Study on the bacterial degradation and detoxification of distillery wastewater pollutants for environmental safety

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

LUCKNOW

BABASAHEB
BHIMRAO
AMBEDKAR
UNIVERSITY

LUCKNOW

प्रज्ञा शील करुणा

ESTABLISHED 1996

FOR THE DEGREE OF

Doctor of Philosophy

IN

ENVIRONMENTAL MICROBIOLOGY

Submitted By

Pankaj Kumar Chowdhary

M.Sc. Biotechnology

(Enrolment no. 635/12)

Under the Supervision of

Dr. Ram Naresh Bharagava

Assistant Professor

DEPARTMENT OF ENVIRONMENTAL MICROBIOLOGY

SCHOOL FOR ENVIRONMENTAL SCIENCES

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

(A Central University, NAAC Accreditation 'A' Grade)

VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW-226 025

UTTAR PRADESH, INDIA

2018

Dedicated to
My Beloved Family

CERTIFICATE

This is to certify that the thesis entitled **"Study on the bacterial degradation and detoxification of distillery wastewater pollutants for environmental safety"** submitted by **Mr. PANKAJ KUMAR CHOWDHARY** 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.) Regulations –1999 as amended in 2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Date: **Supervisor**

Head of Department

STUDENT DECLARATION

This is to certify that the material embodies in the present Ph.D. work entitled **"Study on the bacterial degradation and detoxification of distillery wastewater pollutants for environmental safety"** is original research work done by me. It has not been submitted in part or full for any other diploma or degree in any other University. In this thesis, matter written, data presented and plagiarism, if any, is the sole responsibility of the student Mr. Pankaj Kumar Chowdhary. If any allegations/query/question arises regarding the thesis, I, Mr. Pankaj Kumar Chowdhary, will be solely responsible and answerable.

(Mr. Pankaj Kumar Chowdhary)
Enroll. No. 635/12
***S/o* Mr. Ram Chandra**
Village- Muradiha, Post- Hanumanganj Bazaar
Thana- Ruddhuli
District- Basti
Uttar Pradesh- 272150, India
E-mail- pankaj161089@gmail.com

Acknowledgements

"Success comes only through high ambition, good guidance, strong will, determining and continuous effort."

I am highly indebted to my supervisor ***Dr. RAM NARESH BHARAGAVA*** *for their guidance and constant supervision as well as not only for research work, but also for giving me so many wonderful opportunities related scientific output. Sir has always made himself available to clarify my doubts despite his busy schedules and I consider it as a great opportunity to do my doctoral programme under his guidance and to learn from his research expertise. Thank you Sir, for all your help and support. I am also deeply grateful to* ***Mrs. Bharagava*** *for her warm hospitality.*

I would like to express my gratitude towards all of faculty members of Department of Environmental Microbiology ***Prof. Ram Chandra, Prof. Rajesh Kumar, Prof. Naveen Kumar Arora, Dr. Jay Shankar Singh,*** *and* ***Dr. Vinay Singh Baghel*** *for their kind co-operation support and encouragement, which help me in completion of this research work .*

I am especially grateful and thank to ***Dr. Abhay Raj*** *and* ***Dr. D. K. Patel*** *Scientist CSIR-Indian Institute of Toxicology Research (IITR), Lucknow for LC-MS/MS and metal analysis and also for his valuable suggestion and guidance of this research work.*

I gratefully acknowledge the funding sources that made my research work possible. I was funded by ***University Grant Commission (UGC),*** *Delhi, India*

A special thank of mine goes to my labmates ***Ms. Sujata, Ms. Sandhya Mishra, Ms. Surabhi Zainith,*** *and* ***Mr. Ashutosh Yadav*** *who helped me out in completing the research work, where they all exchanged their own interesting ideas, thoughts and made this possible to complete my research work with all accurate information.*

It's my fortune to gratefully acknowledge the support of my Seniors and friends, ***Mr. Vishvas Hare, Mr. Siddharth Bouddh, Dr. Atul Upadhyay, Dr. Amit Kumar*** *for their support and generous care throughout the research tenure. They*

were always beside me during the happy and hard moments to push me and motivate me.

I extend a warm thanks to all non-teaching staff of Department of Environmental Microbiology and everyone involved directly or indirectly with my this research work.

Finally, but by no means least, thanks go to my father ***(Mr. Ram Chandra),*** *mother* ***(Mrs. Malti Devi),*** *brother* ***(Umesh)*** *and younger sisters* ***(Pinky, Preeti, and Pallavi)*** *for their unbelievable support, selfless love, and care. I would never be able to pay back the love and affection showered upon by my family. They are the most important people in my world and I dedicate this thesis to them.*

Finally, I owe it all to Almighty God for granting me the wisdom, health and strength to undertake this research task and enabling me to its completion.

"Be thankful for each new challenge because it will build your strength and character."

[PANKAJ CHOWDHARY]

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND SYMBOLS

ACN	**:**	Acetonitrile
AC	**:**	Activated Carbon
α	**:**	Alpha
APS	**:**	Ammonium Per Sulphate
ASBR	**:**	Anaerobic Sequencing Batch Reactor
ANOVA	**:**	Analysis of Variance
~	**:**	Approximately
As	**:**	Arsenic
APX	**:**	Ascorbate Peroxidase
ASC	**:**	Ascorbic acid
AAS	**:**	Atomic Absorption Spectroscopy
BCF	**:**	Bioconcentration Factor
BOD	**:**	Biological Oxygen Demand
BMDS	**:**	Biomethanated Distillery Spent Wash
Cd	**:**	Cadmium
CO_2	**:**	Carbon dioxide
CAT	**:**	Catalase
cm	**:**	Centimeter
CTAB	**:**	Cetyl Trimethylammonium Bromide
COD	**:**	Chemical Oxygen Demand
Cr	**:**	Chromium
Co	**:**	Cobalt
CSTR	**:**	Continuous Stirred Tank Reactors
°C	**:**	Degree Celsius
DNA	**:**	Deoxyribonucleic acid
DO	**:**	Dissolved Oxygen
DIs	**:**	Distillery Industries
DWW	**:**	Distillery Wastewater
DMRT	**:**	Duncan's Multiple Range Tests
ETP	**:**	Effluent Treatment Plant
EC	**:**	Electrical Conductivity
ELM	**:**	Emulsion Liquid Membrane

EDCs	**:**	Endocrine Disrupting Compounds
$\triangle H°$	**:**	Enthalpy
$\triangle S°$	**:**	Entropy
FTIR	**:**	Fourier Transform Infrared Spectroscopy
GC-MS	**:**	Gas Chromatography Mass Spectrophotometry
$\triangle G°$	**:**	Gibb's free energy
GSH	**:**	Glutathione
GPx	**:**	Glutathione Peroxidase
g	**:**	Gram
g/L	**:**	Gram per litre
HPLC	**:**	High Pressure Liquid Chromatography
H_2O_2	**:**	Hydrogen peroxide
ICP	**:**	Inductively Coupled Plasma
IUPAC	**:**	International Union of Pure and Applied Chemistry
Fe	**:**	Iron
K	**:**	Kelvin
kDa	**:**	Kilo Dalton
kJ/mol	**:**	Kilo joule per mole
Pb	**:**	Lead
LHCPs	**:**	Light Harvesting Chlorophyll Proteins
LiP	**:**	Lignin peroxidase
LC-MS/MS	**:**	Liquid Chromatography-Mass Spectrometry
MRPs	**:**	Maillard Reaction Products
MG	**:**	Malachite green
MIP	**:**	Manganese Independent Peroxidase
MnP	**:**	Manganese peroxidase
MB	**:**	Methylene blue
µg	**:**	Microgram
µL	**:**	Microlitre
µm	**:**	Micrometer
mL	**:**	Milliliter
µ	**:**	Miu

mm	**:**	Millimeter
MSM	**:**	Mineral Salt Medium
min	**:**	Minute
M	**:**	Molarity
nm	**:**	Nanometer
NEQS	**:**	National Environmental Quality Standards
NIST	**:**	National Institute of Standards and Technology
-	**:**	Negative
NGM	**:**	Nematode Growth Medium
Ni	**:**	Nickel
OD	**:**	Optical Density
%	**:**	Percentage
PPA	**:**	Phenylpyruvic acid
PHB	**:**	Polyhydroxybutyrate
+	**:**	Positive
PTEs	**:**	Potentially Toxic Elements
QA	**:**	Quality Assurance
QC	**:**	Quality Control
ROS	**:**	Reactive Oxygen Species
RO	**:**	Reverse Osmosis
rpm	**:**	Revolution Per Minute
RNA	**:**	Ribonucleic acid
SEM	**:**	Scanning Electron Microscope
SDS-PAGE	**:**	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
SOD	**:**	Superoxide Dismutase
TBA	**:**	Thiobarbituric acid
TDS	**:**	Total Dissolved Solids
TS	**:**	Total Solids
TSS	**:**	Total Suspended Solids
TF	**:**	Translocation Factor
TEM	**:**	Transmission Electron Microscopy
TCA	**:**	Trichloroacetic acid
UF-MBR	**:**	Ultra Filtration Membrane Bioreactors

UV	**:**	Ultra Violet
U/mL	**:**	Unit per milliliter
UASB	**:**	Up-Flow Anaerobic Sludge Blanket
H_2O	**:**	Water
w/v	**:**	Weight over volume
Zn	**:**	Zinc

CHEMICALS & GLASSWARES

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

1. Media from **Hi-media and Merck Millipore, Mumbai**

2. Glassware from **Borosil, Mumbai**

3. Chemical Reagents from **Merck, Mumbai**

4. Plastic wares from **Merck Millipore, Mumbai**

Chapter 1
General Introduction

Chapter 1

General Introduction

In the 21st century a major human society faces serious environmental issues like climate change, pollution and extinction of plants and animals. These all problems are interrelated and have originated from human detonation, uncontrolled use natural resources, urbanization and industrialization. Distillery industries are, belonging to one of the world's leading premium drinks companies, has turned to reed bed treatment systems to remove copper traces from the effluent produced. Distillery industries (DIs) are the key contributors to the world's economy, but these industries also considered as one of the major source of environmental pollution worldwide. In India, there are ~ 319 distilleries with annual production of 3.25×10^9 L of alcohol and 40.4×10^{10} L of wastewater (Chandra et al., 2012; Uppal, 2004). The global production of bioethanol in 2007 was 50 billion liters whereas, in 2008, it reached ~ 60 billion liters representing almost 4% of the world's gasoline consumption (Mussatto et al., 2010; Balat and Balat, 2009). DIs generate a huge volume of dark colored wastewater characterized by its dark brown color, acidic pH (5.4-4.5), high BOD (40,000-50,000 mg/L), COD (80,000-100,000 mg/L), total dissolved solids (TDS), total solids (TS), total suspended solid (TSS), with high nitrogen, potassium, phosphates, calcium, and sulfate content. The high BOD and COD values of DWW are mainly due to the presence of high organic content such as proteins, reduced sugars, polysaccharides, lignin, melanoidins, and waxes along with a complex mixture of recalcitrant organic pollutants (Chowdhary et al., 2017).

In distillery wastewater (DWW), melanoidins are the major coloring pollutants causing serious environmental problems and health threats in human and animals (Tamanna and Mahmood, 2015; Saranraj and Dtella, 2014). Melanoidins are recalcitrant compounds of sugar and amino acids, formed during the processing of sugar cane juice in sugar factories and molasses in distillery industries (Arimi et al., 2015; Saranraj and Dtella, 2014;

Onyango et al., 2012; Wang et al., 2011; Agarwal et al., 2010; Mohana et al., 2009; Plavsic et al., 2006). However, the characteristics of DWW are largely depended on the raw materials, chemicals used and processes adopted by DIs (Arimi et al., 2015; Satyawali and Balkrishnan, 2008). Arimi et al. (2014) described in details the various steps of wastewater generation in DIs utilizing sugarcane molasses as raw material for alcohol production (Fig. 1.1) (Arimi et al., 2014).

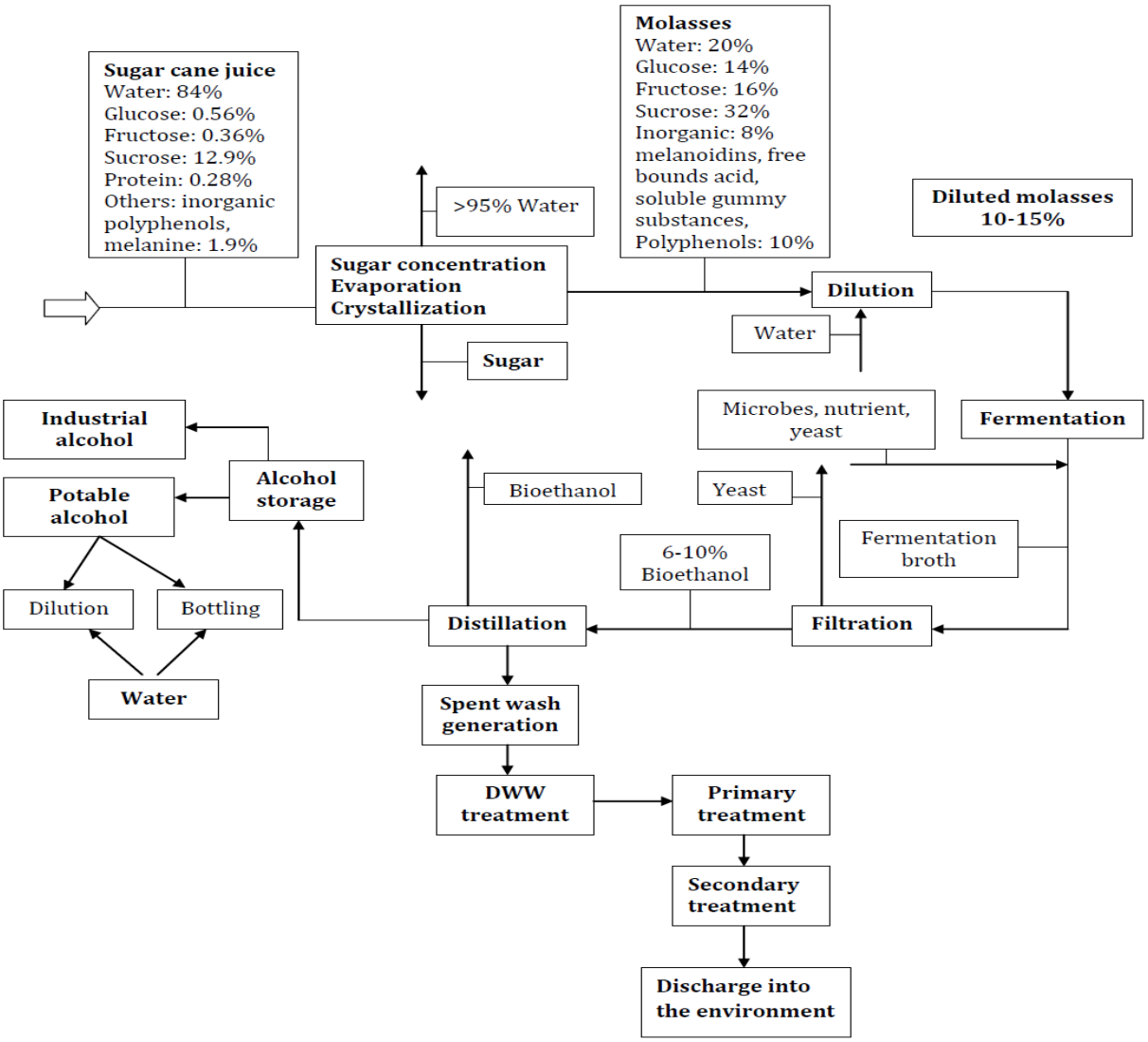


Figure. 1.1 Steps during alcohol production and wastewater generation in distillery industry (Modified from Arimi et al., 2014).

Besides sugarcane molasses, DIs also use grains, grapes, sugarcane juice, and barley malt etc. for alcohol production, which mainly accomplished into four steps such as feed

preparation, fermentation, distillation, and packaging (Satyawali and Balkrishnan, 2008; Skerratt, 2004; Berg, 2004; Tano and Buzato, 2003).

When untreated/partially treated DWW discharged into the environment, it causes serious ecotoxicological and health threats. In water bodies, it reduces the penetration power of sun light causing a reduction in photosynthetic activity and depletion in dissolved oxygen (DO) content (Saranraj and Dtella, 2014; Chandra et al., 2008a) whereas in soil system, it reduces the fertility of agricultural land. Due to these environmental and health threats, DWW should be adequately treated for the degradation and detoxification of organic and inorganic pollutants prior to its final discharge into the environment. Various physico-chemical methods reported for the treatment of DWW are not feasible to meet the discharge standards set by various environmental protection agencies. On other hands, the biological methods like aerobic/anaerobic treatment processes were found somewhat capable to reduce BOD/COD load of DWW, but the substantial concentration of organic and inorganic pollutants and dark color left behind require further treatment (Safari et al., 2013).

But, the detail information about the recalcitrant organic and inorganic compounds remained in distillery wastewater even after the secondary treatment process, their toxicological effects in environment as well as suitable approach/method for their biodegradation and detoxification for environmental safety is not completely known. Thus, there is an urgent need to develop the cost effective and environmental friendly biological techniques for the effective degradation and detoxification of distillery wastewater pollutants for safe disposal into the environment.

As it is well known that effluent generated from distillery wastewater (DWW) causes adverse effect on environment. In order to assess its environmental effect on soil, water and crop plants. A significant number of studies have been done on the treatment of DWW

treatment. This wastewater is a major source of aquatic pollution due to high levels of Maillard complex of sugar and amino acid (melanoidins), color, biological oxygen demand (BOD), chemical oxygen demand (COD), phenolics, total solid (TS), sulphate, nitrogen, and heavy metals such as manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni), lead (Pb) (Chowdhary et al., 2018; Chandra et al., 2008; Kumar and Chandra, 2004; Chandra 2003). Bharagava and Chandra (2010) have reported the biodegradation (upto 70%) of melanoidins, a major color containing compound present in DWW by an aerobic bacterial consortium comprising *Bacillus licheniformis* (DQ79010), *Bacillus* sp. (DQ779011) and *Alcaligenes* sp. (DQ779012) and also characterized the metabolic products. Further, Yadav and Chandra (2013) have detected a variety of persistent organic compounds from biomethanated distillery spent wash (BMDS) and also reported the decolorization of BMDS up to 76% within 192 h along with the degradation of persistent organic compounds by specific consortia comprising *Proteus mirabilis* (FJ581028), *Bacillus* sp. (FJ581030), *Roultella planticola* (GU329705) and *Enterobacter sakazakii* (FJ581031).

The physico-chemical methods suggested for treatment of spent wash are not efficient for decolorization (Jain et al., 2002). However, fate and extent of toxicity of anaerobically treated DWW still remains unknown in the environment (Chandra et al., 2004). Rani et al. (2013) found that biological methods are more efficient for the removal of recalcitrant compound in compare to conventional method. *Candida tropicalis* RG-9 a thermotolerant yeast capable for decolorization (75% at 45 °C) of recalcitrant organic pollutants from DWW (Tiwari et al., 2012).

In a study by Ramana et al. (2002), the germination percent in five crops decreased with increase in concentration of the wastewater. The germination was inhibited in all the five crops studied with concentration exceeding 50%. At the same time, organic wastes contained in DWW are valuable source of plant nutrients especially N, P, K and organic

substrates if properly utilized (Pathak et al., 1999). For instance, DWW in combination with bioamendments such as farmyard manure, rice husk and Brassica residues was used to improve the properties of sodic soil (Kaushik et al., 2005).

The spent wash is highly acidic in nature and has a variety of organic and inorganic compounds such as melanoidins, phenolics and metal sulphides that are mainly responsible for the dark brown colored molasses spent wash (MSW). An average molasses based distillery generates 15 L of spent wash L^{-1} of alcohol produced (Beltran et al., 2001). Melanoidins are dark brown to black colored natural condensation product of sugar and amino acids, recalcitrant compounds (Plavsic et al., 2006). Biological methods like anaerobic digestion treatment reduce BOD load of the spent wash, but substantial amount of organic components and dark brown color left behind requires secondary treatment (Gadre et al., 1986). Internationally, Gonzalez et al. (2000) have isolated and characterized the different organic fractions of DWW from different stages of biological treatment process.

Accordingly, the method for removal of melanoidins before discharging into the environment is necessary. Currently, the decolorization of melanoidins in wastewater is based mainly on physical and chemical methods such as ozonation, flocculation, chemical coagulation, precipitation, activated carbon adsorption and advanced oxidation of the wastewater (Inanc et al., 1999; Pena et al., 2003). Hence, this study was aims to isolation, characterization and development of potential bacterial strains consortia capable for the decolorization/detoxification of DWW pollutants and reduce or remove the wastewater toxicity for the environmental safety. To complete this aim, this research work has been divided into Six (06) objectives.

Objective first (01) of this work comprise sample collection, isolation, screening and characterization of ligninolytic enzyme producing bacterial strains capable for the

degradation of DWW pollutants. Firstly, wastewater and sludge sample were collected in pre-sterilized container (capacity 10 lit.) and conical flasks (Capacity 2 lit.) from the outlet of Effluent Treatment Plant (ETP) of distillery industry located in Unnao Uttar Pradesh, India. Further, nine (09) morphologically distinct bacterial strains (DS, DS1, DS2, DS3, DS4, DS5, DS6, DS7, and DS8) were isolated on nutrient agar plate and also by nutrient enrichment technique from distillery wastes. In second step, these isolated bacterial strains were screened on the basis of growth on melanoidins amended agar plate and manganese peroxidase (MnP) activity on GPYM agar plate amended with melanoidins. Finally, out of nine four bacterial strains (DS1, DS3, DS4, and DS5) were selected as most potential for DWW pollutants decolorization/degradation. Further, out four consortia were developed by the potential strains (DS3, DS4, and DS5) for decolorization/degradation of DWW.

Objective second (02) of this work includes development of consortia by the selected potential bacterial strains for the decolorization of DWW pollutants. In this experiments, initially bacterial strains were checked for compatibility among selected bacterial strains. Compatibility among bacterial strains is most important for the efficient bacterial growth as well as decolorization/degradation experiments. Further, the equal volume (1 mL) of each bacterial strain (48 h grown culture), containing cell density 3.9×10^4 cells mL^{-1} were be used for the preparation of different bacterial combination is develop the most effective bacterial consortium. The axenic culture (single strain) DS3, DS4, and DS5 decolorized DWW only 53.31%, 63.26%, and 49.69%, respectively. Subsequently these bacterial strains were used for the development of bacterial consortia to achieve effective degradation or degradation of distillery wastewater pollutants. Further, developed bacterial consortia were optimized for various environmental parameters such as pH and temperature. In addition, nutritional parameters such as carbon source (glucose, sucrose, lactose, maltose and starch 0.5%) and nitrogen source (yeast extract, peptone, urea,

ammonium sulphate, and sodium nitrate 0.1%) each were optimized for the optimum decolorization/detoxification of distillery wastewater pollutants.

Objective third (03) of this work includes characterization of potential bacterial strains on the basis of shape/morphology, biochemical identification, and 16S rDNA gene sequence analysis, these selected potential bacterial strains i.e. DS3, DS4, and DS4 were identified as *Staphylococcus saprophyticus, Bacillus megaterium* sp., and *Alcaligenaceae* strain with accession no. MF182113, MF967441, and MF182114, respectively. Further, these identified bacterial strains were checked for the ligninolytic enzyme laccase and manganese peroxidase (MnP). The SDS-PAGE of laccase and MnP has yield band 54 kDa for laccase, which ranges from 50-97 kDa and MnP 43 kDa, which ranges from 37-46 kDa reported in various report.

Objective four (04) of this work comprised the degradation and detoxification of distillery wastes pollutants by the developed potential bacterial consortia. The axenic culture (single strain) DS3, DS4, and DS5 decolorized DWW only 53.31%, 63.26%, and 49.69%, respectively. Whereas, consortium (mixed culture) DS3+DS4+DS5 has decolorized DWW 76.12% at optimized condition. In addition, the scanning electron microscope (SEM) analysis has done of identified bacterial strains before and after its use in distillery wastewater treatment process. The aim of SEM analysis was to identify the structural alterations in bacterial cell shape occurs during the degradation of distillery wastewater pollutants.

Objective five (05) of this work includes the characterization of distillery wastewater pollutants and their metabolites before and after bacterial degradation process. In this section

first of all complete physico-chemical analysis of DWW before and after bacterial treatment has done and reported. Result revealed that distillery wastewater before bacterial

treatment was dark brown in color, alkaline (7.71±0.18), and carrying high BOD (11985.67± 387.51), COD (29860.67±317.87), TDS (10147±262.24), TS (33610±361.32), TSS (21639± 245.84), Phosphate (1184.66±25.00), and chloride (613.33±16.04) with Potential Toxic Elements (PTEs) such as Cu (1.38 ± 0.05), Cd (0.42±0.03), Fe (434.95 ±46.63), Mn (34.36 ± 0.65), Ni (0.18 ± 0.01), Pb (0.26 ± 0.02), and Zn (6.14±0.31). Whereas after bacterial treatment the pH (6.99), color was transformed into light yellow and other parameters were also considerably reduced such as BOD, COD, TDS, TS, TSS, phosphate, and chloride up to 70.13, 81.85, 80.27, 89.90, 90.24, 65.10, and 20.86% respectively. The selected bacterial strains were also found effective to reduce PTEs concentration. In addition, various analytical techniques were used such as Fourier transform infrared spectroscopy (FTIR), and liquid chromatography-mass spectrometry (LC-MS/MS). The characterization and identification of pollutants (organic compounds) by the spectrophotometrically of untreated and bacterial treated has shown the various compound such as Hence, on the basis of FT-IR and LC-MS/MS analysis, the untreated DWW was found to contain a number of compounds having different functional groups. The compounds detected in control and bacterial treated samples were characterized as $[M+K^+]$ aduct of maltose, $[M+Na^+]$ aduct of maltose, Benzyl-3,4-ethylenedioxypyrrol-2,5-dicarboxylate, Petunidine-(6-coummaryl)-3-glucoside, Paeonidine-(6 acetyl)-3-glucoside, Diethyl-3,4-ethylenedioxypyrrole-2,5-dicarboxylate (270), Palmitic acid, 1-(tert-Butyl)-2-methylpyrrole-1,2-dicarboxylate, 1-(p-tolysulfonyl) pyrrole, 2-(Trichloroacetyl) pyrrole, 3-heptyl-5-methyl-2,3H-furanone ($C_{12}H_{22}O_2$), Trans-2-Tridecenal, Dihydroxyconiferyl alcohol ($C_{10}H_{14}O_3$) (182), 2-nitroacetophenone ($C_8H_7NO_3$) (165), Anhydrohexose from $[M-H]^-$, 2,6-dimethoxyphenol ($C_8H_{10}O_3$) (154), 4-vinyl-2-methoxyphenol ($C_9H_{10}O_2$) (150), p-chloroanisol (C_7H_7ClO), 4-methyl guaicol, N-methyl indane (135), 5-(hydroxymethyl)-2- furfural ($C_6H_6O_3$) (126), 2-methoxyphenol ($C_7H_8O_2$), Indole, 5-

methyl-2-furancarboxaldehyde ($C_6H_6O_2$), 2- methylhexane (C_7H_{16}), Furfuryl alcohol ($C_5H_5O_2$) (98), Butenoic acid ($C_4H_6O_2$) (86), 3-hydroxypropanal, Pyrroline (C_4H_7N) and acetic acid ($C_2H_4O_2$), respectively.

Objective six (06) was the final objective of this work and includes toxicity assessment of distillery wastes (wastewater and sludge) before and after bacterial treatment. In this section, first of all to investigate the toxic effects of heavy metals on biochemical parameters in wheat (*Triticum aestivum* L.) and mustard (*Brassica juncea* L.) plants growing at distillery wastewater contaminated sites. This study examined contaminated sites i.e. distillery wastewater/sludge contains high concentration of undesirable heavy metals (Co, Cr, Ni, Mn, Zn, Fe, Pb etc.) and physico-chemical parameters (pH, EC, organic matter, moisture, and chloride) beyond the standard/permissible limit. It can be also concluded that wheat and mustard plants growing at distillery wastewater contaminated site have high metals accumulation potential in different parts i.e. root, shoot, and leaves. This high metal accumulation in plants may directly or indirectly hamper various metabolic activities as well as oxidative damage by altering the structure of enzymes, transporters or regulatory enzymes owing to their strong affinity as ligends to sulfhydryl and carboxylic groups. The biochemical analysis of the selected plant i.e., wheat and mustard revealed increased lipid peroxidation, non- enzymatic and enzymatic antioxidant activities such as MDA, H_2O_2, ASC, SOD, APX, CAT and GPX. In addition, DWW showed the presence of many compounds having different functional groups, but after bacterial treatment, most of compounds get diminished and the toxicity of DWW was reduced significantly. The nile red staining of *Caenorhabditis elegans* exposed to untreated and bacteria treated DWW revealed that worms exposed to untreated DWW showed sharp reduction in total fat content having more profound effects, suggesting the

diminished Nicotinic acetylcholine receptors (nAchR) signalling as compare to treated DWW.

Thus, this study concluded that the inadequate disposal of untreated DWW can cause transfer of toxic substances into the environment and receiving water bodies and disturbs the flora and fauna. Hence, overall work found that distillery wastewater is more toxic and prior to final disposal into open environment or in green belt from the industries the wastewater must be treated (physico-chemically or biologically) to reduce its toxicity parameters within the standard limit set by various environmental protection agencies. The overall study of this thesis is divided into eleven (11) chapter. Further, to know the background information or state of art, each part of this thesis has been reviewed in details and all information is elaborated and discussed in review of literature chapter (chapter 2) with following objectives:

Objective(s)

1. **Isolation, screening and characterization of ligninolytic enzyme producing bacteria capable for the degradation of distillery wastewater pollutants.**
    - Collection of DWW sample.
    - Isolation purification of distillery wastewater degrading bacteria.
    - Screening of isolated bacterial strains.
    - Morphological Characterization.
2. **Development of a bacterial consortium for the degradation and detoxification of distillery wastewater pollutants.**
    - Compatibility test of isolated bacterial strains.
    - Development of consortia by the isolated bacterial strains.
    - Distillery wastewater decolorization by axenic culture.
    - Optimization of nutritional and environmental parameters.

3. **Characterization and identification of distillery wastewater pollutants degrading bacteria.**
    - Biochemical Characterization of isolated bacterial strains.
    - Molecular characterization of bacterial isolates.
    - Detection and characterization of ligninolytic enzymes (laccase and MnP).
4. **Degradation and detoxification of distillery wastewater pollutants by the developed bacterial consortium.**
    - Degradation and detoxification of DWW pollutants by isolated bacterial strains in axenic and mixed culture conditions.
    - Physico-chemical analysis of untreated and treated DWW for reduction in pollution parameters.
5. **Characterization of distillery wastewater pollutants and their metabolites before and after bacterial degradation process, respectively by GC-MS/ LC-MS-MS analysis.**
    - Detection and characterization of DWW pollutants and their metabolites.

    (i) IR and LC-MS/MS analysis.
6. **Toxicity assessment of distillery wastewater pollutants before and after bacterial degradation and detoxification process for environmental safety.**
    - Translocation of toxic metals and their impact on biochemical parameters in Wheat and Mustard plants growing at distillery and tannery wastewater contaminated site.
    - Toxicity evaluation of distillery wastewater pollutants and their metabolites after bacterial treatment by using *Caenorhabditis elegans* as terrestrial test models.

Chapter 2
Review of Literature

Chapter 2

2.1. Distillery wastewater pollutants

In developing countries, including India management of wastes is a thrust need for research, because these generated wastes arises many environmental problem. It is well known that nature and composition of wastewater varies from industries to industries. Distilleries industry is one of the most alarming dangerous that faces living being including human.

The major color contributing pollutants present in DWW are melanoidins, an amino-carbonyl polymer, produced during the processing of sugarcane juice in sugar industries and molasses in DIs (Fig. 2.1). Melanoidins are produced by a series of non-enzymatic chemical reactions known as Maillard reactions and products produced as Maillard Reaction Products (MRPs). Melanoidins are the mixture of low and high molecular weight compounds ranging from 40 - 40000 kDa (Chandra et al., 2008a). The elemental composition, structure as well as the molecular weight of MRPs is largely depends on the nature and molar concentration of reacting molecules and reaction conditions i.e. pH, temperature, and reaction time etc. (Silvan et al., 2006; Chandra et al., 2008a). The size of MRPs may vary from small molecules to very large polymers (Wang et al., 2011; Wagner et al., 2002). Various authors have reported that low and high molecular weight MRPs isolated from dry heated glucose-glycine systems (125 °C, 2 h) and aqueous sugar-lysine model systems (121 °C, 1h, pH 9.0) have genotoxic and cytotoxic effects on cells at higher concentration (Glosl et al., 2004; Jing and Kitts, 2000). Besides MRPs, a variety of mutagenic, carcinogenic, cytotoxic and endocrine disrupting chemicals are also reported in DWW (Table 2.1) (Dixit et al., 2015; Yadav and Chandra, 2012; Alam et al., 2010).

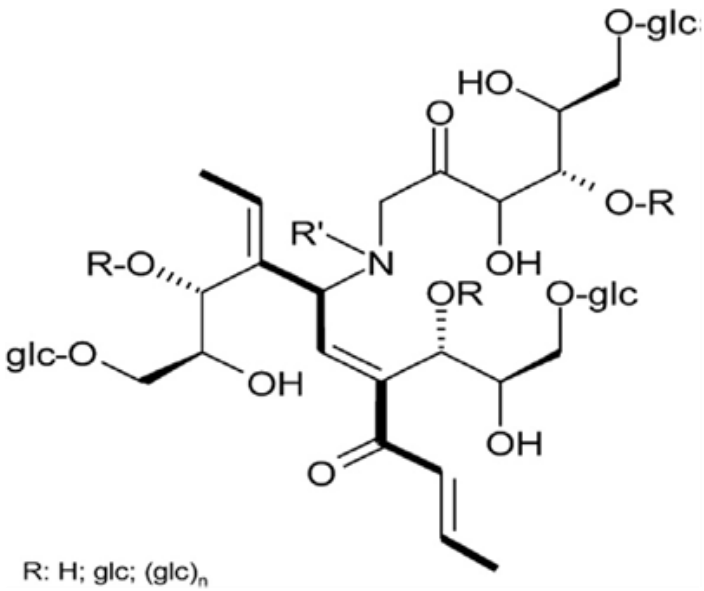


Figure 2.1. Basic structure of melanoidin (Adapted from Cammerer et al., 2002).

Endocrine disrupting chemicals (EDCs) are the chemical agents, which interferes the synthesis, secretion, transport, binding, or elimination of natural hormones in human and animal body that play a key role in various physiological and cellular functions such as homeostasis, reproduction, development and behavioural activities (Kavlock et al., 1996). There are a number of organic compounds in DWW, which have been identified as EDCs such as di-butyl phthalate, di-n-octyl phthalate, butanedioic acid and 2-hydroxysocaproic acid etc. (Table 2.1) (Chandra and Kumar, 2017b; Yadav and Chandra, 2012). These EDCs can alter the regulation of various hormonal activities, which play a significant role in metabolism, sexual development, hormones production and their utilization in growth, stress response, gender behaviour, and reproduction processes (Kabir et al., 2016; Somm et al., 2009). Phthalates have been well documented to induce the lipid peroxidation, oxidative stress, and interference with insulin receptor, altered glucose tolerance induction and reduced glucose oxidation. These also undergo a rapid transformation process in normal environment rather than in abiotic environment (Kabir et al., 2016). Therefore, there is an urgent need for awareness and critical research on EDCs present in industrial wastewaters.

Table 2.1: Organic pollutants identified by various researchers in distillery wastewater

S. No.	Compound Name
1.	3-Amino-2-oxazolidinone
2.	Cyclopropylmethanol acetate
3.	4-Pyridinecarboxlic acid
4.	2-Ethylpyridine
5.	3-(2-Pyridyl)-1-propanol
6.	n-Methyl-2-nitro-3-pyridinamine
7.	3-Ethylpyridine
8.	Nicotinic acid, propyl ester
9.	Isonicotinyl formaldoxime
10.	3-Octadecene
11.	Phthalic acid
12.	DI-N-octyl phthalate
13.	Phthalic acid, butyl-4-octyl ester
14.	Dibutyl phthalate
15.	n-Hexadecanoic acid
16.	1-Eicosanol
17.	13-Tetradecen-1-O-acetate
18.	5,5-Dimethyl hexane
19.	Hexadecamethyl octasiloxane
20.	Benzyl butyl phthalate
21.	1-Hexacosanol
22.	Phthalic acid, dodecyl octyl ester
23.	1,2-Benzenedicarboxylic acid
24.	Phenol
25.	Methylbenzene (toluene)
26.	Butenoic acid
27.	Furfuryl alcohol
28.	2-Hydroxymethylfuran
29..	2-Methoxyphenol (guaiacol)
30.	Methylphenol
31.	Methylbenzaldehyde
32.	Indole
33.	2,6-Dimethoxyphenol (syringol)
34.	1-Hexadecanol
35.	Palmitic acid
36.	Methylindole
37.	2-Ethyl-5-methylfuran
38.	Hydroxypropanone
39.	1,2,3- triethoxy-5-methyl benzene
40.	3,4,5-trimethoxy phenol
41.	2-phenyl ethanol
42.	4,4-dimethyl- 3-(3-oxobutyl)cyclohex-2-enone
43.	2, 2’-bifuran

Adopted from (Fagier et al., 2015; Chandra et al., 2012; Yadav and Chandra, 2012; Bharagava and Chandra, 2010; Wu and Zhou, 2010; Gonzalez et al., 2002)

2.2. Detection and characterization of DWW pollutants by available analytical techniques

DWW contains a number of organic and inorganic pollutants produced during the alcohol production processes in DIs, which can be detected, characterized and identified by using various analytical techniques such as high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS/MS), infrared spectroscopy (IR), protonic nuclear magnetic resonance (^{1}H NMR), fast atom bombardment-mass spectrometry (FAB-MS), matrix assisted lesser desorption ionization-time of flight (MALDI-TOF), and atomic absorption spectroscopy (AAS) and inductively coupled plasma mass spectrometry (ICP-MS) etc. (Table 2.2).

Table 2.2: Various analytical techniques used for the detection and characterization of distillery wastewater pollutants

Distillery wastewater pollutants	**Analytical techniques**	**References**
Organic pollutants	HPLC, GC-MS, Ion-pair RP-HPLC, CEC UV-detection, HPLC differential refractometry detection, HPLC involving derivatization HPAEC coupled electrochemical, and/or DAD, FAB-MS, ESI coupled HPLC and EC, MALDI-TOF, LC-MS, LC-MS with ESI, NBT, ELISA, Ion-pair RP-HPLC, CEC UV-detection, Ion-exchange chromatography, FAB-MS, Colorimetric and fluorimetric methods, FAST, HPLC with UV and fluorescence detection, HPLC-DAD, UV, IR spectrometry, MALDI-TOF mass spectrometry.	Chandra and Kumar, 2017b; Wu and Zhou, 2010; Chandra et al., 2008a; Silvan et al., 2006
Inorganic pollutants	AAS, ICP, Ion chromatography, Flame atomic absorption spectroscopy (FAAS)	Hamza et al., 2017; Chandra and Kumar, 2017b; Chandra et al., 2008b

The HPLC can be used for the separation, identification as well as quantification of organic pollutants present in a complex industrial wastewater (Bharagava et al., 2009). The GC-MS is being used for the characterization and identification of low molecular weight compounds (volatile compounds) from industrial wastewaters, which are soluble in organic solvents such as dichloromethane, ethyl acetate, diethyl ether, methanol, acetone, n-hexane etc. (Chandra and Kumar, 2017b). LC-MS/MS technique is used for the characterization and identification of high molecular weight compounds, which are not soluble in organic solvents, but soluble in water (Chandra et al., 2012; Bharagava and Chandra, 2010a). Infrared (IR) spectroscopy can be used for the identification of functional groups such as alcoholic (-OH), -C-H, ketonic (=C=O), aldehydic (-CHO), carboxylic (-COOH), carbon carbon double bond (-C=C-) and an asymmetric -NO_2 group etc., respectively in the form of stretching frequencies. On the other hand, the ^{1}H NMR showed the presence and position of protons in organic pollutants (Chandra et al., 2012). FAB-MS can also be used for the detection and characterization of organic pollutants from wastewaters and this method is very simple as the samples are directly introduced into the ion source. But, the drawback of this technique is that it can't be used for the detection and characterization of many organic pollutants at a time and thus, this technique has been replaced by electro-spray ionization (ESI) technique that offers the advantage of a very soft ionization. MALDI-TOF technique is used for the detection and characterization of proteinaceous compounds. On the other hand, the AAS and ICP-MS are used for the detection and quantification of metallic (Cu, Cr, Zn, Fe, Ni, Mn, Pb, Hg, As etc.) and non-metallic pollutants from industrial wastewaters (Chandra et al., 2008a; Chandra et al., 2008b).

2.3. Ecotoxicological and health hazards of DWW pollutants

DWW contains a high concentration of recalcitrant organic pollutants generated during the processing of sugarcane juice in sugar industries and alcohol production in DIs. DWW

also contains natural color contributing compounds such as polyphenols, caramels, melanoidins and alkaline degradation products of hexoses (ADPH) etc. (Arimi et al., 2014; Dai and Mumper, 2010). These polyphenolic compounds have antioxidant, anti-microbial, anti-carcinogenic, free radical scavenging and metal chelating properties (Silvan et al., 2006; Borrelli et al., 2003). Phenolic compounds are also reported to react with proteins during beer storage and form high molecular weight compounds and hazes (Siqueira et al., 2011; Dai and Mumper, 2010; Jimoh et al., 2008).

The presence of polyphenols in DWW is largely depends on the source of molasses and sugar content in feed flow (Bustamante et al., 2005; Jimenez et al., 2004; Martin et al., 2003). Polyphenols are categorized into three broad classes: phenolic acids, flavonoids, and tannins. The phenolic compounds detected in molasses based DWW includes benzoic acid and its derivatives (e.g., gallic acid), cinnamic acid and its derivatives (e.g., coumaric acid, caffeic acid, chlorogenic acid and ferulic acid) (Incedayi et al., 2010; Payet et al., 2006). Besides these polyphenols, DWW also contains melanoidins as major recalcitrant coloring compounds (Arimi et al., 2015; 2014).

2.3.1. Ecotoxicity caused by distillery wastewater

The discharge of DWW in water bodies without adequate treatment causes severe water pollution. Due to its high BOD, COD values, high sulphate, phosphate, and nitrogen content, it causes eutrophication of contaminated water resources (Ramakritinan et al., 2005; Mahimaraja and Bolan, 2004). For DWW, Mahimaraja and Bolan (2004) have estimated the LC_{50} value of 0.5% by using a bio-toxicity test on fresh water fish *Cyprinus carpio var. communis*. Subsequently, it was reported by some other researchers that respiratory process in *Cyprinus carpio* under DWW stress get affected resulting in a shift towards the anaerobic conditions at organ level during the sublethal intoxication (Ramakritinan et al., 2005).

DWW also causes soil pollution and acidification in the case of inappropriate land discharge. Further, it is also reported by various researcher that it inhibits seed germination, reduce soil alkalinity, cause soil manganese deficiency and reduces the growth and yield of crop plants (Chowdhary et al., 2017; Onyango et al., 2012; Bharagava and Chandra, 2010b; Agrawal et al., 2010; Mohana et al., 2009). In addition, Bharagava and Chandra (2010b) have also reported that post methanated distillery effluent (PMDE) have deleterious effects on seed germination and seedling growth parameters in *Phaseolus mungo* (L). The inhibition in seed germination at higher PMDE content might be attributed to high salt concentration and TDS, which increases high osmotic pressure (OP) and anaerobic conditions, respectively. These conditions affect various biochemical and physiological activities such as movement of solute, respiration and enzymatic process of seed germination. It has been also reported that high PMDE concentration also acts as an inhibitor for plant growth hormone(s) (auxin and gibberline), which play an important role in plant growth and development (Subramani et al., 1997). Moreover, Bharagava et al. (2010b) have reported that at higher PMDE concentration, the entrance of potentially toxic trace elements into the protoplasm may result in the reduction of intermediate metabolites, which are responsible for the reduction in plant growth parameters.

2.3.2. Various health effects by distillery wastewater

Besides soil and water pollution, the residents of DWW contaminated area also face severe health problems such as irritation of eyes, skin allergies, headache, fever, vomiting sensation, and stomach pain etc. All these problems might be due to the presence of high concentration of dissolved impurities like carbonates, bicarbonates, sulphates, calcium chloride, magnesium, iron, sodium, and potassium along with the colloidal impurities like coloring compounds, organic waste, finely divided silica and clay (Chaudhary and Arora, 2011).

DWW due to the presence of a number of anti-nutritional and toxic MRPs such as melanoidins has been also reported to cause severe health problems in human and animals directly/indirectly (Taylor et al., 2004). Melanoidins present in DWW in high concentration have mutagenic, carcinogenic and cytotoxic effects on cells (Silvan et al., 2006; Somoza, 2005). Some researchers have reported that excessive glycation process also destroys the essential amino acids, inactivation of enzymes, cross-linking of glycated extra-cellular matrix, inhibition of regulatory molecule binding sites, altered macromolecular recognition, abnormalities in nucleic acid function, endocytosis and increased immunogenicity etc. (Silvan et al., 2006; Taylor et al., 2004). In addition, melanoidins were also found to be involved in the progression of various diseases such as cardiovascular complications, diabetes mellitus and Alzheimer's disease (Somoza, 2005).

However, the genotoxic compounds can act at various levels in cells (causing gene, chromosome, or genome mutations), necessitating the use of a range of genotoxicity assays designed to detect these different types of mutations (Bartling et al., 2005; Taylor et al., 2004). Brands et al. (2000) have demonstrated that heated sugar-casein model melanoidins consisting variable sugars exhibit different mutagenic activity. For example, ketose sugars (fructose and tagatose) showed a remarkably high mutagenic activity as compared to their aldose isomers (glucose and galactose) and generated reactive oxygen species results in the breaking of DNA strands and mutagenesis. In addition, some other MRPs were also reported to induce chromosomal aberrations in *Chinese hamster* ovary cells and gene conversion in yeast cells. The mutagenicity and DNA strand breaking activity of glucose-glycine model melanoidins was also demonstrated by Hiramoto et al. (1997), who reported that LMW fractions act as lipid sink and induced DNA damage, where the effect increases with increase in concentration. An overview of distillery wastewater impact on environment, management and treatment technologies (Fig. 2.2).

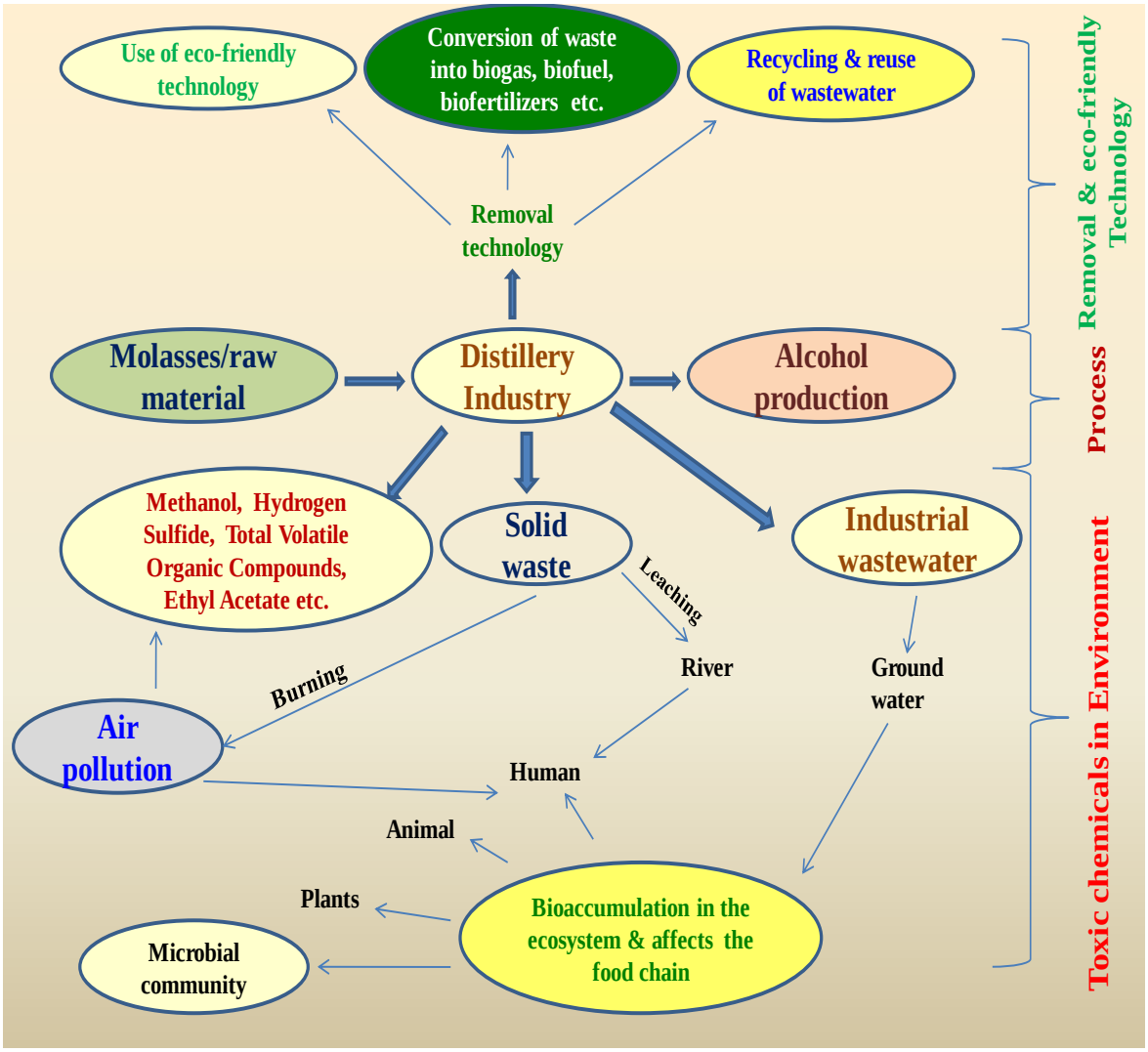


Fig. 2.2. Environmental impact of distillery wastewater and technologies to combat the threat.

2.4.Treatment approaches for DWW

This can be achieved by using various physical, chemical and/or biological treatment processes either alone or in combination for the effective treatment.

2.4.1. Physico-chemical treatment methods

2.4.1.1. Coagulation/flocculation

Coagulation is the destabilization of colloidal particles by neutralizing the forces that keep them apart by using coagulating agents and sometimes by the coagulant aids (e.g. activated silica, bentonite, polyelectrolytes, starch etc.). As a result, the particles collide to form larger particles (flocs) whereas flocculation is the action of polymers to form bridges

between the flocs, and bind particles to form large agglomerates or clumps. A number of coagulants such as aluminium sulfate ($AlSO_4$), ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$), alum, iron aluminum, calcium salts, polyaluminium chloride (PACl) etc. are reported to be used in the treatment of DWW. These coagulants are reported to reduce the organic load (COD) and suspended solids (SS) from DWW (Wagh and Nemade, 2015; Prajapati and Chaudhari, 2015; Arimi et al., 2014; Agarwal et al., 2010; Satyawali and Balakrishnan, 2008; Pandey et al., 2003).

However, coagulants are pH specific and their effectiveness depends on their type, concentration, and characteristics of wastewater to be treated. Chaudhari et al. (2005) have reported 72.5%, 60% and 55% COD reduction and 92%, 86% and 83% color reduction from DWW using polyaluminium chloride (PACl), $AlCl_3$ and $FeCl_3$. Sowmeyan and Swaminathan (2008) have tested $FeCl_3$ and $AlCl_3$ for the effective treatment of DWW and reported 93% & 76% reduction in color and total organic carbon, respectively. Moreover, the highest color removal (upto 98%) from biologically treated DWW was reported by using the conventional coagulants such as ferrous sulfate, ferric sulfate, and alum under alkaline conditions (Pandey et al., 2003). Further, Prajapati et al. (2015) have reported 80%, 90%, 70%, and 92% COD reduction and 81.8%, 80.64%, 74.19% and 81.8% color reduction from DWW by $FeCl_3$, alum, $AlCl_3$, and $FeSO_4$ at concentration of 60mM/dm^3 at pH 5, 5, 6, and 5, respectively. David et al. (2015) have also applied a green methodology for DWW treatment using *Moringa oleifera* seed extract as the coagulant in conjunction with chemical coagulants i.e. aluminium sulphate and calcium sulphate and found 97% color reduction.

2.4.1.2. Adsorption

Adsorption is a surface based physical phenomenon used for the removal of organic pollutants from industrial wastewaters. Extensive literature is available on the studies

using adsorbents such as chemically modified sugarcane bagasse, powdered activated carbon (PAC), activated charcoal, pyochar, chitosan etc. for DWW treatment (Prajapati and Chaudhari, 2015; Agarwal et al., 2010; Satyawali and Balakrishnan, 2008; Mandal et al., 2003; Lalov et al., 2000; Chandra and Pandey, 2000). Activated carbon (AC) has been reported as an efficient adsorbent due to its extended surface area, microporous structure, high adsorption capacity as well as high degree of surface reactivity (Arimi et al., 2014; Agarwal et al., 2010; Satyawali and Balakrishnan, 2008). AC is widely used for the removal of color, polyphenols and specific organic pollutants from various industrial wastewaters (Prajapati and Chaudhary, 2015; Satyawali and Balakrishnan, 2008).

Chandra and Pandey (2000) has reported >99%, 70% and 90% reduction in color, BOD, and COD, respectively by using commercial activated charcoal having a surface area of 1400 m^2/g from anaerobically treated distillery spent wash. Lalov et al. (2000) used chitosan as an adsorbent at the concentration of 10 g/l for 30 min contact time for the effective treatment of DWW and found 98% and 99% reduction in color and COD, respectively. Further, Mane et al. (2006) have reported 50% color reduction from DWW using chemically modified bagasse (0.5 g/100 mL wastewater) using 2-diethylaminoethyl (DEAE), chloride hydrochloride and 3-chloro-2-hydroxypropyl trimethyl ammonium chloride (CHPTAC) for the effective treatment of DWW. Shivayogimath and Inani (2014) have also reported 95.4%, 62.83% and 89.8% COD, color, and TDS reduction, respectively from DWW by using bagasse activated carbon.

2.4.2. Biological treatment of distillery wastewater

Biological treatment approaches are eco-friendly methods for the mitigation of industrial pollutants and involve the stabilization of wastes by degrading them into harmless substances either by anaerobic or aerobic processes.

2.4.2.1. Anaerobic process

The anaerobic digestion is the most appropriate approach for the mitigation of high organic carbon content such as distillery and pulp and paper industry wastewater. The anaerobic digestion process is mainly used to produce biogas from spent wash. The high organic content of molasses spent wash makes the anaerobic treatment more attractive in comparison to direct aerobic treatment process (Satyawali and Balakrishnan, 2008; Mohana et al., 2007). The anaerobic digestion is a process in which the organic compounds present in DWW are digested by microorganisms to produce biogas (CH_4 60% and CO_2 40% approximately). On an average, $1m^3$ of spent wash produces ~38-40 m^3 of biogas. The other products of anaerobic digester include treated spent wash and digested sludge, which is highly rich in nutrients. This digested sludge can be used as green manure since, it contains the high nutrient content (Nandy et al., 2002).

The limitations of anaerobic treatment processes are the requirement of high dilution due to the presence of many antimicrobial compounds such as 2,3-dimethylpyrazone, 2,2-bifuran-5-carboxylic acid, 2-nitroacetophenone, 2,2-bifulan, 2-methylhexane, methylbenzene, 2,3-dihydro-5-methylfuran, p-chloroanisole, 3-pyrroline and acetic acid etc. These compounds can be transformed by bacteria into other products including: 2-nitroacetophenone, p-chloroanisole, indole, 2- methylhexane and 2,3-dihydro-5-methylfuran etc. (Jimenez et al., 2004; Bharagava and Chandra, 2010a). Despite of high COD removal from diluted DWW, the chemical inhibitors remain in DWW even after the anaerobic digestion process.

Table 2.3: Performance efficiency of various anaerobic reactors for the treatment of distillery wastewater

Reactors	**COD Reduction (%)**	**BOD removal (%)**	**Retention time (days)**	**Reference**
Upflow anaerobic fixed film bioreactor	64%		8	Acharya et al., 2008
Upflow Anaerobic Sludge Blanket (UASB)	90-95%	-	-	Moletta, 2005
Anaerobic granular sludge reactor	80-90	-	1	Collins et al., 2005
Thermophilic UASB reactor	87	-	0.3	Syutsubo et al., 1997
Downflow fluidized bed reactor with ground perlite	85	-	3.3-1.3	Garcia-Calderon et al., 1998
Upflow anaerobic sludge blanket (UASB) reactor	39-67	80	-	Harada et al., 1996
UASB	75	-	-	Sanchez Riera et al., 1985
UASB	90	-	-	Wolmarans and de Villiers, 2002
UASB	93	-	20-39h	Wolmarans and de Villiers, 2002
Granular bed anaerobic baffled reactor (GRABBR)	82-90	90	-	Akunna and Clark, 2000
Anaerobic filter and UASB	90	-	1.3d	Blonskaja et al. 2003
Anaerobic contact filter	73-98	-	4	Vijayaraghavan and Ramanujam, 2000
Diphasic (Upflow) fixed film reactor (granular activated carbon support)	67.1		4	Goyal et al., 1996

Therefore, a further treatment is required to remove the remaining dark color and COD, BOD etc. Another strategy is the pre-treatment of DWW with ozone, UV light plus titanium dioxide before the aerobic digestion in order to improve the efficiency of anaerobic treatment processes (Arimi et al., 2014; Jimenez et al., 2004; Martin et al., 2002). It is thus, preferable to treat the DWW anaerobically first and then with other treatment methods. Arimi et al. (2015) have used natural manganese oxides (MnOx) in anaerobic digestion process to remove DWW pollutants. Further, more information on anaerobic digestion processes can be found in Table 2.3 and other reviews (Satyawali and Balakrishnan, 2008; Melamane et al., 2007; Wilkie et al., 2000).

2.4.3. Conventionally used treatment approaches

DWW treatment has been reported in single and as well as biphasic system, resulting in a significant reduction in pollution parameters such as COD and BOD with the provision of a gas collection (Jo et al., 2015; Mendez-Acosta et al., 2010; Mohana et al., 2009; Pathade, 2003). However, the hydraulic retention time in a CSTR- type reactor is determined by the growth rate of microorganism growing in the system. It means to achieve the high degradation rate of DWW, a very high HTR value will be required because the high HTR values make the CSTR concept less feasible and less effective for the treatment of DWW. As CSTR requires long retention time and less gas yield during the treatment process (Siddique, 2012; Kleerebezem and Macarie, 2003).

2.4.3.1. Single and biphasic system

The treatment of DWW in an anaerobic system can be controlled by the single or biphasic system. In single phasic systems, only one reactor involve in the microbial degradation of organic pollutants, whereas biphasic system has two reactors i.e. one for acidogenic and other for methanogenic microorganisms. In biphasic reactors, the most promising thing is that fermentation steps can be optimized at each stage in separate

fermenters. Due to this, the effectiveness and kinetics of biphasic reactors become much higher in comparison to single phasic reactors because in this system all process occurs in same environmental conditions. In both phases (primary and secondary), the end products produced are acetate, lactate, ethanol, CO_2, H, C_3, higher volatile fatty acid and methane, CO_2, respectively (Mohana et al., 2009; Gosh, 1990). Thus, the biomethanation using biphasic system seems to be most appropriate treatment method for DWW because of its multiple advantages such as easy maintenance of optimal conditions for buffering between the production of organic acid and their utilization, steady performance, and high methane gas production.

2.4.3.2. High rate anaerobic reactors

2.4.3.2.1. Upflow anaerobic sludge blanket (UASB) reactors

The UASB reactors have become more popularized in the recent years for the treatment of various types of wastewaters including DWW (Petta et al., 2017). The USAB reactors are high rate anaerobic wastewater treatment reactors, which are extensively used for the treatment of DWW worldwide. The UASB reactors have four main components such as sludge bed, sludge blanket, gas solid separator, and settlement compartment. The biomass layers settled at the bottom of the reactors are called as sludge bed whereas the suspension of sludge particles mixed with produced gas is called as sludge blanket. However, the operation of UASB is mainly dependent on the formation of active and settleable granules (Fang et al., 1994). The function and efficiency of USAB reactors are dependent on several factors like temperature, pH, wastewater composition, and organic loading rate. Recently, Petta et al. (2017) have observed that the UASB reactors combined with the anoxic-aerobic ultra filtration membrane bioreactors (UF-MBR) achieve the treatment efficiency up to 97% with the production of methane 340 L of CH_4/kg COD. The efficiency UASB depends on the active and settleable granules that contain the aggregation of anaerobic bacteria, self-immobilized into a compact form. These granules

enhance the settleability of biomass leading to an effective retention of bacteria in UASB reactors (Akunna and Clark, 2000). However, the most attractive features of UASB reactor design include, its independence from the mechanical mixing of digester contents, recycling of sludge biomass as well as the ability to deal with the perturbances caused due to the high loading rates and temperature fluctuations (Sharma, and Singh, 2000). For the successful performance of UASB reactors, it should be operated at a low loading rate of 4-8 kg COD $m^{-3}d^{-1}$ and COD removal rate should be monitored carefully. Wolmarans and de Villiers (2002) have reported that USAB reactors can achieve 90% COD removal from DWW under high loading rate.

2.4.3.2.2. Anaerobic batch reactors

The anaerobic batch reactors have not been generally used for the treatment of DWW and thus, the potential, operational feasibility and scale-up of such reactors need to be studied. However, Moletta (2005) has achieved 90-95% COD reduction during the anaerobic digestion with the organic loading between 5-15 kg COD/m^3 of digester/day with biogas production from 400-600 per kg COD removal with 60-70% methane content. Recently, Tansengco et al. (2016) have reported 60% and 86% COD and BOD reduction along with the generation of 72% methane gas during the treatment of DWW in Anaerobic Sequencing Batch Reactor (ASBR), at 8 h of reaction time. In addition, a semi continuous batch digester was also designed to study the biomethanation of DWW within the range of mesophilic and thermophilic temperatures (Banerjee and Biswas, 2004). In this study, authors have reported 86.01% BOD reduction with 73.23% methane gas production at the BOD loading rate of 2.71 kg m^{-3} and 50 °C.

2.4.3.2.3. Anaerobic filters

The anaerobic filters are more popular in comparison to aerobic wastewater treatment methods because these generate less amount of solid residue. The anaerobic filters are packed column having static medium to support the colonization of anaerobic microbial

consortium for wastewater treatment. These filters are based on an attached growth process, which immobilizes microorganisms on the surface of packing materials to produce a biofilm (de Lemos Chernicharo, 2007). Thus, in anaerobic filters, the selection of packing materials is important because it plays an important role in the effective performance of anaerobic filters as various characteristics of filter media such as porosity, and surface area has significant effects on the attachment of biomass (Loupasaki and Diamadopoulos, 2013).

The anaerobic filters work in up and downflow mode were the latter achieves better sustained and reliable operation because the downflow has the capacity to reduce the clogging of packed material during the treatment of wastewater carrying the very high content of suspended solid (Nicolella et al., 2000). Yu et al. (2006) achieved 82% COD removal from DWW under laboratory condition by upflow anaerobic filter at a temperature ranging from 19-27 °C, BV = 37.68 kg COD/(m^3.d) and HRT of 8h. Further, a lab scale anaerobic reactor packed with small sized and low-density polyethylene (0.93 g/cm^3, Bioflow 30) as supporting materials resulted in 80% COD removal at BV of 30 kg COD m^3.d. However, the biomass retention capacity obtained was 4-6 g dry solids per g support representing a fixed biomass of 57 g solids/L of reactor volume (Thanikal et al., 2007).

2.2.3.2.4. Bihydrogen production

Industrial wastewaters are well reported to have a high organic load, BOD, and COD, which causes various harmful effects on the environment, but these parameters, can also act as a source of beneficial by-products generation. Approximately, 5.2 million tons of solid waste is generated per day worldwide, which can be used for the generation of useful by-products (Modak, 2011). Many investigators have proposed and selected hydrogen gas as an alternative renewal source of energy and also looking toward the new alternatives to

generate hydrogen gas from organic pollutants by using microorganisms (Choudri and Baawain, 2016; Fountoulakis and Manios, 2009; Wang and Zhao, 2009). Recently, many authors have reported the hydrogen gas production utilizing DWW as C, N, and energy source by anaerobic treatment process (Wicher et al., 2013; Mishra and Das, 2014; Mishra et al., 2015). However, the main advantages of microbiological methods of hydrogen generation rely on the possibility of utilization of industrial and municipal wastewaters, significant decrease of costs of production and simplicity of the processes.

2.4.4. Aerobic process

2.4.4.1. Bacterial treatment of distillery wastewater

Bacterial degradation and decolorization of industrial wastewaters is an environment-friendly and low-cost alternative to the physico-chemical treatment processes of wastewaters. Jiranuntipon et al. (2008) have reported 9.5, 1.13, 8.02, and 17.5%, color removal from Viandox sauce (13.5% v/v), caramel (30% w/v), beet molasses wastewater (41% v/v), and sugarcane molasses wastewater (20% v/v) within 2 days by using a consortium of *Klebsiella oxytoca*, *Serratia mercescens*, and *Citrobacter* sp. In addition, they also achieved 26.5% color removal from DWW by using the consortium of *Acinetobacter* sp., *Pseudomonas* sp., *Comamonas* sp., *Klebsiella oxytoca*, *Serratia marcescens*, and unidentified bacterium in 48 h under aerobic condition (Jiranuntipon et al., 2009). However, a detailed list of bacteria used by various researchers in the treatment of DWW is given in Table 2.4.

Table 2.4: Microorganisms capable for decolorization of distillery wastewater

Reported Microorganisms	Comments	Color Removal (%)	References
Bacterial species			
Pediococcus acidilactici B-25	Glucose are used as a primary supplementary carbon source	79	Tiwari et al., 2013
Pseudomonas putida	Glucose concentration was critical for decolorization and improved color removal efficiency was obtained by periodic replenishment of glucose	24	Ghosh et al., 2009
Pseudomonas putida	Glucose used as a carbon source, for the production of H_2O_2, which reduced the colour	60	Ghosh et al., 2002
Pseudomonas Fluorescens	The organism performed decolorization with cellulose carrier coated with collagen. Reuse of decolorized cells reduced the decolorization efficiency	94	Dahiya et al., 2001a
Pseudomonas Aeruginosa	The three strains were part of a consortium which decolorized the anaerobically digested spent wash in presence of basal salts and glucose	67	Mohana et al., 2007
Pseudomonas sturzeri	The organism required sugar especially, glucose for decolorization of distillery wastewater	≤ 60.00	Ramachandra, 1993
Pseudomonas sp.	The organism used glucose and fructose as carbon source for decolorization	56.00	Chavan et al., 2006
Bacillus thuringiensis	1% glucose are used as a supplementary carbon source	22	Kumar and Chandra, 2006
Xanthomonas fragariae	The organism used glucose as carbon source and NH_4Cl as nitrogen source.	76	Jain et al., 2002
Acinetobacter sp.	All these organisms were isolated from an air bubble column reactor treating winery wastewater after 6 months of operation. Most isolates from	-	Petruccioli et al., 2000

	the colonized carriers belonged to species of the genus Bacillus		
Acetobacter acetii	The organism required sugar especially, glucose and fructose for decolorization of MWWs	76.4	Sirianuntapiboon et al.,2004
P. aeruginosa	Glucose used as carbon source	67.00	Sarayu et al., 2005
P. aeruginosa	Glucose are used as a supplementary carbon source	69	Pal and Vimala, 2012
Fungal species			
Penicillium sp.	All fungi produced decolorization from first day of incubation, with maximum being shown by *P. decumbent* at fourth day with a reduction of 70% of the phenolic content of the wastewater	30	Jimnez et al., 2003
Aspergillus niger UM2	Decolorization was more by immobilized fungus and it was able to decolorize up to 50% of initial effluent concentrations	80	Patil et al., 2003
Flavodon flavus	MSW was decolorized using a marine basidiomycete fungus. It also removed 68% benzo(a) pyrene, a PAH found in MSW	80	Raghukumar and Rivonkar, 2001; Raghukumar et al., 2004
P. chrysosporium	Phenolic concentration and color were decreases under two different growth conditions	56.8 1	Potentini and Rodriguez, 2006
Phanerochaete chrysosporium JAG-40	This organism decolorized synthetic and natural melanoidins when the medium was supplemented with glucose and peptone	80	Dahiya et al., 2001
Aspergillus niveus	The fungus could use sugarcane bagasse as carbon source and required other nutrients for decolorization	56	Angayarkanni et al., 2003

Williopsis saturnus strain CBS 5761	Yeast isolates from a rotating biological contactor (RBC) treating winery wastewater. Only 43% COD removal could be achieved		Malandra et al., 2003
Coriolus versicolor sp no. 20	10% diluted spent wash was used with glucose @ 2% added as carbon source	34.5	Chopra et al., 2004
Phanerochaete Chrysosporium	Sugar refinery effluent was treated in a RBC using polyurethane foam and scouring web as support	55	Guimaraes et al., 2005
Marine Basidiomycete NIOCC # 2a	Experiment was carried out at 10% diluted spent wash	100	D'souza et al., 2006
Citeromyces sp. WR-43-6	Organism required glucose, Sodium nitrate and KH_2PO_4 for maximal decolorization	68.91	Sirianuntapiboon et al., 2003
Pleurotus florida	Various fungi grown under solid-state fermentation using agro-residue	86.3	Pant and Adholeya, 2009
Yeast			
Candida tropicalis RG-9	-----	75	Tiwari et al., 2012
Citeromyces sp.	The organism required sugar especially, glucose and fructose for decolorization	75.00	Sirianuntapiboon et al., 2004
Cyanobacteria			
Oscillotoria boryana	The organism required sugar especially, glucose and fructose for decolorization	60.00	Kalavathi et al., 2001

2.4.4.2. Fungal treatment (Mycoremediation)

There are a number of fungal species such as *Aspergillus fumigatus* G-2-6, *Emericella nidulans var. lata, Geotrichum candidum, Trametes* sp., *Aspergillus niger, Citeromyces* sp., *Flavodon flavus* etc., which have been used by various worker for the treatment of DWW (Bezuneh, 2016; Pal and Vimla, 2012; Raghukumar et al., 2004; Patil et al., 2003; Gonzalez et al., 2000).

Fungal treatment is used to reduce COD, BOD, and degradation of organic compounds as well as to obtain some valuable byproducts such as protein-rich, fungal biomass, which can be used as animal feed or some other specific fungal metabolites. Filamentous fungi have lower sensitivity to variations in temperature, pH, nutrients, and aeration and have lower nucleic acid content in biomass (Satyawali and Balakrishnan, 2008).

Ravikumar et al. (2011) have reported that *Cladosporium cladosporioides* was capable to reduce 52.6% color and 62.5% chemical oxygen demand from DWW at optimum conditions i.e. 5 g/L of fructose, 3 g/L of peptone, 5 pH and 35 °C. Further, these authors again used *Cladosporium cladosporioides* at different conditions i.e. fructose concentration 7 g L^{-1}, peptone 2 g L^{-1}, 6 pH and 10% (w/v) inoculum concentration and found 62.5% and 73.6% reduction in color and COD, respectively (Ravikumara et al., 2013). In addition, Shukla et al. (2014) also reported 97.2% color reduction from DWW by using *Aspergillus niger* (ATCC No. 26550 and NCIM No. 684) with the help of combined coagulants.

However, some white rot fungi also reported to secret ligninolytic enzymes (LiP, MnP & Laccases), which are capable of degrading xenobiotics and organometalic-pollutants (Chandra and Chowdhary, 2015). Moreover, various fungal species investigated for their ability to degrade/decolorize DWW are given in Table 2.4.

2.4.4.3. Algal treatment (Phycoremediation)

The treatment of DWW with microalgae attracts the researchers not only by treating the waste, but also by its products/byproducts, which are in high demands for social welfare (Sankaran et al., 2014). Solovchenko et al. (2014) have investigated the possibilities of DWW bioremediation along with a new *Chlorella sorokiniana* sp. cultivated in a semi-batch mode in a high-density photobioreactor. Microalgal treatment becomes effective only after the anaerobic treatment of spent wash, since the process is energy efficient and has ability to fulfil its nutrients requirement from biomethanated spent wash and energy requirement from sun light. The treatment of anaerobically treated 10% DWW using the microalgae *Chlorella vulgaris* followed by *Lemna minuscula* resulted in 52% color reduction (Valderrama et al., 2002). Further, Kalavathi et al. (2001) examined the degradation of 5% melanoidins by a marine cyanobacterium *Oscillatoria boryana* BDU 92181.

Saha et al. (2005) observed that *Oscillatoria willei*, when grown under lower nitrogen content, but with optimum phenolic compounds, showed an increased oxidative stress with an increase in ligninolytic and anti-oxidative enzymes such as lignin peroxidase, laccase, polyphenol oxidase, superoxide dismutase, catalase, peroxidase and ascorbate peroxidase. This study concluded that these enzymes were responsible for the decolorization of substrate phenol upto 52% in 7 days by the Cyanobacterium *O. willei*.

Sankaran et al. (2014) have given the phycoremediation mechanism of DWW (Fig. 2.3). Thus, coupling microalgae biomass production with nutrient removal/pollutant degradation may represent an important milestone in the bioenergy goals since the wastewater market is immense (Sankaran et al., 2014).

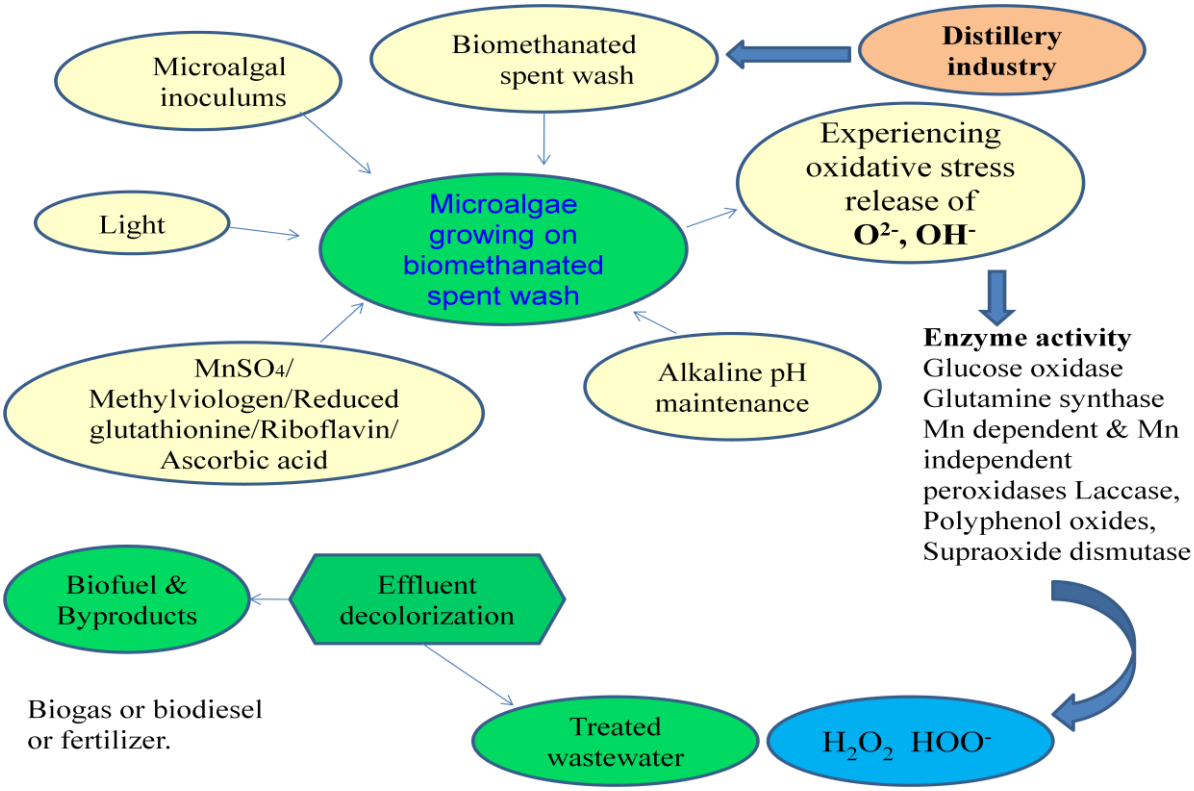


Figure 2.3. The mechanism of melanoidin containing biomethanated spent wash treatment using microalgae (Modified from Sankaran et al., 2014).

2.4.5. Constructed wetlands (CWs)

Plants have high metal accumulation potential from the contaminated sites, which was observed by Transmission Electron Microscopy (TEM) analysis of various naturally growing plants (Fig. 2.4) (Chandra and Kumar, 2017a).

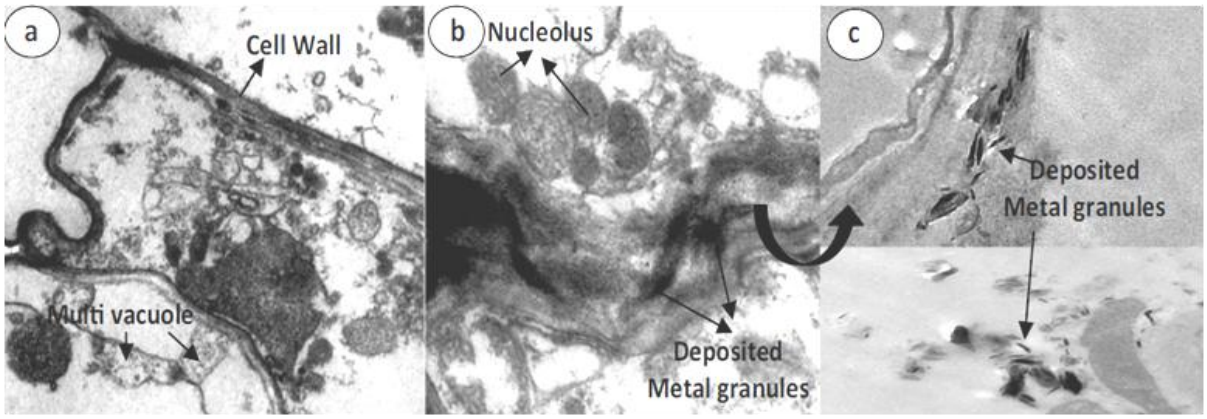


Figure 2.4. TEM images of native plants root after phytoextraction of heavy metals. a-c Congress grass (*Parthenium hysterophorous*) (Adapted from Chandra and Kumar, 2017a).

Constructed wetland as a natural process, environment friendly with a simple construction and low maintenance is one of the interesting technique. The treatment of DWW through constructed wetlands is the most biological active ecosystem worldwide (Sayadi et al., 2012; Choudhary et al., 2011). Mulidzi et al. (2010) showed the impact of shorter retention time on the performance of constructed wetlands in terms of BOD, COD and other elements removal. The results had shown an overall 60% COD removal throughout the year. Results also showed the significant removal of other elements namely; potassium, nitrogen, electrical conductivity, calcium, sodium, magnesium, and boron from DWW wastewater by constructed wetlands.

Billore et al. (2001) have demonstrated a four-celled horizontal subsurface flow (HSF) CW for the treatment of DWW after anaerobic treatment. The post-anaerobic treated effluent had BOD of 2500 mg/L and COD 14,000 mg/L. A pre-treatment chamber filled with gravel was used to capture the suspended solids. All the cells were filled with gravel up to varying heights and cells, third and fourth were planted with *Typha latipholia* and *Phragmites karka,* respectively. The overall retention time was 14.4 d and the treatment resulted in 64%, 85%, 42%, and 79% reduction in COD, BOD, total solids, and phosphorus, respectively.

2.4.6. Biocomposting

In this process, press mud generated from sugar mills is utilized to produce compost by mixing with DWW (Torres-Climent et al., 2015). Both anaerobic and aerobic composting systems are being used for the treatment of DWW. In some treatment plants, composting with effluent treated through the bio-methanation plant is also practiced. Biocomposting is one of the most valuable thermophilic processes, resulting in a product rich in humus, which is used as fertilizer in agriculture fields. The spent wash, either directly, or after biomethanation is sprayed in a controlled manner on sugarcane pressmud. The latter is the

filter cake obtained during the juice clarification in sugar industries. Jimnez and Borja, (1997) reported that the aerobic pre-treatment of beet and molasses spent wash with *Penicillium decumbens* resulted ~74% and 40% reduction in phenolics content and color, respectively. This is a popular option adopted by several Indian distilleries attached to sugar mills with adequate land availability.

2.4.7. Distillery wastewater decolorization by enzymatic mechanism

There are several enzymes (e.g., peroxidases, oxidoreductases, cellulolytic enzymes, cyanidase, proteases, amylases, etc.) reported from different sources to play an important role in waste treatment processes (Chandra and Chowdhary, 2015; Dec and Bollag, 1994). The ligninolytic system consists of two main groups of enzymes: peroxidases (lignin peroxidases and manganese peroxidases) and laccases (Chandra and Chowdhary, 2015; Baldrian, 2006). The bacterial laccases play an important role in bioremediation of industrial waste because these oxidize both toxic and non-toxic substrates. Laccases are also included in the cleaning of industrial effluents, mostly from paper and pulp, textile and DIs. Among the biological agents, laccases represent an interesting group of ubiquitous oxidoreductase enzymes showing great potential for biotechnological applications (Chandra and Chowdhary, 2015; Sangave and Pandit, 2006; Gianfreda et al., 1999). On DWW decolorization, many studies have suggested the involvement of various enzymes with different mechanisms as Watanabe et al. (1982) have reported the involvement of an intracellular enzyme produced by *Coriolus* sp No. 20 that requires active oxygen molecule and sugars for its activity. This intracellular enzyme was identified as sorbose oxides with molecular weight 2,00,000 kDa. The purified enzyme was found capable to decolorize DWW in presence of glucose, galactose, sarbose, xylose, and maltose. DWW is reported to be decolorized by the active oxygen species (O_2^-, H_2O_2) produced by the reactions catalyze by oxidases because the reaction with pure enzymes

was accompanied by the oxidation of glucose into gluconic acid. It could be due to the production of sugar oxidases rather than the sorbose oxidase because the crude preparation utilizes arabinose, fructose, and mannitol while sarbose oxidase does not utilize these sugars. Further, Aoshima et al. (1985) have reported the decolorization of DWW by *Coriolus versicolor* Ps4a, which might be due to an intracellular enzyme induced by DWW pollutants. This intracellular enzyme is reported to consist of two major components i.e. 1st a sugar independent enzyme that forms two-third part while other is sugar independent part that contributes one-third part of the enzyme. Ohmomo et al. (1985) purified a DWW decolorizing enzyme from *Coriolus versicolor* Ps4a and reported that this was an intracellular enzyme consisting of a major P-fraction and a minor E-fraction. The P-fraction consist at least five enzymes, which were of two types that may/may not require sugar for their decolorizing activity. In addition, Miyata et al. (1998) have also studied the DWW decolorizing by *Coriolus hirsutus* pellet, which was mainly due to the production of extracellular hydrogen peroxide (H_2O_2) and peroxidases. The culture filtrate was found to have two major extracellular peroxidases, one manganese independent peroxidase (MIP) and other is manganese dependent peroxidase (MnP). Since both MIP and MnP exhibited DWW decolorizing activity in presence of H_2O_2 and thus, it can be concluded that the decolorization of DWW by *C. hirsutus* involved the production of extracellular H_2O_2 and peroxidases. Therefore, the knowledge of enzymes in bioremediation of various industrial wastes will open many opportunities for large-scale application.

2.4.8. Miscellaneous approaches for color removal from DWW

Sirianuntapiboon et al. (2004) have isolated a strain No. WR-43-6 (*Citeromyces* sp.), which showed the highest decolorization yield i.e. 68.91% from a solution containing molasses pigment in presence of glucose 2.0%, sodium nitrate 0.1% and KH_2PO_4 0.1%,

respectively at 30 °C for 8 days. Further, this bacterium also found capable for removal of color (75%), BOD (76%), and COD (100%) from the stillage of an alcohol factory.

Satyawali and Balakrishnan (2007) have prepared 19 carbon samples by the acid and thermal activation of various agro based by-products such as bagasse, bagasse fly ash, saw dust, wood ash, and rice husk ash for the color removal from the biomethanated distillery effluent. They found that phosphoric acid carbonized bagasse B (PH) has resulted maximum color removal (50%). In addition, various commercial activated carbons AC (ME) and AC (LB) have resulted 80% color removal from biomethanated DWW. Besides color removal, these activated carbons were also found effective for the reduction in COD, TOC, phenol, and total nitrogen content.

Kaushik and Thakur (2009) have isolated 5 different bacterial strains from a distillery mill site and tested for their COD and color removal efficiency. Out of these 5 bacterial strains, one bacterium (*Bacillus* sp.) was found capable for 21% and 30% color and COD reduction, respectively from distillery spent wash. Further, under the optimized parameters such as pH, temperature aeration, carbon, nitrogen, inoculum size, and incubation time by the Taguchi approach, the same bacterium was found effective for 85%, and 90% color, and COD reduction respectively within 12 h of incubation period.

Apollo et al. (2013) achieved maximum color reduction (88%) from DWW by the combined treatment with anaerobic up-flow fixed bed reactor and annular photocatalytic reactor (as post-treatment technique). They also found that during single (UV photodegradation) treatment process, the color reduction was 54% and 69% from DWW and MWW, respectively. But, when UV photodegradation apply as pre-treatment to the anaerobic digestion process, it reduced the biogas generation and also COD reduction. Farshi et al. (2013) have reported 97-98% color reduction from DWW by using electrochemical treatment at different optimized conditions i.e. electrode distance 1 cm,

pH 4, current density 2 A/dm^2 for 3 h. The removal of melanoidins form stimulated and real wastewaters (biologically treated and untreated) was studied by coagulation/flocculation method by Liakos and Lazaridis (2014). In this study, the authors achieved 90% color removal at pH 5 by coagulation method with different concentration of ferric ions. However, the real wastewater could be decolorized by 100 mM [Fe^{3+}] while stimulated wastewater by 300 mM [Fe^{3+}]. After the completion of flocculation experiment, the generated ferric hydroxide residue was washed, solubilised and re-used in new cycle. The maximum color reduction from the real treated, real untreated, and stimulated effluent was 95%, 90%, and 45%, respectively by applying 0.5 A current intensity (Liakos and Lazaridis, 2014).

David et al. (2015) have reported that *Pseudomonas aeruginosa*, which produces Polyhydroxybutyrate (PHB) in presence of excess carbohydrate source. PHB is an intercellular polymer, which is utilized by microorganisms as an energy storage molecule when common energy sources are available in limited amount and this bacteria in presence of PHB resulted in resulted 92.77% color removal from DWW. DWW mainly consist of recalcitrant coloring compound (melanoidins), and other organic colorant, which are not easily degraded in biological treatment process. Arimi et al. (2015) achieved significant reduction in color, dissolve organic carbon, and melanoidins 92.7%, 63.3%, and 48%, respectively at pH 5 and a concentration of 1.6 g/L. In this experiment, the above mentioned physico-chemical parameters were reduced by using six coagulants, out of which, ferric chloride was found to be more effective resulting 92.7% color reduction. In another study, Arimi et al. (2015) have developed an effective polishing step for the removal of colorants from melanoidin-rich DWW by using natural manganese oxides. In this process, low molecular weight coloring compounds removed first followed by high molecular weight colorant removal with a significant dependence on pH.

Georgiou et al. (2016) have reported the decolorization of DWW by the immobilized laccase enzyme. In this study, authors have immobilized the laccase enzyme covalently on alumina or controlled pore glass-uncoated particles and achieved 71% and 74% decolorization, respectively in 48 h of incubation period. In addition, immobilized laccase on glass achieved 68% degradation of baker's wastewater in 24 h. Chen et al. (2016) achieved 97.1% color reduction from 50% (v/v) DWW by combined micro-electrolysis process with the help of biological treatment method. In this study, fungal biomass and ligninolytic enzyme (LiP, MnP, and laccase) are also played an important role in enhancing the DWW de-colorizing efficiency. El-Dib et al. (2016) achieved 78% and 83% reduction in color and chemical oxygen demand by using organic-inorganic nanocomposite (chitosan immobilized bentonite with chitosan content). In this study, the used modified chitosan immobilized bentonite (mCIB) and Bentonite (mbent) were prepared by intercalating cetyl trimethylammonium bromide (CTAB) as a cationic surfactant. Further, FTIR, XRD and SEM were used to study the interlayer structure and morphology of prepared samples. Out of all the used sorbents, the modified CIB_3 was found to be more effective in decolorization of distillery wastewater. Santal et al. (2016) isolated *Paracoccus pantotrophus* and found that these bacterial strains were highly effective to decolorize melanoidins up to 81.2 ± 2.43% in presence of carbon (glucose), and nitrogen (NH_4NO_3) source.

Recently, Zhang et al. (2017) achieved ~94.0% color reduction and ~78% reduction of dissolve organic matter from DWW with the treatment by ferric chloride ($FeCl_3$) as coagulant. During treatment process, this coagulant was found to react preferably with melanoidins (major colorant) via either surface complexation or neutralization of electric charge or by both mechanisms. Krzywonos et al. (2017) achieved 38% color reduction from vinasse by using *Bacillus megaterium* ATCC 14581 and medium component

$(NH_4)_2SO_4$, KH_2PO_4, yeast extract, peptone glucose, and vinasse. Out of these factors, four promising factors were chosen as follows: $(NH_4)_2SO_4$, KH_2PO_4, glucose, and vinasse for further optimizing process for color removal. Nure et al. (2017) have reported the significant reduction in color (64%) and chemical oxygen demand (61%) from melanoidins solution by using activated carbon, which was produced from bagasse fly ash (BFA). In this study, the surface area of used BFA was determined as 160.9 ± 2.8m^2/g with about 90% of particle < 156.8 µm in size. However, BFA was characterized by using Fourier transform infrared spectroscopy (FTIR) and showed the carbonyl (R-C=O) and hydroxyl (OH^-) groups, while X-ray diffraction and scanning electron microscopy analysis showed amorphous nature and heterogeneous and irregular shape of pores, respectively. In addition, microbial fuel cells (MFCs) are also becoming as promising technology, which produce electricity with simultaneous removal of pollutants in terms of COD, color and total dissolved solids etc. from the wastewaters (Feng et al., 2008; Wen et al., 2010; Samsudeen et al., 2015).

2.4.9. Emerging treatment approaches of distillery wastewater

2.4.9.1. Oxidation process

There is a number of oxidation processes, which are being used for the treatment of DWW such as ozone, hydrogen peroxide, Fenton's reagent and ozone combined with hydrogen peroxide (Asaithambi et al., 2015; Arimi et al., 2014; Afify et al., 2009; Dwyer et al., 2008). Ozone treatment alone reduces 76% color, where ozone in presence of low concentration of hydrogen peroxide removes 89% color (Santal et al., 2013; Dwyer et al., 2008). But, bicarbonate ions are reported to have the inhibitory effects on these decolorizing reactions (Coca et al., 2005). The sonication of DWW as a pre-treatment step, converts complex molecules into a more utilizable form by cavitation process and thus, significantly enhances the decolorization of DWW (Sangave and Pandit, 2006).

Vineetha et al. (2013) found that photodegradation of DWW by solar radiation resulted in 79% color reduction under the optimum conditions of H_2O_2, pH, and catalyst. In a recent study, Asaithambi et al. (2015) found that ozone-photo Fenton system was effective to reduce 100% color and chemical oxygen demand (COD) within 4 h.

2.4.9.2. Membrane treatment

In recent years, membrane processes have been widely used in various applications, especially for the treatment of wastewaters. The use of membrane technologies is accompanied with a high removal efficiency, optimal costs and simple devices handling (Prodanovic and Vasic, 2013).

A two-stage biological treatment followed by membrane modules has been recently developed for the effective treatment of DWW, which have following functions:

a. Biological removal of organic pollutants is carried out in bioreactor by the adapted microbial communities;

b. The membrane module performs the separation of microorganisms from treated wastewater. The membranes constitute a physical barrier for all the suspended solids and therefore, enable not only the recycling of activated sludge to the bioreactor, but also the production of permeate that is free from suspended solids, bacteria, and viruses.

Rai et al. (2008) reported that tertiary treatment of aerobically treated DWW by nano-filtration (NF) technique was carried out in a spiral wound NF membrane module under different conditions and resulted in COD, TDS, and color removal within the range of 96-99.5%, 85-95%, and 98-99.5%, respectively.

The total membrane area was $0.2m^2$ and the system was operated at a fluid velocity of 6.08 m/s, and 0.5 bar transmembrane pressure. Besides the COD reduction, the pre-treatment also improved the efficiency of anaerobic process possibly due to the removal of inhibitory substances. Kumaresan et al. (2003) employed the emulsion liquid membrane

(ELM) technique in a batch process for spent wash treatment. In another study, the treatment of vinasse from beet molasses by electrodialysis using a stainless steel cathode, titanium alloy anode and 4% (w/v) NaCl as electrolytic agent resulted in 88% COD reduction at pH 9.5, but it decreased drastically at higher feeding rates (Vlyssides et al., 1997). In addition, reverse osmosis (RO) has been also employed for DWW treatment. In a recent study, Nataraj et al. (2006) reported a pilot trial on distillery spent wash using a hybrid nanofiltration (NF) and RO process. Both, the NF and RO stages employed a thin film composite (TFC) membrane in spiral wound configuration with module dimensions of 2.5 inches diameter and 21 inches length. NF was primarily effective in removing color and colloidal particles accompanied by 80%, 95% and 45% reduction in total dissolved solids (TDS), conductivity and chloride concentration, respectively at an optimum feed pressure of 30-50 bars. The subsequent RO operation at a feed pressure of 50 bar resulted in 99% reduction each in COD, potassium and residual TDS (Prodanovic and Vasic, 2013; Satyawali and Balakrishnan, 2008).

2.5. Challenges in treatment of distillery wastewater pollutants

The DIs is reported to produce only ~7-9% of alcohol from sugarcane molasses and major portion ~91-93% contribute as wastewater. This huge volume of wastewater requires a long time for treatment due to the non-availability of fast and feasible treatment techniques. Due to very high BOD, COD and TDS values, the Effluent Treatment Plant (ETP) remains to fail to reduce these pollution parameters within the permissible limits set by various environmental protection agencies. DWW contains high melanoidins content, the major coloring compounds, which are highly recalcitrant in nature i.e. resistant to biological/microbial degradation. The management of large amount of sludge generated during the physical, chemical and biological treatment of DWW is also a big challenge for DIs. Further, the lack of advanced and feasible treatment techniques for the effective

treatment of DWW within a limited time is a major challenge for sustainable development. In addition, the poor capacity utilization also leads to the higher financial cost and overheads charges. Moreover, the very high expenditure on operation and maintenance of wastewater treatment plants is also not affordable and hence, the Governments should also provide the financial support to industries for sustainable development. Despite of these treatment technologies for DWW there is also need to know about the merits and demerits (Table 2.5).

Table 2.5: Various treatment approaches/technologies with their merits and demerits used for the treatment of distillery wastewaters

Treatment Technology	Advantages	Disadvantages
Physico-chemical Treatment		
Adsorption	Simultaneous adsorption and degradation of many pollutants	Temperature and pH sensitive High cost of commercial adsorbents is their main drawback for application
Coagulation/ Flocculation	Simple, and cost-effective Widely accepted Separates many kinds of particles from wastewater Enhances filtration process Uses abundant and low cost chemicals	pH sensitive As^{+3} and As^{+5} must be fully oxidized High energy lost Excess use of chemicals Large amount of sludge generated
Oxidation process	Broad range of organic compounds are oxidized The method has advantages over AOP since it can be used in either the pretreatment step or in the final treatment step	Ozone can selectively attack the double bonds (e.g. C=C, N=C) and functional groups (e.g. $-OCH_3$, -OH, and $-CH_3$) in acid or neutral conditions with limited concentrations, High cost
Membrane treatment	Significant color removal Removal of multiple contaminants	Membrane fouling, clogging, scaling and cleaning Poor production efficiency, Requires pretreatment
Evaporation and Combustion	Due to potassium rich ash it can be used for land application	Poor efficiency

Biological Treatment		
Aerobic treatment/Anaerobic treatment reactors	Eco-friendly and cost effective	Requires high dilution Slow process
Bacterial treatment		Time consuming
Fungal treatment		It acquires large space for treatment
Treatment by other microorganisms		
Microalgae	No need to add nutrients Yield biogas or biodiesel or fertilizer are by-product	Light dependent process
Cyanobacteria	Energy obtain from photosynthesis	Slow growth rate
Yeast	Produced ethanol for biofuel industry	Slow growth rate
Enzymatic treatment		
Laccases Peroxidases Oxidoreductases Cellulolytic enzymes Cyanidase Proteases Amylases	Enzyme are naturally produced by microorganism which is eco-friendly Reusable in nature Enzymatic biotransformation of industrial pollutants	Slow process and thus, cannot be applicable at large scale application

Chapter 3

Isolation, screening and characterization of ligninolytic enzyme producing bacteria capable for the degradation of distillery wastewater pollutants

Chapter 3

3.1. Introduction

The bioremediation process of industrial waste can be making more efficient by using ligninolytic enzymes like laccase which is obtained from fungus, bacteria, higher plant, insect and also in lichen etc. In aerobic process various types of microorganisms are involved such as bacteria, fungi, actinomycetes etc. are used due to their inherent capacity to breakdown a variety of complex compounds for degradation/decolorization of toxic and recalcitrant compounds present in various industrial wastes for environmental safety. Various researcher have reported that bacteria play an important role in decolorization or degradation wastewater pollutants including degradation or biotransformation of other xenobiotics compounds form the contaminated site. In pollutants removal or biotransformation process microbial enzymes are also play an dominant role.

In the ligninolytic enzyme lignin peroxidase (LiP) and manganese peroxidases (MnP), is lignin-oxidizing enzyme and are mainly obtained from lignin degrading fungi and belong to the family oxidoreductase. Laccases, which is used in the bioremediation of industrial waste and also used in various fields like biotechnology, synthetic chemistry, and in medical applications. Laccases are widely distributed in many species in fungi, higher plants, and bacteria, insect and also in lichens (Arakane et al., 2005; Riva, 2005) MnP and Laccases have been predominantly reported in fungi (Pant and Adholeya, 2009; Raghukumar et al., 2004) but at large scale applications have limitation due to slow growth rate, unfavourable submerged aquatic environment and low pH range. For the different category of waste water treatment involving bacteria is however considered to more stable as bacteria generally, tolerate a broader range of habitat and grow faster than fungi.

Microbial treatments employing pure bacterial culture have been reported frequently in past and recent years. Bacterial degradation/decolorization of industrial wastes is an eco-

friendly and cost effective alternative to chemical decomposition process of wastes minimization. The second factor hampering aerobic wastewater biotechnology is the relatively low density of the microbial biomass in the reactor. Aerobic treatment of DWW was also performed in activated sludge process and bio kinetics coefficients were evaluated (Shah et al., 1989).

Thus, this chapter contains the isolation, screening and characterization of ligninolytic enzyme producing bacteria capable for the degradation of distillery wastewater pollutants.

3.2. Material and Methods

3.2.1 Collection of DWW sample

The DWW used in this study was collected in pre-sterilized plastic Carboy container (capacity 10 L) from the anaerobic digestion Effluent Treatment Plant (ETP) of *M/s* Unnao Distilleries and Breweries (26° 33'15.92"N, 80° 30′ 38″ E), Uttar Pradesh, India (Fig. 3.1). The collected wastewater samples were brought to laboratory, and stored at stored at 4 °C. Further, the collected sample were used in physico-chemical analysis, bacterial treatment, and metabolites characterization as well as in toxicity assessment tests.

3.2.2. Media composition

In this study, a modified medium containing glucose (0.5%), peptone (0.1%), K_2HPO_4 (0.1%) and $MgSO_4.7H_2O$ (0.05%) in double distilled water was used. To this medium, DWW was added to obtain optical density 2.8 at 475 nm and pH was adjusted at 7.3 ± 0.1.

3.2.3. Extraction of melanoidins from distillery wastewater sample

For the extraction of melanoidins from the distillery wastewater use equal volume of isopropanol and distillery wastewater (1:1) by liquid-liquid extraction and the resulting pigment was air dried and used for laboratory experiments (Kalavathi et al., 2001).

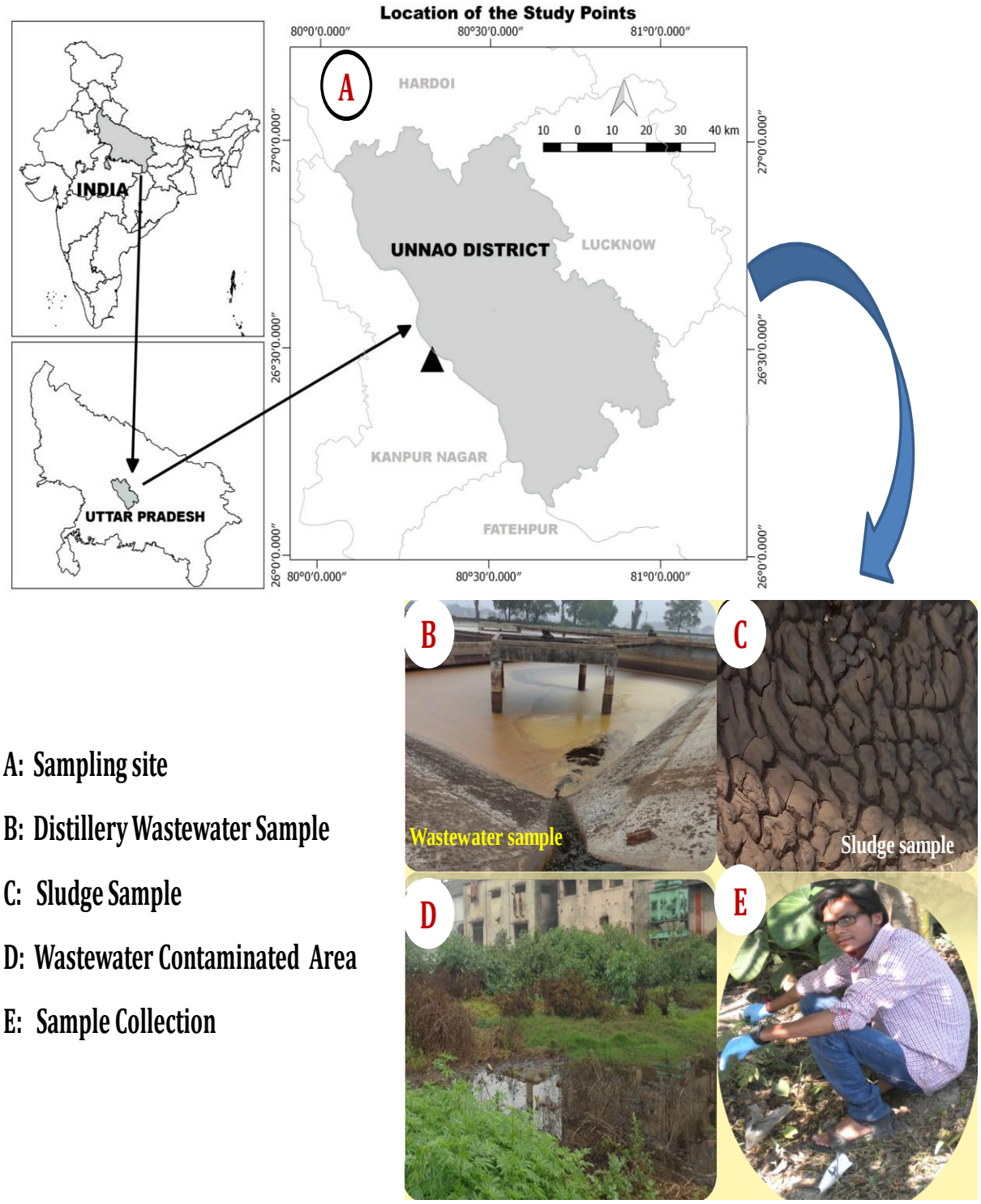


Fig. 3.1. Collection of wastewater and sludge sample from the anaerobic digestion Effluent Treatment Plant (ETP) of *M/s* Unnao Distilleries and Breweries.

3.2.4. Nutrient enrichment technique, isolation purification of distillery wastewater degrading bacteria

For the isolation of distillery wastewater degrading bacteria, sludge/soil samples collected from the distillery wastewater contaminated site *M/s* Unnao distillery and brewery, Ltd Unnao (U.P), India. In the collected sludge/soil samples have more possibility to get potential bacterial strains were used for the isolation of bacteria capable for the decolorization and detoxification of distillery wastewater pollutants.

A fraction of sludge/soil sample (5 g) was added to an Erlenmeyer flask (250 mL) containing 50 mL of autoclaved modified GPYM media with optical density 2.8 at 475 nm and pH was adjusted at 7.3 ± 0.1 followed by the incubation at 32±1 °C for seven days for enrichment (Bharagava et al., 2009; Ghosh et al., 2004). Further, the bacteria with distillery wastewater decolorizing or degrading ability were isolated from enrichment culture by serial dilution method and purified on GPYM agar plate. In addition, purity of each bacterial strains were checked by microscopic method. The above discussed method are shown in Fig. 3.2.

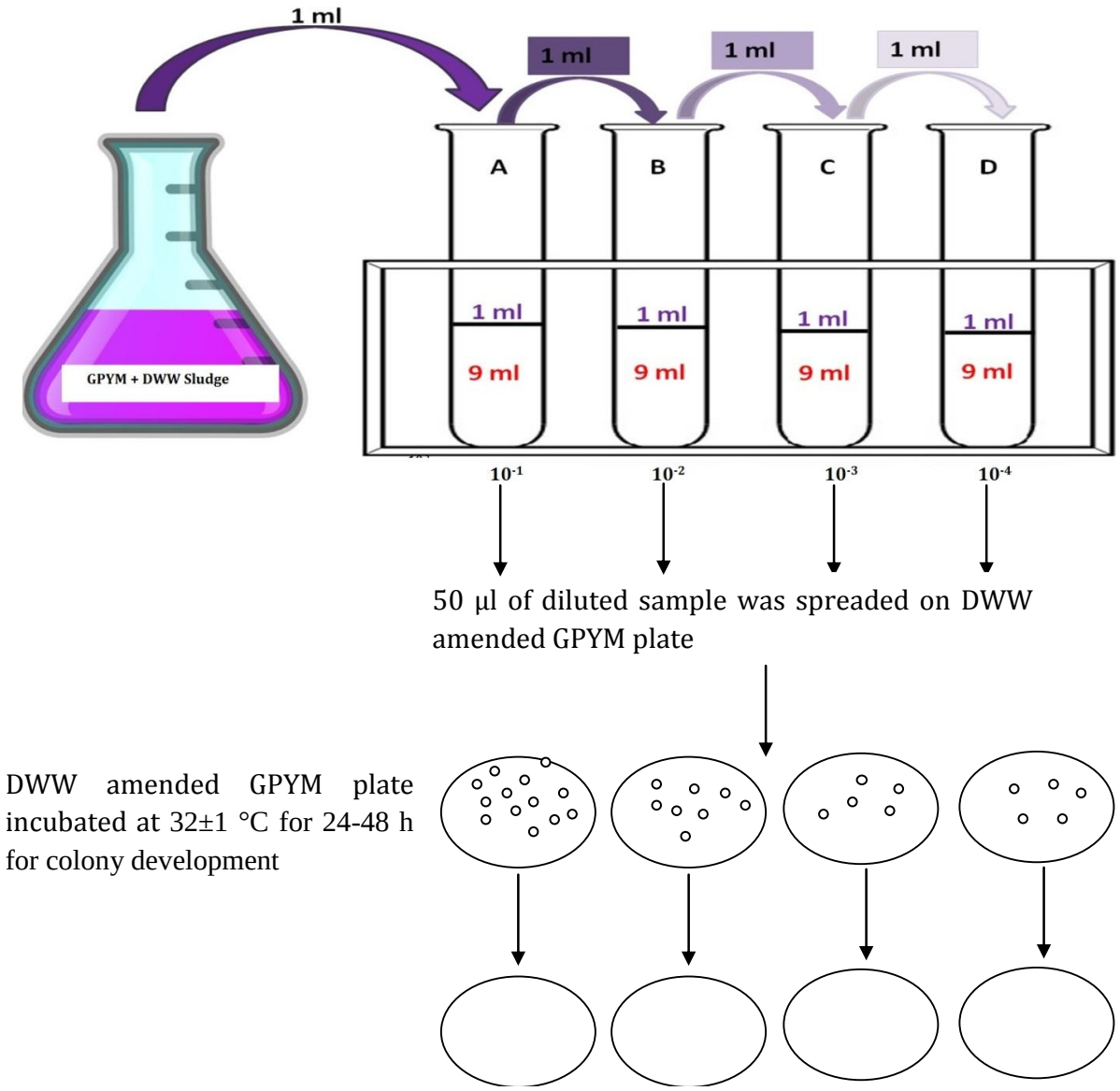


Figure 3.2. Serial dilution of sample for isolation and purification of distillery wastewater degrading bacterial strains.

3.2.5. Screening of isolated bacterial strains

Screening of isolated bacterial strain was done by two way screening process. First, in initial step of screening the pollutants degrading bacterial isolates was done by growing in Mineral Salt Medium (MSM) amended with different concentrations of distillery wastewater at pH 7.5, temperature 37 °C for 144 h incubation period (Pfennig and Lippert, 1966). The bacterial isolates growing at higher concentration of distillery

wastewater was selected as the most potential isolates for further studies. Further, these bacterial strains were also strike on different concentration of melanoidins amended agar plate. In second step of screening process, the isolated bacterial strains capable to degraded melanoidins compounds were further screened on modified GPYM agar plate amended with melanoidin 32±1 °C by nutrient enrichment techniques on basis of their growth performance and manganese peroxidase (MnP) activity (Bharagava et al., 2009). The isolated bacterial strains were grown on GPYM agar plate amendment with extracted melanoidins with optical density 2.8 at 475 nm, 1.0% agar and 0.1% phenol red (w/v) (Bharagava et al., 2012; Silva et al., 2008; Miyata et al., 2000) to visualize the manganese peroxidase activity. Further, the streaked plates were incubated at 32±1 °C for 0-120 h. The bacterial strains showing maximum or fast growth and production of manganese peroxidase were used in further experiments.

3.2.6. Morphological Characterization

3.2.6.1. Gram Staining

Introduction

The most widely used staining procedure in microbiology is the Gram stain, discovered by the Danish scientist and physician Hans Christian Joachim Gram in 1884. Gram staining is a differential staining technique that differentiates bacteria into two groups: gram-positives and gram-negatives. The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. When the bacteria is stained with primary stain Crystal Violet and fixed by the mordant, some of the bacteria are able to retain the primary stain and some are decolorized by alcohol.

Principal

The cell walls of gram positive bacteria have a thick layer of protein-sugar complexes called peptidoglycan and lipid content is low. Decolorizing the cell causes this thick cell

wall to dehydrate and shrink, which closes the pores in the cell wall and prevents the stain from exiting the cell. So the ethanol cannot remove the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan of gram positive bacteria and appears blue or purple in colour. Whereas in case of gram negative bacteria, cell wall also takes up the CV-Iodine complex but due to the thin layer of peptidoglycan and thick outer layer which is formed of lipids, CV-Iodine complex gets washed off. When they are exposed to alcohol, decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells. Then when again stained with safranin, they take the stain and appears pink in color.

Materials Required

1. Clean glass slides
2. Inoculating loop
3. Bunsen burner
4. Bibulous paper
5. Microscope
6. Lens paper and lens cleaner
7. Immersion oil
8. Distilled water
9. 18 to 24 hour cultures of organisms

Reagents

Ammonium oxalate-crystal violet stain

Solution A

Crystal violet : 20 g

Ethanol (95%) : 100 mL

Solution B

Ammonium oxalate : 1 g

Distilled water : 100 mL

Solution A + Solution B = Crystal violet

Lugol's iodine

Iodine : 5 g

Potassium iodide (KI) : 10 g

Distilled water : 100 mL

Ethyl alcohol 95%

95 mL absolute alcohol + 5 mL distilled water

Safranine

Safranine : 2.5 g

Ethyl alcohol 95% : 10 mL

Prepare Reagents

a. Gram Crystal Violet Solution: Dissolve 20 g of crystal violet in 100 mL of ethanol to make a crystal violet stock solution. Similarly, dissolve 1 g of ammonium oxalate in 100 mL of water to make an oxalate stock solution. Working solution is obtained by mixing 1 mL of the crystal violet stock solution with 10 mL of water and 40 mL of the oxalate stock solution. Store the working solution in a drop bottle.

b. Methylene Blue Solution: Dissolve 1 g of methylene blue, 90% dye content, in 100 mL of ethanol; this is Solution A. Mix 0.03 g of KOH in 300 mL of water; this is Solution B. Mixing Solutions A and B yields the working solution.

c. Gram Iodine Solution: dissolve 1 g of iodine, 2 g of potassium iodide, and3 g of sodium bicarbonate in 300 mL of water.

d. Gram Decolorizer Solution: Mix equal volumes of 95 % ethanol and acetone.

e. Gram Safranin Solution: Dissolve 2.5 g of safranin O in 100 mL of 95% ethanol to make a stock solution. Working solution is obtained by diluting one part of the stock solution with five parts of water.

Procedure

- A thin smear of bacterial culture has to be made on clean glass slide
- Allow it to air dry and fix it with flame
- Slides are placed on support for staining
- Smear should be first flooded with crystal violet and allow it for 30 sec
- Smear washed with distilled water gently
- Again stain with Gram's iodine for a min
- Washed with 95% ethyl alcohol. (Added drop wise, until no more colour flows from smear)
- Again washed with distilled water and drained properly
- The smear was finally stained with counter stain safranin for 30sec
- Again washed with distilled water and dried properly
- Observe the dried slide under low and high power objectives of compound microscope.

Interpretation

Pink for gram negative and **purple** for gram positive cells.

3.3. Result and discussion

3.3.1. Isolation and screening of bacterial strains

From the collected distillery wastewater and sludge sample nine (09) morphologically distinct bacterial strains (DS, DS1, DS2, DS3, DS4, DS5, DS6, DS7, and DS8) were isolated (Fig. 3.3) and purified by the repeated plate streak method on distillery wastewater amended GPYM agar plates.

Initially screening the pollutants degrading bacterial isolates was done by growing in Mineral Salt Medium (MSM) amended with different concentrations of distillery wastewater at pH 7.5, temperature 37 °C for 144 h incubation period (Pfennig and Lippert, 1966). The bacterial strain DS1, DS3, DS4, and DS5 were found higher resistance capability and grow at maximum concentration (500-2500 ppm) of distillery wastewater amended MSM agar plates (Table 3.1) and was selected as the most potential isolates for further studies.

Table 3.1: Maximum tolerance limit of isolated bacterial strains on modified MSM media amended with melanoidin (extracted from distillery effluent) at different concentration

S. No.	Bacterial Strains	Distillery effluent (Melanoidin containing)				
		Melanoidin (ppm)				
		500	1000	1500	2000	2500
1.	DS	+	-	-	-	-
2.	DS1	+	+	+	+	+
3.	DS2	+	+	-	-	-
4.	DS3	+	+	+	+	+
5.	DS4	+	+	+	+	+
6.	DS5	+	+	+	+	+
7.	DS6	+	-	-	-	-
8.	DS7	+	-	-	-	-
9.	DS8	+	-	-	-	-
10.	DS9	+	+	-	-	-

DS: distillery Sludge; **(+)** presence of growth; **(-)** absence of growth

Further, these bacterial were screened for their distillery wastewater degradation capability on the basis of growth manganese peroxidase activity shown on phenol red containing GPYM agar plates. This study shown that among nine isolated bacterial strains, four bacterial strain DS1, DS3, DS4, and DS5 have shown rapid growth and high MnP activity on phenol red containing GPYM agar plates amended with distillery wastewater in

terms of change in color from deep orange to yellow (Table 3.2 & Fig. 3.4) within 48 h incubation periods.

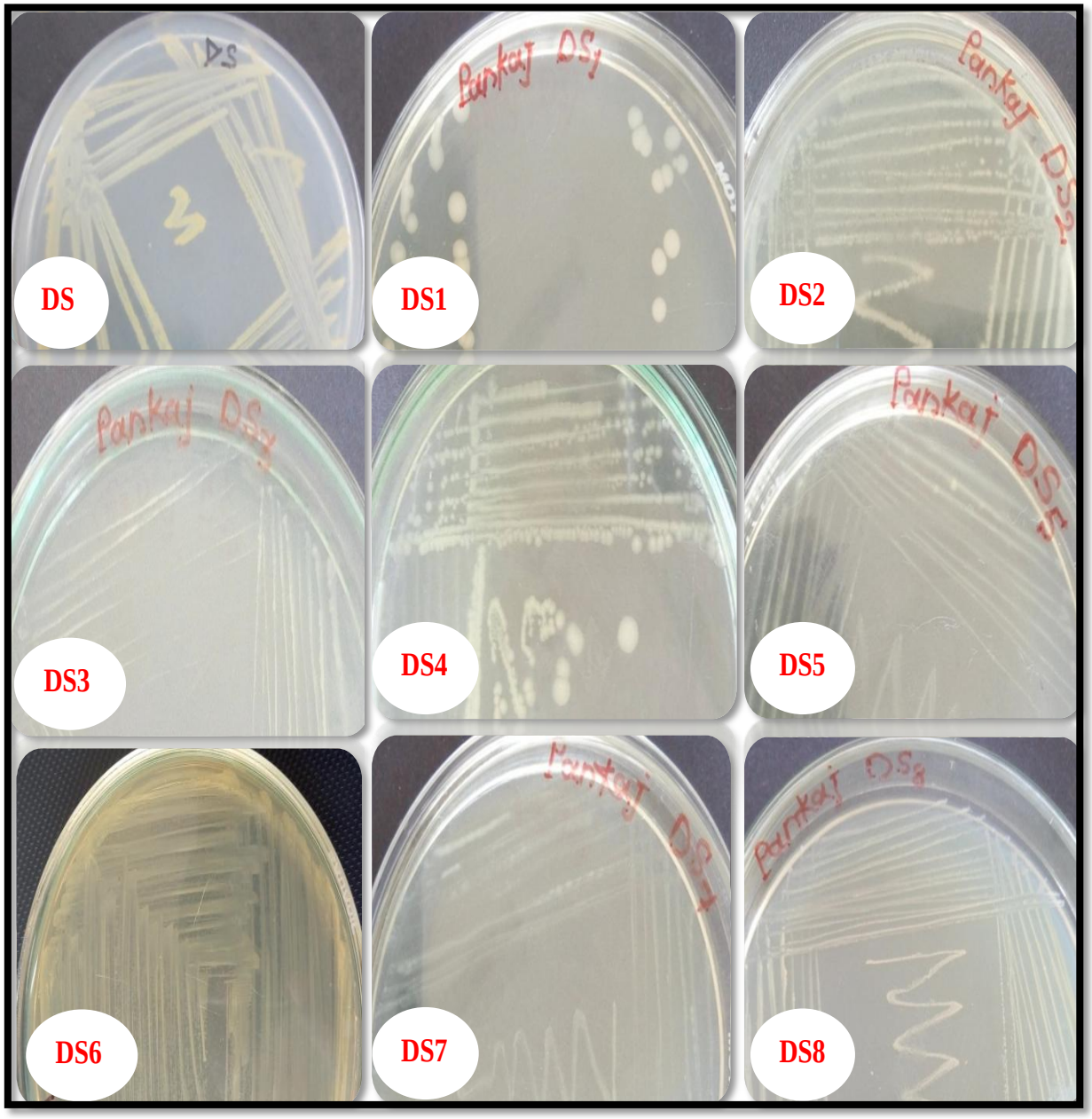


Figure 3.3. Isolation and purification of bacterial strains from distillery wastewater and sludge sample on nutrient agar plates.

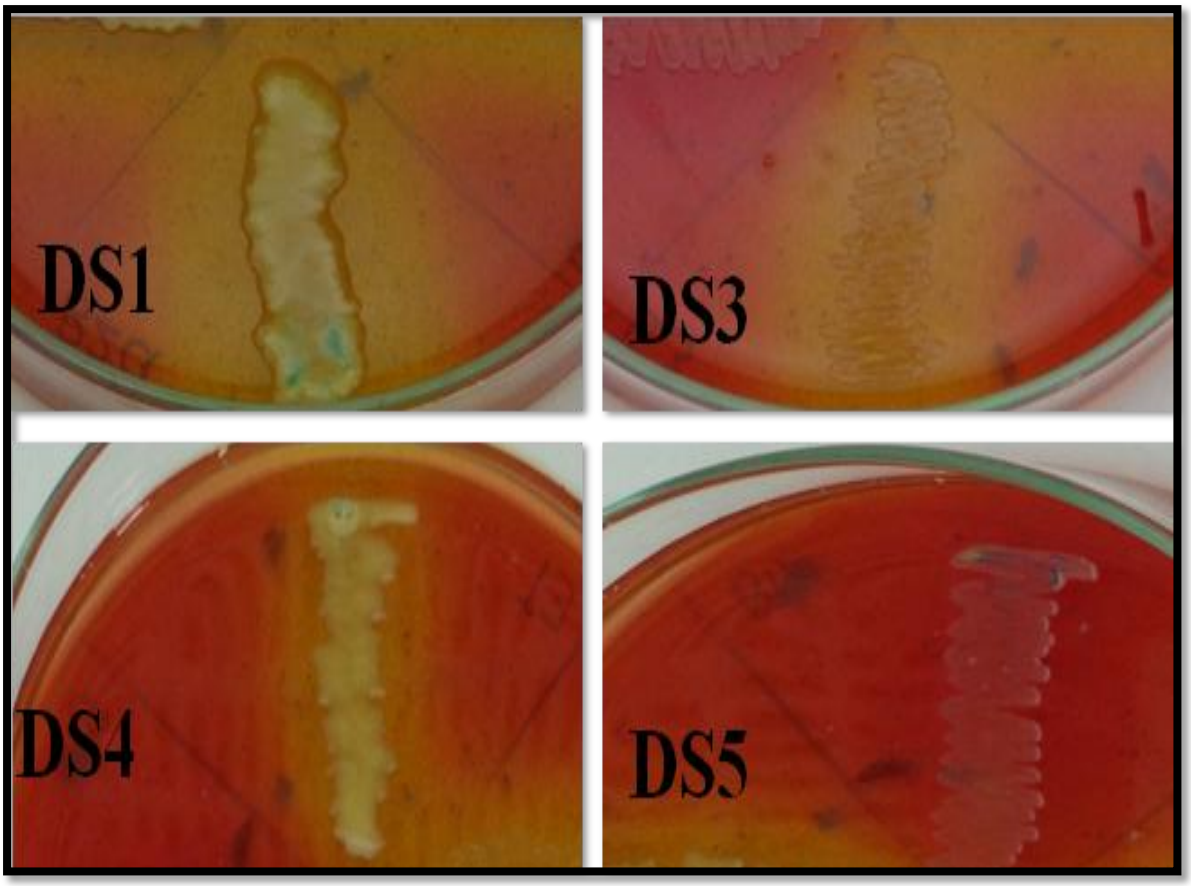


Figure 3.4. Bacterial strain (DS1, DS3, DS4, and DS5) showing MnP activity on phenol red containing modified GPYM agar plate amended with distillery wastewater.

In this study, bacterial strain DS5 showed normal growth, but moderate MnP activity on phenol red containing modified GPYM agar plate amended with distillery wastewater. Modified GPYM agar plates amended with distillery wastewater and phenol red has been used as indicator of MnP activity. Further, these bacterial strains were used for the initial biochemical identification such as morphological/microscopic study.

Table 3.2: Manganese peroxidase (MnP) activity of potential bacterial strains after different time incubation

S. No.	Bacterial strains	Enzymes	Time incubation (h)				
			24	48	72	96	120
1.	**DS1**	**MnP**	*	***	***	**	*
2.	**DS2**	**MnP**	-	**	**	*	-
3.	**DS3**	**MnP**	**	***	****	***	**
4.	**DS4**	**MnP**	**	***	***	*	*
5.	**DS5**	**MnP**	*	*	**	**	-

****very high enzyme activity; ** moderate enzyme activity; ** slower enzyme activity; *very slow enzyme activity; - no enzyme activity

3.3.2. Morphological identification of bacterial strains

The term "colony morphology" refers to the visible characteristics of a bacterial colony. Colonies that differ in appearance are typically different bacterial strains, species, or genera. However, colony morphology is not a reliable way to identify bacteria, as many different types of bacteria have similar colony morphology. This study revealed that bacterial strains DS3 and DS4 were identified as gram positive and cell was coccus and rod shape, respectively whereas DS5 were identified as gram negative and rod shape (Fig. 3.5 and Table 3.3).

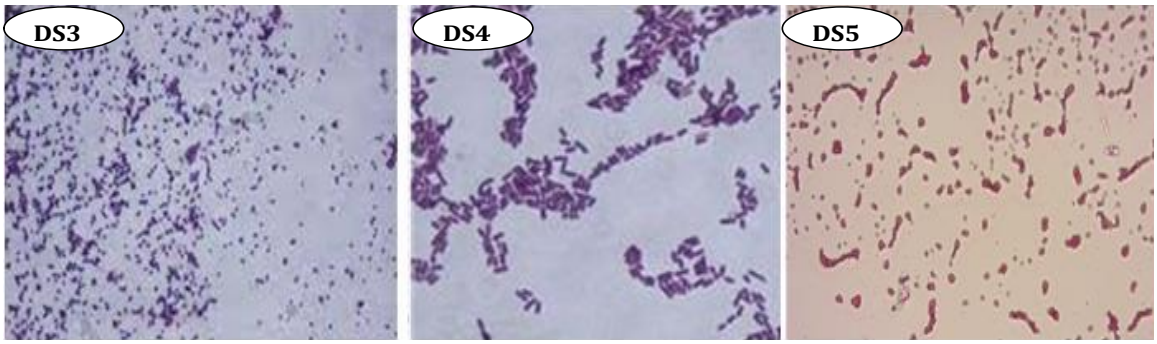


Figure 3.5. Morphological/microscopic identification of isolated bacterial strains DS3 (gram +ve), DS4 (gram +ve), and DS5 (gram -ve).

Table 3.3: Morphological characteristics of isolated bacterial strains from distillery wastes

S. No.	Characteristics	Bacterial strains		
	Morphology	**DS3**	**DS4**	**DS5**
1.	Color	White	Creamy white	Creamy white
2.	Gram stain	+ve	+ve	-ve
3.	Cell shape	coccus	rod	rod
4.	Surface texture	Normal	rough	Normal
5.	Margin	Entire	Irregular	Entire
6.	Elevation	Convex	Flat	Flat

Conclusion

The present study concluded that distillery wastes (wastewater and sludge) have many potential bacterial strains are present. Initially nine (09) bacterial strains (DS, DS1-DS8) were isolated by nutrient enrichment technique, among of these four bacterial strains DS1, DS3, DS4, and DS5 screened on the basis of their growth on different concentration of DWW and manganese peroxidase activity shown on phenol red containing GPYM agar plates amended with DWW. Further, the isolated bacterial strains were identified on the basis of morphologically/microscopically. The bacterial strains DS3 and DS4 were identified as gram positive and cell was coccus and rod shape, respectively whereas DS5 were identified as gram negative and rod shape.

Chapter 4

Development of a bacterial consortium for the degradation and detoxification of distillery wastewater pollutants

Chapter 4

4.1. Introduction

The worldwide production of bioethanol was above 50 billion litres in 2007 and over 60 billion litres in 2008 which represents almost 4% of world gasoline consumption (Sanchez and Cardona, 2008; Balat and Balat, 2009; Mussatto et al., 2010). The distillery wastewater discharged into the environment causes serious soil as well as aquatic pollution. The biochemical oxygen demand (BOD) and chemical oxygen demand (COD), the index of its polluting character, typically range between 35,000–50,000 and 100,000–150,000 mg L^{-1}, respectively (Nandy et al., 2002).

So far several studies has exposed the survival of number of organism in mixed culture conditions capable of decolorization/detoxification of distillery wastewater pollutants. Combination of bacteria (consortia) are usually used for bioremediation of several xenobiotics compounds, which are harmful for flora and fauna. The use of microbial consortia has many advantage for the application in technology development for wastewater treatment. This is because of treatment though microbial consortia no need for sterilized conditions thus decreasing tremendously the overall charge. in addition, the consortia are able to change in temperature, pH, and nature of chemical nature of waste when it compare to pure culture.

Therefore, this chapter contains the information on potential bacterial strains which were capable for the degradation of distillery wastewater pollutants.

4.2. Material and Method

4.2.1. Media composition

In this study, a modified medium containing glucose (0.1%), peptone (0.1%), K_2HPO_4 (0.1%) and $MgSO_4.7H_2O$ (0.05%) in double distilled water was used. To this medium, DWW was added to obtain optical density 2.8 at 475 nm and pH was adjusted at 7.3 ± 0.1. A bacterial consortium comprising of bacterial strains DS3, DS4 and DS5, isolated from

distillery sludge by the nutrient enrichment technique was used for the degradation and detoxification of DWW pollutants.

4.2.2. Compatibility test of isolated bacterial strains

In the compatibility test of isolated bacterial the culture were streaked on nutrient agar (NA) plates in such a way that for every single bacterial strains in the centre of the agar plate, other strains are streaked by cross previous one from the centre. In addition, in second method i.e. by formation of well, in this method single bacterial strains was spread on nutrient agar plate and remaining were inserted into the well. Further, plates were incubated at 35±1 °C for 48 h and zone inhibition was observed and recorded.

4.2.3. Development of consortia by the isolated bacterial strains

In this study, three isolated bacterial strains DS3, DS4, and DS5 that demonstrated good growth and manganese peroxidase activity on phenol red containing GPYM agar plates amended with DWW chosen to construct consortia for DWW pollutants degradation. The isolates were grown separately and inoculated in 150 mL Erlenmeyer flasks containing sterilized 50 mL in modified medium containing glucose (0.1%), peptone (0.1%), K_2HPO_4 (0.1%) and $MgSO_4.7H_2O$ (0.05%) amended with DWW and incubated at 35 ± 1°C and 110 rpm. Further, after 48 h 1 mL culture from the each flask containing cell density 3.9×10^{-4} cells mL^{-1} was used for the preparation of different bacterial combination (Bharagava and Chandra, 2010; Yadav and Chandra, 2012). The developed consortia, bacterial isolates was inoculated in modified medium and the flasks were incubated at 35 ± 1 °C at 110 rpm for 24 h.

4.2.4. Distillery wastewater decolourization by axenic culture

The distillery wastewater decolorization/degradation experiments were performed in triplicate in 250 mL Erlenmeyer flask containing 150 ml of sterile GPYM broth amended with distillery wastewater (optical density 2.8 at 475 nm). The culture containing flask

were inoculated with 1% (v/v) overnight grown axenic culture i.e. DS3, DS4, and DS5 and incubated at 35 ± 1 °C at 110 rpm (LSI-3016R & Labtech) for six (06) days. The degradation/decolorization of DWW pollutants was measured by spectrophotometer (Evolution 201, Australia) at the regular interval of 24 h in terms of bacterial growth and reduction in color intensity at 620 nm and 475 nm, respectively (Bharagava and Chandra, 2009; Kumar and Chandra, 2006). The percentage decolorization was calculated using formula given as below:

$$\textbf{Decolorization (\%)} = \frac{\textbf{Initial OD-Final OD}}{\textbf{Initial OD}} \times \textbf{100}$$

Where OD = Optical Density at 475 nm

4.2.5. Optimization of nutritional and environmental parameters

In this study, to achieve the maximum decolorization of distillery wastewater pollutants by the developed bacterial combination i.e. DS3+DS4+DS5, the degradation of distillery wastewater pollutants was studied by the supplementing GPYM broth with various carbon sources such as glucose, fructose, sucrose, galactose, mannose etc. at the concentration of 0.5% (w/v) and organic and inorganic nitrogen sources at the concentration of 0.1% (w/v). Simultaneously, various environmental conditions such as pH and temperature to achieve the maximum degradation of distillery wastewater pollutants by potential bacterial consortia. The following optimization experiment were performed in triplicate form.

4.3. Result and Discussion

4.3.1. Compatibility of bacterial strains

In this study, the selected potential bacterial strains after the screening test were checked for their compatibility from each other. The result revealed that the selected potential bacteria was compatible with each other, because after streaking on agar plate there was no inhibition zone found around the bacterial colony (Fig. 4.1). Hence, all these

potential bacterial strains was suitable for the consortium development. These bacterial strains were used further, in throughout study.

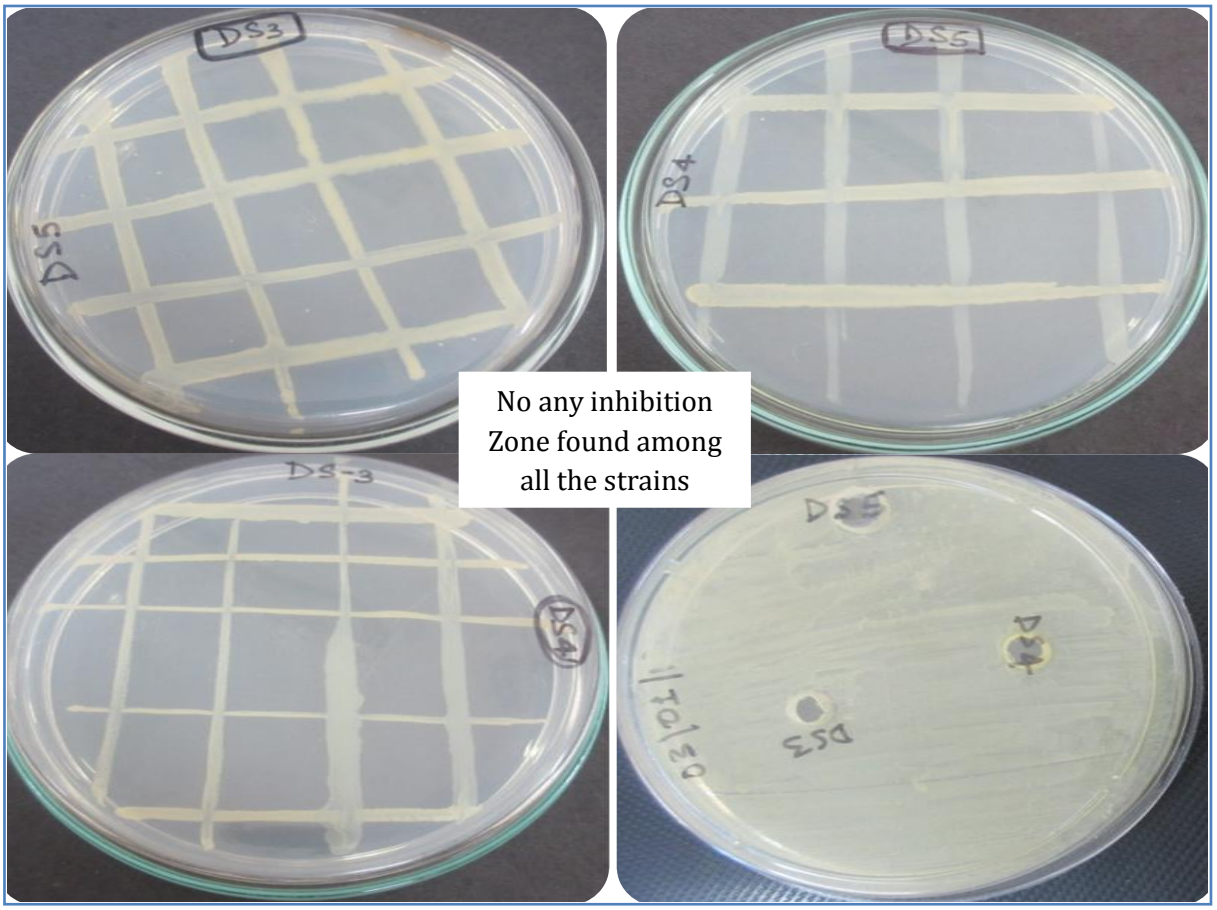


Figure 4.1. Compatibility test of selected potential bacterial strains DS3, DS4, and DS5.

4.3.2. Development of bacterial consortia

Morphologically distinct bacterial strains, having distillery wastewater pollutants decolorizing/degrading capacity, were isolated from sludge/soil samples, collected from the distillery wastewater contaminated site *M/s* Unnao distillery and brewery, Ltd Unnao (U.P), India. The initial identification of the bacterial strains was done on the basis of morphological, whereas biochemical characteristics and 16S rDNA gene sequence (detail discussed in next chapter 5). Hence, these bacterial strains were used in consortia development and for further decolorization and degradation process (Fig. 4.2).

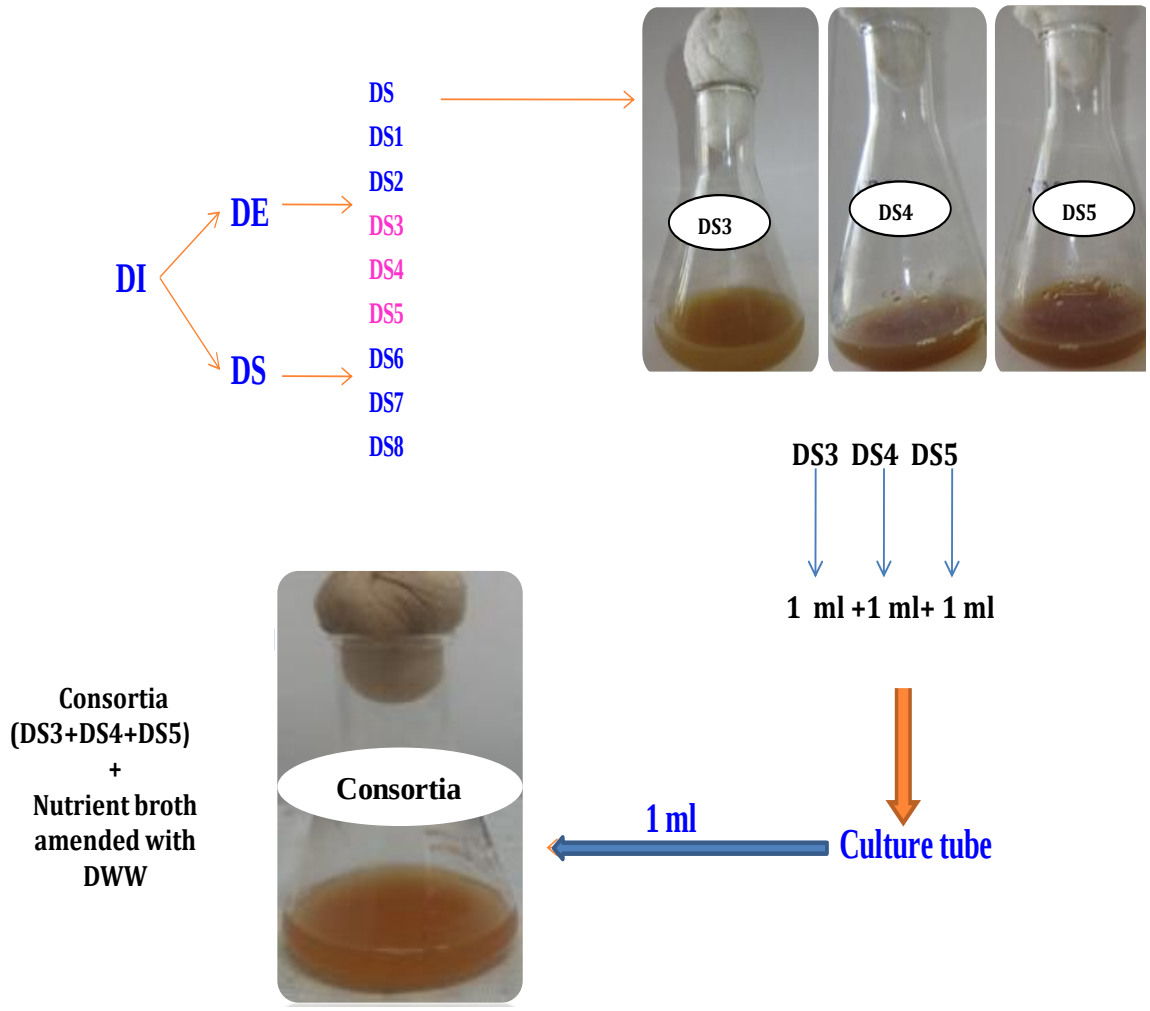


Figure 4.2. Development of potential bacterial consortia by selected bacterial strains.

DI: Distillery industry; DE: Distillery effluent; DS: Distillery sludge; DWW: Distillery wastewater

4.3.3. Decolorization assay by axenic culture

In this study, initially three bacterial strains i.e. DS3, DS4 and DS5 was used in axenic culture condition and found decolorization of DWW 52.31, 63.26, and 49.69%, respectively (Fig. 4.3) with reduction in BOD, COD values, total solids, sulfates, phosphates and phenolic content. During decolorization experiments, an increase in optical density (OD) for bacterial growth was observed indicating the fast growth of bacterial consortium, which reached to optimum at 120 h of incubation time.

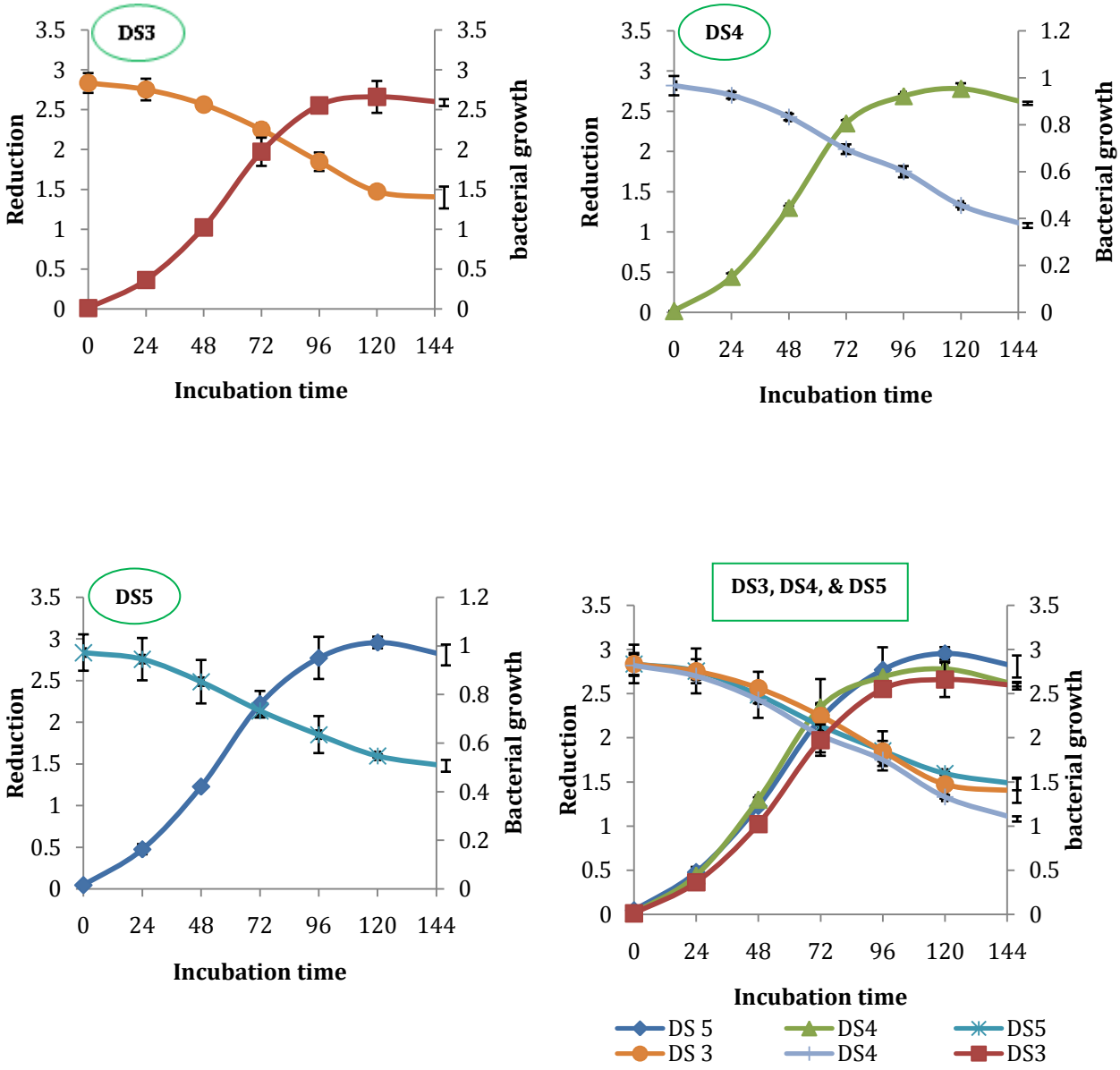


Figure 4.3. Decolorization graph of distillery wastewater by axenic culture strains DS3, DS4 and DS5.

However, initially at 24 h of incubation period, slow reduction in color intensity was observed, which might be due to the utilization of glucose (as primary nutrient source) by bacteria for their establishment and subsequently utilization of DWW pollutants as carbon, nitrogen, and energy source (Bharagava and Chandra, 2010). After it, the reduction in

color intensity was increased up to 120 h of incubation period, which might be due to the utilization of DWW pollutants as carbon, nitrogen, and energy source.

4.3.4. Optimized nutritional and environmental parameters

In this study, result revealed that the selected potential bacterial strains consortia seems to be more effective for the decolorization of distillery wastewater pollutants. During optimization process the effects of various carbon and organic and inorganic nitrogen sources and environmental parameters such as pH and temperature has been optimized for reach to optimum decolorization of distillery wastewater pollutants by developed consortia. The present study showed that the supplementation of carbon and nitrogen source is essential for the effective decolorization/degradation by bacterial consortia.

The decolorization of distillery wastewater pollutants was studied by using various carbon source (glucose, fructose, sucrose, galactose, maltose) at the concentration of 0.5% (w/v) and among these carbon source glucose was found more suitable allowing optimum decolorization 76.12% followed by starch (70.94%), sucrose (60.85%), lactose (60.29%), maltose (56.57%) at 144 h incubation period (Fig. 4.4).

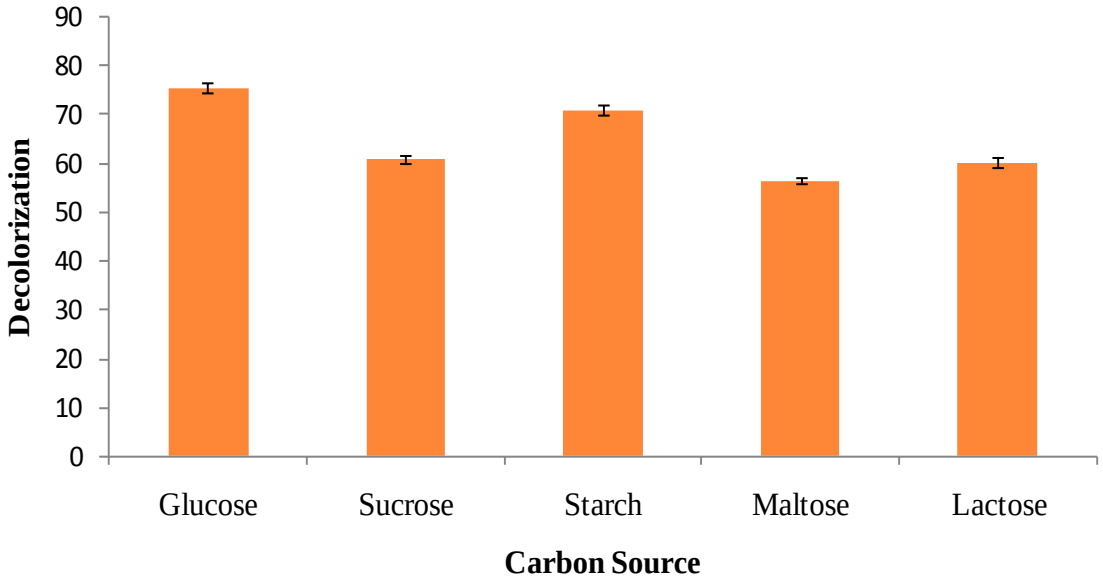


Figure 4.4. Graphical representation of decolorization of distillery wastewater pollutants by the mix bacterial consortia at different Carbon sources

However, maltose was found relatively as a poor co-substrate allowing only 56.57% decolorization of distillery wastewater pollutants. Similar finding were reported by Bharagava et al. (2009) and Bharagava and Chandra (2010), who have reported 0.5%-3.0% glucose in 7 days by the bacterial consortia.

Further, the effect of various organic and inorganic and inorganic nitrogen sources were observed for the decolourization of distillery wastewater pollutants. Among all the nitrogen sources (yeast extract, peptone, urea, ammonium sulphate, and sodium nitrate) at the concentration of 0.1% (w/v) Yeast extract found more suitable for decolourization with 76.12% followed by ammonium sulphate (68.56%), Sodium nitrate (65.28%), Peptone (63.65%), urea (57.66%) (Fig. 4.5). In similar type of study various author have reported that addition of inorganic nitrogen sources (sodium nitrate and ammonium nitrate enhance the decolorization value whereas organic nitrogen source have adverse effect on decolorization of distillery wastewater pollutants (Bharagava et al., 2009; Mohana et al., 2007; Miyata et al., 2000).

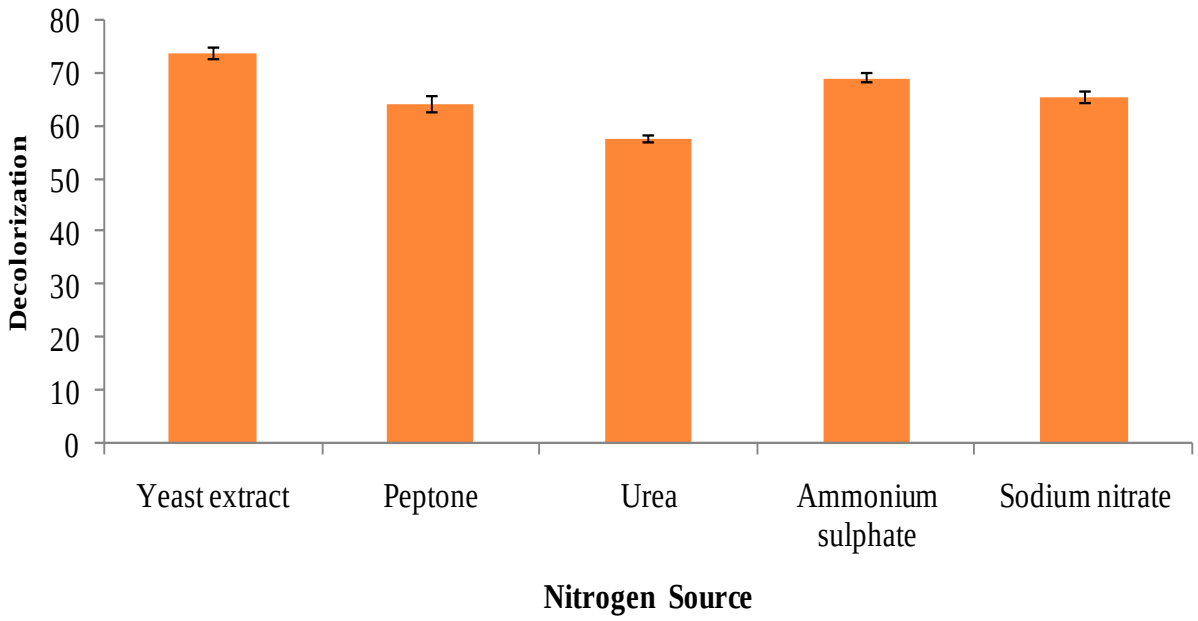


Figure 4.5. Graphical representation of decolorization of distillery wastewater pollutants by the mix bacterial consortia at different Nitrogen sources.

4.3.5. Environmental factor (pH and temperature effect on DWW pollutants decolourization

In this study, it was observed that environmental factors such as pH and temperature play an important role in the distillery wastewater pollutants decolorization/degradation. Hence, in this experiment during decolorization process bacterial consortia achieve optimum decolorization, 76.12% of distillery wastewater pollutants at pH 7.0 (Fig. 4.6A) followed by 8, 9, 6, and 5 decolorization was 64.96, 61.56, 57.35, and 39.62%, respectively. Among all the pH, 5.0 pH showed the minimum decolorization in comparison to other. Therefore, pH higher or lower than 7.0 showed reduction in decolorization percentage because the solubility of some distillery wastewater pollutants such as melanoidins is less soluble in acidic pH than in alkaline (Bharagava et al., 2009; Mohana et al., 2007).

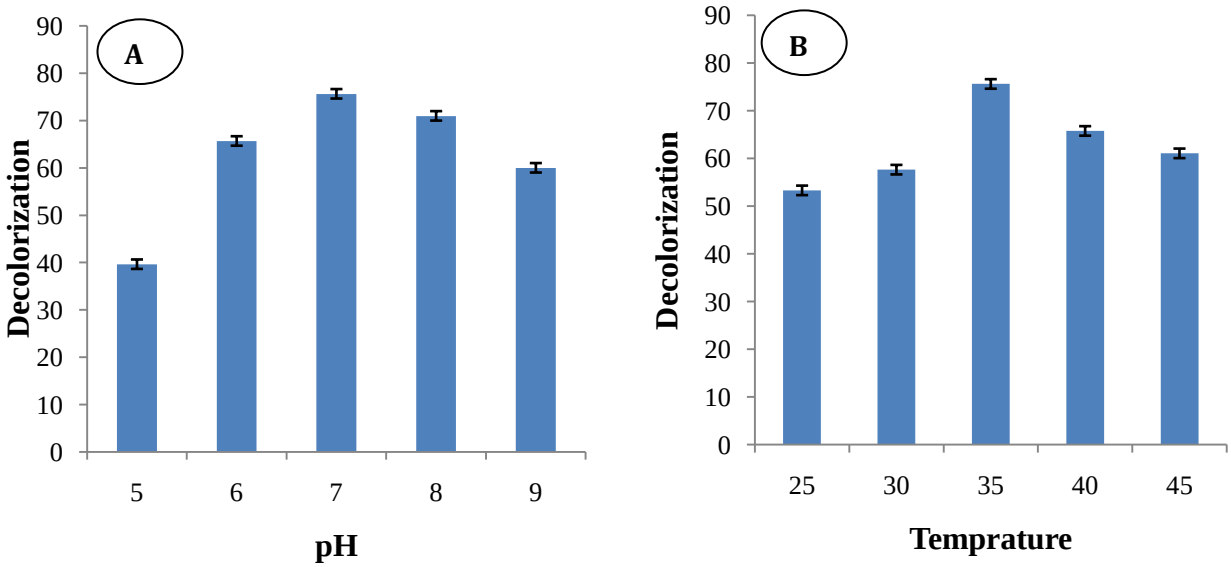


Figure 4.6. Graphical representation of decolorization of distillery wastewater pollutants by the mix bacterial consortia at different environmental conditions; (A): pH (B): and Temperature.

Conclusion

In this study, result concluded that the selected bacteria was compatible with each other, because after streaking on agar plate there was no inhibition zone found around the bacterial colony. All these potential bacterial strains was suitable for the consortium development. Further, decolorization assay in axenic conditions was not much more effective for distillery wastewater decolorization or degradation. Because in axenic culture condition selected bacterial strains i.e. DS3, DS4, and DS5 showed decolorization of distillery wastewater was 52.31, 63.26, and 49.69%, respectively. And also physico-chemical parameters was also not reduced significantly by the axenic bacterial treatment. In addition, the decolorization of distillery wastewater pollutants was also studied by the various environmental factor such as pH and temperature and several carbon source (glucose, fructose, sucrose, galactose, maltose) and nitrogen source (yeast extract, peptone, urea, ammonium sulphate, and sodium nitrate) for the optimum decolorization of distillery wastewater. Therefore, for the effective decolorization of distillery wastewater through consortia was needed.

Chapter 5

Characterization and identification of distillery wastewater pollutants degrading bacteria

Chapter 5

5.1. Introduction

The key microorganisms in biological, wastewater treatment system are the bacteria, eubacteria and archaebacteria. Detection of the difference between bacteria and archaea, these two groups of microorganism is relatively recent, and it is common for species of both groups to be referred to as bacteria. Microorganism like bacteria enter wastewater treatment system through fecal waste and as soil and water organism. This chapter also includes the effects of abiotic and biotic factors upon the organism including their activity and growth i.e. wastewater treatment effectiveness. Biological treatments units are simply biological amplifiers that is, the decolorization or degradation of waste result in an enlarge in the number of organism. As a result acceptable activity and growth of the organism or biomass is acceptable wastewater treatment.

Therefore, this chapter contains identification and characterization of selected pure bacterial isolates were performed by morphological, biochemical tests such as shape, arrangement, colonies, temperature, growth, form, margin, elevation, indole production test, methyl red and Voges-Proskauer test, citrate utilization, starch hydrolysis, urease test, hydrogen sulfide production, catalase test, lactose, glucose, sucrose fermentation tests.

5.2. Material and Method

5.2.1. Biochemical Characterization of isolated bacterial strains

The bacterial strains DS3, DS4, and DS5 were characterized morphological and biochemically as per the methods described in Cowan and Steel's Manual for Identification of Medical Bacteria (Barrow and Feltham 1993).

A. Bacterial motility test

Introduction

It is well known that bacterial strains have ability to move by itself is called motility. Motility is closely linked with chemotaxis, the ability to orientate along certain chemical

gradients. Eukaryotic cells can move by means of different locomotor organelles such as cilia, flagella, or pseudopods. Prokaryotes move by means of propeller-like flagella unique to bacteria or by special fibrils that produce a gliding form of motility. Almost all spiral bacteria and about half of the bacilli are motile, whereas essentially none of the cocci are motile.

Principle

Bacterial strains may be flagellated or non-flagellated. When they bear flagella, termed as motile bacteria then it shows motility or movement whereas those which doesn‘t have flagella, termed as non-motile bacteria and there is no motility observed.

Motility Test Medium (g/l)

Peptone	:	10 g
Meat extract	:	3 g
NaCl	:	5 g
Agar	:	4 g
Gelatin	:	80 g
Distilled water	:	1000 mL

Final pH (at 25 °C): 7.5 ±0.5

Procedure

In distilled water soaked the gelatin for 30 min, added other constituent. Heat to boiling to dissolve the medium completely. Dispense in tubes and sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. Allow tubed medium to cool in an upright position. Inoculation is done by stabbing the top of the medium to a depth of about 5 mm through the centre of the medium. Incubate at appropriate temperature for 24-48 h.

Interpretation

Non-motile organisms grow only along the line of inoculation whereas motile organisms grow away from the line of inoculation or may show growth even throughout the medium.

B. Catalase test

Introduction

Hydrogen peroxide is a by-product of respiration and is lethal if it accumulates in the cell. The enzyme catalase degrades the hydrogen peroxide in the cell before it can do any cell damage. It splits the H_2O_2 to free oxygen (bubbles) and water. Generally, the test reaction is very fast and obvious bubbles will be seen. This test is devised by Gaynon, Hunting and Esselen (1959) and is used for the primary characterization of bacteria.

Principle

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old. Bacteria thereby protect themselves from the lethal effect of Hydrogen peroxide which is accumulated as an end product of aerobic carbohydrate metabolism.

$$\mathbf{H_2O_2} \xrightarrow{\textbf{Catalase}} \mathbf{H_2O + O2}$$

- Pick the inoculum from a plate culture or slant culture and place it on a slide.
- Add one drop of H_2O_2 and look for immediate bubbling.

Interpretation

Usually most often lots of bubbles. Slight bubbles indicate a positive reaction whereas no or very few bubbles produced showed Negative result

C. Citrate Utilization test

Principle

Citrate utilisation test is used to detect the ability of an organism to utilize sodium citrate as a sole source of carbon and ammonium salt as a sole source of nitrogen. Bacteria

that grow in the medium turn the medium alkaline. This is indicated by the change of color of bromothymol blue indicator from green to blue.

Composition of Simmon's Citrate Agar

Sodium Chloride	: 5.0 g
Sodium Citrate (dehydrate)	: 2.0 g
Ammonium Dihydrogen Phosphate	: 1.0 g
Dipotassium Phosphate	: 1.0 g
Magnesium Sulfate (heptahydrate)	: 0.2 g
Bromothymol Blue	: 0.08 g
Agar	: 15.0 g
Deionized water	: 1000 mL

Procedure

Dissolve the above salts compositions in deionized water and adjust pH to 6.9. Add agar and Bromothymol blue. Further, gently heat, with mixing, to boiling until agar is dissolved. Dispense 4.0 to 5.0 mL into 16-mm tubes. Autoclave at 121 degree C under 15 psi pressure for 15 minutes. Cool in slanted position (long slant, shallow butt). Tubes was stored in a refrigerator to ensure a shelf life of 6-8 weeks.

Interpretation

Positive Reaction: Growth with color change from green to intense blue along the slant.

Negative Reaction: No growth and No color change; Slant remains green.

D. Lysine Utilization Test

Principle

The lysine decarboxylase is an enzyme that attack the carboxylic group of the amino acid lysine, with formation of amine cadaverine. The sugar contained in the medium is fermented by all the entrobacteria with consequent initial color change of indicator system from purple to yellow. The acid medium supports the amino acid decarboxylation reactions with formation of cadaverine. the amines production alkalinize the medium and

induces a new indicator color change from yellow to purple. the negative reaction is shown by the appearance of yellow color in the tube while the positive reaction is shown by a purple color.

Composition

Ingredients	**g/l**
Peptic digest of animal tissue	: 5.000
Yeast extract	: 3.000
Dextrose	: 1.000
L-Lysine hydrochloride	: 5.000
Bromocresol purple	: 0.020
Final pH (at 25°C)	: 6.8±0.2

Procedure

Suspend 14.02 g in 1000 mL distilled water. Heat, if necessary to dissolve the medium completely. Dispense 5 mL amount into screw-capped test tubes. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. Cool the tubed medium in an upright position and overlay with 2-3 mL of sterile mineral oil.

Interpretation

During the initial stages of incubation, following inoculation, fermentation of dextrose by the organisms leads to acid production, which causes a subsequent colour change of the bromocresol purple indicator to yellow. The acidic condition thus generated stimulates decarboxylase activity, which leads to decarboxylation of lysine to cadaverine. The alkaline conditions generated due to cadaverine production cause the bromocresol purple indicator (changed to yellow) to revert to purple colour. If the organisms do not produce decarboxylase enzyme, the colour of the medium remains yellow.

Positive Reaction: Appears in purple color

Negative Reaction: Appears in yellow color

E. Ornithine utilization test

Principle

Decarboxylation is the process in which bacteria that possess specific decarboxylase enzyme attack amino acids at their carboxyl end (-COOH) to yield an amine or a diamine and carbon dioxide.

Composition

Ingredients	**g/l**
L-Ornithine monohydrochloride	: 5.0
Yeast extract	: 3.0
Glucose	: 1.000
Bromo cresol purple	: 0.015
Final pH (at 25°C)	: 6.8±0.2

Procedure

Suspend 9.01 grams in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Dispense in test tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. After inoculation overlay the tubes with 2-3 mL mineral oil.

Interpretation

Positive Reaction: Appears in purple color

Negative Reaction: Appears in yellow color

F. Urease Activity

Principle

Urea is a diamide of carbonic acid. It is hydrolyzed with the release of ammonia and carbon dioxide. Many organisms especially those that infect the urinary tract, have an urease enzyme, which is able to split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form

ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.

Composition

Urea	: 20.0 g
Sodium Chloride	: 5.0 g
Monopotassium Phosphate	: 2.0 g
Peptone	: 1.0 g
Dextrose	: 1.0 g
Phenol Red	: 0.012 g
Agar	: 15.0 g
Distilled water	: 1000 mL

Preparation

- Dissolve the constituent in 100 mL of distilled water and filter sterilize (0.45-mm pore size).
- Suspend the agar in 900 mL of distilled water, boil to dissolve completely.
- Autoclave at 121 degree C and 15 psi for 15 minutes.
- Cool the agar to 50 to 55 degree C.
- Aseptically add 100 mL of filter-sterilized urea base to the cooled agar solution and mix thoroughly.
- Distribute 4 to 5 mL per sterile tube (13 × 100 mm) and slant the tubes during cooling until solidified.

Procedure of Urease Test

Initially, streak the surface of a urea agar slant with a portion of a well-isolated colony or inoculate slant with 1 to 2 drops from an overnight brain-heart infusion broth culture. Further, leave the cap on loosely and incubate the tube at 35 -37 °C in ambient air for 48 hours to 7 days.

Interpretation

Finally during the observation development of red color indicated urea hydrolysis.

G. Phenylalanine deamination

Principle

Phenylalanine deaminase test also known as phenylpyruvic acid (PPA) test is used to test the ability of an organism to produce enzyme deaminase. This enzyme removes the amine group from the amino acid phenylalanine and produces phenylpyruvic acid (PPA) and ammonia i.e. oxidative deamination of phenylalanine. Phenylalanine agar, also known as phenylalanine deaminase medium which contains DL-phenylalanine and nutrients is used as a test medium.

Composition

Ingredients	**g/l**
Yeast extract	: 3.0
Sodium chloride	: 5.0
DL-Phenylalanine	: 2.0
Disodium phosphate	: 1.0
Agar	: 15.0
Final pH (at 25 °C)	: 7.3±0.2

Procedure

Suspend 26 grams in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Dispense in tubes and sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. Allow the tubed medium to cool in a slanting position.

Interpretation

Positive test: Production of green colour (Phenylpyruvic acid thus formed reacts with ferric chloride producing a green colored compound thus turning the medium dark green

Negative: No colour change (medium remains straw/yellow color)

H. Nitrate reduction Test

Principle

Some bacteria have the ability to reduce nitrates, as they can produce the enzyme nitrate reductase. Nitrate is a poly-atomic inorganic molecule and its molecular formula is NO_3^- . Nitrate reduction may be shown either by detecting the presence of one of the breakdown products or by or by showing the disappearance of nitrate from the medium. The product of reduction may include nitrite, hyponitrite, hydroxylamine, ammonia, nitrous oxide or gaseous nitrogen. The first test to be applied aim at showing the presence of nitrite. When the test is negative (i.e. nitrite is not detected) the medium is tested to see whether there is residual nitrate, if this test is also negative it confirms that the first stage of the breakdown has been completed and the nitrite further broke down. In uninoculated nitrate broth and with cultures of organisms that do not reduce nitrate, the test for nitrite is negative until zinc dust or other reducing agent is added to the culture medium to reduce the nitrate contained in it. The zinc dust was catalyze the reduction of nitrate to nitrite chemically.

Composition

1. Peptone nitrate broth (KNO_3 1 g + Nutrient Broth 1000 mL)
2. Test culture
3. Reagent A-Sulphanilic acid
4. Reagent B- α-naphthylamine
5. Zinc dust

Dissolved KNO_3 in broth, distributed into test tubes containing inverted inner Durham tubes and sterilized at 115 °C for 20 min, cooled, inoculated and incubated for up to 5 days. Observed any gas formation in the inverted Durham tubes. Added 1 mL of nitrite reagent A followed by 1 mL of reagent B.

Test reagent

Solution A: 0.33% Sulphanilic acid in 5 N acetic acid and it was dissolved by gentle heating.

Solution B: 0.6% Dimethyl α-naphthylamine in 5 N acetic acid.

Or 0.5% α-naphthylamine in 5 N acetic acid

Or 0.13% 1-naphthylamine- 7- sulphuric acid

Dissolved by gentle heating, 10% zinc dust suspended in 1% methyl cellulose solution.

Interpretation

- Development of red colour is observed after addition of reagents then it indicates positive nitrate reduction test.
- Addition of both reagents there is no red colour formation then it may indicate negative nitrate reduction test or false result because there is a possibility that the microflora present in the tube may reduce the nitrate to other byproducts like ammonia, molecular nitrogen, nitrous oxide, nitric oxide and as the test reagents detects only nitrates so it can give a false negative result so to clear this doubt a pinch of zinc dust is added and tube is observed for red colour development .Here zinc ions reduce nitrates to nitrites and we can observe development of red colour as zinc ions confirms presence of residual nitrates and this development of red colour indicates negative nitrate reduction test.

I. H_2S production

Principle

An iron compound and a sulfur compound are included to test for the production of hydrogen sulfide gas. Hydrogen sulfide is produced if the sulfur compound is reduced by the bacterial strain. This happens when the strain either degrades the amino acid cysteine during protein degradation, or when anaerobic respiration shuttles the electrons to sulfur instead of to oxygen. In either case H_2S is produced (hydrogen sulfide gas) which reacts

with the iron compound to form the black precipitate of ferric sulfide. The black color acts as an indicator for the presence of hydrogen sulfide. (If the tube becomes largely black, it may be difficult to read the tube for other tests.)

H_2S production by Triple Sugar Iron Agar (g/l)

Composition

Ingredients	**g/l**
Meat	: 3 g
Yeast extract	: 3 g
Peptone	: 20 g
Glucose	: 1 g
Lactose	: 10 g
Sucrose	: 10 g
$FeSO_4.7H_2O$	: 0.2 g
Or	
Ferric citrate	: 0.3 g
NaCl	: 5 g
$Na_2S_2O_3.5H_2O$	: 0.3 g
Agar	: 20 g
Distilled water	: 1000 mL
Phenol red, 0.2% aq. solution	: 12 mL

All solids were dissolved in water by heating and indicator solution was added, mixed well and dispensed into tubes. Further, these solution were sterilized at 115 °C for 20 min and cooled to form slopes with deep butts about 3 cm long. Cut filter paper strips 5-10 mm wide and 50-60 nm long and impregnated with appropriate solution, dried at 50-60 °C and stored in a screw capped containers.

Detection of H_2S

Lead acetate hot saturated aq. solution (10 g lead acetate in 100 mL hot water). Inoculated a tube of triple sugar iron agar by stabbing the butt and slopes were streaked

with bacterial strains, observed daily for up to 7 days for blacking of the butt only due to H_2S production. Some organism produce a dark pigments on the slope only and this should be mistaken for a positive result. Inoculated the organism in nutrient broth or peptone water and inserted a lead acetate paper between the plug and tube and examined daily for 7 days for blacking of the paper.

A yellow (acidic) color in the slant and butt indicates that the organism being tested ferments dextrose, lactose and/or sucrose.

A red (alkaline) color in the slant and butt indicates that the organism being tested is a nonfermenter.

Hydrogen sulfide production results in a black precipitate in the butt of the tube. Gas production is indicated by splitting and cracking of the medium.

J. Glucose test

Principle

A metabolic process performed by almost all types of bacteria is known as fermentation. This will result in the production of ATP, the ultimate energy source of the organism. This will happen either in the presence or absence of atmospheric oxygen. Bacteria utilize the nutrients in their environment to produce ATP for their biological processes such as growth and reproduction. The enzyme systems in bacteria allow them to oxidize environmental nutrient sources. Bacteria will use different energy sources in the medium depends on the specific enzymes of each bacteria. Many bacteria possess the enzymes system required for the oxidation and utilization of the simple sugar, glucose. Some bacteria have the ability to degrade complex carbohydrates like lactose, sucrose or even polysaccharides. Such bacterium should possess the enzymes that should cleave the glycosidic bonds between the sugar units and the resulting simple carbohydrate can be transported into the cell. Lactose is a disaccharide consisting of the glucose and galactose

connected by glycosidic bond. The bacteria which produce the enzyme lactase will break this bond and thus release free glucose that can be easily utilized by the organism. The characteristics feature of the enzyme production in the bacteria enables them to use diverse carbohydrates and this will aid in the identification of unknown bacteria. Fermentation is best described by the degradation of glucose by Embden- Meyerhof pathway or Glycolytic pathway

Materials required

Phenol Red Carbohydrate Fermentation Broth.

Bacterial culture.

Inoculation loop.

Incubator(37 °C).

Ingredients	**Quantity**
Trypticase	: 1.1 g
Carbohydrate	: 0.5g
Sodium Chloride	: 0.5g
Phenol red	: 0.0189 mg

Preparation and Procedure

- Weigh and dissolve trypticase, Sodium chloride, and Phenol red in 100 mL distilled water and transfer into conical flasks.
- Add 0.5% to 1% of desired carbohydrate into all flasks.
- Insert inverted Durham tubes into all tubes, the Durham tubes should be fully filled with broth.
- Sterilize at 115 °C for 15 minutes.
- Aseptically inoculate each labeled carbohydrate broth with bacterial culture.(keep uninoculated tubes as control tubes).
- Incubate the tubes at 18-24 hours at 37 °C.

- Observe the reaction.

Interpretation

Acid production: Changes the medium into yellow color-organism ferments the given carbohydrate and produce organic acids there by reducing the pH of the medium into acidic.

Acid and Gas production: Changes the medium into yellow color-organism ferments the given Carbohydrate and produce organic acids and gas. Gas production can be detected by the presence of small bubbles in the inverted durham tubes.

Absence of fermentation: The broth retains the red color. The organism cannot utilize the carbohydrate but the organism continues to grow in the medium using other energy sources in the medium.

5.2.2. Molecular characterization of bacterial isolates

5.2.2.1. Genomic DNA Isolation

For the genomic DNA isolation of isolated bacterial strains, each bacterial culture was grown in distillery wastewater amended GPYM broth for 48 h incubation period. The total genomic DNA from overnight grown cultures was isolated following the alkaline lysis method (Kapley et al., 2001). About 5 mL of each log phase bacterial culture was centrifuged at 5,000 rpm for 5 min at 4 °C to pellet the bacterial cells. The obtained bacterial pellet was washed with 200 µL of 10 mM phosphate buffer (pH 7.0) and after washing pellet was resuspended in 200 µL of 0.5 N NaOH by gentle vortaxing to lyses the bacterial cells. The lysate was neutralized by adding the 100 µL 1M Tris (pH 7.5) and centrifuged at 10,000 rpm for 15 min at 4 °C to get pellet from the bacterial cell debris. The supernatant was taken and adjusted to the volume upto 500 µL. The obtained supernatant was clear and colorless, stored at -20 °C and used for further studies.

5.2.2.2. PCR amplification and cloning of 16 S rDNA gene

About 5 µL of genomic DNA was used to amplify the 16S rDNA gene using universal eubacterial primers (27F) 5'-AGAGTTTGATCMTGGCTCAG-3' and (1492R) 5'-CGGTTACCTTGTTACGACTT-3' (Narde et al. 2004) and a 1,500 bp product was amplified. The reaction mixture contained 100 ng template, 1x PCR buffer, 200 µM of each dNTP, 3.0 mM $MgCl_2$, 25 pmol of primer, and 2.5 units of Amplitaq DNA polymerase (Perkin Elmer) in a final reaction volume of 50 µL. The thermocycling steps (Veriti® 96-Well Thermal Cycler, Applied Biosystems, USA) used were as 30 cycles of denaturation at 94 °C for 1 min, followed by annealing at 45 °C for 1 min and extension at 72 °C for 2 min.

The PCR amplified 16S rDNA gene product were electrophoresed through 1.2% (w/v) agarose gel in 1X TAE buffer using 1 Kb DNA ladder (Merk, Biosciences, India) as molecular weight marker and visualised by staining with ethidium bromide (EtBr). The PCR products were gel purified using gel extraction kit (Merk, Biosciences, India) and sequenced using primer 27F. The restriction digestion of purified plasmid DNA made with 10 units of EcoR1 for 2 h at 50 °C in 1X reaction buffer containing 60 mM Tris Tris-Cl, pH 7.9, 1.5 M NaCl, and 60 mM $MgCl_2$ has shown the presence of an expected ~ 1.5 kb insert when subjected to agarose gel electrophoresis.

5.2.2.3. 16S rDNA gene sequencing and phylogenetic tree analysis

The PCR products were gel purified using gel extraction kit (Merk, Biosciences, India) and sequenced using primer 27F and 1492R as described in above section. The PCR amplified product i.e. 16S rDNA gene were gel purified and used as template in sequencing reactions using primer 27 F, carried out using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). The partial sequences obtained were subjected to BLAST analysis using the online option

available at www.ncbi.nlm.nih.gov/BLAST (Altschul et al., 1997) suggesting the identity of bacterial isolates. The phylogenetic tree was constructed by using the neighbour-joining method using Kimura-2-parameter distances in MEGA-4 software and the 16S rDNA sequences obtained was also deposited in GenBank databases under the accession number MF182113, MF967441, and MF182114 for bacterium DS3, DS4 and DS5, respectively.

5.2.3. Detection and characterization of ligninolytic enzymes (laccase and MnP)

5.2.3.1. Preparation of cell free extract

For the obtained bacterial cell free extract strained were grown in 250 mL Erlenmeyer flasks containing 100 mL of medium, pH 7.0 and incubated at 35±1 °C for 24 h. After the incubation period, a sample of 2.0 mL was withdrawn from the flask and harvested by centrifugation at 10,000 rpm at 4 °C for 15-20 min. Further, the obtained supernatant was directly used as extracellular enzymes in order to determine the enzymatic status. These harvested cells (75 mg/mL) were suspended in a potassium phosphate buffer (50 mmol/L) at pH 7.4 and sonicated (Sonics-Vibracell ultrasonic processor, USA), keeping sonifier output at 40 (amps) and giving 7 strokes after each of 30 sec, with 1 min interval at 4 °C. The homogenate was centrifuged at 10,000 rpm for 20 min and supernatant was used as a source of crude enzyme.

The culture supernatant containing crude enzyme (MnP and laccase) was centrifuged at 5,000 rpm for 10 min to remove bacterial biomass. The obtained supernatant was concentrated about 10-fold by passing through a column filled with anhydrous Na_2SO_4. Further, the cold acetone (-20 °C) was added to this crude enzyme solution to give 75% saturation and precipitated protein were collected by centrifugation at 15,000 rpm for 30 min. After removing the acetone, the precipitated proteins were dissolved in the minimal volume of 50 mM malonate buffer, pH 4.5. To purify the soluble proteins were passed

through column (80 cm × 2.0 cm) containing sephadex G-100 previously equilibrated with same buffer and the protein fraction (2.0 mL) eluted at the flow rate of 0.5 mL min^{-1} and stored at -20 °C for further experiments.

5.2.3.2. SDS-PAGE and molecular weight determination of ligninolytic enzyme

The molecular weight of ligninolytic enzyme laccase and MnP was determined by the Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) unit (GX-SCZ2, Genetix Biotech Asia Pvt Ltd) by using 10% polyacrylamide in gel. The constituent of separating gel and stacking gel and the regent preparation has been discussed as below:

(i) Acrylamide-bisacrylamide stock solution

Acrylamide : 29.2 g

N, N' - Methylene bisacrylamide : 0.8 g

Distilled water : 100 mL

Dissolved 29.2 g of acrylamide and 0.8 g of N, N' - Methylene bisacrylamide in 100 mL of distilled water, stirred at magnetic stirrer till complete dissolution. Further, filtered and stored at 4 °C in dark colored bottle.

(ii) SDS, 10%

SDS : 10 g

Distilled water : 100 mL

Dissolved 10 g of SDS in 100 mL of distilled water and kept at 37 °C for complete dissolution.

(iii) Stacking gel buffer (1 M), (pH 6.8)

Tris-HCl : 12.14 g

Distilled water : 100 mL

Dissolved 12.14 g of Tris-HCl in 100 mL distilled water and adjusted pH 6.8 with the help of 0.5 N HCl.

(iv) Resolving/Separating gel buffer (1.5 M), (pH 8.8)

Tris buffer : 18.21 g

Distilled water : 100 mL

Dissolved 18.21 g of Tris buffer in 100 mL of distilled water and adjusted pH 8.8 with the help of 0.5 N HCl.

(v) Electrophoresis buffer/ Tris-glycine buffer (5X), (pH 8.3)

Tris base : 7.55 g

Glycine : 47.0 g

SDS : 2.5 g

Distilled water 500 mL

Dissolved 7.55 g of Tris-base, 47.0 g of glycine and 2.5 g of SDS in 500 mL of distilled water slowly with the help of magnetic stirrer and adjusted pH 8.3 with the help of 0.5 N HCl and used 1X buffer for electrophoresis.

(vi) Sample buffer/Protein loading dye (5X)

250 mM Tris-HCl (pH 6.8) : 2.5 mL 1M

10% SDS : 1 g

0.5% bromophenol blue : 50 mg

50% Glycerol : 5025 mL (60%)

500 mM β-ME : 340 µL

Distilled water : 1.91 mL

Dissolved all the component except β-ME because it degrades within few days. It should be add freshly during sample loading.

(vii) Staining solution

Methanol : 50 mL

Glacial acetic acid : 10 mL

Coomasie Brilliant Blue R-250 (CBRR-250) : 0.1 g

Distilled water : 40 mL

Dissolved 0.1 g of CBBR-250 dye to 100 mL of solution containing 50 mL of methanol, 10 mL of glacial acetic acid and 40 mL of distilled water, shaken slowly to get dissolve the dye and filtered with Whatman No. 42 filter paper.

(viii) Distaining solution

Methanol : 10 mL

Glacial acetic acid : 7 mL

Distilled water : 83 mL

Mixed all the components and stored at room temperature.

(ix) Ammonium per sulphate (APS): 10%

(x) TEMED

Composition of resolving/separating and stacking gel

(i) Resolving gel for SDS-PAGE (10%): 25 mL

H_2O : 9.90 mL

30% Acrylamide : 8.30 mL

1.5 M Tris-buffer : 6.30 mL

10% SDS : 0.24 mL

10% APS : 0.24 mL

TEMED : 0.02 mL

(ii) Staking gel buffer (5%): 5 mL

H_2O : 3.40 mL

30% Acrylamide : 0.83 mL

1.5 M Tris-buffer : 0.63 mL

10% SDS : 0.05 mL

10% APS : 0.05 mL

TEMED : 0.01 mL

Note: APS and TEMED were added just before the pouring of gel solution as TEMED initiate the cross linking of gel.

Sample preparation

- Equal volume (20 µL) of sample buffer (2X) and partially purified enzyme laccase and MnP was taken in an autoclaved eppendrof tube, mixed well and boiled for 5 min in water bath.
- After cooling at room temperature, the samples were centrifuged at 5,000 rpm for 5 min at 4 °C.

Procedure

- Clean and completely dry the glass plates, combs, and any other pertinent materials.
- Place a short plate on top of a spacer plate. Insert both plates into the casting frame on a flat surface. Be sure that the "legs" of the casting frame are down. Clamp the casting frame and check that the plates are level on the bottom.
- Put the casting frame into the casting stand
- Prepare a separating gel in a separate small beaker.
- Add separating gel to a side arm flask, stopper the flask and attach to a vacuum pump equipped with a cold trap. Turn on the vacuum and degas the solution for ~ 10 min. During this period, gently swirl the solutions in the flask.
- Exit the vacuum, open the flask and add 100 µL of ammonium persulfate and 10 µL of TEMED to the solution
- Allow it to polymerize for 20-30 min at room temperature.

- Remove the overlaying solution and wash the gel 2-3 times with distilled water, now pour the staking gel solution (5%) prepared, insert the comb and let it again polymerize.
- Remove the comb and assemble the glass plates containing gels in electrophoresis chamber and filled with electrophoresis buffer.
- In next step load the samples into wells and protein markers into the first lane.
- Fill the region outside of the frame with 1X electrophoresis buffer.
- Cover the tank with the lid aligning the electrodes (black or red) appropriately.
- Connect the electrophoresis tank to the power supply.
- Allow the samples to run at 30 m Amp at the constant voltage 70 until the dye front reaches the bottom of the gel. This can take as long as 1 h.
- When electrophoresis is complete, turn off the power supply and disassemble the apparatus.
- Finally the protein bands were stained with Coomasie Brilliant Blue R-250 dye for overnight and destined with destining solution till band become clear.
- The molecular weight was estimated by comparing with standard protein marker (Molecular Standard Mixture Recombinant, 15-150 kDa; Sigma). The gel visualized under white light and stored in gel documentation system.

5.3. Result and Discussion

5.3.1. Biochemical Characterization of isolated bacterial strains

The isolated bacterial strains from distillery wastewater and sludge samples initially identified on the basis of morphological observation and were characterized as DS3 and DS4 as gram positive (+ve) coccus and rod shape, respectively whereas DS5 as gram negative (-ve) and rod shape, detailed discussed in Chapter 03. Bacterial strains DS3, DS4, and DS5 were giving positive test for motility. Bacterial strains DS3 and DS5 were giving

negative test for catalase, sorbitol whereas DS4 gives positive test but both the strains i.e. DS3 and DS5 have shown the positive test for ornithine utilization. Further, DS3, DS4, and DS5 have shown the negative test for citrate utilization, H_2S, lactose fermentation test. Whereas these strains i.e. DS3, DS4, and DS5 have shown the positive test for urease test but showed variable test for the lysine utilization. In addition, DS3 and DS5 have shown negative test for arabinose whereas DS5 shows variable test. Bacterial strains DS3 have shown the positive test for nitrate reduction but DS4 and DS5 showed the negative nitrate reduction test (Table 5. 1 and Fig. 5.1).

Table 5.1: Secondary biochemical results of isolated potential bacterial strains for initial identification

S. No.	**Biochemical test**	**Bacterial strains**		
		DS3	**DS4**	**DS5**
1.	Catalase	+ve	+ve	+ve
2.	Indole test	+ve	-ve	-ve
3.	Amylase test	+ve	+ve	-ve
4.	Citrate utilization	-ve	-ve	-ve
5.	Lysine utilization	V	V	V
6.	Ornithine utilization	+ve	V	+ve
7.	Urease	+ve	+ve	+ve
8.	Phenylalanine deamination	V	-ve	-ve
9.	Nitrate reduction	+ve	-ve	-ve
10.	H_2S production	-ve	-ve	-ve
11.	Glucose	+ve	+ve	-ve
12.	Adonitol	-ve	-ve	-ve
13.	Lactose	-ve	-ve	-ve
14.	Arabinose	-ve	V	-ve
15.	Sorbitol	-ve	+ve	-ve

(+ve): positive; -ve: (negative); V: variables results

Figure 5.1. Various biochemical reaction shown by isolated potential bacterial strains DS3, DS4, DS5; A: Motility Test; B: Citrate Test; C: Lysine Test; D: Urease Test; E: H_2S Test; F: Catalase Test; G: Indole Test; H: Amylase Test; I: Biochemical Kit (For other Biochemical Test).

5.3.2. 16S rDNA gene sequence analysis

Further, the PCR amplified 1500 bp long 16S rDNA gene (Fig. 5.2A) sequences obtained from DS3, DS4 and DS5 bacterium have shown the closest relatedness with that of *Staphylococcus saprophyticus* strain ATCC15305, *Bacillus megaterium* strain and uncultured *Alcaligenaceae bacterium* clone De 110a, respectively. The phylogenetic tree was constructed by using the neighbour-joining method using MEGA version 4.0 software (Tamura et al., 2007) and ten *Staphylococcus* sp., one *Bacillus* sp., and five *Alcaligenaceae* sp., from GenBank (http://www.ncbi.nlm.nih.gov) (Fig. 5.3). In addition, some non-related sequences were also included in tree as control to demonstrate the linkage distance between the isolates. Hence, based on the 16S rDNA sequence similarity, the bacterial strains DS3, DS4 and DS5 were identified as *Staphylococcus saprophyticus*, *Bacillus megaterium sp.* and *Alcaligenaceae* sp. Further, the partial 16S rDNA gene sequences of DS3, DS4, and DS5 were also submitted to GeneBank public data base and an accession number MF182113, MF967441 and MF182114, respectively.

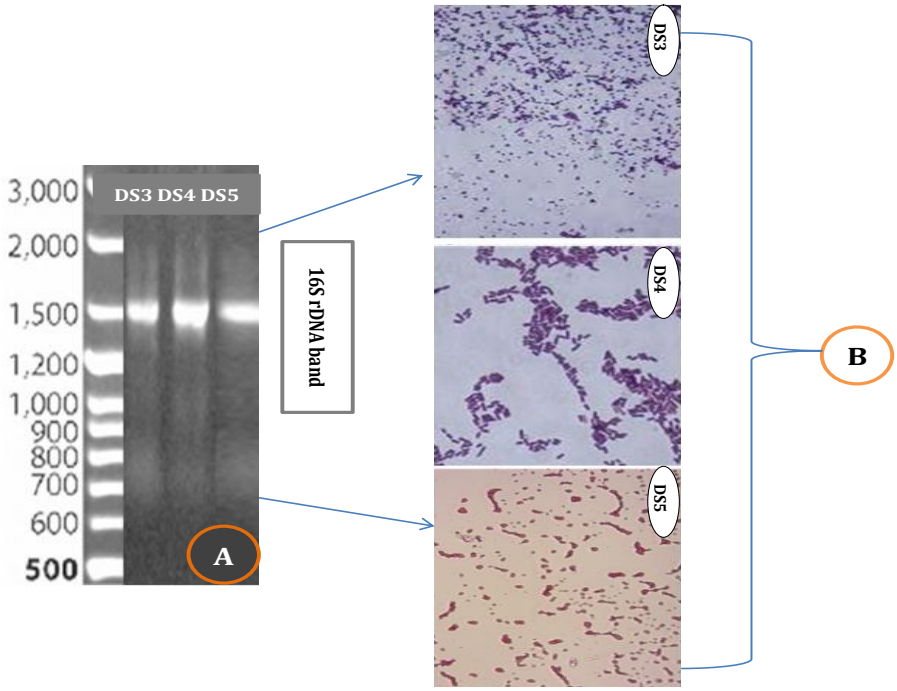


Figure 5.2. (A): PCR amplified 16S rDNA gene band isolated bacterial strains; (B): and morphological characteristics of isolated bacteria strains DS3, DS4, and DS5.

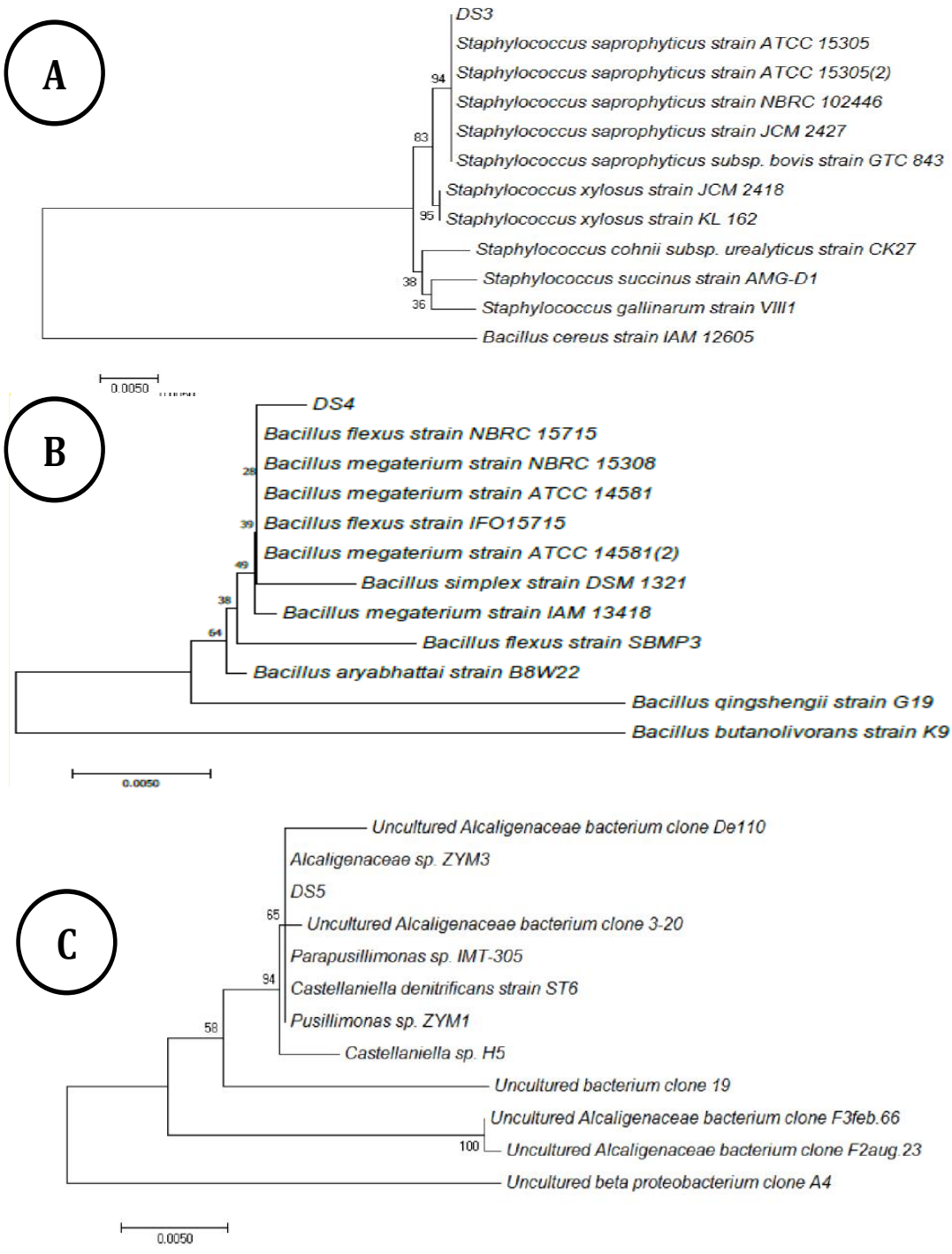


Fig. 5.3. Phylogenetic tree of isolated bacterial strains and their related species based on 16S rDNA gene sequence (A): DS3; (B): DS4); (C): DS5

Hence, in this study based on the 16S rDNA sequence similarity, bacterial strains DS3, DS4, and DS5 were identified as *Staphylococcus saprophyticus, Bacillus megaterium sp.* and *Alcaligenaceae* sp. with accession number MF182113, MF967441 and MF182114, respectively (Bharagava et al., 2009; Bharagava and Chandra, 2010).

5.3.3. Molecular weight determination of ligninolytic enzyme

Result revealed that among nine isolated bacterial strains DS3, DS4, and showed MnP activity by changing the colour from deep orange to light yellow at DWW amended modified media (discussed in previous chapter). Further, the denaturing SDS-PAGE electrophoresis of partially purified enzyme has yield band of laccase and MnP with the molecular weight ~65 and 43 kDa, respectively in all three bacterial strains (Fig. 5.4).

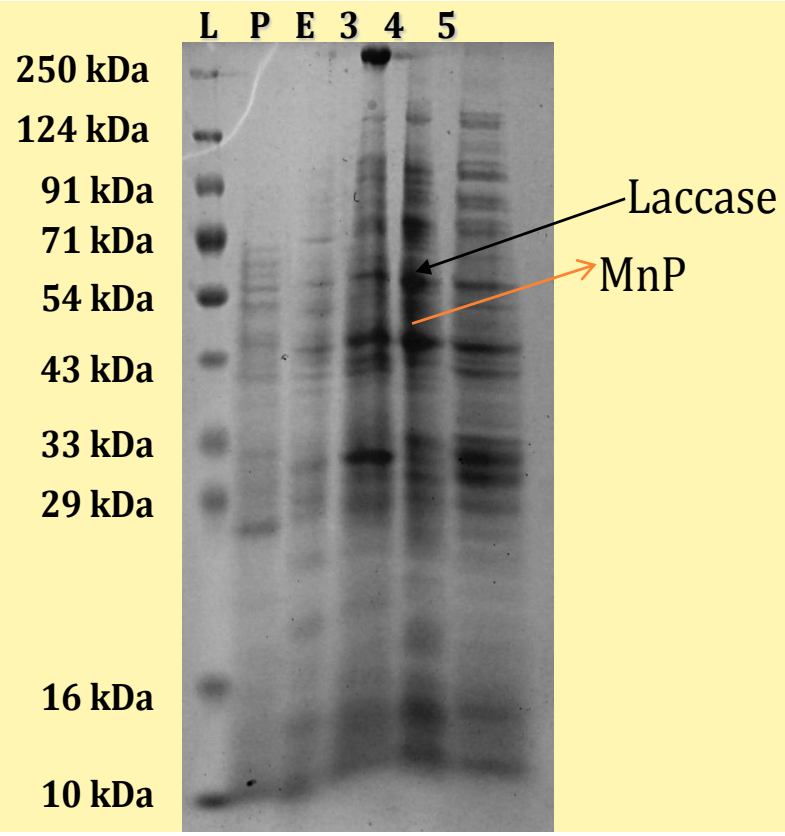


Fig. 5.4. SDS-PAGE analysis of crude enzyme Laccase and MnP production by *Staphylococcus saprophyticus* (DS3), *Bacillus megaterium* sp. (DS4), and *Alcaligenaceae* sp. (DS5)

Laccases have the most potential, with a broad range of specificity, and well-studied ligninolytic enzymatic activities, which is highly versatile in nature with wide variety of industrial applications (Chandra and Chowdhary, 2015). Structurally, laccases contain 15–30% carbohydrate and have molecular masses of 60-90 kDa with acidic isoelectric points around pH 4.0, which shows high enzymatic stability (Baldrian, 2006). Laccases can oxidize a wide range of molecules, and nearly 100 different types of compounds have been identified as substrates, which vary from one laccase to another. Similarly, few bacteria have been reported for the metabolization of distillery wastewater melanoidins by MnP activity (Bharagava et al., 2009).

Conclusion

This study concluded that the potential bacterial strains, which were used for consortia development i.e. DS3, DS4, and DS5 were identified as *Staphylococcus saprophyticus, Bacillus megaterium* sp. and *Alcaligenaceae* sp. with accession number MF182113, MF967441 and MF182114, respectively. Further, these bacterial strains have ability to produce ligninolytic enzyme, which may be responsible for the distillery wastewater pollutants degradation. The ligninolytic enzyme laccase and MnP were identified by SDS-PAGE electrophoresis of partially purified enzyme has yield band of laccase and MnP with the molecular weight ~65 and 43 kDa, respectively. Hence, these enzyme may be involved in the distillery wastewater pollutants degradation such as melanoidins and other phenolic and coloring compounds.

Chapter 6

Degradation and detoxification of distillery wastewater pollutants by the developed bacterial consortium

***Chapter* 6**

6.1. Introduction

DWW is highly colored and acidic with strong and objectionable odour that presents significant disposal or treatment problem (Arimi et al., 2014). The wastewater generation and its characteristic depend upon the use of raw materials and used chemicals in process of alcohol production (Mall and Kumar, 1997; Arimi et al., 2014). Alcohol is mainly produced from cellulosic materials. Raw materials, mainly used in distilleries are sugarcane molasses, grains, grapes, sugarcane juice, and barley malt. Diluted sugarcane molasses is inoculated with yeast and fermented in either batch or continuous mode to yield a broth containing 6-8% ethanol. In the continuous process, the cellulosic materials first delignified and hemi-cellulose and cellulose are subsequently acid hydrolyzed into simple sugars. In this process yeast are mainly responsible for the fermentation of sugar and produced ethanol and CO_2. In addition, cane molasses spent wash contains low molecular weight compounds such as lactic acid, glycerol, ethanol and acetic acid (Wilkie et al., 2000; Bharagava et al., 2010). Spent wash contains about 2% melanoidin which has an empirical formula of $C_{17\text{-}18}H_{26\text{-}27}O_{10}N$ and molecular weight between 5000 and 40,000 kDa (Kalavathi et al., 2001; Martin et al., 2002; Manisankar et al., 2004). These compounds have antioxidant properties, which render them toxic to many microorganisms such as those typically present in wastewater treatment processes (Kumar et al., 1997; Kharayat, 2012).

The distilleries and fermentation industries wastewater are the major source of aquatic and terrestrial pollution due to presence of water soluble recalcitrant coloring compounds called melanoidin (Tiwari et al., 2012). The characteristics of the DWW generated by various processes differ considerably. This is because the final DWW is a function of the

feedstock used as well as the waste management philosophies adopted by individual distillery plants.

Thus, the purpose of this chapter was degradation and detoxification of distillery wastewater pollutants by the developed potential bacterial consortium

6.2. Material and Method

6.2.1. Degradation and detoxification of DWW pollutants by isolated bacterial strains in axenic and mixed culture conditions

The degradation of DWW pollutants was carried out in triplicate in 250 mL Erlenmeyer flasks containing 100 mL of sterile modified medium amended with DWW pollutants (optical density 2.8 at 475 nm). The flasks were inoculated with 1% (v/v) of activated bacterial cultures grown for 48 h in DWW amended medium in axenic and mixed conditions and incubated at 37 °C in incubator shaker at 75 rpm (Innova 4230, New Brunswick, USA) for six consecutive days. The degradation/decolorization of DWW pollutants was measured by spectrophotometer (Evolution 201, Australia) in terms of bacterial growth and reduction in color intensity at 620 and 475 nm, respectively (Bharagava and Chandra, 2009).

6.2.2. Physico-chemical analysis of untreated and treated DWW for reduction in pollution parameters

The physico-chemical analysis of untreated and treated DWW was made as per the Standard Methods for Examination of Water and Wastewater (APHA, 2012). The COD, BOD, TS and TSS were determined by open reflux method, 5 days method, drying and drying filtrate method, respectively. In addition, various heavy metals were also analyzed by the acid digestion ($1HNO_3$:3HCl) method using an inductively coupled plasma (ICP) spectrophotometer (IRIS Interepid II XDL: Thermo Electron, Waltham, Mass, USA) (APHA 2012).

6.2.3. Scanning electron microscopic (SEM) study of bacterial consortium before and after distillery wastewater decolourization

For the SEM analysis of bacterial cell first of all strains were incubated for 2-4 h (4 °C) in glutaraldehyde (1%). Further bacterial cells were centrifuged at 5,000 rpm for 10 min, the pellets were washed thrice with deionized distilled water to remove the medium from the cell. In next step the bacterial cell were fixed with phosphate buffer (0.1 M and pH 7.2) containing 1% glutaraldehyde for 2 h and washed again with distilled water. Subsequently, bacterial cell were refixed in 1% osmium tetroxide (OsO_4) for 30 min and washed two times with phosphate buffer. Fixed cell were then dehydrated with acetone (30, 50, 70, 95 and 100%) for 10 min, at each step. The final dehydration process was repeated with absolute alcohol (100% acetone). Finally, the dried cell were then mounted on metal stub with a paste of colloidal silver paste and coated with a thin conductive film of gold in a sputtering coater and examined under scanning electron microscope (JSM-6490LV, JEOL, Japan). The above mention process were used for both bacterial cell i.e. before and after treatment of distillery wastewater.

6.3. Result and Discussion

6.3.1. Degradation and detoxification of DWW by bacterium DS3, DS4, and DS5 in axenic and mixed culture conditions

In this study, the bacterial strains DS3, DS4, and DS5 was found effective to decolorize DWW upto 76.12% in mixed culture conditions with considerable reduction in BOD, COD values, total solids, sulfates, phosphates and phenolic content as shown in (Table 1). However, in axenic condition, bacterium DS3, DS4 and DS5 was found capable to decolourize only 52.31, 63.26, and 49.69%, respectively with reduction in BOD, COD values, total solids, sulfates, phosphates and phenolic content (Fig. 6.1). During decolorization experiments, an increase in optical density (OD) for bacterial growth was

observed indicating the fast growth of bacterial consortium, which reached to optimum at 120 h of incubation time.

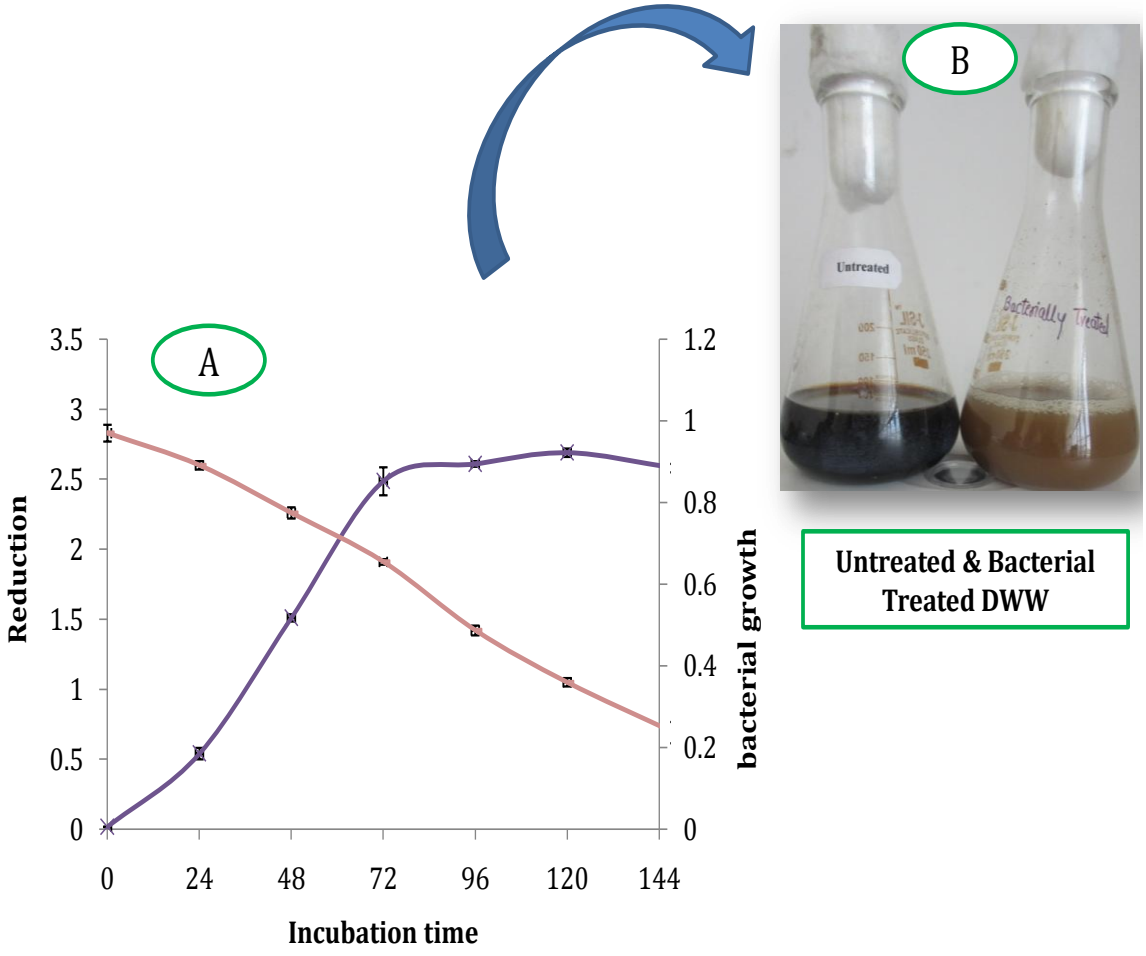


Fig. 6.1. Decolorization of distillery wastewater by axenic and mix culture (consortium); (A): untreated and treated distillery wastewater; (B): graphical representation of bacterial growth and decolorization

However, initially at 24 h of incubation period, slow reduction in color intensity was observed, which might be due to the utilization of glucose (as primary nutrient source) by bacteria for their establishment and subsequently utilization of DWW pollutants as carbon, nitrogen, and energy source (Bharagava and Chandra, 2010). After it, the reduction in color intensity was increased up to 120 h of incubation period, which might be due to the utilization of DWW pollutants as carbon, nitrogen, and energy source.

6.3.2. Physico-chemical characteristics of bacteria treated and untreated DWW

The distillery wastewater analysis showed that it was basic in nature, deep brown color carrying very high values of BOD (11985.67± 387.51), COD (29860.67±317.87), total solids (10147±262.24), phosphate (1184.66±25.00) phenolics, and sulfate. In addition DWW also contains heavy metals such as Fe (434.95 ±46.63), Zn (6.14±0.31), and Ni (0.18 ± 0.01), but after bacterial treatment, these pollution parameters get reduced significantly as shown in Table 6.1.

Analyzed values are more than the standard limit of USEPA 2012. The dark brown color of distillery wastewater is appears due to the complexation of amino carbonyl compound (Maillard product) during the condensation of sugar cane juice for the sugar manufacturing process (Chandra and Kumar, 2017; Arimi et al., 2015). While distillation process, generates huge amount of residual materials, which is responsible for high BOD, COD, TDS, and TS values. Whereas due to the corrosion of pipes, fermentor vessel and plant extract, high concentration of heavy metals are present in distillery wastewater.

The reduction in pollution parameters i.e. BOD, COD, total solids, sulfates, phosphates and phenolic might be attributed to the bacterial degradation of organic and inorganic pollutants present in DWW to meet the nutritional requirements. Further, the bacterial strains were also found much more effective to reduce the metals content (both by extra and intracellular process) and this reduction in metals content might be due to either the bioaccumulation of metals inside the bacterial cells or binding with liposaccharides of extra cellular membranes (Bharagava et al., 2009).

Table 6.1: Physico-chemical characteristics of untreated and treated distillery wastewater with permissible limits

S. No.	Physico-chemical parameters	Distillery Wastewater		
		Untreated	**Bacterial Treated**	**Effluent standard/ Permissible limit**
				USEPA (2000)
1.	pH	7.71±0.18	6.99±0.13	-
2.	Colour	Dark brown	Transparent	-
3.	COD	29860.67±317.87	5418.33±64.84	250.00
4.	BOD	11985.67± 387.51	3579.00±93.78	30.00
5.	TDS	10147.00±262.24	2002.00±76.41	-
6.	TS	33610.00±361.32	3394.33±28.09	-
7.	TSS	21639.00± 245.84	2110.66±77.79	-
8.	Phosphate	1184.66±25.00	413.33±72.62	-
9.	Chloride	613.33±16.04	485.33±20.74	1500
Heavy metals				
10.	Copper	1.38 ± 0.05	0.287±0.01	0.50
11.	Cadmium	0.42±0.03	0.12±0.005	0.01
12.	Iron	434.95 ±46.63	149.66±9.29	2.00
13.	Manganese	34.36 ± 0.65	25.66±1.52	0.20
14.	Nickel	0.18 ± 0.01	0.082±0.003	0.10
15.	Lead	0.26 ± 0.02	0.10±0.006	0.05
16.	Zinc	6.14±0.31	2.22±0.10	2.00

Note: All the values are means of three replicate (n = 3) ± SD in mg l^{-1} except colour and pH. COD: Chemical oxygen demand; BOD: Biological oxygen demand; TS: Total solids; TDS: Total dissolved solids; TSS: Total suspended solids.

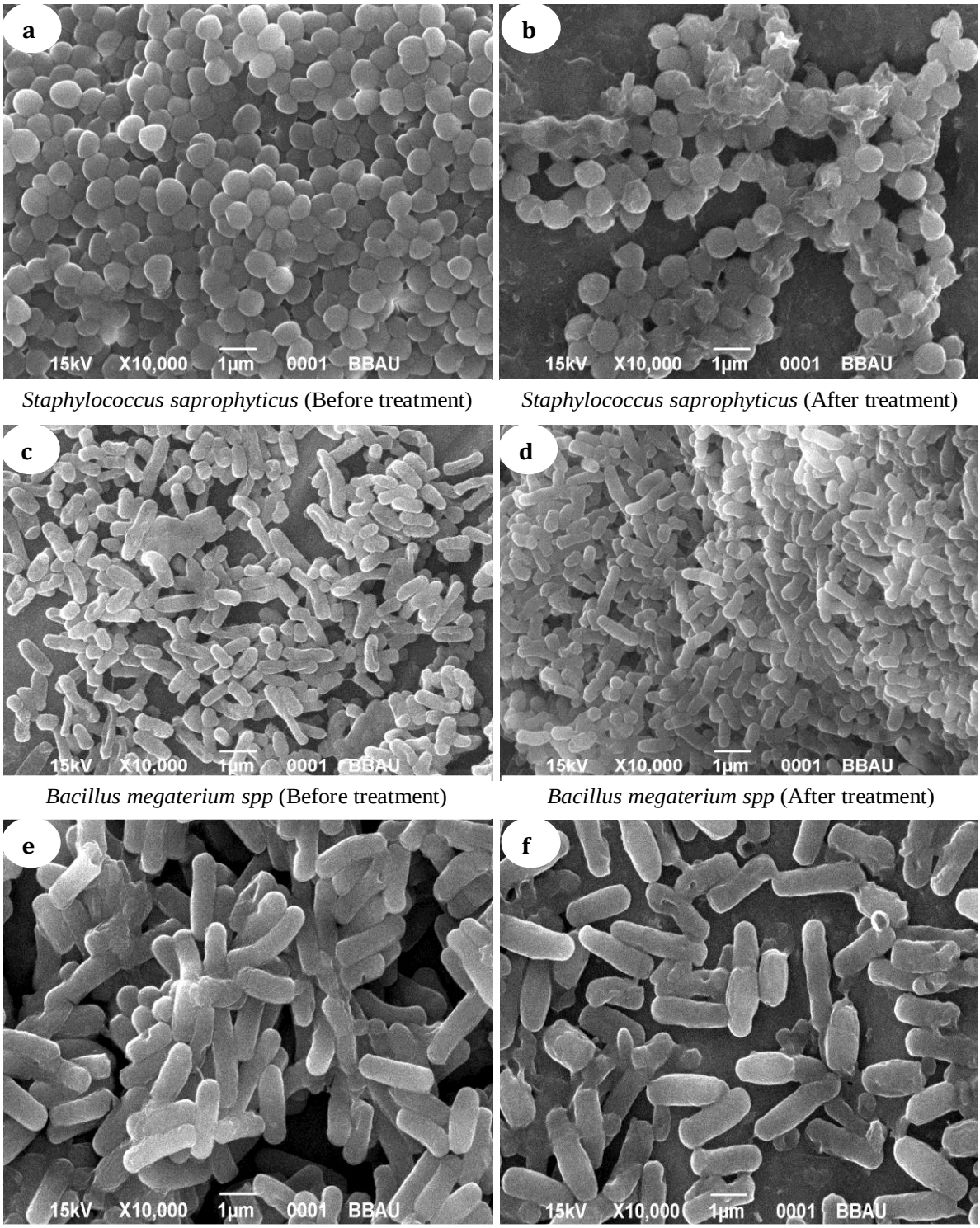
Beside it, distillery wastewater pollutants is largely depends on medium temperature and in this study, it was found that an increase in temperature from 30 to 35 °C favors the decolorization and further, increase in temperature has inhibitory effect of distillery wastewater decolorization. The maximum decolorization was 76.12% at 35 °C, followed by 64.96%, 61.56%, 57.35%, and 53.3% at 40, 45, 30, 25 °C, respectively (Fig 6. 4B).

In similar study, Bharagava and Chandra (2010) found that the bacterial consortium was efficient for 70% color removal in presence of glucose (1.0%) and peptone (0.1%) at pH 7.0 and temperature 37 °C. In addition, they have also found that a noticeable increase in OD of bacterial cell growth at 620 nm it was observed indicating the rapid growth of bacterial mix culture at obtained optimum growth at 120 h of incubation period. This may be due to utilization of glucose by bacteria and then after utilization of color containing compounds as carbon, nitrogen, and energy source. Subsequently they observed a sharp reduction in color intensity in 120 h incubation period and this reduction might be largely attributed to the bacterial degradation of color containing compounds.

6.3.5. Scanning electron microscopic (SEM) of bacterial cell before and after distillery wastewater decolourization

The identified bacterial strains *Staphylococcus saprophyticus*, *Bacillus megaterium* sp. and *Alcaligenaceae* sp. morphological observation under SEM before treatment showed reduced cell size. Whereas, after treatment with distillery wastewater growth of each bacterium cell were observed (Fig. 6.5). This is may be due to the stimulatory effect of coloring compounds (melanoidins).

Similar type of finding were also reported by Yadav et al., 2011. They have reported that morphological observation of bacterial cell under SEM before addition of d-xylose in 1st phase showed reduced cell size. Whereas after addition of d-xylose in 2nd phase ample growth of each bacterium with bigger cell size were observed. this is due to the biostimulatory effect of d-xylose on Sucrose-aspartic acid Maillard product (SAA-MP) decolorization



Staphylococcus saprophyticus (Before treatment) *Staphylococcus saprophyticus* (After treatment)

Bacillus megaterium spp (Before treatment) *Bacillus megaterium spp* (After treatment)

Alcaligenaceae sp. (Before treatment) *Alcaligenaceae* (After treatment)

Figure 6.5. Morphological changes of potential bacterial strains observed under SEM at before and after distillery wastewater treatment; (a-b): *Staphylococcus saprophyticus*; (c-d): *Bacillus megaterium* sp.; (e-f): and *Alcaligenaceae* sp.

Conclusion

The present study concluded that distillery wastewater was dark brown in color carrying very high values of BOD, COD total solids, phosphate, phenolics, and sulfate. In addition DWW also contains heavy metals such as Fe, Zn, Cd, Pb, and Ni etc. Further, by the treatment of bacterial consortium (DS3+DS4+DS5) was found effective to decolorize DWW upto 76.12% in comparison to axenic culture conditions with considerable reduction in BOD, COD values, total solids, sulfates, phosphates and phenolic metal content. Hence, it was observed that potential bacterial consortium showed the maximum decolorization was 76.12% in presence of glucose (0.5%) and peptone (0.1%) at pH 7.0 and temperature 35 °C. Coloring compounds present in distillery wastewater may increases the cell size of identified bacterial strains *Staphylococcus saprophyticus, Bacillus megaterium* sp. and *Alcaligenaceae* sp. under morphological observation with SEM analysis.

Chapter 7

Characterization of distillery wastewater pollutants and their metabolites before and after bacterial degradation process, respectively by GC-MS/LC-MS-MS analysis

***Chapter* 7**

7.1 Introduction

DWW contains a mixture of organic and inorganic pollutants, produced during the alcohol production processes in distillery industries, the present compounds, which can be detected, characterized and identified by using various analytical techniques such as high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS/MS) etc. However, these organic compounds generated during various alcohol production process such as feed preparation, fermentation, distillation, and packaging (Skerratt, 2004; Berg, 2004). In addition, in distillery wastewater there are also some chemicals reported, which are act as endocrine disrupting compounds (EDCs), like phthalate such as DI-N-octyl phthalate and Dibutyl phthalate (Yadav and Chandra, 2012), these EDCs disturb the delicate hormonal balance and compromise the reproductive fitness of living beings and ultimately may lead to carcinogenesis (Dixit et al., 2015).

Thus, the purpose of this chapter was to understand the nature and characteristic of distillery wastewater pollutants, which were generated at various steps during alcohol production process. In this study, detection and identification of pollutant was made possible by performing a GC-MS analysis to identify the compound

7.2. Detection and characterization of DWW pollutants and their metabolites

7.2.1. Extraction of DWW pollutants and their metabolites

For the extraction of DWW pollutants and their metabolites, 300 mL of untreated and bacteria treated DWW samples were centrifuged at 5,000 rpm for 10 min at 4 °C to remove the suspended particles and bacterial biomass from untreated and treated samples, respectively. The supernatant obtained was extracted three times with the equal volume of acetonitrile (ACN) in acidic (2.0), neutral (7.0) and basic (9.0) pH conditions. Further, the ACN extract from untreated and treated samples were pooled, dehydrated over anhydrous

sodium sulfate, and vacuum dried at 40 °C. The dry residues obtained were dissolved in 2 mL of methanol and ACN (HPLC Grade) and used for the analysis of DWW pollutants and their metabolites.

7.2.2. IR and LC-MS/MS analysis

For the characterization and identification of DWW pollutants and their metabolites, the Infrared (IR) spectra were recorded on a Pye Unicam SP3-200 instrument as thin KBr discs and the values were expressed in cm^{-1}. Further, the samples were also analyzed by the liquid chromatography-mass spectrometry (LC-MS/MS) on UPLC system (Acquity-Waters, Miliford, USA) coupled with an API-4000 mass spectrometric system (AB ScieX) with an electro spray ionization (ESI) source to determine the mass of DWW pollutants and their metabolites.

The analysis of samples was performed within 5.0 minutes under an isocratic elution of mobile phases, with 100% - A (0.1% formic acid in water) for positive mode ionization at a constant flow of 0.3 mL/min. Definite sample volume (7.5 µL) was injected using an auto sampler of UPLC system. The column and sample manager temperature was set at 25 °C. However, the instrumental processing was controlled by Analyst^{TM} software (Version 1.6.2, ScieX, Foster city, CA, USA). The ESI was operated in positive as well as in negative mode with source temperature and voltage at 400 °C and 5500 V, respectively for positive mode ionization. The source parameters i.e., nebulizer gas (GS1), turbo gas (GS2), collisionally activated dissociation gas (CAD) and curtain gas (CUR) were programmed at 40 psi, 60 psi, 8 psi and 15 psi, respectively. The acquisition was performed over three times for each sample. The mass spectrometric analysis was executed in multiple reactions monitoring (MRM) mode by keenly optimizing dwell time varied from ≥30 to ≤200 msec. The suitable dwell times were adjusted in order to maintain the appropriate number of data points (≥13) per chromatographic peak. The pause time

and target scan time were set to 3.0 msec and 1.0 sec, correspondingly. The organic pollutants and their metabolites were identified by comparing their *m/z* ratio peaks with previously reported research data (Gonzalez et al., 2000; Yadav and Chandra, 2012; Chandra et al., 2012).

7.3. Result and Discussion

7.3.1. DWW pollutants and their metabolites

The FT-IR analysis of untreated DWW showed the stretching frequencies at 687.0, 1368.7, 1636.5, 2127.0, 2384.8, 3250.9, and 3467.2 cm^{-1} indicating the presence of functional groups such as ethylene (acetylene) (-CH=CH-), CH_3, non-conjugated group, C=CH (terminal), free -SH (thiol group or a sulfhydryl group), -C-H- stretching and intermolecular H-Bonds, respectively**.** Whereas, in bacterial treated DWW showed the stretching frequencies such as 3201.31, 2732.54, 1667.54, 1405.23 cm^{-1} indicating presence of –OH, –C–H, =C=O, and NO_2, respectively (Table 7.1 & Fig. 7.1). Many authors have also reported the presence of various compounds having similar type of functional groups in DWW (Bharagava and Chandra, 2010; Narain et al., 2012; Kadam et al., 2013).

Further, the LC-MS/MS analysis of bacteria treated DWW has shown slight reduction in peak areas compared to untreated DWW indicating the degradation/decolorization of DWW pollutants by the bacterial strains in mixed culture conditions (Fig. 7.2A & B). The reduction in peak areas might be largely attributed to the bacterial degradation of complex organic and inorganic pollutants and their subsequent utilization by bacterial strains as nutrients.

The LC-MS/MS fragmentation pattern of these molecular ion peaks has indicated the generation of several molecular mass (*m/z*) 381, 365, 360, 317, 302, 271, 257, 223, 221,

213, 199, 196, 181, 166, 161, 152, 149, 142, 139, 133, 127, 124, 118, 110, 102, 99, 88, 72, 69, and 59, respectively (Table 7.2).

Table 7.1. FTIR analysis of untreated and bacterial treated distillery wastewater

Wave number (cm^{-1})	**Characterization**
Untreated DWW	
687.0	-CH=CH-(cis)
1368.7	CH_3, COO- in Carboxylic acid salts, SO_2 in sulfonyl chlorides, isopropyl group, NO_2 in aromatic nitro compounds
1636.5	Nonconjugated
2127.0	C=-CH (terminal)
2384.8	Free SH
3250.9	-C-H, Alcohol/Phenol O-H
3467.2	Intermolecular H Bonds
Bacterial treated	
3434.5	-NH2 in aromatic amines, primary amines and amides, -OH in alcohols and phenols
2134.8	-C≡C-, Alkynyl C-H
1640.2	-C=C-, C=N in oximes, C=O and NH2 in primary amides, C=O in ureas, C=C in alkenes,etc, C=O in secondary amides, C=O in benzophenones, C=O in primary amides, C=O in tertiary amides, C=O in ß-ketone esters
1562.9	NH in secondary amides, triazine compounds, NO_2 in aromatic nitro compounds
1456.0	N=N-O in azoxy compounds, CH_2 in aliphatic compounds, CH_3 in aliphatic compounds
1407.8	CH_3 in aliphatic compounds, OH in carboxylic acids, C-N in primary amides
1308.0	COO- in Carboxylic acid salts, SO_2 in sulfonyl chlorides, isopropyl group, NO_2 in aromatic nitro compounds
1112.9	C-O-C in esters, lactones, SO_3H in sulfonic acids, C-O-C in ethers, C-C-N in amines, SO_2Cl in sulfonyl chlorides, C-OH in alcohols, SO_2NH_2 in sulfonamides, SO_2 in sulfones
620.9	C-Cl in Chloro compounds, C-S in sulfonyl chlorides, C-Cl alkyl chlorides, Ar-OH in phenols, C-S in sulfides, O-C=O in carboxylic acids, C-C-CHO in aldehydes, C-OH in alcohols, C≡C-H in alkynes

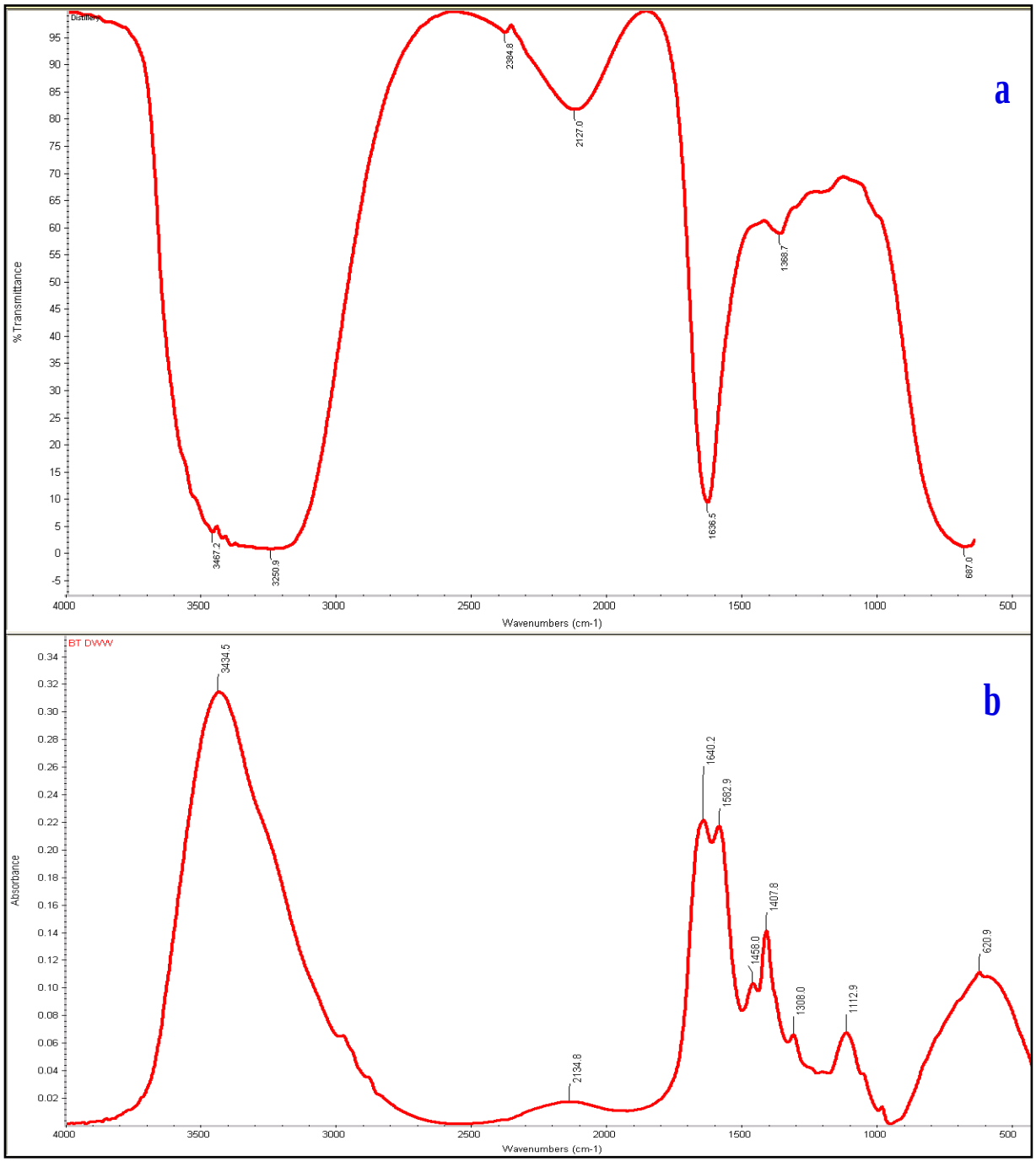


Figure 7.1. FTIR spectra of (a) untreated and (b) bacterial treated distillery wastewater.

Hence, on the basis of FT-IR and LC-MS/MS analysis, the untreated DWW was found to contain a number of compounds having different functional groups. The compounds detected in control and bacterial treated samples were characterized as [M+K^+] aduct of maltose, [M+Na^+] aduct of maltose, Benzyl-3,4-ethylenedioxypyrrol-2,5-dicarboxylate, Petunidine-(6-coummaryl)-3-glucoside, Paeonidine-(6acetyl)-3-glucoside, Diethyl-3,4-ethylenedioxypyrrole 2,5-dicarboxylate (270), Palmitic acid, 1-(tert-Butyl)-2-methylpyrrole-1,2-dicarboxylate, 1-(p-tolysulfonyl) pyrrole, 2-(Trichloroacetyl) pyrrole, 3-heptyl-5-methyl-2,3H-furanone ($C_{12}H_{22}O_2$), Trans-2-Tridecenal, Dihydroxyconiferyl alcohol ($C_{10}H_{14}O_3$) (182), 2-nitroacetophenone ($C_8H_7NO_3$) (165), Anhydrohexose from [M-$H]^-$, 2,6-dimethoxyphenol ($C_8H_{10}O_3$) (154), 4-vinyl-2-methoxyphenol ($C_9H_{10}O_2$) (150), p-chloroanisol (C_7H_7ClO), 4-methyl guaicol, N-methyl indane (135), 5-(hydroxymethyl)-2-furfural ($C_6H_6O_3$) (126), 2-methoxyphenol ($C_7H_8O_2$), Indole, 5-methyl-2-furancarboxaldehyde ($C_6H_6O_2$), 2- methylhexane (C_7H_{16}), Furfuryl alcohol ($C_5H_5O_2$) (98), Butenoic acid ($C_4H_6O_2$) (86), 3-hydroxypropanal, Pyrroline (C_4H_7N) and Acetic acid ($C_2H_4O_2$), respectively. However, similar result have been also reported by many authors (Gonzalez et al., 2000; Bharagava and Chandra, 2009; Yadav and Chandra, 2012). In this study, degradation of distillery wastewater pollutants by bacterial consortium were confirmed through lower RT (min) in LC-MS-MS chromatogram.

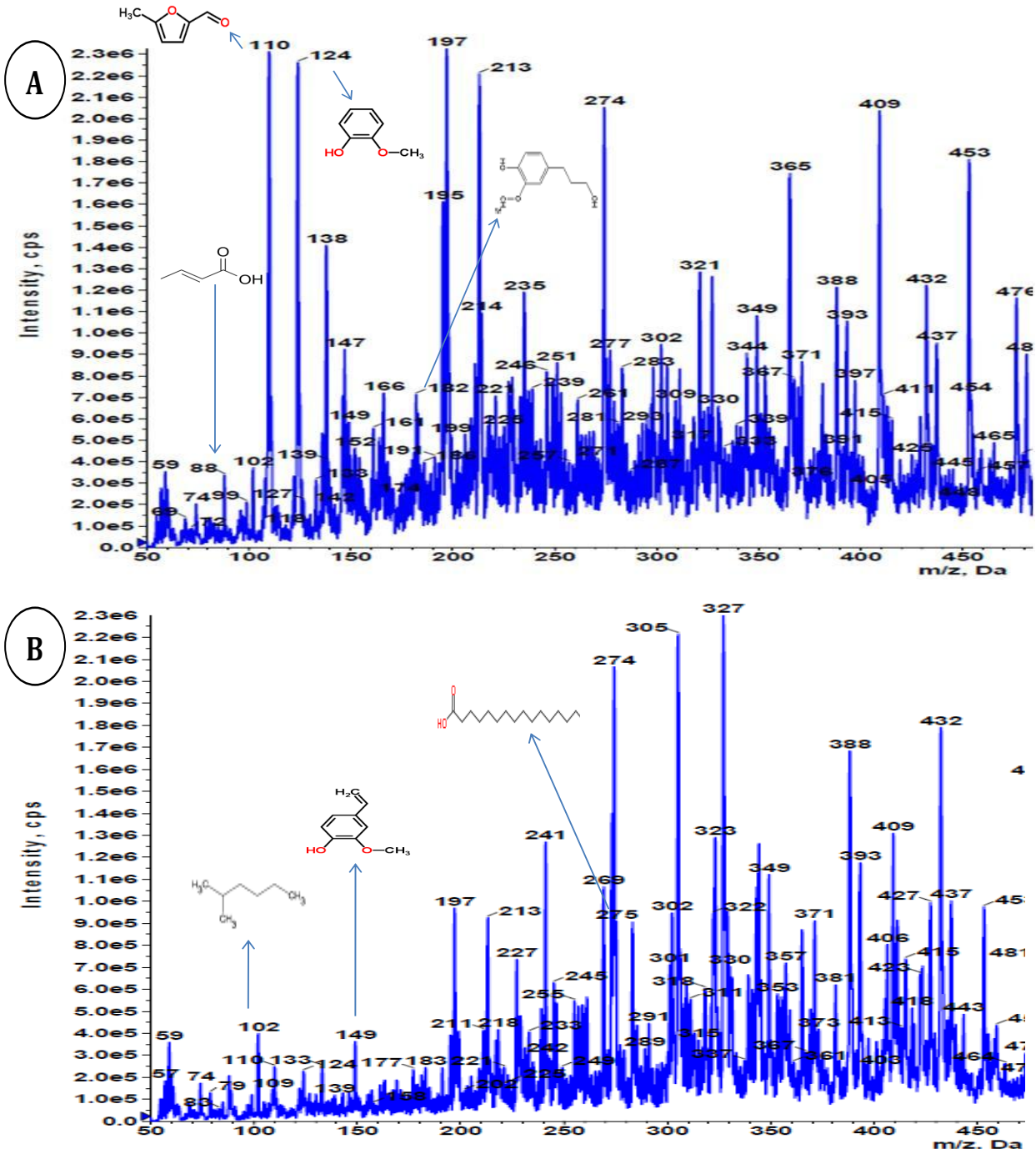
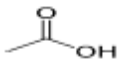
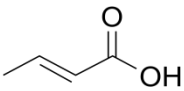
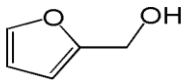
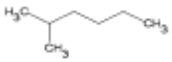
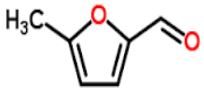
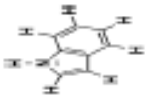
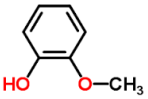
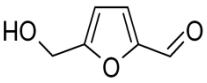
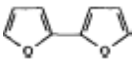
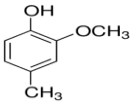
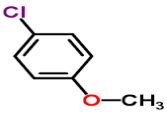
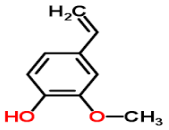
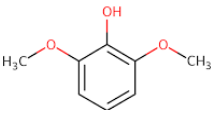
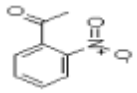
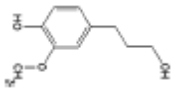
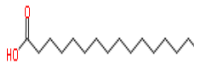


Figure 7.2. Chromatogram of LC-MS/MS analysis of pollutants extracted from distillery wastewater untreated (A) and bacterial treated (B).

Table 7.2: Compounds identified from distillery wastewater by LC-MS/MS technique.

S. No.	*m/z* values of detected compound/ metabolites	Chemical name	Structure	C	BT
1.	59	Acetic acid ($C_2H_4O_2$)		+	+
2.	69	Pyrroline (C_4H_7N)		+	-
3.	72	3-hydroxypropanal ($C_3H_6O_2$)		+	-
4.	88	Butenoic acid ($C_4H_6O_2$)		+	-
5.	99	Furfuryl alcohol ($C_5H_5O_2$)		+	-
6.	102	2- methylhexane (C_7H_{16})		+	+
7.	110	5-methyl-2-furancarboxaldehyde ($C_6H_6O_2$)		+	+
8.	118	Indole (C_8H_7N)		+	-
9.	124	2-methoxyphenol ($C_7H_8O_2$)		+	+
10.	127	5-(hydroxymethyl)-2- furfural ($C_6H_6O_3$)		+	-
11.	133	N-methyl indane		+	-
12.	139	4-methyl guaicol		+	+
13.	142	p-chloroanisol (C_7H_7ClO)		+	-
14.	149	4-vinyl-2- methoxyphenol ($C_9H_{10}O_2$)		+	+
15.	152	2,6-dimethoxyphenol ($C_8H_{10}O_3$)		+	-

16.	161	Anhydrohexose from $[M-H]^-$		+	-
17.	166	2-nitroacetophenone ($C_8H_7NO_3$)		+	-
18.	181	Dihydroxyconiferyl alcohol ($C_{10}H_{14}O_3$)		+	+
19.	196	Trans-2-Tridecenal	-	+	+
20.	199	3-heptyl-5-methyl-2,3H-furanone ($C_{12}H_{22}O_2$)	-	+	+
21.	213	2-(Trichloroacetyl) pyrrole	-	+	+
22.	221	1-(p-tolysulfonyl) pyrrole	-	+	+
23.	223	1-(tert-Butyl)-2-methylpyrrole-1,2-dicarboxylate	-	+	-
24.	257	Palmitic acid		+	-
25.	271	Diethyl-3,4-ethylenedioxypyrrole-2,5-dicarboxylate	-	+	-
26.	302	Paeonidine- (6 acetyl) -3-glucoside	-	+	+
27.	317	Petunidine- (6- coummaryl)-3-glucoside	-	+	+
28.	360	Benzyl -3,4-ethylenedioxypyrrol- 2,5-dicarboxylate	-	+	+
29.	365	$[M+Na^+]$ aduct of maltose	-	+	-
30.	381	$[M+K^+]$ aduct of maltose	-	+	+

Note: Source used for table preparation (Bharagava and Chandra, 2010); **C:** control; **BT:** Bacterial treated +: present, -: absent

Conclusion

This study concluded that FT-IR and LC-MS/MS analysis confirmed the presence of various organic compounds in DWW, in which some compounds were reported as hazardous for living organisms. Bacterial treatment reduces the toxicity of DWW. FT-IR and LC-MS/MS shown the presence of various organic compounds with molecular weight such as 270, 182, 165, 154, 150, 135, 126, 98, 86 that were identified as Diethyl-3,4-ethylenedioxypyrrole-2,5- dicarboxylate, Trans-2-Tridecenal, Dihydroxyconiferyl alcohol ($C_{10}H_{14}O_3$), 2-nitroacetophenone ($C_8H_7NO_3$), Anhydrohexose from $[M-H]^-$, 2,6-dimethoxyphenol ($C_8H_{10}O_3$), 4-vinyl-2- methoxyphenol ($C_9H_{10}O_2$), p-chloroanisol (C_7H_7ClO), 4-methyl guaicol, N-methyl indane, 5-(hydroxymethyl)-2- furfural ($C_6H_6O_3$), Furfuryl alcohol ($C_5H_5O_2$) (98), and Butenoic acid ($C_4H_6O_2$), respectively and some other compounds before and after bacterial treatment. In this study, degradation of distillery wastewater pollutants by bacterial consortium were confirmed through lower RT (min) in LC-MS-MS chromatogram. Therefore, this developed consortium could be useful for the effective decolorization of distillery wastewater pollutants,

Hence, it was clearly observed that after secondary treatment process several toxic chemicals remains in distillery wastewater. Thus, there is an urgent need to address the limitations in the existing treatment methods and to develop the integrated treatment processes that provide a complete solution to the treatment of distillery wastewater.

Chapter 8

Toxicity assessment of distillery wastewater pollutants before and after bacterial degradation and detoxification process for environmental safety

Chapter 8

A. Translocation of toxic metals and their impact on biochemical parameters in Wheat and Mustard plants growing at distillery and tannery wastewater contaminated site

8.1. Introduction

The safe disposal of industrial wastewaters and solid waste is becoming a serious concern worldwide. Industrial waste comprise of various organic and inorganic pollutants in which potentially toxic elements (PTEs) are potent environmental toxicants (Bolana et al., 2014), which disturb physiological and biochemical activities of flora and fauna through the food web and thus, deserve the special attention. Unlike organic pollutants, metals are non-degradable; persist in nature for a long time and causes deleterious effects on human health and environment (Boechat et al., 2016). PTEs are discharge from different industries such as distillery, tannery, textile, electroplating, and paper mills etc. (Chowdhary et al., 2017a, b; Chowdhary et al., 2018).

In India, more than 319 distillery and ~3,000 tannery industries are in function, which generates ~40.4×10^9 L and 7×10^4 tons of wastewater, respectively in one year (Bharagava et al., 2009; Dixit et al., 2015). The waste discharged from distillery and tannery industry are major source of toxic metal(loids) such as lead (Pb), cadmium (Cd), nickel (Ni), cobalt (Co), iron (Fe), zinc (Zn), chromium (Cr), arsenic (As) (Chowdhary et al., 2017a). The toxicity of PTEs in plants is basically associated with the chemical species, concentration, and route of exposure (Tchounwou et al., 2012). The conventional approaches used in the remediation of PTEs involve excavation, disposal to landfill site, dumping and on site management. However, these approaches could not mitigate the pollution level in a sustainable manner. Thus, the big challenge is how to manage the large

volume of wastewater generated from industries. In India being the different investment priorities, the remediation of wastewater is a matter of remote possibility.

Therefore, the best way for management of wastewater is seems to its utilization in irrigation of crop plants. However, the ecotoxicological assessment of plants as well as wastewater toxicity must be imperative (Abbas et al., 2007; Chowdhary et al., 2017a). Wheat and mustard are the most common crops in India. India is the third and fourth largest producer of mustard and wheat in the world accounts for 12% and 8.7%, respectively of the world's total production (Shekhawat et al., 2012). Plants growing near the contaminated sites are more prone to toxicity developed by the waste present in the soil to cope the toxic impact of waste plants are equipped with different antioxidant system (Thakur et al., 2016).

Metal toxicity in plant produced due to the reactive oxygen species (ROS) causes' oxidative damage, electrolyte leakage, membrane damage, DNA distraction and cellular toxicity (Charfeddine et al., 2017). To counter act the ROS induced toxicity plants have different type of antioxidant enzyme like superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPx), peroxidase (POX), and catalase (CAT), which protect plants against different adverse conditions (Jiang et al., 2010; Farooq et al., 2016). Hence, this study was focused on the physico-chemical characteristics of distillery, tannery wastewater and soil/sludge along with the oxidative damage and recovery responses in wheat and mustard growing in contaminated site.

However, less significant research data are available on the accumulation pattern of PTEs with their adverse effects on enzymatic activity in crop plants. This study indicates that industrial wastewaters are the primary sources of accumulation of PTEs in agricultural crops, which upon human and animal consumption may lead to adverse health effects. The accumulation of PTEs in agricultural crops may be risky from health point of view.

Therefore, to prevent the entrance of PTEs into food chain, industrial wastes should not be drained into the rivers and farmlands without proper treatment. Thus, this study aimed (i) To evaluate the uptake, distribution, and accumulation of PTEs in selected plants from industrial wastewater. (ii) To study the toxic effects of PTEs on crop plants. (iii) To investigate the biochemical changes caused by PTEs in plants. (iv) Phytoextraction efficiency of these two plants.

8.2. Material and methods

8.2.1. Collection of industrial wastewater and soil/sludge samples

The wastewater and sludge samples were collected from the two sites i.e. outlet of Effluent Treatment Plant (ETP) of *M/s* Unnao Distilleries and Breweries (26° 33'15.92”N, 80° 30′ 38″ E) (*Site-1*) and tannery industries (26°33'32,32”N latitude, 80°30'49,90"E) (*Site-2*) located in Unnao, Uttar Pradesh, India (Fig. 8.1). The wastewater and sludge samples were collected in Carboy container (capacity 20 L) and in autoclavavel biohazards poly bags, respectively. The soil/sludge samples were collected from the adjoining area away from the wastewater channel. In case of normal irrigated area, soil samples were taken from a depth of 8 cm below the surface. The soil/sludge samples were collected with the help of plastic spatula to avoid any type of metal contamination throughout the sampling.

8.2.2. *Collection and storage of plant samples*

The plant samples (wheat and mustard), were uprooted and randomly collected, at their maturity stage from the contaminated site near the distillery and tannery industry wastewater. However, same plants were also collected from the agricultural land irrigated with normal ground water. The collected plant samples were washed with deionized water. These plant samples were stored at -20 °C for further experimental analysis.

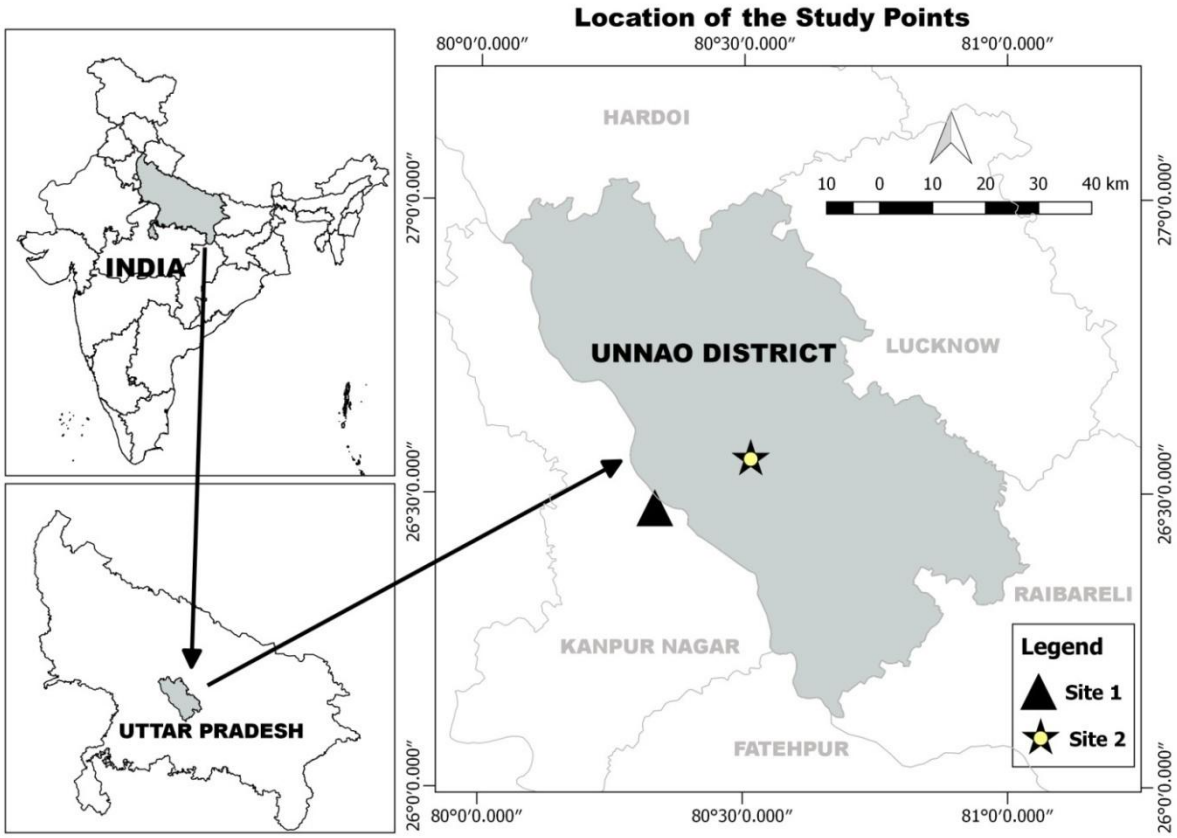


Figure 8.1: Sample collection site of Unnao industrial area; Site 1: *M/s* Unnao Distilleries and Breweries; Site 2: Tannery industries.

8.2.3. Physico-chemical analysis of wastewater and soil/sludge samples

The collected wastewater samples were analyzed for various physico-chemical parameters such as pH, EC, organic matter, chloride, phosphate and PTEs following as per the standard methods for the examination of water and wastewaters (APHA, 2012).

8.2.4. Biochemical analysis

8.2.4.1. Photosynthetic pigments estimation

The photosynthetic pigments (Chl-a & Chl-b), were estimated following the method of Arnon (1949). Leaf samples (100 mg) were crushed in 5 mL of chilled 80% acetone with the help of pestle and mortar and centrifuged at 5,000 rpm for 10 min at 4 °C. The supernatant was used for the estimation of chlorophyll content with the help of spectrophotometer (UV-160, Shimadzu, Japan). The carotenoids content was estimated at

the same process of above and reading the supernatants at wavelength of 480 and 510 nm following the formula given by Duxbury and Yentsch (1956).

8.2.4.2. Protein content

The protein content was estimated as per the Lowry method (Lowry et al., 1951) using bovine serum albumin (Sigma) as standard. For this, 100 mg leaf samples were crushed in 5 mL of 10% chilled trichloroacetic acid (TCA) and centrifuged at 10,000 rpm for 10 min at 4 °C. The obtained pellets were washed and resuspended in 5 mL of 0.1N NaOH and again centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant obtained was mixed with alkaline Cu-solution and Folin-phenolciocalteau reagent and used in protein content estimation at 650 nm (Lowry et al., 1951).

8.2.4.3. Lipid peroxidation content (MDA)

To measure the MDA content, Heath and Packer (1968) method was used in which 200 mg of plant leaves were crushed in 5 mL of 0.1% trichloroacetic acid (TCA) followed by centrifugation at 10,000 rpm for 10 min at 4 °C. The obtained supernatant was mixed with 4 mL of 20% TCA and 0.5% of thiobarbituric acid (TBA) solution, subjected to heating at 95 °C for 30 min, cooled in ice bath and again centrifuged at 10,000 rpm for 10 min and the supernatant was used to measure absorbance at 532 nm.

8.2.4.4. Hydrogen peroxide (H_2O_2)

The plant leaves (250 mg) were homogenized in 2 mL of 5% TCA as per the method of Velikova et al. (2000) method. The homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C. 200 µl of 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. The absorbance was recorded at 350 nm.

8.2.5. Preparation of enzyme extract

To enzyme extract, 250 mg fresh plant leaves were homogenized in 3 mL of 100 µM potassium phosphate buffer (pH 7.5) containing 1mM of ETDA and a pinch of polyvinyl polypyrrolidone (PVP). The homogenate was centrifuged at 12,000 rpm for 10 min at 4 °C and the extract was used to measure the activity of antioxidant enzymes.

8.2.5.1. Superoxide dismutase assay

The activity of SOD was measured by using the enzyme extract as per the method of Nishikimi and Rao (1972). SOD is reported to inhibit the photochemical reductions of nitroblue tetrazolium (NBT and the amount of enzyme required to inhibit the 50% reduction of NBT is expressed as one unit (1U) of enzyme.

8.2.5.2. Ascorbate peroxidase assay

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

8.2.5.3. Ascorbate assay

The estimation of ascorbate assay, the plant sample (50 mg) was homogenized in 2 mL of enzyme extract followed by centrifugation at 2,500 rpm for 15 min at 4 °C and supernatant obtained was used to measure at 520 nm within 2±5 min as per the method of Keller and Schwager (1977).

8.2.5.4. Catalase assay

The catalase (CAT) activity was measured at 240 nm in a reaction mixture containing 50 mM/L phosphate buffer (pH 7.0), 150 mM/L H_2O_2 for 2 min in terms of H_2O_2 decomposition as per the method of Chance and Maehly (1955).

8.2.6. Metal analysis in sludge/soil and plant samples

To analyze PTEs, the sludge/soil was first air-dried, ground, and sieved with a 0.5-mm sieve and then at 65 °C for 48 h followed by using Nitric acid and Perchloric acid in 2:1 ratio (Hesu, 2004). All the steps were performed in triplicate for each sample.

Plant samples were chopped into root, shoot, and leaves and dried in a hot air oven at 70 °C for 5 days. The dried plant parts were crushed in mixer grinder until the fine powder was obtained and this fine powder was ashed in a muffle furnace at 460 °C for 6 h. Now, 500 mg of ash from all samples was digested with 2% HNO_3 and filtered through a 0.45-μm glass fiber filter (AOAC, 2002). Now sieved sediment was again digested with 10 mL of HNO_3 till the appearance of brown fumes followed by addition of 5 mL of HNO_3 and digestion was continued until the generation of brown fumes stopped. Subsequently, the samples were cooled followed by the addition of 2 mL water and 3 mL of H_2O_2 and again digested as per the EPA method 3050-B (EPA, 1996). The concentration of Cr, Zn, Mn, Ni, Co, Fe, Cd, and Pb were measured using an AAS (ZEE nit 700, Analytic Jena, Germany). The AAS was calibrated before each set of measurements using the procedure as per the manufacturer's manual.

8.2.7. Quality Control and Quality Assurance (QA/QC)

In this study, the analytical data was assured by quality assurance (QA) and quality control (QC) methods. The stock standards were used from E-Merck India, and noticeable to National Institute of Standards Technology (NIST), to setup calibration curves that covered the region from 1, 2 and 3 μg g^{-1} levels. For all detected metals, the sensitivity of AAS was 0.1 μg g^{-1}. Analytical data quality of metal was guaranteed with repeated analysis of QC samples (n=3) and the result found ± 2.36, which is certified value. The limit of detection for Cd, Cr Cu, Pb, Ni, Zn, and Fe were 0.054, 0.097, 0.077, 0.031, 0.08, 0.028, and 0.072 (mg/L), respectively.

8.2.8. Metal bioaccumulation studies

8.2.8.1. *Analysis of Bioconcentration factor (BCF)*

The BCF refers to the concentration of any chemical/metal in an organism's tissue divided by its equilibrium concentration in soil/water expressed in equivalent units. The phytoextraction potential of selected crop plants was calculated. The BCF was calculated following the formula of Yoon et al. (2006).

$$\mathbf{BCF = (Metal_{root})/ (Metal_{soil/sludge})}$$

Where,

$Metal_{root}$ = Concentration of metals in root

$Metal_{soil/sludge}$ = Concentration of metal at contaminated site

8.2.8.2. Analysis of Translocation Factor (TF)

In present study, we have also calculated the TF of metals because it plays a key role in the evaluation of various metabolic activities, biochemical and physiological parameters of plants growing at contaminated sites. The TF was calculated following the formula reported by Gupta and Sinha (2008).

$$\mathbf{TF = (Metal_{Shoot})/ (Metal_{Root})}$$

Where,

$Metal_{shoot}$ = metal content in shoot

$Metal_{root}$ = metal content in root

8.2.9. 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, $p \leq 0.05$).

8.3. Result and Discussion

8.3.1. Physico-chemical characteristics of collected distillery and tannery wastewater

The physico-chemical analysis of distillery wastewater showed high pH (8.09 ± 0.0.11) and chloride concentration in comparison to tannery wastewater (Table 8.1). The dark brown color of distillery wastewater is appears due to the association of amino carbonyl compound (Maillard product) during the condensation of sugarcane juice for sugar manufacturing process (Chandra and Kumar, 2017). While distillation process, generates huge amount of residual materials, which is responsible for high BOD, COD, TDS, and TS values. Whereas due to the corrosion of pipes, fermentor vessel and plant extract, high concentration of PTEs are present in distillery wastewater (Bharagava et al., 2008).

The concentration of PTEs in distillery and tannery wastewater along with sludge/soil samples has been also given in Table 8.1. The desire limit of PTEs in distillery and tannery wastewater was significantly higher in comparison to the standard limits set by environmental protection agencies (USEPA, 2000).

Table 8.1: Physico-chemical characteristics of distillery and tannery wastes

Physico-chemical analysis			
	Wastewater		
Parameters			**Permissible Limits**
	Distillery	**Tannery**	(USEPA 2000)
pH	8.09 ± 0.11	7.86±0.35	-
Colour	Dark brown	Turbid transparent	Colorless
COD	53200.00 ± 200.00	5248.00±561.00	250.00
BOD	26566.00 ± 208.16	1491.00±20.20	30.00
TDS	13158.33± 52.04	20380.00±744.00	2100.00
Phosphate mg l^{-1}	14651.33 ± 182.49	15300.00±173.20	-
Chloride mg l^{-1}	2120.00 ± 22.91	1698.66± 35.64	1500.00
Nitrate mg l^{-1}	25.66 ± 0.57	32.89± 0.95	10.00
Heavy metals (mg l^{-1})			
Copper	2.38 ± 0.05	1.23±0.06	0.50
Chromium	2.32 ± 0.07	7.65±0.46	0.05
Zinc	10.35 ± 0.21	1.79±0.03	2.00
Iron	148.46 ± 0.87	5.21±0.52	2.00
Nickel	3.38 ± 0.21	0.36± 0.03	0.10
Manganese	9.56 ± 0.65	1.02± 0.25	0.20
Lead	1.31 ± 0.03	0.56±0.03	0.05
	Contaminated Soil		
	Distillery	**Tannery**	
pH	7.68±0.18	7.33±0.20	-
EC (mS cm^{-1})	2.44±0.06	2.52±0.03	-
OM (%)	6.80±0.50	5.41±0.06	-
Moisture (%)	72.61±1.50	66.73±2.71	-
Chloride mg kg^{-1}	888.66±21.73	4694.00±150.27	-
Heavy metals (mg kg^{-1})			
Cu	46.11±1.20	30.25±1.34	-
Cr	18.67±1.33	193.66±7.09	-
Zn	203.00±8.18	102.66±3.51	-
Ni	53.86±1.02	34.63±4.95	-
Fe	1585.33±52.81	877.66±35.01	-
Mn	1101.00±87.10	792.66±25.42	-
Pb	39.90±1.21	26.31±0.91	-

All the values are mean of triplicate ± S.D. EC: electric conductivity; OM: organic matter

8.3.2. Effects of PTEs on biochemical parameters

8.3.2.1. *Effect on photosynthetic pigments*

An increase in Chlorophyll a, b and carotenoid's content was recorded in wheat and mustard plants growing at wastewater contaminated sites with their respective controls (Fig. 8.2). The increased level of Chl-a and Chl-b in wheat and mustard plants might be associated with the presence of macro and micronutrients i.e Cu- protein, Plastocynin, Fe containg electron carriers in cytocroms complexes as well as various organic and inorganic pollutants (Sheetal et al., 2016). Few time latter, the chlorophyll content started to reduce due to the interference of PTEs in high concentration act as the chlorophyll depredating products i.e. chloroplast stometal products, Cd prevents the synthesis of photoactive proto-chlorophyll reductase enzyme complex and aminolevulinic acid synthesis during the chlorophyll biosynthesis because sulfhydryl groups (-C-SH) of this enzyme are blocked by the complexation of acid active thiol groups by cadmium (Czarnecki and Grimm, 2012; Richter and Grimm, 2013).

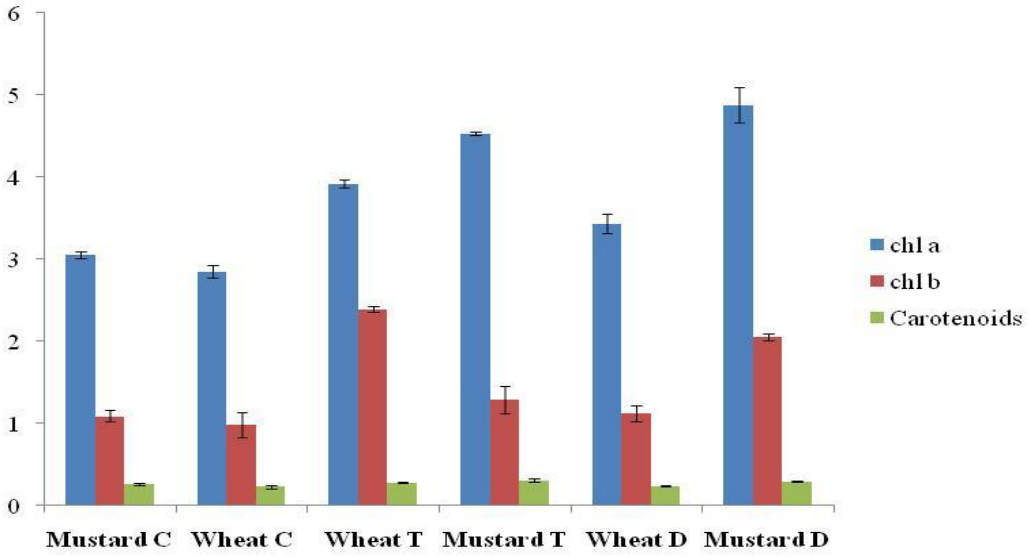


Figure 8.2. Chlorophyll-a, b, and carotenoids content (mg g^{-1}) in leaves of wheat and mustard plants growing at distillery, tannery and normal agriculture land. All values are mean ± S.D. Distillery (D); Tannery (T); Control (C).

The gereral reaction between PTEs and chlorophyll in vitro and vivo have been already reported by various author (Sorrentino et al., 2017; Lismont et al., 2017; Yilmaz et al., 2017; Abbas et al., 2017). However, the content of chl- a, b is increasing might be due to the the low content of light harvesting chlorophyll proteins (LHCPs) (Czarnecki and Grimm, 2012). In addition, the carotenoids contents supposed to act as antioxidant by scavenging the free radicals, electron transfer to double bond structure and minimize the photodamage, cellular damage, destruction of chloroplast membranes and genetic material by photodynamic reaction, quenching, collapsing of membrane and replacing perooxidation from increased accumulation of PTEs and metalloids (Czerpak et al., 2006).

8.3.2.2. ***Effect on protein content***

The protein content in plant leaves of mustard and wheat plants at the site of distillery, and tannery was found higher in comparison to control plants (Fig. 8.3a-d). Further, results also indicated that the protein content in distillery and tannery wastewater affected plants also gets enhanced significantly ($p \leq 0.05$), respectively. The protein content recorded for distillery and tannery wastewater affected wheat plants was 4.190 and 4.372 ug/mL, whereas for distillery and tannery wastewater affected mustard plants, it was 3.771 and 4.267 ug/mL, respectively (Fig 8.3a-d). Previous reports explain the additional supply of macro and micronutrients facilitates the accumulation of amino acids and enhanced the protein contents as well as induces high proline formation, which provides the stress tolerance capacity to plants through various mechanisms such as osmoregulation, and enzyme protection against various stress conditions (Noman et al., 2018). In addition, the high protein content in mustard and wheat plants of might be due to the induction of many stress proteins, growing at distillery and tannery contaminated site as reported by various researchers (Bharagava et al., 2008; Ye et al., 2017).

8.3.2.3. Effect on lipid peroxidation, H_2O_2 and ascorbic acid

The MDA content in distillery and tannery mustard plants enhanced significantly (2.82 and 2.43 mmol g^{-1} fw) in comparison to control plants (2.10 mmol g^{-1} fw) under the potentially harmful elements (PTEs) stress (Fig. 8.3a-d), $p \leq 0.05$). In contrast, the MDA content was reduced in distillery wastewater affected wheat plant, which was 2.50 mmol g^{-1} fw in comparison to tannery wastewater affected wheat 3.19 mmol g^{-1} fw. However, no significant change was observed in distillery wastewater affected mustard plants in comparison to tannery wastewater affected mustard. Enhanced MDA suggests that intracellular generation of excess ROS. These lead to membrane damage by peroxidation of polyunsaturated lipid (Singh et al., 2018) generating malondialdehyde as one of the byproducts of lipid peroxidation. This is in accordance with earlier reports by various authors under PTEs stress such as Ni Cu and Zn (Singh and Agrawal, 2007; Maheshwari and Dubey, 2009).

The reduction in MDA content in control plants in comparison to distillery and tannery wastewater affected plants reveals its detoxification potential. Tannery wastewater induced oxidative damage in wheat and mustard plants was measured in term of H_2O_2 content. A biomarker increases in the label of H_2O_2 (1024.25 nm/gm and 907.52 nm/gm) was observed in tannery wastewater affected wheat and mustard plants in comparison to the control plants. In case of distillery wastewater affected wheat, H_2O_2 content was decrease by 0.069 % in comparison to the tannery wastewater affected wheat. Generally, H_2O_2 content enhanced by the interference of PTEs stress in plants, which eliminates the ROS and suppress the lipid peroxidation. High level of H_2O_2 content in tannery wastewater affected wheat and mustard plants exhibited its toxic effects in form of plasmolysis, electrolytic leakage and membrane damage. Ascorbic acid (ASC) molecules content in

tannery wastewater affected wheat and mustard plants increased with respect to the control plants.

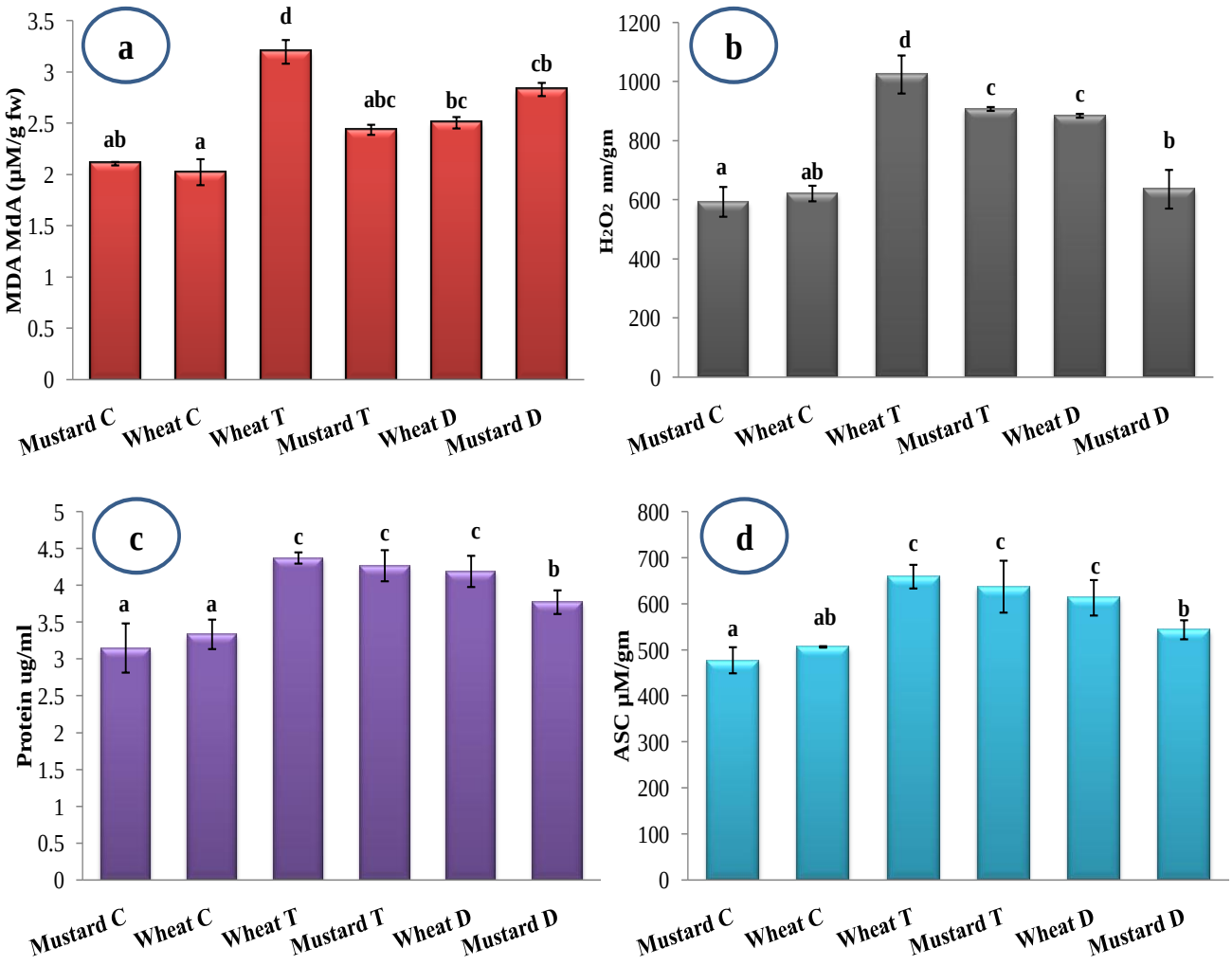


Figure 8.3a-d: Comparative analysis of MDA, H_2O_2, Protein and ASC activity in leaves of wheat and mustard plants growing at distillery, tannery and normal agriculture land. All values are means ± S.D. One-way ANOVA was performed and significant differences in different parameters were tested by DMRT. Identical superscripts denote no significant difference between means according to DMRT ($p \leq 0.05$). Distillery (D); Tannery (T); Control (C)

However, distillery wastewater affected wheat and mustard plants shown a significant decreased 4.6 & 9.4% in ASC content in comparison to tannery wastewater affected wheat and mustard plants (Fig. 8.3a-d). In plants, GSH pool is maintained by the ASC. Hence,

ascorbate is more important non-enzymatic antioxidants acts as the substrate in ASG-GSH cycle. Micro and macronutrients enhanced the activity of GPX enzymes, which maintain the intracellular level of peroxides and increasing the production of GSH (Masella et al., 2008).

8.3.2.4. Effect on enzymatic antioxidants

Tannery wastewater affected wheat and mustard plants showed the more SOD activity 104.14 and 95.44 Unit gm^{-1} fw, respectively. Whereas, distillery wastewater affected wheat and mustard plants showed significant reduction in SOD activity 86.17 and 80.35 Unit gm-1 fw, respectively ($p \leq 0.05$) under potentially harmful PTEs stress in wastewater in comparison to control plants (76.90 and 73.87 Unit gm^{-1} fw). SOD is widely present in plants and algae with a competent biochemical and scavenging machinery (enzymatic and non-enzymatic antioxidants) like as CAT, SOD, APX and Glutathione to control ROS levels to check the toxicity under the wide environmental stress. SOD will catalyze the decomposition of superoxide and detoxification mechanism such as anion in to radical oxygen, hydrogen peroxide and then converted to ground level of O_2 and H_2O. Usually depends on the PTEs cofactors (Peng et al., 2009).

The reduction in SOD activity was possibly due to low concentration of metals in distillery wastewater affected plants either its surface inside the cell and enhanced bio-geochemical cycle within the rhizosphere of plants (Upadhyaya et al., 2016) and by providing a minimum area for metal accumulation. PTEs induced ROS production and oxidative damage have been reported in various plants (Singh et al., 2007). An enhanced APX activity was observed in the tannery wastewater affected wheat plants as well as mustard plants under PTEs stress in comparison to control plants, while the distillery wastewater affected wheat and mustard plants showed the less amount of metal toxicity therefore no significant change in control and distillery wastewater affected plants was

observed. APX in the plants cell shown as an amino acid sequence (Asada et al., 1992). They scavenges the peroxide molecule i.e. H_2O_2 using ascorbate for the management of the photosynthetic machinery and other damage. Enhanced level of APX under PTEs stress showed the contrast role of detoxification of H_2O_2. Catalase (CAT) acts as primary biomarkers for the removal of H_2O_2 produced during the metal stress in form of the peroxisomes (Karuppanapandian et al., 2011).

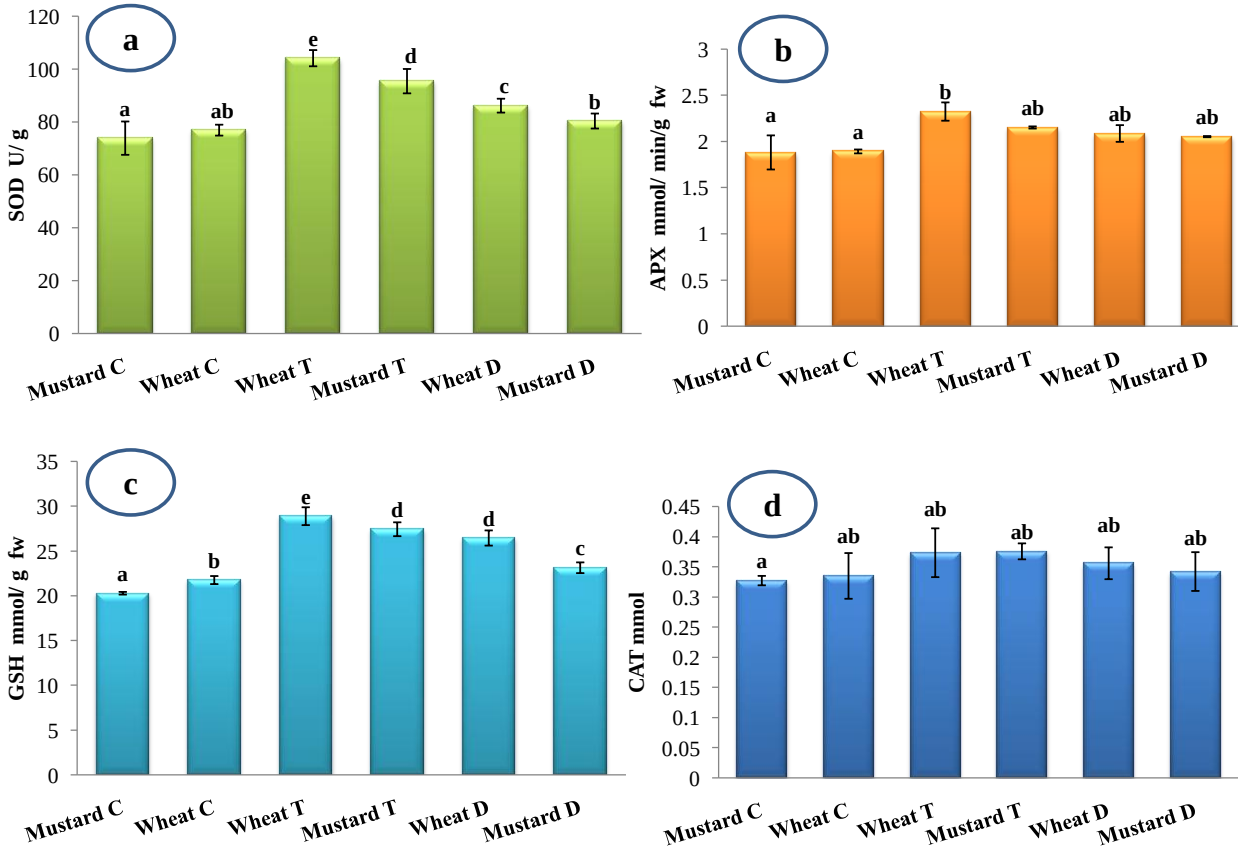


Figure 8.4a-d: Comparative analysis of SOD (U/g), APX (mmol/min/g), GSH (mmol/g) and catalase (mmol) activity in leaves of wheat and mustard plants growing at distillery, tannery and normal agriculture land. All values are means ± S.D. One-way ANOVA was performed and significant differences in different parameters were tested by DMRT. Identical superscripts denote no significant difference between means according to DMRT ($p \leq 0.05$). Distillery (D); Tannery (T); Control (C)

In tannery wastewater affected wheat and mustard plants catalase showed the significantly high-level stress in comparison to the control plants. There is no significant change in distillery wastewater affected wheat and mustard plants 0.002% and 0.002%, respectively in comparison to the control plants was observed. Catalase activity was decrease due to the low concentration of PTEs stress. Glutathione (GSH) activity in tannery wastewater affected wheat and mustard plants showed the increasing trends in comparison to the control plants. While no significant change appears in distillery wastewater affected wheat and mustard plants compare with control plants was observed (Fig 8.4a-d). GSH activity is a key factor in ASC-GSH cycle (Hoque et al., 2007). In which cell involves in removing the peroxide by balancing GSH/GSSG. Change in the level of GSH showed the metal toxicity in plants (Yadav et al., 2010).

8.3.3. Metal accumulation in plants

The analysis of distillery, tannery wastewater affected plants and control plant have revealed that the plants growing on the dross of wastes contaminated site showed a different pattern of metal translocation and accumulation in various plant parts (Table 8.2). The collected wastewater and soil/sludge samples were found to have high content of Zn, Fe, and Mn (Table 8.1). Soil serves as a nutrient medial for the growth of plants. It is well known that soil properties affect metal in various ways. Soil pH is one of the key factors affecting metal availability (Harter, 1983). In addition, organic matter and hydrous ferric oxide have been shown to reduce PTEs availability by the immobilization of these metals (Yi et al., 2007). However, some significant correlation have also been found between PTEs and soil physical properties like water holding capacity and moisture content (Sharma and Raju, 2013). PTEs availability in soils influenced by some other factor such as soil aeration, microbial activity, and mineral composition etc. (Magnuson et al., 2001).

Table 8.2: Metals content in different plant parts collected from distillery and tannery industry contaminated site

S. No.	Plant samples	Plants Parts	Heavy metals							
			Co	**Cd**	**Cr**	**Zn**	**Fe**	**Ni**	**Mn**	**Pb**
		Root	BDL	BDL	4.23±0.10	39.34±0.05	138.00±0.07	0.60±0.50	6.60±0.02	1.40±0.50
1.	**Wheat-T**	Shoot	0.20±0.30	BDL	39.50±0.50	43.56±0.15	298.00±0.05	1.60±0.02	13.40±0.10	2.60±0.10
		Leaves	0.60±.05	BDL	22.00±0.50	61.94±0.50	324.00±0.015	4.60±0.12	32.00±0.50	4.40±0.50
		Total	**0.80**	**-**	**65.73**	**144.84**	**760.00**	**6.80**	**52.00**	**8.40**
	Metal accumulation pattern		**L>S>R**	**-**	**S>L>R**	**L>S>R**	**L>S>R**	**L>S>R**	**L>S>R**	**L>S>R**
		Root	BDL	BDL	153.33±5.13	54.57±0.89	132.66±3.05	8.63±0.45	31.43±1.28	2.73±0.20
2.	**Mustard-T**	Shoot	BDL	BDL	14.86±0.90	37.24±1.54	89.50±1.80	1.61±0.24	11.60±0.30	1.26±0.25
		Leaves	BDL	BDL	79.66±3.51	52.88±1.62	359.00±5.56	6.59±0.87	47.96±0.15	3.50±0.30
		Total	**-**	**-**	**247.85**	**144.69**	**581.16**	**16.83**	**90.99**	**7.49**
	Metal accumulation pattern		**-**	**-**	**R>L>S**	**R>L>S**	**R>L>S**	**R>L>S**	**L>R>S**	**L>R>S**
		Root	BDL	BDL	8.90±0.10	41.64±1.83	361.33±16.50	2.20±0.05	24.00±0.10	2.90±0.03
3.	**Wheat-D**	Shoot	BDL	BDL	42.50±0.50	58.50±1.21	258.00±2.00	2.40±0.01	17.20±0.05	4.20±0.01
		Leaves	BDL	BDL	1.90±0.10	52.84±1.21	216.33±10.59	3.10±0.02	34.00±0.15	4.00±0.05
		Total	**-**	**-**	**53.30**	**152.98**	**835.66**	**7.70**	**75.20**	**11.10**
	Metal accumulation pattern		**-**	**-**	S>R>L	**S>L>R**	R>S>L	**L>S>R**	**L>R>S**	**S>L>R**
		Root	0.73±0.15	BDL	10.86±0.65	53.83±1.92	428.33±9.07	7.00±0.36	51.66±2.02	4.53±0.32
4.	**Mustard-D**	Shoot	BDL	BDL	5.83±0.40	54.29±1.12	210.00±11.13	2.43±0.40	26.46±0.89	2.43±0.35
		Leaves	BDL	BDL	122.00±2.64	51.75±1.45	364.66±11.93	5.79±0.75	85.16±1.11	4.86±0.25
		Total	**0.73**	**-**	**138.69**	**159.87**	**1002.99**	**15.22**	**163.28**	**11.82**
	Metal accumulation pattern		**R**	**-**	**L>R>S**	**S>R>L**	**R>L>S**	**R>L>S**	**L>R>S**	**L>R>S**

Note: Distillery (D); Tannery (T); Control (C)**;** Root (R); Shoot (S); Leaves (L)
BDL (below detection level). All values are mean ± S.D.

However, the accumulation and distribution of metals is reported to depend on metal bioavailability, plant metabolism, as well as on microbes growing in contaminated sites (Balkhair and Ashraf, 2016; Mani and Rayappan, 2014). The order for metal accumulation in distillery and tannery wastewater affected plants was Fe>Zn>Cr>Mn>Pb. However, Fe, Zn, Mn, Pb, and Ni showed same distribution pattern in tannery wastewater affected wheat plants, which was leaf to shoot and root (L>S>R), except Cr (S>L>R) whereas in tannery wastewater affected mustard plants, roots were noted as higher metal accumulator part.

In addition, the high metal content was found in mustard root followed by leaf and shoot (R>L>S), except Mn and Zn, which were higher in leaf followed by root and shoot (L>R>S). It indicated the similarity of metal distribution pattern in mustard plants and indicated the hazardous effects of Pb and Cr. In addition, wheat plants growing at distillery waste contaminated site showed the variable metals distribution pattern such as Zn, Pb (S>L>R), Ni, Mn (higher in leaf), while Cr and Pb were found higher in shoot part. Similar findings have been also reported by various authors indicating that metal accumulation in the lower plants parts (Singh and Sinha, 2005; Sinha and Gupta, 2005).

Cr is a well known to reduce plant growth and development, cause ultra structural alteration in cell membrane and chloroplast, induced chlorosis, damage root cells, reduce photosynthetic pigments, disturb translocation of water and mineral nutrients, and an enzymatic activities in plants (Ali et al., 2015; Farooq et al., 2016). Balkhair and Ashraf, 2016) found that agricultural land contaminated with PTEs causes potential human health hazards related with the edible crops.

8.3.4. Bioconcentration and translocation factors

In present study, we observed that the ratio of metals content in root to soil/sludge (BCF), distillery and tannery wastewater affected plants showed <1, except Zn, which was >1 in tannery wastewater affected mustard plant while the TF for most of metals in

wheat and mustard plants was noted as >1 (Table 8.3). Results showed that experimental plants growing at waste contaminated sites have high metal accumulation potential. Further, the BCF of PTEs, which showed the ratio of root to soil/sludge was as Cr (0.118), Zn (1.29), Fe (0.276), Ni (0.165), Mn (0.249), and Pb (0.133), whereas TF of Cr (10.318), followed by Ni, Mn, Pb, Fe and Zn in tannery wastewater affected mustard plants. Similarly, the BCF of distillery wastewater affected mustard plants was noted as Cr (0.610), Zn (0.741), Fe (0.977), Ni (0.636), Mn (0.527), and Pb (0.314) whereas TF was higher in Ni (2.880) followed by Fe, Mn, Pb, Cr and Zn. Further, the BCF of different metals in distillery and tannery wastewater affected wheat was Cr (0.50), Zn (0.573), Fe (0.824), Ni (0.200), Mn (0.244), Pb (0.201) and Cr (0.003), Zn (0.931), Fe (0.288), Ni (0.001), Mn (0.052), Pb (0.058), respectively.

In addition, the TF of distillery and tannery wastewater affected wheat was Fe (1.400) followed by Mn, Ni, Zn, Pb, Cr, and Zn followed by Pb, Mn, Fe, Cr, respectively. The PTEs tolerant plants have a tendency to confine sludge-root as well as root-shoot transfer, that's why these plants have less accumulation in plant biomass. It is reported that if a plant has BCF <1, then it can be used for metal extraction from contaminated sites (Fitz and Wenzel 2002). However, the metals accumulation from soil to any suitable plants is depends on the PTEs availability, media pH, and chemical nature of other co-pollutants because the basic pH of sludge/soil restricts the metal mobility and thus, inhibits its translocation and accumulation in plants (Gupta and Sinha, 2008; Yoon et al., 2006; Rosselli et al., 2003).

Table 8.3: Translocation and accumulation of heavy metals in different plant samples collected from distillery and tannery industry contaminated site

S. No.	Plant Samples	Bioconcentration Factor (BCF)								Translocation Factor (TF)							
		Co	**Cr**	**Zn**	**Fe**	**Ni**	**Mn**	**Pb**	**Cd**	**Co**	**Cr**	**Zn**	**Fe**	**Ni**	**Mn**	**Pb**	**Cd**
1.	**Wheat-T**	BDL	BDL	0.93	0.29	BDL	0.05	0.06	BDL	BDL	0.11	0.90	0.46	0.38	0.49	0.54	BDL
2.	**Mustard-T**	BDL	0.12	1.29	0.28	0.17	0.25	0.13	BDL	BDL	10.32	0.93	1.48	5.36	2.71	2.17	BDL
3.	**Wheat-D**	BDL	0.50	0.57	0.82	0.20	0.24	0.20	BDL	BDL	0.21	0.71	1.40	0.92	1.40	0.69	BDL
4.	**Mustard-D**	0.125	0.61	0.74	0.98	0.64	0.53	0.31	BDL	BDL	1.87	0.99	2.04	2.88	1.95	1.86	BDL

Note: Distillery (D); Tannery (T); Control (C); BDL (below detection level)

Conclusions

This manuscript reports a study that examined two contaminated sites i.e. distillery and tannery wastewater/sludge contains high concentration of undesirable PTEs (Co, Cr, Ni, Mn, Zn, Fe, Pb etc.) and physico-chemical parameters (pH, EC, organic matter, moisture, and chloride) beyond the standard/permissible limit. It can be also concluded that wheat and mustard plants growing at distillery and tannery wastewater contaminated site have high metals accumulation potential in different parts i.e. root, shoot, and leaves. This high metal accumulation in plants may directly or indirectly hamper various metabolic activities as well as oxidative damage by altering the structure of enzymes, transporters or regulatory enzymes owing to their strong affinity as ligends to sulfhydryl and carboxylic groups. The biochemical analysis of the selected plant i.e., wheat and mustard revealed increased lipid peroxidation, non- enzymatic and enzymatic antioxidant activities such as MDA, H_2O_2, ASC, SOD, APX, CAT and GPX. Thus, it can be concluded that continuous monitoring is too needed near distillery and tannery area of water and soil. However, ecotoxicological analysis/ observation need to be more attention prior its application in crop fields.

8 (B). Toxicity evaluation of distillery wastewater pollutants and their metabolites after bacterial treatment by using *Caenorhabditis elegans* as terrestrial test models

8 (B).1. Introduction

Distillery industries (DIs) are the backbone of agro-industrial economy, but unfortunately, these are also major sources of environmental pollution. DIs generates a huge volume of dark coloured and highly toxic wastewater, which contain very high biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolve solid (TDS) as well as a complex nature of organic and inorganic pollutants (Bharagava and Chandra, 2010; Arimi et al., 2014; Chandra and Kumar, 2017). In India, more than 325 distilleries are in operation, which produce 3.25 x 10^9 l of alcohol and 40.40 x 10^{10} l of wastewater annually (Bharagava and Chandra, 2009; Arimi et al., 2015). An average

molasses based DI generates 12-15 L of spent wash for L^{-1} of alcohol produced. Distillery wastewater (DWW) is highly toxic for aquatic fauna and flora because its dark color reduces photosynthetic activity and depletes dissolved oxygen content in receiving water bodies (Pal and Yadav, 2012; Chowdhary et al., 2017). In DWW, melanoidins are the major recalcitrant coloring pollutants, which cause adverse effects on environment and health problems in human, and (Wang et al., 2011; Arimi et al., 2014; Arimi et al., 2015). DWW is also reported to have a mixture of endocrine disrupting chemicals (EDCs) like phthalate such as DI-N-octyl phthalate and Dibutyl phthalate (Yadav and Chandra, 2012; Arimi et al., 2014). These EDCs disturb the delicate hormonal balance, and compromise the reproductive fitness of animals, and may also lead to the carcinogenesis (Dixit et al., 2015).

In many developing countries including India, farmers are irrigating their crop plants with industrial wastewaters carrying high concentration of various toxic metals due to the non-availability of alternative sources of irrigation water (Chandra et al., 2009). However, many authors have characterized DWW as liquid organic fertilizer as it contains both organic and inorganic nutrients with high potassium content and different trace elements (Ramana et al., 2002a & b) But, the direct use of DWW in agricultural field for irrigational purposes is generally not recommended safe for environmental health and plant growth (Joshi et al., 1998; Mahimaraja and Bolan, 2004) due to high BOD, COD values, high concentration of potentially toxic metals and ions present in DWW.

The fate and toxicity extent of DWW is still remains unknown into the environment (Jain et al., 2002; Yadav and Chandra, 2012). Various authors have used test model *Caenorhabditis elegans* (*C. elegans*) was also used for toxicity evaluation of industrial as well as municipal wastewaters (Tigini et al., 2010; Polak et al., 2014; Imanikia et al., 2016; Jiang et al., 2016). *C. elegans* has several unique features, which make it relevant and

suitable model organism for biological experiments. It is a soil nematode with transparent body, which initially inhabits the liquid phase of soils (Jiang et al., 2016).

Hence, the objectives of this study were to characterize the potential bacterial strains capable for the degradation and detoxification of DWW pollutants and characterization and identification of DWW pollutants and their metabolites. Simultaneously, to evaluate the toxicity of DWW by using *Caenorhabditis elegans* as terrestrial test models before and after bacterial treatment for the protection of environment, human and animal health.

8 (B). 2. Materials and methods

8 (B). 2. 1. Preparation of untreated and bacterial treated DWW samples

For toxicity evaluation, the untreated and bacteria treated DWW samples were centrifuged at 5,000 rpm for 10 min to remove the suspended solids and bacterial biomass, respectively and left over night to settle down the remaining suspended particles. If growth occurs, it was again centrifuged to remove it, stored in screw-cap glass bottle (1 liter) and used in toxicity assessment tests.

8 (B). 2. 2. Toxicity evaluation by using *C. elegans*

8 (B). 2. 2. 1. Culture and maintenance of strains

C. elegans strains, Bristol N2 (wild type), were procured from *Caenorhabditis* Genetics Centre, (University of Minnesota, MN, USA) and grown on Nematode growth medium (NGM), using *Escherichia coli* OP50 as food source. All the experiments were conducted at 22°C.

8 (B). 2. 2. 2. *Relative quantification of acetylcholine (Ach)/acetyl cholinesterase (AchE)*

Ach levels were determined using Amplex Red Ach/AchE estimation kit as indicated in the manufacturer's procedure. The treatment plates added with age-synchronized embryos were incubated at 22 °C for 48 hr. M9 buffer was used to wash adult worms thrice and sonicated in 1x reaction buffer (supplied in the kit) for 3 min. At 7000 rpm,

worm suspension was then centrifuged for 7 min. 100 µL of reaction mixture was prepared by adding 200 µL of 20 mM Amplex Red solution, 100 µL of 200 U/mL HRP solution, 100 µL of 100 U/mL AchE solution, and 100 µL of 20 U/mL choline oxidase solution in 10 mL q.s. of 1x reaction buffer in black well plates in which 100 µL of supernatant was added. The plates were incubated at room temperature for 30 min and fluorescence was read using 96 well plate fluorimeter (BMG Polarstar Galaxy) excitation at 544 nm and emission at 590 nm. The relative fluorescence obtained was normalized with protein content of sample calculated using Bradford method and RFU per µg of protein was calculated to estimate the relative Ach levels (Urra et al., 2016).

8 (B). 2. 2. 3. ***Synaptic Ach levels as deduced through aldicarb assay***

An indirect assay, Aldicarb assay, was used to check the relative effect on neurotransmission and Ach levels within the synapse. Aldicarb is basically a carbamate insecticide, which acts as an AchE inhibitor. Approximately 40 worms were transferred to 0.5 mM aldicarb NGM plates. The worms were keep counted every 30 min for paralysis and were poked using eye lash. As a convention, the worms lacking movement even after poking thrice were considered as paralyzed. Any worms lost or damaged were disregarded from the study. The experiment was done in duplicate sets and percentage of worms paralyzed was calculated (Hosono et al., 1992).

8 (B). 2. 2. 4. ***Effect on nAchR as deduced through levamisole assay***

Levamisole ((S)-6-Phenyl-2,3,5,6-tetrahydroimidazole[2,1-b][1,3]thiazole) a cholinergic agonist is used in Levamisole assay, which is done to check the post synaptic defects in worms. Approximately 20 to 30 worms were transferred to each well of 96 well plates incubated at 22 °C for 48 hr. Equal volume of 50 µM levamisole solution was added to each well and worms were scored for paralysis. The worms that lacked movement were

considered as paralyzed. Percentage of worms paralyzed at every time interval was calculated (Fleming et al., 1997).

8 (B). 2. 2. 5. ***RNA isolation, cDNA synthesis and quantitative real time PCR***

Total RNA from adult age-synchronized animals was extracted by using Trizol reagent (Invitrogen, Life Technologies). 0.1 % DEPC water was used to wash worms from treatment plates. The worms were crushed in 250 µL Trizol reagent using micropestles (Tarson) and for making the final volume up to 1 mL, rest 750 µL of Trizol was added, which was followed by addition of 200 µL of chloroform and was mixed for 2 to 5 min on a vortex mixer. The suspension was then centrifuged at 14000 rpm, 4 °C for 15 min and supernatant was gently pipetted out in fresh vials. RNA was precipitated by addition of 500 µL chilled isopropanol. The vials were kept at room temperature for 10 min and again centrifuged at 14000 rpm, 4 °C for 10 min. The obtained RNA pellets were washed twice with 75 % ethanol (chilled) at 7500 rpm, 4 °C for 5 min. The RNA pellet was finally dissolved in 15 µL of 0.1 % DEPC water and for quantification, RNA absorbance was read using nano drop (QuaWell Q5000).

cDNA synthesis was done from 1 µg of total *C. elegans* RNA in a 96 well thermal cycler (BioRad, C1000) with steps including, incubation at 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min and 4 °C eternally RNA using high capacity cDNA synthesis Kit (Applied Biosystems). Until utilization, quantified cDNA samples by nanodrop were stored at -80 °C. 125 ng of cDNA was used as template for each reaction of qRT-PCR with gpd-1 as housekeeping control using Light Cycler 480 machine (Roche Diagnostics, Germany). For each primer pair, a melting curve analysis was performed. The thermocycling program in brief was an initial incubation of 50 °C for 2 min hold; 95 °C for 10 min followed by 40 cycles of denaturing at 95 °C for 15 s, annealing at 58 °C for 30 s and final extension at 72 °C for 20 s. Differential expression was calculated by $2^{-\Delta\Delta CT}$

method. Gpd-1 was used as internal control and used to normalize ratios between samples (Shen et al., 2001).

Equation for calculation of differential expression:

$$\Delta\Delta CT = \Delta CT_{\text{refrence}} - \Delta CT_{\text{target}}$$

$$\Delta CT = CT_{\text{Refrence in control}} - CT_{\text{Refrence in treatment}}$$

8 (B). 2. 2. 6. *Nile red staining*

The effect of untreated and treated DWW on lipid content in C. *elegans* was studied by staining the worms with a lipid specific dye i.e. Nile Red (9-diethylamino-5-benzo[α] phenoxazinone) as described by Ashrafi et al., (2003) (Ashrafi et al., 2003). The stock solution of Nile red dye was prepared by dissolving 0.5mg Nile red dye in 1 mL of acetone. This stock solution was further diluted to 1:250 and seeded onto NGM plates. Now, age synchronized embryos, isolated through Sodium hypochlorite treatment were transferred onto the Nile red-containing treatment plates and incubated for 48 hrs at 22°C for wild type worms and 48 hrs at 15°C followed by 24 hrs at 25°C for CL4176 worms. The worms were then washed thrice with M9 buffer and anesthetized by adding 10 µl of 100 mM sodium azide in 100 µl of worm suspension. The cover slip was sealed by using transparent nail paint and worms were examined under the fluorescence microscope for visualization of lipid droplets using Rhodamine filter.

8 (B). 2. 2. 7. Statistical analysis

All the experiments were performed in triplicate.

8(B).2.3. Result and Discussion

8(B).2.3.1. Toxicological effects of untreated and bacteria treated DWW on test organisms

A very little variability could be recorded from the total Ach levels and AchE activity of the worms treated with DWW and its metabolites (bacterial treated). A marginal

upsurge in the Ach levels as well as AchE activity was available after the treatment with DWW, which was slightly subsided by the consortia treated DWW. The effect of reduced and non-reduced DWW on the cholinergic transmission using aldicarb and levamisole was also studied. The percentage of worms paralyzed at a particular point of time reflects the synaptic Ach levels. The consortia treated samples reflected increased synaptic Ach levels suggesting increased cholinergic transmission. The cholinergic transmission in worm's muscarinic and nicotinic pathways, where the muscarinic and nicotinic pathways participate the elicit response of acetylcholine. The nicotinic acetylcholine signaling receptor could be studied using levamisole assay. Both treated and untreated DWW samples negatively modulated the nAchR signaling. Overall, the synthetic DWW and

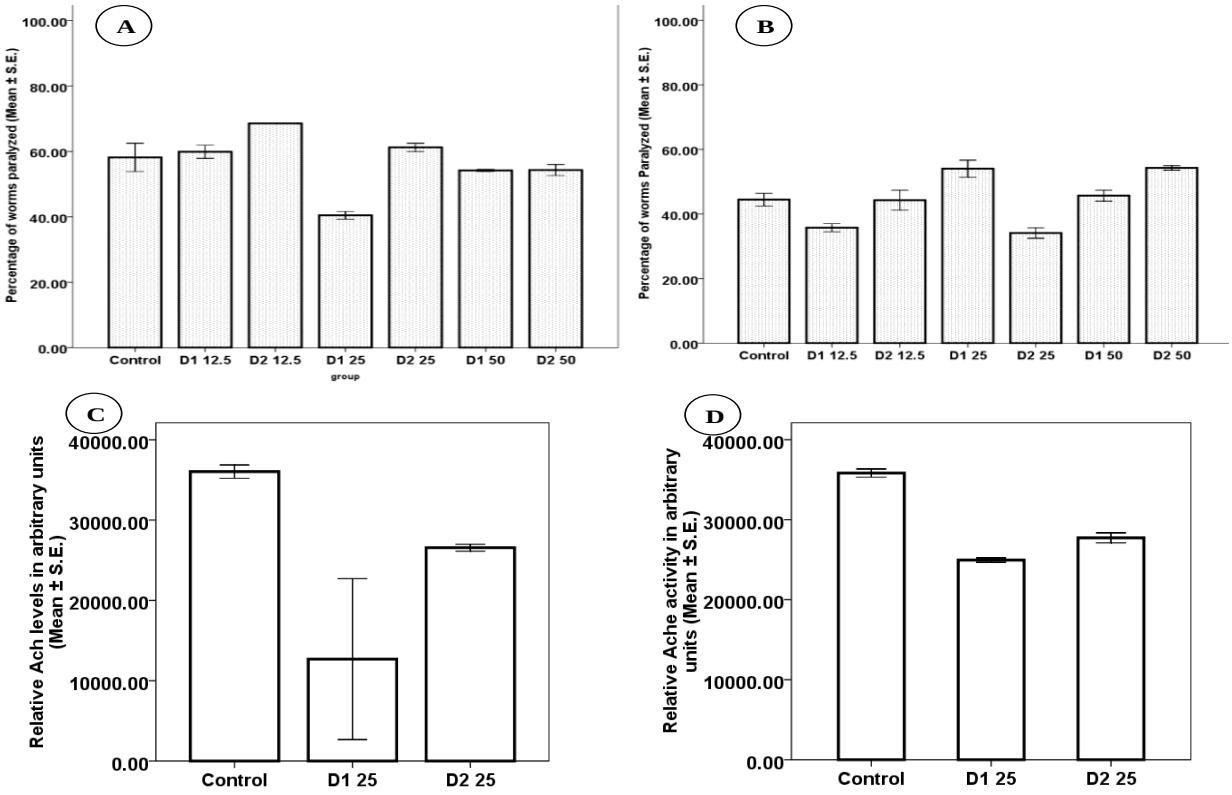


Figure 8B.1. Effect of DWW-UT (Untreated) and DWW-T (Treated) on synaptic Ach (A), nAchR activity (B), gross Ach (C) and AchE levels (D) D1: Untreated DWW; D2: Treated DWW

consortia treated DWW samples increased the overall cholinergic transmission with diminishing effect upon nAchR signaling. Contrary to above, the AchE activity was also slightly increased (Fig. 8B.1).

To find the suitable explanation of this, we performed qPCR for the genes involved in regulation of cholinergic transmission. We observed significant of regulation in the ace2, cho1, cha1 and unc 17. The increased AchE activity could be arbitrated to the up regulation of ace2, cho1, cha1 and is correlated with the findings of aldicarb assay. The above findings are also substantiated by observation of the increased levels of unc17 (gene coding for the Ach transport). The increased unaltered nAchR signalling as recorded after levamisole assay was also substantiated by the genes unc 38 (necessary component of nAchR) and unc50 (participates in nAchR trafficking) (Fig. 8B. 2A).

8(B).2.3.2. Effects of untreated and bacteria treated DWW on lipid content in *C. Elegans*

By allosteric mechanisms, the lipid content influences the functioning of nicotinic receptors by allosteric mechanisms, stabilizing varying proportions of pre-existing resting, open, desensitized, and uncoupled conformations (Baenziger et al., 2013). In fact, lipids stabilize the different conformational states, lipid-nicotinic receptor interactions modulate the receptor functions at the biological synapses, which has been seen through Nile red staining. Nile red staining is established as a suitable and quantitative method to stain the main fat storage in *C. elegans*. Nile red staining of worms exposed to untreated and bacteria treated DWW revealed that the worms exposed for untreated DWW showed sharp reduction/loss in total fat content having more profound effects, suggesting the diminished nAchR signaling as compare to treated DWW. Jiang et al. (2016) have explores the sublethal toxicity of four heavy metals (Cu, Zn, Cd, and Cr) and found highest susceptibility on AChE in *C. elegans*. The exposure of warms to untreated and bacteria

treated DWW diminished the total lipid content with more profound effects in comparison to treated DWW (Fig 8B.2B).

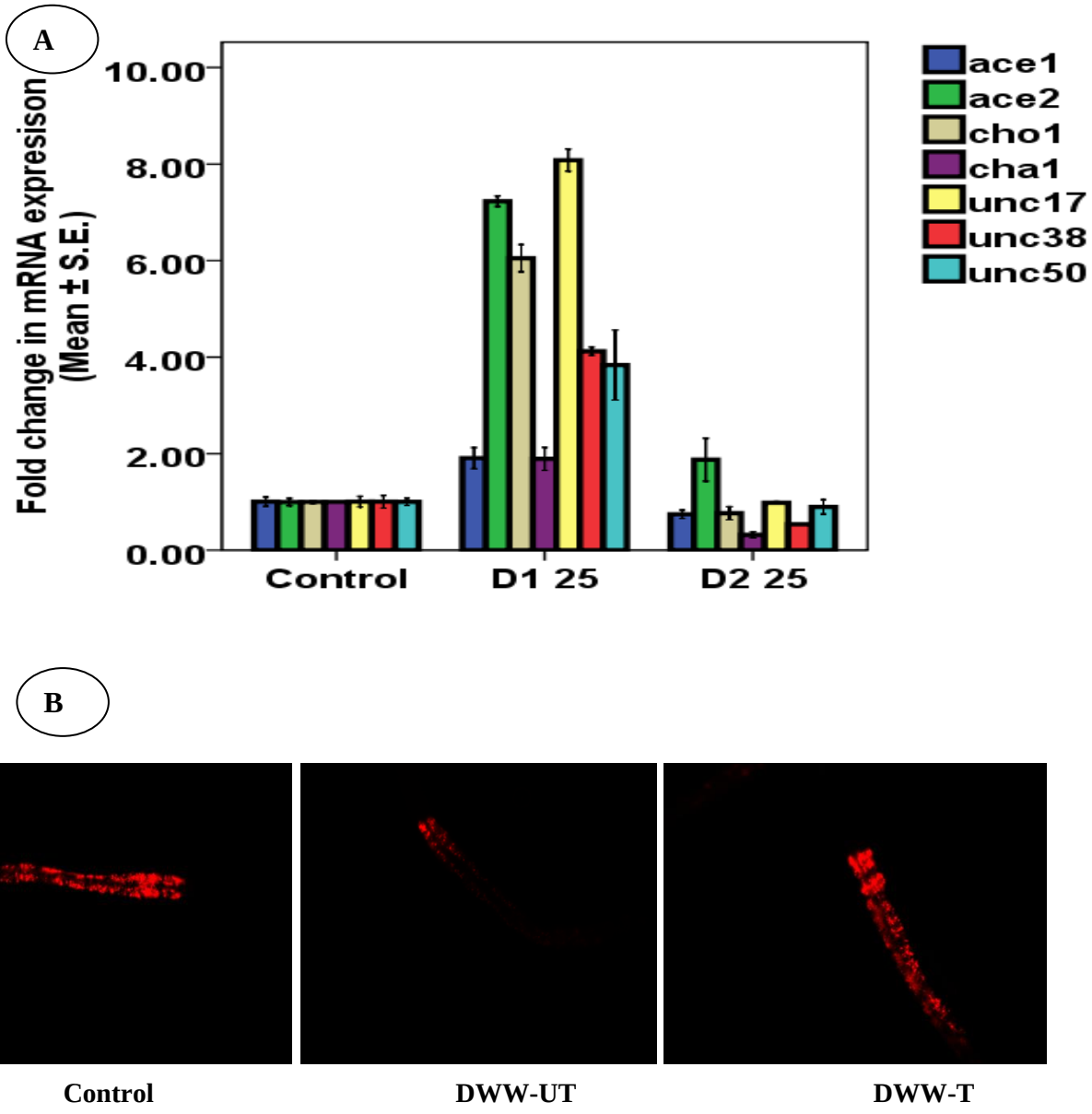


Figure 8B.2. Effect of DWW-UT (Untreated) and DWW-T (Treated) on mRNA expression of genes related to Ach transmission (A) and Nile red staining to study the effect of distillery wastewater on lipid content *C. elegans* (B)

DWW-UT: distillery wastewater-untreated; T: treated; D1: untreated DWW; D2: treated DWW

Conclusion

This manuscript reports a study that examined two contaminated sites i.e. distillery and tannery wastewater/sludge contains high concentration of undesirable PTEs (Co, Cr, Ni, Mn, Zn, Fe, Pb etc.) and physico-chemical parameters (pH, EC, organic matter, moisture, and chloride) beyond the standard/permissible limit. It can be also concluded that wheat and mustard plants growing at distillery and tannery wastewater contaminated site have high metals accumulation potential in different parts i.e. root, shoot, and leaves. This high metal accumulation in plants may directly or indirectly hamper various metabolic activities as well as oxidative damage by altering the structure of enzymes, transporters or regulatory enzymes owing to their strong affinity as ligends to sulfhydryl and carboxylic groups. The biochemical analysis of the selected plant i.e., wheat and mustard revealed increased lipid peroxidation, non- enzymatic and enzymatic antioxidant activities such as MDA, H_2O_2, ASC, SOD, APX, CAT and GPX. Thus, it can be concluded that continuous monitoring is too needed near distillery and tannery area of water and soil. However, ecotoxicological analysis/ observation need to be more attention prior its application in crop fields.

Bacterial treatment reduces the toxicity of DWW. Animal test model (*C. elegans*) are well reported for environmental toxicity, which clearly showed the toxicity of DWW pollutants in terms of percent mortality, morphological changes and alteration in lipid content, respectively. This study concluded that inadequate disposal of untreated DWW can cause the transfer of toxic substances into the environment, disturbing the biological ecosystem near the industry as well as receiving aquatic resources.

Chapter 9
Summary

Chapter 9

Summary

Distillery industry is among of major sources of environmental pollution like water and soil pollution. In India, the number of distilleries has gone up to 319 with annual production of 3.25×10^9 L of alcohol and 40.4×10^{10} L of wastewater. Mainly four steps are involved in alcohol production such as feed preparation, fermentation, distillation and packaging. The DWW has dark brown colored due to the presence of melanoidins and some other coloring compounds. Molasses based distillery generates an average 15 L of spent wash in 1 alcohol production. In DWW, melanoidin is one of the major pollutants causing serious environmental and health problem. Melanoidins are dark brown to the black colored natural condensation product of sugar and amino acids, recalcitrant compounds. DWW contains a complex mixture of organic and inorganic pollutants and acts as a major source of environmental pollution. DWW causes coloration of water resources, reduces photosynthetic activities, and dissolved oxygen content, whereas, in the soil, it reduces soil fertility and seed germination. The organic and inorganic pollutants such as melanoidins and endocrine disrupting compounds (phthalates) present in DWW are well reported to have cytotoxic, genotoxic, carcinogenic and mutagenic effects on human and animal health. The physical and chemical methods suggested for treatment of wastewater are not much effective for decolorization. Biological methods like anaerobic digestion treatment reduce BOD load of the spent wash, but the substantial amount of organic components and dark brown color left behind requires secondary treatment.

Treatment through biological way is an incredible alternate for DWW pollutants due to their low cost, environmental friendly and publicly acceptable treatment. There are various biological processes such as bioadsorption and biodegradation have been reported having prospective application in color removal from spentwash by fungi such as *Coriolus, Aspergillus, Phanerochaete* and certain bacterial sp. as *Bacillus, Alcaligenes* and

Lactobacillus for the bioremediation of spent wash. Microbially treated wastewater may be less toxic and safe device for effluent management.

However, the biological treatment of DWW containing melanoidin largely depends on pH, temperature, concentration of nutrients, oxygen and inoculums size as well as there are some enzymatic system responsible for the degradation of melanoidin consists mainly sugar oxidases and peroxidases as sarbose oxidase, glucose oxidase, etc. and also ligninolytic enzyme (Laccase, MnP and LiP). Since ligninolytic enzyme (MnP and MIP) showed melanoidin decolorizing activity in the presence of H_2O_2 and the decolorizing activity of both sugar oxidases and peroxidases were found optimum at a particular pH, temperature and substrate specific. However, fate and extent of toxicity of anaerobically treated DWW remains unknown in the environment.

Thus, it requires adequate treatment before its final discharge into the environment. Physico-chemical methods available are capable of both color and organic load reduction, but these methods are highly costly and generate a large amount of sludge as secondary pollutants. Hence, biological methods are gaining its momentum in the arena of wastewater treatment methods due to cost effective and eco-friendly nature, but these methods are time-consuming. Therefore, there is an urgent need to address the limitations in existing treatment methods and to develop the integrated treatment processes that can provide a solution to DIs for the management and treatment of generated wastewater.

The first objectives of this study, which detailed in chapter three (03) isolation, screening and characterization of ligninolytic enzyme producing bacterial strains capable for the degradation of DWW pollutants. In this study initially nine (09) bacterial strains (DS, DS1-DS8) were isolated by nutrient enrichment technique, among of these four bacterial strains DS1, DS3, DS4, and DS5 screened on the basis of their growth on different concentration of DWW and manganese peroxidase activity shown on phenol red

containing GPYM agar plates amended with DWW. Further, the isolated bacterial strains were identified on the basis of morphologically/microscopically. The bacterial strains DS3 and DS4 were identified as gram positive and cell was coccus and rod shape, respectively whereas DS5 were identified as gram negative and rod shape.

Chapter four (04) of this study, result concluded that the selected bacteria strains on the basis of melanoidins resistance test and MnP enzyme production activity on plate was compatible with each other, because observed no inhibition zone found around the bacterial colony. Further, all these potential bacterial strains was selected for the consortium development. In addition, decolorization assay in axenic conditions was not much more effective for distillery wastewater decolorization or degradation. Because in axenic culture condition selected bacterial strains i.e. DS3, DS4, and DS5 showed decolorization of distillery wastewater was 52.31, 63.26, and 49.69%, respectively. Finally, it was observed that physico-chemical parameters was also not reduced significantly by the axenic bacterial treatment. Therefore, for the effective decolorization of distillery wastewater through mixed culture consortia was developed and perform decolorization experiment in laboratory.

Chapter five (05) of this study comprised that the potential bacterial strains, which were used for consortia development i.e. DS3, DS4, and DS5 were identified as *Staphylococcus saprophyticus*, *Bacillus megaterium sp.* and *Alcaligenaceae* sp. with accession number MF182113, MF967441 and MF182114, respectively. However, these bacterial strains have ability to produce ligninolytic enzyme, which may be responsible for the distillery wastewater pollutants decolorization/degradation. Subsequently, the ligninolytic enzyme laccase and MnP were identified by SDS-PAGE electrophoresis of partially purified enzyme has yield band of laccase and MnP with the molecular weight ~65 and 43 kDa, respectively. Hence, these enzyme may be involved in the pollutants

degradation such as melanoidins and other phenolic and coloring compounds present in distillery wastewater.

Chapter six (06) summarized that the distillery wastewater was deep brown in color carrying very high values of BOD, COD total solids, phosphate, phenolics, and sulfate. In addition DWW also contains heavy metals such as Fe, Zn, Cd, Pb, and Ni etc. Further, by the treatment of bacterial consortium (DS3+DS4+DS5) was found effective to decolorize DWW upto 76.12% in comparison to axenic culture conditions with considerable reduction in BOD, COD values, total solids, sulfates, phosphates and phenolic metal content. Further, the decolorization of distillery wastewater pollutants was also studied by the various environmental factor such as pH and temperature and several carbon source (glucose, fructose, sucrose, galactose, maltose) and nitrogen source (yeast extract, peptone, urea, ammonium sulphate, and sodium nitrate) for the optimum decolorization of distillery wastewater. Hence, it was observed that potential bacterial consortium showed the maximum decolorization was 76.12% in presence of glucose (0.5%) and peptone (0.1%) at pH 7.0 and temperature 35 °C. This study proves that in distillery wastewater decolorization environmental factor and various nutritional sources are play their role effectively. Further, it was also observed that the coloring compounds such as melanoidins etc. present in distillery wastewater may increases the cell size of identified bacterial strains *Staphylococcus saprophyticus*, *Bacillus megaterium sp.* and *Alcaligenaceae* sp. under morphological observation with SEM analysis.

Chapter seven (07) of this study, concluded that FT-IR and LC-MS/MS analysis confirmed the presence of various organic compounds in DWW, in which some compounds were reported as hazardous for living organisms. Bacterial treatment reduces the toxicity of DWW. FT-IR and LC-MS/MS shown the presence of various organic compounds with molecular weight such as 270, 182, 165, 154, 150, 135, 126, 98, 86 that

were identified as Diethyl-3,4-ethylenedioxypyrrole-2,5-dicarboxylate, Trans-2-Tridecenal, Dihydroxyconiferyl alcohol ($C_{10}H_{14}O_3$), 2-nitroacetophenone ($C_8H_7NO_3$), Anhydrohexose from $[M-H]^-$, 2,6-dimethoxyphenol ($C_8H_{10}O_3$), 4-vinyl-2- methoxyphenol ($C_9H_{10}O_2$), p-chloroanisol (C_7H_7ClO), 4-methyl guaicol, N-methyl indane, 5-(hydroxymethyl)-2-furfural ($C_6H_6O_3$), Furfuryl alcohol ($C_5H_5O_2$) (98), and Butenoic acid ($C_4H_6O_2$), respectively and some other compounds before and after bacterial treatment. Hence, it was clearly observed that after secondary treatment process several toxic chemicals remains in distillery wastewater. Thus, there is an urgent need to address the limitations in the existing treatment methods and to develop the integrated treatment processes that provide a complete solution to the treatment of distillery wastewater.

Finally chapter eight (08) comprised a reports that examined a comparative study at two contaminated sites i.e. distillery and tannery wastewater/sludge contains high concentration of undesirable PTEs (Co, Cr, Ni, Mn, Zn, Fe, Pb etc.) and physico-chemical parameters (pH, EC, organic matter, moisture, and chloride) beyond the standard/permissible limit. It can be also concluded that wheat and mustard plants growing at distillery and tannery wastewater contaminated site have high metals accumulation potential in different parts i.e. root, shoot, and leaves. This high metal accumulation in plants may directly or indirectly hamper various metabolic activities as well as oxidative damage by altering the structure of enzymes, transporters or regulatory enzymes owing to their strong affinity as ligends to sulfhydryl and carboxylic groups. The biochemical analysis of the selected plant i.e., wheat and mustard revealed increased lipid peroxidation, non-enzymatic and enzymatic antioxidant activities such as MDA, H_2O_2, ASC, SOD, APX, CAT and GPX. Thus, it can be concluded that continuous monitoring is too needed near distillery and tannery area of water and soil. However, ecotoxicological analysis/ observation need to be more attention prior its application in crop fields.

In addition, the main objective of this study to check that bacterial treatment reduces the toxicity of DWW or not. This was confirmed by the animal test model (*C. elegans*) with untreated and bacterial treated distillery wastewater. This test organism are well reported for environmental toxicity, which clearly showed the toxicity of DWW pollutants in terms of alteration in mRNA expression of genes related to Ach transmission and lipid content. The overall study concluded that inadequate disposal of untreated DWW can cause the transfer of toxic substances into the environment, disturbing the biological ecosystem near the industry as well as receiving aquatic resources.

Thus, an urgent need to address the limitations in existing treatment methods and to develop the integrated treatment processes that could be provide a solution to DIs for the management and treatment of generated wastewater. In this connection, this study helps to know the nature and characteristics of the recalcitrant organic pollutants in distillery wastewater that remained even after the secondary treatment process. Further, the potential bacterial strains could be useful for the new knowledge generation and technology development for the effective treatment and complete removal of organic pollutants also to know the toxicological effects of such types of wastewaters in soil as well as in animal, which is a serious threat to environment. That's why this study could be useful for the eco-friendly and cost effective treatment of distillery wastewater with sustainable and safe treatment.

Chapter 10
Bibliography

Chapter 10

Bibliography

Abbas, A., Iqbal, Z., Abbas, R.Z., Khan, M.K., Khan, J.A., Mahmood, M.S. and Saleemi, M.K., 2017. In vivo anticoccidial effects of Beta vulgaris (sugar beet) in broiler chickens. Microbial pathogenesis, 111, 139-144.

Abbas, S.T., Sarfras, M., Mehdi, S.M., Hassan, G., Rehman, O.U., 2007. Trace element accumulation in soil and rice plants irrigated with the contaminated water, Soil Tillage Res. 94, 503-509.

Acharya, B.K., Mohana, S., Madamwar, D., 2008. Anaerobic treatment of distillery spent wash - a study on upflow anaerobic fixed film bioreactor. Biores. Technol. 99(11), 4621-6. doi: 10.1016/j.biortech.2007.06.060.

Afify, A.S., Mahmoud, M.A., Emara, H.A., Abdelkreem, K.I., 2009. Phenolic compounds and COD removal from olive mill wastewater by chemical and biological procedures. Aus. J. Basic. Appl. Sci. 3, 1087-1095.

Agarwal, R., Lata, S., Gupta, M., Singh, P., 2010. Removal of melanoidin present in distillery effluent as a major colorant: A Review. J. of Environ. Biol. 31, 521-528.

Ahmad, I., Javed, M.,z Asghar, H.N., Shahid, M., 2016. Differential effects of plant growth-promoting rhizobacteria on maize growth and cadmium uptake. J. Plant Growth Regul. 35, 303-315.

Akunna, J.C., Clark, M., 2000. Performance of a granular-bed anaerobic baffled reactor (GRABBR) treating whisky distillery wastewater. Biores. Technol. 74: 257-261.

Alam, M.Z., Ahmad, S., Malik, A., Ahmad, M., 2010. Mutagenicity and genotoxicity of tannery effluents used for irrigation at Kanpur, India. Ecotoxicol. Environ. Saf. 73(5), 1620-1628.

Ali, S., Chaudhary, A., Rizwan, M., Anwar, H.T., Adrees, M., Farid, M., Irshad, M.K., Hayat, T., Anjum, S.A., 2015. Alleviation of chromium toxicity by glycinebetaine is

related to elevated antioxidant enzymes and suppressed chromium uptake and oxidative stress in wheat (*Triticum aestivum L.).* Environ. Sci. Pollut. Res. 22, 10669-10678.

Altschul, S. F., Madden, T.L., Schaffer, A. A. et al. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402.

Angayarkanni, J., Palaniswamy, M., Swaminathan, K., 2003. Biotreatment of distillery effluent using *Aspergillus niveus*. Bull. Environ. Contam.Toxicol. 70, 268-277.

AOAC, 2002. Official Method of Analysis of AOAC International. In: William, H. (Ed.), 17th edn. AOAC International.

Aoshima, I., Tozawa, Y., Ohmomo, S., Udea, K., 1985. Production of decolourizing activity for molasses pigment by *Coriolus versicolor* Ps4a. Agric. Biol. Chem. 49, 2041-2045.

APHA (American Public Health Association). (2012). Standard Method for Examination of Water and Wastewater, 22nd ed. Washington, DC.

Apollo, S., Onyango, M.S., Ochieng, A., 2013. An integrated anaerobic digestion and UV photocatalytic treatment of distillery wastewater. J. Hazard. Mater. 261 (15), 435-442.

Arakane, Y., Muthukrishnan, S., Beeman, R.W., Kanost, M.R., Kramer, K.J., 2005. Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning, Proceedings of The National Academy of Sciences of the United States of America, 102(32):11337-11342.

Arimi, M.M., Zhang, Y., Geißen, Sven-Uwe., 2015. Color removal of melanoidin-rich industrial effluent by natural manganese oxides. Separa. and Purifi. Technol. 150, 286-291.

Arimi, M.M., Zhang, Y., Götz, G., Geißen, S.-U., 2015. Treatment of melanoidin wastewater by anaerobic digestion and coagulation. Environmental Technology 36(19): 2410-2418.

Arimi, M.M., Zhang, Y., Götz, G., Kiriamiti, K., Geißen, Sven-Uwe., 2014. Antimicrobial colorants in molasses distillery wastewater and their removal technologies. Int. Biodet. & Biodeg. 87, 34-43.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiol. 24,1-15.

Asada, K., 1992. Ascorbate peroxidase–a hydrogen peroxide-scavenging enzyme in plants. Physiologia Plantarum, 85, 235-241.

Asaithambi, P., Saravanathamizhan, R., Matheswaran, M., 2015. Comparison of treatment and energy efficiency of advanced oxidation processes for the distillery wastewater. Int. J. Environ. Sci. Technol. 12, 2213-2220.

Balat, M., Balat, H., 2009. Recent trends in global production and utilization of bioethanol fuel. Appl. Energ. 86, 2273-2282.

Baldrian, P. 2006. Fungal laccases-occurrence and properties. FEMS Microbiol. Rev., 30, 215–242.

Balkhair, K.S., Ashraf, M.A., 2016. Field accumulation risks of heavy metals in soil and vegetable crop irrigated with sewage water in western region of Saudi Arabia. Saudi Journal of Biological Sciences 23, S32-S44.

Banerjee, S., Biswas, G.K., 2004. Studies on biomethanation of distillery wastes and its mathematical analysis. Chem. Eng. J. 102,193-201.

Bartling, B., Rehbein, G., Somoza, V., Silber, R.E., Simm, A., 2005. Maillard reaction product-rich foods impair cell proliferation and induce cell death in vitro. Sig. Transduc. 5, 303-313.

Barrow, G.I., Feltham, R.K.A., 1993. Cowan and steel's manual for the identification of medical bacteria, 3rd edn. Cambridge University Press, Cambridge.

Berg, C., 2004. World fuel ethanol analysis and outlook /http:// www.distill.com/World-Fuel-Ethanol-A&O-2004.html.

Bezuneh, T.T., 2016. The Role of Microorganisms in Distillery Wastewater Treatment: A Review. J. Bioremediat. Biodegrad. 7, 375. doi: 10.4172/2155-6199.1000375.

Bharagava, R.N., Chandra, R., 2010a. Biodegradation of the major color containing compounds in distillery wastewater by an aerobic bacterial culture and characterization of their metabolites. Biodegradation 21, 703-711.

Bharagava, R.N., Chandra, R., 2010b. Effect of bacteria treated and untreated post-methanated distillery effluent (PMDE) on seed germination, seedling growth and amylase activity in *Phaseolus mungo* L. J. Hazard. Mater. 180, 730-734.

Bharagava, R.N., Chandra, R., Rai V., 2008. Phytoextraction of trace elements and physiological changes in Indian mustard plants (*Brassica nigra* L.) grown in post methanated distillery effluent (PMDE) irrigated soil. Bioresource Technology, 99, 8316-8324.

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

Bharagava, R.N., Chandra, R., Singh, S.K., 2008. Characterization of phenolic metabolites from post methanated distillery effluent (PMDE) after degradation with bacterial consortium. Indian J. Environ. Protec. 28(11), 1019-1027.

Bhardwaj, P., Chaturvedi, A. K., Prasad, P., 2009. Effect of enhanced lead and cadmium in soil on physiological and biochemical attributes of *Phaseolus vulgaris* L. Nature and Science.7,63-75.

Billore, S.K., Singh, N., Ram, H.K., Sharma, J.K., Singh, V. P., Nelson, R. M. and Dass, P., 2001. Treatment of molasses based distillery effluent in a constructed wetland in central India. Water Sci. and Technol. 44(11-12), 441-448.

Bingtao, Wu., d, Weili Zhou., 2010. Investigation of soluble microbial products in anaerobic wastewater treatment effluents. J. of Chem. Technolo. and Biotech. 85(12), 1597-1603. doi 10.1002/jctb.2471.

Blonskaja, V., Menert, A., Vilu, R., 2003. Use of two-stage anaerobic treatment for distillery waste. Adv. in Environ. Res. 7(3), 671-678.

Borrelli, R.C., Mennella, C., Barba, F., Russo, M., Russo, G.L., Krome, K., Erbersdobler, H.F., Faist, V., Fogliano, V., 2003. Characterization of coloured compounds obtained by enzymatic extraction of bakery products. Food Chem. Toxicol. 41, 1367-1374.

Bose, S., Bhattacharyya, A.K., 2008. Heavy metal accumulation in wheat plant grown in soil amended with industrial sludge. Chemosphere 70, 1264-1272.

Brands, C.M.J., Alink, G.M, van Boekel, M.A.J.S., Jongen, W.M.F., 2000. Mutagenicity of heated sugar-casein systems: effect of the Maillard reaction. J. Agric. Food Chem. 48, 2271-2275.

Bustamante, M.A., Paredes, C., Moral, R., Moreno-Caselles, J., Perez-Espinosa, A., Perez-Murcia, M.D., 2005. Uses of winery and distillery effluents in agriculture: characterisation of nutrient and hazardous components. Sustain. Viticult. Winery Wastes Manag. 51, 145-151.

Cammerer, B., Jalyschkov, V., Kroh, L.W., 2002. Carbohydrate structures as part of the melanoidin skeleton. Int. Congr. Ser. 1245, 269-273.

Chance, B., and Maehly, A.C., 1955. Assay of catalase and peroxidases. Methods Enzymol. 2, 764-775.

Chandra, R., Bharagava, R.N, Rai, V. 2008. Melanoidins as major colourant in sugarcane molasses based distillery effluent and its degradation. Biores. Technol. 99, 4648-46601.

Chandra, R., Bharagava, R.N., Kapley, A., Purohit, H.J. 2012. Characterization of *Phargmites cummunis* rhizosphere bacterial communities and metabolic products during the two stage sequential treatment of post methanated distillery effluent by bacteria and wetland plants. Biores. Technol. 103, 78-86.

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

Chandra, R., Kumar, V., 2017a. Phytoextraction of heavy metals by potential native plants and their microscopic observation of root growing on stabilised distillery sludge as a prospective tool for in situ phytoremediation of industrial waste. Environ. Sci. Pollut. Res. 24, 2605-2619.

Chandra, R., Kumar, V., 2017b. Detection of *Bacillus* and *Stenotrophomonas* species growing in an organic acid and endocrine-disrupting chemical-rich environment of distillery spent wash and its phytotoxicity. Environ. Monit. Assess. 189, 26.

Chandra, R., Pandey, P.K., 2000. Decolorization of anaerobically treated distillery effluent by activated charcoal adsorption method. Indian J. of Environ. Protec. 21(2), 134-137.

Chandra, R., Yadav, S., Bharagava, R.N., Murthy, R.C. 2008b. Bacterial pretreatment enhances removal of heavy metals during treatment of post-methanated distillery effluent by *Typha angustata* L. J. of Environ. Manage. 88,1016-1024.

Charfeddine, M., Charfeddine, S., Bouaziz, D., Messaoud, R.B. and Bouzid, R.G., 2017. The effect of cadmium on transgenic potato (Solanum tuberosum) plants

overexpressing the St DREB transcription factors. Plant Cell, Tissue and Organ Culture (PCTOC), 128, 521-541.

Charles, David, M., Arivazhagan, M.N., Balamurali, Dhivya, S., 2015. Decolorization of Distillery Spent Wash Using Biopolymer Synthesized by *Pseudomonas aeruginosa* Isolated from Tannery Effluent. doi.org/10.1155/2015/195879.

Chaudhari, P.K., Mishra, I.M, Chand, S., 2005. Catalytic thermal pretreatment (catalytic thermolysis) of biodigester effluent of an alcohol distillery plant. Ind. Eng. Chem. Res. 44, 5518-5525.

Chaudhary, R., Arora, M., 2011. Study on distillery effluent: chemical analysis and impact on environment. Int, J, of Adv, Eng, Technol, 2(2), 352-356.

Chavan, M.N., Kulkarni, M.V., Zope, V.P, Mahulikar, P.P., 2000. Microbial degradation of melanoidins in distillery spent wash by an indigenous isolate. Indian J. Biotechnol. 5, 416-421.

Chen, B., Tian, X., Yu, L., Wu, Z., 2016. Removal of pigments from molasses wastewater by combining micro-electrolysis with biological treatment method. Bioprocess Biosyst Eng. 39, 1867-1875.

Chopra, P., Singh, D., Verma, V., Puniya, A.K., 2004. Bioremediation of melanoidin containing digested spent wash from cane-molasses distillery with white rot fungus, *Coriolus versicolour*. Ind. J. Microbiol. 44, 197-200.

Choudhary, A.K., Kumar, S., Sharma, C., 2011. Organic load removal from paper mill wastewater using green technology. WAC. 103-109.

Choudri, B.S., Baawain M., 2016. Bioenergy from Biofuel Residues and Wastes. Water Environment Research. doi: 10.2175/106143016X14696400495217.

Chowdhary, P., More, N., Raj, A., Bharagava, R. N., 2017b. Characterization and identification of bacterial pathogens from treated tannery wastewater. Microbiol Res Int, 5, 30-36.

Chowdhary, P., Raj, A., Bharagava, R. N., 2018. Environmental pollution and health hazards from distillery wastewater and treatment approaches to combat the environmental. Chemosphere, 194, 229-246.

Chowdhary, P., Yadav, A., Kaithwas, G., Bharagava, R. N., 2017. Distillery Wastewater: A Major Source of Environmental Pollution and Its Biological Treatment for Environmental Safety In: Singh R, Kumar S, (Eds.) Green Technologies and Environmental Sustainability, Springer International, Switzerland, pp 409-435.

Coca, M., Pena, M., Gonzalez, G., 2005. Chemical Oxidation Processes for Decolorization of Brown Colored Molasses Wastewater. Ozone: Sci. and Engineering 27, 365-369.

Collins, G., Foy, C., McHugh, S., Maho, T., O'Flaherty., 2005. Anaerobic biological treatment of phenolic wastewater at 15-18ºC. Water Res. 39, 1614-1620.

Czarnecki and Grimm, 2012. Post-translational control of tetrapyrrole biosynthesis in plants, algae, and cyanobacteria. J of Exp Botany. 63, 1675-1687.

Czerpak, R., Piotrowska, A., Szulecka, K., 2006. Jasmonic acid affects changes in the growth and some components content in alga Chlorella vulgaris, Acta Physiol. Plant 28, 195-203.

D'souza, DT., Tiwari, R., Sah, AK., Raghukumar, C., 2006. Enhanced production of Laccase by a marine fungus during treatment of coloured effluents and synthetic dyes. Enz. Micro. Technol. 38, 504-511.

Dahiya, J., Singh, D., Nigam, P., 2001. Decolorization of synthetic and spentwash melanoidins using the white-rot fungus *Phanerochaete chrysosporium* JAG-40. Biores. Technol. 78, 95-98.

Dai, J., Mumper, R.J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15, 7313-7352.

David, C., Arivazhagan, M., Balamurali, M. N., Shanmugarajan, D., 2015. Decolorization of Distillery Spent Wash Using Biopolymer Synthesized by Pseudomonas aeruginosa Isolated from Tannery Effluent. BioMed Research International. http://dx.doi.org/10.1155/2015/195879.

de Lemos Chernicharo, C.A., 2007. Anaerobic Reactors. IWA Publishing, London, UK.

Dec, J., Bollag, J.M., 1994. Use of Plant Material for the Decontamination of Water Polluted with Phenols. Biotechnol. and Bioeng. 44, 1132-9.

Dixit, S., Yadav, A., Dwivedi, P.D., Das, M., 2015. Toxic hazards of leather industry and technologies to combat threat: A review. J. of Clean. Produc. 87, 39-49.

Duxbury, A.C., Yentsch, C.S., 1956. Plankton pigment monograph. J. Mar. Res. 15, 93-101.

Dwyer, J., Griffiths, P., Lant, P., 2009. Simultaneous colour and don removal from sewage treatment plant effluent: alum coagulation of melanoidin. Water Res. 43, 553-561.

Dwyer, J., Kavanagh, L., Lant, P., 2008. The degradation of dissolved organic nitrogen associated with melanoidin using a UV/H_2O_2 AOP. Chemosphere 71,1745-1753.

El-Dib, F.I., Tawfik, F.M., Eshaq, G., Hefni, H.H.H., ElMetwally, A.E., 2016. Remediation of distilleries wastewater using chitosan immobilized Bentonite and Bentonite based organoclaysInt J Biol Macromol 86, 750-755.

Emamverdian, A., Ding, Y., Mokhberdoran, F., Xie, Y., 2015. Heavy Metal Stress and Some Mechanisms of Plant Defense Response. Scientific World Journal .

EPA, 1996. Test Methods for Evaluating Solid Waste. SW-846 Method 3050-B. U.S. Environmental Protection Agency, Office of research and Development, Cincinnati.

Fagier, M.A., Elmugdad, A.A., Aziz, M.E.A., Gabra, N.M. 2015. Identification of some Organic Compounds in Sugarcane vinasse by Gas Chromatography-Mass Spectrometry and Prediction of their Toxicity Using TEST Method. JCPS. 8, (4).

Fang, H.H.P., Chui, H.K., Li, Y.Y., 1994. Microbial structure and activity of UASB granules treating different wastewaters, Water Sci. Technol. 30, 87-96.

Farhadian, M., Borghei, M., Umrania, V.V., 2007. Treatment of Beet Sugar Wastewater by UAFB Bioprocess. Biores. Technol. 98(16), 3080-3.

Farooq, M., Ali, S., Hameed, A., Bharwana, S., Rizwan, M., Ishaque, W., Farid, M., Mahmood, K., Iqbal, Z., 2016. Cadmium stress in cotton seedlings: physiological, photosynthesis and oxidative damages alleviated by glycinebetaine. S. Afr. J. Bot. 104, 61-68.

Farshi, R., Priya S., Saidutta, M. B., 2013. Reduction of colour and COD of An aerobically Treated Distillery Wastewater by Electrochemical Method, International Journal of Current Engineering and Technology, pp 168-171.

Feng, Y., Wang, X., Logan, B.E., Lee, H., 2008. Brewery wastewater treatment using air-cathode microbial fuel cells. Appl. Microbiol. Biotechnol. 78, 873-880.

Fitz, W.J. and Wenzel, W.W., 2002. Arsenic transformations in the soil–rhizosphere–plant system: fundamentals and potential application to phytoremediation. Journal of biotechnology, 99, 259-278.

Fountoulakis, M.S., Manios, T., 2009. Enhanced methane and hydrogen production from municipal solid waste and agro-industrial by-products co-digested with crude glycerol. Bioresour. Technol. 100(12), 3043-3057.

Garcia-Calderon, D., Buffiere, P., Moletta, R., Elmaleh, S., 1998. Anaerobic digestion of wine distillery wastewater in down-flow fluidized bed. Water Res. 32(12), 3593-3600.

Georgiou, R.P., Tsiakiri, E.P., Lazaridis, N.K., Pantazaki., A.A., 2016. Decolorization of melanoidins from simulated and industrial molasses effluents by immobilized laccase. Journal of Environmental Chemical Engineering 4, 1322-1331.

Ghosh, M., Ganguli, A., Tripathi, A.K., 2002. Treatment of anaerobically digested molasses spent wash in a two-stage bioreactor using *Pseudomonas putida* and *Aeromonas* sp. Process. Biochem. 37, 857-862.

Ghosh, M., Ganguli, A., Tripathi, A.K., 2009. Decolorization of anaerobically digested molasses spent wash by *Pseudomonas putida*. Appl. Biochem. and Microbiol. 45, 68-73.

Glosl, S., Wagner, K.H., Draxler, A., Kaniak, M., Lichtenecker, S., Sonnleitner, A., et al., 2004. Genotoxicity and mutagenicity of melanoidins isolated from a roasted glucose-glycine model in human lymphocyte cultures, intestinal Caco-2 cells and in the *salmonella typhimurium* strains TA98 and TA102 applying the Ames test. Food and Chem. Toxicol. 42, 1487-1495.

Goncalves, J.F., Becker, A.G., Cargnelutti, D., Tabaldi, L.A., Pereira, L.B., Battisti, V., Spanevello, R.M., Morsch, V.M., Nicoloso, F.T., Schetinger, M.R.C., 2007. Cadmium toxicity causes oxidative stress and induces response of the antioxidant system in cucumber seedlings. Braz. J. Plant Physiol. 19, 223-232.

Gonzalez, T., Terron, M.C., Yague, S., Zapico, E., Galletti, G.C., Gonzalez, A.E., 2000. Pyrolysis/gas chromatography/mass spectrometry monitoring of fungal-biotreated distillery wastewater using Trametes sp. I-62 (CECT 20197). Rapid Communications in Mass Spectrometry. 14(15), 1417-1424.

Gosh, S., 1990. Principles and potentials of biphasic fermentation, in: Report: International Conference on Biogas: Technologies and Implementation Strategies, January 10-15, Pune, India.

Goyal, S.K., Seth, R., Handa, B.K., 1996. Diphasic fixed-film biomethanation of distillery spentwash. Biores. Technol. 56(2-3), 239-244.

Guimaraes, C., Porto, P., Oliveira, R., Mota, M., 2005. Continuous decolourization of a sugar refinery wastewater in a modified rotating biological contactor with *Phanerochaete chrysosporium* immobilized on polyurethane foam discs. Process. Biochem. 40, 535-540.

Gupta, A.K., Sinha, S., 2008. Decontamination and/or revegetation of fly ash dykes through naturally growing plants. J. Hazard. Mater. 153, 1078-1087.

Hamza, H. M. C., Duraisamy, P., Periyasamy, S., Pokkiladathu, H., Muthuchamy, M., 2017. Simultaneous Electricity Generation and Heavy Metals Reduction from Distillery Effluent by Microbial Fuel Cell. Indian J. Sci. Technol. 10(13), 10.17485/ijst/2017/v10i13/111203.

Harada, H., Uemura, S., Chen, A.C., Jayadevan, J., 1996. Anaerobic treatment of a recalcitrant wastewater by a thermophilic UASB reactor. Biores. Technol. 55(3), 215-221.

Harter, R. D., 1983. Effect of soil pH on adsorption of lead, copper, zinc, and nickel. *Soil Science Society of America Journal*, 47(1) 47-51.

Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts kinetic and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189-198.

Hiramoto, K., Nasuhara, A., Michikoshi, K., Kato, T., Kikugawa, K., 1997. DNA strand-breaking activity of 2,3-dihydro-3,5-dihydroxy-6- methyl-4H-pyran-4-one (DDMP), a Maillard reaction product of glucose and glycine. Mutat. Res. 395, 47-56.

Hoque, M.A., Banu, M.N.A., Okuma, E., Amako, K., Nakamura, Y., Shimoishi, Y. and Murata, Y., 2007. Exogenous proline and glycinebetaine increase NaCl-induced ascorbate–glutathione cycle enzyme activities, and proline improves salt tolerance

more than glycinebetaine in tobacco Bright Yellow-2 suspension-cultured cells. Journal of plant physiology. 164, 1457-1468.

Hseu, Z.Y., 2004. Evaluating heavy metal contents in nine composts using four digestion methods. Bioresource Technol. 5, 53-9.

Incedayi, B., Tamer, C.E., Copur., U.C., 2010. A research on the composition of Pomegranate molasses. J. of Agri. Fac. 24, 37-47.

Jain, N., Minocha, A.K., Verma, C.L., 2002. Degradation of predigested distillery effluent by isolated bacterial strains. Indian J. of Exp. Biol. 40, 101-105.

Jiang, N., Luo, X., Zeng, J., Yang, Z.R., Zheng, L.N., Wang, S.T., 2010. Lead toxicity induced growth and antioxidant responses in *Luffa cylindrica* seedlings. Inter J Agric Biol 12, 205-210.

Jimenez, A.M., Borja, R., 1997. Influence of aerobic pretreatment with *Penicillium decumbens* on the anaerobic digestion of beet molasses alcoholic fermentation waste water in suspended and immobilized cell bioreactors. J. Chem. Technol. Biotechnol. 69, 193-202.

Jimenez, A.M., Borja, R., Martin, A., 2004. A comparative kinetic evaluation of the anaerobic digestion of untreated molasses and molasses previously fermented with *Penicillium decumbens* in batch reactors. Biochem. Eng. J. 18, 121-132.

Jimnez, A.M., Borja, R., Martin, A., 2003. Aerobic-anaerobic biodegradation of beet molasses alcoholic fermentation wastewater. Process. Biochem. 38, 1275-1284.

Jimoh, F., Adedapo, A., Aliero, A., Afolayan, A., 2008. Polyphenolic contents and biological activities of *Rumex ecklonianus*. Pharma. Biol. 46, 333-340.

Jing, H., Kitts, D.D., 2000. Comparison of the antioxidative and cytotoxic properties of glucose-lysine and fructose-lysine Maillard reaction products. Food Res. Int. 33, 509-516.

Jiranuntipon, S., Chareonpornwattana, S., Damronglerd, S., Albasi, C., Delia, Marie-Line., 2008. Decolorization of synthetic melanoidins-containing wastewater by a bacterial consortium. J. Ind. Microbiol. Biotechnol. 35, 1313-1321.

Jiranuntipona, S., Deliab, Marie-Line., Albasib, Claire., Damronglerdc, S., Chareonpornwattana Supat., 2009. Decolourization of molasses based distillery wastewater using a bacterial consortium. Science Asia 35, 332-339.

Jo, Y., Kim, J., Hwang, S., Lee. C., 2015. Anaerobic treatment of rice winery wastewater in an upflow filter packed with steel slag under different hydraulic loading conditions. Biores.Technol. 193, 53-61.

Kabir, E.R., Rahman, M.S., Rahman, I., 2015. A Review on endocrine disruptors and their possible impacts on human health environmental toxicology and pharmacology. doi.org/doi:10.1016/j.etap.2015.06.009.

Kalavathi, D.F., Uma, L., Subramanian, G., 2001. Degradation and metabolization of the pigment- melanoidin in a distillery effluent by the marine cyanobacterium *Oscillatoria boryana* BDU 92181. Enz. and Microbial. Technol. 29(4-5), 246-251.

Karuppanapandian, T., Moon, J.C., Kim, C., Manoharan, K. and Kim, W., 2011. Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms. Australian Journal of Crop Science. 5,709.

Kaushik, G., Thakur, I.S., 2009. Isolation and characterization of distillery spent wash color reducing bacteria and process optimization by Taguchi approach. International Biodeterioration & Biodegradation 63, 420-426.

Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, LE., Kaattari, S., et al. 1996. Research needs for the assessment of health and environmental effects of endocrine disruptors: A Report of the U.S. EPA-sponsored workshop. Environ Health Perspect. 104(4), 715-740.

Keller and Schwager, H., 1977. Air pollution and ascorbic acid. Eur. J. for. Path. 7, 338-350.

Kleerebezem, R. Macarie, H., 2003. Treating industrial wastewater: ananerobic digestion comes of age, www.che.com.

Krzywonos, M., Chałupniak, A., Zabochnicka-Świątek, Magdalena., 2017. Decolorization of beet molasses vinasse by *Bacillus megaterium* ATCC 14581. Bioremediation Journal, 21, 2, 81-88, DOI: 10.1080/10889868.2017.1312263.

Kumar, P., Chandra, R., 2006. Decolorization and detoxification of synthetic molasses melanoidins by individual and mixed cultures of *Bacillus spp*. Biores. Technol. 97, 2096-2102.

Kumaresan, T., Sheriffa, Begum, K.M.M., Sivashanmugam, P., Anantharaman, N., Sundaram, S., 2003. Experimental studies on treatment of distillery effluent by liquid membrane extraction. Chem. Eng .J. 95(1-3), 199-204.

Lalov, I.G., Guerginov, I.I., Krysteva, A., Fartsov, K., 2000. Treatment of wastewater from distilleries with chitosan. Water Res. 34(5), 1503-1506.

Liakos, T.I., Lazaridis, N.K., 2014. Melanoidins removal from simulated and real wastewaters by coagulation and electro-flotation. Chemical Engineering Journal 242, 269-277.

Lismont, M., Dreesen, L. and Wuttke, S., 2017. Metal-Organic Framework Nanoparticles in Photodynamic Therapy: Current Status and Perspectives. Advanced Functional Materials, 27.

Loupasaki, E., Diamadopoulos, E., 2013. Attached growth systems for wastewater treatment in small and rural communities: a review. J. Chem. Technol. Biotechnol. 88, 190-204.

Lowry, O. H., Rosenbrough, N.M., Farr, A.L., Randall, R.J., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Maheshwari, R. and Dubey, R.S., 2009. Nickel-induced oxidative stress and the role of antioxidant defense in rice seedlings. Plant Growth Regulation. 59, 37-49.

Mahimaraja, S., Bolan, N.S., 2004. Problems and prospects of agricultural use of distillery spentwash in India. Super Soil 2004. 3rd Australian New Zealand Soils Conference. 5-9 December'2004. University of Sydney, Australia.

Malandra, L., Wolfaardt, G., Zietsman, A., Viljoen-Bloom, M., 2003. Microbiology of a biological contactor for winery wastewater treatment. Water Res. 37(17), 4125-34.

Mandal, A., Ojha, K., Ghosh, D.N., 2003. Removal of color from distillery wastewater by different processes. Indian Chem. Eng. Section B 45(4), 264-267.

Mane, J.D., Modi, S., Nagawade, S., Phadnis, S.P., Bhandari, V.M., 2006. Treatment of spentwash using chemically modified bagasse and colour removal studies. Biores Technol 97(14), 1752-1755.

Mani, G.K. and Rayappan, J.B.B., 2014. Novel and facile synthesis of randomly interconnected ZnO nanoplatelets using spray pyrolysis and their room temperature sensing characteristics. Sensors and Actuators B: Chemical. 198, 125-133.

Martin, M., Raposo, F., Borja, R., Martın, A., 2002. Kinetic study of the anaerobic digestion of vinasse pretreated with ozone, ozone plus ultraviolet light, and ozone plus ultraviolet light in the presence of titanium dioxide. Proc. Biochem. 37, 699-706.

Martin, S., Fernandez, Bocanegra, J.L, Martin, A., Garcia, I., 2003. Ozonation of vinasse in acid and alkaline media. J. Chem. Technol. Biotechnol. 78, 1121-1127.

Martins, S.I.F.S., and van Boekel, M.A.J.S. 2004. A kinetic model for the glucose/glycine Maillard reaction pathways. Food Chem. 90(1-2), 257-269.

Masella, R., Di Benedetto, R., Varì, R., Filesi, C. and Giovannini, C., 2005. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. The Journal of nutritional biochemistry. 16, 577-586.

Melamane, X.L., Strong, P.J., Burgess, J.E., 2007. Treatment of wine distillery wastewater: a review with emphasis on anaerobic membrane reactors. S. Afr. J. Enol. Vitic. 28(1), 25-36.

Méndez-Acosta H.O., Snell-Castro R., Alcaraz-González V., González-Álvarez V., PelayoOrtiz C., 2010. Anaerobic digestion of Tequila vinasses in a CSTR-type digester, Biodegradation 21(3), 357-363.

Mishra, P., Das, D., 2014. Biohydrogen production from Enterobacter cloacae IIT-BT 08 using distillery effluent. Int. J. Hydrogen Energy. 39(14), 7496-507.

Mishra, P., Roy, S., Das, D., 2015. Comparative Evaluation of the Hydrogen Production by mixed Consortium, Synthetic Co-culture and Pure culture Using Distillery Effluent. Bioresource Techno., 198, 593-602.

Miyata, N., Iwahori, K., Fujita, M., 1998. Manganese independent and dependent decolourisation of melanoidin by extra cellular hydrogen peroxide and peroxidases from Coriolus hirsutus pellets. J. Ferment. Bioeng. 85 (5), 550-555.

Modak, P., 2011. Waste. Investing in energy and resource efficiency. To war. a green Econ.
Environ.http://www.unep.org/greeneconomy/Portals/88/documents/ger/GER_8_Waste.pdf.

Mohana, S., Desai, C., Datta, M., 2007. Biodegradation and of anaerobically treated distillery spent wash by a novel bacterial consortium. Biores. Technol. 98, 333-339.

Mohana, S., Acharya, B.K., Madamwar, D., 2009. Distillery spent wash: treatment technologies and potential applications. J. Hazard. Mater. 15, 163(1), 12-25.

Moletta, R., 2005. Winery and distillery wastewater treatment by anaerobic digestion. Water Sci. Technol. 51(1), 137-44.

Mourato, M. P., Moreira, I.N., Leitão, I., Pinto, F. R., Sales J. R., and Martins, L. L., 2015. Effect of Heavy Metals in Plants of the Genus Brassica. Int. J. Mol. Sci. 16, 17975-17998.

Mulidzi, A.R., 2010. Winery and distillery wastewater treatment by constructed wetland with shorter retention time. Water Sci. Technol. 61(10), 2611-5.

Mussatto, S.I., Dragone, G., Guimarães, P.M., Silva, J.P.A., Carneiro, L.M., Roberto, I.C., Vicente, A., Domingues, L., Teixeira, J.A., 2010. Technological trends, global market, and challenges of bio-ethanol production. Biotechnol. Adv. 28, 817-830.

Nakano, Y., K. Asada, Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867-880.

Nandy, T., Shastry, S., Kaul., S.N., 2002. Wastewater management in cane molasses distillery involving bioresource recovery. J. of Environ. Manag. 65, 25-38.

Naser A. Anjum, et al., 2016. Catalase and ascorbate peroxidase-representative H_2O_2-detoxifying heme enzymes in plants. Environ Sci. Pollut. Res. 23, 19002-19029

Nataraj, S.K, Hosamani, K.M, Aminabhavi, T.M., 2006. Distillery wastewater treatment by the membrane-based nanofiltration and reverse osmosis processes. Water Res 40(12), 2349-2356.

Nicolella, C., van Loosdrecht, M.C.M., Heijnen, S.J., 2000. Particle-based biofilm reactor technology. Tibtech 18, 312-320.

Nishikimi, M. Rao, N.A., Yagi K, 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem. Biophys. Res. Commun. 46, 849-854.

Noman, A., Ali, Q., Maqsood, J., Iqbal, N., Javed, M.T., Rasool, N. and Naseem, J., 2018. Deciphering physio-biochemical, yield, and nutritional quality attributes of water-stressed radish (*Raphanus sativus* L.) plants grown from Zn-Lys primed seeds. Chemosphere. 195, 175-189.

Nure, J.F., Shibeshi, N.T., Asfaw, S. L., Audenaert W., Hulle S.W.H.V., 2017. COD and colour removal from molasses spent wash using activated carbon produced from bagasse fly ash of Matahara sugar factory, Oromiya region, Ethiopia. Water SA 43(3), http://dx.doi.org/10.4314/wsa.v43i3.12.

Ohmomo, S., Aoshima, I., Tozawa, Y., Sakurada, N., Ueda, K., 1985. Purification and some properties of melanoidin decolourizing enzymes, P-3 and P-4, from mycelia of *Coriolus vericolour* Ps4a. Agric. Biol.Chem. 49, 2047-2053.

Onyango, M.S., Ojijo, V.O., Ochieng, A., Kittinya, J.O., Otieno, F.O., 2012. Simultaneous adsorption and biodegradation of synthetic melanoidin. Afr J Biotechnol 11: 6083-6090.

Orth, A.B., Royse, D.J., Tien, M., 1993. Ubiquity of lignin degrading peroxidases among various wood degrading fungi. Applied and Environmental Microbiology 59,4017-4023.

Pal, S., and Vimala, Y., 2012. Bioremediation and decolorization of Distillery effluent by novel Microbial Consortium. Euro. J. of Experi. Biol. 2(3), 496-504.

Pandey, R.A., Malhotra, S., Tankhiwale, A., Pande, S., Pathe, P.P., Kaul, S.N., 2003. Treatment of Biologically Treated Distillery Effluent- A Case Study Int J of Environ Studies 60(3), 263-275.

Pant, D., Adholeya, A., 2007. Biological approaches for treatment of distillery wastewater: a review. Biores Technol 98, 2321-2334.

Pant, D., Adholeya, A., 2009. Concentration of fungal ligninolytic enzymes by ultrafiltration and their use in distillery effluent decolorization. World J. of Microbiol. and Biotechnol. 25(10),1793-1800.

Pathade, G.R., 2003. A review of current technologies for distillery wastewater treatment, in: P.K. Goel (Ed.), Advances in Industrial Wastewater Treatment, ABD Publishers, Jaipur, India, pp. 180 239.

Patil, P.U., Kapadnis, B.P., Dhamankar, V.S., 2003. Decolorization of synthetic melanoidin and biogas effluent by immobilized fungal isolate of *Aspergillus niger* UM2. AIDA News Letter. 53-57.

Patrick, Lyn., 2009. Thyroid Disruption: Mechanism and Clinical Implications in Human Health. Altern. Med. Rev.14 (4), 326-346.

Payet, B., Shum, Cheong., Sing, A., Smadja, J., 2006. Comparison of the concentrations of phenolic constituents in cane sugar manufacturing products with their antioxidant activities. J. Agric. Food. Chem. 54, 7270-7276.

Peng, J.F., Song, Y.H., Yuan, P., Cui, X.Y. and Qiu, G.L., 2009. The remediation of heavy metals contaminated sediment. Journal of hazardous materials. 161, 633-640.

Petruccioli, M., Duarte, I.C., Federici, E., 2000. High rate anaerobic treatment of winery wastewater using bioreactors with free and imunobilized activated sludge. I. Biosci. Bioeng. 90(4), 381-386.

Petta, L., De Gisi, S., Casella, P., Farina, R., Notarnicola M., 2017. Evaluation of the treatability of a winery distillery (vinasse) wastewater by UASB, anoxic-aerobic UF-MBR and chemical precipitation/adsorption. Journal of Environmental Management 201, 177-189.

Pfennig N., Lippert KD., 1966. Uber das Vitamin B12-Bedurfnis phototropher Schwefelbakterien. Arch Microbiol 55, 245

Plavsic, M., Cosovic, B., Lee, C., 2006. Copper complexing properties of melanoidins and marine humic material. Sci. of Total Environ. 366, 310-319.

Potentini, M.F., Rodriguez-Malaver, A.J. 2006. Vinasse biodegradation by Phanerochaete chrysosporium. J. Environ. Biol. 27(4), 661-5.

Prajapati, A.K., Chaudhari., P.K., 2015. Physicochemical Treatment of Distillery Wastewater-A Review. Chem. Eng. Communi. 202, 1098-1117.

Prodanović Jelena, M., Vesna, M., Vasić., 2013. Application of membrane processes for distillery wastewater purification-a review. Desalination and Water Treatment 51(1-18): 3325-3334. doi: 10.1080/19443994.2012.749178.

Raghukumar, C., and Rivonkar, G., 2001. Molasses spent wash by the white rot fungus, *Flavodon flavus* isolated from marine habitat. *Appl.* Microbiol. Biotechnol. 55, 510-514.

Raghukumar, C., Mohandass, C., Kamat, S., Shailaja, M.S., 2004. Simultaneous detoxification and decolorization of molasses spent wash by the immobilized white-rot fungus Flavodon flavus isolated from a marine habitat. Enzyme Microb. Technol. 35, 197-202.

Raghukumar, C., Raghukumar, S., Sheelu, G., Gupta, S. M, Nagender, Nath, B., Rao, BR., 2004. Buried in time: culturable fungi in a deep-sea sediment core from the Chagos Trench, Indian Ocean. Deep Sea Research Part I: Oceanographic Research Papers 51: 1759-1768.

Rai, U.K., Muthukrishnan, M., Guha, B.K., 2008. Tertiary treatment of distillery wastewater by nanofiltration, Desalination 230, 70-80.

Rakesh Sharma M. S., and Raju, N. S., 2013. Correlation of heavy metal contamination with soil properties of industrial areas of Mysore, Karnataka, India by cluster analysis. International Research Journal of Environment Sciences. 2(10) 22-27.

Ramachandra., 1993. Development of indigenous technology (Microbial strains) for the removal of sulfur compounds and colour from distillery effluent. Annual Progress Report, Industrial Toxicology Res Centre, Lucknow.

Ramakritinan, C.M., Kumaraguru, A.K., Balasubramanian, M.P., 2005. Impact of distillery effluent on carbohydrate metabolism of freshwater fish, *Cyprinus carpio*. Ecotoxicology 14, 693-707.

Rao, K.V.M., and Sresty, T. V., 2000. Antioxidative parameters in the seedlings of pigeonpea (*Cajanus cajan* (L.) Millspaugh) in response to Zn and Ni stresses. *Plant Sci* 157, 113-128.

Ravikumar, R., Vasanthi, N. S., Saravanan, K., 2011. Single factorial experimental design for decolorizing anaerobically treated distillery spent wash using *Cladosporium cladosporioides*. Int. J. Environ. Sci. Tech. 8 (1), 97-106.

Ravikumara, R., Vasanthia, N. S., Saravanan. K., 2013. Biodegradation and Decolorization of Distillery Spent Wash with Product Release by a Novel Strain *Cladosporium cladosporioides*: Optimization and Biokinetics. Chem. Biochem. Eng. Q., 27 (3), 373-383.

Richter A. S., and Grimm, B., 2013. Thiol-based redox control of enzymes involved in the tetrapyrrole biosynthesis pathway in plants.371.

Rosselli, W., Keller, C., Boschi K., 2003. Phytoextraction capacity of trees growing on metal contaminated soil. Plant Soil. 256, 265-272.

S. Riva, 2006. Laccases: blue enzymes for green chemistry, Trends in Biotechnology, 24(5): 219-226.

Safari, G.H., Yetilmezsoy, K., Mahvi, A.H., Mansur, Z., 2013. Post-treatment of secondary wastewater treatment plant effluent using a two-stage fluidized bed bioreactor system. J. Environ. Health Sci. Eng. 11, 10.

Saha, N.K., Balakrishnan, M., Batra, V.S., 2005. Improving industrial water use: case study for an Indian distillery. Resour. Conserv. Recycl. 43, 163-74.

Samsudeen, N., Radhakrishnan, T.K., Matheswaran, M., 2015. Bioelectricity production from microbial fuel cell using mixed bacterial culture isolated from distillery wastewater. Bioresour Technol; 195, 242-7.

Sanchez, Riera F., Cordoba, P., Sineriz, F., 1985. Use of the UASB reactor for the anaerobic treatment of stillage from sugarcane molasses. Biotech. and Bioeng. 27(12), 1710-1716.

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

Sankaran, K., Premalatha, M., Vijayasekaran, M., Somasundaram, V.T., 2014. DEPHY project: Distillery wastewater treatment through anaerobic digestion and phycoremediation-A green industrial approach. Renewable and Sustainable Energy Reviews 37, 634-643.

Santal, A.R, Singh, N., 2013. Biodegradation of Melanoidin from Distillery Effluent: Role of Microbes and their Potential Enzymes. Biodegradation of Hazardous and Special Products. (INTECH Press) 5,71-100.

Santal, A.R., Singh, N.P., Saharan, B.S., 2016. A novel application of *Paracoccus pantotrophus* for the decolorization of melanoidins from distillery effluent under static conditions. Journal of Environmental Management 169, 78-83.

Saranraj, P., Dtella, 2014. Impact of Sugar Mill Effluent to Environment and Bioremediation: A Review. World Appl. Sci. 30(3), 299-316.

Sarayu, M., Chirayu, D., Datta, M., 2005. Biodegradation and decolorization of anerobically treated distillery spentwash by a novel bacterial consortium. Biores. Tech. 98, 333-339.

Satyawali, Y., Balakrishnan, M., 2007. Removal of color from biomethanated distillery spentwash by treatment with activated carbons. Bioresource Technology 98, 2629-2635.

Satyawali, Y., Balakrishnan, M., 2008. Wastewater treatment in molasses-based alcohol distilleries for COD and color removal: a review. J. Environ. Manage. 86, 481-497.

Sayadi, M.H., Kargar, R., Doosti, M.R., Salehi, H., 2012. Hybrid constructed wetlands for wastewater treatment: A worldwide review. Proceedings of the Int. Acad. of Ecol. and Environ. Sci. 2(4), 204-222.

Shahid, M., Shamshad, S., Rafiq, M., Khalid, S., Bibi, I., Niazi, N. K., Dumat, C., Rashid, M. I., 2017. Chromium speciation, bioavailability, uptake, toxicity and detoxification in soil-plant system: A review. Chemosphere. 178, 513-533.

Sharma, J., Singh, R., 2000. Characterization of sludge from UASB reactors operating on molasses based distillery effluent. Ind. J. Microb. 40, 203-205.

Sheetal, K.R., Singh, S.D., Anand, A., Prasad, S., 2016. Heavy metal accumulation and effects on growth, biomass and physiological processes in mustard. Ind J Plant Physiol. 21, 219-223.

Shekhawat, K., Rathore, S.S., Premi, O.P., Kandpal, B.K. and Chauhan, J.S., 2012. Advances in agronomic management of Indian mustard (*Brassica juncea* (L.) Czernj. Cosson): An overview. International Journal of Agronomy.

Shivayogimath, C.B., Inani, S., 2014. Treatment of biomethanated distillery spent wash by adsorption process on bagasse activated carbon. Int J of Appl Sci and Eng Res 3, (6) 2014.

Shukla, S.K., Tripathi, A., Mishra, P.K., Nagar GB., 2014. Fungal decolorization of anaerobically biodigested distillery effluent (ABDE) following coagulant. Int. J. Sci. Environ. Technol. 3(2), 723-34.

Siddique N. I., 2012. Treatment and enhanced degradation of petrochemical wastewater by continuous stirred tank reactor. Civil engineering & earth resources University Malaysia Pahang (Thesis).

Silvan, J.M., Lagemaat, J.V.D., Olano, A., Castillo, M.D.D., 2006. Analysis and biological properties of amino acid derivates formed by Maillard reaction in foods. J. Pharma. Biomed. Anal. 41, 1543-1551.

Singh, R., Upadhyay, A.K. and Singh, D.P., 2018. Regulation of oxidative stress and mineral nutrient status by selenium in arsenic treated crop plant Oryza sativa. Ecotoxicology and environmental safety. 148, 105-113.

Singh, R.P. and Agrawal, M., 2007. Effects of sewage sludge amendment on heavy metal accumulation and consequent responses of Beta vulgaris plants. Chemosphere. 67, 2229-2240.

Singh, S., Sinha, S., 2005. Accumulation of metals and its effects in *Brassica juncea* (L.) czern.(cv. Rohini) grown on various amendments of tannery waste, Ecotoxicol. Environ. Saf. 62, 118-127.

Sinha, S., Gupta, A.K., 2005. Translocation of metals from fly ash amended soil in the plant of Sesbania cannabina L.: Ritz: effect on antioxidants. Chemosphere. 61, 1204-1214.

Siqueira, P.B., Bolini, H.M.A, Macedo, G.A., 2011. Polyphenols and antioxidant properties in forced and naturally aged Brazilian beer. J. of Brewing and Distilling 2(3), 45-50.

Sirianuntapiboon, S., Phothilangka, P., Ohmomo, S., 2004. Decolourization of molasses wastewater by a strain no. BP103 of acetogenic bacteria. Biores. Technol. 92, 31-39.

Sirianuntapiboon, S., Zohsalam, P., Ohmomo, S., 2003. Decolourization of molasses wastewater by *Citeromyces* sp. WR-43-6. Process. Biochem. 39, 917-924.

Skerratt, G., 2004. European distilleries: an overview. In: Tewari, P.K. (Ed.), Liquid Asset, Proceedings of the Indo-EU Workshop on Promoting Efficient Water Use in Agro-Based Industries. (TERI Press), New Delhi, India, 1-11.

Sofo, A., Scopa, A., Nuzzaci, M., Vitti, A., 2015. Ascorbate Peroxidase and Catalase Activities and Their Genetic Regulation in Plants Subjected to Drought and Salinity Stresses. Int. J. Mol. Sci. 16, 13561-13578.

Solovchenko, A., Pogosyan, S., Chivkunova, O., Selyakh, I., Semenova, L., Voronova, E., Scherbakov, P., Konyukhov, I., Chekanov, K., Kirpichnikov, M., Lobakova, E., 2014. Phycoremediation of alcohol distillery wastewater with a novel *Chlorella* sorokiniana strain cultivated in a photobioreactor monitored on-line via chlorophyll fluorescence. Algal Research. 6, 234-241.

Somm, E., Schwitzgebel, V.M., Toulotte, A., Cederroth, C.R., et al. 2009. Perinatal Exposure to Bisphenol A Alters Early Adipogenesis in the Rat. Environ. Health Perspect. 117(10), 1549-1555. doi: 10.1289/ehp.11342.

Somoza, V., 2005. Five years of research on health risks and benefits of Maillard reaction products: An update. Mol. Nutr. Food Res. 49, 663-672.

Sorrentino, M.C., Capozzi, F., Giordano, S. and Spagnuolo, V., 2017. Genotoxic effect of Pb and Cd on in vitro cultures of Sphagnum palustre: An evaluation by ISSR markers. Chemosphere. 181, 208-215.

Sowmeyan, R., Swaminathan, G., 2008. Effluent treatment process in molasses-based distillery industries: A review. J. of Hazard. Mat. 152, 453-462.

Subramani, A., Saravanan, S., Tamizhiniyan, P., Lakshmanachary, A.S., 1997. Influence of heavy metals on germination and early seedling growth of *Vigna mungo* L. Pollut. Resour. 8(7), 360-364.

Syutsubo, K., Harada, H., Ohashi, A., Suzuki, H., 1997. An effective start-up of thermophilic UASB reactor by seeding mesophilically-grown granular sludge. Water Sci. Technol. 36(6), 391-398.

Tamanna, N., Mahmood, N., 2015. Food Processing and Maillard Reaction Products: Effect on Human Health and Nutrition. Hindawi Publishing Corporation Inter. J. of Food Sci. http://dx.doi.org/10.1155/2015/526762.

Tano, M.S., Buzato, J.B., 2003. Effect of the presence of initial ethanol on ethanol production in sugarcane juice fermented by *Zymomonas mobilis*. Brazilian J. of Microbiol. 34, 242-244.

Tansengco, M. L., Herrera, D. L., Tejano, J. C., 2016. Treatment of Molasses-Based Distillery Wastewater in a PilotScale Anaerobic Sequencing Batch Reactor (ASBR). Electronic J. of Biology. 12(4), 367-373.

Taylor, J.L., Demyttenaere, J.C., Abbaspour, Tehrani K., Olave, C.A., Regniers, L., Verschaeve, L., Maes, A., Elgorashi, E.E., van Staden, J., de Kimpe, N., 2004. Genotoxicity of melanoidin fractions derived from a standard glucose/glycine model. J. Agric Food Chem. 52(2), 318-23.

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K. and Sutton, D.J., 2012. Heavy metal toxicity and the environment. In Molecular, clinical and environmental toxicology (pp. 133-164). Springer Basel.

Thakur, S., Singh, L., Ab Wahid, Z., Siddiqui, M.F., Atnaw, S.M. and Din, M.F.M., 2016. Plant-driven removal of heavy metals from soil: uptake, translocation, tolerance

mechanism, challenges, and future perspectives. Environmental monitoring and assessment. 188 , 206.

Thanikal, J.V., Torrijos, M., Habouzit, E., Moletta, R., 2007. Treatment of distillery vinasse in a high rate anaerobic reactor using low density polyethylene supports. Water Sci. Technol. 56(2), 17-24.

Tiwari, S., Gaur, R., Singh, R., 2012. Decolorization of a recalcitrant organic compound (Melanoidin) by a novel thermotolerant yeast, *Candida tropicalis* RG-9. Biotechnology, 12:30. DOI: 10.1186/1472-6750-12-30.

Tiwari, S., Rai, P., Yadav, S.K., Gaur, R., 2013. A novel thermotolerant *Pediococcus acidilactici* B-25 strain for color, COD, and BOD reduction of distillery effluent for end use applications. Environ. Sci. Pollut. Res. Int. Jun. 20(6), 4046-58

Torres-Climent, A., Gomis, P., Martín-Mata, J., Bustamante, M.A., Marhuenda, Egea F.C., Pérez-Murcia, M.D., Pérez-Espinosa, A., Paredes, C., Moral, R., 2015. Chemical, Thermal and Spectroscopic Methods to Assess Biodegradation of Winery Distillery Wastes during Composting. PLOS ONE. DOI:10.1371/journal.pone.0138925.

Upadhyay, A.K., Singh, N.K., Singh, R. and Rai, U.N., 2016. Amelioration of arsenic toxicity in rice: Comparative effect of inoculation of *Chlorella vulgaris* and *Nannochloropsis* sp. on growth, biochemical changes and arsenic uptake. Ecotoxicology and Environmental Safety. 124, 68-73.

Uppal, J., 2004. Water utilization and effluent treatment in the Indian alcohol industry - An overview. In: Liquid Assets, Proceedings of Indo-EU workshop on Promoting Efficient Water Use in Agro-based Industries. (TERI Press), New Delhi, India. 13-19.

USEPA. 2000. U.S. Environmental Protection Agency Endocrine Disruptor Screening 695 Program Universe of Chemicals 2012.

Valderrama, L.T., Del Campo, C.M., Rodriguez, C.M., Bashan, L.E., Bashan, Y., 2002. Treatment of Recalcitrant Wastewater from Ethanol and Citric Acid Using the Microalga *Chlorella vulgaris* and the Macrophyte *Lemna minuscule*. Water Res. 36(17), 4185-92.

Velikova, V., Yordanov, I., and Edreva, A., 2000. Oxidative Stress and Some Antioxidant Systems in Acid Rain Treated Bean Plants: Protective Role of Exogenous Polyamines. Plant Science. 151, 59-66.

Vijayaraghavan, K., Ramanujam, T.K., 2000. Performance of anaerobic contact filter in series for treating distillery spentwash. Biopro and Biosys Eng 22 (2), 109-114.

Vineetha, M.N, Matheswaran, M., Sheeba, K.N., 2013. Photocatalytic colour and COD removal in the distillery effluent by the solar radiation. Sol. Energy 91, 368-373.

Vlyssides, A.G., Israilides, C.J., Loizidou, M., Karvouni, G., Mourafeti, V., 1997. Electrochemical treatment of vinasse from beet molasses. Water Sci and Technol 36 (2-3), 271-278.

Wagh, M.P., Nemade, P.D., 2015. Treatment of Distillery Spent Wash by Using Chemical Coagulation (CC) and Electro-coagulation [EC]. American J. of Environ. Protec. 3(5), 159-163.

Wagner, K.H., Derkits, S., Herr, M., Schuh, W., Elmadfa, I., 2002. Antioxidative potential of melanoidins isolated from a roasted glucose-glycine model. Food Chem. 78, 375-382.

Wang, H., Zhao, Y., 2009. A bench scale study of fermentative hydrogen and methane production from food waste in integrated two-stage process. Int J. Hydrogen Energy 34 (1), 245-254.

Wang, H.Y., Qian, H., Yao, W.R., 2011. Melanoidins produced by the Maillard reaction: structure and biological activity. Food Chem. 128, 573-584.

Watanabe, Y., Sugi, R., Tanaka, Y., Hayashida, S., 1982. Enzymatic decolourisation of melanoidin by *Coriolus* sp. no. 20. Agric. Biol. Chem. 46, 1623-1630.

Wen, Q., Wu, Y., Zhao, L., Sun, Q., 2010. Production of electricity from the treatment of continuous brewery wastewater using a microbial fuel cell. Fuel 89, 1381-1385.

Wicher, E., Seifert, K., Zagrodnik, R.., Pietrzyk, B., Laniecki, M., 2013. Hydrogen gas production from distillery wastewater by dark fermentation. Int. J. Hydrog. Energy. 38, 7767-7773.

Wilkie, A.C., Riedesel, K.J., Owens, J.M., 2000. Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. Biomass and Bioenergy 19 (2), 63-102.

Wolmarans, B, de Villiers, G.H, 2002. Start-up of a UASB treatment plant on distillery wastewater. Water SA 28(1), 63-68

Yadav, S., Chandra, R., 2012. Biodegradation of organic compounds of molasses melanoidin (MM) from biomethanated distillery spent wash (BMDS) during the decolourisation by a potential bacterial consortium. Biodegradation, 23(4): 609-20. doi: 10.1007/s10532-012-9537-x.

Yadav, S., Chandra, R., 2013. Detection of persistent organic compounds from biomethanated distillery spent wash (BMDS) and their degradation by manganese peroxidase and laccase producing bacterial strains. J. of Environ. Biol. 34, 755-764.

Yadav, S., Chandra, R., Rai, V., 2011. Characterization of potential MnP producing bacteria and its metabolic products during decolourisation of synthetic melanoidins due to biostimulatory effect of D-xylose at stationary phase. Process Biochem 46(9):1774-1784.

Yadav, S.K., 2010. Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. South African Journal of Botany. 76, 167-179.

Ye, Y., Ding, Y., Jiang, Q., Wang, F., Sun, J. and Zhu, C., 2017. The role of receptor-like protein kinases (RLKs) in abiotic stress response in plants. Plant cell reports, 1-8.

Yi, L., Hong, Y., Wang, D., Zhu, Y., 2007. Determination of free heavy metal ion concentrations in soils around a cadmium rich zinc deposit. Geochemical Journal. 41(4) 235-240.

Yilmaz, S.H., Kaplan, M., Temizgul, R. and Yilmaz, S., 2017. Antioxidant enzyme response of sorghum plant upon exposure to Aluminum, Chromium and Lead heavy metals. Turkish Journal of Biochemistry. 42, 503-512.

Yoon, J., Xinde, C., Qixing, Z., Ma, L. Q., 2006. Accumulation of Pb, Cu, and Zn in native plants growing on a contaminated Florida site. Sci. Total Environ. 368, 456-464.

Yu, H-Q., Zhao, Q-B., Tang, Y., 2006. Anaerobic treatment of winery wastewater using laboratory-scale multi- and single-fed filters at ambient temperatures. Process. Biochem. 41 (12), 2477−2481.

Zhang, M., Wang, Z., Li, P., Zhang, H., Xie, Li., 2017. Bio-refractory dissolved organic matter and colorants in cassava distillery wastewater: Characterization, coagulation treatment and mechanisms. Chemosphere 178: 259-267.

Chapter 11 Publication and Scientific Output

Chapter 11

PUBLICATION AND SCIENTIFIC OUTPUT:

Books: **01;** Book Chapter**: 11;** Research/Review Papers: **07**; Magazine articles: **02;** Conference paper presented, **01**

Books

Bharagava, R.N., and Chowdhary, P., 2018. Emerging and Eco-friendly Approached for Waste Management. (Edited Book). Springer Nature Singapore Pte Ltd. *ISBN: 9811086680.*

Review/Research

- **Chowdhary, P., Raj, A., Bharagava, R. N., 2018.** Environmental pollution and health hazards from distillery wastewater and treatment approaches to combat the environmental. Chemosphere, 194, 229-246.
- **Chowdhary, P., Bharagava, R. N., 2018.** Degradation and detoxification of distillery wastewater pollutants by *bacillus megaterium* sp. for environmental safety. International Journal of Applied and Advanced Scientific Research. **(Accepted)**
- **Chowdhary, P., et al. 2018.** Stress response of *Triticum aestivum* L. and *Brassica juncea* L. against heavy metals growing at distillery and tannery wastewater contaminated site. Chemosphere. **(Accepted)**
- **Chowdhary, P., Khan N., Bharagava R. N. 2018.** Distillery Wastewater: it's Impact on Environment and Remedies. Environ Anal Eco stud. 1(2). EAES.000507. 2018

Chapters (In Book)

- **Chowdhary, P., Yadav, A., Bharagava, R.N., 2017.** Distillery wastewater: a major source of environmental pollution and it's biological treatment for

environmental safety. In: Singh R & Kumar S (Eds.), Green Technology and Environmental Sustainability. Springer International ISBN 978-3-319-50653-1.

- **Yadav, A., Chowdhary, P., Kaithwas, G., Bharagava, R.N., 2017.** Toxic metals in environment, their threats on ecosystem and bioremediation approaches. In: Das S & Singh HR (Eds.), Handbook of Metal-microbe interaction and bioremediation. CRC press, Taylor & Francis group USA, pp-128-141.
- **Bharagava, R.N., Chowdhary, P., Saxena, G., 2017.** Bioremediation: An eco-sustainable green technology, it's applications and limitations. In: Bharagava RN (Ed.) Environmental pollutants and their bioremediation approaches. CRC press, Taylor & Francis group USA (ISBN 9781138628892)**.**
- **Bharagava RN, Saxena G & Chowdhary P (2017)** Role of constructed wetlands in treatment of industrial wastewaters. In: Bharagava RN (Ed.) Environmental Pollutants and Their Bioremediation Approaches. CRC press, Taylor & Francis group USA (ISBN 9781138628892).
- **Chowdhary, P., More, N., Yadav, A., Bharagava, R.N. 2018.** Ligninolytic Enzymes: An introduction and applications in Food industry. In Enzymes in Food Biotechnology Kuddus M. (Ed). Enzymes in Food Biotechnology. Elsevier Inc. *ISBN: 9780128132807*
- **Chowdhary, P., Yadav, A., Bharagava, R.N. 2018.** Toxicity, beneficial aspect and treatment of alcohol industry wastewater. In Bharagava R N and Chowdhary P (Eds). Emerging and Eco-friendly Approached for Waste Management https://doi.org/10.1007/978-981-10-8669-4_5

Magazine Articles

- **Chowdhary P, Bharagava RN (2015)** Applications of laccase enzyme in biodegradation and bioremediation of industrial wastes. Microbiology World. 3 (1): 9-14.

Seminars/ Conferences/Workshops/Training Programmes etc

(a) Attended Seminars/ Conferences organized by following associations:

1. **Lucknow Science Congress (LUSCON-2015)** at **B. B. Ambedkar University** (A Central University), Vidya Vihar, Raebareli Road, Lucknow- 226 025 (U.P).
2. Seminar on "Environment, Education & Society" at **B. B. Ambedkar University** (A Central University), Vidya Vihar, Raebareli Road, Lucknow- 226 025 (U.P).
3. Participated in **Poster presentation** session of the 57th annual Conference of Association of Microbiologist of India, Organized by department of Botany, Gauhati University, Assam, India, November, 24-27, 2016.
4. Participated in 103rd annual national conference of Indian Science Congress held at University of Mysore, Mysore, January 3-7, 2016.
5. Participated in 104th annual national conference of Indian Science Congress held at Tarakarama Stadium of Sri Venkateswara University in Tirupati in Andhra Pradesh, January 3-7, 2017.

(b) Workshops/ Training Programmes/Seminars/ Conferences

1. International workshop **"Bridge Development Divide for Inclusive Growth through Science, Technology and Innovation" (2013)** at **Babasaheb Bhimrao Ambedkar University** (A Central University), Vidya Vihar, Raebareli Road, Lucknow- 226 025 (U.P).

2. Patent workshop -2013 at **B. B. Ambedkar University** (A Central University), Vidya Vihar, Raebareli Road, Lucknow- 226 025 (U.P).

3. National workshop on **Innovation and Technology Transfer to Industries: Role of Universities (ITTI-2014)** at **B. B. Ambedkar University** (A Central University), Vidya Vihar, Raebareli Road, Lucknow- 226 025 (U.P).

4. National workshop on "**Gene cloning and its expression to produce genetically modified organisms" (2017)** organized by Maharishi University of information technology (Lucknow) in association with Cytogene research & development, Lucknow.

Member of Scientific Society:

1. Life member of **Indian Science Congress Association**, India.

2. Life member of **Association Microbiologist of India** (AMI), India.

Chemosphere 194 (2018) 229–246

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Environmental pollution and health hazards from distillery wastewater and treatment approaches to combat the environmental threats: A review

Pankaj Chowdhary [a], Abhay Raj [b], Ram Naresh Bharagava [a, *]

[a] Laboratory for Bioremediation and Metagenomics Research (LBMR), Department of Environmental Microbiology (DEM), Babasaheb Bhimrao Ambedkar University (A Central University), Vidya Vihar, Raebareli Road, Lucknow, 226 025, Uttar Pradesh, India
[b] Environmental Microbiology Section, CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Post Box 80, M.G. Marg, Lucknow, 226 001, Uttar Pradesh, India

HIGHLIGHTS

- Endocrine disrupting chemicals of distillery wastewater.
- Environmental and health hazards of distillery wastewater pollutants.
- Analytical techniques used for distillery wastewater pollutants analysis.
- Physico-chemical, biological and emerging treatment methods of distillery wastewater.
- Merits and demerits of various distillery wastewater treatment approaches.

ARTICLE INFO

Article history:
Available online 28 November 2017

Handling Editor: Jian-Ying Hu

Keywords:
Melanoidins
Chemical pollutants
EDCs
Environmental problems
Health hazards
Treatment approaches

ABSTRACT

Distillery industries are the key contributor to the world's economy, but these are also one of the major sources of environmental pollution due to the discharge of a huge volume of dark colored wastewater. This dark colored wastewater contains very high biological oxygen demand, chemical oxygen demand, total solids, sulfate, phosphate, phenolics and various toxic metals. Distillery wastewater also contains a mixture of organic and inorganic pollutants such as melanoidins, di-n-octyl phthalate, di-butyl phthalate, benzenepropanoic acid and 2-hydroxysocaproic acid and toxic metals, which are well reported as genotoxic, carcinogenic, mutagenic and endocrine disrupting in nature. In aquatic resources, it causes serious environmental problems by reducing the penetration power of sunlight, photosynthetic activities and dissolved oxygen content. On other hand, in agricultural land, it causes inhibition of seed germination and depletion of vegetation by reducing the soil alkalinity and manganese availability, if discharged without adequate treatment. Thus, this review article provides a comprehensive knowledge on the distillery wastewater pollutants, various techniques used for their analysis as well as its toxicological effects on environments, human and animal health. In addition, various physico-chemicals, biological as well as emerging treatment methods have been also discussed for the protection of environment, human and animal health.

Contents

* Corresponding author.
E-mail addresses: bharagavarnbbau11@gmail.com, ramnaresh_dem@bbau.ac.in (R.N. Bharagava).

https://doi.org/10.1016/j.chemosphere.2017.11.163
0045-6535/

Chapter 18
Distillery Wastewater: A Major Source of Environmental Pollution and Its Biological Treatment for Environmental Safety

Pankaj Chowdhary, Ashutosh Yadav, Gaurav Kaithwas, and Ram Naresh Bharagava

Abstract Distillery industries are one of the major sources of environmental pollution because these industries discharge a huge volume of dark-colored wastewater into the environment. The wastewater discharged contains high biological oxygen demand (BOD), chemical oxygen demand (COD), total solids (TS), sulfate, phosphate, phenolics, and toxic heavy metals. On terrestrial region, distillery wastewater at higher concentration inhibits seed germination, growth and depletion of vegetation by reducing the soil alkalinity and Mn availability, whereas in aquatic region, it reduces sunlight penetration and decreases both photosynthetic activity and dissolved oxygen content damaging the aquatic ecosystem. The large volume of dark-colored wastewater acts as a major source of soil and water pollution and thus requires adequate treatment for its safe discharge into the environment. Therefore, the removal of pollutants and color from distillery wastewater is becoming increasingly important for the environment and sustainable development. Thus, this chapter provides the detailed information on the generation, characteristic, toxicity as well as various biological methods employing bacteria, fungi, microalgae, etc. for the treatment of distillery wastewater. In biological treatment approaches microalgae have a number of applications over the conventional approaches as it is useful in wastewater treatment, CO_2 sequestration, cost-effective, sanitation and also in the production of renewable energy sources such as methane gas, biodiesel, biofuel, glycerol, hydrogen gas, biofertilizers, etc. Furthermore, the merits and demerits of existing processes have been also summarized in this chapter.

P. Chowdhary • A. Yadav • R.N. Bharagava (✉)
Laboratory for Bioremediation and Metagenomics Research (LBMR), Department of Environmental Microbiology (DEM), School for Environmental Sciences (SES), Babasaheb Bhimrao Ambedkar University (A Central University), Vidya Vihar, Raebareli Road, Lucknow 226 025, Uttar Pradesh, India
e-mail: bharagavarnbbau11@gmail.com; ramnaresh_dem@bbau.ac.in

G. Kaithwas
Department of Pharmaceutical Sciences (DPS), Babasaheb Bhimrao Ambedkar University (A Central University), Vidya Vihar, Raebareli Road, Lucknow 226 025, Uttar Pradesh, India

R. Singh, S. Kumar (eds.), *Green Technologies and Environmental Sustainability*, DOI 10.1007/978-3-319-50654-8_18

1 Bioremediation
An Eco-Sustainable Green Technology, Its Applications and Limitations

Ram Naresh Bharagava, Pankaj Chowdhary, and Gaurav Saxena

CONTENTS

Chapter 5
Toxicity, Beneficial Aspects and Treatment *Sorghum cernuum* of Alcohol Industry Wastewater

Pankaj Chowdhary and Ram Naresh Bharagava

Abstract The alcohol industry is one of the most popular among all industries and a key contributor in the world's economic growth, but unfortunately, these industries are also considered one of the major sources of environmental pollution. Alcohol industry wastewater is highly toxic for the aquatic and the terrestrial ecosystem. This wastewater contains a high concentration of biological oxygen demand, chemical oxygen demand, total solids, organic matter, potassium and sulphates and high acidic characteristics. Because of the high content of a toxic nature, it causes an adverse impact on soil structure and water bodies in the case of excessive amounts. In aquatic resources, it reduces the penetration power of sunlight causing a reduction in the photosynthetic activity of aquatic plants, dissolved oxygen content in water bodies and in terrestrial regions, and it causes genotoxic and phytotoxic effects on animal and plants respectively. Additionally, this wastewater may be used for the ferti-irrigation after proper dilution. In this chapter, we discuss in detail the positive and negative aspects of alcohol wastewater and miscellaneous treatment technologies for wastewater treatment. The aim of this chapter is also to provide updated information on the alternative uses of alcohol wastewater, such as energy production, ferti-irrigation, and other value-added products.

1 Introduction

In the twenty-first century, human society faces serious environmental issues such as climate change, pollution and extinction of plants and animals. All these problems are interrelated and have originated from human detonation, uncontrolled use of natural resources, urbanisation and industrialisation. The distillery industries, belonging to one of the world's leading premium drinks companies, have turned to

P. Chowdhary · R. N. Bharagava (✉)
Laboratory for Bioremediation and Metagenomics Research (LBMR), Department of Environmental Microbiology (DEM), Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, Uttar Pradesh, India

R. N. Bharagava, P. Chowdhary (eds.), *Emerging and Eco-Friendly Approaches for Waste Management*, https://doi.org/10.1007/978-981-10-8669-4_5