Development of a bacterial consortium for lignin degradation from pulp and paper mill effluent

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

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Submitted By

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2019

Dedicated to
My Beloved Parents

CERTIFICATE

This is to certify that the thesis entitled "**Development of a bacterial consortium for lignin degradation from pulp and paper mill effluent**" submitted by **Miss. Surabhi Zainith (Enrolment no. 159/09)** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar (A Central) University, Lucknow satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.)* Regulations-1999 as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award of the degree of *Doctor of Philosophy* of the University.

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STUDENT DECLARATION

I, **Surabhi Zainith** hereby declare that the thesis entitled **"Development of a bacterial consortium for lignin degradation from pulp and paper mill effluent"** is an original research work done by me under the supervision of **Dr. Ram Naresh Bharagava,** Assistant Professor, Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (A Central) University, Lucknow, for the award of the degree of **Doctor of Philosophy (Ph.D).** The work has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university and is essentially free from all kinds of plagiarism.

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My deepest gratitude goes to my parents for their continual love and support throughout my life; I am indebted to my father ***Mr. Banwari Lal and*** *mother* ***Mrs. Vinod Kumari*** *for her care and love. I cannot acknowledge my family just by writing few words but, I want to say that this thesis is possible only because of my sweet family blessing.*

Surabhi Zainith

(Surabhi Zainith)

Place: Lucknow
Date: 25/04/2019

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ABBREVIATIONS

~	Approximately
µL	Microliter
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
°C	Degree Celsius
DO	Dissolved Oxygen
G	Gram
g/L	Gram per litre
DNA	Deoxyribonucleic acid
Bp	Base pair
DS	Dissolved solid
H_2O_2	Hydrogen peroxide
GC-MS	Gas Chromatography Mass Spectrophotometry
kDa	Kilo Dalton
LiP	Lignin peroxidase
MnP	Manganese peroxidase
MSM	Mineral Salt Medium
mL	Mililitre
Min	Minute
-	Negative
+	Positive
NIST	National Institute of Standards and Technology
OD	Optical density
%	Percentage

Rpm	Revolution per minute
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
TDS	Total dissolved solids
TS	Total Solids
TSS	Total Suspended Solids
U/mL	Unit per millilitre
w/v	Weight over volume
EtBr	Ethidium bromide
FTIR	Fourier transform infrared
H	Hour
L	Litre
NCBI	National centre for biotechnology information
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulphate
sp.	Species
UV	Ultraviolet
DDW	Double distilled water

CHEMICALS AND GLASSWARES

All the chemicals used throughout this research work were of analytical grade and purchased from:

1. Media from Hi-media and Merck Millipore, Mumbai
2. Lignin alkali purchased from Sigma Aldrich
3. Chemical salts and reagents from Merck, Mumbai
4. Glasswares from Borosil, Mumbai
5. Plastic wares from merck Millipore, Mumbai

INSTRUMENTS

All the instruments used in this research work are listed below:

1. Atomic absorbtion spectrophotometer (AAS) (VARIAN AS240FS)
2. Autoclave (SMI-102 & Indfos)
3. Centrifuge (Universal-320-R& Hettich, Zentrifuge)
4. Fourier transform infrared (FTIR) (Nicolet TM 6700, Thermo Scientific, USA)
5. Hot air oven (LSI-145)
6. Laminar air flow (AEM-915-H)
7. Polymerase chain reaction (PCR) (Verti-R & Applied Biosystems)
8. pH meter (Genei)
9. - 20 ºC Refrigerator (BFS-345 & Cellfrost)
10. Scanning electron microscope (SEM) (JSM-6949LV, JEOL)
11. UV-visible spectrophotometer (Evolution, 201, Australia)
12. Temperature controlled incubator shaker (LSI-3016R & Labtech)
13. Water bath (AEM-54003-Q)
14. Polyacrylamide gel electrophoresis unit (GX-SCZ2, Genetix Biotech Asia, Pvt Ltd.

Chapter 1

Introduction

Chapter 1

1. Introduction

The increasing population and rapid industrialization casuses severe environmental pollution and scarcity of water all over the world. Industrial activities and growing population demand are still putting pressures on the environment and the natural resource base industrial processes play a major role in the degradation of the global environment. According to the Ministry of Environment and Forest (MoEF, 1999), Govt. of India, industries are categorised into red, orange, green and white depending upon their pollution capability. "Red category**"** industries are highly pollution causing followed by orange, green and white industries. Out of 17 red category industries, pulp and paper industry is the sixth largest water polluting industry (CPCB 2001). The pulp and paper industry is one of the oldest and core industrial sector, which plays a very important role in the economic development of any country. It has its own prominence value as in terms of commercial and financial expansion of the country. Pulp and paper industry produces various types of paper products to fulfill our daily requirements. Even though paper has many uses,the most important contribution from earlier days to till now, it's use as a medium to record knowledge.

The origin of word "paper" came from a plant *Papyrus*, in the early Egyptian civilization. Paper is believed to be invented about 105 AD, when Ts'ai Lun, a Chinese Eunuch created a sheet of paper using mulberry and other bast fibres along with old rags and hemp wastes. The first paper mill was established in Baghdad in 793 AD. The first successful paper machine was constructed by Nicolas-Louis Robert in the year 1798. In the year 1844, Charles Fenerty and F.G. Keller invented the full machine i.e. pulping the wood material prior for the paper making process (Bloom.

2001). Mechanized production of paper came in existence in the early 19th century which gave revolutionary changes worldwide.

1.1. Scenario of pulp and paper industries in India

Presently, there are about 759 paper mills in India and it is the important industrial sector to Indian economy. The pulp and paper industries in India have been categorized into large, medium and small-scale on the basis of their production capacity. Those paper industries, which have the capacity more than 24,000 tonnes per annum, are designated as large-scale paper industries while which have below this limit called small scale industries. Out of 759 mills, 114 (15%) are large, 303 (40%) medium and 342 (45%) small scale industries. The paper production from medium and small mills accounts for 60% while 40% comes from large mills.

Based on raw materials, the pulp and paper mills in India can be categorized into three groups (Kulkarni, 2013). :-

a. Wood and forest/bamboo-mills - There are 26 large integrated paper mills using wood and bamboos to produce 3.19 million tonnes of paper which is 31% of the production.

b. Agro based mills - There are 150 mills using agro residues like bagasse, wheat and rice straw etc. to produce 2.2 million tonnes of paper which is 22% of the total production.

c. Recycled fiber mills - These mills using waste paper contribute almost 47% of the country's current production which is 4.72 million tonnes and are 538 mills in operation.

India is the 15th largest paper producer in the world. It provides employment to nearly 1.3 million people and contributes Rs.25 billion to the government's pool (Jain et al., 2009). The installed capacity of 759 paper mills is 12.7 million tonnes and

producing 10.11 million tonnes of paper and paperboards which is about 2.52% of the total world production. Therefore, the present consumption of paper and paperboard is 11.15 million tonnes per annum (Kulkarni, 2013). Due to the limited forest resources, two-third of the raw materials come from non-wood sources like rice straw, wheat straw, bagasse and jute, rags etc. used extensively in paper production process (Maheshwari et al., 2012). About 70% of the total installed capacity of pulp and paper production in India is accounted by Gujarat, West Bengal, Orissa, Andhra Pradesh, Karnataka and Maharashtra. The major paper producing states are represented in (Fig. 1.1).

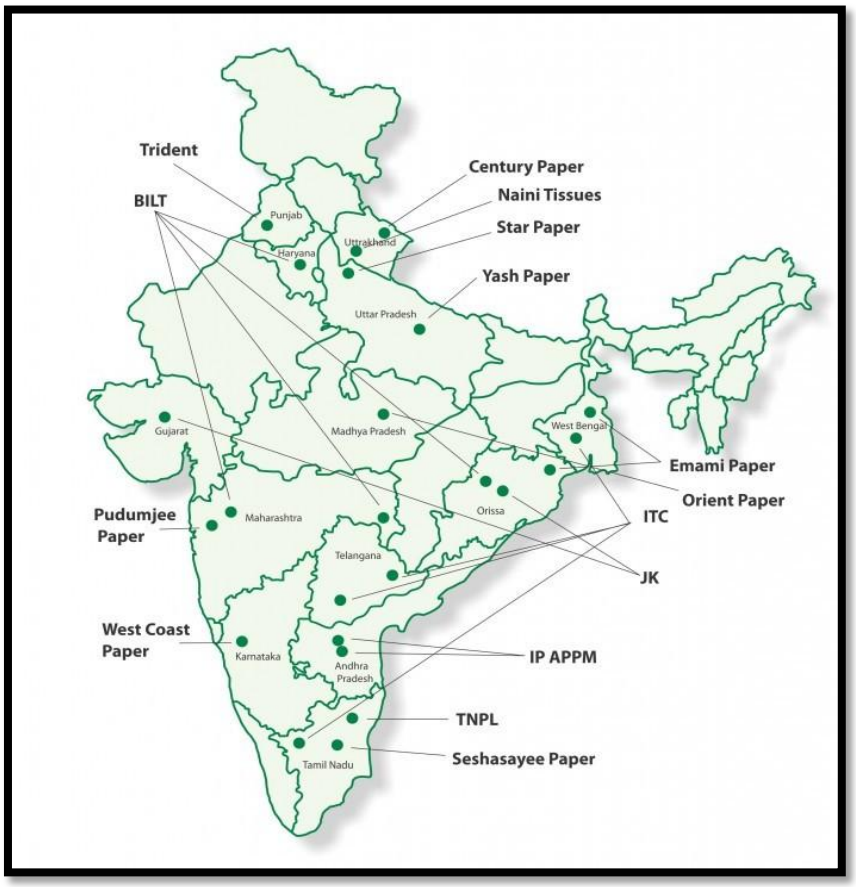


Fig. 1.1 Major states of pulp and paper production in India

1.2. Pulp and Paper Production Processes and generation of wastewater

The raw materials used in paper production process contain cellulosic fibres generally wood, recycled paper, and agricultural residues. In developing countries,

about 60% of cellulose fibres are come from non-wood raw materials such as bagasse, cereal straw, bamboo, reeds, esparto grass, jute, flax, and sisal (Ogunsile and Quintana, 2010). The overall schematic diagram of paper production and the key pollutants generated are presented in Fig. 1.2. The main steps used in pulp and paper manufacturing processes are:

a. Pulping

Pulping is the process commonly started by debarking step in which, the plant fiber converts into smaller pieces called chips and removes soil, dirt, and bark from the raw wood materials (Ali and Sreekrishnan, 2001). In this process, the bonds present in wood structure are ruptured either mechanically or chemically. The pulp yield of mechanical pulping is as high as 90-95% (Smook, 2002) but, the quality of the pulp is low as compared to chemical pulping. In mechanical pulping, mechanical energy uses to weaken and separate cellulose fibers from wood through grinding action. Some methods of mechanical pulping can be modified by several techniques, such as chemo-mechanical pulping (CMP), thermo-mechanical pulping (TMP) and chemical thermo-mechanical pulping (CTMP). Chemical pulping is carried out by either alkaline process (kraft/sulfate pulping) in which, wood chips are cooked in a solution of sodium hydroxide (NaOH) and sodium sulfide (Na_2S) called white liquor, to remove lignin (Sainlez and Heyen, 2013) or acidic process (sulfite pulping) in which wood chips are cooked in a mixture of sulfurous acid (H_2SO_3) and bisulfide ions (HSO_3^-) to dissolve the lignin.

b. Bleaching

This process is commonly used to whitening the brown pulp, which is obtained after pulping process. Several bleaching agents such as chlorine, chlorine dioxide, hydrogen peroxide, oxygen, ozone, etc. may be used either singly or in combination.

c. Washing

In this process, an alkali (caustic soda) is used to extract color and remove the bleaching agents and hardly biodegradable compounds from the pulp.

d. Paper making

This is the final step of pulp and paper production process, in which the washed pulp is combined with dyes, appropriate fillers like titanium dioxide, clay, calcium carbonate, resins and sizing agents such as starch and rosin etc are used to form the paper **(Avsar and Demirer, 2008).**

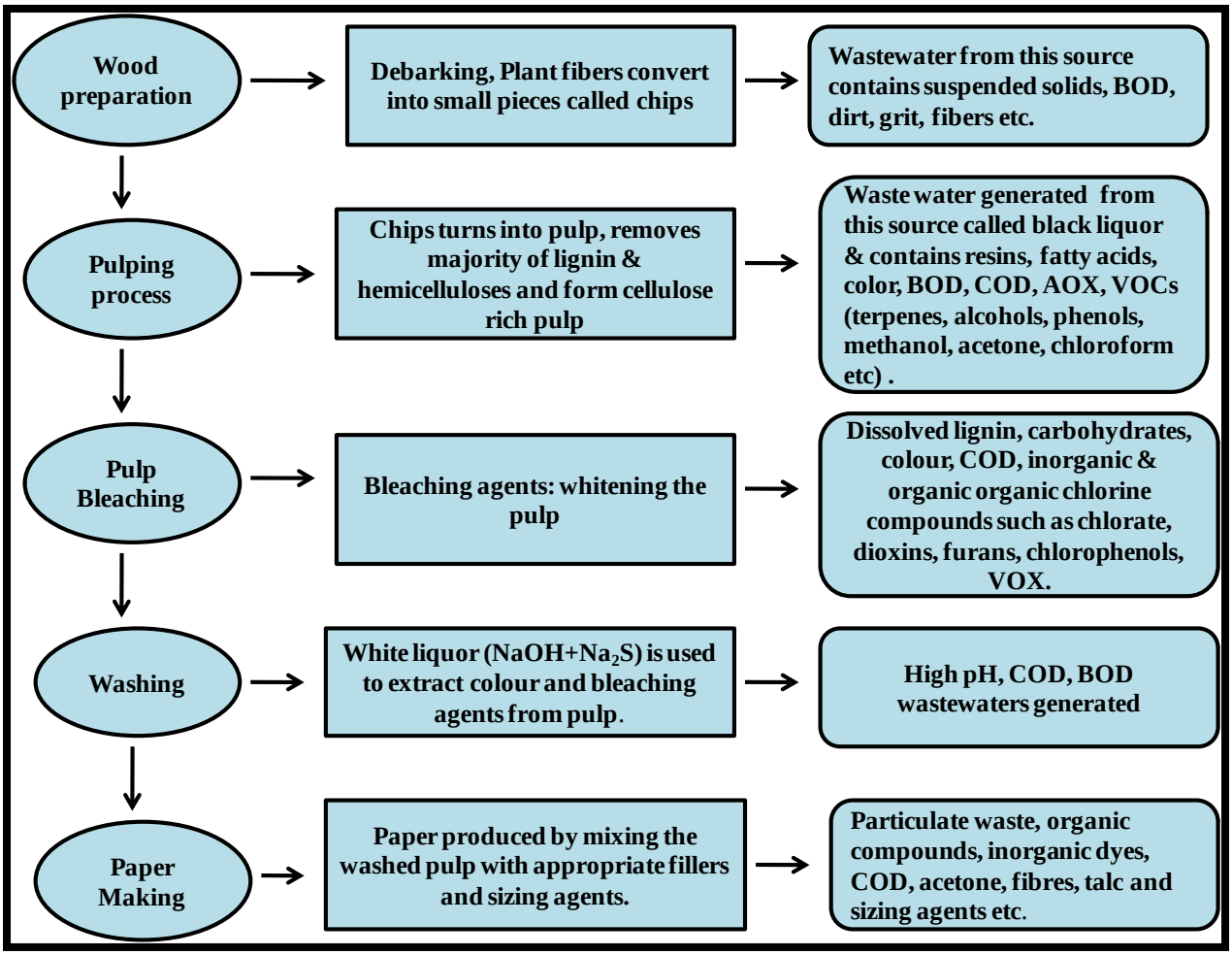


Fig. 1.2 Schematic representation of pollutants generated from various stages of pulping and paper making process

The generation of wastewater from various steps of pulp and paper manufacturing process are highly toxic in nature and give negative effects on environment. Pulping

and bleaching are the two main steps in paper manufacturing process. Pulping process is responsible for generation of large amount of wastewater called "black liquor**"** is brown in colour due to dissolved lignin and its degradation products, hemicelluloses, resins, acids and phenols (Berryman et al., 2004). The effluent generated at bleaching stage contains toxic coloured compounds, chlorinated organic compounds, adsorbable organic halides (AOX) and other derivatives of lignin and hemicelluloses. In this step, lignin, phenols, resin acids, etc. gets chlorinated and transformed into highly toxic xenobiotic compounds. Various xenobiotic compounds formed during pulping and paper making process are chlorinated phenols, chlorolignins, polychlorinated dioxins and furans which are highly persistent and recalcitrant to biodegradation and therefore, maximizing the toxicity of pulp and paper mill effluents which are harmful for the environment (Ince et al., 2011; Badar and Farooqui, 2012; Kamali et al., 2016). Thus, the final discharge of wastewaters increases the level of chemical oxygen demand (COD), biological oxygen demand (BOD), total suspended solids (TSS), total dissolved solids (TDS), phenols and sulphate and causes severe water pollution problems. Lignin and its derivatives that are released during delignification processes of papermaking process impart brown/black colour to the effluent.

1.2.1. Structure of lignin

Lignin is the second most abundant polymer in nature and accounts for 15-30% dry weight of lignocellulose. It is closely associated with cellulose and covalently attached to hemicelluloses. It provides structural support, impermeability, resistance against microbial attack and oxidative stress to the wood. Lignin is a complex heteropolymer, consists of three phenyl propionic alcohols or units: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (p-hydroxy phenyl propanol), and sinapyl alcohol (syringyl propanol) **(**Perez et al., 2001). These basic units of lignin are linked

by C-C and aryl-ether linkages, with aryl-glycerol β-aryl ether. Guaiacyl and syringyl alcohols are the main component of hardwood lignins, whereas coniferyl alcohol is the main component of softwood lignins (Fig. 1.3).

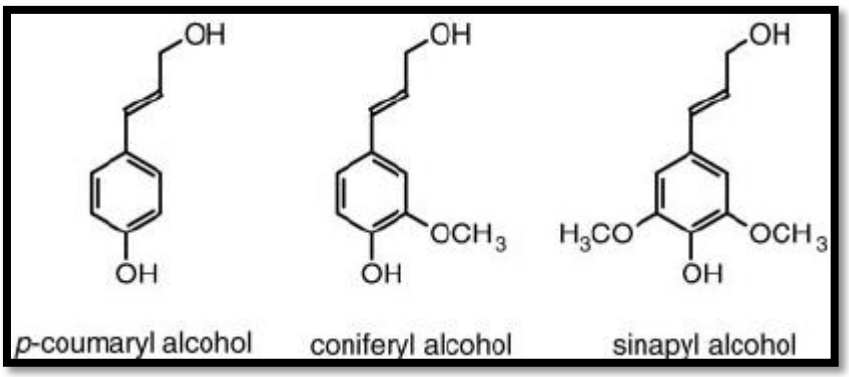


Fig.1.3. Structural units of lignin

1.3. Harmful effects of pulp and paper mill effluent on the environments

The various chemicals used in pulp and paper manufacturing process causes a variety of clastogenic, carcinogenic, endocrinic and mutagenic effects on aquatic as well as on terrestrial environments (Zainith et al., 2019). In aquatic system, the brown color of pulp and paper mill wastewaters, inhibits the photosynthetic process and decreases the dissolved oxygen level in the streams, which adversely affects diversity and abundance of primary as well as secondary and tertiary consumers of aquatic water bodies (Ali and Sreekrishnan, 2001). Many authors reported the detrimental effects of pulp and paper mill wastewater on fishes in the form of respiratory stress, mixed function oxygenase activity, liver damage, genotoxic effects, and lethal effects. A variety of responses occurred in fish populations expose to bleached Kraft pulp paper mills, included delayed sexual maturity, smaller gonads, changes in fish reproduction and a depression in secondary sexual characteristics (Thompson et al., 2001). In the terrestrial ecosystem, pulp and paper mill wastewaters reduces soil fertility and crop productivity and if, the contaminants enter the food chain, affects the general human health and can cause severe metabolic aberration (Tiku et al., 2010).

Mandal and Bandana (1996) reported toxic health impacts such as diarrhea, vomiting, headaches, nausea, and eye irritation on children and workers due to exposure of pulp and paper mill wastewaters. When the treatment processes employed to treat the pulp and paper mill effluent fails results, the release of suspended solids, the loss of nutrients (nitrogen and phosphorus), which can lead to eutrophication in recipient bodies (Thompson et al., 2001). Dioxins and furans are toxic pollutants, causes severe health hazards like cancer, alteration in hormone levels, change in foetus development, suppressed immune system and decreased reproducibility (Kulkarni et al., 2011; Sun et al., 2017). The untreated or inefficiently treated pulp and paper mill wastewater is a major source of ground and surface water pollution, which surge the level of xenobiotic contaminants and heavy metals in the food chain, consequently affects our ecosystem (Chou et al., 2014; Richardson and Kimura, 2017).

Generally, various conventional technologies are used for the degradation and detoxification of pollutants present in pulp and paper mill wastewater including sedimentation and floatation, coagulation and precipitation, filtration, reverse osmosis, adsorption, ozonation and other advanced oxidation processes. But due to the complexity involved in these methods for remediation of pulp and paper mill wastewaters and their contaminated sites, the use of microorganisms has arisen as a potential tool for bioremediation (Kamali and Khodaparast, 2015). Bioremediation is considered to be a cost effective and eco-friendly method for the degradation and decolourization of wastewaters. Microorganisms are advantageous as they are easily grow, produce rapid biomass and are a part of natural environment. The microorganism treats the effluent by many ways: including action of enzymes, biosorption and biotransformation.

Hence, the aim of this study was the isolation and identification of potent lignin degrading bacterial strains and development of a bacterial consortium for the effective degradation and detoxification of pulp and paper mill wastewater for environmental safety. To complete the present study, this research work was divided into seven objectives.

In objective first (01), the pulp and paper mill wastewater was collected from Century Pulp Paper Mill located in Lalkuan; Uttarakhand, (India) was brought to the laboratory for physico-chemical analysis. Further, the effluent was used for the isolation and characterization of lignin degrading bacterial strains capable for the degradation and decolourization of pulp and paper mill effluent. Initially, twenty one bacterial isolates were isolated by enrichment culture technique and these isolates were further, screened and checked for their tolerance limit on MSM agar plates amended with increased concentration of lignin and their ability to decolorize ligninolytic indicator substrate. On the basis of screening, six bacterial isolates (PLP 2, PLP 6, PLP 9, PNP 13, PNP 17 and PNP 19) were selected and out these six, three bacterial isolates (PLP 6, PNP 13 and PNP 19) were showed maximum reduction for lignin and colour. These bacterial isolates i.e. PLP 6, PNP 13 and PNP 19 were characterized and identified on the basis of cellular morphology, biochemical and 16S rDNA gene sequencing analysis as *Bacillus aryabhattai*, *Bacillus megaterium* and *Pseudomonas* sp. with their accession no. MG966493, MG966669 and MH045500, respectively.

Objective second (02) of this work comprised the development of bacterial consortium for the effective degradation and decolorization of pulp and paper mill wastewater. In this objective, the selected lignin degrading bacterial strains (PLP 6, PNP 13 and PNP 19) were checked for their compatibility with each other.

Compatibility among bacterial strains is most important phenomenon for development of effective bacterial consortium regarding efficient growth as well as reduction in colour and lignin content and other parameters of pulp and paper mill effluent. Further, an aliquot of bacterial culture of each bacterial strain was used to develop bacterial consortium (PLP 6+PNP 13+PNP 19) for the effective degradation and detoxification of pulp paper effluent.

In objective third (03), degradation and decolourization of pulp and paper mill wastewater was done by a developed bacterial consortium at optimized conditions of various environmental (pH and temperature) and nutritional parameters including carbon (glucose, sucrose, fructose, maltose and starch) and nitrogen sources (yeast extract, peptone, urea, ammonium sulphate and sodium nitrate).

Objective four (04) of this work comprises the detection and characterization of ligninolytic enzymes by PAGE analysis for the effective degradation of pulp and paper mill wastewater.

Objective five (05) of this work includes the physico-chemical analysis of pulp and paper mill effluent before and after bacterial treatment.

In objective six (06), the detection and characterization of metabolites produced during the bacterial treatment of pulp and paper mill wastewater was done through GC-MS analysis.

Objective seven (07) was the final objective of this research works and include toxicity assessment of pulp and paper mill wastewater before and after bacterial treatment.

Thus, this study concluded that pulp and paper mill wastewater having different type's pollutants and improper disposal of such wastewater increases the toxicity in aquatic as well as in terrestrial environment. Therefore, the overall study of this

research work is divided into ten (10) chapters. Further, to know the background information, each chapter of this thesis is reviewed in detail and all the necessary information is discussed in chapter two (02) review of the literature with the following objectives:

Objectives of the study:

1. **Isolation, screening and characterization of lignin degrading bacterial strains**
   - *Collection of pulp and paper mill wastewater*
   - *Isolation, screening and purification of lignin degrading bacterial isolates*
   - *Biochemical and molecular characterization*
2. **Development of lignin degrading bacterial consortium**
   - *Compatibility test of isolated bacterial strains*
   - *Development of bacterial consortium*
   - *Degradation of pulp and paper mill wastewater by the developed bacterial consortium*
3. **Degradation and detoxification of pulp and paper mill effluent by developed bacterial consortium at optimized nutritional and environmental conditions**
   - *Evaluation of maximum reduction in lignin content colour by a developed bacterial consortium at optimized environmental (pH and temperature) and nutritional (carbon and nitrogen sources) parameters*
4. **Detection and characterization of lignin degrading enzymes by PAGE analysis.**
   - *Preparation of cell-free extract*
   - *Ligninolytic enzyme assay*
   - *SDS-PAGE analysis*

5. **Physico-chemical analysis of pulp and paper mill effluent before and after bacterial treatment.**
   - *Physico-chemical analysis of pulp and paper mill wastewater before and after bacterial treatment*
   - *Lignin estimation and colour measurement before and after bacterial treatment*
6. **Detection and characterization of metabolites produced during the bacterial treatment of pulp and paper mill effluent by HPLC/GC-MC analysis.**
   - *Liquid-liquid extraction*
   - *GC-MS analysis*
7. **Toxicity assessment of pulp and paper mill effluent before and after bacterial treatment by using terrestrial or aquatic test models.**
   - *Toxicity evaluation of pulp and paper mill wastewater by seed germination test before and after bacterial treatment on*
   - *Vigna radiata*
   - *Cicer arietinum*

Chapter 2

Review of Literature

Chapter 2

2. REVIEW OF LITERATURE

2.1. Sources and characteristics of pulp and paper mill waster

Paper is composed of cellulosic fibers. Wood is the most abundant primary source of fiber but some secondary sources such as wheat, rye and rice straws, bagasse, woody stalks from bamboo, flax and hemp, leaf or bast fibers, such as cotton, abaca and sisal are also used for the manufacturing of pulp and paper. The plant wood consists of cellulose (30-50% by weight), hemicellulose (19-45% by weight) and lignin (15-35% by weight) (Pokhrel and Viraraghavan, 2004). Cellulose, hemicellulose and lignin are strongly interlinked and chemically bonded by non-covalent and covalent forces. During paper manufacturing process, the cellulose fiber is separated from hemicellulose and lignin bindings. The various steps of paper production process utilize huge quantity of fresh water and generate a large quantity of wastewater. It is estimated that 273-450 $m^3$of fresh water is used to produce 1 tons of paper and about 60-300 $m^3$of wastewater is generated (CPCB, 2006).

The characteristics of wastewater generated from pulp and paper industry depend upon the type of process, raw materials used, applied production technology, and the amount of water to be used in the particular process. The various steps (wood preparation, pulping, pulp washing, bleaching and finally paper making) involved in paper production process are the most significance source of water pollution (Sharma et al., 2014). Among all, pulping process generates high-strength wastewater especially from chemical pulping. Pulp bleaching discharge highly toxic substances as it uses chlorine for brightening the pulp. Various toxic chemicals such as resin acids, unsaturated fatty acids, diterpene alcohols, juvaniones, and chlorinated resin acids are generated in pulp and paper making process.

Pulp and paper industries produce varieties of complex organic and inorganic pollutants. Many types of pulping process are used to make pulp slurry. Not all pulp and paper mills discharged similar effluents to the surroundings; it varies from mill to mill. Small and medium paper mills (<100t/d) normally generate smaller quantities of wastewater and produced higher pollution load as compared to large mills (>100t/d) (Garg, 2012). This is because, most of the small and medium scale pulp and paper mills do not have an appropriate infrastructure for chemical recovery and thus, discharge them directly into the effluent streams (Tewari et al., 2009).

2.2. Environmental consequences of pulp and paper mill effluent

The pollutants released from pulp and paper industry affect all aspects of environment such as air, water and soil. The effluents from pulp and paper industry causes slime growth, thermal impacts, scum formation, colour problems and loss of aesthetic beauty in the environment (Munkittrick et al., 1997; Pokhrel and Viraraghavan, 2004). They also increase the toxicity level in water causing death of zooplankton and fish, as well as profoundly affect the terrestrial ecosystem (Fentress et al., 2005; Merilainen et al., 2008). Howe and Michael (1999) studied the harmful effects of the treated pulp mill wastewater on irrigated soil in northern Arizona, which showed serious change in soil chemistry. In agriculture, pulp and paper mill wastewaters are used for irrigation purpose, it affects not only the crop growth and soil properties but also the mobility of various ions present in soil which is beneficial to plants (Ugurlu et al., 2008; Kumar and Chopra, 2012).

The toxic compounds released from pulping process are the key pollutant for environmental pollution. In pulping countries, the effect of the generated wastewater on the environment is much greater. Chlorinated organic compounds such as dioxins and furans are supposed to cause skin disorders including skin cancer and also show

reproductive effects in exposed organisms (Nestmann, 1985; Malik et al., 2009), and adsorbable organic halides (AOXs) may bioaccumulate in fish tissues causing a variety of clastogenic, carcinogenic, endocrine and mutagenic effects, which may then also create problems to humans after consumption of the contaminated fish. Chlorinated phenols are also responsible for toxicity to both flora and fauna (Chandra et al., 2009). During treatment process, wood extractives such as resin acids and sterols can be transformed into other toxic compounds, and severe toxicity may occasionally occur.

2.3. Remediation approaches for Pulp and Paper mill wastewater

Several studies showed that P&P mill effluents can potentially induce aquatic toxicity, especially at the reproductive level (Costigan et al., 2012; Waye et al., 2014). Due to the developments occurred in the treatment methods, the toxicity of the pulp and paper effluents has been greatly decreased but some pollutants continue to be found in the final treated effluents (Orrego et al., 2010). This is mainly due to the incomplete degradation as well as economic limitations of some efficient wastewater treatment methods. To overcome these limitations the treatment methods of paper mill wastewater to be of cost benefit and to be able to fulfil all environmental protection rules.

There are several kinds of treatment process for wastewaters are now available. The wastewater treatment normally classified as primary, secondary and tertiary treatment. The wastewater produced from various types of process within the mill is collected for combined treatment by first removing the suspended solids followed by biologically treat and the tertiary treatment of water prior to discharge into the water streams. The removal of solids from wastewater referred to as primary treatment and the latter is biological treatment referred to as secondary treatment.

Various types of treatment process have been developed for the removal of contaminants from wastewaters to reduce their impact on the environment and are grouped into three major categories: Physical, chemical and biological treatment methods. Various treatment technologies and its application are shown in Table 2.1. Pollution from pulp and paper industries can be reduced by various methods as follows:

2.3.1. Physico-chemical treatment methods

The various physico-chemical technologies are now available for the treatment of pulp and paper mill wastewater because of their ability to remove a variety of suspended and floating matters as well as toxic compounds produced from wastewaters.

2.3.1.1. Sedimentation and Flotation

Primary clarification may be attained by either sedimentation or flotation. The basis of the sedimentation process is the removal of suspended solids by settling from water/ wastewater. Suspended particles present in pulp and paper mill wastewaters primarily consist of bark particles, fibres, fillers and coating materials (Pokhrel and Viraraghavan, 2004). Sedimentation was the preferred option in UK and approximately 80% removal of the suspended solids (Thompson et al., 2001). Dissolved air flotation is a conventional method for the removal of suspended solids and has been widely used in treatment of various types of industrial wastewaters. Gubelt et al. (2000) reported that dissolved air flotation method removes 65-95% total suspended solids (TSS), and it was an unstable unit, while Wenta and Hartmen (2002) stated that dissolved air flotation was able to remove 95% of the TSS.

2.3.1.2. Coagulation and Precipitation

Coagulation and flocculation methods are generally used for removing organic materials from wastewaters by partially removing TDS, BOD, COD and colour

(Aguilar et al., 2005). This method mainly works on the principle of addition of a coagulant to the wastewater streams followed by an association between the coagulants and pollutants forming coagulate or flock and subsequently precipitate. The precipitate is then removed by flotation, settling, filtration or other physical techniques (Golob and Ojstrsek, 2005). Wang et al. (2011) used aluminium chloride as coagulant and a modified natural polymer (starch-g-PAM-g-PDMC) as flocculants for the treatment of wastewaters from primary sedimentation tank and concluded that at optimum condition, turbidity, lignin removal efficiency and water recovery were 95.7%, 83.4% and 72.7%. Tong et al. (1999) and Ganjidoust et al. (1997) have done a comparative study between horseradish peroxide (chitosan) and other coagulants such as $Al_2(SO_4)^3$), polyethyleneimine (PEI) and hexamethylene diamine epichlorohydrin polycondensate (HE), to remove colour, total organic carbon (TOC) and adsorbable organic halides (AOX), and observed that chitosan was a more better coagulant in removing these pollutants than others. Dilek and Gokcay (1994) reported that by using alum as coagulant, 96% removal of chemical oxygen demand (COD) from papermaking process, 50% from pulping stage and 20% from bleaching wastewaters were attained. Rohella et al. (2001) stated that polyelectrolytes were more effective than the conventional coagulant alum for the removal of colour, turbidity and COD. Through chemical precipitation method Eskelinen et al. (2010) found that by using used CaO (5 g/L) chemical oxygen demand (COD) reduces up to 90%.

2.3.1.3. Electrochemical Methods

Electrochemical technique is an attractive, alternative and eco-friendly process for treating the wastewaters in large scale (Malkin, 2002; Soloman et al., 2009). This process includes electrocoagulation and electrooxidation. Different types of technical problems arise in pulp and paper industries, which have been solved by

electrochemical method. Electrochemical technologies are of great attention because of their more versatility and environmental compatibility, which makes the treatment of any type of pollutants (liquids, gases and solids) possible. In electrochemical methods, chemicals are not required; the main reagent is the electron, which is a clean reagent (Inan et al., 2004). Chanworrawoot and Hunsom (2012) found that the electrochemical method was much efficient to reduce the pollutants of various types of industries and produce low-density sludge in a very small amount. They also assist the reduction of lignin as well as organic and inorganic compounds, colour, biological oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS) and total dissolved solids (TSD). In their study, the colour, BOD and COD removed upto 98%, 98% and 97%, respectively. The process also requires addition of other chemicals which simultaneously result in the generation of toxic sludge, the disposal of sludge is highly difficult and cost exhaustive.

2.3.1.4. Adsorption

Adsorption is one of the most prominent methods for the treatment and removal of inorganic and organic pollutants present in pulp and paper mill wastewaters. Adsorption is a mass transfer process which involves the accumulation of substances at the interface of two phases, such as, liquid–liquid, gas–liquid, gas–solid, or liquid–solid interface. The substance being adsorbed is the adsorbate and the adsorbing material is termed the adsorbent. The properties of adsorbates and adsorbents are quite specific and depend upon their constituents (Khattri and Singh., 2009). Adsorption has many advantages over other conventional methods like simple design, low cost and land requirement. Recently, the low-cost adsorbents such as agricultural wastes, natural wastes and industrial wastes that have pollutant-binding capacities are used and are easily available. Activated carbon can be used as adsorbent for the

treatment of water and wastewaters (Crini, 2005). Various adsorbents such as coal ash, silica, fuller's earth, activated carbon, etc. have revealed adequate results for decolourization and pollutant removals from pulp and paper mill wastewaters, as reviewed by Pokhrel and Viraraghavan (2004).

Ciputra et al. (2010) investigated the adsorption mechanism of granular activated carbon and ion exchange resin preferentially acts on the hydrophobic and high-molecular-weight fractions and reported 72% and 76% reductions in dissolved organic carbon by using this method. Das and Patnaik (2000) studied the removal efficiency of lignin from blast furnace dust (BFD) and slag by the adsorption mechanism was 80.4% and 61%. Shawwa et al. (2001) used activated coke as an adsorbent that removes 90% of colour, COD and AOX from bleached wastewater through adsorption process. Duan et al. (2010) performed the adsorption method (low-cost bentonite as adsorbent) followed by the coagulation tertiary treatment (polyaluminum silicate chloride as coagulant), and they achieved 60.87% and 41.38% removals of COD and colour, respectively, at optimum doses of adsorbent and coagulant, i.e. 450 mg/l and 400 mg/l.

2.3.1.5. Oxidation

Various oxidation processes are used for the treatment of pulp and paper mill wastewaters. Advanced oxidation processes (AOX) have recognized as highly efficient treatments for the degradation of organic matter. Generation of highly reactive free radicals is the main mechanism of advanced oxidation process. Hydroxyl radicals (OH •) are effectively destroying organic pollutants through the action of electrophiles that react rapidly with all electro-rich organic compounds (Covinich et al., 2014). These free radicals are capable of oxidizing numerous complex organics. They efficiently react with carbon-carbon double bonds and attack the aromatic nucleus, which are prevalent features of refractory organic compounds (Zaviska et al.,

2009). The various oxidation processes are involved in the treatment of pulp and paper mill wastes and effluents:

2.3.1.5.1. Ozonation

Ozone is an efficient oxidising agent with high reactivity through which effective degradation of chlorinated hydrocarbons, phenols, pesticides and aromatic hydrocarbons can be achieved. The combination of ozonation and aerobic biotreatment is demonstrated to be an effective method for destroying lipophilic extractives and hence increases the biodegradability of pulp paper processed effluent before returning to the biotreatment unit (Merayo et al., 2013). Yeber et al. (1999) reported that, after treatment with ozone a substantial reduction in COD, TOC, and toxicity from pulp mill effluent and also increased the biodegradability of the effluent. Freire et al. (2000) reported 12% reduction in total organic carbon, 70% reduction in phenols and 35% reduction in colour of bleached pulp mill effluent after 60 min of ozonation. Ozonation process oxidize chemicals such as guaiacol, syringaldehyde, vainilline, phenol, trichlorophenol, chlorophenol and cinnamic acid derivatives, which present in pulp and paper mill wastewaters (Fontanier et al., 2005; Hermosilla et al., 2014).

2.3.1.5.2. Fenton reagent method

Fenton's reagent is a solution of hydrogen peroxide (H_2O_2) with ferrous iron sulfate ($FeSO_4$) as a catalyst that is used to oxidize contaminants or waste waters. The oxidation system based on the Fenton's reagent has been widely used for the treatment of both organic and inorganic pollutants (Beekeepers, 2000). The Fenton's reagent can be used for the effective removal of colour and absorbable organic halides from the refining wastewater (Mauskan, 2007). Perez et al. (2002) proved that the combinations of fenton and photo-fenton reaction methods are highly effective for the treatment of bleached kraft mill wastewaters. Sevimli (2005) compared ozonation and combination of ozonation with H_2O_2 oxidation and fenton oxidation for the removal

of COD and colour from pulp and paper mill wastewaters and analysed that ozonation and ozonation with hydrogen peroxide successfully remove the colour, while Fenton's oxidation process was more effective in reducing the COD and colour.

Ginni et al., (2014) used solar photo-Fenton process for the treatment of pulp and paper mill wastewater and observed that the complete removal of colour and chemical oxygen demand (COD) under optimal conditions i.e. pH = 4, Fe^{2+} = 1 g/L, H_2O_2 = 5 g/L and irradiation time = 90 min. They also concluded that this process enhances the biodegradability of wastewaters and the level of BOD and total suspended solids below the discharged standard.

2.3.1.5.3. Wet air oxidation

Wet air oxidation is known to possess a large potential for the treatment of wastewaters, which are too concentrated for biological treatment. It can be specified as the oxidation of organic and inorganic substances in an aqueous solution by oxygen or air at elevated temperatures and pressures (Luck, 1999). Process efficiency can be improved by the presence of suitable homogeneous or heterogeneous oxidation catalysts, as well as of extra oxidants such as hydrogen peroxide (Bharagava et al., 2006). Garg et al. (2007) reported 89% reduction in COD of thermally pre-treated pulp and paper mill effluent, using 5% CuO, 95% activated carbon as catalyst through catalytic wet oxidation process. Acid orange 7 is a typical dye used in pulp and paper industries, which is degraded by heterogeneous catalytic wet hydrogen peroxide process (Herney-ramirez et al., 2011).

2.3.1.6. Membrane Technologies

Membrane filtration is a separation process that employs semi permeable membrane to divide the supply wastewater stream into two parts: the first part is permeating that contains the material passing through the membranes and the second is retentate in which the species are being left behind (Mallevialle et al., 1996). Membrane filtration achieved the efficient recovery of waste materials, byproducts

and several impurities discharged from wastewaters. Membrane separation is based on selective filtration through pores of different sizes and consists of four main membrane types: microfiltration, ultrafiltration, nanofiltration, and reverse osmosis. Membrane-based processes, such as ultrafiltration (UF) and nanofiltration (NF) are very important alternatives among the possible effluent treatments. Membrane filtration is also used in pulp and paper industry to achieve the efficient recovery of waste materials, impurities and by-products from effluent before to the discharge (Lin et al., 2013).

Recently, various membrane technologies have been used for the treatment of pulp and paper mill wastewaters. Reverse osmosis is an important method having the ability to destroy the pathogens (Asano and Cotruvo, 2004). Dube et al. (2000) reported that 88% and 89% removal of BOD and COD was attained by reverse osmosis. The most important applications of membrane filtration techniques are the recovery of lignosulfonate from spent sulphite liquor and lignin from kraft black liquor; it also saves energy and is beneficial for the environment (Olsen 1980).

Ciputra et al. (2010) found that nanofiltration technique removes 91% dissolved organic carbon from biologically treated newsprint mill wastewater. Gonder et al. (2011) revealed that at optimized conditions, membrane fouling can be minimized by nanofiltration process. They also investigate by using ultrafiltration membrane method the treatment of pulp and paper mill wastewater which occurs, and they achieved 83%, 97%, 95%, 89% and 50% removal of total hardness, sulphate, spectral absorption coefficient, COD and conductivity, respectively. Krawczyk et al. (2013) recovered high molecular mass hemicelluloses, from chemical thermo-mechanical pulping process wastewater, by using membrane filtration technique.

Table 2.1: Various treatment technologies for the remediation of pulp and paper mill wastewaters

Treatment approaches	Properties	Applications	References
Physico-chemical Treatments			
Sedimentation and floatation	In sedimentation process, gravity is used to separates solid phase from liquid while in floatation process buoyancy is increased of solids by forming gas bubbles.	Industrial (Paper, food, oil and plastic industries) and domestic wastewater treatment.	Ekstrand et al., 2013
Coagulation and flocculation	In coagulation, Particles aggregate with themselves by the influence of a change in pH and in flocculation process the particles aggregate by the use of polymers that binds them together.	Removal of organic matter, Pathogen removal, Removal of inorganics (arsenic and fluoride), All types of wastewater treatment.	Wang et al., 2011
Electrochemical methods	They use electron as unique reagent.	Colour removal in wastewater treatments, Degradation of nonbiodegradable dyes, Do not produce solid waste residues.	Sala and Gutierrez-Bouzan., 2012
Membrane technologies	It generates stable water without the addition of chemicals, low energy use, easy and well-arranged process.	Efficient recovery of waste materials, impurities and by-products from pulp and paper mill effluents prior to discharge. Treatment of industrial wastewaters especially treating wastewater from petrochemical and steel industry and power generation.	Ebrahimi et al., 2016; Chen., 2008; Chen et al., 2009
Adsorption	It is a surface phenomenon. Nature of the bonding between adsorbate and adsorbent: physic sorption (weak Van Der Waals forces), chemisorption (covalent bonding) and electrostatic attraction.	Remove inorganic and organic pollutants i.e. persistent organic pollutants (POPs).	Rashed, 2013

Advanced oxidation process	In this process the main mechanism is the generation of highly reactive free radicals.	Ground remediation, Removal of pesticides from drinking water, Removal of formaldehyde, phenol and reduction in COD from industrial wastewaters.	Ayed et al., 2017
Biological Treatments			
Activated sludge process	Complex mixture of microbiology and biochemistry.	Activated sludge process has been the major treatment method for pulp and paper mill effluents in recent years. It removes organic substances (AOX), nutrients, BOD, COD as well as toxic compounds and pathogens from produced wastewaters.	Ashrafi et al., 2015; Wells et al., 2011
Aerated lagoons		Efficient removal in BOD (over 95%) and chlorinated phenolics (85%) from pulp and paper mill wastewaters and other industrial wastewaters.	Kamali and Khodaparast, 2015
Anaerobic digestion	Biochemical reactions occurs, convert organic polymers from the feedstock into methane (biogas) and nutrient rich digestate. It works best at temperatures of 30 – 60°C.	Used for reducing excess sludge volumes, energy efficient with lower biomass production and is .	Garg and Tripathi, 2011; Metcalf & Eddy et al., 2003
Bacterial treatment	Bacteria show enhanced biodegradation capability, mainly due to the broad pH range tolerability, biochemical versatility, and immense environmental adaptability.	Remove all types of pollutants from industrial wastes and wastewaters.	Chandra and Singh, 2012
Fungal treatment	Produce extracellular enzymes and can survive at higher effluent load.	Degrade lignin/phenolic compounds from pulp and paper mill wastewaters and also reduction in colour, BOD, COD, AOX from different industrial wastewaters (textile, leather, distillery etc).	Sankaran et al., 2010

Algal treatment	Main mechanism for lignin, colour and toxic compounds removal by algae is partially metabolism and transformation rather than adsorption	Treat several types of industrial wastewaters and also remove metals from wastewaters.	Tarlan et al., 2002; Usha et al., 2016
Upflow Anaerobic Sludge Blanket Reactor (UASB)	Form agglomerates (0.5 to 2mm in diameter) and gas forms causes sufficient agitation in the reactor.	Treat various industrial wastewaters like petroleum, distillery, Canning industry, Heavy metals, Paper and Pulp, Tannery, Pharmaceutical, domestic waste water etc.	Kaviyarasan. 2014
Sequencing Batch Reactor (SBR)	Fill and draw activated sludge system for wastewater treatment. Uniquely suited for wastewater treatment applications characterized by low or intermittent flow conditions.	Treat industrial wastewater containing phenolic compounds, such as p-nitrophenol (PNP) which is a hazardous chemical widely used in agricultural, pharmaceutical, and dye industries as a synthetic intermediate in the manufacturing process and also treat both municipal and industrial wastewaters including dairy, pulp and paper, tanneries and textiles.	Dutta and Sarkar, 2015
Constructed wetlands	Use natural functions of vegetation, soil, and organisms to treat different water streams.	They are capable of removing nutrients, biochemical oxygen demand, chemical oxygen demand, total suspended solids, color, metals, and toxic compounds from industrial wastewaters.	Chaudhary et al., 2011
Enzymatic treatments	Remove pollutants by precipitation or transformation to other value-added products.	Application to biorefractory compounds; operation at high and low contaminant concentrations; work over a wide range of pH, temperature and salinity; absence of shock loading effects; reduction in sludge volume (no biomass generated) and the ease of controlling the process. Treat phenolic contaminants and related compounds, pulp and paper wastes, pesticides, cyanide wastes, food processing wastes, removal of heavy metals, Surfactant, Oil and grease degradation.	Karam and Nicell, 1997

2.3.2. Biological Treatment Approaches

Biological methods in which microorganisms such as fungi, bacteria and algae and their enzymes are used, as singly applied or in combination with physical and chemical methods to treat pulp and paper mill wastewaters (Singhal and Thakur, 2009). Most of the conventional treatment methods are not very effective for the removal of colour and degradation of recalcitrant compounds such as lignin (Balcioglu et al**.,** 2007), but compared with physico-chemical methods, biological treatment methods are suitable to reduce COD, BOD and lignin from various types of pulp and paper mill wastewaters (Tiku et al., 2010). Detailed list of microorganisms for the treatment of pulp and paper mill wastewater is given in Table 2.2. Biological processes are divided into two categories: aerobic and anaerobic.

Table 2.2: Microorganisms involved in the treatment of pulp and paper mill wastewater

Microorganisms	**Used for the removal of pollutants**	**References**
Fungal sp.		
Trametes pubescens	Chlorophenols	Gonzalez et al., 2010
Aspergillus niger	Alkaline peroxide mechanical pulping effluent	Liu et al., 2011
Emericella nidulans var. nidulans	Colour and lignin	Singhal and Thakur, 2009
Phanerochaete chrysosporium	Colour, lignin and COD	Saritha et al., 2010
Cryptococcus sp.	Colour, lignin and toxicity of the effluent	Singhal and Thakur, 2009
Trichoderma sp.	Colour	Saravanan and Sreekrishnan, 2005
Aspergillus flavus F10	Colour and lignin	Barapatre and Jha, 2016

Fibrodontia sp. RCK783S	Colour	Kreetachat et al., 2016
Rhizopus aarhizus	Lignin and chlorophenols	Lokeshwari et al., 2015
Bacterial sp.		
Pseudomonas fluorescens	Colour, lignin, COD, phenol, chloride content	Chauhan and Thakur, 2002
Paenibacillus sp.	Colour, lignin, BOD, COD, phenol	Raj et al., 2014
Citrobacter freundii, Serratiia marcescens	TOC, COD, lignin	Abhishek et al., 2015
Alcaligenes faecalis and *Bacillus cereus*	COD	Mehta et al., 2014
Bacillus subtilis sub sp. *inaquosorum*	Lignin, colour, COD	Hooda et al., 2016
Citrobacter freundii and *Citrobacer* sp.	COD, AOX, colour, lignin	Chandra and Abhishek, 2010
Paenibacillus glucanolyticus	Deconstruct pulping waste	Methews et al., 2014
Klebsiella sp., *Alcaligens* sp. and *Cronobacter* sp.	Colour, AOX, TDS, TSS	Kumar et al., 2014
Algal sp.		
Scenedesmus species	Nutrients, organic pollutants, BOD, COD	Usha et al., 2016
Microalgae	Convert secondary waste into value added products	Kouhia et al., 2015
Chlorella	COD, colour, AOX, chlorinated compounds	Tarlan et al., 2002

2.3.2.1. *Aerobic Process*

Aerobic microorganisms require oxygen to perform their metabolic activity. Oxygen is supplied in the form of air by aeration equipment. There are numerous of aerobic systems available for the degradation of various organic compounds in industrial wastewater such as aerated lagoons, activated sludge systems, biofilm

processes etc. (Persson, 2011). Aerobic treatment processes such as activated sludge (AS) and aerated lagoons (AL) are the commonly used treatment methods for pulp and paper mill wastewaters. Activated sludge system is the major treatment method capable of removing huge amount of sludge and secondary pollutants generated from pulp and paper industries (Buyukkamaci and Koken, 2010). Lots of literature has been published for the treatment of pulp and paper wastewaters through activated sludge system. Chandra (2001) reported the efficient removal of colour, BOD, COD, phenols and sulphide by microorganisms such *as Pseudomonas putida*, *Citrobacter* sp. and *Enterobacter* sp. through activated sludge process. Bengtsson et al. (2008) treated pulp and paper mill wastewaters from recycled fibres by activated sludge system and found 95% removal of COD.

Mahmood and Paice (2006) and Ghoreishi and Haghighi (2007) treated various types of pulp and paper wastewaters through aerated stabilization basin and remove 50-70% BOD, 30-40% COD, AOX and chlorinated compounds. Aerated lagoon is the simple and cost-effective biological system which is relevant on both lab scale as well as full scale for pulp and paper mill wastewaters. This system is used to remove BOD, low-molecular-weight AOX and fatty acids at full-scale applications (Bajpai, 2001). Schnell et al. (2000) reported that the aerated lagoon system removes BOD, AOX and phenols. Pokhrel and Viraraghavan (2004) reviewed that aerated lagoon system was efficient in removal of chlorinated phenols (85%) and BOD (>95%) from pulp and paper mill wasters.

2.3.2.2. Anaerobic treatment Process

In anaerobic treatment, anaerobic bacteria (biomass) convert complex organic pollutants into simpler compounds in an oxygen free environment. Under anaerobic conditions, organic matter is degraded by various groups of prokaryotes such as,

fermenters, acetogens, methanogens, and sulfate reducing bacteria (Ahammad et al., 2008). Metabolic interactions between these microorganisms lead to the transformation of complex organic compounds to simple compounds such as methane, carbon dioxide, hydrogen-sulfide, and ammonia. The digestion process is accomplished in four major reaction stages (hydrolysis, acidogenesis, acetogenesis and methanogenesis) involving different microorganisms in each stage (Gunaseelan, 1997). Anaerobic treatment of wastewaters has a number of advantages over aerobic treatment process; the energy input of the system is low, lower production of excess sludge, lower nutrient requirement due to lower biological synthesis, and the degradation of waste organic material leads to the production of biogas, which is a valuable source of energy.

Anaerobic process is considered to be more suitable method for the treatment of high-strength organic wastewaters. A large numbers of literatures have been published on anaerobic process along with microbial communities to treat pulp and paper mill wastewaters (Ince et al., 2007). In recent years, a stable biological process, anaerobic digestion (AD), is used for treatment of high loads of pulp and paper mill wastewaters. This approach has several advantages over various conventional treatment methods such as simple design, reduction of produced sludge volume up to 30–70%, destruction of pathogens in the thermophilic region, cost-effective and eco-friendly in nature (Zwain et al., 2013; Ekstrand et al., 2013). The anaerobic treatment of pulp and paper mill wastewater results in the degradation of pollutants such as lignin and their derivatives, fatty acids, resins and organic compounds, which are produced during the various steps of papermaking process (Sumathi and Hung, 2006).

2.3.2.3. Degradation of pulp and paper mill wastes and wastewaters by Bacteria

Bacteria are the most important group of microorganisms which are responsible for the degradation and detoxification of various types of wastes and wastewaters. Both heterotrophic and autotrophic bacteria are found in wastewater treatment systems the predominant ones are the heterotrophic bacteria. Commonly, heterotrophic bacteria obtain energy from the organic matter present in wastewater effluent and this energy is used for the synthesis of new cells and the conversion of organic matter (Absar, 2005). Several species of bacteria have been used for the remediation of pulp and paper mill wastewaters. Various studies have reported that some bacterial species (anaerobic and aerobic) could metabolize lignin and their related compounds to low-molecular-weight compounds, and this is due to huge adaptability and biochemical versatility of bacterial species (Chandra et al., 2007; Abhishek et al., 2017). However, bacteria are considered most numerous and ubiquitous in nature which have a wider tolerance of temperature, pH, and oxygen limitations than the fungi (Chandra et al., 2011).

Several streptomycetes have been reported to break down lignin and its derivatives, of which the best-studied strain is *Streptomyces viridosporus* T7A. This strain produces several extracellular peroxidases and to catalyse oxidative cleavage of b-aryl ether lignin model compounds (Ramachandra et al., 1988; Vicuna, 1988). Ahmad et al. (2010) isolated two bacterial strains *Pseudomonas putida* mt-2 and *Rhodococcus jostii* RHA1 and found that these two strains act as aromatic and lignin degraders. The activity of these bacterial strains was less than *Phanerochaete chrysosporium* (white rot fungus) but compared to other lignin degrading fungi, these strains were more capable to degrade lignocellulose in small scale incubations and releasing low molecular weight compounds.

Shi et al. (2013) found that low molecular weight compounds such as ferulic acid and cinnamic acid were produce during the degradation of kraft lignin by *Pandoraea* sp. B-6. Chen et al., (2012a) found vanillic acid during the degradation of kraft lignin by *Novosphingobium* sp.B-7.

Thakur (2004) studied the removal of colour and adsorbable organic halogens (AOX) in kraft pulp bleaching effluents by using eight fungal and three bacterial strains. The results showed that the decolourization potency *of Paecilomyces* sp. was maximal (67%) within one day followed by *Phoma* sp. and *Paecilomyces varioti*. Among the various carbon sources used, *Paecilomyces* sp. used 1% dextrose (0.2%) and reduced colour and lignin more than 80% was observed. In the batch reactor, *Pseudomonas aeruginosa*, removed 48% colour from the effluent after one day followed by *Acinetobacter calcoaceticus* (39%) and *Klebsiella pneumonia* (25%). In a two stage sequential bioreactor, two fungal strains *Paecilomyces* sp. and *P. aeruginosa* were able to reduce 68% and 34% colour in one day. The reduction of adsorbable organic halogens (AOX) in effluent was observed by *Paecilomyces* sp. strain.

Singh and Thakur (2006) studied the removal of colour and other pollution parameters of pulp and paper mill effluent by anaerobic treatment used by fungus (*Paecilomyces* sp.) and bacterial strain (*Microbrevis luteum*) separately in two steps bioreactor. The results found that in anaerobic treatment, colour (70%), lignin (25%), COD (42%), AOX (15%) and phenol (39%) were reduced within fifteen days. The anaerobically treated pulp and paper effluent was treated again by fungal strain, *Paecilomyces* sp., and bacterial strain, *Microbrevis luteum* and the results found that the colour, AOX, lignin, COD and phenol by *Paecilomyces* sp. were reduced upto

95%, 67%, 86%, 88% and 63% whereas *Microbrevis luteum* removed 76% of colour, 69% of lignin, 75% of COD, 82% of AOX and 93% of phenol.

Raj et al (2007) worked on the treatment of pulp and paper mill effluent using three lignin degrading bacterial strains: *Paenibacillus* sp., *Aneurinibacillus aneurinilyticus* and *Bacillus* sp. The degradation experiments were performed in Erlenmeyer flasks containing mineral salt medium at pH 7.6 and the flasks were incubated at 30℃ on a rotary shaker (120 rpm) for six days. The results found that all three bacterial strains effectively reduced colour (39-61%), lignin (28-53%), biochemical oxygen demand (BOD) (65-82%), chemical oxygen demand (COD) (52-78%) and total phenol (64-77%) within six days of incubation. However, the highest reduction in colour (61%), lignin (53%), BOD (82%) and COD (78%) was recorded by *Bacillus* sp. while, maximum reduction in total phenol (77%) was recorded with *Paenibacillus* sp. They also characterized the degradation product of lignin after treatment by *Paenibacillus* sp were t-cinnamic acid and ferulic acid, while 3-hydroxy-4-methoxyphenol, vanillic acid and vanillin acid by *A. aneurinilyticus* and gallic acid and ferulic acid by *Bacillus* sp., respectively.

Chandra et al. (2009) isolated two PCP-degrading bacterial strains, *Bacillus cereus* and *Serratia marcescens* were used for the treatment of pulp and paper mill effluent supplemented with glucose (1.0%), peptone (0.5%) and incubate at 30±1℃, 120 rpm for 168 h of incubation. These two bacterial strains effectively reduced colour (45–52%), lignin (30-42%), BOD (40-70%), COD (50-60%), total phenol (32-40%) and PCP (85-90%) within 168 h of incubation individually. However, the highest reduction in colour (62%), lignin (54%), BOD (70%), COD (90%), total phenol (90%) and PCP (100%) was recorded by mixed culture. Further, the metabolites produced during treatment of pulp paper mill effluent were analyzed by

GC-MS analysis and these metabolites increase the bacterium capability for the degradation of lignin and pulp and paper effluent.

Mishra and Thakur (2010) isolated four bacterial strains from contaminated sludge of pulp and paper mill in which one bacterial strain (*Bacillus* sp.) having higher capability to remove colour and lignin at optimized conditions. Optimization was performed by Taguchi approach in which seven factors, carbon %, black liquor %, duration, pH, temperature, stirring and inoculum size, at two levels, applying L-8 orthogonal array were taken. Maximum colour reduction was observed at pH 8, temperature 35℃, stirring 200 rpm, sucrose (2.5%), 48 h, 5% (w/v) inoculum size and 10% black liquor. After optimization process 2-fold increase in colour (25-69%) and lignin (28-53%) reduction was observed. Enzymes produced during decolorization process of effluent were found to be xylanase (54 U/ml) and manganese peroxidase (28 U/ml) respectively. Toxicity of treated effluent was also evaluated by Comet assay by using *Saccharomyces cerevisiae* MTCC 36 as a model organism, which indicated 58% reduction after treatment by *Bacillus* sp.

Tiku et al. (2010) worked on the treatment of pulp and paper mill effluent by three bacterial strains: *Pseudomonas aeruginosa* (DSMZ 03504), *P. aeruginosa* (DSMZ 03505) and *Bacillus megaterium* (MTCC 6544). These bacterial strains reduce BOD and COD level of pulp and paper mill effluents up to permissible limit i.e. 30 mg/l and 250 mg/l respectively within a retention time of 24 h in batch cultures. A concomitant reduction in TDS (total dissolve solid), AOX and colour (76%) was also observed and they also concluded that these bacterial strains reported first time for the holistic bioremediation of pulp mill effluent.

Singh et al (2011) studied the treatment of pulp and paper mill effluent using *Enterobacter* sp. Their results was found that the pollution reduction was significantly

affected by various parameters including the amount of inoculum size, rate of agitation, the reaction temperature and the duration of treatment. Also found that COD/BOD load could rapidly be reduced to 80%; lignin to 73% and colour to 82% within 16 h, using a 10% inoculum size, agitation at 200 rpm and temperature at 35°C.

Chandra and Singh (2012) worked on decolourisation and detoxification of rayon grade pulp and paper mill effluent by mixed culture of three bacterial strains i.e. *Pseudochrobactrum glaciale*, *Providencia rettgeri* and *Pantoea* sp.) isolated from the contaminated site of the industry. The results revealed that the reduction in colour, chemical oxygen demand and biological oxygen demand by mixed culture were found to be 96.02%, 91% and 92.59% within 216 h of the incubation period. The maximum enzyme activity for lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase were recorded at 48, 72 and 144 h of the incubation period, respectively. Phytotoxicity assessment of pulp and paper mill effluent on *Vicia faba* L. after bacterial treatment was also observed which showed 40% reduction in toxicity.

Wang et al. (2013) made lignin degradation bacterial consortium, it could break down lignin 60.9% in reeds at 30 ℃ under static conditions within 15 days of incubation. To analyze the diversity of bacterial consortium, plate isolation, 16S rDNA clone library and ARDRA (Amplified Ribosomal DNA Restriction Analysis) were performed. The results concluded that the bacterial consortium had the capability of lignin degradation and was efficient for pulping, which would provide a new choice for biopulping.

Mathews et al. (2014) isolated a bacterium *Paenibacillus glucanolyticus* was shown to be capable of growth on black liquor as the sole carbon source and this facultative anaerobic bacterium can degrade black liquor as well as cellulose,

hemicellulose, and lignin. GC-MS analysis was identified the products generated by *P. glucanolyticus* during the degradation of black liquor.

Raj et al. (2014) isolated a laccase producing Paenibacillus sp., strain. This bacterium effectively reduced colour (68%), lignin (54%), phenol (86%), BOD (83%) and COD (78%) after 144 h of incubation period at 34±1℃ and 120 rpm. In their study low molecular weight compounds present in control (untreated effluent) samples were reduced after bacterial treatment was analysed through GC-MS analysis.

Tyagi et al. (2014) isolated two bacterial strains, *Bacillus subtilis* and *Micrococcus luteus*, and one fungi, *Phanerochaete chrysosporium*, from pulp and paper mill wastewaters and sludge; these microbes were capable in reducing BOD up to 87.2%, COD 94.7% and lignin content 97% after 9 days; pH was down to neutral, and dissolved oxygen increased from 0.8 to 6.8 mg/L.

Hooda et al. (2015) studied the degradation of pulp and paper mill wastewater by a rod-shaped Gram-positive bacterium, i.e. *Brevibacillus agri*, in batch culture and in semi-continuous reactor. During batch study, this bacterium reduced COD up to 69%, colour 47%, lignin 37% and AOX 39%, while in semi-continuous reactor study, it reduced COD up to 62%, colour 37%, lignin 30% and AOX 40%.

Gaur et al. (2018) worked on different ligninolytic enzymes produced from four novel bacterial strains of *Klebsiella pneumoniae* (*K. pneumoniae* strains NITW715076, NITW715076_1, NITW715076_2 and NITW715076_3) were isolated. The ligninolytic enzymes were characterized by plate assay method and the statistical optimization was done through response surface methodology (RSM). In degradation and decolourization studies, consortia 1 (*K. pneumoniae* NITW715076_2 + *K. pneumoniae* NITW715076_1) reduced lignin upto 82.31% and was found more

effective when compared to axenic culture (*K. pneumoniae* NITW715076_2) which reduced only 74.1%. In RSM studies, Laccase and MnP activities were increased by 20% and 18% i.e. 53338 IU/L and 147900 IU/L, respectively. Further, GC-MS analysis also showed the degradation of different organic pollutants present in the effluent. The seed germination test showed that the toxicity reduced of the effluent upto a significant level and used for agricultural applications.

2.3.2.4. Degradation by fungi

Several strains of wood rotting fungus are capable of oxidizing the various types of complex structures such as lignin and its derivatives, synthetic textile dyes and aromatics compounds. The most studied wood rotting fungus is the white-rot fungus *Phanerochaete chrysosporium*, which is able to decolorize various complex organic compounds. The use of fungus species of the genera *Pleurotus, Bjerkandera, Trametes, Polyporus*, *Aspergillus, Trichoderma*, *Penicillium* and *Rhizopus* have been also investigated (Gassara et al., 2010). Extracellular ligninolytic enzymes produced by fungal species such as: laccase, lignin peroxidase and manganese peroxidase play an important role in degradation and detoxification of complex compounds (Vaithanomsat et al., 2013). Fungi are common in the treatment of pulp and paper mill wastewaters (Yang et al., 2011. In comparison with bacteria, fungi can survive at higher strengths of wastewaters and degrade phenolic compounds of pulp and paper effluent (Singhal and Thakur 2009).

Santos et al. (2002) investigated the treatment of effluent from kraft bleach pulp and paper industry using white-rot fungus *Pleurotus ostreatoroseu*. The experiments were carried out in continuous turbulent-flow bioreactor at different dilutions and concentrations of glucose for the removal of colour, total phenols and lignin/chlorolignins. Their results found that the average removal of colour and total

phenols was 18.6% and 11.6% respectively, after the addition of glucose. The reduction percentage of colour, total phenols and lignin/chlorolignins was increased with increasing glucose concentration and found the maximum removal of colour, total phenols and lignin/chlorolignins was 19.4%, 9.4% and 44.5% respectively.

Prabu and Udayasoorian (2005) studied the removal of colour and chlorinated phenol from paper mill effluent using a white rot fungus *Phanerochaete chrysosporium* isolated from the soil samples of pulp and paper mill effluent. The results found that the addition of carbon and nitrogen sources showed significant effect on colour removal of the effluent. The *Phanerochaete chrysosporium* removed colour (60.7%) with addition of glucose whereas 49.3% and 45.1% of colour removal was achieved with addition of fructose and starch. This fungal strain also responded to nitrogen sources, in presence of ammonium sulphate the percentage of colour removal was 67.4%, whereas 67% and 65% of colour removal achieved with the addition of diammonium phosphate and sodium nitrate, respectively. Moreover, the combination of both nitrogen and carbon sources caused maximum decolourization in colour (84%) and COD (79%) than individual sources. chlorinated phenol was degraded by 91% by this fungal strain when 1% glucose was added as co-substrate.

Malaviya and Rathore (2007) stated the bioremediation of pollutants from pulp and paper mill wastewater by a novel consortium of white-rot and soft-rot fungi which reduced the colour, lignin and COD by 78.6%, 79% and 89.4%. Wood degrading white-rot fungus is very effective for the degradation of lignin and chlorinated compounds, which are mainly responsible for colour and toxicity of pulp and paper mill wastewaters (Saritha et al., 2010). White-rot fungus *T. pubescens* along with TiO2/UV was used for degradation of chlorophenols, and this combination

(biological and advanced oxidation process) allowed up to 100% chlorophenol removal (Gonzalez et al., 2010).

Lokeshwari et al. (2013) isolated a fungal strain *Aspergillus flavus*, white rot fungi was isolated from pulp and paper effluent used for the degradation of lignin and AOX through batch culture technique. The flasks were incubated at temperature 32°C at 200 rpm for eight days. *Aspergillus flavus* sp. was the most effective in the degradation of pollution parameters of pulp industry i.e. lignin 94%, AOX for 62% and chemical oxygen demand levels for 45% after 8 days of incubation. The optimal conditions found were pH 4 and temperature 32℃ for lignin and AOX degradation.

Rajwar and Rai (2015) isolated three fungal strains for the decolourization of kraft black liquor on solid and liquid medium under different concentrations. Qualitative assessment of fungal decolorization was observed by plate assay method. Out of the three fungal strains, *Nigrospora* sp. showed maximum growth and decolorization halos on malt extract agar medium containing 10 % black liquor while slow fungal growth was observed on 20 % black liquor agar medium. In case of liquid medium, the maximum decolorization (61%) and COD removal (58.7%) was observed by *Nigrospora* sp. Moreover, mixed fungal culture enhanced the reduction efficiency of COD and colour removal upto 71.5 % and 73 %, respectively. Their results indicated that the fungal strain *Nigrospora* sp. has huge potential for treatment of kraft black liquor.

Barapatre and Jha (2016) isolated a lignin degrading fungus *Aspergillus flavus* for the treatment of pulp and paper mill effluent. The results of this study showed that this fungal strain effectively reduced the colour and lignin content and other phenolic compounds. It reduces colour up to 31-51 % and lignin content 39-61% within ten days of incubation, while in immobilized condition it reduces more colour and lignin

content within six days of incubation. A significant reduction in colour and lignin concentration was observed after four days of incubation, indicating that *Aspergillus flavus* subsequently utilized chromophoric compounds as reducing the lignin content and colour. They measured the toxicity of paper and pulp effluent in terms of phytotoxicity and showed high germination index indicated that the pollutants of paper mill effluent reduced upto a significant level after treatment of fungal strain.

Rajwar et al. (2017) isolated two fungal strains (*Nigrospora* sp. and *Curvularia lunata*) and made a novel fungal consortium for the treatment of pulp and paper mill effluent. Fungal consortium exhibited enhanced biomass production under optimized medium conditions, i.e., glucose as carbon, sodium nitrate as nitrogen, pH 5, temperature 30 °C, and agitation 140 rpm. They found that the fungal consortium significantly reduced biochemical oxygen demand (85.6%), chemical oxygen demand (80%), colour (82.3%), and lignin content (76.1%). Ligninolytic enzymes, such as laccase, manganese peroxidase and lignin peroxidase were observed to be 13.5, 11.4, and 9.4 IU/ml after the effluent treatment. Scanning electron microscopy of fungal consortium showed their compatibility through intermingled hyphae and spores. FTIR spectra showed the alteration in functional groups during the treatment of effluent. GC-MS analysis showed that the reduction of complex compounds into numerous low molecular weight compounds.

2.3.2.5. Algal treatment

Algae are important bioremediation agents, and they used natural mechanism for the treatment of wastewaters. Tarlan et al. (2002) were found to remove 58% COD, 84% colour and 80% AOX from pulp and paper wastewaters by algae and also showed that they grew mixotrophically and partially metabolized colour and organic compounds (released from pulping stage) to non-coloured and simple molecules.

Several studies have reported wastewater can be used for the cultivation of microalgae (Ramanna et al., 2014). Microalgal cultivation from wastewater has a twin purpose: supply nutrients and minimize the freshwater requirements along with the removal of COD and BOD from wastewaters. Algae can use huge amounts of organic compounds from wastewater for rapid growth in the photoheterotrophic or mixotrophic environment in the presence of light (Li et al., 2011). Usha et al. (2016) stated that microalgal treatment is an efficient tool for the remediation of pulp and paper industry wastewater, and in their lab study, they found maximum removal of BOD (82%) and COD (75%), respectively, through microalgal cultivation in outdoor open pond. Algae have also been used in the removal of heavy metals from wastewaters.

2.3.2.6. Biological reactors used in the treatment of pulp and paper mill effluent

Various types of rectors/digesters including Sequential batch reactor (SBR), Membrane bioreactors (MBR), Upflow anaerobic sludge blanket reactor (UASB) etc. are reported for the degradation and treatment of noxious and deleterious pollutant present in pulp and paper mill wastewater. These reactors are principally based on the anaerobic microbial treatment technology that can efficiently reduce high concentration of pollution load and signifies wastewater quality for its reuse in irrigation and other practices. Khan et al. (2011) observed 87% removal of COD, and the turbidity removal was 95% from pulp and paper mill wastewaters through column-type sequencing batch reactor. The alkalinity and pH of the treated wastewaters were in the permissible range and improved the characteristics of produced sludge.

Kumar and Subramanian (2014) found through SBR system the removal efficiencies of COD, BOD, TDS, TSS and organic compounds reached up to 84%,

83%, 85%, 88% and 80 ± 4.5% under the retention time of 24 h. Various studies showed that high loads of organic pollutants present in pulp and paper mill wastewater reduced through SBR (Milet and Duff, 1996). Muhamad et al. (2013) reported a significant reduction in the pollutants from recovered fibres of pulp and paper mill wastewater by granular activated carbon-sequencing batch biofilm reactor (GAC-SBBR) and also achieved 97.2% removal of COD, 99.4% of NH3-N and 100% of DCP. Buyukkamaci and Koken (2010) stated that the activated sludge process is the most important treatment method for the removal of low and medium strength of pulp and paper mill wastewaters but has some drawbacks, which can be improved in combination with MBRs. Membrane fouling in membrane technologies can increase the maintenance and operational costs, which may also overcome by membrane bioreactors (Le-clech et al., 2006).

Removal of COD by moving bed biofilm reactor (MBBR) and the amount of sludge which is produced in secondary treatment from pulp and paper industries can also be reduced by membrane bioreactors (Jahren et al., 2002). UASB reactor also known as anaerobic reactor was used in the treatment of various industrial wastewaters like tannery, distillery, pharmaceutical, pulp and paper, etc. Microorganisms living in the sludge blanket of UASB having microbial granules of size 0.5-2 mm break down organic matter by anaerobic digestion into simple compounds and biogas and can be used as energy source. Buyukkamaci and Koken, (2010) showed that upflow anaerobic sludge blanket reactor followed by an aeration basin is the most economic and technically feasible treatment for medium and high strength wastewaters. In previous studies, Peerbhoi (2000) investigated that UASB reactor was not feasible, as the pollutants were not properly degraded.

2.3.2.7. Emerging treatment approaches of pulp and paper mill effluent

2.3.2.7.1. Constructed wetlands

Constructed wetlands (CW) are emerging, low-cost and eco-friendly sustainable wastewater treatment systems. These are engineered integrated wastewater treatment systems of plants, water, microorganisms and the environment. Plants, soil, sand and gravels make shallow beds or channels, and a variety of microorganisms grow on these beds or channels to improve the quality of wastewaters (USEPA 2004). Both systems are able to remove high biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), colour, metals and toxic compounds, which are present in various types of industrial wastewaters. Plants directly uptake the nutrients from soil and facilitate indirect aerobic degradation of pollutants. Conventional treatment methods are not very much capable of removing high organic pollutants and colour from pulp and paper mill wastewaters. Constructed wetlands should be a better treatment way for pulp and paper mill wastewaters because their treatment efficiency is higher than conventional treatment methods (Choudhary et al., 2011).

2.3.2.7.2. Biocomposting

Biocomposting is one of the most valuable and green treatment processes for the mitigation of various harmful pollutants of industrial wastewaters. This method is also suitable for the treatment of wastes and sludges especially produced from paper fibres and organic materials. In biocomposting, wastes are dumped with microorganisms; humus like mater is produced which may be used in agriculture, houseplant greenhouse, etc. (Christmas, 2002; Gea et al., 2005). Microorganisms converted organic materials into CO2, humus and heat. The increased temperatures (thermophilic phase) in composts found that the rapid degradation of lignocelluloses

occurs and is mainly degraded by thermophilic micro-fungi and actinomycetes (Tuomela et al., 2000).

2.3.2.7.3. Enzymatic treatments

Several enzymes such as peroxidases, oxidoreductases, cellulolytic enzymes, cyanidase, proteases, amylases, etc. are used to treat industrial wastewaters. Ligninolytic group (laccase, MnP and LiP) of peroxidases from a variety of different sources has been reported to play an important role in waste and wastewater treatment (Chandra and Chowdhary, 2015). Recently the demand of these enzymes (laccase, LiP, MnP) has increased gradually due to their prospective applications in the diverse biotechnological areas. Lignin peroxidase (LiP) and Mn peroxidase (MnP) were reported to be very efficient in decolourization of kraft pulp mill wastewaters (Moreira et al. 2003). Predominantly white-rot fungus and their specific enzymes are required for lignin degradation. *Phanerochaete chrysosporium* is one of the most important white-rot fungus and widely studied model for lignin degradation. Bacterial laccases are most considerable in the remediation of pollutants of industrial wastes. Laccases are also involved in the treatment of various industrial wastewaters such as pulp and paper, textile, tannery, distillery, etc. (Sangave and Pandit**,** 2006; Chandra and Chowdhary, 2015; Mani and Bharagava, 2016; Bharagava and Mishra, 2018; Chowdhary et al., 2017).

Haq et al. (2016a) isolated a lignin-degrading bacterial strain (*Serratia liquefaciens*) capable of decolourising Azure-B dye was isolated from the contaminated site of pulp and paper mill effluent. This strain effectively reduced pollution parameters (colour72%, lignin 58%, COD 85% and phenol 95%) of real effluent after 144 h of treatment at 30◦C, pH 7.6 and 120 rpm. Extracellular lignin peroxidase enzyme produced by *S. liquefaciens* during effluent treatment was purified

to homogeneity by using ammonium sulfate precipitation and DEAE cellulose column chromatography. The molecular weight of the purified lignin peroxidase was estimated to be ~28 kDa and the optimum pH and temperature for purified lignin peroxidase activity were determined as pH 6.0 and 40◦C, respectively. The toxicity of treated effluent was evaluated by alkaline single cell (comet) gel electrophoresis (SCGE) assay using *Saccharomyces cerevisiae* MTCC 36 as model organism. The toxicity reduction in treated effluent was 49.4%.

Sahadevan et al. (2016) isolated a novel lignin degrading *Deuteromycete* from the soil, identified as ‘‘uncultured’’ and coded as MVI.2011 and analysed for Lignin Peroxidase, Manganese Peroxidase and Laccase from 12 h culture of MVI.2011 in optimized mineral medium containing lignin at pH 9.0. Enzyme purification was performed by standard protocols using ammonium sulphate precipitation followed by further purification by Gel Permeation Chromatography. Analysis of total protein, enzyme activity and molecular weight of the purified LiP, MnP and Laccase showed 93.83 µg/ml, 5.27 U/mg, 42 kDa; 78.13 µg/ml, 13.18 U/mg, 45 kDa and 85.81 µg/ml, 4.77 U/mg, 62 kDa, respectively. These purified enzymes possessed high activity on pH (4-11) and temperature (30-55℃).

Patil (2014) worked on lignin peroxidase enzyme produced by the bacteria strain *Bacillus megaterium* at optimized conditions: pH (7), temperature (37℃), inoculum size (6%), agitation speed (180 rpm) and nutritional parameters like carbon (lignin-1.5%) and nitrogen (peptone-2%). Lignin Peroxidase was purified by using ultra filtration, ammonium sulphate precipitation and dialysis. The purity was checked by SDS-PAGE and the molecular weight of the enzyme was estimated to be 65kDa. *Bacillus megaterium* strain decolorized the dyes congo red, methylene blue and malachite green upto 94.90%, 63.23% and 6.40% within 96 h of incubation. *Bacillus*

megaterium effectively acted on pulp and paper, textile red and textile blue samples with decolourization percentage of 93.70%, 48.57% and 31.64% respectively within 96 h.

Kreetachat et al. (2016) worked on the decolorization of pulp and paper mill effluents and the production of ligninolytic enzymes by wood rotting fungus *Fibrodontia* sp. RCK783S. The experimental studies results revealed that laccase was the main enzyme involved in the decolorization of pulp and paper mill effluents. Optimization of laccase was performed as C/N ratio 15.0 to 25.0, CuSO4 of 0.002 to 0.0004 g/L and L-asparagine of 1.563 to 2.813 g/L. The maximum laccase production of 5,145 unit/L was observed at C/N ratio of 18.0, CuSO4 0.0035 g/L and L-asparagine concentration of 2.2256 g/L. The maximum colour and TOC removal efficiency were 61.58 and 48.32% at 5 days of incubation, respectively.

2.3.2.7.4. Biosorption

Sorption is a process in which one substance is attached to another and bio means the involvement of living entities, i.e. biosorption is a physiochemical process that can be defined as the involvement of live entity like fungi or bacteria (adsorbent) and chemical or metal (adsorbate) leading to the removal of substances from solution through biological materials (Aksu, 2002; Gadd, 2009). Biosorption is used to treat wastewaters produced from various industrial sectors, and this treatment is considered clean, efficient, cost-effective and easy to operate (Saiano et al., 2005).

Singhal et al. (2016) isolated a fungal strain, *Emericella nidulans* (anamorph: *Aspergillus nidulans*), from pulp and paper mill effluent contaminated site. This strain was used for biosorption of colour from pulp and paper mill effluent. After the treatment of the effluent fungus turned dark brown in colour. The surface morphology of the fungus was characterized by SEM and FT-IR analysis. Their

study concluded that adsorbents capable of removing pollutants from pulp and paper waste stream.

Chapter 3

Isolation, Screening and Characterization of Lignin Degrading Bacterial Strains

Chapter 3

3. Introduction

Water pollution is the major environmental concern. It is necessary to treat the wastewater through appropriate treatment technology, so that properly treated wastewater is discharged into the environment. Microorganisms play an important role in nutritional chains, which are responsible to maintain the ecological balance of the environment. The microorganisms ability to transform and degrade various types of pollutants, which are present in soil, water, sediments and air, has been widely documented during the last few decades. The major problem associated with pulp and paper industry, is due to the presence of lignin and its derivatives, which provides dark brown colour, high BOD, COD and chlorinated organic pollutants to the wastewater.

Microorganisms are better options for the degradation and detoxification of pollutants present in pulp and paper wastewaters and are considered as cost effective as well as ecofriendly in nature. Thus, the isolation of lignin degrading bacterial strains from sludge and wastewater, attracted towards their potential use in the bioremediation of pulp and paper mill effluent (Hammel and Cullen, 2008).

Many scientists have reported various microorganisms that are proficient for the colour reduction, lignin degradation, and removal of other toxic pollutants in pulp and paper mill wastewater. Microorganisms are natural recyclers, converting toxic organic compounds to harmless products. The majority of basidiomycetous (white rot fungus) fungi such as *Phanerochaete chrysosporium*, *Pleurotusostreatus*, *Trametes versicolor* and *Lentinus edodes* have been the most studied microorganism for the biodegradation of lignin and coloured compounds of pulp and paper wastewaters (Minussi et al., 2007). However, fungal system requires low pH for the treatment of

effluent, while the pH of pulp and paper mill wastewater tends to be neutral to alkaline (i.e. between 7.0-9.0), thus, the pH of the wastewaters must be reduced, prior to the fungal treatment, which adds an additional cost for the treatment approach (Raghukumar et al., 2008; Costa et al., 2017).

In contrast, bacteria survive well in neutral (pH-7.0) to alkaline (pH-9.0) conditions and are suitable for the treatment of pulp and paper mill wastewaters without additional need of pH adjustment (Brown and Chang, 2014; Rahman et al., 2013). Further, several bacterial species like *Bacillus* sp., *Pseudomonas* sp., *Paenibacillus* sp., *Serratia liquefaciens, Citrobacter freundii, Brevibacillus sgri* have been evaluated for lignin degradation (Chandra et al., 2007; Abd-Elsalam and EL-Hanafy, 2009, Hooda et al., 2015) and for pulp and paper mill effluent treatment (Raj et al., 2007; Singh et al., 2011; Chandra and Singh, 2012). Bioremediation of pulp and paper mill effluent by a laccase producing *Paenibacillus* sp. effectively reduced colour 68%, lignin 54%, phenol 86%, BOD 83% and COD 78%, after 144 h of incubation (Raj et al., 2014). Haq et al. (2016a) reduce lignin 53%, colour 72%, COD 85% and phenol 95%.

Thus, this chapter explained about the isolation, screening and identification of lignin degrading bacterial strains that are capable for the effective degradation and detoxification of lignin and pulp and paper mill effluent.

3.1. Materials and Methods

3.1.1. Collection of pulp and paper mill wastewater

The pulp and paper mill effluent was collected in a pre-sterilized plastic Carboy container (Cap. 10 L) from Century pulp and paper mill Ltd., Lalkuan, Uttarakhand (India) situated 8 Km, north east of Pantnagar (29°N latitude, 79.3°E longitude and altitude 243.8 m (Fig. 3.1). The collected wastewater samples were brought to

laboratory and stored at 4 ˚C. Further, the collected samples were used for physico-chemical analysis, isolation of lignin degrading bacterial strains, metabolite characterization and toxicity assessment test.

Figure 3.1: Collection of wastewater and sludge samples from Century Pulp and Paper mill

3.1.1. Media composition

The Kraft lignin used in this experimental study was procured from Sigma-Aldrich (USA). The medium used throughout the study was Mineral Salt Medium agar (MSM) which contained (in gL^{-1}) Na_2HPO_4, 2.4; KH_2PO_4, 2.0; NH_4NO_3, 0.1; $MgSO_4$, 0.01; $CaCl_2$, 0.01; glucose, 10.0; peptone, 5.0 and agar, 15.0 (Hi-media, Mumbai). Medium was adjusted to pH 7.6 prior to autoclaving for sterilization at 121 ^oC for 15 minutes.

3.1.2. Isolation of lignin degrading bacterial isolates from pulp and paper mill effluent

The collected sludge sample was used for the isolation of the potential lignin degrading bacterial strains. Sludge samples of the industry are rich source of microorganisms. Enrichment culture technique followed by serial dilution method was used for the isolation of the bacterial strains. (Chandra et al., 2008; Chandra and Bharagava, 2012). Sludge sample (5 gm) was added in 100 mL sterilized mineral salt medium (MSM), supplemented with lignin (100 mg/L) as carbon source. The flasks were incubated for 24-48 h in an incubator shaker at 35 ºC and 120 rpm. Following incubation, 10 mL of the broth culture was transferred to the freshly prepared MSM broth medium having the same composition and lignin concentration. The same procedure was successively repeated for 2-3 times. The final enriched culture samples (10 mL) were serially diluted and were spread on lignin amended mineral salt medium (L-MSM) agar plates having the same lignin (100 mg/L) concentration and incubated at 35ºC for 24-48 h. Morphologically and phenotypically different bacterial colonies showed on L-MSM plates were selected and purified by repeated streak plate method.

3.1.2.1. Screening of lignin degrading bacterial isolates

The bacterial isolates collected from pulp and paper mill wastewater and sludge samples through serial dilution method and purified by consecutive streaking on the same culture medium. These bacterial isolates were screened by primary and secondary screening tests to obtain most effective and potent bacterial isolates that are capable for the reduction in lignin and colour.

3.1.2.1.1. Primary screening test

The primary screening of selected purified bacterial isolates was done on the basis of their tolerance limit on L-MSM agar plates with increasing concentration of kraft

lignin. The plates were streaked with isolated bacterial strains and incubated at 35 ºC for 24-48 h. The bacterial isolates, that were capable to grow and tolerate higher concentration of lignin, were selected for secondary screening test.

3.1.2.1.2. Secondary screening test

The secondary screening was done by streaking the bacterial isolates on MSM agar plates containing different ligninolytic substrate such as phenol red dye (0.002% w/v) (Archibald 1992), azure B dye (0.002% w/v) (Manji and Ishihara, 2004) and guaiacol (0.005% v/v) (Coll et al., 1993). The plates were incubated for 4-5 days at 35 ºC. The clear decolourization zone appeared around the bacterial colony were screened as positive for ligninolytic enzymes and selected for further studies.

3.1.3. Morphological and biochemical characterization of isolated lignin degrading bacterial strains

On the basis of primary and secondary screening results, three bacterial isolates PLP 6, PNP 13 and PNP 19 were selected. These bacterial isolates were primarily characterized morphologically and biochemically according to the standard protocols of Bergey's Manual of Determinative Bacteriology (Whitman et al., 2012). The morphological characters were determined microscopically by their shape, colour, surface texture, margin and elevation and gram staining. Further, the biochemical characterization was performed based on certain biochemical tests such as: catalase, oxidase, MR-VP, V-P test, starch and gelatine hydrolysis, motility, indole production and citrate utilization etc.

3.1.3.1. Gram's staining

The gram staining is a differential staining process discovered by Dr. Hans Christian Gram (a Danish physician) in 1884. This method is most commonly used for direct microscopic examination of specimens and subculture. This method is very

useful for identifying and classifying bacteria into two major groups: the gram-positive and gram-negative.

Principle:

The gram negative bacterial cell wall is thin complex multi-layered structure and contains relatively high lipid content in addition to protein and muco-peptide. The higher amount of lipid is readily dissolved by alcohol, resulting in the formation of large pores in the cell wall which do not close appreciably on dehydration of cell wall proteins, thus facilitating the leakage of crystal violet iodine (CV-I) complex and resulting in the decolourizing of bacterium, which later takes the counter stain and appears red. In contrast, the gram positive cell walls are thick and chemically simple, composed mainly of proteins and cross linked muco-peptide. When treated with alcohol, it causes dehydration and closure of cell wall pores, thereby not allowing the loss of CV-I complex and cell remains purple.

Procedure:

1. 24 h overnight grown culture was taken.
2. Make a thin smear on glass slide was prepared. Heat fix the smear.
3. Flood the crystal violet on each smear for 30 seconds
4. Wash each slide with distilled water for a few seconds
5. Expose the smear with grams iodine solution for 60 seconds
6. Wash off the iodine solution with 95% ethyl alcohol. Add ethyl alcohol drop by drop, until no more colour flows from the smear. (The gram positive bacteria are not affected while all gram negative bacteria are completely).
7. Wash the slides with distilled water and drain
8. Apply safranin to smear for 30 seconds (counter- staining). Washed with distilled water and blot dry with absorbent paper.

9. Wash and drain the slides and examined under oil-immersion.

Interpretation:

Bacterial cells stained crystal violet colour were gram positive, whereas; cells with red or pink colour stain were gram negative.

3.1.3.2. Biochemical tests

A. Catalase Test

During aerobic respiration, microorganism produces hydrogen peroxide which is lethal to the cell. The enzyme catalase presents in some microorganisms breaking hydrogen peroxide into oxygen molecule and helps them in their survival from the lethal effect of H_2O_2 which is accumulated as an end product of aerobic carbohydrate metabolism.

Procedure:

Take 18-24 h old bacterial culture and place it on slide. Add one drop of 3% H_2O_2 and observe for immediate bubbling.

Interpretation:

Appearance of gas bubbles showed positive catalase reaction, whereas no or very few bubble formation showed negative result.

B. Motility test

Bacterial cells have the ability to move itself is called motility. Prokaryotes move by means of propeller-like flagella unique to bacterial or by special fibrils that produce a gliding form of motility.

Procedure:

Bacterial strain may be flagellated or non-flagellated. When they bear flagella, shows motility or movement otherwise they are included under the category of non motile organisms. Motility of bacteria was checked by stabbing the bacterial culture

into sterilised motility agar medium from top of the medium to a depth of about 5mm and incubated at 37 ºC for 24-48h.

Interpretation:

Motile bacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque, whereas; non-motile bacteria generally give growth that are confined to the stab-line, have sharply defined margins and leave the surrounding medium clearly transparent.

C. Amylase production or starch hydrolysis

Amylase is an exoenzyme that hydrolyses starch, a polysaccharide into maltose, a disaccharide and some monosaccharides such as glucose. These disaccharides and monosaccharides enter into the cytoplasm of bacterial cell through the semi permeable membrane and thereby used by endo-enzymes. Starch is a complex carbohydrate composed of amylase and amylopectin. The ability of microbe to degrade starch is a criterion for the determination of amylase production.

Procedure:

Dissolving the constituent of starch agar medium into 100 mL distilled water. The medium was then autoclaved at 121 ºC (15 psi) for 15 min. The autoclaved agar medium was poured into sterile petriplates to solidify. The plates were then inoculated with bacterial culture using single streak and incubate at 37 ºC for 24-48 h. Flood the surface of the plate with iodine solution for 30 seconds.

Interpretation:

If starch has been hydrolysed, the colour of the medium changed around the line of the organism indicates positive result and no colour change indicates negative result.

D. Citrate utilization test

Citrate utilization test is used to detect the ability of an organism to utilize sodium citrate as a sole carbon source and ammonium salt as a sole source of nitrogen. Bacteria that grow in the citrate medium turn the medium from acidic to alkaline condition. This is indicated by the change of colour of bromothymol blue indicator from green to blue.

The utilization of citrate depends on the presence of an enzyme citrate produced by the organism, which breaks down the citrate to oxalo acetic acid and acetic acid. These products are later converted to pyruvic acid and carbon-di-oxide enzymatically.

The test is usually performed by using an organic synthetic medium, Simmon's citrate agar, where sodium citrate is the only source of carbon and energy and the bromothymol blue is used as an indicator. When the citric acid is metabolized, the CO_2 generated combines with sodium and water to form sodium carbonate, an alkaline product, which changes the colour of the indicator from green to blue resulting positive test.

Procedure:

Dissolved Simmon's citrate agar medium in distilled water (adjust pH to 6.8), autoclaved at 121 ºC (15 psi) for 15 min. Poured 4-5 mL autoclaved medium into sterile boiling tube and solidify in slanted position. Take a 24 h old loopful culture and streaked on citrate medium and incubate at 37 ºC for 48 h.

Interpretation:

Bacterial growth with colour changes of slant from green to intense blue showed positive reaction for citrate utilization, whereas; no colour change showed negative reaction.

E. Casein hydrolysis

Casein is a macromolecule composed of amino acid linked together by peptide bonds CO-NH and it is the major protein found in milk. Some microorganisms have ability to degrade the protein casein by producing proteolytic exo-enzyme called proteinase which breaks CO-NH bond by introducing water molecule, liberating smaller chains of amino acids called peptidase. These are later break down into free amino acids by extracellular or intracellular peptidases and transported through the cell membrane into the intracellular amino acid pool for the synthesis of structural and functional cellular proteins.

Procedure:

Dissolve the skim milk agar medium into 100 mL distilled water. The medium was then autoclaved at 121 ºC (15 psi) for 15 min. The autoclaved agar medium was poured into sterile petriplates to solidify. The plates were then inoculated with bacterial culture using single streak and incubate at 37 ºC for 24-48 h.

Interpretation:

A clear halo-zone around the inoculated organism on skin milk agar plate indicates positive test for casein hydrolysis, and no zone formation indicates negative test.

F. Hydrogen sulphide (H_2S) production test

Sulphur and iron compound are used for the production of hydrogen sulphide gas. Hydrogen sulphide is produced if the sulphur compound is reduced by the bacterial strain. This happens when the bacteria either degrade the amino acid cysteine during protein degradation or when anaerobic respiration shuttles the electrons to sulphur instead of oxygen. The black precipitate of H_2S is also produced when it react with

the heavy metal such as iron or lead to forms ferric sulphide or lead sulphide. The black colour acts as an indicator for the presence of hydrogen sulphide.

Procedure:

Dissolve SIM agar medium into 100 mL distilled water. The medium was then autoclaved at 121 ºC (15 psi) for 15 min. The autoclaved agar medium was poured into sterile test tubes and solidifies in slanted position. Take overnight grown cultures and were streaked on slants. The slants were then incubated at 37 ºC and examined after 48-72 h.

Interpretation:

The appearance of black precipitation in inoculated tubes showed positive test for H_2S production and tubes without black precipitation showed negative test.

G. Indole test

Indole is a volatile chemical compound and can be detected either by testing the medium with (p-dimethylaminobenzaldehyde) Kovac's reagent or with oxalic acid. Tryptophan, an essential amino acid, is oxidized by some bacteria by the enzyme tryptophanase, resulting in the formation of indole, pyruvic acid and ammonia. The indole produced during the reaction is detected by adding Kovac's reagent, which gives a cherry-red reagent layer.

Procedure:

Dissolved peptone broth medium in distilled water and autoclaved at 121 ºC (15 psi) for 15 min. Poured 10 mL autoclaved medium into sterile test tube and cooled. Take 24 h old culture, inoculated into the medium and incubated for 48 h at 37 ºC. After 48 h of incubation, add 1 mL Kovac's reagent drop by drop, shaken well and examined after 1 min.

Interpretation:

Formation of cherry-red layer into the broth medium after addition of Kovac's reagent gives positive indole test, whereas; no formation of cherry red layer gives negative indole test.

H. Gelatin hydrolysis test

This test is used to determine the ability of an organism to produce extracellular proteolytic enzymes gelatinases that hydrolyze gelatin. The gelatin dissolves in water at 50 ºC and exists in liquid form above 25 ºC and solidifies as gel below 25 ºC. The hydrolysis reaction of gelatin carried out in two steps: in first step gelatinases hydrolyze gelatin into polypeptides and in second reaction it converted into amino acids. The amino acid is taken up by the cell used for their metabolic purposes. The presence of gelatin is detected by mercuric chloride ($HgCl_2$) that forms white precipitate with gelatin protein.

Procedure:

Dissolve the gelatin agar medium into 100 mL distilled water. The medium was then autoclaved at 121 ºC (15 psi) for 15 min. The autoclaved agar medium was poured into sterile petriplates to solidify. The plates were inoculated with bacterial culture using single streak and incubate at 37 ºC for 24-48 h. After incubation the plates flooded with $HgCl_2$ solution.

Interpretation:

A clear halo-zone around the bacterial colony on agar plate indicates positive test for gelatin hydrolysis, and no zone formation indicates negative test.

I. Triple sugar iron (TSI) agar test

Triple sugar iron gar test is used to test the ability of microorganisms to ferment sugars (glucose, lactose and sucrose) and produce hydrogen peroxide. It is often used

in the identification of enteric bacteria. TSI is a differential medium that contains lactose, sucrose and small amount of glucose (dextrose), ferrous sulphate and pH indicator phenol red. It is used to differentiate enteric based on the ability to reduce sulphur and ferment carbohydrate.

Procedure:

Prepare triple sugar iron agar medium slants and streak the overnight grown culture into test tube. The tube without streaking served as control. Incubate the tube for 18-24 h at 37 ºC. Examine the colour of both butt and slant of all agar cultures.

Interpretation:

If lactose or sucrose is fermented a large amount of acid is produced, which turns the phenol red indicator in to yellow in both butt and slant.

J. *Oxidase test*

This test is used to determine the presence of cytochrome C and the production of oxidase enzyme in an organism. Cytochromes are catalytic heme containing enzyme that are tightly bound to the plasma membrane of prokaryotic cells. Cytochrome C oxidase is an enzyme that does not react directly with oxygen but its reduced form will be oxidized by cytooxidase. Many bacteria that live in the presence of oxygen produce cytochrome oxidase. Cytochrome oxidase is the final link in the electron transfer system that provides ATP during respiration. This enzyme receives electrons and passes them to oxygen and forming water. Cytochrome oxidase oxidizes p-aminomethylamiline to form a purple pigmented product due to oxidation.

Procedure:

Oxidase discs (Hi-media) impregnated with oxidase reagent was inoculated into 24 h old bacterial broth culture. The inoculated discs were examined within 30-60 sec on the basis of their coloration.

Interpretation:

Appearance of purple-blue coloration of oxidase disc within 30-60 sec considered as a positive reaction. No coloration or coloration that formed later than 1 min considered as negative test.

3.1.4. Molecular characterization and identification of lignin degrading bacterial strains

3.1.4.1. 16S rRNA gene sequence analysis

The molecular characterization of bacterial isolates was done by 16S rRNA sequencing analysis. The genomic DNA was isolated from overnight grown bacterial suspension using alkaline lysis method Kapley et al. (**2001).** About 5µL of genomic DNA (template DNA) was used to amplify the 16S rRNA gene by using universal primers (27F) 5' -AGAGTTTGATCMTGGCTCAG-3' and (1492R) 5' -CGGTTACCTTGTTACGACTT-3' (Narde et al., 2004) to produce □1500 bp**.** The reaction mixture contained 1X PCR buffer, 5µL template DNA, 200 µM of each dNTP, 3.0 mM $MgCl_2$, 25 pmol of primer and 2.5 units of Amplitaq DNA polymerase (Perkin Elmer) in a final volume of 50µL (Bharagava et al., 2009).

The thermocycling reactions were carried out by using Verti® 96- Well Thermal Cycler (Applied Biosystems, USA). 35 cycles were run to amplify the 16S rDNA fragment. The initial denaturation at 95 ºC (5 min), succeeding denaturation at 94 ºC (30s), followed by annealing at 50 ºC (30s), extension temperature at 72 ºC (1.30 min) and the final extension at 72 ºC (7 min). The PCR products were viewed after electrophoresis on 1% agarose gel having 1µg/mL of EtBr and purified by using gel extraction kit (Merk Biosciences, Bangalore) and visualized under UV light. The gel purified PCR products were sequenced by Chromous Biotech, Pvt Ltd. (Bangalore, India) on ABI 3500 Genetic Analyzer, using Big Dye Terminator Version 3.1.

3.1.4.2. Accession no. and Phylogenetic analysis

The sequence was blasted using the online option available at www.ncbi.nih.gov/BLAST (Altschul et al., 1997) to found the closest homology of the bacterium. Phylogenetic trees were constructed using NCBI database online phylogenetic tree builder (http://www.ncbi.nlm.nih.gov). In addition, the sequences were submitted to Gene-bank to obtain the accession number of the isolated bacterial strains.

3.2. Statistical analysis

All the experiments were performed in triplicates (n=3). The results obtained from each set of experiment have been expressed in terms of mean and standard deviation.

3.3 Result and Discussion

3.3.1. Lignin degrading bacteria isolates from pulp and paper mill effluent

Total twenty one morphologically different bacterial strains were isolated and purified on lignin amended MSM agar plates from pulp and paper mill effluent contaminated sludge samples. Primary screening was done on the basis of their tolerance limit of increased concentration of lignin (200-1500 ppm) on L-MSM agar plates. Out of twenty one, six bacterial isolates were selected which found to have maximum tolerance limit of lignin concentration i.e. PLP 2, 1200 ppm; PLP 6, 1300 ppm; PNP 13, 1100ppm; PNP 17, 1200 ppm; PNP 19, 1400 ppm (Fig. 3.2). Further, secondary screening was performed on the basis of their capability to produce ligninolytic enzymes by using dye decolourization plate assay method. Out of six, three bacterial isolates (PLP 6, PNP 13 and PNP 19) were found able to decolorize the dyes i.e. Phenol Red and Azure B (Fig. 3.3). PNP 13, and PNP 19 changed the colour of phenol red and produced yellow zone around the colony at 48 h, indicating the presence of manganese peroxidase enzyme (Chandra and Singh. 2012) while, PLP 6

disappeared the blue colour of Azure-B dye at 72 h incubation period, it confirmed the presence of lignin peroxidase enzyme (Haq et al., 2016a). There was no formation of brown colour decolourization zone around the colony on guaiacol agar plates showed the absence of laccase enzyme. On the other hand no activity was observed on control plates which were devoid of ligninolytic substrates. Thus, on the basis of screening tests three bacterial isolates were selected for further study (Fig. 3.4).

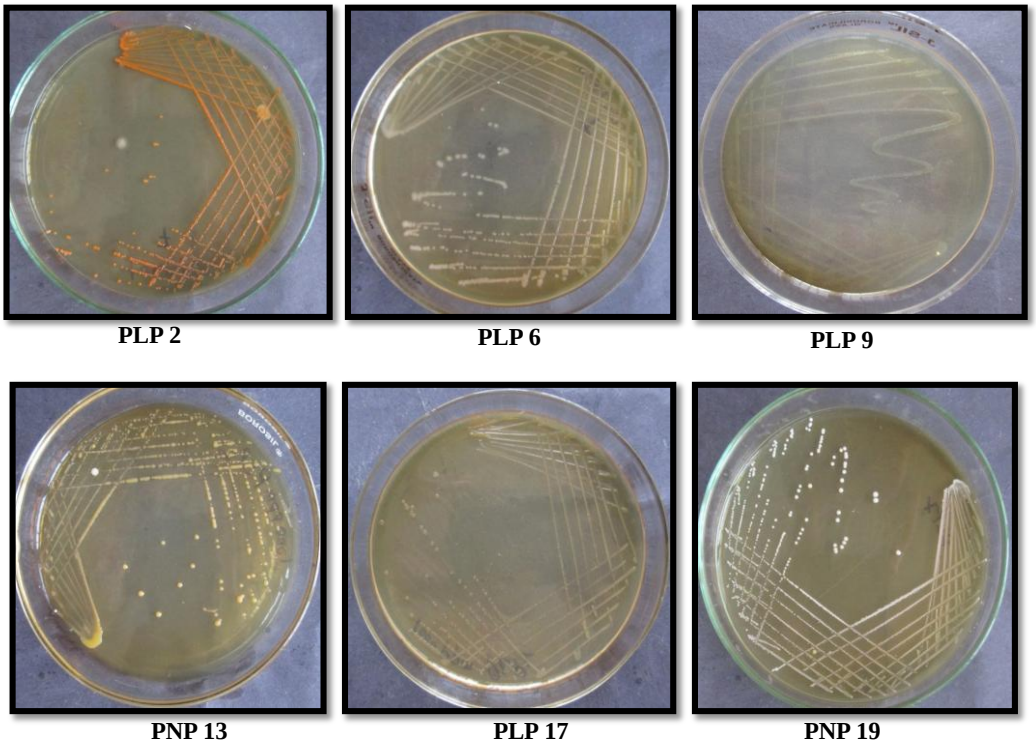
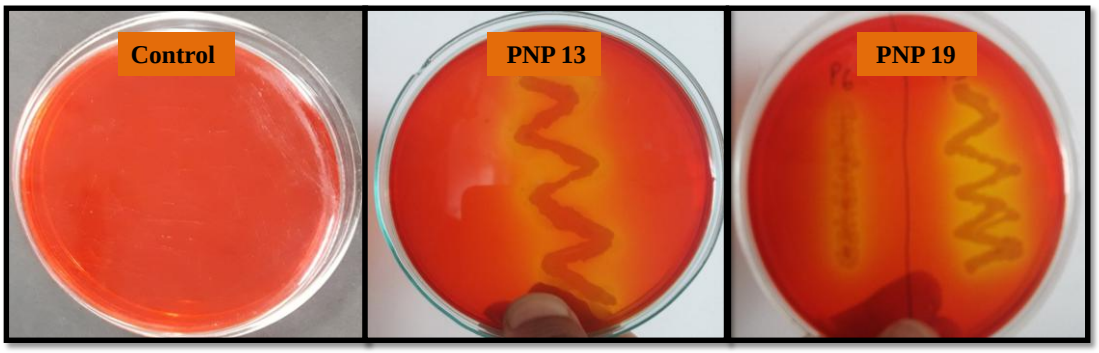
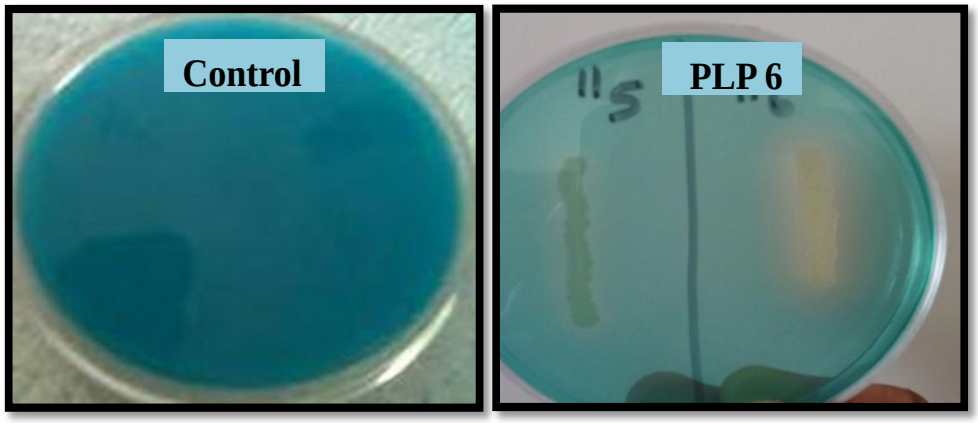


Figure 3.2: Isolation and purification of bacterial strains from pulp and paper mill wastewater and sludge sample on L-MSM agar plates



Manganese peroxidase activity



Lignin peroxidase activity

Figure 3.3: Bacterial strains PLP 6 showing LiP and strains PNP 13 and PNP 19 showing MnP activity on Azure B and phenol red containing MSM agar plates

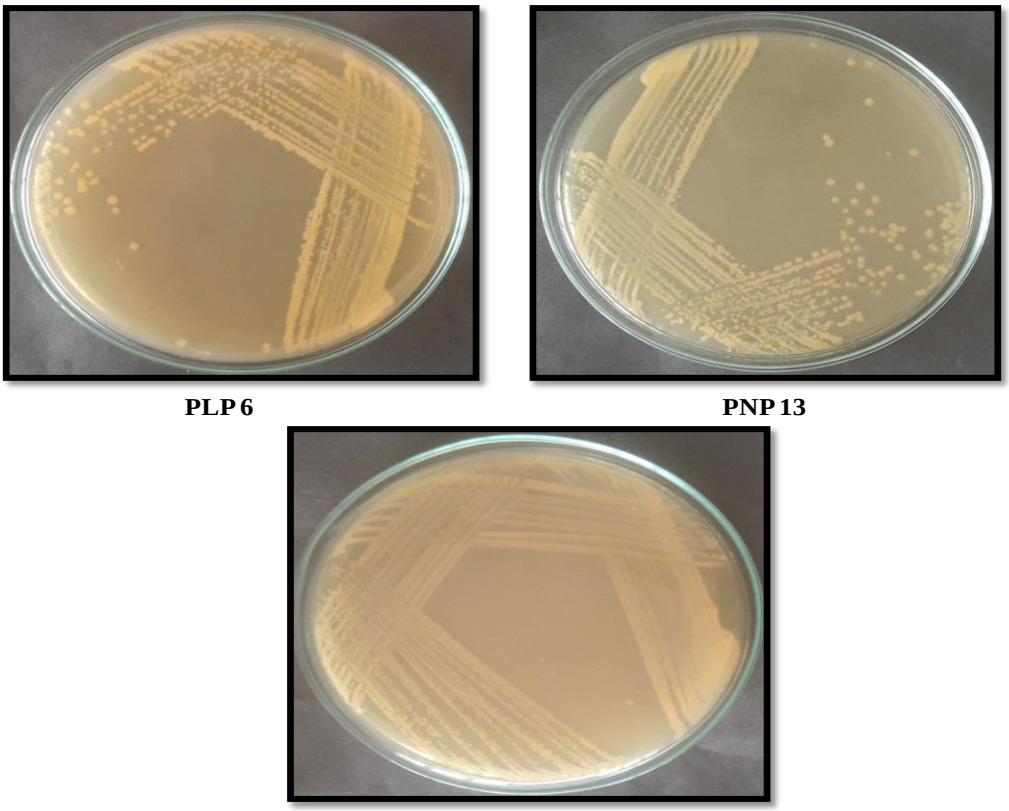


Figure 3.4: Purified cultures of lignin degrading bacterial strains

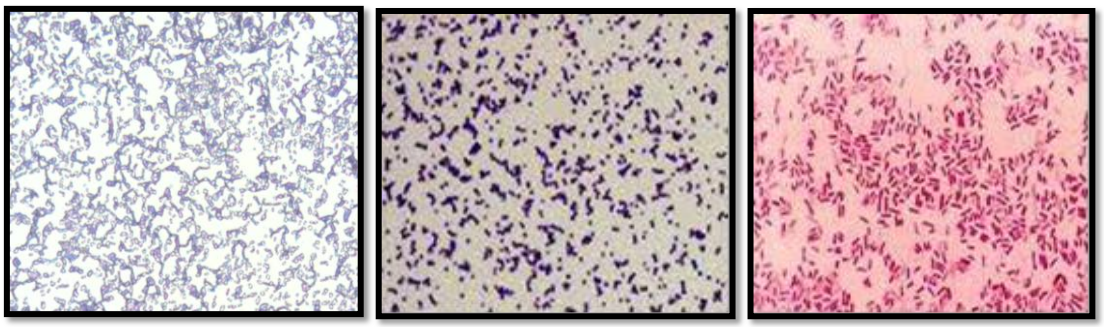
3.3.2. Morphological and biochemical characteristics of lignin degrading bacterial isolates

The lignin degrading bacterial isolates were morphologically different to each other in terms of colour, shape and other characteristics. The morphological characteristics of bacterial isolates were shown in Table 3.1. The isolates PLP 6 and PNP 13 showed light brown colour colony grown on L-MSM agar plates while; PNP 19 showed white colour colony on the same medium. All the bacterial strains showed small and circular colonies and having entire margin. Grams' staining revealed that bacterial isolates PLP 6 and PNP 13 were gram positive, as they stain purple colour whereas PNP 19 was rod shaped and gram negative as it stains red colour respectively (Fig. 3.5).

After morphological characterization, bacterial isolates were further, subjected to various biochemical tests for their characterization. All the three bacterial isolates PLP 6, PNP 13 and PNP 19 had given positive tests for catalase, oxidase, motility and starch hydrolysis. PLP 6 and PNP 13 gives positive tests for casein hydrolysis whereas, PNP 19 showed negative test for casein hydrolysis as it is failed to produce inhibition zone. Only PNP 19 had given positive test for citrate utilization. However, indole test and lipase test was negative for all the three bacterial isolates. Further, the bacterial isolates were also tested for H_2S production; cellulase and several other biochemical tests were represented in Table 3.2 and Fig. 3.6.

Table 3.1: Morphological characteristics of lignin degrading bacterial isolates

S. No.	**Morphological characteristics**	**Bacterial isolates**		
		PLP 6	**PNP 13**	**PNP 19**
1.	Colony colour	Light brown	Light brown	White
2.	Gram stain	Positive	Positive	Negative
3.	Colony shape	Rod	Rod	Rod
4.	Surface texture	Rough	Rough	Normal
5.	Margin	Entire	Entire	Entire
6.	Elevation	Convex	Convex	Convex



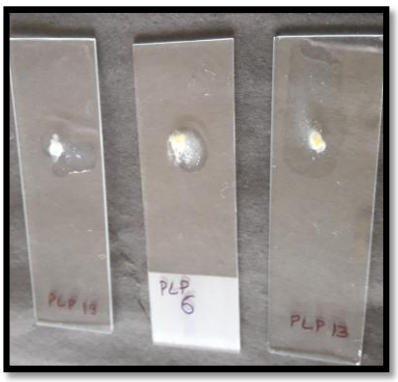
PLP 6 **PNP 13** **PNP 19**

Figure 3.5: Gram staining of lignin degrading bacterial isolates PLP 6 and PNP 13 (gram +ve), PNP 19 (gram -ve).

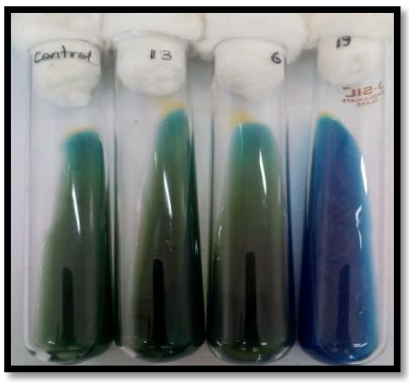
Table 3.2: Biochemical characteristics of isolated bacterial isolates

S. No.	Biochemical test	Bacterial isolates		
		PLP 6	**PNP 13**	**PNP 19**
1.	Catalase	+ve	+ve	+ve
2.	Oxidase	-ve	-ve	-ve
3.	Motility	+ve	+ve	+ve
4.	Casein hydrolysis	-ve	-ve	-ve
5.	Starch hydrolysis	+ve	+ve	+ve
6.	Indole test	-ve	-ve	-ve
7.	Citrate utilization	-ve	-ve	+ve
8.	Cellulase	+ve	-ve	+ve
9.	Gelatinase	+ve	-ve	-ve
10.	H_2S production	-ve	-ve	-ve

(+ve) = Positive, (-ve) = Negative.

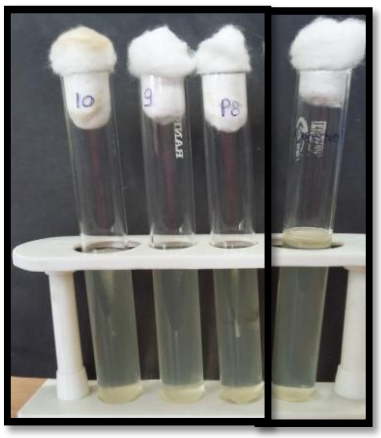


Catalase test



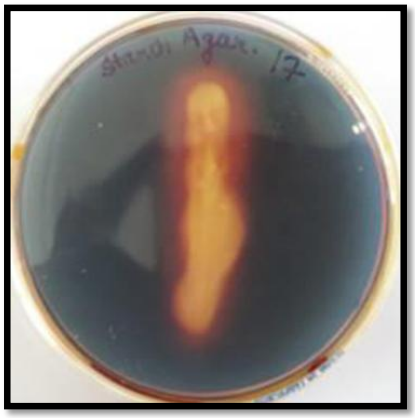
Citrate test

H_2S production



Motility test

Casein hydrolysis



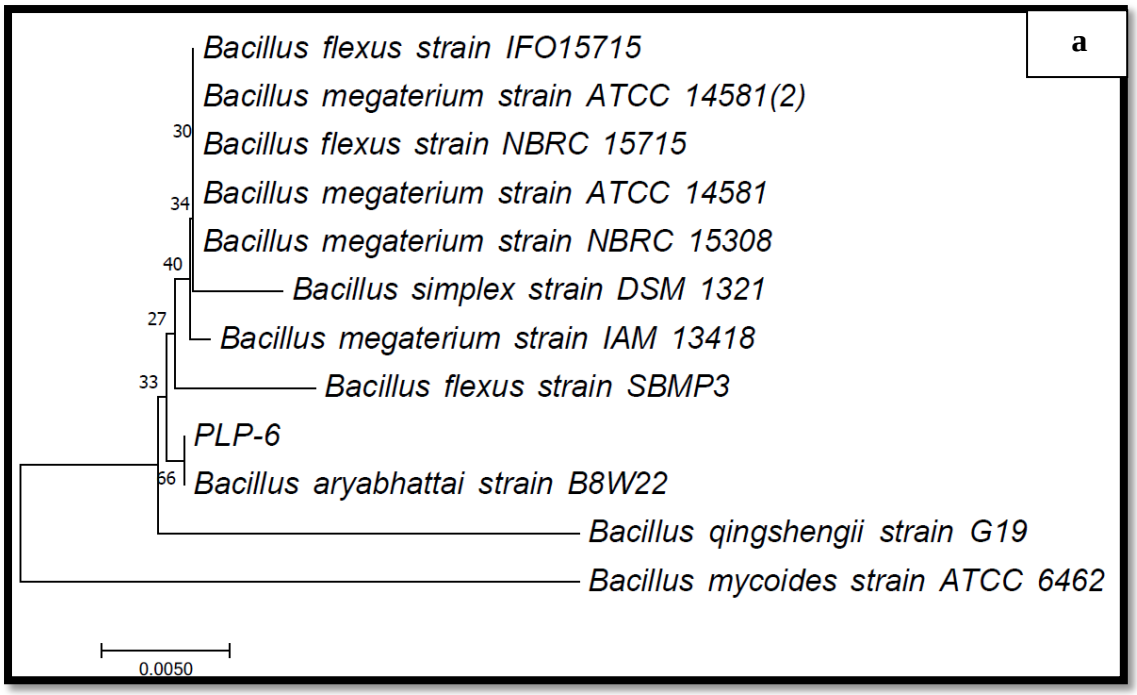
Amylase test

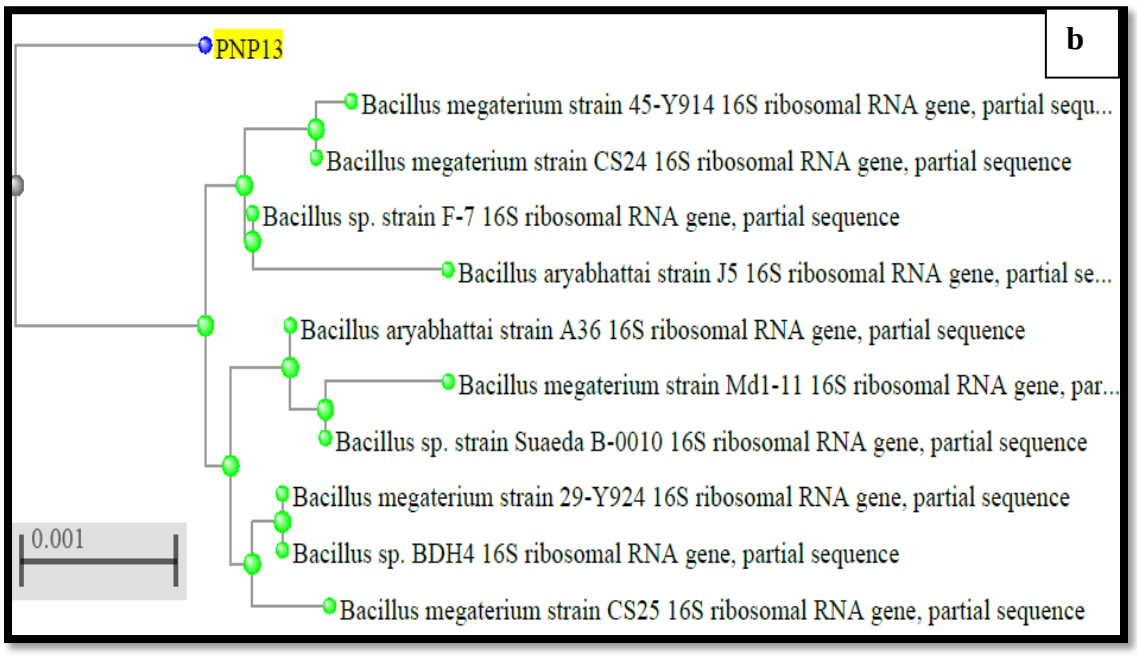
Gelatinase test

Figure 3.6: Biochemical tests shown by lignin degrading bacterial strains

3.3.3. Molecular identification of lignin degrading bacterial

The molecular identification of bacterial isolates was done by 16S rRNA gene sequence analysis. Homology and BLAST (n) analysis revealed that, the bacterial isolates PLP 6, PNP 13 and PNP 19 has closest relatedness with the genus of *Bacillus* and *Pseudomonas*. Thus, on the basis of sequence similarity and BLAST (n) analysis, the bacterial isolates PLP 6, PNP 13 and PNP 19 were identified as *Bacillus aryabhattai, Bacillus megaterium* and *Pseudomonas* sp. The phylogenetic tree was constructed by using the neighbor joining method using MEGA software (version 7.0) and Clustal W programme (Tamura et al., 2011), which showed the closest relatedness sequences of NCBI (www.ncbinlm.nih.gov). Further, the partial 16S rRNA gene sequences of PLP 6 (1232 bp), PNP 13 (1467 bp) and PNP 19 (1441 bp) were submitted to GenBank under the accession number MG966493, MG966669 and MH045500 respectively. The phylogenetic tree of bacterial strains PLP 6, PNP 13 and PNP 19 has been shown in Fig. 3.7 (a, b and c).


a
Bacillus flexus strain IFO15715
Bacillus megaterium strain ATCC 14581(2)
30
Bacillus flexus strain NBRC 15715
34
Bacillus megaterium strain ATCC 14581
Bacillus megaterium strain NBRC 15308
40
Bacillus simplex strain DSM 1321
27
Bacillus megaterium strain IAM 13418
33
Bacillus flexus strain SBMP3
PLP-6
66
Bacillus aryabhattai strain B8W22
Bacillus qingshengii strain G19
Bacillus mycoides strain ATCC 6462
0.0050


b
PNP13
Bacillus megaterium strain 45-Y914 16S ribosomal RNA gene, partial sequ...
Bacillus megaterium strain CS24 16S ribosomal RNA gene, partial sequence
Bacillus sp. strain F-7 16S ribosomal RNA gene, partial sequence
Bacillus aryabhattai strain J5 16S ribosomal RNA gene, partial se...
Bacillus aryabhattai strain A36 16S ribosomal RNA gene, partial sequence
Bacillus megaterium strain Md1-11 16S ribosomal RNA gene, par...
Bacillus sp. strain Suaeda B-0010 16S ribosomal RNA gene, partial sequence
Bacillus megaterium strain 29-Y924 16S ribosomal RNA gene, partial sequence
Bacillus sp. BDH4 16S ribosomal RNA gene, partial sequence
Bacillus megaterium strain CS25 16S ribosomal RNA gene, partial sequence
0.001

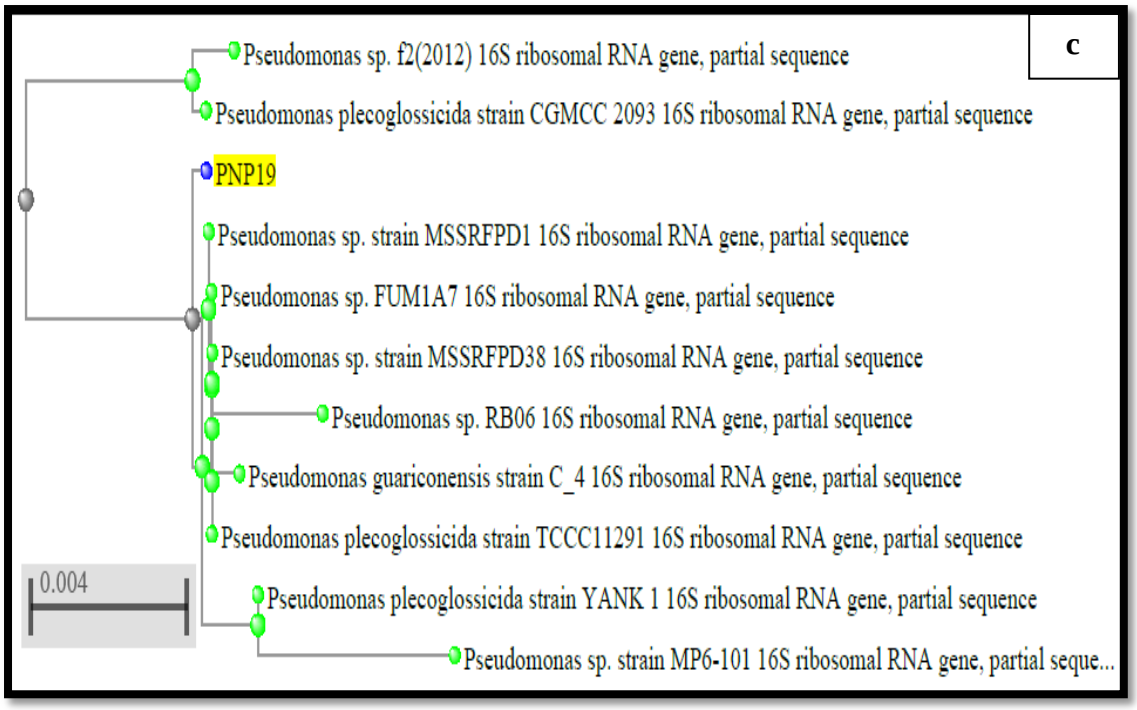


Figure 3.7: Phylogenetic tree of lignin degrading bacterial isolates PLP 6 (a), PNP 13 (b) and PNP 19 (c) constructed by neighbour-joining method based on 16S rRNA gene sequence aligned with NCBI data search.

3.4. Conclusion

Lignin and chlorinated organic compounds present in pulp and paper mill effluent are now considered as a major environmental pollutant due to their toxic effects on environment as well as on human health. The results from the present study concluded that, the isolated bacterial strains *Bacillus aryabhattai* (PLP 6), *Bacillus megaterium* (PNP 13) and *Pseudomonas* sp. (PNP 19) showed higher tolerance of lignin concentration. The dyes such as phenol red and azure B are structurally similar to lignin and the isolated bacterial strains PLP 6, PNP 13 and PNP 19 produced zone of decolorization around bacterial colonies. Thus, the present investigation suggested that all these three bacterial strains *Bacillus aryabhattai* (PLP 6), *Bacillus megaterium* (PNP 13) and *Pseudomonas* sp. (PNP 19) exhibit greater tolerance limit of lignin and

also showed positive test of ligninolytic enzymes on MS agar plates. Therefore these strains can be used as a potential agent for the effective bioremediation of pulp and paper contaminated sites for environmental safety and human/animal health protection.

Chapter 4

Development of Lignin Degrading Bacterial Consortium

Chapter 4

4. Introduction

From last few years, the widespread use of microorganisms in the remediation of organic and inorganic pollutants of industries has gained greater attention over the conventional physico-chemical treatment methods. Degradation of pulp and paper mill effluent along with lignin was studied by a number of pure microbial cultures (Kumar et al., 2012). Recent research has exposed the survival of different variety of organisms in mixed culture capable of degradation and decolourizing a wide range of pulp and paper pollutants. Microbial consortia are usually used for bioremediation in which bacteria play central role (Malaviya and Rathore, 2007). Bacterial consortium is a combination of two or more than two microbial populations, living symbiotically. The use of consortia has several advantages in the application of industrial wastewater treatment. Firstly there is no need for sterile conditions thus decreasing the overall costs. Secondly, consortium systems are more stable to changes in pH, temperature and nature of chemical pollutant when compared to pure cultures (Sahoo and Gupta, 2005).

The microbial consortia are complex in nature and allow microbes to act successfully on a variety of toxic pollutants released from various types of industrial wastewaters. Thus, microbial consortium is better equipped for the removal of contaminants and wastewaters. Several bacterial sp. capable for the degradation of pulp and paper mill effluent either individually or in consortia, has been reported (Chauhan and Thakur, 2002). But, there is little literature is available on the remediation of lignin and its derivatives through bacterial consortium.

Therefore, this chapter contains the information on the reduction of lignin and colour by individual strains as well as by the developed bacterial consortium from the previously isolated bacterial strains.

4.1. Materials and Methods

4.1.1. Media composition

Mineral salt medium broth (MSM) was used in this experimental study, which contained (in gL^{-1}): Na_2HPO_4, 2.4; KH_2PO_4, 2.0; NH_4NO_3, 0.1; $MgSO_4$, 0.01; $CaCl_2$, 0.01; glucose, 10.0 and peptone, 5.0. The medium was dissolved into double distilled water and pH was adjusted to 7.6 ± 0.1 prior autoclaving for sterilization at 121 ˚C (15 psi) for 15 minutes.

4.1.2. Compatibility test for isolated bacterial strains

The lignin degrading bacterial strains were further screened for their compatibility to each other for the development of bacterial consortium used for the degradation and detoxification of pulp and paper mill effluent pollutants. The compatibility test was performed by well-agar diffusion method or by streaking plate method. The isolated bacterial strains grown separately on nutrient broth (NB) amended with 100 mg/L of lignin concentration. In this test culture of one bacterial strain was spread on L-MSM agar plates with the same concentration of lignin, and the culture of other two bacterial strains were inoculated on the wells made on plates after spreading. In addition, in second method i.e. by streaking the bacterial strains on MSM agar plates in such a way that one bacterial strain streaked in the centre of the plate and the other strains were streaked by crossed the previous strain. Further, the plates were incubated at 35 ˚C for 24-48 h and the plates were observed for zone of inhibition.

4.1.3. Development of a bacterial consortium by using isolated bacterial strains

On the basis of screening and compatibility tests, three bacterial strains *Bacillus aryabhattai* (PLP 6), *Bacillus megaterium* (PNP 13) and *Pseudomonas* sp. (PNP 19) were selected to develop consortia for pulp and paper mill wastewater degradation. For developing a bacterial consortium, individual strains were grown overnight and further, added in equal proportion in order to give a final inoculum in reaction mixture. This final concentration of bacterial strains was used for the consortium.

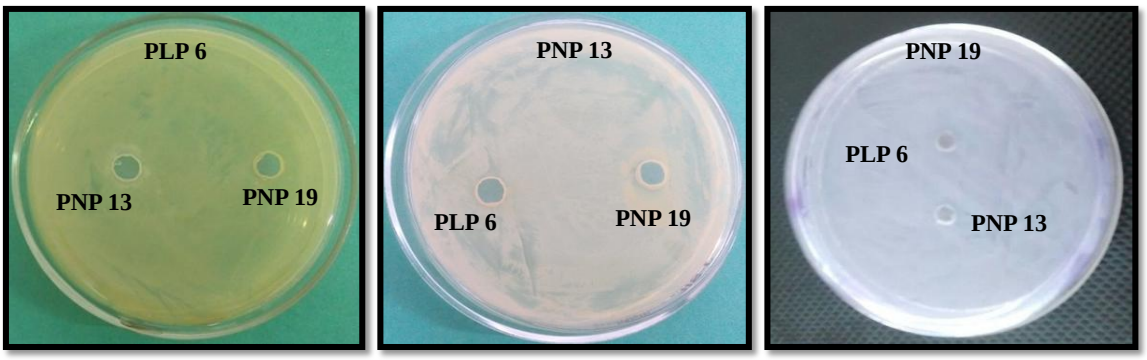
4.2. Statistical Analysis

All the experiments were performed in triplicates (n=3) and the results obtained from each set of experiment have been expressed in terms of mean and standard deviation.

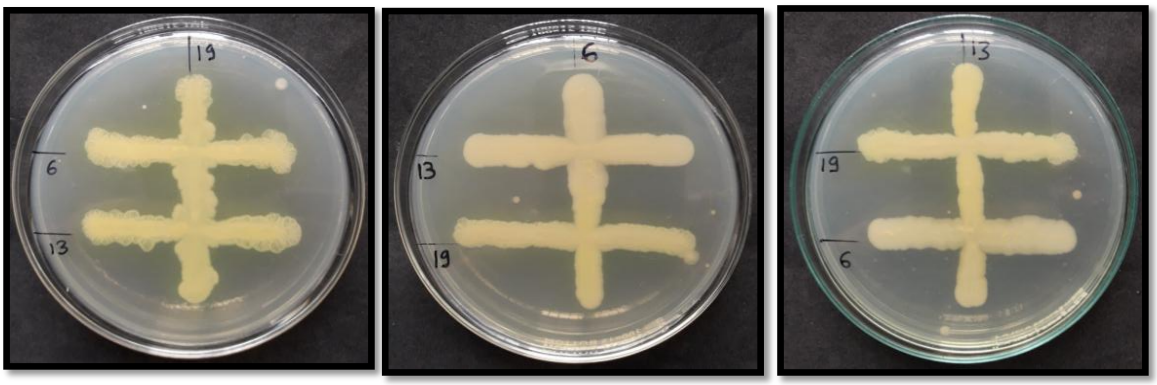
4.3. Results and Discussion

4.3.1. Compatibility test and development of bacterial consortia

In this study, the selected three bacterial strains i.e. *Bacillus aryabhattai* (PLP 6), *Bacillus megaterium* (PNP 13) and *Pseudomonas* sp. (PNP 19), after the screening tests (discussed in previous chapter 3) and their compatibility to each other was used for the development of bacterial consortium. For the development of effective consortium, the compatibility of each bacterial strain was checked to achieve a maximum reduction in pollutants of pulp and paper mill effluent. The results revealed that the selected bacterial strains were compatible to each other, because after inoculation or streaking on the same plates, there was inhibition zone was found around the bacterial colonies (Fig. 4.1). Hence, all these three bacterial isolates were found suitable for the development of consortium. This developed consortium was further used in the remediation of pollutants present in pulp and paper mill effluent.



Ager well plate assay



Streak plate assay

Figure 4.1: Compatibility test of strains PLP 6, PNP 13 and PNP 19 for the development of bacterial consortium by well plate and streak plate assay

4.4. Conclusion

In this study, the results revealed that the bacterial strains PLP 6, PNP 13 and PNP 19 screened after primary and secondary screenings tests were compatible to each other as no inhibition zone was found any well or around the streaking. So these strains were suitable for the development of bacterial consortium. Bacterial consortium is highly effective and active approach for the remediation of pulp and paper mill effluent.

Chapter 5

Degradation and Detoxification of Pulp and Paper Mill Effluent by Developed Bacterial Consortium at Optimized Nutritional and Environmental Conditions

Chapter 5

5. Introduction

Pulp and paper mill is a large industrial sector that generates enormous amount of effluent containing high concentrations of lignin content, gives brown colour to the effluent and results in high BOD and COD values. High levels of organic compounds have been found in pulp and paper effluents. Due to the presence of such contaminants into the effluents, industrial effluents are known to be toxic, mutagenic, bio-accumulating and persistent causes harmful effects on the biological systems. Various conventional methods (physico-chemical) are used for treatment of pulp and paper mill effluent but, they are not feasible not only because of their expensive cost and maintenances but also from environmental safety point of view. Conventional treatment approaches produces secondary pollutants and generating enormous quantity of sludge and disposal of this sludge waste is an another problematic condition for our environment. Instead of conventional methods biological approaches involving microorganisms are highly beneficial and ecofriendly for our green and healthy environment, and plays important role in the management of industrial waste and wastewaters (Bishnoi et al., 2006).

The selection and application of potential pollutant degrading bacterium is an important factor in the bioremediation technology of industrial effluents. The isolation and characterization of potential pollutant degrading microorganisms, along with its optimal growth conditions in different environmental and nutritional parameters helps the microbes to deal with different pollutants under specific stress environments. During bioremediation, microorganisms utilize degradable organic matter as an organic carbon source, resulting in the breakdown of complex compounds to low molecular weight compounds. The attention has to be given to various types of

microorganisms (bacteria, fungi etc.) that dominate treatment system. Some bacterial species are always present in the environment and they multiply only when appropriate condition (pH, temperature, food availability etc.) are available (Chandra et al., 2007; Bharagava and Mani, 2018). Bioremediation can be effective only where environmental conditions permit microbial growth and activity. Its application often involves the manipulation of environmental parameters to allow microbial growth and degradation and detoxification to proceed at a faster rate. So, bioremediation methods have focused on the addition of microorganisms or nutrients concentration and optimal environmental condition in best remediation results. Therefore, optimization of process parameters for microbial growth and activity is essential factor in bioremediation practices for low cost and less time (Yang et al., 2008).

Microorganisms such as bacteria play an important role in converting toxic organic compounds into less toxic or harmless products. Extensive research has been carried out to explore the microbial diversity, particularly for contaminated areas in search of organisms that can degrade a wide range of pollutants released from pulp and paper industries (Prasongsuk et al., 2009). Degradation of pulp and paper mill effluent with microorganisms has recently gained attention as these methods are highly cost effective, produce less amount of solid waste and eco-friendly in nature as compared to physico-chemical treatment methods (Chandra et al., 2006). Fungi (Basidiomycetes) are more proficient for the treatment of lignin and pulp and paper mill effluent, but the immense environmental adaptability and biochemical versatility has made bacteria more effective than fungi for the bioremediation of environmental pollutants. Several bacteria *viz*. *Aeromonas, Bacillus* sp., *Bacillus subtilis, Flavobacterium, Pseudomonas, Aneurinibacillus, Azotobacter, Serratia marcescens, Critobacter* sp., *Nocardia, Novosphingobium* sp. B-7, *Paenibacillus* sp., *Pandoraea, Achromobacter* and *Xanthomonas* are reported to utilize lignocellulosic and chloro-

organic components of pulp and paper effluent and effectively reduced these pollutants (Morii et al., 1995; Gupta et al., 2001; Jain et al., 2006; Chandra et al., 2007; Raj et al., 2007; Bandounas et al., 2011; Bugg et al., 2011; Chandra et al., 2011; Chen et al., 2012a; Raj et al., 2014; Yadav and Chandra, 2015;Haq et al., 2016a; Zainith et al., 2019).

Thus, the main aim of the present study was to optimize developed bacterial consortium at different environmental and nutritional parameters to achieve higher reduction in lignin, colour and other pollutants of pulp and paper mill effluent.

5.1. Materials and Methods

5.1.1. Media composition

For degradation and optimization study, autoclaved P&P effluent (100 mL) was taken, supplemented with mineral salts such as such as: (Na_2HPO_4, 2.4 g/L; KH_2PO_4, 2.0 g/L; NH_4NO_3, 0.1 g/L; $MgSO_4$, 0.01g/L; $CaCl_2$, 0.01 g/L; glucose, 10.0 g/L and peptone, 5.0 g/L) (Hi-media, Mumbai). The media was autoclaved for sterilization at 121 ºC for 15 minutes.

5.1.2. Degradation of pulp and paper mill effluent by individual strains and by developed bacterial consortium

For biodegradation study, overnight grown culture of isolated bacterial strains PNP 6, PNP 13 and PNP 19 having optical density (OD) 1.5, respectively was aseptically transferred into autoclaved Erlenmeyer flask (250 mL) containing 100 mL of effluent supplemented with mineral salts such as: (Na_2HPO_4, 2.4 g/L; KH_2PO_4, 2.0 g/L; NH_4NO_3, 0.1 g/L; $MgSO_4$, 0.01g/L; $CaCl_2$, 0.01 g/L; glucose, 10.0 g/L and peptone, 5.0 g/L). Similarly, in case of bacterial consortium an appropriate amount of each culture to make the final inoculum concentration was inoculated in the same medium. The uninoculated (control) and inoculated flasks were incubated at 35 ºC at 120 rpm for 216 h. The samples (5 mL) were withdrawn from flasks at the regular

time interval of 24h for the analysis of bacterial growth, reduction in colour and lignin content (Chandra and Singh, 2012; Raj et al., 2014).

To calculate the percentage removal of lignin and colour as per the following formula:

$$\textbf{Removal (\%)} = 100 \times (N_t - N_0)/ N_0$$

Where, $\mathbf{N_0}$ **=** the absorbance value of lignin and colour before treatment (untreated)

$\mathbf{N_t}$ **=** is the absorbance value of lignin and colour after bacterial treatment

Lignin estimation

For the estimation of lignin, samples (before and after inoculation of bacterial culture) were centrifuged for 20 min at 8000 × g and the pH of the supernatant was adjusted at 7.0 with 2 M NaOH. The sample (50 ml) was mixed with 1 ml CH_3COOH (10%) and 1 ml $NaNO_2$ (10%). After 15 min, 2 ml of NH_4OH was added and the mixture left for 5 min. The absorbance was measured at 430 nm. For blank, 50 ml distilled water (Instead of sample) was mixed with 1 ml CH_3COOH (10%) and 2 ml NH_4OH. After 15 min, 1 ml of $NaNO_2$ (10%) was added (Pearl and Benson (1940). The absorbance value was transformed into lignin content (ppm) by using the formula:

Lignin (ppm) = Absorbance/0.000247

Colour measurement

Colour of the effluent was measured according to Canadian Pulp and Paper Association (1974) method and the absorbance (465 nm) value transformed into colour units (Pt-Co).

Colour Units (Pt-Co) = 500 A_2/A_1

Where, A_1= absorbance of 500 CU of standard Pt-Co solution (A_{465} = 0.129) and A_2 = absorbance of the effluent sample at 465 nm.

5.1.3. Optimization of parameters

5.1.3.1. Optimization of environmental parameters (pH and temperature)

The effect of physico-chemical parameters like temperature (25-45°C) and pH (5-9) on lignin reduction and colour by a developed bacterial consortium (PLP 6+ PNP 13+ PNP 19) was monitored in flasks containing 100 mL of autoclaved pulp and paper mill effluent, inoculated with fresh culture. The flasks were incubated at 35°C for the optimization of environmental parameters and effective degradation of effluent. The acidic and alkaline pH of the medium was maintained by using hydrochloric acid and sodium hydroxide solution. The optical density of cultures was observed spectrophotometrically at 430 nm for lignin reduction and 465 nm for colour removal at particular pH and temperature condition.

5.1.3.2. Optimization of nutritional parameters (carbon and nitrogen sources)

During optimization study of nutritional parameter, 24 h grown culture was inoculated in Erlenmeyer flasks (250 mL) containing 100 mL autoclaved pulp and paper mill effluent, supplemented with extra carbon and nitrogen sources in order to boost the degradation and detoxification process. The effluent was supplemented with different carbon sources such as glucose, sucrose, starch, maltose and fructose, individually at different concentrations i.e. 0.25%, 0.5%, 1.0%, 1.5% and 2.0% each and various organic and inorganic nitrogen sources such as yeast extract, peptone, urea, sodium nitrate and ammonium sulphate in another set were added at a concentration range of 0.25%, 0.5%, 0.75% and 1.0% each to the effluent for studying the effect on degradation and decolourization process.

5.1.3.3. Effect of static and shaking condition

The effect of shaking and static conditions on the degradation ability of bacterial consortium was studied in 100 mL effluent. The bacterial consortium was inoculated in the effluent and incubated at static (without shaking) and shaking conditions (120 rpm) at 35 ºC for the biodegradation studies.

5.1.4. *Degradation and detoxification of pulp and paper mill effluent by developed bacterial consortium at optimized nutritional and environmental conditions*

In the above experimental studies, we have examine and explored the effect of different pH range from acidic 5.0 to alkaline 9.0 on the degradation of effluent by a developed bacterial consortium and scrutinized that the maximum reduction was taken at pH 7.0 and also, we have investigated the effect of temperature from 25-45 °C range out of which 35 °C was found to be the most appropriate temperature condition for degradation study. In order to intensify the degradation efficiency of bacterial consortium, the medium was also supplemented with different carbon sources such as glucose, sucrose, starch, maltose and fructose at a range from 0.25% to 2% and different nitrogen sources such as yeast extract, ammonium sulphate, sodium nitrate, urea and peptone at a different concentration i.e. 0.25% to 1% respectively. So, here we have studied the degradation efficiency of bacterial consortium at optimized parameters such as pH 7 and temperature 35°C with optimized nutrient sources i.e. glucose (1.0%) and peptone (0.25%) on the degradation and detoxification of pulp and paper effluent.

5.2. Statistical analysis

All the experiments were performed in triplicates (n=3). The results obtained from each set of experiment have been expressed in terms of mean and standard deviation.

5.3. Results and Discussion

5.3.1. Degradation of pulp and paper mill effluent by individual strains and by bacterial consortium

During degradation experiment of pulp and paper mill effluent, the axenic and bacterial consortium growth was observed at 24 h of incubation and the maximum growth of bacterial strains PLP 6, PNP 13, PNP 19 and consortium were observed at 120,168,120 and 144 h, afterwards a slight decline in growth phase was observed

(Fig. 5.1). Significant reduction in colour and lignin content was observed after 2 days of incubation. This indicated that the bacteria utilizes initially carbon (1%) and nitrogen sources (0.5%), which were already supplemented in the medium and also acclimatize itself according to the medium condition and thereafter, started the degradation process. In the present investigation, it was observed that, the bacterial consortium effectively reduces colour and lignin as compared to axenic culture. Bacterial consortium resulted in 75.43% and 56.56% reduction in colour and lignin whereas, axenic culture PLP 6, PNP 13 and PNP 19 reduces lignin 55.03%, 44.09% and 47.00% and colour 68.20%, 67.03%, 69.88% respectively Fig. 5.2 (a and b). In addition, bacterial consortium also reduces BOD (94.58%) and COD (80.67%) effectively, while individual strains PLP 6, PNP 13 and PNP 19 reduces BOD (92.66%, 78.16%, 81.45%) and COD (74.26%, 65.5%, 70.8%) from paper mill effluent respectively in Fig. 5.3 (a and b). The degradation and decolourization of pulp and paper mill effluent by a developed bacterial consortium as shown in Fig. 5.4.

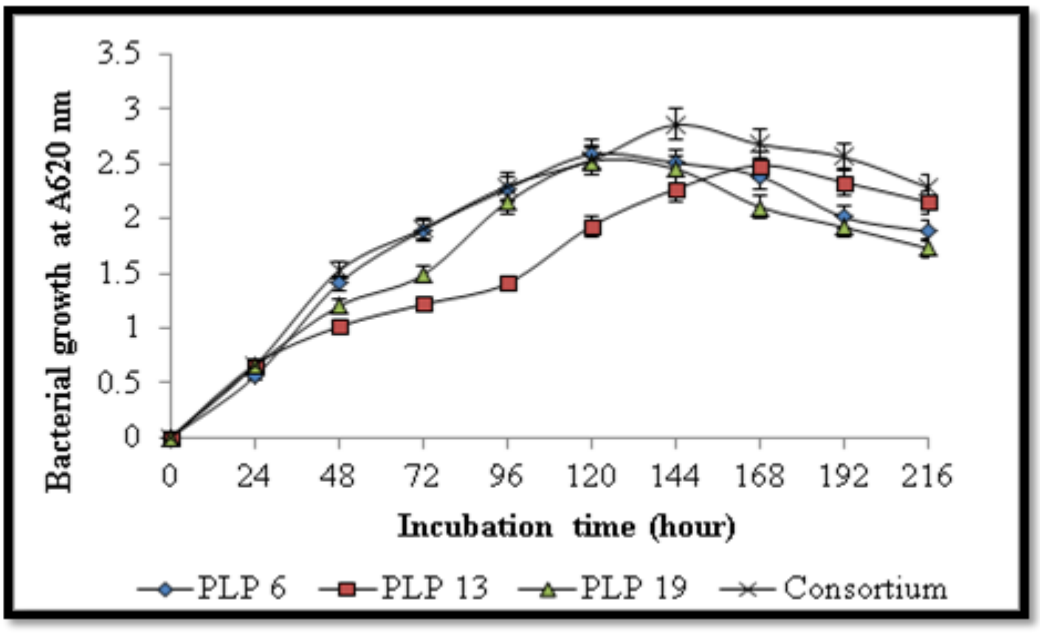


Figure 5.1: Growth curve of lignin degrading bacterial strains PLP 6, PNP 13, PNP 19 and consortium

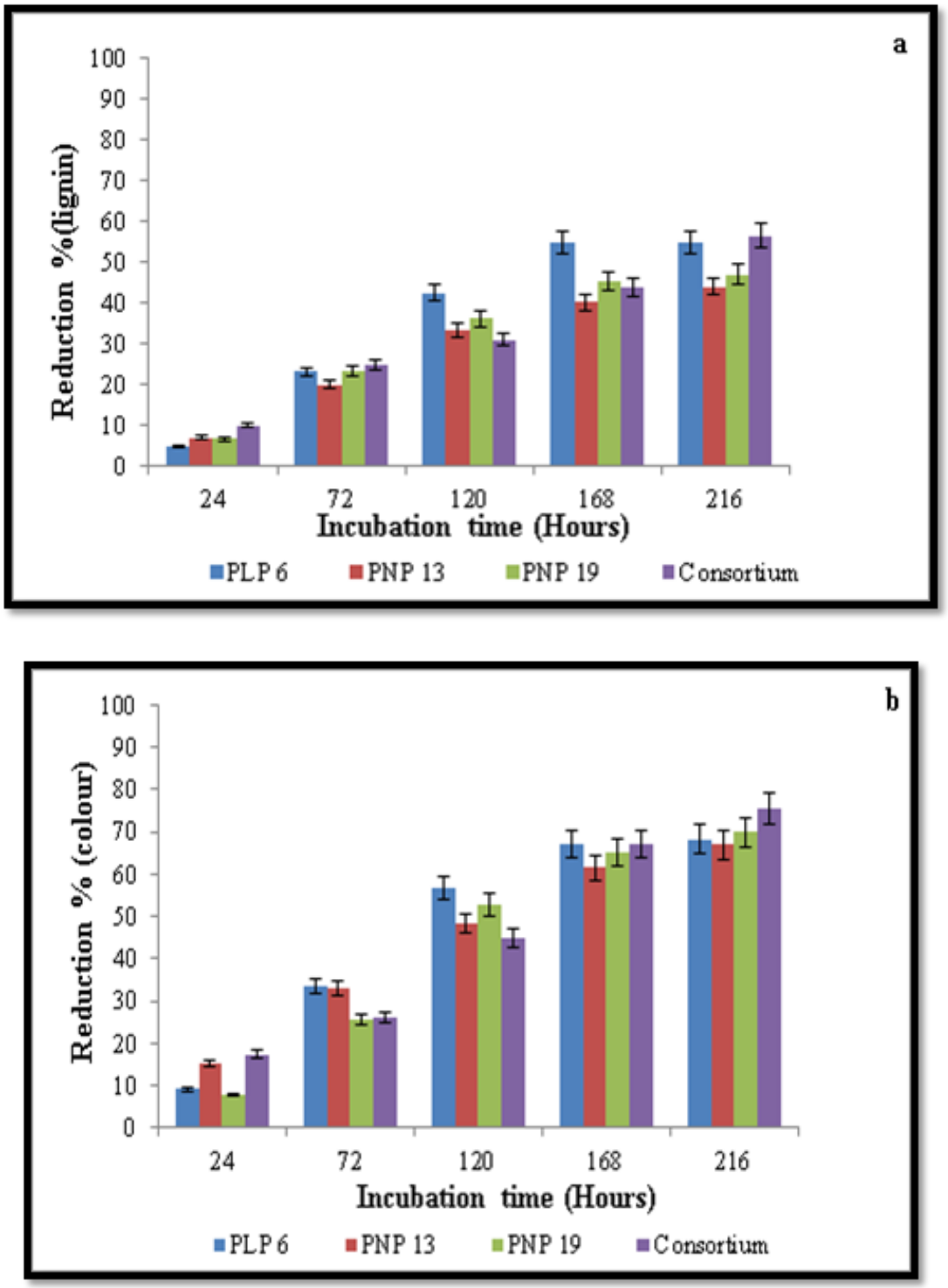
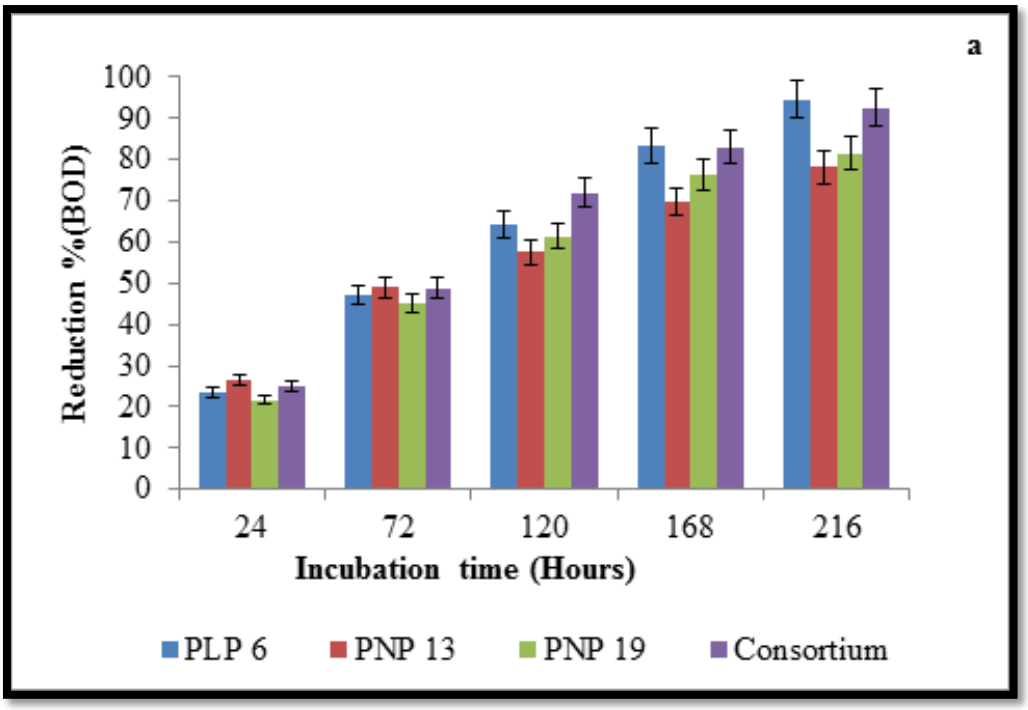


Figure 5.2: Reduction in lignin (a) and colour content (b) by individual strains and bacterial consortium



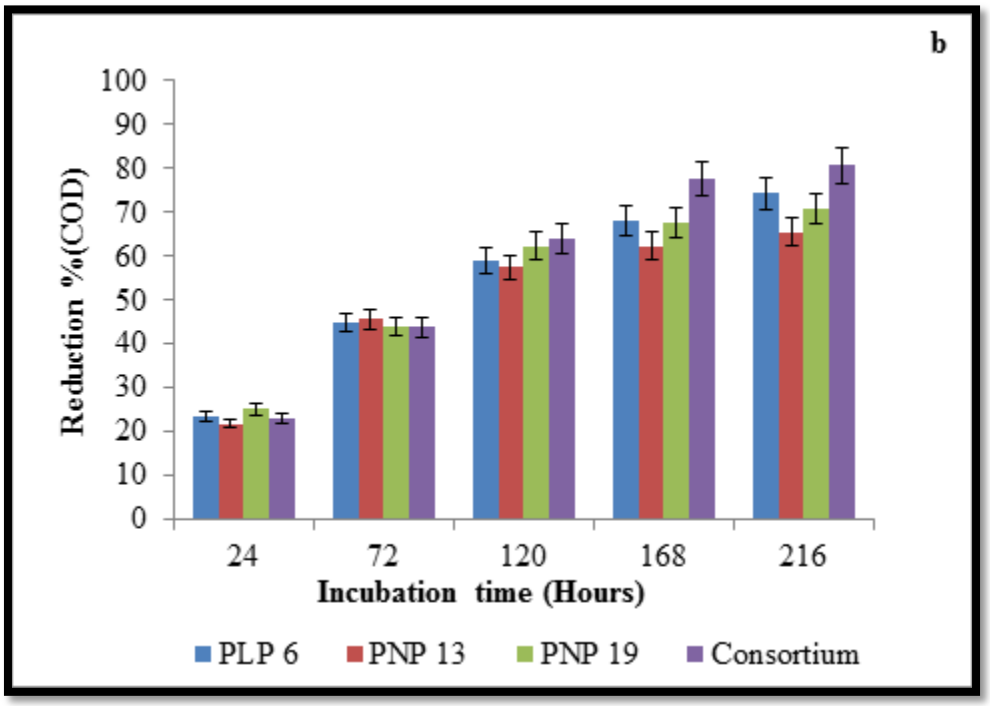


Figure 5.3: Reduction in parameters: BOD (a) and COD (b) by individual strains and bacterial consortium

Figure 5.4: Degradation and decolourization of P&P effluent by a developed bacterial consortium of *Bacillus aryabhattai* (PLP 6), *Bacillus megaterium* (PNP 13) and *Pseudomonas* sp. (PNP 19).

Several authors have also reported similar findings that supported our present study. Raj et al. (2014) found, a laccase producing *Paenibacillus* sp. which reduces colour (68%), lignin (54%), COD (78%), BOD (83%) from pulp and paper mill effluent at 34 ± 1 ºC, 120 rpm and 144 h of incubation time. Haq et al. (2016a) reported a lignin peroxidase producing bacterial strain *Serratia liquefaciens* effectively reduced pollution parameters such as lignin (58%), colour (72%) and COD (85%) from pulp and paper effluent after 144 h of treatment at 120 rpm, at pH 7.6 and temperature maintained at 30 ºC. Moreover, Chandra and Singh, (2012) reported consortium of three bacterial strains (*Pseudochrobactrum glaciale, Providencia rettgeri and Pantoea* sp.) have great potential to reduce BOD (92%), COD (91%), lignin (90%) and colour (96%) of P&P mill effluent within 216 h. Rajwar et al., (2017) found a novel consortium of two fungal strains of *Nigrospora* sp. and *Curvularia lunata,* effectively reduced BOD (85.6%), COD (80%), colour (82.3%) and lignin (76.1%) during degradation of pulp and paper mill effluent at 30 ºC and 140 rpm. A conspicuous reduction in lignin (91.5%), colour (96.1%), BOD (96.7%)

and COD (86.4%) during treatment of pulp and paper effluent by bacterial consortium of two indigenous strains *Bacillus megaterium* and *Pseudomonas plecoglossicida* reported by Paliwal et al., 2015. Hence, the present study showed that the consortium of bacterial strains was found to be more efficient for degradation and detoxification of pulp and paper mill effluent as compared to axenic cultures.

During degradation studies the pH of the medium changed from pH 8.1 to pH 6.6 after two days of incubation, further, it gradually increases up to pH 7.2 at the end of the experiment. The shifts towards acidic pH of the medium might be due to acetate efflux along with other TCA cycle intermediates. The pH of control flasks was remains constant. As the utilization of supplemented glucose and peptone, the scarcity of nutrients in the medium occur then the bacteria forced to utilize lignin and the excreted metabolic intermediates, leading to gradual increase in the pH (Yang et al., 2008).

5.3.2. Optimization of environmental parameters (pH and temperature)

The pH of any medium plays an important role in growth, metabolic activity, and enzyme production and in the degradation of recalcitrant pollutant. Therefore, to examine the effect of pH on degradation ability of bacterial consortium for the remediation of pulp and paper effluent, firstly the pH of the medium was adjusted in a wide range from 5 to 9. The results revealed that reduction ability of bacterium was significantly affected at acidic and alkaline pH range. The maximum reduction percentage in lignin (55.21%) and colour (73.98%) was observed at pH 7.0 after 216 h. However, the reduction showed in lignin (16.36%, 38.23%, 52.89% & 49.64%) and colour concentration (25.43%, 41.89%, 69.86% and 62.75%) at pH 5.0, 6.0, 8.0 & 9.0 respectively. The degradation percentage was markedly found decreased at pH 5.0 due to acidic conditions. Thus, the maximum reduction was observed between pH

7.0-8.0 and neutral pH (7.0) value was considered to be the optimum pH for the bacterial growth and reduction in lignin content and colour as shown in Fig. 5.5.

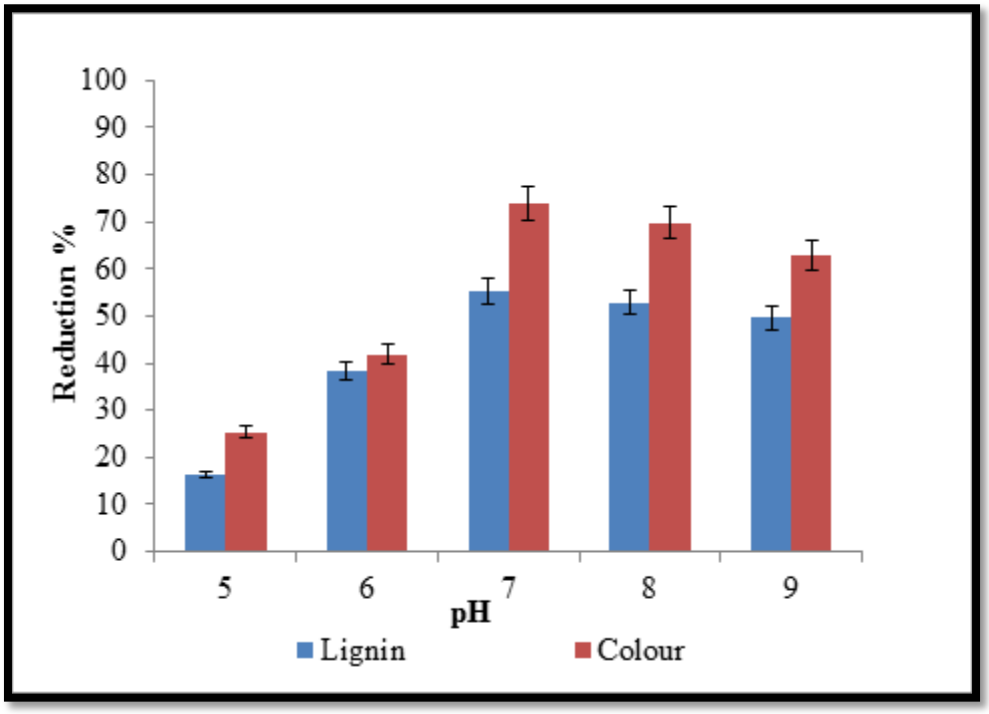


Figure 5.5: Graphical representation of effect of pH on reduction in lignin and colour by developed bacterial consortium

Likewise pH, temperature values from 25-45 ºC was also optimized for bacterial consortium to know the ideal temperature for maximum reduction. Fig. 5.6, showed that the maximum reduction in lignin (56.16%) and colour (76.68%) was observed at 35 ºC and least percentage reduction in lignin (18.42%) and colour (21.85%) was occurred at 25 ºC. 35.97% reduction in lignin and 44.69% reduction in colour was recorded at 30 ºC. Moreover, 50.29% and 46.96% reduction in lignin and 72.83% and 68.74% reduction in colour was recorded at 40 ºC and 45 ºC respectively. This suggested that the temperature increases from lower to higher degree; the reduction potential of bacterial consortium was decreases. The effect of pH and temperature on lignin reduction and colour removal by bacterial consortium was also reported by several investigators (Saraswathi and Saseetharan, 2010; Kumar et al.,

2012).Srivastava et al., 2016 reported the bacterial consortium reduces colour 55%, lignin 25% and COD 64% at optimum temperature 37 ºC and pH 7.0. Mishra and Thakur, (2010) isolated *Bacillus* sp. and found that, the maximum colour was removed at pH 8.0, temperature 35 ºC, and 200 rpm but, after optimization two fold increase in colour and lignin reduction from 25-69% and 28-53%, respectively.

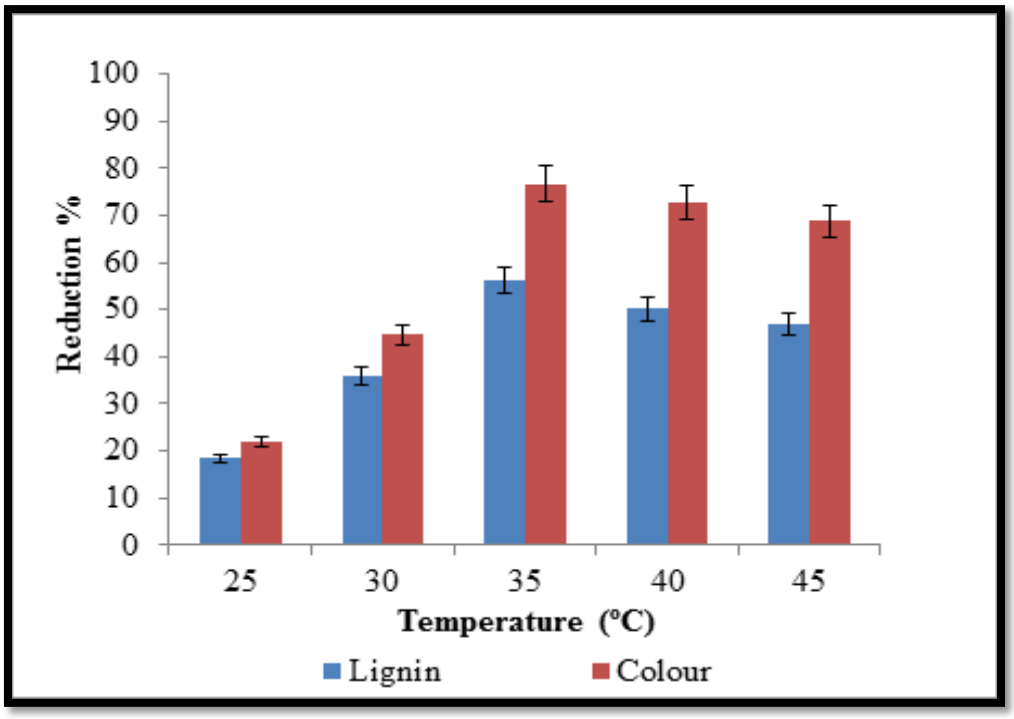


Figure 5.6: Effect of temperature on reduction in lignin and colour by developed bacterial consortium

5.3.3. Optimization of nutritional parameters (carbon and nitrogen sources)

The pulp and paper effluent reduction was also studied with the supplementation of various carbon sources such as: glucose, maltose lactose, sucrose and starch, at different concentration range i.e. 0.25%, 0.5%, 1.0%, 1.5% and 2.0% and among these carbon sources, maximum reduction was observed for lignin content and colour with the addition of glucose (1%) i.e. (55.69% and 73.84%) followed by sucrose (53.48%, 71.92%), maltose (47.86%, 63.69%), lactose (41.78%, 64.28%) and starch (35.98%, 66.28%) as shown in Fig. 5.7. The effect of varying concentration of

organic and inorganic nitrogen sources such as: peptone, yeast extract, ammonium sulphate, urea and sodium nitrate, at different concentration range of 0.25%, 0.5%, 0.75% and 1.0% was also studied and found that in presence of peptone (0.25%) maximum reduction was observed in lignin and colour was 57.92% and 75.21% while, yeast extract reduces lignin (54.89%) and colour (74.76%). Both parameters were more suitable and allowing maximum reduction potentiality to bacterial consortium. Other inorganic nitrogen sources, ammonium sulphate and sodium nitrate reduce lignin 51.85% 47.37% and colour 69.81%, 66.65%, respectively. However, least reduction was observed by urea which reduces lignin and colour only by 34.39% and 51.72% as shown in Fig. 5.8. Chandra et al., (2012) reported 1% dextrose and 0.5% peptone was found to be the best combination of carbon and nitrogen source and showed best degradability of pulp and paper mill effluent. Chandra et al., (2018) demonstrated the effect of carbon and nitrogen source on bacterial strains and found that 2.0% glucose and 0.5% peptone were the optimum for pulp and paper effluent decolourization and degradation.

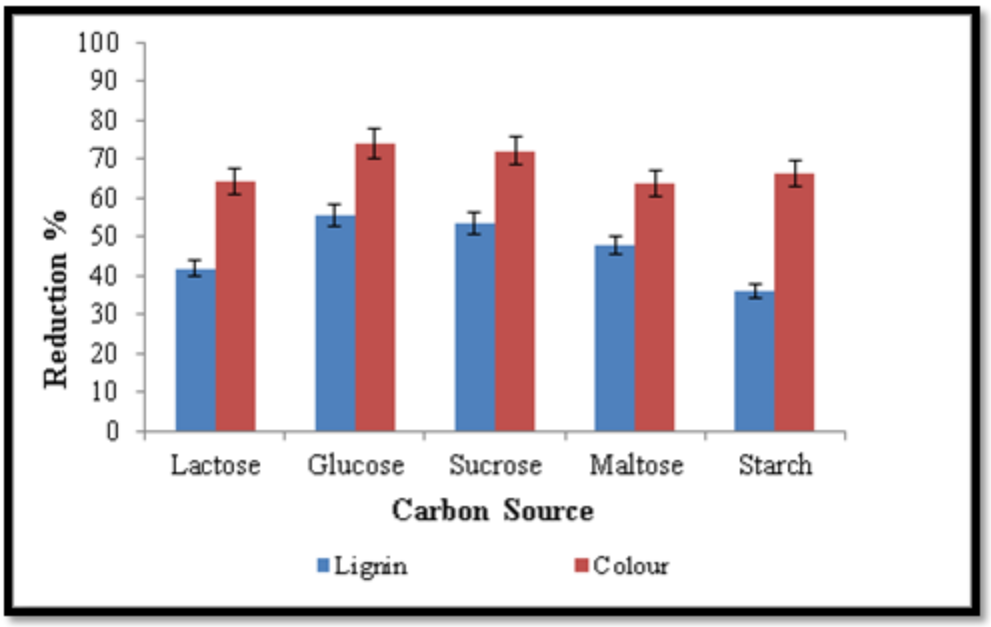


Figure 5.7: Effect of various carbon sources on lignin and colour reduction efficiency of bacterial consortium

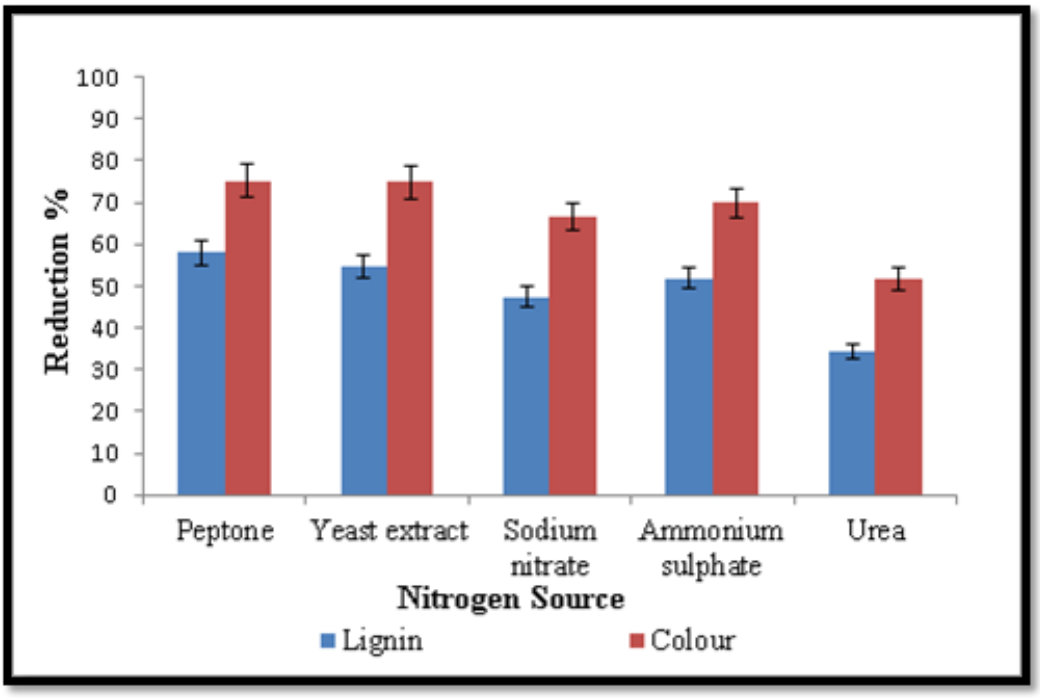


Figure 5.8: Effect of various nitrogen sources on lignin and colour reduction efficiency of bacterial consortium

5.3.4. Effect of static and shaking condition on degradation process

The reduction efficiency of bacterial consortium was evaluated by observing the effect of shaking (agitation) conditions at 120 rpm and resting condition (without shaking). Shaking condition is an important parameter and is complementary to the degradation process. Maximum reduction in lignin (55.37%) and colour (74.28%) was observed at 120 rpm at 35 ºC whereas; only (34.82% and 50.65%) reduction in lignin and colour was achieved under static condition with in the same incubation time as shown in Fig. 5.9. Therefore, it was suggested that, shaking condition is more proficient for the degradation of pulp and paper mill effluent by bacterial consortium.

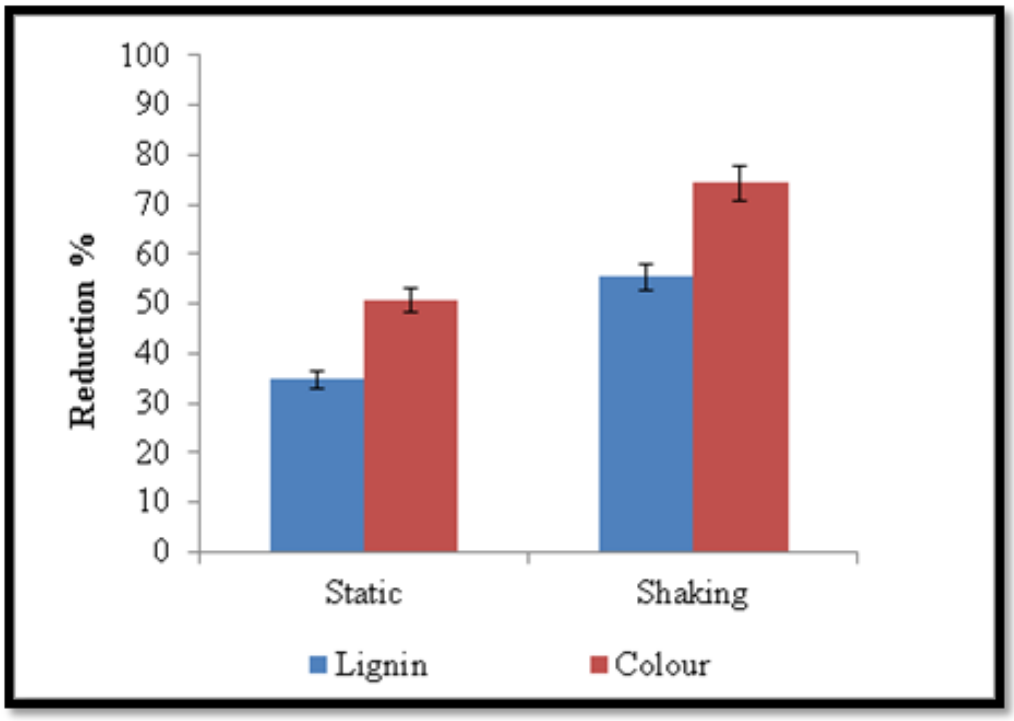


Figure 5.9: Effect of static and shaking condition on reduction in lignin and colour by bacterial consortium

The higher percentage reduction in lignin and colour was attained under shaking condition may be due to the increase in bacterial growth (biomass) which degrades the pollutants present in the effluent whereas in static condition lesser reduction in lignin content and colour was observed (Chandra and Singh, 2012). In static condition improper oxygen transfer between bacterial cells, it may be the reason for lower bacterial growth and lesser reduction percentage. Singhal and Thakur (2009), reported rpm is a significant factor and thus, improved reduction rate of lignin, colour and other pollutants present in pulp and paper effluent.

5.3.5. Degradation of P&P mill effluent by bacterial consortium at optimized and nutritional conditions

The reduction in lignin and colour by a developed bacterial consortium (*Bacillus aryabhattai*, *Bacillus megaterium* and *Pseudomonas* sp.) at optimized nutritional and environmental conditions, that are obtained from optimization studies showed

maximum reduction potential of bacterial consortium to reduce lignin and colour content. The reduction experiment of pulp and paper mill effluent was monitored at different time intervals were set up at optimizes pH (7.0), temperature (35ºC), carbon (glucose) and nitrogen source (peptone). In comparison to normal conditions, optimized condition demonstrated that, when we applied all the optimized conditions, maximum reduction percentage in lignin and colour level was achieved with increased growth of bacterial consortium as shown in Fig. 5.10 (a and b). At all optimized conditions maximum reduction was observed at 35 °C temperature and pH 7.0. However, glucose at 1.0% concentration and 0.25 % concentration of peptone was found to be most suitable carbon and nitrogen sources for the degradation and decolourization of pulp paper effluent by the developed bacterial consortium. The reduction in lignin and colour content was found maximum at optimized environmental and nutritional parameter i.e. 57.89% and 77.64% respectively.

Thus, the results clearly demonstrate that when all the optimized conditions were applied at the same time on consortium culture, the lignin reduction rate and colour removal was much greater than the normal condition and individual strain as discussed above in comparison with consortium culture.

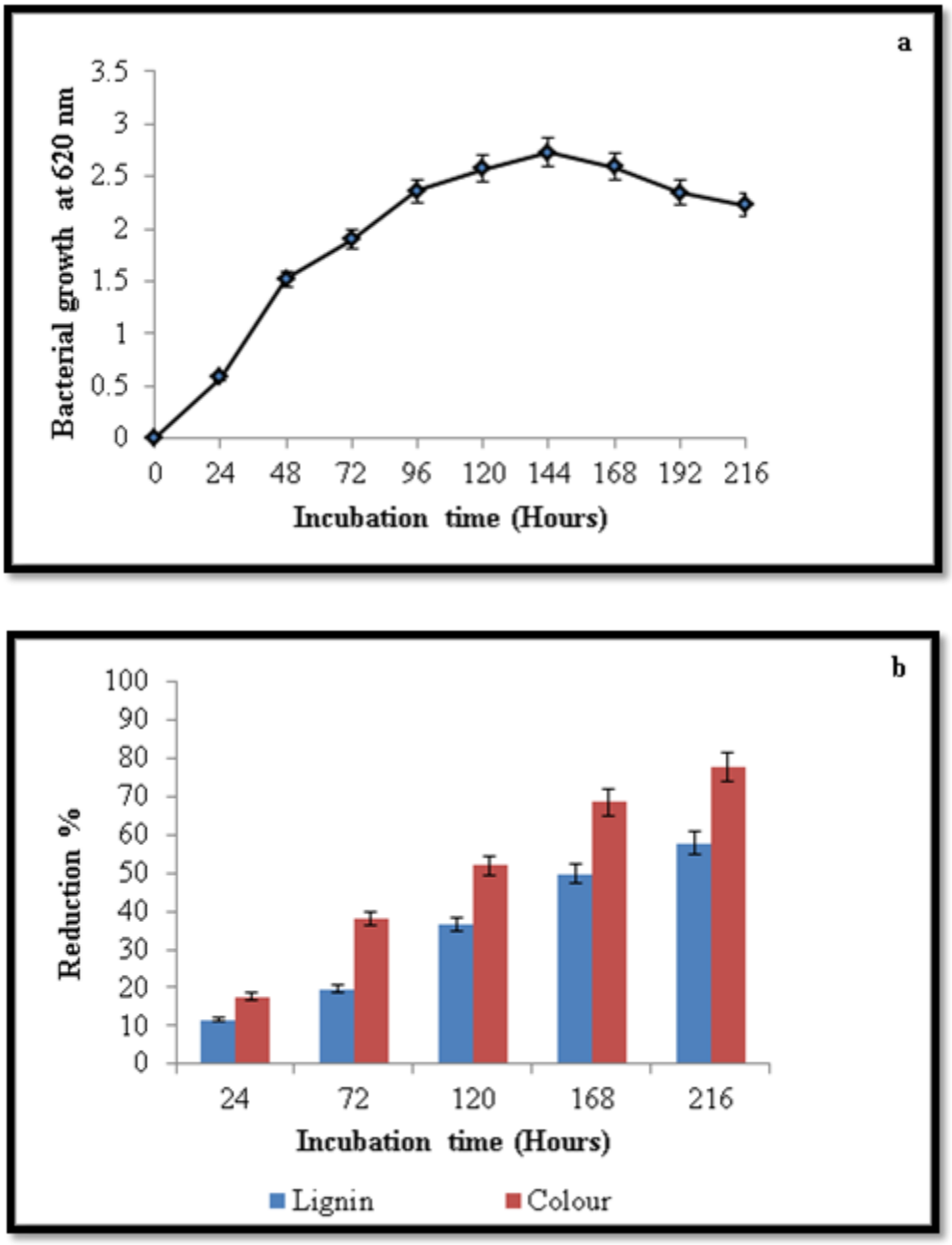


Figure 5.10: Bacterial growth (a) and reduction in lignin and colour (b) of pulp and paper effluent by developed bacterial consortium at optimized conditions

5.4. Conclusion

The use of bacterial consortium for the degradation and detoxification of pollutants present in pulp paper effluent is more useful than the individual cultures. As consortium culture are more efficient and competent to survive under environmental stress condition. The present studies showed that optimization of

environmental i.e. pH and temperature and nutritional parameter (carbon and nitrogen sources) is a pragmatic approach to enhance the reduction capability of consortium cultures. When all the optimized conditions or parameter were applied at one time demonstrated that maximum reduction in lignin and colour concentration was achieved after 216 h of incubation. Thus, reduction in lignin and colour content and detoxification of other toxic pollutants of the effluent at all optimized conditions at the same time by developed bacterial consortium could be a more useful way to remediate pulp paper contaminated environments.

Chapter 6

Detection and Characterization of Lignin Degrading Enzymes by Page Analysis

Chapter 6

6. Introduction

Enzymatic degradation and detoxification of colour and organic pollutants of pulp and paper mill effluent with microbial sources is a good alternative over conventional treatment technologies. Several ligninolytic bacteria are reported, which produces ligninolytic enzymes, that can convert toxic compounds into less toxic forms and thus, reduction by microbial enzymes seems to be feasible for pulp paper effluent bioremediation. Ligninolytic enzymes are applicable in the hydrolysis of lignocellulosic residues, and for the degradation and detoxification of complex and recalcitrant constituent lignin of pulp paper mill effluent. Ligninolytic enzymes are highly versatile in nature, and useful in the treatment of wide range of industrial pollutants (Poonam et al., 1987; Dahiya et al., 1998).

Among various lignin modifying enzymes, manganese peroxidase (MnP), lignin peroxidase (LiP) and Laccase are three main lignin degrading enzymes. These enzymes are lying under the category of peroxidases and oxidases. Peroxidases are heme containing enzymes that require hydrogen peroxide as electron acceptor to catalyse lignin and its related compounds. The molecular weight and isoelectric point of these enzymes range between 35 -60 kDa and 2.8 - 5.4, respectively (Vares et al., 1995; Mester and Field, 1998). Ligninolytic enzymes such as MnP and LiP use hydrogen peroxide (H_2O_2) as co-substrate. These are heme associated enzymes which contain broad substrate spectrum, includes organic and inorganic compounds (Olukunle and Oyegoke, 2016) while, laccases are multi-copper phenol oxidases. It oxidizes phenols and aromatic amines. Rather than H_2O_2, laccase enzymes utilize di-oxygen as an oxidant, reducing it by four electrons to water. Laccases have molecular

weight in the range of 50-300 kDa and have an acidic isoelectric point (Call and Mucke, 1997).

Ligninolytic enzymes have many applications in the field of bio-remediation of industrial effluents, containing recalcitrant and hazardous toxicants (Dwivedi et al., 2010). Intensive studies had been done on white rot fungi, which are able to produce ligninolytic enzymes like LiP, MnP and laccase (Minussi et al., 2007). However, fungal system require narrow pH 4-5 range for the growth and enzyme production, while the pH of pulp paper effluent tends to be neutral or alkaline (between 7 and 9), the requirement to reduce the pH of wastewater prior to the fungal treatment resulting in an additional cost (Raghukumar et al., 2008; Costa et al., 2017). In contrast, bacterial system survive well in neutral to alkaline pH (7-9) and are suitable for the decolourization of effluents and degradation and detoxification of toxic compounds of pulp paper effluent without any need of pH adjustment (Brown and Chang 2014; Rahman et al., 2013)

In this regard bacterial enzymes are more preferred option for remediation process compared to fungi. With the involvement of these enzymes, biological treatment methods are capable of degrading recalcitrant organic pollutants present in pulp paper effluent. Therefore, in this chapter we have discussed about the detection and characterization of ligninolytic enzymes produced during treatment of pulp and paper effluent.

6.1. Materials and Methods

Mineral salt medium broth (MSM) was used in this experimental study, which contained (in gL^{-1}): Na_2HPO_4, 2.4; KH_2PO_4, 2.0; NH_4NO_3, 0.1; $MgSO_4$, 0.01; $CaCl_2$, 0.01; glucose, 10.0 and peptone, 5.0. The medium was dissolved into double distilled water and pH was adjusted to 7.6 ± 0.1 prior autoclaving for sterilization at 121 ºC (15 psi) for 15 minutes.

6.1.1. Preparation of cell free extract

The selected bacterial strain after screening, which showed positive result (showed in previous chapter 3) was grown in 10 mL nutrient broth for overnight (18-24 h) to make pre-culture. One mL of pre-culture was inoculated in Erlenmeyer flask (250 mL) containing 99 mL L-MSM broth (lignin = 100 mg/L). The flask was incubated at 35 ºC and 120 rpm and the pH of broth was maintained at 7.6. Sample (2 mL) was withdrawn from flask and centrifuged at 8,000 rpm for 15 min at 4 ºC. The culture supernatant was obtained from centrifugation, directly used as extracellular enzyme for the determination of enzyme activity. Further, the obtained pellet were resuspended in potassium phosphate buffer of pH 7.4 and sonicated (Sonics-Vibracell Ultrasonic Processor, USA), and give 40 (amps) and give 7 strokes every 30 s, with every 1 min of time interval at 4 ºC and the homogenate was again centrifuged at 10,000 for 15 min and the supernatant was obtained used as crude enzyme.

6.1.2. Enzyme assay

The assay of ligninolytic enzymes was determined during bacterial treatment of pulp paper mill effluent. The crude extract (supernatant) was taken for the estimation of ligninolytic enzyme activity.

6.1.2.1. Lignin peroxidase (LiP) activity

Lignin peroxidase assay was performed by the method as given by Arora et al. (2002), based on the oxidation of Azure B dye. The reaction mixture (3 mL) contained 1 mL sodium tartrate buffer (50 mM, pH 3.0), 1 mL Azure B (32 µM), 500 µL of enzyme extract, 500 µL H_2O_2 (2 µM). H_2O_2 was used to start the reactions in the reaction mixture. After 10 min of incubation OD was taken at 651 nm.

6.1.2.2. Manganese peroxidase (MnP) activity

Manganese peroxidase assay was done as per the method given by Oliveira et al. (2009), based on monitoring the oxidation of phenol red dye in presence of H_2O_2.

Reaction mixture (4 mL) contained 1 mL of enzyme extract, 1 mL phosphate buffer (pH 7.0), 1 mL phenol red (1 mM), 500 µL $MnSO_4$ (1 mM) and 500 µL H_2O_2 (50 µM). 1 mL sample was taken from reaction mixture and 40 µL of NaOH (5M) was used to stop the reaction. OD was taken at every 1 min of time interval at 610 nm.

6.1.3. SDS-PAGE analysis

Denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on polyacrylamide gel electrophoresis unit (GX-SCZ2, Genetix Biotech Asia Pvt. Ltd.) by using 12% polyacrylamide gel. Separating gel and stacking gel used in this experiment and the concentration of the gels has been discussed below:

Materials

- Casting gel unit
- Vacuum chamber for degassing gels
- 1.5 M Tris-HCl buffer, pH 8.8
- 10 % (w/v) SDS
- 10 % (w/v) Ammonium persulfate

Separating gel

- 10 mL of Acrylamide monomer
- 2.6 mL of Tris-HCl Buffer
- 0.1 mL of 10% (w/v) SDS
- 3.8 mL of H_2O

Stacking gel

- 0.67 mL of Acrylamide monomer
- 1.25 mL of Tris Buffer
- 0.05 mL of 10% SDS

Procedure:

- Assemble the slab gel unit with glass sandwich set in the casting mode with 1.5 mm space.
- Prepare a separating gel in a small beaker and add separating gel to a side arm flask, stopper the flask and attach to a vacuum pump equipped with a cold trap.
- Turn on the vacuum and degas the solution for 10 min. During this period gently swirls the solution in the flask.
- Exit the vacuum, open the flask and add 100 μL of ammonium persulfate and 10 μL of TEMED solution.
- Add stopper to flask and degas for additional 2 min with gentle swirl to mix the solution. Now transfer the appropriate amount of degassed solution to casting chamber without any air bubble formation.
- Immediately fill in water to the top of separating gel for preventing formation of meniscus. Let it settle for 20-30 min to gelate.
- Prepare a stacking gel as separating gel preparation method and add 50 μL of ammonium persulfate and 5 μL of TEMED. Pour the stacking gel onto the separating gel.
- Insert the well-forming comb without trapping air and wait for 20-30 min let it to gelate. Take out the comb after complete gelation.
- The prepared samples were mixed with sample buffer and were heated in the boiling water for 5-10 min.
- Now, load the samples into appropriate wells and protein markers into the first lane and cover the top and connect the anodes.

- Set an appropriate volt and run the electrophoresis. After completion of total running time, stop SDS-PAGE running when the down most sign of protein marker reaches foot line of the glass plate.

On completion of electrophoreses, the protein bands on gel was stained with coomassie brilliant blue R-250 dye and destained with destaining solution and left for overnight. The molecular weight was estimated by comparing with standard protein ladder (Molecular Standard Mixture Recombinant, 29-250 kDa; Sigma).

6.2. Statistical Analysis

All the experiments and data analysis were performed in triplicates (n=3) to reduce analytical errors and the results obtained were expressed in terms of mean and standard deviation (SD).

6.3. Results and Discussion

In the present study, the activity of LiP and MnP was observed during bacterial treatment of pulp paper effluent as shown in Fig. 6.1. The enzyme secreted by bacterial strains was measured from the culture supernatant at different time intervals. The maximum enzyme activity was observed for MnP i.e. 10.26 IU/mL and 12.83 IU/mL produced by PNP 13 (*Bacillus megaterium*) and PNP 19 *(Pseudomonas* sp.) respectively after 120 h and 144 h. However, bacterial strain PLP 6 (*Bacillus aryabhattai*) showed maximum LiP enzyme activity 16.64 IU/mL after 168 h respectively. On the other hand, no enzyme activity was observed in control flasks.

The redox potential of LiP and MnP are relatively high and reported for the degradation of both phenolic and non-phenolic compounds. These enzymes are heme containing glycoprotein which requires H_2O_2 as an oxidant. MnP preferentially catalyses the peroxide dependent oxidation of Mn(II) ions (always present in plants and soils) into highly reactive Mn(III), which is stabilized by various chelators such as

oxalate. Oxidised or chelated Mn (III) complex acts as low molecular weight, diffusible redox mediator that attacks phenolic lignin structures ensuing in the formation of unstable free radicals that have a tendency to disintegrate spontaneously (Hofrichter, 2002). Whereas LiP catalyses the oxidation of both non phenolic and phenolic lignin compounds. It oxidises the substrates and forms intermediate phenoxy and veratryl alcohol radical cations. These intermediate radicals endure non enzymatic reactions such as side chain cleavage, radical coupling and polymerization, intramolecular addition and rearrangement and demethylation (Piontek et al., 2001; Dashtban et al., 2010).

The effluent contains lignin and other phenolic compounds that may induce Lip and MnP enzymes during degradation process by the strains *Bacillus megaterium* and *Pseudomonas* sp. similar findings were reported by Chandra and Bharagava, (2012); Haq et al., (2016a); Raj et al., (2014). In a similar study, Chandra and Singh (2012) showed that the induction of MnP, LiP and laccase during the degradation process of pulp paper mill effluent by three bacterial strains *Pseudochrobactrum glaciale, Providencia rettgeri* and *Pantoea* sp.). Zainith et al. (2019) also reported the maximum production of MnP during the bacterial treatment of the effluent by *Bacillus aryabhattai* strain at 72 h. The degradation and decolourization capability of lignin and other organic pollutants present in pulp and paper effluent varies from species to species and most of the researchers have reported fungi to be the most appropriate species through enzyme production. Rajwar et al. (2017), reported two fungal strains *Nigrospora* sp. and *Curvularia lunata* secreted LiP, MnP and laccase during bacterial treatment of pulp and paper mill effluent.

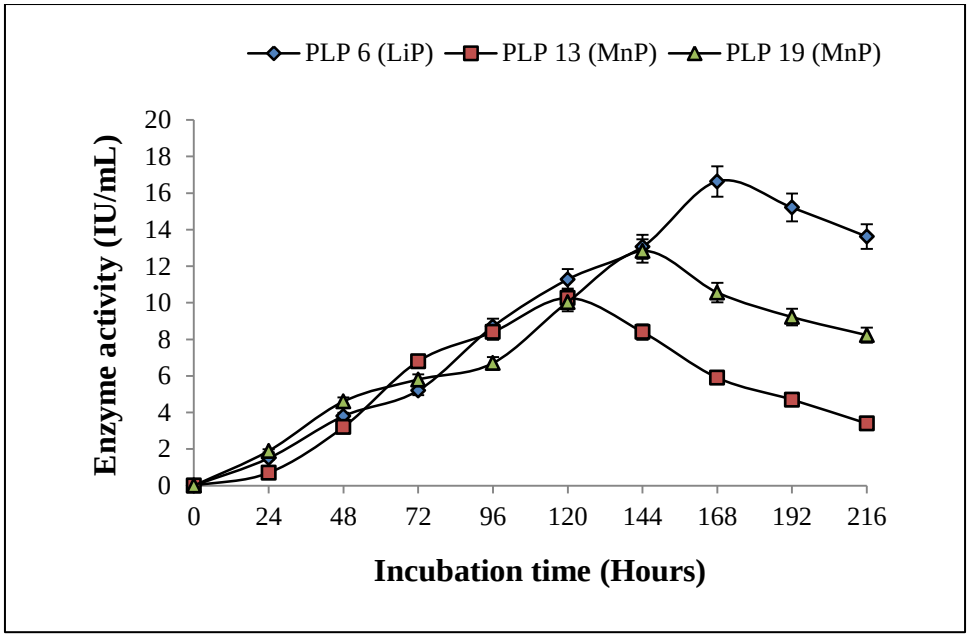


Figure 6.1: Ligninolytic enzyme activity LiP produced by bacterial strain PLP 6 and MnP produced by bacterial strains PNP 13 and PNP 19 during bacterial treatment of pulp and paper mill effluent.

The SDS-PAGE analysis revealed that bacterial strains *Bacillus aryabhattai*, *Bacillus megaterium* and *Pseudomonas* sp. possess ligninolytic enzymes. Protein separated by SDS-PAGE gel was treated for enzyme renaturation and showing that *Bacillus aryabhattai* strain having a protein band that had molecular weight of ~44 kDa, while *Bacillus megaterium* and *Pseudomonas* sp. showed protein band that had molecular weight of ~49 kDa and ~50 kDa. Similarly, lignin peroxidase enzyme was purified from *Bacillus megaterium* produces ~65 kDa protein on SDS-PAGE (Patil. (2014). Oliveira et al., (2009) isolated two bacterial strains *Bacillus pumilus* and *Paenibacillus* sp. were isolated from wood decomposition material and from pulp paper effluent, produces MnP enzyme and the molar masses determined by SDS-PAGE gel electrophoresis were 25 kDa and 40 kDa. Haq et al., (2016a) isolated lignin degrading bacterial strain *Serratia liquefaciens* produced lignin peroxides enzyme and the molecular weight of this purified enzyme was estimated to be ~28 kDa.

The total protein was also calculated before SDS-PAGE analysis i.e. PLP 6 produced 868.9 µg/L of protein, PNP 13 produced 1067.1 µg/L and PNP 19 produced 1943.6 µg/L of protein. The total protein produced from all three bacterial isolates and SDS-PAGE analysis of the strains as shown in Table 6.1 and Fig 6.2.

Table 6.1: Total protein estimation and enzyme activity of all three bacterial strains

Samples	Total Protein (µg/L)	Enzyme activity (IU/mL)
PLP 6	868.9	16.64 (LiP)
PNP 13	1067.1	10.26 (MnP)
PNP 19	1943.6	12.83 (MnP)

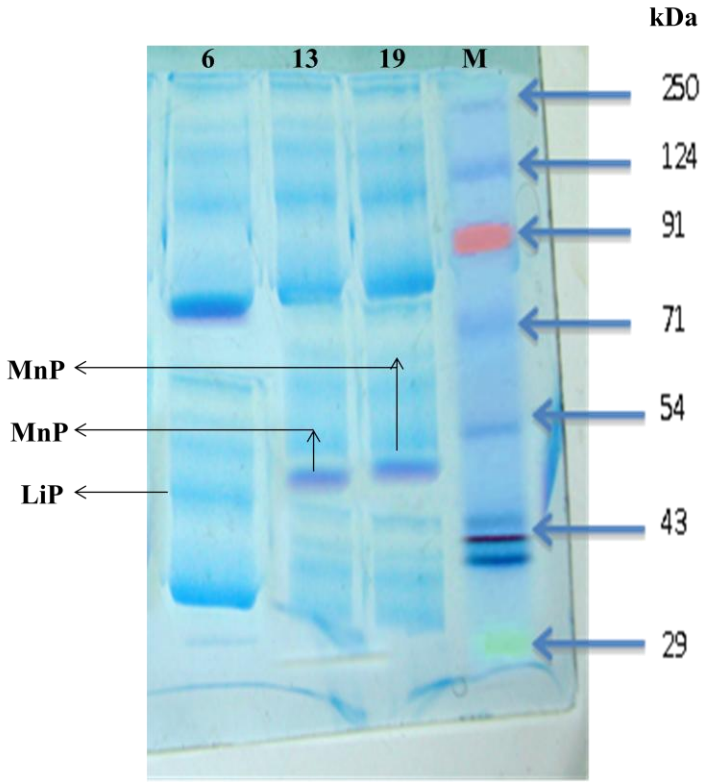


Figure 6.2: SDS-PAGE analysis of crude enzyme manganese peroxidase (MnP) and lignin peroxidase (LiP) produced by *Bacillus megaterium* (PNP 13), *Pseudomonas* sp. (PNP 19) and *Bacillus aryabhattai* (PLP 6).

6.4. Conclusion

This study concluded that microbial reduction of lignin and other organic pollutants is an enzymatic process. The isolated lignin degrading bacterial strains *Bacillus aryabhattai* (PLP 6), *Bacillus megaterium* (PNP 13) and *Pseudomonas* sp. (PNP 19) exhibited remarkable ligninolytic enzyme activity as observed by quantitative analysis. Results obtained from the present investigations showed that *Bacillus megaterium* and *Pseudomonas* sp. having MnP enzyme activity, while *Bacillus aryabhattai* possess LiP activity. SDS-PAGE analysis showed molecular weight of protein around ~44 kDa (PLP 6),~49 kDa (PNP 13) and~50 kDa (PNP 19).

Chapter 7

Physico-Chemical Analysis of Pulp and Paper Mill Effluent Before and After Bacterial Treatment

Chapter 7

7. Introduction

Day by day increased industrialization and population have led to a dramatic proliferation in the production of wastewaters all around the world. This uncontrolled urbanization has caused serious pollution problems, regarding untreated disposal of waste materials in the form of solid and liquid discard from industries directly or indirectly into water bodies. Metal finishing, petroleum refining, iron and steel, pulp and paper, electroplating, tanneries, textile and chemical industries produce various hazardous materials that discharged directly into water systems without any treatment process (Jaggi and Freedman, 1992)**.**

The Physico-chemical parameters are the quality characteristic of any industrial effluent, which represents the pollution profile and toxicity strength. The pollution profile of wastewaters varies from industry to industry and depends on the type of the industry, raw material used and the manufacturing process involved. Physico-chemical analysis is a basic tool that defines the nature and actual pollution potential of various types of industrial effluents. On the basis of physico-chemical characteristics we can predict the environmental fate of the industrial effluents (Somashekar et al, 1984). Therefore, assessment of different physical and chemical factors released from industries, which are responsible for contributing toxicity in environment is very essential.

As pulp and paper mill are the major industry in our country, which releases highly polluted dark coloured effluent with different characteristics. There are two important steps in paper production process: pulping and bleaching. In pulping process about 90% lignin dissolved and 10% lignin remains with the pulp called residual lignin. To remove this residual lignin, sequential bleaching (elemental

chlorine and chlorine dioxide) is carried out by the industries. The effluents generated by these processes are very different and require proper treatment (Zaied and Bellakhal, 2009).

However, the effluent of all types of paper mill contains lignin, chlorinated organic compounds including phenols, which raising the level of biological oxygen demand (BOD), chemical oxygen demand (COD), colour and toxicity to the environment (Bajpai and Bajpai, 1994) causing severe pollution problems not only to the natural flora and fauna of aquatic system but also affects terrestrial ecosystems. Before considering biological system for the treatment of effluent, a comprehensive physico-chemical analysis is necessary to know the concentration of lignin, colour, BOD, COD, inorganic salts, chlorophenols and heavy metals present in pulp paper mill effluents (Devi et al., 2011).

The continuous presence of microorganisms in harsh environments with toxic pollutants develops resistance and reduction mechanism to survive and bioremediate the contaminated environment. Such kind of bacterial strains enriched in the presence of metabolites to toxic compounds with the process of acclimatization and can be used for bioremediation of several compounds present in different, industrial wastes and wastewaters (Mishra et al., 2013). Nowadays, large number of recalcitrant compounds stayed in our environment; just because of superfluous activities of modern industry as well as waste of agricultural practices. Physico-chemical processes such as aerobic oxidation, hydroxylation, photo-decomposition etc., detoxified many industrial waste and wastewaters, but biodegradation process appears to be the major route of their disposal system (Bishnoi et al., 2006). Recent researches have revealed a number of microbial systems i.e. axenic conditions or the consortium

of two or more than two strains capable of transforming or biodegrading recalcitrant compounds of pulp paper mill wastewater.

Hence, this chapter describes the nature and characteristics of pulp and paper mill effluent before (untreated) and after treatment of bacterial consortium.

7.1. Materials and methods

7.1.1. Chemicals and glassware's

All the chemicals and reagents used in this study were of analytical grade. All the glassware used for experimental purpose were washed with nitric acid (10%) and rinsed with double distilled water (DDW) to prevent the impurities. Sterilization was done by autoclaving at 121 ºC, 115 kPa (15 psi) for 15 minutes.

The collection of pulp and paper mill effluent samples and lignin estimation and colour measurement was discussed in previous chapter 3. Erlenmeyer flasks (1000 mL) containing 500 mL effluent sample supplemented with mineral salts (g/L): Na_2HPO_4, 2.4; KH_2PO_4, 2.0; NH_4NO_3, 0.1; $MgSO_4$, 0.01; $CaCl_2$, 0.01; glucose, 10.0; peptone, 5.0, pH was adjusted at 7.5 and sterilised for 15 min, at 121 ºC. After that an appropriate amount of bacterial consortium was inoculate into the effluent sample and incubated at 35 ºC, 120 rpm for 10 days (216 h). The uninoculated flask was taken as control.

7.1.2. Physico-chemical analysis of pulp and paper mill effluent before and after bacterial treatment

The collected P&P mill effluent samples and bacterial treated effluent samples were analysed for physico-chemical parameters such as pH, total alkalinity, BOD (5 day method), COD (open reflux method), totals solids, dissolved and suspended solids (TDS & TSS), chlorides, total phenol (APHA, 1998), total nitrogen, phosphate, sulphate and heavy metals according to the standard protocol and methods for the

examination of water and wastewater (APHA, 2012). Lignin content and colour level measured in the effluent as per the method given by Pearl and Benson, (1940) and CPPA, (1974). All the experiments were performed in triplicates.

7.1.2.1. pH determination

The pH of sample was determined by measuring the electromotive force of a cell comprising of an indicator electrode (an electrode responsive to hydrogen ions such as glass electrode) immersed in the test sample and a reference electrode (usually a mercury/calomel electrode). The electrode is allowed to stand for 2 minutes to stabilize before taking reading for reproducible results.

7.1.2.2. Determination of Total alkalinity

Alkalinity is a measure of the acid-neutralizing capacity of water and wastewater and is usually determined by titration against sulphuric acid (H_2SO_4) to the end point of the acid-base reaction. It is primarily a function of carbonate, bicarbonate and hydroxide content. A 50 mL sample is taken in beaker and pH probe was immersed in the sample. 0.1 N solution of HCl or H_2SO_4 was added drop by drop until the pH of the sample reached 3.7 and the volume of the acid was noted.

Calculation

Alkalinity (mg/L) = (50000 × N of HCl × mL of acid titrated/sample volume (mL)

7.1.2.3. Determination of biological oxygen demand (BOD)

Biological oxygen demand is the amount of dissolved oxygen needed by aerobic biological organisms to break down organic material present in given water and wastewater sample at a certain temperature over a specific time period. A 300 mL of wastewater sample was taken in BOD bottle and kept in BOD incubator for 5 days at 20 ºC. After 5 days, add 2 mL $MnSO_4$, alkali azide and conc. H_2SO_4 to the bottle.

Shake the bottle and out of 500 mL, 200 mL sample was used for the titration with 0.025 N sodium thiosulphate. Starch solution is added as an indicator (gives purple colour) and the end point colour changes from purple to colourless. BOD bottle with aerated water or without wastewater sample was taken as blank.

Calculation

BOD_5 = Blank − titrated sample value 300/ Sample volume

7.1.2.4. Determination of chemical oxygen demand (COD)

Chemical oxygen demand is the amount of oxygen consumed to chemically oxidise organic water contaminants to inorganic end products under specific conditions. In a conical flask, 2.5 mL of wastewater sample was taken and added 1.5 mL of 0.025 N of potassium dichromate, a pinch of mercuric sulphate and 30 mL of conc. HCl into the flask and kept in COD reactor for 2 h at 150 ºC. After cooling, the sample was titrated against 0.1 N ferrous ammonium sulphate (FAS) with ferroin indicator. The Reddish brown colour was appeared at the end point of the result. 2.5 mL of distilled water was taken as blank with same procedure.

Calculation

COD (mg/L) = Blank − titrated sample × N of FAS × 8000/ sample Volume

7.1.2.5. Determination of total solids (TS)

The term solids defined as the total amount of substances soluble, insoluble or suspended in wastewater and residues left upon evaporation and subsequent drying at a defined temperature (103-105 ºC). It includes total dissolved solids (TDS) and total suspended solids (TSS).

Calculation

Total solids (TS) = total dissolved solids (TDS) − total suspended solids (TSS)

7.1.2.5.1. Determination of total dissolved solids (TDS)

Total solid are determined as the residue left after evaporation of the filtered sample. Take the dry weight of evaporating dish (initial weight). Take 50 mL of sample and filter with Whatman no. 4 filter paper. Transfer the filtered sample in evaporating dish and kept it in water bath until dry. After evaporation, the evaporating dish kept in oven for at least 1 h and takes the final weight of the evaporating dish.

Calculation

Total dissolved solid (mg/L) = Final weight – Initial weight 1000 1000/Volume of sample

7.1.2.5.2. Determination of total suspended solids (TSS)

Total suspended solids are determined as the residue left over filter paper after filter the sample. Take the dry weight of the filter paper (initial weight). Take 50 mL of sample and filter with Whatman no. 4 filter paper. The filtrate over the filter paper was dried in oven until it become fully dried and takes the final weight of the filter paper.

Calculation

Total suspended solid (mg/L) = Final weight – Initial weight × 1000 × 1000/Volume of sample

7.1.2.6. Determination of chloride ions

The test for chloride ions is based on precipitation of an insoluble chloride salt. Chloride was determined in water samples by titration against silver nitrate solution using potassium chromate as an indicator. Silver nitrate reacts with chloride ions present in water sample and produces white soluble precipitate of $AgCl_2$. At the end point when all the chloride gets precipitated then free silver ions reacts with chromate

to form reddish brown colour of silver chromate. Distilled water taken as blank followed with same procedure.

Calculation

Chloride (mg/L) = (Sample – Blank) × N of $AgNO_3$ × 35.45 × 1000/ volume of sample

7.1.2.7. Determination of total phenol

Total phenol present in effluent sample was determined by the chloroform extraction method. 3 mL of 4-aminoantipyrine (2%) was added to develop colour and absorbance was measured at 460 nm.

7.1.2.8. Determination of heavy metals present in pulp and paper mill effluent

The determination of heavy metals concentration in collected pulp and paper mill effluent sample was done by Atomic Absorption Spectrophotometer (AAS) (VARIAN AS240FS) through acid digestion method. The acid digestion of water sample was performed by taking 100 mL filtered pulp paper effluent sample in a conical flask and mixed with 6:1 ratio of conc. nitric acid and perchloric acid, respectively. Flask was covered with watch glass and kept in a fume hood on hot plate and digested until the yellow fumes were released and the solution become thick and clear. After cooling, the digested solution was filtered with Whatman filter paper No.4 and volume make up to 10 mL by using distilled water and used for metal analysis with their respective standard metal solution. Distilled water was taken as blank.

7.2. Statistical analysis

All the experiments were performed in triplicates (n=3) and the results obtained from each set of experiment have been expressed in terms of mean and standard deviation.

7.3. Results and discussion

The physico-chemical analysis of pulp and paper mill effluent before and after bacterial treatment as shown in Table 7.1. All the values were found against the minimum standard set by Central Pollution Control Board (CPCB. 2013) guidelines. The effluent was light brown in colour (1065±89.3 Co-Pt) and alkaline in nature (pH = 8.3). In addition to this, various parameters of the effluent were found above permissible limits like: BOD (426 ± 30.61), COD (774 ± 43.75), TDS (859 ± 45.29), TSS (612 ± 24), phosphate (9.1 ± 1.13), sulphate (866 ± 47.03), nitrate (43.6 ± 7.37) and total phenol (39.3 ± 12.05) and chlorides (286 ± 24.63).

Lignin and phenols are the major components of plants that are and presents as foremost pollutants of the effluent. The colour of the effluent exceeds the standard limit and it may be due to the presence of lignin and its derivatives present in cellulose pulp as raw material, which is used in paper manufacturing process. The coloured effluent inhibits the photosynthesis process and creates toxicity in the food chain of aquatic as well as terrestrial ecosystem.

Phenol and its derivatives induce genotoxic, carcinogenic, immunotoxic and physiological effects in fish and other receiving water bodies and have a high bioaccumulation rate along the food chain due to their lipophilicity. Even at very low concentration phenols are toxic to both flora and fauna (Tsutsui et al., 1997). In pulping process white liquor (sodium hydroxide and sodium sulfite) are used to dissolve the lignin and hemicellulose content, which increases the sulfate and pH levels, whereas in bleaching process the release of lignin and its derivatives and chlorides raises the COD level of the effluent (Singhal and Thakur, 2009; Rajwar et al., 2017).

The nitrates present in wastewater mainly associated with lignin. Total solids include organic, inorganic and many dissolved substances, which create toxic environment by changing the ion composition and increase in salinity (USEPA 1986), poses threats to the aquatic system. The high BOD of the effluent indicates that water is highly polluted, which severely affects photosynthetic organisms due to the depletion of dissolved oxygen in the effluent. Various heavy metals such as Cr, Cd, Pb and Mn were also present in trace amount in the effluent (Hakeem and Bhatnagar. 2010). They might be due to the bioaccumulation of plants as wood content used as raw materials and from various chemicals used in paper manufacturing process.

Some parameters such as TDS and metals content were below the permissible limit in untreated wastewater. The quality of the wastewater improved significantly after the treatment of developed bacterial consortium. The results revealed that the consortium effectively reduced pollutant parameters of pulp and paper mill effluent. The main parameters such as colour, lignin, BOD, COD effectively reduced by bacterial consortium up to 73.58%, 56.56%, 94.58% and 75.28%. Other parameters also reduced upto significant level and showed below the permissible limit set by the central pollution control board (CPCB). Several authors reported their findings based on physico-chemical analysis of effluent. Kamalaveni and Karthikeyan (2016) studied the physico-chemical parameters of pulp paper effluent and their results concluded that parameters such as BOD and COD effectively reduced through bacterial strains. Jeenathunisa et al. (2017) found in their study that physico-chemical parameters of untreated pulp paper mill effluent exceed the standard limit, but after bacterial treatment with *Bacillus* sp. and *Pseudomonas* sp. efficiently reduced the pollution parameters.

Table: 7.1 Physico-chemical characteristics of pulp and paper mill wastewater

S. No.	Parameters	Untreated effluent	Bacteria treated effluent	Permissible limit (CPCB, 2010)
1.	pH	8.1	7.5	5-9
2.	BOD (mg/L)	426 ± 30.61	31.59 ± 0.60	30.0
3.	COD (mg/L)	774 ± 43.75	149.61 ± 4.59	250
4.	TDS (mg/L)	859 ± 45.29	428 ± 35.16	2100
5.	TSS (mg/L)	612 ± 24	80.40 ± 15.89	100
6.	Colour (CU)	1065 ± 89.27	261.61 ± 13.88	Colourless
7.	Lignin (mg/L)	529 ± 20.10	229.76 ± 11.12	-
8.	Phosphate (mg/L)	9.1 ± 1.13	3.02 ± 0.32	5.0
9.	Sulphate (mg/L)	866 ± 47.03	498 ± 29.9	1000
10.	Nitrate (mg/L)	43.6 ± 7.37	6.93 ± 2.14	10.0
11.	Chlorides (mg/L)	286 ± 24.63	96.13 ± 5.33	230
12.	Total nitrogen (mg/L)	115 ± 25.23	57.53 ± 8.41	25.0
13.	Total phenol (mg/L)	39.3 ± 12.05	0.509 ± 0.11	1.0
14.	**Heavy metals (mg/L)**			
i.	Cr	BDL		2.0
ii.	Cd	BDL		0.01
iii.	Pb	BDL		0.05
iv.	Mn	0.05 ±0.02		0.20

BDL: Below Detection Limit; **(-):** not specified

7.4. Conclusion

Pulp and paper mill wastewater is highly toxic in nature, which severely affects plants as well as animals growth and metabolism. The results of physico-chemical analysis of collected paper mill effluent sample revealed that, it is highly alkaline in nature, having high BOD, COD and suspended and dissolved solid values. The wastewater was also contaminated with various heavy metals that make wastewater toxic and hazardous. Discharge of such type of contaminated wastewater into fresh water bodies make them unfit for domestic consumption. This could be detrimental for aquatic as well as r terrestrial biota. But after the treatment of bacterial consortium the pollutants present in the effluent reduced upto a significant level and may be suitable for agricultural practices and other purposes.

Chapter 8

Detection and Characterization of Metabolites Produced during the Bacterial Treatment of Pulp and Paper Mill Effluent by HPLC/GC-MC Analysis

Chapter 8

8. Introduction

Lignin is a waste polymer by-product from kraft pulping process released from pulp and paper industries into the environment. It gives dark brown colour to the effluent, and this dark colour brown responsible for the reduction of oxygen level in water streams, causing aquatic pollution and affects aquatic fauna and flora (Gaete et al., 2000). To reduce the colour content of pulp and paper mill wastewater, most of the paper industries have installed chemical recovery of lignin from black liquor (Lara et al., 2003).

Fungi and their ligninolytic enzymes transform high molecular weight organic compounds along with lignin into low molecular weight compounds (Kirk and Farell, 1987). Due to immense environmental adaptability and biochemical versatility bacteria deserves immense ligninolytic potential and hence, bacterial enzyme systems serve as useful tools for the conversion of lignin and organic compounds present in pulp and paper mill effluent into various useful intermediate metabolites (Masai et al., 1999). Earlier studies reported that some bacterial species could metabolize lignin and its derivatives to various low molecular weight compounds (Jokela et al., 1987; Kumar et al., 2001.

Pulp and paper mill effluent contains a mixture of organic and inorganic pollutants, produced during manufacturing process of pulp and paper. Moreover, there are also some hazardous chemicals reported in paper mill effluents, which act as endocrine disrupting compounds (EDCs) such as phthalates, furans and dioxins and their derivatives, disturbs the hormonal balance and reproductive fitness of living organisms and may lead to carcinogenic and mutagenic (Savant et al., 2006).

These compounds present in the effluent can be detected, characterized and identified by various analytical techniques such as high performance liquid chromatography (HPLC), gas chromatography mass-spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS/MS) etc. Thus, the purpose of this chapter was to understand the nature and characteristics of the effluent produced during various steps of pulping and paper manufacturing process before and after bacterial treatment. In this study, detection and characterization of metabolites produced during the bacterial treatment of the effluent through bacterial consortium was analysed by GC-MS.

8.1. Materials and Methods

8.1.1. FT-IR analysis

The FT-IR analysis was carried out to identify or alterations in functional groups of untreated and bacterial treated samples. The untreated and bacterial treated P&P effluent samples were centrifuged at 8000 rpm for 10 min and dried in air at 50 ºC (Kurade et al., 2012). Around 1 mg of dried sample was mixed with pure potassium bromide (400 mg) to form a uniform mixture. The samples were placed in sample disk and gave a manual hydraulic pressure of 100 kg cm^{-2} for 10 min. After that the disk was fixed in FT-IR Spectrophotometer to carry out analysis and the spectrum was recorded in the mid infrared region of 400-4000 cm^{-1} (Bharagava et al., 2018).

8.1.2. Detection and characterization of metabolites produced during the bacterial treatment of pulp and paper mill effluent

8.1.2.1. Liquid-liquid extraction method

For the extraction of pulp paper mill pollutants and their metabolites, 100 mL of untreated and bacterial treated samples were centrifuged at 8000 rpm for 10 min. The obtained supernatant was acidified to pH 1-2 by using 1 N HCl and the acidified

samples were extracted thrice with equal volume (1:1) of ethyl acetate (99.5%) by the solvent extraction method given by Pometto and Crawford, 1988. The extraction process was done in separating funnel by intermittent shaking of acidified samples and ethyl acetate mixture for 20-30 min. The soluble organic layer was separated and the aqueous layer in separating funnel was further extracted to separate the layer. The collected organic layer was dewatered over anhydrous sodium sulphate (Na_2SO_4) with the help of Whatman no. 54 filter paper to absorb the excess water and the samples were vacuum dried. The dried ethyl acetate extracts were derivatized with trimethylsilyl (TMS). In this method, dioxane (100 µL) and pyridine (10 µL) was added in dried samples followed by silylation with 50 µL [BSTFA (*N*,*O*-bis (trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane). After that the mixture heated at 65 ºC in a thermo shaker for 1 h at 14000 rpm until the residue was dissolved completely. Samples were then filtered through syringe filters (0.25 µm) prior to GC-MS analysis.

8.1.2.2. GC-MS analysis

For GC-MS analysis firstly, 1 µL of silylated compounds was injected into the injector port of GC-MS equipped with a PE auto system XL gas chromatograph interfaced with a Turbomass Mass spectrometric mass selective detector. The analytical column connected to the system was a PE-5MS capillary column (20 m × 0.18 mm internal diameter, 0.18 µm film thickness). Helium gas (carrier gas) was used with flow rate of 1 ml/min. The column temperature was programmed as 50 ºC (5 min); 50-300 ºC (10 ºC min, hold time: 5 min). The transfer line and ion source temperature were maintained at 200 and 250 ℃. A solvent delay of 3.0 min was selected. In full scan mode, electron ionization (EI) mass spectra in the range of 30-550 (m/z) were recorded at electron energy of 70 eV. The pollutants present in untreated samples and the metabolites produced during degradation process were

identified by comparing their mass spectra with National Institute of Standards and Technology (NIST) database library and by comparing the retention time with those of accessible authentic organic compounds.

8.2. Statistical analysis

All the experiments and data analysis were performed in triplicates (n=3) to reduce analytical errors and the results obtained were expressed in terms of mean and standard deviation (SD).

8.3. Results and Discussion

8.3.1. FT-IR analysis of degraded products

The bacterial treated pulp paper mill effluent sample was characterized by FT-IR spectra after 216 h of incubation. The spectrum provides information about a change in functional groups after treatment of pulp paper effluent by bacterial consortium and their control samples. The spectrum of lignin compounds has wide absorbance peak in the range from 3570 to 3100 cm^{-1}. These bands are due to the presence of alcoholic and phenolic hydroxyl groups involved in hydrogen bonds of lignin structure give rise to O-H stretching frequencies. A significant difference was observed in the region between 1700 to 1000 cm^{-1} in both untreated and treated samples, which assigned the aromatic ring absorption of lignin (Rajwar et al., 2017).

The spectrum of untreated (Fig. 8.1a) pulp and paper mill effluent sample showed broad peak at 3439 cm^{-1} which allocates carbonylated and hydroxylated functional groups i.e. O-H bond of phenols, poly (vinyl phenol), poly (ethylene oxide), poly (vinyl isobutyl ether). The peak around 1642 cm^{-1} attributed for N-H bond of primary amines, amino acids, C=O stretching for poly-peptides and urease, C=C for cyclic and non-cyclic alkanes and alkenes and NO_2 stretching for nitrates. After treatment with bacterial consortium (Fig. 8.1b), the pollutants present in pulp and paper mill effluent was reduced as the present functional groups are broken and get delinked from other compounds. The peak in the region between 700-600 cm^{-1} showed the stretching

vibration of C-S linkage (Muruganantham et al., 2009). The region 1250-900 cm^{-1} showed the stretching vibration of cyclic ethers caused by the overlapping of C-O bond. These stretching are due to the deformation in lignin structure by different chemicals used in the paper making process.

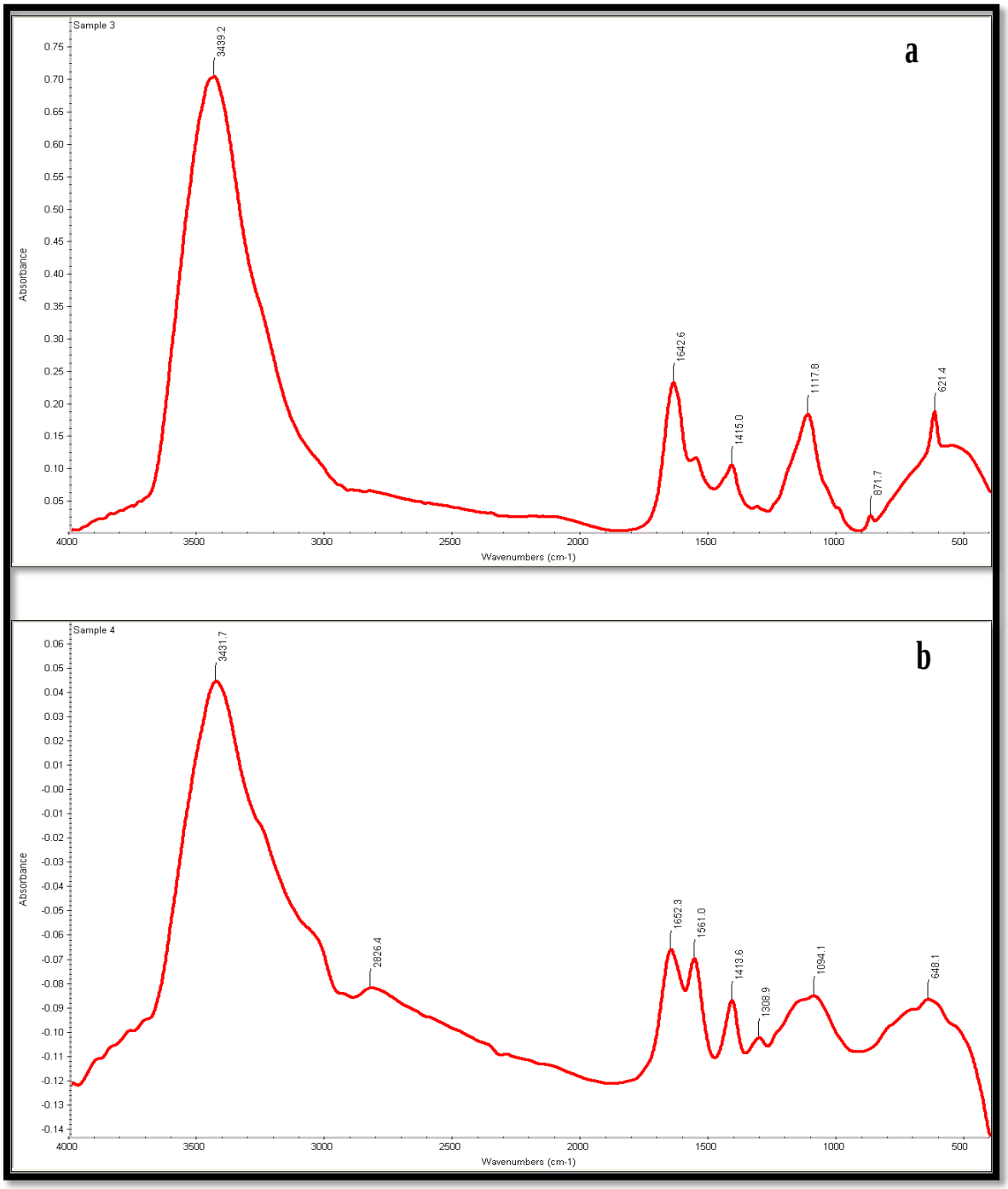


Figure 8.1: FTIR analysis of pulp and paper mill effluent before (a) and after treatment (b) of bacterial consortium

In treated effluent new peaks were found in the region 1400 to 1300 cm^{-1} which gives O-H bending of phenol or a tertiary alcohol. Moreover, increase in the intensity of absorption bands (1652, 1561, 1413, 1308, 1094 cm^{-1}) were also observed, due to the lignin degradation process occurred in the treated sample Pandey and Pitman (2003).

8.3.2. Metabolite characterization through GC-MS

The control and bacterial consortium treated pulp and paper mill effluent samples were analyzed by GC-MS. The results showed that the number of peaks reduced or disappeared in bacterial degraded sample as compared to control samples. In bacterial degraded samples several new compounds were also processed after 216 h (Fig. 8.2 b), indicated the presence of new metabolites. Table 8.1, showed the identified compounds matched from the available standards in the NIST mass spectral database. Major peaks detected in control samples as presented in Fig. 8.2 a at different retention time (RTs) were: 2-methyl-4-keto-pentan-2-ol (RT 7.63), 1-Methyl-2-benzyl-2-phenylpyrrolidine (RT 20.36), 2-(Azidomethyl)-5-phenyltetrahydrofuran (RT 20.56), 1,2-Bis(ç-trimethylsilypropoxy) ethane (21.78), 2,2-Diethyl-4-phenyl-1,3-dioxolane (RT 25.42), 1-[2'-(trimethylsilylmethyl)-cyclopropyl] heptanol (RT 27.66), Benzene-1,2,4-triol,tri (trimethylsilyl) dev (RT 30.54), 1-Methy-2-phenylindole (RT 34.91), (2S,3S)-2,3-Epoxy-1-hexanol (RT 39.85), 2-ethylhydracrylic acid (RT 42.40), 6,7-Dimethoxy-3,4-dihydroisoquinoline - N-Oxide (RT 49.32).

The GC-MS chromatogram of the treated sample with bacterial consortium signifies the release of large amount of low-molecular weight compounds such as hexadecanoic acid (RT 27.68), Octadecane (26.25), 1-Methoxyhexan-2-ol (RT 39.81). Moreover, the low molecular weight alkane compounds like 2-Allylthio-2-methyl-1-

nitropropane (RT 23.20), 2-Fluoro-1-decene (RT 23.46), Octadecane (26.25), 6,13-dihydropentacene and acidic compounds such as 2-Chloroheptylacetate (RT 24.51), Hexadecanoic acid (27.68), 2-ethylhydracrylic acid (RT 42.41) and 1,3,5-Naphthalenetrisulfonic acid (RT 49.89) were identified in the treated effluent sample. The acidic compounds present in the treated effluent sample may be due to the oxidative degradation of lignin and its derivatives (Paliwal et al., 2015; Rajwar et al., 2017). Octadecane (RT 26.25) was detected in treated effluent sample. Presence of this compound in bacterial degraded sample has also been reported by Chandra et al. (2012) in their study. However, the production of octadecane and its role in degradation of pulp paper mill effluent are still unclear whether, it has role as a bacterial metabolite (Yadav and Chandra, 2015).

Most of the compounds discharge from paper mill effluent is toxicants, high molecular weight compounds; fatty acids along with many compounds were detected and are known as endocrine disrupting compounds (Khan and Hall. 2003). Furan derivative was not degraded by bacterial consortium as detected in both untreated (RT 20.56) and treated (RT 36.95) effluent samples which shows its recalcitrant nature. Furan and its derivative could lead to genetic mutations as well as skin disorders to exposed organisms and when it enters the food chain it subsequently affects the trophic level of the life form (Malik et al., 2009). On the other hand, the compound trimethylsilyl remain unchanged, because it is used in the derivatization process of GC-MS analysis as derivatizing agent.

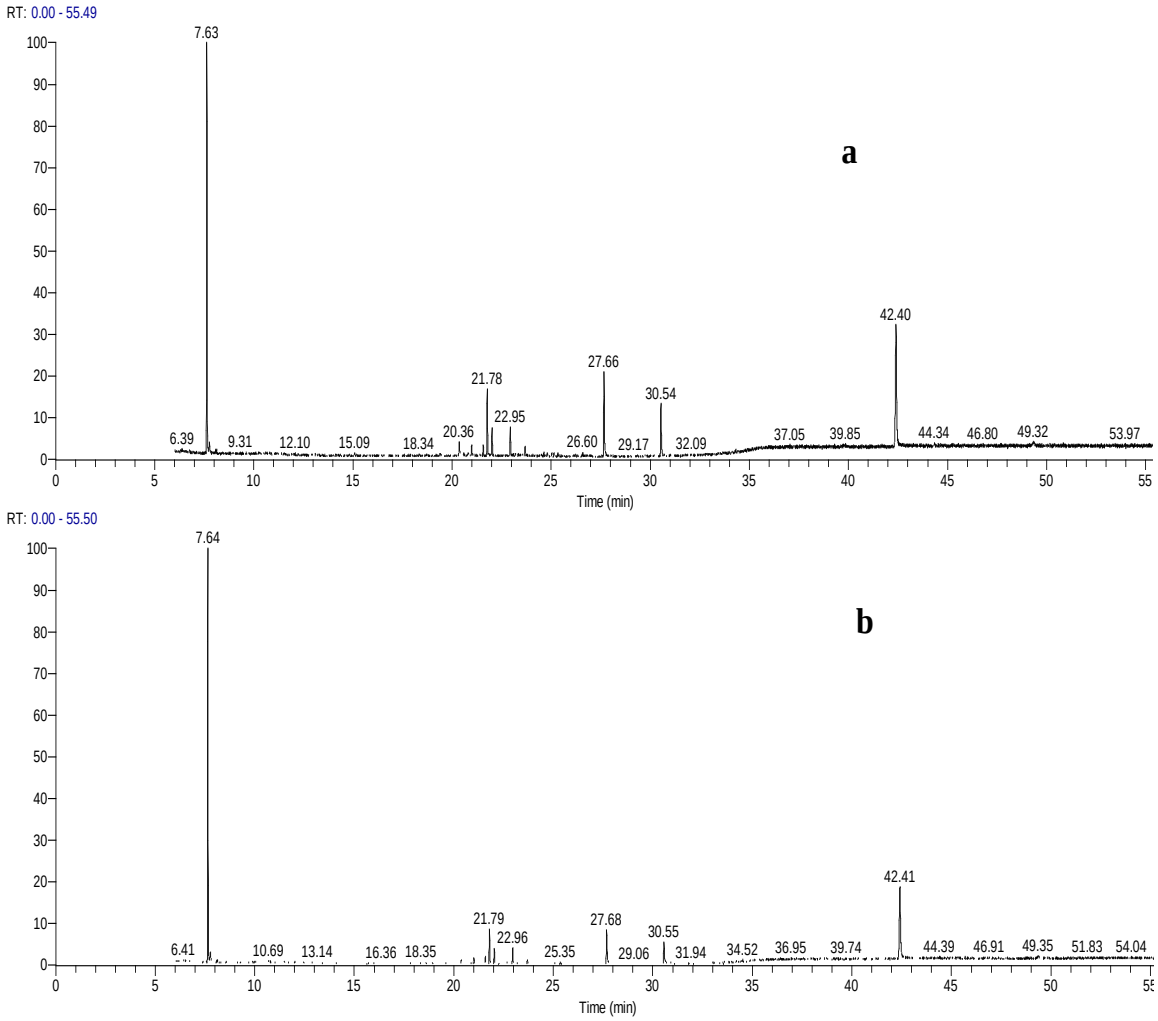


Figure 8.2: GC-MS analysis of pulp and paper mill effluent before (a) and after treatment (b) of bacterial consortium

Table: 8.1 Compounds identified by GC-MS analysis of control (a) and bacterial consortium treated (b) pulp and paper mill effluent extracted with ethyl acetate

Retention time (min)	Compounds	Present/absent in	
		a	B
7.63	2-Methyl-4-keto-pentan-2-ol	+	
10.69	Methyl 3-Iodo-4-hydroxybenzoate		+
20.36	1-Methyl-2-benzyl-2-phenylpyrrolidine	+	
20.56	2-(Azidomethyl)-5-phenyltetrahydrofuran	+	
21.78	1,2-Bis(ç-trimethylsilypropoxy)ethane	+	
21.79	1,2-Bis(ç-trimethylsilypropoxy)ethane		+
23.20	2-Allylthio-2-methyl-1-nitropropane		+
23.46	2-Fluoro-1-decene		+
24.51	2-Chloroheptylacetate		+
25.42	2,2-Diethyl-4-phenyl-1,3-dioxolane	+	
26.25	Octadecane		+
27.68	Hexadecanoic acid		+
30.54	Benzene-1,2,4-triol tri(trimethylsilyl) dev	+	
34.91	1-Methy-2-phenylindole	+	
36.06	(E)-2-Cycloheptyl-1-phenylethene	+	
36.33	6,13-dihydropentacene		+
36.95	5-(2,2-Diphenylvinyl)-4,4-dimethyltetrahydro-2-furanone		+
37.30	1,3-Bis(2-azidophenyl)propane	+	
39.81	1-Methoxyhexan-2-ol		+
39.85	(2S,3S)-2,3-Epoxy-1-hexanol	+	
41.07	4-(2',4'-dimethoxy-6'-propylbenzoyloxy)-2-hydroxy-6-pentylbenzoic acid	+	
42.40	2-Ethylhydracrylic acid	+	+
44.34	(2S,3R)-2-Azido-3-(4-methoxyphenyl)hexanedioic acid 6-tert-Butyl 1-Methyl diester	+	
45.26	2,2-Spiro(pentamethylene-3'-azaacetyl)-3-(3'-oxobutyl)-7-methyl-3,7-diaza-4-oxabicyclo[3.3.0]octane-6,8-dione	+	
45.56	2,3-Dihydro-2-phenyl-2-ethylindole	+	
49.32	6,7-Dimethoxy-3,4-dihydroisoquinoline - N-Oxide	+	
49.35	N-Benzyl-2,2-dimethylpropanamide		+
49.89	1,3,5-Naphthalenetrisulfonic acid		+

8.4. Conclusion

This study concluded that FT-IF and GC-MS analysis confirmed the presence of various organic compounds in pulp and paper mill effluent, in which some compounds were reported as hazardous for aquatic as well as terrestrial environment. Bacterial treatment reduces the toxicity of pulp and paper mill effluent. In this study, bacterial consortium converts various high molecular weight organic compounds in to low molecular weight compounds and the degradation of pulp and paper effluent pollutants were confirmed through lower retention time (RT) in GC-MS chromatogram. Therefore, this developed consortium could be useful for the effective degradation and decolourization of pulp and paper mill effluent wastes and wastewaters.

Chapter 9

Toxicity Assessment of Pulp and Paper Mill Effluent Before and After Bacterial Treatment by Using Terrestrial or Aquatic Test Models

Chapter 9

9. Introduction

The pulp and paper mill effluent contains diverse array of organic and inorganic compounds as well as toxic trace elements, which may accumulate in soils in excessive quantities under long term use. They are toxic in nature, containing higher concentration of various pollutants such as dissolved solids, chlorides, sulphides, phenol, high BOD, COD and various types of toxic metals, which greatly affects both aquatic and terrestrial flora and fauna. They cause various physiological changes and affects plant growth by inhibiting seed germination, root growth and biochemical activity of crop plants. Pulp and paper mill pollutants reduce plant growth by causing various chromosomal abnormalities which eventually reduces mitotic index (Haq et al., 2016b).

Now-a-days, treated industrial effluents can be used for irrigation purpose and is considered as a potential water source, because it contains substantial amount of nutrients, which may beneficial for plant growth (Sahai et al., 1985) and hence, the use of industrial wastewater in agriculture is gaining importance swiftly. The nature of discharged effluent varies from industry to industry and it may or may not be suitable for irrigation purpose. Therefore, the effluent should be assessed properly prior to its application for irrigation. When the effluent is used without any adequate treatment, toxic substances present in the effluent reduces crop yield and gives severe adverse effect on soil properties. Pulp and paper mill effluent increased the concentration of sodium and potassium and disturbed the anionic-cationic balance in plants, thereby reducing the yield and quality of crops (Juwarker et al., 1987; Narwal et al., 2006).

The harmful effects of pulp and paper mill effluent on various crop plants was previously investigated and reported by many researchers. Dutta and Boissya (1997) studied the effect of paper industry on germination of *Oryza sativa L.* and found that; germination percentage and yield in pulp and paper effluent affected area were comparatively less than other normal land. Singh et al. (2002) studied the effect of paper effluent on *Triticum aestivum L*. and found that diluted effluent showed increase in chlorophyll content, plant height, root and shoot biomass and grain yield while concentrated effluent showed a decrease in parameters. Medhi et al., (2008) studied the effect of pulp and paper mill effluent on seed germination and seedling growth of mustard (*Brassica campestris*), pea (*Pisum sativam*) and rice (*Oryza sativa L*) seeds and found that the lower concentration of effluent had a growth promoting effect whereas the germination of seeds and seedling growth gradually declined with increased concentration of effluent. So, this chapter describes the toxicity evaluation of pulp and paper mill effluent before and after bacterial treatment by phytotoxicity assay on *Vigna radiata* and *Cicer arietinum*.

9.1. Materials and Methods

9.1.1. Pulp and paper mill effluent sample preparation for toxicity evaluation

The pulp and paper mill effluent sample collected from Century Pulp Paper Mill, Uttarakhand (India) and treated with bacterial consortium was used in this study. The physico-chemical parameter and lignin concentration in untreated and bacterial consortium treated pulp paper effluent sample was analysed according to (APHA, 2012).

The toxicity assessment of pulp and paper mill effluent, three test solutions; control, untreated and bacterial treated (developed bacterial consortium in this study) pulp and paper effluent sample were taken. The tap water was used as control. The

500 mL untreated pulp and paper effluent sample was treated with developed bacterial consortium of *Bacillus aryabhattai*, *Bacillus megaterium* and *Pseudomonas* sp. for effective degradation of lignin and pulp and paper mill effluent. After then, the sample was centrifuged and filtered to remove bacterial biomass and the left supernatant was used in seed germination experiment for the toxicity evaluation of pulp and paper mill effluent before and after bacterial treatment.

9.1.2. Seed collection

The certified seeds of *Vigna radiata* and *Cicer arietinum* were purchased from the local distributor "M/S Beej Bhandar" Alambagh, Lucknow (U.P.) India.

9.1.3. Phytotoxicity (seed germination) assay

The toxicity assessment of pulp and paper mill wastewater was carried out on two kinds of seeds, *Vigna radiata* and *Cicer arietinum*. Since, pulses are an important crop of Indian agriculture; therefore, the study was carried out on these seeds at room temperature. Ten healthy and uniformed sized seeds of *Vigna radiata* and *Cicer arietinum* were taken and surface-sterilized with 0.1% $HgCl_2$ solution for two min, followed by repeated washes with distilled water to avoid surface contamination. The seeds were then placed into 20 mm × 120 mm petri dishes containing one piece of filter paper, followed by a thin layer of cotton. The dishes were filled with tap water (control), untreated pulp and paper effluent and bacterial consortium treated pulp and paper effluent. After seven days of incubation, the numbers of seed grown were recorded. The seeds of control, untreated and bacterial consortium treated effluent were examined for their germination percentage, root and shoot growth, fresh and dry weight. The seed germination experiment was performed in triplicates.

9.2. Statistical analysis

All the experiments and data analysis were performed in triplicates (n=3) to reduce analytical errors and the results obtained were expressed in terms of mean and standard deviation (SD).

9.3. Results and Discussion

9.3.1. Toxicity evaluation of untreated and bacterial consortium treated pulp and paper mill effluent on seeds of Vigna radiata and Cicer arietinum

Seed germination is a very important physiological and biochemical process in plants. Effect of seed germination and plant growth bioassay is considered as most common technique for evaluating phytotoxicity of industrial wastewaters (Kamlesh. 2016). The toxic effect of untreated pulp and paper effluent results in reduced seed germination percentage, root and shoot growth of seeds. The phytotoxicity analysis of untreated and bacterial consortium treated pulp and paper mill effluent was described in Table and Fig. 9.1 for *Vigna radiata* and in Table and Fig. 9.2 for *Cicer arietinum* respectively. The results of this study showed that the germination was highly affected with untreated effluent in comparison to control and bacterial consortium effluent in both *Vigna radiata* and *Cicer arietinum* seeds. The *Vigna radiata* and *Cicer arietinum* seeds showed (96.66±5.77%) and (93.33±5.77%) in control and (76.66±5.77%) germination in bacterial consortium treated effluent. However, in the case of untreated pulp and paper mill effluent, the germination percentage was significantly inhibited upto (53.33±5.77%) and (56.66±5.77%) and only (46.66±5.77%) and (43.33±5.77%) germination occurred in *Vigna radiata* and *Cicer arietinum* seeds, respectively. The inhibition of seed germination in untreated pulp and paper effluent may be due to the presence of various types of pollutants along with lignin and its derivatives. Moreover, the effect of pulp and paper mill effluent on seedling growth i.e. roots and shoots length of *Vigna radiata* and *Cicer arietinum* seeds were also studied. The reduction in root and shoot growth i.e. 0.36±0.30 cm and 0.88±0.15 cm were observed in *Vigna radiata* seeds, whereas the reduction in root (1.06±0.35 cm) and shoot (0.76±0.11 cm) growth were observed in *Cicer arietinum*

seeds. When both the seeds were treated with untreated effluent, they showed only germination or very little growth.

Table 9.1: Phytotoxic effect of untreated and bacterial consortium treated pulp and paper mill effluent on *Vigna radiata* seeds.

Test parameter	Control	Untreated pulp and paper mill effluent	Bacterial treated pulp and paper mill effluent
Seed germination (%)	96.66±5.77	46.66±5.77	76.66±5.77
Inhibition (%)	3.33±5.77	53.33±5.77	23.33±5.77
Root length (cm)	3.66±0.20	0.36±0.30	3.13±0.20
Shoot length (cm)	9.46±0.97	0.88±0.15	6.63±0.45
Fresh weight (mg)	0.095±0.006	0.032±0.004	0.086±0.004
Dry weight (mg)	0.046±0.006	0.019±0.006	0.036±0.006

Control **Untreated** **Bacterial treated**

Fig. 9.1: Toxicity evaluation of pulp and paper mill effluent by seed germination assay on *Vigna radiata*

However, when these seeds were treated with tap water (control) and bacterial consortium treated effluent, showed better results compared to untreated effluent. The seeds treated with tap water (control) showed 3.66±0.20 cm and 8.76±5.77cm root growth and 9.46±0.97 cm and 4.83±0.55 cm shoot growth in *Vigna radiata* and *Cicer*

arietinum seeds. For bacterial treated effluent the root increase upto 3.13±0.20 cm and 4.36±0.51 cm, while the shoot increased upto 6.63±0.45 cm and 3.96±0.80 cm were observed in the seeds of *Vigna radiata* and *Cicer arietinum* respectively. Lateral root growth was also observed in bacterial consortium treated seeds that was absent in untreated effluent seeds. Likewise, the fresh and dry weight was also affected due to the toxic effect of untreated pulp and paper effluent.

Table 9.2: Phytotoxic effect of untreated and bacterial consortium treated pulp and paper mill effluent on *Cicer arietinum* seeds.

Test parameter	Control	Untreated pulp and paper mill effluent	Bacterial consortium treated pulp and paper mill effluent
Seed germination (%)	93.33±5.77	43.33±5.77	76.66±5.77
Inhibition (%)	6.66±5.77	56.66±5.77	23.33±5.77
Root length (cm)	8.76±0.40	1.06±0.35	4.36±0.51
Shoot length (cm)	4.83±0.55	0.76±0.11	3.96±0.80
Fresh weight (mg)	0.386±0.02	0.183±0.04	0.286±0.02
Dry weight (mg)	0.296±0.03	0.166±0.05	0.243±0.05

Control **Untreated** **Bacterial treated**

Fig. 9.2: Toxicity evaluation of pulp and paper mill effluent by seed germination assay on *Cicer arietinum*

Thus, the present study showed that the isolated bacterial strains (*Bacillus aryabhattai*, *Bacillus megaterium* and *Pseudomonas* sp.) have potential to degrade and decolorize pulp and paper effluent as well as to reduce its toxicity upto a significant level. Our findings are well supported by the earlier reports of (Medhi et al., (2008) on *Brassica campestris*, *Pisum sativum* and *Oryza satival* seeds, Raj et al., (2014) on *Vigna radiata* **,** Bano. (2016) on rice, mustard and pea seeds, Kamlesh. (2016) on *Coriandrum sativum* seeds and Paranthaman and Karthikeyan (2015) on *Vigna mungo* seeds**.**

9.3. Conclusion

The results from present investigation clearly indicate that, seed germination was significantly reduced in untreated effluent. The adverse effect may be due to the higher contaminants along with lignin present in pulp and paper mill effluent. Untreated effluent application showed adverse effect on seedling germination. So, the seed germination experiment revealed that, the seeds exposed to untreated pulp and paper effluent showed very short or no root and shoot development. But, the seeds exposed to bacterial consortium treated pulp and paper effluent showed better development of both root and shoot growth. Hence, the treatment of pulp and paper mill effluent with bacterial consortium could be a useful and ecofriendly approach that effectively reduces its toxic effects

Chapter 10

Summary

Chapter 10

Summary

Pulp and paper mills are one of the main water and energy intensive industries as it is sixth largest water polluting sector in the world. In India about 75% of total fresh water supplied to pulp and paper industries and emerges as waste water. Fresh water requirement in pulp and paper industry is quiet high (150-250 m^3 per ton of product) in comparison to other industries. The problems associated with pulp and paper mill effluents are pH, colour, high levels of biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS), adsorbable organic halides (AOX) etc. Pulp and paper manufacturing process release lignin and chlorinated organic compounds which are the major contaminants formed in the pulp and paper mill effluent. Pollutants released from pulp and paper mills poses harmful effects on aquatic as well as terrestrial environment. Furthermore some pollutants of pulp and paper mill effluent are resistant to biodegradation and can bioaccumulate in the aquatic food chain which indirectly affects human beings. Due to the high chemical diversity of the pollutants of pulp and paper mill effluent, a wide variety of toxic effects on recipient water bodies have been observed. Pulp and Paper mill wastewater treatment is of immense concern for the environment due to this various conventional treatment processes such as adsorption, membrane filtration, ion exchange, chemical precipitation etc have been evaluated. However, these methods are highly cost-intensive and generate enormous amount of toxic sludge and secondary pollutants. Thus, the study of lignin degrading microorganisms for the degradation and detoxification of pulp and paper mill effluent has recently gained importance and offers a safe and eco-friendly option to achieve bioremediation of effluent contaminated environments. So, the aim of present investigation was to isolate and characterize lignin degrading bacterial strains from pulp and paper mill effluent and

develop a potent bacterial consortium that was capable to reduce lignin and colour to explore the reduction potentiality of bacterial consortium. Thus, the findings of the thesis present are summarized below:

1. The pulp and paper mill effluent and sludge sample was collected from Century pulp and paper mill, Lalkuan, Uttarakhand, India. From the collected pulp and paper mill effluent and sludge samples a total of twenty one bacterial isolates were isolated and screened on the basis of their tolerance limit with increased concentration of lignin on L-MSM agar plates. Out of twenty one, six bacterial isolates were selected for ligninolytic enzyme production by using dye decolorization plate assay method, and out of six, three bacterial strains (PLP 6, PNP 13 and PNP 19) were found to decolorize the dyes as PLP 6 decolorize azure B while PNP 13 and PNP 19 changed the colour of phenol red dye. These three bacterial strains were well thought-out for the characterization, based on the Gram's reaction, cell morphology, colony morphology, growth pattern and biochemical tests Further, these bacterial strains i.e. PLP 6, PNP 13 and PNP 19 were characterized and identified on the basis of cellular morphology, biochemical and 16S rDNA gene sequencing analysis as *Bacillus aryabhattai*, *Bacillus megaterium* and *Pseudomona* sp. with their accession no. MG966493, MG966669 and MH045500, respectively. The phylogenetic relationship between the identified isolated bacteria and other intimately related bacteria found in the GenBank database was performed.
2. After screening these three bacterial strains were checked for their compatibility to each other for the development of bacterial consortium. All the three isolated strains were compatible to each other and hence, selected for

development of bacterial consortium and used throughout the study for the effective degradation and detoxification of pulp and paper mill effluent.

3. The reduction ability of bacterial consortium was found better than the individual bacterial strains. The treated pulp and paper mill effluent clearly confirms that, the developed bacterial consortium is capable to remove pollutant parameters such as colour, lignin, BOD and COD. Thus, the use of bacterial consortium instead of single strain is highly effective and active approach for the remediation of pulp paper effluent without any extra additional requirement. The effect of various environmental (pH and temperature) and nutritional (carbon and nitrogen sources) parameters were also studied to achieve much maximum reduction in pollutant parameters by the developed bacterial consortium. The optimization studies revealed that, pH 7.0 and 35 ºC temperature was the most optimum environmental conditions for reduction experiments whereas, glucose (1.0%) and peptone (0.25%) were found optimum carbon and nitrogen sources supplement to broth medium for the effective degradation of pulp paper effluent by consortium culture. In addition to that, the effect of resting and shaking condition was also studied. The results from such studies revealed that, maximum reduction was achieved by bacterial consortium culture under shaking condition. Earlier studies showed that the optimization of environmental (i.e. pH and temperature) and nutritional parameter (carbon and nitrogen sources) is a pragmatic approach to enhance the reduction capability of consortium cultures. But, when all the optimized conditions or parameter were applied at one time on the same medium of bacterial consortium it increases the rate of the degradation process. Thus, degradation and detoxification of pulp paper effluent at all

optimized conditions at the same time by developed bacterial consortium could be a more rapid and useful way to remediate effluent contaminated environments.

4. During the treatment of pulp and paper mill effluent, an increase in the production of ligninolytic enzymes was found indicating its involvement in the degradation process of pulp paper effluent. Thus, the isolated lignin degrading bacterial strains were also studied for ligninolytic enzyme activity. Results obtained from the present investigations, showed that all three bacterial strains *Bacillus aryabhattai* showed lignin peroxidase activity while, *Bacillus megaterium* and *Pseudomona* sp. exhibits manganese peroxidase activity.
5. The results of physico-chemical analysis of pulp and paper mill effluent revealed that it was alkaline in nature, deficient in dissolved oxygen, having high BOD, COD, suspended and dissolved solids values. The tannery wastewater also contained various heavy metals like Cr, Cd, Pb and Mn. Most of the pollutants present in the effluent were more than its recommended permissible limit. But after the treatment by a developed consortium, pollutants of pulp and paper effluent reduced upto a significant level and came under the standard limit set by the central pollution control board.
6. The metabolites produced during the treatment of pulp and paper effluent by the developed bacterial consortium was determined by GC-MS analysis. This study concluded that GC-MS analysis confirmed the presence of various organic compounds in pulp and paper mill effluent, in which some compounds were reported as hazardous for aquatic as well as terrestrial environments. But, after bacterial treatment toxicity of pulp paper effluent reduces. The results showed that the number of peaks reduced or disappeared in bacterial degraded

sample as compared to untreated samples. In treated samples the high molecular weight compounds metabolized into low molecular weight compounds. Some compounds such as furan and its derivatives were not degraded by bacterial consortium as detected in both untreated and treated effluent samples which shows its recalcitrant nature.

7. The toxicity assessment of pulp and paper mill effluent before and after bacterial treatment was also investigated by phytotoxicity on the seeds of two important agricultural pulses viz. *Vigna radiata* and *Cicer arietinum* to show toxic effects of untreated pulp and paper mill effluent. The results from phytotoxicity investigation showed that the germination was significantly reduced in untreated effluent. The seeds that were grown in untreated effluent had shown no or very little shoot and root growth compared to those seeds that grown in control and bacterially treated pulp paper effluent. This indicates that untreated pulp paper effluent had inhibitory effect on seed germination. But, the seeds exposed to bacterial treated pulp paper effluent showed better development of both root and shoot. Thus, treatment of pulp and paper mill effluent with bacterial consortium is an effective approach to reduce the pollutants present in the effluent as well as phytotoxicity.

Thus, the whole work concludes that the developed bacterial consortium from three bacterial strains; *Bacillus aryabhattai*, *Bacillus megaterium* and *Pseudomona* sp. was more effective and promising approach to reduce the toxic pollutants of pulp and paper effluent. Hence, it can be used as a potential agent for the effective bioremediation of pulp and paper mill effluent and also their contaminated environment.

References

References

Abd-Elsalam, H.E., El-Hanafy, A.A., 2009. Lignin biodegradation with ligninolytic bacterial strain and comparison of *Bacillus subtilis* and *Bacillus* sp. isolated from Egyptian soil. Am Eurasian J Agric Environ Sci, 5(1), 39-44.

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

Absar, A.K., 2005. Water and Wastewater Properties and Characteristics. In water Encyclopedia: Domestic, Municipal and Industrial Water Supply and Waste Disposal. Lehr, J.H. &Keeley, J. (eds). John Wiley and Sons, Inc., New Jersey. 903-905.

Aguilar, M.I., Sáez, J., Lloréns, M., Soler, A., Ortuno, J.F., Meseguer, V., Fuentes, A., 2005. Improvement of coagulation–flocculation process using anionic polyacrylamide as coagulant aid. Chemosphere, 58(1), 47-56.

Ahammad, S.Z., Gomes, J., Sreekrishnan, T.R., 2008. Wastewater treatment for production of H2S-free biogas. J. Chem. Technol. Biotechnol. 83(8), 1163-1169.

Ahmad, M., Taylor, C.R., Pink, D., Burton, K., Eastwood, D., Bending, G.D., Bugg, T.D., 2010. Development of novel assays for lignin degradation: comparative analysis of bacterial and fungal lignin degraders. Molecular Biosystems. 6(5), 815-821.

Aksu, Z., 2002. Determination of the equilibrium, kinetic and thermodynamic parameters of the batch biosorption of nickel (II) ions onto Chlorella vulgaris. Process Biochem. 38(1), 89-99.

Ali, M., Sreekrishnan, T.R., 2001. Aquatic toxicity from pulp and paper mill effluents: a review. Adv Environ Res. 5(2), 175-196.

Altschul, S.F., Madden, T.L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25 (17), 3389-3402.

American Public Health Association (APHA). 2012. Standard methods for the examination of water and wastewater. 22nd Edition American Public Health Association, American Water Works Association, Water Environmental Federation, Washington, D.C. pp 981.

American Public Health Association., 1998. American Water Works Association and Water Environment Federation, Standard Methods for the Examination of Water and Wastewater, 20th edn, APHA, Washington, DC.

Archibald, F.S., 1992. A new assay for lignin-type peroxidases employing the dye azure B. Appl Environ Microbiol. 58(9), 3110-3116.

Arora, D.S., Chander, M., Gill, P.K., 2002. Involvement of lignin peroxidase, manganese peroxidase and laccase in degradation and selective ligninolysis of wheat straw. Int Biodeterior Biodegrad. 50(2), 115-120.

Asano, T., Cotruvo, J.A., 2004. Groundwater recharge with reclaimed municipal wastewater: health and regulatory considerations. Water Res. 38(8), 1941-1951.

Ashrafi, O., Yerushalmi, L., Haghighat, F., 2015. Wastewater treatment in the pulp-and-paper industry: A review of treatment processes and the associated greenhouse gas emission. Journal of environmental management, 158, 146-157.

Avsar, E., Demirer, G.N., 2008. Cleaner production opportunity asssment study in SEKA Balikesir pulp and paper mill. J Clean Prod. 16(4), 422-431.

Ayed, L., Asses, N., Chammem, N., Othman, N. B., Hamdi, M., 2017. Advanced oxidation process and biological treatments for table olive processing wastewaters: constraints and a novel approach to integrated recycling process: a review. Biodegradation. 28(2-3), 125-138.

Badar, S., Farooqi, I.H., 2012. Pulp and Paper Industry-Manufacturing Process, Wastewater Generation and Treatment. In Environmental protection strategies for sustainable development, 397-436. Springer, Dordrecht.

Bajpai, P., 2001. Microbial degradation of pollutants in pulp mill effluents. Adv Appl Microbiol. 48, 79-134.

Bajpai, P., Bajpai, P.K., 1994. Biological colour removal of pulp and paper mill wastewaters. J Biotechnol. 33(3), 211-220.

Balcioglu, I.A., Tarlan, E., Kivilcimdan, C., Saçan, M.T., 2007. Merits of ozonation and catalytic ozonation pre-treatment in the algal treatment of pulp and paper mill effluents. J Environ Manag. 85(4), 918-926.

Bandounas, L., Wierckx, N.J., de Winde, J. H., Ruijssenaars, H.J., 2011. Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. Bmc Biotechnol. 11(1), 94.

Bano, S., 2016. Impact of amlai paper mill effluent on growth and development of certain agricultural crops. Int J Biol Res. 1(4), 48-51.

Barapatre, A., Jha, H., 2016. Decolourization and biological treatment of pulp and paper mill effluent by lignin-degrading fungus Aspergillus flavus strain F10. Int J Curr Microbiol App Sci. 5(5), 19-32.

Beekeepers Y (2000) Arising from reactive dyes in textile industry color fenton process remedy with, ITU Institute of Science, M.Sc., Istanbul.

Bengtsson, S., Werker, A., Christensson, M., Welander, T., 2008. Production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater. Bioresour Technol. 99(3), 509-516.

Berryman, D., Houde, F., DeBlois, C., O'Shea, M., 2004. Nonylphenolic compounds in drinking and surface waters downstream of treated textile and pulp and paper effluents: a survey and preliminary assessment of their potential effects on public health and aquatic life. Chemos. 56(3), 247-255.

Bharagava, R.N., Chandra, R., Rai, V., 2009. Isolation and characterization of aerobic bacteria capable of the degradation of synthetic and natural melanoidins from distillery effluent. World J Microbiol Biotechnol. 25(5), 737-744.

Bharagava, R.N., Mani, S., Mulla, S.I., Saratale, G.D., 2018. Degradation and decolourization potential of an ligninolytic enzyme producing Aeromonas hydrophila for crystal violet dye and its phytotoxicity evaluation. Ecotoxicol Environ Saf. 156, 166-175.

Bharagava, R.N., Mishra, S., 2018. Hexavalent chromium reduction potential of Cellulosimicrobium sp. isolated from common effluent treatment plant of tannery industries. Ecotoxicol Environ Saf. 147, 102-109.

Bhargava, S.K., Tardio, J., Prasad, J., Foger, K., Akolekar, D.B., Grocott, S.C., 2006. Wet oxidation and catalytic wet oxidation. Ind Eng Chem Res. 45(4), 1221-1258.

Bishnoi, N.R., Khumukcham, R.K., Kumar, R., 2006. Biodegradation of pulp and paper mill effluent using anaerobic followed by aerobic digestion. J Environ Biol. 37(2), 405-408.

Bloom, J.M., 2001. Paper Before Print: The History and Impact of Paper in the Islamic World, ACLS Humanities E-Book, Yale University Press.

Brown , M.E., Chang, M.C., 2014. Exploring bacterial lignin degradation. Curr Opin Chem Biol. 19, 1-7.

Brown, M.E., Walker, M.C., Nakashige, T.G., Iavarone, A.T., Chang, M.C., 2011. Discovery and characterization of heme enzymes from unsequenced bacteria: application to microbial lignin degradation. J American Chemical Society, 133(45), 18006-18009.

Bugg, T.D., Ahmad, M., Hardiman, E.M., Rahmanpour, R., 2011. Pathways for degradation of lignin in bacteria and fungi. Nat Prod Report. 28(12), 1883-1896.

Buyukkamaci, N., Koken, E., 2010. Economic evaluation of alternative wastewater treatment plant options for pulp and paper industry. Sci Total Env. 408(24), 6070-6078.

Call, H.P., Mucke, I., 1997. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym®-process). J Biotechnol. 53(2-3), 163-202.

Central pollution control board (CPCB) 2013. Pollution assessment: river Ganga. Status of grossly polluting industries (GPI). www.cpcb.nic.in

Central Pollution Control Board (CPCB), 2001. Comprehensive industry document for large pulp and paper industry. COINDS/36/2000-2001.

Central Pollution Control Board., 2006. Report in "Developmental of guidelines for water conservation in pulp and paper sector". Environmental Group, National Productivity Council, New Delhi.

Chandra R., 2001. Microbial decolourization of pulp mill effluent in presence of nitrogen and phosphorous by activated sludge process. J Environ Biol. 22(1), 23-27.

Chandra, R., Chowdhary, P., 2015. Properties of bacterial laccases and their application in bioremediation of industrial wastes. Environ Sci: Processes Impacts. 17(2), 326-342.

Chandra, R., Abhishek, A., 2011. Bacterial decolorization of black liquor in axenic and mixed condition and characterization of metabolites. Biodegrad. 22(3), 603-611.

Chandra, R., Abhishek, A., Sankhwar, M., 2011. Bacterial decolorization and detoxification of black liquor from rayon grade pulp manufacturing paper industry and detection of their metabolic products. Bioresour Technol. 102(11), 6429-6436.

Chandra, R., Bharagava, R.N., 2012. Bacterial degradation of synthetic and kraft lignin by axenic and mixed culture and their metabolic products. J Environ Biol. 34 (6), 991-999.

Chandra, R., Ghosh, A., Jain, R.K., Singh, S., 2006. Isolation and characterization of two potential pentachlorophenol degrading aerobic bacteria from pulp paper effluent sludge. J Gen App Microbiol. 52(2), 125-130.

Chandra, R., Raj, A., Purohit, H.J., Kapley, A., 2007. Characterisation and optimization of three potential aerobic bacterial strains for kraft lignin degradation from pulp paper waste. Chemos. 67(4), 839-846.

Chandra, R., Raj, A., Yadav, S., Patel, D.K., 2009. Reduction of pollutants in pulp paper mill effluent treated by PCP-degrading bacterial strains. Environ Monit Assess. 155(1-4). 1-11.

Chandra, R., Sharma, P., Yadav, S., Tripathi, S., 2018. Biodegradation of Endocrine-Disrupting Chemicals and Residual Organic Pollutants of Pulp and Paper Mill Effluent by Biostimulation. Front Microbiol. 9.

Chandra, R., Singh, R., 2012. Decolourisation and detoxification of rayon grade pulp paper mill effluent by mixed bacterial culture isolated from pulp paper mill effluent polluted site. Biochem Eng J. 61, 49-58.

Chandra, R., Singh, R., Yadav, S., 2012. Effect of bacterial inoculum ratio in mixed culture for decolourization and detoxification of pulp paper mill effluent. J Chem Technol Biotechnol. 87 (3), 598 436-444.

Chandra, R., Singh, S., Reddy, M.M. K., Patel, D.K., Purohit, H.J., Kapley, A., 2008. Isolation and characterization of bacterial strains Paenibacillus sp. and Bacillus sp. for kraft lignin decolorization from pulp paper mill waste. The J Gen App Microbiol. 54(6), 399-407.

Chanworrawoot, K., Hunsom, M., 2012. Treatment of wastewater from pulp and paper mill industry by electrochemical methods in membrane reactor. J Environ Manag. 113, 399-406.

Chauhan, N., Thakur, I.S., 2002. Treatment of pulp and paper mill effluent by Pseudomonas fluorescens in fixed film bioreactor. Pollut Res. 21(4), 429-434.

Chen, P., Zheng, J., Zhou, Y., 2009. The presence and future of membrane industry in china. Environ Prot, 8, 71-74.

Chen, Y.H., Chai, L.Y., Zhu, Y.H., Yang, Z.H., Zheng, Y., Zhang, H., 2012a. Biodegradation of kraft lignin by a bacterial strain Comamonas sp. B-9 isolated from eroded bamboo slips. J App Microbiol. 112(5), 900-906.

Chen, Y., Chai, L., Tang, C., Yang, Z., Zheng, Y., Shi, Y., Zhang, H., 2012a. Kraft lignin biodegradation by Novosphingobium sp. B-7 and analysis of the degradation process. Bioresour Technol. 123, 682-685.

Chen. D., 2008. Application of membrane separation technology in the fine chemical industry.

Chou, P.H., Liu, T.C., Lin, Y.L., 2014. Monitoring of xenobiotic ligands for human estrogen receptor and aryl hydrocarbon receptor in industrial wastewater effluents. J. Hazard. Mat. 277, 13-19.

Choudhary, A.K., Kumar, S. and Sharma, C., 2011. Constructed wetlands: an option for pulp and paper mill wastewater treatment. Electronic J Environ Agri Food Chem. 10(10), 3023-3037.

Chowdhary, P., Yadav, A., Kaithwas, G., Bharagava, R.N., 2017. Distillery wastewater: a major source of environmental pollution and its biological treatment for environmental safety. In Green Technologies and Environmental Sustainability. 409-435. Springer, Cham.

Christmas, P., 2002. Building materials from deinking plant residues–a sustainable solution. In COST Workshop Managing Pulp and Paper Residues, Barcelona, Spain. 14, 465-472).

Ciputra, S., Antony, A., Phillips, R., Richardson, D. and Leslie, G., 2010. Comparison of treatment options for removal of recalcitrant dissolved organic matter from paper mill effluent. Chemos. 81(1), 86-91.

Coll, P.M., Fernandez-Abalos, J.M., Villanueva, J.R., Santamaria, R., Perez, P., 1993. Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971). Appl Environ Microbiol. 59(8), 2607-2613.

Costa, S., Dedola, D.G., Pellizzari, S., Blo, S., Irene, R.I., Pedrini, P., Tamburini, E., 2017. Lignin biodegradation in pulp and paper mill wastewater by elected white rot fungi. Water. 9(12), 1-9.

Costigan, S.L., Werner, J., Ouellet, J.D., Hill, L.G., Law, R.D., 2012. Expression profiling and gene ontology analysis in fathead minnow (*Pimephales promelas*) liver following exposure to pulp and paper mill effluents. Aquat Toxicol. 122, 44-55.

Covinich, L.G., Bengoechea, D.I., Fenoglio, R.J., Area, M.C., 2014. Advanced oxidation processes for wastewater treatment in the pulp and paper industry: a review. Am J Environ Eng. 4(3), 56-70.

CPPA, Technical section standard method H5P, 1974. Canadian Pulp and Paper Association, Montreal, Canada.

Crini, G., 2005. Recent developments in polysaccharide-based materials used as adsorbents in wastewater treatment. Prog Polym Sci. 30(1), 38-70.

Dahiya, J.S., Singh, D., Nigam, P., 1998. Characterisation of laccase produced by Coniothyrium minitans. J Basic Microbiol. 38(5-6), 349-359.

Das, C.P., Patnaik, L.N., 2000. Removal of lignin by industrial solid wastes. Pract Period Hazard Toxic Radioact Waste Manag. 4(4), 156-161.

Dashtban, M., Schraft, H., Syed, T.A., Qin, W., 2010. Fungal biodegradation and enzymatic modification of lignin. Int J Biochem Mol Biol. 1 (1), 36-50.

Devi, N.L., Yadav, I.C., Shihua, Q.I., Singh, S., Belagali, S.L., 2011. Physicochemical characteristics of paper industry effluents-a case study of South India Paper Mill (SIPM). Environ Monit Assess. 177(1-4), 23-33.

Dilek, F.B., Gokçay, C.F., 1994. Treatment of effluents from hemp-based pulp and paper industry. Waste characterization and physico-chemical treatability. Water Sci Technol. 29(9), 161-163.

Duan, X., Liu, T., Duan, W., Hu, H., 2010, June. Adsorption and coagulation tertiary treatment of pulp & paper mills wastewater. In 2010 4th International Conference on Bioinformatics and Biomedical Engineering (pp. 1-4). IEEE.

Dube, M., McLean, R., MacLatchy, D., Savage, P., 2000. Reverse osmosis treatment: effects on effluent quality. Pulp Pap Can. 101(8), 42-45.

Dutta, A., Sarkar, S., 2015. Sequencing batch reactor for wastewater treatment: recent advances. Current Pollution Reports, 1(3), 177-190.

Dutta, S.K., Boissya, C.L., 1997. Effect of Paper Mill Effluent on Germinations of Rice Seed (Oryza Sativa L. Vat Masuri) and Growth Behaviour of its Seedlings. J Ind Pollut Cont. 13, 41-47.

Dwivedi, P., Vivekanand, V., Pareek, N., Sharma, A., Singh, R.P., 2010. Bleach enhancement of mixed wood pulp by xylanase-laccase concoction derived through co-culture strategy. App Biochem Biotechnol. 160(1), 255.

Ebrahimi, M., Busse, N., Kerker, S., Schmitz, O., Hilpert, M., Czermak, P., 2016. Treatment of the bleaching effluent from sulfite pulp production by ceramic membrane filtration. Membranes, 6(1), 7.

Ekstrand, E.M., Larsson, M., Truong, X.B., Cardell, L., Borgstrom, Y., Bjorn, A., Ejlertsson, J., Svensson, B.H., Nilsson, F., Karlsson, A., 2013. Methane potentials of the Swedish pulp and paper industry-A screening of wastewater effluents. App Energy. 112, 507-517.

Eskelinen, K., Sarkka, H., Kurniawan, T.A., Sillanpaa, M.E., 2010. Removal of recalcitrant contaminants from bleaching effluents in pulp and paper mills

using ultrasonic irradiation and Fenton-like oxidation, electrochemical treatment, and/or chemical precipitation: a comparative study. Desalin. 255(1-3), 179-187.

Fentress, J.A., Steele, S.L., Bart Jr, H.L., Cheek, A. O., 2005. Reproductive disruption in wild longear sunfish (Lepomis megalotis) exposed to kraft mill effluent. Environ health perspectives, 114(1), 40-45.

Fontanier, V., Albet, J., Baig, S., Molinier, J., 2005. Simulation of pulp mill wastewater recycling after tertiary treatment. Environ Tech. 26(12), 1335-1344.

Freire, R.S., Kunz, A. and Duran, N., 2000. Some chemical and toxicological aspects about paper mill effluent treatment with ozone. Environmental technology. 21(6), 717-721.

Gadd, G.M., 2009. Biosorption: critical review of scientific rationale, environmental importance and significance for pollution treatment. Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology, 84(1), 13-28.

Gaete, H., Larrain, A., Bay-Schmith, E., Baeza, J., Rodriguez, J., 2000. Ecotoxicological assessment of two pulp mill effluent, Biobio River Basin, Chile. Bullet Environ Contaminat Toxicol. 65(2), 183-189.

Ganjidoust, H., Tatsumi, K., Yamagishi, T., Gholian, R.N., 1997. Effect of synthetic and natural coagulant on lignin removal from pulp and paper wastewater. Water Sci Technol. 35(2-3). 291-296.

Garg, A., 2012. Water pollution from pulp and paper mills. In: Daniels, J. A. (Ed.), Adv Environ Res. vol. 20. Nova Science Publishers, Inc, Hauppauge NY, 245-252.

Garg, A., Mishra, I.M., Chand, S., 2007. Catalytic wet oxidation of the pretreated synthetic pulp and paper mill effluent under moderate conditions. Chemosphere. 66(9), 1799-1805.

Garg, S.K., Tripathi, M., 2011. Strategies for decolorization and detoxification of pulp and paper mill effluent. In Reviews of Environmental Contamination and Toxicology Volume 212, 113-136. Springer, New York, NY.

Gassara, F., Brar, S.K., Tyagi, R.D., Verma, M., Surampalli, R.Y., 2010. Screening of agroindustrial wastes to produce ligninolytic enzymes by *Phanerochaete chrysosporium*. Biochem Eng J. 49(3), 388-394.

Gaur, N., Narasimhulu, K., Setty, Y.P., 2018. Extraction of ligninolytic enzymes from novel *Klebsiella pneumoniae* strains and its application in wastewater treatment. Applied Water Science, 8(4), 111.

Gea, T., Artola, A., Sanchez, A., 2005. Composting of de-inking sludge from the recycled paper manufacturing industry. Bioresour Technol. 96(10), 1161-1167.

Ghoreishi, S.M., Haghighi, M.R., 2007. Chromophores removal in pulp and paper mill effluent via hydrogenation-biological batch reactors. Chem Eng J. 127(1-3), 59-70.

Ginni, G., Adishkumar, S., Rajesh Banu, J., Yogalakshmi, N., 2014. Treatment of pulp and paper mill wastewater by solar photo-Fenton process. Desalin Water Treat. 52(13-15), 2457-2464.

Golob, V., Ojstrsek, A., 2005. Removal of vat and disperse dyes from residual pad liquors. Dyes and pigments, 64(1), 57-61.

Gonder, Z.B., Arayici, S., Barlas, H., 2011. Advanced treatment of pulp and paper mill wastewater by nanofiltration process: Effects of operating conditions on membrane fouling. Sep Purif Technol. 76(3), 292-302.

Gonzalez, L.F., Sarria, V., Sanchez, O.F., 2010. Degradation of chlorophenols by sequential biological advanced oxidative process using *Trametes pubescens* and TiO2/UV. Bioresour Technol 101, 3493-3499.

Gubelt, G., Lumpe, C., Verstraeten, E., Joore, L., 2000. Towards zero liquid effluent at Niederauer Mühle: the validation of two novel separation technologies. Paper technol. 41(8), 41-48.

Gunaseelan, V.N., 1997. Anaerobic digestion of biomass for methane production: a review. Biomass Bioenergy. 13 (1-2), 83-114.

Gupta, V.K., Minocha, A.K., Jain, N., 2001. Batch and continuous studies on treatment of pulp mill wastewater by Aeromonas formicans. J Chem Technol Biotechnol: Int Res Process, Environment Clean Technol. 76(6), 547-552.

Hakeem, A.S., Bhatnagar, S., 2010. Heavy metal reduction of pulp and paper mill effluent by indigenous microbes. Asian J Exp Biol Sci 1 (1), 201-203.

Hammel, E.K., Cullen, D., 2008. Role of fungal peroxidase in biological ligninolysis. Curr Opin Plant Biol., II. 349-355.

Haq, I., Kumar, S., Kumari, V., Singh, S.K., Raj, A., 2016a. Evaluation of bioremediation potentiality of ligninolytic *Serratia liquefaciens* for detoxification of pulp and paper mill effluent. J Hazard Mat. 305, 190-199.

Haq, I., Kumari, V., Kumar, S., Raj, A., Lohani, M., Bhargava, R.N., 2016b. Evaluation of the phytotoxic and genotoxic potential of pulp and paper mill effluent using Vigna radiata and Allium cepa. Adv Bio. http://dx.doi.org/10.1155/2016/8065736

Hermosilla, D., Merayo, N., Gascó, A., Blanco, Á., 2015. The application of advanced oxidation technologies to the treatment of effluents from the pulp and paper industry: a review. Environ Sci Pollut Res. 22(1), 168-191.

Herney-Ramirez, J., Silva, A.M., Vicente, M.A., Costa, C.A., Madeira, L.M., 2011. Degradation of acid orange 7 using a saponite-based catalyst in wet hydrogen peroxide oxidation: kinetic study with the Fermi's equation. App Catal B Environ. 101(3-4), 197-205.

Hofrichter, M., 2002. lignin conversion by manganese peroxidase (MnP). Enzyme Microb Technol. 30 (4), 454-466.

Hooda, R., Bhardwaj, N.K. and Singh, P., 2016. Screening and identification of ligninolytic bacteria for the treatment of pulp and paper mill effluent. Water Air Soil Pollut. 226(9), 305.

Howe, J., Wagner, M.R., 1999. Effects of pulpmill effluent irrigation on the distribution of elements in the profile of an arid region soil. Environ Pollut. 105(1), 129-135.

Inan, H., Dimoglo, A., Şimşek, H., Karpuzcu, M., 2004. Olive oil mill wastewater treatment by means of electro-coagulation. Sep Purif Technol. 36(1), 23-31.

Ince, B.K., Cetecioglu, Z., Ince, O., 2011. Pollution prevention in the pulp and paper industries. In Environmental Management in Practice. Intech Open.

Ince, O., Kolukirik, M., Cetecioglu, Z., Eyice, O., Tamerler, C., Kasapgil Ince, B., 2007. Methanogenic and sulphate reducing bacterial population levels in a full-scale anaerobic reactor treating pulp and paper industry wastewater using fluorescence in situ hybridisation. Water Sci Technol. 55(10), 183-191.

Jaggi, B., Freedman, M., 1992. An examination of the impact of pollution performance on economic and market performance: pulp and paper firms. J Bus Finance Account. 19(5), 697-713.

Jahren, S.J., Rintala, J.A., Odegaard, H., 2002. Aerobic moving bed biofilm reactor treating thermomechanical pulping whitewater under thermophilic conditions. Water Res. 36(4), 1067-1075.

Jain, C.K., Kumar, A., Izazy, M.H., 2009. Color removal from paper mill effluent through adsorption technology. Environ Monit Assess. 149(1-4), 343-348.

Jain, R.K., Mathur, R.M, Thakur, V. V., Verma, P., Kulkarni, A.G. 2006. Implementation of xylanase pre bleaching in Indian paper industry IIPTA Conv. Issue: 65-70.

Jeenathunisa, N., Jeyabharathi, S., Arthi, J., 2017. Bioremediation of Paper and Pulp Industrial Effluent Using Bacterial Isolates. Int J Res App Sci Eng Technol. 5, 692-698.

Jokela, J., Pellinen, J., Salkinoja-Salonen, M., 1987. Initial steps in the pathway for bacterial degradation of two tetrameric lignin model compounds. Appl Environ Microbiol. 53(11), 2642-2649.

Juwarkar, A.S., Bhalkar, D.V., Subrahmanyam, P.V.R., 1987. Effect of pulp and paper mill waste water on soil properties. Indian J Environ Health. 29(4), 313-321.

Kamalaveni, V., Karthikeyan, A., 2016. Characterisation of paper mill effluents and analysis of degradation potential of autochthonous soil bacteria. Asian J Pharm Sci Technol. 6(1), 11-16.

Kamali, M., Gameiro, T., Costa, M.E.V., Capela, I., 2016. Anaerobic digestion of pulp and paper mill wastes-An overview of the developments and improvement opportunities. Chemical Engineering Journal. 298, 162-182.

Kamali, M., Khodaparast, Z., 2015. Review on recent developments on pulp and paper mill wastewater treatment. Ecotoxicol Environ Saf. 114, 326-342.

Kamlesh, K., 2016. Impact of Paper Mill Effluent on Seed Germination and Seedling Growth of Coriander (Coriandrum sativum) Varieties. Int J Environ Agri Biotechnol. 1(4). http://dx.doi.org/10.22161/ijeab/1.4.44.

Kapley, A., Lampel, K., Purohit, H. J., 2001. Rapid detection of Salmonella in water samples by multiplex polymerase chain reaction. Water Environ Res. 73 (4), 461-465.

Karam, J., Nicell, J.A., 1997. Potential applications of enzymes in waste treatment. Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental AND Clean Technology, 69(2), 141-153.

Kaviyarasan, K., 2014. Application of UASB reactor in industrial wastewater treatment-a review. International Journal of Scientific & Engineering Research, 5(1), 584.

Khan, M.Z., Hall, E.R., 2003. Occurrence and removal of plant sterols in pulp and paper mill effluents. J Environ Eng Sci. 2 (1), 17-26.

Khan, N.A., Basheer, F., Singh, D., Faruqi, I., 2011. Treatment of Pulp and paper mill wastewater by column type sequencing batch reactor. J Indust Res Technol. 1(1), 12-16.

Khattri, S.D., Singh, M.K., 2009. Removal of malachite green from dye wastewater using neem sawdust by adsorption. J Hazard Mat. 167(1-3), 1089-1094.

Kirk, T.K., Farrell, R.L., 1987. Enzymatic" combustion": the microbial degradation of lignin. Annu Rev Microbiol. 41(1), 465-501.

Kouhia, M., Holmberg, H., Ahtila, P., 2015. Microalgae-utilizing biorefinery concept for pulp and paper industry: Converting secondary streams into value-added products. Algal Res. 10, 41-47.

Krawczyk, H., Oinonen, P., Jönsson, A.S., 2013. Combined membrane filtration and enzymatic treatment for recovery of high molecular mass hemicelluloses from chemithermomechanical pulp process water. Chem Eng J. 225, 292-299.

Kreetachat, T., Chaisan, O., Vaithanomsat, P., 2016. Decolorization of pulp and paper mill effluents using wood rotting fungus Fibrodontia sp. RCK783S. International Journal of Environmental Science and Development. 7(5), 321.

Kulkarni, H.D., 2013. Pulp and paper industry raw material scenario-ITC plantation a case study. IIPTA, 25(1), 79-90.

Kulkarni, P.S., Crespo, J.G., Afonso, C.A.M. Dioxins., 2011. Encyclopedia of Environmental Health. Reference Module in Earth Systems and Environmental Sciences. 83-92.

Kumar, L., Rathore, Y.S., Srivastava, H.S., 2001. 14 C-[lignin]-lignocellulose biodegradation by bacteria isolated from polluted soil. Ind Jounal Exp Biol. 39, 584-589.

Kumar, R., Subramanian, K., 2014. Treatment of paper and pulp mill effluent using sequential batch reactor. In International Conference on Biological, Civil and Environmental Engineering (pp. 39-42).

Kumar, V., Chopra, A.K., 2012. Fertigation effect of distillery effluent on agronomical practices of Trigonella foenum-graecum L. (Fenugreek). Environ Monit Assess. 184(3), 1207-1219.

Kumar, V., Dhall, P., Kumar, R., Prakash Singh, Y. and Kumar, A., 2012. Bioremediation of agro-based pulp mill effluent by microbial consortium comprising autochthonous bacteria. Sci World J. doi:10.1100/2012/127014

Kumar, V., Dhall, P., Naithani, S., Kumar, A., Kumar, R., 2014. Biological approach for the treatment of pulp and paper industry effluent in sequence batch reactor. J Bioremed Biodeg, 5(3), 1-10.

Kurade, M.B., Waghmode, T.R., Kagalkar, A.N, Govindwar, S.P., 2012. Decolourization of textile industry effluent containing disperse dye Scarlet RR by a newly developed bacterial-yeast consortium BL-GC. Chem Eng J. 184, 33-41.

Lara, M.A., Rodriguez-Malaver, A.J., Rojas, O.J., Holmquist, O., Gonzalez, A.M., Bullon, J., Penaloza, N., Araujo, E., 2003. Black liquor lignin biodegradation by Trametes elegans. Int Biodeterior Biodegrad. 52(3), 167-173.

Le-Clech, P., Chen, V., Fane, T.A., 2006. Fouling in membrane bioreactors used in wastewater treatment. J Memb Sci. 284(1-2), 17-53.

Li, Y., Chen, Y.F., Chen, P., Min, M., Zhou, W., Martinez, B., Zhu, J., Ruan, R., 2011. Characterization of a microalga Chlorella sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. Bioresour Technol. 102(8), 5138-5144.

Lin, H., Peng, W., Zhang, M., Chen, J., Hong, H., Zhang, Y., 2013. A review on anaerobic membrane bioreactors: applications, membrane fouling and future perspectives. Desalin. 314, 169-188.

Liu, T., Hu, H., He, Z., Ni, Y., 2011. Treatment of poplar alkaline peroxide mechanical pulping (APMP) effluent with Aspergillus niger. Bioresour Technol. 102(15), 7361-7365.

Lokeshwari, N., Keshava, J., Sangeetha, M., 2015. Optimization and kinetic studies for the degradation of lignin and chlorophenols by using Rhizopus aarhizus. J Phys Chem Sci 3(1), 1-6.

Lokeshwari, N., Srinikethan, G., Joshi, S.G., Shasikala, I., Srikanth, B., Sushma, L., 2013. Isolation and Screening of Fungi for Aerobic Delignification and Reduction of AOX of Pulp and Paper Mill Effluent. World Academy of Science, Engineering and Technology, Int J Bio Biomol Agri Food Biotechnol Eng. 7(12), 1167-1171.

Luck, F., 1999. Wet air oxidation: past, present and future. Catalysis today, 53(1), 81-91.

Maheshwari, R., Rani, B., Saxena, A., Prasad, M., Singh, U., 2012. Analysis of effluents released from recycled paper industry. J Adv Sci Res. 3(1).

Mahmood, T., Paice, M., 2006. Aerated stabilization basin design and operating practices in the Canadian pulp and paper industry. J Environ Eng Sci. 5(5), 383-395.

Malaviya, P., Rathore, V.S., 2007. Bioremediation of pulp and paper mill effluent by a novel fungal consortium isolated from polluted soil. Bioresour Technol. 98(18), 3647-3651.

Malik, M.K., Kumar, P., Seth, R., Rishi, S., 2009. Genotoxic effect of paper mill effluent on chromosomes of fish *Channa punctatus*. Curr World Environ. 4(2), 353-357.

Malkin, V.P., 2002. Electrochemical methods of treating industrial effluents. Chem Prot Eng. 38(9), 619-622.

Mallevialle, J., Odendaal, P.E., Wiesner, M.R., 1996. Water treatment membrane processes. LyonnaisedesEaux-LdE, New York.

Mandal, T.N., Bandana, T. N., 1996. Studies in physico-chemical and biological characteristics of pulp and paper mill effluent and its impact on human beings. J Freshw Biol. 8(4), 191-196.

Mani, S., Bharagava, R.N., 2016. Exposure to crystal violet, its toxic, genotoxic and carcinogenic effects on environment and its degradation and detoxification for environmental safety. Environ Conta Toxicol. 237, 71-104).

Manji, S., Ishihara, A., 2004. Screening of tetrachlorodibenzo-p-dioxin-degrading fungi capable of producing extracellular peroxidases under various conditions. App Microbiol Biotechnol. 63(4), 438-444.

Masai, E., Shinohara, S., Hara, H., Nishikawa, S., Katayama, Y., Fukuda, M., 1999. Genetic and biochemical characterization of a 2-pyrone-4, 6-dicarboxylic acid hydrolase involved in the protocatechuate 4, 5-cleavage pathway of *Sphingomonas paucimobilis* SYK-6. J Bacteriol. 181(1), 55-62.

Mathews, S.L., Pawlak, J.J., Grunden, A.M., 2014. Isolation of Paenibacillus glucanolyticus from pulp mill sources with potential to deconstruct pulping waste. Bioresour Technol. 164, 100-105.

Mauskan, J.M (2007) Advanced methods for treatment of textile industry effluents. Central Pollution Control Board Ministry of Environment of Forests, New Delhi.

Medhi, U.J., Talukdar, A.K., Deka, S., 2008. Effect of pulp and paper mill effluent on seed germination and seedling growth of mustard (Brassica campestris), pea (Pisum sativam), and rice (Oryza satival) seeds. Pollut Res. 27(4), 437-442.

Mehta, J., Sharma, P., Yadav, A., 2014. Screening and identification of bacterial strains for removal of COD from pulp and paper mill effluent. Adv Life Sci Health, 1(1), 34-42.

Merayo, N., Hermosilla, D., Blanco, L., Cortijo, L., Blanco, Á., 2013. Assessing the application of advanced oxidation processes, and their combination with biological treatment, to effluents from pulp and paper industry. J Hazard Mat. 262, 420-427.

Merilainen, P., Oikari, A., 2008. Exposure assessment of fishes to a modern pulp and paper mill effluents after a black liquor spill. Enviro Monit Assess. 144(1-3), 419-435.

Mester, T., Field, J.A., 1998. Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by Bjerkandera species strain BOS55 in the absence of manganese. J Biol Chem. 273(25), 15412-15417.

Metcalf, Eddy., 2003. (revised by Tchobanoglous G, Burton FL, Stensel HD). Wastewater engineering treatment and reuse, 4th ed. McGraw-Hill, New York.

Milet, G.M.D., Duff, S.J.B., 1998. Treatment of kraft condensates in a feedback-controlled sequencing batch reactor. Water Sci Technol. 38(4-5), 263-271.

Minussi, R.C., Pastore, G.M., Durán, N., 2007. Laccase induction in fungi and laccase/N–OH mediator systems applied in paper mill effluent. Bioresour Technol. 98(1), 158-164.

Minussi, R.C., Pastore, G.M., Duran, N., 2002. Potential applications of laccase in the food industry. Trends Food Sci Tech. 13(6-7), 205-216.

Mishra, M., Thakur, I.S., 2010. Isolation and characterization of alkalotolerant bacteria and optimization of process parameters for decolorization and detoxification of pulp and paper mill effluent by Taguchi approach. Biodegradation, 21(6), 967-978.

Mishra, S., Mohanty, M., Pradhan, P.C., Das, H. K., R., Sahoo, S., 2013. Physico-chemical assessment of paper mill effluent and its heavy metal remediation using aquatic macrophytes-a case study at JK Paper mill, Rayagada, India. Environ Monit Assess. 185(5), 4347-4359.

Moreira, M.T., Feijoo, G., Canaval, J., Lema, J.M., 2003. Semipilot-scale bleaching of Kraft pulp with manganese peroxide. Wood Sci Technol. 37(2), 117-123.

Morii, H., Nakamiya, K., Kinoshita, S., 1995. Isolation of a lignin-decolorizing bacterium. J Ferment Bioeng. 80(3), 296-299.

Muhamad, M.H., Abdullah, S.R.S., Mohamad, A.B., Rahman, R.A., Kadhum, A.A. H., 2013. Application of response surface methodology (RSM) for optimisation of COD, NH3–N and 2, 4-DCP removal from recycled paper wastewater in a pilot-scale granular activated carbon sequencing batch biofilm reactor (GAC-SBBR). J Environ Manag. 121, 179-190.

Munkittrick, K.R., Servos, M.R., Carey, J.H., Van Der Kraak, G.J., 1997. Environmental impacts of pulp and paper wastewater: evidence for a reduction in environmental effects at North American pulp mills since 1992. Water Sci Technol. 35(2-3), 329-338.

Muruganantham S, Anbalagan G, Ramamurthy N (2009) FT-IR and SEM-EDS comparative analysis of medicinal plants, ECLIPTA ALBA HASSK and ECLIPTA PROSTRATA LINN. Romanian J Biophysics. 19 (4), 285-294.

Narde, G., Kapley, A., Purohit, H.J., 2004. Isolation and characterization of Citrobacter strain HPC 255 for broad range substrate specificity for chlorophenol. Curr Microbiol. 48(6), 419-423.

Narwal, R.P., Singh, A., Dahiya, S.S., 2006. Effect of paper mill effluent's irrigation on soil and plants health–a case study. In The 18th World Congress of Soil Science, Philadelphia, USA.

Nestmann, E.R., 1985. Detection of genetic activity in effluent from pulp and paper mills: mutagenicity in *Saccharomyces cerevisiae*. Testing in environmental pollution control. 105-117.

Ogunsile, B.O., Quintana, G., 2010. Modeling of soda-ethanol pulps from Carpolobia Lutea. Bioresour. 5(4), 2417-2430.

Oliveira, P.L.D., Duarte, M.C.T., Ponezi, A.N., Durrant, L.R., 2009. Purification and Partial characterization of manganese peroxidase from Bacillus pumilus and *Paenibacillus* sp. Braz J Microbiol. 40(4), 818-826.

Olsen, O., 1980. Membrane technology in the pulp and paper industry. Desalination. 35, 291-302.

Olukunle, O.F., Oyegoke, T.S., 2016. Biodegradation of Crude-oil by Fungi Isolated from Cow Dung contaminated soils. Nig J Biotechnol. 31(1), 46-58.

Orrego, R., Guchardi, J., Krause, R., Holdway, D., 2010. Estrogenic and anti-estrogenic effects of wood extractives present in pulp and paper mill effluents on rainbow trout. Aquat toxicol. 99(2), 160-167.

Paliwal, R., Uniyal, S., Verma, M., Kumar, A., Rai, J.P.N., 2015. Process optimization for biodegradation of black liquor by immobilized novel bacterial consortium. Desalination Water Treat. 57 (40), 18915-18926.

Pandey, K.K., Pitman, A.J., 2003. FTIR studies of the changes in wood chemistry following decay by brown-rot and white-rot fungi. Int Biodeterior Biodegradation. 52 (3), 151-160.

Paranthaman, S.R., Karthikeyan, B., 2015. Bioremediation of paper mill effluent on growth and development of seed germination (vigna mungo). CIBTech J Biotechnol. 4 (1), 22-26.

Patil, S.R., 2014. Production and purification of lignin peroxidase from Bacillus megaterium and its application in bioremediation. CIBTech J. Microbiol., 3, 22-28.

Pearl, I.A., Benson, H.K., 1940. The determination of lignin in sulphite pulping liquor. Paper Trade J. 105-117.

Peerbhoi, Z., 2000. Treatability studies of black liquor by UASBR. University of Roorkee, India.

Perez, J., Munoz-Dorado, J., Dela Rubia, T.D.L. R., Martinez, J., 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int microbial. 5(2), 53-63.

Persson, P.O., 2011. Cleaner Production: Strategies and Technology for Environmental Production, Stockholm, Royal Institute of Technology-Industrial Ecology.

Piontek, K., Smith, A.T., Blodig, W., 2001. Lignin peroxidase structure and function. 29, 111-116.

Pokhrel, D., Viraraghavan, T., 2004. Treatment of pulp and paper mill wastewater-a review. Sci Total Environ, 333(1-3), 37-58.

Pometto III, A.L., Crawford, D.L., 1988. High-performance liquid chromatography of aromatic fragments from lignin degradation. In Methods in enzymol (Vol. 161, pp. 183-190). Academic Press.

Poonam, N., Pandey, A., Prabhu, K.A., 1987. Ligninolytic activity of two basidiomycetes cultures in the decomposition of bagasse. Biol Wastes. 21(1), 1-10.

Prabu, P.C., Udayasoorian, C., 2005. Decolorization and degradation of phenolic paper mill effluent by native white rot fungus Phanerochaete chrysosporium. Asian J Plant Sci. 4(1), 60-63.

Prasongsuk, S., Lotrakul, P., Imai, T., Punnapayak, H., 2009. Decolourization of pulp mill wastewater using thermotolerant white rot fungi. Sci Asia. 35, 37-41.

Raghukumar, C., D'Souza-Ticlo, D., Verma, A., 2008. Treatment of colored effluents with lignin-degrading enzymes: an emerging role of marine-derived fungi. Crit Rev Microbiol. 34(3-4), 189-206.

Rahman, N.H.A., Aziz, S.A., Hassan, M.A., 2013. Production of ligninolytic enzymes by newly isolated bacteria from palm oil plantation soils. Bioresour. 8(4), 6136-6150.

Raj, A., Kumar, S., Haq, I., Singh, S.K., 2014. Bioremediation and toxicity reduction in pulp and paper mill effluent by newly isolated ligninolytic Paenibacillus sp. Ecological Eng. 71, 355-362.

Raj, A., Reddy, M.K., Chandra, R., 2007. Decolourisation and treatment of pulp and paper mill effluent by lignin-degrading Bacillus sp. J Chem Technol Biotechnol. 82(4), 399-406.

Rajwar, D., Paliwal, R., Rai, J.P.N., 2017. Biodegradation of pulp and paper mill effluent by co-culturing ascomycetous fungi in repeated batch process. Environ Monit Assess. 189 (482), 1-16.

Rajwar, D., Rai, J.P.N., 2015. Kraft black liquor decolorization by fungi isolated from contaminated pulp and paper mill sludge. Int J Recent Sci Res. 6, 7770-7775.

Ramachandra, M., Crawford, D.L., Hertel, G., 1988. Characterization of an extracellular lignin peroxidase of the lignocellulolytic actinomycete Streptomyces viridosporus. Appl Environ Microbiol. 54(12), 3057-3063.

Ramanna, L., Guldhe, A., Rawat, I., Bux, F., 2014. The optimization of biomass and lipid yields of Chlorella sorokiniana when using wastewater supplemented with different nitrogen sources. Bioresour Technol. 168, 127-135.

Rashed, M.N., 2013. Adsorption technique for the removal of organic pollutants from water and wastewater. In Organic pollutants-monitoring, risk and treatment. Intech Open.

Richardson, S.D., Kimura, S.Y., 2017. Emerging environmental contaminants: challenges facing our next generation and potential engineering solutions. Environ Technol Innov. 8, 40-56.

Rohella, R.S., Choudhury, S., Manthan, M., Murty, J.S., 2001. Removal of colour and turbidity in pulp and paper mill effluents using polyelectrolytes. Indian J Environ Health. 43(4), 159-163.

Sahadevan, L.D.M., Misra, C.S., Thankamani, V., 2016. Characterization of lignin-degrading enzymes (LDEs) from a dimorphic novel fungus and identification of products of enzymatic breakdown of lignin. 3 Biotech, 6(1), 56.

Sahai, R., Shukla, N., Jabeen, S., Saxena, P.K., 1985. Pollution effect of distillery waste on the growth behaviour of Phaseolus radiatus L. Environmental Pollution Series A, Ecological and Biological, 37(3), 245-253.

Sahoo, D.K., Gupta, R., 2005. Evaluation of ligninolytic microorganisms for efficient decolorization of a small pulp and paper mill effluent. Process Biochem. 40(5), 1573-1578.

Saiano, F., Ciofalo, M., Cacciola, S.O., Ramirez, S., 2005. Metal ion adsorption by Phomopsis sp. biomaterial in laboratory experiments and real wastewater treatments. Water Res. 39(11), 2273-2280.

Sainlez, M., Heyen,G., 2013.Comparison of supervised learning techniquesfor atmospheric pollutant monitoring in a Kraft pulp mill. J Comput Appl Math. 246, 329-334.

Sala, M., Gutierrez-Bouzan, M.C., 2012. Electrochemical techniques in textile processes and wastewater treatment. International Journal of Photoenergy.

Sanchez, C., 2009. Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv. 27(2), 185-194.

Sangave, P.C., Pandit, A.B., 2006. Enhancement in biodegradability of distillery wastewater using enzymatic pretreatment. J Environ Manag. 78, 77-85.

Sankaran, S., Khanal, S.K., Jasti, N., Jin, B., Pometto III, A.L., Van Leeuwen, J.H., 2010. Use of filamentous fungi for wastewater treatment and production of high value fungal byproducts: a review. Critical reviews in environmental science and technology, 40(5), 400-449.

Santos, A.Z., Tavares, C.R.G., Gomes-da-Costa, S.M., 2002. Treatment of the effluent from a kraft bleach plant with the white-rot fungus Pleurotus ostreatoroseus Sing. Braz J Chem Eng. 19(4), 371-375.

Saraswathi, R., Saseetharan, M.K., 2010. Effects of temperature and pH on floc stability and biodegradation in paper and pulp mill effluent. J Eng Res Studies. 1(2), 166-176.

Saravanan, V., Sreekrishnan, T.R., 2005. Bio-physico-chemical treatment for removal of colour from pulp and paper mill effluents. J Sci Ind Res. 64, 61-64.

Saritha, V., Maruthi, Y.A., Mukkanti, K., 2010. Potential fungi for bioremediation of industrial effluents. Bio Resour Com. 5(1), 8-22.

Savant, D.V., Abdul-Rahman, R., Ranade, D.R., 2006. Anaerobic degradation of adsorbable organic halides (AOX) from pulp and paper industry wastewater. Bioresour Technol. 97(9), 1092-1104.

Schnell, A., Hodson, P.V., Steel, P., Melcer, H., Carey, J.H., 2000. Enhanced biological treatment of bleached kraft mill effluents-II. Reduction of mixed function oxygenase (MFO) induction in fish. Water Res. 34(2), 501-509.

Sevimli, M.F., 2005. Post-treatment of pulp and paper industry wastewater by advanced oxidation processes. Ozone Sci Eng. 27(1), 37-43.

Sharma, R., Chandra, S., Singh, A., Singh, K., 2014. Degradation of pulp and paper mill effluents. The IIOAB Journal, 5(3), 6.

Shawwa, A.R., Smith, D.W., Sego, D.C., 2001. Colour and chlorinated organics removal from pulp wastewater using activated petroleum coke. Water Res 35(3), 745-749.

Shi, Y., Chai, L., Tang, C., Yang, Z., Zheng, Y., Chen, Y., Jing, Q., 2013. Biochemical investigation of kraft lignin degradation by *Pandoraea* sp. B-6 isolated from bamboo slips. Bioprocess Biosyst Eng. 36(12), 1957-1965.

Singh, A., Agrawal, S.B., Rai, J.P.N., Singh, P., 2002. Assessment of the pulp and paper mill effluent on growth, yield and nutrient quality of wheat (*Triticum aestivum* L.). J Environ Biol. 23(3), 283-288.

Singh, P., Thakur, I.S., 2006. Colour removal of anaerobically treated pulp and paper mill effluent by microorganisms in two steps bioreactor. Bioresour Technol. 97(2), 218-223.

Singh, Y.P., Dhall, P., Mathur, R.M., Jain, R.K., Vadde, T.V., Kumar, V., Kumar, R., Kumar, A., 2011. Bioremediation of Pulp and Paper Mill Effluent by Tannic Acid Degrading Enterobacter sp. Water Air Soil Pollut. 218(1-4), 693-701.

Singhal, A., Jha, P.K., Thakur, I.S., 2016. Biosorption of pulp and paper mill effluent by *Emericella nidulans*: isotherms, kinetics and mechanism. Desalin Water Treat. 57(47), 22413-22428.

Singhal, A., Thakur, I.S., 2009. Decolourization and detoxification of pulp and paper mill effluent by *Emericella nidulans var. nidulans.* J Hazard Mat. 171(1-3), 619-625.

Smook, G.A., 2002. Handbook for pulp & and paper technologists. Angus Wilde Publ.

Soloman, P.A., Basha, C.A., Velan, M., Balasubramanian, N., Marimuthu, P., 2009. Augmentation of biodegradability of pulp and paper industry wastewater by electrochemical pre-treatment and optimization by RSM. Sep Purif Technol. 69(1), 109-117.

Somashekar, R.K., Gowda, M.T.G., Shettigar, S.L.N., Srinath, K.P., 1984. Effect of industrial effluents on crop plants. Ind J Environ Health. 26(2), 136-146.

Srivastva, N., Gunja, Jain, P., Kumar, D., Sharma, C.K., Janbade, A., Jain R.K., 2016. Characterization of pulp and paper mill effluent and its biological treatment by isolated bacterial consortia. Int J Curr Res. 8(7), 33855-33858.

Sumathi, S., Hung, Y.T., 2006. Treatment of pulp and paper mill wastes Waste treatment in the process industries. Wang LK, Hung YT, Lo HH et al 453-497. Taylor & Francis. 0-8493-7233-X. Boca Raton.

Sun, X.L., Kido, T., Honma, S., Koh, E., Okamoto, R., Manh, H.D., Maruzeni, S., Nishijo, M., Nakagawa, H., Nakano, T., Takasuga, T., 2017. The relationship between dioxins exposure and risk of prostate cancer with steroid hormone and age in Vietnamese men. Sci Total Environ. 595, 842-848.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution, 28(10), 2731-2739.

Tarlan, E., Dilek, F. B., Yetis, U., 2002. Effectiveness of algae in the treatment of a wood-based pulp and paper industry wastewater. Bioresour Technol. 84(1), 1-5.

Tewari, P.K., Batra, V.S., Balakrishnan, M., 2009. Efficient water use in industries: cases from the Indian agro-based pulp and paper mills. J Environ Manage. 90(1), 265-273.

Thakur, I.S., 2004. Screening and identification of microbial strains for removal of colour and adsorbable organic halogens in pulp and paper mill effluent. Process Biochem. 39(11), 1693-1699.

Thompson, G., Swain, J., Kay, M., Forster, C.F., 2001. The treatment of pulp and paper mill effluent: a review. Bioresour Technol. 77(3), 275-286.

Tiku, D.K., Kumar, A., Chaturvedi, R., Makhijani, S. D., Manoharan, A., Kumar, R., 2010. Holistic bioremediation of pulp mill effluents using autochthonous bacteria. Int Biodeterior Biodegrad. 64(3), 173-183.

Tong Z, Wada S, Takao Y et al (1999) Treatment of bleaching wastewater from pulp-paper plants in China using enzymes and coagulants. J Environ Sci. 11(4), 480-484.

Tsutsui, T., Nobuko, H., Heiji, M., James, H., Barrett, J. C., 1997. Benzene-, catechol-, hydroquinone-and phenol-induced cell transformation, gene mutations, chromosome aberrations, aneuploidy, sister chromatid exchanges and unscheduled DNA synthesis in Syrian hamster embryo cells. Mut Res/Fund Mol Mech Mut. 373(1), 113-123.

Tuomela, M., Vikman, M., Hatakka, A., Itavaara, M., 2000. Biodegradation of lignin in a compost environment: a review. Bioresour Technol. 72(2), 169-183.

Tyagi, S., Kumar, V., Singh, J., Teotia, P., Bisht, S., Sharma, S., 2014. Bioremediation of pulp and paper mill effluent by dominant aboriginal microbes and their consortium. In J Environ Res. 8(3), 561-568.

Ugurlu, M., Gurses, A., Dogar, Ç., Yalçın, M., 2008. The removal of lignin and phenol from paper mill effluents by electrocoagulation. J Environ Manag. 87(3), 420-428.

United States Environmental Protection Agency (USEPA) 1986. Office of Water Quality criteria for water (gold book). EPA 440/5-86-001. Washington DC.

USEPA (Environmental Protection Agency) (2004) Constructed treatment wetlands. Office of water 843-F-03-013

Usha, M.T., Chandra, T.S., Sarada, R., Chauhan, V.S., 2016. Removal of nutrients and organic pollution load from pulp and paper mill effluent by microalgae in outdoor open pond. Bioresour Technol. 214, 856-860.

Vaithanomsat, P., Sangnam, A., Boonpratuang, T., Choeyklin, R., Promkiam-on, P., Chuntranuluck, S., Kreetachat, T., 2013. Wood degradation and optimized laccase production by resupinate white-rot fungi in northern thailand. BioResour. 8(4), 6342-6360.

Vares, T., Kalsi, M., Hatakka, A., 1995. Lignin Peroxidases, Manganese Peroxidases, and Other Ligninolytic Enzymes Produced by Phlebia radiata during Solid-State Fermentation of Wheat Straw. Appl Environ Microbiol. 61(10), 3515-3520.

Vicuna, R., 1988. Bacterial degradation of lignin. Enz Microbiol Technol. 10(11), 646-655.

Wang, J.P., Chen, Y.Z., Wang, Y., Yuan, S.J., Yu, H.Q., 2011. Optimization of the coagulation-flocculation process for pulp mill wastewater treatment using a combination of uniform design and response surface methodology. Water Res. 45(17), 5633-5640.

Wang, Y., Liu, Q., Yan, L., Gao, Y., Wang, Y., Wang, W., 2013. A novel lignin degradation bacterial consortium for efficient pulping. Bioresour Technol. 139, 113-119.

Waye, A., Annal, M., Tang, A., Picard, G., Harnois, F., Guerrero-Analco, J.A., Saleem, A., Hewitt, L.M., Milestone, C.B., MacLatchy, D.L., Trudeau, V.L., 2014. Canadian boreal pulp and paper feed stocks contain neuro active substances that interact in vitro with GABA and dopaminergic systems in the brain. Sci Total Environ. 468, 315-325.

Wells, G.F., Park, H.D., Eggleston, B., Francis, C.A. and Criddle, C.S., 2011. Fine-scale bacterial community dynamics and the taxa–time relationship within a full-scale activated sludge bioreactor. Water research, 45(17), 5476-5488.

Wenta, B., Hartman, B., 2002. Dissolved Air Flotation System Improves Wastewater Treatment at Glatfelter. Pulp & Paper. 76(3), 43-46.

Whitman, W.B., Goodfellow, M., Kampfer, P., Busse, H.J., Trujilo, M.E., Ludwig, W., Suzuki, KI., 2012. Bergey's Manual of Systematic Bacteriology, 2nd edition. Springer-Verlag, New York, NY.

Yadav, S., Chandra, R., 2015. Syntrophic co-culture of Bacillus subtilis and Klebsiella pneumonia for degradation of kraft lignin discharged from rayon grade pulp industry. J Environ Sci. 33, 229-238.

Yang, C., Cao, G., Li, Y., Zhang, X., Ren, H., Wang, X., Feng, J., Zhao, L., Xu, P., 2008. A constructed alkaline consortium and its dynamics in treating alkaline black liquor with very high pollution load. PLoS One, 3(11), p.e3777.

Yang, Q., Angly, F.E., Wang, Z. and Zhang, H., 2011. Wastewater treatment systems harbor specific and diverse yeast communities. Biochem Eng J. 58, 168-176.

Yeber, M., Rodríguez, J., Freer, J., Baeza, J., Durán, N., Mansilla, H.D., 1999. Advanced oxidation of a pulp mill bleaching wastewater. Chemosphere. 39(10), 1679-1688.

Zaied, M., Bellakhal, N., 2009. Electrocoagulation treatment of black liquor from paper industry. J Hazard Mat. 163(2-3), 995-1000.

Zainith, S., Purchase, D., Saratale, G.D., Ferreira, L.F.R., Bilal, M., Bharagava, R.N., 2019. Isolation and characterization of lignin-degrading bacterium Bacillus aryabhattai from pulp and paper mill wastewater and evaluation of its lignin-degrading potential. 3 Biotech. 9 (3), 92.

Zaviska, F., Drogui, P., Mercier, G., Blais, J.F., 2009. Procédés d'oxydation avancée dans le traitement des eaux et des effluents industriels: Application à la dégradation des polluants réfractaires. Revue des sciences de l'eau. J Water Sci. 22(4), 535-564.

Zwain, H.M., Hassan, S.R., Zaman, N.Q., Aziz, H.A., Dahlan, I., 2013. The start-up performance of modified anaerobic baffled reactor (MABR) for the treatment of recycled paper mill wastewater. J Environ Chem Eng. 1(1-2), 61-64.

List of Publications

List of Publications

Research/Review paper

Zainith, S., Purchase, D., Saratale, G.D., Ferreira, L.F.R., Bilal, M., Bharagava, R.N., 2019. Isolation and characterization of lignin degrading bacterium *Bacillus aryabhattai* from pulp and paper mill wastewater and evaluation of its lignin-degrading potential. 3 Biotech. 9 (3), 92.

Zainith, S., Bharagava, R.N., 2019. Isolation and Screening of a Bacterial Strain for the Degradation and Decolourization of Pulp and Paper Mill Wastewater has been accepted in the Journal of International Journal of Emerging Technologies and Innovative Research.

Book Chapter (s)

Zainith, S., Sandhya, Sujata, Saxena, G., Bharagava, R.N., 2016. Microbes: An ecofriendly tools for the treatment of industrial wastewaters. Microbes and Environmental Management. Studium Press (India) Pvt. Ltd. (ISBN: 978-93-80012-83-4).

Zainith, S., Chowdhary, P., Bharagava, R.N., 2018. Recent advances in physico-chemical and biological techniques for the management of pulp and paper mill waste. (Bharagava and Chowdhary (Eds). Springer

Mishra, S., Bharagava, R.N., More, N., Yadav, A., **Zainith, S**., Mani, S. and Chowdhary, P., 2019. Heavy Metal Contamination: An Alarming Threat to Environment and Human Health. In Environmental Biotechnology: For Sustainable Future (pp. 103 125). Springer, Singapore.

Papers and chapters communicated

Zainith, S., Bharagava, R.N., "Biodegradation of pulp and paper mill effluent by a developed bacterial consortium through batch process and its toxicity evaluation" (communicated) in Ecotoxicology and Environmental Safety (**Research paper**).

Workshop and conferences

- Participated in Poster Presentation session of the 55thannual national conference of Association Microbiologist of India. Organized by department of Agriculture Microbiology, TNA University, Coimbatore. November, 12-14-2014.
- Attended three days' workshop on Hands-on-training SEM, FTIR, FPLC and Ion chromatography organized by USIC, BBAU, Lucknow February, 18-20, 2015.
- Participated in Poster Presentation session of the 57th annual conference of Association of Microbiologist of India (AMI 2016) organized by Department of Botany, Guwahati University, Assam, India, November 24-27, 2016.
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ORIGINAL ARTICLE

Isolation and characterization of lignin-degrading bacterium *Bacillus aryabhattai* from pulp and paper mill wastewater and evaluation of its lignin-degrading potential

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Abstract

This study reports the degradation and decolourization capability of a manganese peroxidase enzyme producing bacterium isolated from pulp and paper mill wastewater. The isolate was identified as *Bacillus aryabhattai* based on biochemical analysis and 16S rRNA gene sequencing. The strain was designated MG966493. This bacterium was able to reduce 67% and 54% colour and lignin, respectively, from the pulp and paper mill wastewater after 144 h of treatment at 32 °C, pH 7.6 and 120 rpm. Further, FT-IR analysis showed that during the lignin degradation process a number of metabolites were produced comprising different functional groups such as carbonyl (C=C), carboxyl (–COOH), alkene (C=C), amines ($–NH_2$), sulphonic ($–SO_3$) and nitro ($–NO_2$). In addition, the SEM analysis showed that the bacterial cells exposed to pulp and paper mill wastewater have rough surfaces with reduced size as compared to the unexposed cells with smooth surfaces. This study concluded that the isolated bacterium *B. aryabhattai* has significant potential for the bioremediation of pulp and paper mill wastewater and thus, can be applied for their treatment at an industrial scale.

Keywords Pulp and paper mill wastewater · Bacterial degradation · Lignin reduction · SEM analysis · FT-IR analysis

Introduction

Pulp and paper industry is the third most wastewater producing industry in the world and key contributors to the industrial water pollution (Asghar et al. 2008). According to the Indian Ministry of Environment and Forest (MOEF), pulp and paper industry is categorized as one of the "Red Category" of 17 listed industries, which causes severe environmental pollution. To minimize the environmental pollution, the industry must follow various effluent discharge standards set by the Central Pollution Control Board (CPCB 2010) and other agencies.

Pulp and paper industry discharges a dark brown coloured wastewater produced during the various stages of papermaking process into the environment. This deeply coloured wastewater is reported to have high pollution parameters such as biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS) along with toxic chlorinated compounds, tannins, resin acids, sulfur compounds, lignin and its degradation products (Haq et al. 2016a, b; Pokhrel and Viraraghavan 2004). This dark-coloured wastewater, if discharges into the environment without

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Isolation and screening of a bacterial strain for the degradation and decolourization of pulp and paper mill wastewater

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Abstract

Pulp and paper mill effluent contains organic, inorganic and coloring compounds which have high pH, biological oxygen demand (BOD), chemical oxygen demand (COD), total solids (TS), and phenols along with lignin. When untreated or poorly treated pulp and paper mill effluents discharged into the environment, it causes adverse effects on aquatic as well as terrestrial environments. Due to the heavy pollution load of pulp and paper mill industries, the removal of pollutants present in the effluent is very necessary for environmental safety. Thus, in this study deals with the bioremediation of pulp and paper mill effluent by the bacterial strain PLP 2 was isolated from contaminated site of pulp and paper mill industry. The isolated bacterial strain PLP 2 was effectively reduced colour (69%) and lignin (44%) after 144 h of treatment at 35°C, pH 7.5 and 120 rpm in presence of glucose (1.0%) and peptone (0.5%) as additional carbon and nitrogen source. Therefore, this study showed that the isolated bacterial strain can be useful for the effective bioremediation of pulp and paper mill effluent.

Key Words: Pulp and paper mill effluent, Bioremediation, Pollutants, Lignin

Introduction

The pulp and paper industry uses large amounts of fresh water and different types of chemicals during manufacturing process of paper making and generates large quantities of recalcitrant toxic compounds and coloured effluents which have an adverse effect on the environment (Subramanium. 1976; Saraswathi and Saseetharan 2010). Pulping stages produced 40-45% toxic effluent and these effluents are heavily loaded

Chapter 13
Recent Advances in Physico-chemical and Biological Techniques for the Management of Pulp and Paper Mill Waste

Surabhi Zainith, Pankaj Chowdhary, and Ram Naresh Bharagava

Abstract Pulp and paper industries are one of the major sources of environmental pollution that discharge enormous amount of wastewaters containing recalcitrant pollutants into the environment. Wastewaters have high biological oxygen demand (BOD), chemical oxygen demand (COD), total solids (TS), phenols, lignin and its derivatives. High strength of wastewaters containing dark colour and toxic compounds from pulp paper industries causes serious aquatic and soil pollution. On terrestrial region, pulp and paper mill wastewater at high concentration reduces the soil texture and inhibits seed germination, growth and depletion of vegetation, while in aquatic system, it blocks the photosynthesis and decreases the dissolved oxygen (DO) level which affects both flora and fauna and causes toxicity to aquatic ecosystem. The high pollution load from pulp and paper industrial wastewater gradually increases, and hence, there is a need for adequate treatment to reduce these pollution parameters before final discharge into the environment. Thus, this chapter gives detailed information about sources, characteristics, toxicity and physico-chemical and biological methods for the treatment of pulp and paper mill wastes and wastewaters.

Keywords Pulp and paper mill wastewater · Recalcitrant pollutants · Bioremediation

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Microbes an Ecofriendly Tools for the Treatment of Industrial Wastewaters

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ABSTRACT

Industries play a major role in the economic growth of developing countries. However, these are also the major source of environmental pollution because all types of industries discharge a huge volume of wastewater into the environment, which causes serious soil and water pollution as well as serious health threats in living beings. There are many types of industrial wastewaters based on different industries such as distilleries, tanneries, textile, pulp and paper industries, pharmaceuticals, electroplating, iron and steel, mine and quarries, etc. Each industry produces its own particular combination of organic and inorganic pollutants such as melanoidins, lignin, dyes, pesticides, pigments, phenols, chlorophenol, toxic heavy metals and a many recalcitrant pollutants. Industrial wastewater contains a variety of organic and inorganic pollutants and if discharged into the environment without adequate treatment, causes serious environmental problems and health hazards in all living organisms. Therefore, proper treatment of industrial wastewaters is essential for environmental safety. Thus, this chapter provides the detail knowledge on the nature and characteristics of different industrial wastewaters, major organic and inorganic pollutants present in different industrial wastewaters, their toxicological effects in environment and health hazards as well as various treatment approaches using microbes for the sustainable environment. In addition, the advances, merits, and demerits of various microbial treatment methods used for the treatment of industrial wastewaters are also discussed.

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Chapter 5
Heavy Metal Contamination: An Alarming Threat to Environment and Human Health

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