Isolation of Lignin-converting Microbes Contributing Towards Recalcitrant Carbon Degradation in Boreal Forest System

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ABSTRACT

Plant biomass, composed of cellulose, hemicellulose and lignin, are being explored as renewable carbon feedstock that could replace a significant amount of petroleum-derived chemicals and other products. Lignin, a component of wood, is the second most abundant natural organic polymer after cellulose. It provides strength and rigidity to plants and is highly recalcitrant to degradation due to its complex, three-dimensional structure. The valorization of lignin is essential for viable and sustainable uses of lignocellulosic biomass for the production of renewable fuels and chemicals. In order to achieve this, microbial degradation is extensively researched as microorganisms have evolved different enzymatic and/or non-enzymatic strategies to utilize biomass (Janusz et al., 2017). In boreal forest ecosystem, brown rot fungi are dominant players for decomposing and recycling carbon sources sequestered in tree biomass, but the carbohydrate-selective nutritional mode of these fungi does not allow them to consume all the forms of carbon in wood, with the undecayed lignin residues creating a recalcitrant carbon pool in the forest ecosystem. In this research, we investigated up to 200 functional microbes, including fungi and bacteria, involved in the breakdown of lignin components in this distinctive brown rot niche. Preliminary screening of the microbes was performed using indicator dyes, resulting in eight fungi and fourteen bacteria which were then screened to be further characterized. These isolates were identified using DNA extraction, PCR and sequencing and were further evaluated for their ability to degrade and metabolize lignin and aromatic lignin monomers. We anticipate understanding the lignin-degrading characteristics of microbes occurring naturally and its role in industrial lignin conversion or bioremediation of related recalcitrant aromatic hydrocarbons.

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INTRODUCTION

There are three main components of plant biomass – cellulose, hemicellulose, and lignin. Lignin, the second most abundant biopolymer after cellulose, is a highly complex aromatic heteropolymer. It is estimated to constitute 30-35% of the Earth's non-fossil organic carbon (Aarti et al., 2015). It is located in the middle lamella of plant cell walls and encompasses the cellulose and hemicellulose in plant cell walls and protects the plant against microbial attack while also providing structural rigidity and mechanical strength (Kumar et al., 2021). Lignin is formed from the radical polymerization of three different phenolic monomers – *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Brown and Chang, 2014). After polymerization, these get incorporated into lignin polymers as *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, and its composition varies among different species and within tissues of the same plant (Kumar et al., 2021). These alcohol precursors form a complex three-dimensional structure that consists of several linkages within the lignin structure with the β-O-4 bond being the most common bond among other ether and carbon-carbon linkages.

There are several sources of lignin such as native lignins that are found naturally in plant biomass and industrial lignins which are by-products from several industrial pretreatment and separation processes. Depending on the pretreatment methods (such as chemical, biological, or thermochemical) different types of lignin are generated with varying properties. Kraft lignin, lignosulfonates, soda and organosolv lignin are different types of lignin from the pulping industries. They have several uses in binder, adhesives, resins, etc. and are also used as an energy source in pulping processes. Removal of lignin is essential in industries such as pulp and paper and biofuels production. In the pulp and paper industry, the wood chips are treated with either chemical, biological, or thermochemical methods to alter the lignin in order to access the cellulose and hemicellulose required to make paper. Lignocellulosic biomass is also used in the production of biofuels such as ethanol which involves delignification to remove the lignin that is encasing the cellulose and hemicellulose, depolymerization to carbohydrate to produce free sugars and, fermentation of the sugars to ethanol (Abdel-Hamid et al., 2013). Moreover, removal of lignin is not only an essential step in the industries but also important for carbon cycling. Lignin metabolism is crucial to the carbon cycle on earth as lignin accounts for about 50% of the dry weight of cellular carbon stored in the biosphere (Zeikus, 1981).

The growing need for fuel and food, oil scarcity and environmental concerns have raised the interest in utilizing lignin as a feedstock for value-added products. Lignin is one of the main residues from the pulp and paper industry as well as ethanol production and is used to provide energy to these processes via combustion. Its removal is essential for the bioavailability of cellulose for efficient production of products from pulp and paper and biorefineries. The structural complexity and heterogeneity make lignin polymer highly recalcitrant to degradation. Several chemical methods such as chemical oxidation, hydrogenolysis, gasification and hydrolysis or thermochemical processes such as pyrolysis are used to breakdown lignin. However, the use of biological methods involving microbial enzymes are found to be less energy intensive, cost effective and environmentally friendly. Valorization of lignin has been researched intensively over the years which has resulted in several strategies for lignin depolymerization. While lignin is highly recalcitrant to microbial attack, several microorganisms such as fungi and bacteria have evolved to use mechanisms to depolymerize lignin using extracellular oxidative enzymes, as well as to uptake the aromatic species generated during depolymerization. These enzymes provide a more specific and effective alternative to breaking down lignin for value-added products and other processes. White-rot fungi are the most extensively studied lignin-degrading microbes. These produce ligninolytic enzymes such as lignin peroxidases, manganese peroxidases and laccases. The other two types of wood decaying fungi are brown-rot and soft-rot fungi which are also known to produce lignin degrading enzymes. Depolymerization by fungi results in low-molecular-weight aromatic compounds such as guaiacol and coniferyl alcohol among others (Masai et al., 2007). Ligninolytic bacteria are less studied compared to fungi, but several bacteria have demonstrated the ability to produce lignin-degrading enzymes and utilize lignin. Lignin-degrading bacteria are found in three main groups – actinomycetes, α-proteobacteria and γ-proteobacteria, that produce enzymes such as dye-decolorizing peroxidases and laccases (Janusz et al., 2017). Bacteria are also known to mineralize lignin-derived low molecular weight aromatic compounds (Bandounas et al., 2011).

This study aims to identify lignin-degrading microorganisms that are isolated from a forest ecosystem. It also evaluates their ability to produce lignin-degrading enzymes, depolymerize or utilize lignin for growth and also uptake degradation products. Specifically, 8 fungi and 14 bacteria screened from up to two hundred isolates were characterized for their lignin-converting capacities. The findings in this study will provide novel lignin-degrading fungi and bacteria and their characteristics that can be utilized for effective valorization of lignin, industrial use of lignin and bioremediation of related aromatic pollutants.

OBJECTIVES AND HYPOTHESIS

This study focuses on identifying and characterizing lignin-converting microbes, including fungi and bacteria, that are isolated from the boreal forest ecosystem in Northern Minnesota. These microbes are found in the distinctive brown rot fungi niche that are found in boreal forests and possess the ability to convert lignin residues left by the brown rot fungi. The objective is to identify the microbes, evaluate their role in the carbon cycle and assess their ability to depolymerize lignin, secrete ligninolytic enzymes, and uptake aromatic compounds.

The novelty of the study is the microbes that are isolated from this brown rot niche that have not been evaluated for their ability to convert lignin or utilize aromatic monomers. Replicating the environmental conditions from which these microbes were isolated, gives an insight to their ability to convert lignin and participate in lignin degradation. It will also help us to study the interaction and characteristics of the microbial community found in forests.

Lignin is a complex aromatic polymer and has the potential to be used as a renewable resource for several industries. In this study, it is hypothesized that microbes from the same niche can convert and recycle recalcitrant lignin residues left by the activity of the primary brown rot fungal colonizers on wood biomass. The main question that this study aims to answer is how the recalcitrant lignin residues, that are generated by brown rot fungi, processed, and utilized by the microbes present in this niche.

CHAPTER 1: LITERATURE REVIEW

1.1 Lignin – Occurrence, Function, Structure and Properties

Plant biomass consists of three major components – cellulose, hemicellulose, and lignin. Lignin is the most abundant naturally occurring polymer after cellulose. The highest amount of lignin occurs in conifers (35-40%). Its content is higher in softwoods (27-33%) than in hardwoods (18-25%) and grasses (17-24%), but is not found in algae, lichens, or mosses (Saake and Lehnen, 2007).

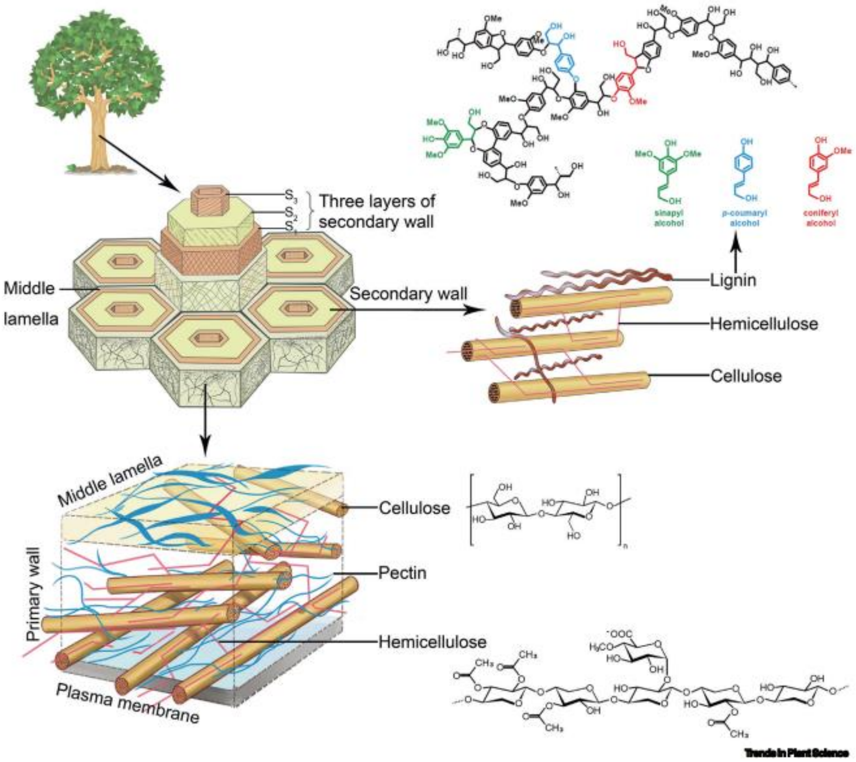


Figure 1: Illustration of plant cell wall structure and its composition (Zhao et al., 2019)

In a plant cell, lignin is found in the secondary cell wall, middle lamella (between adjacent cell walls) and at the cell corners as depicted in the figure above. Lignin provides rigidity to the cells walls in woody parts and performs important functions in xylem tissue of plants. Lignin along with hemicellulose, creates a binder between the cells that provides great strength and elasticity. The lipophilic character of lignin decreases the permeation of water across cell walls which enables the transport of nutrients and metabolites in the xylem tissue (Saake and Lehnen, 2007). The presence of lignin is also essential in resisting attacks by microorganisms that penetrate the cell walls with destructive enzymes.

Lignin is a complex, aromatic polymer that is made up of phenylpropanoid aryl-C_3 units that form a three-dimensional structure linked via ether and carbon-carbon linkages (Bugg et al., 2011) with β-O-4 ether bonds as the main linkage (de Souza et al., 2020). These linkages are not susceptible to hydrolytic attack making lignin highly resistant to degradation. It is formed by radical polymerization of the precursors of coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol, which are guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) units, respectively. The structure of these phenylpropanoid units is shown in the figure below.

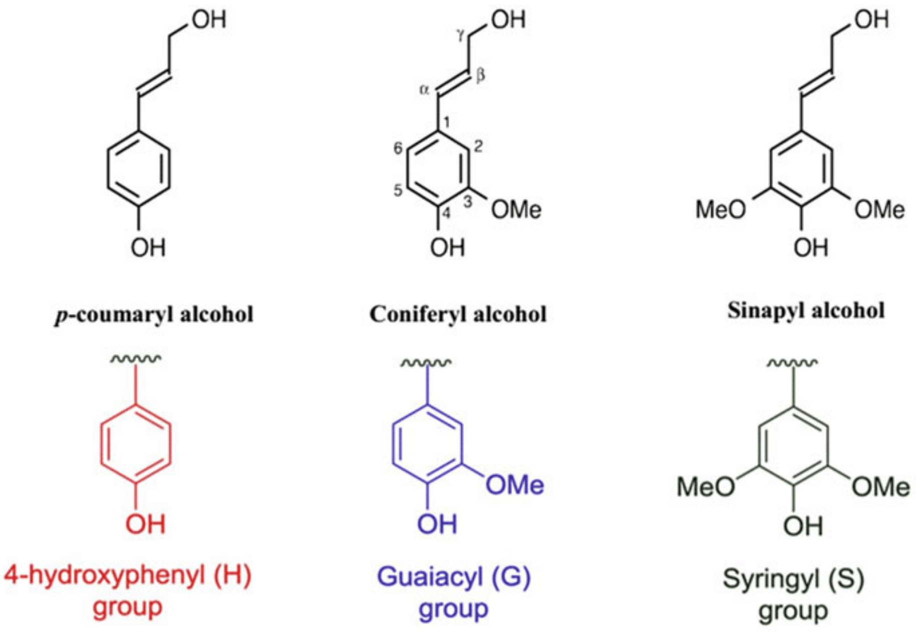


Figure 2: Lignin monomers and its polymerization units that form lignin (Kumar et al, 2021)

The ratio of G:S:H units varies among different species. Hardwood lignin consists of G and S units and low levels of H units, conifer lignin has higher level of G units and low levels of H units, and grasses consist of G, S and H units (de Souza et al., 2020).

Given the complex structure of lignin, the molar mass of isolated lignin depends on the isolation conditions whereas molar mass of lignin *in situ* is unknown. For example, spruce milled wood lignin has an average molar mass of 11,000 g/mol which was estimated using ultracentrifugation (Saake and Lehnen, 2007). Lignin, in native state, is insoluble in all neutral solvents at room temperature. Its solubility in organic solvents in not clear and depends on variables such as chemical structure, molecular weight, and the presence of hydrophilic moieties in the lignin molecule (Shukry et al., 2008).

Lignin is an amorphous thermoplastic polymer, and its glass transition temperature is affected by moisture content and hydrogen bonding. The most reactive functional groups in lignin are the phenolic, ketone, aldehyde, benzyl alcohol and noncyclic benzyl ether group, and are prone to condensation reactions under acidic and alkaline conditions (Saake and Lehnen, 2007).

1.2 Current Uses of Lignin and Challenges

Lignin residue is a by-product of several pretreatment or separation process in industries such as pulp and paper or biofuel, in which lignocellulosic biomasses are used as raw materials. In the production of biofuels such as ethanol, lignocellulosic biomass is pretreated to remove lignin in order to access cellulose and hemicellulose used in these processes and break it down to simple sugars and the subsequent conversion of these sugars to usable fuels. Approximately 50 million tons of lignin are produced worldwide annually, of which 98-99% is incinerated to produce energy and steam in the pulp mills and biorefineries (de Souza et al., 2020).

The main types of lignin produced by the pulping processes are Kraft lignin, lignosulphonates, soda lignin and organosolv lignin. The Kraft process in the pulp and paper industry uses sodium hydroxide (NaOH) and sodium sulfide (Na_2S) for delignification of lignocellulosic feedstock and eventually leads to the production of Kraft lignin. This lignin consists of sulfur impurities along with small amounts of ash and carbohydrates. More than 500,000 tons of Kraft lignin is produced per year and is mainly used in the form of combustion for heat recovery, although, there have been emerging studies on high-value utilization of Kraft lignin (Chen, 2015). Lignosulfonates comes mainly from the sulfite pulping process in the paper industry in which a mix of alkaline earth metal sulfite performs the delignification process. Lignosulfonates are water soluble and have unique colloidal properties which makes it useful as binders, adhesives, additives for cements and can be used in several other industries (de Souza et al., 2020). Organosolv lignin is lignin extracted by using organic solvents for delignification process which causes low environmental pollution compared to other pulping methods and high purity of the derived lignin is observed. However, it is not a widely used form of lignin (Chen, 2015). Soda lignin, on the other hand, is produced from a soda-based process similar to Kraft process but is a sulfur-free. It is used for the production of phenolic resins, dispersants and for polymer synthesis. However, in the pulping industry, this process yields low pulp and is therefore rarely used (de Souza et al., 2020).

In addition to its utilization in several industries, lignin removal plays an important role in carbon cycling in land ecosystems, given its abundance. The breakdown process of lignin involves oxidation and removal of the side chains followed by the oxidation of the methoxyl (CH_3O) group to carbon dioxide. The depolymerization step removes the phenolic units of the side chains using non-specific enzymes from lignin-degrading microbes. These units are now available to the soil microbial community and transported in their cells for metabolism. Any hydroxyl groups present on phenolic rings are cleaved. Most of the carbon is either evolved as carbon dioxide which is released back into the atmosphere or incorporated into soil organic matter (SOM) (Fuhrmann and Zuberer, 2021). The carbon cycle is essential to life on Earth. A general pathway describing the importance of lignin removal in carbon cycle is shown in the figure below.

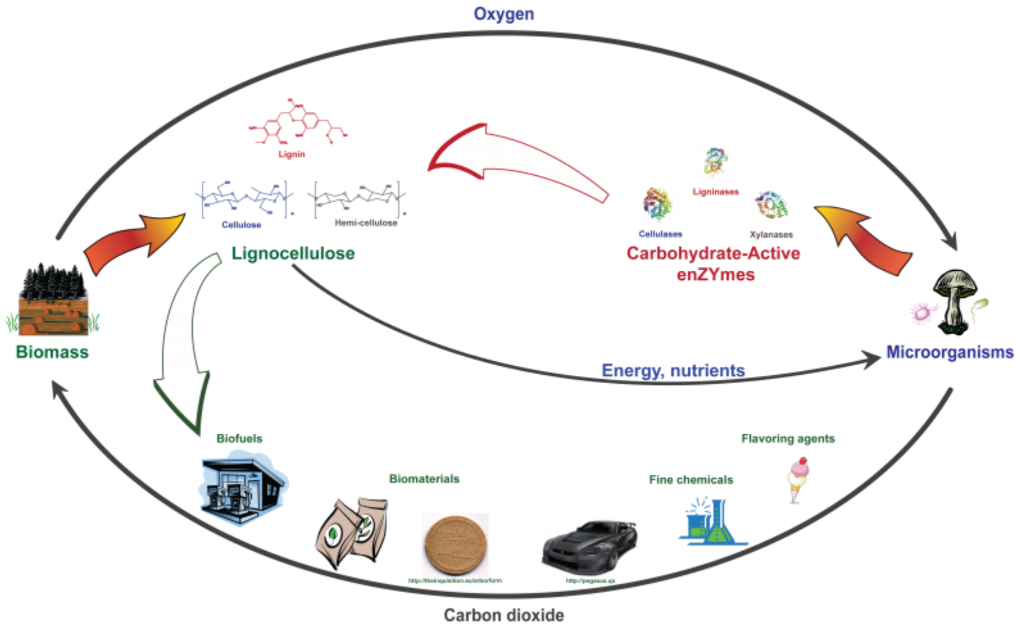


Figure 3: General pathway for carbon cycle (Eltis, L. *Bacterial Lignin Degradation)*

Given the importance of lignin in the nature and its industrial application, efficient valorization of lignin using cost-effective methods for selective lignin degradation are in high demand. Biological treatment methods for lignin valorization using microorganisms, such as fungi and bacteria, have shown promising results. Fungi and bacteria are capable of producing ligninolytic enzymes and can provide a specific yet effective alternative for breakdown of lignin. In addition, biocatalytic and biodegradative processes take place under milder conditions in comparison to various chemical and thermochemical methods, which in turn lowers the energy input and environment impact of effective pretreatment and utilization of lignin and its degradation products.

1.3 Biodegradation of Lignin using Fungi and Bacteria

Lignin is highly recalcitrant to both chemical and biological degradation due to the ether and C-C linkages present in its structure which are not susceptible to hydrolytic attack (Bugg et al., 2011). Removal of lignin is an essential step in utilization of plant carbohydrates and cellulosic biomass as renewable feedstocks in industries such as bioethanol production, paper and other cellulose-based products and chemicals. Microbes, such as fungi and bacteria, have evolved to break down lignin using metalloenzyme-dependent radical pathways (Brown and Chang, 2014).

Enzymes Involved in Microbial Degradation of Lignin

Enzymes are essential in the microbial degradation of recalcitrant lignin. The enzymes involved in fungal lignin degradation are extracellular heme peroxidases: lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). These enzymes catalyze several oxidative reactions using hydrogen peroxide as the electron acceptor. LiPs are monomeric hemoproteins that catalyze the H_2O_2-dependent oxidative depolymerization of lignin. MnP, on the other hand is a heme-containing glycoprotein that oxidizes Mn^{2+} to Mn^{3+}, which in turn can oxidize a large number of phenolic substrates. VP enzymes can oxidize Mn^{2+} and high-redox potential non-phenolic compounds like MnP and LiP, respectively (Abdel-Hamid et al., 2013). Dye decolorizing peroxidase or DyP have been identified in both fungi and bacteria and can oxidize high-redox potential dyes as well as some lignin compounds (Sugano, 2009). Laccase is a copper-containing oxidase which utilizes molecular oxygen as oxidant and also oxidizes phenolic rings to phenoxy radicals. It can also oxidize nonphenolic compounds with redox mediators such as ABTS (Hatakka, 1994). Laccases, glutathione S-transferases, ring cleaving dioxygenases are reported to be involved in bacterial lignin breakdown (Bandounas et al., 2011). Bacterial peroxidases are less oxidatively powerful compared to their fungal counterparts; however, they contain oxidative enzymes that could either modify lignin or breakdown by hydroxylation and methylation (Brown and Chang, 2014). Aryl-alcohol oxidase and glyoxal oxidase and other accessory enzymes are sources of extracellular H_2O_2 to aid LiPs and MnPs in lignin breakdown. The role of these enzymes is illustrated in the figure below.

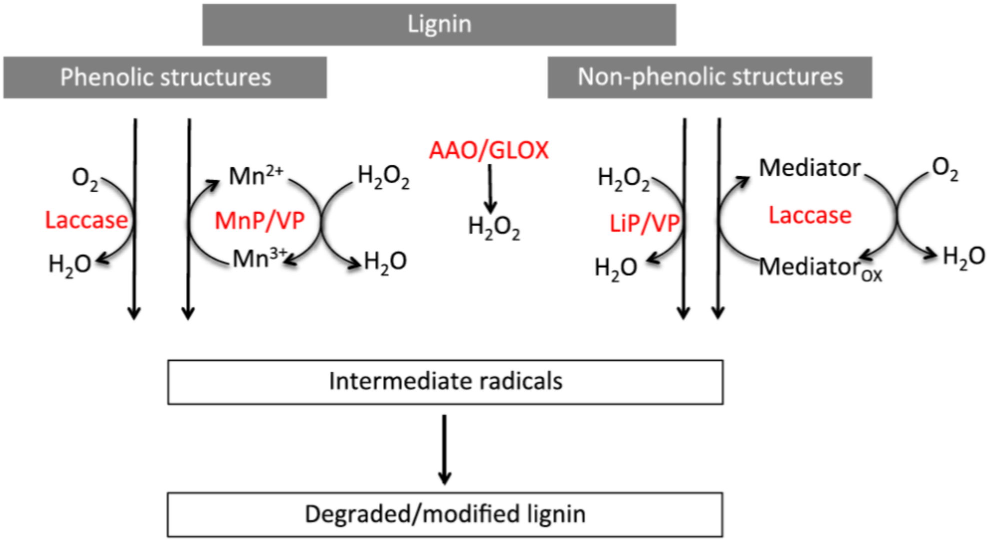


Figure 4: Reaction catalyzed by lignin-degrading enzymes. LiP: lignin peroxidase, MnP: manganese peroxidase AAO: aryl alcohol oxidase, GLOX: glyoxal oxidase (Abdel-Hamid et al, 2013).

Lignin-degradation by fungi

The ligninolytic capacity of filamentous fungi have been widely studied by researchers over several decades. There are three types of wood-decaying fungi that have been studied for lignin degradation – white-rot, brown-rot, and soft-rot fungi. Degradation by fungi is dependent on several environmental factors such as, temperature, pH, and C:N ratio, which are necessary for fungal growth, as well as the enzymes required and/or produced during the degradation process (Abdel-Hamid et al, 2013).

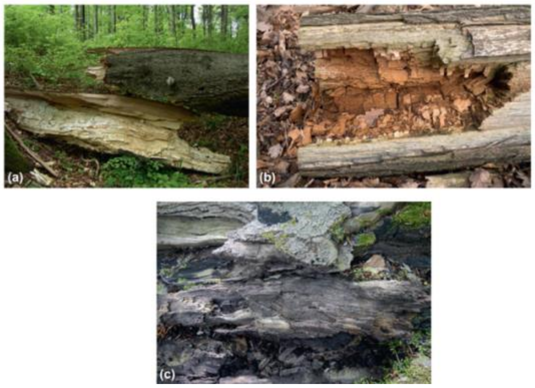


Figure 5: Wood decay observed by the attack of (a) white-rot (b) brown-rot and (c) soft-rot fungi

White-rot fungi are Basidiomycetes that produce extracellular lignin-modifying enzymes such as LiP, MnP and laccase. *Phanerochaete chrysosporium* is one of the best studied lignin-degrading fungi that can efficiently depolymerize, degrade, and mineralize cellulose, hemicellulose and even the recalcitrant lignin by producing ligninolytic enzymes. Glyoxal oxidase, in *P. chrysosporium*, uses products from lignocellulose degradation to generate hydrogen peroxide required by the ligninolytic peroxidases (Kersten and Cullen, 2007). Laccase from *Trametes versicolor* can catalyze C-C cleavage and aromatic ring cleavage in the presence of 1-hydroxybenzotriazole (Kawai et al., 2002). The residues from wood decay by white-rot fungi are whitish in color and fibrous in texture. White-rot fungi are capable of complete degradation of wood compounds by producing polysaccharide- and lignin-degrading enzymes.

Brown rot fungi degrade wood by producing cellulosic enzymes rather than producing lignin-degrading enzymes. They represent up to 10% of wood-decaying Basidiomycetes. Brown-rot fungi partially modify lignin while completely degrade polysaccharides using Fenton redox cycle. This was proposed in *Gloeophyllum trabeum* which uses two quinones: 2,5, -DMBQ and 4,5-DMBQ, which reduce Fe^{3+} to Fe^{2+} and forms oxalate complex which reacts with hydrogen peroxide to generate hydroxyl radicals, which attack lignin (Bugg et al., 2011). The decay by brown-rot fungi is brownish in color and crack into cubical pieces as shown in Figure 5(b).

Soft-rot fungi are Ascomycetes fungi, such as *Aspergillus niger* and *Penicillium chrysogenum*, that can tolerate a wide range of temperature, humidity, and pH conditions. Their hyphae secrete cellulases to break down cellulose in wood tissues (Langer et al., 2021). There are two types of attack by soft-rot fungi: some produce cavities within secondary cell walls of wood following microfibrillar orientation of cellulose that impacts different cell wall layers, while others erode the secondary cell wall completely without attacking the middle lamella. *A. niger* shows the cavity formation type decay whereas *P. chrysogenum* shows the erosion type decay (Hamed, 2013).

Lignin degradation by bacteria

Although lignin degradation has been extensively studied in fungi, especially in white-rot fungi, there are several studies on the lignin-degrading ability of bacteria. Bacteria involved in lignin-degradation can be found in diverse environments such as soil, digestive system of herbivores, wood-eating insects (termites), effluents from paper industry, sludge, etc. (Kumar et. Al, 2021). There are three classes of bacteria known to degrade lignin - actinomycetes (such as *Rhodococcus* sp., *Streptomyces* sp.), α-proteobacteria (such as *Sphingomonas* sp.*)* and γ-proteobacteria (such as *Pseudomonas* sp.). These bacteria produce enzymes such as laccases, DyPs, glutathione S-transferases, ring cleaving dioxygenases, monooxygenases, and phenol oxidases (Bandounas et al., 2011). The heme peroxidases, such as DyPs, from ligninolytic bacteria are less oxidatively powerful compared to their fungal counterparts. However, several oxidative enzymes found in ligninolytic bacteria can modify or break down lignin by hydroxylation or demethylation (Brown and Chang, 2014).

Streptomyces viridosporus T7A is a well-studied actinobacteria that is similar to fungi given its filamentous form and the ability to produce extracellular lignin-degrading enzymes. It forms an intermediate, acid-precipitable polymeric lignin (APPL) upon culturing it on corn-stover lignocellulose. *Amycolatopsis* sp. 75iv2 produced similar results on grass lignocellulose (Lee et al., 2019). *Rhodococcus* species such as *Rhodococcus jostii* RHA1 can utilize Kraft lignin and wheat straw lignocellulose as the sole carbon source to generate bio-products such as vanillin and other low molecular weight phenolic products. (Ahmad et al., 2010). *Amycolatopsis* sp., *Pseudomonas putida* and *Acinetobacter* ADP1 can depolymerize high molecular weight lignin species and can be used as hosts for metabolic engineering in consolidated bioprocessing of lignin which involves simultaneous depolymerization of lignin and uptake of the generated aromatic species for carbon and energy sources (Salvachua et al., 2015).

CHAPTER 2: ISOLATION AND IDENTIFICATION OF LIGNIN-DEGRADING MICROBES

2.1 Sample Collection and Isolation

Cedar Creek Ecosystem Science Reserve field, located in East Bethel in Minnesota, is a University of Minnesota biological field station that comprises of several ecosystems and species found in North American forests. Brown rot fungi are found in this forest and are the dominant players involved in the decomposing and recycling of carbon sources sequestered in tree biomass. From this field, five wooden logs that were extensively rotted by brown rot fungi were randomly selected and labeled as L1, L2, L3, L4 and L5 for sampling for microbial activity investigation. From each wooden log, three samples were collected from the interior part (I), adjacent to (A) and 2 meters away (C) from each rotted log, to represent high, moderate, and low-lignin environments, respectively. It is expected that the microbes isolated from interior part of the rotted wood, where the lignin content is high, are capable of utilizing lignin for growth or producing lignin-degrading enzymes. The experimental design is illustrated in the figure below.

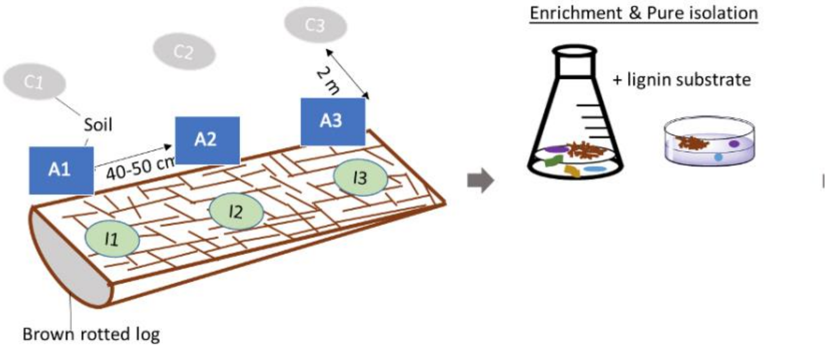


Figure 6: Experimental design to isolate the lignin-converting microbes

The samples were collected using 15 mL sterilized Corning tubes, transported at 4°C with ice packs and stored at -20°C before the isolation of microbes. The microbes were cultured within three weeks after sample collection. Lignin-converting microbes were isolated using 1 g of the collected rotted logs or soil and were dissolved in 9 mL of sterilized physiological salt (0.9% NaCl). The mixture was vortexed and settled for one hour at 4°C. The supernatant of the sample was used for microbial isolation. One loop of the supernatant was streaked onto culture plates of potato dextrose agar (PDA) or Luria broth (LB) with agar. PDA is used for the cultivation of fungi and can be supplemented with acid or antibiotics to inhibit bacterial growth, whereas LB is used for the growth of bacteria. The emerging fungal and bacterial colonies with different morphological phenotypes were selected after 3-4 days of incubation at 30°C and transferred to PDA and LB plates, respectively. Two consecutive transfers were then made to isolate pure cultures, which obtained about 100 fungal and bacterial isolates each. The fungal isolates were labeled as L(1-5)-I/A/C-F(1-n), while bacterial isolates were labeled as L(1-5)-I/A/C-B(1-n), where, L represents the log from which the microbes were isolated, I, A and C represent the location on or around the rotted wood logs from where the microbes were isolated, F(1-n) represents the number of fungi and B(1-n) represents the number of bacteria. These isolated strains were screened for their lignin- degrading and utilizing abilities and then identified by gene sequencing.

2.2 Microbial Screening

The isolated microbes were screened for their lignin-degrading and/or utilizing abilities. The screening process involved the use of lignin and decolorizing dyes that change color based on the production of lignin-degrading enzymes. The decolorizing dyes used in the screening process were 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), reactive black 5 (RB5), and Remazol brilliant blue R (RBBR). ABTS is a nonphenolic dye that is oxidized by the enzyme laccase to a more stable ABTS•+ cation radical resulting in a blue-green color and correlating to enzyme activity (More et al., 2011). Decolorizing of both RBBR and RB5 is a result of the production of peroxidases such as LiP, MnP or VP (Shin and Kim, 1998) (Hadibarata et al., 2013). The screening process allows for the selection of microbes that can produce lignin-degrading enzymes and/or utilize lignin and be further characterized.

<u>Screening for Fungal Isolates</u>

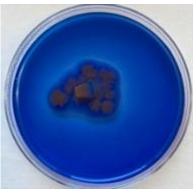
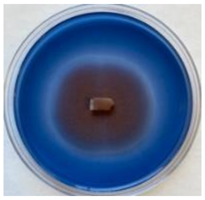
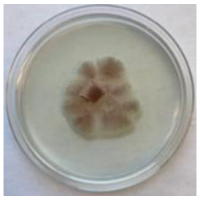
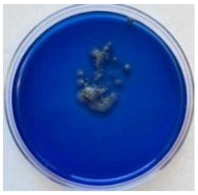
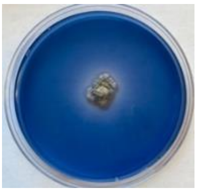
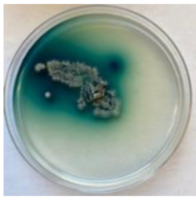
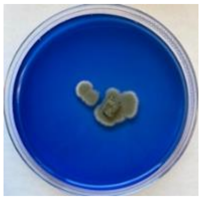
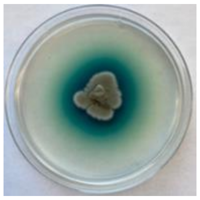
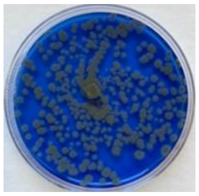
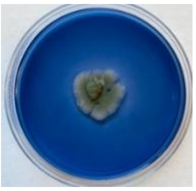
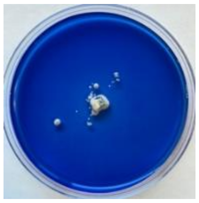
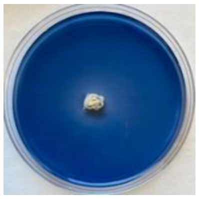
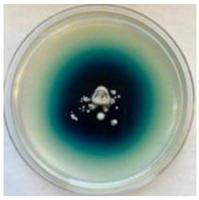
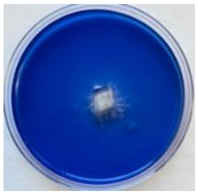
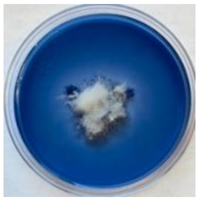
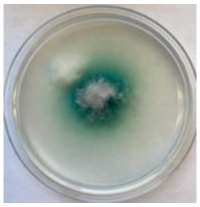
Materials and Methods

For testing the lignin-utilizing abilities, the fungal isolates were cultured on plates containing Highley's Minimum Medium (HMM) and Kraft lignin (Sigma) as the sole carbon source. HMM consists of 2 g NH_4NO_3, 2 g KH_2PO_4, 2 g NH_4NO_3, 2 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.57 mg H_3BO_4, 0.036 mg $MnCl_2.4H_2O$, 0.31 mg $ZnSO_4.7H_2O$, 0.039 mg $CuSO_4 \cdot 5H_2O$, 0.018 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.015 mg $FeSO_4 \cdot 7H_2O$, and 0.001 g thiamine hydrochloride, per liter; and was supplemented with 5 g/L Kraft lignin and 1.2% agar to solidify the medium. The media was autoclaved for 20 mins at 121°C. The chemicals used in this experiment were purchased from Sigma-Aldrich. The isolates were cultured at 30°C for 2 weeks and the pH was maintained between 6 and 7. The growth was visualized for day 7 and 14 after inoculation and recorded.

For testing for the ligninolytic enzyme production of the fungal isolates, the isolates were cultured on agar plates containing HMM supplemented with low concentration of glucose (0.2%) as carbon source and decolorizing dyes consisting of 2 mM ABTS, 50 mg/L RB5 or 200 mg/L RBBR. The dyes were filtered through a 0.2 μm filter for sterilization and added to the medium before pouring the plate. The plates were cultured at 30°C for 2 weeks and the decolorization was recorded. The pH was maintained between 6 and 7. To select the isolates for further analysis, the results of dye decolorization and growth on lignin were ranked on an evaluation scale of 0-2, in which 0 represents no growth or decolorization, 1 represents change in the color of ABTS plates or growth on lignin plates and 2 represents decolorization of RBBR/RB5 plates. The growth on lignin and ABTS production are lower on the scale as production of peroxidases is a better indicator of lignin degradation in fungi than that of laccases. Additionally, the lignin-degrading capacity does not necessarily correlate with efficient growth on lignin, therefore, the growth of lignin is lower on the evaluation scale (Bandounas et al., 2011).

Results and discussion

The screening method was applied on 76 isolated fungal samples. Of the 76, 14 samples ranked high on the evaluation scale (not shown). Removing the redundancy based on phenotypical appearance of the fungal mycelia, 8 fungal isolates were selected for identification and characterization. The enzyme production by the selected fungal isolates can be visualized in Table 1.

Isolates	**Enzyme Production Indicating Dyes**		
	RBBR	RB5	ABTS
L2-I-F8			
L3-A-F4			
L3-I-F3			
L3-I-F10			
L4-I-F6			
L5-I-F4			

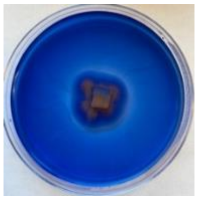
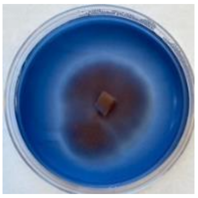
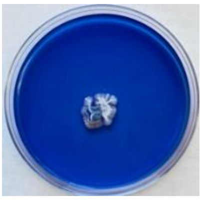
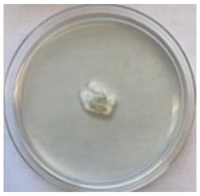
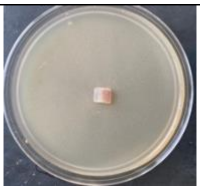
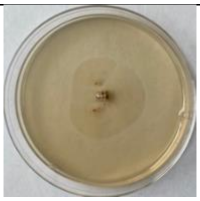
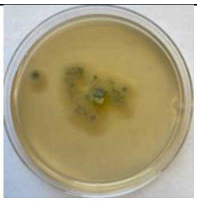
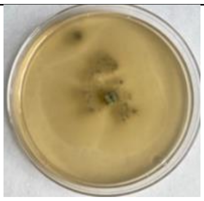
L5-I-F6			
L5-I-F10			

Table 1: Decolorization/change of color in dye-containing plates for the selected 8 fungal isolates

The growth on culture plates using HMM and 5g/L Kraft lignin as sole carbon source to test for lignin-utilization ability of the isolates, is also shown in the Table 2.

Isolates	**Growth on Kraft lignin**	
	Day 7	Day 14
L2-I-F8		
L3-A-F4		
L3-I-F3		

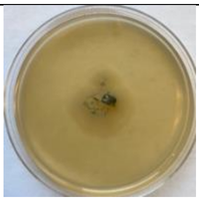
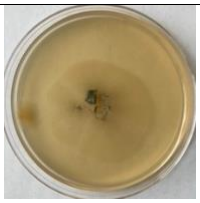
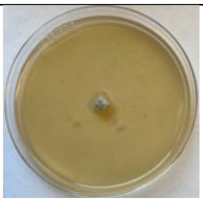
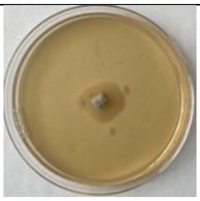
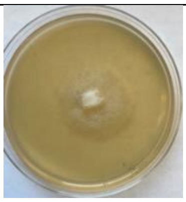
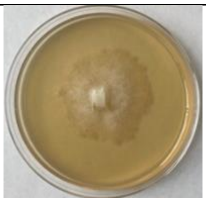
L3-I-F10		
L4-I-F6		
L5-I-F4		
L5-I-F6		
L5-I-F10		

Table 2: Fungal mycelia growth observed on day 7 and 14 of inoculation on culture plates containing Kraft lignin as sole carbon source

The screening on dye-containing plates indicates that the eight isolates shown here produced varying amounts of lignin-degrading enzymes such as peroxidases and laccases over a period of incubation of 7 days in the nutrient HMM media at 30°C. The other isolates were also able to produce lignin-degrading enzymes by this screening process but are not shown here. Several of the isolated fungi were able to grow on lignin containing plates however the growth rate seemed slow. In the table above, there is significant growth observed on day 14 compared to day 7 for the eight isolates with the degree of growth varying among them. Overall, it can be concluded that although a slow growth rate, several of the isolated fungi were able to utilize Kraft lignin for growth and produce lignin-degrading enzymes. It can also be observed that majority of the isolates that indicated growth on lignin or produced lignin-degrading enzymes were isolated from the interior of the wooden log wherein the lignin concentration is expected to be higher. The fungal isolates collected from the soil 2 m away from the wooden log had little to no success in producing lignin-degrading enzymes or growing on culture plates with lignin as the sole carbon source.

Screening for Bacterial Isolates

Materials and Methods

The screening process for bacteria involved both solid and liquid cultures. The recipe for the M9 media used for bacterial screening was provided by Dr. Davinia Salvachua, NREL. The M9 media is a minimal media that consists of 6.78 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 2 g $(NH_4)_2SO_4$, 2 mL 1M $MgSO_4.7H_2O$, 100 μL 1M $CaCl_2.2H_2O$ and 1 mL 5 g $FeSO_4.7H_2O$, per liter. To solidify, 1.2% agar was used, and the media was autoclaved for 20 mins at 121°C. For the solid cultures, the bacterial isolates were streaked on culture plates containing M9 supplemented with 5 g/L Kraft lignin as the sole carbon source. The pH was adjusted to 7 using 0.1M HCl. The bacteria isolates were incubated for 7 days at 30°C and the isolates that produced the most colonies on the lignin culture plates were selected for the liquid culture screening.

The liquid cultures used the same M9 media as described above and was supplemented with 5g/L Kraft lignin to test for lignin-utilizing ability and with 50 mg/L RBBR dye to test for the production of lignin degrading enzymes. For this experiment, *Amycolatopsis sp., Rhodococcus jostii* and *E. coli* were used as a control group. The first two bacteria are positive controls that have demonstrated the ability to depolymerize high molecular weight lignin and catabolize low molecular weight metabolites (Salvachua et al, 2015). The *E. coli* DH5a strain is a negative control and is not expected to show ligninolytic characteristics. The bacterial isolates were pre-cultured overnight in LB and centrifuged. The cell pellet was washed with sterilized DI water and used to inoculate in 250 mL Erlenmeyer flask containing 20 mL of the medium. The cultures were incubated at 30°C and 125 rpm and the pH was maintained at 7. Samples were drawn on the 4th, 7th, and 10th day of incubation for optical density (OD) measurements at 600 nm to measure growth. Additionally, for the RBBR cultures, spectrophotometric analysis of the culture supernatant was performed at 595 nm to observe decolorization of RBBR by the bacterial isolates (Bandounas, 2011).

Results and Discussion

The initial screening method using the solid phase cultures was applied to 108 bacterial isolates. While several isolates were able to develop colonies over the 7-day incubation period, fourteen of the isolates produced numerous colonies with the set culture conditions. These isolates were then screened in liquid cultures to quantify its growth on lignin and its ability to produce lignin-degrading enzymes.

The growth of the bacterial isolates measured by the OD values at 600 nm on media supplemented with 5 g/L lignin as the sole carbon source is depicted in Fig. 7. It can be observed that significant growth occurred at a longer incubation period with the degree of growth varying for the different isolates.

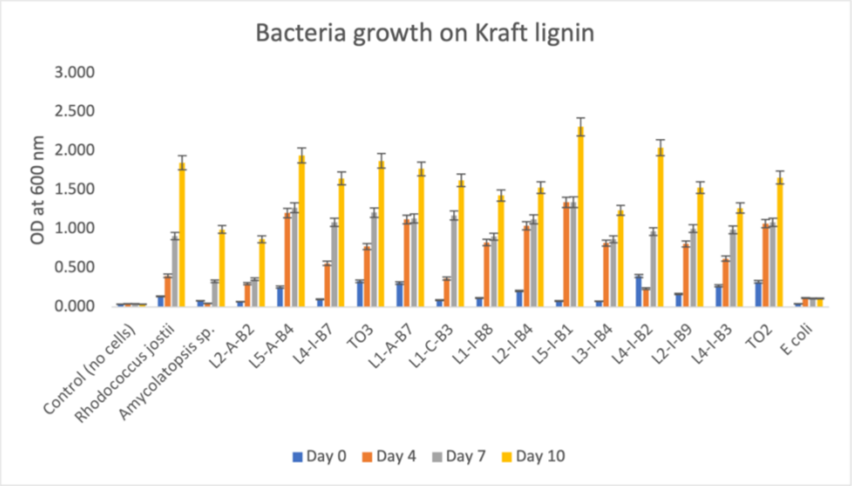


Figure 7: Bacterial growth on lignin observed over an interval of 3 days

The ligninolytic potential of the bacterial isolates were observed by inoculating the isolates in liquid culture supplemented with decolorizing dyes. RBBR, which is an indicator for the production of peroxidases, was used and the decolorization is recorded in the figure below.

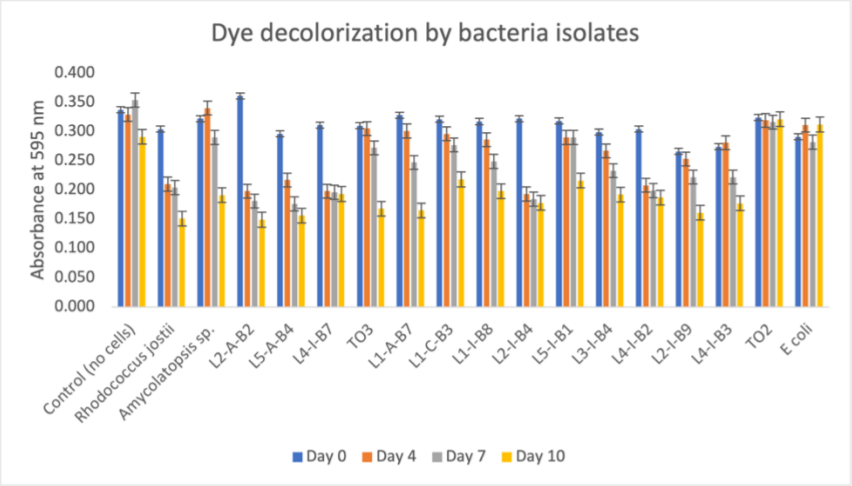


Figure 8: Decolorization of RBBR dye by the bacterial isolates

The absorbance at 595 nm reduced over time for several of the isolates and no decolorization was observed by the control species *E. coli*. It should be noted that RBBR appeared to be adsorbed to the cells in some of the cultures rather than degraded. This indicates that the dye-decolorizing potential of the isolates could either be extracellular or cell-associated. There is a possibility of choosing other indicator dyes showing decolorization on enzyme production since bacterial peroxidases are different from fungal peroxidases. However, this screening process was helpful in selecting isolates that indicated lignin utilization and degradation potential and can be further studied for characterization.

2.3 Sequencing and Identification of Isolates

Following the screening process is the identification process for these isolated fungi and bacteria. This process gives an insight into novel microorganisms participating in lignin-degradation and utilization and understand the microbial community present in the boreal forest ecosystem.

Materials and Methods

The pure cultures of selected fungi and bacteria were used to isolate DNA for sequencing, targeting the fungal (ITS) and bacterial (16S rRNA) marker genes, respectively. A small colony (from bacteria) and mycelium (from fungi) were scratched off into a 1.5 mL centrifuge tube and 100 μL of MightyPrep® reagent for DNA (Takara Bio) was added and vortexed to mix thoroughly. The mixture is heated on a heat block at 95°C for 10 mins. The mixture was centrifuged at 13,000 rpm for 10 mins and the supernatant was collected.

To prepare the extracted DNA for polymerase chain reaction (PCR), a mixture containing 25 μL EmeraldAmp® MAX PCR Master Mix (Takara Bio), 1 μL of forward and reverse primers each, 22 μL ddH_2O and 1 μL of extracted DNA was mixed to a homogeneous solution. The 50 μL PCR solution was put in Bio-Rad T100™ Thermal Cycler using 95°C for 2 mins (pre-denature step), 39 cycles of 95°C for 30 seconds (denature dsDNA), 55.5°C for 30 seconds (primer annealing), 72°C for 1 min (polymerase extends DNA from primers), 72°C for 5 mins (final extension) and holding at 4°C, as the PCR conditions. The primers (5' → 3') used for the fungal isolates were: ITS-1: TCC GTA GGT GAA CCT GCGG and ITS-4: TCC TCC GCT TAT TGA TAT GC; primers (5' → 3') for bacterial isolates were 16S rRNA Forward: AGA GTT TGA TCC TGG CTC AG and 16S rRNA Reverse: ACG GCT ACC TTG TTA CGA CTT. The primers used and the PCR conditions were selected based on literature data for ligninolytic microbes' identification.

Gel electrophoresis was performed to confirm the amplification of extracted DNA by PCR using 1% agarose gel. The gel is immersed in TAE buffer, PCR product is mixed with loading dye and loaded on to the gel. The voltage is set to 120V for 30 mins. The gel was observed using a UV transilluminator. The PCR products were cleaned using NucleoSpin® Gel and PCR Clean-up kit from Macherey-Nagel and sent for sequencing to ACGT, Inc. A Basic Local Alignment Search Tool (BLAST) analysis was performed on the resulting sequences to determine the identity of the fungal and bacterial isolates. Additionally, a phylogenetic analysis was performed using Common Taxonomy Tree tool from NCBI and the FigTree software to infer any evolutionary relationship between the different fungal and bacterial isolates and to identify the microbial community present in the boreal forest ecosystem.

Results and Discussion

The BLAST analysis performed on the selected fungi and bacteria isolates for identification. The most probable BLAST hit along with its accession number, and percent sequence identity was recorded. The results for the isolated fungi and bacteria are summarized in the tables below.

Isolate Name	**Most probable BLAST hits**	**% Sequence Identity**
L2-I-F8	*Umbelopsis dimorpha* NR_111664.1	96.70
L3-A-F4	*Penicillium rolfsii* var. *sclerotiale* NR_153216.1	99.80
L3-I-F3	*Penicillium citreosulfuratum* NR_153252.1	99.63
L3-I-F10	*Penicillium grancanariae* NR_138348.1	99.62
L4-I-F6	*Penicillium pancosmium* NR_121506.1	99.65
L5-I-F4	*Mortierella gemmifera* NR_111559.1	99.33
L5-I-F6	*Umbelopsis angularis* NR_137072.1	99.49
L5-I-F10	*Spencermartinsiella ligniputridi* NR_155842.1	99.80

Table 3: Identification of isolated fungi by ITS rRNA gene sequencing

Isolate Name	**Most probable BLAST hits**	**% Sequence Identity**
L1-A-B7	*Lelliottia amnigena* NR_024642.1	98.01
L1-C-B3	*Bacillus toyonensis* NR_121761.1	99.37
L1-I-B8	*Bacillus wiedmannii* NR_152692.1	98.51
L2-A-B2	*Bacillus cereus* NR_112630.1	97.32
L2-I-B4	*Bacillus mycoides* NR_036880.1	87.07

L2-I-B9	*Bacillus thuringiensis* NR_043403	98.28
L3-I-B4	*Bacillus paramobilis* NR_175556.1	98.78
L4-I-B2	*Bacillus mobilis* NR_157731.1	96.02
L4-I-B3	*Bacillus proteolyticus* NR_157735.1	99.13
L4-I-B7	*Bacillus pacificus* NR_157733.1	98.61
L5-I-B1	*Bacillus sanguinis* NR_175555.1	99.81
TO2	*Brucella intermedia* NR_113812.1	99.05
TO3	*Stenotrophomonas maltophilia* NR_041577.1	96.44
L5-A-B4	*Rhodococcus qingshengii* NR_145886.1	99.04

Table 4: Identification of bacterial isolates by 16S rRNA gene sequencing

The sequencing resulted in an identity threshold of >95%. While the marker gene sequencing result is enough to identify the species, to assign the strain at species level or for the taxonomic characterization, further studies with DNA hybridization using standard strains is required which was not performed for this study.

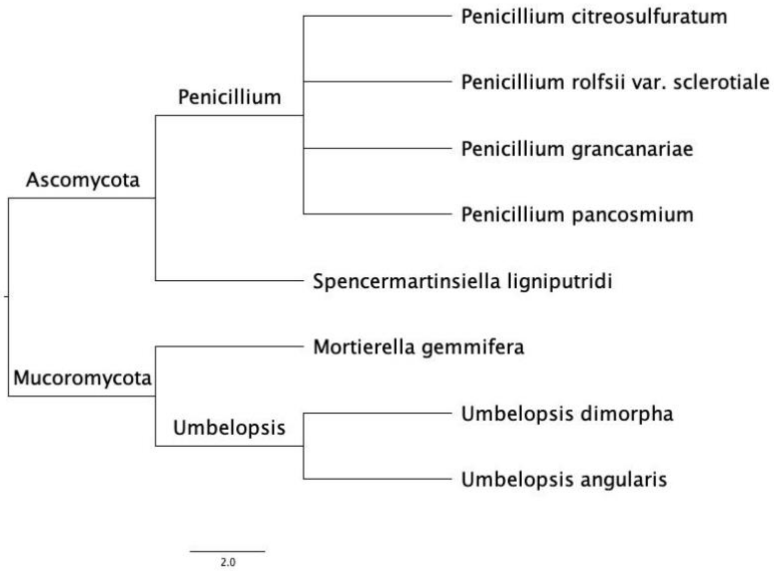


Figure 9: Phylogenetic tree of the isolated fungi

From the phylogenetic analysis of the isolated fungi, it can be observed that several of the selected fungal isolates are Ascomycetes (such *as P. citreosulfuratum, P. rolfsii* var *sclerotiale, P. grancanariae, P. pancosmium* and *S. ligniputridi*), along with a few Mucoromycetes (*M. gemmifera*, *U. angularis* and *U. dimorpha*). As mentioned in Chapter 1, Ascomycetes are wood decaying soft-rot fungi. They can degrade lignin in "recently-produced" lignocellulosic materials, such as Kraft lignin, which was used in this study, by producing lignin-degrading enzymes which was observed during the preliminary screening of isolates (Ferrari et al., 2021). Mucoromycetes, that were identified among the fungal isolates were able to produce peroxidases (observed during preliminary screening), with *Mortierella gemmifera*, producing laccases instead of peroxidases. Mucoromycetes have not been extensively studied for their role in lignin degradation, however, they have shown success as plastic-degrading fungi in recent studies (Ekanayaka et al., 2022).

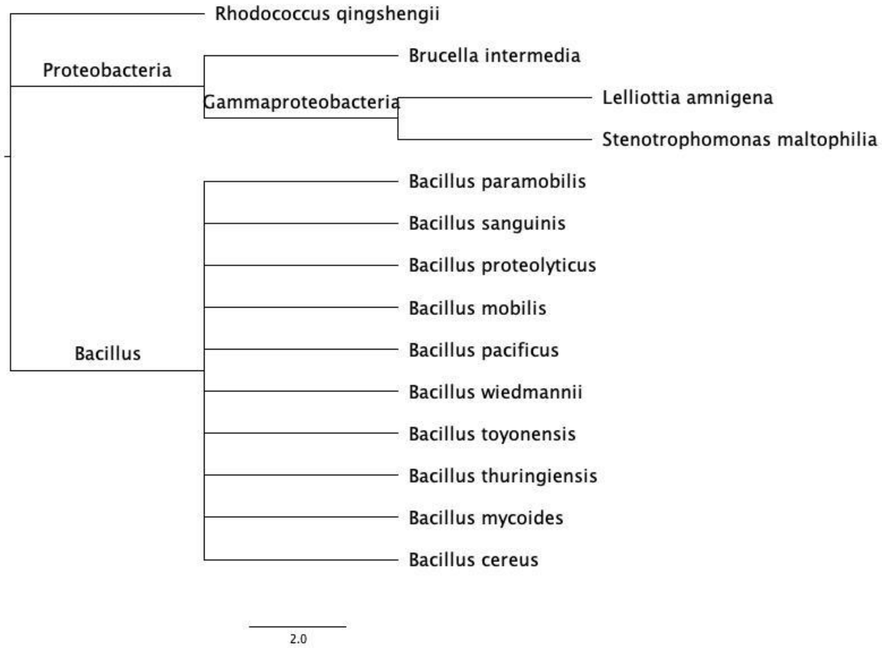


Figure 10: Phylogenetic tree of the isolated bacteria

For the bacterial isolates, there was a mixture of actinomycetes (such as the *Bacillus* sp.), α-proteobacteria (such as *Brucella intermedia*), and γ-proteobacteria (such as *Lelliottia amnigena* and *Stenotrophomonas maltophilia*). These are common soil bacteria that can degrade aromatic compounds (Bugg et al., 2011). Additionally, these isolates have been shown to utilize lignin in the preliminary screening process used in this study, therefore providing a possible link between aromatic degradation and lignin degradation. The sequencing and identification of the isolated fungi and bacteria recognized novel species that have not been explored for lignin degradation but have the capacity to participate in the process.

CHAPTER 3: CHARACTERIZATION OF IDENTIFIED MICROBES USING AROMATIC LIGNIN MONOMERS

Depolymerization of lignin results in a mixture of aromatic compounds which can be utilized as a sustainable alternative to petrochemical-based aromatic feedstocks (Azubuike et al., 2022). As mentioned in Chapter 2, lignin-degrading capacity does not necessarily correlate with efficient growth on lignin as several microbial species may not be able to efficiently metabolize or utilize lignin and its degradation products. As observed from the preliminary screening, several of the identified fungal isolates were Ascomycetes. It is evident in literature that lignin degradation is most successfully observed in Basidiomycetes but the ability of ascomycetes to degrade different types of lignin is less emphasized. In the case of actinomycetes, it has been reported that despite being unable to mineralize lignin, it can solubilize and modify lignin and its degradation products (Bandounas et al., 2011). In this section, the isolated fungi and bacteria were evaluated for the capability to utilize aromatic lignin monomers that are involved in lignin-degradation, for cell growth.

3.1 Fungal Culture on Lignin Aromatic Monomers

Materials and Methods

For assessing the ability of the fungal isolates to utilize aromatic monomers, a solid-phase culture was used. The lignin aromatic monomers used for this experiment were vanillyl alcohol, 4-hydroxybenzoicacid (4-HBA) and syringaldehyde. The concentrations of the monomers tested were 0.5 mM and 5 mM, respectively. The culture media consisted of HMM along with aromatic monomer as carbon source and 1.2% agar for each of the isolates. For the control, the mycelia growth was also evaluated on culture plates with no C source, low-concentration glucose and 5 g/L lignin in HMM. The isolates were pre-cultured on PDA plates for 7 days at 30°C and 3 mm mycelium plug was transferred to the center of the culture plates containing the aromatic monomers. The cultures were incubated for 14 days. The mycelia growth in mm per day of the fungal mycelia for each culture condition was evaluated after 7 days of incubation. *Gloeophyllum trabeum* and *Trametes versicolor*, were used as control group. These are model brown-rot and white-rot fungi, respectively, that have been extensively studied for their efficient breakdown of lignin and therefore used as control group in the characterization experiments.

Results and Discussion

The growth of fungal mycelia was observed over a 7-day incubation period in culture plates containing 0.5 mM and 5mM concentration of aromatic monomers.

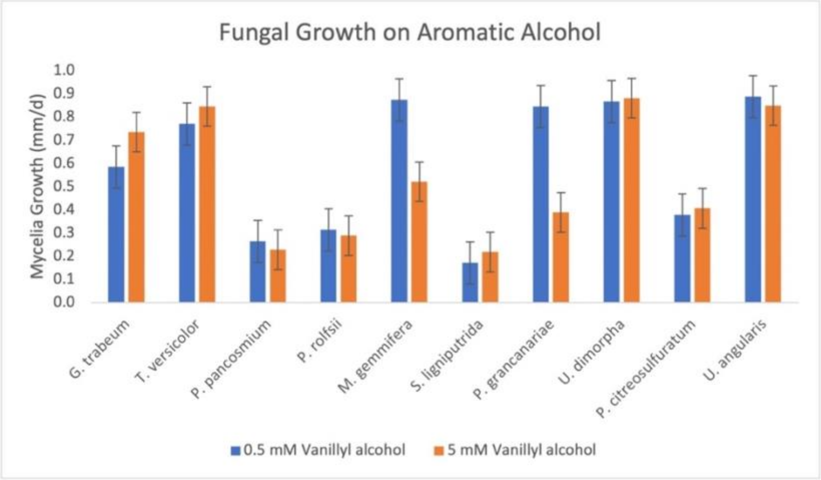


Figure 11: Mycelia growth on vanillyl alcohol at 0.5 mM and 5 mM conc.

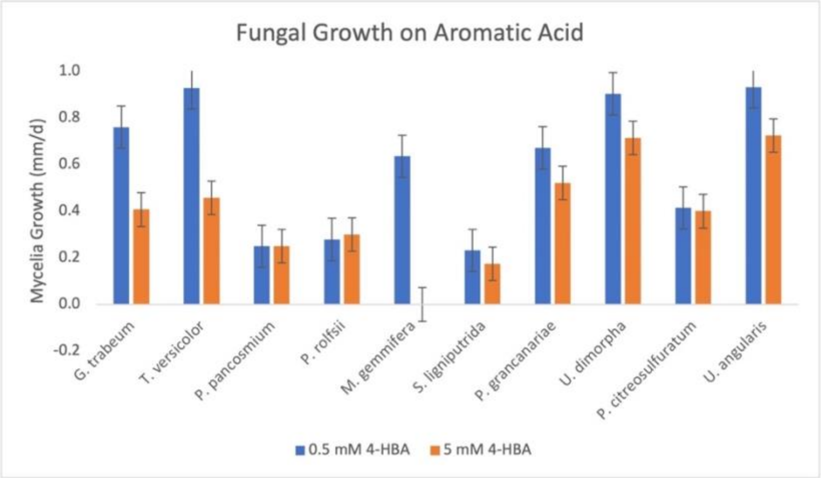


Figure 12: Mycelia growth on 0.5 mM and 5mM 4-HBA

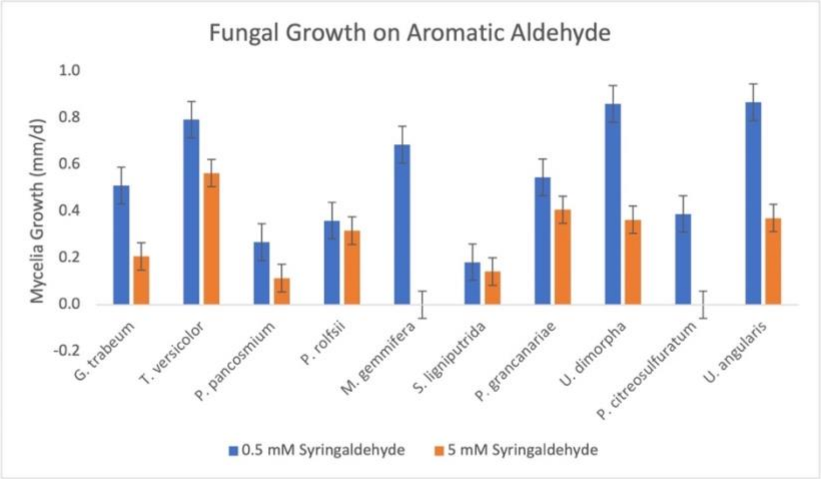


Figure 13: Mycelia growth on 0.5 mM and 5mM syringaldehyde

Most of the fungal isolates were able to utilize vanillyl alcohol for growth with *P. pancosmium*, *P. rolfsii* var. *sclerotiale* and *S. ligniputrida* growing especially slowly compared to the others. While it was expected for the growth percent to be higher in low concentration of the aromatic alcohol, an opposing trend was observed in several of the fungal isolates. The growth on the aromatic acid and aldehyde showed that all the isolates utilized the 0.5 mM concentration culture condition well however, at 5 mM concentration of both acid and aldehyde, the growth of *M. gemmifera* was inhibited. Additionally, the growth of P. citreosulfuratum was inhibited at 5mM of syringaldehyde. The utilization of aldehyde was to a limited extent compared to that of the aromatic acid and alcohol.

The mycelia growth was also evaluated on culture plates with no C source, low-concentration glucose and 5 g/L lignin in HMM and shown in the heatmap in Fig. 14.

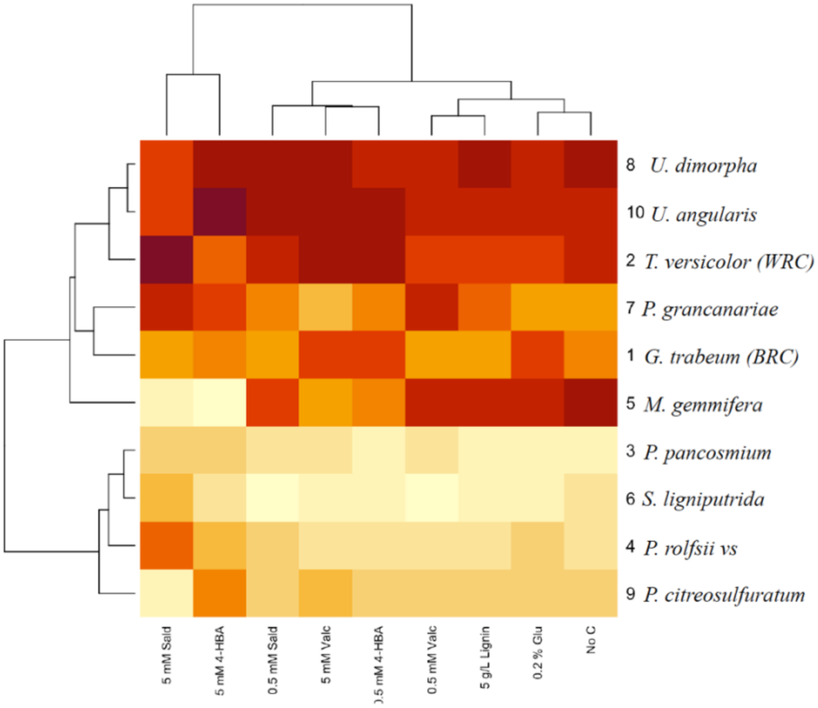


Figure 14: Heatmap comparing the mycelia growth in variable media. Scale: Yellow is low growth of mycelia (mm/d) whereas red is high growth of mycelia.

The growth observed on culture plates with no carbon source indicate that these fungi are most likely using the agar and media nutrients for growth. This is a limitation of using the solid-phase culture for screening as it is unclear whether the fungi solely utilized the aromatic monomers as carbon sources for growth or if the growth was supplemented by the agar present. Regardless, these observations suggest that the isolated fungi are fairly capable for aromatics degradation and/or utilization.

3.2 Bacterial Culture on Aromatic Lignin Monomers

Materials and Methods

To assess the ability of the isolated bacteria to utilize aromatic lignin monomers, the isolates were pre-cultured overnight in liquid media in culture tubes containing 5 mL of LB and 100 μL of stored bacteria isolates each at 30°C. The lignin monomers used for this experiment were: aromatic acids – vanillic acid, ferulic acid, *p*-coumaric acid, syringic acid and 4-HBA; aromatic alcohols – vanillyl alcohol, veratrole alcohol and syringol; aromatic aldehyde – syringaldehyde. The bacteria culture was transferred to centrifuge tubes and centrifuged at 13,000 rpm for 10 mins. The supernatant was discarded, and the cells washed using autoclaved DI water and transferred to a fresh culture media consisting of M9 with the aromatic monomers as carbon sources. Two different concentrations of the monomers were used – 0.5 mM and 5 mM, to compare the utilization of low and high concentration of the monomers by the isolates, respectively. For the control, culture tubes containing M9 along with isolated colonies and no carbon source was used. The cultures were incubated in a shaking incubator for 7 days at 30°C and 175 rpm, and the cell growth was quantified by measuring the OD values at 600 nm. A heatmap was created, using R software, to compare the different OD values observed for a total of 20 variables involved in bacterial isolate characterization. *Rhodococcus jostii*, *Amycolatopsis sp*. and *E. coli* were used as control groups.

Results and Discussion

Several lignin monomers, that could be utilized for growth by bacteria, were tested and recorded. The overall bacterial growth on the 20 variables is shown in Figure 18. To establish characterization trends from the 20 variables, one of each aromatic alcohol, acid and aldehyde was selected based on the overall result observed in Figure 18. The figures below are observations from 0.5 mM and 5 mM of vanillyl alcohol, 4-HBA and syringaldehyde.

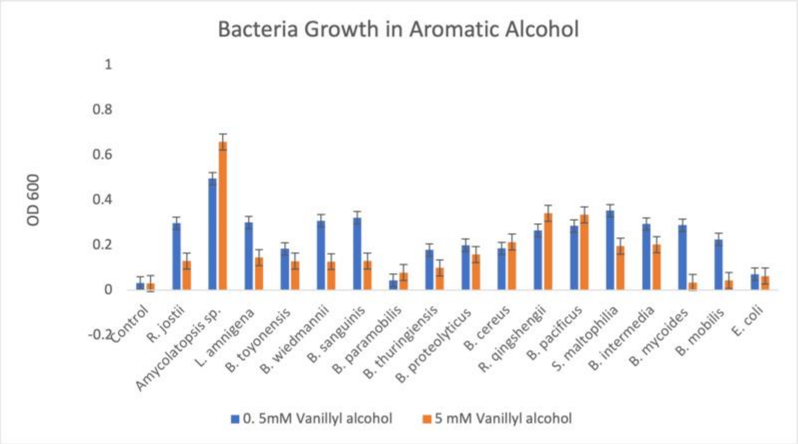


Figure 15: Bacteria cell growth measured by OD 600 with vanillyl alcohol as sole C source

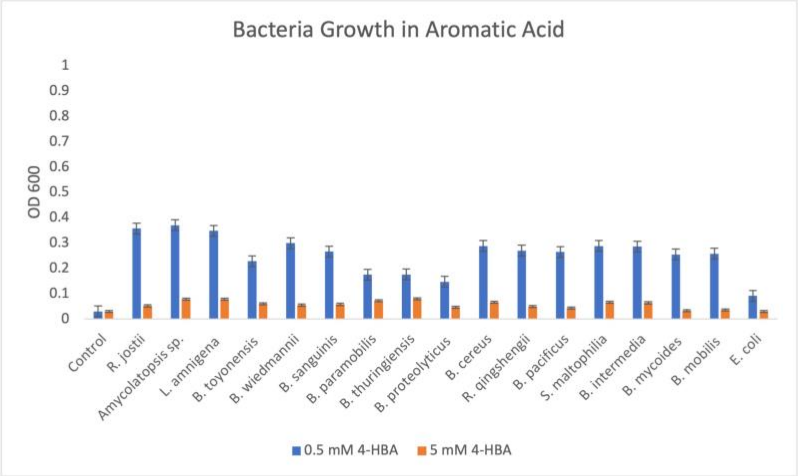


Figure 16: Bacteria cell growth measured by OD 600 with 4-HBA as sole C source

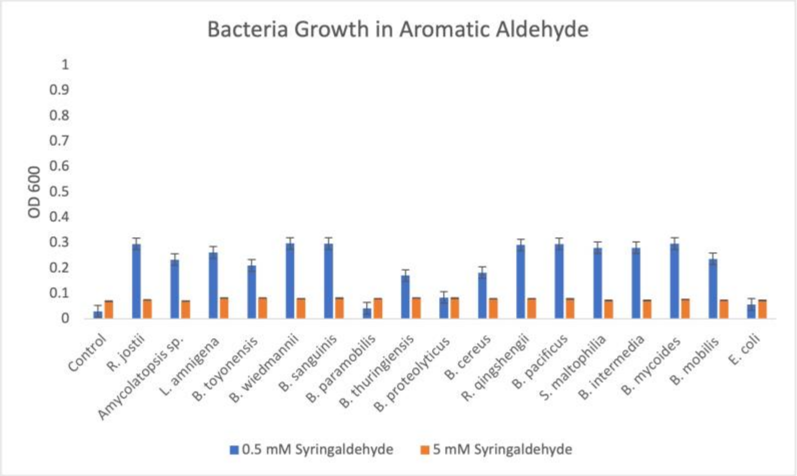


Figure 17: Bacteria cell growth measured by OD 600 with syringaldehyde as sole C source

Several trends could be observed from the culture of isolated bacteria on aromatic lignin monomers as carbon sources. For the aromatic alcohol, several of the *Bacillus* species, such as *B. wiedmannii, B. sanguinis, B. cereus, B. pacificus,* and *B. mycoides,* utilized low concentration of vanillyl alcohol for growth. *B. cereus, R. qingshengii* and *B. pacificus* were able to use both high and low concentrations for growth however, inhibitory effect was observed for *B. paramobilis*, *B. mycoides* and *B. mobilis* at 5 mM of vanillyl alcohol. For the aromatic acid, it was evident that the 5 mM concentration of 4-HBA was inhibitory whereas, the 0.5 mM concentration was utilized fairly well for growth, with slow growth observed for *B. paramobilis*, *B. thuringiensis* and *B. proteolyticus*. For the aromatic aldehyde, similar inhibitory effect was observed at higher concentration of the aromatic monomer. The *Bacillus* species and the *R. qingshengii*, *S. maltophilia*, *Brucella* sp., utilized the aromatic aldehyde fairly well with a few exceptions. Overall, for each of the aromatic monomer highlighted above, several bacteria had similar growth rate regardless of the C source. The control species, both positive and negative, showed expected results. The figure below summarizes the growth observed on all the variables including the control variables.

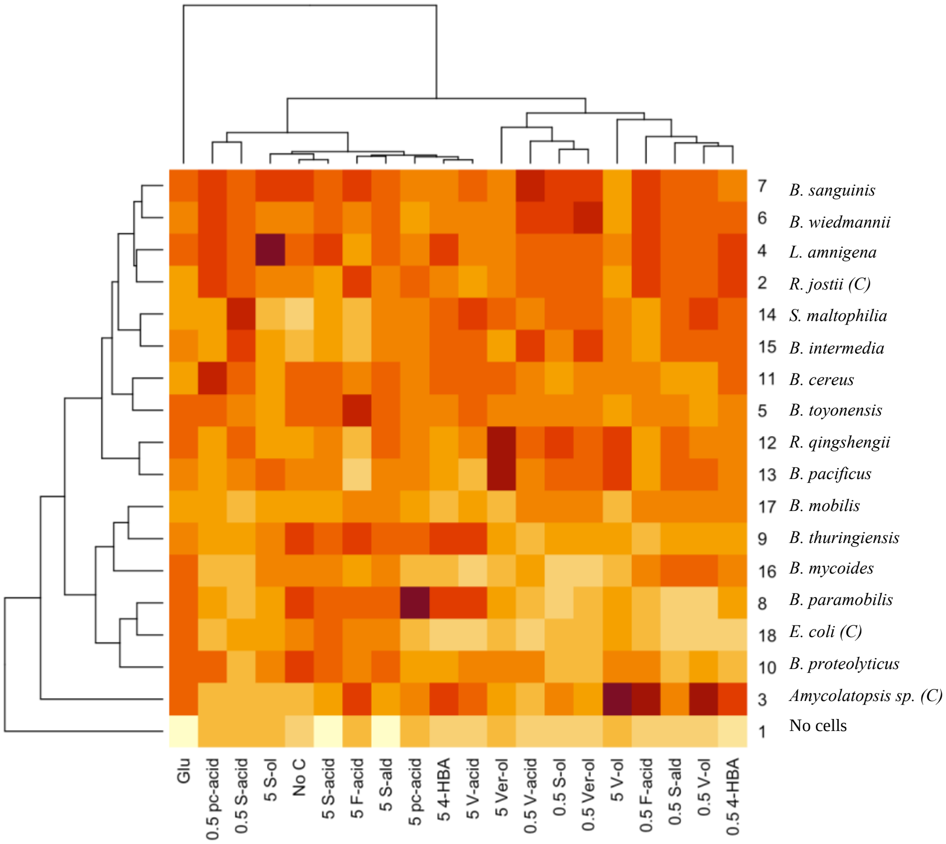


Figure 18: Comparison of cell growth of bacteria in different C sources. Scale: Yellow is low growth of cells whereas red is high growth of cells.

It can be observed that the bacteria isolates were able to utilize several of the aromatic monomers for growth. Almost all of the isolates grew fairly well in low concentration of monomers compared to the higher 5 mM concentration. Similar to the trend observed among the fungal isolates, the bacteria isolates grew well in aromatic alcohols. Among the aromatic acids, highest growth was observed in 4-HBA and lowest in *p*-coumaric acid. In the case of aromatic aldehyde, little to no growth was observed in the high concentration culture condition. It was also observed that several of the bacteria were able to utilize the culture media that did not contain any C source. While this was not expected, a possible source of error could be the washing step of the pre-cultures before its transfer to the new media. The cells could contain residual nutrient LB that could have been utilized for growth in the media containing no C source. However, with or without the supplemental residual LB, the bacterial isolates utilized the aromatic monomers for growth fairly well.

To summarize, the characterization of the isolated fungi and bacteria on aromatic lignin monomers suggested that the isolates were able to utilize the monomers for growth. This provides an insight into the degradation mechanisms undertaken by these isolates during lignin depolymerization. While the processes used for the characterization for this study is useful and successful in identifying microbial growth, several other processes that are more efficient can be applied to characterize it. This includes developing enzymatic assays to identify the enzymes produced and also to study the degradation products. With both the fungal and bacterial isolates able to utilize aromatic alcohol successfully for growth suggests that these microbes have the required alcohol dehydrogenase activity to oxidize alcohols to acid forms. This is useful in establishing the degradation pathway followed by these microbes. It also suggests that microbes from the same niche have similar interactive enzymes that can participate in lignin depolymerization.

CONCLUSION AND FUTURE WORK

This study identified eight fungi and fourteen bacterial species from up to two hundred microbes that were isolated from the boreal forest ecosystem found at the Cedar Creek Ecosystem Science Reserve Field located in East Bethel, MN. The microbes were selected based on a preliminary screening method that evaluated its ability to produce ligninolytic enzymes by decolorizing or changing color of indicator dye-containing cultures. The microbes were also evaluated for their lignin-degrading and/or utilizing ability in a nutrient media with added Kraft lignin in both solid and liquid cultures. Several of the fungal and bacterial isolates were able to utilize Kraft lignin for growth and/or produce lignin-degrading enzymes such as peroxidases and laccases. The identification procedure revealed that the isolated fungi belonged to ascomycota and mucoromycota phylum, which are a representative of soft-rot wood decaying fungi, whereas the bacterial isolates were a mix of actinomycetes, α-proteobacteria and γ-proteobacteria. This study identified the potential of several microbes that have not been extensively studied for their ability to depolymerize lignin and its degradation products and explore the possibility of novel enzymes which can be used by the microbes to breakdown lignin. These findings are important to the effective valorization of lignin using microbes as microbes have evolved to produce the enzymes required to depolymerize this recalcitrant biopolymer and lignin is a potential renewable carbon feedstock that could replace petroleum-derived products. The characterization of these isolated microbes revealed that the isolated fungi and bacteria are not only capable of producing lignin-degrading enzymes and utilizing lignin for growth but also fairly capable of utilizing the aromatic lignin monomers.

There have been several studies recently to enhance the degradation of lignin by microorganisms. While both fungi and bacteria have evolved to develop unique metabolic pathways to convert lignin, there are challenges to lignin-degradation by using fungi or bacteria alone. Fungi and bacteria co-inhabit several environments and their interactions can be manipulated for effective breakdown of recalcitrant compounds. Fungal degradation involves oxidative enzymes which are not as powerful in bacterial degradation. Bacterial systems can however provide accessory enzymes that could act synergistically with the major oxidative fungal enzymes, providing efficient depolymerization of lignin in a co-culture or consortia (Brown and Chang, 2014). A recent study identified the bacteria species *Sphingomonads* to degrade low-molecular-weight lignin produced during the decomposition of high-molecular-weight lignin by white-rot fungi in addition to other bacteria that may also assist in lignin degradation (Iimura et al., 2021). While the interaction among fungi and bacteria can be used for breakdown of lignin, there have also been studies to utilize this method for removing organic pollutants from the environments that are recalcitrant and structurally similar to lignin. In a study, a consortium between a brown-rot fungus and a bacterium was able to degrade DDT, an organic pollutant structurally similar to lignin, using the combined and inductive effect of various enzymes (Purnomo el al., 2020).

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