Study of bacterial communities in two step treatment of post methanated distillery effluent by bacteria and constructed wetland plant treatment system

THESIS

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Chapter-10

Summary

Sugarcane-molasses based post methanated distillery effluent (PMDE) is a threat to environment for its safe disposal due to presence of complex polymer containing heterocyclic nitrogenous compounds of aldehyde-amines, various heavy metals, phenolic compounds and plant derived resins and fatty acids. Melanoidin is a major colorant of distillery effluent. It has also been reported that melanoidins have net negative charges; therefore, various heavy metals strongly binds with melanoidins to form large organo-metallic complex molecules. Among the different process available for treatment of spent wash, biomethanation is a popular anaerobic convention process which produces methane to meet a part of the power requirement in distilleries. The effluent after anaerobic treatment (methanogenesis) becomes more viscous and dark due to complexation of organic and inorganic pollutants present in spent wash. The discharged effluent after anaerobic treatment causes environmental problems in aquatic and soil ecosystem which ultimately affect to aquatic flora and fauna. Therefore, adequate treatment of the distillery effluent is warranted prior to its discharge into environment. Hence, the detailed chemical properties of various pollutants present in distillery effluent before and after anaerobic treatment have yet to be elucidated.

The above information has been systematically elaborated in the first chapter of thesis as introduction. Subsequently, second chapter has mentioned objectives of thesis. Further, the review of literature of the topic has been elaborated in chapter three, while chapter four has mentioned the physico-chemical analysis and identification of various persistent organic compounds present in PMDE before and after anaerobic treatment. The result has revealed that DSW showed high BOD (42,000), COD (90,000), TDS (77,776), TS (83,084), chloride (2200), phenol (4.20), sulfate (5760), and phosphate (5.36 mg L^{-1}) levels with acidic pH (4.07), and dark brown color. In addition, several major heavy metals were detected, including Fe (163.947), Mn (4.556), Zn (2.487), and Ni (1.175 mg L^{-1}). Subsequently, the physico-chemical characteristics of PMDE showed high BOD (6500), COD (10,864), TDS (10,764), TS (12,248), chloride (19,993), phenol (3.98), sulfate (4850), and phosphate (5.12 mg L^{-1}) levels with alkaline pH (8.17), and dark brown color. The GC-MS analysis of DSW revealed the presence of toxic organic acids (butanedioic acid bis(TMS)ester; 2-hydroxysocaproic acid; benzenepropanoic acid, α-[(TMS)oxy], TMS ester; vanillylpropionic acid, bis(TMS)), and other recalcitrant organic pollutants (2-furancarboxylic acid, 5-[[(TMS)oxy] methyl], TMS ester; benzoic acid 3-methoxy-[(TMS)oxy], TMS ester; and tricarballylic acid 3TMS), which are listed as endocrine-disrupting chemicals. While, The GC-MS analysis of PMDE revealed the presence of hexadecanoic acid, TMS ester; octadecanoic acid, TMS ester; β–Sitosterol TMS ester; benzeneacetic acid, α,4-bis[(TMS)oxy], TMS ester; benzoic acid, 2,5-bis(TMSoxy)-TMS ester; Stigmasta-5,22-Dien-3-ol (3β, 22E); phenol,4,4'-thiobis[2-(1,1dimethylethyl)-6-methyl. The detected compounds have been listed as potential EDCs by the USEPA. Further, phytotoxicity assay of DSW and PMDE

with *Phaseolus mungo* L. and *Triticum aestivum* revealed the presence of toxic organic compounds.

Due to presence of sucrose and glutamic acid, which abundantly present in sugarcane juice and represent the major composition of molasses-melanoidins, we have selected sucrose and glutamic acid for the degradation study. The sucrose glutamic acids-Maillard reaction products (SGA-MRPs) are predominantly present in PMDE responsible for color of distillery effluent as environmental pollutant due to its recalcitrant nature. The chapter five of thesis has mentioned the characterisation of potential manganese peroxidase (MnP) and laccase producing bacteria capable for degradation of sucrose glutamic acid-maillard reaction products at different nutritional and environmental conditions. The result revealed that twenty four morphologically different aerobic bacterial strains (IITRCS01 to IITRCS024) were isolated by nutrient enrichment technique from distillery sludge by streak plate method. Further, these bacterial strains were screened on the basis of MnP and laccase and melanoidins tolerance activity. Out of 24 bacterial strains, four aerobic bacterial strains IITRCS01, IITRCS06, IITRCS07, and IITRCS11 were showed maxium MnP and laccase producing activity on phenol red amended GPM medium and guiacol amended B&K agar medium plated. Further, these bacterial strains were also showed higher melanoidins (3500 mg L^{-1}) tolerance activity. On the basis of 16S rRNA gene sequence analysis potential MnP and laccase producing bacterial strains IITRC01, IITRC06, IITRC06, and IITR11, were identified as *Klebsiella pneumoniae* (KU726953), *Salmonella enterica* (KU726954), *Enterobacter aerogenes* (KU726955), *Enterobacter cloaceae* (KU726957), respectively. The identified aerobic bacterial consortium consisting *K. pneumoniae* (KU726953), *S. enterica* (KU726954), *E. aerogenes* (KU726955), *E.* cloaceae (KU726957) showed optimum production of MnP and laccase at 120 and 144 h of growth, respectively. The potential bacterial consortium showed decolourisation of SGA-MRPs up to 70% in presence of glucose (1%), peptone (0.1%) at optimum pH (8.1), temperature (37 °C) and shaking speed (180 rpm) within 192 h of incubation. The reduction of colour of Maillard product correlated with shifting of absorption peaks in UV–Vis spectrophotometry analysis. UV-Vis spectrophotometric analysis of SGA-MRPs showed many absorption peaks between 200 and 450 nm and their absorption maximum peak was noted at 250 nm in spectrophotometric detection. Further, the changing of functional group in FT-IR data showed appearance of new peaks and GC–MS analysis of degraded sample revealed the depolymerisation of complex MRPs. The toxicity evaluation using seed of *P. mungo* L. showed reduction of toxicity of MRPs after bacterial treatment. Thus, this consortium might be useful for decolourisation of industrial wastewater containing high concentration of melanoidins.

Based on potentiality of isolated bacterial strains the developed bacterial consortium was used to assess degradability of molasses-melanoidins. The study was conducted for optimal decolourisation and degradation of molasses-melanoidins under optimised environmental and nutritional conditions which constitute as chapter six of my thesis. The result revealed that molasses-melanoidin extracted from PMDE with mixture of isopropanol and PMDE (1:1 v/v) showed presence of Mn (8.20), Cr (2.97), Zn (16.61), Cu (2.55), Fe (373.95), Pb (2.59) and Ni

(4.18 mg L^{-1}) along with mixture of other organic compounds which have endocrine-disrupting chemicals (EDCs) properties as per USEPA. The aerobic bacterial consortium consisting with four bacterial strains *K. pneumoniae* (KU726953), *S. enterica* (KU726954), *E. aerogenes* (KU726955), and *E. cloacae* (KU726957) isolated from distillery sludge sample to degrade synthetic SGA-MRPs was used in this study. A consortium of aerobic bacteria comprising *K. pneumoniae* (KU321273), *S. enteric* (KU726954), *E. aerogenes* (KU726955), and *E. cloacae* (KU726957) in ratio of 2:1:2:2 showed the optimum decolourisation of molasses-melanoidins up to 81% through co-metabolism in presence of glucose (1.0%) and peptone (0.2%) as a carbon and nitrogen source, respectively. The absorption spectrum scanning by UV-Visible spectrophotometer between 200-700 nm revealed reductions of absorption spectrum of organic compounds present in bacterial degraded sample of melanoidins in range of 200-450 nm compared to control. The degradation and decolourisation of melanoidins by bacterial consortium was noted by induction of manganese peroxidase and laccase activities in sample supernatant. Further, the TLC and HPLC analysis of bacterial decolourised melanoidins also showed degradation and reduction of absorption peak at (295nm), respectively. Furthermore, FT-IR and GC-MS analysis also showed the change of functional group and disappearance of ion peaks. This indicated the degradation and depolymerisation of melanoidins and cleavage of C═C, C=O and C≡N conjugated bonds which resulted in reduction of colour. The metabolic analysis also showed the disappearance of some organic compounds and generation of new metabolites. Further the seed germination test using *P. mungo* L. showed toxicity reduction in decolorized effluent. Thus, the result revealed that the developed bacterial consortium could be used to scale up the decolourisation, degradation and detoxification process of PMDE for industrial application.

The nature of complex pollutants at distillery waste contaminated sites and the potential for microbial strains to grow such environment are still unknown. A study was conducted to reveal the endemic bacterial communities growing in specific environments that might be responsible for amelioration of these pollutants, eventually leading to biological succession and bioremediation for eco-restoration of polluted sites. Therefore, the chapter seven of thesis has mentioned the dominant bacterial communities and metabolic products of spent wash and post methanated distillery sludge during *in*-situ bioremediation. The result revealed the presence of toxic organic acids (butanedioic acid bis(TMS)ester; 2-hydroxysocaproic acid; benzenepropanoic acid, α-[(TMS)oxy], TMS ester; vanillylpropionic acid, bis(TMS)), and other recalcitrant organic pollutants (2-furancarboxylic acid, 5-[[(TMS)oxy] methyl], TMS ester; benzoic acid 3-methoxy--[(TMS)oxy], TMS ester; and tricarballylic acid 3TMS) in spent wash, which are listed as potential EDCs. Subsequently, the dominant autochthonous bacterial communities were investigated by the RFLP method using a metagenomic approach to reveal the microbial niche in this polluted environment. Bacterial community analysis by RFLP revealed that *Bacillus* and *Stenotrophomonas* were dominant autochthonous bacterial communities belonging to the phylum *Firmicutes* and *γ-Proteobacteria,* respectively grown in distillery spent wash. The presence of *Bacillus* and *Stenotrophomonas* species in highly acidic environments indicated its broad range

adaptation. Further, phytotoxicity assay of DSW with *P. mungo* L. and *T. aestivum* revealed that *T. aestivum* was more sensitive than *P. mungo* L. in the seed germination test. Moreover, GC-MS analysis of distillery sludge and leachate both showed 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, 1-phenyl-1-propanol, 5-methyl-2-(1-methylethyl) cyclohexanol, and 2-ethylthio-10-hydroxy-9-methoxy-1,4 anthraquinone as major androgenic and mutagenic organic pollutants along with heavy metals (mg kg^{-1}): Fe (2403), Zn (210.15), Mn (126.30, Cu (73.62), Cr (21.825), Pb (16.33) and Ni (13.425). In a simultaneous analysis of bacterial communities using the RFLP method the dominance of *Bacillus* sp. followed by *Enterococcus* sp. as autochthonous bacterial communities growing in this extremely toxic environment was shown, indicating a primary community for bioremediation. A toxicity evaluation showed a reduction of toxicity in degraded samples of sludge and leachate, confirming the role of autochthonous bacterial communities in the bioremediation of distillery waste *in*-situ. These findings indicated that these autochthonous bacterial communities were pioneer taxa for *in*-situ remediation of hazardous distillery waste during ecological succession. The results of this study may be useful for monitoring and toxicity assessment of sugarcane molasses-based distillery waste at disposal sites.

Since, the phytoremediation of complex industrial waste by native plants is an emerging green technology for eco-restoration of polluted site. Hence, before construction of wetland plant treatment system some potential native plants have been assessed for phytoextraction of heavy metals from stabilised post methanated distillery sludge. Therefore, in chapter eight of thesis has showed in detail result of phytoextraction of heavy metals by twenty four native plants species (weeds and grasses) (i.e. *Dhatura stramonium, Achyranthes* sp., *Kalanchoe pinnata, Trichosanthes dioica, Parthenium hysterophorous, Cannabis sativa, Amaranthus spinosus* L. *Croton bonplandianum, Solanum nigrum, Ricinus communis, Setaria viridis, Blumea lacera, Argemone mexicana, Saccharum munja, Cynodon dactylon, Pennisetum purpureum, Chenopodium album, Rumex dentatus, Tinospora cordifolia, Calotropis procera,* and *Basella alba).* These plants were collected based on dominant species luxuriantly growing on disposed distillery sludge. These native plants species were uprooted with associated sludge samples 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. 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 USEPA. However, the phytoextraction potential of growing

native weeds and grasses 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. Anatomical observations through TEM in the root of various potential native plants showed apparent formation of multi-nucleolus, multi-vacuoles and deposited metal granules in the cellular components of plants. This indicated the variable adaptive characteristics of these plants growing at a hazardous waste polluted site. Hence, these native plants may be used as a tool for *in*-situ phytoremediation and eco-restoration of industrial waste contaminated site.

Further, as per objective of study, the bacterial degraded PMDE was integrated with designed horizontal subsurface flow-constructed wetland (HSSF-CW) plant treatment system. The PMDE degradation was assessed at different treatment stages. Therefore, the chapter nine of thesis has mentioned the dominant rhizospheric bacterial communities of *Phragmites communis* characterised through metagenomic approach to reveal the microbial community structure of rhizobacteria of *P. communis* during PMDE degradation and decolourisation in HSSF-CW plant treatment process. It was also interesting to note that the bacterial pretreatment (for 168 h) followed by phytoremediation with *P. communis* wetland plant rhizosphere (for 168 h) has also improved the physico-chemical properties of effluent resulting reduction in colour, BOD, COD, TS, TDS, TSS, Total nitrogen, chloride, sulfate, and phenol up to 84.03, 91.81, 92.84, 94.09, 94.93, 91.66, 92.19, 88.83, and 88.51%, respectively. Moreover, the constructed wetland plant rhizosphere treated PMDE sample has also shown reduction of various heavy metals such as Fe, Zn, Ni, Mn, Pb, Cu, and Cd up to 97.16, 89.01, 86.66, 82.84, 98.57, 99.75, 0.00%. The absorption spectrum scanning by UV-Visible spectrophotometer between 200-700 nm revealed reductions of absorption spectrum of organic compounds present in bacterial degraded and constructed wetland rhizosphere treated sample of PMDE in range of 200-450 nm compared to control. The HPLC analysis confirmed the reduction of peak lead to colour reduction. Further, the GC-MS analysis has shown that most of the compounds detected in untreated PMDE were diminished from bacterial and wetland plant treated samples. The disappearance of compounds from bacteria and wetland plant treated sample could be related with degradation and color removal from PMDE. The sludges from the rhizopheric zone of *P. communis* were collected and the microbial communities were analyzed by Illumina high-throughput sequencing of V3-V4 hypervariable reagions of 16S rDNA. The most abundant phylum was *Proteobacteria* (50%) followed by *Bacteriodetes* (33%), *Firmicutes* (5%), *Gemmatimonadetes* (2%), *Choloroflexi* (2%), and *Tenericutes* (2%). At the class level, the most predominant bacteria affiliated with *Gammaproteobacteria* (36%) followed by *Sphingobacteria* (19%), *Aplhaproteobacteria* (11%), *Flavobacteria* (9%), Actinobacteria (6%), *Bacilli* (5%), *Cytophagia* (5%), *Betaproteobacteria* (3%), *Mollicutes* (2%), and *Deltaproteobacteria* (1%). At the genus level, the Illumina MiSeq analysis showed the presence of *Rheinheimera* (21%), *Sphingobacterium* (17%), Idiomarina

(8%). *Acidothermus* (4%), *Pseudomonas* (2%), *Flavobacterium* (2%), Uncultured bacterium (2%), *Parapedobacter* (2%), *Alcanivorax* (2%), Uncultured (4%), *Bacillus* (3%), *Acholeplasma* (2%), *Hyphomonas* (1%), and *Aquamicrobium* (1%), as dominant rhizospheric bacterial communities of *P. communis* plant which were beneficial for the degradation of toxic constituents present in the PMDE. This is the first time to identify the microbial communities of *P. communis* during PMDE treatment in HSSF-CW plant treatment system by Illumina high-throughput sequencing. The decolourization of PMDE is combined phenomenon by *P. communis* and rhizosphere bacteria. Bacteria helped in the degradation of toxic wastes and plant accumulates the heavy metals in its tissues. Both of them played an important role in the bioremediation of environmental pollutants. Constructed wetland can be an effective technology for bioremediation of organic and inorganic pollutants in two step treatment of PMDE.

Based on work, six original research papers have been published in high impact journal and one research paper is under consideration. While eleven conference papers presented in national and international conferences.