Study on the bacterial population and residual organic pollutants present in tannery wastewater after secondary treatment process and it's toxicity assessment

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

LUCKNOW

BABASAHEB
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Submitted By

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SCHOOL FOR ENVIRONMENTAL SCIENCES

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

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2019

Dedicated to
My Beloved Mom

Certificate

This is to certify that the thesis titled "**Study on the bacterial population and residual organic pollutants present in tannery wastewater after secondary treatment process and it's toxicity assessment**" submitted by **Mr. Ashutosh Yadav** 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.) University regulations 1999 as amended in 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 the Department

STUDENT DECLARATION

This is to certify that the material embodies in the present Ph.D. work entitled **"Study on the bacterial population and residual organic pollutants present in tannery wastewater after secondary treatment process and it's toxicity assessment"** 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 Ashutosh Yadav. If any Allegations/query/question arises regarding the thesis, I, Mr. Ashutosh Yadav, will be solely responsible and answerable.

Place:
Date **(Ashutosh Yadav)**
Ph.D. Scholar

Acknowledgements

*“**Completion of a creative work among time constrains seems to be a day-dream in absence of proper guidance**”*

*Life is a journey where every individual with their own experience lays milestones. This is a righteous opportunity to mention the name of those wonderful and caring persons who have shown me the right way to achieve the ultimate Milestone. First of all, I would like to express my profound feeling of reverence and extend my heartfelt gratitude to **Dr. Ram Naresh Bharagava**, my supervisor for his exemplary guidance and dynamic initiation, continuous encouragement, thoughtful discussions, and untiring supervision throughout the work. It gives me immense pleasure in expressing my gratitude to **Dr. Abhay Raj**, **Senior Scientist**, Environmental Microbiology Laboratory, CSIR-Indian Institute of Toxicology Research Lucknow. His concern, constant encouragement and appreciation have helped me in accomplishing my target on time. He is the figure without whom it would be quite difficult to shape this work in present format.*

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*The whole credit of my achievement goes to my **parents,** who were always there in my difficulties. It was their unshakable faith in me that has helped me to proceed further.*

Place: Lucknow **Ashutosh Yadav**

Date:

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ABBREVIATIONS & SYMBOLS

α	Alpha
~	Approximately
µ	Miu
APHA	American Public Health Association
AAS	Atomic Absorption Spectroscopy
ANOVA	Analysis of Variance
ASP	Activated Sludge Process
bp	Base Pair
BOD	Biological Oxygen Demand
CO2	Carbon dioxide
CA	Chromosomal abbreviation
CETP	Common Effluent Treatment Plant
CFU	Colony Forming Unit
COD	Chemical Oxygen Demand
Cm	Centimetre
CPCB	Central Pollution Control Board
Cr	Chromium
CO_2	Carbon dioxide
°C	Degree Celsius
DCM	Dichloromethane
DO	Dissolved Oxygen
DNA	Deoxyribonucleic acid
EC	Electrical Conductivity
GC-MS	Gas Chromatography Mass Spectrophotometry
g	Gram
g/L	Gram per litre
H_2O	Water
H_2O_2	Hydrogen peroxide
HPLC	High Pressure Liquid Chromatography
IUPAC	International Union of Pure and Applied Chemistry
K	Kelvin
LLE	Liquid-Liquid Extraction

M	Molarity
MPN	Most Probable Number
MLD	Million Litter Per Day
MIC	Minimum Inhibitory Concentration
Min	Minute
MI	Mitotic Index
mL	Millilitre
mg/l	Milligram per litter
nm	Nanometre
-	Negative
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NEQS	National Environmental Quality Standards
NIST	National Institute of Standards and Technology
OD	Optical density
+	Positive
%	Percentage
rpm	Revolution per minute
RNA	Ribonucleic acid
ROPs	Residual organic pollutants
SPC	Standard Plate Count
TDS	Total dissolved solids
TS	Total Solids
TSS	Total Suspended Solids
TWW	Tannery wastewater
UT	Untreated
UV	Ultra violet
U/mL	Unit per milliliter
w/v	Weight over volume
WHO	World Health Organization
USEPA	United State Environmental Protection Agency

A) 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. Glasswares from **Borosil, Mumbai**

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

4. Plasticwares from **Merck Millipore, Mumbai**

B) INSTRUMENTS

All the instruments used in this research work are listed below:

1. Autoclave (SMI-102 & Indfos)

2. Centrifuge (Universal-320-R & Hettich, Zentrifugen)

3. Dry Air Oven (LSI-145)

4. Fridge for storing cultures (BFS-345 & Celfrost)

5. GC-MS (**Shimadzu Model Number: QP2010S)**

6. Laminar Air Flow (AEM-915-H)

7. PCR (Verti-R & Applied Biosystems)

8. Spectrophotometer (Evolution 201, Australia)

9. Temperature Controlled Incubator Shaker (LSI-3016R & Labtech)

10. Water Bath (AEM-54003-Q)

Chapter 1
General Introduction

1. General Introduction

1.1. Tannery Industry: Historical Background

Global trade started getting liberalized right through the 1970s and 80s. From a global turnover of $4 billion, the leather and leather products industry grew to an estimated $70 billion in 2000 (UNIDO, 2010). Leather is an intermediate industrial product, with numerous applications in down-stream sectors of the consumer products industry. For the latter, leather is often the major material input, and is cut and assembled into shoes, clothing, leather goods, furniture and many other items of daily use (Ballard, 2001; Lofrano et al., 2013; Yadav et al., 2016a). Different applications require different types of leather for other assorted goods migrated from industrialized countries in the West to the developing countries of the East in a big way, primarily motivated by cost considerations.

Rapid industrialization and modernization around the world have produced the unfortunate consequences of releasing various types of wastes containing different types of toxic pollutants into the environment (Mishra and Bharagava, 2016). Water is the most important and essential element on the mother earth for sustainable life. Industrialization and extraction of natural resources have resulted in a large-scale environmental contamination and severe health hazards to animal and human beings. The contamination of soils, groundwater, sediments, and surface water with toxic metals and chemicals is one of the major problems facing the world today (Alam et al., 2009).

Alarming growth in population, urbanization, industrialization, agricultural activities, climate change, socio-economic growths, along with high living standards, have generated ever-lasting demands for water resources (Mishra et al., 2018). However, due to this heavy population load and human activities quality of our water resources is deteriorating continuously. Every year, millions of tons of tannery wastes containing thousands of organic (Phthalates, biphenyls, phenols, tannin oils, greases), inorganic (heavy metals, compounds containing, fluoride, phosphate, sulfate, nitrate) and biological pollutants or contaminants (virus, bacteria, fungi, algae, amoebas, and planktons) have been released to our water resources (Haydar and Aziz, 2009; Saxena et al.., 2017. Chowdhary et al., 2018)

Nowadays tannery industries occupy place of pride, due to its massive potential for employment export and growth, play an important role in the economy of many developing countries (Leta et al., 2004; Lefebvre et al., 2006).

The tannery industry is a major industry on an international scale and is of significant economic importance as an agro-based sector producing a host of products in one of the world's finest natural materials. The conversion of animal hides/skin into useful leather artifacts may be man’s oldest technology that is why tanning is claimed to be the second oldest profession in the world. India is the third largest producer of leather and its valuable products around the world (Ramteke et al., 2010). The production of leather and their products by the use of raw skins/hides is one of the oldest technologies of human civilization. The processing of leather is a high water consuming process, utilising large volume of fresh water and only 30% of this fresh water is used in leather manufacturing and rest of 70% water is discharged into the environment as a wastewater containing various toxic pollutants and thus, becomes a great challenge for environment (Chowdhury et al., 2013).

Our earth is getting progressively polluted with different types of inorganic and organic compounds, primarily as a result of anthropogenic activities (Kumar et al., 2019; Kumar et al., 2017a, b). Natural resources (soil, air, and water) have faced a tremendous amount of pressure because of the rising human population and their associated activities. Uncontrolled discharge of effluent from industries in water led to a rapid increase in effluent concentration which alters the nature of ecosystem and adversely affects the health of human beings, plant, and animals (Kumar et al., 2019; Kumar et al., 2014, 2015). There are several ways by which huge amount of toxic compounds enter into the environment.

Tannery industries are one of the most polluting industries mainly causing soil and water pollution in environment. India is the third largest leather producer in the world (Ramteke et al., 2010). There are more than 3,000 tanneries in India, and most of them (nearly 80%) are engaged in chrome tanning process (Shukla et al., 2009).

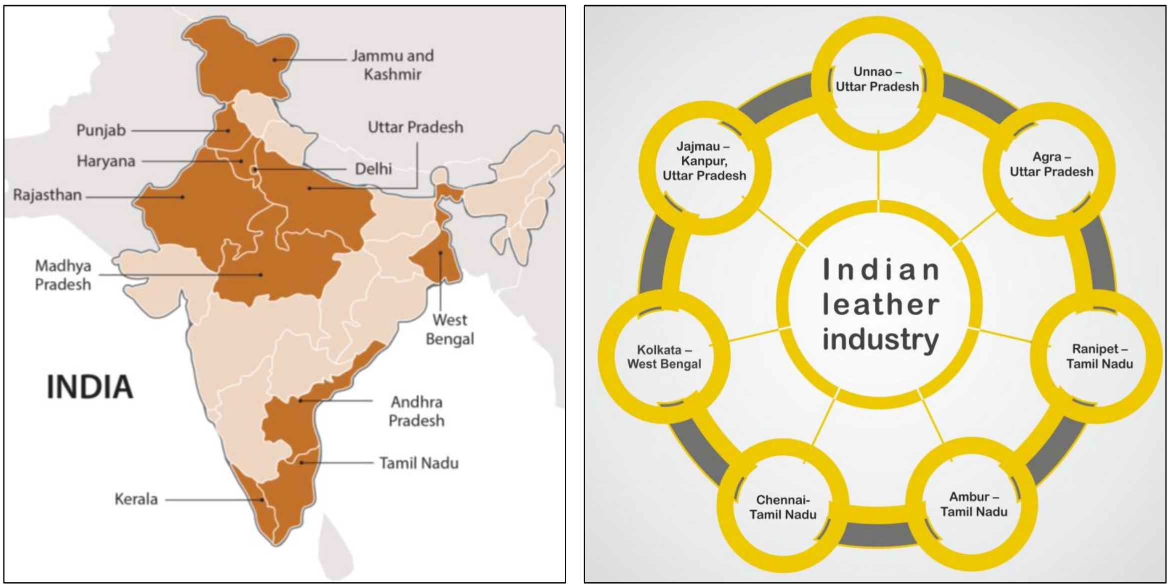


Figure 1.1: Map showing the major production hubs for leather in diffrent state (India)

A large portion of the world's tanning industry operates in low- and middle-income countries, contributing to light and heavy leather materials and many of these tannery sites are clustered together, creating heavily polluting industrial areas in many countries (Figure 1.1). Tanning Industry is considered to be a major source of pollution and tannery wastewater in particular, is a potential environmental concern as the process of making leather has always been associated with odour and water pollution (Taylor et al., 1998; Khwaja et al., 2001).

During the tanning process, a number of chemical compounds (mainly chromium salts) are used to convert the raw hides/skins into leather and generate large volume of wastewater containing huge amount of organic matter, phenolics, tannins, a variety of organic pollutants and toxic heavy metals mainly chromium making wastewater unsuitable for irrigation and aquatic life (Haydar et al., 2007; Chandra et al., 2011). The organic pollutants present in tannery wastewaters do not degrade much during the primary and secondary treatment process in industries and goes into the environment causing serious soil and water pollution along with serious threat to human and animals health in different ways (Yadav et al., 2019). The residual organic pollutants present in tannery wastewater (TWW) may provide chance to a variety of pathogenic microbes to flourish and contaminate the receiving water bodies as well as surrounding environments because they may act as a source of nutrients for a diversity of microbes (Yadav et al., 2016a; Verma et al., 2008; Viti et al., 2003). However, various authors have reported that numerous residual organic pollutants and pathogenic microbes also remain in wastewater discharged from different industries even after the secondary treatment process and goes in environment and persist in nature for long period (Chandra et al., 2011; Naraian et al., 2012; Kapley et al., 2001).

Waterborne pathogens infect around 250 million people each year resulting in 10-20 million deaths (Saxena et al., 2015). Many of these infections occur in developing nations which suffer from lower levels of sanitation, problems associated with low socio-economic conditions, and less public health awareness than in more developed nations. The risk from microbial pathogens in water necessitates the proper monitoring of wastewaters and contaminated aquatic resources for various types of organic pollutants and microbial pathogens (Yadav et al., 2016).

In addition, the wastewater released from tannery industries may contain a variety of organic pollutants and millions of pathogenic and non-pathogenic bacteria per milliliter including coliform, *Streptococci*, *Staphylococci*, anaerobic spore forming bacilli, and many other types of health hazards organisms (Chandra et al., 2011; Naraian et al., 2012; El-Lathy et al., 2009). The presence of microbial pathogens in treated wastewater as well as in polluted aquatic resources poses serious threats to the general public health. Despite large advances in water and wastewater treatment processes, waterborne diseases still pose a major worldwide threat to public health. Thus, the high concentration and low biodegradability of pollutants as well as a variety of pathogenic and non-pathogenic microbes present in tannery wastewater is a matter of serious environmental concern and hence, it becomes very essential to adequately treat the TWW before its final disposal into the environment.

1.2. Cr (VI) as major pollutant in tannery wastewater

Environmental damage caused by tannery discharge has created a critical problem in India and signifies a technical challenge for an efficient and safe cleaning process. Chromium, a brittle, hard, steel grey and shiny metal present in environment in combined form around 0.1-0.3 mg/kg of earth’s surface. It exists in several oxidation states (–2 to +6) and the most stable are Cr (III) and Cr (VI) (Mishra and Bharagava, 2016; Molokwane et al. 2008). Cr (III) solubility is affected by the formation of oxides and hydroxides. Chromium is mainly employed in the metallurgy industry, particularly stainless steel production. Other Cr salts are used for manufacturing of pigments, leather tanning, metal finishing etc. Tanned hide is approximately 80-90% made from chromium compounds (Yadav et al., 2016; Papp 2004).

Discharged effluents from tanneries contain about 40% of Cr as Cr (III) and Cr (VI). For each 200 kg of hide, more than 600kg of waste is produced by a tannery (Khan 2001). Cr chemicals have also been used for the production of metal castings and mortars, refractory bricks and as wood preservative. Conversely, the U.S. Environmental Protection Agency (USEPA) has prohibited the use of Cr (VI) compounds as a wood preservative regarding health issues.

Figure 1.2: Hexavalent chromium (Cr^{+6}) effect on human health like skin ulceration and allergic contact dermatitis

Because of wide applications, huge amount of Cr wastes is released into the environment each year. In 2003, USEPA declared about 32,589.6 metric tons of Cr compounds were disposed off and half of the quantity was land filled in the surroundings (U.S. Environmental Protection Agency 2005). Potable water guidelines by the WHO states 0.05 mg/l as the maximum permissible limit for total chromium. Cr is hazardous, but also spreads fast over aquatic systems and underground waterways. Consequently, Cr has been recognized as toxic environmental pollutant by US EPA (Narayani and Shetty 2013).

Chromate is present naturally but anthropogenic activities give rise to Cr (VI) pollution in the environment. Natural sources contribute 54,000 tons of chromium.

Studies showed that atmospheric Cr comes back to soil and water bodies by rain. Cr estimated time in the atmosphere is less than ten days (Agency for Toxic Substance and Diseases Registry (ATSDR) 2015). Chromate present in soils can seep into surface water because of its highly soluble and mobile nature (Coetzee et al. 2018). It is a common practice to irrigate agricultural land by wastewater. Tannery effluents has large content of valuable nutrients, however also contains toxins such as Cr that might damage soil quality and crop production (Alvarez-Bernal et al. 2006). High percentage of Cr in soils can prevent germination of seeds and growth of seedling. The toxic effects of Cr are less apparent on seed development than on growth of seedling. Barley seeds were able to germinate in soil under chromate stress of 100 mg/kg. However it showed slow growth due to Cr (VI) inhibition of diastase that is necessary for mobilizing the starch reserved for early growth (Zayed and Terry 2003). In plants, toxicity of Cr greatly dependents on ionic species of element. Cr (VI) and Cr (III) were supplied in the range of 0 to 100 mg/kg. When chromate (100 mg/l) stress was applied to plants, up to 3000–5000 mg/kg of Cr(VI) was accumulated and was up to 300-400 mg/kg when Cr(III) (100 mg/l) stress was applied in hydroponic culture. These high levels of chromium caused leaf chlorosis, reduced root and shoot growth, stimulation of chitinase activity and low levels of water content in leaves (Chowdhary et al., 2018; Zayed and Terry 2003).

1.3. Persistant organic pollutants in tannery wastewater

Persistant organic pollutants are a group of chemical compounds originated from different anthropogenic activity, but have some common characteristics like semi-volatility, lipophilicity, bioaccumulation, which make the POPs resistant to photolytic, biological as well as chemical degradation over a reasonable period of time and these persist in the environment for a long period of time and have potential significant impacts on human health and the environment (Chandra and Chaudhary, 2013; Samaranda and Gavrilescu 2008) due to their very high toxicity, their prolonged persistence in polluted ecosystems, and their extremely limited biodegradability (Chandra and Chaudhary 2013).

These properties include aqueous solubility, vapor pressure, partition coefficients between water: solid and air: solid or liquid, and half-live in air, water and soil (Saxena and Bharagava et al., 2015).

These chemicals are genotoxic nature and compounds can act at various levels in the cell (causing gene, chromosome, or genome mutations), necessitating the use of a range of genotoxicity assays designed to detect these different types of mutations (Chowdhary et al., 2017; Bartling et al., 2005; Taylor et al., 2004).

Several treatment technologies have been developed and applied for tannery wastewater treatment including biological, precipitation, co-precipitation, solvent extraction, adsorption, coagulation, flocculation, ion-exchange and membrane technology. These techniques are exploited to reduce pollutants or contaminants from tannery wastewater and to remove toxic chemicals from tannery wastewater and to recover the quality of raw wastewater for irrigation purpose.

Thus, the overall study of this research work is divided into seven (07) chapters. Further, to know the background information, each chapter of thesis was reviewed in detail and all the necessary information is elaborated and discussed in chapter (02) review of literature with following objectives.

Objectives (s)

1. **Physico-chemical analysis of tannery wastewater collected from CETP after secondary treatment process.**
    - ✓ Collection of tannery wastewater samples

- ✓ Physico-chemical analysis of collected tannery wastewater

2. **Detection, enumeration and characterization of bacterial population by conventional and PCR based molecular methods from tannery wastewater after secondary treatment process.**
   - ✓ Multiple-tube fermentation test or most probable number (MPN) of treated tannery wastewater for bacterial population analysis
   - ✓ **Isolation, purification and characterization of pathogenic and non-pathogenic bacteria from tannery wastewater samples**
   - ✓ Isolation and purification of bacteria
   - ✓ Biochemical and molecular characterization
   - ✓ Antibiotic sensitivity pattern
   - ✓ Determination of minimum inhibitory concentration
   - ✓ **Bacterial community analysis in tannery wastewater by 16S rRNA based metagenomics analysis using Illumina platform (Next Generation Sequencing-NGS)**
   - ✓ Isolation, Qualitative and quantitative analysis of gDNA
   - ✓ Preparation of libraries for 2 x 250 bp Run Chemistry
   - ✓ Cluster Generation and Sequencing and data analysis
   - ✓ Phyla of associated bacteria in activated sludge
   - ✓ Distribution of bacterial community at class level
   - ✓ Distribution of bacterial community at Order level
   - ✓ Distribution of bacterial community at Family
   - ✓ Distribution of bacterial community Genus level
   - ✓ OTU Abundance Heatmap
   - ✓ Krona Graph

3. **Detection and characterization of residual organic pollutants from tannery wastewater after secondary treatment process by using different solvents system and HPLC/ GC-MS/MS analysis**
    - ✓ Extraction of residual organic pollutants from collected tannery wastewater by using different solvents systems
    - ✓ GC-MS/MS analysis
4. **Toxicity assessment of tannery wastewater after secondary treatment process by using terrestrial and aquatic test models**
    - ✓ Phytotoxicity test using *Vigna radiata L*
    - ✓ Phytotoxicity test using *Allium cepa L*
    - ✓ Genotoxicity studies
    - ✓ Animal toxicity study using *C. Elegans*
    - ✓ Nile red staining

Chapter 2
Review of Literature

2. Review of literature

The tannery industry is a major industry on an international scale and is of significant economic importance as an agro-based sector producing a host of products in one of the world's finest natural materials. Rapid industrialization and modernization in developing countries, different types of industrial wastes (solid and liquid) containing a large number of organic pollutants and toxic metals in high concentration are directly or indirectly discharged into the environment without adequate treatment (Dixit et al., 2015; Chandra et al., 2009). In India tannery industries play prominent role and contributes 15% of total production capacity of world. There are more than 3000 tanneries, produce about 500000 tons of hides and 314 of skin (Verma et al., 2007). In Unnao, Kanpur CEPT for tannery, of cost 19.3 million under World Bank line credited and operated by Unnao Pollution Controls Co .Ltd. which includes primary and secondary treatments but various organic pollutants do not degrade in secondary and primary treatment and enters the major water source and contaminates water and soil.(Yadav et al., 2016).

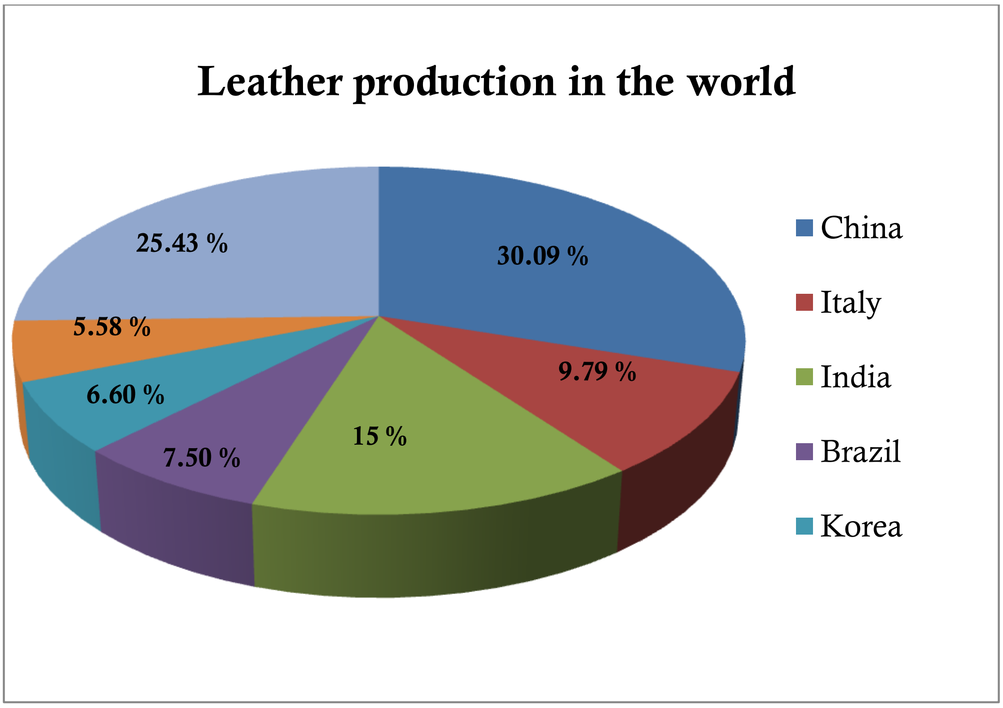


Figure 2.1: Leather production in the world

Major tanneries are located in Tamil Nadu, West Bengal Uttar Pradesh other are shown in the tabulate form (Table 2.1.).

Table 2.1: Detail of Leather industry in India

State	Number of tanneries	Percentage (%)
Tamil Nadu	934	44.60
West Bengal	538	25.70
Uttar Pradesh	378	18.00
Punjab	79	3.80
Maharashtra	33	1.60
Haryana	18	0.80
Bihar	17	0.80
Karnataka	16	0.80
Other states	63	2.75

For the past few years, anthropogenic activities have contributed to ecological contamination, causing an increase in concentration of various heavy metals for example chromium, lead, cadmium and Nickel etc. Industrial waste is disposed off in the nearby water bodies and ultimately absorbed in the surroundings. These heavy metals are utilized in several industries such as tanneries, electroplating, mining, textiles, pesticide industries etc. (Mishra et al., 2018; Vendruscolo et al., 2017). Environmental pollution has affected various illnesses as they have crossed the recommended threshold limit value given by WHO (World Health Organization) (Witek-Krowiak et al. 2011; Joshi 2018). These industries are releasing their effluents continuously in their surroundings, which is a leading threat to environmental safety. Because chromium is a non-degradable pollutant, therefore it persists in the environment (Ran et al. 2016).

The organic and inorganic pollutants present in tannery wastewaters do not degrade much during the primary and secondary treatment process in industries and goes into the environment causing serious soil and water pollution along with serious threat to human and animals health in different ways. The organic pollutants present in tannery wastewater (TWW) may provide chance to a variety of pathogenic microbes to flourish and contaminate the receiving water bodies as well as surrounding environments because they may act as a source of nutrients for a diversity of microbes.

Gauthier and Archibald., (2001) has studied the ecology of "fecal indicator" bacteria commonly found in seven Canadian pulp and paper mill wastewater systems and showed that the organic pollutants present in wastewater support the growth of numerous coliforms, especially *Klebsiella spp*., *Escherichia coli*, *Enterobacter spp*., *Citrobacter spp*. (Filali et al., 2000) have isolated and characterized several antibiotic and heavy metal resistant bacteria such as *Pseudomonas fluorescens*, *Pseudomonas aeruginosa, Klebsiella pneumoniae, proteus mirabilis and staphylococcus sp.* from the sewage water of Casablanca, which is an industrial city in Morocco.

Thus, the high concentration and low biodegradability of pollutants as well as a variety of pathogenic and non-pathogenic microbes present in TWW is a matter of serious environmental concern and hence, it becomes very essential to adequately treat the TWW before its final disposal into the environment. Therefore, this chapter provides the detailed information on the various types of organic and inorganic pollutants as well as different types of pathogenic and non-pathogenic microbes present in tannery wastewater and various treatment approaches, which can be used for the removal of above mentioned pollutants for the safety of environment as well as human and animal health.

2.1. Various steps and chemicals used in leather production process:

In India, tanning industries are leading cause of pollution due to disposal of unprocessed wastes in water bodies and on land. Developing countries have been observed with an increase in leather production as compared to developed nations. In tanning process, animal hides are transformed through various chemical reactions to leather (Figure 2.2).

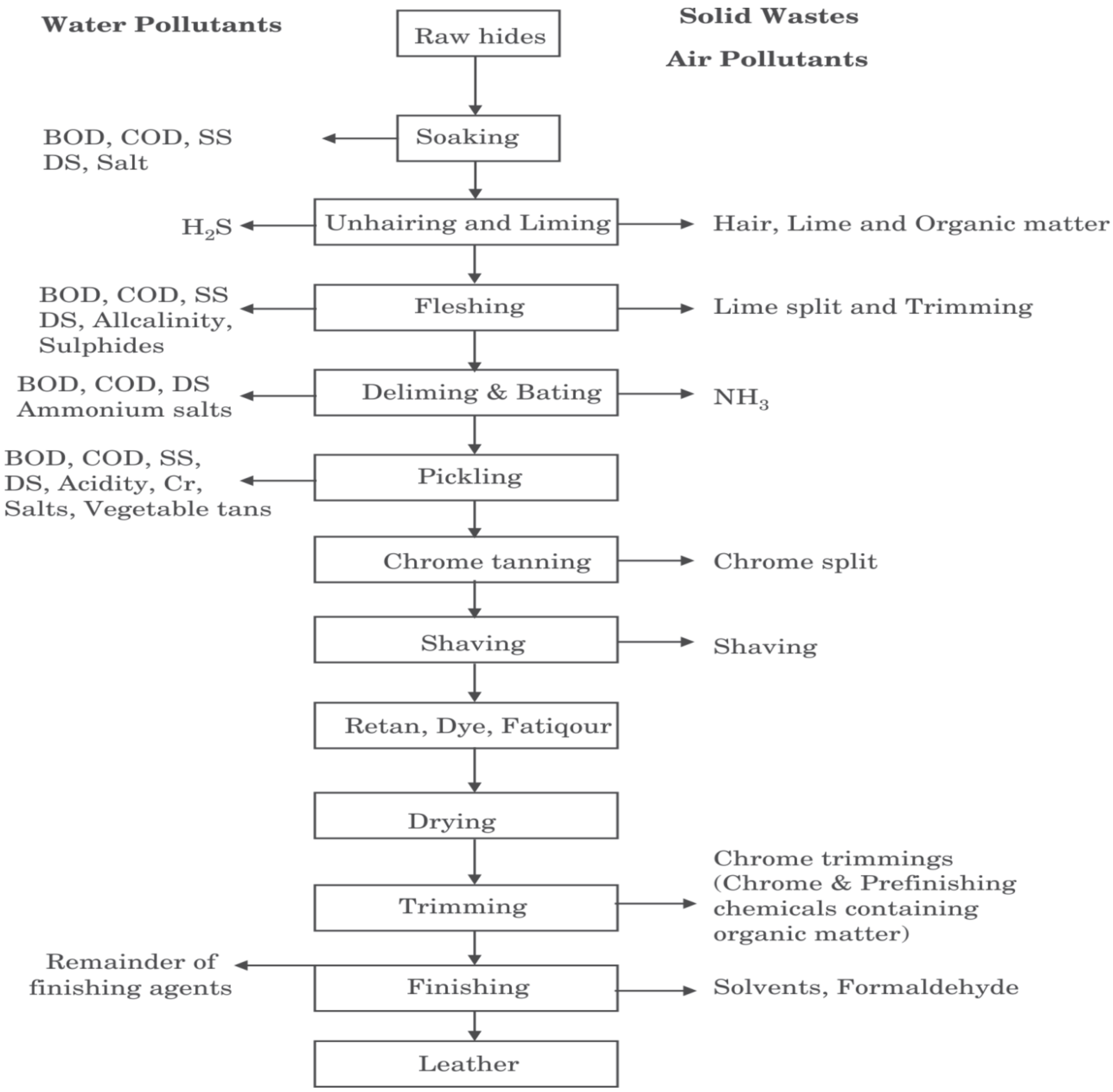


Figure 2.2: Different steps used in a typical tanning industry to obtain finished leather from raw hides/skins (Source: UNEP 1994)

The hide is processed through various chemicals such as NaOCl, NaOH, H_2SO_4, chlorides, enzymes, lime, formic acid, chlorobenzene, ammonium salts, kerosene, and tenso-active compounds to remove fat, meat and hair. The hide is then subjected to mineral salts, chromium [Cr (III)] and dyes to leather. Thus, effluent generated has a large quantity of Cr (III), sulphates, sodium and chlorides. However, the end products of each step produce different kind of waste materials and concentration may vary (Tunay et al. 1995). Basaran et al. (2008) and Saravanabhavan et al. (2004) described similar procedures for leather tanneries situated in India and Turkey respectively.

The tanning process aims to transform skins in stable and imputrescible products namely leather (Table 2.2).

Table: 2.2: Quantity of pollution load in tannery wastewater contributed by individual operations of leather tanning process

Pollution load	Pollution Load (kg/T of raw hide/skins processed operations)						
	Soaking	Unhairing/ liming	Deliming and bating	Chrome tanning	Post tanning	Finishing	Total Load
Waste water generated (m3/T)	9-12	4-6	1.5-2	1-2	1.-1.5	1-2	17.5-25.5
Suspended solids	11-17	53-97	8-12	5-10	6-11	0-2	83-149
COD	22-33	79-122	13-20	7-11	24-40	0-5	145-231
BOD	7-11	28-45	5-9	2-4	8-15	0-2	50-86
Chromium	-	-	-	2-5	1-2	-	3-7
Sulphides	-	3.9-8.7	0.1-0.3	-	-	-	4-9
NH_3-N	0.1-0.2	0.4-0.5	2.6-3.9	0.6-0.9	0.3-0.5	-	4-5.8
Total nitrogen	1-2	6-8	3-5	0.6-0.9	1-2	-	11.6-17.9
Chlorides	85-113	5-15	2-4	40-60	5-10	-	137-202
Sulphates	1-2	1-2	10-26	30-55	10-25	-	52-110

Source: Modified from Dixit et al. (2015); Sabumon, (2016)

There are four major groups of sub-processes such as Pretanning, Tanning, Wet Finishing and Finishing Operations are applied obtain the finished leather: (U.S. EPA, 1986; Lofrano et al., 2013).

2.1.1. Pretanning (Beamhouse Operations)

Pretanning process is also known as Beamhouse operation as it is carried out in Beamhouse for cleaning and condtioniorng of raw hides/skins leading to the generation of the major part of effluent.

2.1.1.1. Soaking

Raw skins are treated with water and small quantities of imbibing substances in order to hydrate the skin proteins, to solubilize the denatured proteins, to eliminate the salt used in the preservation step, to hydrate and to open the contract fibers of the dried skins, and eliminate the residuals of blood, excrement, and earth attached to the skin.

2.1.1.2. Fleshing and Trimming

Extra tissue is removed. Unhairing is done by the chemical dissolution of hair and epidermis with an alkaline medium of sulfide and lime. After skinning at the slaughterhouse the hide appears to contain excessive meat and fleshing usually precedes the Unhairing and liming this process produce the wastewater containing very high COD value (Lofrano et al., 2013).

2.1.1.3. Deliming and Bating

The unhaired, fleshed and alkaline hide are neutralized with acid ammonium salts and treated with enzymes, similar to those found in the digestive system to remove the hair remnants and to degrade proteins. During this process hair roots and pigments are removed contradiction the major parts of the ammonium salts load in the generated wastewater.

2.1.1.4. Pickling

Pickling increases the acidity of the hides to up pH 3 by addition of acid liquor and salts, enabling chromium tannins to penetrate the hide. However the value salts are added to prevent the hide from swelling. For preservation purposes, 0.03- 2% by weight fungicides and bactericides are used.

2.1.1.5. Degreasing

It is used in remove the excess of material fatty substances from skins by using the organic solvents /surfactant.

2.2. Tanning (Tanyard Operation)

Tanning allows the stabilization of collagen fibers through a cross- linking action. The tanned hides and skins are tradable intermediate products (wet- blue). Tanning agents can be categorized in three main groups namely mineral (chrome) tanning agent; vegetable tanning agents; and alternative tanning agents (e. g. Syntans, aldehydes, and oil tanning agents).

2.1.2. Chrome Tanning (CT)

Chrome tanning is the most common type of tanning process used in most of tanning industry in the world (Chandra et al., 2011). After pickling, when the pH value is low, chromium (III) salts are added. To fixate the chromium, the pH is slowly increased through addition of a base. The chrome tanning process is based on the cross- linkage of chromium ions with free carboxyl groups in the collagen fibers. It makes the hides resistant to bacteria and high temperature. Chrome tanned leather are characterized by top handling quality, high hydro thermal stability, user specific properties and versatile applicability. Waste chrome from leather manufacturing, however, poses a significant disposal problem.

2.1.3. Vegetable Tanning (VT)

Vegetable tanning is usually accomplished in a series of vats with increasing concentrations of tanning liquor, Vegetable tanning produces relatively dense, pale brown leather that tends to darken on exposure to natural light. Vegetable tanning is frequently used to produce sole leather, belts, and other leather goods. Unless specifically treated, the vegetable tanned leathers have low hydrothermal stability, limited water resistance, and are hydrophilic in mature.

2.1.4. Alternative Tanning

Tanning with organic tanning agents, using polymers or condensed plant polyphenols with aldehydic cross linkers, can produce mineral free leather with high hydrothermal stability similar to chrome- tanned leather. However, organic all tanned leather usually is more filled (e. g. leather with interstices filled with a filler material) and hydrophilic than chrome free leather, with equally high hydrothermal stability. This tanning process is carried out with a combination of metal salts, preferable but not exclusively aluminum (III), and a plant polyphenol containing pyrogallol groups, often in the form of hydrolysable tannins

2.1.5. Wet Finishing (Post Tanning)

Post tanning operations involved neutralization and bleaching, followed by retanning, dyeing, and fat liquoring. These processes are mostly carried out in a single processing vessel. Specialized operations may also be performed to add certain properties to the leather product (e. g. Water repellence or resistance, oleophobicity, gas permeability, flame retardancy, abrasion resistance, and anti electrostatic properties).

2.1.6. Finishing

The crust resulted after the retanning and drying process is subjected to a number of finishing operations. The purpose of these operations is to make the hide softer and to mask the small mistake. The hide is treated with an organic solvent on water based dye and varnish. Environmental aspects are mainly related to the finishing chemicals, which can also reach the wastewater generated.

Table 2.3: Chemical compounds used at different steps in leather tanning process

Tanning stage	Chemical used	Functions
Pretanning	Surfactants	Help in the wetting back of the hides or skins
	Biocides	Prevent bacterial growth which can damage the hides or skins during soaking
	Swell regulating agents	Prevent uneven swelling of hides or skins during liming
	Formic acid	Lower the pH of hides or skins
	Sodium sulphide	Destroys hairs on hides or skins
	Sodium hydrosulphide	Destroys hairs on hides or skins
	Low sulphide unhairing agents	Reduce the amount of sulphides used thus reducing the environmental impact of tanneries
	Caustic soda	Help in swelling of hides or skins during soaking or liming
	Ammonium sulphate	Helps to remove lime from hides or skins
	Ammonium chloride	Helps to remove lime from hides or skins during the deliming
	Sodium metabisulphite	Prevent formation of toxic hydrogen sulphide gas during deliming. It also acts as a bleaching agent
	Degreasers	Remove natural fats and greases from the hides or skins
	Sodium formate	Assist penetration of chrome tanning salts into hides or skins
	Aldehyde tanning agents	Tanning agents used to make wet white
	Chromium sulphate	Act as tanning agent used to make wet blue
	Magnesium oxide	Raises the pH of hide or skin to allow chromium or aldehydic to chemically bind to the skin protein
	Fungicide	Prevent moulds or fungal growth on tanned hides or skins
Dye house	Degreasers	Remove grease or fats that may be present on the wet blue as a result of the wet blue coming into contact with machinery
	Surfactants / Wetting agents	Help in getting back of wet blue in dye house
	Sodium formate	Raise the pH during neutralization process
	Sodium bicarbonate	Raise the pH during neutralization process
	Chrome syntans	Improve softness of final leather during rechroming process
	Dyeing auxiliaries	Disperse dyes evenly
	Polymers	Give fullness and a tight leather grain to final leather
	Dyes	Give a colour to final leather desired by customer
	Chromium sulphate	Improve softness of final leather during rechroming process
	Fat liquors	Give softness to final leather
Finishing	Butadiene resins	Give specific properties like good coverage to leather finish
	Acrylic resins	Give properties like adhesion, water resistance to final leather
	Fillers	Fill small blemishes on the leather surface
	Polyurethane resins	Give properties like toughness and light fastness to leather finish
	Handle modifiers	h Give the leather surface a waxy or slippery feel
	Cross linkers	Toughen the leather finish and improve water resistance
	Dullers	Reduces the gloss of the finis
	Nitrocellulose lacquers	Used in the top coat of a leather finish
	Acrylic lacquers	Used in the top coat of a leather finish
	Polyurethane lacquers	Used in the top coat of a leather finish
	Viscosity modifiers	Used to increase the viscosity of a finish mixture

2.2. Generation of tannery wastewater and its characteristics

An average of 30-35 m^3 of wastewater is produced per ton of rawhides However, processed the wastewater production varies in wide range (10-100 m^3 per ton hide) depending on the raw material, the finishing product and the production processes (Tunay et al., 1995). Organic pollutants (proteic and lipidic components) are originated from skins (it is calculated that the raw skin loss of 30%organic material during the working cycle) or they are introduced during the processes**.** The Beamhouse wastewater is characterized by an alkaline pH and by a very acidic pH as well as high COD value. Lofrano et al. (2013) reported COD value of 27,600 mgL^{-1} for Beamhouse wastewater and this very high COD value in mostly highest amount of the salt load occurs in the Beamhouse area. About 15 to 40% (w/w) of common salt is used for preserving animal skins and it is removed during the soaking process (Sundarapandiyan et al., 2010). Lime and sodium sulfide or sulfydrate are normally used during the liming-unhairing operation.

Organic solvents are the most widely used chemicals in degreasing step, which contribute the major part of environmental pollutions by the emission of volatile compounds with the generated wastewater (Cassano et al., 2001). If a tannery is processing salted hides, then sodium chloride is the biggest salt component in the wastewater is always the from hide and skin preservation. The wet-end re-tanning, dyeing and fat liquoring processes have only a minor impact on the total salt load dominantly originated from the hides during the initial pre-soak and main soak process. The tannery wastewater contains the highest concentration of chromium (up to 4950 mgL^{-1}) (Cooman et al., 2003). Coloring is usually involves the combining of dyes with the tanned skin fibers to form an insoluble compound. Retanning and wet finishing streams contribute relatively low BOD and TSS, but high COD (Table 2.4**)**. (Lofrano et al., 2013)

Table 2.4: Physico-chemical characteristics of CETP treated tannery wastewater

Parameters	Treated wastewater	Effluent discharge standards		
		CPCB (2013)	**ISI standard (2000)**	**USEPA (2002)**
pH	8.7	6–9	6–9	-
Color	Dark brown	-	-	-
Temperature (°C)	32 °C	40 °C	-	-
Conductivity (moles/ cm)	11,050	850	-	-
EC (mS Cm/L)	-	0.85	-	-
Alkalinity (mg/L)	680	500	500	-
Total solids (TS; mg/ L)	2500	2,200	-	-
Total dissolved solids (TDS; mg/ L)	2,219	2,100	2100	-
Total suspended solids (TSS; mg/ L)	281	100	100	-
BOD (mg/L)	250	30	30	40
COD (mg/L)	449	250	250	120
Phosphate(mg/L)	5.4	5	-	-
PCP(mg/L)	14.9	0.1	-	-
Sulfate (mg/L)	2,490	5	-	-
Total nitrogen (mg/L)	234.50	100	-	-
Magnesium(mg/L)	234	200	-	-
Chloride	354	600	600	-
Nitrate	20.08	10	-	-
Fluoride (mg/L)	3.9	2.01	-	-
Phenol (mg/L)	10	1	-	0.5
Oil and grease	17	10	-	-
Heavy metal concentration				
Cr (mg/L)	19.50	2	-	0.05
Cu (mg/L)	1.75	3.0	-	0.50
Mn (mg/L)	1.62	2.0	-	0.20
Zn (mg/L)	3.6	5.0	-	2
As (mg/L)	0.60	0.2	-	0.010
Pb (mg/L)	0.1	0.1	-	0.05
Cd (mg/L)	0.5	2.0	-	0.05
Fe (mg/L)	3.0	3	-	2
Co (mg/L)	0.32	1.5	-	-
Ni (mg/L)	3.4	3	-	0.10

Modified from Verma et al., 2008; Verma and Mauraya et al., 2013; Chowdhury et al., 2013; Dixit et al., 2015; Kumari et al., 2016

2.3. Organic and inorganic pollutants present in tannery wastewater

The environmental pollution due to wastewater discharged from tannery industries causes serious problems in environment as well as health threats to both humans and animal. Since, it contains a complex mixture of both organic and inorganic pollutants, which do not degrade much during the secondary treatment process in industries and goes into the environment causing serious problems.

2.3.1. Organic pollutants present in Tannery Wastewater

During the end of the 20^{th} century, the global environment became polluted with a number of industrial pollutants. The pollution of global environment with a complex mixture of organic pollutants has resulted from industrial discharges anthropogenic activities, as well as the inadvertent formation of by-products of various industrial processes (Masood and Malik, 2013). Organic pollutants are a group of chemical compounds that originated from different anthropogenic activities but have some common characteristics such as semi volatility, lipophilicity, bioaccumulation, which make them resistant to biological photolytic, as well as chemical degradation and persist in the environment for a long period of time (Yadav et al., 2017; Samarandaand Gavrilescu, 2008).

Most of the organic pollutants have three common characteristics: (i) one or more cyclical ring structures of either aromatic or aliphatic nature, (ii) a lack of polar functional groups, and (iii) a variable amount of halogen atoms usually chlorine. If some key properties of organic pollutants are known, then the environmental chemists can make predictions about their fate and behavior in the natural environment. These properties include aqueous solubility, vapor pressure, partition coefficients between water: solid and air: solid or liquid, and half-live in air, water, and soil (Chandra and Chaudhary, 2013). A large amount of waste discharged from tannery industries such as, includes a variety of gaseous, liquid, and solid waste, which persist for long period of time into the environment and causes serious threats to the environment ,human, animal and as well as plants.(Chandra and Chaudhary, 2013).

In tannery industry during the tanning process, a number of chemical compounds (mainly chromium salts) are used to convert the raw hides/skins into leather and generate large volume of wastewater containing huge amount of organic matter, phenolics, phthalates, tannins, salts a variety of organic pollutants and toxic heavy metals mainly chromium making wastewater unsuitable for irrigation and aquatic life (Chandra et al., 2011). Most of the compounds detected (Table 2.5) in present study have also been reported by various authors by using different solvent systems (Kumari et al., 2016; Masood and Malik, 2013; Chandra et al., 2011; Alam et al. 2010; 2009).

Table 2.5: Previously reported organic pollutants identified in tannery wastewater using different extraction solvents through GC-MS/MS analysis

Solvents	Identified organic Pollutants (Many authors)
Acetonitrile + acetone	Benzene, 2,2,3-Trimethyl oxepane
Methanol	3-Nitropthalic acid,1, 2-Benzenedicarboxylic acid, di-isooctyl ester, 1, 3-Hexadien-5-yn
Diethyl ether + Chloroform	2-phenylethanol, Nonadec-1-ene, bis (2-methoxyethyl)phthalate, Hexatriacontane, Heneicosane, 2,4-Di-tert-butylphenol, Tricosane2,3-Epoxypinane
Choloform+Hexane	Dibutyl phthalate, Hexatriacontane, Bis(2-ethylhexyl) phthalate, bis (2-methoxyethyl) phthalate,1,2-Benzenedicarboxylic acid, diisooctyl ester (diisooctyl phthalate)
Dichloromethane (DCM)	Trimethyl1(2,6 ditert-butylphenoxy) silane, Dibutyl phthalate, Phenyl N-methylcarbamate, Di-n-octyl phthalate,1,2-Benzenedicarboxylic acid, diisooctyl, Oleic acid,2,4-bis(1,1-dimethyl) phenol, aminobiphenyl,4-nonylphenol, Hexadecanoic acid, Phenol, 2,4-bis(1,1-dimethylethyl), hexachlorobenzene, 4-Trimethylsiloxyphenylphenoxysulfone
Ethyl acetate	1,1-dimethylethyl-2-phenylethiazole, Acetic acid, Benzene,3-methoxy-4-benzaldehyde ,Benzoic acid ,Decanoic acid, Benzene propanoic acid, 2-hydroxy-3-methyl-butanoic acid

2.3.1.1. Total Kjeldahl nitrogen (TKN)

Several components in TWW contain nitrogen as part of their chemical structure. The most common chemicals are ammonia (from deliming materials) and the nitrogen contained in proteinaceous materials (from liming/unhairing operations).

These nitrogen sources pose two direct problems:

1. Plants require nitrogen in order to grow, but the high concentration of nitrogen substances containing over-stimulate the plant growth. Water-based plants and algae grow too rapidly, where upon water ways become clogged and flows are impaired. As the plants die, a disproportionately high amount of organic matter has to be broken down and if the load outstrips the natural supply of oxygen from the river plants, fish and aerobic bacteria die and ultimately anaerobic conditions develop (Chowdhary et al., 2018).

2. The nitrogen released through protein and the deliming process is in the form of ammonia. The latter can be converted by bacteria over several stages into water and nitrogen gas, which is ultimately released into the atmosphere. Both of these degradation products are non-toxic, yet large volumes of oxygen are needed in the process. If oxygen demand is greater than the supply natural then, toxic anaerobic conditions develop rapidly .The nitrogenous compounds can be broken down by combining intensive aerobic and anaerobic biological treatment processes. The oxygen demand is very high and, thus leads to high operational requirement and energy. Calculations showed that typical TWW, 40%of the oxygen requirements are spent on the removal of nitrogen compounds.

2.3.1.2. Sulphide (S_2-)

The sulphide content in TWW mainly resulted from the sodium sulphide and sodium hydrosulphide, used during the unhearing process as well as from the breakdown of hairs.

The sulphide content may cause: pose:

Under alkaline conditions, the sulphides remain largely in solution. If the pH of effluent drops below 9.5, hydrogen sulphide evolves from the wastewater and lower the pH, the higher will be the rate of evolution, which is characterized by the smell of rotten eggs. Comparable toxicity of hydrogen cyanide, even at a low level of exposure to gas induces headaches and nausea, as well as possible damage to the eye. At higher levels, death can rapidly set in and countless deaths attributable to the build-up of sulphide in sewage systems have been recorded (Tare et al., 2003).

Hydrogen sulphide is soluble gas. When absorbed, weak acids can form and cause corrosion. This weakens the metal roofing, girders and building supports. In industries, major problems can arise as metal fittings, structural reinforcements and pipe work corrode. If discharged to surface water, even low concentrations pose the toxicological hazards. Sulphides can be oxidized into non-toxic compounds by certain bacteria in rivers but if generate anaerobic condition causing harm is aquatic life. Sulphides in effluent can be determined in several ways. One of the most accurate methods relies on the acidification of suitable quantities of wastewater to generate hydrogen sulphide. This is flushed through the apparatus using nitrogen gas where after, it is trapped and converted into zinc sulphide, which is then determined by the titration process.

2.3.1.3. Neutral salts

Two common types of salts are supposed to be present in TWW.

2.3.1.3.1. Sulphates (SO42-)

Sulphates are a component of TWW, orignated from the use of sulphuric acid or products with a high (sodium) sulphate content. Many auxiliary chemicals contain sodium sulphate as a by-product of their manufacture. For example, chrome-tanning powder contains high levels of sodium sulphate, as do many synthetic retanning agents. An additional source is created by removing the sulphide components from wastewater by aeration since, the oxidation process creates a whole range of substances, including sodium sulphate. These sulphates can be precipitated by calcium-containing compounds to form calcium sulphate, which has a low level of solubility. Problems arise only with soluble sulphates, due to, for two main reasons:

1. Sulphates cannot be removed completely from a solution by chemical means. Under certain biological conditions, it is possible to remove the sulphate from a solution and bind the sulphur on to microorganisms. However, the sulphate remains either as sulphate or is get broken down by the anaerobic bacteria to produce malodorous hydrogen sulphide. This process occurs very rapidly in effluent treatment plants, sewage systems and water courses, if wastewater remains static. This bacterial conversion of sulphate in to hydrogen sulphide in sewage treatment plants results in the corrosion of metal parts, and unless sulphate-resistant concrete will gradually erode.

2. If no degradation occurs, the risk of increasing the total concentration of salts in the surface and groundwater runs is incurred. Sulphate analysis is performed by adding barium chloride solution to a sample of filtered wastewater. The sulphates get precipitated as barium sulphate and filtrate is dryed and calculation determines the sulphate level.

2.3.1.4. Chlorides (Cl^-)

The major source of chloride ions in TWW is sodium chloride, which is used in large quantities for the hides and skin preservation or during the pickling process. Being highly soluble and stable, chloride ions remains unaffected by wastewater treatment process and, thus, remain as a burden on the environment. However, considerable quantities of salts are produced by the industry and the increased salt content in groundwater, especially in areas of high industrial density, is now becoming a serious environmental problem.

2.3.2. Inorganic pollutants present in Tannery Wastewater

The rapid industrialization and urbanization has given rise to the problem of heavy metal contamination into the environment. The environment (soil, water and air) have been severely contaminated with inorganic pollutants because different types of pollutants are released into the environment through tannery industry. A number of toxic metals such as chromium (Cr), copper (Cu), cadmium (Cd), zinc (Zn), nickel (Ni), mercury (Hg), arsenic (As), lead (Pb), and cobalt (Co), etc. are known as serious environmental pollutants and act as a potentially toxic or carcinogenic agents in nature at very low concentrations and cause serious health hazards in human and animals, if enter into the food chain (Hutchinson and Meema, 1987; Jarup, 2003; Khan et al., 2008).

Nowadays, the contamination of soil and water ecosystems with toxic metals is a major environmental problem. They are number of natural and anthropogenic activity, which act as a major sources metal contamination in the environment. However, most significant natural sources of metal contamination in environment are weathering of minerals, erosion and volcanic activity, while the anthropogenic sources are largely depend upon the human activities such as mining, smelting, electroplating, use of pesticides and discharge of phosphate fertilizer, as well as biosolids (e.g. livestock manures, composts, and municipal sewage sludge), and atmospheric deposition etc.

A mass balance of heavy metals in the soil environment can be expressed by using the formula (Khan et al., 2008):

$$M_{total} = (M_p + M_a + M_f + M_{ag} + M_{ow} + M_{ip}) - (M_{cr} + M_l):$$

Where M is the heavy metal, ***p*** is the parent material, ***a*** is atmospheric deposition, ***f*** is fertilizer source, ***ag*** is agrochemical source, ***ow*** is organic waste source, ***ip*** is inorganic pollutant, ***cr*** is crop removal and ***l*** is losses by leaching, volatilization and other processes. It is estimated that emission of several heavy metals in atmosphere from anthropogenic sources is one to three times higher than that of natural sources.

In the environment, the heavy metals enter naturally from pedogenic processes of weathering of parent materials and also through various anthropogenic sources. There are different sources of heavy metals contamination in the environment as shown in Fig 1.

2.3.2.1. Chromium (Cr) compounds

Chromium metal compounds are not biodegradable. Thus, they can be regarded as long-term environmental features. Since, they also have accumulative properties, and thus are the subjects of close attention. Two forms of chrome are associated with the tanning industry, whose properties are often confused.

Chromium is a *d*-block transition metal placed in-group VIB in the periodic table and has atomic number 24, atomic mass 52, density 7.19 g cm^{-3}, melting point 1875 °C and boiling point 2665 °C. It is one of the less common elements and does not occur naturally in elemental form, but only in compounds. Chromium is mined as a primary ore product in the form of mineral chromate ($FeCr_2O_4$). In nature, chromium mainly exits in trivalent and hexavalent forms. Chromium is a naturally occurring heavy metal that exists in air, water, soil and food. It is now considered as one of the major environmental pollutants due to its toxicity for ecological, nutritional and environmental reasons.

2.3.2.1.1. Chrome^{3+} (trivalent chrome, chrome III)

Chromium is mainly found in the waste can be generated from the chrome tanning process. It occurs as a part of the retanning system and is displaced from leathers during the retanning and dyeing processes. This chrome is discharged from industry in soluble form. However, when mixed with TWW from other processes (especially with proteins), the reaction is very rapid. Precipitates are formed mainly protein-chrome, which adds to the sludge generation. However, as discussed in section 1.1.b, very fine colloids are also formed, which are then stabilized by the chrome in effect, the protein has been partially tanned. The components are thus become highly resistant to the biological breakdown, and thus, the biological process in both surface waters and treatment plants is inhibited. Once successfully broken down, chromium hydroxide gets precipitated and persists in the ecosystem for an extended period of time. If chrome discharges are excessive, the chromium might remain in the solution. Even in low concentration, it has a toxic effect on *daphnia* and thus, thus disrupting the food chain for fish and possibly inhibiting the photosynthesis.

2.3.2.1.2. Chrom $^{6+}$ (hexavalent chrome, chrome VI)

Tannery wastewater is unlikely to contain chromium in this form. Dichromates are toxic to fish life since they swiftly penetrate the cell walls. They are mainly absorbed through the gills and the in effect is accumulative. Analysis is highly specialized.

Cr (VI) is present as either dichromate ($Cr_2O_7^{2-}$) in acidic environment or as chromate (CrO^-_4) in alkaline environments (Srinath et al., 2002). In environment chromium is found in two stable states: (1) Cr (III) and (2) Cr (VI). The first is not very soluble and is immobilized by precipitation as hydroxide, the later is toxic, soluble, and get easily transported to water resources. Microbial communities as well as reduced their growth retarding the bioremediation process and if Cr^{6+} enters in food chain, it causes skin irritation, eardrum perforation, nasal irritation, ulceration and lung carcinoma in humans and animals along with accumulate in placenta impairing the fetal development in mammals (Verma et al., 2001; Srinath et al.,2002; Cheung and Gu, 2007).

2.3.2.1.3. Health hazards

Cr (VI) can enter the body when people breathe air, eat food, or drink water contaminated with it. Cr(VI) is also found in house dust and soil, which can be ingested or inhaled out of the various forms of chromium, Cr (VI) is the most common form and toxic in nature. Many Cr (VI) compounds have been found to be carcinogenic in nature, but the evidence to date indicates that the carcinogenicity is site specific and limited to lung and sinonasal cavity and dependent on the exposure intensity (Salem et al., 2000). Inhaling relatively high concentration of Cr (VI) can cause a runny nose, sneezing, itching, nosebleeds, ulcers, and holes in nasal septum. Short-term high-level inhalational exposure can cause adverse effects at the contact site, including ulcers, irritation of the nasal mucosa, and holes in nasal septum. Ingestion of very high Cr (VI) doses can cause kidney and liver damage, nausea, irritation of the gastrointestinal tract, stomach ulcers, convulsions, and death. While dermal exposures may cause skin ulcers or allergic reactions. Cr (VI) is one of the most highly allergenic metals, second to nickel and studies on mice given high doses of Cr (VI) have shown reproductive abnormalities including reduced litter size and decreased fetal weight (ATSDR, 2000; Mishra and Bharagava, 2015).

2.3.2.2. Cadmium (Cd)

Cadmium occurs naturally in ores together with zinc, lead and copper. Cadmium compounds are used as stabilizers in PVC products, colour pigments, several alloys and most commonly in re-chargeable nickel and cadmium batteries. Metallic cadmium has been mostly used as an anticorrosing agent (cadmiation). Cadmium is also present as a pollutant in phosphate fertilizers. Cadmium containing products are rarely re-cycled, but frequently dumped together with household wastes, thereby contaminating the environment, especially if the waste is incinerated.

2.3.2.2.1. Health hazards

Inhalation of cadmium fumes or particles can be life threatening. Although acute pulmonary effects and deaths are uncommon, but sporadic cases are still occurring (Seidal et al., 1993; Barbee and Prince, 1999). Cadmium exposure may also cause kidney damage. The first sign of the renal lesion is tubular dysfunction, which is evidenced by an increased excretion of low molecular weight proteins [such as β2-microglobulin and α1-microglobulin (protein HC)] or enzymes [such as N-Acetyl-β-D-glucosaminidase (NAG) (WHO 1992; Jarup et al., 1998). It has been suggested that tubular damage is reversible, but there is overwhelming evidence that the cadmium induced tubular damage is indeed irreversible (Jarup et al., 1998).

According is WHO, a urinary excretion of 10 nmol/mmol creatinine (corresponding to *circa* 200 mg Cd/kg kidney cortex) would constitute a 'critical limit' below which the kidney damage would not occur (WHO, 1992). Several reports have shown that lower cadmium level may also cause kidney damage and/or bone effects (Jarup, 2003).

2.3.2.3. Mercury (Hg)

Mercury is a chemical element with symbol Hg and atomic number 80. Metallic mercury is used in thermometers, barometers and instruments used to measure blood pressure. Mercury is largely used in the electrochemical process of chlorine manufacturing, where mercury is used as an electrode in chlor-alkali industry.

2.3.2.3.1. Health hazards

Acute mercury exposure may give rise to lung damage. Chronic poisoning is characterized by neurological and psychological symptoms such as tremor, changes in personality, restlessness, anxiety, sleep disturbance and depression (Jarup, 2003). Metallic mercury may cause kidney damage, which is reversible if exposure has stopped. It has been also possible to detect proteinuria at relatively low levels of occupational exposure.

2.3.2.4. Lead (Pb)

Lead is a chemical element in the carbon group with symbol Pb, atomic number 82, atomic mass 207.2, density 11.4 gcm^{-3}, melting point 327.4 °C, and boiling point 1750 °C. It is a naturally occurring, bluish gray metal usually found in the form of minerals combined with other elements such as sulphur (i.e. PbS, $PbSO_4$), or oxygen ($PbCO_3$), and it's concentration ranges from 10 to 30 mg kg^{-1} in the earth's crust. Lead is a soft, malleable and heavy post-transition metal. Metallic lead has a bluish-white color after being freshly cut, but it soon turned into a dull greyish color when exposed to air. The general population gets exposed to lead from air and food in roughly equal proportions. However, the occupational exposure to inorganic lead mainly occurs in mines and smelters as well as welding of lead painted metal, and in battery plants, whoever low or moderate exposure may take place in the glass industry. High levels of air emissions may pollute areas near the lead mines and smelters. Airborne lead can be deposited on soil and water and thus, finally reaches into the human or animal's body.

2.3.2.3.1. Health hazards

The symptoms of acute lead poisoning are headache, irritability, abdominal pain and various disorders related to the nervous system (Steenland and Boffetta, 2000). Lead encephalopathy is characterized by the sleeplessness and restlessness. Children may be affected by behavioral disturbances, learning and concentration difficulties. In severe cases of lead encephalopathy, the affected person may suffer from acute psychosis, confusion and reduced consciousness. People who have been exposed to lead for a long period of time may suffer from memory deterioration, prolonged reaction time and reduced ability to understand. Individuals with average blood lead levels under 3 μmol/l may show signs of peripheral nervous symptoms with reduced nerve conduction velocity and reduced dermal sensibility.

2.3.2.4. Arsenic (As)

Arsenic is a widely distributed metalloid, which occurs in rock, soil, water and air. Inorganic arsenic is a metalloid present in group VA and period 4 of the periodic table that occurs in a wide variety of mineral ores as As_2O_3 and can be recovered by processing of ores containing mostly Cu, Pb, Zn, Ag and Au etc. It is also present in ashes from coal combustion. Arsenic has the following properties: atomic number 33, atomic mass 75, density 5.72 g cm^{-3}, melting point 817 °C, boiling point 613 °C, and exhibits fairly complex chemistry and can also be present in several oxidation states (−III, 0, III, V). In aerobic environments, As (V) is dominant, usually in arsenate (AsO_4^{3-}) forms in various protonation states: H_3AsO_4, $H_2AsO_4^{-}$, $HAsO_4^{2-}$, and AsO_4^{3-}. Arsenate and other anionic forms of arsenic behave as chelater and can precipitate in presence of metal cations.

Since, arsenic is often present in anionic form, it does not form complexes with simple anions such as Cl^{-} and SO_4^{2-}. Arsenic speciation also includes organ metallic forms such as methyl arsenic acid (CH_3) AsO_2H_2 and dimethyl arsenic acid $(CH_3)_2AsO_2H$. Many As compounds adsorb strongly to soils and are therefore transported only over short distances in groundwater and surface water. Arsenic is reported to cause skin damage, increased risk of cancer and problems with circulatory system.

2.3.2.4.1. Health hazards

Inorganic arsenic is acutely toxic and intake in large quantities may leads to the gastrointestinal symptoms, severe disturbances in cardiovascular and central nervous systems and even death also. Arsenic exposure through drinking water is reported to cause skin, lungs, kidney and bladder cancer (WHO, 2001). In affected person, the skin cancer in preceded by directly observable precancerous lesions. Uncertainties in the estimation of past exposures are important while assessing the exposure-response relationships, but the arsenic contamination in drinking water at the level of 100 μg/l leds to the development of cancer whereas the concentration of arsenic from 50-100 μg/l is found to be associated with the precursors of skin cancer. The relationships between the arsenic exposure and health effects are less clear. But, there is strong evidence for hypertension and cardiovascular disease, but the evidence is only suggestive for diabetes and reproductive effects and weak for cerebrovascular disease, long-term neurological effects, and cancer at the sites other than lung, bladder, kidney and skin etc.

2.3.2.5. Other metals

Other metals which might be discharged from the tannery industries in wastewaters include aluminum and zirconium. Depending on the chemical species, these metals have differing toxicities that are also affected by the presence of other organic matter, complexing agents and the pH of water .Aluminum, in particular, appears to inhibit the growth of green algae and crustaceans, which are sensitive to low concentrations.

2.4. Pathogenic bacteria spp. present in tannery wastewater

Waterborne pathogens infect around 250 million people each year resulting in 10-20 million deaths (El-Lathy et al., 2009; Toze, 1999). Many of these infections occur in developing countries, which suffer from lower levels of sanitation, problems associated with low Socio-economic conditions and less public health awareness than in developed countries. A number of chromium-resistant microorganisms have been reported, such as *Pseudomonas* spp., *Proteus spp.*., *Enterobacter spp Enterobacter* spp., *Escherichia coli*, and *Bacillus* spp. (Naraian, et al; 2012) by various authors as known in table

Table 2.6: Various pathogenic bacteria reported in tannery wastewater and their health hazards in living organisms

Bacterial species	Health hazards	References
Escherichia coli	Food poisoning gastroenteritis, urinary tract infections and neonatal meningitis	Verma et al., 2004; Verma et al., 2008
Proteus spp.	Urinary and septic infections (nosocomial)	Naraian et al., 2012
Salmonella spp.	Typhoid fever, paratyphoid fever, and food poisoning	Naraian et al., 2012
Serratia spp.	Nosocomial infections	Naraian et al., 2012
Pseudomonas spp.	lung infection (Ventilator-associated pneumonia), nosocomial infections	Naraian et al.,2012
Enterococcus spp.	Urinary tract infections, Bacteremia, Bacterial endocarditis, Diverticulitis, and Meningitis	Naraian et al., 2012
Enterobacter spp.	Opportunistic infections in immunocompromised,	Naraian et al.,2012
Aeromonas spp.	infectious diarrhea (Gastroenteritis)	Naraian et al., 2012
Vibrio spp.	Diarrhea, Vomiting and gastroenteritis, Septicemia (fever, increased heart rate, increased breathing rate)	Ramteke et al.,2010
Bacillus spp.	Foodborne illness(nausea, vomiting, and diarrhea)	Chandra et al., 2011
Cronobacter spp.	Bacteraemia, Meningitis and Necrotising enterocolitis	Chandra et al., 2011
Shigella spp.	Bacillary dysentery, foodborne illness (Shigellosis).	Ali et al., 2015
Klebsiella spp.	Pneumonia, urinary tract infections, diarrhea septicemia and meningitis	Noorjahan, 2014

The microbial pathogens in water necessitate the proper monitoring of wastewaters presence of contaminated aquatic resources for various types of organic pollutants and microbial pathogens. For an appropriate risk assessment, the type of organic pollutants and microbial pathogen present in wastewater and this relative numbers need to be determined. This is particularly important for the effective treatment of industrial wastewaters and its safe reuse /recycle or disposal into the environment.

In this literature study for current and emerging conventional and molecular approaches for characterizing bacterial community, composition and structure in water and wastewater processes in Figure 2.3 (Gilbride et al., 2006). During the recent year to molecular techniques have supplied for examining microbial diversity and detecting specific microorganisms. The wastewater released from tannery industries may contain a variety of organic pollutants and millions of pathogenic and non-pathogenic bacteria per milliliter including coliform, *Streptococci*, *Staphylococci*, anaerobic spore forming bacilli, and many other types of health hazards organisms (Naraian et al., 2012).

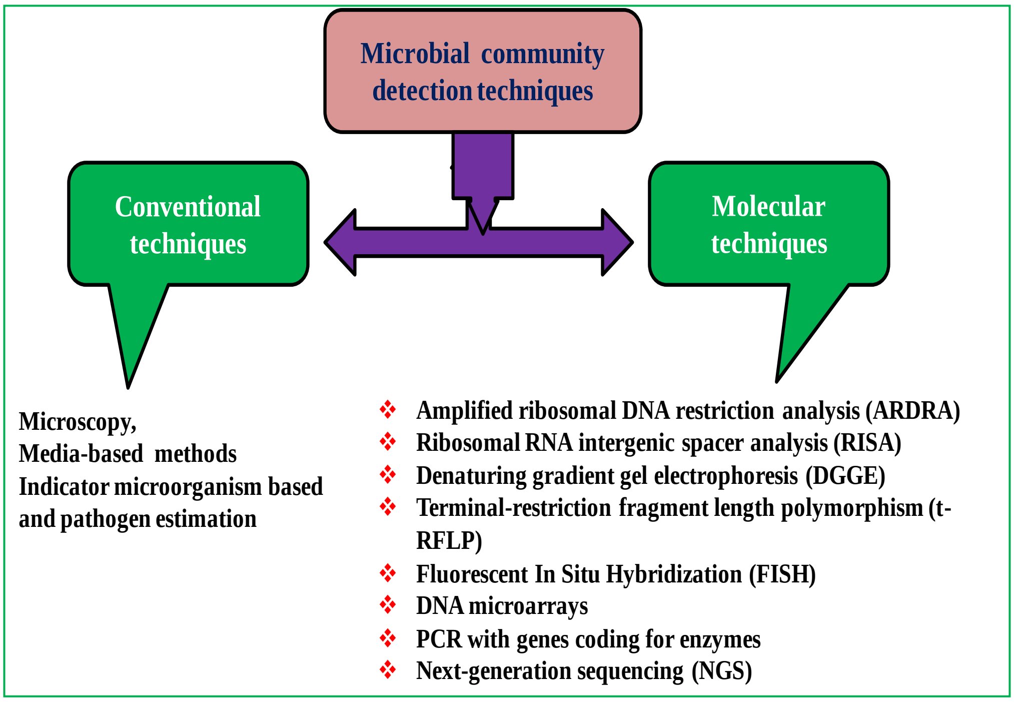


Figure 2.3: Current and emerging conventional and molecular techniqes used to detected wastewater microorganisms

The presence of microbial pathogens in treated wastewater as well as in polluted aquatic resources poses a considerable health risk to the general public health. Despite large advances in water and wastewater treatment processes, waterborne diseases still pose a major world-wide threat to public health.

There are several reports available on the presence of a variety of organic pollutants and pathogenic microbes in various types of industrial wastewaters as El-Lathy et al., (2009) have reported the presence of various pathogenic bacteria like *Salmonella*, *Vibrios*, and *Listeria* in raw and treated wastewater samples collected from the inlet and outlet of oxidation ponds in El-Sadat city, Egypt. Previous studied many authors have detected and characterized various antibiotic resistant bacteria such as *Citrobacter*, *Enterobacter*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Escherichia cloacae, Pseudomonas aeruginosa, Citrobacter freundii, Serratia marcescens, Shigella flexneri, Shigella sonnei and proteus mirabilis* from industrial wastewater systems (Verma et al.2004; Chandra et al., 2011; Naraian et al., 2012; Ramteke et al., 2010).

2.5. Environmental health hazards from tannery wastewater

Tannery industries are considered as one of the major source of pollution soil and water because the wastewater generated from these industries poses serious environmental impacts on soil, and water, and atmospheric system (Mwinyihija, 2010). Usually tanning, industries discharge their wastewater into nearby rivers and are indirectly being used for the irrigation of crops and vegetables. This practice has ultimately led to the movement of potentially toxic metals from water to plants and finally to human beings (Sinha et al., 2008). Organic pollutants (OPs) are mainly because of their very low solubility and high lipid solubility leads to their bioaccumulation in tissues (Guzzella et al., 2005). They enter the body through food and get transferred to all the trophic levels of the ecosystem.

Organic pollutants are highly toxic in nature and have a wide range of chronic effects such as endocrine disrupting activities mutagenicity, and carcinogenicity (Sultan et al., 2001; Lee et al., 2006). Organic pollutants ate also able to persist in the environment for decades causing serious health hazards such as cancer, birth defects, learning disabilities, immunological, behavioral, neurological impairment, developmental abnormalities and reproductive discrepancies in human and animals. (Sweetman et al., 2005). It is well reported that TWW is the major source of pollution in the environment as it has very high concentration of chromium, which goes in soil and water and create serious health hazards in the environment. The Cr^{6+} is a potent carcinogen to humans and animals as it enters the cells *via* surface-transport system and gets reduced to Cr^{3+} inducing genotoxicity (Matsumoto et al., 2006).

Thus, the use of Cr-loaded wastewater for irrigation practices disrupts the several physiological and cytological processes in cells (Shanker et al., 2005; Chidambaram et al., 2009) leading to reduced root growth, biomass, seed germination, early seedling development (Irfan and Akinici, 2010), and induces chlorosis,b photosynthetic impairment, and finally leading to plant the death (Akini and Akini, 2010). In the environment, chromium (Cr^{6+}) contamination alters the structure of soil microbial communities as well as reduce their growth retarding the bioremediation process and if Cr^{6+} enters in food chain, it causes skin irritation, eardrum perforation, nasal irritation, ulceration, and lung carcinoma in humans and animals along with accumulating in placenta impairing the fetal development in mammals (Chandra et al., 2011). The extensive use of chromium salts in tanning industries have resulted in chromium contamination in soil and ground water at production sites, which pose a serious threat to human health, fish, and other aquatic biodiversity (Turick et al., 1996).

There are a number of studies that highlighted the number of health hazards from tanning industry including occupational exposures (Battista et al., 1995), water and land contamination affecting the seed germination in various crop plants (Asfaw et al., 2012), aquatic and terrestrial biota, and humans (Barnhart, 1997), as well as acute toxicity in *Vibrio fischeri* (Jochimsen and Jekel, 1997) and the micro crustacean *Daphnia magna* (Tisler et al., 2004), sea urchin (De Nicola et al., 2007), and marine micro algae (Mericet al., 2005). Verma et al. (2008) have conducted the quality assessment of treated TWW with special emphasis on pathogenic Escherichia coli detection through serotyping.

2.6. Strategies for the removal of organic pollutants and pathogenic bacteria from Tannery Wastewater

2.6.1. Physico-chemical Treatment

2.6.1.1. Coagulation and Flocculation

Various authors have investigate the coagulation and flocculation of TWW has been investigated by using various inorganic coagulants such as aluminum sulfate ($AlSO_4$), ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$) to reduce the total organic load (BOD and COD), total solids (TDS and TSS) as well as to remove the toxic metals such as chromium before the biological treatment of TWW (Song et al., 2004; Lofrano et al., 2006; Gaurav and Bharagava; 2015). However, each coagulant operates most effectively at specific pH and the extent of pH range largely depends on the nature of coagulants, as well as on the dosage of the coagulant characteristics of the wastewater to be treated (Song et al., 2004).

There are several studies that have been conducted to investigate the effectiveness of different coagulants used for the treatment of TWW in terms of COD and chromium removal (Song et al., 2004). However various physicochemical methods are found to be effective, but their application is limited due to large amount of chemicals, used, huge quantity of sludge generation, and disposal problems in environment, high installation as well as operating cost. Thus the biological treatment method since, to be viable cost effective and environment friendly alternative of physicochemical method for the treatment industrial wastewater.

2.6.2. Biological Treatment

2.6.2.1. Aerobic Processes

Biological treatment processes are generally used for the treatment of industrial wastewaters to reduce the organic content as these processes have many economic advantages over the physicochemical treatment methods. But, the high concentration of tannins and other poorly degradable compounds as well as toxic metals present in tannery wastewater have negative effects on the biological treatment processes (Lofrano et al., 2013). Stasinakis et al. (2002) have observed a significant inhibition in growth of heterotrophic in presence of 10 mg/L Cr^{6+}.

A typical sequencing batch reactor (SBR) has been proved to be more capable for carrying out the biological processes such as nitrification and denitrification in presence of inhibitors due to the selection and enrichment of particular microbial species. (Murat et al., (2006). Have studies performance of SBR for nitrogen removal in TWW, with a wide range of temperature (7–30°C), was studied and achieved full nitrification and denitrification was by the adjustment of sludge age for each temperature range

The biodegradation of naphthalene-2-sulfonic acid, which is a main component of the naphthalenesulfonate, by *Arthrobacter*sp. 2AC and *Comamonas*sp. 4BC was reported and these two bacterial strains were isolated from tannery activated sludge (Song et al., 2005). Song and Burns (2005) described the degradation of all components of the condensation product of 2-naphthalenesulfonic acid and formaldehyde (CNSF) by fungus *Cunninghamella*

Polymorpha and suggested that the combination of *C. polymorpha*and *Arthrobacter*sp. 2 AC or *Comamonas*sp. 4BC was effective for the treatment of TWW. However, conventional cultures could not treat saline wastewaters of values higher than 3% - 5% (weight/volume, w/v) and shift in salt concentration causes significant reactor failures in the system performance. Senthil kumar et al. (2008) have isolated *Pseudomonas aeruginosa, Bacillus flexus, Exiguobacteriumhomiense,* and *Staphylococcus aureus* from soak liquor, marine soil, salt lake saline liquor, and seawater, respectively, and studied the biodegradation of tannery soak liquor by these halotolerant bacterial consortia. An appreciable COD removal (80%) was observed at 8% (w/v) salinity for mixed salt tolerant consortia, but increase in salt concentration to 10% (w/v) resulted in a decrease in COD removal efficiency.

The presence of sulfide, chromium, chloride, and fluctuations in pH and temperature has adverse effects on the nitrification process. The impact of temperature on the organic carbon and on the efficiency a full-scale industrial-activated sludge plant nitrogen removal had been studied during the treatment of TWW (Gorgun et al., 2007). It was observed that temperature changes had a minor influence on COD removal efficiency (4%–5%), while the total nitrogen removal was affected significantly by the temperature. Insel et al. (2009) also investigated the performance of intermittent aeration type of operation when temperature was fluctuated between 21°C and 35°C and they found that an increase in the aeration intensity improved the nitrification performance and the application of intermittent aeration also improved the total nitrogen removal up to 60%.

2.6.2.1. 1. Activated Sludge Process (ASP)

The ASP is the most generally applied biological (aerobic) wastewater treatment method that primarily removes the dissolved organic solids as well as settleable and non-settleable suspended solids (SSs). In ASP, a suspension of bacterial biomass (the activated sludge) is mainly used for the removal of organics. These organisms are cultivated in aeration tanks, where they are provided with dissolved oxygen (DO) and food from the wastewater. Depending on the design and specific applications, an activated sludge treatment plant can remove organic nitrogen (N) removal and phosphorus (P), besides the of organic carbon substances.

Processes used for the biological treatments of TWW in CETPs. In India, the ASP and upflow anaerobic sludge blanket (UASB) process on the most common (Jawahar et al., 1998). However, the biological treatment of TWW using ASP has been reported by many workers (Ramteke et al., 2010; Hayder et al., 2007; Tare et al., 2003).

2.6.2.2. Anaerobic Process

Anaerobic treatment processes are the subset of processes in which microorganisms break down the biodegradable material in the absence of oxygen, and used for the management of industrial or domestic waste and/or to release energy. There has recently been growing interest within the scientific community regarding the anaerobic treatment of TWW due to the several drawbacks of its application (Lofrano et al., 2013):

(i) The implementation of adequate technology for H_2S desorption and treatment is required due to the consistent production of sulfide as a result of the reduction of sulfate, which occurs in absence of alternative electron acceptors such as oxygen and nitrate;

(ii) High protein content affects the selection of biomass, slow kinetics of hydrolysis, and also inhibits the granular sludge formation.

The anaerobic treatment of TWW is mainly performed by using the anaerobic filters (AF) composed of both upflow anaerobic filters (UAF) and down flow anaerobic filters (DAF) and UASB reactors (Lefebvre et al., 2006; El-Sheikh et al., 2011). Only a few experiments have referred to the expanded granular sludge bed and anaerobic baffled reactor (ABR) (Zupancic and Jemec, 2010).

A number of bacterial species such as *Bacillus* sp., *Pseudomonas* sp., *Alcaligene s*sp., *E. coli*, and *Shewanella*alga are reported to have Cr^{6+} detoxification capability due to the presence of reductases soluble in cytosol (Srinath *et al.,* 2002). In *Pseudomonas maltophilia* and *Bacillus megaterium*, Cr^{6+} reduction is associated with membrane cell fractions (Srinath et al., 2002; Shukla et al., 2009). During the bacterial treatment of tannery wastewater in aeration lagoons at CETP, Cr^{6+} reduction commonly occurs in two or three steps in which Cr^{6+} initially get reduced to a short-lived intermediates Cr^{5+} and/ or Cr^{4+} before further reduction to the thermodynamically stable end product Cr^{3+} (Cheung and Gu, 2007). However, at present it is unclear that whether the reduction of Cr^{5+} to Cr^{4+} and Cr^{4+} to Cr^{3+} is spontaneous or enzyme mediated. The NADH, NADPH and electrons from the endogenous reservoir are implicated as electron donors in Cr^{6+} reduction process.

During the reduction process, the enzyme Cr^{6+} reductase (ChrR) transiently reduces Cr^{6+} with a one-electron shuttle to form Cr^{5+} followed by a two-electron transfer to generate Cr^{3+} (Ackerley*et al.,* 2004). Although a proportion of the Cr^{5+} intermediate is spontaneously reoxidized to generate reactive oxygen species (ROS), its reduction through two electron transfer catalyzed by ChrR reduces the opportunity to produce harmful radicals. Several facultative anaerobes such as *P. dechromaticans, P. chromatophila, Aeromonas dechromatica, Microbacterium* spp, *Geobacter metallireducens, Shewanella putrefaciens, Pantoea agglomerans,* and *Agrobacterium radiobacter* EPS-916 are also reported to catalyze the biotransformation of Cr^{6+} to Cr^{3+} under anoxic conditions. But, unlike to Cr6+ reductases isolated from aerobes, the Cr^{6+} reducing activities of anaerobes are associated with their electron transfer systems ubiquitously catalyzing the electron shuttle alone.

2.6.2.4. Emerging Treatment Technologies

2.6.2.4.1. Membrane Processes

In recent years, membrane technologies have been focused and their cost is continuing to reduce while the application possibilities are ever extending. The use of membrane technologies applied to the leather industry represents an economic advantage, especially in the recovery of chromium from residual waters of leather tanning process. Several studies have shown that cross flow microfiltration, ultrafiltration, nanofiltration, reverse osmosis (RO), and supported liquid membranes can be applied in leather industry for the recovery of chromium from spent liquors (Ashraf et al., 1997; Cassano et al., 2001; Labanda et al.,2009), reuse of wastewater and chemicals of deliming/bating liquor (Gallego-Molina et al., 2013), reduction in polluting load of unhairing and degreasing removal of salts, and in biological treatment of TWW in the light of their reuse. Reverse osmosis with a plane membrane has been used as post treatment process to remove refractory organic compounds (chloride and sulfate) by De Gisi et al. (2009). The high quality of permeate produced by the RO system with a plane membrane allowed the reuse of TWW within the production cycle, and thus reducing the groundwater consumption.

2.6.2.4.1.1. Membrane Bioreactors

Membrane Bio-Reactor (MBR) has been attracting much attention from scientists and engineers for TWW treatment due to the numerous advantages over CASP, such as elimination of settling basins, independence of process performance from filamentous bulking, or other phenomena affecting settleability (Suganthi et al., 2013). MBR systems essentially consists of a combination of membranes and biological reactor systems. The separation of biomass from wastewater by membranes also allows the concentration of MLSS in bioreactor to be increased significantly. However, from the studies of Munz et al. (2009), it is possible to infer how the kinetics of nitrification are effectively reduced by the presence of

tannins, without large differences between biomass selected with either the CASP or the MBR. One of the main drawbacks of membrane application is a significant fouling due to the clogging, adsorption, and cake layer formation by the pollutants onto the membrane. In recent years, extensive work is in progress to reduce the befouling phenomenon.

2.6.2.4.2. Advanced Oxidation Processes

There has been increasing interest in studying on advanced oxidation processes (AOPs) to treat TWW for the removal of organic pollutants and pathogenic microbes. AOPs refers to the set of the chemical treatment processes that uses strong oxidizing agents O_3, hydrogen peroxide (H_2O_2)] and/or catalysts (Fe, Mn, TiO_2) and also sometimes supported in activity by high-energy radiation, for example, UV light (Schrank et al., 2004). All these processes are based on the production and utilization of hydroxyl radicals, which are very powerful oxidants that quickly and unselectively oxidize a broad range of organic compounds. The scientific interest toward AOPs application to high strength wastewater has increased remarkably in the last 20 years. AOPs can reduce the concentration of pollutants several hundred ppm to less than 5 ppm and therefore significantly brings down the level of COD and TOC, which earned it the credit of "wastewater treatment processes of the 21st century" (Munter, 2001).

Generally, AOPs can be used to treat wastewater generated after secondary treatment processes, which is then called as tertiary treatment process (Audenaert et al., 2011). The pollutants are converted to a large extent into stable inorganic compounds such as water, carbon dioxide, and salts, they undergo mineralization. However, most studies have efficiency evaluated the treatment by COD removal, but TOC remains the more suitable parameter to investigate the state of mineralization ccurring in the treatment process (Schrank et al., 2004).

A goal of wastewater purification by means of AOP procedures is the reduction of the chemical pollutants and the toxicity to such an extent that the cleaned wastewater may be reintroduced into the receiving streams or into a conventional sewage treatment process. AOPs still have not been put into commercial use on a large scale (especially in developing countries) even up to today mostly because of the relatively high costs. However its high oxidative capability and efficiency make the AOPs a popular technique for the tertiary treatment of wastewaters in which the most recalcitrant organic and inorganic pollutants are to be eliminated. The increasing interest in wastewater reuse and more stringent regulations regarding the water pollution are currently accelerating the implementation of AOPs at full scale.

2.6.2.4.3. Plant / phytoremediation

Phytoremediation refers to the use of plants and associated microorganisms to partially or completely remove the selected contaminants from soil, sludge, sediments, wastewater and ground water etc. It can also be used for the removal of radionuclides, organic pollutants as well as toxic metals from the contaminated sites (Saxena et al., 2019; Dixit et al., 2015). Phytoremediation utilizes a variety of plant species and processes and the physical characteristics of plants to aid in the remediation of contaminated sites (Table 2.7). Over the recent years, a special emphasis has been given on phytoremediation since, this technique can be exploited for the remediation of toxic metals polluted soils, as it is a cost-effective, eco-friendly and efficient in-situ remediation technology driven by solar energy. The phytoremediation technique includes a number of different processes such as phytoextraction, phytofiltration, phytostabilization, phytovolatilization and phytodegradation etc. as shown in Figure 2.4.

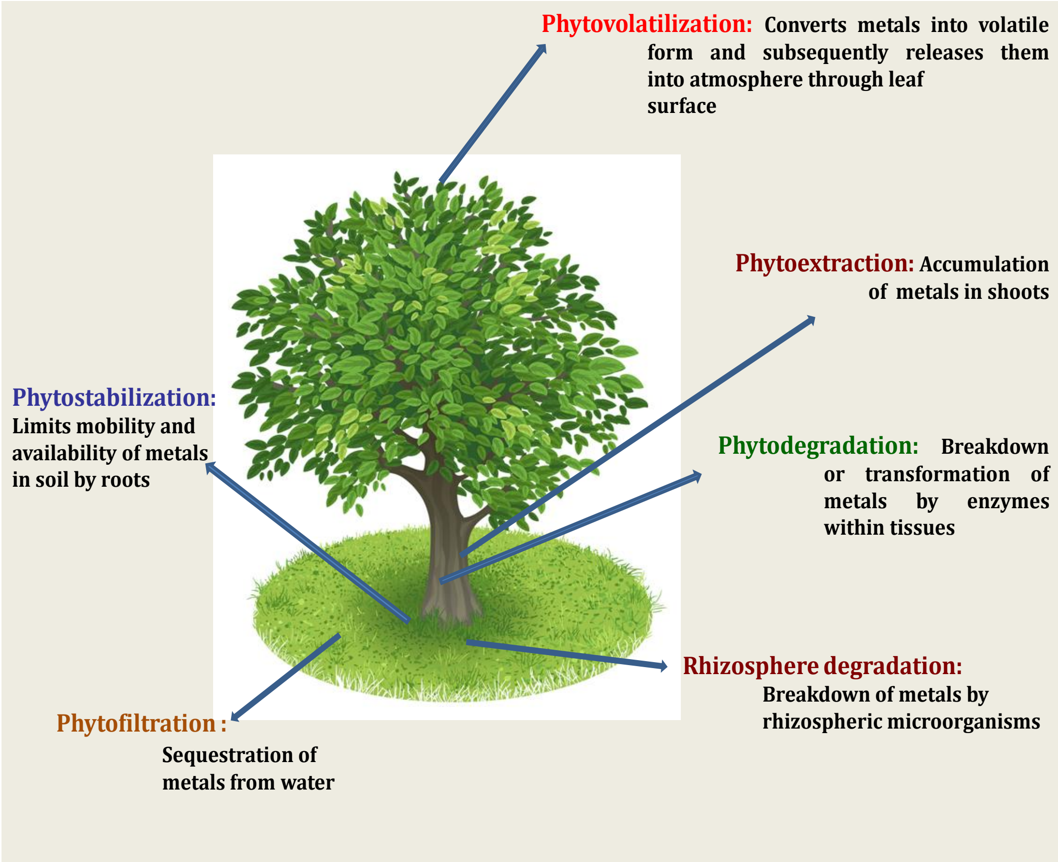


Figure 2.4: Various phytoremediation processes used by plants to remove toxic metals from contaminated sites

Table 2.7: Various plant species reported capable for the phytoremediation of different heavy metals

Heavy metals	Plants Species	References
Cd, Cr	Castor (*Ricinus communis*)	Huang et al.(2011), Pandey (2013)
Cd, Pb, Zn	Corn (*Zea mays*)	Meers et al.(2010)
Cd, Cu, Pb, Zn	*Populus* spp. (*Populus deltoides, Populus nigra, Populus trichocarpa)*	Ruttens et al.(2011); Abhilash et al (2012)
Cd, Cu, Pb, Zn	*Salix* spp. (*Salix viminalis, Salix fragilis*)	Pulford and Watson(2003); Volk et al.(2006); Ruttens et al.(2011)
Cd, Cu, Ni, Pb	Jatropha (*Jatropha curcas* L.)	Abhilash et al.(2009) ; Jamil et al.(2009)
Hg	*Populus deltoides*	Che et al.(2003)
Zn	*Populus canescens*	Bittsanszkya et al.(2005)

2.6.2.4.3.1. Phytoextraction

Phytoextraction is also known as phytoaccumulation in which metals are removed from the contaminated sites by taking the advantages of plants ability to (hyper-) absorb and accumulate or translocate metals or/metalloids and by concentrating them within the biomass. The purpose of this type of remediation is to reduce the metals concentration in contaminated soils, so that they can be used profitably for agriculture, forestry, horticulture, and grazing etc.

2.6.2.4.3.2. Phytostabilization

Phytostabilization is also known as phytoimmobilization, in which plants in combination with soil additives mechanically stabilize the sites and reduce the transfer of metals to the other compartments of ecosystem and finally into the food chain. The "stabilized" organic or inorganic compound is normally get incorporated into plant lignin or into the soil humus. The basis for phytostabilization is that metals do not degrade, so capturing them in-situ is often the best alternative. This approach is particularly applicable when low-concentration, diffused, and vast areas of contamination are to be treated. Plants restrict the metal pollutants by creating a zone around the roots where the pollutant is precipitated and stabilized. When phytostabilization is undertaken, the plants used do not absorb the targeted pollutant(s) into plant tissue.

2.6.2.4.3.3. Phytostimulation

In phytostimulation plant, roots promote the development of rhizospheric microorganisms that are capable for the degradation of metal contaminants and microbes utilize plant root exudates as a carbon and energy source.

2.6.2.4.3.4. Phytovolatilization/rhizovolatilization

Phytovolatilization/ rhizovolatilization employ the metabolic capabilities of plants and associated rhizospheric microorganisms to transform metal pollutants into the volatile compounds that are released into the atmosphere. Some ions such as elements of subgroups II, V, and VI of the periodic table like mercury, selenium, and arsenic etc.

are absorbed by roots, get converted into less toxic forms and released into the surrounding environment.

2.6.2.4.3.5. Phytodegradation

In phytodegradation, the metal contaminants are get degraded or mineralized by the specific plant enzymes or exudates and then the organic component is utilized by the rhizospheric microbes as carbon and energy source while metal component is taken up by the plant roots and used in various metabolic activities. However, phytoremediation being as cost-effective and eco-friendly technology also has many limitations due to which this technology is not much effective for a wide range of metal contaminated sites.

2.6.2.4.3.6. Rhizofiltration

Rhizofiltration is the use of plants to absorb, concentrate, and/or precipitate metal contaminants in the aqueous system. Rhizofiltration is also used to partially treat the industrial and agricultural runoff.

Plants that can accumulate large quantities of metals by natural methods have been identified and are used to remediate the metal contaminated sites. These plants are called as hyper-accumulators and are often found growing in areas having elevated metal concentrations in soil. But, unfortunately at high metals content even hyper accumulating plants grow slow and attain only a small size. Thus, high metals content inhibit the plant growth even that are capable for hyper accumulation of metals. However, depending upon the amount of metals at a particular site and the type of soil, even hyper-accumulating plants may require 15-20 years to remediate the contaminated sites. This time frame is usually too slow for practical application. Hence, there is a need to search the plant species, which can grow fast, and accumulate the greater amounts of biomass in addition to being tolerant to one or more metals.

2.7. Emerging trends, Future prospects and challenges

The major problem with the tannery wastewater is its complex nature due to the organic pollutants such as tannins and other poorly degradable compounds that can inhibit the biological treatment process. Besides organic pollutants, tannery wastewater also contains many toxic metals ions such as Cu^{2+},Cr^{6+},Cd^{2+},Fe^{3+},Zn^{2+},Ni^{2+}, and Pb^{2+}, all these metals ions also have high inhibitory and antimicrobial activity reducing the anaerobic digestion of TWW.

Bioremediation approach such as in situ remediation is applied to reduce or eliminate residual organic pollutants and toxic heavy metals that have led to environmental hazard and risks. In situ remediation involves direct inoculation of microbes and reagents into the polluted aquifer and is becoming progressively common technique. The cost effectiveness, simplicity of procedure and least interference of the site give further advantage for the application of this technique. The removal processes that utilize permeable reactive barriers is also gaining acceptance. No single technique is adequate for the removal of majority of the pollutants that might exist at a site or to accomplish compliance with cleanup standards. To accomplish the goals, the use of treatment train strategy is frequently required. For instance, inorganic reductants might be applied for mass removal of chromate contaminants, followed by the use of anaerobic bioremediation and/or to additionally check natural reduction.

Recently, the sequential applications of bacteria and wetland plants have been also reported to be very promising for the degradation and detoxification of TWW, but this has to be optimized yet with the detailed microbiology of wetland plants,

plants rhizosphere, interaction, and detoxification mechanisms. Moreover, the nature of residual organic pollutants in tannery waste water and extent of their toxicity need to be explored in detail for their complete degradation and detoxification during the treatment process at CETPs.

Chapter 3

Physico-chemical analysis of tannery wastewater collected from CETP after secondary treatment process

Physico-chemical analysis of tannery wastewater collected from CETP after secondary treatment process

3.1. Introduction

Tannery industries are one of the most polluting industries mainly causing soil and water pollution in environment (Dixit et al., 2015; Bharagava and Mishra, 2018). Over the preceding two decades, various treatment technologies such as physical and chemical methods have been engaged for the exclusion from tannery wastewater. But, these methods have certain limited applications, since these produce large amount of sludge, solid wastes and are very expensive (Srinath et al., 2002; Chowdhury et al., 2013; Haydar and Aziz, 2009)

Unnao is one of the major industrial towns in the UP and most famous for leather industrial area worldwide. Unnao industrial area is situated near Kanpur in northern side of River Ganga having more than 40 industrial units mainly tannery units and the wastewater discharged by the tannery industries after treatment at CETP is finally discharged into the River Ganga (Figure 3.1).

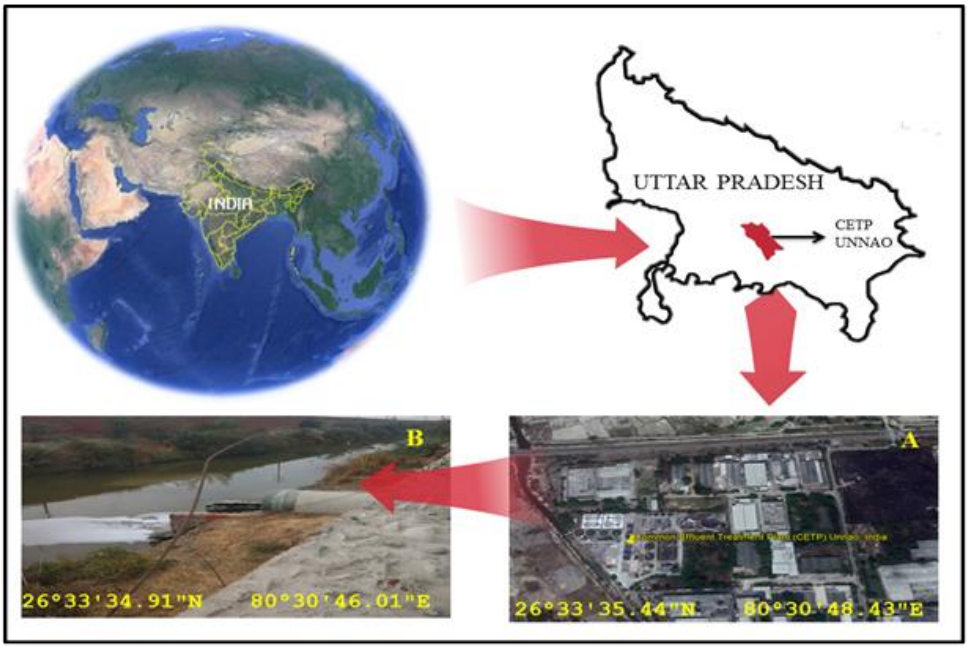


Figure 3.1: Location of sampling site (CETP, Unnao) and discharge of treated tannery wastewater through a drain into the environment

The wastewater discharged from tannery industries is characterized by high chemical oxygen demand (COD, 1244 ± 5.2 mg/L), biochemical oxygen demand (BOD, 560 ± 20 mg/L), total dissolved solids (TDS, 7020 ± 31 mg/L) and total chromium (6.4 ± 0.12 mg/L), sulphate (7.8 ± 0.4 mg/L), phosphate (12.5 ± 0.5 mg/L), chloride (732 ± 2.2 mg/L) along with the other highly toxic organic and inorganic pollutants (Haydar and Aziz, 2009; Raj et al., 2014; Dixit et al. 2015; Kumari et al. 2016). Large amount of different chemicals used in lather tanning process such as chrome salts, vegetable and synthetic tannins, pthalates, phenolic compounds, azo dyes, surface-active compounds, pesticides, sulphonated oils, grease etc. (Lofrano et al. 2013; Yadav et al. 2016a). These chemicals are not fully taken up by hide/skins and thus, endup in wastewater and susbiquently contaminate environment and pose serious health threats to living beings (Matsumoto et al. 2006; Lofrano et al. 2013; Dixit et al. 2015; Saxena et al. 2017). The tannery wastewater characteristic in India indicates that the pollutants are of high BOD, COD, sulfide, chromium, suspended particulate matter, and salt concentration.

Therefore, this chapter studies was analysis for physico-chemical parameters as well as different types of metal analysis present in tannery wastewater.

3.2. Material and methods

3.2.1. Collection of tannery wastewater samples from CETP Unnao

The wastewater samples collected from the inlet (untreated) and outlet of CETP-Unnao, located in Unnao district of Uttar Pradesh, India (Figure 3.2) in pre-sterilized plastic containers (capacity 5L) were brought to the laboratory and stored 4° C. The CETP Unnao is in operation since 1994. This is an activated sludge process (ASP) based CETP treating ~1.9 MLD wastewater received from a cluster of ~25 tanneries located in nearby areas against a design flow of ~2.35 MLD. The quality of treated wastewater often fails to conform the prescribed limit recommended by various pollution controlling bodies of India (CPCB, 2013).

Therefore, we have chosen this site for the study. The collected samples were immediately processed for analysis of physico-chemical parameters, detection and characterization of residual organic pollutants, microbial analysis as well as for toxicity evaluation tests. Analyzed parameter included were pH, BOD, COD, TDS, TSS, total chloride, phenolics, nitrate, phosphate and sulfate (APHA, 2012).

Figure 3.2: Collection of treated tannery wastewater samples at CETP Unnao and discharge wastewater through drain

3.2.2. Physico-chemical analysis of tannery wastewater samples

Following standard methods of the examination of water and wastewaters (APHA, 2012), tannery wastewater samples were analyzed for colour, pH, total solid (TS), total dissolved solid (TDS), total suspended solid (TSS) by drying method, BOD by 5 days method, COD by open reflux method, total nitrogen (TN) by Kjeldhal method and phosphate and sulphate was measured by the vanadomolybdo-phosphoric acid colorimetric and BaCl2 precipitation methods, respectively by UV-spectrophotometer and chloride by AgNO3 titration method. Digested samples (100 ml) in a digestion mixture of nitric-perchloric acid (5:1) were used to determine total chromium and other metals with AAS (GBC, Avanta Sigma, Australia) (APHA, 2012).

3.3. Result and discussion

Tannery is one of the higest environmental polluting industry due to the discharge of wastewater containing high concentration of hazardous waste including heavy metal like chromium. Over the years, many new chemicals has been introduced in tanning processes. Hence, detail analysis of wastewater generated from tanneries are essential for better understanding of the toxicity and chemical nature of the effluents.

The results of collected tannery wastewater for physico-chemical analysis of untreated and CETP treated tannery wastewater is summarized in Table 3.1.

The inlet and outlet tannery wastewater collected from CETP was found to have high concentration of BOD, COD, TDS, nitrogen, phenolics, sulfate, phosphate, and chloride. The wastewater was found to have high concentration of BOD (680.00 ± 20 mg/L), COD (1300.00 ± 45 mg/L), EC (4.40±0.2 Ms/cm), TDS (3850.00 ± 10.0 mg/L), (566.00 ± 12.5 mg/L), sulfate (8.64 ± 0.42 mg/L), phosphate (26 ± 2 mg/L), nitrate (12.3 ± 0.3 mg/L), chloride (1434 ± 12 mg/L) and phenolic (10.5 ± 0.5 mg/L). The nature of wastewater was alkaline of pH (8.45 ± 0.18). Besides, high concentration of total chromium (7.39 ± 0.06 mg/L) was also observed in treated tannery. The values of the various physico-chemical parameters were higher than the permissible limits for effluent discharge as suggested by CPCB (CPCB, 2013 and USEPA (USEPA, 2004) and (Table 3.1) which clearly indicates the toxic nature of wastewater treated by the CETP in Unnao district.

Table 3.1: Physico-chemical characteristic of untreated and CETP treated tannery wastewater

Parameters	**Collected wastewater values**		**Permissible limits (CPCB, 2013)**
	untreated (UT)	treated (CETP)	
Color	Dark brown	Light coloured	Colorless
pH	9.2	7.8	5.5-9
Temperature	30°C	32°C	40°C
Alkalinity (mg/L)	824	625.33	500
BOD (mg/L)	1810	680	30
COD (mg/L)	3210	1300	250
TS (mg/L)	9840	5024	2200
TDS (mg/L)	8532	3850	2100
TSS (mg/L)	2839	566	100
Sulfate (mg/L)	80	8.64	5
Chloride(mg/L)	1803	832	600
Phosphate (mg/L)	114	26	5
Nitrate (mg/L)	17.24	12.3	10
Phenolics (mg/L)	36.24	10.5	1-5
Heavy metals concentration			
Cr (mg/L)	24.44	7.39	2.0
Zn (mg/L)	0.12	0.002	1.0
Cd (mg/L)	1.24	0.032	2.0
Cu (mg/L)	0.78	0.34	3.0
Fe (mg/L)	3.22	2.34	3.0
Ni (mg/L)	2.4	0.56	3.0

The discharge of partially treated wastewater from CETP, affects the flora and fauna of the aquatic ecosystem by blocking the sunlight penetration in receiving water bodies and photosynthetic activity, thus, negatively affects the aquatic life (Sukumaran et al., 2008; Deepa et al., 2011). The high TDS value is also toxic to aquatic lives by causing osmotic stress and affecting the osmoregulatory functions of organisms (Thakur and Srivastava, 2011). The high BOD and COD values of tannery wastewater might be due to the presence of high organic contents and salts in the wastewater (Mishra and Bharagava, 2016). High salts are responsible for acidification, reduced soil fertility and increased salinity of groundwater and rivers.

The high sulfate, phosphate and nitrate content in tannery wastewater might be associated with the use of sulfuric acid and sulfide in dehairing process during the tanning process (Yadav et al., 2016a). The high TDS level in wastewater directly indicates the presence of metal ions in the system.

Presence of monosodium, disodium phosphates, polyphosphates used in leather treatment processes and ammonium salts in deliming and bating process, are responsible for the eutrophication which disturbed the normal ecological functioning of receiving water bodies (Saxena et al., 2016). Phenolics which are listed as the "priority pollutant" by the USEPA (2014) owing to its toxic, genotoxic and carcinogenic effects in plants, animals and human beings are also high in wastewater due to its utilization in preservation of raw hides/skins and leather finishing (Mishra and Bhragava, 2016). Chromium used in leather tanning as fastening agent for marking and surfacing of leather was also found to be in high (7.39 mg/L) (Lofrano et al., 2013; Yadav et al., 2016b) than permisible limit (2 mg/L). High Cr level causes toxic, genotoxic, mutagenic, and carcinogenic effects on humans, animals, plants, and microbes as reported by various authors (Mishra and Bharagava, 2016; Chowdhary et al., 2018).

Chapter 4

Detection, enumeration and characterization of bacterial population by conventional and PCR based molecular methods from tannery wastewater after secondary treatment process

Detection, enumeration and characterization of bacterial population by conventional and PCR based molecular methods from tannery wastewater after secondary treatment process.

4.1. Introduction

The elimination of pollutants and wastes from the environment is an absolute requirement to promote the sustainable development of our society with low environmental impact. The effluent discharged from these tanneries after treatments is still left with high level of BOD, COD, TDS and other specific pollutants such as chromium [Cr(III) & Cr(VI)], pentachlorophenol, surfactant, synthetic tannins, azo dyes, chloride, sulphate and oil and grease (Singh et al., 2013; Thakur and Srivastava, 2011; Chandra et al., 2011). This wastewater containing a variety of toxic pollutants when discharged into the water bodies poses a serious threat to the living organisms inhabiting respective ecosystem and also tends to be accumulated in food chain. (Ramteke et al., 2010; Flores et al., 2012)

The organic pollutants remained in tannery wastewater after the secondary treatment process provide chance to a variety of pathogenic and non-pathogenic microbes to flourish and contaminate the aquatic environments, whereas toxic metals induce genotoxic and mutagenic changes in bacterial communities making them resistant against a wide spectrum of antibiotics and toxic metals (Viti et al., 2003; Malik and Jaiswal, 2000; Filali et al. 2000).

In previous studies, wastewater contamination is reported as a major source of pathogenic bacteria in water resources, but few authors also suggested that besides sewage contamination, the wastewaters discharged from different industries such as distillery, pulp paper mills and tannery industries etc. also act as a good source of nutrients and support the growth of pathogenic microbes in receiving water bodies (Chandra et al., 2011; Ramteke et al., 2010; El-Lathy et al., 2009). However, the detail information about the pathogenic microbes that remained in tannery wastewater even after the secondary treatment process is not available so far.

In addition, the wastewater released from tannery industries may contain a variety of organic pollutants and millions of pathogenic and non-pathogenic bacteria per millilitre including coliform, fecal coliform, anaerobic spore forming bacilli, and many other types of health hazards organisms. The presence of microbial pathogens in treated wastewater as well as in polluted aquatic resources poses serious threats to the general public health. Despite large advances in water and wastewater treatment processes, waterborne diseases still pose a major worldwide threat to public health.

Hence, this chapter was aimed to detection, enumeration and characterization of bacterial community by conventional and PCR based molecular methods from tannery wastewater after the secondary treatment process, so that the treatment processes can be improved /modified accordingly for the adequate treatment of tannery wastewater for its safe disposal into the environment.

4.2. Material & methods

4.2.1. Detection, enumeration and characterization of bacterial population by conventional methods from collected tannery wastewater samples

4.2.1.1. Bacteriological analysis

For the quantitative as well as qualitative analysis, the collected tannery wastewater samples were serially diluted and analyzed for the presence of total heterotrophs, total coliforms, and fecal coliforms in three steps by multiple tube fermentation technique as well as by the standard plate count (SPC) method on plate count agar (PCA), specific media and different chromogenic media respectively (APHA, 2012; El-Lathy et al., 2009; Ramteke e al., 2010; Chandra et al., 2006).

4.1.1.1.1. Multiple-tube fermentation test or most probable number (MPN) of treated tannery wastewater for bacterial population analysis

Qualitative analysis for bacterial population have detected in treated wastewater through the most probable number (MPN) technique. Multiple tube fermentation technique was carried for members of the coliform group (APHA, 2012). The MPN test were used to detect coliform (coliforms are defined as facultative anaerobic, gram-negative, non-sporing, rod shaped bacteria that ferment lactose with the production of acid and gas within 24 hrs of incubation at 35 °C).

The test is performed sequentially in three stages: presumptive, confirmative and completed test. Coliform bacteria were detected by presumptive inoculation into tubes of Lactose broth and their incubation at 37±2°C for 48h. The results of positive tubes were sub cultured into levine's EMB agar for confirmation. Subsequently, positive growth on plates were inoculated into Brilliant green bile broth (BGLB) and incubated at 37°C for 48h for complete test. Gas production in BGLB was used for the detection of coliforms after 48h incubation. MPN of coliforms were found in terms of index/100 ml by using standard tubes. All the results were compared with the standard MPN chart and the results were expressed as the total number of coliform/100 ml of the water. The complete test Positive results on MacConkey agar were tested for the biochemical properties in the BGLB broth finally *E. coli* were confirmed. FC was analyzed by direct specific test method (APHA, 2012) for the group of bacterial population taken a criterion for indicating coliform of fecal origin and simultaneously TC were determined in three steps by multiple tube fermentation technique (APHA, 2012).

Requirements

- Wastewater sample

- Petridishes
- Durham tubes (15)
- Test tubes (15)
- Sampling bottle (sterile)
- MacConkey or lactose broth medium
- MacConkey agar
- Test tube stand
- Bunsen burner/sprit lamp
- Micropipette

4.1.1.2. Isolation, purification and characterization of pathogenic and non-pathogenic bacteria

For isolation and purification of pathogenic bacteria, an aliquot (50 µl) of collected tannery wastewater sample was serially diluted (Figure 4.1**)** with sterile-distilled water was spread on different types of chromogenic media such as HiCrome Vibrio agar plates, HiCrome Klebsiella agar base, and HiCrome Bacillus agar plate followed by incubated at 32° C for 48 hours for colony appearance. The different colonies appeared on different agar plates were picked up and purified by repeated streak plate method. The different colonies appeared on different agar plates were picked up, purified by repeated streak plate method and in morphological, biochemical and molecular characterization studies.

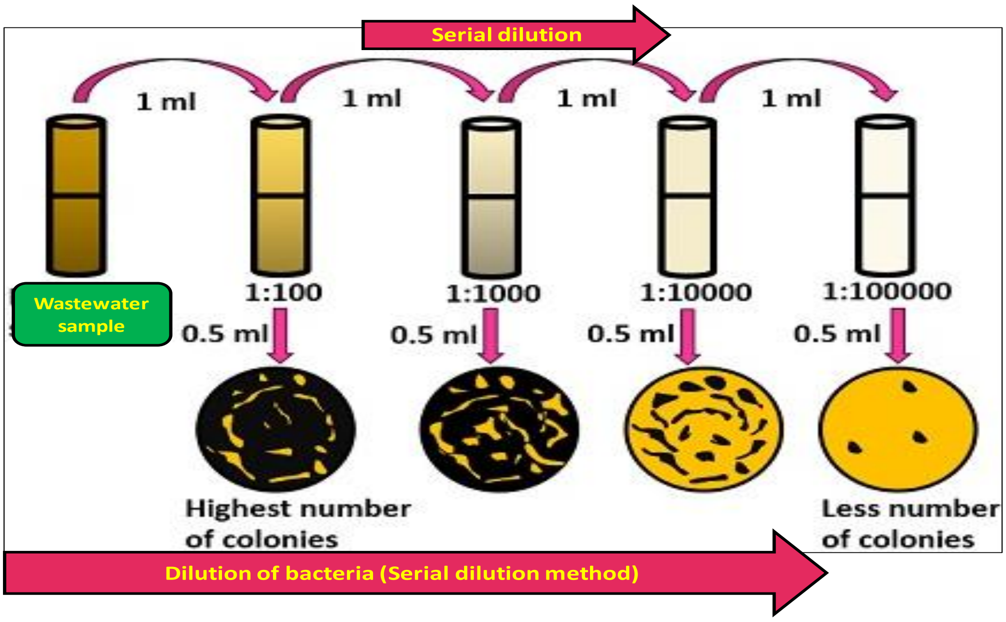


Figure 4.1. Serial dilution methods for isolation of bacteria

4.1.1.2.1. Morphological and biochemical characterization

Bacterial strains isolated from tannery wastewater were characterized morphologically and biochemically characterized according to the standard procedures of Bergey's Manual of Determinative bacteriology (Whitman et al., 2012) and Cowan and Steele Manual for the identification of medical microbiology (Barrow and Feltham, 1993).

The morphological characteristics examined include gram staining, shape, color, and surface. Biochemical tests were performed by manually, include catalyze, oxidase, and motility. Other tests were performed by Enterobacteriaceae (KB003) biochemical characterization Kit such as ONPG, lysine utilization, Urease test nitrate reduction test, hydrogen sulphide test, indole production test, Vogas proskauer test, methyl red test, and citrate utilization test.

1) Primary biochemical test

A) Colony morphology

Bacterial strains grow on solid media as colonies. A colony is defined as a visible mass of microorganisms all originating from a single mother cell, therefore a colony constitutes a clone of bacteria all genetically alike. The different isolated bacterial strains were produced different type colonies. The color, size, type, margin, height, surface, and pigmentation of different bacteria were observed by growing separately on different agar plates (chromogenic media).

A) Gram's staining

Gram's staining techniqes was discovered over 100 years ago by Dr. Hans Christian Gram, a Danish physician, in 1884. This techniqes was most commonly used for direct microscopic examination of specimens and subculture. It is very useful stain for identifying and classifying bacteria into two major groups: the gram-positive and gram-negative.

Principle:

The first step of the process involves staining with the colouring dye crystal violet. This is the primary stain. This is then treated with iodine solution, which acts as mordant; that is, it increases the interaction between the bacterial cell and the dye so that the dye is more tightly bound or the cell is more strongly stained.

The smear is decolourized by washing it with an agent such as 95% ethanol. Gram-positive bacteria retain the crystal violet-iodine complex when washed with the decolourizer, while gram-negative bacteria lose their crystal violet-iodine complex and lose color. Finally, the smear is counterstained with a basic dye, which differs in color compared to the crystal violet. This counter stain is usually safranin and used the colourless, gram-negative bacteria to pink but does not alter the dark purple colour of the gram-positive bacteria. The end result is that gram-positive bacteria are deep purple in colour and gram-negative bacteria are pinkish to red in colour.

- Make thin smear of bacterial strains on separate glass slides.
- Let the smear air dry.

- With inoculating loop, a drop of the culture was transferred on to the slide.
- Heat fixes the smears.
- The smear was fixed by passing the slide rapidly over the flame (film-side up).
- The glass slide (smears) were stained with crystal violet solution (Primary stain) for 30 second.
- The Gram's iodine solution (Mordant) was applied and allowed to react for 60 second.
- Wash with distilled water and drain carefully (Do not blot.)
- Wash with 95% alcohol (decolorizing agent) for 30 seconds.
- Wash with distilled water at the end of 30 seconds to stop the decolorization.
- Counter stain with 0.25% safranin for 30 seconds.
- Wash off the slide dries with blotting paper.
- Observed under microscope.

Observations:

- Examine the slides microscopically using oil-immersion objective.
- Identify the gram reaction of both the cultures and classify them.
- Make sketches for morphology cultures.
- Describe the morphology and arrangement of the cells.
- Gram-positive bacteria appear purple and Gram-negative bacteria appear pink or red.

2) Secondary biochemical tests

A) Catalase test

In order to survive, organisms must rely on defence mechanisms that allow them to repair or escape the oxidative damage of hydrogen peroxide (H_2O_2).

Principle:

Catalase enzyme converts hydrogen peroxide into water and oxygen. This enzyme indicates either the organism is aerobic or facultative anaerobe. Catalase neutralizes the bactericidal effects of hydrogen peroxide and therefore, the catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Its concentration in bacteria is correlated with pathogenicity.

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + O_2 \text{ (bubbles)}$$

<u>Procedure:</u>

- Fresh bacterial culture was transferred to slide with sterilized inoculating loop
- 1 drop of 3% H_2O_2 was placed onto the organism on the microscope slide.
- Oxygen bubbles are observed.

B) Motility test

Evidence for presence of bacterial flagella is obtained by observing motility.

<u>Principle:</u>

The ability to move by own is called motility flagella are present to perform this function. On the basis of presence of flagella they are called motile or non motile. In other method, bacteria are inoculated into a semi solid medium; they migrate from the inoculation site and form a characteristic pattern of growth, which indicates mobility.

<u>Procedure:</u>

- Motility media is prepared and sterilized by autoclaving at 121°C for 15 min at 15 lbs
- The motility medium is poured in test tube
- Stabbing is done into the test tube
- The test tubes are incubated for 24 to 48 hours.

4.1.1.2.1. PCR based detection and confirmation of pathogenic bacteria from the collected tannery wastewater samples

Besides the biochemical identification of different bacteria isolates were also confirmed and identified by the PCR on the basis of 16S rRNA gene sequence analysis using the specific primers as reported by the various authors for different pathogenic bacterial strains (El-Lathy et al., 2009; Chandra et al., 2011).

4.1.1.2.2. 16S rRNA gene sequencing analysis and gene-bank accession number

The total genomic DNA from isolated bacteria was extracted from the overnight grown culture using DNA isolation kit (Qiagen, Germany) and 2 µl DNA was used to amplify the 16S rRNA gene using the forward and reverse primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), respectively. The reaction mixture contained 2 µl of template DNA, 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 volume of 50 µl. The thermocycling steps (Applied Biosystems, USA) used were as follows: 35 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 product was gel purified using QIA gel extraction kit (Qiagen, Germany). Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

4.1.1.2.2. Study of antibiotic and heavy metal resistant property of bacteria isolated from tannery wastewater after secondary treatment process

4.1.1.2.2.1. Antibiotic resistance property of isolated bacterial strains

The antibiotic resistance property for the isolated bacterial strains was performed by the disk diffusion method using Muller-Hinton agar medium against the different antibiotics such as Gen: Gentamicin (10 mcg); Amp: Ampicillin (10 mcg); Ciprofloxacin (5 mcg); Le: Levofloxacin (5 mcg); T: Tetracycline (30 mcg); VA: Vancomycin (30 mcg); NX: Norfloxacin (10 mcg); S: Streptomycin (10 mcg); P: Penicillin G (10 mcg); E: Erythromycin (15 mcg). The plates were swabbed with a faintly opalescent culture, and then the antibiotic disks were applied and incubated at 32° C for 24 h (Jain et al., 2009). The inhibition zone was measured after 24 h of incubation period and the isolated bacterial strains was classified as resistant, intermediate, or sensitive based on zone size as per the standard antibiotic disc sensitivity testing method (DIFCO, 1984).

4.1.1.2.1.2. Minimum Inhibitory Concentration (MIC) of heavy metals for isolated bacterial strains

The minimum inhibitory concentration (MIC) of Cr, Cu, Zn, and Ni for the isolated bacterial strains was determined in nutrient broth amended with the increasing concentrations (0-500µg ml^{-1}) of Cr, Cu, Zn, and Ni. The stock solutions of the analytical grade salts of $K_2Cr_2O_7$, $CuSO_4$, $ZnSO_4$ and $NiCl_2$, for Cr^{6+}, Cu^{2+} Zn^{2+} and Ni^{2+} ions, respectively were prepared in Millipore water and autoclaved.

The experiment was performed in 20 ml tubes containing 10 ml of autoclaved nutrient broth supplemented with the increasing concentrations (0-1000 µg ml^{-1}) Cr^{6+}, Cu^{2+} Zn^{2+} and Ni^{2+} and 100 µl of bacterial culture followed by incubation for 48 h at 35º C and 125 rpm in shaking incubator (Jain et al., 2009; Bharagava et al., 2014). The bacterial growth was monitored by measuring the optical density at 600 nm (Dynamica, Australia). The tubes containing 10 ml of autoclaved nutrient broth supplemented with the increasing concentrations (0- 500 µg ml^{-1}) of above metal ions without bacterial culture served as control. The MIC of different toxic metals for the isolated bacterial strains was designated as the minimum concentration of metal ions at which no growth of bacteria was observed.

4.2.2. Bacterial community analysis in tannery wastewater by 16S rRNA based metagenomics analysis using Illumina platform (Next Generation Sequencing-NGS)

The tannery wastewater sample was collected from CETP (Figure. 3.2) in sterilized flask was used for the analysis of microbial community associated with tannery wastewater treatment. The samples were centrifuged to obtain activated sludge in solid form. NGS high-throughput sequencing technologies have been developed to compressively identify the microbial diversity in different samples including activated sludge, soil, wastewater and food. In this study, microbial community in contaminated wastewater samples from CETP was investigated by NGS. This study was conducted in order to elucidate the microbial community structure associated with tannery wastewater treatment.

4.2.2.1. Isolation, qualitative and quantitative analysis of gDNA

The isolation of genomic DNA from the wastewater sample was carried out using Qiagen DNA Kit. Quality of genomic DNA was checked on 0.8% agarose gel (loaded 3µl) for the single intact band. The gel was run at 110 V for 30 mins.1µl of each sample was used for determining the concentration of genomic DNA.

1.1. 16S rRNA bacterial amplification and preparation of libraries

The 16S rRNA gene in this study was amplified using the bacterial primer set V3-F (CCTACGGGNBGCASCAG) and V4-R (GACTACNVGGGTATCTAATCC).

Primers for the amplification of the V3-V4 hyper-variable region of 16S rDNA gene of bacteria and archaea were designed in Xcelris Labs ltd. These primers were synthesized in Xcelris PrimeX facility. The amplicon library was prepared using Nextera XT Index Kit (Illumina Inc.) as per the 16S metagenomics Sequencing Library preparation protocol (Part # 15044223 Rev. B).

4.2.2.2. Preparation of libraries for 2 x 250 bp Run Chemistry

The amplicon library was prepared using Nextera XT Index Kit (Illumina Inc.) as per the 16S Metagenomic Sequencing Library was prepared using standard protocol. Primers for the amplification of the V3-V4 (Prokaryote V3-Forward - CCTACGGGNBGCASCAG)-(Prokaryote V4-Reverse- GACTACNVGGGTATCTAATCC) hyper-variable region of 16S rDNA were used. The amplicon with the Illumina adaptors were amplified by using i5 and i7 primers that add multiplexing index sequences as well as common adapters required for cluster generation (P5 andP7) as per the standard illumina protocol. 1 X AMpureXP beads, checked on Agilent DNA1000 chip on Bioanalyzer2100 and quantified by Qubit Fluorometer 2.0 using Qubit dsDNA HS Assay kit (Life Technologies), purified the amplicon libraries.

4.2.2.2. Cluster generation and sequencing and data analysis

After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyzer profile, library was loaded onto Illumina platform at appropriate concentration (10-20pM) for cluster generation and sequencing. Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions on Illumina platform. The kit reagents were used in binding of samples to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment. The generated multi-million reads were trimmed and assembled using QIIME (Quantitative Insight Into Microbial Ecology) software.

4.2. Result and discussion

4.2.1. Bacteriological analysis

The bacteriological analysis of treated tannery wastewater samples has shown the presence of a diverse group of bacterial population as shown in (Table 4.1), in which the MPN value of coliforms in contaminated samples and found polluted with fecal indicator bacteria. A higher standard plat count (SPC) standard on plate count agar (PCA) method for TC counts heterotrophs ($3.0 \times 10^{5,}$ cfu/ml) was observed in tannery wastewater samples.

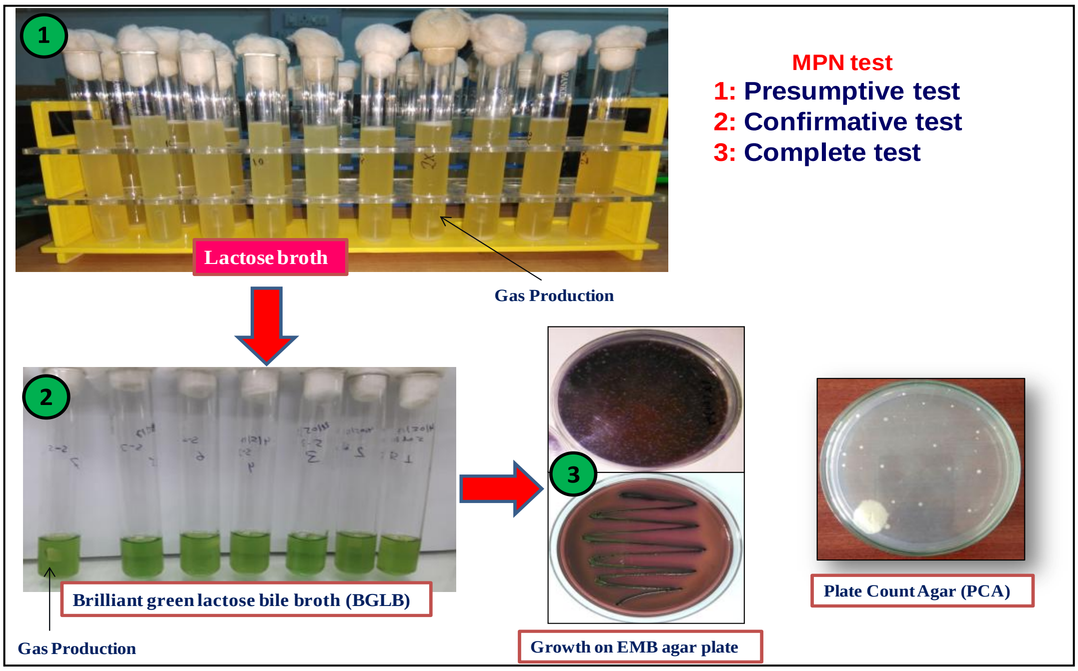


Figure 4.2: Multiple-tube fermentation test or most probable number (MPN) test performed in diffirent steps of contaminated wastewater samples for bacterial population analysis

Table 4.1: Bacterial population analysis in CETP treated tannery wastewater (cfu/ml)

Sr. no.	Bacterial Population	Tannery wastewater contaminated water samples
1.	Total heterotrophs	3.0×10^5 (cfu/ml)
2.	Total coliforms	2.1×10^5 (cfu/ml)
3.	Fecal coliforms	1.3×10^5 (cfu/ml)

In the present study coliform bacteria was also showed irregular pattern of their occurrence in treated tannery wastewater sample. The existence of other members of the Fecal Coliforms (FC) group (*Klebsiella*, *Enterobacter* and *Citrobacter*) has been reported for non-fecal origin (Chandra et al., 2006). This studied has found coliforms and fecal coliforms with pollutants in treated tannery. The presence of detected coliform bacteria such as *E.coli, Vibrio spp., and Klebsiella spp.* and *Bacillus spp.* in water and wastewater has led to several water-related diseases.

4.2.2. Isolation, purification and characterization of bacteria from contaminated wastewater samples

The bacterial strains were isolated of tannery wastewater sample collected from CETP. The isolated strains was further streaked successively on HiCrome Klebsiella selective agar plates , HiCrome vibrio agar plates, and HiCrome Bacillus agar plates for several times to get distinct and pure colonies. Four (04) bacterial strains were isolated from CETP-treated tannery wastewater samples (Figure 4.3.). The isolated bacterial strains were streaked successively for several times to get distinct and pure colonies.

The microscopic observation of the isolated pathogenic and non-pathogenic bacteria has revealed that all isolates were rod shaped, gram negative, except TWW-2, which was gram positive. Further, all isolates also showed positive reaction for motility and catalase activity as shown in Table 3. The pure bacterial strains were maintained at 4 °C and used for screening test.

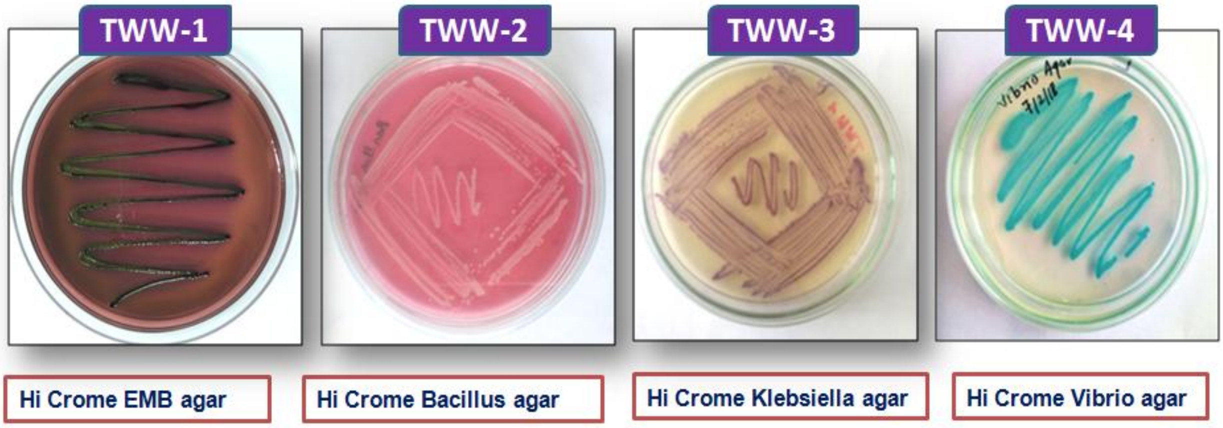


Figure 4.3: Isolation of bacteria on the basis of colony colour pigmentation on chromogonic media agar plates

4.2.2.1. Charectrization and identification of isolated bacterial strains

4.2.2.1.1. Biochemical charetерization of isilated bacerial strains

The isolates were confirmed by microscopic, cultural and standard biochemical tests (Indole, MR-VP, citrate utilization, urease, motility, catalase oxidase test, nitrate reduction, and H_2S production etc.) according to Berge's manual of determinative bacteriology (9^{th} edition, 1994) for further analysis.

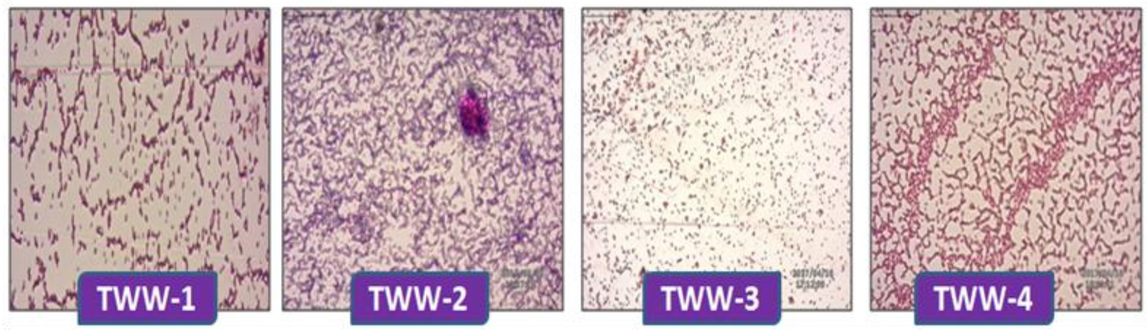


Figure 4.4: Microscopic view for gram staining of isolated bacteria strains

Herewith, based on the biochemical reactions, these bacterial strains (TWW-1, TWW-3, and TWW-4) were found to be were rod shaped, gram negative, except TWW-2, which was gram positive and all catalase positive with many other biochemical reactions as shown in Figure 4.5. & Table 4.2.

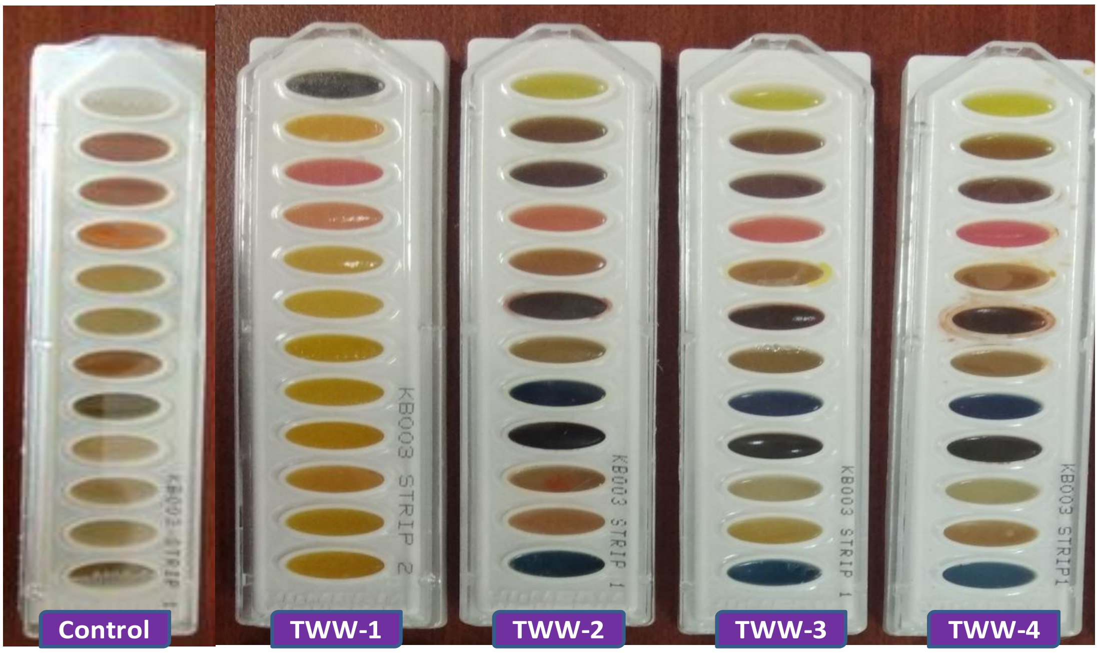


Figure 4.5: Biochemical characteristics performed on KB003 Enterobacteriaceae (KB003) biochemical characterization Kit

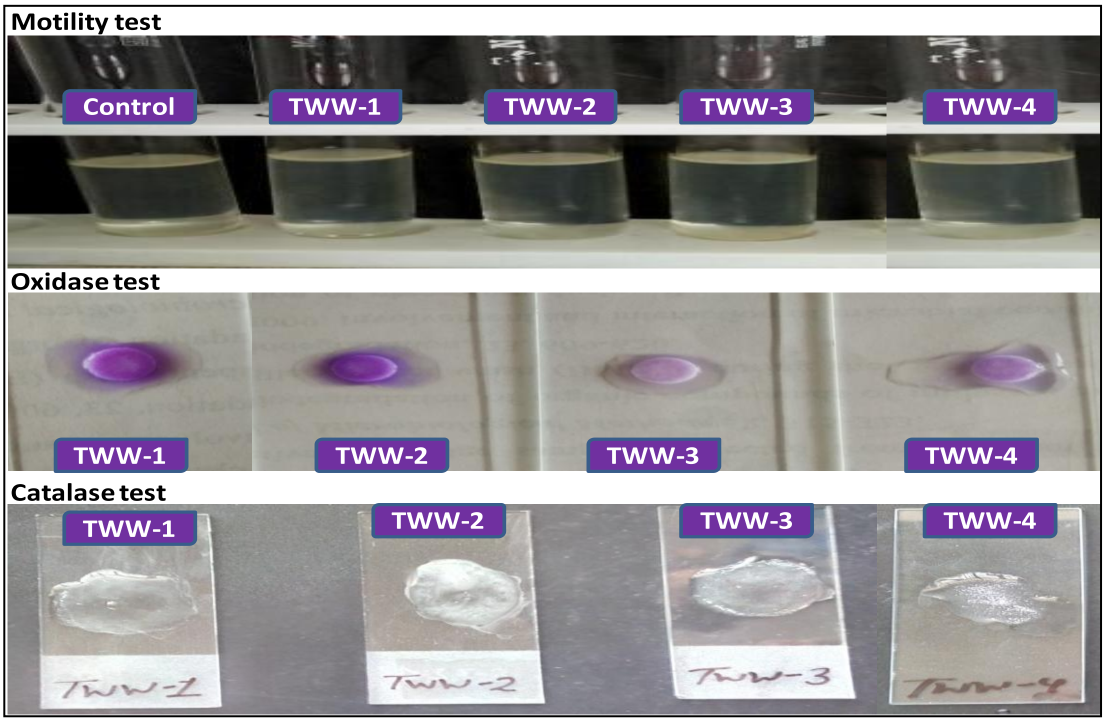


Figure 4.6: Pictorial representation of biochemical tests performed (Motility, Oxidase and Catalase test)

Table 4.2: Morphological and biochemical characteristics of isolated bacterial strains

Biochemical Characteristics	**TWW-1**	**TWW-2**	**TWW-3**	**TWW-4**
Gram reaction	-	+	-	-
Shape	rod	rod	rod	rod
Colony colour	pink	purple	pink	Pink
Motility	+	+	+	+
Catalase	+	+	+	+
Oxidase	+	+	+	+
ONPG	+	+	+	+
Lysine utilization	-	-	-	-
Urease test	+	+	+	-
Nitrate reduction	+	+	+	+
H_2S Production	-	-	-	+
Citrate utilization	+	-	+	+
V-P Test	+	-	+	+
Methyl red	+	+	+	+
Indole Production	+	-	+	+

+ = positive; - = Negative; ONPG= V-P=Voges Proskauer

4.2.2.1.2. Molecular characterization of isolated bacterial strains

The genomic DNA was PCR amplified and ~1500 bp long 16S rRNA gene were partially sequenced. Hagstrom et al., (2000) reported that a 16S rRNA sequence similarity of ≥97 % is a reasonable level for grouping the bacteria into species. Further, the PCR amplified 1500 bp long 16S rRNA gene (Figurre. 4.7) sequences of bacterial isolates TWW-2 and TWW-3 have shown the closest relatedness (99%) with *Bacillus tropicus* and *Klebsiella pneumonia*, respectively. The partial 16S rRNA gene sequences of 566 bp, and 1427 bp were submitted to GenBank and an accession number MH762877 and MH559818 were assigned to TWW-2 and TWW-3 strains, respectively.

The partial consensus nucleotide sequence of TWW-2 (566bp) and TWW-3 (1427bp) generated from forward and reverse sequence data using aligner software were used to carry out BLAST with the database of NCBI GenBank database (http://www.ncbi.nlm.nih.gov/). Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

The PCR amplification of 16S rRNA gene of isolated bacteria has been shown in (figure 4.7.) and the phylogenetic tree of TWW-2 and TWW-3 has been shown below in fig 4.8a, and 4.8b.

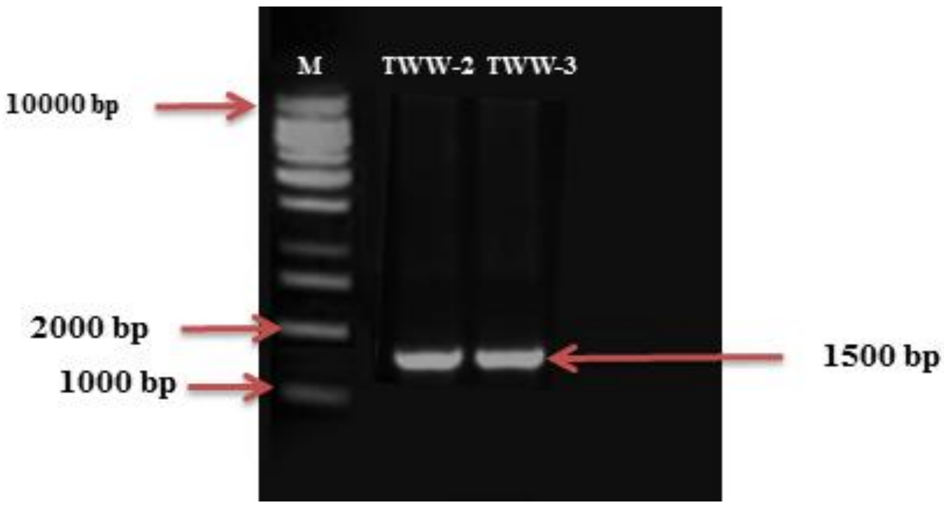


Figure 4.7: PCR amplification of 16S rRNA gene of bacterial isolates; Lane M: 1 kb Ladder Marker

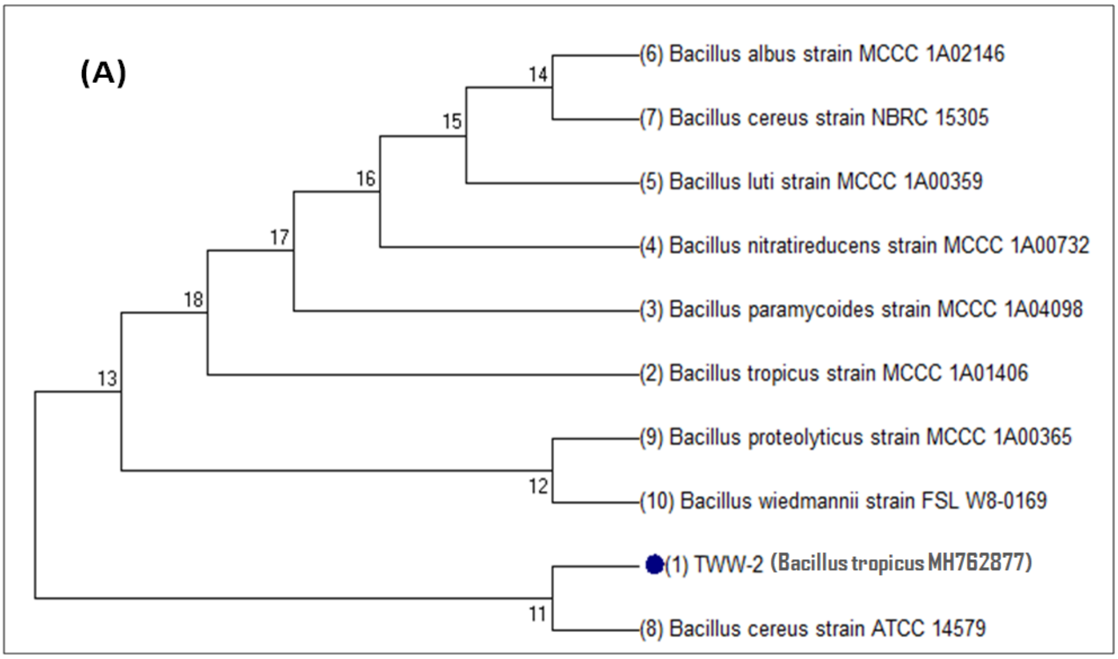


Figure 4.8a: Phylogenetic tree showing the relationship of isolated bacterium TWW-2 with its neighbouring species

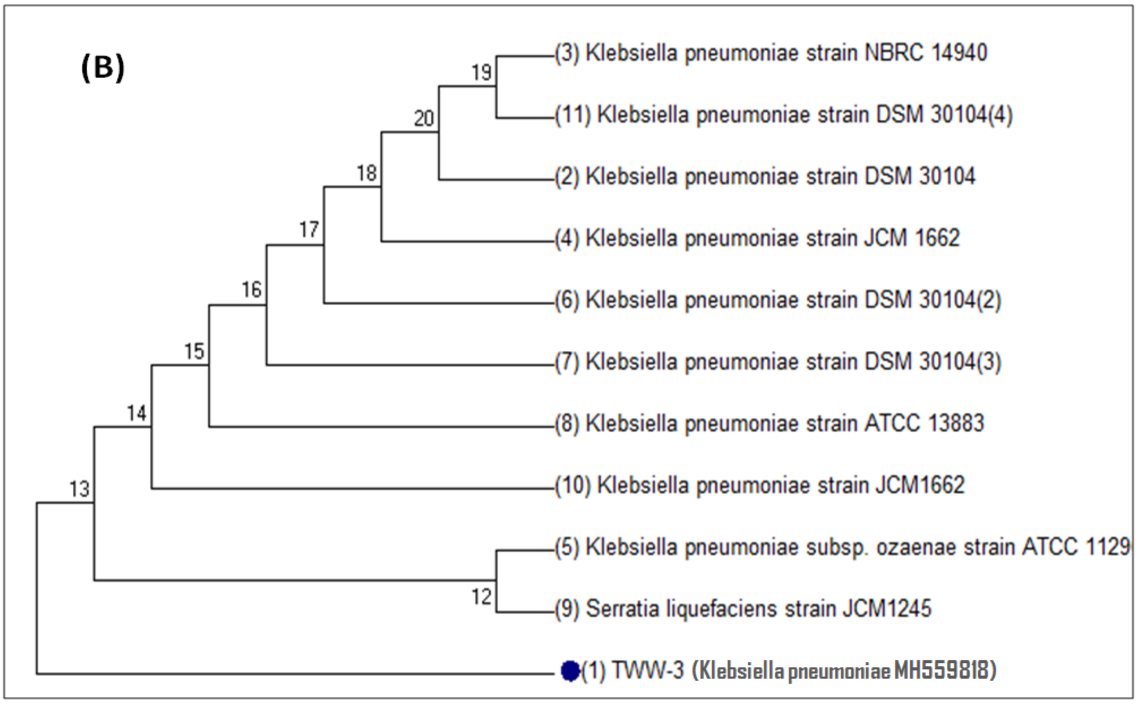


Figure 4.8b: Phylogenetic tree showing the relationship of isolated bacterium TWW-3 with its neighbouring species

2.2. Antibiotic susceptibility of isolated bacterial strains

In present study, results revealed that most of the isolates have shown resistance, sensitive and intermediate for other antibiotics used in (Figure 4.9).

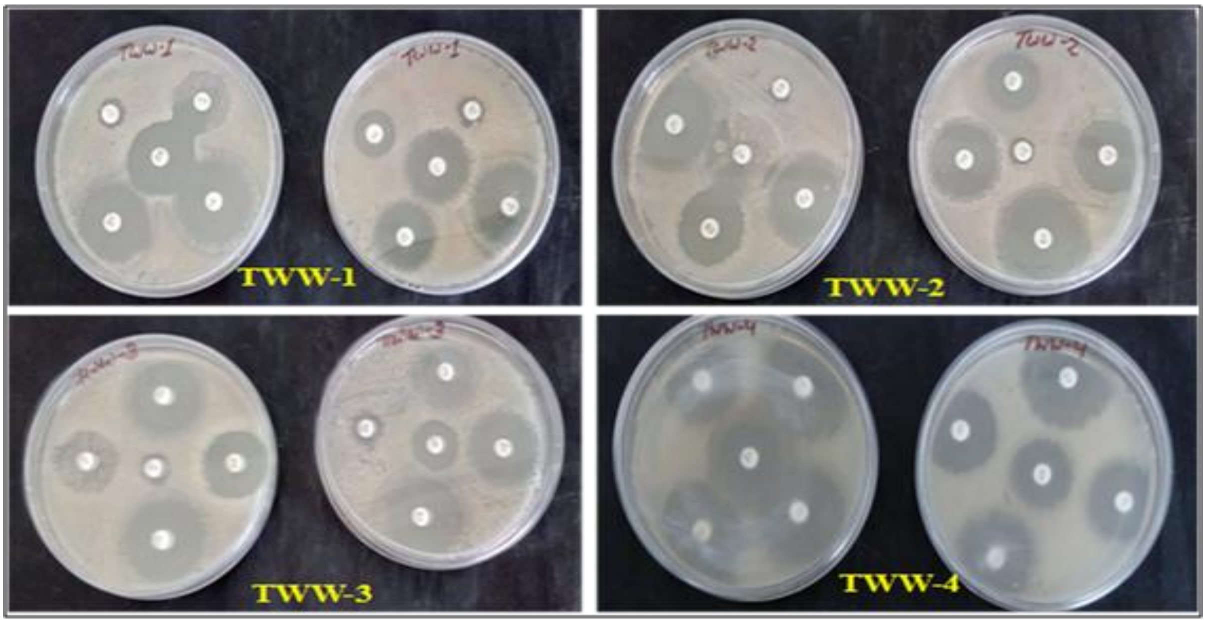


Figure 4.9: Antibiotic resistance property of bacterial isolates

The results revealed that the isolated bacterial strains was sensitive for Gen: Gentamicin (10 mcg); Amp: Ampicillin (10 mcg); Ciprofloxacin (5 mcg); Le: Levofloxacin (5 mcg); T: Tetracycline (30 mcg); VA: Vancomycin (30 mcg); NX: Norfloxacin (10 mcg); S: Streptomycin (10 mcg); P: Penicillin G (10 mcg); E: Erythromycin (15 mcg) as shown in Table 4.3 & Figure 4.9.

Table 4.3: Antibiotic resistance property of bacterial isolates

Antibiotics	TWW-1	TWW-2	TWW-3	TWW-4
GEN (10 mcg)	R	R	R	R
AMP (10 mcg)	I	S	I	R
CIP (5 mcg)	R	R	R	R
LE (5 mcg)	R	R	R	R
TE (30 mcg)	R	R	R	R
VA (30 mcg)	R	R	R	R
NX (10 mcg)	R	R	R	R
S (10 mcg)	R	R	R	R
P (10 mcg)	I	I	I	R
E (15 mcg)	S	S	R	R

This indicates that tannery wastewater is organically enriched medium and supports the fast growth and spreading of multi-drug and multi-metal resistant microbes in aquatic environments.

2.3. Minimum inhibition concentration (MIC) of tested metal ions for isolated bacterial strains

The isolated bacterial strains also showed a wide range of MIC values for tested metals ranging from (0-500 μg ml^{-1}) for Cr, Cu, Zn and Ni, respectively (Table 4.4 & Figure 4.10 a, b, c, d).

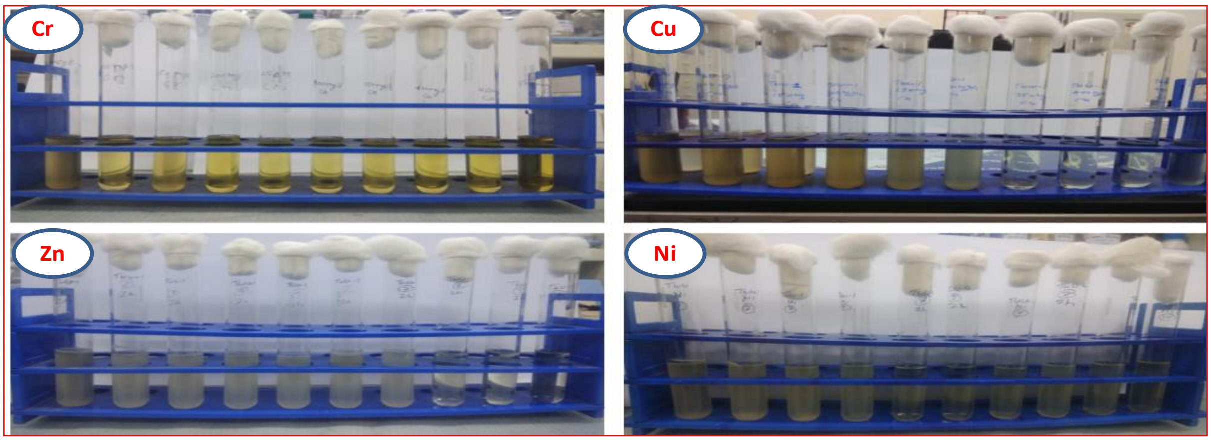


Figure 4.10a: Minimum Inhibitory Concentration (MIC) of diffirent metal ions for the isolated strain (TWW-1)

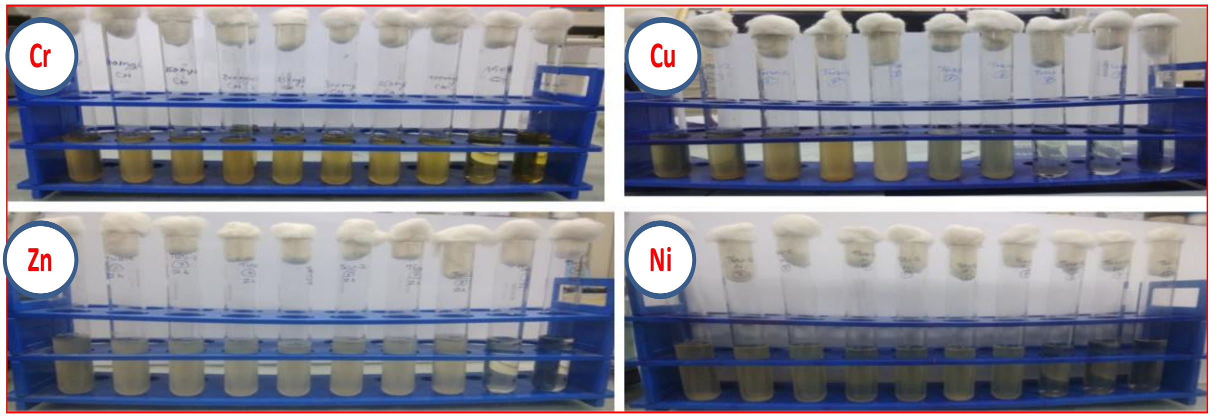


Figure 4.11b: Minimum Inhibitory Concentration (MIC) of diffirent metal ions for the isolated strain (TWW-2)

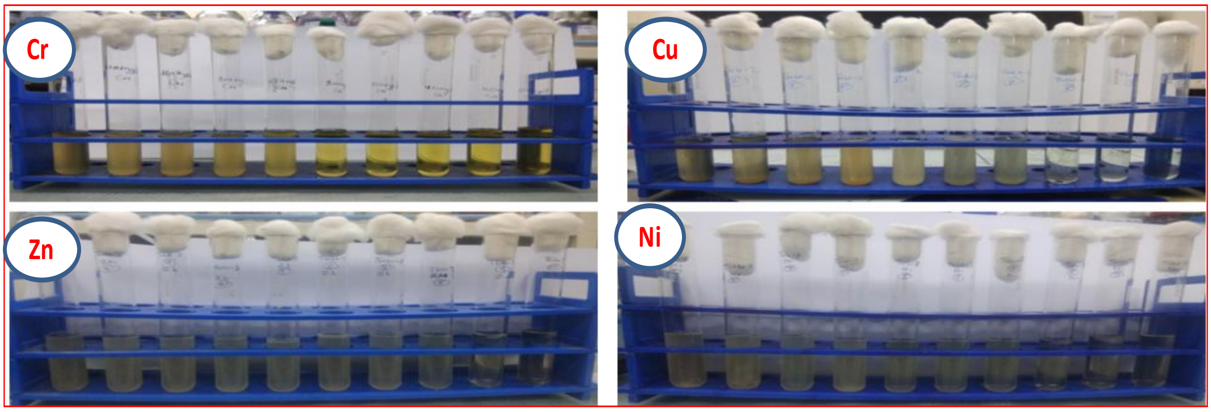


Figure 4.10c: Minimum Inhibitory Concentration (MIC) of diffirent metal ions for the isolated strain (TWW-3)

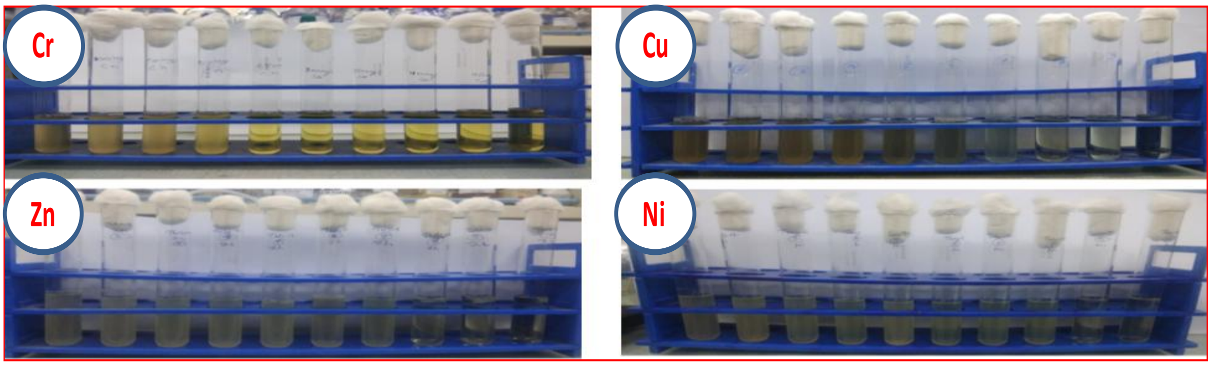


Figure 4.10d: Minimum Inhibitory Concentration (MIC) of diffirent metal ions for the isolated strain (TWW-4)

The resistance for toxic metals in bacteria probably reflects the degree of environmental contamination with toxic metals and may be directly related to the exposure of bacterial cells with the toxic metals (Dhakephalkar and Chopade, 1994). However, the unpolluted environments may also harbour metal resistant organisms or organisms that readily adapt to high concentrations of toxic metals. Malik and Jaiswal (2000) have suggested that the incidence of a high metal resistant population resulted from increasing environmental pollution. They also reported that plasmid-bearing strains are more in polluted sites than unpolluted sites. However, the bacterial resistance to heavy metals is an important consideration when bacteria are to be introduced into soils for enhancing the bioremediation of metal contaminated soils.

Although some heavy metals are required in low concentrations for normal metabolic activities. But, at elevated levels, these metals act as carcinogenic, mutagenic or teratogenic agents (Feuerpfeil et al., 1999).

Table 4.4: Minimum inhibitory concentration (MIC) of different metal ions for the isolated bacterial strains

Bacterial isolates	Minimum inhibitory concentration (MIC) (µg mL^{-1}) of various metal ions for isolated bacterial strains			
	Cr	Cu	Zn	Ni
TWW-1	50	350	350	200
TWW-2	400	300	400	350
TWW-3	250	350	350	200
TWW-4	200	300	400	250

4.2.3. Bacterial community analysis of tannery wastewater

The gDNAextracted from tannery wastewater was quantified by using 0.8% Agarose gel, in 1X TAE buffer, followed by electrophoresis by 110 volt D.C. for 30 minutes (Fig. 4.11) lane 1). The quantification of gDNA was done using Qubit Fluorometer dsDNA HS Assay and the recorded yield was 2.6 µg and A260/280 OD was 1.89.

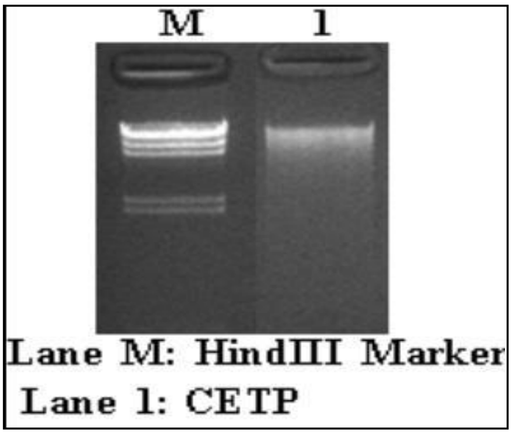


Figure 4.11: gDNA quantification on 0.8% agarose; 1X TAE buffer

4.2.3.1. Bacteria composition in tannery wastewater

Total number of amplicon sequences reads obtained from tannery wastewater was 1,113,718 reads. The operational taxonomy unit (OTU) recorded was 9,525.

4.2.3.1.1. Phyla of associated bacteria in tannery wastewater

Taxonomic analysis of the V3 16S rRNA gene amplicon reads yielded a total of eighteen classifiable phyla (Fig.4.12a). Five of which, were dominant in the entire sample namely; *Firmicutes* (32.53%), *Bacteroidetes* (24.18%), *Proteobacteria* (12.51%), *Synergistetes* (10.35%) and *Chloroflexi* (7.65%) and *Thermotogae* (3.38%). While, the highest percentage of *Firmicutes* observed was observed in the tannery wastewater sample. From the figure it can be inferred that Firmicutes is most abundant followed by *Bacteroidetes*.

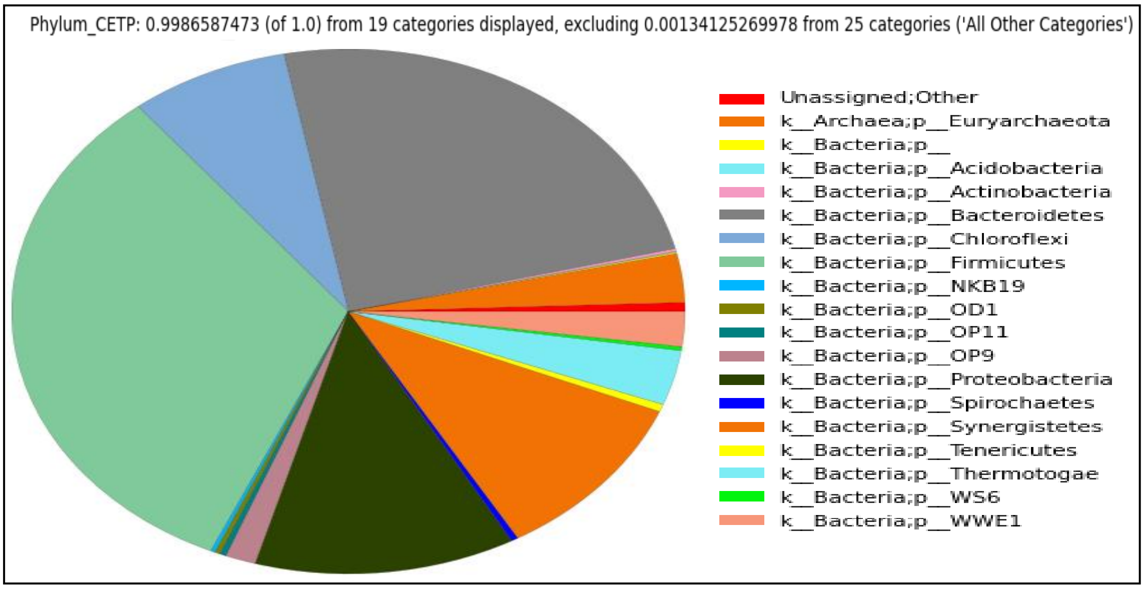


Figure 4.12a: Pie-chart of sample CETP showing the relative abundance of each phylum within each bacterial community.

4.2.3.1.3. Distribution of bacterial community at class level

The distribution of bacterial community at class level in this study, a total of 19 different classes of bacteria was identified in this study (Fig 4.12b). However, most of these classes were about 1% or less of the total bacterial isolated, hence, limiting their resolution (Fig. 4.12b). In general, the *Clostridia* represented about 30.95%. While the *Bacteroidia* and *Synergistia* were found at 22.36% and 10.35%. *Synergistia* (10.35%) in the phyla Prote-bacteria were detected at 2.6% and less than 1%, respectively. *Gammaproteobacteria* and *Anaerolineae* were also found at more than 5%. However, Thermotogae, *Methanomicrobia* and *Cloacamonae* bacteria were found at less than 5%.

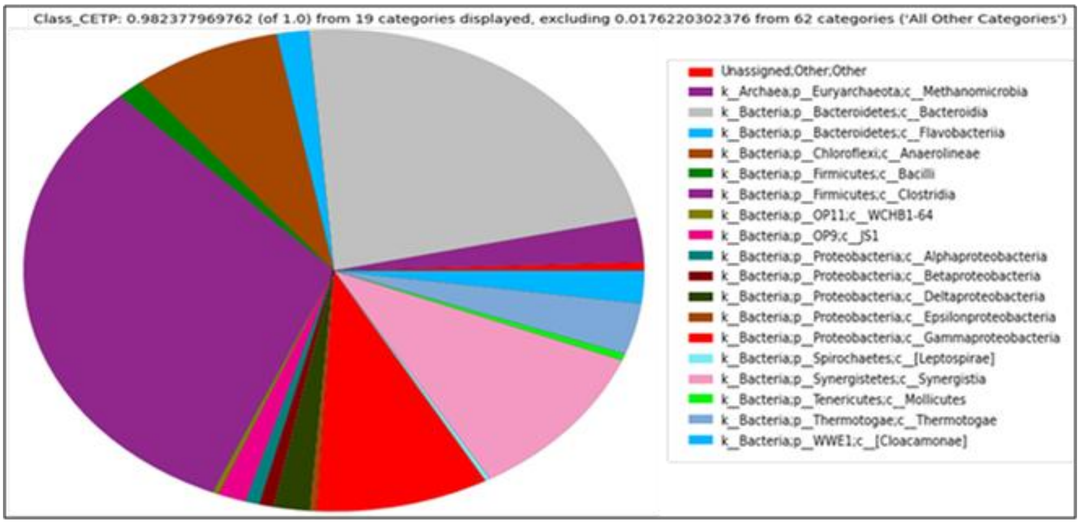


Figure 4.12b: Pie-chart of sample CETP showing the relative abundance of each class within each bacterial community

4.2.3.1.3. Distribution of bacterial community at Order level

To assess the microbial composition, the most abundant class observed; *Clostridia* were categorized into different order level (Fig. 4.12c). The *Gammaproteobacteria*, were the largest chunk of *Proteobacteria* observed in this study, and with the largest number of orders isolated. The *Clostridiales* in all the sample groups were dominated by the Burkholderiales, While, the *Bacteroidales* (22.36%), *Synergistales* (10.35%), *Anaerolineales* (7.60%), *Oceanospirillale* (4.06%) *Thermotogales (3.38*%), *Alteromonadales* (2.87%), *Methanosarcinales (2.76%)* and *Cloacamonales (2.14%)* are orders found in the tannery wastewater sample. These two phyla were earlier reported to be among the dominant isolated phylum after *Proteobacteria* in this study.

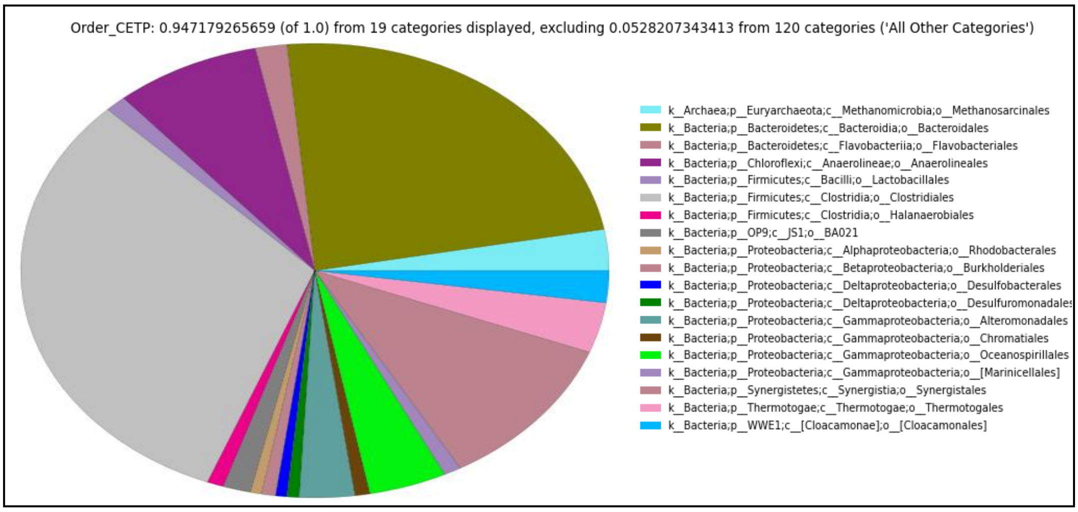


Figure 4.12c: Pie-chart of sample CETP showing the relative abundance of each order within each bacterial community

4.2.3.1.4. Distribution of bacterial community at Family level

A total of 19 families of bacteria were identified in the wastewater (CETP) sample respectively (Fig.5.12d). However, nine bacteria high families (52.97%) were found in CETP sample. While, the *Tissierellaceae* (13.40%), *Clostridiaceae* (8.89%), *Anaerolinaceae* (7.60%). *Porphyromonadaceae* (5.29%), *Dethiosulfovibrionaceae* (4.84%), *Halomonadaceae* (3.67%), *Thermotogaceae* (3.38%), *Thermovirgaceae* (3.15%) and *Methanosaetaceae* (2.75%) are families found in the CETP wastewater sample.

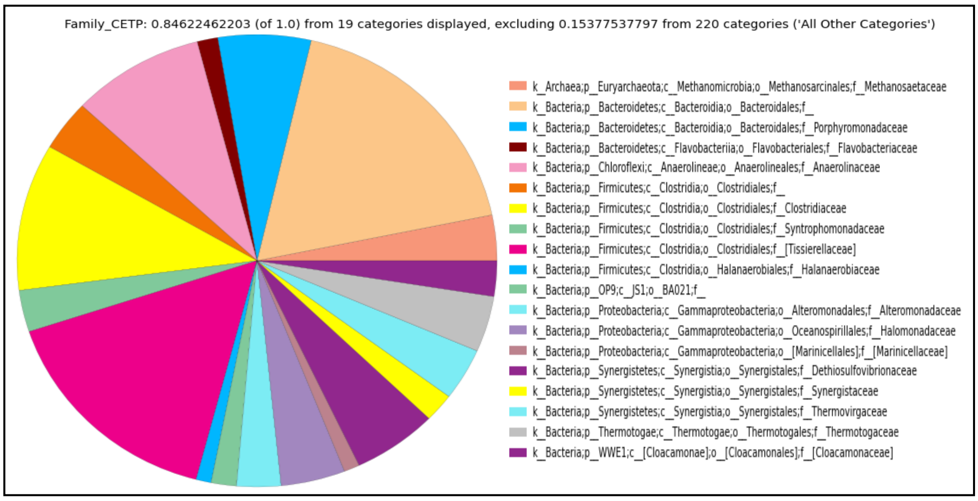


Figure 4.12d: Pie-chart of sample CETP showing the relative abundance of each genus within each bacterial community

4.2.3.1.5. Distribution of bacterial community at Genus level

A total of 19 Genus of bacteria were identified in the wastewater (CETP) respectively (Fig.4.2e). However, ten bacteria high families (41.48%) were found in CETP sample. While, the Tissierella_Soehngenia (7.90%), T78 (7.20%), Proteiniclasticum (5.58%). HA73 (3.97%), Halomonas (3.60%), Clostridium (2.83%), Methanosaeta (2.75%), ecb11 (2.64%) Syntrophomonas (2.52%) and Marinobacter (2.49%) are families found in the CETP wastewater sample.

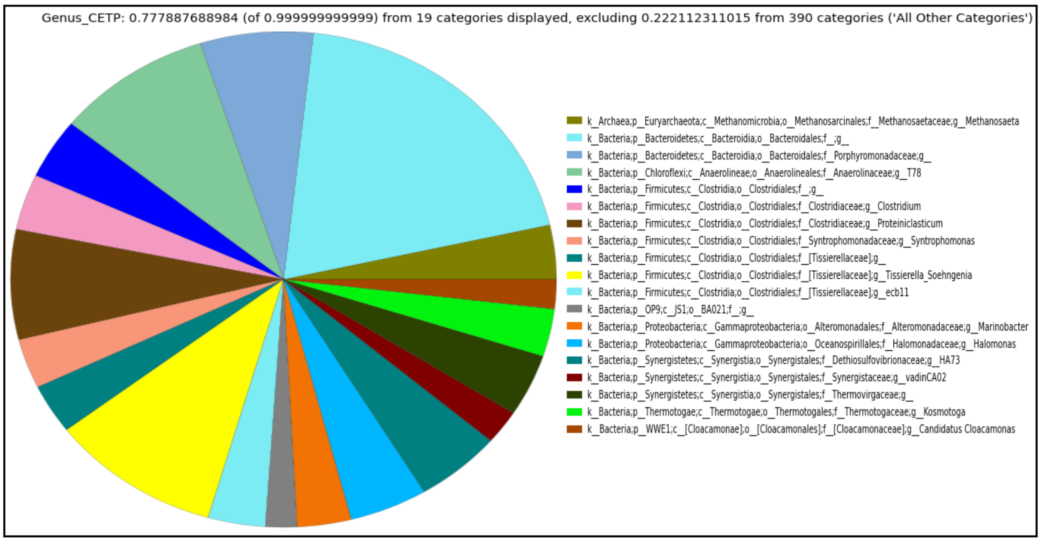


Figure 4.12e: Pie-chart of sample CETP showing the relative abundance of each genus within each bacterial community

4.2.3.2. α-Diversity

α-Diversity or within-sample diversity is calculated using QIIME in term of OUT richness. Alpha diversity summarizes the diversity or richness of organisms in a sample with a single number using different metrics (observed species, and chao1). The below table summarizes the α-Diversity, where the columns correspond to alpha diversity metrics and the rows correspond to samples and their calculated diversity measurements.

Sample	shannon	observed species	chao1
CETP	8.33399415033	4628.0	4628.0

4.2.3.3. Rank Abundance Plot

The rank abundance curve representing species richness and species evenness is shown in Figure below. Species richness can be viewed as the number of different species on the chart and species evenness is derived from the slope of the line that fits the graph. A steep gradient indicates low evenness as the high ranking species have much higher abundances than the low ranking species. The curve is a 2D chart with relative abundance on the Y-axis, usually measured on a log scale, and the abundance rank on the X-axis. The most abundant species is given rank.

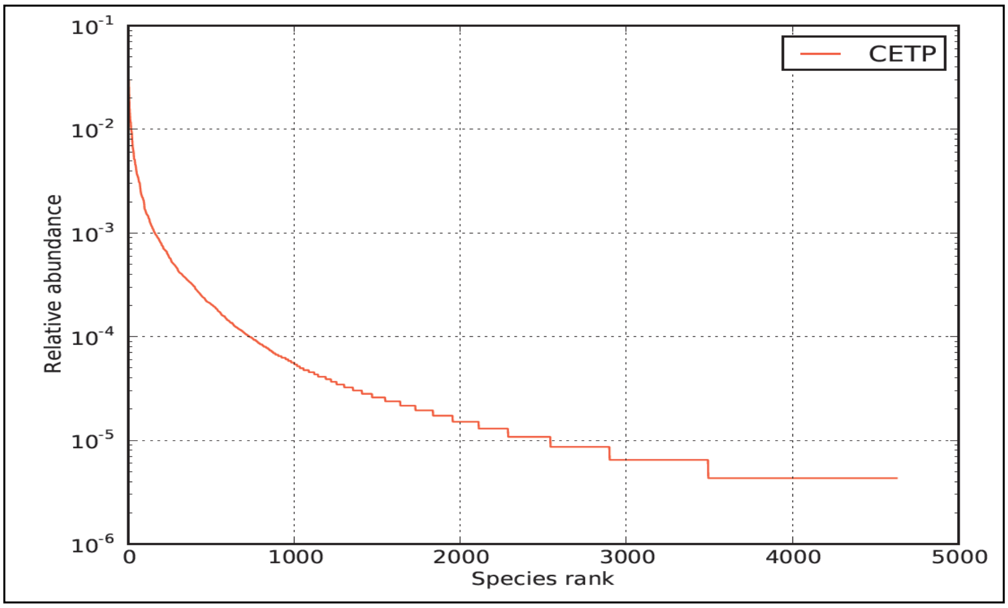


Figure 4.13: Rank abundance plot of CETP Samples

4.2.3.2. *OUT (Operational Taxonomic Unit) Abundance Heatmap*

In these heatmap each row corresponds to an OTU, and each column corresponds to a sample. The higher the abundance of an OTU in a sample, the more intense the color at the corresponding position in the heatmap. By default, the OTUs (rows) were clustered by UPGMA hierarchical clustering, and the samples (columns) were presented in the order in which they appear in the OTU table (Fig. 13).

Consensus Lineage	CETP	#OTU ID
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__;s__	2360	denovo0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__;s__	9784	denovo248
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Tissierella_Soehngenia;s__	2579	denovo285
k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__T78;s__	3978	denovo444
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__;s__	2106	denovo511
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae;g__HA73;s__	4470	denovo625
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae;g__HA73;s__	3180	denovo869
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae;g__vadinCA02;s__	4310	denovo995
k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__T78;s__	14618	denovo1077
k__Bacteria;p__WWE1;c__[Cloacamonae];o__[Cloacamonales];f__[Cloacamonaceae];g__Candidatus Cloacamonas;s__	3027	denovo1813
k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__T78;s__	4765	denovo1817
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__[Marinicellales];f__[Marinicellaceae];g__Marinicella;s__	2087	denovo1955
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__ecb11;s__	5702	denovo2145
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__;s__	5870	denovo2148
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Ulvibacter;s__	2354	denovo2391
k__Bacteria;p__Thermotogae;c__Thermotogae;o__Thermotogales;f__Thermotogaceae;g__SC103;s__	2771	denovo2495
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Proteiniclasticum;s__	2205	denovo3237
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Halomonadaceae;g__Halomonas;s__campisalis	3935	denovo3554
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Tissierella_Soehngenia;s__	4659	denovo3651
k__Bacteria;p__OP9;c__JS1;o__BA021;f__;g__;s__	2065	denovo3914
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__;s__	9563	denovo4930
k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanosarcinales;f__Methanosaetaceae;g__Methanosaeta;s__	12650	denovo5222
k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__T78;s__	3119	denovo5506
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__;s__	2245	denovo5537
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Proteiniclasticum;s__	6584	denovo5867
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__;s__	21517	denovo5938
k__Bacteria;p__Thermotogae;c__Thermotogae;o__Thermotogales;f__Thermotogaceae;g__Kosmotoga;s__mrcj	7129	denovo6010
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__;s__	2900	denovo6024
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Proteiniclasticum;s__	2107	denovo6303
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Syntrophomonadaceae;g__Syntrophomonas;s__	5380	denovo6333
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__ecb11;s__	2394	denovo6443
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__	2380	denovo6573
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__;s__	4560	denovo6613
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__;s__	2359	denovo6651
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Tissierella_Soehngenia;s__	15316	denovo6670
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__;s__	7720	denovo7219
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Halomonadaceae;g__Halomonas;s__	3012	denovo7314
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Tissierella_Soehngenia;s__	5382	denovo7358
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Thermovirgaceae;g__;s__	5878	denovo7797
k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__T78;s__	2468	denovo7975
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae;g__Marinobacter;s__	4428	denovo8031
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__;s__	2845	denovo8085
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Proteiniclasticum;s__	5478	denovo8514
k__Bacteria;p__Thermotogae;c__Thermotogae;o__Thermotogales;f__Thermotogaceae;g__Kosmotoga;s__mrcj	3194	denovo9103
k__Bacteria;p__OP9;c__JS1;o__BA021;f__;g__;s__	3684	denovo9159
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae;g__HA73;s__	7376	denovo9347

Figure 4.14: An OTU table heat map showing taxonomy assignment for each OUT from sample. The OTU heatmap displays OTU counts per sample, where the counts are colored based on the contribution of each OTU to the total OTU count present in the sample (blue: contributes low percentage of OTUs to sample; red: contributes high percentage of OTUs). The table based on taxonomy assignment is filtered the OTU table by number (2000) of counts per OTU.

4.2.3.3. Krona graph

Krona graph displays abundance and hierarchy simultaneously using a radial space-filling display. The Krona chart features a red-green colour gradient signifying average value within each taxon. Krona graph was plotted with Krona-Tools-2.7 using taxonomy summary provided by QIIME. Below krona graph is provided at order level, however krona for at other level can be plotted using HTML file provided in data deliverables folder.

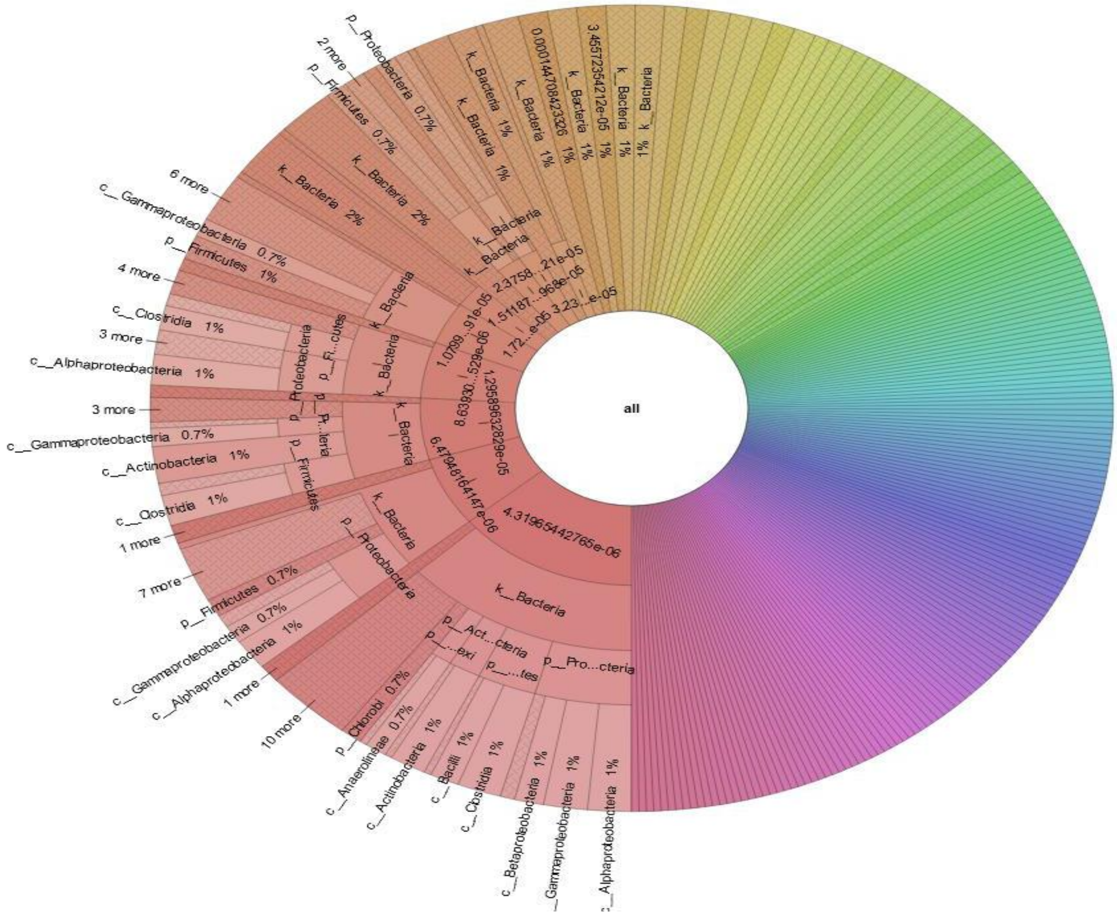


Figure 4.15: The Krona graph showing the relative abundance of CETP sample at class level

Chapter 5

Detection and characterization of residual organic pollutants from tannery wastewater after secondary treatment process by using different solvents system and HPLC/ GC-MS/MS analysis

Detection and characterization of residual organic pollutants from tannery wastewater after secondary treatment process by using different solvents system and HPLC/ GC-MS/MS analysis.

5.1. Introduction

Leather industries are one of the well-developed economic sectors in many developing countries including India. But, these industries are also the major source of environmental pollution due to discharge of huge volume of potentially toxic and hazardous wastewater, which creates negative impact of leather industries in public society (Lofrano et al. 2013; Chowdhury et al. 2015; Bharagava et al. 2017; Priac et al. 2017). Thus, making the wastewater unfit for irrigation and aquatic life (Bharagava and Mishra 2016; Chowdhary et al. 2018). Most of tanneries are small-scale industries and cannot afford to Effluent Treatment Plants (ETPs) to treat the generated wastewater.

The continuous discharge of the tannery wastewater into the environment is of serious eco-toxicological concerns (Matsumoto et al., 2006; Bharagava and Mishra, 2018). The chemicals used in tannery for the tanning process include synthetic organic pollutants like tannins, phthalates, phenolic compounds, azo dyes, surface active compounds, pesticides, sulphonated oils and grease that are not completely degraded through secondary CETP and are released untreated. Continuous releases of residual organic pollutants in tannery wastewater into the Ganga River through the drains have been a growing environmental concern (Verma et al., 2001; Tare et al., 2003; Alam et al., 2009; Chandra et al., 2009) and require urgent attention for the protection of environment and human health. The nature and characteristics of the residual organic pollutants in tannery wastewater, which are not significantly degrade during the secondary treatment process at CETP, have yet to fully investigated.

Hence, there is an urgent need of the detection and characterization of residual organic pollutants remained in treated tannery wastewater for the development of an effective treatment process as well as the toxicity evaluation for the safety of environment, human and animal's health. Thus, the present study aimed to characterize and identify the residual organic pollutants remained in tannery wastewater even after the secondary treatment process carried out at CETP.

5.2. Material methods

5.2.1. Characterization and identification of residual organic pollutants from treated tannery wastewater

5.2.2. Extraction of residual organic pollutants from collected tannery wastewater by using different solvents systems

The extraction of residual organic pollutants from wastewater was performed by liquid-liquid extraction (LLE) method using different combination of organic solvents (Minuti et al., 2006). Briefly, centrifuged (8000 x g for 20 min at 4º C) samples (200 ml) were acidified to pH=2.0 using 1N HCl and then extracted three times with the equal volume of solvent system containing 100 ml of dichloromethane (DCM) and 100 ml of diethyl ether (solvent system-1) and 100 ml of dichloromethane (DCM) and 100 ml of n-haxane (solvent system-2) in a separating funnel (500 ml) by intermittent shaking (Haq et al., 2017). The solvent layer containing residual organic pollutants was separated and evaporated to dryness under vacuum at 40º C. The extracts were dissolved in 2 ml of DCM and passed through syringe filter (0.22 µm). All the organic solvents used were of HPLC grade (purity >99%).

5.2.3. GC-MS/MS analysis

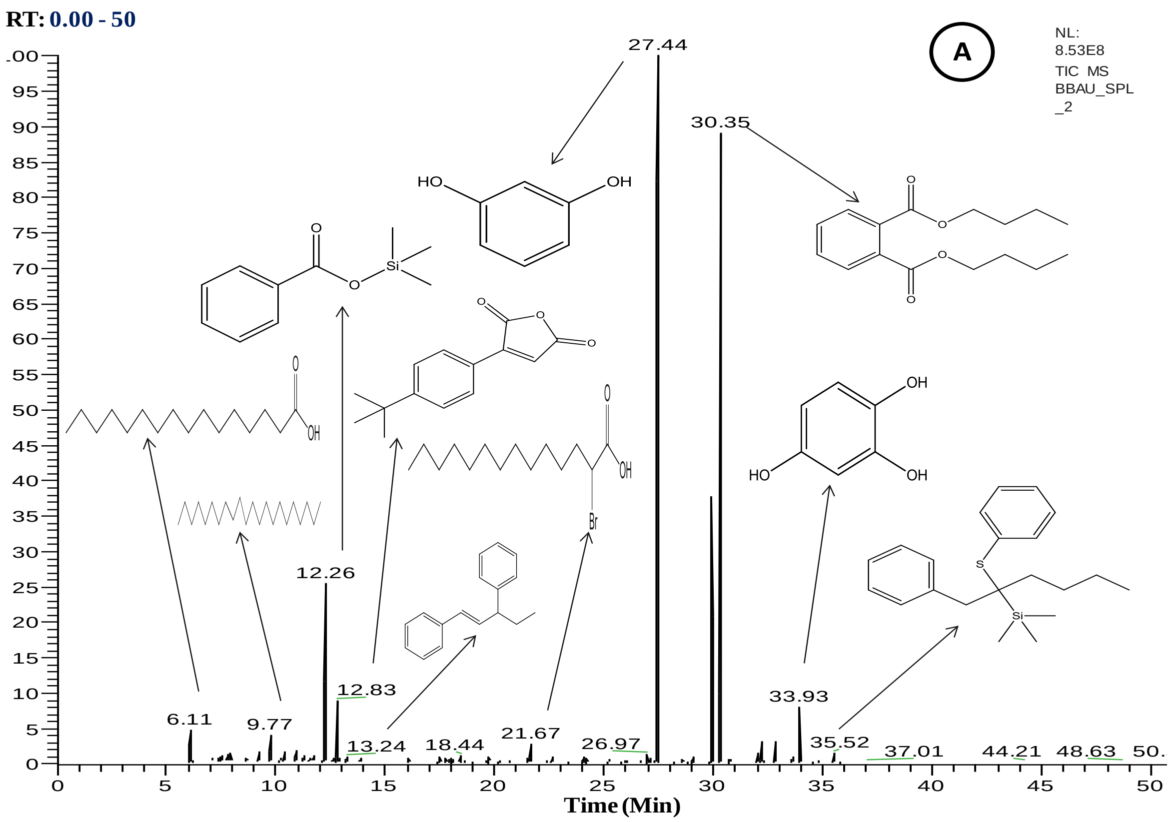
The extracts were derivatized using trimethyl silyl (BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) TMCS) at 60º C for 15 min (Marco et al., 2007). Silylated samples (1 μl) were injected in GC-MS (PerkinElmer, Waltham, MA, USA) equipped with a PE auto system XL gas chromatograph interfaced with a Turbomass mass spectrometric mass selective detector. Helium gas was used as carrier gas with flow rate of 1 ml min^{-1} in column, which was programmed as: 50º C (5 min); 50-300º C (10º C min^{-1}, hold time: 5 min). The electron ionization (EI) mass spectrum was recorded in full-scan mode in the range of 30-550 (*m/z*) at 70 eV. To identify the compounds, the mass spectra of peaks were compared with that of National Institute of Standards and Technology (NIST) library available with the equipment.

5.3. Result and discussion

5.3.1. Residual organic pollutants (ROPs) present in treated tannery wastewater

Tannery wastewater has complex mixture of various chemicals pollutant, which is not possible to extract by single solvent extraction method. Therefore, combinations of different solvent system were used to extract majority of organic pollutants through GC-MS analysis. The ROPs identified by GC-MS in the extract of DCM-diethyl ether (1:1) using NIST library were mainly the derivatives of fatty acids and organic acids (Figure 5.1A & Table 5.1).

In GC-MS analysis, various major peak were observed and identified ROPs at different retention time (RT) viz., RT 6.11 (hexadecanoic acid), 9.77 (Docosane), 12.26 (benzoic acid), 12.83 (3-[4'-(T-Butyl) Phenyl] furan-2-5-dione), 18.44 (benzeneacetamide), 27.44 (resorcinol), 30.35 (dibutyl phthalate), 33.93 (benzene 1,2,4 triol), and 35.52 (1-phenyl-2-phenylthio), respectively. Minor peaks at RT 13.24 (1-pentene1,3-diphenyl), 21.67 (2-bromotetradecanoic acid), 26.67 (phosphoric acid), 37.01 (9-octadecanoic acid), 44.21 (octadecanoic acid) and 48.63 (monopalmitin), respectively.



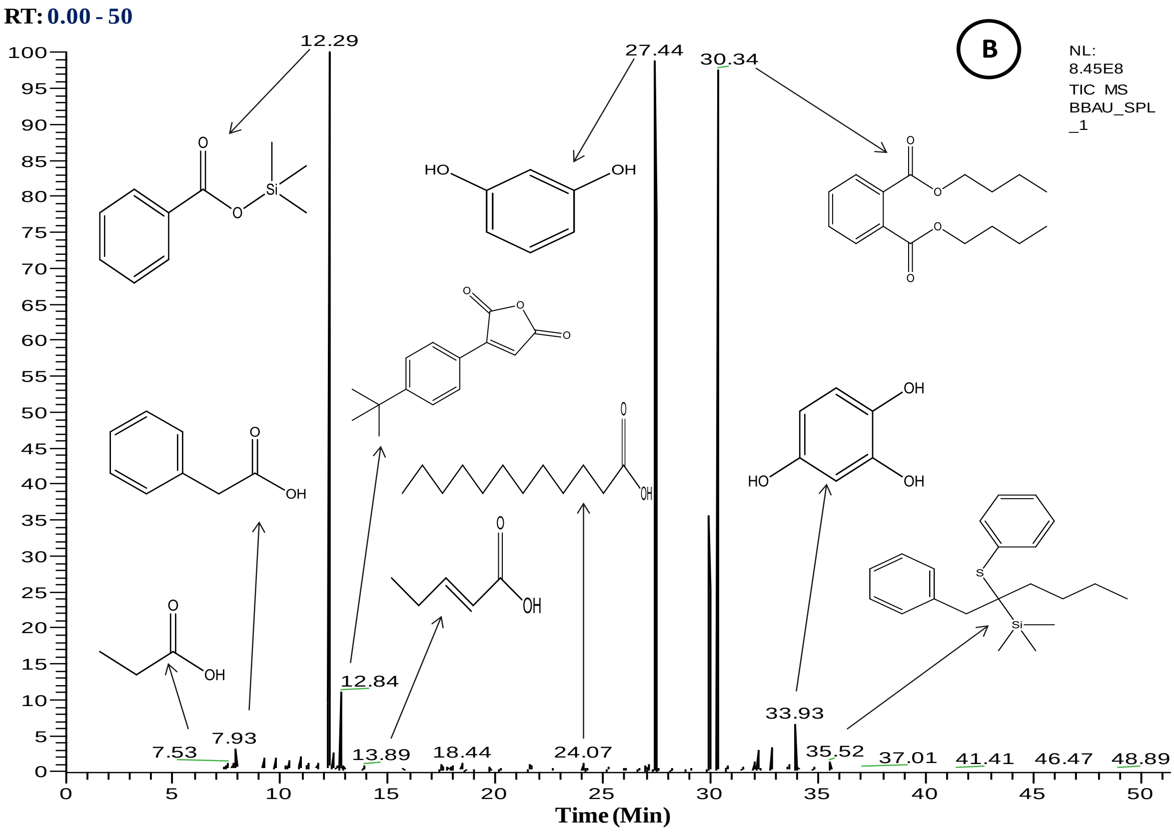


Figure 5.1: GC-MS chromatogram of dichloromethane + diethyl either (A) and dichloromethane + n-hexane (B) extracts of CETP treated TWW showing the presence of various residual organic pollutants (ROPs).

Table 5.1: Residual organic pollutants identified as TMS (Trimethylsilyl) derivatives by GC-MS/MS analysis of CETP treated tannery wastewater extracted with solvent system containing dichloromethane + diethyl either

Retention time (min)	Molecular formula	Identified residual organic compounds
6.11	$C_{19}H_{40}O_2$	Hexadecanoic acid
9.77	$C_{22}H_{46}$	Docosane
12.26	$C_{10}H_{14}O_2$	Benzoic acid
12.83	$C_{14}H_{14}O_3P$	3-[4'-(T-Butyl)Phenyl]furan-2-5-dione
13.24	$C_{20}H_{26}O$	1-pentene,1,3-diphenyl
18.44	C_6H_9NO	Benzeneacetamide
21.67	$C_{14}H_{27}BrO_2$	2-Bromotetradecanoic acid
26.97	H_3PO_4	Phosphoric acid
27.44	$C_{12}H_{22}O_2$	Resorcinol
30.35	$C_{16}H_{22}O_4$	Dibutyl phthalate
33.93	$C_{15}H_{30}O_3$	Benzene 1,2,4 triol
35.52	$C_{12}H_{32}O_2$	1-Phenyl-2-phenylthio
37.01	$C_{18}H_{34}O_2$	9-octadecanoic acid
44.21	$C_{18}H_{36}O_2$	Octadecanoic acid
48.63	$C_{25}H_{54}O_4P$	Monopalmitin

Further, GC-MS analysis of extracts of DCM-n-hexane (1:1) solvent system showed the presence of fatty acids and carboxylic acids (Fig. 5.1B & Table 5.2). The major peaks at RT 7.53 (propanoic acid), 7.93 (banzeneacetic acid), 12.29 (benzoic acid), 12.84 (3-[4'-(T-Butyl) Phenyl]furan-2-5-dione), 13.89 (2-pentenoicacid), 18.44 (benzeneacetamide), 24.07 (dodecanoic acid), 27.44 (resorcinol), 30.34 (dibutyl phthalate), 33.93 (benzene 1,2,4 triol) and 35.52 (1-phenyl-2-phenylthio) were identified compunds, respectively. Compounds at minor peaks at RT 37.01, (9-octadecanoic acid), 41.41 (10-undecynoic acid), 46.47 (Docosanoic acid 1,2,3-propanetriyl) and 48.89 (acetic acid) were identified, respectively.

The detection of compunds in treated tannery wastewaters is clearly indicates (Table 5.1) recalcitrant nature of the compounds as these were not degraded completely during the secondary treatment at CETP and get discharged into the environment along with the wastewater (Chandra et al., 2011).

Table 5.2: Residual organic pollutants identified as TMS (Trimethylsilyl) derivatives by GC-MS/MS analysis of CETP treated tannery wastewater extracted with solvent system containing dichloromethane + n-hexane

Retention time (min)	Molecular formula	Identified residual organic compounds
7.53	$C_9H_{20}O_2$	Propanoic acid
7.93	$C_8H_8O_2$	Benzeneacetic acid
12.29	$C_{10}H_{14}O_2$	Benzoic acid
12.84	$C_{14}H_{14}O_3P$	3-[4'-(T-Butyl)Phenyl]furan-2-5-dione
13.89	$C_{18}H_{38}O_3$	2-pentenoic acid
18.44	C_6H_9NO	Benzeneacetamide
24.07	$C_{12}H_{24}O_2$	Dodecanoic acid
27.44	$C_{12}H_{22}O_2$	Resorcinol
30.34	$C_{16}H_{22}O_4$	Dibutyl phthalate
33.93	$C_{15}H_{30}O_3$	Benzene 1,2,4 triol
35.52	$C_{12}H_{32}O_2$	1-Phenyl-2-phenylthio
37.01	$C_{18}H_{34}O_2$	9-octadecanoic acid
41.41	$C_{12}H_{32}O_2$	10-undecynoic acid
46.47	$C_{69}H_{134}O_6$	Docosanoic acid, 1,2,3-propanetriyl
48.89	$C_{19}H_{38}O_2$	Acetic acid

Fatty acids such as hexadecanoic acid, dodecanoic acid, and octadecanoic acid might be originated as a result of processing of raw hide/skins (Saxena et al., 2016). Phthalate (such as dibutyl phthalate: DBP), benzoic acid and resorcinol are used as plasticizers to increase the flexibility and pliability of leather products as biocide in raw hide/skins preservation and as surfactants, respectively in leather industries (Lyche et al., 2009; TFL, 2010; Dixit et al., 2015).

Phthalates such as, dibutyl phthalate, diethyl phthalate, butylbenzyl phthalate, and diethylhexyl phthalate and phthalic acid have been listed as priority pollutants by USEPA (Lyche et al. 2009; He et al., 2015). These compounds create serious toxicological effects in aquatic organisms, such as animals and fishes etc. as result of bioaccumulation and thus cause toxic, genotoxic effects, and endocrine disruption, as well as disturb the antioxidant defense system (Alam et al. 2009; Chen et al. 2014; Venali et al. 2016; Saxena et al., 2016). Benali et al. (2016) also reported that phthalate bioaccumulation leads to genotoxic effects, endocrine disruption, disruption of antioxidant defense system in plants and human being. Phenolic compounds and phthalates are reported as potential endocrine disrupting chemicals (EDCs).

Benzoic acid, a well known EDCs was detected in the tannery wastewater byin GC-MS analysis (William et al. 2017; Lyu et al. 2018). It has been classified as a Group B2, a probable human carcinogen and higly toxic to aquatic organisms (Kumari et al., 2016; USEPA, 2012). Benzene is known carcinogen was also observed and its presence in tannery wastewater might be associated with the use of phthalate and phenolics compounds in leather industries (Lyche et al., 2009; USEPA, 2014; Dixit et al., 2015) (Table 5.2). Further, recently classified EDCs such as resorcinol, hexadecanoic acid, and octadecanoic acid were also detected in tannery wastewater (USEPA, 2012).

The result of the present studies suggests that combination of DCM + n-hexane organic solvents was able to extract maximum number of ROPs and thus, might be useful in the extraction of organic pollutants from tannery and other wastewater. Previous studies have indicated that the combination of different solvents such as dichloromethane, chloroform, n-hexane, acetonitrile, acetone, and ethyl acetate may be most suitable for the extraction of most of the organic pollutants from wastewaters (Masood and Malik, 2013; Alam et al. 2010; 2009).

In the last few years, leather-tanning has adapted an eco-friendly, non/less toxic and biodegradable chemicals as per strict regulations to limit the pollution level. However, the quality of treated wastewater has not yet improved, which is apparent from present study.

Chapter 6

Toxicity assessment of tannery wastewater after secondary treatment process by using terrestrial and aquatic test models

Toxicity assessment of tannery wastewater after secondary treatment process by using terrestrial and aquatic test models.

6.1. Introduction

The leather industry is an important economic sector in many developing countries including India. However, it is also a major source of environmental pollution due to the discharge of huge volume of potentially toxic and hazardous wastewater into the receiving water body, which negatively affect societies (Dixit et al., 2015; Montalvão et al., 2017; Chowdhary et al., 2018). Wastewater generated by leather industries has very high pollution parameters due to the presence of a complex mixture of organic and inorganic pollutants even after treatment at the Common Effluent Treatment Plant (CETP) and disturb the ecological flora and fauna. Plants play important ecological role/functions in providing habitat for natural world protect soil from erosion and also provide enormous immensity of organic matter to soil, which is important for maintaining its fertility. These toxic chemicals are reported to disturb the delicate hormonal balance and compromise the reproductive fitness and heath of all living being and ultimately may lead to carcinogenesis (Matsumoto et al., 2006; Dixit et al., 2015).

The plant *A. cepa L.* has been regarded as a suitable plant model to assess the chromosomal damage and disturbances in the mitotic cycle due to the presence of good chromosome conditions such as large chromosomes and in a reduced number (2n = 16). The *A. cepa* test, a relatively easy, rapid, sensitive and highly reproducible plant model is strongly recommended to be included in the battery of tests to carry out the toxicity/genotoxicity evaluation of environmental contaminants present in water, wastewater, sludge and soils (Fiskesjo, 1985; Leme and Marin-Morales, 2009; Haq et al., 2017).

There are increasing scientific proofs that these toxic chemicals interfere with the normal neurological functioning of brain (Rabelo et al., 2016). Studies in humans have shown the relationship between the exposure to tannery wastewater and related problems, such as skin, eye, respiratory, and neural problems adversely affecting the cell signalling, neurochemicals pathways and cell metabolism (Siqueira et al., 2011; Guimaraes et al., 2016). A recent report has also submitted that parental exposure to tannery wastewater can precipitate behavioral abnormalities like anxiety and depression in the offspring; suggesting long term detrimental effects of tannery wastewater.

So, this chapter describes about to evaluate toxicity assessment of these residual organic pollutants present in tannery wastewater after the secondary treatment process carried out at CETP in Unnao, using agriculture crop *Vigna radiata* and *Allium cepa* (*A. cepa*) and animal toxicity on *Caenorhabditis elegans (C. elegans).*

6.2. Material methods

***6.2.1.. Phytotoxicity test using Vigna radiata* L.**

Mung bean (*Vigna radiata* L.) seed germination and seedling growth test was performed as per established protocol (OECD, 2003; Bharagava et al., 2010). Mung bean seeds were purchased from local certified seed seller shop and healthy seeds were surface sterilized with 0.2% (*w/v*) $HgCl_2$. Six test solutions of treated wastewaters were prepared (6.25%, 12.5%, 25%, 50%, 75% and 100%, *v/v*) with distilled water. Petri dishes (20 mm × 120 mm) containing 10 seeds were irrigated with 5 ml test solutions and seeds irrigated with tap water were treated as control. The test Petri dishes were incubation at 28 ± 1 °C in a BOD incubator. The number of seeds germinated was recorded after 48 hrs and expressed in terms of percentage (%) germination. Seedlings growth parameters (root and shoot length) were measured after 5 days of treatment (Bharagava and Chandra, 2010; Kumari et al., 2014). The studies were experimented in triplicate.

The seedlings growth parameters (root and shoot length) were measured by using the centimetre scale after 5 days treatment (Bharagava and Chandra 2010; Vibhuti et al. 2015). The experiments were performed in triplicate. The % inhibition in seed germination was calculated following the formula given by Ogbebor and Adekunle (2005) as below:

% Inhibition =100 (Control-Experimental)/Control

6.2.2. Phytotoxicity test using Allium cepa L.

Onion (*Allium cepa* L.*)* root growth inhibition test was carried out by growing onion bulbs in tannery wastewaters. The onion bulbs purchased from local market of Lucknow were healthy and equal-sized. The outer layer of onion bulbs and the dry bottom plate was removed, while taking care of root primordial.

Five onion bulbs were placed over 50 mL Falcon tubes filled with test solutions (6.25%, 12.5%, 25%, 50% and 100%, v/v). The tubes were kept in an incubator at 23 °C for 5 days. The test solutions stored at 4 °C were refilled morning and evening to ensure the contact between onion bulbs and samples present in the tubes. After 5 days, the onion bulbs exposed at each concentration were observed for the root growth and lengths. The inhibition in root growth was measured and was correlated with an index of degree of toxicity (Fiskesjo, 1985).

6.2.2.1. Genotoxicity studies

The genotoxicity of treated wastewaters were measured in term chromosomal aberrations in root tip cells of *A. cepa*. This study was conducted using three test solutions (6.25%, 12.5% and 25% v/v) of treated wastewaters. Five onion bulbs were initially rooted in tap water for 48 h till root lenth reached 1-2 cm and then transferred to test solutions for 24 h, to complete the cell cycle in meristematic cells of *A. cepa* roots within 24 h. To evaluate the cyto and genotoxicity, the root tips were fixed in alcohol and glacial acetic acid (3: 1) fixative for 12 h at 60-70 °C and after washing with distilled water, root tips were hydrolyzed with 1N HCl at 60 - 70 °C for 5 min. After proper washing root tips were processed for slide preparation using haemotoxylin as the stain (Chauhan and Sundararaman, 1990; Haq et al., 2017). Mitotic Index was determined by scoring approximately 4000 cells (500-1000 cells per slide). All the slides were microscopically analysed to calculate the mitotic index (MI) and chromosomal aberrations (CA). MI and CAs were calculated using following formula:

Mitotic Index (%) = (Number of dividing cells/Number of total observed cells) x100

Chromosomal Aberrations (%) = (Total aberrant cells/ Number of total observed cells) x100

6.3.3. Animal toxicity study using C. elegans

6.3.3.1. Culture and maintenance of strains

Nematode (*Caenorhabditis elegans*) a useful test model for neurotoxic effects in developmental toxicological research. Therefore, this study is mainly focused on the comparative assessment of the toxic pollutants of untreated and CETP treated tannery wastewater using *Caenorhabditis elegans (C. elegans)* as a terrestrial test model. *C. elegans* strains, Bristol N2 (wild type), was growing on Nematode growth medium (NGM), using *Escherichia coli* OP50 as a food source and procure from *Caenorhabditis* Genetics Centre (USA) and. The UT (untreated) and CETP-T tannery wastewater for experiments were conducted at 23°C.

6.3.3.2 Nile red staining

The effect on lipid content of *C. elegans* was studied by staining worms with a lipid specific dye, Nile Red (9-diethylamino-5-benzo[α]phenoxazinone) as described by

Ashrafi et al 2003. Briefly, a stock solution of Nile red was prepared by dissolving 0.5mg Nile red dye in 1 ml of acetone. The stock solution was further diluted to 1:250 and was seeded onto NGM plates. 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 were anesthetized by adding 10 µl of 100 mM sodium azide in 100µl of worm suspension. The coverslip was sealed using transparent nail paint. The worms were observed in fluorescence microscope (Lieca) for visualization lipid droplets using Rhodamine filter.

6.3.4. Statistical data analysis

All the experiments were in triplicates and values are presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by Dunnett's post multiple comparison tests was also performed for MI and CA values. The value of $p < 0.05$ was considered significant. Statistical analysis was performed using IBM SPSS Statistics-20.0 software.

6.3. Result and discussion

The toxicity tests combined with physico-chemical analysis are essential in the evaluation of effluent quality. Hence, treated wastewaters were assessed for phytotoxic and genotoxic nature. The phytotoxicity using seed germination test is considered as one of the simplest short-term, sensitive and cost effective method of toxicity evaluation for wastewaters (Rusan et al., 2015; Liu et al., 2018). Seed germination is a very sensitive process likely to be disturbed by the substances present in the environment.

In present study, mung bean (*Vigna radiata L.*) seeds germination test (48h) was tested in different concentrations of treated tannery wastewater. The result of mung seed germination inhibition upon exposure to different concentrations of wastewater is given in Table 6.1, which showed 50% seed germination inhibition at 50% wastewater concentration. Hence, the noted value of IC50% for seed germination was 50% (v/v) concentration (data not shown). At 75 and 100% (v/v) wastewater concentrations, the percentage of seed germination was 10 and 30%, respectively.

Table 6.1: Effect of different concentrations of CETP treated tannery wastewater on seed germination, root length, and shoot length in mung bean (*Vigna Radiata*) plant

Wastewater (%)	Germination (%)	Root length (cm)	Shoot length (cm)
0	100±0.0	1.5±0.3	5.7±0.2
6.25	100±0.0	0.9±0.1	4.6±0.2
12.5	90±0.5	0.5±0.1*	3.1±0.2*
25	70±0.5	0.37±0.05*	1.5±0.5*
50	50±0.9	0.06±0.6*	0.8±0.1*
75	0±0.0	0±0.0*	0±0.0*
100	0±0.0	0±0.0*	0±0.0*

Values are mean ± SD (n=3). The *refers to statistically significant difference from control ($p<0.05$)

The effect of wastewater on early seeding growth (5-seedling) after 5 days is apparent in Table 6.1. Seedling growths were gradually decreased with increasing concentration treated wastewaters. However, compared to controls, root lengths of seedling were highest at 12.5% (v/v) and thereafter gradually decreased with increasing concentrations (Table 6.1 & Figure 6.1).

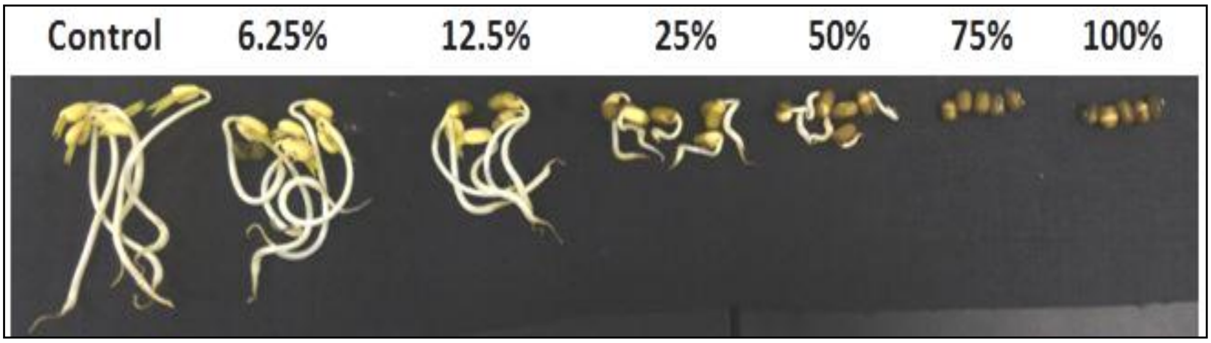


Figure 6.1: Effect of different concentrations of treated tannery wastewater on early seedling growth of mung bean after 48 h

Notable reduction in root length, and shoot length were observed at 75 and 100% wastewater concentrations, respectively, which might be due to the effect of high salts and phenolics and ROPs present in the treated wastewater (Kumari et al., 2016). Oliveira, (2012) reported that the inhibition of seed germination percentage was associated with high TDS and Cr ion in wastewater causing the osmotic stress and toxicity in plants (Kasoobi et al., 2017). Phenolics content in wastewater alters the homeostatic of plants through the over production of reactive oxygen species as reported earlier (William et al., 2017; Lyu et al., 2018).

Further, the phytotoxic effect of tannery wastewater was measured in term of root growth inhibition test using *Allium cepa*. Root growth inhibition in *Allium cepa* root has been considered as a toxicity indicator since it may result from inhibition of the cell division (Fiskesjo, 1985; Egito et al., 2007). The effect of different concentrations of tannery wastewater on root growth and length of *A. cepa* is shown in figures 6.2 (a) and 6.2 (b). Initially, the onion bulbs were rooted in different concentrations of wastewater (0-100% v/v) to observe the root growth of *A. cepa* and results showed that wastewater beyond 25% was inhibitory for root growth. The inhibition was more pronounced at 50%. The IC50% value of wastewater for root growth inhibition was 10% (v/v) wastewater concentration (data not shown). The recorded mean root lengths after 5 days treatment were 0.6, 2.2, 5.6, and 7.1 cm when grown in 50%, 25%, 12.5%, 6.25% and 0%, respectively (Figure 6.2b).

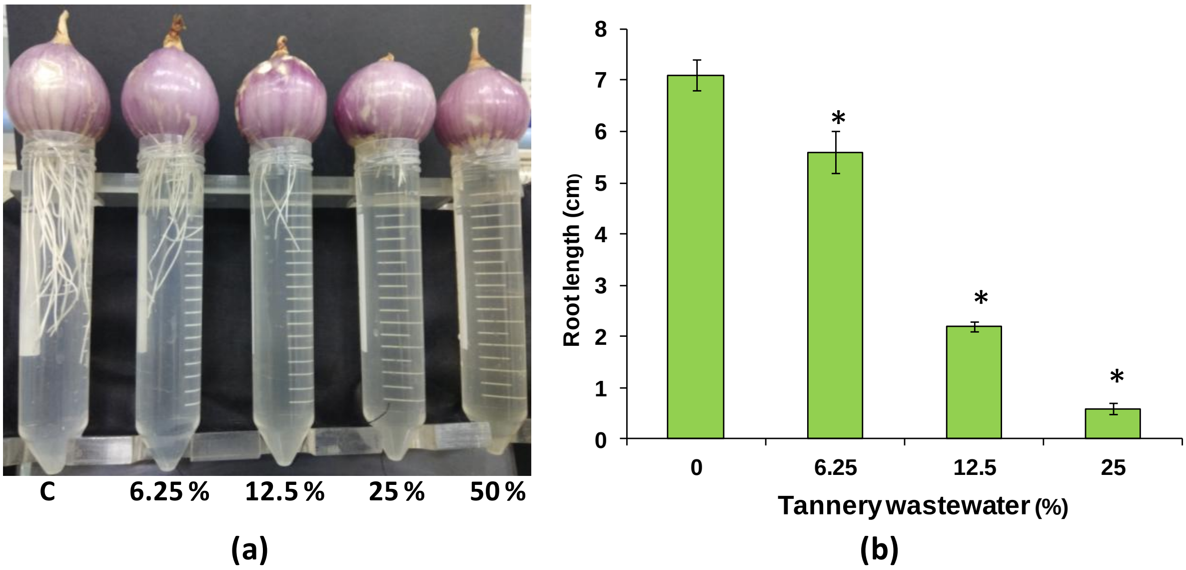


Figure 6.2. Effect of different concentrations of CETP-treated tannery wastewater on root growth (a) and root length (b) of *A. cepa*. Values are mean ± SD of three samples. $p < 0.05$, significant when compared to control using ANOVA

The prevention of root growth above 25% wastewater concentration is indicative of the presence toxic pollutants in tannery wastewater.

3.4. Cytotoxicity and genotoxicity of tannery wastewater

The cytotoxic and genotoxic effects of treated tannery wastewater were evaluated on the basis of mitotic index (MI) and chromosomal aberrations (CA) in root tip cell of *A. cepa*.

3.4.1. Mitotic index

Mitotic index (MI) is a good experimental method to assess the cytotoxic effect of variety of pollutants in the cell division. MI measures the proportion of cells in mitotic phase of cell cycle and its inhibition could be interpreted as cellular death (Rojas, 1993). The cytotoxic effect of treated tannery wastewater in *Allium cepa* roots is summarized in Table-6.2.

Table 6.2: Mitotic index (%) of root tip cells of *A. cepa* following with different treatments

Exposure (24 h)		Treatment	Total cells	Dividing cells	MI%
Water	Control	–	570	375	66±7.0
Wastewater	6.25%	TWW	542	170	31±8.0*
	12.5%	TWW	590	135	23±1.0*
	25%	TWW	506	80	16±2.0*

Values are mean ± SD (n=3). The *refers to statistically significant difference from control ($p<0.05$), TWW = Tannery wastewater

Results revealed that the percent mitotic index (MI%) value of plant root was in the order of 31%, 23%, and 16% as compared to control (66%) at the concentration of 6.25, 12.5%, and 25% wastewater respectively. The MI% decreased progressively with increasing wastewater concentrations indicating presence of various cytotoxic residual organic pollutants in treated tannery wastewaters. These pollutants may interfere with normal process of mitosis, thus preventing a number of cells from entering the prophase and blocking the mitosis cycle during interphase (Srivastava 2015; Haq et al., 2017). The inhibition of MI% may be attributed due to the effect of pollutants on DNA/protein synthesis. The results are agreement with the earlier studies of declined MI% has been reported when *A. cepa* root cell exposed to wastewaters (Rojas et al., 1993; Haq et al., 2017).

3.4.2. Chromosomal aberrations

Chromosomal aberration (CA) analysis of root tip cells of *A. cepa* is considered as an efficient test to investigate the genotoxic, clastogenic and aneugenic potential of chemical agents and industrial wastewaters. CA has been characterized by changes in either of chromosomes structure, which can occur both spontaneously and as well as result of the exposure to physical or chemical agents (Kumari et al., 2016; Papa et al., 2016). Various types of chromosomal aberrations are considered over the four stages of the cell cycle (prophase, metaphase, anaphase, and telophase) as depicted in Table 6.3 and Figure 6.3.

Table 6.3: Different chromosomal and nuclear abnormalities observed in root tip cells of *A. cepa* exposed with different concentrations of CETP treated tannery wastewater

Assay	**Water**	**Tannery wastewater**		
		6.25%	12.5%	25%
Chromosomal aberrations				
Stickiness	0.0±0.0	2.3±0.5	5.0±1.0	7.0±1.0
Vagrant	0.0±0.0	3.0±1.0	3.0±1.0	4.3±1.5
Chromosomal loss	0.0±0.0	2.0±1.0	2.7±0.6	4.3±0.6
C-mitosis	0.0±0.0	2.7±1.5	3.3±1.5	4.3±1.5
Binucleated	0.0±0.0	2.3±0.6	4.0±1.0	5.3±1.5
Micronuclei	0.0±0.0	3.0±1.0	3.0±1.0	5.3±1.5
Aberrant cells (%)	0.0±0.0	9.0±1.2*	15±1.7*	38.3±2.0*

Values are mean ± SD (n=3). Chromosomal aberrations were scored on 500–1000 cells per slide. The *refers to statistically significant difference from control ($p<0.05$), TWW= Tannery wastewater

Results showed that no chromosomal abnormalities were observed in control cells treated with tap water. But, on the other hand, the treatment with different concentrations of tannery wastewater induced various types of chromosomal aberrations and nuclear abnormalities (Fig. 6.3). The observed aberrations were chromosome loss (Fig. 6.3a), vagrant chromosome (i.e. moving/wondering chromosomes having no defined place) (Fig. 6.3b), sticky metaphase (i.e. clumping of chromosomes in metaphase stage) (Fig. 6.3c), c-mitosis (i.e. induced abortive nuclear division leading to the doubling in chromosome numbers) (Fig. 6.3d), binucleated (i.e. cell having two nuclei) (Fig. 6.3e) and micronuclei (i.e. small nucleus formed whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei during cell division) (Fig. 6.3f). The most frequent aberrations were c-mitosis, vagrant, and stickiness chromosomes at all the tested wastewaters concentration.

The percentage of aberrant cells was concentration-dependent and it was highest (38.3%) at 25% wastewater concentration. The induction of various chromosomal aberrations in the root tip cells of *A. cepa* was possibly due to the presence of residual organic pollutants as detected by GC-MS and heavy metals including chromium (Kumari et al., 2016).

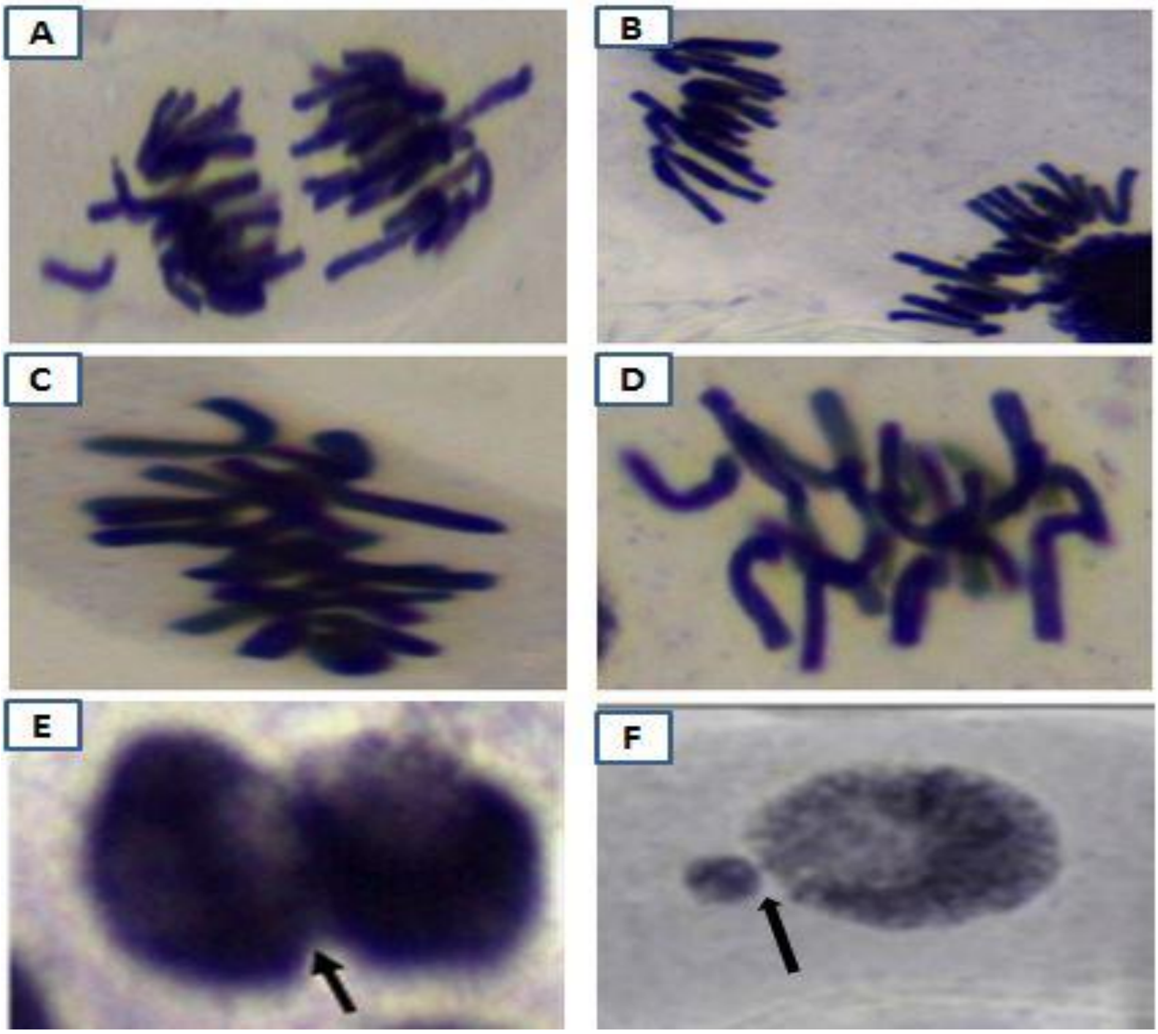


Figure 6.3: Chromosomal aberrations observed in root tip cells of *A. cepa* exposed with different concentrations of CETP treated tannery wastewater. (a) chromosome loss, (b) vagrant chromosome, (c) Sticky metaphase, (d) c-mitosis (e) Binucleated (f) Micronucleated

Stickiness is considered a common sign of toxic effects on chromosomes probably leading to cell death (Rojas, 1993). Stickiness of chromosomes may also occur due to either increased chromosomal contraction and condensation or depolymerization of DNA and partial dissolution of nucleoproteins (Turkoglu, 2007). The occurrence of chromosomal loss and vagrant chromosomes suggests spindle failure (Haq et al., 2017). Colchicine mitosis (c-mitosis) is defined as the inactivation of spindle followed by random scattering of chromosomes around the cells. The wastewater induced a high frequency of c-mitosis, which has been also shown by other studies indicating that wastewater is comparable toxic to colchicine and thus capable to induce C-mitosis.

The chromium and GC-MS detected other residual organic pollutants such as benzoic acid, 3-[4'-(T-butyl) Phenyl] furan-2-5-dione, benzeneacetamide, resorcinol, dibutyl phthalate, benzene-1,2,4-triol, and 1-Phenyl-2-phenylthio detected in tannery wastewater are earlier reported to cause cell division, change in chlorophyll contents, which directly influences the root growth, length and biomass of plant (Salminen and Karonen, 2011; Gao and Ven, 2016; Lyu et al., 2018).

9.3.2. Animal toxicity study

The results of collected tannery wastewater for animal toxicity indicated that most of the of organic pollutants detected in UT samples were diminished in CETP-T samples. The disappearance of most of organic compounds from UT tannery wastewater has revealed that the bacteria present in CETP might be utilizing these organic compounds as sole source of C, N, and energy.

Lipids influence nicotinic receptor function by allosteric mechanisms, stabilizing varying proportions of pre-existing resting, open, desensitized, and uncoupled conformations (DOI: 10.1007/s12551-012-0078-7). Infact, lipids stabilize different conformational states and lipid–nicotinic receptor interactions modulate receptor function at biological synapses and same elucidate through the nile red staining. Nile red staining is established as a suitable and quantitative method to stain the main fat stores in *C. elegans*. Nile red staining of the UT and CETP-treated worms was evident for decrease in the total fat content with UT having more profound effect, suggesting diminished nAchR signaling further endorsing findings reported in the preceding sections. Treatment with UT and CETP-T diminished the total lipid content with more profound effect by UT.

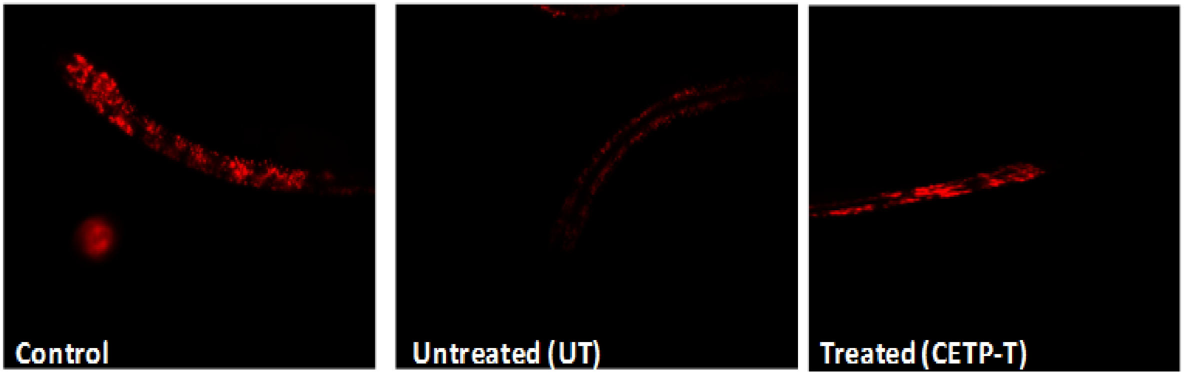


Figure 6.5: Effect of UT (Untreated) and CETP-T (Treated) tannery wastewater on lipid content.

Chapter 7
Summary & Conclusion

Summary and Conclusion

The leather industry is an important economic sector in many developing countries including India. However, it is also a major source of environmental pollution due to the discharge of huge volume of potentially toxic and hazardous wastewater into the receiving water body, which negatively affect societies. The wastewater discharged from leather industries are characterized by high pH, chemical oxygen demand (COD), biochemical oxygen demand (BOD), total dissolved solids (TDS), chromium, sulfate, phosphate, chloride and highly toxic organic pollutants that makes the wastewater unfit for irrigation and poses serious damage to plants and human being.

The majority of small-scale tanneries cannot afford their own effluent treatment plant, instead they depends on a central facility, the Common Effluent Treatment Plant (CETP), to manage their wastewater. In the CETP, combined effluent from nearby tanneries is brought to a central place for the treatment. More than 150 have been set-up so far under the Indian government scheme for the treatment of industrial wastewater. The continuous discharge of the tannery wastewater into the environment is of serious eco-toxicological concerns. The chemicals used in tannery for the tanning process include synthetic organic pollutants like tannins, phthalates, phenolic compounds, azo dyes, surface active compounds, pesticides, sulphonated oils and grease that are not completely degraded through secondary CETP and are released untreated.

Continuous releases of residual organic pollutants in tannery wastewater into the Ganga River through the drains have been a growing environmental concern and require urgent attention for the protection of environment and human health. The nature and characteristics of the residual organic pollutants in tannery wastewater, which are not significantly, degrade during the secondary treatment process at CETP, have yet to fully investigated.

The tannery wastewater samples were collected from the Common Effluent Treatment Plant (CETP) located at Unnao, Uttar Pradesh, India. The wastewater samples viz. raw effluent (a-before treatment), after secondary treatment were collected for physicochemical analysis and pollutant detection as well as bacterial population analysis. This is an activated sludge process (ASP) based CETP, treating ~1.9 MLD wastewater received from a cluster of ~ 30 tanneries located in nearby areas against a design flow of ~2.15 MLD Unnao is one of the major industrial towns adjacent to Kanpur having most of the leather, slaughterhouse, textile, steel and other industries.. Unnao industrial area is situated near Kanpur in northern side of River Ganga having more than 40 industrial units mainly tannery units and the wastewater discharged by the tannery industries after treatment at CETP is finally discharged into the River Ganga. The quality of the treated wastewaters often fails to conform to the prescribed limits recommended by the pollution control bodies of India (CPCB, 2013). Therefore, we have chosen this site for the study.

Over the years, many new chemicals has been introduced in tanning processes. Hence, detail analysis of wastewater generated from tanneries are essential for better understanding of the toxicity and chemical nature of the effluents. The discharge of partially treated wastewater from CETP, affects the flora and fauna of the aquatic ecosystem by blocking the sunlight penetration in receiving water bodies and photosynthetic activity, thus, negatively affects the aquatic life .

The wastewater was found to have high concentration of BOD (680.00 ± 20 mg/L), COD (1300.00 ± 45 mg/L), EC (4.40±0.2 Ms/cm), TDS (3850.00 ± 10.0 mg/L), (566.00 ± 12.5 mg/L), sulfate (8.64 ± 0.42 mg/L), phosphate (26 ± 2 mg/L), nitrate (12.3 ± 0.3 mg/L), chloride (1434 ± 12 mg/L) and phenolic (10.5 ± 0.5 mg/L). The nature of wastewater was alkaline of pH (8.45 ± 0.18). Besides, high concentration of total chromium (7.39 ± 0.06 mg/L) was also observed. The values of the various physico-chemical parameters were higher than the permissible limits for effluent discharge as suggested by CPCB and USEPA. High Cr level causes toxic, genotoxic, mutagenic, and carcinogenic effects on humans, animals, plants, and microbes.

The wastewater released from tannery industries may contain a variety of organic pollutants and millions of pathogenic and non-pathogenic bacteria per milliliter including coliform, fecal coliform, anaerobic spore forming bacilli, and many other types of health hazards organisms. The presence of microbial pathogens in treated wastewater as well as in polluted aquatic resources poses a considerable health risk to the general public health. Despite large advances in water and wastewater treatment processes, waterborne diseases still pose a major world-wide threat to public health.

In this study Four (04) bacterial strains were isolated on HiCrome Bacillus Agar (TWW-2) and Klebsiella Selective agar base selective media *(*TWW-3) Vibrio agar and(TWW-4) from CETP- treated tannery. The isolates bacterial strains were further characterized to confirm their identified on the basis of morphological and biochemical test. Result of the microscopic observation of the isolated pathogenic and non-pathogenic bacteria has revealed that isolates (TWW-1, TWW-2 and TWW-3) were rod shaped, gram negative, except TWW-2, which was gram positive. Further, all isolates also showed positive reaction for motility and catalase activity. The tannery wastewater act as enriched media to many microbes including pathogens. It also spread growth of bacterial species resistant to may metals and antibiotic.

Based on the results obtained in this study, it was concluded that the contamination of soil and water resources with industrial wastewaters containing toxic metals and a variety of residual organic pollutants are the major sources of multi-drug and multi-metal resistant pathogenic microbes in environment.

Further, the PCR amplified 1500 bp long 16S rRNA gene sequences of bacterial isolates have shown the closest relatedness (99%) with newly *Bacillus tropicus* (TWW-2) and *Klebsiella pneumonia* (TWW-3) respectively. The partial 16S rRNA gene sequences of 566 bp, and 1427 bp were submitted to GenBank and an accession number MH762877 and MH559818 were assigned to TWW-2 and TWW-3 strains, respectively.

NGS high-throughput sequencing technologies have been developed to compressively identify the microbial diversity in different samples including activated sludge, soil, wastewater and food. In this study, microbial community in treated tannery wastewater from CETP was investigated by NGS. This study was conducted in order to elucidate the bacterial community structure associated with tannery wastewater treatment. Total number of amplicon sequences reads obtained from tannery wastewater was 1,113,718 reads. The operational taxonomy unit (OTU) recorded was 9,525.

Tannery wastewater has complex mixture of various chemicals pollutant, which is not possible to extract by single solvent extraction method. Therefore, combinations of different solvent system were used to extract majority of organic pollutants through GC-MS analysis. The GC-MS/MS analysis of the CETP treated tannery wastewater was also performed. In GCMS/ MS analysis of tannery wastewater sample, we have detected many peaks of harmful compounds. The result of the present studies suggests that combination of DCM + n-hexane organic solvents was able to extract maximum number of ROPs and thus, might be useful in the extraction of organic pollutants from tannery and other wastewater. Almost 16 types of residual organic pollutants were detected by GC-MS, which indicates incomplete treatment of tannery effluent by CETP.

Based on the results, it was concluded that the collected CETP treated tannery wastewater had very high values for BOD, COD, TDS, sulfate and phenolics along with a variety of residual organic pollutants such as benzoic acid, 3-[4'-(T-butyl) Phenyl] furan-2-5-dione, benzeneacetamide, resorcinol, dibutyl phthalate, benzene-1,2,4-triol, and 1-Phenyl-2-phenylthio etc. In the last few years, leather-tanning has adapted an eco-friendly, non/less toxic and biodegradable chemicals as per strict regulations to limit the pollution level. However, the quality of treated wastewater has not yet improved, which is apparent from present study.

The toxicity tests combined with physico-chemical analysis are essential in the evaluation of treated tannery using *Vigna radiata* L. and *Allium cepa* L. The *A. cepa* test, a relatively easy, rapid, sensitive and highly reproducible plant model has been strongly recommended for the toxicity/genotoxicity evaluation of environmental contaminants present in water, wastewater, sludge and soils. The phytotoxicity using seed germination test is considered as one of the simplest short-term, sensitive and cost effective method of toxicity evaluation for wastewaters.

The toxicological studies showed the phytotoxic nature of wastewater as it inhibited the seed germination in *Vigna radiata* L. and root growth of *A. cepa*. Genotoxicity was evidenced in root tip cell of *A. cepa* where chromosomal aberrations (stickiness, chromosome loss, C-mitosis, and vagrant chromosome) and nuclear abnormalities like micronucleated and binucleated cells were observed. Thus, results suggested that the discharge of partially treated wastewater into the environment is not safe for environment.

The chemically contaminated wastewater discharged into the environment causing serious soil and water pollution along with serious threat to human health in different ways. Thus, it becomes very essential to develop and optimize a biological process (bacterial process) for the adequate degradation and detoxification of toxic pollutants present in tannery wastewater for the safety of aquatic resources. Over all the study indicated that there is a need to adopt a proper treatment and bioremediation strategies to reduce the pollution load of tannery wastewater for the safety of environment.

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Scientific Publications and Achievements

List of Publications

Research article

- **Yadav, A**., Raj, A., Purchase, D., Ferreira, LFR., Saratale, GD., Bharagava, RN. 2019. Phytotoxicity, cytotoxicity and genotoxicity evaluation of organic and inorganic pollutants rich tannery wastewater from a Common Effluent Treatment Plant (CETP) in Unnao district, India using *Vigna radiata* and *Allium cepa*. ***Chemosphere*** 224, 24-332 **(IF- 5.105)**
- Bharagava, RN., **Yadav A**., Chowdhary P., Raj A. 2019. Residual organic pollutants in treated tannery wastewater and its toxicity evaluation by using *Vigna radiata* and *Allium cepa.* ***Environmental Sustainability*** (Under revision)
- Chowdhary, P., **Yadav, A**., Singh, R., Chandra, R., Singh, DP., Raj, A. and Bharagava, RN. 2018. Stress response of *Triticum aestivum L.* and *Brassica juncea L.* against heavy metals growing at distillery and tannery wastewater contaminated site. ***Chemosphere***, 206, 122-131 **(IF- 5.105)**
- Kumari, V, **Yadav A,** Haq I, Kumar S, Bharagava, RN , Singh SK, and Raj A. 2016. Genotoxicity evaluation of tannery effluent treated with newly isolated hexavalent chromium reducing *Bacillus cereus*. ***Journal of Environmental Management*.** 183:204-211 **(IF- 4.8)**
- **Yadav, A**, Raj A, and Bharagava, RN. 2016. Detection and Characterization of a Multi-drug and Multi-metal Resistant Enterobacterium *Pantoea sp*. from Tannery Wastewater after Secondary Treatment Process**. *International Journal of Environment and Botany***, 1(2), 37-42

Book chapters (National/International)

- Mishra S., Bharagava RN, More N, **Yadav A,** Zainith S, Mani S and Chowdhary P. 2019. Heavy Metal Contamination: An Alarming Threat to Environment and Human Health. (ISBN 978-981-10-7284-0) Environmental Biotechnology: For Sustainable Future. Sobti RS, Arora NK and Kothari R. Springer Nature Singapore Pte Ltd. pp-103-125
- Chowdhary P., More N, **Yadav A** and Bharagava RN. 2019. Ligninolytic Enzymes: An Introduction and Applications in the Food Industry. (ISBN 978-0-12-813280-7) Enzymes in Food Biotechnology Production, Applications,

and Future Prospects. Mohammed Kuddus, **Elsevier, Academic Press** (USA) pp-181-195

- **Yadav A**, Chowdhary P, Kaithwas G, and Bharagava RN. **2017.**Toxic metals in environment, threats on ecosystem and bioremediation approaches.(ISBN 9781498762427) Handbook of Metal-Microbe Interactions and Bioremediation,Surjit Das and Hirak Ranjan Singh, **CRC Press, Taylor & Francis Group**, Boca Raton, Florida (USA), 2017 pp 128-141
- Chowdhary P, **Yadav A**, Kaithwas G, and Bharagava RN. **2017.** Distillery wastewater: a major source of environmental pollution and it's biological treatment for environmental safety. ISBN (978-3-319-50653-1) Green Technologies and Environmental Sustainability, Ritu **Singh**, and Sanjeev **Kumar**, (Eds.), **Springer (USA)**, 2017 pp 409-435
- **Yadav A**, Mishra S, Kaithwas G, Raj A and Bharagava RN**. 2016.** Organic pollutants and pathogenic bacteria in tannery wastewater and their removal strategies. Microbes and Environmental Management. Jay Sankar Singh and DP Singh, **Studium Press (India)** Pvt. Ltd, 2015 pp 101-127
- Tripathi, S., **Yadav, A.,** and Tripathi, DM. 2016. Plastic Waste: Environmental Pollution, Health Hazards and Biodegradation Strategies. Bioremediation of Industrial Pollutants. Bharagava RN and Saxena G, **Write & Print Publications New Delhi** (India) pp-152-177

Papers and Chapters communicated

Seminars/ Conferences/Workshops/Training Programmes etc.

- Attended four days National Conference **103rd Indian Science Congress** to be organized by University of Mysore, Mysore from 3-7 January 2016
- Attended four days National Conference **104th Indian Science Congress** to be organized by SV University, Tirupati from 3-7 January 2017
- **Poster presentation** in **Fourth Lucknow Science Congress**, on Conference on Science Technology & Innovation for Sustainable Development, Organized by Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow (U.P). March, 3rd-4th 2017
- Attended three days **workshop** on Bioinformatics: Database and Sequence Analysis, Organized by Department of Microbiology and department of

Mathematical and Computer applications, Bundelkhand University, Jhansi (U.P). March 17-19, 2017

- ❖ **Poster presentation** in **National Conference** on Bio-degradation of Wildlife, Environment and Biodiversity, Organized by Department of Zoology, Gandhi Faiz-e-Azam Collage, Shahjahanpur (U.P), March 19-20, 2017
- ❖ Attended two days **National Symposium** on IPRs in Agricultural Research to be organized by Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow (U.P), and U.P. Council of Agricultural Research Lucknow (U.P) August 30-31, 2017
- ❖ **Oral presentation** in **International Conference** on Emerging Trends in Protein Science & Proteomics, Organized by Department of Biotechnology, Invertis University, Bareilly (U.P), September 15th-16th, 2017
- ❖ **Poster presentation** in **International Conference** on Advance in Agricultural and Biodiversity Conservation Sustainable Development, Organized by Department of Horticulture, C.C.S University , Meerut (U.P) & Agricultural Technology development society (ATDS) Ghaziabad, (U.P), October 27th-28th, 2017
- ❖ **Poster presentation** in **International** Conference on Microbes for sustainable development: Scope & Applications (AMI-2017) Organized by Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow (U.P), November 16rd-19th 2017
- ❖ Attended **Seven days workshop** on Gene Cloning & its Expression, to Produce Genetically Modified Organism**,** 17th, 18th, & 19th (March, 2017), Organized by School of Science, Maharishi University of Information Technology, Lucknow (U.P). November 20th- 26th 2017

Professional Memberships:

- ❖ Life member of **AMI (Association of Microbiology of India)** with Membership No. **3861-2014**
- ❖ Annual member of session (2017-2018) **ISCA (Indian Science Congress Association)** with Membership No. **SLM776**

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Phytotoxicity, cytotoxicity and genotoxicity evaluation of organic and inorganic pollutants rich tannery wastewater from a Common Effluent Treatment Plant (CETP) in Unnao district, India using *Vigna radiata* and *Allium cepa*

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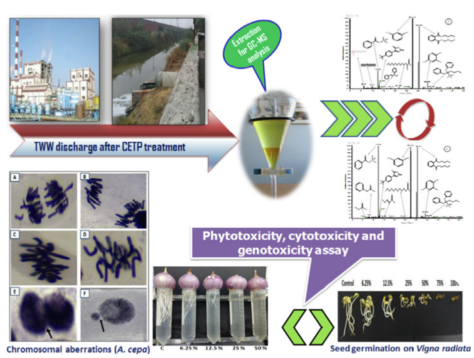
HIGHLIGHTS

- Tannery wastewater rich in organic and inorganic pollutants.
- Tannery wastewater possesses genotoxic and cytotoxic pollutants.
- Phytotoxic nature of tannery wastewater was evaluated by *Vigna radiata* seeds.
- *Allium cepa* chromosomal tests revealed genotoxic nature of treated tannery wastewater.
- Wastewater also induced chromosomal aberrations and nuclear abnormalities in cells.

GRAPHICAL ABSTRACT



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ABSTRACT

The leather industry is a major source of environmental pollution in India. The wastewater generated by leather industries contains very high pollution parameters due to the presence of a complex mixture of organic and inorganic pollutants even after the treatment at a Common Effluent Treatment Plant (CETP) and disturbs the ecological flora and fauna. The nature, characteristics and toxicity of CETP treated wastewater is yet to be fully elucidated. Thus, this study aims to characterize and evaluate the toxicity of CETP treated tannery wastewater collected from the Unnao district of Uttar Pradesh, India. In addition to measuring the physico-chemical parameters, the residual organic pollutants was identified by GC-MS analysis and phytotoxicity, cytotoxicity and genotoxicity of the treated wastewater was evaluated using *Vigna radiata* L. and *Allium cepa* L. Results showed that the treated wastewater contained very high

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pollution parameters (TDS 3850 mg/L, BOD 680 mg/L, COD-1300 mg/L). GC-MS analysis revealed the presence of various types of residual organic pollutants including benzoic acid, 3-[4·-(T-butyl) Phenyl] furan-2-5-dione, benzeneacetamide, resorcinol, dibutyl phthalate, and benzene-1,2,4-triol. Further, toxicological studies showed the phytotoxic nature of the wastewater as it inhibited seed germination in *V. radiata* L. and root growth of *A. cepa*. Genotoxicity was evidenced in the root tip cell of *A. cepa* where chromosomal aberrations (stickiness, chromosome loss, C-mitosis, and vagrant chromosome) and nuclear abnormalities like micronucleated and binucleated cells were observed. Thus, results suggested that it is not safe to discharge these wastewater into the environment.

1. Introduction

The leather industry is an important economic sector in many developing countries including India. However, it is also a major source of environmental pollution due to the discharge of huge volume of potentially toxic and hazardous wastewater into the receiving water body, which negatively affect societies (Dixit et al., 2015; Montalvão et al., 2017; Chowdhary et al., 2018). The wastewater discharged from leather industries are characterized by high pH, chemical oxygen demand (COD), biochemical oxygen demand (BOD), total dissolved solids (TDS), chromium, sulfate, phosphate, chloride and highly toxic organic pollutants that makes the wastewater unfit for irrigation and poses serious damage to plants and human being (Kumari et al., 2016; Bharagava and Mishra, 2018). In India, there are more than 2500 tanneries, of these, nearly 80% are based on chrome tanning process which account for 15% of the total worldwide leather production (Shukla et al., 2009; Chandra et al., 2011). The majority of small-scale tanneries cannot afford their own effluent treatment plant, instead they depends on a central facility, the Common Effluent Treatment Plant (CETP), to manage their wastewater. In the CETP, combined effluent from nearby tanneries are brought to a central place for the treatment (Pathe et al., 2004). More than 150 have been set-up so far under the Indian government scheme for the treatment of industrial wastewater.

The continuous discharge of the tannery wastewater into the environment is of serious eco-toxicological concerns (Matsumoto et al., 2006; Bharagava and Mishra, 2018). The chemicals used in tannery for the tanning process include synthetic organic pollutants like tannins, pthalates, phenolic compounds, azo dyes, surface-active compounds, pesticides, sulphonated oils and grease that are not completely degraded through secondary CETP and are released untreated. Continuous releases of residual organic pollutants in tannery wastewater into the Ganga River through the drains have been a growing environmental concern (Tare et al., 2003; Alam et al., 2009; Chandra et al., 2009) and require urgent attention for the protection of environment and human health. The nature and characteristics of the residual organic pollutants in tannery wastewater, which are not significantly degrade during the secondary treatment process at CETP, have yet to fully investigated.

A. cepa L. has been regarded as a suitable plant model to assess chromosomal damage and disturbances in the mitotic cycle due to the presence of good chromosome conditions such as large chromosomes and in a reduced number (2n = 16). The *A. cepa* test, a relatively easy, rapid, sensitive and highly reproducible plant model has been strongly recommended for the toxicity/genotoxicity evaluation of environmental contaminants present in water, wastewater, sludge and soils (Fiskesjo, 1985; Leme and Marin-Morales, 2009; Haq et al., 2017).

The present study aims to characterize and identify the residual organic pollutants remained in tannery wastewater after the secondary treatment process carried out at a CETP in the Unnao district of Uttar Pradesh, India and to evaluate the phytotoxicity, cytotoxicity and genotoxicity assessment of these residual organic pollutants present in tannery wastewater using agriculture crop *Vigna radiata* and *Allium cepa*.

2. Materials and methods

2.1. Collection of treated tannery wastewater and its physico-chemical characterization

The wastewater samples collected from the outlet of CETP-Unnao, located in the Unnao district of Uttar Pradesh, India (Fig. 1) in pre-sterilized plastic containers (capacity 5-L) were brought to the laboratory and stored at 4 °C. CETP-Unnao was in operation since 1994. This is an activated sludge process (ASP) based CETP, treating ~1.9 MLD wastewater received from a cluster of ~25 tanneries located in nearby areas against a design flow of ~2.15 MLD. The quality of the treated wastewaters often fails to conform to the prescribed limits recommended by the pollution control bodies of India (CPCB, 2013). Therefore, we have chosen this site for the study. The collected samples were immediately processed for physico-chemical parameters analysis, residual organic pollutants detection and charactertization as well as toxicity evaluation tests. The analysed parameters included pH, BOD, COD, TDS, TSS, total chloride, phenolics, nitrate, phosphate and sulfate (APHA, 2012). The pH was measured with a digital pH meter (Metrohm, USA). Digested samples (100 mL) in a digestion mixture of nitric-perchloric acid (5:1) were used to determine total chromium

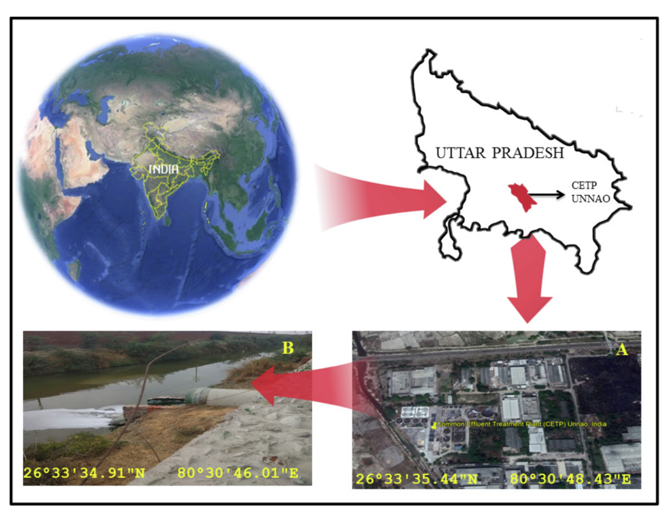

Fig. 1. Location of sampling site (CETP, Unnao) and the discharge of treated tannery wastewater through a drain into the environment.

using atomic absorption spectroscopy (AAS) (GBC, Avanta Sigma, Australia) (APHA, 2012).

2.2. Characterization and identification of residual organic pollutants from treated tannery wastewater

2.2.1. Extraction of residual organic pollutants from collected tannery wastewater by using different solvents systems

Extraction of the residual organic pollutants from wastewater was performed by liquid-liquid extraction (LLE) method using different combination of organic solvents (Minuti et al., 2006). Briefly, centrifuged (8000×g for 20 min at 4 °C) samples (200 mL) were acidified to pH 2.0 using 1 N HCl and then extracted three times with the equal volume of solvent system containing 100 mL of dichloromethane (DCM) and 100 mL of diethyl ether (solvent system-1) and 100 mL of dichloromethane (DCM) and 100 mL of n-haxane (solvent system-2) in a separating funnel (500 mL) by intermittent shaking (Haq et al., 2017). The solvent layer containing residual organic pollutants was separated and evaporated to dryness under vacuum at 40 °C. The extracts were dissolved in 2 mL of DCM and passed through syringe filter (0.22 μm). All the organic solvents used were of HPLC grade (purity >99%).

2.2.2. GC-MS/MS analysis

The extracts were derivatized using trimethyl silyl (BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) TMCS) at 60 °C for 15 min (Marco et al., 2007). Silylated samples (1 μL) were injected into the GC-MS (PerkinElmer, Waltham, MA, USA) equipped with a PE auto system XL gas chromatograph interfaced with a Turbomass mass spectrometric mass selective detector. Helium gas was used as carrier gas with flow rate of 1 mL min^{-1} in column, which was programmed as: 50 °C (5 min); 50–300 °C (10 °C.min^{-1}, hold time: 5 min). The electron ionization (EI) mass spectrum was recorded in full-scan mode in the range of 30–550 (*m/z*) at 70 eV. To identify the compounds, the mass spectra of peaks were compared with that of National Institute of Standards and Technology (NIST) library available with the equipment.

2.3. Toxicological evaluation of CETP treated tannery wastewater for environmental safety

2.3.1. Phytotoxicity test using Vigna radiata L

Mung bean (*Vigna radiata* L.) seed germination and seedling growth test was performed as per the established protocol (OECD, 2003; Bharagava and Chandra, 2010). Mung bean seeds were purchased from a local certified seed seller shop and healthy seeds were surface sterilized with 0.2% (*w/v*) $HgCl_2$. Six test solutions of treated wastewaters were prepared (6.25%, 12.5%, 25%, 50%, 75% and 100%, *v/v*) with distilled water. Petri dishes (20 mm × 120 mm) containing 10 seeds were irrigated with 5 mL test solutions and seeds irrigated with tap water were treated as control. The test Petri dishes were incubation at 28 ± 1 °C in a BOD incubator. The number of seeds germinated was recorded after 48 h and expressed in terms of percentage (%) germination. Seedlings growth parameters (root and shoot length) were measured after 5 days of treatment (Bharagava and Chandra, 2010; Kumari et al., 2014). The studies were experimented in triplicate.

2.3.2. Phytotoxicity test using Allium cepa L

Onion (*Allium cepa* L.) root growth inhibition test was carried out by growing the onion bulbs in tannery wastewaters. The onion bulbs purchased from a local market of Lucknow were healthy and equal-sized. The outer layer of onion bulbs and the dry bottom plate was removed, while taking care of root primordial.

Five onion bulbs were placed over 50 mL Falcon tubes filled with test solutions (6.25%, 12.5%, 25%, 50% and 100%, v/v). The tubes were kept in an incubator at 23 °C for 5 days. The test solutions stored at 4 °C were refilled morning and evening to ensure the contact between onion bulbs and samples present in the tubes. After 5 days, the onion bulbs exposed at each concentration were observed for the root growth and lengths. The inhibition in root growth was measured and was correlated with an index of degree of toxicity (Fiskesjo, 1985).

2.3.3. Genotoxicity studies

The genotoxicity of treated wastewaters were measured in terms of chromosomal aberrations in the root tip cells of *A. cepa*. This study was conducted using three test solutions (6.25%, 12.5% and 25% v/v) of treated wastewaters. Five onion bulbs were initially rooted in tap water for 48 h till root lenth reached 1–2 cm and then transferred to test solutions for 24 h, to complete the cell cycle in meristematic cells of *A. cepa* roots within 24 h. To evaluate the cyto and genotoxicity, the root tips were fixed in alcohol and glacial acetic acid (3: 1) fixative for 12 h at 60–70 °C and after washing with distilled water, the root tips were hydrolyzed with 1 N HCl at 60–70 °C for 5 min. After proper washing the root tips were processed for slide preparation using haemotoxylin as the stain (Chauhan and Sundararaman, 1990; Haq et al., 2017). Mitotic Index was determined by scoring approximately 4000 cells (500–1000 cells per slide). All the slides were microscopically analysed to calculate the mitotic index (MI) and chromosomal aberrations (CA). MI and CAs were calculated using following formula:

Mitotic Index (%) = (Number of dividing cells/Number of total observed cells) ×100.

Chromosomal Aberrations (%) = (Total aberrant cells/Number of total observed cells) ×100.

2.3.4. Statistical data analysis

All the experiments were carried out in triplicates and values are presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by Dunnett's post multiple comparison tests was also performed for MI and CA values. The value of $p < 0.05$ was considered significant. Statistical analysis was performed using IBM SPSS Statistics-20.0 software.

3. Results and discussion

3.1. Physico-chemical characteristics of treated tannery wastewater

Tannery is one of the higest environmental polluting industry due to the discharge of wastewater containing high concentration of hazardous waste including heavy metal like chromium. Over the years, many new chemicals has been introduced in tanning processes. Hence, detail analysis of wastewater generated from tanneries are essential for better understanding of the toxicity and chemical nature of the effluents. The results of physico-chemical analysis of the CETP treated tannery wastewater is summarized in Table 1. The wastewater was found to have high concentration of BOD (680.00 ± 20 mg.L^{-1}), COD (1300.00 ± 45mg.L^{-1}), EC (4.40 ± 0.2 MS.cm^{-1}), TDS (3850.00 ± 10.0 mg.L^{-1}), (566.00 ± 12.5 mg.L^{-1}), sulfate (8.64 ± 0.42mg.L^{-1}), phosphate (26 ± 2 mg.L^{-1}), nitrate (12.3 ± 0.3 mg.L^{-1}), chloride (1434 ± 12 mg.L^{-1}) and phenolic (10.5 ± 0.5 mg.L^{-1}). The nature of wastewater was alkaline (pH 8.45 ± 0.18). High concentration of total chromium (7.39 ± 0.06 mg.L^{-1}) was also observed. The values of the physico-chemical parameters were higher than the permissible limits for effluent discharge as suggested by CPCB (CPCB, 2013 and USEPA (U.S. Environmental Protection Agency (USEPA), 2004) and Table 1 clearly indicates the toxic nature of wastewater treated

Table 1
Physico-chemical characteristics of CETP treated tannery wastewater with reference to the national and international standards.

Parameters	Collected wastewater values	Effluent standards	
		(CPCB, 2013)	(USEPA, 2004)
Color	Light yellowish	-	-
Temperature	32 °C	40 °C	-
pH	8.45 ± 0.18	5.5–9.0	-
EC (mS/cm)	4.4 ± 0.2	0.4	-
BOD (mg.L^{-1})	680.00 ± 20.0	30.00	40.00
COD (mg.L^{-1})	1300.00 ± 10.0	250.00	120.00
TS (mg.L^{-1})	4416.00 ± 14.0	2200.00	-
TDS (mg.L^{-1})	3850.00 ± 10.0	2100.00	-
TSS (mg.L^{-1})	566.00 ± 12.5	100.00	-
Sulfate (mg.L^{-1})	8.64 ± 0.42	5	-
Chloride(mg.L^{-1})	1434.00 ± 12	600.00	-
Phosphate (mg.L^{-1})	12.5 ± 0.5	5	-
Nitrate (mg.L^{-1})	12.3 ± 0.3	10.00	-
Phenolics (mg.L^{-1})	10.5 ± 0.5	1–5	0.50
Heavy metal concentration			
Chromium (mg.L^{-1})	7.39 ± 0.03	2	0.05

All the values are mean of triplicates (n = 3) ± SD; BOD: Biological oxygen demand; COD: Chemical oxygen demand; TS: Total solids; TDS: Total dissolved solids; TSS: Total suspended solids.

by the CETP-Unnao.

The discharge of wastewater from CETP affects the flora and fauna of the aquatic ecosystem by blocking the sunlight penetration in receiving water bodies and photosynthetic activity, thus, negatively affects the aquatic life (Sukumaran et al., 2008; Deepa et al., 2011). The high TDS value is also toxic to aquatic lives by causing osmotic stress and affecting the osmoregulatory functions of organisms (Thakur and Srivastava, 2011). The high BOD and COD values of the tannery wastewater might be due to the presence of high organic contents and salts in the wastewater (Mishra and Bharagava, 2016). High salts are responsible for acidification, reduced soil fertility and increased salinity of groundwater and rivers. The high sulfate, phosphate and nitrate content in the tannery wastewater could be associated with the use of sulfuric acid and sulfide in dehairing process during the tanning process (Yadav et al., 2016a). The high TDS level in wastewater directly indicates the presence of metal ions in the system. Monosodium, disodium phosphates, polyphosphates used in leather treatment processes and ammonium salts in deliming and bating process, are responsible for eutrophication that disturbes the normal ecological functioning of receiving water bodies (Saxena et al., 2016). Phenolics, listed as the "priority pollutant" by the U.S. Environmental Protection Agency (USEPA) (2014) owing to its toxic, genotoxic and carcinogenic effects in plants, animals and human beings, are also high in wastewater due to its utilization in the preservation of raw hides/skins and leather finishing (Mishra and Bharagava, 2016). Chromium used in leather tanning as fastening agent for marking and surfacing of leather was also found to be in higher (7.39 mg/L) (Lofrano et al., 2013; Yadav et al., 2016b) than the permisible limit (2 mg/L). High Cr level causes toxic, genotoxic, mutagenic, and carcinogenic effects on humans, animals, plants, and microbes as reported by various authors (Mishra and Bharagava, 2016; Chowdhary et al., 2018).

3.2. Residual organic pollutants (ROPs) present in treated tannery wastewater

Tannery wastewater has complex mixture of various chemicals pollutants, they are not possible to extract using single solvent extraction method. Therefore, combinations of different solvent systems were used to extract the majority of organic pollutants for GC-MS analysis. The ROPs identified by GC-MS in the extract of DCM-diethyl ether (1:1) using NIST library were mainly derivatives of fatty acids and organic acids (Fig. 2A). In the GC-MS analysis, various major peaks were observed and ROPs were identified at different retention time (RT) viz., RT 6.11 (hexadecanoic acid), 9.77 (Docosane), 12.26 (benzoic acid), 12.83 (3-[4ʹ-(T-Butyl) Phenyl] furan-2-5-dione), 18.44(benzeneacetamide), 27.44 (resorcinol), 30.35 (dibutyl phthalate), 33.93(benzene 1,2,4 triol), and 35.52 (1-phenyl-2-phenylthio), respectively. Minor peaks at RT 13.24 (1-pentene1,3-diphenyl), 21.67(2-bromotetradecanoic acid), 26.67 (phosphoric acid), 37.01 (9-octadecanoic acid), 44.21 (octadecanoic acid) and 48.63 (monopalmitin), respectively.

Further, GC-MS analysis of extracts of DCM-n-hexane (1:1) solvent system showed the presence of fatty acids and carboxylic acids (Fig. 2B & Table 2). The major peaks at RT 7.53 (propanoic acid), 7.93 (banzeneacetic acid), 12.29 (benzoic acid), 12.84 (3-[4ʹ-(T-Butyl) Phenyl]furan-2-5-dione), 13.89 (2-pentenoicacid), 18.44 (benzeneacetamide), 24.07 (dodecanoic acid), 27.44 (resorcinol), 30.34 (dibutyl phthalate), 33.93 (benzene 1,2,4 triol) and 35.52 (1-phenyl-2-phenylthio) were the identified compunds. Compounds at minor peaks at RT 37.01, (9-octadecanoic acid), 41.41 (10-undecynoic acid), 46.47 (Docosanoic acid 1,2,3-propanetriyl) and 48.89 (acetic acid) were also identified.

The detection of compounds in the treated tannery wastewaters clearly indicates (Table 2) the recalcitrant nature of these compounds as they were not degraded completely during the secondary treatment in the CETP and were discharged into the environment along with the wastewater (Chandra et al., 2011). Fatty acids such as hexadecanoic acid, dodecanoic acid, and octadecanoic acid might be originated as a result of processing of raw hide/skins (Saxena et al., 2016). Phthalate (such as dibutyl phthalate: DBP), benzoic acid and resorcinol are used as plasticizers to increase the flexibility and pliability of leather products, as biocide in raw hide/skins preservation and as surfactants, respectively in leather industries (Lyche et al., 2009; TFL, 2010; Dixit et al., 2015).

Phthalates such as, dibutyl phthalate, diethyl phthalate, butylbenzyl phthalate, and diethylhexyl phthalate and phthalic acid have been listed as priority pollutants by USEPA (He et al., 2015). Discharge of these phthalates causes water pollution and serious toxicological effect in aquatic organisms, such as animals and fishes etc (Alam et al., 2009; Chen et al., 2014; Saxena et al., 2016). Benli et al. (2016) also reported that phthalates bioaccumulation leads to genotoxic effects, endocrine disruption, disruption of antioxidant defense system in plants and human being.

Phenolic compounds and phthalates are reported as potential

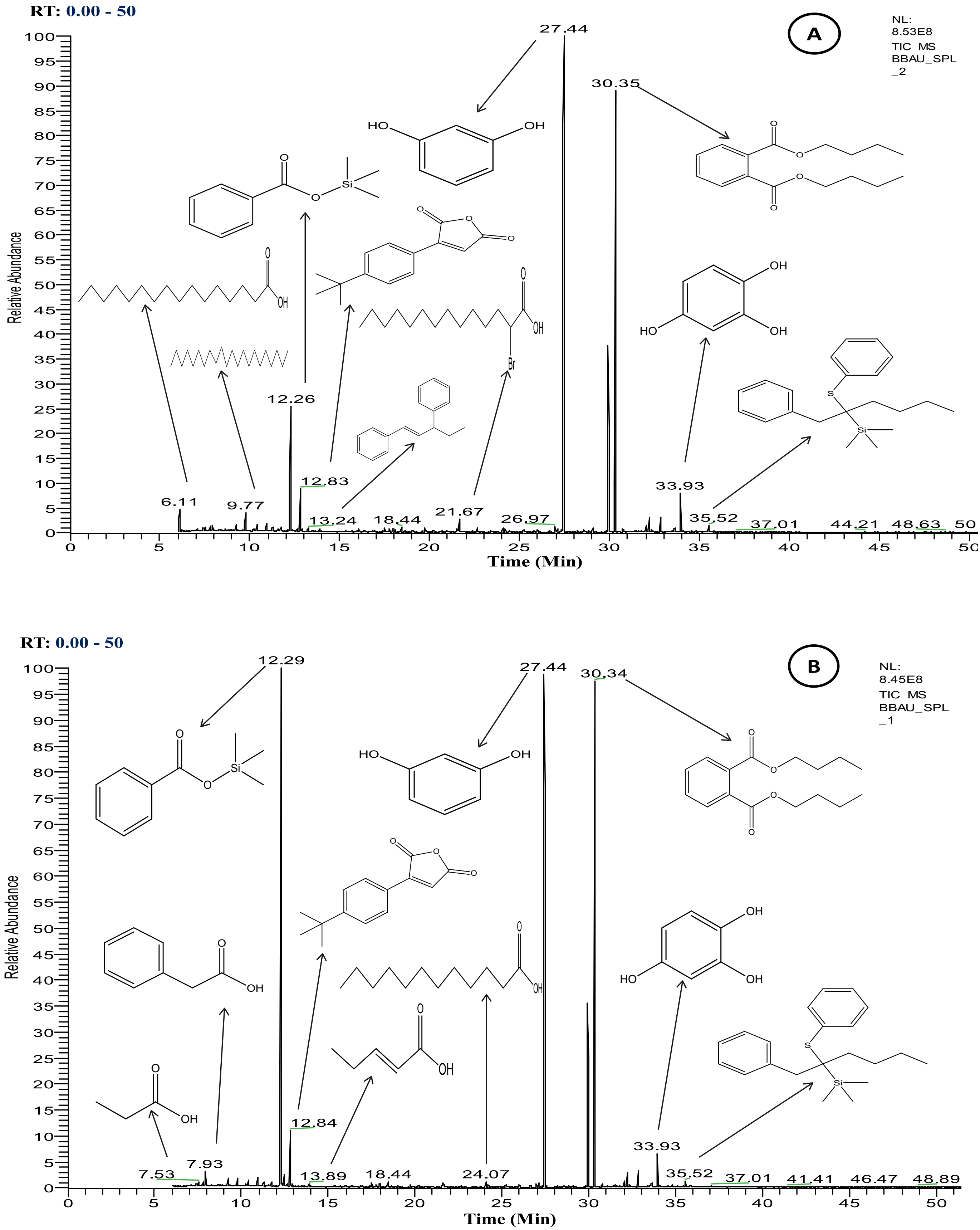

Fig. 2. GC-MS chromatogram of dichloromethane + diethyl either (A) and dichloromethane + n-hexane (B) extracts of CETP treated TWW showing the presence of various residual organic pollutants (ROPs).

Table 2
Residual organic pollutants identified as TMS (Trimethylsilyl) derivatives by GC-MS/MS analysis of CETP treated tannery wastewater extracted with solvent system containing dichloromethane + diethyl either (A) and dichloromethane + n-hexane (B).

Retention time (min)	Molecular formula	Identified residual organic compounds	A	B
6.11	$C_{19}H_{40}O_2$	Hexadecanoic acid	+	-
7.53	$C_9H_{20}O_2$	Propanoic acid	-	+
7.93	$C_8H_8O_2$	Benzeneacetic acid	-	+
9.77	$C_{22}H_{46}$	Docosane	+	-
12.26	$C_{10}H_{14}O_2$	Benzoic acid	+	+
12.83	$C_{14}H_{14}O_3P$	3-[4'-(T-Butyl)Phenyl]furan-2-5-dione	+	+
13.24	$C_{20}H_{26}O$	1-pentene,1,3-diphenyl	+	-
13.89	$C_{18}H_{38}O_3$	2-pentenoic acid	-	+
18.44	C_6H_9NO	Benzeneacetamide	+	+
21.67	$C_{14}H_{27}BrO_2$	2-Bromotetradecanoic acid	+	-
24.07	$C_{12}H_{24}O_2$	Dodecanoic acid	-	+
26.97	H_3PO_4	Phosphoric acid	+	-
27.44	$C_{12}H_{22}O_2$	Resorcinol	+	+
30.34	$C_{16}H_{22}O_4$	Dibutyl phthalate	+	+
33.93	$C_{15}H_{30}O_3$	Benzene 1,2,4 triol	+	+
35.52	$C_{12}H_{32}O_2$	1-Phenyl-2-phenylthio	+	+
37.01	$C_{18}H_{34}O_2$	9-octadecanoic acid	+	+
44.21	$C_{18}H_{36}O_2$	Octadecanoic acid	+	-
41.41	$C_{12}H_{32}O_2$	10-undecynoic acid	-	+
46.47	$C_{69}H_{134}O_6$	Docosanoic acid, 1,2,3-propanetriyl	-	+
48.63	$C_{25}H_{54}O_4P$	Monopalmitin	+	-
48.89	$C_{19}H_{38}O_2$	Acetic acid	-	+

endocrine disrupting chemicals (EDCs). Benzoic acid, a well known EDCs was detected in the tannery wastewater by GC-MS. It has been classified as a Group B2, a probable human carcinogen and highly toxic to aquatic organisms (Kumari et al., 2016; (USEPA, 2012). Benzene is known carcinogen was also observed and its presence in tannery wastewater might be associated with the use of phthalate and phenolics compounds in leather industries (Lyche et al., 2009; U.S. Environmental Protection Agency (USEPA), 2004; Dixit et al., 2015) (Table 2). Further, recently classified EDCs such as resorcinol, hexadecanoic acid, and octadecanoic acid were also detected in tannery wastewater (U.S. Environmental Protection Agency (USEPA), 2004). The result of the present studies suggests that combination of DCM + n-hexane organic solvents was able to extract maximum number of ROPs and thus, will be useful in the extraction of organic pollutants from tannery and other wastewater. In the last few years, leather-tanning has adapted an eco-friendly, non/less toxic and biodegradable chemicals as per strict regulations to limit the pollution level. However, the quality of treated wastewater has not yet improved, which is apparent from present study.

3.3. Phytotoxicity of tannery wastewater

The toxicity tests combined with physico-chemical analysis are essential in the evaluation of effluent quality. Hence, treated wastewater was assessed for its phytotoxic and genotoxic nature. The seed germination test is considered as one of the simplest short-term, sensitive and cost effective method of phytotoxicity evaluation for wastewaters (Rusan et al., 2015; Lyu et al., 2018). Seed germination is a very sensitive process, likely to be disturbed by the substances present in the environment. In the present study, mung bean (*Vigna radiata L.*) seeds germination test (48 h) was carried out in different concentrations of treated tannery wastewater. The result of mung seed germination inhibition upon exposure to different concentrations of wastewater is given in Table 4, which showed 50% seed germination inhibition at 50% wastewater concentration. Hence, the noted value of IC50% for seed germination was 50% (v/v) concentration (data not shown). At 75 and 100% (v/v) wastewater concentrations, the percentage of seed germination was 10 and 30%, respectively.

Table 3
Effect of different concentrations of CETP treated tannery wastewater on seed germination, root length, and shoot length in mung bean (*Vigna Radiata*) plant.

Wastewater (%)	Germination (%)	Root length (cm)	Shoot length (cm)
0	100 ± 0.0	1.5 ± 0.3	5.7 ± 0.2
6.25	100 ± 0.0	0.9 ± 0.1	4.6 ± 0.2
12.5	90 ± 0.5	$0.5 \pm 0.1^*$	$3.1 \pm 0.2^*$
25	70 ± 0.5	$0.37 \pm 0.05^*$	$1.5 \pm 0.5^*$
50	50 ± 0.9	$0.06 \pm 0.6^*$	$0.8 \pm 0.1^*$
75	0 ± 0.0	$0 \pm 0.0^*$	$0 \pm 0.0^*$
100	0 ± 0.0	$0 \pm 0.0^*$	$0 \pm 0.0^*$

Values are mean ± SD (n = 3). The *refers to statistically significant difference from control ($p < 0.05$).

The effect of wastewater on early seeding growth (5-seedling) after 5 days is apparent in Table 3. Seedling growths gradually decreased with increasing concentration of treated wastewaters. However, compared to the controls, the root lengths of seedling were highest at 12.5% (v/v) and thereafter gradually decreased with increasing concentrations of treated wastewater (Table 3). Notable reduction in root length, and shoot length were observed at 75 and 100% wastewater concentrations, respectively, which might be due to the effect of high salts and phenolics and ROPs present in the treated wastewater (Kumari et al., 2016). Oliveira (2012) reported that the inhibition of seed germination percentage was associated with high TDS and Cr ion in wastewater causing the osmotic stress and toxicity in plants (Kasoobi, 2017). Phenolics content in wastewater alters the homeostatic of plants through the over production of reactive oxygen species as reported earlier (William et al., 2017; Lyu et al., 2018).

Further, the phytotoxic effect of tannery wastewater was measured in term of root growth inhibition test using *Allium cepa*. Root growth inhibition in *Allium cepa* root has been considered as a toxicity indicator since it may result from inhibition of the cell division (Fiskesjo, 1985; Egito et al., 2007). The effect of different concentrations of tannery wastewater on root growth and length of *A. cepa* is shown in Fig. 3(a) and (b). Initially, the onion bulbs were rooted in different concentrations of wastewater (0–100% v/v) to observe the root growth of *A. cepa* and results showed that wastewater beyond 25% was inhibitory for root growth. The

Table 4
Effect of different concentrations of CETP treated tannery wastewater on Mitotic index (%) of root tip cells of *A. cepa*.

Exposure (24 h)		Total cells	Dividing cells	MI%
Water	Control	570	375	66 ± 7.0
Wastewater	6.25%	542	170	31 ± 8.0*
	12.5%	590	135	23 ± 1.0*
	25%	506	80	16 ± 2.0*

Values are mean ± SD (n = 3). The *refers to statistically significant difference from control ($p < 0.05$), TWW = Tannery wastewater.

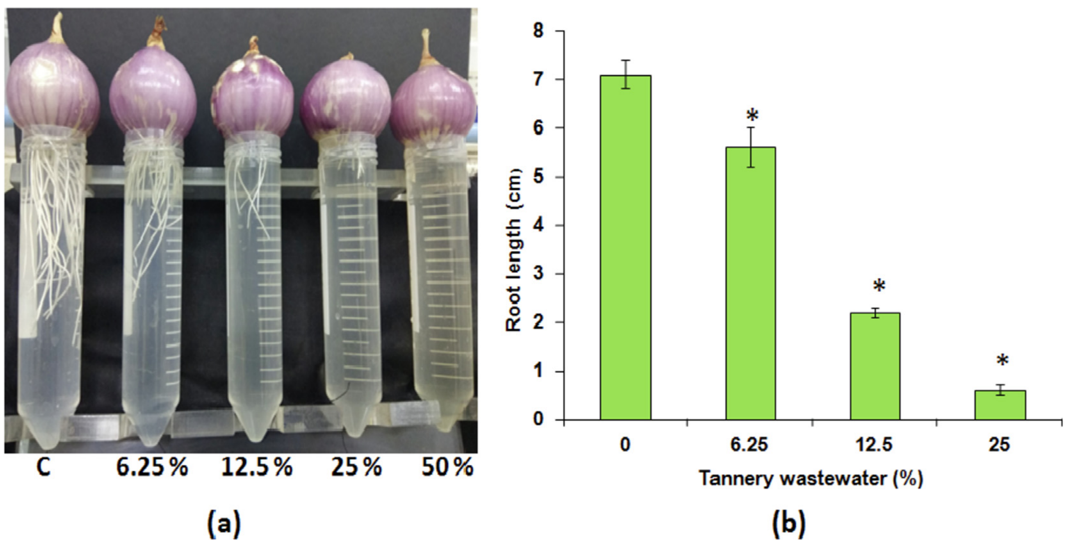

Fig. 3. Effect of different concentrations of CETP-treated tannery wastewater on root growth (a) and root length (b) of *A. cepa*. Values are mean ± SD of three samples. $p < 0.05$, significant when compared to control using ANOVA.

inhibition was more pronounced at 50%. The IC50% value of wastewater for root growth inhibition was 10% (v/v) wastewater concentration (data not shown). The recorded mean root lengths after 5 days treatment were 0.6, 2.2, 5.6, and 7.1 cm when grown in 50%, 25%, 12.5%, 6.25% and 0%, respectively (Fig. 3b). The prevention of root growth above 25% wastewater concentration is indicative of the presence toxic pollutants in tannery wastewater. The chromium and GC-MS detected other residual organic pollutants such as benzoic acid, 3-[4'-(T-butyl) Phenyl] furan-2-5-dione, benzeneacetamide, resorcinol, dibutyl phthalate, benzene-1,2,4-triol, and 1-Phenyl-2-phenylthio detected in tannery wastewater are earlier reported to cause cell division, change in chlorophyll contents, which directly influences the root growth, length and biomass of plant (Salminen and Karonen, 2011; Gao and Wen, 2016; Lyu et al., 2018).

3.4. Cytotoxicity and genotoxicity of tannery wastewater

The cytotoxic and genotoxic effects of treated tannery wastewater were evaluated on the basis of mitotic index (MI) and chromosomal aberrations (CA) in root tip cell of *A. cepa*.

3.4.1. Mitotic index

Mitotic index (MI) is a good experimental method to assess the cytotoxic effect of variety of pollutants in the cell division. MI measures the proportion of cells in mitotic phase of a cell cycle and its inhibition could be interpreted as cellular death (Rojas et al., 1993). The cytotoxic effect of treated tannery wastewater in *A. cepa* roots is summarized in Table 4. Results revealed that the percent mitotic index (MI%) value of plant root was in the order of 31%, 23%, and 16% as compared to control (66%) at the concentration of 6.25, 12.5%, and 25% wastewater respectively. The MI% decreased progressively with increasing wastewater concentrations indicating the presence of various cytotoxic residual organic pollutants in the treated tannery wastewaters. These pollutants may interfere with normal process of mitosis, thus preventing a number of cells from entering the prophase and blocking the mitosis cycle during interphase (Srivastava, 2015; Haq et al., 2017). The inhibition of MI% may be attributed to the effect of pollutants on DNA/protein synthesis. The results are in agreement with earlier studies where *A. cepa* root cells were exposed to wastewaters (Rojas et al., 1993; Haq et al., 2017).

3.4.2. Chromosomal aberrations

Chromosomal aberration (CA) analysis of the root tip cells of *A. cepa* is considered as an efficient test to investigate the genotoxic, clastogenic and aneugenic potential of chemical agents and industrial wastewaters. CA has been characterized by changes in either of chromosomes structure, which can occur both spontaneously and as well as result of the exposure to physical or chemical agents (Kumari et al., 2016; Papa et al., 2016). Various types of chromosomal aberrations are considered over the four stages of the cell cycle (prophase, metaphase, anaphase, and telophase) as depicted in Table 5 and Fig. 4.

Results showed that there was no chromosomal abnormalities in the control cells treated with tap water. On the other hand, treatment with different concentrations of tannery wastewater induced various types of chromosomal aberrations and nuclear abnormalities (Fig. 4). The observed aberrations were chromosome loss (Fig. 4a), vagrant chromosome (i.e. moving/wondering chromosomes having no defined place) (Fig. 4b), sticky metaphase (i.e. clumping of chromosomes in metaphase stage) (Fig.4c), c-mitosis

Table 5
Different chromosomal and nuclear abnormalities observed in root tip cells of *A. cepa* exposed with different concentrations of CETP treated tannery wastewater.

Assay	Water	Tannery wastewater		
		6.25%	12.5%	25%
Chromosomal aberrations				
Stickiness	0.0 ± 0.0	2.3 ± 0.5	5.0 ± 1.0	7.0 ± 1.0
Vagrant	0.0 ± 0.0	3.0 ± 1.0	3.0 ± 1.0	4.3 ± 1.5
Chromosomal loss	0.0 ± 0.0	2.0 ± 1.0	2.7 ± 0.6	4.3 ± 0.6
C-mitosis	0.0 ± 0.0	2.7 ± 1.5	3.3 ± 1.5	4.3 ± 1.5
Binucleated	0.0 ± 0.0	2.3 ± 0.6	4.0 ± 1.0	5.3 ± 1.5
Micronuclei	0.0 ± 0.0	3.0 ± 1.0	3.0 ± 1.0	5.3 ± 1.5
Aberrant cells (%)	0.0 ± 0.0	9.0 ± 1.2*	15 ± 1.7*	38.3 ± 2.0*

Values are mean ± SD (n = 3). Chromosomal aberrations were scored on 500–1000 cells per slide. The *refers to statistically significant difference from control ($p < 0.05$), TWW = Tannery wastewater.

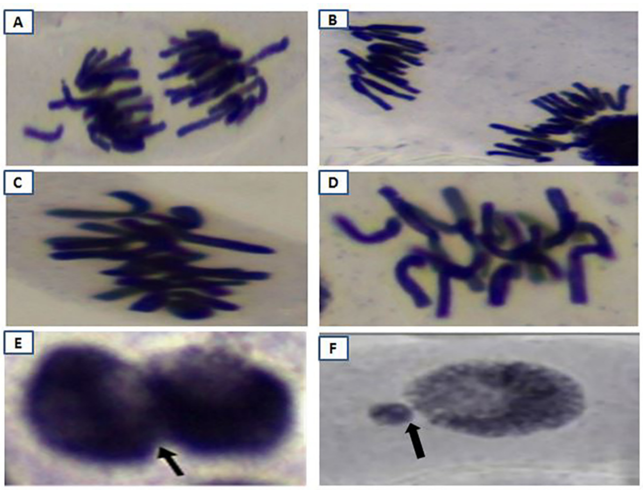

Fig. 4. Chromosomal aberrations observed in root tip cells of *A. cepa* exposed with different concentrations of CETP treated tannery wastewater. (a) chromosome loss, (b) vagrant chromosome, (c) Sticky metaphase, (d) c-mitosis (e) Binucleated (f) Micronucleated.

(i.e. induced abortive nuclear division leading to the doubling in chromosome numbers) (Fig. 4d), binucleated (i.e. cell having two nuclei) (Fig. 4e) and micronuclei (i.e. small nucleus formed whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei during cell division) (Fig. 4f). The most frequent aberrations were c-mitosis, vagrant, and stickiness chromosomes at all the tested wastewaters concentration.

The percentage of aberrant cells was concentration-dependent and it was the highest (38.3%) at 25% wastewater concentration. The induction of various chromosomal aberrations in the root tip cells of *A. cepa* was possibly due to the presence of residual organic pollutants as detected by GC-MS and heavy metals including chromium (Kumari et al., 2016).

Stickiness is considered a common sign of toxic effects on chromosomes probably leading to cell death (Rojas et al., 1993). Stickiness of chromosomes may also occur due to either increased chromosomal contraction and condensation or depolymerization of DNA and partial dissolution of nucleoproteins (Turkoglu, 2007). The occurrence of chromosomal loss and vagrant chromosomes suggests spindle failure (Haq et al., 2017). Colchicine mitosis (c-mitosis) is defined as the inactivation of spindle followed by random scattering of chromosomes around the cells. The wastewater induced a high frequency of c-mitosis, which has been also shown by other studies indicating that wastewater is comparable toxic to colchicine and thus capable to induce C-mitosis.

4. Conclusions

The Residual organic pollutants and toxicity characterization studied of CETP treated tannery reveals toxic nature of wastewater with the following observations:

- CETP treated wastewater was found to have very high BOD, COD, TDS, sulfate and phenolics which are above the prescribed limits.
- The wastewater contained high level of toxic chromium (7.39 $mg.L^{-1}$) and a variety of residual organic pollutants such as benzoic acid, 3-[4'-(T-butyl) Phenyl] furan-2-5-dione, benzeneacetamide, resorcinol, dibutyl phthalate, benzene-1,2,4-triol, and 1-Phenyl-2-phenylthio.
- The wastewater was phytotoxic, as it inhibited seed germination, root and shoot *in V. radiata* upon exposure to diluted samples.
- It inhibited mitotic index and induced chromosomal aberration in root tip cells of *A. cepa*. The observed chromosomal aberration in root tip cells of *A. cepa* were stickiness, chromosome loss, vagrant chromosome and C-mitosis etc.

This study indicated that there is a need to adopt a proper treatment and bioremediation strategies to reduce the pollution load of tannery wastewater for the safety of environment.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Research article

Genotoxicity evaluation of tannery effluent treated with newly isolated hexavalent chromium reducing *Bacillus cereus*

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ABSTRACT

In this study, the efficiency of free and immobilized cells of newly isolated hexavalent chromium [Cr(VI)] reducing *Bacillus cereus* strain Cr1 (accession no. KJ162160) was studied in the treatment of tannery effluent. The analysis of effluents revealed high chemical oxygen demand (COD-1260 mg/L), biological oxygen demand (BOD_5-660 mg/L), total dissolved solids (TDS-14000 mg/L), electrical conductivity (EC-21.5 mS/cm) and total chromium (TC-2.4 mg/L). The effluents also showed genotoxic effects to *Allium cepa*. Treatment of tannery effluent with isolated *B. cereus* strain led to considerable reduction of pollutant load. The pollutant load reduction was studied with both immobilized and free cells and immobilized cells were more effective in reducing COD (65%), BOD (80%), TDS (67%), EC (65%) and TC (92%) after 48 h. GC-MS analysis of pre and post-treatment tannery effluent samples revealed reduction of organic load after treatment with free and immobilized cells. An improvement in mitotic index and reduction in chromosomal aberrations was also observed in *A. cepa* grown with post-treatement effluent samples compared to untreated sample. Results demonstrate that both methods of bacterial treatment (free and immobilized) were efficient in reducing the pollutant load of tannery effluent as well as in reducing genotoxic effects, however, treatment with immobilized cells was more effective.

1. Introduction

Environmental pollution by effluents of tannery industries is a major concern throughout the world. Plethora of chemicals such as acids, alkalis, chromium salts, tannins, biocides, solvents, sulfides, dyes etc. are used during leather processing and remain in the effluent. These are difficult to treat by conventional activated sludge process due to high toxicity. Their discharge in to water bodies poses a serious threat to the living organisms inhabiting respective ecosystem and also tends to be accumulated in food chain. There are about 3000 major tanneries in India, located in different parts of the country. Most of them (nearly 80%) are engaged in chrome tanning process (Shukla et al., 2009). The effluent discharged from these tanneries after treatments is still left with high level of BOD, COD, TDS and other specific pollutants such as chromium [Cr(III) & Cr(VI)], pentachlorophenol, surfactant, synthetic tannins, azo dyes, chloride, sulphate and oil and grease (Singh et al., 2013; Thakur and Srivastava, 2011). The discharge of inadequately treated tannery effluent causes water and soil pollution and affects plant and animal health. The genotoxic and mutagenic effect of tannery effluents from leather processing industry is well documented (Raj et al., 2014; Gupta et al., 2012; Alam et al., 2009).

Over the years, various physical and chemical processes including advance oxidation processes using ozone, electrochemical treatment, fenton, photocatalysis (UV/TiO2) and membrane processes have been developed to enhance pollutants removal from effluent (Lofrano et al., 2013). Most of these processes removed the majority of colloidal organic substances and suspended materials; but, refractory compounds still remained in the effluent. To over-come these problems bio-based remediation

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strategies have evolved as a promising tool. Bioremediation of tannery effluents is an environment friendly, safe, cost effective alternative to traditional physical and chemical methods. Bioremediation of chromium in tannery effluents using bacteria or fungal mycelia as bioabsorbent has been studied earlier (Saranraj and Sujitha, 2013).

Immobilization of microbial cells has received increasing interest in the field of waste treatment (Martins et al., 2013) as it offers advantages such as high biomass, high metabolic activity, stability and strong resistance to toxic chemicals. Immobilized microorganisms can be reused several times without significant loss of activity, making them cost effective. The present study was undertaken to isolate potential Cr(VI) reducing bacteria and study the bioremediation potential of free with immobilized cells for tannery effluent treatment.

2. Materials and methods

2.1. Collection of tannery effluents and soil samples

Effluent samples were collected from a common effluent treatment plant (CETP) at district Unnao, Uttar Pradesh, India. It is an activated sludge process-based common effluent treatment plant receiving effluent from 21 tanneries with an inflow of 2.15 million liters per day (MLD). Effluent samples were collected from outlet point of CETP in plastic bottles of 0.5 lit capacity. Samples were brought to laboratory in icebox and stored at 4 °C. Similarly, soil samples were collected from effluent receiving drain in pre-sterilized collection tubes.

2.2. Isolation and screening of chromium (VI) reducing bacteria

Isolation of chromium resistant bacteria from soil sample was done by an enrichment culture technique in mineral salt medium (MSM) supplemented with 100 mg/L of Cr(VI). The MSM contained (g/L): NaCl: 1; $CaCl_2.2H_2O$: 0.1; $MgSO_4.7H_2O$: 0.5; KH_2PO_4: 1; Na_2HPO_4: 1, yeast extract: 4 and pH = 6.0 (Mahmood et al., 2013). Erlenmeyer flasks containing autoclaved MSM (99 mL) were supplemented with filter-sterilized Cr(VI) as $K_2Cr_2O_7$ @ 100 mg/L and were inoculated with 1 g of soil. The flasks were incubated at 37 °C under shaking condition (120 rpm) in an orbital shaker (Innova-4230, New Brunswick, USA). After 7 days of enrichment, culture broth was serially diluted and plated on MSM agar plate containing 100 mg/L of Cr(VI). The plates were incubated at 37 °C for 48 h. Potential isolates were tested for Cr(VI) reduction in MSM.

2.3. Growth tolerance and chromium (VI) reduction

Growth tolerance and Cr(VI) reduction assay was performed by growing the isolate in 50 mL LB broth supplemented with 0, 25, 50, 100, 150, 200, 250, 300, 350 and 400 mg/L of Cr(VI). The flasks were incubated at 35 °C under shaking (120 rpm). After 24 h of growth, culture broth from each test concentration was centrifuged (8000 rpm for 15 min) and pellet was re-suspended in same volume of distilled water. The optical density (OD_{600}) of bacterial suspension was measured spectrophotometrically (Techcomp, Korea). Cr(VI) concentration of centrifuged supernatant was measured by 1, 5-diphenylcarbazide (DPC) method at 540 nm (APHA, 2005) and was quantified using a standard plot prepared from $K_2Cr_2O_7$ in the range of 1–10 mg/L. The percent reduction of Cr(VI) was calculated using the formula:[(Ci-Cf)/(Cix100), where, Ci = initial Cr(VI) conc. (mg/L) and Cf = final Cr(VI) conc. (mg/L)].

2.4. Characterization of culture supernatant, cell free extract and membrane fraction for the localization of chromium reduction and chromium reductase activity

Isolate was grown in LB broth containing 80 mg/L Cr(VI) at 35 °C and 120 rpm for 24 h. Cells (30 mL) were harvested by centrifugation (5000 rpm for 30 min) and supernatant was stored at 4 °C. Cell pellet was re-suspended in 30 mL phosphate buffer (100 mM, pH = 7.0) and cells were disrupted by sonication (Sonics VCX 750, USA) with 15-s pulses at 15-s interval for 30 min. The resultant homogenate was centrifuged at 15,000 rpm for 15 min at 4 °C and cell free extract was collected and stored. The remaining fraction containing cell wall fraction was re-suspended in 30 mL phosphate buffer. Total chromium, Cr(VI), chromium reductase activity and total protein content of all three fractions (culture supernatant, cell free extract and cell wall fraction) was estimated.

2.5. Identification of selected bacterial isolate

Morphological and biochemical tests were conducted as per standard method (Barrow and Feltham, 1993). The molecular characterization of the isolate was done by 16S rRNA gene sequencing. DNA was extracted using UltraClean Microbial DNA isolation Kit (MO BIO, USA) according to the manufacturer's instructions. PCR amplification of the 16S rRNA gene was performed with 16S rRNA universal Primers: 27F (5′-AGAGTTTGATCCTGGCT-CAG-3′) and 1492R (5′-TACGGTTACCTTGTTAC G ACTT-3′)at annealing temperature of 56 °C (35 cycles). The PCR product was purified by gel extraction (Gel extraction Kit, Qiagen) and was sequenced in an ABI 3130 genetic analyzer using Big Dye Terminator version 3.1 cycle sequencing kit. The nucleotide sequences of 16S rRNA gene were compared with available sequences using NCBI-BLAST.

2.6. Scanning elcelctron microscopy (SEM) analysis

SEM analysis was carried out to observe the morphological changes on the cell surface of isolate exposed to Cr(VI). Cells grown in LB broth (with and without 100 mg/L of Cr(VI)) at 120 rpm, 35 °C for 24 h were harvested by centrifugation at 5000 rpm for 30 min. The pellets were washed thrice with phosphate buffered saline (PBS) and pre-fixed with 2.5% glutaraldehyde for 2 h at 4 °C. The pre-fixed cells were washed with PBS twice and post-fixed with 1% osmium tetraoxide for 1 h. After washing with PBS thrice dehydration process was performed with 15, 30, 60, 90 and 100% (v/v) of acetone. The fixed cells were dried and gold-coated using Mini sputter coater (Model- SC7620, Quorum, Technologies, UK) and then examined with a field emission of SEM (Quanta 450 FEG, FEI, Netherland).

2.7. Preparation of bacterial cell suspension

Bacterial cells were grown in MSM (200 mL) at 35 °C under shaking (120 rpm) for 24 h and afterwards were separated by centrifugation (8000 rpm for 15 min). The pellet was re-suspended in 20 mL of 0.1 M phosphate buffer (pH = 7.0) at a final concentration of 215 mg wet cells/mL and used in bioremediation studies.

2.8. Immobilization of cells

Ten mL of bacterial cell suspension was mixed with 10 mL of 4% sterile sodium alginate solution (final alginate concentration of 2%). The alginate-bacterial mixture was then dropped gently in calcium chloride (0.1 M) solution using a sterile syringe to get equal sized beads. The beads were kept in the same solution for 30 min at 4 °C for hardening (Champagne et al., 1992).

2.9. Bioremediation of tannery effluent using free and immobilized cells

Flasks containing 100 mL of sterilized tannery effluent were inoculated with 10 mL of bacterial cell suspension (free cell) or beads (immobilized cells). The flasks were incubated at 35 °C and 120 rpm for 48 h. Uninoculated flask and flask inoculated with beads (without cells) were treated as control and incubated under the same conditions. Important parameters such as pH, colour, TDS, EC, BOD, COD and total chromium were measured pre and post-treatment (48 h) using standard methods (APHA, 2005). All the values were recorded in triplicate and the data are reported as mean ± SD.

2.10. GC-MS analysis

The control and treated samples were analyzed by GC-MS to study the effect of treatment on organic pollutants and their degradation products. Extraction and derivatization of samples was performed using ethyl acetate and trimethyl silyl (BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) TMCS) (Chandra et al., 2011). Silylated samples (1 μL) were injected in GC-MS (Ultra TSQ Quantum XLS Mass spectrometer, ThermoScientific, USA) equipped with Elite 5MS capillary column (30 m × 0.25 mm), 0.25 μm film thickness of stationary phase, 5% phenyl and 95% dimethyl polysiloxane. This was operated in splitless injection mode with an injector temperature of 250 °C. Helium was used as a carrier gas with a flow rate of 1.1 mL min^{-1}. The GC oven temperature was programmed as 65 °C (hold for 2 min), increased to 230 °C at a rate of 6 °C min^{-1}, and finally reached to 290 °C (hold for 20 min) at a rate of 10 °C min^{-1}. Total run time was 55 min. The ion source and interface temperature were set at 220 °C and 300 °C, respectively. The mass spectrometer was operated at electron energy of 70 eV. All the samples were analyzed in full scan mode of mass in the range of m/z 50–600. The identification of organic compounds and their degradation products was done by comparing their mass spectra with NIST library.

2.11. Toxicity evaluation of tannery effluent before and after bacterial treatment using Allium cepa bioassay

2.11.1. Root growth inhibition test

Onion (*Allium cepa* L.) root growth inhibition test was carried out for the phytotoxicity assessment of pre and post-treatment tannery effluents. *A. cepa* bulbs used in this study were purchased from local market. The root growth inhibition test was conducted using healthy and equal-sized onion bulbs. Five onion bulbs were placed over 50 mL Falcon tubes filled with the different concentration of effluent samples (0, 6.25, 12.5, 25, 50 and 100%, v/v) and incubated at 23 °C for 5 days. Dechlorinated tap water was used as control and for dilution of effluents. The test solutions stored at 4 °C were replaced every 24 h. At the end of the study, the root length of onion bulbs at each concentration were measured.

2.11.2. Genotoxicity studies

The studies were carried out at 6.25%, 12.5% and 25% (v/v) concentration of effluents. Five onion bulbs per effluent concentration were initially rooted (1–2 cm) in tap water for 48 h and then transferred to test solutions for 24 h (Chauhan and Sundararaman, 1990). The root tips were fixed in Carnoys fixative (3 alcohol: 1 acetic acid) for 12 h and washed with distilled water to remove residual fixatives. They were hydrolyzed with 1 N HCl at 60–70 °C for 5 min. After hydrolysis, the roots were washed with distilled water and approximately 1–2 mm of the root tips were cut and processed for staining with hematoxylin (Fiskesjo, 1985; Chauhan and Sundararaman, 1990). All slides were examined microscopically to calculate the mitotic index (MI) and score chromosomal aberrations present. MI was determined by the examination of 1000 cells per concentration (100 cells per slide) (Fiskesjo, 1985). The MI (%) was calculated as number of dividing cells in mitosis/total number of observed cells. The number of chromosomal aberrations was recorded from 300 to 500 dividing cells for each concentration.

3. Results and discussion

3.1. Isolation and screening of chromium (VI) reducing bacteria

Chromium contaminated soil, sludge and effluent are common source of chromium-resistant bacteria. In this study, tannery wastewater contaminated soil collected from the CETP-Unnao (U.P.), India was enriched in MSM supplemented with 100 mg/L of Cr(VI) to isolate potential Cr(VI)-reducing bacteria. Three morphologically distinct Cr(VI)-resistant bacteria (named as Cr1, Cr2 and Cr3) were isolated on Cr(VI) amended MSM agar plate. These bacterial isolates were tested for Cr(VI) reduction in MSM amended with different concentrations of Cr(VI). Isolate Cr1 which exhibited maximum Cr(VI) reduction at all tested concentrations, was selected for further studies.

The growth tolerance and simultaneous reduction of Cr(VI) by selected strain Cr1 at different concentrations (25–400 mg/L) is shown in Fig. 1. Compared to control (without Cr(VI)), the growth of isolate was stimulated in presence of up to 75 mg/L of Cr(VI). Afterwards, growth declined with increasing concentration of Cr(VI). The strain exhibited minimum inhibitory concentration (MIC) for Cr(VI) at 400 mg/L. Further, this strain exhibited concentration-dependant Cr(VI) reduction. With an initial Cr(VI) concentration of 25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L, 200 mg/L and 300 mg/L, Cr(VI) reduction of 99%, 84%, 69%, 59%, 39% and 11%, respectively, was observed after 24 h. There were negligible amount of Cr(VI) reduction in un-inoculated controls. The decrease in chromium reduction with increasing initial Cr(VI) concentration may be due to the mutagenic and toxic effects of chromium for culture metabolism (Thacker et al., 2006).

3.2. Location of the chromium reductase activity and chromium reduction

Cell fractionation studies showed that chromium reductase activities (IU/min/mg protein) of 1.1 ± 0.1 and 0.2 ± 0.05, were present in both cell wall and cell free extract fractions (Table 1). The cell

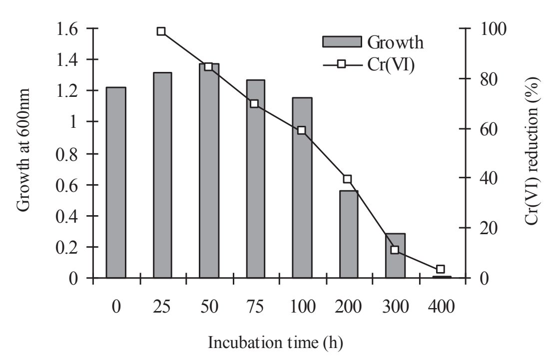

Fig. 1. Growth tolerance and Cr(VI) reduction (%) by *B. cereus* at different initial concentration of Cr(VI) after 24 h.

Table 1
Determination of chromate reductase activity, Cr(VI) and total chromium and in the cell wall fraction, cell free extract and culture supernatant.

Fraction	Specific chromate reductase activity (IU/min/mg protein)	Cr(VI) (mg/L)	Total Cr (mg/L)
Cell wall fraction	1.1 ± 0.1	0.0	0.0 ± 0.0
Cell free extract	0.2 ± 0.05	0.0	0.2 ± 0.1
Culture supernatant	0.8 ± 0.1	4.0	75.8 ± 0.2

wall fraction contained 85% of the total chromium reductase, suggesting a cell wall or membrane-bound chromium reductase in isolate. Chromium reductase activity (0.8 ± 0.1 IU/min/mg protein) was also observed in culture supernatant, suggesting that although chromium reductase in this strain is cell wall or membrane bound, it is also secreted into the medium. This generalised pattern of chromium reductase activity of this strain corroborates the earlier findings (Ilias et al., 2011) with bacterial isolates *Staphylococcus aureus* and *Pediococcus pentosaceus*. Both the membrane bound (Mangaiyarkaras et al., 2011) and soluble (Sultan and Hasnain, 2007) proteins have been shown to reduce Cr(VI). However, bacteria with extracellular chromium reductase are also reported (Ilias et al., 2011; Priester et al., 2006).

To investigate the location of Cr(VI) reduction, total chromium and Cr(VI) was determined in all three fractions. The result (Table 1) showed that Cr(VI) was detected in the culture supernatant (4.0 mg/L), but not in the cell wall and cell free fractions. AAS results revealed a total chromium of 0.2 ± 0.1 and 75.8 ± 0.2 mg/L in the cell free extract and culture supernatant fractions, respectively. This suggests that Cr(VI) was almost completely reduced externally by extracellular chromium reductase, as very negligible amount of total chromium was detected in the cell free fraction.

3.3. Characterization of chromium (VI) reducing isolate

The strain Cr1 is gram-positive, spore-forming, rod shaped and showed a positive reaction for catalase, oxidase and motility. PCR amplification with 16S rRNA gene specific primers resulted in ~1500 bp fragment. The sequencing and BLAST analysis 100% identity with *Bacillus cereus*. Hence, this bacterium was identified as *B. cereus*. The 16S rDNA sequence has been deposited in NCBI GenBank (Accession no. KJ162160).

The SEM images of *B. cereus* cells grown without and with 100 mg/L of Cr(VI) in LB broth are presented in Fig. 2a and b. In SEM images, the bacterial cells without Cr(VI) were rod shaped with smooth surface. However, upon exposure to 100 mg/L of Cr(VI) for 24 h, the cells became rough along with depressions in the surface of few cells. This artifact in the cell surface appears to be associated with Cr(VI) induced stress. Cr(VI) is known to cause several morphological changes such as rough, porous, coagulated surface, irregular surface with the appearance of wrinkles and elongated cells with production of more capsular material upon contact to bacterial cell (Rida et al., 2012; Liu et al., 2006; Thacker and Madamwar, 2005).

3.4. Physicochemical characteristics of tannery effluent before and after treatments

The physicochemical analysis of pre and post treatment tannery effluent is summarized in Table 2. The untreated tannery effluent was light yellow in colour with alkaline pH (8.4 ± 0.2). Also, its TDS (14,000 ± 150 mg/L), EC (21.5 ± 1.0 mS/cm), BOD (660 ± 45 mg/L), COD (1260 ± 102 mg/L) and total chromium (2.41 ± 0.2 mg/L) were higher than the permissible limit (Table 2). No hexavalent chromium [Cr(VI)] was detected by DPC method. The high EC and TDS in effluent indicates the presence of inorganic substances and salts. The EC, which bear direct relation with salinity affects seed germination and plant growth (Sultan and Hasnain, 2007). The high TDS is toxic to aquatic lives by causing osmotic stress and affecting the osmoregulatory functions of the organisms (Thacker et al., 2006). High BOD indicates poor dissolved oxygen in the effluent, which may cause hypoxia with consequent adverse effect on aquatic biota (Sukumaran et al., 2008). High level of COD in the tannery effluent may also adversely affect the downstream water bodies due to reduction in dissolved oxygen content affecting the survival of aquatic organisms (Deepa et al., 2011). The tannery effluent characteristics reported in present study were in agreement with the previous studies (Naaz and Pandey, 2010; MoWR, 2013).

The physicochemical characteristics of tannery effluent after treatments with free and immobilized bacterial cells showed reduction of analyzed parameters (Table 2). After treatment, the colour of effluent became light yellow to colorless. This was possibly achieved by degradation of the dye molecules present in effluent. The pH of effluent was reduced from 8.4 ± 0.2 to 8.0 ± 0.4 and 7.2 ± 0.1 following the treatment with free and immobilized

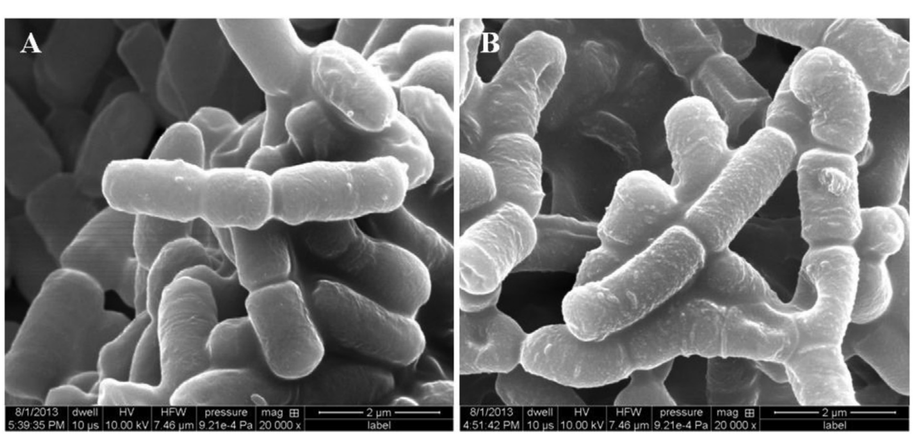

Fig. 2. SEM images of *B. cereus* cells grown in LB broth without Cr(VI)(A) and with 100 mg/L of Cr(VI) (B) for 24 h.

Table 2
Physicochemical characteristic of tannery effluent before and after treatment (48 h) with *B. cereus*.

Parameters	Mean ± SD and % reduction	Before treatment	Free cells treated	Immobilized cells treated	Effluent standard (CPCB, 1995)
pH	Mean ± SD % Reduction	8.4 ± 0.2	8.0 ± 0.4 (6%)	7.2 ± 0.1 (14%)	5.5–9.0
TDS	Mean ± SD (mg/L) % Reduction	14,000 ± 150	7020 ± 310 (50%)	4630 ± 200 (67%)	2100
EC	Mean ± SD (mS/cm) % Reduction	21.5 ± 1	10 ± 0.5 (54%)	7.6 ± 0.5 (65%)	0.4
BOD	Mean ± SD (mg/L) % Reduction	660 ± 45	169 ± 8.5 (74%)	130 ± 4 (80%)	30
COD	Mean ± SD (mg/L) % Reduction	1260 ± 102	566 ± 28 (55%)	435 ± 20 (65%)	250
Chromium	Mean ± SD (mg/L) % Reduction	2.41 ± 0.2	0.7 ± 0.03 (73%)	0.2 ± 0.1 (92%)	2

cells. Treatment of tannery effluent with free cells resulted in the reduction of TDS, EC, BOD, COD and chromium by 50%, 54%, 74%, 55% and 73%, respectively after 48 h at 35 °C and 120 rpm. The reduction in TDS, EC BOD and COD might be attributed to the bacterial degradation of complex organic and inorganic pollutants to meet the nutritional requirements (Chandra et al., 2011). Under similar conditions, immobilized cells reduced 67%, 65%, 80%, 65% and 92% of TDS, EC, BOD, COD and chromium, respectively. Compared to free cells, immobilized cells of present isolate were more effective in the treatment of tannery effluent. Our findings of the degradation of tannery effluent by free and immobilized cells are in agreement with earlier study by Ramkrishan and Sivasubramanian (2013). The increased degradation by the immobilized cells might be due to factors such as accelerated reaction rates caused by high local density and stabilization of cells. Immobilization of cells may lead to their protection resulting in better biodegradation rate (Martins et al., 2013). The level of COD reduction obtained in present study after 48 h was higher than those obtained by Mohamed et al. (2011) as they reported only 19.42% COD reduction of tannery effluent after 72 h treatment with *Aspergillus niger*.

Chromium [Cr(III) & Cr(VI)] are one of the highly toxic components of tannery wastewater and their microbial remediation offers an ecofriendly and cost-efective approach. In this study, removal of chromium from effluent by free and immobilized cells was 73% and 92%, respectively after 48 h. The reduction in chromium metal content might occur either by bioaccumulation inside cells and/or biosorption on cell surface (Srinath et al., 2002). Biosorption is a metabolism-independent process. It depends on mechanisms such as complexation, ion exchange, coordination, adsorption, chelation and microprecipitation and can be performed by both living and dead cells (Srinath et al., 2002). Also, in some microbes like *Zoogloea ramigera*, *Klebsiella aerogenes*, *Arthrobacter viscosus* and *Pseudomonas* sp., the exopolymers of capsule/slime layer are the major site of metal biosorption. The Cr(VI) uptake is thought to be mediated by "acid adsorption" mechanism in which liquid should have enough protons to cause anion exchange and after accumulation, Cr(VI) may act as terminal electron acceptor, is reduced to Cr(III) and binds to cell wall (Cabrera et al., 2007; Srinath et al., 2002).

The microbial bioaccumulation comprises of two phases: an initial rapid phase involving physical adsorption or ion exchange at cell surface and by a subsequent slower phase involving active metabolism-dependent transport of Cr(VI) into the bacterial cells. During the bioaccumulation process, many features of a living cell like intracellular sequestration followed by localization within specific organelles, metallothionein binding, particulate metal accumulation, extracellular precipitation and complex formation can occur (Shukla et al., 2009; Cabrera et al., 2007; Chandra et al., 2011).

GC-MS analysis of ethyl acetate extracts from untreated and treated (free and immobilized cells) samples are presented in Table 3 and Fig. 3a-c. The compounds identified by GC-MS analysis in present study were almost similar to earlier reports (Chandra et al., 2011; Naumczyk and Rusiniak, 2005). GC-MS analysis of untreated tannery effluent revealed the presence of various types of organic compounds including benzene (RT = 8.8), hexanoic acid (RT = 9.8), 4-methylvaleric acid (RT = 9.9), 3-methylbutanoic acid (RT = 11.4), benzoic acid (RT = 12.6), eicosane (RT = 13.5), decanoic acid (RT = 13.9), 2-butenoic acid (RT = 14.8), benzene propanoic acid (RT = 15.5), 2-oxovaleric acid (RT = 16.04), 1,1-dimethylethyl-2-phenylethiazole (RT = 16.9), 3-methoxy-4-benzaldehyde (RT = 17.6) and acetylthiocarbamic acid (RT = 20.3) (Fig. 3a). GC-MS analysis of post-treatement tannery effluent by both methods (free and immobilized cells) showed presence of new peaks suggesting that these peaks belong to degradation products (Table 3, Fig. 3b–c). Also, the initial peaks observed in untreated sample were mostly absent or were at a much reduced levels. Effluent treated by immobilized cells showed presence of benzene (RT = 8.8) and heptadecane (RT = 10.6) (Fig. 3c). Caprolactum at RT = 6.3 was detected in both free and immobilized cells treated samples. The study confirms that pollutants were actively being metabolized by the bacterial cells and isolated *Bacillus cereus* strain Cr1 was capable of using the effluent being discharged from CETP for its growth and metabolism and can be used for bioaugmentation of CETP for effective effluent bioremediation.

Table 3
Organic pollutants and their degradation products identified as trimethyl silyl (TMS) derivatives in ethyle extracts of untreated (a) and free (b) and immobilized (c) cells treated tannery effluent samples as given in Fig. 3.

Retention time (in min)	Compounds	Present/ absent in		
		a	b	c
6.3	Caprolactum	−	+	+
8.8	Benzene	−	−	+
9.7	Octacosane	−	+	−
9.8	Hexanoic acid	+	−	−
9.9	4-methylvaleric acid	+	−	−
10.3	not identified	−	−	+
10.4	10-methylnonadecane	−	+	−
10.6	Heptadecane	−	−	+
11.3	Tetratetracontane	−	+	−
11.4	3-methylbutanoic acid	+	−	−
12.6	Benzoic acid	+	−	−
13.5	Eicosane	+	−	−
13.9	Decanoic acid	+	−	−
14.8	2-butenoic acid	−	+	−
15.5	Benzene propanoic acid	+	+	−
16.04	2-oxovaleric acid	−	+	−
16.3	1,2-benzenedicarboxylic acid	−	+	−
16.9	1,1-dimethylethyl-2-phenylethiazole	+	−	−
17.6	3-methoxy-4-benzaldehyde	+	−	−
20.3	Acetylthiocarbamic acid	+	−	−

3.5. Toxicity and genotoxicity evaluation of tannery effluent before and after bacterial treatments

The *Allium* test has often been used for the determination of cytotoxic and/or genotoxic potential of a mixture of pollutants

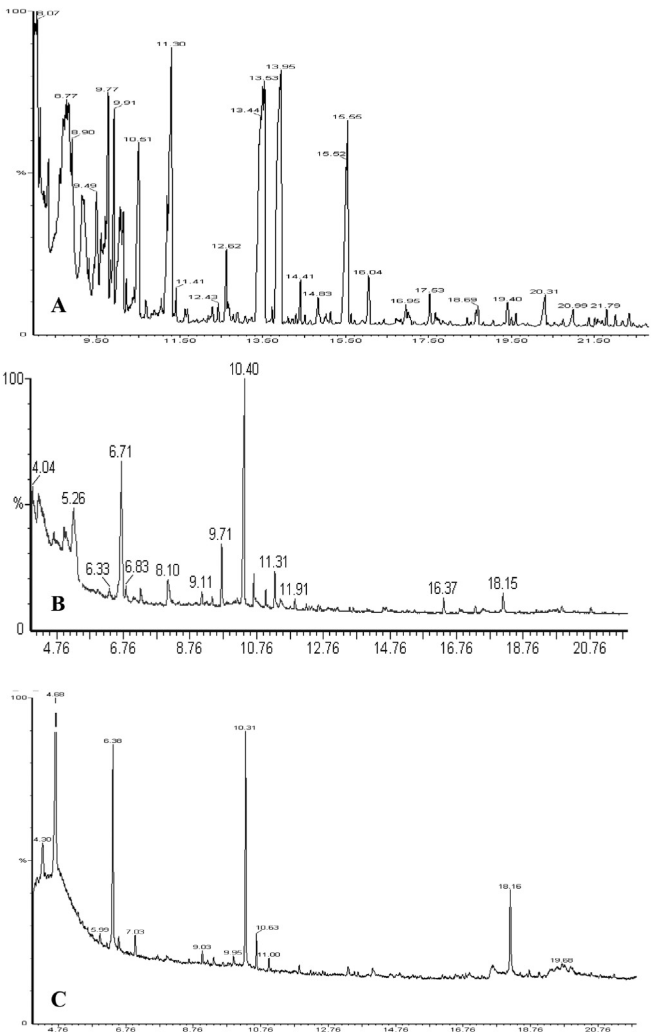

Fig. 3. GC–MS chromatograms of compounds extracted with ethyl acetate from control (a), free cell treated (b) and immobilized cell treated (c) tannery effluents. The MS-identified compounds with respect to their retection time are listed in Table 3.

including heavy metals, hydrophilic and lipophilic chemicals and industrial effluents (Leme and Marin-Morales, 2009). It is considered to be a standard procedure for quick testing and detection of toxicity and pollutant load of environmental samples. In this study, *A. cepa* root tip cells were used to measure phytotoxic, cytotoxic and genotoxic effects of untreated and bacterial treated tannery effluent by analyzing root growth, mitotic index and the number of chromosomal aberrations, respectively.

The effect of different concentrations of untreated and bacterial treated tannery effluent (free and immobilized cells) on the root growth of *A. cepa* was analyzed (Fig. 4), which showed concentration dependent root growth inhibition. Compared to control (8.8 cm), *A. cepa* exposed to untreated and treated (free and immobilized cells) effluent had 91%, 58% and 32% less root length, respectively. The strong inhibitory effect of untreated tannery effluent on root growth might be due to the high salt load, which induces high osmotic pressure and affected root growth. This observation was in accordance with the data reported earlier (Gupta et al., 2012).

Mitotic index (MI) shows the proliferation status of a cell and its inhibition could be interpreted as cellular death or a delay in the cell proliferation (Rojas et al., 1993). The result of our study showed that the MI decreased progressively with the increasing

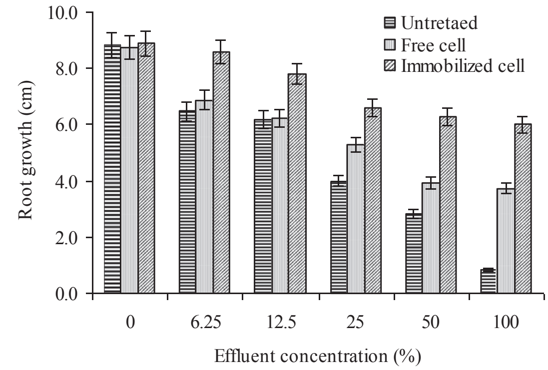

Fig. 4. Effect of different concentrations of untreated and bacterial treated (free and immobilized cell) tannery effluent on root growth of *A. cepa* after 120 h.

concentration of tannery effluent (Table 4). This supports our earlier observation of decrease in root tip length with increasing concentration of effluent. The *A. cepa* root tip cells exposed to 25% v/v concentration of untreated and treated (free and immobilized cells) effluent had 81%, 69% and 57%, respectively, compared to control. The strong decrease in MI by untreated effluent suggested presence of cytotoxic compounds which might also be affecting DNA and proteins synthesis and thus preventing the cell from entering mitosis. Compared to untreated effluent, treated (free and immobilized cells) effluent induced higher MI values. This again confirms our earlier observation in GC-MS study of decrease in pollutants load due to biodegradative activity of bacteria. The decreased pollutant load also leads to decrease in cytotoxic effect and an increase in MI value. Various heavy metals and effluent including tannery effluent are reported to reduce root growth and MI of the root tip cells of *A. cepa* (Gupta et al., 2012; Shrivastava, 2015; Aslam et al., 2014; Olusegun et al., 2010).

Induction of chromosomal aberrations were observed in roots tip cells of *A. cepa* exposed to tannery effluent (Table 4 and Fig. 5). No chromosomal aberrations were observed in control cells exposed to tap water. Although, exposure to untreated and treated effluents induced chromosomal aberrations, the degree of aberration varied with maximum aberrations being observed with untreated effluent. The observed chromosomal aberrations were: sticky metaphase (Fig. 5a), anaphase bridge (Fig. 5b), tripolar anaphase (Fig. 5c), unequal separation of chromosome at anaphase stage (Fig. 5d), anaphase break (Fig. 5e), binucleate (Fig. 5f), micronucleated cell (Fig. 5g), c-mitosis (Fig. 5h) and laggard anaphase (Fig. 5i) in all treatments. The most frequent aberrations were bridges and sticky chromosome and the frequency was highest with untreated effluent (Table 4). Chromosomes with vagarant and tripolar anaphase were also in considerable number. Binucleated cells were observed in all effluents, while chromosomal break, c-mitosis and micronucleated cell were only detected in untreated effluent at 12.5% and 25%, v/v of treatment. The induction of high frequency of chromosomal aberrations by untreated effluent occurs primarily by their effect on mitotic spindle, which results in disorientation of chromosomes at various stages of cell cycle (Sudhakar et al., 2001). The frequency was lower in bacterial treated tannery effluents. Especially, severe chromosomal aberrations stages like chromosome break, c-mitosis and micronucleated cells were not detected in both treatments. This again confirmed our earlier observation of biodegradation of tannery effluent leading to reduced cytotoxicity and also reduction of genotoxic substances.

Sticky chromosomes were most frequent in all tested concentrations. According to Odeigah et al. (1997), sticky chromosomes

Table 4
Effect of tannery effluents on mitotic index and chromosomal aberrations in root tip cells of *A. cepa*.

Treatments	Conc. (%)	Mitotic Index (%)[a]	Chromosome aberrations								
			Stickiness	Bridge	Vagrant	Tripolar	Bionucleated	Break	c-metaphase	Micronuclei	%Aberrant cells (±SD)
Tap water	100	13.8	0	0	0	0	0	0	0	0	0.0 ± 0.0
Tannery effluent	6.25	7.1	15	10	5	8	5	0	0	0	4.3 ± 0.2
	12.5	5.4	28	20	8	10	8	1	1	3	7.5 ± 0.4
	25	2.7	55	25	12	12	10	3	5	8	10.1 ± 0.5
Free cells treated tannery effluent	6.25	9.5	10	7	3	5	3	0	0	0	2.7 ± 0.1
	12.5	7.0	19	15	5	8	5	0	0	0	5.2 ± 0.3
	25	4.3	35	19	8	7	6	0	0	0	7.1 ± 0.4
Immobilized cells treated tannery effluent	6.25	11.5	4	3	2	3	2	0	0	0	1.3 ± 0.1
	12.5	8.2	10	12	3	5	2	0	0	0	3.1 ± 0.2
	25	6.0	28	15	5	3	2	0	0	0	5.1 ± 0.3

[a] Mitotic index was *calculated as (number* of dividing cells/number of total observed cells) x 100.

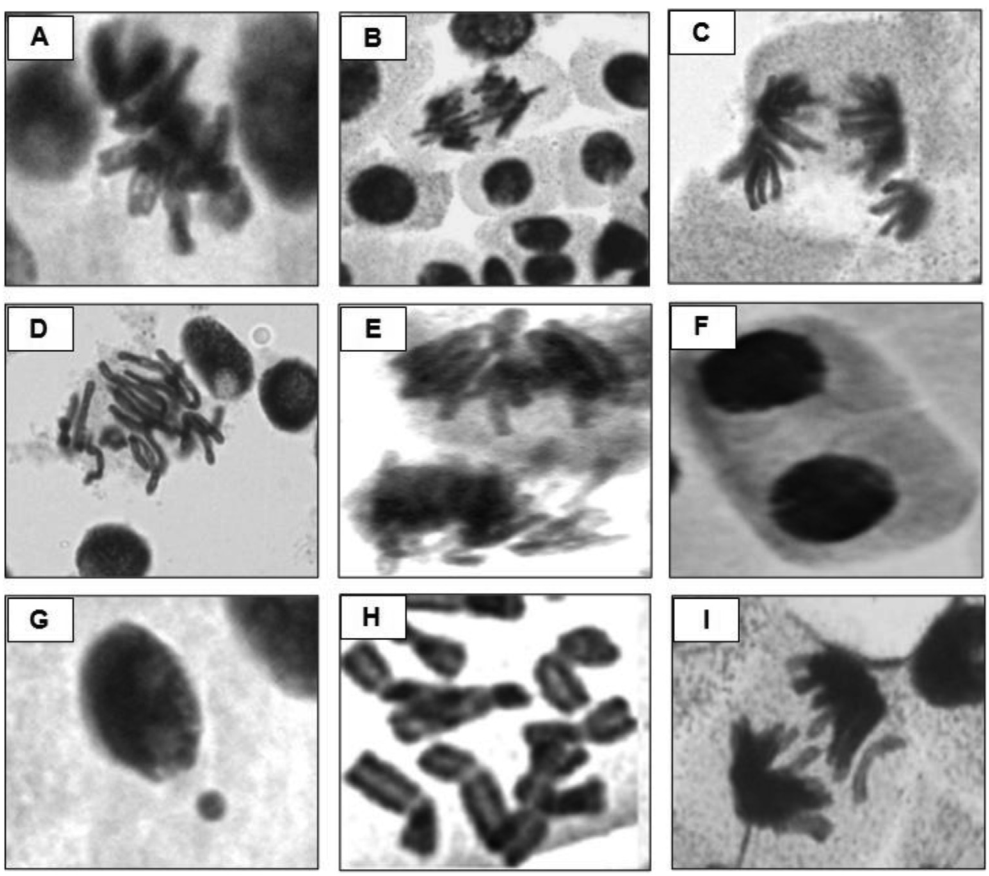

Fig. 5. Chromosome aberrations observed in root tip cells of *A. cepa* exposed to tannery effluents (6.25–25% conc.) A-sticky metaphase, B-anaphase Bridge, C-tripolar anaphase, D-unequal separation of chromosome at anaphase stage, E-anaphase break, F- bionucleate, G-micronucleated cell, H- C-mitosis and I-laggard anaphase.

are indicative of a highly toxic, usually irreversible effect, leading to cell death. Chromosome stickiness is caused probably by DNA-DNA or DNA-protein cross-linking (Amin, 2002). Sticky chromosome may also occure due to incomplete replication of chromosomes by defective enzymes (Bennet, 1977). Chromosome bridges result from chromosome and/or chromatid breaks, indicating the mutagenic event in the cell, whereas vagarant chromosomes and c-metaphase increases the risk of aneuploidy (Leme and Marin-Morales, 2009). The small micronuclei arising from chromosomal breaks and detected after exposure to untreated tannery effluent are indicative of clastogenic action. Various heavy metals and industrial effluents are reported to be induce chromosome breaks, c-metaphase, fragments, and micronucleus formation in root tip cells of *Allium* (Gupta et al., 2012; Shrivastava, 2015; Aslam et al., 2014; Olusegun et al., 2010) and their effects were emphasized to be the result of formation of DNA–DNA and DNA-protein cross-link (Costa et al., 1994).

4. Conclusions

The present study reports the isolation and characterization of a Cr(VI)-reducing *B. cereus* strain Cr1 from tannery effluent contaminated soil. Treatment of tannery effluent by free and immobilized cells of *B. cereus* resulted in the reduction of TDS, EC, BOD, COD and chromium. GC-MS analysis revealed that most of the compound present in untreated tannery effluent were biodegraded after treatment with free and immobilized cells. Further, the genotoxicity of treated tannery effluent was reduced and the treated effluent was able to support the root growth of *A. cepa* even in undiluted form suggesting improved properties of biologically treated effluent. The present study demonstrates that isolated *Bacillus cereus* strain Cr1 was capable of using the effluent being discharged from CETP for its growth and metabolism. Also, present bacterial isolate can be used for bioaugmentation of CETP for effective effluent bioremediation. Also, immobilized bacterial cells were better in treating the effluent.

Acknowledgements

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Stress response of *Triticum aestivum* L. and *Brassica juncea* L. against heavy metals growing at distillery and tannery wastewater contaminated site

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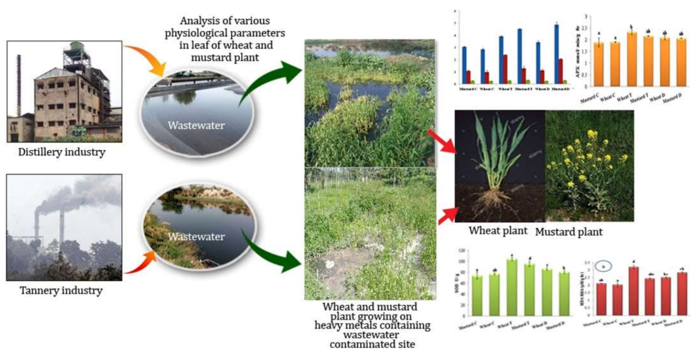
HIGHLIGHTS

- Industrial wastes contains high metals content and BOD, COD values.
- Metals induces antioxidant enzymes SOD, APx, GPX, MDA, H_2O_2, and CAT.
- Mustard plant exhibited high anti-oxidant responses.
- Translocation factor >10 (10.31) of Cr in tannery mustard plants.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aimed to investigate the effects of potentially toxic elements on biochemical parameters in wheat (*Triticum aestivum* L.) and mustard (*Brassica juncea* L.) plants growing at distillery and tannery wastewater contaminated sites. The analysis of plants showed the highest accumulation of Fe (361 mg kg^{-1} in wheat root and 359 mg kg^{-1} in mustard leaves) followed by Zn, Cr and Mn in leaf>-shoot>root. Further, the Chl-a, b, and carotenoids content was also found high in plant samples. Results also showed that photosynthetic content in wheat and mustard growing at tannery wastewater contaminated sites was Chl-a 3.92, 4.53 (mg g^{-1} fw), Chl-b 2.39, 1.29 (mg g^{-1} fw) and carotenoids 0.28, 0.32 (mg g^{-1} fw), respectively. Whereas, photosynthetic content in these plants with distillery waste was as Chl-a 3.43, 4.88 (mg g^{-1} fw), Chl-b 1.12, 2.05 (mg g^{-1} fw) and carotenoids 0.24, 0.29 (mg g^{-1} fw), respectively. In addition, the activity of plant enzymes such as SOD, APx, GPX, MDA, H_2O_2, and CAT was also higher in selected plants in comparison to control plants. Moreover, the high bioconcentration factor of Zn > 1 (1.29) and translocation factor >10 (10.31) of Cr in tannery wastewater affected mustard plants. This study concluded that industrial wastewaters are the primary sources of metal accumulation in agricultural crops and thus, it should not be discharged into the environment before its proper treatment.

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Detection and Characterization of a Multi-drug and Multi-metal Resistant Enterobacterium *Pantoea* sp. from Tannery Wastewater after Secondary Treatment Process

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Abstract

In this study, an enterobacterium was isolated from treated tannery wastewater and characterized as gram-negative, rod shaped, motile, and lactose fermenting bacterium. Further, based on the 16S rRNA gene sequence analysis, the bacterium was identified as *Pantoea* sp. with accession number KJ576899. The antibiotic and heavy metal resistant property of isolated bacterium was investigated by the disk diffusion method on Muller-Hinton and nutrient agar medium amended with the increasing concentrations of various toxic metal ions, respectively. Results showed that the isolated bacterium was sensitive for amikacin, ampicillin, cefepime, cetriaxone, chloramphenicol, levofloxacin, meropenem, nalidixic acid, piperacillin and tobramycin, resistant for aztreonam, carbenicillin, cefotaxime, ceftazidime, ciprofloxacin, cotrimoxazole, imepenam, moxifloxacin, streptomycin and tetracycline, but intermediate for amoxicillin and gentamicin. In addition, the bacterium also showed the Minimum Inhibitory Concentration (MIC) values of 250, 500, 160, 190, 600, 700, 500, 380, 600 and 350 $\mu g\ ml^{-1}$ for Cu, Cr, Cd, Co, Zn, Fe, Ni, Pb, Mo and As, respectively with a plasmid DNA of 33.5 kb. This multi-drug and multi-metal resistant bacterium can be used as a potential agent for the bioremediation of metal contaminated sites.

1. Introduction

Tannery industries are one of the major sources of soil and water pollution mainly causing chromium pollution in environment. In India, there are more than 2,500 tanneries and most of them (nearly 80%) are engaged in chrome tanning process (Chandra *et al.*, 2011). During the tanning process, chromium salt is used to convert hides into leather and the wastewater generated contains huge amount of organic matter, phenolics, tannins and toxic metals mainly chromium (Chandra *et al.*, 2011). This wastewater containing a variety of toxic pollutants when discharged into the environment causes serious soil and water pollution along with serious health hazards to humans, animals as well as plants (Ramteke *et al.*, 2010; Flores *et al.*, 2012).

The organic pollutants present in tannery wastewater do not degrade much during the treatment process carried out at Effluent Treatment Plant (ETP) in industries as various authors have reported the presence of numerous recalcitrant organic pollutants and pathogenic microbes in wastewater discharged from industries even after the secondary treatment process (El-Lathy *et al.*, 2009; Ramteke *et al.*, 2010; Chandra *et al.*, 2011). The organic pollutants remained in tannery wastewater after the secondary treatment process provide chance to a variety of pathogenic and non-pathogenic microbes to flourish and contaminate the aquatic environments, whereas toxic metals induce genotoxic and mutagenic changes in bacterial communities making them resistant against a wide spectrum of antibiotics and toxic metals (Filali *et al.* 2000; Malik and Jaiswal, 2000; Viti *et al.*, 2003).

In literature, sewage contamination is reported as a major source of pathogenic bacteria in water resources, but few authors also suggested that besides sewage contamination, the wastewaters discharged from different industries such as distillery, pulp paper mills and tannery industries etc. also act as a good source of nutrients and support the growth of pathogenic microbes in receiving water bodies (El-Lathy *et al.*, 2009; Ramteke *et al.*, 2010; Chandra *et al.*, 2011). However, the detail information about the pathogenic microbes that remained in tannery wastewater even after the secondary treatment process is not available so far.

5

Organic Pollutants and Pathogenic Bacteria in Tannery Wastewater and their Removal Strategies

ASHUTOSH YADAV[1], SANDHYA MISHRA[1], GAURAV KAITHWAS[2], ABHAY RAJ[3] AND RAM NARESH BHARAGAVA[1*]

ABSTRACT

Tannery industries are one of the major sources of environmental pollution as the wastewater discharged from industries contain high concentration of organic pollutants, organic matter, phenolics, tannins and heavy metals mainly chromium as environmental pollutants. The organic pollutants present in tannery wastewater are not completely degraded even after the secondary treatment process and support the growth of pathogenic microbes, which goes into the aquatic environment along with tannery wastewater and cause serious health hazards to living organisms. Therefore, it is very essential to treat the tannery wastewater adequately for the complete removal of organic pollutants and pathogenic microbes for the safety of aquatic resources and human as well as animal health. Thus, this book chapter provides the detailed information on the various types of organic pollutants and pathogenic bacteria that remained even after the secondary treatment process as well as various types of environmental problems and health hazards contributed by these organic pollutants and pathogenic bacteria. In addition, this chapter also covers the recent advancements, merits and demerits of various treatment methods used for the treatment of tannery wastewaters to remove organic pollutants and pathogenic bacteria at common effluent treatment plant of tannery industries.

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9 Toxic Metals in the Environment

Threats on Ecosystem and Bioremediation Approaches

Ashutosh Yadav, Pankaj Chowdhary, Gaurav Kaithwas, and Ram Naresh Bharagava

CONTENTS

Chapter 5
Heavy Metal Contamination: An Alarming Threat to Environment and Human Health

Sandhya Mishra, Ram Naresh Bharagava, Nandkishor More, Ashutosh Yadav, Surabhi Zainith, Sujata Mani, and Pankaj Chowdhary

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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.

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CHAPTER

12

Ligninolytic Enzymes: An Introduction and Applications in the Food Industry

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12.1 INTRODUCTION

Ligninolytic enzymes are highly versatile and ubiquitous in nature, known for their role in the degradation of various complexes and recalcitrant polymers (Chandra and Chowdhary, 2015; Chowdhary et al., 2016; Wong, 2009). Naturally, there are a number of microbes that are capable of producing ligninolytic enzymes, but among these, white-rot fungi is more effective to produce many enzymes such as manganese peroxidase (MnP) and lignin peroxidase (LiP). The activity of laccase, MnP, and LiP is further enhanced due to the cooperative activity of some other enzymes such as glyoxal oxidase, aryl alcohol oxidase (veratryl alcohol oxidase), pyranose 2-oxidase (glucose 1-oxidase), cellobiose/quinone oxidoreductase, and cellobiose dehydrogenase. Laccases are also known as polyphenol oxidases and enhance the oxidation of several aromatic compounds (phenolic and nonphenolics), especially those having electron donor groups such as anilines and phenolics using molecular oxygen as a terminal electron acceptor (Gianfreda et al., 1999). The food processing industries produce a huge volume of wastewater globally, which can cause various environmental problems. Food industry wastewater is highly rich in derivatives of sugars, which, due to their organic nature, get easily utilized by microbes as C, N, and energy sources. Hence it is preferred as a cheap raw material for the production of secondary metabolites of industrial importance (Piontek et al., 2002). The Laccase, MnP, and LiP play a key role in different industrial processes such as

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