

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

SUMMARY

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

(A CENTRAL UNIVERSITY)

LUCKNOW



FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

ENVIRONMENTAL MICROBIOLOGY

SUBMITTED BY

POOJA SHARMA

Enrolment No. 1402/16

UNDER SUPERVISION OF

PROF. RAM CHANDRA

DEPARTMENT OF ENVIRONMENTAL MICROBIOLOGY

SCHOOL FOR ENVIRONMENTAL SCIENCES

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

(A CENTRAL UNIVERSITY)

VIDYA VIHAR, RAE BARELI ROAD,

LUCKNOW- 226025 (U.P.) INDIA

2020

Introduction

The wastewater discharged from pulp paper industry contain a high number of diverse compounds and containing with more than 250 identified chemicals, including resin acids, fatty acids, tannins and sterols. In addition, pollutants are generated during different stages of paper making process, such as wood debarking, pulp washing, and bleaching. Moreover, one of the major sources of water pollution in the pulp and paper manufacturing industries is the bleaching process is generated large number of chemical compounds. However, the paper is a cellulose fibre generated after digestion of wood using alkali which requires bleaching to impart whiteness to the preparation of the better quality of paper from industry. The bleaching agent conventionally used is elemental chlorine, which is reasonably cost-effective but it causes a group of toxic compounds in effluents. The demand for paper is rising sharply, now exceeding 330 million tons of paper per year worldwide. The components in pulp mill wastewaters are often aromatic, including lignin and its chlorinated derivatives. The findings also revealed that mixing effluent from the pulp paper industry not only contributed to phytoplankton and zooplankton toxicity by enhancing BOD, COD and other pollution parameters. But this also induce the growth of faecal coliform bacteria, which is creates as health hazards in living being. Therefore, the objective of my study is as below.

- 1.** Detection of various residual organic pollutant present in various pulp and paper mill industrial waste after secondary treatment
- 2.** Heavy metal accumulation pattern in different part of native and crop plants growing at disposal site of pulp and paper mill waste
- 3.** Investigation of the molecular mechanism of hyperaccumulation plant growing at pulp and paper waste disposal site
- 4.** Profiling of dominant bacterial community growing in pulp and paper mill sludge containing heavy metal and organic pollutant
- 5.** Characterization of potential bacterial community growing in the rhizospheric zone of native plants at the polluted site of pulp paper industry waste
- 6.** Assessment of detoxification and degradation of pulp and paper mill effluent by biostimulation and bioaugmentation process growing at pulp and paper mill contaminated site

Physicochemical analysis of effluent and sludge

The physics-chemical analysis of discharged waste of pulp paper mill wastewater (PPMWw) showed the presence of a high amount of color, TDS (1789 ± 1560), TSS (73 ± 51), COD (19100 ± 17890), BOD (4180 ± 3653), phenolics compound along with nitrogen and phosphorus. Moreover, 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 to aquatic organism in the aquatic ecosystem. However, high COD leads to the toxic state of the PPMWw along with the presence of biologically resistant organic substances. The EC value of the pulp paper industry waste was observed as $1400 \mu\text{hos cm}^{-1}$ which was very high than the EPA guidelines i.e. $1000 \mu\text{hos cm}^{-1}$. Moreover, higher EC might be due to salt and ions content of pulp paper industry waste. 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 sulfate to most plants and aquatic life. Moreover, lignin contents were so high (39000 mg l^{-1}) in pulp paper industry waste which might be the source of the dark color of the effluent. The dark color of pulp paper industry waste adversely affects the lower organisms in the food chain. Furthermore, the significant amount of Fe ($87\text{-}79$), Zn ($34\text{-}22$), Cu (3.28), Cd ($1\text{-}0.36$), Mn ($16\text{-}15$) and Ni ($6\text{-}5 \text{ mg l}^{-1}$) was present in PPMWw which are hazardous to the environment. 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 sulphur. Carboxylic acid (-COOH) and amino (-NH₂) 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 pulp paper industry waste due to the presence of absorption maxima between 250 to 380nm and > 250nm, respectively. Some peaks were also observed after 380 nm which showed the turbidity of the effluent. This study showed that this pulp paper industry waste is not safe for irrigation, human and animals.

Detection and characterization of various residual organic pollutants

To investigate the residual organic pollutants from pulp paper mill effluent sample from different industries i.e. K R pulp paper mill, Century paper mill. Further, the characterization of different organic pollutants present in effluent and sludge by GC-MS analysis of the ethyl acetate extract showed various peaks at different retention times (RT) in mass spectra (m/z). Further, the major pollutants present in pulp paper industry i.e. Nonacosane; Heptacosane; 6-Benzoamido- 3- [2- [1-(phenylmethyl) - 4-piperidinyl] ethyl] -1, 2-benzisoxazole; hexadecanoic acid, 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 mg l⁻¹), COD (19100 mg l⁻¹) and reduced DO. 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. Most predominant fatty acids were Octadecanoic (C18), hexadecanoic (C16) and Propanoic acid (C3) was also detected in PPMWw. Further, the Stearic acid, 3-(octadecyloxy) propyl ester (CAS) was also detected from effluent which has been reported as B and T cell lines toxicity, cancer, neurotoxicity, organ toxicity and irritation. The phenolic compound such as 1-(5-Ethyl-tetrahydrofuran-2-yl)-3, 3-dimethyl-butane-2-one was also found in effluent and sludge after secondary treatment which has been reported as a highly toxic compound. However,

Hexadecanoic acid or palmitic acid was the most prevalent. In addition, the toxicity evaluation of the discharged waste by seed germination and genotoxicity analysis is done by the *Phaseolus mungo* and *Allium cepa*, respectively. Furthermore, germination index 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. In germinating seeds, starch degradation is initiated by α -amylase producing soluble oligosaccharides. These are then hydrolyzed by α -amylase into maltose and glucose providing energy to germinating seeds.

Heavy metals accumulation in different part of native plants

The analysis of discharged pulp paper sludge showed the presence of various heavy metals as complex organo-metallic pollutants after secondary treatment along with other organic compounds. The study has revealed to special attention towards health hazardous of the waste for pollution prevention for aquatic resources. The potential native plants growing on pulp paper sludge altered their Physico-chemical properties by the accumulation of heavy metals in their different parts of tested parts as an evidence of Phytoextraction tools. 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, H₂O₂, 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.

Profiling of dominant bacterial community on contaminated site

To investigate the microbial community from polluted site and their role for remediation of organic pollutants by using metagenomics techniques is done. The focus of residual organic pollutants biodegradation has been done on the use of a unique microorganism community which has the genes of interest. Recent advancements in genomics, transcriptomics and proteomics improve investigations of bacterial communities in degradation of industrial waste. The significance of assessing the biological and structural diversity of the soil directly from the polluted site is that we can define a natural process of bioremediation. Newly, a single molecular real-time (SMRT) DNA sequencing system has been developed by Pacific Biosciences (PacBio) and applied to the microbial community and metagenome analyses. 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. In order to obtain so much information on the functional and genetic structure of soils in this research, it was agreed to use two methods: the technique of NGS (V3–V4 16S rRNA gene region) and the

method of physiological profiling at community level (CLPP). 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 253556 total OTUs, 21102 OTUs with less than 5 read were removed and 4254 OTUs were selected further analysis. 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. In pie chart showing 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. In several environments, as many as 99% of microorganisms cannot be cultivated by standard techniques, and the uncultured fraction contains various organisms that are only remotely related to those that are cultivated. Therefore it is very important to understand exactly the genetic structure and function of microbes at the contaminated site, for a better understanding of the bioremediation pathways and also as a source of inoculum in the management of these areas. Moreover, bacterial community analyses by DNA extraction and amplification method were dominant bacterial communities belonging to the phylum *Proteobacteria* and *Firmicutes*, respectively. The PCR-based approach was successfully used to classify microorganisms which act as pollutant degraders in soil, such as β -*Proteobacteria* naphthalene, salicylate or benzoate degraders. The aim of this research was to determine the functional and structural diversity of bacterial communities in soils with long-term effects of pulp paper industry waste pollution. Moreover, determining the function and availability of bacteria in the industrial waste site is the justification for further research to select the strains which are active in bioremediation, and in future bioremediation processes, such bacteria could be used in the industry for environmental safety.

Identification of plant growth promoting rhizobacteria in rhizospheric zone of *Phragmites communis* for phytoextraction

Further, the identification and 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.

Assessment of detoxification and degradation of pulp and paper mill effluent by biostimulation and bioaugmentation process

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 ($37\pm 1^{\circ}\text{C}$) 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 calcoeticus 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.

Conclusion

This study concluded that PPMWw after secondary treatment contains various residual organic pollutants which showed chromosomal aberration, genotoxicity, cytotoxicity and oxidative damage of DNA in *A. cepa* and *P. mungo* plant. After secondary treatment is most dominant and persistent organic pollutants i.e. Nonacosane, Heptacosane, Hexadecanoic acid, Stearic acid, 3-(octadecyloxy) propyl ester (CAS), Octadecane, 3-ethyl-5-(2-ethylbutyl)-(CAS), benzoic acids and Octadecanoic has been detected by GC-MS analysis. Majority of these compounds has been reported as bacteriostatic and bactericidal effects on the microbial community. In addition, some plant steroids and other detected residual organic pollutants are reported as EDCs compounds. The native plants growing at contaminated site showed high potentiality for Phytoextraction of heavy metals from organometallic complex along with phytoremediation for the Eco restoration of polluted site. A diversity of microbial community from rhizosphere has been also detected as evidence for bacteria assisted phytoremediation. The presence of mutagenic and androgenic compounds as persistent pollutants even after secondary treatment of PPMWw makes the aquatic ecosystem and environment more attentive to tertiary treatment for pollution prevention.