Evaluation of Lignin Degradation Potential of Fungi Isolated from Pulp and Paper Mill Effluent.

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

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By

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Dedication

This thesis is dedicated to my loving and supporting parents whose love and support has led me to pursue my dreams, my elder brother, and my sisters.

Declaration

I hereby declare that no part of this thesis has been previously submitted to this or any other University as part of the requirement for a higher degree. The contents of this thesis are the results of my own work unless otherwise acknowledged in the text or by reference. The research work presented in this thesis was carried out by me in the Applied, Environmental and Geo-Microbiology (AEG) Laboratory, Department of Microbiology, Faculty of Life Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

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Certificate

This thesis submitted by Asma Mukhtiar titled, "*Evaluation of Lignin Degradation Potential of Fungi Isolated from pulp and Paper Mill Effluent*" is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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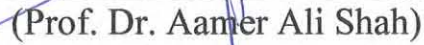

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List of Contents

List of Tables viii
List of Figures ix
List of Abbreviations xii
Acknowledgments xiii
Abstract xiv
1. Introduction 1
2. Literature Review 14
3. Materials and Methods 38
4. Results 53
5. Discussion 88
6. Conclusion 93
References 95
Appendix 1: Turnitin Originality Report 111

List of Tables

Sr. No	Title	Page. No.
1	Production volume of paper and paperboard worldwide from 1961 to 2022.	16
2	Pollutants discharge from P&P making process.	20
3	Characteristics of lignin-degrading enzymes.	33
4	Composition of reaction mixture for enzyme assay	41
5	Composition of reaction mixture for the enzyme assay	42
6	Composition of reaction mixture for enzyme assay.	43
7	Shows bacterial and fungal isolates from black liquor and sludge samples.	53
8	showing the values of COD (First Trial)	80
9	Showing the values of COD (Second Trial)	81
10	Shows the phytotoxicity of sample with Tap water, Untreated and Treated water samples with 5%Black liquor Concentration (First Trial)	83
11	Shows the phytotoxicity of sample with Tap water, Untreated and Treated water samples with 5%Black liquor Concentration (Second Trial).	85

List of Figures

Sr. No	Title	Page. No.
1	Pakistan pulp and paper annual production.	18
2	Diagram depicts the procedure of paper making and mitigation of different compounds that are generated in each stage.	26
3	Gallic acid standard curve.	50
4	Shows Fungal strains (a) SL-6(b) SST-1 growth on L-MSM containing lignin in different concentrations.	54
5	Shows (a) Laccase Plate assay (b) Azure-B Plate assay (c) Crystal violet plate assay before culturing.	54
6	Appearance of brown color around growth on guaiacol agar plates show laccase by fungal strain (a) SST-1 and (b) SL-6 Fungal strains on minimal salt medium plate containing Crystal violet dye shows clear zone around colonies indicates the production of lignin peroxidase.	55
7	Quantitative assay for Ligninolytic Enzymes Production after culturing.	55
8	Production of manganese peroxidase, laccase, and lignin peroxidase by SST-1.	56
9	Production of manganese peroxidase, laccase, and lignin peroxidase by SL-6.	57
10	Growth of Fungal strains on PDA media (a) SL-6 (b) SST-1	57
11	Microscopic Identification of fungal strains (a) SL-6 and (b) SST-1 with lactophenol cotton blue staining under 100X.	58
12	Phylogenetic tree of *Aspergillus fumigatus* SL-6 by Neighbor-Joining method constructed using MEGA-X software.	59
13	Phylogenetic tree of *Trametes hirsuta* SST-1 by Neighbor-Joining method constructed using MEGA-X software.	60
14	Evaluation of lignin reduction (%) at different conc. by *Aspergillus fumigatus* SL-6.	61
15	Evaluation of color reduction (%) at different conc. by *Aspergillus fumigatus* SL-6.	62
16	Evaluation of lignin reduction (%) at different conc. by *Trametes hirsuta* SST-1.	62
17	Evaluation of color reduction (%) at different conc. by *Trametes hirsuta* SST-1.	63
18	Effect of different pH on lignin degradation by *Aspergillus fumigatus* SL-6.	64

19	Effect of different pH on color reduction by *Aspergillus fumigatus* SL-6.	64
20	Effect of different pH on lignin degradation by *Trametes hirsuta* SST-1.	65
21	Effect of different pH on color reduction by *Trametes hirsuta* SST-1.	65
22	Effect of different temperature on lignin degradation by *Aspergillus fumigatus* SL-6.	66
23	Effect of different temperature on color reduction by *Aspergillus fumigatus* SL-6.	67
24	Effect of different temperature on lignin degradation by *Trametes hirsuta* SST-1.	67
25	Effect of different temperature on color degradation by *Trametes hirsuta* SST-1.	68
26	Effect of different carbon sources on lignin degradation by *Aspergillus fumigatus* SL-6.	69
27	Effect of different carbon sources on color reduction by *Aspergillus fumigatus* SL-6.	69
28	Effect of different carbon sources on lignin degradation by *Trametes hirsuta* SST-1.	70
29	Effect of different carbon sources on color reduction by *Trametes hirsuta* SST-1.	70
30	Effects of different nitrogen sources on lignin degradation by *Aspergillus fumigatus* SL-6.	71
31	Effect of different nitrogen sources on color reduction by *Aspergillus fumigatus* SL-6.	72
32	effect of different nitrogen sources on lignin degradation by *Trametes hirsuta* SST-1.	72
33	Effect of different nitrogen sources on color reduction by *Trametes hirsuta* SST-1.	73
34	Schematic diagram of Lab-scale sequential bioreactor for paper and pulp effluent treatment.	74
35	Bioreactor before Experiment. (a) Fungal chamber (b) Sand filter (c) fungal chamber (d) Sand filter.	74
36	Shows Biofilm formation on wood chips (a) fungal Biofilm *Trametes hirsuta* SST-1 (b) Fungal biofilm *Aspergillus fumigatus* SL-6.	75
37	Shows Treatment of BL through Sequential Bioreactor	76
38	Lignin degradation of sample after treatment in Bioreactor (First Trial)	77
39	Color reduction of sample after treatment in Bioreactor (First Trial) Results of the second trial (consortium)	77
40	Lignin reduction (%) of sample after treatment in Bioreactor (Second Trial)	78

41	Color reduction (%) of sample after treatment in Bioreactor (Second Trial)	78
42	Gallic acid standard curve	79
43	Shows phenolic contents reduction (%) after treatment in Bioreactor (First Trial	79
44	Shows phenolic contents reduction (%) after treatment in Bioreactor (Second Trial)	80
45	Shows reduction of COD (%) of sample after treatment in Bioreactor (First Trial	81
46	Shows reduction of COD (%) of sample after treatment in Bioreactor (Second Cycle).	82
47	Phytotoxicity of Black Liquor (a), (b) Before Treatment and (c), (d) After Treatment in First Bioreactor Cycle, with 5% BL.	84
48	Shows the phytotoxicity of sample (a), (b) Before Treatment and (d) After Treatment in Second Bioreactor Cycle (Consortium), with 5% BL.	85
49	shows the mortality of brine shrimps when exposed to untreated BL and treated BL by *Aspergillus fumigatus* SL-6 and *Trametes hirsuta* SST-1.	86
50	shows the mortality of brine shrimps when exposed to untreated BL and treated BL by Fungal Consortium.	87

List of Abbreviations

PDA	Potato dextrose Agar
AEG	Applied Environmental and Geomicrobiology lab
MSM	Minimal salt medium
COD	Chemical oxygen demand
BOD	Biological oxygen demand
FC	Folin-Ciocalteau reagent
TDS	Total Dissolved Solids
TSS	Total Suspended Solids
TPC	Total Phenolic Contents
MBR	Membrane Biofilm Reactor
SBR	Sequential Batch Reactor
PAMs	Polyacrylamides
NMR	Nuclear Magnetic Resonance
rpm	Revolution per minute
DO	Dissolved Oxygen
UV	Ultraviolet
KBL	Kraft Black Liquor
LCFAs	Long Chain Fatty Acids
TMP	Transmembrane Pressure
PAHs	Polycyclic Aromatic Hydrocarbon
Mnp	manganese peroxidase
Lip	lignin peroxidase

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Abstract

Pulp and paper mill industries release an excessive amount of wastewater containing hazardous compounds which have an impact on every aspect of life. Effluent is required to be treated to minimize its severe effects on the environment. The biological approach is the most popular among all treatment techniques. Main goal of the study was to focus on the treatment of effluents from pulp and paper mills using native fungal strains. Sample was taken from pulp and paper mill industry located in district Kasoor, Lahore, Pakistan. Two strains SST-1 and SL-6 were selected after initial screening on the basis of their ability to degrade lignin using L-MSM as growth medium. In qualitative and quantitative enzyme screening, both fungal isolates exhibited positive activity for Ligninolytic enzymes production. Both the fungal strains were identified through morphologic, microscopic, and molecular method. Strain SST-1 was identified as *Trametes hirsuta* while SL-6 was identified as *Aspergillus fumigatus*. It was observed that both the strains grow well at pH 6.0 and temperature 30°C. *Trametes hirsuta* SST-1 and *Aspergillus fumigatus* SL-6 were observed to show maximum lignin degradation using ammonium sulfate and peptone as nitrogen sources respectively. A lab scale biological reactor was used for black liquor treatment. During first trial two separate reactors were run for both the fungal strains. *Trametes hirsuta* SST-1 demonstrated 47% reduction in lignin, 62% reduction in color, 59% reduction in phenol and 58% reduction in COD. Similarly, *Aspergillus fumigatus* SL-6 exhibited 41% reduction in lignin, 56% reduction in color,47% reduction in phenol and 45% reduction in COD. However, in second trial, co-cultivation of both strains showed enhanced activities with notable 74% reduction in lignin, 80% reduction in color, 84% in phenol and 67% reduction in COD. In phytotoxic activity germination rate of 90% and 80% was observed for the treated effluent of the first trial. Comparatively, seed germination activity of 100% was observed for the effluent of second trial. Moreover, in cytotoxic assay *Trametes hirsuta* and *Aspergillus fumigatus* treated black liquor showed 60% and 40% viability respectively whereas treated black liquor by the consortium showed viability rate of 70%. Both the strains exhibited significant lignin degradation ability and toxicity of the black liquor was considerably reduced.

1. Introduction

Environmental ambience and standards of life in it are dependent on the overall features of the industrial setup and its management in that particular environment. It has been observed that generation of waste depends upon the overall size of industrial setup. A bigger industry will end up generating more waste impacting the surrounding environment at greater pace as compared to small scale industries. Recently, demand of production of paper and pulp have risen and according to food and agriculture organization (FAO) the demand for paper industry has risen considerably in the past few years, but the problem with these industries is that they are consuming a great sum of energy and water resources (FAO, 2016). In Europe, pulp and paper mill coincide of about ¼ of the manufacturing industry in the world and respectively generate about 90 million tons of pulp and paper in a year (Costa et al., 2017). In several industries, the pulp and paper industrial setups are among the voluminous polluting industries (Liu, 2018). Pulp and paper are made from cellulosic fibers mainly as well as some other plant materials. Maximum of the papers are synthesized entirely from fibers which are extracted from woods, but several other sources for example sugar can residues are also utilized for papers making. However, in some cases virgin fibers are also added depending upon the nature and quality of used paper. Furthermore, wood pulp can also be utilized for making several other products including packaging films. In composition of wood there is 50% ratio of cellulose which has a great demand in paper industries (P. Bajpai et al., 2015). In paper manufacturing procedure intense amount of water is utilized to get a consistent structure and without water the process completion cannot be possible (Swamy et al., 2011). The paper mill wastewater characteristically contains color, Chemical Oxygen Demand (COD). An elevated amount of Biochemical Oxygen Demand (BOD), because of the presence its derivatives and lignin from the chlorinated compounds, raw cellulosic materials, tannins, sulphur and fatty acids etc.(Swamy et al., 2011).

The P&P (pulp and paper) mill effluents color not solely only responsible for the aesthetic change, but also blocks the passage of light from the sun to enter into the water and impedes the occurrence of photosynthesis, which not only decreases oxygen level of water making life arduous for organisms but also affects the vegetation of water (Ebrahiem et al., 2017). Insufficiently treated waste is discharged by most of these industries into the rivers or streams, which creates significant problem to flora-fauna and other aquatic life. That's why, economic development, is important for the effluent discharged. Due to lack of proper management in developing countries even if less amount of water is reused, huge amount of water that cannot be utilized any further are produced and treatment facility of which is poorly organized. However, practice of generating amount of wastewater has been reduced in the mills that are carrying out their operations in developed countries and biological methods for treatment are being employed (FPAC, 2009).

This effluent which releases from industries mostly used for irrigation purposes, toxic components enter the food chain, affect the food badly and leading to effect terrestrial and aquatic life (Schwarzenbach et al., 2010). The main hazardous polluting contents that have the ability to pollute environment and which are released from pulp and paper industry are lignin and chlorinated phenols. Lignin is mostly accountable for the offensive color which restricts the growth of phototrophic organisms by reducing the sunlight transmission in water. Chlorinated phenols are the major chemical species that are held accountable for harming flora and fauna. Chlorinated organic components that contain furans and dioxins, in exposed organism's furans and dioxins are also presumed to induce genetic mutations (Raj et al., 2014). A specific type of densely black color liquid product from the paper mill industries and pulp during the Kraft lignin process contains many extractive releases during the removal from cellulose fibers like tannins, hemicellulose, cellulose and many other components (Stenius et al., 2000). Black liquor is contemplated as one of the chief by-products and highly viscous aqueous waste being released from pulp industry. Black liquor is severely colored and toxic effluent of paper

pulping, containing, inorganic chemicals that are utilized in different processes and sand organics that are taken from biomass. Black liquor solid content varies by weight between 15% and 40% whereas the total solid composition, lignin accounts for 30–45% (Jin et al., 2013). Paper and pulp can be segregated as straw pulp and wood pulp. The black liquor proceeding technology is quite develop in wood alkaline pulping, including chemical regeneration which use lignin as mean of getting energy in soda recovery boiler. However, the wood resources are not mainly in use as there is a dearth of these resources in China, where straw is mainly utilized as raw material for paper pulp. In black liquor annually, million tons of lignin is produced, however, minimum amount of which was utilized for the synthesis of activated carbon as a precursor (Fu et al., 2013). In black liquor if the waste lignin is used for example as a feedstock for bio-oil production, a significant opportunity would be produced for increasing the economic viability, overall operational efficiency, carbon conversion rate, and sustainability of chemical production and befouls, in conformity with the need of sustainable development and also resource conserving economy. Thus it is very crucial to characterize and separate lignin from straw pulping black liquor (Tian et al., 2015).

The characteristics of effluent discharge from various industries based on the raw material utilized but black liquor is mostly densely brown in color and have high alkaline nature that makes it difficult to degrade, high in demand for COD and also makes it rich in toxicity (Pola et al., 2021).

A different significant class of chemicals known as rosins can be found in the by-products of the paper and pulp industry and could be an attractive source of bio based polymer precursor. According to Gandini et al. (2015), rosin is a crucial candidate for achieving polymerizable structures in both cross-linked and linear materials. Over a million tons of rosin are generated each year. Although rosin primarily serves as a component of inks, cosmetics, varnishes, paper sizes, adhesives, and medications, it can

also be suggested that rosin and its derivatives can serve as reactive monomers in the synthesis of polymers (John et al., 2019).

The 2nd predominantly plentiful polymer in the worldwide is lignin, the pulp and paper industry provides about 2% of significant lignin that is utilized to manufacture useful products and approximately 50 million tons of lignin is generated and the rest produce a huge amount of energy, which is substantially 98% and utilized for the purpose of producing energy.

The term 'Lignin' refers to a broad class of hard, aromatic, and impermeable polymers formed through the oxidative coupling of the 4 hydroxyl phenylpropanoids, which are predominantly present in woody plants (Vanholme et al., 2010). The 3-D, random phenyl propanoid (C9) polyphenol found in lignin is primarily linked by aryl glycerol ether interactions between the monomeric phenolic units of p-coumaryl (H), sinapyl alcohol (S), and coniferyl (G). The structure of lignin is shaped through a pathway which is biosynthetic that takes place by the monolignols oxidative radicalization ensued by another pathway radical coupling where two monomer radicals make a dehydrodimer. At monolingual β positions coupling is favored that results in pinoresinol (β−β'), aryl glycerol-β-aryl ether (β-O-4'), phenylcoumaran (β-5'), diphenyl ethane (β-1') and spirodienone (SD) dimers formation. Coupling at the site 4 and 5 may occur in principle dilignol utilizing diaryl ether (4-O-5') and another diphenyl (5-5') dimers development wise coupling mode. Coupling via (5-5) and (4-O-5) links the two lignin oligomers. These 5-5'subunits in turn subsequently experience α−β-O-4-4' bonding to dibenzodioxocine units (5-5'-O-4) (Sette et al., 2011). In the following process the dimer is initially dehydrogenated to form phenoxy radical and later in an end, can make a bond with the other monomer radical. Lignin, contributing to structural support and hydrophobic characteristics to the cell wall of plant, that makes lignocellulosic biomass from hemicellulose and cellulose. Even though hemicellulose and cellulose are carbohydrate polymers that get stabilized through strong hydrogen bonds however,

microbial enzymes can still degrade it, because of the ether bonds along with carbon-carbon bonds in its structure, lignin is highly resistant to hydrolysis). Both hemicellulose and cellulose are encapsulated by lignin crafting a sturdy 3-dimensional intricate known as lignin-carbohydrate complex (LCC), thus this results in the structural component becoming insoluble in water (Ozsefil et al., 2013).

There are three ways to separate lignin: physically, chemically, and biologically. Technical lignin is modified chemically by the following five procedures. These five steps primarily concentrate on the ways of degradation as well as the chemical processes that are essential to converting technically isolated lignin into useful (chemical) form. Technical lignin strategy employed to decrease lignin content has a considerable effect on the structure of synthesized product, this includes Kraft lignin (KL). The second technique includes Lignosulfonates (LS), which are obtained by cooking or treating in an acidic or neutral solution and are taken from sulfite process. The next process uses anthraquinones as a catalyst to create soda lignin (SL), a co-product of straw, flax, and nonwood fibers. Another technique is for treating pulp with an organic solvent to produce Organosolv lignin (OL). This procedure, which acts as a substitute for conventional pulping technology, uses solvents to extract lignin without the need for either alkaline or acidic conditions.

The final technique is called Steam-explosion lignin (SEL), and it originated with a steam process (Pezzana et al., 2011). Lignin removal can be achieved by different Physiochemical treatments such as Coagulation and Adsorption, and flocculation, Chemical oxidation, Ultrafiltration, Ozonation, etc. Although these are well-developed methods, there are few drawbacks that include harsh operating conditions, Environment impact, Energy intensive, Complexity, production of toxic compounds, and many more. The products obtained through chemical degradation of lignin are complex to valorize because these products are variable and highly heterogenous. Moreover, the by-products generated during physicochemical treatment cause serious reproductive and genetic

disorders, respiratory chronic diseases, skin irritation, and many other diseases (Parveen et al., 2022). There are various procedures available for the treatment of wastewater, however they consume great amount of energy. Therefore, they are less desirable as compared to biological processes and are often most cost-effective. There is a demand of an energy efficient, environment friendly, and affordable technologies (Raj et al., 2014). Treatment of biological methods involves the use of bacteria, algae, fungi and enzymes as a single step treatment or in combination with some other chemical and physical methods. The microorganism treats the effluent usually by two methods: biosorption and enzymatic action. The important enzymes taking part in process of treating paper and pulp mill effluent include lignin peroxidase, laccase and manganese peroxidase. Microorganisms that are producing enzymes at enhanced level have the capability to effectively deal with effluent. Biological treatment systems are notably appealing, this is because along with reduction in color they also reduce the COD and BOD of the effluent (Sharma et al., 2014).

As they facilitate the conversion of toxic substances to less toxic forms in an efficient and environmentally friendly manner, biological technologies often successfully eliminate the deficiencies that are left during the physiochemical processes (Madan et al., 2018). Bacterial treatment techniques were found helpful in the treating of Pulp and Paper discharge. Furthermore, the bacterial populations in two aerated lagoons undergoing processing P&P mill effluent also, molecular methods were employed to identify commercially available inoculums (Bailón-Salas, Ordaz-Díaz et al., 2017). There are numerous bacteria which are capable to degrade lignin. Streptomyces viridosporus T7A has the capacity foe the depolymerizing of lignin, utilizing an enzyme lignin peroxidase which is extracellular and there are also reports that lignin breakdown can be done through soil bacteria including Rhodococcus and Nocardia, identified through an assay incorporating 14C-labelled lignin (Bugg et al., 2011). R. jostii RHA1 and P. putida mt-2 were found to degrade lignocellulose, discharging phenolic product which has low molecular weight (Ahmad et al., 2010). The fly-ash, effluent, and activated sludge in the

sequential Batch Reactor (SBR) were mixed in an in-situ technique showing an effective elimination of several components from P&P mill effluent where fly-ash demonstrate significant removal of COD, Lignin, etc. Fly-ash also plays a crucial role in the settling of the sludge in the wastewater (Chen et al., 2017). Also, Planococcus sp was found to be useful in the elimination of lignin and as a result COD and color was also removed (Priyadarshinee et al., 2017).

Fungi are eukaryotic, achlorophyllous and heterotrophic organisms, can treat the complex constituents of the effluent discharge from the various industries including the P&P industry, since fungi owing to their capability to produce many enzymes in the extracellular environment such as Laccase, manganese peroxidase and Lignin peroxidase necessitate for the treatment of P&P effluent (Díaz, Laca et al., 2022). Various factors in the surrounding enhance the break-down of lignin by enhancing fungal growth and its overall metabolism. Carbon, temperature, nitrogen sources and low pH are the main significant parameters that affect fungal growth. Lignin scarification is high in white-rot fungi for example fungal species that belong to basidiomycetes. *P. chrysosporium* took two days to downgrade 1 gram of various separated lignins under the conditions where there is no availability of oxygen. Moreover, *Trichoderm viride* is another fungal strain that have the potential to be utilized for degradation of lignin (56%) leading to an improvement in the enzymatic digestibility of biomass (Ghorbani F et al., 2015). Similarly, treatment of wheat straw by *Pleurotus ostreatus* resulted in 34% reduction of lignin in the initial wheat straw, however in an untreated sample only 12% lignin reduction has occurred (Madadi et al., 2017). White-rot fungi can reduce pollution at the high concentration. It has also been observed that several genes are produced by fungi which can transform lignin into hydrophilic compounds (Hatakka et al., 2011). Different types of biological reactors are reported such as MBR, FBR, SSR, SBR, and UASB, etc., which are identified to be useful in the elimination and treatment of noxious and toxic constituents of the effluent including effluent from P&P industries. The effluents are treated under anaerobic condition in these reactors, that not only decreases the high

number of pollutants from the wastewater but also makes it effective for irrigation and for other purposes as well. Column-type SBR is found to significantly treat the P&P mill effluent that showing the results 87% COD and 95% elimination of the turbidity in conjunction with acceptable pH range and characteristic sludge generation (Khan et al., 2011). A sequential Batch Reactor (SBR) found to predominantly eliminate the organic pollutants from P&P industrial effluent on a wide scale. After 24 hours according to the studies SBR found to efficiently remove the following components from P&P effluents such as TSS 88%, BOD 83%, TDS 85% and COD 84%, (Sivasubramanian et al., 2015). During biological fungal treatment the optimum temperature changes with the type of microorganism being used. Various white rot fungal species that are belonging to ascomycetes thrive at 39°C however the white rot fungal species that belong to basidiomycetes thrive at temperature of round about 27°C. Excessive volume of heat is produced by these fungal strains during metabolic processes in solid form media which can inhibit the growth of fungi. Difference in optimized temperature for the biological pre-treatment of biomass is because of fungal strain, fungal physiology and nature of substrate (Rouches et al., 2016). Sequential Bio rectors (SBR) are crucial in the treatment of the effluent and elimination of pollutants from P&P mill wastewater. The Effluent undergoes a step-wise process, progressing through treatment in different.

The study aims to address the harmful effects of effluents, investigating the impact of releasing of sewage from pulp and paper mill on the surrounding environments along with methods to reduce hazardous effects associated with these with the help of prominent microorganisms using lab sale bioreactor. Fungal strains were isolated and different culture conditions were optimized to enhance the degradation process. Following optimization, a laboratory- scale sequential bioreactor was devised for effluents treatment from paper and pulp mill effluents employing chosen fungal strains.

Aims and Objectives

Aim:

Investigation of lignin degrading abilities of fungal species isolated from pulp and paper mill effluents.

Objectives:

- Isolation, identification, and screening of fungal isolates for their ligninolytic potential.
- Optimization of different parameters for lignin degradation through selected fungal isolates.
- Treatment of paper and pulp effluents in a lab scale bioreactor and monitoring of different parameters such as color reduction, lignin reduction, phenol reduction and COD periodically.
- Phytotoxic and cytotoxic evaluation of treated effluents.

2. Literature Review

2.1. Background

The pulp and paper industry has been regarded the largest consumers of resources that are occurring naturally (water, wood) and energy (electricity and fossil fuels) and as a significant contributor to tax revenue while discharging pollutants into the environment. Pulp and paper mills, they are classified as a fundamental segment industry, also a 5th biggest source of contamination in polluting water as it utilizes more water. Pulp and paper mills produce several of toxicants based on the specific pulping process employed. Pulp and paper mill effluents contaminate air, soil and water, causing a significant environmental hazard (Sharma *et al.,* 2014). The paper manufacturing process generates substantial amount of wastewater, reaching up to 60 m3/ton of paper produced (Costa *et al.,* 2017). The generation of lignocellulosic waste from the industries: Paper and pulp industry (effluent 150–200 m3/ton), agricultural waste (200 billion tons annually), and the food industry annually (1.3 billion tons) causes a primary threat to the human health and environment. Within this lignocellulosic waste, lignin is an unwanted polymer, and it is eliminated in the course of pulping process due to its presence, it contributes to the yellowness of paper and also has an impact on durability, however it utilizes considerable amount of chemicals and energy to eliminate it during pulping process. Industries that are involved in processing of paper and pulp utilize an extensive volume of fresh water and inorganic substances (chlorinated compounds, Sodium carbonate and Sodium hydroxide), and discharges highly contaminated effluent that usually appears dark brown and has high COD and BOD alongside 30 to 35% Inorganic substances (inorganic salts, toxic chlorinated compounds and other salts coupled with organic matter) and approximately 60 to70% organic matter (lignin, starch, resins, along with other low molecular compounds) amid the washing procedures, pulping and bleaching. For about 90% of pulp production is attributed to the Kraft process (Dessbesell *et al.,* 2020). Black liquor is an extremely viscous unwanted product that is produced during the alkaline Kraft process

that takes place during wood pulping. The black liquor varies in solid content by weight between 15% and 40%, Meanwhile lignin constitutes 30 to 45% of the overall solid composition. On the contrary, there is substantial potential to make black liquor's lignin economically significant and to enhance its usage either in generation of activated carbons or to exploit its true potential in synthesis of fuels like methane (Jin *et al.,* 2013). Lignin has great potential in economic and environmental sectors and that's why multiple biological techniques are utilized to reduce the adverse effects induced by industries that are processing paper and pulp. For degradation of effluents that are coming out of these mills almost all methods that are involving microorganisms have been recognized that are involved in the degradation of mill effluents. Biological procedures encompass microorganisms that are capable of degrading pulp and paper waste in natural environments. The biological color eliminating procedure utilizes various classes of microorganism algae, fungi and bacteria to breakdown the polymeric lignin derived chromophoric material. Various techniques have been employed by several researchers all over the world for eliminating of color from paper and pulp mill effluents (Sharma *et al.,* 2014).

2.2. Pulp and Paper Industry

2.2.1. Global Paper Industry

Even in the digital era the role of paper remains significant, as this ever-present material continues to be utilized daily for various reasons worldwide. In fact, the annual global production of paper and cardboard exceeds 400 million metric tons. Packaging paper and board is the most produced type of paper, due to surge in online shopping; its demand has been increased in recent years. China and the United States are the two largest paper manufacturing countries in the world. However, in recent decades the paper production has been declining in the United States. In China paper production has witnessed a huge increased and reached to a high peak of 117 million metric tons in 2020. This was approximately double the output of the United States that year.

As per the available data China holds the title of the largest producer of paper and cardboard, in major countries from 2009 to 2018 for about 1,000 metric tons' paper and cardboard were produced.

Table 1: Production volume of paper and paperboard worldwide from 1961 to 2022 (Statista, 2023).

S. No	Country	Paper and Pulp Production (million metric tons)
1	China	86,310
2	U.S	71,642
3	Japan	26,273
4	Germany	20,870
5	Canada	12,943
6	Sweden	10,932
7	Finland	10,602
8	Italy	8449
9	France	8331
10	UK	4293

In 2021 the global production of paper and paperboard was approximately 417.3 million metric tons. Compare to the preceding year this was an increase of about four percent. Since 2010, worldwide production of paper and paperboard has maintained a relatively balance trend, hovering approximately 400 million metric tons annually. In 2021, the valuation of the global pulp and paper market was at 351.53 billion U.S. dollars, and in 2022 a further increase was expected. Projection of market indicate a compound annual

growth rate (CAGR) of 0.72 percent from 2022 to 2029 aiming to achieve a value of about 373 billion U.S. dollars (Statista, 2023).

2.2.2. Paper Industry in Pakistan

Paper industry in Pakistan is consists of more than 57 mills that are processing paper along with pulp, boasting a total installed capacity of about 1,050,499 metric tons/year (Pakistan Bureau of Statistics, 2019). In Pakistan forests are covering 4.8% of total area as per the World Bank (2022) report, which indicates a great decrease of land area that is covered by forest. Bagasse, crop straws (rice and wheat) and cotton linter are among the important raw materials, and in several conditions, their mixture has the potential to be used in the production of paper (World Bank, 2022). In this context, wheat straw constitutes for about 46% of the essential raw materials along with wastepaper that accounting approximately for 29% and roughly 10% accounting of the total imported pulp, all of which is utilized completely in the manufacturing of specialty-grade products. Cotton linter, rice straw and bagasse constitutes the remaining 15 percent of agricultural-based raw materials employed. As the chemical method is the highly suitable process utilized for the manufacturing of pulp from these raw materials, in Pakistan in 2019 for about 96% of total pulp was manufactured via the Neutral Sulphite Semi-Chemical (NSSC) method. Despite an increase in demand of paper manufacturing in the country, in the previous 10 years as shown in the given Figure. The effective manufacturing of around 703,863 metric tonnes in 2019 indicates only 67 percent of the country's overall installed capacity (Shabbir *et al.,* 2022).

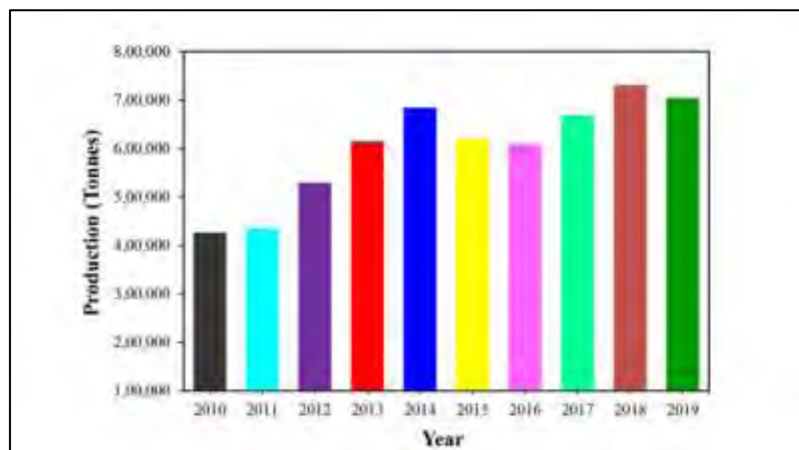

Figure 1: Pakistan pulp and paper annual production (Pakistan Bureau of Statistics, 2019).

Because of Covid 19, Pakistan's paper and pulp industry experienced a lot and has suffered an economical loss up to some extent. In 2017 the highest export of paper and paperboard was about US$100 Million, which decreases to approximately US$90 in 2019 and in 2020 for about US$46.17, due to the pandemic and restarted to increase to approximately US$64.1 in 2021 (Schmid *et al.,* 2023).

Natural Water Pollution

Water is a natural resource mostly employed in various industrial processes. Petrochemical plants petroleum refineries, chemical and pulp and paper industries use an extensive amount of water. The water utilized in these plants is directly linked to the generation of effluent (water plus contaminants), which needs to be released back to the environment. Industries that are involved in processing paper along with pulp produce excessive amount of polluted water (Francisco *et al.,* 2014). The discharged lignin gave the mill water effluent a dark brown color during the digestion of wood materials. Furthermore, being an aesthetic concern, color also has an impact on natural process of photosynthesis in the watercourse by hindering the uptake of sunlight (Ebrahiem *et al.,* 2017). Human interference is the main reason of water pollution, with the bulk of

pollutants emitting from industries such as heavy metals waste, many herbicides and pesticides, fertilizers, oils, and sewage (Schwarzenbach *et al.,* 2010). The harm to the ecosystem is irreversible which is because of polluted water that is coming. The Inadequacy of a substantial treatment method and immediate discharge of these hazardous effluent into the sewerage drainage pollute the groundwater and also other substantial water bodies, leading to adverse outcomes equally on aquatic and terrestrial life including food materials and crops as well (Aulakh *et al.,* 2009). Experts have not approved zero discharge policy for water consuming industries with a major increase cross-media environmental impact. Specialists in water resources are mainly concentrating on the release of environmentally friendly industrial wastewater discharge into the water bodies and rivers for the river system' stewardship. The present conventional treatment systems for Paper and Pulp effluent can eliminate pollutants significantly nevertheless; the existence of chloro-organic compounds in the treated discharge indicates the requirement for additional improvement (Jamil et al., 2011).

Wastewater Generated by Paper and Pulp Mills

Technological advancement has led to the greater water consumption for industry (Ebrahiem et al., 2017). The Paper and Pulp industry has consistently been the largest water intensive industry, necessitating approximately 50–60 m3 of water (Pathak et al., 2021). Typically produce one metric ton pulp and paper product. Each stage in paper manufacturing, that is from raw materials synthesis to finalizing and coating needs a significant volume of water. Water containments have a vigorous influence on paper standard throughout the manufacturing of pulp and paper by intervention with procedures like coloring, sizing, and bleaching (Jiang et al., 2021). Cellulose pulp manufacturing is accountable for the production of huge quantity of effluents arising from cooking the raw materials, specifically when utilizing some sulphur-containing reagent (for example, in the kraft pulping procedures). In the course of wood synthesis, bark, soil, and dirt eliminated from the raw materials. As a result, the wastewater is densely colored containing tannin content and resin acids. Generated pulp is of brown color which is then

exposed to bleaching in order to enhance its color. Resultant effluent from this process contains several hazardous substances including resin acids and unsaturated fatty acids etc. (Kamali et al., 2015).

Table 2: Pollutants discharge from P&P making process (Kamali et al., 2015).

Process stage	Wastewater		
	Volume	Pollution load	Effluent content
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.
Paper-making	Depends on the extent of the recycling effluents	Low	Particulate wastes, organic and inorganic compounds, COD, and BOD.

Majority of such industrial setups are releasing inadequately processed effluent into the rivers water bodies which is posing a significant issue to aquatic organisms (Kesalkar et al., 2012). Therefore, these industrial setups have been compelled to make a transition from traditional method of treating wastewater to further advanced methods which permit them to fulfill the present environmental standards. In developed countries, amount of generated waste water has been minimized considerably through biological treatments (Toczyłowska-Mamińska et al., 2017).

Black Liquor

During digesting process of wood into pulp, Black liquor is produced as a by-product (Radoykova et al., 2013). The composition of this waste primarily based on two substantial factors: (1) the raw material utilized for paper manufacturing, such as fibrous plants, softwood and hardwood and (2) pulping state. In terms of lignin and hemicellulose concentration, the composition of black liquor varies significantly, if the raw material utilized in pulping is non- wood or wood materials (Esmaeeli et al., 2013). For example, Agricultural based residue black liquor contains for about 8% to18%

hemicellulose and approximately 28% to 32% lignin, while eucalyptus black liquor incorporates 40% to 42% lignin and only 1 or 2% hemicellulose. Furthermore, in both of these types of black liquor, the leftover components remain the same (Bajpai et al., 2018).

Black liquor was utilized to directly discharge into the water bodies in the initial kraft pulp factories. Black liquor is highly toxic to marine life as it transforms the water into a deep caramel hue. A primary step forward for the kraft method was the recovery boiler, invented by G.H. Tomlinson in 1930 (Sjöholm et al., 2000). The methoxyl groups exist in lignin are substituted by hydroxyl groups, during pulping steps such as sulfite or kraft pulping, free radicals are produced by this reaction, which then mix lignin to generate volatile chemicals such as methanol and mercaptans etc., that emit a foul odor. These ionizing groups mainly depends on pH; they forfeit their ionic status, and instantly deionize resulting in the precipitation of lignin at low pH for example at pH (2-4) (Melro et al., 2020). The kraft pulping approach manufactures the most toxic waste stream, which is known as Kraft Black Liquor (KBL) (Toczyłowska-Mamińska et al., 2017). White liquor, is mainly a blend of two chemicals sodium hydroxide (NaOH) and sodium sulfide (Na_2S), is utilized in the kraft pulping technology to degrade the wood chips in elevated temperature in conjunction with high pressure. In this method, the cellulose fibers and lignin are disconnected, and the ensuing pulp, which is actually semi-solid and remnant containing a solvent nature is assembled. This pulp is subsequently purified by specific means of bleaching agents and utilized in following production stages, Meanwhile the liquid phase is utilized to craft KBL through the combination of the liquid residue remaining after washing the pulp (Vickers et al., 2017).

Pulp mills can be transform into more economical through the inclusion of chemical recovery cycle, that utilizes black liquor for repurposing the chemicals which can be reutilized in the procedure once again and additionally as an energy source (Cardoso et al., 2009). Typically, it is concentrate to between 65 percent and 80% by multi-effect evaporators, prior to combustion in a recovery boiler to retrieve the cooking

chemicals and produce electricity, elevation in viscosity are met by an accompanying rise in concentration. Roughly approximately 50 to 55% quantity of salt can be dissolved. Majority of the Pulp and Paper mills produce energy to steam and reutilize the chemicals used in the cooking procedures, that fulfill the power requirement of the industry and chemicals for reutilization with the help of recuperation methods such as Na2S and NaOH. This method of electricity production permits pulp and paper mills to almost entirely fulfill their energy requirements on-site, lowering off-site discharges, chemical used and water pollution (Mufson et al., 2009). This wastewater has biodegradability up to some extent, because it is exceedingly alkaline, and possesses a significant chemical oxygen demand. Dark viscous black liquor and alkaline waste black liquor comprises of different essences while washing which incorporates fatty acids, tannins, lignins, cellulose, resins and phenolic. The alkaline toxic waste comprising only 10 to15% of total wastewater, but adds to nearly surpassing 90 to 95 percent constituting the entire pollution load in terms of elevated pH, color, COD, and BOD which make it subsequently hazardous to the environment.

Composition of P&P mill Effluent

Pulp and paper waste water comprises of intricate inorganic and organic components. Lignocellulosic waste discharge from raw material is approximately 55–60% and the chemical makeup of these effluents includes resins, tannins, cellulose, lignin, phenols, and fatty acid. The pulp and paper industry effluents consists of an evaluated quantity of organic matter for example biocides, lignosulphonic acid, chlorinated hydrocarbon, chlorinated resin acids, chlorinated and phenols (Singh et al., 2019). Most of the pollutants in pulping effluents derived from the pulping of wood and the chemicals that are often Sulphur based, utilized to help in the pulping process. Substances that are mostly derived from organic chemicals like catechol, resin acids and terpenes, and catechol that have been segregated from wood (Dsikowitzky et al., 2014).

Role of Resin Acids and Fatty Acids in Making of Paper

Role of resin along with fatty acids in paper manufacturing procedure has been thoroughly researched. These components are derived from additives (i.e. surfactants) and raw materials. The paper manufacturing procedure discharges these components during their operational procedures including bleaching and pulping. These are the different steps in paper manufacturing process that uses in a unique combination; the amount of resin and fatty acids in the procedure based on the operational efficiency. Resin acids that are most often originated in polluted water during paper manufacturing process are separated into two: the abietanes which include dehydroabietic acid along with other components and pimaranes. Dehydroabietic acid is frequently common and also stables (specially the ring in structure which is aromatic in nature) resin acid exists in the paper manufacturing process waters and effluents. It also constitutes for the large part of wastewater hazardous nature as it can be turned into more hazardous components like retene. Among others, resins acids have been considered as the most prominent contributory factor in the overall lethal impact of polluted water that is generated during softwood pulping. The impact of resin acid detrimental effects on fish has undergone through extensively studied over the course of decades. Wastewater components can gather in the fish and have an impact on reproduction (Valto et al., 2012).

Phytosterols

In wood derivatives phytosterols are the compounds present in it. Wastewater with phytosterols amount in a specific volume as low as 0.26- 1.0 mg/L have documented a long term harmful effects. In treated (bleached kraft pulp mill) BKPM effluents stigmasterol and b-sitosterol have been found. Daphnids such as D. magna have utilized for examination of acute and chronic hazardous nature of wastewater and pure component. Campesterol, b-sitosterol, stigmasterol, campestanol, stigmastanol and b-sitostanol are the major phytosterols included in BKPM wastewater. Phytosterols have

been interlinked to endocrine disturbance on fishes. Amount of phytosterol as low as 0.26- 1.0 mg/L possibly has an impact on fishes (Xavier et al., 2006).

Lignin

Lignin generally constitutes approximately 15 to 30 percent of the weight of plant biomass and it is considered as the second most abundant biopolymer. It is recalcitrant and a very complex polymer, encased within the cell wall of plants (Schutyser et al., 2018). The word "lignin" refers to an extensive category of aromatic, impermeable and hard polymers generated by combining through oxidation of 4 hydroxy phenylpropanoids, which are mainly exist in woody plants (Vanholme et al., 2010). Approximately 50 to 70 million tons of lignin is produced/year during production of paper throughout the globe, the predominance of which is actually kraft lignin. In the pulp and paper the major lignin exist in effluent generated is burned as low value fuel to generate heat and electricity and less than 2 percent is utilized to produce significant chemicals such as dispersants, adhesives, surfactants, and other standard worth products. Due to its high stability, and complicated structure, it is not extensively examined for industrial applications (Sharma et al., 2020). So, it is quite difficult to breakdown polymers of lignin in a productive, directed, and also in a commercial manner.

Kraft Lignin

Lignin is handling by industrial setups that are involved in processing of paper along with pulp. Kraft pulping is the most prevalent chemical pulping method so far, manufacturing greater than 90 percent of chemical pulp (Tran et al., 2008). In this process, aqueous solution of sodium hydroxide is used for the processing of wood chips along with NaS_2 (white liquor). During this treatment process, alkaline liquor is used for the extraction and solubility of lignin. A prominent role is played by nucleophilic reactions among the sulfide anions and lignin, that carry out the processes related to lignin fragmentation, sulfur integration, and the process of re-forming polymers. Lignin that contains black liquor is separated rendering pulp isolated which is followed by a sequence of

evaporators are used to concentrate the liquor. Subsequently, the concentrated black liquor is reduced to ashes through incineration in a recovery boiler. Salts that are derived from the recovery boiler are subjected to slaked lime to restore the white liquor. In conclusion, a lime kiln supplies a continuous recycling of lime mud (Calcium carbonate) converted back to Calcium oxide (Tran et al., 2008).

Process of Paper Production

Paper production procedure comprises various phases: first phase involves making of raw material which is followed by pulping then comes paper manufacturing and bleaching. Significant amount of hazardous waste is generated during all these process which is ultimately released from the industry in form polluted water (Chandra et al., 2018).

Debarking process

Pulp making initiated with conveying wood originating from the forest to an area where wood piling takes place, known as wood yard. Following this by wood skin peeling utilizing the drum of debarking, a drum for pairing tool which has a lifting bar that uses for turning motion. There are holes in the wall slot through which Peeled skin will fall (Rullifank et al., 2020).

Pulping Process

During pulping phase, digestion of wood logs takes place at temperature ranging from 160 to 170 degree centigrade using Na_2S in combination with NaOH that results in formation of wood chips which are then cooked in solution containing HSO^{3-} and H_2SO_3.

Bleaching Phase

Pulping process is followed by bleaching phase of pulp which is important for the production of white paper. Chlorination and oxidation are utilized for bleaching, also the fibers delignification takes place further by solubilizing the extra lignin from cellulose.

Different chemicals are utilized during this phase including bisulfite and sulfur dioxideborol etc.

In last step of paper making, bleached paper is transformed into various products including tissues, papers, and toweling. Waster water that is generated during this whole process contains various toxic chemicals and pollutants along with hemicellulose and cellulose (Singh et al., 2019).

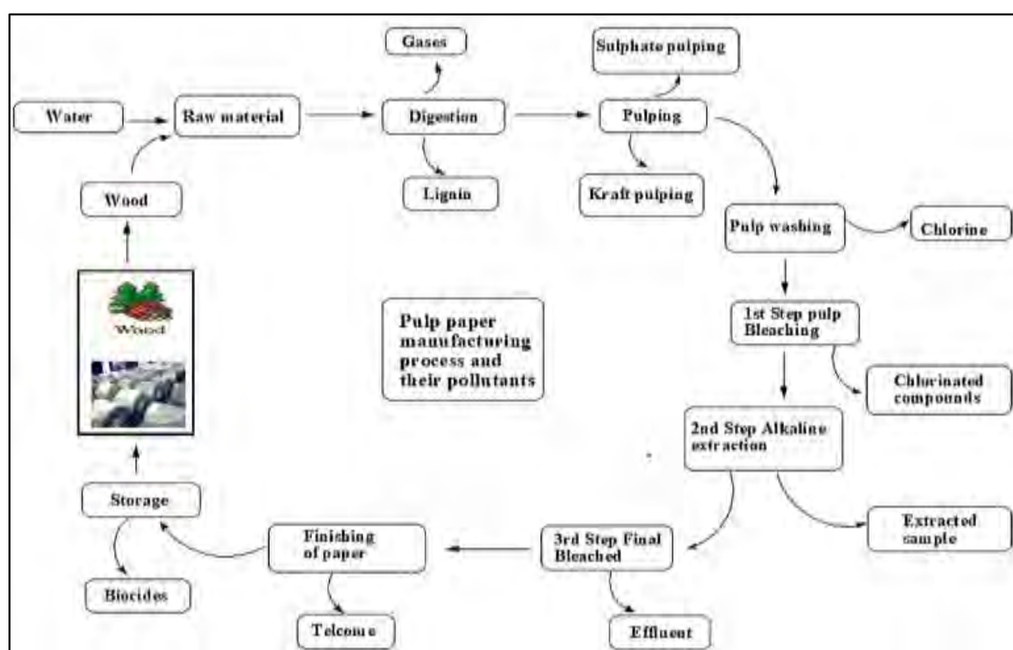

Figure 2: Diagram depicts the procedure of paper making and mitigation of different compounds that are generated in each stage (Singh et al., 2019).

2.2.4. Toxicity to the Environment

Effects on humans

Wastewater from the pulp and paper industry comprises of a complex inorganic and organic compounds. These pollutants are released when bleaching and pulping process occur while the paper making. The major pollutants that are gaseous include, methyl

mercaptans, chlorine dioxide, sulfur, hydrogen sulfides, and sodium sulfide, these gaseous pollutants are mainly responsible for causing chronic respiratory disorder, nausea, headache, eyes and cardiac problem (Singh et al., 2019). These toxic components penetrate humans through the eating of fish and it causes grave issues of health like the alteration of several metabolic activities. Which include increased excretion of 17-hydroxycorticosteroid, an increase in lymphocyte level and g-glutamyl trans peptidase activity. These hazardous compounds can also be causing dermal diseases like folliculitis, dermatitis, hepatomegaly and chlorane. Lignin in bleaching process contain large amount of chlorinated organic compounds. These compounds when consumed by human can have a profound effect on them, specifically one of the significant chemicals that is 2,3,7,8-tetrachlorodibenzo-p-dioxin, it is the most toxic compound which is mostly responsible for causing severe acne in males that mainly known as (acneiform dermatitis), (Abhishek et al., 2017). The wastewater of pulp and paper industry is not only toxic to our environment but it also has a major impact on human's life, when the effluent is discharged directly in the environment it can cause several acute and chronic diseases in workers, kids, diseases like vomiting, eye irritation and diarrhea etc. (Bhagawati et al., 2017).

Bottom-Water and Sediment Deoxygenation

Primary pollutants that are discharged from paper making industries and which have the potential to negatively affect the environment are lignin and chlorinated phenols. Lignin is accountable for the offensive color and growth inhibition of phototrophic organisms by reducing the transfer of light that is coming from the sun into streams and rivers. The major chemicals accountable for the lethal impact on animals and plants are chlorinated phenolic. Genetic mutations in exposed organisms may be caused by the chlorinated organic compounds, which comprises of furans and dioxins. These compounds not only have these impacts but can also have hazardous effect on aquatic organisms for example chlorinated compounds can bio accumulate in the tissue of fish and can cause variety of mutagenic, endocrinic, clastogenic and carcinogenic effects (Raj et al., 2014).

It has been reported by various scientists that effluent discharging from paper industry comprises approximately of 40%-45% organic chemicals, like pesticides, chlorophenols, chlorinated resins, lignocellulose, chlorolignins, chlorinated phenols, fatty acids and biocides. Aquatic organisms are severely affected because of these organic compounds and mostly inhibit the growth of flora and fauna. Various articles have mentioned, endocrine disruption, cellular damage, anoxia-induced oxygen depletion and delayed sexual maturation (Sharma et al., 2021). Pentachlorophenol (PCP) is actually a potential carcinogen and a widely recognized detrimental pollutant. Mostly fish and invertebrates are the target of these compounds and have an adverse or toxic effect on these organisms. As per USEPA (the United States Environmental Protection Agency) it is a major toxicant and its minute quantity of about 1.0 mg/L is not even regarded as secure for the environment (Singh et al., 2019).

Other than the pollution and worsening in water quality, there is a growing scarcity of water. As the wastewater is very toxic and contain variety of organic compounds and in order to mitigate its impact on overall environment there is a critical requirement for effective management of water in these sectors. In paper mill effluents approximately 500 diverse chlorinated organic compounds have been recognized. The high chemical compounds of these containments causes diverse range of mutagenic, clastogenic, carcinogenic, and endocrinic effects on fishes and other aquatic organisms in recipient rivers and streams. Exposure to the wastewater negatively affects variety and ample quantity of zoo benthos, phytoplankton and zooplankton, causing disturbance to invertebrate communities and benthic algal. Hence before discharging it is obligatory to treat the effluent (Sharma et al., 2014).

2.2.5. Treatment of P&P Mill Effluent

One of the major sources of environmental pollution is the huge volume of effluent from the pulp and paper industry, which contain recalcitrant toxicants and some other pollutants to the environment. The effluent has an elevated amount of BOD, total solids,

phenols, lignin, COD, and its extractives which induce a potential toxicity. Treatment of discharge coming out from paper making industry pulp is important to decrease its negative impact on the surroundings. Various techniques can be used to treat the effluent of pulp and paper industry, which include biological method, chemical and physical methods of wastewater (Zainith et al., 2019).

Physiochemical Treatment

The elimination of lignin from polluted water of paper making industry is done through implementation of various methods. These techniques include, advance oxidation process, ozonation, coagulation, filtration and precipitation, adsorption and reverse osmosis etc. (Wang et al., 2011).

Coagulation and Flocculation

The process of coagulation is divided into four stages initiating with enmeshment, the second step is adsorption, followed by charge neutralization and the last stage is precipitation. In simple way the primary steps are sweep flocculation and charge neutralization. Contaminants are entangled and adsorbed to cationic metal hydroxides in sweep coagulation resulting in the precipitation of organic toxicants. The second is the charge neutralization where negatively and positively charged particles are covered through counter-ionic particles. Different chemicals as per study are the most significant in removing AOX, color and total organic carbon. These chemicals include hexamethylene diamine, epichlorohydrin polycondensate (HE), horseradish peroxide (chitosan) and polyethyleneimine (PEI) (Haq et al., 2020). Other inorganic coagulants such as alum and ferric chloride are also researched and found good in eliminating organic pollutants due to their efficiency and effectiveness. However, these traditional coagulants are not able to remove organic waste efficiently and this is the major reason researchers are studying and searching for a natural polymer as a coagulant which can remove the organic waste efficiently. Furthermore, a study reported about the calcium lactate–APAM, calcium lactate–magnesium hydroxide, calcium lactate–APAM, calcium

lactate–polyDADMAC and calcium lactate alone can remove lignin approximately 44%, 64%, 50% and 60%, in the corresponding order (Wang et al., 2011). Where as in the flocculation method by the use of polymers the particles aggregate that binds them together, a natural polymer (starch-g-PAM-g-PDMC) which is modified, it is utilized as flocculants for the wastewater treatment. For better treatment of the effluent that flocculation should be utilized that are prepared the PAMs consisting of various charges like neutral, positive and negative which is economical and gives better settling properties, for the flocculation of the suspended particles existing in the pulp and paper mill wastewater the double bond is responsible (Zainith et al., 2019).

Adsorption

Distinguishing method of reducing pollutants found in polluted water coming out from paper making industry is Adsorption. Adsorption comes with various benefits and has comparatively low cost, simple design, and less requirement of land. Currently, these cost-effective adsorbents are utilized as they are readily accessible and are used to deal with natural waste, and waste that is produced by agricultural and industrial sectors. An effective adsorbent, activated carbon, has been used for water and wastewater treatment. Other adsorbents that are used in paper making industry include ash, coal, and silica.

The mechanism of adsorption of ion exchange resin and granular activated carbon respectively acts on high-molecular-weight and hydrophobic fractions and documented that 72 percent and 76 percent decrease in dissolved organic carbon by utilizing adsorption process. The blast furnace dust (BFD) and slag has the removal effectiveness through the adsorption mechanism was approximately 80.4% and 61% (Li et al., 2020).

Chemical treatment

Chromophoric and non chromophoric contaminants both are present in paper and pulp mills wastewater and to destroy them several advanced oxidation techniques are employed which include the following photo-oxidation, wet oxidation, photo catalysis,

Fenton-type reactions and ozonation (Ribeiro et al., 2020). There are various advantages of chemical treatment as they are substantially cost-effective than secondary treatment and were effectively removing color and it was observed that large amount of TSS and COD were decreased during this method (Irfan et al., 2017).

Membrane technologies

Lately from the previous years, the membrane techniques have been used widely when it was observed for water and wastewater treatment. However, because of some disadvantages and its very elevated cost, utilizing them at large scale is very challenging, as they have some technical limitation (Greenlee et al., 2010). Through the employing of pretreatment techniques, the efficiency of this system can increased. As per a research, utilizing a flocculants could decrease approximately 75 percent of COD from polluted water of paper making industrial setups (Li et al., 2011). Another study reported that in a membrane electrochemical reactor BOD, COD, TDS and, color of the effluent from paper manufacturing industry was considerably eliminated (Chanworrawoot et al., 2012). Conversely, ultrafiltration membranes employing has been done to treat the wastewater and it was reported that approximately 89% COD, 97% sulfate, 83%total hardness ,95% spectral absorption coefficient, and 50% conductivity at pH 10 (G nder et al., 2012).

Ozonation

Many scientists have proposed the usage of O_3 for elimination of color, hazardous components, and COD from polluted water of various industries. Usage of O_3 in treating polluted water of industries that are involved in making of papers reduces TOC and BOD. The use of ozone methods and photo catalysis to treat the wastewater of bleached mill decreases COD, color, and TOC. Moreover, the process of ozonation and activated sludge treatment were found to be advantageous in treating the nonfiltered wastewater. In another study more than 50 percent decrease in reduction and turbidity in color and lignin content was observed as the amount of ozone dose was raised (Ruas et al., 2007). Furthermore, it was observed in a research study that there was round about 40 percent to

96 percent reduction in lignin content when amount of O_3 was raised from 0.1 to 3.6 mgO_3/mg (G nder et al., 2012).

Biological Treatment

Various types of pollutants based upon the type of the pulping method are generated by Pulp and paper mills. Pulp and paper mill wastewater contaminate soil, water, and air, resulting in a significant threat to the environment. Moreover, the other treatment methods like chemical and physical methods are on the right track of treatment, however they cannot be compare with biological treatment due to its cost ineffectiveness and lingering impact. In reducing the organic load and toxic effect of kraft mill effluent biological treatment is considered to be advantageous. In the present available conventional methods, none of them are permanent and environmentally friendly disposal solution. To degrade effluents of pulp and paper mill biological methods have been acknowledged. Biological processes include all those microorganisms that are able to degrade waste of pulp and paper in natural environments. Many classes of microorganisms are utilized for the degradation of pulp and paper mill effluents such as fungi, bacteria, and algae to breakdown the polymeric lignin extracted from chromophoric material. Many processes have been employed by different researchers all over the world for the elimination of the color from the effluent of pulp and paper mill (Sharma et al., 2014).

Fungal Treatment

Wood decaying by fungi takes place through hyphae and spores and then to attack plant cell wall fungi secrete extracellular enzymes, that leads to the breakdown of lignocellulose (Chen et al., 2017). The main group of fungi that are mainly able to decay wood are Basidiomycetes which are grouped into three classes including Soft-rot fungi, white-rot fungi, and brown-rot fungi. Lignin in biomass is deconstructed by *Phanerochaete chrysosporium,* which is a primary white-rot fungus, shows a high effectiveness of up approximately %, which it gains by generating manganese dependent

peroxidases, laccases, and lignin peroxidases. It has also been observed that production of these enzymes varies from one fungal strain to another (Rodríguez-Couto et al., 2017).

Table 3: Characteristics of enzymes involved in breaking down of lignin (Rahul *et al.,* 2017; Fakoussa and Hofrichter, 1999).

E.C.	Laccases 1.10.3.2	Manganese peroxidase (MnP) 1.11.1.13	Lignin peroxidase (LiP) 1.11.1.14	Versatile peroxidases (VPs) 1.11.1.16
Structure	Monomer, dimer, tetramer, glycoprotein	Monomer, glycoprotein	Monomer, glycoprotein	Monomer, glycoprotein
Catalytic center	Four copper atoms	Fe-protoporphyrin	Fe-protoporphyrin	Fe-protoporphyrin
pH Range	2.0-8.5	2.0-6.0	2.0-5.0	3.0-5.0
H_2O_2 dependence	No	Yes	Yes	Yes
The ability of C-C bond cleavage	No	Yes	Yes	Yes
Cofactor	O_2	H_2O_2	H_2O_2	H_2O_2
Substrate	polyphenols, polyamines, lignins, and aryl diamines	Lignin and phenolic compounds	Halogenated phenolic compounds, polycyclic aromatic compounds	Aromatics, phenols and non-phenolic, VA and RB5

Other than white-rot fungi, Brown-rot fungi also have the capability to breakdown lignin that mainly target cellulose; however comparatively these have less efficacy to degrade lignin. Moreover, lignin is also modified through addition of methyl during this process. According to a research study, expression of enzymes has been observed by fungal strain in order to degrade lignin (Colonia et al., 2019).

Similar study reported that *Aspergillus fumigatus* notably have five times greater lignin degrading activity than the *Coriolus versicolor*. Enzymes that are having lignin degrading ability are too large and have an immediate access to the cell wall. Because of this they first loosen the matrix of the lignin, after which enzyme is efficiently able to act on lignin (Hernández-Ortega et al., 2012). A study has carried out to explore white rot fungi ability of lignin degrading which includes *P. chrysosporium* and *B. adusta* that is grown in media most probably containing (1) mineral salts and glucose (2) mineral salts and a dairy residue (3) only a dairy residue. These both fungi are then utilizing as an inoculum to treat industrial pulp-and-paper mill and synthetic wastewater. On synthetic wastewater, lignin degradation by *P. chrysosporium* and *B. adusta,* approximately 97% and 74%,

have been achieved. Wherase 100% delignification of polluted water of industries was achieved by both strains in 8–10 days, with a significant reduction of total organic carbon (TOC). Results of study have shown great possibility of *P. chrysosporium* and *B. adusta* for highly proficient lignin elimination in industrial wastewater and can open the way to further industrial operations on wide scale. It has also been studied those fungi that are in the black *Aspergillus* category are efficiently capable of degrading waste in effluent for the paper and pulp industry (Costa et al., 2017).

Fungi have been broadly researched for their effective ability to break down lignin into monomers. Due to the secretion of several ligninolytic enzymes, they are interesting bio agents for the detoxification of wastewater from Pulp and Paper mills. The broadly researched fungi are *Merulius aureus, Phanerochaete chrysosporium, Aspergillus niger*, *Fusarium sambucinum*, and *Rhizopus oryzae*. White rot fungi have the potential to completely degrade lignin. In 1977 Fukuzumi first reported white-rot fungus application in the treatment of effluent coming out from paper producing industries. The cultivation of white rot fungi was done in submerged culture parameters utilizing a liquid medium that contained vitamins, spent liquor from alkali extraction, nutrients, and vitamins.

Bacterial Treatment

Lignin degrading enzymes in bacteria are fewer in numbers, that are primarily less effective degraders of lignin than fungi. Furthermore, bacteria are very sensitive to genetic alteration to enhance lignin reduction. Classes actinomycetes, alphaproteo bacteria and gammaproteobacteria, are the groups where lignin-degrading bacteria are found (Xu et al., 2019). However, the mode of action employed by filamentous fungi described above, is similar to the fundamental mechanism of lignin degradation by bacteria, where oxide reductases significantly contribute. As the research progresses, in bacteria metabolic pathways and the enzymes of lignin degradation were characterized, incorporating hydroxylation and catalytic oxidation, in addition to the depolymerization lignin (Mallinson *et al.,* 2018).

Most of these bacteria which have an efficient lignin degrading ability have been utilized in huge amount in wastewater treatment from the pulp and paper industry including *Bacillus atrophaeus*, *Bacillus pumilus* and *Mycobacterium smegmatis*. *Bacillus atrophaeus*, *Bacillus pumilus* have been observed to produce laccase (Zhang et al., 2019; Hooda et al., 2018; Huang et al., 2013).

Bioreactors

Frequently utilized biotechnologies for the treatment of wastewater encompass bioreactors. In bioreactors, a bacterium sometimes uses to treat effluent, but the use of fungi has received generally less attention. Considerably because of the plentifulness of degrading enzymes secreted by fungus and their ability to recover and adapt adverse environmental condition, incorporating changing pollution level, low nutrient concentrations, low pH, and fungal bioreactors are advantageous (Hooda et al., 2015).

Fluidized bed reactor

The fluidized bed reactor that can be used to carry out wastewater treatment, specifically biological treatment, and Advanced Oxidation Processes, indicates an exceptional opportunity for economical treatment of effluent comprising stubborn pollutants. The biological wastewater treatment through fluidized bed reactor is well with various grand-scale plants in existing, its application in advanced oxidation methods is mostly at laboratory-scale. A stationary bed of solid particles at a sufficient superficial velocity that is enough to suspend the particles and make them to behave as like they were fluid, this is the fundamental concept of fluidization, where the attachment of microorganisms occurred, which is initially located at the bottom of the reactor and play crucial role in the breakdown of wastewater and move upward by the help of fluidized bed containing the microbes attached (Bello et al., 2017).

Stir tank reactor (STR)

The reactor sizes that are utilized from the laboratory level to the industrial scale for large and bulk treatment methods are of various types, for example initiating from an about dm3 up to 100dm3. As per the research, it is reported that stir tank reactor is found to be economical and gives better treatment results. Mostly, on lab scale about 20 L of the reactor is utilized which is generally made up of glass material, while those reactors utilized for the treatment of significant capacity of the wastewater at an industrial scale are usually made of steel material. The Height and material type of the reactor is varied based on the conditions (Younesi et al., 2008).

Membrane bioreactors

In the treatment of pulp mill effluent, Paper and various domestic wastes, the membrane bioreactors are gaining their interest and due to various domestic wastes, by utilizing the membranes to filter the wastewater and also eliminate most of the components and activated sludge which predominantly addresses the wastewater. It is considered that the effective dimensions of the pores exist in the membrane is approximately 0.1 micrometer (Santos et al., 2011). After the treatment when the wastewater is examined for treatment of various components, it shows significant removal of the water reclamation, COD, BOD and suspended solids as well. The fouling of the membrane which needs to be changed after every step is the one disadvantage (Malik2009).

Sequencing Batch reactor (SBR)

There are four basic steps that take place in sequence such as filling, settling, withdrawing, and idling. During the filling stage, the wastewater is added into the chamber, and soon after the completion of filling aeration is supplied to the reactor to carry out the degradation in the presence of oxygen. Then settling process takes place to allow the settling of the biomass and about 3rd part of the treated effluent is withdrawn from the reactor then the reactor is left for second cycle or set idle for the next use. In this

study, the same sequencing batch reactor is used consisting of four chambers made of Pyrex glass two of them for treatment of Pulp and Paper effluent and the remaining two for sand filtration. Starting with the fungal chamber containing wood chips having fungal biofilm (SL-6) where initial treatment of effluent takes place then followed by the sand bed and after filtration to the 3rd chamber having another fungal strain (SST-1) for treatment of effluent and then passed to the sand bed. The treated effluent is collected at the end of the cycle.

3. Materials and Methods

The current study was conducted in Applied, Environmental and Geo-Microbiology (AEG) Laboratory, Department of Microbiology, Faculty of Life Sciences, Quaid-i-Azam University, Islamabad, Pakistan from February 2023 to February 2024.

3.1. Sample collection

Samples were taken from Century Papers and Board Limited District Kasur, Punjab Pakistan. The manufacturing plant, responsible for paper making, consisted of seven paper machines. The plant was however also dealing with effluent treatment and was involved in generation of electricity. For this study, samples were collected from two points: Black liquor and Sludge.

3.1.1. Samples Taking Procedure

Pulp and paper effluent was collected in a 5- liter gallons aseptically. After collection the samples were placed on ice packs and were properly labeled. Collected samples were brought to lab in 24 hours and were placed in refrigerator at 4°C until use for analysis.

3.2. Isolation of fungal strains from pulp and paper effluent

Isolating a fungi strain from pulp and paper effluent involves a series of steps to obtain pure cultures of the desired fungal strains. Isolation of fungal strains was carried out on Potato Dextrose Agar (PDA) media. The 90mm agar plates were prepared by pouring 25ml of media in each plate with PDA for fungal strain isolation.

3.2.1. Serial dilution

The serial dilution of the sample (Black liquor) collected from the Pulp and Paper mill effluent was carried out from 10^{-1} – 10^{-9} by adding 1ml of sample in first test tube containing 9ml of sterilized distilled water and taking 1ml from first tube to the next one and so on.

3.2.2. Spread Plate Method

Different test tubes were selected (10^{-1}, 10^{-3}, 10^{-6} and $10^{-9)}$ and 100µl of the sample was taken from each test tube and spread on the PDA plates. The plates were then placed in incubator at 30°C and were given incubation time of 3-4 days. After incubation time, single and isolated colonies were taken using sterilized inoculating loop and were transferred onto the fresh plates containing PDA media for culture purification. This Process was repeated to get purified fungal strains.

3.3. Assays for Enzymes Screening on Agar Plates

3.3.1. Qualitative Screening

The qualitative screening was performed to detect the presence of lignin peroxidase, laccase and manganese peroxidase. Three assays were performed for the detection of these enzymes that are Azure B plate assay, Guaiacol plate assay and Methylene Blue.

a. Azure B Plate Assay

Azure B Plate assay was used for the detection of lignin peroxidase. Lignin peroxidase is an enzyme that plays role in degrading lignin in plant material. To qualitatively screen for lignin peroxidase Azure B dye is used as an indicator. A concentration of 0.01% of Azure-B was added in minimal salt medium (MSM) media for Fungal strain for lignin peroxidase screening. The PDA media was poured in plates and solidified under sterile condition and after that, spot inoculated plates were placed at 37 ºC and were given incubation time of 7-10 days.

b. Guaiacol Plate Assay

Guaiacol assay is used to detect the presence of laccase enzyme. In PDA media 0.004% guaiacol was added and autoclaved at 121◦c at 15psi. Guaiacol is a colorless compound that, when oxidized by laccase enzyme it forms a brown or reddish-brown product. The PDA media containing Guaiacol was poured in plates and solidified in laminar flow

hood, after the spot inoculation plates were placed at 37 ºC and were given incubation time of 7-10 days.

c. Methylene Blue Assay

Methylene blue assay is used to assess the detection of manganese peroxidase (MnP). To perform this experiment 0.0025g methylene blue was used in 100ml of PDA media and autoclaved at 121 ºC at 15psi. After that PDA was poured in 25ml plates and solidified under the sterile conditions, spot inoculation was done. Plates were then placed at 37ºC and were given incubation time of 7-10 days. The decrease in methylene blue absorbance serves as an indirect indicator of MnP presence.

3.3.2. Quantitative Screening

Quantitative assay was made to detect the ability of a fungal strain to degrade lignin as a source of carbon as well as to measure the activity of these enzymes in a sample. For this purpose, Minimal salt medium (MSM) was prepared for the quantitative screening of lignin peroxidase, laccase and manganese peroxidase.

a. Preparation of Minimal Salt Medium (MSM)

For the preparation of MSM media peptone 3g/l, glucose 10g/l, KH2PO4 0.6g/l, ZnSO4 0.001g, K2HPO4 0.0005g/l, FeSO4 0.0005g/l, MnSO4 0.05g, MgSO4 0.05g/l and lignin 0.3g/l was added. Guaiacol was used as an inducer.

MSM media preparation was similar for lignin peroxidase and Manganese peroxidase as both have the exact same composition, moreover in Laccase lignin was not added but here too guaiacol was used as an inducer. When the weighing process was completed, the salts were separately dissolved in distal water (This was done in order to avoid the precipitation of salts) while peptone and glucose were mixed together in a flask and autoclaved at 121◦c at 15psi. After this the MSM media was mixed and equally separated in 500ml flasks. Two flasks were for manganese peroxidase and lignin peroxidase and two were for laccase.

The flasks were inoculated with the fungal strains and were labeled and placed in a 37°C shaking incubator for 10 days. However, after 48 hours first sample was collected and processed. Enzymes assays quantifications were differently and separately processed, as all have their own method, they are the following.

b. Enzyme Assay for Lignin Peroxidase

Lignin peroxidase activity was checked by azure B oxidation method (Archibald, 1992). Various reagents needed for enzymes assays were prepared accordingly. 0.160 azure B solution, 2mM hydrogen peroxide solution and 125mM sodium tartrate buffer, for further use solution was kept at room temperature.

Procedure

Fungal strains that were producing lignin peroxidase were further quantitatively screened for enzyme activity. For this purpose, fungal strains were grown in liquid medium and for the determination of enzyme activity samples were taken regularly. The optical density of enzyme activity was measure at 310nm. Following are the reaction mixture for the enzyme assay given in table:

Table 4: Composition of reaction mixture for enzyme assay

S.No	Components	Concentration	Quantity
1	Azure B	0.160mM	500 ul
2	Sodium tartrate buffer	125 Mm	1 ml
3	Hydrogen peroxide	2mM	500 ul
4	Culture supernatant	-	500 ul

c. Enzyme Assay for Mnp Activity

The activity of manganese peroxidase was determined by the oxidation of guaiacol as described by the paszczvnski and coworkers (paszczvnski.et.al, 1998). For this reason, fungal strains were grown in liquid medium and for the determination of enzyme activity samples were taken regularly. Composition of reaction mixture for the enzyme assay given in table:

Table 5: Composition of reaction mixture for the enzyme assay

S. No	Components	Quantity
1	Guaiacol	20ul
2	MnSO4	2ul
3	Sodium Potassium Tartarate Buffer	500ul
4	H2O2	10ul
5	Enzyme	500ul

Before taking the optical density at 465nm, incubate the reaction mixture for 30 minutes at 40˚C.

d. Guaiacol assay for laccase enzyme

Guaiacol assay method can be used to measure the activity of the enzyme laccase. Laccase catalyzes the oxidation of guaiacol, producing a colored product. For this reason, fungal strains were grown in liquid medium and for the determination of enzyme activity samples were taken regularly. The increase in absorbance at a specific wavelength is proportional to laccase activity. The optical density of enzyme activity was measure at 450nm. Composition of reaction mixture for the enzyme assay is given in table:

Table 6: Composition of reaction mixture for enzyme assay.

S. No	Components	Concentration	Quantity
1	Sodium acetate buffer	10mM	3ml
2	Guaiacol	2mM	1ml
3	Enzyme source (fungal supernatant)		1ml

e. Blank preparation for enzyme assays

Blank preparation was done that contained 1ml of distilled water instead of enzyme. The mixture was incubated at 30ºC for 15 min and the absorbance was taken at 450nm UV spectrophotometer. Enzyme activity was expressed as international unit, where 1ul is the amount of enzyme required to oxidize 1umol of guaiacol per min.

3.4. Identification of Fungal Isolates

3.4.1. Microscopic Examination of Fungal Isolates

Morphological identification of these fungal strains was done through microscopic analysis and colony morphology. Color, shape, and size of the colonies were thoroughly examined. However, for the sake of microscopy, lacto phenol cotton blue staining was used.

3.4.2. DNA Extraction and Molecular Identification of Fungal Isolates

For fungal DNA extraction, the isolates were grown on PDA plates for about 6-7 days and the DNA was extracted by CTAB method using the following protocol.

a. DNA Extraction Protocol

Take fungal mycelium and add it to 500ul CTAB Buffer. Freeze the mycelium in freezer and then crush it in mortar and pestle gently. Thaw the Eppendorf at 90 ℃ in already set water bath. Add proteinase K and Lysozyme10 ul each and 30ul SDS (30%). Incubate at 37°C for 1 hour. Add 100ul 5M NaCl and incubate at 65°C for 20-30 minutes. Add 500ul phenol: chloroform: isoamylalcohal in ratio of 25:24:1 and centrifuge at 10000rpm for 20 minutes. Take upper layer, add to new centrifuge tube, and repeat the PCI step again. Take upper layer in new tube and add 30ul sodium acetate (3M) and 500ul Isopropanol and incubate at -20°C for 1 hour. Centrifuge the tube at maximum speed for 10 minutes. Discard supernatant and add 500ul 70% ethanol to pellet. Centrifuge at 10000 rpm for 5 minutes and discard ethanol. Dry the pellet at 37°C and add 40ul T.E buffer to dissolve the DNA. DNA purification was visualized through gel electrophoresis.

b. Gel Electrophoresis

The purified sample of DNA was run on 1% agarose gel for DNA confirmation. In order to prepare 1 % agarose gel, 0.45g of agarose powder was weighed and mixed with 45 ml TBE buffer. The mixture was heated in microwave for 1 minute after which 5 µL of ethidium bromide was added. The mixture was then poured in the electrophoresis tray along with comb until it gets solidified. After the gel was solidified the comb was removed and plates were placed in the chamber with well in direction of negative end of chamber. The 1µL of 6X loading dye was mixed with 5µL DNA sample and loaded in wells with the help of pipette. The electrophoresis was run for 40 minutes at 120 volts. The DNA in gel was then viewed under the U.V transilluminator.

c. Phylogenetic Analysis

The amplification of extracted DNA was carried out through PCR and then PCR products were sent for sequencing. The obtained sequences were trimmed to remove unwanted sequences using Bioedit-7.2. The trimmed sequences were BLAST (Basic local alignment search tool) searched in NCBI (National Centre for Biotechnology

Information) data bases (http://www. ncbi.nlm.nih.gov/BLAST/) to find the most homologous species and then closely related sequences were downloaded. Mega-X software was used to construct phylogenetic trees by the neighbor-joining method.

3.5. Optimization of different culture Parameters

The optimization of different parameters like PH, temperature, lignin Concentration, carbon and nitrogen sources was made to increase the degradation process. On the basis of primary screening, the best fungal strains were selected and all the conditions were optimized. All the flasks were placed in horizontal shaking incubator. To examine the impact of initial pH, temperature, lignin concentration, carbon source and nitrogen source on the process of bioconversion of lignin by these isolated different experiments were conducted.

3.5.1. Optimization of Initial Lignin Concentration

The lignin optimization was performed on using different concentration of lignin such as 1g/l, 1.5g/l, 2.0g/l and 2.5g/l, this experiment was performed in the 250ml flask containing 100ml of distilled water along with the minimal salt media to support the growth of the fungal strains. After that autoclaved fungal strains were added into the flasks and were kept in shaking incubator at 35˚C temperature for 12 days. First sample was taken after 48 hours and was checked for lignin and color reduction through spectrophotometer.

3.5.2. pH Optimization

The experiment for monitoring the optimum pH was performed by adjusting different pH conditions such as pH 4, pH 6, pH 8, pH 10 with 2% BL concentration. For performing this experiment, 1 liter MSM media was prepared that was covered with aluminum foil and autoclaved. After that fungal strains were added into the flasks and were kept in shaking incubator at 35˚C temperature. First sample was taken after 48 hours and was checked for lignin and color reduction through spectrophotometer.

3.5.3. Temperature Optimization

1-liter MSM media was prepared, covered with aluminum foil and autoclaved. After that flasks were inoculated with fungal strains except control. The optimization of temperature was made by using different temperature ranges such as 25°C, 30°C, 37°C and 40°C with 2% black liquor concentration, and placed in different shaking incubators accordingly. First sample was taken after 48 hours and was checked for lignin and color reduction through spectrophotometer.

3.5.4. Carbon source optimization

The carbon source optimization was made by using different carbon sources, such as glucose, sucrose, dextrose, starch and cellulose. This experiment was performed in the 250ml flask containing 100ml of distilled water along with the minimal salt media to support the growth of the fungal strains. After that fungal strains were added into autoclaved flasks and were kept in shaking incubator at 35°C temperature for 12 days. First sample was taken after 48 hours and was checked for lignin and color reduction through spectrophotometer.

3.5.5. Nitrogen source optimization

Nitrogen source optimization was made by using different nitrogen sources, such as peptone, yeast, casein hydrolysate, tryptone and ammonium sulfate. This experiment was performed in the 250ml flask containing 100ml of distilled water along with the minimal salt media to support the growth of the fungal strains. After that fungal strains were added into autoclaved flasks and were kept in shaking incubator at 35°C temperature for days. First sample was taken after 48 hours and was checked for lignin and color reduction through spectrophotometer.

3.6. Design and Construction of Sequential Bioreactor

The sequential bioreactor is actually a series of reactors that is made of Pyrex glass, where the treatment of paper and pulp effluent takes place. There were two chambers

of equal size and dimensions. They were organized in a manner that allowed water to flow to the next chamber naturally due to gravity. This reactor made up of Plexiglas. Dimension of each chamber was width =25cm, length=45cm and height= 30cm.The first chamber was used as fungal batch reactor where the black liquor was treated for 15 days through selected fungal strain. Next chamber was used as Sand filter to avoid any mycelial flow to the next fungal chamber. Wood chips having width 7cm and length 5cm were used in all the chambers as packing materials. After the treatment was completed, there was an outlet about 2-3 inches above the base of each reactor which was used to transfer the effluent from one reactor to another. A pipe which was about 2cm in diameter was adhere for sample transferring and at its end a valve was fixed to prevent the outflow of effluent during treatment.

3.6.1. Study Design

To treat pulp and paper mill Wastewater by using indigenous microorganisms, a lab scale sequential biological reactor was designed. The fungal biomass was grown on the wood chips having 7cm width and 5cm length were used as inert media. To detect the efficiency of the bioreactor for the treatment of Pulp and paper wastewater various parameters were determined such as lignin, color, COD and Phenolic contents.

Trials performed with 5% BL concentration.

Trial 1st: First trial was run for 15 days at 30 °C temperature with the help of an aquarium heater. The black liquor concentration was kept as 5% (360ml BL in 12L distilled water).

Trial 2nd: Second trial was run for 15 days and the temperature was maintained using an aquarium heater. The black liquor concentration was also kept as 5% (360ml in 12L distilled water) to check the combine effect of both fungal strains.

3.7. Fungal biomass development

Two fungal strains *Trametes hirsuta* SST-1 and *Aspergillus fumigatus* SL-6 were used for the treatment of black liquor. Wood chips of same sizes length 5cm and width 7cm were used as a surface for the development of fungal biomass. Two sterile fish tanks were taken for this purpose, autoclaved wood chips were added to these fish tanks and various supplements like glucose and yeast extract were autoclaved and added to fish tanks. These fish tanks were then inoculated with fungal strains and left for about 10 days until enough fungal biomass was formed. *Trametes hirsuta* SST-1 and *Aspergillus fumigatus* SL-6 fungal strains were added to two separate fish tanks.

3.7.1. Analysis of different parameters

b. Lignin Estimation

Pulp and Paper mill effluent contain a main component in it that is lignin which needs to be estimated before and after the treatment of effluent sample. The Lignin contents present in the effluent was determine according to the method that was reported by the Pearl and Benson (Pearl and Benson, 1940).

Procedure

In this method about 1ml of 10% CH3COOH and 1ml of 10% NaNO3 were added to the 2ml of the sample that was treated. After this 15 minutes of reaction time was given, later NH4OH was added to the sample and given a time of 5minutes, after that at 430nm absorption was measured. The Blank was prepared for the detection lignin by adding about 1ml of 10% CH3COOH, 2ml of the NH4OH added to the 2ml of Distilled water sample. 15mins of incubation was given after that 1ml of the NaNO3 was added to the mixture and absorbance of the sample was taken at 430nm after 5minutes.

c. Measurement of Color Unit

According to the Canadian Pulp and Paper Association (Yang et al., 1974), the color units of the treated sample was detected. For this reason, the samples were centrifuged for 30 minutes at 10000rpm to remove the suspended particles. The spectrophotometry was then performed and measured at 465nm, and for blank distilled water was used. The formula given below were used for the calculation of color units.

CU (PtCO) = 500 A2/A1

A1 = Absorbance of 500 CU of standard platinum-cobalt solution (0.1214)

A2 = Absorbance of the sample

3.8. Phenol Content Detection

The total phenolic contents of the wastewater before and after the treatment were determined by the method containing the use of FC reagent as an oxidizing agent and Gallic acid was used as the standard. Total phenolic content of SST-1and SL-6 sample was found using Folin ciocalteau (Fc) reagent by following procedure as described by (Kaur & Kapoor, 2002) with little changes. FC reagent's working solution was prepared in the ratio of 1:1 with distilled water. Then, 1 ml of the sample was added into the autoclaved test tubes. In this experiment of determining the total phenolic content of fungal sample (pulp and paper effluent), Gallic acid was used as a standard and different concentration of Gallic acid (50 µl- 500 µl) were taken in test tubes. This was followed by the addition of 800 µl distilled water. The test tubes were thoroughly shaken to mix the reaction mixture. 1.5 FC reagent was added to all the test tubes and were vortexed. The reaction mixtures in test tubes were then given incubation time of 15 minutes and then 1 ml of 20% Na_2CO3 solution was added to the test tubes, containing the reaction mixture. Test tubes were then left in dark conditions for 1 hour and after 1-hour absorbance was measured at 710nm against the blank which contained all the chemical ingredients of the reaction except standard (gallic acid) or sample. Total phenolic content was calculated from the calibration curve and the outcomes were presented as milligrams of gallic acid equivalent per gram of dry weight.

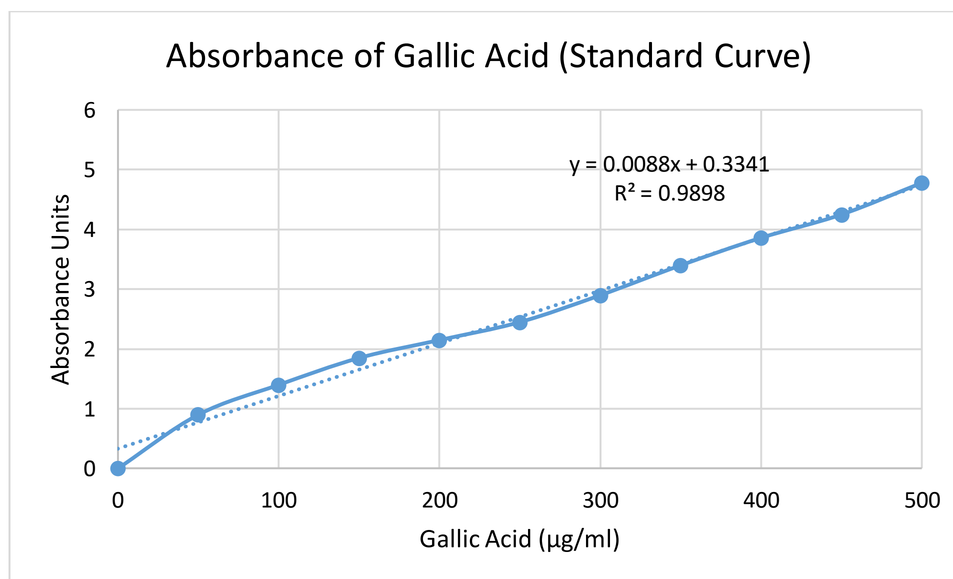

Figure 3: Gallic acid standard curve.

3.8.1. Reagents and Apparatus

- Folin-Ciocalteau reagent (10 times diluted)
- 20% Sodium Carbonate solution
- Gallic acid (standard)
- Micropipette (10-100µl)
- Pipette (1ml)
- Spectrophotometer.

3.8.2. Total phenolic content Calculation

The total phenolic content was calculated by the help of the formula given below:

$$C = X (v/m)$$

Where, C = Total phenolic contents

X = concentration of gallic acid calculated from Standard curve (y=mx=c)

V = Volume of the solution,

m = amount of the sample in solution

3.9. Measurement of Chemical oxygen demand (COD)

COD vials were used to measure the COD of the samples before and after the treatment. The measuring range of the COD vials lies from 25-1500mg/L. To measure the COD, the effluent was diluted with distilled water to lower the COD up to 1500mg/L.

Procedure

Take 3ml of the diluted sample and pour it into the COD vials using pipette by allowing the sample to run down through the walls of the vial. The screw cap was tightly fixed and mix the contents present in vial vigorously until it was heated. After mixing the COD vial was placed in a reactor at 148◦c for 2 hours. The hot vial was removed from the reactor and placed in a test tube rack for cooling for about 10-30 min without the use of water. The measurement was carried out in Spectro quant.

3.10. Phytotoxicity Experiment

This bioassay was performed to detect the toxicity of the wastewater before and after the treatment of wastewater. The procedure for this experiment is described as below.

Procedure

The wastewater which is used for the detection of the phytotoxicity was first filtered by using 0.4µm thin filter paper. This experiment is performed on the petri plates having 90mm in size by using the filter paper of equal size to fit inside the petri plate, then about 4mm of the sample, which needs to be detected was placed on the filter paper until it is completely wet. About 10 wheat seeds were placed on the wet filter

paper and lid was closed. There were about 3 dishes per sample to detect the phytotoxicity. To prevent the evaporation and transfer of volatile components between the treatment each of these plates were wrap in the polyethylene bag. This experiment was performed at room temperature 25◦c over the period of about 5 days, performed in the darkness and 3 types of samples were used such as tap water as control, untreated sample, and treated sample. (Saadi, Laor et al. 2007)

3.11. Cytotoxic assay

Cytotoxicity of treated pulp and paper effluent was determined using brine shrimp lethality test (BST) by following protocol described by Meyer, with slight modifications (Meyer et al., 1982). The procedure for this experiment is described as below.

Procedure

17g of sea salt and 3mg yeast extract was dissolved in 500ml distilled water to make artificial sea water. Water was then shifted to a container and brine shrimp eggs (*Artemia salina*) were added to it. The eggs were then incubated for 48 hours under continuous illumination (100-Watt lamp) to hatch. After 48 hours, the nauplii were seen to be released from the eggshells and were rapidly moving in the container. Nauplii were collected using a micropipette and were transferred to glass tube (10 nauplii per glass tube) containing artificial seawater and different concentrations of prepared bacterial metabolic extract ranging from 20µl – 100µl. Cytotoxic effect of extracts was observed after 24 hours by counting number of alive and dead nauplii. Nauplii were termed as alive if they exhibited any movement during 15 seconds of observation. Mitomycin-C was used as positive control and black liquor was taken as negative control. The experiment was repeated three times and mean values were taken.

4. Results

This research work was performed in the Applied, Environmental, and Geo microbiology Laboratory, Quaid I Azam University Islamabad. The basic purpose of this research was the treatment of Black liquor from Pulp and paper mill industry, Lahore, Kasoor, Pakistan. The Indigenous strains of fungi isolated from paper mill effluents were used in this research.

4.1. Isolation of Fungal Strains from Pulp and Paper Mill Effluents

During the research work, black liquor samples were processed collected from different collection points from Pulp and Paper mill. These collection points were Inlet point (IP), Aeration tank 1 (AT1), Aeration tank 2 (AT2), Black liquor (BL), Secondary sedimentation tank (SST), Final sedimentation tank (FST). Based on our research work Black liquor (BL) sample were proceed. 2 fungal strains were isolated which are shown in the table below.

Table 7: Shows fungal strains isolated from black liquor samples.

Sr. No	Samples	Fungal isolates
1.	BL	02

4.2. Screening of The Lignin Degrading Fungal Isolates

This experiment was performed to determine the ability of fungal strains to degrade lignin. These strains were grown on the lignin amended MSM media containing only lignin as sole source of carbon. The lignin was provided in different concentrations such as 1g/l – 2.5g/l. The incubation time for Fungal strains was 30°C for 14 days. The results shown that these strains can grow at the L-MSM media, determine the capability to degrade lignin. These selected fungal strains (SST-1 and SL-6) were selected because they were equally capable of growing at 2.5g/l lignin concentration.

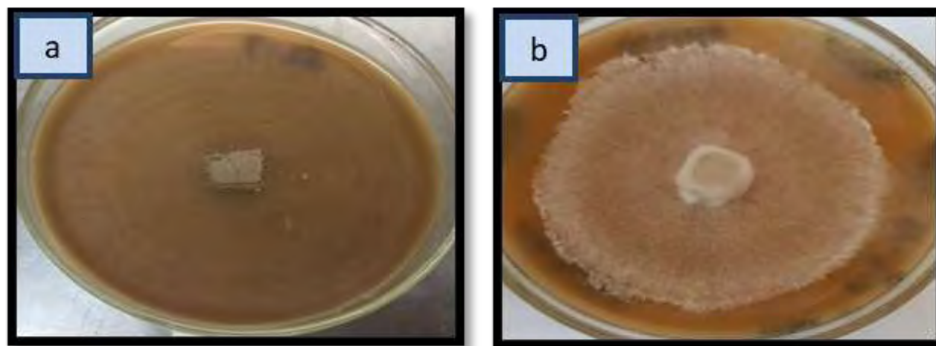

Figure 4: Shows Fungal strains (a) **SL-6** (b) **SST-1** growth on L-MSM containing lignin in different concentrations.

4.3. Plate assay for Ligninolytic enzymes

The plate assays were performed for the identification of Fungal strains to screen for ligninolytic enzymes. This screening was performed by using different indicators such as guaiacol, Crystal Violet and Azure-B were provided in the petri plates.

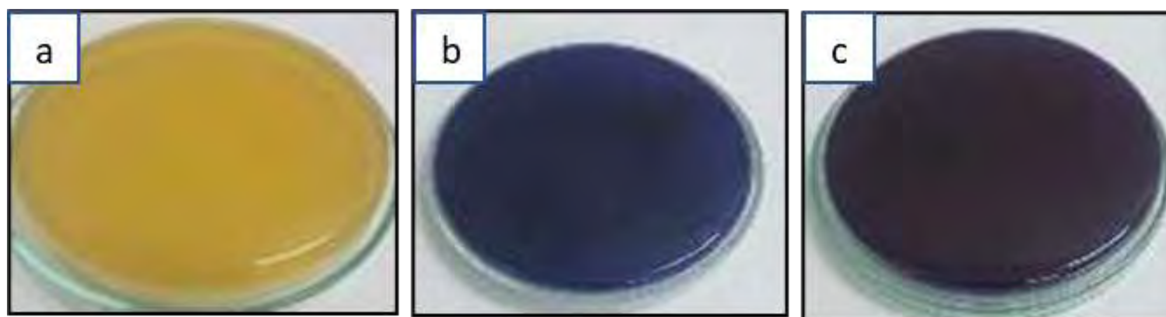

Figure 5: Shows **(a)** Laccase Plate assay **(b)** Azure-B Plate assay **(c)** Crystal violet plate assay before culturing.

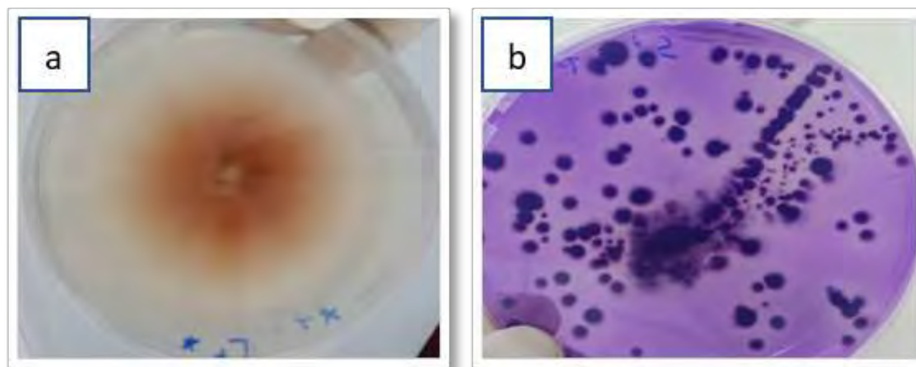

Figure 6: Appearance of brown color around growth on guaiacol agar plates show laccase by fungal strain (a) SST-1 and (b) SL-6 Fungal strains on minimal salt medium plate containing Crystal violet dye shows clear zone around colonies indicates the production of lignin peroxidase.

4.4. Quantitative assay for Ligninolytic Enzymes Production

The quantitative assay were performed for the identification of Fungal strains to screen for ligninolytic enzymes. Selected fungal strains were allowed to grow in minimal nutrient broth. for laccase, Guaiacol was used as a substrate MnP involved oxidation of guaiacol in the presence of MnSO4 and LiP involves oxidation of Azure B.

Figure 7: Quantitative assay for Ligninolytic Enzymes Production after culturing.

The production of laccase enzyme by SST-1 rose to a maximum after 8 days of incubation, at 9th day maximum 5.36819 U/ML and then decreased, so Laccase enzyme

activity was found on 9th day. LiP enzyme production was investigated by the oxidizing property of LiP enzyme; it oxidizes Azure B in the presence of hydrogen peroxidase, the absorbance was read at 310nm. After the 10th day of incubation maximum 2.367662 U/ML LiP enzyme activity was found and then the activity decreases. MnP enzyme involves the oxidation of guaiacol in presence of MnSO4. The maximum 1.9112 U/ML MnP enzyme activity was found on day 7th.

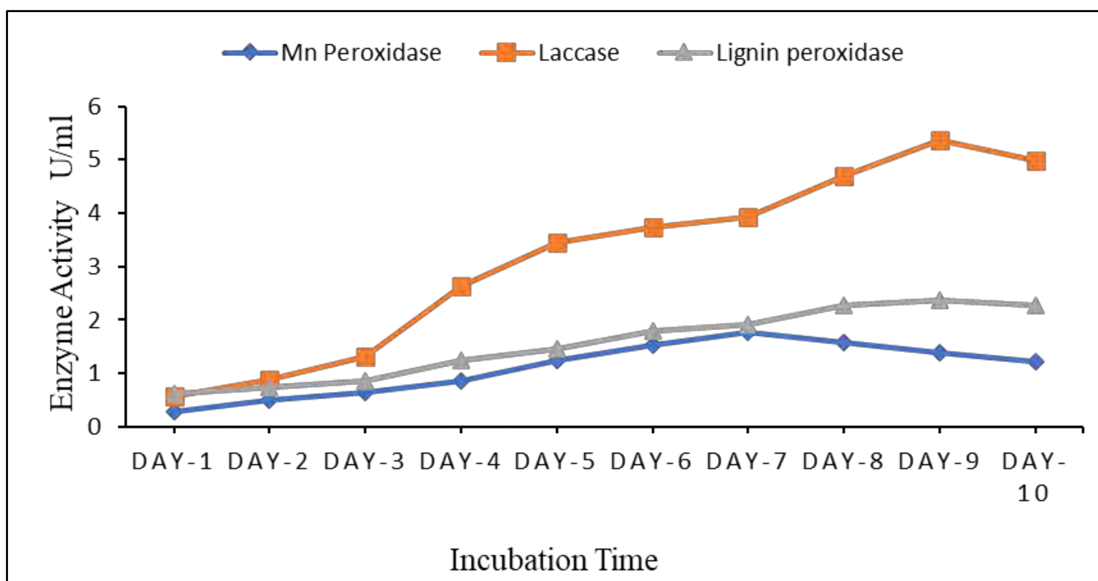

Figure 8: Production of manganese peroxidase, laccase, and lignin peroxidase by SST-1.

The ligninolytic enzyme production was significantly lower in SL-6 fungal strain as compared to SST-1 fungal strain. The maximum 2.78283 U/ML enzyme activity of laccase was found on day 8th of incubation and highest value 1.7112 U/ML enzyme activity of LiP was recorded. Manganese peroxidase maximum 1.36837 U/ML enzyme activity was observed at 7th day of incubation.

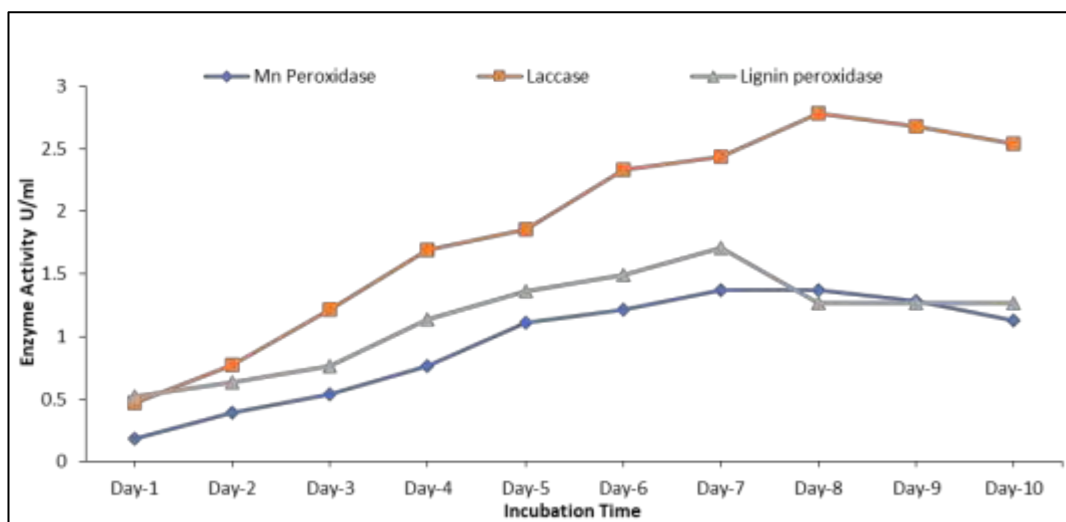

Figure 9: Production of manganese peroxidase, laccase and lignin peroxidase by SL-6.

4.5. Morphology

Fungal isolates were grown on PDA plates and different culture characteristics were observed. SST-1 appeared as white soft, cottony mycelium demonstrates a spongy texture and covered the substrate as the time passes. SL-6 had a greenish powdery appearance and produces many small, airborne spores.

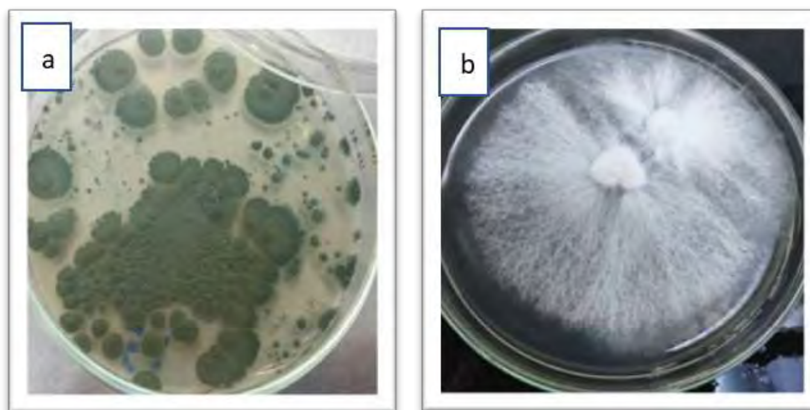

Figure 10: Growth of Fungal strains on PDA media (a) SL-6 (b) STT-1.

4.5.1. Microscopy

The cellular morphology of fungal strains SST-1 and SL-6 was observed under microscope through lactophenol cotton blue staining. The microscopic analysis revealed fungal strain SST-1 has aseptate and branched hyphae. The hyphae are very tiny and cannot be seen with naked eyes. A microscopic view of SL-6 reveals that it has smooth colored conidiophores and conidia. The conidiophores are protrusions from a septate and unbranched hypha. The conidial heads appeared radial, and they were split into columns.

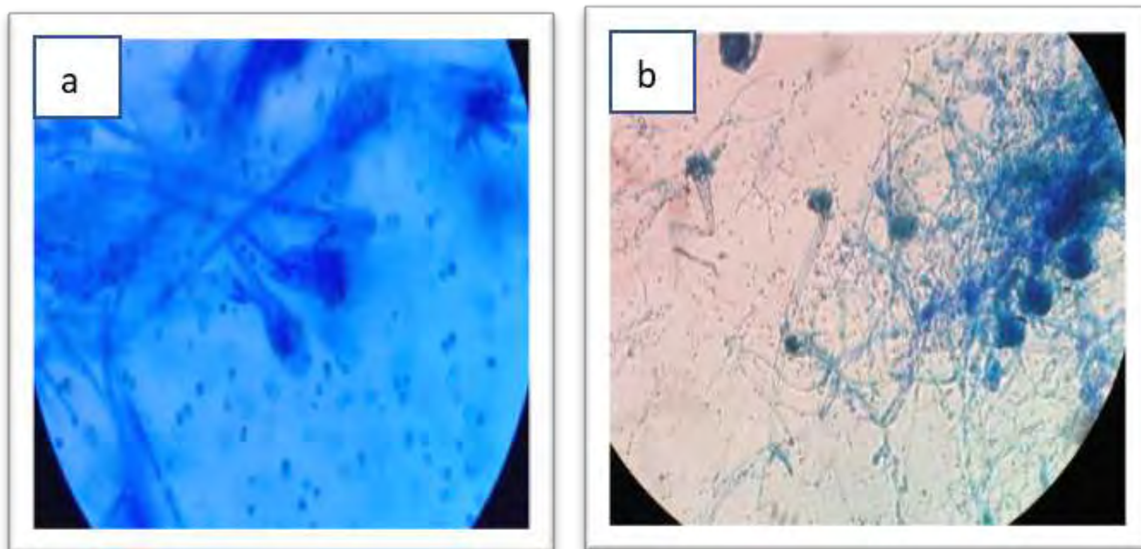

Figure 11: Microscopic Identification of fungal strains (a) SL-6 and (b) SST-1 with lactophenol cotton blue staining under 100X.

4.6. Molecular identification

The amplification of extracted DNA was carried out through PCR and then PCR products were sent for sequencing. For homology search, the final sequences were subjected to blasting on NCBI. From NCBI the similar sequences were downloaded. By using MEGA6 software the genomic elements sequences were arranged in alignment and then method of Neighbor- Joining method was used in order to construct phylogenetic tree. To estimate Significance of produced tree, the 1000 bootstraps replicates were used.

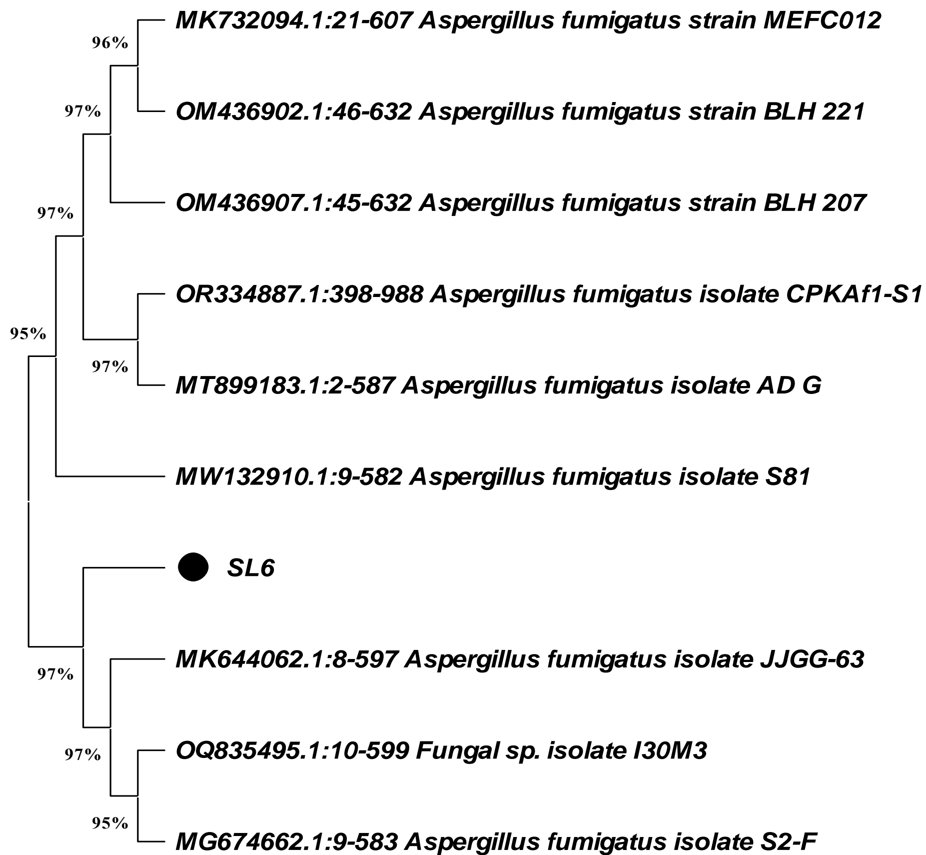

Figure 12: Phylogenetic tree of *Aspergillus fumigatus* SL-6 by Neighbor-Joining method constructed using MEGA-X software.

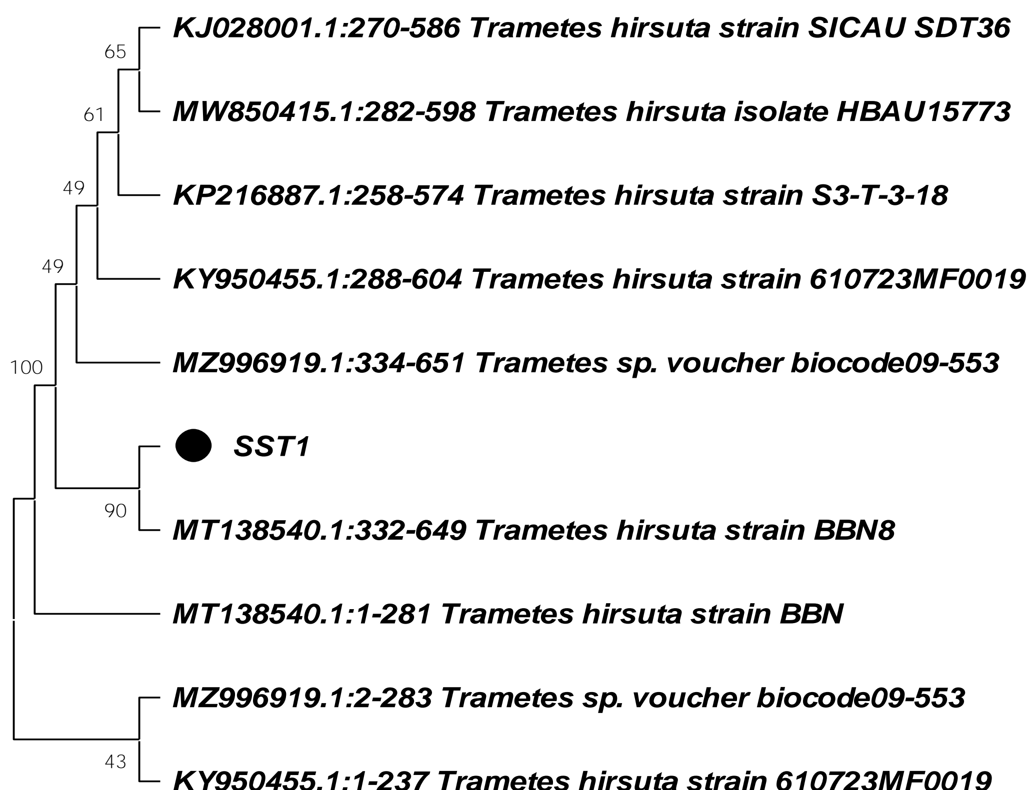

Figure 13: Phylogenetic tree of *Trametes hirsuta* SST-1 by Neighbor-Joining method constructed using MEGA-X software.

4.7. Optimization of different culture conditions for Fungal strains

For optimization, we perform different experiments in the AEG Laboratory to determine suitable conditions for the best degradation of lignin by these bacterial and fungal strains.

4.7.1. Effect of different lignin concentration on lignin reduction and color reduction

To optimize different lignin concentration, we performed experiment by using different concentrations of lignin such as 1g/l, 1.5g/l, 2g/l and 2.5g/l. The experiment was carried out at flask level containing Lignin amended MSM. Time for this experiment was 12

days at 35°C temperature under continuous agitation where daily analysis of the sample was performed to determine the best degradation results. Control was prepared for each condition for comparison.

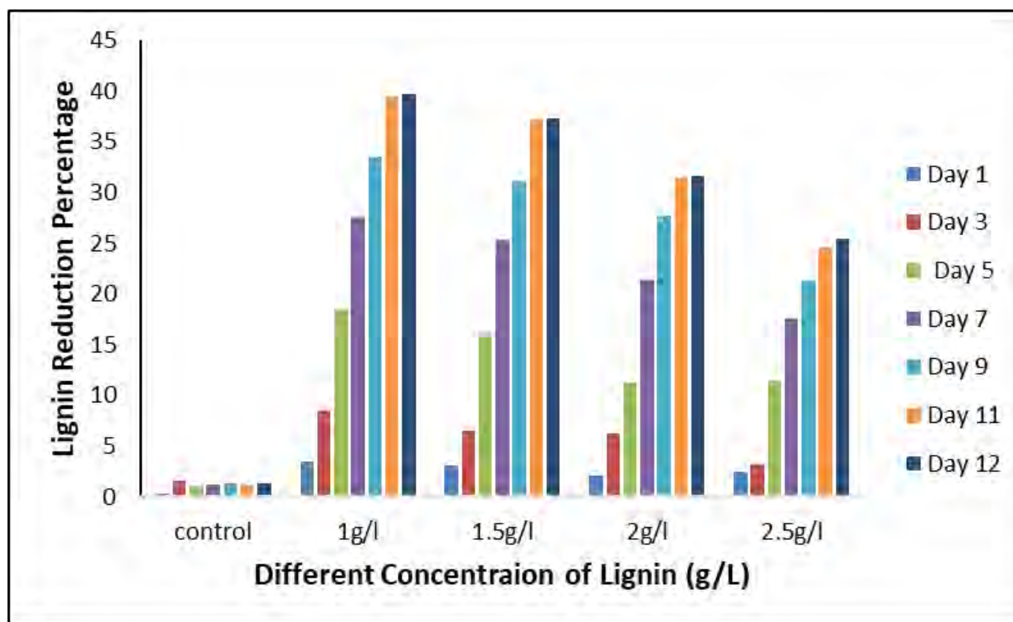

Figure 14: Evaluation of lignin reduction (%) at different conc. of Lignin (g/L) by *Aspergillus fumigatus* SL-6.

Thus, *Aspergillus fumigatus* SL-6 shows maximum lignin reduction of 39% at 1g/l. The % color reduction of 46% in case of *Aspergillus fumigatus* SL-6 was observed by adding 3 ml of phosphate buffer in 1ml of supernatant which is shown in graph below.

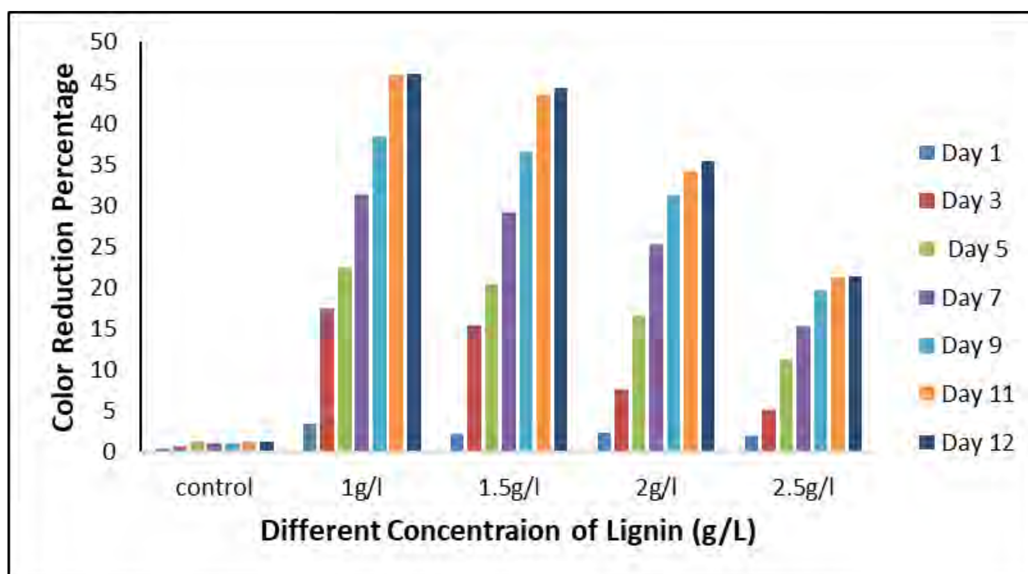

Figure 15: Evaluation of color reduction (%) at different conc. of Lignin (g/L) by *Aspergillus fumigatus* SL-6.

The following are the trends for the lignin and color reduction of SST-1 at different concentration of Lignin. SST-1 shows maximum lignin reduction of 44% at 1.5g/l concentration of lignin and 51% was color reduction at 1.5g/l.

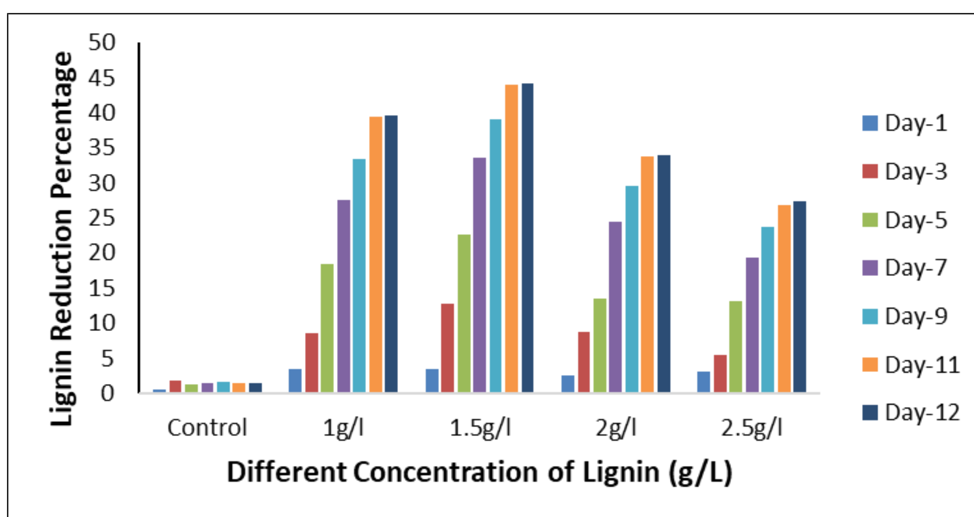

Figure 16: Evaluation of lignin reduction (%) at different conc. of Lignin (g/L) by *Trametes hirsuta* SST-1.

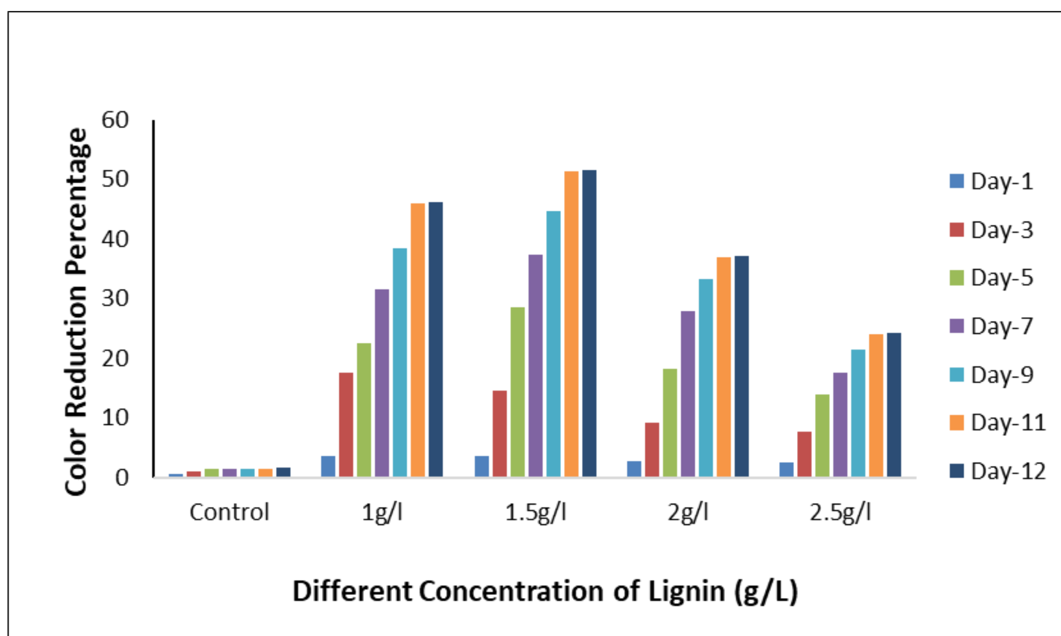

Figure 17: Evaluation of color reduction (%) at different conc. of lignin (g/L) by *Trametes hirsuta* SST-1.

4.7.2. Effect of pH on lignin and color reduction

Different pH conditions were used for determining the value of optimized conditions such as pH 4 to pH 10. Control was prepared for each condition for comparison. All the flasks were incubated at 35°C under continuous agitation for 12 days. The pH optimized for this condition was 6 the results of which are shown below for both fungal strains.

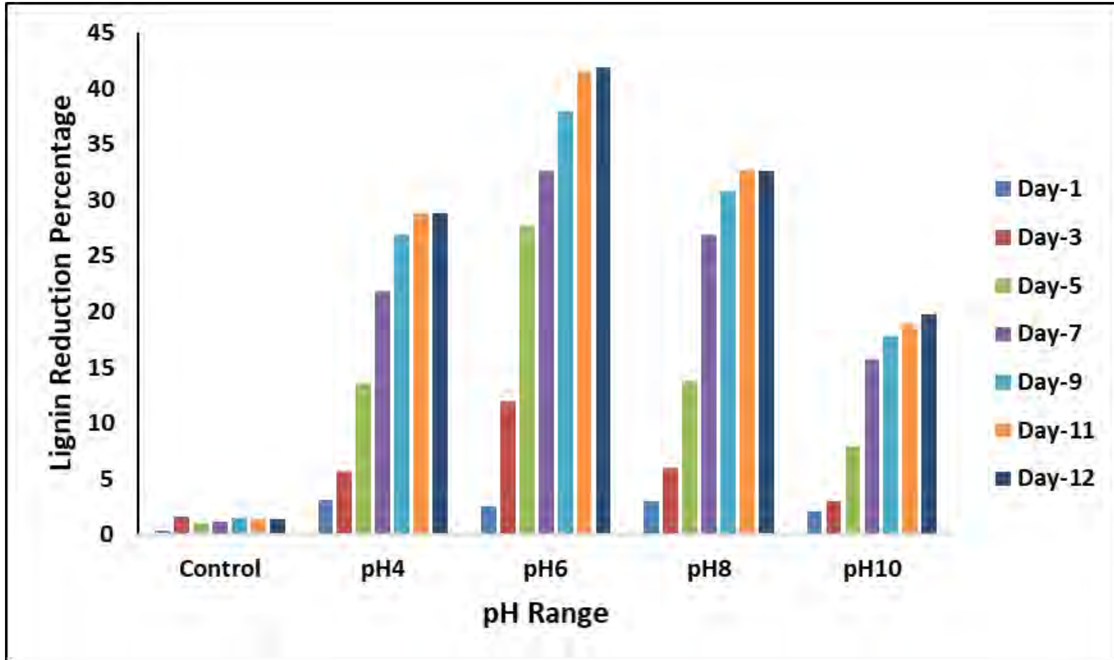

Figure 18: Effect of different pH on lignin degradation by *Aspergillus fumigatus* SL-6.

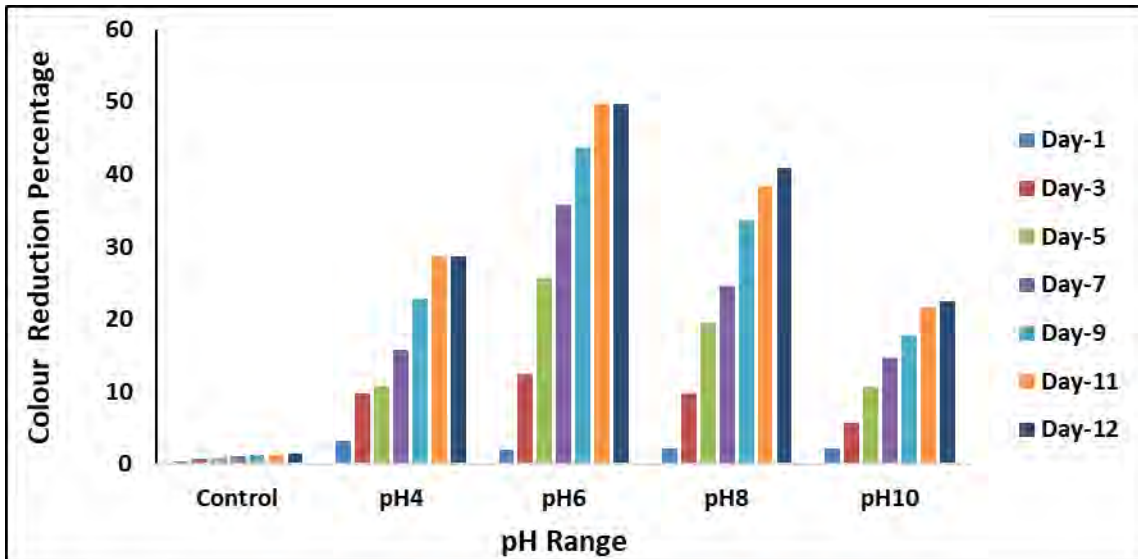

Figure 19: Effect of different pH on color reduction by *Aspergillus fumigatus* SL-6.

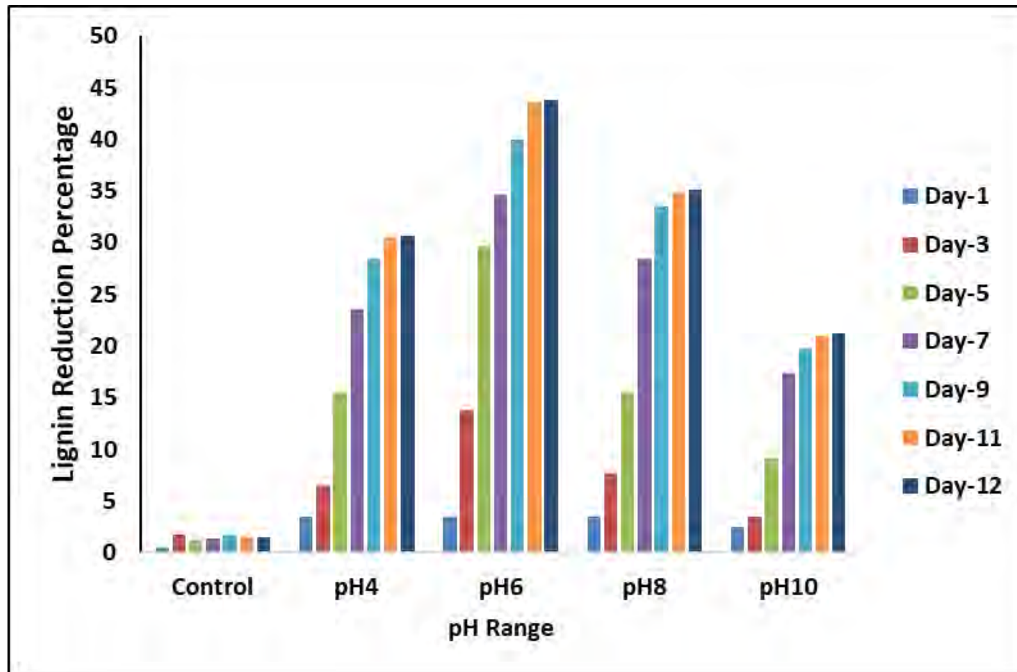

Figure 20: Effect of different pH on lignin degradation by *Trametes hirsuta* SST-1.

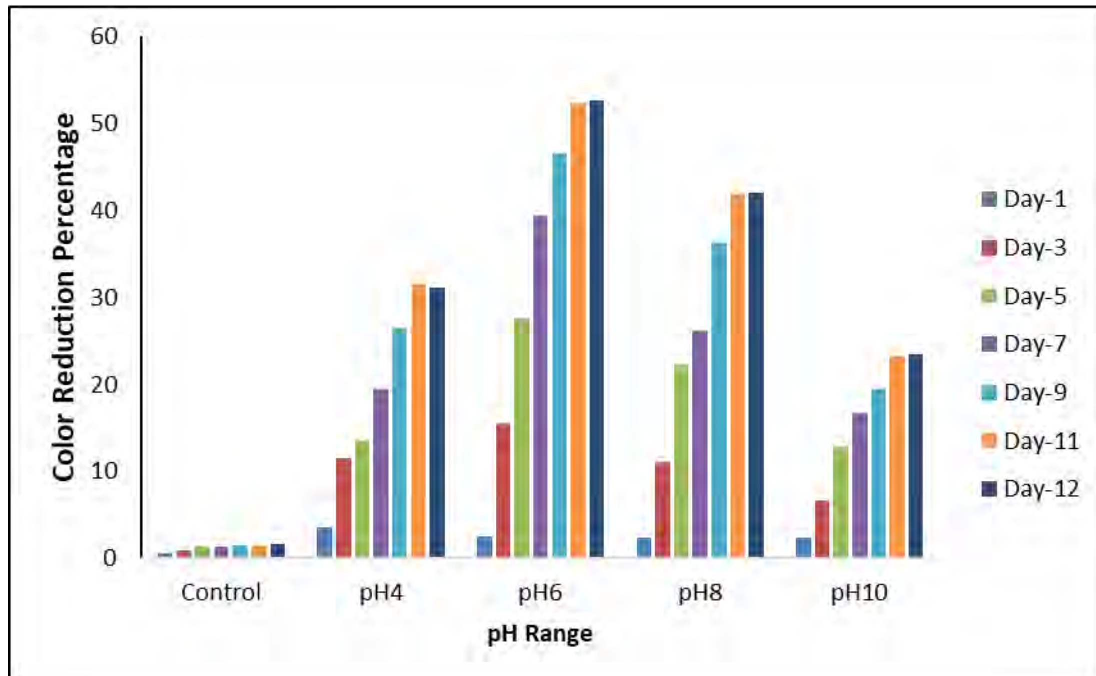

Figure 21: Effect of different pH on color reduction by *Trametes hirsuta* SST-1.

4.7.3. Effect of incubation temperature on lignin reduction and color reduction

Optimization of temperature was performed by using different temperature conditions such as 25°C, 30°C, 35°C, and 40°C. Control was also prepared for each value of Temperature. For lignin reduction and color reduction optimum temperature was 30°C for both fungal strains.

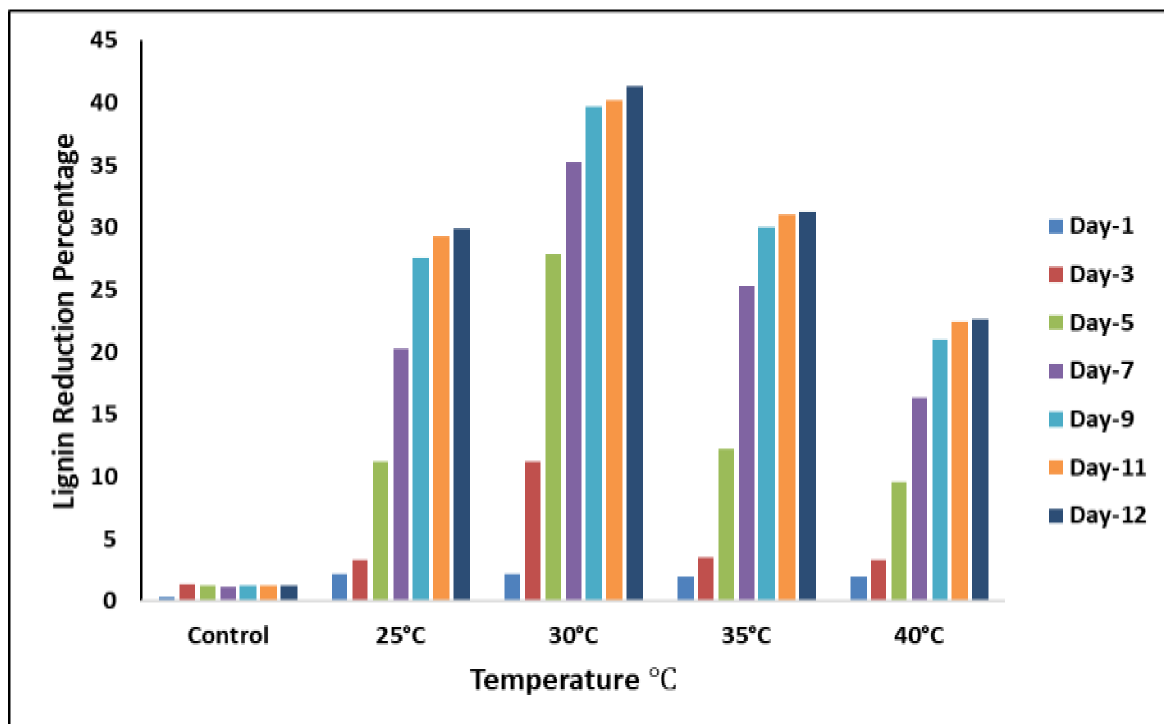

Figure 22: Effect of different temperature on lignin degradation by *Aspergillus fumigatus* SL-6.

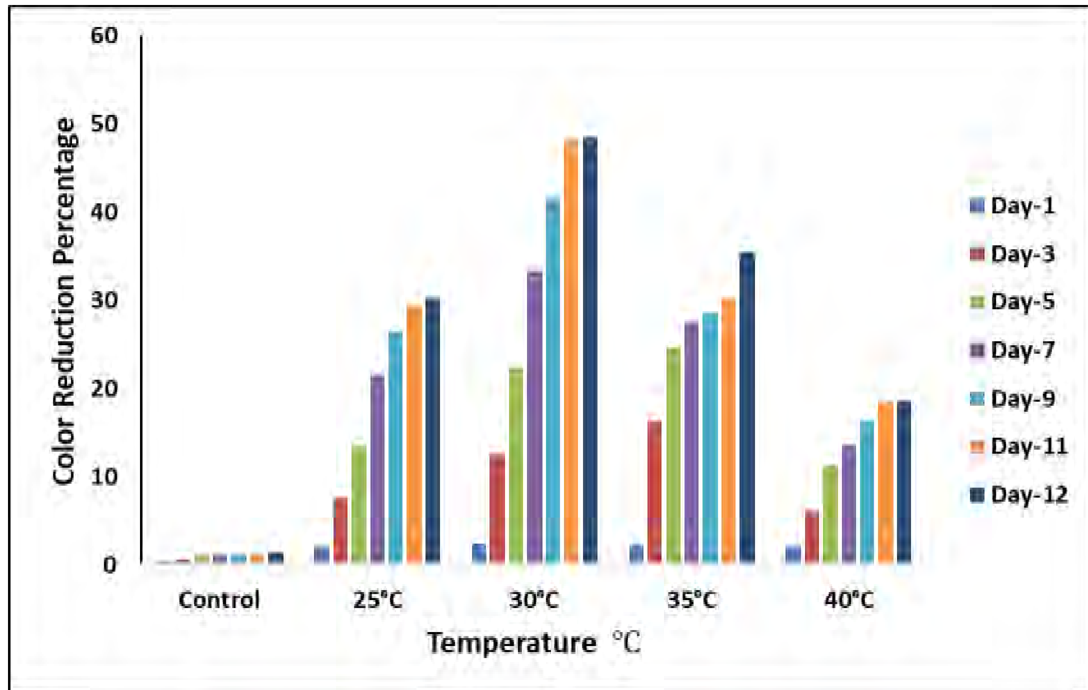

Figure 23: Effect of different temperature on color reduction by *Aspergillus fumigatus* SL-6.

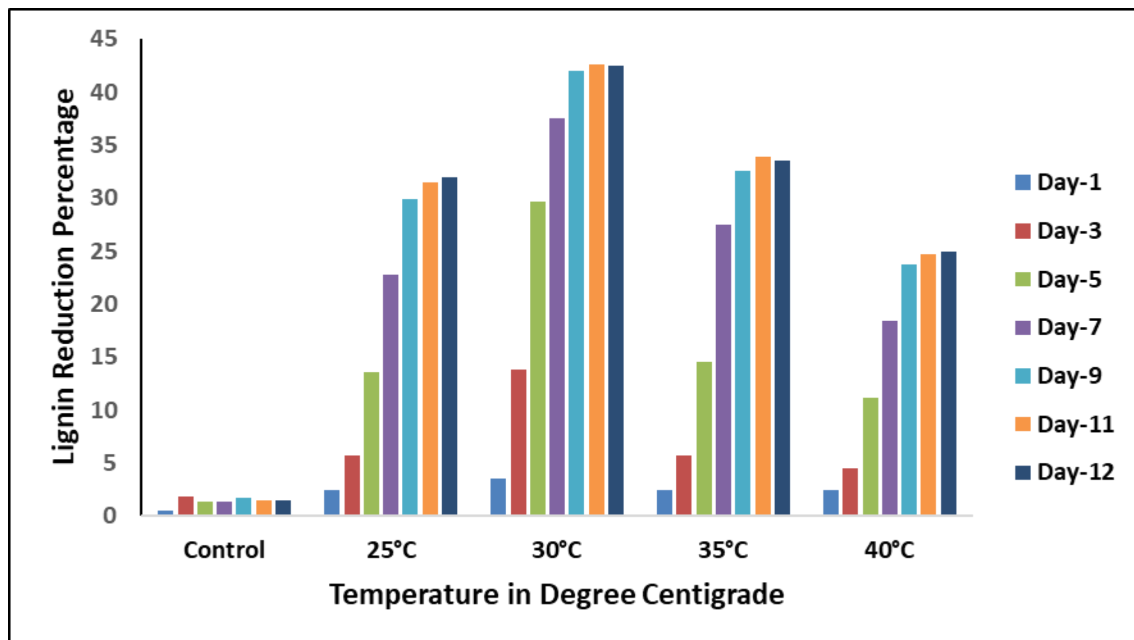

Figure 24: Effect of different temperature on lignin degradation by *Trametes hirsuta* SST-1.

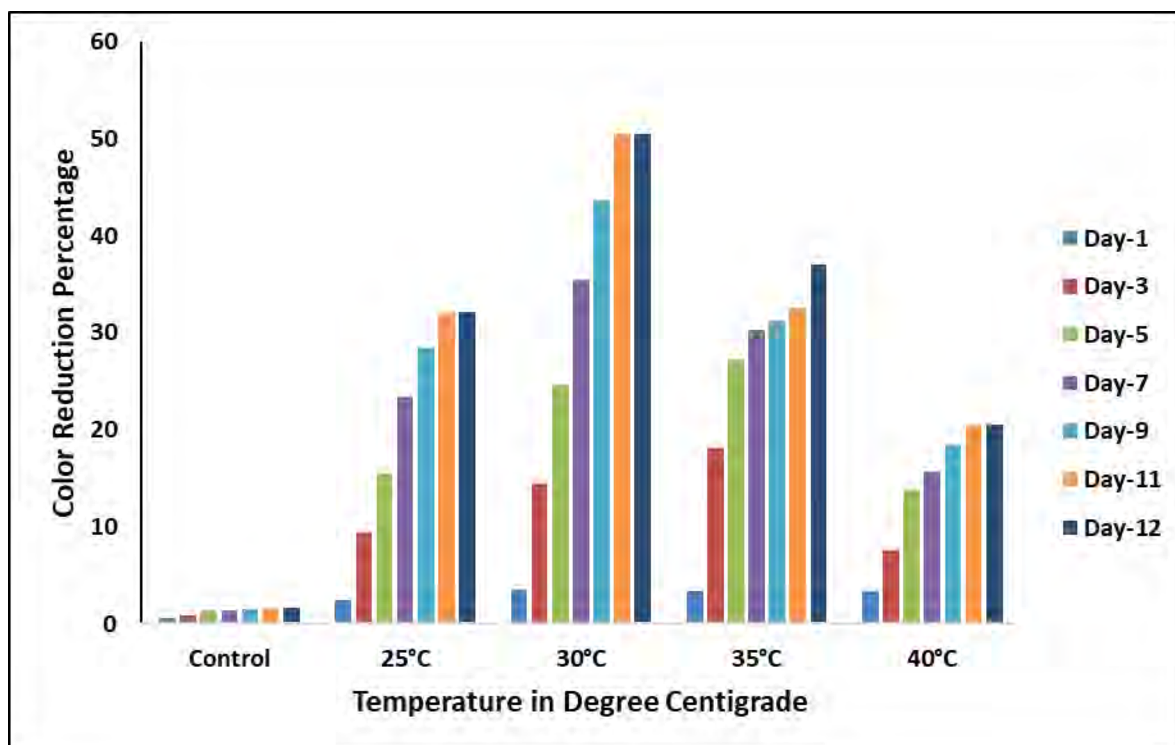

Figure 25: Effect of different temperature on color degradation by *Trametes hirsuta* SST-1.

4.7.4. Effect of carbon sources on lignin and color reduction

A classical method of optimization was followed, varying parameters one by one in a series of experiments and maintaining the previously optimized at constant level. The effect of different carbon and nitrogen sources were studied. Additional carbon sources (glucose, dextrose, cellulose, sucrose and starch) were investigated on lignin reduction and color reduction.

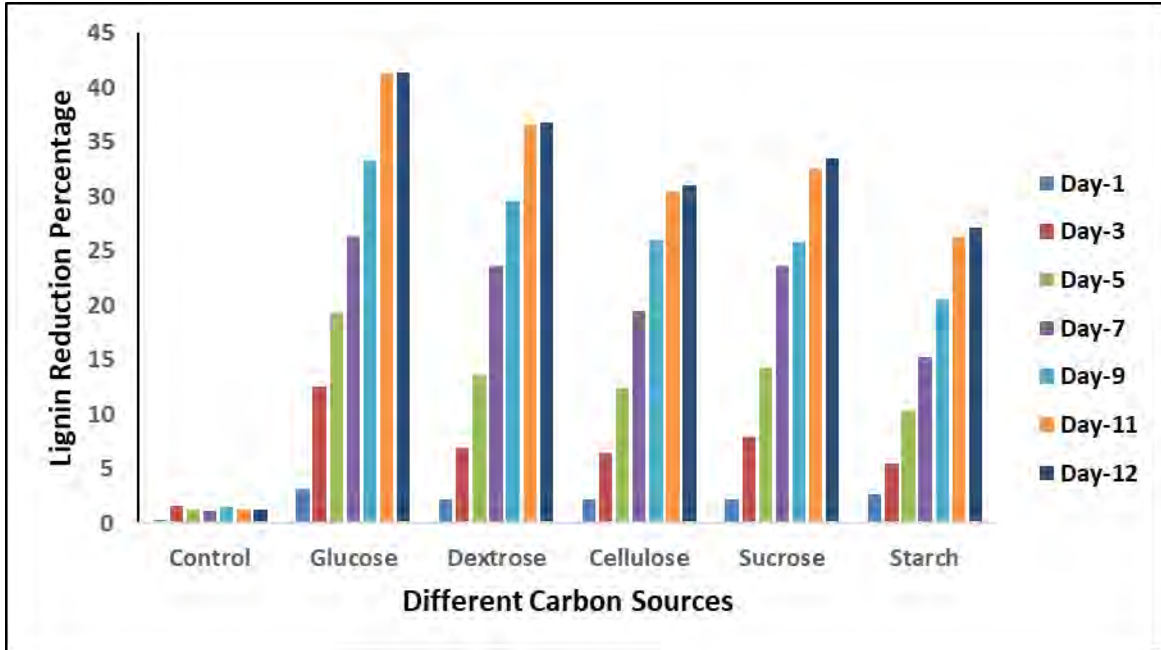

Figure 26: Effect of different carbon sources on lignin degradation by *Aspergillus fumigatus* SL-6.

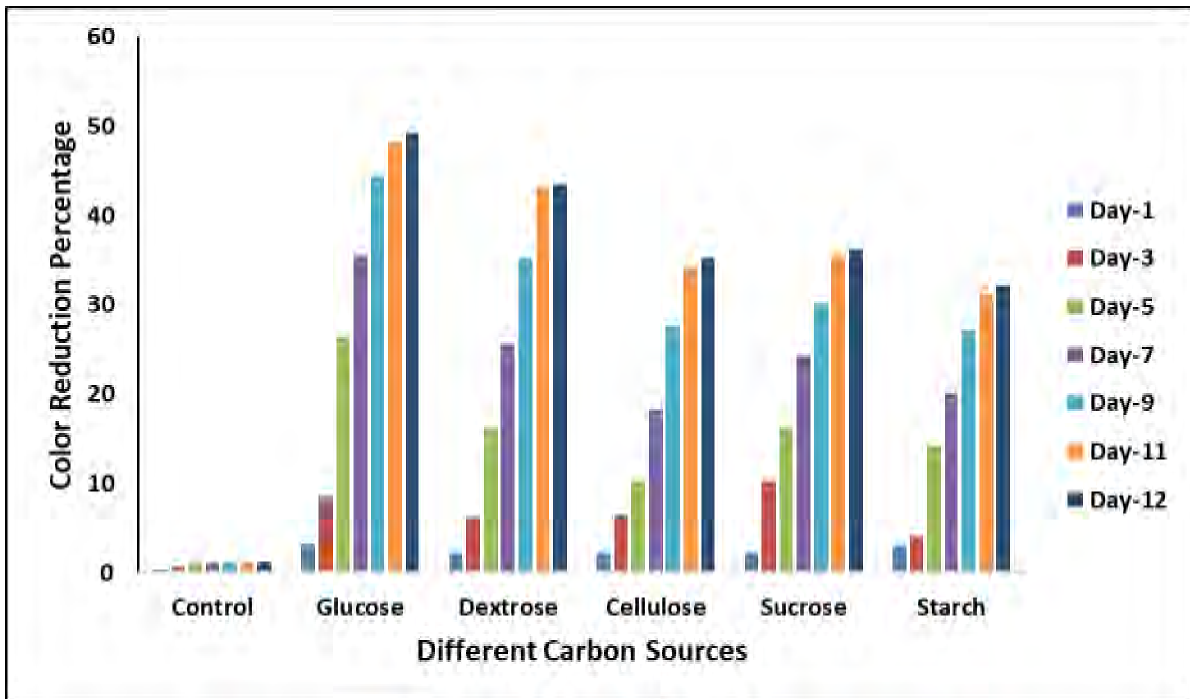

Figure 27: Effect of different carbon sources on color reduction by *Aspergillus fumigatus* SL-6.

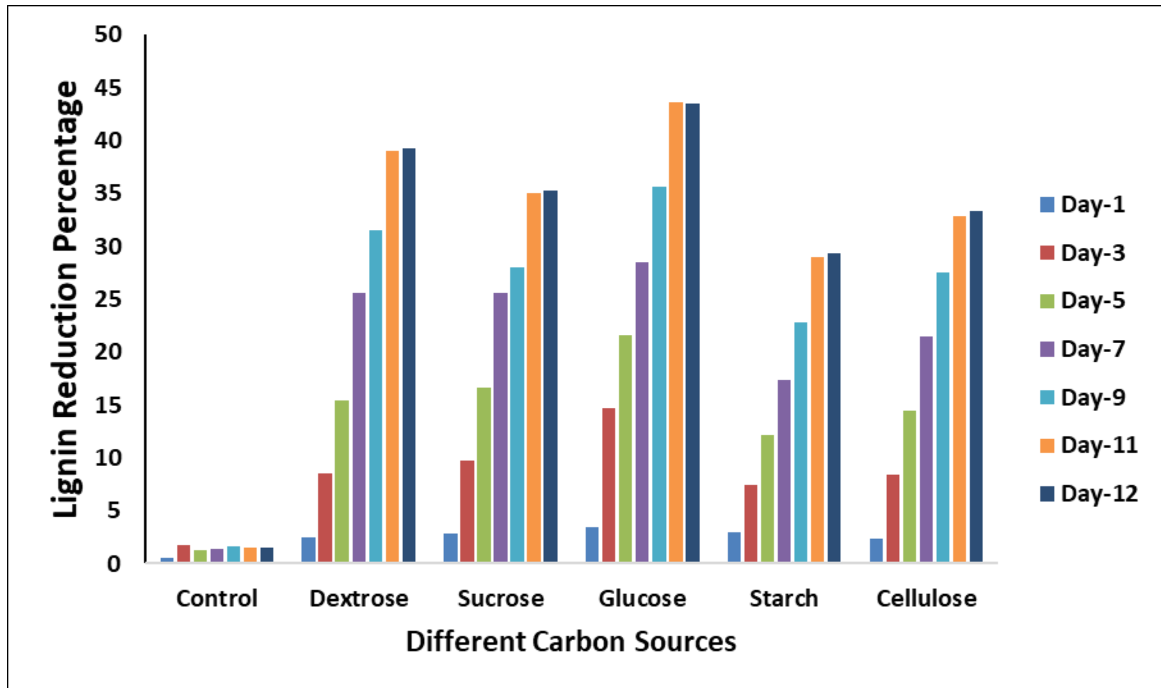

Figure 28: Effect of different carbon sources on lignin degradation by *Trametes hirsuta* SST-1.

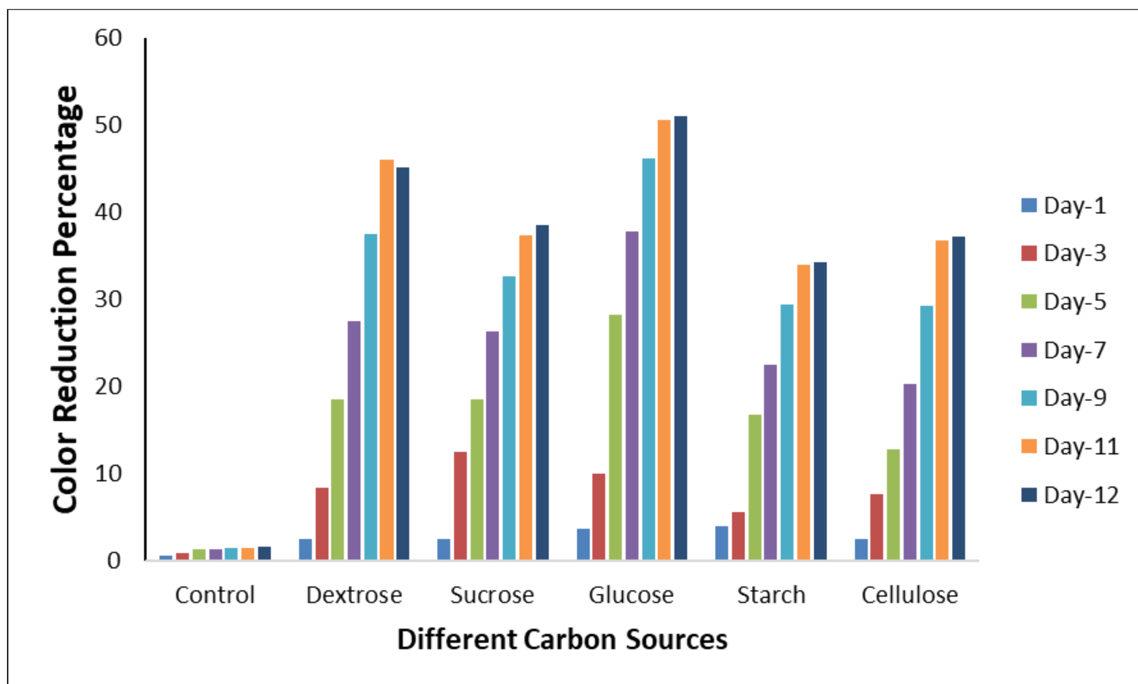

Figure 29: Effect of different carbon sources on color reduction by *Trametes hirsuta* SST-1.

4.7.5. Effect of nitrogen sources on lignin and color reduction

For process parameter optimization like nitrogen, different nitrogen sources were tested peptone, ammonium sulfate, yeast, urea and casein. All the flasks were incubated at 35°C under continuous agitation for 12 days.

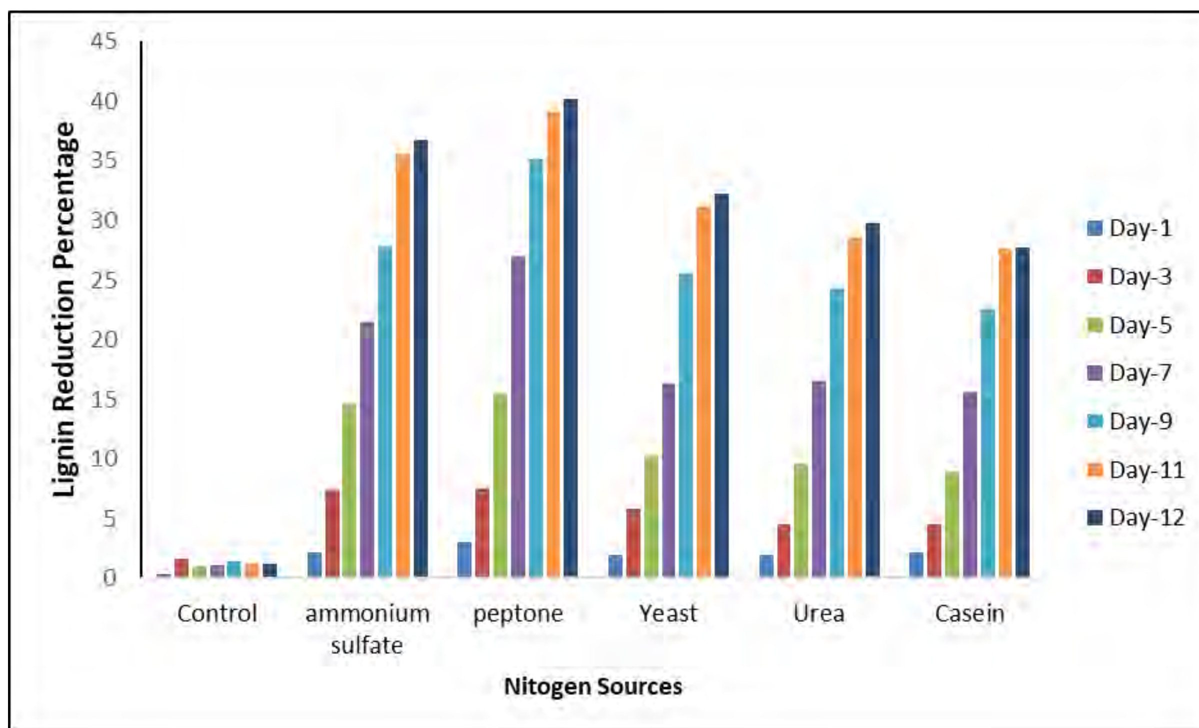

Figure 30: Effects of different nitrogen sources on lignin degradation by *Aspergillus fumigatus* SL-6.

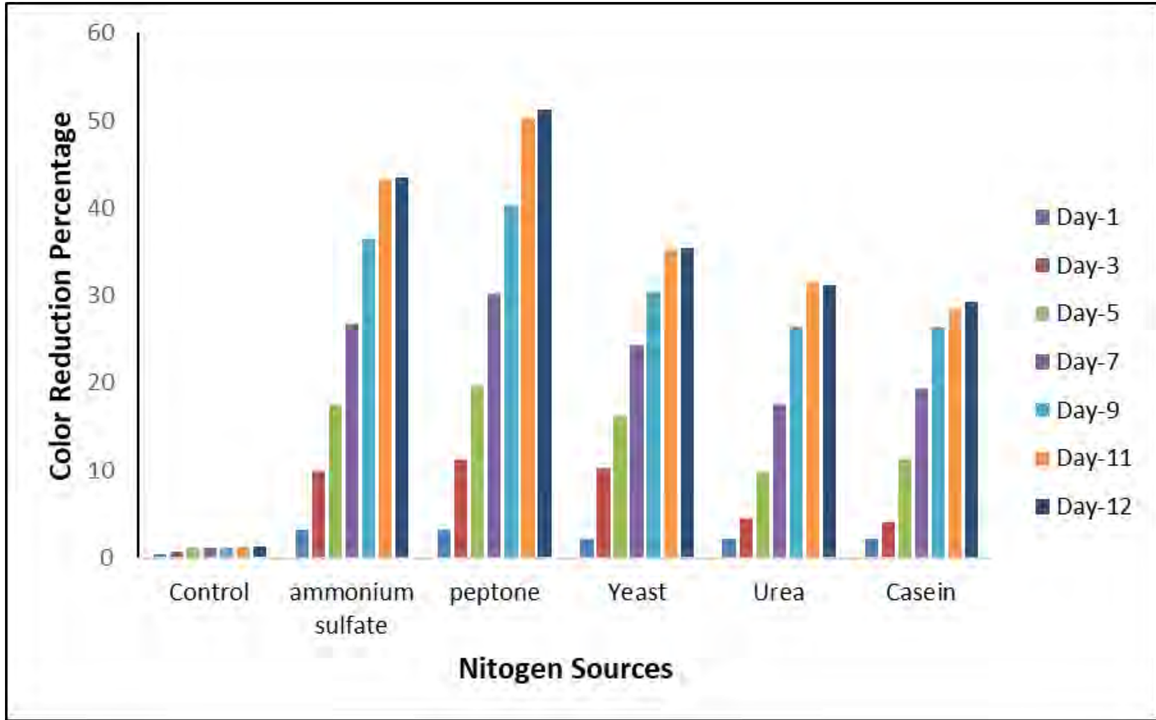

Figure 31: Effect of different nitrogen sources on color reduction by *Aspergillus fumigatus* SL-6.

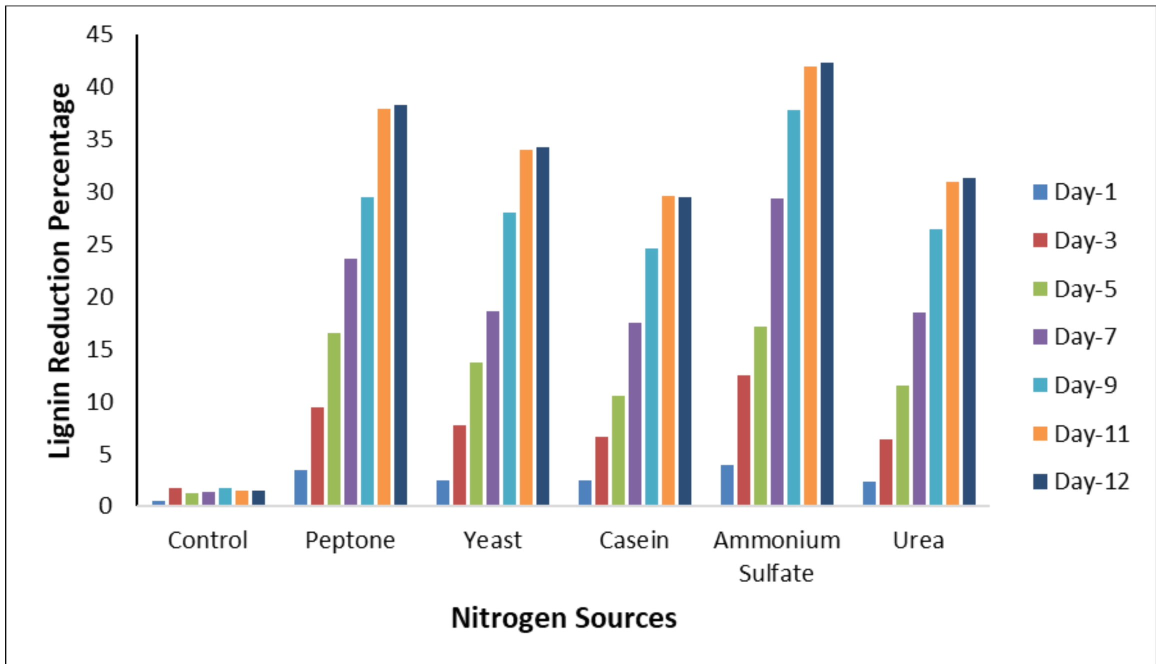

Figure 32: effect of different nitrogen sources on lignin degradation by *Trametes hirsuta* SST-1.

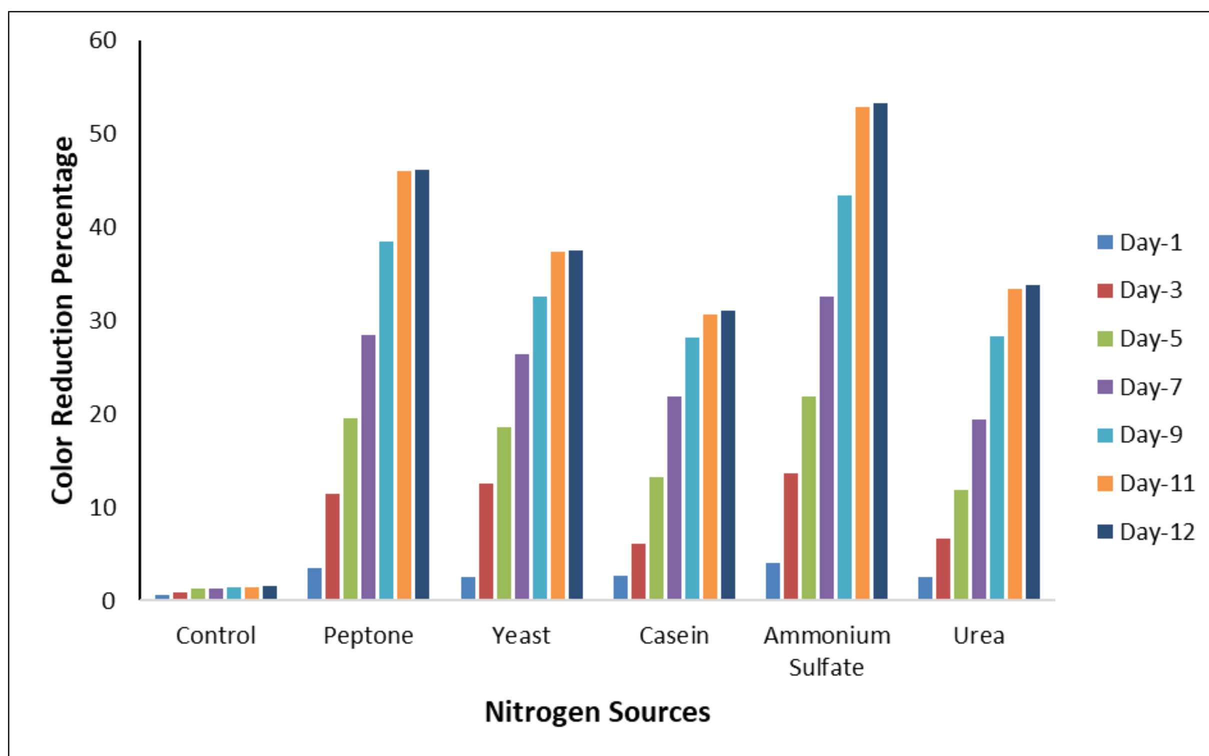

Figure 33: Effect of different nitrogen sources on color reduction by *Trametes hirsuta* SST-1.

4.8. Bioreactor

The sequencing batch reactor (SBR) was used in this study consisting of four chambers of 45cm in width and 30 cm in length, made up of Pyrex glass, containing outlet at the base of each chamber, where effluent is shifted from one reactor to another after processing. The pipes at the outlet were stopped with the help of valve to prevent the outflow of effluent during treatment.

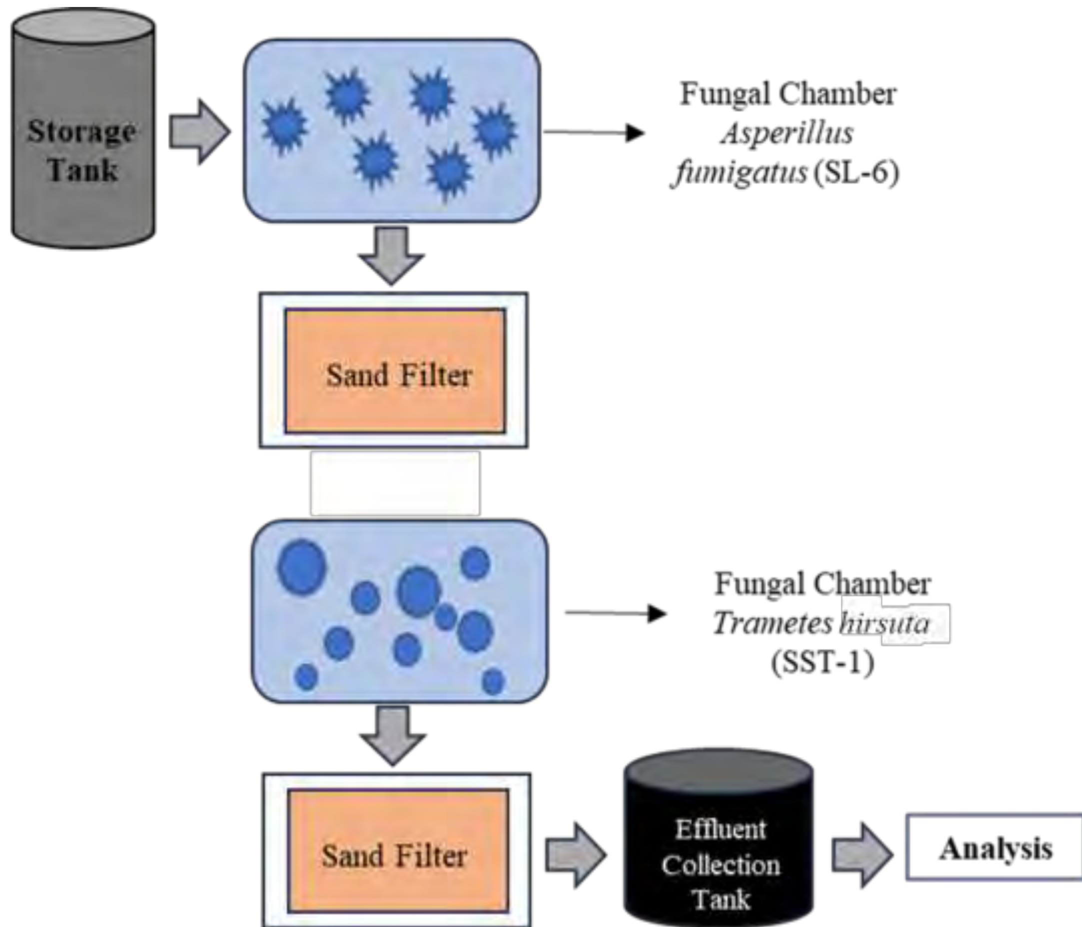

Figure 34: Schematic diagram of Lab-scale sequential bioreactor for paper and pulp effluent treatment.

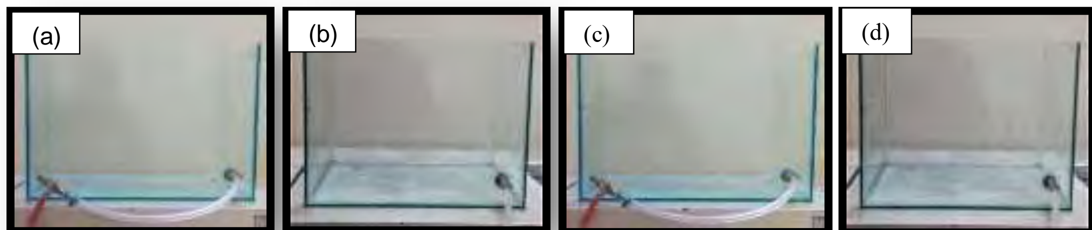

Figure 35: Bioreactor before Experiment. (a) Fungal chamber (b) Sand filter (c) fungal chamber (d) Sand filter.

4.8.1. Experimental setup

During the experiment, we run the reactor by using optimized conditions of lignin concentration, pH, glucose as carbon source, ammonium sulfate as nitrogen source and 5% BL conc., pH 6, and 30℃ temperatures.

4.8.2. Bioreactor Cycle(first)

During the first trial the reactor was run with 5% BL, pH 6, glucose as carbon source, ammonium sulfate for *Trametes hirsuta* SST-1 and peptone for *Aspergillus fumgiatus* SL-6 as nitrogen source and 30℃ temperature. Aquarium heater was used to adjust the temperature as at that time room temperature was not suitable for the reactor. The bioreactor was set in order to determine the reduction of different components in BL sample. In first reactor both fungal strains were separately evaluated.

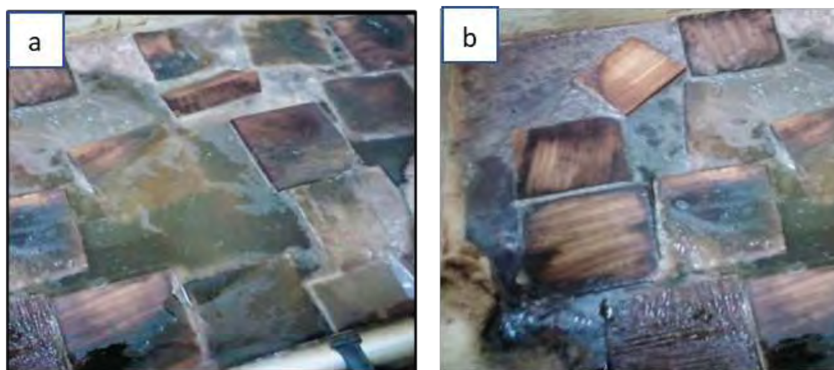

Figure 36: Shows Biofilm formation on wood chips (a) fungal Biofilm *Trametes hirsuta* SST-1 (b) Fungal biofilm *Aspergillus fumigatus* SL-6.

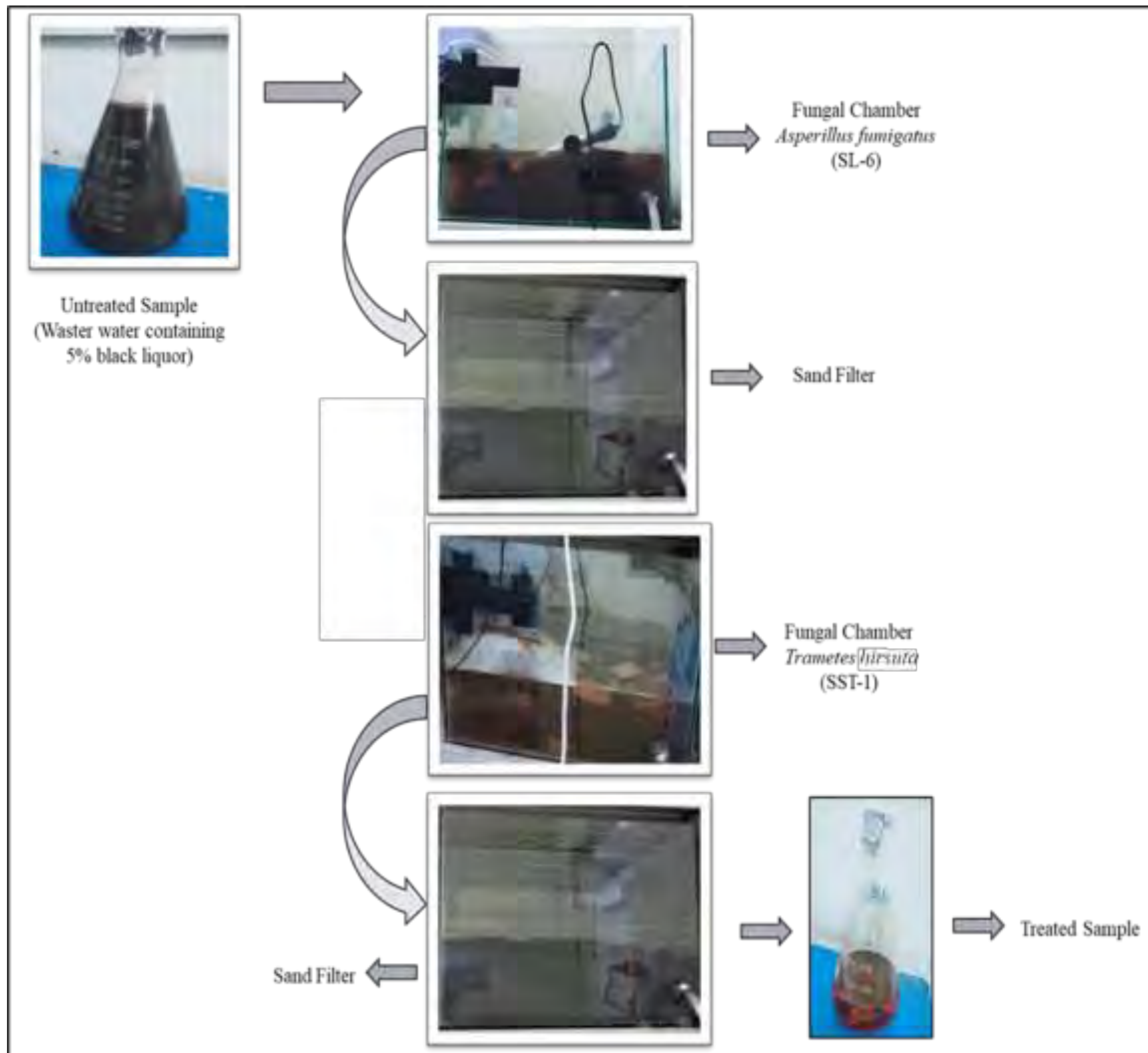

Figure 37: Shows Treatment of BL through fungal bioreactor.

The results of the first trial are given below:

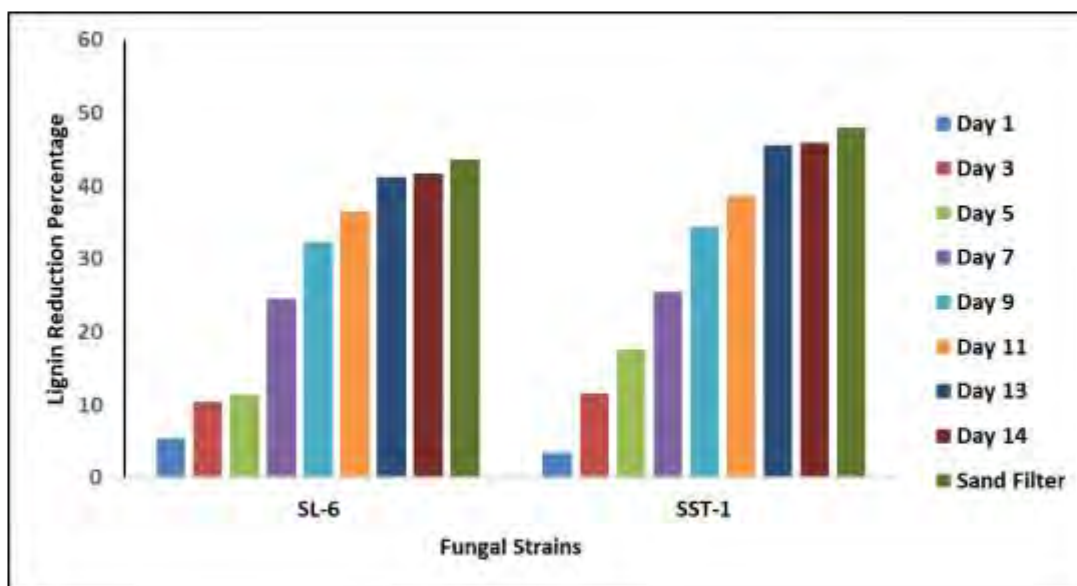

Figure 38: Lignin degradation of sample after treatment in Bioreactor (First Trial).

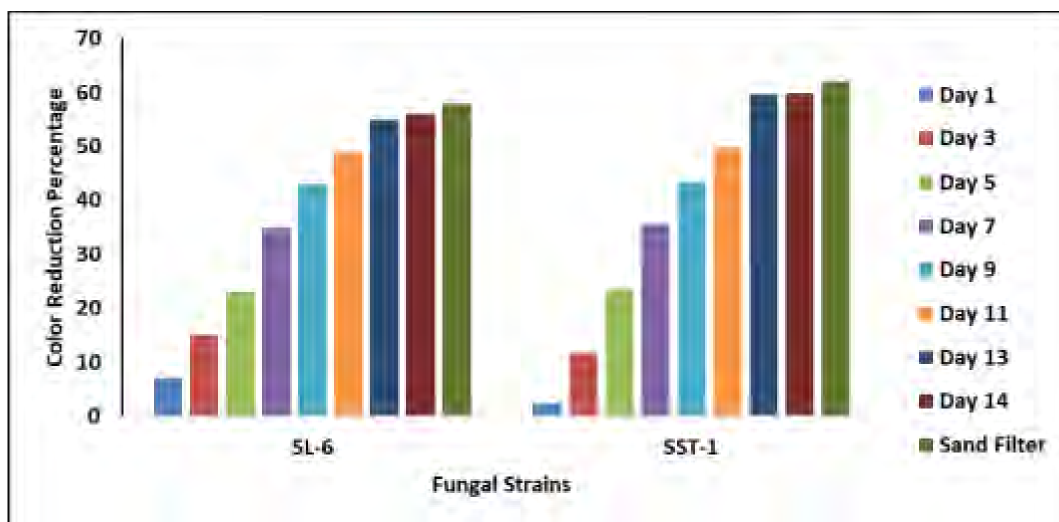

Figure 39: Color reduction of sample after treatment in bioreactor (First Trial).

Results of the second trial (consortium)

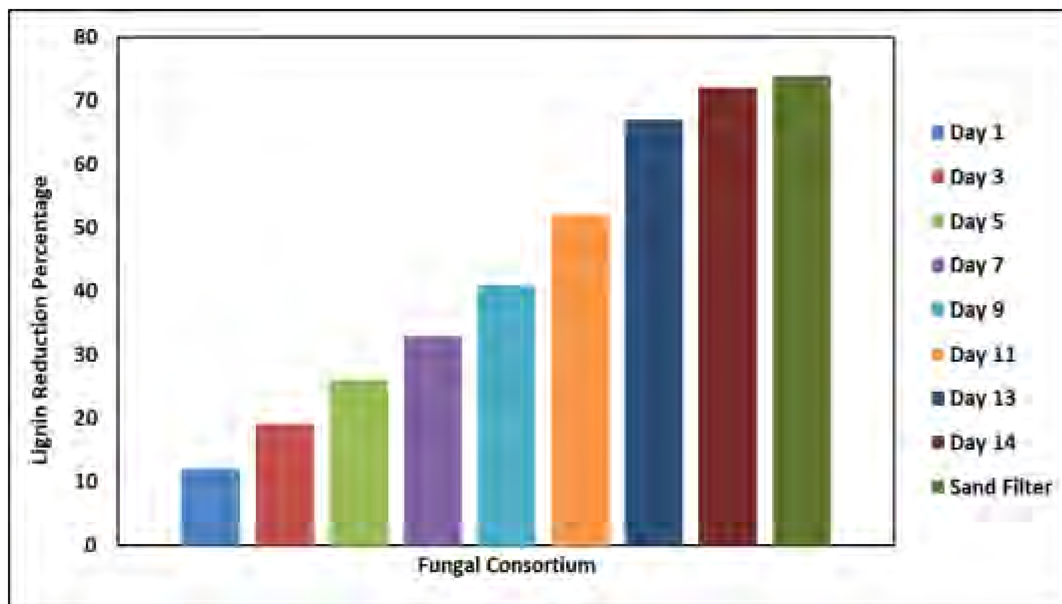

Figure 40: Lignin reduction (%) of sample after treatment in Bioreactor (Second Trial).

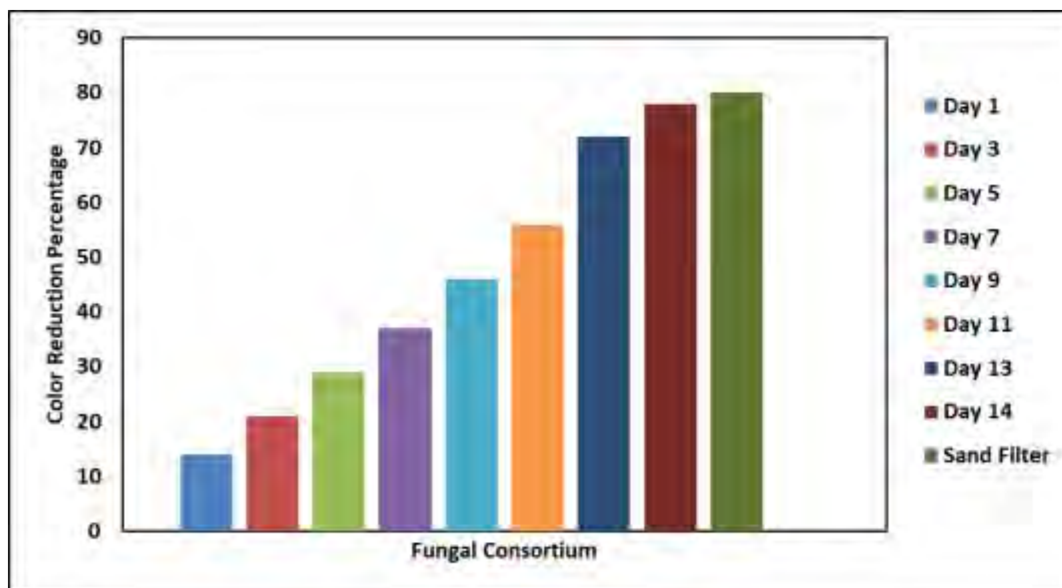

Figure 41: Color reduction (%) of sample after treatment in Bioreactor (Second Trial).

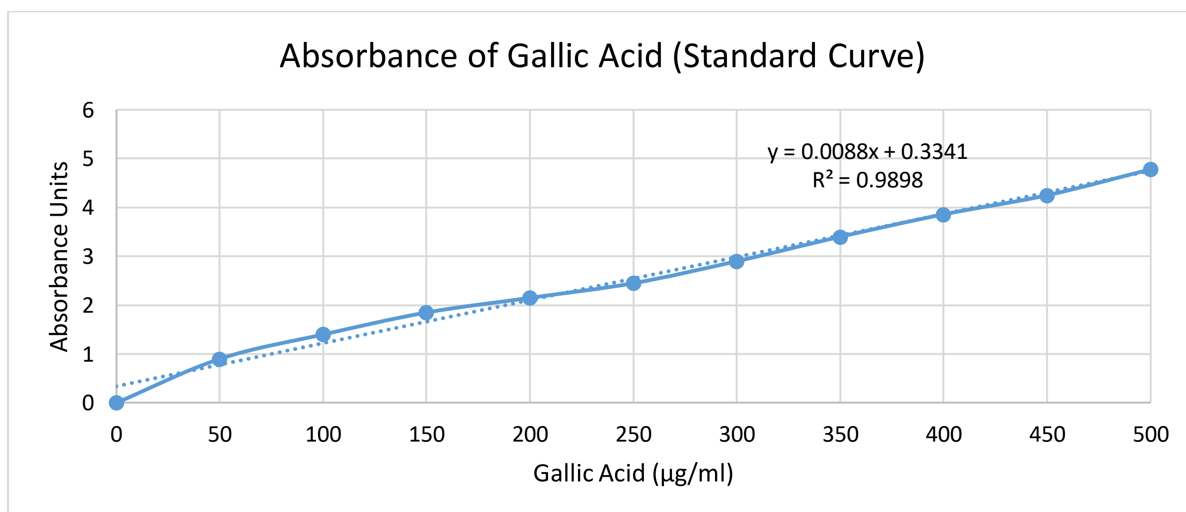

Figure 42: Gallic acid standard curve.

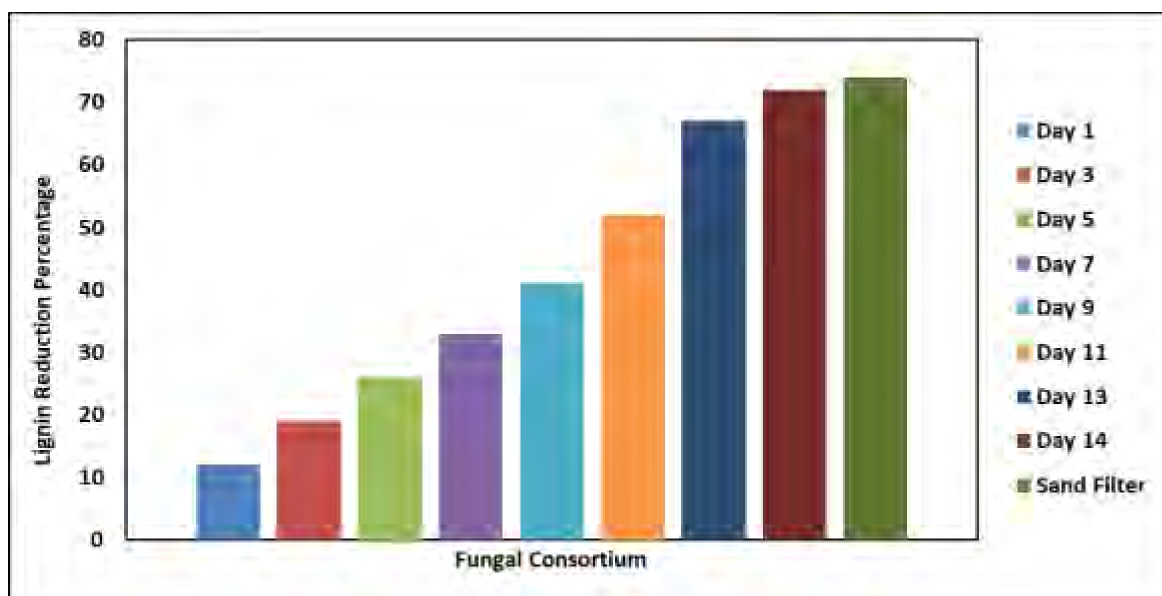

Figure 43: Shows phenolic contents reduction (%) after treatment in Bioreactor (First Trial)

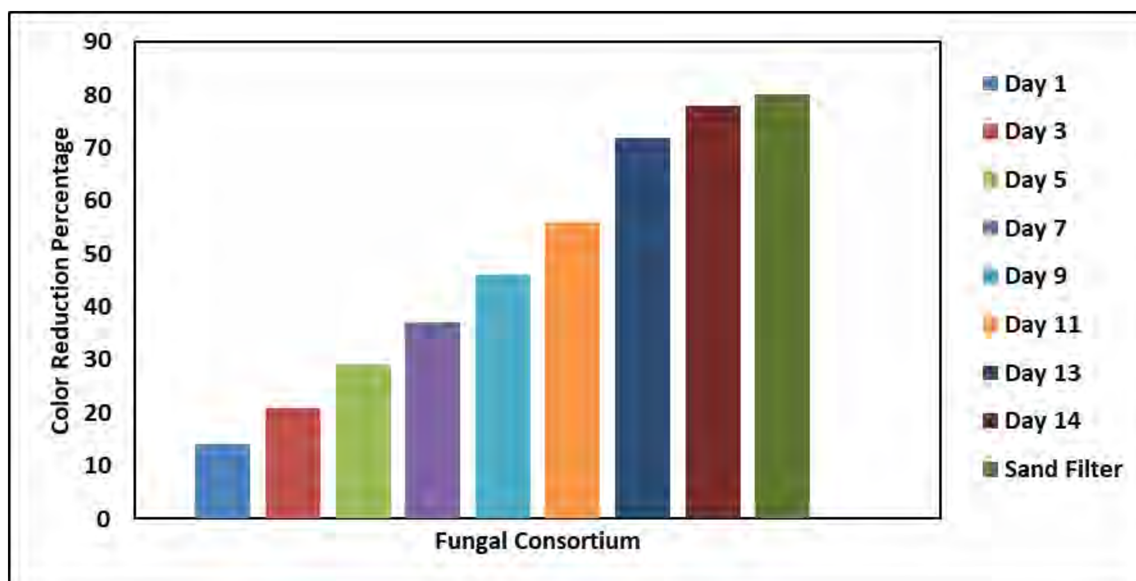

Figure 44: Shows phenolic contents reduction (%) after treatment in Bioreactor (Second Trial).

Following are the results of chemical oxygen demand COD

Table 8: showing the values of COD (First Trial)

COD	**Values**	
	5% Black Liquor	
	Aspergillus fumigatus (SL-6)	Trametes hirsuta (SST-1)
Initial COD	1278 mg/l	1245 mg/l
COD value after treatment for 7 days in Bioreactor having Fungal Strain	947 mg/l	746 mg/l
COD value after treatment for 15 days in Bioreactor having Fungal Strain	697 mg/l	512 mg/l

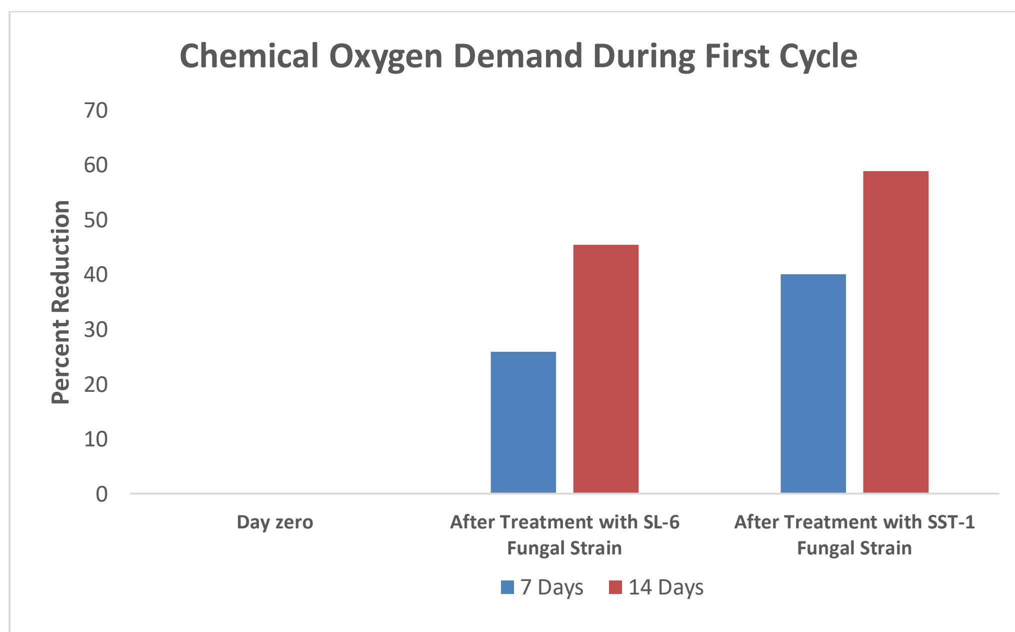

Figure 45: Shows reduction of COD (%) of sample after treatment in Bioreactor (First Trial).

Table 9: Showing the values of COD (Second Trial).

COD	**Values** 5% Black Liquor Fungal Consortium
Initial COD	1267 mg/l
COD value after treatment for 7 days in Bioreactor having Fungal Strain	632 mg/l
COD value after treatment for 15 days in Bioreactor having Fungal Strain	411 mg/l

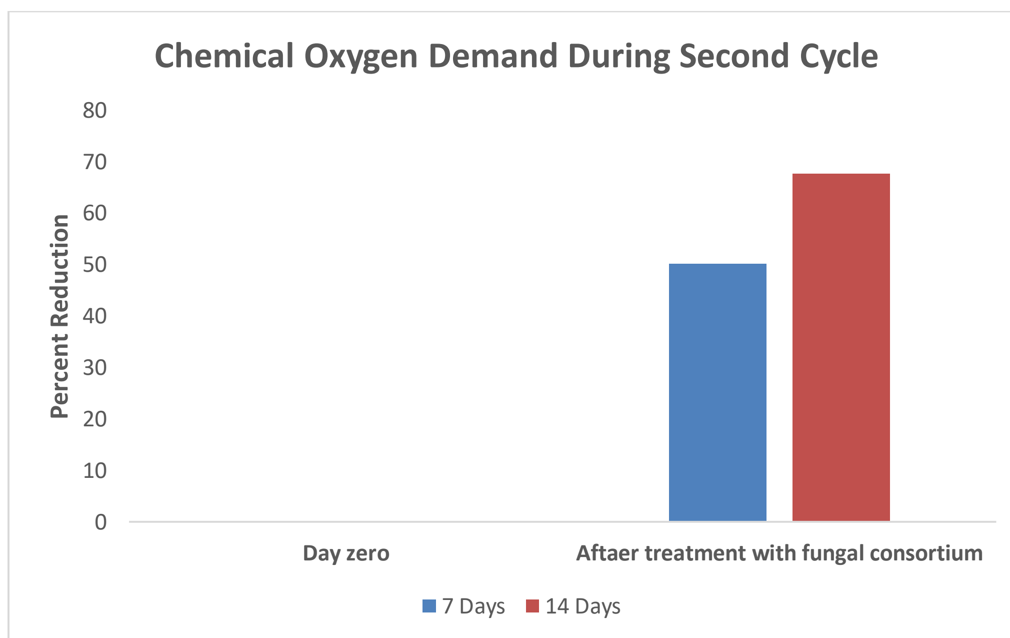

Figure 46: Shows reduction of COD (%) of sample after treatment in Bioreactor (Second Cycle).

4.9. Phytotoxicity

4.9.1. Seed Germination

The Seed Germination test was performed to check toxicity of untreated black liquor and treated black liquor. For this 5% black liquor was subjected to treatment by *Trametes hirsuta* SST-1 and *Aspergillus fumigatus* SL-6 for 5 days. The seeds 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. After the growth the result was obtained in such a way the root and shoot length was measured by the help of scale.

Table 10: Shows the phytotoxicity of sample with Tap water, Untreated and Treated water samples with 5%Black liquor Concentration (First Trial)

Growth	Tap Water	Untreated Sample (BL)	Treated Sample	
			***Aspergillus fumigatus* (SL-6)**	***Trametes hirsuta* (SST-1)**
Average Root Length	7cm	2cm	6cm	6.5cm
Average Shoot Length	2.5cm	0.5cm	2.6cm	3.2cm
Number of Germinated Seeds	10/10	5/10	8/10	9/10
Germination Rate	100%	50%	80%	90%

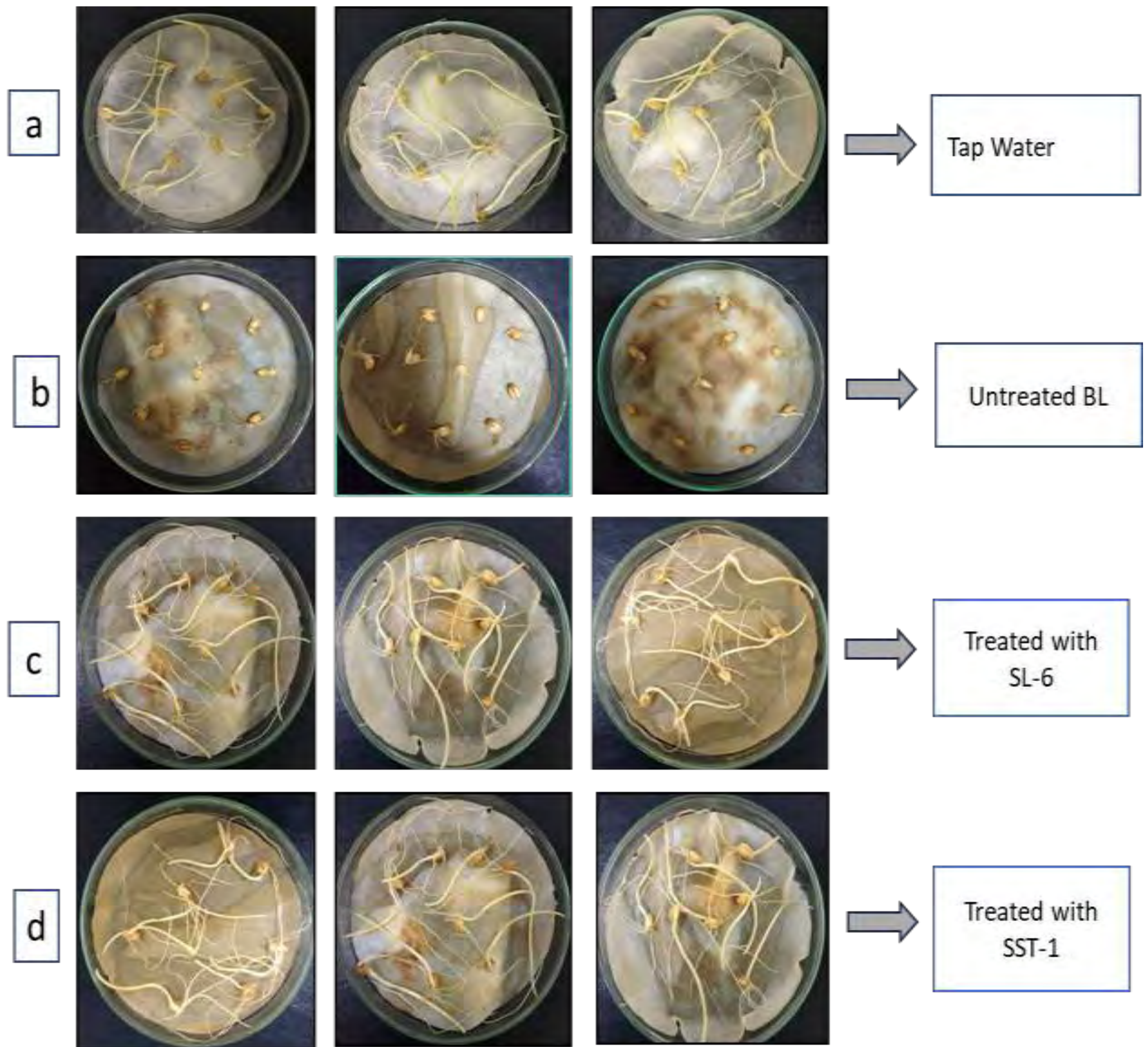

Figure 47: Phytotoxicity of Black Liquor (a), (b) Before Treatment and (c), (d) After Treatment in First Bioreactor Trial, with 5% BL.

4.9.2. Phytotoxicity of second trial

Table 11: Shows the phytotoxicity of sample with Tap water, Untreated and Treated water samples with 5%Black liquor Concentration (Second Trial).

Growth	Tap Water	Untreated Sample (BL)	Treated Sample (Fungal Consortium)	
Average Root Length	7cm	2cm	6cm	6.5cm
Average Shoot Length	2.5cm	0.5cm	2.6cm	3.2cm
Number of Germinated Seeds	10/10	5/10	8/10	9/10
Germination Rate	100%	50%	80%	90%

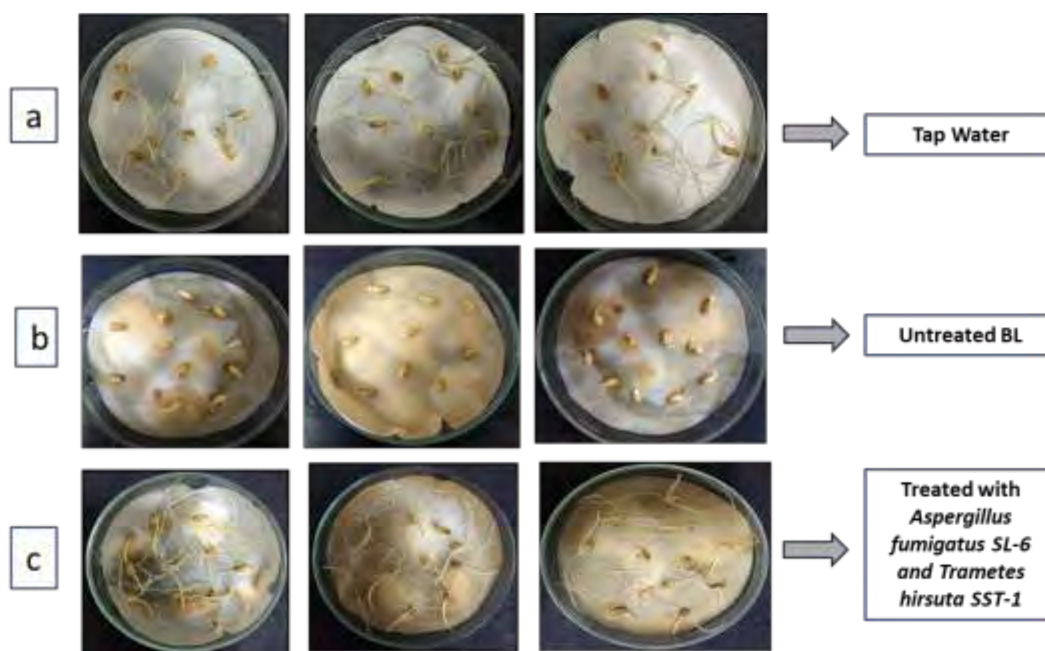

Figure 48: Shows the phytotoxicity of sample (a), (b) Before Treatment and (d) After Treatment in Second Bioreactor Cycle (Consortium), with 5% BL.

4.10 Cytotoxicity testing by Brine Shrimps

Cytotoxicity of the treated and untreated black liquor was investigated via brine shrimp lethality assay. Cytotoxic effect of the *Trametes hirsuta* SST-1 treated black liquor were low with shrimps viable rates of 60% at the concentration of 300µI/ml. However, in the control (untreated BL) mortality rate was 100% at a similar concentration. In the second trial, the cytotoxic impact of black liquor treated by the combined actions of both strains resulted in a viable shrimp rate of 70% at a concentration of 300µI/ml. However in the control (untreated BL)mortality rate was 100% at a similar concentration. Indicating improved outcomes as compared to the first trial.

The following graph shows the % mortality rate of brine shrimps when exposed to different concentrations of treated and untreated BL

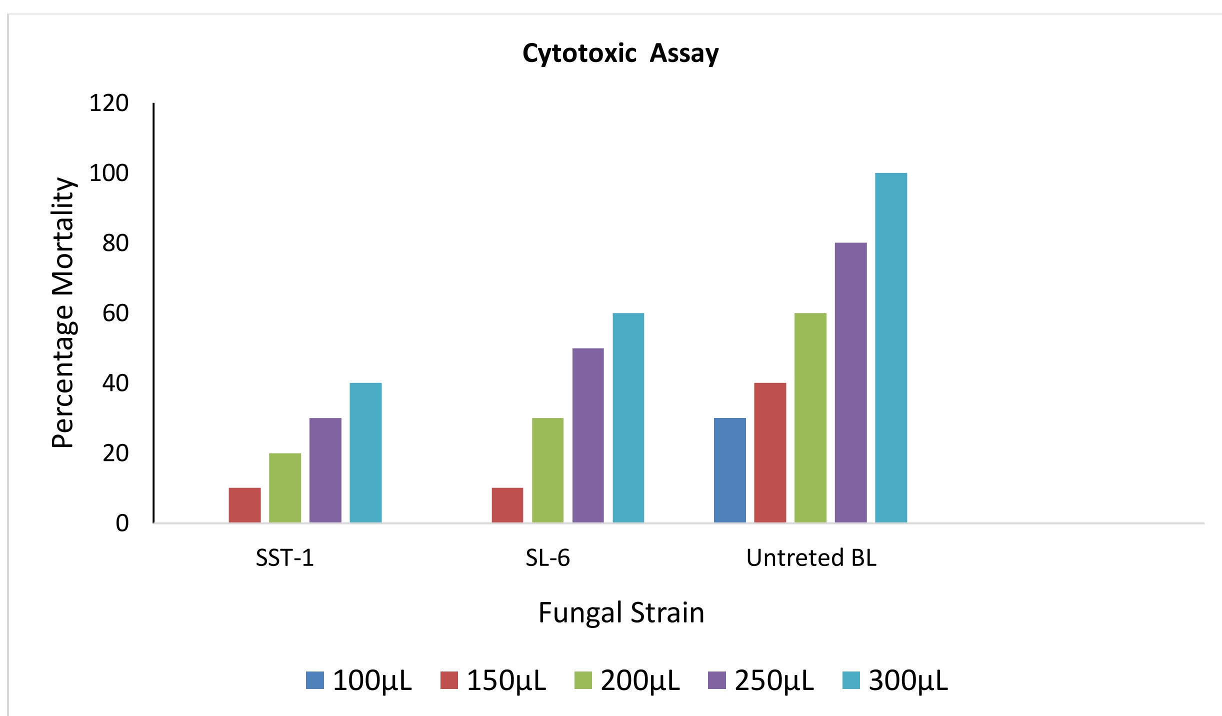

Figure 49: shows the mortality of brine shrimps when exposed to untreated BL and treated BL by *Aspergillus fumigatus* SL-6 and *Trametes hirsuta* SST-1.

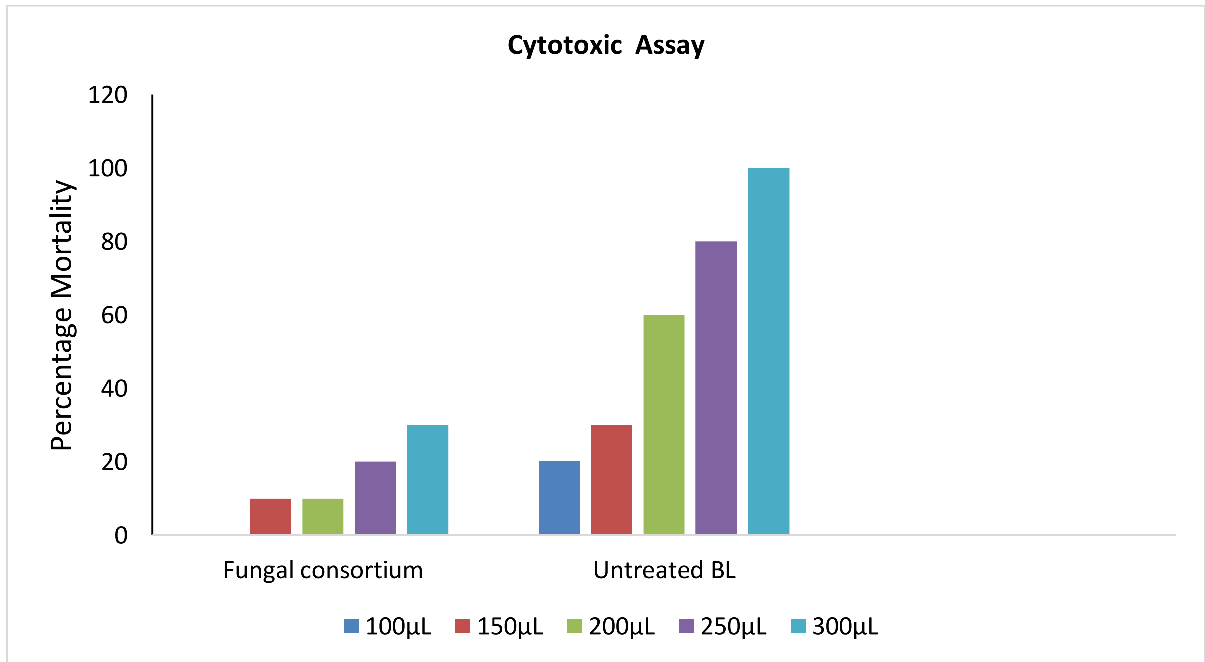

Figure 50: shows the mortality of brine shrimps when exposed to untreated BL and treated BL by Fungal Consortium.

5. Discussion

Currently, the pulp and paper industry is significant to the global economy (Rullifank et al., 2020). Paper and pulp mills constitute a substantial and rapidly growing industry within the global economy. Global paper and pulp production has been growing over time and is expected to do so in the near future. A plentiful and reliable supply of water is available to the majority of these enterprises because they are situated near rivers (Tariq, A., & Mushtaq, 2023). Up to 60 m3/ton of paper manufactured is produced during the paper-making process, which results in large amounts of effluent. These untreated wastewaters, sometimes known as "black liquor," have the potential to be extremely polluting. The main issue with P&P mill effluent treatment is the difficulty in breaking down lignin. It has been discovered that biological treatment is successful, economical, and efficient when compared to other treatment modalities including physiochemical treatments.

The sample was gathered, placed in a sterile container, and refrigerated at 4°C. It was then transported to the laboratory within a day. After collection, the sample was examined for physiochemical traits including TSS and TDS as well as other parameters like COD, color, and lignin. The same findings were also published by (Zheng et al., 2014). Although the pulp and paper mill sample was gathered from seven distinct locations, the treatment of the black liquor sample was the primary focus of this study. Consequently, two or more fungal strains were identified using PDA medium for fungal strain isolation. After that, these strains were tested to determine whether they were able to break down lignin on the MSM medium that had been changed with lignin as the only carbon source. Various lignin concentrations, including 1 g/l, 1.5 g/l, 2 g/l, and 2.5 g/l, were utilized for this purpose (Olajuyigbe et al., 2022). Only two fungal strains, *Trametes hirsuta* SST-1 and *Aspergillus fumigatus* SL-6, were able to reproduce successfully in the lignin-containing substrate and tolerate it. High amounts of lignin are only tolerated by strong fungi because lignin and the aromatic compounds it is produced from damage cells by breaking membranes, disrupting DNA, and blocking enzymes.

Following that, the fungus strains were purified on PDA medium, in turn. Both qualitative and quantitative analysis was used to further investigate the enzymatic capacity to breakdown lignin. The guaiacol plate test was used in this test to measure laccase activity. *Trametes hirsuta* SST-1 was screened using PDA that has guaiacol as the laccase substrate. Brown color development is a sign of favorable results for the fungal strain. The oxidative depolymerization of the substrate (guaiacol) by laccase is shown by the development of brown hue. (Illuri et al., 2021) also screened the fungus based on their ability to oxidize, and as a result of oxidizing guaiacol, they discovered that P. djamor had the highest laccase activity (red dish brown zone diameter, 8.2 cm). Similarly, the use of Azure-B and crystal violet as indicators allowed for the detection of lignin peroxidase synthesis. Positive outcomes are demonstrated by the fungus strain *Aspergillus fumigatus* SL-6, which forms clean zones surrounding the colony. The breakdown of the indicator is what causes the development of clear zones (Hooda et al., 2015) investigated into ligninolytic bacteria and found similar results. It was also looked into how to measure ligninolytic enzymes quantitatively. In this work, after 8 days of incubation, the synthesis of laccase enzyme by *Trametes hirsuta* SST-1 increased to a maximum of 5.36819 U/ML on the ninth day, and then fell, resulting in the observation of laccase enzyme activity on the ninth day. The oxidizing ability of LiP enzyme—which oxidizes Azure B in the presence of hydrogen peroxidase—was used to study the synthesis of LiP enzyme. The maximal 2.367662 U/ML LiP enzyme activity was found after the tenth day of incubation, and after that, the activity started to decline. In the presence of MnSO4, guaiacol is oxidized by the MnP enzyme. On day seven, the MnP enzyme activity reached a maximum of 1.9112 U/ML. In comparison to the *Trametes hirsuta* SST-1 fungal strain, the *Aspergillus fumigatus* SL-6 fungal strain produced much less ligninolytic enzyme. On the eighth day of incubation, the maximum laccase enzyme activity of 2.78283 U/ML was discovered, and the highest LiP enzyme activity value of 1.7112 U/ML was noted. On the seventh day of incubation, manganese peroxidase maximal 1.36837 U/ML enzyme activity was noted. Similar research on the production of ligninolytic enzymes by white rot fungi was conducted by Agrawal et al. (2017). They observed that *Podoscypha elegans* strain FTG4 was effective in secreting ligninolytic

enzymes and also proposed that ligninolytic enzymes are important for the breakdown of organic pollutants.

The *Trametes hirsuta* SST-1 and *Aspergillus fumigatus* SL-6 fungal strains were designed to perform well under various conditions, including temperature, lignin concentration, carbon and nitrogen sources, and pH. Remarkably, *Aspergillus fumigatus* SL-6 reaches optimal degradation at lignin concentration of 1g/l, whereas *Trametes hirsuta* SST-1 has greater degradation capacity at 1.5g/I. The aim of this experiment was to determine the parameters that, if fulfilled, allow fungi to significantly reduce lignin and color, and which may then be used to conduct bioreactor cycles. The optimal temperature for fungal strains (*Trametes hirsuta* and *Aspergillus fumigatus*) was found to be 30°C, which was also the temperature at which the maximum lignin degradation was observed. In a recent study, *P. chrysosporium* stated that the optimal temperature was 35–40°C (Vandana et al., 2018). Fungal strains like *Trametes hirsuta* SST-1 and *Aspergillus fumigatus* SL-6 exhibit maximum lignin breakdown at pH 6 when pH optimization is applied. pH 5 was found to be the ideal environment for *Chrysosporium* in a prior study (Gassara et al., 2010). The best carbon source for both strains was glucose, while the best nitrogen sources for *Aspergillus fumigatus* SL-6 and *Trametes hirsuta* SST-1 were peptone and ammonium sulfate, respectively. Nevertheless, in a previous study, sodium nitrite was examined to be an effective nitrogen source and glucose was reported as the best carbon source (Vandana et al., 2018).

The sequencing bioreactor's first and second trials were conducted in the lab at 5% BL under optimum conditions. In the first trial, two different reactors were used to run the *Aspergillus fumigatus* SL-6 and *Trametes hirsuta* SST-1 strains. It was observed that *Trametes hirsuta* SST-1 showed a considerable reduction in lignin (45.94%), color (59.98%), COD (58.87%), and phenolic contents (57.26%) in the first cycle with 5% BL. Sand filters improved the reduction even further, resulting in lignin reduction of 47.94%, color reduction of 62%, and phenol reduction of 59.23%. In contrast, *Aspergillus fumigatus* SL-6 showed reductions of 41.64% in lignin, 56% in color, 47% in phenol, and

45.46% in COD. Following the use of sand filters, reductions of 43.64% in lignin, 58% in color, and 49% in phenol were noted. A significant 72% reduction in lignin, 78% reduction in color, 82% reduction in phenol, and 67.56% reduction in COD was seen during the second cycle of the consortium of *Trametes hirsuta* SST-1 and *Aspergillus fumigatus* SL-6, which also produced enhanced activity. Following the use of sand filters, the reductions were 74% in lignin, 80% in color, and 84% in phenol. In comparison to their solo activities in the first trial, both fungal strains demonstrated comparatively significant degradation activities when combined in the second simultaneously, the combined activity is higher than what was observed in the first study, suggesting improved efficacy. *Aspergillus niger* was reportedly employed in a prior study to batch-treat actual pulp mill effluent and remove over 60% of the phenolic compounds (Sharma et al., 2021). For *Aspergillus fumigatus* SL-6, the maximum color decrease was 61%. highest lignin degradation recorded by Parveen et al. (2022) on the third day of incubation was 17.58%. Additionally, it was found that 72% of COD was removed in the anaerobic-aerobic treatment system (Kathawala et al., 2021).

Fungal consortiums were plentiful and accelerated the decomposition of lignin, while microbial consortiums enhanced the activities of lignin degradation enzymes. Through the synergy of fungal consortia, my work highlighted the potential exploitation of microbial consortia, which may overcome the limitations of traditional lignin biodegradation when utilizing a single strain. Zhang et al. (2021) conducted a similar investigation on microbial consortiums and provided an explanation for their increased lignin breakdown efficiency. Microbial consortia also have the advantage of being able to adapt to a variety of conditions, including changing amounts of lignin.

Furthermore, the germination of seeds and the lethality assay of brine shrimp (*Arthemia salina*) were used to assess the phytotoxic and cytotoxic effects of treated black liquor. The results showed that treated black liquor's toxicity had considerably dropped, which may have been brought about by the biological treatment's biodegradation of hazardous substances. Ren et al. (2022) conducted an evaluation of the toxicity of pulp mill effluent

and found 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. These findings support our hypothesis that treating BL using *Trametes hirsuta* SST-1 and *Aspergillus fumigatus* SL-6 can significantly improve wastewater characteristics and reduce wastewater toxicity.

6. Conclusion

- Two strains SST-1 and SL-6 were selected after initial screening on the basis of their ability to degrade lignin and were identified as *Aspergillus fumigatus* SL-6 and *Trametes hirsuta* SST-1 through 16S rRNA sequencing.
- In qualitative and quantitative enzyme assays, both fungal isolates exhibited positive activity for ligninolytic enzymes production.
- Both the fungal strains showed maximum degradation activity at pH 6, and temperature of 30℃.
- *Trametes hirsuta* SST-1 showed good degradation ability at a lignin concentration of 1.5g/L, while *Aspergillus fumigatus* SL-6 achieves optimal degradation at a lignin concentration of 1g/L.
- During first trial of lab scale bioreactor, *Trametes hirsuta* SST1 demonstrated 47% reduction in lignin, 62% reduction in color, 59% reduction in phenol and 58% reduction in COD.
- *Aspergillus fumigatus* SL-6 exhibited 41% reduction in lignin, 56% reduction in color,47% reduction in phenol and 45% reduction in COD during first trial.
- During second trial co-cultivation of both the strains showed enhanced activities with notable 74% reduction in lignin, 80% reduction in color, 84% in phenol and 67% reduction in COD.
- In phytotoxic assay germination rate of 90% and 80% was observed for the treated effluent of the first trial and 100% for the effluent of second trial.
- The cytotoxic assay *Trametes hirsuta* SST-1 and *Aspergillus fumigatus* SL-6 treated black liquor showed 60% and 40% viability respectively whereas treated black liquor by the consortium showed viability rate of 70%.

7. Future Prospects

Prospects for Lignin Biodegradation in the Future Using Lab Scale Significant potential exist for using bioreactors to solve technical, financial, and environmental issues. The following are some of the main possibilities for the future:

- To learn more about the isolated fungal strain's capacity to degrade lignin, more research and characterization are needed.
- Moreover, the maximum lignin, color, phenol, and COD reductions must be attained by optimizing the growth parameters of the fungal isolate.
- To avoid having a severe negative influence on the environment, reactors can be further improved and optimized for pulp and paper mill effluent at the industrial level.
- Moreover, ligninolytic enzyme isolation and genetic engineering hold the key to developing robust and effective enzyme combinations.

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