Delhi Technological University, Delhi, India

Screening of lignin degrading microbes and their morphological and biochemical characterization

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Submitted By

NEHA BANSAL

(2K15/IBT/08)

Under the Supervision of

Dr. SMITA RASTOGI VERMA
(ASSISTANT PROFESSOR)
&
Prof. D. KUMAR
(HEAD OF DEPARTMENT)

DEPARTMENT OF BIOTECHNOLOGY
DELHI TECHNOLOGICAL UNIVERSITY
(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Bawana Road
Delhi-110042, INDIA

CERTIFICATE

This is to certify that the M. Tech thesis entitled "**Screening of lignin degrading microbes and their morphological and biochemical characterization**" submitted by **Neha Bansal (2K15/IBT/08)** in the partial fulfilment of the requirements for the reward of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under our guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

Date:

Dr. SMITA RASTOGI VERMA
Supervisor
ASSISTANT PROFESSOR
Department of Biotechnology
Delhi Technological University
(Formerly Delhi College of Engineering, University of Delhi)

Prof. D. KUMAR
Co-Supervisor
Head of Department
Department of Biotechnology

DECLARATION

I declare that my major project entitled "**Screening of lignin degrading microbes and their morphological and biochemical characterization**" submitted to Department of Biotechnology, Delhi Technological University is a result of the work carried out by me at Environmental Biotechnology Laboratory, Department of Biotechnology, as major project.

DATE: NEHA BANSAL

PLACE: DELHI

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2K15/IBT/08

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Screening of lignin degrading microbes and their morphological and biochemical characterization

Neha Bansal

Nhbansal06@gmail.com

Delhi Technological University

ABSTRACT

Lignin is found in abundance in nature. It is an aromatic polymer found as a noteworthy part of lignocellulose in plant cell walls and is to a great degree impervious to chemical or any kind of biochemical breakdown. Research on lignin biodegradation has quickened enormously amid the previous 20 years, primarily in view of the significant potential uses of bio-ligninolytic frameworks in pulping, bleaching, changing over lignin to valuable items and treating of horticultural squanders utilizing microbes. Disconnection and distinguishing proof of natural neighbourly bacteria for lignin degradation turns into a fundamental, since all the past information was primarily focused on utilizing white-rot fungi. Be that as it may, bacteria appear to assume a main part in breaking down lignin since wood degenerating bacteria have a more extensive resilience of temperature, pH and oxygen restrictions than fungi. Hence, in this investigation soil and decaying wood samples were gathered from wood industry and utilized for segregation of lignin degenerating microorganisms on the basis of their laccase and lignin peroxidase producing potential. Four bacterial strains were isolated by screening methodology in view of their action on guaiacol and Azure B. Based on lignin degrading enzymatic analyses, four bacterial isolates (two each from decaying wood and soil samples) with high lignin degrading activity were selected and characterized. These isolates were tentatively identified as *Serratia* spp., *Streptococccus* spp., *Bacillus subtilis* and *Pseudomonas* spp. Fungal isolates were also partially characterized and identified tentatively as *Alternaria* spp., *Aspergillus* spp. and *Helminthosporium* spp.

INTRODUCTION

One of the major renewable resources of energy available on earth is wood. The trees are abundant sources of wood. The woods serve as a fuel for our needs. The wood is rich in lignocellulosic biomass.

Every year tons of lignocellulosic biomass is generated from agricultural fields, timber industries and paper and pulp industries which pose a huge environmental threat. This biomass which is regarded as waste is of huge commercial importance if utilized properly. This waste can be converted into a number of by products like biofuel, improved feedstock for animals, various chemicals, cost effective source for fermentation etc. This lignocellulosic biomass comprises of lignin, celluloses and hemicelluloses. Out of these the lignin is the most recalcitrant.

Celluloses and hemicelluloses are found in the cell wall of plants. Cellulose is a homopolymer which consists of β-(1,4)-linked glucopyranose units. It provides support to the cell wall. On the other hand, hemicellulose is a heteropolymer composed of pentoses, hexoses and sugar acid units, which provide more flexibility to the cell wall of plants. Both of these components are present in the plant in the form of crystalline insoluble fibres. They are comparatively easy to degrade than lignin but their degradation involves several steps and various enzymes [1].

Lignin is a complex aromatic compound which consists of three phenylpropanoid units, differing in their degree of methoxylation [2]. The units are connected by various ether and carbon-carbon linkages [3]. These linkages make lignin difficult to degrade and digest. They also help in providing the plant mechanical strength and resistance to microbial degradation [4, 5]. Lignin degradation is a tedious process. By linking to both hemicelluloses and cellulose, lignin acts as a barrier to any solutions or enzymes and prevents penetration of lignocellulolytic enzymes to the interior lignocellulosic structure. The lignin degradation process involves methods such as gasification, oxidation by chemicals, pyrolysis etc. [6]. But, these processes put a great load on nature in terms of pollution and energy [7]. Therefore, environmental friendly processes are required to be able to degrade lignocellulosic biomass. Enzymatic degradation is one such eco-friendly approach which requires less input in terms of energy [8, 9]. A lot of research has been going on in past few years to identify microorganisms capable of degrading

lignocellulosic biomass. The most significant of them are wood degrading bacteria and white rot fungi.

The white rot fungi were studied extensively for their lignin degrading properties. They produce lignin degrading enzymes extracellularly. The enzymes produced by them are classified under three classes, namely, Lignin peroxidases (EC 1.11.1.14) (LiP), Manganese peroxidases (EC 1.11.1.13) (MnP) and Laccases (EC 1.10.3.2) [10]. Though for industrial applications application of fungi is not a suitable option because of their filamentous growth and conditions like temperature, humidity etc. required for their growth are not similar with the environment required for industrial processing [11].

Recently a lot of research focus has been on lignin degrading bacteria whose potential to degrade lignin was largely unexplored. When compared to fungi, bacteria are more adaptable to environment and are very versatile biochemically [12].

Therefore, this study is aimed to isolate, screen and characterize novel lignin degrading bacteria and fungi from soil and decaying wood based on their potential to produce lignin oxidising enzymes of significant importance [13].

Objectives

- To isolate bacteria and fungi from decaying wood and soil samples.
- To perform plate assay to isolate putative bacteria and fungi with lignin degrading potential.
- To characterize bacterial and fungal isolates on the basis of morphology.
- To perform biochemical characterization of bacterial and fungal isolates.
- To perform laccase and lignin peroxidase enzyme assay for bacterial isolates.

REVIEW OF LITERATURE

This literature review contains information on the chemical composition of lignocellulosic biomass, lignocelluose degrading microbes, major bacterial enzymes involved in lignin degradation and microbial lignin degradation pathway.

2.1Chemical composition of lignocellulosic biomass.

Wood comprises of around 45% of cellulose, 20 – 30 % hemicelluloses and 20 - 40 % lignin relying upon the wood species, their parts and age [14].

2.1.1 Cellulose

Cellulose, the significant part of the fiber wall, is a homopolysaccharide made completely out of D-glucose connected together by β-1,4-glycosidic bonds with level of polymerization extending from 1,000 in blanched kraft pulps to 10,000 in local wood. Every glucose unit is pivoted 180^0 with respect to the adjoining one and the tiny reiterative unit of cellulose is cellobiose, two glucose units. Cellulose is a straight structure that has a solid inclination to frame intra or intermolecular hydrogen bonds bringing about the development of cellulose microfibrils which advance accumulation into crystalline, high order regions. Cellulose and microfibrils arrangement can be viewed in plant cell wall in **Figure 1**. The areas inside microfibrils with less order are named amorphous. The manner in which the crystalline and amorphous cellulose are arranged brings about intriguing properties of firmness and inflexibility on one hand and adaptability on the other. This structural arrangement of cellulose with its hydrogen bond makes it insoluble in many solvents and is mostly the main reason behind the resistance of cellulose against microbial degradation [15].

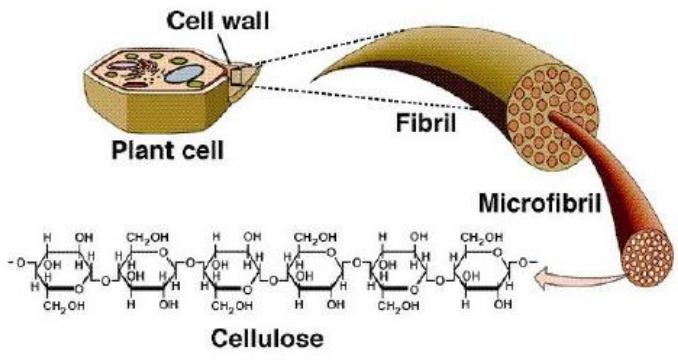


Figure 1: Arrangement of fibrils, microfibrils and cellulose in plant cell wall

Degradation of cellulose and hemicellulose is performed by a specific set of enzymes belonging to class hydrolases. These enzymes perform degradation by hydrolysis of glycosidic bonds. Such enzyme system hydrolysing cellulose is known as cellulases. This system consists of β-glucosidase, endoglucanase and cellobiohydrolase. The two enzymes endoglucanase and cellobiohydrolase work together by hydrolysing 1,4-β-D-glycosidic linkages in cellulose. The hydrolysis of cellulose by these enzymes releases cellobiose from non-reducing ends, degradation of cellobiose is then performed by β-glucosidase as demonstrated in **Figure 2**.

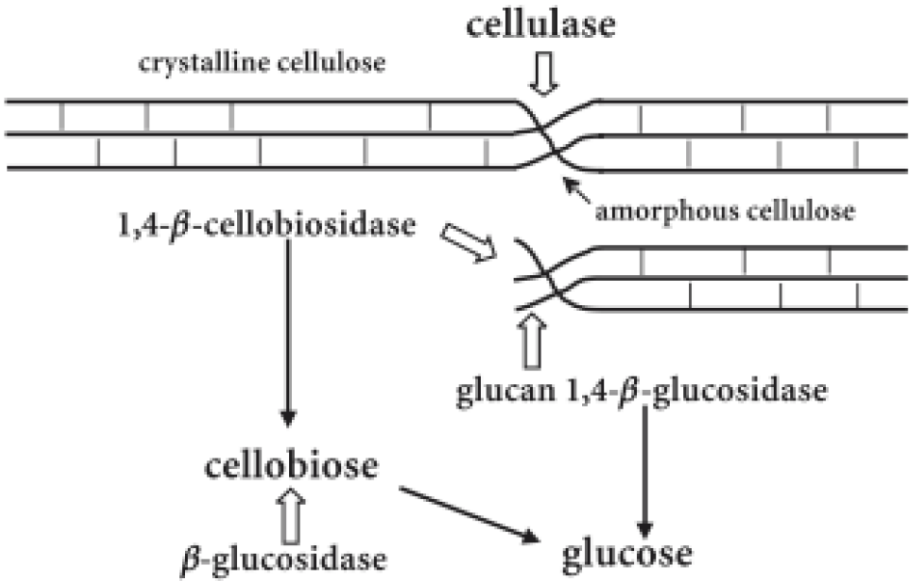


Figure 2: Degradation of cellulose by enzymes.

2.1.2 Hemicellulose

Hemicelluloses are complicated heterogeneous polysaccharides comprised of various monomeric units, for example, D-xylose, D-glucose, D-arabinose, D-mannose and D-glucuronic acid. Degree of polymerization (DP50 – 300) of hemicellulose is less as compared to cellulose, they have side chains that can be acetylated and are basically amorphous. They are arranged by the monomeric sugar in the foundation of the polymer, e.g. mannan (β-1,4-connected mannose) or xylan (β-1,4-connected xylose) hemicelluloses. The primary chain of glucose and mannose deposits are normally associated with β-(1,4) bond while the side anchor is appended to principal chain by means of α-(1,6) bonds in galactoglucomannan as seen in **Figure 3**.

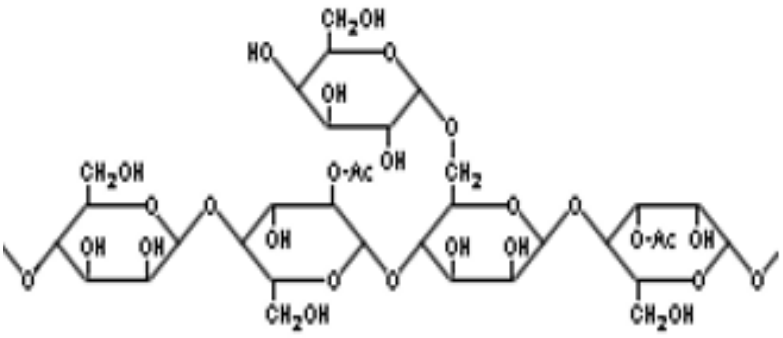


Figure 3: Schematic structure of *O*-galacto-glucomannan

Because of the more heterogenous nature of hemicellulose contrasted with cellulose, a complicated blend of compounds is required for its degradation, for example, endoxylanases, *β*-xylosidases, endomannanases, *β*-mannosidases, *α*-L-arabinofuranosidases and α-galactosidases. The degradation of hemicellulose is more typical in fungi than in bacteria.

2.1.3 Lignin

The third principal constituent in wood, lignin, is a complicated macromolecule framed by the dehydrogenative polymerization of three phenyl propane units (**Figure 4**), in particular *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol joined mainly through ether bonds. The structure of lignin shifts generally inside species. It gives compressional quality to the cell wall of plant while cellulose furnishes the plant with adaptable quality. In spite of cellulose, lignin is an irregular, three-dimensional macromolecule and is very hydrophobic. It shapes a nebulous complex with hemicelluloses encasing cellulose and therefore keeps the microbial degradation of open starches inside wood cell wall. In addition, its aromatic rings make it more hard to degrade [14]. Lignin helps in the smooth transportation of water from the roots to reach upto leaves, by acting as a glue sticking cells together and hardening the cell wall of xylem. The free carboxyl groups in hemicellulose and the benzyl groups in lignin are linked by ester linkages forming a lignin-carbohydrate complex (LCC). Due to this complex formation the cellulose gets enclosed thereby providing protection against any sort of degradation by microbes and chemicals. Cellulose and hemicellulose are fairly effectively

degradable, while lignin is impervious to degradation by most microorganisms because of its phenylpropane units in the structure and the stubborn linkages between them. In this way, there is a great room for discovering microbes with the potential for separating lignin and in the cleaving of the linkages that exist amongst lignin and hemicelluloses (LCC). These microbes may be capable of producing enzymes that have the potential to transform plant biomass.

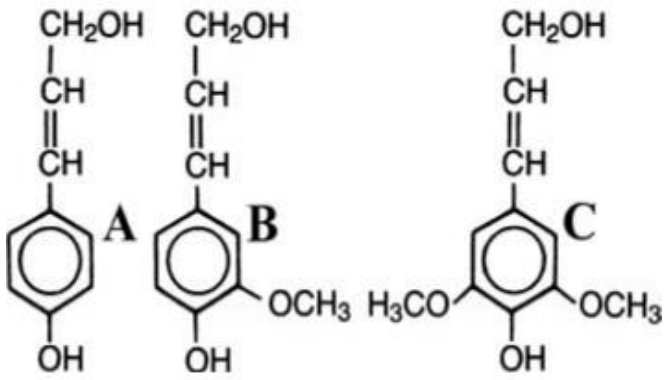


Figure 4: Building units of lignin. (A) *p*-coumaryl alcohol, (B) coniferyl alcohol, (C) sinapyl alcohol

Lignin can be successfully degraded by a group of filamentous fungi known as white-rot fungi, which are a potent producer of enzymes that can attack phenolic structures, some of them are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase, also they are capable of producing number of oxidoreductase.

2.2 Lignocellulose degrading microbes

Unspecific and extracellular enzymes are vital for the biodegradation of lignin because of its irregular structure and high sub-atomic mass. The product produced as a result of the degradation of lignocellulose is lignin, which gets accumulated in the environment. Lignin is recalcitrant in nature but in environment it is presumably hydrolysed by a group of microorganisms. An extensive variety of microorganisms including fungi and bacteria have a potential to secrete cellulases and hemicellulases yet just a set number of these microorganisms have potential to degrade lignin degrading compounds. Among these microorganisms, filamentous fungi have shown immense capability of being the most proficient producers of enzymes degrading lignin [15]. The capacity to secrete particular

enzymes for degradation of various carbon and nitrogen sources is because of the differing habitat in which they are discovered [16].

2.2.1 Fungi

Degradation of lignin relies on different ecological parameters. Degradation of lignin is promoted by natural variables responsible for the development and metabolism of the fungi. Temperature, pH, carbon and nitrogen sources are amongst the most impacting parameters influencing fungal development. The most crucial parameter in the fungal growth is the high and low level of nitrogenous compounds. Fungi hydrolyse hemicelluloses more effortlessly than cellulose hydrolyzation. White-rot fungi are the most capable lignin degraders in nature. White-rot fungi have the capability to degrade lignin more quickly than some other microorganisms and therefore they account for the major lignin degradation in nature. The most commonly found white-rot fungi are Basidiomycetes.

There are several advantages offered by the system utilized by white-rot fungi for degrading pollutants at higher concentrations . The system utilized for lignin degradation is usually free-radicals based and non-specific in nature, hence the white-rot fungi are capable to degrade many pollutants. Degradation of low concentration of pollutant by white-rot fungi is advantageous over bacteria. The cultivation of fungi requires liquid media and cost effective substrates. Fungi produce such enzymes that can effectively convert lignin into water soluble compounds.

2.2.2 Bacteria

There have been few reports of degradation of lignin effectively by pure bacterial strains. This is because of the specific nature of bacteria to degrade lignin. Only one type of bond per bacterial species can be cleaved in lignin. Thus digestion of lignocellulose by bacteria occurs in mixed cultures or along with fungi. The tolerating capacity of wood degrading bacteria is higher than fungi as it can tolerate a wide range of abiotic factors. But by using only bacterial population for the degradation of wood will take longer duration as they are extremely slow. The bacteria are mostly associated with the decay of water-logged wood.

The two forms of bacteria that are capable to degrade exracted lignin are Actinomycetes and Eubacteria. Actinomycetes have immense potenial to alter and degrade lignin structure broadly, yet in restricted way. The degradation of lignin by Actinomycetes varies from white-rot fungi. The mode of action of Actinomycetes is to degrade lignin by primary metabolic activity on the other hand white-rot fungi uses secondary metabolic activity for degradation. Actinomycetes mostly process lignin polymer to acid precipitable polymeric lignin (APPL). APPL comprises small amounts of proteins, carbohydrates, organic,inorganic substances, as well as lignin polymer.

There are also some species that are able to degrade lignin in soil like *Streptomyces* and *Nocardia.*

2.3 Lignin degrading enzymes

Because of the expanding, massive three-dimensional structure and the C-C and C-O ether linkage heterogeneity of lignin, hydrolytic catalysts cannot cleave lignin [17]. Also, low-potential oxidoreductases, for example, the plant oxidases that start lignin polymerization, cannot oxidize the non-phenolic aromatic lignin subunits. These complexities have restricted the progressive diversity of lignin-degrading microbial phenotypes to a specific set of fungi and bacteria.

Many classes of enzymes with ligninolytic activity have been identified within lignin degrading bacteria and fungi [17-19]. While facts encompassing the enzymology and conveyance of these oxidative enzymes are starting to be explained, the knowledgebase of ligninolytic catalysts still falls a long way behind the knowledge of cellulases.

2.3.1 Peroxidases

Peroxidases (EC 1.11.1.7) are haem-containing enzymes that catalyze a number of oxidative reactions and hydroxylations, using hydrogen peroxide (H_2O_2) as the electron acceptor (**Figure 5**) [20]. Peroxidases exist in bacteria, fungi, plants, and animals. In consideration of the sequence similarity and structural divergence, they are viewed as belonging to a super-family consisting of three major classes: Mitochondrial yeast cytochrome c peroxidase, chloroplast and cytosol ascorbate peroxidases, and gene duplicated bacterial peroxidase (class I); secretory fungal peroxidases (class II); classical, secretory plant peroxidases (class III). Peroxidases are enzymes defined as

oxidoreductases using hydroperoxides as electron acceptor, and are able to catalyze the oxidation of a large variety of substrates such as phenol, aromatic amines, and other compounds such as alkyl peroxides and aromatic per acids. Most of them have a common catalytic cycle:

$$\text{Native peroxidase}(Fe^{3+}) + H_2O_2 \rightarrow \text{Compound} - I + H_2O \quad \text{(1a)}$$

$$\text{Compound} - I + AH_2 \rightarrow \text{Compound} - II + AH^{\bullet} \quad \text{(1b)}$$

$$\text{Compound} - II + AH_2 \rightarrow \text{Native peroxidase}(Fe^{3+}) + AH^{\bullet} + H_2O \quad \text{(1c)}$$

Figure 5: Catalytic cycle of peroxidases

In the first reaction (1a), there is a two-electron oxidation of the ferriheme prosthetic group of the native peroxidase by H_2O_2 (or organic hydroperoxides). Compound-I (oxidation state + 5) is an intermediate derived from the first reaction, consisting of oxyferryl iron (Fe^{4+}= O) and a porphyrin π cation radical. In the following reaction (1b), compound-I is reduced by the first electron donor AH2, receives one electron, and forms compound-II (oxidation state + 4). Then compound-II accepts an additional electron from AH2 in the third step (1c), whereby the enzyme is returned to its native resting state, ferriperoxidase. During the peroxidase reaction, different oxidation products are formed, depending on the various natures of the substrates. Electron donors such as aromatic amines and phenolic compounds are oxidized to free radicals, AH* (reactions (1b) and (1c)). When reaction (1a) occurs on an electrode surface, compound-I can be directly reduced into ferriperoxidase by a heterogeneous electron transfer (ET) which comes from the electrode material, instead of redox mediators. The aim of these approaches is to lead to a reduction current correlated with the concentration of peroxide in the solution. At high concentrations of peroxide, the peroxidases turn into an enzymatically inactive form, denoted as compound-III (oxidation state + 6). Because lignin peroxidase can catalyze the oxidation of substrates with a reduction potential greater than 1.3 volts, the range of its substrates is extremely broad. The enzymes have been proven to utilize lignin monomers, dimers, and trimers as well as polycyclic aromatic compounds as substrates. Side-chain fragmentation (C-C cleavage) caused by the radicals (compounds I and II) results in the breakdown of the lignin polymer. They can also catalyze lipid

peroxidative pathways, which are capable of oxidizing substrates such as lignin-model dimers and polycyclic aromatic hydrocarbons.

2.3.1 (a) Lignin peroxidase

Lignin peroxidase is a haem containing glycoprotein. It performs non-enzymatic oxidative cleavage of β-O-4 bond of side chains, C-C bonds and other bonds as shown in **Figure 6.** It also performs oxidation of benzyl alcohol to ketones and aldehydes. It is capable of degrading both phenolic and non-phenolic units. The mechanism by which it degrades lignin utilizes one electron oxidation to produce aryl radical cations which are unstable in nature. H_2O_2 acts as terminal electron acceptor [14].

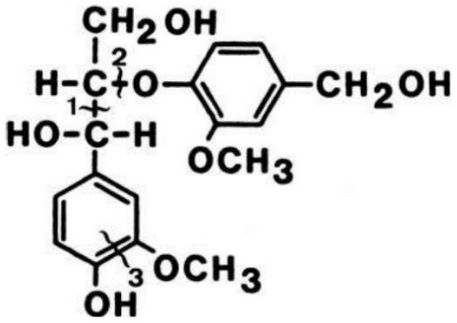


Figure 6: Reactions initiated by lignin peroxidase (1) Cleavage of C-C bond (2) cleavage of β-O-4 bond of side chain (3) Cleavage of aromatic ring

2.3.1 (b) Manganese peroxidase

Manganese peroxidase (MnP) is a glycoprotein that contains haem. It degrades lignin by mainly attacking phenolic units. MnP is mainly released into environment by Basidiomycetes and some wood degrading white-rot fungi. MnP oxidises Mn^{+2} to Mn^{+3} by utilizing H_2O_2 as electron acceptor. The Mn^{+3} is a strong oxidant and thus oxidises phenolic structures. The H_2O_2 binds to ferric enzyme to form iron-peroxide complex (**Figure 7**). This initiates the catalytic cycle of MnP [21].

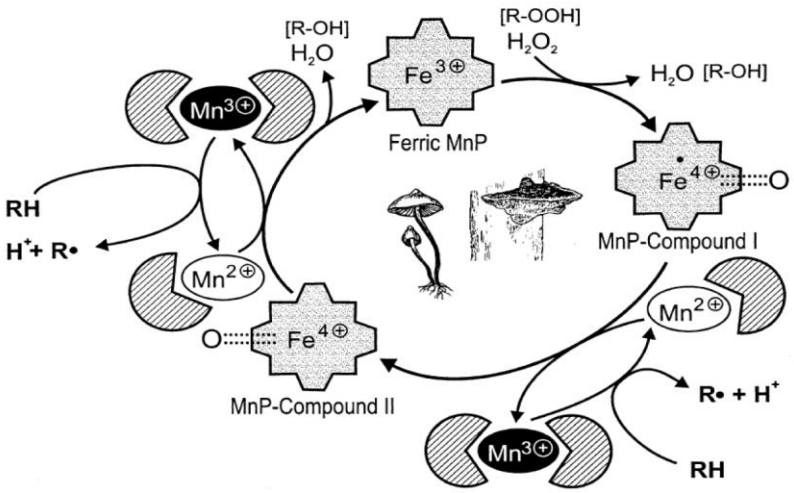


Figure 7: Catalytic cycle of MnP

2.3.1 (c) Versatile peroxidases

These peroxidases possess mixed properties of both manganese peroxidase (MnP) and lignin peroxidase (LiP). They are able to oxidise substrates specific to both MnP and LiP respectively. VPs are glycoproteins capable of oxidising a wide range of substrates including phenolic, non-phenolic, both low redox and high redox potential substrates. This is possible because of its hybrid molecular structure consisting of multiple binding sites for substrates. The catalytic versatility of VP makes them a better and superior peroxidase for lignin degradation. They are capable of oxidising phenolic compounds in absence of veratryl aclcohol unlike LiP and in absence of Mn(II) unlike MnP respectively.

2.3.2 Laccases

Laccases (EC 1.10.3.2) are multi-copper oxidases and one of the most important enzyme responsible for lignin degradation. Laccases are universal in nature, being found in fungi, insects, bacteria and plants. They are frequently discharged as extracellular catalysts and commonly perform polymerization or depolymerization reactions [22]. Laccases are capable of oxidising a wide variety of phenolic substrates including mono-, di- and polyphenols, methoxy phenols, aromatic amines, amino phenols and ascorbate. The by-product of this oxidation is water produced as a result of the reduction of molecular oxygen [23]. Laccases are mostly isolated from the fungi.

Laccases have usually four copper atoms per monomer but few laccases have been reported to have two copper atoms and one pyrroloquinoline quinone as prosthetic

group. These copper atoms are classified on the basis of UV\visible or electron paramagnetic resonance (EPR) spectroscopy into three different types.

(i) Type I copper- it has an absorption maxima around 600nm and is responsible for the intense blue color of the enzyme. It is EPR detectable.

(ii) Type (II) copper- it is colourless but EPR detectable

(iii) Type (III) copper- it comprises a pair of copper atoms that are weakly absorbed near the UV spectrum also it gives no EPR signal.
The copper sites of type (II) and type (III) copper are in close proximity and together forms a trinuclear centre where binding dioxygen and four-electron reduction to water occur.

The mode of action of laccase to degrade lignin is restricted to its phenolic subunits. The attack on phenolic subunits cause oxidation of Cα and cleavage of Cβ along with aryl-alkyl cleavage (**Figure 8**). Catalysis by cleavage involves these steps:

(i) reduction of the type I copper by reducing substrate

(ii) internal transfer of electron form type I copper to type 2 and type 3 copper

(iii) reduction of molecular oxygen to water at type 2 and type 3 copper site.

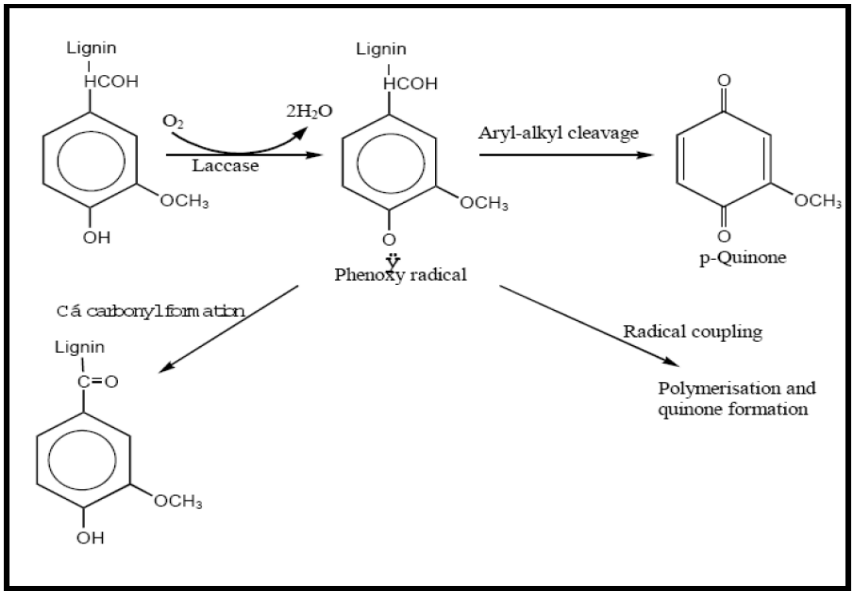


Figure 8: Oxidation of phenolic subunits of lignin by laccase

The oxidation of reducing substrate by laccase involves the removal of a single electron and formation of a free radical. The radical being unstable in nature may undergo further laccase-catalyzed oxidation or non-enzymatic reactions like polymerization, hydration etc. Redox potential difference might contribute in controlling the transfer of electron from substrate to type I copper. A higher redox potential of laccase or a lower redox potential of lignin frequently leads to a higher rate for substrate oxidation.

Sometimes due to the large size or high redox potential of substrate laccases are unable to oxidize them. In such cases a suitable chemical mediator is included which acts as an intermediate substrate for laccase, this is known as laccase mediator system (LMS). An example of one such mediator is 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). This mediator also aids in extending the effect of laccase to even non-phenolic substrates [24].

The LMS works by first getting oxidised by laccase and then diffusing into the cell wall to further oxidise lignin which was inaccessible by laccase alone (without mediator) (**Figure 9**). The oxidation of non-phenolic lignin subunits by LMS can follow any mechanism like an ionic mechanism, a radical hydrogen atom transfer, an electron transfer depending on the mediator [25].

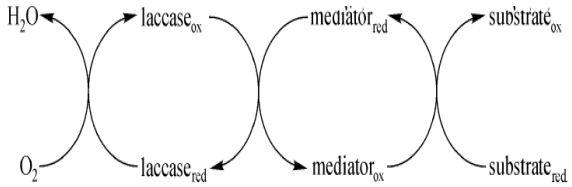


Figure 9: The redox cycle of the oxidation of LMS by laccase

Laccases are mechanically alluring biocatalysts, as not at all like many oxidoreductases, they do not require the expansion of cofactors. Additionally, unique in relation to most oxidases, they do not deliver lethal hydrogen peroxide as by product. Laccases have been utilized in bioremediation procedures to sterilize modern wastewaters, in sustenance industry for the adjustment of refreshments and change of the organoleptic properties of nourishment, in the combination of pharmaceuticals and other fine chemicals, in material color change, and in the delignification of wood [26]. Laccases can be additionally utilized in the pretreatment of softwood tests with the point of enhancing the consequent hydrolysis treatment [27].

2.4 Microbial lignin degradation pathway

Lignin degradation plays an important part in earth's carbon cycle, as the maximum renewable carbon is present in it or in lignocellulose which is recalcitrant in nature. Due to the structural complexity lignin is hard to degrade. In recent years a lot of research has been done on the microbial degradation of lignin. Amongst microorganisms, degradation of lignin by white-rot basidiomycetes is studied extensively. Though a number of reports has suggested that bacteria are also capable of degrading lignin. The process of lignin degradation is slow due to its complex structure but it's of burgeoning interest commercially.

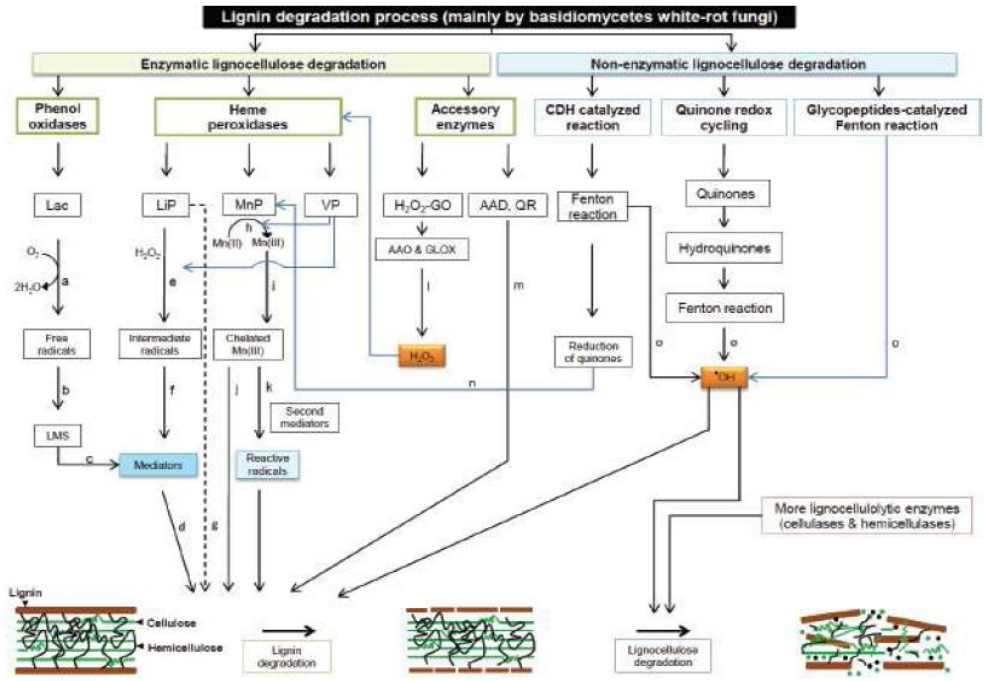
2.4.1 Lignin degradation by fungi

White rot fungi are the most rapid as well as the most extensive degraders of lignin. They can degrade lignin either in selective or non-selective manner. In selective degradation, hemicellulose and lignin components of lignocellulose are degraded remarkably more than cellulose on the other hand in non-selective degradation all the three components of lignocellulose are degraded equally [28, 29]. The degradation by fungi is non-specific and oxidative. It cleaves aromatic rings, reduces phenolic and aliphatic content of lignin and generates new carbonyl groups. This leads to lignin depolymerization and formation of carbon dioxide. Various enzymes are produced by white-rot fungi capable of degrading lignin including manganese peroxidase, laccase which is produced by almost all of them and lignin peroxidase produced by only few of the white-rot basidiomycete (**Figure 10**) [30]. In addition to these enzymes some accessory enzymes are also produced by white-rot fungi like glyoxal oxidase that generate H_2O_2 required by peroxidases (**Figure 11**).

Enzyme	Type of enzyme	Proposed role in lignin degradation	Acts together with/ co-factor
LiP[a] (EC 1.11.1.14)	Peroxidase	Degradation of non-phenolic units	H_2O_2
MnP[b] (EC 1.11.1.13)	Peroxidase	Degradation of phenolic units, and non-phenolic units with lipids	H_2O_2, lipids
Laccase (EC 1.10.3.2)	Phenol oxidase	Oxidation of phenolic units, and non-phenolic units with mediators	O_2, mediators, such as 3-hydroxybenzotriazole (3-HBT)
Others	Various H_2O_2 generating oxidases	H_2O_2 production	Peroxidases

[a]lignin peroxidase
[b]manganese peroxidase

Figure 10: Table of ligninolytic enzymes produced by white-rot fungi



Lac: laccase, LMS: laccase-mediator system, LiP: lignin peroxidase, MnP: manganese peroxidase, VP: versatile peroxidase, H_2O_2-GO: H_2O_2-generating oxidases, AAO: aryl-alcohol oxidase, GLOX: glyoxal oxidase, H_2O_2: hydrogen peroxide AAD: aryl-alcohol dehydrogenases, QR: quinone reductases and $^{\cdot}OH$: free hydroxyl radicals.

Figure 11: An overall schematic representation of lignin degradation by white-rot basidiomycete

2.4.2 Lignin degradation by bacteria

In nature, most lignin is degraded by fungi but several reports have suggested that bacteria are also potent lignin degraders. The enzymology of bacterial lignin degradation is not well-studied but the catabolic pathways involved in the breakdown of lignin components are studied extensively in bacteria. There are three major classes of bacteria involved in lignin degradation, the α-proteobacteria, γ-proteobacteria and the actinomycetes. These bacteria are capable of modifying and degrading biphenyl structure of several lignin dimeric compound to low molecular weight compounds such as syringic acid, vanillate and 3-methyl gallic acid. The best studied bacterial strain capable of degrading lignin is *Streptomyces viridosporus* T7A. This bacterial strain produces various peroxidases extracellularly which cleaves β-aryl ether bonds leading to depolymerization of lignin and release of several low molecular weight phenols.

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Source of lignin degrading microbes

The decaying wood and the soil sample containing lignin degrading microbes were collected from the backyard of a woodmill near Subhash Chowk, Gurgaon.

These samples were screened for the presence of both lignin degrading bacteria and fungi.

3.1.2 Chemicals

All the chemicals used in this study were of analytical grade and were obtained from Hi-media.

3.2 METHODS

3.2.1 Isolation of bacteria

The decaying wood sample and the soil sample were screened for the presence of bacteria individually on the basis of laccase (Lac) and lignin peroxidase (LiP) plate assays. The positive bacteria possessing lignin degrading ability were observed for the formation of reddish brown zone in case of Lac plate assay and clear zones with white colonies in case of LiP plate assay.

(i) Media preparation

Lignin-degrading microorganisms were screened on the principle of laccase and lignin peroxidase plate assay. For this Nutrient agar medium (**Table 1**) was prepared with a pH of 7.0. This medium was supplemented with agar 2% and autoclaved at 15 psi for 15 min. Autoclaved medium was poured in sterile petriplates (25 ml/plate) under laminar flow hood and permitted to solidify. Then 2 mM sterile guaiacol (in diethylether) was spread on the solidified medium for Lac plate assay and 0.05% Azure B for LiP plate assay respectively.

Table 1: Composition of nutrient agar

Ingredients	Composition (100 ml)
Peptone	0.5 g
Yeast extract	0.3 g
$CuSO_4$	10 mg
Agar	2 g
Final pH	7.0

(ii) Serial dilution of samples

The decaying wood was finely crushed into powder form. Then 1 g of this finely crushed wood was added to test tube containing 9 ml saline (0.8% NaCl) and mixed properly. This was 10^{-1} dilution. Now further 1 ml of this solution was taken and transferred to another test tube containing 9 ml saline. This process is repeated till 10^{-8} dilution is achieved.

The similar procedure was done for the soil sample, 1 g of soil was added to test tube containing 9 ml saline (0.8%NaCl) and mixed properly. And similarly, the dilution was done upto 10^{-8}.

(iii) Inoculation and incubation

100 µl of all the dilutions of decaying wood sample were put on nutrient agar plates. Plates were supplemented with guaiacol for lac plate assay and with Azure B for LiP plate assay respectively. These dilutions were then spread with a sterile glass spreader onto the nutrient agar plates supplemented with substrates. The plates were then kept for incubation in inverted position at 37°C for upto 72 hours.

Similarly, 100 µl of all the dilutions of soil sample were put on nutrient agar plates and the same protocol was followed.

(iv) Subculturing

Best growth was observed with 10^{-6} and 10^{-7} dilutions. Positive colonies with lignin degrading potential showed reddish brown colonies on guaiacol containing medium and white colonies with clear zones were observed in Azure B containing medium. These positive cultures were subcultured in fresh medium to obtain pure cultures. A total of 12 colonies were picked, six from the decaying wood sample and six from soil sample respectively, subcultured and incubated for 72 hours at 37^0C.

3.2.2 Morphological characterization of bacteria by Gram staining

To characterise the isolated bacteria morphologically, the foremost step is their authentic identification on the basis of their shape, size, form as well as their Gram positive and Gram negative behaviour. This was determined with the help of Gram staining technique. For this technique a Gram's staining kit is required which consists of Crystal violet (a violet colour primary stain), Safranin (a red colour counterstain), Acetone/Ethyl Alcohol (a decolourizer) and Iodine (a mordant).

Principle

The basic principle behind Gram staining is the difference in the cell wall of Gram positive and Gram negative bacteria (**Figure 12**). Initially the cells are stained with crystal violet dye followed by mordant (Iodine). This mordant allows crystal violet to adhere to the cell wall of bacteria. After this the decolorizer is added to the cells which causes some cell to lose the primary stain while some are able to retain it.

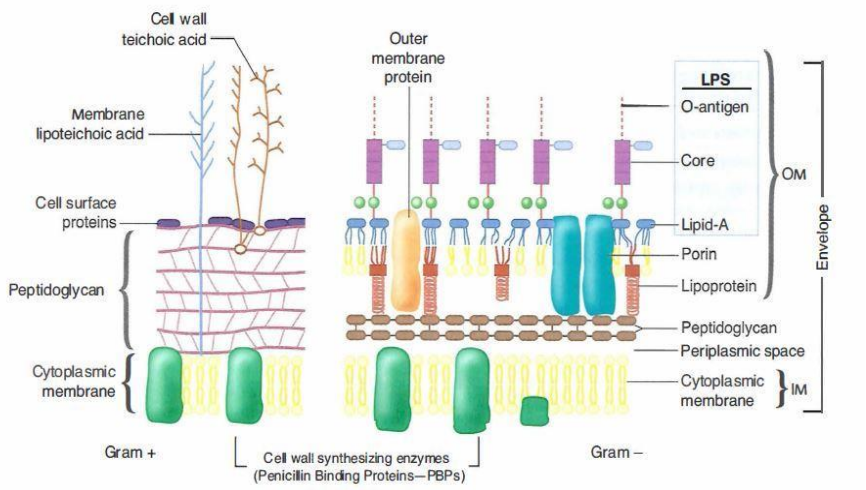


Figure 12: Schematic diagram of cell wall of Gram positive and Gram negative bacteria

The cell walls of Gram positive microscopic organisms have a thick layer of protein-sugar edifices called peptidoglycan and lipid content is low. Decolorizing the cell causes this thick cell wall to get dried out and recoil, which shuts the pores in the cell wall and keeps the stain from leaving the cell. So, the ethanol cannot evacuate the crystal violet iodine complex that is bound to the thick layer of peptidoglycan of Gram positive microbes and seems blue or purple in colour.

If there should be an occurrence of Gram negative microscopic organisms, cell wall likewise takes up the CV-Iodine complex however because of the thin layer of peptidoglycan and thick external layer which is comprised of lipids, CV-Iodine complex gets washed off. When they are exposed to alcohol (decolorizer), it breaks down the lipids in the cell wall, which permits the CV-Iodine complex to filter out of the cells. At that point when again recolored with safranin, they take the stain and seems red in colour.

Procedure

The procedure was followed:

The subcultured pure colonies were inoculated each in 5 ml nutrient broth (**Table 2**) in test tubes. A control was also prepared without inoculation. The tubes were incubated at 37^0C on an incubator shaker overnight. The tubes were observed for growth by observing the turbidity in tubes, the control did not show any turbidity.

Table 2: Composition of nutrient broth

Ingredients	Composition (100 ml)
Peptone	0.5 g
Yeast extract	0.3 g
$CuSO_4$	10 mg
Final pH	7.0

Clean glass slides were taken, wiped with alcohol and exposed to flame. The slides were marked as W1-W6 for isolates from decaying wood sample and S1-S6 for isolates from soil sample. The colonies were picked by a sterile loop and smeared on glass slides. The smear was first air dried and then fixed to the glass slide by heating. The slides were then

first flooded with crystal violet stain for about 1 minute. After that the slides were washed under running tap water gently. Then the slides were flooded with iodine for about 1 minute. The slides were then washed under running tap water. The third step was the addition of decolorizer. It was added drop by drop to the slides for about 5-10 seconds and then the slides were immediately washed with water. The last step was the addition of counter stain safranin. The slides were flooded with it and kept for about 45 seconds and then washed with water. The glass slides were then blotted dry.

These slides were then observed under light microscope under 40x and 100x magnification. For viewing under 100x magnification immersion oil was used.

3.2.3 Biochemical characterization of bacteria

The positive colonies which gave positive results in Lac and LiP plate assay were then further subjected to biochemical tests like catalase production test, Indole test, Nitrate reduction test and Methyl red test. These tests are necessary because the basic differences regarding shape, size of microscopic organisms is not enough to identify the species. For a more better understanding and identification of microorganism biochemical tests are performed. Therefore, at last, the identification of microbes is for the most part in light of the distinctions in their biochemical activities. Each types of microorganisms have an all-around characterized set of metabolic activities unique in relation to every single other species. These biochemical fingerprints are properties controlled by the bacterial compounds. Differences in concrete enzymatic activities educate us concerning the biology, the physiology or the normal habitat of the microorganism.

(i) Catalase production test

This test is perfomed to confirm the existence of enzyme catalase in bacteria. Catalase catalyzes the decomposition of hydrogen peroxide (H_2O_2) to oxygen and water. It is utilized to distinguish those bacteria that produces enzyme catalase, for example, staphylococci, from non-catalase producing bacteria, for example, streptococci. Typically, 3% H_2O_2 is utilized for the standard culture.

Principle

The compound catalase intervenes the breakdown of hydrogen peroxide into oxygen and water. To confirm the presence of the compound in a bacterial isolate, a small amount of bacterial culture is brought in contact with H_2O_2 and quick effervescence occurs. A weak or no effervescence indicates the absence of catalase in the microorganism. For this test, the bacterial culture used should be an overnight culture and not older than that.

This is a defence mechanism for aerobic bacteria to protect themselves from the toxic effect of H_2O_2 which is the end product of carbohydrate metabolism.

Procedure

The 12 positive bacterial isolates were picked individually by a sterilized loop. These colonies were then added in 12 different tubes containing 2 ml of sterile nutrient broth (**Table 3**) each. Aseptic conditions were maintained throughout.

Table 3: Composition of nutrient broth medium

Ingredients	Composition (30 ml)
Peptone	0.15 g
Yeast extract	0.09 g
$CuSO_4$	30 µg
Final pH	7.0

The 3% H_2O_2 was made by dissolving 3 ml H_2O_2 in 97 ml distilled water. A loopful of culture in nutrient broth was then transferred to a clean slide. After that 3% H_2O_2 was added in a little amount to the drops of bacterial culture. Effervescence was observed in case of catalase positive bacteria.

(ii) Methyl red test (MR)

The methyl red (MR) test distinguishes the bacteria on the basis of their ability to perform mixed acid fermentation when supplied with glucose. The genera of bacteria can also be

differentiated on the basis of the type of fermentation product produced as well as the product ratio.

Principle

A few microscopic organisms can use glucose and change it over to a stable acid like lactic acid, acidic acid or formic acid. These microbes at first utilize glucose to pyruvic acid, which is additionally processed through the 'mixed acid pathway' to deliver the stable acid. The type of acid generated varies from species to species and relies upon the particular enzymatic pathways present in the microbes. The acid so generated diminishes the pH to 4.5 or beneath, which is demonstrated by change in the color of pH indicator dye methyl red from yellow to red (**Figure 13**).

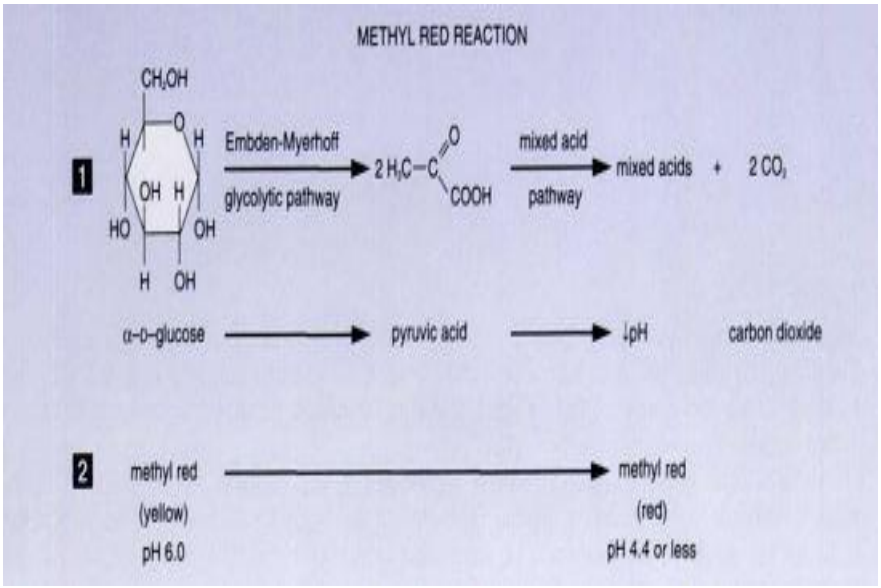


Figure 13: Methyl red reaction depicting conversion of glucose to mixed acids

Procedure

The 12 positive bacterial isolates were picked individually by a sterilized loop and added to different test tubes containing 5ml of buffered glucose broth (**Table 4**) each and one test tube was kept uninoculated as control. Aseptic conditions were maintained throughout. The test tubes were incubated at 37^0C for 72 hours in an incubator shaker. The turbidity was checked in the test tubes within 72 hours. Turbidity indicated bacterial growth. Now, added few drops of methyl red solution which was prepared by dissolving 0.1 g of methyl red in 300 ml of 95% ethyl alcohol and made the volume up to 500 ml using distilled water. On addition of methyl red, recorded for colour change in the test

tubes immediately. The appearance of red color in the test tubes containing bacteria was an indicator of positive MR test.

Table 4: Composition of buffered glucose broth

Ingredients	Composition (100 ml)
Protease peptone	0.5 g
D-glucose	0.5 g
$NaHPO_4$	0.5 g
Final pH	7.0

(iii) Indole test

This test shows the capacity of specific microorganisms to decay the amino acid tryptophan to indole, which accumulates in the medium. The production of indole in this test is crucial for the identification of Enterobacteria. The hydrolysis of tryptophan to produce indole as end product is performed by a series of intracellular enzymes called tryptophanase. The strains of *Escherichia coli, Proteus vulgaris, Providencia rettgeri*, *Morganella morganii* and *Providencia* species are capable of producing tryptophanase.

Principle

Bacteria that expresses tryptophanase enzyme gives positive result for this test, as this enzyme performs reductive deamination of tryptophan an amino acid. Tryptophanase removes the amine (- NH_2) group resulting in the production of indole, pyruvic acid, ammonium ions and energy (**Figure 14**). The production of indole is not direct, prior to its formation the formation of an intermediate called indolepyruvic acid occurs.

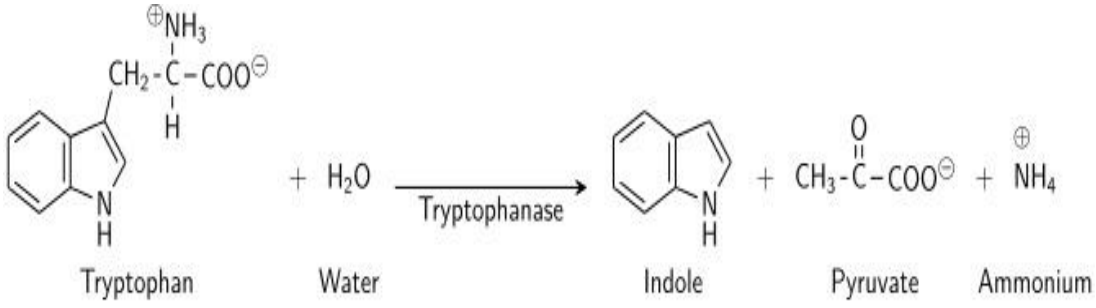


Figure 14: Reductive deamination of tryptophan by tryptophanase

When Kovac's reagent (which contains hydrochloric acid and *p*-dimethylaminobenzaldehyde in amyl alcohol) is added to the inoculum containing indole, the medium turns cherry red from yellow. This red color is observed in the top layer, as amyl alcohol is insoluble in water.

Procedure

The colonies of 12 laccase or lignin peroxidase positive bacterial isolates were incubated at 37^0C in tryptone water. The tryptone water was made by adding NaCl and tryptone to distilled water (**Table 5**). This was then autoclaved and allowed to cool down. 3 ml tryptone per test tube was added in 12 test tubes for 12 different colonies. The colonies were then inoculated to it and were allowed to incubate for 72 hrs at 37°C on a orbitary shaker.

Table 5: Composition of Tryptone water

Ingredients	Composition (50 ml)
Tryptone	0.5 g
NaCl	0.25 g

After 48 hrs checked growth in test tubes. If growth was there, prepared Kovac's reagent. It was prepared by dissolving *p*-dimethylamino benzaldehyde in amyl alcohol, then adding conc. HCl slowly to this mixture (**Table 6**). Once this reagent is prepared add 1ml of it to the test tubes containing tryptone water. Immediate appearance of bright pink color in the top layer was indicative of a positive test.

Table 6: Composition of Kovac's reagent

Ingredients	Composition (15 ml)
p-dimethylamino benzaldehyde	0.075 g
Amyl alcohol	1.125 ml
Conc. HCl	0.375 ml

(iv) Nitrate reduction test

This test distinguishes the bacteria on the basis of the presence of nitrate reductase enzyme. This enzyme is required to perform the hydrolysis of nitrate (NO_3^-) to nitrite (NO_2^-). Nitrite is then further broken down to release several nitrogen products like ammonia, nitrous oxide, nitrogen oxide. The further degradation of nitrite varies with the enzyme system of bacteria and with its surrounding.

Principle

The bacterial isolates are inoculated in nitrate broth. After 14-18 hours, a few drops of sulfanilic acid is added to check for the formation of nitrite. If nitrate is reduced to nitrite then formation of nitrous acid in the medium will occur by nitrites. At the point when sulfanilic acid is introduced to the medium, it will react with the nitrous acid to form diazotized sulfanilic acid. This in turn reacts with the α-naphthylamine to form a red-colored compound. This is viewed as a positive outcome for nitrate reduction (**Figure 15**).

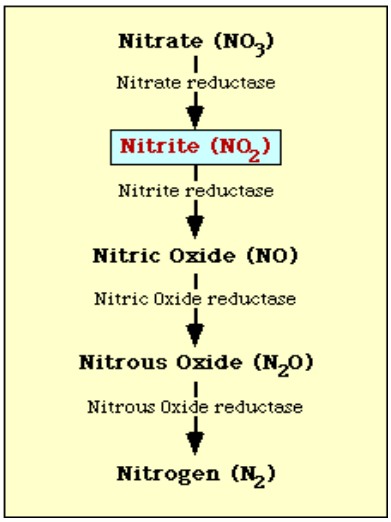


Figure 15: A schematic representation of nitrate reduction

If the medium does not turn red after the addition of the reagents, it can imply that the micro-organism is not able to reduce the nitrate, or it can imply that the organism can denitrify the nitrate or nitrite to form ammonia or atomic nitrogen.

Procedure

The nitrate reduction broth was prepared by adding peptone, KNO_3 and yeast extract and making up the volume to 30ml by distilled water (**Table 7**). The broth was then autoclaved and allowed to cool down. Under laminar hood the 12 bacterial colonies were then inoculated in the sterile broth in 12 different test tubes. These test tubes were then kept for incubation for 72 hrs at 37°C.

Table 7: Composition of nitrate reduction broth

Ingredients	Composition (30 ml)
Peptone	0.12 g
Yeast extract	0.09 g
KNO_3	0.03 g
Final pH	7.0

After incubation, 1 ml each of sulphanilic acid (0.8% in 5N acetic acid) and α-naphthylamine (0.5% in 5N acetic acid) was added into the tubes. The 5N acetic acid was prepared by adding 11.5 ml glacial acetic acid in 28.5 ml of distilled water. 20 ml of this mixture was then poured into 2 different test tubes. In one test tube 0.16 g of sulpanilic acid was added to 5N acetic acid (5.75 ml glacial acetic acid and 14.25 ml distilled H_2O) and was labeled as A. In the other test tube 0.1 ml of α-naphthylamine was added to 5N acetic acid and was labeled as B. The appearance of red color was an indication of the positive test for nitrate reduction.

3.2.4 Methods to analyse the lignin degrading enzyme activities

(i) Media preparation

The 12 bacterial isolates tested positive on plate assays were inoculated individually in sterile nutrient broth medium (10 ml/ test tube) and incubated at 37^0C at 120 rpm on an orbitary shaker overnight.

(ii) Preparation of crude protein sample

The above grown overnight cultures of 12 bacterial colonies were taken and then filtered through Whatman no. 1 filter paper. This was performed by keeping the sterile empty test tubes in ice packs and then filtering the culture one by one into each test tube kept at 4^0C. The filtrate collected in the test tube was considered as the crude protein sample.

(iii) Protein content determination by Lowry's method

The protein solution was first prepared for the determination of protein content by Lowry's method. This was done by taking 500 µl of bacterial culture in a microfuge tube. The protein present in that culture was then precipitated with 500 µl of ice-cold 20% trichloroacetic acid (TCA) and kept at 4°C overnight. Next day the bacterial culture along with TCA is centrifuged at 12,000 rpm for 5 min at room temperature to recover the pellet, the supernatant so obtained was discarded. The pellet was then washed with 0.1 ml ice cold 10% TCA followed by washing with 0.1 ml ice-cold acetone. The pellet was dissolved in 0.5-1.0 ml of 0.1 N NaOH according to its size. The pellet along with NaOH was subjected to heating for 5 min in hot water bath and vortexed vigorously. This was considered as protein solution and the protein content was determined by Lowry's method.

Principle

The guideline behind the Lowry's method for determining protein concentration lies in the reactivity of the peptide nitrogen[s] with the copper [II] particles under soluble conditions and the ensuing reduction of the Folin Ciocalteau (phosphomolybdic phosphotungstic acid) to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. The Lowry's method is sensitive to pH changes which was kept at 10 - 10.5.

Reagents

1. Solution A: 2% Na_2CO_3 in 0.1N NaOH

2. Solution B: 2% Sodium potassium tartrate in H_2O

3. Solution C: 1% $CuSO_4.5H_2O$ in H_2O

4. Reagent I: 100 ml of A, 1 ml of B, 1 ml of C

5. Reagent II: 0.25 ml of 1.0N Folin's reagent (available commercially)

BSA Standard- 2 mg/ml (stock solution)

Procedure

(a) Preparation of standard curve for BSA

Required aliquot of BSA in different test tubes was taken from the stock (2 mg/ml) ranging from 0.1 mg/ml to 1 mg/ml. BSA aliquots in required volume were added to the test tubes and made the volume up to 1 ml in each test tube using distilled water. Test tube with no BSA and 1 ml distilled water served as blank. Added 2.5 ml of reagent I to each tube and mixed thoroughly. Incubated test tubes for 10 minutes. Added 0.25 ml of reagent II to each tube while shaking the tube. Incubated tubes for 30 minutes at room temperature. Absorbance was taken at 660 nm using spectrophotometer Standard graph was plotted.

(b) Protein content determination using standard graph

The standard curve for BSA was plotted (absorbance at 660 nm at Y-axis vs amount of BSA at X-axis). The amount of BSA used for standard curve preparation ranged between 0.1 to 1.0 mg/ml. 1 ml of protein solution (0.5 ml crude protein + 0.5 ml of distilled water) was used to set the reaction for calculation of protein concentration. The concentration of unknown solution was determined using the standard curve.

The reagents added in setting the reactions are given in **Table 8**

Table 8: Reagents added for measurement of OD at 660nm

S.No.	Protein sample (ml)	Reagent I (ml)		Reagent II (ml)	
1	1 ml	2.5 ml	Incubate for 10 min at room temperature	0.25 ml	Incubate for 30 min at room temperature
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					

3.2.5 Determination of lignin degradation potential by enzymatic analysis

(i) Determination of laccase (lac) enzyme activity.

The screening of bacteria for the presence of laccase enzyme was done by using ABTS (2,2'-azinobis-3-ethylbenzothiozoline-6-sulfonic acid) as substrate.

Principle

Laccases are mostly multi copper oxidases that catalyzes the oxidation of many phenolic substrates by one electron oxidation. The terminal electron acceptor is molecular oxygen which gets reduced to water. There are three types of copper which can be differentiated as type I, type II and Type III on the basis of their absorbance at 660 nm. The type I copper binds to two nitrogen atoms present in two histidines and one sulphur atom in cysteine. The bond of type I copper with sulphur is responsible for the characteristic blue colour of laccase enzyme.

Procedure

A total of 3 ml reaction mixture was prepared per test tube for 12 different samples. The reaction mixture consisted of 1 ml of 50 mM sodium acetate buffer (pH 5.0) which was prepared by weighing 0.5 g of sodium acetate and adding it to 220 µl of glacial acetic acid and making up the volume upto 200 ml by using distilled water, 1 ml of 2 mM ABTS in acetate buffer (prepared by dissolving 54 mg of ABTS in 50 ml of sodium acetate buffer) and at last 1 ml of enzyme extract i.e. crude protein sample was added to the test tube.

The reaction mixture was blended thoroughly and then kept at room temperature for about 15-30 minutes to check for the oxidation of ABTS which was indicated by the change in color to green.

Once the green color was observed the absorbance of all the samples were taken at 420 nm against a reagent blank. One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1 mole of ABTS per minute. The substrate containing buffer without supernatant was used as standard. The experiment was performed in triplicates.

(ii) Determination of lignin peroxidase activity

The screening of bacteria for the presence of lignin peroxidase enzyme was done by using Azure B as substrate.

Principle

Lignin peroxidase are haem containing peroxidases. Their activity can be determined by H_2O_2 dependent oxidation of several non-phenolic lignin model compounds, β-O-4 linked lignin model compounds, C-C cleavage and aromatic ring cleavages. The oxidation of dye azure B involves removal of two electron per unit of azure B in presence of H_2O_2 resulting in the formation of two water molecules. This results in the decolorization of azure B measured by change in absorbance at 651 nm.

Procedure

A total of 3 ml reaction mixture was prepared per test tube for 12 different samples. The reaction mixture consisted of 1 ml of 50 mM sodium tartrate buffer (pH 4.5) (prepared by weighing 0.736 g of Sodium tartrate and adding it to 0.27 g of tartaric acid and making the volume up to 100 ml by using distilled water), 0.1 ml of 100 µM Azure B in acetate buffer (prepared by dissolving 13.7 mg of azure B in 250 ml distilled water), 0.1 ml of

H_2O_2, 1 ml of enzyme extract and 0.8 ml of distilled water. The reaction mixture was blended thoroughly and then kept at room temperature for about 1 hour.

After 1 hour the absorbance of all the samples were recorded at 651 nm against a reagent blank. One unit (U) of LiP activity was defined as the activity of an enzyme that catalyzes the conversion of 1 μmole of azure B per minute. The substrate-containing buffer, without supernatant, was used as a standard. The experiment was performed in triplicates.

3.2.6 Isolation of fungi capable of lignin degradation

The decaying wood sample and the soil sample were screened for the presence of fungi individually on the basis of laccase (Lac) and lignin peroxidase (LiP) plate assays. The positive fungi possessing lignin degrading ability were observed for the formation of reddish brown zone in case of Lac plate assay and clear zones with white colonies in case of LiP plate assay.

(i) Media preparation

Lignin-degrading fungi were screened on the principle of laccase and lignin peroxidase plate assay. For this Olga *et al.* medium (**Table 9**) was prepared with a pH of 6.0. This medium was supplemented with agar 2% and autoclaved at 15 psi for 15 min. Autoclaved medium was poured in sterile petriplates (25 ml/plate) under laminar flow hood and permitted to solidify. Then 0.02% sterile guaiacol (in diethylether) was spread on the solidified medium for Lac plate assay and 0.05% Azure B for LiP plate assay respectively.

Table 9: Composition of Olga *et al.* medium

Ingredients	Composition (100 ml)
Peptone	0.3 g
Glucose	1.0 g
KH_2PO_4	0.03 g
$ZnSO_4$	0.001 g
K_2HPO_4	0.004 g
$FeSO_4$	0.00005 g
$MnSO_4$	0.0005 g
$MgSO_4$	0.0005 g
Agar	2.0 g
Guaiacol/ Azure B	0.02% / 0.05%
Final pH – 6.0	

(ii) Serial dilution of samples

The decaying wood was finely crushed into powder form. Then 1 g of this finely crushed wood was added to test tube containing 9 ml saline (0.8% NaCl) and mixed properly. This was 10^{-1} dilution. Now further 1ml of this solution was taken and transferred to another test tube containing 9 ml saline. This process is repeated till 10^{-8} dilution is achieved.

The similar procedure was done for the soil sample, 1 g of soil was added to test tube containing 9 ml saline (0.8% NaCl) and mixed properly. And similarly, the dilution was done upto 10^{-8}.

(iii) Inoculation and incubation

100 µl of all the dilutions of decaying wood sample were put on Olga *et al.* medium. Plates were supplemented with guaiacol for lac plate assay and with Azure B for LiP plate assay respectively. These dilutions were then spread with a sterile glass spreader onto the Olga plates supplemented with substrates. The plates were then kept for incubation in inverted position at 37°C for upto 72 hours.

Similarly, 100 µl of all the dilutions of soil sample were put on Olga medium plates and the same protocol was followed.

(iv) Subculturing

Best growth was observed with 10^{-6} and 10^{-7} dilutions. Positive colonies with lignin degrading potential showed reddish brown colonies on guaiacol containing medium and white colonies with clear zones were observed in Azure B containing medium. These positive cultures were subcultured in Sabouraud agar medium (**Table 10**) to obtain pure cultures. A total of six colonies were picked, three from the decaying wood sample and three from soil sample respectively, subcultured and incubated for 72 hours at 37^0C.

Table 10: Composition of Sabouraud agar medium

Ingredients	Composition (200 ml)
Peptone	2 g
Dextrose	8 g
Agar	7 g
Final pH	5.0

3.2.7 Morphological identification of fungi by lactophenol cotton blue staining

Principle

The lactophenol cotton blue (LPCB) wet mount procedure is the most generally utilized and the simplest one to stain and observe fungi. The procedure has three parts:

1.Phenol: destroys any living organism
2. Lactic acid: It prevents destruction of fungi by phenol,
3. Cotton blue: It is the dye that stains the chitin present in cell walls of fungi.
Lactophenol cotton blue solution is a stain that is utilized to prepare the slides for the microscopic examination of fungi. The structural components of fungi are strongly stained blue.

Procedure

The clean coverslips with the help of sterile forceps were inserted in tilted position over the fungal growth in the Sabouraud agar media containing plates. After the fungal growth was observed over coverslip, they were gently lifted with the help of sterile forceps to be placed over a clean glass slide. The glass slides were wiped with alcohol and were exposed to flame. The glass slides were numbered as W1-W3 for decaying wood fungal colonies and S1-S3 for soil fungal colonies. A drop of lactophenol cotton blue was added to each glass slide. The coverslips containing fungal growth were placed facing down on the glass slide containing stain according to the numbering.

These slides were then observed under light microscope at 40X magnification.

3.2.7 Biochemical characterization of lignin degrading fungi

(i) Starch hydrolysis test

Starch agar is a differential medium that tests the capacity of microbes to release certain exoenzymes, including an amylase and oligo-1,6-glucosidase, that hydrolyze starch. Starch atoms are too bulky to enter the fungal cell, so a few microbes release exoenzymes to degrade starch into subunits that would then be able to be used by the microorganisms.

Principle

Starch agar is a basic nutritive medium. Since no color change happens in the medium when living beings hydrolyze starch, iodine is added to the plate after incubation. If starch is present in the medium and is not hydrolysed then the iodine will react with starch to form a complex which imparts blue black color to the medium. A clearing around the fungal development shows that the fungi has hydrolyzed starch.

Procedure

Starch agar media was prepared as described in the **Table 11** below:

Table 11: Composition of starch agar media

Ingredients	Composition (200 ml)
Peptone	1 g
Yeast extract	0.6 g
Starch	4 g
Agar	4 g
Final pH	7.0

The media was autoclaved at 15 psi for 20 minutes and then cooled to room temperature. The media was poured into 6 sterile petri plates and allowed to solidify. The solidified plates were then inoculated with a sterile loop with fungal colonies from both wood and soil sample. The plates were then kept at 25^0C in inverted position for 5-7 days. Once the growth was observed after 5-7 days the surface of the plates is flooded with iodine solution. The iodine solution was prepared by dissolving 10 g of potassium iodide in 50 ml distilled water then adding 5 g iodine to it and making up the volume to 100 ml by distilled water. The solution was swirled thoroughly until all the iodine crystal dissolved. The iodine solution was then flooded over the surface of starch agar plates. The appearance of clear zones in the midst of dark plates was indicative of positive starch hydrolysis test.

(ii) Cellulose hydrolysis test

Cellulose is the most abundant organic compound present in plants. It is a linear polysachharide linked together by β-1,4 glycosidic bonds. Degradation of cellulose is significant in the biological cycle of carbon, since it is added as vegetation in great quantity to the soil.

Principle

Cellulose is degraded mostly by fungi, though bacteria are also capable of degrading it. They produce a complex system of enzyme called cellulases. This enzyme system is composed of β-glucosidase, endoglucanase and exoglucanase. For the complete degradation of cellulose to glucose the co-operative action of these three components is required. The utilization of cellulose in the medium is detected by a reagent called hexadecyltrimethyl ammonium bromide. It interacts with carboxymethyl cellulose and precipitates it causing appearance of clear zones around fungal colonies.

Procedure

Czapek-mineral salt agar medium was prepared as shown in the **Table 12** below:

Table 12: Composition of Czapek-mineral salt agar medium

Ingredients	Composition (200 ml)
Peptone	0.2 g
Carboxymethyl cellulose	1 g
Agar	6 g
KCl	0.1 g
K_2HPO_4	0.2 g
$NaNO_3$	0.4 g
$MgSO_4.7H_2O$	0.1 g
Final pH	6.5

The media was autoclaved at 15 psi for 20 minutes and allowed to cool down. The media was then poured to sterile petri plates and allowed to solidify The solidified media plates were then inoculated with fungal cultures and kept at 35^0C in inverted position for 2-5 days. When the growth was observed in 2-5 days the surface of the plates was flooded with 1% aqueous solution of hexadecyltrimethyl ammonium bromide for 30 seconds.

The plates were observed for the appearance of a clear zone around the fungal growth.

RESULTS AND DISCUSSION

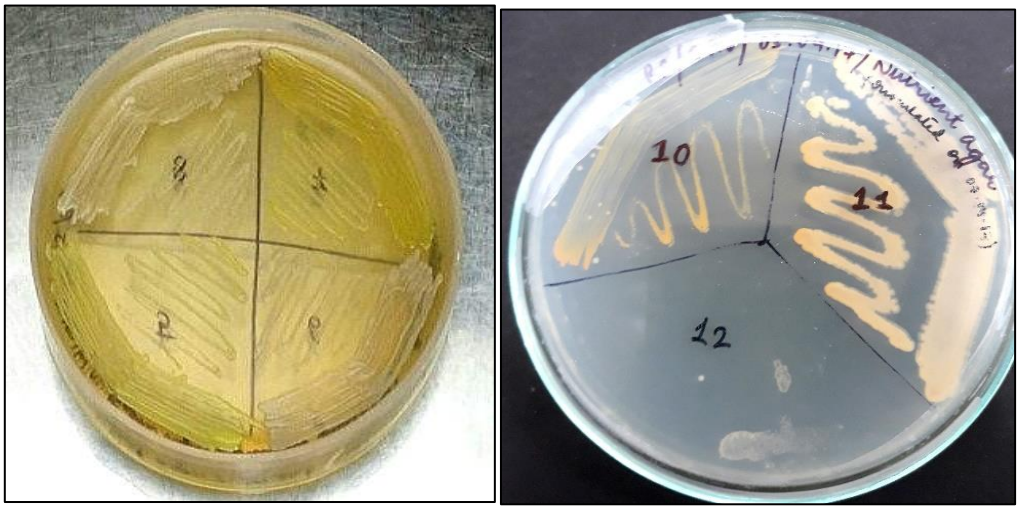
Results

4.1 Isolation of lignolytic bacteria

The decaying wood and the soil sample were collected from the backyard of a woodmill near Subhash chowk, Gurgaon. Both these samples were screened for the presence of both lignin degrading bacteria and fungi on the basis of laccase and lignin peroxidase plate assays. These enzymes are implicated in lignin degradation pathways. Laccase positive bacteria were detected on the basis of their capability of forming reddish brown coloration in the presence of guaiacol. Likewise, lignin peroxidase positive bacteria were detected on the basis of their ability to form white colonies with clear zones in the presence of Azure B.

A total of 12 isolates, six from each decaying wood sample and soil sample, with lignin degrading potential were isolated as pure cultures. These were named as W5, W6, W7, W8, W10, W11 and S4, S5, S6, S7, S8, S9 from decaying wood and soil samples respectively.

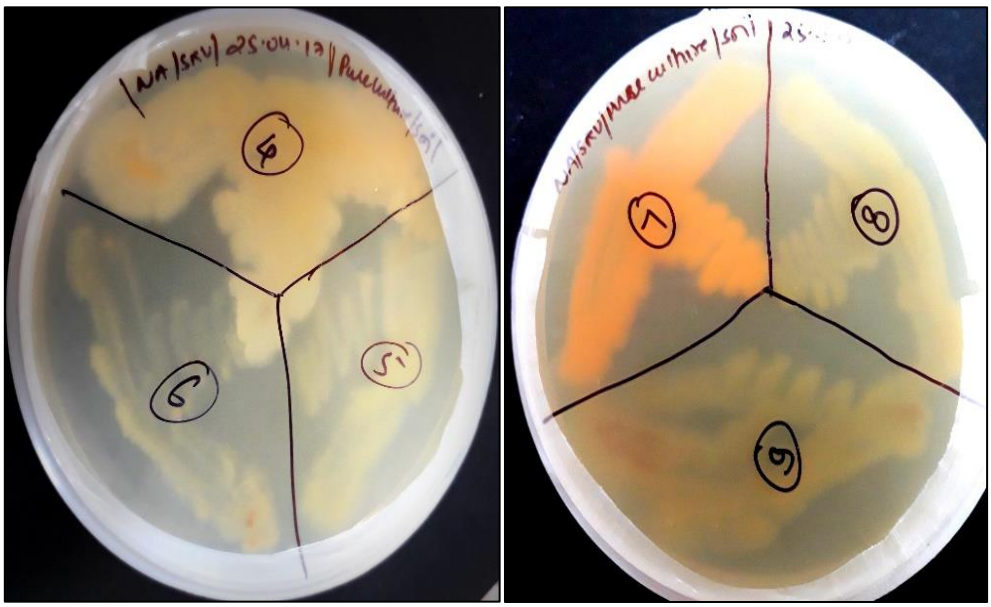
Six putative bacterial isolates with lignin degrading potential were isolated from the decaying wood sample and subcultured (**Figure 16**):



Isolates 5, 6, 7, 8 Isolates 10, 11

Figure 16: Bacterial isolates tested positive for laccase plate assays from decaying wood

Six bacterial isolates with lignin degrading ability on the basis of laccase plate assay were isolated from soil sample and subcultured (**Figure 17**):



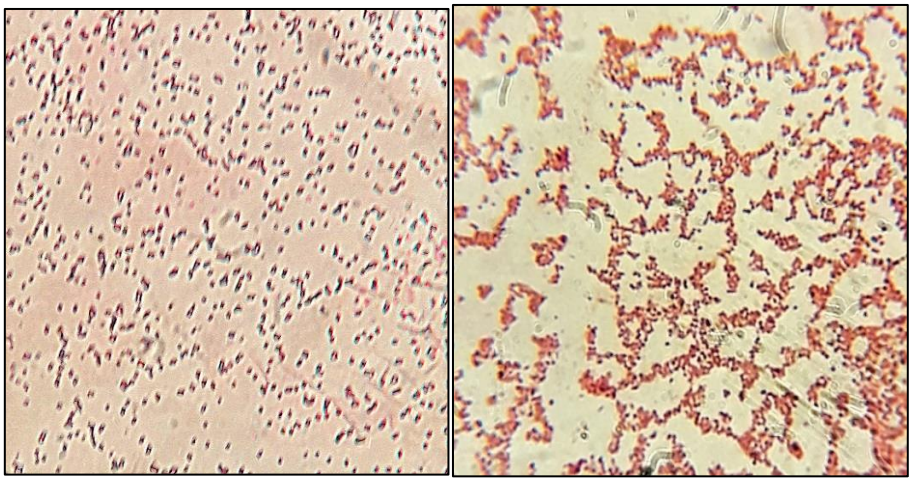
Isolates 4, 5, 6 Isolates 7, 8, 9

Figure 17: Bacterial isolates tested positive for laccase plate assay from soil sample

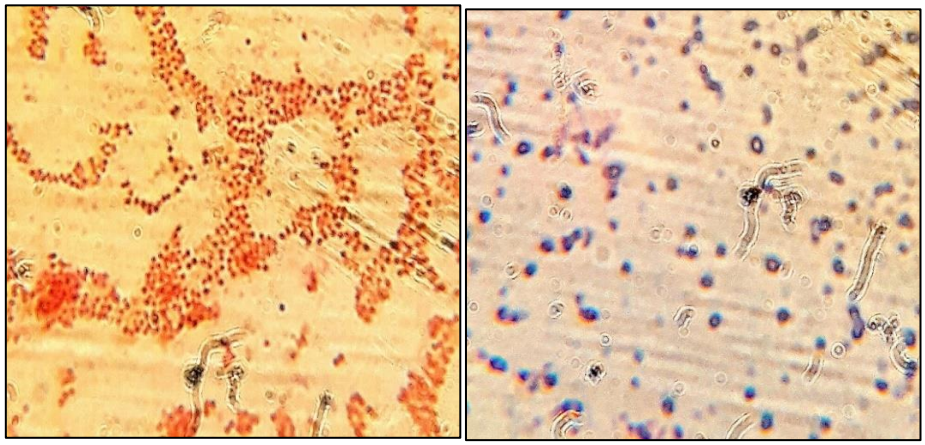
These twelve positive isolates were subcultured to maintain their vitality for the ongoing research. The isolates from the decaying wood and the soil were then subjected to morphological and biochemical characterization to understand their morphology and nature so as to determine the type of species isolated from the respective samples. Morphological characterization was done by Gram's staining.

4.2 Morphological characterization of bacterial isolates

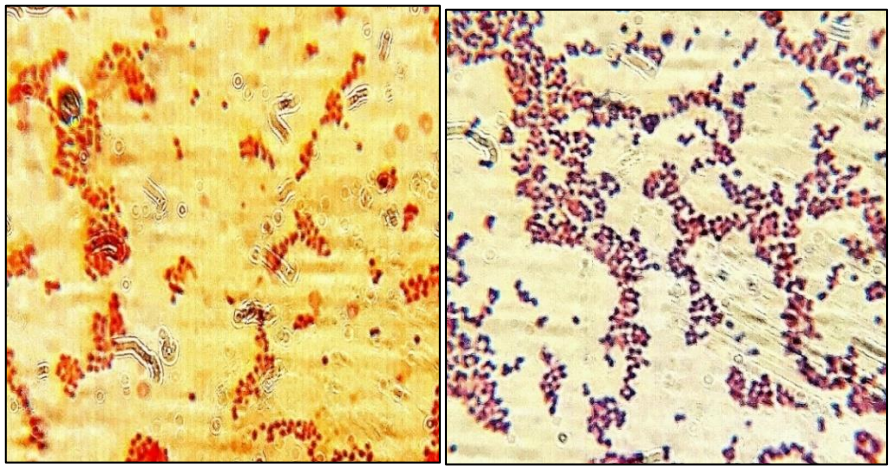
The results of Gram staining for the isolates obtained from the decaying wood sample are shown in **Figure 18**:



Isolate W5 Isolate W6



Isolate W7 Isolate W8



Isolate W10 Isolate W11

Figure 18: Morphological characterization of six bacterial isolates from decaying wood sample on the basis of Gram's staining

The results of Gram staining for the isolates obtained from soil sample are shown in **Figure 19:**

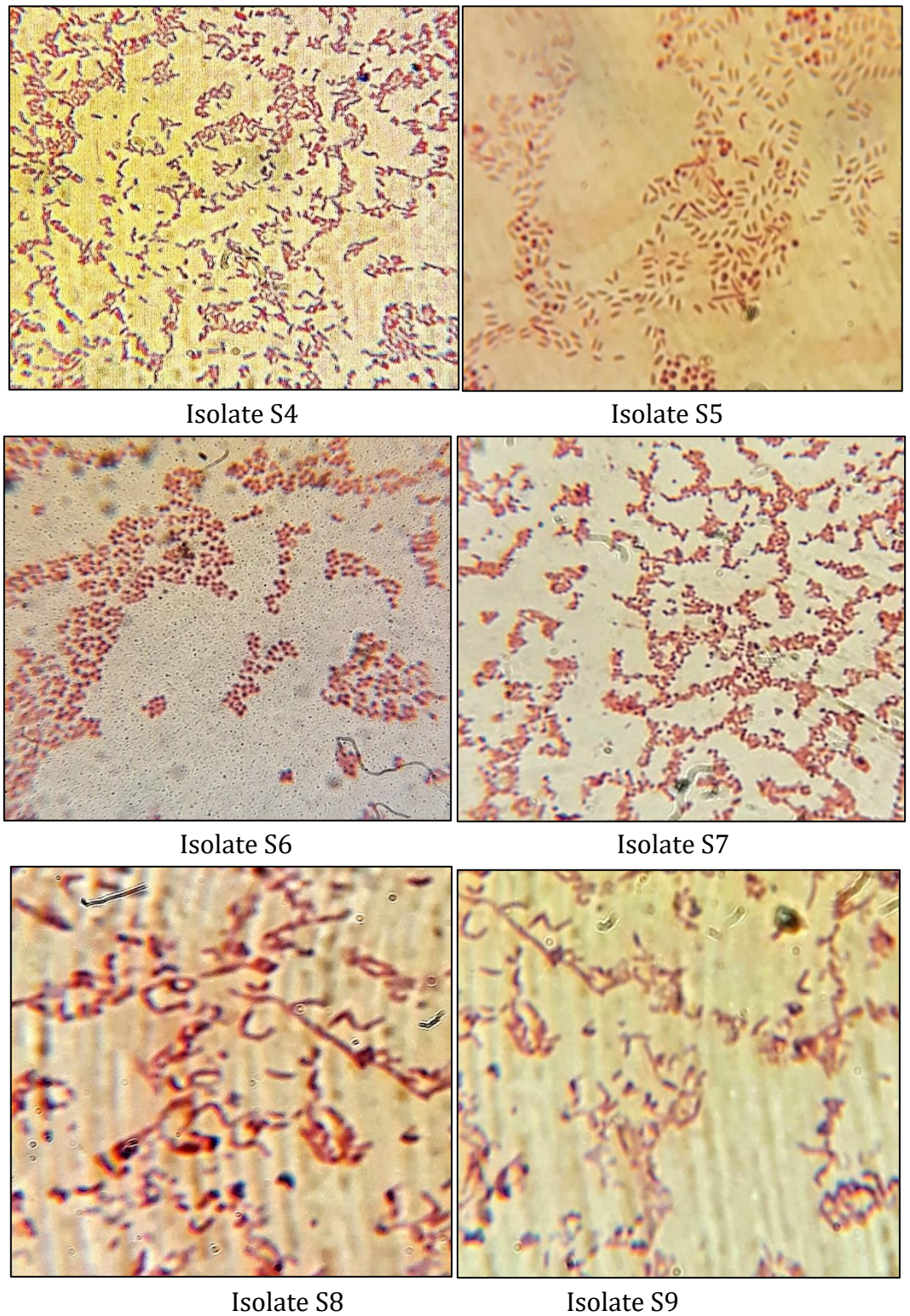


Figure 19: Morphological characterization of six bacterial isolates from soil sample on the basis of Gram's staining

Thus, the results of Gram's staining led to morphological characterization of the bacterial isolates from both the decaying wood sample and soil sample. Thus, the shape and Gram property of all twelve isolates was determined. Isolates W5, W8, W11, S4, were Gram positive rest all were Gram negative. All the isolates from decaying wood sample and S6 isolated from soil sample were cocci , S4, S7, S8, S9 were bacillus and S5 was cocco-bacillus. The results are tabulated in **Table 13**.

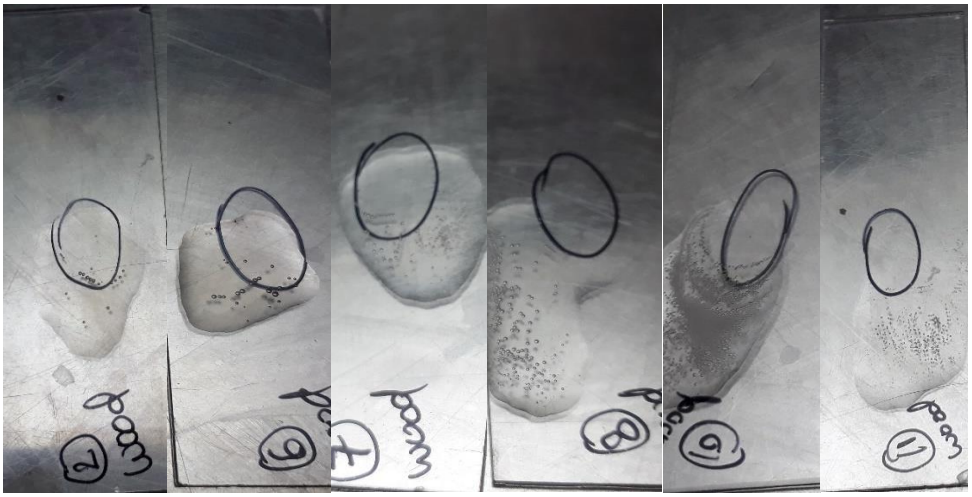
Table 13: Shape and Gram property of bacterial isolates obtained from decaying wood and soil samples

ISOLATE NUMBER	SHAPE	GRAM POSITIVE / NEGATIVE
W5	Cocci	Gram positive
W6	Cocci	Gram negative
W7	Cocci	Gram negative
W8	Cocci	Gram positive
W10	Cocci	Gram negative
W11	Cocci	Gram positive
S4	Bacilli	Gram positive
S5	Cocco-bacillus	Gram negative
S6	Cocci	Gram negative
S7	Bacilli	Gram negative
S8	Bacilli	Gram negative
S9	Bacilli	Gram negative

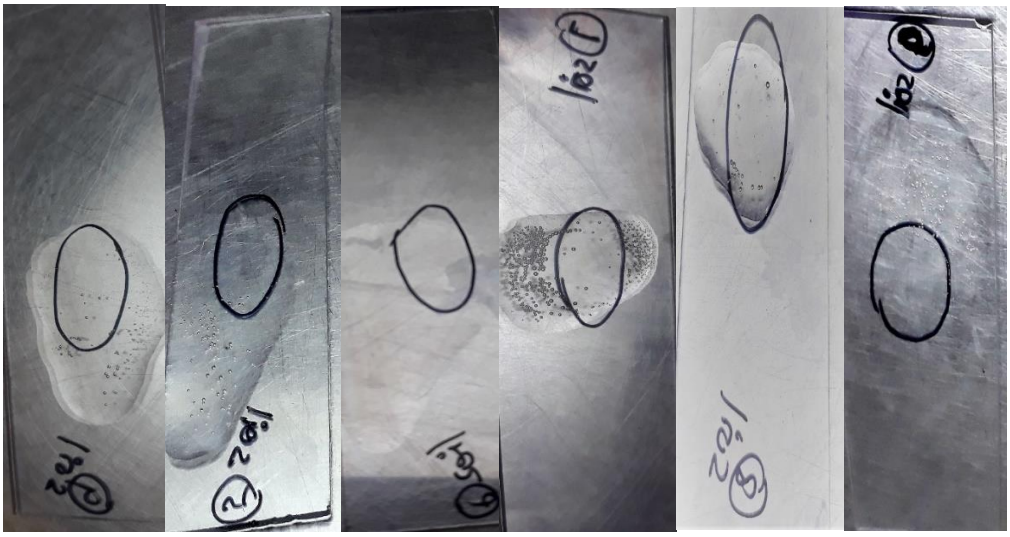
4.3 Biochemical characterization of bacterial isolates

Biochemical characterization of twelve bacterial isolates was done by performing different biochemical tests including catalase test, indole test, methyl red test and nitrate reduction test.

(i) Catalase test- Results of catalase test of bacterial isolates from decaying wood and soil sample are shown in **Figure 20.** Effervescence or bubbles formation indicated positive catalase test while no bubbles or less bubbles indicated negative test.



W5 W6 W7 W8 W10 W11



S4 S5 S6 S7 S8 S9

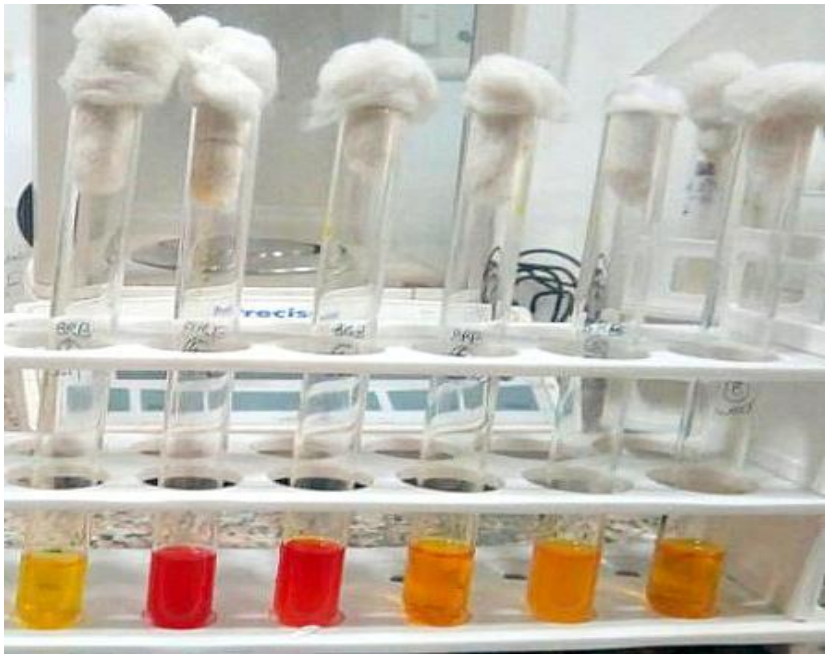
Figure 20: Biochemical characterization of twelve bacterial isolates from decaying wood and soil samples on the basis of catalase test

Analysis of bacterial isolates indicated that all the isolates from decaying wood sample except W5, were catalase positive. Isolates S4, S5, S7, S8, S9 from soil sample were also catalase positive. The results are tabulated in **Table 14.**

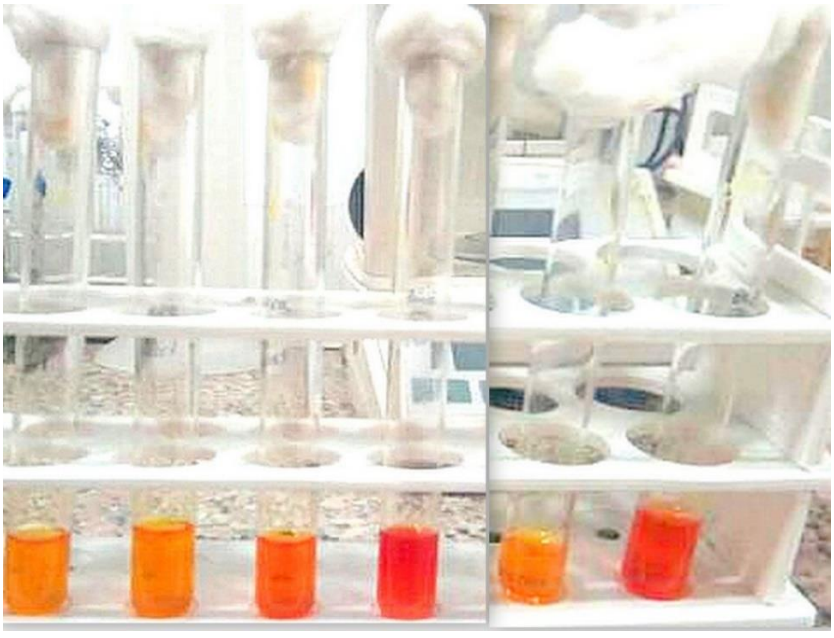
Table 14: Catalase test of bacterial isolates from decaying wood and soil samples

ISOLATE NUMBER	CATALASE POSITIVE / NEGATIVE
W5	Negative
W6	Positive
W7	Positive
W8	Positive
W10	Positive
W11	Positive
S4	Positive
S5	Positive
S6	Negative
S7	Positive
S8	Negative
S9	Positive

(ii) Methyl red test- Results of methyl red test of bacterial isolates from decaying wood and soil sample are shown in **Figure 21:**



W5 W6 W7 W8 W10 W11



S4 S6 S8 S5 S9 S7

Figure 21: Biochemical characterization of twelve bacterial isolates from decaying wood and soil sample on the basis of methyl red test

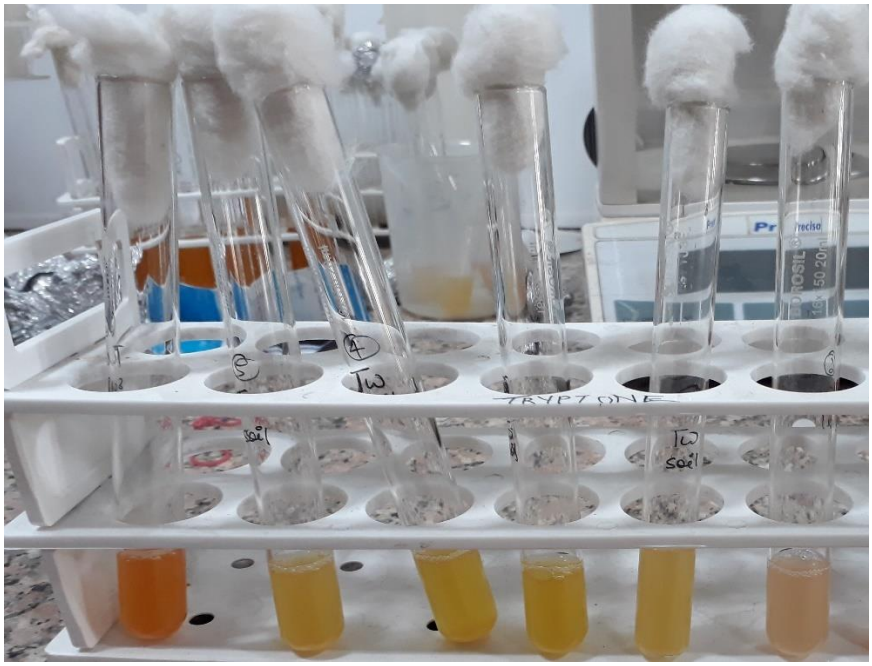
From the test it was observed that isolates W6, W7, S5, S7 gave red coloration with methyl red indicating the release of acid and hence gave positive methyl red test while the remaining isolates did not show any color change and hence gave negative test. Results of methyl red test are presented in **Table 15.**

Table 15: Methyl red test of bacterial isolates from decaying wood and soil samples

ISOLATE NUMBER	METHYL RED POSITIVE / NEGATIVE
W5	Negative
W6	Positive
W7	Positive
W8	Negative
W10	Negative
W11	Negative
S4	Negative
S5	Positive
S6	Negative
S7	Positive
S8	Negative
S9	Negative

(iii) Indole test- Results of indole test of bacterial isolates from decaying wood and soil sample are shown in **Figure 22:**

W6 W5 W7 W10 W11 W8



S6 S5 S4 S7 S9 S8

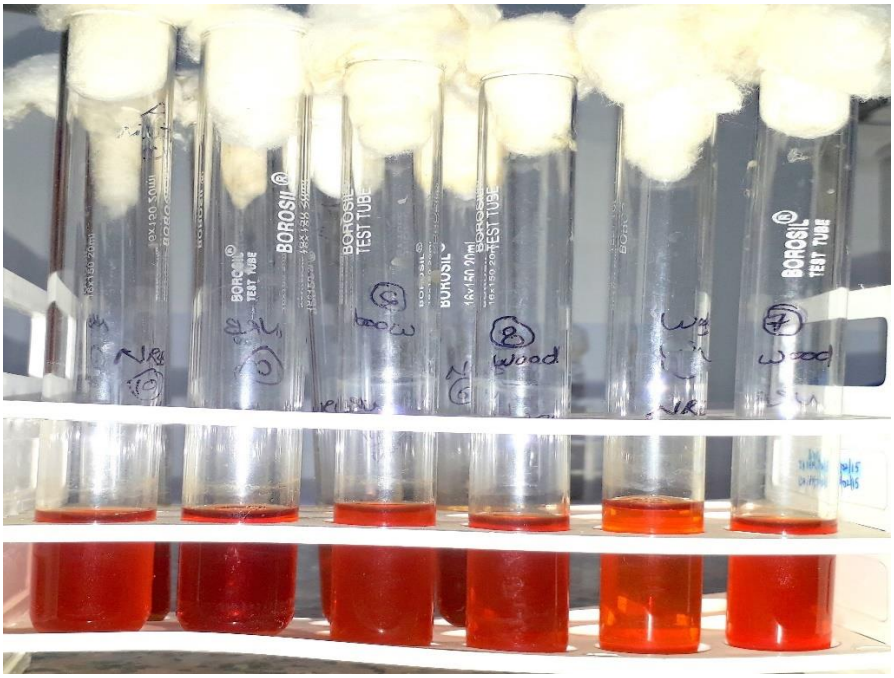
Figure 22: Biochemical characterization of twelve bacterial isolates from decaying wood and soil sample on the basis of indole test

Thus, the results of indole test led to biochemical characterization of the bacterial isolates from both the decaying wood sample and soil sample. Pink color formation by isolates W5 and S6 indicated positive test while no color change in the rest of the isolates indicated negative test. The results are tabulated in **Table 16**.

Table 16: Indole test of bacterial isolates from decaying wood and soil samples

ISOLATE NUMBER	INDOLE POSITIVE / NEGATIVE
W5	Positive
W6	Negative
W7	Negative
W8	Negative
W10	Negative
W11	Negative
S4	Negative
S5	Negative
S6	Positive
S7	Negative
S8	Negative
S9	Negative

(iv) Nitrate reduction test- Results of indole test of bacterial isolates from decaying wood and soil sample are shown in **Figure 23:**



W10 W11 W9 W8 W5 W7

S5 S8 S6 S4 S7 S9

Figure 23: Biochemical characterization of twelve bacterial isolates from decaying wood and soil sample on the basis of nitrate reduction test

Thus, the results of nitrate reduction test led to biochemical characterization of the bacterial isolates from both the decaying wood sample and soil sample. Red color formation by isolates W6, W8, W10, W11, S4, S6, S7, S8, S9 indicated positive test while no color change by the rest of the isolates indicated negative test. The results are summarized in **Table 17**.

Table 17: Nitrate reduction test of bacterial isolates from decaying wood and soil samples

ISOLATE NUMBER	NITRTAE REDUCTION POSITIVE / NEGATIVE
W5	Negative
W6	Positive
W7	Negative
W8	Positive
W10	Positive
W11	Positive
S4	Positive
S5	Negative
S6	Positive
S7	Positive
S8	Positive
S9	Positive

4.4 Determination of protein concentration of bacterial isolates

The twelve isolates screened on the basis of laccase and azure B plate assays were then subjected to quantitative analysis of laccase and lignin peroxidase enzyme activities. The aim was to determine the isolates with the highest lignin degrading potential.

In order to determine the protein content by Lowry's method, BSA was used as standard and a standard curve was plotted between OD at 660 nm versus BSA concentration. Protein concentration in various bacterial samples was then determined using this standard plot (**Figure 24**).

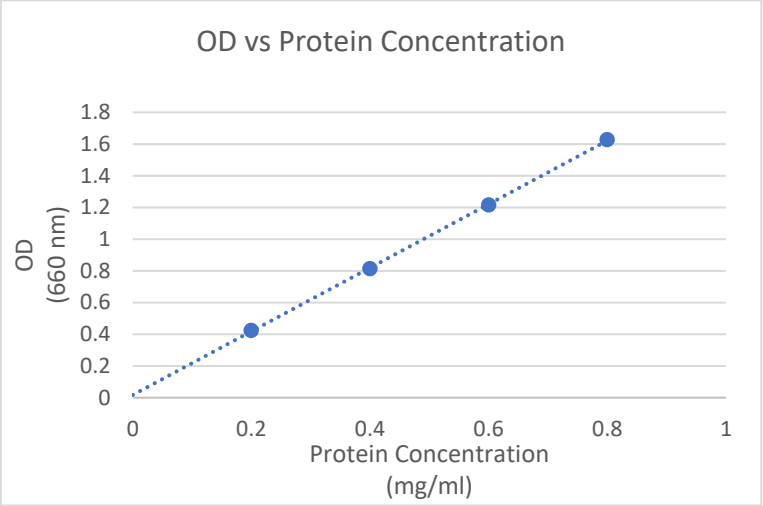


Figure 24: Standard graph between OD (660 nm) and BSA concentration

The protein concentrations of various bacterial isolates are shown in **Figure 25** and **Figure 26**

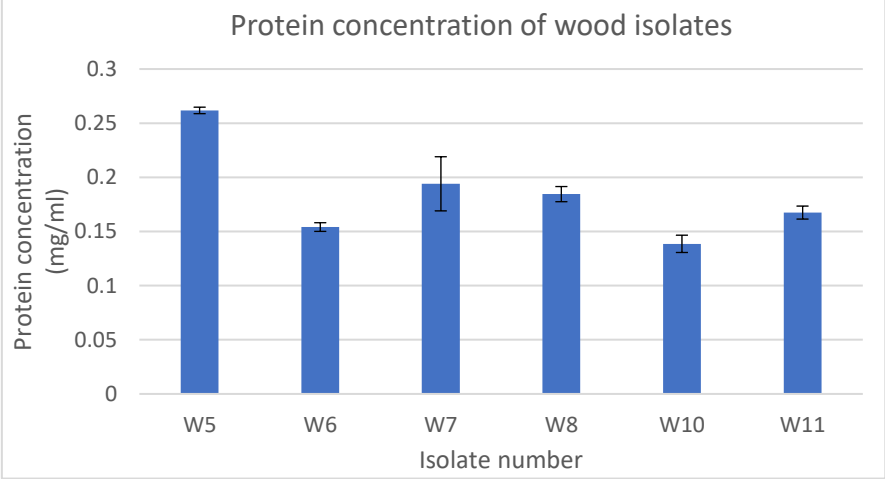


Figure 25: Protein concentrations of bacterial isolates from decaying wood

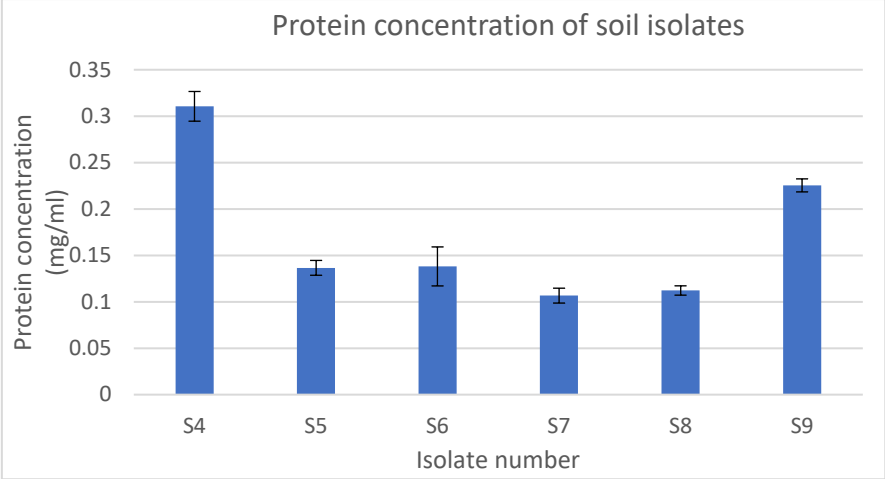


Figure 26: Protein concentrations of bacterial isolates from soil

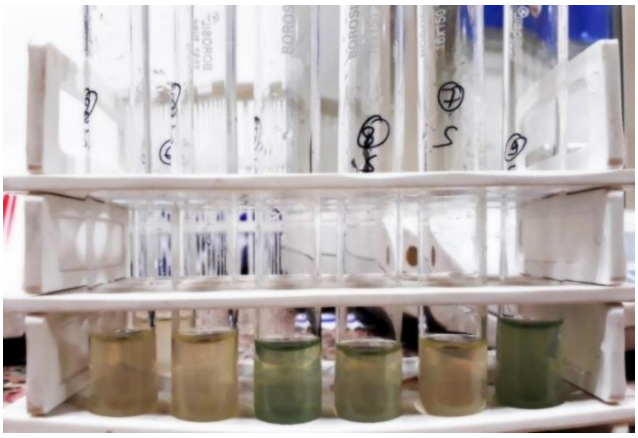
Thus isolate S4 exhibited maximum protein concentration, followed by W5, S9, W7, W8, W11, W6, W10, S6, S5, S8 and isolate S7 had minimum protein concentration.

4.5 Analysis of lignin degrading enzymes from bacterial isolates

4.5.1 Laccase enzyme activity

Laccase enzyme activities in the crude enzyme extracts of all bacterial isolates were determined spectrophotometrically using ABTS (2,2'-azinobis-3-ethylbenzothiozoline-6-sulfonic acid) as noncatalytic cooxidant. In the reaction ABTS is oxidized to the stable cation radical, ABTS·+, which accumulates. The oxidation of ABTS leads to green coloration, which is indicative of laccase enzyme activity (**Figure 27**). The absorbance of the reaction mixture prepared for the analysis of laccase enzyme activity was taken at 420 nm. The substrate-containing buffer, without enzyme, was used as a standard. One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1 mole of ABTS per minute.

W6 W5 W7 W8 W10 W11



S5 S6 S4 S8 S7 S9

Figure 27: Analysis of laccase enzyme activity of decaying wood and soil sample isolates

Thus, from the analysis it was observed that the deep green colour formation by W5, W7, W11, S4, S8 and S9 isolates indicated high laccase enzyme activity while for the rest of the isolates low laccase activity was observed.

The laccase enzyme activity of bacterial isolates from decaying wood and soil sample is calculated and is summarized in **Table 18**. Laccase enzyme activity ranged between 0.031 µmol/ml/min to 0.133 µmol/ml/min.

Table 18: Laccase enzyme activity of bacterial isolates from wood and soil sample

Isolate number	Laccase activity (µmol/ml/min)	Laccase specific activity (U/mg protein)
W5	0.102	0.389 ± 0.006
W6	0.051	0.331 ± 0.003
W7	0.079	0.407 ± 0.008
W8	0.054	0.293 ± 0.007
W10	0.031	0.224 ± 0.001
W11	0.071	0.425 ± 0.004
S4	0.097	0.311 ± 0.002
S5	0.049	0.361 ± 0.005
S6	0.041	0.297 ± 0.001
S7	0.033	0.311 ± 0.003
S8	0.053	0.473 ± 0.002
S9	0.133	0.591 ± 0.004

The histograms of the isolates from the decaying wood and soil samples were drawn for the laccase enzyme activity as shown in the **Figure 28** and **Figure 29** respectively.

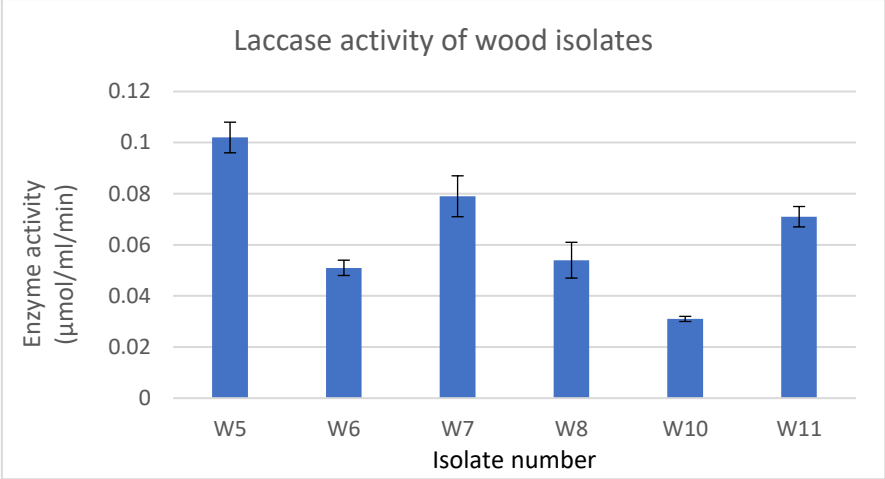


Figure 28: Laccase enzyme activity in bacterial isolates from decaying wood sample

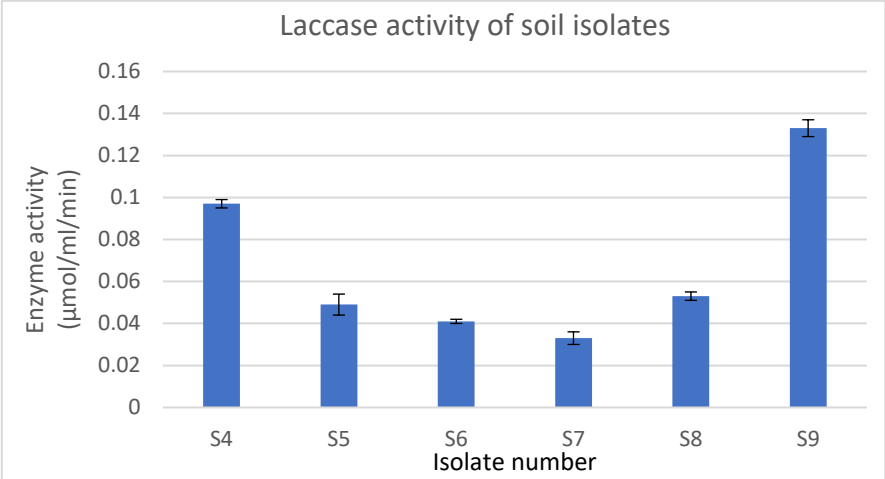
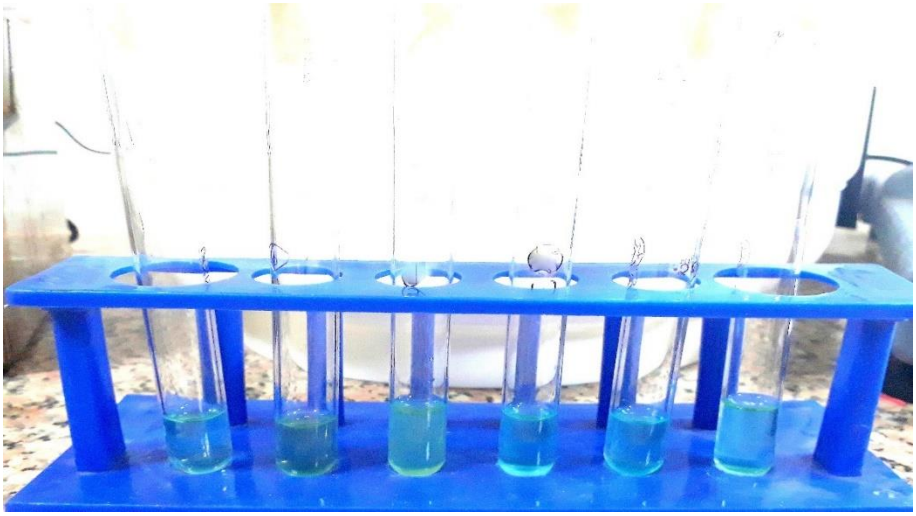


Figure 29: Laccase enzyme activity in bacterial isolates from soil sample

Laccase enzyme activity was recorded to be the highest in isolate S9 (0.133 µmol/ml/min) followed by W5 (0.102 µmol/ml/min). Thus, S9 and W5 possessed a very high lignin degrading potential. Isolates W7 and S4 also showed good enzyme activity amounting to 0.079 µmol/ml/min and 0.097 µmol/ml/min respectively. Isolates W10 and S7 showed low laccase activity.

4.5.2 Lignin peroxidase enzyme activity

Lignin peroxidase enzyme activities in the crude enzyme extracts of all bacterial isolates were determined spectrophotometrically using Azure B as noncatalytic cooxidant. In the reaction Azure B is oxidized in presence of H_2O_2 resulting in formation of water molecule. The oxidation of Azure B leads to discoloration of blue color solution, which is indicative of lignin peroxidase enzyme activity (**Figure 30**). The absorbance of the reaction mixture prepared for the analysis of lignin peroxidase enzyme activity was taken at 651 nm. The substrate-containing buffer, without enzyme, was used as a standard. One unit of lignin peroxidase activity was defined as activity of an enzyme that catalyzes the conversion of 1 µmole of Azure B per minute.



W5 W10 W11 W8 W7 W6

S9 S4 S6 S8 S5 S7

Figure 30: Analysis of LiP enzyme activity of wood and soil sample isolates

Thus, from the analysis it was observed that the discoloration of the reaction mixture by W5, W6, W7, W8, S4 and S9 isolates indicated high lignin peroxidase enzyme activity while for the rest of the isolates low activity was observed.

The lignin peroxidase enzyme activity of bacterial isolates from decaying wood and soil sample is calculated and is summarized in **Table 19**. Lignin peroxidase enzyme activity ranged between 0.016 µmol/ml/min to 0.102 µmol/ml/min.

Table 19: Lignin peroxidase enzyme activity of bacterial isolates from wood and soil sample

Isolate number	LiP activity (µmol/ml/min)	LiP specific activity (U/mg protein)
W5	0.084	0.321 ± 0.006
W6	0.058	0.376 ± 0.002
W7	0.073	0.376 ± 0.005
W8	0.053	0.288 ± 0.004
W10	0.032	0.231 ± 0.002
W11	0.016	0.095 ± 0.003
S4	0.084	0.271 ± 0.005
S5	0.048	0.352 ± 0.001
S6	0.019	0.137 ± 0.001
S7	0.030	0.282 ± 0.003
S8	0.038	0.339 ± 0.002
S9	0.102	0.453 ± 0.004

The histogram of the isolates from the wood and soil sample was drawn for the lignin peroxidase enzyme activity as shown in the **Figure 31** and **Figure 32** respectively.

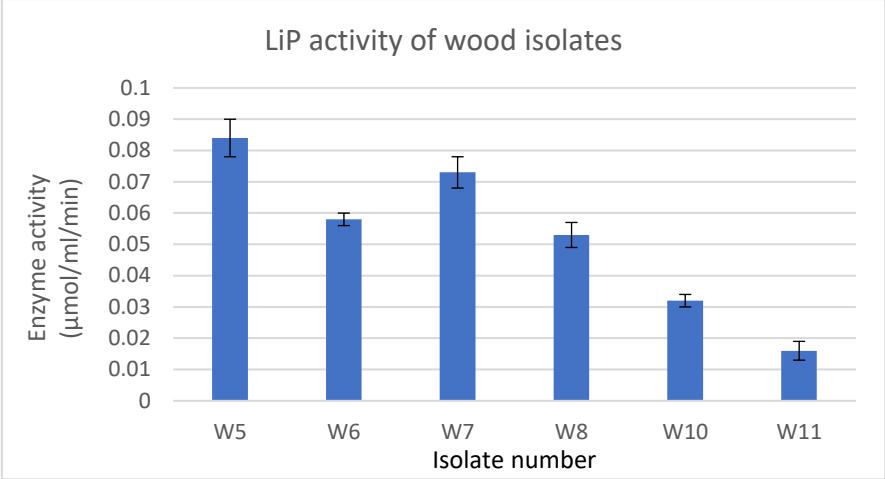


Figure 31: Lignin peroxidase enzyme activity in bacterial isolates from decaying wood sample

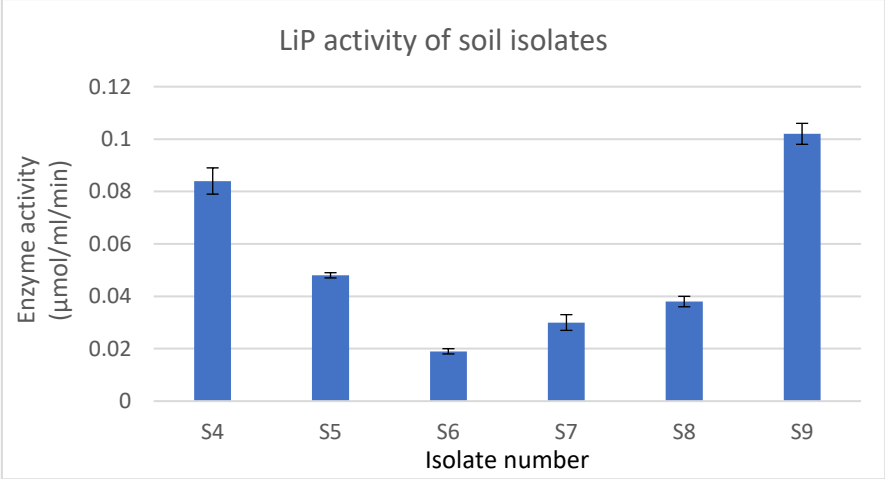
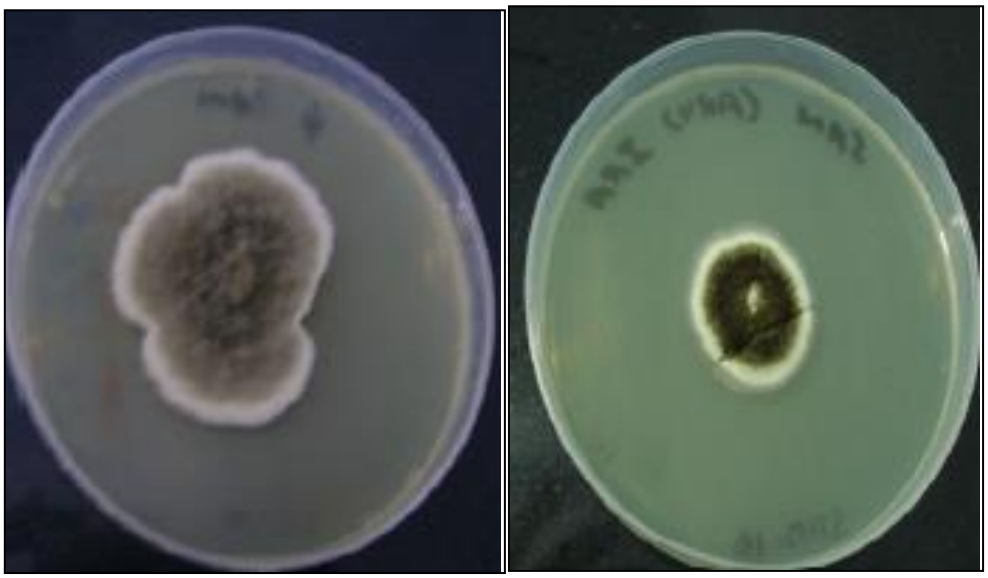


Figure 32: Lignin peroxidase enzyme activity in bacterial isolates from soil sample

Lignin peroxidase enzyme activity was recorded to be the highest in isolate S9 (0.102 µmol/ml/min) followed by W5 (0.084 µmol/ml/min). Thus, S9 and W5 possesed a very high lignin degrading potential. Isolates W7 and S4 also showed good enzyme activity amounting to 0.073 µmol/ml/min and 0.084 µmol/ml/min respectively. Isolates W11 and S6 showed low lignin peroxidase activity.

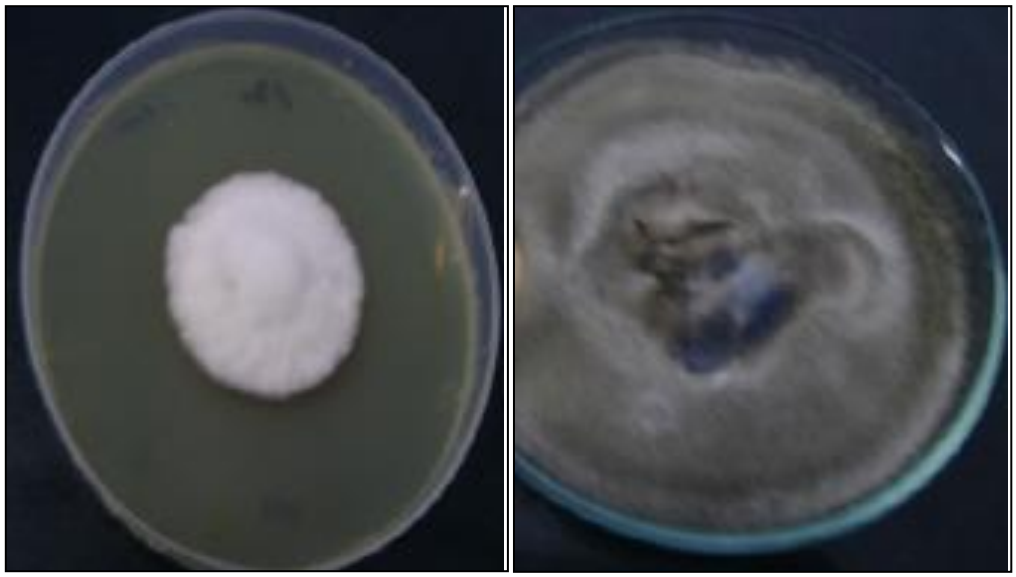
4.6 Isolation and characterization of fungi with lignin degrading potential

A total of four putative ligninolytic fungal isolates, two from each decaying wood sample and soil sample were isolated as pure cultures. These were named as W1, W2 and S1, S2 from decaying wood and soil samples, respectively (**Figures 33 and 34**).



Isolate W1 Isolate W2

Figure 33: Fungal isolates tested positive for laccase plate assays from decaying wood

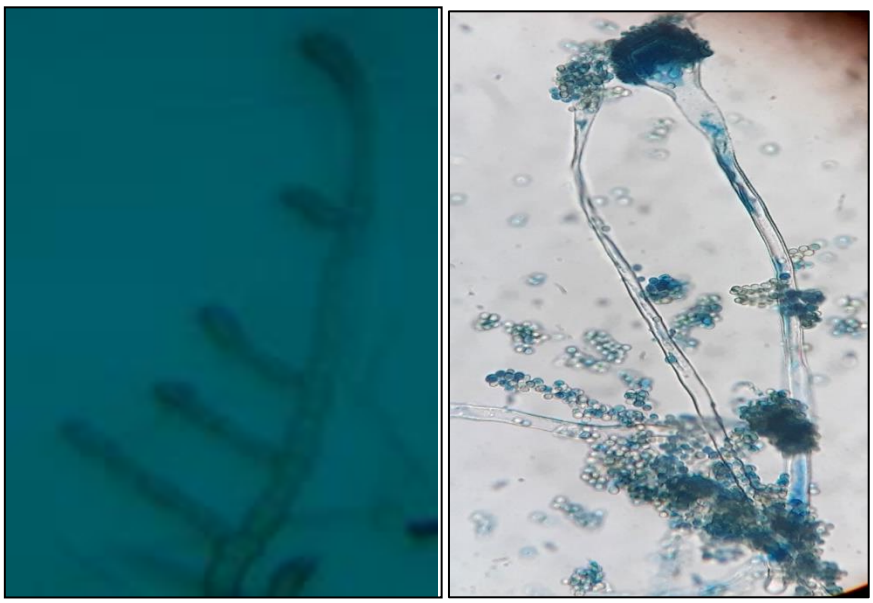


Isolate S1 Isolate S2

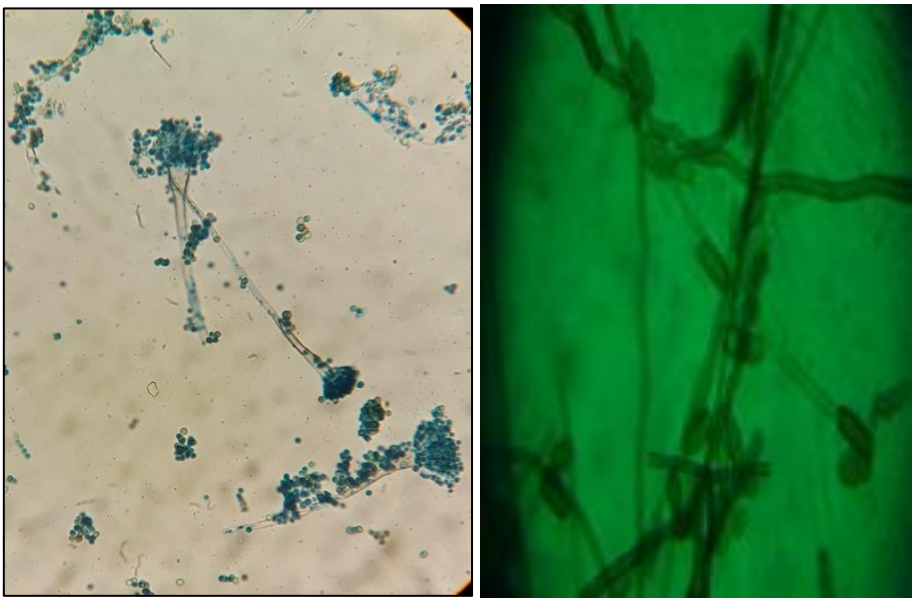
Figure 34: Fungal isolates from soil tested positive for laccase plate assays

These four positive isolates were subcultured to maintain their vitality for the ongoing research. The isolates from the decaying wood and the soil were then subjected to morphological and biochemical characterization to understand their morphology and nature so as to determine the type of species isolated from the respective samples. Morphological characterization was done by plating on Sabouraud's agar medium and lactophenol cotton blue staining procedure. The conidial and hyphae structures were

observed by lactophenol cotton blue staining for the isolates obtained from the decaying wood and soil sample are shown in **Figure 35.**



Isolate W1 Isolate W2



Isolate S1 Isolate S2

Figure 35: Morphological characterization of four fungal isolates from decaying wood and soil sample on the basis of lactophenol cotton blue staining

All these fungal isolates were analyzed to exhibit starch and cellulose hydrolysis activities. The results are presented in **Tables 20 and 21**, respectively.

Table 20: Starch hydrolysis test for fungal isolates from decaying wood and soil samples

COLONY	STARCH HYDROLYSIS POSITIVE/ NEGATIVE
W1	Positive
W2	Positive
S1	Positive
S2	Positive

Table 21: Cellulose hydrolysis test for fungal isolates from decaying wood and soil samples

COLONY	CELLULOSE HYDROLYSIS POSITIVE/ NEGATIVE
W1	Positive
W2	Positive
S1	Positive
S2	Positive

Discussion

This world is full of diverse microrganisms which have the ability to degrade the lignocellulosic rich biomass. Henceforth an endeavor was made to screen and portray ligninolytic organisms isolated from decaying wood and soil. The first attempt was made to isolate bacterial and fungal colonies from the soil and decaying wood sample. Initially, serial dilution was performed on both the samples. Bacterial and fungal species with lignin degrading potential were screened by two Laccase and lignin peroxidase plate assay procedures.

A total of twelve bacterial colonies were isolated, six each from the decaying wood sample and soil sample. These colonies were subjected to morphological and biochemical characterization to know their basic identity. Further these colonies were then subjected to protein content determination followed by analysis of their enzymatic activity. These colonies were assayed for two different enzymatic activities namely laccase and lignin peroxidase, implicated in lignin degradation. On the basis of the laccase enzyme activity only four colonies, two from decaying wood sample (isolates W5 and W7) and two from soil sample (isolates S4 and S9) were selected to be the potential lignin degrading bacteria with high lignin degradation activities. These bacterial species were tentatively characterized as *Serratia* spp. and *Streptococcus* spp. from wood sample and *Bacillus subtilis* and *Pseudomonas* spp. from soil sample.

Similarly, fungal isolates with lignin degradation potential were isolated and characterized. A total of four isolates were screened on the basis of laccase plate assay. Colony morphology and hyphae and conidia structure analyses led to tentative identification of these fungal isolates as *Alternaria* spp. (W1), *Aspergillus* spp. (W2), and *Aspergillus* spp. (S1) and *Helminthosporium* spp. (S2).

Further investigation and research can reveal a lot about the potential of microbes capable of degrading lignin. These microbes have potential use in paper industry and in the field of bioenergy production, where removal of lignin from lignocellulose biomass is a major concern. The enzymes like laccase and peroxidases from these microbes if quantified properly can prove very effective in variety of applications such as for bioconversion, for food processing and in field of biosensors.

CONCLUSION

This project contains the information on the lignin degrading ability of fungi and bacteria. In the present study, lignin-degrading microbes (bacteria and fungi) from soil and decaying wood samples were isolated and distinguished these strains on the basis of their laccase and lignin peroxidase activities. These enzymes produced by lignin degrading microbes have specialized applications in various industries like pulp and paper industries, textile, dye industries. Still a lot of research is required to make these enzymes from microbes to be used at industrial level. The need of the hour is to search and isolate more specific and broad-spectrum microbes (bacteria and fungi) capable of degrading lignin. With such environmental friendly and sustainable microbial approach, a new era of safe and better environment can be established.

REFERENCES

1. Kuhad R C, Singh A (1997), Microorganisms and enzymes involved in the degradation of plant fiber cell walls. *Adv Biochem Eng Biotechnol*., Vol 57: 45-125

2. Argyropoulos D S, Menachem S B (1997), Biotechnology in the paper and pulp industry. *Adv Biochem Eng Biotechnol*., Vol 57: 127–158

3. Brunow G, Hofrichter M (2001), Methods to reveal the structure of lignin. *Wiley-VCH*., Vol 1: 89–116.

4. Kumar R, Singh S, Singh O V (2008), Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *Journal of industrial microbiology & biotechnology.*, Vol 35: 377-391.

5. Rubin E M (2008), Genomics of cellulosic biofuels. *Nature*., Vol 454: 841-845.

6. Pandey M P, Kim C S (2011), Lignin Depolymerization and Conversion: A Review of Thermochemical Methods. *Chem Engin Technol.,* Vol 34:29-41.

7. Ward P O, Singh A (2002), Bioethanol technology: developments and perspectives. *Advan. Appl. Microbiol*., Vol 51: 53-80.

8. Perez J, Munoz-Dorado, J de la Rubia T, Martinez J (2002), Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *Int. Microbiol.,* Vol 5: 53-63.

9. Sun Y, Cheng J (2002), Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource technology*, Vol 83: 1-11.

10. C srinivasan, T M d'souza, K boominathan and C A reddy (1995), Demonstration of laccase in the white rot basidiomycete *Phanerochaete chrysosporium*. *Applied and environmental microbiology*, Vol. 61: 4274–4277.

11. Crawford D L, Muralidhara R (2004), Bacterial extracellular lignin peroxidase. *United states Patent* 5200338.

12. Kalyani D C, Phugare S, Shedbalkar U, Jadhav J P (2008), Purification & Characterization of a bacterial peroxidase from the isolated strains, Pseudomonas sp. SUK1 & its application for textile dye decolourization. *Bioresource Technology* Vol 81: 4635-4641.

13. Timothy D H, Ahmad M, Elizabeth M H and Singh R (2011), The emerging role for bacteria in lignin degradation and bioproduct formation. *Environmental biotechnology*, Vol 22: 394–40.

14. Schmidt O (2006), Wood and Tree Fungi: Biology, Damage, Protection, and Use. *Springer*. Vol 12: 334

15. Jørgensen H (2003), Production and characterization of cellulases and hemicellulases produced by Penicillium strains. *Anal Biochem*, Vol 317: 85-93.

16. Prathumpai W (2003), Enzyme production in Aspergilli on different carbon sources. *Appl Microbiol Biotechnol*, Vol 63: 748-753

17. Foyle T, Jennings L, Mulcahy P (2007), Compositional analysis of lignocellulosic materials. *Bioresour Technol* , Vol 98: 3026–3036.

18. Ruiz-Dueñas F J, Martínez Á T (2009), Microbial degradation of lignin: How a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. *Microbial Biotechnology*, Vol 2: 164–177.

19. Faix O (1991), Classification of lignins from different botanical origins by FT-IR spectroscopy. *Holzforschung*, Vol 45: 21–28.

20. Ralph J, Akiyama T, Kim H *et al.* (2006), Effects of coumarate 3-hydroxylase down-regulation on lignin structure. *J Biol Chem.* Vol 281: 8843–8853.

21. Hofrichter M (2002), Review: lignin conversion by manganese peroxidase (MnP). *Enzyme and Microbial Technology,* Vol. 30: 454-466.

22. Ralph K (2005), Actinomycetes and lignin degradation. *Advances in Applied Microbiology*, Vol 58: 125–168.

23. Galhaup C Goller, Peterbauer C K, Strauss J, and Haltrich D (2002a), Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. *Microbiology*, Vol 148: 2159-2169

24. Galli C, Gentili P (2004), Chemical messengers: mediated oxidations with the enzyme laccase. *Journal of Physical Organic Chemistry*, Vol 17: 973–977.

25. Barreca A M, Sjögren B, Fabbrini M, Galli C, Gentili P (2004), Catalytic efficiency of some mediators in laccase-catalyzed alcohol oxidation. *Biocatalysis and Biotransformation*, Vol 22: 105–112

26. Santhanam N, Vivanco J M, Decker S R, Reardon K F, (2011), Expression of industrially relevant laccases: prokaryotic style. *Trends Biotechnol.* Vol 29: 480–489.

27. Shraddha, Sheker R, Sehgal S, Kamthania M, Kumar A (2011), Laccases: microbial sources, production, purification, and potential biotechnological applications. *Enzyme Res.,* Article ID 217861.

28. Blanchette R A (1995), Degradation of lignocellulose complex in wood. *Can. J. Bot.,* Vol 73: 999-1010.

29. Hatakka A (2001), Biodegradation of lignin. *Wiley VCH*, Vol 1: 129-180.

30. Hatakka A (1994), Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiol,* Vol 13: 125-135.