Bacterial Assisted Phytoremediation of Organic Pollutants for Detoxification of Pulp and Paper Mill Effluent after Secondary Treatment

THESIS

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CERTIFICATE

This is to certify that the thesis titled **"Bacterial Assisted Phytoremediation of Organic Pollutants for Detoxification of Pulp and Paper Mill Effluent after Secondary Treatment"** submitted by **Ms. Pooja Sharma** 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 University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulation-2013 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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DECLARATION

I, **Pooja Sharma** hereby declare that the work which is presented in the thesis entitled ***"Bacterial Assisted Phytoremediation of Organic Pollutants for Detoxification of Pulp and Paper Mill Effluent after Secondary Treatment"*** in fulfillment of the requirements for the award of the Degree of Doctor of Philosophyand submitted in the Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (a central) University, Lucknow, Uttar Pradesh is an authentic record of my own work carried out during the period from November, 2016to October, 2019 under the supervision of **PROF. RAM CHANDRA**, Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow.

The matter presented in this thesis has not been submitted by me for the award of any other degree in any other University/Deemed University without proper citation. I also declared that the thesis is essentially free from all kinds of plagiarism.

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CONTENTS

List of Figure

(k) normal anaphase (l) normal telophase

List of Table

ABBREVIATIONS AND SYMBOLS

α	**:**	Alpha
Al	**:**	Aluminium
$AlCl_3$		Aluminium chloride
amu	**:**	Atomic mass unit
ANOVA		Analysis of variance
AAS	**:**	Atomic absorption spectrophotometer
ATP	**:**	Adenosine triphosphate
APS	**:**	Ammonium per sulfate
ASS	**:**	Ammonium salt sugars
~	**:**	Approx
β	**:**	Beta
BD	**:**	Bacterial decolorized
$BaSO_4$	**:**	Barium sulfate
BLAST	**:**	Basic local alignment search tool
BOD	**:**	Biological oxygen demand
BSA	**:**	Bovine serum albumin
BSTFA	**:**	N,O-bis(trimethylsilyl)trifluoroacetamide
C	**:**	Carbon
$CaCl_2$	**:**	Calcium chloride
CaO	**:**	Calcium oxide
Cd	**:**	Cadmium
CEC	**:**	Cation exchange capacity
CFU	**:**	Colony forming unit
cm	**:**	Centimeter
COD	**:**	Chemical oxygen demand
Cr	**:**	Chromium
Cu	**:**	Copper
$CuSO_4$	**:**	Copper sulfate
CW	**:**	Constructed wetland
°C	**:**	Degree centigrade
DO	**:**	Dissolved oxygen
dNTP	**:**	Deoxynucleotide triphosphate
dATP	**:**	Deoxyadenosine triphosphate
dGTP	**:**	Deoxyguanosine triphosphate
dTTP	**:**	Deoxythymidine triphosphate
dCTP	**:**	Deoxycytidine triphosphate
DNA	**:**	Deoxyribo nucleic acid
DAD	**:**	Diode array detector
DSW	**:**	Distillery spent wash
eV	**:**	Electron volt
EC	**:**	Electrical conductivity
EDCs	**:**	Endocrine disrupting chemicals
EDTA	**:**	Ethylenediamine tetra acetic acid
EtBr	**:**	Ethidium bromide
F	**:**	Forward
Fe	**:**	Iron
$FeCl_3$	**:**	Ferric chloride

$FeSO_4$	**:**	Ferrous sulfate
Fig.	**:**	Figure
FT-IR	**:**	Fourier transform-infrared spectroscopy
λ	**:**	Wavelength
g	**:**	Grams
g L^{-1}	**:**	Gram per liter
GC-MS	**:**	Gas chromatography-mass spectrometery
GPM	**:**	Glucose peptone melanoidins
h	**:**	Hours
H_2S	**:**	Hydrogen sulfide
H_2O_2	**:**	Hydrogen peroxide
HCl	**:**	Hydrochloric acid
$HgCl_2$	**:**	Mercuric chloride
HNO_3	**:**	Nitric acid
HPLC	**:**	High performance liquid chromatography
HRT	**:**	Hydraulic retention time
IU	**:**	International unit
kV	**:**	Kilovolt
Kb	**:**	Kilo base
Kbp	**:**	Kilo base pair
kDa	**:**	Kilo Dalton
K_2HPO_4	**:**	Dipotassium hydrogen orthophosphate
L	**:**	Liter
LiP	**:**	Lignin peroxidase
Ltd.	**:**	Limited
M	**:**	Mol
mm	**:**	Millimeter
mM	**:**	Milimolar
m/z	**:**	Mass-to-charge ratio
µg	**:**	Microgram
µl	**:**	Microlitre
µm	**:**	Micrometer
mg	**:**	Milligrams
mL	**:**	Milliliter
mmol L^{-1}	**:**	Milimolar per liter
m^2/g		Metre square per gram
m^3d^{-1}	**:**	Cubic metre per days
mg L^{-1}	**:**	Milligram per liter
mg kg^{-1}	**:**	Milligram per kilogram
MEGA	**:**	Molecular evolutionary genetics analysis
$MgSO_4$	**:**	Magnesium sulfate
$MgCl_2$	**:**	Magnesium chloride
Mn	**:**	Manganese
MnP	**:**	Manganese dependent peroxidase/Manganese peroxidase
$MnCl_2$	**:**	Manganese chloride
MRPs	**:**	Maillard reaction products
MIP	**:**	Manganese independent peroxidase
MR	**:**	Methyl red
Min	**:**	Minutes

MW	**:**	Molecular weight
N	**:**	Normality
NaOH	**:**	Sodium hydroxide
Na_2SO_4	**:**	Sodium sulfate
ng·µL^{-1}	**:**	Nanogram per microliter
ng	**:**	Nanogram
nm	**:**	Nanometer
Ni	**:**	Nickel
NaCl	**:**	Sodium chloride
NaOH	**:**	Sodium hydroxide
NIST	**:**	National Institute of Standard and Technology
NCBI	**:**	National Center for Biotechnology Information
O	**:**	Oxygen
OTU	**:**	Operational taxonomic unit
OD	**:**	Optical density
(O-F) test	**:**	Oxidative-fermentative
OsO4	**:**	Osmium tetroxide
%	**:**	Percent
p	**:**	Para
pmol	**:**	Picomole
pUC	**:**	Plasmid vector UC
Pvt.	**:**	Private
pH	**:**	Potential of hydrogen
Pb	**:**	Lead
PAGE	**:**	Polyacrylamide gel electrophoresis
PCR	**:**	Polymerase chain reaction
PMDE	**:**	Post methanated distillery effluent
PMDS	**:**	Post methanated distillery sludge
QIIME		Quantitative Insights Into Microbial Ecology
R_f	**:**	Retardation factor
R	**:**	Reverse
RFLP	**:**	Restriction fragment length polymorphism
rRNA	**:**	Ribosomal ribonucleic acid
rDNA	**:**	Ribosomal deoxyribonucleic acid
RDP	**:**	Ribosomal database project
rpm	**:**	Revolution per minute
RT	**:**	Retention time
SEM	**:**	Scanning electron microscope
EDS	**:**	Energy dispersive X-ray spectrometer
***Sau*3A**	**:**	*Staphylococcus aurous* restriction enzyme 3A
Sp.	**:**	Species
Sec	**:**	Seconds
SDS	**:**	Sodium dodecyl sulfate
SD	**:**	Standard deviation
SCB	**:**	Sodium cacodylate buffer
SPSS	**:**	Statistical package for Social Sciences
T4	**:**	Bacteriophage T4
***Taq*1**	**:**	*Thermus aquaticus* type I
TMCS	**:**	Trimethylchlorosilane

TLC	**:**	Thin layer chromatography
TOC	**:**	Total organic carbon
TDS	**:**	Total dissolved solids
TS	**:**	Total solids
TSS	**:**	Total suspended solids
TN	**:**	Total nitrogen
TEM	**:**	Transmission electron microscope
TMS	**:**	Trimethylsilyl
TAE	**:**	Tri-acetate-EDTA
TEMED	**:**	N, N,N'N'-tetramethylethylenediamine
U	**:**	Unit
U mL^{-1} min^{-1}	**:**	Unit per milliliter per minute
UV	**:**	Ultraviolet
V	**:**	Volt
VS	**:**	Volatile solid
V3	**:**	Variable region third
V4	**:**	Variable region four
v/v	**:**	Volume/ volume
w/v	**:**	Weight/volume
X-Gal	**:**	5-Bromo-4-chloro-3-indoyl-β-D-galactopyranosides
Zn	**:**	Zinc
$ZnSO_4$	**:**	Zinc sulfate
3′	**:**	Three prime
5′	**:**	Five prime
<	**:**	Less than
>	**:**	Greater than
SOD	**:**	Superoxide dismutase
CAT	**:**	Catalase
APX	**:**	Ascorbate peroxidase
POD	**:**	Peroxidase
H_2O_2	**:**	Hydrogen peroxide
MDA	**:**	Lipid peroxidation content
WHO	**:**	World Health Organization
SEPA	**:**	State environmental protection agency
PLFA	**:**	Phospholipid-derived fatty acids

Chapter-One

Introduction

1. Introduction

Pulp paper industry waste (PPIW) is among the world's most polluting industries and is a potential source of air, soil and groundwater pollution that could cause environmental and human health hazards showed in Fig.1.1. (Azevedo et al., 2017). Approximately 411 million tons of paper is generated worldwide in 2017, most of the countries like the US, European countries, Brazil, India, and China (Demirel and Altin, 2017).The Agency of Environmental Protection Agency (EPA, USA) reports that over 250 million tons of municipal solid waste (MSW) is discharged every year, while 30% of only discharged from the pulp paper industry after secondary treatment. It would be estimated which approximately 0.4 tons of PPIW is generated of each ton of paper produced (Toczylowska-Maminska, 2017). Moreover, the pulp paper industry is rank 6^{th} among the most polluting industries in the world which are generating potential toxic elements on a very large scale after paper manufacturing (Ugurlu et al., 2007). There are about 800 pulp paper industries in India which generate 55–60% (1800–2400 m^3) sludge waste containing nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), iron (Fe), cadmium (Cd), magnesium (Mg), copper (Cu), silicon (Si), zinc (Zn) and manganese (Mn) compounds 1800–2400 m^3 sludge per tons of paper production annually, while only 40–45% of pulp is obtained during the pulping process present in both wastewater and sludge that can accumulate in different part of crop plants and soil and lead to cause health hazards through food chain (Chandra et al., 2011; Singh and Chandra, 2019).The total producing 6 million tons of paper, by using a different variety of raw materials such as forest wood, agricultural residues, rice straw, bagasse, and wheat straw with use a large number of different chemicals such as sodium hydroxide, solvents chlorine compounds during paper manufacturing processes. The discharged waste colored due to the presence of lignin with high TDS, TSS, BOD, COD, tannins, resin acids sulfur, and lignin along with heavy metals above the permissible limit (Pokhrel and Viraraghavan, 2004). Moreover, 190-200 m^3 of freshwater is utilized per ton of paper production and sludge is about 0.04–0.5 m^3 dry weight of sludge in North American paper mills (Bajpai, 2015; Chandra and Singh, 2012).The main source of heavy metals in the environment i. e.

metallurgical work, urbanization, and industrialization, particularly in highly populated developing countries such as India and China (UN-HABITAT, 2004). In addition, lignocellulosic waste and humic substances have a powerful binding inclination with heavy metals which reduce the availability of metals to growing species of plants. Moreover, heavy metal remains decreased at the alkaline condition indisposed of fresh sludge. Furthermore, the luxurious growth of some indigenous plants on PPIW stated the potential for phytoextraction of respective pollutants and the bioremediation of complex hazard compounds contains sludge of different metals and persistent organic co-pollutants (Chandra et al., 2017). The waste generated by the pulp paper industry during paper manufacturing is divided into four categories of sludge as follows: sludge generated during production of virgin wood fibers is primary sludge; sludge generated by removal of the fiber ink is destination sludge ; activated sludge process from the microorganism treatment is secondary sludge; sludge generated from paper production for biological purposes is combined sludge, this is a biological treatment process in which microorganisms transform the organic matter in the sludge into a type of soil fertilizer (Boni et al., 2004). In addition, Furans and other methylated compounds have been reported as a cleavage product of ferulic acid from PPIW. Similarly, these compounds either generated in bleaching or pulping process during the wastewater treatment in industry level this still need deep research through various stages of the extracted sample (Chandra et al., 2017; 2018). The persistent organic pollutants (POPs) are identified from the sludge of pulp paper industry such as Hexadecanoic acid, tetradecanoic acid, Pentadecanoic acid, and hexadecane is mostly phytosterols of plants and they are detected from humic substances also (Reveille et al., 2003).But, these compounds are listed under endocrine-disrupting chemicals (EDCs) as per the USEPA and Endocrine disruptor screening program (EDSP, 2012). Moreover, some organic pollutants detected from sludge and effluent of PPIW i.e. sulfurous acid (RT-18.63), pentadecanoic acid, phthalic acid (22.56) is reported as EDCs compounds. Further, the effluent sample extracted with chloroform detected several phenolic and non-phenolic compounds i.e. Decane-1-Bromo-2-methyl (RT-13.51), pentadecanone (RT-15.71), 2-pentadecanone (RT- 16.16), benzene dicarboxylic acid (RT-16.37), Dibutyl phthalate (RT- 17.32), 1-Decanol, 2-hexyl (RT- 18.63), respectively are major source of environmental pollution

(Chandra et al., 2017) and these compound is carcinogenic and mutagenic in nature and causes aquatic toxicity in ecosystem. Moreover, recalcitrant chlorogenic compounds even in this effluent tend to persist in nature for long term, are highly poisonous to aquatic life, and have the capability to migrate extensively throughout the ecosystem, ultimately accumulating in individuals fatty tissues. These compounds are generated during the biological treatment process by using microbes of PPIW in the treatment plant. But, these pollutants are responsible in aquatic resources are reported as EDCs in fish (Chandra et al., 2018).The observation of the toxicity of PPIW in different fishes has been noted an adverse effect on their reproductive system i.e. masculinization, lower plasma sex hormone, reduced gonad size, and reduced vitellogenin in the female, circulating sex hormone, fecundity, delayed maturity and change in secondary sex characteristics (Gustavo et al., 2015). The effect of PPIW discharge on caged fish rainbow trout (*Oncorhynchus mykiss*) was found as biomarker responses on the development of secondary sexual characteristics and induced intersex characteristics in juvenile rainbow trout (*Oncorhynchus mykiss*) along with the pollution gradient of effluent discharge in the river of Chile, Canada and Argentina (Gustavo et al., 2015). The masculinization and reproductive effects in western mosquito fish (*Gambusia affinis*) after long-term exposure to androstenedione has been reported from China and other countries. In addition, India is a developing country where farmers do not have sufficient resources for irrigation of agricultural crops; therefore, they use industrial wastewater as a source of water with a higher level of toxic organic and inorganic compounds such as heavy metals (Chandra et al., 2018). The research also revealed that PPIW not only contributed to the toxicity of phytoplankton and zooplankton by improving BOD, COD and other pollution parameters. However, it also induces the growth of fecal coliform bacteria that generates health risks in a living being (Gauthier and Archibald 2001; Chandra et al., 2006). However, due to a lack of proper knowledge regarding the pollutants discharged from PPIW and their health hazards. This literature has focused on the various categories of pollutants discharged from the pulp paper industry after secondary treatment and their toxicity to the environment.

1.1. Classification of pollutants discharged from the pulp paper industry

The pulp and paper industry is known as one of the world's most polluting industries and there are two main steps in the production process i.e. pulping and bleaching (Sumathi and Hung, 2006; Singh and Chandra, 2019). In addition, pulping is the initial stage and bleaching the final cause of the most polluting release in the world. In the process of pulping wood chips used as raw material is to remove lignin and improve fibers for papermaking and bleaching is the final step of the process aimed at whitening and lightening the pulp. These industry whole processes are very energy and water-intensive in terms of freshwater consumption (Pokhrel and Viraraghavan, 2004).

1.1.1. Organic, inorganic and gaseous pollutants and their health-hazardous

The environmental problems pulp and paper industry are not limited to high water usage but to generating wastewater, solid waste, including sludge produced by wastewater treatment plants, and cause air pollution. The major organic pollutants released in the bleaching process are adsorbable organic halides (AOX), ammonium nitrogen ($NH4^{+-}N$), chlorophenols, ditolyethane, di-iso-propyl naphthalene, bis-(methyl phenoxy)-ethane, bis (methyl phenoxy) ethane, terphenyl, benzyl naphthyl ether, chloromethyl-phenoxy-ethane, benzyl-biphenyl and grease (Sponza, 2003). Even after secondary treatment, pulp paper mill waste has a high pollution load of pathogenic bacteria and complex organic compounds that have been reported to be clastogenic, carcinogenic, endocrinic, and mutagenic to fishes and other aquatic communities (Karrasch et al., 2006; Chandra et al., 2006; 2009; 2012). Furthermore, in separate studies, high amounts of lignin, sulfate, phenolic, nitrate-compounds and another fatty acid monomer along with plant steroids are detected during the chlorine bleaching procedure (Chandra et al., 2011). Therefore, recalcitrant chloro-organic compounds present in effluent continue to persist in nature, become toxic to aquatic life, are genotoxic and have the potential to migrate widely throughout the ecosystems and ultimately accumulate in organism’s fatty tissues (Yadav and Chandra, 2018). Several researchers have used gas chromatography mass spectroscopy (GC-MS) analysis to separate, identify and quantify these types of pollutants in PPIW after secondary treatment (Chandra et al. 2009; Martin et al., 2012; Yadav and Chandra, 2015; 2018). Moreover, the significant discharges of heavy metals from the pulp paper industry are Cd, Cr, Cu, Fe, Ni, Mn, and non-metallic elements are

K^+, Na^+, Cl^-, P, sulfate, Ca, and Mg etc. (Chandra and Abhishek, 2010; Chandra and Yadav, 2017; Sangeeta and Chandra, 2018). In addition, increased toxicity of Cu the aquatic ecosystem has reported in the European eels (*Anguilla* L.) (Gravato et al., 2006). There is a generation of different gaseous pollutants i.e. nitrous oxide, sulfur dioxide, and particulate matter in the calcination process, where total reduced sulfur gases are

Fig.1.1. Pulp paper Industrial view and discharged waste in the environment after secondary treatment. (a). Pulp paper industry view (b-d) Discharged waste site and calcium carbonate generate from pulp paper industry

produced due to poor separation of Na_2S from lime mud and the primary source of volatile organic compounds depends on the total resin acid content in the wood during the defiberization technique (Pohanish, 2002).

1.2. Treatment techniques of pulp paper industry waste

There is a high amount of liquid and solid waste generated in the paper industry is produced during the pulping and bleaching process even after secondary treatment.

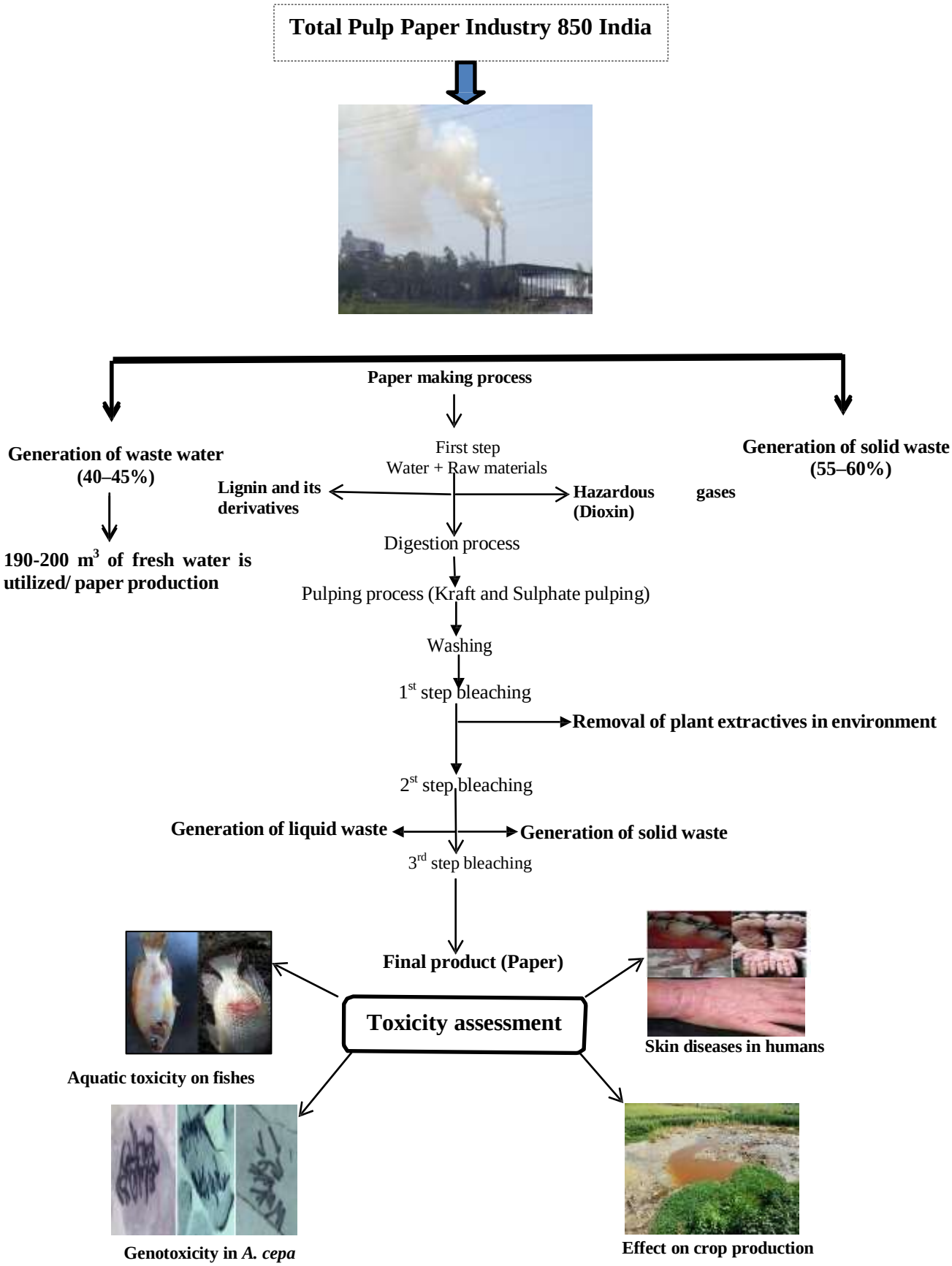


Fig.1.2. Graphical abstract of Paper Making Process and their Toxicity in Environment

The pulp and paper industry produces huge amounts of highly heterogeneous wastewater with wood or other raw material compounds, process chemicals and compounds formed during processing. Moreover, high water uses ranging from 20,000 to 60,000 gallons per ton of product (Nemerow and Dasgupta, 1991). The increased awareness of the fate of such contaminants is forcing the industry to manage waste effluents as they require and strict laws are established by multiple regulatory authorities such as provincial and federal agencies before discharging to the environment. Furthermore, both primary and secondary sludge from wastewater treatment plants in the pulp and paper industry is appropriate for anaerobic digestion. In removing soluble biodegradable organic pollutants, combinations of anaerobic and aerobic treatment processes are found to be effective.

1.2.1. 1. Adsorption

The adsorption technique is very good for the removal of color from the effluent of pulp paper industry waste after secondary treatment. Moreover, high color removal through carbon activation from sludge and effluent (Murthy et al., 1991). However, the adsorption process uses activated oil as an adsorbed for 90% color, COD, DOC and AOX removals from bleached wastewater (Shawwa et al., 2001). The efficiency of lignin removal by the blast furnace (BFD) and slag adsorption mechanism and throughout the study, lignin was removed by BFD and slag by 80, 4 % and 61 % respectively (Das and Patnaik, 2000). Adsorption is usually the preferred term in carbon-based processes and strictly speaking, adsorption defines binding as a physical rather than a chemical process on the surface (Dabrowski et al., 2005).

1.2.2. Precipitation and coagulation

Coagulation and flocculation are usually used during the treatment of wastewater in pulp paper mills and not usually used in primary treatments. Moreover, comparative study of chitosan peroxide (Al_2 $(SO_4)_3$), hexamethyl diamine polycondensate epichlorohydrin (HE), polyethylene (PEI), adsorbable organic halides (AOX), total organic carbon (TOC) and color removable (Tong et al., 1999;Ganjidoust et al., 1997). Moreover, 96% separation of COD from the paper machine, 50% separation from pulp and 20% removal by bleaching effluents using alum as a coagulant (Dilek and Gokcay, 1994). The removal

of turbidity, COD, and the color polyelectrolytes was much better than the conventional coagulant alum (Rohella et al., 2001). Application of coagulants, including Polyethylene oxide (PEO), increased settlement, turbidity, suspended solids and COD concentrations in effluent and between 25 and 250 ppm (Wang and Pan, 1999). The best solution for color removal from the sulfate and sulfite wood from pulp paper industry waste is coagulation using aluminum sulfate or altered adsorbents (Chernoberezhskii et al., 1994).

1.2.3. Membrane filtration and Ozonation

Treatment of membrane filtration for paper coating color effluent treatment suggests an important impact on color composition efficiency. The results revealed that 54%, 88%, 100% of total organic carbon (TOC), color and total suspended solids (TSS) were removed by ultrafiltration alone, respectively. Moreover, revealed reverse osmosis (RO) of 88 % and 89 % biological oxygen demand (BOD) and chemical oxygen demand (COD) removal (Dube et al., 2000). Membrane separated methods for removing AOX, COD, and color from pulp and paper mills had been indicated to be suitable. While, the considerable extraction of COD, TOCs, and toxicity from pulp mill effluent and reduced wastewater biodegradability has been obtained subsequent ozone treatment (Yeber et al., 1999). Furthermore, 90% EDTA reduction and 65% COD reduction by ozone treatment of pulp mill wastewater (Korhonen et al., 2000). None of EDTA and no diethylene triamine pentaacetic acid (DTPA) are biodegraded there under aerobic environmental conditions (Hinck et al., 1997). Moreover, high COD and dissolved organic carbon (DOC) from removed by ozone treatment from pulp effluent were previously reported by Oeller et al., (1997). Therefore, a 12 % decrease in total organic carbon, 70% decreased in total phenols and 35 % decreased in effluent colors after 60 minutes of ozonation (Freire et al., 2000). However, previously reported that bio treated kraft effluents produced a considerable reduction in the biologically recalcitrant residual AOX, transferred COD to BOD and produced a significant decrease in color (Roy-Arcand and Archibald, 1996).

1.2.4. 4. Chemical oxidation

In this treatment the three hours of reaction time, of hydrogen peroxide to decolorize Kraft effluent in 50% respectively. Moreover, a studied reduction is carried out using modern oxidation processes such as O_2/ZnO/UV, O_3 and O_2/TiO_2/UV photocatalysis for

the phenolic and polyphénolic compounds present in the bleaching effluent (Zamora et al., 1998). However, the Fenton and photo-Fenton reactions combined have been highly efficient in the treatment of kraft mill bleaching effluent (Perez et al., 2002). Improving effluent biodegradation by moist oxidation technique from 30% to 70% (Verenich et al., 2000). Feral obtained from natural clay sources, which comprise 2 % ferric sulfates and 6% aluminum sulfate and ferric sulfate have been obtained for color removal by mixed oxidation with ozone and Fenton reagents, and for pH 4–5 for color removal (Hassan and Hawk yard, 2002). The oxidation by catalytically increased oxidation of total reduced sulfur (TRS) odor-free products (Dufresne et al., 2000).

1.3. Biological treatment

1.3.1. 1. Aerated lagoons

The 70% removal of the AOX from the aerated lagoon was attributed to a short residence time section of the treatment system where the chlorinated stage effluents were mixed with general mill wastewaters (Stuthridge and Mcfarlane, 1994). The 67% removal of ammonia from black liquor spill at temperatures of 22-35 ºC, pH near 7.3 in an aerated lagoon (Bryant et al., 1997).Moreover, removal of BOD-7 ranging between 50% and 75% and chlorinated phenolics 10–50% by an aerated lagoon (Junna and Ruonala, 1991).

1.3.2. 2. Activated sludge process

The pulp paper industry is available throughout the world and is up to 30 % annually. Several studies evaluated specific characteristics of the formation of strong industrial solid and liquid waste, allowing the best final disposal technique to be simply screening in time. Furthermore, the use of chemical additives to generate the paper for hygiene purposes outcomes in organic and heavy metal which are present in large quantities (around 8%). It is estimated that more than 250 million tons of municipal solid waste (MSWs) are produced every year by the United States Environmental Protection Agency, of which about 30% is related to pulp paper industry waste. The changes in pH, temperature, and H_2O_2 and DTPA in the results of the sludge activated process (Ginkel et al., 1999). By a two-stage activated sludge technique, a large decrease in BOD and soluble COD (Knudsen et al., 1994). However, the activated sludge plant with the addition, from 51 % to 90% and from 70 to 93 %, of Floobeds or floating biological beds in the sequence that enhanced COD and BOD removal, respectively reported by

Hansen et al., (1999). In addition, the removal from oxygen activated sludge treatment plant of sludge and effluent of chlorinated phenols, 1, 1-dichloro dimethyl-Sulfone (DDS) Mohamed et al., (1989). The AOX removal by activated sludge processes and 90% BOD, 70% COD, 40%-60% AOX and 60% – 95% chlorinated phenols removal by activated phenols (Demirbas et al., 1999).

1.4. Chemical treatment

The pulp paper industry waste will probably face stronger requirements on the quality of receiving water. The use of tertiary treatment in the UK is limited, even though similar constraints imposed by the municipal wastewater treatment directive on COD may in the future be needed. Further, the paper mill wastewater can contain significant levels of COD, BOD, TSS, and TS, even after completely biological treatment. This mechanism is a high-pressure separation based on a membrane selective permeability which can serve as a further clarification for the final separation of the liquid and solids. In addition, various physicochemical techniques have been established for the removal of a range of toxic products from pulp effluent and the reduction of color and COD limitations.

1.5. Bacteria assisted phytoremediation

The indifferent and uncontrolled discharge of industrial and urban waste into the environmental dump has become a main global issue for the country. The pulp paper industry waste one of the important categories of this kind of compounds that really damage the environment of our precious water resource. Although advanced strategies including the use of bacteria, fungi, yeast, and their combinatorial technologies were shown to be useful for decolorizing effluent and contaminant remediation, we have practical problems when viewed in the field implementations. The use of plants might be an extremely exciting, sustainable and environmentally sustainable approach to the treatment of effluent and sludge (Govindwar and Kagalkar, 2010). Consequently, phytoremediation techniques focused on the mixed activity of plants and bacterial community within the rhizosphere suggest water and land remedial work (Glick, 2010). Moreover, phytoremediation has also been proposed used it to treat several kinds of pollutants, including heavy metals, radionuclides, chlorinated solvents, polyaromatic hydrocarbons (PAH), oil and crude oil, pesticides, explosives and leachates from wastewater (Shaffiqu et al., 2002; Ghosh and Singh 2005; Pilon-Smits, 2005; Nwoko,

2010). The number of microbes including such bacteria, ligninolytic and nonligninolytic fungi and ascomycetes has also been recommended for both the independent role of microorganisms throughout the degradation of complex pollutants. In addition, bacterial consortia and even fungal-bacterial consortia have been used to enhance and efficiently discolor and degrade pulp paper effluent discharged after secondary treatment. The capacity of plant growth-promoting bacteria (PGPB) to behave as biocontrol agents against phytopathogens and thus potentially stimulate plant growth may consequence to any of a multitude of mechanisms such as the production of antibiotics, depletion of iron from the rhizosphere, enhanced systemic resistance, development of fungal cell wall lysing enzymes and competition for root binding sites.

1.6. Phytoremediation of heavy metals and other co-pollutants from pulp paper industry waste

Phytoremediation is also an advancing green, cost-effective technology used only to remove huge amounts of heavy metals from soil and store them in a harvestable part from the contaminated site of pulp paper industry waste. The biomass can be treated to recover the toxin after hyper accumulating plants are grown at a contaminated site. Several plants can accumulate tissue contaminants including *Eleocharis acicularis, Solanum nigrum, Rumex dentatus, Alternanthera philoxeroides,* and *Cammelina benghalensis* might well accumulate As, Cu, Zn, and Pb from the pulp paper industry disposal site. Furthermore, inorganic contaminants could not be deteriorated Inorganic pollutants must be improved in the soil to make them less bioavailable, i.e. phytostabilization; removed, transferred and accumulated in plant tissues, i.e. phytoextraction; or transformed into volatile forms, i. e. phytovolatilization (Pilon-Smits, 2005).

1.7. Interaction between plant and bacteria for remediation of organic and inorganic pollutants

The technique of promoting PGPB with phytoremediation is ideal for some complex hazardous pollutants remedial work. A wide number of PGPB were reported, such as metal-resistant and PGPB were isolated by metal-polluted soil. Some potential isolated strains were identified such as *Enterobacter sp.* and *Klebsiella sp.,* which are incubated into Brassica napus for heavy metal accumulation and these bacteria are also responsible for Cd, Pb and Zn, and plant growth. Heavy metals are removed by the Phyto-bacterial

system instead of phytoremediation alone. The extent of heavy metal absorption is dependent on the pH, bacteria, metal oxidation and the large percentage of cases of solvent phosphate and siderophores-producing bacteria, 1-aminocyclopropane-1-carboxylate deaminase (ACC), and Indole-3-acetic acid (IAA), in order to improve plant growth and convert heavy metals to soluble and bioavailable forms.

1.8. Biodegradation of residual organic pollutants by bacterial strains

Many soil bacteria have also been recognized to degrade hazardous organic compounds from the contaminated site of pulp paper industry waste (Chakrabarty, 1981; Chandra et al., 2018). Some potential bacterial strains have been reported as more effective than fungal strains for bioremediation of complex environmental pollutants due to their good environmental adaptability and biochemical versatility for their growth (Chandra et al., 2018). This has been reported that bacteria isolated from compost soil, i.e. *Azotobacter* and *Serratia marcescens*, are capable of degradation and decoloration of lignin compounds (Morii et al., 1995). Moreover, *Pseudomonas sp.* is by far the most popular community of soil microorganisms that biodegrade complex organic compounds, a process that usually requires several different enzymes to work around each other. With both the finding of a variety of soil microorganisms capable of degrading xenobiotic chemicals such as herbicides, pesticides, refrigerants, solvents, and other organic compounds, the concept gained credibility in which microorganisms' degradation would provide a reasonable and effective means of removal of highly toxic waste. The comparative study of lignin degradation by Bacillus subtilis and Bacillus sp. isolated from soil was reported (Abd-Elsalam and El-Hanafy, 2009). Even though the introduction of anaerobic decomposition bacteria to polluted soils such as bioremediation is generally effective in facilitating the breakdown of pollutants in a laboratory environment. Consequently, three potential aerobic bacterial strains were isolated from pulp and paper mill sludge for kraft lignin degradation, identified i.e. *Paenibacillus sp*, (AY952466), *Aneurini bacillus* (AY856831), and *Bacillus sp*. (AY952465) (Chandra et al., 2007). Moreover, *Bacillus sp.* and *Serratia marcescens* have also been reported as degradation of pentachlorophenol from waste of pulp and paper mill effluent up to 94% in the presence of nutrients i.e. carbon (1% glucose) and nitrogen (0.5% peptone m/v) under in-vitro conditions (Singh et al., 2008). Such observations provide strong evidence

for bacterial consortia to degrade and detoxify chlorolignins containing wastewater from pulp and paper mills (Chandra and Singh, 2012). In addition, isolated potential autochthonous bacteria were identified as *Klebsiella pneumonia* IITRCP04 (KU715839), *Enterobacter cloacae strain* IITRCP11 (KU715840), *Enterobacter cloacae* IITRCP14 (KU715841), and *Acinetobacter pittii* strain IITRCP19 (KU715842) are responsible for bioremediation of lactic acid, benzoic acid, vanillin, and other residual chlorolignins compounds (Chandra et al., 2018).

1.9. Environmental impact of pulp paper industry waste

Toxic hazardous pollutants releases from the wastewater of the pulp paper industry escape to the environment via different pulping and bleaching processes can cause an adverse effect on human and animal health. The previous study revealed that wastewater generated from the various pulp paper industry throughout India, causes toxicity to both terrestrial and aquatic ecosystems, which is a severe problem, and challenges to the researchers for understanding the level and build up the suitable technology for environment safety and eco-restoration. In addition, the concentration of metals is present in a high amount of PPIW is responsible for damage to various parts of the human body even at very low concentrations. The phytotoxicity effect of PPIW in crop plants has been previously published by various researchers (Chandra et al., 2018; Yadav and Chandra, 2018). The toxicity assessment in *P. mungo* and *T. aestivum* at different concentrations (10, 20, 40, 60, 80 and 100%) of PPIW and result is shown the maximum inhibition of seed germination after exposure is above than 90% is a comparison to control reported by Yadav and Chandra, (2018). The analysis is confirmed that the presence of different carcinogenic, mutagenic and EDCs compounds in PPIW. Furthermore, The *P. mungo* and *T. aestivum* is an important edible crop for human beings but after the irrigation PPIW effect on the production, seed size, plant growth as well accumulation of different organometallic compounds causes the health hazards. In addition, *P. mungo L.* and *T. aestivum* after treatment germination index with more than 80% were observed to be severely affected by toxic compounds. Moreover, in this review showed the evidence of Phytotoxicity from PPIW. The cell toxicity and gene toxicity of PPIW in *A. cepa* root showed on the basis of chromosomal aberration. The continue discharged of PPIW in the environment is affecting the balance of the ecosystem and cause

mutation to plant and animal. The cytotoxicity and genotoxicity of *A. cepa* are showing the abnormality of chromosome shape and size at different stages of meiosis and different concentration of PPIW was (Yadav et al., 2018). The examine of toxicity of PPIW

Table.1.1. List of plant growth-promoting bacteria growing at the contaminated site and help in remediation of different heavy metal for enhancing phytoremediation

Bacterial isolates	**Valuable features**	**Associated plant**	**Heavy metal**
Meshorhizobium huakuii sub sp. rengei strain B3	Metallothionein (MT) and Phytochelatine (PC) production	-	Cd
Pseudomonas putida KT2440	Phytochelatin production (PCs) development	*Triticum aestivum*	Cd, Hg, Ag
Enterobacter cloacae CAL2	IAA, ACC deaminase, siderophores	*Brassica napus*	As
Meshorhizobium huakuii sub sp. rengei strain B3	Metallothionein (MT) and Phytochelatine (PC)	-	Cu, Cd, Zn, and As
Meshorhizobium huakuii sub sp. rengei strain B3	Metallothioneins (MTs)	*Astragalus sinicus*	Cd2+
Pseudomonas putida 06909	Production of a metal-binding peptide	*Helianthus annuus*	Cd

Metaphase, disturbed anaphase, Anaphase Bridge, anaphase chromosome, sticky anaphase, c-mitosis, ring chromosome, and vacuolated nucleus. Furthermore, chromosomal fragments and bridges were also induced at anaphase, indicating mutagenic events in the cell. Moreover, the loss of the telomeric side of chromosomes resulted in ring chromosomes. This can be attributed to the alteration in the spindle. Moreover, chromosome aberrations are demonstrated by a change in the genetic structure or chromosome number that can occur due to long-term exposure to PPIW. The PPIW contains several compounds such as pentadecanoic acid Hexadecanoic acid, β-Sitosterol trimethylsilyl ether, and 2-Methyl-4-keto-2-pentane-2-of 1TMS are the main source of cause mutation in cells and androgenic compounds are responsible for cell shrinkage. These compounds are listed under EDCs (USEPA, 2012). Several factors can cause the

breakdown of DNA, inhibition of DNA synthesis, and replication of altered DNA, which consequently induce structural chromosomal changes/abnormalities. Moreover, the *A.cepa* test also confirmed several types of chromosomal aberrations at different stages of cell division i.e. prophase, metaphase, anaphase, and telophase, etc. This test can be performed through detailed knowledge of the cell division phase and associated potential abnormalities.

1.10. Problem and challenges

Due to the release of complex hazardous gaseous, organic and inorganic pollutants after secondary treatment from the pulp paper industry that is still not much known, the various studies have shown one of the most polluting industries in the country is the pulp paper industry (Chandra et al., 2018; Yadav and Chandra 2008; Haq et al., 2017). The waste generated from various pulp paper industries revealed that the morphological and physiological properties of flora and fauna are adversely affected (Kim, 1996). The most common gaseous pollutants including methyl mercaptan, sulfur dioxide, sodium sulfide, hydrogen sulfide, sulfur, nitrogen, nitrogen dioxide, sulfur oxide, hydrogen peroxide, and chlorine dioxide and organic pollutant 2-methyl-4-keto- 2-pentane-2-ol 1TMS 3,7-dioxane-2,8- disiloxane-2,2,8,8-tetramethyl-5- hexadecanoic acid, Trimethylsilyl ester, or palmitic acid TMS; and Octadecenoic acid, trimethylsilyl ester, [(trimethylsilyl) oxy] 2-methyl-4-keto-2-trimethyl siloxy pentane are not removed during secondary treatment. These are the main source of occupational health hazards, while lignocellulosic and chlorolignins compounds are found in the discharged wastewater. Similarly, some organic compounds i.e. Chlorophenols, chloroguaiacols, chlorosyringols, chlorocatechols, octacosane, pentane, silane, β-sitosterol, Octadecenoic acid amide, octylphenol, chlorovanillins, terpenes, nonylphenol, trimethylsilyl ether, and estradiol are listed under EDCs by USEPA 2012. All through bleaching and paper making processes, the mixture of various metallic and non-metallic contaminants including nickel, iron, chromium, lead, sulfur, phosphorus, zinc and arsenic is discharged are toxic to aquatic and terrestrial ecosystem. Different aquatic organisms and plants have illustrated the estrogenic and androgenic impact of such pollutants is reported by several researchers. The study of my thesis has been compiled into the following chapters**:**

The chapter first has introduced the basic information related to my Ph.D. thesis topic. The information related to the total number of pulp paper industries in India, generation of total liquid and solid waste and their toxicity on human health and environment. Further, this chapter also provides comprehensive information on native hyperaccumulators plants growing at the contaminated sites and their role for remediation of organometallic complex containing waste of pulp paper industry.

Chapter second of this thesis summarized all of the objectives of the study as well as described the review of the new technique and experimental results for scale-up to develop a new approach for industrial application.

Chapter three of this thesis has described the review and literature related to pulp paper industry waste, generation of effluent and sludge, category of pollutants, and their toxicity on plant and human health. Moreover, the study also concluded the properties i.e. Physico-chemical properties of effluent/sludge and detection of several organic and inorganic pollutants release from the pulp paper industry after secondary treatment. In addition to toxicity assessment of discharged waste was done by using *Allium cepa* genotoxicity or chromosomal aberrations.

Chapter four characterized the Physico-chemical properties and the identification of recalcitrant organic pollutants from effluent and sludge in accordance with authorized standard methods. In addition, this chapter also identified the characterization of different persistent organic pollutants from sludge and effluent following liquid-liquid and liquid-solid extraction and examined by GC-MS to demonstrate the nature of the pollutants present in this waste. To investigate the surface study of effluent and sludge samples by using scanning electron microscopy analysis. Moreover, seed germination and chromosomal aberration testing of *Phaseolus mungo* L. and *Triticum aestivum* were used to determine the impact on the environment of pulp paper industry waste.

Chapter five of the thesis has investigate the pollution parameter of discharged waste from pulp paper industry and their remediation or detoxification strategy by native grass and weeds plants growing at the contamination site of pulp paper industry waste disposal site. Moreover, the toxicity on crop plants was also evaluated using *Trigonella* plant after irrigated with effluent in duration of 120 days. The collected native plants i.e. *Tribulus Terrestris* (Zygophyllaceae), *Parthenium hysterophorus* Asteraceae),

Momordica doica (Cucurbitaceae), *Alternanthera sessilis* (Amaranthaceae), *Cannabis sativa* (Cannabaceae), *and Calotropis procera* (Apocynaceae), are showed the potentiality for in-situ phytoextraction. Moreover, the study of accumulation and distribution of metals in different parts of native hyperaccumulators and their potentiality by atomic absorbance spectroscopy (AAS) and the histological observation for storage of heavy metal in their root tissue by transmission electron microscopy (TEM). In addition, the observation of pollutants remediation before and after in-situ phytoextraction through Gas chromatography-mass spectrometry (GC-MS) analysis. In addition, the bioaccumulation coefficient factor/bioconcentration factor (BCF) and translocation factor (TF) for various heavy metals in different parts of native plants were evaluated. While, the effect on crop plants i.e. morphological changes, the effect on antioxidants, chlorophyll content, the effect on photosynthetic organelles, the effect on root nodule formation by nitrogen fixating bacterial community, estimation of different heavy metals and estimation of total protein in the root, shoot and leaves after and before irrigation with effluent at different concentration. In addition, the Fourier transform infrared spectroscopy (FTIR) analysis of sludge effluent is showed the variability in results is confirming evidence of in- situ phytoextraction. Furthermore, the result was concluded with the harmful effect of discharged waste after secondary treatment.

Chapter six has described the effect of pulp paper mill effluent on crop plant after secondary treatment. For this analysis the pot experiment deign with different concentration of effluent was irrigated with *T. foenum-graecum L* plant. After the 90 days exposure the plant showed various type of abnormalities in their root, leaves and root nodule. The study is confirmed the presence of toxic compounds in effluent and their toxicity plant.

Chapter seven has described the molecular mechanism of collected potential native hyperaccumulators plants based on their luxuriant growth and development on sludge bed of discharged waste from pulp paper industry waste. consequently, the total plants were collected from contaminated site i.e. *Brassica campestris L.* (Brassicaceae) and *Chenopodium album L.* (Amaranthaceae), *Ricinus communis* (Euphorbiaceous), *Ranunculus sceleratus* (Ranunculaceae), *and Rumex dentatus* (Polygonaceae) were collected on the basis of abundantly growing on sludge. Further, complete analysis of

sludge and effluent from pulp paper industry waste near the plant root zone and distance the plant found the increases in absorption peaks (200-700 nm) are correlated by UV-Vis spectrophotometric analysis for structural changes and color decrease by FT-IR investigative process; and GC-MS analysis before and after phytoextraction potential. In addition, the estimation of different antioxidants i.e. superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and catalase (CAT) value is treated with plants and control (from normal soil).

Chapter eight is described as the profiling of bacterial communities in the sludge samples from the contaminated site of the pulp paper industry. In this chapter described the different microbial community based on 16sRNA growing on complex organometallic containing compounds of sludge. Investigation of GC content in sludge and their Reads and Operational taxonomic units (OTUs) distribution by using a Metagenomics technique strategy to uncover the microbial niche in this polluted environment to investigate the dominant autochthonous bacterial communities.

Chapter nine has described the plant growth-promoting rhizobacteria (PGPR) activity and role of *Phragmites communis* (Poacaae) for remediation and detoxification of pulp paper industry waste after secondary treatment. For the complete study, we have collected the plant, sludge and rhizosphere soil form the disposal site of the pulp paper industry. Further, the Physico-chemical analysis before and after in-situ bioremediation, although the detection of the organic compounds before and after in-situ bioremediation by GC-MS analysis showed the potentiality of PGPR. Moreover, isolation and identification of PGPR bacteria and their screening for investigate their potentiality in stress environment. The total seven potential isolates are identified i.e. *Bacillus sp*. PS-2 (MN238724.1), *Escherichia coli* strain PS-3 (MN238725.1), *Brevundimonas sp*. PS-4 (MN238722.1), *Bacillus sp*. PS-6 (MN238714.1), *Aeromonas salmonicida* strain BBAUPS-1 (MN294457), *Aeromonas salmonicida* strain BBAUPS-2 (MN294456), and *Stenotrophomonas maltophilia* strain BBAUPS-3 (MN294458), respectively. While the heavy metals accumulation in *P. communis* root shoot and leaves by AAS and cellular observation of metal storage in root tissue by TEM analysis.

Chapter ten described the treatment technology of discharged waste from the pulp paper industry after secondary treatment by biostimulation and bioaugumentation

process. The discharged waste is fully loaded with hazardous organic and inorganic pollutants in effluent and sludge. Moreover, the assessment of toxicity and nature of pollutants before and after in-situ bioremediation is evaluated by UV-Vis spectrometry and FTIR analysis. Consequently, after the biostimulation and bioaugumentation process confirmed the pollutant remediation or break down was measured by GC-MS analysis. In biostimulation processes, they use nitrogen and carbon supplementation in ratio i.e. glucose 1.0, 1.5 and 2.0 % and peptone 0.5, 1.0 % enhanced the degradation mechanism of discharged waste from the pulp paper industry. The degraded sample after the biostimulation process, however, showed either the disappearance or the generation of new metabolic compounds during optimized conditions, i.e. temp (37±1ºC), rpm (150), after 3 and 6 days of bacterial incubation. In addition, the total isolated potential autochthonous bacteria i.e. *Enterobacter cloacae* strain IITRCP11 (KU715840), *Klebsiella pneumonia IITRCP04* (KU715839), *Enterobacter cloacae* IITRCP14 (KU715841), and *Acinetobacter calcoceticus* strain IITRCP19 (KU715842) have been identified. Furthermore, the study also demonstrated that certain value-added products were generated during the detoxification of effluent from residual chlorolignins chemicals in the biostimulation method. Moreover, toxicity measurement by seed germination and chromosomal aberration test before and after biostimulation and bioaugumentation process is evidence of in-situ bioremediation of residual organic pollutants from the disposal site of pulp paper industry waste.

Chapter eleven has summarized the findings of my Ph.D. work and their importance for detoxification and degradation of pulp paper industry waste after secondary treatment with the help of bacteria and native plants.

Chapter twelve represented the sources of reference mentioned in the whole thesis as per university reference format. The section of the reference was written in a standard format and included all the relevant references related to the topic

The title page of published papers and other research data was added to Chapter twelve with all details. Also included is the cover page for all published original research papers, book chapter and conferences in the national and international journals.

Chapter-Two

Objectives

2. Objectives:

The objectives of my thesis are as following:

1. **Detection of various residual organic pollutant present in various pulp and paper mill industrial waste after secondary treatment**
    - Collection of sample from different pulp paper industry waste
    - Extraction and characterization of residual organic compounds by GC-MS analysis
    - Toxicity assessment by seed germination (*Phaseolus mungo* L) and chromosomal aberration (*Allium cepa)*
2. **Heavy metal accumulation pattern in different part of native and crop plants growing at disposal site of pulp and paper mill waste**
    - Collection of different native plants from different industry
    - Estimation of heavy metals in different part of native plants
    - Histological observation of plant root tissues by TEM analysis after accumulation of heavy metals
3. **Investigation of the molecular mechanism of hyperaccomulation plant growing at pulp and paper waste disposal site**
    - Estimation of stomata morphology condition in stress environment condition
    - Measurement of different biochemical parameter and their antioxidants activity of native hyperaccumulators plants compare to control (normal land) plants
4. **Profiling of dominant bacterial community growing in pulp and paper mill sludge containing heavy metal and organic pollutant**
    - Extraction and purification of DNA from environmental sample
    - Analysis of uncultured bacterial communities grown in sludge from disposal site of pulp paper industry basis on the 16sRNA genome sequence by Metagenomics analysis
5. **Characterization of potential bacterial community growing in the rhizospheric zone of native plants at the polluted site of pulp paper industry waste**
    - Assessment of PGPR activity of isolated bacteria from rhizospheric zone *Phragmites communis*
    - Identification of bacterial strains by 16sRNA sequencing
6. **Assessment of detoxification and degradation of pulp and paper mill effluent by biostimulation and bioaugumentation process growing at pulp and paper mill contaminated site**
    - Assessment of degradation of organic pollutants by bioaugumentation process
    - The toxicity evaluation of pulp paper mill effluent after biostimulation process

Chapter-Three

Review Of Literature

3.1.Review of literature

3.1.1.Bacteria assisted phytoremediation of environmental pollutants

Microorganisms are found almost everywhere in the environment, growing and tolerating the circumstances of soil, air, water, desert and extreme temperatures (Vidali, 2001).The soil near to plant roots, i.e. the rhizospheric zone is a significant environment and ecosystem for microorganisms, which include bacteria, fungi, algae, and protozoa.Moreover, bacteria cells can produce and sense signal proteins, allowing the entire population to grow over the root surface as a biofilm and start a concerted action after a specific population density has been achieved (Daniels et al., 2004).Bacteria that seem to be extremely valuable and play a significant part in nutrient supply and therefore can decrease the hazardous effects of metals on plants and most of these bacteria can operate straight on organic and inorganic pollutants using their own degradation capacities, including volatilization, conversion, and biodegradation.Some organic compounds can be excreted such as remediated by bacteria which might be either found in or added to the soil, in the lack of plants, this process is usually slow and inefficient, in part as a consequence of the comparatively low numbers of such degradative microorganisms in the soil. In addition, bacterial plasmids have genes that are also resistant to many other toxic/heavy metals and metalloids such as Ag^{+}, Cd^{2+}, Hg^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Sb^{3+} (Rosen, 2002; Silver and Phung, 2009).Further, bacteria, especially plant growth-promoting bacteria (PGPB), could well reduce the number of restricting factors in phytoremediation technology, such as metal solubility, contamination level, and soil chemistry (Tangahu et al., 2011).Several general bacterial diversity assessments of activated sludge and biofilm technologies have been investigated since 1995 based on 16S ribosomal ribonucleic acid (rRNA) gene database analyzes.However, metal-resistant bacteria reported belonged to a broad range of bacterial species, such as *Arthrobacter, Bacillus, Clostridium, Curtobacterium, Enterobacter, Leifsonia, Microbacterium, Paenibacillus, Pseudomonas, Xanthomonadaceae, Staphylococcus, Stenotrophomonas, Sanguibacte, and Microsphaeropsis, Mucor, Phoma, Alternaria, Peyronellaea, Steganosporium,* and *Aspergillus*are reported as bioremediation process.

3.1.2. Microorganisms for the degradation of lignocellulosic waste

Microbial bioremediation is increasingly considered a reasonable and effective method for removing environmental contaminants, due to the discovery of a number of microorganisms or their enzymes that are capable of degrading organic pollutants. The previously mentioned microbes for bioremediation of organic pollutants have been applied where the microbes can utilize methane, phenol, or toluene as growth substrates (Chang and Alvarez-Cohen, 1995).

3.1.3.Application of microbial bioremediation

Bacterial surface-active agents or biosurfactants, with a wide spectrum in applications in several industries, are important biotechnological products. A significant issue currently is the accumulation and persistence of toxic materials in water and soil (Gadd, 1999;2002). The pathways by which micro-organisms affect shifts in metal evolution and mobility are basic building blocks of atmospheric circulation cycles for metals as well as all other components, such as carbon, nitrogen, sulfur, and phosphorus, with additional advantages for plant efficiency and human health. Molecular and genetic evaluation now improves knowledge of bacterial metabolisms, such as those elements that are relevant to the environmentand biotechnology e (Nies, 1999; Chen et al., 1999). Moreover, methylation of Hg, As, Se, Sn, Te, and Pb even under aerobic and anaerobic environmental conditions still can be achieved by a variety of bacteria and fungi. Moreover, methyl groups were transferred enzymatically to the metal and a species may change a number of unique components. Further, bacterial methylation of selenium, resulting in volatilization, was effectively used for in situ bioremediation of selenium-containing water and soil in Kesterson Reservoir, California, lowering selenium levels to acceptable standards s (Thompson-Eagle and Frankenberger, 1992). Where metal is reduced to a lower redox state, mobility and toxicity could be decreased with applications for bioremediation (Lovley, 2001; Finneran et al., 2002). Although, peptidoglycan carboxyl groups were the principal binding site in Gram-positive bacterial cell walls with such an important contribution in Gram-negative species (Beveridge and Doyle, 1989).

3.1.4. Mechanisms of plant growth-promoting bacteria in phytoremediation

Plant useful bacteria that strongly affect plant healthy development has been generally referred to as PGPB and function as biocontrol agents and potentially enhance plant growth and development through their suppressive phytopathogens behavior. However, they also may synthesize plant hormones including auxins, cytokinins, and

gibberellins, which could improve multiple phases of plant growth; synthesize iron chelators called siderophores, simultaneously enhancing plant metabolism or inhibiting phytopathogens i.e. synthesize 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity which might decrease plant ethylene concentration and solubilize minerals (Glick, 2002;2010). In addition, PGPB could still supply plants with important plant hormones to improve nutrient accumulation and metabolism by influencing root development mechanisms (Ma et al., 2016). In addition, IAA production is also common between plant-associated bacteria in comparison to its synthesis.

Table.3.1. Showing the relationship between organic pollutants and microorganism from the pulp paper industry waste and their toxicity effect on different microorganisms and aquatic organisms.

Experimented species	**Organic pollutants**	**Source**	**Toxicity**
Salmonella typhimurium	Trichlorotrihydroxy benzenes	Spent bleach lecher	Mutagenicity
Fish	Octylphenol, nonylphenol, bisphenolA,estrone, estradiol, triclosan,	Effluent	Inhibition of development, death
Fish	PCBs, PAHs, PCDD/PCDF	Effluent	Increase the rate of mortality
Fish	Terpenes, methanol, acid, abietic acid, dehydroabietic acid,	Effluent/sludge	Carcinogenic
Fish	Phenol, alkylated phenols, decline, benzoic	Effluent/sludge	Carcinogenic, androgenic
Oncorhynchusmykiss	Chlorinated pterostilbene	Effluent/sludge	The activity of hepatic mixed-function of oxygenase
Saccharomyces cerevisiae	Methyl dehydroabietic, ethyl dehydroabietic,	Effluent	Anti-estrogenic activity
Vibrio fischeri	Octadecenoic acid amide, linoleic acid, Linoleic acid isomer	Effluent	Luminescence Inhibition
Salmonella Typhimurium	Trichlorohydroxyfuranone	Effluent	Mutagenicity
Brown bullhead	Linoleic acid isomer	Effluent	Reduced elevated peptide hormone concentrations, no gonad size change
Eelpout	Benzoic acid	Effluent	A larger percentage of male embryos
Largemouth bass	Tetradecanoic acid	Effluent	Decreased gonad size, reduced sex hormones in plasma, and decreased female vitellogenin
Goldfish	Pentadecanoic acid	Effluent	Reproductive organs reduced

in plant tissues and maintains a significant part in plant-bacteria relationships (Singh et al., 2012). In addition, bacterial IAA might well boost the percentage of root hairs, the number of lateral roots, and the total root surface, resulting in increased root exudation and soil mineral absorption. The arsenic-resistant

*Brevundimonasdiminuta*NBR1012 strain IAA was used to defend rice plants from the adverse effects of arsenic stress and to enhance the phytostabilization capability of rice plants (Singh et al., 2012).The development of bacterial IAA is among the most essential strategies to promote plant growth as it instantly stimulates plant cell elongation and cell division (Wang et al., 2013).For instance, *Pseudomonas* 102 Zhaoyu Kong and Bernard R. Glick brassicacearum strain Zy-2-1, which also indicates copper resistance and generates IAA, siderophores, and ACC deaminase operations, has now been co-inoculated with Sinorhizobiummeliloti to support *Medicagolupulina* development and Cu accumulation (Kong et al., 2013). These results indicate which endophytic bacteria producing IAA might well play a major part in the Mn-stressed plants' development and Mn tolerance. In addition, the production of IAA.Bacterial modulates protein expression associated with plant growth and metabolism (Rajkumaret al., 2012). However, both cytokinins and gibberellins play a significant role in the regulation of plant growth and development (Frugier et al., 2008; Romanov, 2009). Several bacteria particularly PGPB could produce either cytokinins or gibberellins or both. For instance, in the cell-free medium of *Pseudomonas, Rhizobium, Azotobacter, Azospirillum, Bacillus,* and *Arthrobacter,* bacterial cytokinins have been observed (Tsavkelova, et al., 2006). In addition, gibberellins have been produced by *Azospirillum, Bacillus, Pseudomonas, Rhizobium, Azotobacter, Arthrobacterium, Agrobacterium, Clostridium, Flavobacterium* and *Xanthomonas* bacteria (Tsavkelova et al., 2006). In addition, several bacteria producing cytokinins or gibberellins have indicated enhancing plant growth (Joo et al., 2006; Kang, et al., 2013; Lorteau et al., 2001). For instance, through gibberellins they produced, *Bacillus pumilus* and *Bacillus licheni*formare promoted plant growth and yield (Gutierrez-Manero et al., 2001). Including auxin, ethylene is an important regulator of multiple elements of plant growth and development, in specific plant reactions to biotic and abiotic stress (Gamaleroand Glick, 2012; Hao, et al., 2007; Wang, Knill, Glick, andDefago, 2000). Moreover, the improvement in metal absorption by roots has been much larger than by shoots; reducing the factor of Cu translocation. These conclusions indicate the prospective use of ACC deaminase-overproducing rhizobial species in the phytostabilization of copper. Although, iron among the most common elements on earth, is largely unsuitable for direct absorption by plants and microbes because it exists mainly as Fe^{3+} in nature and is generally available in the form of insoluble hydroxides and

oxyhydroxides (Prasad andFreitas, 2010). Except maybe iron, siderophores can also form permanent complexes with other metal ions, such as Al, Cd, Cu, Ga, In, Pb and Zn, and with U and Np radionuclides (Kiss andFarkas, 1998; Neubauer et al., 2000). Moreover, siderophores bacteria should stimulate plant growth either directly by improving plant Fe nutrition or indirectly by limiting the iron supply of plant pathogens in the rhizosphere (Beatriz Jorge, andManero, 2008; Ma Prasadet al, 2011). For instance, mungo bean plants incubated with the siderophores-producing bacterium *Pseudomonas putida* KNP9 reported increase biomass and enhanced chlorophyll concentration in the presence of Pb or Cd compared to uninoculated plants (Tripathi et al., 2005). Furthermore, the siderophores generated by Streptomyces tendance F^4 considerably stimulated sunflower development and consumption of Fe and Cd by *Helianthus annuus*(Dimkpa et al., 2009).

Table.3.2.Showing the interaction between bacteria and plant and their mechanism for accumulation and remediation of heavy metals from the contaminated site of the pulp paper industry

Bacterial strains	**Plant name**	**Mechanism**	**Heavy metals**
Pseudomonasfluorescens	*Brassica napus*	Increase biomass, metal accumulation, stress tolerance	Lead
Azotobacterchroococcum	*Indian mustard*	Increased plant survival and lead uptake	Lead
Pseudomonas sp. Bacillus sp.	*Lycopersiconesculentum*	Increased biomass and metalbioavailability	Zinc, Cadmium
Enterobacteraerogenes,	*Indian mustard*	Increase biomass and siderophores, IAA, ACC deaminase	Cadmium, Lead
Pseudomonas sp.	*Cicerarietinum*	Increased germination, biomass, andmechanism unknown	Copper
P. aeruginosa	*Vigna mungo*	Increased biomass and decreased metal uptake	Nickel
Enterobacter sp.	*Indian mustard*	Increased biomass and rooting, anddecreased cadmium uptake;	Cadmium
Serratianematodiphila,	*Solanumnigrum L.*	Increased biomass and metal uptake; IAA,siderophores, ACC deaminase, phosphatesolubilization	Nickel, Chromium
Brevundimonasdiminuta,	*Eichhorniacrassipes*	Increased biomass; IAA, ACC deaminase,	Nickel, Copper,
Methylobacteriumoryzae, Burkholderia sp.	*Lycopersiconesculentum*	Increased metal uptake	Chromium
Actinobacterium	*Salix caprea*	Promoted plant growth andreduced accumulation	Cadmium
Bacillus thuringiensis	*Alnus firma*	Production of siderophoresand ACCD enhanced plant growth and metal accumulation inleaves	Cadmium and
Bradyrhizobium sp.	*Mung bean*	Production of IAA,siderophores, ACCD	Arsenic, Cadmium
Serratia sp.	*Nigrum L.*	Increased nodule number and plantnutrition; IAA, siderophores	Nickel, Zinc
Rhizosphere bacteria	*Thlaspicaerulescens*	Bioaccumulation or removal of metals in both single-ion and multi-ions systems	Cadmium, Chromium,
Graminaceae grasses	*Rhizosphere bacteria*	Increased zinc uptake; mechanismunknown	Zinc
Pseudomonas fluorescens	*Helianthus annuus*	IAA, siderophores, ACC deaminase	Cadmium, Zinc, Nickel
Indian mustard	*Bacillus sp.*	Increased growth mechanism unknown	Arsenic
P. putida	*Sunflower*	Increased metal uptake depending onspecific metal–bacteria combination;mechanism unknown	Selenium,Cadmium,
Bacillussp, Mycobacteriumsp	*Thlaspicaerulescens,*	Increased cadmium uptake and decreasedtoxicity; bacterium expresses a metal-binding peptide	Cadmium

In addition, bacterial siderophores should increase metal mobility and bioavailability for plant absorption in addition to reducing the herbal iron content. Phosphate (P) is one of the important macronutrients for plant growth and development but has a limited bioavailability and was seen as one of the elements limiting crop production (Brıgido and Glick, 2015). The current research showed a potential use of Phosphate solubilizing microbes including PGPB to reduce chemical P applications by 50 percent without a substantial in crop production (Jilani et al., 2007; Yazdani et al., 2009). Furthermore, phosphate-solutionization bacteria should increase plant phytoremediation by enhancing their development and health even though hazardous metals are available (Ahemad, 2014).

3.2. Persistent organic pollutants discharged from the pulp paper industry

Historically, the pulp and paper mill has been a major consumer of natural resources, i.e. fossil fuels, electricity for energy, wood, and water; consequently, a major contributor to environmental pollutant discharges. The commonly associated pollutants of pulp and paper mill effluents include pesticides, phenols, lignin, fatty acids, resin acid, lignin, and other chlorinated compounds. These chlorophenols compounds are contributed from the pulping and bleaching process. Moreover, the derived Chlorophenolic compounds from the action of chlorine and its hydrolyzed derivatives. These compounds are formed particularly during the breakdown of lignin in the pulping process. Further, the formation of chlorophenols does not depend on the ratio of chlorine dioxide and chlorine alone, but also on the ratio of hard and softwood types. These chlorophenols may also act as precursors of highly toxic compounds such as dioxins and furans. The natural properties of chlorophenols make them lipophilic and therefore tend to absorb on solids and accumulate in soils, sediments, and sludge.

After secondary treatment, the discharged effluent typically has a dark brown color; however, it depends on the raw material of industry and final product. The effluent pulping step creates color-rich streams, probably due to the dissolution of TSS, TDS, and lignin with other organic polymer compounds (Ali and Sreekrishnan, 2001). After secondary treatment, the brown color mostly appears in effluent which can be attributed to the lignin and its derivatives, and other polymerized tannin acid, fatty acid, and plant derivatives. After bleaching, the dark brown color is converted to a bright one. Besides, the wood debarking effluents are also observed to be dark in color. The origin compounds responsible for such color are lignin, lignin derivatives,

and tannins. These toxic compounds are well-known for their toxicity to bacteria and aquatic lives (Chandra et al., 2018).

During the chemical processing of wood, the paper and pulp industry releases high amounts of lignin, and which persists in the environment even after the secondary treatment. Moreover, lignin is the product of a pulping process based on kraft in paper mills. This is mainly used as black liquor in the pulp mill for power generation, processing steam and recovery. The pulp and paper mill disposal site that lignin and its derivatives not only give color but also cause an unpleasant smell (Sastry et al., 1986). In effluent, when lignin exceeds the allowable limits, it inhibits the photosynthesis process by absorbing the solar light. Bio-stimulation and bioaugumentation used to remove color, EDCs and organic compounds at different environmental condition effluent (Chandra et al., 2009). Next, to cellulose, the lining is the second most commonly used natural organic polymer in paper and pulp mills. Moreover, along with natural lignin, other derivatives such as chlorinated lignin are also used in pulping and bleaching processes (Ali and Sreekrishnan, 2001).

Effluent and sludge, obtained from secondary treatment, contains a significant quantity of dioxins and similar compounds like dioxin-like compounds (DLCs) are present in the effluent after secondary treated. These substances are already listed in the category of highly hazardous organic pollutants. In particular, aromatic halogenated hydrocarbons such as polychlorinated dibenzodioxins, dibenzofurans, and biphenyls are of great concern in this regard.These compounds are lipophilic in nature and have resistance to biological/chemical degradation (Vallejo et al., 2015). The common compounds used as a precursor in this industry are generated from the wood treatment, where it works as a bacterial/fungicidal agent (Vallejo et al., 2015). Due to low solubility, although the natural concentrations of dioxins and furans generally are low, it can enter into the water environment through effluent released from the sewage/municipal treatment plants. Among various wastewater treatment methods, solar light-based photocatalysis methods have been reported effective in the removal/degradation of dioxins and furans.

A typical paper and pulp mill are also known for producing various types of wood extracts. Further, the extractive type is also depending on the wood species, its various parts, and grows conditions. For example, trees grown in warm climates are able to produce appreciable amounts of fatty acid with less variation throughout the season. Among various process streams, secondary effluents and wasted sludge are

the most dominating sources of wood extracts such as alcohols, terpenes, and low molecular weight carboxylic acids. These compounds can be extracted through organic solvents such as dichloromethane, methanol, acetone, diethyl ether, ethanol, etc. Furthermore, these extracted can also be degraded or transformedby the activated sludge of bioreactors.

3.2.1. Endocrine-disrupting chemicals detected from pulp paper industry waste

Effluent discharged from the secondary treatment of paper and pulp mill, and wasted sludge is observed to be rich in resin acids. Such acids reported being toxic for aquatic plants, lives, as well as for humans. These acids i.e. tricyclic and diterpenic carboxylic acids etc. are mainly released from tree bark and softwood trees and non-volatile and hydrophobic in nature (Mc Martin et al., 2002). The concentration of this resin may reach as high as hundreds of parts per million, however, double concentration can be expected in hardwood trees (Ali and Sreekrishnan, 2001). Literature review revealed that acids like abietic acid, neoabietic acid, dehydroabietic acid, pimaric acid, and isopimaric acid, etc. are generally released from paper and pulp mills. The further effect depends upon the pH and the solubility of these substances. The lower pH values promote high toxicity than their dissociated counterparts. Generally, fats and waxes are defined as esters of carboxylic acids or fatty acids with glycerol or alcohol (Ali et al., 2001). The number of dry wood fats was observed to be in the range of 0.3 to 0.4%. With respect to wood type, the heartwood is found to be having greater content than sapwood. The extractives used in paper and pulp mill processes also contain fatty acid. To extract this fat and waxes from wood, organic solvents such as diethyl ether and acetone, are used. So far, around 20 saturated/unsaturated fatty acids have been reported in the literature, which is isolated from softwoods. Some of these acids are removed in chemical pulping or saponification, and the esters and waxes are hydrolyzed. Further, long-chain fatty acid, which enters into wastewater treatment plants, inhibits the growth of bacterial communities (Ali et al., 2001). These acids can be degraded anaerobically, but only in low concentrations. Various types of aerobic and anaerobic bacteria, fungi, and yeast are involved in the paper manufacturing process. Different types of biocide such as 2-(thiocyanomethylthio)-Benzothiazoles (TCMTB) and MBT are used in paper and pulp mills. Due to their high anti-fungal activity, these compounds are used to preserve wood from algal, fungal, and microbial growth, hence works as a replacement of traditional chlorophenols. Some of the examples of biocides, which work as oxidizing agents too, are chlorine dioxide,

hydrogen peroxide, and thiocyanates, isothiazole, and cyclobutane. Further, these compounds may also be classified on the basis of mode of action of their chemical structure such as membrane-active biocides, cytotoxic, and genotoxicity agents. Benzothiazoles are also used in paper and pulp industry, along with algaecides and fungicides. These compounds are also expected to enter an air environment through air or water means.

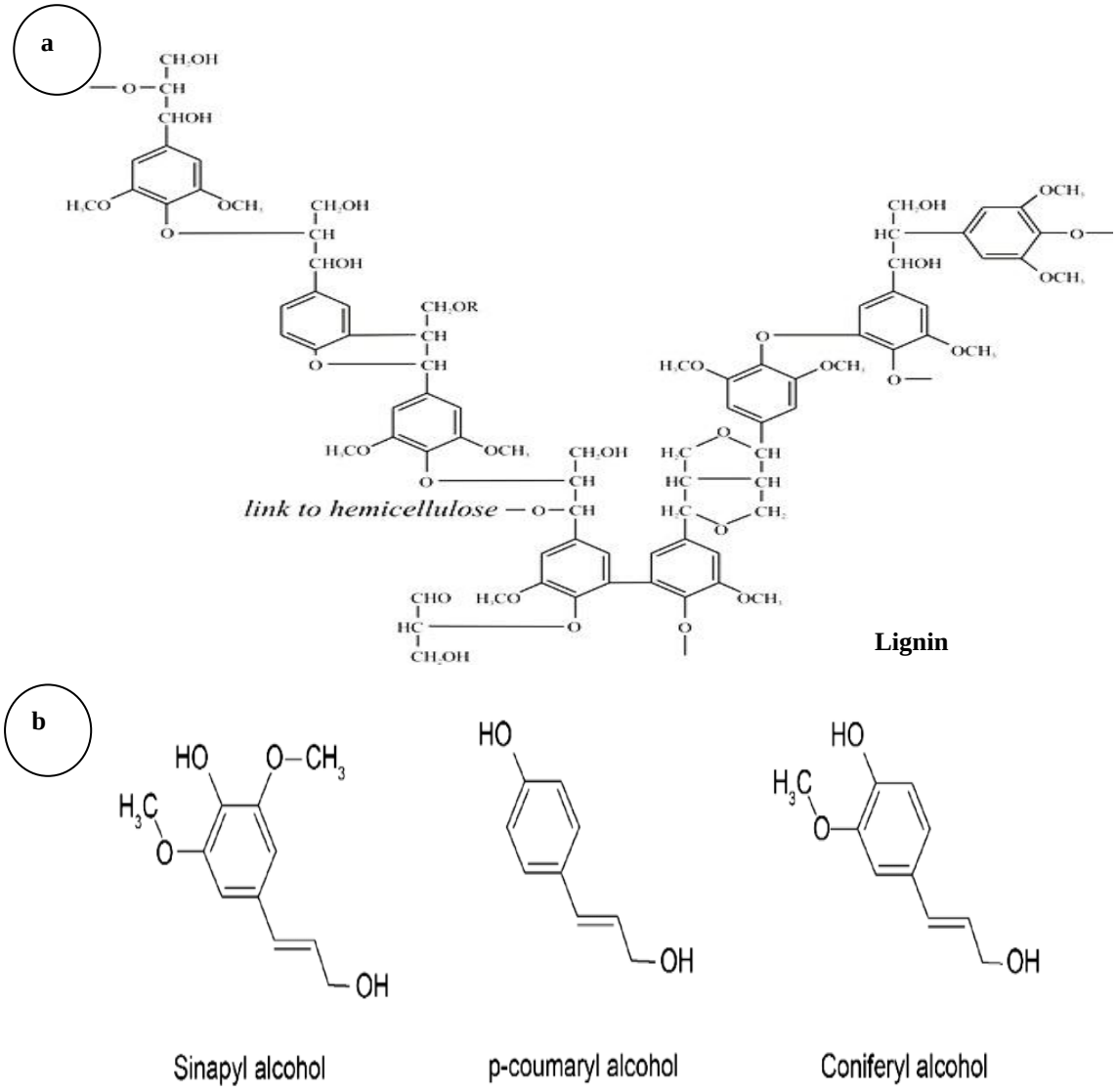


Fig. 3.1.Showing the chemical structure of lignin and its derivatives (a). Lignin (b). Sinapyl alcohol, p-comuaryl and coniferyle alcohol

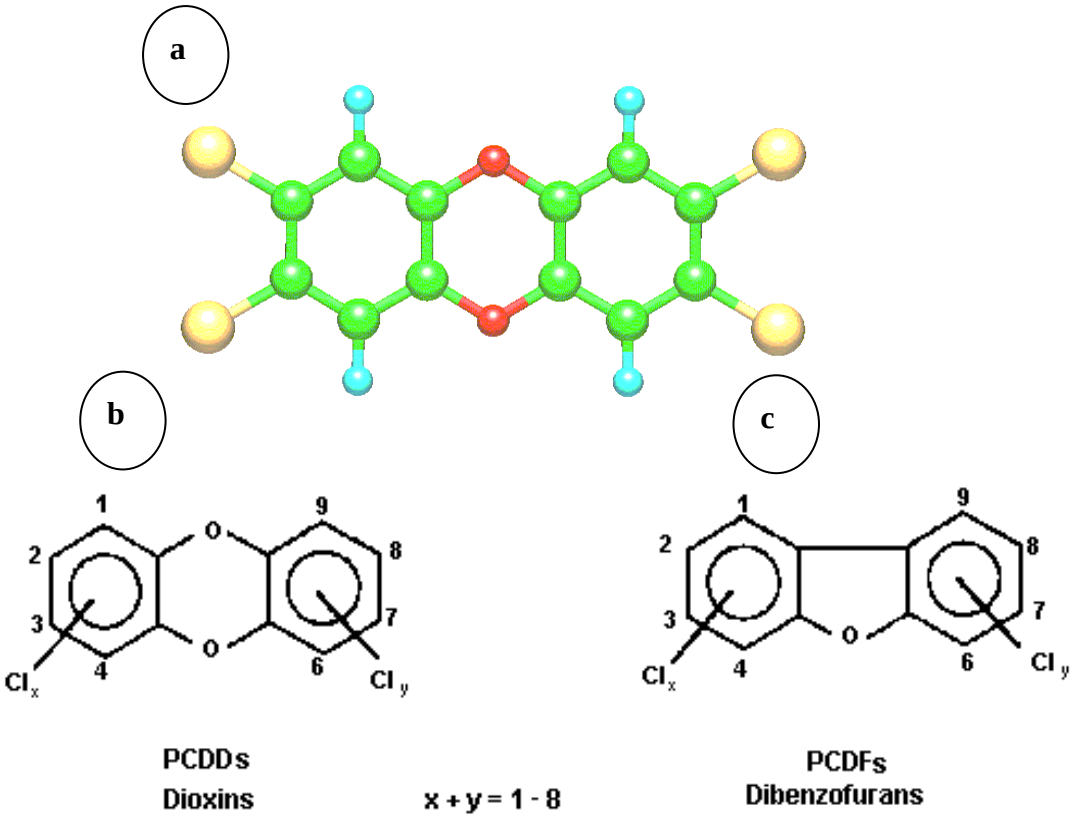


Fig. 3.2.The chemical structure of dioxin and other gaseous pollutants discharged from pulp paper industry waste

3.2.2. Diversity of complex organic pollutants degrading PGPB

The PGPB are bacterial strains isolated from different environments with a capacity to have a positive effect on several parameters of plant growth and output. The revegetation use of PGPB has many advantages; (i) reduce bioremediation expenses by reducing fertilizer and compost quantities, and (ii) enhances plant health and development in eroded areas and improves drought and salinity tolerance, (iii) Allows plants to be established in eroded areas where they had grown. The PGPB deserves special attention among the rhizosphere microorganisms involved in plant interactions with the metal-contaminated soil environment. In various ways, plant growth is Affected by microbes, some microbes cause disease and inhibit plant growth; others can directly or indirectly encourage development through a multitude of nitrogen fixation mechanisms, phosphate Solubilization, siderophores, phytohormones production, and ACC deaminase.In addition, positive impacts due to the inoculation of bacterial endophytes are physiological changes in plants, including accumulation of osmolytes and osmotic adjustment, stomata regulation, decreased membrane

potential, and changes in the phospholipid content of cell membranes. Plant species survive in a bacteria-rich environment in nature and will need to interact with a multitude of pathogenic, commensal, and useful microorganisms.The plant immune response composed of a complex system of signals, transcriptional systems, and hormonal crosstalk; at least two types of microorganism's stimuli should activate it. The processes through which micro-organisms affect changes in metal evolution and movement are basic building blocks of biogeochemical cycles for metals as well as all other elements, including carbon, nitrogen, sulfur, and phosphorus, with additional consequences for plant productivity and public health.Microorganisms should transfer metals via autotrophic and heterotrophic leaching, bacterial metabolite and siderophores chelation, and methylation which can contribute to volatilization.Many autotrophic leaching is conducted by chemolithotrophic, acidophilic bacteria that replace carbon dioxide and receive energy from ferrous iron oxidation or decreased sulfur compounds which cause metal Solubilization as a result of Fe (III) and H_2SO_4 production. Under aerobic and anaerobic environments, methylation of Hg, As, Se, Sn, Te, and Pb can be achieved by a range of bacteria and fungi.Bio sorption could be characterized by physical and chemical mechanisms including adsorption as the microorganism's absorption of organic and inorganic metal species, both soluble and insoluble. Peptidoglycan carboxyl groups were the primary binding site for cations in Gram-positive bacteria cell walls with an important contribution in Gram-negative organisms with phosphate groups (Beveridge and Doyle, 1989; McLean et al., 2002).

3.2.3. Bacteria assisted Phytoremediation

Moreover, PGPB may change the accumulation potential and its translocation in plants through decreasing Phytotoxicity and modifying the phytoavailability of heavy metals in polluted soils through its multiple plant growth-promoting characteristics including metal resistance, detoxification, accumulation, transformation, and sequestration (Ma et al., 2016). The lead resistant Bacterium *Bacillus sp,* MM3–4 that is resistant to isolated from the roots of the metal hyperaccumulator *Alnus firma* company improved the phytoremediation ability via extracellular sequestration and intracellular accumulation, reducing the phytotoxic effects of metals (Shin et al., 2012). In addition, *Pseudomonas sp.* A3R3 is nickel-resistant, the biomass of *Brassica juncea* and accumulation of Ni in hyperaccumulator *A. Serpyllifolium* grown in Ni- contaminated soil.

Table.3.3.List of organic pollutants discharged from pulp paper industry waste after secondary treatment and their toxic effect on plant and fish.

Pollutants name	**Source**	**Test model**	**Nature of pollutants**	**Toxicity effect**
β-Sitosterol trimethylsilyl ether	Effluent	*Allium cepa*	EDCs, carcinogenic	Chromosomal aberration
2-Methyl-4-keto-2-pentan-2-ol	Effluent	*Allium cepa*	EDCs, mutagenic effect	DNA damage
Octadecanoic acid	Bleached kraft effluent	White sucker	Carcinogenic, EDCs	Reduced gonad size, sex hormones circulating and fecundity, delayed maturity
Pentadecanoic acid	Bleached kraft effluent	Mummichog	Androgenic, EDCs	Recessions of testosterone
Tetradecanoic acid	Kraft paper mill	Female mosquitofish	EDCs, cytotoxic	Masculinization of the female fish.
	Paper mill	Mosquitofish		Masculinized female eastern mosquitofish, *Gambusia holbrooki*
Mg/Ca	Pulping stage	Catfish	Heavy metals	Neurotoxic effects, juvenile toxicity
Cr	Sulfite pulping	Human/salmon Oncorhynchustshawytscha	Heavy metals	Carcinogenic, genotoxicity, the effect on gill and kidney, DNA damage
As	Digestion of wood	Human	Heavy metals	Carcinogenic, inner cancers, neurological issues, pulmonary disease, vascular peripheral illness, cardiovascular disease and hypertension
Cr	Sulphite pulping	Human	Heavy metals	Carcinogenic DNA damage, genotoxicity
K	Kraft pulping	Human	Heavy metals	Diseases of the cardiovascular, liver and pulmonary
Zn	Chemical pulping	Human/plant	Heavy metals	Gill > liver > ovary > muscle accumulation
Hexadecanoic acid, trimethylsilyl ester or Palmitic acid TMS	Pulping and beaching	Aquatic ecosystem	EDCs	Alteration in chromosome
Phenol,4-ethyl-2-Methoxy	Pulping	Aquatic ecosystem	EDCs	Effect on hormones
Lactic acid, trimethylsilyl ether,	Beaching	Fishes	Carcinogenic, EDCs	Hormonal imbalance

Throughout the last twenty years, researchers had already obtained a far better understanding of the contribution of numerous PGPB to plant growth and consequently, enhance phytoremediation efficacy. In addition, PGPB Improved phytoremediation has become a successful strategy to tackle plant stress difficulties in heavy metal-contaminated soils. During most of the Bioaugumentation assessment process with PGPB the interaction of the inoculated strain with the indigenous

microbial community, the survival or colonization of the non-indigenous strains in the host plants, and the response of the indigenous microbial community structure and function to the implementation of exotic PGPB should be defined. Moreover, organic acids produced by plant-associated microorganisms play a significant part in the complexation of toxic and essential ions.

3.3. Approaches for phytoremediation

Toxic metal-contaminated in soil threatens both the environment and human health. Generally, leakage may have existed through human activities including mining and smelting metalliferous ores, burning fossil fuels, disposing of sewage and municipal waste, or using fertilizers and pesticides in agriculture. The hazardous metals should be removed or made unavailable to return this land for use in agriculture or amenities. However, phytoremediation is a potential phenomenon where green plants have been used to decontaminate contaminated sites.

3.3.1. Phytoextraction or Phytoaccumulation

Phytoextraction, also called phytoaccumulation, refers to the uptake and translocation of metal contaminants in the soil by plant roots into the above-ground components of the plants. Some plants, widely known as hyperaccumulators, help absorb extremely large quantities of metals compared to other plants and the ambient metals. Two fundamental phytoextraction approaches are being established: chelate-assisted phytoextraction, which we consider phytoextraction induced; and long-term phytoextraction constantly.

3.3.2. Phytostabilization or phytoimmobilization

Phytostabilization requires those species of plants to immobilize soil contaminants via root absorption and accumulation, root adsorption or precipitation within the root zone, and physical stability of the soil. Moreover, phytostabilization, in which plants consolidate instead of absorbing contaminants by metal retention of plant roots. Phytostabilization is much more successful in fine-textured soils with high organic content, but it is suitable for managing an environment where large areas are contaminated by soils (Cunningham et al., 1997). Moreover, several heavily contaminated sites were not appropriate for phytostabilization since it is difficult to grow and survive (Berti and Cunningham, 2000). Phytostabilization has strengths over other techniques in soil remedial work in that it is less costly, simpler to enforce and aesthetically preferable (Berti and Cunningham, 2000; Schnoor, 2000). Though if decontamination different approaches are completely unworkable due to the extent of

the contaminated area or absence of appropriate financing, phytostabilization is economically beneficial (Berti and Cunningham, 2000).

3.3.3. Phytovolatization

There may be some metal contaminants in such as As, Hg, and Se, as gaseous form species release in the environment from pulp paper industry waste. In the latest years, research scientists have indeed originally sought natural or genetically modified crops fully capable of absorbing elementary types of these metals from the soil, converting them biologically into plant-based gaseous species and releasing them into the atmosphere is called phytovolatilization.

3.3.4. Phytofiltration

Phytofiltration is the use of the plant roots in sequence to absorb or adsorb pollutants, mainly metals, from water and aqueous waste streams (Prasad and Freitas, 2003). In aerated water, plant roots or seedlings absorb, precipitate and concentrate toxic metals from polluted effluents (Dushenkov and Kapulnik, 2000; Elless et al., 2005). Moreover, Biosorption mechanisms include chemical adsorption, complexation, exchange of ions, micro precipitation, bio surface condensation of hydroxide and surface adsorption (Gardea-Torresdey et al., 2004).

3.3.5. Phytohydraulics

Using of long-rooted plants-generally trees -to contain, sequestrate or degrade pollutants of groundwater that come into contact with their roots. Microorganisms are generating a range of specific and non-specific metal-binding compounds.Eventually, it must be highlighted that such a study area also provides an understanding of the biogeochemistry of metalloid cycling in the environment and the important role microorganisms play in influencing metal mobility and transferring between distinct biotic and abiotic sites.

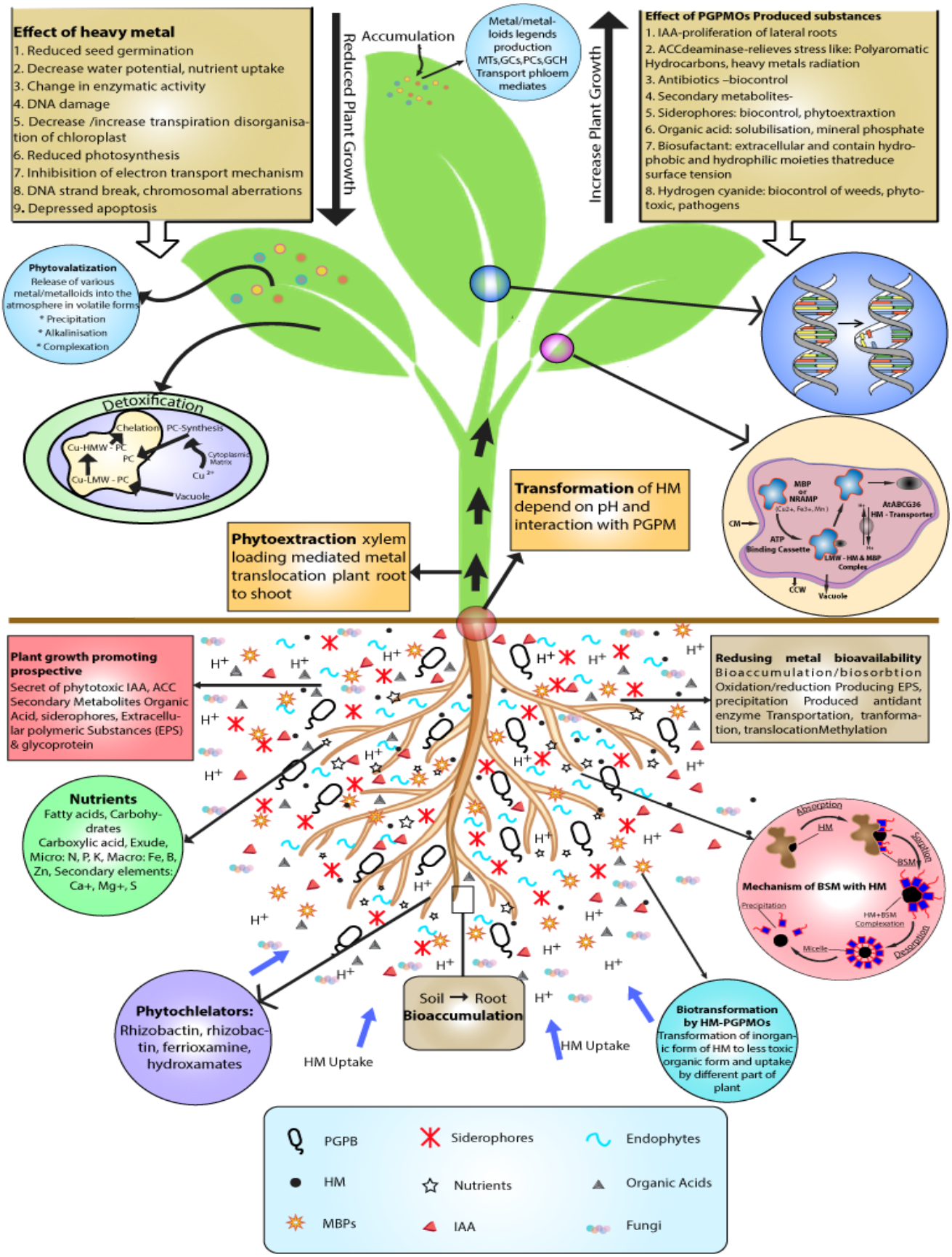


Fig. 3.3. Relationship between plant and microbes for tolerance and detoxification of heavy metals from pulp paper mill wasteafter secondary treatment

3.4. The following conclusions are made based on the above review of the literature

a. The wastewater from all types of pulp and paper mills waste can be treated both aerobic and anaerobic treatment technologies except that bleaching Kraft effluents are less suitable to process.
b. Combinations of phytoremediation and natural phenomena with application optimization provide such a long-term solution for treating wastewater from the pulp paper industry.
c. Bacteria played significant roles in the environmental fate of heavy metals and metalloids with a multiplicity of physicochemical and biological processes which transform soluble and insoluble stages.
d. To accelerate the process and optimize the rate of mobilization or absorption or accumulation of pollutants, the role of soil micro biota, specifically Rhizospheric and entophytes microorganisms, in the growth of phytoremediation technologies must be illustrated.
e. In this respect, advantageous bacteria might be used to change metal bioavailability in order to enhance the phytoremediation of huge-scale metal pollutants in the environment.
f. This study revealed the conclusions of the cultural bacterial niche in the rhizosphere of *P. communis*, which were useful for the degradation of toxic metals and other complex pollutants constituents in the pulp paper industry waste, further confirmed by the bioassay of decolorisation with the lowest final color value.

Chapter-Four

Detection of various residual organic pollutant present in various pulp and paper industry waste after secondary treatment

Detection of Various Residual Organic Pollutant Present in Various Pulp and Paper Industry Waste after Secondary Treatment

4.1. Introduction

Pulp paper mill wastewater (PPMWw) is an important source of environmental contamination due to the release of the huge amount of wastewater containing chlorinated lignosulphonic acid, chlorinated resin acid, chlorinated phenol, and chlorinated hydrocarbon yet after secondary treatment (Kirk et al., 2018). Bayik and Altin, (2017) reported that worldwide paper manufactured in 2017 was approximately 411 million tons, mainly in the USA, European countries, Brazil, India, and China. Approximately 190-200 m^3 of freshwater can be used for the annual production of one-ton paper (Chandra et al., 2012). In India, approximately 100 million kg of hazardous pollutants is produced annually from pulp paper industries (Dey et al., 2013). Approx 40-45% of pulp are generated from the initial raw material weight. The waste discharged from these industries is extensively loaded with approximately more than 500 different organic chlorinated compounds including chlorinated hydrocarbons, phenols, catechols, guaiacols, furans, dioxins, syringols, vanillins, lignosulphonic acids, chlorolignins, chlorinated resin acids, chlorinated phenols, chlorinated hydrocarbons, various surfactants and biocides (Ali and Sreekrishna, 2001; Antizar Ladislao, 2004; Lacorte et al., 2003; Savant et al., 2006). In addition, chlorinated compounds measured as AOX present in PPMWw may cause a variety of clastogenic, carcinogenic, endocrine and mutagenic effects in fish tissue (Farooqi and Basheer, 2017). High organic and inorganic wastewater loads have mainly affected the aquatic ecosystem, especially fish and certain bacteria, in multiple respects, for example, localized benthic community damage, hypoxia in major areas and various changes in fish increased biochemical changes, genotoxicity, and physiology. Due to various chlorinated bleaching processes, many discharged toxic organic chlorinated compounds have been reported in PPMWw that receive water and biota of the aquatic system is not yet known which is difficult to degrade (Dey et al., 2013; Sharma et al., 2007). However, resin acids are responsible for fish larvae teratogenicity and accumulate in sediments and become bioavailable to fish. While short-term exposure

to low concentrations of erythrocytic micronuclei and nuclear abnormalities induced genotoxic effects in sea bass reported by Oikari et al., (2002). The impacts of bleached wastewater in fingerling *Micropterus salmoides* on selected physiological and hematological endpoints indicated that in-stream exposure to elemental-chlorine-free PPMWw generates a widespread response to stress, leading to potential immune suppression. The late sexual development, cellular damage and negative effect on the biochemical parameters have been noted in fishes due to depletion of oxygen in waste and anoxia to the aquatic organism. Some hazardous persistent organic pollutants (POPs) and heavy metals might have been harmfully affected by top predators, including plants and humans by the bioaccumulation and magnification. Although, POPs are among the various chemicals that are currently associated with human and wildlife concerns about EDCs and chlorophenols are toxic chemicals with estrogenic, mutagenic and carcinogenic effects (Farooqi and Basheer, 2017). The toxicity of PPMWw derives from the presence of several organic, xenobiotic compounds in different stages of the papermaking process (Yadav and Chandra, 2018). Moreover, they can also inhibit oxidative phosphorylation and ATP synthesis. The PPMWw also containing a large number of tannins, these tend to absorb more light, heat and retain less oxygen, thereby negatively affecting the aquatic flora and fauna. This was also observed to be discharged PPMWw in species living downstream of delayed sexual maturity, smaller gonads, improvements in fish reproduction and anxiety in phonotypical sexual characteristics in the aquatic ecosystem. The majority of environmental toxicity due to PPMWw is monitored by other counties, but cytotoxic and mutagenic effects are not clear. Most of the pollutants are reported as androgenic and mutagenic pollutants, most of which influence the zooplankton and phytoplankton directly or indirectly, including soil and microbial communities. But, detail knowledge regarding the nature of pollutants discharged from pulp paper industries in India is unknown. Moreover, fish toxicity reported by other countries but the exact toxicity of pollutants present in PPMWw is also unknown. Results of a study on *Anabustestudineus, Channapunctatus* and *Clariasbatrachus* showed that *Anabustestudineus* was most susceptible to paper mill waste, whereas *Channa punctatus* and *Clarias batrachus* were relatively resistant (Nanda et al., 2002). Hence, the present study is focused on the detection of androgenic and mutagenic pollutants present in PPMWw. Genotoxicity and mutagenetic effect of PPMWw are also

evaluated through chromosomal aberration in *Allium cepa.* Furthermore, conventional toxicity tests were also carried out to assess the toxicity of PPMWw on *Phaseolus mungo L.* and *A. cepa*. This information will be useful in managing and monitoring POPs in contaminated sites of PPMWw.

4.2. Material and Methods

4.2.1. Sample Collection

PPMWw for this study was collected from the pulp paper industry discharged site of M/s K.R. Pulp and Papers Limited, Shahjahanpur, U.P. India (27°50'31.8"N 79°51'15.7"E) Fig. 4.1. This industry uses Eucalyptus and bamboo woods as raw materials and pulping through the Kraft process, followed by multistage chlorine bleaching to make white papers. This site is well known for high pollution with organic and inorganic pollutants reported earlier by various researchers. All the study parameters i.e. Physico-chemical analysis, pollutant extraction, and toxicity were done within 48 hrs.

Fig. 4.1.Pulp paper industry view and sample collection from the disposal site (a). Collection of sample (b) sample collection site of K R pulp paper industry

4.2.2. Physico-chemical Analysis of PPMWw

The Physico-chemical analysis of PPMWw total solid (TS), TSS, total dissolved solid (TDS), COD, BOD, total phenols, total nitrogen (Micro Kjeldahl), sulphate (gravimetric method), phosphate and color (visual color comparison method) were analyzed as per methods described in American Public Health Association (APHA, 2012). The pH, chloride, sodium, and potassium of the PPMWw were also analyzed with the respected selective ion electrode of Orion Thermo (Model 960) and lignin has been measured by Pearl and Benson, (1990) method. In this method, the sample (50 ml) was mixed with 1 ml CH_3COOH (10%) and 1 ml ($NaNO_2$: 10%) after adjusting the pH 7. NH_4OH (2 ml) was added and absorbance (OD) was measured at 430 nm. The absorbance (ppm) using the following formulae.

Lignin (ppm) = Absorbance/0.000247

4.2.3. Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDX) Analysis of PPMWw

Freshly collected PPMWw was completely dried at 50°C in the hot air oven (Thermo scientific). After that dried samples were converted into powder form. For SEM analysis, 10 mg PPMWw Powder was spotted on high purity aluminum stubs for gold coating. The gold coating was done on the powder sample using a vacuum coating unit called gold sputter coater (SPI-MODULE). This made the sample electrically conductive. Then the sample was placed in the SEM-EDX chamber. The operating condition was set to 15 kV voltages and Images were taken at different ranges. While, for elemental analysis of the PPMWw a specific point was chosen and elements examined through a high-resolution scanning electron microscope equipped with an EDAX system (SEM, JEOL, JSM 6490LV, Tokyo, Japan).

4.2.4. FTIR analysis of root tips

For FTIR analysis root tips of each plant treated with PPMWw *A. cepa* and *P. mungo L.* untreated controls were dried in a hot-air sterilizer, FTIR Perkin Elmer Spectrum II spectrometer at 65°C for 48 h (Ahmed et al., 2017). Dried roots were powdered using mortar and pestle and sieved through a mesh. KBr discs were made with IR grade KBr in a mortar in the ratio of 1:100 (sample: KBr). Moreover, FTIR analysis was performed in attenuated total reflectance (ATR) mode in the range of 4000-400 cm-1 using Spectrum Two FTIR spectrometer (Perkin Elmer, UK).

4.2.5. Extraction of Residual Organic Pollutants from PPMWw and characterization of pollutants by GC-MS Analysis

Various organic solvents i.e. ethyl acetate, isopropyl alcohol, n-hexane, and methanol were tested to compare extractability of residual organic pollutants and ethyl acetate was found to be optimal. The residual organic pollutants from PPMWw were extracted three times with the equal volume of ethyl acetate in a separating funnel having capacity 500 ml by intermittent shaking (Fig.4.2). The solvent layer containing organic pollutants was separated and evaporated under vacuum at 40°C for dryness. The obtained dried residue was dissolved in 1.0 ml of HPLC grade acetonitrile, filtered through a syringe filter (0.22 µm) and used for further analysis. The GC-MS analysis of the ethyl acetate extracts was performed following the method described by Chandra et al., (2009) for the characterization of the residual organic pollutants. The persistent organic contaminants are detected by comparing their mass spectra with those given with the instrument in the National Institute of Standards and Technology (NIST) library.

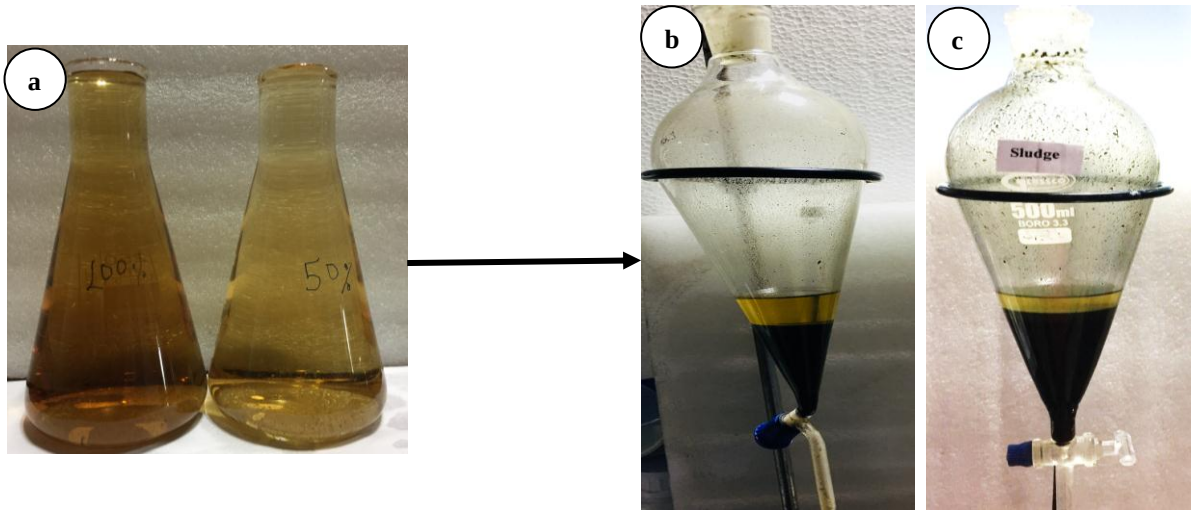


Fig.4.2.Showing the extraction of pulp paper mill waste by using different solvent based on their solubility

4.2.6. Estimation of antioxidants in *P. mungo* and *A. cepa*

The removal of enzyme from *P. mungo* and *A. cepa* treated with PPMWw was homogenized by 220 mg of fresh plant leaves in a 3 mL 100 µM potassium phosphate buffer (pH 7.5) containing 1 mM of ETDA and a pinch of polyvinylpolypyrrolidone

(PVP). The activity of SOD in the extract was measured as per the method of Nishikimi et al. (1972). The CAT estimated as per the method described by Chance and Maehly (1955). Moreover, hydrogen peroxide (H_2O_2) and APx were analyzed by the standard method described by Velikova et al., (2000) and Nakano and Ascada (1981), respectively.

4.2.7. Phytotoxicity Assessment of PPMWw with *Phaseolus mungo* L and *Allium cepa*

The toxicity evaluation of PPMWw was analyzed as per the guidance of the Environmental Protection Agency (EPA), US, using the seed germination bioassay tests. The toxicity of the PPMWw was also examined through a seed germination test with green gram (*P. mungo*) following the method described by Yadav and Chandra (2015). Ten sterile seeds of *P. mungo* L were placed on three layers of filter papers (Whatman No.1) in a glass Petri dish (9 cm dia).The filter paper was moistened with different concentrations of PPMWw i.e. 20, 40, 60, 80 and 100% along with tap water as a control. While *A. cepa* were placed on the top of the test tube (50 ml capacity) filed with different concentration of PPMWw in such a manner so that the bottom of *A. cepa* was deep in the effluent. Growth of the bulbs exposed to effluent solutions *A. cepa* and *P. mungo* was measured in terms of their germination and root length. Moreover, root length in cm was observed after 24 hrs interval.

4.2.8. Anatomical Microscopic Study of *A. cepa* Treated with PPMWw

Apical parts of the roots grown in absence and presence of PPMWw (100%) were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 12 h (overnight) at 4°C with intermittent stirring. Samples were dehydrated through a graded series of acetone (30%, 50%, 70%, 90%, 95% and 100% once for 15 min at each step). Roots were then transferred to a critical point dryer using CO_2 as transitional fluid (Fig.4.3).

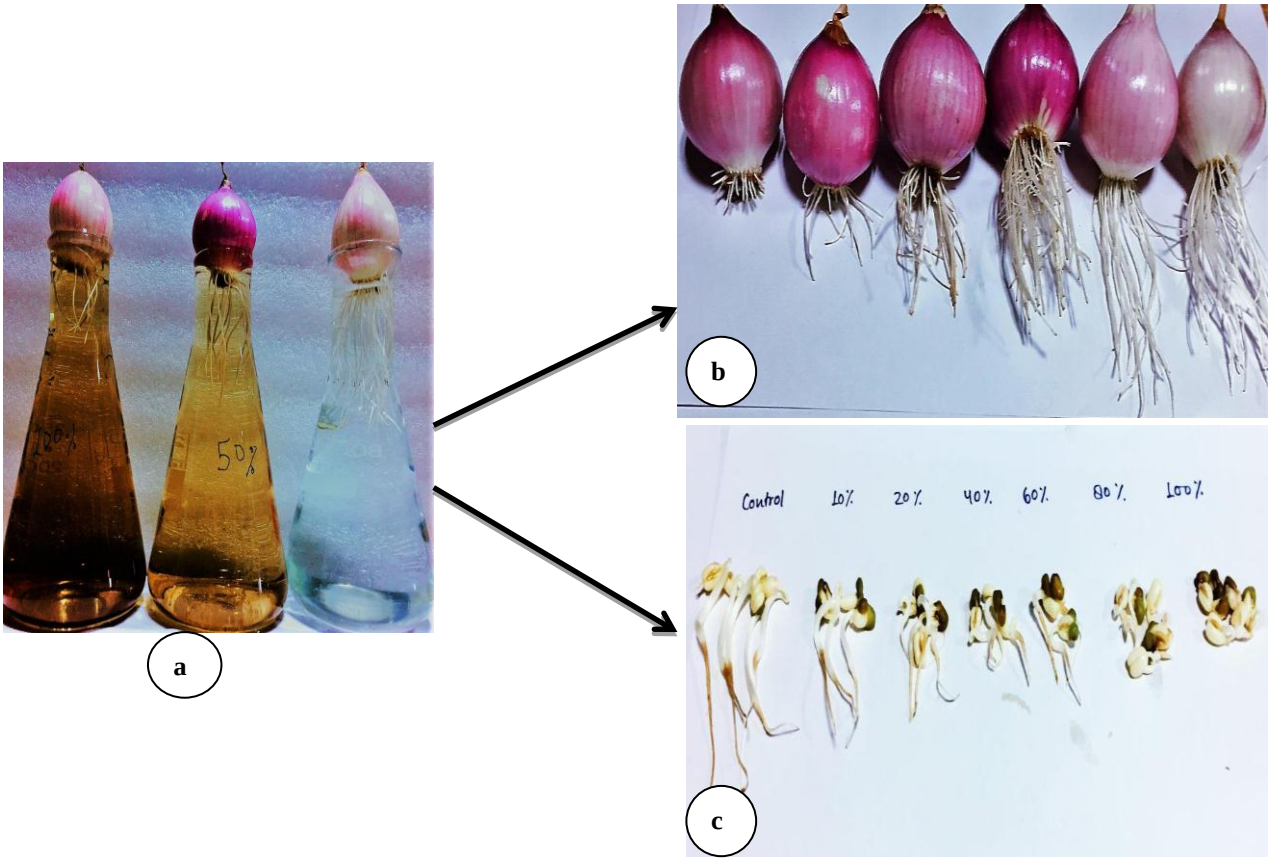


Fig.4.3.The environmental toxicity evaluation of direct exposure with pulp paper industry waste (a). Exposure with effluent (b). Effect of root length of *A.cepa* after exposure (c). Seed germination test

This removes water from the specimen and avoids unwanted damage due to the surface tension generated by a liquid/gas interface. Dehydrated roots were then put on the two-sided carbon tape fix on SEM stub and visualized under JSM 6490LV scanning electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 10 kV. For light microscopy, thin sections (1µm) were cut with an ultra-microtome (Ultracut, Reichert-Jung, Wien, Austria) and stained with safranin, being then examined (Phase Contrast microscope; Nikon; Japan).

4.2.9. Root Cell Viability Test of *A. cepa* by 2, 3, 5-triphenylte tetrazolium chloride (TTC) Assay

For testing the viability of *A. cepa* root tips after PPMWw treatment were stained with TTC as a histopathological stain. Moreover, metabolically active and inactive root cells under the exposure of 20, 60, 80 and 100% effluent of pulp and paper mill were

detected using the method of Shaymurat et al., (2012). The test was as follows: 5 ml of 0.5% solution of TTC was added to sterile test tubes containing 10 root tips; the temperature was kept at 35±1°C the test was performed in dark due to the instability of TTC to light. After 5 h incubation in the dark, the TTC solution was removed with a syringe and root tips were thoroughly rinsed with distilled water and then examined. The red-colored root tips were considered to be viable and others were non-viable or dead.

4.2.10. Chromosomal Aberration on *A. cepa* for Genotoxicity Assessment of PPMWw

Onion bulbs (*A.cepa* L., 2n=16) of the purple variety of average size (15-22 mm diameter) were purchased in the local market of Lucknow, India. Bulbs were made germinated in common portable water (without any growth factors) during the course of 2-4 days. Then the bulbs were carefully removed without any damage to the roots. Infectious bulbs are discarded for experimental purposes. The outer scales of onion bulbs and brownish bottom plates were removed without injuring the ring of root Primordia. Onion bulbs with good root growth were selected. For root growth inhibition evaluation, freshly prepared stock extracts were diluted into 50 and 100%. Three healthy onion bulbs with roots were utilized for each concentration and the control as tap water. The base of each of the roots of the bulbs was suspended in the dark for 6 to 24 h on the extracts inside 100 ml beakers and five root tips were cut and fixed in ethanol at the end of the exposure period: glacial acetic acid (3:1, v / v). These were hydrolyzed in 1N HCl at 60°C for five minutes after which they were washed in distilled water. Three root tips were squashed on each slide, stained for 10 minutes with 2% hematoxylin and covered to prevent air bubbles. The coverslips were sealed on the slides with clear fingernail polish. Slides were monitor under phase-contrast microscopy (Phase Contrast microscope; Nikon; Japan).

4.2.11. Toxicity Assessment Through α-amylase Activity

The bioassay of an acute toxicity test was performed to evaluate the detoxification of effluent using the seed germination method of *P. mungo* L. For toxicity evaluation PPMWw was diluted with tap water at different concentrations i.e., 20, 40, 60, 80 and 100% (v/v). The toxicity was expressed in terms of % inhibition of their amylolytic activity (Bharagava and Chandra, 2010). For the α-amylase assay, twenty seeds from

each treatment were homogenized with 0.1 mol sodium acetate buffer (pH 4.8 filtered through two layers of cheesecloth to remove large particles and the supernatant obtained was centrifuged at15, 000×g for 20 min. All the preparations were carried out at 4°C. The supernatant obtained was used as a crude enzyme extract for α-amylase assay. For enzyme assay, the reaction medium (3 mL) contained 1 mL of 0.1 mol acetate buffer, pH 4.8, 0.5 mL of enzyme extract diluted to 1 mL using acetate buffer, and 1 mL of 0.1% soluble starch solution. During the enzyme assay, the enzyme extract was diluted to obtain an absorbance range of less than one and the reaction medium was incubated at room temperature for 10 minutes, then the reaction was stopped by adding 1 mL of 0.1% iodine reagent and 3 mL of 0.05 mol l^{-1}HCl. In terms of amylase activity, the absorbance was measured at 620 nm and decreased in absorbance (Beri and Gupta, 2007).

4.2.12. SDS-PAGE and Molecular Weight Determination of Enzyme

Seeds treated with different concentration PPMWw and tap water was crushed with chilled acetone. The supernatant containing crude -amylase enzyme was concentrated by adding the double volume of cold acetone (-20°C) and the centrifugation of the precipitated proteins was 15,000 g for 20 minutes. After the acetone removing, the precipitated proteins were dissolved in a fixed concentration of 0.1 M sodium acetate buffer (pH 4.8).To purify, the soluble proteins were passed through a column (80 cm × 2.0 cm) containing Sephadex G-100 (Bharagava and Chandra, 2010) previously equilibrated with the same buffer and the protein fractions (2.0 ml) eluted at the flow rate of 0.5 ml min^{-1} were stored at -20 °C. The effect of PPMWw through the pattern of amylase bands was determined by denaturing SDS-PAGE performed on 10% polyacrylamide gel. The catalase (240 kDa), bovine albumin (67 kDa) and ovalbumin (43 kDa) enzyme purchased from Sigma–Aldrich, USA were used as standards. The protein bands are stained with Coomassie Brilliant Blue R-250 coloring after gel electrophoresis and destained with a destaining solution. The gels are visualized and processed in a gel documentation system after destaining (Syngene, UK).

4.2.13. Statistical Analysis for Data Variability

All data were reported as means ± SD for triplicate samples. To confirm the data variability and results from validity, all the data were subjected to Statistical analysis. Tukey's test (Ott, 1984) using the Graph Pad software (Graph Pad Software, San Diego, Calif.) was used for statistical analysis.

4.3. Results and Discussion

4.3.1. Physico-chemical analysis and UV-Scanning Spectra of PPMWw

Physico-chemical characteristics of the PPMWw after secondary treatment are shown in Table.4.1. The physics-chemical analysis of discharged PPMWw showed the presence of a high amount of color, TDS, TSS, COD, BOD, phenolics compound along with nitrogen and phosphorus. TDS is found to be higher than the permissible limits of waste discharge into surface water. Higher TDS may increase the salinity of the wastewater and thus it showed unfit for irrigation and drinking. The higher concentration of TDS has been reported to cause disorders of the respiratory system, alimentary canal, nervous system, coronary system and causing miscarriage in the aquatic ecosystem (Reddy and Rao, 2007).Moreover, high COD leads to the toxic state of the PPMWw along with the presence of biologically resistant organic substances. The EC value of the PPMWw was observed as 1400 µmhos cm^{-1} which was very high than the EPA guidelines i.e. 1000 µmhoscm^{-1}. Higher EC might be due to salt and ions content of PPMWw (Deepali et al., 2009).Moreover, in the present study high sodium content (94 mg l^{-1}) was observed as compared to the permissible limit of effluent discharge. Sodium is an important cation that occurs in less than 20 ppm in all-natural freshwater sources (EPA 2002). An increased level (17.80 mg l^{-1}) of potassium was also observed in the PPMWw. Similarly, chloride is the most troublesome anion also found in PPMWw which is generally more toxic than sulfates to most plants and aquatic life. Moreover, lignin contents were so high (39000 mg l^{-1}) in PPMWw which might be the source of the dark color of the effluent. The dark color of PPMWw adversely affects the lower organisms in the food chain. Furthermore, the significant amount of Fe (87-79), Zn (34-22), Cu (3.28), Cd (1-0.36), Mn (16-15) and Ni (6-5mg l^{-1}) was present in PPMWw which are hazardous to the environment. This observation is corroborated with the findings with others (Chandra et al., 2011; Madan et al., 2018).The sources of heavy metals in PPMWw might be due to the corrosion activity of alkaline black liquor produced during wood digestion as it passes through iron pipes. Although, some heavy metals, such as Zn, Cu, and Cu are described to be essential in the aquatic environment because of their roles in several biochemical processes. They become detrimental, when present in high concentrations. The incorporation of heavy metals into food chains could lead to

accumulation in aquatic organisms to a level that affects their biological and physiological processes. Most of the heavy metals are known to be toxic and carcinogenic; they represent a serious threat to human flora and fauna of receiving water bodies. The high concentration of these metals also affects soil permeability and texture which leads to a productivity loss of soil. Metals also disrupt the enzyme function due to their tremendous affinity toward sulfur. Carboxylic acid (-COOH) and amino ($-NH_2$) groups of proteins are also chemically bound by heavy metals. Some metals hindering transport processes through the cell wall because it also binds with the cell membranes. Similar to physical-chemicals results, UV scanning spectra of PPMWw showed the presence of organics and nitrates in the PPMWw due to the presence of absorption maxima between 250 to 380nm and > 250nm, respectively (Fig.4.2a). Some picks were also observed after 380 nm which showed the turbidity of the effluent. This study showed that this PPMWw is not safe for irrigation, human and animals.

4.3. 2. Surface Morphological Studies through SEM-EDX Analysis

The images of SEM analysis of PPMWw upstream and downstream samples are shown in Fig. 4.4a_2 and b_2. SEM image indicated that the PPMWw was composed of irregular shapes which provide a large surface area for the adsorption of various pollutants along with heavy metals and lignin compounds. The underivatized lignin showed a granulated structure with grains or oval particles of lightweight in different sizes. The crystal shape showed the presence of different heavy metals and various particles. The crystal and irregular shape of metals and lignin have been reported recently (Liu et al., 2013; Batista et al., 2018). The EDAX analysis was also performed in both samples for the confirmation of elemental constituent in PPMWw (Fig. 4.4a1 and b1). Figure 4.4a1 and b1 indicates that the iron (12.10%, 11.43%), oxygen (70.44%, 64.32%) and silica (16.30%, 12.85%) content was in considerable amount followed by manganese and potassium. The high content of aluminum was observed in the sample might be due to aluminum stub used for gold coating of the sample. The images of SEM analysis of PPMWw upstream and downstream samples are shown in Fig. 4.4a_2 and b_2. Similar to physico-chemicals results, UV scanning spectra of

Table. 4.1. Physico-chemical characteristics of discharged Pulp Paper mill waste and their heavy metals content collected from M/s K R Pulp Paper Ltd. Shahjahanpur, Uttar Pradesh, India. All the values are means of triplicate (n=3) ±SD. Unit of all parameters are in mgl^{-1} except pH, color (Co-Pt Unit) and EC ($\mu mhoscm^{-1}$) Statistical significant between the value of both samples was evaluate with their respective control through ANOVA. Significant level a=P<0.001, b=P<0.01, c=P<0.05, ns =P>0.05

SN	Parameters	Wastewater site-1	Wastewater site-2	Permissible limit (EPA 2002)
1.	pH	8.4±0.42	7.9 ± 0.47^{ns}	5-9
2.	Colour	2345±143	2100 ± 105^{ns}	Dark Brown
3.	Total solid (TS)	1968±162	1611 ± 102^{ns}	-
4.	Total dissolved solid (TDS)	1789±42.56	1560 ± 31.25^{ns}	-
5.	Total suspended solid (TSS)	73±2.30	51 ± 3.21^{ns}	35
6.	Chemical oxygen demand (COD)	19100±754	17890 ± 821^{b}	120
7.	Biological oxygen demand (BOD)	4180±209	3653 ± 285^{b}	40
8.	Electrical conductivity (EC)	1762 ±86	1400 ± 84^{c}	1000
9.	Total Phenols	432±43.90	389 ± 22.23^{ns}	0.50
10.	Total nitrogen	234±5.76	125 ± 4.10^{ns}	143
11.	Ammonical Nitrogen			
12.	Sulphate	2098±89	1926 ± 97^{b}	250
13.	Phosphorus	173±5.98	150 ± 5.84^{ns}	200
14.	Cl^{-}	5.43±0.40	3.12 ± 0.20^{ns}	1500
15.	Na^{+}	331±15.87	294 ± 14.20^{ns}	200
16.	K^{+}	19.05±0.70	17.8 ± 0.80^{ns}	-
17.	Ca^{++}			
18.	Lignin	39000±1110	38950 ± 1124^{ns}	-
19.	Chlorophenol	431±12.76	311 ± 10.23^{a}	3.0
	Heavy metals			
20.	Iron (Fe)	87 ±1.89	79 ± 1.48^{b}	2.00
21.	Zinc (Zn)	34±1.35	22 ± 1.40^{a}	2.00
22.	Copper (Cu)	3.28±0.17	2.57 ± 0.19^{c}	0.50
23.	Cadmium (Cd)	1.90±0.09	0.36 ± 0.02^{a}	0.01
24.	Manganese (Mn)	16±0.77	15 ± 0.57^{ns}	0.20
25.	Nickel (Ni)	6±0.38	5 ± 0.34^{a}	0.10
26.	Chromium (Cr)	4±0.09	3 ± 0.08^{a}	-
27.	Lead (Pb)	41.23±2.15	36.54 ± 1.54^{c}	-

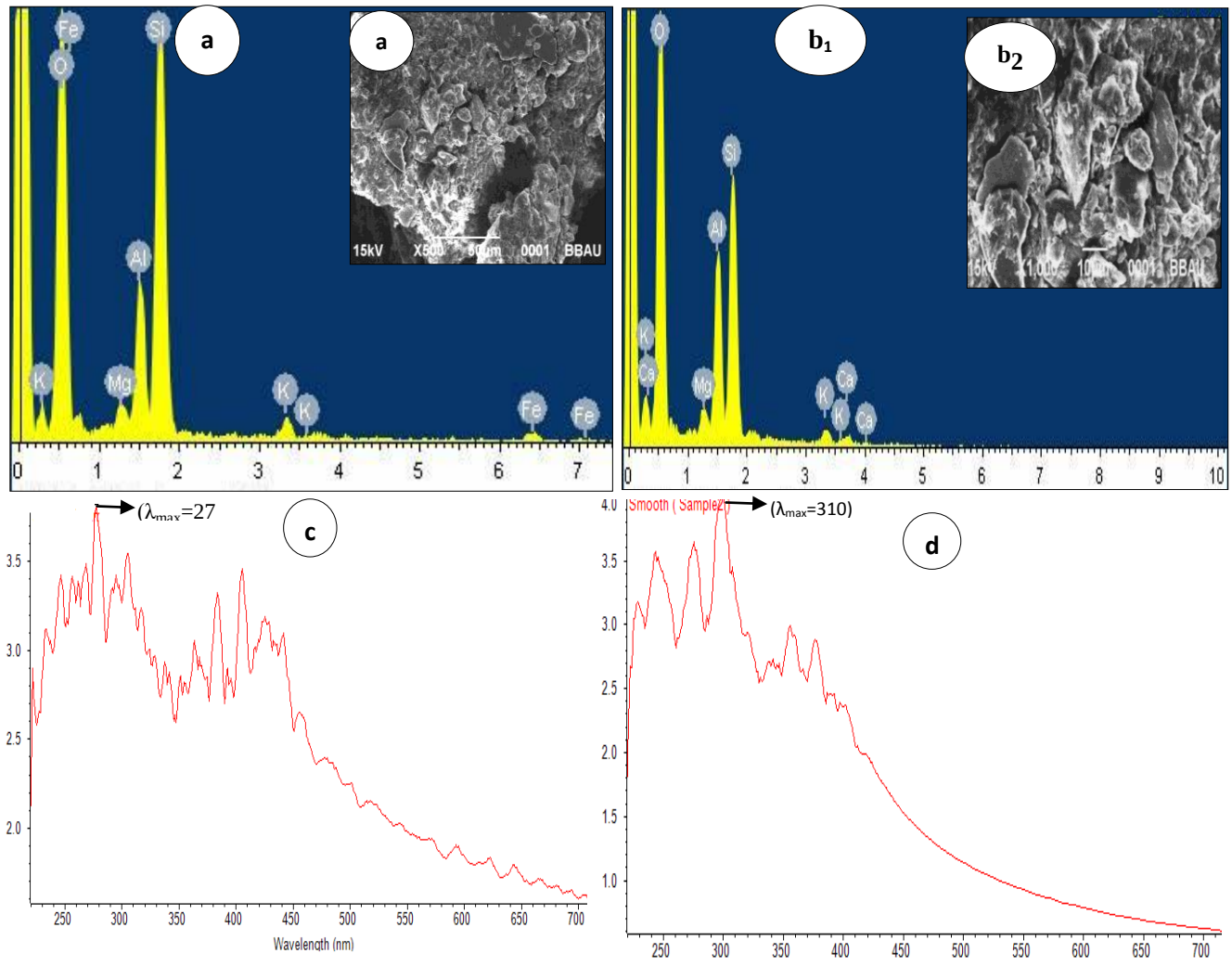


Fig 4.4. EDX, scanning electron microscopy of PPMWw collected from site 1 (upstream; $a_{1,}$ a_2) and site 2 (downstream; $b_{1,}$ b_2). UV spectra of upstream (c) and downstream (d) PPMWw

Upstream PPMWw and downstream PPMWw showed the presence of organics and nitrates in the wastewater due to the presence of absorption maxima between 270 to 310nm and > 250nm, respectively (Fig. 4.4c and d). In addition, the upstream PPMWw might have different stable peaks ranging from 270 to 450 nm, while downstream PPMWw could have different stable peaks ranging from 220 to 350 nm, respectively. Based on our results, the formation of various peaks in the UV and visible region revealed the presence of a complex mixture of organometallic compounds in upstream and downstream PPMWw (Yadav and Chandra, 2018). Some picks were also observed after 380 nm in both samples which showed the turbidity of

the effluent. This study showed that PPMWw is not safe for irrigation, human and animals.

4.3.3. FTIR analysis

FTIR spectra were obtained to detect conformational alterations and functional group content differences which contributed protein and root lipids from both plants tested. The average spectra in the 4000-600 cm^{-1} wavenumber region of control and treated samples. The spectra typically consist of several bands resulting from the vibration of various groups of proteins, lipids, carbohydrates, and nucleic acids, showing that the leaves are rich in biochemical components. The broad peak at 3399.9 cm^{-1} was assigned to O-H (H-bonded), usually broad, N-H (2°-amines) of proteins and polysaccharides/water and show alcohols, amines and phenols functional group with strong bond intensity. The peak at 2919.4 and 1645.9 cm^{-1} was due to O-H and C=C stretch asymmetric of carboxylic and alkenes group of strong and weak medium intensity. While the peak located at 3406.8 and 2924.5 cm^{-1} was due to N-H stretch and C-H stretching vibrations that are caused by lipids and functional groups present in amides and alkyl halides with strong intensity. Overall, the spectrum of control and PPMWw treated samples differ in the figure of the absorption spectrum, suggesting apparent changes in the structure and contents of biological components due to PPMWw treatment. Pollutants present in PPMWw might adhere and enter into the root cells then interact with DNA, RNA, protein, lipids, etc to change the biological component.

4.3.4. Assessment and Characterization of Metabolites

The GC-MS chromatogram of ethyl acetate extracted organic pollutants from the discharged PPMWw after secondary treatment collected from two sites is shown in Fig 4.5. The detail identified compounds at different retention time (RT) and relative abundance has been listed in Table 4.2 and 4.3. The GC-MS spectrum of ethyl acetate extract of PPMWw exhibited three major peaks at retention time 9.08, 12.89, 13.73. NIST library search shows a probability above 90%. At RT9.08, a compound is obtained which shows 91.10% similarity with Heptacosane. The molecular weight of this compound is 380.745 g mol^{-1}. At RT-12.89 a compound is obtained which shows 92.50% similarity with 3', 5'-Dimethoxyphenyl1, 8-Dibromo-4, 5-di isopropoxy anthraquinone-2-carboxylate. RT-13.37was showed a similarity of 90.08% with

Nonacosane. Its molecular mass is 408.80 g mol^{-1}. These compounds have been reported as toxic and recalcitrant (Kim et al., 2016). However, the GC-MS spectra of PPMWw of site 2 showed four major peaks at RT 13.73, 27.97, 30.81 and 36.48.A similar compound of site 1 was also detected in site 2 at RT 13.73 which shows 91.10% similarity with Nonacosane. Moreover, at RT 27.97 a compound is obtained which shows 95% similarity with Hexadecanoic acid, trimethylsilyl ester. The molecular mass of this compound is 328.612 g mol^{-1}. The compound of RT 30.81 showing the similarity of 94% with Octadecanoic acid, trimethylsilyl ester. However, at RT 36.48 a compound is obtained which shows a 60% similarity with 6-Cyclopentyloxy-2, 3-bis (hydroxymethyl)-1-(2-chloro-4-pyridyl)-7-methoxynaphthalene. Moreover, some minor picks were also obtained in both samples collected from different PPMWw contaminated areas. However, some compounds at RT 9.08, 12.89, 13.73 and 45.20 were also detected in both sites as Heptacosane; 3', 5'-Dimethoxyphenyl1, 8-Dibomo-4, 5-di isopropoxy anthraquinone-2-carboxylate; Nonacosane and Octadecane, 3-ethyl-5-(2-ethyl butyl)- (CAS), respectively. The GC-MS analysis of both sites showed the presence of N_2 containing the aromatic heterocyclic organic compound, alkane, aromatic organic compound, polycyclic aromatic hydrocarbon, organic compounds, aromatic carboxylic acid, fatty acid, phenolics in which most of the pollutants are toxic and mutagenic in nature. This study showed that the PPMWw consists of highly toxic organic pollutants that can cause significant damage to body organs and systems, such as the nervous, respiratory, circulatory, immune, reproductive, sensory and endocrine systems. This finding is corroborated with findings of Yadav and Chandra, (2018) in which they showed the presence of endocrine-disrupting chemicals in pulp and paper mill effluent contaminated sludge. At site 1,1-(2-trimethylsiloxy-1,1-dideuteriovinyl)-4-trimethylsiloxy-benzene (100%, RT= 13.73) was present in large amount followed by Heptacosane (76%, RT=9.08), 6-(4-Chlorophenyl)-2,5,5-triphenyl-5,8-6H-azeto [1,2a] [1,3] thiazolo [4,5-d] pyrimidine (58%, RT=7.23);3',5'-Dimethoxyphenyl1,8-Dibomo-4,5-diisopropoxyanthraquinone-2-carboxylate(47%, RT=12.89); N-Methylleuconolane (32%, RT=36.08);Stearic acid, 3-(octadecyloxy)propyl ester (CAS) (38%, RT=34.88) etc. While, at site 2 Hexadecanoic acid, trimethylsilyl ester (RT=27.97) was present in 76% followed by 6-Cyclopentyloxy-2, 3-bis (hydroxymethyl)-1-(2-chloro-4-pyridyl)-7-methoxynaphthalene (60%, RT=36.48),

Octadecanoic acid, trimethylsilyl ester (42%, RT=30.81), Nonacosane (37%, RT=13.73), Benzene acetic acid,3, 4-tris [(trimethylsilyl) oxy]-, trimethylsilyl ester ((30%, RT=27.61).Tetrabromo-2-(3-hydroxy-1,2-dihydro-quinol-2-ylidene)-2,3-dihydro-1H-benz[f]indene, Ergostan-6-one, 3,25-bis(acetyloxy)-5-hydroxy-(3a,5a)-CAS detected at site 2 was reported as EDCs. Moreover, EDCs are also known as exogenous agents that interfere with the synthesis, secretion, transport, action, and binding of natural hormones in the body that are responsible for the maintenance of development reproduction behavior and homeostasis (USEPA, 2012). The presence of endocrine disruptors in PPMWw that share sufficient structural similarity with the endocrine hormones to interact with animal endocrine receptors sites and trigger negative effects on reproductive success and long-term survival of sensitive aquatic populations. Aside from these pollutants ester of benzoic acid (RT=16.55) was found in the PPMWw of site 1 might be coming from the raw material used by industry because benzoic acid occurs naturally in many plants and serves as an intermediate in the biosynthesis of many secondary metabolites. However, as per several reports, it has been causing gastric pain, nausea, vomiting, and possible allergic reactions (USEPA, 2012).A series of n-alkanes from C16 to C29 was also detected in PPMWw. Nonacosane (C29, RT=13.73) was the most abundant (37%) followed by Octadecane, 3-ethyl-5-(2-ethyl butyl)-(CAS) (RT=32.13, 25%) and Hexadecane, 2, 6, 10, 14-tetramethyl-(CAS) (RT=22.91. The presence of several hydrocarbon pollutants in PPMWw is known to lead to several health and environmental impacts, which are of great concern for the environment and health. Benzene acetic acid or Phenylacetic acid (RT=27.61) was also detected in PPMWw which is a catabolite of phenylalanine. Phenylalanine is the precursor of lignin. Further, the peak at RT 34.88 was identified as Stearic acid, 3-(octadecyloxy) propyl ester (CAS) which has been reported as B and T cell lines toxicity, cancer, neurotoxicity, organ toxicity and irritation (Table.4.2).The phenolic compound such as 1-(5-Ethyl-tetrahydrofuran-2-yl)-3, 3-dimethyl-butane-2-one was also found in PPMWw at site 1 which has been reported as a highly toxic compound. Octadecanoic acid, trimethylsilyl ester of stearic acid was also detected in PPMWw. Most predominant fatty acids were Octadecanoic (C18), hexadecanoic (C16) and Propanoic acid (C3) was also detected in PPMWw. However, Hexadecanoic acid or palmitic acid was the most prevalent, hence in agreement with observations as Morrison and Akin, (2001) confirmed that palmitic

acid was the main fatty acid contained in fiber extracts of several flux cultivars. However, a very less amount of Aconoridine-Methyl Ether at RT= 20.22 was also detected in site 2 samples.

4.3.5. Effect on antioxidants

The effect of PPMWw on the *P. mungo L* and *A. cepa* showed less SOD activity 56.89 and 41.89 unit gram^{-1} fresh weight, respectively as compared to control of *P. mungo* (71.38 89 unit gram^{-1} and *A. cepa* (68.45 unit gram^{-1}). This might be due to the presence of harmful heavy metals and other POPs in PPMWw. However, SOD is usually present in plants and algae with a competent biochemical and scavenging mechanism, i.e. non-enzymatic and enzymatic antioxidants including CAT, APx and H_2O_2, SOD is usually found to regulate ROS concentrations in order to regulate toxicity under wide environmental stress. SOD could catalyze the decomposition and detoxification process of superoxide such as anion into radical oxygen, hydrogen peroxides and then transform it to surface level O_2 and H_2O. In addition, after exposure to PPMWw, the CAT activity in P. *mungo* (0.58 mmole) and A. *Cepa* (0.49 mmole) was also found less as compared to their respected control. CAT acts as the primary biomarkers for removing H_2O_2 produced during metal stress in the form of peroxisomes reported by Karuppanapandian et al., (2011). They scavenge the molecule of peroxide, i.e. H_2O_2 use ascorbate for photosynthetic machinery management and other damage. Similarly, APx content significantly decreased after treatment with PPMWw as a comparison to control. APx scavenges the peroxide molecule i.e. H_2O_2 using ascorbate for management of the photosynthetic machinery and other damage. Reduce the activity of APx also showed the failure of the detoxification machinery of potential plants. Hence, these observations showed that PPMWw adversely affected both tested plants.

4.3.6. Toxicity Assessment by Seed Germination Test with *P. mungo* L and *A. cepa*

A seed germination test, germination index (GI), inhibition of root elongation has been used as a rapid, reliable and reproducible technique to indicate the damaging effect of different industrial waste on plant growth (Yadav and Chandra 2018). Furthermore, GI combines effect on germination and root growth has proved to be very sensitive parameters, low toxicity of waste affect root growth and increased

toxicity affects germination. The responses of *A. cepa* and *P. mungo* to the toxicity of PPMWw in terms of root length were found different as shown in Fig 4.7. Root length of *A.cepa* (2.41 cm) and *P. mungo* (2.05 cm) treated with 20% PPMWw and their respected control 2.50 and 2.22 cm, respectively was almost the same. This showed a lower concentration of PPMWw may be growth supporting. This might be due to the presence of an essential element present in low concentration in the PPMWw at 20% dilution. While>20% of PPMW was significantly reduced the root length of both crops (Fig. 4.7). This could be due to the increased accumulation of toxic organic pollutants that impair seed germination. Further, it has been reported that high salt load and metals content act as an inhibitor for plant hormones (amylases, auxins, gibberlines, and cytokinins), which are mainly required for seed germination, seedling growth and development of plants, respectively (Bucker-Neto et al., 2007). This reduction was significant for *P. mungo* then *A. cepa* which showed a high sensitivity of *P. mungo* than *A. cepa.* The GI of *A.cepa* was found 76.22, 43.15, 35.62, 16.73 and 4.74 for 20, 40, 60, 80 and 100% PPMWw. The result showed a concentration-dependent GI reduction. Seed germination is reduced at higher concentrations of the effluent due to high osmotic pressure caused by high salt concentrations in the PPMWw. This observation corroborated with the earlier findings of Ilic et al., (2015) which showed the different concentrations of lead and the different degrees of permeability of seed coat led to a different degree of germination inhibition. Similarly, the seed germination index of *P. mugo* in different concentrations (20, 40, 60, 80 and 100%) of PPMWw was 73.63, 36.24, 28.63, 13.84 and 2.85 respectively (Fig.4.7a). Only a 9.06% decrease in GI was found at 20% PPMWw, but a significant decrease ($p<0.01$) of 96.45% was noticed at 100% of the effluent concentration in comparison to control. These results clearly indicate that the toxicity of effluent was increased as the concentration of effluent was increased.

4.3.7. Scanning Electron Microscopy of PPMWw Treated Roots

Exposure of 100% PPMWw with *A. cepa* induced morphological changes visually observed under phase-contrast microscopy and scanning microscopy are shown in Fig.4.6a. The surface of the untreated root as control of *A. cepa* showed a clear, smooth and intact root surface along with normal root cap (Fig.4.6a, A1 and B1). However, significant aberrations, fissures, and fractured tissues were observed on the

surface of roots treated with 100 % PPMWw (Fig. 4.6a A2-A4). Moreover, root cells were become crumbled, shrink and ruptured after treatment with PPMWw observed during SEM analysis (Fig. 4.6a B2-B4).

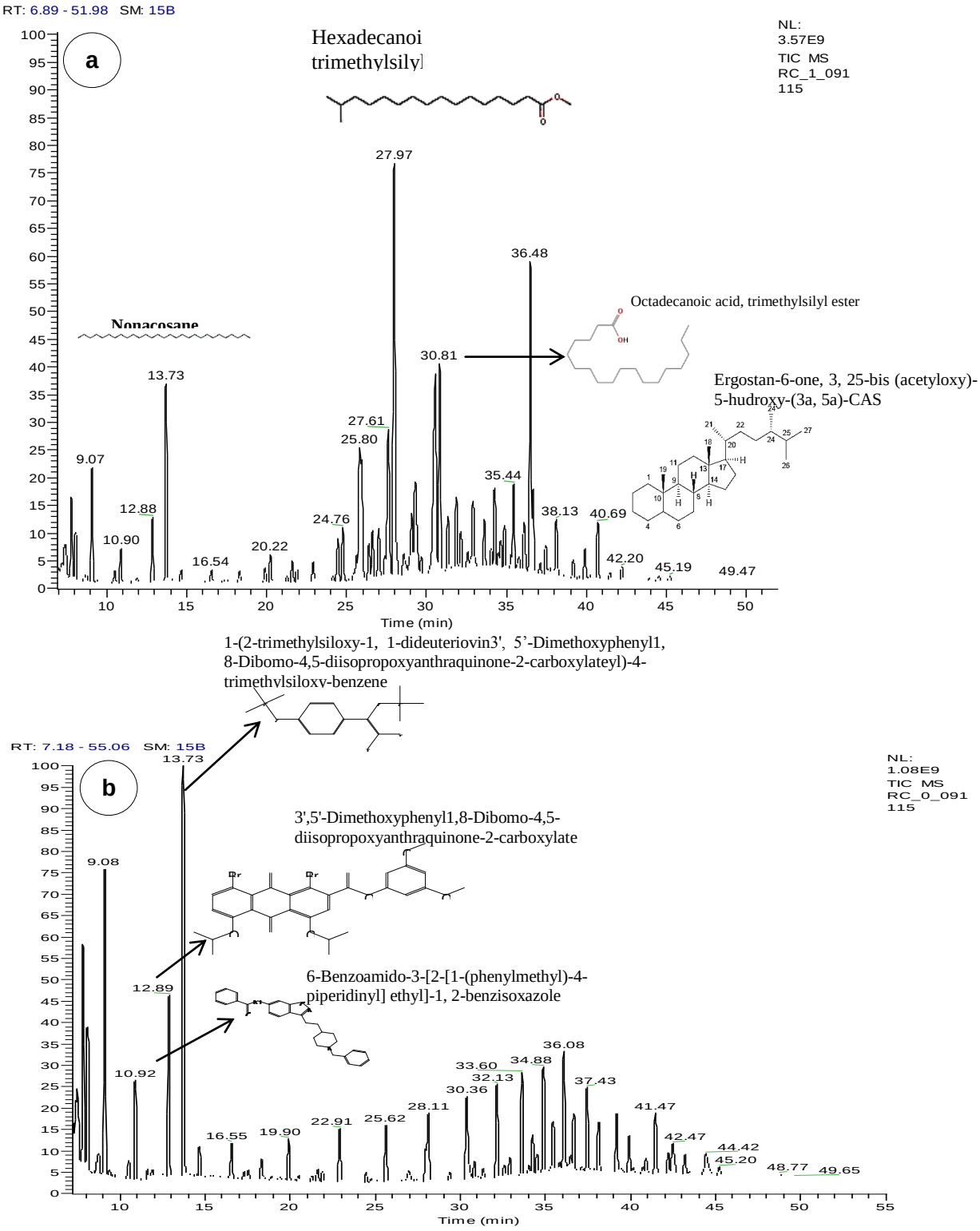


Fig. 4.5. Total Ion Chromatogram (TIC) of TMS derivative detected residual Organic Pollutants from ethyl acetate extract of PPMWw collected from site 1 (upstream) (a) site 2 (downstream) (b)

Table. 4.2. Identified Residual Organic Pollutants by GC-MS in the TMS derivative ethyl acetate extracts of PPMWw from the site- 1.

RT	Compounds	% with NIST Library	Nature of compounds	Toxicity
7.23	6-(4-Chlorophenyl)-2,5,5-triphenyl-5,8-6H-azeto[1,2a][1,3]thiazolo[4,5-d]pyrimidine	37.01	N_2 containing aromatic heterocyclic organic compound	Histopathological changes, mutagenic, and carcinogenic effects, POPs, effect on liver & immune system
9.08	Heptacosane	91.10	Alkane	Aquatic and terrestrial toxicity
13.73	Nonacosane	90.08	Organic compounds	Oral, dermal and inhalation toxicity
16.55	Benzoic acid,2-[(trimethylsilyl)oxy]-,methyl ester	37.60	Aromatic carboxylic acid	Gastric pain, nausea, vomiting, and possible allergic reactions
19.90	Hexadecanoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis	95.20	Fatty acid	DNA damage, human fibroblasts
22.91	Hexadecane, 2,6,10,14-tetramethyl- (CAS)		Alkane hydrocarbon	Irritation, CNS depression, and gastrointestinal tract irritation
25.62	1-(5-Ethyl-tetrahydrofuran-2-yl)-3,3-dimethyl-butan-2-one	47.30	Phenolics	Irritating and highly *toxic*
28.11	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	81.01	Alkane hydrocarbon	Irritation, CNS depression, and gastrointestinal tract irritation
30.36	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-	74.22	Fatty acid	Gastrointestinal *Toxicity,* eye and dermal irritation effects
32.13	Octadecane, 3-ethyl-5-(2-ethyl butyl)- (CAS)	86.23	Alkane hydrocarbon	Respiratory tract irritation, skin & Eyes eye irritation.
33.60	3-[3-(1,5-Dimethylhexyl)-7-(2-hydroxy-1-methylethyl)-3a,6,9b-trimethyl-2,3,3a,4,5,6,7,8,9,9b-decahydro-1H-cyclopenta[a]naphthalen-6-yl]propano ic acid, methyl ester	68.01	Fatty acid	Hemoglobinuria or myoglobinuria
34.88	Stearic acid, 3-(octadecyloxy)propyl ester (CAS)	78.20	Fatty acid	*Toxicity* B and T cell lines, cancer, neurotoxicity, organ *toxicity*, and irritation.
36.08	N-Methylleuconolane	48.40	-	*Metabolic acidosis, neurologic squeal, and even death,*
37.43	2,5-Bis(methylthio)-3-Phenyl-7-(6-phenyl-1,3,5-hexatrienyl)pyrazolo[1,5-a]pyrimidine	69.10	-	Fluorouracil toxicity
45.20	Octadecane, 3-ethyl-5-(2-ethylbutyl)- (CAS)	83.10	-	EDCs

Table.4.3 Identified Residual Organic Pollutants by GC-MS in the TMS derivative ethyl acetate extracts of PPMWw from site 2.

RT	Compound	% similarity with NIST Library	Nature of compounds	Toxicity
9.07	2,3-Dibromo-[1]benzopyrano[4,3-b]pyrrol-4(1H)-one	72.30	Aromatic heterocycle	Carcinogenicity
10.90	Tetrabromo-2-(3-hydroxy-1,2-dihydro-quinol-2-ylidene)-2,3-dihydro-1H-benz[f]indene	67.10	-	EDCs, Reproductive *toxicity*
12.88	3',5'-Dimethoxyphenyl1,8-Dibomo-4,5-diisopropoxyanthraquinone-2-carboxylate	53.30	-	Methemoglobinemia , Acute and chronic*inhalation*, Fatigue , weakness, dyspnea, headache
13.73	Nonacosane	85	hydrocarbon	Human disorder
16.54	Octadecane,2,2,4,15,17,17-hexamethyl-7,12-bis(3,5,5-trimethylhexyl)- (CAS)	93.01	Alkane	Very highly *toxic*
20.22	Aconoridine - Methyl Ether	44.20	Alkaloid	Carcinogenicity
24.76	Phthalic acid, butyl nonyl ester	48.30	ester	
25.80	1,2-Benzenedicarboxylic acid, butyl octyl ester	57.10	ester	Acute *toxicity*,
27.61	Benzene acetic acid, 3, 4-tris [(trimethylsilyl) oxy]-, trimethylsilyl ester	89.10	organic compound	Chronic oral *toxicity* study of *erythritol* in dogs.
27.97	Hexadecanoic acid, trimethylsilyl ester	95	saturated fatty acids	Subchronic or chronic
30.81	Octadecanoic acid, trimethylsilyl ester	94	saturated fatty acids	Acute Toxic
35.44	6-Chloro-2-methyl-1,2-dihydroisoquinoline-3-carbaldehyde	66.04	Aldehyde	Less *toxic* therapeutic agents
36.48	6-Cyclopentyloxy-2, 3-bis (hydroxymethyl)-1-(2-chloro-4-pyridyl)-7-methoxynaphthalene	63	organic compound	Harmful in contact with skin, Acute *toxicity*, dermal,
38.13	Glycocholic Acid Methyl Ester TMS	57	-	Toxic
40.69	Ergostan-6-one, 3,25-bis(acetyloxy)-5-hydroxy-(3a,5a)-CAS		-	EDCs, Genotoxicity, and sub chronic toxicity
45.19	Octadecane, 3-ethyl-5-(2-ethyl butyl)- (CAS)	61	organic acid	Gastrointestinal tract and through the lungs and almost completely oxidized by tissues.

Root cap was also damaged during PPMWw effluent treatment. This might be due to the presence of various Types of carcinogenic, mutagenic and androgenic compounds including heavy metals present in PPMWw. This type of pollutants can inhibit the auxin which plays a major role in root cap formation. This showed that PPMWw contained highly toxic and hormone-disrupting compounds.

4.3.8. TTC Assay for Root Viability

The TTC tests showed that the effects of PPMWw on root tips varied with different concentrations 20, 60, 80 and 100% applied and time of exposure (Fig. 4.6b). For 12-h treatment, all root tips were colored red including control which showed cells were viable at initial stages of treatment. But, the viability of root becomes reduced with time at all concentrations. Moreover, roots were completely dead after 72 hrs exposure to all tested concentrations of PPMWw except control. The living tissues have the capacity to reduce the TTC (colourless) into formazan (colored compound) by the enzyme dehydrogenases through H transfer during the respiration. Formazan is a non-diffusible stain so that living tissues have become red when incubated the tissue in the solution of this chemical. This result showed that the pollutants present in PPMWw were completely blocked the respiration lead to non-viability of *A. cepa* tissues.

4.3. 9. Chromosomal Aberration in Root of *A. cepa*

The qualitative effect of PPMWw on chromosomes at different phases of the cell cycle (prophase, metaphase, anaphase, and telophase) in untreated and treated root meristematic cells is shown in (Fig.4.8). The genotoxic study result reveals a concentration-dependent reduction of MI the *A. cepa* root tip meristem cells treated with 50 and 100% PPMWw. The MI, characterized by the total number of dividing cells in the cell cycle, and has been used as a parameter to assess the cytotoxicity of several agents. Both the reduction and the increase in MI are important indicators in monitoring environmental pollution, especially for the assessment of contaminants that have toxic and cytotoxic potential. The study has reported that trace metals and other organic pollutants were considered responsible for the diminished MI of *A. cepa* exposed to PPMWw. Further, the cytogenic effect might be due to the presence of Genotoxicity compounds in the PPMWw. The PPMWw induced chromosome abnormalities in *A. cepa*. The observed chromosome abnormalities included, disturb metaphase, c-mitosis, Chromosome Bridge, sticky chromosome, laggard chromosome, polyploidy cell, and apoptotic bodies**.** The explanation for such an impact might be due to the presence of toxic substances in the PPMWw which could disrupt the division causing a relatively high number of aberrations. The frequency of cells with laggard and stickiness chromosome significantly increased with increase PPMWw concentration. The number of histones or other proteins responsible for

controlling the proper structure of nuclear chromatin was disturbed by exposure of the organic pollutants. These observations are in conformity with previous reports of the detection of androgenic, mutagenic compounds present in distillery sludge (Chandra and Kumar, 2017). The reason for such an effect could be due to the presence of toxic substances in the PPMWw which may disturb the division, causing a relatively high number of aberrations. The frequency of cells with laggard and stickiness chromosome significantly increased with increase PPMWw concentration. The organic pollutants disturbed the balance in the number of histones or other proteins responsible for controlling the proper structure of nuclear chromatin. These observations are in conformity with the previous report of the detection of androgenic, mutagenic compounds present in distillery sludge (Chandra and Kumar, 2017). Chromosome stickiness reflects a highly toxic effect, probably leading to cell death (Leme et al., 2009).

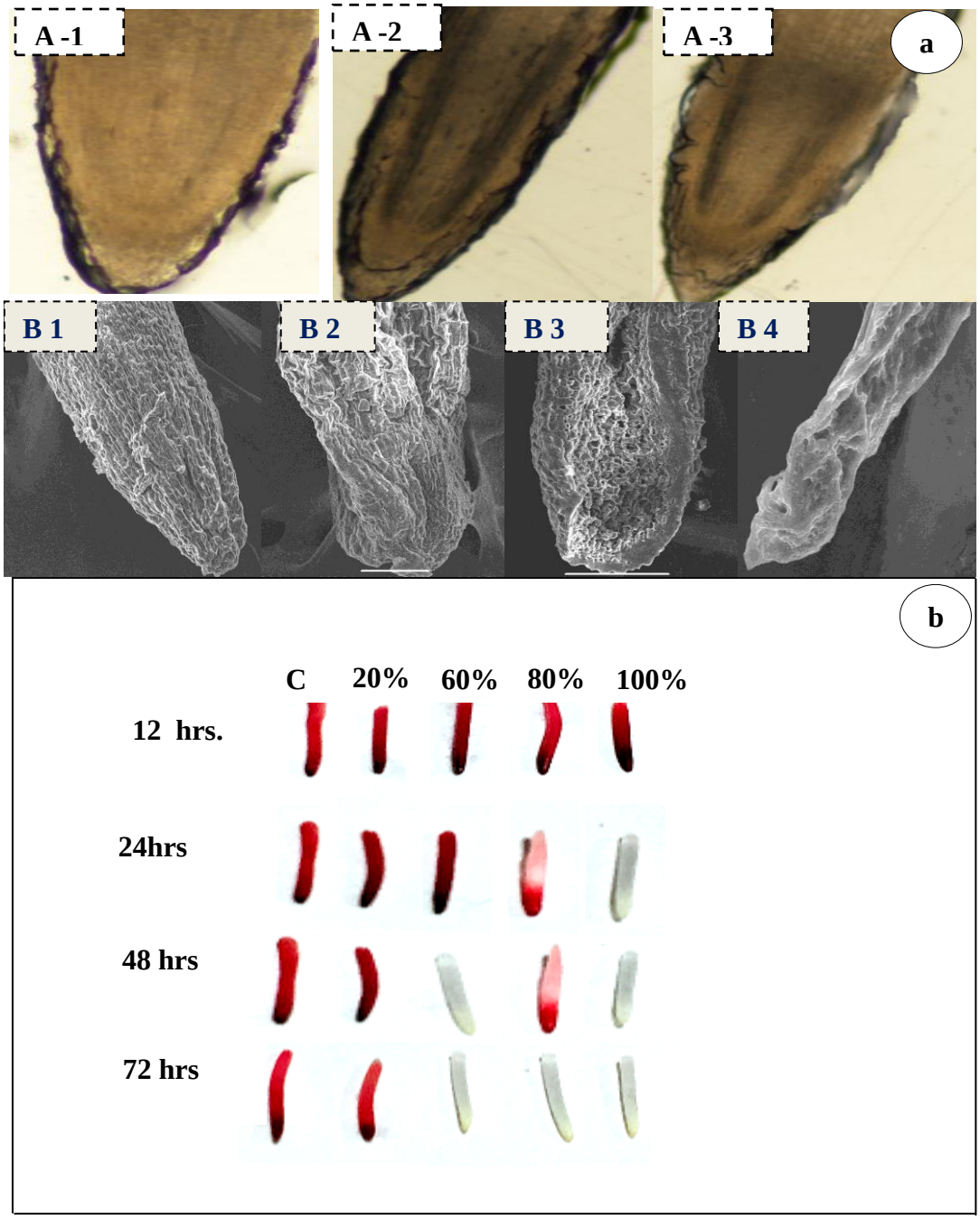


Fig.4.6. (a) Phase contrast (A1 to A3) and scanning electron microscopy (B1 to B4) of *A. cepa* roots treated with pulp and paper mill effluent along with control (A1, B1). Fissures and fractured tissues at root surface are shown by arrows and root cap damage with * (b) TTC test for different concentrations and time (hrs) of PPMWw by *A. cep*a root during treatment.

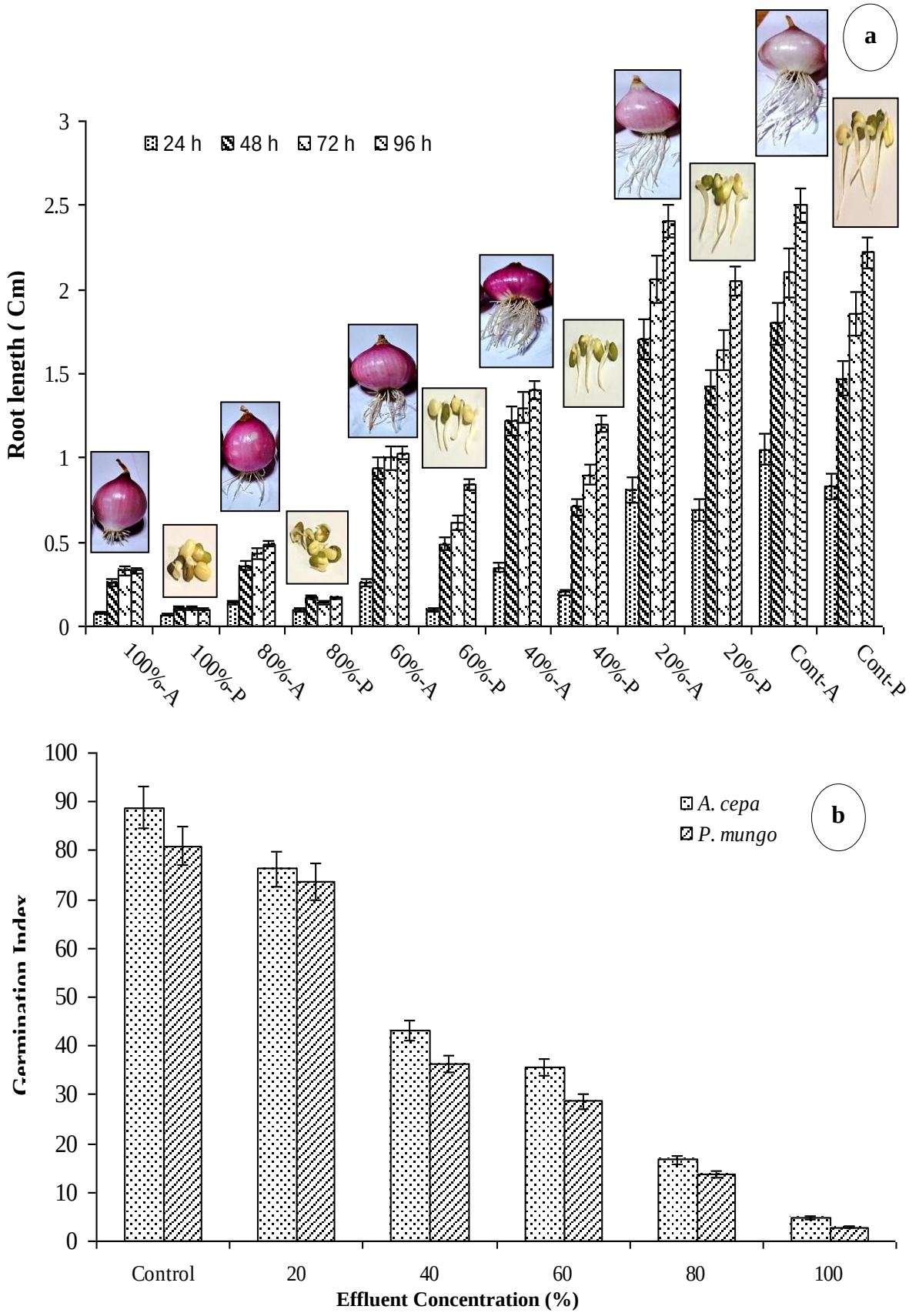


Fig.4.7. Effect of different concentration of PPMWw on root length (a) and seed germination index (b) of *P. mungo* L (P) and *A. cepa* (A). Inserted figures show the root length of *A. cepa* (upper) and *P. mungo* L (lower).

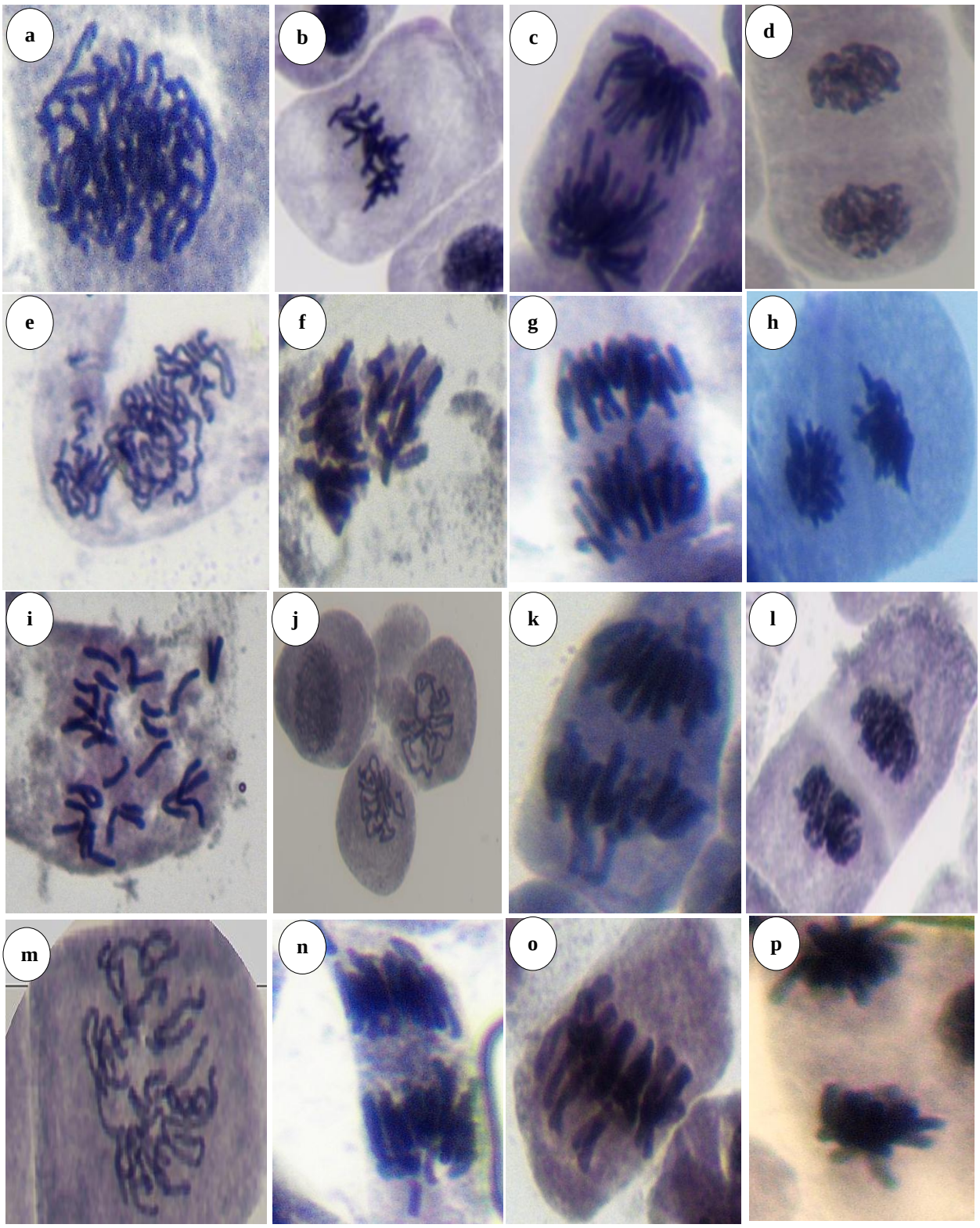


Fig.4.8. Microscopic analyses of chromosomal damage in A. cepa root meristem cells induced by 100% and 50% of PPMWw in A. cepa root meristem cells at different cell division stages of chromosome damage (a) Normal prophase, (b) Normal metaphase, (c) Normal anaphase, (d) normal telophase, (e) Metaphase with ring chromosome, breaks and gaps, (f) vagrant chromosome, (g) Irregular single chromosome bridge at anaphase, (h) Chained anaphase showing pulverization,, (i) chromosome fragment at metaphase, (j) lagging chromosome, (k) laggard chromosome, (l) Sticky anaphase ,(m) Scattered polyploidy cell, (n) shrink chromosome, (o) c-metaphase (p) sticky chromosome at anaphase

The improved stickiness also contributes to Chromosome Bridge being built. However, the frequency of cells with Chromosome Bridge significantly increased with increased concentration of PPMWw. It involves one or more chromosomes. Chromosome Bridge was originated from the dicentric chromosome at anaphase. This could have occurred due to the misrepair of DNA, telomere end fusions or even chromosome adherence. The most likely reason for the high genotoxicity and cytotoxicity of PPMWw in the complex assortment of organic pollutants present in raw material or produced during the pulping process. In addition, *Allium cepa* chromosomal aberration due to municipal water with constructed wetlands was also evaluated by Firbas and Amon (2013).

4.3.10. Toxicity Evaluation Through α-amylase Enzyme

Seed germination is a complex physiological and biochemical process in plants that can be affected severely by several environmental factors. Degradation of starch is essential for seed germination. In germinating seeds, starch degradation is initiated by α-amylase producing soluble oligosaccharides from starch. These are then hydrolyzed by α-amylase to liberate maltose and finally, glycosidase breaks down maltose into glucose providing energy to germinating seeds. α- amylase activity was measured in the *P. mungo* treated with different concentrations of PPMWw along with tap water as a control. Results showed that PPMWw inhibited amylase activity and thereafter a continuous decline in α-amylase activity was observed at the higher concentration at a different time interval. However, the seeds treated with tap water and 20% PPMWw have shown 0.56 and 0.53 unit gain^{-1}activity, respectively. While 100% PPMWw reducedα-amylase activity upto0.037 unit gain^{-1}.This revealed that the no adverse effect on amylase at lower concentration might be due to the presence of an optimum level of organic nutrients essential for plant growth. The reduction in amylase activity at a higher concentration of PPMWw might be due to the high pollution content affecting various physiological and biochemical processes during seed germination. Results indicated that the concentration of amylase enzyme (i.e. the intensity of the band) decreased gradually as the concentration of PPMWw increased as compared to control. A similar pattern was also detected on native PAGE which again showed a reduction in amylase quantity as the concentration of PPMWw was increased (Fig 4.9). A very light band of α-amylase was observed in the seeds treated with 100%

PPMW. Further, the analysis showed the presence of a single α-amylase with an estimated molecular weight of 67 kDa with standard proteins marker. Toxic pollutants concomitantly affected amylase activity

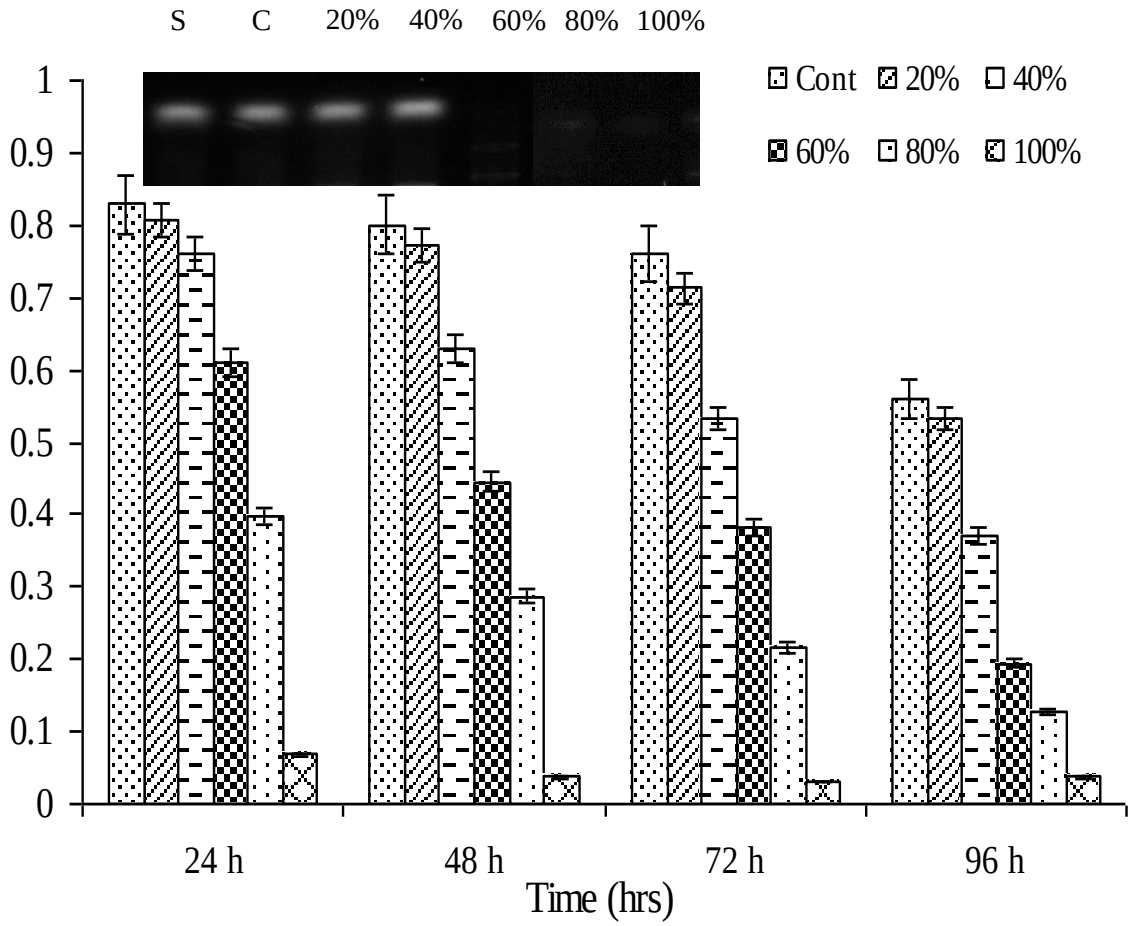


Fig. 4.9. α- Amylase enzyme activity in *P. mungo L*. with different concentration PPMWw. The inserted figure showed different band patterns of amylase protein of *P. mungo L*. treated with various concentration effluents on SDS PAGE. S: Standard amylase (bovine albumin; 67 kDa), C: control treated with tap water

Documented by previous reports in various plants (Bharagava and Chandra, 2010; Fendri et al., 2012).

Conclusions

This study revealed that discharged PPMWw contained Nonacosane(oral, dermal and inhalation toxicity);Heptacosane(aquatic and terrestrial toxicity); 6-(4-Chlorophenyl)-2,5,5-triphenyl-5,8-6H-azeto[1,2a][1,3]thiazolo[4,5-d]pyrimidine (mutagenic and carcinogenic); 6-Benzoamido-3-[2-[1-(phenylmethyl)-4-

piperidinyl]ethyl]-1,2-benzisoxazole; Hexadecanoic acid or palmitic acid (chronic); and Octadecenoic acid as residual organic pollutants after secondary treatment. This showed that these compounds are not degraded by bacterial communities in secondary treatment. Further, the study also showed that these detected pollutants may produce foul odors and induced hermaphroditism, Hepatotoxicants, carcinogens and endocrine disrupter for aquatic fauna. In addition to these persistent compounds, heavy metals (iron, copper, and zinc) beyond the permissible limits may contribute to bacterial growth inhibition and toxic vulnerability of effluent. The toxicity assessment through seed germination with *P. mungo* and cytotoxicity with *A. cepa* also showed inhibition of α-amylase and reduction in the mitotic index. Disturb metaphase, c-mitosis, Chromosome Bridge, sticky chromosome, laggard chromosome, and polyploidy cells lead to lethal effects on plant cells. The SEM analysis of PPMWw treated and untreated root of *A. cepa* showed cellular damage and fracture in tissue. This study also showed the concentration and exposure time-dependent environmental toxicity. Thus, this study concluded that PPMWw contains various mutagenic and endocrine-disrupting chemicals, which adversely affect the soil and aquatic ecology. Hence, this study recommended the tertiary treatment is essential prior to the discharge of waste in the environment.

Chapter-Five

Heavy metal accumulation pattern indifferent part of native plants growing at disposal site of pulp paper mill waste

Heavy Metal Accumulation Pattern in Different Part of Crop and Native Plants Growing at Disposal site of Pulp Paper Mill Waste

5. Introduction

Heavy metal pollution is a worldwide environmental issue due to indiscriminate discharged in the environment from various sources i.e. metallurgical work, pesticides, solvent, and industrial activity. Moreover, various agro-based industry i.e. tannery, distillery, and pulp paper industry are also reported as a notable source of heavy metal discharged above the permissible limit. Furthermore, the heavy metal remains a complex form indisposed of fresh sludge at the alkaline condition. But recently, the luxurious growth of some native plants on pulp paper effluent sludge indicated that the phytoextraction potential of various heavy metals from complex organo-metallic source and the bioremediation of complex hazardous sludge containing the mixture of different heavy metals (Chandra et al., 2016). In addition, lignocellulosic waste and humic substances have a powerful binding capacity with heavy metals that reduces the accessibility of metals to growing plant species. Previous studies also reported that pulp paper mill sludge (PPMS) pollution not only contributed toxicity to phytoplankton and zooplankton by enhancing the parameters of TDS, TSS, BOD, COD, and other organic pollutants along with metals (Chandra et al., 2018). The making of white papers are used by various hazardous dyes, bleaching agents, salts, acids, dichloroguicol, trichloroguicol, tetrachloroguicol, and chlorinated phenols are major contaminants and they cause delayed sexual maturation, cell damage and depletion of oxygen in aquatic ecosystem (Zahrim et al., 2007; Maria et al., 2002)**.** With the exception of non-hyperaccumulator plants that maintain most of the heavy metal taken from the soil in root cells, detoxify them by cytoplasm chelation or store them in vacuoles, hyperaccumulator convert these elements quickly and effectively to the shoot via the xylem (Rascio and Navari, 2011). Some of these heavy metals are not essential since they do not perform any known physiological role in plants, such as Hg, Cd, As, Pb or Se, but PPMS contains all metals, so their remediation is a concern. In addition, plants

use a sequence of defense mechanisms to regulate and detoxify its absorption, accumulation, and translocation of these hazardous metals by excluding free ionic forms from the cytoplasm. The detoxification mechanisms of hyperaccumulator plants mainly consist of metal complexation with receptors and their extraction from metabolically active cytoplasm by transferring them to inactive compartments, primarily vacuoles and cell walls (Chandra and Kumar, 2017). Some researchers recently reported that the newly identified wild plants as potential hyperaccumulators, for example, *Prosopis* sp. (Aldrich et al., 2003) and *Salsola kali* and *Salsola kali*. The knowledge of heavy metals for in-situ phytoextraction is still unknown through the native hyperaccumulator; hence the immediate need for these studies is to investigate the heavy metal phytoextraction potential by native hyperaccumulator plants growing on chlorolignins sludge. The bioavailability of the metals is above the permissible limits, a major problem for crop quality, food chain and its harmful impact on human health (Mani et al., 2014a). This manuscript focuses on the remediation of heavy metals by native hyperaccumulator plants and their possible conversion into the food chain from the PPMS.

5.1. Material and method

5.1.1. Sample collection

Sample collected from M/s K.R. pulp paper mill, Shahjanpur, Uttar Pradesh. Collection of native six native plants based on luxuriantly growing native plant species i.e. *Tribulus Terrestris* (Zygophyllaceae), *Parthenium hysterophorus* Asteraceae), *Momordica doica* (Cucurbitaceae), *Alternanthera sessilis* (Amaranthaceae), *Cannabis sativa* (Cannabaceae), and *Calotropis procera (*Apocynaceae*)*, were collected based on their abundant number. Collected plant sample washing with calcium chloride solution thoroughly to remove the adherent sludge particles and after were washed the plant root was cut into small pieces and fixed into the 2.5 % glutaraldehyde. For confirmation and authenticity of scientific data, this process has been repeated three times in different seasons from the same location.

Fig 5. 1. View of the polluted site of the pulp paper industry. (a) Collection of plant and sludge (c and d) growth of native hyperaccumulators plant near the contaminated site of pulp paper industry sludge

5.1.1.2. Physico-chemical Assessment

To evaluate the pollutant load in PPMS, all Physico-chemical analysis i.e. EC, pH, TDS, TSS, BOD, COD, total phenols, total nitrogen, ions i.e. Na^+, K^+, Cl^-, potassium, chlorophenols and heavy metals was estimated within 48 h as per described method in APHA, 2012.

5.1.1.3. Scanning Electron Microscopy and Energy-Dispersive X-ray Spectroscopy Analysis

For SEM analysis, PPMS samples were completely dried at 50°C in the hot air oven (Thermo scientific) and converted into powder form for analysis were described the method by Yadav and Chandra (2018). Moreover, samples were finally mounted on aluminum stubbing with double-sided carbon tape, sputter-coated with platinum coater (Auto Fine Coater JFC- 1600 JEOL, Japan). The sample was analyzed by JEOL JSM 6490 LV (Tokyo, Japan) scanning electron microscope at different magnifications and

accelerating 15 kV voltages. For the element composition analysis in the sludge sample, a selected area was investigated by SEM with an EDAX system JEOL JSM 6490 LV (Tokyo, Japan).

5.1.1.4. UV-Vis Spectral Analysis and FT-IR

To observe the absorbance parameter of different organic pollutants present in the PPMS. The scanning of absorption spectra was done in between 200-700 nm at room temperature (Thermo Fisher Scientific Shanghai spectrophotometer Evolution 2001, China) (Yadav and Chandra, 2018). For FT-IR analysis the sample of PPMS centrifugation and washed twice using double distilled water and pellets were dried for 6 hrs in the oven at 65°C to remove moisture content. The 100 mg of potassium bromide (KBr) and 1 mg of sample were mixed (KBr, 1:10). The KBr based pellet was compressed into a thin disk using a hydraulic press (CAP–15T) by establishing 15 tons pressure. The disks were fixed in an FT-IR spectrometer (Thermo-Nicolet 6700) and analyzed in the spectral region 4000-400 cm^{-1} with KBr pallets.

5.1.2. Extraction and characterization of Residual Organic Pollutants

5.1.2.1. Solid-liquid extraction

The organic compounds and other EDCs compounds present in PPMS were extracted from sludge using dichloromethane (DCM) to identify a wide range of organic compounds present in PPMS before and after phytoextraction (Chandra and Kumar, 2017a). The fresh pulp paper sludge sample (10 g) was weighed and put into an Erlenmeyer flask (500 ml), added 25 ml of DCM in each flask separately and mix vigorously and separation of solvent with an organic compound. The extraction of organic compounds from sludge has been repeated three times for the authenticity of data. Since dissolving the dry residue collected in 2.0 ml DCM and filtering through 0.22 μM syringe filters Millipore Ltd., Bedford, Massachusetts, USA), the sample was finally made up of 3.0 ml for GC-MS analysis.

5.1.2.2. Characterization of organic pollutants through GC-MS

After the extraction samples 200 µl) were transferred into GC vials and dry with nitrogen gas. For the organic compounds, characterization was performed by adding 50 µl of pyridine to the sample and then silylating it with 80 µl BSTFA and TMCS. Furthermore, the sample mixture was heated with intermittent shaking at 70 ° C for 30 minutes, after which it was subjected to GC-MS analysis. The organic pollutants were identified by comparing their mass spectra (*m/z*) with recorded at different retention times (RT) in the NIST library (Chandra et al., 2011).

5.1.2.3. Digestion of plant for Metal estimation

To estimate the concentration of different heavy metals in the aerial parts of the potential native hyperaccumulator plants. The plants were subsequently segregated into roots, shoot and leaves and cut into small pieces. The sample was incubated at 70ºC for 7 days for dry. The dried sample was then ashed in a muffle furnace at 460ºC for 6 h. The weighed ash from these samples was digested in 2% HNO_3 and filtered through a 0.45 µM glass fiber filter (AOAC, 2002). Five gram of dried sample was digested with 10 ml 2% HNO_3. When brown fumes longer appeared, added 5.0 ml of HNO_3 and digestion continued until brown fumes were gone method by 3050-B (EPA, 1996). The concentrations of heavy metals in pulp paper sludge before and after phytoextraction i.e. Mn, Pb, Cd, Zn, Cr, Fe, Cu, Ni and As were measured by AAS (ZEEnit 700 Analytical Jena, Germany).

5.1.2.4. Bioconcentration factor

The Bioconcentration factor (BCF) was determined as the following formula to measure the in-situ phytoextraction potential by increasing native hyperaccumulator plants which were also mentioned earlier by Yoon et al., (2006).

$$BCF = \frac{\text{The Metal concentration of plant roots}}{\text{The Metal concentration of sludge}} \text{ X100 ..... (i)}$$

5.1.2.5. Translocation factor

The translocation factor (TF) is evaluating the metabolism and health activities of native plants growing on contaminated PPMS site. TF of metals was calculated by the ratio of metal concentration in plant shoot and metal concentration in pulp paper sludge growing plant root as mentioned in previous studies Gupta et al., (2008).

$$TF = \frac{\text{The metal concentrations in plant shoot}}{\text{The metal concentrations in plant root}} \quad X100 \ldots\ldots\ldots\ldots\ldots\ldots \text{(ii)}$$

5.1.2.6. Estimation of antioxidants and Microscopic observation of stomata

For the estimation of antioxidants enzymes from collected native hyperaccumulator plants, in 3 ml of 100 µM potassium phosphate solution, the fresh leaves 250 mg are homogenized (pH 7.5) containing 1mM of ETDA and a pinch of polyvinylpolypyrrolidone (PVP).The sample was centrifuged for 10 min at 4°C at 12,000 rpm and the extract was used to estimate antioxidant enzyme activity. For the estimation of superoxide dismutase (SOD) assay was done by using the enzyme extract method of Nishikimi and Yagi, (1972). After 15 min incubation, optimum density (OD) was recorded at 560 nm. While the activity of the Ascorbate peroxidase APX enzyme was measured method of Nakano and Ascada, (1981). In APX enzyme the oxidation of ascorbate in the presence of H_2O_2 was observed at 250 nm in terms of decreases in absorbance. The CAT activity monitoring by spectrophotometrically at 37ºC the decrease in absorbance at 240 nm resulting from the decomposition of H_2O_2 as per the previously described method of Chance and Maehly, (1955). For the estimation of H_2O_2 content, the fresh plant leaves 250 mg were homogenized in 2 ml of 5% TCA and centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was appropriately diluted in a test tube containing 200 µl of potassium phosphate buffer 10 mM) and 500 µl of 1M KI solution and mixed vigorously and absorbance was recorded at 350 nm as per the described (Velikova et al., 2000). For the estimation of malondialdehyde (MDA) content in native hyperaccumulator was used 200 mg fresh plant leaves and chopped in 5 ml of 0.1% trichloroacetic acid (TCA). Furthermore, the samples were centrifuged at 10000 rpm for 12 min at 4°C. The supernatant was collected and mixed with 5 ml of 0.5% of

thiobarbituric acid (TBA) and 20% TCA solution Heath and Packer, (1968). The solution is mixing proper and heat at 95°C for 30 min and put in an ice bath and again centrifuge at 10000 rpm for 10 min at 4°C. The supernatant was collected and absorbance was measured at 532 nm. In addition, the Observation of stomata condition at the contaminated site of PPMS by light microscope from collected native plants to prepare the sample for stomata observation the leaves peel was removed from the upper surface of the leaves preserved in a watch glass containing distilled water. Consequently, after 3-4 minutes it was taken out from watch glass and placed on a clean glass slide. The observation of stomata was done under the phase-contrast microscope at low power magnification.

5.1.2.7. Histological observations of root tissues by TEM

For the histological observation of heavy metal accumulation inside the plant root tissue, root tips. The plant roots were cut approximately 2.0 mm for fixed 2.5 % glutaraldehyde solution (Khan et al., 1984). Fixed root dehydrates with acetone in different concentrations (30, 50, 60, 70, 80, 90, 95, and 100%) Araldite-DDSA (Ladd Research Industries, Williston. TEM Section observation was performed under TEM (FEI Tecnai TM G2 Spirit Twin, Hillsboro, USA) with an 80 KV voltage velocity.

5.1.3. Statistical analysis

In-situ phytoextraction of heavy metals by various native plants as hyperaccumulator compared with the original PPMS. The calculation was done using was calculated using Student t-test ($P < 0.001$) by comparing it with the control plant sample. The mean concentration of metals in the statistical analysis of the plant using the SPSS analytical program (version 17.0; SPSS Inc., Chicago, IL, USA) to confirm the quality of the data obtained and the accuracy of the tests.

5.2. Results and Discussion

5.2.1. Physico-chemical characteristic

The physicochemical characteristics of PPMS showed the presence of the organic and inorganic pollution load along with alkaline pH (8.0) containing nitrogen, sulfate, and phosphorus as shown in Table.5.1**.** Due to the presence of various organic pollutants along with heavy metals before and after in-situ phytoextraction of sludge which was found above then the permissible limit and contributed toxicity to PPMS. TDS is found to be a high amount before the phytoextraction but after the phytoextraction, the TDS is reduced. The source of TDS is dissolved of cellulosic material during the alkali pulping (USEPA, 2002). The waste of pulp paper industry is shown a high level of COD and BOD because during the biological treatment some of the low molecular weight and high molecular weight compounds are broken down and they can increase the concentration of COD and BOD in effluent and reduce the oxygen level in aquatic system. The EC value of the PPMS was very high than the EPA guidelines i.e. 1000 µmhos cm^{-1}.While, the Higher EC might be due to salt and ions content of effluent (Deepali et al., 2009). Moreover, lignin and phenolic compounds come directly from plant material during the chemical treatment and thy dissolved in effluent at high pH and also contribute the whole waste toxicity (WWT) in the pulp paper mill. While phenols are very harmful to aquatic organisms even at relatively low concentrations and inhibition of photosynthesis of microorganisms. Sodium is an important cation that occurs in less than 20 ppm in all-natural freshwater (EPA, 2002). Moreover, the content sodium and phosphorus were observed above the permissible limit, which might be released from the bleaching and pulping process. The properties of Chlorophenol are tending to absorb in soil, sludge, and sediments due to their lipophilic properties (De Morais et al., 2012). In addition, the before and after phytoextraction of sludge showed the concentration of the metals was reduced i.e. Fe, (95.35-47.23) Zn (48.40-32.01), Cu (3.28-1.03), Cd (9.36-5.32), Mn (19.00-11.23) and Ni (4.24-2.41 mg L^{-1}), which are above the permissible limit. The presence of heavy metals might be from the alkali pulping and bleaching process in industry and bioaccumulation by plants which are used as raw materials (Chandra et al., 2011). Since the metals have strong binding properties with lignocellulosic materials a cationic

molecule. Therefore, during the treatment of waste, some organic pollutants might be degraded and metals have been released as residual pollutants (Chandra et al., 2009).

5.2.2. SEM and EDX investigation

The SEM images analysis of the PPMS showed the structure of the lignocellulosic organic polymer and organometallic compounds along with metals are shown in Fig. 1a-b. The result showed complex irregular structure mixed with an elongated rod or cylindrical shaped bodies (Fig.5.2) the organic polymer i.e. lignin along with a heterogeneous compound on the surface of sludge. The rod shape structure might be cellulose present in PPMS. A similar observation for the granulated appearance of lignin with the complete structure of different size has been reported in an earlier study (Liu et al., 2013). Furthermore, the irregular shape is also indicating the lignin complexation with different heavy metals and another carbonyl, hydroxyl and phenolic compounds (Demirbas, 2007). The EDX analysis showed the different toxic elements in the sludge shown in Fig. 5, c-d. The figure also represented that there was an inconsiderable amount of iron (1.99-3.86), oxygen (64.52-51.83), Mg (1.36-1.48), Al (10-9.27) and silica (17.21-28.90) content in effluent and sludge of PPMS. The presence of a higher amount of different elements reported in the previous study by hazardous metals i.e. Fe, Cu, Zn are absorbed in groundwater and would affect human health. These findings collaborate with the previous study of PPMS from another site (Yadav and Chandra, 2018).

5.2.3. UV-Vis Spectral Analysis and FT-IR

UV-Vis spectral wavelength range of 250-700 nm analysis is asses the availability of dissolved organometallic compound present in sludge before and after phytoextraction is used method (Martins and Boekel, 2003). The result revealed that indicate of high of different peak absorption in UV-region and their maximum absorbance was noted λ_{max} 310 on before phytoextraction in sludge as shown in Fig. 5.3.c. While some peak disappeared and showed new peak maximum absorbance was noted λ_{max} 320 after phytoextraction as shown in Fig. 5.3 c. The change of peak area and height is indicating the conversation of compounds into complex metabolites (Chandra et al., 2018). Furthermore, FT-IR spectra cover a wide range of functional groups with strong and weak bonds of organic compounds and polymers from two different samples i.e. SS-1

(sludge before phytoextraction) and SS-2 (sludge after phytoextraction). To reveal the presence of a different functional group of various organic pollutants present in PPMS. The FT-IR analysis was one in the range of IR region from wave number 4000 to 500 cm^{-1} as shown in Fig. 5.3 c.

Table.5.1. Physico-chemical characteristics of discharged PPMW along with heavy metals content collected from M/s K R Pulp Paper mill Ltd. Shahjahanpur, Uttar Pradesh, India. All the values are means of triplicate (n=3) ±SD. The unit of all parameters is in mg L^{-1} except pH; color (Co-Pt. Unit).

Parameters	**Sludge Values (mean) before phytoextraction**	**Sludge Values (mean) after phytoextraction**	**Permissible limit (EPA 2002)**
pH	8.4±0.24	6.4±0.21	5-9
Color	2340±105	1647±103	Dark Brown
TS	1618±108	853±1.11	-
TDS	1430±31.25	840±0.10	-
TSS	73±1.21	41±3.14	35
COD	24670±254.00	14260±1.24	120
BOD	8974±172	3574±85	40
EC	1570 ±84.00	652±20	1000
Total Phenols	456±22.23	254±35.10	0.50
Total nitrogen	183±4.10	89±0.21	143
Sulphate	2137±09.70	1241±0.01	250
Phosphorus	161±5.84	46±0.04	200
Cl^-	4.51±0.20	3.24±1.11	1500
Na^+	331±11.20	154±10.11	200
K^+	19.8±0.80	14.01±3.11	-
Lignin	42100±114.21	21420±2.14	-
Chlorophenol	324±10.23	84.78±1.14	3.0
Heavy metalsB			
Iron (Fe)	95.35±1.89	47.23±0.41	2.00
Zinc (Zn)	48.40±0.40	32.01±0.12	2.00
Copper (Cu)	3.28±0.07	1.03±0.94	0.50
Cadmium (Cd)	9.36±0.01	5.32±0.21	0.01
Manganese (Mn)	19.00±0.27	11.23±1.11	0.20
Nickel (Ni)	4.24±0.04	2.41±1.22	0.10

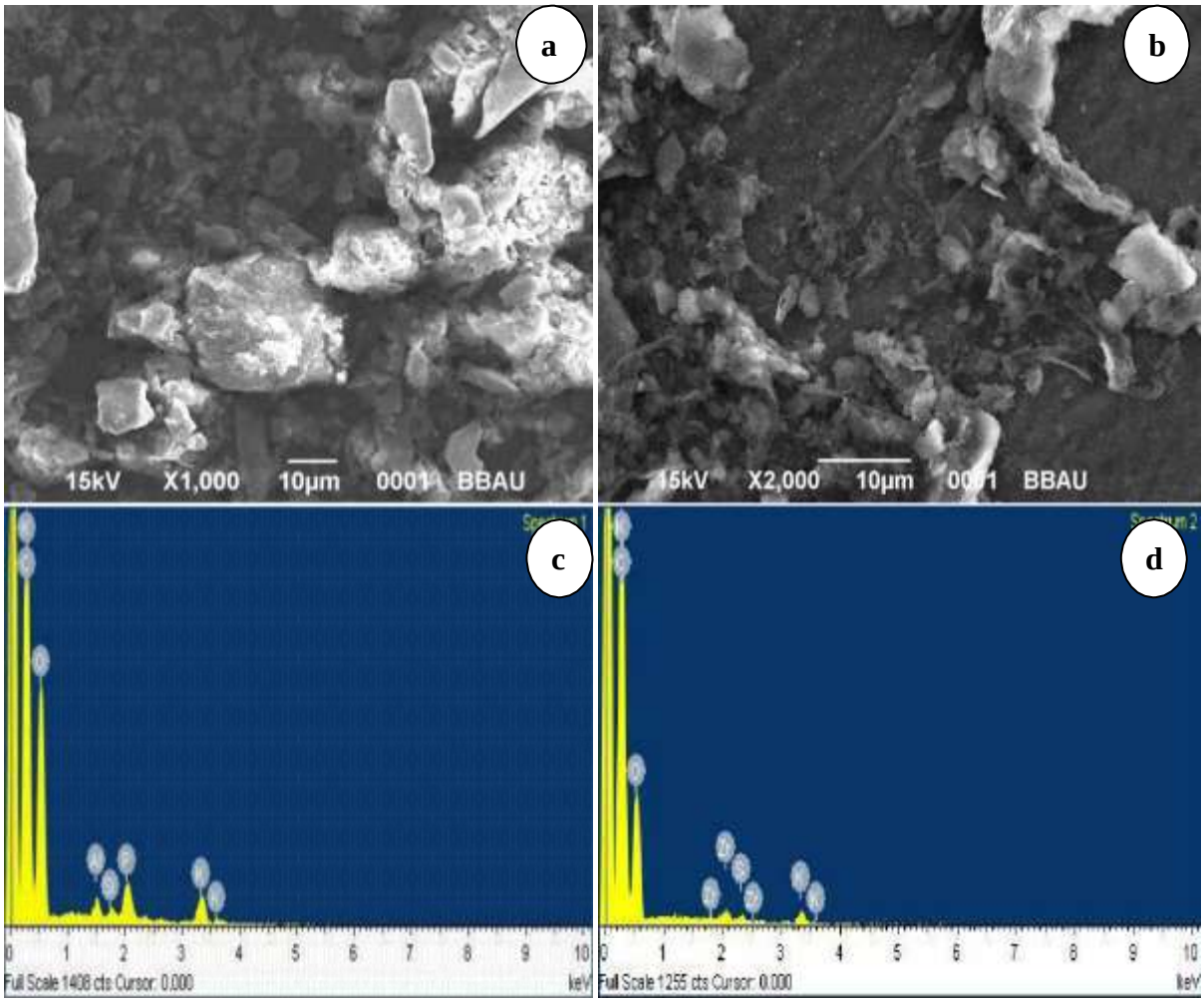


Fig.5.2**.** SEM and EDX analysis of PPMS (a) effluent (b) sludge, EDX analysis of circle area of the figure is effluent and sludge

In addition, the result showed SS-1 broadband was noted at 3436 cm^{-1} absorptions is containing functional group alcohol and phenol bonds are O-H with strong intensity indicates the presence of various organic compounds in PPMS. The broadband region 2923 cm^{-1} absorption containing functional group alkanes C-H, alkyls C=C and alkene and bonds is strong and often weak in nature showed the fatty acid present in PPMS.

The contain of different fatty acid is a by-product of the pulping process during papermaking. While 1021cm^{-1} absorption is contained functional group is sulfur S=O sulfoxide and ether linkage =C-O-C system, and bonds are strong and medium-strong. Ether deformation is grafting of epichlorohydrin onto the lignin from the pulp paper industry. Furthermore, the comparative analysis of SS-2 is near the plant root sludge sample is showed the broadband i.e. 3480,781 and 681. The peak was decreased at 3480 cm^{-1} is related to the functional group of O-H is alcohols and organic acids are noted. The broadband at 687 cm^{-1} functional group is aromatic compounds bonds is Ar C-H stretch is medium strong while alkynes bond is a =C-H stretch is strong and sharp. The FT-IR results indicate the change of function group because of the exchange of metabolites during in-situ phytoextraction by native hyperaccumulators.

5.2.4. Identification of organic compounds

The GC-MS analysis of extracted organic pollutants from the discharged PPMS after secondary treatment before and after phytoextraction has shown the presence of different lignocellulosic metabolic product as shown in Fig.5.4a-b. All of these persistent compounds might be generated either during the alkali pulping process with Na_2S or during the bacterial biotransformation and during effluent treatment. The GC-MS chromatogram of main hazardous compounds before phytoextraction in sludge at major RT 9.06, RT 10.90, RT 13.73, RT 20.55, and RT 27.96 were identified which listed in Table 2. Out of these, some were with EDCs nature compound identified as 2-Methyl-4-keto-2-pentane-2-of TMS (RT 9.06), octadecanoic acid (13.73), trimethylsilyl ester, Hexadecanoic acid, trimethylsilyl ester (RT 10.90), and tetradecanoic methyl ester (RT 27.64) is reported as mutagenic. However, these compounds are identified as endocrine-disrupting chemicals in the United States EPA and EDSP, (2012). Octadecenoic acid was detected from *Eucalyptus camaldulensis* and it is also reported as extractive of ethanol or benzene (Peng and Wu, 2008). The tetradecanoic acid and hexadecanoic acid were essentially plant-based fatty acids in humic substances which were also reported by Reveille et al., (2003). Moreover, Eicosane (CAS) (RT 37.45) detected from sludge before the phytoextraction was also reported as EDCs nature by USEPA, (2012).

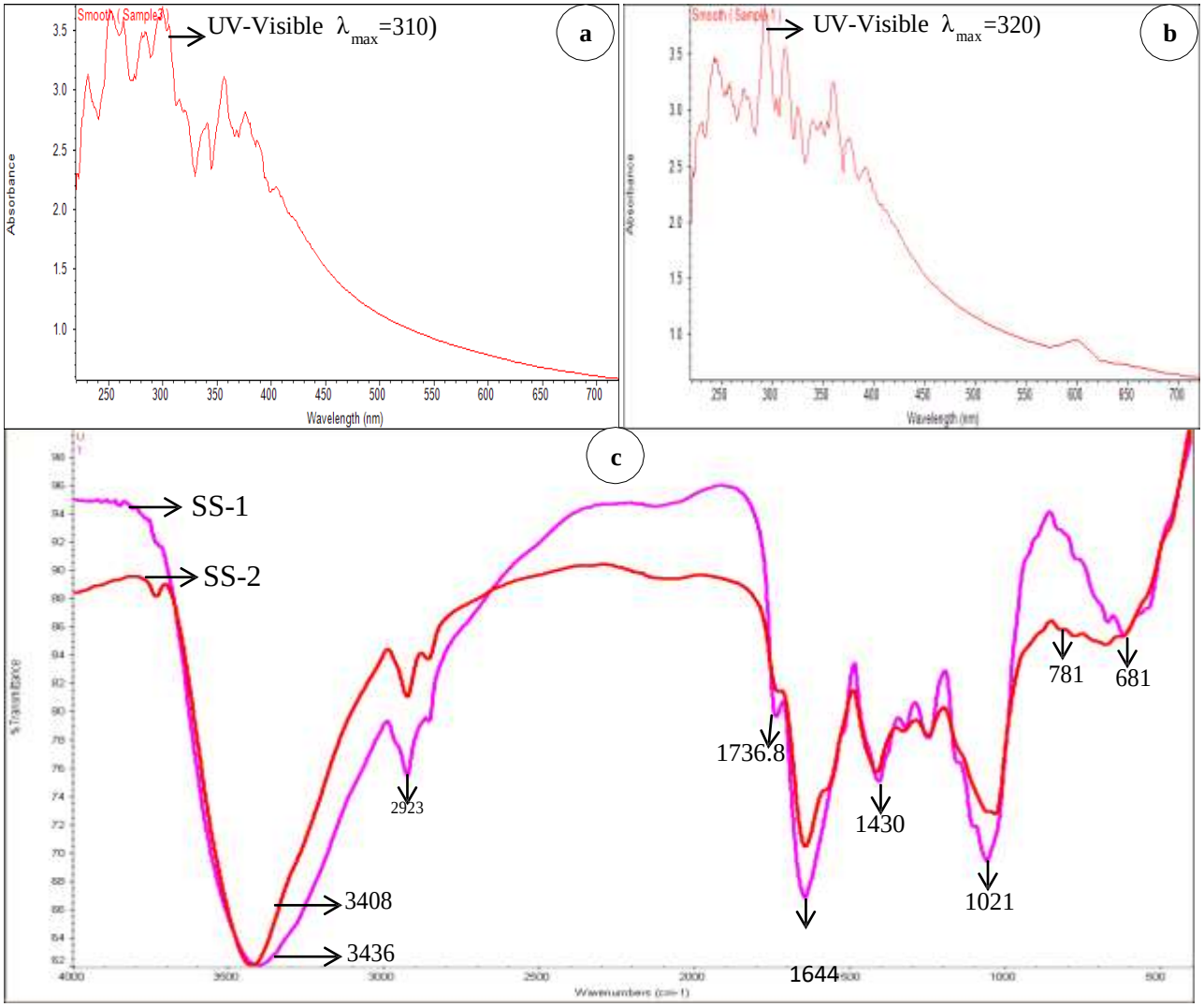


Fig.5.3.Assessment of IR-spectrum (KBr) PPMS of chemical constituents by FTIR analysis. UV-scanning (200-700 nm) analysis contaminated site (a-b). FTIR analysis (c), Sludge before phytoextraction (SS-1), Sludge after phytoextraction (SS-2).

The detail identified compounds at a different retention time with the help of the NIST library and their relative abundance along with similar percentage has been shown in Table.5.2. The detailed nature of the Identified compound indicated as various metabolic products of lignin from wood raw material during pulping and bleaching process there is generated generate various chlorinated toxic organic waste. Moreover, Eicosane is detected in PPMS is an alkane group compound generated during the pulping and bleaching process after secondary treatment. In addition, β- Sitosterol (RT 48.77) is

identified from the sludge sample after the biological treatment of the industry. β-Sitosterol is a plant sterol with a similar chemical structure is the same as cholesterol which might be generated during the wood bleaching process in the industry at chemical treatment. These compounds are reported as EDC, damage to DNA and carcinogenic is previously reported by Chandra et al., (2017). The chromatogram of organic pollutants after phytoextraction from the sludge sample is shown in Fig. 3b. The major RT peak has been noted at RT 25.54 (1- monopalmitin-ditms), RT 28.81 (1, 2, diphenyl-s (t-butyl) acephenanthrylene), RT 33.32 (2', 6'-Dihydroxyacetophenone) and RT 42.27 (Cinnamic acid-α-phenyl-trimethylsilyl ester) but there was a difference with the previous chromatogram which was detected before phytoextraction in chromatogram is showed in Table 2. The compounds of the initial peak have been disappearing and major peak noted at RT 9.06,13.17, and 20.55 also disappeared after phytoextraction. After phytoextraction, the detected compound at RT 19.56 (Lactic acid, trimethylsilyl ether, trimethylsilyl) Lactic acid was observed in alkaline conditions after phytoextraction of sludge. In addition, it is produced due to the fermentation and hydrolysis of cellulose components of the wood during the pulping process. The Cinnamic acids are a byproduct of lignin and hemicellulose was detected after in-situ phytoextraction at RT 42.27. The ester bonds break down during the alkaline pulping cycle in the chemical treatment and Cinnamic acid is attached to the lignin by ether links (Hernandez et al., 1997) and broken down through phytoremediation. Cinnamic acids are produced by cleavage of ester linkages in guaiacols (Shi et al., 2013) and this is also produced ether linkages and ester by the reaction of their carboxyl and phenolic groups (Jeffries, 1990). Furthermore, RT 47.17 were Ethanedioic acid, bis (trimethylsilyl) ester or oxalic acid is an organic compound and related to the carboxylic group detected after phytoextraction from the contaminated site of PPMS but this compound is unknown. The result has concluded that the native hyperaccumulator plants have the capacity to remediation the toxic organic pollutants present in sludge.

5.2.5. Heavy metals accumulation analysis

Potential hyperaccumulators plants collected from the sludge bed from PPMS site were characterized using standard taxonomic methods and revealed by the plant taxonomist of the genera and families according to Dutch flora of Indo-Gangetic plains. These plants showed the accumulation capacity of heavy metals from PPMS in their different parts i.e. root, shoot, and leaves shown in Fig.5.5 and Table.5.2. The hyperaccumulation characteristics of these plants from PPMS might be due to the existence of lignocellulosic particles and polymer contents such as nitrogen and phosphate, which are very conducive to plant development owing to natural vegetation due to the various lignocellulosic bacteria i.e. *Clostridium thermocellum, Clostridium aldrichii, Serratia marcescens, Bacillus cereus, Achhromobacter, Azotobacter, Azospirillum, Enterobacter, Pseudomonas,* etc. might be easily degrading the cellulose and providing source of carbon for the growing plants (Kato et al., 2004; Chandra et al., 2011).

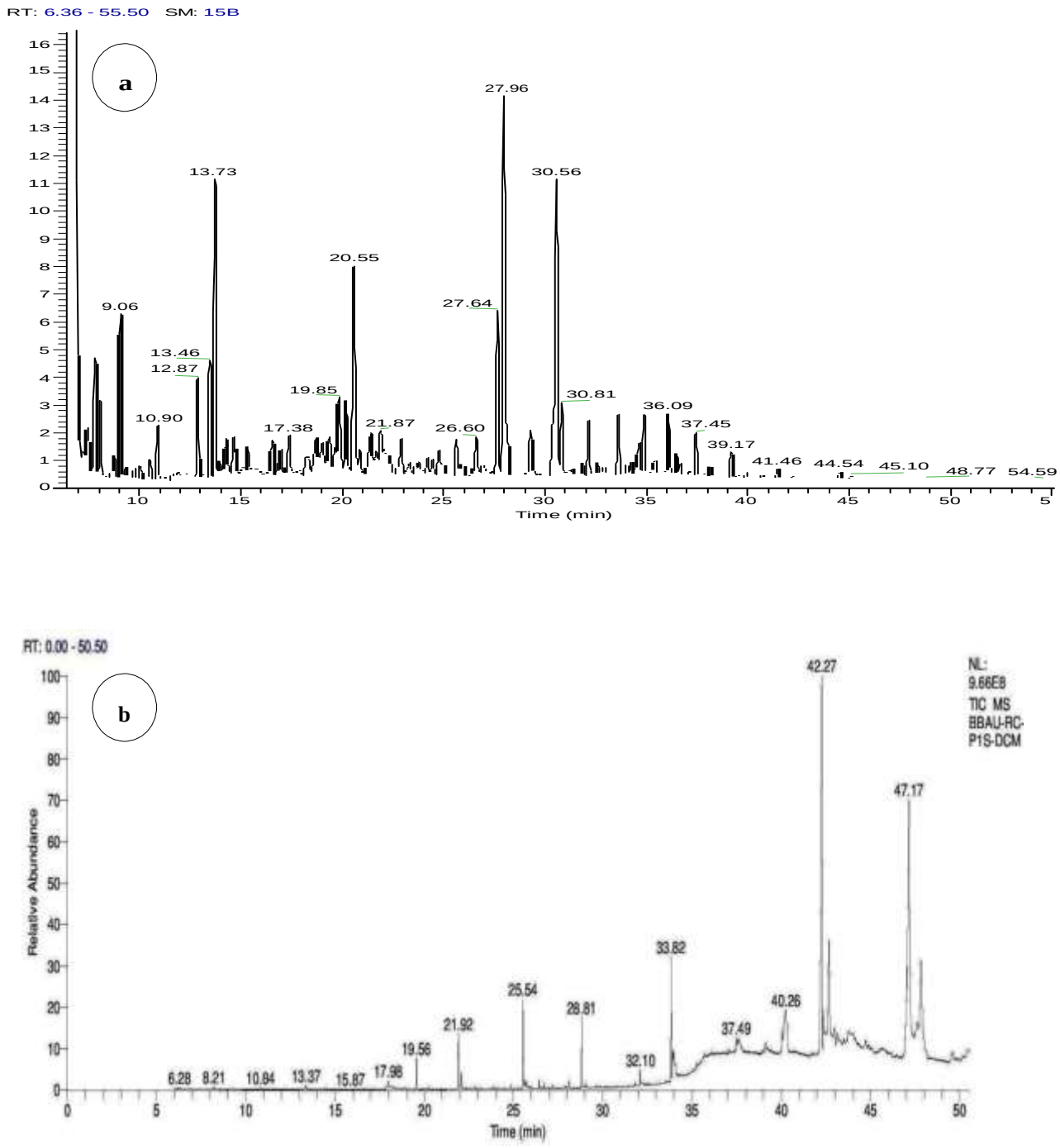


Fig.5.4. Total Ion Chromatogram (TIC) of TMS derivative detected residual Organic Pollutants from DCM extract of PPMS after secondary treatment (a) before the phytoextraction sludge (b) After the phytoextraction sludge

Table. 5.2. Identified Organic Pollutants by GC-M in the TMS derivatized dichloromethane (DCM) extracts of PPMW (sludge after and before phytoextraction) secondary treatment

RT	Compound Name	Relative abundance	Nature of compounds	% similarity with NIST Library	Toxicity
Sludge before phytoremediation					
9.06	2-Methyl-4-keto-2-pentan-2-ol 1TMS	41	Organic compound	67.18	Endocrine distrusting chemicals (EDC)
10.90	Hexadecanoic acid, trimethylsilyl ester	68	Organic compound	78.01	Mutagenic and Carcinogenicity
13.46	D-Lactic acid- DITMS	80	Fatty acid	93.11	EDCs
13.73	Octadecanoic acid, trimethylsilyl ester	71	Saturated fatty acids	93.12	Ecological *toxicity skin irritation*
13.46	Phenol-4-ethyl-2-methoxy or 4-Ethylguaiacol	41	Organic compound	57.01	Carcinogenicity Reproductive *Toxicity*
19.85	(Z)-2, 2'-Dibromo-4, 4'-di-n-pentylstilbene	56	Organic compound	98.23	EDCs and fish reproduction toxicity
20.55	Dimethyl2, 2', 4, 4', 5, 5'-Hexamethoxy	36	Organic compound	90.02	Acute *toxicity*
27.64	Tetradecanoic methyl ester	84	Saturated fatty acids	97.13	EDCs, Animal *toxicity*
27.96	Pentacarbonyl {[2'-(mesitylethynyl) phenylamino]-(p-tolyl) carbene}-chromium	78	Organic compound	92.05	EDCs, comedogenic
36.09	Pentadecanoic acid, ethyl ester	84	Organic compound	74.35	EDCs,
37.45	Eicosane (CAS)	57	Acyclic, alkane	34.54	EDCs
45.10	Benzoicacid,2,6-bis[(trimethylsilyl)oxy]	81	Organic compound	95.14	Carcinogenic, Mutagenic or *EDCs*
48.77	β- Sitosterol trimethylsilyl		Organic compound	71.23	DNA damage, Genotoxicity
54.59	Hexadecane		Alkane hydrocarbon	41.00	EDCs, Cytotoxicity Genotoxicity
Sludge after phytoremediation					
19.56	Lactic acid, trimethylsilyl ether, trimethylsilyl	94	Fatty acid	79.34	EDC, hyperkeratosis
21.92	4-Mercaptobenzoic acid	93	Sulfonic benzoic	97.24	Unknown
25.54	1- Monopalmitin-DITMS	80	Fatty acid	93.11	Data not reported
28.81	1,2,diphenyl-s (t-butyl) acephenanthrylene	81	Organic compound	89.74	Carcinogenic, mutagenic
32.82	2',6'-Dihydroxyacetophenone	31	Organic compound	71.74	Induced toxicity in male rats
37.49	Phenol-4-ethyl-2-methoxy or 4-Ethylguaiacol	54	Organic compound	76.34	
40.26	9,12-octadecadienoic acid (z,z)-2,3-dihydroxypropyl ester	81	Organic compound	96.03	Hepatotoxicants and carcinogens
47.17	Ethanedioic acid,bis(trimethylsilyl)ester	42	Organic compound	24.22	Unknown

Our study, total concentration of Fe highest in *P. hysterophorus* leaves at in rang (526.4 mg kg^{-1}), followed by the root of *T. Terrestris is* (495.6 mg kg^{-1}) and the shoot accumulation highest in *P. hysterophorus* (237.8 mg kg^{-1}) respectively. Since Fe is a significant micronutrient, It plays an important role constitute of several enzymes and some pigments and assisted in sulfate and nitrate reduction and energy production within the plants. A similar accumulation pattern of Zn concentration in the plant was found in variables ranged in different native plants. The Zn in the root of *M. dioica* (38.76 mg kg^{1}), leaves in *T. Terrestris* (33.46 mg kg^{-1}) and shoot in *A. sessilis* (43.68 mg kg^{-1}). The Zn is a crucial micronutrient free ion that may also be confirmed as a molecule of proteins and Zn acts as a functional, the structural or regulatory cofactor of a large number of enzymes (Peck and McDonald, 2010). Recent evidence recommended that Zn plays an important role in stabilizing RNA and DNA structure, in preserving the activity of DNA synthesizing enzymes and controlling the activity of RNA degrading enzymes and may play a role in controlling gene expression. The higher concentration of Cu present in the root of *M. dioica* (55.41 mg kg^{-1}) shoots of *P. hysterophorus* (33.25 mg kg^{-1}) and leaves of *A. sessilis* (22.356 mg kg^{-1}). Cu contributes to numerous physiological and Cellular activities *i.e.,* photosynthetic electron transport and is an essential cofactor for many metalloproteinases. In the previous study also similar observations have been reported by Chandra et al., (2017). The concentrations of Cd naturally occurring low. Reports indicate that concentrations of Cd in non-contaminated soil vary from 0.01 to 5 mg soil kg^{-1} and Cd are mobilized in the food chain that affects producers and consumers (Kabata-Pendias, 2004; Veltman et al., 2008), but the PPMS contain a high amount of Cd. The concentration of Cr is highest in root and shoot of *C. procera* (6.23 mg kg^{-1} to 12.65 mg kg-1) is show the high accumulation capacity. Cr enters in plants through root exudates decrease and complexation such as organic acids, which increase Cr mobility through xylem root (Bluskov et al., 2005). The accumulation of Pb is highest in the root of *C.sativa* (9.24 mg kg^{-1}), leaves of *A. sessilis* (16.45 mg kg^{-1}) and root of *T. terrestris* (2.34 mg kg^{-1}). In addition, Pb is not an important

element this element is compelled on root surfaces by carboxylic groups of uronic mucilage acids (Morel et al., 1986; Sharma and Dubey, 2005), Very few studies have been published on the transport of lead in the food chain. The result showed that the metal tolerance capacity is widely present in collected native hyperaccumulator plants when they will grow on the organometallic containing sludge of disposal site of PPMS. All the analyses of hyperaccumulator plants showed the accumulation of heavy metals is higher than non-hyperaccumulator.

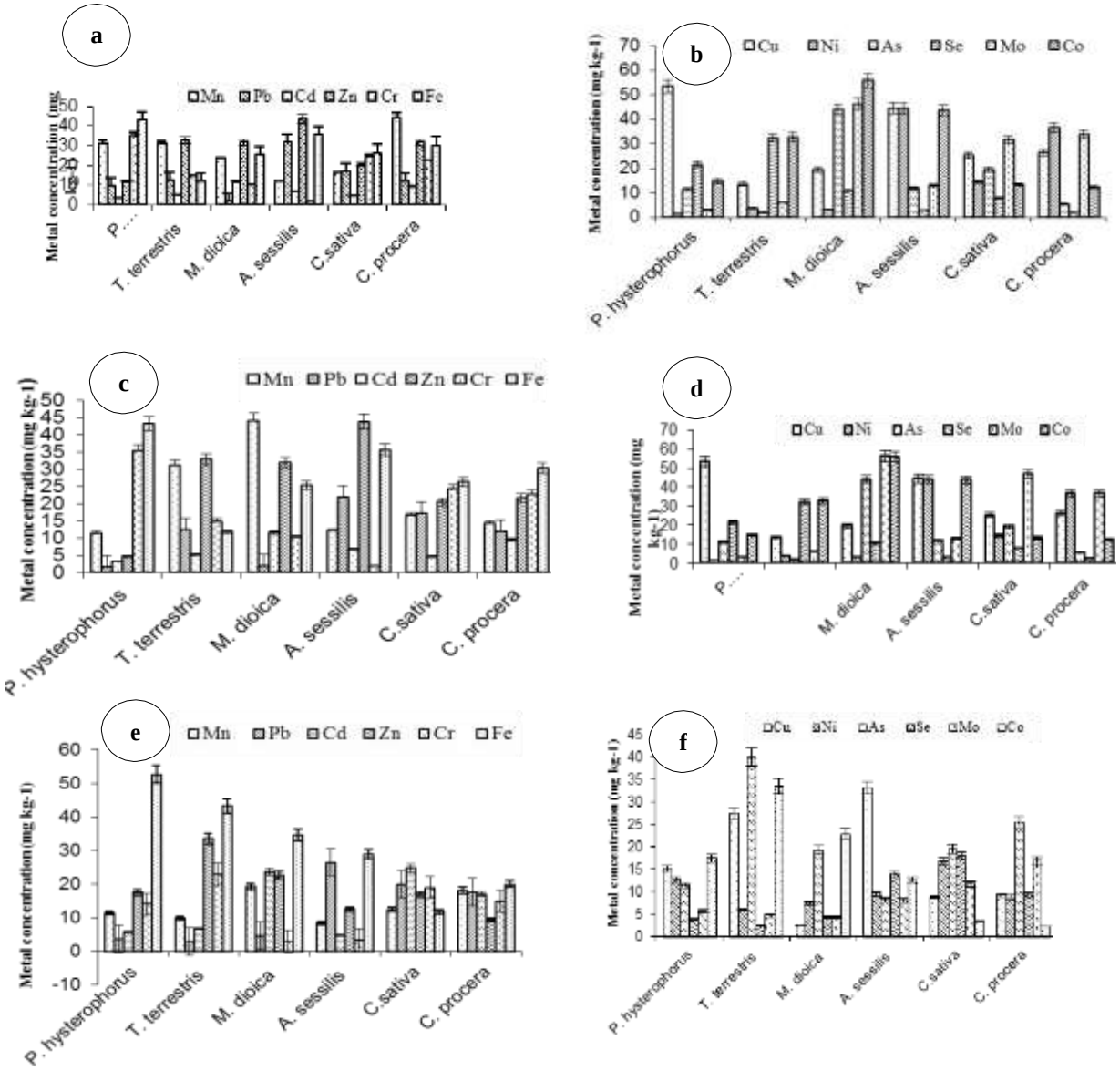


Fig.5.5.Comparative analysis of heavy metal accumulation pattern in the root by native Hyperaccumulators plants growing on PPMS (A-B) Root (C-D) Shoot (E-F) Leaves

Table.5.3. Heavy metal accumulation (mg kg^{-1} DW) in the root, shoot, and leaves of various hyperaccumulator plant species growing contaminated site of pulp paper sludge. All the values are mean of three replicates (n=3) ±standard deviation (SD), BDL: Below detection limit, R: Root, S: Shoot, L

Plant name	Plant part	Mn	Pb	Cd	Zn	Cr	Fe	Cu	Ni	As
P. hysterophorus	***Root***	14.65±0.1	1.97± 0.5	2.64± 0.1	27.78±0.1	2.43± 0.4	237.4± 0.1	21.23± 0.3	1.36± 0.1	2.03± 0.1
	Shoot	11.49±0.3	Nil	Nil	14.76±0.1	12.20± 0.5	341.8± 0.5	33.25± 0.3	0.83± 0.5	2.36± 0.4
	Leaves	10.38±0.2	3.75± 0.1	1.26±0.2	**47.49±0.4**	4.02± 0.3	526.4± 0.1	14.59± 0.2	2.59± 0.1	1.03± 0.1
	Total	36.52±*0.6*	5.72±*0.6*	3.9±*0.3*	60.03±*0.6*	18.65±*1.1*	1105.6±0.7	68.07±*0.8*	4.78 ± *0.7*	5.42±*0.6*
Accumulation pattern		**R> S> L**	***L > R>S***	***L> R> S***	***R> L >S***	***S>L> R***	***R> S> L***	***S > R > L***	***L> R> S***	***S> R > L***
T. terrestris	***Root***	53.02±0.1	3.83± 0.4	0.169±0.2	21.38±0.3	5.206± 0.5	495.6± 0.3	7.466± 0.1	2.698± 0.2	2.300± 0.1
	Shoot	Nil	**2.34 ± 0.2**	0.995±0.5	32.88±0.2	6.702± 0.5	111.8± 0.5	13.046± 0.5	0.752± 0.2	1.546± 0.3
	Leaves	9.89± 0.3	Nil	0.323±0.1	33.46±0.1	4.850± 0.1	212.2± 0.2	17.426± 0.4	1.976± 0.3	1.234± 0.4
	Total	62.91±0.4	6.17±0.6	1.487±0.8	87.72±0.6	16.758±1.1	819.6±1.1	37.938±1.0	5.426±0.7	5.08±0.8
Accumulation pattern		***R>L***	***R > S***	***S> L> R***	***L > S> R***	***S> R> L***	***R> L > S***	***L > S > R***	***R> L> S***	***R>S>L***
M. dioica	***Root***	46.76±0.3	0.36 ± 0.2	0.521±0.3	**38.76±0.2**	3.496± 0.3	371.4± 0.3	55.41± 0.4	3.194± 0.3	43.4± 0.3
	Shoot	44.01±0.3	Nil ± 0.3	1.23± 0.3	31.78±0.3	2.558± 0.3	Nil ± 0.3	19.044± 0.3	3.294± 0.3	44.01± 0.3
	Leaves	**59.25±0.3**	Nil ± 0.3	4.25± 0.3	22.7± 0.3	2.911 ± 0.3	211.4± 0.3	11.079± 0.3	1.592± 0.3	19.25± 0.3
	Total	110.02±0.9	0.36±0.8	6.001±0.9	95.24±0.8	8.965±0.9	582.8±0.9	85.533±1.0	8.08±0.9	106.66±0.9
Accumulation pattern		***R>S>L***	***BDL***	***L>S>R***	***R>S>L***	***R>L>S***	***R>S***	***R >S >L***	***S>R>L***	***S>R>L***
A. sessilis	***Root***	15.03±0.3	5.23± 0.1	Nil ± 0.2	14.23±0.3	0.57± 0.4	477.2± 0.6	8.582±0.1	22.36± 0.1	6.21± 0.3
	Shoot	12.04±0.2	Nil ± 0.3	Nil ± 0.3	**43.68±0.5**	Nil ± 0.3	156.3± 0.4	14.521± 0.2	4.21± 0.3	12.04± 0.2
	Leaves	8.36± 0.1	**16.45±0.3**	Nil ± 0.3	12.56±0.4	Nil ± 0.3	245.5± 0.5	22.356± 0.3	1.258± 0.1	8.36± 0.3
	Total	35.42±0.6	21.68±0.7	0.0±0.8	70.47±1.2	0.57±1.0	879.0±1.5	45.459±0.6	27.828±0.5	26.61±0.8
Accumulation pattern		***R>S>L***	***L>R***	***BDL***	***S>R>L***	***BDL***	***R>L>S***	***L > S> R***	***R>S>L***	***S>L>R***
C. sativa	***Root***	18.89±0.6	**9.24±0.5**	1.23±0.5	16.21±0.5	**7.21**±0.5	40.25±0.5	35.23±0.5	19.65±0.5	19.41±0.5
	Shoot	16.54±0.5	1.23±0.4	1.02±0.3	20.54±0.5	11.23±0.5	26.33±1.4	15.23±0.4	14.56±0.5	17.50.5
	Leaves	12.43±0.5	9.86±0.6	4.76±0.4	6.87± 0.5	**8.95± 0.5**	21.78±0.5	18.76± 0.5	6.77± 0.5	15.41±0.6
	Total	47.86±1.6	20.33±1.5	7.01±1.2	43.68±1.5	27.39±1.5	88.36±1.4	69.22±1.4	4.98±1.5	52.32±1.6
Accumulation pattern		***R>S>L***	***L>R>S***	***L>R>S***	***S>R>L***	***S>L>R***	***R>S>L***	***R > L>S***	***R>S>L***	***R>S>L***
C. procera	***Root***	19.25±0.5	8.26±0.4	1.21±0.5	12.32±0.6	6.23± 0.5	42.36±0.4	6.89± 0.4	17.54 ±0.5	5.44 ± 0.5
	Shoot	12.36±0.5	1.98±0.4	1.54±0.5	21.65±0.5	12.65±0.5	30.21±0.4	16.32±0.5	16.56±0.5	4.74± 0 .5
	Leaves	18.09±0.5	7.68±0.5	6.97±0.5	9.43± 0.5	8.90± 0.5	19.98±0.5	9.07± 0.5	8.76± 0.4	3.43± 0.3
	Total	46.7±1.5	17.92±1.4	9.72±1.5	43.4±1.6	27.78± 1.5	92.55±1.3	32.28±1.4	42.86±1.4	13.61±1.3
Accumulation pattern		***R>L>S***	***R>L>S***	***L>R>S***	***S>L>R***	***S>L>R***	***R>S>L***	***S>L>R***	***R>S>L***	***R>S>L***

5.2.6. Bioconcentration factor and Translocation factor

The study we observe the ratio of heavy metals concentration in plants root to sludge by using the Bioconcentration factor (BCF) showed in Table 5.4. The capacity of native potential hyperaccumulator plants to accumulate the metals which might be a tool for in-situ phytoextraction of heavy metals mixed with organic pollutants from PPMS. In addition, *P. hysterophorus* showed maximum BCF which showed the ratio of metals in root and soil was more than one, Mn (74.40 mg kg^{-1}), Zn (15.75 mg kg^{-1}), showed in Table 4. While the Measure the plant ability to transfer metals from the root to the shoot by TF is >10 highest in Fe *C. procera* (18.65 mg kg^{-1}) Cu *T. terrestris* (41.29 mg kg^{-1}) and As in *C. sativa* (18.34 mg kg^{-1}) respectively. High concentration accumulation of metals might be established as a detoxification mechanism dependent on ion sequestration in the vacuole by binding with ligands i.e. protein, organic acid and peptides in the presence of which may act at a high level of metalicilous conditions (Yang et al., 2005). According to MacFarlane et al. (2007), the translocation value was evaluated the phytostabilization and phytoextraction of the metals by native hyperaccumulator growing at the disposal site of PPMS. In addition, the accumulation of metals by plants from sludge to root depends on the chemical nature of element, pH, and other co-pollutants of sludge is inhibit the mobility of metals so it inhibits the accumulation and translocation in plants (Gupta and Sinha, 2008; Yoon et al., 2006; Rosselli et al., 2003). The comparison of BCF and TF by native hyperaccumulator plants to absorb heavy metal from sludge to root and root to shoot. The BCF and TF values are less than one is unacceptable for phytoextraction (Fitz and Wenzel, 2002).

Table.5.4. Showing BCF and TF of different Heavy metal accumulation (mg kg^{-1} DW) by various hyperaccumulator plants of a different part in the root, shoot and leaves on disposal sludge bed

Native hyperaccumulators plants	**Bioconcentration Factor (BCF)**							
	Mn	**Pb**	**Zn**	**Cr**	**Fe**	**Cu**	**Ni**	**As**
P. hysterophorus	**74.40**	13.50	**15.75**	17.72	12.33	**95.12**	1.539	1.832
T. terrestris	14.65	16.12	7.123	19.25	14.23	56.23	6.254	5.632
M. dioica	53.2	**48.15**	8.258	14.26	15.14	47.19	4.369	**8.243**
A. sessilis	46.76	37.68	4.244	**23.85**	19.56	23.84	4.218	5.346
C. sativa	15.03	11.63	6.764	17.52	28.49	21.59	7.243	4.283
C. procera	20.46	17.54	**10.65**	22.36	**31.51**	54.82	**9.243**	6.364
	Translocation Factor (TF)							
	Mn	**Pb**	**Zn**	**Cr**	**Fe**	**Cu**	**Ni**	**As**
P. hysterophorus	**10.23**	5.251	9.175	**9.246**	16.24	36.12	0.0	2.36
T. terrestris	4.025	2.312	7.123	6.249	15.24	**41.29**	0.245	5.36
M. dioica	4.022	**8.015**	8.258	2.561	14.27	27.56	0.558	2.94
A. sessilis	4.076	7.468	4.244	6.254	12.14	17.81	**1.374**	8.63
C. sativa	5.203	1.603	6.764	7.560	11.23	11.49	1.297	**11.34**
C. procera	8.24	6.124	**9.182**	9.266	**18.65**	19.32	0.0	6.64

5.2.7. Response of antioxidants and stomata against pollutants

Native Hyperaccumulator reveals the more antioxidants activity is a comparison to control plants growing at the organometallic sludge bed of PPMS as shown in Fig.5.6. The study has shown a higher SOD activity in P. hysterophorus (237.11 unit gm^{-1}) comparison to the control crop (2013-1977 gm^{-1}). In addition, SOD catalyzes and detoxifies the mechanism of decomposing and detoxification of the superoxide, including hydrogen peroxides, and anion into radical oxygen and then converts it to ground level O_2 and H_2O. In addition, APX is the highest concentration in *C. sativa* plant in comparison to control (unpolluted site). The higher APX amount of under PPMS stress revealed the conflicting role of detoxification with H_2O_2. The APX scavenges the peroxide molecule, i.e. H_2O_2 requires ascorbate for the management of photosynthetic machinery and other injuries. Generally, H_2O_2 content enhanced by the interference of toxic heavy metals, which eliminates the ROS and suppresses lipid peroxidation. High level of H2O2 content in affected plants exhibited its toxic effects in the form of plasmolysis, electrolytic leakage and membrane damage. Metal toxicity in plants generated due to the reactive oxygen species (ROS) actually causes oxidative damage, leakage of electrolytes, harm to cells, DNA inhibition and mitochondrial toxicity (Charfeddine et al., 2017). There are also signs of antioxidant enzymes to prevent the toxicity crops caused by reactive oxygen species (ROS), such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), which protect plants from multiple adverse conditions (Jiang et al., 2010; Farooq et al., 2016). The concentration of MDA was highest in *A. sessile* showed the high cytotoxic of lipid peroxidation and indicate the peroxidation of membrane lipids in plants. The level of MDA formation is high in *A. sessile* is indicate the high free radical production and help the lipid peroxidation. The similar finding showing an increased concentration of MDA content in leaves of *Helianthus annuus* after treatment with the solution of different metals (Gallego et al., 1996). Similarly, excessive accumulation of metals may also result in an increased level of MDA content in roots and leaves of mustard and wheat plants. The pollutants present in PPMS damage to cuticular waxes layer by which they enter the leaves through stomata and directly affected on plant morphology, physiology, and anatomical

properties, and reduce the transpiration rate during stress condition but these native hyperaccumulator plants show the high tolerance.

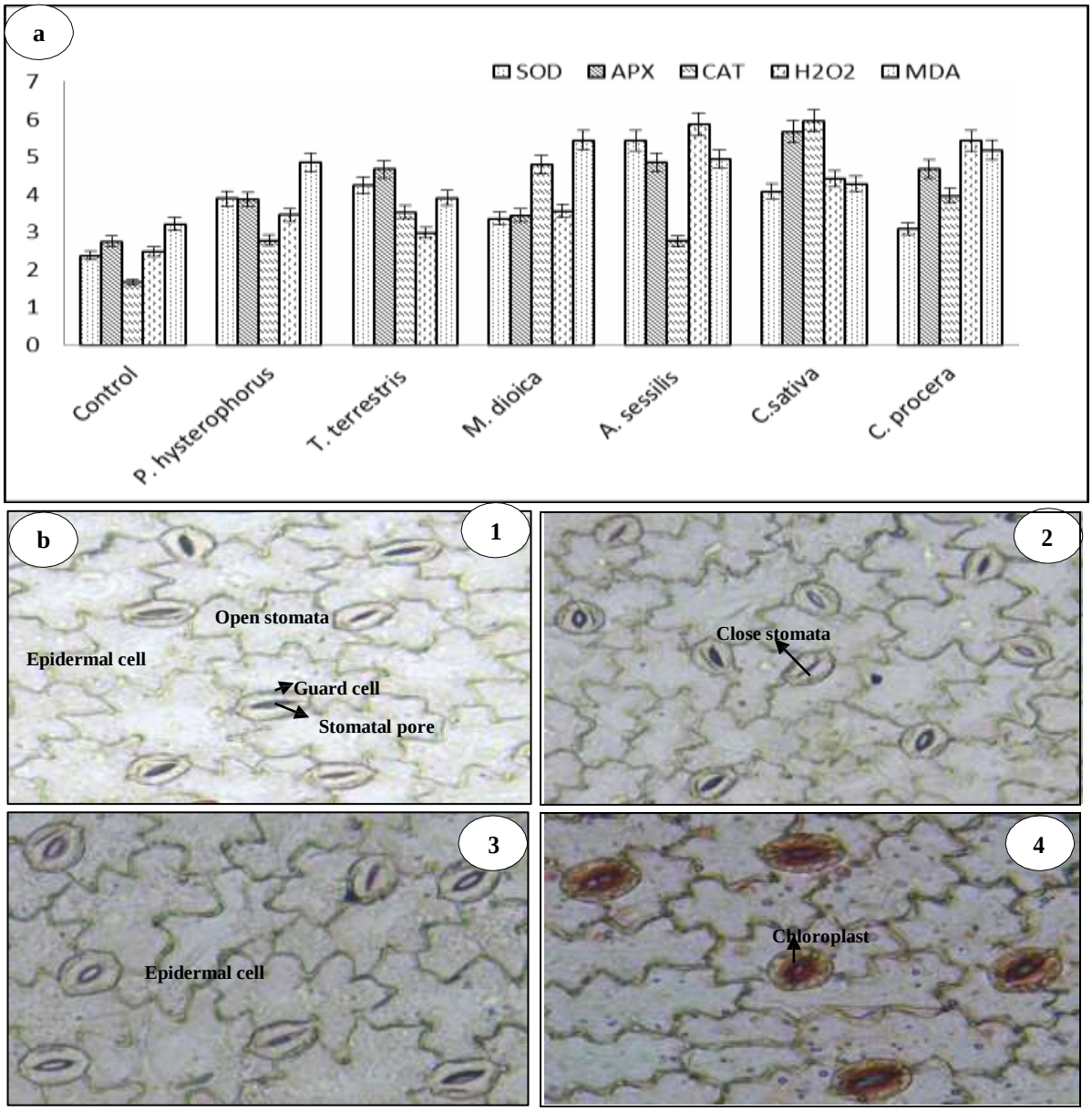


Fig.5.6. Comparative analysis of SOD, APX, H_2O_2 and MDA activity in leaves of native plants growing at PPMS and normal agriculture land (Control) (a). Stomata observation by light Microscopic in leaf portions (abaxial epidermal layer) showed the opening status of the stomata apparatus of different hyperaccumulator plants grow on the contaminated site of PPMS (b)

Plant function for cytokinins and auxins have also been shown to regulate stomata behavior that controls open and close mechanisms and has no impact on transpiration after exposure of organometallic sludge (Jewer and Incoll, 1980).

5.2.8. Histological observations by TEM analysis

The transmission electron microscopy (TEM) analysis of root tissue of collected native hyperaccumulator plants is showed the presence of metals in their intracellular space, cytoplasm,

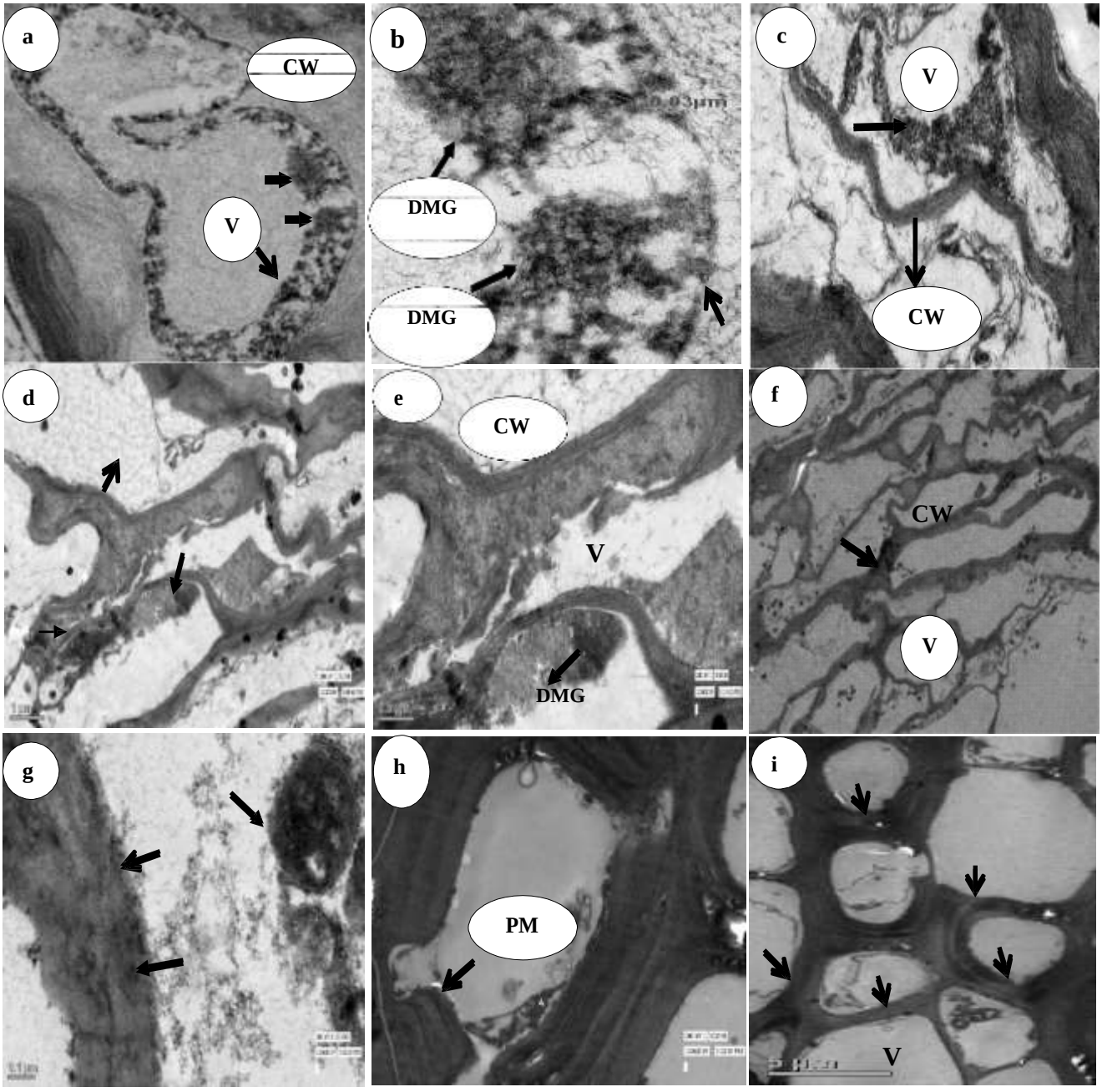


Fig 5.7.The TEM analysis of native hyperaccumulator plants root after in-situ phytoextraction of heavy metals. CW- cell wall, V- vacuole, PM-Plasma membrane, DMG-Deposited metal granule (a-b) *T. Terrestris*, (c-d) *M. dioica* (e-f) *A. sessilis.* (g) *P. hysterophorus* (h) *C. sativa* (i) *C. procera*

Vacuole and cell wall shown in Fig. 5.7. Bini et al., (2012) reported that understanding physiological and morphological conditions after exposure of heavy metals from the contaminated site is a good tool for examining the accumulation and storage of heavy metals in various plant cells. Metal deposition near the cell wall in *M. dioica* plays a significant role in the tolerance of heavy metals by preventing free metal ions from circulating in the cytosol. This observation of potential native plants shows new information on heavy metal detoxification at the contaminated site. In addition, some antioxidants work as a substratum for phytochelatins synthesis and play a crucial role in high concentration detoxification of hazardous heavy metals such as Ni and Cd. Similarly, *A. sessilis* root tissue showed information about middle lamella metal accumulation, cell wall, cytoplasm, and vacuole is useful knowledge to detoxify heavy metal from PPMS. Tong et al., (2004) reported a mechanism of plant accumulation and detoxification of metals in larger amounts in their cell tissues through the formation and deposition of metal granules through multi-vacuoles in the cell wall. Anatomical observation of *C. sativa* root tissue has been shown too thick the cell wall after heavy metal accumulation and the plant has shown no cell damage or noticeable change is evidence of plant tolerance mechanism. Since different parameter analysis, the collected native plants show strong evidence that the capacity of heavy metal contains persistent organic pollutants and stress tolerance and accumulation for their growth and development in rich. This study is strong evidence for the evaluation and monitoring of eco-restoration hazardous pollutants.

Conclusion

The result concluded that PPMS content mixture of various organo-metallic pollutants above the permissible limit of environmental regulation. Some pollutants are listed under EDC compounds by USEPA, 2012. Therefore, the discharged waste is a source of environmental pollutant. In the residual pollutants i.e. Eicosane (CAS), Hexadecane, Benzoic acid, 2, 6-bis [(TMS) oxy], dodecane, 1-iodo, and D-Lactic acid- DITMS which are listed as EDC compound and after phytoextraction some compounds disappear. But the phytoextraction potential of some native plants for heavy metals from complex

pollutants of pulp paper waste showed as evidence of in-situ phytoremediation of complex waste for the eco-restoration polluted site. The antioxidants analysis by collected hyperaccumulator plants i.e. SOD, APx, H_2O_2, and MDA also showed show in higher concentration compared to control. Thus these plants can be recommended as biotechnological tools for the affected site of the pulp and paper industry.

Chapter-Six

Heavy metal accumulation pattern in a different part of Trigonella foenum-graecum L. plant irrigated with effluent from pulp paper mill

Heavy Metal Accumulation Pattern in a Different Part of *Trigonella foenum-graecum L.* Plant Irrigated with Effluent from Pulp Paper Mill

6. Introduction

Currently, agriculture and the aquatic environment have been highly polluted by hazardous organic pollutants including EDCs compounds and toxic heavy metals released from pulp paper industry after secondary treatment. The pulp paper industry is extremely water-intensive, uses up 100-250 m^3 of freshwater per ton of papermaking and generates 75-225 m^3 of wastewater in the environment without proper treatment (Thompson, 2001 and Tewari et al., 2009). In addition, some bacteria strains i.e. *Aeromonas, Bacillus subtilis, Pseudomonas* and *Xanthomonas* are reported to utilize lignocellulosic and chloro-organic components of pulp paper effluent (Vora et al., 1988; Jain et al., 1997; Gupta et al., 2001) and other bacteria isolated from compost soil viz. *Azotobacter* and *Serratia marcescens* were found capable of the degradation and decolourization of lignin (Morii et al., 1995). The *Trigonella foenum-graecum* L also known as fenugreek is an annual plant belonging to the Leguminosae family and very well-known spices in human food for several diseases and medicinal plants. Moreover, has pharmaceutical characteristics including antimicrobial, anti-cholesterol emic, carminative, emollient, febrifugal, laxative, restorative, uterine, expect oral, galactagogue, anti-carcinogenic, anti-inflammatory, antiviral, antioxidant, demulcent and hypotensive characteristics (Moradi, 2013). The wastewater discharge from the pulp paper industry is a major problem for agriculture land due to elevated effluent and sludge quantity generation and limited land-based treatment and disposal space. In the process of wood digestion, where wood chips are transformed into fiber masses by reacting at high temperature and pressure with sodium hydroxide (NaOH) and sodium sulphite (Na_2SO_4). Moreover, seed germination is particularly susceptible to metal pollution because of the lack of defence mechanisms (Xiong and Wang, 2005). Furthermore, heavy metals can bind and inactivate biomolecules to structurally substantial domains for example; the result might be enzymatic response immune

response and metabolism disturbance (Van Assche and Clijsters, 1990). There are many possible reasons for vegetative cell changes by the first target cupric ion excess seems to be the cell membrane described by Wecke and Clijsters,(1996). The formation of harmful free radicals can be catalyzed by various hazardous metals, a redox-active metal, through reactions of Fenton and Haber Weiss (Van Assche., 1995). Moreover, excess copper may cause chlorosis as a result of alterations in the photosystem. Indeed, several authors have reported that photosynthesis is greatly affected by the availability of Cu causing a decline in photosynthesis activity (Frankart et al., 2002). Heavy metals can bind and inactivate biomolecules to structurally substantial domains for example; the result might be enzymatic response inhibition and metabolism disturbance (Van Assche and Clijsters, 1990). The aim of this study is to provide information on discharged effluent toxicity after chemical and biological treatment in industry through physico-chemical analysis and detection of several organic pollutants by GC-MS analysis. In addition, the exposure of pulp paper mill effluent on fenugreek showed accumulation of high concentrations of heavy metals and affects their biochemical and physiological parameters. Moreover, pollution from heavy metals is disturbing fenugreek physiological process resulting in stomata, chlorosis in the formation of root nodule, and also affects the level of antioxidants after irrigation with pulp paper industry effluent. The TEM analysis is investigate the metals accumulation in root tissue of fenugreek after exposure with different concentration of pulp paper mill effluent.

6.1. Material methods

6.1.1. Sample collection and Experimental design

The effluent sample collected from M/s K.R. Pulp and Papers Limited, Shahjahanpur, U.P. India (27 ° 50'31.8"N 79 ° 51'15.7"E) for this study (Fig.6.1). The field study was conducted in the experimental garden of the Department of Microbiology school for Environmental Science, BBA University Lucknow (29^{o}55'10.81 " N and 78^{o}07'08.12 'E) to study the effect of the paper mill effluent on *T. foenum-graecum L* (fenugreek). The experiment was conducted under a designed and replicated four times completely randomized in December 2018, to March 2019. For maximum crop

performance, the proper distance was maintained between each replicate (30 cm), between each therapy (60 cm) and plant-to-plant (5 cm) (Fig.6.2).

Fig.6.1. View of pulp paper industry and they are discharged in the environment after secondary treatment (a) pulp paper industry view (b) collection of effluent from the discharged site (c, d) soil pollution due to pulp paper industry wastewater after secondary treatment

6.1.5. Physiological and biochemical parameter

In the pot experiment, the treated and untreated (control) fenugreek plants were harvested after 30, 60 and 90 days and washed with distilled water to remove any contiguous particles. For the estimation of photosynthetic pigments including chlorophyll a (Chl a) and chlorophyll b (Chl. b) content (Arnon, 1949). While the protein content was estimated in different parts of plants i.e. roots, shoots, leaves (Lowry et al., 1951). To estimate the different biochemical parameter i.e. SOD, CAT, POD from freshly harvested leaves, shoot and root of fenugreek grow in soil irrigated with effluent after 30, 60, and 90 days of growth (Aebi, 1983;Oktay et al., 1995; Kumar et al., 2012).

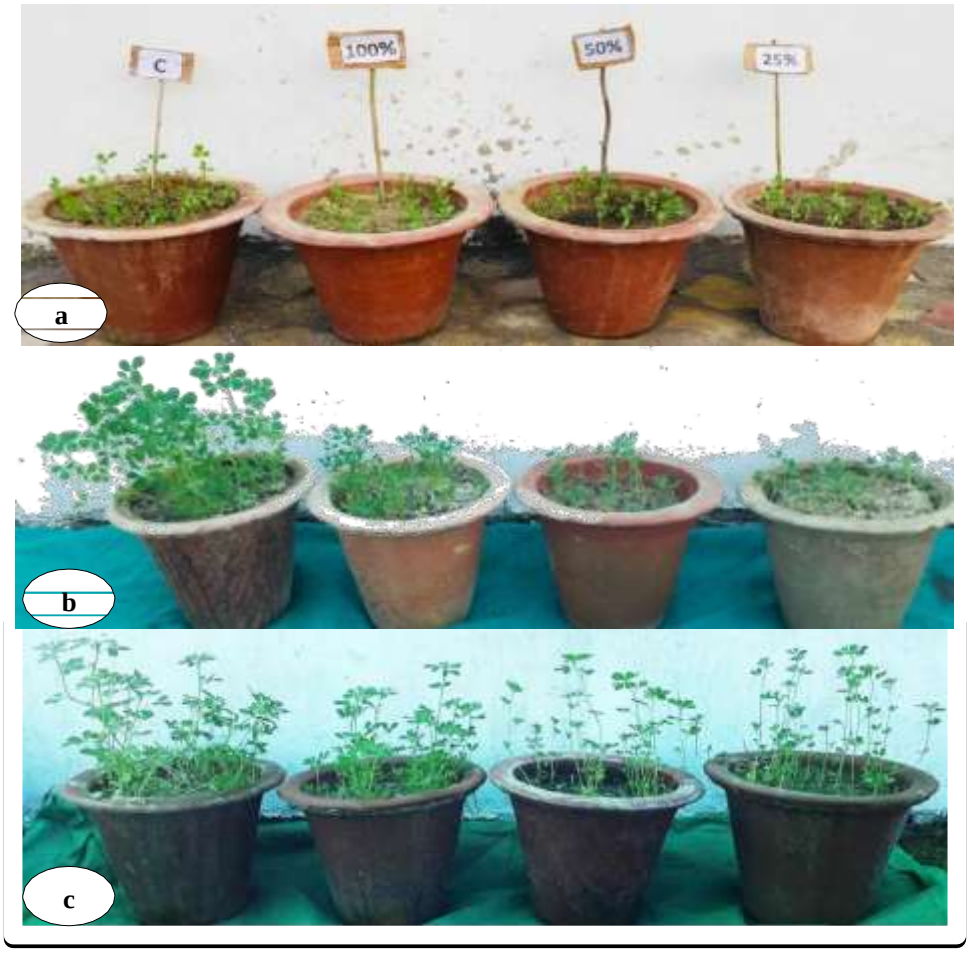


Fig.6.2. Experiment in pot irrigated with pulp paper industry effluent at different concentration (25%, 50%, and 100%) in fenugreek

6.1.6. SEM analysis for stomata and root and root nodule surface study

The Observation of stomata condition after exposure with pulp paper effluent by SEM analysis to prepare the sample for stomata observation the leaves peel was removed from the upper surface of the leaves and root, and root nodule washed with tap water and cut into small pieces and fixed in 2.5 % glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 12 h (overnight) at 4ºC. Consequently, samples were dehydrated through a graded series of acetone (30 %, 50 %, 70 %, 90 %, 95 % and 100 %) for 30 min at each step root and root nodule were then transferred to a critical point dryer using CO_2 as a transitional fluid that removes water from the specimen and prevents unwanted damage due to liquid/gas surface tension (Ahmad et al., 2016). The dehydrated roots were then placed on the two-sided carbon tape fix on the SEM stub and

displayed under JSM 6490LV SEM, (JEOL, Tokyo, Japan) with a 10 kV accelerating voltage.

6.1.7. Genotoxicity assessment

For genotoxicity evaluation of fenugreek after irrigated with effluent was measured. The root tip cut and fixed root tips in fixative solution (ethanol: glacial acetic acid 3:1) overnight and remove and washed with DW four-time ten minutes each (Chandra et al., 2018). Consequently, after washing with distilled water remove the root tip and put it into the1N HCl solution for five minutes at room temperature and washed again with DW four times. For the stained of the nucleus add 2% hematoxylin for ten minutes. In addition, two root tips were squashed on each slide with 45 % of acetic acid and covered slips carefully lowered to exclude the air bubble. The coverslips were sealed with clear fingernail polish on the slides and the slides were monitored under phase-contrast microscopy (Phase Contrast Microscope; Nikon; Japan).

6.1.8. Estimation of heavy metals from the root, shoot and leave

Harvesting the various parts fenugreek plant after 90 days and washed thoroughly with deionized water to remove soil and process for estimation of heavy metal (Fig.6.3) by previous described method in chapter five.

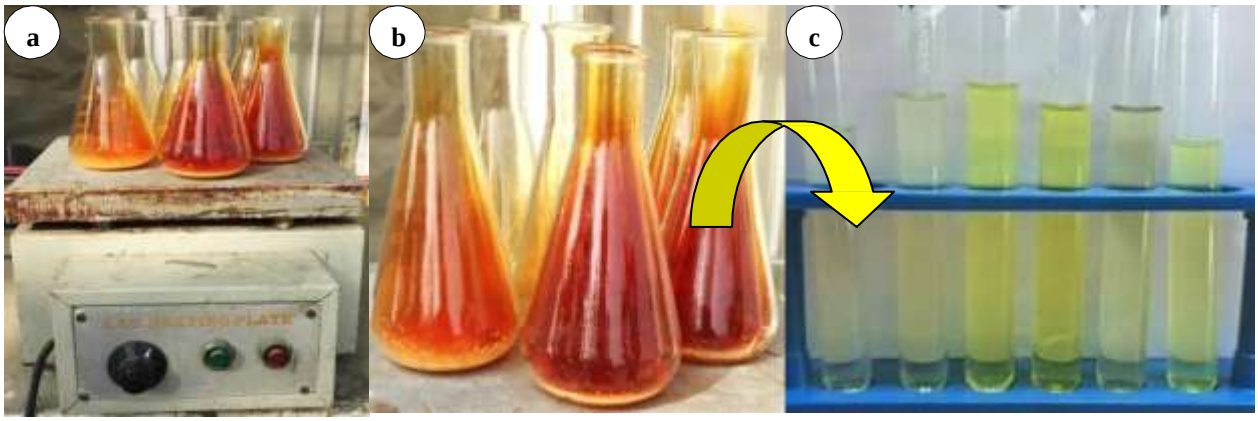


Fig 6.3. Showing the digestion of heavy metals presents in fenugreek and their estimation by AAS (a-b). Digestion of plant material (c). After digestion sample ready for metal analysis

6.1.9. Histological observation by TEM analysis

After irrigated with effluent in pot experiment the mature fenugreek root and root nodule cut into segments and fixed into glutaraldehyde as per previous described method in chapter five.

6.1.10. 10. Statistical Analysis

All data for triplicate samples were reported as ± SD means and confirm data variability and validity of results; all data have also been subjected to statistical analysis.

6.2. Results and discussion

6.2.1. Effect on physiological and biochemical parameter

In the present study, it was observed that up to 100% concentration of effluent decrease in percent germination while 25% is showing less inhibit percentage (Fig.6.4). Because they contain several mutagenic compounds along with heavy metals above the permissible limit, the 25, 50 and 100% effluent did not support seed germination in the present investigation. The 100% effluent showed reduced germination fenugreek probably due to the presence at these concentrations of high salt in the effluent. Seeds take up water during germination and stored food material for hydrolysis and may inhibit seed germination to activate enzymatic systems and during germination salts. Moreover, NaCl seed germination inhibition mechanism may be associated with radical emergence due to insufficient water absorption or toxic effects on the embryo. Seeds that absorb an insufficient quantity of water can accumulate a large quantity of Cl^- when the osmotic pressure of the substratum is increased by salt concentration and as a result, the seeds emerged slowly and do not germinate at higher concentrations (Patterson, 2008). However, high concentrations are usually most harmful to young plants, but not necessarily at germination, although high concentrations of salt may slow or inhibit germination for several days. Evaporation moves salts to the soil surface where they accumulate and harden the soil surface delays germination as soluble salts move readily with water (Singh et al., 2002). Embryonic roots fenugreek is closed with a thick cell wall that forms the boundary between the root cap and the rest of the root apex content of the

pigments is affected by exposure of fenugreek to various kinds of hazardous compounds stresses present in the effluent after secondary treatment. Moreover, the leaves of fenugreek are completely yellow, the increasing number of chlorophyll in stomata shown in Fig.7a is shown after irrigation with effluent the maximum number and size of the leaves in 100% of the effluent treated. The parameters of plant growth i.e. the length of the root, the length of the shoot, fenugreek plants with different effluent concentrations of 25, 50 and 100 % showed a significant increase in their control plant as shown in figure 7a. There was an increase in the content of Chl-a, b, total chlorophyll in leaves fenugreek Plants grown in agricultural land irrigated with mixed effluent after 30.60 and 90 days of growth compared to their control. The protein content in the root, shoot and leaves after effluent irrigation with effluent has been observed increases over control over the entire growth period. The measurement of antioxidants after irrigation of the effluent at different concentrations increases day by day. Antioxidants in the presence of metal ions are considered to play an important role in detoxifying toxic oxygen species. However, plants have adopted several non-enzymatic cellular entities such as ascorbic acid, cysteine, and no protein thiol, etc. to protect against oxidative stress conditions induced by free radicals. The parameters of plant growth i.e. the length of the root, the length of the shoot, in tested fenugreek plants with different effluent concentrations of 25, 50 and 100% showed a significant increase in their control plants respectively.

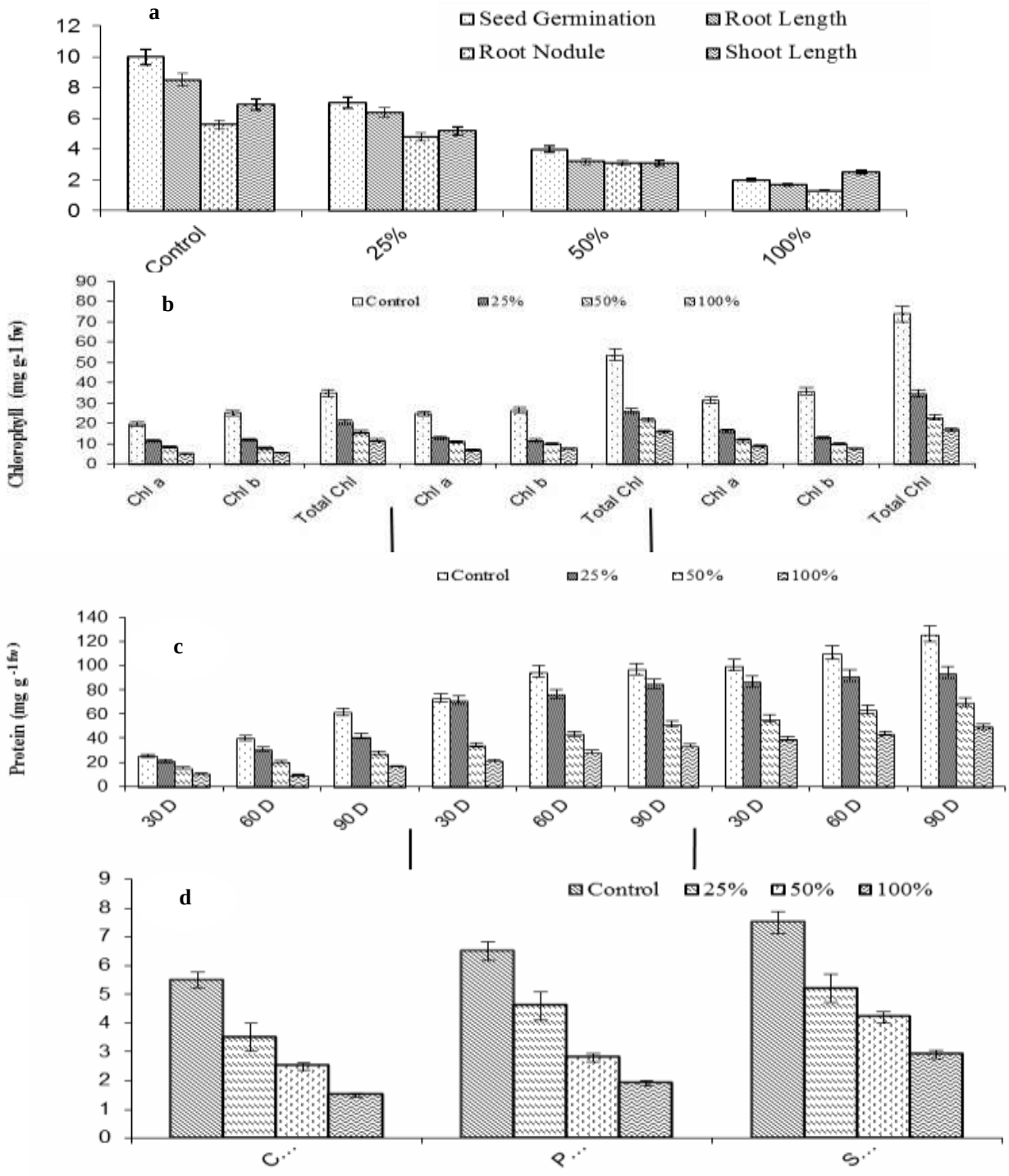


Fig.6.4. Estimation of the different parameters in fenugreek after irrigated with pulp paper industry effluent. (a) Seed germination, root length, root nodule and shoot length (b) Chlorophyll-a, b, and total chlorophyll (mg g^{-1}) (c) estimation of total protein (mg g^{-1}) after irrigated with pulp paper effluent

6.2.2. Surface Damage Study of Root, Root nodule and stomata

The Fenugreek is treated with different concentrations of 25, 50 and 100 % of pulp and paper mill waste discharged after treatment for the entire plant study in pot experiments. Surface root surface damage, cell shrink and Change in morphology study carried out after effluent exposure visually observed scanning microscopy is shown in Fig.6.5. *T.* Fenugreek untreated root surface together with normal root cap Fig.4a-c showed clear, smooth and intact root surface. Significant aberrations, fissures, and fractured tissues were observed on the surface of effluent-treated roots and root cells crumbled, increased in size and ruptured after effluent treatment during SEM analysis. This could be due to the presence in the effluent of different types of carcinogenic, mutagenic and androgenic compounds including heavy metals. The auxins that play a major role in root cap formation can be inhibited by this type of pollutants. This study showed that the effluent contained compounds that have been highly toxic and disrupted by hormones. The mature root nodule of fenugreek is divided into four characteristic regions cortex nodule, meristem nodule vascular system, and Bacteroid region. Bacteroid tissue increases in quantity by the fresh invasion of new meristem-generated cells, but after treatment, root nodule number and size formation is affected in Fig.6.5b. Rhizobium genus bacteria induce the formation of nodules on the nitrogen fixing roots. Many phases involve the formation of nodules (Vincent, 1980). For the fixation of nitrogen to the root and symbiotic association with bacteria, the function of the root nodule is very important for sustained growth. The high toxic effect of fenugreek physiology and genotoxicity is shown in 100 % effluent, so this manuscript provides comprehensive information on effluent toxicity and its effect on crop plants for human health safety. In addition, stomata studies after treatment are found to affect the hormonal signalling mechanism directly and the rate of transpiration. The metals have the highest concentrations, i.e. Cd and Zn are found in epidermal cells of the leaf, four times the concentration of mesophyll cell. Therefore, this preferred storage in the leaves epidermal cells could be associated with heavy metal damage to photosynthesis, even though epidermal cells lack chloroplasts including guard cell shape. This manuscript contains information on the entire pulp paper mill effluent physiological and biochemical effects after irrigation.

6.2.3. Chromosomal aberration

Identified some mutagenic and EDCs compounds, i.e. Hexadecanoic acid, trimethylsilyl ester acid, Stimgmasterol trimethylsilyl ether and β-sitosterol trimethylsilyl ether from effluent creates chromosomal changes in different cell divisions (Fig.6.7). This is strong evidence for evaluating the toxicity of mitotic index and chromosomal aberrations in fenugreek present in the effluent after biological treatment. In addition, chromosome abnormalities are induced by cytotoxic and mutagenic compounds i.e. chromosome breaks, lagging. The residual organic pollutants have disturbed the balance in the number of histones and this report confirmation of androgenic, mutagenic compounds present in the pulp paper industry sludge (Chandra et. al., 2017; Leme et al., 2009). The toxicity effect represents the stickiness of both the chromosome and is likely to result in cell death. The chromosome decreased in size, or break and nucleus show abnormalities in shape after becoming irrigated with 100% effluent. In conclusion, as mentioned above, discharged effluent have adverse effects on fenugreek plant growth and development.

6.2.4. Accumulation of metals in fenugreek plants

In the pulp paper mill discharged effluent site some agricultural land is used as growing crops and formers use the effluent directly in crops so that the effluent is not only toxic to crops but also affects human health. Accumulation of heavy metals in the root, shoot leaves and seed part of fenugreek plants are shown Fig.6.6. Moreover, Fe and Cu concentration in shoot organs of the plants was higher than comparison to other metals. In addition, the Cr also detected from fenugreek plants after exposure to pulp paper mill effluent, thus Cr interfering with several metabolic processes, causing in plant toxicity due to reduced seed germination (Sharma et al., 1995). However, metabolic modification has also been reported in plants as the direct effect on enzymes and metabolites or their ability to produce reactive oxygen species (Shankar et al., 2005).

6.2.5. Ultrastructure observations of root tissues

Fenugreek seeds and green leaves are used in both food and medicinal applications, which are the old practice of human history but continue to industrialize affect nutrient quality. Moreover, the treatment with root effluent in fenugreek showed cell organelle

damage, tissue, protein and antioxidant activity affected. After exposure with pulp paper effluent the result revealed of TEM analysis showed nucleus change, mitochondria, and endoplasmic reticulum completely disappears from the cell Fig 6.8. This study indicates that the heavy metals and other complex pollutants affected the growth and development of fenugreek. Although the study of the accumulation of heavy metals in fenugreek root, shoot and leaves are above the permissible limit is harmful to food and another growth factor of plant. Ultrastructure fenugreek root tissue observation showed metal granules deposition in the cell wall, cell membrane, cytoplasm, nucleoplasm and mitochondria at low and high magnification. This plant root could see the heavy metal deposition inside the vacuoles, middle lamella, and cell wall. The development of a larger number of nucleolus and vacuoles at a high level of heavy metals increases ribosome and mRNA production which ultimately improves the development of new proteins which leads to metal resistance, but after disruption to mitochondria and chromosome, the tolerance potential is low after treatment.

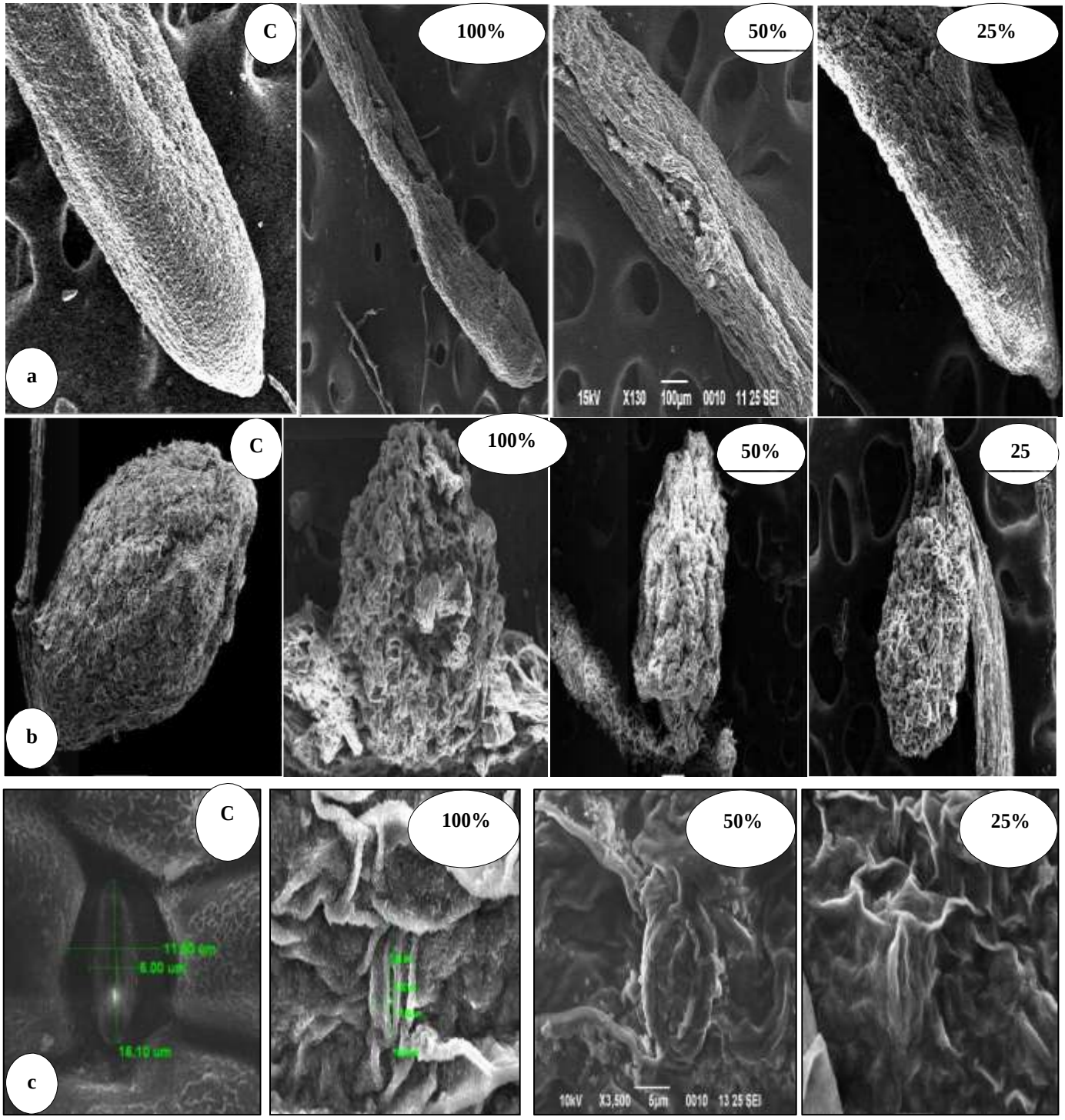


Fig. 6.5.Scanning Electron Microscopy analysis of root, root nodule and stomata cells of fenugreek after exposure with pulp paper mill effluent (a). Root surface study (b). Root nodule, (c). Stomata cell on epidermal.

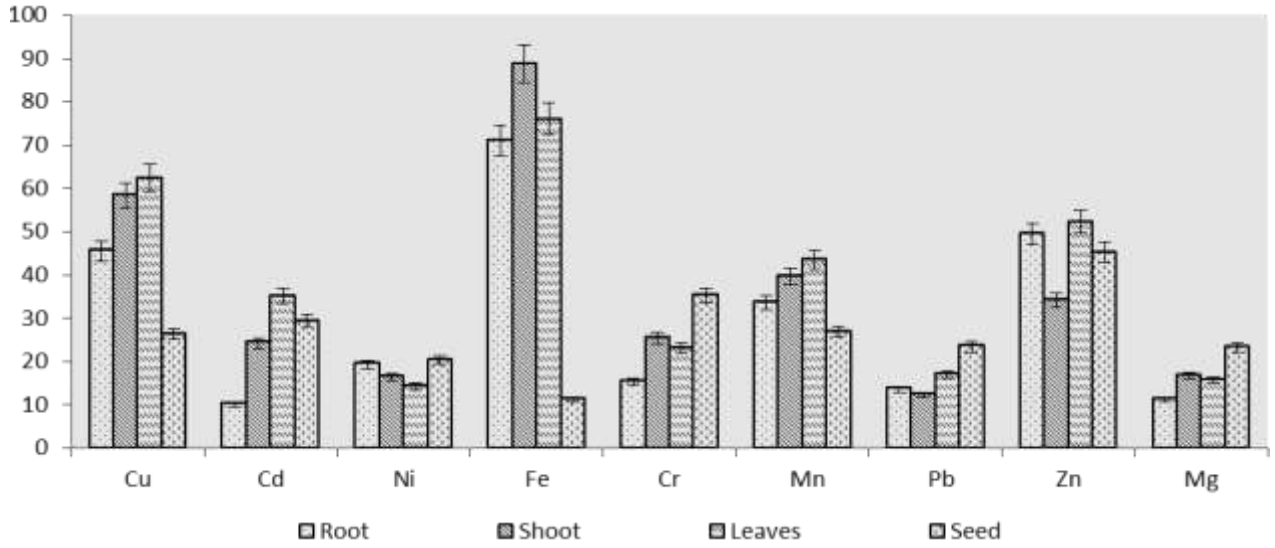


Fig. 6.6. Graph showed the accumulation of different heavy metals in their root, shoot, leaves, and seed of fenugreek after secondary treatment

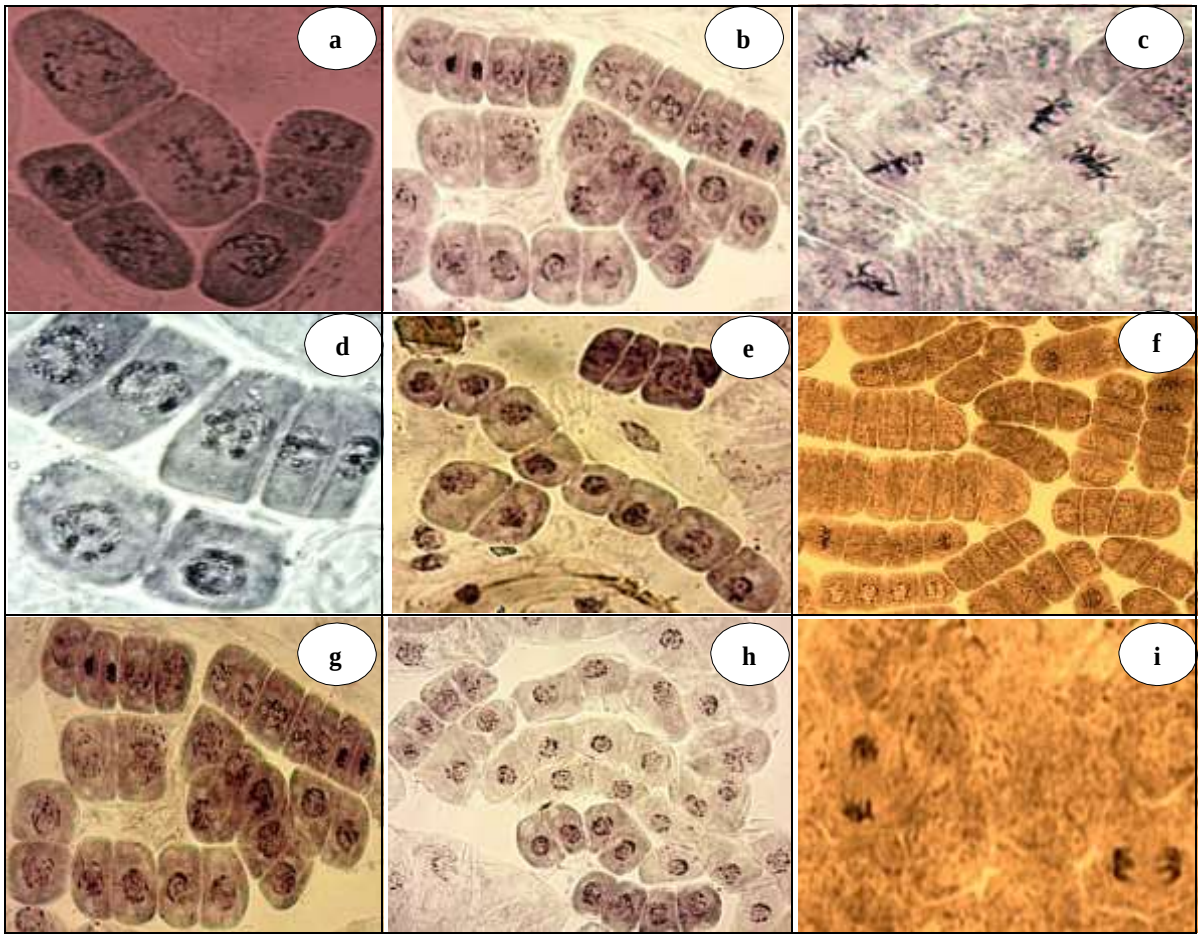


Fig. 6.7. The different phase of chromosomal aberration induced by pulp paper mill effluent after treatment in fenugreek at different phase.

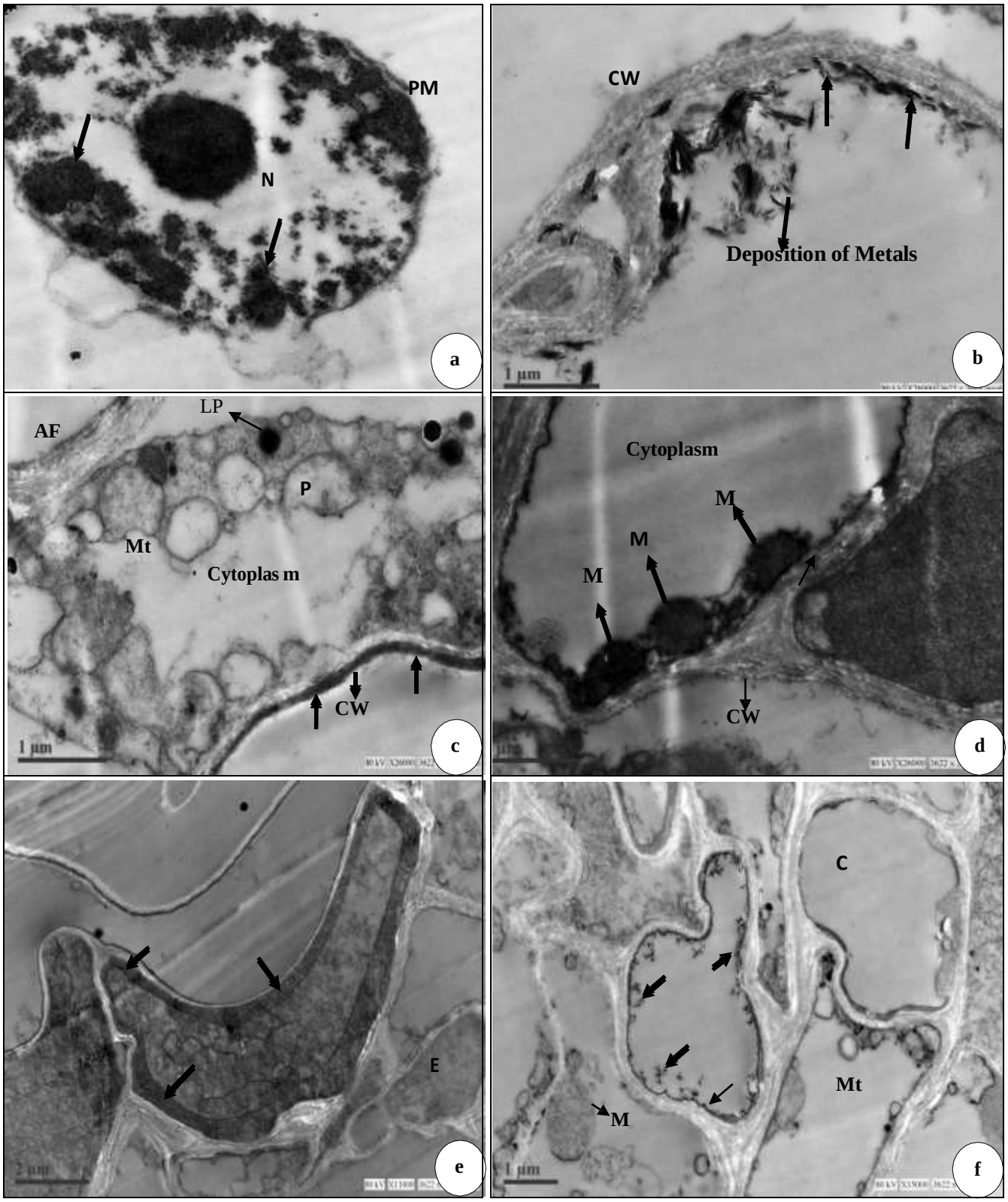


Fig. 6.8. TEM analysis of *T. foenum-graecum L* roots after in-situ accumulation of heavy metals in root tissue of fenugreek. CW- cell wall, V- vacuole, PM-Plasma membrane, DMG-Deposited metal granule.

Conclusions

The discharged effluent from the pulp paper industry is not safe for crop irrigation heavy loaded with different organometallic pollutants along with BOD, COD and other physico-chemical parameter. It indicated that selected agricultural land and mixed effluent to irrigate agricultural land were not appropriate for agricultural practices even though, due to their strong affinity as legends to sulfur, high metal content can directly or indirectly interfere with fenugreek by changing protein conformation, e.g. enzymes, transporters or regulatory proteins. Consequently, several organic pollutants along with mutagenic and androgenic compounds i.e. Hexadecanoic acid, Tetradecanoic methyl ester, Pentadecanoic acid, methyl ester Octadecanoic acid, and β-Sitosterol trimethylsilyl ether are also identified through GC-MS analysis in my chapter four. Moreover, TEM analysis showed strong evidence of heavy metal storage and damage to mitochondria, endoplasmic reticulum disappearance, nucleus, and other cell organs. After 90 days of treatment with 100% effluent, stomata and chlorophyll are highly affected due to disturbance of phytohormones signals. In addition, released effluent is not appropriate for agricultural property or irrigation, and this information is helpful for knowledge of hazardous compounds even after secondary treatment in the effluent.

Chapter-Seven

Investigation the molecular mechanism of hyperaccumulator plant growing at pulp and paper waste disposal site

Investigation of Molecular Mechanism of Metal Hyperaccumulation in Plants Growing at Pulp and Paper Waste Disposal Site

7. Introduction

Heavy metal pollution is spread throughout the world, damaging the ecosystem and causing serious health problems to humans and animals. The main source of heavy metals in the environment i.e. metallurgical work, urbanization, and industrialization, particularly in highly populated developing countries such as China and India (UN-HABITAT, 2004). Further, the huge amount of waste is generated during the biological and physical treatment process in the pulp paper industry, ranging from 0.3 to 1 m^3 of primary sludge per ton of manufacturing of paper (Priadi et al., 2014). Furthermore, the environmental protection agency (EPA) in United State reports more than 250 million tonnes of municipal solid waste (MSW) are generated every year, which approximately 30% are related to pulp paper industry waste, while one tone of paper produced generate approximately 0.4 tons of waste discharged in the environment without proper treatment (IPPC, 2001; EPA, 2002; CANMET, 2005; Toczylowska- Maminska, 2017). In addition, the sludge waste generated during the paper manufacturing from the paper industry is divided into four categories as follows: first is the primary sludge wastes generated by the production of virgin wood fibers, second is the destination sludge wastes generated by removal of the fiber ink, activated waste after biological treatment is secondary sludge, waste from paper production for biological purposes is complex sludge. In the biological process in which microorganisms transform the organic matter in the sludge waste into a type of soil fertilizer (Boni et al., 2004). Numerous techniques are already used to clean up the environment from such type pollutants, but most of them are expensive and far from their optimum performance (Rakhshaee et al., 2009). Further, the low molecular weight compounds discharged from pulp paper industry wastewater like as biocides, chlorophenols, and other organic chlorinated compounds are released in the environment during the pulping process, while this is the main source of mutagenicity in fishes. These compounds are properties of bioaccumulation due to their hydrophobic nature and their ability to penetrate cell membranes (Savant et al., 2006). Furthermore, soil–crop systems

provide a classic example of abiotic and biotic interactions in the environment. In addition, BCF and TF of various heavy metals and metalloids in crop plant to soil interaction, primarily in important worldwide crops such as wheat and maize, have been reported as a significant criterion for evaluating global health problems (Wang et al., 2017a, 2017b,). Moreover, several heavy metals have adversely effect on different enzymes including acid phosphatases, proteases, and α-amylases and protein profiles involved in germination. For instance, heavy metals decreased the starch content, reduced the nutrient content, impaired the PS-II of the chloroplast, and induced the expression of heat shock proteins and proline (Rai, 2016; Seneviratne et al., 2017). The heavy metals enter into the crop plant's root cells via the activity of metal cheaters or transporters like phytosiderophores (Guerinot, 2000; Shenker et al., 2001; Perfus-Barbeoch et al., 2002; Eide, 2004; Babula et al., 2008). The objective of this study to evaluate the potential of the phytoremediation strategy for the treatment of polluted heavy metal site, to provide a brief description of the processes used by native hyperaccumulators plants. Furthermore, the aim of this study was to investigate the pollutants of pulp paper industry sludge (PPIS) and accumulation, uptake, of heavy metals in selected native hyperaccumulators plants from the disposal site of the pulp paper industry.

7.1. Material methods

7.1.1. Sample collection

The collection of sample is two different pulp paper industry i.e. M/s Century pulp paper mill Ltd (PPI-1) is located (29ºN, 79.3ºE) at Lalkua, India, and M/s K.R pulp paper mill Ltd (PPI-2) located (27°50'31.8"N 79°51'15.7"E) in Shahjanpur, India. For this study samples were collected in a 20 kg (Tarson Production Pvt. Ltd., USA) sterile plastic bag from the pulp paper mill disposal site. After collection, the bags were kept at 4ºC until further use. Furthermore, five characteristic plant species i.e. *Brassica campestris L.* (Brassicaceae) and *Chenopodium album L.* (Amaranthaceae), Ricinus *communis* (Euphorbiaceous), *Ranunculus sceleratus* (Ranunculaceae), *and Rumex dentatus* (Polygonaceae) were collected on the basis of abundantly growing on sludge from PPI-1 and PPI-2 (Fig.7.1). In addition, the study of normal plant (as control) growth plant samples were collected from normal agriculture land. For the confirmation and

authenticity of scientific data, this process has been repeated three times in different seasons.

7.1.2. Physico-chemical analysis of sludge

The Physico-chemical analysis of PPI-1 and PPI-2 was performed different Physico-chemical parameters including pH, EC, TS, TDS, TSS, BOD, COD, described method in (APHA, 2012). Moreover, the various ions i.e. chloride (Cl^-), potassium (K^+) and sodium (Na^+) along with heavy metals by the previous described method in chapter five.

7.1.3. Scanning Electron Microscopy and UV-Vis Spectral Analysis

For the surface structure investigation of the sludge sample of PPI-1 and PPI-2 is done by SEM analysis. The sample was completely dried in the hot air oven (Thermo Scientific) at 50°C, after the dried sample was converted into 10 mg powder form, were described the method by Yadav and Chandra, (2018).

7.1.4. FT-IR analysis of sludge of PPI-1 and PPI-2

The FT-IR analysis of different functional groups and their bond intensity of sludge sample discharged from the pulp paper industry is the previous described method (Yadav and Chandra, 2018).

7.1.5. Detection of residual organic pollutants from sludge by GC-MS analysis

7.1.5.1. Solid-liquid extraction

The detection of a broad range of different residual organic compounds and other endocrine-disrupting chemicals compounds present in discharged sludge of both pulp paper industry i.e. PPI-1 and PPI-2 were extracted using dichloromethane (DCM) solvent by previously described method (Chandra and Kumar, 2017, Yadav and Chandra, 2018).

7.1.5.2. Characterization of organic pollutants through GC-MS

For the detection of organic pollutants present in sludge, sample (200 µl) was extracted and transferred into GC vials, and dry with nitrogen gas. The organic pollutants were identified by comparing their mass spectra (m/z) with recorded at different retention times (RT) in the NIST library (Chandra et al., 2011).

7.1.5.3. Estimation of biochemical parameters

7.1.5.3.1. Protein content and Photosynthetic pigments

The measurement of protein content in plants grows on contaminated site (treated) and agriculture land (control) by using bovine serum albumin (Sigma) as per the described standard method (Lowry et al., 1951). For the estimation of protein using 200 mg, fresh leaves samples added 10 ml of 10% chilled trichloroacetic acid (TCA) were crushed with the help of mortar and pastel.

7.1.5.3.2. Preparation of enzyme extract

For the preparation of enzyme extract for estimation of antioxidants analysis, described by the above method.

7.1.5.3.3. CAT assay

The estimation of CAT enzyme activity in treated plants compared to control was measured in a reaction mixture added 50 mM/L phosphate buffer (pH 7.0), 150 mM/L H_2O_2 for 2 min OD was recorded at 240 nm Chance and Maehly, (1955). In addition, the POD activity was determined by using the 4-methyl catechol substrate. While, the increase absorbance caused by oxidation of 4-methyl catechol by H_2O_2, was measured at 420 nm spectrophotometrically (UV-160, Shimadzu, Japan). The mixture of a reaction containing 5 mM 4-methylcatechol, 100 mM sodium phosphate buffer (pH 7.0), 500 µL of crude extract in a total volume of 3.0 mL at room temperature (Onsa, 2004). The sample was centrifuged at 10,000 rpm for 10 min at 4°C and estimation of total protein by described the previous method (Lowry et al., 1951). Frequently, photosynthetic pigment estimation i.e. Chl-a and Chl-b, the 100 mg fresh leaf sample were crushed with pestle and mortar in 5 ml of chilled 80 percent acetone (Arnon, 1949). The sample was centrifuged for 10 min at 4ºC at 5,000 rpm, collecting the supernatant and measuring the chlorophyll content in a spectrophotometer .In addition, the carotenoid content was also calculated at the same step above (Duxbury and Yentsch, 1956).

7.1.5.3.4. Lipid peroxidation content and hydrogen peroxide

For the estimation of Lipid peroxidation content in plant sample, 200 mg of fresh leaves added with ml of 0.1% trichloroacetic acid (TCA) and crushed in a mortar and pestle Heath and Packer, (1968). Moreover, the absorbance at 532 nm in a spectrophotometer (UV-160, Shimadzu, Japan). In addition, the estimation of H_2O_2 is described method of Velikova et al., (2000).

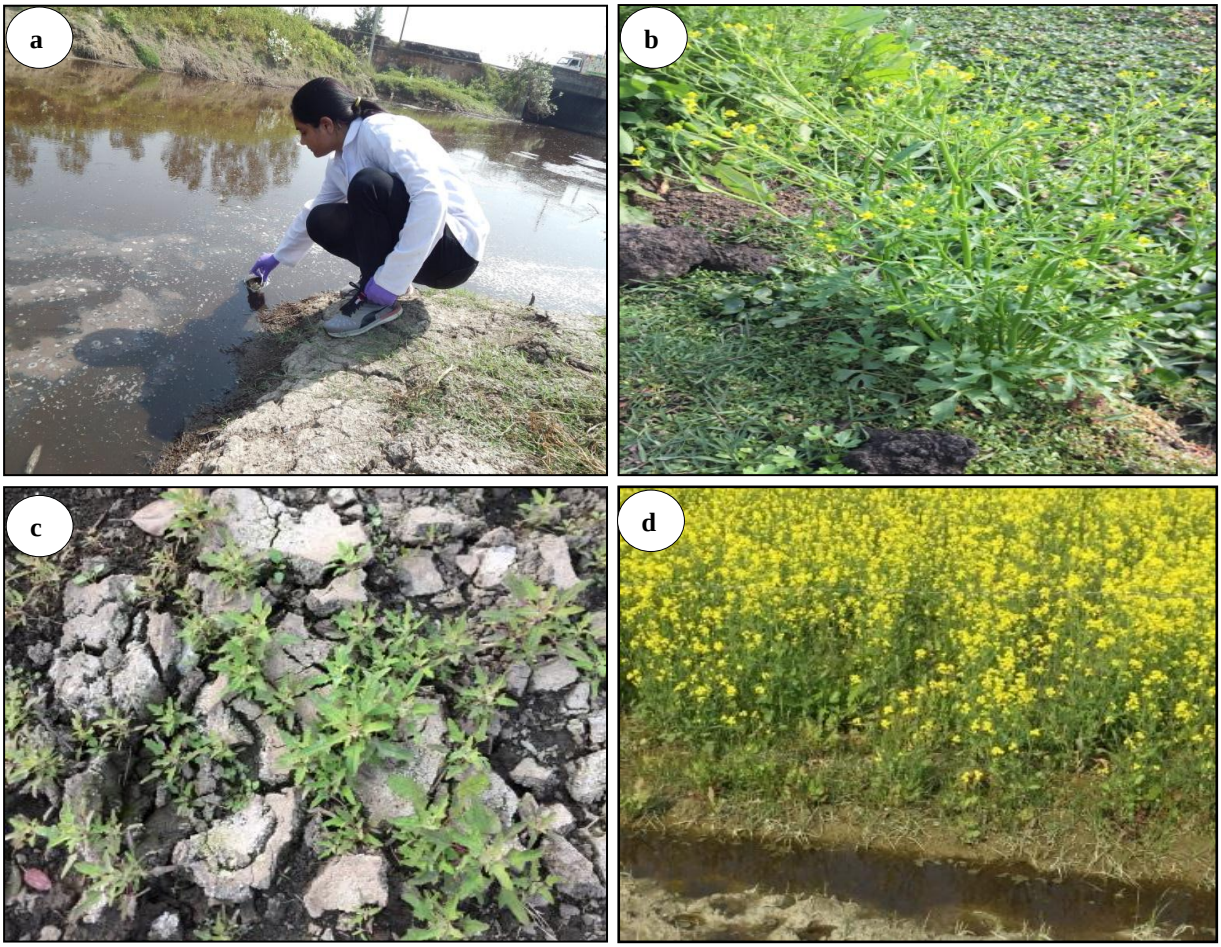


Fig.7.1.Showing the discharged of pulp paper industry waste indirectly environment. (a-b). Collection of the sample (c-d) collection of native plants growing at pulp paper industry contaminated site

7.1.5.3.5. Ascorbate assay

The estimation of ascorbate from treated plant and control, the 50 mg of fresh plant leaves sample was homogenized with 2 ml of enzyme extract. Further, the sample was centrifugation at 2,500 rpm for 15 min at 4°C and supernatant collected was used to measure Ascorbate activity at 520 nm within 2±5 min as per the described method of Keller and Schwager, (1977).

7.1.5.3.6. SOD assay

The activity of SOD was measured by the inhibition in photo-reduction of nitroblue tetrazolium (NBT) by the absorbance was recorded at 560 nm using a spectrophotometer (Nishikimi and Rao, 1972).

7.1.5.3.7. APX assay

The estimation of the APX enzyme has measured the oxidation of ascorbate in presence of H_2O_2 was recorded at 250 nm in terms of decreases in absorbance and the activity of APX was expressed in terms of mM ascorbate oxidized min-$1g^{-1}$ of weight (Nakano and Ascada, 1981).

7.1.5.3.8. Estimation of heavy metal in plant

To estimate the concentration of different heavy metals in the different parts of the *Brassica campestris* L. *Chenopodium album* L. *Ricinus communis, Ranunculus sceleratus, and Rumex dentatus* plants growing at the contaminated site of pulp paper industry. Moreover, calculate the concentrations of different heavy metals i.e. Cu, Cd, Ni, Fe, Cr, Mn, Pb, Zn, and Mg were measured by Coupled Plasma (ICP) spectrophotometer (IRIS Intrepid II XDL: Thermo Electron, Waltham, Mass., USA) (Chandra et al.,2017).

7.1.5.3.9. The efficiency of metal accumulation

For the evaluation of metal accumulation efficiency in collected native hyperaccumulators plants growing on organometallic complex sludge bed, calculate the bioconcentration factor (BCF) and translocation factor (TF) respectively. Moreover, the methodology of calculation of BCF and TF described in my previous chapter.

BCF= Metals in root/Metal in sludge…… (i)

TF= Metals Shoot/ Metal Root…… (ii)

7.1.5.3.10. Histological observations of root tissues by TEM

For the histological observation of heavy metal accumulation inside the plant root tissue, the root tips were cut approximately 2.0 mm for section cutting and fixed 2.5 % glutaraldehyde solution as described in chapter five (Khan et al., 1984).

7.1.6. Statistical analysis

All the experiments were performed in triplicates. Further, to confirm the validity of data, an analysis of variance (ANOVA) was performed and significant differences in different parameters were verified by Duncan's multiple range tests (DMRT, $p \leq 0.05$).

7.2. Results and Discussion

7.2.1. Physico-chemical characteristics of the sludge of PPI-1 and PPI-2

The Physico-chemical analysis of both industries i.e. PPI-1 and PPI-2 sludge showed the presence of high pH (8.2±8.6) and different ions i.e. Na^+ (547±745), Cl^- (6.34±8.54), and K^+ (23.65±35.65), concentration is beyond the permissible limit shown in Table.7.1. The black color of sludge is appeared due to the presence of lignin and another organic compound during the pulping and bleaching process in the industry. Furthermore, the process of papermaking is a different stage of physical, chemical and biological treatment, generate a huge amount of wastewater is contains high TS (1845±2678), TDS (1456±2756), TSS (395±189), COD (15169±43587), BOD (18961±1569) and EC (1896 ±2067) value. Moreover, the concentration of total phenol and lignin was also higher in sludge might be planted constituents and a major source of water pollution and cause serious problems for flora and fauna (Yadav and Chandra, 2018). The higher concentration of phenol is responsible for inhibiting the photosynthesis of algae, diatoms and other microorganisms in the aquatic ecosystem (Kostya, 1973).

7.2.2. SEM and UV-Vis Spectral Analysis

The SEM analysis of the sludge sample of PP-1 and PPI-2 revealed the presence of the lignocellulosic organic polymer and organometallic compounds along with metals are shown in Fig. 7.2a-b. The result showed that the sludge image is irregular, elongated rod or cylindrical shaped bodies of different organic polymer is present in the waste (Yadav and Chandra, 2018). Moreover, the release of different lignin and fibers component in waste during the bleaching process and rod shape structure might be cellulose or lignin in sludge. A similar observation for the granulated appearance of lignin with the complete structure of different size has been reported in an earlier study (Liu et al., 2013). Furthermore, the irregular shape is also indicating the lignin complexation with different heavy metals and another carbonyl, hydroxyl and phenolic compounds (Demirbas, 2007). In addition, the UV-Vis Spectral wavelength range of 250-700 analysis of both sludge sample PPI-1 and PPI-2 is to assess the availability of dissolved organometallic compound present in sludge is used method (Martins and Boekel, 2003). The result

indicated the height of different peak absorption in UV-region and their maximum absorbance was noted λ_{max} 320 in the sludge of PPI-1 as shown in Fig. 7a. Although some new peak showed in PPI-2, maximum absorbance was noted λ_{max} 310 showed in Fig.6b. Further, the change of peak area and height is different in both samples indicating the difference of compound conversation into complex metabolites in nature from the contaminated site of the pulp paper industry (Chandra et al., 2018).

7.2.3. FT-IR analysis assessment

The discharged waste containing lignocellulose material mainly biopolymer of cellulose, hemicellulose, and lignin are rigid and complex structures in the sludge of the pulp paper industry after secondary treatment (Zheng et al., 2014). Furthermore, the result revealed that FT-IR spectra cover a wide range of functional groups with strong and weak bonds of organic compounds and polymers from two different samples i.e. PPI-1 (sludge) and PPI-2 (sludge) for investigation of organic and inorganic pollutants. The contains different fatty acid is a by-product of the lignin during pulping and bleaching process in papermaking. While 1059.0 cm^{-1} absorption is contained functional group is ethers and C-O-C stretch system, and bonds are strong. Consequently, ether deformation is grafting of epichlorohydrin onto the lignin from pulp paper waste. In addition, the comparative analysis of PPI-2 is a sludge waste sample is showed the broadband i.e. 2850.7 cm^{-1} absorptions belongs to the functional group of carboxylic acids with O-H strong bond. In addition, the result showed PPI-1 broadband was noted at 2919.4 cm^{-1} absorptions is containing functional group Carboxylic acids and derivatives bonds are O-H with strong intensity indicates the presence of various organic compounds in PPIS. The broadband region 1408.6 cm^{-1} absorption containing functional group Aromatic Compounds and C=C stretch bonds is strong and often weak in nature showed the fatty acid present in PPI-1. Furthermore, 2924.9 cm^{-1} function group is alcohols, amines, and phenols with C-H stretch and strong bond intensity. Although, the other peak at 1544.9 cm^{-1} is related to the functional group of O-H is alcohols and organic acids are noted. The broadband at 687 cm^{-1} functional group amides and N-H bend with medium-strong bond intensity. The FT-IR results investigate the function group and their bond intensity because of the exchange of metabolites during in-situ remediation of pulp paper sludge waste.

Table.7.1.Physico-chemical characteristics of discharged Pulp Paper mill Effluent along with heavy metals content collected from M/s K R Pulp Paper Ltd. Shahjahanpur, Uttar Pradesh, India All the values are means of triplicate (n=3) ±SD. Unit of all parameters is in mgl^{-1} except pH, color (Co-Pt. Unit) and EC ($\mu mhoscm^{-1}$)

Parameters	**PPI-1value (mean)**	**PPI-2 value (mean)**	**Permissible limit (EPA 2002)**
pH	8.2±0.22	8.6±0.25	5-9
Color	2741±101	2534±105	Colorless
TS	1845±104	2678±147	2100.00
TDS	1456±24.21	2756±0.05	-
TSS	395±1.21	189±0.13	35
COD	58961±232	43587±230	250
BOD	18961±141	15169±191	30
EC	1896 ±70	2067 ±88	1000
Total Phenols	823±23.58	1027±19.34	0.50
Total nitrogen	279±3.33	654±0.21	143
Sulfate	1854±0.31	3358±1456	250
Phosphorus	181±6.71	457±7.28	200
Cl^-	6.34±0.21	8.54±0.21	1500
Na^+	547±11.33	745±10.31	200
K^+	23.65±0.71	35.65±0.81	-
Lignin	44587±117.02	49678±141.33	-
Chlorophenol	427±11.31	561±14.61	3.0
Heavy metals (mg kg^{-1})			
Cu	154.21±1.28	211.±0.23	2.00
Cd	64.23±0.51	72.03±0.21	2.00
Ni	561±0.05	661±0.11	0.50
Fe	2.34±0.01	3.67±0.05	0.01
Cr	18.34±0.22	21.30±0.21	0.20
Mn	8.34±0.05	9.37±0.25	0.10
Pb	3.14±0.11	4.21±0.20	-
Zn	9.36±0.15	11.37±0.18	-
As	2.37±0.24	3.69±0..33	-
Mg	7.38±0.22	9.37±0.18	-

7.2.4. Identification of persistent organic pollutants from sludge

The identification of persistent organic pollutants present in two different pulp paper industry sludge i.e. PPI- and PPI-2 extracted with DCM in detail at different retention time (RT) based on mass spectra (*m*/*z*) in chromatogram showed in Fig.7.3 and Fig.7.4 The result revealed that PPI-1 sludge waste contained a large number of residual organic pollutants in the range of (6.88 to 49.48) characterized by GC-MS Table.7.2.

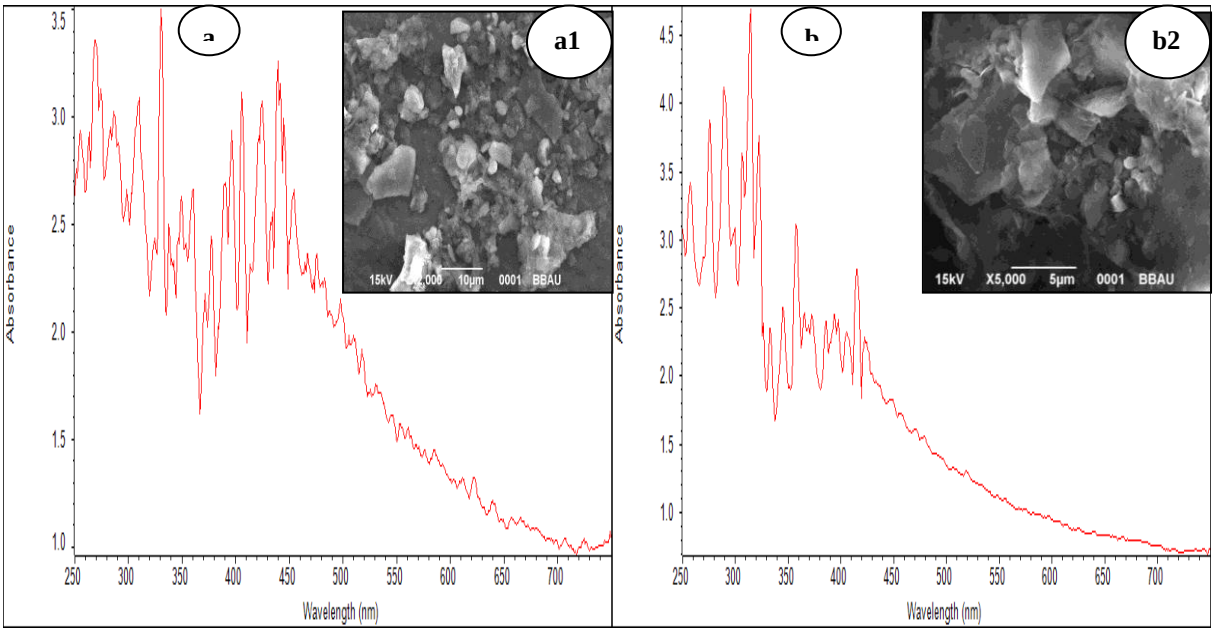


Fig.7.2 SEM image showing of pulp paper waste site from PPI-1 and PPI-2. (a-b) SEM image shows the content of heavy metals indicated by arrow and lignin (c-d) UV-Vis (250-700)

For the PPI-1 are detected major compounds as saturated fatty acids and EDCs compounds such as hexadecanoic acid, trimethylsilyl ester (RT-10.59), 1-tetradecane (15.10), pentadecanoic acid, ethyl ester (25.25), hexadecane (31.35) and hexadecanoic acid, trimethylsilyl ester or palmitic acid TMS (48.46) respectively. The hexadecanoic acid, trimethylsilyl ester, Pentadecanoic acid, ethyl ester, and hexadecane is characterized in our result; it is planted fatty acids and antiquorum sensing molecules of the bacterial product reported by Singh et al., (2013). In addition, some studies have been reported that the toxicity of compounds in aquatic ecosystems and inducers of DNA damage, human melanoma cell line (Kamaya et al. 2003; de Sousa Andrade et al. 2005). Moreover, these compounds are also listed under EDCs as per USEPA, (2012) guideline. In addition, the huge amount of existing carbon was due to the cellulose throughout the sludge from the production process and its procedures (Sutcu and Akkurt, 2009). Consequently, these compounds have been as key constituents of environmental pollutants responsible for dermal irritation and eye. In addition, our finding we have extracted some sample near the root zone of *Brassica* and *Chenopodium* and the result is interesting some hazardous organic compounds are disappearing and detected new metabolites in rang of (6.64 to 49.65) at different peak shown in Fig.7.2. Furthermore, GC-MS chromatogram obtains from PPI-1 sludge waste is in-situ remediation the disappearance of many peaks and

formation of some new peaks i.e. 2 ethyls 4-6 dimethyl-1,3,5-trioxane (RT-6.64), Eicosane, 3-methyl (RT-8.42) and 1-(1-Ethoxyethyl)-2,3,4,5,6,7,8-heptaethylporphyrin (RT-11.89). These new compounds generate after in-situ remediation and Eicosane is also detected in sludge waste is an alkane group compound generated during the pulping and bleaching process after secondary treatment. This finding revealed that the disappearance of major toxic organic compounds has occurred along with the simultaneous transformation of some new metabolites in the nature of carcinogenic, mutagenic, and EDCs. During the in-situ remediation process higher molecular weight compounds break into the low molecular weight compounds and further degraded by the aromatic ring cleavage. Additionally, six major peak compounds identification from PPI-2 sludge waste such as 2 ethyl 4-6 dimethyl-1,3,5-trioxane (RT-6.61), 1- monopalmitin-ditms (RT-7.07), cinnamic acid-α-phenyl-trimethylsilyl ester (RT-8.28), heptane, 2, 2, 3, 3, 5, 6, 6-heptamethyl (RT- 9.95), tetradecanoic methyl ester (RT-11.88) and Hexanoic acid (RT- 32.08) respectively. The phenolic compounds present in pulp paper sludge might introduce the dangerous effect of microbial and plant life in the soil and water. The Cinnamic acids are a by-product of lignin and hemicellulose is generate dunging alkali bleaching process and this is also produced ether linkages and ester by the reaction of their carboxyl and phenolic groups (Jeffries, 1990). These complex pollutants in pulp paper sludge are generated during the pulping and bleaching process in industrial. Moreover, several studies are confirmed that these compounds have also been reported in PPIS and cause serious health problems with mutagenic and carcinogenic nature (Yadav and Chandra, 2018; Chandra et al., 2018). In addition, some other hazardous organic compounds present in PPI-2 sludge waste i.e. β- Sitosterol (12.85), Decanol, 2-hexyl (23.23), and Benzene dicarboxylic acid (37.24). In addition, some phenolic and non-phenolic compounds extracted i.e. benzene dicarboxylic acid has also been obtained in sludge waste and responsible for skin disease. In an earlier study, reported that aerobic and anaerobic microbes are able to transform β-sitosterol and other sterols into androgenic hormones, i.e. 5-β-androstane 3, 17-dione and androstane 4-en-3, 17-dione (Taylor et al. 1981). The result indicated that persistent organic pollutants present in PPIS and their toxicity were very high for crop plants.

7.2.5. Effect of heavy metals on biochemical parameters

7.2.5.1. Effect on Protein content and Photosynthetic pigments

In our study revealing the content of chlorophyll a, b and carotenoids were recorded in *Brassica* and *Chenopodium* leaves growing at the disposal site of PPI-1 and PPI-2 sludge bed comparisons to control. The increased concentrations of Chl-a and Chl-b in *Brassica campestris L. Chenopodium album L., Ricinus communis*, *Ranunculus sceleratus, and Rumex dentatus* respectively, plants can be connected with the existence of macro and micronutrients, i.e. Cu Plastocynin, protein, Fe contains electron transports in cytochrome chains as well as multiple organic and inorganic contaminants (Sheetal et al., 2016). In addition, the concentration of carotenoids expected to behave as antioxidants by scavenging free radicals, transferring electrons to dual bond structure and eliminating light damage, cell damage, destruction of chloroplast membranes and genetic material by photodynamic response, quenching, membrane collapse and replacement of peroxidation by enhanced accumulation of hazardous metals and metalloids (Czerpak et al., 2006). Furthermore, the estimation of protein content in *Brassica* and *Chenopodium* leaves of plants at the contaminated site PPI-1 and PPI-2 sludge was found also higher in comparison to normal agriculture land plants. In addition, the analysis investigates that the protein content in plants is also significantly enhanced after exposure to PPIS (p. 0.05), respectively.

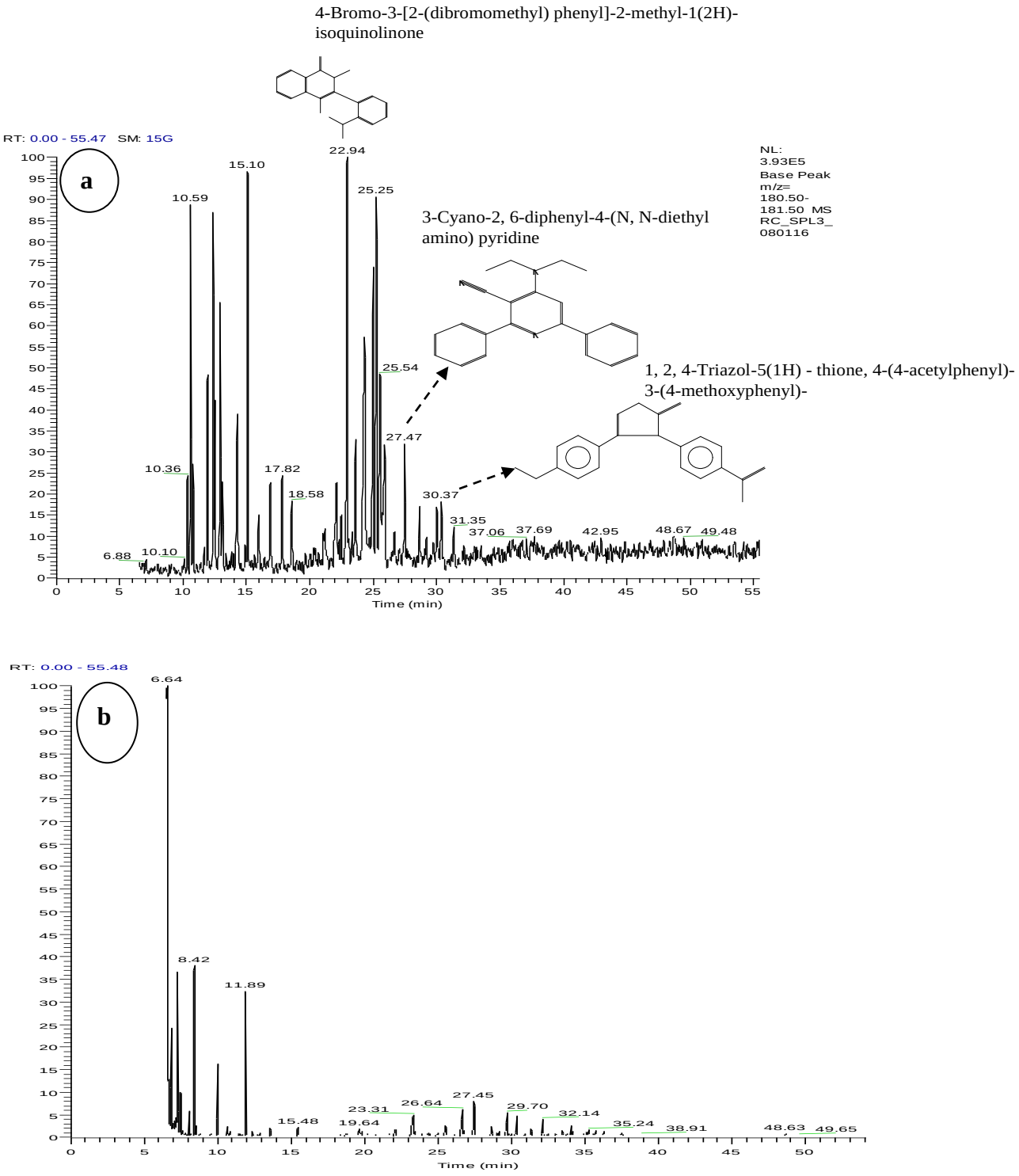


Fig. 7.3. Total Ion Chromatogram (TIC) of TMS derivative identification of organic pollutants from DCM extract of pulp paper sludge from PPI-1 after secondary treatment (a) before phytoaccumulation (b) after phytoaccumulation.

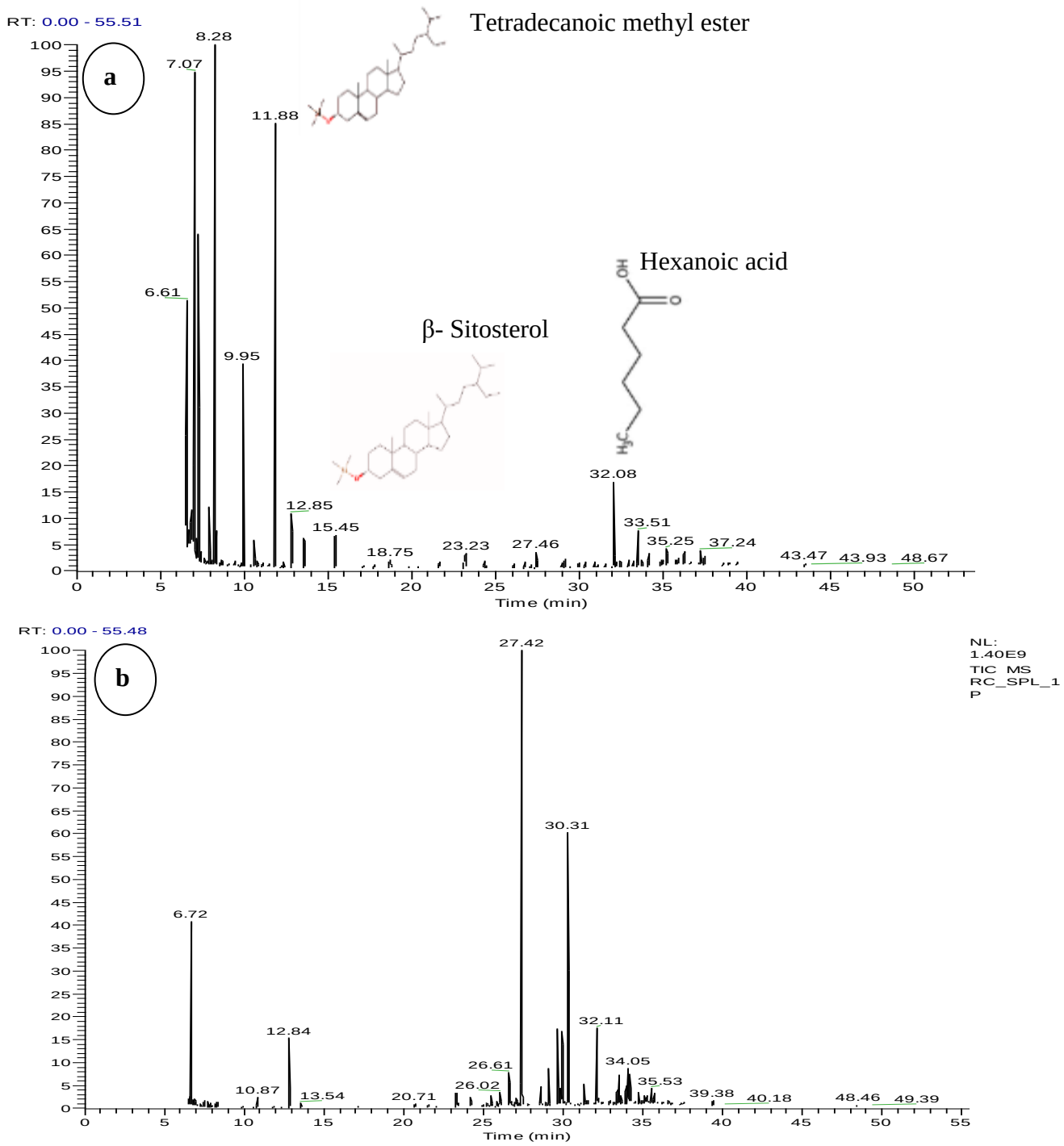


Fig. 7.4. Total Ion Chromatogram (TIC) of TMS derivative identification of organic pollutants extract from DCM of pulp paper sludge from PPI-2 (a) before phytoaccumulation (b) PPI-2, after phytoaccumulation

Table.7.2.Identified residual organic pollutants by GC-M in the TMS derivatized dichloromethane (DCM) extracts of pulp paper sludge after secondary treatment PPI-1 and PPI-2

RT	Compound name	Toxicity
	PPI-1	
6.88	2-Methyl-4-keto-2-pentan-2-ol 1TMS	Endocrine distrusting chemicals (EDCs)
10.10	Furan 2,5-dimethyl	Tumors in rats
10.36	Ethyl guaiacol	Reproductive Toxicity Carcinogenic
10.59	Hexadecanoic acid, trimethylsilyl ester	Mutagenic, carcinogenic
15.10	1-Tetradecene	Nervous system affected, EDC, and odor nuisance
18.58	Eicosane (CAS)	EDCs
25.25	Pentadecanoic acid, ethyl ester	EDCs
27.47	3-Cyano-2, 6-diphenyl-4-(N, N-diethyl amino) pyridine	Data not available
30.37	β- Sitosterol trimethylsilyl	Genotoxicity and DNA damage,
31.35	Hexadecane	EDCs, Cytotoxicity Genotoxicity
48.67	Hexadecanoic acid, trimethylsilyl ester or Palmitic acid TMS	EDCs,
	PPI-2	
6.61	2 ethyl 4-6 dimethyl-1,3,5-trixane	Data not reported
7.07	1- Monopalmitin-DITMS	Data not reported
8.28	Cinnamic acid-α-phenyl-trimethylsilyl ester	Data not available
9.95	Heptane,2,2,3,3,5,6,6-heptamethyl	Data not available
11.88	Tetradecanoic methyl ester	EDC, Comedogenic
12.85	β- Sitosterol	EDC, genotoxic and cytotoxic
23.23	Decanol,2-hexyl	Data not available
32.08	Hexanoic acid	EDC, carcinogen
33.51	Bicycle(2.2.1)heptan-2-one,4-hydroxy-1,7,7-trimethyl	Data not available
37.24	Benzene dicarboxylic acid	Data not available

The protein content recorded for PPIS affected *Chenopodium* plants was 5.173 and 5.367 ug/ml, whereas for PPIS affected *Brassica* plants, it was 4.697 and 5.349 ug/ml, respectively. Increased supply of micro and macronutrients promotes amino acid accumulation and enhances protein content, as well as supplying high proline formation which provides stress resistance by multiple processes such as osmoregulation and enzyme defense against distinct stress circumstances (Noman et al., 2018).Furthermore, the high content of proteins in *Brassica* and *Chenopodium* show the induction of many stress protein growing at chlorolignins contain sludge from pulp paper mill.

7.2.5.2. Effect on MDA and H_2O_2 content

The MDA content in PPIS *Chenopodium album L., Ricinus* communis, *Ranunculus sceleratus, and Rumex dentatus* respectively ,plants enhanced significantly (3.76, 4.21,3.02 and 4.51mmol g^{-1} fw) in comparison to control plants (2.10 mmol g-1 fw) under the potentially toxic hazardous metal stress condition ($p \leq 0.05$). In contrast, the MDA content was reduced in pulp paper sludge affected *Brassica* plant, which was 3.13 mmol g^{-1} fw in comparison control *Brassica* 2.29 mmol g^{-1} fw. Enhanced MDA suggests the generation of excess ROS intracellular. However, these result in membrane disruption as one of the lipid peroxidation by peroxidation of polyunsaturated lipid which generates malondialdehyde (Singh et al., 2018). Furthermore, various earlier research reports by several authors under potential toxic metals stress such as Ni, Cr, Zn, Cu and Cd (Singh and Agrawal 2007; Maheshwari and Dubey, 2009).

7.2.5.3. Effects on antioxidants enzyme

7.2.5.3.1. Catalase, Peroxidase and Ascorbate assay

Comparative pollutants and their toxicity analysis of both industries i.e. PPI-1 and PPI-2 sludge waste are affected *Chenopodium album* L., *Ricinus* communis, *Ranunculus sceleratus, and Rumex dentatus* respectively, plants showed more CAT activity acts as primary biomarkers for the removal of H_2O_2 produced during the metal stress in form of the peroxisomes is previously described by Karuppanapandian et al., (2011). Furthermore, the catalase activity decreased due to the low concentration of hazardous pollutants from pulp paper sludge stress. In addition, the concentration of the POD id increase in *Brassica* and *Chenopodium* in the disposal site of pulp paper sludge is comparing to control (Fig 7.5). The high POD levels enhanced the elimination of H_2O_2 and thus diminished the formation of hydroxyl radicals and preventing the damage of chlorophylls.

7.2.5.3.2. Superoxide dismutase and Ascorbate peroxidase assay

The collected plants i.e. *Brassica* and *Chenopodium* grow on PPI-1 sludge waste affected showed maximum SOD (214.21 and 112.29 Unit gm^{-1} fw) in a comparison to control (growing on agriculture land), respectively. Moreover, the PPI-2 sludge waste *Brassica* and *Chenopodium* plants revealed a significant reduction in SOD activity (106.23 and 98.20 Unit gm^{-1} fw), respectively ($p \leq 0.05$) under potentially harmful toxic element stress in PPIS in comparison to control plants Fig 7.5. Moreover, Peng et al., (2009) reported that

the SOD could catalyze superoxide decomposition and detoxifying processes including anion into radical oxygen, hydrogen peroxide and then transform to anion into radical oxygen, hydrogen peroxide and then converted to the ground level of O_2 and H_2O and usually depends on the potentially toxic metals cofactors. In addition, the activity of APX improvement was demonstrated in Chenopodium plants impacted by PPI-1 sludge waste as well as in Brassica plants under prospective toxic metal stress compared to control plants, whereas PPI-2 sludge wastes affected by Chenopodium and Brassica plants showed less metal toxicity, therefore no significant change in control and PPI-2 was demonstrated (Asada et al., 1992).

7.2.5.3.3. Accumulation of metals in plants

The analysis of PPI-1 and PPI-2 sludge in concentration different heavy metals affected plant growth and showed translocation and accumulation of metals in their root, shoots and leaves parts of *Brassica campestris L. Chenopodium album L.,* Ricinus communis, *Ranunculus sceleratus, and Rumex dentatus* showed in Table.7.3 and 7.4 and Fig.7.6. The collected sludge sample from PPI-1 and PPI-2 were found to have a high concentration of different heavy metals i.e. Cu, Cd, Ni, Fe, Cr, Mn, Pb, Zn and Mg are above the permissible limit. Although, the soil is important to factor for nutrient medium in plant growth and development and the toxic metals have affected the various properties of soil i.e. pH, moisture, organic matter, and humic substances in various ways. Further, the soil pH plays an important role in metal availability and immobility (Harter, 1983). Moreover, the accumulation and distribution of metals in the plant are depending on metal bioavailability and plant metabolism, as well as on the growth of microbes in contaminated sites responsible for uptake of metals in a plant (Balkhair and Ashraf, 2016; Mani and Rayappan, 2014). In addition, the order for metal accumulation in *Chenopodium* and *Brassica* from the contaminated site of *PPI*-1 and PPI-2 sludge bed affected plants. Therefore, Cr is known to impair plant growth and to cause ultra-structural changes in the cell membrane, chloroplast, mediated chlorosis, damage to root cells, decrease photosynthetic enzymatic activity in plants (Ali et al., 2015; Farooq et al., 2016). Furthermore, the agricultural land contaminated with potentially toxic heavy metals causes human health risk related to edible crops Balkhair and Ashraf (2016). The accumulation of heavy metals from PPI-1 is highest in *Brassica* root of Fe (201.02), Zn (49.44), Cu (45.56)

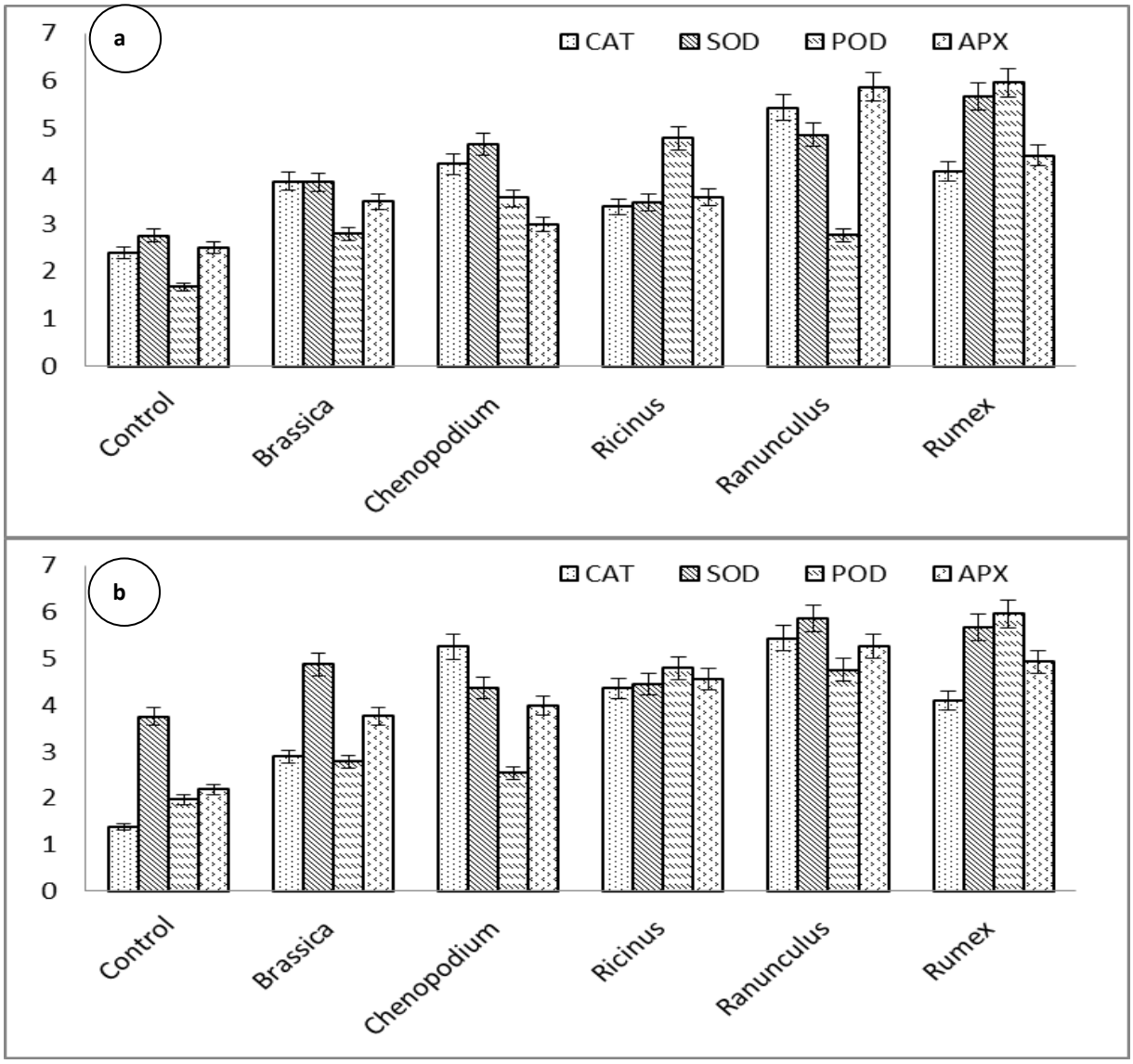


Fig. 7.5. Comparative analysis of CAT, SOD, POD, and APx, activity in leaves of native plants growing at pulp paper industry waste and normal agriculture land (Control) (a). Pulp paper industry-1 (PPI-1) (b). Pulp paper industry-2 (PPI-2)

and Mn (33.64) respectively. Similarly, the PPI-2 in the highest of Fe in *Brassica* leaves of (194.00), and shoots (181.07). The comparative analysis of heavy metals accumulation PPI-1 and PPI-2 in *Brassica* is Cr (S<R<L) and Pb (L<R<S) and Ni (R<S<L) respectively. While the highest accumulation of Fe (228.00) in leaves of *Chenopodium* and shoot (186) < root (117) in PPI-1. Furthermore, the PPI-2 is Pb (R<S<L), Cr (L<R<S) and Ni (L<S<R) similarly.

Table.7.3. Heavy metal accumulation (mg/kg^{-1} DW) in the root, shoot, and leaves of plant species growing contaminated site of pulp paper industry sludge-2. All the values are mean of three replicates (n=3) ±standard deviation (SD), BDL: Below detection limit, R: Root, S: Shoot, L: Leave

	Plants name	**Plants parts**	**Heavy metals**								
			Cu	**Cd**	**Ni**	**Fe**	**Cr**	**Mn**	**Pb**	**Zn**	**Mg**
PPI-1	***Brassica campestris***	**Root**	45.56±1.20	BDL	9.36±0.30	201.02±0.05	5.33±0.10	33.64±0.50	3.56±0.25	49.44±0.05	11.33±0.10
		Shoot	58.36±0.50	BDL	6.37±0.20	188.00±0.05	25.41±0.20	39.67±0.54	2.34±0.20	34.21±0.04	16.74±0.20
		Leaves	62.37±1.27	BDL	4.22±0.20	361.00±0.05	BDL	43.37±0.67	3.99±0.20	52.26±1.16	15.69±0.25
	Accumulation pattern		**L<S<R**	**-**	**R<S<L**	**L<R<S**	**S<R<L**	**L<S<R**	**L<R<S**	**L<R<S**	**S<L<R**
	Chenopodium album	**Root**	43.58±1.21	BDL	4.31±0.10	127.00±0.05	39.61±0.44	31.24±0.20	4.22±0.17	37.41±0.05	9.41±0.10
		Shoot	61.23±1.73	BDL	6.44±0.11	152.00±0.05	34.24±0.31	37.24±0.26	2.31±0.11	42.01±0.05	12.59±0.20
		Leaves	43.64±1.18	1.23±0.04	6.11±0.17	226.00±0.05	41.37±0.53	45.37±0.63	3.29±0.22	55.64±1.10	15.74±0.20
	Accumulation pattern		**L<S<R**	**-**	**R<S<L**	**L<R<S**	**S<R<L**	**L<S<R**	**L<R<S**	**L<R<S**	**S<L<R**
	Ricinus communis	**Root**	41.37±1.27	10.31±0.02	5.34±0.11	127.00±0.05	38.24±0.41	32.21±0.22	5.37±0.16	41.27±0.05	10..49±0.17
		Shoot	64.71±1.94	12.35±0.02	5.36±0.12	171.00±0.05	36.94±0.32	37.56±0.25	4.35±0.15	46.02±0.05	14.78±0.21
		Leaves	59.80±1.23	2.36±0.02	7.24±0.17	223.00±0.05	51.07±0.51	45.33±0.68	5.37±0.21	58.57±1.11	15.61±0.290
	Accumulation pattern		**L<S<R**	**-**	**R<S<L**	**L<R<S**	**S<R<L**	**L<S<R**	**L<R<S**	**L<R<S**	**S<L<R**
	Ranunculus sceleratus	**Root**	48.31±1.21	9.36±0.02	5.37±0.16	145.00±0.05	39.61±0.48	33.21±0.26	5.39±0.19	44.26±0.05	15.34±0.10
		Shoot	55.37±1.95	8.31±0.02	6.31±0.11	194.00±0.03	35.64±0.46	38.64±0.27	3.65±0.17	46.37±0.05	16.33±0.23
		Leaves	52.34±1.23	4.37±0.01	7.28±0.17	248.00±0.04	46.37±0.53	45.66±0.62	6.31±0.21	58.37±1.13	15.64±0.21
	Accumulation pattern		**L<S<R**	**-**	**R<S<L**	**L<R<S**	**S<R<L**	**L<S<R**	**L<R<S**	**L<R<S**	**S<L<R**
	Rumex dentatus	**Root**	43.15±1.26	3.34±0.02	5.37±0.15	136.00±0.05	49.21±0.44	49.37±0.22	8.37±0.18	64.45±0.05	13.64±0.10
		Shoot	56.34±1.97	4.31±0.02	6.37±0.11	149.00±0.05	41.26±0.36	41.29±0.26	4.34±0.16	54.04±0.05	15.37±0.20
		Leaves	53.24±1.21	5.34±0.02	6.97±0.19	248.00±0.05	63.54±0.52	43.61±0.68	5.69±0.21	57.61±1.10	18.67±0.20
	Accumulation pattern		**L<S<R**	**-**	**R<S<L**	**L<R<S**	**S<R<L**	**L<S<R**	**L<R<S**	**L<R<S**	**S<L<R**

Table.7.4. Heavy metal accumulations (mg/kg−1 DW) in the root, shoot, and leaves of collected plant species growing contaminated site of pulp paper industry sludge-1. All the values are mean of three replicates (n=3) ±standard deviation (SD), BDL: Below detection limit, R: Root, S: Shoot, L: Leave

	Plants name	**Plants parts**	**Heavy metals**								
PPI-2			**Cu**	**Cd**	**Ni**	**Fe**	**Cr**	**Mn**	**Pb**	**Zn**	**Mg**
	Brassica campestris	**Root**	45.56±1.20	BDL	9.36±0.30	201.02±0.05	5.33±0.10	33.64±0.50	3.56±0.25	49.44±0.05	11.33±0.10
		Shoot	58.36±0.50	BDL	6.37±0.20	188.00±0.05	25.41±0.20	39.67±0.54	2.34±0.20	34.21±0.04	16.74±0.20
		Leaves	62.37±1.27	BDL	4.22±0.20	361.00±0.05	BDL	43.37±0.67	3.99±0.20	52.26±1.16	15.69±0.25
	Accumulation pattern		**L<S<R**	**-**	**R<S<L**	**L<R<S**	**S<R<L**	**L<S<R**	**L<R<S**	**L<R<S**	**S<L<R**
	Chenopodium album	**Root**	32.67±1.11	BDL	5.34±0.10	124.00±0.05	71.63±0.40	30.23±0.20	4.31±0.12	61.22±0.05	29.49±0.11
		Shoot	51.39±1.19	BDL	4.81±0.11	153.00±0.05	61.47±0.33	36.54±0.24	2.34±0.10	58.34±0.06	25.79±0.22
		Leaves	24.35±1.22	3.13±0.01	4.64±0.10	82.00±0.05	43.31±0.53	42.37±0.60	3.69±0.20	22.37±1.11	19.64±0.20
	Accumulation pattern		**L<S<R**	**-**	**R<S<L**	**L<R<S**	**S<R<L**	**L<S<R**	**L<R<S**	**L<R<S**	**S<L<R**
	Ricinus communis	**Root**	42.36±1.23	9.2±0.00	44.37±0.10	157.00±0.05	56.11±0.41	46.21±0.23	4.31±0.10	69.49±0.05	15.49±0.11
		Shoot	54.74±1.94	19±0.00	45.41±0.13	142.00±0.05	51.57±0.32	36.30±0.23	2.34±0.10	63.04±0.05	10.79±0.21
		Leaves	49.87±1.21	16±0.00	26.52±0.18	121.00±0.05	23.01±0.16	22.31±0.61	3.69±0.20	34.67±1.10	9.64±0.22
	Accumulation pattern		**L<S<R**	**-**	**R<S<L**	**L<R<S**	**S<R<L**	**L<S<R**	**L<R<S**	**L<R<S**	**S<L<R**
	Ranunculus sceleratus	**Root**	63.24±1.20	29.02±0.01	64.37±0.10	87.00±0.05	26.11±0.40	38.23±0.21	74.31±0.12	49.49±0.05	29.49±0.10
		Shoot	71.54±1.26	19.03±0.00	55.41±0.13	58.00±0.05	21.57±0.33	26.54±0.25	32.34±0.10	33.04±0.05	21.79±0.20
		Leaves	47.77±1.23	12.23±0.4	26.51±0.18	43.00±0.05	33.31±0.50	18.37±0.61	13.69±0.20	27.67±1.10	13.64±0.20
	Accumulation pattern		**L<S<R**	**-**	**R<S<L**	**L<R<S**	**S<R<L**	**L<S<R**	**L<R<S**	**L<R<S**	**S<L<R**
	Rumex dentatus	**Root**	72.30±1.21	21.23±0.04	54.34±0.10	77.00±0.05	66.12±0.43	65.21±0.21	44.31±0.12	29.49±0.05	89.49±0.10
		Shoot	64.71±1.12	11.23±0.04	65.47±0.13	81.00±0.05	51.53±0.37	56.51±0.21	22.34±0.10	13.04±0.05	41.79±0.20
		Leaves	29.80±1.11	5.23±0.04	16.55±0.18	18.00±0.05	23.34±0.51	12.32±0.61	13.69±0.20	6.67±1.15	33.64±0.20
	Accumulation pattern		**L<S<R**	**R<S<L**	**R<S<L**	**L<R<S**	**S<R<L**	**L<S<R**	**L<R<S**	**L<R<S**	**S<L<R**

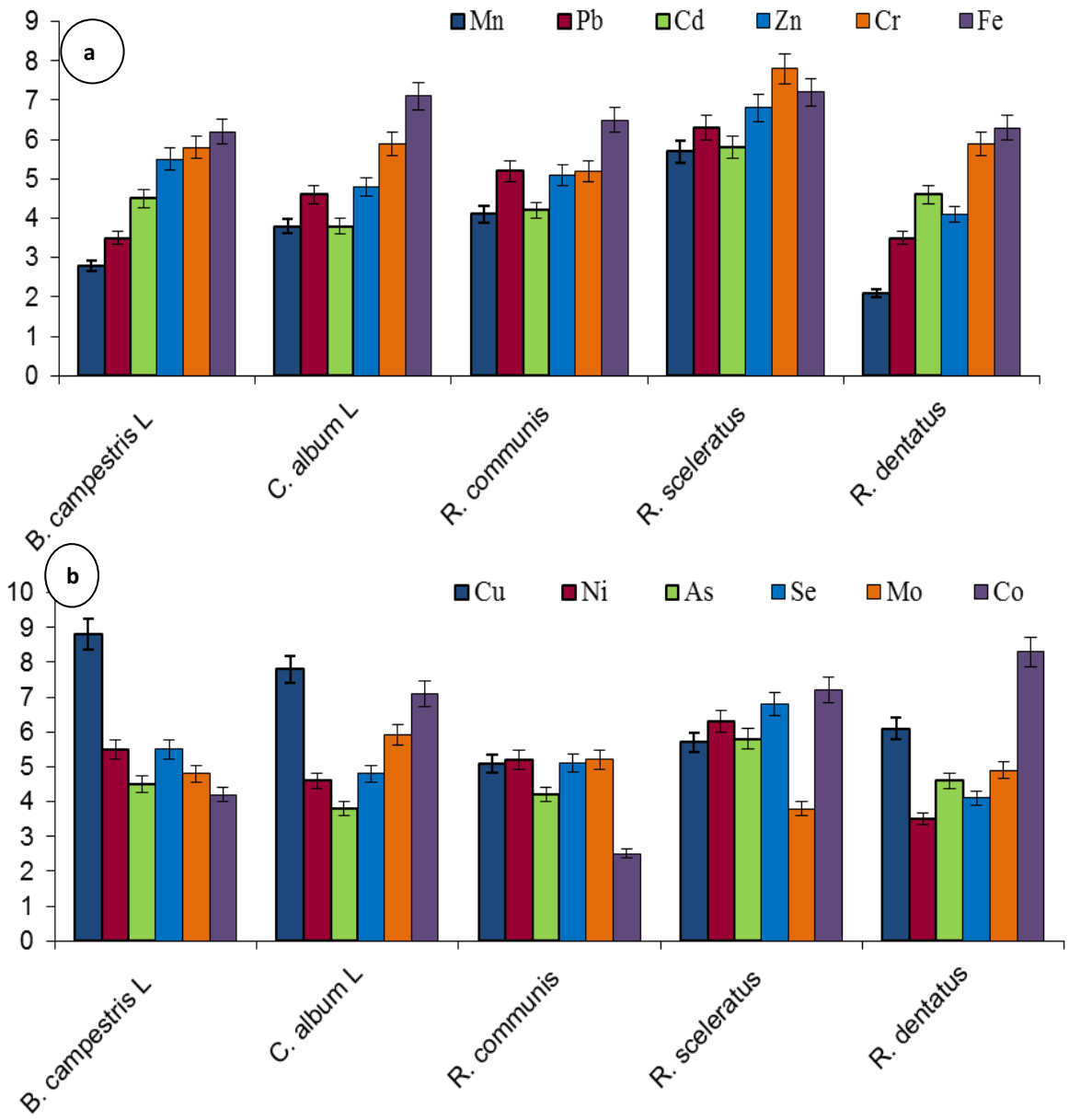


Fig.7.6. Showing the metal concentration root, shoot and leaves of collected native hyperaccumulator plant

6.2.5.3.4. BCF and TF

The bioconcentration factor and translocation factor refer to the most significant function of the plant and metal absorption, mobilization into different plant tissues and storage in their leaves and shoot. In addition, we found in this study that the metal content ratio in root to sludge by BCF analysis from PPI-1 and PPI-2 waste affected plants shows < 1. Showed in Table.7.5. In addition, the results revealed that experimental plants growing on PPIS disposal sites have high metal accumulation potential via phytoaccumulation.

Table.7.5. BCF and TF heavy metal in various plants from contaminated sites with the pulp paper industry

Plant name	**BCF**								
	Mn	**Pb**	**Zn**	**Cr**	**Fe**	**Cu**	**Ni**	**As**	**Cd**
PPI-1									
Brassica campestris L.	0.97	1.47	0.87	**10.95**	1.25	0.84	1.22	1.36	0.54
Chenopodium album L.	0.91	**10.08**	1.28	0.61	1.66	1.34	0.47	1.08	**BDL**
Ricinus communis	1.23	1.79	1.21	**10.91**	1.21	1.27	1.56	1.42	1.34
Ranunculus sceleratus	1.68	**10.13**	1.41	1.39	1.62	1.37	1.36	1.19	**1.20**
Rumex dentatus	1.27	**10.25**	1.31	1.20	1.84	1.33	1.51	1.01	**1.33**
PPI-2	TF								
Brassica campestris L.	0.84	1.54	1.07	0.76	1.71	1.47	0.65	**1.34**	**BDL**
Chenopodium album L.	0.93	1.31	**10.39**	0.49	**2.04**	1.38	0.87	1.24	0.68
Ricinus communis	1.95	1.41	1.24	**10.91**	1.41	1.57	1.35	1.66	1.33
Ranunculus sceleratus	1.36	**10.11**	1.23	1.60	1.68	1.35	1.40	1.57	**1.25**
Rumex dentatus	1.29	**10.23**	1.44	1.30	1.66	1.33	1.37	1.48	**1.22**

Moreover, comparative analysis of PPI-1 and PPI-2 the BCF of potentially toxic metals which showed the ratio of root to sludge in *Brassica* was as Mn (1.02-0.97), Pb (0.79-1.47), Zn (1.28-0.87), Cr (0.59-0.95), Fe (1.69-1.25), Cu (1.20-0.84), Ni (1.51-1.22), As (0.61-1.36) and Cd (BDL-0.54), while TF of Mn (0.97-0.84), followed by Cd (0.54), Ni (1.22-0.65), Fe (1.25-1.71), Cr (0.95-0.76), in PPIS waste. In addition, the BCF value of PPI-1 and PPI-2 in *Chenopodium* Pb (0.94-0.83), Cd (BDL-0.68), Ni (1.68-0.61), and Fe (2.54-2.12) respectively. Similarly the TF value in *Chenopodium* Pb (1.08-1.31), Ni (0.47-0.87), and Fe (1.66-2.04) respectively.

7.2.5.3.5. Cellular observation of metal in root tissue

The TEM analysis of *Brassica* and *Chenopodium* in root tissue showed seemingly metals deposition is near the mitochondria, chloroplast, cell wall, intercellular spaces and thinning of the cell wall is showed in Fig.7.7. It is shown that the toxic effect of heavy metals released from PPI-1 and PPI-2 on collected native plants is an important factor in understanding the physiological and morphological modifications caused by excessive metals due to the complementary structure and function reported by Bini et al., (2012).

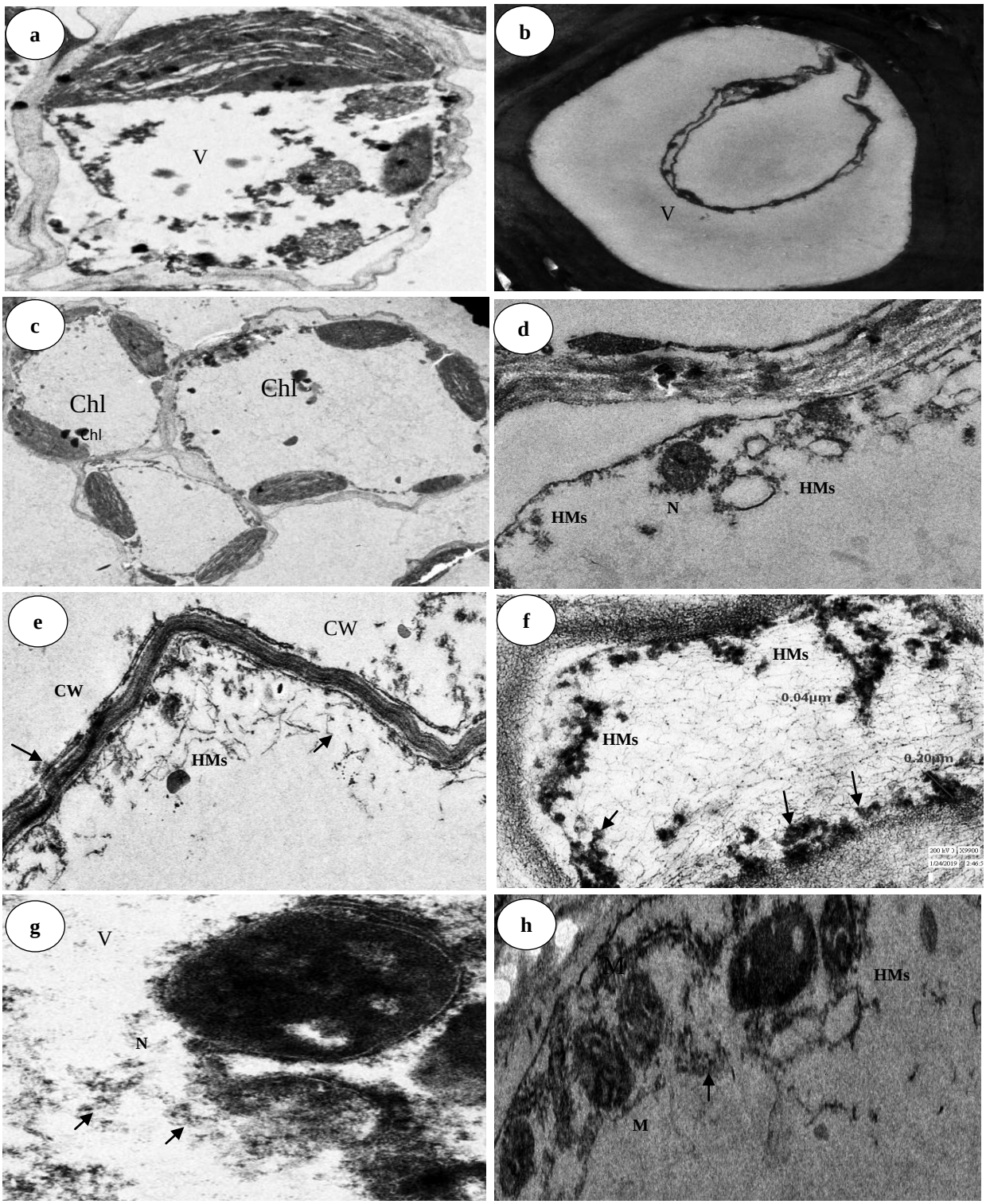


Fig.7.7. Electron micrographs of transverse section of plants root (a–h) *Brassica campestris L.* (a–d) and *Chenopodium album L.* (e-h) V: Vacuole; PM: Plasma membrane; P: Peroxisome; CW: Cell wall; CWI: Cell wall invagination CM: Cell membrane; N- Nucleolus Chl: Chloroplast; ML: Middle lamella; N: Nucleus; Arrow (→) indicated metals deposition; IS: Intercellular space.

In addition, the root tissue of *Brassica* showed a reduction of intercellular spaces, plastids and abnormal cell shape (Fig.7.7a-d). The above observation proved that *Brassica* will have more metal tolerance capacity. Similar observations were also reported for a

reduction in intercellular spaces in *Brassica juncea* leaf by Sridhar et al., (2005). The change of shape of chloroplast because of abiotic and biotic stresses is the result of an increased volume of the stroma and disorganization of the thylakoid membranes which have been previously reported in the literature (Vijaranakul et al., 2001). In addition, the root tissue of Chenopodium demonstrated the ability of phytoaccumulation to deposit electron-dense metal granules in the cell cytoplasm (Fig. 7.7e-h).

Conclusion

This manuscript concludes to the study of environmental pollutants discharged from the pulp paper industry waste which is directly or indirectly link with human health through the food chain. The current study revealed that Physico-chemical (TDS, TSS, pH, phenols, BOD and COD) analysis of PPI-1 and PPI-2 sludge waste consist of potentially toxic elements (Pb, Zn, As, Cr, Fe, and Mn) along with organic compounds which are above the acceptable limit. Moreover, various androgenic and mutagenic compounds are also present i.e. Hexadecanoic acid, trimethylsilyl ester, β- Sitosterol trimethylsilyl and Eicosane (CAS) was detected from sludge by GC-MS analysis. In addition, this can also be concluded that PPI-1 and PPI-2 sludge waste *Brassica campestris L. Chenopodium album L., Ricinus communis, Ranunculus sceleratus,* and *Rumex dentatus* plants have high metal accumulation and allocation in their aerial parts. Furthermore, the antioxidant enzyme revealed increased MDA, H_2O_2, CAT, POD, SOD, and APX respectively. Therefore, there is a need for continuous monitoring near the pulp paper industrial area of soil and water condition for eco-restoration and environmental safety. Moreover, human health threats are extensive research on a worldwide scale, but only a few studies have used adequate observational strategies. Consequently, the treatment technology of discharge waste is needed for environmentally safe disposal and public health, like phytoextraction is an eco- friendly, eco-feasible and cost-effective technology for moderately contaminated soils.

Chapter-Eight

Profiling of dominant bacterial community growing in pulp and paper mill sludge containing heavy metal and organic pollutants

Profiling of Dominant Bacterial Community Growing in Pulp and Paper Mill Sludge Containing Heavy Metal and Organic Pollutants

8. Introduction

Technological advancements in Next Generation Sequencing (NGS) technology over the past few decades have revolutionized biological sciences. Soil contamination by environmental pollution is a widespread environmental problem that often requires the contaminated site to be cleaned up (Whittaker et al., 1995). During the past several decades, soil contaminated by heavy metals and other organic pollutants discharged from pulp paper industry waste has become a serious phenomenon in India (Chandra et al., 2017, 2018; Singh and Chandra et al., 2019) In addition, the several molecular techniques i.e. Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) has allowed for a more complete analysis of microorganisms at a molecular level (Chodak et al., 2013; Epelde et al., 2012; Jousset et al., 2010). However, most pollutants and organic compounds are transformed into less toxic compounds by local microbial communities, particularly in sludge and soil during the in-situ bioremediation process (Staley et al., 2015). Although, fungi, bacteria, protozoa, and nematodes are a few microorganisms which have shown the potential to degrade persistent complex compounds containing contaminants (Fang et al., 2014). This research of microbial communities in the polluted site of pulp paper industry, therefore, reflects the level of toxicity through which this precise information could be used as a tool to estimate and monitor their natural degradation rates (Jain et al., 2005; Tavares et al., 2016). So, therefore, the fundamental research findings of these studies are in line with the notion that landfills contain extremely complex microorganism's communities such as Proteobacteria, formicates and bacteroidetes as the dominant phyla and archaea population observed includes the methanogen species (Kochling et al., 2015). Microorganisms respond immediately to changes, enabling them to adapt quickly to environmental conditions, allowing monitoring and growth of bacterial responses. Besides, advanced molecular techniques provide us with an interesting opportunity to succeed in improving necessity, hence

strongly increasing our knowledge of microbial diversity and environmental characteristics. Such techniques are based on the classification of genetic components including nucleic acids, proteins, fatty acids and other taxa specific compounds (Rossello-Mora and Amann, 2001). A further benefit of culture-independent genomic characterization was the opportunity to maintain in situ metabolic function and the structure of the microbial community by maintaining samples immediately (Moller et al., 1998; Wilson et al., 1999a). Many studies have taken different approaches to understand the profiles of community fatty acids (Haack et al., 1994). Moreover, some researchers are improvements in microbial population profiles from metals polluted soils using phospholipid-derived fatty acids (PLFA) (Frostegard et al., 1993). In addition, PLFA profiles of particular bacteria reduced significantly as the amount of chromium contamination increased, suggesting that chromium concentration in tannery waste contaminated soil had a significant impact on the composition of the microbial community (Kamaludeen et al., 2003). However, microbial identification and characterization of diversity have been improved by the use of the extremely conserved 16S rRNA gene that would be omnipotent in all microorganisms. In addition, PCR-based techniques have been used to identify and quantify microorganisms present in soil and water (Wilson et al., 1999). However, PCR amplification depends on separating and purification of nucleic acids of adequate results and quality environmental samples. The physico-chemical analysis of discharged sludge from pulp paper industry is highly loaded with different organic and inorganic pollutants. The above study represents an original study of the bacterial community using 16S rDNA along with an imputed assessment of Metagenomics.

8.1. Material and methods

8.1.1. Sample collection

The sludge sample was collected aseptically from M / s K.R. Pulp Papers mill Limited, Shahjahanpur, U.P. India (27°50'31.8"N 79°51'15.7"E) in pre-sterilized plastic containers (capacity 20L Tarson Production Pvt. Ltd., USA). The collected sludge samples were

transferred to the laboratory and then used to examine Physico-chemical parameters and the metagenomics analysis of microbial communities in sludge (Fig.8.1).

8.1.2. Physico-chemical analysis of sludge

All analyses of Physico-chemical parameters of sludge and leachate samples, i.e. pH, EC TDS, TSS, pH, EC, salinity, chloride, sodium, nitrate, ammonia nitrogen, was estimated by the previously described method in chapter four.

Fig.8.1.The image showing the view of the discharged of waste in the environment after secondary treatment. (d) View of sludge (1) Effluent (2) and leachate (3)

8.1.3. Scanning electron microscopy analysis of sludge

The SEM analysis of pulp paper mill sludge was used to perform a sample that was done by the previously described method in chapter five.

8.1.4. Preparation of leachate of sludge

The leachate sludge was prepared by stirring it for approximately 48 h in distilled water (1:1 w / v) permitting the sludge suspension to stand still for 6 hours in an Erlenmeyer flask (250 mL). The pure supernatant was drained out and purified into Whatman filter paper and freshly prepared sludge leachate was taken as 100 percent, with different concentrations prepared from it by adding distilled water to a final concentration of 1.0 %, 2.5 %, 5 % and 10 % for evaluation of specific Physico-chemical parameters, organic and inorganic pollutant (Fig.8.1d).

8.1.5. Study of uncultured bacterial communities growing in the sludge of pulp paper mill sludge

8.1.5.1. Extraction and purification of DNA

The total genomic DNA from the collected pulp paper sludge sample was extracted using the DNeasy kit (Qiagen, USA) and 2% CTAB conventional DNA extraction method as per the described protocol. The DNA concentration was estimation using Qubit Fluorimeter (V.3.0). The V3-V4 region of 16sRNA was amplified using specific V3 Forward primer CCTACGGGNBGCASCAG and V4 Reverse primer GACTACNVGGGTATCTAATCC. The amplified product was checked on 2% agarose gel and gel purification was doe to remove non-specific amplification. 5gn of amplified product was used for library preparation using the NEBNext Ultra DNA library preparation kit. The library quantification and quality estimation were done in Agilent 2200 Tapestation. The prepared library was sequenced in Illumia Hiseq 2500 with 250 cycle chemistry. The library preparation steps are illustrated.

8.1.5.2. Sequencing of cloned 16S rDNA PCR fragments

Based on differences in the RFLP profiles generated, 10 bands generated by the digestion of sludge by *Taq*I and *Sau*3AI were selected for sequence analysis. Selected bands were gel purified using gel extraction kits (Merck Biosciences, Mumbai, Maharashtra, India) and sequenced using the M13 forward (5′-GTAAAACGACGGCCAGT 3′) and reverse universal primers (5′-CAGGAAACAGCTATGAC-3′) (Shi et al., 2007) and an ABI

PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Waltham, MA, USA). The samples were then sequenced using an automatic DNA sequencer (ABI PRISM® 310 Genetic Analyzer, USA). The partial sequences obtained were subjected to BLAST analysis using the online option available at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

8.2. Results and discussion

8.2.1. Physicochemical analysis of sludge

The physicochemical analysis sludge and leachate are shown in the Table. 8.1. The pH was high (8.2-7.8) TS (1594-1857), TDS (1742-1967), TSS (69-84) is found to be higher than the permissible limits discharged after secondary treatment. Further, the BOD (8146-7569), COD (20130-18541), EC (1549-1657), total phenol (421-367), total nitrogen (143-124), respectively. Higher EC might be due to salt and ions content of sludge and leachate (Deepali et al., 2009). Moreover, Na^+ and K^+ increase the salinity of the wastewater and thus it showed unfit for irrigation and drinking and also responsible for disorders of the respiratory system, alimentary canal, nervous system, coronary system and causing miscarriage in the aquatic ecosystem (Reddy and Rao, 2001). Similarly, chloride is the most troublesome anion was also found in sludge and leachate which is generally more toxic than sulfate to flora and fauna. Moreover, lignin contents were noted very high (4710-3854 mg l^{-1}) in sludge and leachate which might be the source of the dark color of the sludge and leachate. The dark color of sludge and leachate adversely affected aquatic flora and fauna along with microorganisms. Furthermore, the significant amount of Fe (94-75), Zn (23-21), Cu (3.29-4.57), Cd (0.39-1.01), Mn (19-14), Cr (4.2-4.9), Pb (3.54-8.9) and Ni (6.2-5.4 mg l^{-1}) was present in sludge and leachate which are hazardous to the environment. This observation corroborated with previous findings (Chandra et al., 2011; Madan et al., 2018). The heavy metal sources in sludge and leachate might be due to alkaline black liquor corrosion activity generated during wood digestion as it passes through iron pipes. However, Zn, Cu, and Cu are described as vital in the aquatic environment due to their role in several biochemical mechanisms but they become detrimental, when present in high concentrations. The inclusion of heavy metals into food chains could result in a level of accumulation in aquatic

Table.8.1. Physico-chemical characteristics of discharged waste from pulp paper industry and their heavy metals content collected from M/s K. R. Pulp Paper Ltd. Shahjahanpur, Uttar Pradesh, India

Parameters	**Sludge value**	**Leachate value**	**Permissible limit (EPA 2002)**
pH	8.2±0.40	7.8±0.43	5-9
Color	3120±105	2937±140	Dark Brown
TS	1594±102	1857±154	-
TDS	1742±31.21	1967±42.12	-
TSS	69±3.22	84±2.31	35
COD	20130±854	18541±453	120
BOD	8146±251	7569±247	40
EC	1549±74	1657 ±71	1000
Total Phenols	421±23.21	367±41.37	0.50
Total nitrogen	143±4.09	124±6.37	143
Sulphate	1894±88	1957±25	250
Phosphorus	153±6.32	149±5.97	200
Cl^-	4.21±0.22	6.37±0.43	1500
Na^+	354±11.21	289±15.84	200
K^+	19.2±0.83	18.4±0.60	-
Lignin	4710±1110	3854±1257	-
Chlorophenol	421±10.29	357±12.71	3.0
Heavy metals			
Fe	94 ±1.80	75±1.40	2.00
Zn	23±1.23	21±1.31	2.00
Cu	3.29±0.19	4.57±0.14	0.50
Cd	0.39±0.02	1.01±0.07	0.01
Mn	19±0.74	14±0.51	0.20
Ni	6.2±0.21	5.6±0.34	0.10
Cr	4.2±0.05	4.9±0.04	-
Pb	3.54±2.11	8.94±1.51	-

Organisms influencing their biological and physiological mechanisms. Most of the heavy metals are known to be toxic and carcinogenic; they pose a serious threat to human flora and fauna. Moreover, hazardous metals lead to the formation of reactive oxygen species

(ROS) such as superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radicals (Verma et al., 2008).

8.2.2. Morphological view of the bacterial community in SEM analysis

The SEM analysis revealed the microorganisms; they can be found individually and collectively or in aggregates colonies in shape and size (Chandra et al., 2018). The sludge sample as the functioning is affected by microbial interactions. Moreover, our study is showed that the different colonies of bacteria are present in sludge samples i.e. *cocci sp.* *bacillus sp.* and others for working as in-situ bioremediation of complex organic pollutants in the contaminated site of the pulp paper industry after secondary treatment shown in Fig.8.3. In addition, high pH and electrical conductivity values of pore water in wood ash may affect the microbial community (Bang-Andreasen et al., 2017). Further, some other structures are also showed that the confirmation of complex organic pollutants and other heavy metals.

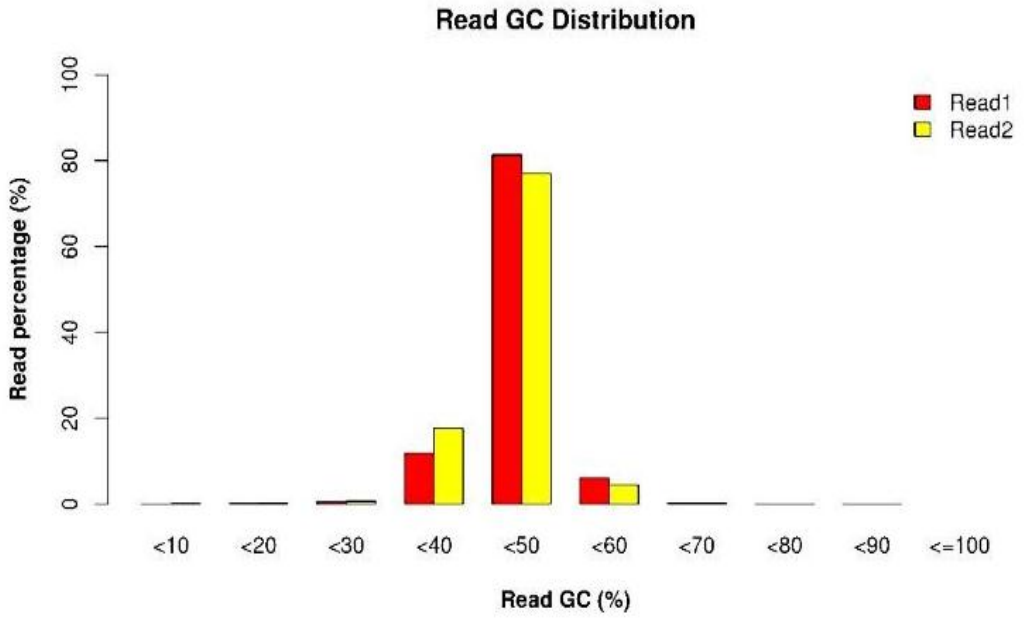
8.2.3. Characterization of bacterial communities using 16S rRNA gene analysis

8.2.3.1. GC content distribution

Bacterial genomes reveal a broad range of compositional diversity, most spectacularly represented by variation in the GC content of the genome, averaging from as low as 17% to as high as 75% in different species. Moreover, Culture-independent approaches are required to understand many species genetic diversity, population structure and ecological roles. Differences in GC content respectively prokaryotic genomes represent largely on or are motivated by, the GC content of protein-coding sequences that usually occupy most of the genome. The composition of nucleotides in the sequence read for each sample is shown in Fig.8.2. Various factors that include both neutral and selective processes form the simplest and most commonly used indicator of nucleotide composition, the abundance of guanine plus cytosine (%GC).The x-axis represents a sequencing cycle and the y-axis represented nucleotide parentage. The base composition of the left and right end of the paired-end read sequences is calculated.

8.2.3.2. Reads and Operational taxonomic units (OTUs) distribution

In order to assign operational taxonomic units in the form of species, genera or phyla, the majority of Metagenomics analysis is completed using the 16S rRNA sequencing. The majority of Metagenomics analyses are carried out using 16S rRNA sequencing in order to assign OTUs in the form of species, genera and phyla. A total of 25356 OTUs were identified from 273654 reads. From 253556total OTUs, 21102 OTUs with less than 5 read were removed and 4254 OTUs were selected further analysis. Also, it should be noted that the 16S rRNA approach is well suited to examine a large number of micro biome samples, but has a minimal taxonomic and functional resolution. Moreover, sludge metals content collected from M/s K. R. Pulp Paper Ltd. Shahjahanpur, Uttar Pradesh,



(a) K1

India

Fig. 8.2. The graph showed the concentration of GC content in the sludge sample (K)

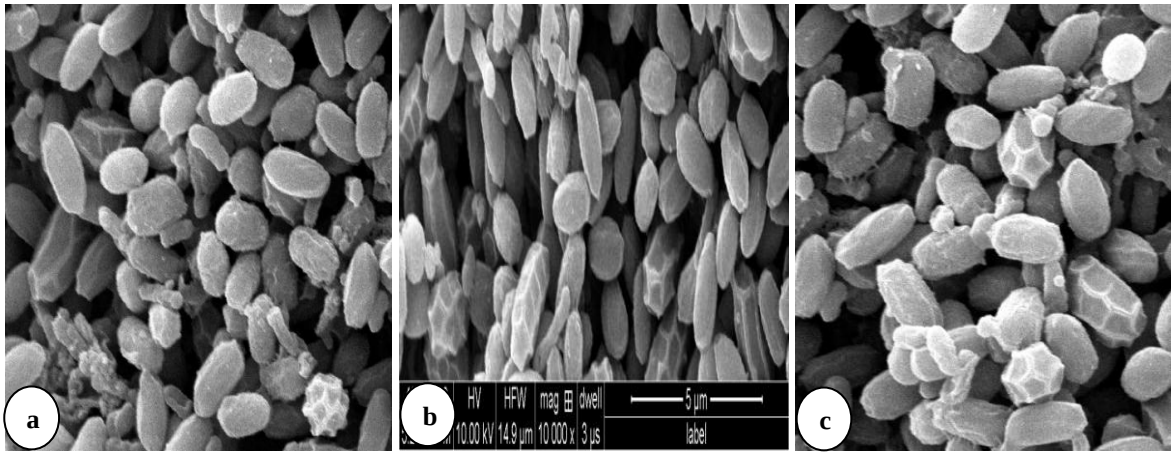
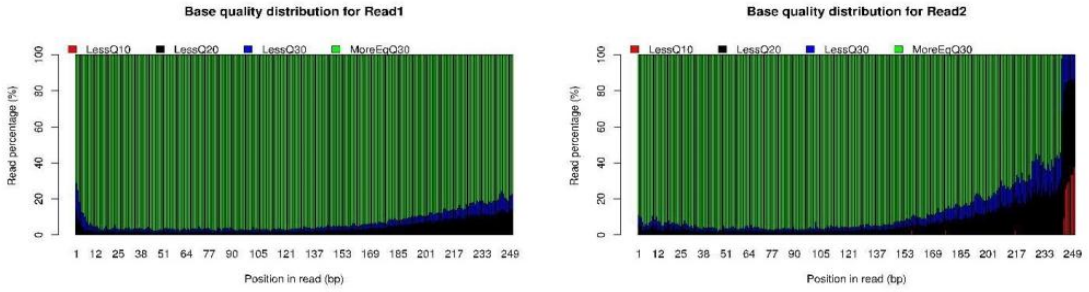
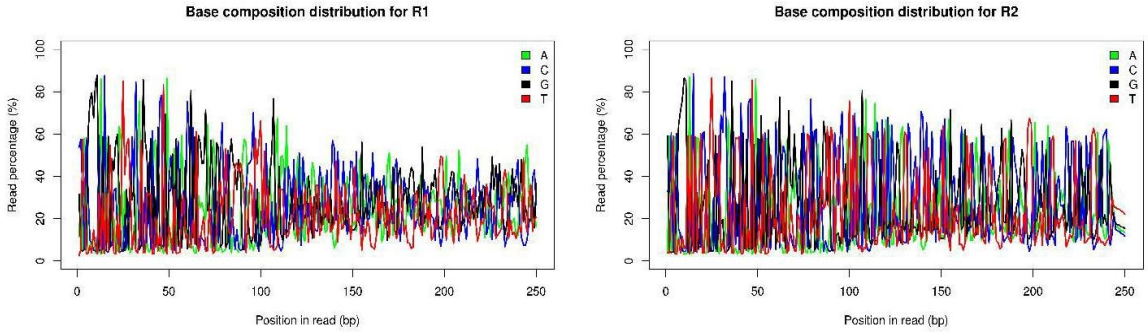


Fig.8.2. SEM analysis of sludge microbial community from the disposal site of the pulp paper industry



(a) K1 (R1) (b) K1 (R2)



(a) K1 (R1) (b) K1 (R2)

Fig. 8.3. Showing Base quality and base composition distribution of the sludge sample from the contaminated site of the pulp paper industry.

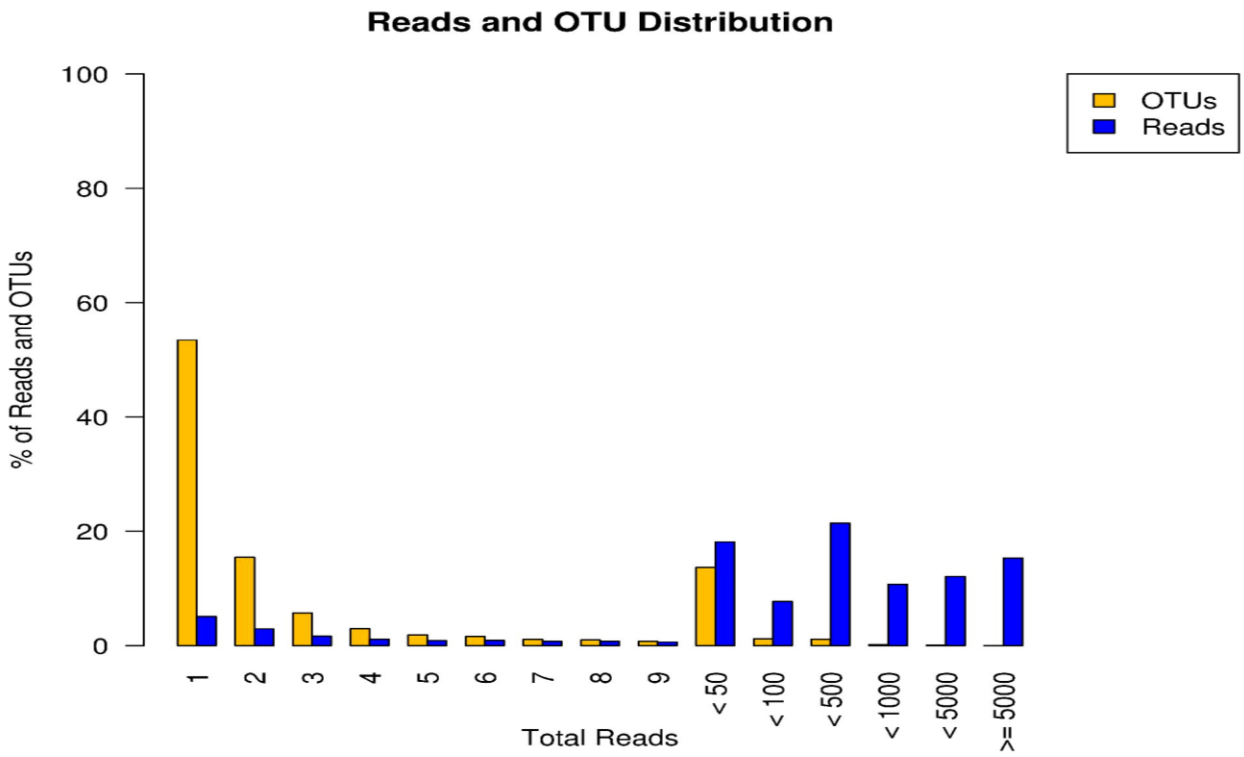


Fig. 8.4. Bar plot represents the relative reads and operational taxonomic unit proportion

8.2.3.3. Microbial community structures at the genus phylum, and class level

The various domains of bacterial community from pulp paper sludge after secondary treatment from the contaminated site clearly illustrated in Fig. 8.7. Taxonomic affiliation at different levels has been studied to better understand the microbial community structure of pulp paper industry waste in sludge. Furthermore, we have seen significant changes in the bacterial community structure of the sludge sample using the Illumina sequencing of the V3-V4 hyper-variable region of bacterial 16S rRNA genes and Metagenomics library analysis. The relative abundance of bacteria in the sludge can be seen in fig at phylum, group and class levels. However, the result revealed that sludge has significantly different social structures and the abundance of species.

8.2.3.4. Phylogenetic of heat map

Phylogenetic heat maps, a new approach to precise visualization of differences in sequence composition between two groups of sequences that contain the same phylogenetic groups (Fig.8.6 and 8.7). Here we define such a technique in metagenome sequence statistics produced by different sequencing platforms to detect sequence-based

bias. In these sequencing, we calculated nucleotide word frequencies and showed the outcomes using a phylogenetic heat map and main component analysis showed in fig. 8.4.

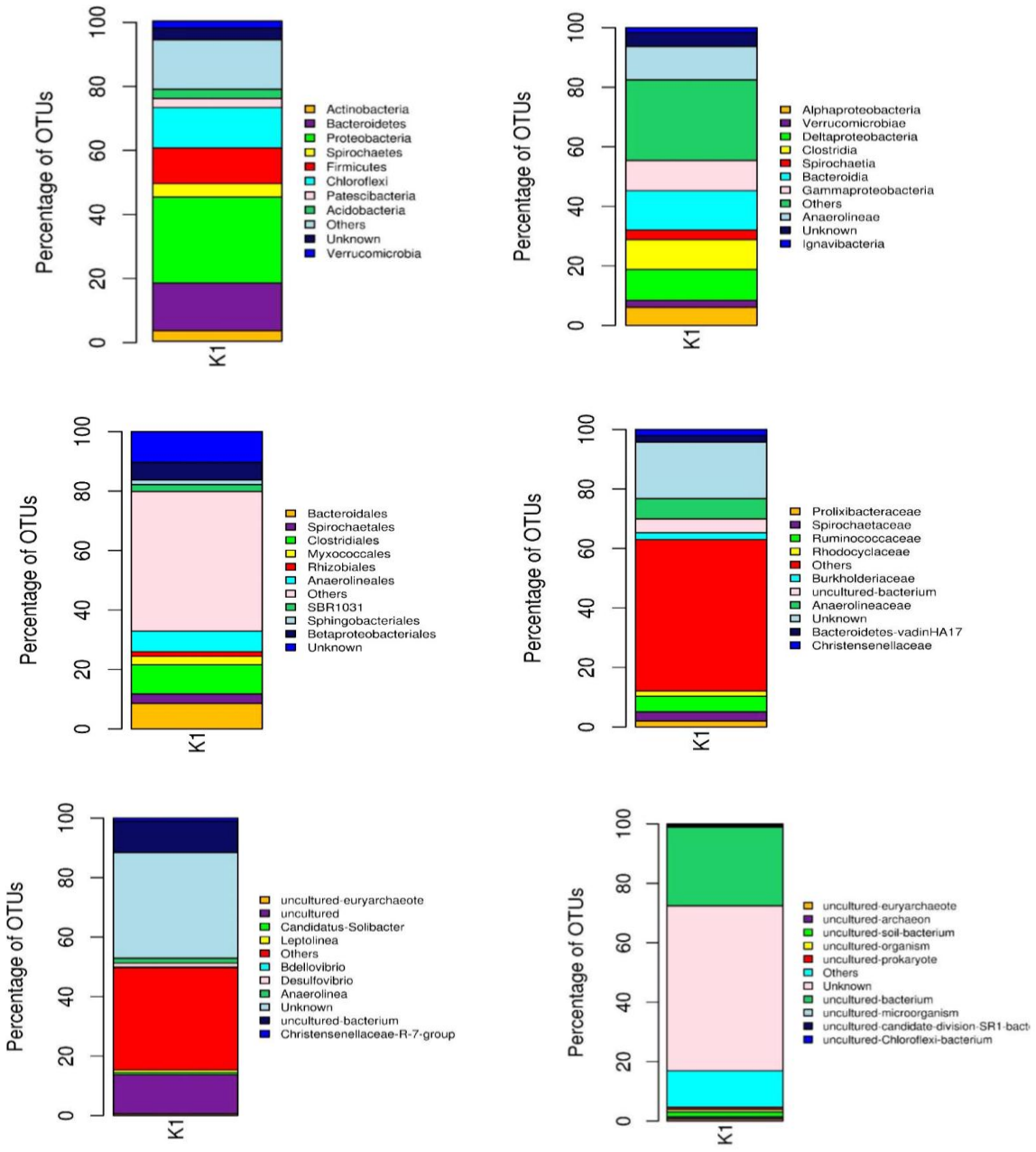


Fig. 8.5. Relative abundance at Phylum, Class, Order, Family, Genus, and Species level (OTUs).

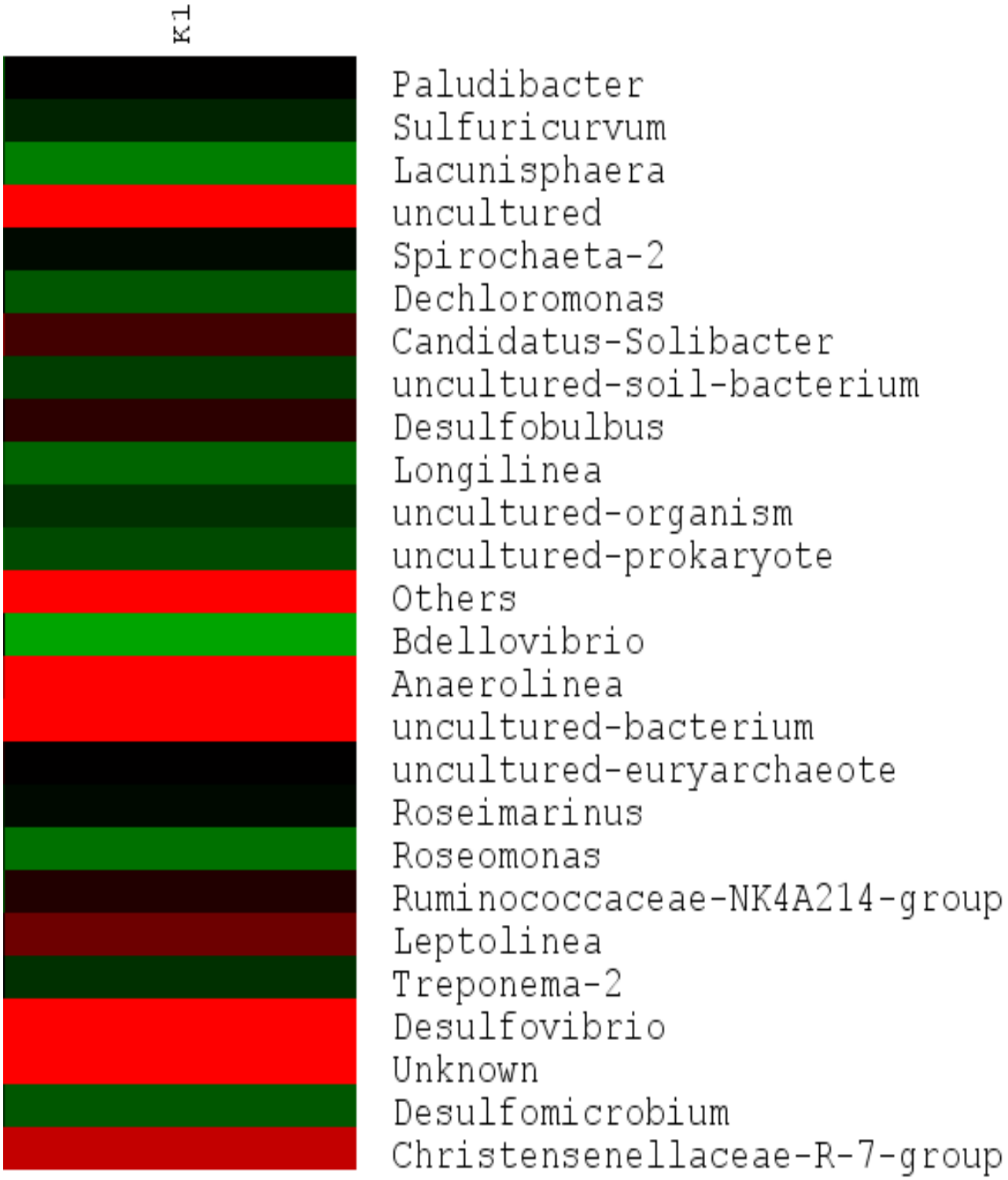


Fig.8.6.Showing Heat maps representing the relative abundance (% of total sequences) of bacterial genera from sludge sample of pulp paper industry.

Fig. 8.7. Showing Heat map representing the relative abundance (% of total sequences) of bacterial genera from sludge sample of pulp paper industry.

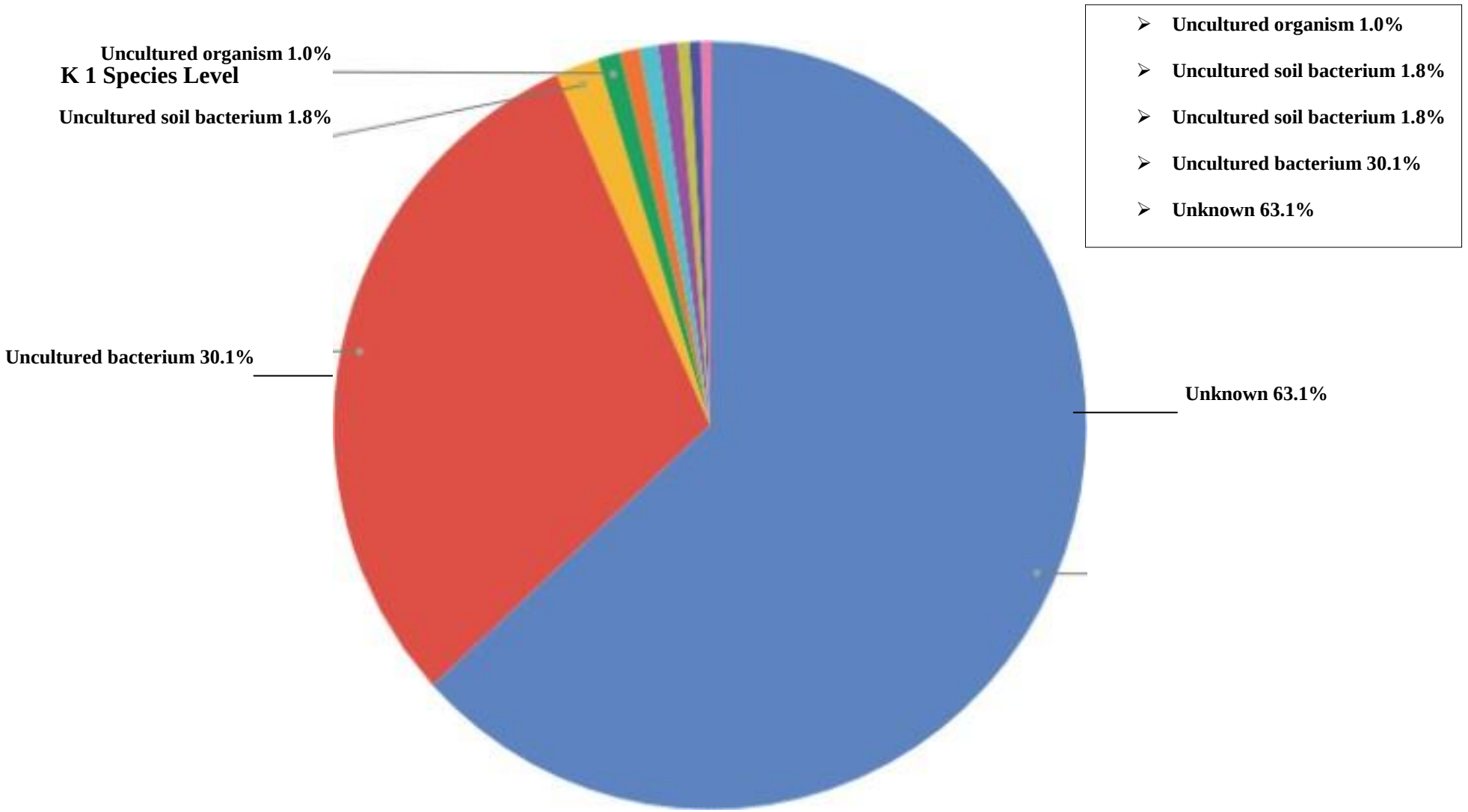


Fig.8.8.Pie chart showing the taxonomic distribution of OUTs at the different phylogenetic levels in pulp paper industry sludge based on Metagenomics sequencing data

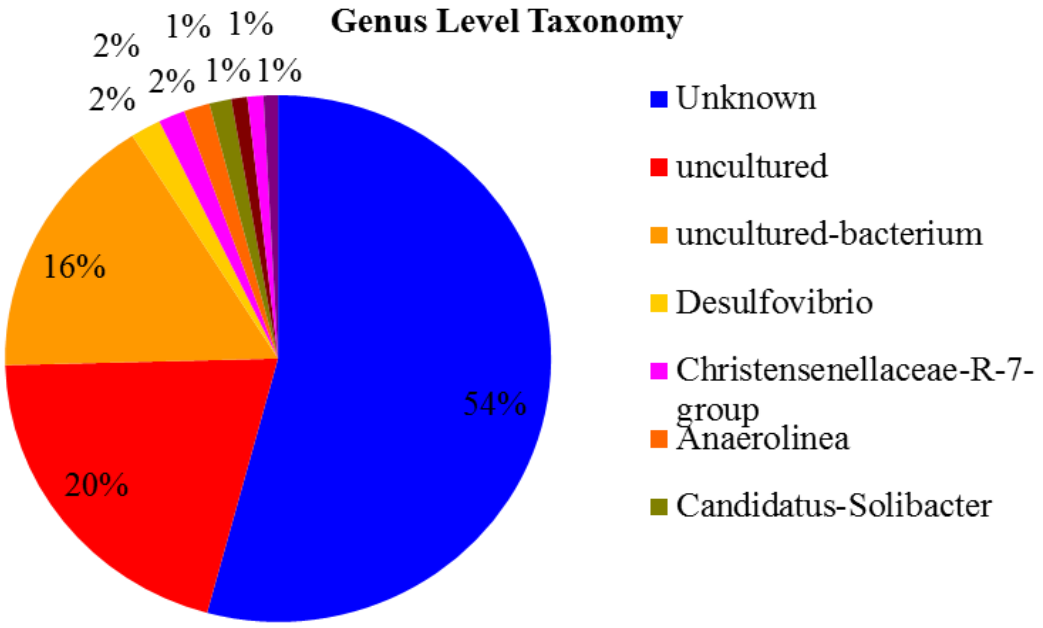


Fig.8.9.Pie chart showing the taxonomic distribution of OUTs at different genus-level taxonomy in pulp paper industry sludge based on Metagenomics sequencing data

In pie chart showing (Fig.8.8) the taxonomic distribution of OUTs at the different phylogenetic levels after metagenomics analysis of sludge sample is total uncultured organism 1.0%, uncultured soil bacterium 1.8%, uncultured soil bacterium 1.8%, uncultured bacterium 30.1% and Unknown 63.1%, respectively.

Conclusion

This study has revealed that the pulp paper industry sludge and its leachate contain several residual persistent organic pollutants along with heavy metals. Moreover, in my previous study detected the majority of environmental pollutants i.e. Octadecanoic acid, tetradecanoic acid, Nonacosane, hexadecanoic acid, heptadecanoic acid, β-sitosterol, and Nonacosane respectively. The presence of these compounds are mutagenic and carcinogenic compounds and after the hormonal disturbances in aquatic organism. Nevertheless, they are discharged into systems where they may impact biota and benthic organisms. Moreover, the dominate bacterial community is identified by metagenomics analysis is proteobacteria are showed the potential in contaminated site of pulp paper industry. The findings of the present study will be useful for monitoring and managing the pulp paper industry waste for environmental safe and eco-restoration of polluted sites.

Chapter-Nine

Characterization of potential bacterial community growing in the rhizospheric zone of native plants at polluted site of pulp paper industry waste

Characterization of Potential Bacterial Community Growing in the Rhizospheric Zone of Native Plants at Polluted site of Pulp Paper Industry Waste

9. Introduction

Sustained worldwide industrialization may have caused extensive environmental and human health problems and detected a wide variety of toxic substances including heavy metals, pesticide residues, chlorinated solvents, and so on. However, during the manufacturing process of pulp and paper industries use lignocellulosic components of plants and chemicals and cause environmental pollution due to discharged huge amounts of waste (Chandra et al., 2012). The pulp paper industry effluent is categorized by its dark brown color, high temperature, strong odor, low alkaline pH, high COD-200,000 mg/l) and BOD-40,000–50,000 mg/l, respectively. The treatment technologies are needed to decolorize and detoxify the effluent of pulp and paper mills as it causes an adverse effect on humans system like respiratory problems, oxidation stress, organ damage and carcinogenicity. In addition, phytoremediation is the use of plants to remediate contaminated soils by industrial waste, an environmentally sustainable and cost-effective, eco-friendly green technology that is receiving important global attention (Glick, 2010). In addition, lignin and chlorinated phenols are the major environmental pollutants discharged from the pulp and paper industry. In addition, the presence of lignin in effluent provides offensive color and inhibits the growth and development of phototrophic organisms by reducing sunlight transmission in the aquatic system (Karrasch et al., 2006). However, plant growth-promoting rhizobacteria (PGPR) are the play key role in the rhizospheric and are directly or indirectly involved in plant growth promotion via secretion of certain regulatory chemicals in the rhizospheric zone (Lugtenberg and Kamilova, 2009). However, there is a way to maximize the chances of success of phytoremediation by utilizing PGPR, which are soil microbes that inhabit the rhizospheric. The PGPR bacteria provide the root exudates such as free amino acids, proteins, carbohydrates, alcohols, vitamins, and hormones, which are important sources of their nutrition and enhance the phytoextraction capability of plant. The rhizosphere has high concentrations of root-exuded nutrients and attracts more bacteria than does bulk soil (Han et al., 2005). They fix atmospheric nitrogen and supply it to plants and

synthesize different secondary metabolites like siderophores that can solubilize and sequester iron from the soil and provide it to plant cells. Moreover, PGPR is introduced to a contaminated site; they increase the potential for plants that grow there to sequester heavy metals and to recycle nutrients, maintain soil structure, detoxify chemicals, and control disease. Moreover, PGPR also decrease the toxicity of metals by changing their bioavailability in plants. Moreover, PGPR also synthesizes several different phytohormones, including IAA, ACC and cytokinins, which enhance plant growth and solubilize minerals such as phosphorus, thereby rendering it more readily available for plant growth. Moreover, PGPR contains enzymes that modulate plant growth and development (Sheng and Xia, 2006; Ma et al., 2009). The importance of rhizosphere mechanisms in the phytoremediation of inorganic pollutants, particularly metals/metalloids, and there are only a few studies on this subject (McGrath et al., 2001; Fitz and Wenzel, 2002; Wenzel et al., 2004).

Therefore, the aims of this study were isolate and identify bacteria from the rhizospheric zone of *Phragmites communis* from the contaminated site of the pulp paper industry and optimize all the PGPR parameters and established the best possible technique for remediation and detoxification discharged waste from pulp paper industry.

9.1. Material method

9.1.2. Site description and sample collection

The sludge rhizospheric soil and *Phragmites communis* samples were collected from M/s K.R. pulp and paper mill Ltd., located at Shahjanpur, Uttar Pradesh, India (27°50'31.8"N 79°51'15.7"E). The effluent, sludge, and rhizospheric soil samples were collected using a sterile plastic container and stored at 4°C until use.

9.1.3. Physico-chemical analysis of sludge and rhizospheric soil

For the physicochemical analysis of effluent, sludge and rhizospheric soil are different parameters i.e. TDS, TS, TSS, BOD, COD, total phenols, total nitrogen EC, pH, chloride, potassium, and sodium (Micro kjeldahl), phosphate and color (visual color comparison method) as per methods described in APHA (2005).

9.1.4. SEM and EDAX analysis of sludge and rhizospheric soil

For an analytic process of SEM and EDAX analysis of the collected sample by the previously described method in chapter seven.

9.1.5. Isolation and purification of PGPR

The dominant PGPR isolation from the sludge and rhizospheric soil of pulp and paper mill waste contaminated site. The bacteria were isolated from 1g. Sample transfer in the distilled water of 9 ml for serial dilution, and 5 min mixed by serial dilution and plate streak method. After serial dilution 0.1 ml suspension was spread over pre-sterilized and cooled down nutrient agar plates in triplicates. The inoculated plates were incubated at 30±1ºC for 24-48 h. On the basis of preliminary investigation twenty isolates (PS1-PS12) were selected and maintained on the agar medium (NAM) slants at 4ºC for further use. Dominantly growing purified bacterial strains were identification as per Cowan and Steels Manual for the identification of medical bacteria (Barrow and Feltham, 1993).

9.1.6. Screening of PGPR activity

9.1.6.1. .1. Ligninolytic enzyme assay

The screening of ligninolytic enzyme activity monitoring by different standard protocols. For the lignin peroxidase (Lip) and Laccase enzyme assay was done according to Arora et al 2002. Further, manganese peroxidase (MnP) enzyme assay was performed by the method as described by de Oliveira et al., (2009). In addition, for the Lip enzyme screening, bacterial isolates to the further screen have been used as a methylene blue dye indicator. Further, isolated bacteria were streaked on methylene blue marker dye (0.25 g / L) containing Luria Bertani (LB) agar plate and the plates were incubated at 30ºC for 72 hours. For the growth of bacteria and decoloration of methylene blue dyes, the agar plates have been monitored daily (Bondounas et al., 2011). Moreover, for the Mnp enzyme screening bacterial isolates to the further screen have been used as a phenol red dye indicator. The media was selected minimal salt media (MSM) composition is K_2HPO_4, 4.55g; NH_4NO_3, 5g; H_3BO_3, 0.5g; $CaCl_2$, 0.01g; KH_2PO_4, 0.53g; Trace element solution 1 ml ($ZnSO_4.7H_2O$, 2.2g; Mn acetate, 0.5g; $FeCl_3$, 0.5g; $CuSO_4.6H_2O$, 0.16g; Molybdic acid, 0.11g; Na_2 EDTA, 5g; and distilled water, 1000 ml) and distilled water 1000 ml

(Sasikumar et al., 2014). The cleared zone is observed after 24 hrs is showed the degradation of lignin. For the estimation of laccase enzyme production was measured by Isolated bacteria were maintained on nutrient agar medium in slants at 4 °C. The medium were contained (w/v): beef extract (1%), peptone (1%), tryptophan (1%) and NaCl (0.05 %), agar 0.3 %, and guaiacol 0.1% respectively. The cultures were incubated at 37°C at room temperature after 72 hrs the zone is clear (Koschorreck et al., 2009).

Media composition

Luria Bertani (LB) agar

Casein enzymic hydrolysate	10g/L
Yeast extracts	5g/L
Sodium chloride	10g/L
Agar	15 g/L

Peptone - Peptides, Amino acids, Nitrogen

Skim milk (SM) powder

Skim milk powder	28.000
Tryptone	5.000
Yeast extract	2.500
Dextrose (Glucose)	1.000
Agar	15.000
Final pH (at 25°C)	7.0±0.2

9.1.7.2. Hydrolytic enzyme assay

For evaluation, the activity of hydrolytic enzymes such as α-amylases and proteases by methods of Cappuccino and Sherman, (2005). In addition, determining the production of α-amylase and protease enzymes from bacteria, one loopful of bacterial cell suspension was streaked on peptone-containing starch agar plate media-5 g, beef extract- 3 g, soluble starch- 10g, agar-15 g, distilled water-1000 mL and skim milk agar plate containing skim milk- 100g, peptone-5g, agar -15g, water-1000 mL, respectively. The plate was observed for the clear area around the streak after 48 h of incubation at 28ºC. In addition, the media was prepared to determine the production of pectinase and cellulose by adding 1% pectin and cellulose to the basal medium containing $NaNO_3^{-1}$ g, $K_2HPO_4^{-1}$ g, KCl^{-1} g, $MgSO_4$-0.5 g, yeast extract-0.5 g, $glucose^{-1}$ g, distilled water, 1000mL, and Agar-15 g. One

loopful of the bacterial cell suspension was streaked on the medium and incubated for 5 days. Moreover, a gram iodine solution was poured in the pectin agar and a zone of clearance was observed against the dark blue background. The cellulose medium was flooded with 0.01% Congo red solution for 15 min and the plates were destained using a 1% NaCl solution for 5 min. The clear zone against the red background indicated that the rhizobacteria were positive for pectinase and cellulase production.

9.1.7.3. .3. Indole-3-acetic acid production

For the estimation of Indole-3-acetic acid (IAA) production in isolated bacterial strains' ability to produced IAA according to described methods of Sawar and Kremer, (1995). The test organism is metabolized by the addition in 5 mL of sterile tryptone / peptone-yeast extract broth of 50µL of cell suspension containing with peptone/tryptone- 10 g, beef extract-3 g, NaCl- 5 g, l-tryptophan- 0,204 g, distilled water-1 L, pH-7 in 15 mL tubes and incubated for 72 hrs at 28ºC in darkness . Subsequently, the 1.5 mL of this broth was centrifuged at12, 850 g for 10 minutes, followed by the 1 mL of Salkawaski (50 mL, 35 percent of Perchloric acid, 1 mL 0.5 M of $FeCl_3$solution) addition of the Salkawaski reagent in the 2 mL Eppendorf tube. The culture tubes were then incubated for 1 hour in dark at 37ºC and red color formed in the medium of indicated bacteria produced IAA.

9.1.7.4. .4. Siderophores production

The siderophores productions from bacterial isolates have been determined on Chrome-azurol S (CAS) medium described method of Schwyn and Neilands, (1987). Further, the bacterial strains (24 hrs old cultures) were spotted separately on CAS medium and incubated at 28±1ºC for 48–72 hrs. The formation of orange to yellow halo around the colonies confirmed the production of siderophore.

9.1.7.5. Phosphate Solubilization

For determining of Phosphate Solubilization, the cultures were inoculated on to Pikovskaya's agar medium (Hu et al., 2006). The media composition of the Pikovsakya agar medium for 1000 ml i.e. calcium phosphate- 5 g, glucose-10 g, potassium chloride-0.2 g, ammonium sulfate-0.5 g, magnesium sulfate-0.1 g, yeast extract-0.5 g, agar- 15 g, distilled water- 1000 m L. One loop full of the 24 h broth culture was spot inoculated on the Pikovsakya culture plate. The plates were incubated at 28ºC for 96 hrs and were

observed for the zone of clearance around the bacterial colony, which indicated the Solubilization of Phosphate. The Solubilization zone was determined by subtracting the diameter of the bacterial colony from the diameter of the total zone (Gaur, 1990).

9.1.7.6. Zinc Solubilization

Zinc Solubilization ability of the isolates was detected by spotting the log phase culture of bacterial strains on mineral salts agar (MSA) medium plates having zinc phosphate, and zinc carbonate as a source of insoluble inorganic zinc. Moreover, the inoculated plates were then incubated at 28±1ºC for 3 days and observed for the clearing zone around the colonies. Zn Solubilization was determined in MSA medium (Glucose-10 g, $(NH_4)_2SO4$-1.0 g, KCl – 0.2 g, K_2HPO_4-0.1 g, $MgSO_4$- 0.2 g and H_2O-1000 Ml with pH 7.0) amended with 0.1% of insoluble zinc oxide (ZnO) or zinc carbonate ($ZnCO_3$). The actively growing cultures (5µL) were spot inoculated onto the medium, incubated at 28ºC and Solubilization zone was measured 15 days after inoculation and clearing zone was expressed as the area in cm^2 (Venkatakrishnan et al., 2003).

9.1.7.7. .7. Hydrogen cyanide determination

Hydrogen cyanide (HCN) production was determined by the modified method of Bakker and Schippers, (1987). Exponentially grown cultures (10^{-8} cells ml^{-1}) of strains were streaked on solid agar plates supplemented with or without 4.4 g glycine l-1 with simultaneous addition of filter paper soaked in 0.5% picric acid in 1% Na_2CO_3 in the upper lids of plates along with inoculated control. The plates were sealed with parafilm and incubated at 28±1ºC, the development of color from yellow to light brown, moderate brown or strong brown was examined for putative HCN production.

9.1.7.8. Morphological characterization by gram staining

The isolated bacterial strains were identified using the standard procedure depending on phenotypic traits (Barrow and Feltham, 2003). The method is used as a tool for differentiating gram-positive and gram-negative bacteria to determine the identification of a specific bacterial sample as a first step.

9.1.8. Identification of isolated bacterial strain

After the screening of different parameters of PGPR strains along with primary biochemical characterization was done for the identification of isolated as per Cowan and Steels Manual for the identification of medical bacteria (Barrow and Feltham, 1993).

Further, for 16S rRNA sequencing the bacterial culture was inoculated in Luria Bertani broth (Himedia Pvt Ltd). Overnight grown bacterial culture was used for total DNA isolation using a genomic DNA extraction kit (real Biotech Corporation). Universal primers 16Sf (CAGCAGCCGCGGTAATAC) and 16S RNA (TACGGCTACCTTGTTACG) were used for amplification of the 16S rRNA gene. The PCR reaction mixture contained and assay buffer 5µl, forward primer 1 µl, reverse primer 1µl, dNTP 1µl, template 2µl, tag polymerase 1µl, and final total volume was made up 50µl with Milli Q. Polymerase chain reaction was performed in thermo cycler (BIORAD) under the following conditions, denaturation at 94°C for 1 min, followed by annealing at 55°C for 1 min and extension at 72°C for 2 min, for 35 repeated cycles. Approximately 1500 bp region of the gene was amplified and the amplification product was gel purified using QIA gel extraction kit and sequenced. The sequence data were analyzed by BLAST and identified based on closet similarity with the reported sequenced data.

9.1.9. Estimation of heavy metal from *Phragmites communis*

The estimation of metals concentration in different parts of *P. communis* i.e. root, rhizome, shoot and leaves by the described method in chapter five.

9.1.10. Bioconcentration and Translocation factor

It is important to evaluate the BCF and the TF in order to determine the phytoextraction potential of native *P. communis* plant growing on any polluted site of the pulp paper industry (Yoon et al., 2006; Gupta et al., 2008).

9.1.11. Statical data analysis

All data for triplicate samples were reported as means ± SD. All data were subjected to variance analysis to confirm the data variability and validity results (ANOVA).

9.2. Results and discussion

9.2.1. Physico-chemical characterization

A comparative Physico-chemical analysis of the sludge and rhizospheric soil value of the pulp paper mill polluted site is showed in Table.9.1. In addition, the pH value of sludge and rhizosphere soil was substantially more alkaline (pH 8.6 ±8.4) and the alkaline pH for sludge and rhizosphere soil could be caused by the digestion by sector of wood chips containing sodium hydroxide and sodium sulfite. Due to the dissolved lignin, the value of

the color is also high above the recommended limit. The EC (1574) value of sludge was found to be high, while rhizospheric soil EC value (1178) was less comparison to sludge value after in-situ bioremediation. However, EC is an important indicator of soil health and shows the salinity of the soil. Increased salinity is insufficient for agriculture soil because saline should affect crop production, accessibility of nutrients and activity of soil microbes. Furthermore, some studies have also shown that pollution from the pulp paper industry might be the primary cause of abnormally elevated organic matter and EC is the sediment of the disposal site (Bajpai 2015; Yadav and Chandra, 2015; 2018). In addition, in sludge and rhizospheric soil, the concentration of phosphate, sodium, potassium, and chloride was significantly greater. Therefore the most disturbing anion, in general, is chloride, which for most plants and aquatic life is much more toxic than sulfates. Significant amounts of various heavy metals i.e. Fe, Zn, Cu, Cd, Mn, and Ni were also detected in sludge in high amount content of Fe was the highest, followed by that of Cu and Zn, but after the in-situ bioremediation near the rhizospheric zone of *P. communis* the reduced of metal concentration and the (Table.9.1). Moreover, the discharge of the various mentioned metals could be due to the black alkaline oxidation activity generated during most of the digestion of wood when moving via iron pipes. These metals have an elevated concentration that influences the soil permeability, texture as well as relative efficiency. Although, this effluent was used for irrigation by farmers who live around industrial zones; this research has shown that this is hazardous for both humans and animals.

9.2.2. SEM and EDAX Analysis of sludge and rhizospheric soil

The SEM method is a useful strategy to illustrate the structure and morphology of solids and sludge showed in Fig .9.1. The present SEM images of sludge and rhizospheric soil from the pulp paper disposal site showed the organic polymer and microorganism present in the sample. Moreover, the micrographs show that the sludge surface seems to have a rough, rounded, permeable structure that provides the adsorption of metal and other complex biological elements with a heterogeneous particle allocation.

Table.9.1.Physico-chemical characteristics of discharged Pulp Paper mill Effluent and their heavy metals content collected from M/s K R Pulp Paper Ltd. Shahjahanpur, Uttar Pradesh, India. All the values are means of triplicate (n=3) ±SD. Unit of all parameters are in mgl^{-1} except pH, color (Co-Pt Unit) and EC ($\mu mhoscm^{-1}$)

Parameters	Sludge values (mean)	Rhizospheric soil Values (mean)	Permissible limit (EPA 2002)
pH	8.6±0.24	8.4±0.24	5-9
Colour	2634±105	2289±105	Dark Brown
TS	1965±101	1582±110	-
TDS	1478±31.25	983±11.22	-
TSS	67±1.21	23±1.31	35
COD	19340±254.00	8234±125.00	120
BOD	7954±172	4521±120	40
EC	1574 ±84.00	1178 ±79.00	1000
Total Phenols	954±22.23	342±21.24	0.50
Total nitrogen	145±4.10	92±4.10	143
Sulphate	1725±09.70	1738±9.70	250
Phosphorus	147±5.84	192±6.23	200
Cl^-	4.52±0.20	4.23±0.10	1500
Na^+	514±14.20	485±17.23	200
K^+	14.65±0.80	17.8±0.80	-
Lignin	40265±114.21	36300±124.20	-
Chlorophenol	426±10.23	432±10.23	3.0
Fe	91.36±1.81	91.25±1.75	2.00
Zn	23.15±0.40	32.45±0.20	2.00
Cu	4.21±0.07	4.32±0.06	0.50
Cd	0.36±0.01	0.47±0.01	0.01
Mn	1654±0.26	17.32±0.25	0.20
Ni	6.32±0.04	6.32±0.02	0.10

Further, the physical and chemical adsorption on surface quantitative EDX analysis of both samples might be used to adsorb metal or other complexions to the soil. This semi- quantitative analysis of sludge and rhizospheric soil showed the presence of O, Al, Ca, and Si as major elements.

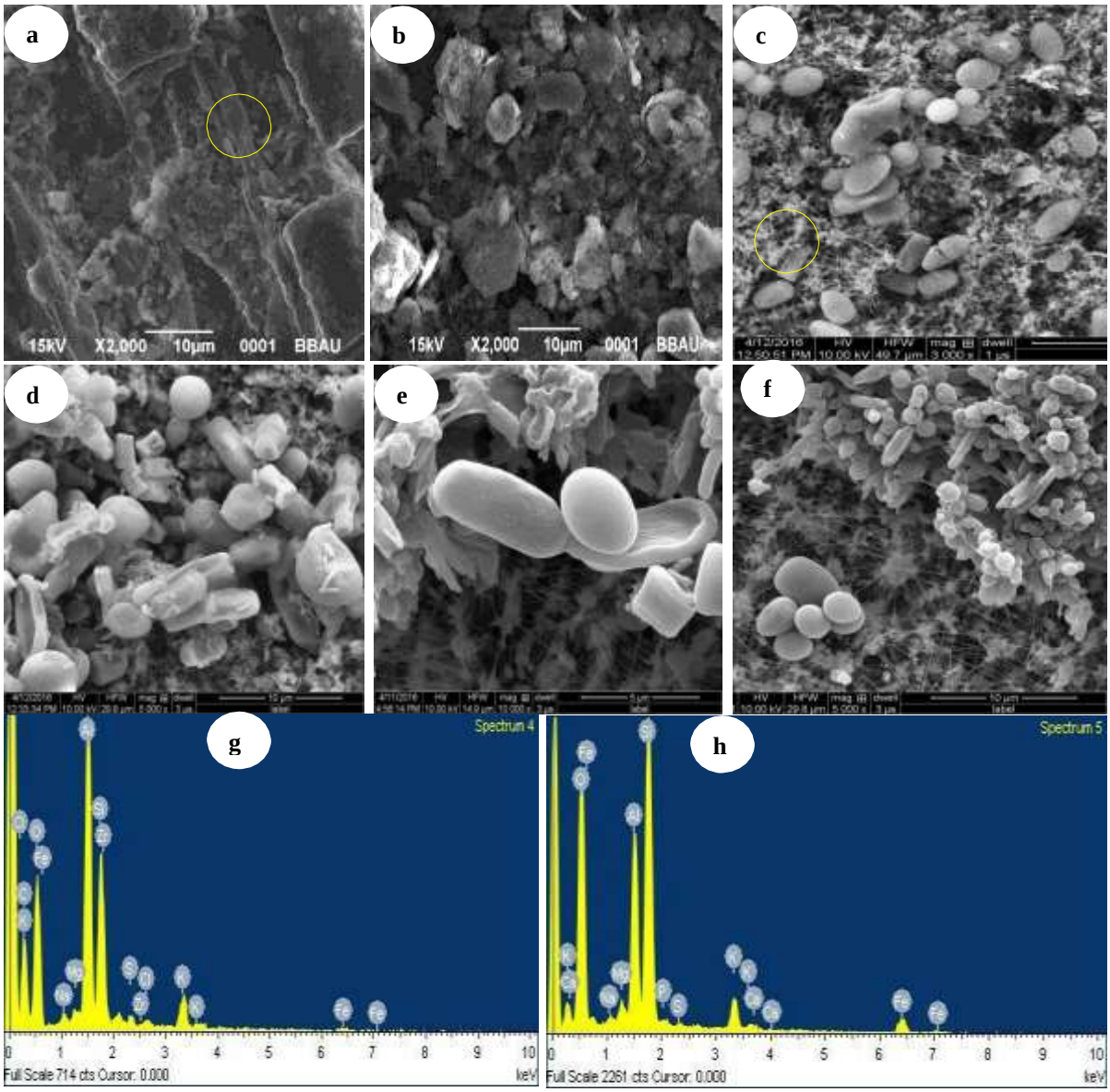


Fig.9.1.Showing the SEM and EDX analysis of different polymer from effluent and sludge of pulp and paper mill waste. The circled area shows the element composition in atomic % and weight %. (a-f) SEM analysis of sludge sample showing the different microbial community (g-h) EDX analysis

9.2.3. Screening of PGPR activity

The total twelve bacteria are isolated from, rhizospheric soil of *P. communis* from the contaminated sites of the pulp paper industry showed in Fig.9.3. Out of theses total isolates, seven isolates (PS-2 MN238724.1, PS-3 MN238725.1, PS-4 MN238722.1, PS-6 MN238714.1, BBAUPS-1 MN294457, BBAUPS-2 MN294456, and BBAUPS-3 MN294458) are highly potential for producing ligninolytic enzyme and hydrolytic enzyme screening. Their microscopic

observation shown in Fig.9.4.In addition, each plant rhizosphere acts as a unique ecological niche and those beneficial plant-related bacteria are referred to as bacteria-promoting plant growth.

Fig.9.2. Purification of isolated PGPR bacterial strains PS-1 to PS-12 (1-12) on Hi-chrome specific media and their microscopic observation

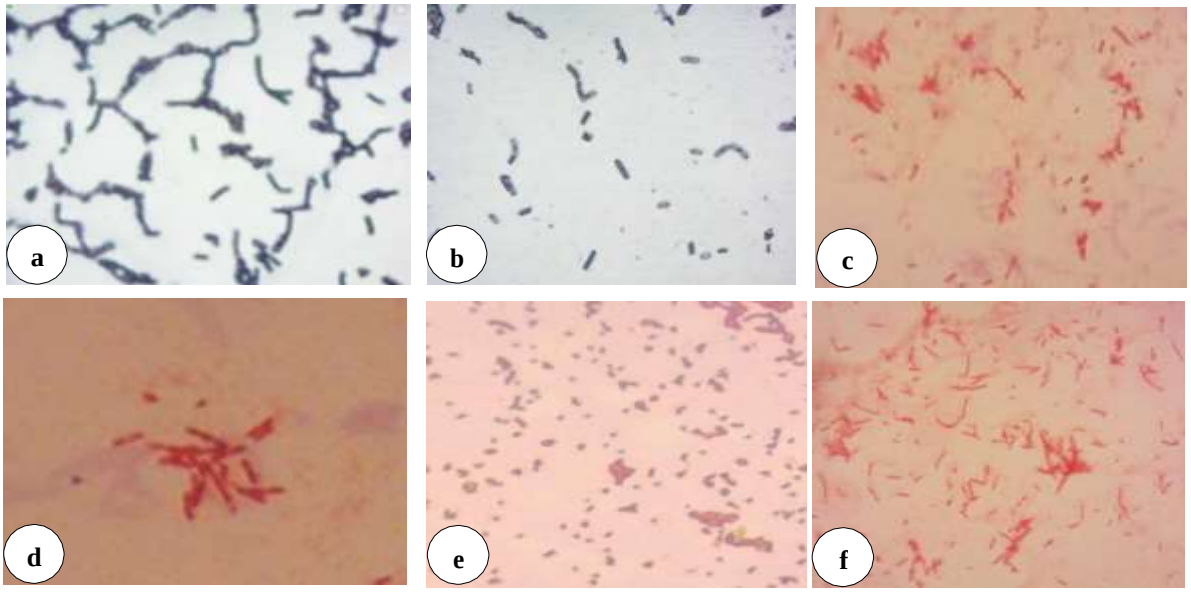


Fig.9.3. Morphological characteristics of isolated PGPR bacterial strains and their microscopic observation (a-f)

isolated bacterial strains was measured by comparing the diameter of the colony and yellow-brown circles Fig.9.6c. The results revealed that the yellow circle diameter percentage and bacterial colony diameter are the largest for 24 to 48 hrs.

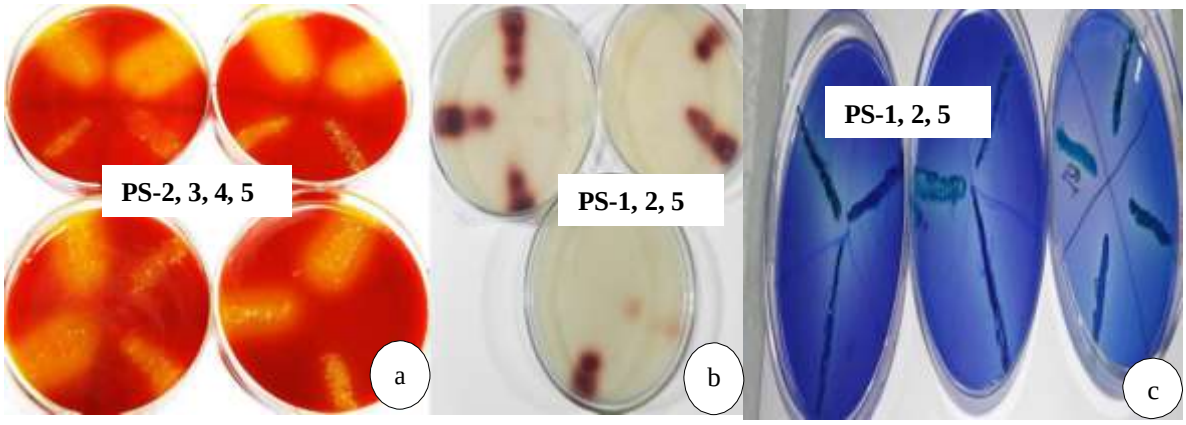


Fig.9.4. The clear zone around the bacterial colony confirms the secretion of ligninolytic enzymes by the potential isolate showing Mnp on the Phenol red plate (a), Laccase on guaiacol plate (b) and, Lip on Azure-B plate (c).

9.2.4. Phosphate solubilization

The favoured ecological niche of plant rhizosphere is considered for different soil microorganisms due to wealthy nutrient availability. The result showed the growth of bacterial culture formation of the hallo zone around the culture inoculation. In addition, the Pikovsakya media containing Bromo Thymol blue (BTB) changed the color from blue to yellow because of the decrease in the pH of the media (Fig.9.7a). Moreover, phosphorus, the second essential nutrient to limit the growth of plants after nitrogen, is accessible organically as well as inorganically rich soils.

9.2.5. Zinc solubilization

Plants might well take up Zinc as a divalent cation, but a very small quantity of complete zinc is available as a soluble form in soil solution (Kabata-Pendias and Pendias, 2001) PGPR have always been soil-borne, rhizocolonizing bacteria, multiplying and competing for plant growth with other bacteria shown in Fig.9.6b.

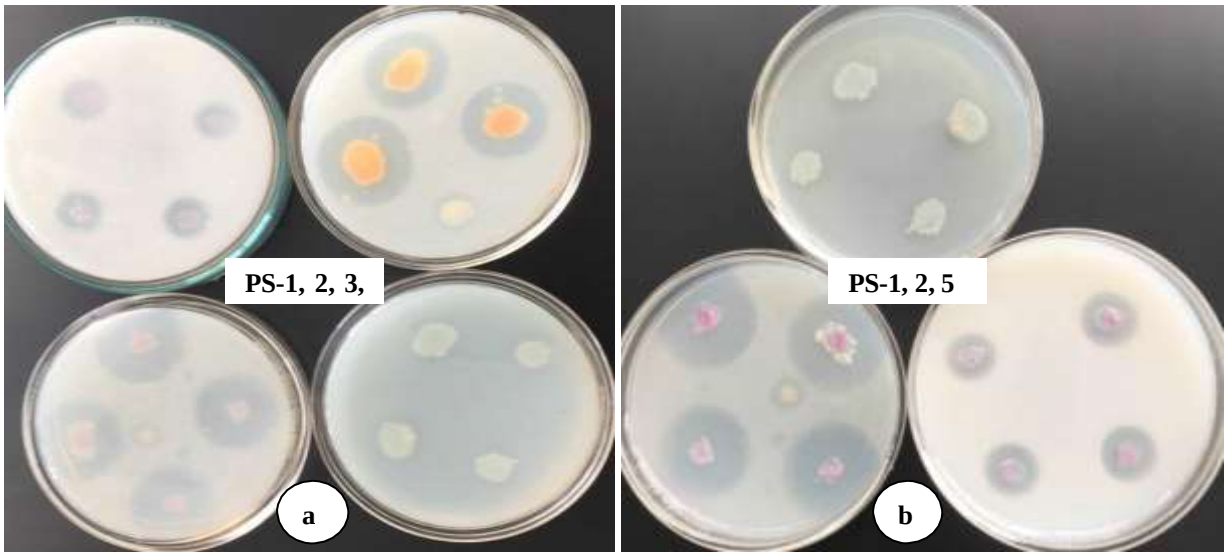


Fig.9.5. Zinc solubilization activity showed by potential PGP bacterial strains based on nutrient solubility test.

9.2.6. Hydrolytic enzyme assay

A study on qualitative analysis of the Hydrolytic enzyme indicated a strong production of Hydrolytic enzyme in PS-1 to PS-12 was estimated. The production of α-amylase, and protease by selected promising bacterial strains, *Bacillus sp.* (MN238724.1 and MN238714.1), and the factors affecting enzyme production, including incubation period, pH, temperature and incubation time shown in Fig.9.7.

9.2.7. 9. IAA production

All the total isolate showed the production of IAA developed pink color in medium Table. A total of four selected isolates i. e. PS-2, PS-, 3 PS-4, and PS-6 were tested for the quantitative estimation of IAA (Table.9.2). Moreover, color growth first became visible at the highest IAA concentration within minutes and continued to rise in frequency for a period of 30 min. In addition, IAA is the key member of the plant-produced auxins family as it plays a significant role in a variety of plant activities such as seed forming, embryo growth, root initiation, and production, abscission i.e. flower falling, phototropism, geotropism, fruit development, etc. Moreover, IAA helps to increase the root length by increasing the number of root branches root hairs and root laterals which continue to absorb the relevant nutrient.

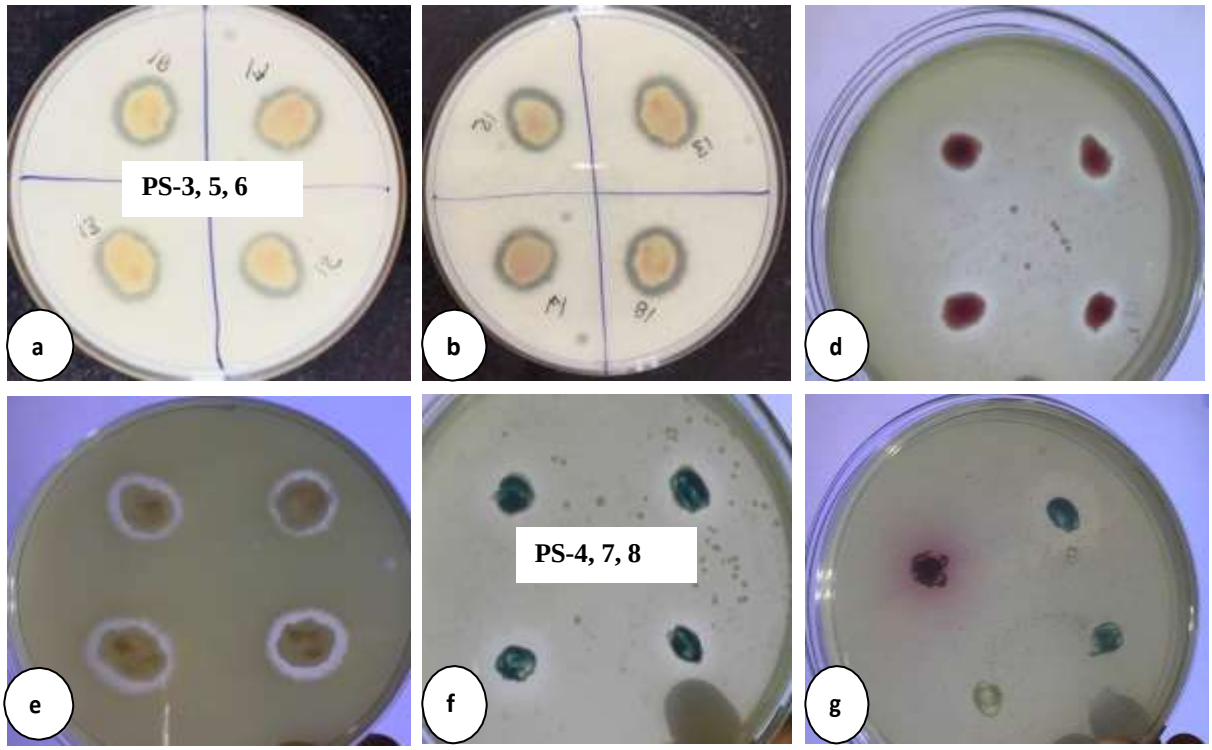


Fig.9.6.Proteases (a,b and e)and α-amylases enzyme (d, f and g) activity assay on plates hydrolysis halos produced on the plate by touching and incubated at 30ºC for 24 hrs.

9.2.8. Siderophore production

Four isolates were siderophore positive showing color change from greenish blue to yellow the production of siderophores is yet another significant feature of PGPR, which could influence plant growth. Optimum siderophores yield has been noted at 36 hrs after incubation and began to decline as production its stationary level. In addition, siderophores can form metal complexes with reduced affinity than $Fecl_3$ Baysse et al., (2000). However, metals including Cd_2 as, Hg_2 as, and Co_2 as revealed an inhibitory impact on both productivity and siderophores growth that might have been owing to competitive metal binding, thus increasing oxidative stress in bacterial cells (Dimkpa et al., 2009). The improvement in development with an increase in iron content expressed a need for iron in metabolic processes by the strain.

9.2.9. HCN production

HCN production was detected for any of the strains and production of ammonia was checked for 71% of the total isolate isolates. The *Bacillus sp.* genus is studied extensively and the elevated frequency of *Bacillus sp.* identified in this experiment can be assigned to the potential to form endospores that allow bacteria to flourish in adverse circumstances such as heat, radiation, dehydration, and hunger. One of the original HCN studies found

which HCN may be harmful to plant pathogens. The statistical technique used to calculate the (CN^-) in the liquid media has been based on a modified colorimetric methemoglobin technique, which is used in the extracellular and dissolved free non-complex cyanide.

9.3. Construction of phylogenetic tree

The 16S rRNA gene sequences data showed that isolated bacteria i.e. *Bacillus sp*. PS-2-(MN238724.1), *Escherichia coli* strain PS-3 (MN238725.1), *Brevundimonas sp*. PS-4 (MN238722.1), *Bacillus sp*. PS-6(MN238714.1), *Aeromonas salmonicida* strain BBAUPS-1 (MN294457), *Aeromonas salmonicida* strain BBAUPS-2 (MN294456), and *Stenotrophomonas maltophilia* strain BBAUPS-3 (MN294458), respectively (Table.9.3. and Fig.9.8), constructed a phylogenetic tree by using Mega 6.0 software. Moreover, Phylogenetic analysis like morphological, physiological, and bionomic characteristics, allozyme and RFLP data were widely used to infer the evolutionary relationship between organisms showed in Fig.9.8.

9.4. Estimation of heavy metal from *Phragmites communis*

The concentration of different heavy metals in the root, shoot, and leaves of *P. communis* was higher in the disposal site of the pulp paper industry. In addition, our study, total concentration of Fe is highest in *P. communis* of rhizome in rang (71.56 mg kg^{-1}), followed by the root (56.46 mg kg^{-1}) and the shoot (44.21mg kg^{-1}), respectively (Table. 9.4). However, Fe is an important micronutrient and contributes the several enzymes and metabolites of plants (Fig.9.9).The similar pattern of metal accumulation pattern of Cu concentration highest in the rhizome (21.65 mg kg^{-1}), while the concentration of Ni is highest in the shoot (31 mg kg^{-1}), respectively. The Cu and Zn concentration is highest in the root (24.77 mg kg^{-1}) of *P. australis* showed their accumulation potential. Consequently, Zn is also an important micronutrient and plays a crucial role in protein function. Although, Cu contributes to numerous physiological and cellular activities i.e. photosynthetic electron transport and is an essential cofactor for many metalloproteinases. In the previous study also similar observations have been reported by Chandra et al., (2017).

Table.9.2. Showing the different PGPR activity from bacterial isolate on the contaminated site of pulp paper industry waste.

Bacterial strains	IAA	P solubilization	Zn solubilization	Ligninolytic Enzyme			Hydrolytic Enzyme	
				Mnp	Lip	Laccase	α-amylase	Protease
PS-1	+++	+++	+++	+++	+++	+++	+++	---
PS-2	---	+++	---	+++	---	+++	---	---
PS-3	+++	---	+++	+++	+++	---	+++	+++
PS-4	---	+++	---	+++	---	---	---	+++
PS-5	+++	+++	+++	+++	+++	+++	---	---
PS-6	+++	+++	+++	+++	+++		+++	---
PS-7	---	---	+++	---	---	+++	---	+++
PS-8	---	+++	---	+++	+++	---	+++	+++
PS-9	+++	+++	+++	+++	+++	---	+++	---
PS-10	---	---	+++	+++	---	+++	---	---
PS-11	+++	+++	+++	+++	---	---	+++	+++
PS-12	---	---	---	+++	+++	---	---	+++

Isolates code	Bacterial species	Accession number	Similarity
PS-2	*Bacillus sp.*	MN238724.1	99.32%
PS-3	*Escherichia coli*	MN238725.1	99.05%
PS-4	*Brevundimonas sp.*	MN238722.1	97.07%
PS-6	*Bacillus sp.*	MN238714.1	98.97%
BBAUPS-1	*Aeromonas salmonicida*	MN294457	96.76%
BBAUPS-2	*Aeromonas salmonicida*	MN294456	94.75%
BBAUPS-3	*Stenotrophomonas maltophilia*	MN294458	99.08%

Table.9.3.Showing the complete detail of bacterial isolate and their accession number in NCBI

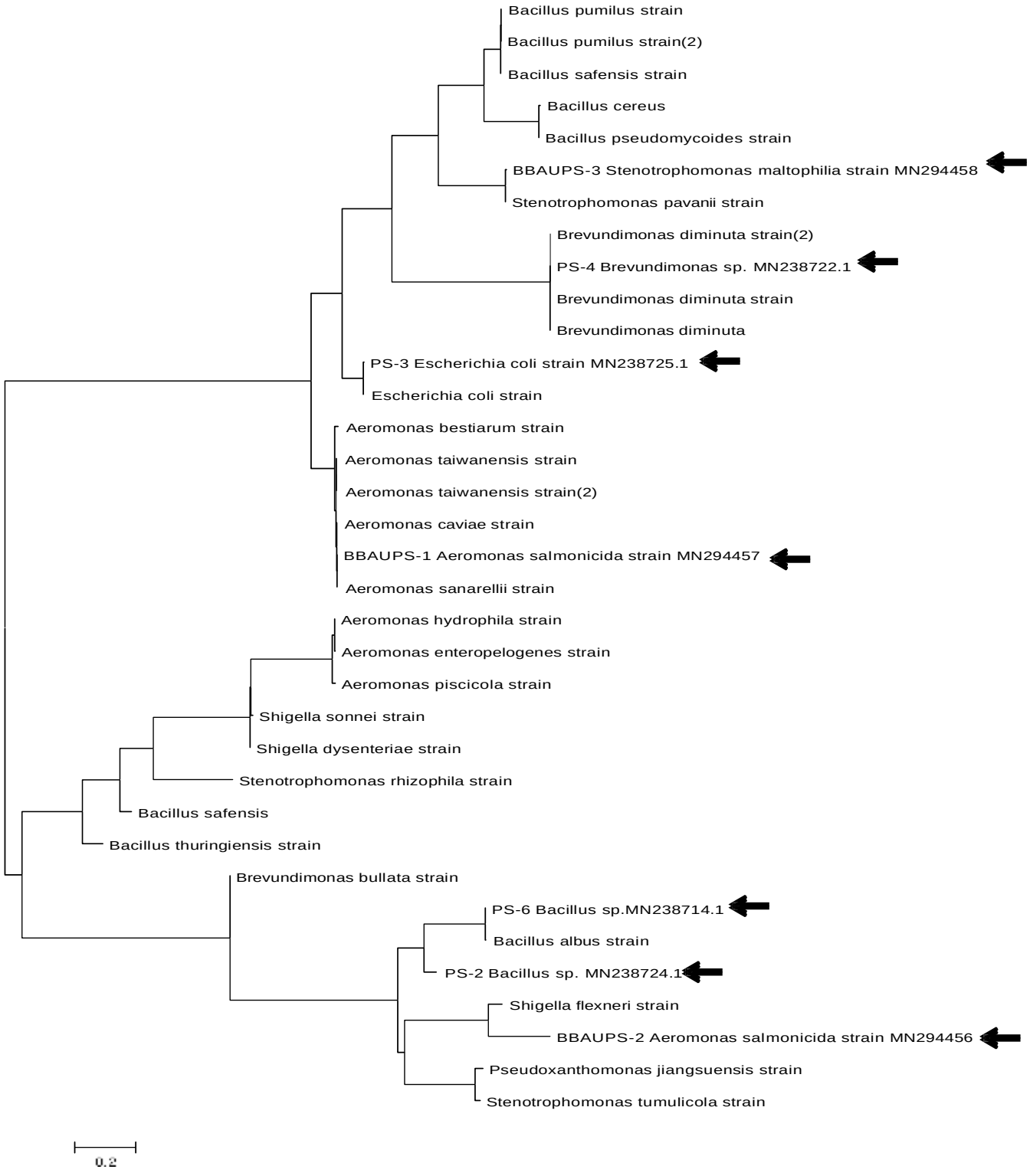


Fig.9.7.Phylogenetic tree showing the relationship of bacterial communities growing on the sludge sample of the pulp paper industry. The accession numbers for all strains used in tree construction are indicated in the figure in square brackets.

Recent evidence recommended that Zn plays an important role in stabilizing RNA and DNA structure, in preserving the activity of DNA synthesizing enzymes and controlling the activity of RNA degrading enzymes and may play a role in controlling gene expression. Cu contributes to numerous physiological and cellular activities *i.e.*, photosynthetic electron transport and is an essential cofactor for many metalloproteinases. In the previous study also similar observations have been reported by Chandra et al., (2017). The result showed that the metal tolerance capacity is widely present in collected native hyperaccumulator plants when they will grow on the organometallic containing sludge of disposal site of the pulp paper industry.

9.5. Bioconcentration and Translocation factor

The study we observe the ratio of heavy metals concentration in plants roots to sludge by using the BCF shown in (Fig.9.5). The capacity of native *P. communis* potential plants to accumulate the metals which might be a tool for in-situ phytoextraction of heavy metals mixed with organic pollutants from pulp paper industry waste after secondary treatment. In addition, *P. communis* showed maximum BCF which showed the ratio of metals in soil was more than one, in rhizome Fe (7.354 mg kg^{-1}), Cu (1.957 mg kg^{-1}), Ni (1.647 mg kg^{-1}), Cr (1.024 mg kg^{-1}), and in root of Zn (1.567 mg kg^{-1}), showed in Table. 9.7. However, metals from the root to the shoot by TF is >10 highest in Fe in the shoot (1.746mg kg^{-1}), Mn (1.846mg kg^{-1}) and Cu in the rhizome (2.514 mg kg^{-1}) and respectively shown in Table. In addition, the accumulation of metals in high concentration could be developed detoxification technique depending on ion sequestration in vacuole by binding with

Table. 9.4. Showing the concentration of different metals in *P. communis in* the root, shoot and leaves and their BCF and TF

Metals (mg kg−1)	**Different Parts of *Phragmites communis***			
	Root	**Rhizome**	**Shoot**	**Leaves**
Cu	19.65± 0.32	21.65± 0.22	13.53± 0.44	10.23±0.84
Cr	11.84± 0.13	33.45± 0.11	19.87± 0.56	5.00± 0.77
Ni	18.22± 0.43	26.13± 0.46	31.26± 0.89	9.12± 0.57
Pb	12.33± 0.52	23.24± 0.78	18.99± 0.64	14.46± 0.90
As	16.32± 0.51	14.52± 0.90	12.67± 0.83	10.23± 0.13
Fe	59.46± 0.18	71.56± 0.53	44.21± 0.19	36.21± 0.56
Mn	46.21± 0.21	39.34± 0.20	33.64± 0.46	31.26± 0.34
Cd	10.30± 0.71	11.24± 0.47	10.49± 0.90	8..23± 0.42
Mg	15.33± 0.67	19.64± 0.98	14.62± 0.23	12.34± 0.89
Zn	24.77± 0.34	21.46± 0.48	19.37± 0.43	16.38± 0.65
Mo	31.22± 0.97	35.61± 0.14	27.54± 0.90	24.31± 0.32
Se	17.54± 0.38	22.54± 0.18	15.64± 0.45	12.34± 0.52
Co	13.64± 0.91	19.47± 0.93	16.34± 0.14	12.36± 0.90
	BCF			
	Root	**Rhizome**	**Shoot**	**Leaves**
Cu	**1.876**	**1.957**	**1.235**	**1.214**
Cr	0.844	**1.024**	0.647	0.026
Ni	**1.241**	**1.647**	**1.514**	**1.023**
Pb	0.234	0.564	0.624	0.444
As	0.145	0.268	0.364	0.251
Fe	**5.324**	**7.354**	**4.265**	**3.258**
Mn	0.957	0.952	0.741	0.562
Cd	0.024	0.056	0.081	0.059
Mg	0.094	0.456	0.364	0.561
Zn	**1.567**	**1.268**	**1.024**	**1.002**
Co	0.023	0.062	0.023	0.014
	TF			
	Root	**Rhizome**	**Shoot**	**Leaves**
Cu	**2.330**	**2.514**	**1.248**	**1.124**
Cr	0.214	0.654	0.859	0.740
Ni	0.745	0.854	0.954	0.880
Pb	0.546	0.751	0.654	0.549
As	0.762	0.969	0.741	0.721
Fe	**1.364**	**1.258**	**1.746**	**1.561**
Mn	**1.254**	**1.843**	**1.846**	0.871
Cd	0.124	0.249	0.241	0.231
Mg	0.749	0.849	0.854	0.746
Zn	0.015	0.194	0.147	0.122
Co	0.042	0.097	0.049	0.045

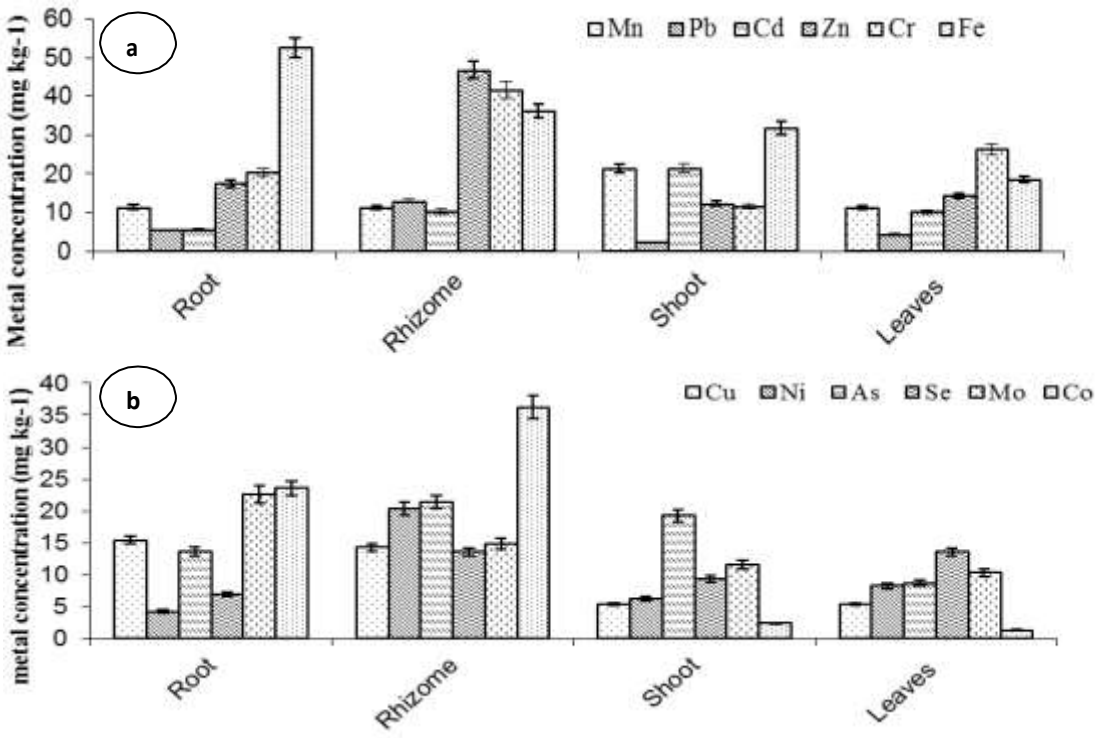


Fig.9.8. Metal accumulation in *P. communis* in their root, rhizome, shoot, and leaves on the contaminated site of the pulp paper industry

Ligands i.e. protein, organic acid and peptides in the presence of which can operate at a high rate of meticulous circumstances (Yang et al., 2005). MacFarlane et al., (2007), described the translocation value was evaluated the phytostabilization and phytoextraction of the metals by native plant growing at the disposal site of the pulp paper industry. In addition, the accumulation of metals by plants from sludge to root depends on the chemical nature of element, pH, and other co-pollutants of sludge is inhibit the mobility of metals so it inhibits the accumulation and translocation in plants (Gupta and Sinha, 2008; Yoon et al., 2006; Rosselli et al., 2003). In addition, native hyperaccumulator plants compare BCF and TF to extract heavy metal from sludge to root and root to shoot. The BCF and TF values are less than one is unacceptable for phytoextraction (Fitz and Wenzel, 2002).

Conclusion

This study underlines the importance PGPR strains in the rhizospheric zone of *P. communis* and evaluating the in-situ phytoextraction of hazardous pollutants present in contaminated site of pulp paper industry after secondary treatment.

Chapter-Ten

Assessment of Detoxification and Degradation of Pulp and Paper Mill Effluent by Biostimulation and Bioaugumentation Process from Pulp and Paper Mill Contaminated site

Assessment of Detoxification and Degradation of Pulp and Paper Mill Effluent by Biostimulation and Bioaugumentation Process from Pulp and Paper Mill Contaminated site

10. Introduction

Pulp paper industries are one of the major sources of aquatic pollution due to discharge of large amount of complex chlorolignins compounds (Singh and Chandra, 2019). The presence of organic pollutants in pulp paper industry waste after final discharge that the microbial community is unable to degrade the pollutants due to no availability of adequate nutrients. Hence, the optimization of bacterial growth conditions by the addition of nutrient or environmental conditions may be an effective approach for detoxification or of final discharged effluent (Chandra et al., 2018). However, due to the lack for the understanding of controlling factors for biodegradation of residual organic pollutants in-situ represent significant obstacles to the application of bioremediation as a remedial option in several polluted sites with complex organic pollutants. In-situ bioremediation has therefore been proposed as an alternative method in order to reduce time and cost for any polluted site restoration. However, prior to selection for the mode of bioremediation, the name of the microbial community and required environmental condition should be detected. In general three types of bioremediation processes are selected for in-situ bioremediation of any complex industrial pollutants i.e. natural attenuation; bio-stimulation and bio-augmentation are commonly used. The simplest method of bioremediation is to implement is natural attenuation where contaminated sites are only monitored for the concentration of the pollutant. To assure the controlling factors that natural processes of pollutant degradation are active which bio-stimulation requires adjustment to the polluted site in order to provide bacterial communities with a favourable environment in which they can effectively degrade pollutants (Kaplan and Kitts, 2004). This includes the addition of nitrogen, phosphorous for the proliferation of indigenous bacterial communities and the required pH is also adjusted for enhancement of bioremediation process (Salanitro et al., 1997) but in case this is low number of degrading bacterial communities or absent at the polluted site, where the natural communities of degrading bacteria are present in low numbers or absent. This requires

the addition of competent bacteria to speed up the degradation process which is called a bio-augmentation process (Vanlimbergen et al., 1998). But, some competent bacteria have been reported more effective than fungi for bioremediation of environmental pollutants due to their immense environmental adaptability and biochemical versatility. Bacteria isolated from compost soil, viz. Azotobacter and Serratia marcescens were capable of degradation and decolorization of lignin (Morii et al., 1995). Moreover, bacteria such as *bacillus subtilis* and *bacillus sp.* have also been tested for kraft-lignin degradation (Abd-Elsalam and El-Hanafy, 2009). Previously, three potential bacterial strains of *Panibacillus sp. Aneurini bacillus aneurinilyticus* and *Bacillus sp*. were isolated from pulp and paper sludge for degradation and decolorization of synthetic lignin at 500 mg/l and characterized their metabolic products by GC–MS analysis (Chandra et al., 2007; Raj et al., 2007). Furthermore, the *Bacillus sp.* and *Serratia marcescens* have also been reported for degradation of pentachlorophenol from pulp paper mill effluent in presence of supplement i.e. carbon (1% glucose) and nitrogen (0.5% peptone w/v) source up to 94% at optimized conditions in the laboratory. Thus, these studies are given a strong clue for the degradation and detoxification capability by bacteria for chlorolignin containing pulp paper mill wastewater (Chandra and Singh, 2012). However, the detoxification and in-situ bioremediation of pulp paper mill effluent after secondary treatment are not reported so for. Therefore, the present studying has focused on the detection of residual organic pollutants and the number of microbial counts and their nutritional requirements and environmental conditions for detoxification of discharge pulp paper mill effluent. This will lead to developing in-situ bioremediation technology for pollution prevention.

10.1. Materials and Methods

10.1.1. Sample collection

Pulp and paper mill effluent from the paper mill effluent discharged site of M/s Century Pulp Paper Mill Ltd, Lalkua, Uttarakhand, India (29ºN, 79.3ºE) were collected. M/s Century pulp paper mill is located at the foothills of the Himalayas. The mill produces 524 tons of fine quality pulp per day and discharges 48426 m^3 effluents in total. All the Physico-chemical analysis was done within 48 hrs.

Fig. 10.1.Sludge discharged from pulp paper industry (a-b) discharged view from industry (c-d) sludge waste at dumping site of M/s K.R. pulp paper mill

10.1.2. Physico-chemical analysis

The Physico-chemical analysis of pulp and paper mill effluent as such as a control sample and bio stimulated samples were analyzed for TS, TDS, TSS, BOD, COD, total phenols, total nitrogen (Micro Kjeldahl), sulphate (gravimetric method) phosphorus and color (visual color comparison method) as per standard methods described in APHA (2005). The pH, chloride, sodium, and potassium of the medium were also analyzed with the respected selective ion electrode of Thermo Orion (Model 960). While lignin was estimated according to the method of Pearl and Benson, (1990). The concentration of different heavy metals i.e. Fe, Zn, Cu, Cr, Cd, Mn and Ni were also measured by means of acid digestion, following the standard method for the examination of water and wastewater using inductively coupled plasma spectrophotometer (Thermo Electron; Model IRIS Intrepid II XDL, USA) (APHA, 2005).

10.1.3. Colony forming unit (CFU), biomass formation and total protein estimation

The total microbial load of the degraded effluent was determined with the help of the standard plate count method. The degraded samples were serially diluted 10-fold, and 15 µl of the diluted sample was spread over plate count agar (PCA, Himedia, India)

containing Casein enzymic hydrolysate 5.0 g/l, yeast extract 2.5 g/l, Dextrose 1.0 g/l and agar 15.0 g/l. The plates were incubated at 37°C for 24 h, after which the total colony count was determined. For biomass estimation, 1 ml of bacterial growth sample was centrifuged and dried in the pre-weighed Eppendorf tube. The cells were completely drying at 50°C, took the post-weight of Eppendorf. Post weight minus pre-weight of Eppendorf was considered as bacterial biomass per ml. However, an aliquot of the culture filtrates was used for the estimation of extracellular protein content according to the method of Lowry et al., (1951). Bovine serum albumin was used as a protein standard.

10.1.4. SEM study of bacterial consortium during biostimulation

Bacterial cells were centrifuged (6500×g) for 20 min at 6 and 12 days bacterial incubation; the pellets were washed thrice with distilled water to remove the medium contents. Subsequently, the bacterial cells were fixed with 0.1M phosphate buffer (pH 7.2) containing 1% glutaraldehyde for 2 h and washed again with distilled water. Fixed cells were then dehydrated using a series of acetone solutions (15, 30, 60, 90 and 100%) for 20 min, as the standard method described by Sangeeta et al., (2011). The final dehydration process was repeated twice. The dried cells were then mounted on the metal stubs being coated under vacuum with approximately 25 nm of high purity carbon and examined under the scanning electron microscope (SEM, QUANTA FEG 450, FEI, and Netherland).

10.1.5. Ligninolytic enzyme activity in potential bacterial strains

The LiP assay was done by monitoring the oxidation of dye Azure B in the presence of H_2O_2. The reaction mixture contained sodium tartrate buffer (50 mmol, pH 3.0), Azure B (32 mM), 500 ml of culture filtrate, 500 ml of H_2O_2 (2 mM). OD was taken at 651 nm after 10 min (Arora et al., 2002). MnP assay was performed by the method as described by de Oliveira et al. (2009) which is based on the oxidation of phenol red. The reaction mixture (4 ml) contained 1 ml of potassium phosphate buffer (pH 7.0), 1 ml of enzyme extract, 500 ml of $MnSO_4$ (1 mmol), 1 ml of phenol red (1 mmol) and 500 ml H_2O_2 (50 mmol). 1 ml sample was removed from the reaction mixture and 40 ml of 5 mol NaOH was added to stop the reaction. Consequently, OD was taken at 610 nm at every 1 min interval. The absorption at 610 nm was measured against a blank without any manganese in the reaction mixture. Absorption difference per min was converted to U/L using an

extinction coefficient of the oxidized phenol red that is 22 m/mol/cm. Moreover, laccase activity was detected by taking the absorbance at 450 nm. The reaction mixture was prepared by 3.8 ml of acetate buffer (10 mmol, pH 5.0), 1 ml of guaiacol (2 mmol) and 0.2 ml of enzyme extract. Then the reaction mixture was incubated at 25°C for 2 hr (Arora et al., 2002). One international unit (IU) of enzyme activity was defined as the activity of the enzyme that catalyzed the conversion of 1 m mol of substrate/min.

10.1.6. Pulp and paper mill effluent degradation through biostimulation

The biostimulation studies were performed in pulp and paper mill effluent samples in 250 ml Erlenmeyer flask amended with different concentrations of glucose (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) and 0.5% peptone (w/v) as additional carbon and nitrogen source. Each sample was autoclaved by subjected these flasks to high pressure saturated steam at 121°C for around 15-20 min. Autoclaved samples were incubated at 37±1°C in an incubator shaker (Orbitek, Scigenics Biotech, India) at different rpm 130, 140 and 150 rpm up to 144 h. The culture sample was removed under aseptic conditions. The growth of the mixed culture was determined by measuring optical density at 620 nm at every 24 h interval up to 144 h incubation period. Pulp and paper mill effluent (without inoculum) was used as control during the experimental period. The samples were centrifuged at 8000g for 30 min and absorbance was measured at an optical density at 620 and 465 nm for bacterial growth and color reduction by using UV–VIS spectrophotometer (Evaluation 20, Thermo Fisher Scientific, India) at every 24 h interval up to 144 h. The inoculated media were used as control throughout the degradation period. Among aforesaid conditions, 2.0% glucose and 0.5% peptone at 150 rpm was found optimum for bacterial growth and decolorization. Hence, this condition was used throughout the study.

10.1.7. Identification of Residual Organic Pollutants

10.1.7.1. Extraction of organic pollutants

Various organic solvents i.e. n-hexane, methanol, isopropyl alcohol, and ethyl acetate were tested to compare extractability of residual organic pollutants and ethyl acetate was found to be optimal. The selected operating process was as follows: 50 g air-dried sludge samples were dissolved in 500 ml distilled water having pH 8.0 and left for 24 h on 100 rpm. The samples were centrifuged at 3000 X g for 20 min to remove suspended particles. The supernatant obtained was extracted three times with the equal volume of

ethyl acetate in a separating funnel having capacity 500 ml by intermittent shaking. The solvent layer containing organic pollutants was separated and evaporated under vacuum at 40°C for dryness. The obtained dried residue was dissolved in 1.0 ml of HPLC grade acetonitrile, filtered through a syringe filter (0.22 µm) and used for further analysis.

10.1.7.2. GC-MS analysis for Characterization of residual organic pollutants

The GC–MS analysis of ethyl acetate extracts were analyzed as per the method described by Chandra et al., (2009). 100 µl dioxane and 10 µl pyridine were added to samples followed by silylation with 50 µl BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane)]. To dissolve the residue the solution was heated at 60°C for 15 min with periodic shaking. An aliquot (1 µl) of silylated samples were injected in GC–MS (PerkinElmer, UK) equipped with a PE auto system XL gas chromatograph. The analytical column connected to the system was a PE-5MS capillary column (20 m 0.18 mm internal diameter, 0.18 mm film thickness). Helium gas was used as a carrier gas with a flow rate of 1 ml/min. The column temperature was programmed as 50°C (5 min); 50-300°C (10°C per min, hold time: 5 min). The transfer line and ion source temperature were maintained at 200 and 250 ºC, respectively. A solvent delay of 3.0 min was selected. The electron ionization (EI) mass spectra were recorded in a range of 30–550 (m/z) at 70 eV. The residual organic pollutants were identified by comparing their mass spectra with that of the National Institute of Standards and Technology (NIST) library available with instruments.

10.1.7.3. Evaluation of bioremediation through HPLC and GC–MS analysis

The bacteria degraded and undegraded (control) samples of pulp paper mill effluent were centrifuged at 5,000 rpm and acidified (pH 1–2) by 0.1 N HCl and alkaline (pH 8.5) by NaOH pellets. The precipitate was extracted thrice with ethyl acetate and dewatered over anhydrous sodium sulfate. The residues were dried under a stream of nitrogen gas and dissolved in acetonitrile (HPLC grade), filtered through Whatman no. 54 filter paper for HPLC analysis, The samples were analyzed using a Waters, 515 HPLC, equipped with a 2487 UV/VIS detector, via millennium software. Samples (20 µl) were injected followed by the implementation of HPLC grade acetonitrile/water (70:30) at the rate of 1 ml min^{-1}. Reverse phase C-18 column (250 mm 9 4.6, particle size 5 lm) at 27°C were used to analyse the compounds at 250 and 320 nm. For GC–MS analysis, the dry residues of

ethyl acetate extracts were derivatized with trimethylsilyl [BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) TMCS] (Raj et al., 2007). An aliquot of 1µl of silylated compounds was injected into the GC–MS equipped with a PE Auto system XL gas chromatograph interfaced with a Turbo mass spectrometric mass selective detector. The analytical column connected to the system was a PE 5MS capillary column (20 m 9 0.18 mm i.e., 0.18 lm film thickness). Helium gas with a flow rate of 1 ml/min was used as the carrier gas. The column temperature was programmed as 50°C (5 min); 50–300°C (10°C per min, hold time: 5 min).

10.1.7.4. Isolation and Identification of Autochthonous Bacteria

The dominant autochthonous bacteria were isolated by serial dilution and plate streak method. For bacterial isolation plates were prepared using effluent extract mixing with carbon (1% dextrose), nitrogen (0.5% peptone) and agar (15%) in 250 ml distilled water. Dominantly growing purified autochthonous bacterial strains were identification as per Cowan and Steels Manual for the identification of medical bacteria (Barrow and Feltham, 1993). Further, for 16S rRNA sequencing the bacterial culture was inoculated in Luria Bertani broth (Himedia Pvt Ltd). Overnight grown bacterial culture was used for total DNA isolation using the genomic DNA extraction kit (real Biotech Corporation). Universal primers 16Sf (5’ CAGCAGCCGCGGTAATAC 3’) and 16 Sr (5’ TACGGCTACCTTGTTACG 3’) were used for amplification of 16S rRNA gene. The PCR reaction mixture contained and assay buffer 5µl, forward primer 1 µl, reverse primer 1 µl, dNTP 1 µl, template 2 µl, tag polymerase 1 µl and final total volume was made up 50 µl with Milli Q. Polymerase chain reaction was performed in thermo cycler (Sure Cycler 8800; Agilent Technologies, Malaysia) under the following conditions, denaturation at 94°C for 1 min, followed by annealing at 55°C for 1 min and extension at 72°C for 2 min, for 35 repeated cycles. Approximately 1500 bp region of the gene was amplified and the amplification product was gel purified using QIA gel extraction kit and sequenced. The sequence data were analyzed by BLAST and identified based on closet similarity with the reported sequenced data. A phylogenetic tree was generated using MEGA-6.0 software (Tamura et al., 2013). All query sequences and other homologous sequences available online in the NCBI (National Centre for Biotechnology Information) nucleotide database were saved in a single FASTA file format after retrieval.

Furthermore, all sequences were saved in one FASTA format file and then subjected to multiple sequence alignment using MEGA-6.0, which was subsequently used to reconstruct phylogenetic trees by the Neighbor-Joining method using the MEGA-6.0 Draw Tree tool (Larkin et al., 2007) with a bootstrap value of 1,000 replicates.

10.1.8. Phytotoxicity Evaluation

10.1.8.1. Seed germination test with green gram (*Phaseolus mungo*) and wheat (*Triticum aestivum*)

Bacterial treated pulp and paper mill effluent was centrifuge at 7000 rpm for 20 min and the supernatant was autoclaved, filter through the 0.2 µm membrane for seed germination study. Different concentrations of bacterial treated and untreated effluent i.e. 50 and 100% were prepared with the help of tap water. As such supernatant was treated as 100% and tap water as a control. Ten seeds of *P. mungo* and *T. aestivum* were placed on three layers of filter papers (Whatman No.-1) in a glass Petri dish (9 cm dia). The filter paper was moistened with different concentrations of effluent and tap water. Seed germination was observed after 24 hrs interval.

10.1.8.2. Genotoxicity evaluation on root tips of *Allium cepa*

Onion bulbs (*Allium cepa* L., 2n=16) of the purple variety of average size (15-22 mm diameter) were purchased in the local market of Lucknow, India. Bulbs were made germinated in common portable water (without any growth factors) during the course of 2-4 days. Then the bulbs were carefully removed without any damage to the roots. Infectious bulbs are discarded for experimental purposes. The outer scales of onion bulbs and brownish bottom plates were removed without injuring the ring of root Primordia. Onion bulbs with good root growth were selected. For root growth inhibition evaluation, freshly prepared stock extracts were diluted into 50 and 100%. Three onion bulbs (with roots) were utilized for each concentration and the control (tap water). The base of each of the bulbs (roots) was suspended on the extracts inside 100 ml beakers in the dark for 6 to 24 h. At the end of the exposure period, five root tips from these bulbs were cut and fixed in ethanol: glacial acetic acid (3:1, v/v). These were hydrolyzed in 1N HCl at 60°C for five minutes after which they were washed in distilled water (Fiskesjo G 1985, 1997, 1993). Two root tips were then squashed on each slide, stained with 2% hematoxylin for

10 min and coverslips carefully lowered on to exclude air bubble. The coverslips were sealed on the slides with clear fingernail polish.

10.1.9. Statistical Analysis

All data were reported as means ± SD for triplicate samples. To confirm the data variability and results from validity, all the data were subjected to analysis of variance (ANOVA). Turkey's test (Ott, 1984) using the Graph Pad software (Graph Pad Software, San Diego, Calif.) was used for statistical analysis.

10.2. Result and Discussion

10.2.1. Physico-chemical Analysis

Physico-chemical characteristics of the pulp and paper mill effluent after secondary treatment are shown in Table.10.1. The Physico-chemical analysis of pulp and paper mill effluent after secondary treatment revealed the presence of a high amount of color, TDS, TSS, COD, BOD, phenolics compound along with nitrogen and phosphorus. Besides, sodium, potassium and chloride ion are also present in the effluent but this shows that autochthonous bacterial community unable to utilize the nutrient for their growth. Therefore, supplementary carbon and nitrogen was effective as glucose (2%) and peptone (0.5 %). Moreover, there was a significant amount of Fe, Zn, Cu and Mn in the effluent which is hazardous to the environment. However, after bacterial treatment in the bio-stimulation process, all the parameters were reduced drastically. This revealed that the bacterial populations were the potential to degrade the residual organic compound.

10.2.2. Biomass Production and SEM Analysis

The measurement of colony-forming unit CFU and biomass showed a continuous increase along with incubation time compared to the control sample (Fig.10.2). In addition, the periodic optical density at 620 nm of the control sample and bacterial growing sample also apparently supported the data for increased biomass. Furthermore, the periodic SEM analysis of degrading samples in the biostimulation process also showed the increase of bacterial population and diversity also (Fig. 10.1b and c). Hence, these findings are also given strong evidence that there might be sequential bacterial species responsible for the detoxification of various pollutants present in the pulp and paper mill effluent after secondary treatment. However, this study needs a more detail study regarding the identification of the autochthonous bacterial community and the

compounds of EDCs nature present in the effluent of pulp and paper in the industry. Simultaneously, the bacterial treated sample also showed the reduction of color compared with control. Hence, this established that the growing autochthonous bacterial populations were potential enough for degradation of the residual organic complex compound in the presence of optimum nutrient and environmental conditions. However, such a study regarding the detoxification of complex compounds from domestic and industrial waste for safe disposal.

Table.10.1. Physico-chemical characteristics of discharged pulp and paper mill effluent and their heavy metals content collected from M/S century pulp paper Ltd. Lalkuan, Nainital, and Uttarakhand, India.

Parameters	Effluent Values (mean)	Degraded effluent	Permissible limit (EPA 2002)
pH	8.1±0.20	7.0±0.20 ^{ns}	5-9
Colour	2500±125	625±24.35*	
TS	616±120	136±4.21	
TDS	560±13.25	110±2.12	
TSS	56±2.13	26±1.02	
COD	17999±205.00	3000±64.87*	
BOD	6000±127	2700±60.00*	
Total Phenols	413±18.23	389±18.14 ^{ns}	-
Total nitrogen	143±6.10	103±5.20 ^{ns}	143
Sulphate	1692±13.70	1280±15.67*	250
Phosphorus	180±6.60	172.3±6.40^{ns}	180
Cl^-	2.04±0.10	1.230±0.10*	1500
Na^+	64±19.90	25.00±20.50*	200
K^+	7.8±0.20	1.380±0.90*	-
Lignin	46000±14.21	1550±12.06*	-
Chlorophenol	203±20.30	195±20.00^{ns}	3.0
Heavy metals			
Fe	67.53±2.00	1.05±0.30*	2.00
Zn	13.90±0.30	0.27±0.01*	2.00
Cu	2.15±0.06	0.09±0.01*	0.50
Cr	2.30±0.06	0.11±0.01*	0.05
Cd	0.255±0.01	0.02±0.01*	0.01
Mn	11.00±0.30	0.07±0.01*	0.20
Ni	3.30±0.02	0.19±0.01*	0.10

10.2.3. Ligninolytic Enzyme Activity Assessment and Total Protein

To detect the role of extracellular enzyme activity by the autochthonous bacterial community during the degradation of pulp and paper mill effluent are shown in Fig.10.4. The enzymes secreted by bacteria were measured from the culture supernatant. Moreover, LiP and MnP were recorded as dominating enzymes at the initial stage of bacterial growth for the degradation of organic pollutants. The highest LiP was recorded 40.0 IU/ml and MnP 45 IU/ml at 48 hrs incubation.

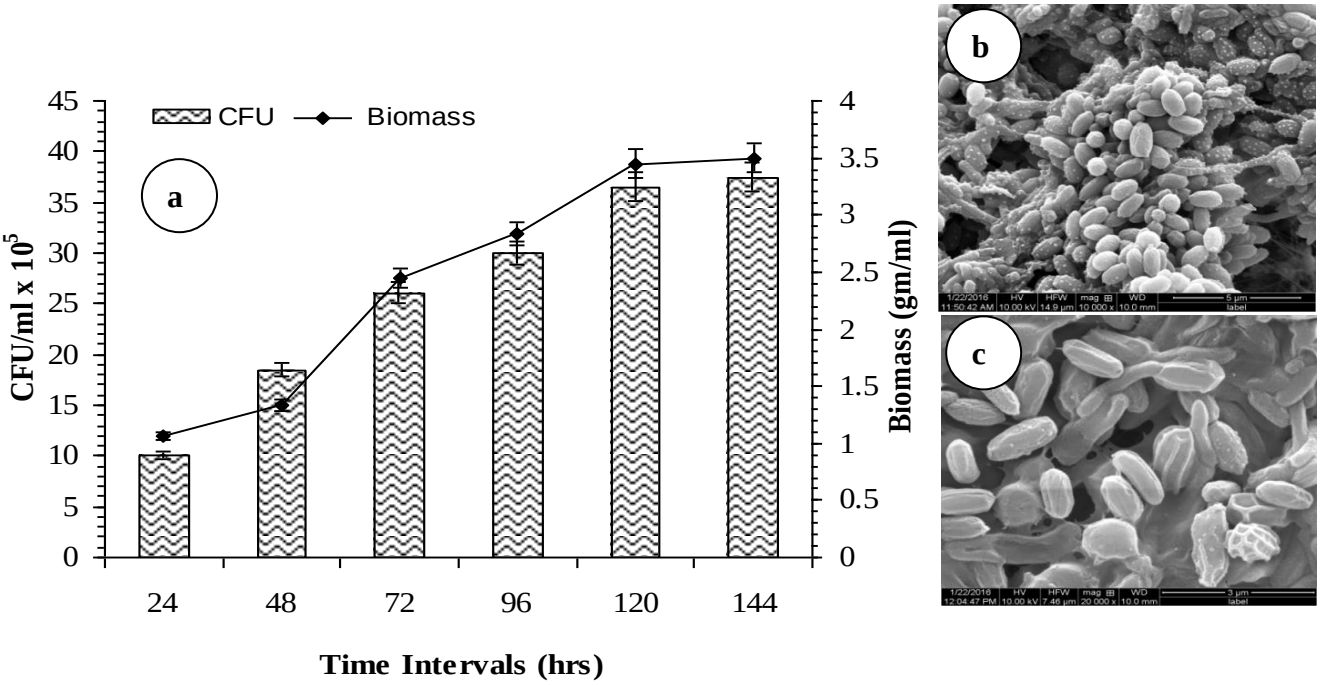


Fig.10.2 Pattern of Biomass and CFU in the pulp paper mill effluent decolourization after Biostimulation of autochthonous bacteria by 2% glucose and 0.5% peptone (a). Morphological view of bacterial strains observed under SEM at 3 days (b) and 6 days (c) incubation at 10000x magnification

This revealed that the LiP enzyme was predominantly present at the initial growth phase of the autochthonous bacterial population. Therefore, several lignin monomer compounds were either generated in the initial phase or diminished in the treated sample. Subsequently, the induction of MnP in the highest amount also given strong evidence for the presence of several pollutants including the metabolite where MnP could play a very vital role. While the induction of Laccase at the later stage of growth phase i.e. at 120 h indicated that there was the dominance of phenolic compounds where Laccase could

contribute a very vital role and maximum induction of Laccase (39.0 IU/ml) at 120 h. The presence of all three extracellular ligninolytic enzymes in the autochthonous bacterial community is very important for detoxification of broad range organic compounds where the nutrients play a very crucial role. The increasing pattern of total protein content at different incubation time was similar to enzyme activities (Fig.10.4). This supported the data for enzyme-mediated biodegradation of organic pollutants present in pulp and paper mill effluent.

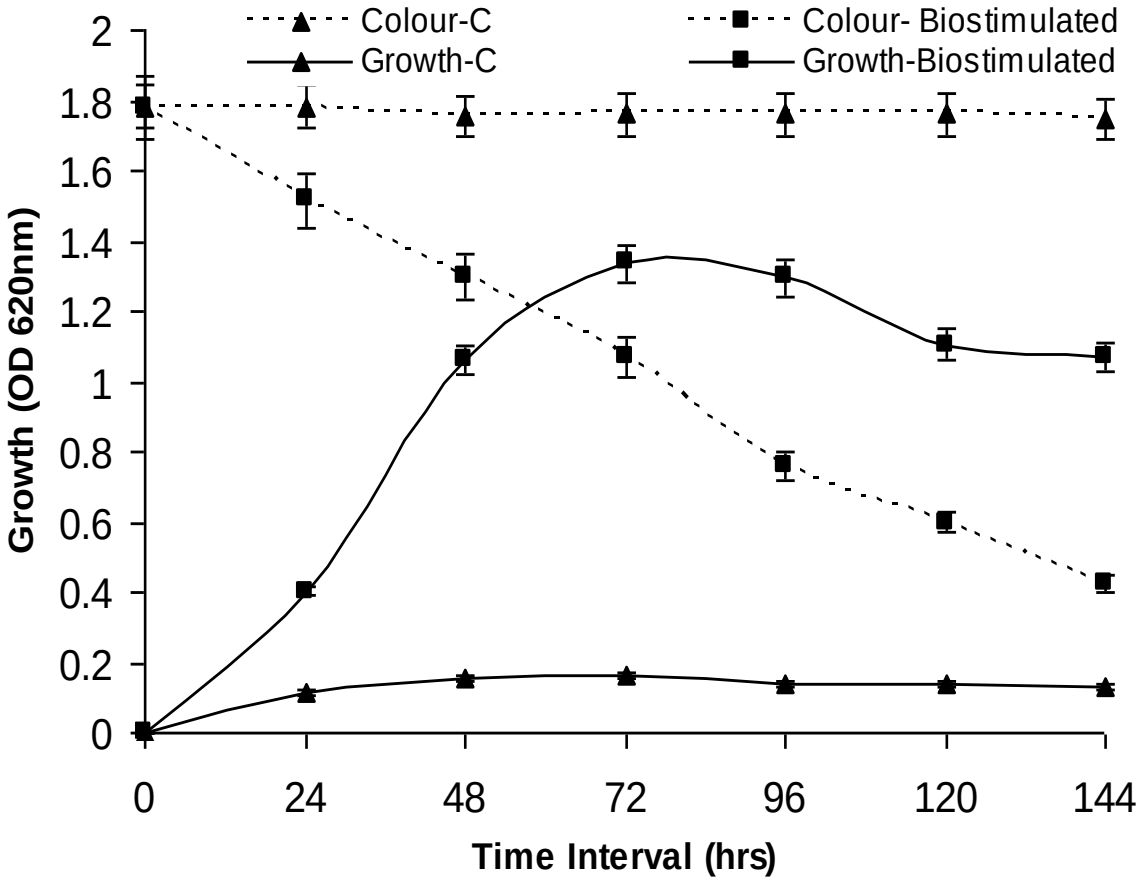


Fig.10.3. the pattern of bacterial growth and color reduction in the pulp and paper mill effluent in biostimulation condition by 2% glucose and 0.5% peptone

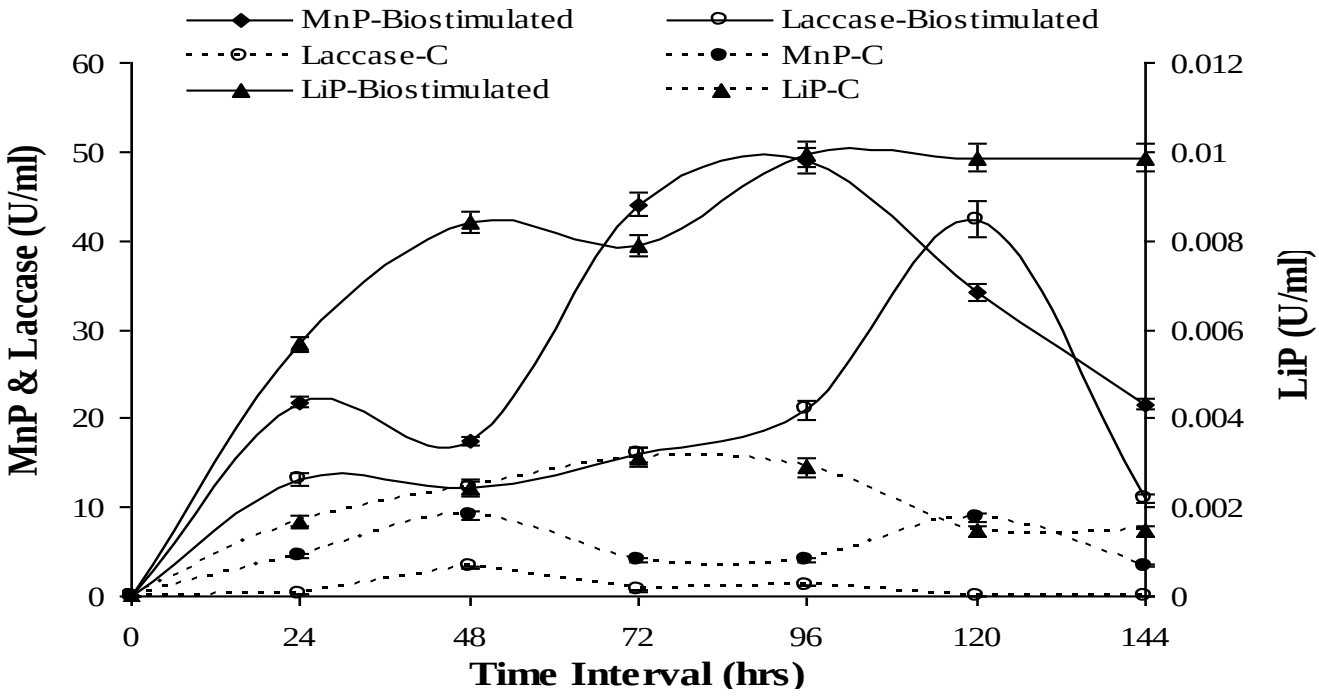


Fig.10.4.The pattern of Ligninolytic enzyme during pulp and paper mill effluent decolorization after Biostimulation. MnP: Manganese Peroxidase, LiP: Lignin Peroxidase

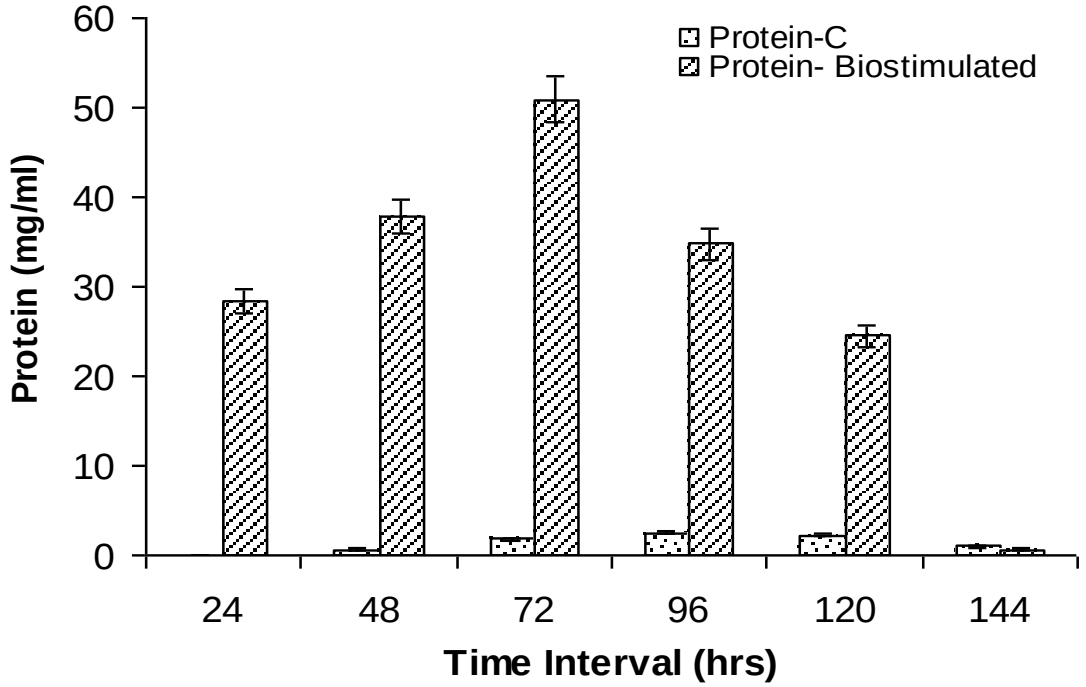


Fig. 10.5 The pattern of Ligninolytic enzyme during pulp and paper mill effluent decolorization after Biostimulation. MnP: Manganese Peroxidase, LiP: Lignin Peroxidase

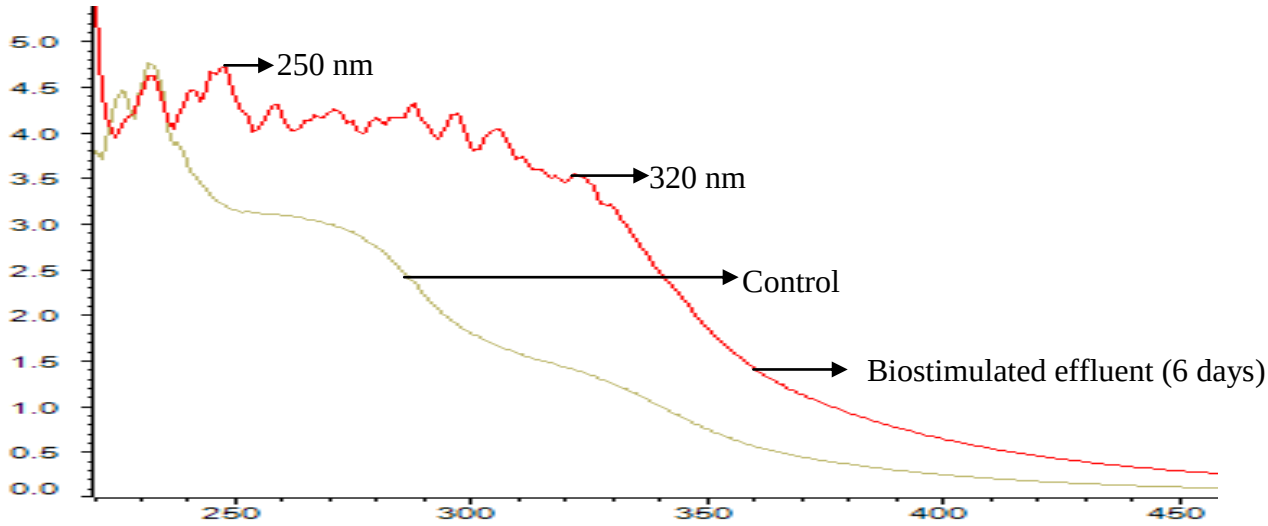


Fig.10.6. UV-Visible scanning spectra (200-450nm) of degraded pulp and paper mill effluent after biostimulation

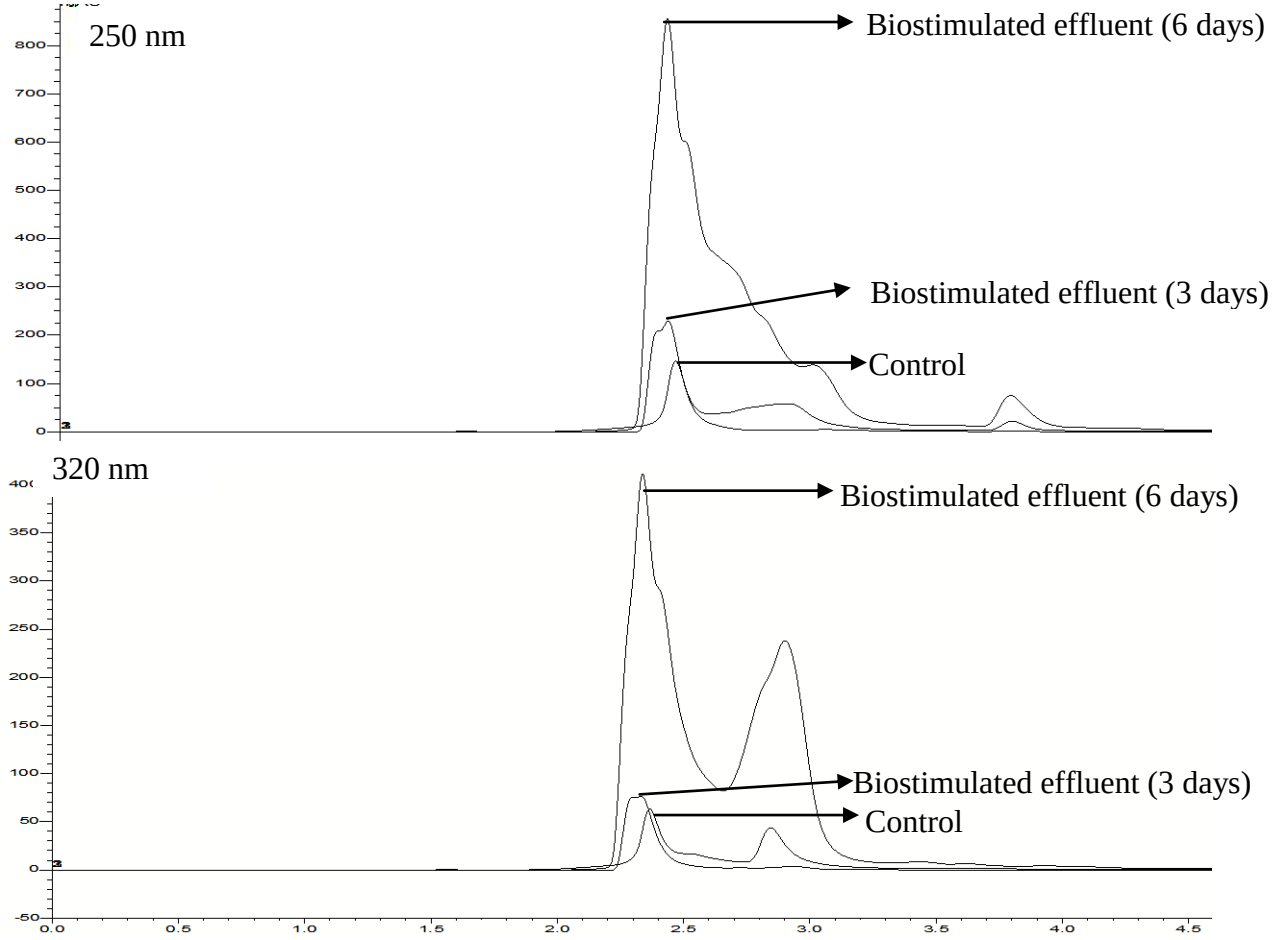


Fig. 10.7. HPLC analysis of decolorized pulp and paper mill effluent at different time interval on 250 nm (a) and 320nm absorbent (b)

10.2.4. Assessment of Degradation and Characterization of Metabolites

To assess the degradability scanning absorption spectrum by UV-Vis spectrophotometers and HPLC analysis showed an elevation overall along with the generation of scan peaks (Fig.10.6 and 10.7), while the absorption spectrum of the control sample showed the very complete and smooth lining. Thus, this indicates the depolymerization of the compound present in the effluent. Further, the compression HPLC chromatograph of control and degradation sample at 320 nm revealed an apparent reduction of peaks at periodic bacterial incubation showed in Fig.10.7. This given strong evidence for the reduction of organic pollutants present in the effluent. A similar pattern of HPLC data has been also recorded in our previous study for degradation of lignocellulosic compound present in pulp and paper mill waste (Chandra and Singh, 2012; Chandra et al., 2012). Further, the GC-MS analysis (Fig. 10.8 and 10.9), have shown the GC-MS chromatogram of extracted organic pollutants from the discharged pulp and paper mill effluent after secondary treatment in ethyl acetate at alkaline (pH 8.5) and acidic (pH 2.0) condition this was considered as control. The detail identified compounds at different RT has been listed in Table. 10.2. The major identified TMS derivatized products with relative contributions (%) extracted with ethyl acetate alkaline and acidic pH. The residual organic pollutants after degradation through the biostimulation process were also extracted with similar conditions of control. The phenol and phenolic compounds such as 2,3,6-trimethyl phenol; phenol-4-ethyl-2-methoxy; 2-methoxyphenyl; Phenol,2,6-dimethoxy; phenol-2-methoxy-4-(1-propenyl); Methoxy cinnamic acid; 2 Methoxy phenol, 1-hydroxy-2-methoxy-4-methylbenzene, 4-ethyl guaiacol, 4-propylguaiacol, 4 hydroxy-3-methoxy Benz aldehyde, 2-methoxy-4-(2- propenyl) phenol, 1-(4-hydroxy-3 methoxyphenyl) ethanone, (4- hydroxy-3-methoxyphenyl)-2-propanone, 4-hydroxy-3 Methoxy benzene acetic acid, etc. All of these compounds have a 2-methoxy phenol group in their structures but have a different substituent at the position that is opposite to the hydroxyl group on the aromatic ring, the majority of these compounds are the monomer of lignin compounds. While most of these compounds were disappeared after biostimulation treatment. Moreover, 2-Butoxyethanol was detected in degraded effluent after 6 days of biostimulation, implying that they are likely degraded compounds from

lignin. Lignin is a polymeric natural product arising from an enzyme initiated dehydrogenative polymerization of three primary precursors: trans-coniferyl, trans-sinapyl, and trans-p-coumaryl. Among which the trans-coniferyl precursor also has the 2-Methoxy phenol group in its structure. Aside from these phenolic compounds, there were also some compounds (e.g. 2-butoxyethanol, 2-methyl- 1-one-2-cyclopentene, 2, 5-hexanedione and 5-methyl-2-furancarboxaldehyde) that were likely initially derived from cellulosic components via a series of reactions such as hydrolysis, dehydration. Similar observations were also reported by Kruse et al. (2007). Lactic acid was observed in the pulp and paper mill effluent at both alkaline and acidic extract. This might be produced due to softwood hydrolysis and fermentation of the cellulose component of wood. In recent research have been reported that lactic acid may be used as the value-added product from pulp and paper mill industries. Moreover, Benzoic acid i.e. benzoate was also detected in bio-stimulated effluent after 3 and 6 days incubation; this might be due to the utilization of lignin. Fungal lignin depolymerization usually results in a variety of low molecular weight aromatic compounds such as guaiacol, coniferyl alcohol, p-coumarate, ferulate, protocatechuate, p-hydroxybenzoate and Vanillate (Harwood and Parales, 1996; Masai et al., 2007). Citral was also detected in pulp and paper mill effluent at acidified conditions. This was completely degraded during biostimulation process. This is a type of terpenoids, which contribute to the perfume of eucalyptus oil. This might be extracted from eucalyptus wood during the pulping process. The monomer of lignin i.e. guaiacol and 4-ethyl guaiacol were also detected in the pulp and paper by degraded effluent of biostimulation. This might be due to the degradation of complex lignin into the monomer unit of lignin present in the effluent. In addition, syringols was also detected in the pulp and paper mill effluent and this was disappeared after degradation biostimulation process. In addition, guaiacol, syringeal, and its derivate were also characteristic products of lignin.

Further, the peak at RT 15.7 was identified as Pthalatic anhydride. The phthalate derivatives such as butyl phthalate and bis (2-Ethylhexyl) phthalate had been detected by fungal peroxidase degradation of lignosulfonate (Shin and Lee, 1999) and also during Photodegradation of black liquor lignin (Ksibi et al., 2003). Phenol-2-methoxy-4-(1-propenyl) or isoeugenol was noted in pulp paper mill effluent. It is propenyl-

substituted guaiacol. The result of GC-MS data has shown that Acetyl vanillin was completely utilized by the bacteria through the biodegradation of lignin. Vanillin is a phenolic aldehyde as an organic compound with the molecular formula $C_8H_8O_3$. Its functional groups include aldehyde, ether, and phenol. Vanillin is one of the most popular flavoring agents in the food industry and its wide range of applications is in the fields of perfumery and pharmaceutical intermediates as a value-added product. Vanillin has been reported as a byproduct of the pulp and paper industry by the oxidative break down of lignin in several reports (Jose et al., 2010). Some saturated fatty acid i.e. octadecanoic acid, trimethylsilyl ester of stearic acid was also detected in control pulp paper effluent and finally, it was degraded by bacteria. It has been reported that stearic acid can be easily chlorinated and it is one become a toxic form. Several detected organic compounds were diminished in the bacterial process treatment. Hydrocarbon i.e. Pentadecane was completely degraded during biostimulation. Moreover, cinnamic acids, which is known as a by-product of lignin and hemicellulose fraction of lignocellulose, was detected in biostimulation extract. P-Coumaric acid (4-hydroxycinnamic acid) and ferulic acid (4-hydroxy-3-methoxy cinnamic acid) are bifunctional. They are able to form ester and ether linkages by the reaction of their carboxyl and phenolic group, respectively (Jeffries, 1990). During the alkaline extraction of the pulping process, most of the ester linkages are broken, but some cinnamic acid still remains bound to the lignin by ether linkages (Hernandez et al., 1997), which has been broken down during biostimulation treatment.

Nonacosane was also detected in degraded pulp paper mill effluent after 3 days of incubation. Nonacosane is a straight-chain hydrocarbon with a molecular formula of $C_{29}H_{60}$. Formation of this showed the degradation of complex compounds into the simplest compounds which were completely degraded after 6 days of bacterial incubation in the detected organic compounds. There were several esterified fatty acids ranged from C16 to C30, and the esterified fatty alcohols ranged from C20 to C38. The acid moiety of the waxes was exclusively constituted by saturated fatty acids with even carbon numbers. Among these, the most predominant ones were Octadecanoic (C18) and hexadecanoic (C16) acids followed by eicosanoid (C20) and Docosanoic (C22) acids. Among the alcohol moiety, the most predominant was tetratriacontanol (C34) followed by hexatriacontanol (C36) and dotriacontanol (C32). The predominant wax was C52, mostly

constituted by octadecanoic acid (C18) esterified to tetratriacontanol (C34), followed by wax C50, mostly constituted by hexadecanoic acid (C16) esterified to tetratriacontanol (C34) and by wax C54 mostly constituted by o y octadecanoic acid (C18) esterified to hexatriacontanol (C36). Free fatty acids were also identified in the flax fibers ranging from C14 to C32, with a strong even-over-odd predominance. The series was dominated by the saturated counterparts although the unsaturated C18:1, C18:2 and C16:1 was also identified. Hexadecanoic acid (palmitic acid) was the most abundant, thus in agreement with finding as Morrison et al., (2001) who reported that palmitic acid was the major fatty acid found in the extracts from fibers of several flux cultivars. Stearic and oleic acids were also found in significant amounts. It is interesting to note that although unsaturated fatty acids were present in free form in flax fibers; they were not found esterified with fatty alcohols forming waxes. A series of n-alkanes (from C21 to C31) were also identified in the flax fibers with a strong odd-over-even carbon atom number predominance; Nonacosane (C29) was the most abundant. N-Alkanes with even carbon atom numbers (C26, C28, and C30) were also identified albeit in lower amounts. N-Fatty alcohols ranging from C16 to C32 were present in the fiber extracts with strong even-over-odd carbon atom predominance; octacosane (C28) was the most abundant. Octacosane was also has been reported as the major fatty alcohol found in the flax fibers by Morrison et al., (2001). Interestingly, the series of free fatty alcohols do not parallel the series of esterified fatty alcohols. Moreover, a series of fatty alcohols with odd carbon numbers (C27, C29, C31, C33, C35, and C37) were found esterified with long-chain fatty acids but were not found in free form. Free alcohol composition is often different from that of esterified alcohols (Tulloch, 1976); therefore, the analysis of saponified extracts does not give reliable information about the composition of esters of fatty alcohols. A series of n-aldehydes ranging from C21 to C32 were identified in the flax fibers with strong even carbon atom predominance with octacosane (C28) predominating. The distribution of the aldehyde series correlates to that of free alcohols, as usually occurs in the plant kingdom suggesting that aldehydes are intermediates in the biosynthesis of alcohols from fatty acids (Tulloch, 1976; Bianchi and Waxes, 1995).

10.2.5. Identification of Autochthonous Bacterial Species

Purified isolated autochthonous bacterial strains were identified based on 16S rRNA sequencing. The 16S rRNA sequence data were submitted to NCBI using the BLAST tool and the obtained sequences were then further compared with sequences of bacteria from known taxa and a phylogenetic tree was constructed (Fig.10.10). Further, based on 16S rRNA sequencing isolated strains IITRCP04, IITRCP11, IITRCP14 and IITRCP19 showed closed relatedness with *Klebsiella pneumonia*, *Enterobacter cloacae* strain, *Enterobacter cloacae* strain, and *Acinetobacter calcoceticus*, respectively. Furthermore, the partial sequences of bacterial diversity growing during biostimulation were deposited to Gen Bank public database under the accession number KU715839, KU715840, KU715841, and KU715842, respectively

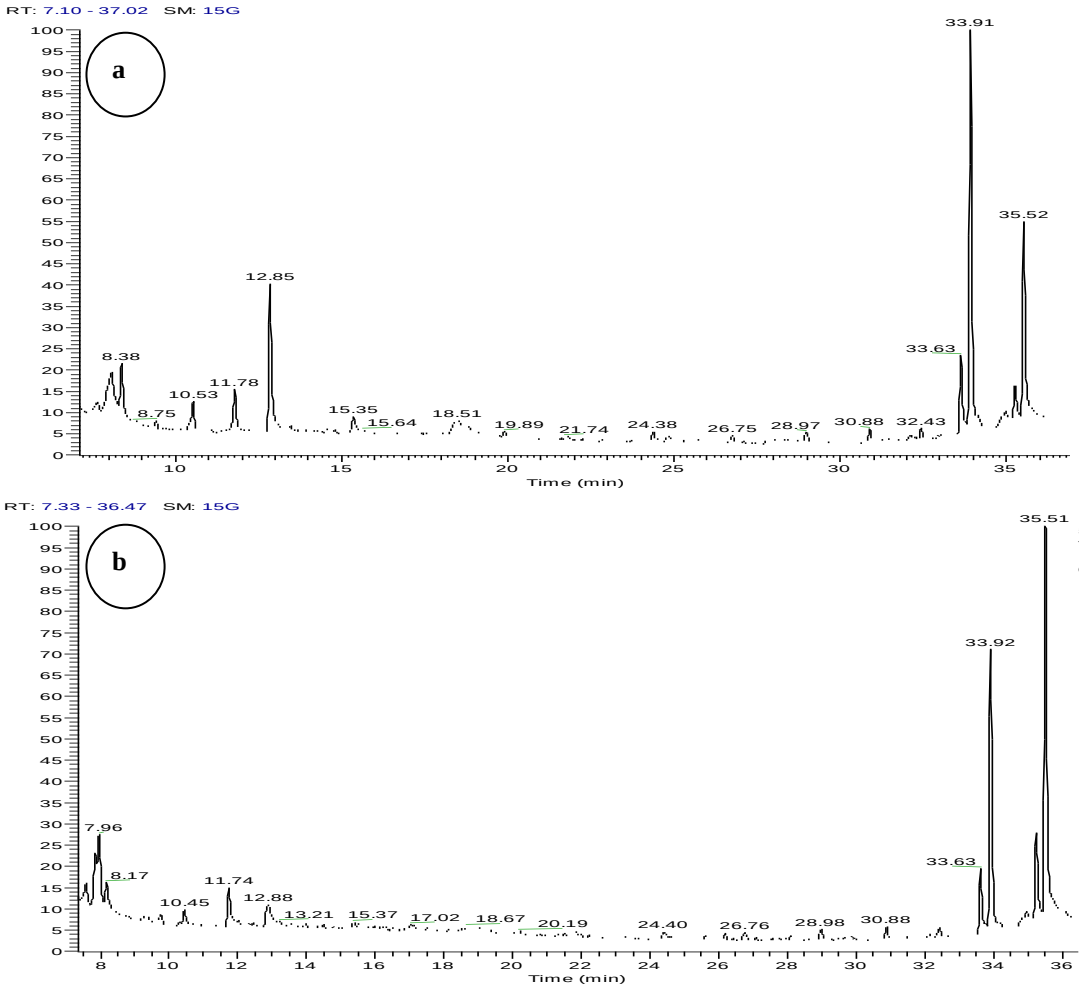


Fig.10.8.Total Ion Chromatogram (TIC) of TMS derivatized detected residual Organic Pollutants from ethyl acetate extract of pulp and paper mill effluent after secondary treatment in (a) alkaline pH (8.5) (b) acidic pH

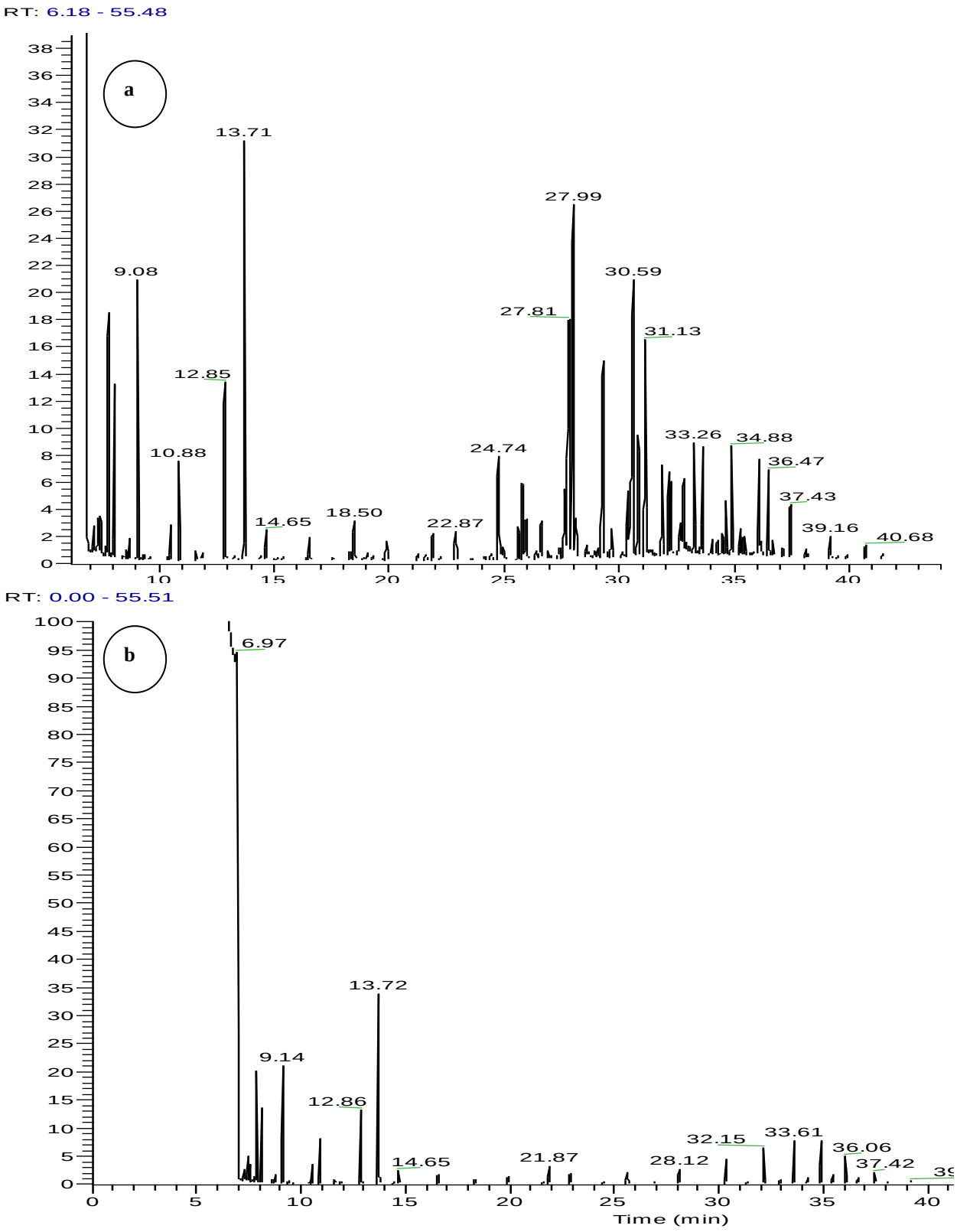


Fig.10.9. Total Ion Chromatogram (TIC) of TMS derivatized detected residual organic pollutants from ethyl acetate extract of biostimulation effluent after 3 days (a) and 6 days incubation (b)

Table.10.2. Identified residual organic pollutants by GC-MS in the TMS derivatized ethyl acetate extracts of pulp and paper mill effluent after secondary treatment in alkaline (pH, 8.5), acidic (pH 2), 3 days and 6 days of bacterial treatment.

Retention Time (RT)	**Identified compounds**	**pH 8.5**	**pH 2.0**	**3 days degradation**	**6 days degradation**
6.97	2-Butoxyethanol	-	-	-	+
7.96	Thymol- TMS	-	+	-	-
8.17	D-Lactic acid- DITMS	-	+	-	-
8.38	D-Lactic acid- DITMS	+	-	-	-
8.75	2,3,6-trimethyl phenol	+	-	-	-
9.08	benzoic acid trimethylsilyl ester	-	-	+	-
9.14	Benzoic acid, Trimethylsilyl ester	-	-	-	+
10.45	Citral	-	+	-	-
10.53	2',6'-Dihydroxyacetophenone, bis(trimethylsilyl) ether	+	+	+	-
11.78	2',6'-Dihydroxyacetophenone, bis(trimethylsilyl) ether	+	-	-	-
12.85	Glycerol- tri-TMS ether	+	+	+	+
13.71/13.8	phenol-4-ethyl-2-methoxy or 4-Ethylguaiacol	-	-	+	+
14.66/14.68	2-methoxyphenol or guaiacol	-	-	+	+
15.35	Phenol,2,6-dimethoxy or syringol	+	+	-	-
15.64/15.7	Pthalatic anhydride	+	-	-	-
17.02	phenol-2-methoxy-4-(1-propenyl or isoeugenol	-	+	-	-
18.51	9-decenoic acid, trimethylsilyl ester	+	+	+	-
19.89/19.92	Benzyldehyde,4-(acetyloxy)-3-methoxy or acetylvanillin	+	-	-	-
20.19/20.17	Octadecanoic acid, trimethylsilyl ester or stearic acid	-	+	-	-
21.74	1,2-benzenedicarboxylic acid,bis(2-ethylhexyl)ester	+	-	-	+
22.87	Acetic acid [(trimethylsilyl)oxy]trimethyle ester	-	-	+	-
24.38/24.50	Methoxy cinnamic acid	+	-	-	-
24.40	9,12 octadecadienoic acid,(2-phenyl 1,3 dioxolan-4-yl)methyl ester trans	-	+	-	-
24.87	n-pentadecanoic acid,trimethylsilyl ester	-	-	+	-
26.75	9,12-octadecadienoic acid,(2-phenyl-1,3-dioxolan-4-yl]methyl ester cis	+	+	-	-
27.81	2,6-bis[trimethylsilyl]-3,4-dimethylphosphinine	-	-	+	-
27.99	Hexadecanoic acid,trimethylsilyl ester	-	-	+	+
28.97/28.98	Pentadecane	+	+	-	-
30.88	Octadecanoic acid,trimethylsilyl ester	+	+	+	-
31.13	Cinnamic acid-α-phenyl-trimethylsilyl ester	-	-	+	-
32.15	Cinnamic acid,α-phenyl,trimethylsilyl ester	-	-	-	+
32.43	Cis,13-docosenoic acid,trimethylsilyl ester	+	-	-	-
33.26	9-[2,6-diethylphenyl]2,8-dimethyl-9-h-purin-6-amine	-	-	+	-
33.63	2-Monopalmitin TMS ether	+	+	-	+
33.91	1- Monopalmitin-DITMS	+	+	-	-
34.88	1,2,diphenyl-s (t-butyl) acephenanthrylene	-	-	+	-
35.52/35.5	1-Monostearin – DITMS/Cyclotetracosane	+	+	-	-
36.06	Octacosane	-	-		+
36.47	Squalene/2,6,10,14,18,22-tetracosahexane,2,6,10,15,19,23-heaxamethyl-[all-E]	-	-	+	-
37.43	5,8-dimethoxy-6-methyl-2,4-bis(phenylmethyl)naphthalen-1-ol	-	-	+	+
39.16	1,2-benzendicarboxylic acid disononyl ester	-	-	+	-
40.68	Nonacosanol	-	-	+	-

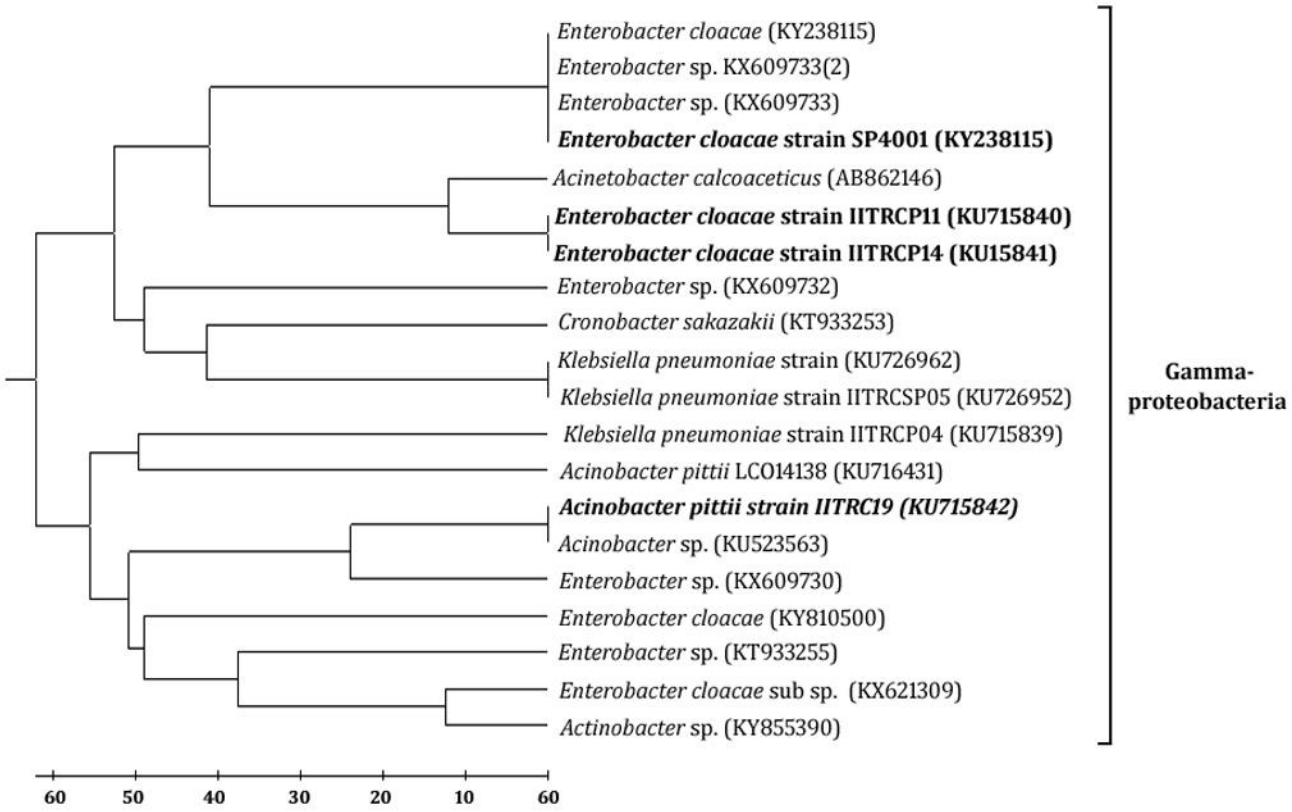


Fig.10.10.Phylogenetic tree showing the relationship of bacterial communities growing during biostimulation of pulp and paper mill effluent degradation. The accession numbers for all strains used in tree construction are indicated in the figure in square brackets.

10.2.6. Toxicity Assessment of Bacterial Treated Samples

The comparative toxicity assessment of bio stimulated and a control sample with seed germination on test showed a 60% reduction of toxicity in both tested seed i.e. *Triticum aestivation* and *Phaseolus mungo* (Fig.10.11.a-b and c). Further, the result also revealed that *Phaseolus mungo* was found more sensitive than *Triticum aestivum*. In addition, the comparative cytotoxic and genotoxic effect of pulp and paper mill effluent before and after bio stimulated *on A. cepa* roots were also determined on the basis of chromosomal aberrations. The root cells of *A. cepa* treated with before and after bio stimulated effluent are showed various types of chromosomal aberrations (Fig.10.12). Under microscopic observation onion root tips treated with pulp and paper mill effluent showed abnormal and vagrant metaphase, diagonal anaphase, chromosome laggards at anaphase, ring chromosome and sticky anaphase were observed.

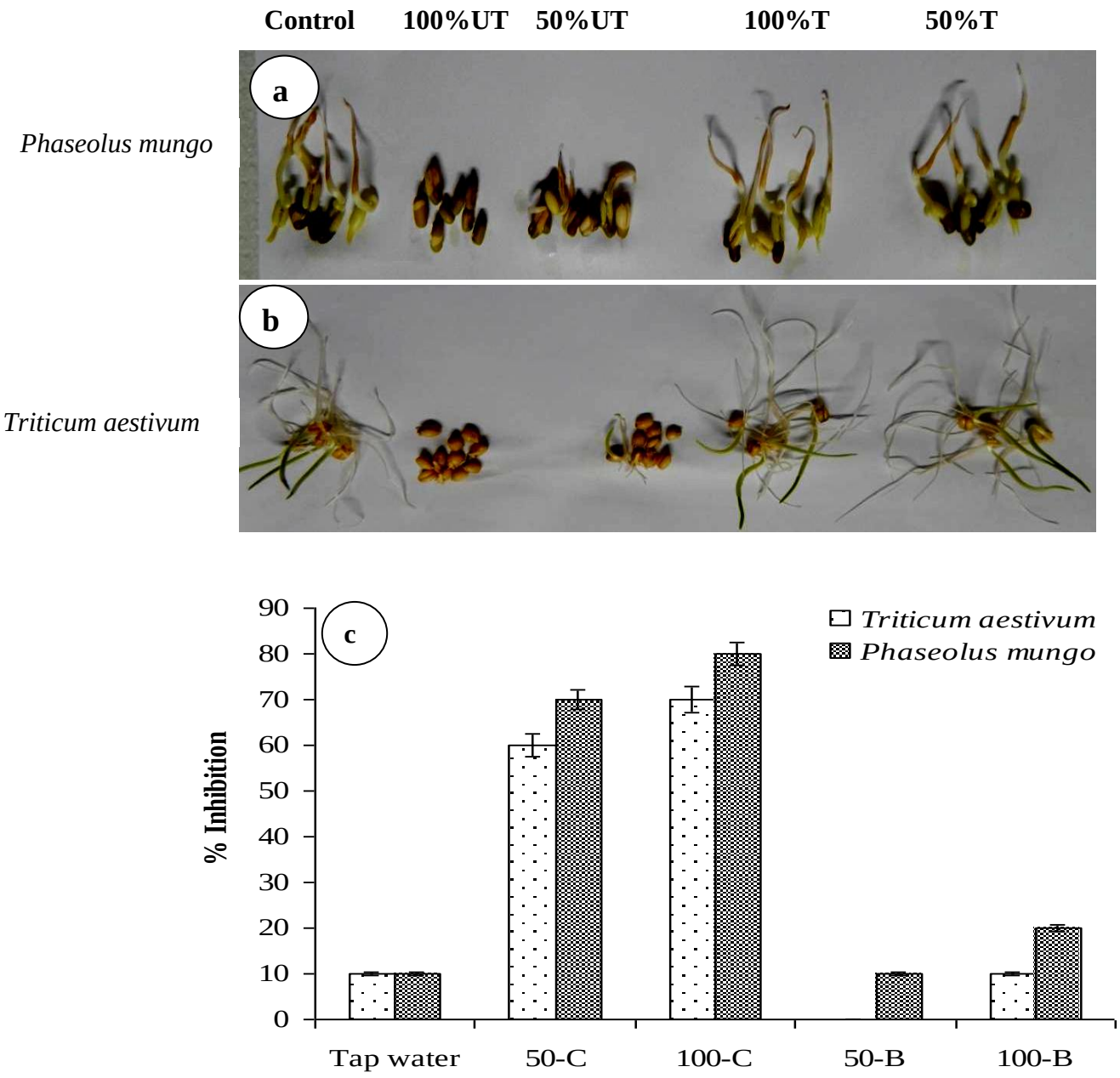


Fig. 10.11. Seed germination test at 50 and 100% concentration of pulp and paper mill untreated (UT) and bacterial degraded sample (T) on *Phaseolus mungo* (a) and *Triticum aestivum* (b) and percent inhibition of seed germination (c)

CONCLUSION

The study has concluded that in the optimized bio stimulated process most of the toxic residual organic compounds of pulp and paper effluent were disappeared while several other compounds were generated as a byproduct of bacterial degradation which was as value compound of lignocellulosic waste material.

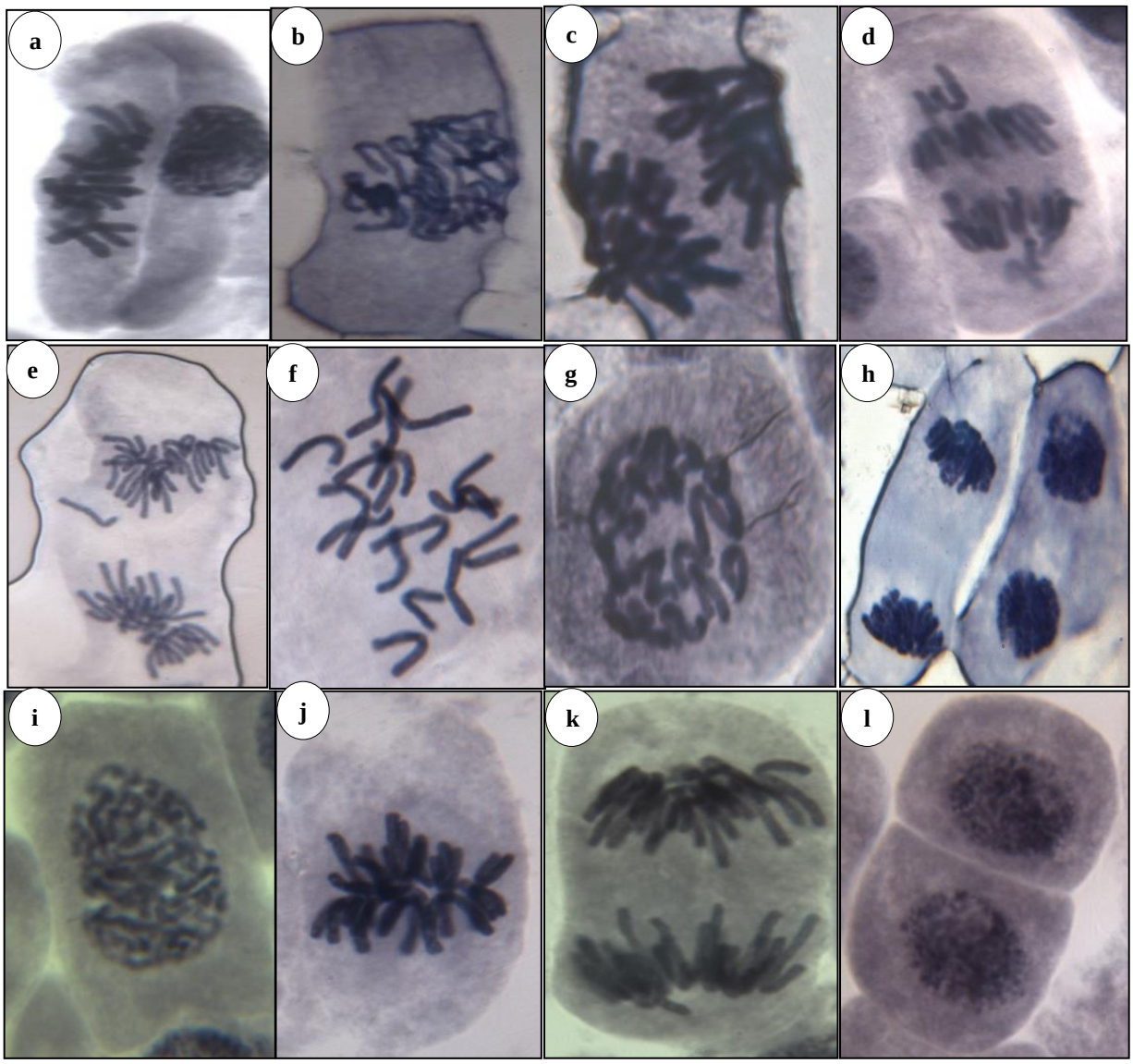


Fig.10.12.Different chromosomal aberration induced by pulp paper mill effluent before (a-h) and after (i-l) bacterial treatment. (a) disturbed pole to pole arrangement of chromosomes at metaphase (b) vagrant chromosome at metaphase (c) diagonal anaphase (d) arrow shows laggard chromosome (e) vagrant chromosome in anaphase-telophase (f) diploid chromosome (g) ring chromosome (h) sticky anaphase (i) normal prophase (j) normal metaphase (k) normal anaphase (l) normal telophase

Thus, this is evidence of pulp and paper mill effluent detoxification process after secondary treatment for safe disposal. In addition, the generation of some value-added products as metabolites also an advantageous feature this may be as commercial importance of this technique.

Chapter-Eleven

Summary

Summary

The pulp paper industry discharged waste containing several complex pollutants including lignin, chlorolignins, organic compounds along with heavy metals is above the permissible limit after secondary treatment. The discharged effluent and sludge are highly color and release several other gaseous pollutants i.e., dioxin, hydrogen peroxide (H_2O_2), chlorine dioxide (ClO_2), sulphur oxide (SO_2), hydrogen sulphide (H_2S), and Methyl mercaptan (CH_3SH) in the environment. In addition, the discharged waste is also containing a high amount of heavy metals (Cu, Cr, Pb, Fe, Zn, Ni, Mn, and Mg) and they have a strong binding capacity with other co-pollutants in soil and affect the fertility of soil and plant growth. Presently, many physicochemical and biological technologies were used to remediate metal-contaminated soils neighbouring the pulp paper industrial area. While decontamination of metal-polluted soils utilizing plant phytoremediation, phytoextraction or phytostabilization has shown promising performance, this strategy has limitations for contaminated areas in which the concentration of metal is extremely high. However, some potential native hyperaccumulators plants and bacterial strain are observed capable of detoxifying and degradation of these wastes in their natural site of contaminated sites of the pulp paper industry. For, degradation mechanisms of organisms such as bacteria and plants have been found in several environmental factors and several studies has given the evidence of biological activity enhanced the detoxification and degradation of industrial waste after secondary treatment. Moreover, phytoremediation of metals in association with phosphate-solubilizing bacteria significantly eliminates the useful disadvantages imposed on plants by metal stress. The bacterial degradation of complex organic pollutants from the disposal site of the pulp paper industry occurred by aerobic and anaerobic metabolism. Further, the anaerobic metabolism activity has been used as a very important common technique for bioremediation, in this condition the degradation of chlorinated organic compounds involves a reductive dechlorination process in which chlorinated compounds are used as electron acceptors**.** The using of the different potential bacterial community for enhancement of phytoremediation process needs the assessment of plant microbes' interaction. Moreover, bacteria facilitated phytoremediation is a green technology for remediation of persistent organic

pollutants from the disposal site of pulp paper industrial waste. In addition to the above-mentioned plant growth strategies, many other bacterial characteristics could be used to enhance metal phytoremediation. Though, one study showed that a bacterial strain that assisted phytoremediation generated bio surfactants, once more, potentially working to make the metals much more bioavailable.

The toxic compounds of pulp paper mill wastewater (PPMW) discharged from pulp paper and paper industry is a major source of environmental toxicity which is not yet much known. Further, the analysis of waste showed the presence of Nonacosane; Heptacosane; 6-Benzoamido- 3- [2- [1-(phenylmethyl) - 4- piperidinyl] ethyl] - 1, 2-benzisoxazole; Hexadecanoic acid and Octadecenoic acid as residual organic pollutants. The majority of these pollutants are androgenic and mutagenic in nature derived from plant steroid, fatty and resin acids of the plant during pulping processes which were not degraded by bacterial communities during biological treatment of wastewater. Moreover, presence of recalcitrant pollutants along with a mixture of heavy metals (Fe, 87.35; Zn, 22.40; Ni, 5.24; Cu, 3.28 and Cd, 0.36 mg l^{-1}) here also detected which were found beyond the permissible limit these contributed elevated BOD (7880 mgl^{-1}), COD (19100 mgl^{-1}) and reduced DO. The seed germination test with *Phaseolus mungo* and chromosomal aberration and cytotoxicity test with *Allium cepa* showed the inhibition of α-amylase at >20% concentration of PPMW and disturbed chromosomal segregation during cell division of metaphase and anaphase which showed c-mitosis, sticky chromosome, laggard chromosome and polyploidy cells in *A. cepa* treated with PPMW. Further, the SEM observation of the root of *A. cepa* treated with PPMW showed fissures and fractured tissues of root cap might be due to the inhibition of auxins responsible for root cap this formation. In addition, the enzymatic activity SOD, CAT, H_2O_2, and APx were decreased after PPMW treatment as a comparison to control. Hence, this study will be a global interest in the monitoring of recalcitrant pollutants discharged after biological treatment from the pulp paper industry for aquatic pollution prevention.

Since the phytoremediation potentiality and toxicity on crop plants of complex industrial waste by native plants is an emerging green technology for eco-restoration of the polluted site. The analysis of discharged pulp paper sludge showed the presence of various unknown complex organo-metallic pollutants after secondary treatment along with androgenic compounds. Therefore it is globally reported as health hazards for the aquatic and terrestrial ecosystem. The native potential plants growing on pulp

paper sludge altered their Physico-chemical properties by the accumulation of heavy metals in different parts of tested plants. The GC-MS analysis revealed the remediation of organic pollutants after the plant growth and various metabolic products were also detected. The analysis of heavy metals in various parts of plants showed the phytoextraction potential of different metals i.e. Fe (95.35-47.23), Zn (48.40-32.01), Cu (3.28-1.03), and Mn (19.00-11.23) which showed properties of hyperaccumulators plants. The comparative analysis of different heavy metals accumulation revealed that Pb was highest in leaves of *A. sessilis* (16.45 mg kg^{-1}), leaves of *T. terrestris* (2.34 mg kg^{-1}) and root of *C. sativa* (9.24 mg kg^{-1}) followed by Cr, Mn, and Zn in leaves>shoot>root. Further, bioconcentration factor (BCF) was noted greater than one for accumulated metals i.e. Mn and Cu in *P. hysterophorus* (74.40-95.12 mg kg^{-1}), Pb in *M. dioica* (48.15 mg kg^{-1}), Zn and Fe was *C. procera* (10.65-31.51 mg kg^{-1}). While the translocation factor >10 of Cu (41.29 mg kg^{-1}), and Fe (18.65 mg kg^{-1}), was tested in *T. terrestris*> *C. procera* > *C. sativa* in growing on pulp paper sludge bed. In addition, TEM analysis showed high metal deposition in the root cell wall, cytoplasm, and vacuole as strong evidence of in-situ phytoremediation for the eco-restoration of a polluted site. Furthermore, the activity of plant antioxidants such as SOD, APx, H_2O_2, and MDA was also observed higher in hyperaccumulators plants in comparison to control plants. Moreover, the guard cell shape of stomata was also observed normally in the growing plant even after heavy metal accumulation. The discharged effluent release organic pollutants along with heavy metals above the permissible threshold after biological and chemical treatments. The different Physico-chemical parameters are highly increased in effluent i.e. Total phenols, lignin, TDS, TSS, BOD, COD, total nitrogen affects the phototrophic of aquatic organisms, while the color of effluent and turbidity is because of this. Identification of carcinogenic mutagenic and EDC compounds i.e. Hexadecanoic acid, trimethylsilyl ester acid, 2, 3-Butanediol, bis-O-(trimethylsilyl), and β-Sitosterol trimethylsilyl ether by GC-MS analysis and estimation of heavy metals in *T. foenum-graecum* L by AAS. The physiological and cytological changes of *T. foenum-graecum* L grown in soil irrigated with pulp and effluent (50%, 75%, and 100 %,) were studied after 30, 60 and 90 days after compared to their respective controls. Furthermore, the study of physiological effect i.e. roots, shoot length, root nodule and surface study are damaged after exposure of effluent is done by SEM. The total chlorophyll, protein and antioxidants contents highly increase with 100 %

concentration of effluent irrigation. The mitochondrial and chromosomal damage induced by the cytotoxic and mutagenic compounds present in the effluent, this finding confirmed that the Phytotoxicity is caused in *T. foenum-graecum* L. besides finding also showed the effect of the pollutants on *T. foenum-graecum* L development and direct effect on human health.

The discharged waste from the pulp paper industry contains several androgenic and mutagenic compounds even after secondary treatment, which causes toxicity on agricultural land and crop plants. Moreover, after the Physico-chemical analysis of two different pulp paper industry contaminated site i.e. PPI-1 and PPI-2, it was also found that the value of pH, TDS, TSS, COD, BOD, and lignin was beyond the permissible limit (USEPA, 2012). Moreover, major persistent organic pollutants (POPs) compounds i.e. Eicosane (CAS), β- Sitosterol trimethylsilyl and Hexadecanoic acid, trimethylsilyl ester or Palmitic acid TMS respectively detected from the sludge are also listed under EDCs in USEPA 2012**.** The studies showed the effect of heavy metals on antioxidants enzyme of five native potential plants *Brassica campestris L.* (Brassicaceae) and *Chenopodium album L.* (Amaranthaceae), *Ricinus communis* (Euphorbiaceous), *Ranunculus sceleratus* (Ranunculaceae), *and Rumex dentatus* (Polygonaceae) plants growing on organometallic sludge waste was also found high. Furthermore, results revealed that the chlorophyll content in *Brassica* and *Chenopodium* was Chl-a (4.57-5.21 mg g^{-1} fw), chl-b (5.29-5.89 mg g^{-1} fw), while the content of carotenoids (0.84-1.07 mg g^{-1} fw) was also high, respectively. In addition, the high bioconcentration factor of *Brassica* was Fe, (1.69), Ni (1.51) >1 and *Chenopodium* Fe (2.54), Zn (1.69) > 1. Moreover, the translocation factor >10 (10.95) of Cr was *Brassica and Chenopodium was* >10 (10.39) *Zn* respectively. The study concluded that the main source of heavy metals in soil from pulp paper industrial waste. Consequently, *Brassica sp.* and *Chenopodium sp.* plants growing on pulp paper mill sludge are health hazards for humans and the environment.

Subsequently, profiling of dominate bacterial community in sludge sample of pulp paper industry. Further, the dominant bacterial communities were investigated by using a Metagenomics approach to reveal the microbial niche in this polluted environment from the pulp paper industry site. Moreover, bacterial community analyses by DNA extraction and amplification method were dominant bacterial communities belonging to the phylum *γ-Proteobacteria* and *Firmicutes,* respectively. In addition, the toxicity evaluation showed toxicity in degraded samples of sludge and

leachate the results of this study may be useful for monitoring and toxicity assessment of pulp paper industry waste at disposal sites.

Further, the characterization of plant growth-promoting bacteria (PGPB) and the mechanism for detoxification and degradation of pulp paper industrial waste. Moreover, the result revealed that the total twelve morphologically different aerobic bacterial strains (PS-1 to PS-2) were isolated by nutrient enrichment technique from the pulp paper industry sludge by streak plate method. Further, these bacterial strains were screened ligninolytic enzyme activity on the basis of MnP and Lip and laccase tolerance activity. Out of 12 bacterial strains, six aerobic bacterial strains PS-2, PS-3, PS-4, PS-6, BBAUPS-1, BBAUPS-2, and BBAUPS-3 were showed maximum MnP and laccase producing activity on phenol red amended MSM medium and guaiacol amended B and K agar medium plated. Further, these bacterial strains were also found higher lignin concentration at different ppm (parts per million) (1400ppm/ L^{-1}) tolerance activity respectively. While the potential isolates also show the activity of plant growth-promoting bacteria (PGPR) at different media. The activity of IAA was showing the PS-2, PS-3, and BBAUPS-1 isolates. In addition, the nutrient solubility activity i.e. Zn Solubilization and K Solubilization are also observed by bacterial isolates on the Pikovsakya agar plate. On the basis of 16S rRNA gene sequence analysis potential bacterial strains PS-2, PS-3, PS-4, PS-6, BBAUPS-1, BBAUPS-2, and BBAUPS-3, were identified as *Bacillus sp.* (MN238724.1), *Escherichia coli strain* (MN238725.1), *Brevundimonas sp.* (MN238722.1), *Bacillus sp.* (MN238714.1), *Aeromonas salmonicida* (MN294457) , *Aeromonas salmonicida* (MN294456), *Stenotrophomonas maltophilia* (MN294458) respectively. These strains showed the optimum production of PGPB activity 12 and 144 h of growth, respectively.

The pulp and paper mill effluent showed a source to discharge out various recalcitrant and androgenic compounds even after secondary treatment, but detail knowledge is not available yet regarding the properties of organic pollutants and their bioremediation process. Therefore, the study has been focused to detect the residual organic pollutants of pulp and paper mill wastewater after secondary treatment and their degradability in the biostimulation process. The major identified compounds were as 2, 3, 6-Trimethyl phenol; 2-methoxyphenyl or guaiacol; phenol,2,6-dimethoxy or syringe; Methoxy cinnamic acid; Pentadecane; Octadecanoic acid, trimethylsilyl ester; cyclotetracosane; 5,8- dimethoxy-6-methyl-2,4-bis

(phenylmethyl) naphthalene-1-ol and 1,2-benzene dicarboxylic acid isononyl ester. While the majority of these compounds are known as environmental toxicants as endocrine-disrupting chemicals. Some of these compounds were lignin monomer which revealed the necessity of treatment for the detoxification of discharged effluent. The supplementation of carbon (glucose 1.0%) and nitrogen (peptone 0.5%) which stimulated the degradation process. Therefore, degraded samples after the biostimulation process showed either disappearance or generation of metabolic products at optimized conditions i.e. rpm (150), temp (37±1^{o}C) after 3 and 6 days of bacterial incubation. Isolated potential autochthonous bacteria were identified as *Klebsiella pneumonia* IITRCP04 (KU715839), *Enterobacter cloacae* strain IITRCP11 (KU715840), *Enterobacter cloacae* IITRCP14 (KU715841) and *Acinetobacter calcoceticus* strain IITRCP19 (KU715842). In addition, the study also revealed that there was a generation of some value-added products during the detoxification of effluent in the biostimulation process from residual chloro-lignin compounds. This also supported the commercial importance of this process.

Based on the study under this thesis two original research papers has been published in high impact journals while two other original research paper is under revision. Related with thesis of study six conference papers also has been presented in various national and international conferences. One best posted presentation award has been also received in 58th annual conference of association of Microbiologist of India (AMI). Beside, one book chapter has been also published related with my work.

Chapter-Twelve

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Scientific Publications and Achievements:

(A) Research Papers published high impact journals of International Repute: Seven

1. Ram Chandra, **Pooja Sharma**, Sangeeta Yadav and Sonam Tripathi (2018). Biodegradation of Endocrine-Disrupting Chemicals and Residual Organic Pollutants of Pulp and Paper Mill Effluent by Biostimulation. **Frontier in Microbiology.** 9:1-15.
2. Ram Chandra, Vineet Kumar, Sonam Tripathi, Pooja Sharma (2018). Heavy Metal Phytoextraction Potential of Native Weeds and Grasses from Endocrine-Disrupting Chemicals Rich Complex Distillery Sludge and their Histological Observations During In-situ phytoremediation. **Ecological Engineering**. 111:143-156.
3. **Pooja Sharma**, Sonam Tripathi, Ram Chandra (2020). Accumulation and Histological Observation of Heavy Metal in *Brassica campestris* L. and *Chenopodium album* L. Growing on Sludge Waste of Pulp Paper Industry after Secondary Treatment. **Journal of Experimental Biology and Agriculture Science**.8:1-14.
4. **Pooja Sharma**, Sonam Tripathi, Ram Chandra (2020). Characterization of Autochthonous Bacteria Capable for Degradation of Residual Organic Pollutants of Pulp Paper Mill Effluent by Biostimulation Process. **Journal of Pure and Applied Microbiology**. 14:1-14.
5. **Pooja Sharma**, Sonam Tripathi, Ram Chandra (2020). Environmental Impacts of Pulp Paper Mill Effluent: Potential Source of Chromosomal Aberration and Phytotoxicity. **International Journal of Applied Environmental Sciences**. 15: 77-92.
6. **Pooja Sharma**, Sonam Tripathi, Shailesh Kumar Patel, Kuldeep Dhama, Ram Chandra (2020). SARS-CoV-2/COVID-19 and its Transmission, Prevention, Treatment and Control -An Update. **Journal of Pure and Applied Microbiology**. 14:1-12.
7. **Pooja Sharma**, Sonam Tripathi, Ram Chandra (2020). Phytoremediation potential of heavy metal accumulator plants for waste management from pulp paper industry. **Heliyon** (Accepted In Press)

(B) Research Paper Communicated: Two

1. **Pooja Sharma**, Diane Purchase, Ram Chandra (2020).Residual Pollutants in Treated Pulp Paper Mill Wastewater and their Phytotoxicity and Cytotoxicity in *Allium cepa*. **Environmental Geochemistry and Health.**
2. **Pooja Sharma** and Ram Chandra (2020). Phytoremediation of Heavy Metals (Pb, Cd, Fe, Cu, Cr Ni, Zn, Mn and As) from Mixed Persistent Organometallic Pollutants of Pulp Paper Industry Sludge by Selective Native Plants: A Green Remediation Technology. **Journal of Environmental Management**.

(C) Book Chapters: one

Ram Chandra, Vineet Kumar**,** Sonam Tripathi, **Pooja Sharma (2018).** Phytoremediation of Industrial Pollutants and Life Cycle Assessment. In: Phytoremediation of Environmental Pollutants, Ram Chandra, N.K. Dubey, Vineet Kumar (Eds), CRC Press, USA, pp-441-469.

(D) Awards

BEST Poster Award in Environmental Microbiology at 58th Annual Conference of Association of Microbiologists of India (AMI) & International Symposium on "Microbes for Sustainable Development: Scope and Application", Babasaheb Bhimrao Ambedkar University, Lucknow, UP, INDIA (16-19 Nov. 2017).

(E) Memberships of Scientific Societies

(1) Life Member of **Indian Science Congress Association (ISCA)**

(F) Research Paper Presented in National /International Symposium and conferences

1 **Pooja Sharma,** Sonam Tripathi and, Ram Chandra (**2016**). **"**Bioremediation and Detoxification of Endocrine Disrupting Chemicals (EDC) and Residual Organic Pollutants of pulp paper mill effluent after secondary treatment in Biostimulation process for environmental safety". 57th Annual Conference of **Association of Microbiologists of India** (AMI) & International Symposium on "Microbes and Biosphere: What's New what"s Next", Guwahati University, Guwahati, Assam, INDIA (24-27 Nov. 2016) (**Poster Presentation**)

2 **Pooja Sharma,** Vineet Kumar, Sonam Tripathi and Ram Chandra **(2016).** "Heavy metal phytoextraction potential of common Indian aquatic weeds and grasses from pulp & paper mill effluent after secondary treatment and their accumulation pattern in different parts" **International Conference on Current Trends in Biotechnology** ICCB-2016, organized by the School of Biosciences and technology [SBST], VIT University, Vellore, in association with The Biotech Research Society, India [BRSI] (8-10 Dec 2016). (**Poster Presentation**)

3 **Pooja Sharma,** Sonam Tripathi and Ram Chandra (**2017**). **"**Bioremediation of Endocrine Disrupting Chemicals (EDC) and refractory organic pollutants of pulp paper mill effluent after secondary treatment in Biostimulation process for environmental safety". 58th Annual Conference of **Association of Microbiologists of India** (AMI) & International Symposium on "Microbes for Sustainable Development: Scope and Application", Babasaheb Bhimrao Ambedkar University, Lucknow, UP, INDIA (16-19 Nov. 2017). (**Poster Presentation-BEST Poster Award**).

4 **Pooja Sharma,** Sonam Tripathi and Ram Chandra (**2017**). **"**Detection of Residual Organic Pollutants of Pulp and Paper Mill Waste and Its Detoxification by Potential Bacterium Consortium". **4th Lucknow Science Congress [LUSCON-2017]** on Science Technology and Innovation for Sustainable Development, Organized by Babasaheb Bhimrao Ambedkar University, Lucknow, UP, INDIA 3-4 March 2017. (**Poster Presentation).**

5 **Pooja Sharma,** Ram Chandra **(2017)** in **"National Symposium** on **IPRs in Agriculture Research"** organized by B.B. Ambedkar University Lucknow held from August 30-30, 2017, Lucknow.

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(G) Training and Skill Development Program:

1. **Institute:** CSIR-Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India.
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Biodegradation of Endocrine-Disrupting Chemicals and Residual Organic Pollutants of Pulp and Paper Mill Effluent by Biostimulation

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Effluent discharged from the pulp and paper industry contains various refractory and androgenic compounds, even after secondary treatment by activated processes. Detailed knowledge is not yet available regarding the properties of organic pollutants and methods for their bioremediation. This study focused on detecting residual organic pollutants of pulp and paper mill effluent after biological treatment and assessing their degradability by biostimulation. The major compounds identified in the effluent were 2,3,6-trimethylphenol, 2-methoxyphenol (guaiacol), 2,6-dimethoxyphenol (syringol), methoxycinnamic acid, pentadecane, octadecanoic acid, trimethylsilyl ester, cyclotetracosane, 5,8-dimethoxy-6-methyl-2,4-bis(phenylmethyl)napthalen-1-ol, and 1,2-benzendicarboxylic acid diisononyl ester. Most of these compounds are classified as endocrine-disrupting chemicals and environmental toxicants. Some compounds are lignin monomers that are metabolic products from secondary treatment of the discharged effluent. This indicated that the existing industrial process could not further degrade the effluent. Supplementation by carbon (glucose 1.0%) and nitrogen (peptone 0.5%) bio-stimulated the degradation process. The degraded sample after biostimulation showed either disappearance or generation of metabolic products under optimized conditions, i.e., a stirring rate of 150 rpm and temperature of 37 ± 1°C after 3 and 6 days of bacterial incubation. Isolated potential autochthonous bacteria were identified as *Klebsiella pneumoniae* IITRCP04 (KU715839), *Enterobacter cloacae* strain IITRCP11 (KU715840), *Enterobacter cloacae* IITRCP14 (KU715841), and *Acinetobacter pittii* strain IITRCP19 (KU715842). Lactic acid, benzoic acid, and vanillin, resulting from residual chlorolignin compounds, were generated as potential value-added products during the detoxification of effluent in the biostimulation process, supporting the commercial importance of this process.

Keywords: biostimulation, chromosomal aberration, lignolytic enzyme, phytotoxicity, pulp paper effluent, refractory pollutants

INTRODUCTION

The pulp and paper industry is an important source of aquatic and soil pollution due to its discharge of significant amounts of chlorolignin-containing compounds. Approximately 190–200 m^3 of fresh water is consumed per ton of paper production (Orrego et al., 2009; Chandra and Singh, 2012). From the original mass of raw material, about 40–45% reports to pulp, while the remainder is released in effluent that contains cellulose, hemicelluloses, tannins, resin acids, and chlorophenols, as well as chloro-lignins produced by bleaching and washing of the pulp. Chlorinated resin acids and phenols, chlorinated hydrocarbons, several surfactants, biocides, and plasticizers used in pulp and paper production are also discharged as common pollutants (Ali and Sreekrishnan, 2001).

Primary treatment of the effluent involves screening of pulp fibers and pH adjustment. The resulting mixed wastewater is then subjected to a secondary effluent treatment process using activated sludge. A recent study revealed that complex organic pollutants are retained as residual organic pollutants in discharged pulp and paper mill wastewater, even after secondary treatment (Chandra and Singh, 2012). The common residual complex organic pollutants detected were dibenzoparadoxin and dibenzofurans, 4-isopropoxybutyric acid, 3,7,11,15,18-pentaoxa-2,19-disila nonicosane, butane-1-ol, hexahydropyrrole (1,2A) pyrazine-1,4-dione, 6-chlorohexanoic acid, 2,5-3-6-bis(phenylmethyl)piperazinedione, 1-chlorooctadane, 1,2-benzene carboxylic acid, 3-tetradecyl ester benzene acetic acid, 2- hydroxymethylcyclopropane carboxylic acid, 2-methoxyphenol phthalic anhydride, 2,6-dimethoxyphenol, 2-methoxy-4-ethylphenol, 3-allyl-6-methoxyphenol, and 2-methoxy-4(1-propenyl) (Chandra and Singh, 2012). This study also indicated that these compounds exhibited an endocrine-disrupting effect on aquatic organisms due to the presence of endocrine-disrupting chemicals (EDCs) (Orrego et al., 2009; Chandra and Kumar, 2017); however, detailed knowledge regarding the estrogenic and androgenic potential of these compounds toward aquatic organisms is lacking. The presence of organic pollutants in the final effluent discharge indicated that the microbial community present in the wastewater was unable to degrade them due to lack of adequate nutrients; hence, optimization of bacterial growth conditions by addition of nutrient or environmental conditions may be an effective approach for detoxification of such effluents.

In-situ bioremediation has been reported as an alternative method to reduce time and cost of restoration of polluted sites (Olaniran et al., 2006); however, owing to lack of knowledge regarding the controlling factors for in-situ biodegradation of persistent organic pollutants, there are significant obstacles to the application of bioremediation techniques at polluted sites with complex organic pollutants.

Prior to selecting a mode of bioremediation, the type of microbial community and required environmental conditions should be determined. Three types of bioremediation processes are commonly used for in-situ bioremediation of complex industrial pollutants, i.e., natural attenuation, bio-stimulation, and bio-augmentation. The simplest method of bioremediation is natural attenuation, where contaminated sites are only monitored for pollutant concentrations. Using biostimulation, the controlling factors in natural processes of pollutant degradation require adjustment at the polluted site to provide bacterial communities with a favorable environment in which they can effectively degrade pollutants (Kaplan and Kitts, 2004). This may include the addition of nitrogen or phosphorus for the proliferation of indigenous bacterial communities and the pH may be adjusted to enhance the bioremediation process (Salamitou et al., 1997). Cases where natural communities of degrading bacterial communities are present in low numbers or absent require addition of a competent bacterial inoculums to accelerate the degradation process: this is known as bio-augmentation (Vanlimbergen et al., 1998).

Certain potential bacterial strains have been reported as more effective than fungal strains for bioremediation of complex environmental pollutants due to their good environmental adaptability and biochemical versatility for their growth. Bacteria isolated from compost soil, viz., *Azotobacter* and *Serratia marcescens*, were reported capable of degradation and decolorization of lignin compounds (Morii et al., 1995). A comparative study of lignin biodegradation under aerobic conditions by *Bacillus subtilis* and *Bacillus* sp. isolated from soil was reported (Abd-Elsalam and El-Hanafy, 2009). Three potential aerobic bacterial strains were isolated from pulp and paper mill effluent sludge for kraft lignin degradation, identified as *Paenibacillus* sp. (AY952466), *Anoxybacillus amylolyticus* (AY856831), and *Bacillus* sp. (AY952465) (Chandra et al., 2007). These bacterial strains showed decolorization of kraft lignin at 500 mg/l and their metabolic products were characterized by gas chromatography with mass-spectrometric detection (GC–MS) (Raj et al., 2007). *Bacillus* sp. and *Serratia marcescens* have also been reported as effective for pentachlorophenol and pulp and paper mill effluent degradation up to 94% in the presence of sources of supplemented carbon (1% glucose) and nitrogen (0.5% peptone m/v) under optimized laboratory conditions (Singh et al., 2008). These studies provide strong evidence for the degradation and detoxification of chlorolignin-containing pulp and paper mill wastewater by bacterial consortia (Chandra and Singh, 2012); however, the detoxification and in-situ bioremediation of pulp and paper mill effluent after secondary treatment has not yet been reported. The present study therefore focused on persistent organic pollutants, the number of microbial counts and their nutritional requirements, and environmental conditions for detoxification and reuse of discharged pulp and paper mill wastewater. This study may assist development of an in-situ bioremediation technology for pollution prevention, and potentially provide an economic opportunity for recycling and application of degraded and decolorized effluents after discharge.

MATERIALS AND METHODS

Sample Collection

Discharged pulp and paper industry wastewater after secondary treatment were collected in sterile plastic containers from Century Pulp Paper Mill, Ltd., Lalkuan, Uttarakhand, India,

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Heavy metal phytoextraction potential of native weeds and grasses from endocrine-disrupting chemicals rich complex distillery sludge and their histological observations during in-situ phytoremediation

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ABSTRACT

Sugarcane-molasses based distillery waste is a threat to environment for its safe disposal due to complexation of endocrine-disrupting chemicals (EDCs) containing mixture of organic pollutants. This study revealed that distillery sludge contains not only mixture of complex organic pollutants but also retains high quantity of Fe (5264.49), Zn (43.47), Cu (847.46), Mn (238.47), Ni (15.60), and Pb (31.22 mg kg^{-1}) which enhances the toxicity of sludge to the environment. The major identified organic compounds were benzene, 1-ethyl-2-methyl, benzene, 1-ethyl-4-methyl benzoic acid, 3,4,5-tris(TMS oxy), TMS ester; hexanedioic acid, dioctyl ester; stigmasterol TMS ether; 5α-cholestane,4-methylene; campesterol TMS; β-sitosterol and lanosterol. These compounds are listed under the EDCs also as per U.S. Environmental Protection Agency. However, the phytoextraction potential of growing native weeds and grasses i.e. *Argemone mexicana, Saccharum munja, Cynodon dactylon, Pennisetum purpureum, Chenopodium album, Rumex dentatus, Tinospora cordifolia, Calotropis procera* and *Basella alba* revealed the high accumulation of Fe, Zn, Cu, Mn, Ni, and Pb in their root and leaves compared to shoot. This indicated high accumulation and translocation capabilities of these plants. Further, the bioaccumulation coefficient factor (BCF) and translocation factor (TF) was found > 1 for majority of plants for various metals. Thus, this given strong evidence for hyperaccumulation tendency of these native weeds and grasses from complex polluted sites. Furthermore, the ultrastructural observations of root tissues also revealed the deposition of heavy metals at various cellular components without any apparent toxic effects. This indicated the variable adaptive characteristics of these plants growing at a hazardous waste polluted site. Thus, the study given a strong evidence for application of these weeds and grasses as tools for in-situ phytoremediation and eco-restoration of polluted sites.

1. Introduction

Sugarcane-molasses based distillery waste is well known as source of complex environmental pollutants due to various heavy metals containing complex organic pollutants (Chandra et al., 2008; Chandra and Kumar, 2017a, 2017b). In India, there are more than 397 sugarcane molasses based distilleries releasing approximately 3.5×10^{13} kL spent wash annually (AIDA, 2016). There is an average sludge generation of 1500 tons per day during anaerobic digestion of spent wash (Kansal et al., 1998). This reflects the magnitude of the environmental pollution caused by the waste generated from distillery sector all over India. The sludge generated from distilleries also contain mainly dodecanoic acid, octadecanoic acid, *n*-pentadecanoic acid, hexadecanoic acid, β-sitosterol, stigmasterol, β-sitosterol trimethyl ether, heptacosane, dotriacontane, lanosta-8, 24-dien-3-one, 1-methylene-3-methyl butanol, and 1-phenyl-1-propanol as androgenic and mutagenic compounds (Chandra and Kumar, 2017a), which are listed under the endocrine-disrupting chemicals (EDCs) list of USEPA (2012). The study has revealed that these organic pollutants makes organo-metallic complex with various heavy metals which are mainly iron (Fe), zinc (Zn), copper (Cu), chromium (Cr), cadmium (Cd), manganese (Mn), nickel (Ni), and lead (Pb) present in high quantity i.e. (Fe: 2403.64), (Zn: 210.624), (Cu: 73.63), (Cr: 21.84), (Cd: 1.446), (Mn: 126.292), (Ni: 13.425), (Pb: 16.332 mg kg^{-1}) (Chandra and Kumar, 2017c). The concentrations of these metals are for above than the prescribed limit in environment as per USEPA (2002) and European Union (2002).

Generated effluent after distillation process also a major source of aquatic pollution due to high level of maillard products generated due

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to complexation of sugar and amino acid at elevated temperature (Chandra and Kumar, 2017c). It has been reported that melanoidins have net negative charges, hence, different heavy metals (Cu^{2+}, Cr^{3+}, Fe^{3+}, Zn^{2+} and Pb^{2+} etc.) strongly bind to form organo-metallic complex with melanoidins (Migo et al., 1997). The high metal binding tendency of melanoidins also enhances vulnerability of organo-metallic complex towards its toxicity in environment. Consequently, the sludge generated after anaerobic digestion contains mixture of various complex organic pollutants along with heavy metals, phenolics, and EDCs which are generated in sugar production and molasses fermentation (Chandra and Kumar, 2017a, 2017c). The study have shown the toxicity of heavy metals at the genetic and cellular level both due to generation of reactive oxygen species under metal stress which can seriously disrupt normal metabolism of the plant and animal both (Nagajyoti et al., 2010; Jaishankar et al., 2014). Oxyradicals can cause lipids peroxidation, inactivation of enzyme and membrane damage leads to cause cellular toxicity in plants and human (Kumar et al., 2013a, 2013b; Tian et al., 2012). Consequently, the contamination of distillery waste to aquatic resources not only affects the water quality but also adversely affect to aquatic flora and fauna (Bhargava and Chandra, 2010; Ayyasamy et al., 2008). In addition, the presence of androgenic and mutagenic compounds in the industrial sludge not only changes the soil quality but also adversely affect to soil microbial communities which are essential for elemental recycling and maintenance of soil fertility (Jeswarkar and Dutta, 1989). But, the recent studies have reported that to mitigate the oxidative damage, plants have developed a complex defense antioxidant system including low molecular weight antioxidants (cystein, ascorbic acid, and protein thiol) as well as enzyme such as superoxide dismutase, catalase and peroxidase (Kumar et al., 2013a, 2013b; Tian et al., 2012). Thus, these antioxidants play an important role in the cellular defense strategy of plants against oxidative stress, inducing resistance for metals by protecting labile macromolecules (Chandra and Yadav, 2010). However, the knowledge regarding the presence of androgenic and mutagenic compounds in distillery waste is still not well known to majority of the environmentalist. Therefore, the development of detoxification device of distillery waste including the sludge is essential prior to its disposal in the environment for sustainable development.

There are certain hyperaccumulator plants with characteristic properties of fast growth and production of high biomass of shoot along with diversified rhizospheric microbial communities, which facilitate the bioavailability of plant nutrient and mineralization of complex organic pollutants (Jesmkh et al., 2013; Rajkumar et al., 2012; Gan and Coulight, 2014). The bacterial assisted phytoremediation of organic and inorganic pollutants is regulate by soil texture, pH, temperature and other environmental factors which regulated the heavy metal accumulation pattern in the different parts of plant and detoxification mechanism of organic pollutants (Ma et al., 2011; Sinha et al., 2007). Though, few reports have highlighted the ability of some wetland plants to remove the heavy metals from the organic pollutants containing wastewater in natural and constructed wetland (Weis and Weis, 2004; Deng et al., 2004). A study from Kolkata (India) also showed that 10 common regional wetland plant species from a wetland site accumulated metals like Cd, Cr, Cu, Pb, Zn, Mn and Fe (Chatterjee et al., 2011). The study reported water hyacinth (*Eichhornia crassipes*), water spinach (*Ipomoea Aquatica*), watercress (*Wolffia arrhiza*), water chestnut (*Trapa bispinosa*), water lettuce (*Pistia stratiotes*), common arum (*Colocasia esculenta*), common sedge (*Cyperus rotundus*), bulrush (*Scirpus sp.*), arrowhead weed (*Sagittaria montevidensis*), bermuda grass (*Cynodon dactylon*) for heavy metal accumulation efficiency in their different parts. Further, they also reported that such plants were naturally ameliorating metal contamination from the wetland site, thus acting as sustainable and cost effective natural effluent treatment system for bioremediation. Some studies on phytoextraction potential of various plants and their histological observations have been reported globally from pure metal solution in vitro conditions only (Maruthi Sridhar et al., 2005; Najeeb et al., 2011; Alkhatib et al., 2013; Daud et al., 2013). However, the histological observations of these plants due to heavy metal accumulation in their tissue are not revealed so far. Furthermore, the phytoextraction capabilities of native weeds and grasses growing at complex organic polluted site is not yet reported for heavy metal accumulation to explore in-situ phytoremediation potential for eco-restoration of polluted sites with complex organic waste. Moreover, the heavy metals phytoextraction potential by native weeds and grasses growing on complex organo-metallic compounds which are rich with androgenic and mutagenic compounds listed under EDC groups (USEPA, 2012).

Therefore, the present study has been focused on detail investigation of complex organic pollutants present in distillery sludge by Gas chromatography-mass spectrometry (GC–MS) analysis and microscopic histological observations of root of growing weeds and grasses by transmission electron microscope (TEM) for heavy metal accumulation in their parts to reveal the hyperaccumulation mechanism of these potential plant species in presence of complex pollutants.

2. Materials and methods

2.1. Site description

The test site of the experiments selected for soil and plant sampling was located in Unnao, Uttar Pradesh (26°32′0″ N, 80°30′0″E), India (Fig. 1). The samples were taken from the sludge dumping site of M/s Unnao Distilleries & Breweries. This site is well known for high pollution with organic and inorganic pollutants reported earlier by various researchers (Chandra et al., 2008; Chandra and Kumar, 2017c)

2.2. Collection of plant and distillery sludge samples

Nine representative native plants species (weeds and grasses) were collected based on dominant species luxuriantly growing on disposed distillery sludge. These plants species were identified from different genera and families according to Dutch flora of Indo-Gangetic plains, where three species *Saccharum munja* (munja), *Cynodon dactylon* (bermuda grass), and *Pennisetum purpureum* (elephant grass) belong to Gramineae family while other five plants namely *Argemone mexicana* (mexican poppy), *Chenopodium album* (goosefoots), *Rumex dentatus* (toothed dock), *Tinospora cordifolia* (giloy), *Calotropis procera* (madhaar), and *Basella alba* (pui) belonging from Papaveraceae, Amaranthaceae, Polygonaceae, Menispermaceae, Asclepiadaceae, and Basellaceae, respectively. These plants species were uprooted with associated sludge samples and carried in pre-sterilized polythene bags for the analysis of accumulated heavy metal in different parts of growing plants. Besides, the fresh disposed dried distillery sludge cakes were collected in clean pre-sterilized polythene bags from sludge dumping site of distillery plant located inside the premises of industry. The fresh as well as ameliorated distillery sludge after plant growth was collected randomly in triplicate from three different points of same location. This process was repeated three times in different seasons from same place which was protected from any outside interference of human and animals.

2.3. Physico-chemical analysis of distillery sludge

The physico-chemical parameters of distillery sludge sample i.e. pH, electrical conductivity (EC), chloride (Cl^-), sodium (Na^+), and nitrate were estimated according to the method described by Kalra and Maynard (1991). The phenol contents in sludge were analyzed as per standard methods described in APHA (2012). The pH and EC values (sludge:water = 1:2.5 w/v) of sludge samples were measured by using Orion meter (Model 960, Thermo Scientific, FL, USA) and Orion conductivity meter, respectively (Chandra et al., 2008). The total content of Fe, Zn, Cu, Mn, Ni, and Pb in dry weight sample of sludge was measured

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RESEARCH ARTICLE OPEN ACCESS

Characterization of Autochthonous Bacteria Capable for Degradation of Residual Organic Pollutants of Pulp Paper Mill Effluent by Biostimulation Process

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Abstract

The purpose of this study is remediation of residual organic pollutants from effluent by autochthonous bacterial community in biostimulation process. Discharged effluent showed high TDS (549 mg L^{-1}), TSS (59 mg L^{-1}), COD (20349 mg L^{-1}) and BOD (25946 mg L^{-1}), value. The level of total phenol (421 mg L^{-1}), nitrogen (156 mg L^{-1}), sulphate (1854 mg L^{-1}), phosphorus (176 mg L^{-1}) chlorine (2.01 mg L^{-1}), sodium (75 mg L^{-1}) and potassium (8.4 mg L^{-1}) along with various heavy metals (Fe, 75.23; Zn, 15.60; Cu, 4.1; Cr, 3.12; Cd, 0.324; Mn, 13.24; and Ni, 4.01 mg L^{-1}) were noted above the permissible limit of Environmental Protection Act. The result revealed that the reduction of the physico-chemical parameter of pollutants were above 50% after biostimulation process, this confirmed the potentiality of growing autochthonous bacterial community responsible for bioremediation. The comparative UV-Vis spectroscopy showed reduction in the absorption spectra of degraded sample. Further, GC-MS analysis showed major organic pollutants i.e. Octadecanoic acid, Hexadecanoic acid, citral, benzoic acid, and 2, 6'-Di-hydroxy acetophenone, bis (trimethylsilyl) ether, were detected in control few compounds were degraded while there was formation of some new metabolic products also. Few pollutants persisted in the degraded sample as recalcitrant toxicant and causes environmental toxicity and hormonal imbalance as endocrine-disrupting chemicals (EDCs). But the detailed knowledge and characterization of organic pollutants are not available yet regarding their properties. The SEM image showed the diversity of bacterial community in biostimulation responsible for utilisation of various detected compounds. The growing bacterial communities were identified as potential bacterial strains as *Aeromonas salmonicida,* BBAUPS-1 (MN294457.1) and *Bacillus* sp. BBAUPS-2 (MN238724.1) responsible for the remediation of residual organic pollutants. Further, Evaluation of toxicity parameter of effluent by seed germination test of *Triticum aestivum* and *Cicer arietinum* inhibited the seed germination upto 80%. Hence, this study revealed that the biostimulation process is a good technique for detoxification and degradation effluent.

Keywords: Environmental toxicity, endocrine-disrupting chemicals, organic pollutants, phytotoxicity, effluent detoxification

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INTRODUCTION

The discharged effluent from the pulp paper industry contains more than 700 organic and inorganic pollutants that are directly responsible to cause the soil and water pollution after biological and chemical treatment[1]. The pulp paper industry ranks 6th among the world's most polluting industries and generate large-scale hazardous pollutants during the making of papers[2]. Pulp paper industry effluents contain high number of complex matrices along with many other diverse compounds[3]. Moreover, the effluents may contain more than 250 identified chemicals and harmful components like sterols and asresin acids[4, 5]. These compounds may end up as sediments in the water bodies and serve as food for fish and benthos[6]. Although, many harmful substances are removed before release by modern wastewater purification processes but some are released unintentionally into the environment. Moreover, untreated effluent with heavy nutrient load and wood derived bioactive substances if released into the natural water bodies may cause oxygen depletion and subsequent decline in the biodiversity[7].

In India, there are approximately 650 small and large scale paper industries discharging approximately 190-200 m^3 effluent per ton of paper production as aquatic pollutants[8]. Furthermore, the recent study has also revealed that some of these compounds showed EDCs effects on aquatic organisms. Moreover, there is still a lack of detailed knowledge on the estrogenic and androgenic compounds from pulp paper industry effluent and their toxicity in the aquatic organism. Therefore, optimizing bacterial growth conditions by adding different nutrients and providing good environmental conditions might be an effective strategy for detoxification. However, there is lack of understanding on the controlling factors for biodegradation of complex organic pollutants derived from several polluted sites. In-situ bioremediation has been introduced as an cost effective and less tedious approach in re-developing any contaminated site. Additionally, the identity of the microbial community and required environmental conditions should be detected before specification for bioremediation function. Three types of bioremediation processes are generally used for the in-situ bioremediation process of any complex industrial contaminants viz. natural attenuation, bio-stimulation and bio-augmentation.

Bio-stimulation process involves adaptation to the contaminated site to provide a favourable environment for bacterial communities for effective natural degradation of different pollutants[9]. Several persistent organic pollutants (POPs) are present in industrial wastes which are known to be silent environmental killers due to their bio accumulative and long-lasting existence[10, 11]. Furthermore, isolated bacterial strains from compost soil i.e. *Azotobacter sp.* and *Serratia marcescens* might degrade and decolorize lignin-containing effluents while *Bacillus subtilis* and *Bacillus sp.* are responsible for kraft-lignin degradation[12]. The quality of water is highly detoriated by surpassing TDS, TSS, BOD and COD values. Moreover, the dark colour and high turbidity of effluent due to suspended solids, TSS, TDS and TS can further cause river pollution and compromise the drinking water quality. The pulp paper industry effluent absorbs more light and reduces the oxygen concentration in water thereby affecting the aquatic life due to presence of tannins and resin acids[13]. While the change in the colour and water quality nearby the industrial area makes it unsafe for drinking purpose. The leaching property of different effluent pollutants is a major source of ground pollution. Pollution of our environment by heavy metals particles is a very dangerous and challenging problem for the country[14, 15]. Earlier, three potential bacterial strains viz. *Panibacillus sp. Aneurinibacillus aneurinilyticus* and *Bacillus sp.* were identified and characterized from sludge for synthetic lignin degradation and metabolic properties using gas chromatography-mass spectrometry (GC-MS) analysis respectively[16]. Similarly, the *Bacillus sp.* and *Serratia marcescens* were also reported to cause degradation of the pentachlorophenol containing effluent in the presence of about 94% nutrients in optimized in-vitro conditions. Thus, these studies report the degradation and detoxification capability of bacteria for chlorolignins containing effluent[17]. The removal of heavy metals from contaminated water and soil by employing electro-coagulation for the waste management methods has been previously described[18, 19]. Therefore, the present study was designed to focus on the characterization and identification of residual organic pollutants and

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ACCUMULATION AND HISTOLOGICAL OBSERVATION OF HEAVY METAL IN *Brassica campestris L.* AND *Chenopodium album L.* GROWING ON SLUDGE OF PULP PAPER INDUSTRY AFTER SECONDARY TREATMENT

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KEYWORDS:

Heavy Metals

Sludge Waste

GC-MS analysis

Health risks

Antioxidant enzymes

ABSTRACT

The discharged sludge from the pulp paper industry contains several organic and inorganic pollutants even after secondary treatment, which might cause toxicity in plants. The present study was designed to investigate the accumulation and histological findings of heavy metal in *Brassica campestris* and *Chenopodium album* growing on sludge waste of pulp paper industry after secondary treatment. The physico-chemical analysis of sludge viz., pH, total solid (2678 mg L^{-1}), total dissolved solid (2756 mg L^{-1}), total suspended solid (189 mg L^{-1}), chemical oxygen demand (43587 mg L^{-1}), biological oxygen demand (1569 mg L^{-1}), and electric conductivity (2067 ms cm^{-1}) contents in it were found beyond the permissible limit. The result of study revealed that the heavy metal content in sludge viz., Cu (59), Ni (66), Fe (153), Mn (9.37) Zn (12.31) and Mg (11.8 mg L^{-1}). Furthermore, the chlorophyll and carotenoids contents in *B. campestris* and *C. album* was (Chl-a 4.57-5.21 mg g^{-1} fw), (chl-b 5.29-5.89 mg g^{-1} fw), and (carotenoids 0.84-1.07 mg g^{-1} fw) also high. The effect of heavy metals on antioxidants enzyme of *B. campestris* and *C. album* growing on organometallic containing sludge waste was observed to be high. Further, the concentrations of heavy metals in *B. campestris* and *C. album* were reported in descending order as Fe (211-208)> Cu (62.37-49.87)>Zn (56.67-34.26) > Mn (36.37-42.37) > Mg (35.69-13.64)> Ni (9.36-6.51 mg kg^{-1}) root> shoot> leaves respectively. Hence the result of study concluded that pulp paper industry sludge is the main source of metals accumulation in crop plants, and need to be appropriately treated before discharge in the environment for human and animal health safety.

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I Introduction

Environmental pollution and contamination by heavy metals is a threat to the environment and is of serious concern to plants. Rapid industrialization and urbanization have caused pollution of the environment owing to heavy metals, and their rate of mobilization, transport and accumulation in the environment has accelerated significantly since the 1940 (Khan et al., 2004). The main sources of heavy metals in the environment include metallurgical work, urbanization, and industrialization, particularly in highly populated developing countries such as China and India (Un-Habitat, 2004). Moreover, in the year 2017, approximately 411 million tons of worldwide paper production accounted mostly in countries such as India, China, Brazil, European country and USA (Buyuk Demirel & Altas, 2017). The pulp paper industry might be releasing 55–60% sludge wastes containing nitrogen, potassium, calcium, phosphorus, iron, magnesium, copper, silicon, zinc and manganese compounds, although during pulping process only 40-45% of the pulp is obtained (Ali & Sreekrishnan, 2001). Consequently, approximately 190-200 m^3 of freshwater is utilized per ton of paper production and sludge is about 0.04–0.5 m^3 dry weight of sludge in North American paper mills (Chandra & Singh, 2012; Bajpai, 2015). Furthermore, the environmental protection agency (EPA) in United States reports more than 250 million tonnes of municipal solid waste to be generated every year, of which approximately 30% are related to pulp paper industry waste, while one ton of paper produced generate approximately 0.6 tons of waste discharged in the environment without proper treatment (IPPC, 2001; EPA, 2002). In addition, the sludge waste generated during the paper manufacturing from the paper industry is divided into four categories as follows: first is the primary sludge wastes generated by the production of virgin wood fibers, second is the destination sludge wastes generated by removal of the fiber ink, third include activated waste after biological treatment that is secondary sludge; fourth is the waste from paper production for biological purposes which is complex sludge. In the biological process, microorganisms transform the organic matter of the sludge waste into a type of soil fertilizer (Ikoni et al., 2004). The discharged sludge waste contains chlorinated compounds measured as adsorbable organic halides (AOX) that can bio-accumulate in fish tissue causing a range of clastogenic, endocrine and mutagenic impacts (Savant, et al., 2006).

India is a developing country where farmers do not have sufficient resources for irrigation of agricultural crops; therefore, they use industrial wastewater as a source of water with a higher level of toxic heavy metals. Moreover, several heavy metals have adverse effects on different enzymes including acid phosphatases, proteases, and α-amylases and protein profiles involved in germination of seeds. For instance, heavy metals have been reported to decrease the starch content, reduce the nutrient content, impair the PS-II of the chloroplast, and induce the expression of heat shock proteins and proline (Seneviratne et al., 2019). Similar observation of heavy metal accumulation and effect of production, biomass and physiological processes in mustard was also reported by Sheetal et al., (2016).

Wild plants are important components of ecosystem as their distribution help in keeping the environment clean. *Chenopodium album* constitutes common weeds of cultivated fields and widely distributed in many parts of the world. Information on metal accumulation in Chenopodiaceae species has been widely reported among weeds with these characteristics (Turker et al., 2019). Similarly, *Brassica* species are [illegible] agricultural crops in various parts of the world and are also considered to be heavy metal (Fe, Cu, Zn, Ni, Pb, [illegible] Cd and Mg) accumulators (Monarto et al., 2015). This study aimed to analyze accumulation and histological findings of heavy metals in *B. campestris* and *C. album* growing commonly on sludge waste of pulp paper industry after every secondary treatment at industrial level. In the present study, the effect of heavy metals on antioxidants enzyme of *B. campestris* and *C. album* growing on organometallic containing sludge waste was found to be high. Furthermore, this manuscript discusses the toxicity of pulp paper industry sludge and metals as accumulated by plants that causes health risks for humans and animal via food-chain consumption. Brassica species are significant in the production of oil, both for human food and for the production of biodiesel; the potential contamination of seeds and oil from these plants was also the objective of some studies, as this could lead to pollution of the food chain and the environment. The study included accumulation, uptake, effect, and distribution of heavy metals in selected plants from the disposal site of the pulp paper industry waste. Consequently, the biochemical changes in plants by the stress of metals and their phytoextraction efficiency could be deleterious.

2 Material and Methods

2.1 Collection of sludge and plant samples

The fresh effluent samples were collected from the M/s KR Pulp Paper Industry, Shahjahanpur, Uttar Pradesh, India (27°50'31.8"N 79°51'15.7"E). For this study, sludge samples were collected in a 20 kg (Tarson Production Pvt. Ltd., USA) sterile plastic bag from disposal site. The effluent samples were collected using a sterile plastic container and stored at 4°C until further use. Furthermore, two characteristic plant species i.e. *B. campestris* (Brassicaceae) and *C. album* (Amaranthaceae) were identified and collected on the basis of abundantly growing on sludge. In addition, the study of normal plant (as control) growth plant sample was collected from normal agriculture land. This process was repeated three times in different seasons from the same place for the confirmation and authenticity of scientific data.

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Environmental Impacts of Pulp Paper Mill Effluent: Potential Source of Chromosomal Aberration and Phytotoxicity

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Abstract

The toxic compounds of pulp paper mill effluent after secondary treatment discharged is a major source of environmental toxicity which is not yet known. The physico-chemical analysis of discharged pulp paper mill effluent showed their beyond permissible limit. Furthermore, the BOD, COD ratio of discharged pulp paper mill effluent indicated the < 0.2 showed not biodegradable effluent. It persistent longer in environment and caused toxicity to aquatic resources. The analysis of effluent showed the presence of endocrine disrupting chemicals along with genotoxicity compound i.e. Hexadecanoic acid and Octadecenoic acid. Majority of these pollutants are androgenic and mutagenic in nature derived from plant steroid, fatty and resin acids of the plant during pulping processes which were not degraded by bacterial communities during biological treatment of wastewater. Presence of recalcitrant pollutants along with a mixture of heavy metals i.e. Fe (81 ±1.80), Zn (31±1.41), Cu (4.05±0.15), Cd (1.26±0.08), Mn (19±0.68), Ni (6.03±0.33), Cr (4.21±0.07) and Pb (43.61±2.01) beyond the permissible limit contributed elevated BOD, COD and reduced DO. The seed germination test with *Triticum aestivum* and chromosomal aberration test with *Allium cepa* showed the inhibition and disturbed chromosomal segregation during cell division of metaphase and anaphase which showed sticky chromosome, laggard chromosome and polyploidy cells in *A. cepa* treated with pulp paper mill effluent. Hence, this manuscript will be global interest for monitoring of recalcitrant pollutants discharged after biological treatment from pulp paper industry for aquatic pollution prevention.

Keywords: Effluent, Chlorolignins; Heavy metals; Toxicity; Seed germination

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INTRODUCTION

Pulp paper mill effluent is an important source of environmental pollution due to the high content of chlorinated compounds chlorolignins, chlorinated hydrocarbons along with resin acid, tannin acid, phenolics, lignosulphonic acids, various surfactants, plasticizers , biocides, waxes, fatty acids, heavy metals and other complex organic, inorganic compounds (Chandra et al., 2012; Pokhrel et al., 2004). In India there are more than 800 pulp papers industries out of these 45 are large industries that manufacture the writing papers and uses the pulping and bleaching process (CPPRI 2016). In general there is discharged 100-190 m^3 of wastewater per ton of paper production (Petra et al., 2015). This reflects the magnitude of the environmental problem caused due to pulp paper industry in India. In addition, several potentially toxic compounds like dibenzo-p-dioxins and dibenzofurans are unintentionally generated during paper production and processing. Thus, the effluents discharged from these industries are heavily loaded with a mixture of several unknown organic and inorganic compounds. Various workers have reported that pulp paper effluent contains more than 200 organic and 700 inorganic compounds (Lacorte, et al., 2003; Sunito et al., 1988). These compounds increase the toxicity of effluent substances as well as Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD) and Total Dissolved Solids (TDS) of the receiving aquatic resources, which consequently imbalances the aquatic life. Therefore, globally various researcher have reported fish toxicity to receiving aquatic resources (Singh and Chandra, 2019). This revealed to delayed sexual maturation, cellular damage and adverse effects on biochemical parameters in fish due to oxygen depletion and anoxia was created due to mixing of pulp paper mill effluent (Maria et al., 2002). The masculinization, androstenedione and adverse effect on the reproductive system have been observed in western mosquito fish (*Gambusia affinis*) in China. The study has revealed the adverse effects on health, development, and reproduction in both male and female *G. affinis* (Li-Ping et al., 2018). In addition, China and Germany, the study also reported the high COD fraction and toxic effect of PPMWw after secondary treatment (Huang et al., 2014). The chronic toxic effects of PPMWw are also observed through the Microtox test in microbes by estimating the inhibition of bioluminescence of *Vibrio fischeri* (McMartin et al., 2002). Besides, the toxic effect has also been reported on the micro plankton and the benthic organisms which reduced the self-purification capacities of rivers and other aquatic resources (Karrasch et al., 2005). The toxic effect of PPMWw has further reported on the terrestrial ecosystem (Iqbal et al., 2013). In the recent past due to the advancement of analytical facilities, some researchers have detected a complex mixture of organic and inorganic compounds as residual recalcitrant pollutants present in pulp paper mill effluent after secondary treatment (Yadav and Chandra, 2018; Chandra et al., 2018). These compounds not only contribute the toxicity and increase COD, but some are even also carcinogenic, mutagenic and endocrine-disrupting properties along with metabolic constituents which disturb the food chain and adversely affect to human health also (Gustavo et al., 2015; Savant et al., 2006; Sangeeta et al., 2018, USEPA 2012). The discharged pollutants indicated that the operating techniques in the industry are not able to degrade these residual pollutants. Hence, prior to its

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REVIEW ARTICLE OPEN ACCESS

SARS-CoV-2 / COVID-19 and its Transmission, Prevention, Treatment and Control – An Update

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Abstract

Coronavirus Disease 2019 (COVID-19), caused by Severe Acute Respiratory Syndrome Virus-2 (SARS-CoV-2), pandemic has caused huge panic, havoc and global threats worldwide. The origin of this virus has been linked to animals, intermediate host is still to be identified, and studies are being carried out that how it got transmitted to humans and acquired rapid human-to-human transmission. Within a short time period of only 05 months, SARS-CoV-2 has spread to 213 countries, and till 28th May, 2020, nearly 5.8 million confirmed cases have been reported while taking lives of 0.36 million persons. Seeing the current situation of rapid increase in COVID-19 cases daily in many countries, this seems to be the deadliest pandemic after the 1918 Spanish Flu. There is currently no specific effective treatment for COVID-19 and also in absence of vaccine the radical cure of the disease is far away. Researchers are pacing high to design and develop effective vaccines, drugs and therapeutics to counter COVID-19, however such efforts, clinical trials, necessary approvals and then to reach the level of bulk production of many millions of doses may still take much time. Prevention and control of COVID-19 outbreaks requires an evidence-based, multi-factorial and effective mitigation strategy to be adopted. The current review discusses on the research advancements, challenges and opportunities in COVID 19 management with a focus on its transmission, prevention, treatment and control.

Keywords: COVID-19, SARS-CoV-2, transmission, prevention, treatment, control

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INTRODUCTION

A novel coronavirus named as Severe Acute Respiratory Syndrome Virus-2 (SARS-CoV-2), the causative agent of Coronavirus Disease 2019 (COVID-19), emerged in Wuhan, China in December month of the last year (2019). It attained the status of public health emergency of international concern on January 30, 2020, and later on March 11, 2020 was declared as a global pandemic by World Health Organization[1]. The International Committee on Taxonomy of Viruses (ICTV) recognized the virus as SARS-CoV-2 (earlier known as nCoV-2019) and the disease was named as coronavirus disease 2019 (COVID-19) by WHO[2]. The virus has now caused huge panic and very high global threats to the lives of mass population across the globe. The global risk of COVID-19 is increasing progressively for the general public and considered to be very high for the communities with identified risk factors. The virus appears to be highly infectious and has spread rapidly throughout the globe. A meeting was held on this issue on 30th January 2020 under the International Health Regulations (IHR, 2005) and WHO declared the outbreak as Public Health Emergency of International Concern (PHEIC) because it had spread to 18 countries with four countries reporting human-to-human transmission. Currently, nearly 06 million individuals from 213 countries and territories are affected from the SARS-CoV-2 infection with over 0.35 million deaths worldwide[3]. Presently, the USA, Brazil, Russia, Spain, UK, Italy, France, Germany, Turkey and India are among the list of top 10 COVID-19 affected countries, which overload the healthcare system due to high patient burdens needing intensive care and treatment. As on 28th May, 2020, 4,534 deaths out of a total of 1,58,086 confirmed cases of COVID-19 have been reported from India[4].

Moreover, earlier coronaviruses (CoVs) viz. Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) and Middle East Respiratory Syndrome coronavirus (MERS-Cov) also induced lethal outbreaks but the currently ongoing SARS-CoV-2 pandemic is the deadliest among all the zoonotic CoVs. On 20 January 2020, China announced the disease as a second-class infectious disease, but followed prevention and control strategies for the first-class infectious disease, the most risky category of infectious diseases[5]. The COVID-19 was first reported as pneumonia of unknown origin in China and then spread like wildfire to the rest of the world, posing a noteworthy global threat to the public health globally [6, 7]. Presently, there is no effective vaccine or treatment option available for COVID-19, and for this purpose high efforts are continuously being made by researchers from many countries.

In particular, reports showed that 2 % population are healthy carriers of CoVs and these viruses contribute for 5 to 10 % of acute respiratory infections. A total of seven CoVs have been reported to infect human's viz., HCoV-229E, HCoV-NL63, HCoV-HKU1 and HCoV-OC43, capable of causing mild respiratory symptoms alike to that of common cold, while SARS-CoV-2, SARS-CoV and MERS-CoV have been associated with fatal infections[8,9]. SARS-CoV (2003) has previously infected 8096 people with 774 deaths at a case fatality rate of 9.6% around the world, although the rate of survival is higher among the COVID-19 patients but the rate of infection is growing exponentially which may have a larger impact. Even though COVID-19 is spreading rapidly through airborne route, air disinfection of towns and communities was not found to be effective enough in disease control. This review presents current knowledge and advances on SARS-CoV-2 / COVID-19 management and specifically highlighting its transmission, prevention and control strategies.

Characteristics of SARS-CoV-2

Coronaviruses (CoVs) belong to the family *Coronaviridae* in the order of Nidovirales. The SARS-CoV-2 represents crown-like spikes on the envelope of the virus; hence named coronavirus[10]. Coronavirinae consists of four main sub-groupings / genera, Alpha-, Beta-, Gamma- and Delta-coronavirus, and that the first two host human infecting viruses (Human Coronavirus, HCoV): HCoV-229E and HCoV-NL63 (alpha coronavirus) and HCoV-HKU1, HCoV-OC43, Middle East coronavirus respiratory syndrome (MERS-CoV), extreme acute coronavirus respiratory syndrome (SARS-CoV) (beta coronavirus)[11,12]. The CoVs including the SARS-CoV-2 have a non-segmented, single stranded, positive sense RNA genome of around 30 kb[11,13] (Fig. 1). The SARS-CoV-2 is a member of the sub-family *Orthocoronavirinae*

18

Phytoremediation of Industrial Pollutants and Life Cycle Assessment

Ram Chandra, Vineet Kumar, Sonam Tripathi, and Pooja Sharma

CONTENTS

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This certificate is awarded to

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ISME-AMI Mini- Workshop

On *Art of Publication and Communication*

held on December 11, 2018

during 50th International Annual Conference of Association of Microbiologists of India (AMI-2018)

at University of Hyderabad, Hyderabad, Telangana, India

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on

" IPRs in Agricultural Research "

August 30-31, 2017

Jointly Organized by:

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&

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कागज कारखानों से उत्सर्जित विषैले अपशिष्टों का पर्यावरण पर दुष्प्रभाव तथा सुरक्षात्मक निस्तारण की चुनौतियाँ

राम चन्द्रा, विनीत कुमार, सोनम त्रिपाठी एवं पूजा शर्मा

पर्यावरणीय सूक्ष्म जैविकी प्रभाग, पर्यावरण विषविज्ञान समूह
सी.एस.आई.आर.– भारतीय विषविज्ञान अनुसंधान संस्थान
विषविज्ञान भवन, 31, महात्मा गाँधी मार्ग लखनऊ–226001, उत्तर प्रदेश भारत

भारत को कागज के उत्पादन में विश्व के 10 वें सबसे बड़े देश के रूप में जाना जाता है। वर्तमान में हमारे देश में लगभग 618 कागज के कारखाने हैं जिसमें 565 कारखाने स्थाई रूप से तथा 58 कारखाने अस्थाई रूप से कार्यरत हैं। प्रायः 01 टन सफेद कागज बनाने हेतु 100 से 200 घन मीटर ताजे जल की आवश्यकता होती है, जिसके कारण इससे निकलने वाला बहिस्राव बहुत भारी मात्रा में निकलता रहता है। प्रायः लकड़ी को कागज बनाने में कच्चे माल के रूप में प्रयोग किया जाता है उसके मूल संरचना के करीब 40–45 प्रतिशत ही लुग्दी के रूप में सेल्यूलोज के रेशे बच पाते हैं जबकि बाकी लकड़ी के मूल

कोई आँकड़े उपलब्ध हैं।

कागज उत्पादन हेतु मुख्यतः विभिन्न पेड़ों की लकड़ियाँ जैसे–यूकेलिप्टस, पापलस, बाँस तथा चीड़ के अलावा पुनः चक्रित (रिसाईकिल्ड पेपर) पुराने कागज, गन्ने की खोई, या अन्य पौधों के रेशे को कच्चे माल के रूप में उपयोग में लाया जाता है। कागज बनाने की प्रक्रिया प्रायः दो से तीन चरणों में पूरी होती है। पहले चरण में लट्ठे को छोटे–छोटे टुकड़ों में काटकर उसे लुग्दी बनाने हेतु बड़े–बड़े डाइजेशन टैंक में कास्टिक सोडा और सोडियम सल्फाइड के मिश्रण के साथ उबाला जाता है। जिसके फलस्वरूप लकड़ी के विभिन्न अवयव घुल जाते हैं। इन अवयवो

अंतरराष्ट्रीय वैज्ञानिक संगोष्ठी
पर्यावरण प्रदूषण : चुनौतियाँ एवं रणनीतियाँ
सीएसआईआर-भारतीय विषविज्ञान अनुसंधान संस्थान एवं
नगर राजभाषा कार्यान्वयन समिति, (कार्यालय-3), लखनऊ

प्रमाण पत्र

प्रमाणित किया जाता है कि पूजा शर्मा, बी.बी.ए.यू., लखनऊ ने सीएसआईआर-आईआईटीआर में दिनांक 11-13 अक्टूबर, 2017 को पर्यावरण प्रदूषणः चुनौतियाँ एवं रणनीतियाँ विषय पर हिंदी में आयोजित तीन दिवसीय अंतरराष्ट्रीय वैज्ञानिक संगोष्ठी में व्याख्यान प्रस्तुत किया/पोस्टर प्रस्तुत किया/सक्रिय भाग लिया।

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योगेश्वर शुक्ला
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प्रोफेसर आलोक धावन
निदेशक
सीएसआईआर-आईआईटीआर

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&
International Symposium on "Microbes and Biosphere: What's New What's Next"

November 24 - 27, 2016, Gauhati University, Guwahati, Assam, India

Organized by : Department of Botany, Gauhati University
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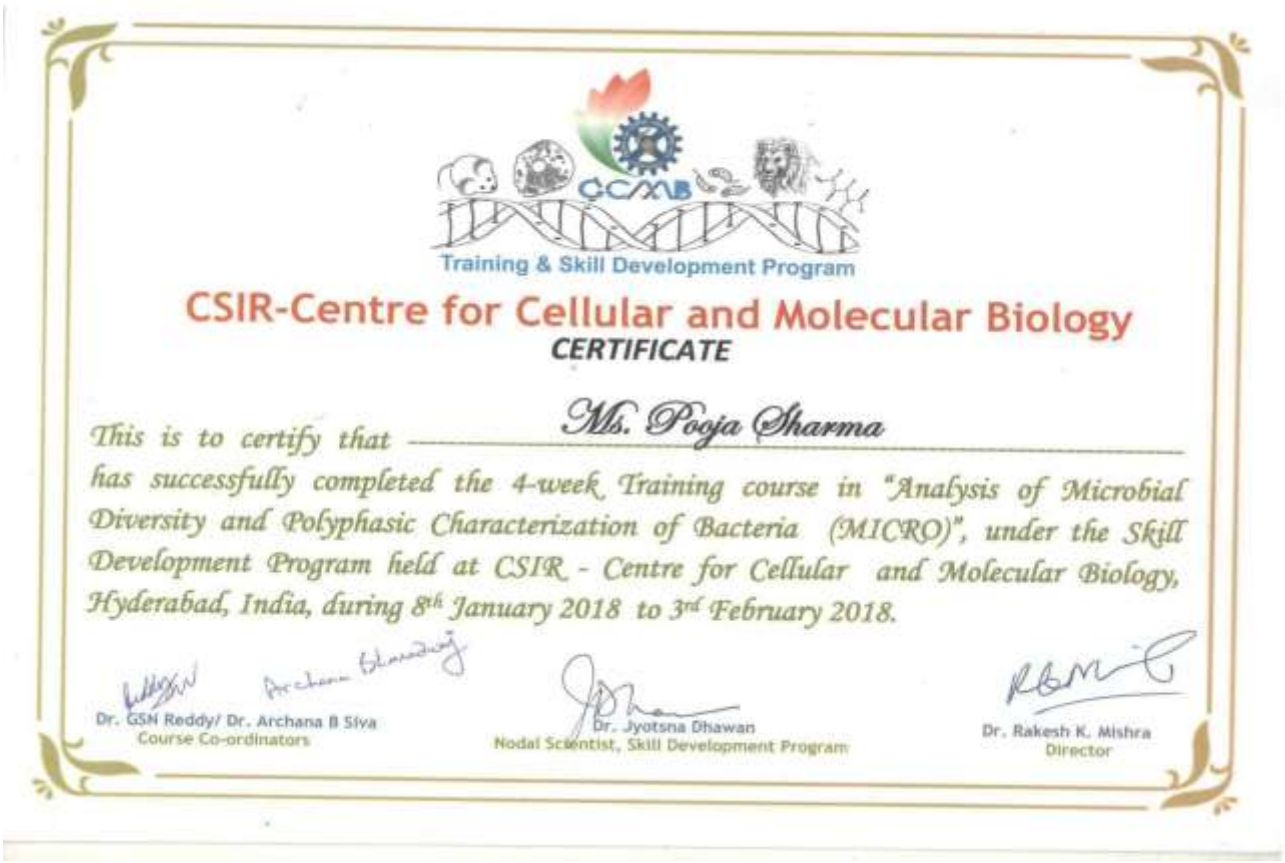

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