Evaluation of Lignin Degrading Efficiency of Selected Fungal Strains Isolated from Pulp and Paper Industry Wastewater

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A thesis submitted in partial fulfillment of the requirements for the Degree of

Doctor of Philosophy

In

Microbiology

By

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Islamabad

2023

Dedicated

TO MY PARENTS

and my elder brother

Mutsil Munir

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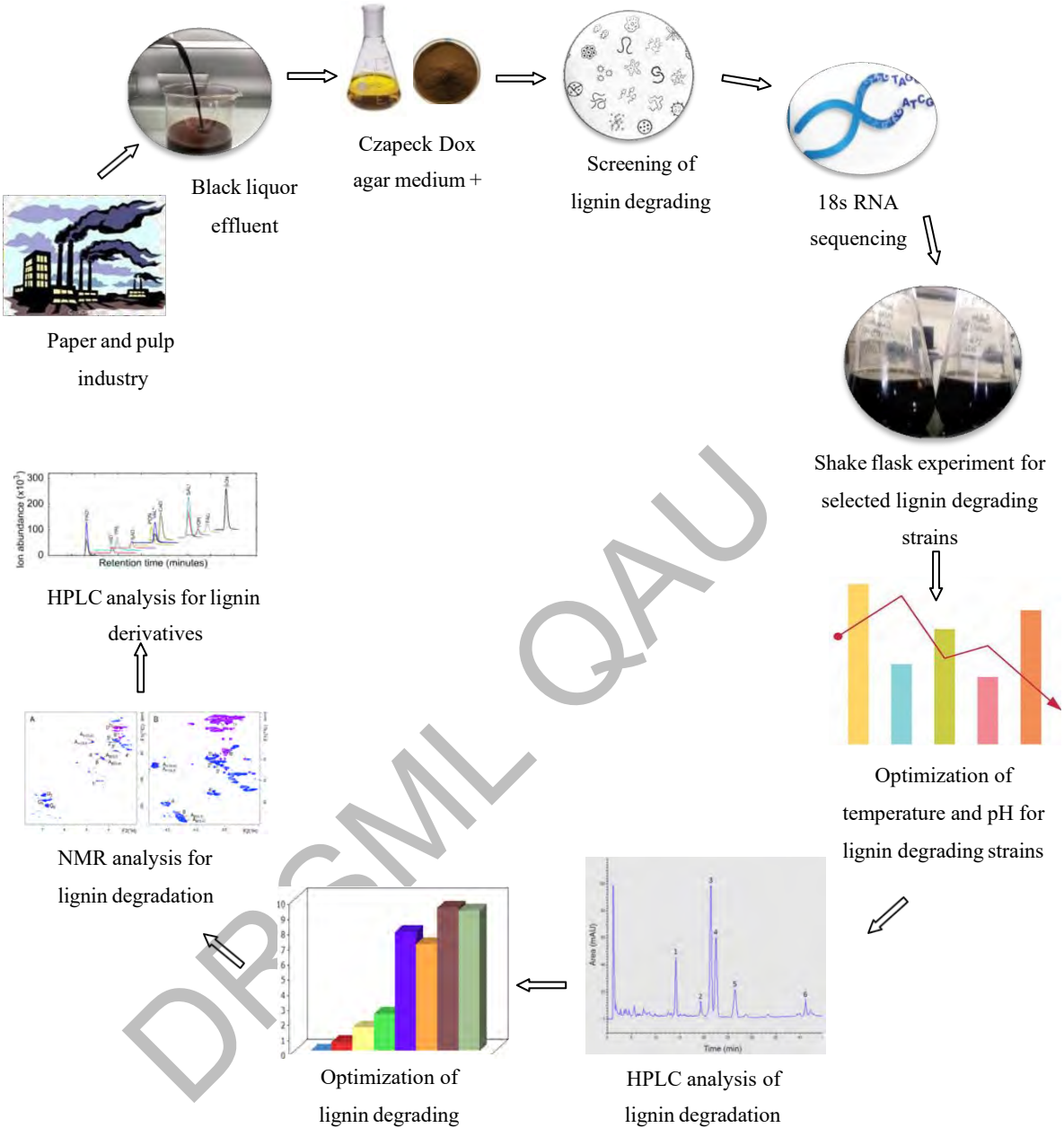
Graphical Abstract



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Abstract

Lignin is the second most abundant natural biopolymer on the land having complex and heterogenic chemical structures. In recent years, biodegradation of the lignin polymers and its transformation into value added products have been gaining ample scientific attraction. In present research four potent fungal strains were isolated and screened from pulp and paper industry for their possible role in biodegradation of commercial lignin and lignin in the industrial waste water. The enzymatic potential of the fungal isolates was also determined for the bio-depolymerisation of lignin and their suitability to produce peroxidises. During first phase of the research, black liquor (BL) was obtained from the pulp and paper mill effluent and its physiochemical analysis was carried out. In addition, BL was also used for the screening of lignin degrading fungi. Two step screening strategy was employed for the isolation and screening of the potent fungal strains having enzymatic capability to use and degrade lignin. For enrichment and screening of the fungi Czapek Dox agar media was made with the following composition was used (g/L); Sodium Nitrate; 2.0g/L, Dipotass ium Phosphate; 0.35g/L, Magnesium Sulfate; 0.5g/L, Potassium Chloride; 0.5g/L, Ferrous Sulfate; 0.01g/L, 200 mg/L lignin and 2% agar. Initially thirteen fungal strains showed rich growth on the aforementioned media and subjected for secondary screening in the liquid MSM using black liquor as only source of carbon. On the basis of higher growth, lignin degradation and colour reduction, four fungal strains were screened namely M-1, M-2, M-3 and M-4. These four fungal strains were then identified on the basis of 18s RNA gene sequencing as *Aspergillus terrus*M-1*, Dipodascus autraliensis*M-2*, Geotrichum candidum*M-3*, Aspergillus fumigatus* M-4*.* The lignin degradation efficiency was determined and it was revealed that the strain *Aspergillus terrus* M-1 caused biodegradation of lignin in the BL up to 73%, *Dipodascus autraliensis* M-2 84%*, Geotrichum candidum* M-3 84% and *Aspergillus fumigates* M-4 80%. The optimum reaction conditions for the maximum biodegradation of lignin by these strains were different. However, most of the fungi were able to perform best at 41 °C and pH above 7. The highest lignin degradation efficiency showed by stains M-2 and M-3 were recorded at 41°C and pH9; and 47°C and pH9 respectively. In the next phase, these fungi were evaluated for the biosynthesis of different peroxidises and laccases including Lignin peroxidase (LiP) and Managnese peroxidase (MnP) under submerged fermentation conditions. The

reaction parameters such as pH, temperatures, substrate concentration and different carbon sources were also checked for their effect on the fungal growth and enzyme production rates. The fungal strains showed varying metabolic efficiency under different reaction conditions. Maximum activity of laccase (139 U/mL) was observed from *Aspergillus terrus* M-1, whereas highest LiP and MnP were observed in case of *Geotrichum candidum* M-3, 115U/mL and 106 U/mL respectively. The other fungal strains produced relatively less amount of peroxidises and laccases as compared to the M-1 and M-3 strains. The molecular weights of the different peroxidise and laccases was determined for all four strains. The enzymes were extracted from the growing cultures of the M-1-M-4 strains and SDS Gel electrophoresis was performed. It was noted that the molecular weight of the lignin isoenzymes was found to be in the range of 35 kDa and for laccases it was in the range of 130 kDa.

The effect of different types and concentrations of lignin (insoluble and soluble) on the growth of fungal strains was also studied. Our results indicated that the fungal strains showed varying capacity to use lignin variants as sole source of carbon and energy. In case of avicel, the highest growth was observed by *Geotrichum candidum* M-3 *and Aspergillus fumigatus* M-4. Whereas strain *Aspergillus terrus* M-1 and *Dipodascus autralienses* M-2, showed comparatively less growth on the avicel. The growth pattern of the fungi on Mannans indicated that all strain has the metabolic capacity of using this carbon source efficiently except *Aspergillus terrus* M-1. Further, *Aspergillus terrus* M-1was also found less competent to use pectin, starch and xylan as compared to the other fungal strains. In the next phase the analysis of various degradation products of the lignin was evaluated using H-^{13}C HSQC Nuclear magnetic resonance. Different intermediate compounds were detected during lignin degradation by the action of our selected fungal strains. In case of *Aspergillus terrus* strains M-1 the chemical shift was observed at 3.5 to 4.0 ^{1}Hppm and the chemical shift at ^{13}C, ppm was around 57 to 60. These results depicted the methoxyl group formed in the result of lignin degradation. *Dipodascus australiensis* M-2 and *Geotrichum candidum* M-3 after degradation liberated xylan from the lignin-carbohydrate complex. *Dipodascus australiensis* M-2 showed the chemical shift of ^{1}H, ppm at 4 – 5.1 indicated lignin transformation into carbohydrates. The strain *Geotrichum candidum* M-3 were more active in metabolizing residual monomers such as vanillin, acetovanillone and coniferyl alcohols. In addition, these strains

significantly reduced the linkage content in the lignin fraction giving the chemical shift of ^{1}H ppm at 4.9 to 5.1 which represented the formation of carbohydrates, 6.2 to 6.7 exhibited the aromatic-H in syringyl and 6.7 to 7.1 aromatic-H guaiacyl. The chemical shift at ^{13}C ppm by this fungus provided the evidences for a LiPhatic and aromatic groups. Relatively, both of these strains M-2 and M-3 showed cleavage not only of *β*-aryl-ether (*β*-O-4), but also of resinol (*β*-*β*) and of the less prevalent phenylcoumaran (*β*-5) linkages for biodegradation of lignin. *Aspergillus fumigatus* strain M-4 had less pronounced activity than strains. This strain showed the chemical shift of methoxy and slightly for the aliphatic groups.

In the last phase of the research, the biodegradation of intermediate compounds detected by NMR results was studied. Reverse Phase High pressure liquid chromatography (RP- HPLC) was used to track down degradation of intermediate products. The guaiacol, hydroxybenzaldehyde, syringaldehyde and vaniline were used as substrate and after six days of experiments the results were analysed. HPLC analysis of control and fungal mediated degraded samples showed reduction and shifting of peaks were revealed for vanillin and hydroxybenzaldehyde for the strain *Geotrichum candidum* M-3 and *Aspergillus fumigatus* M-4. However, the fungal strains did not show any significant degradation of syringaldehyde and guaiacol since slight shift of peaks were obtained. On the basis of the results, it can be concluded that the fungi isolated and screened in the present study showed excellent metabolic efficacy to grow and degrade lignin both in the industrial waste water and pure lignin. Further, these strains produced various lignin degrading enzymes using lignin as sole source of carbon and energy. Therefore, the fungal strains *Aspergillus terreus, Dipodascus australiensis, Geotrichum candidum* and *Aspergillis fumigatus* can be used for the bioremediation of pulp and paper industrial waste and for the biotransformation of lignin containing waste into value added commercial products.

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List of Abbreviation

Abbreviation	Full form
LCB	Lignocellulosic biomass
KL	Kraft Lignin
MnP	Manganese Peroxidase
LiP	Lignin Peroxidase
VP	Versatile Peroxidases
COD	Chemical Oxygen Demand
BOD	Biological Oxygen Demand
LPF	Lignin modified Phenol Formaldehyde
H2SO4	Sulphuric acid
HCl	Hydrochloric acid
CBD	Cellulose Binding Domain
CipA	Cellulosome integrating protein A
TDS	Total Dissolve Solids
TSS	Total suspended solids
VS	Volatile solids
µ	Micro
Ml	Milliliter
Mg	Milligram
L	Liter
M	Molar
CTAB	Acetyl trimethyl ammonium bromide
SDS	Sodium dodecyl sulphate
HPLC	High performance liquid chromatography
LC	Liquid chromatography
NMR	Nuclear Magnetic Resonance
C-C bond	Carbon-Carbon bond
C-O bond	Carbon-Oxygen bond
H2O	Water
H_2O_2	Hydrogen Peroxide
kDa	Kilodalton
Cu	Copper

BL	Black Liquor
pI	Isoelectric point
Mn	Manganese
PAH	Polycyclic aromatic hydrocarbons
CO_2	Carbon dioxide
HBT	Hydroxybenzatriole
EBTS	Ethylbenzothiazoline-6-sulfonic acid
GC-MS	Gas chromatography- mass spectrometry
5C	Five carbon sugars
6C	Six carbon sugars
BaCl2	Barium Chloride
NaOH	Sodium Hydroxide
UV	Ultraviolet radiations
MgSO4	Magnesium sulphate
KCl	Potassium chloride
$FeSO_4$	Ferrous sulphate
$NaNO_3$	Sodium nitrate
KH_2PO_4	Potassium dihydrogen phosphate
mM	milliMolar
U/ml	Units per millilitre
Ppm	Parts per million
DMSO	Dimethyl sulphoxide
MHz	Mega hertz

1 Introduction

The pursuit of environmental and industrial sustainability has become the most important agenda of the current global policy. On the other hand, growing population and rapid industrialization have been associated with generation of huge amounts of liquid and solid wastes. In particular liquid industrial wastes are being directly added into the fresh water bodies without any prior treatment causing structural and functional changes in the ecosystem. Considering the eco-toxicity imposed by these chemicals, there is great push to develop innovative and cost affective waste management technologies in order to either degrade or transform these chemicals into value added products (Azubuike *et al*., 2022).

Lignin is naturally occurring complex aromatic polymeric compound with high abundance in the ecosystem (Wang *et al*., 2021). About 20 % of carbon fixed through the photosynthesis process and stored in plant biomass as lignin making it abundantly available biochemical after polymeric cellulose (Parakh *et al*., 2020). Complexity and recalcitrance of the lignin molecules is basically associated with its physiological function of plant protection against the hydrolytic attack by plant pathogens and saprophytic microorganisms (Shu *et al*., 2021). These features make lignin highly recalcitrant natural compound towards chemical and biological degradation. On the other hand, lignin degrading microbes are continued to evolve in terms of their lignin metabolism abilities (Kamimura *et al*., 2019). These microorganisms are playing a vital role in biological processes i.e., carbon cycle by degrading complex lignin molecules into relatively simpler compounds enabling their subsequent utilization by the other microorganisms (Xu *et al*., 2019). The woody biomass is not the only source of the lignin in the ecosystem. Industrial processes such as pulp and paper industry also produces huge amount of highly contaminated lignin containing waste water (black liquor) which is considered as a serious environmental pollutant (Baghel *et al*., 2020). The kraft lignin, a by-product, is formed during alkaline sulphide pre-treatment of LCB in pulp and paper mills. It different from lignin synthesized in industries due to number of reactions including cleavage of oxygen bond in aryl-alkyl ether, side chain modifications, and a variety of reactions that cause the polymer disintegrate into shorter alkali soluble components. This kraft lignin effluents from paper and pulp mill is the cause of serious environmental deterioration with intense colouring for water

bodies that block the sun light pass (Haq & Raj, 2020) to the depths of water bodies and effect photosynthesis, resulting in anaerobic conditions that severely effects the marine life, causing bad odour and results in increase in toxicity of aquatic systems. Lignin gives dark brownish colour and cause harmful effect by shifting the water pH and can cause rise in BOD and COD in water thus seriously affecting the ecosystem (Zainith *et al*., 2019). Therefore, before sewage of these industries release their sewage into water bodies, a sequential process is required to treat this water to save the aquatic system and reduce the contamination of water(Almuktar *et al*., 2018). Industrial removal of lignin involved certain physicals and chemical are energy intensive and associated with other harmful environmental effects like greenhouse gas emissions (Okolie *et al*., 2021). Therefore, biodegradation of lignin through microbial means has been gaining significant interests because of two particular reasons;

a) Maintenance of the biogeochemical cycle and ecological sustainability and,
b) Transformation of lingo-cellulosic waste into value added by products for the industrial applications such as polyhydroxy alkanoates, vanillin and muconic acid.

Several microorganisms are widely used for lignin degradation and related substrates while white rot fungi has been proves as the powerful cellular resource for de-polymerisation of lignin (Grosse, 2018).They are capable of mineralising lignin components to CO_2 and H_2O while using them as their growth substrate under ambient conditions. Degradation of wood from ascomycota species was studied in detail and designated as “soft rot” by Savory (1954). Nilsson *et al*. (1989) confirmed that, a numerous higher ascomycetes’ species, chiefly *Daldinia concentrica* that degraded Aspen wood up to the same extent as *Trametes versicolour*, a basidiomycete in general classified as white rot fungus (Schilling *et al*., 2020). *Chrysonilia sitophila,* an ascomycete, produces number of cellulolytic and ligninolytic enzymes and could degrade *Pinus radiata* bark products and rice hull (Ferraz *et al*., 2000; Sharma & Aggarwal, 2020).

Chemically lignin has a complex molecular structure and poses challenges for their biodegradation both in natural and industrial wastes (Vashi *et al*., 2018). The first molecular information of the lignin model structure was revealed in 1970s by Nimz, list1974; Adler, 1977. However, with the advancement of new analytical techniques,

new substructures of the lignin are continued to emerge describing the heterogeneity and natural complexity of the lignin (del Río *et al.,* 2007). The biodegradation of the lignin, both synthetic and model compounds, has been studied in order to investigate the underlying mechanism of the microbial degradation process using white-rot basidiomycete (Eriksson *et al.,* 1990). The initial studies indicated that a diverse group of enzymes are required for degradation of different lignin substructures that release varying breakdown products (Chen and Chang, 1985; Kirk and Farrell, 1987). However, these studies are not conclusive to understand the exact role of lignin degradation enzymes. Lignin structure represents a dimeric model compound and has been extensively studied for biodegradation studies. Lignin is phenolic heteropolymer compound made up of hydroxycinnamyl alcohol through radical coupling. The C-C and C-O-C linking bonds in phenylpropane units gives resistance to microbial degradation (Meenakshisundaram *et al*., 2022). Because of the extremely complex chemical structure, it is largely established that irregular biosynthesis pathways lead to formation of lignin constructed via three fundamental phenylpropanoid monomer, guaiacyl (G), p-hydroxyphenyl (H), and syringyl (S) units which are originated from coniferyl, p-coumaryl, and sinapyl alcoholic precursors, respectively (Perkins *et al*., 2019). The essential components are linked with β-aryl ether linkages. The significance of model compounds to demonstrate polymeric lignin degradation has been evaluated and criticized as these model compounds are water soluble and known to be broken down by intracellular substrate specific enzymes that are contradictory to the enzymes which depolymerize water insoluble lignin polymer (Giummarella et al., 2019) . However, presence of both soluble and insoluble lignin substrates particularly in the industrial waste water need further research for their removal from the environment (Singh, Bilal, Iqbal, Meyer, *et al*., 2021). On the other side, lignin transformation into value added compounds face many challenges, foremost of which is bond cleavage. The C-C or C-O ether linkages between aryl-C_3 units in polymeric lignin are not easily susceptible to hydrolytic cleavage (Darie-Nita et al., 2022), so unusual enzymatic chemistry is required for its breakdown. Another challenge is the insolubility of organosol lignin in H_2O or diverse range of organic solvents. Lignosulfonates and kraft lignin have high water solubility and produce dark brown colour in the freshwater-wastewater (Araújo et al., 2018). The hydrophobic characteristics of lignin polymers results in paralysis of the catalytic machinery of the biodegrading microorganisms and becomes bottleneck of the process (Singh *et al*.,

2020). The chemical heterogeneity, irregular structures and generation of low molecular weight intermediates are few other constraints associated with the lignin biodegradation (Agarwal et al., 2018).

Biodegradation of the lignin has been investigated in various members of white rot fungi owing to their metabolic capacity of producing different extracellular catalytic enzymes including manganese peroxidase, lignin peroxidase and laccases. Over the last three decades extensive research has been performed on lignin degrading fungi and their secretory enzymes. The *Phanerochaete chrysosporium* was reported for producing lignin depolymerizing enzymes particularly lignin peroxidase (LiP) (Sadaqat *et al*., 2020). This was the first fungi belongs to basidiomycete subjected for whole genome sequencing owing to the interest in microbial degradation of lignin and related compounds (Martínez *et al*., 2004). Later on, versatile peroxidases (VP) were added into the list of lignin degrading enzymes identified from *Pleurotus* and *Bjerkandera* species (Martínez *et al*., 1994). The LiP is an extracellular enzyme which mainly depends on the H_2O_2 which contains heme group in glycoprotein, produced by white rot fungi. The redox potential of LiP is 700–1400 mV, pH 3.0 to 4.5 having the potential to degrade substrates like veratryl alcohol, methoxy benzenes and non-phenolic lignin compounds and some essential organic compounds (Singh, Bilal, Iqbal, & Raj, 2021). It catalyzes the oxidative cleavage of β-O-4 linkages, Cα–Cβ linkages, and other complex linkages present in lignin and its model compounds. This extracellular enzyme catalyzes the benzyl alcohol oxidations, side-chain cleavages, demethoxylation, oxidative de-chlorination and ring-opening reactions (Liu *et al*., 2019). It degrades broad spectrum of lignin derivatives including catechol, vanillyl alcohol, syringic acid and guaiacol comparably faster than non-phenolic substrates. LiP is a glycoprotein of 38 to 46 kDa catalyzes the H_2O_2 dependent oxidative degradation of lignin. Catalytic cycle of LiP includes oxidation of ferric enzyme by hydrogen peroxide to produce the two-electron oxidized intermediate, LiP-I (Mousavi *et al*., 2021).

Other important class of the enzyme that is responsible for the depolymerization of the lignin and complex biopolymers is microbial laccases (Curran *et al*., 2021). They are copper-containing polyphenol oxidases and are extensively distributed in microorganisms. Aromatic compounds are generally oxidized by this enzyme with the

help of electron donating groups like anilines (NH_2) and phenols (OH) and oxygen as an electron acceptor through a mechanism involving radicals (Kulikova & Perminova, 2021). These radicals make laccases capable to catalyzed reaction as well as non-enzymatic reaction including polymerization. Thus, laccases have the tendency to oxidize wide range of substrates including both phenolic and non-phenolic compounds. The average molecular weight of a laccase enzyme is 50–97 kDa. Laccases consist of four copper (Cu) atoms in their active sites that contribute in production of water molecules by reduction of molecular oxygen (Arregui *et al*., 2019). The lignin structure is transformed by laccase enzyme through Cα– Cβ and aryl cleavages. Consequently, phenoxy radicals degrade the lignin by causing oxidation of the α-carbon or by the break the bond between the α and β-carbon atoms. Laccase leads to the formation of numerous phenoxy radicals by the catalyzing the one electron substraction from phenolic hydroxyl groups of phenolic lignin compounds, such as syringaldehyde and vanillyl glycol, 4,6-di (t-butyl) guaiacol (Kumar & Chandra, 2020).

The manganese peroxidase (MnP) is among the most prevalent microbial peroxidase having the ability to alter the lignin structure and found in most of the wood-colonizing basidiomycetes. MnP is a glycosylated heme protein having molecular weights ranging between 40 to 50 kDa (Rekik *et al*., 2019). Interestingly, production of MnP is largely associated with certain basidiomycetous fungi. This metabolic potential to secrete the respective enzyme is restrained by other microorganisms. Most of the data regarding the biosynthesis of MnP has been derived from two groups of the basidiomycetes including rot causing wood-decaying fungi and dead organic matter decomposing fungi. These fungi produce multiple molecular forms of MnP into their extracellular environment. For instance, eleven different isoforms of the Mnp have been reported from *C. Subvermispora* (Andlar *et al*., 2018). The distinction between these isoforms originates from their different isolectric points (p*I*s) categorizing these as acidic, slightly acidic and neutral variants. The catalytic action of MnP resembles with other peroxidises involving oxidation of Mn^{2+} ions into Mn^{3+} which is chelated with oxalic acid. In this state, Mn^{3+} ion have the ability to act as diffusible redox-mediator to attack phenolic lignin causing alkyl aryl cleavage and α-carbon oxidation in lignin and generates relatively unstable free radicals. Besides availability of wealth of information, there are several limitations in understanding the

biodegradation of lignin including less production of enzymes by the natural and recombinant hosts, stability of enzymes under hostile industrial conditions of pH and temperature and understanding of optimum fermentation conditions. The search for the new candidate fungi having higher enzymes production rate and characteristic stability under varying operation conditions considered important for future implications of ligninolytic fungi in the areas of environmental remediation and production of valuable chemicals (Alao & Adebayo, 2022).

The Ascomycetes fungal strains have long been studied for their lifecycle characteristics and shorter time span of growth, now getting attention for biotechnological significance (Mukherjee *et al*., 2020). Generally, fungi are acidophilic organisms with their metabolic apparatus aligned to perform best between pH 4-6 in order to carry out lignin decomposition (Cequier et al., 2019). Moreover, a single fungal strain cannot produce all three types of aforementioned enzymes. This specific limitation becomes important while considering the application of these fungi for bioremediation of lignin containing wastes particularly under alkaline conditions or biotransformation of lignin into value added commercial products (Wu et al., 2022). Most of the past studies have only been directed towards the members of white rot fungi. However, the members of Ascomycetes fungi have been ignored besides their excellent ecological diversity (Charria-Girón*et al*., 2022). Pulp and paper industry use extensive quantity of wood for making pulp and paper. Being one of the largest industrial sectors, this industry generates huge amounts of highly alkaline aqueous waste known as black liquor. This waste is blackish in colour and contains high quality of lignin (30-45 %) along with other contaminants and its treatme nt has been quite challenges because of higher pH and temperature (Tomé et al., 2022). On the other side, black liquor could be an important source for the isolation of fungi having ability of growth under extreme conditions of temperature and pH. Many previous findings suggested isolation and characterisation of wood digesting fungi from the black liquor and waste stream of the paper industry (Song et al., 2022). Isolation of the fungi with desired stability for industrial applications like waste water treatment and bioconversion of lignin into valuable chemicals, could be very useful in promoting bio-refining of waste into opportunity (He et al., 2020). Considering the metabolic traits of the fungi and future biotechnological scope of these microbial cell factories, current research was focused to isolate potent ligninolytic fungi from pulp

and paper mill waste water. These fungal isolates were screened to withstand higher pH and temperatures for biodegradation of lignin. The process for the lignin degradation and production of peroxidises from these microorganisms was optimised under laboratory conditions. The analysis of degradation products was also monitored by NMR and HPLC techniques.

Scope of the research:

Lignin is a recalcitrant compound and poses significant challenge for its biodegradation under natural conditions. On the other hand, the wood utilizing industries specifically pulp and paper mills generate high quantities of waste water containing lignin and related hydrocarbons. These wastes are continuously being added into our ecosystem increasing the carbon footprints. The living organisms, specifically fungi are known to degrade these complex biopolymers and convert them into simple substrates that could be used for improving the quality and biology of the soil. Pakistan is an agriculture country and produces huge amounts of solid wastes and due to unavailability of the appropriate waste remediation technologies, these wastes are causing serious environmental pollution. In addition, development of cost effective and eco-friendly industrial waste water treatment systems for most polluting sectors is a need of time considering the current sustainability agendas. Therefore, current research is typically designed to investigate the role of indigenous fungi to biodegrade the complex lignin polymers into simple products for environmental safety, reducing impact of waste and development of future bio-refining tools to transform waste into an opportunity.

Aim:

Biodegradation of lignin by selected fungi isolated from pulp and paper industry waste water.

Objectives:

- Screening of lignin degrading fungi from pulp and paper mill effluent
- Characterisation of fungi for biodegradation of alkali lignin and related compounds
- Characterisation of lignin degrading enzymes (Lignin peroxidises, laccases, Mn peroxidases from the selected fungi
- Optimisation of the bioprocess variables for increasing enzyme production
- Identification of degradation products of lignin using NMR and HPLC techniques

2 Literature Review

Lignin is the second most prevalent biopolymer exists in the universe. It is chemically complex molecule ring structure containing synaphyl alcohol, conferyl alcohol p-coumaryl-, coniferyl- and sinapyl-alcohols. In recent years biodegradation of the lignin and biopolymers has gained the renewed biotechnological interest because of the possibility to transform into various value added products including bio plastics, blended biofuels, vanillin and variety of other important products (Ashokkumar *et al*., 2022). Another important aspect of biodegradation of lignin is its possible application of lignin biodegradation in bioremediation of industrial waste specifically pulp and paper industry (Dixit *et al*., 2021). In nature variety of microorganisms are present with innate ability to degrade/ transform the complex biopolymers into various simpler products. These microorganisms secrete a wide range of the peroxidases in their micro environment to facilitate depolymerization of the complex substrates. The enzyme secretes includes laccase, LiP, Mnp (Chan *et al*., 2020). Besides extensive research in the past few decades lignin valorization and the role of these enzymes have not been translated into commercial products. In addition, the information of the bioprocess and the variable effecting growth and metabolism of the fungi is not largely available due to copyright and patent issues. Therefore, this chapter will provide an insight into the recent development in lignin biodegradation by microorganisms, their metabolic pathways (peroxidase production and their future biotechnological role.

2.1 Biodegradation of LCB (lignin, hemicellulose and cellulose): an overview

Lignocellulose is the main part of biomass. It constitutes about 50% of the organic matter generated by chemosynthesis. It is made up of different forms of polymers i.e., cellulose (chief component), hemicellulose and third component is lignin –all of these polymers are tightly attached with one another either by non-covalent bonds or covalent cross linkages between the layers (Albornoz-Palma *et al*., 2020). Different kinds of microorganisms i.e., bacteria and fungi are present that can break these polymeric substrates by using a variety of oxidative or hydrolytic enzymes. The cross linkages of the polymers oppose their degradation because of their structural complexity and presence of poly aromatic rings. Significant advancements have been observed at genetics level of biomass degrading microbial systems during the late

1990s (Liu *et al*., 2020). Some of these hydrolytic enzymes have been successfully cloned, sequenced, and then expressed both in homologous and in heterologous hosts providing remarkable details of the biomass de-polymerisation.

In order to explain the enzymatic and degrading systems of cellulose, hemicellulose, and lignin, it is essential to describe briefly about the structure and components of cell wall. Naturally lignocellulosic biomass has been obtained from agricultural residues, wood, forestry wastes, grass and municipal solid waste. On the basis of cellulose, hemicelluloses and lignin degradation, Several biological methods have been discovered to recycle lignocelluloses (Ho *et al*., 2019). Among these methods, composting is the most frequently cited and economically feasible approach (Østby *et al*., 2020). Many of these alternative and green technologies bring in lignocellulose enzymes as pre-treatment during pulp and paper manufacturing. These technologies are efficient and allow diminishment of pollutants in the sewage from these industries. Pre-processing of biomass in the form of agricultural residues through ligninolytic fungal strains allows it to utilize as primal matter for paper production.

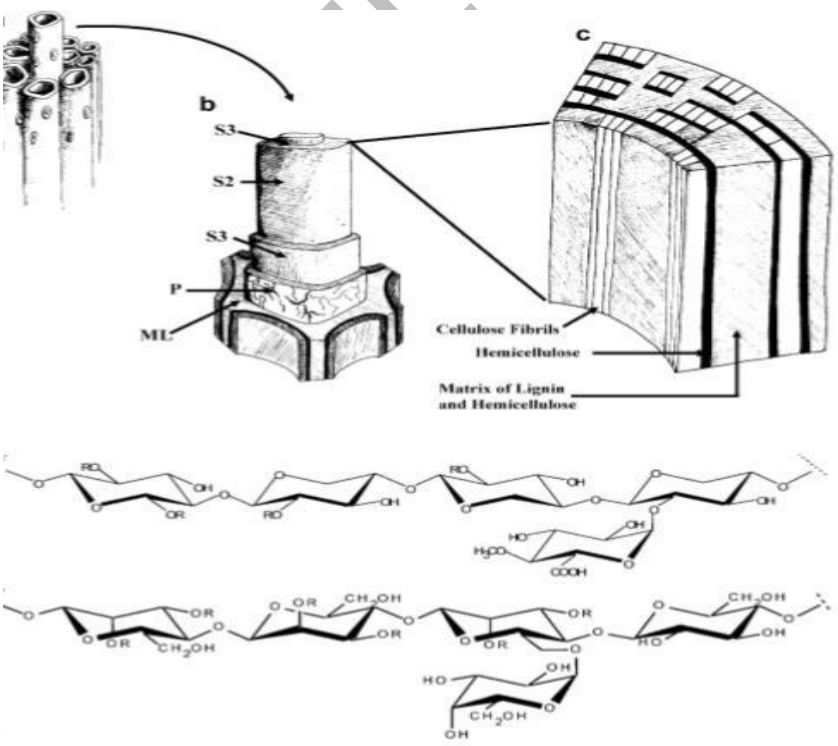

Figure 2. 1: Composition of lignocellulosic biomass and recalcitrant structure of the wood (Zabed et al., 2019)

Cellulose is the vital part of lignocellulose, followed by hemicellulose and lignin respectively (Yousuf *et al*., 2020). Lignin is primarily an aromatic (ring compound) produced from phenyl-propanoid monomers, while cellulose and hemicelluloses both are macromolecules derived from different sugars (glucose, xylose, arabinose etc) (Kumar *et al*., 2021). The composition in biomass and percentages of all these polymers in one plant is different from another and vice versa. Moreover, the general composition of these polymers in a plant depends on age, its stage of growth, and some other physiological factors. Wood is formed by cells covered by a cellular wall. The cellular wall is a complicated structure that acts as plant skin as well as backbone. Cellulose consists of about 45% of the dry weight of wood biomass. Cellulose is composed of cellobiose molecules consisting of glucose subunits interlinked by β-1,4 glycosidic linkages (Abdeshahian *et al*.). These molecules form polymers (elemental fibrils) interlinked by van der Waals forces and hydrogen bonds (Bulmer *et al*., 2021). These microfibrils are enveloped by lignin and hemicellulose. At specific growth level of cell wall the orientation of these microfibrils also vary. One configuration of cellulose is the crystalline cellulose that appears in crystalline form. Another cellulose form called as amorphous cellulose constitutes of a disordered cellulose chain but in amorphous form, cellulose is more vulnerable to enzymatic degradation (Bregado *et al*., 2019).

Naturally cellulose is found to be linked with other plant substances and this linkage plays an important role in its biodegradation. About 25–30% of wood dry weight is made up of hemicelluloses, which is a complex carbohydrate polymer. Its molecular weight is less than cellulose (Naidjonoka *et al*., 2020). It constitutes of D-xylose, D-galactose, D- mannose, L-arabinose, D- glucose, D-galacturonic, D-glucuronic acids and 4-O-methyl- glucuronic (Kobetičová & Nábělková, 2021).

Structure of hemicelluloses is extensively studied. The major difference between cellulose and hemicelluloses is that hemicellulose consists of branches with short chains, different kinds of sugars are found on these chains (Brigham *et al*., 2018). Hemicelluloses can easily be hydrolysed as compare to celluloses. They do not form clumps, even when they are interlinked with cellulose chains (Phan *et al*., 2020). Lignin is present in cell wall and is the most abundant polymer found in nature. It is

responsible to confer structural support, impermeability, and also act as a strong shield against oxidative stress and microbial degradation.

Lignin, a heteropolymer, impermeable in water and is optically inactive; structurally it is made up of units connected through different kinds of linkages, these units are called as phenylpropane units (Wang *et al*., 2020). In soft wood lignin, the major component is coniferyl alcohol, whereas in hard woods the main component is guaiacyl and syringyl alcohols (Inkrod *et al*., 2018). As a result of this polymer formation a heterogeneous structure is formed, subunits of this structure are linked by carbon-carbon and aryl-ether linkages, with aryl- glycerol, β-aryl ether being the predominant structure.

The biological metabolism of different components of biomass like hemicellulose, cellulose and lignin is the most promising field of microbiology and biotechnology (Houfani *et al*., 2020). Major problem found in the studies concerning enzymatic breakdown of cellulose and hemicelluloses is their highly diverse nature. In comparison to other microorganisms, Fungi has the maximum capability to depolymerize these three polymers (Saldarriaga-Hernández *et al*., 2020). Insoluble nature of these polymers requires primarily extracellular biodegradation. Hydrolytic and ligninolytic systems are generally possessed by the microbial strains which secretes extracellular enzymes (Sindhu *et al*., 2022). Hydrolytic system, composed mainly of hydrolases, is involved in biodegradation of cellulose and hemicellulose; whereas ligninolytic system depolymerises lignin and related biopolymers. The following section of the chapter will provide highlights biochemistry and molecular genetics of the enzymes used in the de-polymerisation of cellulosic and lignocellulosic materials.

2.2 Structure-related properties of lignin

Lignin, a phenolic compound, comprises of large portion of cell wall in plants, most importantly in woody stem. In comparison to other biopolymers, lignin is a complex network that results from the dehydrogenative radical long chains of p-coumaryl-, coniferyl- and sinapyl-alcohols (Domínguez-Robles *et al*., 2021), which are connected via ether and carbon–carbon linkages. Amount of monolignol units in cell wall depends on the species of plants. In softwood, the structure is formed chiefly by

coniferyl units (95%), while p-coumaryl units makes the rest of the structure and only minute quantities of sinapyl alcohol units are present, while in comparison, hardwood different ratios of sinapyl/coniferyl subunits have been reported (Rico-García *et al*., 2020). Lignin consists of different functional groups, such as hydroxyl groups (e.g. phenolic or alcoholic functional groups), methoxyl, carbonyl and carboxyl, in various amounts that generally depends on the origin of the group and isolation process that has been applied (Souza *et al*., 2020).

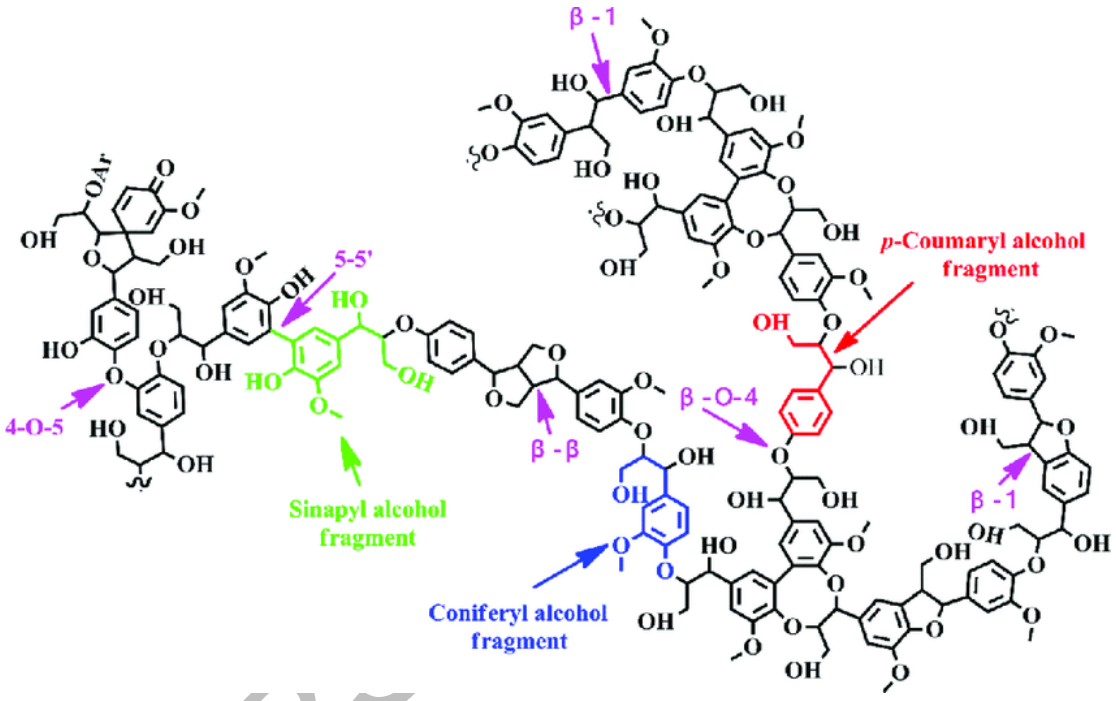

Figure 2. 2 : Representation of the chemical structure of lignin

(Cao et al., 2018)

Several extraction methods have been used to extract lignin having variable chemical properties from different sources. Commercial kraft and sulfite processes produce kraft lignin and lignosulfonates as residues respectively (Demuner *et al*., 2021). Pulp precipitation technique in alkaline conditions release lignin compounds free of sulphur content. During other delignification processes, steam under high pressure or different solvents are used to obtain lignin from biomass. Despite of all modifications in the processes it is not possible to extract pure intact lignin (Muley *et al*., 2019). All the treatments either physical, biological or chemical degrades or solubilizes the lignin from various morphologically unidentified regions and the resultant product is a mixture of various degraded polymers. (Shen *et al*., 2019).

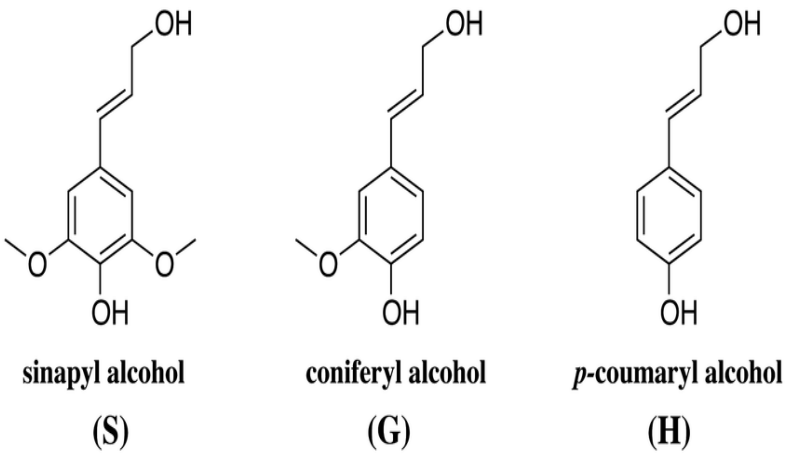

Figure 2. 3: Chemical structure of lignin monomers

2.3 Lignin biodegradation

High molecular weight of lignin with its recalcitrant structure and its impermeability in water makes it difficult to degrade (Melati *et al*., 2019). Lignin depolymerisation is initiated by different types of oxidative reactions, these reactions are catalysed by the products released from extracellular, oxidative, and non specific enzymes. ‘‘Enzymatic combustion’’ is the oxidation procedure of lignin by these non-specific processes. Among fungi, White-rot fungi is considered to be the most efficient to degrade lignin from wood (Rodríguez-Couto *et al*., 2019). *Phanerochaete chrysosporium* is the pioneer fungi to sequenced whole genome owing to its capability of producing lignin degrading enzymes.

Tremendous research has highlighted the biodegradation process of lignin by white rot fungal strains (K ijpornyongpan *et al*., 2022). White rot fungi cause degradation of complex structure of lignin with the aid of two different extracellular enzymes referred to as: laccases and most important class of enzyme peroxidases. Both of these enzymes use low molecular weight components to breakdown lignin (Rekik *et al*., 2019). Based on these ligninolytic enzymes fungi are now divided into several different classes. Some of these classes of fungi produce all major types of enzymes involved in lignin degradation, others may produce one or two types of these enzymes (Andlar *et al*., 2018). Ligninolysis has primarily been done by some reductive enzymes which include aryl alcohol oxidases, aryl alcohol dehydrogenases and cellobiose oxidizing enzymes. Two major types of peroxidises that have been studied extensively include: lignin peroxidases (LiPs) and manganese peroxidases (MnPs).

There are many different kinds of white rot fungi from which LiP can easily be isolated. The oxidative cycle of LiP is much similar to those of other peroxidises. Lignin Peroxidase is glycoprotein in nature, it contains heme group in its active site (Chaurasia & Bhardwaj, 2019). Its molecular weight varies from 38 to 43 kDa and its isoelectric point ranges from 3.3 to 4.7. Due to the large size of LiP its entry into the plant cell becomes difficult, so its degradation takes place only in specific regions of lumen. However, many research conducted on lignin depolymerization find out that white rot fungi degrade lignin inside cell wall (Del Cerro *et al*., 2021). This degradation suggests that low molecular weight LiP can easily penetrate into the cell wall, causing its breakdown (Chukwuma *et al*., 2020). But this concept is not considered to be appropriate because intermediate products such as veratryl alcohol radicals have short lifespan in this whole process hence can't perform their function as mediators.

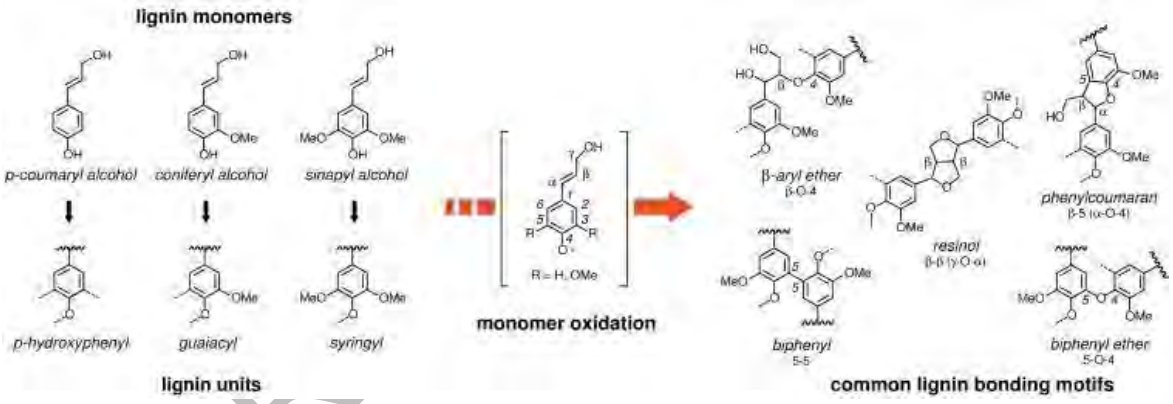

Figure 2. 4: Schematic representation of the biodegradation of lignin linkages

(Bilal & Iqbal, 2020)

Molecular structure of MnPs is similar to that of LiPs, and like LiP, MnPs is also a glycosylated protein, but the molecular mass of MnP is slightly higher than that of LiP ranging from 45 to 60 kDa (Kuppuraj *et al*., 2021). The function of MnPs is to oxidize the Mn (II) to the Mn (III). Microbial strains have a peroxidase catalytic biochemical cycle, but they can use only Mn (II) as a substrate (Obinger, 2022). Before oxidation of Mn (II) it must be chelated with the help of any organic acid chelator, this help to stabilize the end product which is oxidized Mn (III) (Cao *et al*., 2020). This oxidized Mn (III) formed during the process act as a strong oxidizing agent that can substantially oxidize the phenolic compounds, but it lacks the ability to

degrade the non-phenolic components of polymer. Phenoxy radicals produce from MnP go through various types of reactions which results in the depolymerization of lignin molecule (Saikia *et al*., 2022). Mn (II) also acts as a cofactor in the presence of MnP to catalyse the oxidation process of non-phenolic lignin molecules through peroxidation of unsaturated lipids.

An innovative peroxidase enzyme has been identified known as versatile peroxidase (VP), which contain the properties of both MnP and LiP and also have the ability for lignin biodegradation (Wang *et al*., 2021). VP can also use Mn (II) as cofactor and oxidize hydroquinone even when exogenous H_2O_2 is not available. As structurally complex ligninolytic enzymes are unable to penetrate into the cell wall and the presence of Mn(II) enhance the chemical oxidation of hydroquinones, so the availability of Mn(II) is considered to be most important in wood biodegradation (Biko *et al*., 2020). Laccase enzymes are blue-copper phenol-oxidases that are responsible to carry out oxidation of phenolic compounds; they also have ability to oxidize non phenolics in the presence of some mediator compounds. Phenolic compounds are strongly oxidized by releasing one electron from the structure thus generating phenoxy free radical products, which results in cleavage of the polymer (Duan *et al*., 2018).

Laccases are produced mainly from brown and white rot fungi, but can also be isolated from many other groups of fungi i.e., *Aspergillus* and the *Myceliophora thermophila* and *Chaemotium thermophilium* specie (Parakh *et al*., 2020), (Prakash *et al*., 2019). Currently, bacterial strains have also been identified that are capable of producing laccase enzymes like proteins. With the help of these enzymes, from compost low molecular weight fragments can be produced suggesting there (laccases) involvement in humification during composting. In lignin biodegradation laccases play an important role that has been discussed recently. There are many microbiological and biotechnological uses of these ligninolytic enzymes (Chowdhary *et al*., 2019).

Biopulping and bleaching are the two main applications of these enzymes. Many types of dangerous and toxic xenobiotics can be degraded by white rot fungi, these toxic xenobiotics include PAH (polycyclic aromatic hydrocarbons), chlorophenols, dyes, nitrotoluenes, polychlorinated and biphenyl compounds (Kathiravan &

Gnanadoss, 2021) . Major application of these enzymes includes in situ bioremediation of these compounds in the contaminated soils. Other important areas under study and research are the valorisation of these fungal strains as biocatalyst to produce various fine chemicals and other natural flavors for example vanillin, and biological treatment of sewage containing lignin- like polymer such as bleach plant effluents (Pulp and paper industry), effluents released from dye industries and olive oil mill sewage (Periasamy *et al*., 2019). From the genus actinobacteria and streptomyces some of these lignin degrading enzymes have been discovered and isolated. Lignin degradation involves strict aerobes during the whole process, but current studies reveal that facultative anaerobes in the rumen may de- lignify some proportions of plant lignin (Rajeswari *et al*., 2021).

2.4 Fungal and enzymatic treatments of wood chips and pulps: bio-pulping and bio-bleaching

The term bio pulping can be described as an opposite pre-treatment of wood residues through lignin degrading fungal strains preceding to paper production process (Lehr *et al*., 2021). The biological treatment in the process of pulping not only diminish energy depletion but it also ameliorate paper quality and eliminates wood residues which lead to more advantages like as pitch issues reduces and lesser waste toxicological properties (Gupta & Gupta, 2019). The paper and pulp industry practices mechanical and chemical processes or both in combination to produce pulp (Gopal *et al*., 2019). The mechanical process implies mechanical force to detach wood fibres. The paper produced through this process is of fine quality and have high yield. On the other hand, chemical pulping process exhibited low yield but it enhances strength of pulp material. The pre-treatments of wood residual compounds for physical and chemical processing of pulping using various white rot fungal isolates have been established under laboratory conditions (Wagle *et al*., 2022). From economical perspective, biomechanical pulping process has been verified practicable using microorganism.

The worldwide concerns for the conservation of natural plant biodiversity and eradication of pollution from these industrial effluents has shifted focused on substitute fibrous materials like wood and biomass for manufacturing paper for instance agricultural residues or non-woody plants (Abd El-Sayed *et al*., 2020). The bio-pulping of non-woody plants using *Pleurotus, C. subvermispora* and other

members of class basidiomycota lessens the utilization of electrical power utilized for purifying up to 30%, and enhance paper quality to a certain extent. Fungal pre-treatments obliterate some of the compounds or alters their chemical composition (Abo *et al*., 2019). This is possibly due to their improved chemical diffusion and thus lessens the use of chemicals. This technique is also advantageous for other practices as for instance; organ solving, dissolving, and sulphite pulp production. The Kraft pulping practice using fungal treatment gained less consideration (Vidal *et al*., 2021). Though, numerous studies pointed out the importance of white-rot fungi in bio Kraft pulping industry as it enhances the pulp yield and strength while reducing the cooking time (Mboowa, 2021). Data from various studies suggested bio pulping equally an environmental friendly, cost effective technique as a substitute method (Ingle *et al*., 2020). Bio bleaching is another alternative practice in which pulp bleaching occurred through extracellular enzymes or ligninolytic fungi. The process significantly reduces the use of chemical bleach entailed to acquire a advantageous brightness of pulp (Sharma *et al*., 2020). Treating Kraft pulps with fungal hemicellulases was first reported to minimize successive chlorine bleaching requirements. The chlorine demand can be reduced by 6-15% with xylanase treatments. Presently, approximately 15 patents regarding enzymatic treatments that improve kraft pulp has been released. Different hypothesis have been postulated to elucidate the contrivance of hemicellulose pre-bleaching. The hydrolysis of re-precipitated and relocated and expose the xylan on pulp surface so that the fibre condenses the pulp and makes it more penetrable.

The second model for pulping industry demonstrated the production of chromophores or lignin during Kraft reaction with carbohydrates moieties (Liao *et al*., 2020). The lignin extraction can be increased when hemicellulose extricate lignin by liberating xylan-chromophore fragments. Both enzymes (i.e., mannanases and xylanases) interact synergistically to ameliorate biobleaching particularly in softwood. Initially during the process mannanases appear to be less efficacious than xylanases. A number of xylanases enzymes obtained from various microorganisms have been proved to be effectual for bio-bleaching of pulps (Gao & Fatehi, 2019). The xylanases obtained from microorganisms have been evaluated on laboratory scale. The commercially prepared xylanases are derived from *Trichoderma species*(Amorim *et al*., 2019). The ligninolytic fungal isolates have also been evaluated for the process of bio bleaching

of pulp using their ligninolytic biochemical system (Bilal & Iqbal, 2020). But, they have cofactors of relatively smaller molecules which include veratryl for Lignin Peroxidase, Mn (manganese) and some common organic acids for activation of MnP. Laccase and Manganese Peroxidase appear to be most important in bio-bleaching. While the part of Lignin Peroxidase persistently unidentified as this enzyme was not purified and identified during this process. The most potent fungi for bio-bleaching are *Trametes versicolour*, *Phlebia radiate, Lentinus tigrinus*, and *Bjerkandera sp*. Likewise, the lignocellulosic enzymes such as endoglucanases from *Paenibacillus sp*., has been extensively used in bio-refining industry as a substitute to mechanical refining.

2.5 Lignin degrading microorganisms

Microorganisms including bacterial and fungal strains are able to biodegrade lignin. In case of bacteria lignin polymer degradation is restricted to very slower rate. Depolymerisation of lignin has been studied in bacteria and fungi (Li & Zheng, 2020). The following genera of bacteria are reported extensively to deolymerize lignin.

- Actinomycetes
- α proteobacteria
- γ proteobacteria

These bacterial strains range from terrestrial to aquatic ecosystems. These genera of bacteria resembles with filamentous fungi in some general features i.e., (Chauhan, 2020).

- Branching and re-branching during growth
- Aerial mycelia production
- Morphogenetic developments

Table 2. 1; Classification of microorganisms having capability of lignin degradation

Microbial Class	Representative Microorganisms	References
Bacteriaodetes	*Sphingobacterium* *Microbacterium phyllosphaerae* *Microbacterium marinilacus* *Microbacterium oxydans* *Micrococcus luteus*	(Wang *et al*., 2018) (Woo & Hazen, 2018) (Bugg *et al*., 2021) (Qin *et al*., 2021)
α – proteobacteria	*Brucella, Ochrobactrum, Sphingobium and Sphingomonas* *Rhizobiales* bacterium *Ochrobactrum rhizosphaerae* *Ochrobactrum pseudogrignonense*	(Tsegaye *et al*., 2019) (Mendes *et al*., 2021) (Granja-Travez *et al*., 2018) (Arteaga-Cuba *et al*., 2021)
γ - proteobacteria	*Pseudomonas fluorescens, P.putida, Enterobacter lignolyticus, E.coli*	(Jaiswal *et al*., 2020) (Chattopadhyay, 2022) (Wu *et al*., 2018)
Actinomycetes	*Streptomyces viridosporus* *S.paucinobillis* *Rhodococcus jostii*	(Rashid *et al*., 2015) (Anthony *et al*., 2019)

Genus Streptomyces is the class of several filamentous bacterial strains that secretes number of lignin degrading enzymes. Genus Actinomycetes also comprises of certain bacterial species including *S. griseus, S. psammoticus* and *S. coelicolour* that are well known for some secretory enzymes that are pivotal for lignin biodegradation (Olawale, 2018). The catalytic activity of LiP and Laccase was studied and clearly observed in two extensively studied strains i.e, *S, cinnamomeus , S. viridosporus*. These strains were distinguished as laccase and peroxidase producers as extracellular enzymes which performed better catalytic activity. *S. ipomoea* have also been known for the laccase production. From all of the research and findings, it is suggested bacterial strains are also a good source of enzymes and lignin degradation from effluents.

The total numbers of fungal strains that are capable of lignin depolymerization are still not well investigated but it is reported by Gilbert that more than 1600 wood valorising fungal species are currently found only in North America. In areas where

anthropogenic activities are maximum fungal strains usually survive as saprobes or in the form of mild parasites. *Ustilago maydis*, a phytopathogen, has been studied at genetic level to code the genes which are responsible for enzyme secretion. Coprophylic fungi for example *Coprinopsis friesii* and *Panaelus papilionaceus* also produce peroxidases and laccases for degradation. These fungi play an important role for the decay of organic matter to decompose the litter (Frey, 2019). Class of saprophytic fungi, generally decomposes the organic matter, can be further classified into three categories, white rot fungi, soft rot and brown rot fungi. The most common group of fungi that completely degrades organic matter into soluble organic and inorganic compounds i.e., CO_2 and H_2O is white rot mycelium.

Variety of substrates have been utilized by the species of fungal class Basidiomycota such as plant roots, leaves litter, grass and crops (He *et al*., 2022). Members of white rot fungal genera are generally Agaricales which includes *Pleurotus spp.* And *Lentinula edodes.* Members of brown rot fungi, *Ganoderma spp, Gleophyllum trabium* and *Phlebia radiata* are extensively studied and can decay the wood during construction process which can cause severe damages. White rot fungal specie, *Phanerochaete Chrysosporium,* produces several enzymes which includes isoenzymes and some quantities of manganese peroxidases (Shi *et al*., 2021). This strain mostly founds during last stages as this can grow more rapidly in nitrogen deficient conditions (Singhania *et al*., 2022).

Botrytis cinereal, a laccase producing fungus starts a soft rot like degradation in garden ornamental plants. In *Vitis vinifera*, they initiate grey rot. Smaller fungal strains have not been studied much but these strains have proven best candidates for the wood decaying process (Zhao et al., 2022). Zygomycota, another class of fungi, does not have much ability to use lignin and cellulose. To initiate the decay process Basidiomycota firstly covers the area and then ascomycetes spreads their hyphae over the surface. *Xylariaceae* member of class ascomycota chiefly resides within the live wood (Osono, 2020).

2.6 Degradation of lignin by fungi

The degradation of lignicellusic biomass by fungi depends on two forms of extracellular enzymatic systems. Firstly, fungal strains involved in secretion of

hydrolases to degrade the structurally present lignocellulosic polysaccharides. Secondly, they acquire a distinctive enzyme ligninolytic systems which involved in degradation or modification of lignin (Cairns *et al*., 2021). The predominance of these enzymatic systems changes along with the type of fungus. The decay of fungal wood is decay distinguished in three forms : white, brown and soft rot (Sista Kameshwar & Qin, 2018). Wood decayed which presents the white rot fungi is pale yellow in colour and usually affects the fibrous texture. Frequently found white-rot fungi include *Phanerochaete chrysosporium, Pleurotus ostreatus* and *Ceriporiopsis subvermispora* (Ding et al., 2019). The fungus attack the lumens of the wood cells along with enzyme discharge that cause lignin degradation in addition to linked polysaccharides (Schoenherr *et al*., 2018). White rot fungi however most commonly cause degradation of deciduous wood (hardwood). On the contrary, brown rot fungi mainly grow on the coniferous wood (softwood) due to this property they are categorizing as softwood degraders. Both of these groups white rot and brown rot fungi demonstrate host choices. In brown rot fungi typically an uniqueness has been observe that prior to lignocellulosic polysaccharides is established the lignocellulose should be lignified, and then degradation made achievable (Yadav *et al*., 2022). As lignin is not directly degraded by them, but to a certain extent they modify it through partial oxidization and subsequently degrade the polysaccharides. Therefore, wood that decomposed by means of brown rot fungi is regarded as brown colour which arises from lignin residues. Besides the colour changes, wood decayed through brown rot fungi cause shrinking and shatter as brick shaped pieces which deteriorate to a brown powder (Sealy, 2022). The third group involved in wood-decaying are the soft rot fungi. Soft rot fungi cause wood decay in water saturated systems and the vicinity with random moisture levels (Hamed, 2018). They are however less destructive than white-rot and brown rot fungi, and preferably make use of polysaccharides, but they are also involved in lignin decomposition to a certain degree.

2.7 Enzymes involved in biodegradation of lignin

Decomposition of lignin successfully accomplished only by the implementation of multiple extracellular enzymes due to having the complexity and heterogeneity in structure and the diverse range of chemical linkages. Thus, the degradation of wood constituent most specifically lignin is extensively explained as a multi-enzymatic

route that also produces many intermediates. Though, fungal degradation of lignin does not achieve by only one set of specific set of dedicated enzymes. However, the decomposition of lignin by the use of multiple enzyme also depends on fungal type (Iram *et al*., 2021).

Lignin depredating enzymes are known as ligninases. The one catagaory of these enzymes is "copper-containing laccases" and other is reffered as "heme peroxidases", which includes lignin peroxidases, manganese peroxidases, versatile peroxidases and dye decolourizing peroxidases. Most of the lignin degrading enzymes are supported by some fungal enzymes. An important group of enzymes is oxidases, for example aryl-alcohol oxidase and glyoxal oxidase. These are known to produce peroxide, important for peroxidase activity (Tamilarasan *et al*., 2018). Laccases are known to catalyze the oxidation of polyphenols and phenols by producing free radicals. The oxidation step is normally fixed along the reduction of molecular oxygen into water. Due to existence of redox mediators, laccases can also tend to non-phenolic lignin breakdown, together with the cleavage of β-O-4 linkages. Generally mediators involve in laccase mediator systems include 2,20-azino-bis (3-ethylbenzothiazoline-6sulfonic acid) (ABTS), 1-hydroxybenzotriazole (1-HBT) and another natural mediator acetosyringone (4-hydroxy-3,5-dimethoxyacetophenone) (Singh & Gupta, 2020).

Lignin peroxidases are liable to catalyse the oxida tive de-polymerization of lignin along with H_2O_2 which act as an oxidizing agent. This group of enzymes is comparatively nonspecific and can be consequently oxidize both phenolic and non-phenolic aromatic substrates of lignin. The significance of lignin peroxidases for depolymerization of lignin exhibit their capability to cause cleavage α, β and β-ether bonds that leads to the efficient lignin degradation into mono-aromatic structures. For instance, the synthetic hardwood lignin with approximate molecular weight > 1800 Da was tended to degrade in fragments equally with average molecular weight as low as ~170 Da (Biko *et al*., 2020). Manganese peroxidases make use of H_2O_2 for Mn^{2+} oxidation, found in wood and soils, as a result generate reactive Mn^{3+} ions. This is stabilized by the exploitation of chelators and used as charge transfer mediators capable for the oxidation of several phenolic substrates. This catalytic mechanism is comprised of two consecutive one-electron driven oxidation steps yielding

intermediate cation radicals. Contrary to lignin peroxidases, manganese peroxidases are not able to cause oxidation of other recalcitrant non-phenolic structures under normal conditions. (Chowdhary *et al*., 2019). Versatile peroxidases coalesce the characteristics of lignin peroxidases and manganese peroxidases, presents the catalytic versatility. They are capable to oxidize Mn^{2+} to Mn^{3+} like manganese peroxidases, but are not able to oxidize non-phenolic compounds in the same manner as lignin peroxidases.

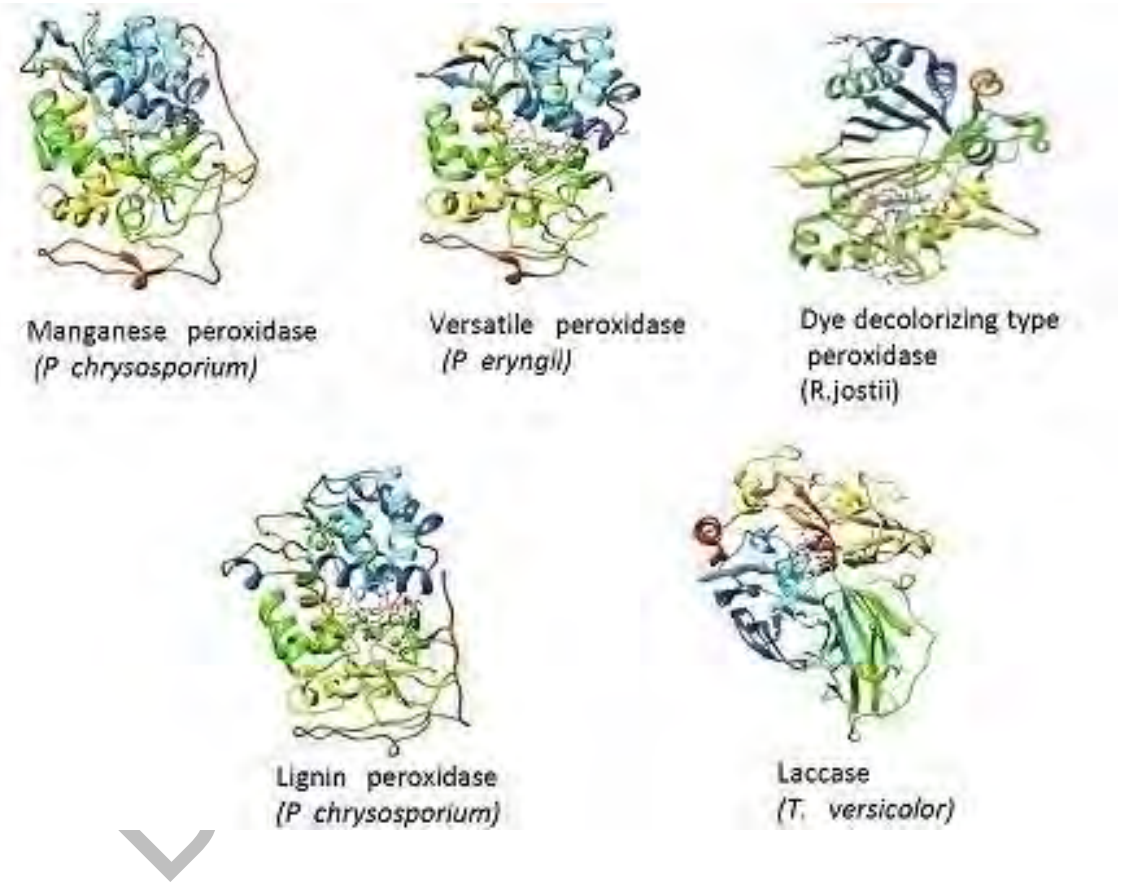

Figure 2. 5: 3D structure of lignin degrading enzymes

(Ayeronfe et al., 2018)

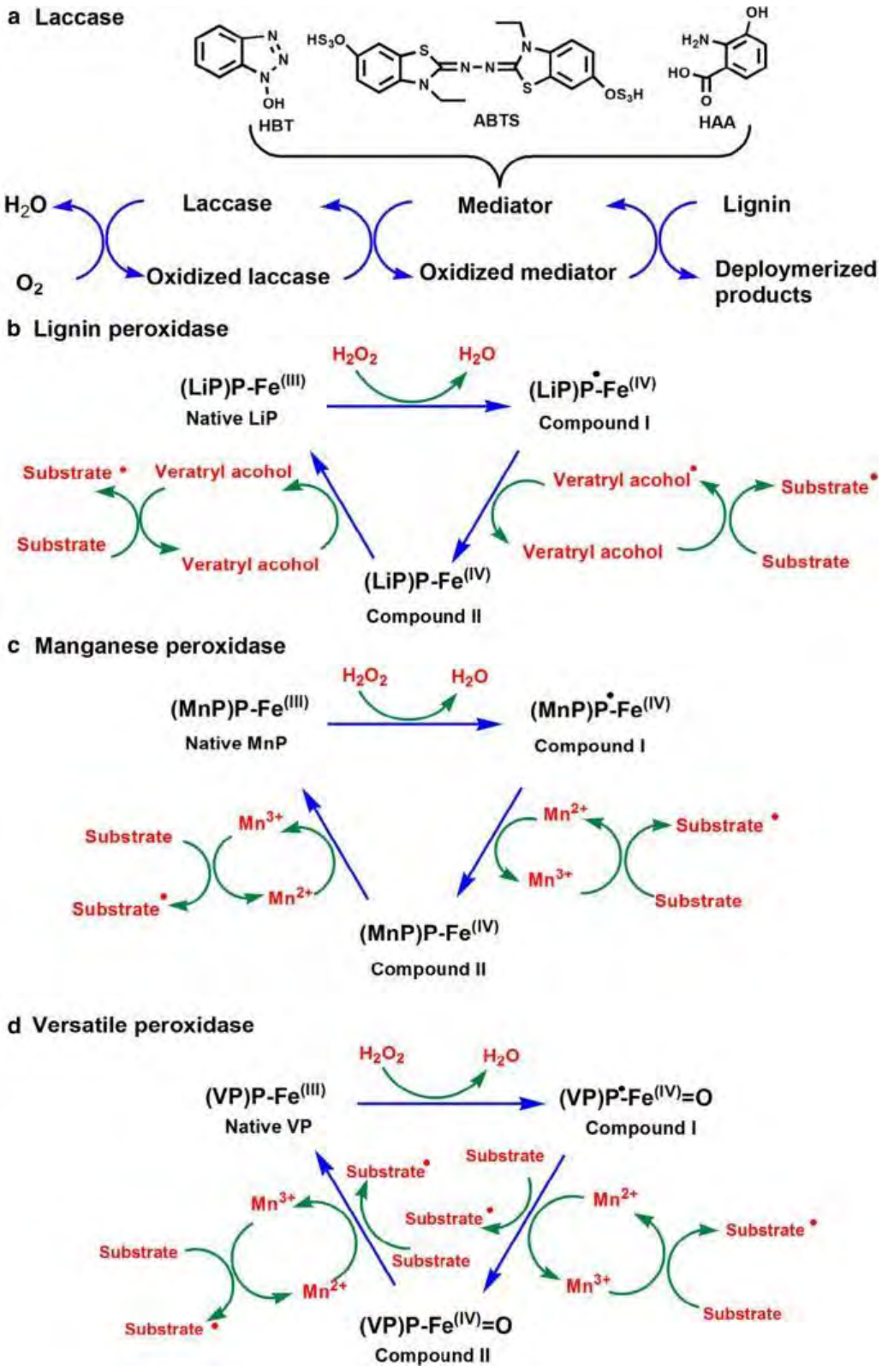

Figure 2. 6: Putative pathway of peroxidases and laccases in lignin degradation

(Weng et al., 2021)

2.8 Characterization of lignin

Lignocellulosic biomass is the most abundantly available form of biomass to make renewable energy. Worldwide annual supply of lignocellulosic biomass is 200 billion metric tons. Lignocellulosic is mainly composed of plant cell wall and it is primarily comprised of three basic polymers; cellulose, hemicellulose and lignin. The composition and structure of lignocellulose is significantly depends on specie, part and growth conditions of the plant etc.(Yousuf *et al*., 2020) Lignin is an important phenolic polymer which is formed by the oxidative incorporation of the three major C6–C3 units of phenylpropanoid which are named as syringyl alcohol (S), guaiacyl alcohol (G), and p-coumaryl alcohol (H), these all form a three dimensional randomly present network inside the cell wall. They all are linked by the aryl-aryl units. In addition to this lignin is also comprised of 20 different kind of the bonds, lignin consider to be predominantly linked with the hemicellulosic polysaccharides. Additionally, a minute amount of lignin units are considered as phenolic, only linked by C-C bonds. Despite of the fact this phenolic moiety signifies a small (and variable) portion of the entire lignin, it be capable to influence strongly the reactivity of the polymer (Ralph *et al*., 2019).

Numerous procedures have been utilized in order to extract lignin and for the purpose to manufacturing of pulp and paper from lignocellulosic materials. Pulping is considered as one of the most abundantly used process for delignification for the non-woody materials. Consequently to the pulping process, lignin is dissolved by the raw material, and being removed in the liquor form which is rich in phenolic compounds that are characterize as effluent of pulping process (Kihlman & Gustavsson, 2021). The effluent obtained from industry is dark blackish brown in colour. Black liquor effluent of the paper and pulp industry comprised of different chemical compounds, lignin and its derivatives. Usually pulp industry generate energy by make use of lignin which is dissolved in black liquor. On the contrary, the lignin can also be extracted and utilized as the preliminary material to make number of valuable products. Lignin which is obtained after fractionation and pulping procedures, have also been considered for numerous applications (Pola *et al*., 2022).

2.9 Applications of lignin degradation/depolymerisation

The most useful application of the lignin is the synthesis of different polymeric compounds. Lignin also helpful for production of thermostable blends, for example the blend form with thermoplastic polymer i.e., polypropylene shown the excellent mechanical and thermostable characteristics (Tian *et al*., 2022). Lignin is also considered as compatibilizing agent for thermoplastics and some natural fibres.

Lignin has also been used as an alternative to phenol for the generation of ligninmodified phenolformaldehyde (LPF) resins because of structural similarity. Lignin also goes through the chemical modification for the preparation of polyurethanes, (Ma *et al*., 2021) acrylates, epoxies, polymer blends, and composites (Grossman & Vermerris, 2019). Although, main issues with the operation of lignin obtained from industry may also occur in some application fields , like in synthetic polymers, the heterogeneity of industrial lignin along with the be deficient in of efficient methods for separation of the pure lignin. It is considered that an improved method is required for industrial lignin fractional extraction and structural characterization, to make its optimum use. Conservatively, the Kraft lignin has also been obtained from black liquor by appropriate implementation of methods at lab scale or at industrial sections, with the help of acids like H_2SO_4 or HCl precipitation is done, after that filtration and washing is performed which is followed by purification. The intermediates form during lignin degradation process can further utilized for formation of value added compounds.

2.9.1 Vanillin

Vanillin is the major component of the extract of vanilla beans (Banerjee *et al*., 2019). It widely used in pharmaceutical products, foods and beverages. It is the only phenolic product that is produced from biomass on industrial level (Martău *et al*., 2021). Aerobic oxidation process of degrading lignin results in formation of vanillin (Zhu *et al*., 2020). According to a modern research, various lignin monomers have been employed for the production of lignin. Various carbon sources and pH have been used for optimal production of vanillin. Major sources of carbon include glucose, sucrose, fructose, xylitol and mannitol (Ma *et al*., 2022). Fructose is known to produce the most yield of vanillin as compared to others (Gou *et al*., 2022). Among the nitrogen sources used for vanillin production, peptone, yeast extract, urea and ammonium

sulphate are common (Chattopadhyay *et al*., 2018). It has been observed that beef extract is a valuable nitrogen source for the production of vanillin as compared to others. Different values of pH have also been take into account and it has been seen that values near to 9 are more suitable for vanillin production (Paul et., 2021). The maximum yield of vanillin depends on optimum conditions of the reaction and the nature of the substrate. Yields are better when the temperature is raised to higher values. Lignin fractionation results in the formation of propanol which in turn is oxidized to vanillin. Apart from lignin, other substrates including eugenol, ferulic acid can also be used for vanillin production. Production up to 56.24×10^{-2}g of vanillin has been achieved on lab scale in the past (Sales et ., 2007). Temperatures ranging up to 160°C are shown to be effective in the production of vanillin (Li *et al*., 2020). The following figure describes the process of vanillin production briefly.

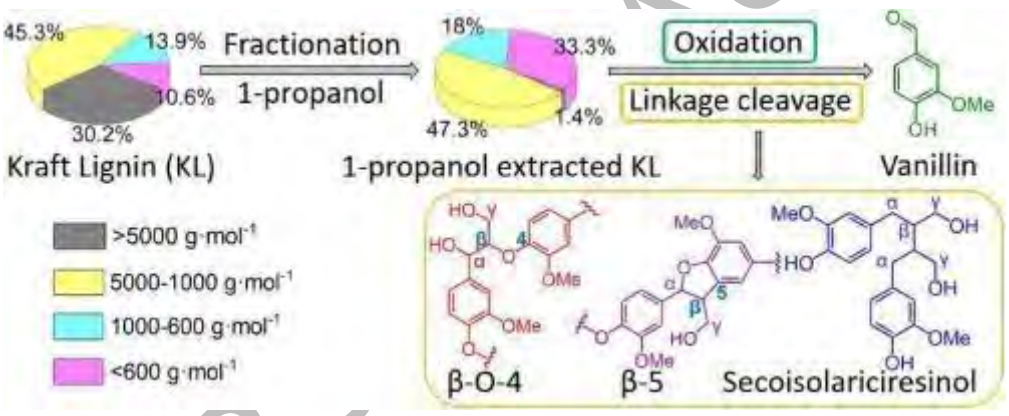

Figure 2. 7: Biosynthesis pathway of vanillin from kraft's lignin

(Mei et al., 2020)

2.9.2 Hydroxybenzaldehyde

Hydroxybenzaldehyde is an important intermediate formed during lignin degradation. It is used in polymer synthesis and other products in chemical industry. It is used on blends formation and is employed in the preparation of various synthetic products. Its conversion to vanillin has been achieved in various research studies and conditions have been optimized for production (Tarabanko *et al*., 2017). It has also been evaluated as a plant metabolite and its functioning is currently under study. Various microbes have been used for the production of this useful compound and substrates

other than lignin such as acetone have also been employed for studying the production of hydroxybenzaldehyde. The yield of hydroxybenzaldehyde from lignin has been observed to be higher at temperatures up to 180ºC (Li *et al*., 2020).

2.9.3 Syringaldehyde

Syringaldehyde is an important intermediate product of lignin degradation pathway (Li *et al*., 202). It is a phenolic aldehyde possessing unique properties. It has been studied with respect to antifungal properties, inhibitor of enzymatic hydrolysis, applications in food, pharmaceutical companies and cosmetics (Ibrahim *et al*., 2012). Various enzymes have been employed for the prepared of these useful compounds and varying conditions of temperature show that temperature up to 190ºC is effective for the production of syringaldehyde. According to a study, 50.01×10^{-2}g yield of syringaldehyde has been achieved on lab scale (Sales *et al*., 2007) and efforts are being made to look for alternative substrates that can be utilized for the production of syringaldehydes. (Ibrahim *et al*., 2012)

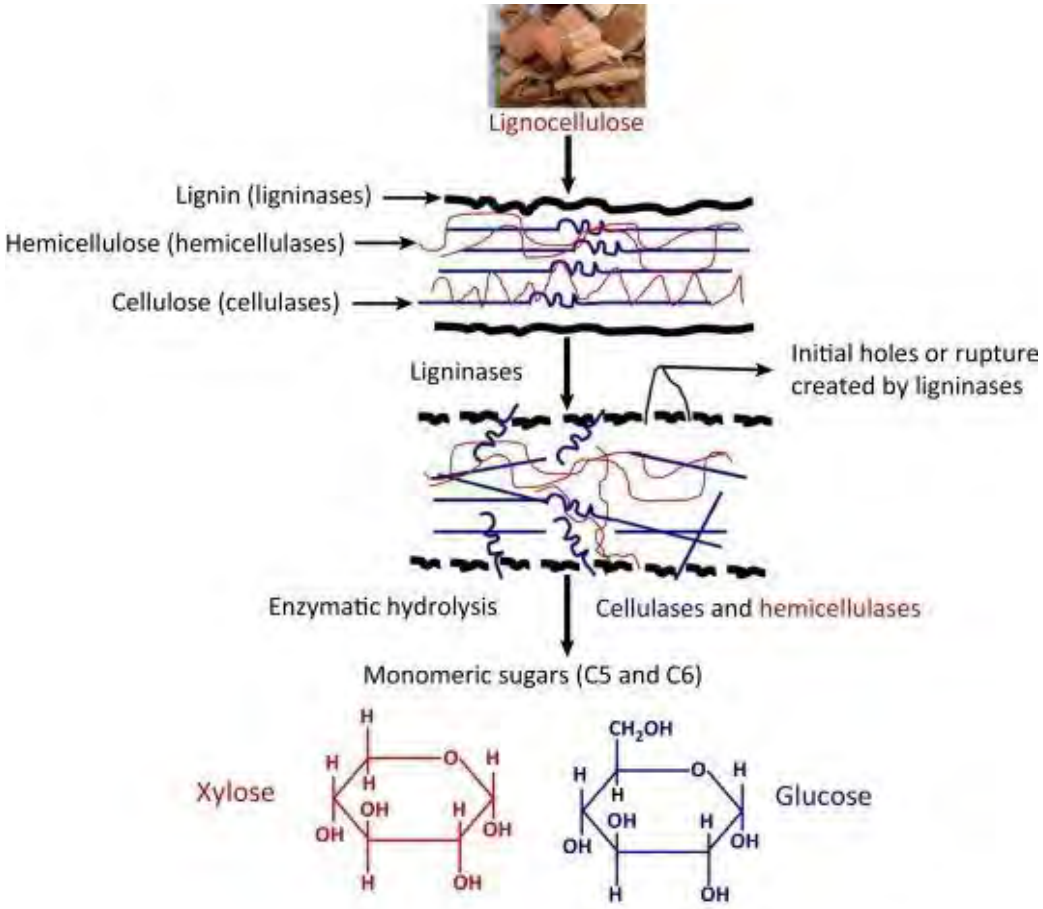

Figure 2. 8: Schematic diagram for complete biodegradation of lignocellulosic biomass

(Den et al., 2018)

2.10 Decolourization of black liquor

The manufacturing of pulp in paper and pulp industries involves two important steps i.e. wood digestion followed by bleaching. The wood digestion process comprises of chemical cooking of the wood chips followed by high temperature and pressure treatment. The treatment of wood chips with soda yields fine fibers. Consequently, a complex aqueous waste of dark colour containing high concentration of lignin, cellulose and different phenolic compounds produced as waste, technically known as black liquor (Kim *et al*., 2019). Black liquor is highly alkaline effluent of the pulp and paper industry which results in higher BOD and COD of the waste water and contribute significantly in the water pollution up to 90-95%. Therefore its treatment prior to the discharge is required to curtail its negative environmental impacts.

A number of the waste water treatment options has been reported so far for pulp and paper mill effluent including electrocoagulation (Buftia *et al*., 2018), ozonation,(Zhou *et al*., 2019) ultrafiltration (Mendes *et al*., 2022). These method are coined with different other engineering techniques to mitigate the complex hydrocarbons present in the said waste (Moussa *et al*., 2017). The most efficient approach has been reported as combustion followed by sodium recycling. During these processes the black liquor (BL) is subjected to increase its viscosity and subsequent treatment of incineration. These processes are not environment friendly because of less process efficiency and involvement of heat energy. In this prospective when the world is already facing energy crisis such processes pose additional burden both in terms of their impact and recurrent cost. Considering the sustainability drive the microbial decontamination of the waste has been gaining ample scientific attraction in addition to 35% less cost than the conventional methods. The microorganisms capitalize their metabolic potential to degrade these xenobiotics by implying heir extra cellular enzymes such as peroxidases. These peroxidases depolymerize the complex lignin polymers and other related compounds in order to facilitate their bioremediation (Estrada-Vázquez *et al*., 2020).

In past three decades extensive research for the development of pulp and paper remediation techniques provided various options involving the application of nature's catalysts (microorganisms). As stated previously these microorganisms are present within the natural environment and waste streams having excellent adaptations to use toxic hydrocarbons to satisfy their cell growth thereby causing their removal from the environment. Fungi is the prominent cellular resource having metabolic diversity of producing variants of peroxidases including LiP, MnP and laccases (Kamimura *et al*., 2019). Other advantages of the use of indigenous fungal strains includes: High performance under chemical stress, pH tolerance and increased expression of peroxidases. Yadav *et al* 2022 reported the metabolic efficiency of *Panibacillus, Bacillus*, *Aneurinibacillus* to degrade and decolourize commercial lignin. In another study *Citrobacter sp.* was isolated and used for the degradation of azo dyes and other organic pollutants in the waste streams (Schmidt *et al*., 2019; Selvaraj *et al*., 2021).

2.11 Overview of biodegradation of alkali lignin by bacteria

Lignin has cyclic hetero-polymeric structure. Lignin has similar structure to cellulose and hemicellulose; both of them are part of plant cell walls. Phenylpropanoid aryl-C3 monomers bind together with different ethers to form the lignin structure. Due to its complexity and irregularity in its structure much of lignin structure is unexplained. (Ponnusamy *et al*., 2019) showed that during the pre-treatment of lignocellulosic aromatic compounds are produced that stop that further degradation and fermentation process needed to generate biofuels. Because of these harmful effects it has become a challenge to convert lignin to useful products. (Liao *et al*., 2020).

Up to date many microbes have been found to degrade lignin. Fungi that belong to class basidiomycota produce enzymes such as Mn-peroxidases, lignin peroxidases and laccases. Different bacteria are reported to have ability to degrade lignin but their metabolism is still less defined. These bacteria belong to actinomycetes, α- and γ-protobacteria including *Rhodococcus jostii, Streptomyces viridosporus T7A, Sphingobium sp. SYK-6, Nocardia, Pseudomonas, Comamonas, Bacillus*, and sulfate-reducing bacteria from sources like soil,animals and insect guts, etc. (Nandal *et al*., 2021). Bacteria have various oxidative enzymes like dye-decolourizing peroxidase (DyP), cytochrome P450 monooxygenases (P450s), manganese superoxide dismutase and laccases, that change lignin through demethylation or hydroxylation (Venkatesagowda & Dekker, 2021). Bacteria like *Rhodococcus jostii* RHA1 use β-ketoadipate pathway to degrade lignin aryl rings into metabolites of TCA cycle using nine enzymes and various intermediate products. (Wang *et al*., 2020).

2.12 Biological treatments of lignocellulosic biomass (LBC)

Extensive research has been made to convert LCB into biofuels which are strong candidates to be used as an alternative to traditional fossil fuels. Cellulose and lignocellulosic residues has been widely used as a raw material to convert it into bioethanol and biogas (Robak & Balcerek, 2018). Industrial scale application of this bioconversion is the production of bioethanol from corn and other high starch containing crops. Bioethanol blends with gasoline (10:90) are currently used in automobile industry which reduces carbon monoxide emissions to great extent. Use of lignocellulosic biomass is also cost effective as compared to the starch-based crops. In

the last two decades, lot of advancement has been made to produce bioethanol through enzymatic conversion, and this product is so cost effective that nowadays ethanol can replace gasoline. The conversion of lignocellulosic biomass into ethanol takes place in two steps:

(1) Hydrolysis of biomass and delignification to liberate cellulose and hemicellulose from their complex mixture with lignin, and breakdown of carbohydrate polymer to liberate fermentable sugars; and

(2) Use of liberated sugars (pentoses and hexoses) in the fermentation process to produce ethanol. In traditional biorefineries, lignin present in the raw biomass and liberating fermentable monomeric sugars are removed by chemical and/or thermal pre-treatment processes followed by dilute acidic or enzymatic hydrolysis.

However, biological pre-treatments have been proved best alternative the physicochemical treatment processes or for removal of specific inhibitors before the fermentation process. The previously mentioned ligninolytic microorganisms including Streptomyces and white rot fungal strains can significantly delignify the biomass (Suryadi *et al*., 2022). A better substitute to the bioconversion is the to step operation i.e, concurrent saccharification and fermentation, where hydrolytic enzymes (e.g. *T. reesei cellulases*) and microorganisms (fermentative) are present in the specific reactor. In case of lignocellulosic biomass as a substrate, utilization of xylan for biological processes is a vital step. Xylanases produced by fermentative microbes catalyse xylan and convert it into xylose in moderate reaction conditions where small amounts of substrate can be lost as well (Guido *et al*., 2019). One of the major drawbacks of this enzymatic hydrolysis is that this process is not cost effective and due to recalcitrant nature of LCB extent of enzymatic hydrolysis is also a limiting factor. Hemicellulose that is composed of different sugars can be hydrolysed by dilute acid pre-treatment. Nevertheless, they comprise of just 15% of total biomass sugars, 45% of total hydrolysis recoverable sugars can be attained by hydrolysis of hemicellulose. Over a long period of time, the bioconversion of liberated sugars into ethanol was sectional to 6-C sugars (hexoses) because xylose cannot be fermented by traditional fermenting strains. Xylose (5C sugar) can be fermented into ethanol by genetically engineered strains or xylitol (artificial sweetener).

In most yeast strains, aerobic condition is required to initiate the fermentation of sugars for bioethanol production but even in this case the final ethanol yield is quite low. Genetic engineering, a recent advancement in molecular biology, has changed the scenario up to some extent by providing with the strains capable of utilizing both pentoses (xylose) and hexoses (glucose) sugars (Keshav *et al*., 2021). During the last decade of previous century, many researchers around the globe tried to obtain the ethanol directly from lignocellulosic materials by using anaerobic thermophilic microorganisms. *Clostridium thermocellum* proved as a best candidate because it can hydrolyze cellulose and, at the same time, also ferment the liberated sugars into bioethanol (Abo *et al*., 2019). Moreover, *C. thermocellum* is a thermophilic-bacteria can grow at elevated temperature range, depicts higher growth rates, fast metabolic activities, and increased enzymatic stability. The cellulosome of *C. thermocellum* consists of variety of hydrolase enzymes, including cellulases, and hemicellulases, all of them are attached to a long polypeptide chain called scaffoldin or CipA (for cellulosome integrating protein). CipA contains various functional components (modules). One is a single cellulose binding domain (CBD), and there are nine recurrent domains, called cohesins, which are interconnected with cellulosomes (hydrolases). In addition, Due to a specific domain in scaffoldin, it has the ability to attach to the surface of cell. Wild type yeast strains have narrow range of toleration towards ethanol concentration. Genetic engineering of wild type strains will help to mitigate this issue.

BMP of biomass has been analysed to convert lignocellulose into methane; however, further improvement, such as increasing the enzyme concentration for the conversion of cellulose and hemicellulose into fermentable sugars such as glucose and xylose, and genetically engineered organisms that endure alleviated pH levels, are required to attain economic benefits.

This review provides an insight of lignin degradation fungal strains and their extracellular enzyme production. Ascomycetes have never been studied for lignin degradation process owing to them in capability. This study based on ascomycetes fungal strains isolation from pulp and paper waste water and screening for their lignin degradation potential. This study optimized and determined lignin degradation by using advanced analytical techniques. It will add up the existing knowledge of the

lignin degradation to the extent to stabilize the carbon cycle of ecosystem. Still door to knowledge will never close and in future more will be added to make this planet a home to live.

3 Material and Methods

Research Phase I

3.1 Sampling site and collection of wastewater

The effluent (Black Liquor; BL) samples were collected from Kraft section outlet of Bulleh Shah Packages Limited, Lahore, Pakistan, in a sterile container. These samples were stored in ice box and transferred to Microbiology Research laboratory, Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, for further processing and analyses.

3.2 Physiochemical characterization of the Black Liquor

Chemical oxygen demand (COD) of the BL samples were determined in 16× 100mm culture tubes. In order to measure the COD, 3.5 ml sulphuric acid reagent, 2.5 ml standard solution and 1.5 ml digestion solution were added in each tube containing 3ml of sample. All tubes were incubated at 150°C for 2 hours. After cooling, COD was measured by using spectrophotometer at wavelength of 600nm (PGI/T60UV/VIS Spectrophotometer). In order to determine the Total Dissolve Solids (TDS), a pre-weighed filter paper and evaporating dish was used. Then, 50 ml of sample was filtered and filtrate was transferred to evaporating dish. The filtrate was evaporated in evaporating dish and was then dried at 105°C. The TDS was calculated by using the formula,

$$T.D.S\ (\frac{mg}{l}) = \frac{(A-B)x1000}{ml\ of\ sanple} \quad \ldots\ldots \quad \text{Equation 1}$$

Where A= weight of the dried residue + dish (mg) and B= weight of the dish (mg)

For the determination of Total Suspended Solids (TSS), standard method was used, which involve usage of HACH TSS filters. 100ml of sample was passed through the pre weighed filter paper and then filter paper was dried in an oven at 105°C for one hour.

$$T.S.S\ (\frac{mg}{l}) = \frac{(A-B)x1000}{ml\ of\ sanple}$$

Where, A = Weight of filter+ dried residue (mg) and B = Weight of filter (mg).

To measure the colour of the BL sample, it was centrifuged at 10,000 rpm for 10 minutes to remove suspended solids. 1 ml of supernatant was washed with 3 ml of phosphate buffer and its pH was adjusted between 7-7.2. Spectrophotometer (PGI/T60UV/VIS Spectrophotometer) was used to measure the absorbance at 465 nm using distilled water as blank. The absorbance values were transformed into colour units (PCU) as follows,

$$Color\ (PCU) = \frac{500xA2}{A1} \quad \text{.....Equation 2}$$

Where, A1 = absorbance of standard platinum – cobalt solution and A2 = absorbance of the effluent samples

For the measurement of total nitrogen present as nitrates, 0.5ml of 1N HCl was mixed with 25 ml of sample and was digested. Spectroquantm (Spectroquant® Pharo 300) was used to determine the level of nitrate-nitrogen. Gravimetric method was used for determination of Total Sulphates present in the sample. For this purpose, 250ml of the water sample was taken in 500 ml of Erlenmeyer flask and 3ml of 1M HCL was added. Solution was then left for heating on hot plate and 80 ml of 0.5 M $BaCl_2$ was added in the solution. Presence of cloudiness in the flask indicates reaction. The precipitates were kept on digesting for 30 minutes. About 1-2 drops of $BaCl_2$ was added to complete the test. Upon reaction completion 40 ml of $BaCl_2$ was added in the solution and it was allowed to digest for 20 minutes. A weighed piece of filter paper was taken and solution was filtered through it. Filter paper was oven dried and precipitate of $BaSO_4$ was dried and weighed.

Weighed of SO_4^{-2} = Weight of $BaSO_4$ x gravimetric factor

= Weight of $BaSO_4$ x **a** (gram formula weight of SO_4-2) **b** (gram formula weight of $BaSO_4$)

3.3 Measurement of the lignin contents in the black liquor samples

To weigh the lignin content in the sample method presented by Ulmer in 1983 was used. In this method 4.5 ml of 0.55% of NaOH was added in 0.5 ml of sample. The sample was centrifuged at 8000rpm for 30 minutes was diluted by adding 3.0 mL phosphate buffer and absorbance was measured at 280 nm on a UV-visible spectrophotometer (PGI/T60UV/VIS Spectrophotometer). The degraded lignin percentage was calculated by using below equation;

$$Lignin\ degradation(\%) = \frac{Initial\ A280 - Final\ A280}{Initial\ A280} \text{ X } 100 \text{ ….. Equation 3}$$

3.4 Isolation and characterization of fungal biomass from black liquor samples

3.4.1 Measurement of fungal biomass (dry cell weight)

In order to measure the fungal biomass/dry weight, the fungi were grown for 3-4 days on shaking condition and then filter the media through *Whatman filter paper No. 1*. After this keep the filter paper along with fungal filtrate were dried at 40-50°C for 42-72 hours in an incubator.

$$Dry\ Cell\ weight \frac{g}{100ml} =$$

$$(Total\ weight - \text{W}eight\ filter\ paper\ (without\ moisture) \text{ …… Equation 4}$$

3.4.2 Enrichment of the black liquor samples

The basic theme of the enrichment of the samples was to activate/promote the vegetative growth of the microbial biomass in the collected samples. For this purpose, a black liquor sample was enriched for a period of 10 days. For the enrichment of fungi Sabouraud dextrose broth (Mycological Peptone 10.0 g, Dextrose 20.0 and final pH 5.6) was used. The medium was autoclaved and after sterilization and cooling, 1mL of the black liquor was added into flask. The flask was then placed in a rotary shaker for 10 days at 30°C and 120 rpm.

3.4.3 Isolation of microorganisms from the enriched black liquor samples

After ten days of the enrichment, the samples were taken from flasks, and serially diluted. The process involves the dilution of the cultures in a series of test tubes

having 9ml normal saline mixed with 1 ml of 10 days old inoculums in order to get isolated fungal colonies. From each dilution, 0.1 mL samples were taken under sterile conditions and spread over the prepared Sabouraud dextrose agar plates. After spreading, the plates were incubated at 30°C in an incubator till the growth of visible colonies on the agar plates. After incubation, the general form of the colony, mycelial growth and the shape of the edge or margin were examined. Each colony with different morphology was picked up and then streaked onto a fresh medium to obtain pure cultures.

3.4.4 Primary screening of fungal strains

The screening of the fungal strains was carried out to check their potential for using lignin as sole source of carbon and energy. Initially thirteen fungal strains were isolated and purified. These strains were labeled as M-1 to M-9 and MF-1 to MF-4. The screening media (Czapek Dox agar) was made with the following composition; Sodium N itrate, Dipotassium Phosphate, Magnesium Sulfate, Potassium Chloride, Ferrous Sulfate, Sucrose, 200 mg/L lignin and 2% agar. After the preparation of the plates, each fungal strain was point inoculated in the centre of the plate and incubated at 37˚C for 8 days. Plates were monitored at regular interval throughout the ten days to check the zone of visible fungal colony and results were recorded.

3.5. Secondary Screening of lignin degrading fungi

3.5.1. Lignin degradation and colour reduction assay in shake flask experiments

In order to evaluate the efficiency of fungal isolates to degrade the lignin, the shake flask experiments were designed. Submerged fermentation was carried out using the MSM media. After preparation of the media in 250 mL flasks, each fungal strain was inoculated and experiments were set under varying condition of pH, temperatures and lignin concentrations. The flasks were incubated for 8 days and lignin degradation and colour reduction were monitored after every 24 hours interval. The colour reduction and lignin degradation were recorded for each fungal strain.

3.5.2. Colour reduction assay

For the measurement of the colour reduction, same samples were centrifuged at 10,000 rpm and change in the colour of the media was determined by using spectrophotometry at PtCob. 0.1214 standard solutions at 465 nm (Equation 2)

3.5.3. Lignin degradation assay

The lignin degradation was determined by using USA standard Pt. Cob01214 standard solution. For this purpose, sample from the same flasks were centrifuge at 8000 rpm for 30 minutes, followed by discarding the pellet and mixing the supernatant with 3ml of phosphate buffer (pH7.6) to measure the absorbance at 280 nm with UV-visible spectrophotometer (PGI/T60UV/VIS Spectrophotometer). (Equation 3)

3.5.4. High performance liquid chromatographic (HPLC) analysis

For extraction of lignin, 30 ml of fungal treated and untreated sample were centrifuged at 10000 rpm for 15 minutes. Then the supernatant was taken and acidification (pH1-2) was done by using 0.1N HCl. After that solvent extraction was performed (3times) with equal volume of ethyl acetate i.e. 30ml sample and 30ml ethyl acetate. The upper layer, also called organic layer, was collected and dewatered over anhydrous Na_2SO_4. After that the residues were dried under nitrogen. The dried residues were dissolved in acetonitrile and then HPLC (LC1620A) was performed using C-18 column (reverse phase column). For HPLC, LC pump 250 was used and the detector was UV/VIS LC 295. The column was C-18-WP 100, 4.6 mm×250 mm, 5 um. The solvent A used was methanol and solvent B used was distilled water o.1%, acetic Acid 30%. HPLC was done at the wavelength of 280 nm. The samples (20 µl) were injected followed by implementation of HPLC grade methano l: water (70:30) at the flow rate of 1 ml/minute (Ali et al., 2022).

3.5.5. Preservation of microbial culture

The fungal strains showed better results and were preserved in the glycerol medium at -20°C for further use.

3.6. Molecular identification of the isolated fungal strains

3.6.1. Genomic DNA extraction

For molecular identification of fungal strains genomic DNA was extracted. The fungal cultures were inoculated in their relevant media and incubated for 4 days. The next day after getting the significant growth, 2 mL of culture from each sample was collected in Eppendorf tubes and vortex for 2 minutes at 150 rpm. After that, 1.5ml of TE Buffer, 5µl of proteinase K and 30 µl of 10% sodium dodecyl sulphate (SDS) were added. The vials were incubated in water bath at 37°C for 1 hour. After the incubation 100 µl of acetyl trimethyl ammonium bromide (CTAB) buffer and 80 µl 5M sodium chloride (NaCl) was added and incubated again in water bath at 65°C for 10 minutes. Then 500 µl phenyl chloroform I (PCI) was added into the sample and centrifuged at 10,000 rpm for 20 minutes at 4°C. The upper layer was taken and transferred into new Eppendorf tubes. 500 µl PCI was again added into it and re-centrifuged at 10,000 rpm for 10minutes at 4°C. The upper layer was taken again and added the 500 µl isopropanol and 300 µl sodium acetate and placed overnight in refrigerator. After overnight incubation the samples were centrifuged again at 10,000 rpm at 4°Cfor 20 minutes. The supernatant was discarded and then 200 µl of 70% ethanol was added into the pellet followed by centrifugation for 10 minutes at 4°C. The supernatant was discarded and 100 µl TE buffer was added to dissolve the pellet. The partially purified DNA samples were stored in freezer at -20°C (Rodrigues et al., 2018).

3.6.2. Gel electrophoresis

For the purpose of gel electrophoresis, agarose gel was prepared by adding 27 ml of water and 3 ml of 10X TE buffer and heated in oven until the colour of the solution gets transparent. Then added 4 µl ethidium bromide and mixed it well. The comb was fixed carefully in gel tank and poured the gel into the tank. The gel was left to solidify for 25 minutes. After the solidification of gel, 3 µl of purified DNA sample was taken with the help of micropipette and mix it with 3 µl of loading dyes. The comb was then removed from the gel and the samples were carefully loaded in the wells of gel. The loaded gel was set into the chambers of gel tank. After 30 minutes of the process, the bands were visualized in order to check the accuracy of the gel electrophoresis.

3.6.3. DNA sequencing and phylogenetic analysis

For sequencing, the DNA of fungal strains (M-1, M-2, M-3 and M-4) were sent to Macrogen Sequencing Center, Korea. The MEGA 7.0 software was used to make the phylogenetic trees for each sample. Phylogenetic analysis was done for all the identified strain and similarity index of the selected strain was determined with all the available relevant species.

Research Phase II

3.7. Enzyme production from selected fungal strain

3.7.1. Fermentation media for the production of fungal extracellular enzymes

For this purpose, MSM was prepared for both strains in 250ml flasks according to Czapeck's original recipe containing monopotassium phosphate, $MgSO_4$.,KCl , $FeSO_4$, $NaNO_3$ and 200mg/ml of lignin. The prepared media was then inoculated with all four isolated fungal strains (2% inoculum) and placed in shaking incubator at 150 rpm for eight days. After incubation, the extracellular enzymes (Laccases, LiP, MnP) produced by each strain were measured by the methods given in the consequent text.

Table 3. 1 Composition of Czapeck agar recipe supplemented with lignin agar (Singh *et al*., 2015)

Reagents	Quantity (gram/L)
KH_2PO_4	0.35
KCl	0.5
$MgSO_4$	0.5
$NaNO_3$	2.0
$FeSO_4$	0.01
Lignin	2.0 %

3.7.2. Enzyme assays

3.7.2.1. Lignin peroxidase assay

The lignin peroxidase enzyme assay was carried out using 250 mM sodium tarterate buffer with a pH of 5.5, verateryl alcohol in the concentration of 10mM, and 4mM H_2O_2 (30%). This enzyme assay would be accomplished in silica cuvette. Later on, its absorbance would be measured by UV-Visible Spectrophotometer at 310nm.This spectrophotometer absorbance started to monitor on 0 time and then after each of 30 seconds observe at ambient condition. According to Beer's Law, the enzyme activity would be calculated as

$$A = \varepsilon dc$$

By rearranging the equation to solve for concentration and adding the thickness results in:

$$C = \frac{A}{s}(d = 1cm)$$

This 1cm would not affect the calculation and this will be ignored as units of E could be corrected. For measuring activity of enzyme, change in concentration over time can be determined as:

$$\frac{\Delta C}{\Delta t} = \frac{(\Delta As - \Delta Ab)}{s.\Delta t}$$

3.7.2.2. Manganese peroxidase assay

MnP peroxidase assay was done by the method of Nguyen *et al*., 2017. Briefly, 100 micro liter of culture supernatant was incubated with prescribed amount of malonate buffer containing $MgSO_4$. The reaction was supported by the addition of H_2O_2, followed by incubation at 35C for 30 minutes. The malonate Mg complex was quantified with the help of visible spectrophotometer at 270nm. Where one enzyme unit is defined as the amount of enzyme used to produce 1mol of product (Rekik et al., 2019).

3.7.2.3. Laccases assay

The laccase activity was measured by ABTS method described by (Muthuvelu et al., 2020). 0.5mM ABTS and 0.1 molar Na acetated were mixed together with the enzyme. The oxidation of ABTS was measured in relation to increase in absorbance of reaction mixture at 420nm.The reaction mixture contains ABTS and sodium acetate buffer were mixed with 100microliter supernatant culture and absorbance was measured at 420nm.

3.7.3. Effect of carbon source on the production of the peroxidases enzymes by selected microbial isolates (Laccases, Mn Peroxidase, Lignin peroxidase)

The four fungal strains which showed significant peroxidases enzyme production in MS broth amended with 2% lignin at 37^0C, 120 rpm for eight days were further analyzed by using different carbon source. Three different carbon sources i.e. glucose, sucrose and lignin were evaluated for the ability to maximize the production of peroxidases enzymes by selected fungal strains. After eight days of incubation period, each fungal strain was tested for the production lignin peroxidase, Mn peroxidase and laccase with the methods already mentioned in section 3.7.2.

3.7.4. Effect of nitrogen source on the production of the peroxidases enzymes by selected microbial isolates (Laccases, Mn Peroxidase, Lignin peroxidase)

Two different nitrogen sources i.e. peptone and sodium nitrate $NaNO_3$were used four fungal strains (M-1, M-2, M-3 & M-4) to enhance their ability to produce lignocellulytic enzymes. For this purpose, broth amended with 2% lignin and varying concentration of peptone or sodium nitrate was inoculated with 2% inoculum separately, and incubated at 37^0C, 120rpm for eight days. After eight days of incubation period, each fungal strain was tested for the production lignin peroxidase, Mn peroxidase and laccase with the methods already mentioned in section 3.7.2.

3.7.5. Optimization of fungal peroxidases under different experimental conditions in shake flask experiment

3.7.5.1. Optimization of bioprocess for enzyme production:

In order to maximize the production of fungal peroxidases, 250ml of the Minimal salt media was used and the flasks were inoculated with 2% inoculums of fungal strains (M-1, M-2, M-3 & M-4) separately with optimum quantity of sucrose and peptone. Flasks were incubated in a shaker incubator, 120rpm for eight days at varying range of temperature (37°C, 41°C and 47°C) and pH 7-9.

3.8. Purification and precipitation of Enzymes

3.8.1. $(NH_4)_2 SO_4$ precipitation's optimization

Ammonium sulfate ($(NH_4)_2SO_4$) was added in different concentrations (30,40,50,60,70,90) to 100ml of the crude enzyme aliquots that was kept on the crushed ice. By centrifugation (Kokosan Model H-251) the precipitates were collected at 10,000 rpm at 4°C for 30 minutes. The protein content and peroxidase activity of the pellete and supernatant were checked and on the basis of the activity of peroxidase by the enzyme assay the optimum concentration of Ammonium sulfate was determined.

3.8.2. Gel filtration chromatography using Sephadex G-75

From 10mM Tris buffer (pH 7.5) in the 500ml, the 10.0 g Sephadex was soaked that contains sodium azide (0.1g) that act as bacteriostatic agent. In a sonicator the gel homogenized for 20 minutes was packed in a column (0.9×60 cm) and until fully packed the gel was washed with a 10mM Tris Buffer (pH 7.5). To the column the enzyme that was precipitated was applied (2ml) and with excess volume of the buffer (10mM tris (pH 7.5)) it was eluted. The fractions were collector and after that absorbance of the samples were recorded at 280nm. The high absorbance fractions were analyzed further for protein estimation and the activity of enzyme.

Determination of molecular weight of the peroxidases (SDS-PAGE)

Now next step was molecular mass determination of peroxidase enzymes. First s tep was protein extraction.0.5M NaCl was used, 1 ml of it was taken in an Eppendorf and enzyme was poured in it, then it was mixed by agitation, then micro centrifuged at 13000 rpm for 10 minutes. Pellet was used leaving the supernatant for further processing.1 ml distilled water was used for pellet washing purpose repeatedly. Then, Gel sample buffer of 50 µl concentrations was added to pellet and it was water bathed for 10 minutes at 100°C. After this it was cooled in ice for 10 minutes, again micro centrifuged for 1 minute at 13,000 rpm. The SDS-PAGE method uses 12.25% polyacrylamide gel for protein analysis. At the bottom pool of apparatus, the electro buffer was poured and placement of gel pates also occurs provided no air bubbles present under gel. Then protein samples (supernatant) were loaded of 15 µl volume was loaded into gel wells. In the same way protein markers of known molecular

weight which was pre-stained were loaded. Constant voltage of 100Vand 20 mA was used for gel run purpose until at the end of gel plates front dye should be reached. After this step, battery was switched off and gel plates were removed.

Now the next step was protein detection which involves steps of separation of gel plates and their staining and destining. Spatula was used for gel plate's separation purpose and stacking gels were also removed then those separated gels were transferred into box rotary in which staining solution was present and placed on a shaker for full night. Then staining step was finished when staining solution was removed and by washing gel 2 or 3 times with distilled water. Next step was de-staining which started by adding de-staining solution and shaking till gel background disappears. After this step in the light box gel was placed and its snapshot wa s taken. Then was the final step of analysis in which samples molecular weight and variations were recorded by comparison with standard marker protein/enzyme

Research Phase III

3.9. Preparation of lignin samples

The commercially available Sigma alkali lignin was subsequently washed with water to separate insoluble lignin residues from soluble one. The insoluble lignin residues are water-extracted in a concentration of 2% (w/w) at 40^0C through centrifugation at 4000rpm for 10 min until lignin gives no more colour to water. The obtained insoluble lignin residue then treated with 70% ethanol for 24hr by keeping it on rotary shaker to sterilize the sample. After 24hr sample centrifuged and washed multiple times with 70% ethanol at aforementioned conditions until the A_{280} gives 0.00 absorbance. Dried out the sample in sterile conditions and stored insoluble residues of lignin stored at 4^0C for further use.

3.10. Fungal growth in media containing lignin:

Media used for the growth of fungal strain is modified Czapeck Dox medium (Potassium phosphate monobasic 1g/L; Sucrose 2g/L; Magnesium sulphate 0.5g/L; Potassium Chloride 0.5g/L; Ferrous Sulphate 0.01g/L; Sodium Nitrate 2g/L, Agar 2g/L) at optimized growth condition 30^0C. By using all these components 200 ml of media was prepared in 500ml of flask and autoclaved, different concentration of lignin pure, water soluble and water insoluble were added in autoclaved media. Three sets of experiments were conducted with different concentrations of lignin pure, water soluble, insoluble with varying carbon concentration. First set contain 0.2% water soluble lignin (2g/l) with 0.2% of sucrose in triplicates and 0.4% of water soluble lignin with no sucrose. Second set contains 0.2% of water insoluble lignin with 0.2% of sucrose and 0.4% of water insoluble lignin with no carbon source. Third set contain 0.2% of pure lignin and sucrose and 0.4% of water insoluble lignin without any other carbon source. All the experiments were performed in triplicates and incubated in static incubator at 30^0C for 8 days. Growth was determined at 8th day of experiment.

3.11.Effect of different carbon sources on the growth of fungi:

Fungal strains are optimized to grow on different carbon sources varied from simpler to complex carbon component, including Starch, Xylan, Apfelpekin, Cellulose, Manan and lignin with 0.5% concentrate of each by using the aforementioned media component, sterilized the media by autoclaving along with following carbon sources

soluble starch, Xylan, Manan, apfelpektin. Lignin was used in its soluble form and sterilized by syringe filtration and Cellulose (Avicel) was used after washing with MilliQ water and 70% ethanol, after drying it was added to the medium.

3.12.Effect on different lignin variants on the growth of fungi:

Lignin degradation by different fungal isolates were also optimized on different derivatives of lignin, the idea was behind to check which component or derivative is being degraded by fungal strains. Six different lignin derivatives along with control of lignin and cellulose were grown on optimized fungal media with 0.2% concentration in 1000ml. The results were remarkable, as the strains shows different growth pattern on different substrate, this idea anticipate the information from NMR spectroscopy, as it was defining the degradation comparatively well as compare to whole lignin.

3.13.Analysis of biodegradation of lignin by NMR

3.13.3. Sample preparation for NMR

By growing fungal strains on different lignin derivative as well as different carbon sources, it narrows down our knowledge for determine the lignin degradation pattern in the right direction. Fungal strains were then grown on soluble, insoluble and ligno-sulfonic acid to understand the depth of lignin degradation that which functional group change and at which point the lignin degradation occurred. Samples were obtained at 8th day of fungal growth; centrifuge separated the pellet freeze the culture at -80^{0}C and then freeze dried the sample and NMR analysis was conducted by dissolving 600 micro liters of freeze-dried sample into DMSO (Shayesteh & Zamanloo, 2020).

3.13.3 Determination of lignin degradation by NMR analysis

Kraft lignin was incubated in the absence or in the presence of the isolated lignolytic yeast strains in aforementioned modified Czapeck Dox medium for 8 days. The liquid fraction of the culture was collected by centrifugation and was subsequently condensed. Of this condensed fraction, 30 mg were extracted into 550 µl DMSO-d_6 and the resultant sample was transferred to a 5 mm NMR sample tube. All NMRspectrawereacquired at 323 K on an 800 MHzBrukerAvanceIII HD instrumentequippedwitha 5 mm TCIcryoprobe and a Sample Jet sample changer. Sensitivity enhanced ^{1}H-^{13}CHSQC spectra were acquired by sampling 1024×512

complex data points for 128ms and 7.7ms in the direct and indirect dimension, respectively. For *Geotrichum candidum* strain M-3 and the substrate incubated in the absence of microorganisms, additional multiplicity-edited ^{1}H-^{13}C HSQC spectra were acquired, sampling 1024×128 complex data points for 79.9ms and 4.5ms in the direct and indirect dimension, respectively. ^{1}H-^{1}HCOSY was acquired to gain insight into products formed with strain M-3 by sampling 2048 × 512 complex data points for 255.6ms and 31.9ms in the direct and indirect dimension, respectively. ^{1}H-^{1}HTOCSY (2048 × 256 complex data points sampling 255.6ms and 31.9ms in the direct and indirect dimension, respectively) was acquired with a 10 kHz spin lock field and a mixing time of 80 ms. ^{1}H-^{13}CHSQC spectra of 5 mg authentic standard compounds (including vanillin, acetovanillone and benzylalcohol) were acquired in 550 µl DMSO-d^{6}. All NMR spectra were processed with a shifted sine-bell apodization function and ample zero filling in both dimensions using Bruker Topspin 3.5 pl6 software.

3.14. High performance liquid chromatographic (HPLC) analysis of lignin degradation:

To determine the degradation process by selected isolates for different lignin monomers Reverse phase HPLC analysis was performed with different derivatives used as substrate for growth. The samples were incubated for 6 days on different substrate including guaiacol, hydroxybenzenealdehyde, syringaldehyde. Interval of every 2 days 1ml of all the samples with inoculated substrate and control were centrifuged at 10000 rpm for 15 minutes. Then the supernatant was taken and acidification (pH1-2) was done by using 0.1N HCl. After that solvent extraction was performed (3times) with equal volume of ethyl acetate i.e. 30ml sample and 30ml ethyl acetate. The upper layer, also called organic layer, was collected and dewatered over anhydrous Na_2SO_4. After that the residues were dried under nitrogen. The dried residues were dissolved in acetonitrile and then HPLC (LC1620A) was performed using C-18 column (reverse phase column). For HPLC, LC pump 250 was used and the detector was UV/VIS LC 295. The column was C-18-WP 100, 4.6 mm×250 mm, 5 um. The solvent A used was methanol and solvent B used was distilled water o.1%, acetic Acid 30%. HPLC was done at the wavelength of 280 nm. The samples (20 µl)

were injected followed by implementation of HPLC grade methanol: water (70:30) at the flow rate of 1 ml/minute.

4 Results

Results Phase I

In present research biodegradation of the lignin was studied using fungal isolates obtained from pulp and paper industry waste water. At first phase of the research, the samples were subjected for microbial isolation and physiochemical analysis in order to find out microbial diversity present in the black liquor and the level of organic pollution. Microbial strains were isolated, screened and optimized for their lignin degrading metabolic potential. In the next phase, the extra cellular enzymes production for lignin degradation was optimized at different concentration of lignin and nutrients. In the third phase, lignin biodegradation by fungal strains *Aspergillus terrus*, *Dipodascus australiensis*, *Geotrichum candidum* and *Aspergillus fumigatus* on different lignin substrate was determined by NMR and HPLC analysis.

4.1 Sampling and physiochemical characterization of the Black Liquor

The wastewater (black liquor) samples obtained from paper and pulp industry, Kraft process outlet was characterized. It was noted that the BL sample was slightly alkaline with high lignin contents. Furthermore, total dissolved and suspended solids were found to be 622 and 460 mg/L respectively, with 17082 mg/L chemical oxygen demand (COD). The nitrates and sulphates in the BL sample were 26671 and 877 mg/L. The following results (Table 4.1(a,b) gives the analysis of lignin degradation and other pollutants before and after treatment by selecting microbial isolates.

Table 4. 1 . Analysis of Black Liquor obtained from Bhulle Shah Packages Limited (Before treatment)

Sample	pH	COD mg/L	Sulphates mg/L	TDS mg/L	TSS mg/L	Colour PCU	Nitrates mg/L	Lignin mg/L
Black Liquor	7.9	17082	26671	622	460	1033	877	4413

Table 4. 2. Analysis of Black Liquor obtained from Bhulle Shah Packages Limited (After Treatment)

Sample	pH	COD mg/L	Sulphates mg/L	TDS mg/L	TSS mg/L	Colour PCU	Nitrates mg/L	Lignin mg/L
Black Liquor	7.9	16564	22875	548	386	934	877	1089

4.2 Isolation and screening of the lignin degrading fungal strains

The wastewater samples were used for the isolation of the lignin degrading fungal strains. Since, sample have sufficient carbon source in the form of lignin, therefore, support growth of diverse fungal communities. After serial dilution of the samples, 13 fungal strains were obtained. These fungal cultures were then analyzed for their metabolic potential to use and degrade lignin on agar plate containing lignin. Out of these 13 strains, 7 fungal strains (M-1, M-2, M-3, M-4, MF-1, MF-2 and MF-3) showed better potential for lignin degradation on the agar plate assay and produces higher degradation zones. However, the zones for other 6strains (M-5, M-6, M-7, M-8, M-9 and MF-4) were comparatively smaller. On the basis of these results, aforementioned 7 strains were subjected to secondary screening assay by using the specific media czapek Dox. It is selective media for isolation of fungal lignin degraders. This media is reported specifically for the isolation of fungal strains from waste water including *Aspergillus niger, Penicillium camembert*ias well as saprophytic fungi including *Aspergillus candida, Penicillium sp* and *Paecilomyces.*(Salvachúa *et al.*, 2016).

Table 4. 3 Zones of lignin degradation and growth of fungal strains

Fungal Strain	Zone of lignin degradation (mm)	Growth
M-1	15	+++
M-2	13	+++
M-3	11	+++
M-4	9	+++
M-5	8	++
M-6	5	++
M-7	3	+
M-8	2	++
M-9	11	++
MF-1	7	+++
MF-2	9	++
MF-3	11	+++
MF-4	6	++

+++ rich growth, ++, medium growth, + less growth

4.2.1. Secondary screening

The results of the secondary screening revealed the ability of the fungal isolates to use lignin as the substrate and show growth in the presence of lignin along with other nutrients. Initially seven fungal strains (M-1, M-2, M-3, M-4, MF-1, MF-2 and MF-3) were screened out as a result of primary screening. But after rescreening, four isolates (M-1, M-2, M-3and M-4) were found to have great ability to grow in the presence of

varying concentrations of the lignin and sugar source i.e. sucrose. Among these four stains, M-1 showed maximum growth of 68% at 37°C and pH7, M-2 showed maximum fungal growth of 89% at 47°C and pH7, M-3 showed maximum growth of 71% at 37°C and pH9 while M-4 showed maximum growth of 70% at 37°C and pH10. These results indicate that M-3 is the most robust fungal strain in terms of growth at varying range of pH and temperature. Growth of these fungal strains at pH range of 7-10 also depicts that these strains can survive in the raw effluent, which has alkaline nature. Growth at varying temperature suggests that M-2 strain is neutrophilic slightly thermophile, strains M-2 and M-3 are neutrophilic alkaLiPhiles and strain M-1 is mesophilic in nature.

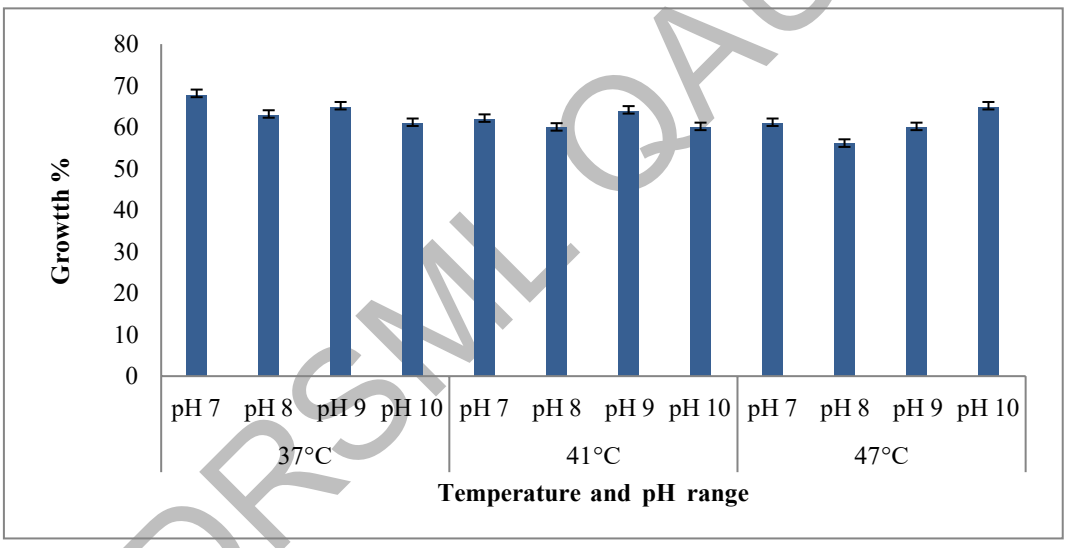

Figure 4. 1 : Growth pattern of *Aspergillus terrus* (M-1) under varying pH and temperatures

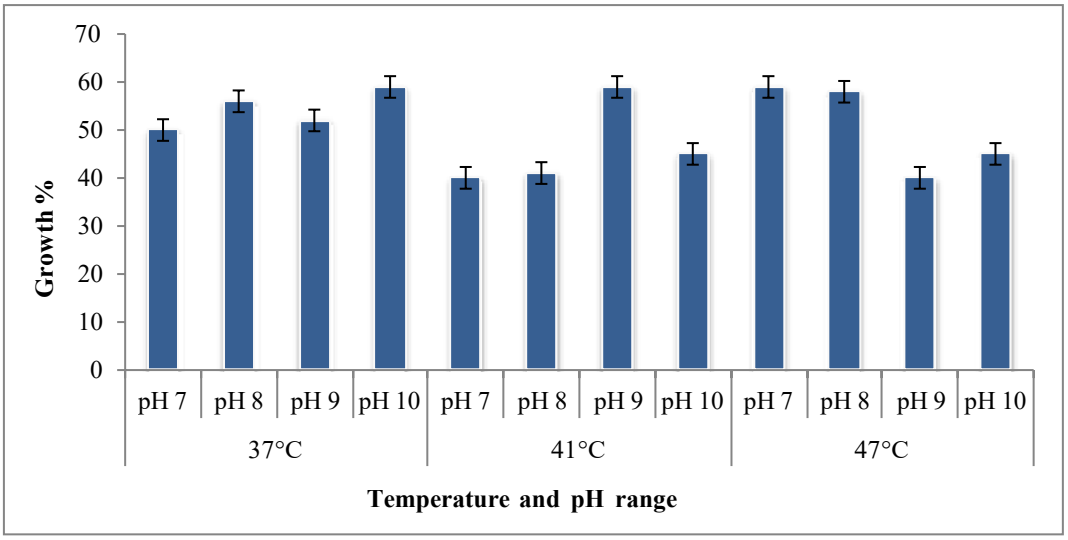

Figure 4. 2: Growth pattern of *Dipodascus autraliensis* (M-2) under varying pH and temperatures

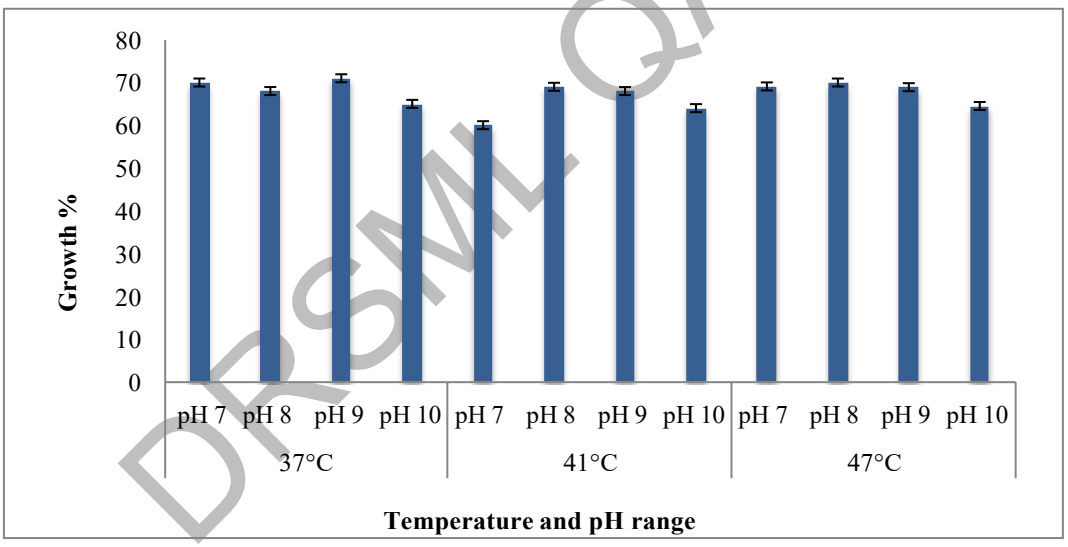

Figure 4. 3: Growth pattern of *Geotrichum candidum* (M-3) under varying pH and temperatures

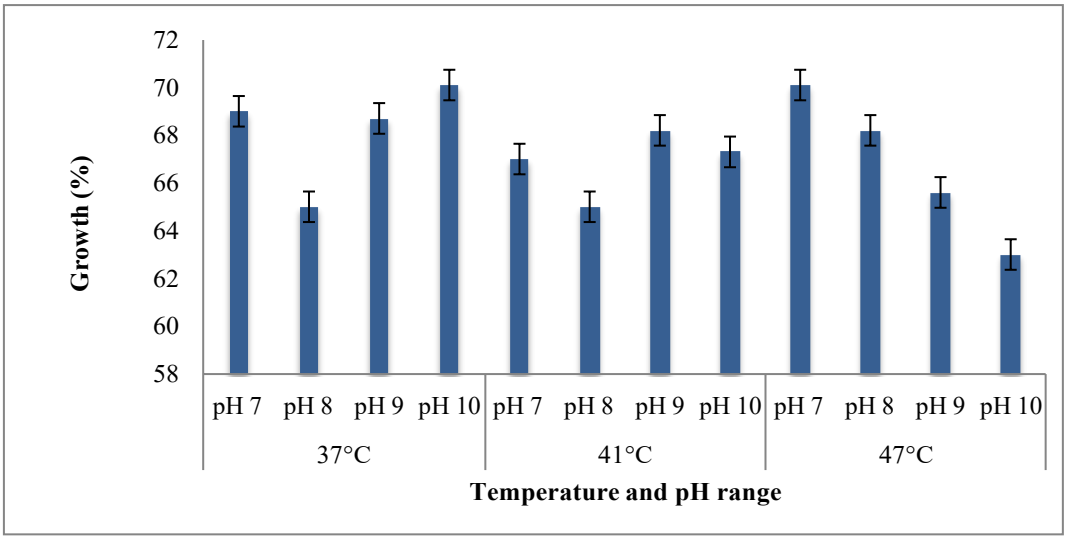

Figure 4. 4: Growth pattern of *Aspergillus fumigatus* (M-4) under varying pH and temperatures

4.3 Lignin degradation assay

In the present research, M-1 showed maximum lignin degradation (73%) at two different environmental conditions i.e. at 37°C at pH8 and 47°C and pH9. Similarly, both fungal strains M-2 and M-3 showed 84% lignin degradation at 41°C and pH9 and 47°C and pH9 respectively, while M-4 showed 80% lignin degradation at 41°C and pH10. Among all four strains, M-2 and M-3 showed maximum capacity to degrade lignin under alkaline and slightly thermophilic conditions. Despite the resistance of lignin to degradation, numerous fungi are able to cause breakdown of lignin.

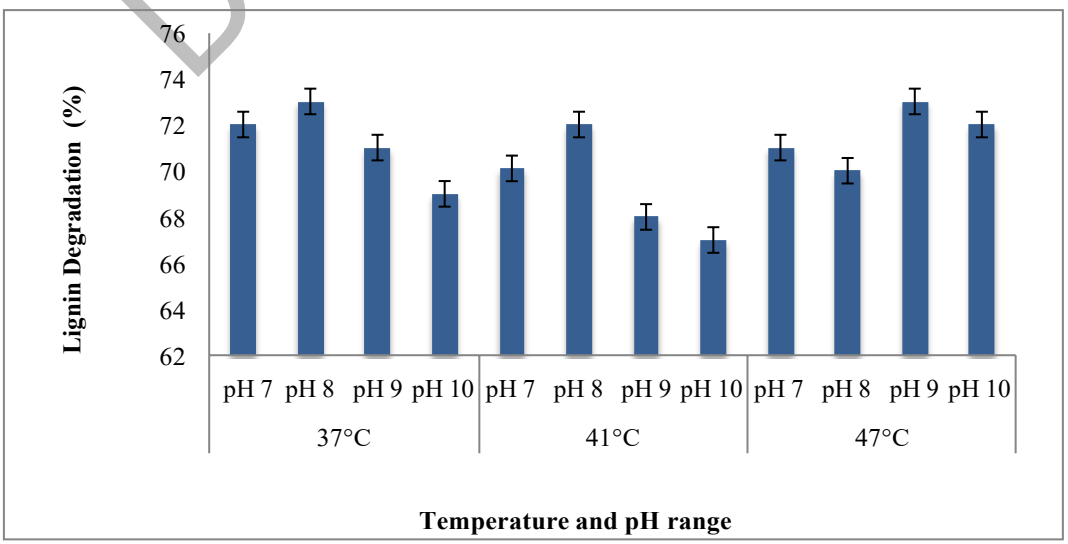

Figure 4. 5: Lignin degradation (%) by *Aspergillus terrus* (M-1) under varying pH and temperatures

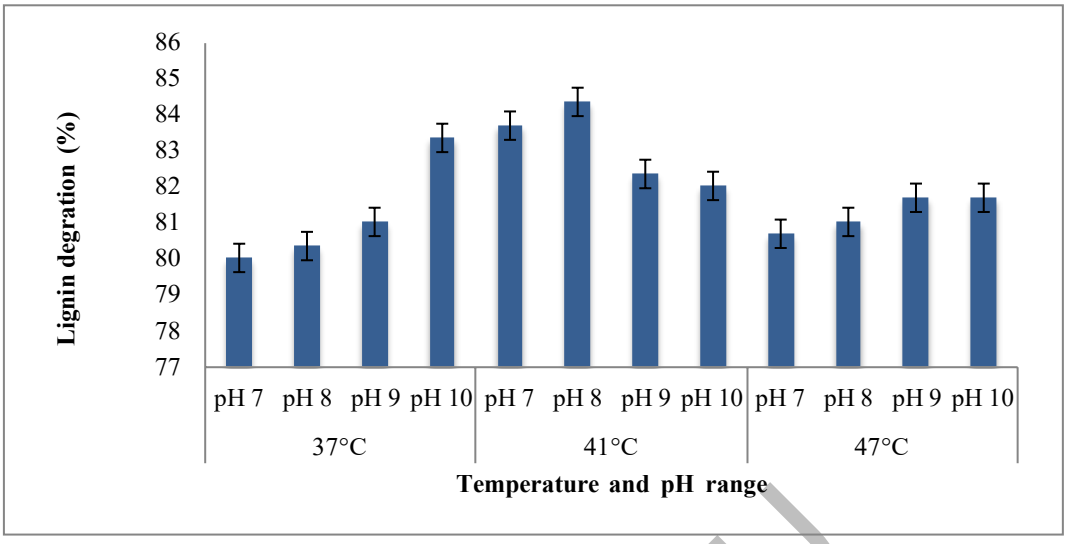

Figure 4. 6Lignin degradation (%) by *Dipodascus autraliensis* (M-2) under varying pH and temperatures

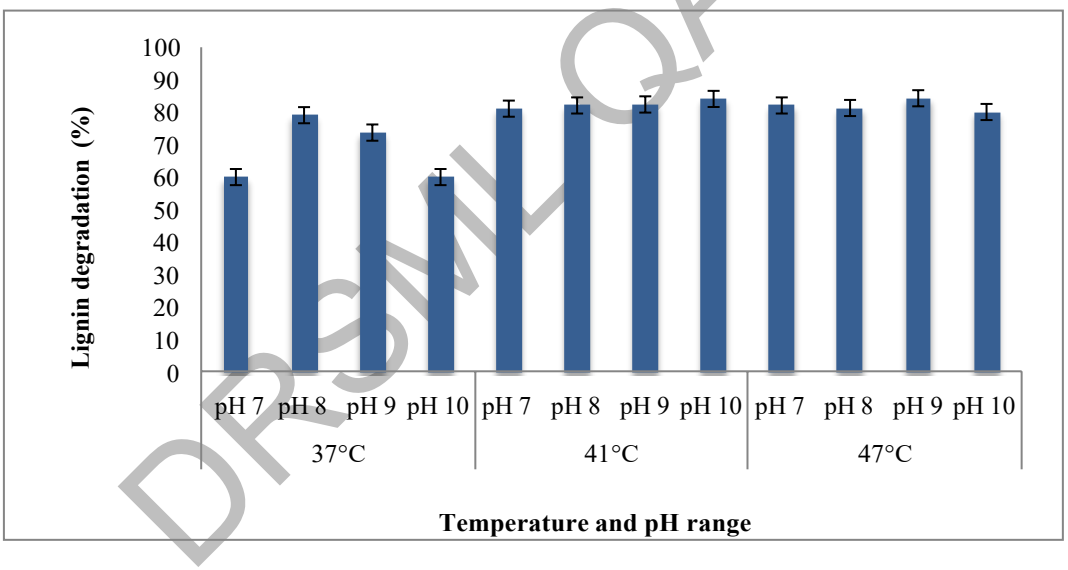

Figure 4. 7 Lignin degradation by *Geotrichum candidum* (M-3) under varying pHand temperatures

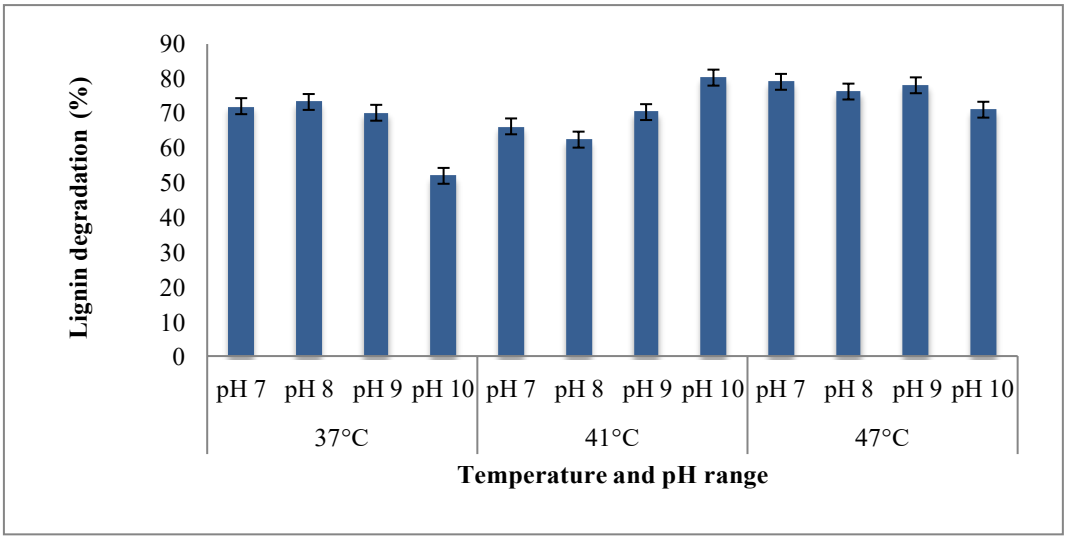

Figure 4. 8. Lignin degradation by *Aspergillus fumigatus* (M-4) under varying pH and temperatures

4.4 Colour reduction assay

Currently, M-1, M-2, M-3 and M-4 showed colour reduction of 19% (at 47°C and pH 10), 11.33% (at 41ºC and pH 10),11% (at 41ºC and pH 9) and 15% (at 37ºC and pH 9) respectively (Figure 4.3). This colour reduction was due to presence of lignin degrading enzymes such as lignin peroxidase (LiP) and manganese peroxidase (MnP) produced by *G. candidum* (Asses *et al*., 2009).

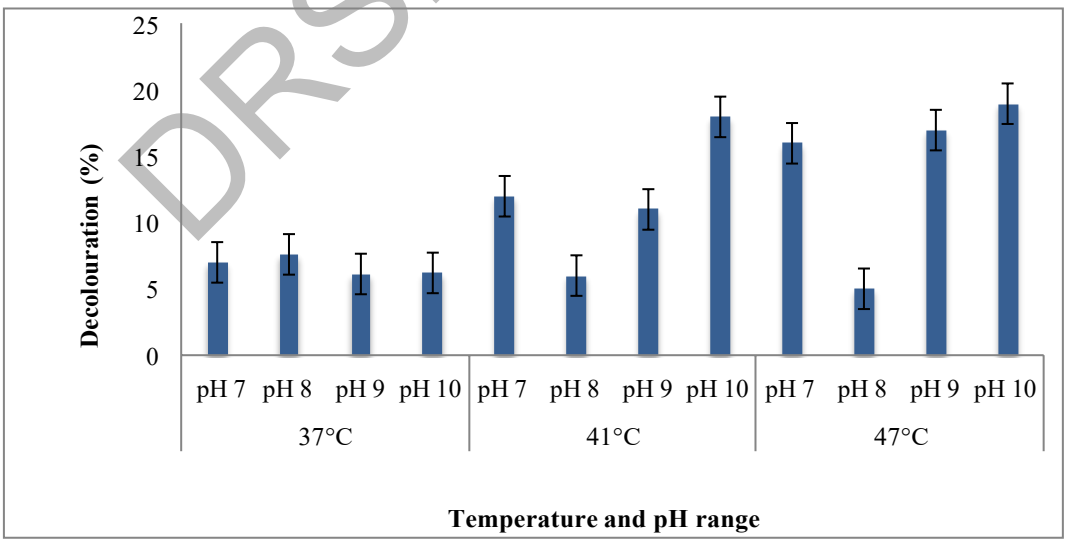

Figure 4. 9: Decoloration lignin by *Aspergillus terrus* (M-1) under varying pH and temperatures

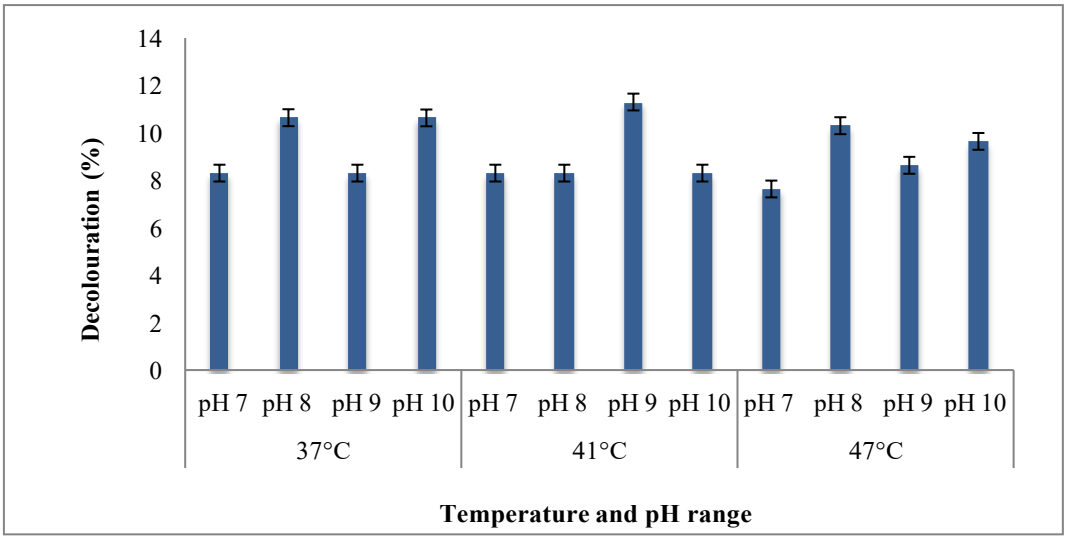

Figure 4. 10: Decoloration lignin by *Dipodascus autraliensis* (M-2) under varying pHand temperatures

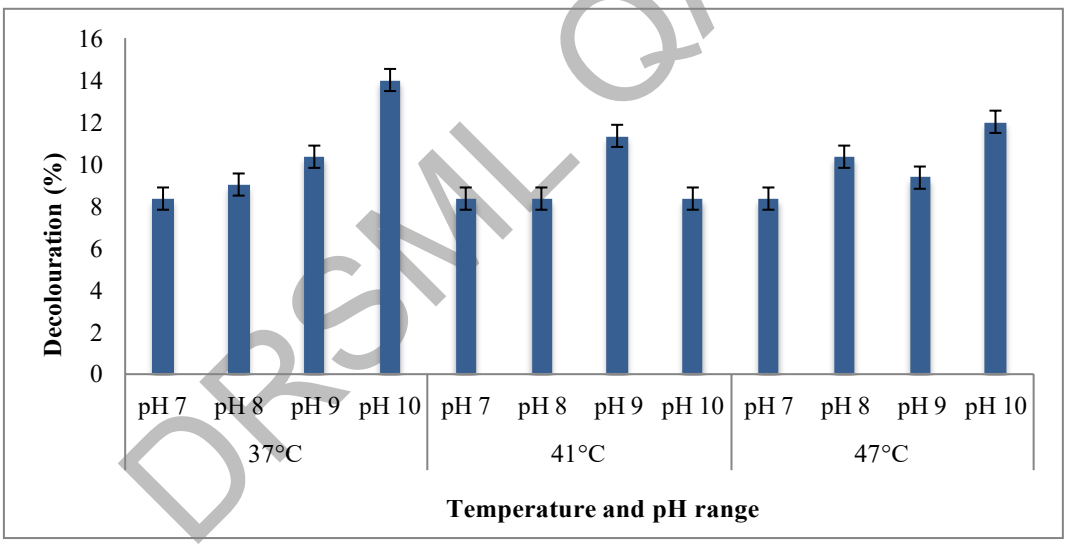

Figure 4. 11: Decoloration by *Geotrichum candidum* (M-3) under varying pH and temperatures

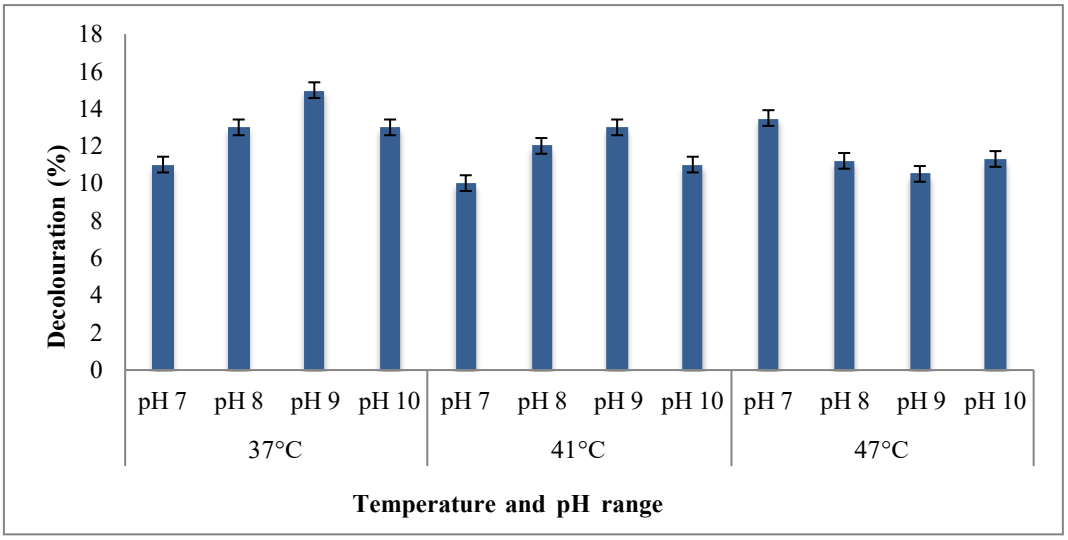

Figure 4. 12: Decolouration (%) by *Aspergillus fumigatus* (M-4) under varying pH and temperatures

4.5 Gel electrophoresis of the DNA samples of the isolated fungal strains

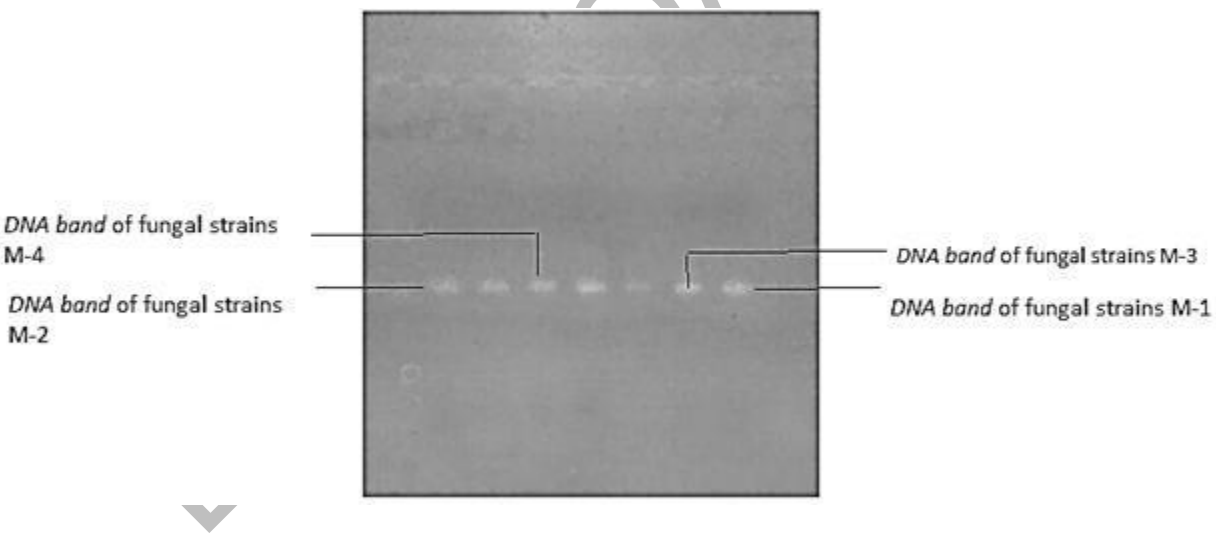

Figure 4. 13: DNA band of fungal strains M-1, M-2, M-3 and M-4

4.6 Molecular Identification of the isolated fungal strains

DNA of four fungal samples i.e., M-1, M-2, M-3 and M-4 were sequenced for the identification. First ABI files were open for each sequence and trimmed the redundant peaks of chromatogram in Mega 10 Software then the trim sequences were blast. The maximum homology was recorded about 97-99%. All the sequences of maximum

homology were collected in a note pad file and then a phylogenetic tree was constructed showing that our target sample was how much similar to the all other sequences found in NCBI database. Strain M-1 is identified as *Aspergillus terreus* (Figure 4.5) and M-2 is *Dipodasceus australiansis* (Figure 4.6) which is novel strain as it not been reported so far for the degradation of the lignin or for the treatment of black liquor (BL). While, M-3 and M-4 were identified as, *Geotrichum candidum*(Figure 4.7) and *Aspergillus fumigates* (Figure 4.8) respectively. In strain M-1,*Aspergillusterreus*an enzyme, alkaline manganese peroxidase have reported previously for lignin degradation and colour reduction of black liquor (Kanayama *et al*., 2002). Strain, M-3, *Geotrichum candidum* showed 10% increase in colour reduction when black liquor is amended with veratryl alcohol. This decolourization ability of *Geotrichum candidum* is due to presence of lignin peroxidase enzyme (Ayed *et al*., 2005).Strains of *Aspergillus* has been reported to reduce the colour of black liquor and lignin content. A decrease in colour content was about 39-61% and colour reduction was about 51% in ten days of incubation in free state and significant increase in the results were observed when *Aspergillus* cultures were immobilized and incubated for six days (Barapatre & Jha, 2016).

Table 4. 4. Percentage homology of *Aspergillus terrus* (M-1) with relevant species

Description	% Similarity	Max Score	Total Score	Accession #
Aspergillus terreus isolate M-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1066	1066	100	MW130259.1
Aspergillus terreus strain MBL1414 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	977	1293	97.23	KM924436.1
Aspergillus terreus isolate MD11_2(1) 18S ribosomal RNA	977	977	97.23	JQ697519.1

gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence				
Aspergillus terreus isolate XGSF-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	977	977	97.23	MT141149.1

Table 4. 5. Percentage homology of *Dipodascus australiensis* (M-2) with relevant species

Description	% Similarity	Max Score	Total Score	Accession #
Dipodascus australiensis isolate M-2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene an	100	621	621	MW130293
Dipodascus australiensis 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	98.50	577	577	AF157596
Dipodascaceae sp. CPK3512 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and	97.65	573	573	FJ827630

28S ribosomal RNA gene, partial sequence				
Dipodascaceae sp. DX-2011b isolate J8M-55 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	99.06	571	571	JN227041

Table 4. 6. Percentage homology of *Geotrichum candidum* (M-3) with relevant species

Description	% Similarity	Max Score	Total Score	Accession #
Geotrichum candidum isolate M-3 internal transcribed spacer 2 and large subunit ribosomal RNA gene, partial sequence	100	401	401	MW227567.1

Table 4. 7. Percentage homology of *Aspergillus fumigatus* (M-4) with relevant species

Description	% Similarity	Max Score	Total Score	Accession #
Aspergillus fumigatus isolate M-4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	100	1048	1048	MW130257.1
Aspergillus fumigatus strain sM-3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	99.645	1029	1029	KU561918.1
Aspergillus fumigatus isolate FR18 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	99.645	1029	1029	KP689196.1
Aspergillus fumigatus isolate 16 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	99.821	1027	1027	MH345856.1

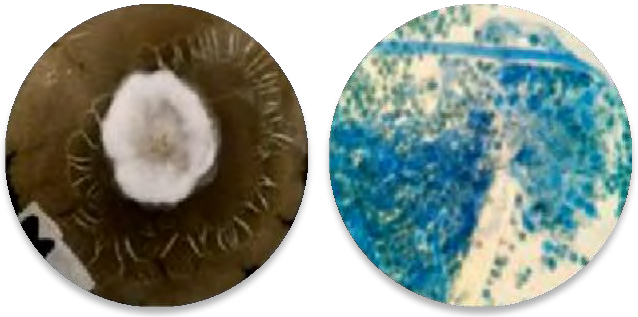

Figure 4. 14. Growth pattern of M-1 (*Aspergillus terrus*)

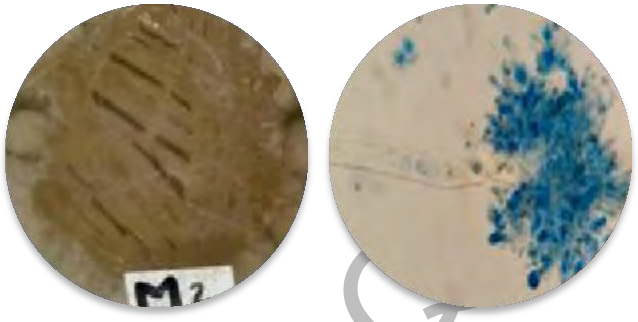

Figure 4. 15. Growth pattern of M-2 (*Dipodascus australiensis*)

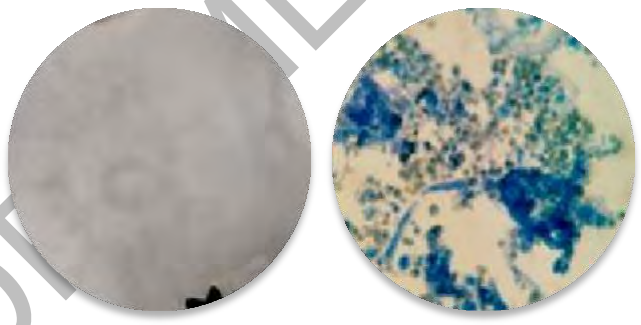

Figure 4. 16. Growth pattern of M-3 (*Geotricham candidum*)

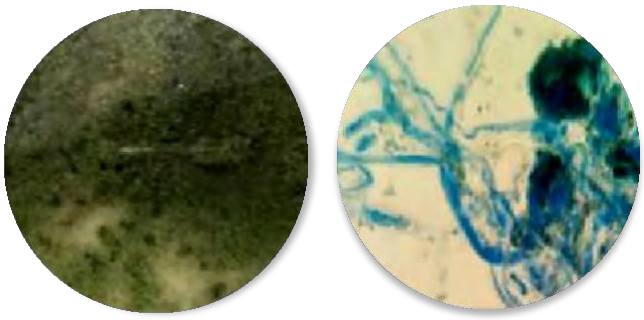

Figure 4. 17. Growth pattern of M-4 (*Aspergillus fumigatus)*

4.7 High performance liquid chromatographic analysis

High pressure liquid chromatography (HPLC) is an analytical technique used to quantify individual components of the mixture. In present study, HPLC is used as a conformational tool for lignin degradation. Furthermore, HPLC analysis of control and fungal strains degraded showed reduction in the lignin content and shifting of peaks is observed, which indicates transformation of compounds in the black liquor. The results obtained from *Aspergillus terrus* strain M-1 demonstrated highest peak at the retention time 5.38 representing degradation of lignin complex structure into an intermediate polymer, syringaldehyde. The other important peaks were observed at retention times of 4.73, 3.84, 3.38 showed transformation of lignin into veratryl alcohol, syringic acid and vanilic acid respectively. In the case of strain *Dipodascus australiensis* M-2 the results showed slight shift in the highest peak to 5.26 retention time. This represents the lignin structure conversion into *p*-courmaric acid. The conversion of lignin structure into polymers *p*-hydroxybenzaldehyde at 4.01 retention time. Figure 10 (c) exhibited the degradation pattern of lignin from *Geotrichum candidum* strain M-3 by HPLC. The prominent peak was observed at 4.80 retention time. This peak was comparatively different from the previous peaks obtained by aforementioned fungal strains. The lignin intermediate degrading polymer 4-Ethoxy-3-methoxybenzaldehyde was formed. The biodegradation of *Aspergillus fumigatus*strain M-4 by HPLC was recorded. The strain showed the breakdown of lignin heteropolymer to degrading intermediate polymer *p*-hydroxybenzaldehyde at retention time 4.01. Other peaks obtained were 4.73, 3.84 and 3.38 that represent the veratryl alcohol, syringic acid and vanilic acid respectively.On the basis of the HPLC analysis results of lignin degradation suggests these fungal strains have the ability to convert complex lignin hetero-polymeric structure to relatively simpler ones.

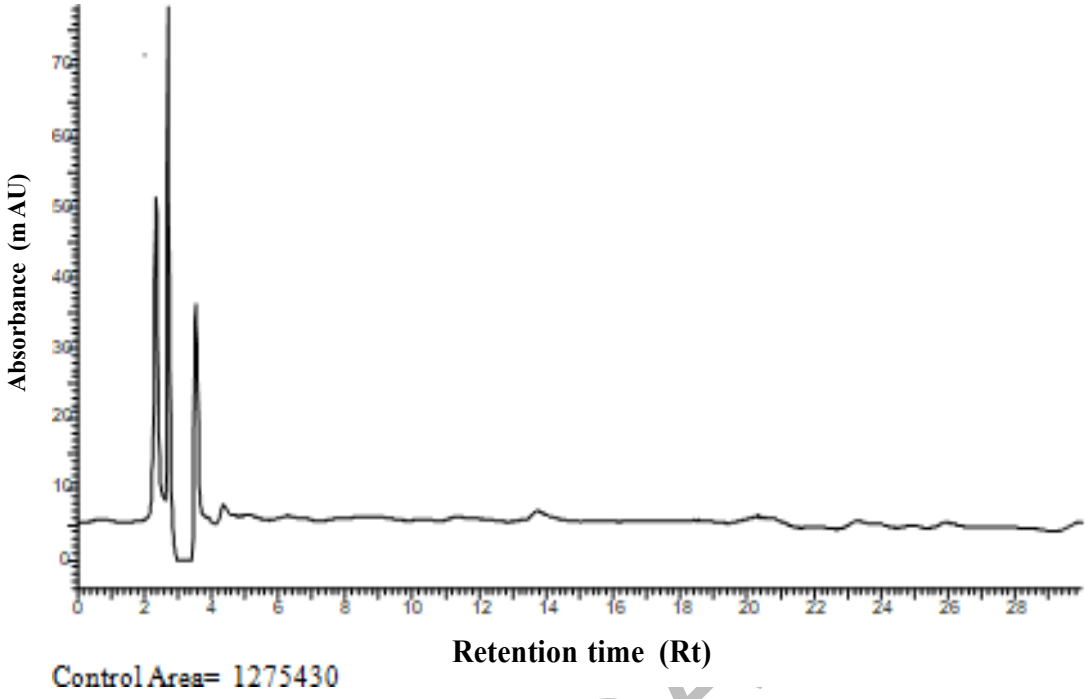

Figure 4. 18: HPLC of untreated Lignin Control (pure Lignin)

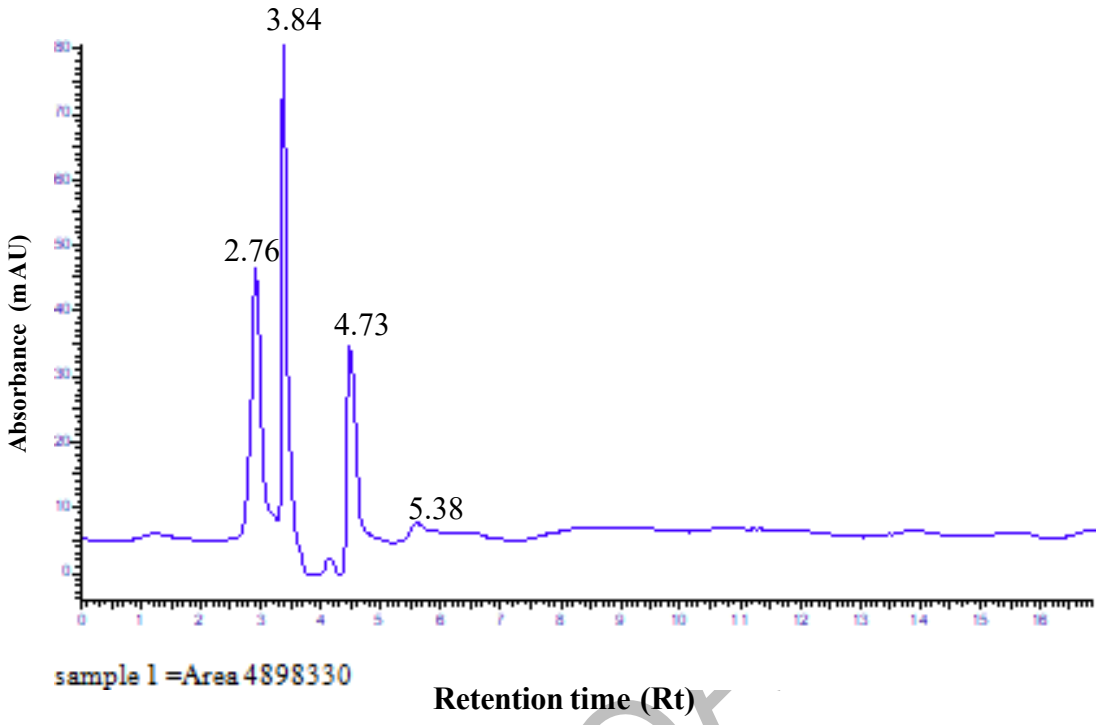

Figure 4. 19: HPLC analysis of lignin biodegradation by *Aspergillus terrus* (M-1)

Table 4. 8. Lignin degraded compounds of *Aspergillus terrus* (M-1) detected by HPLC at specific retention times

Compound	Retention Time
Syringaldehyde	5.38
Syringic acid	3.84
Vanilic acid	3.38
Veratryl alcohol	4.73
Catechol	2.76

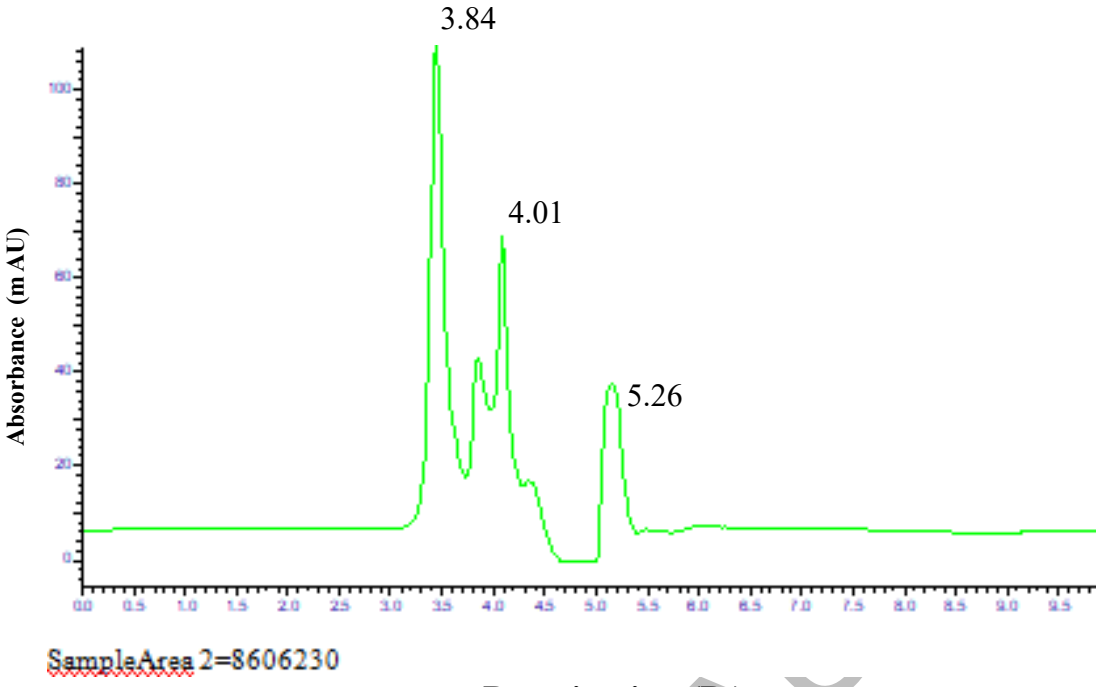

Figure 4. 20: HPLC analysis of lignin biodegradation by *Dipodascus australiansis* (M-2)

Table 4. 9. Lignin degraded compounds of *Dipodascus australiansis* (M-2) detected by HPLC at specific retention times

Compound	Retention Time
p-coumaric acid	5.26
Syringic acid	3.84
Vanilic acid	3.38
p-hydroxybenzaldehyde	4.01

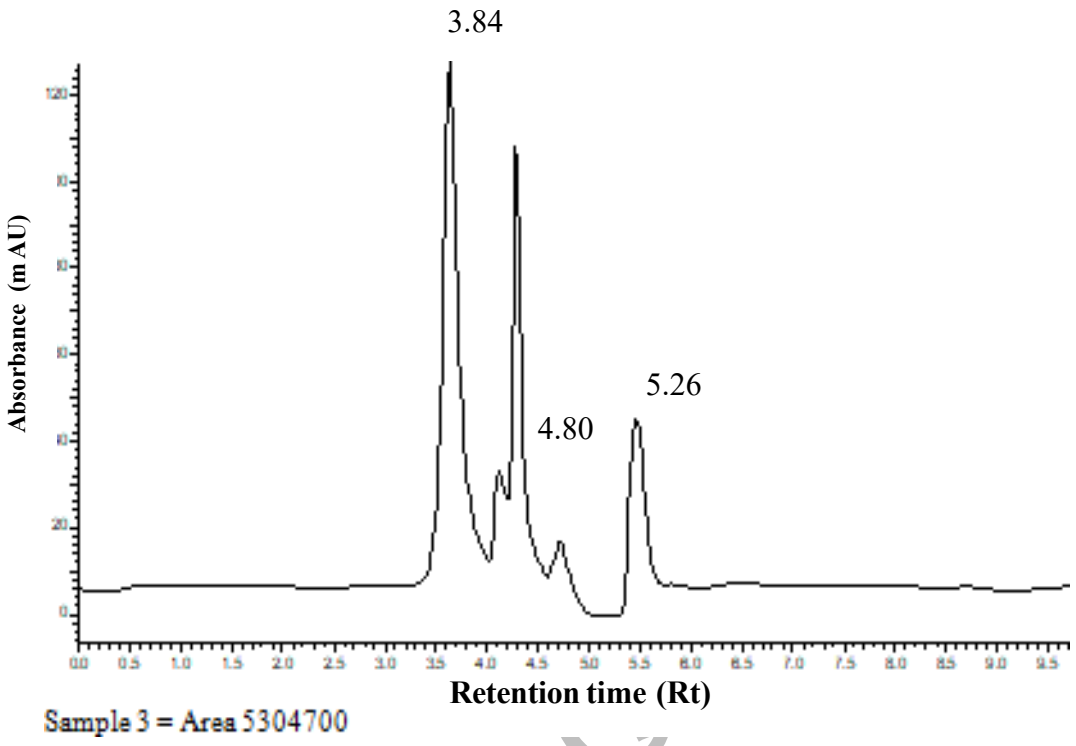

Figure 4. 21: HPLC analysis of lignin biodegradation by *Geotricham candidum* (M-3)

Table 4. 10. Lignin degraded compounds of *Geotricham candidum* (M-3) detected by HPLC at specific retention times

Compound	Retention Time
P-coumaric acid	5.26
Syringic acid	3.84
4-Ethoxy-3-methoxybenzoic acid	4.80

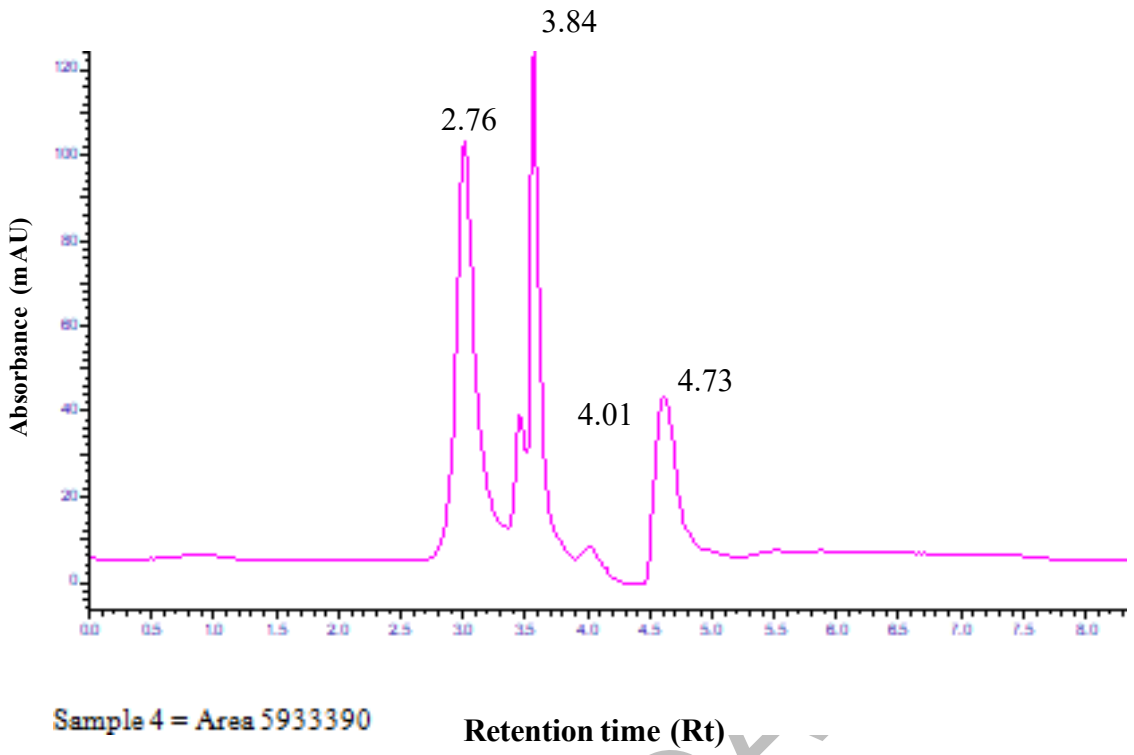

Figure 4. 22: HPLC analysis of lignin biodegradation by *Aspergillus fumigatus* (M-4)

Table 4. 11. Lignin degraded compounds of *Aspergillus fumigatus* (M-4) detected by HPLC at specific retention times

Compound	Retention Time
Syringic acid	3.84
Vanilic acid	3.38
p-hydroxybenzaldehyde	4.01
Veratryl alcohol	4.73

Results Phase II

4.8 Production of the peroxidases enzymes by selected microbial isolates (Laccases, Mn Peroxidase, Lignin peroxidase) in shake flask fermentation

The production of extracellular peroxidases were studied and the results described as the four fungal strains (M-1, M-2, M-3 & M-4) showed significant growth and production of microbial enzymes in broth amended with 2%, lignin at 37^0C, 120 rpm for eight days. Maximum activity of Laccases was observed by M-1 which is 139 U/mL while for Lignin peroxidase (LiP) and Managnese peroxidase was observed in fungal strains M-3, 115U/mL and 106 U/mL respectively as shown in the Table 4.12.

Table 4. 12. Production of laccases, LiP and MnP by fungal strains

Fungal Strain	Laccases (U/mL)	Lignin Peroxidase(U/mL)	Mn Peroxidase(U/mL)
M-1	139	88	98
M-2	98	91	76
M-3	89	115	106
M-4	49	37	95

4.8.1 Effect of carbon source on the production of the peroxidases enzymes by selected fungal isolates (Laccases, Mn Peroxidase, Lignin peroxidase)

The four fungal strains which showed significant peroxidases enzyme production in broth amended with 2% lignin at 30ºC, 120rpm for eight days were further analyzed by using different carbon source. Three different carbon sources i.e. glucose, sucrose and lignin were evaluated for the ability to maximize the production of peroxidases enzymes by selected fungal strains.

4.8.2 Effect of glucose on the production of the peroxidases enzymes by selected fungal isolates (Laccases, Mn Peroxidase, Lignin peroxidase)

Fungal strain M-1was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37℃, 120rpm for eight days with varying concentration of glucose. Glucose in the broth was provided as carbon source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.23 illustrates that fungal strain M-1 produced highest concentration of laccases 157 U/mL at glucose concentration of 5.5g/L. Similarly, highest concentration of Lignin peroxidase (LiP) 147 U/mL and manganese peroxidase (MnP) 137 U/mL was produced at glucose concentration 5 g/L and 5.5 g/L respectively.

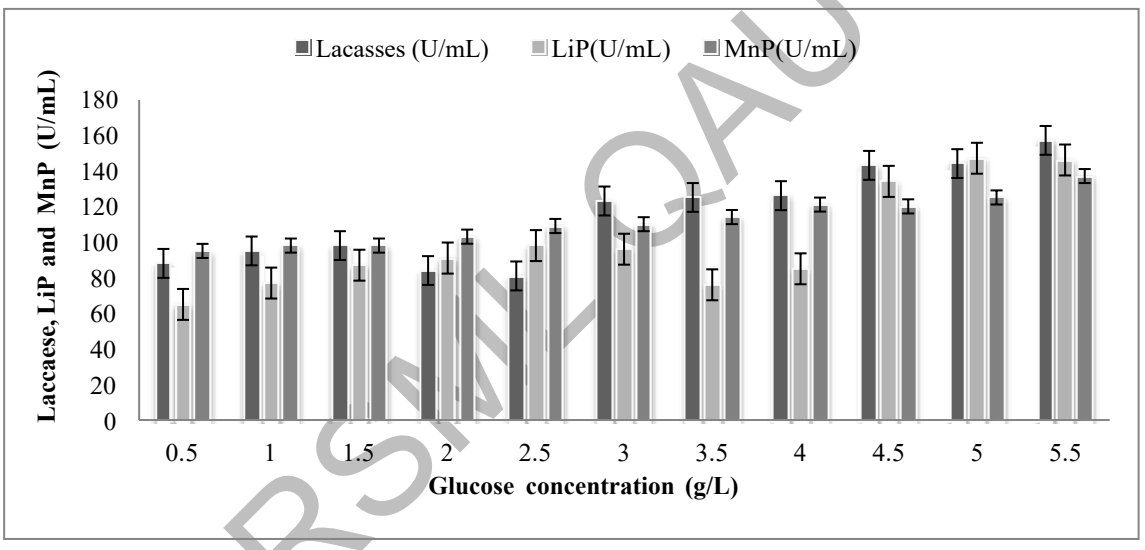

Figure 4. 23: Production of Laccases, LiP and MnP by *Aspergillus terrus* (M-1) at varying concentration of glucose

Fungal strain M-2was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37℃, 120rpm for eight days with varying concentration of glucose. Glucose in the broth was provided as carbon source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.24 illustrates that fungal strain M-2 produced highest concentration of laccases 165 U/mL at glucose concentration of 5.5g/L. Similarly, highest concentration of Lignin peroxidase (LiP) 155 U/mL and manganese peroxidase (MnP) 145 U/mL was produced at glucose concentration 5.5 g/L and 5 g/L respectively.

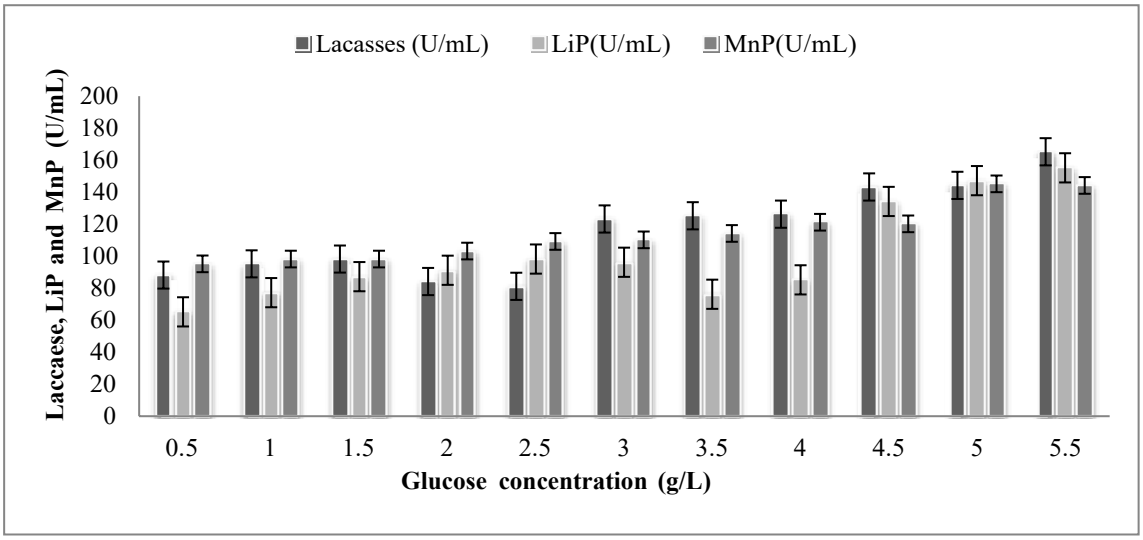

Figure 4. 24: Production of Laccases, LiP and MnP by *Dipodascus australiensis* (M-2) at varying concentration of glucose

Fungal strain M-3was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37^0C, 120rpm for eight days with varying concentration of glucose. Glucose in the broth was provided as carbon source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.25 shows that fungal strain M-3 produced highest concentration of laccases 144 U/mL at glucose concentration of 4.5 g/L. Similarly, highest concentration of Lignin peroxidase (LiP) 155 U/mL and manganese peroxidase (MnP) 125 U/mL was produced at glucose concentration of5.5 g/L.

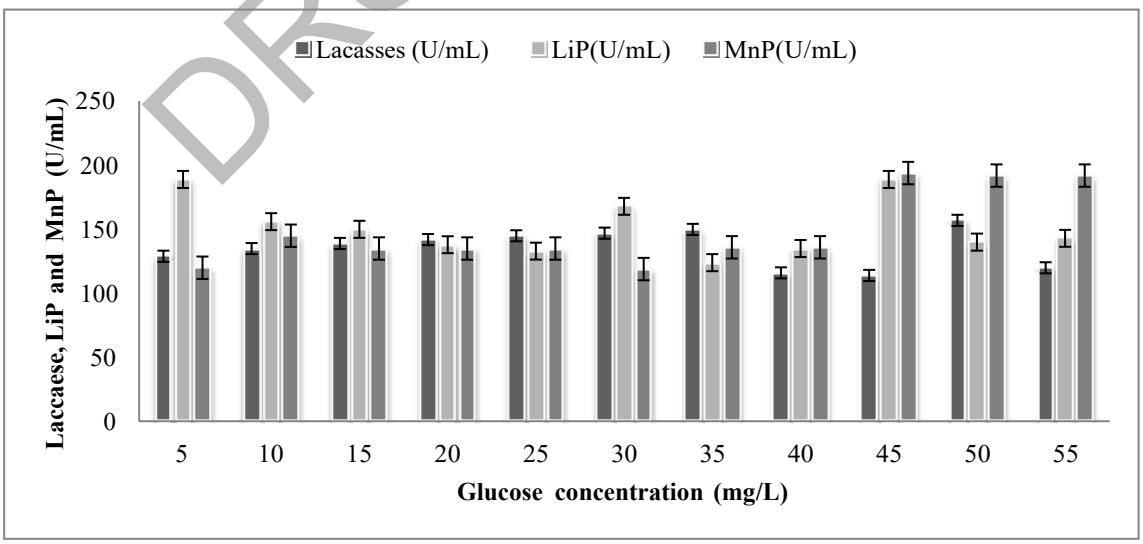

Figure 4. 25: Production of Laccases, LiP and MnP by *Geotrichum candidum* (M-3) at varying concentration of glucose

Fungal strain M-4was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37℃, 120rpm for eight days with varying concentration of glucose. Glucose in the broth was provided as carbon source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.26 shows that fungal strain M-4 produced highest concentration of laccases 141 U/mL at glucose concentration of 4.5 g/L. Similarly, highest concentration of manganese peroxidase (MnP) 125 U/mLand Lignin peroxidase (LiP) 147 U/mL was shown at glucose concentration 5.5 g/L and 5 g/L respectively.

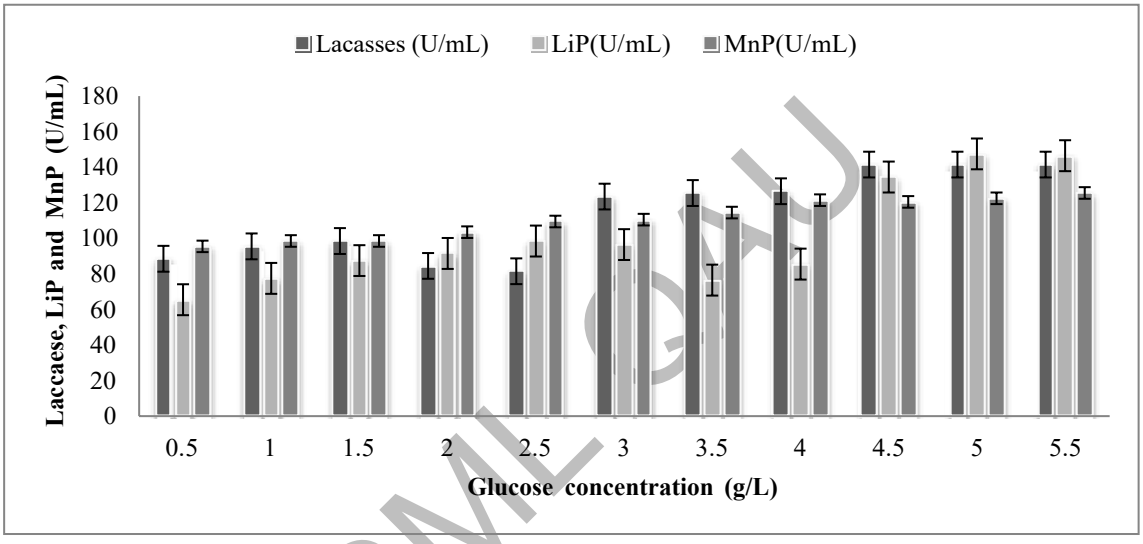

Figure 4. 26: Production of Laccases, LiP and MnP by *Aspergillus fumigatus* (M-4) at varying concentration of glucose

4.8.3 Effect of lignin on the production of the peroxidases enzymes by selected fungal isolates (Laccases, Mn Peroxidase, Lignin peroxidase)

Fungal strain M-1was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37^0C, 120rpm for eight days with varying concentration of lignin. Lignin in the broth was provided as carbon source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.27 illustrates that fungal strain M-1 produced highest concentration of laccases 125 U/mL at lignin concentration of 2.5g/L. Similarly, highest concentration of Lignin peroxidase (LiP) 99 U/mL and manganese peroxidase (MnP) 104 U/mL was produced at lignin concentration 3.0 g/L and 3.5g/L respectively.

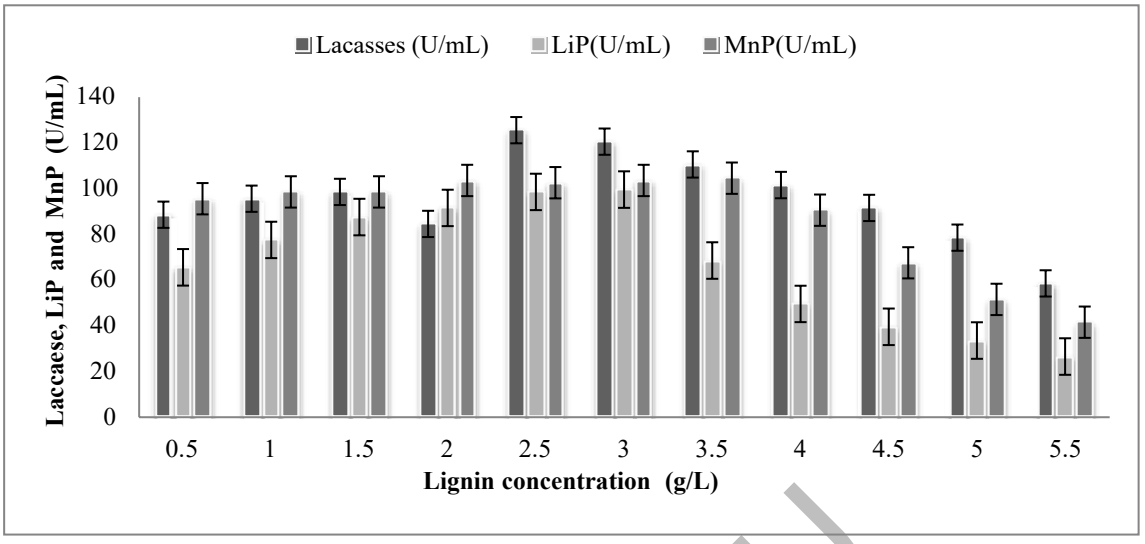

Figure 4. 27: Production of Laccases, LiP and MnP by *Aspergillus terrus* (M-1) at varying concentration of lignin

Fungal strain M-2was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37^0C, 120rpm for eight days with varying concentration of lignin. Lignin in the broth was provided as carbon source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.28 shows that fungal strain M-2 produced highest concentration of laccases 144 U/mL at lignin concentration of 3.5g/L. Similarly, highest concentration of Lignin peroxidase (LiP) 98 U/mL and manganese peroxidase (MnP) 78 U/mL was produced at lignin concentration 2.5 g/L respectively.

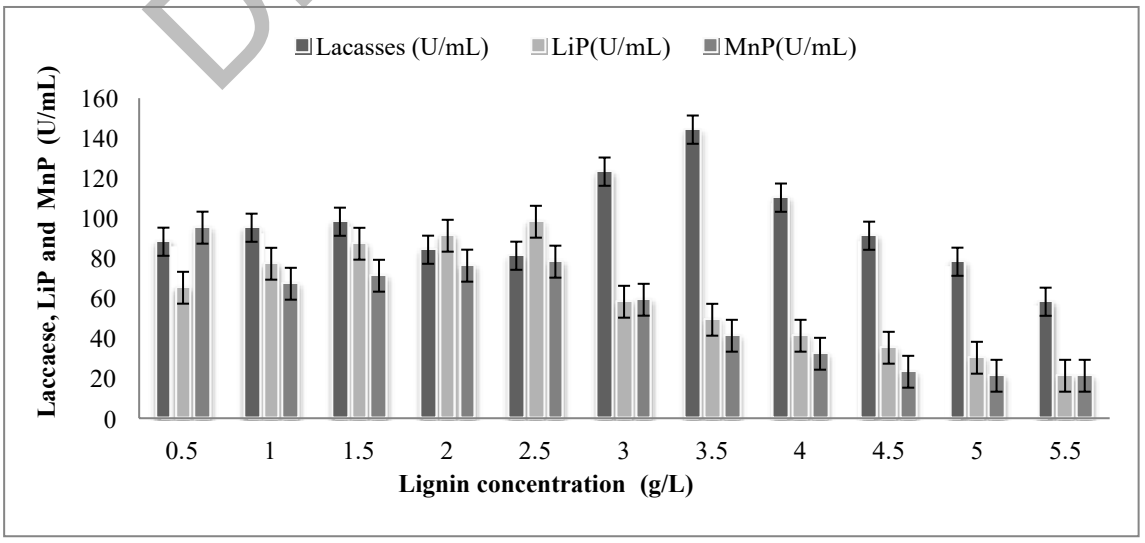

Figure 4. 28: Production of Laccases, LiP and MnP by *Dipodascus australiensis* (M-2) at varying concentration of lignin

Fungal strain M-3 was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37^{0}C, 120rpm for eight days with varying concentration of lignin. Lignin in the broth was provided as carbon source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.29 illustrates that fungal strain M-3 produced highest concentration of laccases 98 U/mL at lignin concentration of 1.5g/L. Similarly, highest concentration of Lignin peroxidase (LiP) 95 U/mL and manganese peroxidase (MnP) 66 U/mL was produced at lignin concentration 2.5 g/L and 2g/L respectively.

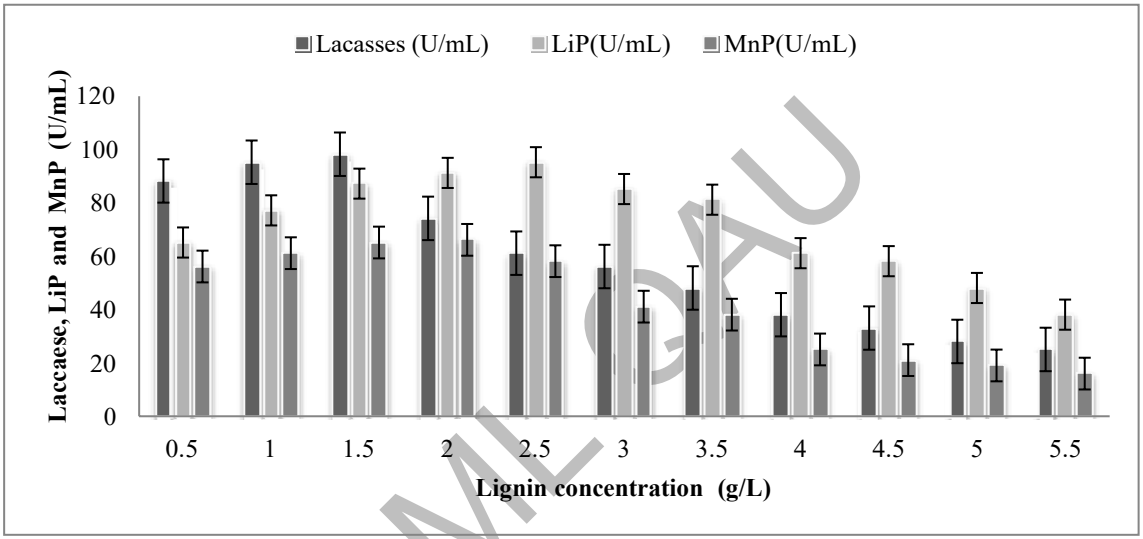

Figure 4. 29: Production of Laccases, LiP and MnP by *Geotrichum candidum* (M-3) at varying concentration of lignin

Fungal strain M-4was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37ºC, 120rpm for eight days with varying concentration of lignin. Lignin in the broth was provided as carbon source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.30 shows that fungal strain M-4 produced highest concentration of laccases 125 U/mL at lignin concentration of 3g/L. Similarly, highest concentration of Lignin peroxidase (LiP) 71 U/mL and manganese peroxidase (MnP) 112 U/mL was produced at lignin concentration 2 g/L and 3 g/L respectively.

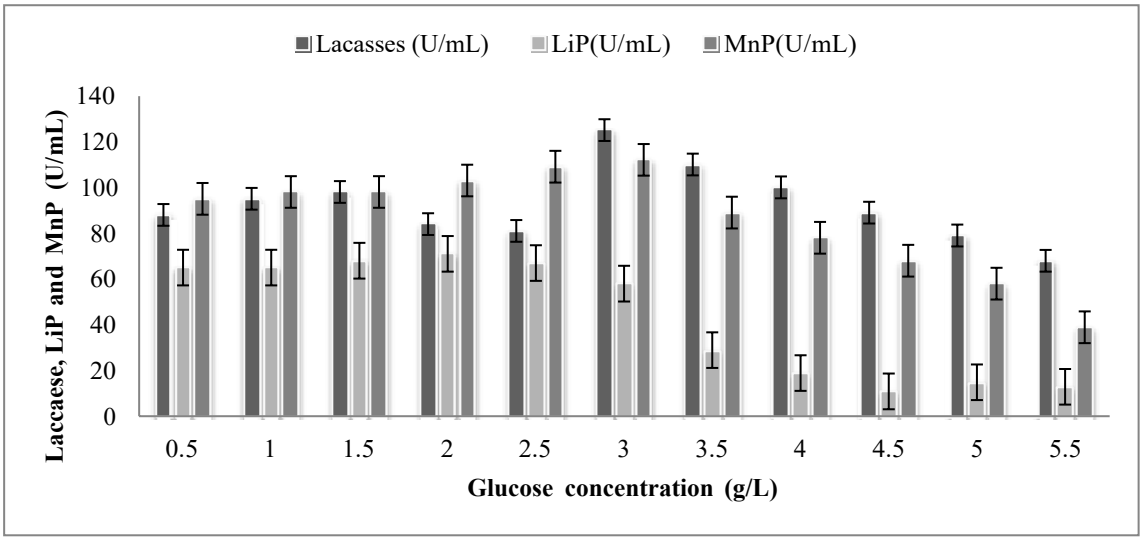

Figure 4. 30: Production of Laccases, LiP and MnP by *Aspergillus fumigatus* (M-4) at varying concentration of lignin

4.8.4 Effect of sucrose on the production of the peroxidases enzymes by selected microbial isolates (Laccases, Mn Peroxidase, Lignin peroxidase)

Fungal strain M-1was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37ºC, 120rpm for eight days with varying concentration of sucrose. Sucrose in the broth was provided as carbon source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.31 illustrates that fungal strain M-1 produced highest concentration of laccases 281 U/mL at sucrose concentration of 5.5 g/L. Similarly, highest concentration of Lignin peroxidase (LiP) 252 U/mL and manganese peroxidase (MnP) 210 U/mL was produced at sucrose concentration 5.5 g/L respectively.

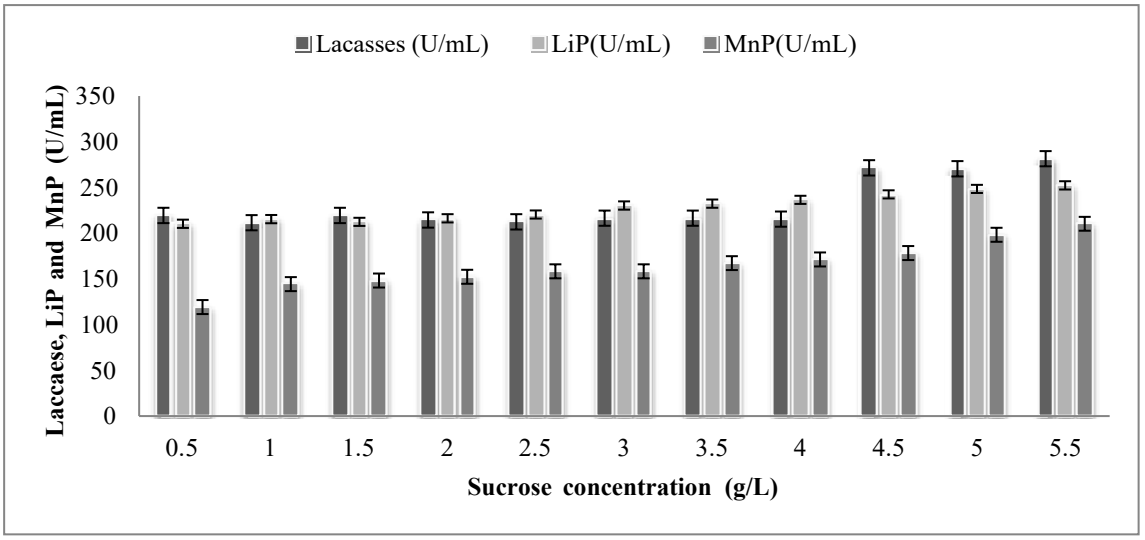

Figure 4. 31: Production of Laccases, LiP and MnP by *Aspergillus terrus* (M-1) at varying concentration of sucrose

Fungal strain M-2 was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37ºC, 120rpm for eight days with varying concentration of sucrose. Sucrose in the broth was provided as carbon source and to stimulate the ability of strain to produce the higher concentrations of enzymes. Figure 4.32 depicts that fungal strain M-2 produced highest concentration of laccases 288 U/mL at sucrose concentration of 5.5g/L. Similarly, highest concentration of Lignin peroxidase (LiP) 268 U/mL and manganese peroxidase (MnP) 280 U/mL was produced at sucrose concentration 5.5 g/L respectively.

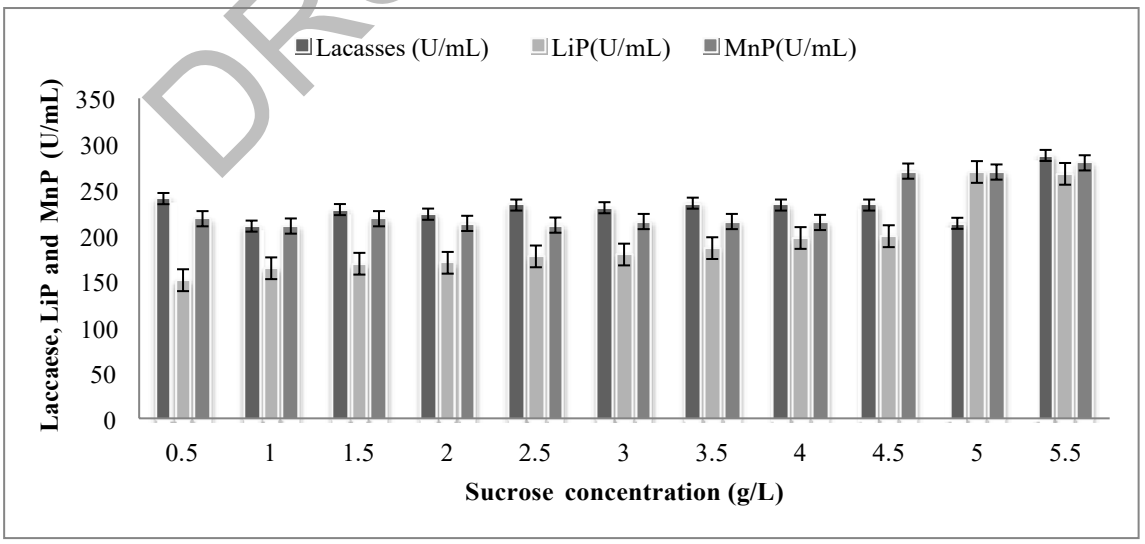

Figure 4. 32: Production of Laccases, LiP and MnP by *Dipodascus australiensis* (M-2) at varying concentration of sucrose

Fungal strain M-3was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37ºC, 120rpm for eight days with varying concentration of sucrose. Sucrose in the broth was provided as carbon source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.33 shows that fungal strain M-3 produced highest concentration of laccases 255 U/mL at sucrose concentration of 50mg/L. Similarly, highest concentration of Lignin peroxidase (LiP) 199 U/mL and manganese peroxidase (MnP) 193 U/mL was produced at sucrose concentration 40 mg/L and 45 mg/L respectively.

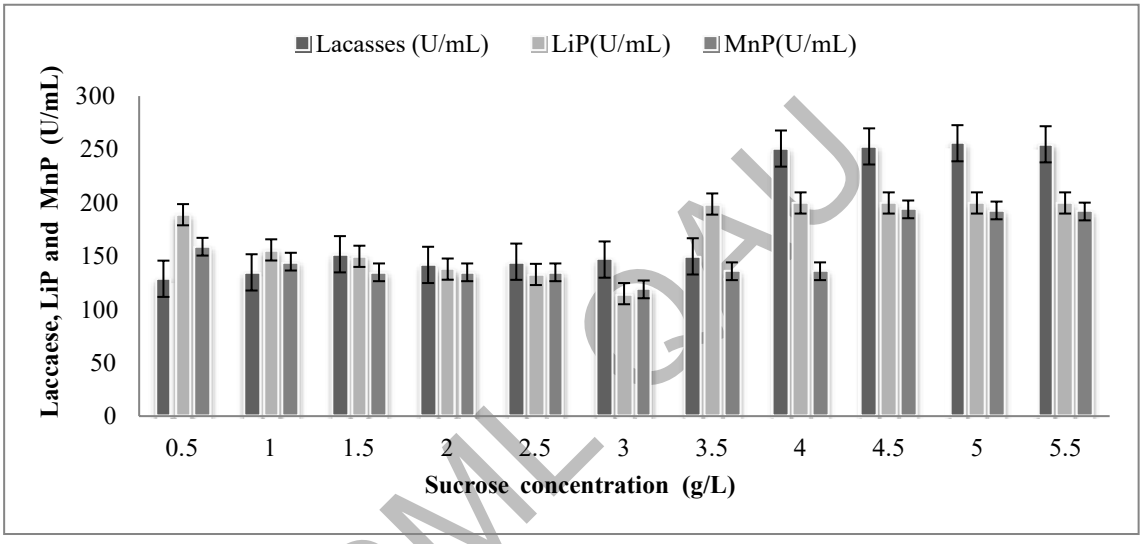

Figure 4. 33: Production of Laccases, LiP and MnP by *Geotrichum candidum* (M-3) at varying concentration of sucrose

Fungal strain M-4 was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37ºC, 120rpm for eight days with varying concentration of sucrose. Sucrose in the broth was provided as carbon source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.34 shows that fungal strain M-4 produced highest concentration of laccases 256 U/mL at sucrose concentration of 5g/L. Similarly, highest concentration of Lignin peroxidase (LiP) 211 U/mL and manganese peroxidase (MnP) 210 U/mL was produced at sucrose concentration 5 g/L and 5.5 g/L respectively.

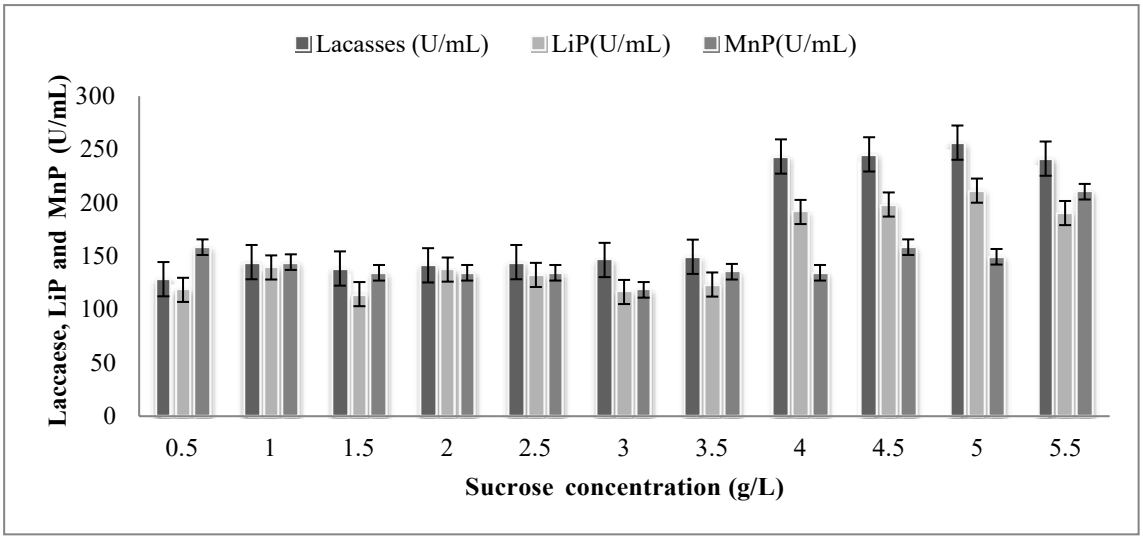

Figure 4. 34: Production of Laccases, LiP and MnP by *Aspergillus fumigatus* (M-4) at varying concentration of sucrose

4.8.5 Effect of Nitrogen source on the production of the peroxidases enzymes by selected fungal isolates (Laccases, Mn Peroxidase, Lignin peroxidase)

The four fungal strains which showed significant peroxidases enzyme production in broth amended with 2%, lignin at 37ºC, 120rpm for eight days were further analyzed by using $NaNO_3$ or peptone as nitrogen source. The nitrogen sources i.e. $NaNO_3$ was evaluated for the ability to maximize the production of peroxidases enzymes by selected fungal strains. All fungal strains (M-1, M-2, M-3 and M-4) showed direct relationship with production of peroxidases and nitrogen source, $NaNO_3$. All fungal strains showed maximum production of peroxidases at highest concentration of $NaNO_3$ i.e. 5.5g/L as shown in the figure 3.32, 3.33, 3.34 and 3.35 respectively.

Fungal strain M-1 was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37ºC, 120rpm for eight days with varying concentration of peptone. Peptone in the broth was provided as nitrogen source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.35 shows that fungal strain M-1 produced highest concentration of laccases 282 U/mL at sucrose concentration of 5g/L. Similarly, highest concentration of Lignin peroxidase (LiP) 252 U/mL and manganese peroxidase (MnP) 211 U/mL was produced at peptone concentration 5 g/L and 5.5 g/L respectively.

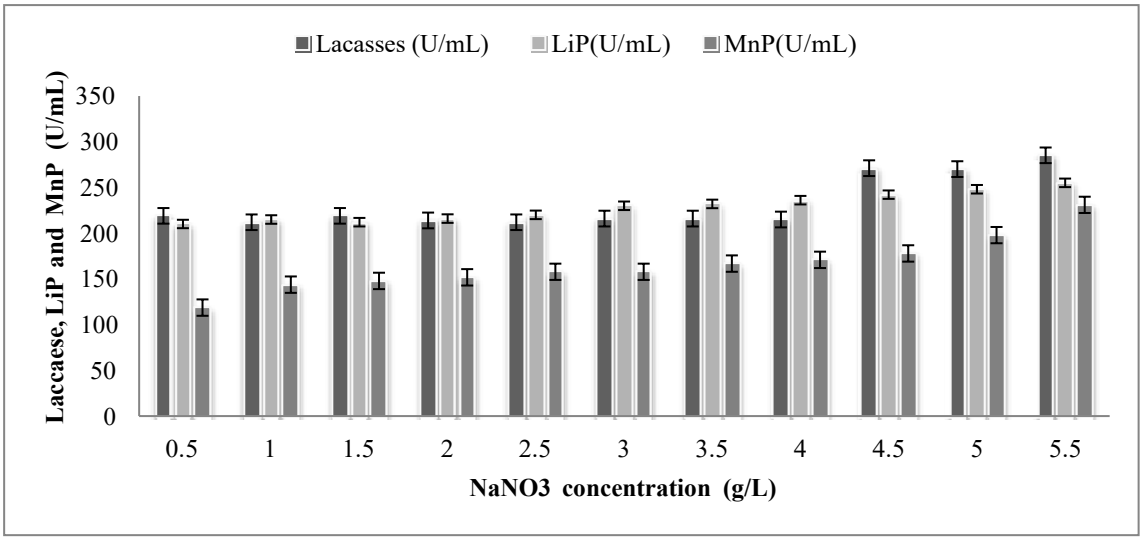

Figure 4. 35: Production of Laccases, LiP and MnP by *Aspergillus terrus* (M-1) at varying concentration of $NaNO_3$

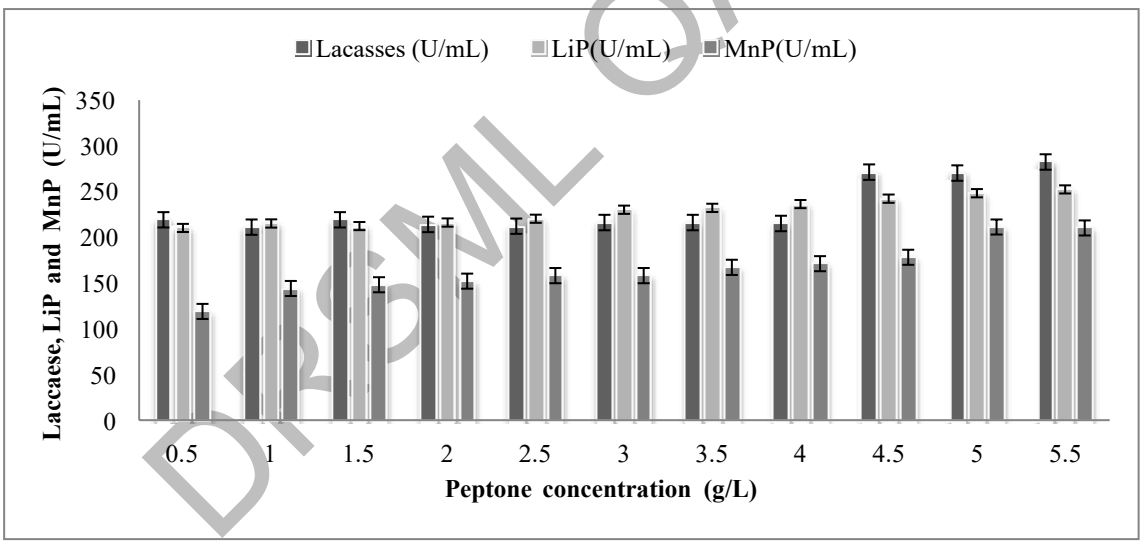

Figure 4. 36: Production of Laccases, LiP and MnP by *Aspergillus terrus*(M-1) at varying concentration of peptone

Fungal strain M-2 was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37 ºC, 120rpm for eight days with varying concentration of peptone. Peptone in the broth was provided as nitrogen source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.37 shows that fungal strain M-2 produced highest concentration of laccases 291 U/mL at sucrose concentration of 5g/L. Similarly, highest concentration of Lignin peroxidase

(LiP) 272 U/mL and manganese peroxidase (MnP) 292 U/mL was produced at peptone concentration 5 g/L and 5.5 g/L respectively.

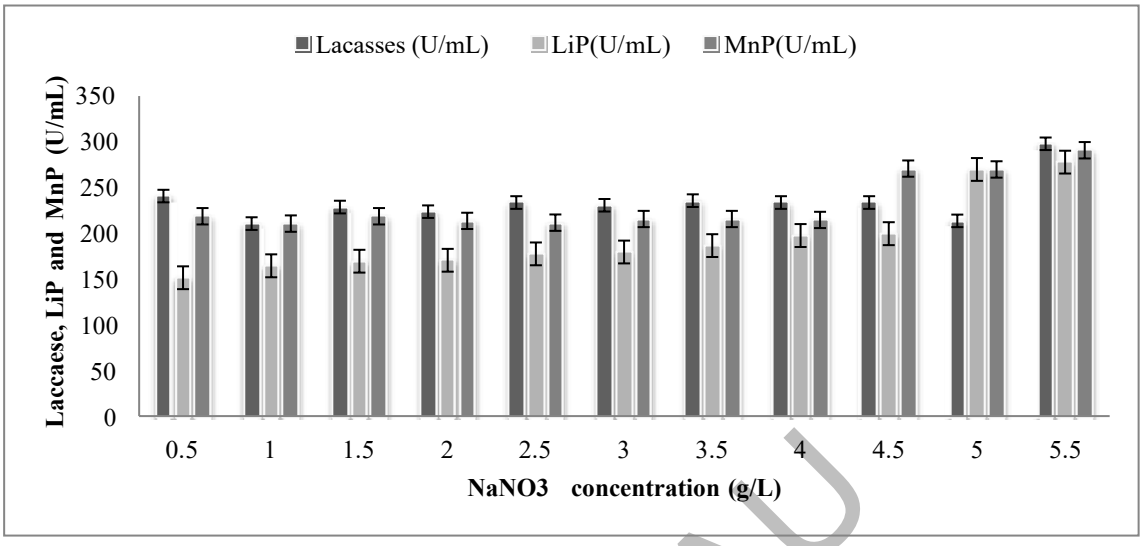

Figure 4. 37: Production of Laccases, LiP and MnP by *Dipodascus australiensis* (M-2) at varying concentration of $NaNO_3$

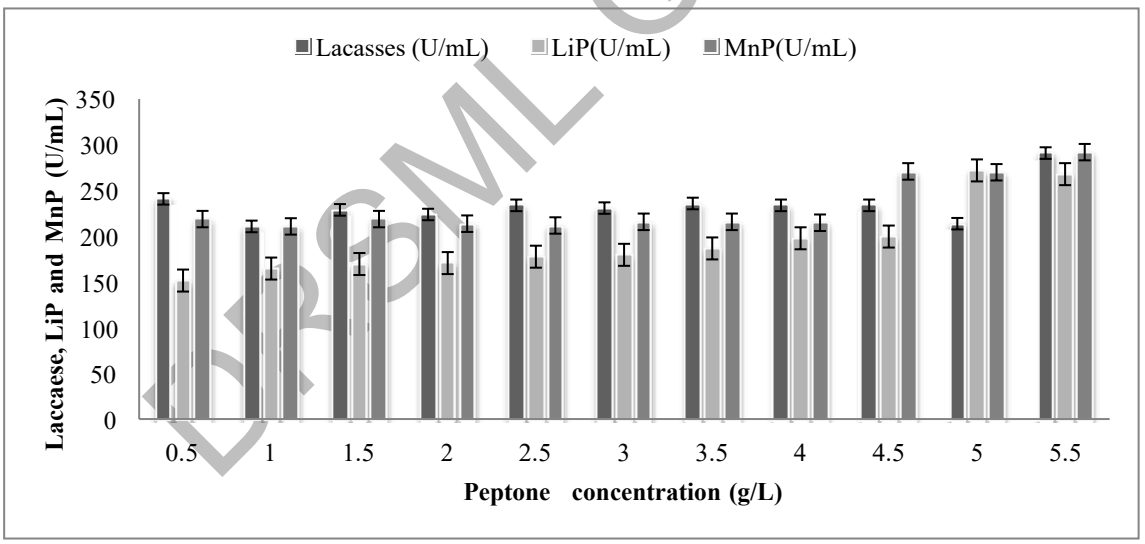

Figure 4. 38: Production of Laccases, LiP and MnP by *Dipodascus australiensis* (M-2) at varying concentration of peptone

Fungal strain M-3 was also observed for peroxidases enzyme production in broth amended with 2% lignin at 37ºC, 120rpm for eight days with varying concentration of peptone. Peptone in the broth was provided as nitrogen source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 4.39 shows that fungal strain M-3 produced highest concentration of laccases 298 U/mL at sucrose concentration of 5g/L. Similarly, highest concentration of Lignin peroxidase

(LiP) 213 U/mL and manganese peroxidase (MnP) 199 U/mL was produced at peptone concentration 5 g/L and 5.5 g/L respectively.

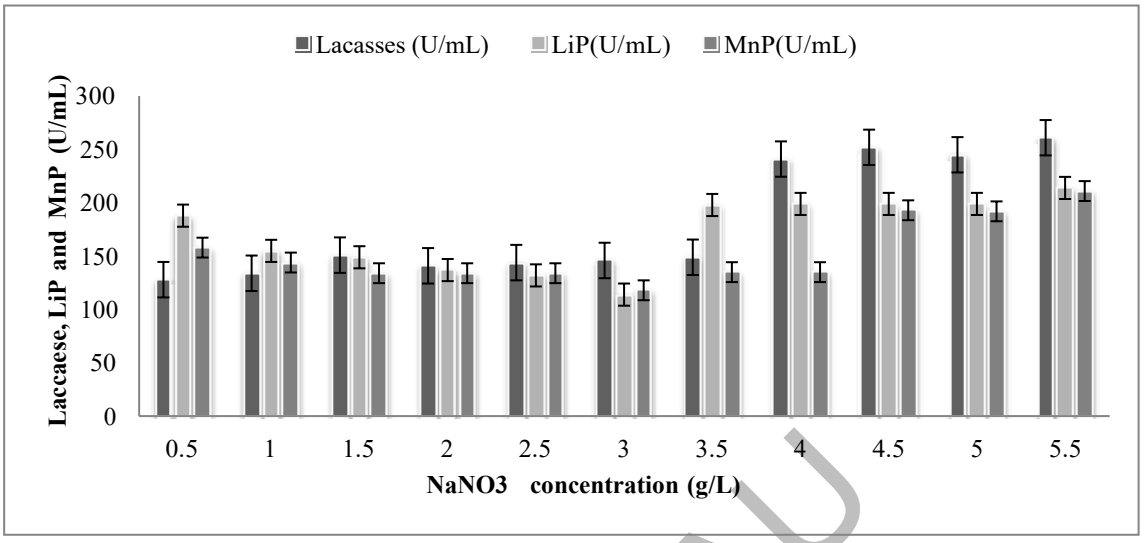

Figure 4. 39: Production of Laccases, LiP and MnP by *Geotrichum candidum* (M-3) at varying concentration of $NaNO_3$

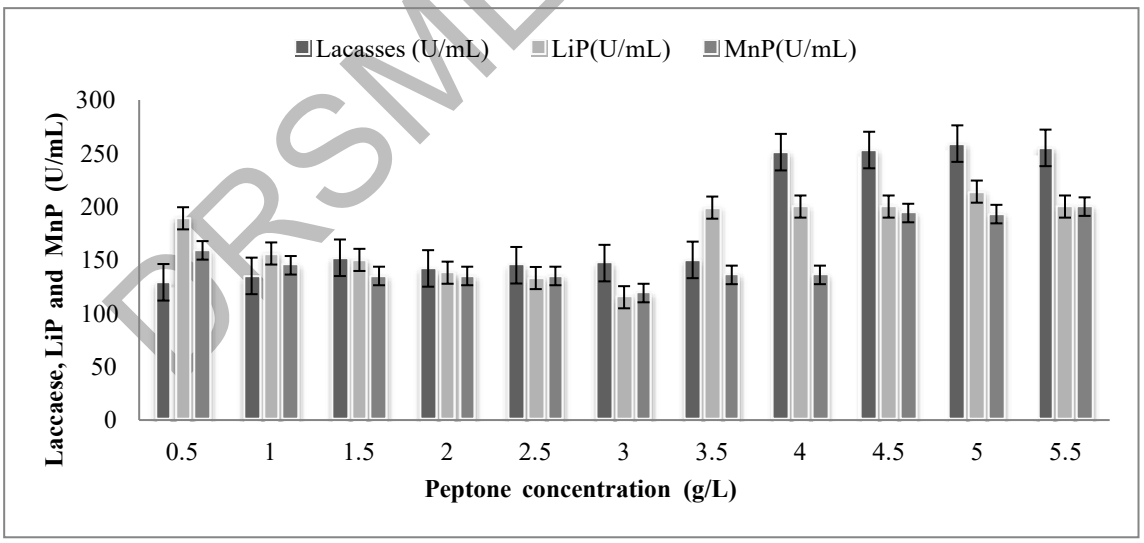

Figure 4. 40: Production of Laccases, LiP and MnP by *Geotrichum candidum* (M-3) at varying concentration of peptone

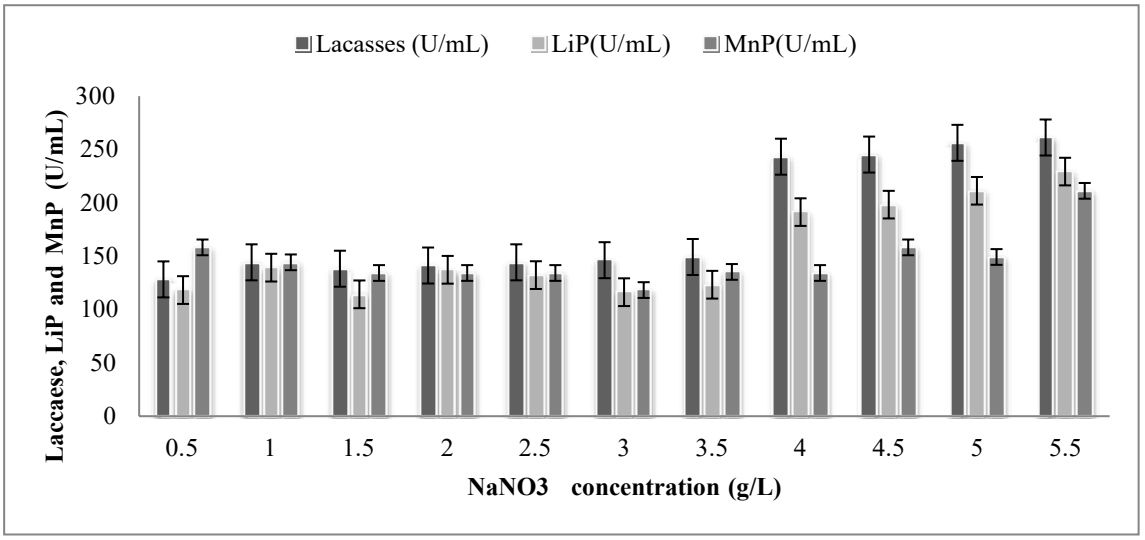

Figure 4. 41: Production of Laccases, LiP and MnP by *Aspergillus fumigatus* (M-4) at varying concentration of NaNO3

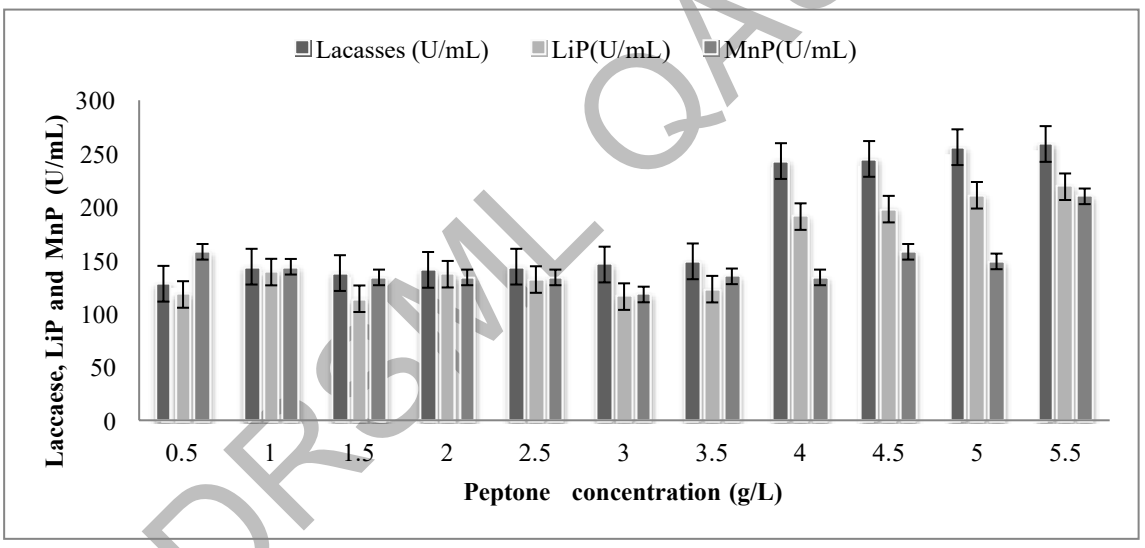

Figure 4. 42: Production of Laccases, LiP and MnP by *Aspergillus fumigatus* (M-4) at varying concentration of peptone

Fungal strain M-4 was also observed for peroxidases enzyme production in broth amended with 2%, lignin at 37ºC, 120rpm for eight days with varying concentration of peptone. Peptone in the broth was provided as nitrogen source and to stimulate the ability of strain to produce the higher concentrations of enzyme. Figure 3.42 shows that fungal strain M-4 produced highest concentration of laccases 259 U/mL at sucrose concentration of 5g/L. Similarly, highest concentration of Lignin peroxidase (LiP) 219 U/mL and manganese peroxidase (MnP) 216 U/mL was produced at peptone concentration 5 g/L and 5.5 g/L respectively.

4.9 Production of fungal peroxidases under different experimental conditions in shake flask experiment

For shake flask experiment four fungal strains M-1, M-2, M-3 and M-4 were selected and exposed to different experimental setups with varying range of pH and temperature to check their ability to produce peroxidases i.e., Laccases, LiP and MnP, growth, lignin degradation and colour reduction. Among the three fungal strains M-1, M-2 and M-3, the fungal strain M-2 showed maximum production Laccasses 241 U/mL, LiP 239 U/mL and MnP 215 U/mL at 37C and pH8. However, a slight increase in pH causes reduction in production of fungal peroxidases as shown in the figure 3.36, 3.37 and 3.38 respectively.

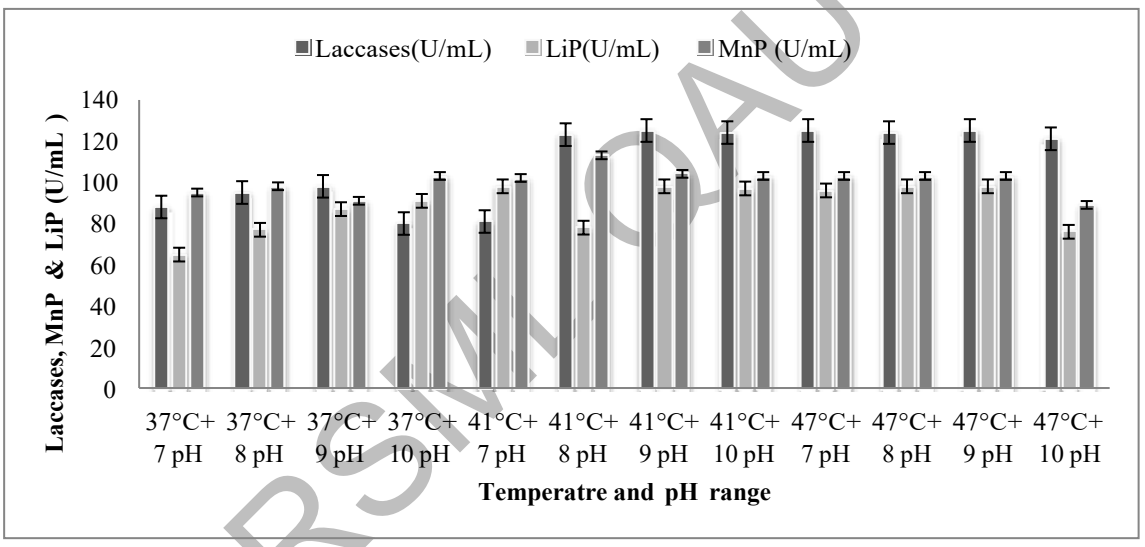

Figure 4. 43: Production of Laccases, LiP and MnP by *Aspergillus terrus* (M-1) at varying pH and temperatures

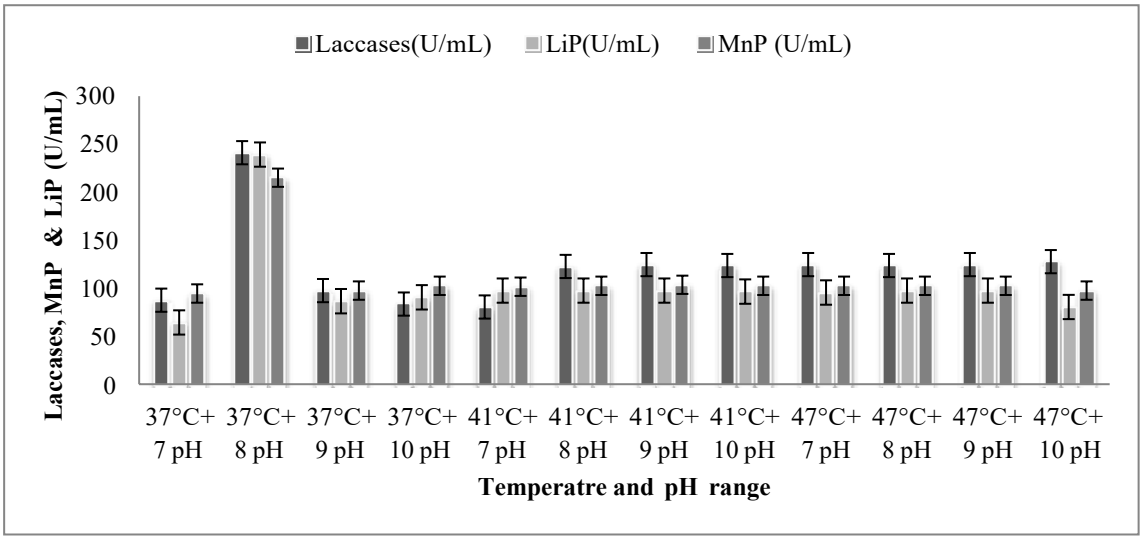

Figure 4. 44: Production of Laccases, LiP and MnP by *Dipodascus autraliensis* (M-2) at varying pH and temperatures

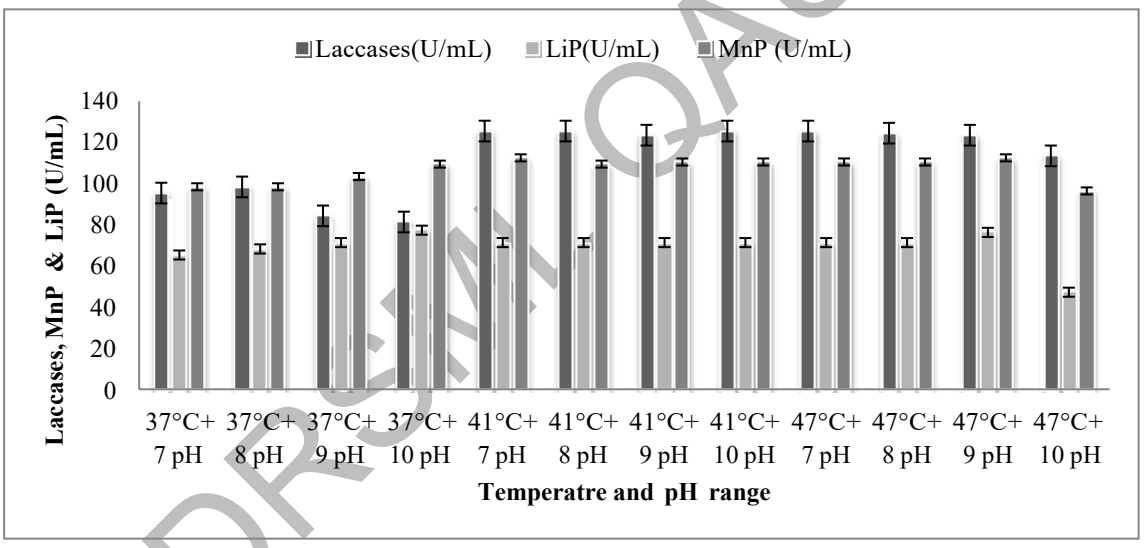

Figure 4. 45: Production of Laccases, LiP and MnP by *Geotrichum candidum* (M-3) at varying pH and temperatures

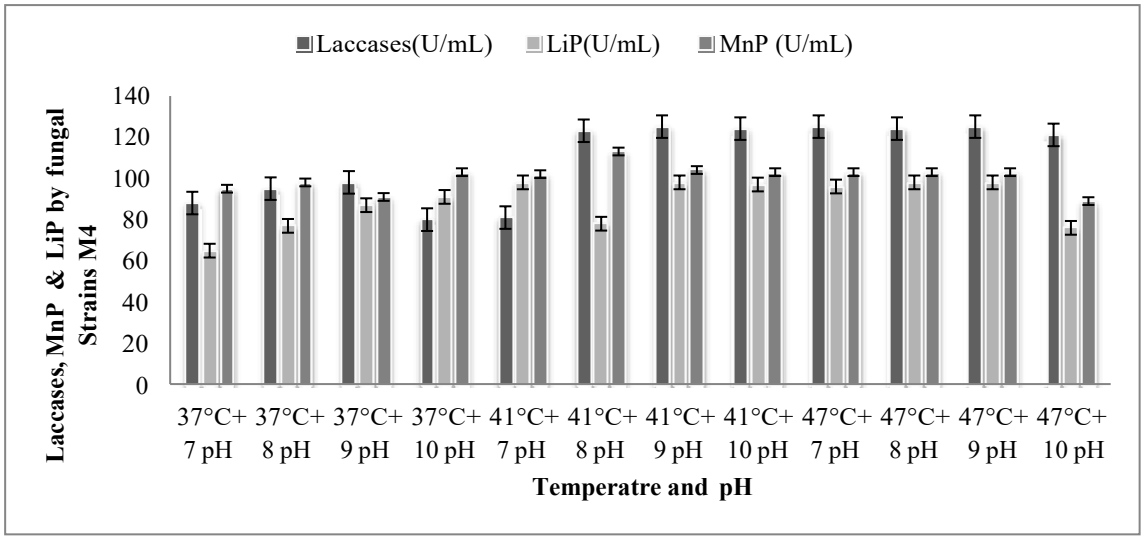

Figure 4. 46: Production of Laccases, LiP and MnP by *Aspergillus fumigatus* (M-4) at varying pH and temperatures

4.10 SDS polyacrylamide gel electrophoresis of fungal enzymes

Presence of various forms of the lignin degrading enzymes is indicated for fungal strains (M-1, M-2, M-3 and M-4). The SDS-PAGE analysis of the fungal samples revealed that molecular weight of the lignin isoenzymes was found to be in the MW of 35 kDa and laccases was found to be 130 kDa. These findings agree with various previous reports in which different peroxidases of fungal origins fall in the similar molecular weight range.

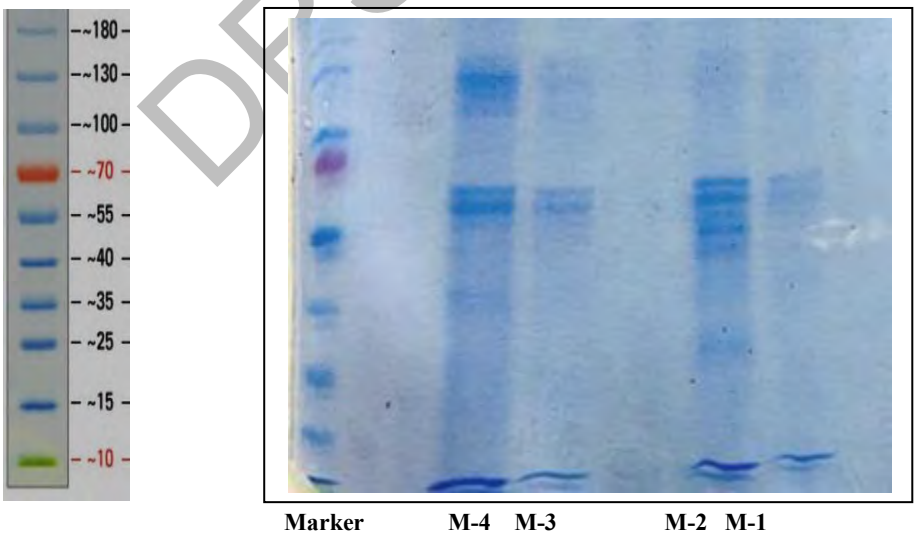

Figure 4. 47: Molecular weight of purified ligninolytic enzymes from fungal strains*Aspergillus terrus* (M-1), *Dipodascus autraliensis* (M-2), *Geotrichum candidum* (M-3) and*Aspergillus fumigatus*(M-4)

Results Phase III

In the third phase of the study, different lignin variant were created (soluble and insoluble) in order to investigate the enzymatic potential of fungal isolates for using these substrates for their growth.

4.11 Effect of lignin on the growth of fungal strains:

Effect of lignin on the growth of fungal strains *Aspergillus terrus* M-1*, Dipodascus autralienses* M-2, *Geotrichum candidum* M-3and *Aspergillus fumigatus* M-4 was investigated using varying concentrations of the mentioned substrate. The results were recorded for a period of 8 days and are presented in table 4.12. It was noted that dense mycelia growth was observed in case of the insoluble lignin on the SDA plates for *Geotrichum candidum* M-3 followed by *Dipodascus autralienses* M-2 and *Aspergillus terrus*M-1. The growth of *Aspergillus fumigatus* M-4 was found comparable with the strain M-3 (Figure 4.20). In contrast, the growth of the aforementioned fungi was quite rich while using soluble lignin for all four strains, with comparatively less growth of M-1 only.

Table 4. 13. Growth of fungal strains on soluble and insoluble lignin

Strains	1% Insoluble	0.4% Insoluble	0.2% soluble
M-1	+	+	++
M-2	++	++	+++
M-3	+++	+++	+++
M-4	+++	+++	+++

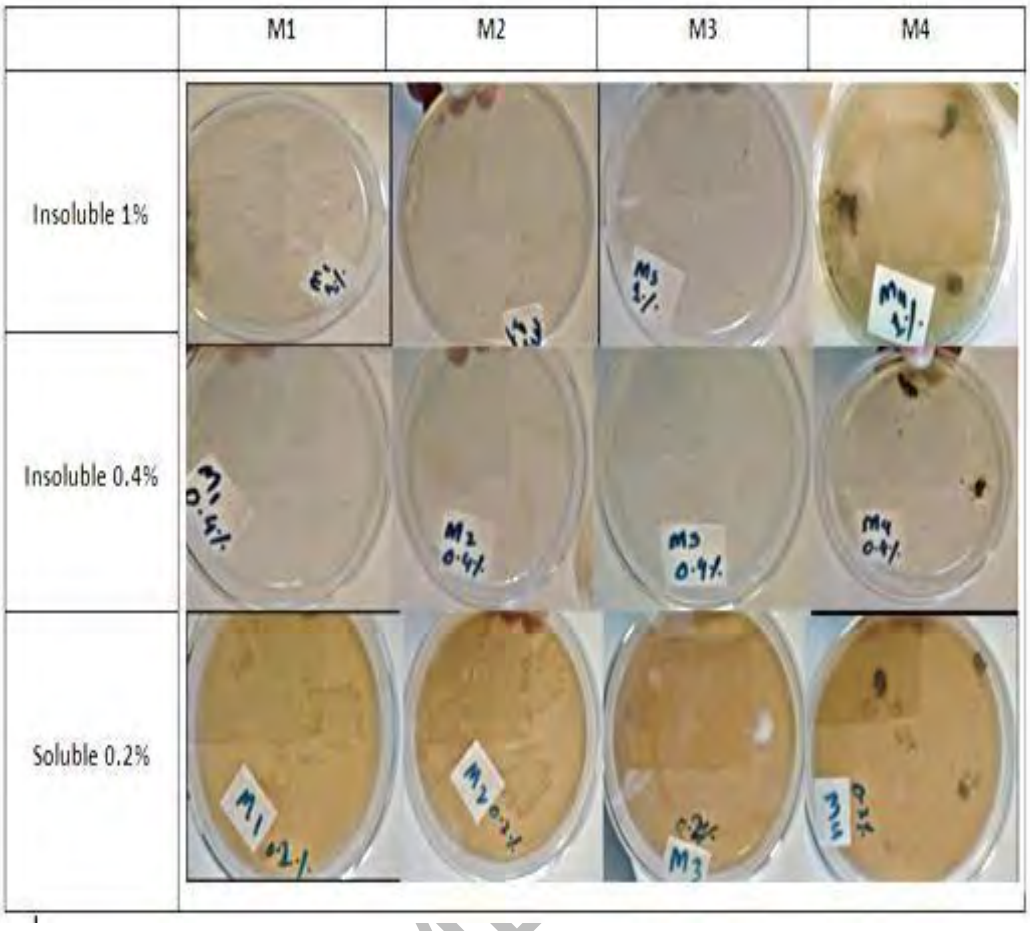

Figure 4. 48: Growth of fungal strains on different concentration of lignin substrates

4.12 Effect of different carbon sources on the growth of the fungi

The effect of different carbon sources on the growth of the fungal strains M-1, M-2, M-3 and M-4 was observed. The results suggested that fungal strains can growth on different types of carbons substrates with different efficiency. In case of avicel, the higest growth was observed by *Geotrichum candidum* M-3 *and Aspergillus fumigatus* M-4. Whereas, the *Aspergillus terrus* M-1and *Dipodascus autralienses* M-2 showed comparatively lesser growth on the avicel (Table 4.13). The growth of the medium containing lignin as carbon sources suggested that all aforementioned fungal strains can efficiently use and grow this carbon source. The growth pattern of the fungi on Mannans indicated that all strain have the metabolic capacity of using this carbon source efficiently except *Aspergillus terrus.* Further,*Aspergillus terrus*was also found less competent to use pectin, starch and xylan as compared to the other fungal strains.

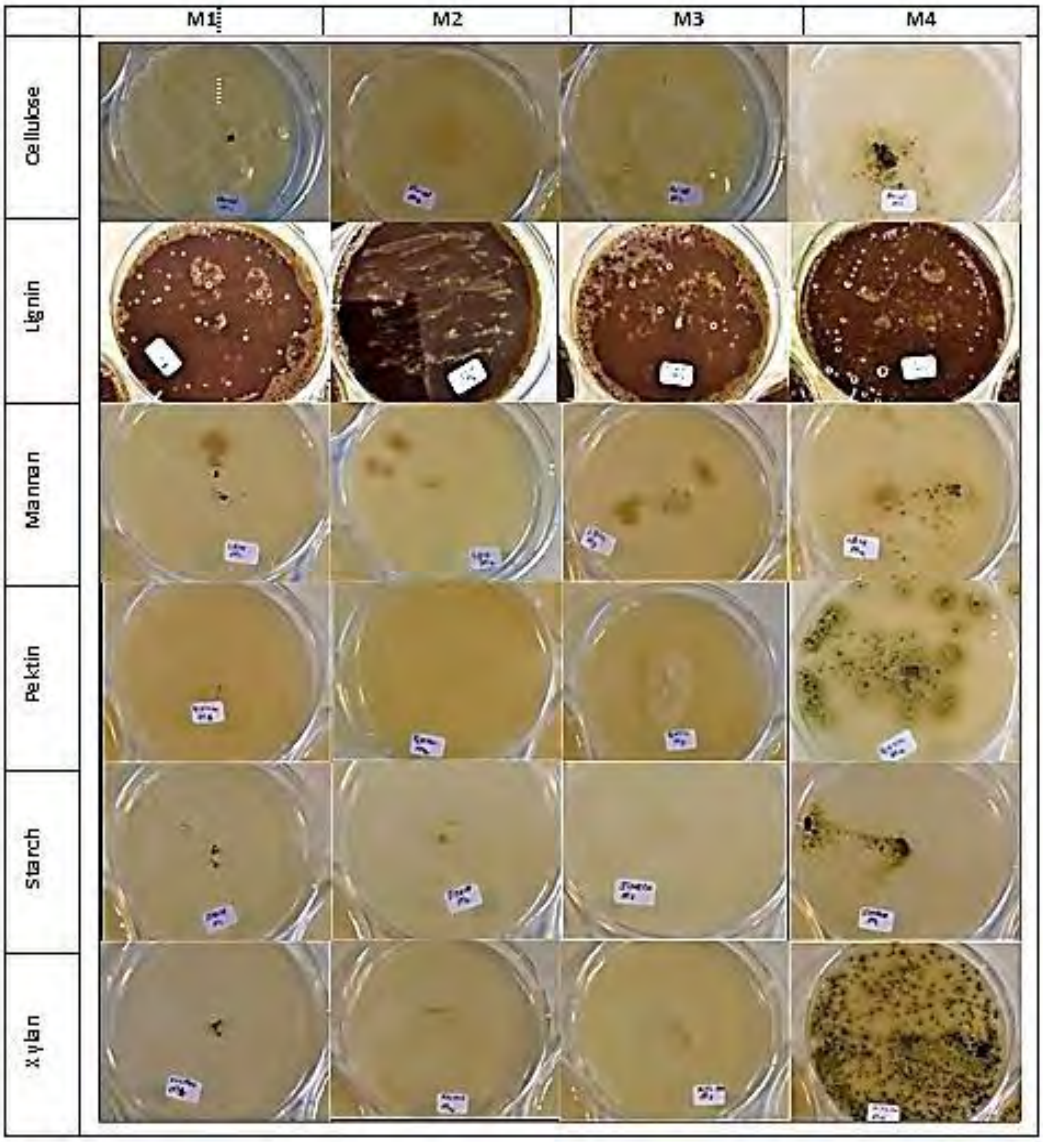

Figure 4. 49: Effect of growth on different carbon sources by fungal strains (Day 3)

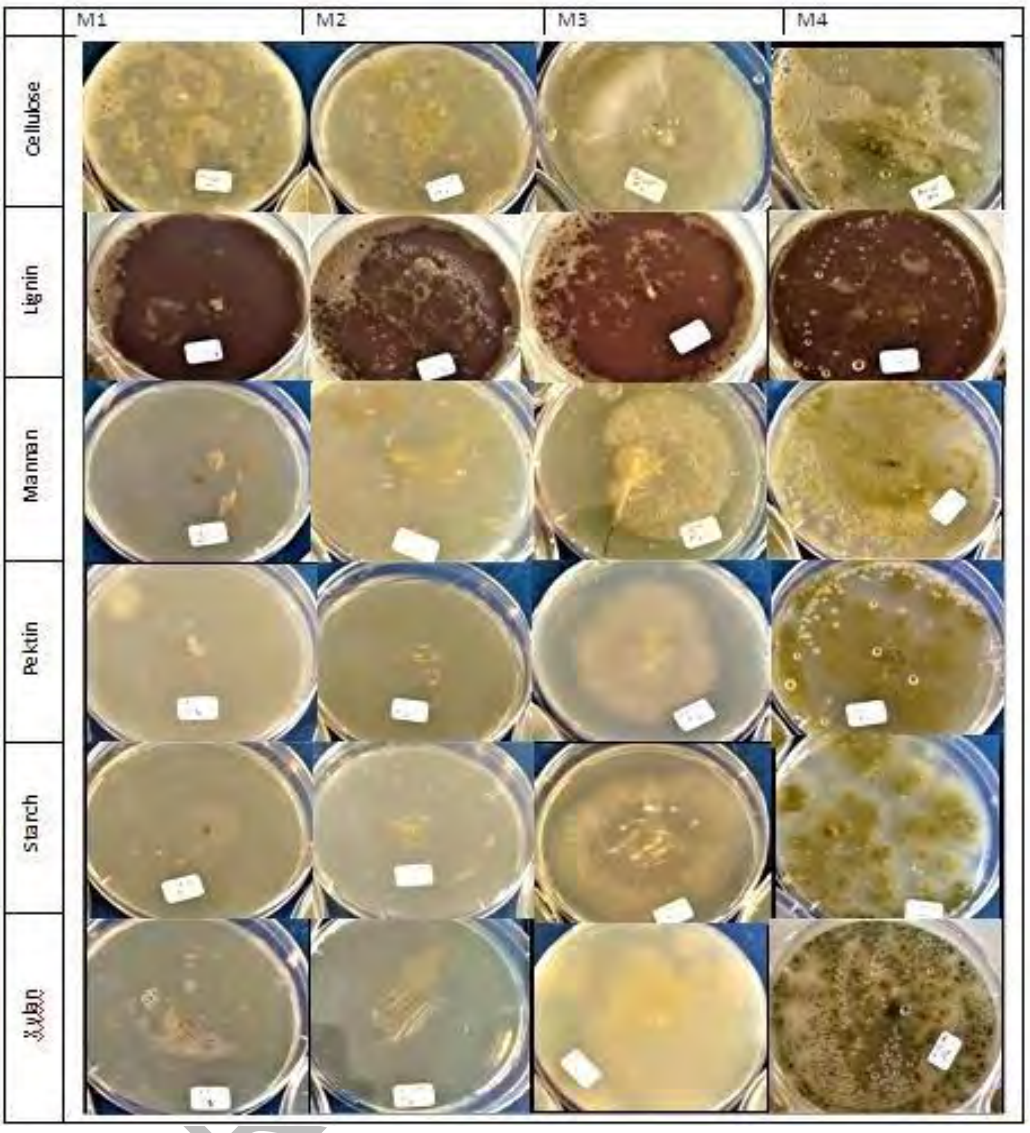

Figure 4. 50: Effect of growth on different carbon sources by fungal strains*Aspergillus terrus***(M-1),***Dipodascus autraliensis***(M-2),***Geotrichum candidum* (**M-3**)**,***Aspergillus fumigatus* (**M-4**)

(Day 6*)*

Table 4. 14. Growth of fungal strains *Aspergillus terrus, Dipodascus australiensis, Geotrichum candidum*and*Aspergillus fumigatus*on different carbon sources

Strains	Avicel		Lignin		Mannan		Pectin		Starch		Xylan	
	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6
Aspergillus terrus M-1	--	+	+	+	--	--	--	+	+	+	--	+
Dipodascus australiensis M-2	++	++	+++	++	+++	++	++	++	++	++	++	++
Geotrichum candidum M-3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++
Aspergillus fumigatus M-4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++

4.13 Heteronuclear Single Quantum Coherence (HSQC) Nuclear magnetic resonance analysis for lignin biodegradation by selected fungal strains

Efficiency of lignin biodegradation by fungal strains was analysed by ^{1}H-^{13}C HSQC Nuclear magnetic resonance. Different monomers were formed during lignin degradation by our selected fungi as depicted by NMR analysis. In case of *Aspergillus terrus* strains M-1 the chemical shift was observed at 3.5 to 4.0 ^{1}H ppm and the chemical shift at ^{13}C, ppm wasaround 57 to 60 (Figure 4.23). These results depicted the methoxyl group formed in the result of lignin degradation. *Dipodascus australiensis* M-2 and *Geotrichum candidum* M-3 on after degradation liberated xylan from the lignin-carbohydrate complex. *Dipodascus australiensis* M-2 showed

the chemical shift of ^{1}H, ppm at 4 – 5.1, this indicated the lignin transformation into carbohydrates. For chemical shift of 67 to 78 ^{13}C, ppm represents aLiPhatic Cα-O and Cβ-O (Figure 4.24). The strain *Geotrichum candidum* M-3 were more active in metabolizing residual monomers such as vanillin, acetovanillone and coniferyl alcohol (Fig. 4.25). In addition, these strains significantly reduced the linkage content in the lignin fraction giving the chemical shift of^{1}H, ppm at 4.9 to 5.1 which represented the formation of carbohydrates, 6.2 to 6.7 exhibited aromatic-H in syringyl and 6.7 to 7.1 aromatic-H guaiacyl. The chemical shift at ^{13}C, ppm by this fungus provided the evidences for a LiPhatic and aromatic groups. Relatively, both of these strains M-2 and M-3 showed cleavage not only of *β*-aryl-ether (*β*-O-4), but also of resinol (*β-β*) and of the less prevalent phenylcoumaran (*β*-5) linkages for biodegradation of lignin. *Aspergillus fumigatus*strain M-4 had less pronounced activity than strains (Figure 4.26). This strain showed the chemical shift of methoxy and slightly for the aLiPhatic groups.

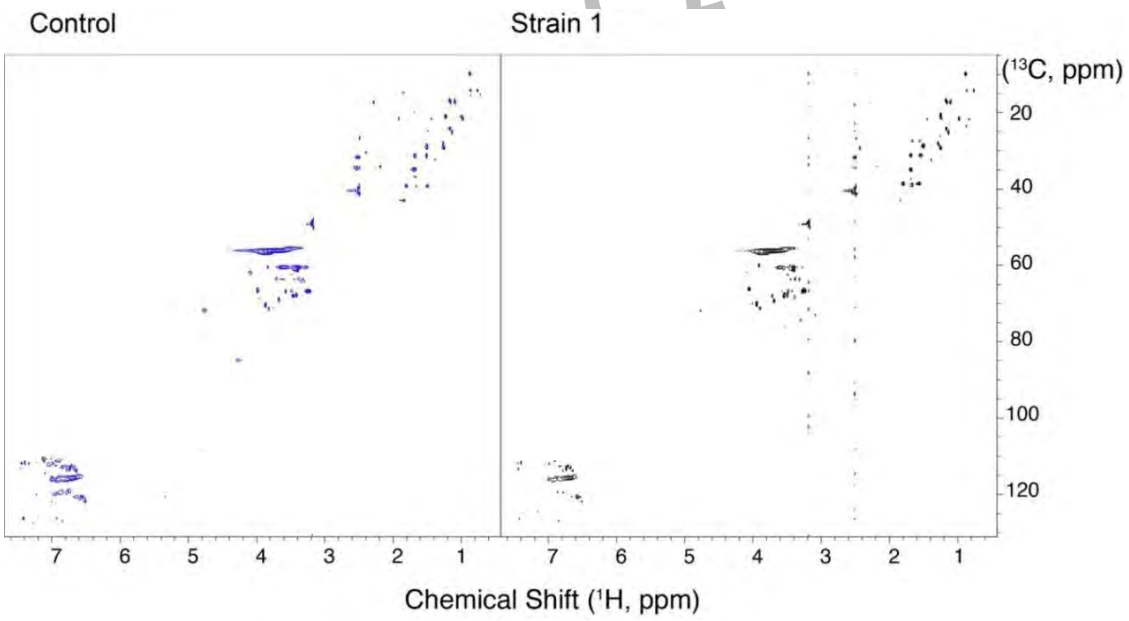

Figure 4. 51: Full ^{1}H-^{13}C HSQC NMR spectra of Kraft lignin in the absence (left) and in the presence (right) of fermentation with the isolated yeast *Aspergillus terrus* (M-1). Spectra were acquired with an 800 MHz instrument at 323 K by dissolving 30 mg condensed lignin medium in DMSO-d_6.

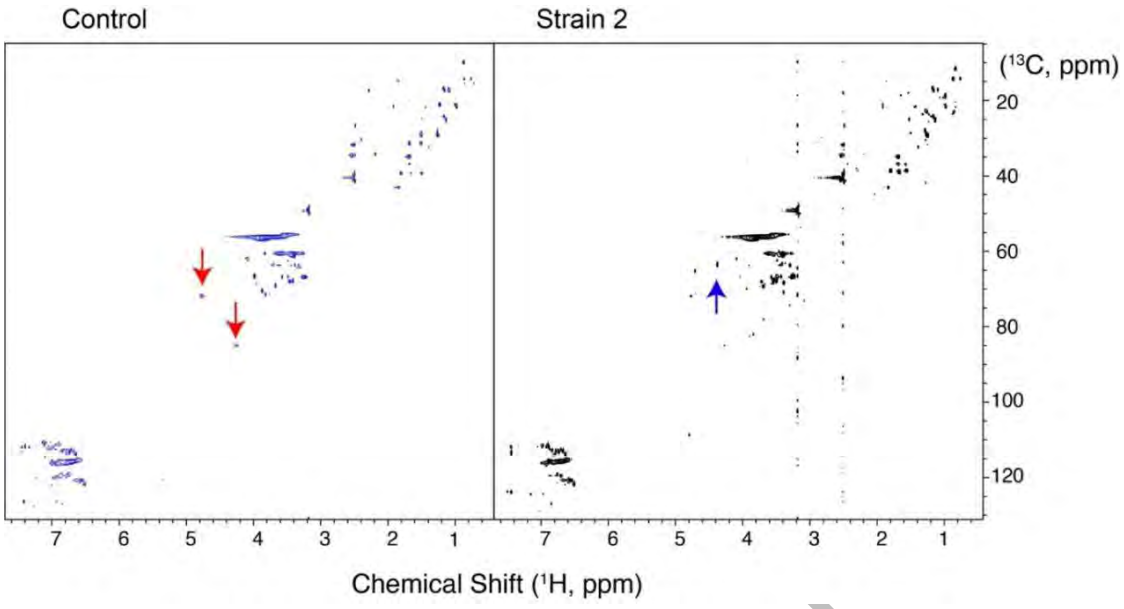

Figure 4. 52: Full ^{1}H-^{13}C HSQC NMR spectra of Kraft lignin in the absence (left) and in the presence (right) of fermentation with the isolated yeast *Dipodascus australiensis* (M-2). Spectra we re acquired with an 800 MHz instrument at 323 K by dissolving 30 mg condensed lignin medium in DMSO-d_6. Red arrows indicate the loss of linkage signals, while the blue arrow exemplifies the formation of an aromatic alcohol product.

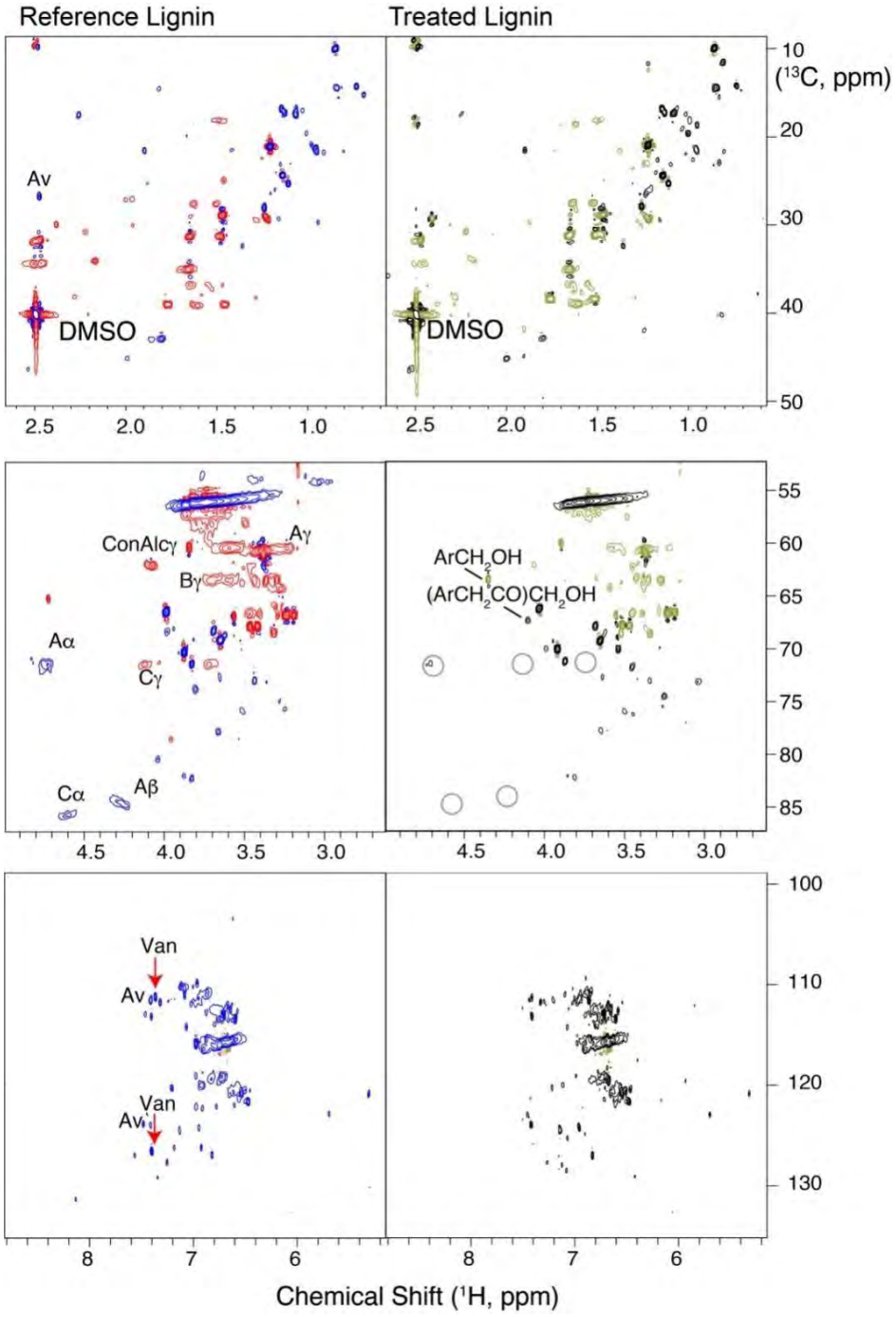

Figure 4. 53: Multiplicity-edited ^{1}H-^{13}C HSQC NMR spectra of the aLiPhatic (top), linkage (middle) and aromatic (bottom) regions of Kraft lignin in the absence (left) and in the presence (right) of fermentation with the isolated yeast *Geotrichum candidum* (M-3). Spectra were acquired with an 800 MHz instrument at 323 K by dissolving 30 mg condensed lignin medium in DMSO-d_6.

Abbreviations are: Van: vanillin, Av: acetovanillone, ConAlc: coniferyl alcohol, A: β-O-4 linkage, B: β -5 linkage, C: $\beta - \beta$ linkage.

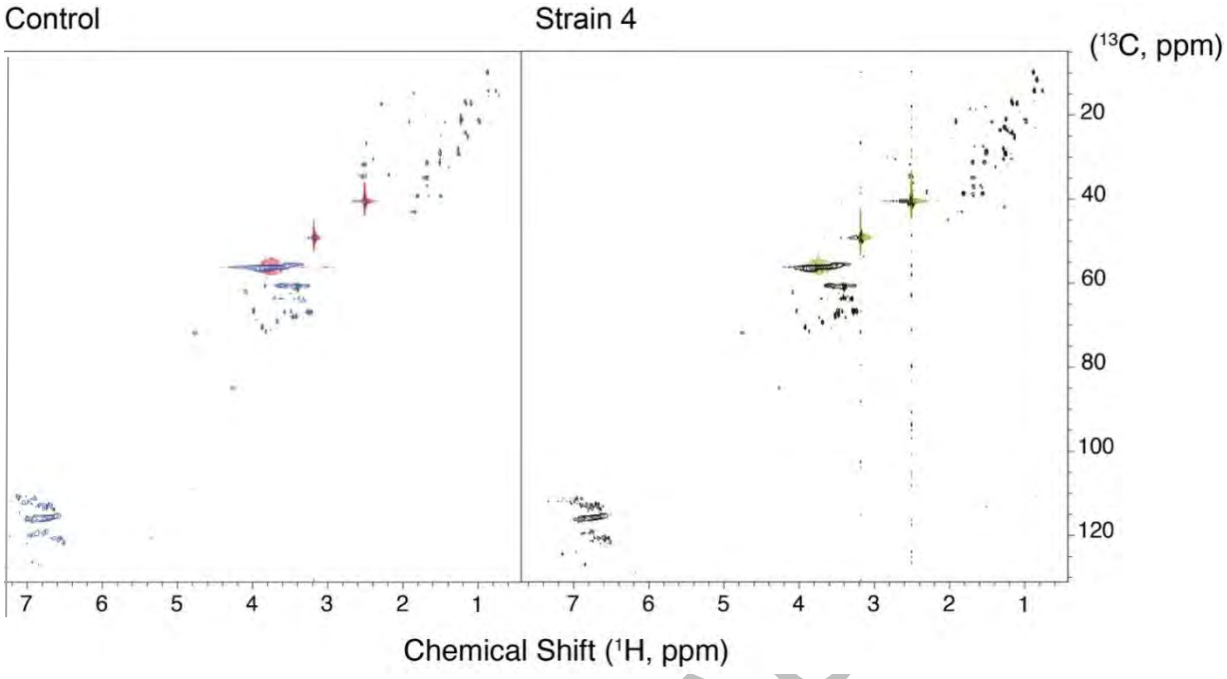

Figure 4. 54: Full ^{1}H-^{13}C HSQC NMR spectra of Kraft lignin in the absence (left) and in the presence (right) of fermentation with the isolated yeast *Aspergillus fumigatus* (M-4). Spectra were acquired with an 800 MHz instrument at 323 K by dissolving 30 mg condensed lignin medium in DMSO-d_6. Strain 4 shows a limited activity in the conversion of lignin.

Table 4. 15. Assignments of signals in ^{1}H NMR spectrum of typical functional groups

Chemical shift/ppm	Assignment
9.7-9.9	Cinamaldehyde and benzaldehyde
6.7-7.1	Aromatic-H in guaiacyl
6.2-6.7	Aromatic-H in syringyl
5.8-6.2	Benzyl OH in β-O-4 and β-1
4.9-5.1	Carbohydrates
3.3-4.0	Methoxyl
3.0- 3.1	Hβ in β-1
2.2-2.4	Phenolic OH
1.6-2.2	ALiPhatic OH

Table 4. 16. Assignments of signals in ^{13}C NMR spectrum to typical functional groups in lignin

Chemical shift/ppm	Assignment
167-168	Unconjugated- COOH
162-168	Conjugated- COOH
140-155	C3,C4 aromatic ether or hydroxyl
127-140	C1, aromatic C-C
123-127	C3, aromatic C-C
117-123	C6, aromatic C-H
114-117	C3, aromatic C-H
106-114	C2, aromatic C-H
78-90	ALiPhatic Cβ-O
67-78	ALiPhatic Cα-O
54-57.5	Methoxyl

4.14 Effect of different lignin derivatives on the growth of fungi

Lignin degradation of Ascomycetes strains (M-1-M-4) were also optimized on different derivatives of lignin including cellulose, guaiacol, hyroxybenzaldehyde, syringaldehyde and vanillin. The results suggested that strains show different growth

pattern on different substrate, this idea anticipate the information from NMR spectroscopy, as it was defining the degradation comparatively well as compare to whole lignin. The HPLC analysis of these derivatives was performed to check which component is more significantly degraded by selected fungal strains. It will help in deep understanding of the fact that which polymer is more effectively degraded by ascomycetes strains.

Table 4. 17. Degradation of lignin derivatives by fungal isolates M-1, M-2, M-3 and M-4

Strains	Cellulose	Guaiacol	Hydroxybenzaldehyde	Syringaldehyde	Vanillin
M-1	+	+	+	+	+
M-2	+	+	+	+	+
M-3	++	++	+	+	++
M-4	++	++	+++	++	+++

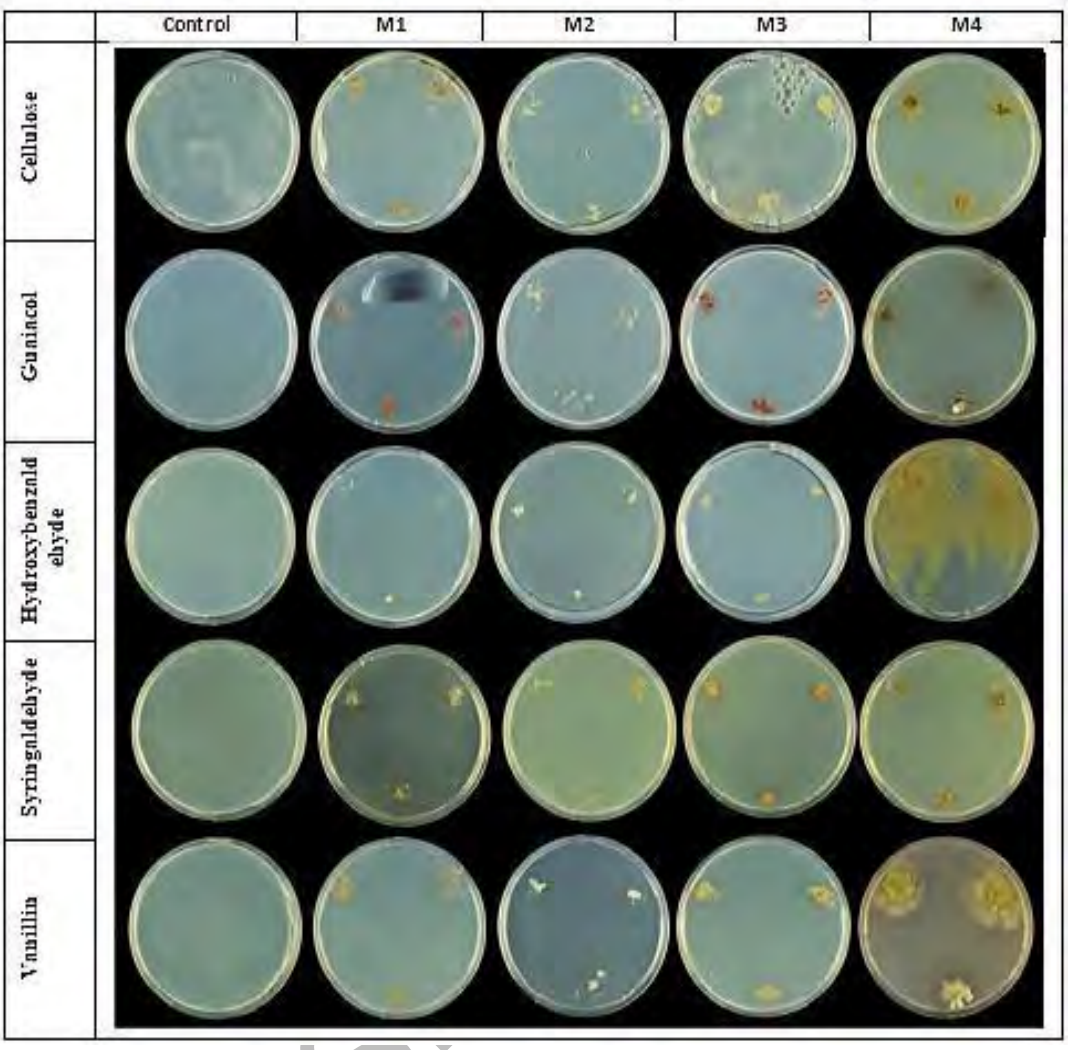

Figure 4. 55: Growth of fungal strains on different lignin derivatives

4.15 Analysis of biodegradation of lignin intermediates by Reverse-phase high pressure liquid chromatography

Reverse Phase High pressure liquid chromatography (HPLC) used to determine the degradation of intermediate products obtained from NMR. Following lignin derivatives were used including guaiacol, hydroxybenzaldehyde, syringaldehyde, vaniline.. The degradation pattern of the aforementioned molecules for was observed for a period of six days. HPLC analysis of control and fungal mediated degraded samples showed reduction and shifting of peaks were revealed for vaniline and hydroxybenzaldehyde for the strain *Geotrichum candidum* M-3 and *Aspergillus fumigatus*M-4. However, the fungal strains did not show any significant degradation of syringaldehyde and guaiacol since slight shift of peaks were obtained. Collectively,

it can be suggested that these intermediate products of lignin were modified/transformed into simple compounds (figures 4.28, 4.29, 4.30 and 4.31)

4.16 Degradation of Guaiacol by selected fungal strains

The lignin derivative product guaiacol is used to determination further degradation of this intermediate to any other component. These results depict that probably the guaiacol degradation process is intracellular process and cellular metabolic machinery was involved to its further break down. Therefore, the conversion of guaiacol was observed as the highest peak from the control has been shifted to some lower peaks. The retention time of catechol is 15.7 and it was observed in the case of all strains. However, the peak formed at retention time 12.46 was observed by *Geotrichum candidum* M-3 and *Aspergillus fumigatus* M-4 was slightly different and represented the production of resorcinol a much simpler compound. (Figure 4.28 a,b,c,d,e)

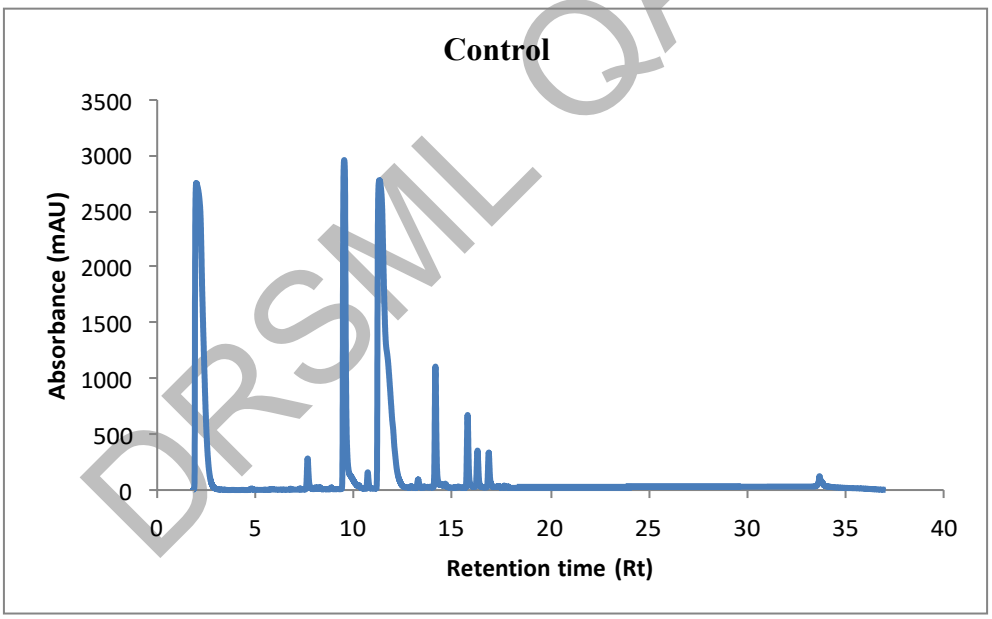

Figure 4. 56: HPLC spectra of lignin derivative compound Guaiacol (Control)

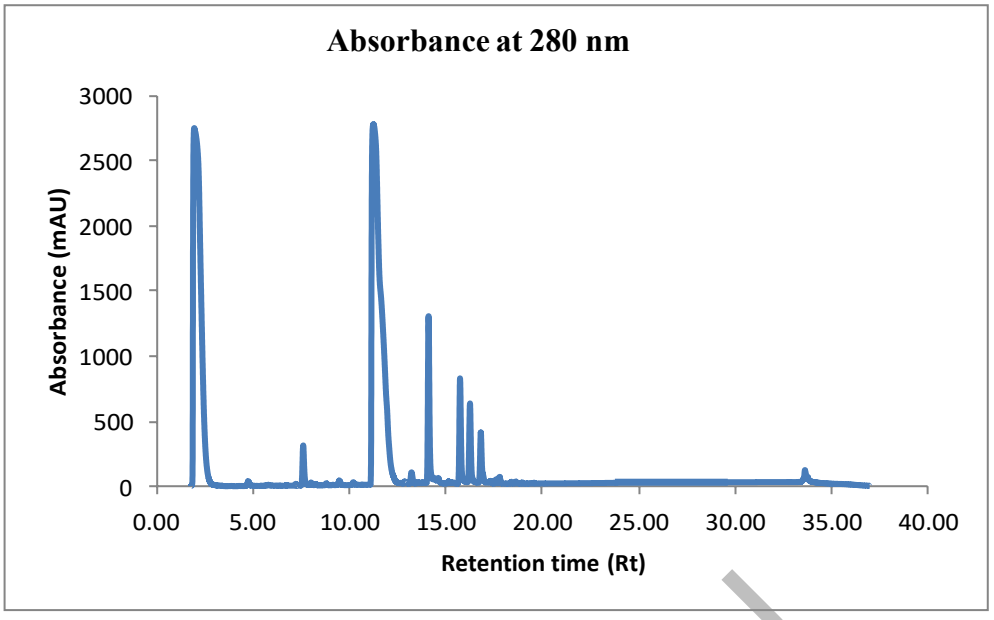

Figure 4. 57: Biodegradation of Guaiacol by *Aspergillus terrus* (M-1)

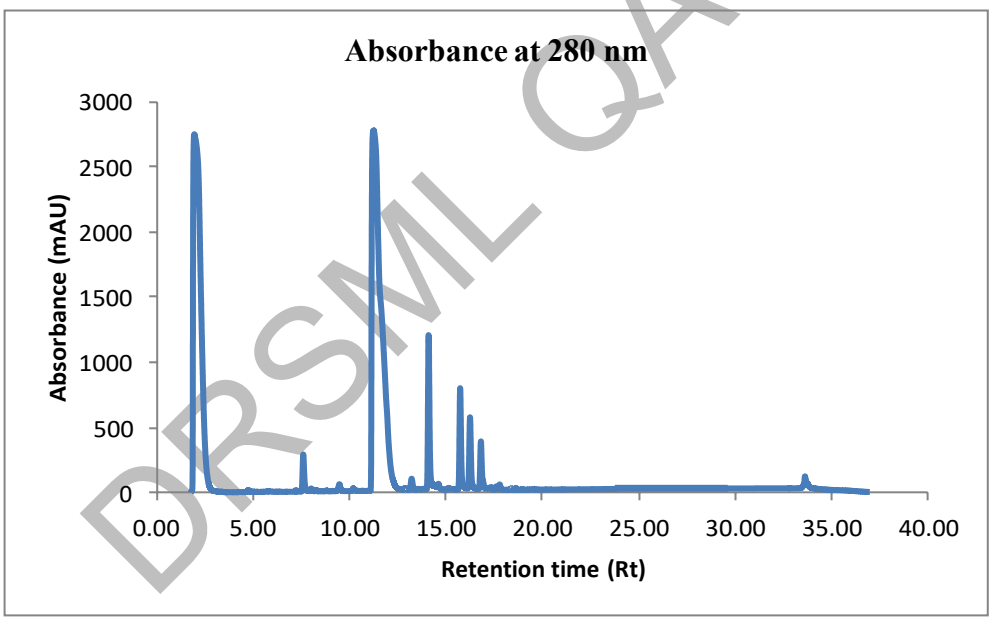

Figure 4. 58: Biodegradation of Guaiacol by *Dipodascus australiensis* (M-2)

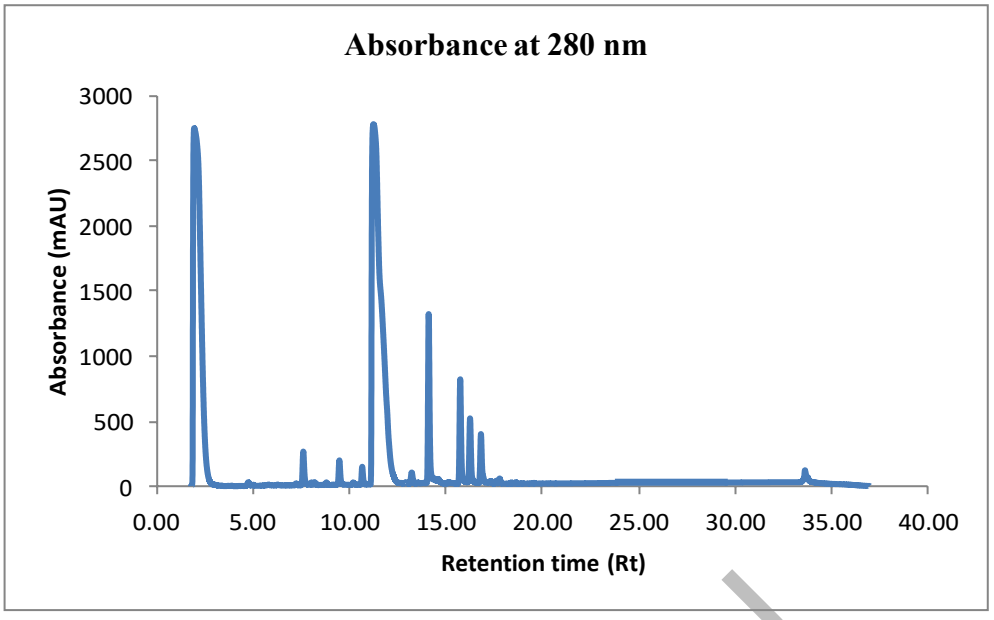

Figure 4. 59: Biodegradation of lignin intermediate Guaiacol by *Geotrichum candidum* **(M-3)**

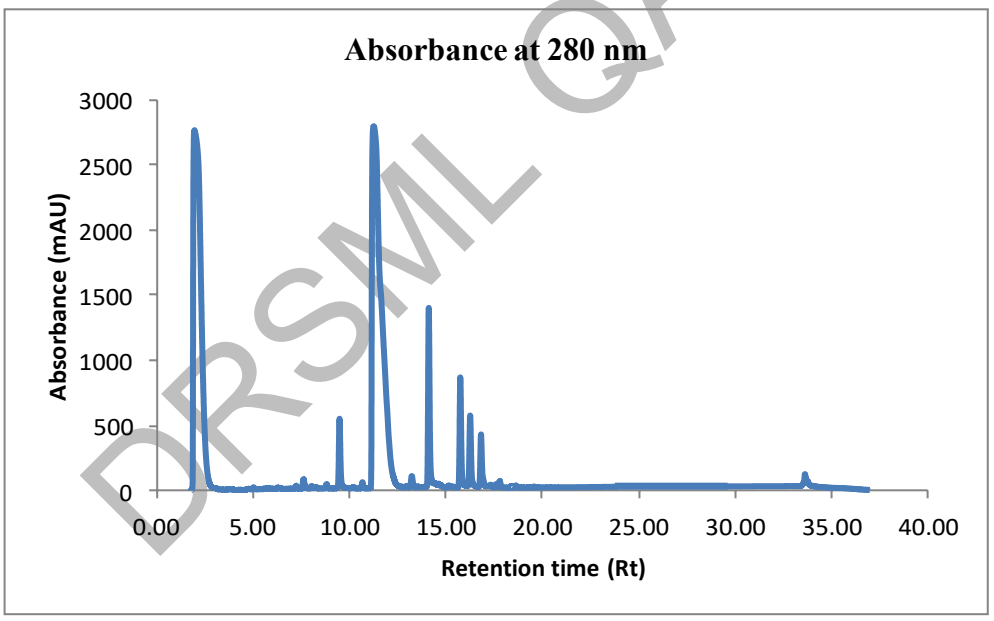

Figure 4. 60: Biodegradation of lignin intermediate Guaiacol by *Aspergillus fumigatus***(M-4)**

4.17 Degradation of Hydroxybenzaldehyde (lignin derivative) by fungi

The effect of degradation of lignin derivative product hydroxybenzaldehyde into simpler compound was analysed at the interval of every two days for six days. The slight shift of peaks was observed from control to strains *Dipodascus australiensis* M-2, *Geotrichum candidum* M-3, *Aspergillus fumigatus* M-4 at 6th day. The new peak was formed at retention time 8.6 to 9 shows the production of gallic acid and 11.9

retention times for M-3 and M-4 shows benzoic acid for the strains, results depicts the attribute to a loss in some conjugated groups with the aromatic ring (e.g., hydroxyl groups). (Figure 4.29 a,b,c,d,e)

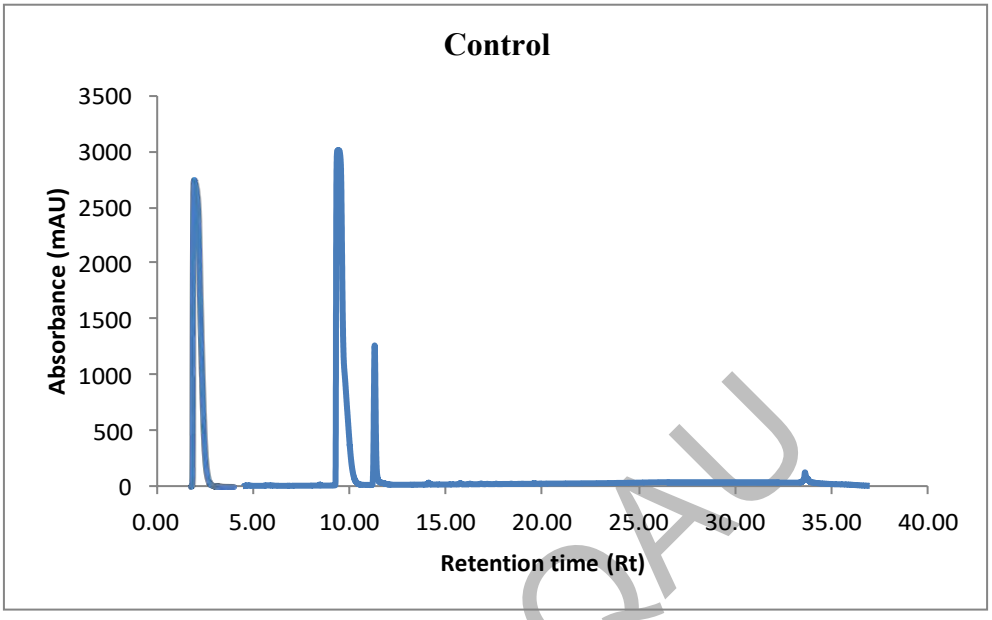

Figure 4. 61: HPLC spectra of lignin derivative compound Hydroxybenaldehyde (Control)

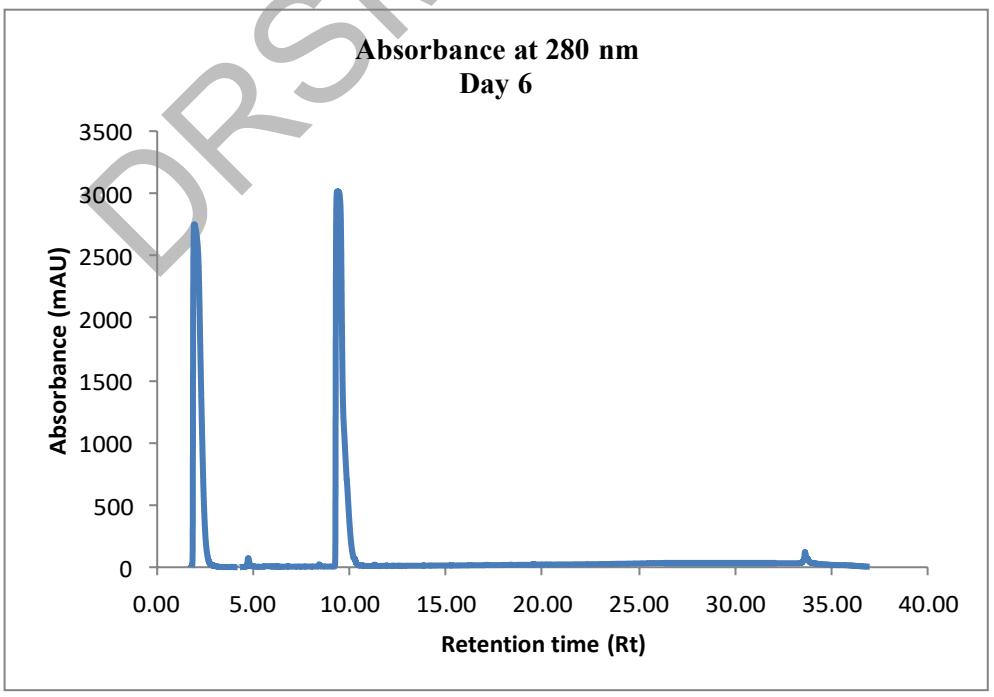

Figure 4. 62: Biodegradation of lignin intermediate Hydroxybenaldehyde by *Aspergillus terrus* (M-1)

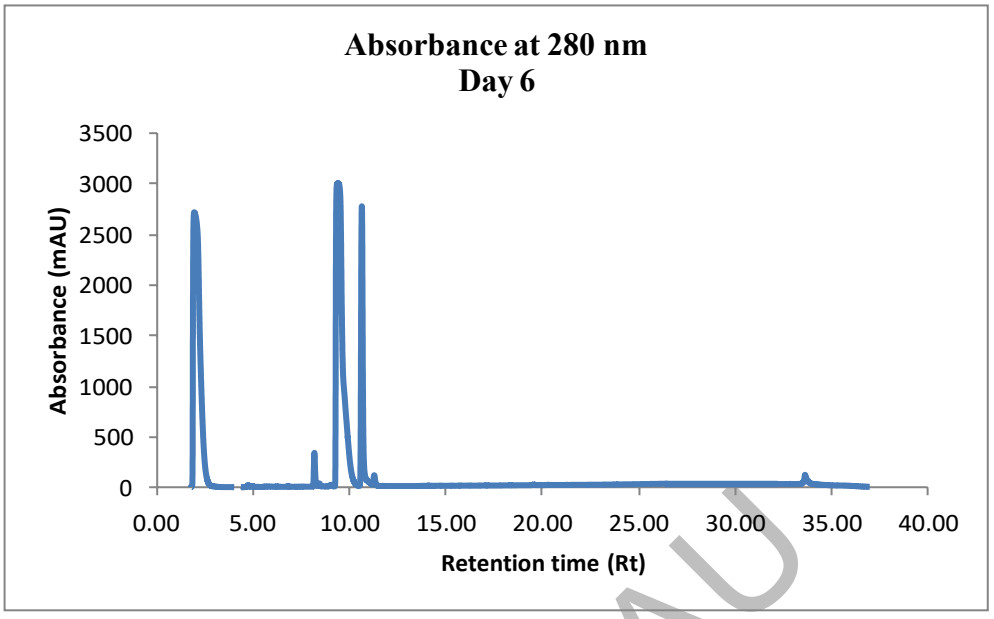

Figure 4. 63: Biodegradation of lignin intermediate Hydroxybenzaldehyde by *Dipodascus australiensis***(M-2)**

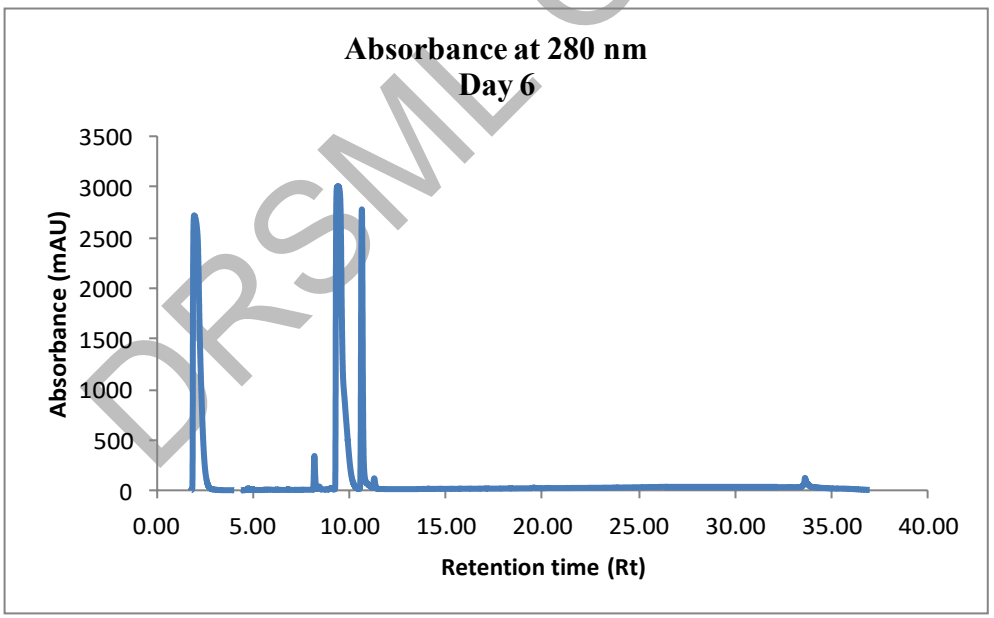

Figure 4. 64): Biodegradation of lignin intermediate Hydroxybenzaldehyde by *Geotrichum candidum* **(M-3)**

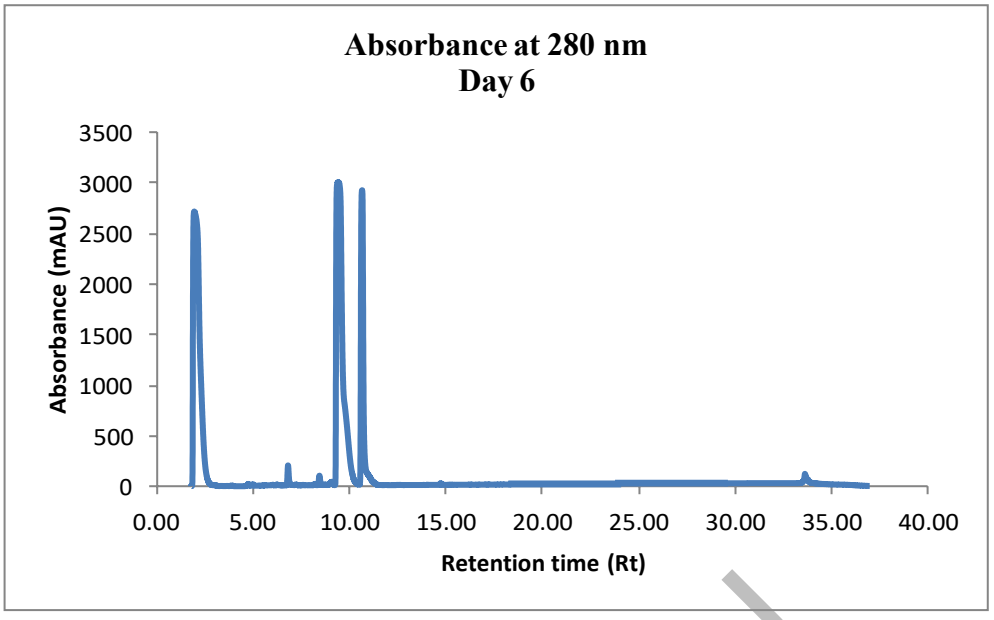

Figure 4. 65: Biodegradation of lignin intermediate Hydroxybenzaldehyde by *Aspergillus fumigatus*(M-4)

4.17.1 Degradation of Syringaldehyde (lignin derivative) by fungi

The lignin degradation for the derivative product syringaldehyde was analysed to observe the formation of simpler compounds from intermediates. The slight shift of peaks was observed by strain M-1 *Aspergillus terrus* and strain M-2 *Dipodascus australiensis* from control. For strains M-3 *Geotrichum candidum* at 6th day, and for M-4 *Aspergillus fumigatus* the conversion of compounds was observed from 2nd day to 6th day. The new peak was formed at retention time 8.7 to 10.3 the strains, results depicts the attribute to a loss in some conjugated groups with the aromatic ring (e.g., hydroxyl groups). This retention time showed the production of phenol and aldehydes. (Figure 4.30 a,b,c,d,e)

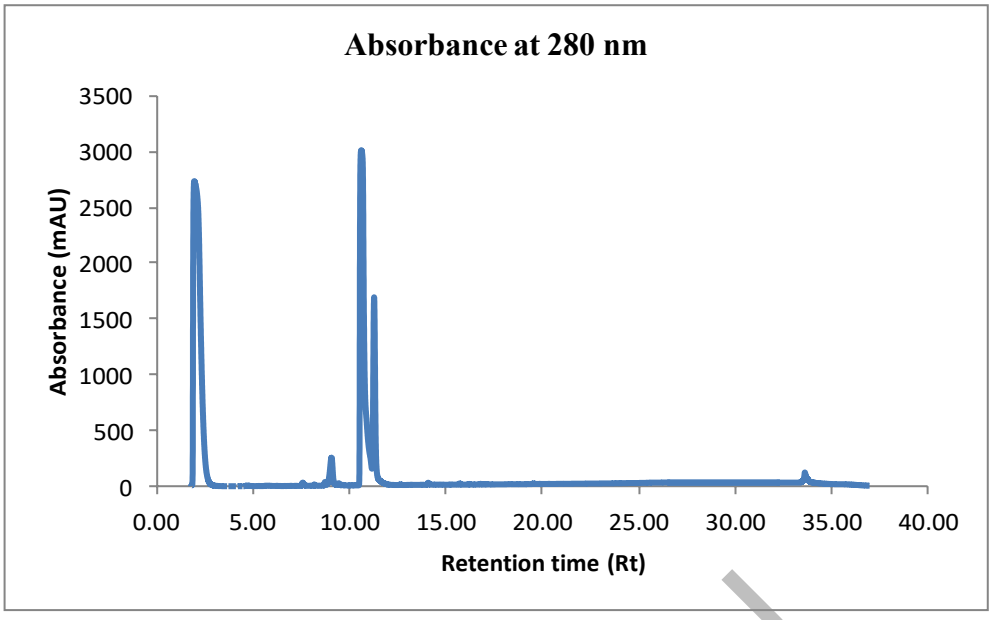

Figure 4. 66: HPLC spectra of lignin derivative compound Syringaldehyde (Control)

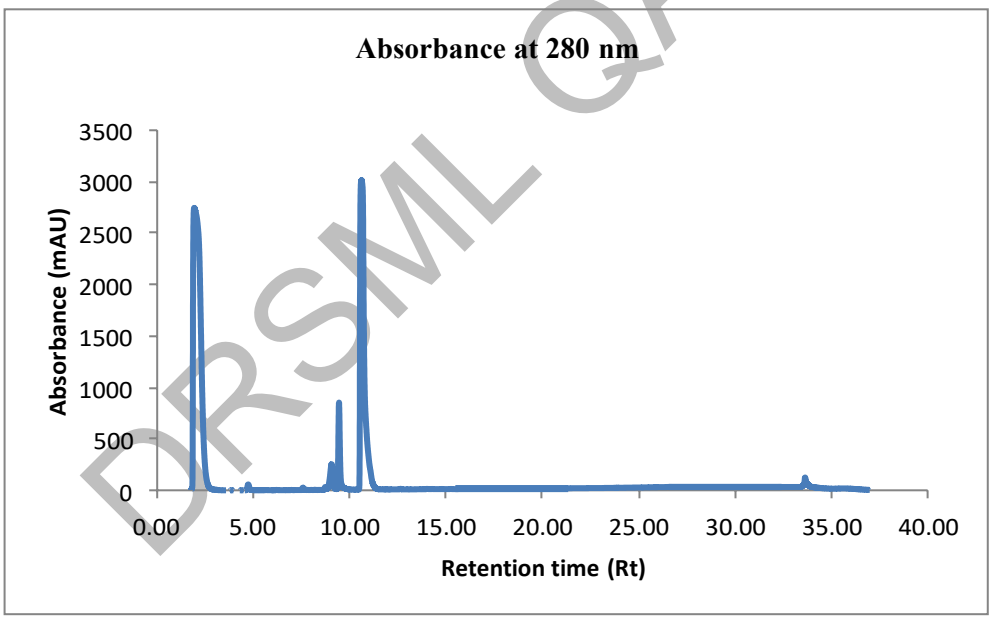

Figure 4. 67: Biodegradation of lignin intermediate Syringaldehyde by *Aspergillus terrus* (M-1)

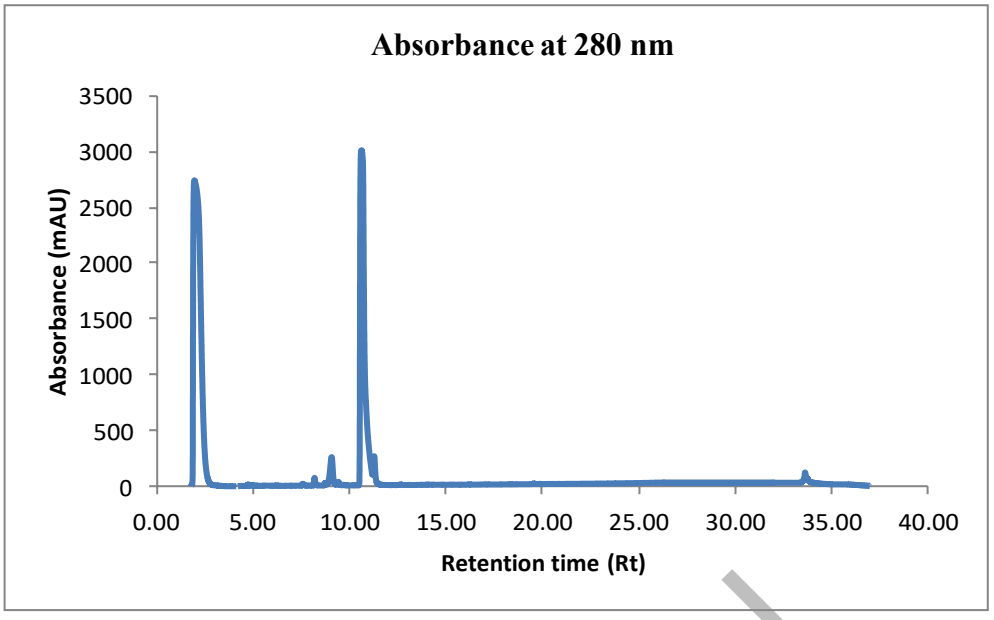

Figure 4. 68: Biodegradation of lignin intermediate Syringaldehyde by *Dipodascus australiensis* (M-2)

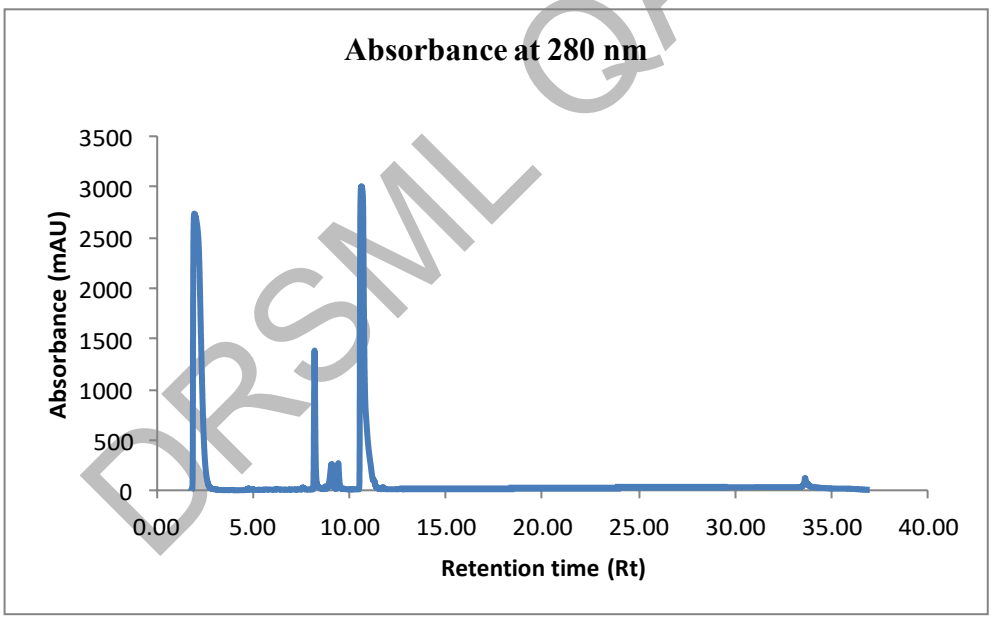

Figure 4. 69: Biodegradation of lignin intermediate Syringaldehyde by *Geotrichum candidum* (M-3)

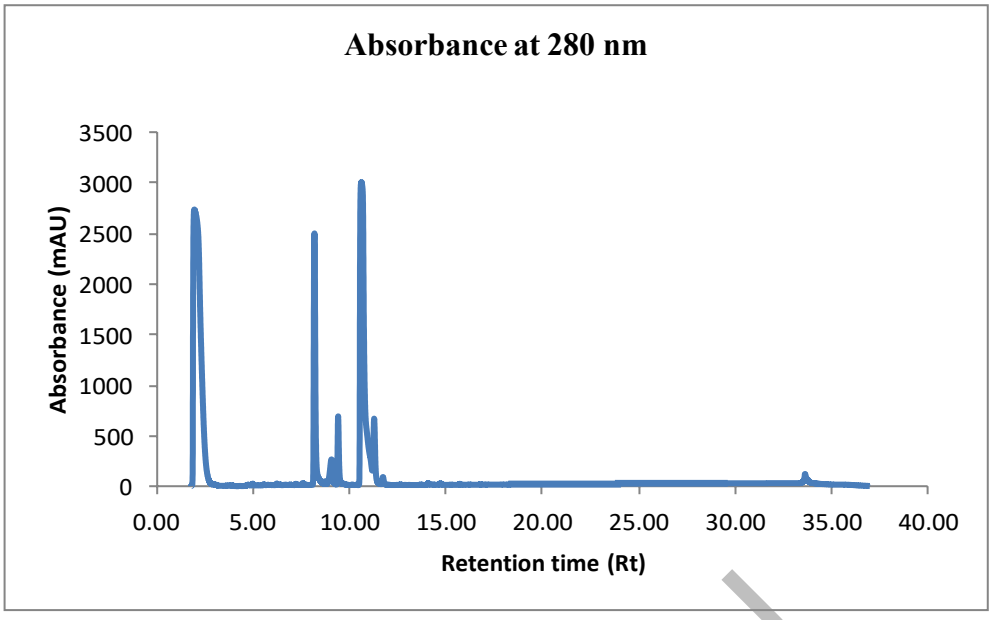

Figure 4. 70: Biodegradation of lignin intermediate Syringaldehyde by *Aspergillus fumigatus* (M-4)

4.17.2 Degradation of Vanilin (lignin derivative) by fungi

The lignin derivative product vanilin is used to determine further degradation of this intermediate to any other component. The shift of peak was observed by *Aspergillus terrus* M-1 at 6th day of incubation. The peak formed at control was not showed for this strain. This depicted the transformation of the intermediate vanillin to some simpler monomers. For the strain *Dipodascus australiensis* M-2 the results obtained were relevant to that of M-1 *Aspergillus terrus.* The very small shift of peaks was observed from control to strains M-3 *Geotrichum candidum* at 6th day at retention time 8.3 to 9 represents the conversion of vaniline to some phenyl groups and for M-4 *Aspergillus fumigatus*the conversion to compounds was observed from 2nd day to 6th day. The new peak was observed at retention time 8 to 10.3 and represents propanoate and poly (ethylene glycol) and phenolic groups. From all the strain M-4 *Aspergillus fumigatus* provided the comparatively better results in terms of clear formation of new peaks. (Figure 4.70.,4.71, 4.72, 4.73,4.74)

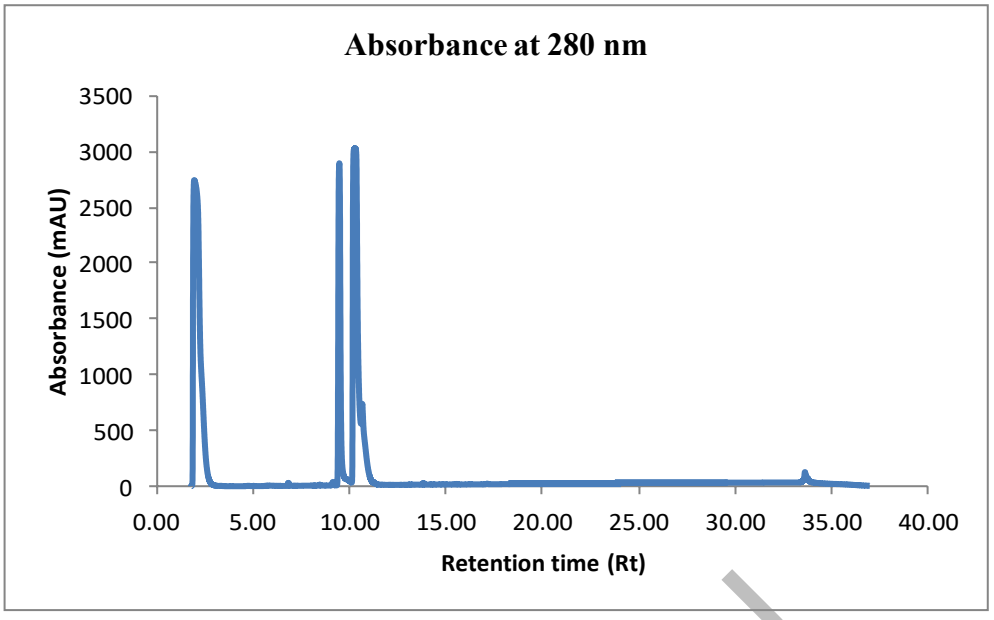

Figure 4. 71: HPLC spectra of lignin derivative compound Vanillin (Control)

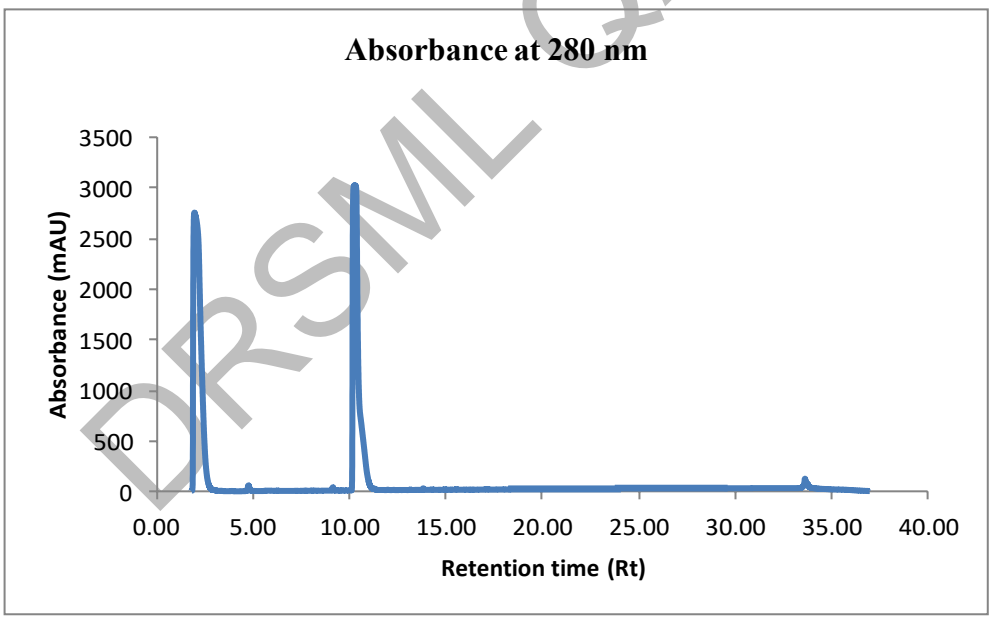

Figure 4. 72: Biodegradation of lignin intermediate Vanillin by *Aspergillus terrus*(M-1)

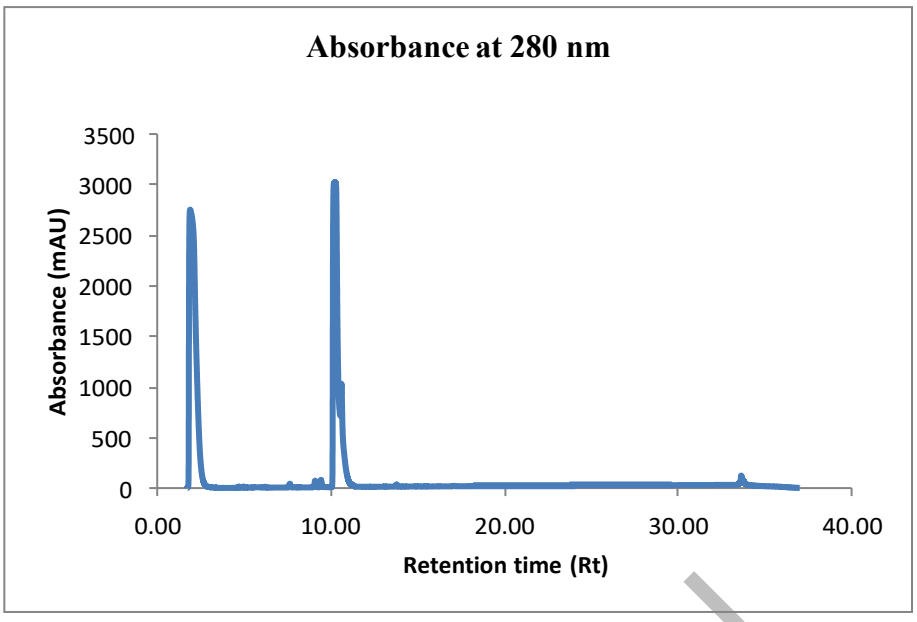

Figure 4. 73: Biodegradation of lignin intermediate Vanillin by *Dipodascus australiensis***(M-2)**

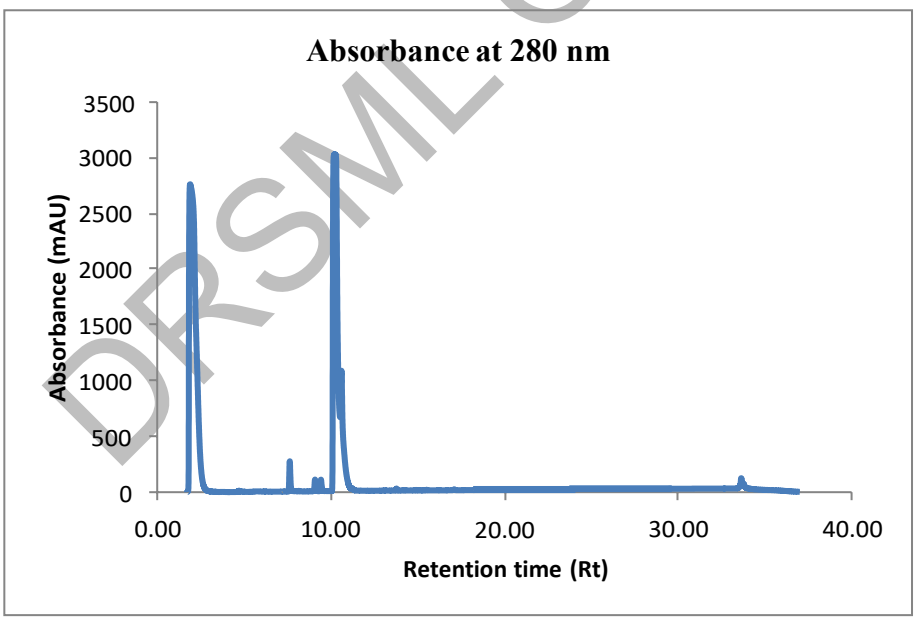

Figure 4. 74: Biodegradation of lignin intermediate Vanillin by *Geotrichum candidum***(M-3)**

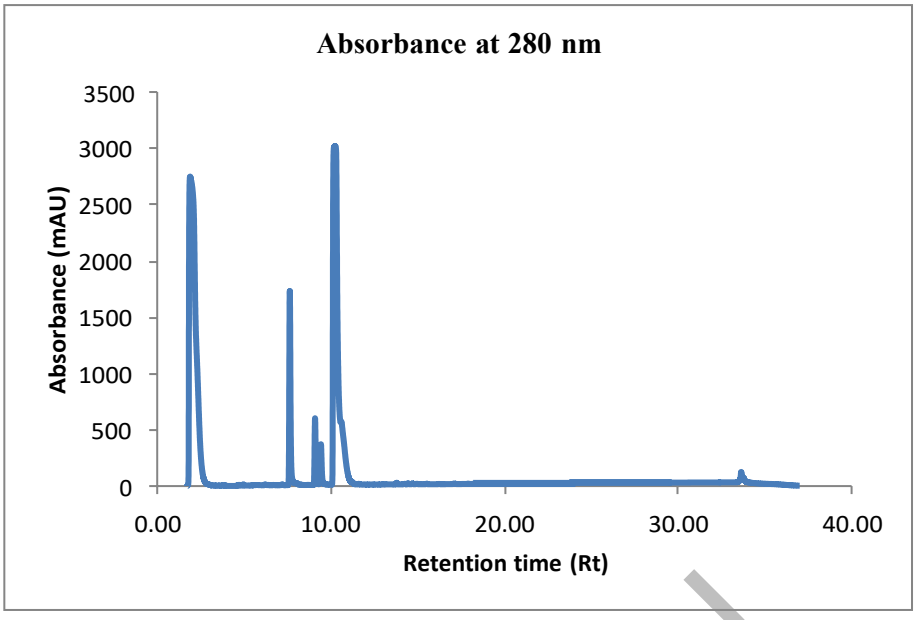

Figure 4. 75: Biodegradation of lignin intermediate Vanillin by *Aspergillus fumigatus***(M-4)**

Discussion

Lignin is the second most abundant source of carbon and only few microorganisms have been identified to break down its complex structure completely. The degradation of lignin and other complex natural polymers is a complex process involving different cellular catalysts under suitable environmental conditions (Kaczmarek *et al*., 2020). Besides extensive literature and research, the mechanisms underlying the biolo gical depolymerisation of lignin still remains unclear owing to its structural heterogeneity. Lignin is a second most prevalent biopolymer found in nature (Tribot *et al*., 2019). Other important source of lignin into the environment is the industrial processes using huge amount of lignocellulose biomass in paper and pulp manufacturing industry (Chukwuma *et al*., 2020). These industries generate huge amount of the highly alkaline lignin containing wastewater into the environment posing a significant thread to the ecosystem (Hoque *et al*., 2018). Many microorganisms have the potential to degrade lignin and related complex biopolymers and convert them in to simple molecules (Banwell *et al*., 2021). During this process a number of the commercially important by products are formed creating an opportunity to transform lignocellulosic waste into an opportunity. In the prospective of the global sustainability the bio refining of the lignocellulosic waste has gained a considerable attraction making biodegradation research as an important component of the modern biotechnological industries (Jain *et al*., 2022). Considering the aforementioned implications and future scope of the biodegradation of the complex lignin polymers the search of potent microbial strains having the metabolic ability to transform these compounds into valuable resources comply notion of circular economy. In present work four potent fungal strain were isolated from pulp and paper mill effluent to determine their metabolic efficiency to degrade lignin under optimized conditions.

In the first phase of the research the wastewater was analysed. The results indicated that black liquor obtained from paper and pulp industry have high BOD and COD indicating the presence of high pollution load and organic matter (Chamorro *et al*., 2022) (Table 4.1). In addition many inorganic pollutants were also found in high quantities. In previous research various scientist have reported that due to the involvement of variety of chemical. The organic pollution load of the black liquor was quite higher as compare to the other liquid waste (Pola *et al*., 2022). This could be due

to involvement of various processes and chemical for the manufacturing of wood pulp. Usually 30 to 35% of lignin is present in black liquor giving it darkish brown to black colour (Putri *et al*., 2019). Similar findings were observed for our sample. With respect to the microbial load, the waste water contained a number of fungi and bacterial strains. In a previous study the isolation of *Bacillus* strains is reported from the paper and pulp mill effluent and posses the lignin degradation ability (Narra *et al*., 2020). Therefore black liquor considered as an important source for isolation and screening of microbial strains with efficient biodegradation abilities.

Based on the initial, experiment a rich diversity of bacteria and fungi can be detected from black liquor (Mathews *et al*., 2019). However, the screening of fungal strains due to their greater stability, faster growth rate and excellent scretomes is one of the most criteria for the selection of these microbes for any specific biotechnological application (Aslam *et al*., 2018). A total of 13 different fungal strains were screened and presented in the table 4.2 showing the comparative growth in medium containing black liquor. Among these strains the isolates number M-1, M-2, M-3 and M-4 performed better in terms of their growth and lignin degradation abilities. Various qualitative and quantitative methods have been proposed for the isolation of the lignin degrading microorganisms from the environmental and waste samples. These methods demonstrated the use of black liquor as sole source carbons in the mineral salt media. Some of the compounds used as a single carbon source in a lignin selection medium were alkali lignin or lignin kraft (Mendes *et al*., 2021). In present study, black liquor was used for the enrichment and isolation of the lignolytic fungi because it is readily available as well as less expensive substrate. The major content in black liquor is lignin which provides a ready source for their growth. In principle, only fungi which can utilize lignin as a carbon source that will be able to grow on this medium (Del Cerro *et al*., 2021). This ability is only possessed by fungi that can decompose lignin polymers into simple compounds so that they can be metabolized by fungal cells to produce energy to grow. Numerous studies have also reported that lignolytic fungi can grow well in media containing black liquor as their primary carbon source. (Ramadhani et al., 2019; Singh & Arya, 2019). Todate, a number of whate rot fungi have been isolated from different samples including *P. chrysosporium*, *C. versicolour, T. versicolour*. *P. ostreatus* and *C. Subvermipora* having the metabolic traits to degrade lignin and other related chemicals. Yingjie *et al* 2017, isolated sixty three

strains of the lignin degrading fungi and suggested that *Myrothecium verrucaria* as the most efficient for having exceptional enzyme production and biodegradation rates. Djanira *et al*., 2015, reported 13 white rot fungi isolated from black liquor, among them *Bjerkandera adusta* about 10 % decolourization activity on the medium containing black liquor. In present work, thirteen fungal strains were initially showed positive results on medium containing black liquor. Therefore, black liquor is considered as a important waste to isolate potential fungal strains with excellent lignin degradation ability.

The further screening suggested that the strain M-1, M-2, M-3 and M-4 were more efficient lignin degraders as compared to the others on the basis of secondary screening. These strains were identified as *Aspergillus terrus, Dipodascus australiensis, Geotrichum candidum* and *Aspergillus fumigatus.* White rot fungi is considered to be dominant creator in waste containing lignocellulosic biomass. Being saprophytic in nature these microorganism are coined with metabolic machineries for the degradation of complex lignin biopolymers (Cain, 2018). In contrast of the previous findings the most of the strains screened in the present study belong to Ascomycota as compare to other wood decaying fungi. This could possibly because of the difference of the isolation source, type of wood material and local environmental conditions. These strains showed varying capacity of lignin biodegradation and colour reduction when subjected to varying black liquor concentrations. Different strains of the *Basidiomycetes* have been reported for the biodegradation of the lignin. The studies reported the most efficient known lignin *degrader was white rot fungi i.e., Phanerochaete chrysosporium* (Loi *et al*., 2020). This white rot fungus belongs to Basidiomycetes and show good metabolic potential for depolymerisation of lignin into simpler intermediates (Nandal *et al*., 2021). However, very few members of Ascomycota have been previously reported for lignin biodegradation. Most of the previous studies demonstrates the role of different members of Ascomycota in the biodegradation of cellulose and other biopolymers (An *et al*., 2022; Kumar & Chandra, 2020)**.** In contrast to the present studies, all the four isolated strains having the b iodegradation potential for lignin and related compounds belonged to the Asomycetes indicating the distribution and adaptation of the these fungi in the Lahore region. However, further research is required to identify

biodiversity and functional traits of the Ascomycetes particularly with reference to their lignin degrading ability.

In other set of experiments, the biochemical efficiency of the four selected fungal strains was observed under varying pH and temperatures. It was noted that the fungal strains showed different growth, lignin degradation capacity and colour reduction efficiency under the effect of varying pH and temperatures. The strain *Aspergillus terrus* M-1 showed relatively similar growth trend under the effect of different temperatures and pH (Figure 4.1 a). However, this strains proved to be a better lignin degrader under relatively higher temperature (41^0C) and slightly alkaline pH (Figure 4.2 a). It seems that *Aspergillus terrus* adopted well in the complex environment of the black liquor. Practically, its ability to strive in alkaline medium with elevated temperature could be attributed to the higher pH of the black liquor and higher process temperature. Subsequently, the membrane of the fungal cells could have been modulated their membrane associated transported to withstand the harsh conditions of the environment (Rapoport *et al.,*2019). Similar trends have been found for the other isolated fungi including *Dipodascus australiensis* M-2, *Geotrichum candidum* M-3 and *Aspergillus fumigatus* M-4. It was interesting to note that *Dipodascus australiensis* M-2 showed the slight difference in growth on varying temperature and pH (Figure 4.1 b). Recently, Zhao*et al*., have reported relative abundance and role of *Dipodascus australiensis* in the composting of food waste. However, the role of *D. Australiensis* has not been reported so far in the biodegradation of the lignin and other related compounds. In present study, we have reported for the first time the potential of the *Dipodascus australiensis* for biodegradation of lignin and production of ligninlytic enzymes (Parveen *et al*., 2022). Moreover, high temperature tolerance of *Dipodascus australiensis* could be associated with its wide spread distribution to high temperatures zones (Berber-Villamar et al., 2018; Zhu et al., 2021). The strain *Geotrichum candidum* M-3 also showed a similar trend of growth and biodegradation efficiency under arying temperature and pH (Figure 4.1 c). Similar findings have been previously reported by (Catucci et al., 2020), which described that these fungi have been changing their growth condition and metabolic capacity for lignin degradation owing to change in the environment (Gad *et al*., 2022). The colour reduction efficiencies of the aforementioned fungal strains were also revealed somewhat similar trends with highest by *Geotrichum candidum* M-3. The colour reduction of the media

containing lignin (even in the black liquor) is an indication of the biodegradation of the complex biopolymers by the action of cellular machines. Various reports suggested that both bacteria and fungi alter chemical composition of the lignin and reduce its colour. It is also interesting to learn that all of the four fungal isolates showed better growth, lignin degradation and colour reduction under alkaline conditions. In contrast to our results, (Eggert et al., 1996) demonstrated that fungi are more inclined towards acidic environment and prefer low hydrogen concentration for their growth, substrate accession and metabolism. In another study (Suryadi *et al*., 2022) reported that the fungal growth and lignin peroxidise production was the function of slightly acidic conditions mostly around 5-6 pH. Again, the strains used in the present study dictated higher efficiency at elevated temperature and pH which could be associated with their isolation source which was alkaline in nature (Xu *et al*., 2018). pH plays critical role both in the growth and metabolism of the microorganisms. It has strong influence on their cell structure and function. In a general prospective, both higher and lower pH is considered inhibitory for microbial growth and metabolism. It has been suggested that change in pH fungi are dominating in the ecosystem over the bacteria because of their better growth under acidic conditions. Johannes Rousk*et al*., 2009, reported that due to environmental changes the soils are becoming more acidic and fungi are increasing their dominance in the ecosystem by altering their membrane ergosterol and phospholipid fatty acids. These changes could alter the overall biogeochemical cycles. Regarding the effect of temperature, microorganisms are altering their membranes and enzymatic machinery to perform best under these conditions. The previous studies revealed that lignin degrading white rot fungi, particularly, *Phanerochaete chrysosporium* and *Phlebia radiate* were able to perform under slightly thermophilic conditions (Tuomela *et al*., 2000). Collectively, the four fungal strains screened possess excellent metabolic potential to withstand higher pH and temperature and can be used for various applications where processes are performed under hostile conditions.

The metabolic efficiency of fungal strains screened in the present study was evaluated through the production of different peroxidises and laccases. Biodegradation of lignin was determined with reference to the enzyme laccases (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP) (Shi *et al*., 2021; Zhang *et al*., 2022). The production of the enzymes by four selected strains was studied under the effect of

temperature, pH, and varying carbon and nitrogen substrates. Maximum activity for laccase was observed by *Aspergillus terrus* M-1. For LiP and MnP maximum activity was observed by *Geotrichum candidum* M-3 followed by *Dipodascus australiensis* M-2. In contrast, *Aspergillus fumigatus* M-4 yeilded higher amount of MnP (Table 4.11). These results suggested that *Aspergillus terrus* M-1 was capable to use lignocellulosic biomass efficiently and therefore laccase enzyme was released to metabolize related groups. The previously reported study by (Hasanin *et al*., 2019) exhibited the reduction of lignin content by *Aspergillus* strains by production of lignin degrading enzymes. The fermentation media was optimized for improving the yield of these enzymes under the effect of various variables. Results suggested that glucose was proved to be a potent substrate for producing more extracellular enzymes owing to its simple structure and easy assimilation profile (Figure 4.11). It has been reported that microorganisms are more efficient is utilizing simpler carbons source for their growth and metabolism (Lee *et al.,* 2019). Moreover, cells use simple substrates at the first in order to increase their growth and for the production of extracellular enzymes. Soon after utilisation of glucose, the complex carbons sources such as lignin can be degraded and by the extracellular enzymes in the medium and thus enhance their biodegradation. Previously many researchers have suggested the application of simple carbons addition along with complex substrates to boost the biodegradation efficiency of the microorganisms (Kiran *et al*., 2019).

Enzyme production on varying concentration of lignin was optimized by using lignin conc. from 0.5 to 5.5 g/L. The optimum range suggested by the results was from 2 to 2.5g/L for *Aspergillus terrus* M-1, *Dipodascus australiensis* M-2 and *Aspergillus fumigatus* M-4 (Figure 4.12). However, for *Geotrichum candidum*, at lignin concentration from 0.5 to 3, almost same results was obtained that suggested efficiency of this strain to degrade lignin. For different concentration of sucrose similar results were obtained as in the case of glucose. The lignin degrading enzyme production increased with increase in simpler carbon source concentration (Figure 4.13). (Taherzadeh-Ghahfarokhi *et al*., 2019) provided the similar results for lignin degradation by adding carbon source in media. Different carbon sources have been evaluated for the production of various enzymes such as laccases (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP). For laccases, prominent carbon sources are fructose, xylose, glucose, starch and cellulose. Kumar *et al*., 2016 showed

that laccase production varied significantly with change in pH, carbon and nitrogen sources. It was observed that maximum yield of laccases was obtained at a pH of 7, significant values were obtained for cellulose concentration (8%) as carbon source and peptone (2%) as nitrogen source. 35ºC was observed as effective temperature for the production of these enzymes in the research. In our study, maximum laccase production was observed to be 139 U/mL for glucose as carbon source. For LiP, highest activity was recorded as 115 U/mL while for MnP the activity was 106 U/mL. According to Nayana*et al*. 2020 optimized conditions for maximum LiP production include temperature up to 30ºC, dextrose as the carbon source, ammonium tartarate as the nitrogen source and pH value of 5. For MnP, optimized conditions for laccase production include pH value of 5 and carbon source as sucrose (Sijinamanoj et al., 2021) . Production of up to 1972 IU/mL has been obtained for MnP using substrates such as rice straw (Rana et al., 2019). In this study the maximum activity regarding laccase obtained was 106 U/mL. The maximum lignin degradation was observed at temperatures of 41ºC and 47ºC and pH values of 9 respectively. Different carbon sources utilized for lignin degradation directly impact the carbon to nitrogen ratio. Any alteration in the C:N ratio can lead to decreased biodegradation of lignin molecules. Among the mentioned carbon sources, glucose has shown to increase the lignin degradation with increased efficiency (Rybczyńska-Tkaczyk et al., 2018). Carbon sources can directly affect the molecular uptake by the cells and their passage through the cell membrane. Optimal degradation of lignin occurs at C:N ratio of 25. When this ratio is disturbed, proton pumps functioning are affected and it leads to a decrease in microbial uptake of nutrients (Zhang et al., 2022).

The relationship between the nitrogen source and lignin degradation potential was established in this study. The results suggested the direct relation of nitrate concentration to that of ligninolytic enzyme activity (Figure 4.14, 4.15.4.16.4.17). Since the enzymatic activity by all the fungal strains was increased with increasing nitrate ions and peptone. The production of lignin degrading enzymes depends on the modifiers, the availability of nitrogen sources increased the chances of utilizing modifiers which in results increased the lignin degrading activity (Jędrzejczak *et al*., 2021; Khatoon *et al*., 2019; Tatiane *et al*., 2021).

Effect of physiochemical parameters such as temperature and pH were studied to optimized the ligninolytic enzyme activity at different conditions (Sosa-Martínez *et al*., 2020). *Aspergillus terrus* M-1 showed quite a same range for enzyme production at varying pH and temperature. However, results suggested the increased in enzymatic activity with increased in pH and temperature (Figure 4.18). These result suggested the adaptation of this fungus at higher pH and temperature due to its isolating background. For strain *Dipodascus australiensis* M-2 highest degradation enzyme production was observed at 37^0C and 8pH for all the other no prominent activity was observed. The possibility of this could be its unique metabolic machinery, as this strain has never been reported previously in any study with respect to lignin degradation potential. Its metabolic pathways need to be elucidated in detail for degradation of lignocellulosic biomass (Bissaro *et al*., 2018). For other two fungal strains results obtained was similar to that of M-1 *Aspergillus terrus*, suggested the adaptation of these fungal strains to the alkaline and slight thermophilc environment due to evolving of their cellular machinery to these conditions. Previously few studies have been reported got thermophilc fungi but lignin degradation specially by Ascomycetes is still need to be elucidate (Patel & Rawat, 2021). The possible reason could be that they have changed their membrane transport system to survive in the high alkaline environment.

In the next phase of the study, NMR was performed to analyse the monomers formed during lignin biodegradation. Various fungal enzymes have the ability to act on the monomers formed and degrade them. Among the intermediates, guaiacol is known to be degraded by microbially produced monoxyenases For the degradation of this intermediate microbes utilize glucose as a carbon source, tryptone, and yeast extract as nitrogen source (Ravi et al., 2019). Hydroxylbenzaldehyde is degraded with the help of p-hydroxybenzaldehyde dehydrogenases and CO_2 is used as a carbon source in this type of degradative reaction (Feng, 2022). The third product syringaldehyde was catabolized by a family of enzymes known as oxidoredcutases (Morya et al., 2021). This product is degraded by microbes which utilize glucose as the sole carbon source (Freitas et al., 2020). Vanillin is another chemical compounds produced during the biodegradation of lignin by the action of dioxygenases enzymes(Iram et al., 2021). These products are further broken down into various simpler components. It has been observed that certain phenolic compounds can also be used as carbon sources by the

fungi to degrade intermediates including vanillin. In this study, degradation of guaiacol produced catechol and resorcinol as shown by HPLC analysis. For hydroxylbenzaldehyde, simpler product such as galic acid was formed. Phenols and aldehydes were formed as a result of syringaldehyde degradation. Vanillin was shown to produce phenyl groups, propionate and ethylene glycol. Previously, some researchers have reported conversion of the Kraft lignin into various value added products like polyurethanes, syringaldehyde, and vanillin (Sun *et al.,* 2018, Fadlallah *et al.,* 2021). Vanillin is an important lignin degradation product that can be used as flavouring agent, ripening agent and anti- foaming agent, besides its application in perfumes making (Zhao *et al.,* 2022, Banerjee *et al.,* 2019, García-Hidalgo *et al.,* 2020). White rot fungi, including *Fomes fomentarus, Coriolus versicolour,* have been reported for the conversion of lignin into vanillin using kraft lignin from *Eucalyptus grandis* (Radhika *et al.,* 2022). It is also important to mention that no previous report is found with respect to biodegradation of lignin into value added products like vanillin from the Acscomycetes strains used in the present study. Industrial vanillin production is generally carried out by aerobic oxidation process using NaOH (Hosoya *et al.,* 2020). However, due to toxicity and environmental implications of the aforesaid process, trends are re-shifting towards the application of fungi and their enzymes for the bioconversion of the lignin and lignocellosic waste into vanillin. In present work, the fungal straoins M-1, M-2, M-3 and M-4 were able to generate vanillin as one of the biodegradation products; therefore, these fungal strains present excellent opportunity to generate vario us value added molecules from the lignin and from the black liquor (Nguyen *et al.,* 2021, Liu *et al* 2019). However, further studies are required to develop bioprocess for the transformation of BL and Kraft lignin from different resources for the commercial production of high value biomolecules like vanillin. Another important degradation product found in case of the present research was guaiacol (Nowakowska *et al*., 2018). This compound has been known to give wine a typical smoky aroma. The formation of the guaiacol has been associated with the saprophytic action of the fungi during lignin degradation. The guaiacol, in particular, generated during biodegradation of vanillic acid as consequence of non oxidative decarboxylastion process (Niu et al., 2021; Wang et al., 2020). In some previous reports, guaiacol generation was reported from *B. megaterium* (Dhankhar et al., 2021) , *Rhodotorula rubra* (Ayadi et al., 2019), and *Nocardia* sp. (Bosco et al., 2018). However, in relation with the *Aspergillus, Geotrichum* and *Dapodascus* strains, the

pathway of lignin degradation and its exact biochemical information still remains elucidative.

Different variants of lignin were use to analyse the lignin degradation capability of the fungal strains including soluble, insoluble (0.2%, 0.4%). Depending on mass analysis it showed that the fungus had degraded and removed substantial amount of the substrates (Alessi *et al*., 2018). The selected fungal, as *Aspergillus terrus*M-1*, Dipodascus autraliensis*M-2*, Geotrichum candidum*M-3*, Aspergillus fumigatus* M-4*,* have the ability to degrade lignin both soluble and incalculable. Although ligninolytic activity of some ascomycetes has been previously reported, this is the first evidence that the strains of Ascomycetes were capable to degrade aromatic biopolymer. The ligninolytic capability of these strains i.e., the extent of lignin degradation during growth of 8 days was high specifically for an ascomycete fungus, as compared to previous studies that represent the degradatio n of lignin by basidiomycetes (Table 4.12). (Dicko *et al*., 2020; Illuri *et al*., 2021; Kowalczyk *et al*., 2019). The fungal strains were grown on soluble and insoluble alkali kraft lignin under submerged fermentation conditions as well as in solid state incubatio n. Both conditions revealed the marvellous growth of fungal strains specially M-3 *(Geotrichum candidum)* using lignin as solo carbon source. The other strains M-2 (*Dipodascus autralienses)* M-4 (*Aspergillus fumigatus)* showed slightly lesser degradation, M-1 (*Aspergillus terrus)* was slowest amongst all four strains. The observed extents of lignin degradation suggested predominant alternations in the structural characteristics of the lignin residues. Several studies demonstrated that white-rot fungal- strains approximately double the total Cα-oxidized moieties depending on the extents of delignification (van Erven *et al*., 2018). Study reported that strain *C. subvermispora* observed to increase 16-fold of diketone markers. This interpretation would recommend that there are diverse routes of degradation. In addition to increase Cα-oxidized substructures, the action of fungal strains causes considerable decline of vinyl products. These are formed as a resultant product of decarboxylation of hydroxycinnamates upon pyrolysis (Nastasiienko *et al.,* 2021). Specifically, the reduction of 4- vinylphenol, mainly caused by p-coumarate which proposed there importance as targeted moieties during the cycle of fungal growth (Cao *et al*., 2020). Similarly, the decline of 4-vinylguaiacolis occurred due to subtraction of ferulic acid moieties. The fungal strains were also analysed for their growth on different carbon sources to reveal their specific

pattern of growth on lignin. The results obtained suggested that strains have shifted their enzymatic machinery to complex carbohydrate. The growth on simpler carbohydrate like starch was significantly very low as compare to avicel where they showed remarkably efficient growth. This study was conducted to check the extent and difference of growth on different carbon sources in order to determine degradation pattern. (4.13)

The information obtained from the NMR data indicated that the fungal strains used in the present study had metabolic capability to degrade lignin into different intermediates as described in the previous section of the discussion. In order to track down the pathway of the fungal biodegradation of the lignin, further experiments were performed using the guaiacol, hydroxylbenzaldehyde, syringaldehyde and vanillin was studies in shake flask experiments and results are presented in the last section of the results chapter. The reverse phase-HPLC was used to analyse the biodegradation of aforementioned lignin intermediates. The results showed that guaiacol was further degraded to simple monomers indicated by the transformation of HPLC peaks as compared to the control. Most promising results were obtained by *Geotrichum candidum* and *Aspergillus fumigatus* in degradation of vanillin and *p*-hydroxybenzaldehyde. The vanillin degradation by the application of microorganisms has captured significant attraction in the past few years owing to its importance in food and fragrance industries (Braga *et al.,* 2018. Bajwa *et al.,* 2019). Besides various effectors, there is scarcity of the data regarding the vanillin biodegradation by the fungi. Hao-Ping Chen *.,* 2012, described the biodegradation of vanillin using *Rhodococcus jostii*. They reported that the degradation of the vanillin by the bacteria was attributed to vanillin dehydrogenase and vanillin O-demethylase encoded by *vdh* and *vanAB* genes. Further, it was suggested that these enzymes convert vanillin into vanillate in an NAD+ dependant manner. The enzyme *O*-demethylase futher concerts this substrates into protocatechute. Henderson and Farmer 1995, reported similar types of finding where they suggested decomposition of *p*- hydroxybenzaldehyde, syringaldehyde and vanillin by the application of *Aspergillus* sp, *Botrytis* sp. *Penicillium* and *Trichoderma* strains. These fungi were able to transform vanillin to vannillic acid and syringaldehyde into syringic acid. Therefore, we propose that the biodegradation of the vanillin and other compounds could be a function of vanillin dehydrogenase and vanillin *O*-demethylase enzymes; however, further research in

biodegradation pathway of vanillin by white rot fungi is needed to elucidate the underlying mechanism of vanillin degradation. Interestingly, the information regarding the biodegradation of intermediate lignin products have not been studies so far particularly in case of *Dipodascus australiensis* and *Geotrichum candidum*. It could be suggested that the strains fungal strains used in the present studies provides new opportunity to convert the lignocellulosic biomass into value added products of commercialimportance.

Conclusion

Biodegradation of lignin through microbial means has been gaining significant interests. Several microorganisms are widely used for lignin degradation and related substrates while white rot fungi have been proved as the powerful cellular resource for de-polymerisation of lignin. In present work, four strains of Ascomycetes were screened for their potential to degrade lignin and production of peroxidises and lacesses under laboratory conditions. On the basis of two step screening strategy, four potent fungal strains were selected for the further studies. These strains were identified as *Aspergillus terrus* M-1*, Dipodascus autraliensis* M-2*, Geotrichum candidum* M-3*, Aspergillus fumigates* M-4. The lignin degradation efficiency was found to be 73 % for *Aspergillus terrus* M-1, 84 % for *Dipodascus autraliensis* M-2, 84 % for *Geotrichum candidum* M-3 and 80 % in case of *Aspergillus fumigates* M-4 using black liquor and lignin. These strains were also effected colour reduction of the lignin containing media. Moreover, these fungi were able to tolerate alkaline pH and high temperatures. The fungal strains were able to produce lignin peroxidises and laccases under different variants and concentrations of the lignin. The extracellular enzymes production was optimised for all four fungal strains with highest production rate of laccase (139 U/mL) was observed from *Aspergillus terrus* M-1, whereas highest LiP and MnP were observed in case of *Geotrichum candidum* M-3, 115U/mL and 106 U/mL respectively. These fungal strains were able to use both soluble and insoluble forms of lignin and some related compounds such as avicel, Mannans pectin, starch and xylan. During biodegradation of lignin these fungi generated various simple degradation products such guaiacol, hydroxybenzaldehyde, syringaldehyde and vaniline which were further degraded into more simple monomers. On the basis of the results, it can be concluded that the fungi isolated and screened in the present study showed excellent metabolic efficacy to grow and degrade lignin both in the industrial waste water and pure lignin. Further, these strains produced various lignin degrading enzymes using lignin as sole source of carbon and energy. Therefore, the fungal strains *Aspergillus terreus, Dipodascus australiensis, Geotrichum candidum* and *Aspergillis fumigatus* can be used for the bioremediation of pulp and paper industrial waste and for the biotransformation of lignin containing waste into value added commercial products.

Future Prospects

In present research four potent strains of the fungi genus Ascomycetes i.e. *Aspergillus terrus, Dipodascus autraliensis, Geotrichum candidum* and *Aspergillus fumigates* were studies for their metabolic efficiency to degrade lignin and related compounds in shake flask experiments. Besides a comprehensive work on their growth, biochemistry, enzymology and biodegradation efficiency, some important aspects were not covered due to paucity of time. Therefore, following areas are presented for the upcoming researchers for future investigations.

- The effect of various growth inhibitors should be studied in order to maximise the growth and metabolic efficiency of these isolates.
- The enzymatic potential of these isolates should be studies for other biopolymers and synthetic polymers.
- The purification of the lignin peroxidises, their molecular structures and protein chemistry needs further investigations.
- The whole genome sequencing of these isolates should be done in order to understand overall metabolic traits of these fungal strains
- Proteome analysis, particularly secretome, should be carried out for their application in various other biotechnological applications related to food and medicines
- The bioprocess for the treatment of lignin containing waste water should be designed using our screened fungi in future.
- Since our fungal isolates generate various commercially important degradation products of lignin, their potential should be investigated for the production of value added materials such as bioplastics and vanillin.

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Optimizing biocatalytic potential of *Dipodascus australiensis* M-2 for degrading lignin under laboratory conditions

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ABSTRACT

In present research, a potent fungal strain was isolated from paper mill effluent (black liquor) in order to investigate its potential for the biodegradation of lignin. Two step strategy was used to screen most efficient fungal strain having ability to growin MSM-black liquor medium and to degrade alkali lignin.The results of initial screening indicated that the strain M-2 produced comparatively higher ligninolytic zone on MSN agar plates supplemented with black liquor (BL) and alkali ligninase compared to the other isolates.The results of 18S rRNA gene sequencing revealed that strain M-2 showed ≥ 99% sequence homology with *Dipodasceus australiansis*.The process for the biodegradation of lignin was optimized using Taguchi Orthogonal Array design. Under optimized conditions of pH 9, 40 °C and 4% inoculum, a maximum of 89% lignin was degraded with 41% color reduction after 8 days of incubation period by *Dipodasceus australiansis M-2*. The pH and temperature were found to be significant terms with the *p*-values of 0.002 and 0.001 respectively. The laccase activity of the *Dipodascus australiensis* was found to be maximum of 1.511 U/mL. The HPLC analysis of lignin biodegradation indicated sharp transformation of peaks as compared to the control. Our results suggested that the strain *Dipodascus australiensis M-2* possess excellent lignin degradation and color reduction capability and can be applied in waste treatment systems for pulp and paper mill effluent. In present work we are reporting first hand information regarding biodegradation of lignin by a potent strain of *Dipodascus australiensis* and statistical optimization of the bioprocess.

1. Introduction

Pulp and paper manufacturing is one of the major industrial sectors in worldwide with an annual production capacity of 434,740 tons (Shabbir and Mirzaeian, 2017). This sector has been facing serious challenges of waste management in order to meet the sustainability regulations set by the local and international agencies. According to the reliable reports, about 200 $m^3$of water is consumed for the production of one ton of pulp. Due to the addition of various process chemicals, pulp and paper mill produces highly contaminated and toxic wastewater known as black liquor (Kujur, 2017; Younas et al., 2020). Black liquor (BL) is a complex aqueous mixture of lignin, polysaccharides and resinous compounds along with various soluble salt ions. BL contains about 35–45% of lignin making this waste water a serious environmental problem (da Silva et al., 2020; Fahim et al., 2019). Owing to the presence of myriad of pollutants, eco-toxicity,higher pH, dark color, pulp and paper effluent need to be treated before its final discharge into the environment (Haq and Raj, 2020). Since most of the color of the black liquor is due to lignin contents, therefore, removal of lignin derivatives from the pulp and paper effluent is considered most important strategy for its bioremediation. Lignin is a high molecular weight complex organic phenolic biopolymer and acts as a major environmental pollutant generated by the pulp and paper industry (Sharma et al., 2020). A range of physicochemical methods has been proposed for the

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