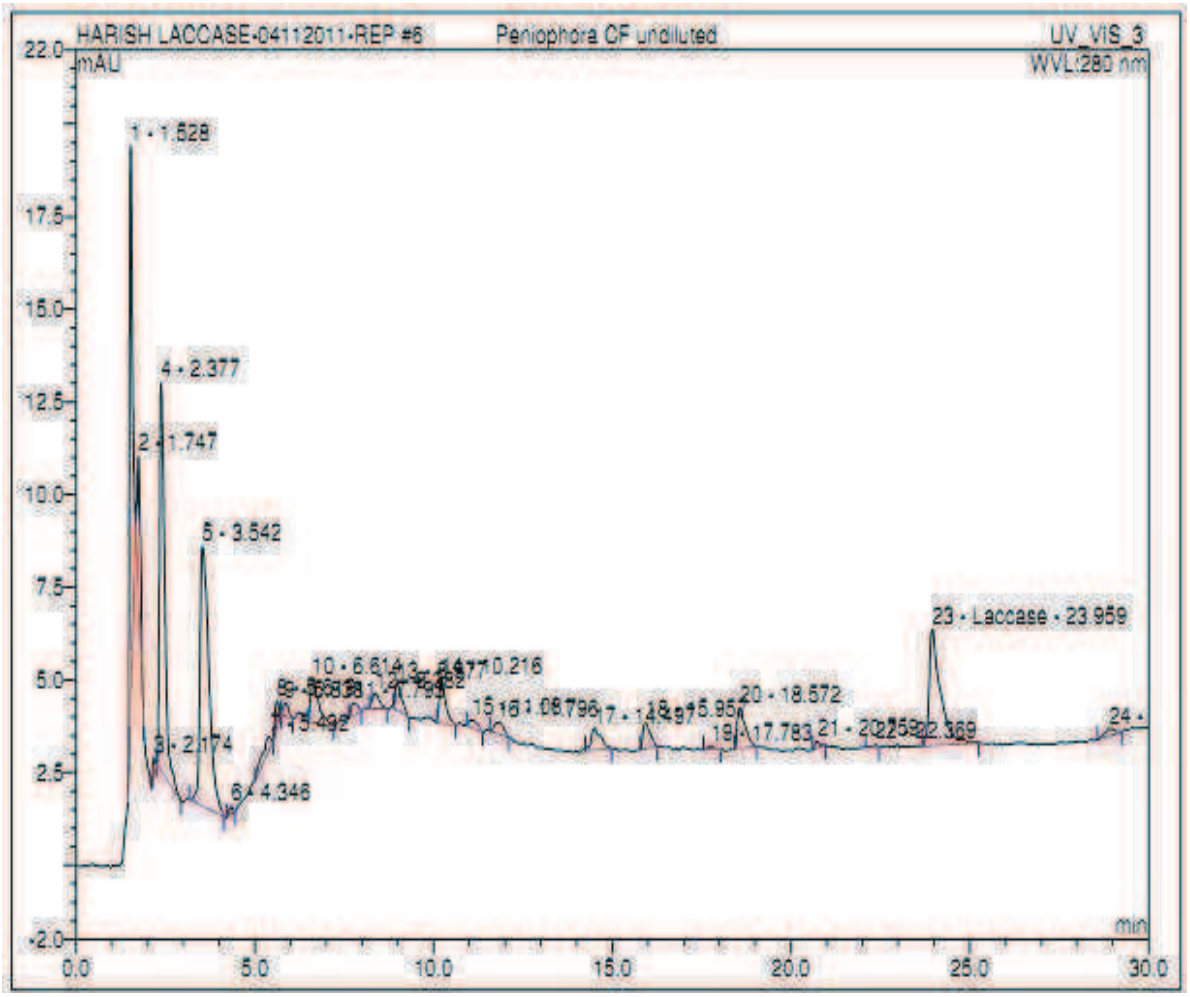

Fig. 7.15 RP-HPLC chromatogram of crude culture filtrate from *Peniophora sp.* hpF-04

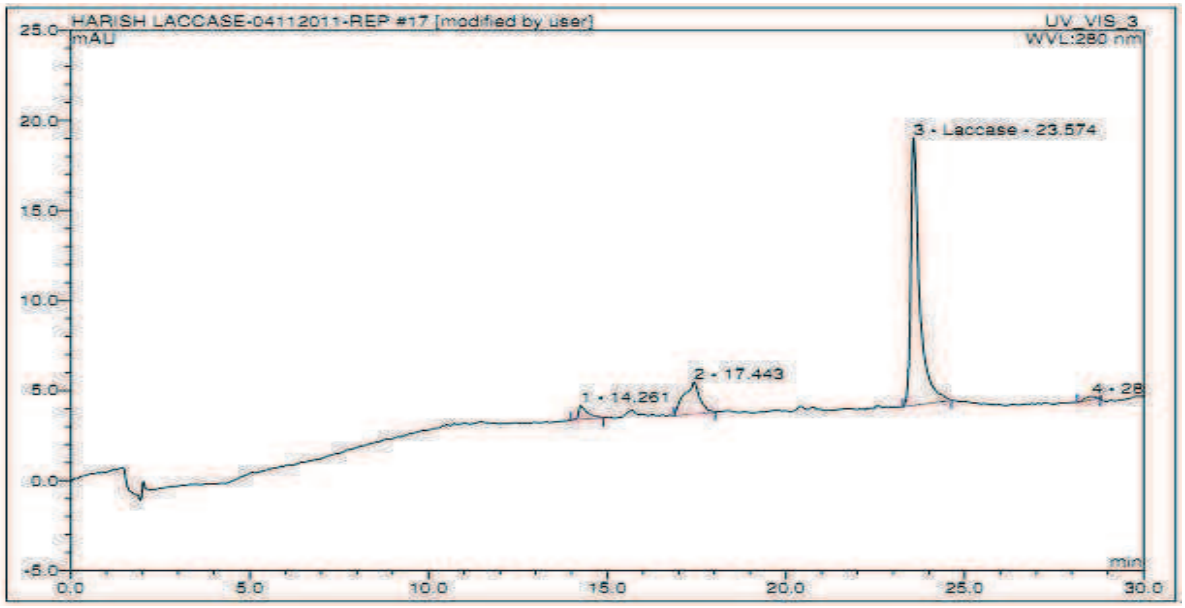

Fig. 7.15 RP-HPLC chromatogram of TPP purified laccase from *Peniophora sp.* hpF-04

7.3.2.7 Laccase spectroscopic studies and determination of copper and carbohydrate content

The presence of copper was confirmed by atomic absorption spectrophotometer and scanning spectrum. However, the absorption spectrum of laccase in the range 300-800nm lacked the absorption maximum at 610 nm (Fig. 7.16), characteristic of fungal laccases that corresponds to copper atom type I (Thurston 1994; Baldrian 2006). However, the spectrum showed two shoulders at 400nm and 300nm. The shoulder at around 300nm suggests the presence of the type III binuclear Cu^{2+} center. Similar spectrum with absorption maximum at 400nm was also reported in *Pleurotus ostreatus* laccase isoform (Palmieri et al. 2000).

The concentrated enzyme solution was yellowish-brown in colour. The purified laccase contained 5.12 µg of copper per mg enzyme, determined by atomic absorption spectrometry, which corresponds to 3.12±0.2 copper atoms per molecule. The occurrences of laccases that do not display a typical characteristic spectrum and the lack

of blue colouration have been reported earlier. A "white" laccase was said to be isolated from *Pleurotus ostreatus* (Palmieri et al. 2000), while Leontievsky et al. (1997) reported the presence of "yellow" laccases. The loss of the absorption peak at 600 nm of the "white" laccase was attributed to the presence of only a single copper atom in the metal cluster, the other three atoms being replaced by two Zn and one Fe atom (Kumar et al. 2010). Recently Zhao et al. (2012) reported a novel white laccase from *Myrothecium verrucaria* NF-05 having similar properties. The fungus contained 3.08±0.3 Cu and 0.95±0.2 Fe atoms with an atypical spectrum lacking peak around 600nm. Leontievsky et al. (1997) attributed the loss of this peak in the case of "yellow" laccases to copper atoms being present in their reduced state. The laccase was found to be glycosylated as it was determined to contain 0.12mg of carbohydrate per mg of protein (11.5%).

Thus, we found that *Peniophora sp.* hpF-04 laccase is a glycosylated copper-containing monomer with a molecular weight 67 kDa, like most fungal laccases (Thurston 1994; Baldrian 2006). However, unlike laccases described before, the enzyme was light yellow and its absorption spectrum lacked the maximum at 610 nm characteristic of typical blue laccases. According to these features, we classified *Peniophora sp*. hpF-04 laccase with so-called yellow laccases, recently found in basidiomycetes (Leontievsky et al. 1997; Palmieri et al. 2000; Pozdnyakova et al. 2006).

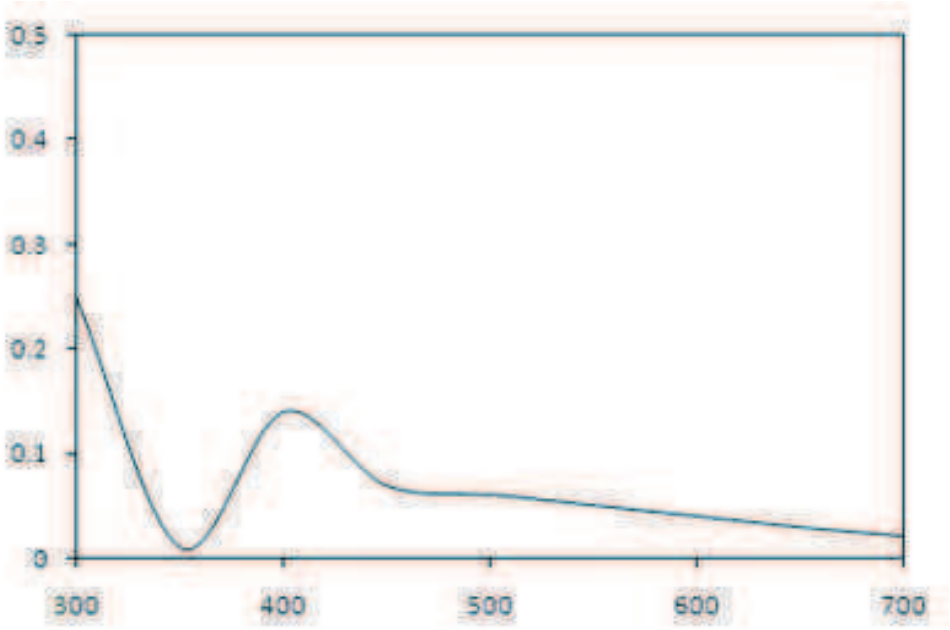

Fig. 7.16 UV–visible absorption spectrum of the TPP purified laccase. The assay contained 200 µg/ml laccase in acetate buffer

To sum up, the present study describes a simple, cost-effective purification procedure such as three phase partitioning to purify extracellular yellow laccase from the crude extract of *Peniophora* sp. hpF-04. The laccase was characterized in order to define its enzymatic properties and compared it with several other laccases found in fungal culture in the literature reports. Due to its higher stability at acidic pH and elevated temperatures, this laccase could be an ideal candidate as a biocatalyst in harsh biotechnology processes. However, further studies on chemical and molecular level properties of the enzyme would be necessary for elucidation of the novelty of this laccase.

Chapter 8

PARTIAL PURIFICATION AND CHARACTERIZATION OF LACCASE PRODUCED BY *PHELLINUS NOXIUS* HPF-17

8.1. Introduction

In the previous chapter, we described the separation and characterization of laccase from a newly isolated basidiomycetae *Peniophora sp*. hpF-04 for the first time. Together with this fungus, another fungal isolate, *Phellinus noxius* hpF-17 showing significant production of laccase was discovered. . Hence, the main aim of this study is to separate and purify laccase from *Phellinus noxius* hpF-17 and also to study its biochemical and catalytic properties.

There are only two reports cited in scientific literature with respect to laccase characteristics of the fungal genera *Phellinus*. Geirger et al. (1986) studied and reported the molecular mass of the laccase from *Phellinus noxius* for the first time in 1986. Min et al. (2001) also reported some properties of laccase from *Phellinus ribis.* Moreover, both the study exploited conventional separation techniques to separate and purify laccase. Hence, in this study we deal with the separation and purification of laccase by advanced methods such as membrane filtration and three phase partitioning which were very successful in separating and purifying *Peniophora sp.* hpF-04 laccase as described earlier.

While purifying laccase from *Peniophora sp.* hpF-04, TPP parameters for maximum laccase yield and purity was optimized by traditional one-factor-at-a-time method, which was found to be very tedious and extremely time consuming. To overcome these difficulties, a statistical approach by response surface methodology (RSM) was used as an alternative tool. The RSM approach for optimization requires first an experimental design and then fitting experimental data into an empirical model equation to determine the optimum conditions. Box-Behnken of response surface methodology was used in the present study for optimization of different TPP variables affecting separation. This design is an efficient and creative 3-level composite design for fitting second-order response surfaces. It is an independent quadratic design. The methodology is based on the construction of balance designs which are rotatable and enable each factor level to be tested several times. Each factor or independent variable

can be placed at one of three equally spaced values (coded as –1, 0, and +1). In this design, the treatment combinations are at the midpoints of edges of the cubical design region and at the center. Box-Behnken designs provide excellent predictability within the spherical design space and require fewer experiments compared to the full factorial designs or central composite designs. The number of required experiments for Box-Behnken design can be calculated according to $N = k^2 + k + cp$, where k is the factor number and cp is the replicate number of the central point.

The purified enzyme was then characterized with respect to its activity and stability at various pH and temperature ranges. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and determination of kinetic parameters (K_m and V_{max}) were also carried out. This is the first report on the purification of laccase from *Phellinus noxius* hpF-17 by using three-phase partitioning.

8.2. Materials and Methods

8.2.1 Culture maintenance and Production of laccase by *Phellinus noxius* hpF-17

The fungus *Phellinus noxius* hpF-17 was maintained in 2% MEA slants and sub-cultured in the same media before use. Previously optimized medium for maximum laccase production was used for the production of laccase by the fungus. The optimized medium of *Phellinus noxius* hpF-17 consisted of (g/l); glucose (20.0), ammonium tartarate (2.25), urea (5.0), Tween 80 (2.08ml), KH_2PO_4 (1.0), KCl (0.5), $MgSO_4$ (0.5) and 10 ml/l of trace elements containing g/l of distilled water: EDTA (0.5), $FeSO_4$ (0.2), $ZnSO_4.7H_2O$ (0.01), $MnCl_2.4H_2O$ (0.003), H_3BO_4 (0.03), $CoCl_2.6H_2O$ (0.02), $CuCl_2.2H_2O$ (0.001) and $Na_2MoO_4.2H_2O$ (0.003). Mycelial agar plugs (4-5 plugs of 9mm diameter) were cut along the edge of the actively growing colony and used as the inocula. The fungus was cultivated at 30°C on a rotary shaker (150 rev/min). After six days, the mycelium-free culture filtrate was collected and used for the experiments.

8.2.2 Concentration of culture filtrate using tangential flow filtration (TPP)

Concentration of protein was carried out using tangential flow filtration, as described earlier for *Peniophora sp.* hpF-04.

The tangential flow filtration was carried out as follows

1. Clean containers and tubing connections were arranged as shown in Fig. 7.1
2. The feed container was filled with 120ml of crude filtrate solution of 6 days old culture.
3. The pump was turned on with a flow rate of 30-50ml/min
4. The solution was filtered till the volume reduced to 70ml and around 50 ml of waste was collected.
5. The pump was turned off. The tubing connections were removed. The retentate (concentrate) as well as permeate (waste) were collected in clean containers.
6. The membrane was cleaned by pumping 0.5N NaOH till 250ml of waste was collected in the waste container.
7. The samples were analyzed for laccase activity, protein content and specific activity.
8. The % yield, fold purification and concentration factor were calculated as described earlier in section 7.2.2.

8.2.3 Precipitation of proteins by TPP

All TPP experiments were carried out using the crude fungal extract. The combinations of separation parameters used in one step TPP had not proved to be the best combination for separation of *Phellinus noxius* hpF-17 laccase. One step TPP resulted in low protein purification fold and purity. Hence, statistical optimization was used to get the best separation combinations for better protein yield.

8.2.3.1 Three phase separation by response surface methodology design

In the present study, the effects of operating parameters of TPP were optimized using RSM. Based on economic feasibility, Box-Behnken Design (BBD) was employed.

The experimental factors were ammonium sulphate saturation; 20-80% (w/v), ratio of crude extract to *tert*-butanol; 1.0:0.5 – 1.0:2.5 (v/v) and temperature; 20–60°C as shown in Table 8.1. The total number of experimental runs was 17 with replications and the yield (%) and purity (fold) of laccase was taken as the dependent variable or response as shown in Table 8.2.

Table 8.1 Experimental factors and coded levels in the three factor three-level response surface design used for optimizing the purity and yield of laccase

Experimental factor	Symbol	Coded unit		
		−	0	+
$(NH_4)_2SO_4$ saturation (%)	A	20	50	80
Ratio of crude extract to *tert*-butanol (v/v)	B	1:0.5	1:1.5	1:2.5
Temperature (°C)	C	20	40	60

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included the fisher's F-test (overall model significance), its associated probability P (F), determination coefficient R^2 which measures the goodness of fit at regression models. For each variable, the quadratic model was represented as contour plates (3D) and response surface curves were generated using Design expert 8.0 trial version.

Table 8.2 Box Behnken design matrix for the experimental design and predicted results for laccase purity and yield

Run Order	Factor			Response	
	$(NH_4)_2SO_4$ (w/v)	*tert*-butanol to crude extract (v/v)	Temperature (°C)	Yield (%)	Purity (fold)
1	20	01:00.5	40	28.2	0.54
2	80	01:00.5	40	63.12	2.03
3	20	01:02.5	40	34.2	1.76
4	80	01:02.5	40	60.38	2.15
5	20	01:01.5	20	30.5	1.6
6	80	01:01.5	20	53.14	1.32
7	20	01:01.5	60	32.8	0.65
8	50	01:01.5	60	64.59	2.65
9	50	01:00.5	20	53.1	1.98
10	50	01:02.5	20	59.6	2.21
11	50	01:00.5	60	54.21	1.88
12	50	01:02.5	60	83.12	2.98
13	50	01:01.5	40	79.43	2.95
14	50	01:01.5	40	91.25	3.01
15	50	01:01.5	40	93.4	2.99
16	50	01:01.5	40	89.8	2.25
17	50	01:01.5	40	88.93	3.04

8.2.3.2 Effect of pH on laccase separation

Effect of pH was studied separately on separation after other three variables such as temperature (^oC), ammonium sulfate saturation (%) and ratio of crude extract to *tert*-butanol was optimized through RSM.

1. Crude enzyme was buffered at different pH values (3–9). The buffered solutions were saturated with 57% (w/v) ammonium sulphate.
2. It was followed by addition of *tert*-butanol in volumetric aqueous phase-to-*tert*-butanol ratio of 1:1.7 (v/v).
3. The mixtures were allowed to stand at 44°C for 1h, after which precipitate and organic layer were separated.
4. The resultant interfacial precipitate was analyzed for laccase activity and protein content.

8.2.4 Characterization of laccase

8.2.4.1 Effect of pH on activity and stability of the purified laccase

The effect of pH on the activity of the purified laccase was determined by assaying the enzyme activity at different pH values ranging from 2.5 to 9.0 using 0.1M of the following buffer systems: acetate (3.0, 4.0 and 5.0), phosphate (pH 6.0 and 7.0) and Tris–HCl (pH 8.0 and 9.0) buffer systems. The pH stability of *Phellinus noxius* hpF-17 laccase was investigated at pH 3.0. Therefore, 1 ml of the enzyme was mixed with 1ml of the buffer solutions mentioned above and incubated at room temperature for 20h. Afterwards aliquots of the mixtures were taken to measure the laccase activity (%) with respect to control, under standard assay conditions.

8.2.4.2 Effect of temperature on activity and stability of the purified laccase

The effect of temperature on the activity of the purified laccase was determined by performing the standard assay procedure at different temperatures ranging from 20 to 90^{o}C. Before the addition of enzymes, the substrate (50 mM) ABTS and acetate buffer (pH 3.0) were pre-incubated at the respective temperature for 10 min. Thermal stability studies were conducted without any additives. The enzyme solution was incubated at 75^{o}C and 85^{o}C in a temperature-controlled water bath for 15-30h and the relative enzyme activity was measured at regular intervals of time. The relative activity was calculated as

the percentage ratio of activity at a given temperature to the activity at optimum temperature.

8.2.4.3 Effect of metal ions, organic acids and various compounds on the laccase activity

Metal compounds (10 mM) ($CoCl_2$, NaCl, $NiCl_2$, $MgCl_2$, $ZnSO_4$, $MnCl_2$, CuCl and $AgCl_2$), organic acids (10 mM) (citric acid and oxalic acid) and various compounds EDTA (10 mM), TEMED (0.1%), SDS (0.01%) and β-mercaptoethanol (0.1%), p-chlorophenol (0.1%), sodium azide (0.005 mM)) were tested for their effect on purified laccase activity in acetate buffer, pH 3.0. The enzyme was pre-incubated for 1h at 45°C with listed components.

8.2.4.4 Protein determination and enzyme activity

Protein concentration was determined by the Lowry's method using bovine serum albumin as the standard. The laccase activity was determined using ABTS as the substrate. Enzymatic activity was expressed as 1U=1µmol of ABTS oxidized per min under specific condition.

8.2.4.5 Determination of Kinetics parameters

The kinetic constants (V_{max} and K_m) were determined using Lineweaver–Burk double reciprocal (1/v versus 1/S) plot, where different concentrations of guaiacol (0.1 to 2.0 mM), ABTS (0.002 to 0.1 mM), and 2,4-dimethoxyphenol (2, 4-DMP) (0.2 to 1.0 mM) were used as substrate at pH 3.0 and temperature at room temperature.

8.2.4.6 Molecular weight determination by SDS-PAGE

For calculation of the protein molecular mass, SDS-PAGE was carried out with a 10 % gel, as described by Laemmli et al. (1971). After running the gel, the proteins were stained by Coomassie brilliant blue R. Standard molecular markers of range 3 kDa to 250 kDa obtained from (Genie) was used for comparison. Crude culture filtrate and partially purified laccase were used as samples.

8.2.4.7 Laccase purity analysis using RP-HPLC studies

Purity of the enzyme was confirmed by RP-HPLC analysis using Dionex HPLC system. RP-HPLC was carried out according to the method of Divakar et al. (2010). The crude and TPP purified laccase was applied on to C-18 column (Zorbax C-18, 4.6mm×250mm i.d., 5µm particle size, Agilent technologies). For elution, buffer C consisted of 0.1% (v/v) trifluroacetic acid (TFA) in water and buffer D consisted of 0.1% (v/v) TFA in acetonitrile. After loading, the column was washed with 2% (v/v) buffer D for 2 min, to elute any unbound protein. The bound proteins were eluted from column using a 25 min linear gradient from 2 to 100% (v/v) buffer D at constant flow rate of 1ml/min. Column temperature was maintained at 25°C and column eluent was monitored at 280nm.

8.2.4.8 Laccase spectroscopic studies and determination of copper and carbohydrate contents

The absorption spectrum of purified laccase was determined at room temperature (28^{o}C) in the range of 300-700nm (Kumar et al. 2010).

The copper content of the protein was determined by atomic absorption spectrometry (AAS) (GBC Avanta, Australia).

Amount of carbohydrate present in the protein was determined using phenol sulphuric acid method (Sadashivam and Manikckam 2003) (Appendix IV).

8.3 Results and Discussion

8.3.1 Concentration of crude enzyme solution by TFF

Concentration is a simple process that involves removing fluid from a solution while retaining the solute molecules. The concentration of the solute increases in direct proportion to the decrease in solution volume, *i.e.* halving the volume effectively doubles the concentration. Concentration of protein (enzyme) was carried out by TFF system

using ultrafiltration membrane with NMWCO of 10KDa. Choosing ultra filtration membrane with a Molecular weight cut-off (MWCO) value that is substantially lower than the molecular weight of the molecules to be retained is vital in TFF process. This is important in order to assure complete retention and high recovery of the target molecule. A good general rule is to select a membrane with a MWCO that is 3 to 6 times lower than the molecular weight of the molecules to be retained. Most of the fungal laccases were reported with molecular weights more than 40KDa (Baldrian 2006).

During the process, TFF system was initialized (typically flushed with water and tested for water filtrate flow rate and integrity) and 120 ml of crude culture filtrate having enzyme activity 90.0U was added and cross flow was established. Around 70 ml of retentate was collected and checked for specific activity, yield (%) and purity fold. The TFF process resulted in 97.5% laccase yield with purity fold of 2.42 in the retentate (Table 8.3). However, presence of 1.75 U laccase activity in permeate showed certain loss of enzyme through waste.

Table 8.3 TFF Concentration and purification of laccase produced by *Phellinus noxius* hpF-17

Volume (ml)	Activity (U/ml)	Activity (U/Vol)	Protein (mg/ml)	Protein (mg/Vol)	Specific activity	Purity (fold)	Yield (%)
120.00	0.75	90.00	0.45	54.00	1.67	--	--
68.00	1.29	87.72	0.32	21.76	4.03	2.42	97.47
50.00	0.04	1.75	--	--	--	--	--

8.3.2 Purification of laccase produced by *Phellinus noxius* hpF-17 by TPP

Separation and purification of proteins by TPP is dependent on different process parameters like salt concentration, crude filtrate to *tert*-butanol ratio, temperature, pH, etc. Hence, an efficient separation to get highest yield and purity relies on the optimum levels of these parameters. To overcome the disadvantages of traditional one-factor-at-a-

time method, statistical methods of optimization using Box Behnken design was carried out.

8.3.2.1 Box-Behnken design for separation and purification of proteins from crude extract

The experiment was carried out with three variables, $(NH_4)_2SO_4$ saturation, ratio of crude extract to *tert*-butanol and temperature. However, another factor pH was not included to reduce the number of experiments and was optimized after the optimum levels of these three variables were obtained. A central composite design (CCD) was employed within a range of –α (-1.68) and +α (+1.68) in relation to the laccase yield (%) and protein purity. According to the design, 17 runs replicated three times at central points were performed and experimental and predicted responses were obtained as shown in Table 8.2. Relationship between the variables was determined by fitting a second order polynomial equation to the data obtained from the 17 runs.

The predicted and observed responses along with design matrix are presented in Table 8.4 and the results were analyzed by ANOVA. The second-order regression equation provided the levels of laccase yield and purity as a function of $(NH_4)_2SO_4$ saturation, ratio of crude extract to *tert*-butanol and temperature which can be presented in terms of coded factors as in the following equation:

$$\text{Laccase yield (\%)} = +88.56+14.44*A+4.83*B+4.80*C-2.19*A*B+2.29*A*C+5.60*B*C-29.67*A^2-12.42*B^2-13.64*C^2 \quad (8.1)$$

$$\text{Laccase purity (fold)} = +2.85+0.45*A+0.33*B+0.13*C-0.28*A*B+0.57*A*C+0.22*B*C- 0.97*A^2-0.26*B^2-0.33*C^2 \quad (8.2)$$

Where, A, B, and C are $(NH_4)_2SO_4$ saturation, ratio of crude extract to *tert*-butanol and temperature.

The statistical significance of Equation 8.1 and 8.2 was checked by F-test and the analysis of variance for response surface quadratic model is shown in Table 8.4.

ANNOVA of regression model demonstrates that the model is highly significant as it is evident from the Fischer test with very low probability value. The value of lack of fit, Model F and model P>F for yield was found to be 1.72, 22.92 and <0.05 and for purity 0.04, 15.8 and <0.05 respectively, indicating that model was significant. Fisher F-test with a very low probability value (Pmodel>F = 0.0002) and also lack of fit was insignificant that demonstrates a very high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient (R^2). The mathematical adjustment of those values generated $R^2 = 0.9672$ (96.72%) for laccase yield and $R^2 = 0.9067$ (90.67%) for purity, revealing that the model was unable to explain 3.28% and 9.33% of the overall effects, demonstrating it to be a good statistical model. Adjusted R^2 value of 0.9250 (92.50%) for yield and 0.8928 (89.28%) for purity suggested that model was significant. A very low value of coefficient of variation (CV %) for laccase yield (11.02) and purity (10.57) clearly indicate precision and a good deal of reliability of experimental values. From the Table 8.4, values of "Prob > F" less than 0.0500 indicate model terms are significant.

Table 8.4 ANNOVA for laccase yield (%) and purity (fold)

Source	Yield (%)					Purity (fold)				
	Sum of Squares	Df	Mean Squares	F-value	p-Value	Sum of Squares	Df	Mean Squares	F-value	p-Value
Model	7817.15	9	868.57	22.92	0.0002	9.45	9	1.05	15.8	0.0007
A-$(NH_4)_2SO_4$ Saturation (%)	1668.4	1	1668.4	44.03	0.0003	1.62	1	1.62	24.39	0.0017
B-ratio of crude extract to t-butanol	186.92	1	186.92	4.93	0.0618	0.89	1	0.89	13.41	0.008
C-Temperature(^oC)	184.13	1	184.13	4.86	0.0633	0.14	1	0.14	2.07	0.193
AB	19.1	1	19.1	0.5	0.5007	0.3	1	0.3	4.55	0.0703
AC	20.93	1	20.93	0.55	0.4816	1.3	1	1.3	19.56	0.0031
BC	125.55	1	125.55	3.31	0.1115	0.19	1	0.19	2.85	0.1353
A^2	3706.19	1	3706.19	97.81	<0.0001	3.94	1	3.94	59.36	0.0001
B^2	649.34	1	649.34	17.14	0.0044	0.29	1	0.29	4.29	0.077
C^2	782.91	1	782.91	20.66	0.0027	0.45	1	0.45	6.71	0.036
Residual	265.25	7	37.89			0.47	7	0.066		
Lack of Fit	149.56	3	49.85	1.72	0.2996	0.014	3	0.0046	0.041	0.9875
Pure Error	115.69	4	28.92			0.45	4	0.11		
Cor Total	8082.4	16				9.91	16			
Model fitting										
C.V = 9.87%						C.V = 12.17%				
R-Sq = 96.72%						R-Sq = 90.67%				
R-Sq(adj) = 92.50%						R-Sq(adj) = 89.28%				

The relationship between the actual and predicted laccase yield (%) and purity (fold) (responses) is shown in Fig. 8.1. The cluster of measurements near the diagonal line in the parity plot indicates a good fit of the model and demonstrates a satisfactory correlation between the actual and predicted values. The minimum response of 28.2% yield was obtained with $(NH_4)_2SO_4$ saturation (20%), ratio of crude extract to *tert*-butanol (1:0.5) and temperature (40°C). The maximum response of 93.4 % yield was obtained

with $(NH_4)_2SO_4$ saturation (50%), ratio of crude extract to *tert*-butanol (1:1.5) and temperature (40°C).

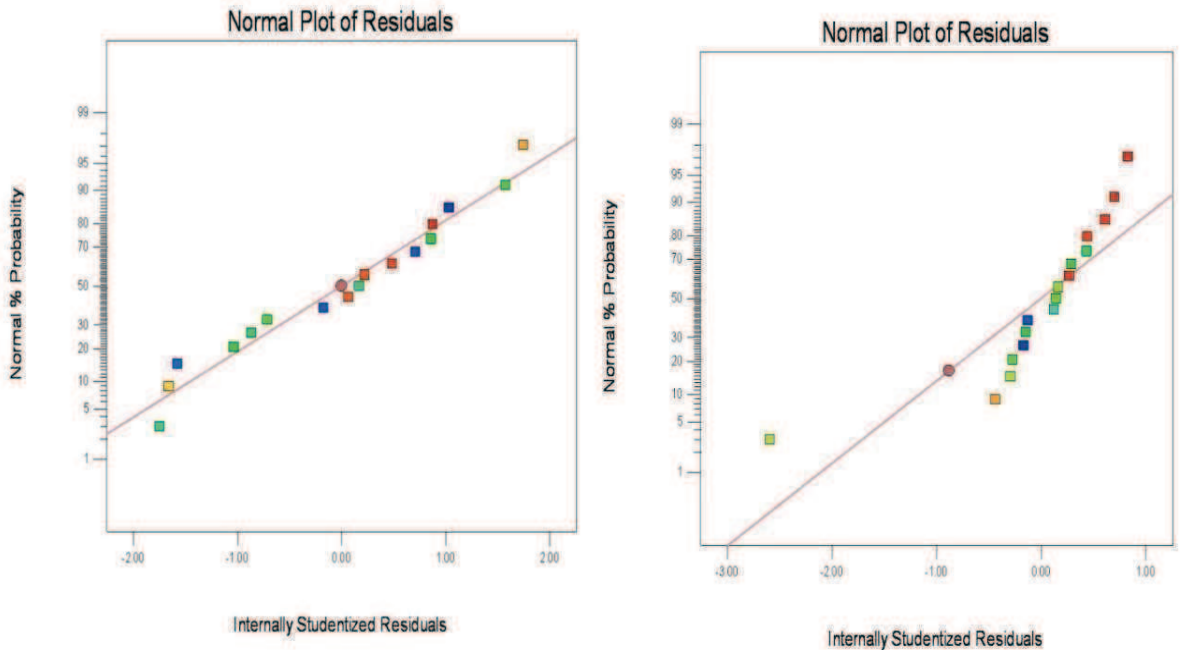

Fig. 8.1 Actual and predicted laccase yield for *Phellinus noxius* hpF-17

The optimum values of the selected variables were also obtained by analyzing response surface contour plots. Response surface plots as a function of two factor at a time maintaining all other factors at a fixed level (zero for instance) are more helpful in understanding both the main and interaction effects of the two factors. The plots can be easily obtained by calculating the data from the model. The values were taken by one factor, where the second varies with constant of a given Y -values. The yield values of the different concentrations of the variable can also be predicted from respective response surface plots (Fig. 8.2 and Fig. 8.3). The coordinates of the central point within the highest contour levels in each of these figures corresponded to the optimum concentrations of the respective components.

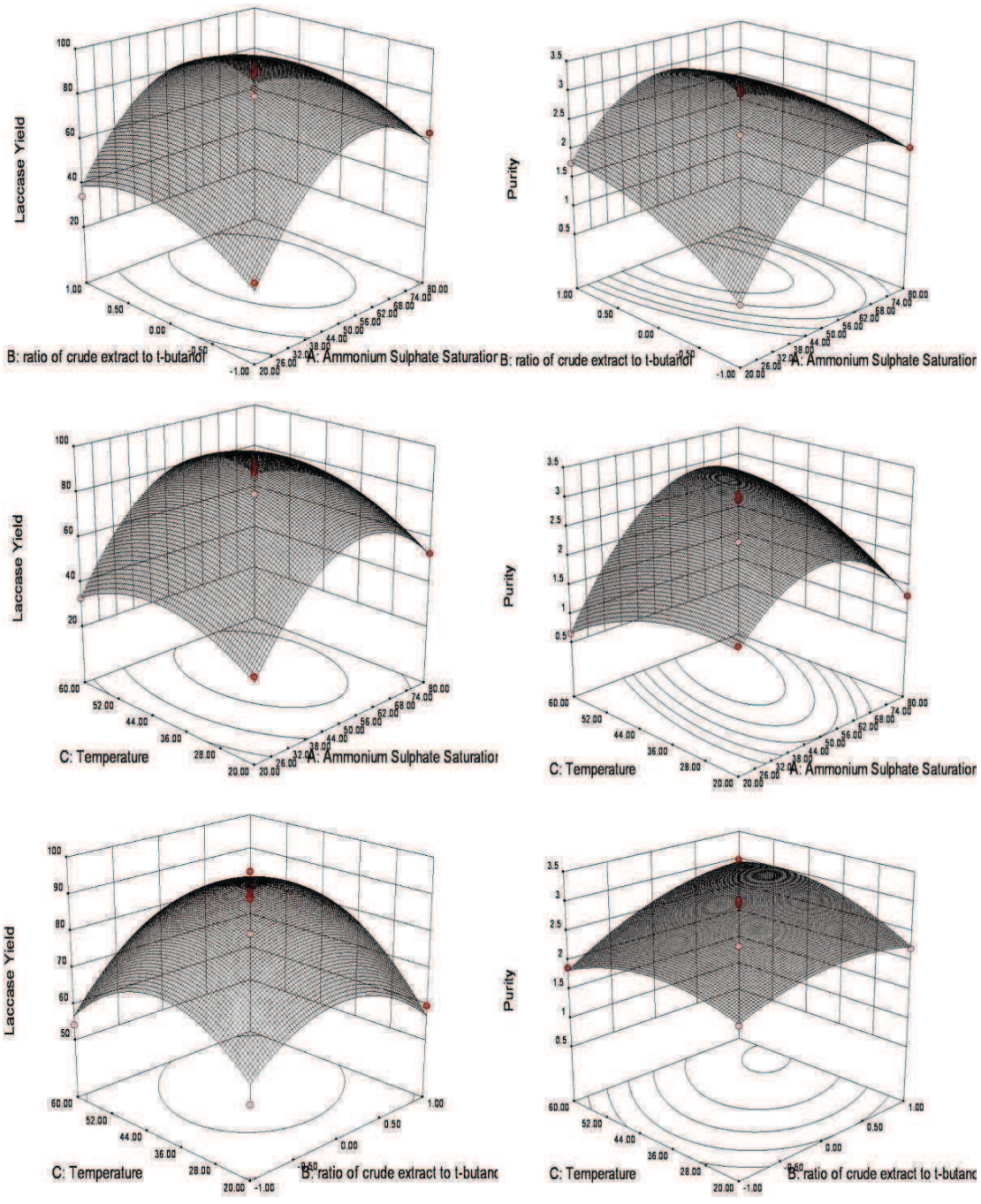

Fig. 8.2 3D response surface plot for the effect of variables for the purification of laccase produced by *Phellinus noxius* hpF-17

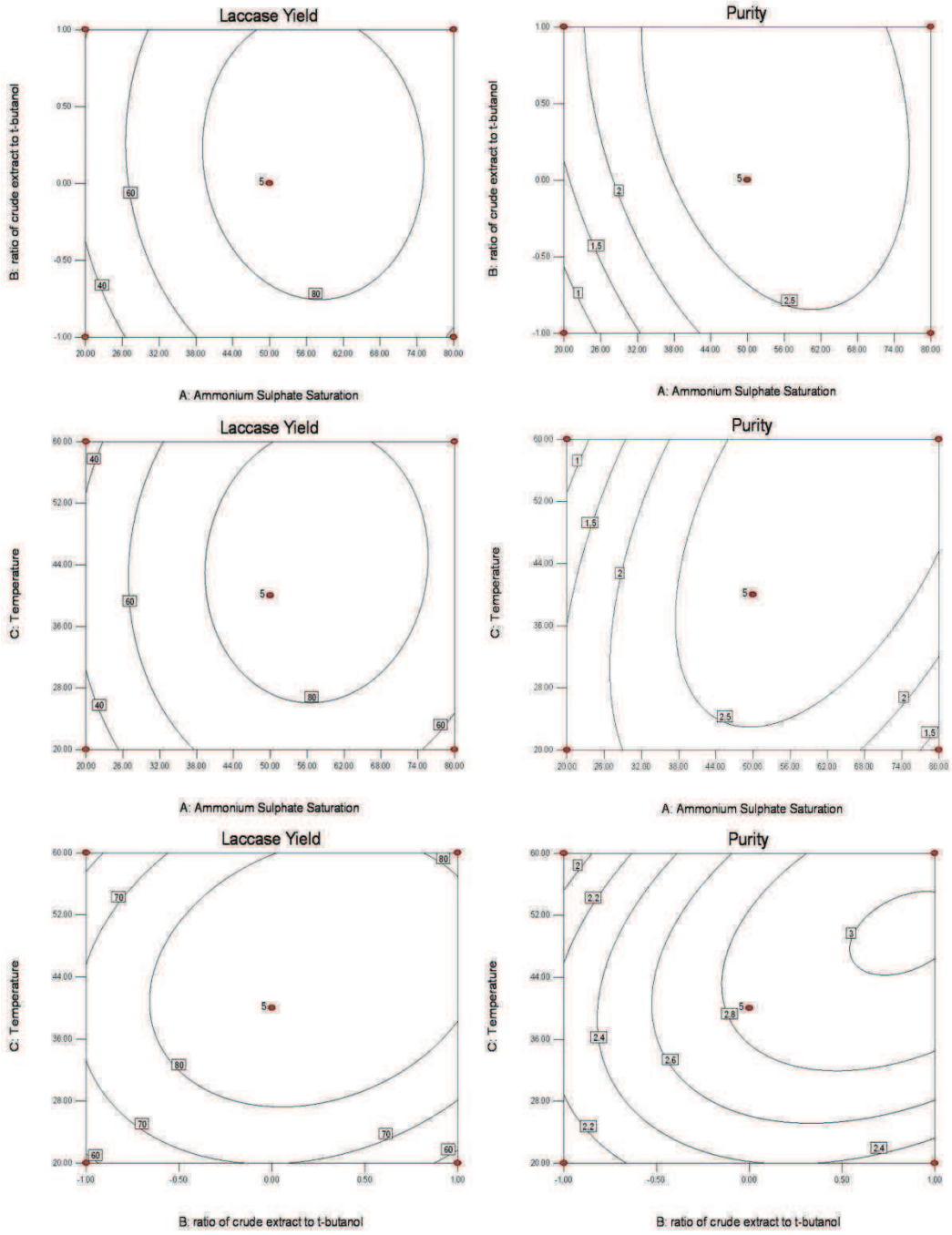

Fig. 8.3 3D counter plot for the effect of variables for the purification of laccase produced by *Phellinus noxius* hpF-17

The predicted optimum levels of the tested variables, namely, $(NH_4)_2SO_4$ saturation (A), ratio of crude extract to *tert*-butanol (B) and temperature(°C) were

obtained by applying regression analysis on Equation 8.1 and 8.2. The optimal levels were as follows: A=57%, B= 1:1.7, C= (44.13^{o}C) with the corresponding, laccase Yield (Y) = 91.43% and purity fold of 2.98. Verification of the predicted values was conducted by using optimal medium in inoculation experiments. The practical corresponding response obtained was 89 % with purity fold of 2.78.

8.3.2.2 Effect of pH on laccase separation

The optimization of TPP process parameters for separation of proteins was carried out by employing only three factors while carrying the experiments with the constant pH of the original crude filtrate (*i.e.*5.2-5.5). Once suitable combination of three factors was obtained by design experiments, the fourth factor pH was optimized by carrying out the experiments by adjusting pH ranging from 3-7 with $(NH_4)_2SO_4$ saturation (57%), ratio of crude extract to *tert*-butanol (1:1.7) and temperature (44^{o}C). The effect of pH on separation was shown in Fig. 8.4. There was not much effect of pH on laccase separation. Highest yield of 85.4% with purity fold of 2.87 was obtained at pH.5.0.

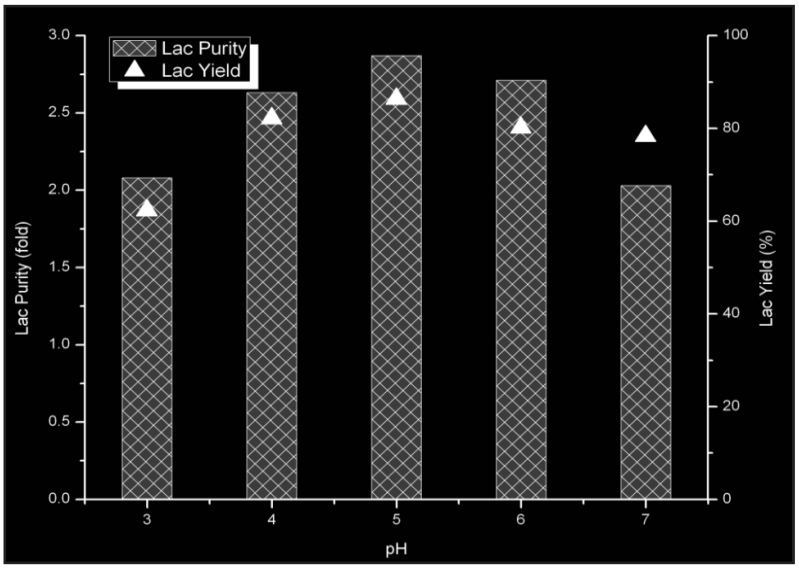

Fig. 8.4 Influence of pH on laccase separation

TPP is an efficient purification technique, where remarkable increase in the yield of various enzymes were reported previously like invertase (1000%) by Dennison and

Lovrein (1997), Ipomea peroxidase (976%) by Narayan et al. (2008) and *Calotropis procera* latex protease (165%) by Rawdkuena et al. (2010). Statistical optimization by Box-Behnken Design was also reported by Diwakar et al. (2012) for the separation of laccase from *Pleurotus ostreatus*. In the present study, Box-Behnken design was successfully employed for the optimization of TPP process variables for efficient separation of laccase from *Phellinus noxius* hpF-17. Fig. 8.5 shows the visible observation of three phase partitioning of laccase from the crude filtrate after incubating the mixture at optimum levels of factors obtained by RSM.

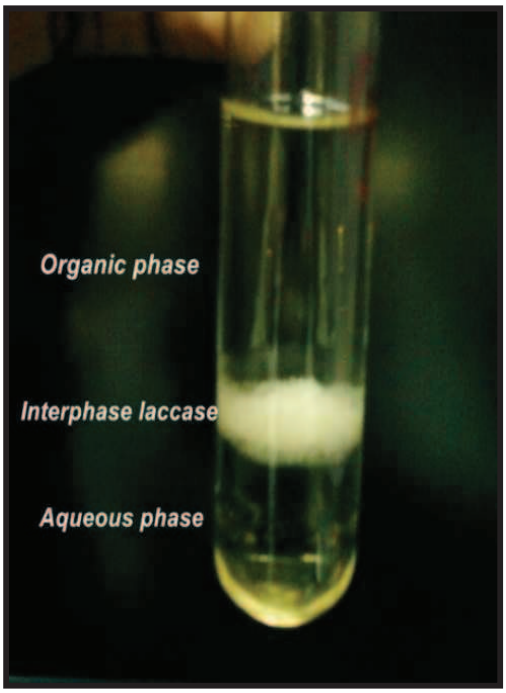

Fig. 8.5 TPP separation of laccase under optimized condition

8.3.3 Characterization of purified laccase from *Phellinus noxius* hpF-17

Laccase from *Phellinus noxius* hpF-17 was purified by three phase partitioning. The purified laccase was then characterized in terms of its molecular weight, optimum pH and temperature as well as stability, carbohydrate and copper contents. In addition, kinetic parameters using different substrates were also analyzed.

8.3.3.1 Effect of pH on laccase activity and its stability

Laccase activity was studied at varying pH (2.2 to 9.0) by varying the assay pH using buffers as described in section 8.2.4.1. The optimum pH of purified laccase produced by *Phellinus noxius* hpF-17 was found to be 3.0 for ABTS oxidation (Fig. 8.6). A further rise in pH was found to deactivate laccase protein. This was not accordance with the optimum pH obtained for *Phellinus ribis,* a fungus from the same genus. Min et al. (2001) reported the optimum pH of *P.ribis* was 5.0. However, Heinzkill et al. (1998) reported that pH optima for fungal laccase was more acidic and was found in the range between pH 3-5 for ABTS substrate. In our study, when purified laccase was incubated for 20h at room temperature at optimum pH of 3.0, it remained quiet stable. The enzyme retained 44.3% of its original activity at this pH for 20h of incubation at room temperature as shown in Fig. 8.7.

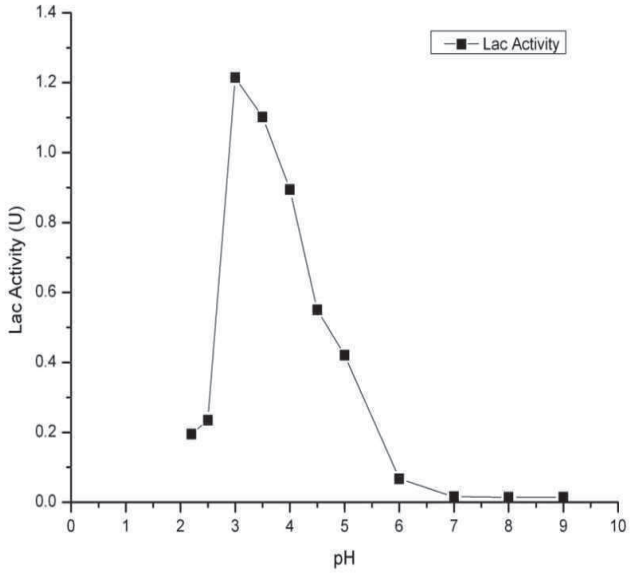

Fig. 8.6 Effect of pH on purified *Phellinus noxius* hpF-17 laccase activity

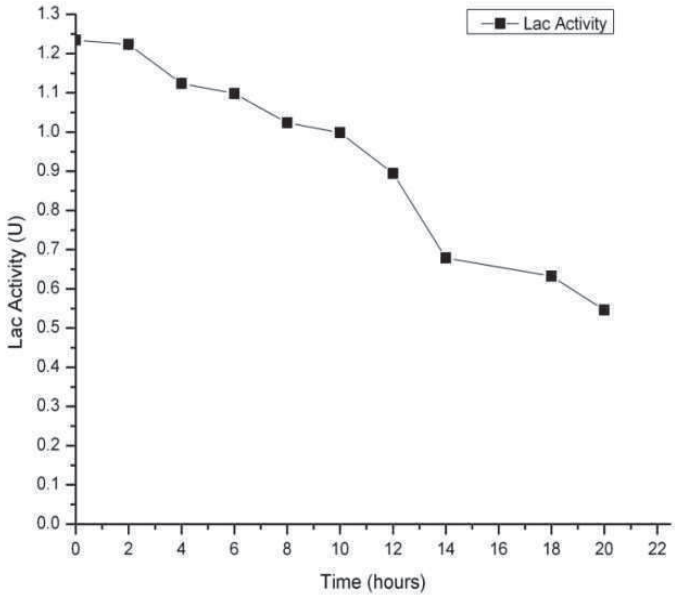

Fig. 8.7 Stability of purified *Phellinus noxius* hpF-17 laccase at its optimum pH

The findings of the present study were in correlation with several fungal laccase pH optima of 3.0-3.6 for ABTS. Laccases having pH optima at acidic pH have been reported in many fungi. The optimum pH for *Coniothyrium minitans* laccase was reported to be 3.5 (Dahiya et al. 1998). Laccase from *Mauginiella sp.* had optimal activity at acidic pH of 2.4 and remained reasonably stable within 4-8 pH range after 24h incubation (Palonen et al. 2003). Boer et al. (2004) reported pH 4.5 as optimum pH of *Lentinula edodes.* Crude laccase from *T. versicolor* showed its optimum pH at 4.5 (Stoilova et al. 2010).

In 1996, a study was conducted by Feng Xu to elucidate the reason for the different pH dependencies of laccases with different substrates. The dependence of laccase on pH usually renders a bell-shaped profile. As it can be seen from Fig. 8.6, similar bell-shaped curve was obtained in the present study. This bi-phasic profile is the result of two opposing effects. The first is due to the redox potential difference between a reducing substrate and type 1 copper centre of laccase, where the substrates dock. Here, the electron transfer rate is favoured for phenolic substrate at high pH. The second is

generated by the binding of hydroxide anion to the type 2/type 3 copper centre of laccase, which inhibits the binding of O_2, the terminal electron acceptor, and therefore, inhibits the activity at higher pH because of the increased amount of OH^- ions.

8.3.3.2 Thermal effects on laccase activity and its stability

The *Phellinus noxius* hpF-17 laccase was found to be highly thermostable with broad temperature range of 65-85^oC with an optimum temperature of 75^oC (Fig. 8.8). At 75^oC enzyme was very stable even after 72h of incubation (Fig. 8.9). But at 85^oC, the enzyme retained only 4% of its original activity after 24 h of incubation (Fig. 8.9). When compared to other fungal laccases, laccase from this strain showed high temperature stability at 75^oC and 85^oC. This was a first case of fungal laccases showing high thermo stability at more than 80^oC. There was an absolute loss in activity only when the enzyme was heated at boiling temperature, which is surprisingly high for an enzyme from a mesophilic, wood-decaying fungus. Laccase from a mesophilic wood rot, *Rigidoporus lignosus* also found to have high melting point temperature of 92.5^oC (Ragusa et al. 2002). Based on scanning calorimetric curves, the *Coriolus (Trametes) hirsutus* and *C. zonatus* laccase reported by Koroleva et al. (2001) represents exceptionally thermostable enzyme showing thermal transitions at 87 and 92^oC. Thermal transition of the laccase from the unidentified basidiomycete PM1 (CECT 2971) was observed as low as 70^oC (Coll et al. 1993).

Several physico-chemical factors are suggested to be the causes for protein thermostability (e.g. protein packing, hydro-phobicity, increased helical fold content, density of internal hydrogen bonds and salt bridges, distribution of charged residues on the surface, proportion of certain amino acids) (Kumar and Nussinov 2001; Sterner and Liebl 2001). In the majority of the thermophilic proteins so far studied, a general increase in the content of internal salt bridges and hydrogen bonds, as well as an enhanced proportion of amino acid residues in a-helical conformation have been observed (Vogt et al. 1997; Sterner and Liebl 2001). The compact protein structure together with metal-

binding at the three copper sites are involved in creating the thermal properties of *R. lignosus* laccase (Ragusa et al. 2002)

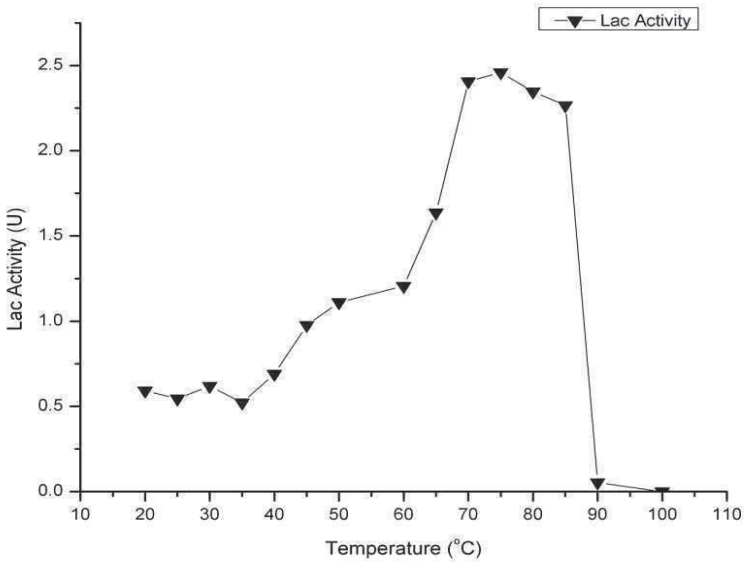

Fig. 8.8 Effect of temperature on purified *Phellinus noxius* hpF-17 laccase activity

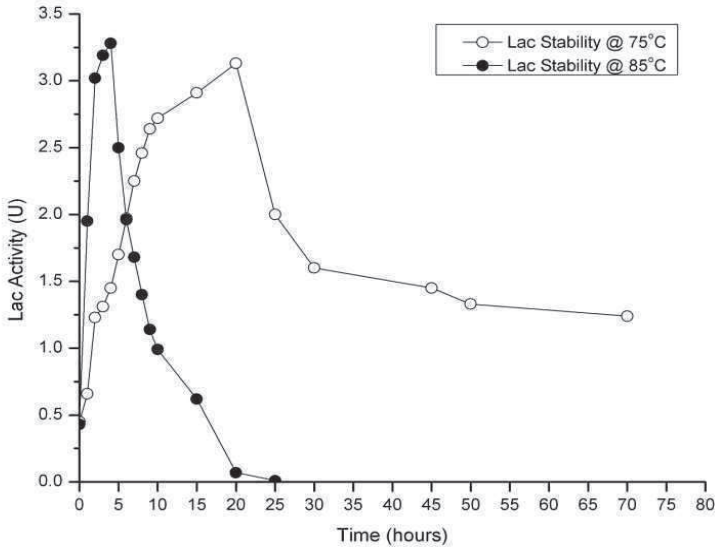

Fig. 8.9 Stability of purified *Phellinus noxius* hpF-17 laccase at high temperature

In the present study, another important observation made was thermal activation of laccase. As seen in Fig. 8.9, pre-incubation of enzyme at higher temperatures at 75^{o}C and 85^{o}C was found to activate the enzyme considerably. This might be due to the unfolding of active sites of the enzyme. As a result, stimulation of enzyme occured which might have enhanced the activity up to certain extent. However, on further incubation, denaturuation of the enzyme structure might have occured; hence decline in the enzyme activity was observed. Such result of thermal activation of enzyme was already reported in the basidiomycete PM1 laccase (Coll et al. 1993) and *P.ribis* laccase (Min et al. 2001).

8.3.3.3 Determination of kinetic parameters

The reaction kinetics of enzyme was determined by using ABTS, guaiacol and 2,4-DMOP. Enzyme activities were measured under standard assay conditions and results were used to construct reciprocal plot using Line-Weaver and Burk equations. The reciprocal of laccase activity (1/[V]) in IU/ml was plotted against the reciprocal of substrate concentrations (1/[S]) in mM (Fig. 8.10).

The values of kinetic parameters Vmax and Km are shown in Table 8.5. For guaiacol, V_{max} and K_m values were 0.036 mM and 3.42 mM/min; for ABTS, 6.67 mM 0.022 mM/min and for 2,4-DMP 8.33 mM and 0.48 mM/min were obtained, respectively. The relationship between rate of the reaction and concentration of the substrates depends on the affinity of the enzyme for its substrates expressed as Km and high Vmax value for ABTS reflects its higher affinity for the enzyme.

Table 8.5 Kinetics of purified laccase from *Phellinus noxius* hpF-17

Substrate	**V_{max} (mM)**	**K_m (mM/min)**
Guaiacol	0.036	3.42
ABTS	6.67	0.022
2,4-DMP	8.33	0.48

To confirm the thermal activation process, the kinetic parameters were determined at 85°C using ABTS as substrate. Enzyme was pre-incubated at 85°C and ABTS kinetics was determined at time interval of 2, 4 and 6 h. The increase in V_{max} and decrease in K_m value was observed which aids the thermal activation of laccase from *Phellinus noxius* hpF-17.

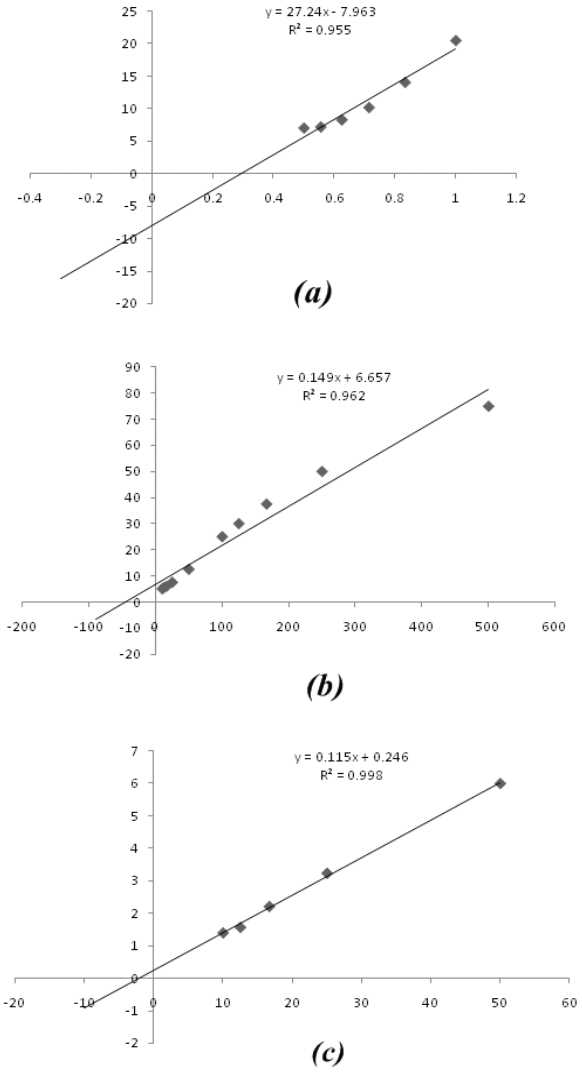

Fig. 8.10 LB plots for K_m amd V_{max} determination of laccase produced by *Phellinus noxius* hpF-17 for (a) Guaiacol. (b) ABTS and (c) 2,4-DMP

8.3.3.4 Effect of compounds and metal ions

Unlike *Peniophora sp*. hpF-04 laccase, laccase produced by *Phellinus noxius* hpF-17 was inhibited by more compounds employed in the study. Compounds like β-mercaptoethanol and TEMED completely reduced the activity. Sodium azide was also very effective in reducing the laccase activity by inhibiting the enzyme. On the other hand, in the presence of metallic compounds like $NiCl_2$, $CoCl_2$ and CuCl, irreversible inhibition effect was observed (Fig. 8.11). In the case of CuCl, 8 fold increase in laccase activity was observed. Thus, the presence of copper enhanced the laccase activity in *Phellinus noxius* hpF-17 laccase. The organic compounds such as citric acid and oxalic acid were also effective in reducing the laccase activity.

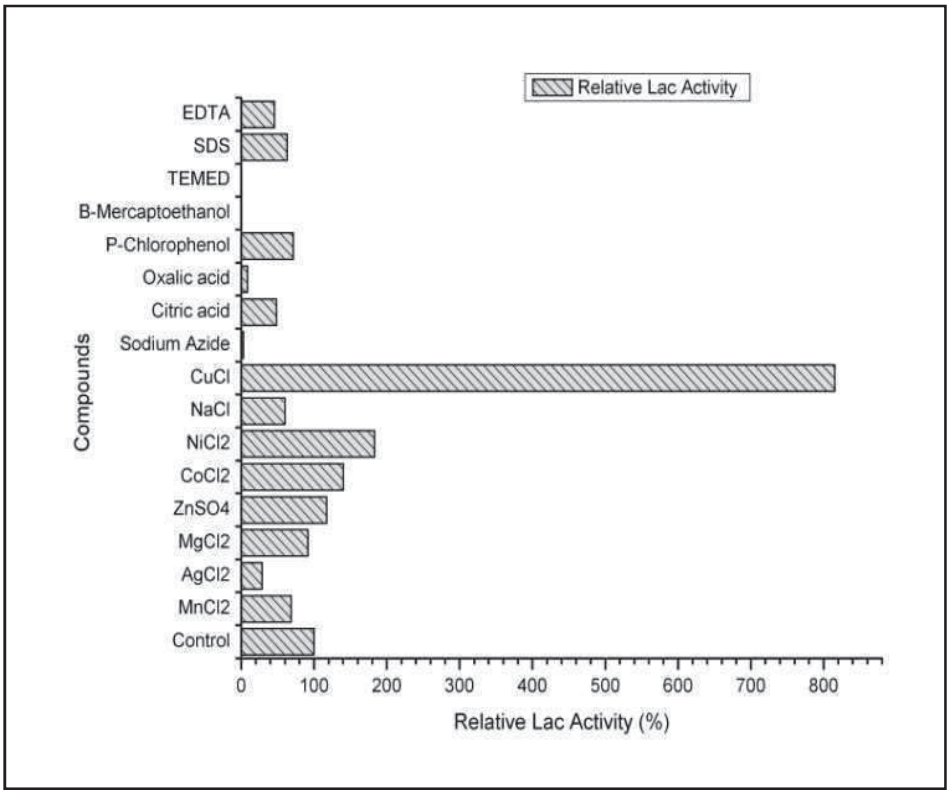

Fig. 8.11 Effect of inhibitor on *Phellinus noxius* hpF-17 laccase activity.

Laccase inhibition might occur through amino acid residue modification, copper chelation, or conformational changes of the enzyme. Generally sodium azide complexes

to the copper in the active site, EDTA exhibit metal chelating properties and halides (Cl^-) are known to inhibit laccase at the type 2/3 trinuclear copper site. It is in this site where the molecular oxygen is reduced to two molecules of water. Hence, oxygen is prohibited from being reduced, causing the break in the terminal electron acceptance and then, leading to a decrease in the redox potential difference between the two copper sites.

8.3.3.5 SDS-PAGE analysis for molecular weight determination

SDS–PAGE analysis of the purified laccase resulted in a single band of protein. The approximate molecular mass of the laccase was determined by calibration against broad range (3kDa to 205kDa) molecular weight markers (Genie), which contained the proteins myosin, rabbit muscle (205 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6.5 kDa) and insulin (α and β chain). The molecular mass of the *Phellinus noxius* hpF-17 laccase was estimated to be 75,000Da by SDS polyacrylamide gel electrophoresis (Fig. 8.12). The similar result was obtained for *P.ribis* laccase which was reported to have molecular mass of 76,000Da by Min et al. (2001).

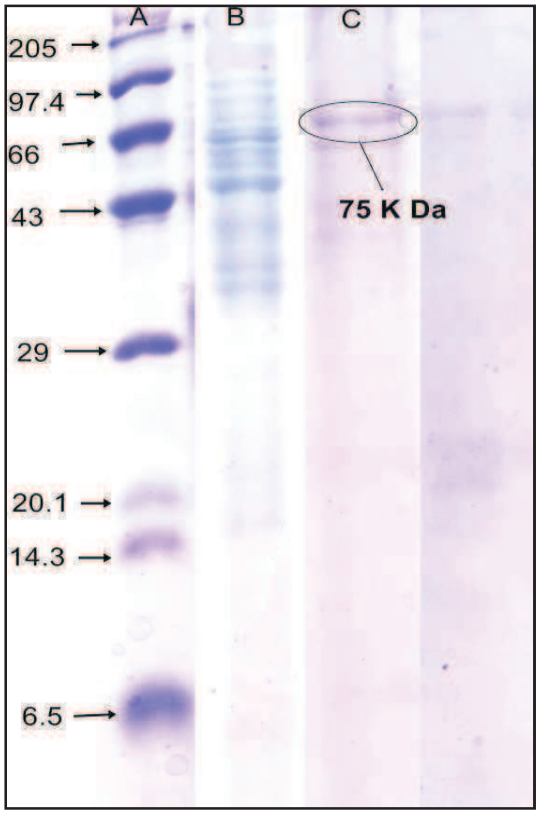

Fig. 8.12 SDS-PAGE of TPP purified laccase. Lane A: Molecular weight standard, lane B: Crude enzyme; Lane C Interfacial precipitate. Molecular weight markers contained the proteins myosin, rabbit muscle (205 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa) and aprotinin (6.5 kDa)

8.3.3.6 Laccase purity analysis using RP-HPLC

The purity of the laccase separation by TPP was checked by HPLC studies. The analysis was carried out in multistep gradient using buffer A (0.1% TFA in water) and buffer B (0.1% TFA in ACN) for 30 min at 25^{o}C with a flow rate of 1.0ml/min.. The absorption was monitored at 280nm.

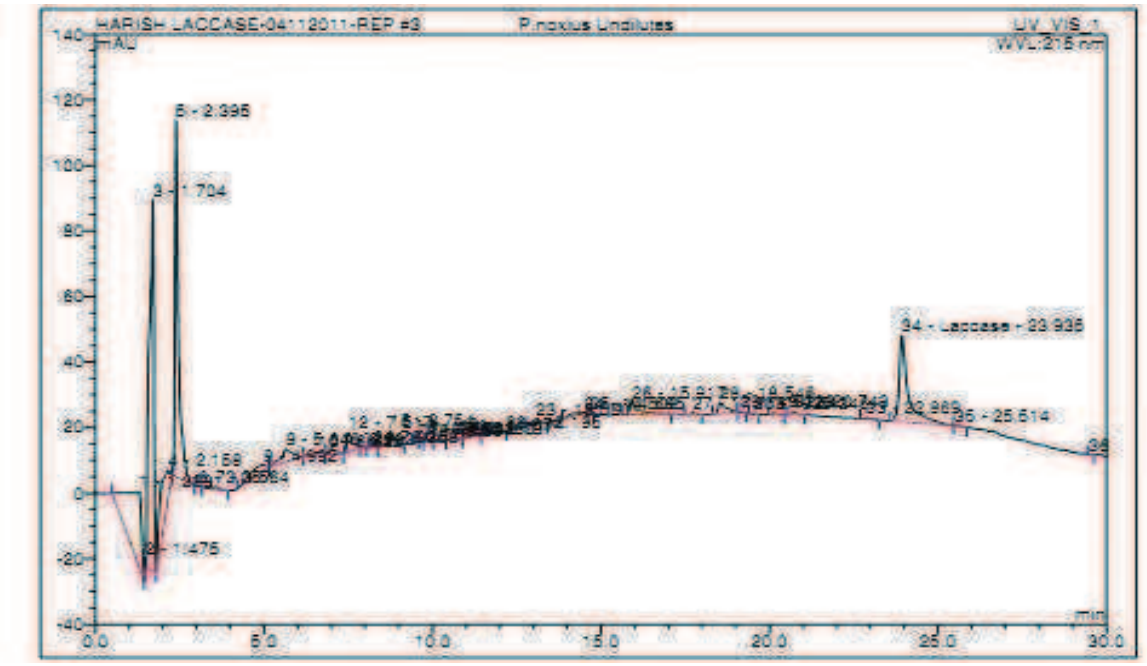

Fig. 8.13 RP-HPLC chromatogram of crude laccase produced by *Phellinus noxius* hpF-17

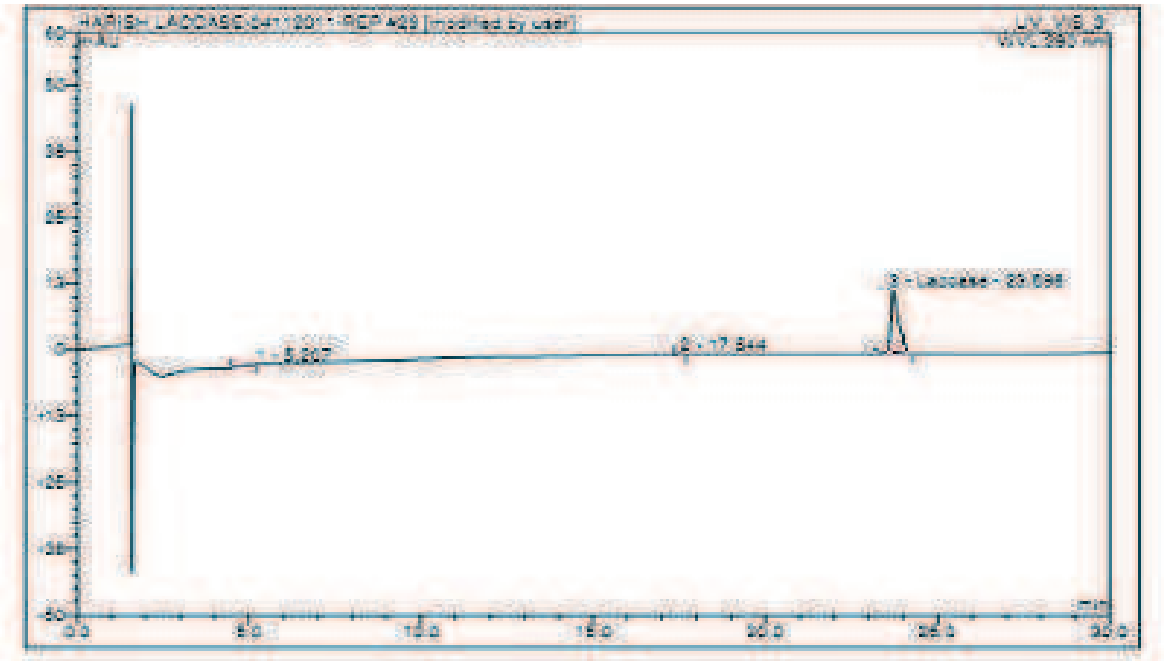

Fig. 8.14 RP-HPLC chromatogram of TPP purified laccase from *Phellinus noxius* hpF-17

The chromatographs of crude enzyme (Fig.8.13) and purified enzyme (Fig. 8.14) was obtained and compared. The crude enzyme chromatograph showed many minute peaks of unwanted proteins which were eliminated in the purified sample. A major peak at 23.57 min was confirmed as laccase peak which explains the purity of laccase separated by TPP.

8.3.2.7 Laccase spectroscopic studies and determination of copper and carbohydrate content

The purified enzyme lacks an absorption peak near 600 nm which indicates the absence of type I copper and exhibits a minor shoulder near 330 nm, perhaps responsible for the type-3 binuclear copper site (Fig. 8.14). However, metal content analysis revealed the presence of 1.44 µg of copper per mg enzyme, determined by atomic absorption spectrometry, which corresponds to 0.863(€1.0) copper atoms per molecule. Hence, only only one copper atom per protein molecule was estimated, whereas two copper ions Cu (II) are necessary for the type-3 binuclear copper center. The protein may contain additionally two other atoms like zinc or manganese or iron that may act in place of a copper ion at the catalytic site. Similar findings were reported for the laccase from *Phellinus ribis* by Min et al. (2001), Palmieri et al. (1992) for *Pleurotus ostreatus* white laccase and Pozdnyakova et al. (2006) for yellow laccase from *P. ostreatus* D1. The yellow laccase from *Peniophora sp*. hpF-04 was also found to have similar characteristics.

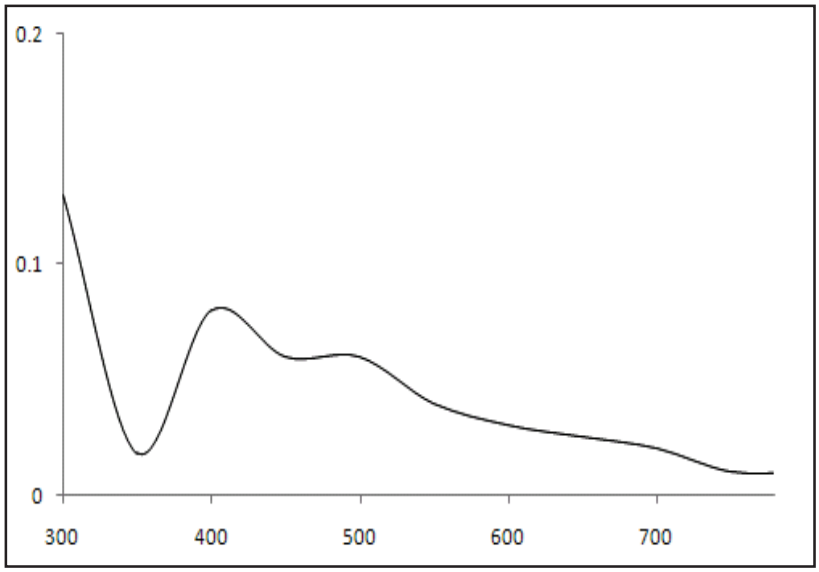

Fig. 8.14 UV–visible absorption spectrum of the TPP purified laccase. The assay contained 100 µg/ml laccase in acetate buffer

The measured copper content of the purified laccases varies between four and about two atoms per enzyme molecule (subunit). There are two reasons why the lower values must be regarded with caution. Purified preparations of some fungal laccases include a fraction of molecules that are demonstrably damaged and therefore, unlikely to retain their full complement of copper. Secondly, as discussed by Reinhammar and Malmstrom (1981), although the copper ions in these proteins are regarded as firmly bound, selective depletion of one copper centre (type 2) can be achieved experimentally and this might occur during purification.

In the present study, the *Phellinus noxius* hpF-17 laccase was found to be highly glycosylated as it was estimated to contain 0.27mg of carbohydrate per mg of protein (26.5%). The carbohydrate content of fungal laccases usually ranges between 10 and 25% (Solomon et al. 1996). However, the laccase from *Phellinus ribis* was reported to glycosylated up to 28% (Min et al. 2001) and laccase from *Botrytis cinerea* was glycosylated up to 49% (Slomczynski et al. 1995). This high glycosylation content may aid for the thermal stability of the enzyme. Glycosylations are suggested to have a

general non-specific effect on protein properties such as stabilization of conformation, sensitivity to proteases and secretion of proteins (Wang et al. 1996). The high glycosylation rate (49%) of *B. cinerea* laccase has been proposed to protect the enzyme from heat denaturation (Slomczynski et al. 1995). Accordingly, differences within thermal stability of *Ceriporiopsis subvermispora* laccases was suggested to be due to carbohydrate content (Fukushima and Kirk 1995). However, several studies have indicated that thermal stability of laccases is not solely explained by glycosylation stage (Li et al. 1999; De Souza and Peralta 2003; Koschorreck et al. 2008).

To sum up, in the present study, a 75KDa laccase monomer was separated and purified successfully by optimized three phase partition method. Isolated laccase does not show any blue colouration which was confirmed by the lack of absorption peak at 610 nm under spectral scan. The laccase showed high stability at acidic pH and elevated temperatures. The enzyme has thermotolerance (transient ability to maintain catalytic activity at elevated temperatures) or thermostability (ability to resist irreversible inactivation at high temperatures for a prolonged period of time) properties which is one of the general prerequisites for an enzyme to be applicable in industrial processes. The catalytic properties of the laccase are similar to the corresponding enzymes of other related group. The longer stability and higher temperature and acidic pH, lack of inhibition makes this enzyme suitable for industrial purpose/or bioremediation processes. Further studies on the structural and catalytic properties of the enzyme would be necessary for elucidation of the novelty of *Phellinus noxius* hpF-17 laccase.

Chapter 9

SUMMARY AND CONCLUSIONS

The findings of the present research is summarized as follows

- The present study reports the isolation and screening of autochthonal fungal stains of basidiomycetous fungi from village forests of Mangalore region of Karnataka, India.

- Pure cultures of 45 indigenous fungal strains were isolated and studied for their ligninolytic activities. Among them, 12 strains exhibited azure-B dye decolourization and 24 strains showed the oxidation of guaiacol on solid agar plates within 20 days of incubation period.

- Based on their rate of decolourization of dye and guaiacol oxidation, 12 efficient azure-B decolourizers and 5 guaiacol oxidation hyperproducers were selected.

- The selected strains were cultivated as agitated cultures on LMM and SMM media for the production of laccase and peroxidase enzymes, respectively. Only six out of 17 strains were able to produce significant amounts of laccase, MnP and LiP in liquid agitated cultures. The isolates identified as *Peniophora sp., Polyporous badius, Pleurotus sp., Phellinus sp., Panus sp. and Trametes sp.* showed significant production of LME's.

- The strain *Tramatus sp*. hpF-35 produced highest amount of MnP, in which production of significant amount of LiP and laccase was also observed. The strain hpF-33 *Panus sp.* produced the highest amount of LiP. Traces of MnP could be detected in this strain but no laccase production was observed.

- The highest level of laccase production was observed in the strain *Peniophora sp.* hp-F04, which also produced MnP but no LiP. The strain *Phellinus sp.* hpF-17 also showed significant laccase activity and traces of lignin peroxidase activity but no manganese peroxidase activity.

- The screening study resulted only in efficient laccase producers and no significant LiP and MnP producers were found. Hence, the study was continued with the aim

of laccase production, medium optimization, its purification and characterization from the selected fungi. Two novel isolates *Peniophora sp*.hpF-04 (DDBJ Accession number AB639021) and *P.noxius* hpF-17(DDBJ Accession number AB639022) were selected for this study.

- The time course production of laccase in basal medium showed similar enzyme production curves in both fungi. They showed maximum laccase activities and biomasses within 6-8 days of incubation period. *Peniophora sp* hpF-04 showed laccase activity of 1051U/l and *P.noxius* hpF-17 showed laccase activities of 545U/l on 6^{th} day of incubation period respectively.

- Results from the effect of temperature showed that, as the incubation temperature increased fungal mycelial biomass yield and laccase activity decreased. Temperature of 30°C was observed to be optimum incubation temperature for laccase production in *Peniophora sp*.hpF-04 (1100.5 U/l) and *Phellinus noxius* hpF-17 (658.9 U/l).

- The initial medium pH 6.0, prior to sterilization was found to be suitable medium pH for the production of laccase in these fungi.

- Experiments on effect of different carbon and nitrogen sources showed that the presence of dissimilar carbon and nitrogen sources altered fungal laccase production. The fungus *Peniophora sp.*hp-F04 showed maximum laccase activity in the medium containing sucrose (1249 U/l). Presence of CMC as a carbon source also showed laccase activity of 1060 U/l in this culture. Glucose and cellobiose showed almost similar laccase activities of 735 U/l and 697 U/l, respectively. In starch and xylan, the activity was reduced to 32.5 U/l and 34.6 U/l, respectively.

- On the other hand, in *P.noxius* hpF-17, maximum laccase activity was found in glucose containing media (557 U/l). Detectable levels of activities were observed in case sucrose (53.8 U/l) and cellobiose (36.90U/l) supplemented media. In case

of starch, barely measureable laccase activity could be detected (1.95 U/l). However, in the case of complex carbon sources like CMC and xylan, fungi grew but no laccase activities were detected.

- *Peniophora sp.* hpF-04 showed maximum laccase activity in the presence of ammonium chloride (1082.1 U/l). Detectable amounts of laccase activities could be detected in the presence of organic nitrogen sources. But *Phellinus noxius* hpF-17 showed highest laccase activity in the presence of ammonium tartarate (557 U/l). Low level of enzyme activities were found in media containing organic nitrogen sources such as yeast extract, aspargine and urea.

- Inducers like veratryl alcohol, guaiacol and 2,5-xylidine suppressed the production of laccase in both the fungi. But on increasing the concentrations of copper in the media, there was an increase in the production of laccase in *Peniophora sp.* hpF-04 as well as in *Phellinus noxius* hpF-17.

- The optimization of medium components for enhancement of laccase production by these fungi was carried out by statistical methods using Placket-Burman Design (PBD) and Central Composite Design of Response Surface Methodology (CCD-RSM). Initial screening of medium components was performed using a PBD and the variables with statistically significant effects on laccase production were identified. The interactions among the screened variables were studied by CCD.

- Variables such as carboxy methyl cellulose, ammonium chloride and copper sulfate were found to influence the laccase production significantly in *Peniophora sp.* hpF-04. Optimum values of tested variables for maximum laccase production were found to be; carboxy methyl cellulose (15 g/l), ammonium chloride (1.35 g/l) and copper sulphate (807 μM) by RSM. By using this optimal fermentation medium, the laccase yield increased up to 1900 U/l, an approximate 1.81 fold

improvement was achieved over the previous yield (1051 U/l) with un-optimized medium.

- Conversely, variables such as glucose, ammonium tartarate and Tween 80 were found to influence laccase production significantly in *Phellinus noxius* hpF-17. Optimum values of tested variables for maximum laccase production such as glucose (20 g/l), ammonium tartarate (2.25 g/l) and Tween 80 (2.08 ml/l) were obtained by RSM. By using this optimal fermentation medium, the laccase yield was increased up to 780 U/l, an approximate 1.43 improvement in laccase production as compared to the previous yield with un-optimized medium.

- Present investigation also aimed to exploit locally available, inexpensive agro-industrial wastes as substrate for laccase production under solid State fermentations (SSF). Seven residues *viz;* sugar cane bagasse (SCB), wheat bran (WB), rice bran (RB), corn stover (CS), saw dust (SD), grass powder (GP) and Jatropa seed cake (JSC) were evaluated as potential solid state substrates for laccase production by *Peniophora sp.* hpF-04 and *Phellinus noxius* hpF-17.

- In *Peniophora sp.* hpF-04, no laccase activities (very negligible lac activities in Sugar Cane Bagasse and Rice Bran) were found during SSF without nutrient supplements in any of the substrates on 5^{th} day. On 10^{th} day, increase in laccase activities were observed in all the substrates and on further incubation, i.e on 15^{th} day, the laccase production gradually reduced in many substrates. Among all, a high level of laccase activity was found in Jatropa seed cake. Contrastingly, detectable amounts of laccase activities were found in all the substrates of SSF samples when fermentation was carried out with the supplements of nutrients in 5^{th} day. Furthermore, maximum levels of laccase production were observed in 5^{th} day samples in all the substrates which were later reduced on 10^{th} and 15^{th} day samples, being highest in Jatropa seed cake.

- Similar results were obtained in SSF using *Phellinus noxius* hpF-17 also. Highest laccase production was observed in JSC, in both cases of with and without nutrients supplements. In the absence of nutrients, laccase productions of 19.6, 79.92 and 67.82U/g were observed, respectively during 5^{th}, 10^{th} and 15^{th} day sample. In the presence of medium nutrients, the fungus showed laccase productions of 24.02, 128.5 and 105.32 U/g, respectively on 5^{th}, 10^{th} and 15^{th} day samples. With the supplements of nutrients, on day 5, sugar cane bagasse produced laccase activity of 1.45 U/g of the substrate; wheat bran showed laccase of 49.8 U/g, saw dust 75.53 U/g, grass powder 94.58 U/g and rice bran showed laccase activity of 54.88 U/g.

- Among the seven agro-industrial wastes employed for SSF, only JSC was found to be potential subdtrate for laccase production in both fungi without the nutrient supplements. Hence, a detailed study of SSF with JSC was carried out to study time course production profile, effect of pH, effect of moisture on SSF on laccase production.

- Among the two fungal startins, *Phellinus noxius* hpF-17 produced laccase in higher titre than the *Peniophora sp.* hpF-04. It showed laccase activity of 79.92 U/g on 10^{th} day while *Peniophora sp*. hpF-04 showed laccase activity of 14.32 U/g on the same day. *Phellinus noxius* hpF-17 produced maximum laccase activity at 70% moisture level where as *Peniophora* sp. hpF-04 showed its maximum laccase at 80% of moisture level. Fungus *Phellinus noxius* hpF-17 produced maximum laccase at pH 5.0 (*i.e.*83 U/l) whereas *Peniophora sp*. hpF-04 produced maximum laccase within pH range of 5-6.

- Purification and characterizations of laccases produced by *Peniophora sp.* hpF-04 and *Phellinus noxius* hpF-17 was also investigated in this research. The purification was achieved by simple methods such as a combination of ultrafiltration and three phase partitioning (TPP). A single step purification of TPP was carried out to purify laccase produced by *Peniophora sp.* hpF-04.

However, TPP parameters had to be optimized for *Phellinus noxius* hpF-17 laccase separation due to its low recovery in single step process.

- Concentration by ultrafiltration achieved 94.41% yield of laccase with a 2.28-fold purification in *Peniophora sp.* hpF-04.

- By single step TPP process, crude enzyme solution of pH 4.0 saturated to 55% (w/v) ammonium sulphate with a crude extract to *tert*-butanol ratio of 1:1.5 (v/v) at 45°C resulted in 96.4% recovery of laccase with 2.61fold purification. The HPLC analysis resulted in a single peak in TPP purified sample which revealed the purity of the separation.

- Optimum pH and temperature for maximum activity were determined as pH 4.0 and 45-50°C for *Peniophora sp.* hpF-04 laccase. The kinetic constants (V_{max} and K_m) of this laccase was measured using Lineweaver–Burk double reciprocal (1/[v] vs 1/[S]) plots, the values were 30.3 and 0.06 for ABTS, 8.77 and 0.27 for 2, 4-dimethoxy phenol as well as 1.49, 0.28 for guaiacol, respectively.

- SDS-PAGE profiling revealed a single band of laccase with molecular weight of 67KDa. The laccase contained 3.12±0.2 Cu with an atypical spectrum lacking peak around 600 nm.

- The *Peniophora sp.* hpF-04 laccase was completely inhibited by β-mercaptoethanol, oxalic acid. Sodium azide also showed around 98% of reduction in laccase activity. Interestingly, compounds like TEMED, EDTA and SDS, which were proved to be inhibitors of enzymes of different types, did not show any inhibition of laccase activity in case of *Peniophora sp.* hpF-04. Inhibition by other compounds used in the study was also insignificant.

- Concnetration by TFF process resulted in 64% laccase yield with purity fold of 1.58 in *Phellinus noxius* hpF-17 laccase.

- BBD was employed with experimental factors; ammonium sulphate saturation; 20-80% (w/v), ratio of crude extract to *tert*-butanol; 1.0:0.5 – 1.0:2.5 (v/v) and temperature; 20–60°C. A combination of 57% (w/v) of ammonium sulphate with 1:1.7 (v/v) ratio of crude extract to *tert*-butanol at pH 5.0 and incubation temperature of 44°C was found to be good for the maximum recovery of *Phellinus noxius* laccase.

- Isolated laccase did not show any blue colouration which was confirmed by the lack of absorption peak at 610 nm under spectral scan. This laccase found to be larger than the *Peniophora* laccase with molecular weight of 75KDa as determined by SDS-PAGE analysis.

- The laccase showed high stability at acidic pH and elevated temperatures. The optimum pH of purified laccase produced by *Phellinus noxius* hpF-17 was found to be 3.0 for ABTS oxidation. When purified laccase was incubated for 20h at room temperature at optimum pH opf 3.0, it remained quiet stable.

- The laccase was found to be highly thermostable with broad temperature range of 65-85^{o}C with an optimum temperature of 75^{o}C. At 75^{o}C enzyme was very stable even after 72 h of incubation. But at 85^{o}C, the enzyme retained only 4% of its original activity after 24 h of incubation.

- The catalytic properties of the laccase are similar to the corresponding enzymes of other related group. Like *Peniophora* laccase, this enzyme also showed high affinity towards ABTS substrates.

- Compounds like β-mercaptoethanol and TEMED, sodium azide very effective in reducing the laccase activity. In case of CuCl, 8 fold increase in laccase activity was observed.

Conclusions

1. The present investigation reports the new indigenous fungal species capable of producing ligninolytic enzymes. Two fungal strains, *Peniophora sp*. hpF-04 and *Phellinus noxius* hpF-17 are reported for the first time as the efficient producers of laccase.

2. The high production of laccase during SSF of Jatropa seed cake would prove a new approach of utilizing the seed cake as fermentation feed stock for the production of laccase enzyme.

3. The use of Jatropa seed cake as a substrate not only solves the problem of seed cake discharge but also forms a cheap and economical method of producing laccase by these fungi.

4. The laccase produced by submerged fermentation was successfully separated and purified by a simple and economical three phase artitioning (TPP) method. This would be the first report on purification of thermostable laccase from *Peniophora sp*. and *Phellinus sp*. by three phase partitioning (TPP).

5. Due to the atypical characteristics of these laccases, both the fungal species could be listed into recently classified groups of fungi producing yellow or white laccases.

6. The laccases produced by both isolates were found to be acid stable and thermostable, unlike most of the other fungal enzymes.

7. The longer stability and higher temperature and acidic pH, lack of inhibition makes these enzyme suitable for industrial purpose/or bioremediation practices dealing with harsh process.

8. Further studies on the structural and catalytic properties of the enzyme would be necessary for elucidation of novelty of these laccases.

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APPENDIX

APPENDIX-I

MEDIA COMPOSITION

a. Malt extract agar-Isolation medium

Malt extract	20
Agar	20
Chloremphenicol	0.25

The components of the medium were dissolved in 1 litre of water and the pH of the medium was set to 6.0. The medium was sterilized by autoclaving at 15 psi pressure for 20 minutes at 121°C.

b. Laccase screening medium (LSM) (Viswanath et al. 2008)

Glucose	10.0
Peptone	3.0
KH_2PO_4	0.6
K_2HPO_4	0.001
$ZnSO_4$	0.4
$FeSO_4$	0.005
$MnSO_4$	0.05
$MgSO_4$	0.5
Agar	20.0
Guaiacol	0.2ml
pH	5.5-6.0

The components of the medium were dissolved in 1 litre of water and the pH of the medium was set to 5.5 to 6.0. The medium was sterilized by autoclaving at 15 psi pressure for 20 minutes at 121°C.

c. Ligninolytic basal medium (LBM) for screening LiP and MnP (Pointing 1991)

Yeast extract	1.0
$C_4H_{12}N_2O_6$	0.5
KH_2PO_4	0.01
$CuSO_4.5H_2O$	0.001
$MgSO_4.7H_20$	0.5
$Fe_2(SO_4)_3$	0.001
$CaCl_2.2H_2O$	0.01
$MnS0_4.H_2O$	0.001
Agar	20
Azure-B dye	0.1
pH	5.5-6.0

The components of the medium were dissolved in 1 litre of water and the pH of the medium was set to 5.5 to 6.0. The medium was sterilized by autoclaving at 15 psi pressure for 20 minutes at 121°C.

d. Laccase modifying media (LMM) - production medium for laccase

Glucose	10.0
Ammonium tartarate	2.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.5
Yeast extract	1.0
KCl	0.5
$CuSO_4.5H_2O$	150 µM
Trace elements*	10 ml
pH	5.5-6.0

***Composition (g/l):** EDTA (0.5), $FeSO_4$ (0.2), $ZnSO4.7H_2O$ (0.01), $MnCl_2.4H_2O$ (0.003), H_3BO_4 (0.03), $CoCl_2.6H_2O$ (0.02), $CuCl_2.2H_2O$ (0.001) and $Na_2MoO_4.2H_2O$ (0.003).

The components of the medium were dissolved in 1 litre of water and the pH of the medium was set to 5.5 to 6.0. The medium was sterilized by autoclaving at 15 psi pressure for 20 minutes at 121°C.

e. LiP and MnP production medium (SMM) (Dhouib et al. 2005)

Glycerol	10.0 ml
Ammonium tartarate	1.84
Sodium tartarate	2.3
Kh_2po_4	2.0
$Mgso_4.7H_2O$	0.7
$Cacl_2.2H_2O$	0.14
$Feso_4.7H_2O$	0.07
$Znso_4.7H_2O$	0.046

$Mnso_4.7H_2O$	0.035
$Cuso_4.5H_2O$	0.007
Thiamine	0.0025
Yeast extract	1.0
Veratryl alcohol	0.067
Tween 80	0.5ml
pH	5.5-6.0

The components of the medium were dissolved in 1 litre of water and the pH of the medium was set to 5.5 to 6.0. The medium was sterilized by autoclaving at 15 psi pressure for 20 minutes at 121°C.

f. Optimized medium for laccase production by *Peniophora sp.* hpF-04

CMC	15.0
Ammonium Chloride	1.35
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.5
Yeast extract	1.0
KCl	0.5
$CuSO_4.5H_2O$	807 µM
Trace elements*	10 ml
pH	6.0

g. Optimized medium for laccase production by *Phellinus noxius* hpF-04

Glucose	20.0
Ammonium Chloride	2.25
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.5
KCl	0.5
$CuSO_4.5H_2O$	150 µM
Urea	5.0
Tween 80	2.08 ml
Trace elements*	10 ml
pH	6.0

APPENDIX II

PREPARATION OF REAGENTS, STANDARDS AND BUFFER

a. Acetate Buffer (pH range 3-6)

Solutions required

i) 0.1 M acetic acid: Dissolved 5.8 ml of acetic acid in 1l of distilled water

ii) 0.1 M sodium acetate trihydrate: dissolved 13.6 g of sodium acetate trihydrate in 1l of distilled water

Mixed the following proportions to get the buffer of desired pH

pH	Vol. of 0.1M acetic acid (ml)	Vol. of 0.1 M sodium acetate (ml)
3	982.3	17.7
4	847.0	153.0
5	357.0	643.0
6	52.2	947.8

b. Phosphate Buffer (pH range 7-11)

Solutions required

i) 0.1 M disodium hydrogen phosphate: Dissolved 14.2 g of disodium hydrogen phosphate in 1l of distilled water

ii) 0.1 M Hydrochloric acid: dissolved 8.9 ml of conc. HCl in 1l of distilled water

iii) 0.1 M sodium hydroxide: dissolved 4.0 g NaOH in 1l of distilled water

Mixed the following proportions to get the buffer of desired pH

pH	Vol. of 0.1M Na_2HPO_4 (ml)	Vol. of 0.1 M HCl (ml)	Vol. of 0.1 M NaOH (ml)
7	756.0	244.0	--
8	955.1	44.9	--
9	955.0	45.0	--
10	966.4	--	33.6
11	965.3	--	34.7

c. Prpeparation of 50mM ABTS-2,2'-azino-di-(3 ethylbenzthiazoline sulfonic acid), Sigma A1888

Dissolved 274mg ABTS to 1000ml buffer to get the substrate solution of 50mM ABTS.

d. Prpeparation of 0.216 mM Syringaldazine ($C_{18}H_{20}N_2O_6$, Mol. Weight 360.36)

Dissolved 7.8 g of syringaldazine in 100ml of absolute methanol

e. Prpeparation of 1.0 mM 2,6-Dimethoxyphenol (C8H10O3, Mol. weight 154.16)

Dissolved 3.3 g of 2,6-Dimethoxyphenol in 100ml of distilled water.

f. Prpeparation of 1.0 mM o-dianisidine ($C_{14}H_{16}N_2O_2$, Mol. weight 244.293)

Dissolved 5.3 g of o-dianisidine in 100ml of distilled water

g. Preparation of 2mM guaiacol (CH_3O) C_6H_4OH, Mol. weight 124.14)

Mixed 5.4 ml of guaiacol with distilled water and made up to 100 ml

h. SDS reagents preparation

i) 10% resolving gel

1.5 ml 1.5 M Tris-HCl, pH 8.8

2 ml 30% acrylamide

0.06 ml 10% SDS

2.44 ml water

Added last to initiate reaction:

30 µl 10 % ammonium persulfate and 5 µl TEMED

ii) Stacking gel

0.875 ml 1.0 M Tris-HCl, pH 6.8

0.583 ml 30% acrylamide

0.035 ml 10% SDS

2.007 ml water

Added last to initiate reaction:

30 µl 10 % ammonium persulfate and 5 µl TEMED

iii) 5x Sample Buffer

60 mM Tris-HCl, pH 6.8

25% glycerol

2% SDS

14.4 mM b-mercaptoethanol

1% bromophenol blue

iv) Electrophoresis tank buffer

25 mM Tris

192 mM glycine, pH 8.8

0.1% SDS

v) Coomassie staining solution

10% Acetic acid,

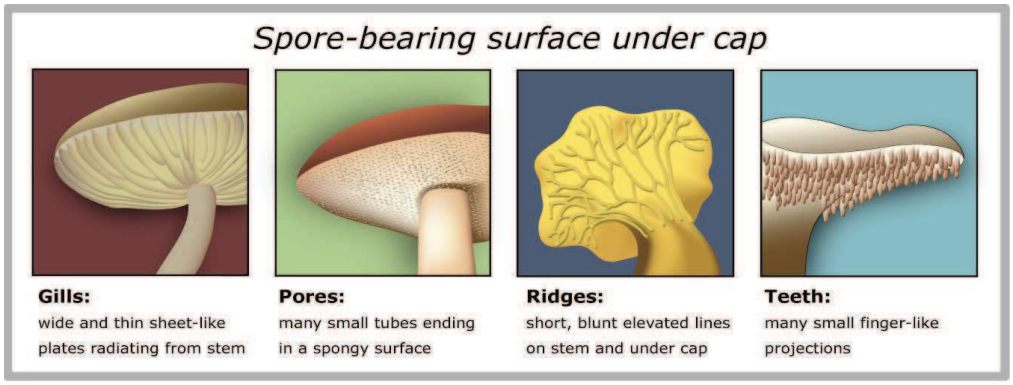
25% Methanol,

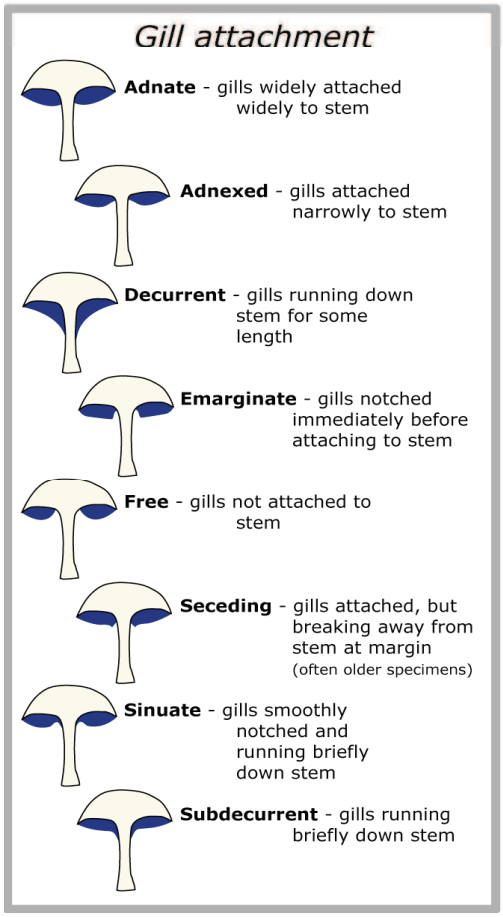
0.05% Coomassie R- 250 or Bio-Safe Coomassie staining solution

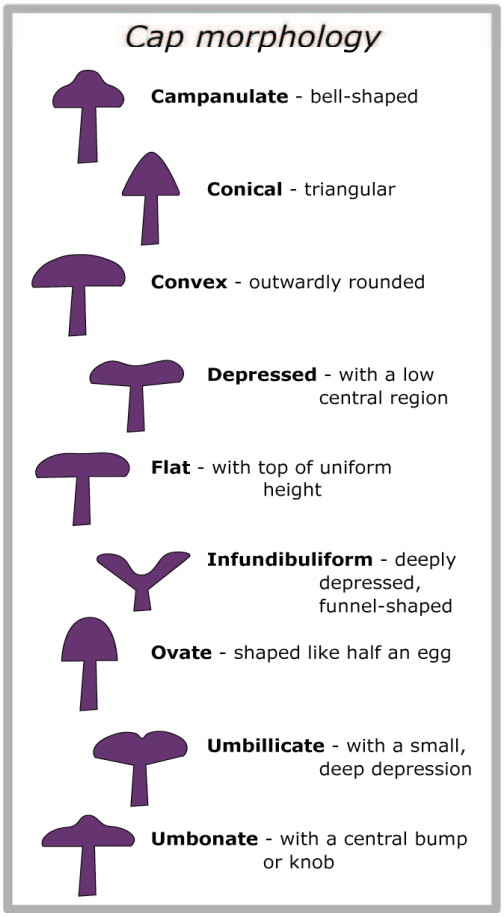
APPENDIX III

MUSHROOM IDENTIFICATION KEY

Figure below shows the key features for mushroom identification







APPENDIX IV

GLUCOSE ESTIMATION BY DNS METHOD

The glucose concentration was estimated by 3, 5-dinitrosalicylic acid (DNS) method using UV/VIS spectrophotometer.

Principle

3, 5-DNS (yellow colour) in alkaline solution is reduced to 3-amino, 5-nitro salicylic acid (orange-red).

Reagents

1. Dinitrosalicylic Acid Reagent (DNS Reagent): 1.0 g of dinitrosalicylic acid, 200 mg of crystalline phenol and 50 mg of sodium sulphite were dissolved in 100 ml of 1% NaOH. The reagent was stored at 4°C.

2. 40% Rochelle salt solution (Potassium sodium tartarate).

3. Glucose standard: Aliquots of solution ranging from 0-500 µg/ml were prepared.

Procedure

1. 0.1 ml of test samples were pipetted out into test tubes and the volume was made upto 3 ml with water in all the tubes.

2. 3.0 ml of DNS reagent was added and the contents were heated in a boiling water bath for 5 minutes.

4. When the contents of the tubes were warm, 1.0 ml of 40% Rochelle salt solution was added.

5. The test tubes were cooled and the intensity of dark red colour was read at 510 nm.

6. A calibration curve of concentration of glucose versus optical density was plotted. Concentration range of 0-500 µg/ml gave a linear curve as shown in Fig. 1.

Standards	Concnentartion of glucose (µg/ml)	Absirbance at 540 nm
STD 1	0	0
STD 2	200	0.194
STD 3	400	0.37
STD 4	600	0.58
STD 5	800	0.758
STD 6	1000	0.982

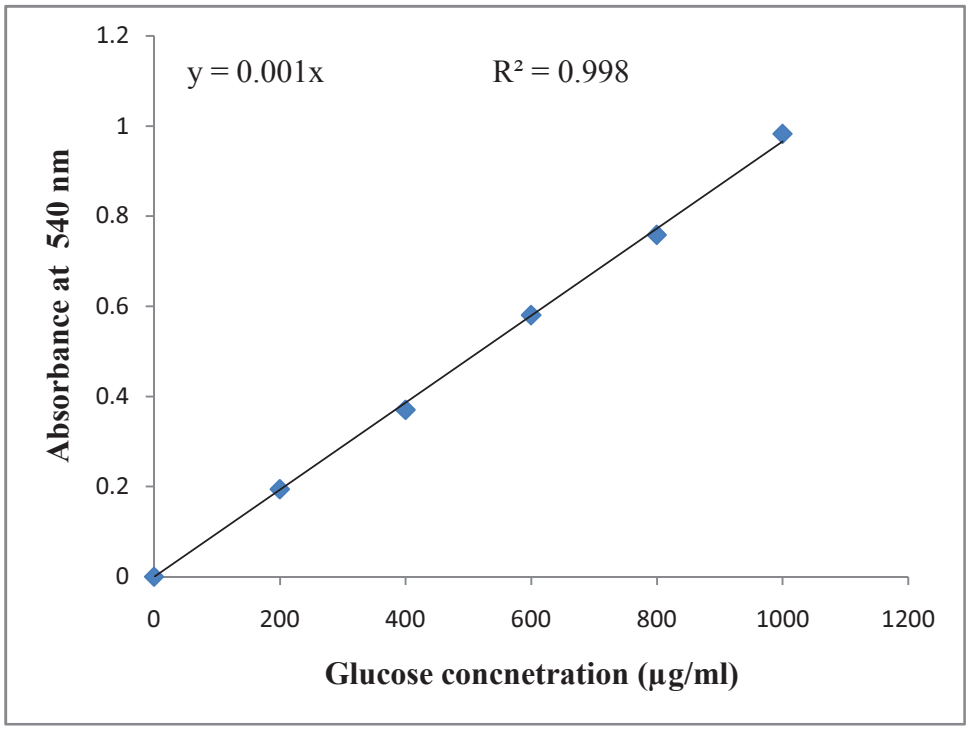


Fig. 1: Calibration curve for the DNS assay for the determination of glucose concentration expressed in µg/ml

APPENDIX V

PROTEIN ESTIMATION BY LOWRY'S METHOD

The concentration of proteins was determined by Lowry's method using UV/VIS spectrophotometer (Thimmaiah 1999).

Principle

The phenolic group of tyrosine and trytophan residues (amino acid) in a protein will produce a blue purple colour complex in an alkaline condition with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent.

Reagents

Reagent A - 2.0 g of sodium carbonate was dissolved in 100 ml of 0.1 N NaOH.

Reagent B - 0.5 g of copper sulphate was dissolved in 100 ml of freshly prepared sodium potassium tartarate (1%).

Reagent C - 50 ml of Reagent A was mixed with 1.0 ml of Reagent B to make the alkaline copper reagent at the time of use.

Reagent D - Folin-Ciocalteau reagent which is commercially available was diluted with water in 1 :1 ratio at the time of use.

Bovine serum albumin (BSA) standard: Aliquots of solution ranging from 0-200 µg/ml were prepared

Procedure

1. 0.5 ml of the test solutions were pipetted out into test tubes and made up to 1.0 ml with sterile distilled water.

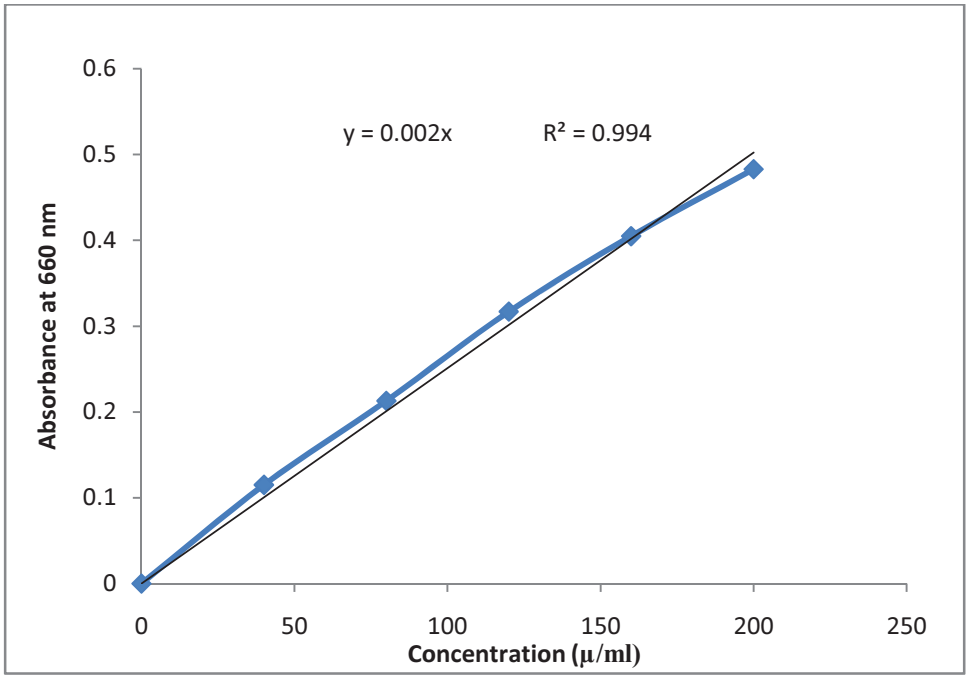
2. 5.0 ml of reagent C was added to all the aliquots, mixed well and incubated at room temperature for 10 minutes.

3. 0.5 ml of reagent D was added and incubated in dark for 20 minutes at room temperature.

4. The mixture was mixed well and O.D. was read at 660 nm in an UV/VIS spectrophotometer.

5. A calibration curve of optical density versus concentration of BSA was plotted. Concentration range 0-200 µg/ml gave a linear curve (Fig. 4).

Standards	Concentration of protein (mg/ml)	Absorbance at 660nm
STD 1	0	0
STD 2	40	0.115
STD 3	80	0.213
STD 4	120	0.317
STD 5	160	0.405
STD 6	200	0.483



APPENDIX VI

ESTIMATION OF TOTAL CARBOHYDRATES BY PHENOL-SULPHURIC ACID METHOD

The total carbohydrate concentration was estimated by phenol-sulphuric acid method using UV/VIS spectrophotometer.

Principle

In hot acidic medium, glucose is dehydrated to form hydroxymethyl furfural. This forms a green coloured product with phenol.

Reagents

5% Phenol: 5.0 g of phenol was dissolved in water and made upto 100 ml.

96% Sulphuric acid

Glucose standard: Aliquots of solution ranging from 0-200 µg/ml were prepared.

Procedure

1. 0.1 ml of the test solutions were pipetted out into test tubes and the volume in each tube was made up to 1 ml with water.

2. 1.0 ml of phenol solution was added to each tube.

3. 5.0 ml of 96% sulphuric acid was added to each tube.

4. After 10 minutes, the contents in the tubes were mixed and placed in a water bath at 25-30°C for 20 minutes.

5. The absorbance of the samples was read at 490 nm.

6. The amount of total carbohydrate present in the sample solution was calculated using the standard graph (as shown in Fig. 2).

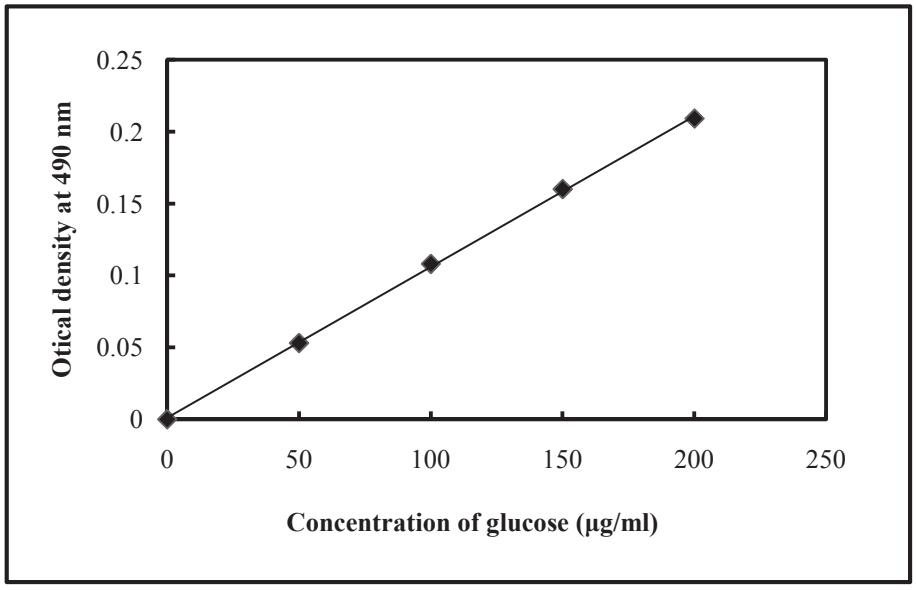


Fig. 2: Calibration curve for the phenol-sulphuric acid method for the determination of total carbohydrate concentration expressed in µg/ml

APPENDIX VII

DETERMINATION OF MOLECULAR WEIGHT OF ENZYME BY SDS-PAGE

Molecular Marker	Molecular wt of marker (kDa)	Migration (cms)	log (MW)	Rf
	solvent front	10		
Myosin Rabbit Muscle	205	0	2.31	0
Phosphorylase B	97.4	0.5	1.99	0.05
Bovine Serum Albumin	66	1.1	1.82	0.11
Ovalbumin	43	1.7	1.63	0.17
Carbonic hydrolase	29	3.5	1.46	0.35
Soyabean trypsin inhibitor	20.1	5.1	1.30	0.51
Lysozyme	14.3	5.8	1.16	0.58
Aprotinin	6.5	7.2	0.81	0.72
Insulin	3	10.8	0.48	1.08