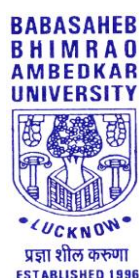


**Study the Degradation Mechanism of Distillery Waste by
Potential Bacterial Consortium for its safe Disposal and
Re-cycling in Environment**

SUMMARY

SUBMITTED TO

**BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
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Study the Degradation Mechanism of Distillery waste by Potential Bacterial Consortium for its safe Disposal and re-cycling in Environment

Summary

In India a wide network of distilleries has been established one of the most polluting agro - based industries producing enormous amounts of complex distillery effluent. The distillery effluent contains organic and inorganic pollutants which have been reported to be beneficial and have a beneficial effect at low concentration on crop yields. Melanoidin is known as a major distillery wastewater colorant. Chemically melanoidins are complex due to polymerization of amino-carbonyl complex due to polymerization of amino acid and carbohydrate at elevated temperature. It has also been stated that melanoidins have a net negative charge; thus to form large organo-metallic complex molecules. Consequently, different heavy metals strongly bind to melanoidins in environment. Biomethanation is a common convention anaerobic method that produces methane to meet out a part of the power requirement in distilleries among the various processes available for the treatment of spent wash. Owing to the complexity of organic and inorganic pollutants present in waste, the effluent becomes more pungent and black during anaerobic treatment called methanogenesis produces PMDE (Post methanated Distillery Effluent). After anaerobic treatment, the discharged effluent causes environmental issues in marine and surface water that eventually impact aquatic flora and fauna. Therefore, prior to its discharge into the atmosphere, proper care of the distillery effluent is warranted. Hence, before and after anaerobic treatment, the precise chemical properties of different contaminants present in the distillery effluent are still to be investigated.

In the first chapter of the study, the above information was systematically revealed as an introduction. Subsequently, the objectives of the study were stated in the second chapter. Furthermore the literature review of the subject was expanded in Chapter Three, while the investigation of physicochemical properties and characterization of the different persistent organic compounds present in distillery effluent has been addressed in Chapter Four. Anaerobically treated molasses-based distilleries spent wash which is also known as post

methanated distillery effluent (PMDE) is a major source of terrestrial and aquatic pollution due to the mixture of the various unknown organometallic compounds in discharged waste. The nature of pollutants present in effluent has to be characterized prior to the evaluation of their fate in the environment at various trophic levels. The absorption maxima (λ_{max}) were obtained at 295 nm of pollutants present in spent wash which indicated that melanoidins are a major colorant along with other organic compounds. Abundantly identified compounds with GC-MS in effluent were found: 1,2,4,5-tetrahydro-2-methyl-3H-2-benzazepine-3-one, 6-(Trifluoromethyl)-1H-imidazole [4,5-c] pyridine, 4,6-di-*t*-butyl-1H, 3H-thieno[3,4-c] thiophene, butanoic acid, trimethylsilyl ester, silane, (dodecyloxy)trimethylsilyl ester, 4-styryl [2,2] paracyclophane, 4-(*p*-cumylphenoxy) phthalonitrile, hexadecanoic acid, trimethylsilyl ester, butyl ester, and squalene. Some of these compounds are known with mutagenic and androgenic properties. The toxicity test of post-methanated distillery effluent (PMDE) showed direct toxic effects on catfish (*Heteropneustes fossilis*) even at 1% (v/v) concentration and caused degeneration of primary and secondary lamellae of the gill and the epithelial layer. Further, higher concentrations between 5 and 10% of PMDE showed loss of primary and secondary lamellae of gills, and liver catalase activity was also increased dramatically in *Heteropneustes fossilis* fish, indicating the generation of oxidative free radicals. Similarly, inhibition of α -amylase activities in germinating *Zea mays L.* (Maize) seeds were also noted at 10% PMDE. This study confirmed the environmental toxicity of effluent with freshwater fish and *Zea mays* seeds. The chapter five of thesis has mentioned the Isolation & characterization of potential bacterial community responsible for bio-stimulation and bio-augmentation process during detoxification of Distillery waste. The analysis showed that sixteen morphologically different bacterial strains (ACS1-ACS16) were isolated in Biostimulation process and twenty morphologically different aerobic bacterial strains (IITRCS01-IITRCS020) were isolated by nutrient enrichment technique from distillery sludge by streak plate method. The tolerance behaviour of MnP, laccase, and Lip was also tested on these bacterial strains. Total out of 36 overall bacterial strains, two aerobic bacterial strains (AS1 and AS7) in biostimulation and (IITRCS10 - IITRCS12) were showed maximum MnP and laccase producing activity on phenol red amended GPM medium and guaiacol amended B&K agar medium plated. Further, these bacterial strains were also showed higher

melanoidins (3500 mg L^{-1}) tolerance activity. The potential bacterial consortium showed 40% decolourisation in biostimulation sample and up to 70% in bioaugmented consortium in presence of glucose (1%), peptone (0.1%) at optimum pH (8.1), temperature ($37 \text{ }^\circ\text{C}$) 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 distillery effluent 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 organometallic compounds. The toxicity evaluation using seed of *P. mungo* L. showed reduction of toxicity of effluent after bacterial treatment in Bioaugmentation and biostimulation process. Thus, this consortium might be useful for decolourisation of industrial wastewater containing high concentration of melanoidins.

In the Chapter six, the created bacterial consortium was employed for the assessment of TDS reduced wastewater, based on potential isolated bacterial strains. The study was conducted for optimal decolourisation and degradation of wastewater after TDS reduction under optimised environmental and nutritional conditions which constitute as chapter six of my thesis. Thus, constructed wetland systems planted with potential wetland plant have been optimized as an effective device at pilot scale. Combination of wetland treatment process with bacterial degradation at reduced TDS of PMDE offers an excellent system for removal of colour from PMDE and further reduction of BOD, COD for safe disposal wherein the increased microbial population has been indicated for the degradation of organic chemical contaminated soil. In this objective the role of rhizosphere bacteria for a degradation of industrial wastewater is reported wetland plants growing at polluted site. The Optimization of TDS and potential growth of many microphytes has been observed in distillery effluent contaminated site. 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 after TDS reduction in range of 200-450 nm compared to control. The degradation and decolourisation of TDS reduced PMDE 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 TDS reduced PMDE for industrial application.

In Chapter 7, this is the first report of the microbial community present in sugarcane molasses-based distillery sludge and their relationships with the organo-metallic pollutants present. Samples were collected from the discharge point, 1 and 2 km meters downstream (D1, D2, and D3, respectively) and their physico-chemical properties characterised. The complex physico-chemical characteristics of distillery sludge have significant impact on the diversity and relative abundance of the bacterial community. Using QIIME, taxonomic assignment for the V3 and V4 hypervariable regions of 16S rRNA was performed. The phyla *Fermicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Euryarchaeota*, *Tenericutes* and *Patescibacteria* were the predominant bacteria in samples collected from all three sites. *Spirochaetes*, *Sinergistetes* and *Cloacimonetes* were only detected in samples from site D1. Shannon, Simpson, Chao1, and Observed-species indices indicated that site D1 (10.18, 0.0013, 36706.55 and 45653.84, respectively) has higher bacterial diversity and richness than D2 (6.66, 0.0001, 25987.71 and 49655.89, respectively) and D3 (8.31, 0.002, 30345.53 and 30654.88, respectively), suggesting the organo-metallic pollutants provided the stressor to favour the survival of microbial community that can biodegradation and detoxification them in the distillery sludge. This finding provides important information for toxicity assessment on bacterial communities in distillery industry and selection of bioremediation candidates. These bacteria are able to tolerate extreme condition and survive in the hostile environment of the distillery sludge. When the environmental pressure was reduced through mixing and dilution of the receiving water, the competitive advantage was removed and was reflected in

reduced bacterial diversity. Many of the compounds identified in the sludge samples are hazardous to the environment and human health, the current treatment process is not sufficiently effective to reduce the level of organic and metal pollutants to an acceptable level, additional steps are required to improve the treatment of the distillery discharge. Bacteria detected in the most pollution site, D1, are potential candidates to remediate the organic and metal pollutants present in distillery sludge and should be further explored as part of the solution to improve the chemical quality of the discharge. 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. The study investigated the heavy metal accumulation by herbaceous traditional medicinal and food plants (*Achyranthus aspera*, *Amaranthus viridis*, *Basella alba*, *Sesbania bispinosa*, *Pedaliium murex*, and *Momordica doica*) grown on complex distillery waste containing a mixture of organometallic compounds. The results revealed bioaccumulation of Mn, Cd, Fe, Cr, Cu, As, Se, Mo, and Co in their root, shoot and leaves in levels higher than the surrounding sludge. *A. aspera* was noted as root accumulator for Mn (16.95 ± 0.1 mg/kg-1), Zn (30.12 ± 0.3 mg/kg--1), Fe (240.4 ± 0.1 mg/kg- -1), Co (3.19 ± 0.2 mg/kg--1), while Se (4.07 ± 0.3 mg/kg--1), Mo (4.36 ± 0.2 mg/kg- -1), was accumulated selectively in the shoot of the plant. Similarly, *S. bispinosa*, *P. murex*, and *M. doica* were found as root accumulators for Mn, Fe, and Ni. *A. viridis* accumulated Cd, Zn, and Cu in the shoot and leaves of the plant. In another study the phytoremediation potential of *Ricinus communis* L. was evaluated for heavy metals remediation via rhizospheric bacteria for distillery waste detoxification and management. Results showed that the wastewater containing high metals and other Physico-chemical parameters, which cause environmental pollution and aquatic toxicity. The identified bacterial strain produced siderophores, indole acetic acid, and ligninolytic enzymes resulted in provides nutrients and help in the mineralization of metals in plants during stress conditions as plant growth promoting rhizobacteria (PGPR). The translocation factor and Bioconcentration factor of all the metals were < 1 , which reveals that these metals are stabilized in the root portion of *Ricinus*

communis L. The ability of *Ricinus communis* L. to grow well in metals containing distillery waste suggests that it can be used for the removal of heavy metals. The use of *Ricinus communis* L. is an eco-friendly tool to deal with heavy metal pollution and improvement in productivity of agricultural systems.

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 total dissolved solid (TDS) was optimized with using various coagulants such as aluminum sulfate, aluminum chloride, sodium aluminate, ferric chloride, ferric sulfate, ferrous sulphate, lime etc, at variable concentration (10%, 20%, 40%,50% & 100%) of PMDE. But the ferric chloride showed optimum decolorization in presence of 0.34% within 6-8hrs. The (80%) decolorization period was also influence with 97% TDS reduction with mixing of ferric chloride in PMDE. Fast and prolong mixing enhanced the decolorization process. Further the efficiency of decolorization and TDS reduction was evaluated with rapid mixing variable pH and the coagulant dosage which determined the hydrolysis species for effective treatment of PMDE. The decolorized supernatant of PMDE was separated from optimized dose of coagulant. The optimum TDS reduction resulted 80% supernatant and 20% colloidal precipitated material. The PMDE supernatant was assessed for bacterial growth in presence of variable nitrogen (0.5%, 1.0%, 1.5% & 2.0%) and carbon (0.5%, 1.0%, 1.5% & 2.0%) to evaluate the bacterial growth in biostimulation for reduction of colour from supernatant of PMDE. The optimum decolorization of PMDE was noted 96% in biostimulation process while the reduction of BOD & COD was noted 98% & 97% respectively. Further, the biomass separated supernatant was (96%). The bacterial consortium of four identified bacteria were inoculated in 30% PMDE collected after extended aeration in presence of 1% glucose as additional carbon source and 0.5% peptone as nitrogen in 800 L capacity bioreactor with working capacity of 500 L at pH 8.0 ± 0.5 , 120 ± 2 rpm and 37 ± 2 °C temp. This was incubated in tank up to 96 hrs. This showed 98% bacterial decolorization along with reduction of various pollution parameters (BOD, COD, TS, TDS, sulphate and heavy metals) up to 70-99%. Subsequently, the biomass was separated and supernatant was injected (passed through) with constructed wetland treatment system, upstream flow rate 2 L min⁻¹ versus 1.5 L min⁻¹ downstream having mixed vegetation of *Phragmites cummunis* & *Typha angustata*. The

constructed wetland channel was in zigzag manner to maximize the retention time in minimum area having three vertical layers of gravel and concrete from upper to deeper side for each layer. The dimension of constructed wetland treatment system was 60x72x1000 cm (width x depth x length) having 0.88 % slopes. Retention time of wetland treatment was 10 days. The reduction of BOD and COD was observed up to 97 and 98%. While the colour was reduced up to 98-99%, sulphate and heavy metals was also reduced up to 95%.the colour reduction was also validated with HPLC analysis which showed the comparative reduction of colouring peak. The Physico-chemical analysis of decolorized PMDE after biofilters with constructed wetland treatment was found environmentally safe with seed germination test using *Phaseolus mungo* and ferti-irrigation to *Vicia fabae* (L.) as terrestrial test model approved by OECD,1995; USEPA, 1980.

Therefore, novel technique for biological decolorization and detoxification of post methanated distillery effluent (PMDE) in combined bacterial and constructed wetland plant treatment system for environmental safety is novel because of following reasons;

1. This is new process of post methanated distillery effluent decolorization with integration of bacterial treatment followed by constructed wetland for pollution prevention and environmental safety.
2. There is reduction of colour (up to 98%) along with COD (97%), BOD (95%), TS (95%), phenol (93%), sulphate (90%), nitrogen (82%) and heavy metals at alkaline pH in 70-80% post methanated distillery effluent (PMDE).
3. The decolorization of distillery effluent and reduction of pollution parameters is achieved up to environmental safe limit. This can be used for ferti-irrigation in India.
4. There is a production of useful biomass of wetland plants during the wastewater treatment process which can be used for various small scale industries.
5. This is cost effective technique which will be applicable at industrial scale.

As per objective for development of feasible technology for sustainable development a patent has been granted as innovative technology entitled “**Decolorization and Detoxification of Distillery Wastewater with Combined Use of Bacteria and Constructed Wetland Treatment (2021101010)**”.

Conclusion

This study concluded that Distillery Effluent 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 Biostimulation and Bioaugmentation process 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 and degraded various treatment process. 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 distillery effluent makes the aquatic ecosystem and environment more attentive to tertiary treatment for pollution prevention. The proposed technology is very helpful for our nation/country and Environment for sustainable development.

Based on work, fifteen (23) original research papers have been published in high impact journal and three research papers is under consideration. While seven conference papers presented in national and international conferences. Two book chapters are published in firm of reputed publisher. Two awards and two Patent related to my PhD Topic are also published.