Isolation of Thermophilic Bacteria from Hot Spring and Exploring its Role in Lignin Valorization

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“IN THE NAME OF ALLAH, THE MOST BENEFICENT, THE MOST GRACIOUS, THE MOST MERCIFUL”

DEDICATION

This research work is dedicated to the
unfathomable love
and ever strengthening prayers of my beloved
parents, siblings, friends, and all those encouraged me to
fly toward my dreams.

Declaration

This thesis contains my own work in terms of information and content. I have never submitted any part of this work before for any other degree.

Noor-ul-Huda

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List of Acronym/Abbreviations

AhR	Aryl hydrocarbon receptor
CH_3COOH	Acetic acid
NH4OH	Ammonium hydroxide
NH_4NO_3	Ammonium nitrate
AOP	Advance oxidation procedure
5-O-4	Biphenyl ether linkages
BOD	Biological Oxygen Demand
BL	Black liquor
COD	Chemical Oxygen Demand
CaCl2	Calcium chloride
K_2HPO_4	Dipotassium phosphate
FTIR	Fourier Transform Infrared Spectrometer
GCMS	Gas Chromatography mass Spectrometry
GIT	Gastrointestinal tract
MnP	Manganese peroxidase
$MgSO_4$	Magnesium sulphate
MgO	Magnesium oxide
P&P	Pulp and Paper
KH_2PO4	Potassium dihydrogen phosphate
KCL	Potassium chloride
PCP	Polychlorinated phenol
RO	Reverse Osmosis
β- β	Resinol Linkage
NaOH	Sodium hydroxide
Na_2S	Sodium sulfide
$NaNO_2$	Sodium nitrite
L-MSM	Lignin amended minimal salt media
Lip	Lignin peroxidase
ZnO	Zinc oxide

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Noor ul Huda

Abstract

Lignin, the second-most prevalent natural polymer, is mainly produced as a byproduct of the pulp and paper industry. About 50 million tons of it are produced annually, and only 2% of it is utilized for high-value products, and the rest of it is released as a waste in the form of black liquor. The effective breakdown of lignin at high temperatures is essential for the effective removal of pulping effluent. Therefore, there is an urgent need to find appropriate eco-friendly techniques to get beyond this environmental limit and utilize this waste for the production of value-added products. The current study focuses on the potential of novel and unidentified thermophilic bacteria SP2 (10) isolated from hot springs, Chitral in lignin degradation, and its role in lignin valorization. The morphological depictions and biochemical characterization suggest that the strain SP2(10) belongs to family Enterobacteriaceae. The strain SP2(10) effectively reduced the target parameters (lignin 44% and color 47%) of BL effluent at 50◦C and pH 8 which was further revealed by FTIR. Moreover, Gas chromatography-mass spectrometry analysis of the untreated and treated BL samples revealed the reduction of the majority of toxic chemicals and the production of high-value products after biological treatment with SP2(10). Furthermore, toxicological studies have also supported the bacterial detoxification of black liquor. Moreover, the active fractions of the Lignin biodegradable extracts were separated via column chromatography, and the phenolic compounds in the active fractions were evaluated using HPLC. HPLC analysis detected the presence of gallic acid and caffeic acid in the fractions C and D of EAE with a specific RT. These results highlight the importance of this unknown strain in relation to sustainable bioprocessing and lignin valorization. By unlocking the bacterium's lignin-degrading abilities, new and intriguing methods for transforming lignin, a historically underutilized waste product, into a regenerative source of useful compounds are made possible.

1.1 Introduction

Out of many industries, the pulp and paper industry are one of the largest polluting and energy-consuming industries. Paper is a felted or matted sheet made up of pulp i.e., cellulose fibers, and is the main tool for communication. The pulp that is used in papermaking is primarily made up of wood, but it can also be composed of various plant fibers in many cases such as grains, flex, wheat, cotton, kenaf, and hemp (Liu et al., 2018). In ancient times, the paper was made by the fibrous layers present in the stem of papyrus reed, and with another set of layers, these layers were arranged at right angles and pressed and dampened. After drying the sticky sap of the plant that serves the function of glue sticks the layers together. Modern methods like defibering and indispensable elements were not used in papermaking at those times.

Production of pulp and paper is increasing globally and will do so in the coming years. In 2021, China became the world's largest paper producer with approximately 125 million metric tonnes of paper production making the US the second largest paper-producing country with a production of about 67.5 million metric tonnes (Statista, 2023b). Moreover, The paper industry is among the important industries in Pakistan and primarily serves the internal or domestic market. The types of paper that are produced in Pakistan are packaging board, corrugate medium, liner board, banknote paper, writing paper, and tissues. Whereas the raw materials that are used in papermaking include non-woody raw materials (cotton linter, grasses, rags, etc.), domestic OCC/ONP, and imported wooden pulps. Paper mills like Century Paper and Board Mills and Packages in Pakistan are spending a huge sum on R&D and are constantly improving their products. With the Covid 19 pandemic, due to a shortage of raw materials, the paper industry of Pakistan is likely to suffer, and the use of alternative sources lead to an increase in the prices of the products.

The P&P industry is considered the third-largest wastewater-producing industry globally and an important contributor to industrial water pollution. According to the MOEF (Ministry of Environment and Forest) of India, the P&P industry is designated as a “Red Category” among 17 listed industries, which is causing severe environmental pollution. The wastewater generated by this industry and released into the environment is dark brown

and produced during the many steps of the paper-making process. The P&P industry consumes a significant amount of freshwater i.e.,273 to 455 m 3 per tonne paper, and in turn produces almost 220-280 m 3 per ton paper of contaminated wastewater. This colored wastewater reportedly has significant levels of hazardous chlorinated chemicals, tannins, resin acids, sulfur compounds, BOD, COD, suspended solids, and lignin and its by-products. The lignin and its derived compounds make the largest of P&P waste effluent and give it a characteristic brown color and if this dark-colored effluent is released into the environment untreated or just partially treated, it can result in undesired coloring of aquatic resources, along with an increase in COD and BOD, a decrease in the amount of dissolved oxygen in the water, and a decrease in the photosynthetic activities of aquatic life.

Lignin is considered the second-most prevalent organic compound found on the Earth. Regarding production, lignin was already the major industrial by-product by the first half of the 20th century. The production of lignin as a residual by the P&P industry alone was anticipated to be close to 50 million tons per year, with around 98-99% of that amount being used for energy production (Pezzana et al., 2021). Unfortunately, less than 2% of the lignin biomass was employed as the raw material for the production of value-added products. It is predicted that by 2030, this figure would rise by 225 million tons annually as the production of lignin, a byproduct of biofuel production (Ozsefil et al., 2023), increases annually since the RFS program requires the production of almost sixty billion gallons of biofuel.

Lignin is the most prevalent and complex biopolymer found in nature. It has a variety of structural variations on the basis of removal method and the presence of different functional groups. It is a highly complex natural polymer, is composed of three common 4-hydroxy phenylpropanoids referred to as monolignols (p-coumaryl, coniferyl, and sinapyl alcohols), varies in the methoxylation of the aromatic ring and give rise to p-hydroxyphenyl, guaiacyl, and syringyl units. Moreover, It consists of several common functional groups and inter-unit linkages among the aromatic components. A few of these include β-O-4, α-O-4, dibenzodioxocin, β-β, 5-5, β-1, 4-O-5, and β-5. The lignin structure's density is determined by the ratio of the monomers, the composition of the structure, and the pattern of linkages

between the monomers. β-O-4 linkage is one of the prominent linkages (about 40-60%) present in the polymer (Fang et al., 2021).

The modification in the lignin's structure can be done via two processes: (i) extraction process and (ii) chemical modification. The extraction of lignin from lignocellulose materials is a method that is mostly employed in the pulp industry. The structure of lignin may alter as a result of the extraction techniques, changing the lignin's characteristics. Further alterations made to the extracted lignin form the basis of the second procedure, chemical modification of the extracted lignin. Technical lignin is typically divided into five categories: kraft lignin, lignosulfonates, soda lignin, organosolv lignin, and dissolved lignin. These types of lignins are acquired using a variety of technical routes. Different solvents, including acid, alkaline, and organosolv, are utilized to treat lignin during the chemical extraction method. The pulping processes account for the majority of the lignin that is chemically removed. The linkages between cellulose and lignin are broken by the solvents used in pulping process leading to small molecules that are soluble in alkali or acid or alkali.

Lignin can be removed by various Physicochemical treatments: Adsorption, Coagulation and flocculation, Ultrafiltration, Chemical oxidation, Ozonation, etc. Although these are well-established methods, there are some disadvantages such as harsh operating conditions, Energy intensive, Environment impact, production of toxic compounds, Complexity, and many more. The products derived via chemical degradation of lignin are difficult to valorize because these products are highly heterogenous and variable. Moreover, the by-products produced during physicochemical treatment cause serious genetic and reproductive disorders, skin irritation, respiratory chronic diseases, and many more.

One of the most practical and widely used techniques for lignin removal and color from industrial effluents is a biological method that includes the treatment of wastewater with microbial enzymes. These enzymes include laccase, xylanase, and peroxidases. These enzymes are environment-friendly, also cost-effective, and have high productivity. White-rot fungi are considered to have great potential for the degradation of lignin as compared to other microbes because of having powerful degrading enzyme system. The P&P mill

effluent have been treated for the degradation of lignin by the use of certain fungal cultures which are *Phanerochaete chrysosporium, Tinctoria borbonica, and Schizophyllum commune (Parveen et al., 2022*). However, due to its high complexity and lack of ability to survive in diverse environments, many researchers are more focused on the lignin degradation by bacteria. Using pure bacterial strains, many studies have been conducted for lignin biodegradation. The maximum amount of COD removal and degradation of lignin achieved from *Aeromonas formicans*, *Pseudomonas putida, Bacillus sp,* and *Acinetobacter calcoaceticus* is about 70-80%. The study on thermophilic bacteria for lignin degradation found in P&P mill effluent is relatively rare as compared to mesophiles. Some thermophilic bacteria *Serratia sp*. AXJ-M, *Bacillus licheniformis*, and *Bacillus Subtilis* were reported to degrade the Kraft lignin found in P&P Effluent.

Degradation of lignin occurs in two steps (1) non-specific extracellular depolymerization of lignin to aryl and biaryl compounds, and (ii) mineralization of the latter by particular catabolic pathways and enzymes (Radhika et al., 2022). Degradation of these aromatic compounds supplies microbes with both energy, and carbon resources and might aid in the increased transformation of lignin to high-value products. The majority of lignin is typically composed of β-O-4 bonds, which makes the β-Aryl ether (β-O-4) pathway crucial to the depolymerization of lignin. Certain ligninolytic enzymes produced by microorganisms facilitate the removal of the benzyl hydroxyl group, leading to the formation of free benzyl cation. Once the structural unit of the β-aryl ether loses the benzyl hydroxyl group, the benzyl cation will create a new C-C cross-linkage. Finally, the ring cleavage compounds are transformed into organic acids that may enter the microbes' central carbon metabolism.

Lignin valorization has drawn a lot of interest due to its potential to build a more sustainable biobased economy. Transformation of lignin into value-added products can lessen the environmental effect of industries that generate lignin as a byproduct and provide substitutes for valuable chemicals. There are two approaches to obtaining these products: one is by pretreating the lignin, and the second is by entering the metabolic pathways. The pretreatment may be chemical/enzymatic to extract the lignin derived aromatic compounds. However, Microorganisms are the best choice to handle the variability, toxicity, and

inherent recalcitrance of lignin. These compounds will then be converted through the metabolic pathway. Lignin-derived products can be divided into three categories based on their present and potential uses: low-molecular-weight aromatic chemicals (such as Vanillin, gallic acid, Syringaldehyde, ferulic acid, caffeic acid, etc.), macromolecules (such as bioplastics) and power, fuel, and syngas products (such as lipids, biodiesel, etc.). Overall, the economic effectiveness of lignin valorization depends on the low prices of lignin removal and downstream separation procedures. The development of biotechnological technologies like metabolic engineering and synthetic biology for extracting high-value chemicals from lignin could serve as a driving force to increase their production yields and design new bioconversion pathways, lowering the cost of the finished products and broadening and diversifying the range of lignin-derived products.

Aim and Objectives

The aim of this study was to investigate the role of thermophilic bacteria isolated from hot spring in lignin Valorization.

Objectives

The objectives of the study are:

- Screening of thermophilic ligninolytic bacteria from hot spring.
- Evaluation of the selected strains of bacteria for their lignin degradation efficiency.
- Toxicological studies of untreated and treated black liquor
- Evaluation of lignin-derived metabolites via GC-MS
- Extraction of value-added products from lignin and their analysis through HPLC

Literature review

2.1 Background

The Pulp and Paper (P&P) industry is among the most significant sectors in the world. Cellulosic fibers and other plant-based sources are used in the production of paper, while some synthetic elements could be added to this process to give the finished product with special qualities. Paper pulp is a raw ingredient for making paper that contains natural, mineral, or synthetic fibers. Wood fibers are commonly used in the production of paper that serve as a source of cellulosic fibers, however, other materials, such as bagasse (a sugarcane residue), rags, cotton liners, and flax, can sometimes also be used for this purpose. Wood fiber is composed of cellulose, hemicellulose, lignin, and extractives. By dry weight, cellulose makes up around 50% of the wood, which is crucial in paper production (Debnath et al., 2021).

The P&P industry is one of the biggest producers of wastewater globally and a major contributor to water pollution. The production of lignocellulosic waste from the industries: agricultural waste (200 billion tons annually), P&P industry (effluent 150–200 m3/ton), and the food industry (1.3 billion tons annually) possess a major threat to the environment and human health. Among this lignocellulosic waste, lignin is an undesirable polymer, and it is removed during the pulping process because its presence contributes to the yellowness of paper and also decreases its durability, but it takes a lot of energy and chemicals to remove it during pulping. The P&P industry utilizes a tremendous amount of fresh water and inorganic substances (Sodium carbonate, Sodium hydroxide, and chlorinated compounds), and produces extremely contaminated discharges effluent that is dark brown and has high BOD and COD along with 30- 35% Inorganic substances (toxic chlorinated compounds, inorganic salts and other salts combined with organic matter) and about 60-70% organic matter (resins, starch, lignin, and various other low molecular compounds) during the pulping, bleaching, and washing procedures. The kraft pulping accounts for about 90% of pulp production (Dessbesell et al., 2020). This process generates lignin in the form of BL. Although there is considerable potential for recovery of lignin from this waste stream, it is typically burned in recovery boilers to produce steam energy and recover chemicals used further for pulping. In the P&P industry, lignin of about 50 million tons is

produced annually as a byproduct of the pulping process. However, only 2% of it is utilized for production of value-added products, and the remainder is burned as an inefficient fuel (Singhania et al., 2022). Therefore, to reduce waste, GHG emissions, and reliance on fossil fuels, researchers must focus on lignin valorization, which may present a compelling opportunity to advance the production of environmentally friendly and high-value products.

2.2 Pulp and Paper Industry

2.2.1 Global Paper Industry

Due to a change in consumer behavior and growing digitalization, the paper sector has undergone significant transformation in recent decades. Newsprint production has decreased to less than 4% as a result of the shift towards electronic media platforms just 20 years ago. Moreover, writing and printing papers have also been negatively affected by the 25 % decrease in annual worldwide production since 2010 and drastically decreased at the time of Covid 19 pandemic. However, Despite being in the digital age, paper still plays a significant role because it is a material that is widely utilized every day for a variety of purposes globally. Paper and cardboard are produced greater than 400 million metric tons annually on a global scale. Nowadays, Packaging paper is the most produced type of paper as its demand has risen recently as a result of the rise in online shopping. In 2021, The global production of paperboard and packing paper rise to 264 million metric tons (Statista, 2023), and its market size was valued at around US$ 203 billion and is anticipated to reach about US$ 243 billion by 2030. In total, the market size for paper products grew at a CAGR of 6.4% from US $974.14 billion in 2022 to US $1036.59 billion in 2023 grew.

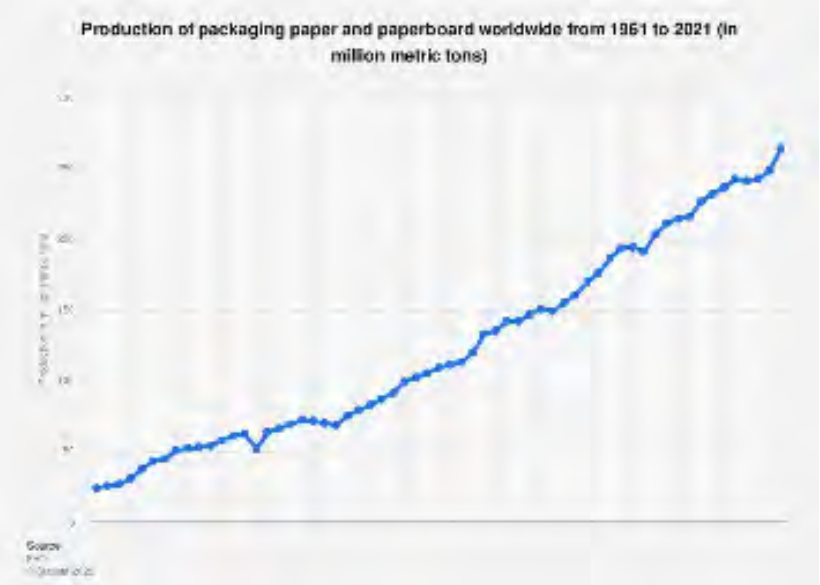


Figure 2.1 Paper and paperboard production trends globally from year 1961-2021 (ReportLinker,2023)

2.2.2 Paper Industry in Pakistan

The paper industry of Pakistan comprises more than 57 P&P mills in Pakistan, with a total installed capacity of approximately 1,050,499 metric tons per annum (Shabbir et al., 2022). Some of the major Pakistani paper industries are Premier Paper Mill Ltd, Century Paper & Board Mills Ltd, and Packages. According to the World Bank report, Pakistan's forest area accounts only 4.8% of the nation's total land area (World Bank, 2022), indicating a dearth of forest resources required for paper production. Therefore, Argo-based raw materials such as crop straws, cotton linter and bagasse, are the frequently and, in mostly cases, a mixture of these raw materials is used in the production of paper. In this regard, wheat straw makes up approximately 46% of the fundamental raw materials, followed by wastepaper 29%, and imported pulp make-ups around 10% of the total. All of these commodities are extensively used in the manufacturing of specialty-grade products.

Punjab accounts for more than 70% of P&P mills, followed by Sindh with 20% and Khyber Pakhtunkhwa with 10%. The type of papers produced in Pakistan are Printing and writing paper, Liner board, Packaging paper, tissue paper, Corrugate medium, and Continuous computer reels.

Pakistan is among the few nations in the world where paper and paperboard products are produced using agricultural waste and a Neutral sodium sulfite cooking procedure. Due to Covid 19, Pakistan's paper and pulp industry suffered a lot. The maximum export of paper and paperboard was about US$100 Million in 2017, which decline to about US$90 in 2019 and US$46.17 in 2020 because of the pandemic and again started to rise to about US$64.1 in 2021 (Trading Economics, 2023). However, the paper packaging market has remained largely constant. The segment's demand remained essentially constant despite the COVID-19 pandemic's effects on the economy because of its position in the supply chains of numerous crucial goods. In addition, the country's ban on plastic bags has aided the market. The market for paper packaging is anticipated to expand at a CAGR of 3.5% from FY20 to FY26. The output of paper has increased by about 2% annually over the past five years, as shown in the graph. Additionally, steady output levels have been evident since FY18. Given that it accounts for a sizeable share of the production of all paper and board, illustrating the stability of the paper packaging industry.

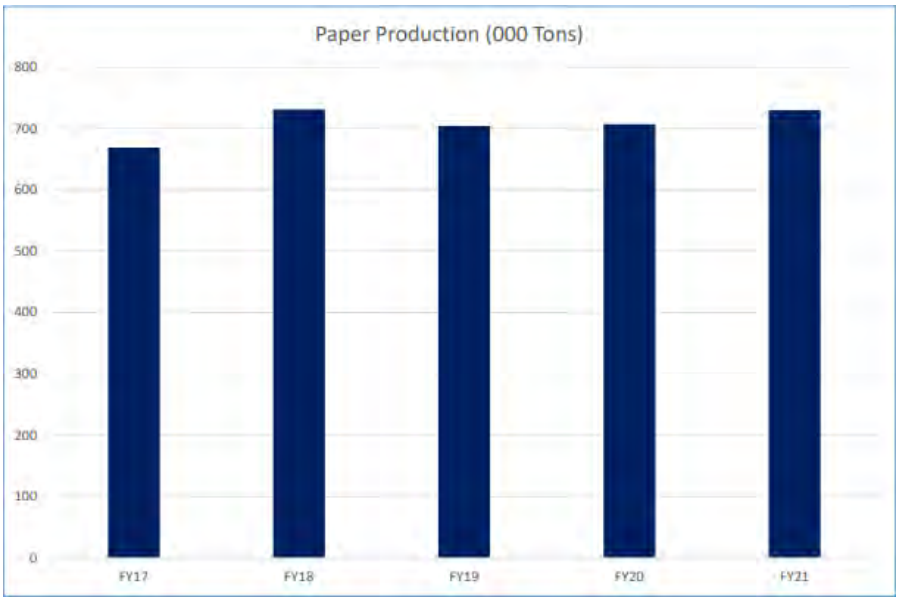


Figure 2.2 Graph showing the paper production trends from year 2017-2021 in Pakistan (Pacra, 2021)

2.2.3 Water consumption in Pulp and Paper industry

The P&P industry has always been the biggest water-consuming industry, requiring about 50–60 m3 of water (Pathak and Sharma, 2021) on average to produce one metric ton of product (pulp or paper). Every step-in paper production, i.e., from raw materials preparation to finishing and coating requires a substantial amount of water. Water impurities impact directly on paper quality during the production of pulp and paper by interfering with processes like bleaching, sizing, and coloring (Shiyue et al., 2021). Therefore, the P&P industry not only requires water in abundance but also of a high quality that is on par with or better than that of municipal sources. The P&P industry relies on two main sources of water: surface water and groundwater. However, the shortage of water and the high cost of treating wastewater for reuse due to energy-intensive processes put pressure on the sector to improve water efficiency and reduce its water impact.

2.2.4 Waste discharge from Papermaking process

The P&P industry used a large amount of energy as well as resources such as wood and water. These mills generate approximately 220 to 380m3 of highly colored, potentially contaminated, and hazardous wastewater per ton of paper produced (Rather et al., 2022). These pollutants are released directly into the water bodies without any treatment as a result, there are significant effects on aquatic life., human life, and the atmosphere. The additives, the feeding water, and the fibrous raw material used in paper production are major sources of contamination in the water that produces wax, lignin and its derivatives, dioxins, resins, organic chlorides, and many more. It is widely known that the paper manufacturing process pollutes the environment to a high degree both in terms of discharge volumes and composition.

Pulping is the first step and main source of pollution in this industry. The process of pulping involves purely chemical or physical methods or effectively combining physical and chemical processes to break down and separate wood fibers into pulp and then turn the pulp into a finished product. However, the process yields a dark brown toxic by-product known as "black liquor."

Table 2.1 Showing the pollutants present in wastewater released in different paper production stages (Sharma et al., 2020).

Process stages	Wastewater (v)	Pollution load	Pollutants
Raw material preparation	Low	Low	Suspended solids including bark particles, fiber pigments, dirt, grit, BOD and COD
Pulping	Low	High	Color, bark particles, soluble wood materials, resin acids, fatty acids, AOX, VOCs, BOD, COD and dissolved inorganics
Bleaching	High	High	Dissolved lignin, color, COD, carbohydrate, inorganic chlorines, AOX, EOX, VOCs, chlorophenols, and halogenated hydrocarbons
Papermaking	Depends on the extent	Low	Particulate wastes, organic and inorganic compounds, COD and BOD

2.3 Black liquor Composition

Black liquor is composed of two main components: organic and inorganic. The organic content makes up 2/3 of its composition, with inorganic making up the remaining 1/3. The main organic constituents found in black liquor include extractives, lignin, and polysaccharides. Among the inorganic compounds are sodium thiosulfate, sodium sulfide, sodium chloride, sodium hydroxide, and sodium carbonate.

The composition of this waste mainly dependent on two significant factors: (1) the raw material used for paper production, such as hardwood, softwood, or fibrous plants, and (2) pulping condition. The composition of black liquor differs significantly, in terms of lignin and hemicellulose concentration, if the raw material used in pulping is wood or non-wood (Esmaeeli et al., 2023). For instance, Agro-residue black liquor includes approximately 8 to18% hemicellulose and 28 to 32% lignin, whereas eucalyptus black liquor comprises 40-42% lignin and just 1-2% hemicellulose. Moreover, the remaining components remain the same in the case of both these types of black liquor (Bajpai, 2018),

Hardwood contains high methoxy content (21%) than softwood (15 – 16%) and is commonly used in the P&P industry (Lourenço and Pereira, 2017). During pulping steps such as sulfite or kraft pulping, the methoxyl groups present in lignin are replaced by hydroxyl groups. This reaction produces free radicals, which then dissolve lignin to produce volatile chemicals like mercaptans, methanol, etc, that give off an unpleasant odor.

These ionizing groups rely on pH; they lose their ionic characteristic, and immediately unionize leading to the precipitation of lignin at lower pH (2-4) (Melro et al., 2020). This is crucial in determining how viscous black liquor is; an increase in pH results in an increase in viscosity and vice versa (Morya et al., 2022).

2.4 Effects of P&P Industrial Effluent on the Benthic Environment

2.4.1 Bottom-Water and Sediment Deoxygenation

Pulp mill effluents after their discharge into the marine environment may have both short- and long-term hydrographic effects on areas that are receiving them. The dissolved organic content causes an immediate BOD, whereas the suspended solids result in long-term BOD. Both components have major consequences in regions when the value of effluent BOD is high or when the volume of water that is receiving it is small. The complete eradication of benthic fauna because of the formation of an anaerobic blanket deposit of wood chips and fibers at the sediment surface is one of the most significant consequences. Even if the conditions improve, i.e., increase in oxygen content or reduction in pollution, the presence of this anaerobic blanket will keep the anoxic conditions at the bottom. This same case happened on the Canadian west coast inlets.

2.4.2 Acute Toxicity

The negative impact of P&P mill effluent on living organisms and the environment is well-studied. The discharged wastewater contains 40 to 45% organic contaminants, such as lignocellulose, chlorolignins, chlorophenols, chlorinated resins, chlorinated phenols, fatty acids, biocides, and pesticides. These organic compounds might have serious toxic effects on the Aquatic life. Several studies reported cellular damage, anoxia-induced oxygen depletion, delayed sexual maturation, and endocrine disruption (Sharma et al., 2021). Studies reported that the refractory chemical compounds, such as chlorolignins, chlorophenols, and biocides in the effluent, bioaccumulate in the fatty tissues of tubifex worms and readily penetrate the cell membrane leading to the harmful consequences on the reproduction, physiology, and growth of these worms (Kumar et al., 2020). Moreover, exposure to higher concentrations can lead to the death of these worms. In addition to bioaccumulation, these derivatives can also reach the trophic level of the natural ecosystem

via the food chain. Numerous researchers have reported the presence of various chemicals in addition to chlorolignin compounds that are androgenic and carcinogenic. For example, Pentachlorophenol (PCP) is a well-known harmful contaminant and a potential carcinogen. These compounds have adverse or toxic effect effects on invertebrates and fish. USEPA (the United States Environmental Protection Agency) declared it a major pollutant and its concentration of about 1.0 mg/L is not considered safe for the environment (Singh and Chandra, 2019). Another study reported the toxicity of copper channel catfish and rainbow trout in the presence of calcium and high pH in the effluent of a Pulp mill (Dixit et al., 2019).

Moreover, Waste from the P&P industry has a direct or indirect impact on the food chain in relation to agriculture and fisheries. The effluent discharge in the waterbodies affects the growth and development of small fishes, plankton, and microbes. Several studies reported the presence of mutagens in the seals, roaches, and zooplanktons near P&P industry outlets.

2.4.3 Discoloration of Water

According to numerous studies, KL is a significant component of wastewater that contributes to toxicity and color appearance. Because of the complex three-dimensional structure and various ether and carbon-carbon bonds, similar to that of natural lignin, KL is particularly resistant to biodegradation or chemical degradation. KL is visible in water even at low concentrations (25 mg L-1), in contrast to the paper industry's effluent, which normally includes >200–600 mg L-1 KL and hence reduces the clarity of the water. This causes far more serious environmental issues in freshwater than it does in the sea but when these effluents are released into the fjords, there is a severe reduction in the phytoplankton population because of a decrease in the transparency of water (Panwar et al., 2020). Similarly, aquatic plants and algae growth are both affected by highly colored water because it reduces the process of photosynthesis as well as the oxygen level in the water (Singh et al., 2019). As a result, a brightly colored body of water would be unable to support aquatic life, potentially harming the ecosystem.

2.5 Effects of Paper and Pulp Effluent on Human Health

The wastewater generated by different processes such as pulping, bleaching, and digestion contains various hazardous compounds which enter the food chain and induce biomagnification. It is reported that the pollution generated by paper and pulp mills mainly affects the people living near these mills (Shah et al, 2021). These hazardous compounds enter humans via consuming fishes and cause serious health issues, which include modification of some metabolic activities such as an increase in lymphocyte levels, g-glutamyl transpeptidase activity, and increased excretion of 17- hydroxycorticosteroid. These toxic compounds also cause some skin diseases such as dermatitis, and folliculitis.

Lignin generates large amounts of chlorinated organic compounds in the bleaching process. These compounds induce bad effects when consumed by humans. 2,3,7,8-tetrachlorodibenzo-p-dioxin, the most hazardous chemical molecule, is responsible for acne in males (acneiform dermatitis). These compounds also enter in the cytoplasm and bind with the aryl hydrocarbon receptor (AhR). Moreover, a study reported the acute toxicity of KL, an active component of paper mill effluents, in Human keratinocyte cell line as evaluated by Reactive oxygen species production and cytotoxicity assay (Abhishek et al., 2017).

2.6 Lignin

Lignin is considered as the second most abundant biopolymer on the earth., makes up 30% of total non-fossil organic carbon (Yao et al., 2022). The P&P industry is the main producer of lignin. About 50 to 70 million tons of it are generated annually during paper production globally, the majority of which is kraft lignin.

The major lignin found in effluent produced by the P&P industry is burned as low-value fuel to produce electricity and heat, and < 2% is used to produce specialty chemicals like adhesives, surfactants, dispersants, and other high-value products. It is not extensively studied for industrial applications because of its high stability, and complicated structure (Sharma, et al., 2020). Thus, it is quite challenging to depolymerize lignin in an efficient, directed, and commercial manner.

Lignin is one of the significant components of plant's secondary cell walls along with cellulose, and hemicellulose, as it maintains the rigidity and integrity of plants by providing mechanical support, disease resistance, and stress response. Additionally, it is essential for the transportation of water and nutrients and water as well as for protecting plants from microorganisms. The relative percentage of lignin varies with plant types, their growth conditions, source, and biomass maturation. For instance, grass contains 17–24%, softwood 18–25 %, and hardwood 27–33% lignin content, respectively (Bajwa et al., 2019). These polymers are arranged into complex, and non-uniform 3D patterns where lignin acts as a natural glue and holds the lignocellulosic matrix together.

2.6.1 Monolignols

Chemically speaking, Biosynthesis of lignin occur via enzymatically catalyzed radical polymerization of its fundamental building blocks i.e., three phenylpropane units, namely monolignols (Khan and Ahring, 2019). These three monolignols include: p-coumaryl, coniferyl, and sinapyl alcohol, corresponding to the H unit (p-hydroxyphenyl), G unit (guaiacyl), and S unit (syringyl), respectively (Sun, 2020). The combination of these monolignols with unique patterns results in the diverse and complex lignin structure in various types of plants. The molecular structure of lignin found in grass and softwood is almost similar, however, in the case of hardwood, it differs significantly. The S/G ratio is frequently used to identify the various structural variations of lignin in hardwood, like condensation level, content of functional groups, and number of certain dimers. Accordingly, the S/G ratio in hardwood lignin is higher with greater methoxy groups as compared to softwood lignin. In hardwoods, the fraction of sinapyl (S) (45-75%) is higher than the coniferyl (25-50%), however, coniferyl (G) is entirely dominating (90–95%) in softwood (Haq et al., 2020).

In addition to the above-mentioned units that are the main building block of lignin, several functional groups found in its side chains have a significant impact on its reactivity (Wang et al., 2023). These include benzyl alcohol methoxyl, carbonyl, phenolic hydroxyl, and various terminal aldehydes.

2.6.2 Inter-unit linkages

In contrast to many natural polymers having only a single inter monomeric bond, lignin is an amorphous 3D copolymer in which its monomeric units are connecting in multiple patterns via several ether bonds (C-O-C) i.e., condensed bonds like α-O-4, β-O-4' and 4-O-5' and non-condensed bonds such as 5–5', β–β', β-5' and β--1'.

In lignin, β-O-4 is the highly abundant linkage, occurring roughly 45–50% in softwoods and 60–85% in hardwoods. This high abundance is most probably because of the extreme reactivity of β-carbon and phenoxy oxygen, and this linkage is easier to cleave. The β-1 (diphenyl ethane dimers) is highly stable and makes up about 5 to 10% of the total linkages present in lignin. The 5-5' undergoes α-β-O-4-4 coupling and forms dibenzodioxocine. The 4-O-5 oligomers and dibenzodioxocine act as branching points and give lignin molecules their characteristic helical shape.

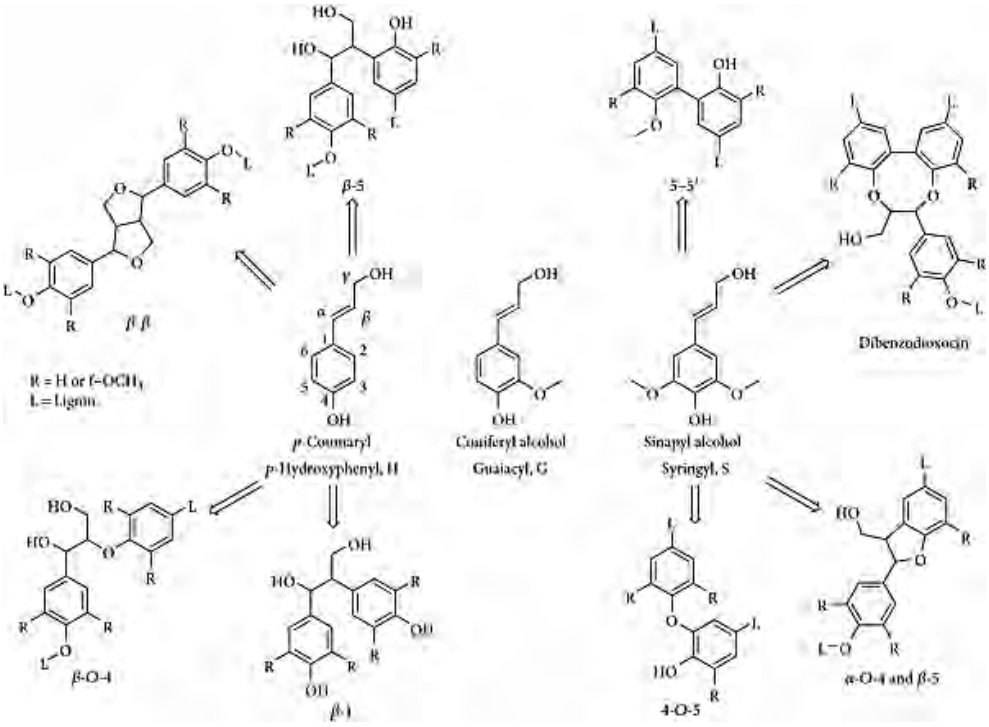


Figure 2.3 Showing the monolignols and Inter-unit linkages found in lignin (Melro et al., 2021)

Table 2.2 Showing the % of different Linkages found in Hardwood and softwood (Ahmad et al., 2020)

Linkages	Structures	Hardwood (%)	Softwood (%)
β-O-4	β-Aryl ether	45–65	42–50
β-5′	Phenylcoumaran	5–6	9–12
5-5′	Biphenyl	4–10	9.5–25
4-O-5′	Diaryl ether	6–7	3.5–8
α-O-4′	α-Aryl ether	4–8	6–12
β-1′	1,2-Diaryl propane	5–7	3–10
β-β′	Resinol	3–6	2–3
Dibenzodioxocin	Diary propane diether	1–2	3–7
Spirodienone		3–5	1–2

2.7 Different types of lignin found in black liquor

In paper and pulp industries, Different types of lignin with different chemical properties such as molecular structure, molecular weight, and composition may be produced, depending on the types of methods used to remove lignin. Additionally, they differ in terms of solubility, hydrophilicity, and hydrophobicity (de Souza et al., 2020). Pulp mills are one of the primary industrial sources of lignin. Its extraction processes, depending on the technique used, may take place via various organic solvents or acidic or alkaline media. Through these processes, the lignin is gradually catabolized into fragments of low-molecular weight. A considerable degradation and modification in the native lignin structure may occur, depending on the technique utilized, with a reduction in the quantities of aliphatic β-β, β-O-4 bonds, and OH groups. Furthermore, it is possible to produce degradation products such as phenolic hydroxyl groups, carbonyl groups, and carboxylic acids. Therefore, the extraction process and the source raw material (grasses, hardwood or softwood, hardwood) both have a major impact on the structure of technical lignin.

2.7.1 Kraft lignin

It is the most prevalent type of industrial lignin, accounts for around 85% of total lignin generated globally (Sameni et al., 2020). For lignin removal from other lignocellulosic biomass, the kraft process uses a mixture of several alkaline cooking chemicals (sodium

hydroxide and sodium sulfide), often termed as white liquor, as the principal reagent, at extremely high temperatures (140–170 °C). Due to the usage of various chemical reagents during the kraft pulping, KL may have some contaminants in its structure, such as sulfur that sometimes makes its use unfeasible.

Generally speaking, KL differs from native lignin in a number of ways, such as condensation at the C5, the primary alcohol γ-elimination, the stilbene and vinyl ethers formation in the phenolic unit, the β-aryl ether cleavage, the α-ethers hydrolysis in the phenolic unit and a greater number of phenolic hydroxy groups (Giummarella et al., 2019). Moreover, the KL structure possesses a very low number of hydrophilic groups due to which its solubility in water is low. Up to 30% of Ash is also found in kraft lignin, which is further reduced by treatment with sulfuric acid (Marson et al., 2023). Due to the increased commercial availability of kraft lignin in recent years, research, and investigations on it have increased.

2.7.2 Soda Lignin

It is a byproduct of the soda anthraquinone process, industrialized in the 19th century for non-wood plant fibers (like flax, straw, etc.) applications. It is a sulfur-free process; therefore, its composition is more similar to natural lignin as compared to KL. In developing nations (Asia and South America), these materials have been employed as pulp sources and are currently used to produce high-yield pulps for packaging papers and boards (Tribot et al., 2019).

In the soda pulping process, lignin is removed from lignocellulosic material by using 13 to 16% NaOH at pH 11 to 13 (Kazzaz and Fatehi, 2020). It is reported that all soda lignin possesses a low-molecular weight, high phenolic hydroxyl groups, and a somewhat low (and varied) glass transition temperature. Moreover, it has higher solubility in water as compared to Kraft lignin. Nevertheless, the contaminant concentration of soda lignin (such as sugar and ash) affects its thermal behavior. These contaminants could serve as plasticizers or anti-plasticizers. The conditions and origin of feedstock may also affect its thermal behavior. For instance, wheat straw yields soda lignin with a higher Tg than hemp. Additionally, it may contain silica and nitrogen since non-wood fibers are the main crops

utilized in the process. Therefore, high silica content makes it difficult to recover soda-lignin from these fibers as it may co-precipitate with lignin leading to the reduction of its quality and purity. Soda lignin has been used in the manufacture of dispersants, polymers, and phenolic resins. However, the use of soda pulping in pulp mills is declining because of its poor pulp yield performance.

2.7.3 Sulfite Lignin

It is another widely used method, based on cooking (acidic or neutral) of wood chips with sulfites or bisulfates salts and bases. These reactions are typically carried out at temperatures 140-160 °C. During this process, lignin is cleaved, resulting in a formation of sulfonated molecule with a range of functional groups that provide it with unique colloidal characteristics. Certain types of bonds, such as α -O-4 can be selectively broken down by this process while keeping a considerable fraction of the β-O-4 bonds intact. Due to the reaction mechanism of this process, lignosulfonate fragments possess a high molecular weight, high ash content, and high-water solubility as compared to lignin generated from other delignification techniques (Vásquez-Garay et al., 2021). After kraft pulping, it is the most commonly used industrial process, generating around 7 million tons of sulfite lignin per year. However, the rise of kraft pulping has replaced sulfite lignin production in Europe, North America, and Japan, but it is increasing in China and India. Until recently, LS was utilized solely as a dispersant in the manufacture of coal briquettes, ceramics, and resins (Lugovitskaya, 2022). The shift to green technologies has significantly boosted the research developments related to examining the structure and properties of LS, consequently broadening the scope of application. On the basis of LS, it is now possible to develop drug delivery systems such as graphene-like 2D films, an electrochemical material for the storage of energy, plant growth bioregulators, biodegradable conductors, and so on.

2.7.4 Organosolv Lignin

Organosolv pulping is the extraction of lignin from lignocellulosic biomass by utilizing organic solvents or their solutions. Various organic solvents, like methanol, acetone, ethanol, or their mixtures, are employed to dissolve lignin at elevated temperatures (100-250 °C) (Brosse et al., 2017). In this process, the α-O-aryl ether linkages cleaved easily (a

rate-controlling step) as compared to β-O-aryl ether linkages, which are generally cleave under more extreme conditions, particularly at high acid concentrations. Lignin extracted via the organosolv procedure may have a high molecular weight (2000–9000 Da), chemical reactivity, high purity, sulfur-free, and non-toxic. Moreover**,** It possesses a low molecular weight, a polydisperse structure, and a homogenous structure, similar to native lignin. Typically, lignin extracted using this method is less contaminated than lignin extracted using other methods. Additionally, organosolv lignins are hydrophobic, highly soluble in organic solvents but nearly insoluble in water**.** The method for extracting this lignin as well as the expense of solvent recovery is prohibitively expensive, making it difficult to scale up to an industrial scale. According to some research, increasing the intensity of organosolv processes reduces the molecular weight of recovered lignin by 36-56% as compared to untreated lignin.

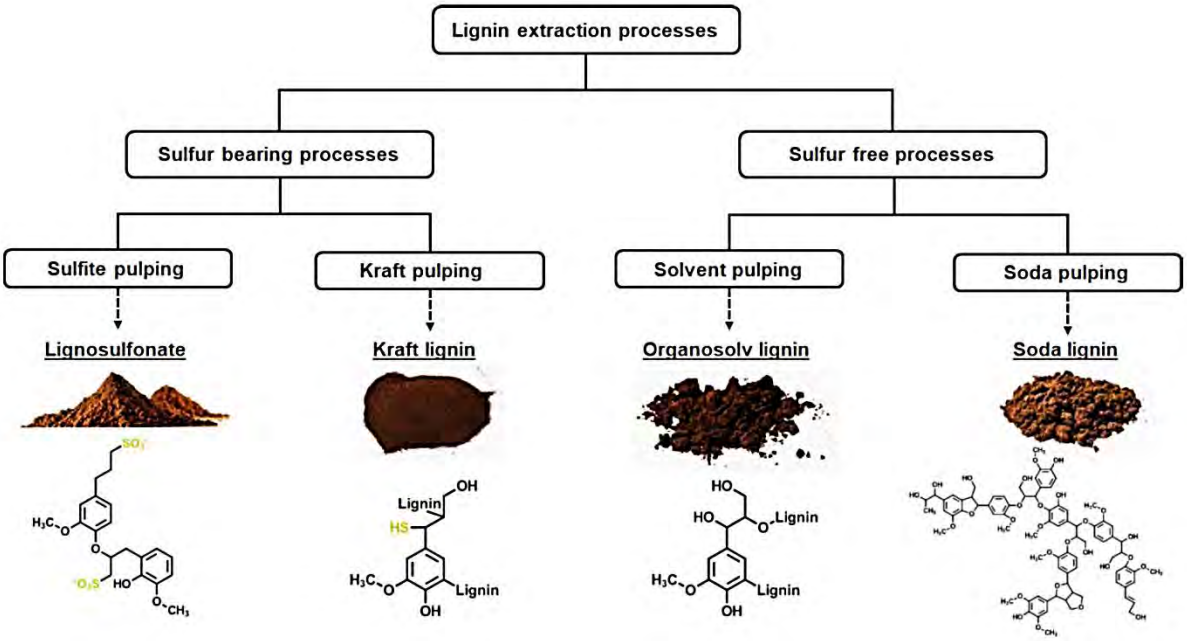


Figure 2.4 Showing the different types of lignin found in Black liquor (Fabbri et al., 2023).

2.8 Methods for Lignin Removal

Wastewater discharges from the P&P industries are extremely contaminated with Complex hazardous compounds. A high color index is the primary indicator of visible contamination.

This color comes from wood, tannins, resins, synthetic dyes, lignin, and its degradable products. Lignin is responsible for turbidity, a high color index, and a high COD. Lignin is an aromatic molecule that is challenging to biochemically oxidize in the effluent treatment facilities present at almost all paper and pulp plants. Lignin, due to various conformations, has a variety of removal methods. For lignin recovery and removal of lignin, many strategies have been developed, which are categorized into physicochemical and biological methods.

2.8.1 Physico Chemical Methods

For the lignin removal from the effluent, various physicochemical procedures such as filtering, adsorption, precipitation, ozonolysis, adsorption, advanced oxidation process, and reverse osmosis have been used, which are as follows:

2.8.1.1 Coagulation and Flocculation

The principle of this technology is the usage of metal salts to create larger flocs from small particles to remove contaminants easily. Among all other chemical methods, this method is regarded as one of the most simple, economical, and effective to remove lignin from wastewater. It has been widely used to treat wastewater from the P&P mill, textile wastewater, palm oil mill effluent, and abattoir wastewater.

The coagulation process is divided into four steps starting with enmeshment, followed by adsorption, charged neutralization, and precipitation. In a more simplified way, it consists of two major steps i.e., charged neutralization and sweep flocculation. In sweep coagulation, contaminants are enmeshed and adsorbed to cationic metal hydroxides, leading to precipitation of organic pollutants. Charge neutralization occurs When positively or negatively charged particles are blanketed by counter-ionic particles. Several chemicals such as polyethyleneimine (PEI), horseradish peroxide (chitosan), epichlorohydrin polycondensate (HE), and hexamethylene diamine have been reported as effective chemicals for the removal of color, AOX, and total organic carbon (Surabhi Zainith et al., 2018). Inorganic coagulants like Ferric chloride and alum are also studied to remove organic contaminants due to their effectiveness. These traditional coagulants, however, are unable to meet the rising need for organic waste removal, which is why

researchers started to look into natural polymers as coagulants. Moreover, A study reported Poly (ethylene oxide) as an effective flocculant for pre-hydrolysis liquor (Takahashi et al., 2020). Additionally, using an acidification process along with flocculants such as Poly (ethylene oxide) may result in a reduction in the number of flocculants needed to generate the precipitation and even an improvement in the removal of lignin (Domínguez-Robles et al., 2019). Similarly, it is reported that corn stover pretreatment using metal catalysts such as MgO, Fe2O3, NIO, ZnO, and CuO reduced sugar depletion and boost lignin removal (Li, 2020). MgO (Magnesium oxide) was shown to be the most effective catalyst for the pretreatment of biomass among the five metal oxides.

Nowadays, electrochemical technologies are increasingly in demand for wastewater treatment using physicochemical approaches. Electrocoagulation is a method that involves electro-dissolving the soluble anodes to form a metallic hydroxides floc inside the wastewater. The advantage of electrocoagulation over conventional coagulation and flocculation technique is that it is capable of coagulating the smallest particles because of electric fields that cause them to move (Pandey and Thakur, 2020). The removal of phenol and lignin from pulp and paper industry effluent by using aluminum (Al) electrodes is found to be more effective. Electrocoagulation is characterized by simple operation, a reduced amount of sludge, minimal equipment for adding chemicals, and a short reactive retention period.

2.8.1.2 Adsorption

Adsorption is another technique for treating industrial effluent, however, it seems to be employed rarely in the P&P industry. It is a method used for the removal of both organic and inorganic pollutants from waste effluent. In this process, the contact between the adsorbable solute-containing solution and the solid (highly porous surface) results in the deposition of solute molecules that were present in the solution at the solid surface because of a liquid-solid intermolecular force of attraction. The solute that is deposited on the solid is termed as adsorbate,whereas that solid is termed as adsorbent. The various adsorbents used in this process are activated carbon, activated coke, coal ash, activated charcoal fuller’s earth, silica, etc.

Activated charcoal is reported to be the most efficient adsorbent Although high material cost prevents widespread its application. A study reported 57% and 90% maximum adsorption of lignin onto activated charcoal respectively. Likewise, fly ash was reported to remove lignin by 41% and 91% respectively (Andersson et al., 2011). Moreover, A study reported that slag and blast furnace dust can remove lignin up to 61% and 80.4%, respectively (Li et al., 2022). Similarly, it is reported that almost 89% of color and 84% of COD is removed from bleached water by using absorbent i.e., activated coke (Mehmood et al., 2019). About 36.75% lignin removal was reported after XAD-16N adsorption, which quickly rose and reached 89.84% at 5 g/20 mL (Li et al., 2020).

2.8.1.3 Chemical Oxidation

In paper and pulp mills, several advanced oxidation techniques, such as ozonation, Fenton-type reactions, photocatalysis, wet oxidation, photo-oxidation, etc., are employed to destroy both non-chromophoric and chromophoric contaminants. These advanced oxidation processes aimed to degrade recalcitrant organic compounds which are even difficult for microbes to break. These processes have been found to lower the toxicity, organic load, and content of refractory chemicals such as AOX from P&P Effluent. Such remediation methods involve the production of non-selective and extremely reactive hydroxyl (OH) radicals that attack these organic contaminants. The significant drawback of using AOP is the substantial consumption of chemical and electrical energy.

For the bleaching kraft mill effluent treatment, the photo-Fenton and Fenton processes are reported to be quite successful. The Fenton process is a rapid and effective AOP that works by catalytic dissociation of O2 (the oxidant) into hydroxyl (OH) radicals, which can then oxidize strong organic contaminants (Ribeiro et al., 2020). The principal drawbacks of this process are its high chemical costs and the production of iron-oxidized sludge, which necessitates a management plan and additional costs to handle or dispose of. Similarly, the effectiveness of ozone towards color, COD, and hazardous chemicals is investigated by many researchers. About 95-97% of color reduction can be noticed if, during ozonation, the concentration of ozone is kept high for up to 15 minutes. Moreover, TiO2 is one of the most commonly used and investigated photoanode materials due to its low cost, non-toxicity, and potential oxidizing capacity (Ahmed et al., 2022). It is reported that TiO2

together with Au has the potential to increase photo electrocatalytic activity, resulting in a 63.5% COD reduction and a 44.4% TOC decrease (Vidal et al., 2021).

2.8.1.4 Membrane Technologies

One of the most promising approaches for the recovery of lignin is membrane technology. The ability of membranes to recover lignin from wastewater has been investigated in several research. The membrane-based systems are found to be highly effective at removing color, COD, inorganic and organic matter, microorganisms (viruses and bacteria), TDS, AOX, salts, and heavy metals. The most common membrane filtration techniques are nanofiltration, microfiltration, ultrafiltration, electrodialysis, and reverse osmosis. In the first step, lignin is precipitated by the addition of chemicals such as sulfuric acid, chlorine dioxide, and carbon dioxide. After precipitation, lignin is isolated via membrane filtration. These techniques differ in the type of pollutant, their capacity, and the strength of the driving force utilized to promote separation.

With the typical effectiveness of membrane technologies being 50-90%, reverse osmosis was able to achieve the highest removal of 89% of COD5 and 88% of BOD (Dagar et al., 2023). On the other hand, the maximum color reduction and AOX achieved via ultrafiltration was reported to be 92% and 72%, respectively (Esmaeeli et al., 2023). Meanwhile, nanofiltration is reported to remove color and AOX by more than 90% (Mänttäri et al., 2021).

2.8.2 Biological Treatment

In terms of freshwater consumption, the P&P industry is relatively water-consuming industry. The growing requirement for the lesser use of water and to comply with severe environmental regulations has led pulp and paper companies to treat their wastewater using modern treatment technologies for safe disposal. Moreover, The physicochemical methods face significant obstacles, including the significant production of sludge, high capital expenditure, production of secondary byproducts, and reliance on energy sources, which limits their implementation. In response to the zero-discharge policy, industries are now employing modern, environmentally friendly, sustainable, and effective treatment methods. One of the practical choices is the biological treatment of wastewater which

includes the usage of microbes for lignin removal from effluent or the pretreatment of the lignocellulosic substrate. The biodegradation of lignin is regarded as a green and environmentally friendly method Since microorganisms do not produce secondary pollutants.

Microbes have a remarkable potential to transform waste effluents into energy and high-value products. These methods are environmentally friendly, cost-effective, very efficient, and do not require any chemicals as compared to physiochemical processes (Patel et al., 2021). Lignin, a major component of P&P mill effluent, is a recalcitrant compound due to its complex structure, as it contains random covalent bonds that make it difficult for microorganisms to degrade it. However, some Microorganisms have a special strategy to get over this barrier for complicated lignin compounds, though. Therefore, to lower the burden of organic compounds in the wastewater, it becomes necessary to investigate the potential of ligninolytic microorganism.

2.8.2.1 Fungal Treatment

Fungi have been widely studied for their ability to degrade lignin into associated monomers. They are interesting bioagents for the detoxification of effluent from P&P mills due to the production of various ligninolytic enzymes. With the help of extracellular enzymes they secrete, fungi can thrive in sludge and effluent containing significant chemical loads. The most widely studied fungi are *Aspergillus niger*, *Fusarium sambucinum*, *Phanerochaete chrysosporium*, *Rhizopus oryzae,* and *Merulius aureus.* Among them, *Phanerochaete chyrosporium* and a few other brown rot fungi are commonly employed to remediate the effluent from P&P mills. White rot fungi are reported to be the only microbes that completely degrade the lignin, making them the best lignin degraders. The application of white-rot fungus in the of P&P mill wastewater treatment was first reported by Fukuzumi in 1977. It was cultivated in submerged culture conditions using a liquid medium that contained nutrients, vitamins, and spent liquor from alkali extraction. (Hou et al., 2020) studied the synergistic effect of the Biological method, and physiochemical method for the lignin degradation. Enhanced degradation was reported to degrade lignin at rates of 89%, 86%, and 82% at 4 V, respectively.in the presence of applied voltage for the Fenton reaction 4V and white-rot fungi. *L. edodes, T. versicolor, P.*

chrysosporium, and *L. edodes*. These rates were about 65-70% greater than those obtained with fungus or applied voltage alone. Moreover, (Yang et al., 2019) reported enhanced lignin degradation after the inoculation of *P. ostreatus* S18 and *T. hirsuta* S13 to Tabacco stalk composting. Following LDM inoculation, LiP, MnP, and Lac activity increased by 3.5, 2.9, and 3.1 folds, respectively. As a result, the lignin degradation ratio increased from 23.7% to 41.1%.

Coriolus Versicolor possesses the feature of degrading wood components which include hemicellulose, cellulose, and lignin at the same pace. It is reported that in the presence of 0.5 percent sucrose, *Coriolous versicolor* in liquid culture eliminated nearly 80% of the effluent color in six days, without any changes in molecular weight or COD. Similarly (Li et al., 2020) reported the biodegradability of *Aspergillus Flavus* F-1. Following optimization, the 44.6% rate of lignin degradation was achieved within 3 days at pH 7.0, temperature 30 °C, 2 g L-1 lignin, and 0.5 g L-1 glucose. Moreover, (Lokeshwari et al., 2013) reported that after 8 days of incubation, the fungus *Aspergillus flavus* effectively biodegrades 94% of lignin and reduces 45% of COD levels. Similarly, (Kamali & Khodaparast, 2015) reported about a 52% decrease in lignin level in wastewater and 42% in color by *Gliocladium virens*. Another white-rot fungi *Bjerkandera adusta* belongs to the division Basidiomycota first found in Europe has gained biotechnological interest due to its ability to break down aromatic xenobiotics. (Costa et al., 2017) reported that this fungus begins lignin degradation i.e.,1 g/L per day (at optimum pH) without any lag phase. Moreover, (Rajwar et al., 2017) investigated the biodegradation of P&P effluent by co-culturing ascomycetous fungi, *Nigrospora sp,* and *Curvularia lunata*., in a repeat batch process. He reported a significant increase in biomass while the decrease of Lignin, chroma, BOD, COD, and lignin by 76.1%, 82.3%, 85.6%, and 80%, respectively.

Although fungi possess a very efficient ligninolytic system for the biodegradation of lignin but different factors such as pH, temperature, nutrient requirements, and production of toxic products lead to the reduction of their efficacy for lignin degradation. This is one big drawback of using fungi for wastewater treatment. Therefore, To take full advantage of the benefits of employing fungi for lignin breakdown sustainably and effectively, it is imperative that these issues be addressed.

2.8.2.2 Bacterial Treatment

As bacteria inherent "biological funneling" processes, bacterial systems have recently attracted increased attention on lignin valorization. As a result, the difficulties associated with lignin heterogeneity may be overcome. As compared to fungi, bacteria not only possess the ability of bioremediation but can also adjust themselves to unfavorable and diverse environments and that is the only major reason for giving priority to bacteria on fungi. The most commonly employed bacterial species that can degrade kraft lignin effectively *are Bacillus sp., Streptomyces, Pseudomonas fluorescens, Nocardia, Aeromonas formicans, Ochrobactrum*, and many more.

(Tan et al., 2022) investigated the potential of *Streptomyces thermocarboxydus* strain DF3-3 for lignin degradation by growing cells in a medium containing alkali lignin. He reported that The alkaline lignin degradation rate continued to increase from day 1 to 14 and reaches a maximum rate of 31% at day 15. Similarly, (Mei et al., 2020) reported 69.35% of COD reduction after 7 days of incubation and 28.55% of straw lignin after 14 days of incubation by *Bacillus amyloliquefaciens SL-7.* Similarly, *Bacillus ligniniphilus* L1 was reported to degrade about 38.9% (Zhu et al., 2017) of alkali lignin after the incubation of 7 days. (Chandra et al., 2012) studies the detoxification and decolorization of P&P wastewater at a laboratory scale by using mixed bacterial culture in different ratios which include 3 strains of bacteria i.e., *S. liquefaciens, S. marcescens, and Bacillus cereus.* Two ratios i.e., 1:4:1 and 4:1:1 was found to be very effective against wastewater pollutants. According to GC-MS and HPLC analysis, about 98% chlorophenol and 95% of lignin degradation by 4:1:1 ratio of mixed bacterial culture and about 84% and 58% of lignin and chlorophenol degradation after the incubation of 7 days was observed. Likewise, (Gaur et al., 2018) compared the lignin biodegradation and decolorization efficiency of consortia with axenic culture. Axenic culture (*K. pneumoniae* NITW715076_2) was found to be less effective i.e., 74.1% as compared to consortia (*K. pneumoniae* NITW715076_2 + *K. pneumoniae* NITW715076_1) i.e, 82.31%. Moreover, The mixed and axenic culture of *Citrobacter* sp. and *Citrobacter freundii* was studied for lignin, COD, and color AOX reduction. After the incubation of 144 hours, about 82% COD, 79% AOX, 60% lignin, and 60% lignin removal were observed by mixed culture (Shah, 2020).

The potential of psychrophilic bacteria for lignin-degradation is currently an active area of research. To overcome the issue of black liquor bioremediation in P&P industries at lower temperatures, a psychrotrophic ligninolytic bacteria was used in BL treatment for the very first time by (Wang et al., 2022). In this study, *Arthrobacter* sp. C2 showed remarkable lignin degrading abilities, with a lignin biodegradation rate of 65.5% for lignin (3g/L) at 15 °C. Additionally, strain C2 was able to remove 100% of the nitrate nitrogen (NO3--N) and 96.4% of the color from papermaking black liquid after 15 days at 15 °C.

2.8.2.2.1 Role of thermophilic bacteria

In recent years, bacterial lignin degradation in black liquor has received a lot of attention due to the bacteria's rapid growth, abundance of acquisition sources, biochemical diversity, environmental adaptability, Ligninolytic enzymes stability, and ease of industrial manufacturing and genetic engineering. Various ligninolytic bacteria have been identified, including *Pseudomonas, Bacillus, Paenibacillus, Comamonas, Rhodococcus, Pandoraea,* and *Anaerocellum.* For instance, *Bacillus altitudinis* SL7 isolated from P&P industry effluent was reported to degrades lignin at rates of 26% and 44%, respectively, at 40 °C (Khan et al., 2021); in addition, *Bacillus aryabhattai* reported to degrades lignin at rates of 54% at 32 °C (Singh et al., 2022). These bacterial species are unsuitable for treating effluent from papermaking since they do not thrive in high temperatures and harsh environmental conditions. Furthermore, thermophilic bacteria release extremely stable enzymes. Thus, thermophilic bacteria may be the best candidates for BL treatment in papermaking. However, the studies on these extremophiles' potential to degrade lignin are very limited. Recently (An et al., 2023) isolated the thermophilic ligninolytic bacteria *Serratia* sp. AXJ-M from P&P mill effluent that reported to degrade lignin 70.5% at 50°C.

2.9 Bacterial Ligninolytic Enzymes

The waste generated from many different industries is causing serious environmental pollution. The chemical methods somehow failed in the complete removal of these pollutants and these methods also generated secondary pollutants that are more toxic than the previous ones. One of the safe and alternative methods for the removal of these

pollutants is bioremediation in which microorganisms are used for the degradation. These microorganisms release extracellular enzymes that degrade these toxic compounds into nontoxic forms. The main ligninolytic enzymes that play an important role in lignin biodegradation are Peroxidases and laccase. To break down recalcitrant compounds, these enzymes possess their mechanism.

2.9.1 Manganese Peroxidase

Manganese Peroxidase is a heme-containing oxidoreductase enzyme having a molecular mass of about 40–50kD. MnP enzyme consists of 350 amino acids, 1 minor and 10 major helices, and a heme sandwiched between two domains. Its active site is maintained by 5 disulfide bridging elements and 2 calcium ions. Different amino acids such as aspartic acid residue bonded with hydrogen, proximal histidine ligand and arginine residues, and catalytic His in a distal side peroxidase-binding pocket are present in its active site (Kumar & Arora, 2022).

The oxidation of Mn+2 into Mn+3 is done in multiple steps by ligninolytic microorganisms. MnP is H_2O_2 dependent, and its catalytic activity starts with the oxidation of the heme group by H_2O_2 that results in the production of MnP compound 1 (oxyferryl porphyrin cation radical and H_2O_2). MnP compound 2 (oxyferryl chemical species) and a free radical ion is produced by the oxidation of substrate molecule via MnP compound 1. Next is the oxidation of Mn+2 into Mn+3 via compound 2. The final step is the Mn+3 dissociation from the enzyme and its stabilization via various organic acids (chelators) which are oxalate, glyoxylate, fumarate, and malate. Mn3+ is responsible for the oxidation of a variety of aromatic compounds via one electron or proton abstraction. Generally, the production of Mn3+ is via the oxidation of phenolic compounds, lignin and its derivatives, whereas in the case of non-phenolic compounds, the second mediator is required (Zainith et al., 2020). The overall catalytic process of MnP is as follows:

$$MnP + H_2O_2 \rightarrow MnP\ compound\ 1 + H_2O$$

$$MnP\ compound\ 1 + Mn^{2+} \rightarrow MnP\ compound\ 2 + Mn^{3+}$$

$$MnP\ compound\ 2 + Mn^{+2} \rightarrow MnP + Mn^{3+} + H_2O$$

2.9.2 Lignin Peroxidase

Lignin peroxidase is one of the most important lignin degrading enzymes, and its activity is detected in various bacterial species such as *Streptomyces* and *Acinetobacter.* It is also heme-containing enzyme just like MnP and is glycosylated and monomeric containing 343 amino acid residues, two calcium ions, and four carbohydrates having a molecular weight of 40–68 kDa (Chowdhary et al., 2018). It also consists of 2 anti-parallel beta-sheets, eight helices, and two domains present on both sides heminic group.

The catalytic mechanism of Lip is based on two steps and is H_2O_2 dependent. Just like MnP, there is the involvement of native enzyme's ferric resting state which are compound 1 (Singh et al., 2021). The main difference between Lip and MnP is that in the case of Lip the oxidation of non-phenolic compounds does not require mediators whereas MnP requires a mediator. The catalytic activity of Lip begins with the formation of compound 1 by the oxidation of native Lip enzyme i.e., two-electron oxidation via H_2O_2. The 2nd step involves the reduction of compound 1 by receiving one electron from a reducing substrate and converting it into compound 2. The last step is the reduction of compound 2 again into the native state by receiving one electron from a substrate (Solbiati et al., 2013). Sometimes, compound 2 converts into compound 3 when the level of H_2O_2 is high, and this compound 3 is the enzyme's inactive form.

The excellent substrate of Lip is Veratryl alcohol as it prevents the enzyme inactivation and oxidizes to Veratryl alcohol cation radical. The overall catalytic process of Lip is as follows:

$$[Lip] – Fe\ (III) + H_2O_2 \rightarrow [Lip]^{++}\text{- } Fe\ (IV) + H_2O$$

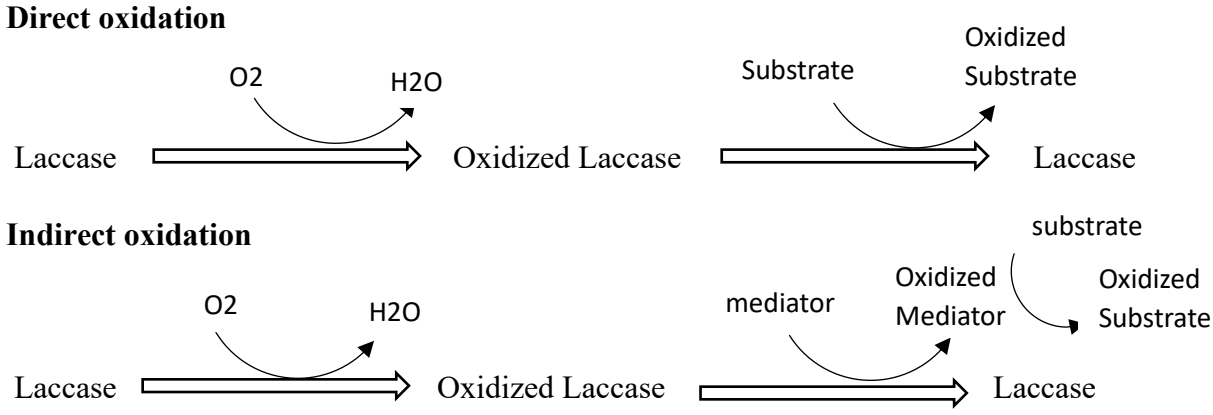
$$[\ Lip]^{++}\text{- } Fe\ (IV) + A \rightarrow [Lip]\text{-}Fe\ (IV) + A^{\cdot +}$$

$$[Lip]\text{-}Fe\ (IV) + A \rightarrow [\ Lip] – Fe\ (III) + A^{\cdot +}$$

2.9.3 Laccase

Laccase, an extracellular enzyme, belongs to the family of multicopper oxidase and consists of glycoproteins i.e., monomeric, dimeric, and trimeric. It is also known as p-diphenol oxidases or dioxygen oxidoreductase. Laccase enzyme has around three domains, 500 amino acid residues, and a Greek key barrel structure. According to molecular characterization, 150 amino acids are present in the 1st domain, the 2nd domain consists of 150 and 300 amino acids, whereas 300 to 500 amino acids are present in the 3rd domain. Four different types of copper i.e., blue Cu (T1), normal Cu (T2), and coupled binuclear Cu (T3) are found in laccase that maintains the amino acids active site. Laccase is the only enzyme that liberates only H2O as a byproduct, and it can oxidize a vast variety of compounds which is why it is referred to as "Green Catalysts". Recently some bacterial strains have been reported that secrete this enzyme, which are *Marinomonas mediterranea, Bacillus* sp., *S. lavendulae,* and *Pseudomonas* sp. (Ayodeji et al., 2022). Microbial laccases have higher efficiency as compared to plant laccases because they exhibit higher redox potential.

Two types of reactions are catalyzed by laccase. They are direct substrate oxidation and indirect substrate oxidation. In the first type, there is direct contact of the substrate with the copper that results in the substrate oxidation into a comparable radical. In the 2nd type i.e., indirect substrate oxidation, first a mediator is catalyzed by enzyme, and then the substrate is oxidized by this mediator.



2.10 Bacterial Lignin Degradation pathways

Bacteria have evolved numerous metabolic pathways to break down lignin and assimilate its aromatic component blocks. Similar to fungi, certain bacteria degrade lignin via various enzymes, including laccases, peroxidases, β-etherase , superoxide dismutases, cytochrome P450s, non-heme iron enzymes, and dioxygenase Although several well-known lignin-degrading fungi have a higher capacity for depolymerizing lignin than do bacteria, some of these bacteria are capable of making efficient use of depolymerized lignin, including lignin monomers, dimers, and various low-molecular-weight aromatic compounds. The degradation of lignin -derived aromatic compounds provides bacteria with energy and Carbon resources and aids in the increased conversion of lignin to value-added products. As the β-O-4 bonds often make up the majority of lignin structure, making the β-Aryl ether pathway a significant role in the biodegradation of lignin.

2.10.1 *β*-Aryl ether (β-O-4) Catabolic Pathway

The biodegradation of lignin-derived aromatic compounds has been investigated using oligomers and monomers that are frequently present in lignin. In lignin, the β-O-4 linkages predominate regardless of S, G, and H ratios. Thus, the β-aryl ether (β-O-4) pathway serves a crucial part in the breakdown of lignin-derived aromatics.

One of the most extensively researched bacteria involved in the biodegradation of lignin-derived compounds is *Sphingobium* sp. strain SYK-6 which has the potential to thrive on a wide range of aromatic compounds, each with their distinctive interunit linkages, found in plant lignins. For the cleavage of β-β-aryl ether linkages, β -etherase is reported to be the most promising enzyme. The β-etherase system, consisting of 4 stereoisomers, carry out three-step reactions, 1) α-hydroxyl group oxidation by a Cα-dehydrogenase, ether-cleavage by glutathione S-transferases, and 3) cleavage of ether linkages in β--aryl ether-type dimeric units by glutathione-removing GSTs, via oxygen-independent reactions (Kamimura et al., 2019). This enzyme system was initially discovered in *Sphingobium* sp. SYK-6 and its enzymatic characteristics and catalytic processes have undergone extensive characterization. The Multiple β-etherases (GST family member) were found in. strain SYK-6 such as ligE, ligF, and ligP (Cui et al., 2022). Cα-dehydrogenase of LigN/LigL and LigD oxidizes β-aryl ether stereoisomers (αR and αS) and generate Cα-keto compounds.

The resulting βR and βS enantiomers undergo ether cleavage by glutathione's nucleophilic attack, which is catalyzed by LigF and LigE/LigP, respectively. Finally, LigG cleaves the thioether bond in the βR isomer of the glutathione conjugate that results from LigF's ether cleavage generating glutathione disulfide and γ-hydroxypropiovanillone

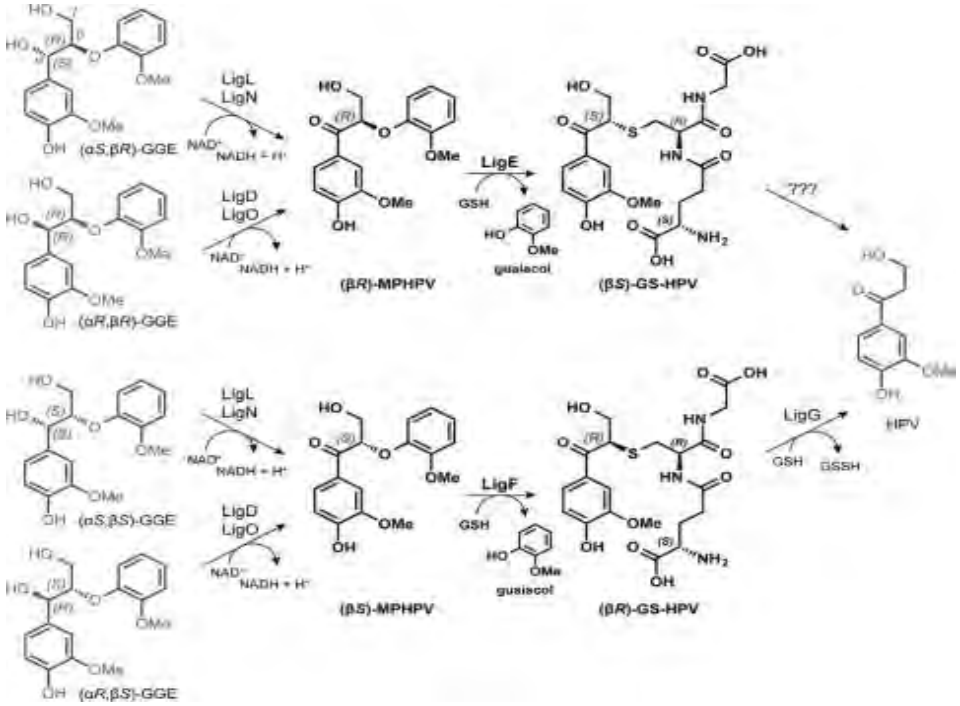


Figure 2.5 Showing the β-etherase pathway in *Sphingobium* sp. SYK-6 (Helmich et al., 2016

2.10.2 Biphenyl Catabolic Pathway

Depending on the source of lignin, the biphenyl component of lignin, which may account for up to 10% of its structure, must undergo a metabolic transformation before being broken down. Bacterial genera include *Pseudomonas*, *Sphingomonas, Rhodococcus, Achromobacter*, *Burkholderia, Ralstonia, Comamonas, Bacillus*, and Acinetobacter, have been reported to degrade biphenyl (Kamimura & Masai, 2022). Among them, *Sphingobium* sp. SYK-6, one of the best-studied ligninolytic bacteria, converts the lignin-related biphenyl compound DDV (5,5′-dehydrodivanillate) into vanillate via various catabolic genes i.e., ligZ, ligY, lig X, ligW, and ligw1 (DH Bugg et al., 2010).

In SYK-6, this pathway is initiated by O-demethylation of DDVA with LigX that results in the generation of OH_DDV which is then followed by the meta cleavage of the aromatic ring by LigZ. The ring fission product is then transformed by the LigY into 5-carboxy

vanillic acid, and 4-carboxy-2-hydroxypentadienoic acid. The final step is the transformation of 5CVA by the LigW and LigW2 into the metabolic core intermediate vanillate or vanillic acid for the production of byproducts.

Given that many soil microorganisms are biphenyl degraders and that lignin degradation accounts for the majority of the biphenyl-containing chemicals in soil, it seems likely that there is some relationship between lignin biodegradation and biphenyl degradation. Recent research demonstrates that the PCB-degrading *Rhodococcus jostii RHA1* exhibits lignin degradation ability (Navas et al., 2022). In addition, a study of bacteria that break down lignin discovered that many of these species also break down aromatic compounds.

Figure 2.6 Showing bi-phenyl degradation Pathway in *Sphingobium* sp. SYK-6 (Mori et al., 2018).

2.10.3 β-ketoadipate Pathway

The β-ketoadipate pathway is a well-studied convergent pathway for the breakdown of aromatic compounds and is extensively distributed in bacteria and fungi found in soil. It's one branch transforms protocatechuate, which comes from phenolic substances like 4-hydroxybenzoate, p-cresol, and many lignin monomers, into β-ketoadipate while the other branch transforms catechol, which is produced from different lignin monomers, amino aromatics, and aromatic hydrocarbons, to β-ketoadipate. Catechol and protocatechuate undergo ortho-cleavage by dioxygenase and are converted to cis, cis-muconate, and 3-carboxy-cis, cis-muconate which are further converted into β -ketoadipate, which interacts with succinyl-CoA to produce succinate and -ketoadipyl-CoA (Weng et al., 2021). This pathway is found exclusively in *Pseudomonas* species, highlighting the significance of the catabolism of aromatic compounds. Biochemical studies reported Four enzymes and four-step reactions in the conversion of Protocatechuic acid to β-ketoadipate. These are

Protocatechuic acid cleavage to 3-carboxy-cis, cis-muconic acid via protocatechuate 3,4-dioxygenase followed by the conversion of 3-carboxy-cis, cis-muconic acid to 4-carboxymuconolactone via 3-carboxy-cis, cis-muconate cycloisomerase, which further converts into β-ketoadipate enol-lactone by 4-carboxymuconolactone decarboxylase. Lastly, β-ketoadipate enol-lactonase converts β-ketoadipate enol-lactone to β-ketoadipate.

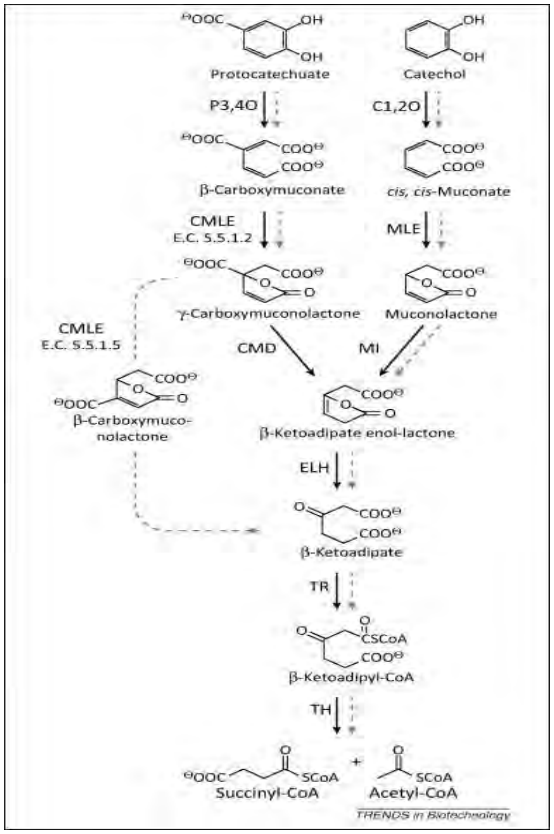


Figure 2.7 Showing β-ketoadipate pathway for the degradation of aromatic compounds (Sgro et al., 2023).

2.11 Lignin Valorization to High Value Products

According to the Economic Analysis, using lignin simply for energy production is not economically advantageous when compared to using it to create high-value chemicals and materials, particularly in the long term. For instance, lignin is worth about $150 per ton when utilized as energy solid fuel as an alternative to natural gas. The economics of its

applications could be considerably improved by the valorization of lignin into aromatic compounds and polymers, valued at over $1000-1200 per tonne (Charles Xu et al., 2020).

Lignin-derived organic compounds are a sustainable alternative to chemicals produced in petroleum refineries. In terms of current and future uses, lignin-derived products can be classified into three groups: low molecular weight aromatic compounds, macromolecules and power, fuel, heat, and syngas products.

2.11.1 Lignin valorization to Aromatic compounds

Globally, Natural aromas and flavors are in high demand due to their widespread application in the various industries. A very small number of flavors are produced from natural sources, whereas the majority are produced chemically. Chemical synthesis can produce large yields, but there are many drawbacks including health risks, the generation of unwanted byproducts, the production of poor-quality products, and environmental effects. Vanillin is among the most significant flavors, and it is commonly employed in the food industry.

Vanillin, 4-hydroxy-3-methoxybenzaldehyde, is a flavoring and fragrance ingredient that is frequently used in food, cosmetics, and pharmaceuticals such as papaverine, cyclovalone, and Levodopa. There are two types of vanillin found (i) synthetic vanillin that is made from petrochemicals such as glyoxylic acid and guaiacol (about 85%) or lignin (about 15%), and (ii) vanilla extract made from dried beans of tropical Vanilla orchids (<1%). Considering the environmental sustainability and economic importance, Lignin is recognized as an attractive source of vanillin in comparison to the other two sources. (Harshvardhan et al., 2017) reported the production of vanillin as a major product (0.9 ± 0.03 mg/mL), from lignin by natural bacterial consortium. Similarly, (Baghel & Anandkumar, 2019) reported the high production of vanillin (75.55mg/l) by mixed culture (*Staphylococcus* as a dominant species) from kraft lignin on 6th day of incubation. Moreover, (Sainsbury et al., 2013) reported the production of vanillin (about 96mg/L) by Gene (vanillin dehydrogenase gene) deleted *R. jostii RHA1* strain.

Due to the complicated nature of KL's structure, its toxicity, and a greater variety of enzymes associated with lignin bioconversion, the literature does not provide any specific information on the bio-depolymerization process for the synthesis of vanillin from KL. In general, bacteria are unable to produce ferulic acid, an essential component in the production of vanillin, directly from the complicated lignin structure. However, when microorganisms use the phenylpropanoid eugenol, produced from the guaiacol group, as a carbon source, ferulic acid can then be produced as a transient intermediary.

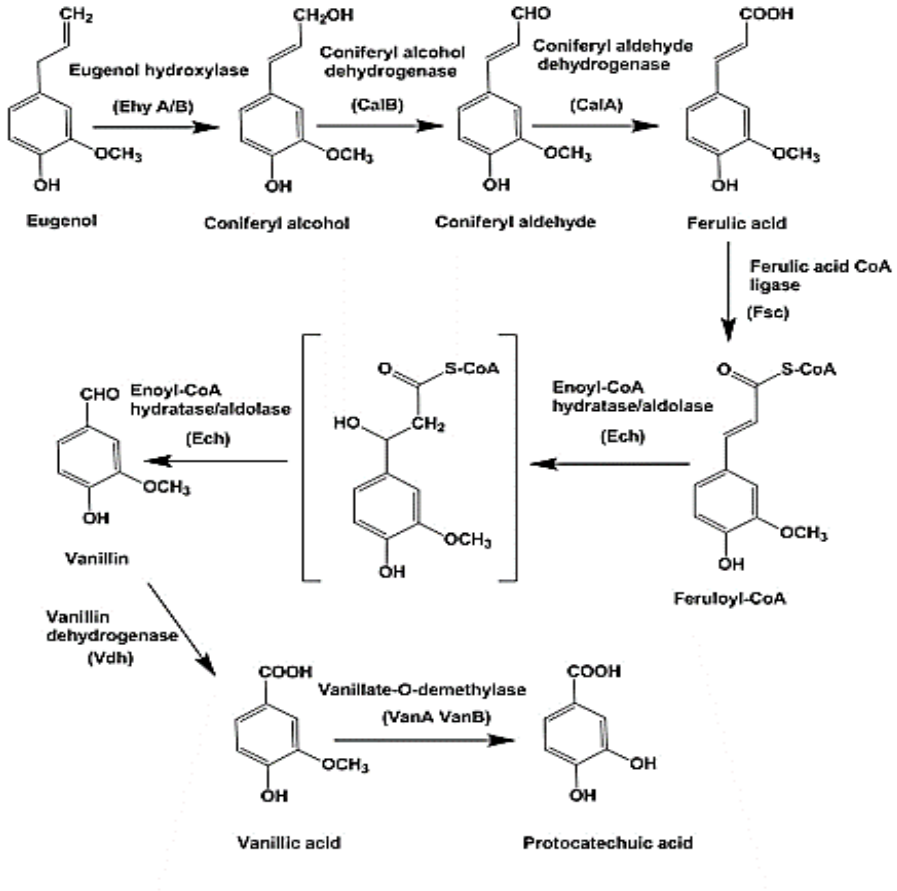


Figure 2.8 Showing Vanillin production from Eugenol in *Pseudomonas*

Syringaldehyde, 3,5-dimethoxy-4-hydroxybenzaldehyde, is another common aromatic compound produced by lignin biodegradation. Just like Vanillin, it is used in the fragrance and flavoring industries (Tarabanko & Tarabanko, 2017). Even though much less widely used commercially than vanillin, it has recently gained popularity as a lignin-derived chemical, particularly since it was found to be a crucial precursor to several medications

used to treat bacterial infections, including Bactrim, trimethoprim, and Biseptal. Vanillin is also a precursor for the production of trimethoprim, but syringaldehyde has the advantage of possessing two methoxyl groups.

Another significant central intermediate node is **gallic acid**, produced by enhancing the hydroxylation of p-hydroxybenzoic acid or PCA in *R. opacus and E. coli* from lignin-derived aromatics. Gallic acid (3,4,5-trihydroxybenzoic acid), a phenolic acid and antioxidant, is present in varying levels in several plants. Catechol and PCA are significant key intermediates in biological funneling pathways and also serve as biosynthetic precursors of other significant aromatics like gallic acid. Both have been produced from lignin-derived aromatics by blocking the aromatic ring opening genes in bacteria such as *R. jostii* RHA1 and *P. putida* KT2440.

Table 2.3 Showing the production of lignin-derived aromatics compounds by different strains

Products	Strain	Substrate	Product	Fermentation	Gene manipulation
PCA	*P. putida* KT2440	EDL	6.73 mg/L	Batch	Deleting pcaGH
Catechol	*E. coli*	Vanillin	8 mg/L	Batch	Overexpressing CouP, LigV, LigM and Aro under ADH7 promoter
Gallic acid	*R. opacus*	APL	0.40 g gallic acid/g APL	Batch	Integrating biological hydroxylation, *O*-demethylation, and aryl side-chain oxidation
Gallic acid	*E. coli*	Ferulic acid and *p*-CA	19.57 mM gallic acid from 20 Mm FA; 19.96 mM gallic acid from *p*-CA	Fed-batch	Overexpressing FCS, ECH, HFD1, VanAB, and $PobA^{Y385F}$; overexpressing FCS, ECH, HFD1, HpaBC, and $PobA^{Y385F}$
Vanillin	*P. putida* KT2440	Ferulic acid	86% yield	Batch	Deleting *vdh*, molybdate transporter gene; overexpressing *fcs, ech* gene
p-Hydroxybenzoic acid	*B. glumae* BGR1	*p*-CA	2.73 g/L	Batch	Deleting *phb3h, bcl* gene and overexpressing *phcs* II
Vanillic acid	*E. coli*	Vanillin	~ 400 mg/L	Batch	Overexpressing CouP, LigV, LigM and Aro driven by ADH7 promoter
p-Hydroxystyrene	*E. coli*	*p*-CA	266 mM/L	Packed-bed reactor	Overexpressing phenolic acid decarboxylase from *B. amyloliquefaciens*

2.11.2 Lignin valorization to Bioplastics

Polyhydroxyalkanoates are naturally occurring polymers with promising properties. Because of their biodegradability and biocompatibility, they can be used in a various industrial areas, including biomaterials and medicinal applications (Nguyen et al., 2021). Numerous bacteria have evolved metabolic pathways in nature that allow them to convert lignin into PHAs with short, medium, or long chains. Bacteria can produce PHAs by

metabolizing lignin-derived aromatics through a β-ketoadipate pathway. Previous research has investigated extensively how P. putida converts certain carbon sources into PHA, such the lignin-derived monomers like vanillic acid, vanillin, and benzoate.

P. putida can therefore accommodate the degradation of many substrate types for both lignin-derived monomers and glycerol (Xu et al., 2021).

Table 2.4 Showing the production of PHA by different *P. putida* strains

Strains	Highlights	Gene manipulation	Substrates	Fermentation	Titer	References
P. putida KT2440	Bacterium produce PHA from lignin	–	Alkaline pretreated liquor	Shake-flask batch	252 mg/L	(Linger et al., 2014)
P. putida A514	Three-component design enables the efficient lignin bioconversion to PHA	Overexpression heterogenous *dyp2* fused with peptides *pelb*; overexpression and *vanAB*, *phaJ4* and *phaC1*	Insoluble Kraft lignin	Shake-flask batch	75 mg/L	(Lin et al., 2016)
Engineered *P. putida* KT2440	Combinatorial pretreatment, fermentation optimization	–	Lignin from combined pretreatment	Fed-batch	1.0 g/L	(Liu et al., 2017)
P. putida $A_{ayl,alkKphaGC1}$	Strengthening the PHA biosynthesis, fermentation optimization	Overexpressing *phaG*, *alkK*, and *phaC1*	Vanillic acid	Shake-flask batch	246 mg/L	(Wang et al., 2018)
P. putida KT2440-AG2162	Knocking out the PHA competitive pathway and strengthening the PHA biosynthesis pathways	Overexpressing *phaG*, *alkK*, *phaC1* and *phaC2* gene and deleting *phaZ*, *fadBA1* and *fadBA2*	*p*-Coumaric acid	Shake-flask fed batch	953 mg/L	(Salvachua et al., 2020a)
P. putida NX-1	Newly isolated *P. putida* NX-1 produce PHA from lignin	–	Kraft lignin	Shake-flask batch	114 mg/L	(Xu et al., 2021)
Engineered *P. putida* KT2440	Effective fractionation design, process optimization	–	Soluble lignin	Fed-batch	4.5 g/L	(Liu et al., 2021)

2.11.3 Lignin Valorization to Lipids

Due to the growing global population and the depletion of fossil fuels, the demand for biofuel is expected to rise. Microbes can convert lignin into lipids that can be used as biofuel. Oleaginous microorganisms are reported to produce high biomass with a lipid content of over 20%. The metabolic pathway for lignin bioconversion to lipids consists of four steps: (1) low-molecular-weight lignin degradation into its derivatives (2) degradation of the aromatic compounds into protocatechuate or catechol; (3) production of acetyl-CoA via β-ketoadipate pathway; and (4) lipid biosynthesis. The oleaginous *Rhodococci* receive special attention for biotransformation of lignin-derived aromatics to lipids, because of their fast metabolism, tolerance of aromatics generated from lignin, and relative adaptability to genetic manipulation (Wang et al., 2022).

Table 2.5 Showing the lignin valorization to lipids *Rhodococcus sp.*

Strains	Carbon source	Innovative strategies	Yield	References
Rhodococcus opacus DSM 1069	Kraft lignin	O_2-pretreatment under alkaline environment	0.067 mg/ml	Wei et al. (2015)
Rhodococcus opacus	Corn stover	Ammonia fiber expansion-pretreated	32 mg/L	Wang Z. et al. (2019)
Rhodococcus opacus PD630 and *Rhodococcus jostii* RHA1 VanA	Corn stover	Co-fermentation of wild and engineered bacteria	0.39 g lipid/g CDW	He et al. (2017)
Rhodococcus pyridinivorans CCZU-B16	Alkali lignin	Screening of new strains	0.52 g lipid/g CDW	Chong et al. (2017)
Rhodococcus opacus PD630	Corn stover	Multi-stage pretreatment method ALK-AHP	1.3 g/L	Le et al. (2017)
Rhodococcus jostii RHA1	Benzoate (Lignin-degradation products)	Nitrogen-limiting condition	55% of CDW	Amara et al. (2016)
Rhodococcus opacus DSM 1069	Pine organosolv pretreatment effluent	Organic solvent pretreatment	26.99 ± 2.88% of CDW	Wells et al. (2015)

Methodology

The present study was conducted at Applied, Environmental and Geomicrobiology Laboratory, Department of Microbiology, Quaid-I-Azam University, Islamabad, Pakistan.

3.1 Sampling

The sludge samples were collected from the sites around the hot spring, Chitral. Two sludge samples named SP1 and SP2 were collected by using a sterile spatula from the surface to a depth of between 5-10 cm. The samples were kept in sterile zip-lock bags and stored at 4 °C for future research.

3.2 Isolation of Bacterial strains

The isolation of the bacterial strains from sludge samples was carried out on nutrient agar medium by serial dilution and spread plate method.

3.2.1 Serial Dilution

Serial dilution is a process of reducing the number the bacteria by serially transferring the sample into a fixed volume of autoclaved distilled water.

Procedure

Suspend 1g of Sludge samples in 50 ml autoclaved nutrient broth and incubate them at 40°C for 48 hours. Pipette 9ml of autoclaved distilled water in all the 9 sterile tubes and label them properly. Pipette 1ml of Sample into tube 1 and mix it properly. Now transfer 1ml of sample from tube 1 to tube 2 and make dilutions up to 10^{-9}.

3.2.2 Spread plate Method

The Spread plate method is used for isolating and purifying bacteria easily by spreading a sample containing bacteria on a media.

Procedure

Pour the autoclaved nutrient agar media into plates and allow them media to solidify. Pipette 100 µl sample from the dilutions 10^{-1}, 10^{-3}, $10^{-5,}$ 10^{-7} and 10^{-9}**.** Spread the sample via a spreader. properly. Incubate the plates at 40°C for 48 hours. Streak the plates for the

isolation of pure strain after incubation. For this heat the wire loop until it turns red hot. Pick different isolated colonies and streak them on the freshly prepared nutrient agar plates via the streak plate method and then incubate these plates at 40°C for 24 hours.

3.3 Screening of Ligninolytic Bacteria Using L-MSM (Lignin-Minimal Salt Medium)

L-MSM is used to screen isolated bacteria that are capable of degrading lignin by using it as a sole source of carbon. The composition of L-MSM (g/L) was followed as: $CaCl_2.2H_2O$ 0.01 g, K_2HPO_4 2.0 g, KH_2PO_4 2.3 g, $MgSO_4$ 0.01 g, NH_4NO_3 0.1 g, agar, and lignin. The isolated bacterial strains were inoculated on MSM having different lignin content g/L i.e., 1 g, 2 g and 3 g.

Procedure

Prepare L-MSM of 1000ml in 3 flasks and each flask contains different lignin content. Label the flasks and adjust the pH of medium to 7.6. Autoclave the medium at 121°C, 15 psi for at least 30 minutes. Pour each media into autoclaved petri plates under sterilized conditions. Streak the purified bacterial strains by using sterilized wire loop and Incubate them at 40°C for 7-10 days and note the bacterial growth daily.

3.4 Measurement of Lignin Degradation by Ligninolytic Bacteria

The lignin degradation by ligninolytic bacteria is measured by using lignin amended minimal salt media (L-MSM). The composition of L-MSM g/L, in this case, is K_2HPO_4 2g, $Na_{2H}PO_4$ 2.4g, $MgSO_4$ 0.01g, $CaCl_2$ 0.01 g, NH_4NO_3 0.1g, glucose 10 g, peptone 5g, and black liquor 1%.

Procedure

In Erlenmeyer flasks of 1000 ml, add salts, black liquor, glucose, and peptone of the required quantity in each flask containing and adjust the pH at 7, 8 and 9. Autoclave the media at 121°C and 15lbs. Inoculate one flask with the overnight grown inoculum called a sample and uninoculated one is labelled as control. Incubate the flasks at 40°C, 45°C and 120 rpm for 168 hours. Samples were withdrawn daily for measurement of lignin degradation.

3.5 Determination Of Optimum Growth Conditions

The optimum growth conditions were determined by examining two physical factors, namely temperature and pH, that were noted for each bacterial strain.

3.5.1 Temperature

For optimum temperature, Erlenmeyer flasks of 1000ml having BL-MSM were inoculated with isolated ligninolytic bacteria. The flasks were then incubated are 40°C, 45°C and 50°C for 168 hours. Samples were taken out daily for the measurement of lignin degradation and color reduction.

3.5.2 pH

Similarly for optimum pH, BL-MSM were prepared, and their pH were adjusted at 7, 8 and 9. Prepared media were autoclave and then inoculated with the isolated ligninolytic bacteria. Samples were taken out daily for the measurement of lignin degradation and color reduction.

3.6 Measurement of Lignin Content By Pearl-Benson Method

For the estimation of lignin degradation, Make a stock solution of 10% acetic acid,10% $NaNO_2$ and 2N NH_4OH. Take 50 ml of sample and control in falcons and centrifuge them under the conditions of 10,000 rpm and 10 mins. Discard the pellet and collect the supernatant. Next, add 1 ml of CH_3COOH to the samples, control, and distilled water, followed by the addition of 1 ml of $NaNO_2$ in the samples as well as in control and 2 ml NH_4OH in distilled water. Mix them properly and incubate them at room temperature for 15 minutes. After incubation, add 2 ml of NH4OH in the sample as well as in control and 1 ml of $NaNO_2$ in dH_2O. Mix properly and again incubate them at room temperature for 10 minutes. Take OD at 430 nm. Percentage lignin degradation was calculated by:

$$\% \text{ lignin degradation} = \frac{(C1-C2)}{C1} \text{ x } 100$$

Where, C1 is the alkali lignin concentration in Control, and C2 is lignin concentration in samples.

3.7 Color Reduction Estimation

The CPPA standard procedures were used to determine the color reduction of the degrading media. 1 mL of sample was taken at 24-hour intervals and centrifuged for 30 minutes at 8,000 rpm. Collect the supernatant and discard the pellet. in a supernatant, 3ml of phosphate buffer (pH 7.6) was added and a UV-visible spectrophotometer was used to measure its absorbance at 465 nm. The following formula was used to convert absorbance values into color units (CU):

$$CU = 500\,A_2/A_1$$

Where, A1 is the absorbance of standard solution, and A2 is the absorbance of the sample.

3.8 Ligninolytic Enzyme Assays

3.8.1 Qualitative Assays

The potential of isolated lignin-degrading bacteria to produce ligninolytic enzymes (lignin peroxidase, manganese peroxidase, and laccase) were determined using qualitative plates assays i.e., guaiacol plate assay and methylene blue decolorization plate assay.

3.8.1.1 Guaiacol Plate Assay

This plate assay is used to screen isolated bacterial strains. The medium used for this assay is composed of g/L agar 2%, broth 16g, and guaiacol 0.02%. Guaiacol derived from guaiacum is a yellow-colored aromatic compound. Pyrolysis of lignin present in wood results in the production of guaiacol.

Procedure

In a nutrient agar medium containing 0.02% filtered guaiacol (sterilized), the isolated bacterial strain was inoculated using the spot inoculation method in which microbes are simply transferred to the medium by touching the wire loop instead of dragging it which ensures that a single colony will grow from a single point. Incubate the plates at 40°C for 168hours. The formation of zones around the bacterial colonies determines the laccase activity.

3.8.1.2 Methylene Blue Dye Decolorization

This plate assay is used to determine the activity of peroxidases. The medium used for this plate assay is composed of agar 2%, broth 16 g/L, and methylene blue(indicator) 0.025 g/L.

Procedure

Autoclave the nutrient agar medium and then filter methylene blue dye solution by a syringe filter in the flask containing nutrient agar. Pour the media into the plates and allow them to solidify. Inoculate the isolated bacterial strain by spot inoculation method just like in guaiacol plate assay. Incubate the plates at 40°C for 168 hours (7 days). Decolorization zones will appear around the peroxidase producing bacterial colonies. The zones are due to the formation of colorless amines due to azo bonds' reductive cleavage.

3.9 Identification of Bacterial Strain

3.9.1 Morphology

The ligninolytic bacterial strains were identified on the basis of morphological characteristics such as size, shape, texture, color, and margins.

3.9.2 Gram Staining

Gram staining is the most common method for distinguishing between two broad bacterial groups on the basis of the content of their cell wall. Gram-positive bacteria retain the crystal violet (a primary dye) followed by a mordant (iodine) as they possess a thick peptidoglycan. The primary dye and mordant forms a complex called CV1 complex which remains within the cells even after applying decolorizer due to which stained cells appear purple in color.

Similarly, Gram-negative bacteria possess a thin layer of peptidoglycan due to which the CV 1 complexes do not firmly bound within the peptidoglycan and washed off during the application of decolorizer and thus cells appear pink or red after the staining with safranin.

Procedure

Take a clean glass microscopic slide and by using a loopful sample, make a smear. Heat fixes the bacterial cells by passing the slide 2-3 times above the flame and then allow it to dry. After it, stain the cells with primary dye i.e., crystal violet for about 1 minute, followed by rinsing the slide to remove excess stain. After rinsing, add the mordant i.e., gram's Iodine for 1 minute, and again rinse with water. Cells are decolorized by washing with alcohol (a differential step). In the last step, the slide is flooded with secondary strain i.e., safranin for 20-30 seconds. Again, rinse the slide with water and allow it to dry. Now the slide is ready to be seen under the microscope.

3.9.3 Biochemical Tests

3.9.3.1 MacConkey Agar test

MacConkey agar is a differential media used to isolate enteric gram-negative bacteria and also used to differentiate bacteria that are able to ferment lactose from those that are not. In this medium, lactose monohydrate is used as a carbohydrate source.

Procedure

Autoclave the media and pour it into petri plates. Allow the media to solidify and then by streak plate method, inoculate the bacteria. Incubate the plates at 40°C for 24 hours. Growth on media indicates that the experimental organism is gram-negative whereas no growth shows negative results.

3.9.3.2 Eosin Methylene Blue Agar Test

EMB agar is a differential medium used for differentiating among lactose fermenting organisms (e.g., *E. coli*) and those that do not (e.g., *Shigella, Salmonella*) by providing a color indicator. Moreover, It somewhat limits the growth of Gram-positive bacteria. Lactose-fermenting gram-negative bacteria produces acid by fermenting lactose, which lowers the pH, and the dye generates a dark purple complex that is typically accompanied by a green metallic sheen. This fecal coliforms-typical metallic green sheen implies active lactose or sucrose fermentation capabilities. Moreover, non-lactose fermenters raise the

lactose or sucrose fermentation capabilities. Moreover, non-lactose fermenters raise the pH by deaminating proteins and produce light pink or colorless colonies.

Procedure

Weigh and suspend dehydrated media in 1L distilled water. Sterilize the media by autoclaving at 121°C and 15 lbs of pressure, for 15 minutes. Shake the medium and allow it to cool to oxidize the methylene blue and then pour it into petri plates. Streak the bacteria and incubate them at 40°C fo4 24 hours. After incubation, observe the colony morphology. If the results are negative, repeat the incubation process.

3.9.3.3 Oxidase Test

This test is used to find whether the bacteria are producing the cytochrome oxidase enzyme or not. This enzyme plays an important part in the electron chain reaction of aerobic respiration as this enzyme accepts electrons from reduced cytochrome c and donates them to oxygen. In this test, an artificial electron acceptor i.e., p-amino dimethylaniline oxalate is used which is usually pink in color which in turn oxidized by Oxidase converts into a dark substance.

Procedure

Wet a section of the oxidase test strip that is to be tested. Smear a bacterial paste onto the moistened area with the help of a sterile inoculating loop. A positive test is indicated by the purple or blue color appearance within 30 seconds. On the other hand, no color change after 30 seconds indicates a negative result.

3.9.3.4 Catalase Test

This test distinguishes bacteria on the basis of the production of the catalase enzyme. This enzyme readily degrades hydrogen peroxide into water and oxygen. Hydrogen peroxide is a toxic compound, produces in aerobic respiration and its accumulation results in the death of cells.

Procedure

Transfer a loopful sample onto a clean slide. Add 2-3 drops of 3% hydrogen on the slide. The formation of bubbles is the indication that oxygen production, and degradation of hydrogen peroxide.

3.9.3.5 Citrate Utilization test

This test is used to detect whether bacteria are utilizing citrate as a carbon source or not in the absence of Glucose or lactose. Bacteria use citrate as a source of carbon and converts it into sodium carbonate thus creating an alkaline pH. This change in pH converts the bromothymol blue indicator used in this test from green to blue.

Procedure

Inoculate the Simmons citrate agar with a bacterial sample by streaking onto its slant. Incubate the tubes at 40 °C for 2-3 days. Note the change in color. Change in color from green to Prussian blue indicates a positive result, whereas no change in color indicates a negative result.

3.9.3.6 Triple Sugar Iron Test (TSI)

This test is used to determine the fermentation capabilities of bacteria along with hydrogen sulfide production. Fermentation is the anaerobic breakdown of carbohydrates and alcohols with the production of organic acids. Along with this, hydrogen gas or carbon dioxide are also produced. The fermented carbohydrates turn phenol red to yellow color due to acid production. Lack of carbohydrate fermentative ability means the bacteria are utilizing other substrates like peptones in the media.

- **Red slant and yellow butt with or without production of gas:**
  The organisms degrade glucose first. Only glucose degradation has occurred. The utilization of peptones gives the red color to the slant.

- **Yellow slant and yellow butt with or without gas production:**
  Fermentation of lactose and/or sucrose has occurred. Due to their higher concentration, the reaction is maintained in slants and butt.

- **Red slant and red butt or no change butt:**

  Fermentation has not occurred. Only utilization of peptones has taken place.

Procedure

Inoculate the experimental microorganism into its appropriately labeled tube by means of the stab or streak method aseptically. Incubate the tubes at 40°C. for 18-24 hours. Note the color in both butt and slant. The test is positive when sources of sugars other than glucose are being utilized and dark yellow color observed. Whereas the color yellow and red indicate negative results.

3.10 Toxicological Studies of Untreated and Treated Black Liquor

3.10.1 Phytotoxicity testing Seed Germination

This test is frequently employed to evaluate the potential toxic effects of chemicals present in water samples on seed growth and development. Seeds are exposed to the water sample under test, and during a predetermined time period, their germination and growth are observed.

The toxicity of treated and untreated black liquor was carried out using chickpea seed germination. The potential toxicity of an effluent sample on plant growth can be detected by the inhibition of amylase activity. Amylase is an enzyme that converts starch into simpler metabolized sugars, supplying energy to the seedling during germination. The inhibition of amylase can interfere the normal metabolic functions, which can impede seedling growth.

Procedure

Surface Sterilized the wheat seeds with 1-5% Sodium Hypochlorite for minutes and then rinse with distilled to remove any traces of bleach. After surface sterilization, place the Whatman type 2 filter papers in sterilized petri plates and soak them with the control (untreated 1% black liquor) and treated sample (after 7 days of bacterial treatment). Cover the petri plates and place them in incubator at 21-27°C. Set a timer to keep track of the germination progress. Monitor the seeds in each petri plate for germination after a

predetermined amount of time (often 3–7 days). The germinated seedlings were removed carefully from each petri plate and placed on a clean surface.

3.10.2 Cytotoxicity testing by Brine Shrimps

The brine shrimp bioassay, also known as Artemia salina assay or the brine shrimp lethality assay, is a typical bioassay used to evaluate the cytotoxicity or toxicity of various substances. Brine shrimps are Small crustaceans that are frequently employed in this kind of bioassay because they are simple, readily available, and sensitive to toxins.

Procedure

Dissolve marine salt in distilled water to create a brine solution with a salinity of around 25–35 ppt. Add the appropriate quantity of Brine shrimp eggs to the brine solution. Heat the solution (23-28°C) containing the shrimps' eggs by placing the container under small lamp. The eggs will begin to hatch within 24-48 hours and observe tiny nauplii swimming in the water. Now add the harvested brine shrimps (appr. 10) in untreated water (1% black liquor) and treated sample (after 7 days of bacterial treatment) of different concentrations. The exposed shrimps are incubated usually for 24 to 48 hours under controlled conditions including temperature and light. The number of brine prawns that have survived in untreated and treated sample following the incubation period was counted. Higher survival rates of brine shrimp are correlated with decreased toxicity, while lower survival rates are correlated with higher toxicity.

3.11 Analysis of Lignin degradation by FT-IR

FTIR spectroscopy is an effective analytical technique used to examine the chemical makeup and structural modifications of compounds such as lignin during their biodegradation.

Protocol

The samples were taken from lignin degrading media and centrifuged at 8000rpm for 15 minutes. The cell palate was discarded, and supernatant was poured in petri plates and dried properly. The dried lignin form both control and samples were scratched from plates and transferred carefully to Eppendorf. About 2mg of dried lignin from control and samples

were mixed with 200mg of Potassium bromide. The mixture was pressed using hydraulic press into thin pellets. The pellets were then evaluated using FT-IR spectra in the range of 4000 to 400cm-1.

3.12 Analysis of Lignin degradation products by GC-MS

Detection and isolation of lignin-derived metabolites was performed through GC–MS. About 50ml of the lignin degradation samples were collected after 0, 2, 4 and 6 days of incubation with strain SP2(10) under optimum conditions and centrifuged at 10,000rpm for 15 minutes. pH of supernatant was adjusted to 2 via 6M HCl, after which the lignin derived compounds were extracted using a threefold volume of ethyl acetate. The organic layer that is formed on the top of aq. Medium was collected from the separating funnel and is dehydrated by using anhydrous Na_2SO_4. The samples were filtered by using Whatman filter paper and then evaporated within rotary vacuum evaporator.

The dried residues were derivatized by adding dioxane (100µL) and pyridine (10µL), followed by the addition of trimethylsilyl (50µL). The derivatized samples were heated at 60°C for 15 minutes and were dissolved by continuous shaking. 3 µL of derivatized samples were injected into the GCMS injector port, equipped with DB-FFAP capillary column (30m x 0.25µm x 0.25mm). The column temperature was set to 120 to 280°C with flow rate of 1.5mL per minute. The solvent removal time was set to 3 min and mass spectra in the range of 50-750m/z. The detection of lignin degradation products was accomplished by comparing the RT and mass spectra of products in test sample with available mass spectra in the National Institute of Standards and Technology (NIST) library.

3.13 Lignin Valorization

3.13.1 Preparation of Extracts

The extraction of compounds from treated BL samples was performed by Liquid-Liquid extraction. The sequential extraction was done by employing four solvents i.e., hexane, ethyl acetate, ethanol, and methanol, starting from low polarity to high. About 100ml of BL sample was centrifuged at 10,000rp m for 15 min to remove the biomass. The extraction of lignin-derived phenolic compounds was done by adding three times the volume of

hexane in a separating funnel. Collect the organic layer and concentrate it via rotary vacuum evaporator. The same protocol was performed for the other solvents. The amber vials containing the dried crude extracts were kept in a refrigerator

3.13.2 Column chromatography

The extracts were subjected to column chromatography for the purpose of isolating different compounds. For the fractionation, a vertical glass column made of borosilicate material was employed. The column was thoroughly cleaned with acetone and dried completely before packing. A small piece of cotton ball was set at the base of the column with the aid of a glass rod. A fine layer of Sea sand was added on top of that cotton ball to a height of 1 cm and then rinsed it down by adding a solvent (hexane) up to 3/4th level of column. Now we will prepare the silica slurry by adding hexane to the silica and pouring it into the column while simultaneously draining the solvent to help the column pack properly. The Extract was mixed with a small amount of hexane and then loaded the prepared sample to the top of the column. After loading the loading, the sample, add another layer of sea sand (1cm) as a protective layer and again fill up our column with an eluent. Now run the column and collect the fractions. The gradient elution method was used to separate the EAE fractions by utilizing solvents ranging in polarity from low to high (such as hexane to methanol) in different ratios.

3.13.3 High-Performance Liquid Chromatography of Fractions

HPLC is an essential analytical technique frequently used to identify and measure the components of liquid samples. HPLC profiles of Ethyl acetate extract fractions were obtained by Using two separate mobile phases chosen on the basis of varied gradations of solvent systems in specified retention times and elute detection. All samples were analyzed with the reverse-phase analytical column, SPD-M10A VP PDA, injection volume 10.00µl, run time 10 minutes, and detection wavelength 320 nm. EAE fractions were dissolved in methanol for the analysis. Prior to use, the solvents for the mobile phases were degassed and filtered through Millipore. Gallic acid and caffeic acid were employed as standard in this analysis.

Results

4.1 Isolation of Bacterial Strains from Sludge

For the isolation of bacterial strains, dilution was made up to 10^{-9} by serial dilution method. The samples from different dilutions were spread on nutrient agar plates by spread plate methos and then incubated at 40 °C for 48 hours. In case of SP1, the plate with dilution 10^{-7} and 10^{-8} and in SP2, the plate with dilutions 10^{-5} and 10^{-7} gives isolated colonies. Therefore, these plates were selected as the best plates for further screening of lignin degrading bacterial isolates.

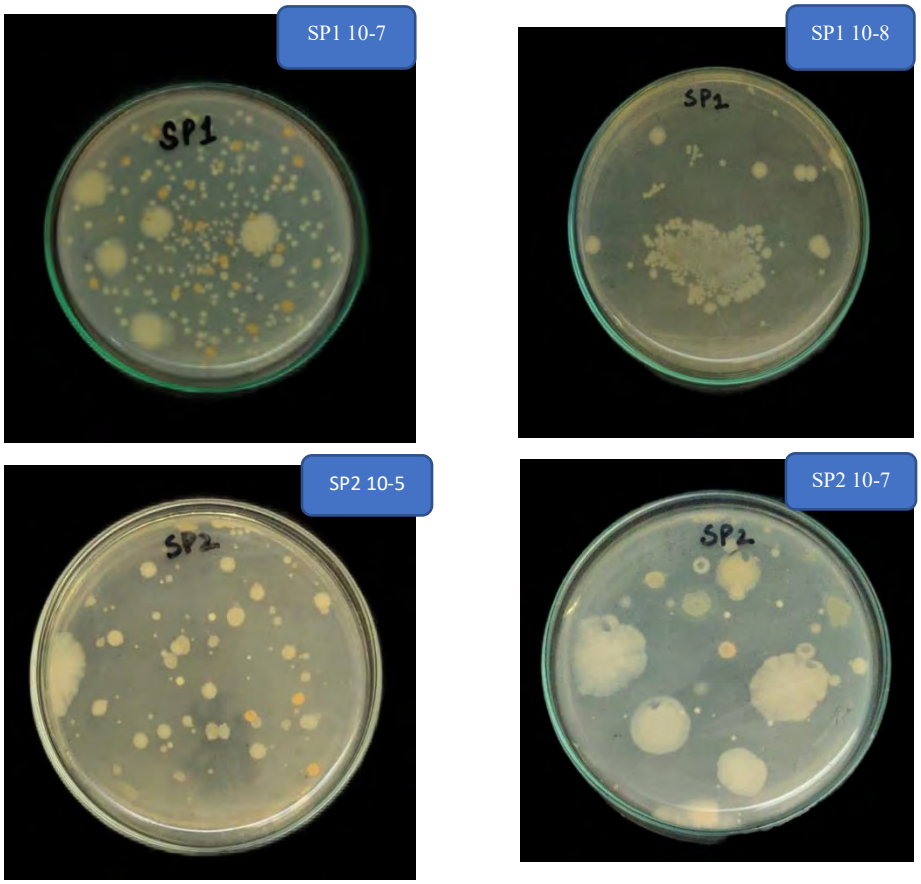


Figure 4.1 Serially diluted plates of Two sludge sample SP1 and SP2

About 7 different isolated colonies from SP1 and 13 from SP2 were selected from the plates shown above, and these isolated colonies were further purified by streak plate method. The results obtained are:

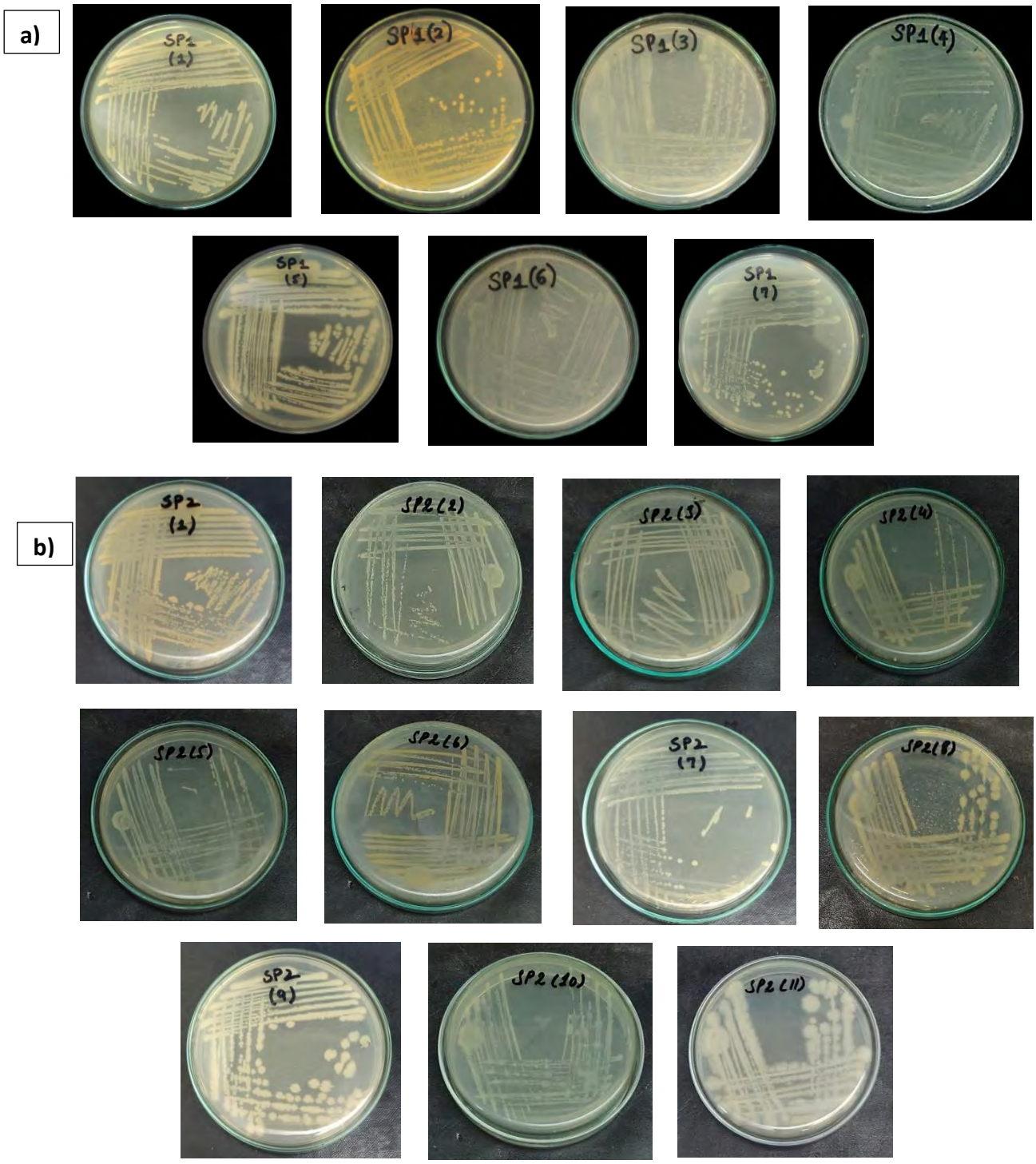


Figure 4.2 a) Showing Seven different bacterial strains isolated from SP1 diluted plates b) Showing thirteen different bacterial strains isolated from SP2 diluted plates.

4.2 Screening of Ligninolytic Bacteria Using L-MSM

These purified bacterial strains were screened on the basis of lignin degradation ability. These strains were grown on MSM having different lignin content g/L i.e., 1 g, 2 g, and 3 g. It was observed that SP1(4) and SP1 (6) shows positive growth in case of 2 g/L and 3 g/L lignin content. A growth was observed in 1g/L lignin content however, the growth was so minimal, and it can only be seen with naked eye and was not possible to capture it. Whereas in case of SP2, SP2(4), SP2 (8) and SP2 (10) shows maximum growth on L-MSM plates. Therefore, these 5 thermophilic bacterial strains were selected for potential to degrade lignin and its conversion to high-value products.

Table 4.1 Summary of SP1 bacterial strains showing growth on MSM plate containing lignin

	1g/L		2g/L		3g/L	
	SP1 (4)	**SP1 (6)**	**SP1 (4)**	**SP1(6)**	**SP1 (4)**	**SP1(6)**
Day 01	–	–	–	–	–	–
Day 02	–	–	+	+	+	+
Day 03	+	+	++	++	+	+
Day 04	+	+	++	++	++	++
Day 05	+	+	++	++	++	++
Day 06	+	+	++	++	++	++
Day 07	+	+	++	++	++	++

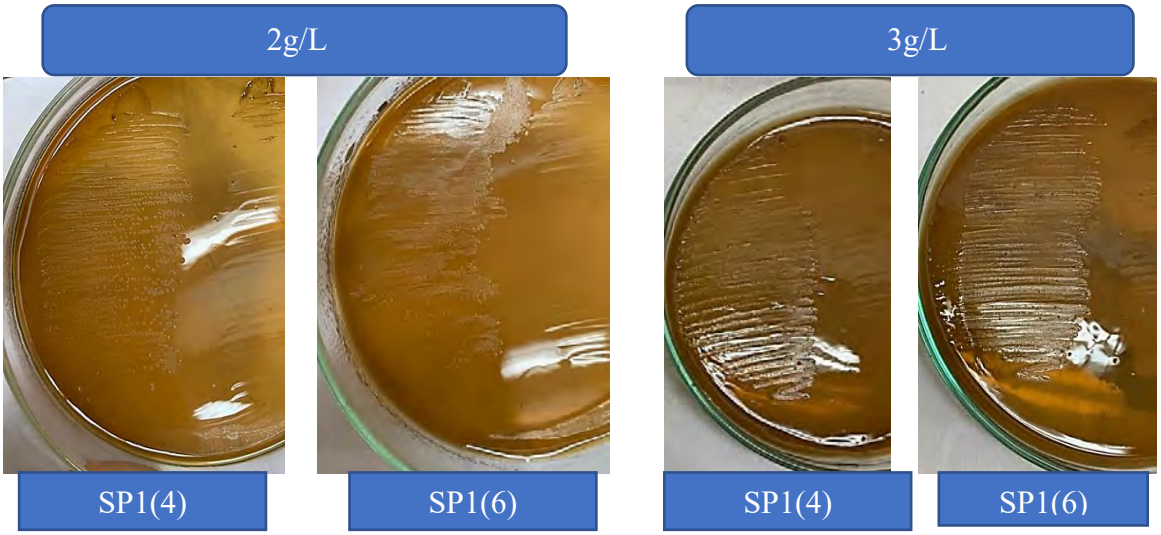
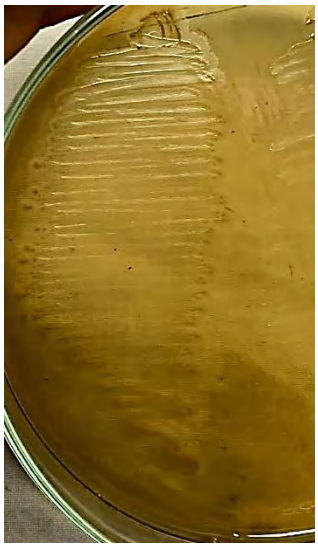


Figure 4.3 Bacterial growth on MSM containing different lignin content i.e., 2 g/L, and 3 g/L

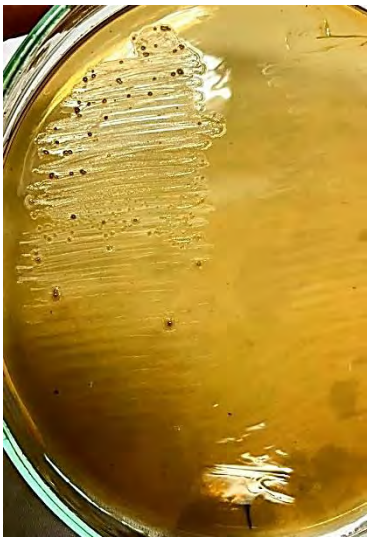
Table 4.2 Summary of SP2 bacterial strains showing growth on MSM plate containing lignin

	1g/L			2g/L			3g/L		
	SP2 (4)	**SP2(8)**	**SP2 (10)**	**SP2 (4)**	**SP2(8)**	**SP2 (10)**	**SP2 (4)**	**SP2(8)**	**SP2 (10)**
Day 01	–	–	–	–	–	–	–	–	–
Day 02	–	–	–	–	–	+	+	+	+
Day 03	+	+	+	+	+	+	++	+	++
Day 04	+	+	+	+	++	++	++	++	++
Day 05	+	++	++	++	++	++	++	++	++
Day 06	++	++	++	++	++	++	++	++	++
Day 07	++	++	++	++	++	++	++	++	++

1g/L



SP2(4)



SP2(8)

SP2(10)

2g/L

SP2(4)

SP2(8)

Sp2(10)

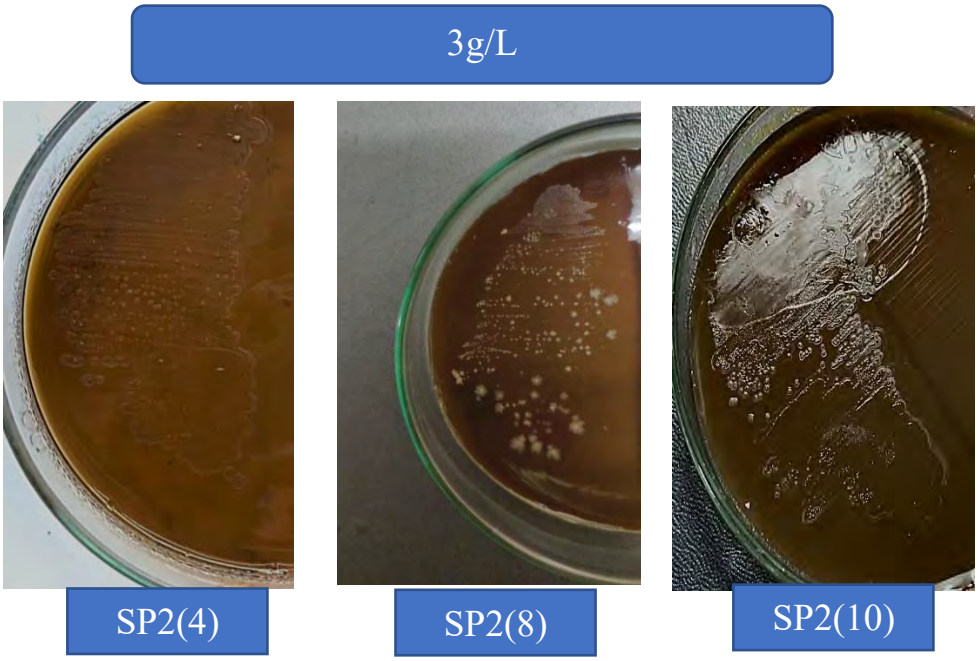


Figure 4.4 SP2 Bacterial strains growth on MSM containing different lignin content i.e., 1 g/L, 2 g/L, and 3 g/L

4.3 Biodegradation of Kraft Lignin

Among 07 bacterial strains from Sludge sample 1 (SP1) and 13 from Sludge sample 2 (SP2), only five bacterial strains named as SP1(4), SP1(6), SP2(4), SP2(8) and SP2(10) shown growth on MSM containing different lignin content i.e., 1g/L, 2g/L., and 3g/L. These bacterial strains were selected and further examined for their lignin degradation potential. According to Pearl and Benson method, among five bacterial strains, strain SP2(10) showed maximum lignin reduction, which were selected for further analysis.

4.3.1 Effect of temperature and pH on the lignin degrading ability of SP2(10)

The effect of temperature and pH on the lignin degradation ability of SP2 (10) was determined via shaking flask experiment. The experiment was performed at different temperatures (40-50°C) and pH (7-9) to determine the optimum conditions at which these bacterial strains degrade lignin at maximum level. The media was incubated for 7-10 days and samples were withdrawn on daily basis for lignin content analysis.

Following graphs shown the % lignin reduction of SP2(10) observed after 7 days at temperature (40-50°C) and pH (7-9).

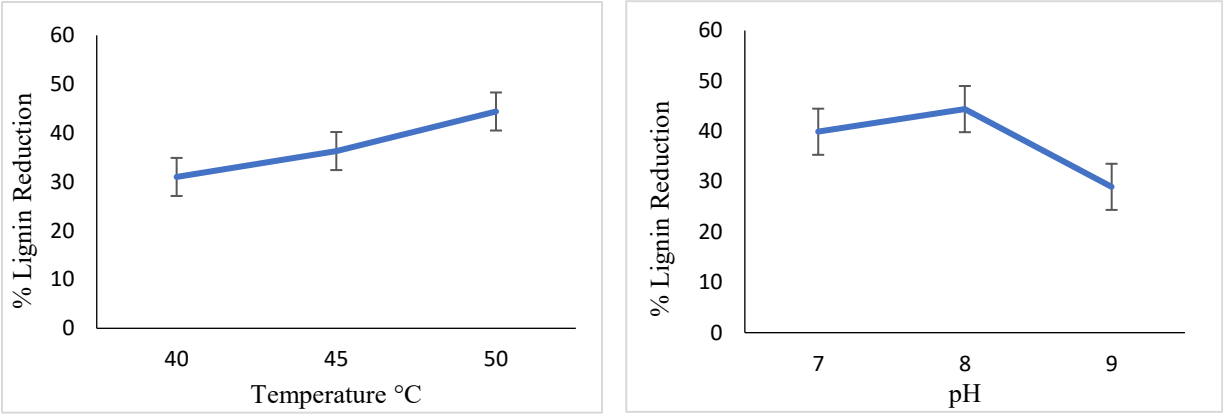


Figure 4.5 Effect of temperature and pH on lignin degradation (%) by bacterial strain SP2 (10)

Thus, SP2(10) shows maximum lignin reduction of 44.4% at 50°C and pH 8. The following are the trends for the lignin and color reduction of SP2(10) at this optimum temperature and pH.

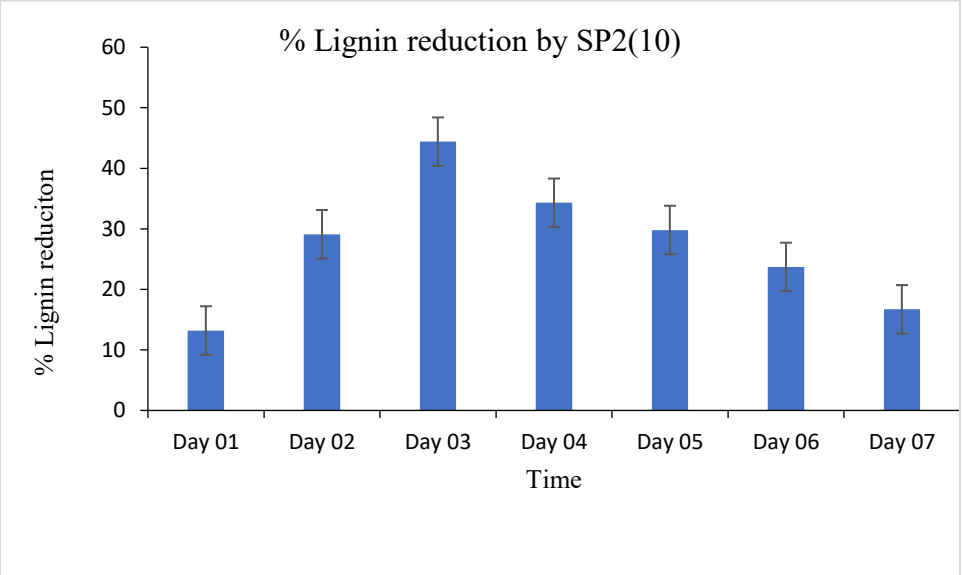


Figure 4.6 Evaluation of lignin degradation (%) by bacterial strain SP2 (10) over 168h at 50°C

The % color reduction of 47.2% in case of SP2(10) was observed by adding 3 ml of phosphate buffer in 1ml of supernatant which is shown in graph below.

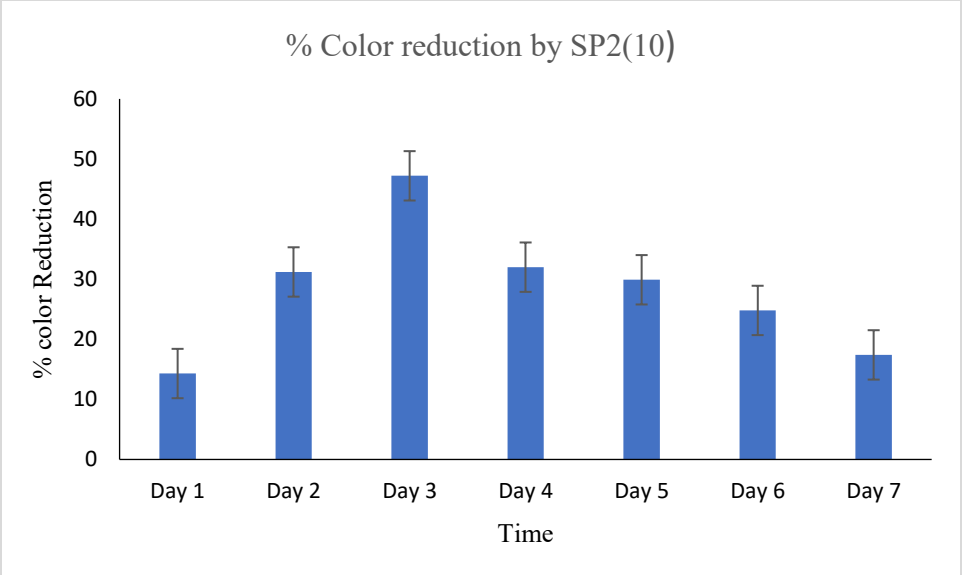


Figure 4.7 Evaluation of color reduction (%) by bacterial strain SP2 (10) over 168h at 50°C

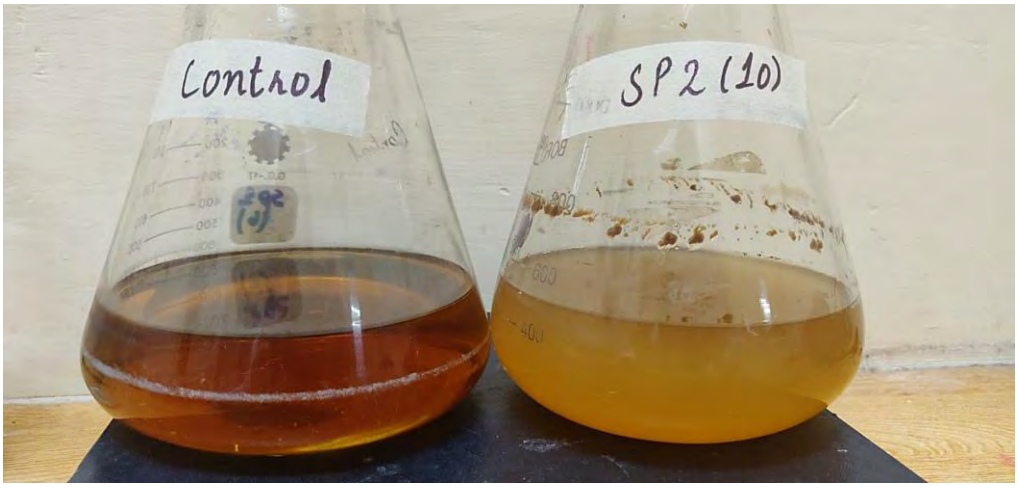


Figure 4.8 Comparison of color of untreated BL and treated BL by bacterial strain SP2 (10) over 168h at 50°C

4.4 Ligninolytic Enzyme Assays

4.4.1 Qualitative Assays

The potential of SP2(10) to produce ligninolytic enzymes were examined by guaiacol plate assay (for laccase) and methylene blue dye decolorization (for peroxidases).

4.4.1.1 Guaiacol plate assay

Isolated bacterial strain SP2(10) was inoculated on nutrient agar containing guaiacol (sterilized) and incubated for 7 days. After incubation, a brown color was observed that indicates the enzyme laccase activity. The brown colored colonies of SP2(10) are as follows:

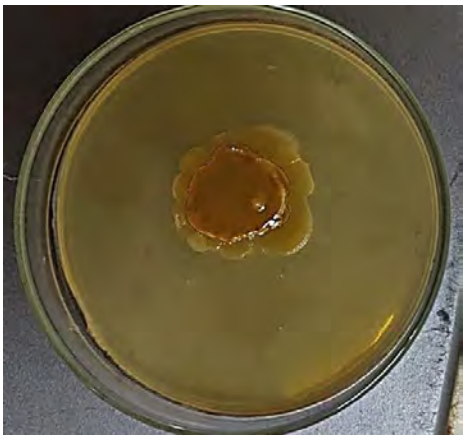


Figure 4.9 Laccase activity from SP2(10) on Guaiacol agar plate, formation of brown color around bacterial growth is indication of laccase activity.

4.4.1.2 Methylene Blue Plate Assay

Isolated Bacterial strain was inoculated on nutrient agar containing indicator i.e., methylene Blue and incubated for 7 days. Decolorization zones were observed around the colonies which is an indication of peroxidases activity. Bacterial strains forming clear zone around the colonies is shown in figure:

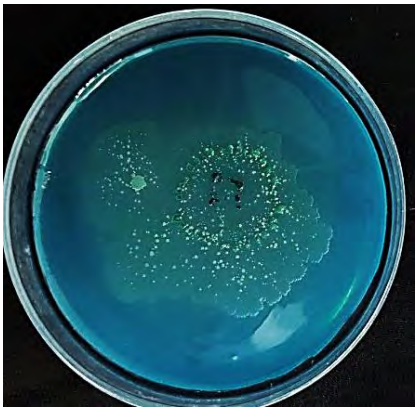


Figure 4.10 Peroxidase activity from Sp2(10) on Methylene blue agar plate, formation of clear zones around bacterial growth is indication of peroxidase activity.

4.5 Physical characterization of Selected Ligninolytic Bacteria

4.5.1 Morphology

The selected bacterial strain SP2(10) is circular, display yellowish-white colonies, have smooth rounded edges (entire), and is moist and shiny in texture.

4.5.2 Gram staining

Under the microscope, SP2 (10) bacterial cells display a pink color and exhibit a morphology characterized by cocci shape. It means that the primary stain is washed away after applying the decolorizer. Hence our bacteria are identified as Gram-negative cocci.

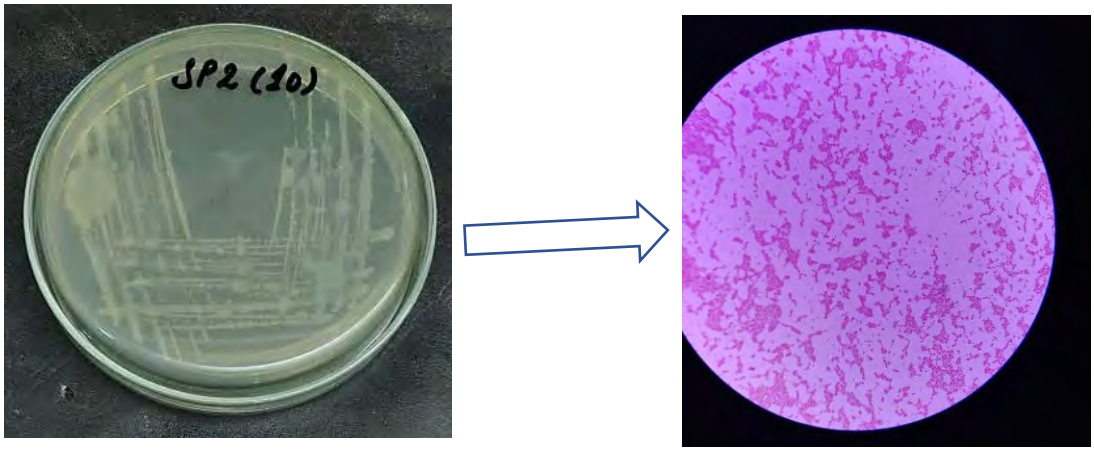


Figure 4.11 Appearance of Pink color under light microscope indicates gram negative cells

4.5.3 MacConkey Agar test

SP2 (10) colonies appear pink on MccConkey Agar indicating that our selected bacterial strain is gram-negative Lactose fermenter.

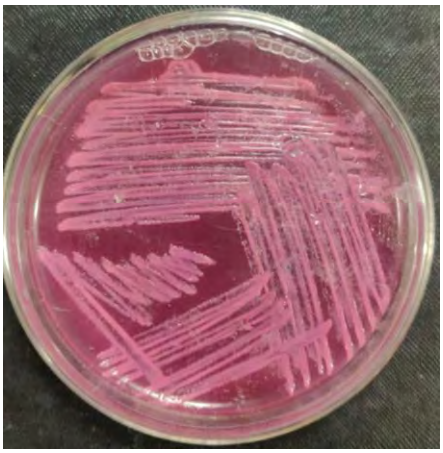


Figure 4.12 Appearance of Purple color on MacConkey agar indicates gram-negative lactose fermenter

4.5.4 Eosin Methylene Blue agar test

SP2 (10) appears dark purple and displays slight green metallic sheen on EMB agar indicating that our strain is vigorous lactose fermenter.

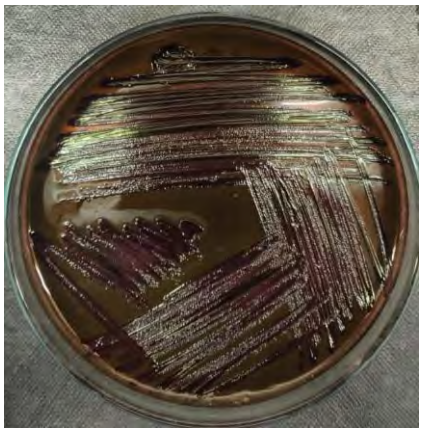


Figure 4.13 Appearance of dark purple color with slight metallic sheen on EMB indicates a vigorous fermenter

4.5.5 Catalase

Bubbles formed after the addition of H_2O_2 indicating Positive result. It means that Sp2(10) produces enzyme catalase that converted H_2O_2 into O_2.

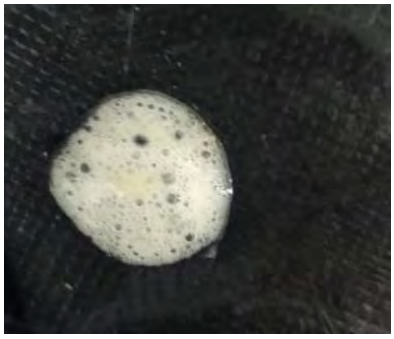


Figure 4.14 Formation of oxygen bubbles indicate positive catalase test

4.5.6 Citrate Utilization Test

Color changes from green to Prussian blue which indicates positive result. It means that SP2(10) can use citrate as a carbon source in the absence of glucose or lactose.

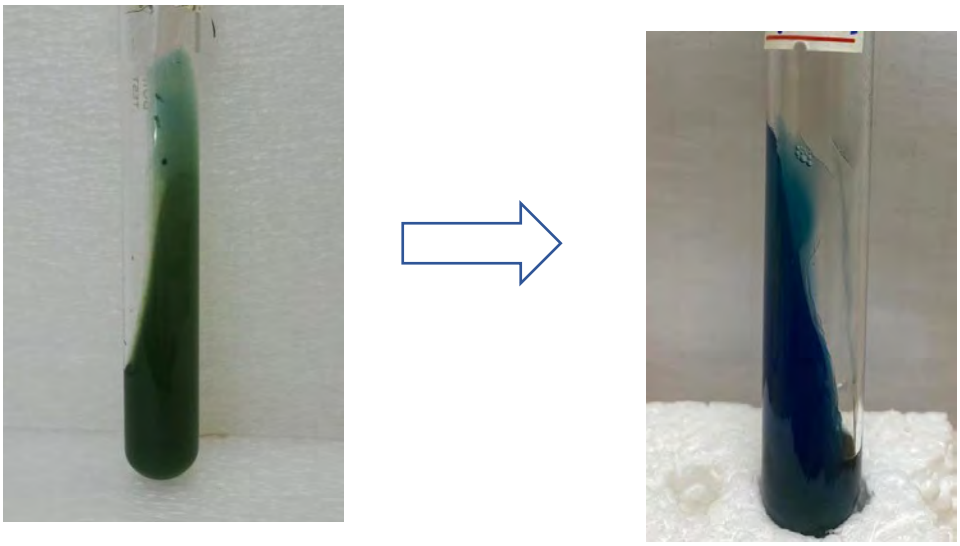


Figure 4.15 Changing of color from green to Prussian blue indicates positive citrate test

4.5.7 TSI agar test

Yellow (butt) / Yellow(slant) (A/A) with gas production was observed. It indicates positive result meaning SP2(10) can be able to ferment lactose sucrose and/or dextrose

.**Figure 4.16** Slant yellow and Butt yellow with gas production indicates that SP2(10) can ferment lactose and/or sucrose

Table 4.3 Summary of Identification of Bacterial Strain SP2 (10)

B4	**Results**
Morphology	Circular, yellowish white colonies, transparent and smooth, moist, shiny texture
Gram staining	Gram Negative rods
Catalase test	Positive
Oxidase test	Negative
TSI test	Positive
Citrate utilization test	Positive
MacConkey agar test	Pink Colonies
EMB test	Dark purple colonies with slight metallic sheen

4.6 Phyto toxicity and Cytotoxicity of Black liquor

4.6.1 Seed Germination

The Seed Germination test was performed to check toxicity of untreated black liquor and treated black liquor. For this 1% black liquor was subjected to treatment by Sp2(10) for 7 days. The seeds (chickpea) were then exposed to untreated and treated wastewater. Initially, the seeds exposed with untreated wastewater failed to germinate, however, when exposed to treated wastewater, they are capable of germinating.

a) b)

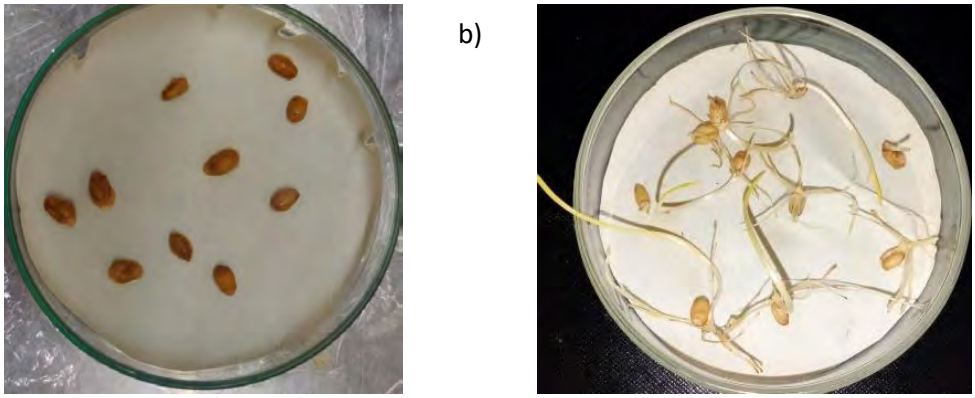


Figure 4.17 showing a) the seeds exposed with untreated water (control) fails to germinate b) the seeds exposed with treated wastewater germinates

4.6.2 Cytotoxicity testing by Brine Shrimps

The cytotoxicity of the treated and untreated black liquor was investigated via brine shrimp lethality assay. In this study, cytotoxic effects of the treated black liquor were low with shrimps' viable rates of $\geq 60\%$ at the concentration of 500 µL/ml. However, in the control (Untreated BL), mortality rate was 100% at a similar concentration. The following graph shows the % mortality rate of brine shrimps when exposed to different concentrations of treated and untreated BL shows the % mortality rate of brine shrimps when exposed to different concentrations of treated and untreated BL.

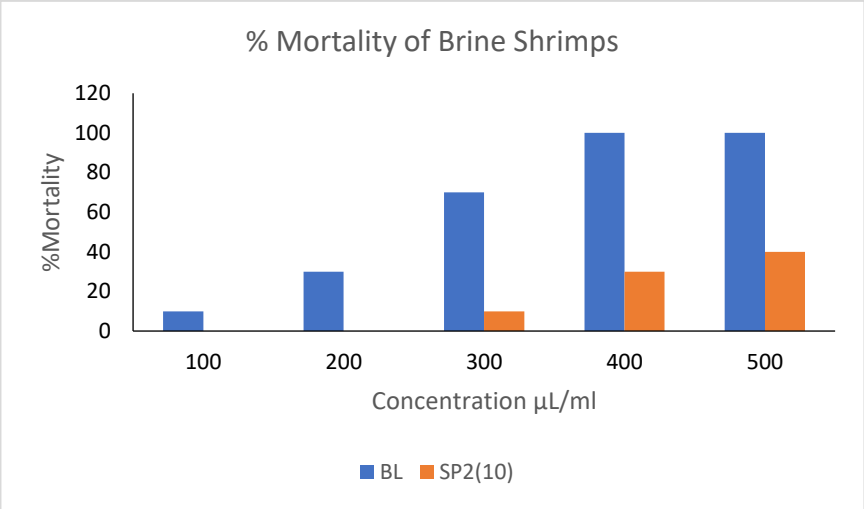


Figure 4.18: Graph showing the % mortality of brine shrimps when exposed to untreated BL and treated BL by SP2 (10)

4.7 Analysis of Lignin degradation by FT-IR

FTIR spectroscopy was used to examine the sample for modifications in the polymeric structure of lignin following treatment with SP210. The test sample's spectrum revealed a slight decrease in absorbance at wave numbers 3500–3000 cm^{-1} which is related to the lignin's phenol and alcohol -OH bond stretching frequency. Moreover, The stretching of C=C bonds in the region of wavenumber 1600-1400 cm^{-1} relates to changes in the aromatic structure of lignin. The appearance of new peaks during degradation from this region demonstrated that SP210 modified and degraded the lignin's aromatic skeleton in addition to oxidizing the side chain.

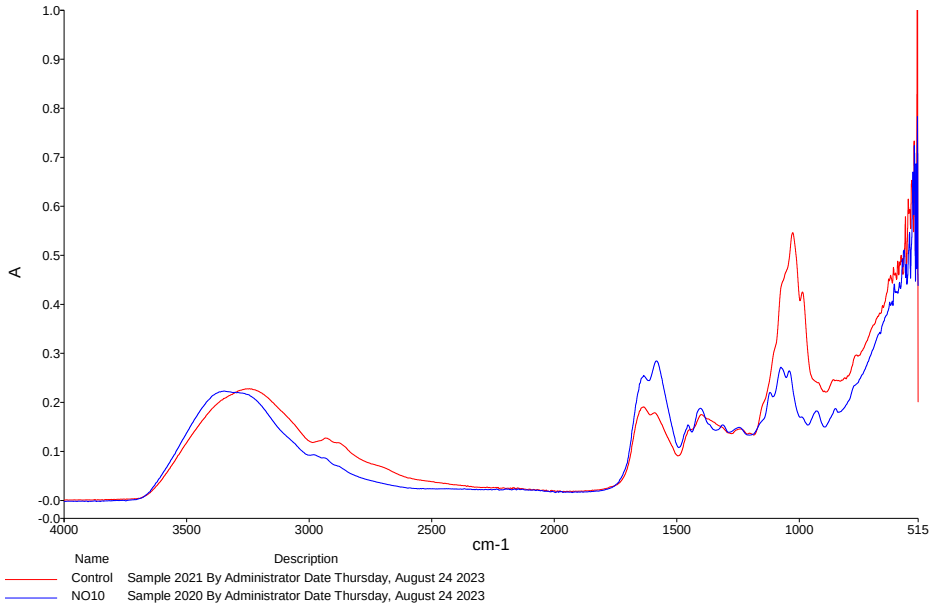
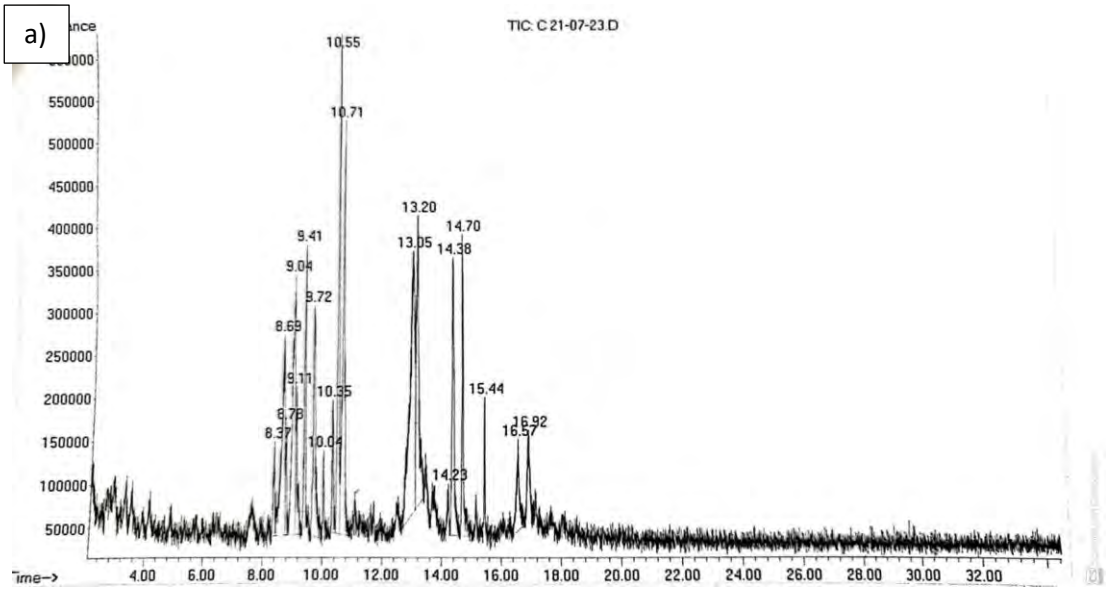


Figure 4.19 FTIR Spectra of BL after treatment with SP2(10)

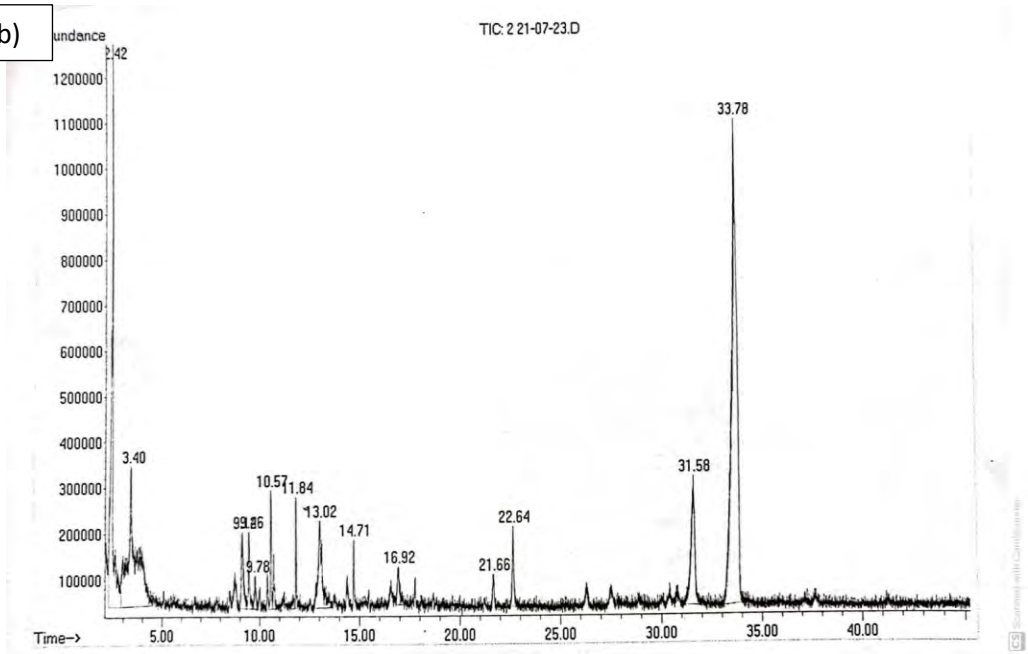
4.8 Metabolite characterization through GC–MS

To explore the lignin biodegradation and analysis of its metabolic products, both control and treated BL samples were analyzed by GC–MS. The GC-MS analysis of the control revealed prominent peaks with a retention time between 8 to 16. A detailed list of the compounds produced as a result of the breakdown of lignin and its derivatives is provided in the table below. The results showed a significant qualitative and quantitative difference in the pattern of products obtained through lignin degradation by bacteria as compared to control. the TIC (total ion chromatogram) of the treated sample revealed significant compound consumption, indicating that the bacteria have a strong ability to use the constituents as its carbon, and energy source. Some lignin-derived compounds such as 2-Hexanol (RT 2.433min), 2- Butanol (RT 3.398min), 2,5-Cyclohexadien-1-one, 3,5-dihydroxy-4, 4-dimethyl, 3 Penten-2-ol, 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-Benzo[b]dihydropyran, 6-benzyloxy-4, 4-dimethyl-, P-Hydroxybiphenyl, Pyrimidine-4, 6(3H,5H)-dione, 2,3-dihydro-2-(4-chlorophenylimino)-5,5-di-1-methyl, (E)-1, 3-Butadien-1-ol, Valeraldehyde, dimethylhydrzone, 2- Butenoic acid, 2,3-dimethyl-, 6-Oxabicyclo[3,1,0]hexa-3-one, 2,2,4,4-tetramethyl- were detected. Total ion gas chromatogram of products obtained from untreated and treated BL is shown below:

a)


TIC: C 21-07-23.D
10.55
10.71
13.20
13.05
14.70
14.38
9.41
9.04
9.72
8.69
9.11
10.35
8.78
8.37
10.04
15.44
16.92
16.57
14.23
Time-->
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
32.00

b)


TIC: 2 21-07-23.D
2.42
33.78
3.40
31.58
10.57
11.84
13.02
14.71
22.64
16.92
21.66
9.78
Time-->
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00

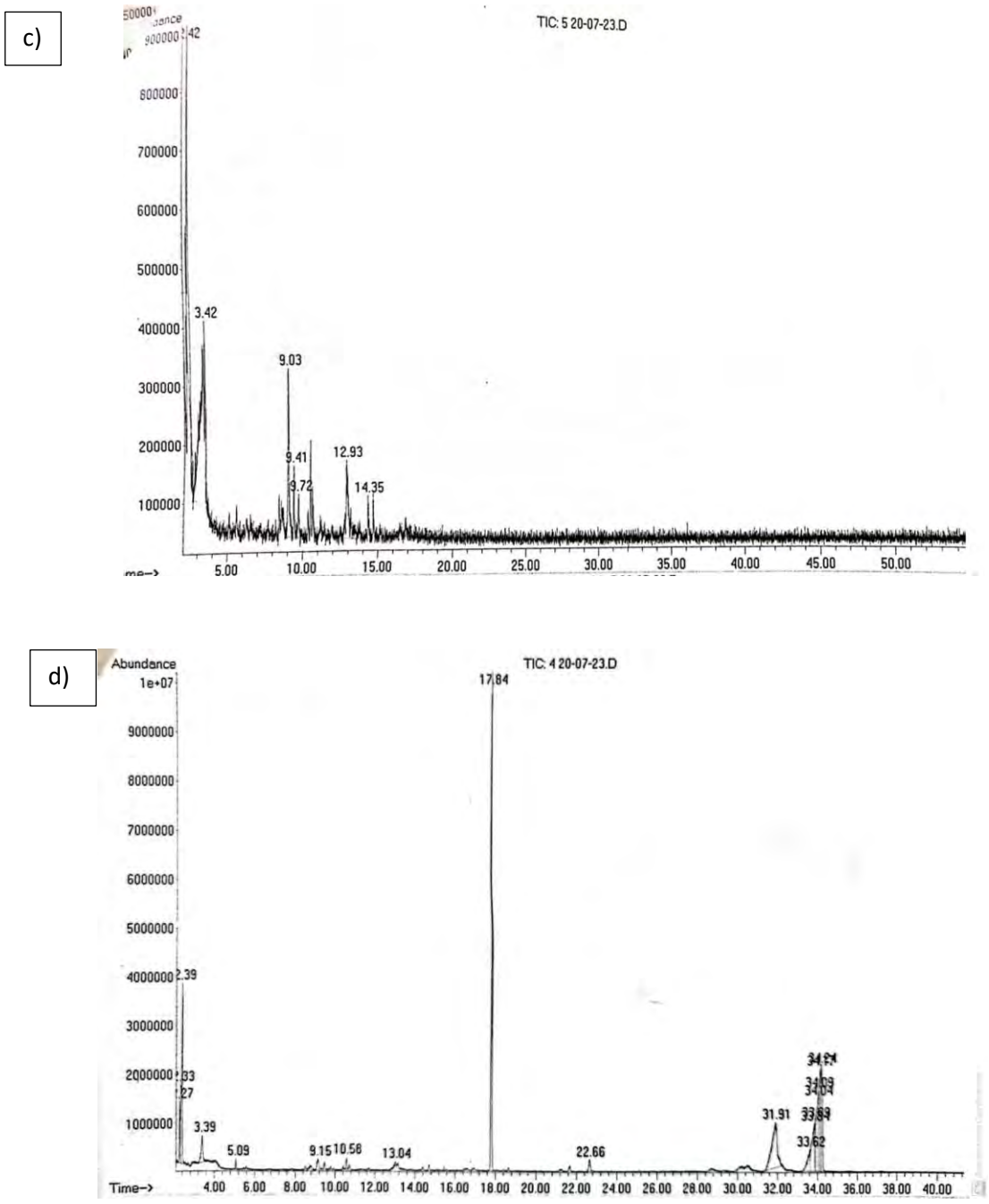


Figure 4.20 Total ion gas chromatogram of products obtained from Black liquor at a) 0-hour b) 24 hours c) 62 hours d) 110 hours

Table 4.4 Compounds identified in treated and untreated BL by GC–MS analysis

Compound Name	**RT (min)**	**Control**	2d	4d	6d
2-Pentanol, 3-methyl-	2.391	_	-	+	-
2-Hexanol	2.433	-	+	-	-
2 –Ethoxypentane	3.393	-	-	+	+
2- Butanol, 3-methyl-, (S)	3.398	-	+	-	-
Hydrazine, propyl-	3.432	-	-	-	+
Butane, 1-methoxy	5.084	-	-	+	-
Valeraldehyde, dimethylhydrzone	8.697	+	-	-	-
2- Butenoic acid, 2,3-dimethyl-	9.114	+	-	-	-
Borane, diethylpropyl-	9.124	-	+	-	-
Pyrimidine-4, 6(3H,5H)-dione, 2,3-dihydro-2-(4-chlorophenylimino)-5,5-di-1-methyl	9.144	-	-	+	-
6-Oxabicyclo [3,1,0] hexa-3-one, 2,2,4,4-tetramethyl-	9.379	+	-	-	-
Urea, 1-(4-chlorophenyl_-3-(4,6-dimethoxypyrimidin-2-yl)-	9.411	-	-	-	+
2-Butenal	9.711	+	-	-	-
(E)-1, 3-Butadien-1-ol	9.718	-	-	-	+
2-Hydroxy-3, 5, 5-trimethyl-cyclohex-2-enone	10.547	+	-	-	-
2,5-Cyclohexadien-1-one, 3,5-dihydroxy-4, 4-dimethyl-	10.582	-	+	-	-
5-Isopropylidene-3, 3-dimethyl-dihydrofuran-2-one	10.717	+	-	-	-
3 Penten-2-ol	11.831	-	+	-	-

2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	13.019	-	+	-	+
2-Hexanone, 3,3-dimethyl-	13.036	-	-	+	-
P-Hydroxybiphenyl	13.196	+	-	-	-
2(1H)-pyrinethione, 4-methyl-	14.350	-	-	-	+
2-Ethyl-N-(4-methylphenylsulfonyl) azetidine-3-one	14.364	+	-	-	-
Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	14.703	+	+	+	+
Benzo[b]dihydropyran, 6-benzyloxy-4, 4-dimethyl-	16.922	-	+	-	-
Squalene	17.820	-	-	+	-
Vitamin E	22.643	-	+	-	-
D,.alpha,-Tocopherol	22.661	-	-	+	-
Acetic acid, 13-acetoxy-4, 4,6a,6b,8a,11,12,14b-octamethyldocosahydropicen-3-yl-ester	31.578	-	+	-	-
5H-3, 5a-Epoxynaphth[2,1-c] oxepin, dodecahydro-3, 8,8, 11a-tetramethyl-, alpha., 5a, alpha., 7a, alpha., 11a, beta., 11b.alpha.)]-	31.912	-	-	+	-
Lanosta-8, 24-dien-3-ol, acetate, (3 beta)-	33.762	-	+	+	-

4.8 Fractions obtained via column Chromatography

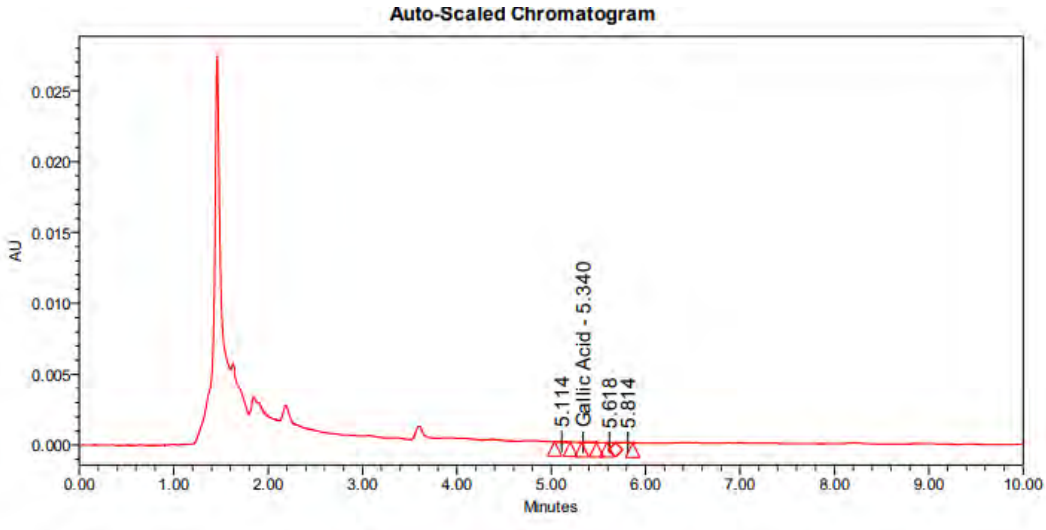
Following are the fractions obtained through Column Chromatography and these fractions are analyzed for Gallic acid and Caffeic acid via HPLC.

Table 4.5 Experimental yield of Ethyl acetate Extract of Lignin biodegradable sample

Number of elutes	Solvent System	Fractions
1-13	H: EA (100:0)	Fraction A
13- 30	H: EA (75:25)	Fraction B
30-48	H: EA (50:50)	Fraction C
48-59	H: EA (25:75)	Fraction D
59-66	H: EA (0:100)	Fraction E

4.9 HPLC

HPLC profiles of fractions of Ethyl acetate extract were analyzed for two phenolic compounds viz gallic acid, and caffeic acid. The separation was achieved using water/methanol/acetic acid (87:8:5) as the mobile phase with an injection volume 10.00 ul and run time 10.00 minutes. Both gallic acid (RT 5.365) and caffeic acid (RT 4.015) were detected in Fraction C and fraction D. The HPLC profile of gallic and caffeic acid are as follows:



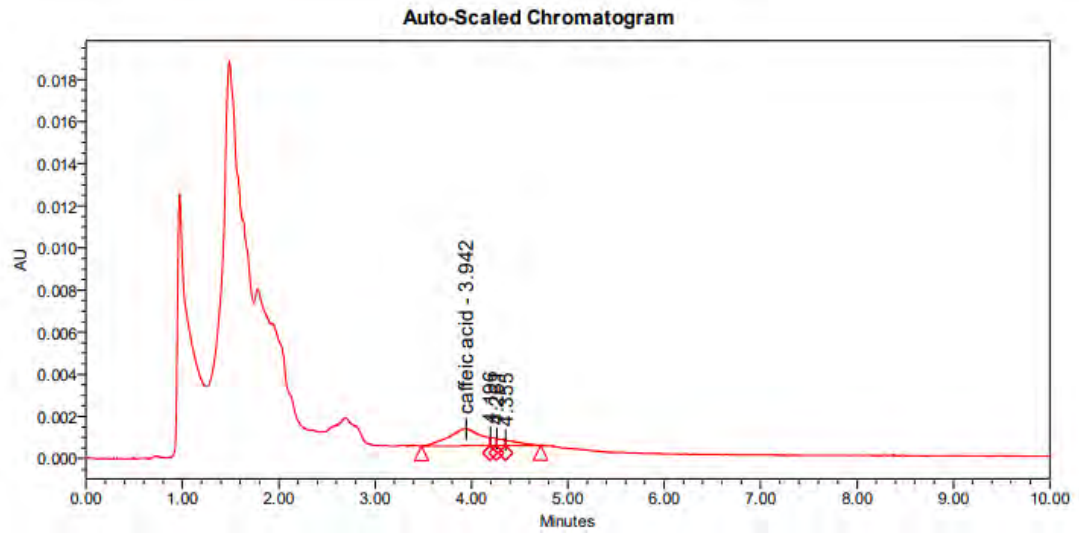
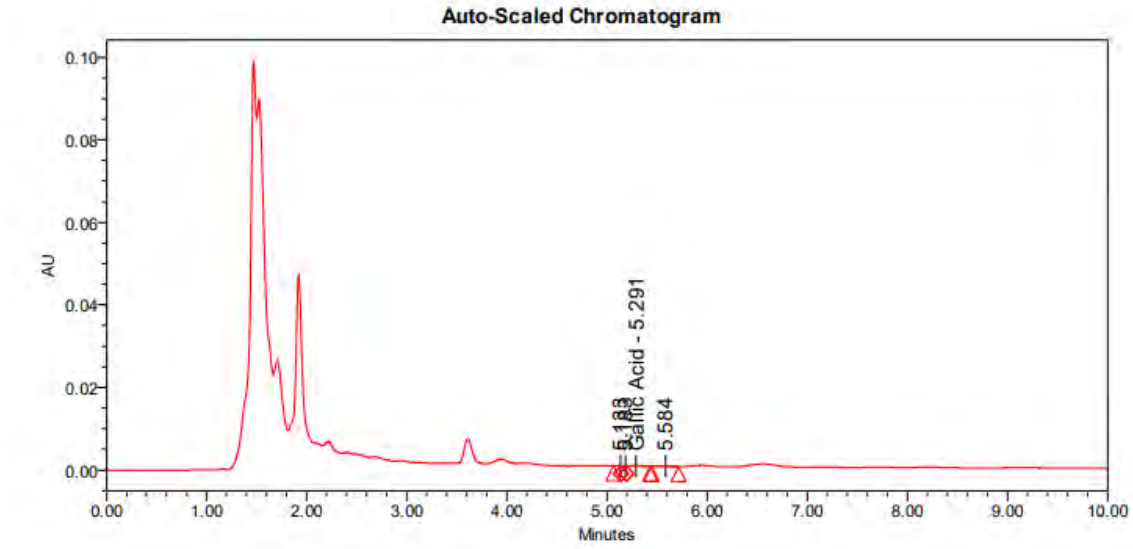


Figure 4.21 Graph showing the HPLC profile of Gallic acid and Caffeic acid in Fraction C



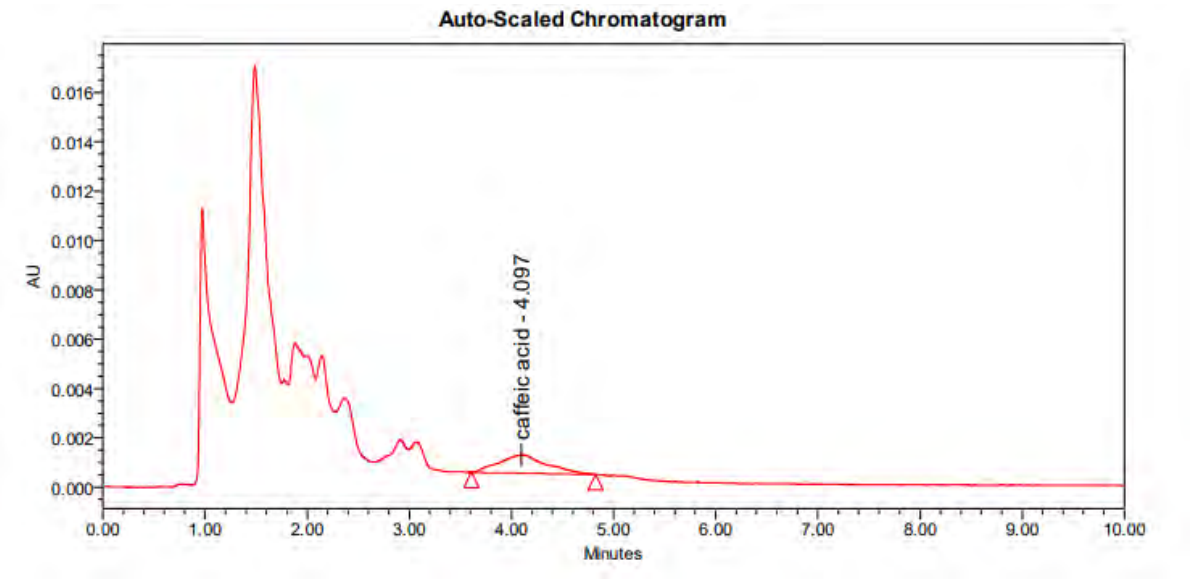


Figure 4.22 Graph showing the HPLC profile of Gallic acid and Caffeic acid in Fraction D

Table 4.6: Showing the RT and Number of phenolic compounds detected in Fraction C and D

Extract or Fractions	**Identified Compounds**	**Retention time**	**Area**	**Height**	**Amount mg/ml**
Fraction C (H: EA) (50:50)	Gallic acid	5.340	81	14	0.004671
	Caffeic acid	3.942	18427	811	0.008209
Fraction D (H: EA) (25:75)	Gallic acid	5.365	170	36	0.009101
	Caffeic acid	4.097	24159	748	0.009866

Discussion

Paper and pulp mills are a significant and rapidly expanding sector of the global economy. The production of paper and pulp is increasing with time globally and will continue to do so in the near future. Most of these industries are located close to the waterways and have access to a plentiful and consistent supply of water (Tariq & Mushtaq, 2023). These mills release the wastewater into the waterways after pulp and paper manufacture. Effluent from these industries contains high organic content in which lignin and chlorinated phenols are of major concern. Lignin gives water a brown color that can be seen from a considerable distance. This dark color prevents the entry of sunlight into water which limits the growth of phototrophic organisms. Similarly, lignin generated many chlorinated compounds after its reaction with chlorine. These chlorinated compounds are highly toxic to aquatic life. Around 80% of lignin produced globally is kraft lignin. However, only a small percentage of this lignin (around 1-2%) is used to produce high-value products, while the other 98-99% is burned to generate heat and energy (Pinar Karagoz et al., 2023). By considering the toxic effect of lignin and its limited consumption to produce value-added products, the current study is focused on lignin degradation and its valorization to high-value products by thermophilic bacteria isolated from hot springs.

In this study, the sludge sample of Hot Spring was used to isolate lignin-degrading bacteria. Isolating bacteria from hot springs can offer numerous benefits for our lignin valorization research. Hot springs are well-known for their harsh conditions, including high temperatures and a wide range of pH levels. These circumstances encourage the development of distinctive and varied microbial communities that have developed to adapt to such environments (Mohammad et al., 2017). These extremophiles can have special metabolic abilities that might be used for lignin degradation. By isolating microorganisms from hot springs, we gain access to a wide range of microorganisms with specialized metabolic pathways and enzymatic systems that could be more effective in degrading lignin (Choure et al., 2021). Moreover, thermophilic bacteria have faster metabolic rates at high temperatures, which speeds up enzymatic reactions and can lead to effective lignin degradation and conversion to useful compounds. Elevated temperatures may speed up

reactions and improve the effectiveness of lignin degradation. However, the investigation of the potential of thermophilic bacteria isolated from hot springs for lignin valorization is a somewhat untapped avenue.

The contemporary Investigation involves the isolation of 7 different bacterial strains from SP1 and 13 from SP2 on nutrient agar plates via serial dilution and spread plate method. To determine the potential of these bacterial strains to degrade lignin, these strains were screened on L-MSM having different lignin content i.e., 1 g/L, 2 g/L, and 3 g/L. Only five bacterial Strains i.e., two from SP1 and three from SP2 were able to tolerate it and showed growth in the media containing lignin. Only potent bacteria can be able to tolerate high lignin concentrations as lignin and its derived aromatics have negative effects on cells by destroying membranes, damaging DNA, and inhibiting enzymes.

The lignin-degrading ability of these selected bacterial strains was further studied by growing them in a media that contains black liquor (1%) as the only carbon source. Samples were withdrawn daily, and the lignin reduction was analyzed by Pearl and Benson method. (Singh et al., 2019) and (Yadav et al., 2023) also used this conventional method to estimate lignin degradation. Among the other strains, Sp2 (10) showed the maximum lignin reduction i.e., about 31% at 40°C. therefore, this strain was selected for further studies.

Physical parameters such as temperature and pH greatly affect the ligninolytic activity of bacteria, therefore we further study the effect of temperature and pH on the lignin degradation by SP2(10). The effects of different temperatures (40-50°C) and pH (7-9) on lignin degradation were assessed, and it was found that our strain SP2(10) showed the maximum lignin degradation i.e., about 44% and color reduction i.e., 47% at 50 °C and pH 8. Currently, a number of bacterial strains isolated from multiple ecological niches are being investigated to remove the color and toxicity of P&P mill effluent (Mohammad & Bhukya, 2022, Majumdar et al., 2019, (Kumar & Chandra, 2021)). However, most of these bacteria are able to adapt to moderate temperatures, and their pH range is limited. These strains are not appropriate for the wastewater treatment produced during paper production due to their harsh environmental conditions and high lignin content. Reports on lignin-degrading strains that are simultaneously thermophilic and alkali tolerant that can be

utilized to treat BL are very rare. (An et al., 2021) investigated the novel thermophilic and alkali-tolerant bacterial strain Serratia sp. AXJ-M for its ability to bioremediate and detoxify BL produced in papermaking and reported the maximum lignin reduction of 70.7% and color reduction of 80% at 50 °C. In contrast to this earlier reported higher lignin degradation, the relative lower lignin reduction seen in our study could be the result of a number of factors. First of all, Naturally occurring bacteria in black liquor have likely endured selected pressures through time, leading to greater lignin-degrading capabilities specific to that environment, as compared to bacteria found in hot springs, which may not have been subjected to the same level of environmental conditions. Additionally, Our research focused exclusively on temperature and pH, while the previous study has taken a wider range of factors into account, including nutrition availability, salinity level, glucose concentration, and other co-factors necessary for lignin-degrading enzymes. Moreover, (Mittal et al., 2023) studied *Bacillus sonorensis* NAM5 for the production of polyhydroxy butyrate (pHB) from P&P mill effluent. It was reported that the NAM5 showed maximum growth and PHB production at 50°C.

Enzymatic potential to degrade lignin was examined further by both qualitative assays. In this assay, laccase activity was determined by guaiacol plate assay. SP2(10) was screened on a nutrient agar plate containing guaiacol as a substrate for laccase. The bacterial strain showed positive results which are indicated by brown color formation. The formation of brown color depicts the Oxidative depolymerization of the substrate (Guaiacol) via laccase. (Sharma et al., 2019) also reported similar results during their investigation regarding the production of thermotolerant laccase from the thermophilic bacterium *Bacillus sp*. PC-3 and Bacillus *licheniformis* VNQ isolated from Tattapani hot springs, India. Moreover, The peroxidase activity was examined by methylene blue dye decolorization assay in which SP2(10) was grown in a nutrient agar containing the indicator methylene blue. The strain showed positive results which are indicated by decolorization zones. These zones are due to the formation of colorless amines. (Wu et al., 2022) investigated the decolorization of methylene by Ligninolytic bacteria and reported similar results. Furthermore, the morphology and physiology of bacteria were studied. It was observed that SP2(10) was Gram-negative, cocci, oxidase negative, catalase positive, TSI positive, Citrate positive,

and shows pink colonies on MacConkey agar and Dark purple color with slight metallic sheen on EMB. These results are consistent with species belonging to the Enterobacteriaceae family. (Cortes-Tolalpa et al., 2020) reported the ligninolytic bacteria C. freundii so4 isolated from microbial consortia that could break down raw wheat straw. Similarly, (dos Santos Melo-Nascimento et al., 2020) reported the bacteria *K. variicola* P1CDI isolated from soil sediments, at Chapada Diamantina National Park capable of lignin degradation.

Moreover, the phytotoxicity and cytotoxicity of treated black liquor were analyzed via seed germination and brine shrimp lethality assay. The findings indicated that the toxicity of treated black liquor decreased, which might have been due to the biodegradation of toxic chemicals during the biological treatment. The toxicity of pulp mill effluent was also evaluated by (Ren et al., 2022), and reported that the germination rate of Vigna unguiculata seeds grown in untreated effluent significantly decreased from 92.3% (distilled water) to 66.67% and the radicle length decreased from 1.57 to 0.68 cm, demonstrating the severe toxicity of the untreated wastewater in nature, which supports our hypothesis that treatment of BL using Sp2 (10) can greatly reduce wastewater toxicity and significantly enhance wastewater characteristics.

After the evaluation of lignin degradation by SP2(10), its derived metabolites were identified via GC-MS. The results demonstrated significant differences between untreated and treated black liquor. Additionally, the results indicated a high degree of compound consumption in comparison to the control group; this can be attributed to the strain Sp2 (10) remarkable ability to utilize its constituents as the only source of energy, carbon, and nitrogen. Many compounds such as P-Hydroxybiphenyl, 2- Butenoic acid, 2,3-dimethyl-**,** 6-Oxabicyclo [3,1,0] hexa-3-one, 2,2,4,4-tetramethyl-, and 2-Butenal were detected in control. These are the degradation products of lignin, which may have been generated by industrial processes. Compounds like Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- remain unchanged both in the control and treated sample. It might be because it possesses an antimicrobial property (an antibiotic agent) that could possibly explain its resistance to degradation. Antibiotic compounds are mostly resistant to degradation to carry out their intended functions of eradicating or preventing the growth of

Bacteria. In addition, 2- Butanol, 2-Hexanol, Acetic acid, 2,5-Piperazinedione, 3,6-bis(2-methyl propyl)-, Benzo[b]dihydropyran, 6-benzyloxy-4, 4-dimethyl-, Pyrimidine-4, 6(3H,5H)-dione, 2,3-dihydro-2-(4-chlorophenylimino)-5,5-di-1-methyl, (E)-1, 3-Butadien-1-ol, and Valeraldehyde, dimethylhydrzone were also detected in samples of day 2, 4 and 6. (Lin et al., 2020) have reported the presence of Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)-, 2,5-Cyclohexadien-1-one, 3,5-dihydroxy-4, 4-dimethyl and 6-Oxabicyclo[3,1,0]hexa-3-one, 2,2,4,4-tetramethyl- in the extracted sewage sludge bio-oil. Bio oils are reported to be produced from kraft lignin by hydrothermal liquefaction (Lyckeskog et al., 2017). Moreover, Suman et al., 2016) reported the presence of acetic acid in the lignin extract treated with strain IIPTG13. Likewise, other previous reports have detected the presence of 2,5-Piperazinedione, 3,6-bis(2-methylpropyl), Butenoic acid (Singh et al., 2019b), 1,3-butanediol (Chen et al., 2012) (Senthilvelan et al., 2017) in the treated KL sample extract.

The lignin-derived phenolic compounds were separated via column chromatography. For the extraction of phenolic compounds, ethyl acetate, ethanol and methanol extracts were selected as hexane is non-polar and is effective in solubilizing non-polar compounds like lipids, oils, fats, etc. (Cravotto et al., 2022) As lignin biodegradable samples can be highly complex and contain a variety of compounds with variable polarities therefore, the hexane extract might have a higher proportion of non-polar components, which is not relevant to our research goals. (Gini & Jeya Jothi, 2018) also performed separated the phenolic compounds present in the Salvinia molesta Mitchell via column chromatography of extracts from the same above-mentioned solvents.

HPLC detected the presence of two phenolic compounds i.e., gallic acid and caffeic acid in fraction B and Fraction E of ethyl acetate extract. The amount of both gallic acid and caffeic acid increases as the solvent composition moves toward the high polarity. (Raj et al., 2006) detected the presence of gallic acid in the treated KL sample by *Aneurinibacillus aneurinilyticus*. The detection of these phenolic compounds was an indicator of lignin degradation, as these were thought to represent the fundamental structural constituents of the lignin polymer. Although the concentration is small, the production of gallic acid and caffeic acid sets the stage for possible scalability. These compounds are not detected in

GC-MS because the Black liquor contains a variety of non-polar and polar compounds, present in different concentrations that can affect the accuracy and precision of analysis. Moreover, GCMS is more suited for compounds present in high concentration, non-polar and volatile; on the other hand, HPLC is highly precise and detects compounds even in the lowest concentration and are polar.

Conclusion

Depending on the sort of processes employed, pulp-and-paper mills produce a variety of contaminants as well as a substantial amount of effluent. The Effluent discharge from Pulp and paper mills has a significant pollution load and contains compounds that contribute to color, which seriously pollute the water and land. A potential thermophilic ligninolytic bacterial strain SP2(10) was isolated from Hot Spring, Chitral. This bacterium significantly degrades the lignin and reduces the color by effectively producing Ligninolytic enzymes. Additionally, the toxicity of the treated effluent was found to be greatly reduced. Moreover, the present research has reported the presence of some phenolic compounds in the EAE fractions of lignin biodegradable sample, including gallic acid and caffeic acid. The investigation of lignin biodegradation and its valorization by SP2(10) has enormous promise for converting a copious waste product into a useful resource. We have the unique opportunity to reshape industries, support sustainable development objectives, and pave the road for a more economically viable and environmentally conscious future by taking advantage of this bacteria's intricate capabilities.

Future Prospects

The Future prospects for lignin biodegradation and valorization hold significant potential for addressing technological, economic, and environmental problems. Some of the key future prospects are as follows:

- More investigation and characterization of isolated bacterial strain to understand its lignin degrading potential.
- Optimizing the growth conditions of lignin-degrading bacterial isolate to achieve the highest lignin degradation and highest lignin-derived products.
- The isolation and genetic engineering of ligninolytic enzymes could lead to the formation of more effective and efficient enzyme cocktails.
- Expanding the lignin valorization to more value-added products, such as bioplastics, composite materials, and lipids, offers Exciting opportunities for developing new markets and lowering reliance on fossil fuels.

References

Abhishek, A., Dwivedi, A., Tandan, N., & Kumar, U. (2017). Comparative bacterial degradation and detoxification of model and kraft lignin from pulp paper wastewater and its metabolites. Applied Water Science, 7(2), 757–767

Ahmad, A., Khan, S., Khan, M., Luque, R., Jalalah, M., & Alsaiari, M. A. (2022). Microwave Assisted Preparation of Barium Doped Titania (Ba/TiO2) as Photoanode in Dye Sensitized Solar Cells. Applied Sciences, 12(18), 9280.

Ahmad, Z., Al Dajani, W. W., Paleologou, M., & Xu, C. (Charles). (2020). Sustainable Process for the Depolymerization/Oxidation of Softwood and Hardwood Kraft Lignins Using Hydrogen Peroxide under Ambient Conditions. Molecules, 25(10), 2329.

Ahmed, M., Mavukkandy, M. O., Giwa, A., Elektorowicz, M., Katsou, E., Khelifi, O., Naddeo, V., & Hasan, S. W. (2022). Recent developments in hazardous pollutants removal from wastewater and water reuse within a circular economy. *Npj Clean Water*, *5*(1).

An, X., Li, N., Zhang, S., Han, Y., & Zhang, Q. (2023). Integration of proteome and metabolome profiling to reveal heat stress response and tolerance mechanisms of Serratia sp. AXJ-M for the bioremediation of papermaking black liquor. *Journal of Hazardous Materials*, *450*, 131092.

An, X., Zhong, B., Chen, G., An, W., Xia, X., Li, H., Lai, F., & Zhang, Q. (2021). Evaluation of bioremediation and detoxification potentiality for papermaking black liquor by a new isolated thermophilic and alkali-tolerant Serratia sp. AXJ-M. *Journal of Hazardous Materials*, *406*, 124285.

Andersson, K. I., Eriksson, M., & Norgren, M. (2011). Removal of Lignin from Wastewater Generated by Mechanical Pulping Using Activated Charcoal and Fly Ash: Adsorption Isotherms and Thermodynamics. *Industrial & Engineering Chemistry Research*, *50*(13), 7722–7732.

Ayodeji, F. D., Shava, B., Iqbal, H. M. N., Ashraf, S. S., Cui, J., Franco, M., & Bilal, M. (2022). Biocatalytic Versatilities and Biotechnological Prospects of Laccase for a Sustainable Industry. *Catalysis Letters*.

Baghel, S., & Anandkumar, J. (2019). Biodepolymerization of Kraft lignin for production and optimization of vanillin using mixed bacterial culture. *Bioresource Technology Reports*, *8*, 100335.

Bajpai, P. (2018). *Biermann's Handbook of Pulp and Paper - 3rd Edition*. Shop.elsevier.com.

Bajwa, D. S., Pourhashem, G., Ullah, A. H., & Bajwa, S. G. (2019). A concise review of current lignin production, applications, products and their environmental impact. *Industrial Crops and Products*, *139*, 111526.

Brosse, N., Hussin, M. H., & Rahim, A. A. (2017). Organosolv Processes. *Advances in Biochemical Engineering/Biotechnology*, 153–176.

Chandra, R., Singh, R., & Yadav, S. (2012). *Effect of bacterial inoculum ratio in mixed culture for decolourization and detoxification of pulp paper mill effluent*. ResearchGate; Journal of Chemical Technology & Biotechnology.

Chen, Y., Chai, L., Tang, C., Yang, Z., Zheng, Y., Shi, Y., & Zhang, H. (2012). Kraft lignin biodegradation by Novosphingobium sp. B-7 and analysis of the degradation process. *Bioresource Technology*, *123*, 682–685

Cheng, Y., Huang, M., Shen, X., & Jiang, C. (2022). Enhanced cornstalk decomposition by a psychrotrophic bacterial consortium comprising cellulose, hemicellulose, and lignin degraders with biochar as a carrier for carbonneutrality. *Bioresource Technology*, *344*, 126259.

Choure, K., Parsai, S., Kotoky, R., Srivastava, A., Tilwari, A., Rai, P. K., Sharma, A., & Pandey, P. (2021). Comparative Metagenomic Analysis of Two Alkaline Hot Springs of Madhya Pradesh, India and Deciphering the Extremophiles for Industrial Enzymes. *Frontiers in Genetics*, *12*.

Chowdhary, P., Shukla, G., Raj, G., Ferreira, L. F. R., & Bharagava, R. N. (2018). Microbial manganese peroxidase: a ligninolytic enzyme and its ample opportunities in research. *SN Applied Sciences*, *1*(1).

Chunbao Charles Xu, Dessbesell, L., Zhang, Y., & Yuan, Z. (2020). Lignin valorization beyond energy use: has lignin's time finally come? *Biofpr*, *15*(1), 32–36.

Cortes-Tolalpa, L., Wang, Y., Salles, J. F., & van Elsas, J. D. (2020). Comparative Genome Analysis of the Lignocellulose Degrading Bacteria Citrobacter freundii so4 and Sphingobacterium multivorum w15. *Frontiers in Microbiology*, *11*.

Costa, S., Dedola, D., Pellizzari, S., Blo, R., Rugiero, I., Pedrini, P., & Tamburini, E. (2017). Lignin Biodegradation in Pulp-and-Paper Mill Wastewater by Selected White Rot Fungi. *Water*, *9*(12), 935.

Cravotto, C., Fabiano-Tixier, A.-S., Claux, O., Abert-Vian, M., Tabasso, S., Cravotto, G., & Chemat, F. (2022). Towards Substitution of Hexane as Extraction Solvent of Food Products and Ingredients with No Regrets. *Foods*, *11*(21), 3412.

Cui, L., Wang, Z., Zeng, Y., Yang, N., Liu, M., Zhao, Y., & Zheng, Y. (2022). Lignin Biodegradation and Its Valorization. *Fermentation*, *8*(8), 366–366.

Dagar, S., Singh, S. K., & Gupta, M. K. (2023). Integration of Pre-Treatment with UF/RO Membrane Process for Waste Water Recovery and Reuse in Agro-Based Pulp and Paper Industry. *Membranes*, *13*(2), 199. https://doi.org/10.3390/membranes13020199

de Souza, R. E., Gomes, F. J. B., Brito, E. O., Lelis, R. C. C., Batalha, L. A. R., Santos, F. A., & Junior, D. L. (2020). *A review on lignin sources and uses*. ResearchGate; Journal of Applied Biotechnology and Bioengineering.

Debnath, B., Haldar, D., & Purkait, M. K. (2021). A critical review on the techniques used for the synthesis and applications of crystalline cellulose derived from agricultural wastes and forest residues. *Carbohydrate Polymers*, *273*, 118537.

Dessbesell, L., Paleologou, M., Leitch, M., Pulkki, R., & Xu, C. (Charles). (2020). Global lignin supply overview and kraft lignin potential as an alternative for petroleum-based polymers. *Renewable and Sustainable Energy Reviews*, *123*, 109768.

DH Bugg, T., Ahmad, M., Hardiman, E. M., & Singh, R. (2010). *Sci-Hub | The emerging role for bacteria in lignin degradation and bio-product formation. Current Opinion in Biotechnology, 22(3), 394–400 | 10.1016/j.copbio.2010.10.009*.

Dixit, M., Gupta, G. K., Liu, H., & Shukla, P. (2019). Pulp and paper industry based pollutants, their health hazards and environmental risks. *Current Opinion in Environmental Science & Health*, *12*.

Domínguez-Robles, J., Valle, del, Sánchez, R., Javier Mauricio Loaiza, Espinosa, E., Rosal, A., & Rodríguez, A. (2019). Coagulation–Flocculation as an Alternative Way to Reduce the Toxicity of the Black Liquor from the Paper Industry: Thermal Valorization of the Solid Biomass Recovered. *Waste and Biomass Valorization* , *11*(9), 4731–4742.

dos Santos Melo-Nascimento, A. O., Mota Moitinho Sant´Anna, B., Gonçalves, C. C., Santos, G., Noronha, E., Parachin, N., de Abreu Roque, M. R., & Bruce, T. (2020). Complete genome reveals genetic repertoire and potential metabolic strategies involved in lignin degradation by environmental ligninolytic Klebsiella variicola P1CD1. *PLOS ONE*, *15*(12), e0243739.

Esmaeeli, A., Mohammad-Hossein Sarrafzadeh, Siavash Zeighami, Masoud Kalantar, Saeed Ghasemzadeh Bariki, Fallahi, A., Hashem Asgharnejad, & Ghaffari, S.-B. (2023). A Comprehensive Review on Pulp and Paper Industries Wastewater Treatment Advances. *Industrial & Engineering Chemistry Research*, *62*(21), 8119–8145.

Fabbri, F., Bischof, S., Mayr, S., Gritsch, S., Jimenez Bartolome, M., Schwaiger, N., Guebitz, G. M., & Weiss, R. (2023). The Biomodified Lignin Platform: A Review. *Polymers*, *15*(7), 1694.

Fang, Y., Yin, L., Yang, H., Gong, X., Chen, Y., & Chen, H. (2021). Catalytic mechanisms of potassium salts on pyrolysis of β-O-4 type lignin model polymer based on DFT study. *Proceedings of the Combustion Institute*, *38*(3), 3969–3976.

Fortune Business Insights. (2022, July). *Pulp and Paper Market Size, Share, Analysis | Industry Trends, 2027*

Gaur, N., Narasimhulu, K., & Pydi Setty, Y. (2018). Extraction of ligninolytic enzymes from novel Klebsiella pneumoniae strains and its application in wastewater treatment. *Applied Water Science*, *8*(4).

Gini, T. G., & Jeya Jothi, G. (2018). Column chromatography and HPLC analysis of phenolic compounds in the fractions of Salvinia molesta mitchell. *Egyptian Journal of Basic and Applied Sciences*, *5*(3), 197–203.

Giummarella, N., Lindén, P. A., Areskogh, D., & Lawoko, M. (2019). Fractional Profiling of Kraft Lignin Structure: Unravelling Insights on Lignin Reaction Mechanisms. *ACS Sustainable Chemistry & Engineering*, *8*(2), 1112–1120.

Haq, I., Mazumder, P., & Kalamdhad, A. S. (2020). Recent advances in removal of lignin from paper industry wastewater and its industrial applications – A review. *Bioresource Technology*, *312*, 123636

Harshvardhan, K., Suri, M., Goswami, A., & Goswami, T. (2017). Biological approach for the production of vanillin from lignocellulosic biomass (Bambusa tulda). *Journal of Cleaner Production*, *149*, 485–490.

Helmich, K. E., Jose Henrique Pereira, Gall, D., Heins, R. A., McAndrew, R., Bingman, C. A., Deng, K., Holland, K. C., Noguera, D. R., Simmons, B. A., Sale, K. L., Ralph, J., Donohue, T. J., Adams, P. D., & Phillips, G. N. (2016). Structural Basis of Stereospecificity in the Bacterial Enzymatic Cleavage of β-Aryl Ether Bonds in Lignin. *Journal of Biological Chemistry*, *291*(10), 5234–5246.

Hou, L.-P., Ji, D., Dong, W., Yuan, L., Zhang, F., & Li, Y. (2020). The Synergistic Action of Electro-Fenton and White-Rot Fungi in the Degradation of Lignin. *Frontiers in Bioengineering and Biotechnology*, *8*.

Kamali, M., & Khodaparast, Z. (2015). Review on recent developments on pulp and paper mill wastewater treatment. *Ecotoxicology and Environmental Safety*, *114*, 326–342.

Kamimura, N., & Masai, E. (2022). *Lignin Bacterial Catabolism of a Lignin-derived β-5 Dimer*. The Lignin Society.

Kamimura, N., Sakamoto, S., Mitsuda, N., Masai, E., & Kajita, S. (2019). Advances in microbial lignin degradation and its applications. *Current Opinion in Biotechnology*, *56*, 179–186.

Kazzaz, A. E., & Fatehi, P. (2020). *Technical lignin and its potential modification routes: A mini-review*. ResearchGate; Industrial Crops and Products .

Khan, S. I., Zarin, A., Ahmed, S., Hasan, F., Belduz, A. O., Çanakçi, S., Khan, S., Badshah, M., Farman, M., & Shah, A. A. (2021). Degradation of lignin by Bacillus altitudinis SL7 isolated from pulp and paper mill effluent. *Water Science and Technology*, *85*(1), 420–432.

Kumar, A., & Arora, P. K. (2022). Biotechnological Applications of Manganese Peroxidases for Sustainable Management. *Frontiers in Environmental Science*, *10*.

Kumar, A., & Chandra, R. (2021). Biodegradation and toxicity reduction of pulp paper mill wastewater by isolated laccase producing Bacillus cereus AKRC03. *Cleaner Engineering and Technology*, *4*, 100193.

Li, J. (2020). *Biomass Pretreatment by Metal Oxides for Reducing Sugar Degradation and Water Consumption in Biofuel Production - ProQuest*.

Li, S., Wang, H., Chen, J., Zhu, H., Yao, R., & Wu, H. (2020). Degradation and Transformation of Lignin by a Fungus Aspergillus Flavus Strain F-1. *Iranian Journal of Biotechnology*, *18*(3), e2461–e2461.

Li, Y., Fang, S., Zhou, X., Zhao, Z., Li, F., & Liu, P. (2020). Adsorption Study of Lignin Removal from Recycled Alkali Black Liquor by Adsorption Resins for Improved Cellulase Hydrolysis of Corn Straw. *Molecules*, *25*(19), 4475.

Lin, Y.-Y., Chen, W.-H., & Liu, H.-C. (2020). Aging and emulsification analyses of hydrothermal liquefaction bio-oil derived from sewage sludge and swine leather residue. *Journal of Cleaner Production*, *266*, 122050.

Liu, Z., Wang, H., & Hui, L. (2018). Pulping and Papermaking of Non-Wood Fibers. In *www.intechopen.com*. IntechOpen. https://www.intechopen.com/chapters/62223

Lokeshwari, N., Srinikethan, G., Joshi, S. G., Shasikala, I., Srikanth, B., Bashirahmed, & Sushma, L. (2013). Isolation and Screening of Fungi for Aerobic Delignification and Reduction of AOX of Pulp and Paper Mill Effluent. *International Journal of Environmental and Ecological Engineering*, *7*(12), 1167–1171.

Lourenço, A., & Pereira, H. (2017). Compositional Variability of Lignin in Biomass

Lugovitskaya, T. N. (2022). Sulfite Lignin Nanoparticles and Nanovesicles as Biologically Active Crop Growth Stimulants. *ACS Applied Nano Materials*.

Lyckeskog, H., Mattsson, C., Olausson, L., & Andersson, S.-I. (2017). *Accelerated aging of bio-oil from lignin conversion in subcritical water*. TAPPI Journal 123-133. =

Majumdar, S., Priyadarshinee, R., Kumar, A., Mandal, T., & Dasgupta Mandal, D. (2019). Exploring Planococcus sp. TRC1, a bacterial isolate, for carotenoid pigment production and detoxification of paper mill effluent in immobilized fluidized bed reactor. *Journal of Cleaner Production*, *211*, 1389–1402.

Mänttäri, M., Nyström, M., Nuortila-Jokinen, J., & Kallioinen, M. (2021). Nanofiltration in the Pulp and Paper Industry. In *Nanofiltration: Principles, Applications, and New Materials, I, Second Edition* (pp. 599–620).

Marson, A., Samec, J. S. M., & Manzardo, A. (2023). Consequential life cycle assessment of kraft lignin recovery with chemical recycling. *Science of the Total Environment*, *882*, 163660.

Mehmood, K., Kashif Ur Rehman , S., Wang, J., Farooq, F., Mahmood, Q., Jadoon, A. M., Javed, M. F., & Ahmad, I. (2019). Treatment of Pulp and Paper Industrial Effluent Using Physicochemical Process for Recycling. *Water*, *11*(11), 2393.

Mei, J., Shen, X., Gang, L., Xu, H., Wu, F., & Sheng, L. (2020). A novel lignin degradation bacteria-Bacillus amyloliquefaciens SL-7 used to degrade straw lignin efficiently. *Bioresource Technology*, *310*, 123445

Melro, E., Filipe, A., Sousa, D., Valente, A. J. M., Romano, A., Antunes, F. E., & Medronho, B. (2020). Dissolution of kraft lignin in alkaline solutions. *International Journal of Biological Macromolecules*, *148*, 688–695.

Mittal, M., Bhuwal, A., Sharma, P., & Aggarwal, N. K. (2023). Utilization of pulp and paper industrial wastewater for production of polyhydroxybutyrate by Bacillus sonorensis NAM5. *Systems Microbiology and Biomanufacturing*.

Mohammad, B. T., Al Daghistani, H. I., Jaouani, A., Abdel-Latif, S., & Kennes, C. (2017). Isolation and Characterization of Thermophilic Bacteria from Jordanian Hot Springs: Bacillus licheniformis and Thermomonas hydrothermalis Isolates as Potential Producers of Thermostable Enzymes. *International Journal of Microbiology*, *2017*, 1–12.

Mohammad, S. H., & Bhukya, B. (2022). Biotransformation of toxic lignin and aromatic compounds of lignocellulosic feedstock into eco-friendly biopolymers by Pseudomonas putida KT2440. *Bioresource Technology*, *363*, 128001.

Mori, K., Niinuma, K., Fujita, M., Kamimura, N., & Masai, E. (2018). DdvK, a Novel Major Facilitator Superfamily Transporter Essential for 5,5′-Dehydrodivanillate Uptake by Sphingobium sp. Strain SYK-6. *Applied and Environmental Microbiology*, *84*(20).

Morya, R., Kumar, M., Singh, S. S., & Thakur, I. S. (2019). *Genomic analysis of Burkholderia sp. ISTR5 for biofunneling of lignin-derived compounds*. ResearchGate; Biotechnology for Biofuels.

Morya, R., Kumar, M., Tyagi, I., Kumar Pandey, A., Park, J., Raj, T., Sirohi, R., Kumar, V., & Kim, S.-H. (2022). Recent advances in black liquor valorization. *Bioresource Technology*, *350*, 126916.

Navas, L. E., Zahn, M., Bajwa, H., Grigg, J. C., Wolf, M. E., Chan, A. C. K., Murphy, M. E. P., McGeehan, J. E., & Eltis, L. D. (2022). Characterization of a phylogenetically distinct extradiol dioxygenase involved in the bacterial catabolism of lignin-derived aromatic compounds. *Journal of Biological Chemistry*, *298*(5), 101871.

Nguyen, L. T., Phan, D.-P., Sarwar, A., Tran, M. H., Lee, O. K., & Lee, E. Y. (2021). *Sci-Hub | Valorization of industrial lignin to value-added chemicals by chemical depolymerization and biological conversion. Industrial Crops and Products, 161,*

Ozsefil, I. C., Miraloglu, I. H., Ozbayram, E. G., Uzun, O., Ince, B., & Ince, O. (2023). Is a floodplain forest a valuable source for lignin-degrading anaerobic microbial communities: A metagenomic approach. *Chemosphere*, *339*, 139675.

Pacra. (2021). *Paper & Packaging Sector Study.*

Pandey, N., & Thakur, C. (2020). Study on treatment of paper mill wastewater by electrocoagulation and its sludge analysis. *Chemical Data Collections*, *27*, 100390.

Parveen, S., Ali, M. I., Aslam, M., Ali, I., Jamal, A., Al-Ansari, M. M., Al-Humaid, L., Urynowicz, M., & Huang, Z. (2022). Optimizing biocatalytic potential of Dipodascus australiensis M-2 for degrading lignin under laboratory conditions. *Microbiological Research*, *265*, 127179

Patel, K., Patel, N., Vaghamshi, N., Shah, K., Duggirala, S. M., & Dudhagara, P. (2021). Trends and strategies in the effluent treatment of pulp and paper industries: A review highlighting reactor options. *Current Research in Microbial Sciences*, *2*, 100077.

Pathak, P., & Sharma, C. (2021). *Processes and problems of pulp and paper industry: an overview*. ResearchGate; Physical Sciences Reviews.

Pezzana, L., Malmström, E., Johansson, M., & Sangermano, M. (2021). UV-Curable Bio-Based Polymers Derived from Industrial Pulp and Paper Processes. *Polymers*, *13*(9), 1530.

Pinar Karagoz, Sansanee Khiawjan, Marco, Samir Santzouk, Timothy, & Lye, G. J. (2023). Pharmaceutical applications of lignin-derived chemicals and lignin-based

materials: linking lignin source and processing with clinical indication. *Biomass Conversion and Biorefinery*

Radhika, N. L., Sachdeva, S., & Kumar, M. (2022). Lignin depolymerization and biotransformation to industrially important chemicals/biofuels. *Fuel*, *312*, 122935.

Raj, A., Chandra, R., Reddy, M. M. K., Purohit, H. J., & Kapley, A. (2006). Biodegradation of kraft lignin by a newly isolated bacterial strain, Aneurinibacillus aneurinilyticus from the sludge of a pulp paper mill. *World Journal of Microbiology and Biotechnology*, *23*(6), 793–799.

Rajwar, D., Paliwal, R., & Rai, J. P. N. (2017). Biodegradation of pulp and paper mill effluent by co-culturing ascomycetous fungi in repeated batch process. *Environmental Monitoring and Assessment*, *189*(9).

Rather, M. A., Dolley, A., Hazarika, N., Ritse, V., Sarma, K., Jamir, L., Satapathy, S. S., Ray, S. K., Deka, R. C., Biswal, A. K., Doley, R., Mandal, M., & Namsa, N. D. (2022). Glycome Profiling and Bioprospecting Potential of the Himalayan Buddhist Handmade Paper of Tawang Region of Arunachal Pradesh. *Frontiers in Plant Science*, *13*.

Ren, J., Li, X., Zhang, W., Li, Z., Wang, Q., Li, S., Wang, S., & Hong-ya, L. (2022). Evaluation of application potential of dye-decolorizing peroxidase from Bacillus amyloliquefaciens in bioremediation of paper and pulp mill effluent. *Frontiers in Microbiology*, *13*.

ReportLinker. (2023). *Paper Products Global Market Report 2023*. ReportLinker.

Ribeiro, J. P., Marques, C. C., Portugal, I., & Nunes, M. I. (2020). AOX removal from pulp and paper wastewater by Fenton and photo-Fenton processes: A real case-study. *Energy Reports*, *6*, 770–775.

Sainsbury, P. D., Hardiman, E. M., Ahmad, M., Otani, H., Seghezzi, N., Eltis, L. D., & Bugg, T. D. H. (2013). Breaking Down Lignin to High-Value Chemicals: The Conversion of Lignocellulose to Vanillin in a Gene Deletion Mutant of Rhodococcus jostii RHA1. *ACS Chemical Biology*, *8*(10), 2151–2156.

Sameni, J., Jaffer, S. A., Tjong, J., & Sain, M. (2020). Advanced Applications for Lignin Micro- and Nano-based Materials. *Current Forestry Reports*, *6*(2), 159–171.

Senthilvelan, T., Kanagaraj, J., & Panda, R. C. (2017). Enhanced Biodegradation of Lignin by Laccase through HOBT Mediator: Mechanistic Studies Supported by FTIR and GCMS Studies. *Environmental Processes*, *4*(1), 201–217

Sgro, M., Chow, N., Farnaz Olyaie, Arentshorst, M., Geoffrion, N., Arthur F.J. Ram, Powlowski, J., & Tsang, A. (2023). Functional analysis of the protocatechuate branch of the β-ketoadipate pathway in Aspergillus niger. *Journal of Biological Chemistry*, *299*(8), 105003–105003.

Shabbir, I., Mirzaeian, M., & Sher, F. (2022). Energy efficiency improvement potentials through energy benchmarking in pulp and paper industry. *Cleaner Chemical Engineering*, 100058.

Shah, M. P. (2020). Microbial Bioremediation & Biodegradation. In *Google Books*. Springer Nature.

Shah, S. N., Manzoor, S., & Asim, M. (2021). Impact of industrial pollution on our society - *ProQuest*.

Sharma, N., Bhawana, Godiyal, R. D., & Thapliyal, B. P. (2020). A Review on Pulping, Bleaching and Papermaking Processes. *Journal of Graphic Era University*, 95–112.

Sharma, P., Purchase, D., & Chandra, R. (2021). Residual pollutants in treated pulp paper mill wastewater and their phytotoxicity and cytotoxicity in Allium cepa. *Environmental Geochemistry and Health*, *43*(5), 2143–2164

Sharma, V., Ayothiraman, S., & Dhakshinamoorthy, V. (2019). Production of highly thermo-tolerant laccase from novel thermophilic bacterium Bacillus sp. PC-3 and its application in functionalization of chitosan film. *Journal of Bioscience and Bioengineering*, *127*(6), 672–678.

Shiyue, J., Binjie, L., & Yufei, S. (2021). The Influence of Pulp and Paper Industry on Environment. *E3S Web of Conferences*, *308*(308), 02007.

Singh, A. K., Bilal, M., Iqbal, H. M. N., & Raj, A. (2021). Lignin peroxidase in focus for catalytic elimination of contaminants — A critical review on recent progress and perspectives. *International Journal of Biological Macromolecules*, *177*, 58–82.

Singh, A. K., & Chandra, R. (2019). Pollutants released from the pulp paper industry: Aquatic toxicity and their health hazards. Aquatic Toxicology, 211, 202–216.

Singh, A. K., Kumar, A., & Chandra, R. (2020). Detection of refractory organic pollutants from pulp paper mill effluent and their toxicity on Triticum aestivum; Brassica campestris

AND Tubifex-tubifex. ResearchGate; Journal of Experimental Biology and Agricultural Sciences.

Singh, A. K., Yadav, P., Bharagava, R. N., Saratale, G. D., & Raj, A. (2019a). Biotransformation and Cytotoxicity Evaluation of Kraft Lignin Degraded by Ligninolytic Serratia liquefaciens. Frontiers in Microbiology, 10.

Singh, A. K., Yadav, P., Bharagava, R. N., Saratale, G. D., & Raj, A. (2019b). Biotransformation and Cytotoxicity Evaluation of Kraft Lignin Degraded by Ligninolytic Serratia liquefaciens. Frontiers in Microbiology, 10.

Singh, A., Kumar, R., Maurya, A., Chowdhary, P., & Raj, A. (2022). Isolation of functional ligninolytic Bacillus aryabhattai from paper mill sludge and its lignin degradation potential. *Biotechnology Reports*, *35*, e00755.

Singh, S., Panwar, S., Jain, R. K., & Tripathi, Y. C. (2020). Assessment of Physicochemical Characteristics of Effluents from Paper Mill in the State of Uttar Pradesh, India. ResearchGate; International Journal of Engineering Research & Technology.

Singhania, R. R., Patel, A. K., Raj, T., Chen, C.-W., Ponnusamy, V. K., Tahir, N., Kim, S.-H., & Dong, C.-D. (2022). Lignin valorisation via enzymes: A sustainable approach. *Fuel*, *311*, 122608.

Statista. (2023a). Global packaging paper & board production 1961-2021. Statista.

Statista. (2023b). Production volume of paper and paperboard in selected countries worldwide in 2010 and 2021. Statista.

Suman, S. K., Dhawaria, M., Tripathi, D., Raturi, V., Adhikari, D. K., & Kanaujia, P. K. (2016). Investigation of lignin biodegradation by Trabulsiella sp. isolated from termite gut. International Biodeterioration & Biodegradation, 112, 12–17.

Sun, R. (2020). Lignin Source and Structural Characterization. ChemSusChem, *13*(17), 4385–4393.

Surabhi Zainith, Chowdhary, P., & Ram Naresh Bharagava. (2018). Recent Advances in Physico-chemical and Biological Techniques for the Management of Pulp and Paper Mill Waste. Emerging and Eco-Friendly Approaches for Waste Management , 271–297.

Takahashi, S., Nge, T. T., Takata, E., Ohashi, Y., & Yamada, T. (2020). Flocculation properties of polyethylene glycol-modified lignin. Separation and Purification Technology, 253, 117524.

Tan, F., Cheng, J., Zhang, Y., Jiang, X., & Liu, Y. (2022). Genomics analysis and degradation characteristics of lignin by Streptomyces thermocarboxydus strain DF3-3. Biotechnology for Biofuels and Bioproducts , *15*(1).

Tarabanko, V., & Tarabanko, N. (2017). Catalytic Oxidation of Lignins into the Aromatic Aldehydes: General Process Trends and Development Prospects. *International Journal of Molecular Sciences*, *18*(11), 2421.

Tariq, A., & Mushtaq, A. (2023). Untreated Wastewater Reasons and Causes: A Review of Most Affected Areas and Cities. International Journal of Chemical and Biochemical Sciences, *23*(1), 2023.

Trading Economics. (2023). Pakistan Exports of paper and paperboard, articles of pulp, paper and board - 2023 Data 2024 Forecast 2003-2021 Historical. Tradingeconomics.com.

Tribot, A., Amer, G., Abdou Alio, M., de Baynast, H., Delattre, C., Pons, A., Mathias, J.-D., Callois, J.-M., Vial, C., Michaud, P., & Dussap, C.-G. (2019). Wood-lignin: Supply, extraction processes and use as bio-based material. European Polymer Journal, 112, 228–240.

Vásquez-Garay, F., Carrillo-Varela, I., Vidal, C., Reyes-Contreras, P., Faccini, M., & Mendonça, R. T. (2021). A Review on the Lignin Biopolymer and Its Integration in the Elaboration of Sustainable Materials. Sustainability, 13(5), 1–15.

Vidal, G., González, Y., Piña, B., Jarpa, M., & Gómez, G. (2021). Minimization of Environmental Impact of Kraft Pulp Mill Effluents: Current Practices and Future Perspectives towards Sustainability. Sustainability, 13(16), 9288.

Wang, H., Peng, X., Li, H., & Giannis, A. (2022). Recent Biotechnology Advances in Bio-Conversion of Lignin to Lipids by Bacterial Cultures. ResearchGate; Frontiers in Chemistry.

Wang, L., Ren, W., Zhang, X., Zeng-lin, H., & Li, J. (2022). Modification of Basic Oxygen Furnace Slag Using Iron Ore Tailing and Blast Furnace Dust. 12th International Symposium on High-Temperature Metallurgical Processing, 121–132.

Wang, X., Xu, W., Zhang, D., Li, X., & Shi, J. (2023). Structural Characteristics–Reactivity Relationships for Catalytic Depolymerization of Lignin into Aromatic Compounds: A Review. International Journal of Molecular Sciences, 24(9), 8330.

Wang, Y., Chen, X., Wu, B., Ma, T., Jiang, H., Mi, Y., Jiang, C., Zang, H., Zhao, X., & Li, C. (2022). Potential and mechanism for bioremediation of papermaking black liquor by a psychrotrophic lignin-degrading bacterium, Arthrobacter sp. C2. *Journal of Hazardous Materials*, *439*, 129534.

Weng, C., Peng, X., & Han, Y. (2021). Depolymerization and conversion of lignin to value-added bioproducts by microbial and enzymatic catalysis. *Biotechnology for Biofuels*, *14*(1).

World Bank. (2022). Forest area (% of land area) - Pakistan | *Data*. Data.worldbank.org.

Wu, K., Shi, M., Pan, X., Zhang, J., Zhang, X., Shen, T., & Tian, Y. (2022). Decolourization and biodegradation of methylene blue dye by a ligninolytic enzyme-producing Bacillus thuringiensis: Degradation products and pathway. Enzyme and Microbial Technology, *156*, 109999

Xu, Z., Pan, C., Li, X., Hao, N., Zhang, T., Gaffrey, M. J., Pu, Y., Cort, J., Ragauskas, A. J., Qian, W., & Yang, B. (2021). Enhancement of polyhydroxyalkanoate production by co-feeding lignin derivatives with glycerol in Pseudomonas putida KT2440. Biotechnology for Biofuels , 14(1).

Yadav, S., Tripathi, S., Purchase, D., & Chandra, R. (2023). Development of a biofilm-forming bacterial consortium and quorum sensing molecules for the degradation of lignin-containing organic pollutants. Environmental Research, 226, 115618.

Yang, L., Yuan, H., Yang, Y., Wang, R., Wang, C., Wei, X., Chen, S., Jae Hyeon Yu, & Ma, X. (2019). Enhanced Lignin Degradation in Tobacco Stalk Composting with

Inoculation of White-Rot Fungi Trametes hirsuta and Pleurotus ostreatus. Waste and Biomass Valorization , 11(7), 3525–3535.

Yao, H., Wang, Y., Liu, J., Xu, M., Ma, P., Ji, J., & You, Z. (2022). Review on Applications of Lignin in Pavement Engineering: A Recent Survey. *Frontiers in Materials*, *8*.

Zahrim, A. Y., Nasimah, A., & Hilal, N. (2015). Coagulation/flocculation of lignin aqueous solution in single stage mixing tank system: Modeling and optimization by response surface methodology. Journal of Environmental Chemical Engineering, *3*(3), 2145–2154.

Zhu, D., Zhang, P., Xie, C., Zhang, W., Sun, J., Qian, W.-J., & Yang, B. (2017). Biodegradation of alkaline lignin by Bacillus ligniniphilus L1. Biotechnology for Biofuels, *10*(1).