Bacterial degradation of persistent organic pollutants from tannery wastewater after secondary treatment process

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

LUCKNOW

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प्रज्ञा शील करुणा

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2019

Dedicated to My Beloved Family

CERTIFICATE

This is to certify that the thesis entitled **"Bacterial degradation of persistent organic pollutants from tannery wastewater after secondary treatment process"** submitted by **Mr. Gaurav Saxena** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) Regulations - 1999 as amended in 2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Date:

Supervisor

Head of Department

DECLARATION

I, **Gaurav Saxena** hereby declare that the work which is being presented in the thesis entitled **"Bacterial degradation of persistent organic pollutants from tannery wastewater after secondary treatment process"** in the partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (Central) University, Lucknow, Uttar Pradesh (India) is an authentic record of my own work carried out during the period from October 2013 to April 2019 under the supervision of **Dr. Ram Naresh Bharagava,** Assistant Professor, Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (Central) University, Lucknow, Uttar Pradesh (India).

The thesis is essentially free from all kinds of plagiarism and the work has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university."

(GAURAV SAXENA)

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"The three great essentials to achieve anything worthwhile are, first, hard work; second, stick-to-itiveness; third, common sense."

Thomas Alva Edison, American Inventor (1847-1931)

Gaurav Saxena

Babasaheb Bhimrao Ambedkar (Central) University
Lucknow, Uttar Pradesh, India, April 2019

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List of Abbreviations

LLE	**:**	Liquid-liquid extraction
BSTFA	**:**	N, O-bis (trimethylsilyl) trifluoroacetamide
N	**:**	Normal
ASP	**:**	Activated sludge process
ASTP	**:**	Activated sludge treatment process
AFP	**:**	Advanced facultative pond
AIWTPs	**:**	Advanced integrated wastewater treatment pond system
AOPs	**:**	Advanced oxidation processes
ABR	**:**	Anaerobic baffled reactor
AF	**:**	Anaerobic filters
AP	**:**	Anaerobic pond
BDL	**:**	Below detection limit
BBP	**:**	Benzyl butyl phthalate
BAT	**:**	Best available techniques
BLAST	**:**	Basic local alignment search tool
BOD	**:**	Biological oxygen demand
COD	**:**	Chemical oxygen demand
BT	**:**	Bioremediation technology
BT-TWW	**:**	Bacterially treated tannery wastewater
CASTP	**:**	Conventional activated sludge treatment process
CETPs	**:**	Common effluent treatment plants
CTs	**:**	Clean technologies
cm	**:**	Centimeter
Conc.	**:**	Concentration
CWs	**:**	Constructed wetlands (CWs)
CPCB	**:**	Central pollution control bard
CTTs	**:**	Combined treatment technologies
dATP	**:**	Deoxyadenosine triphosphate
dCTP	**:**	Deoxycytidine triphosphate
DGGE	**:**	Denaturation gradient gel electrophoresis
dGTP	**:**	Deoxyguanosine triphosphate
DEHP	**:**	Di-(-2ethyl hexyl)phthalate
DBP	**:**	Dibutyl phthalate
DCM	**:**	Dichloromethane
DO	**:**	Dissolve oxygen
DW	**:**	Distilled water
DAF	**:**	Down-flow anaerobic filters
dTTP	**:**	Deoxythymidine triphosphate
EC	**:**	Electrical conductivity
EDCs	**:**	Endocrine disrupting chemicals
EPA	**:**	Environmental protection agency

ETPs	**:**	Effluent treatment plants
ETTs	**:**	Emerging treatment technologies
ECHA	**:**	European chemical agency
EGSB	**:**	Expanded granular sludge bed
FAS	**:**	Ferrous Ammonium Sulphate
FT-IR	**:**	Fourier transform-infrared
GC	**:**	Gas chromatography
GC-MS	**:**	Gas chromatography-mass spectroscopy
GI	**:**	Germination index;
GP	**:**	Germination percentage
GTs	**:**	Greener technologies
HMs	**:**	Heavy metals
HP-LC	**:**	High performance-liquid chromatography
HMBR	**:**	Hybrid membrane bioreactor
IR	**:**	Infrared
MP	**:**	Maturation pond
MEGA	**:**	Molecular evolutionary genetics analysis
MBR	**:**	Membrane bioreactor
MTs	**:**	Membrane technologies
MF	**:**	Microfiltration
MLD	**:**	Million liter per day
MOEF&CC	**:**	Ministry of environment, forest and climate change
MRVP	**:**	Methyl red voges proskauer's
MSM	**:**	Mineral salt medium
NAM	**:**	Nutrient agar medium
NF	**:**	Nanofiltration
NCBI	**:**	National council for biotechnological information
NIST	**:**	National institute of standards and technology
ND	**:**	Not detected
NPE	**:**	Nonyl ethoxyphenol
NP	**:**	Nonylphenol
OECD	**:**	Organization for economic cooperation & development
ONPG	**:**	o-nitrophenyl-ß-D-galactopyranoside
OPs	**:**	Organic pollutants
PCMC	**:**	*p*-chloro-*m*-cresol
PCR	**:**	Polymerase chain reaction
PCP	**:**	Pentachlorophenol
PAFC	**:**	Poly-aluminium ferric chloride
PaSiC	**:**	Poly-aluminium silicate (PASiC)
PAHs	**:**	Polyaromatic hydrocarbons (PAHs
PCBs	**:**	Polychlorinated biphenyls
PCR	**:**	Polymerase chain reaction
POPs	**:**	Persistent organic pollutants
PP	**:**	Phytotoxicity percentage

RAPD	**:**	Random amplified polymorphic DNA
ROPs	**:**	Recalcitrant organic pollutants
RT	**:**	Retention time
RO	**:**	Reverse osmosis
RL	**:**	Root length
rpm	**:**	Revolution per minute
rRNA	**:**	Ribosomal Ribonucleic acid
RSR	**:**	Root-shoot ratio
SD	**:**	Standard deviation
SFP	**:**	Secondary facultative pond
SBR	**:**	Sequencing batch reactor
SL	**:**	Shoot length
SLM	**:**	Seedling mortality
SVHC	**:**	Substances of very high concern
SLMs	**:**	Supported liquid membranes
SVI	**:**	Seed vigour index
STs	**:**	Syntans
TPCB	**:**	Tamilnadu pollution control board
TWW	**:**	Tannery wastewater
TMCS	**:**	Trimethylchlorosilane
TMS	**:**	Trimethyl silyl
TOC	**:**	Total organic carbon
TDS	**:**	Total dissolved solids
TS	**:**	Total solid
TSS	**:**	Total suspended solids
TP	**:**	Tap water
USPHS	**:**	United States public health service
UAE	**:**	United Arab Emirates
UK	**:**	United Kingdom
UF	**:**	Ultrafiltration
UNIDO	**:**	United nations industrial development organization
UAF	**:**	Upflow anaerobic filters
UASB	**:**	Upflow anaerobic sludge blanket
USA	**:**	United States of America
USEPA	**:**	United States environmental protection agency
UT-TWW	**:**	Untreated tannery wastewater
UV	**:**	Ultra violet
WWTPs	**:**	Wastewater treatment Plants
Amu	**:**	Atomic mass unit
OD	**:**	Optical density
PAGE	**:**	Polyacrylamide gel electrophoresis
SDS	**:**	Sodium dodecyl sulfate
TEMED	**:**	N, N,N’N’-tetramethylethylenediamine

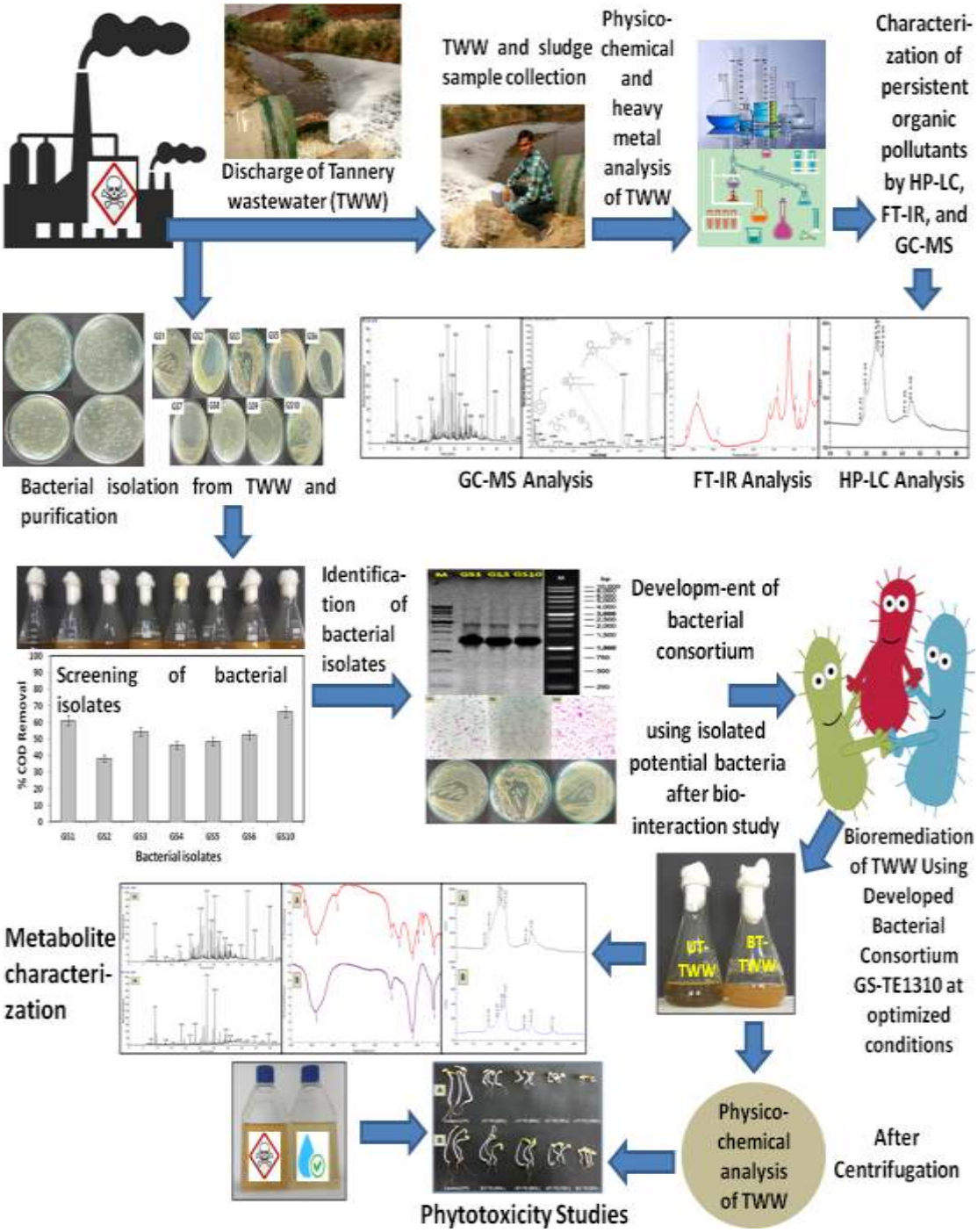
List of Symbols

~	**:**	Approximately equal
<	**:**	Less than
>	**:**	Greater than
=	**:**	Equal
±	**:**	Plus - minus
≥	**:**	Greater than or equal to
°C	**:**	Degree Celsius
%	**:**	Percentage
µl	**:**	Microliter
Co	**:**	Copper
Cr(III)	**:**	Trivalent chromium (Cr^{3+})
Cr(VI)	**:**	Hexavalent chromium (Cr^{6+})
Cr	**:**	Chromium
eV	**:**	Electron volt
Fe	**:**	Iron
Fig	**:**	Figure
g	**:**	Gram and gradient
g/L	**:**	Gram per liter
h	**:**	Hour
K	**:**	Potassium
M	**:**	Molar
M sq. feet	**:**	Meter square feet
m^3	**:**	Cubic meter
Mg	**:**	Magnesium
mgL^{-1}	**:**	Milligram per liter
IN	**:**	India
µS/cm	**:**	micro-siemens per centimeter
mS/cm^{-1}	**:**	Millisiemens per centimeter
min	**:**	Minute
ml	**:**	Milliliter
Mm	**:**	Millimolar
mM	**:**	Micromolar
Mn	**:**	Manganese
Ng	**:**	Nanogram
nm	**:**	Nanometer
P	**:**	Phosphorous
pH	**:**	Power of hydrogen
US$	**:**	United States dollar (USD)
v/v	**:**	Volume by volume
v	**:**	Volume
w/v	**:**	Wight by volume

Zn	**:**	Zinc
µl	**:**	Microliter
µm	**:**	Micromolar
α	**:**	Alpha
λ	**:**	Wavelength
Kbp	**:**	Kilo base pair
kDa	**:**	Kilo dalton
m/z	**:**	Mass-to-charge ratio
3′	**:**	Three prime
5′	**:**	Five prime

Pictorial Travelogue

Graphical Abstract



सारांश

चमड़े के करखानों से निकालने वाला अनुपचारित / आंशिक रूप से उपचरित अपशिस्ट जल हमारे प्राकृतिक संसाधनों (जल और मिट्टी) को भारी रूप से प्रदूषित व नष्ट कर रहा है। इसलिए, पर्यावरण और सार्वजनिक स्वास्थ्य की सुरक्षा के लिए टेनरी अपशिष्ट जल के पर्याप्त जैवउपचार / जैवविषहरण की आवश्यकता होती है। इस वर्तमान अध्ययन में, एक नए जीवाणु कंसोर्टियम, जीएस-१३१० को तीन प्रदूषकों को छोटे-छोटे भाग में तोड़ने ब उनका विषहरण करने वाले जीवाणुओं, *ऑक्रोबैक्ट्रम इंटरमीडियम* जीएस १, *माइक्रोकॉकस लाइली* जीएस ३, और *स्टीनोट्रोफोमोनास एसिडामिनिफिला* जीएस १० का उपयोग करके विकसित किया गया था और टेनरी अपशिस्ट जल के छय और विषहरण के लिए इस्तेमाल किया गया था। परिणामों से पता चला था कि नए विकसित किया गए जीवाणु कंसोर्टियम, जीएस-१३१० द्वारा टेनरी अपशिस्ट जल का एक प्रभावी ढंग से १२० (७ पीएच, १२० आरपीएम, और ३५◦C तापमान) घंटे में जैवउपचार किया गया था। और ७६.१२, ८५.३२, ७१.८९, ४८.५९, ७८.८१, ६९.५३, ७१.२२, और ८८.७०% की कमी प्रदूषण मानकों जैसे रासायनिक ऑक्सिजन मांग (सीओडी), जैवरासायनिक ऑक्सिजन मांग (बीओडी), पूर्णतः घुले हुए ठोंस पदार्थ (टीडीएस), फॉस्फेट, सल्फ़ेट, नाइट्रेट, क्रोमियम और फिनोल, क्रमश: में दर्ज की गयी थी। एचपी-एलसी, एफटि-आईआर, और जीसी-एमएस विश्लेषण से पता चला था कि अनुपचारित टेनरी अपशिष्ट जल में पाए जाने वाले अधिकांश दृढ़ कार्बनिक प्रदूषकों को नए विकसित जीवाणु कंसोर्टियम, जीएस-टीई १३१० ने अनुकूलित स्थितियों (७ पीएच, ३५◦C तापमान, ०.५% ग्लूकोज और अमोनियम क्लोराइड (डब्लू/वी), १२० आरपीएम (आंदोलन दर), और २० मिलीलीटर इनोकुलम मात्रा) में पूरी तरह से खनिज/छोटे-छोटे भागों में नए मेटाबोलाइट के रूप में तोड़ दिया था। इसके अलावा, टेनरी अपशिस्ट जल के जैवउपचार के दौरान, केटेखोल १,२ डाइऑक्सीजीनेस गतिविधि का पता चला था। यह स्पष्ट रूप से इंगित करता है कि केटेखोल १,२ डाइऑक्सीजीनेस एंजाइम द्वारा कार्बनिक प्रदूषकों के क्षरण में एक महत्वपूर्ण भूमिका निभाई गयी है। केटेखोल १,२ डाइऑक्सीजीनेस का आणविक भार एसडीएस-पेज पर ~ ३२ केडीए निर्धारित किया गया था। इसके अलावा, जीवाणु-उपचारित टेनरी अपशिस्ट जल का उपयोग पौधों पर विषाक्तता के मूल्यांकन के लिए फेसेओलस ऑरियस का उपयोग एक स्थलीय मॉडल जीव के रूप में किया गया था। परिणामों से पता चला था कि जीवाणु-उपचारित टेनरी अपशिस्ट जल में विषाक्तता की भरी कमी हुई और बीजों के अंकुरण को ७०% तक बड़ा दिया था, और इस प्रकार, टेनरी अपशिस्ट जल के प्रभावी क्षरण / विषहरण की पुष्टि हुई। कुल मिलाकर, इस नए विकसित जीवाणु कंसोर्टियम जीएस-टीई १३१० ने पर्यावरणीय सुरक्षा के लिए टेनरी अपशिस्ट जल का कुशलतापूर्वक उपचार / विषहरण करने की आश्चर्यजनक क्षमता दिखाई।

कुंजीशब्द: टेनरी अपशिस्ट जल; सीओडी निष्कासन; जीवाणु कंसोर्टियम; जैवउपचार; फाइटोक्सिसिटी; पर्यावरणीय सुरक्षा

Abstract

The untreated/partially treated effluent discharged from leather tanning industries is heavily polluting/destroying our natural resources (water and soil) Hence, the adequate biotreatment/biodetoxification of tannery wastewater (TWW) is required to safeguard the environment and public health. In the present study, a new bacterial consortium GS-TE1310 was developed using three pollutants degrading/detoxifying bacteria, *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, and *Stenotrophomonas acidaminiphila* GS10 and used for the degradation and detoxification of TWW. Results revealed that an effective bioremediation of real TWW was attained by this newly developed bacterial consortium GS-TE1310 within 120 h (at 7 pH, 120 rpm, and 35°C) with 76.12, 85.32, 71.89, 48.59, 78.81, 69.53, 71.22, and 88.70% reduction in pollution parameters such as COD (1428 ± 5.56 mgL^{-1}), BOD (436 ± 4.58 mgL^{-1}), TDS (4064 ± 3.46 mgL^{-1}), phosphate (118.66 ± 5.03 mgL^{-1}), sulphate (6.75 ± 0.27 mgL^{-1}), nitrate (14.05 ± 0.16 mgL^{-1}), Cr (6.88 ± 0.02 mgL^{-1}), and phenol (8.68 ± 0.04 mgL^{-1}), respectively. The HP-LC, FT-IR and GC-MS analysis showed that most of the persistent organic pollutants detected in the untreated TWW were completely mineralized/degraded into new metabolites in the treated TWW by the newly developed bacterial consortium GS-TE1310 at the optimized conditions (7 pH, 35°C temperature, 0.5% glucose and ammonium chloride (w/v), 120 rpm (agitation rate), and 20 ml inoculum volume)). Moreover, during the bioremediation of TWW, catechol 1,2 dioxygenase activity was detected. This clearly indicated that the catechol 1,2 dioxygenase enzyme played an important in the degradation of persistent organic pollutants by the isolated bacterial strains. The molecular weight of catechol 1,2 dioxygenase was determined as ~ 32 kDa on denaturing SDS-PAGE. Further, the bacterially treated TWW was used for the phytotoxicity assessment using *Phaseolus aureus* L as a terrestrial model organism. Results revealed that the toxicity of bacterially treated TWW was reduced significantly allowing the 70% germination of the seeds, and thus, confirmed the effective degradation/detoxification of leather TWW. Overall, this newly developed bacterial consortium GS-TE1310 showed a astounding potential to efficiently treat/detoxify the leather TWW for environmental safety.

Keywords: Tannery wastewater; COD removal; Bacterial consortium; Bioremediation; Phytotoxicity; Environmental safety

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Chapter-01
General Introduction

Introduction

Chronologically, the birth of leather in India dates back to 3,000 years B.C. Leather and its products are the unique items known for their versatility, style, and fashion. Currently, leather is one of the most widely traded items in the world. The place where leather is produced is technically termed as the leather industry (LI). The leather industrial sector comprises tanneries (where hide and skins are transformed into leather/leather products) and manufacturing units (where leather footwear, garments and outerwear, and assorted leather goods are made). These production facilities are predominantly spread over the unorganized (mostly family owned) units/production centers which contribute almost 80% of the total production. Leather industries (LIs) are specialized in the processing of hide (skins of large animals such as cows, buffaloes, and horses) and skins (skins of small animals such as sheep, goats, and calves) for leather production. The hide/skins are available from animals that died naturally or are the by-products of meat and meat products industry. In India, the leather is mainly prepared from the raw hide/skin of different animals like buffalo, goat, cow, and sheep. Majorly, buffalo skin and goat skin are used to make leather products that are exported to the other countries. It is recorded that from total leather exports, 40% of buffalo and 30% of goat rawhide skins are used for leather production (https://www.ibef.org/blogs/leather-exports-from-india-going-strong). LIs use a variety of highly toxic chemicals during the processing of hide/skins to produce leather or leather products and chemicals used are discharged in the effluent that causes serious environmental pollution and toxicity in the environment. An overview of a leather industry with all its functions is depicted in Fig. 1.1.

LIs are the key economic drivers of many developing nations that significantly earn foreign exchange through leather exports and create employment opportunities for economically weaker sections. Approximately, 22,700.5 M ft^2 of leather is being made each year worldwide (FAO 2008) and the estimated world trade for the leather sector is US$100 billion/year (UNIDO 2010). The leather industry is one of the oldest industries in India. Indian leather industry is the 6^{th} largest in the World, 2^{nd} largest producer of footwear and leather garments, 3^{rd} largest producer of saddlery and harness items (ILTIP 2010). India leather industry generates employment for 2.5 million people, mostly from the weaker sections with 30% women predominance. Indian leather sector stands at USD 17.85 billion (Exports: USD 5.85 billion,

Domestic Market: USD 12 billion) (http://www.makeinindia.com/sector/leather). Nearly 60-65% of the leather is mainly produced in the small/micro leather industrial sectors in India. India is also the 5th largest exporter of leather goods and accessories in the world with a lion's share of 24.27% in the country's export of leather & leather products (ILTIP 2010).

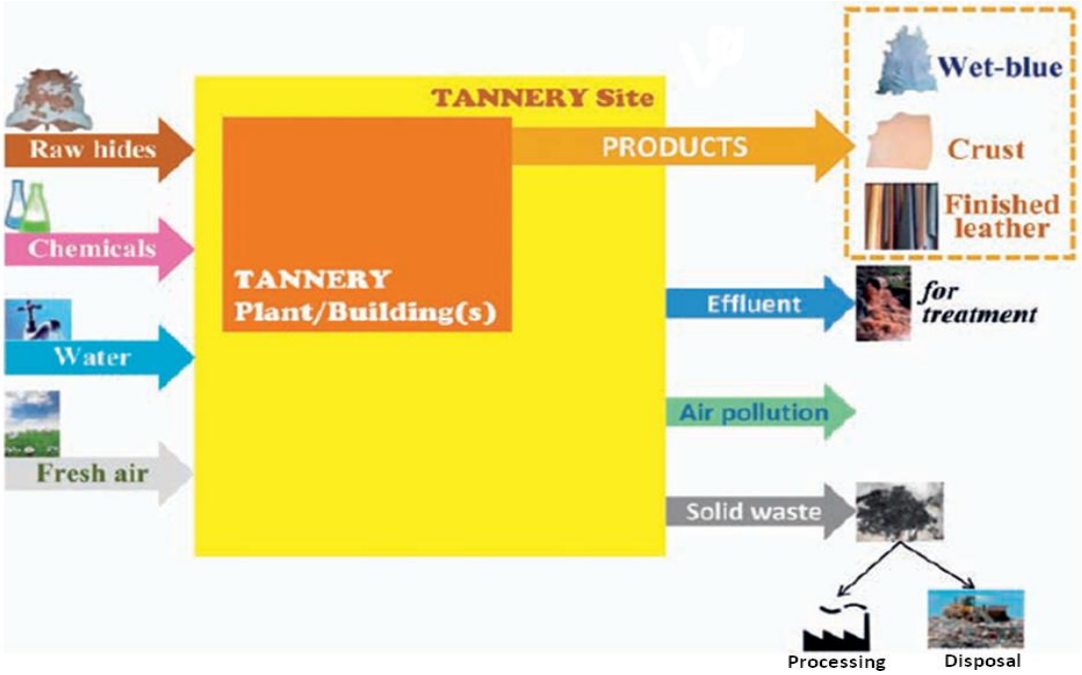


Fig. 1.1 An overview of a leather industry with all the functions (adapted from UNIDO 2011)

LIs are the key economic drivers of many developing nations that significantly earn foreign exchange through leather exports and create employment opportunities for economically weaker sections. Approximately, 22,700.5 M ft^2 of leather is being made each year worldwide (FAO 2008) and the estimated world trade for the leather sector is US$100 billion/year (UNIDO 2010). European Union is one of the major markets for the Indian leather products and shares 65.57% of all India exports of leather and leather products whereas Italy is the second largest market in the world for Indian leather products with a share of 12.82 % (ILTIP 2010). In addition to this, Germany (14.12%), UK (11.48%), USA (9.98%), Hong Kong (6.61%), Spain (6.09%), France (6.14%), Netherlands (4.13%), UAE (2.38%) and Australia (1.55%) comprise 75.30% of India's total leather products export (Fig. 1.2).

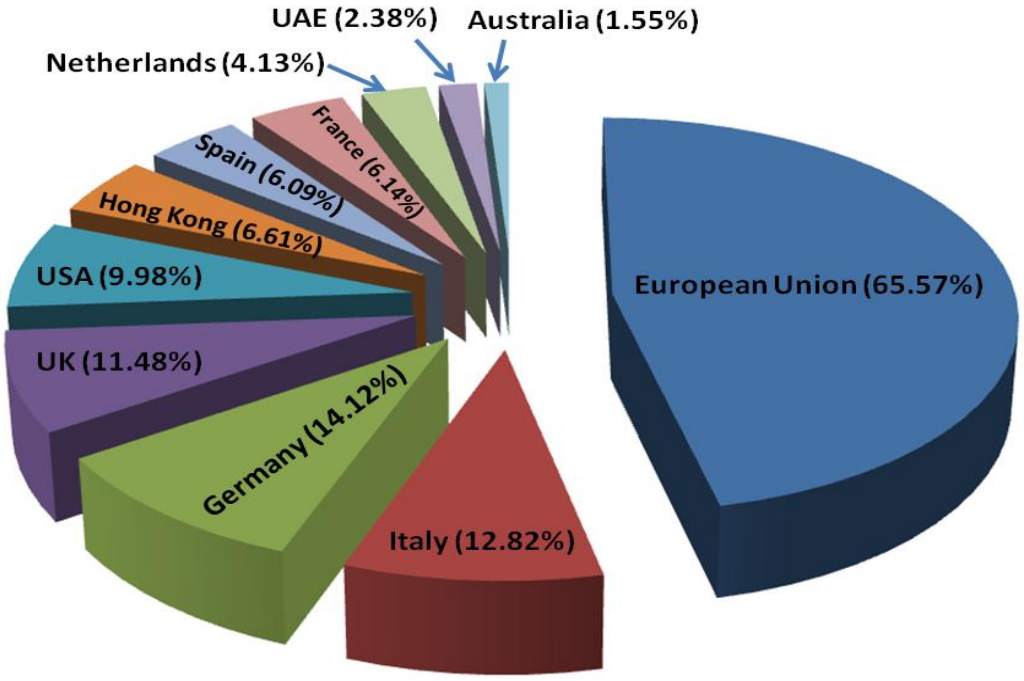


Fig. 1.2 Country-wise share in the total leather & leather products exports from India

Indian leather industry ranks amongst the five topmost export-oriented industries of the country and is among the top ten export earning industries of the country. The major production centres of leather and leather products are located mainly in the eight states of India namely Tamil Nadu (Chennai, Ambur, Ranipet, Vaniyambadi, Trichy and Dindigul), West Bengal (Kolkata), Uttar Pradesh (Kanpur and Agra), Punjab (Jalandhar), Delhi, Andhra Pradesh (Hyderabad), Karnataka (Bangalore) and Maharashtra (Mumbai). Tamil Nadu, by the number of production centers located, is the biggest leather exporter of the country (Fig. 1.3).

About 75% of the tanneries are running in the cottage and small-scale sector, about 20% are running in the medium and only about 5% are running in the medium/large sector. India is the fifth-largest exporter of leather goods and accessories in the world. Leather sector is dominated by micro and small units with bigger units accounting for just around 5% of total manufacturing units. The leather industry is spread in different segments, namely, tanning and finishing, footwear and footwear components, leather garments, leather goods including saddlery and harnesses (Fig. 1.4).

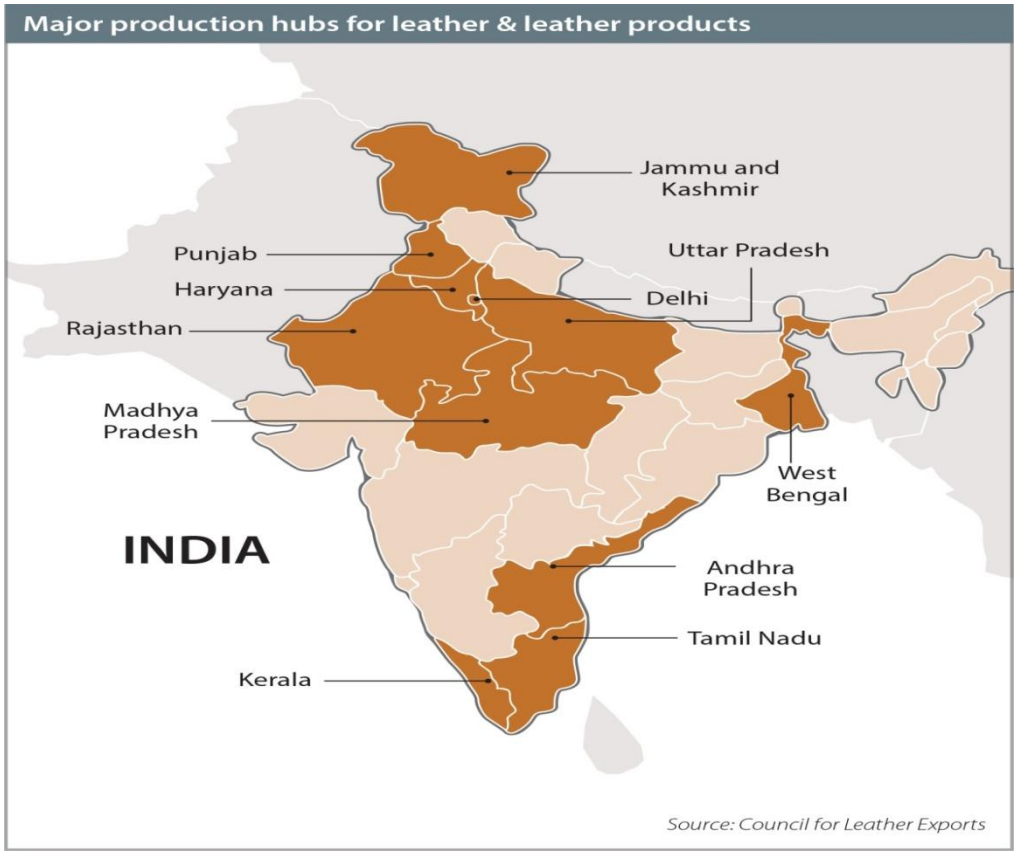


Fig. 1.3 State-wise distribution of the leather industry among the different states of India

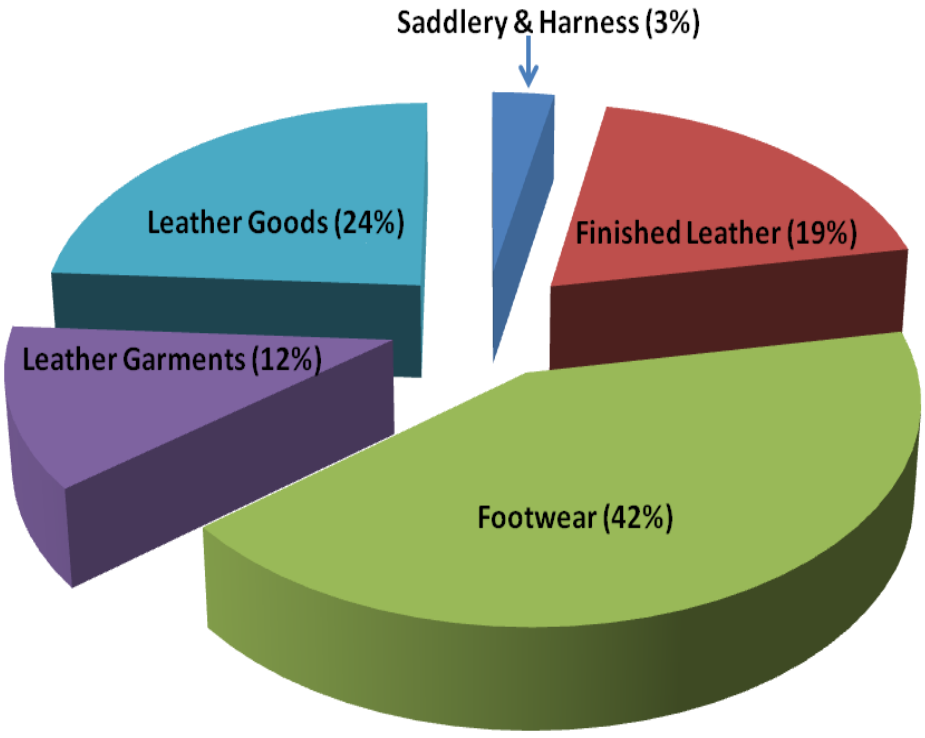


Fig. 1.4 Product wise contribution of the leather industry in Indian export basket

Nonetheless, LIs are also viewed as the most pollution creating industries as these releases a high volume of potentially toxic and hazardous effluent, which creates a negative image of LIs in society. However, the adequate treatment and management of tannery wastewater (TWW) is a challenging task worldwide. LI is one of the oldest industries of the world and the problem of treatment and disposal of liquid effluent is as old as the industry itself. TWW is ranked as the highest pollutants among all industrial wastes. The inherent nature of the tanning process is such, that large quantity of water is consumed. Approximately, 30-35 m^3 of effluent is being discharged during the processing of one ton of hide/skins in LIs (Lofrano et al. 2013). The wastewater discharged from LIs is characterized by dark brown colour, objectionable odour, high pH, and a high pollution parameters such as chemical oxygen demand (COD), biochemical oxygen demand (BOD), total dissolved solids (TDS), chromium, sulphate, phosphate, nitrate and a variety of highly toxic organic chemicals and heavy metals (HMs) (Suganthi et al. 2013; Chandra et al. 2011; Haydar and Aziz 2009). However, its physicochemical characteristics may vary according to, from industry to industry depending upon the size of leather industry, raw materials used, chemicals used for a specific process, amount of water consumed, type of final product, and the production processes applied in the LIs (Rameshraja and Suresh 2011; Lofrano et al. 2013). In India, there are more than 2500 tanneries, which mostly rely on the chrome tanning process and afford 15% of the total worldwide leather production (Shukla et al. 2009; Alam et al. 2009). In the leather tanning process, a huge amount of highly toxic chemicals such as chrome salts, vegetable and synthetic tannins, phenolic compounds, azo dyes, surface-active compounds, pesticides, sulphonated oils and a variety of persistent organic pollutants (POPs) are being used to convert the raw hide/skins into the commercial leather or leather products (Saxena et al. 2016; Dixit et al. 2015). These highly toxic POPs and inorganic chemical compounds such as heavy metals are not fully uptaken by the hide/skins during leather processing and also do not degrade/detoxify much during the secondary treatment process at common effluent treatment plants (CETPs) of LIs and goes into the environment causing serious soil and water pollution along with serious environmental threats and severe toxic hazards. Further, the nature and characteristics of different POPs present in TWW after secondary treatment process have yet to be studied to understand their mechanism of toxicity and to protect the human health and environment and thus, their removal from wastewater is urgently required.

The discharge of untreated/partially treated TWW causes serious soil and water pollution in the environment. TWW causes (a) a huge foaming problem on surface waters; (b) inhibits the nitrification process; (c) blocks sunlight penetration due to its dark brown color and, thus, reduces the photosynthetic activity and oxygenation of receiving water bodies and, hence, becomes detrimental to aquatic life; (d) causes depletion in dissolved oxygen that encourages the anaerobic condition, which leads to the putrefying odor of receiving water bodies; (e) causes eutrophication of water bodies, thus adversely affecting the ecological functioning of aquatic resources; (f) causes salinisation of soil (acidification, reduce soil fertility) and water due to its highly saline nature (salt concentration); (g) causes groundwater pollution due to leaching of highly toxic chromium (Cr) to the deeper layer of soil; (h) causes the deficiency of some micronutrients in soil such as zinc (Zn), copper (Cu), iron (Fe), etc.; and (i) alters the composition and structure of microbial communities in soil and reduces their growth and finally retards the bioremediation process due to its high chromium content (Saxena et al. 2016; Bhattacharya et al. 2016).

TWW also causes serious toxicity in living beings. It has been reported to: (a) cause genotoxicity and mutagenicity in fish, *Oreochromis niloticus* (Matsumoto et al. 2006) (b) disturb protein metabolism in fresh water teleost fish, *Cirrhinus mrigala* (Ham.) (Afaq and Rana (2009) (c) cause hematotoxicity in the common fish, *Tilapia mossambica* and fresh water fish, *Labeo rohita* (Hamilton) (Sounderraj et al. 2012; Praveena et al. 2013) (d) interfere metabolic processes by altering the activity of oxidative enzymes in different organs of guppy fish, *Poecilia reticulata* (Aich et al. 2015; 2011) (e) disrupt the several physiological and cytological processes in plants (Saxena et al. 2016) (f) disturb the delicate hormonal balance (endocrine disruption in rats) and compromise the reproductive fitness of living beings (Kumar et al. 2008) (g) cause embryonic toxicity coagulation of fertilized eggs, detachment of tail bud from the yolk sac, yolk sack edema, malformation of the tail, scoliosis, and deformation of swim bladder in the embryos of zebra fish, *Danio rerio* (Rocha and De Oliveira 2017) (h) cause reduction in the diversity of macroinvertebrates (Wosnie and Wondie 2014) (i) cause acute embryotoxicity and developmental defects in the sea urchins (*Paracentrotus lividus* and *Sphaerechinus granularis*) and serious toxicity in *Daphnia magna* (Oral et al. 2007) (j) cause detrimental changes in the biochemical parameters, damage to gonad and mantle tissues, and also genotoxic effects in the snail, *Pila*

globosa (Bhattacharya et al. 2016). In developing countries, TWW is also being used as a liquid fertilizer by the local farmers to irrigate their food crops in the agricultural land (soil pollution). This uncontrolled and illegal practice paves a way for the bioaccumulation of toxic heavy metals like Cr at sequentially higher trophic levels in the food chain *via* consumption by human/animals and thus, resulting in the severe health threats (Goutam et al. 2018). Further, TWW has been also reported to be eliciting toxic effects in agricultural plants. For example, the irrigation of agricultural crops with the HM-rich TWW has been reported to cause a reduction in root/shoot growth and biomass, seed germination, and seedling growth and also induce chlorosis and photosynthetic impairment (Chandra et al. 2009; Bharagava et al. 2017). Moreover, TWW has been also proved to be e mutagenicity, genotoxicity, cytotoxicity, and endocrine disruption in the exposed organisms (Montalvao et al. 2017; Kumari et al. 2016; Kumar et al. 2008; Matsumoto et al. 2006). Such toxic effects caused by TWW make it a serious pollutant, and hence, it's adequate treatment to its final disposal into the environment is required to combat the environmental threats and protect the public health.

The conventional wastewater treatment plants (WWTPs), which mostly relied on the conventional activated sludge treatment process (CASTP) do not efficiently remove the color and persistent organic pollutants (POPs) from TWW. The presence of poorly degradable tannins, recalcitrant metals like Cr and high salt concentration inhibit the biological treatment of TWW (Saxena et al. 2016). In addition, the high energy demand, operation, and maintenance cost, post-treatment requirement for TWW and excess sludge production associated with CASTP also discredit its application at large scale. Several physico-chemical treatment technologies (such as coagulation/flocculation, adsorption, microfiltration, sedimentation, ozonation, photocatalysis, etc.) have been also developed by the time to improve the quality of treated TWW. However, these treatment technologies are of limited scope because these use a huge amount of chemicals, cause secondary pollution and a high operational and treatment cost and thus, are environmentally and economically not suitable for the effluent treatment (Saxena et al. 2016; Lofrano et al. 2013). Emerging treatment technologies (ETTs) such as membrane technologies, membrane bioreactors, anammox technology and oxidation processes (electrochemical treatment, Fento, etc.) are also available for the treatment of TWW. The applications of ETTs at

large scale are also uneconomical due to associated high energy demand and operation cost and some other serious drawbacks (Saxena et al. 2016) and thus, are less preferred in developing countries. Furthermore, to improve the pollutants removal from TWW, various combined treatment technologies (CTTs: biological treatment followed by physico-chemical treatment) has been also developed (Saxena et al. 2016). However, CTTs require major changes to the existing wastewater treatment plants (WWTPs) available with LIs. Therefore, an environmentally and economically feasible effluent treatment option with high treatment efficiency will be more preferable and acceptable to LIs.

Bioremediation technology (BT) has been increasingly recognized as an eco-friendly, safe, and cost-effective solution for industrial wastewater treatment. BT employs microbes and plants or their enzymes to degrade/detoxify the noxious organic and inorganic contaminants present in the industrial effluents. Several biological agents including bacteria (Kumari et al. 2016), fungi (Sharma and Adholeya 2011), yeast (Okoduwa et al. 2017), algae (Ajayan et al. 2015), and plants (Gregorio et al. 2015) have been reported for the degradation and detoxification of TWW. In past, most of the studies were focused on the removal of specific pollutants like Cr (Bharagava and Mishra 2018), phenol (Paisio et al. 2012), naphthalene-2-sulfonic acid (Song et al. 2005) and pentachlorophenol (Srivastava et al. 2007) from TE by using microbes. Moreover, TWW is highly complex in nature and contains a mixture of POPs and inorganic metals and hence, a monoculture of any biological agents could not efficiently degrade/detoxify contaminants present in real TWW. Conversely, the application of microbial consortia is more suitable over pure cultures to efficiently degrade/detoxify tannery effluent due to intensive metabolic activities of microbes and their catabolic enzymes that can effectively degrade/detoxify a mixture of POPs and inorganic metals (Kurade et al. 2012).

To date, there is very limited detail available on the application of microbial consortia in the degradation and detoxification of real TWW (Sivaprakasam et al. 2008) and some authors have used undefined microbial consortia (Sul et al. 2016; Kim et al. 2013). Keeping in view of all facts, the present study was to develop a new bacterial consortium using identified potential pollutant degrading bacterial strains for the degradation and detoxification of POPs in real TWW for environmental safety and human health protection. Prior to developing the bacterial consortium, the study was

focused on the physico-chemical analysis of TWW and characterization of various persistent organic pollutants (POPs) in TWW by Fourier-transform infrared (FTIR) spectroscopy, High Performance-Liquid Chromatography (HP-LC) and Gas Chromatography-Mass Spectroscopy (GC-MS) analysis to know their nature and characteristics. Further, a potential bacterial consortium was developed by using potential bacterial strains isolated from collected TWW and sludge sample for the effective degradation and detoxification of POPs and metals to protect the environment and human health. In addition, the environmental and nutritional factors were also optimized in order to enhance the pollutants removal efficiency in the real TWW. Further, the degradation and detoxification of POPs and heavy metals in TWW was carried out at the optimized conditions for the efficient bioremediation and metabolic products were further characterized by FT-IR, HP-LC, and GC-MS analysis. The prime objective of bioremediation is to lessen the toxicity of industrial effluents and hence, the phytotoxicity of TWW before and after consortial treatment was also assessed to evaluate the environmental safety. This study is perhaps the first attempt on the development of a new bacterial consortium with identified potential bacterial strains and its application in the bioremediation and toxicity reduction in the TWW after the secondary treatment. Finally, this study would be useful to develop a bacteria-based bioremediation process for WWTPs treating TWW for environmental protection.

The present study has been compiled into the following chapters:

The chapter first has introduced the basic information on the topic of the thesis and mainly focused on the introduction to the Indian leather industry, structure and its role in the national economy of the country and employment generation. This chapter also introduced the pollution and toxicity profile of the leather industry and the status of effluent treatment and also highlights the need for the adequate treatment and detoxification of TWW.

Chapter two of this thesis has described the objectives of this study, which were targeted to complete this research work successfully in a more organized and manageable way. Chapter three of this thesis has described the review of literature that provides comprehensive information on leather industry, leather processing, pollution and toxicity profile of TWW, various chemicals used in leather production,

and various physico-chemicals, biological (i.e. aerobic and anaerobic), and emerging techniques used for the treatment of TWW.

Chapter four has described the physico-chemical characteristics of TWW as per approved standard methods to actually define the strength of pollution. Chapter five described the characterization of various persistent organic pollutants (POPs) in TWW even after secondary treatment process using liquid-liquid extraction method and analyzed by FT-IR, HP-LC and GC-MS techniques to reveal their nature and characteristics.

Chapter six has focused on the isolation, purification, screening, and characterization of bacteria capable of the degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater. Chapter seven has described the development and optimization of a potential bacterial consortium at different nutritional and environmental parameters, and inoculum volume and agitation rate for the optimum degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater.

Chapter eight described the biodegradation and bioremediation of persistent organic pollutants in TWW by the newly developed bacterial consortium at the optimized conditions and characterization of new metabolites produced during the degradation and detoxification of TWW. This chapter also deals with the complete physico-chemical analysis of TWW before and after treatment with the newly developed bacterial consortium.

Chapter nine described the detection and characterization of the catabolic enzyme responsible for the degradation of persistent organic pollutants (POPs) in TWW produced by the potential bacterial strains. Chapter ten described the toxicity assessment of tannery wastewater before and after treatment with the newly developed bacterial consortium.

Chapter eleven has summarized the findings of the thesis. This section has mentioned the brief findings of each chapter. Chapter twelve described the concerned references cited in the whole thesis. The reference section has been written in a standard format and all the important references related to the topic have been included.

2

Chapter-02
Objectives

Objectives

The objectives of this study are as follows:

1. **Physico-chemical analysis of tannery wastewater (TWW) collected from CETP after secondary treatment process**
   - *1.1. Collection of tannery wastewater samples from CETP after the secondary treatment process*
   - *1.2. Physico-chemical analysis of tannery wastewater (TWW) collected from CETP after the secondary treatment process*
   - *1.3. Analysis of heavy metals in tannery wastewater (TWW) collected from CETP after the secondary treatment process*

2. **Detection of persistent organic pollutants from tannery wastewater by HPLC/GC-MS-MS/ LC-MS-MS analysis.**
   - *2.1. Sample preparation and liquid-liquid extraction of persistent organic pollutants from TWW*
   - *2.2. Characterization of persistent organic pollutants from TWW by HP-LC analysis*
   - *2.3. Characterization of persistent organic pollutants from TWW by FT-IR analysis*
   - *2.4. Characterization of persistent organic pollutants from TWW by GC-MS analysis*

3. **Isolation, purification, screening and characterization of bacteria capable for the degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater**
   - *3.1. Isolation and purification of bacterial strains from tannery wastewater and sludge and culture conditions*
   - *3.2. Screening of potential bacterial strains for salt tolerance*
   - *3.3. Screening of potential bacterial strains for COD removal efficiency*
   - *3.4. Identification and characterization of potential bacterial isolates.*
     - *3.4.1. Morphological characterization*
     - *3.4.2. Biochemical characterization*
     - *3.4.3. Molecular characterization*

4. Development and optimization of a potential bacterial consortium for the effective degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater

4.1. Bio-interaction study of the isolated potential bacterial strains for the development of bacterial consortium

4.2. Development of a potential bacterial consortium for the effective degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater

4.3. Optimization of the newly developed bacterial consortium at various environmental parameters (pH and temperature) for the effective degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater

4.4. Optimization of the newly developed bacterial consortium at various nutritional parameters (carbon and nitrogen sources) for the effective degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater

4.5. Optimization of the newly developed bacterial consortium at various inoculum volume and agitation rate for the effective degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater

5. Characterization of metabolites from bacterially treated tannery wastewater

5.1. Bioremediation experiment for the degradation of persistent organic pollutants (POPs) in tannery wastewater by the newly developed bacterial consortium at the optimized conditions

5.2. Physico-chemical analysis of tannery wastewater after treatment with the newly developed bacterial consortium

5.3. Liquid-liquid extraction

5.4. Characterization of metabolites in the bacterially treated TWW by HP-LC analysis

5.5. Characterization of metabolites in the bacterially treated TWW by FT-IR analysis

5.6. Characterization of metabolites in the bacterially treated TWW by GC-MS analysis

6. Detection and characterization of catabolic gene/enzyme responsible for the degradation of persistent organic pollutants (POPs)

6.1. Preparation of cell free extract

6.2. Enzyme assays

6.3. SDS-PAGE analysis

7. Toxicological assessment of tannery wastewater before and after bacterial treatment process

7.1. Effect of untreated and bacterially treated tannery wastewater on the seed germination and seedling growth of Phaseolus aureus L.

7.2. Assay for the determination of α-amylase activity in the seeds irrigated with untreated and bacterially treated tannery wastewater

3

Chapter-03
Review of Literature

3. Introduction

Leather industries (LIs) play an important role in the national economy of many developing countries like India, China, Turkey, Brazil, Ethiopia, Pakistan and Bangladesh (Leta et al. 2004; Lefebvre et al. 2006; Kurt et al., 2007; Verma et al. 2008; Haydar and Aziz 2009; Lofrano et al. 2013; Chowdhury et al. 2013; Wang et al. 2014). Approximately, 22700.5 M sq. feet of leather is produced annually in the world (FAO 2008), whereas the world trade for leather sector is estimated as US$100 billion per year (UNIDO 2010). The demand for leather and leather products is ever increasing and independent of supply. The United States, Germany and other European countries are the major importers whereas the countries like India, China, Pakistan, Egypt, Brazil, Thailand and Indonesia are the major exporter of leather and leather products.

Unfortunately, LIs are also one of the major polluters worldwide because of their complex nature of wastewater. During leather production processes, a variety of chemicals with large volume of water are used to convert the raw hide/skins into leather or leather products and generates a large volumes of high strength wastewater, which is a major source of environmental pollution. The wastewater generated is characterized by a high chemical oxygen demand (COD), biological oxygen demand (BOD), Total dissolved solids (TDS), Total suspended solids (TSS), chromium (III) and phenolics with high pH, strong odor and dark brown color (Durai and Rajasimman 2011; Suganthi et al. 2013; Dixit et al. 2015). Apart from high organic content, tannery wastewater (TWW) also contains various nutrients such as nitrogen and phosphorus that can lead to eutrophication of water bodies (Rai et al. 2005; Durai and Rajasimman 2011; Raj et al. 2014). In addition, the dark brown color of wastewater hinders the photosynthesis process by blocking the sunlight penetration and it is therefore deleterious to aquatic life (Aravindhan et al. 2004; Rai et al. 2005; Kongjao et al. 2008; Mwinyihija 2010; Durai and Rajasimman 2011). However, the major pollutants present in TWW include chromium, tannins or syntans (STs), phenolics, phthalates and azo dyes (Kumar et al. 2008; Lofrano et al. 2013; Dixit et al. 2015).

The high concentration and low biodegradability of pollutants present in TWW is a major cause of serious environmental concern (Di Iaconi et al. 2002; Schrank et al.

2009) and thus, it is imperative to adequately treat the TWW before its final disposal in the environment. However, the increasingly stringent environmental regulations are also forcing the LIs to improve the treatment processes applied at wastewater treatment plants (WWTPs) and also explore the alternative methods for the better treatment and management of TWW.

Therefore, this literature of review highlighted the environmental impacts and toxicity profile of TWW and chemicals and a detailed review on the existing treatment approaches for its safe disposal into the environment. The emerging treatment approaches have been discussed with their merits and demerits. Further, the emerging anammox technology for the removal of ammonia from TWW and constructed wetlands (CWs) for wastewater treatment has been also discussed. In addition, the clean technologies (CTs) for waste minimization, control and management in LIs are also discussed. Moreover, the international legislation scenario on discharge limits for TWW and chemicals has also been discussed country wise with discharge standards to prevent the environmental pollution.

3.2. Leather production and chemicals used in tanning process

LIs are specialized in processing of hide (skins of large animals like cows, buffaloes and horses) and skins (skins of small animals like sheep, goats and calves) for leather production. The tanning process used to convert the hide/skins (a highly putrescible material) into stable and imputrescible products termed as leather, which is used for various purposes (Dixit et al. 2015). Tanning processes are classified into vegetable or chrome tanning depending on the type of tanning reagent (tannins or chromium) applied (Ram et al. 1999; Mannucci et al. 2010) (Table 1). The steps and overall process of leather production is well described in literature (Thanikaivelan et al. 2005; ILTIP 2010; Lofrano et al. 2013; Dixit et al. 2015). Moreover, the contaminations events and how TWW causing pollution and toxicity in environment as a result of leather production processes in leather industry is depicted in Fig. 1. However, the tanning process involves different steps and chemicals for different end products and the kind and amount of waste generated may vary in a wide range of quantity and nature (Lofrano et al. 2013).

Table 3.1 Comparison between vegetable tanning and chrome tanning process

S. No.	Parameters	Vegetable tanning	Chrome tanning
1.	Tanning agent	Vegetable tannins (VTs)	Chromium salt
2.	Nature	Organic tanning	Inorganic (mineral) tanning
3.	Action	Slow process	Fast process
4.	Cost	Costly affairs	Cost effective
5.	Time	Time consuming	Less time consuming
6.	Geographical use	Used in developed countries and few developing countries	Used in developing countries
7.	Products	Heavy leather like shoe soles, luggage, saddlery and belt etc.	Light weight leathers like shoe uppers, garments and bag etc.
8.	Product characteristics	Higher thermal stability and water resistant	Softer and more pliable leather
9.	Processing steps	All the steps are same as in chrome tanning process	Additionally, retanning, dyeing and fatliquoring are usually performed to produce finished leather and a preliminary degreasing step may be necessary when using animal skins, such as ship skins
10.	Environmental Impact	Does not require prior preparation of pickling and therefore contribution to pollution load from sulfate salts are lower hence ecofriendly, but VTs are hard to biodegrade. Thus, waste bearing VTs degrade slowly	Generation of chromium containing sludge and wastewater is still a major environmental problem of chrome tanning process

During tanning process, a large amount of chemicals such as acids, alkalis, chromium salts, tannins, sulfates, phenolics, surfactants, dyes, auxiliaries, sulphonated oils and biocide etc. are used to convert the semi-soluble protein "collagen" present in hide/skins into highly durable commercial forms of leather, and the chemicals used

are not completely fixed by the hide/skins and goes in wastewater (Lofrano et al. 2008; Mannucci et al. 2010). The poor uptake of chromium salt (50-70%) during the tanning process results in the material wastage on one hand and disturb the ecological balance on the other hand (Saravanbahavan et al. 2004; Dixit et al. 2015). Moreover, the sulphonated oils and synthetic tannins or syntans (STs) (an extended set of chemicals such as phenol, naphthalene, formaldehyde, melamine and acrylic resins) are also used in tanning/retanning process to make the leather more softer (Lofrano et al. 2008; Lofrano et al. 2013).

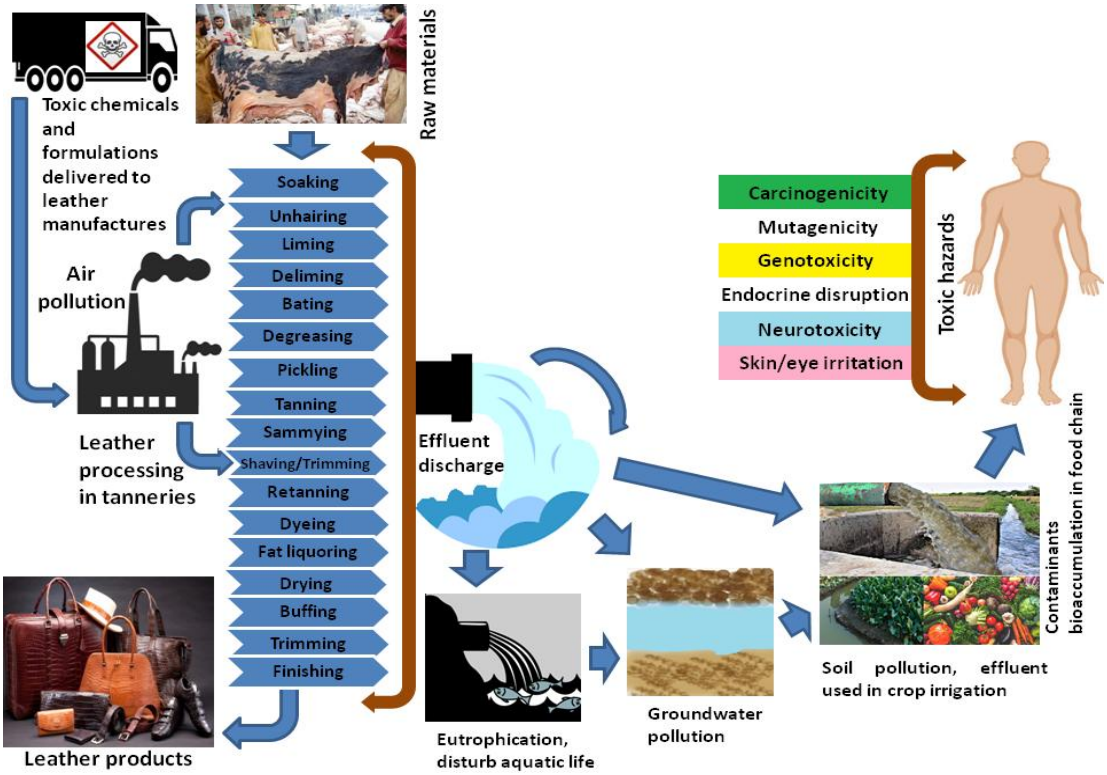


Fig. 3.1 Leather processing in leather industries and wastewater generation as a source of environmental pollution and toxicity

Many regulations have been passed to avoid the use of hazardous chemicals in industrial processes such as Integrated Pollution Prevention and Control Directive (96/61/EC 1996; 2008/1/EC 2008). The Directive (REACH (EC 1907/2006) for European Regulatory Framework on chemicals namely Registration, Evaluation, Authorization and Restriction of Chemical substances directed the LIs to avoid the use of those leather auxiliaries and basic chemicals, which are not registered and listed in the Safety Data Sheet (Lofrano et al. 2013). Moreover, the Directive (2003/53/EC) restricted the marketing and use of products/product formulations

containing > 0.1% of nonyl ethoxyphenol (NPE) or nonylphenol (NP) and their use in making of the leather products in Europe (Lofrano et al. 2008). In addition, the Directive (1999/815/EC) has directed the industries to label the products if contain > 0.5% phthalates (benzyl butyl phthalate, di-butyl phthalate and di-ethyl hexyl phthalate) due to its reproductive toxic potential (EU 2003). The use of o-phenyl phenol is restricted for leather finishing due to its carcinogenic potential (EPA 2007) as well as the use of formaldehyde (a cross liker casein top coats) due to its carcinogenic potential has been also restricted (EU 1998). The inorganic compounds such as cadmium sulfate and lead chromate (fastening agents) are highly toxic in nature (IARC 2004; ATSDR 2008). Further, the EU Azo Colorants Directive (2002) has prioritized several azo dyes and restricted their use in LIs due to higher toxicity but there is no any particular restriction to use STs yet in LIs worldwide (Dixit et al. 2015).

3.3. Tannery wastewater: nature and characteristics

Water is crucial for life and also used in many industrial processes. In tanning process, a large quantity of water and chemicals are used to treat raw hide/skins and approximately 30-35 m^3 of wastewater is generated per ton of raw hide/skins processed (Lofrano et al. 2008; Islam et al. 2014). However, the wastewater generation depends on the nature of raw material, finishing product and production processes applied (Tunay et al. 1995; Lofrano et al. 2013). This presents two major problems for LIs: First, the availability of good quality of water and second is the adequate treatment of such a large volume of high strength wastewater.

Tannery wastewater (TWW) is a basic, dark brown coloured waste having COD, BOD, TDS, chromium (III) and phenolics with high pH and strong odor (Durai and Rajasimman 2011; Suganthi et al. 2013; Dixit et al. 2015). However, the characteristics of TWW may vary from industry to industry, raw materials and chemicals used, type of final product and the production processes adopted by LIs (Apaydin et al. 2009; Rameshraja and Suresh 2011; Lofrano et al. 2013).

During leather production, the beamhouse and tanning operation are the highly pollution causing steps because beamhouse operation contribute high organic and sulfide content whereas tanning operation contribute high salts (of chloride, ammonium, chromium and sulfate) concentration in TWW (Cooman et al. 2003;

Rameshraja and Suresh 2011). Hence, the beamhouse wastewater is characterized by an alkaline pH and tanning wastewater by a very acidic pH as well as a high COD value (Lofrano et al. 2013). Generally, TWW is highly rich in nitrogen, especially organic nitrogen, but very poor in phosphorous (Durai and Rajasimman 2011). The retanning and streams relatively have a low BOD and TSS (Total suspended solids), but high COD and contain trivalent chromium (III), tannins, sulfonated oils and spent dyes whereas the wet finishing, retanning, dyeing and fat liquoring processes contribute low fraction of salt in TWW that is predominantly originated from the hide/skins in the soak liquor (USEPA 1986; Lofrano et al. 2013). Further, BOD_5/COD (due to inhibitors) or BOD_5/TOC (due to high sulphide and chloride concentration) ratio is used for the biodegradation study of TWW (Lofrano et al. 2013). The data on wastewater generation and pollution load of each step during the processing of raw hide/skins are presented in Table 3.2.

Table 3.2 Pollution load and quantity of wastewater generated during the processing of per ton raw hide/skins

Pollution load	**Processing operation (load kg/ton of raw hide/skins)**					
	Soaking	**Unhairing/ liming**	**Deliming and bating**	**Chrome tanning**	**Post-tanning**	**Finishing**
Wastewater generated (m^3)	9.0-12.0	4.0-6.0	1.5-2.0	1.0-2.0	1.0-1.5	1.0-2.0
TSS	11-17	53-97	8-12	5-10	6-11	0-2
COD	22-33	79-122	13-20	7-11	24-40	0-5
BOD	7-11	28-45	5-9	2-4	8-15	0-2
Cr	-	-	-	2-5	1-2	-
Sulphides	-	3.9-8.7	0.1-0.3	-	-	-
NH_3-N	0.1-0.2	0.4-0.5	2.6-3.9	0.6-0.9	0.3-0.5	-
TKN	1-2	6-8	3-5	0.6-0.9	1-2	-
Chlorides	85-113	5-15	2-4	40-60	5-10	-
Sulfates	1-2	1-2	10-26	30-55	10-25	-

Adapted from Dixit et al. (2015)

3.4. Environmental pollution and toxicity profile of tannery wastewater

TWW is ranked as one of the major environmental pollutant among all the industrial wastewaters (Verma et al. 2008; Gupta et al. 2012). The presence of a variety of toxic and hazardous chemicals such as chromium, chlorophenols, formaldehydes, STs, oils, resins, biocides, detergents and phthalates etc. in TWW creates a negative image of

LIs (Lofrano et al., 2013; Dixit et al. 2015). The toxicity of chemicals used during leather processing is summarized in Table 3.3. The wastewater generated from Common Effluent Treatment Plant (CETP) contains high BOD, COD, TDS and a variety of toxic heavy metals especially chromium, which makes it potentially toxic for humans and other living beings (Mondol et al. 2012; Lofrano et al. 2013; Dixit et al. 2015). In addition, TWW also contains a mixture of chemical compounds, which are used during leather processing and are not get properly degraded even after the conventional treatment and have a negative impact on living organisms and environment (Alvarez-Bernal et al. 2006; Oral et al. 2007; Kumar et al. 2008; Tigini et al. 2011; Siqueira et al. 2011; Shakir et al. 2012; Lofrano et al. 2013; Saxena and Bharagava 2015).

TWW is a major source of water and soil pollution. The dark brown color blocks the sunlight penetration, and thus, reduces the photosynthetic activity and oxygenation of receiving water bodies and hence, becomes detrimental to aquatic life (Song et al. 2000; Kongjao et al. 2008; Bakare et al. 2009; Mwinyihija 2010; Carpenter et al. 2013). In addition, the depletion in dissolved oxygen encourages the anaerobic condition, which leads to the putrefying odour of receiving water bodies (Rai et al. 2005; Sahu et al. 2007; Verma et al. 2008). TWW also causes eutrophication of polluted water bodies and thus adversely affecting the ecological functioning of aquatic resources (Rai et al. 2005; Durai and Rajasimman 2011; Schilling et al. 2012; Dixit et al. 2015). The high concentration of heavy metals in sediments of Ganga river and its tributaries has been reported (Singh et al. 2003; Tare et al. 2003; Bhatnagar et al. 2013). The increase in the salinisation of rivers and groundwater has led to the reduction in soil fertility and quality of drinking water in Tamil Nadu, India (Money 2008). It has been estimated that over 55,000 ha of land has been contaminated by TWW and around 5 million peoples are affected by low quality of drinking water and social environment (CSIRO 2001; Sahasranaman and Jackson 2005). TWW is also reported to inhibit the nitrification process (Szpyrkowicz et al. 2001; Trujillo-Tapia et al. 2008; Lofrano et al. 2013) as well as to cause a huge foaming problem on surface waters (Schilling et al. 2012).

Moreover, the treated/partially treated TWW causes severe toxic effects in fishes and other aquatic organisms. The genotoxicity and mutagenicity of water polluted with TWW has been evaluated by micronucleus test and comet assay by using fish

Oreochromis niloticus (Matsumoto et al. 2006). De Nicola et al. (2007) have studied the toxicity of mimosa tannin and phenol-based syntans on sea urchin (*Paracentrotus lividus* and *Sphaerechinus granularis*) during the early developmental stages and on marine algal cell growth (*Dunaliella tertiolecta*) and reported the sea urchin embryogenesis was affected by vegetable tannins and syntan water extracts at level of 1 mgL^{-1}. Afaq and Rana (2009) also studied the impact of leather dyes (Bismarck brown and acid leather brown) on the protein metabolism in fresh water teleost, *Cirrhinus mrigala* (Ham.) and reported a significant decrease in total protein content in teleost treated with leather dyes. In addition, the toxic effects of TWW on the survival and histopathological parameters in the different organs of fishes *Channa punctatu*s and *Oreochromis mossambicus* have been studied (Mohanta et al. 2010; Navaraj and Yasmin 2012). However, the toxic effects of TWW on the hematological parameters of a common fish *Tilapia mossambica* and fresh water fish, *Labeo rohita* (Hamilton) has also been recently studied (Lesley Sounderraj et al. 2012; Praveena et al. 2013). Further, TWW also reported to interfere with the metabolic processes by altering the activity of oxidative enzymes in different organs of guppy fish, *Poecilia reticulate* and thereby causing cellular injury as a result of exposure (Aich et al. 2011; 2015).

Further, the presence of pathogens in water and wastewater environment has been reviewed by many workers (Bharagava et al 2014; Saxena et al. 2015). TWW also highly rich in organic and inorganic constituents and thus, may provide a chance to a variety of pathogenic bacteria to flourish and contaminate the receiving water bodies as these constituents may act as a source of nutrients (Verma et al. 2008; Bharagava et al. 2014). Recently, Chandra et al. (2011) have reported the presence of various types of organic pollutants (OPs) and bacterial communities in two aeration lagoons of a CETP used for the degradation and detoxification of TWW in India and also tested the toxicity of TWW on mung bean (*Phaseolus mungo*) in terms of seed germination and seedling growth. In addition, various authors have also assessed the bacteriological quality of TWW and reported the presence of a variety of pathogenic bacteria remained in TWW even after the secondary treatment process (Verma et al. 2008; Ramteke et al. 2010; Bharagava et al. 2014).

Generally, LIs discharges their wastewater into nearby canals/rivers, which is directly/indirectly is being used by farmers for the irrigation of agricultural crop

(Trujillo-Tapia et al. 2008; Gupta et al. 2012). This practice leads to the movement of potentially toxic metals like chromium from water to crop plants and ultimately reached into the human/animals body and cause toxicity (Sinha et al. 2008; Chandra et al. 2009). However, the chromium toxicity is mainly depends on the chemical speciation and thus, the associated health effects are influenced by the chemical forms of exposure (Rameshraja and Suresh 2011). It is well reported that chromium (VI) is a potent carcinogen for humans, animals, plants as well as microbes as it enters the cells *via* surface transport system and get reduced into chromium (III) form and causes various genotoxic effects (Ackerley et al. 2004; Aravindhan et al. 2004; Matsumoto et al. 2006; Tripathi et al. 2011; Raj et al. 2014). Thus, the use of Cr loaded TWW for the irrigation of agricultural crops disrupts the several physiological and cytological processes in cells (Shanker et al. 2005; Chidambaram et al. 2009; Gupta et al. 2012) leading to the reduction in root and shoot growth and biomass, seed germination, seedling growth (Lopez-Luna et al. 2009; Hussain et al. 2010), and also induces the chlorosis, photosynthetic impairment and finally leading to the plant death (Akini and Akini 2010; Asfaw et al. 2012). However, the effect of TWW on seed germination and seedling growth is governed by its concentration and it is crop-specific. In a recent study conducted on mung bean (*Vigna radiate* (L.) wilczek) by Raj et al. (2014), the percent inhibition of seed germination was 90% and 75%, when seeds were treated with 25% untreated and treated TWW, respectively. Moreover, it is also reported that treated and adequately diluted TWW can be used for the irrigation of agricultural crops as it provide a reliable source of water supply to farmers and contains valuable plant nutrients especially N, P, K and also add organic matter to soil (Trujillo-Tapia et al. 2008; Durai and Rajasimman 2011; Asfaw et al. 2012; Sangeetha et al. 2012; Kohli and Malaviya 2013). Further, the genotoxic and mutagenic effects of TWW and agricultural soil irrigated with TWW has been recently studied (Alam et al. 2009; 2010).

In addition, the inappropriate discharge of TWW also leads to the significant level of soil pollution as well as acidification because of high salt load in wastewater (Chowdhury et al. 2004; Alvarez-Bernal et al. 2006; Mwinyihija 2010; Raj et al. 2014). High sulphide content in TWW also causes the deficiency of some micronutrients in soil such as Zn, Cu and Fe etc (Raj et al. 2014). However, Cr(VI) alters the structure of soil microbial communities and reduced their growth and finally

retarding the bioremediation process and if it enters in food chain, causes skin irritation, eardrum perforation, nasal irritation, ulceration and lung carcinoma in humans as well as animals along with accumulation in placenta impairing the fetal development in mammals (Cheung and Gu 2007; Chandra et al. 2011; Asfaw et al. 2012). In addition, the exposure to chlorinated phenols particularly pentachlorophenol (PCP), which is highly carcinogenic, teratogenic and mutagenic in nature and causes toxicity to living beings by inhibiting the oxidative phosphorylation, inactivating the respiratory enzymes and damage the mitochondrial structure (Jain et al. 2005; Verma and Maurya 2013; Tripathi et al. 2011). The high concentration of PCP can also cause the obstruction in circulatory system of lungs, heart failure and damage to central nervous system (USDHHS 2001; Tewari et al. 2011; Dixit et al. 2015).

In addition, TWW also contain azo dyes that are highly persistent in nature due to their complex chemical structure and xenobiotic nature leading to the environmental pollution (Nachiyar and Rajkumar 2003; Gurulakshmi et al. 2008; Mahmood et al. 2013; Baccar et al. 2011; Patel et al. 2012; Preethi et al. 2013; Dixit et al. 2015). Thus, the removal of azo dyes from TWW is essential because of their high mutagenicity, carcinogenicity and intense coloration problems of contaminated aquatic resources (Osugi et al. 2009; Saratale et al. 2010). The discharge of azo dyes into the surface water also leads to the aesthetic problems and obstruct the light penetration and oxygen transport into the water bodies and finally affecting the aquatic life (Khalid et al. 2008; Chen et al. 2011). Moreover, these dyestuffs have been also reported to cause some other serious problems such as dermatitis, skin and eye irritation and respiratory problems in human beings (Keharia and Madamwar 2003).

Further, there has been an increasing concern regarding the release of many endocrine disrupting compounds (EDCs) along with TWW in environment. EDCs disturb the delicate hormonal balance and compromise the reproductive fitness of living beings and ultimately may lead to carcinogenesis (Dixit et al. 2015). Kumar et al. (2008) have detected many EDCs like nonylphenol (NP), 4-aminobiphenyl, hexachlorobenzene and benzidine in TWW collected from northern region of India and tested their toxicity on reproductive system of male rats. However, the presence of phthalates (EDCs) such as bis(2-ethylhexyl)phthalate (DEHP), Dibutyl phthalate (DBP), bis(2-methoxyethyl)phthalate in TWW has been also reported (Alam et al.

2009; 2010). Therefore, the adequate treatment of TWW prior to its final disposal into the environment is required.

Table 3.3 Toxicity profile of organic and inorganic pollutants used in leather processing

Name of chemicals	**Applications**	**LD_{50} in rats (oral mg/kg)**	**Target organs**
Pentachlorophenol (PCP, a carcinogen)	Applied as a biocide in preservative for raw hides/skins	2000	Eyes, nose, skin, respiratory tract, blood, kidney, liver, immune system and reproductive system
Di-butyl phthalate (DBP, a endocrine disrupting chemical)	Applied as a plasticizer in artificial leather manufacturing	7499	Eyes, lungs, gastrointestinal (GI) tract and testes
Benzyl butyl phthalate (BBP, a endocrine disrupting chemical)	Applied in preparation of micro-porous artificial leather coating/water vapour-permeable sheet materials	2330	Eyes, lungs, liver and reproductive system
Bis(2-ethylhexyl) phthalate (DEHP, a endocrine disrupting chemical)	Applied as a plasticizer in artificial leather manufacturing	30.000	Liver and testes
Short chain, chlorinated paraffin's	Additive for leather treatment (gives smoothness), leather clothing and belts and as oiling agent	3090	Liver, kidney and thyroid
Anthracene (a carcinogen)	Additive during tanning	16,000	Kidneys and liver
Nonyl phenol (a endocrine disrupting chemical and xenoestrogen)	Applied during finishing	1475	Blood. Lungs, eyes, skin, central nervous system (CNS), kidneys and testes
N-methyl pyrrolidone	Applied as a coalescene, plasticizers and wetting agents	3914	Eyes, kidneys, lymphatic system, liver, lung and testes
Methyl isothiazolinone (a carcinogen)	Applied as biocide	1800	Skin and eyes
Organotin compounds (Dibutyl tin) (a carcinogen)	Applied as a catalyst	175	GI tract and liver
Azo dyes (Orange II, a carcinogen)	Applied as a dyeing agent	3418	Blood, liver and testes
Chloromethyl benzene or benzyl chloride	Applied to inhibit the growth of molds and mildews on hides/skins, facilitates leather softening, wetting, and to color finished leather in combination with dyes	-	Moderately aquatic toxicant and identified as a Group 2A carcinogen

4-chloro-3-methyl phenol or *p*-chloro-*m*-cresol (PCMC)	Applied as biocide in raw hide/skins preservation	-	Potential endocrine disruptor by U.S. Environmental Protection Agency
Hexachlorobenzene (a carcinogen)	Applied for raw hide/skins preservation	10,000	Reproductive system
Chromium (a carcinogen)	Applied as a tanning agent	3250	Kidneys, CNS and hematopoietic system
Formaldehyde (a carcinogen)	Applied in finishing of leather	100	Eyes and lungs
Arsenic (a carcinogen)	Applied in finishing of leather	763	Liver, kidneys, skin, lungs and lymphatic system
Sodium dichromate	Applied in preparation of chrome-tanning salts	-	Blood, kidneys, heart, lungs and eyes
Cobalt dichloride	Applied in dyeing and finishing	80	Skin, lungs, liver, kidney and heart
Cadmium sulfate (Pigment)	Applied as fastening agents and used in marking and surfacing of material.	280	Lungs, liver, tissues and reproductive system
Lead chromate (pigment)	Applied as fastening agents and used in marking and surfacing of material.	1000	Lungs, liver, tissues and reproductive system

Adapted from Bharagava et al. (2017); Saxena et al. (2016); Dixit et al. (2015); Kumar et al. (2008)

3.5. Treatment approaches for tannery wastewater and chemicals

TWW is a major source of soil and water pollution and it is therefore essential to adequately treat the TWW prior to its safe disposal into the environment. This can be achieved by using physical, chemical and biological methods either alone or in combination.

3.5.1. Physico-chemical treatment approaches

3.5.1.1. Coagulation/flocculation

Coagulation is the destabilization of colloids by neutralizing the forces that keep them apart. Cationic coagulants provide positive charge to reduce the negative charge (zeta potential) of the colloids. As a result, the particles collide to form larger particles (flocs) whereas flocculation is the action of polymers to form bridges between the flocs, and bind the particles to form large agglomerates or clumps. There are a number of coagulants such as aluminium sulfate ($AlSO_4$), ferric chloride ($FeCl_3$), ferrous

sulfate ($FeSO_4$) etc. are used to reduce the organic load (COD) and total suspended solids (TSS) as well as to remove toxic metals mainly chromium from TWW (Lofrano et al. 2013).

However, coagulants are pH specific and their effectiveness is largely depends on their type and concentration and characteristics of wastewater to be treated (Song et al. 2004). Ates et al. (1997) reported >70% removal of COD and <5 mgL^{-1} of total chromium from TWW using alum and $FeCl_3$ based-CF. Song et al. (2004) also reported 30-37% removal of total COD, 74-99% of chromium and 38-46% of TSS by using 800 mgL^{-1} of alum at pH 7.5 from TWW containing 260 mgL^{-1} of suspended solids, 16.8 mgL^{-1} of chromium, 3300 $mgL^{-1}$of COD at pH 9.2 and finally concluded that $FeCl_3$ based CF proved better results than alum based-CF. Chowdhury et al. (2013) have reported 92% removal of COD and 96% of chromium from TWW using $FeCl_3$ at the concentration of 150 mgL^{-1} at pH 7 followed by sand-stone filtration process. In addition, Shegani (2014) also reported 81.60%, 98.34%, 92%, 75.00%, 70.00%, 69.20% and 50% removal of COD, ammonia, nitrate, hexavalent chromium, phosphate, chloride and H_2S, respectively by using coagulants $Ca(OH)_2$ and $FeSO_4$ $.7H_2O$, but a low reduction in sulfate (19.00%) and TSS (13.00%) and an increase in TDS (15.60%) were observed.

Moreover, some coagulants such as poly-aluminium chloride (PAC), poly-aluminium silicate (PASiC) and poly-aluminium ferric chloride (PAFC) ($[Al_2(OH)nCl_6\text{-}n]m.[Fe_2(OH)nCl_6\text{-}n]m$) have been developed with improved coagulation efficiency to minimize the residual coagulants in treated wastewater (Gao et al. 2004; Lofrano et al. 2013). Lofrano et al. (2006) reported >75% removal of COD and >95% of TSS from TWW at all doses of alum (800-900-1000-1200 mgL^{-1}) using PAFC (900 mgL^{-1}) at pH 8.5. However, Yoganand and Umapathy (2013) have also applied a green methodology for the recovery of chromium (VI) from TWW using newly synthesized quaternary ammonium salt and reported 99.99% removal of chromium (VI) from TWW.

3.5.1.1. Adsorption

Adsorption is typically used for the removal of toxic metals especially chromium from TWW. There are a number of studies available on the use of adsorbents such as bentonite clay, cement kiln dust, activated carbon etc. for the treatment of TWW

(Fadali et al. 2004; Fahim et al. 2006; Tahir and Naseem 2007). Further, the use of chitin-humic acid based hybrid and ground shrimp shells as adsorbent for the significant removal of Cr(III) from TWW has been reported (Santosa et al. 2008; Fabbricino et al. 2013). Moreover, the use of lime/bitten based coagulants and activated carbon as a post treatment of TWW is also suggested (Ayoub et al. 2011)

3.5.2. Biological treatment approaches

Biological approaches are the eco-friendly methods for the treatment of industrial wastewaters and involve the stabilization of waste by decomposing them into harmless inorganic solids either by aerobic or anaerobic processes. The most commonly used processes for the biological treatment of TWW are the Activated sludge process (ASP) and Upflow Anaerobic Sludge Blanket (UASB) process (Durai and Rajasimman 2011).

3.5.2.1. Aerobic treatment

In aerobic treatment process, the waste decomposition rate is fast and also not characterized by unpleasant odours but a large amount of sludge is generated. There are several studies on the aerobic treatment of TWW using ASP has been reported earlier by many workers (Jawahar et al. 1998; Eckenfelder 2002; Tare et al. 2003; Vidal et al. 2004; Hayder et al. 2007; Ramteke et al. 2010) and some of the important findings are summarized in Table 3.4.

Table 3.4 Microorganisms used for the bioremediation of tannery wastewater

References	Microorganisms	COD removal (%)	BOD removal (%)	Cr removal (%)
Bharagava and Mishra (2018)	*Cellulosimicrobium* sp.			62.28
Okoduwa et al. (2017)	*Saccharomyces cerevisiae*	54.2	60.7	
	Torulaspora delbrueckii	90.6		
Kumari et al. (2016)	*Bacillus cereus*	65	80	92
Kim et al. (2014)	*Brachymonas denitrificans*	98.3	-	88.5
Noorjahan (2014)	*E. coli*			63.8
	Bacillus sp.	95.4	95.4	73.5
Elmagd and Mahmoud	Mixed culture	98.3	98.4	98.3

(2014)				
Sharma and Malviya (2014)	*Fusarium chlamydosporium* SPFS2-g	71.80	-	-
Yusuf et al. (2013)	*B. subtilis*	87.6	-	-
	P. fragi	85.2		
El-Bestawy et al. (2013)	*Providencia vermicola* W9B-11, *Escherichia coli* O7:K1 CE10, *Bacillus sp.* 58, *Bacillus amyloliquefaciens* T004, *Pseudomonas stutzeri* M15-10-3, *Bacillus sp.* PL47	79.16	94.14	93.66
Ajayan et al. (2012)	*Scenedesmus* sp.	-	-	96
Mandal et al. (2010)	*Thiobacillus ferrooxidans*	69	72	5
Sharma and Adholeya (2011)	*Paecilomyces lilacinus*			96.84
Nanda et al. (2010)	*Nostoc* sp.	37.8	48.6	-
Ramteke et al. (2010)	*E. coli*	98.46	90	-
	Vibrio sp.	87.5		
	Pseudomonas sp.	96.15		
Sivaprakasam et al. (2008)	*P. aeruginosa, B. flexus, E. homiense, S. aureus*	80	-	-
Vankar and Bajpai (2008)	*Trichoderma* sp.	-	-	97.93
Onyancha et al. (2008)	*S. condensate*	-	-	>75
	R. hieroglyphicum			
Srivastava et al. (2007)	*Acenetobacter* sp.	-	-	90
Rajasimman et al. (2007)	Mixed culture	46-85	65-93	-
Wang et al. (2007)	*A. thiooxidans*	-	-	99.7
Srivastava and Thakur (2006)	*Aspergillus* sp.	-	-	
	Hirsutella sp.			70
Lefebvre et al. (2005)	Halophiles	95	-	-
Thanigavel (2004)	Mixed culture	89.5	-	-
Shakoori et al. (2000)	Bacterial strain	-	-	87

TWW is highly saline in nature due to high load of salts, which are used for the preservation of raw hides/skins (Sundarapandiyan et al. 2010) and therefore, causes

some serious problems in the biological treatment of TWW. The major problems include (Sivaprakasam et al. 2008): (a) limited adaptation of conventional cultures due to higher salt concentration (>3-5% w/v), and therefore could not effectively treat TWW (b) salt adaptation of cultures is easily lost when subjected to salt free medium, and (c) changes in the ionic strength (salt concentration from 0.5-2% w/v) cause cell disruption even with the acclimatized cultures and finally leads to system failure.

However, the high concentration of poorly biodegradable compounds such as tannins and other toxic metals inhibit the biological treatment processes (Schrank et al. 2004). Cr(VI) is well reported to inhibit the growth of heterotrophs as well as nitrifying/denitrifying bacteria (Stasinakis et al. 2002; Farabegoli et al. 2004). To overcome this problem, a Sequencing Batch Reactor (SBR) is highly efficient to carry out the biological treatment and nitrogen removal from TWW in presence of inhibitors due to its low cost, flexible operation and selection and enrichment of a particular microbial species (Farabegoli et al. 2004; Ganesh et al. 2006; Murat et al. 2006; Durai and Rajasimman 2011; Rameshraja and Suresh 2011; Faouzi et al. 2013; Lofrano et al. 2013).

Moreover, the fluctuation in temperature range also has adverse effects on the nitrification process. The fluctuation in temperature range significantly affects the removal of organic carbon and nitrogen from TWW whereas have a minor influence on COD removal efficiency (4-5%) that has been studied for a full-scale activated sludge process based treatment plant used for TWW (Gorgun et al. 2007). Further, the improvement in the performance of nitrification process through increased aeration and total nitrogen removal efficiency (up to 60%) at a temperature range between 21-35 °C during an intermittent aeration type of operation has been reported (Insel et al. 2009).

3.5.2.2. Anaerobic treatment

The use of anaerobic treatment processes to treat TWW is an interesting option as compared to aerobic treatment process because of low energy consumption and sludge production however its full scale applications has several drawbacks (Mannucci et al. 2010): i) continuous production of sulfide (from sulfate reduction) in absence of alternative electron acceptors such as oxygen and nitrate; ii) high protein content affects the selection of biomass, slow down the kinetics of hydrolysis and also

inhibit the sludge formation, and iii) requirement of an additional aerobic treatment to meet the high COD removal.

The sulfide mainly inhibits the methanogenesis process during the anaerobic treatment of TWW and this is might be due to the direct toxicity of sulfide, substrate competition between the sulfate reducing bacteria and methanogenic bacteria and precipitation of trace elements (Midha and Dey 2008; Rameshraja and Suresh 2011; Mannucci et al. 2014). However, the mechanisms of sulfide toxicity are not well understood.

The anaerobic treatment of TWW is mainly performed by using either the anaerobic filters (AF) composed of both upflow anaerobic filters (UAF) and down-flow anaerobic filters (DAF) or Upflow Anaerobic Sludge Blanket (UASB) reactors (Lefebvre et al. 2006; Rajasimman et al. 2007; El-Sheikh et al. 2011; Dixit et al. 2015). Beside these, the use of expanded granular sludge bed (EGSB) and anaerobic baffled reactor (ABR) for the treatment of TWW is also suggested (Zupancic and Jemec 2010).

In addition, the anaerobic treatment of TWW is more favorable in tropical countries having higher temperatures such as India, Pakistan, China, and Brazil etc. as compared to European countries (Durai and Rajasimman 2011; Mannucci et al. 2014). In these countries, the spread of new and large industrial area to establish the LIs favor the development of centralized WWTPs. However, the application of anaerobic treatment processes at large scale makes it possible to balance the high operation and management costs with energy saving over the traditional aerobic treatment processes.

3.5.2.3. Constructed wetlands and treatment ponds

The constructed wetlands (CWs) are the man-engineered, eco-friendly systems designed to remove the pollutants from highly polluted industrial and municipal wastewater. The use of CWs for the treatment of industrial wastewater has developed rapidly in current years and is now successfully employed to remove a diverse array of pollutants from wastewaters.

The proper functioning of a wetland system depends on the complex relationship between the plants, microorganisms, soil, wastewater characteristics and operational parameters (Aguilar et al. 2008). In this regard, several efforts have been made to

select the suitable plant species capable to tolerate and remove the pollutants from TWW (Mant et al. 2004; Calheiros et al. 2007; 2008; 2012), selecting the suitable supporting media/substrate for proper growth and development of wetland plants (Calheiros et al. 2008) as well as to study the bacterial community dynamics in CWs (Aguilar et al. 2008; Calheiros et al. 2009a,b). The plant roots and rhizomes are the major sites of microbial degradation/transformation of pollutants and subsequently to the purification of wastewater because microbes form a biofilm on root surface and substrates (Stottmeister et al. 2003; Gagnon et al. 2007; Munch et al. 2007). However, the availability of nutrients or other environmental parameters affect the biofilm formation (Kierek-Pearson and Karatan 2005). Therefore, the detailed profiling of complex microbial populations is required to understand the proper functioning of CWs and phytoremediation processes (Chandra et al. 2015). Culture-dependent techniques are known to be insufficient to study the microbial community structure because numerous microorganisms are unculturable in lab conditions (Ward et al. 1990). Hence, molecular techniques such as random amplified polymorphic DNA (RAPD), polymerase chain reaction (PCR) and denaturation gradient gel electrophoresis (DGGE), is used for the study of microbial community structure, composition and diversity in CW system (Calheiros et al. 2009a; Calheiros et al. 2012).

Mant et al. (2004) have studied the phytoremediation potential of *Penisetum purpureum*, *Brachiaria decumbens* and *Phragmites australis* in CWs for the removal of chromium (ranging from 10 and 20 $mgCrdm^{-3}$) from TWW. In addition, the potentials of *Canna indica*, *Typha latifolia*, *P. australis*, *Stenotaphrum secundatum* and *Iris pseudacorus* in CWs for the treatment of TWW under two different hydraulic loading rates at 3 and 6 cmd^{-1} has been studied and found that only *P. australis* and *T. latifolia* were able to establish successfully (Calheiros et al. 2007). Further, they also evaluated *Arundo donax* and *Sarcocornia fruticosa* in two series of horizontal subsurface flow CWs used to treat TWW received from a conventional biological treatment plant and reported the removal of COD (51 and 80%) and BOD_5 (53 and 90%) for COD inlet: 68-425 mgL^{-1} and for BOD_5 inlet: 16-220 mgL^{-1} (Calheiros et al. 2012). In addition, the use of TWW as a growth medium for *Arthrospira* (*Spirulina*) has been recently suggested (Dunn et al. 2013). However, the chromium salt can be retained in wetlands with non-specialized supporting media (Dotro et al. 2012).

On the other hand, the use of treatment ponds for the treatment of TWW can also be an effective approach. The effect of different environmental parameters like pH, temperature and dissolved oxygen on the efficiency of a pilot-scale advanced integrated wastewater treatment pond system (AIWTPSs) used to treat TWW has been reported by Tadesse et al. (2004). They also suggested a combination of advanced facultative pond (AFP), secondary facultative pond (SFP) and maturation pond (MP) in a series for the effective treatment of TWW. Recently, Kumar and Sahu (2013) have designed the anaerobic pond (AP) for the treatment of TWW in Egypt.

3.5.3. Emerging treatment approaches

The TWW discharged even after the conventional treatment process still contains many refractory and recalcitrant organic pollutants (ROPs) and thus, require further treatment for environmental safety. Therefore, in order to overcome this problem, the use of emerging treatment technologies is increasing in the recent years.

3.5.3.1. Membrane technologies

Membrane technologies (MTs) are used for the mechanical separation/purification of industrial wastewater with the help of permeable membranes. MTs operate without heating and therefore use less energy than conventional thermal separation processes such as distillation, sublimation or crystallization. The use of MTs in LIs is becoming popular in current years because of continually reducing cost and ever extending application possibilities.

The MTs offers many economic benefits to leather industry, especially the recovery of chromium from TWW (Labanda et al. 2009; Ranganathan and Kabadgi 2011) and are used for purification/reuse of wastewater and chemicals of deliming/bating liquor (Gallego-Molina et al. 2013), reduction of pollution load due to unhairing and degreasing (De Pinho 2009; Wang et al. 2011), removal of salts as well as in the biological treatment of TWW for its reuse (Lofrano et al. 2013). Several membrane-based technologies such as cross flow microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO) and supported liquid membranes (SLMs) can be used for the removal of pollutants from TWW (Lofrano et al. 2013; Dixit et al. 2015). However, the use of reverse osmosis (RO) with a plane membrane has been suggested as a post treatment for the removal of refractory compounds such as chlorides and sulfates, resulted in the production of high quality of permeate that

allowed the reuse of tannery wastewater within the production cycle and thus, reducing the groundwater consumption (De Gisi et al. 2009). In addition, the economical evaluation of membrane filtration technologies has been discussed in detail by Scholz and Lucas (2003). However, the successful integration of MTs in a conventional purification process for TWW streams has been recently reported by Stoller et al. (2013)

3.5.3.2. Membrane bioreactors

Membrane bioreactor (MBR) is the combination of a membrane process like microfiltration or ultrafiltration with a suspended growth bioreactor, and is now widely used for municipal and industrial wastewater treatment**.** MBRs offers several advantages over the conventional activated sludge treatment process (CASTP) such as elimination of sludge from settling basins, independence of process performance from filamentous bulking or other phenomenon that affect the sludge settleability (Munz et al. 2008; Suganthi et al. 2013; Dixit et al. 2015). The presence of tannins in TWW reduces the kinetics of nitrification without large differences between the biomass selected with either the CASTP or the MBR used (Munz et al. 2009). However, the major drawbacks of membrane application are the significant fouling due to clogging, adsorption and formation of cake layer by pollutants like residual organics, dyes, and other impurities onto the membrane (Srinivasan et al. 2012; Stoller et al. 2013). However, the extensive work is in progress to reduce the bio-fouling problem in MBRs. Further, a hybrid membrane bioreactor (HMBR), which is the integration of various treatment technologies, may be a solution to overcome the bio-fouling problem of MBRs. More recently, the efficiency of HMBR (activated sludge process + electro-coagulation) for the effective removal of COD and color from TWW satisfying the discharge limits set by Tamilnadu Pollution Control Board (TPCB) India has been evaluated (Suganthi et al. 2013).

3.5.3.3. Anammox technology

The anammox technology is used for the anaerobic removal of ammonia from TWW and it is currently emerging because of its low cost and energy consuming nature (Anjali and Sabumon 2014). It involves the anoxic oxidation of ammonia with nitrite as a preferred electron acceptor and consumes 50% less oxygen, 100% less organic carbon and saves 90% of operational costs in sludge disposal as compared to the

conventional nitrification/denitrification processes (Anjali and Sabumon 2014). Therefore, industries, producing wastewaters having high concentration of ammonia, are showing increased interest in anammox process. However, the long start-up time and inhibitive nature in the presence of organic carbon and NH_4-N limits its field applications. Therefore, it is imperative to develop the mixed consortium capable of anammox in the presence of organic compounds. Further, the development of mixed microbial consortium consisting of ammonia oxidizing bacteria, anammox bacteria, and denitrifying bacteria is also expected to treat the wastewaters containing both ammonia and organic carbon.

3.5.3.4. Advanced oxidation processes

Advanced oxidation processes (AOPs) refers to the set of chemical treatment processes that uses strong oxidizing agents (O_3, H_2O_2) and/or catalysts (Fe, Mn, TiO_2) and sometimes also uses the high-energy radiation, e.g., UV light (Schrank et al. 2004; Naumczyk and Rusiniak 2005; Srinivasan et al. 2012; Dixit et al. 2015). AOPs are based on the production and utilization of hydroxyl radicals, which are strong oxidizing agents and quickly and non-selectively oxidize a broad range of organic pollutants in less time (Dixit et al. 2015). Generally, the AOPs are used to treat the secondary treated wastewater and therefore known as tertiary treatment (Audenaert et al. 2011). In this, most of the pollutants get converted into stable inorganic compounds such as H_2O, CO_2 and salts, i.e. they undergo mineralization (Rameshraja and Suresh 2011). The treatment efficiency of AOPs is mostly evaluated in terms of COD removal however TOC is a more suitable parameter to study the state of mineralization (Schrank et al. 2004; 2005; Costa et al. 2008; Monteiro Paschoal et al. 2009). There are various types of AOPs such as fenton oxidation, photo-oxidation, photo-fenton oxidation, ozonation, photocatalysis and electrochemical treatment processes that are applied to treat the TWW (Rameshraja and Suresh 2011; Lofrano et al. 2013; Dixit et al. 2015). The overall goal of AOPs used for TWW treatment is to reduce the pollution load and toxicity to such an extent that the treated TWW may be reintroduced into the receiving water bodies or reused during the process. The important findings of various AOPs applied to treat the TWW are presented in Table 3.5.

Table 3.5 Findings of some advanced oxidation processes (AOPs) applied for the treatment of tannery wastewater

References	AOPs	Wastewater type	Influent COD (mgL^{-1})	Operation parameters and reduction in pollutants
Modenes et al. (2012)	Photo-Fenton ($UV/Fe^{2+}/H_2O_2$)	Equalized tannery wastewater	11,878	COD removal (90%), TSS removal (50%), Fe^{2+} (0.4 g L^{-1}) and H_2O_2 (15 g L^{-1}), Irradiation time (540 min)
Houshyar et al. (2012)	Ozone	Pre-alkalized tannery wastewater	2177	COD removal (30-70%), Time (120 min), Ozone flow rate (1-8 g/h)
Di Iaconi et al. (2010)	Ozone	Biologically treated tannery wastewater	2900	COD removal (97%), TSS removal (96%), TKN removal (91%), Surfactants removal (98%), Color removal (96%)
Sundarapandiyan et al. (2010)	Electrochemical treatment	Synthetic tannery wastewater	10,715	COD removal (89%), pH 3-9, Current density (0.006-0.024 A cm^{-2}), Time (120 min)
Preethi et al. (2009)	Ozone	Raw tannery wastewater	5000	COD removal (60%), O_3 flow rate (2×10-3 m^3 min^{-1}), Time (20-120 min) and pH (4)
Espinoza-Quinones et al. (2009)	Electrochemical treatment	Equalized tannery wastewater	17,618	COD removal (51-56%), TSS removal (30-70%), Electric current flow rate (0-10A at 0-30V), Time (30-45 min)
Costa et al. (2008)	Electrochemical treatment	Equalized tannery wastewater	1005 (TOC)	Maximum phenol removal (83.9%), Maximum TOC removal (40.5%), Time (5 h of electrolysis)
Kurt et al. (2007)	Electrochemical treatment	Raw tannery wastewater	2810	COD removal (70%), Electric current (15.0 W), Time (10 min) and pH (3)
Pokrywiecki Saur et al. (2006)	UV/H_2O_2	Coagulated tannery wastewater	200-800	COD removal (60%), H_2O_2 (0.5 hL^{-1}), Time (4 h)
Schrank et al. (2005)	Fenton reagent	Coagulated tannery wastewater	130	COD removal (80%), H_2O_2/Fe^{2+} (500/100 w/w), Time (2 h)
Schrank et al. (2004)	Photocatalysis (UV/TiO_2)	Coagulated/Flocculated tannery wastewater	2365	COD removal (6% at pH 3), TOC removal (11% at pH 3), BOD removal (15% at pH 7)
Dogruel et al. (2004)	Ozone	Biologically treated tannery wastewater	835	COD removal (30%), Ozone flow rate (42.8 mg min^{-1}), Time (5 min)
Dantas et al. (2003)	Fenton reagent	Raw tannery wastewater	1803	COD removal (70%), Time (20 min), pH (2.5) and Temperature (25 °C)

Despite of a broad range of applications, AOPs also have some drawbacks that should also be considered before its applications. The presence of scavenger compounds such as an excess amount of H_2O_2 sometime can act as a hydroxyl scavenger instead of hydroxyl radical source, which interferes with the COD determination and reduces the reaction kinetics making the process uneconomical (Kang 2002; Lofrano et al. 2013). Further, the TWW also contains a significant amount of chromium, which may be oxidized from trivalent to hexavalent form, a more toxic form during oxidation treatment and thus, it is highly recommended to evaluate the possible effects of oxidation on the transformation of chromium atoms in different oxidation states (De Laat et al. 2004; Dogruel et al. 2006; Rameshraja and Suresh 2011; Lofrano et al. 2013). For these reasons, AOPs should be applied more properly to the segregated streams of wastewater containing high amount of aromatic compounds for fenton treatments or high content of salts for electrochemical treatment.

Moreover, AOPs still have not been put commercially at large scale (especially in the developing countries) even upto today mostly because of the relatively high costs. Nevertheless, its high oxidative capability and efficiency make AOPs popular techniques for the tertiary treatment of recalcitrant organic and inorganic pollutants. However, the increasing interest in wastewater reuse and more stringent regulations regarding the water pollution prevention and control are currently accelerating the implementation of AOPs at large scale.

3.5.4. Combinatorial treatment approaches

In previous section, various treatment approaches applied for TWW have been discussed. However, these treatment approaches have some serious limitations that need to be addressed further. The presence of residual organics, dyes, and other impurities in TWW even after the biological treatment processes followed by the RO based membrane technologies have been reported as the major drawbacks leading to the membrane fouling and finally failure of treatment processes (Srinivasan et al. 2012). Therefore, a combined application of physico-chemical treatment methods with biological treatment methods or various oxidation processes is generally preferred for the effective TWW treatment. Some of the combined treatment methods applied for TWW is presented in Table 3.6.

Table 3.6 Combined treatment approaches reported for tannery wastewater

References	Combined treatment applied	Pollutants	Optimum parameters
Suganthi et al. (2013)	Hybrid membrane bioreactor	COD and color	Electric current density (15 mA/cm^2), Electrocoagulation time (15 min), Membrane area (0.0143 m^2), Membrane spacing (0.22 µm), pH (7.4 and 9)
Srinivasan et al. (2012)	Biological treatment with ozonation	COD and color	Ozone flow rate (3 g/h), Time (24 h), pH (12), Hydraulic retention time (36 h), sludge age (10 days)
Mandal et al. (2010)	Biological treatment with fenton oxidation	COD, BOD, Chromium, Sulphide and Color	Fenton reagent (6 g $FeSO_4$ and 266 g H_2O_2), Time (30 min: fenton oxidation, 72 h: biological oxidation), pH (2.5), Temperature (30 °C)
Iaconi et al. (2009)	SBBR with ozonation	COD, BOD, TSS, TKN and color	Sludge production (0.4 kg TSS/kg COD), Time (5760 h and 2160 h)
Rodrigues et al. (2008)	Photo-electrochemical treatment with electrodialysis	COD and NH_4-N	Electric current density (36 mA/cm^2), Ti electrode, Membrane area (1.72 dm^2), Membrane spacing (0.75 mm)
Dogruel et al. (2006)	Biological treatment + ozonation with biological treatment	COD	Ozone flow rate (20 g/h), Reaction time (30 min)
Naumczyk et al. (2005)	AOP with fenton reagent	COD and Ammonia	Fenton reaction time (30 min)
Szpyrkowicz et al. (2005)	Electrochemical treatment with biological treatment	COD and Ammonia	Sludge production (1.37 kg/m^3/day), Electrolysis time (49 min)
Kennedy et al. (2004)	CAACO system	COD, BOD, Sulphide and sulfate	Volumetric loading rate (0.7376 m^3/m^3 day), Surface loading rate (0.2438 m^3/m^3/day)
Iaconi et al. (2004)	SBBR with ozone oxidation	COD, TKN and TSS	Sludge production (0.05 kg VSS/kg COD)
Iaconi et al. (2003)	SBBR with ozonation	COD, TKN and TSS	Sludge production (4 kg/kg COD), Organic loading (2.6 kg COD/m^3/day)
Iaconi et al. (2002)	SBBR with ozone oxidation	COD, Ammonia and SS	O_3 flow rate (8.7 mg O^3/min), Sludge production (4 kg TSS/kg COD)

3.6. Waste minimization, operation, treatment and management in leather industries

3.6.1 Solid waste generation, treatment and management

In LIs, apart from liquid waste, a large amount of chromium containing tanned solid waste (non-biodegradable sludge) is also generated during leather processing (Dixit et al. 2015). The waste generated finds very limited applications and its disposal cause serious environmental problems (Mwinyihija 2010; 2012). The types and quantity of solid waste generated during the processing of 1 ton of raw hide/skins have been presented in Table 3.7.

Table 3.7 Nature and quantity of solid waste generated during the processing of 1 ton of raw hide/skins

Nature of solid waste generated	Quantity (kg)
Salt from handshaking	80
Salt from solar pans (not realized)	220
Hair (pasting ovine)	100
Raw trimmings	40
Lime sludge (mostly bovine)	60
Fleshing	120
Wet blue trimmings (grain splits)	30
Chrome splitting (bovine)	65
Chrome shaving (mostly bovine)	95
Buffing dust (including shaving bovine after crust)	65
Dyed trimmings	35
Dry sludge from CETPs	125

Adapted from Rao et al. (2004) & Thanikaivelan et al. (2005)

However, the conventional treatment and disposal of solid waste is not environmentally feasible because of transformation and leaching of Cr(III) from tanned waste to Cr(VI) and groundwater, emission of nitrogen oxide (NO_x), hydrogen cyanide (HCN) and ammonia (NH_3) (Fatima et al. 2012; Dixit et al. 2015). Therefore, the combination of aerobic treatment (for degradation of low molecular weight compounds) with anaerobic treatment (for further degradation of metabolites) may be

a suitable treatment option for tannery waste. The methodologies for the treatment of liquid tannery waste using solid tannery waste have been recently suggested by Fatima et al. (2012). Further, after treatment the remaining waste can be recycled and utilized as useful by products and raw materials. Some of the technological options, which are proposed for the handling and management of solid waste, are presented in Fig 3.2.

3.6.2 Gaseous emission and control

The emission of gaseous waste such as ammonia (during deliming, unhairing and drying), hydrogen sulphide (released in TWW from sulphides if pH is > 8), particulate matter (containing chromim from reduction of chromate or from buffling), and volatile organic compounds (hydrocarbons, amines and aldehydes) from leather industries during the different steps of tanning processes may also cause the pollution of atmosphere (Dixit et al. 2015). Therefore, the proper control of gaseous emission should be required.

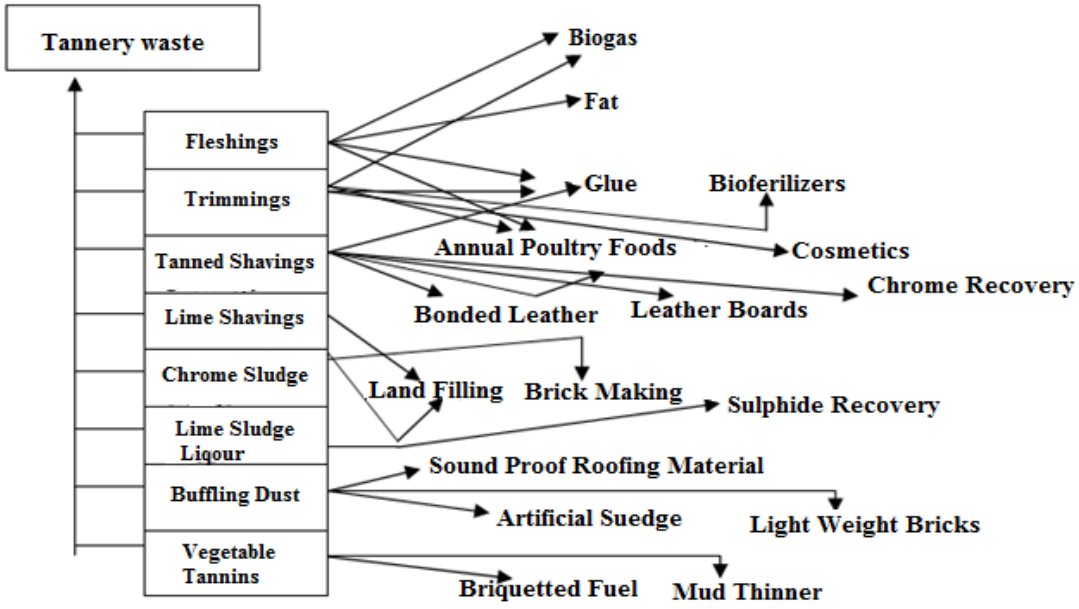


Fig 3.2 Technological options for handling and management of solid waste generated during leather production (adapted from ILTIP, 2010)

3.6.3 Clean technologies for hazards minimization

Environmental pollution due to leather industry is a major cause of concern and its mitigation requires some cleaner technologies (CTs) or also regarded as greener

technologies (GTs) for pollution prevention and hazards minimization. CTs utilize the processes that avoid the use of harmful chemicals or promote the use of eco-friendly chemical and cut or eliminate the gaseous emissions and wastes and therefore are cost-effective. Various CTs for the tannery waste minimization and control has been reviewed by many workers (Thanikaivelan et al. 2005; Lofrano et al. 2013; Islam et al. 2014; Dixit et al. 2015).

The development and implementation of CTs at large scale require (a) careful auditing and assessment of the toxicological effects of chemicals used in leather processing, (b) to avoid the use of environmentally susceptible chemicals, (c) to ensure the maximum uptake of chemicals used, (d) assessment of environmental impact of waste generated during leather processing, and (e) optimization of processes for the best economic returns. However, the success of CTs depends on the following parameters: (a) reduction of pollution load in terms of quantity and quality, (b) tanner's benefit in terms of leather quality and/or cost reduction, (c) reproducibility of the process, (d) economic feasibility of process (e) wide market opportunities. Further, the use, assessment and selection of best available techniques (BAT) for the tanning of hides and skins have been discussed (IPPC 2013).

3.7. International legislations scenario for tannery wastewater and chemicals

3.7.1. Legislations for discharge limits of tannery wastewater

In developing countries, according to the environmental pollution control regulations set by various national and international environment protection agencies, LIs are forced to set up the WWTPs either individually as ETP or collectively as CETP and the treated wastewater should comply with the discharge standards. The compliance with the discharge standards has not always been practical either because the laws are too ambitious or unrealistic in case of certain parameters, or they have lacked the effective instrumentation and institutional support. Some environment protection laws have not succeeded because they do not match the technical requirements and economic reality of the country or they do not have the institutional support to implement them into consideration.

In India during 1990's, several LIs were ordered to close their units as these could not meet the discharge standards, while many of them paid huge compensation for the damage caused due to the groundwater contamination (CSIRO 2001). For the sake of

LIs, Indian government has offered subsidies to construct Common Effluent Treatment Plants (CETPs) for the treatment of TWW. Notwithstanding, the pollution problems are still common due to high operation and management cost associated with CETPs and thus causing illegal dumping of wastewater (Beg and Ali 2008). In Uganda, the main leather industry was found to dump its wastewater directly into a wetland adjacent to Lake Victoria (The Monitor 2009) whereas in Croatia, the pollution abatement cost exceeded the compensation cost against the irresponsible behaviour of LIs (EcoLinks 2001).

The environmental regulations regarding the discharge standards for TWW has become a serious environmental concern in recent years. For pollution prevention from TWW and its chemicals, the United Nations Industrial Development Organization (UNIDO) has compiled the standard limits for the discharge of TWW into water bodies and sewers from several countries worldwide (UNIDO 2000; 2003). However, the discharge standards for some of the countries are presented in Table 3.8. The discharge limits for TWW may vary from country to country and are either related to the quality of treated wastewater or the quality of receiving water bodies (Dixit et al. 2015).

Table 3.8 Discharge limits for tannery wastewater into water bodies and sewers in some countries

S. No.	Parameter	Italy		Turkey		Netherlands		Argentina		Brazil		Egypt		China		Vietnam		Indonesia		Bangladesh		India		Pakistan	
		S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	*S^a	S^b	S^a	S^b	S^a	**S^b
1.	pH	5.5-9.5	5.5-9.5	6-9	6-10	6-10	6.5-10.0	5.5-10	5.5-10	5.0-9.0		6.0-9.0	6.0-9.0	6.0-9.0	6.0-9.0	5.5-9.0	5.5-9.0	6.0-9.0				5.5-9.0	5.5-9.0	6.0-9.0	6.0-9.0
2.	Temperature °C	30-35	30-35		40		40	45	45	<40	40	35	0		35	40	45					40-45	40-45	40	
3.	Conductivity (µs/cm)																								
4.	Suspended solids (mg/l)	40-80	200	150	350	150	350					30	500	70-150	400	100	200	150	150		500	100	600	200	
5.	Settleable solids							0.5	0.5	1.0		*	5-10		10										
6.	BOD_5 (O_2 mg/l)	40	250	100	250	5	250	50	200	60		20-30	400	20-100	600	50	100	150	150		250	30	500	80	
7.	COD (mg/l)	160	500	200	800	*	*	250	700			30-40	700	100-300	1000	100	400	300	300		400	250		150	
8.	TDS (mg/l)											800-1200	2,000									2100	2100		
9.	Sulphide (S^{2-} mg/l)	1	2	1	2	*	*		1	0.2	5	1	10	1	10	0.5	1.0				2.0	2	2	1	
10.	Chrome (III) (mg/l)		4				1				5			1.5	2.0	1.0	2.0					2	2		
11.	Chrome (VI) (mg/l)	0.2	0.2	0.3		*	*							0.5	0.5							0.1	0.1		
12.	Total Chrome (mg/l)	2	4	2	5	0.05	2	0.5	2	0.5		0.05	5-10	1.5	1.5	2.0	2.0	2	2		2.0	2	2	1	
13.	Chloride (mg/l)	1200	1200			200	*	*	*			*	*									1000	1000	1000	
14.	Sulfates (mg/l)	1000	1000		1700	3		*	1000			*	*									1000	1000	1000	
15.	Ammonia (mg N/l)	10-15	30					3	10	5		100	100					10	10			50	50	40	
16.	TKN (mg N/l)				100	*	*	10	30	10		*	*			60	60								
17.	Phosphorous (mg									1															

	P/l)																					
18	Oil/grease (mg/l)	20	40	20	100			100	100	20-30	100	100	100	10-15	100	10	30	5	5	20	10	20	10
19.	Phenol (mg/l)	0.5	1		10	*	*	0.5	0.5	0.1-0.5		0.001-0.002	*	0.5	2.0				1		5-50	5-50	0.3
20.	Detergents (mg/l)																		1.5				
21.	Solvents (mg/l)																						
21.1.	Hydrocarbons (mg/l)	0.2	0.4																				
21.2.	Nitrogenous (mg/l)	0.1	0.2																				
21.3.	Chlorinated (mg/l)	1	2					1	2	5													

Foot note:

S^a: Surface, S^b: Sewer, *S^a: Bangladesh has no discharge standards for tannery wastewater into surface water, **S^b: Pakistan has no discharge standards for tannery wastewater into sewer. *Spaces left blank indicate that parameters which are not specified and considered as specific requirements that need to be fulfilled.

3.7.2. Legislations for leather chemicals

A variety of chemicals are used during the leather processing, which are highly toxic to living beings and cause environmental pollution. In this view, some countries have also made regulations for the production, import and sale of leather products containing harmful chemicals. The chemicals and their permissible limits in leather and leather products approved in some countries are summarized in Table 3.9. However, the European Chemical Agency (ECHA) has also prioritized and restricted the use of few chemicals in LIs under Substances of Very High Concern (SVHC), which are considered to be hazardous for environment and human beings (UK REACH 2009). However, all the chemicals are still used in leather making and therefore their proper control is urgently required.

Table 3.9 Maximum permissible limits of chemicals of leather products in some countries (adapted from Dixit et al. 2015)

Residual substances limits for chemicals	**EU**	**Germany**	**Austria**	**Denmark**	**France**	**Netherlands**	**Switzerland**
Azodyes*	30 ppm						
PCP	30 ppm	5 ppm	30 ppm		30 ppm	30 ppm	30 ppm
Phthalates	0.1%	0.1%		0.05%			
PCBs and PCTs***	Not to be used						
Biocides**	5 ppm	5 ppm	5 ppm		5 ppm	5 ppm	10 ppm
Hexavalent Chromium	3 ppm	10 ppm					
Cadmium	100 ppm		75 ppm			100 ppm	100 ppm
Arsenic	Nil						
Lead	90 ppm						
Organotin Compounds	Nil						
Specific Flame Retardants	<0.1 %						
Formaldehyde		>1500 ppm	>1500 ppm		200-400 ppm	120 ppm	

Foot note:

*Azo dyes: Biphenyl-4-ylamine; 4-aminobiphenyl xenylamine; Benzidine; 4-Chloro-o-toluidine; 2-Naphthylamine; o-aminoazotoluene; 4-amino-2', 3-dimethylazobenzene; 4-o-tolylazo-o-toluidine; 5-Nitro-o-toluidine; 4-chloroaniline; 4-methoxy-m-phenylenediamine; 4,4'-methylenediani-line; 3,3'-dichlorobenzidine; 3,3'-dimethoxybenzidine o-dianisidine; 3,3'-dimethylbenzidine 4,4-bi-o-toluidine; 4,4-methylenedi-o-toluidine; 6-methoxy-m-toluidine; p-cresidine; 4,4'-methylene-bis-(2-

chloroaniline); 4,4'-oxydianiline; 4,4'-thiodianiline; o-toluidine; 2-aminotoluene; 4-methyl-m-phenylenediamine; 2,4,5-trimethylaniline; o-anisidine 2-methoxyaniline; 4-amino-azobenzene.

**Biocides (23 approved): Human hygiene biocidal products; Private area and public health area disinfectants and other biocidal products; Veterinary hygiene biocidal products; Food and feed area disinfectants; Drinking water disinfectants; Preservatives; In-can preservatives; Film preservatives; Wood preservatives; Fibre, leather, rubber and polymerised materials preservatives; Masonry preservatives; Preservatives for liquid-cooling and processing systems; Slimicides; Metalworking-fluid preservatives; Pestcontrol; Rodenticides; Avicides; Molluscicides; Piscicides; Insecticides, acaricides and products to control other arthropods; Repellents and attractants; Other biocidal products; Preservatives for food or feedstocks; Antifouling products; Embalming and taxidermist fluids; Control of other vertebrates.

***PCBs: Polychlorinated biphenyls; PCTs: Polychlorinated terphenyls.

3.8. Challenges and future prospects

Today's the LIs are facing some serious challenges from public and government mainly due to the environmental pollution and there is a public outcry against the industry. The major challenges faced by LIs include:

(a) Increased cost of leather production per unit area due to the stringent environmental regulations.

(b) Increasing demand of raw material i.e. raw hides, skins and semi-finished leathers.

(c) Lack of advanced processing techniques and waste treatment technologies in developing countries.

(d) Lack of specific dedicated industrial areas for the positioning of LIs.

(e) Poor capacity utilization leading to the higher financial cost and overheads charges.

(f) Lack of financial support from government.

The mitigation of these challenges requires the financial supports at large scale from the government for the upgradation of LIs, especially small scale industries (Xu and Zhiping 2011). Hence, there is a need to revisit the leather processing again for making the continue sustainability of LIs in near future because LIs are the key drivers of many nation's economy.

3.9. Conclusion and recommendation

(a) LIs are one of the major sources of environmental (soil, water, air) pollution.

(b) TWW is a highly polluted wastewater among all the industrial wastewater.

(c) Currently, the processes used for leather making in several developing countries are traditional and required to be optimized for chemical and water consumption.

(d) The search for some other suitable tanning agents to replace the chromium is urgently required for eco-sustainable tanning process.

(e) Sulfide is highly toxic but the mechanism of toxicity is not well understood and implementation of adequate technology for H_2S desorption is required.

(f) Membrane bioreactors and constructed wetlands are the eco-friendly options for treatment of TWW and its management but have some limitations that need to be addressed in future.

(g) The combinatorial approaches involving physical or chemical with biological treatment process to treat the TWW may give the satisfactory results as compared to the individual treatment process but require major changes at the WWTPs that usually industry are not ready to adapt because of high treatment and operation cost.

(h) The emerging treatment approaches like membrane filtration and oxidation processes are also currently using/under analysis.

(i) AOPs are much promising to remove the recalcitrant organic pollutant but there is a still need to optimize these for best economic returns as these are costly to adapt.

(j) The emerging anammox technology for the anaerobic removal of ammonia from TWW is under research and further investigation is required.

(k) A complete understanding of toxicity profile of TWW may also be helpful in achieving the appropriate treatment solutions for future tanneries.

(l) Locating LIs in a planned industrial area is another common approach to abate the environmental pollution in parallel to strengthen the discharge limits for TWW.

(m) The use of eco-friendly chemicals, water minimization technologies and wastewater treatment/purification and recycling as per the EU integrated pollution prevention strategy and greening policy will be fruitful for solving the environmental problems.

Thus, we can say that there is no treatment method at its best to treat TWW and its chemicals. However, it is clear that continuous efforts are required in order to search for the better treatment approaches for TWW in near future. Further, bioremediation technology could be a suitable alternative for the efficient treatment/detoxification of TWW to apply as the best pollution prevention solution for the future tanneries.

4

Chapter-04

Physico-Chemical Analysis of Tannery Wastewater (TWW) Collected from CETP After Secondary Treatment Process

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

4.1. Introduction

The untreated or partially treated tannery wastewater (TWW) discharged from leather industries (LIs) is heavily polluting our natural resources (water/soil) available on the earth. A huge volume of wastewater with noxious chemicals is released from the different steps of leather processing in the LIs. During the processing of raw hide/skins, a huge amount of fresh and clean water and chemicals are used for the treatment of raw hide/skins. TWW is a basic, dark brown colored waste having an offensive odor and high pollution parameters such as COD, BOD, TDS, Cr, and phenolics with a variety of hazardous organic and inorganic chemicals (Durai and Rajasimmam 2011; Suganthi et al. 2013; Dixit et al. 2015). However, the characteristics of TWW may vary from industry to industry, raw materials and chemicals used, type of final product and the production processes adopted by LIs (Apaydin et al. 2009; Rameshraja and Suresh 2011; Lofrano et al. 2013).

TWW has been reported to cause eutrophication in water bodies (Rai et al. 2005), groundwater pollution (Money 2008) and soil pollution (Sahasranaman and Jackson 2005; CSIRO 2001) and various types of toxicity such as endocrine disruption (Kumar et al. 2008), neurotoxicity (Almeida et al. 2016), cytotoxicity (Montalvao et al. 2017), mutagenicity (Matsumoto et al. 2006), and genotoxicity (Kumari et al. 2016) in the living beings. Hence, the proper treatment and regular monitoring of TWW is required to safeguard the environment and public health. Moreover, LI is heavily suffering from the negative impact caused by its highly toxic wastewater that causes serious damage to the receiving environment and enormous pressure from the pollution control authorities to regulate and minimize the load of pollution parameters in the discharged TWW. Therefore, in view of the above, the present study was to characterize the TWW for various physico-chemical parameters to know its pollution profile for environmental safety.

4.2. Materials and methods

4.2.1. Chemicals and reagents used

The chemicals and reagents were used in the physico-chemical analysis of TWW are of analytical grade (highest purity ≥ 99%) and purchased from Sigma-Aldrich (St.

Louis, MO, USA). Whatman® Grade GF/C filter papers (pore size 1.2 µm) (Whatman, England, UK) were used for the filtration of TWW.

4.2.2. Collection of tannery wastewater samples from CETP after the secondary treatment process

The TWW samples (after secondary treatment) were collected in pre-sterilized clean carboy containers (capacity 20 l; Tarson Production Pvt. Ltd., USA) from the outlet of CETP located at Unnao (26.48^{o} N, 80.43^{o} E), Uttar Pradesh, India (Fig. 4.1, 4.2).

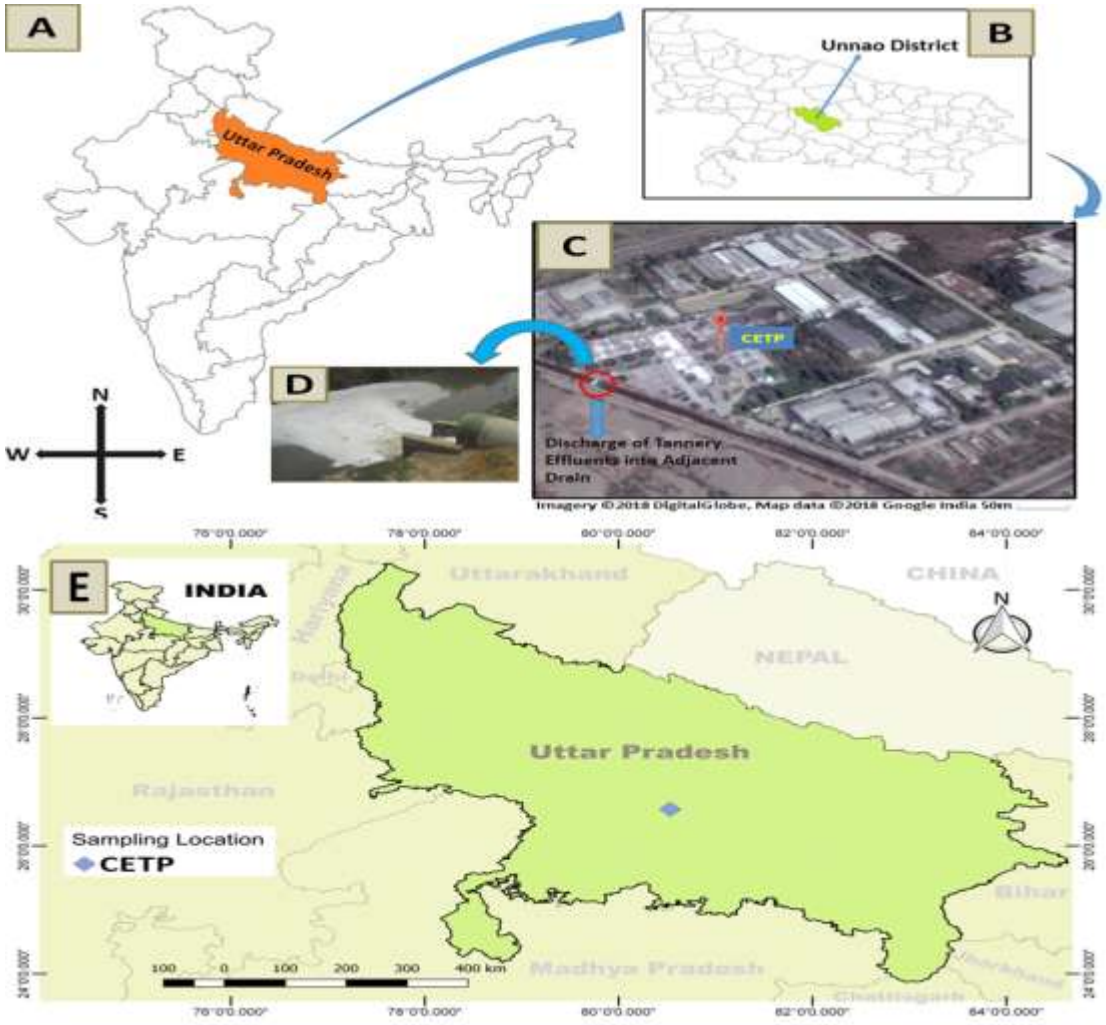


Fig. 4.1 Sampling site and discharge of tannery effluent into an adjacent drain from the outlet of a common effluent treatment plant (CETP) of tannery industries in Unnao, Uttar Pradesh, India (A-D) and geographic location of CETP in Unnao district used for the treatment of TWW (E).

Unnao is a large industrial city in the UP and famous for its industrial sectors worldwide. Tanning is the biggest industry in Unnao. Unnao is known for its leather industry and leather goods. The CETP is relied on the activated sludge treatment process (ASTP) and used to treat ~1.9 MLD of wastewater against a designed flow of 2.15 MLD. The CETP receives wastewater from a cluster of ~21 TIs located in nearby areas through pipelines and tanks, and the wastewater treatment is carried out round the year (365 days). The collected TWW samples were immediately brought to the laboratory, stored at 4°C and used for the analysis of physico-chemical parameters and heavy metals in TWW.

Fig. 4.2 Pics showing TWW treatment, discharge and its collection as well as sludge generation from CETP (A - I)

4.2.3. Physico-chemical analysis of collected tannery wastewater sample from CETP after the secondary treatment process

The collected TWW samples were analyzed in three replicates for various physico-chemical parameters to define the strength of pollution as per the standard protocols outlined in the "Standard Methods for Examination of Water and Wastewater" (APHA 2012). The Orion ion meter (Orion 096000 960 Titrator PLUS System, Thermo Fisher Scientific, USA), 5 day dilution methods, open reflux method, drying method, TOC-V_{csn} analyzer (Shimadzu, Japan), naphthylethylenediamine reagent method, 4-aminoantipyrene method, vanadomolybdo-phosphoric acid colourimetric method and $BaCl_2$ precipitation method were used for the measurement of pH, BOD, COD, total solids (TS), nitrate, total phenol, phosphate and sulphate in TWW samples, respectively. All the physico-chemical parameters reported in the present study were analyzed at Indian Institute of Toxicology research (CSIR-IITR), Lucknow (UP) India.

4.2.3.1. Determination of pH

The negative log of the hydrogen ion concentration is called pH ($pH= - \log_{10} [H^+]$). The pH is an important quality parameter for both the water and wastewater. The determination of pH plays an important role in the wastewater treatment process as it has a direct influence on wastewater treatability – regardless of whether treatment is physical/chemical or biological. In the present study, pH of the TWW collected after secondary (biological) treatment at CETP was determined by the Orion Ion pH Meter (Orion 096000 960 Titrator PLUS System, Thermo Fisher Scientific, USA) according to the manufacturer instructions.

4.2.3.2. Determination of temperature

Temperature is a very important parameter because of its effect on chemical reactions on reaction rates, aquatic life, and the solubility of essential gases such as oxygen in the water. The temperature (°C) of the TWW collected after secondary (biological) treatment at CETP was determined by using a Lab Pro Laboratory Analog chemical Thermometer (Lab Pro Inc., CA, USA).

4.2.3.3. Determination of electrical conductivity

Electrical conductivity (EC) is a measure of how well a material can conduct electricity. This ability is directly related to the concentration of ions present in the

water or wastewaters. These conductive ions come from dissolved salts and inorganic materials such as alkalis, chlorides, sulfides, and carbonate compounds. Compounds that dissolve into ions are also known as electrolytes. The more ions that are present, the higher the conductivity of water. Likewise, the fewer ions that are in the water or wastewater, the less conductive it is. EC is usually measured in micro-or millisiemens per centimeter (µS/cm or mS/cm^{-1}). The EC of the TWW collected after secondary (biological) treatment at CETP was determined by an electrochemical method using an HQ14D Portable Conductivity Meter (Hach Company, Colorado, USA).

4.2.3.4. Determination of biochemical oxygen demand

Biochemical oxygen demand (BOD) is the most widely used test to know the index of organic pollution, the approximate quantity of oxygen that will be required to biologically stabilize the organic matter, efficiency of some treatment processes and determine the compliance with wastewater discharge permits. It is on the principle that if sufficient oxygen is available, aerobic biological decomposition (i.e., stabilization of organic waste) by microorganisms will continue until all waste is completely consumed. It is also known as "BOD_5" since it is based on the accurate measurement of dissolved oxygen (DO) at the beginning and end of a five-day period in which the sample is placed in the dark to prevent the possibility of photosynthetic production of DO and incubated at 20°C. The change in concentration of DO over five days represents the "oxygen demand" for respiration by the aerobic biological microorganisms in the sample. If the BOD of a wastewater sample is high, it means the wastewater contains too much of bio-degradable organic compounds and hence, responsible for polluting the receiving water highly. Therefore, the determination of BOD is very important for water and wastewater.

The BOD of the TWW collected after secondary (biological) treatment at CETP was determined by BOD_5 method. For this, following reagents were used: (a) Phosphate Buffer Solution: dissolved 8.5 g of KH_2PO_4, 21.75 g of K_2HPO_4, 33.4 g of Na_2HPO_4.7H2O and 1.7 g of NH_4Cl in about 500 ml distilled water (DW) and diluted to 1000 ml, the pH should be 7.2 without further adjustment, (b) Magnesium Sulfate Solution: dissolved 22.5 g of $MgSO_4.7H_2O$ in DW and diluted to 1000 ml, (c) Calcium Chloride Solution: dissolved 27.5 g of $CaCl_2$ in DW and diluted to 1000 ml, (d) Ferric Chloride Solution: dissolved 0.25 g of $FeCl_3.6H_2O$ in DW and diluted to 1000 ml, (e) Sodium Sulfite Solution: dissolved 1.575 g of Na_2SO_3 in 1000 ml DW,

(f) Manganese Sulphate Solution: dissolved 480 g of $MnSO_4.4H_2O$ in 1000 ml DW, (g) Alkaline-iodide-azide Reagent: dissolved 500 g of NaOH and 135 g of NaI in 1000 ml DW, (h) Starch: dissolved 2 g of starch in 1000 ml DW, (i) Standard Sodium Thiosulphate: dissolved 6.025 g of $Na_2S_2O_3.5H_2O$ and 0.4 g of NaOH in 1000 ml DW.

For BOD determination, firstly dilution waters were prepared. TWW sample was taken in the suitable BOD bottle (capacity: 300 ml) and 1 ml of phosphate buffer, $MgSO_4$, $CaCl_2$ and $FeCl_3$ solutions/l of water were added and seeded 1 ml L^{-1} water dilution. The temperature was maintained at 20 ± 1 ºC. Further, to these bottles, 1.0 ml of $MnSO_4$ solution was added followed by the addition of 1.0 ml of alkaline-iodide-azide reagent and mixed properly by inverting the bottle a few times. Further, kept the bottles to precipitate and settle the sample sufficiently and 1.0 ml of conc. H_2SO_4 was again added and mixed properly by inverting the bottle several times until dissolution was completed, titrated with 0.025 M $Na_2S_2O_3$ solution to pale straw color and then, initial DO was determined. In addition, few drops of the starch solution were added and titration was done until the disappearance of blue color was achieved. Afterward, BOD bottles containing 1000 × dilution, seed controls, and dilution water blanks were incubated at 20 ± 1 ºC BOD bottles. Further, final DO after 5 day incubation period was determined as per the method described above.

The calculation of BOD was done according to the following equation:

$$BOD_5\ (mgL^{-1}) = (D_1 - D_2) - (B_1 - B_2)\ f / P \quad \text{..... (1)}$$

[Where, D_1 = DO of diluted TWW sample immediately after preparation (mg L^{-1}), D_2 = DO of diluted TWW sample after 5 day incubation at 20 ºC (mg L^{-1}), P = Decimal volumetric fraction of TWW sample used, B_1 = DO of seed control before incubation in mg L^{-1}, B_2 = DO of seed control after incubation in mg L-1, f = Ratio of seed in diluted TWW sample to seed in seed control].

4.2.3.5. Determination of chemical oxygen demand

Chemical oxygen demand (COD) is the most popular alternative test to BOD for establishing the concentration of organic matter in wastewater samples. The COD test only takes a few hours (3 h) to complete, giving it a major advantage over the 5-day BOD test. Wastewater treatment system personnel can use COD as an almost real-time operational adjustment parameter. COD can test wastewater that is too toxic for

the BOD test. The COD test should be considered an independent measure of the organic matter in a wastewater sample rather than a substitute for the BOD_5 test. The COD test uses a chemical (potassium dichromate in a 50% sulfuric acid solution) that "oxidizes" both organic (predominate) and inorganic substances in a wastewater sample, which results in a higher COD concentration than BOD concentration for the same wastewater sample since only organic compounds are consumed during BOD testing.

For COD determination, TWW was refluxed with a strong acid solution with a known excess of potassium dichromate ($K_2Cr_2O_7$) in a reflux apparatus consisting of 250 mL Erlenmeyer flasks with ground-glass 24/40 neck and 300 mm jacket Liebig, west or equivalent condenser with 24/40 ground-glass joint. After digestion, the remaining unreduced ($K_2Cr_2O_7$) is titrated with ferrous ammonium sulfate to determine the amount of ($K_2Cr_2O_7$) consumed and the oxidizable organic matter is calculated in terms of oxygen equivalent.

Following chemical and reagents were used for COD determination: (a) Standard Potassium Dichromate Solution, 0.0417 M: dissolved 12.259 g of $K_2Cr_2O_7$, previously dried at 103 ^oC for 2 h, in DW and diluted to 1000 ml, (b) Sulphuric Acid Reagent: added 5.5 g Ag_2SO_4 in 1000 g of sulphuric acid (H_2SO_4), (c) Ferrion indicator solution: dissolved 1.484 g of phenanthroline monohydrate and 695 mg of $FeSO_4.7H_2O$ in DW and diluted to 100 ml, (d) Mercuric Sulphate: powdered mercuric sulphate ($HgSO_4$), and (e) Standard Ferrous Ammonium Sulphate (FAS), titrant, 0.25N: dissolved 98 g of ferrous ammonium sulphate [$Fe(NH_4)_2(SO_4)_2.6H_2O$] in DW. To this, 20 ml of concentrated sulphuric acid (H_2SO_4) was added, diluted to 100 ml, and standardized the solution against standard $K_2Cr_2O_7$ solution as per method as follows: 10 mL standard $K_2Cr_2O_7$ was diluted up to 100 ml and to this, 30 ml of concentrated hydrogen sulfides was added, heated, cooled and titrated with FAS titrant using 0.10 to 0.15 ml (2 to 3 drops) ferrion indicator.

Molarity of FAS solution = Volume 0.0417 M $K_2Cr_2O_7$ solution titrated (ml) × 0.25 / volume of FAS used in titration (ml)……. (2)

For COD determination, TWW sample (20) was taken and diluted to 50 ml in a 250 ml refluxing flask, added 1.0 g $HgSO_4$, some glass beads and 5 ml sulphuric acid reagent was added very slowly with proper mixing to dissolve $HgSO_4$. Afterward,

cooled while mixing the sample to avoid the loss of volatile materials and then added 25 mL of 0.0417 M $K_2Cr_2O_7$ solution and mixed well. Attached the flask to a condenser and turned on cooling water. Remaining sulphuric acid reagent (70 ml) was added through the open end of the condenser, continued swirling and mixing while adding the sulphuric acid reagent. The open end of the condenser was covered with a small beaker to prevent foreign material from entering refluxing mixture and refluxed for 2 h. Cooled and washed down condenser with DW. A disconnected reflux condenser and diluted mixture to about twice its volume with DW and then cooled to room temperature and titrated excess $K_2Cr_2O_7$ with FAS using 2-3 drops (0.10 to 0.15 ml) ferrion indicator. The first sharp change from blue-green to reddish brown was taken as an endpoint of the titration. In the same manner, refluxed and titrated a blank containing the reagents and a volume of DW equal to that of the sample. Further, the COD of the TWW collected after secondary (biological) treatment at CETP was calculated by the following equation:

$$COD\ (mgl^{-1}) = (A - B) \times M \times 8000 / \text{volume of sample (ml)} \quad (3)$$

[Where, A = volume of FAS used for blank (ml), B = volume of FAS used for sample (ml), M = molarity of sample]

4.2.3.6. Determination of total solids

The term 'total solid (TS)' refers to the matter either filterable or non-filterable that remains as residue upon evaporation and subsequent drying at a defined temperature. The residue left after the evaporation and subsequent drying in an oven at a specific temperature (103 – 105 °C) of a known volume of the sample are TSs.

For TSs determination, firstly heated the clean evaporating dish at 103 to 105 ºC for 1 h and then cooled in a desiccator and weighed immediately. Further, 20 ml of well mixed TWW sample was taken in a pre-weighed evaporating dish and then evaporated to dryness in a drying oven at approximately 2 ºC below boiling to prevent splattering. Dried evaporated sample for at least 1 h in an oven at 103 to 105 ºC, cooled the evaporating dish in a desiccator to balance temperature and then weighed. Repeated cycles of drying, cooling, desiccating and weighing were performed till a constant weight was obtained, or until weight change was less than 4% of the previous weight or 0.5 mg. The TSs in the TWW collected after secondary (biological) treatment at CETP was calculated by the following equation:

TSs (mg^{-l}) = (A – B) × 1000 / Volume of the sample (ml)…….. (4)

[Where, A = weight of dried residue (mg) + dish and B = weight of dish (mg)]

4.2.3.7. Determination of total dissolved solids

A well mixed TWW sample is filtered and the filtrate is evaporated to dryness in a weighed evaporating dish and then dried to constant weight at 180 °C. The increase in the weight of the evaporating dish represents the total dissolved solids (TDSs). This procedure may be used for drying at other temperatures.

For the determination of TDSs, firstly heated the clean dish at 103 to 105 ºC for 1 h and then cooled in a desiccator and weighed immediately. Further, Now 20 ml of the well-mixed TWW sample was taken in a pre-weighed evaporating dish and evaporated to dryness in a drying oven at approximately 2 ºC below boiling to prevent splattering. Afterward, the dried and evaporated TWW sample for at least 1 h in an oven at 103 to 105 ºC, cooled the evaporating dish in desiccators to balance the temperature and weighed immediately. Repeated cycles of drying, cooling, desiccating and weighing were performed till a constant weight was obtained, or until weight change was less than 4% of previous weight or 0.5 mg. The TDSs in the TWW collected after secondary (biological) treatment at CETP was calculated by the following equation:

TDSs (mg^{-l}) = (A – B) × 1000 / Volume of sample (ml)……... (5)

[Where, A = weight of filter (mg) + dried residue and B = weight of filter (mg)]

4.2.3.8. Determination of total suspended solids

The determination of total suspended solids (TSSs) was done by subtracting the reading of total dissolved solids (TDSs) by total solids (TSs) and calculated according to the following equation:

TSS (mgl-1) = TS-TDS……..... (6)

4.2.3.9. Determination of phenol

Phenol is defined as a hydroxy derivative of benzene and its condensed nuclei may occur in domestic and industrial wastewaters. Steam-distillable phenols react with 4-Aminoantipyrine at pH 7.9 ± 0.1 in the presence of potassium ferricyanide to form a colored antipyrine dye. This dye is extracted from aqueous solution with $CHCl_3$ and

the absorbance is measured at 460 nm. This method covers the phenol concentration ranging from 1.0 μg L^{-1} to over 250 μg L^{-1} with a sensitivity of 1 μg L^{-1}.

Following apparatus were used in the experiment: spectrophotometer, filter funnels (Buchner type with fritted disk), filter paper (11 cm filter paper for filtering chloroform (CHCl3) extracts), pH meter, separatory funnels (1000 ml Squibb form, without ground-glass stoppers and TFE stopcocks).

Following reagents (prepared in DW and free of phenols and chlorines) were used in the experiment: (a) Stock Phenol Solution: dissolved 100 mg of phenol in freshly boiled and cooled distilled water and diluted to 100 ml, (b) Intermediate Phenol Solution: diluted stock phenol solution (1 ml) in freshly boiled and cooled DW to 100 ml [1 mL = 10 μg phenol], (c) Standard Phenol Solution: diluted 50 ml of intermediate phenol solution to 500 ml with freshly boiled and cooled DW [1 ml = 1 μg of phenol, prepared within 2 h of use], (d) Bromate-bromide Solution: dissolved 2.784 g of anhydrous potassium bromo-oxide ($KBrO_3$) in DW and the, added 10 g of potassium bromide (KBr) crystals, dissolved and diluted to 1000 ml, (e) Hydrochloric Acid: concentrated hydrochloric acid (HCl), (f) Ammonium Hydroxide (NH_4OH), 0.5 N: diluted 35 ml of fresh concentrated NH_4OH to 1000 ml with DW, (g) Standard Sodium Thiosulphate Titrant, 0.025 M: dissolved 6.025 g of $Na_2S_2O_3.5H_2O$ and 0.4 g of NaOH in 1000 ml DW, (h) Starch Solution: dissolved 2.0 g of laboratory-grade soluble starch and 0.2 g of salicylic acid as a preservative, in 1000 ml hot DW, (i) Phosphate Buffer Solution: dissolved 104.5 g of K_2HPO_4 and 72.3 g of KH_2PO_4 in water and dilute to 1000 ml DW (pH = 6.8), (j) 4-Aminoantipyrine Solution: dissolved 2.0.g of 4-aminoantipyrine in water and diluted to 100 ml, (k) Potassium Ferricyanide Solution: dissolved 8.0 g of $K_3Fe(CN)_6$ in water and diluted to 100 ml, filtered, if necessary, it was stored in brown glass bottle or prepared freshly, (l) Chloroform: laboratory grade chloroform ($CHCl_3$), (m) Sodium Sulphate: anhydrous sodium sulphate (Na_2SO_4), and (n) Potassium Iodide: laboratory grade potassium iodide (KI) crystals.

For the determination of phenol, TWW sample was taken in a distillation flask and its pH was adjusted to 2-3 using 1N hydrochloric acid and removed oil and grease from the sample by transferring it in a separatory funnel and extracted oil and grease with 25 ml of chloroform. Repeated this process twice to ensure complete removal of oil and grease, then added four drops of orthophosphoric acid and three drops methyl

orange indicator to the sample. Distilled the solution and placed 500 ml distillate in a 1000 ml flask. Prepared a 500 ml blank and a series of 500 ml of phenol standards (5, 10, 20, 30, 40, and 50 µg phenol). The treated samples, blank and standards are as follows: added 12.0 ml 0.5 N NH_4OH and immediately adjusted pH to 7.9 ± 0.1 with phosphate buffer (10 ml) and transferred it to a 1000 ml separating funnel. Added 3 ml of 4-aminoantipyrine solution, mixed well followed by addition of 3 ml $K_3Fe(CN)_6$ solution. Mixed the solution well and let color to develop for 15 min. Extracted immediately with 50 ml of chloroform each time. Shaken the separating funnel many times (10 times), let $CHCl_3$ settle, shaken again and let the $CHCl_3$ to settle again. Filtered each $CHCl_3$ extract through filter paper or fritted glass funnels containing a 5 g layer of anhydrous Na_2SO_4. The dried extract was collected in clean test tubes or cells for absorbance measurements. Read absorbance of samples and standard against the blank at 460 nm. Constructed calibration curve by plotting absorbance against the micrograms of phenol concentration and calculated the amount of phenol in samples using this curve.

The concentration of phenol in the TWW collected after secondary (biological) treatment at CETP was calculated by the following equation:

Phenol (mg^{-l}) = (A × 100) / B……..…….…….. (7)

[Where, A = phenol in sample (mg) from calibration curve, B = volume of sample (ml)]

4.2.3.10. Determination of sulphate

Sulfate is precipitated in hydrochloric acid (HCl) solution as barium sulfate ($BaSO_4$) by the addition of barium chloride ($BaCl_2$). The precipitation is carried out near the boiling temperature, and after a period of digestion, the precipitate is filtered, washed with water until free of Cl^-, ignited or dried, and weighed as $BaSO_4$.

Following apparatus were used in the experiment: steam bath, drying oven, equipped with thermostatic control, muffle furnace, with temperature indicator, desiccator, analytical balance, capable of weighing to 0.1 mg, and filter (fritted-glass filter, fine (‘‘F’’) porosity, with a maximum pore size of 5 µm or membrane filter, with a pore size of about 0.45 µm or vacuum oven).

Following reagents were used for the determination of sulphate: (a) Methyl Red Indicator Solution: dissolve 100 mg of methyl red sodium salt in DW water and dilute

to 100 ml, (b) Hydrochloric Acid (HCl), (c) Barium Chloride Solution: dissolve 100 g of $BaCl_2.2H_2O$ in 1 litre DW, filter through a membrane filter or hard- finish filter paper before use; 1 ml is capable of precipitating approximately 40 mg of SO_4^{2-}, and (d) Silver Nitrate-nitric Acid Reagent: dissolve 8.5 g of $AgNO_3$ and 0.5 ml of conc. HNO_3 in 500 ml DW.

For the determination of sulphate: (a) precipitation of barium sulfate: TWW sample was taken in a flask and pH was adjusted to 4.5 to 5.0 with HCl using a pH meter or the orange color of methyl red indicator. Added 1 to 2 ml HCl and heated to boiling and while stirring gently, slowly added warm $BaCl_2$ solution until precipitation appeared to be completed, then added about 2 ml in excess, digested precipitate overnight at 80 to 90 ºC. (b) filtration and weighing: filtered $BaSO_4$ through a pre-weighed membrane filter at room temperature. Washed precipitate with several small portions distilled water until washings are free of Cl^- as indicated by testing with $AgNO_3$-HNO_3 reagent. Added a few drops of silicone fluid to the suspension before filtering, to prevent adherence of precipitate to holder. Dried, filter and precipitated in a conventional oven at a temperature of 103 to 105 ºC. Cooled in a desiccator and weighed.

The concentration of sulphate in the TWW collected after secondary (biological) treatment at CETP was calculated by the following equation:

$$\text{Sulphate } (mg^{-l}) = (BaSO_4 \times 411.5) / A \quad \ldots\ldots (8)$$

[Where, A = volume of sample (ml)]

4.2.3.11. Determination of phosphate

Organic phosphates are formed primarily by biological processes. They are contributed to sewage by body wastes and food residues and also may be formed from orthophosphate in biological treatment processes or by receiving water biota. Molybdo-phosphoric acid is formed and reduced by stannous chloride to intensely colored molybdenum blue. The minimum detectable concentration is about 3 µg phosphate/l. The sensitivity at 0.3% absorbance is about 10 µg P/l for an absorbance change of 0.009. Following apparatus were used in the experiment: spectrophotometer (400-490 nm), filtration apparatus and filter paper (Whatman No. 42), and acid washed glassware (with hot diluted HCl and rinsed well with DW).

Following reagents were used in the determination of phosphate: (a) Phenolphthalein Indicator Aqueous Solution, (b) Strong-acid Solution: slowly added 300 ml of concentrated H_2SO_4 to about 600 ml DW, when cold, added 4.0 ml concentrated HNO_3 and diluted to 1000 ml, (c) Ammonium Molybdate Reagent I: dissolved 25 g of $(NH_4)_6MO_7O_{24}.4H_2O$ in 175 ml DW, cautiously added 280 ml of concentrated H_2SO_4 to 400 ml DW, cooled and added molybdate solution and diluted to 1000 ml, (d) Stannous Chloride Reagent I: dissolved 2.5 g of fresh $SnCl_2.2H_2O$ in 100 ml of glycerol, heated in a water bath and stirred with a glass-rod to hasten dissolution. The reagent was stable and required neither preservative nor special storage, (e) Standard Phosphate Solution: dissolved 219.5 mg of anhydrous KH_2PO_4 in DW and diluted to 1000 ml; 1.00 ml = 50.0 μg PO_4^{3-} - P, (f) Benzene-iso-butanol Solvent: mixed equal volumes of benzene and isobutyl alcohol, (g) Ammonium Molybdate Reagent II: dissolved 40.1 g of $(NH_4)_6MO_7O_{24}.4H_2O$ in approximately 500 ml DW, slowly added 396 ml of ammonium molybdate reagent I, cooled and diluted to 1000 ml, (h) alcoholic sulphuric acid solution, cautiously added 20 mL of conc. H_2SO_4 to 980 ml methyl alcohol with continuous mixing, (i) Diluted Stannous Chloride Reagent II: mixed 8 mL of stannous chloride reagent I with 50 ml of glycerol. The reagent was stable for at least 6 months.

For the determination of phosphate, TWW sample was taken and diluted to 100 ml with distilled water and added 4.0 ml molybdate reagent I and 0.5 ml (10 drops) stannous chloride reagent I. After 10 min, but before 12 min, measured colour photometrically at 690 nm and compared with a calibration curve using distilled water blank. The concentration of phosphate in the TWW collected after secondary (biological) treatment at CETP was calculated by the following equation:

Phosphate (mg^{-1}) = P (in approx. 104.5 ml final volume) / A × 1000……. (9)

[Where, A = volume of sample (ml)].

4.2.3.10. Determination of nitrate

Nitrate is often found in the wastewaters of biological treatment plants because it represents the final form of nitrogen from the oxidation of organic nitrogen compounds. The U.S. Public Health Service (USPHS) has designated the safe limit for nitrogen in nitrates to be 10 mgl^{-1}. Nitrates in drinking water are particularly dangerous to small children, infants, and fetuses. Following apparatus were used in

the experiment: spectrophotometer set at 550 nm and filter paper and vacuum filtration apparatus. The reagents used in the experiment were: (a) stock potassium nitrate solution: 50 mgL^{-1}, (b) HCl (c) Sulfanilic acid, (d) Zn/NaCl, (d) N-1-naphthylethylenediamine dihydrochloride reagent, (e) Sodium acetate solution.

The determination of nitrate was accomplished in the following steps: preparation of standard: prepare a 5 point calibration curve using the stock KNO_3 solution above. Prepared the concentrations in the range of 0 - 15 mg^{-1}, analysis of sample: TWW sample was taken in a 250 ml Erlenmeyer flask added 1.0 ml if HCl, and 1.0 mL of sulfanilic acid reagent and mix thoroughly. In a dry 10 ml graduated cylinder, measure 1 ml of Zn/NaCl and add it to the Erlenmeyer flask. Swirl the flask for 7 min. Filter with a vacuum flask after the seven minutes and rinse the Erlenmeyer flask well with DW and pour the water sample back into the flask, color development: added 1.0 ml of the naphthylethylenediamine reagent to the filtered sample and mix. Properly, added 1.0 ml of the 2M sodium acetate solution and mix. Properly, allow 5 min (or more) for color development, spectrophotometric Measurement: measure the color intensity with the spectrophotometer set at 550 nm.

4.2.4. Analysis of heavy metals in tannery wastewater (TWW) collected from CETP after the secondary treatment process.

The detection and quantification of heavy metals (HMs) in TWW sample was done by nitric-perchloric acid digestion method discussed in the "Standard Methods for Examination of Water and Wastewater" (APHA 2012). Briefly, 20 ml of filtered TWW was taken in a beaker (250 ml) and to this, 10 ml of digestion solution (a mixture of HNO_3 + $HClO_4$ in 6:1 ratio) was added and kept for digestion at 85 °C to oxidize the oxidizable matter until the dense white fumes comes out followed by the formation of white precipitates, an indication of complete digestion. After cooling, the obtained precipitates were dissolved in double distilled water (10 ml), filtered through Whatman no. 42 filter paper (Whatman, England, UK), transferred to a volumetric flask and the final volume make up to the mark with double distilled water and finally used for HMs analysis carried out by inductively coupled plasma spectrophotometer (Thermo Electron; Model IRIS Intrepid II XDL, USA). The concentration of HMs in the TWW collected after secondary (biological) treatment at CETP was calculated by the following equation:

Concentration of element (mg L^{-1}) = (Observed concentration - Blank) × Dilution factor….. (10)

4.2.5. Quality control and quality assurance

The reference stock standard of metals (E-Merck, Germany) was used for the calibration and quality assurance for each analytical batch. The analytical data quality of metals (Cr, Cd, Cu, Zn, Ni, Pb, As, Fe, and Mn) was ensured by using EPA samples in water and the results were found to be within the prediction intervals. The blanks were run in triplicate to check the precision of the method with each set of samples.

4.2.6. Statistical analysis

All the laboratory experiments were performed in triplicates (n=3). The statistical calculations were done as per the standard methods outlined (Steel and Torrie 1992) and the results were expressed as mean ± SD values.

4.3. Results and discussion

The physico-chemical analysis of TWW has revealed that it has high BOD (436 ± 4.58 mgl^{-1}), COD (1428 ± 5.56 mgl^{-1}), TSS (2216 ± 2.64 mgl^{-1}), TDS (4064 ± 3.46 mgl^{-1}), sulfate (6.75 ± 0.27 mgl^{-1}), phosphate (118.66 ± 5.03 mgl^{-1}), nitrate (14.05 ± 0.16 mgl^{-1}) and phenol (8.68 ± 0.04 mgl^{-1}) content with alkaline pH (8.2 ± 0.05), dark brown color and objectionable odour. Besides these, a high concentration of different HMs such as Cr (6.88 ± 0.02 mgl^{-1}), Cd (1.18 ± 0.03 mgL^{-1}), and Pb (0.38 ± 0.03 mgl^{-1}) was also detected in TWW. All the values for various physico-chemical parameters obtained in this study were found higher than the recommended permissible values for industrial discharge (Table 4.1). The objectionable odour might be due to the presence of sulphides in TWW whereas azo dyes used in coloring of leather might be a cause for its dark brown color, which blocks the sunlight penetration in receiving water bodies and reduces photosynthetic activity, dissolved oxygen content and thus, negatively affects the aquatic life (Mahmood et al. 2013; Saxena et al. 2016).

Table 4.1 Physico-chemical characteristics of collected TWW sample

Physico-chemical parameter	**Recorded values**	**Effluent discharge standards**	
		(CPCB, 2010; MoEF&CC 2016)	**(USEPA 2002)**
Color	Dark brown color	-	-
Odor	Objectionable	-	-
pH	8.2 ± 0.05	6.0-9.0	-
Temperature (°C)	32 ± 0.57	<35	-
EC (mS $cm^{-1)}$	11.65 ± 0.08	0.85	-
BOD (mgl^{-1})	436 ± 4.58	30.00	40.00
COD (mgl^{-1})	1428 ± 5.56	250.00	120.00
TS (mgl^{-1})	6280 ± 3.60	-	-
TDS (mgl^{-1})	4064 ± 3.46	2100.00	-
TSS (mgl^{-1})	2216 ± 2.64	100.00	-
Phosphate (mgl^{-1})	118.66 ± 5.03	5.0	-
Sulfate (mgl^{-1})	6.75 ± 0.27	-	-
Nitrate (mgl^{-1})	14.05 ± 0.16	10.0	-
Phenol (mgl^{-1})	8.68 ± 0.04	1.0	0.50
Heavy metals (mgl^{-1})			
Cr	6.88 ± 0.02	2.0	0.05
Cd	1.18 ± 0.03	0.05	0.01
Cu	1.72 ± 0.05	3.0	0.50
Zn	0.96 ± 0.03	5.0	2.00
Ni	0.68 ± 0.02	3.0	0.10
Pb	0.38 ± 0.03	0.1	0.05
As	BDL	0.2	0.010
Fe	2.86 ± 0.30	3.0	2.00
Mn	0.72 ± 0.04	2.0	0.20

Footnote: All the values are mean of triplicates (n = 3) ± SD, EC: Electrical conductivity; BOD: Biological oxygen demand; COD: Chemical oxygen demand; TS: Total solids; TDS: Total dissolved solids; TSS: Total suspended solids; BDL: Below detection limit.

The dark brown colored TWW causes a variety of serious health hazards to both vertebrates and invertebrates animals in the receiving water bodies. TWW contaminated water has been reported to induce nuclear abnormalities in the erythrocytes of fish, *Oreochromis niloticus* (Matsumoto et al. (2006). TWW is responsible for embryonic toxicity and reported to cause coagulation of fertilized eggs, detachment of tail-bud from the yolk sac, yolk sack edema, malformation of the tail, scoliosis, and deformation of the swim bladder in the embryos of zebrafish, *Danio rerio* (Rocha and De Oliveira 2017). TWW has been also reported to interfere with the metabolic processes by altering the activity of oxidative enzymes in different organs of guppy fish, *Poecilia reticulata* and thus, causes cellular injury as a result of exposure (Aich et al. 2011, 2015). Wosnie and Wondie (2014) assessed the downstream effect of Bahir Dar TWW on macroinvertebrates populations in the Blue Nile River and reported a drastic reduction in their diversity using different indexes. In addition, TWW has been also reported to cause acute embryotoxicity and developmental defects in the sea urchins (*Paracentrotus lividius* and *Sphaerechinus granularis*) and serious toxicity in *Daphnia magna* (Oral et al. 2007). Further, TWW has been reported to cause detrimental changes in the biochemical parameters like antioxidants, protein, carbohydrate, and amino acid, considerable damage in the gonad and mantle tissues and also genotoxic effects in the snail, *Pila globosa* (Bhattacharya et al. 2016).

The high BOD, COD and TDS values of TWW might be due to the high organic content, unknown POPs, dissolved minerals, and salts and thus, the collected wastewater is of very high strength, which is responsible for serious soil and water pollution. When the untreated or partially treated TWW with high BOD, COD, TDS and salt load discharged on land or utilized for irrigation practices, it causes a significant level of soil pollution. High salinity (salt load) of TWW is the most brutal cause of reduced soil fertility because salt gets accumulated in the soil capillaries resulted in the increased soil osmotic pressure and thus, interfere with the plant nutrient absorption and ultimately, reduce agricultural farming efficacy. High salt concentration also causes a reduction in the activity and biomass of soil microbes, which play an important role in the mineralization of organic matter and thus, contribute to the reduced soil fertility (Yan et al. 2015). Moreover, TWW also adversely affect the soil chemical and biological characteristics as well as its high

sulfide content also causes the deficiency of some essential micronutrients in the soil such as Zn, C, and Fe, etc. (Raj et al. 2014; Alvarez-Bernal et al. 2006). In addition, the highly polluted TWW also causes serious water pollution when discharged into the nearby canals, which further join rivers where it adds a huge amount of salts to fresh water and thus, increases the salinization of rivers. Furthermore, the increased salinization of groundwater mainly occurs due to the presence of a large amount of Cr salt in TWW. Cr^{6+} is highly toxic in nature due to its high solubility as compared to Cr^{3+} and thus leach out to the deeper layer of soil and ultimately, causes groundwater pollution resulting in the severe health hazards (Saxena et al. 2016).

A high concentration of total suspended solids (TSS) in the untreated TE was might be due to the presence of HMs. Suspended solids act as carriers for HMs and other sources of contaminants in the aquatic system. The high sulfate, phosphate, and nitrate content in TWW might be associated with the use of sulfuric acid and sulfide in the dehairing process; monosodium, disodium phosphates as well as polyphosphates in leather treatment and processing and ammonium salts in the deliming and bating process, respectively, which are responsible for the eutrophication and disturbed the normal ecological functioning of receiving water bodies. The high phenol, Cr, Cd, and Pb content might be due to the excess use of phenolic compounds [(pentachlorophenol (C_6HCl_5O) and nonylphenol ($C_{15}H_{24}O$)] in preservation of raw hides/skins and leather finishing, basic chromium sulphate [$Cr_2(SO_4)_3$] in leather tanning, Cd-based pigments and dyes used in the leather coloring, and lead chromate [$PbCrO_4$] as fastening agent for marking and surfacing of leather, respectively (Saxena et al., 2016). Phenolic compounds, Cr and Pb are listed as the "priority pollutant" by the United States Environmental Protection Agency (USEPA) and reported to cause severe toxic, genotoxic and carcinogenic effects in plants, animals and human beings (https://www.epa.gov/sites/production/files/2015-09/documents/priority-pollutant-list-epa.pdf). In present study, the physico-chemical parameters of TWW were well corroborated with those reported in the earlier study (Tripathi et al. 2011). However, the findings reported in the present study were also supported by previous studies (Yadav et al. 2019; Bharagava and Mishra 2018; Kumari et al. 2016; Chandra et al. 2011)

4.4. Conclusion

The present study was designed to characterize TWW for various physico-chemical parameters after secondary treatment at the CETP of LIs, Unnao, India to know the pollution profile of collected wastewater. The physico-chemical characterization of TWW reported that it has very high pollution parameters beyond the permissible limits of industrial discharge and was highly polluted and hence, is not suitable for discharge in the receiving environment because it may cause serious environmental risks and toxic effects in the exposed organisms. Therefore, regular monitoring and assessment of TWW are required before discharge into the environment; however, its adequate treatment is a dire need for environmental and public health protection. Overall, the CETP is not complying with the discharge standards; however, the findings reported in the present study will be useful to modify/improve the existing CETP accordingly for the adequate treatment and management of TWW not only for the pollution prevention and control but also public health protection.

5

Chapter-05

Detection of Persistent Organic Pollutants from Tannery Wastewater By HPLC/ GC-MS-MS/LC-MS-MS Analysis

Detection of persistent organic pollutants from tannery wastewater by HPLC/GC-MS-MS/ LC-MS-MS analysis

5.1. Introduction

Thousands of chemical compounds are released and find their way into the environment, i.e. air, land, groundwater and surface water, by the industrial activity, agriculture practices, domestic activity, etc. Environmental pollutants released from the wastewater treatment plants (WWTPs) are widespread in the industrialized countries, causing the direct pollution of aquatic resources and soils and indirect pollution of the groundwater. Hence, the elimination of these pollutants from our natural environment is an absolute requirement to promote the sustainable development of our society with low environmental impact.

Persistent organic pollutants (POPs) are defined as highly toxic and hazardous organic chemical compounds that are resistant not easily degraded by biological, chemical or physical means and thus, remains in the environment for a long period of time and causes serious environmental pollution and toxicity in the environment (Saxena and Bharagava 2015). Many POPs are currently being used are pesticides, solvents, chlorinated phenols, pharmaceuticals, and industrial chemicals. During the leather tanning process, a variety of highly toxic and hazardous persistent organic chemicals are currently being used along with inorganic heavy metals (HMs) to convert the raw hide/skins into the commercial form of leather or leather products (Bharagava et al. 2018; Saxena et al. 2018). These persistent organic chemicals do not degrade much during the secondary (biological) treatment process and ultimately, discharged along with TWW into the receiving environment causing serious soil and water pollution along with the serious threat to human health in different ways. The composition of organic pollutants in TWW is very complex. Proteins, mainly collagen and their hydrolysis products - amino acids derived from the skin are predominant, while others such as fats are in low concentrations. The most important organic chemicals used in the tanning of raw hide/skins are tannins both natural and synthetic, dyes, fatty aldehydes and quinones (Lofrano et al. 2013). LIs also use several toxic chemical compounds like aliphatic amines, biocides, non-ionic surfactants, sulphonated oils, dyes, pigments, etc. during leather production (Lofrano et al. 2013). Most of these pollutants are available in a soluble form, but a lot of them exist in suspension and only a few are colloids (Ates et al. 1997; Cassano et al. 2001; Di

Iaconi et al. 2002). Furthermore, there has been an increasing environmental concern regarding the release of various POPs in TWW, which do not degrade during the secondary treatment process at the common effluent treatment plant (CETP) of LIs. Hence, the information about the nature and characteristics of POPs remained in TWW even after the secondary treatment process at CETP is urgently required to understand their mechanism of toxicity and to protect the human health and environment.

Therefore, the present study was aimed to characterize and identify the unknown POPs remained in TWW even after the secondary (bacterial) treatment process at CETP of LIs. To do so, liquid-liquid extraction (LLE) method with the different combination of organic solvents was used for the extraction of POPs from TWW, which were further identified by High-Pressure Liquid Chromatography (HP-LC), Fourier Transform Infrared (FT-IR) Spectroscopy and Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

5.2. Materials and methods

5.2. Chemicals, reagents, and solvents used

All the required chemicals, reagents and solvents were used in the experiments are of highest purity (purity ≥ 99%) and analytical grade. The chemicals, reagents, and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Whatman® Grade GF/C filter papers (pore size 1.2 μm) (Whatman, England, UK) were used to filter collected TWW. After filtration, the TWW sample was used for the detection and characterization of persistent organic pollutants to know their nature and characteristics by various analytical techniques such as HP-LC, FT-IR, and GC-MS.

5.2.1. Sample preparation and characterization of persistent organic pollutants in TWW by HP-LC analysis

Liquid-liquid extraction (LLE) method was used for the preparation of TWW sample for HP-LC analysis as per the method described earlier (Bharagava et al. 2018). For this, the collected TWW sample (50 ml) was centrifuged (5000 × g, 10 min, 4 °C) to eliminate the microbial biomass and other suspended solids. The supernatant obtained was acidified (pH ≤ 2.0) with 1 N hydrochloric acid and extracted three times with the equal volume of ethyl acetate (50 ml, HPLC & GC grade, ≥ 99.9%) followed by

dichloromethane (DCM, 50 ml, HPLC & GC grade, ≥ 99.9%) in a separating funnel (500 ml). The solvent layer containing POPs was collected in a beaker and the extracts were combined and evaporated in a Rotavapor (Rotavapor RE 120, Buchi, Flawil, Sweden) at ≤ 40 °C until the solvent is completely evaporated. The dried residues obtained were dissolved in DCM (3.0 ml), filtered through syringe filters (0.22 µm) (Millipore Ltd., Bedford, MA, USA) and the final extract obtained was used in the high-performance liquid chromatography (HP-LC) analysis.

The HP-LC analysis of POPs present in the collected TWW sample was performed on 515 HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Diode Array Detector System (1100 series, Agilent Technologies, USA) and reverse phase C18 column (250 × 4.6 mm, 5 µm particle size) at 27 °C and 2487 Absorbance UV-Vis Detector *via* Millennium® Software (v32). For this, the final extract (20 µl) was injected into the HPLC system and monitored at wavelength 224 nm (absorption maxima) to characterize the POPs in the collected TWW sample. The mobile phase consisted of Milli-Q® water (Millipore Corp., Billerica, MA, USA) and acetonitrile in the volume ratio of 70:30 (v/v) and the flow rate was set at the rate of 1.0 ml min^{-1}.

5.2.2. Sample preparation and characterization of persistent organic pollutants in TWW by FT-IR analysis

To know the functional group of toxic organic chemicals, the TWW sample was oven dried at 105 °C, and obtained pellets were mixed with KBr to obtain the absorption spectra of the wastewater sample. For this, the dried sample was mixed with KBr (IR Grade; purity ≥ 99%) in ratio of 1:30, and the mixture was finely ground and fused into a thin pellet (13 mm diameter and 1 mm thickness) prepared under vacuum condition using a PCI Hydraulic Press (Manual) with a capacity of 15 tons. Further, the absorbance spectrum was recorded by a Nicolet FT-IR Spectrometer (Model Nicolet 6700, Thermo Fisher Scientific, USA). The analysis was carried out in the mid-infrared region from 4000-400 cm^{-1} with a resolution of 4 cm^{-1}. Scanning was performed to obtain the spectrum and measured in the ambient air against the pure KBr as a background spectrum. The data processing was performed using the software OMNIC™ (v7.4). The assignment of the absorption peaks observed in the FT-IR spectrum was done on the basis of those outlined in the "Introduction to Organic Spectroscopy" (Lambert 1987).

5.2.3. Sample preparation and characterization of persistent organic pollutants in TWW by GC-MS analysis

Liquid-liquid extraction (LLE) method was used for the TWW sample preparation for GC-MS analysis. For this, the collected TWW sample (50 ml) was centrifuged (5000 × g, 10 min, 4 °C) to eliminate the microbial biomass and other suspended solids. The supernatant obtained was acidified (pH ≤ 2.0) with 1 N hydrochloric acid and extracted three times with the equal volume of dichloromethane (DCM, 100 ml, HPLC & GC grade, ≥ 99.9%) followed by *n*-Pentane and chloroform (100 ml, HPLC & GC grade, ≥ 99.9%) and dichloromethane (DCM, 50 ml, HPLC & GC grade, ≥ 99.9%) followed by ethyl acetate (50 ml, HPLC & GC grade, ≥ 99.9%) in a separating funnel (500 ml). The solvent layer containing POPs was collected in a beaker and the extracts were combined and evaporated in a Rotavapor (Rotavapor RE 120, Buchi, Flawil, Sweden) at ≤ 40 °C until the solvent is completely evaporated. The dried residues obtained were dissolved in DCM (3.0 ml), filtered through syringe filters (0.22 µm) (Millipore Ltd., Bedford, MA, USA) and the final extract obtained was used in the gas chromatography-mass spectroscopy (GC-MS) analysis.

Afterward, the obtained extract (300 µl) was dried in the GC vials using nitrogen gas and derivatized according to the standard protocol outlined (Gatidou et al. 2007). Briefly, 100 µl of dioxane and 10 µl of pyridine was added to the sample extract and silylated with 50 µl trimethyl silyl [BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane)] by heating the mixture at 70 °C for 30 min with intermittent shaking to dissolve the dried residues and kept for cooling at the ambient temperature.

After cooling, the dissolved residues (in DCM) were analyzed as trimethylsilyl (TMS) derivatives as previously outlined (Bharagava et al. 2008). An aliquot (2.0 µl) of derivatized sample was injected into a DB-5MS capillary column (30 m length × 0.18 mm internal diameter × 0.18 mm film thickness: 5% phenyl + 95% methylpolysiloxane, carrier gas: helium, flow rate: 1 ml min^{-1}) fitted with a Thermo Scientific Trace GC Ultra Gas Chromatograph equipped with a TriPlus auto sampler coupled to a TSQ Quantum XLS triple quadrupole mass spectrometer (Thermo Fisher Scientific, FL, USA). At the start, the column temperature was 60 °C (hold time: 2 min) and increased up to 290 °C min^{-1} (hold time: 20 min) at the rate of 10 °C. The MS transfer line and ion source temperatures were kept at 200 and 250 °C,

respectively. Full scan mode was used to operate MS whereas 3.0 min solvent delay was selected to record the mass spectra within a range of 30-550 (m/z units) at 70 eV (energy). The National Institute of Standards and Technology (NIST, USA) mass spectral library (v1.0.0.12) available with the instrument was used to identify the persistent organic compounds by comparing their mass spectra with that of their retention time (RT).

5.3. Results and discussion

5.3.1. Characteristics of persistent organic pollutants in TWW by HP-LC analysis

A variety of organic pollutants are used in the leather industry during leather production processes, which do not degrade much during the treatment of TWW during its secondary (biological) treatment. These compounds are mainly recalcitrant in nature and may cause serious toxic effects in the environment and hence, the analysis of organic pollutants is required for environmental safety. According to HP-LC analysis, the collected TWW contained a mixture of POPs as revealed by the several peaks obtained at different retention time (RT: 2.31, 2.44, 3.16, 3.33, 3.65, 5.16, and 5.58) (Fig. 5.1). These organic compounds, which are detected at different RT, were recalcitrant in nature and further characterized by FT-IR and GC-MS analysis to know their nature, characteristics, and toxicity in the environment.

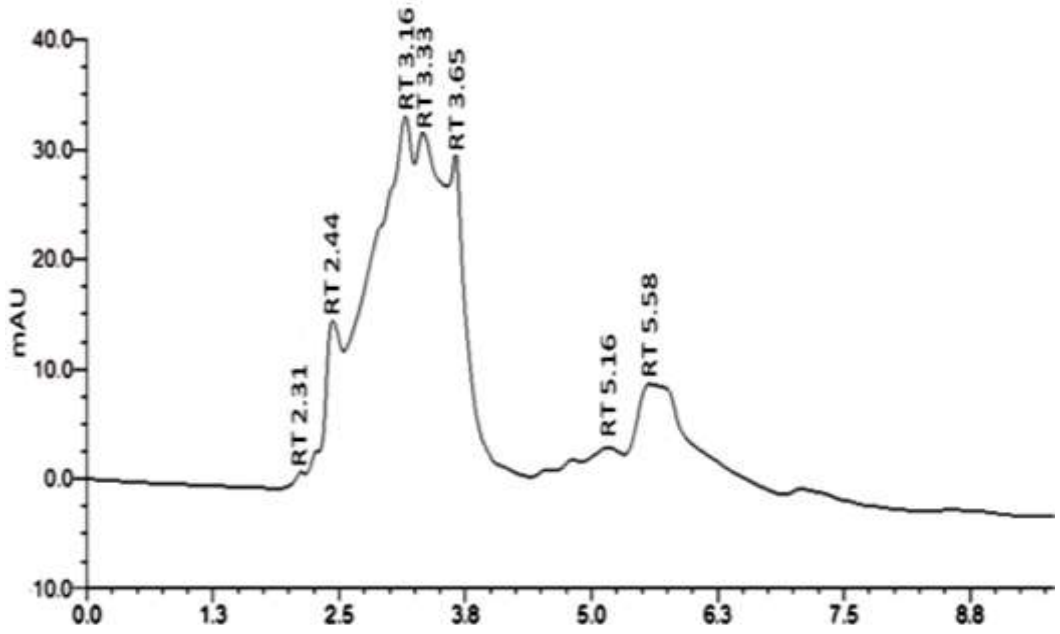


Fig. 5.1 HP-LC chromatogram of the collected TWW sample

5.3.2. Nature and characteristics of persistent organic pollutants in TWW by FT-IR analysis

A typical FT-IR spectrum recorded for CETP treated TWW sample is presented in Fig. 5.2. The assignment of the absorption peaks observed in the FT-IR spectrum was done on the basis of those outlined in the "Introduction to Organic Spectroscopy" (Lambert 1987) to know the relevant chemical bonds and functional groups of toxic organic compounds and is presented in Table 5.1.

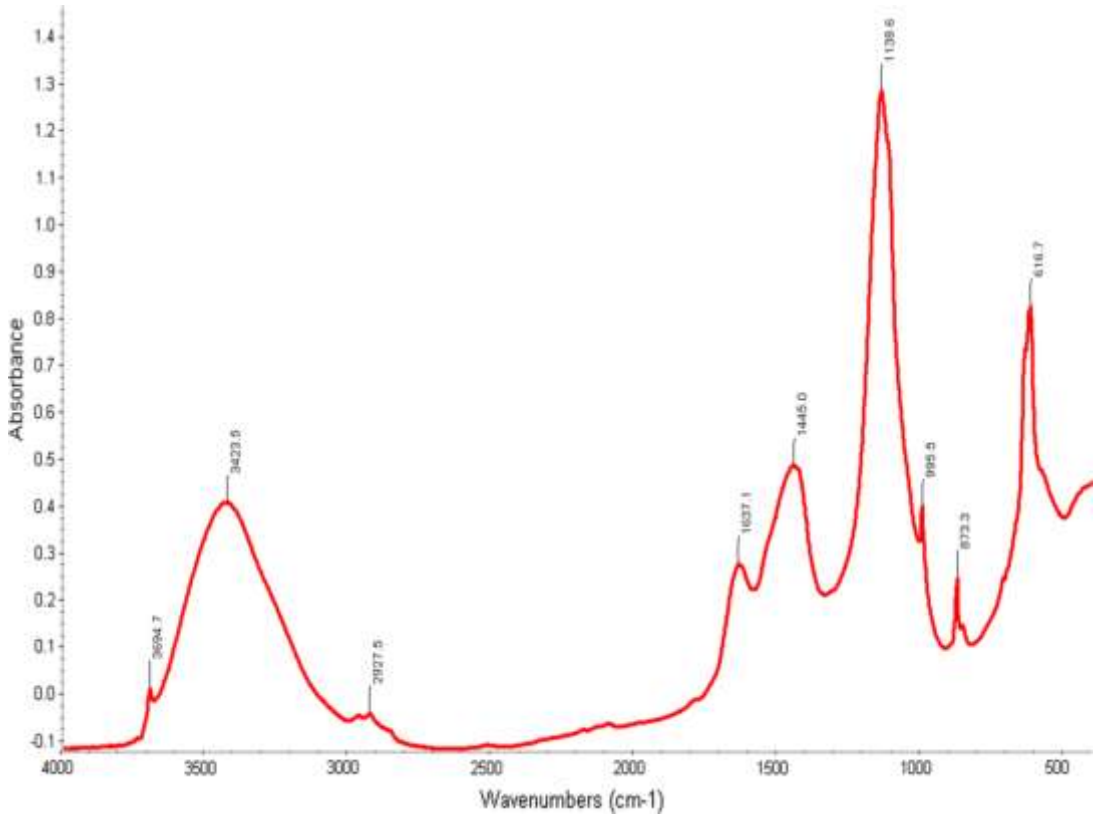


Fig. 5.2 Typical FT-IR spectra of TWW

The absorption peak at 3694.7-3423.5 cm^{-1} indicated the –OH group (O-H stretching) with strong intensity corresponded to the existence of alcohol and phenol derivatives in the TWW sample, the absorption peak at 2927.5 cm^{-1} is attributed to C–H stretching corresponded to the presence of long chain aliphatic compounds such as fatty acids, surfactants, and diazo compounds might be arisen from azo dyes used in leather coloring, and absorption peak 1637.1 cm^{-1} indicated the N–H bending of amines and amides. Further, absorption peak at 1445.0 cm^{-1} indicated the CH_3 bending, while absorption peak at 1138.6 cm^{-1} indicated the C–N stretching of

aliphatic amines, the absorption peak at 995.5 cm^{-1} attributed to P–O–alkyl organophosphorus compounds while absorption at 873.3 cm^{-1} corresponds to the presence of 1,2,4-trisubstituted benzene, the absorption at 616.7 cm^{-1} indicated the O–C=O bending of carboxylic acids. Overall, the absorption spectrum of CETP treated TWW showed the presence of some of the alcohols, phenols, amines and aromatic skeleton.

Table 5.1 FT-IR spectral data of TWW with their corresponding peak assignments

Observed frequencies (cm^{-1})	Tentative peak assignments
3694.7	O–H stretching
3423.5	O–H stretching
2927.5	C–H stretching
1637.1	N–H bending
1445.0	CH_3 bending
1138.6	C–N stretching
995.5	P–O–alkyl organophosphorous compounds
873.3	1,2,4-trisubstituted benzene
616.7	O–C=O bending

5.3.3. Nature and characteristics of persistent organic pollutants characterized and identified in DCM + n-Pentane extract of TWW by GC-MS analysis

The GC-MS analysis of DCM + *n*-Pentane extract has revealed the presence of various POPs, fatty acids, aromatic alcohols and organic acids at different RTs identified using NIST mass spectral library (Fig. 5.3). The major peaks were recorded at RT 6.19, 6.99, 10.46, 26.27, 27.35 and 31.91, which corresponded to the presence of acetic acid, trimethylsilyl ester; benzyl chloride (chloro-methylbenzene); benzyl alcohol; dibutyl phthalate, dibutyl ester; cis-9-hexadecanoic acid, trimethylsilyl ester and benzyl butyl phthalate, benzyl butyl ester, respectively, based on the match with NIST database.

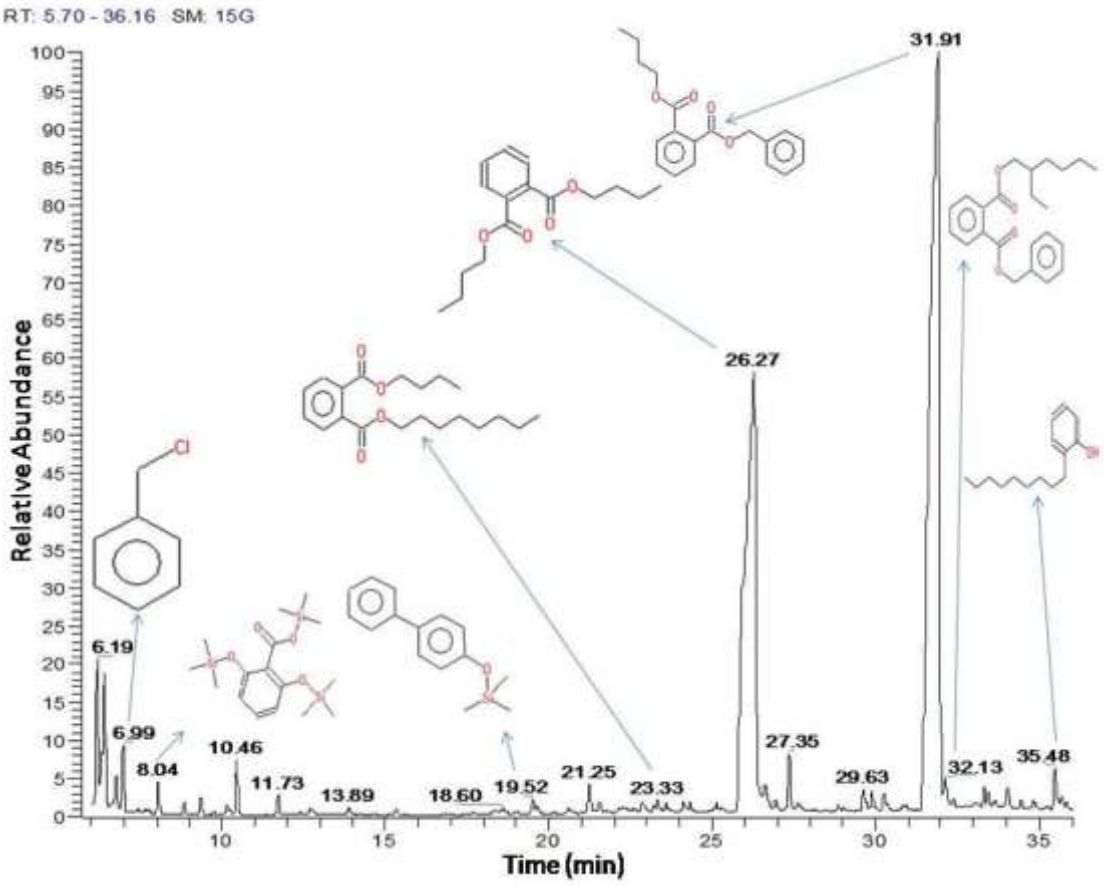


Fig. 5.3 GC-MS chromatogram of dichloromethane (DCM) + *n*-Pentane extract of TWW

Several minor peaks were also recorded at RT 8.04, 11.73, 13.89, 19.52, 21.25, 23.33, 29.63, 32.13 and 35.48, which corresponded to the presence of 2,6-Dihydroxybenzoic acid 3TMS; 2'6'-dihydroxyacetophenone, bis(trimethylsilyl)ether; 4-chloro-3-methyl phenol, trimethylsilyl ether; 4-biphenyltrimethylsiloxane; 1,2-benzenedicarboxylic acid, bis(trimethylsilyl)ester; Butyl octyl phthalate, butyl octyl ester; trans-9-Octadecanoic acid, trimethylsilyl ester; phthalic acid, benzyl 2-ethylhexyl ester and nonylphenol (NP), respectively with one unknown compound at RT 18.60 was also reported on the basis of match with NIST database.

Most of the compounds detected in TWW were of recalcitrant in nature because these were not degraded much during the secondary treatment of TWW at CETP and are discharged into the environment along with the wastewater (Table 5.2). These organic pollutants might be originated from the hide/skins or introduced during the leather tanning process. Fatty acids (such as cis-9-Hexadecanoic acid and trans-9-Octadecanoic acid) might be originated in TWW during the processing of raw

hide/skins in leather industries. Phthalates (such as dibutyl phthalate: DBP and benzyl butyl phthalate: BBP), 4-chloro-3-methyl phenol or *p*-chloro-*m*-cresol (PCMC) and NP 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 (TFL Eco Guidelines 2010; Saxena et al. 2016). NP is a highly toxic persistent organic pollutant (POP), which exists ubiquitously in the environment (Qian et al. 2011). NP and phthalates have been reported as potential endocrine disrupting chemicals (EDCs), benzyl chloride has been classified as a Group B2, probable human carcinogen as well as PCMC, benzyl alcohol, dihydroxybenzoic acid, 4-biphenyl and 2'6'-dihydroxyacetophenone have been listed as EDCs and also known to cause acute toxicity in aquatic organisms (USEPA 2012; https://www.epa.gov/sites/production/files/2016-09/documents/benzyl-chloride.pdf) and thus, are of serious environmental concern.

Table 5.2 POPs identified by GC-MS analysis in dichloromethane (DCM) + *n*-Pentane extract of TWW

Sr. No.	RT (min)	Identified compounds
1.	6.19	Acetic acid, trimethylsilyl ester ($C_6H_{20}O_3Si_2$)
2.	6.99	Benzyl chloride (chloro-methylbenzene) (C_7H_7Cl)
3.	8.04	2,6-Dihydroxybenzoic acid 3TMS ($C_{16}H_{30}O_4Si_3$)
4.	9.37	2-Methyl-1,2-propanediol 2TMS ($C_{10}H_{26}O_2Si_2$)
5.	10.46	Benzyl alcohol ($C_{10}H_{16}OSi$)
6.	11.73	2'6'-Dihydroxyacetophenone, bis(trimethylsilyl)ether ($C_{14}H_{24}O_3Si_2$)
7.	12.68	2-(4-Methoxyphenyl)-2-(4-trimetthoxysiloxane) ($C_{19}H_{26}O_2Si$)
8.	13.89	4-chloro-3-methyl phenol, trimethylsilyl ether ($C_{10}H_{15}ClOSi$)
9.	18.60	Unknown compound
10.	19.52	4-Biphenyltrimethylsiloxane ($C_{15}H_{18}OSi$)
11.	20.59	Dodecanoic acid, trimethylsilyl ester ($C_{15}H_{32}O_2Si$)
12.	21.25	1,2,Benzenedicarboxylic acid, bis(trimethylsilyl)ester ($C_{14}H_{22}O_4Si_2$)

13.	23.33	Butyl octyl phthalate, butyl octyl ester ($C_{20}H_{30}O_4$)
14.	24.10	Tetradecanoic acid, trimethylsilyl ester ($C_{17}H_{36}O_2Si$)
15.	26.27	Dibutyl phthalate, dibutyl ester ($C_{16}H_{20}O_4$)
16.	26.60	Chloroacetic acid, 4-hxadecyl ester ($C_{18}H_{35}ClO_2$)
17.	27.35	cis-9-Hexadecanoic acid, trimethylsilyl ester ($C_{19}H_{38}O_2Si$)
18.	29.63	trans-9-Octadecanoic acid, trimethylsilyl ester ($C_{21}H_{42}O_2Si$)
19.	31.91	Benzyl butyl phthalate, benzyl butyl ester ($C_{19}H_{20}O_4$)
20.	32.13	Phthalic acid, benzyl 2-ethylhexyl ester ($C_{23}H_{28}O_4$)
21.	35.48	Nonylphenol ($C_{15}H_{24}O$)

5.3.4. Nature and characteristics of persistent organic pollutants characterized and identified in DCM + Chloroform extract of TWW by GC-MS analysis

The GC-MS analysis of DCM + Chloroform extract of TWW also showed the presence of various persistent organic chemicals, fatty acids, aromatic alcohols and carboxylic acids at different RTs identified using NIST mass spectral library (Fig. 5.4). The major peaks were recorded at RT 6.11, 6.34, 6.94, 10.45, 26.50, 27.38, 32.32 and 35.50, which corresponded to the presence of acetic acid, trimethylsilyl ester; 2,6-Dihydroxybenzoic acid 3TMS, bis(trimethylsilyl)ether, benzyl chloride (chloro-methylbenzene), (Benzyloxy)trimethylsilane, dibutyl phthalate, dibutyl ester, hexadecanoic acid, trimethylsilyl ester, benzyl butyl phthalate, benzyl butyl ester, and benzyl 2-ethyl hexyl ester, respectively based on the match with NIST library.

In addition, several minor peaks were also recorded at RT 8.01, 9.36, 11.73, 13.89, 19.52, 21.55, 23.59, 29.01, 30.26, 33.50 and 35.72 corresponding to the presence of trimethyl(4-(1,1,3,3-tetramethylbutyl)phenoxy)silane; 2-methyl-1,2-propanediol 2TMS; 2'6'-dihydroxyacetophenone, bis(trimethylsilyl)ether; 4-chloro-3-methyl phenol, trimethylsilyl ether; 4-biphenyltrimethylsiloxane; phthalic acid, ethyl isopropyl ester; 1,2-benzenedicarboxylic acid, ethyl(trimethylsilyl)ester; trans-9-Octadecanoic acid, trimethyl silyl ester; octadecanoic acid, trimethyl silyl ester; di-(-2ethyl hexyl)phthalate and di-benzyl phthalate, dibenzyl ester, respectively with two

unknown compound at RT 15.33 and 17.76 were also reported based on the match with NIST database (Table 5.3).

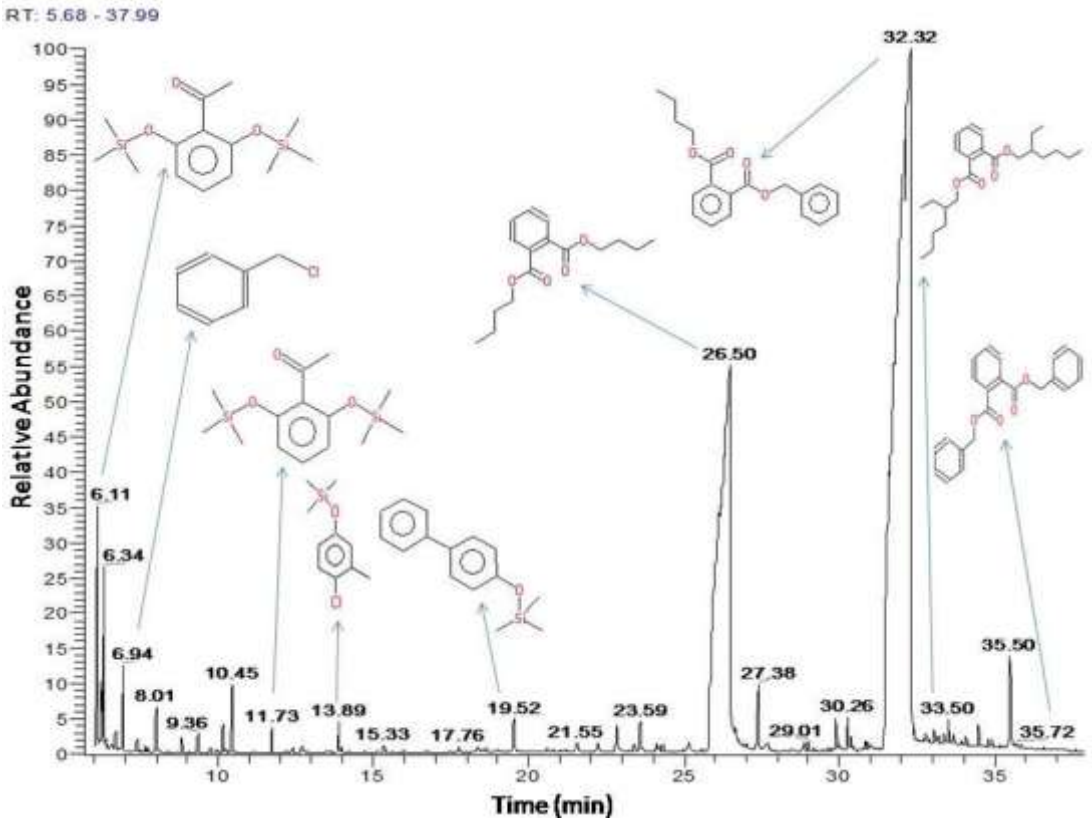


Fig. 5.4 GC-MS chromatogram of dichloromethane (DCM) + chloroform extract of TWW

Most of the organic pollutants detected at the major peaks in GC-MS data analysis were identified as endocrine disrupting phthalate esters, carcinogens, and aquatic toxicants, which are classified as "priority pollutants" due to their severe toxicity in living beings (USEPA 2012; https://www.epa.gov/sites/production/files/2015-09/documents/priority-pollutant-list-epa.pdf). Phthalates such as dibutyl phthalate: DBP, benzyl butyl phthalate: BBP, di-(-2ethyl hexyl)phthalate: DEHP, benzyl 2-ethyl hexyl phthalate ester, dibenzyl phthalate are applied as a plasticizer in artificial leather manufacturing, in preparation of micro-porous artificial leather coating/water vapour-permeable sheet materials and also used as plasticizer in artificial leather manufacturing in leather industries, respectively and thus, end up in TWW, which create serious ecotoxicological concern in the receiving environment (Saxena et al. 2016). Phthalates discharged along with industrial wastewaters causes water pollution and disturb the ecology of the receiving water bodies by creating serious toxicity to aquatic organisms such as fishes, as a result of bioaccumulation and thus, causes

toxic, genotoxic effects and endocrine disruption as well as disturb the antioxidant defense system (Benli et al. 2016; Chen et al. 2014; Zhao et al. 2014; Aoki et al. 2011). Phthalates are also reported to cause endocrine disruption in humans and animals upon long-term exposure (USEPA 2012). 2,6-Dihydroxybenzoic acid might be raised in TWW as a key metabolite of biodegradation of polyaromatic hydrocarbons (PAHs) during wastewater treatment at CETP, which are used in the degreasing and dyeing of finished leather in leather industries (Li et al. 2010). 2,6-Dihydroxybenzoic acid has been reported as a serious aquatic toxicant, which reduces the algal growth in aquatic systems and thereby reduce photosynthesis and hence, ultimately disturbs the ecological functioning of receiving water bodies (water pollution) (Lee and Chen 2009). Chloromethyl benzene or benzyl chloride is used to inhibits the growth of molds and mildews on hides/skins, facilitates leather softening, wetting and also used in the coloring of finished leather in combination with dyes and hence, finished up in TWW. It is considered to be moderately aquatic toxicant and poses moderate to low toxicity to aquatic animals such as fishes and also listed as a Group 2A carcinogen (IARC 1999). 4-chloro-3-methyl phenol or *p*-chloro-*m*-cresol (PCMC) is a highly recalcitrant chlorophenol widely used as biocide in raw hide/skins preservation in leather industries and reported to cause water pollution and pose serious toxicity to aquatic animals such as fishes (TFL Eco Guidelines 2010). PCMC has been also reported as a potential endocrine disruptor by US Environmental Protection Agency (USEPA 2012). Moreover, hexadecanoic acid and octadecanoic acid might be originated in TWW during the processing of raw hide/skins in leather industries and have been recently identified as EDCs (USEPA 2012). Further, phthalic acid is also used as a plasticizer in leather industries and thus, ends up in TWW and has been reported to cause mutagenicity, developmental toxicity and reproductive toxicity in animals (Bang et al. 2011)

In the present investigation, the combination of DCM + Chloroform (organic solvents) has reported the maximum number of POPs and thus, recommended as the excellent combination for the extraction of persistent organic pollutants from TWW. However, the toxicity profile of several other organic compounds detected in TWW has not been explored till date and represents a remaining part of the further investigation.

Table 5.3 POPs identified by GC-MS in dichloromethane (DCM) + chloroform extract of TWW

Sr. No.	RT (min)	Identified compounds
1.	6.11	Acetic acid, trimethylsilyl ester ($C_6H_{20}O_3Si_2$)
2.	6.34	2,6-Dihydroxybenzoic acid 3TMS ($C_{16}H_{30}O_4Si_3$)
3.	6.94	Benzyl chloride (chloro-methylbenzene) (C_7H_7Cl)
4.	8.01	Trimethyl(4-(1,1,3,3-etramethylbutyl)phenoxy)silane ($C_{17}H_{30}OSi$)
5.	9.36	2-Methyl-1,2-propanediol 2TMS ($C_{10}H_{26}O_2Si_2$)
6.	10.45	(Benzyloxy) trimethylsilane ($C_{10}H_{16}O_{Si}$)
7.	11.73	2'6'-Dihydroxyacetophenone, bis(trimethylsilyl)ether ($C_{14}H_{24}O_3Si_2$)
8.	13.89	4-chloro-3-methyl phenol, trimethylsilyl ether ($C_{10}H_{15}ClOSi$)
9.	15.33	Unknown compound
10.	17.76	Unknown compound
11.	19.52	4-Biphenyltrimethylsiloxane ($C_{15}H_{18}OSi$)
12.	21.55	Phthalic acid, ethyl isopropyl ester ($C_{13}H_{16}O_4$)
13.	23.59	1,2,Benzenedicarboxylic acid, ethyl(trimethylsilyl)ester ($C_{13}H_{18}O_4Si$)
14.	24.12	Tetradecanoic acid, trimethylsilyl ester ($C_{17}H_{36}O_2Si$)
15.	24.32	Diisobutyl phthalate ($C_{16}H_{22}O_4$)
16.	26.50	Dibutyl phthalate, dibutyl ester ($C_{16}H_{22}O_4$)
17.	27.38	Hexadecanoic acid, trimethylsilyl ester ($C_{19}H_{40}O_2Si$)
18.	29.01	trans-9-Octadecanoic acid, trimethylsilyl ester ($C_{21}H_{42}O_2Si$)
19.	30.26	Octadecanoic acid, trimethylsilyl ester ($C_{21}H_{42}O_2Si$)
20.	32.32	Benzyl butyl phthalate, benzyl butyl ester ($C_{19}H_{20}O_4$)
21.	33.50	Di-(-2ethyl hexyl)phthalate ($C_{24}H_{38}O_4$)
22.	35.50	Benzyl 2-ethyl hexyl ester ($C_{23}H_{28}O_4$)
23.	35.72	Di-benzyl phthalate, dibenzyl ester ($C_{22}H_{18}O_4$)

5.3.5. Nature and characteristics of persistent organic pollutants characterized and identified in DCM + Ethyl Acetate extract of TWW by GC-MS analysis

The GC-MS analysis of DCM + ethyl acetate extract of TWW also showed the presence of various persistent organic chemicals, fatty acids, aromatic alcohols and carboxylic acids at different RTs identified using NIST mass spectral library (Fig. 5.5). The major peaks were recorded at RT 9.24, 21.69, 25.36, 27.57, 29.26, 30.47, 31.99, 34.04, 41.90, 44.54 and 49.84, which corresponded to the presence of benz[a]anthracene,7,12-dimethyl-, p-trimethylsiloxynitrobenzene, 1,2,Benzenedicarboxylic acid, ethyl(trimethylsilyl)ester, hexadecanoic acid, trimethylsilyl ester, trans-9-Octadecanoic acid, trimethylsilyl ester, benzyl butyl phthalate, benzyl butyl ester. 3-Chloropropionic acid, heptadecyl ester, 17-pentatriacontene, tetradecanoic acid, trimethylsilyl ester, phthalic acid, benzyl isobutyl ester, and dodecanoic acid, trimethyl ester, respectively based on the match with NIST library.

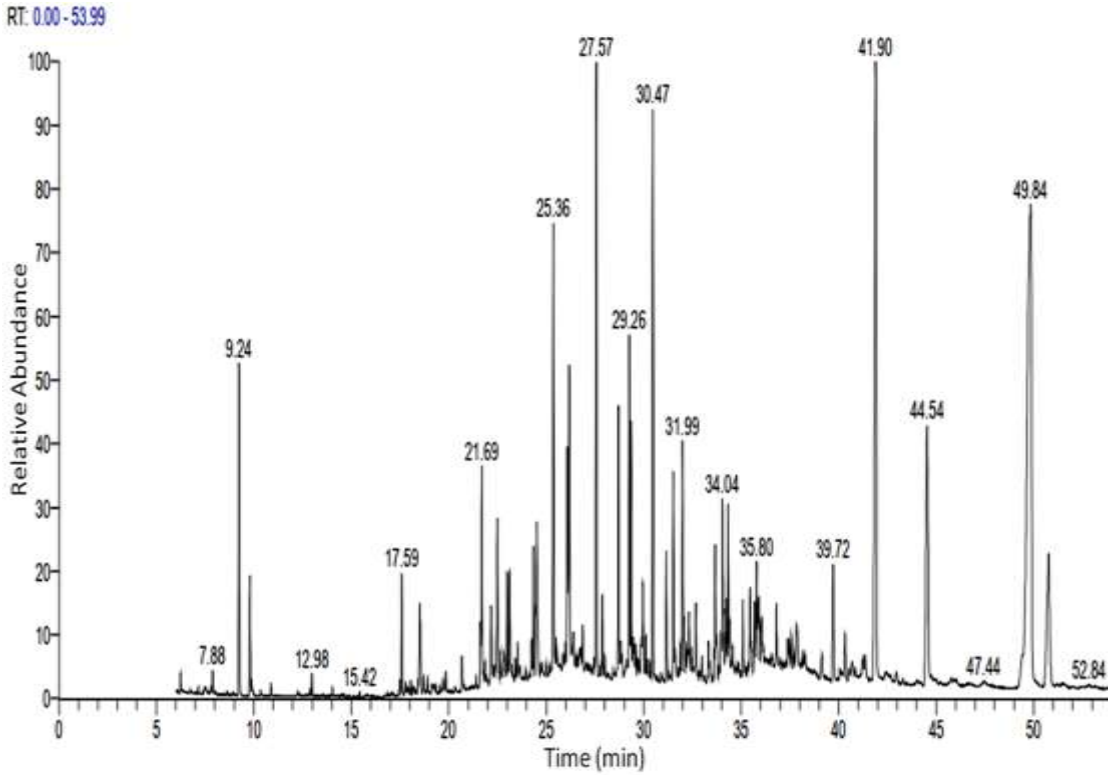


Fig. 5.5 GC-MS chromatogram of dichloromethane (DCM) + ethyl acetate extract of TWW

In addition, several minor peaks were also recorded at RT 7.88, 12.98, 15.42, 17.59, 35.80, 39.72, 47.44, and 52.84, which corresponded to the presence of

Heptanoic acid, 7-phenoxy-, trimethyl ester, Dodecane, 4,6-dimethyl-, benzoic acid, trimethylsilyl ester, Octadecane, Di-benzyl phthalate, dibenzyl ester, 4-(2,4-dimethylheptan-3-yl)phenol, and Stearic acid, 3-(octadecyloxy)propyl ester, respectively with one unknown compound at RT 52.84 was also reported based on the match with NIST database (Table 5.4).

Table 5.4 POPs identified by GC-MS in dichloromethane (DCM) + ethyl acetate extract of TWW

Sr. No.	RT (min)	Compounds identified
1.	7.88	Heptanoic acid, 7-phenoxy-, trimethyl ester ($C_{16}H_{26}O_3Si$)
2.	9.24	Benz[a]anthracene,7,12-dimethyl- ($C_{20}H_{16}$)
3.	12.98	Dodecane, 4,6-dimethyl- ($C_{14}H_{30}$)
4.	15.42	Benzoic acid, trimethylsilyl ester ($C_{16}H_{30}O_4Si_3$)
5.	17.59	Octadecane ($C_{18}H_{38}$)
6.	21.69	p-Trimethylsiloxynitrobenzene ($C_9H_{13}NO_3Si$)
7.	25.36	1,2,Benzenedicarboxylic acid, ethyl(trimethylsilyl)ester ($C_{13}H_{18}O_4Si$)
8.	27.57	Hexadecanoic acid, trimethylsilyl ester ($C_{19}H_{40}O_2Si$)
9.	29.26	Trans-9-Octadecanoic acid, trimethylsilyl ester ($C_{21}H_{42}O_2Si$)
10.	30.47	Benzyl butyl phthalate, benzyl butyl ester ($C_{19}H_{20}O_4$)
11.	31.99	3-Chloropropionic acid, heptadecyl ester ($C_3H_5ClO_2$)
12.	34.04	17-Pentatriacontene ($C_{35}H_{70}$)
13.	35.04	Phthalic acid, 1-phenylpropyl butyl ester ($C_{21}H_{24}O_4$)
14.	35.80	Di-benzyl phthalate, dibenzyl ester ($C_{22}H_{18}O_4$)
15.	39.72	4-(2,4-dimethylheptan-3-yl)phenol ($C_{15}H_{24}O$)
16.	41.90	Tetradecanoic acid, trimethylsilyl ester ($C_{17}H_{36}O_2Si$)
17.	44.54	Phthalic acid, benzyl isobutyl ester ($C_{19}H_{20}O_4$)
18.	47.44	Stearic acid, 3-(octadecyloxy)propyl ester ($C_{39}H_{78}O_3$)
19.	49.84	Dodecanoic acid, trimethyl ester ($C_{15}H_{32}O_2Si$)
20.	52.84	Unknown compound

Most of the persistent organic compounds detected in the secondary treated TWW were of recalcitrant in nature because these were not degraded much during the

treatment of TWW at CETP and are discharged into the environment along with the wastewater (Table 5.2). These organic pollutants might be originated from the hide/skins or introduced during the leather tanning process in LIs. Fatty acids (such as Trans-9-Octadecanoic acid, Hexadecanoic acid, Tetradecanoic acid, Stearic acid, and Dodecanoic acid) were might be originated in the TWW during the processing of raw hide/skins in leather industries. Phthalates (such as benzyl butyl phthalate: BBP, Di-benzyl phthalate, and Phthalic acid) are used as plasticizers to increase the flexibility and pliability of leather products and hence, are discharged in the TWW. Benz[a]anthracene is also used as a leather tanning agent in LIs and is highly toxic in nature and also considered as a group B2 probable human carcinogen (https://cfpub.epa.gov/ncea/iris/iris_documents/documents/subst/0454_summary.pdfb b). Benzoic acid is used in the treatment of raw hide/skins and has been reported to disturb the ecological functioning of water bodies (water pollution) (Lee and Chen 2009). p-Trimethylsiloxynitrobenzene is also used in the dressing of leather and preparation of aniline-based dye used in the coloring of leather and hence, is discharged in the TWW as a metabolic product during the secondary treatment at CETP. Exposure to nitrobenzene irritates the skin, eyes and respiratory tract and can result in methemoglobinemia causing fatigue, dyspnea, dizziness, disturbed vision, shortness of breath, collapse and even death (https://pubchem.ncbi.nlm.nih.gov/compound/nitrobenzene). Nitrobenzene also damages the liver, spleen, kidneys and central nervous system (https://pubchem.ncbi.nlm.nih.gov/compound/nitrobenzene). Nitrobenzene is a possible mutagen and is reasonably anticipated to be a human carcinogen (https://pubchem.ncbi.nlm.nih.gov/compound/nitrobenzene). Phthalates have been reported as potential endocrine disrupting chemicals (EDCs) (USEPA 2012; https://www.epa.gov/sites/production/files/2016-09/documents/benzyl-chloride.pdf) and thus, are of serious eco-toxicological concern.

Some of the highly toxic chemicals, which are detected and characterized in the present study, were also reported by many researchers in TWW in their previous studies and thus, our findings are well corroborated with them (Yadav et al. 2019; Bharagava et al. 2018; Alam et al. 2010; Lofrano et al. 2008). Further, LIs claim that they use eco-friendly, non- or less toxic and biodegradable chemicals in leather processing as per the strict regulations, but actually, that was not the case as the

presence of many POPs reported in TWW further provided the evidence of its highly toxic and hazardous nature.

5.4. Conclusion

The present study was to characterize the various POPs present in the TWW discharged after secondary (biological) treatment carried out at the CETP of LIs, Unnao, India. According to the present study, the collected secondary treated TWW reported to contain a variety of POPs as confirmed by the HP-LC, FT-IR and GC-MS analysis. In the present study, some of the reported chemical pollutants are EDCs, carcinogens, and aquatic toxicants, which negatively affect the flora and fauna in the environment. Therefore, the discharge of this highly polluted and toxic wastewater in the receiving environment is not suitable with reference to environmental health and thus, requires regular monitoring and assessment before its final disposal. Hence, there is a need to develop an eco-friendly treatment/remediation solution for the degradation and detoxification of TWW containing such highly toxic POPs to safeguard the environment and public health.

6

Chapter-06

Isolation, Purification, Screening and Characterization of Bacteria Capable for the Degradation and Detoxification of Persistent Organic Pollutants (POPs) from Tannery Wastewater

Isolation, purification, screening, and characterization of bacteria capable for the degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater

6.1. Introduction

Undoubtedly, leather industries are the key contributors to the economy of many developing countries. However, these are also the major pollution causing industries worldwide, generating large volumes of high-strength wastewater having high pH, dark brown color, unpleasant odor, and a high BOD, COD, TDS, Cr, phenolics with a variety of persistent organic pollutants (POPs) which do not much degrade during the secondary treatment at CETP and thus, causes serious environmental pollution and toxicity in the living beings (Saxena et al. 2016; Dixit et al. 2015; Lofrano et al. 2013). It reduces sunlight penetration in aquatic resources which in turn decreases both photosynthetic activity and dissolved oxygen concentration affecting aquatic life; however, on land, it causes a reduction in soil alkalinity and inhibition of seed germination (Saxena et al. 2016). Moreover, it has been also reported to cause a variety of severe toxic effects in living beings upon exposure (Saxena et al. 2016). Therefore, it becomes necessary to adequately treat/detoxify the contaminants present in the TWW to protect the environment and living beings.

Currently, various physicochemical treatment approaches are being used to treat the TWW, but these are environmentally destructive and costly and may cause secondary pollution and, hence, are not economically feasible (Saxena et al. 2016). However, the application of microbes could be an alternative approach to effectively degrade/detoxify the contaminants in TWW (i.e. bioremediation) for environmental protection and sustainable development of the society. A variety of bacterial strains such as *Cellulosimicrobium* sp. (Bharagava and Mishra 2018), *Bacillus cereus* (Kumari et al. 2018), *Bacillus subtilis* (Yusuf et al. 2013), *Bacillus amyloliquefaciens* (El-Bestawy et al. 2013), *Pseudomonas stutzeri* (El-Bestawy et al. 2013), *Providencia vermicola* (El-Bestawy et al. 2013), *Pseudomonas fragi* (Yusuf et al. 2013), *Brachymonas denitrificans* (Kim et al. 2014), *Thiobacillus ferrooxidans* (Mandal et al. 2010), *Pseudomonas aeruginosa* (Sivaprakasam et al. 2008), *Bacillus flexus* (Sivaprakasam et al. 2008), *Acinetobacter* sp. (Srivastava et al. 2007), Rhodococcus sp. (Paisio et al. 2012), and *A. thiooxidans* (Wang et al. 2007) have been reported for the degradation and detoxification of contaminants in TWW. Microbes play an

important role in the biodegradation and detoxification of environmental pollutants from industrial wastes due to their enzymes that have high specificity to a broad range of substrates (pollutants). In addition, search for the pollutants degrading/detoxifying novel microbes cold pave the way towards the sustainable treatment and management of industrial wastewaters. Therefore, the present study was to isolate, screen and characterize the potential bacterial capable for the degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater for environmental safety.

6.2. Materials and methods

6.2.1. Chemicals and media's

All the chemicals used in the experiments were of highest purity (purity ≥ 99%) and analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Microbiological media was purchased from HiMedia Laboratories (Mumbai, MH, IN). Mineral salt medium (MSM g/L, Na_2HPO_4 2.4; K_2HPO_4 2.0; NH_4NO_3 0.1; $MgSO_4$ 0.01; $CaCl_2$ 0.01, Agar 1.5) was used to isolate the potential bacterial strains for degradation and detoxification of persistent organic pollutants (POPs) from TWW. Nutrient agar medium (NAM g/L, yeast extract 2 g/L; meat extract 1.0; NaCl 5.0; peptone 5.0; agar 15.0) was used for the screening of potential bacterial strains on the basis of salt tolerance. Whatman® Grade GF/C filter papers (pore size 1.2 μm) (Whatman, England, UK) were used to filter TWW.

6.2.2. Isolation of bacterial strains and culture conditions

Serial dilution method was used for the isolation of bacterial strains from the collected TWW and sludge as per standard protocol (Aneja 2007). The scheme of serial dilution for the isolation of bacterial strains is depicted in Fig. 6.1. Briefly, to isolate the bacterial strains for the degradation of real TWW, the MSM-broth (100 ml + 1% glucose and 0.5% peptone (w/v) as carbon and nitrogen source, pH 7.0)) was prepared using distilled water in an Erlenmeyer flask (250 ml), sterilized (at 121 °C for 15 min) in the autoclave (SM-102, S M Scientific Instruments Pvt. Ltd., UP, IN), and then, kept for cooling at room temperature. After cooling, to this medium, 20 ml of undiluted TE + 1 g of tannery sludge was added, shaked well, and kept for enrichment and incubated at 35 °C under shaking condition (120 rpm) in an temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA) for successive

five days. Afterward, the developed bacterial suspension (1 ml) was serially diluted (10^{-4} - 10^{-5}, 50 µl) and spreaded on the MSM-agar plates, which were kept for incubation (48 h) in the temperature controlled incubator shaker fitted with tray for the development of bacterial colonies.

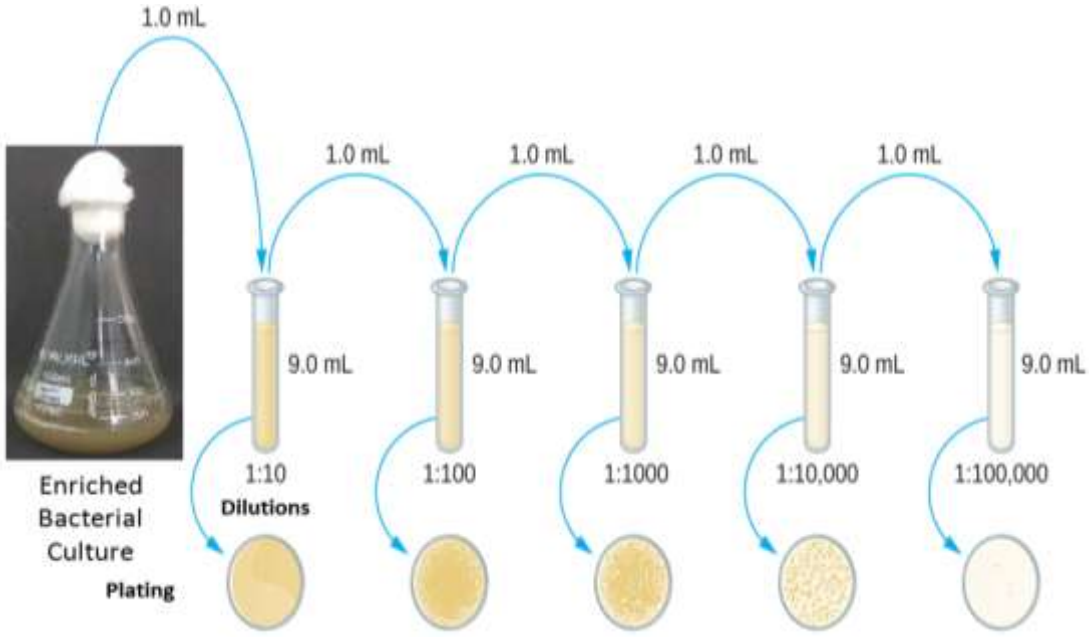


Fig. 6.1 Scheme of serial dilution method used for the bacterial isolation from enriched bacterial suspension in MSM-broth

Further, the morphologically distinct bacterial colonies grown on the MSM-agar plates were selected, picked up and purified by repeated streaking method for the further screening of potential bacterial strains.

6.2.3. Screening of potential bacterial strains

6.2.3.1. Primary screening

For the better effluent treatability, the isolated bacterial strains were screened for salt tolerance because TWW contains an appreciable amount of salts and recalcitrant pollutants. For the primary screening, a loopfull culture of the purified bacterial strains were streaked on the NAM-plates amended with varying concentrations of salt (NaCl 0-10%, w/v) and incubated (at 35 °C for 48 h) in the temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA) fitted with tray for the growth and development of bacterial strains. The screening was done on the basis of salt tolerance limit and time of adaptation as suggested by Sivaprakasam et al. (2008).

6.2.3.2. Secondary screening

The bacterial strains that showed tolerance to the high salt concentration were further selected for the secondary screening on the basis of COD removal efficiency as suggested by Sivaprakasam et al. (2008). For this, a loopfull culture of the selected purified bacterial strains was cultured in MSM-broth (50 ml, pH 7.0) supplemented with glucose (0.5%, w/v) as a carbon source. Afterwards, 20 ml of the overnight grown bacterial precultures were inoculated in TWW (undiluted, 80 ml, pH 7.0) in the Erlenmeyer flasks (250 ml) which were incubated (at 35 °C) under shaking condition (120 rpm) in an temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA). The bacterially treated TWW samples were taken out every 24 h for successive five days, centrifuged at 10000 × g for 10 min in a refrigerated centrifuge (REMI Instruments Pvt. Ltd., Mumbai, MH, IN) and then, the supernatant was used for the COD measurements. The bacterial strains that showed maximum COD removal from TWW samples were selected for further studies. All the bacterial cultures were maintained on MSM-agar plates (supplied with glucose, 1%, and w/v) at 35 °C.

6.2.4. Identification of potential bacterial strains

6.2.4.1. Morphological and biochemical characterization

Based on the results of salt tolerance and COD removal efficiency, only three potential bacterial strains were selected and identified by various morphological and biochemical tests as outlined in the "Bergey's Manual of Determinative Bacteriology (Whitman et al. 2012).

6.2.4.1.1. Gram Staining

(a) Principle

Christian Gram discovered the gram staining in (1884), a differential staining method which differentiates bacteria either into Gram "positive" or Gram "negative". Staining is based on the principle of a component of the cell wall of a bacterial cell. The Gram-negative bacterial cell wall is thin, complex, multilayered structure and contains relatively high lipid contents in addition to protein and mucopeptides. The high lipid content is readily dissolved by alcohol resulting in the formation of large pores in the cell wall, which does not close appreciably on dehydration of cell wall protein. Thus,

facilitating the leakage of crystal violet-iodine complex and resulting in the decolorization of the bacterium that later takes the counter strain and appears pink. In contrast, the gram-positive bacterial cell wall is thick and chemically simple, mainly composed of protein and cross-linked by mucopeptides. This cell wall, when treated with alcohol causes dehydration and closure of cell wall pore thereby not allowing the loss of crystal- iodine complex and cells remain violet.

(b) Reagents

Ammonium oxalate-crystal violet stain

Solution A

Crystal violet : 10 g

Ethanol (95%) : 100 ml

Mixed and dissolved

Solution B

Ammonium oxalate : 1 g

Distilled water : 100 ml

For use, mixed 2 ml of solution A and 80 ml of solution B

Solution A + Solution B = Crystal violet

Lugol's iodine

Iodine : 5 g

Potassium iodide (KI) : 10 g

Distilled water : 100 ml

Dissolved the iodide and iodine in some of the water and adjusted to 100 ml with distilled water.

Ethyl alcohol 95 %

95 ml ethyl alcohol + 5 ml distilled water

Safranin

Safranin : 2.5 g

Ethyl alcohol 95% : 10 ml

These were added in 100 ml distilled water

(d) Procedure

Bacterial cells were grown on nutrient agar plates to mid-log phase and a smear of cells was prepared on a clean microscopic slide. The slides were flooded with ammonium oxalate-crystal violet stain for one min and then wash with distilled water.

Now, apply Lugol's iodine solution for half min and after then the iodine solution was drained off but do not wash. The smear was decolorized with a few drops of acetone and washed thoroughly with water. Counterstain the slides with 0.5% safranin for a half min, washed again and stand slide on end to drain or blot dry and then observed microscopically.

(e) Interpretation

Violet color: Gram-positive; **Pink color:** Gram-negative

6.2.4.1.2. Motility

(a) Principle

A bacterial strain may be flagellated or non-flagellated. When bacterial strains are flagellated then it shows motility or movement.

(b) Composition of Motility Media (gL^{-1})

Peptone	:	10 g
Mean extract	:	3 g
NaCl	:	5 g
Agar	:	4 g
Gelatin	:	80 g
Distilled water	:	1000 ml

Soaked the gelatin in water for 30 min, added other ingredients, heated to dissolve and sterilized at 115 ºC for 20 min.

(c) Procedure

The motility medium was stab-inoculated with a straight needle to a depth of about 5 mm. The tube was left overnight incubation (at or below the optimum growth temperature).

(d) Interpretation

Motile organisms migrate throughout the medium, which becomes turbid in semi-solid media was scored positive for motility. Growth of non-motile organisms is confined to the stab inoculum was showing negative.

6.2.4.1.3. Catalase

(a) Principle

Catalase acts as a catalyst to breakdown the hydrogen peroxide into oxygen and water. An organism is tested for catalase activity by bringing it into contact with hydrogen peroxide. Oxygen bubbles are released which is the gaseous product of the enzymatic activity, which indicated the liberation of oxygen and presence of bacterial catalase enzyme. Hydrogen peroxide forms as one of the oxidative end products of aerobic carbohydrate metabolism. H_2O_2 is lethal to bacterial cells.

$$H_2O_2 \xrightarrow{\text{Catalase}} H_2O + O_2 \quad \text{..... (11)}$$

(b) Procedure

The test was done by placing a drop of 3-6% H_2O_2 on a microscope slide. Then, by using an applicator stick, touch the colony and formed a smear on the slide. The slide was observed for the formation of bubbles.

(c) Interpretation

Rapid effervescence or production of gas bubbles (molecular oxygen) has indicated the positive test.

6.2.4.1.4. Citrate utilization test

(a) Principle

Carbon source utilization and their application to identification are limited mainly to tests for the utilization of citrate. Other citrate media, such as Christensen's contain additional nutrients.

(b) Reagents and media

The test was performed by using the HiMedia Rapid Biochemical Identification kit, [Enterobacteriaceae Identification Kit (KB002 HiAssorted®)] for the gram-negative rod. KB002 is the comprehensive test system used for identification of gram-negative Enterobacteriaceae species and other non-fastidious, Gram-negative rods.

(c) Procedure

The biochemical strips were inoculated with isolated bacterium suspension and incubated at 37 °C for 24 h. The indices obtained after reading and results were interpreted using the HiMedia result interpretation chart supplied with Biochemical Identification kit. The organisms were identified to species level. The bromothymol

blue pH indicator is a deep forest green at neutral pH. If citrate is present, a degradation product is produced which increase in medium pH to above 7.6, bromothymol blue changes to blue. The blue color indicates a positive result.

(c) Interpretation

Blue color and streak of growth: Citrate utilized

The original green color of medium: Citrate not utilized

6.2.4.1.5. Lysine utilization

(a) Principle

The purpose is to see if the microbe can use the amino acid lysine as a source of carbon and energy for growth. Use of lysine is accomplished by the enzyme lysine decarboxylase. This enzyme attacks the carboxylic group of amino acid lysine, with the formation of amine cadaverine. These by-products are sufficient to raise the pH of the media so that the broth turns purple.

(b) Reagents and media

The reagent and media used in this test were the same as described in section 6.2.4.1.4.

(c) Procedure

The strips were inoculated with isolated bacterium suspension and incubated at 37 °C for 24 h. The indices obtained after reading the results were interpreted using the HiMedia result interpretation chart supplied with Biochemical Identification kit.

(d) Interpretation

If the inoculated medium is light purple, or if there is no color change, the organism is decarboxylase-negative for that amino acid. If the medium turns dark purple, the organism is decarboxylase-positive for that amino acid.

6.2.4.1.6. Ornithine utilization

(a) Principle

Ornithine Decarboxylase is used for the detection ability of microorganisms to decarboxylate ornithine. Decarboxylation is the process in which bacteria that possess specific decarboxylase enzyme attack amino acids at their carboxyl end (-COOH) to yield an amine or a diamine and carbon dioxide. The amino acid L-ornithine is

decarboxylated by the enzyme ornithine decarboxylase to yield the diamine putrescine and carbon dioxide.

(b) Reagents and media

The reagent and media used in this test were the same as described in section 6.2.4.1.4.

(c) Procedure

The procedure of this test was the same as described in section 6.2.4.1.5.

(d) Interpretation

The production of this amine elevates the pH of the medium towards alkalinity, changing the color of the indicator from light purple to dark purple. If the organism does not produce the appropriate enzyme, the medium remains light purple.

6.2.4.1.7. Urease activity

(a) Principle

The test organisms were cultured in a medium containing urea and indicator phenol red. If the stain is urease producing, the enzyme will break down the urea by hydrolysis to give ammonia and CO_2 with the release of ammonia. The medium becomes alkaline as shown by a change in color of the indicator to red-pink.

$(NH_2)_2CO + H_2O \longrightarrow CO_2 + 2NH_3$ …… (12)

(b) Reagents and media

The reagent and media used in this test were the same as described in section 6.2.4.1.4.

(c) Procedure

The procedure of this test was the same as described in section 6.2.4.1.5.

(c) Interpretation

Pink color and streak of growth: Urea Utilised
Organish yellow color of medium: Urea not utilized

6.2.4.1.8. Phenylalanine deaminase

(a) Principle

This test determines whether the microbe produces the enzyme phenylalanine deaminase, which is needed for it to use the amino acid phenylalanine as a carbon and energy source for growth.

(b) Reagents and media

The reagent and media used in this test were the same as described in section 6.2.4.1.4.

(c) Procedure

The procedure of this test was the same as described in section 6.2.4.1.5.

(d) Interpretation

If phenylalanine deaminase is present, a degradation product is produced from phenylalanine. The product combines with iron compounds in an acidic environment to produce a green color. The green color indicates a positive result.

6.2.4.1.9. Nitrate reduction

(a) Principle

Nitrate reduction may be shown either by detecting the presence of one of the breakdown products or by showing the disappearance of nitrate from the medium. The products of reduction may include nitrite, hyponitrite, hydroxylamine, ammonia, nitrous oxide or gaseous nitrogen. The first test to be applied aims at showing the presence of nitrite. When this test is negative (i.e. nitrite is not detected) the medium is tested to see whether there is residual nitrate, if this test also is negative it confirms that the first stage of the breakdown has been completed and the nitrite further is broken down. In uninoculated nitrate broth and with cultures of organisms that do not reduce nitrate, the test for nitrite is negative until zinc dust or other reducing agent is added to the culture medium to reduce the nitrate contained in it. To detect small amounts of residual nitrate, the amount of zinc added may be critical. The tests are very sensitive and it is important to check the uninoculated medium for nitrite, which should not be present.

(b) Reagents and media

The reagent and media used in this test were the same as described in section 6.2.4.1.4.

(c) Procedure

The procedure of this test was the same as described in section 6.2.4.1.5.

(d) Interpretation

The appearance of pinkish red color, which showed the presence of nitrite and thus shows that nitrate has been reduced, indicates a positive reaction. Tubes not showing red color within 5 min, added powdered zinc and allowed to stand.

Pinkish Red color: Nitrate present in the medium (i.e. not reduced by the organism).
Absence of red color (colorless): Nitrate absent in medium (i.e. reduced by the organism to nitrite, which in turn was itself reduced).

6.2.4.1.10. Hydrogen sulfide (H_2S) production test

(a) Principle

The H_2S test is one that can be made as sensitive as required with an adequate sulfur source (cysteine) and a delicate indicator (lead acetate papers) almost all the enteric bacteria can be shown to be able to produce H_2S. Tested in this way an accurate estimation can be obtained of an organism's catabolic power in relation to sulfur compounds, but it is not possible to distinguish readily between those organisms with much and those with little ability to produce H_2S. With a poor medium or a less sensitive (ferrous chloride or lead acetate in the medium), only the strong H_2S producer are detected.

(b) Reagents and media

The reagent and media used in this test were the same as described in section 6.2.4.1.4.

(c) Procedure

The procedure of this test was the same as described in section 6.2.4.1.5.

(d) Interpretation

Black color: H_2S produced by the organism
Organish Yellow color (colorless): H_2S not produced by the organism

6.2.4.1.11. Malonate

(a) Principle

An organism that simultaneously can utilize sodium malonate as its carbon source and ammonium sulfate as its nitrogen source produce alkalinity due to the formation of sodium hydroxide. The medium used for malonate utilization test is malonate broth. It contains mineral salts, sodium malonate for carbon, and ammonium sulfate for its nitrogen source. The pH indicator is bromothymol blue, which is green at neutral pH, yellow at acidic pH < 6.0 and turns blue at alkaline (basic) pH > 7.6. Organisms which simultaneously utilize malonate and ammonium sulfate produce sodium hydroxide which thereby results in an alkaline reaction and changes the indicator from its original green color to light blue or Prussian blue. Organisms which cannot utilize malonate and ammonium sulfate and do not ferment dextrose produce any color change. Organisms which are malonate-negative but do ferment dextrose result in the development of a yellow color due to increased acidity in the medium.

(b) Reagents and media

The test was performed by using HiMedia Rapid Biochemical Identification kit, [Bacillus Identification Kit (KB013 HiBacillus^{TM}] for gram-positive rod. KB013 is a standardized, colorimetric test system used for the identification of Gram-positive bacteria.

(c) Procedure

The procedure of this test was the same as described in section 6.2.4.1.5.

(d) Interpretation

Dark Blue: Positive reaction is given by organism
Bluish green: Negative reaction given by organism

6.2.4.1.12. Voges Proskauer's

The Voges-Proskauer test determines the capability of some organisms to produce non-acidic or neutral end products, such as acetyl methyl carbinol, from organic acids that result from glucose metabolism. The reagent used in this test is Barritt's reagent, consists of a mixture of alcoholic α-naphthol and 40% potassium hydroxide solution. Detection of acetyl methyl carbinol requires this end product to be oxidized to a diacetyl compound. This reaction will occur in the presence of α-naphthol catalyst and a guanidine group that is present in peptone of MRVP medium.

(b) Reagents and media

The reagent and media used in this test were the same as described in section 6.2.4.1.11.

(c) Procedure

The procedure of this test was the same as described in section 6.2.4.1.5.

(d) Interpretation

Pinkish Red: Positive reaction is given by organism
Colorless/Slight Copper: Negative reaction given by organism

6.2.4.1.13. Arginine

(a) Principle

The arginine decarboxylase is an enzyme that attacks the carboxylic group of the amino acid arginine, with the formation of amine putrescine. The sugar contained in the medium is fermented by all the enterobacteria with the consequent initial color change of indicator system from purple to yellow. The acid medium supports the amino acid decarboxylation reactions with formation of putrescine. The amines production alkalinize the medium and induce a new indicator color change from yellow to purple. The negative reaction is shown by the appearance of yellow color in the tube, while the positive reaction is shown in purple color.

(b) Reagents and media

The reagent and media used in this test were the same as described in section 6.2.4.1.11.

(c) Procedure

The procedure of this test was the same as described in section 6.2.4.1.5.

(d) Interpretation

Dark purple: Positive reaction given by organism
No change in color/Yellow: Negative reaction given by organism

6.2.4.1.14. ONPG

(a) Principle

The o-nitrophenyl-ß-D-galactopyranoside (ONPG) test is used to determine the presence or absence of the enzyme ß-galactosidase in an organism. The presence of

two enzymes, permease, and ß-galactosidase, are required to demonstrate lactose fermentation. Permease allows the lactose to enter the bacterial cell. In lactose fermenting bacteria the breakdown of lactose to glucose and galactose involves the enzyme beta-galactosidase. True lactose non-fermenters do not possess either of these enzymes. Late lactose fermenting organisms do not have permease but do possess ß-galactosidase. ONPG is similar in structure to lactose. If ß-galactosidase is present, the colorless ONPG is split into galactose and o-nitrophenol, a yellow compound

(b) Reagents and media

The reagent and media used in this test were the same as described in section 6.2.4.1.11.

(c) Procedure

The procedure of this test was the same as described in section 6.2.4.1.5.

(d) Interpretation

Yellow: Positive reaction given by organism
Colorless: Negative reaction is given by organism

6.2.4.1.15. Sugar fermentation test

(a) Principle

The sugar fermentation tests were performed for the detection of acid and gas production by an isolated bacterium. The test was performed in a fermentation tube as well as a kit that contained Durham tube (a small tube placed in an inverted position in the fermentation tube) for the detection of gas production, as an end product of metabolism.

(b) Reagents and media

The reagent and media used in this test were the same as described in section 6.2.4.1.4 and 6.2.4.1.1.4.

(b) Procedure

To perform sugar fermentation test (i.e. Glucose, adonitol, lactose, arabinose, sorbitol, sucrose, mannitol, and trehalose) the biochemical strips were inoculated with isolated bacterium suspension and incubated at 37 °C for 24-48 h. The indices obtained after

reading the results were interpreted using the HiMedia result interpretation chart supplied with Biochemical Identification kit.

(d) Interpretation

Yellow color: Positive reaction shown fermentation by bacteria

Pinkish Red/Red: Negative reaction showed no fermentation by bacteria

6.2.5. Molecular characterization

Further, the identity of the isolated bacterial strains was also confirmed by 16S rRNA gene sequence analysis. For this, the extraction and preparation of genomic DNA were performed according to the protocol described earlier (Kathiravan et al. 2011).

For the molecular identification of the isolated bacterial strains, the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R (5'-CGGTTACCTTGTTACGACTT-3') were used for the PCR amplification of the 16S rRNA gene products. PCR amplifications were performed in 50 ml of reaction mixture contained the template DNA (100 ng), forward primer 0.3 µM, reverse primer 0.3 µM, and master mixture 25 µl (Promega Corporation, Madison, WI, USA; 50 units/ml of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), dATP 400 µM, dGTP 400 µM, dCTP 400 µM, dTTP 400 µM, $MgCl_2$ 3 mM) in the nuclease-free water.

The thermocycling reactions were executed in Veriti™ 96-Well Thermal Cycler (Applied Biosystems™ Inc., CA, USA). The 16S rRNA gene was amplified using 35 PCR cycles (initial denaturation, 95 °C for 2 min; subsequent denaturation, 95 °C for 30 min; annealing temperature, 52 °C for 30 sec; extension temperature, 72 °C for 2 min; and final extension, 72 °C for 15 min). The electrophoresis of PCR products was done on 1% agarose gel and the band of interest was excised and purified with DNA Extraction Kit (Merck Life Science Pvt. Ltd., Bengaluru, KA, IN). Afterward, the purified PCR products were sequenced on the ABI 3500 Genetic Analyzer using Big Dye terminator software (v3.1). Further, the obtained partial nucleotide sequences were analyzed at National Council for Biotechnological Information (NCBI, USA) server using BLAST software available online at https://blast.ncbi.nlm.nih.gov/Blast.cgi and the corresponding sequences were downloaded and aligned using the Clustal-X program (Altschul et al. 1997). The phylogenetic tree was constructed by the neighbor-joining method using MEGA

software (v7.0) online available at www.megasoftware.net. In addition, the partial sequences were also submitted to the GeneBank database to receive the accession numbers for the isolated bacterial strains.

6.3. Results and discussion

6.3.1. Salt tolerance and COD removal efficiency of isolated bacterial strains

In the present study, morphologically distinct bacterial colonies grown on the MSM-agar plates were selected, picked up and purified by repeated streaking method for the further screening of potential bacterial strains (Fig. 6.2, 6.3).

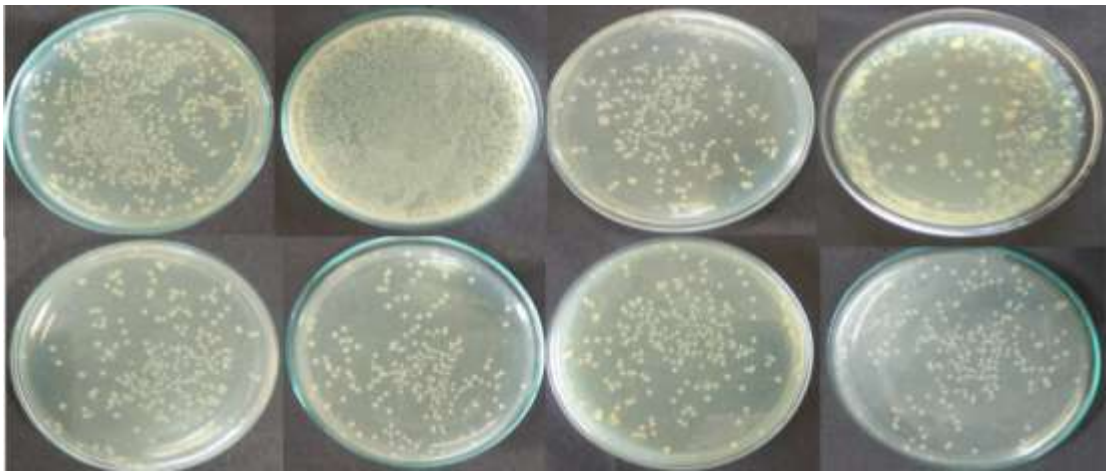


Fig. 6.2 Bacterial colonies developed on the MSM-agar plates

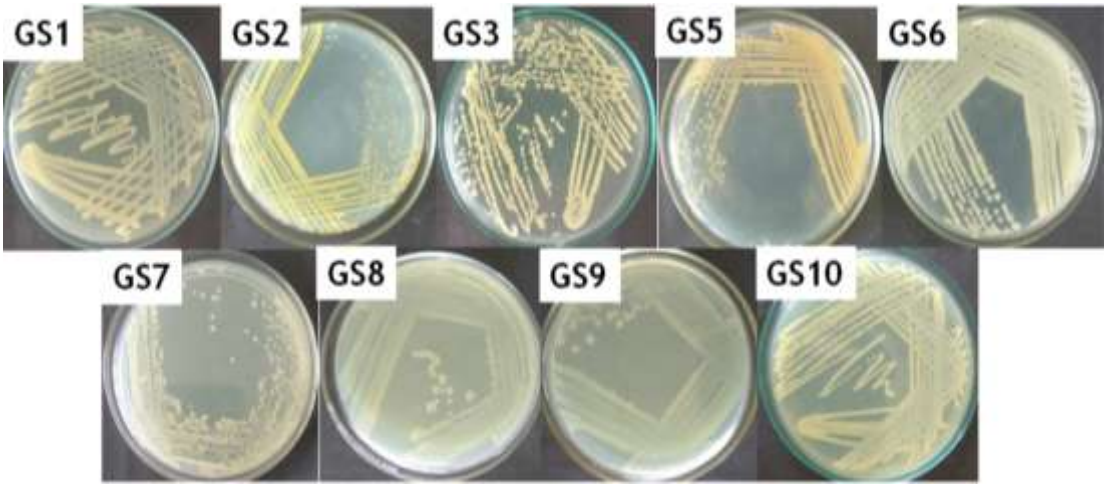


Fig. 6.3 Purified isolated bacterial strains (GS1-GS10) on the MSM-agar plates

Table 6.1 Salt tolerance pattern for the bacterial isolates

Salinity	Bacterial Strains									
	GS1	**GS2**	**GS3**	**GS4**	**GS5**	**GS6**	**GS7**	**GS8**	**GS9**	**GS10**
1%	+	+	+	+	+	+	+	+	+	+
2%	++	++	++	++	++	++	-	-	-	++
3%	++	++	+++	+	++	++	-	-	-	++
4%	+++	+	++	-	+	-	-	-	-	+++
5%	+++	-	-	-	-	-	-	-	-	+++
6%	++	-	-	-	-	-	-	-	-	+++
7%	-	-	-	-	-	-	-	-	-	+++
8%	-	-	-	-	-	-	-	-	-	++
9%	-	-	-	-	-	-	-	-	-	-
10%	-	-	-	-	-	-	-	-	-	-

Key: +: very slow growth; ++: slow growth; +++: fast and luxuriant growth; -: no growth

A total of ten (10) bacterial strains (GS1-10) were isolated from TWW + sludge sample collected from the outlet of CETP, Unnao (UP) India. Further, the isolated bacterial strains (GS1-10) were subjected to the primary screening on the basis of salt tolerance index. In our study, out of ten (10) bacterial isolates (GS1-10), only seven (07) bacterial strains i.e. GS1, GS2, GS3, GS4, GS5, GS6, and GS10 were adapted to tolerate up to 6%, 4%, 4%, 3%, 4%, 3%, and 8% (w/v) salt (NaCl) concentration, respectively, over a wide range (1-10%) of salinity (Table 6.1) and thus, can be used for the treatment of TWW. Further, salt-tolerant bacterial strains have been also reported for the treatment of TWW in a previous study (Sivaprakasam et al. 2008). CO is a major pollution parameter to predict the strength of pollution of any type of industrial wastewaters and used as a water quality criteria (Goutam et al. 2019). Therefore, the isolated bacterial strains (GS1-6 and GS10) were further selected for the secondary screening on the basis of COD removal efficiency to achieve the better effluent treatability. According to our study, only three bacterial strains i.e. GS1, GS3, and GS10 were reported to remove COD up to 61.12%, 54.28%, and 66.32% in real TWW sample within 120 h at 35 °C and 120 rpm (Fig. 6.4). Moreover, these bacterial strains (GS1, GS3, and GS10) were also showed maximum tolerance to salt (NaCl) concentration up to 6%, 4%, and 8% (w/v) and thus, are halotolerant in nature and suitable for the degradation and detoxification of persistent organic pollutants in TWW.

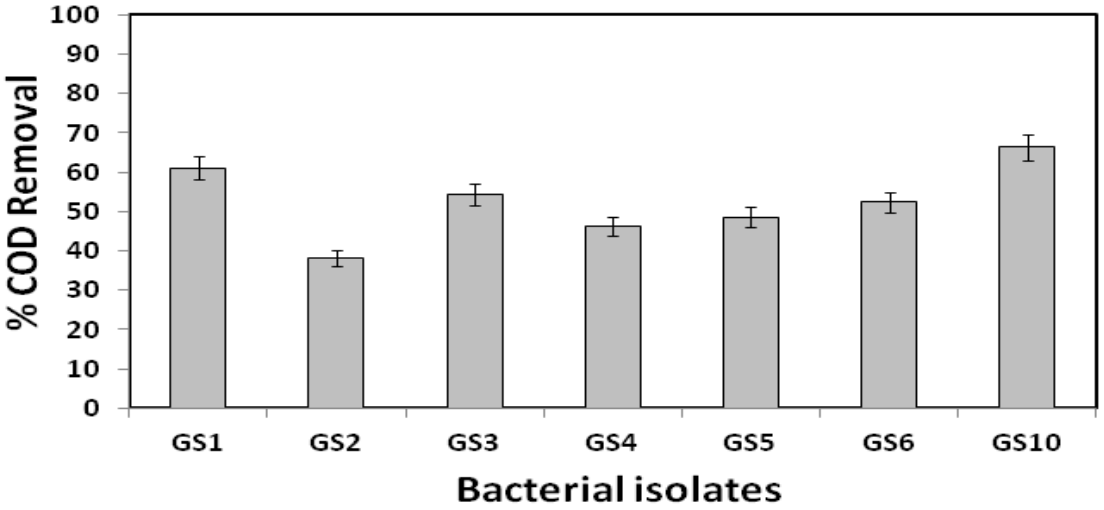


Fig. 6.4 Screening pattern of the bacterial strains for COD removal efficiency

6.3.2. Morphological characteristics of bacterial strains

On the basis of salt tolerance and COD removal efficiency, the three potential bacterial strains i.e. GS1, GS3, and GS10 were finally selected and identified on the basis of various morphological tests. The morphological characters examined in the present study were: Gram staining, shape, pigmentation, surface texture, margin, elevation, and motility. The morphological characteristics of the isolated bacterial strains GS1, GS3, and GS10 are listed in Table 6.2. Results revealed that the bacterial strain GS1 appeared as milky white colonies on MSM-agar plates and was gram-negative, motile, and rod-shaped. Bacterial strain GS3 appeared as white colonies on MSM agar plates and was gram-positive, non-motile, and round-shaped whereas bacterial strain GS10 appeared as greenish colonies on MSM agar plates and was also gram-negative, motile, and rod-shaped. The picture of gram staining, motility, and catalase tests are depicted in Fig. 6.5, 6.6. Further, these bacterial strains were further characterized by several biochemical tests to confirm their identity.

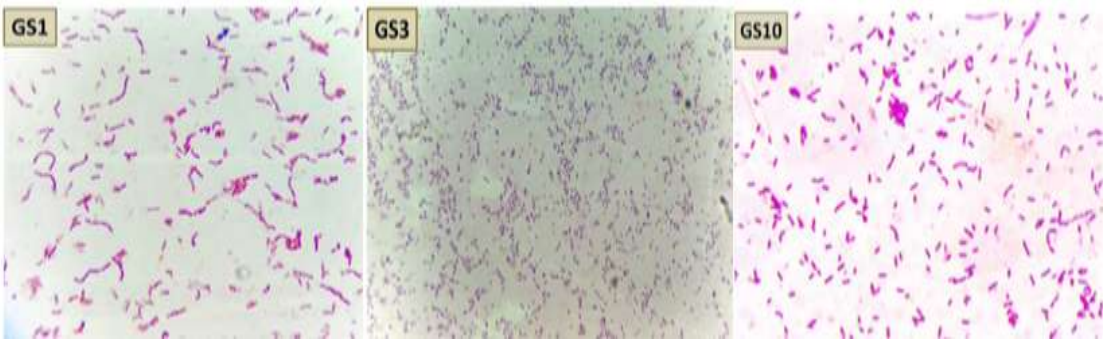


Fig. 6.5 Microscopic observation of the isolated bacterial strains GS1, GS3 & GS10 by Gram staining

Table 6.2 Morphological and biochemical characteristics of the isolated bacterial strains

Strain	GeneBank accessions	Identification	Morphological characteristics	Biochemical reactions
GS1	MK344317	*Ochrobactrum intermedium*	Gram straining (-ve), shape (short-rod (bacilli)), pigmentation (milky white), surface texture (smooth), margin (even), elevation (flat), and motility (motile)	Citrate utilization (+ve), lysine utilization (+ve), onithine utilization (+ve), urease (+ve), phenylalanine deamination (-ve), catalase (+ve), nitrate reduction (+ve), H_2S production (-ve), glucose (+ve), adonitol (+ve), lactose (+ve), arabinose (-ve), and sorbitol (-ve)
GS3	MK344318	*Micrococcus lylae*	Gram straining (+ve), shape (round (spherical)), pigmentation (white), surface texture (rough (granular), margin (even), elevation (convex), and motility (non-motile)	Malonate (+ve), voges proskauer's (-ve), citrate utilization (+ve), ONPG (-ve), nitrate reduction (-ve), catalase (+ve), arginine (-ve), sucrose (-ve), mannitol (-ve), glucose (+ve), arabinose (-ve), and trehalose (-ve)
GS10	MK344319	*Stenotrophomonas acidaminiphila*	Gram straining (-ve), shape (rod (bacilli)), pigmentation (greenish), surface texture (normal), margin (even), elevation (flat), and motility (motile)	Citrate utilization (-ve), lysine utilization (-ve), ornithine utilization (+ve), urease (+ve), phenylalanine deamination (-ve), catalase (+ve), nitrate reduction (+ve), H_2S production (-ve), glucose (+ve), adonitol (+ve), lactose (+ve), arabinose (+ve), and sorbitol (-ve)

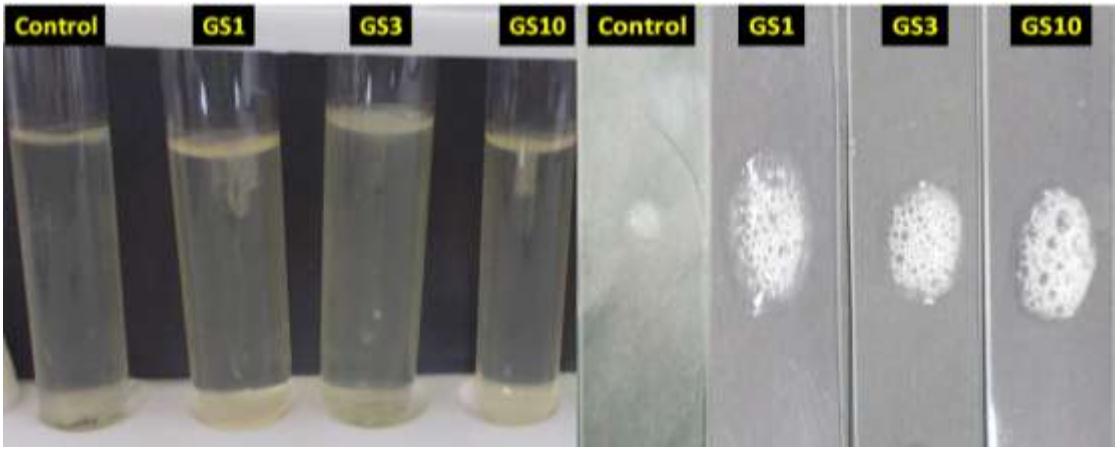


Fig. 6.6 Motility and catalase tests of the isolated bacterial strains GS1, GS3 & GS10

6.3.3. Biochemical characteristics of bacterial strains

On the basis of various morphological tests, the three potential bacterial strains i.e. GS1, GS3, and GS10 were finally characterized on the basis of various biochemical tests done by HiMedia biochemical kits (6.7). The biochemical characteristics of the isolated bacterial strains GS1, GS3, and GS10 are listed in Table 6.2. The bacterial strain GS1 showed positive reactions for citrate utilization, lysine utilization, ornithine utilization, urease, catalase, nitrate reduction, glucose, adonitol, lactose whereas negative reactions for phenylalanine deamination, H_2S production, arabinose, and sorbitol. Bacterial strain GS3 showed positive reactions for malonate, citrate utilization, catalase, glucose whereas negative reactions for Voges Proskauer's, ONPG, nitrate reduction, arginine, sucrose, mannitol, arabinose, and trehalose. Bacterial strain GS10 showed positive reactions for ornithine utilization, urease, catalase, nitrate reduction, glucose, adonitol, lactose, arabinose whereas negative reactions for citrate utilization, lysine utilization, phenylalanine deamination, H_2S production, and sorbitol. On the basis of different morphological and biochemical tests, the isolated bacterial strain GS1, GS3, and GS10 were probably belonged to the *Ochrobactrum*, *Micrococcus* and *Stenotrophomonas* genera, respectively.

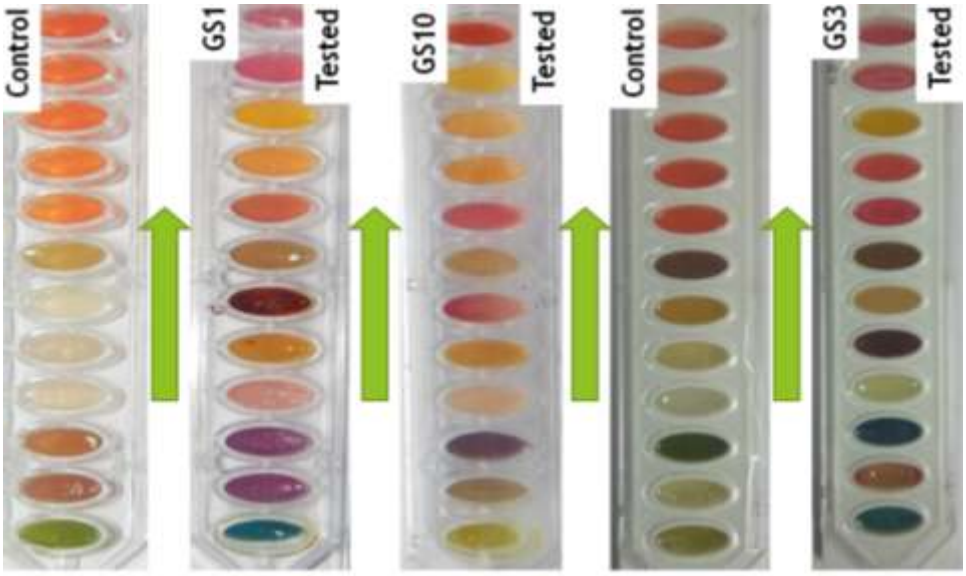


Fig. 6.7 HiMedia Biochemical Kits used for the biochemical characterizations of isolated bacterial strains GS1, GS3 & GS10

6.3.4. Molecular characteristics of bacterial strains

Further, on basis of 16S rRNA gene sequence analysis, the isolated bacterial strains GS1, GS3, and GS10 were identified and confirmed as *Ochrobactrum intermedium* (MK344317), *Micrococcus lylae* (MK344318), and *Stenotrophomonas acidaminiphila* (MK344319) (Fig. 6.8). The nucleotide sequences of the 16S rRNA genes of bacterial strain GS1, GS3, and GS10 were sequenced and used to calculate sequence similarity (Fig. 6.9).

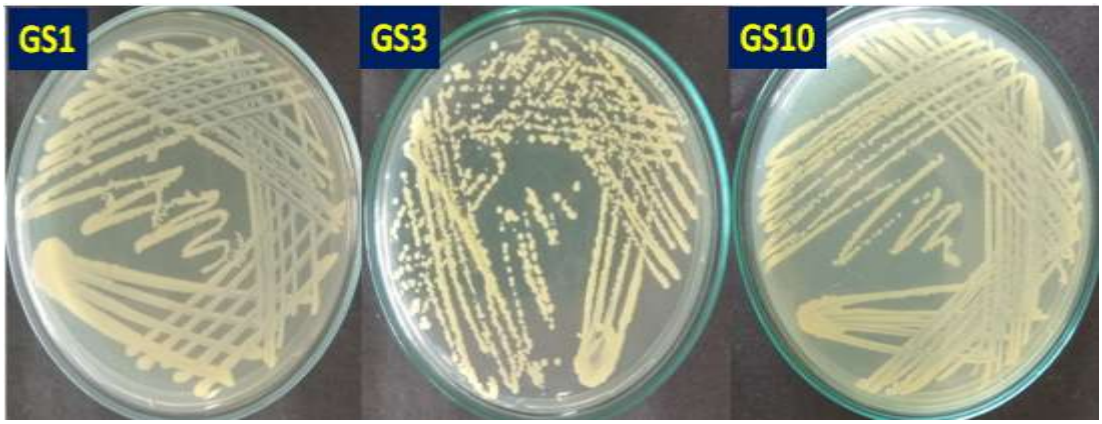


Fig. 6.8 Selected bacterial strains, *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3 & *Stenotrophomonas acidaminiphila* GS10

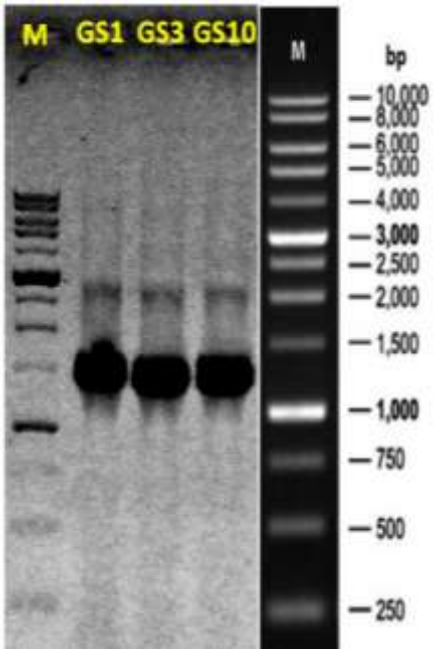
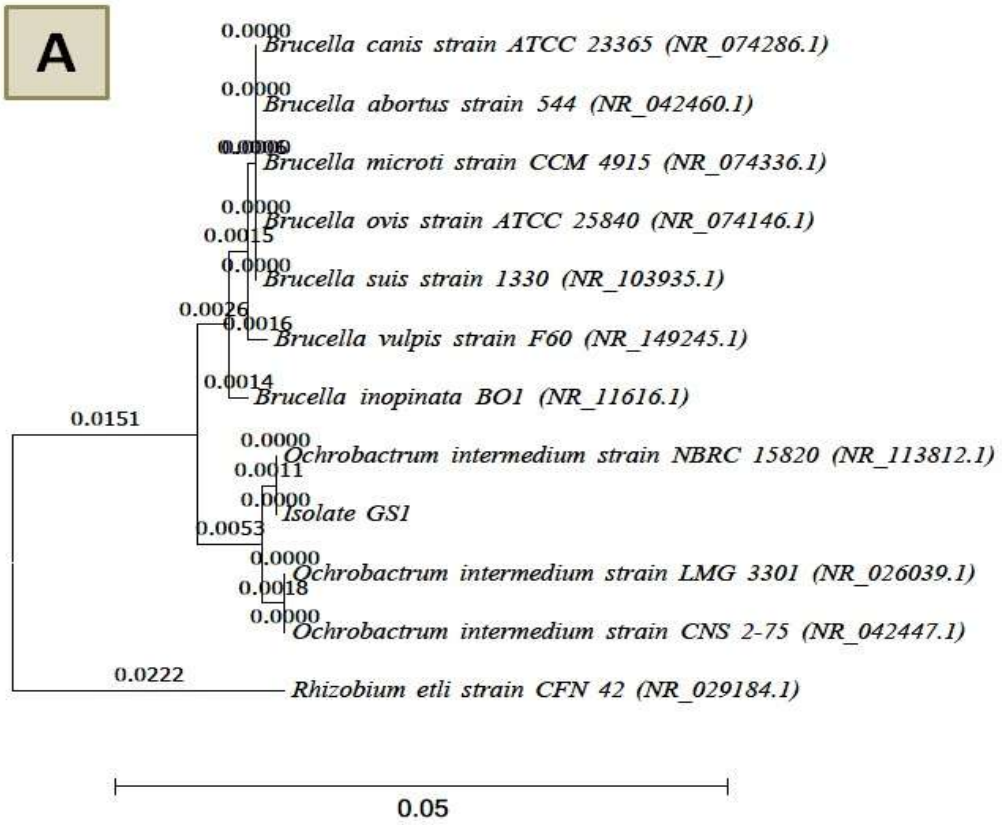
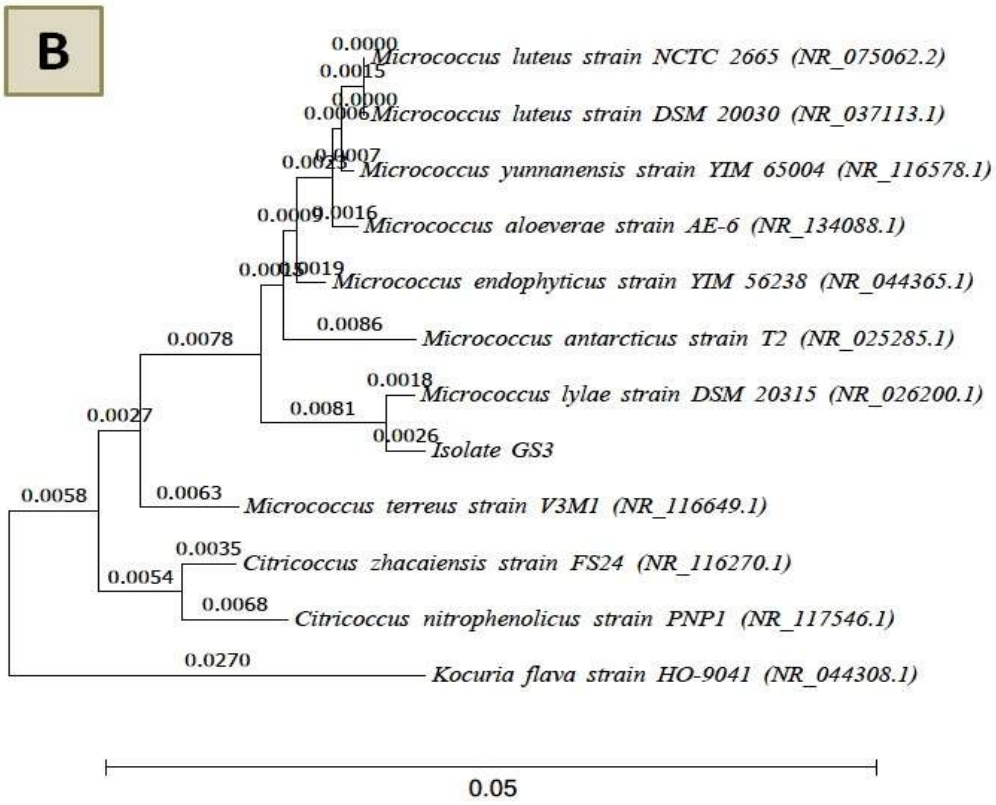


Fig. 6.9 PCR amplification of 16S rRNA gene of isolated bacterial strains; Lane M 10,000 bp DNA ladder

The strain GS1, GS3, and GS10 was homologous to *Ochrobactrum intermedium*, *Micrococcus lylae*, and *Stenotrophomonas acidaminiphila* 16S rRNA gene sequences obtained from the BLAST search with 97-100% of similarity index. Fig. 6.10 A, B, and C shows the phylogenetic tree drawn from the neighbour-joining program by bootstrap consensus test using MEGA software (v7.0) (Tamura et al. 2004; Saitou and Nei 1987). The phylogenetic tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Tamura et al. 2004; Felsenstein 1985). Based on the tree, the bacterial strains GS1, GS3, and GS10 was closely related to the *Ochrobactrum intermedium* (NR113812.1), *Micrococcus lylae* (NR026200.1), and *Stenotrophomonas acidaminiphila* (NR025104.1), respectively. Further, our findings are supported by previous studies that report the potential of *Ochrobactrum*, *Micrococcus*, and *Stenotrophomonas* genera in the biodegradation and bioremediation of environmental contaminants/industrial wastes (Marzan et al. 2017; Chen et al. 2016; Deng et al. 2015; Mangwani et al. 2014; Rida et al. 2012; Kavita and Keharia 2012; Sultan and Hasnain 2007).


A
0.0000
Brucella canis strain ATCC 23365 (NR_074286.1)
0.0000
Brucella abortus strain 544 (NR_042460.1)
Brucella microti strain CCM 4915 (NR_074336.1)
0.0000
Brucella ovis strain ATCC 25840 (NR_074146.1)
0.0015
0.0000
Brucella suis strain 1330 (NR_103935.1)
0.0026
0.0016
Brucella vulpis strain F60 (NR_149245.1)
0.0014
Brucella inopinata BO1 (NR_11616.1)
0.0151
0.0000
Ochrobactrum intermedium strain NBRC 15820 (NR_113812.1)
0.0011
0.0000
Isolate GS1
0.0053
0.0000
Ochrobactrum intermedium strain LMG 3301 (NR_026039.1)
0.0018
0.0000
Ochrobactrum intermedium strain CNS 2-75 (NR_042447.1)
0.0222
Rhizobium etli strain CFN 42 (NR_029184.1)
0.05


B
0.0000
Micrococcus luteus strain NCTC 2665 (NR_075062.2)
0.0015
0.0000
0.0006
Micrococcus luteus strain DSM 20030 (NR_037113.1)
0.0007
0.0021
Micrococcus yunnanensis strain YIM 65004 (NR_116578.1)
0.0009
0.0016
Micrococcus aloeverae strain AE-6 (NR_134088.1)
0.0019
Micrococcus endophyticus strain YIM 56238 (NR_044365.1)
0.0086
Micrococcus antarcticus strain T2 (NR_025285.1)
0.0078
0.0018
Micrococcus lylae strain DSM 20315 (NR_026200.1)
0.0027
0.0081
0.0026
Isolate GS3
0.0058
0.0063
Micrococcus terreus strain V3M1 (NR_116649.1)
0.0035
Citricoccus zhacaiensis strain FS24 (NR_116270.1)
0.0054
0.0068
Citricoccus nitrophenolicus strain PNP1 (NR_117546.1)
0.0270
Kocuria flava strain HO-9041 (NR_044308.1)
0.05

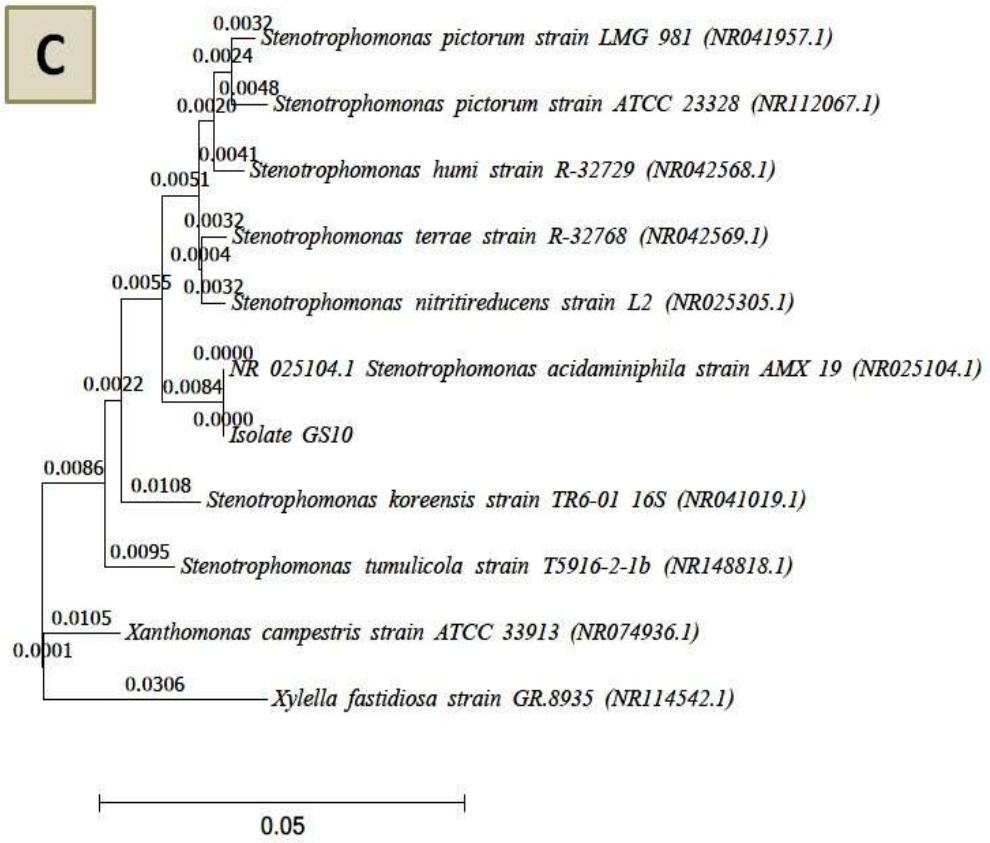


Fig. 6.10 Phylogenetic tree showing the relationship of selected bacteria strains, *Ochrobactrum intermedium* GS1 (A), *Micrococcus lylae* GS3 (B) and *Stenotrophomonas acidaminiphila* GS10 (C) with its neighboring bacterial species. The scale represents the evolutionary branch length and numbers in the bracket represent GeneBank accession numbers

6.4. Conclusion

The present study was aimed to isolate, purify, screen, characterize and identify the potential bacterial strains capable for the degradation of persistent organic pollutants present in the secondary treated TWW for environmental safety. In the present study, a total of ten (10) bacterial strains were isolated from the collected TWW and sludge sample after secondary treatment process and purified by the repeated streaking method. These isolated bacterial strains (GS1-GS10) were further screened for the ability to tolerate high salt concentration and remove COD from real TWW. Results revealed that among all the isolated bacterial strains, only three bacterial strains i.e. GS1, GS3, and GS10 were able to tolerate up to 6, 4, and 8% salt concentration and capable to remove COD up to 61.12, 54.28, and 66.32% from real TWW. Further, these bacterial strains were identified as *Ochrobactrum intermedium* GS1,

Micrococcus lylae GS3, and *Stenotrophomonas acidaminiphila* GS10, respectively, on the basis various morphological and biochemical reactions and 16s RNA gene sequence analysis. Overall, on the basis of bioremediation potential, these bacteria can be used either alone or in combination for degradation and detoxification of contaminants present in the TWW to safeguard the environment and public health.

7

Chapter-07

Development and Optimization of a Potential Bacterial Consortium for the Effective Degradation and Detoxification of Persistent Organic Pollutants (POPs) From Tannery Wastewater

Development and optimization of a potential bacterial consortium for the effective degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater

7.1. Introduction

The treatment and management of industrial wastewater is one of the major challenges in developing countries because of deficient wastewater treatment plants and lack of advanced treatment technologies. Tannery wastewater (TWW) is a major source of environmental pollution and characterized by high pollution parameters such as COD, BOD, TDS, Cr, phenol and a mixture of persistent organic pollutants (POPs) and heavy metals, which extremely polluting our natural resources (soil/water) and also creates severe toxic effects in the exposed organisms (Yadav et al. 2019; Bharagava et al. 2018; Goutam et al. 2018). Various physical and chemical treatment technologies are currently applied to effectively treat and detoxify TWW; however, these approaches are not environmental friendly because of the use of the huge amount of chemicals during treatment and generation of the high amount of contaminated sludge as a secondary pollutant in the environment (Saxena et al. 2016).

Hence, there is a need to replace the physico-chemical methods by the eco-friendly treatment solution for wastewater treatment and management. Bioremediation technology is an eco-friendly remediation solution as it employs microbes and plants or their enzymes for the degradation and detoxification of organic and inorganic pollutants in industrial wastewaters (Saxena and Bharagava 2017a,b). There are several reports are available in the public domain on the microbial degradation and detoxification of contaminants from TWW (Kumari et al. 2018; Bharagava and Mishra 2018; Kim et al. 2014; El-Bestawy et al. 2013; Paisio et al. 2012; Mandal et al. 2010). However, there is a scarcity of literature is available on the use of a microbial consortium, especially bacterial consortia in the removal of contaminants from TWW. TWW is highly complex in nature and contains a mixture of organic and inorganic contaminants and hence, monoculture of any biological agents could not efficiently treat/detoxify it. Conversely, the application of microbial consortia is more suitable over pure cultures to efficiently degrade/detoxify industrial effluents due to the intensive metabolic activities of microbes that can effectively degrade a mixture of organic and inorganic pollutants. Microbes play a central role in the degradation and detoxification of environmental pollutants in industrial

wastewaters (Saxena et al. 2016); however, requires some specific conditions that favor the optimum removal of contaminants from industrial wastewaters. Environmental and nutritional factors, as well as the size of inoculum and agitation rate, are the key process parameters in the microbial degradation/detoxification of contaminants from industrial wastewaters (Anjali and Sabumon 2014; Preethi et al. 2013; Paisio et al. 2012). These process parameters directly or indirectly affect the capability of microbes during the degradation and detoxification of organic and inorganic pollutants from industrial wastewaters.

Therefore, the present study was aimed to develop a new bacterial consortium for the efficient degradation of TWW. Further, the effects of various environmental (pH and temperature) and nutritional (carbon and nitrogen sources) factor, as well as inoculum concentration and agitation rate/shaking speed on the degradation and detoxification of TWW containing persistent organic pollutants by the newly developed bacterial consortium, was studied to achieve the better treatment efficiency.

7.2. Materials and methods

7.2.1. Chemicals and media's

All the required chemicals and reagents were used in the experiments are of highest purity (purity ≥ 99%) and analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Mineral salt medium (MSM g/L, Na_2HPO_4 2.4; K_2HPO_4 2.0; NH_4NO_3 0.1; $MgSO_4$ 0.01; $CaCl_2$ 0.01) was used for the development of bacterial precultures and consortium and bioremediation studies for TWW. The collected TWW sample was used for bioremediation studies. Whatman® Grade GF/C filter papers (pore size 1.2 μm) (Whatman, England, UK) were used for the filtration of TWW.

7.2.2. Bio-interaction study of the isolated potential bacterial strains for the development of a bacterial consortium

For revealing the capability of each strain to existing with other strains without any inconsistency, a compatibility experiment was performed before developing the bacterial consortium. In this test, one bacterial strain was spread on the MSM-agar plate and other two strains were inoculated on the wells made on the plates after spreading just to know that these bacterial are inhibiting the growth of each other or

not on the basis of formation of the zone. The compatibility test was performed as per the method outlined (Poulsen and Currie 2010).

7.2.3. Development of a potential bacterial consortium for the effective degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater

Based on the performance of monocultures in the bioremediation studies for TWW, a new bacterial consortium comprising potential bacterial strains GS1, GS3, and GS10 was developed after a positive compatibility test and named as "GS-TE1310". For the development of bacterial consortium, a loopfull culture of the purified bacterial strains was aseptically transferred in the Erlenmeyer flasks (150 ml) containing 50 ml MSM broth (pH 7.0) supplemented with glucose (0.5%, w/v) as a carbon source and incubated for 24 h at 35 °C under shaking condition (120 rpm) in an temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA). Further, 5 ml of each bacterial pre-culture was inoculated in MSM broth (85 ml, pH 7.0) amended with glucose (1%, w/v) as a carbon source in a Erlenmeyer flask (250 ml) which was incubated (at 30 °C) under shaking condition (120 rpm) in an temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA) for 24 h. Afterward, the performance of the developed bacterial consortium GS-TE1310 was evaluated in the bioremediation of TWW on the basis of COD removal efficiency.

For the performance evaluation, 20 ml of the newly developed bacterial consortium GS-TE1310 was inoculated in TWW (undiluted, 80 ml, pH 7.0) in the Erlenmeyer flasks (250 ml) and kept for incubation (at 35 °C) under shaking condition (120 rpm) in the temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA) for successive five days. Afterward, the bacterially treated TWW sample was taken out every 24 h, centrifuged at 10000 × g for 10 min in a refrigerated centrifuge (REMI Instruments Pvt. Ltd., Mumbai, MH, IN) and the supernatant was used for the COD measurements.

7.2.4. Optimization of the newly developed bacterial consortium at various nutritional parameters for the effective degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater

To enhance the pollutant (COD) removal efficiency, the newly developed bacterial consortium GS-TE1310 was optimized for the various nutritional sources (carbon and

nitrogen sources) for the efficient degradation of persistent organic pollutants (POPs) in real the TWW.

7.2.4.1. Optimization of carbon sources

For the optimization of carbon sources, 20 ml of the newly developed bacterial consortium GS-TE1310 was inoculated in TWW (undiluted, 80 ml, pH 7.0) supplied with different carbon sources (sucrose, glucose, maltose, lactose, and starch; 0.5%, w/v) in the Erlenmeyer flasks (250 ml) and kept for incubation (at 35 °C) with parallel abiotic controls under shaking condition (120 rpm) in the temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA) for successive five days. Afterward, the bacterially treated TWW sample was taken out every 24 h, centrifuged at 10000 × g for 10 min in a refrigerated centrifuge (REMI Instruments Pvt. Ltd., Mumbai, MH, IN) and the supernatant was used for the COD measurements.

7.2.4.2. Optimization of nitrogen sources

For the optimization of nitrogen sources, 20 ml of the newly developed bacterial consortium GS-TE1310 was inoculated in TWW (undiluted, 80 ml, pH 7.0) supplied with different nitrogen (yeast extract, peptone, ammonium chloride, sodium nitrate, and urea; 0.5%, w/v) sources in the Erlenmeyer flasks (250 ml) and kept for incubation (at 35 °C) with parallel abiotic controls under shaking condition (120 rpm) in the temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA) for successive five days. Afterward, the bacterially treated TWW sample was taken out every 24 h, centrifuged at 10000 × g for 10 min in a refrigerated centrifuge (REMI Instruments Pvt. Ltd., Mumbai, MH, IN) and the supernatant was used for the COD measurements.

7.2.5. Optimization of the newly developed bacterial consortium at various environmental parameters for the effective degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater

To enhance the pollutant (COD) removal efficiency, the newly developed bacterial consortium GS-TE1310 was optimized for the various environmental parameters (pH and temperature) for the efficient degradation of persistent organic pollutants (POPs) in real the TWW.

7.2.5.1. Optimization of pH

For the optimization of pH, 20 ml of the newly developed bacterial consortium GS-TE1310 was inoculated in TWW (undiluted, 80 ml) in the Erlenmeyer flasks (250 ml). To this, the optimized concentrations (0.5%, w/v) of carbon and nitrogen source was added, shaked well and kept for incubation at a wide range of pH (5-9) at 35 °C with parallel abiotic controls under shaking condition (120 rpm) in the temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA) for successive five days. Afterward, the bacterially treated TWW sample was taken out every 24 h, centrifuged at 10000 × g for 10 min in a refrigerated centrifuge (REMI Instruments Pvt. Ltd., Mumbai, MH, IN) and the supernatant was used for the COD measurements.

7.2.5.2. Optimization of temperature

For the optimization of temperature, 20 ml of the newly developed bacterial consortium GS-TE1310 was inoculated in TWW (undiluted, 80 ml) in the Erlenmeyer flasks (250 ml). To this, the optimized concentrations (0.5%, w/v) of carbon and nitrogen source was added, shaked well and kept for incubation over a wide range of temperature (25, 30, 35, 45 and 45 °C) with the optimized pH with parallel abiotic controls under shaking condition (120 rpm) in the temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA) for successive five days. Afterward, the bacterially treated TWW sample was taken out every 24 h, centrifuged at 10000 × g for 10 min in a refrigerated centrifuge (REMI Instruments Pvt. Ltd., Mumbai, MH, IN) and the supernatant was used for the COD measurements.

7.2.6. Optimization of the newly developed bacterial consortium at various inoculum concentration and agitation rate for the effective degradation and detoxification of persistent organic pollutants (POPs) from tannery wastewater

To enhance the pollutant (COD) removal efficiency, the newly developed bacterial consortium GS-TE1310 was optimized for the various inoculum volume and agitation rate for the efficient degradation of persistent organic pollutants (POPs) in real the TWW.

7.2.6.1. Optimization of inoculum volume

For the optimization of inoculum concentration, different concentrations (4, 8, 12, 16, and 20%, v/v) of the developed bacterial consortium GS-TE1310 was inoculated in the undiluted TWW amended with the optimized nutrient (C & N source)

concentrations (0.5%, w/v) in the Erlenmeyer flasks (250 ml). Afterward, the flasks were kept with parallel abiotic controls for incubation at the optimized pH and temperature (°C) under shaking condition (120 rpm) in the temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA) for successive five days. Further, the bacterially treated TWW sample was taken out every 24 h, centrifuged at 10000 × g for 10 min in a refrigerated centrifuge (REMI Instruments Pvt. Ltd., Mumbai, MH, IN) and the supernatant was used for the COD measurements.

7.2.6.2. Optimization of agitation rate

For the optimization of agitation rate, the optimized concentration of the developed bacterial consortium GS-TE1310 was inoculated in the undiluted TWW amended with the optimized nutrient (C & N source) concentrations (0.5%, w/v) in the Erlenmeyer flasks (250 ml). Afterward, the flasks were kept with parallel abiotic controls for incubation at the optimized pH and temperature (°C) under different shaking conditions (80, 90, 100, 110, and 120 rpm) in the temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA) for successive five days. Further, the bacterially treated TWW sample was taken out every 24 h, centrifuged at 10000 × g for 10 min in a refrigerated centrifuge (REMI Instruments Pvt. Ltd., Mumbai, MH, IN) and the supernatant was used for the COD measurements.

7.3. Results and discussion

In the present study, a potential bacterial consortium GS-TE1310 was developed using three potential pollutants degrading/detoxifying bacteria, *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, and *Stenotrophomonas acidaminiphila* GS10 on the basis of bio-interaction study or compatibility test among them. Results revealed that all the selected bacterial strains were able to growth with each other without forming any zone of inhibition and hence, used in the development of the new bacterial consortium GS-TE1310 (Fig. 7.1)

The pure monocultures of three isolated potential bacterial strains, *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, and *Stenotrophomonas acidaminiphila* GS10 and their consortium GS-TE1310 was used for the bioremediation of real TWW. During bioremediation studies, the COD removal from real TWW was 61.12%, 54.28%, and 66.32% by the *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, and *Stenotrophomonas acidaminiphila* GS10, respectively, within 120 h at

35 °C and 120 rpm. However, the COD removal from real TWW by the newly developed bacterial consortium GS-TE1310 comprising *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, and *Stenotrophomonas acidaminiphila* GS10 was much higher (74.15%) as compared to the individual bacterial strains within 120 h at 35 °C and 120 rpm (Fig. 7.3 & 7.3). This faster COD removal from real TWW by the newly developed bacterial consortium GS-TE1310 was might be due to the utilization of pollutants as nutrients by the isolated bacterial strains and thus, the degradation was higher.

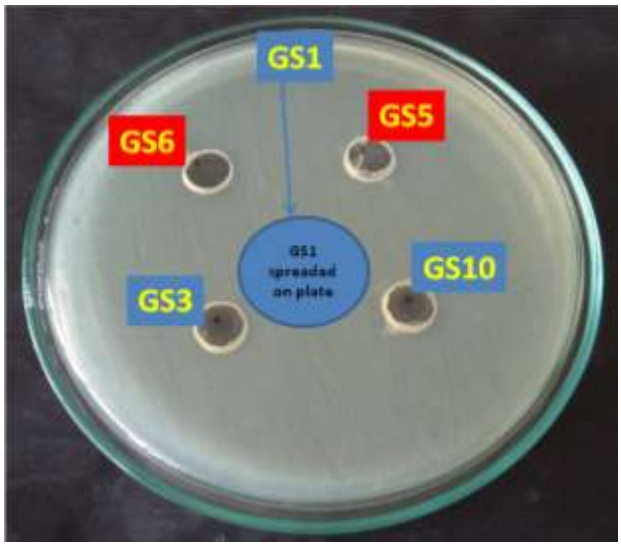


Fig. 7.1 Picture showing the compatibility among the selected bacterial strains GS1, GS3, and GS10 (strains in red color (GS5 & GS6) are excluded from the present study)

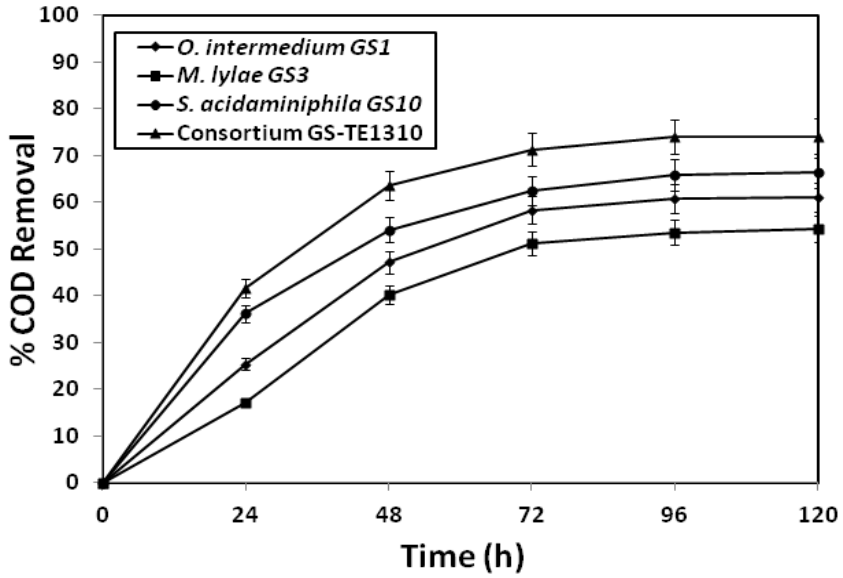


Fig. 7.2 COD removal from real TWW by the newly developed bacterial consortium GS-TE1310 and individual bacterial strains, *O. intermedium*, *M. lylae*, and *S.*

acidaminiphila. Error bars represent the standard deviation calculated from at least three independent experiments performed at the standard conditions (7 pH, 35 °C, and 120 rpm)

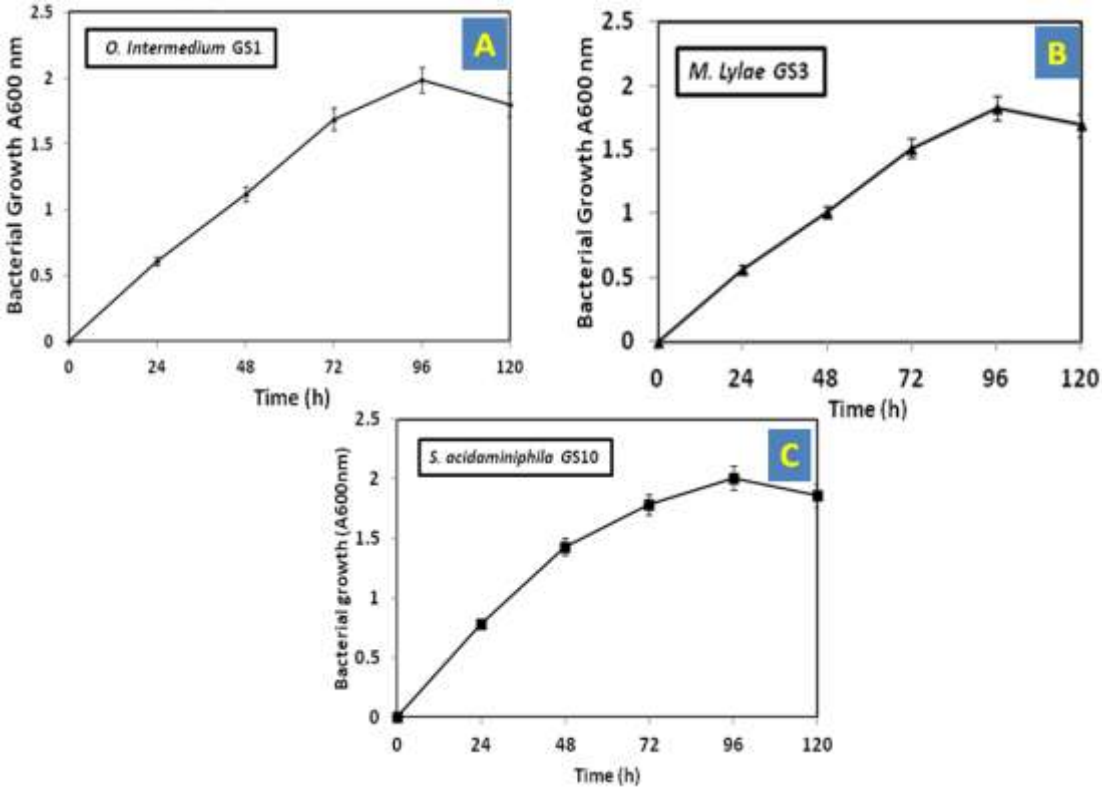


Fig. 7.3 Growth curve of the isolated bacterial strains GS1 (A), GS3 (B), & GS10 (C)

Further, to enhance the COD removal efficiency, the newly developed bacterial consortium GS-TE1310 was optimized for environmental (pH and temperature) and nutritional (carbons and nitrogen sources) parameters, as well as inoculum concentration and shaking speed for effective degradation of real TWW that contained various POPs. The results of the optimization of carbon sources (0.5%, w/v) showed that the maximum COD removal (74.68%) was noted in presence of glucose as additional C-source within 120 h at 35 °C and 120 rpm, followed by lactose (68.48%), maltose (56.32%), sucrose (44.28%), and starch (38.58%) (Fig. 7.4). Glucose was might be acted as an external co-substrate that enhanced the degradation and detoxification of persistent organic and inorganic pollutants present in TWW by the newly developed bacterial consortium GS-TE1310.

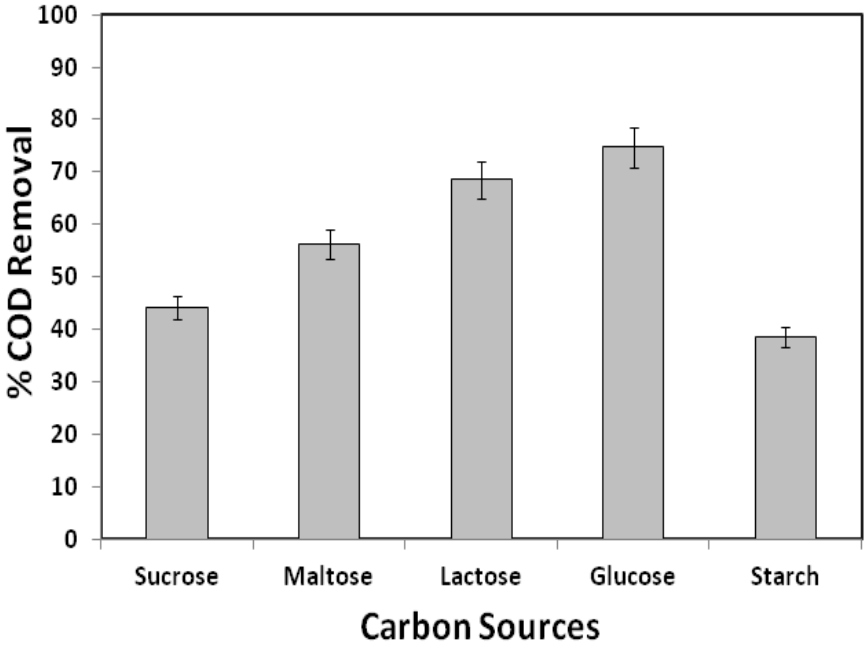


Fig. 7.4 Effect of carbon sources (0.5%, w/v) on COD removal from real TWW using newly developed bacterial consortium GS-TE1310. Error bars represent the standard deviation calculated from at least three independent experiments (performed at 7 pH, 35 °C, and 120 rpm)

On the other hand, in case of nitrogen sources (0.5%, w/v), the maximum COD reduction (74.92%) was recorded in presence of ammonium chloride (NH_4Cl) as a major source of nitrogen within 120 h at 35 °C and 120 rpm, followed by sodium nitrate (72.28%), urea (68.52%), yeast extract (61.39), and peptone (38.68%), respectively (Fig. 7.5).

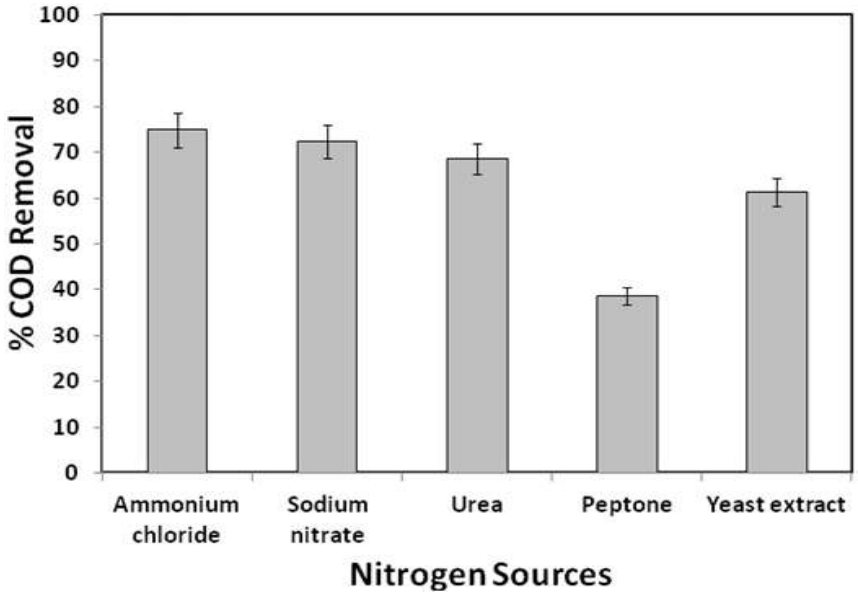


Fig. 7.5 Effect of nitrogen sources (0.5%, w/v) on COD removal from real TWW using newly developed bacterial consortium GS-TE1310. Error bars represent the standard deviation calculated from at least three independent experiments (performed at 7 pH, 35 °C, and 120 rpm)

In addition, the results of the pH optimization showed that the newly developed bacterial consortium GS-TE1310 was able to remove COD within a wide range of pH (5 - 9) (Fig. 7.6). The maximum COD reduction (75.18%) was recorded at pH 7, indicating that the neutral pH highly favored the bacterial growth and metabolism during the biodegradation and detoxification of persistent organic pollutants and heavy metals present in the TWW whereas, at pH 6, 8 and 9, the COD reduction was 54.28%, 68.72%, and 56.12%, respectively. However, the COD removal was least (42.14%) observed at pH 5 due to acidic conditions. The common salt (NaCl) used in soaking operation imparts a neutral pH to the wastewater and hence, this newly developed bacterial consortium GS-TE1310 is suitable for the treatment and detoxification of TWW without any pH correction.

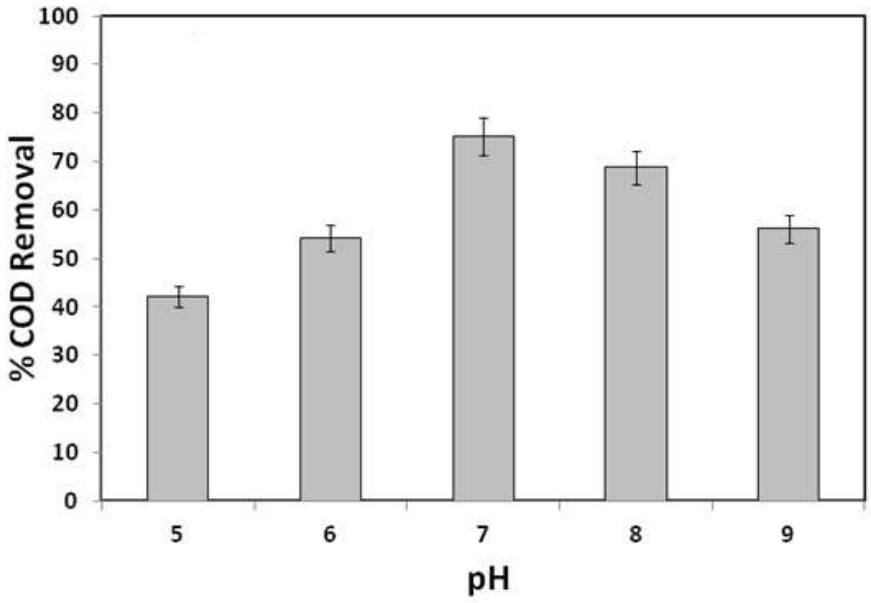


Fig. 7.6 Effect of different pH (5-9) on COD removal from real TWW using newly developed bacterial consortium GS-TE1310. Error bars represent the standard deviation calculated from at least three independent experiments (performed at 35 °C, 120 rpm and 0.5%, w/v glucose, and NH_4Cl)

Further, temperature optimization studies proved that the newly developed bacterial consortium GS-TE1310 was able to remove COD from TWW within a wide

range of temperature (25 - 45 °C). In the present study, the maximum COD reduction (75.52%) was recorded at 35 °C while the least COD reduction (43.36%) was noted at 25 °C (Fig. 7.7). This was might be due to higher bacterial activity at 35 °C; however, ETPs in the tropical countries (like India) are mostly operated at normal day temperature (≥ 30°C) and thus, this newly developed bacterial consortium GS-TE1310 is suitable for the treatment and detoxification of TWW.

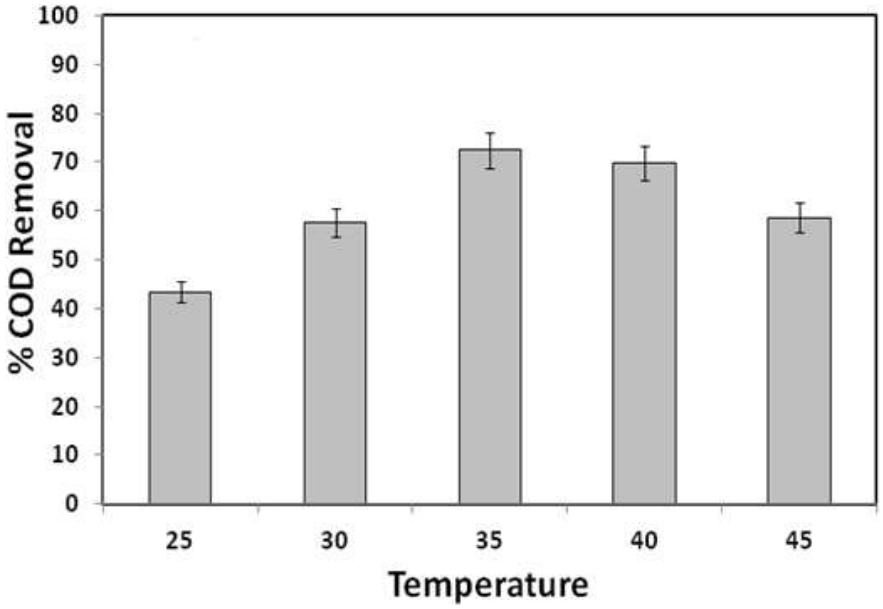


Fig. 7.7 Effect of different temperature (°C) on COD removal from real TWW using newly developed bacterial consortium GS-TE1310. Error bars represent the standard deviation calculated from at least three independent experiments (performed at 7 pH, 120 rpm and 0.5%, w/v glucose, and NH_4Cl)

Further, the results of the inoculum concentration optimization showed that only 20% (v/v) inoculum concentration was found to be optimum inoculum volume for the maximum COD reduction (75.86%) from real TWW by the newly developed bacterial consortium GS-TE1310 (Fig. 7.8). This was might be attributed to the production of higher cellular biomass and thus, higher the metabolic activities of the bacterial strains capable to degrade/detoxify the persistent organic pollutants and toxic metals present in the TWW.

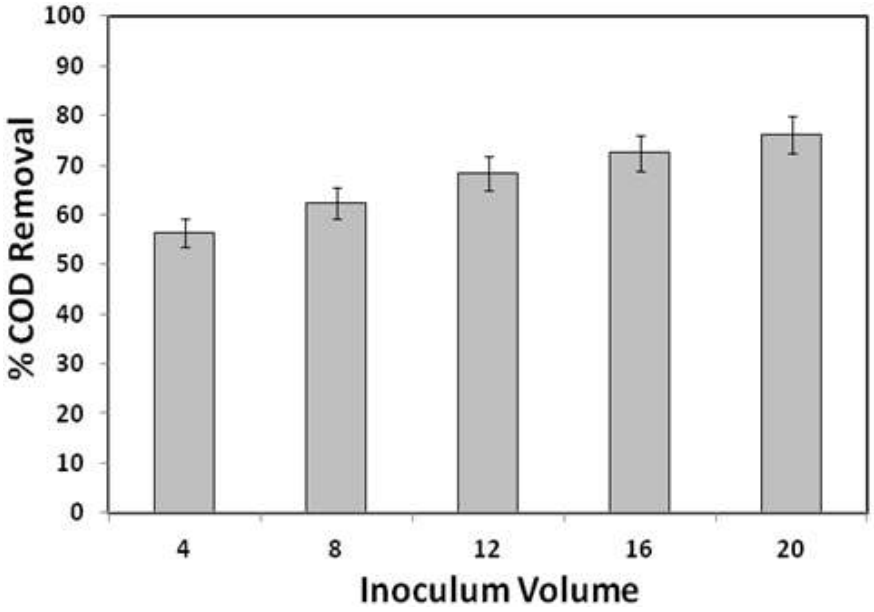


Fig. 7.8 Effect of inoculum concentration (ml) on COD removal from real TWW using newly developed bacterial consortium GS-TE1310. Error bars represent the standard deviation calculated from at least three independent experiments (performed at 7 pH, 35 °C, 120 rpm and 0.5%, w/v glucose, and NH_4Cl)

In addition, the results of the shaking speed optimization showed that only 120 rpm was found to be optimum shaking speed for the maximum COD reduction (76.08%) from real TWW by the newly developed bacterial consortium GS-TE1310 within 120 h at 35 °C and 7 pH (Fig. 7.9). This was might be due to the proper mixing of oxygen in the medium, increase in cellular biomass and oxygen transfer between the cells and medium. Further, an increase in shaking/agitation rate (130-140 rpm) caused an observable fall in COD removal efficiency by 17.30% and was might be due to the effect of shear rate on the cell wall, resulting in the cell damage (mechanical injury to bacterial cells).

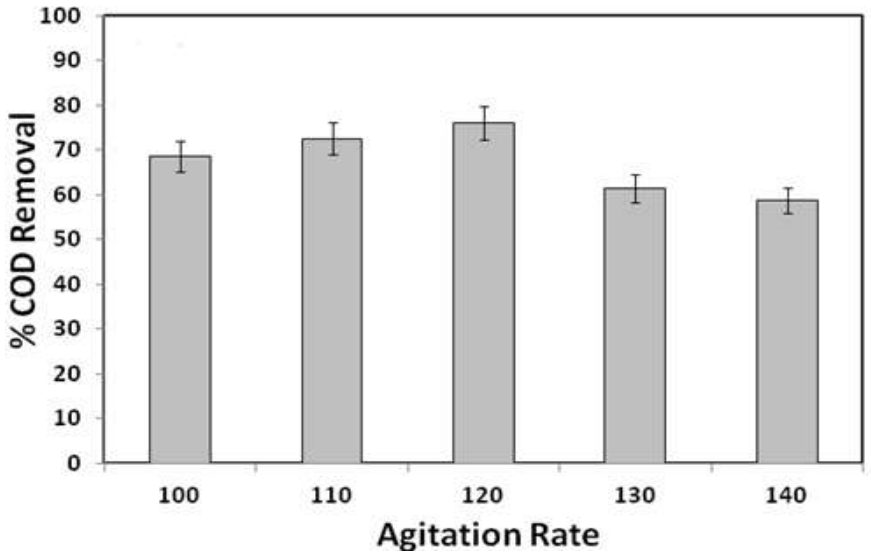


Fig. 7.9 Effect of shaking/agitation (rpm) on COD removal from real TWW using newly developed bacterial consortium GS-TE1310. Error bars represent the standard deviation calculated from at least three independent experiments (performed at 7 pH, 35 °C, 20 ml inoculum concentration, and 0.5%, w/v glucose, and NH_4Cl)

In previous studies, many authors have reported the optimization of nutritional and environmental parameters for the maximum removal of pollutants from TWW. Tripathi and Garg (2013) have reported the co-remediation of pentachlorophenol (PCP) and hexavalent chromium (Cr^{6+}) at different optimized parameters (pH, temperature, carbon and nitrogen sources and agitation rate) by free and immobilized cells of native Bacillus cereus, isolated from secondary treated TWW. Further, Tripathi et al. (2011) have also reported the degradation and detoxification of pentachlorophenol (PCP) and hexavalent chromium (Cr^{6+}) at optimized carbon and nitrogen sources by *Bacillus cereus*, isolated from TWW. Sivaprakasam et al also studied the bioremediation of TWW by salt-tolerant bacterial strains at the optimized environmental parameters (pH, temperature) and inoculum volume as well as agitation rate.

7.4. Conclusion

The present study was aimed to develop a new bacterial consortium for the degradation and detoxification of persistent organic pollutants (POPs) in the real leather TWW. In the present study, a new bacterial consortium GS-TE1310 was developed comprising three potential pollutants degrading bacterial strains, *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, and *Stenotrophomonas*

acidaminiphila GS10 because these bacteria are not inhibiting the growth of each other as there was no zone of inhibition was formed on the MSM-agar plate and thus, the selected bacterial strains were compatible to each other and able to work together during bioremediation study. The newly developed bacterial consortium GS-TE1310 was able to remove COD up 74.15% as compared to individual bacterial strains, *Ochrobactrum intermedium* GS1 (61.12%), *Micrococcus lylae* GS3 (54.28%), and *Stenotrophomonas acidaminiphila* GS10 (66.32%). Further, the newly developed bacterial consortium GS-TE1310 was optimized at various environmental and nutritional parameters as well as inoculum concentration and agitation speed. Results revealed that the optimum pH, temperature, inoculum concentration, and agitation rate were found to be 7, 35 °C, 20 ml and 120 rpm, respectively and the best carbon and nitrogen source was found to be glucose and ammonium chloride, respectively, among the various carbon and nitrogen sources used for the bioremediation of real TWW. After optimization of process parameters, the newly developed bacterial consortium GS-TE1310 was able to remove COD up to 76.08% from real TWW. Overall, this newly developed and optimized bacterial consortium GS-TE1310 can be used for the degradation of persistent organic pollutants (POPs) in the real TWW.

8

Chapter-08

Characterization of Metabolites from Bacterially Treated Tannery Wastewater

Characterization of metabolites from bacterially treated tannery wastewater

8.1. Introduction

Industrial wastewaters are considered as the major sources of environmental pollution and health hazards and thus, their treatment and detoxification are essential to safeguard the environment and public health. The wastewater released from leather industries (LIs) is of high strength and potentially toxic in nature as it has high pollution load such as COD, BOD, TDS, Cr, phenol, sulphate, nitrate, phosphate, etc., and a variety of POPs (Bharagava et al. 2018; Saxena et al. 2016) and Hence, its treatment and detoxification is required for sustainable development.

Microbes are the eco-friendly tools that are currently being used to degrade and detoxify the persistent organic chemicals and toxic metals present in the industries wastewaters (Saxena et al. 2016). There are several reported are available on the bioremediation of TWW by an array of microorganisms such as bacteria, fungi, yeast and algae (Kumari et al. 2016; Sharma and Adholeya 2011; Okoduwa et al. 2017; Ajayan et al. 2015). Microbes can degrade/detoxify highly complex organic pollutants into simpler forms and thus, formed metabolic products are usually non-toxic in nature and hazardous to the environment. However, revealing the nature and characteristics of metabolites formed during the biodegradation of complex organic pollutants accurately provide a clue for the efficient bioremediation processes. Therefore, the present study was to characterize the metabolites formed during the biodegradation of persistent organic pollutants present in the TWW by the newly developed bacterial consortium GS-TE1310. To do so, various analytical techniques such as HP-LC, FT-IR, and GC-MS to know the nature and characteristics of metabolites produced during the bacterial degradation of persistent organic pollutants present and confirm the detoxification of TWW.

8.2. Material and methods

8.2.1. Chemicals, reagents, and solvents used

All the required chemicals, reagents and solvents are used in the experiments were of highest purity (purity ≥ 99%) and analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Mineral salt medium (MSM g/L, Na_2HPO_4 2.4; K_2HPO_4 2.0; NH_4NO_3 0.1; $MgSO_4$ 0.01; $CaCl_2$ 0.01) was used for the bioremediation studies for

TWW. Whatman® Grade GF/C filter papers (pore size 1.2 µm) (Whatman, England, UK) were used for the filtration of TWW.

8.2.2. Bioremediation experiment for the degradation of persistent organic pollutants (POPs) in tannery wastewater by the newly developed bacterial consortium GS-TE1310 at the optimized conditions

For the bioremediation studies, real undiluted TWW amended with the optimized nutrient (C & N source) concentrations (0.5%, w/v) was inoculated with the optimized inoculum concentration (%, v/v) of the developed bacterial consortium GS-TE1310 in the Erlenmeyer flasks (250 ml). Further, the flasks were kept for incubation with parallel abiotic controls at optimized environmental (pH and temp. (°C)) and shaking (rpm) conditions in the temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA) for successive five days. During the experiment, bacterial growth was observed using spectrophotometer (Thermo Scientific™ Evolution 201, Australia) by taking the absorbance at λ_{max} = 620 nm. Thereafter, the bacterially treated TWW sample was taken out every 24 h, centrifuged at 10000 × g for 10 min in a refrigerated centrifuge (REMI Instruments Pvt. Ltd., Mumbai, MH, IN) and the supernatant was used for the COD measurements. The COD was measured using open reflux method (Method No. 5220B) and removal efficiency was calculated according to the following equation:

Removal Efficiency (%) = C_0-C_t/C_0 × 100 ……..… (13)

[Where C_0 = initial concentration of pollutant (mgL^{-1}) in TWW; C_t = pollutant concentration of pollutants (mgL^{-1}) in TWW after biotreatment]

8.2.3. Physico-chemical analysis of tannery wastewater after treatment with the newly developed bacterial consortium GS-TE1310

TWW samples after treatment with the newly developed bacterial consortium GS-TE1310 were analyzed in three replicates for various physico-chemical parameters to define the strength of pollution as per the standard protocols outlined in the "Standard Methods for Examination of Water and Wastewater" (APHA 2012). The Orion ion meter (Orion 096000 960 Titrator PLUS System, Thermo Fisher Scientific, USA), 5 day dilution methods, drying method, TOC-V_{csn} analyzer (Shimadzu, Japan), naphthylethylenediamine reagent method, 4-aminoantipyrene method, vanadomolybdo-phosphoric acid colourimetric method and $BaCl_2$ precipitation

method were used for the measurement of pH, BOD, total solids (TS), nitrate, total phenol, phosphate and sulphate in the bacterially treated TWW samples, respectively. The detailed methodology for the determination of various physico-chemical parameters is described in section 4.2.3 of chapter 04.

8.2.4. Analysis of heavy metals in tannery wastewater after treatment with the newly developed bacterial consortium GS-TE1310

The detection and quantification of various heavy metals (HMs) in the bacterially treated TWW samples were done by nitric-perchloric acid digestion method discussed in the "Standard Methods for Examination of Water and Wastewater" (APHA 2012). The methodology used for the quantification of different HMs is previously described in section 4.2.4 of chapter 04.

8.2.5. Characterization of metabolites in the bacterially treated TWW by HP-LC analysis

Before HP-LC analysis, TWW sample after treatment with the newly developed bacterial consortium GS-TE1310 was extracted by liquid-liquid extraction (LLE) method as per Bharagava et al. (2018). Briefly, the bacterially treated TWW sample (50 ml) was centrifuged (5000 × g, 10 min, 4 °C) to eliminate the microbial biomass and other suspended solids. The supernatant obtained was acidified (pH ≤ 2.0) with 1 N hydrochloric acid and extracted three times with the equal volume of dichloromethane (DCM, 50 ml, HPLC & GC grade, ≥ 99.9%) followed by ethyl acetate (50 ml, HPLC & GC grade, ≥ 99.9%) in a separating funnel (500 ml). The solvent layer containing POPs was collected in a beaker and the extracts were combined and evaporated in a Rotavapor (Rotavapor RE 120, Buchi, Flawil, Sweden) at ≤ 40 °C until the solvent is completely evaporated. The dried residues obtained were dissolved in DCM (3.0 ml), filtered through syringe filters (0.22 μm) (Millipore Ltd., Bedford, MA, USA) and the final extract obtained was used in the high-performance liquid chromatography (HP-LC) analysis.

The HP-LC analysis of POPs and their metabolic products in TWW sample after treatment with bacterial consortium GS-TE1310 was performed on 515 HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Diode Array Detector System (1100 series, Agilent Technologies, USA) and reverse phase C18 column (250 × 4.6 mm, 5 μm particle size) at 27 °C and 2487 Absorbance UV-Vis Detector

via Millennium® Software (v32). For this, the final extract (20 µl) was injected into the HPLC system and monitored at wavelength 224 nm (absorption maxima) to assess the degradation of POPs as well as to characterize their metabolic products produced during the bioremediation of TWW. The mobile phase consisted of Milli-Q® water (Millipore Corp., Billerica, MA, USA) and acetonitrile in the volume ratio of 70:30 (v/v) and the flow rate was set at the rate of 1.0 ml min^{-1}.

8.2.6. Characterization of metabolites in the bacterially treated TWW by FT-IR analysis

The presence of functional groups of the metabolic products produced during the biodegradation/biotransformation of POPs in real TWW after treatment with the newly developed bacterial consortium GS-TE1310 was determined by FT-IR analysis. For this, the bacterially treated TWW sample (10 ml) was filtered and oven dried at 105 °C, and obtained pellets were mixed with KBr to obtain the absorption spectra of the effluent sample. For this, the dried sample was mixed with KBr (FT-IR grade; purity ≥ 99%) in ratio of 1:30, and the mixture was finely ground and fused into a thin pellet (13 mm diameter and 1 mm thickness) prepared under vacuum condition using a PCI Hydraulic Press (Manual) with a capacity of 15 tons. Furthermore, the absorbance spectrum was recorded by a Nicolet FT-IR Spectrometer (Model Nicolet 6700, Thermo Fisher Scientific, MA, USA). The analysis was performed in the mid-infrared region from 4000-400 cm^{-1} with a resolution of 4 cm^{-1}. Scanning was performed to obtain the spectrum and measured in the ambient air against the pure KBr as a background spectrum. The data processing was performed using the software OMNIC™ (v7.4). The assignment of the absorption peaks observed in the FT-IR spectrum was done on the basis of those outlined in the "Introduction to Organic Spectroscopy" (Lambert 1987).

8.2.7. Characterization of metabolites in the bacterially treated TWW by GC-MS analysis

Before GC-MS analysis, TWW sample after treatment with the newly developed bacterial consortium GS-TE1310 was extracted by liquid-liquid extraction (LLE) method as per the methodology described in previous section 8.2.5. For GC-MS analysis, the obtained final extract after liquid-liquid extraction was firstly derivatized using trimethylsilyl [BSTFA (N, O-bis(trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane)] (Bharagava et al. 2018). Afterward, an aliquot (2.0 µl) of

derivatized sample was injected into a DB-5MS capillary column (30 m length × 0.18-mm internal diameter × 0.18 mm film thickness: 5% phenyl + 95% methylpolysiloxane, carrier gas: helium, flow rate: 1 ml min^{-1}) fitted with a Thermo Scientific Trace GC Ultra Gas Chromatograph equipped with a TriPlus autosampler coupled to a TSQ Quantum XLS triple quadrupole mass spectrometer (Thermo Fisher Scientific, FL, USA). At the start, the column temperature was 60 °C (hold time: 2 min) and increased up to 290 °C (hold time: 20 min) at the rate of 10 °C. The MS transfer line and ion source temperatures were kept at 200 °C and 250 °C, respectively. Full scan mode was used to operate MS whereas 3.0-min solvent delay was selected to record the mass spectra within a range of 30-550 (m/z units) at 70 eV (energy). The National Institute of Standards and Technology (NIST, USA) mass spectral library (v1.0.0.12) available with the instrument was used to identify the metabolic products (by comparing their mass spectra with that of their retention time (RT)) produced during the biodegradation/biotransformation of POPs by the newly developed bacterial consortium GS-TE1310.

8.2.8. Quality control and quality assurance

The reference stock standards of HMs (Merck KGaA, Darmstadt, Germany) were used for the calibration and quality assurance for each analytical batch. The analytical data quality of HMs (Cr, Cd, Cu, Zn, Ni, Pb, As, Fe, and Mn) was ensured by using EPA samples in water, and the results were found to be within the prediction intervals. The blanks were run in triplicate to check the precision of the method with each set of samples.

8.2.9. Statistical analysis

All the laboratory experiments were performed in triplicates (n = 3) to confirm the variability and validity of the results expressed as mean ± SD (standard deviation) values, calculated using IBM SPSS Statistics Software (v20.0.0, SPSS Inc., Chicago, IL, USA).

8.3. Results and discussion

TWW contains a mixture of highly toxic organic and inorganic pollutants and thus, its treatment and detoxification are necessary for environmental protection. COD is considered as one of the most important pollution parameters that actually define the strength of recalcitrant industrial effluents and has been widely used as water quality

criteria to measure the pollution profile of water and industrial effluents (Goutam et al. 2018; Sivaprakasam et al. 2008). Industrial effluents with high COD are mainly responsible for the disturbed ecological functioning of receiving water bodies (water pollution) and ultimately adversely affects aquatic life (Saxena et al. 2016). Hence, the removal of COD from TWW is a key requirement for the safety of the environment and public health.

In the present study, the bioremediation of TWW is mainly analyzed in terms of COD reduction according to equation 13. For this, batch studies (in shaking flasks) were performed for the bioremediation of TWW at the optimized conditions with the newly developed bacterial consortium GS-TE 1310 comprising three potential bacterial strains, *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, and *Stenotrophomonas acidaminiphila* GS10. Results revealed that the newly developed bacterial consortium GS-TE1310 was capable for the maximum COD removal up to 76.12% from real TWW within 120 h at the optimized conditions (0.5% glucose and NH_4Cl (w/v), 7 pH, 35 °C, 120 rpm and 20 ml inoculum volume) (Fig. 8.1) and thus, demonstrated tremendous potential to degrade the leather TWW (Fig. 8.2). This high COD removal was might be due to the degradation and detoxification of persistent organic pollutants and heavy metals present in the TWW. Further, a high rate of COD reduction is also considered as an indication of mineralization (Hassan and Hawkyard 2002).

Sivaprakasam et al. (2008) have developed a consortium of four salt-tolerant bacterial strains (*Pseudomonas aeruginosa, Bacillus flexus, Exiguobacterium homiense* and *Staphylococcus aureus*) isolated from marine and tannery saline wastewater for the removal of COD from TWW. According to them, salt-tolerant bacterial mixed consortia showed appreciable biodegradation of TWW with 80% COD reduction in particular at 8% salinity level and suggested that the consortia could be used as suitable working cultures for tannery saline wastewater treatment.

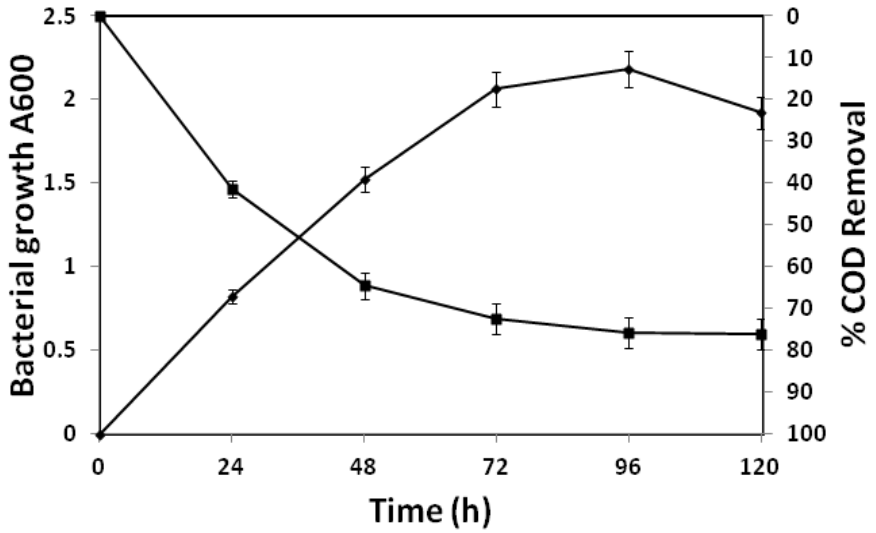


Fig. 8.1 COD removal from real TWW by newly developed bacterial consortium GS-TE1310. Error bars represents the standard deviation calculated from at least three independent experiments **(performed at the optimized conditions: 7 pH, 35 °C, 120 rpm, 20 ml inoculum volume, and 0.5%, w/v glucose and NH_4Cl)**

Fig. 8.2 Untreated (A) and treated TWW (B) by the newly developed bacterial consortium GS-TE1310

In addition, TWW samples after treatment with the newly developed bacterial consortium were used for the physico-chemical analysis to define the pollution profile. The physico-chemical characteristics of TWW before and after treatment with the newly developed bacterial consortium GS-TE1310 are listed in Table 8.1. The

analysis of untreated TWW revealed that it has high BOD (436 ± 4.58 mgL^{-1}), COD (1428 ± 5.56 mgL^{-1}), TSS (2216 ± 2.64 mgL^{-1}), TDS (4064 ± 3.46 mgL^{-1}), sulfate (6.75 ± 0.27 mgL^{-1}), phosphate (118.66 ± 5.03 mgL^{-1}), nitrate (14.05 ± 0.16 mgL^{-1}) and phenol (8.68 ± 0.04 mgL^{-1}) content with alkaline pH (8.2 ± 0.05), dark brown color and objectionable odour. Moreover, a high concentration of different HMs such as Cr (6.88 ± 0.02 mgL^{-1}), Cd (1.18 ± 0.03 mgL^{-1}), and Pb (0.38 ± 0.03 mgL^{-1}) was also detected in untreated TWW. It can be observed that all the values for various physico-chemical parameters recorded in the present study were high and beyond the permissible limits (Table 8.1).

Table 8.1 Physico-chemical characteristics of TWW before and after bacterial treatment

Physico-chemical parameter	Effluent discharge limits*	UT-Tannery effluent	BT-Tannery effluent	Pollutant removal efficiency (%)**
Color	-	Dark brown	Light brown	-
Odour	-	Objectionable	Unobjectionable	-
pH	6.0 - 9.0	8.2 ± 0.05	7.4 ± 0.11^{b}	-
Temperature (°C)	< 35	32 ± 0.57	31 ± 1.15^{ns}	-
BOD_5 (mg/L^{-1})	30.00	436 ± 4.58	64 ± 1.53^{a}	85.32
COD (mgL^{-1})	250.00	1428 ± 5.56	341 ± 2.64^{a}	76.12
TDS (mgL^{-1})	2100.00	4064 ± 3.46	1142 ± 2.64^{a}	71.89
TSS (mgL^{-1})	100.00	2216 ± 2.64	1155 ± 2.88^{a}	47.87
Phosphate (mgL^{-1})	5.0	118.66 ± 5.03	61 ± 2.61^{a}	48.59
Sulfate (mgL^{-1})	-	6.75 ± 0.27	1.43 ± 0.06^{a}	78.81
Nitrate (mgL^{-1})	10.0	14.05 ± 0.16	4.28 ± 0.03^{a}	69.53
Phenol (mgL^{-1})	1.0	8.68 ± 0.04	0.98 ± 0.14^{a}	88.70
Heavy metals (mgL^{-1})				
Cr	2.0	6.88 ± 0.02	1.98 ± 0.12^{a}	71.22
Cd	0.05	1.18 ± 0.03	0.30 ± 0.06^{a}	74.57
Cu	3.0	1.72 ± 0.05	0.25 ± 0.02^{a}	85.46
Zn	5.0	0.96 ± 0.03	ND	-
Ni	3.0	0.68 ± 0.02	0.18 ± 0.02^{a}	73.52
Pb	0.1	0.38 ± 0.03	ND	-
As	0.2	BDL	ND	-
Fe	3.0	2.86 ± 0.30	0.52 ± 0.05^{a}	81.81
Mn	2.0	0.72 ± 0.04	ND	-

EC: Electrical conductivity; BOD: Biochemical oxygen demand; COD: Chemical oxygen demand; TS: total solids; TDS: total dissolved solids; TSS: Total suspended

solids; BDL: Below detection limit; ND: Not detected; UT-TWW: Untreated tannery wastewater; BT-TWW: Bacterially (consortial) treated tannery wastewater.
All the values are mean of three replicates ± SD.
Data were analyzed by Student's t-test [two tailed as compared to untreated sample]
[a]Highly significant at $p < 0.001$
[b]Less significant at $p < 0.05$
[ns]Non significant at $p > 0.05$
*As per Central Pollution Control Board (2010); Ministry of Environment, Forest & Climate Change (2016), India.
**Pollutant removal efficiencies (%) were calculated according to equation 13.

The physico-chemical characteristics of TWW treated with the newly developed bacterial consortium GS-TE1310 showed an appreciable reduction in all the pollution parameters (Table 8.1). After bacterial treatment, the color of TWW has turned from dark brown to light brown and this was might be due to the degradation and decolorization of the azo dyes present in the untreated TWW sample. The pH of TWW was also significantly reduced from 8.2 ± 0.05 to 6.2 ± 0.11. The treatment of TWW with the newly developed bacterial consortium GS-TE1310 resulted in the significant removal of BOD, TDS, TSS, phosphate, sulphate, nitrate, and phenol by 85.324%, 71.89%, 47.87%, 48.59%, 78.81%, 69.53%, 88.70%, respectively after 120 h at 35 °C, 5 pH, and 120 shaking speed. The removal of BOD, TDS, TSS, phosphate, sulphate, nitrate, and phenol from TWW was might be due to the degradation/transformation of persistent organic pollutants and utilization of dissolved minerals and salts to meet the nutritional requirements by the newly bacterial consortium GS-TE1310. Moreover, the HMs like Cr, Cd, Cu, Ni, and Fe were significantly removed by 71.22%, 74.57%, 85.46%, 73.52%, and 81.81% from TWW and their concentration was found within permissible limits (Table 8.1). In addition, other HMs like Pb, As, Zn, and Mn were not detected in the TWW after bacterial treatment. The reduction/removal in the concentration of HMs was might be due to either through metal bioaccumulation inside the bacterial cells or binding with lipopolysaccharides (LPS) of extra cellular membrane (Chandra et al. 2011). Overall, the developed bacterial consortium GS-TE1310 successfully reduced all the pollution parameters in TWW during its bioremediation. Kumari et al. (2016) have also reported the reduction in pollution parameters such as COD (65%), BOD (80%), TDS (67%), EC (65%) and chromium (92%) after 48 h by *Bacillus cereus* isolated from TWW.

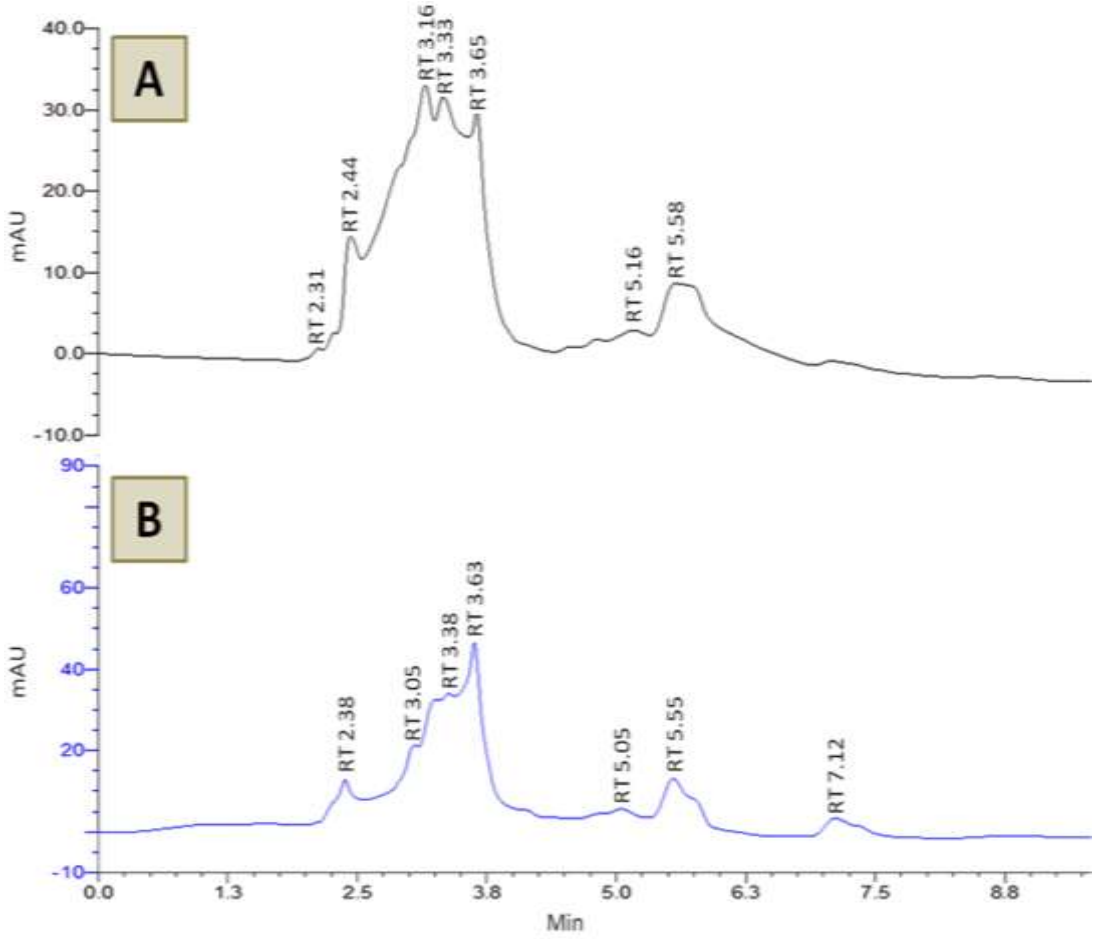


Fig. 8.3 HP-LC chromatogram of untreated TWW (A) and treated TWW (B) by the newly developed consortium GS-TE1310

Further, HP-LC, FT-IR, and GC-MS techniques were used for the characterization of recalcitrant organic pollutants (POPs) and metabolic products formed during the bioremediation of real leather TWW by the newly developed bacterial consortium GS-TE1310. According to HP-LC analysis, the untreated TWW contained a mixture of POPs as revealed by the several peaks obtained at different retention time (RT: 2.31, 2.44, 3.16, 3.33, 3.65, 5.16, and 5.58). However, the reduction in peak area (reduction in pollutants concentration) has clearly indicated the biodegradation/biotransformation of POPs and formation of new metabolic products as confirmed by some additional peaks (at different RT: 2.38, 3.05, 3.38, 3.63, 5.05, 5.55, and 7.12) obtained in the bacterially treated TWW, which were further confirmed by the FT-IR and GC-MS analysis (Fig. 8.3A, B).

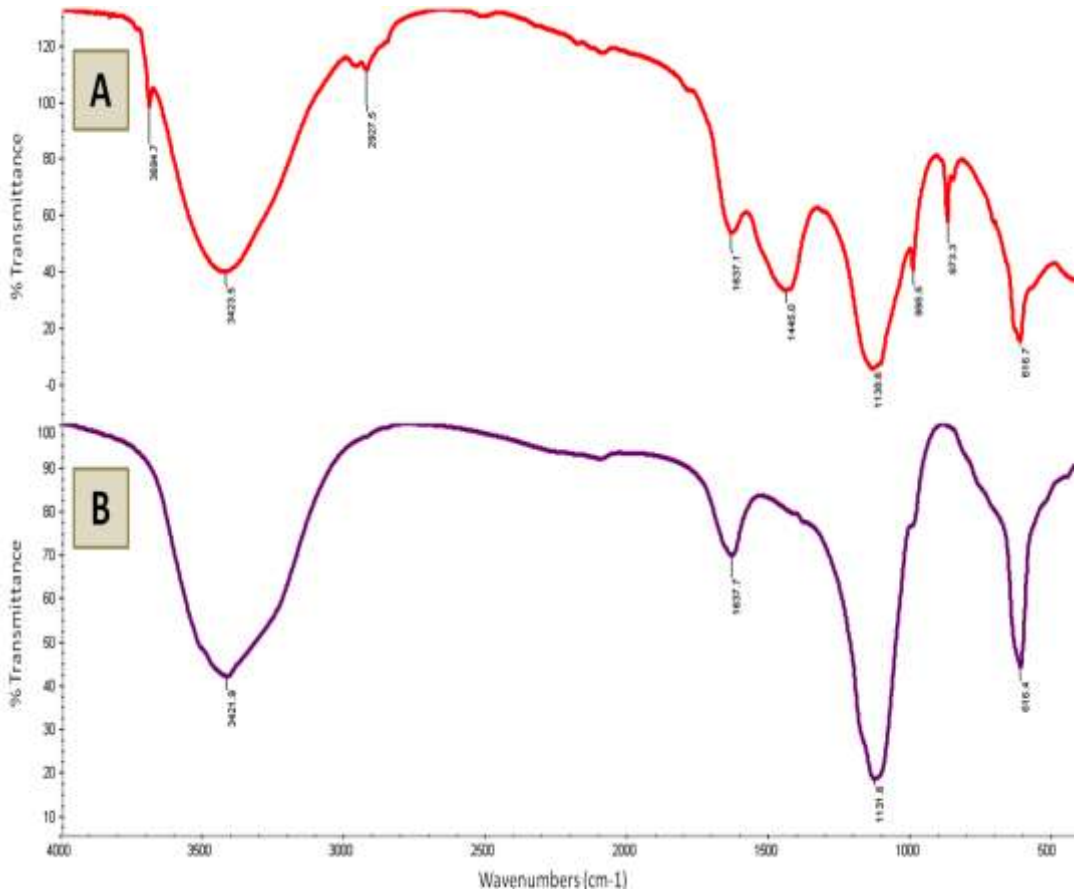


Fig. 8.4 FT-IR spectrum of untreated TWW (A) and treated TWW (B) by the newly developed consortium GS-TE1310

The FT-IR spectrum of untreated TWW showed several peaks which correspond to toxic functional groups in POPs present in the sample. The absorption peak at 3694.7-3423.5 cm^{-1} indicated the –OH group (O–H stretching) with strong intensity corresponded to the existence of alcohol and phenol derivatives, the absorption peak at 2927.5 cm^{-1} is attributed to C–H stretching corresponded to the presence of long chain aliphatic compounds, such as fatty acids, surfactants, and diazo compounds, might be raised from azo dyes used in leather coloring, and absorption peak 1637.1 cm^{-1} indicated the N–H bending of amines and amides. An absorption peak at 1445.0 cm^{-1} indicated CH_3 bending, whereas an absorption peak at 1138.6 cm^{-1} indicated the C–N stretching of aliphatic amines. The absorption peak at 995.5 cm^{-1} attributed to P–O-alkyl organophosphorus compounds, and the absorption at 873.3 cm^{-1} corresponds to the presence of 1,2,4-trisubstituted benzene. The absorption at 616.7 cm^{-1} indicated the O–C=O bending of carboxylic acids. Overall, the absorption spectrum of untreated TWW showed the presence of some of the alcohols, phenols, amines, and aromatic skeleton. However, the disintegration of major peaks corresponding to toxic

functional groups and the appearance of new peaks suggests the biodegradation/biotransformation of the POPs present in the bacterially treated TWW (Fig. 8.4A, B).

Further, it is impossible to predict the metabolism of a particular compound from wastewater having "*n*" number of POPs. The GC-MS analysis of untreated TWW showed the presence of a variety of POPs at different RT. However, most of the POPs detected in the untreated TWW were completely mineralized/degraded into new metabolic products in the treated TWW by the newly developed bacterial consortium GS-TE1310 at the optimized conditions (7 pH, 35°C temperature, 0.5% glucose and ammonium chloride (w/v), 120 rpm (agitation rate), and 20 ml inoculum volume)) (Fig. 8.5A, B, Table 8.2). The disappearance of most of the POPs from untreated TWW revealed that the newly developed bacterial consortium GS-TE1310 utilized these organic chemicals as a source of nutrients and energy and thus, played a crucial role in the degradation/detoxification of leather TWW for environmental safety.

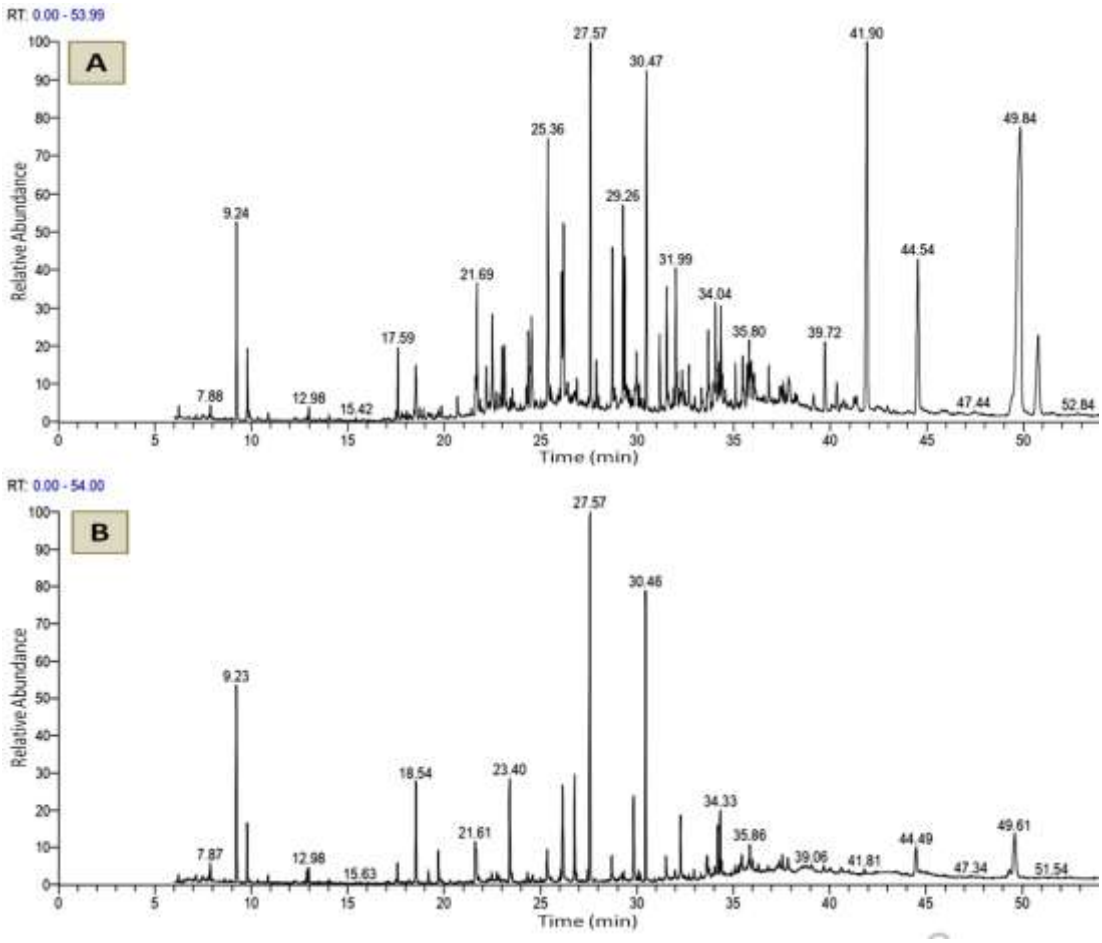


Fig. 8.5 GC-MS chromatogram of untreated TWW (A) and treated TWW (B) by the newly developed consortium GS-TE1310

Table 8.2 Persistent organic pollutants (POPs) and their metabolic products identified as TMS (trimethylsilyl) derivatives by GC-MS analysis in untreated and treated TWW by the newly developed bacterial consortium GS-TE1310

Sr. No.	RT	Compounds identified	Tannery wastewater	
			UT-TE	BT-TE
1.	7.88	Heptanoic acid, 7-phenoxy-, trimethyl ester ($C_{16}H_{26}O_3Si$)	+	-
2.	9.24	Benz[a]anthracene,7,12-dimethyl- ($C_{20}H_{16}$)	+	-
3.	12.98	Dodecane, 4,6-dimethyl- ($C_{14}H_{30}$)	+	+
4.	15.42	Benzoic acid, trimethylsilyl ester ($C_{16}H_{30}O_4Si_3$)	+	-
5.	17.59	Octadecane ($C_{18}H_{38}$, MW 254)	+	-
6.	21.69	p-Trimethylsiloxynitrobenzene ($C_9H_{13}NO_3Si$)	+	-
7.	25.36	1,2,Benzenedicarboxylic acid, ethyl(trimethylsilyl)ester ($C_{13}H_{18}O_4Si$)	+	-
8.	27.57	Hexadecanoic acid, trimethylsilyl ester ($C_{19}H_{40}O_2Si$)	+	+
9.	29.26	Trans-9-Octadecanoic acid, trimethylsilyl ester ($C_{21}H_{42}O_2Si$)	+	-
10.	30.47	Benzyl butyl phthalate, benzyl butyl ester ($C_{19}H_{20}O_4$)	+	-
11.	31.99	3-Chloropropionic acid, heptadecyl ester ($C_3H_5ClO_2$)	+	-
12.	34.04	17-Pentatriacontene ($C_{35}H_{70}$)	+	-
13.	35.04	Phthalic acid, 1-phenylpropyl butyl ester ($C_{21}H_{24}O_4$)	+	-
14.	35.80	Di-benzyl phthalate, dibenzyl ester ($C_{22}H_{18}O_4$)	+	-
15.	39.72	4-(2,4-dimethylheptan-3-yl)phenol ($C_{15}H_{24}O$)	+	-
16.	41.90	Tetradecanoic acid, trimethylsilyl ester ($C_{17}H_{36}O_2Si$)	+	-
17.	44.54	Phthalic acid, benzyl isobutyl ester ($C_{19}H_{20}O_4$)	+	-
18.	47.44	Stearic acid, 3-(octadecyloxy)propyl ester ($C_{39}H_{78}O_3$)	+	-
19.	49.84	Dodecanoic acid, trimethyl ester ($C_{15}H_{32}O_2Si$)	+	-
20.	52.84	Unknown compound	+	-
21.	7.87	D-(-)-Lactic acid, trimethylsilyl ($C_9H_{22}O_3Si_2$)	-	+
22.	9.23	Pentanoic acid ($C_5H_{10}O_2$)	-	+
23.	15.63	Unknown compound	-	+
24.	18.54	Unknown compound	-	+
25.	21.61	2-Methyl-1,2-propanediol 2TMS ($C_{10}H_{26}O_2Si_2$)	-	+
26.	23.40	1-Octadecanol ($C_{18}H_{38}O$)	-	+
27.	30.46	Octadecanoic acid, trimethylsilyl ester ($C_{21}H_{44}O_2Si$)	-	+
28.	34.33	Phosphoric acid, tris(trimethylsilyl) ester ($C_9H_{27}O_4PSi_3$)	-	+
29.	35.86	Butanoic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester ($C_{10}H_{24}O_3Si_2$)	-	+
30.	39.06	Benzyl alcohol-MONOTMS ($C_{10}H_{16}OSi$)	-	+
31.	41.81	Acetic acid, trimethyl silyl ester ($C_8H_{20}O_3Si_2$)	-	+
32.	44.49	Propanoic acid, 2-methyl-2[(trimethylsilyl)oxy]-, trimethylsilyl ester ($C_{10}H_{24}O_3Si_2$)	-	+
33.	47.34	Tris(trimethylsilyl)borate ($C_9H_{27}BO_3Si_3$)	-	+
34.	49.61	1,3-Heptanol, trimethylsilyl ($C_{10}H_{24}OSi$)	-	+
35.	51.54	2-Butene-1,4-diol, bis(trimethylsilyl) ($C_{10}H_{24}O_2Si_2$)	-	+

Key: +: present; -: absent; TMS: trimethyl silyl; RT: retention time; UT: untreated; BT: bacterially treated; TE: tannery effluent

8.4. Conclusion

In the present study, an effective degradation of real TWW was attained by a newly developed bacterial consortium GS-TE1310 within 120 h (at 7 pH, 120 rpm, and 35°C) with 76.12, 85.32, 71.89, 48.59, 78.81, 69.53, 71.22, and 88.70% reduction in pollution parameters such as COD (1428 ± 5.56 mgL^{-1}), BOD (436 ± 4.58 mgL^{-1}), TDS (4064 ± 3.46 mgL^{-1}), phosphate (118.66 ± 5.03 mgL^{-1}), sulphate (6.75 ± 0.27 mgL^{-1}), nitrate (14.05 ± 0.16 mgL^{-1}), Cr (6.88 ± 0.02 mgL^{-1}), and phenol (8.68 ± 0.04 mgL^{-1}), respectively. The HP-LC, FT-IR and GC-MS analysis showed that most of the organic pollutants detected in the untreated TWW were completely mineralized/degraded into new metabolic products in the treated TWW by the newly developed bacterial consortium GS-TE1310 at the optimized conditions (7 pH, 35°C temperature, 0.5% glucose and ammonium chloride (w/v), 120 rpm (agitation rate), and 20 ml inoculum volume)). Thus, the newly developed bacterial consortium GS-TE1310 showed astounding potential for treatment/detoxification of TWW.

9

Chapter-09

Detection and Characterization of Catabolic Gene/Enzyme Responsible for The Degradation of Persistent Organic Pollutants (POPs)

Detection and characterization of catabolic gene/enzyme responsible for the degradation of persistent organic pollutants (POPs)

9.1. Introduction

Environmental pollution is of serious global concern and the public outcry against it continually rising to ensure the safest and healthiest environment for living beings. Industries are the major players in the national economy of every country; however, these are also the major cause of serious environmental pollution. Industries use a variety of highly toxic chemicals in various operations to produce the good quality of products in short a time and after processing, discharge these chemicals along with complex wastewaters responsible for toxicity in living beings. These highly toxic complex wastewaters are the responsible cause for serious soil and water pollution and severe toxic effects in living beings (Bharagava et al. 2018; Goutam et al. 2018), and hence, their adequate treatment is necessary for environmental protection and public health safety.

Currently, physicochemical approaches are being applied to treat and manage hazardous wastewaters. These approaches are environmentally destructive, cost high money to apply, and may cause secondary pollution by generating contaminated sludge as a result of the remediation process (Saxena et al. 2016). Hence, these methods are not compatible with our natural environment and thus, lead to the need for some eco-friendly approaches. Bioremediation is an environmentally friendly approach that can be a suitable alternative to effectively treat and manage the highly toxic complex industrial wastewater and its chemical pollutants. Bioremediation is the use of microbes and plants or their enzymes to degrade and detoxify the organic and inorganic pollutants present in the industrial wastewaters (Paisio et al. 2012; Singh et al. 2013). But, currently, the use of microbial enzymes in the bioremediation of environmental pollutants is an emerging area of research.

Microbial enzymes have diverse industrial applications and have diverse roles in various industrial applications. Microbial enzymes are also useful in bioremediation of environmental pollutants from industrial wastes due to their high specificity to a broad range of substrates (pollutants), use under extreme conditions that microbe cannot thrive, high effectiveness at low pollutant concentration, high activity in the presence of inhibitors of microbial metabolism, and high mobility (small size) than

microorganisms. A variety of enzymes are produced by microorganisms that can be used in the degradation and detoxification of a wide range of organic and inorganic pollutants (Singh et al. 2013; Paisio et al. 2012). Hence, the use of enzyme can be an effective approach for the degradation and detoxification of pollutants in the industrial wastewaters. Therefore, the present work was to detect and characterize the catabolic enzyme responsible for the degradation of persistent organic pollutants present in the TWW.

9.2. Materials and methods

9.2.1. Chemicals and reagents

All the required chemicals, reagents and solvents were used in the experiments are of highest purity (purity ≥ 99%) and analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Mineral salt medium (MSM g/L, Na_2HPO_4 2.4; K_2HPO_4 2.0; NH_4NO_3 0.1; $MgSO_4$ 0.01; $CaCl_2$ 0.01) was used for the bioremediation studies for TWW. Whatman® Grade GF/C filter papers (pore size 1.2 μm) (Whatman, England, UK) were used for the filtration of TWW.

9.2.2. Preparation of cell-free extract

For the expression of enzyme and preparation of cell-free extract, 10 ml of the pre-cultures of selected bacterial strains (i.e. GS1, GS3 and GS10, cultured in MSM broth (50 ml, pH 7.0, supplemented with glucose, 0.5%, w/v)) were inoculated in real TWW (undiluted, 80 ml, pH 7.0) in the Erlenmeyer flasks (250 ml) which were incubated (at 35 °C) under shaking condition (120 rpm) in an temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA). The bacterially treated TWW sample was taken out every 24 h for successive five days of treatment. During the experiment, the bacterially treated TWW sample was taken out every 24 h, centrifuged (6,000 × g for 10 min), and the cell pellet was suspended in Tris-HCl buffer (pH 9), sonicated (Sonics-Vibracell ultrasonic processor, USA), keeping sonifier output at 40 (amps) and giving 7 strokes each of 30 sec, with 1 min interval at 4 °C. The homogenate was centrifuged (6,000 × g for 10 min) to obtain a supernatant which was considered as crude enzyme extract and used for the determination of enzyme activity.

9.2.3. Enzyme assay

The bacterially treated TWW samples were used for the determination of catechol 1,2- and 2,3-dioxygenase activities because of the presence of aromatic compounds and phenol in the untreated TWW as confirmed by GC-MS and physico-chemical analysis (Table 4.1, 5.2, 5.3, 5.4). Catechol 1,2- and 2,3-dioxygenase activities were determined as previously described (Pradhan and Ingle 2007). Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities were determined spectrophotometrically by measuring their reaction products cis,cis-muconic acid at 260 nm and 2-hydroxymuconic semialdehyde at 375 nm, respectively. The reaction mixture consisted of potassium phosphate buffer (0.1 M, pH 7.5), 55 µl of the crude extract, and 7.5 µl of 10 mM catechol as substrate. One unit (U) of enzyme activity was defined as the amount which catalyzed the formation of 1 µmol of product/min at 25 °C. Specific activity was calculated as units per milligram of protein.

9.2.4. SDS preparation

Denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on Polyacrylamide Gel Electrophoresis unit (GX-SCZ2, Genetix Biotech Asia Pvt. Ltd) by using 10% polyacrylamide in the gel. The samples were loaded in duplicates and the concentration of enzyme extract was calculated by comparing with the standard curve at 260 nm absorbance. The composition of separating gel and stacking gel has been discussed below:

Materials

- Casting gel unit for electrophoresis
- Siliconized Pasteur pipettes
- Syringes equipped with blunt, stub nosed, needles
- Vacuum chamber for degassing gels
- Micropipettes (10-300 µl)
- Stock 30 % T: 0.8 % C Acrylamide monomer
- 1.5 M Tris-HCl buffer, pH 8.8
- 10 % (w/v) SDS
- 10 % (w/v) Ammonium persulfate

Separation gel

- 10 ml of Acrylamide monomer
- 2.6 ml of Tris-HCl Buffer, pH 8.8
- 0.1 ml of 10 % (w/v) SDS
- 3.8 ml of H_2O

Stacking gel

- 67 ml of Acrylamide monomer
- 1.25 ml of Tris buffer, pH 8.8
- 0.05 ml of 10 % (w/v) SDS
- 2.975 ml of H_2O

Procedure

1. Assemble the slab gel unit with the glass sandwich set in the casting mode with 1.5 mm space.
2. Prepare a separating gel in a separate small beaker.
3. Add separating gel to a side arm flask, stopper the flask and attach to a vacuum pump equipped with a cold trap. Turn on the vacuum and degas the solution for ~ 10 min. During this period, gently swirl the solutions in the flask.
4. Exit the vacuum, open the flask and add 100 µl of ammonium persulfate and 10 µl of TEMED to the solution.
5. Add stopper to the flask and degas for additional 2 min with gentle swirl to mix the solutions.
6. Transfer appropriate amount of degassed solution to casting chamber without any air bubble formation.

7. Immediately fill in water to the top of separating gel for preventing the formation of the meniscus.
8. Let it settle for 20-30 min to gelate.
9. Prepare a stacking gel as separating gel preparation method and add 0.05 mL of ammonium persulfate and 0.005 mL of TEMED.
10. Pour the stacking gel onto the separating gel.
11. Insert the well-forming comb without trapping air and wait for 20-30 min to let it gelate.
12. Take out the comb after complete gelation.
13. The prepared samples were mixed with sample buffer and were heated in the boiling water for 5-10 min.
14. Now load the samples into wells and protein markers into the first lane. Now cover the top and connect the anodes.
15. Set an appropriate volt and run the electrophoresis.
16. After completion of total running time, stop SDS-PAGE running when the downmost sign of protein marker reaches the foot line of the glass plate.

On completion of Electrophoreses, the protein bands on the gel were stained with Coomassie brilliant blue R-250 dye and destained with a destaining solution and left for overnight. The molecular weight was estimated by comparing with standard protein ladder.

9.2.5. Statistical analysis

All the laboratory experiments were performed in triplicates (n = 3) to confirm the variability and validity of the results expressed as mean ± SD.

9.3. Results and discussion

It is well known that phenol can be metabolized by two major pathways: *ortho* and *meta*-cleavage. Phenol hydroxylase is the first enzyme in the metabolic pathway of

phenol degradation and catalyzes phenol oxidation to form catechol. In the next step, two enzymes can be induced, catechol 1,2- or 2,3-dioxygenase, which belong to the *ortho* and *meta* ring fission pathways, respectively (Dagley 1971; Harayama and Renik 1989). Thus, to distinguish between ortho and meta pathways of aromatic ring cleavage, catechol 1,2-and 2,3-dioxygenase activities were measured. A catechol 1,2-dioxygenase activity was detected during the bacterial treatment of TWW (Fig. 9.1). This indicated that the catechol ring fission was performed through the ortho pathway. However, the catechol 2,3-dioxygenase activity was not detected in the crude extract of the selected bacterial cells.

The enzyme secreted by bacterial strains GS1, GS3, and GS10 was measured from the culture supernatant at different time intervals. The maximum catechol 1,2-dioxygenase enzyme activity was observed for *Stenotrophomonas acidaminiphila* GS10 is 0.32 IU/ml, followed by *Ochrobactrum intermedium* GS1 (0.26 IU/ml) whereas the least activity was recorded for *Micrococcus lylae* GS3 i.e. 0.21 IU/ml after 48 h (Fig. 9.2). On the other hand, no catechol 1,2- dioxygenase enzyme activity was observed in control flasks.

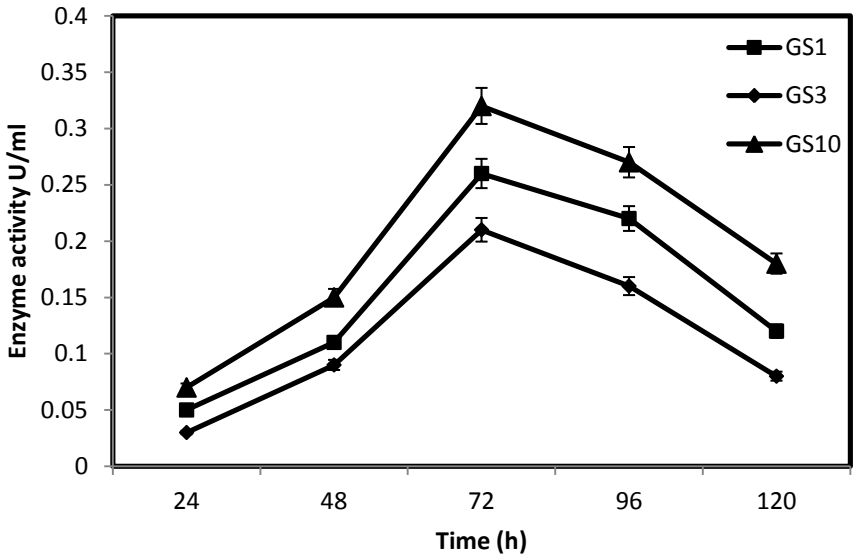


Fig. 9.1 Catechol 1,2- dioxygenase enzyme activity produced by bacterial strain GS1, GS3 and GS10 during bacterial treatment of TWW.

TWW has been reported to contain a variety of aromatic compounds and phenols that might induce the production of catechol 1,2- dioxygenase enzyme during their degradation by the selected bacterial strains, *Stenotrophomonas acidaminiphila* GS10, *Ochrobactrum intermedium* GS1 and *Micrococcus lylae* GS3. This study was strongly

supported by the results reported by Paisio et al. (2012). They reported the induction of catechol 1,2- dioxygenase enzyme during the degradation of phenols present in the TWW by a *Rhodococcus* sp. CS1, isolated from tannery sediments.

Further, the SDS-PAGE analysis also revealed that bacterial strains, *Stenotrophomonas acidaminiphila* GS10, *Ochrobactrum intermedium* GS1 and *Micrococcus lylae* GS3 possess catechol 1,2- dioxygenase enzyme. The protein separated by SDS-PAGE gel was treated for enzyme renaturation and showed that all the selected bacterial strains produced a protein band that had a molecular weight of ~32 kDa (Fig. 9.2). A similar result is reported by Singh et al. (2013). They reported the degradation of pyrene by bacterial strain, *Pseudomonas* BP10 that produced catechol 1,2- dioxygenase enzyme and its molecular weight was exactly estimated to be ~32 kDa.

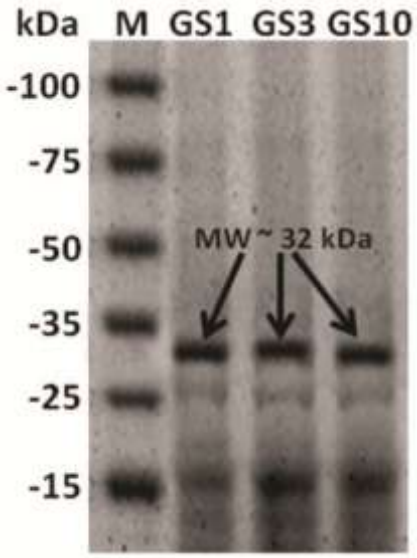


Fig. 9.2 SDS page analysis of crude enzyme produced by bacterial strains, *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, & *Stenotrophomonas acidaminiphila* GS10.

9.4. Conclusion

The present study was to detect and characterize the catabolic enzyme responsible for the degradation of persistent organic pollutants like phenols and other aromatic compounds. Results revealed that the selected bacterial strains, *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, & *Stenotrophomonas acidaminiphila* GS10 exhibited catechol 1,2- dioxygenase enzyme as confirmed by the quantitative

analysis. Further, the SDS-PAGE analysis showed the molecular weight of protein around ~32 kDa in the crude extract of all the selected bacterial strains. Overall, these selected bacterial strains were able to degrade the persistent organic pollutants present in TWW and the developed consortium could be an appropriate option for the bioremediation of tannery effluents or other phenol-containing wastewaters.

10

Chapter-10

Toxicological Assessment of Tannery Wastewater Before and after Bacterial Treatment Process

Toxicological assessment of tannery wastewater before and after bacterial treatment process

10.1. Introduction

TWW (TWW) released from LIs invariably have a dark brown color and offensive smell with high pollution parameters such as chemical oxygen demand (COD), biochemical oxygen demand (BOD), total dissolved solids (TDS), phosphate, nitrate, sulphate, phenol and a blend of potentially toxic organic and inorganic contaminants (Bharagava et al. 2018). During leather processing, a huge quantity of noxious chemical compounds such as chromium (Cr), vegetable tannins, syntans, phenolics, azo dyes, pesticides, sulphonated oils, polychlorinated biphenyls (PCBs), phthalates and a variety of persistent organic pollutants are currently applied to transform the raw hide/skins into leather/leather products (Bharagava et al. 2018; Saxena et al. 2016; Dixit et al. 2015). These noxious chemical compounds are not fully uptaken by the hide/skins and thus, discharged in the TWW, which causes serious environmental threats and severe toxic hazards.

The discharge of untreated/partially treated TWW causes serious soil and water pollution. In an aquatic ecosystem, TWW blocks sunlight penetration due to its dark brown color and reduces photosynthetic activity and oxygenation (depletion in dissolved oxygen) of in the water bodies and therefore, adversely affects flora and fauna (Saxena et al. 2016). In developing countries, TWW is also being used as a liquid fertilizer by the local farmers to irrigate their food crops in the agricultural land (soil pollution). This uncontrolled and illegal practice paves a way for the bioaccumulation of toxic heavy metals like Cr at sequentially higher trophic levels in the food chain *via* consumption by human/animals and thus, resulting in the severe health threats (Goutam et al. 2018). The toxic hazards of TWW in both the aquatic and terrestrial ecosystem have been well documented (Lunardelli et al. 2018; de Souza et al. 2017; Chandra et al. 2009). Thus, it becomes essential to assess the toxicity of TWW to safeguard the environment and public health.

Therefore, the present study was focused on the phytotoxicity assessment of TWW before and after treatment with the newly developed bacterial consortium GS-TE1310 to evaluate its environmental safety. The mung bean (*Phaseolus aureus* L.) was used as a terrestrial model for the phytotoxicity assessment to confirm the

reduction in toxicity in the TWW treated with the newly developed bacterial consortium GS-TE1310.

10.2. Materials and methods

10.2.1. Chemicals and materials

All the required chemicals and reagents were used in the experiments are of highest purity (purity ≥ 99%) and analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Whatman® Grade GF/C filter papers (pore size 1.2 μm) (Whatman, England, UK) were used to filter untreated TWW. The seeds of *Phaseolus aureus* L. were purchased from the local market of Lucknow (UP, IN) and used to study the phytotoxic effects of TWW.

10.2.2. Phytotoxicity assessment of TWW before and after bacterial treatment

The use of higher plants for the ecotoxicological assessment of complex industrial wastewaters is an attractive idea to monitor the ecotoxicological risks and to evaluate their environmental safety. To accomplish this, the recommended phytotoxicity assay is used for the toxicity evaluation of industrial wastewaters/chemicals (OECD 2003). In the present study, mung bean (*Phaseolus aureus* L.) was used as a terrestrial model for the phytotoxicity assessment of TWW before and after treatment with the newly developed bacterial consortium GS-TE1310 for environmental safety. Mung beans were used as per the "OECD Guideline for the Testing of Chemicals" (http://www.oecd.org/chemicalsafety/testing/33653757.pdf) because of high sensitivity to toxic chemicals/hazardous wastewaters and compatibility with the environmental growth conditions within a specific time frame and also as per the recommendation of previous authors (Kaur et al. 2012; Chandra et al. 2011).

Before phytotoxicity evaluation, the bacterially treated TWW was firstly centrifuged at 5000 × g for 20 min to remove bacterial biomass and left it overnight to settle down the remaining suspended particles. If growth occurs, the wastewater was further centrifuged again to remove the bacterial biomass again, stored in a screw-capped glass bottle and used in seed germination experiment.

For the phytotoxicity assessment, seed germination experiment was performed in triplicate using ten (10) uniform size seeds of *Phaseolus aureus* L., which was surface-sterilized with 2.0% $HgCl_2$ solution for 2 min to remove any fungal contaminants followed by three times washing with deionized water. All seeds were

kept on the sterilized Whatman 52 filter papers (Whatman, England, UK) in every sterilized glass Petri dishes and moistened with 10 ml of tap water (control) and with the same volume (i.e., 10 ml) but different concentrations, i.e., 25, 50, 75 and 100% (v/v) of filtered untreated and treated TWW just to check the concentration-dependent inhibitory effect on seed germination and seedling growth parameters. After that, all the Petri dishes were kept at the room temperature for six successive days to document the physiological parameters of seed germination and seedling growth.

The criterion of germination that has been taken was the visible protrusion of radical from the seed coat and it was expressed in percentage. The germinated seeds were counted to the initial appearance of the radical by continuous visual observation for 6 days. The physiological parameters of seed germination and seedling growth were calculated according to Bharagava et al. (2018).

10.2.3. Preparation of enzyme extract and assay for α-amylase activity in seeds

Furthermore, to better elaborate the phytotoxicity of untreated and bacterially treated TWW, the α-amylase assay was performed to check its deleterious effect on α-amylase enzyme activity in the treated seeds of *Phaseolus aureus* L., according to Bharagava and Chandra (2010).

Briefly, the seeds irrigated from each treatment were homogenized with 0.1 M sodium acetate buffer (pH 4.8), filtered through two layers of cheese-cloth to remove large particles and the supernatant obtained was centrifuged at 15,000 × g for 20 min. All the preparations were carried out at 4 °C. The supernatant obtained was used as a crude enzyme extract for α-amylase assay. For enzyme assay, the reaction medium (3 ml) contained 1 ml of 0.1 M acetate buffer, pH 4.8, 0.5 ml of enzyme extract diluted to 1ml using acetate buffer, and 1 ml of 0.1% soluble starch solution. The enzyme extract was diluted to obtain an absorbance change of less than one during the enzyme assay. The reaction medium was incubated for 10 min at room temperature and then the reaction was stopped by adding 1ml of 0.1% iodine reagent and 3ml of 0.05 N HCl. The absorbance was measured at 620 nm and the decrease in absorbance was expressed in terms of amylase activity (Beri and Gupta 2007).

10.2.4. Statistical analysis

All the laboratory experiments were performed in triplicates (n = 3) to confirm the variability and validity of the results expressed as mean ± SD (standard deviation) values.

10.3. Result and discussion

The Ganga River and Unnao area near Kanpur (UP), India are heavily industrialized, with the significant discharge of TWW from LTs to the receiving environment, which causes the harmful impacts on the nearby flora and fauna; hence this study is of particular importance. In the present study, seed germination experiments were performed to explore the toxic effects of TWW on plant growth and development (Fig. 10.1). Results revealed that the untreated TWW was proved to be highly toxic in nature as it showed inhibitory effect on seed germination and seedling growth parameters of *Phaseolus aureus* L, which were significantly improved when seeds were irrigated with TWW treated with the developed bacterial consortium GS-TE1310 (Table 10.1, Fig. 10.2A, B). This was might be due to the bacterial degradation and detoxification of highly toxic organic and inorganic pollutants present in TWW as confirmed by physico-chemical characterization (Table 2) and FT-IR, HP-LC and GC-MS analysis.

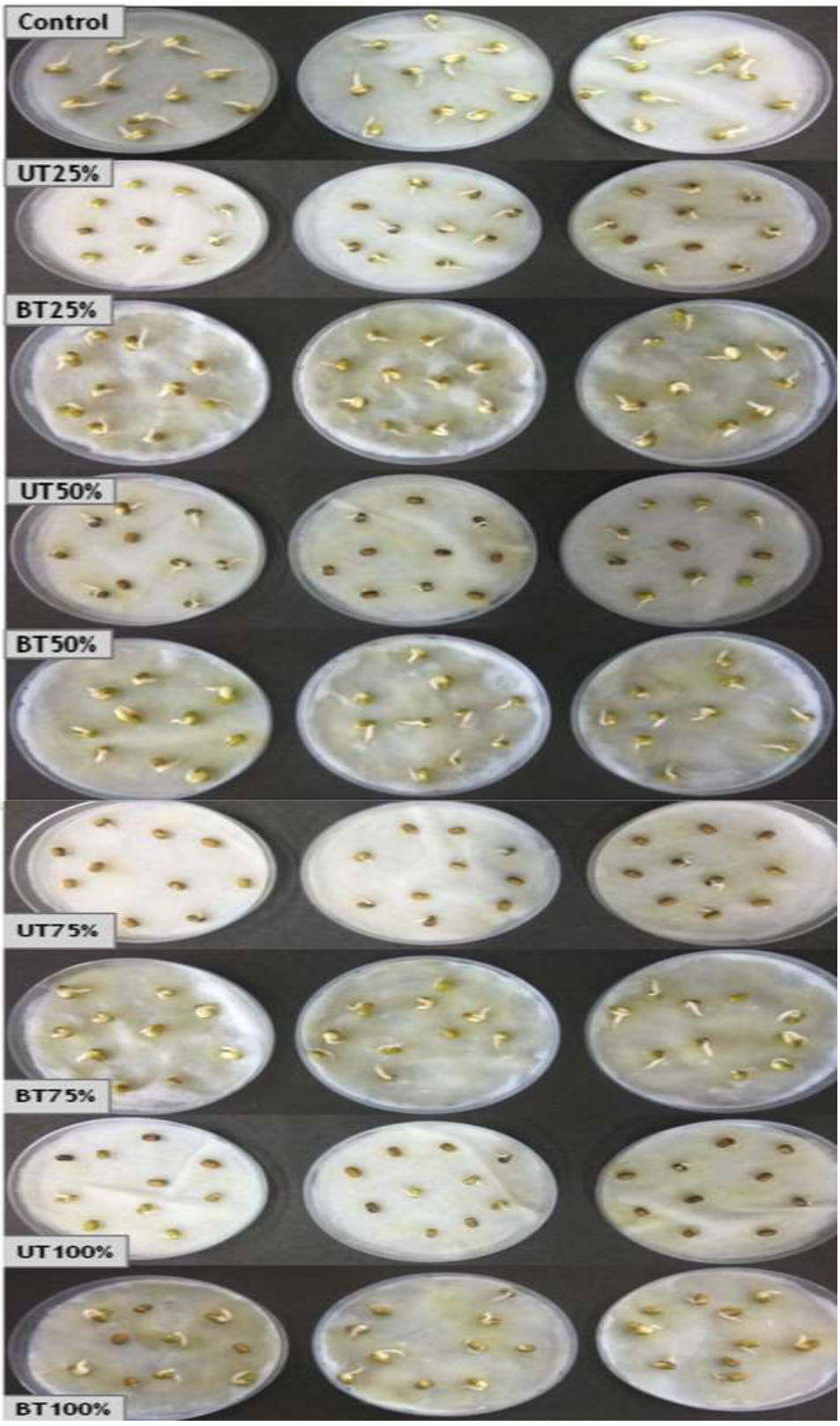


Fig. 10.1 Seed germination experiment on Petri plates (performed at room temperature)

Table 10.1 Phytotoxicity of tannery wastewater before and after bacterial (consortial) treatment

TWW (%)	GP (%)	SLM (%)	GI (%)	SL (cm)	RL (cm)	RSR	SVI	PP (%)	α-amylase activity (unit grain^{-1})
UT-TWW (25%)	90.00	10.00	47.04	2.74 ± 0.36	2.07 ± 0.10	0.75 ± 0.27	432.90	47.72	0.62 ± 0.04
BT-TWW (25%)	100.00	0.00	67.17	4.73 ± 0.41	2.66 ± 0.37	0.56 ± 0.90	739.00	32.82	0.65 ± 0.04
UT-TWW (50%)	70.00	30.00	23.33	2.15 ± 0.10	1.32 ± 0.47	0.61 ± 4.70	242.90	66.66	0.52 ± 0.04
BT-TWW (50%)	100.00	0.00	52.02	4.23 ± 0.20	2.06 ± 0.09	0.48 ± 0.45	629.00	47.97	0.60 ± 0.02
UT-TWW (75%)	53.33 ± 0.47	46.67	9.23	1.07 ± 0.56	0.69 ± 0.14	0.64 ± 0.25	93.86	82.57	0.41 ± 0.05
BT-TWW (75%)	90.00	10.00	37.72	3.33 ± 0.23	1.66 ± 0.23	0.49 ± 1.00	449.10	58.08	0.48 ± 0.03
UT-TWW (100%)	33.66 ± 0.47	66.34	2.75	0.50 ± 0.24	0.33 ± 0.12	0.66 ± 0.50	27.93	91.66	0.34 ± 0.02
BT-TWW (100%)	70.00	30.00	20.50	2.23 ± 0.20	1.16 ± 0.28	0.49 ± 1.40	244.30	70.70	0.42 ± 0.00
Control (TP)	100.00	0.00	100.00	5.80 ± 0.43	3.90 ± 0.29	0.68 ± 1.27	976.00	0.00	0.68 ± 0.01

TWW: Tannery wastewater; UT-TWW: Untreated tannery wastewater; BT-TWW: Bacterially (consortial) treated tannery wastewater; TP: Tap water; GP: Germination percentage; SLM: Seedling mortality; GI: Germination index; SL: Shoot length; RL: Root length; RSR: Root-shoot ratio; SVI: Seed vigour index; PP: Phytotoxicity percentage

All the values are mean of three replicates ± SD.

The germination percentage (GP) was much lower in all the seeds of *Phaseolus aureus* L treated with different concentration (25%, 50%, 75%, and 100%, v/v) of untreated TWW than control (TP). However, the GP was significantly improved in all the seeds irrigated with the different concentration (25% - 100%, v/v) of TWW treated with bacterial consortium GS-TE1310. The phytotoxicity percentage (PP) was varied from 91.66% - 47.72% from higher to lower concentrations (100% - 25%, v/v) of untreated TWW. This trend was might be due to the presence of high salts load and toxic organic pollutants and metals present in the untreated TWW, which induces high osmotic pressure and anaerobic conditions (Bharagava and Chandra 2010). However, the PP was significantly improved when seeds were irrigated with the different concentrations (100% - 25%, v/v) of TWW after treatment with bacterial consortium GS-TE1310 that caused the degradation and detoxification of organic and inorganic pollutants in TWW. Further, the negative impact of industrial effluents on the seed germination and seedling growth parameters depends on its volume and the type of crops to be irrigated (Bharagava et al. 2018).

High salt load and toxic organic pollutants along with metals act as an inhibitor for various phytohormones(s) such as auxins, gibberellins, and cytokinins), which play a key role in seed germination and seedling growth (Chandra et al. 2011). The toxic nature of untreated TWW was also confirmed by SLM, which was maximum (66.34%) at a higher effluent concentration (100%, v/v) as compared to control (TP, 0.00%). However, the SLM was substantially decreased from higher to lower concentrations (100% - 25%, v/v) of TWW after treatment with bacterial consortium GS-TE1310. The germination index (GI) was 100% at control (TP) and varied from 47.04% to 2.75% from lower to higher concentrations (25% - 100%, v/v) of untreated TWW; however, after bacterial treatment, it was significantly improved from higher to lower concentrations (100% - 25%, v/v) of treated TWW. The seeds treated with control (TP) exhibited both normal shoot and root growth whereas seeds treated with 100% untreated TWW exhibited very small shoot (0.50 ± 0.24 cm) and root (0.33 ± 0.12 cm) development. The possible cause for the reduction shoot and root growth were might be the presence of high salts concentration, COD, TSS, recalcitrant organic and inorganic chemicals in the untreated TWW (Table 2). However, after treatment with bacterial consortium GS-TE1310, the shoot and root growth were significantly improved from higher to lower concentrations (100% - 25%, v/v) of

treated TWW. Similarly, the seeds treated with control (TP) showed a very high (976.00) seed vigour index (SVI) as compared to that of seeds irrigated with untreated TWW. However, SVI was significantly improved in seeds which were irrigated with TWW treated with bacterial consortium GS-TE1310.

Fig. 10.2 Effect of untreated (UT) (A) and bacterial treated (BT) (B) tannery wastewater (TWW) at the concentration of 25%, 50%, 75% and 100% on seedling growth of *Phaseolus aureus* L.

Further, the α-amylase activity recorded in the germinating seeds clearly justified the toxicity of untreated TWW on seed germination and seedling growth parameters (Table 4). The optimum α-amylase activity (0.62 ± 0.04 Unit grain^{-1}) was recorded in seeds treated with 25% (v/v) concentration of untreated TWW and thereafter a continuous decline was observed at successively higher TWW concentrations (i.e. 50% - 75%, v/v). High salt load, toxic metals, and recalcitrant organic pollutants were might be affected the expression of amylase, which catalyzes the degradation of starch into sugars and thus, essential for seed germination and seedling growth parameters (Bharagava and Chandra 2010). However, α-amylase activity was significantly improved in the seeds irrigated with higher to lower concentrations (100% - 25%, v/v) of TWW treated with bacterial consortium GS-TE1310. The improved α-amylase activity in the seeds irrigated with treated TWW was might be

due to the degradation and detoxification of recalcitrant organic pollutants and HMs present in the untreated TWW by bacterial consortium GS-TE1310. The results obtained at 25 and 50% concentration (v/v) of bacterially treated TWW were invariably better as compared to untreated leather TWW. Further, these concentrations (25 and 50%, v/v) of bacterially treated TWW might be acted like a liquid fertilizer and proven non-toxic to plant growth and development, and hence, could be used to irrigate the agricultural crops. The results reported in the present study are well corroborated with those reported in the previous studies (Bharagava et al. 2018; Chandra et al. 2011)

10.4. Conclusion

The present study revealed that the phytotoxicity of TWW after treatment with the newly developed bacterial consortium GS-TE1310 was reduced significantly allowing the 70% germination of seeds as compared to seeds irrigated with untreated TWW (100%, v/v). Thus, the bacterially treated TWW could be used as a liquid fertilizer to irrigate agricultural crops. Overall, the present study suggests that the newly developed bacterial consortium GS-TE1310 was more effective in the treatment/detoxification of persistent organic pollutants present in the TWW for environmental protection.

11

Chapter-11
Summary and Conclusions

Summary and Conclusions

Leather industries (LIs) are the key contributors in the economy of many developing countries because they significantly earn foreign exchange through leather exports and create employment opportunities for economically weaker sections. However, unfortunately, they are facing serious challenges from the public and governments due to the associated environmental pollution and toxicity. There is a public outcry against the industry due to the discharge of a highly polluted and potentially toxic wastewater having alkaline pH, dark brown colour, unpleasant odour, high biochemical oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS), phosphate, nitrate, sulphate, phenol, chromium and a variety of persistent organic pollutants (POPs) which are used during leather processing in LIs to produce commercial leather or leather product. Various environment protection agencies and pollution control authorities have prioritized several chemicals as highly toxic and hazardous in nature and restricted their use in leather processing, however; many of these recalcitrant chemicals are used and discharged in wastewater. The highly toxic POPs applied during leather processing are not fully uptaken by the hide/skins and hence, are discharged in the TWW. Moreover, these POPs also do not degrade much during the secondary treatment process at common effluent treatment plants (CETPs) of LIs and goes into the environment where causes serious soil and water pollution along with severe toxic effects in living beings. Therefore, the biodegradation and biodetoxification of POPs in real TWW is required to protect the environment and public human.

Microbes are considered as the eco-friendly tools for the degradation and detoxification of organic and inorganic pollutants in industrial wastewaters. Therefore, in the present study, a total of ten (10) bacterial strains (GS1-GS10) were isolated from TWW and sludge samples collected after secondary treatment process carried out at common effluent treatment plant (CETP), Unnao, Kanpur (UP), India. In addition, the purified bacterial strains (GS-GS10) were primarily screened for the ability to tolerate high salt (NaCl) concentration. Results revealed that among all the isolated bacterial strains (GS1-GS10), only seven bacterial strains i.e. GS1, GS2, GS3, GS4, GS5, GS6, and GS10 were adapted to tolerate up to 6, 4, 4, 3, 4, 3 and 8% salt (NaCl) concentration over a wide range of salinity (1-10%). Thus, it was confirmed that the isolated bacterial strains are halotolerant in nature and suitable for

the treatment and detoxification of real TWW as it contains a high amount of salts. Moreover, these selected bacterial strains were further screened on the basis of pollutants (COD) removal efficiency from real TWW for the degradation and detoxification of persistent organic pollutants and to achieve better effluent treatability. Results revealed that among seven bacterial strains, only three bacteria strains i.e. GS1, GS3, and GS10 were able to remove COD up to 61.12, 54.28, and 66.32% from real TWW during its bioremediation.

The isolated bacterial strains were further characterized to confirm their identity on the basis of various morphological and biochemical tests. Results of the morphological tests revealed that the bacterial strain GS1 appeared as milky white colonies on MSM-agar plates and was gram-negative, motile, and rod-shaped. Bacterial strain GS3 appeared as white colonies on MSM agar plates and was gram-positive, non-motile, and round-shaped whereas bacterial strain GS10 appeared as greenish colonies on MSM agar plates and was also gram-negative, motile, and rod-shaped. Results of the biochemical tests revealed that the bacterial strain GS1 showed positive reactions for citrate utilization, lysine utilization, ornithine utilization, urease, catalase, nitrate reduction, glucose, adonitol, lactose whereas negative reactions for phenylalanine deamination, H_2S production, arabinose, and sorbitol. Bacterial strain GS3 showed positive reactions for malonate, citrate utilization, catalase, glucose whereas negative reactions for Voges Proskauer's, ONPG, nitrate reduction, arginine, sucrose, mannitol, arabinose, and trehalose. Bacterial strain GS10 showed positive reactions for ornithine utilization, urease, catalase, nitrate reduction, glucose, adonitol, lactose, arabinose whereas negative reactions for citrate utilization, lysine utilization, phenylalanine deamination, H_2S production, and sorbitol. On the basis of different morphological and biochemical tests, the isolated bacterial strain GS1, GS3, and GS10 were belonged to the *Ochrobactrum*, *Micrococcus* and *Stenotrophomonas* genera, respectively. In addition to this, the isolated bacterial strains GS1, GS3, and GS10 were identified and confirmed as *Ochrobactrum intermedium* (MK344317), *Micrococcus lylae* (MK344318), and *Stenotrophomonas acidaminiphila* (MK344319) on the basis of 16S rRNA gene sequence analysis.

Further, a new bacterial consortium, GS-TE1310 was developed using these three potential pollutants degrading bacterial strains i.e. *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, and *Stenotrophomonas acidaminiphila* GS10 on the basis of

performance of the mono-cultures in the bioremediation of TWW. For the development of bacterial consortium, the selected bacterial strains (*Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, and *Stenotrophomonas acidaminiphila* GS10) were checked for the compatibility test/ bio-interaction study. Results revealed that these bacterial strains (GS1, GS3 & GS10) were not inhibited the growth of each other as no zone of inhibition was formed on the MSM-agar plate and thus, the selected bacterial strains were compatible to each other and able to work together during bioremediation study. Afterward, the performance of the developed bacterial consortium GS-TE1310 was evaluated in the bioremediation of TWW on the basis of COD removal efficiency. Results revealed that the newly developed bacterial consortium GS-TE1310 was able to remove maximum COD up 74.15% within 120 h as compared to individual bacterial strains, *Ochrobactrum intermedium* GS1 (61.12%), *Micrococcus lylae* GS3 (54.28%), and *Stenotrophomonas acidaminiphila* GS10 (66.32%).

Moreover, the newly developed bacterial consortium GS-TE1310 was also optimized at various environmental and nutritional parameters as well as inoculum concentration and agitation rate to further enhance the pollutants (COD) removal efficiency. Results revealed that the newly developed bacterial consortium GS-TE1310 was able to remove maximum COD up to 76.08% from real TWW at the optimized conditions within 120 h. The optimum pH, temperature, inoculum concentration, and agitation rate were found to be 7, 35 °C, 20 ml and 120 rpm, respectively and the best carbon and nitrogen source was found to be glucose and ammonium chloride, respectively, among the various carbon and nitrogen sources used for the bioremediation of real TWW.

Thereafter, the newly developed bacterial consortium GS-TE1310 was used for the degradation and detoxification of persistent organic pollutants (POPs) present in the TWW. Results revealed that an effective degradation of real TWW was attained by a newly developed bacterial consortium GS-TE1310 within 120 h (at 7 pH, 120 rpm, and 35°C) with 76.12, 85.32, 71.89, 48.59, 78.81, 69.53, 71.22, and 88.70% reduction in pollution parameters such as COD (1428 ± 5.56 mgL^{-1}), BOD (436 ± 4.58 mgL^{-1}), TDS (4064 ± 3.46 mgL^{-1}), phosphate (118.66 ± 5.03 mgL^{-1}), sulphate (6.75 ± 0.27 mgL^{-1}), nitrate (14.05 ± 0.16 mgL^{-1}), Cr (6.88 ± 0.02 mgL^{-1}), and phenol (8.68 ± 0.04 mgL^{-1}), respectively. In addition, HP-LC, FT-IR, and GC-MS techniques

were used to assess the biodegradation as well as to characterize the persistent organic pollutants (POPs) and their metabolites produced during the bioremediation of TWW. Results revealed that most of the persistent organic pollutants detected (POPs) in the untreated TWW were completely mineralized/degraded into new metabolic products in the treated TWW by the newly developed bacterial consortium GS-TE1310 at the optimized conditions (7 pH, 35°C temperature, 0.5% glucose and ammonium chloride (w/v), 120 rpm (agitation rate), and 20 ml inoculum volume)). Thus, the application of this newly developed bacterial consortium GS-TE1310 instead of individual bacterial strains was highly effective in the degradation and detoxification of real TWW.

Moreover, the catabolic enzyme responsible for the degradation of persistent organic pollutants during bacterial treatment of TWW was characterized to confirm the enzymatic degradation process. Results revealed that the selected bacterial strains, *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, & *Stenotrophomonas acidaminiphila* GS10 exhibited catechol 1,2- dioxygenase enzyme as confirmed by the quantitative analysis. Further, the SDS-PAGE analysis also showed the molecular weight of protein around ~32 kDa in the crude extract of all the selected bacterial strains. Overall, these selected bacterial strains were able to degrade the persistent organic pollutants present in TWW and thus, the developed consortium could be used for the bioremediation of TWW.

Further, the prime objective of bioremediation is to lessen the toxicity of industrial effluents and hence, the phytotoxicity of TWW before and after treatment with the newly developed bacterial consortium, GS-TE1310 was assessed to evaluate the environmental safety. In the present study, untreated TWW was proved to be highly toxic in nature as it showed inhibitory effect on seed germination and seedling growth parameters of *Phaseolus aureus* L, which were significantly improved when seeds were irrigated with TWW treated with the newly developed bacterial consortium GS-TE1310. In addition, the phytotoxicity of TWW after treatment with the newly developed bacterial consortium GS-TE1310 was reduced significantly allowing the 70% germination of seeds as compared to seeds irrigated with untreated TWW (100%, v/v). Thus, the bacterially treated TWW could be used as a liquid fertilizer for the irrigation of agricultural crops.

Overall, the present study concludes that the newly developed bacterial consortium GS-TE1310 comprising *Ochrobactrum intermedium* GS1, *Micrococcus lylae* GS3, and *Stenotrophomonas acidaminiphila* GS10, was more effective in the treatment/detoxification of persistent organic pollutants present in the TWW to safeguard the environment and public health. This study was perhaps the first attempt on the development of a new bacterial consortium with identified potential bacterial strains and its application in the bioremediation and toxicity reduction in the TWW. This study would be useful to develop a bacteria-based bioremediation process for WWTPs treating TWW for environmental protection and to promote the sustainable development of our society with less environmental impacts.

12

Chapter-12
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Scientific Publications and Achievements

(A) Scientific Papers

1. **Saxena G**, Chandra R, Bharagava RN (2016) Environmental pollution, toxicity profile and treatment approaches for tannery wastewater and its chemical pollutants. Reviews of Environmental Contamination and Toxicology 240: 31-69. **(IF: 7.00)**

2. Bharagava RN, **Saxena G**, Mulla SI, Patel DK (2018) Characterization and Identification of Recalcitrant Organic Pollutants (ROPs) in Tannery Wastewater and its Phytotoxicity Evaluation for Environmental Safety. Archives of Environmental Contamination and Toxicology 75:259–272**. (IF: 2.467)**

3. Goutam SP, **Saxena G**, Singh V, Yadav AK, Bharagava RN, Thapa KB (2018) Green synthesis of TiO_2 nanoparticles using leaf extracts of *Jatropha curcas* L. for photocatalytic degradation of tannery wastewater. Chemical Engineering Journal 336: 386–396**. (IF: 6.735)**

4. **Saxena G,** Purchase D, Mulla SI, Patel DK, Bharagava RN (2109) Degradation and detoxification of leather tannery effluent by a newly developed bacterial consortium GS-TE1310 for environmental safety. Under Review in Communicated to Chemical Engineering Journal **(IF: 6.735)**

5. **Saxena G** and Bharagava RN (2019) Evaluation of bioremediation potential of a newly isolated *Stenotrophomonas acidaminiphila* for degradation and detoxification of tannery effluent and its validation by phytotoxicity. **(In preparation)**

6. **Saxena G**, Purchase D, Mulla SI, Saratale GD, Bharagava RN (2019) Phytoremediation of Heavy Metal-Contaminated Sites: Eco-Environmental Concerns, Field Studies, Sustainability Issues and Future Prospects. Reviews of Environmental Contamination and Toxicology. doi: 10.1007/398_2019_24 **(IF: 7.00)**

7. **Saxena G**, Bharagava RN, Kaithwas G, Raj A (2015) Microbial indicators, pathogens and methods for their monitoring in water environment. Journal of Water and Health 13(2): 319-339. **(IF: 1.352)**

(B) Book Reviews

1. **Saxena G**, Bharagava RN (2015) Ram Chandra: advances in biodegradation and bioremediation of industrial waste CRC Press, Taylor & Francis Group, Boca Raton/London/New York, 2015, 479 pp, Price: 108.00 £, ISBN: 13: 978-1-4987-0055-9. Clean Techn Environ Policy (2016) 18:979-980. **(IF: 2.337)**

2. **Saxena G**, Kishor R, Purchase D, Bharagava RN (2019) Ram Chandra, Nawal K. Dubey, Vineet Kumar: Phytoremediation of environmental pollutants2017 CRC Press, Taylor & Francis Group, Boca Raton/London/New York, xiv + 510 pp, Hardback 145.00 £, 127 figs , ISBN: 9781138062603 (Hardback), ISBN: 9781351665636 (eBook). Environmental Earth Sciences (Under Review). **(IF: 1.435)**

(C) Book Chapters

1. **Saxena G** and Bharagava RN (2015) Persistent Organic Pollutants and Bacterial Communities Present during the Treatment of Tannery Wastewater. In book: **Environmental Waste Management** by Ram Chandra (Ed.) CRC Press, Taylor and Francis Group, FL, United States, pp. 217-247.

2. Chandra R, **Saxena G** and Kumar V (2015) Phytoremediation of environmental pollutants: an eco-sustainable green technology to environmental management. In Advances in book: **Biodegradation and Bioremediation of Industrial Waste** by Ram Chandra (Ed.) CRC Press, Taylor and Francis Group, FL, United States, pp. 1-30.

3. **Saxena G,** Chowdhary P, Mishra A, Bharagava RN (2016) Xenobiotics: Environmental Pollution, Health Hazards, its Biodegradation and Future Challenges. In book: **Bioremediation of Industrial Pollutants** by Ram Naresh Bharagava and Gaurav Saxena (Eds.) Educationist Press, Write and Print Publications, New Delhi, India, pp. 352-378.

4. **Saxena G** and Bharagava RN (2016) Organic Pollutants in Tannery Wastewater and Bioremediation Approaches for Environmental Safety. In book: **Bioremediation of Industrial Pollutants** by Ram Naresh Bharagava and Gaurav Saxena (Eds.) Educationist Press, Write and Print Publications, New Delhi, India, pp. 1119-151

5. **Saxena G**, Chowdhary P and Bharagava RN (2016) Role of Laccase Enzyme in Bioremediation of Industrial Wastes and its Biotechnological Application. In book: **Bioremediation of Industrial Pollutants** by Ram Naresh Bharagava and Gaurav Saxena (Eds.) Educationist Press, Write and Print Publications, New Delhi, India, pp. 307-331.

6. Zainith S, Sandhya, Sujata, **Saxena** G and Bharagava RN (2016) Microbes: An Eco-Friendly Tools for the Treatment of Industrial Wastewaters. In book: **Microbes and Environmental Management** by Jay Shankar Singh and Devendra Pratap Singh (Eds.) Studium Press (India) Pvt. Ltd., New Delhi, India, pp. 75-100.

7. Bharagava RN, Chowdhary P and **Saxena G** (2017) Bioremediation, An Eco-sustainable Green Technology: Its Applications and Limitations. In book: **Environmental Pollutants and their Bioremediation Approaches** by Ram Naresh Bharagava (Ed.) CRC Press, Taylor and Francis Group, FL, United States, pp. 1-22.

8. **Saxena G** and Bharagava RN (2017) Organic and Inorganic Pollutants in Industrial Wastes, Ecotoxicological Effects, Health Hazards, and Bioremediation Approaches. In book: **Environmental Pollutants and their Bioremediation Approaches** by Ram Naresh Bharagava (Ed.) CRC Press, Taylor and Francis Group, FL, United States, pp. 23-56.

9. Bharagava RN, **Saxena G** and Chowdhary P (2017) Constructed Wetlands: An emerging phytotechnology for the degradation and detoxification of industrial wastewaters. In book: **Environmental Pollutants and their Bioremediation Approaches** by Ram Naresh Bharagava (Ed.) CRC Press, Taylor and Francis Group, FL, United States, pp. 397-426.

10. Gautam S, Kaithwas G, Bharagava RN and **Saxena G** (2017) Pollutants in Tannery Wastewater, Pharmacological Effects, and Bioremediation Approaches for Human Health Protection and Environmental Safety. In book: **Environmental Pollutants and their Bioremediation Approaches** by Ram Naresh Bharagava (Ed.) Taylor and Francis Group, FL, United States, pp. 369-396.

11. Bharagava RN, Purchase D, **Saxena G** and Mulla SI (2018) Applications of Metagenomics in Microbial Bioremediation of Pollutants: From Genomics to Environmental Cleanup. In the book: **Microbial Diversity in the Genomic Era** by Surajit Das and Hirak R. Das (Ed.) Academic Press, Elsevier, USA, pp. 459-477.

12. Bharagava RN, **Saxena G** and Mulla SI (2019) Introduction to industrial wastes containing organic and inorganic pollutants and bioremediation approaches. In book: **Bioremediation of Industrial waste for Environmental Safety – Vol. I: Industrial Waste and Its Management** by G. Saxena and R.N. Bharagava (Eds.) Springer Nature, Singapore. doi: 10.1007/978-981-13-1891-7_1

13. **Saxena G**, Purchase D and Bharagava RN (2019) Microbial enzyme and their role in biodegradation and bioremediation of industrial waste pollutants. In Press in book: **Bioremediation of Industrial waste for Environmental Safety – Vol. I: Industrial Waste and Its Management** by G. Saxena and R.N. Bharagava (Eds.) Springer Nature, Singapore. doi: 10.1007/978-981-13-1891-7_3

14. Mulla SI, Ameen F, Talwar MP, Eqani SAMAS, Bharagava RN, **Saxena G,** Tallur PN, Ninnekar HZ (2019) Organophosphate pesticides: Impact on environment, toxicity and their degradation. In book: **Bioremediation of Industrial waste for Environmental Safety – Vol. I: Industrial Waste and Its Management** by G. Saxena and R.N. Bharagava (Eds.) Springer Nature, Singapore

15. Goutam SP, Roy D, **Saxena G**, Bharagava RN and Yadav AK (2019) Green synthesis of nanoparticles and their applications in water and wastewater treatment. In book: **Bioremediation of Industrial waste for Environmental Safety – Vol. I: Industrial Waste and Its Management** by G. Saxena and R.N. Bharagava (Eds.) Springer Nature, Singapore.

16. **Saxena G**, Saratale GD and Bharagava RN (2019) Genetically modified organisms (GMOs) and their potential in environmental management: Constraints, prospects and challenges. In book: **Bioremediation of Industrial waste for Environmental Safety – Vol. II: Biological Agents and Methods for Industrial Waste Management** by R.N. Bharagava and G. Saxena (Eds.) Springer Nature, Singapore. doi: 10.1007/978-981-13-3426-9_1

17. **Saxena G**, Mulla SI and Bharagava RN (2019) Ecofriendly and emerging technologies for removal of organic and inorganic pollutants from industrial wastewaters. In book: **Bioremediation of Industrial waste for Environmental Safety – Vol. II: Biological Agents and Methods for Industrial Waste Management** by R.N. Bharagava and G. Saxena (Eds.) Springer Nature, Singapore. doi: 10.1007/978-981-13-3426-9_5

18. Bharagava RN and **Saxena G (2019)** Progress in bioremediation technologies for industrial waste treatment and management for environmental sustainability, future

directions and concluding remarks. In book: **Bioremediation of Industrial waste for Environmental Safety – Vol. II: Biological Agents and Methods for Industrial Waste Management** by R.N. Bharagava and G. Saxena (Eds.) Springer Nature, Singapore. doi: 10.1007/978-981-13-3426-9_21

19. Bharagava RN, Kishor R and **Saxena G (2018)** Industrial wastewaters: Major sources of dyes contamination in environment, health hazards and bioremediation approaches. in the book: **Recent Advances in Environment Management** by Ram Naresh Bharagava (Ed.) CRC Press, Taylor and Francis Group, FL, United States.

20. **Saxena G,** Nandkishor M, Kumar N and Bharagava RN (2019) Metagenomics: A genomic tool for monitoring microbial communities during bioremediation. In the book: **Microbes for Sustainable Development: Scope & Applications** by R. C. Sobti and Ram Chandra (Ed.) CRC Press, Taylor and Francis Group, FL, United States **(In press).**

21. Mulla SI, Bharagava RN, Saratale GD, **Saxena G**, Kumar A, Yu C-P and Ninnekar HZ (2019) An Overview of Nitro Group Containing Compounds and Herbicide Degradation in Microorganisms. I**n the book: Microbial Metabolism of Xenobiotic Compounds** by Pankaj Kumar Arora (Ed.) Springer Nature, Singapore **(In press).**

(D) Books

1. Bharagava RN and **Saxena G** (2019) Bioremediation of Industrial Waste for Environmental Safety – Vol. II: Biological Agents and Methods for Industrial Waste Management. Springer Nature, Singapore. **(ISBN: 978-981-13-3425-2)**

2. **Saxena G** and Bharagava RN (2019) Bioremediation of Industrial Waste for Environmental Safety – Vol. II: Biological Agents and Methods for Industrial Waste Management. Springer Nature, Singapore **(ISBN: 978-981-13-1891-7)**

3. Bharagava RN and Saxena G (2016) Bioremediation of Industrial Pollutants. National, Write and Print Publications, New Delhi, India **(ISBN: 978-93-84649-60-9)**

(E) Magazine Articles

1. **Saxena G** and Bharagava RN (2017) Wetland system to combat water pollution. **Dream 2047.**

2. **Saxena G**, Chowdhary P and Bharagava RN (2015) Bioremediation approaches for industrial waste containing organic and inorganic pollutants for environmental safety. **Microbiology World**, 3(2): 29-33.

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

4. **Saxena G** and Bharagava RN (2015) Phytoremediation: A Green Technology for Restoration of Heavy Metal Contaminated Sites. **Everyman's Science**, vol. L, issue 3: 156-159.

5. **Saxena G** and Bharagava RN (2015) Role of microbes in remediation of leather industry wastewater causing environmental pollution. **Kahaar Magazine: A multilingual magazine for common people**, 2(1):48.

6. **Saxena G,** Rohan Kanaujia and Ram Naresh Bharagava (2019) Microbial Indicators: Role in monitoring of drinking water quality and health prospects. **Microbiology World,** 8:5-8.

7. **Saxena G,** Akash Mishra and Ram Naresh Bharagava (2015) Application of molecular technologies for detection of pathogens in water environment. **Microbiology World,** 10:10-15.

8. **Saxena G,** Bharagava RN (2017) Emerging waterborne pathogens: The serious threats to public health. Scientific India: By the Scientists, for the people, 5(2): 34-36.

(F) Hindi Articles

1. **गौरव सक्सेना,** आकाश मिश्रा, राम चन्द्र, अभय राज अँड राम नरेश भार्गव (२०१४) चमड़ा उधोग के अपशिस्टो का पर्यावरणीय दुष्प्रभाव एवं उनका जैवीय अपघटन. **विश विज्ञान संदेश,** सी0 एस0 आई0 आर0-आई0 आई0 टी0 आर0, भारत, pp. ८६-८९.

2. **गौरव सक्सेना**, आकाश मिश्रा व राम नरेश भार्गव (२०१६) एल्कोहौल आसवनी उधोग के अपशिस्टो का पर्यावरण पर दुष्प्रभाव इवान उनका जैवीय अपघटन. **कहार पत्रिका,** ब0 व0 अ0 यू0, लखनऊ, भारत, pp. ३-४: २४-२५.

(G) Newspaper Articles

1. **गौरव सक्सेना** व राम नरेश भार्गव (2019) निर्मित वेटलैंड) आर्द्रभूमि (तकनीक :अपशिष्ट जल उपचार के लिए एक कुशल विकल्प. Communicated in Janodaya Times.

(H) Paper Presented

1. **Gaurav Saxena** and Ram Naresh Bharagava (2017) Bacterial degradation of recalcitrant organic pollutants in tannery wastewater after secondary treatment process and its phytotoxicity evaluation for environmental safety. In: **International: 58th Annual Conference of Association of Microbiologists of India & International Symposium on Microbes for Sustainable Development: Scope & Applications.** Organized by Babasaheb Bhimrao Ambedkar University (BBAU), Lucknow, Uttar Pradesh, India **(Poster Presentation).**

2. **Gaurav Saxena** and Ram Naresh Bharagava (2019) Eco-friendly degradation and detoxification of leather tannery effluent by a novel bacterial consortium GS-TE1310. In: **National Conference on "Biotechnology and Environment for Sustainable Development 2019"** BioESD2019, held on March 29th - 30th, 2019, Organized by B. Lal Institute of Biotechnology, Jaipur (Rajasthan) India **(Oral Presentation).**

(I) Professional Trainings Received

1. Attended and successfully completed a short term training programme on **"Basic and Advanced Tools in Microbiology and Molecular Biology"** under **"Skill India Initiative"** of **"Government of India (GOI)"** organized at **CSIR-Indian Institute of Toxicology Research (IITR), Lucknow, U.P.,** India from **September 05, 2017 to September 27, 2017.**

2. **Attended and successfully** completed a short term training programme on **"Advance Material and Characterization Techniques"** sponsored by **"TEQIP-II"** organized by **"Dr. B. R. Ambedkar National Institute of Technology",** Jalandhar (Pb), India from **June 01, 2015 to June 07, 2015.**

3. **Attended and successfully** completed a short term training programme on **"Biological Treatment of solid Waste"** sponsored by **"TEQIP Programme"** and organized by **"Indian Institute of Technology" Guwahati (IIT-G), Asom,** India from **February 08, 2016 to February 10, 2016.**

4. **Attended and successfully** completed a workshop on **"Chromatography"** organized by **"Department of Botany", University of Calcutta, Kolkata, West Bengal,** India from **January 05, 2016 to January 08, 2016.**

5. **Attended and successfully** completed a seven days workshop on **"Gene Cloning and Its Expression, to produce Genetically Modified Organisms"** jointly organized by **CytoGene™ and MUIT, Lucknow (UP) India from November 20 – 26, 2017.**

6. **Attended and successfully** completed a five short course **"Bioremediation for Environmental Sustainability"** organized under the aegis of **"Global Initiative of Academic Networks (GIAN)"** by **"National Institute of Technology" (NIT-K) Mangalore, Karnataka** from **August 11 – 15, 2018.**

(J) Orientation Programme Attended

1. Attended and successfully completed **"Faculty Development Programme"** on **"Technology Based Research Methodology and Data analysis"** organized by the Department of Commerce, **University of Lucknow**, Lucknow, India from **October 26, 2015 to November 1, 2015.**

(K) Scholarship/Fellowship Received

1. Fellowship received from **"University Grants Commission",** Government of India (GOI), New Delhi for **Doctoral Research Work** from October 23**,** 2013 to October 23, 2017 at the **Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (Central) University,** Lucknow, India.

2. Selected as **DST-Junior Research Fellow (JRF)** and received fellowship from **"Department of Science and Technology",** Government of India (GOI), New Delhi for **Doctoral Research Work** from June 04**,** 2018 to March 31, 2019 at the **Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (Central) University,** Lucknow, India

(L) Competitive Exams Qualified at National Level

1. Qualified "**National Eligibility Test (NET: 2016)"** organized by **"Agricultural Scientists Recruitment Board (ASRB)"** of **"Indian Council of Agricultural Research (ICAR)",** New Delhi, India.

(M) Awards

1. Conferred honorary **"Young Environmentalist Award-2018"** during **International Conference on "Emerging Issues in Agricultural, Environmental & Applied Sciences for Sustainable Development",** which is being held during November 27-29, 2018, organized by **Agro-Environmental Development Society (AEDS)** at **Sam Higginbottom University of Agriculture, Technology and Sciences (SHUATS),** Allahabad, Uttar Pradesh, India
2. Conferred **"InSc Young Achiever Award-2019"** for best paper selected by **Institute of Scholars,** Bengaluru, Karnataka, India.

(N) Membership of Scientific Societies

1. **Life Member** of the **Association of Microbiologists of India (AMI),** India.
2. **Life Member** of the **Indian Science Congress Association (ISCA),** Kolkata, India.
3. **Life Member** of the **Agro-Environmental Development Society (AEDS),** Kolkata, India.
4. **Student Member** of the **American Society of Microbiology (ASM),** USA.

(O) Editor/Reviewer for Scientific Journals/Chapters from Books

1. **Review Editor,** Frontiers in Environmental Science
2. **Review Editor,** Frontiers in Microbiology
3. **Reviewer,** PLOS One
4. **Reviewer,** Scientific Reports, Nature Publishing Group
5. **Reviewer,** International Journal of Microbiology Research
6. **Reviewer,** Indian Journal of Microbiology Research
7. **Reviewer,** Frontiers in Environmental Science
8. **Reviewer,** Chapter from Springer Book: *"Microbes and Enzymes in Soil Health & Bioremediation"*

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Environmental Pollution, Toxicity Profile and Treatment Approaches for Tannery Wastewater and Its Chemical Pollutants

Gaurav Saxena, Ram Chandra, and Ram Naresh Bharagava

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Characterization and Identification of Recalcitrant Organic Pollutants (ROPs) in Tannery Wastewater and Its Phytotoxicity Evaluation for Environmental Safety

Ram Naresh Bharagava[1] · Gaurav Saxena[1] · Sikandar I. Mulla[2] · Devendra Kumar Patel[3]

Received: 14 August 2017 / Accepted: 1 December 2017 / Published online: 14 December 2017

Abstract

Tannery wastewater (TWW) is of serious environmental concern to pollution control authorities, because it contains highly toxic, recalcitrant organic and inorganic pollutants. The nature and characteristics of recalcitrant organic pollutants (ROPs) are not fully explored to date. Hence, the purpose of this study was to characterize and identify the ROPs present in the treated TWW. Gas chromatography–mass spectrometry data analysis showed the presence of a variety of ROPs in the treated TWW. Results unfolded that benzyl chloride, butyl octyl phthalate, 2,6-dihydroxybenzoic acid 3TMS, dibutyl phthalate, benzyl alcohol, benzyl butyl phthalate, 4-chloro-3-methyl phenol, phthalic acid, 2′6′-dihydroxyacetophenone, diisobutyl phthalate, 4-biphenyltrimethylsiloxane, di-(-2ethy hexyl)phthalate, 1,2-benzenedicarboxylic acid, dibenzyl phthalate, and nonylphenol were present in the treated TWW. Due to endocrine disrupting nature and aquatic toxicity, the U.S. Environmental Protection Agency classified many of these as "priority pollutants" and restricted their use in leather industries. In addition, the physicochemical analysis of the treated TWW also showed very high BOD, COD, and TDS values along with high Cr and Pb content beyond the permissible limits for industrial discharge. Furthermore, phytotoxicity assessment unfolds the inhibitory effects of TWW on the seed germination, seedling growth parameters, and α-amylase activity in *Phaseolus aureus* L. This indicates that the TWW discharged even after secondary treatment into the environment has very high pollution parameters and may cause a variety of serious health threats in living beings upon exposure. Overall, the results reported in this study will be helpful for the proper treatment and management of TWW to combat the environmental threats.

Tannery industries (TIs) are one of the well-developed economic sectors in many developing countries. Unfortunately, these also are the major source of environmental pollution due to the discharge of a huge volume of potentially toxic and hazardous wastewater, which creates a negative image of TIs in the society. The wastewater discharged from TIs is characterized by dark brown colour, objectionable odour, high pH, chemical oxygen demand (COD), biochemical oxygen demand (BOD), total dissolved solids (TDS), chromium, sulphate, phosphate, nitrate, and a variety of highly toxic organic chemicals and heavy metals (HMs) (Suganthi et al. 2013; Chandra et al. 2011a; Haydar and Aziz 2009). In India, there are more than 2500 tanneries, which mostly rely on the chrome tanning process and afford 15% of the total worldwide leather production (Shukla et al. 2009; Alam et al. 2009).

In the leather tanning process, a huge amount of highly toxic chemicals, such as chrome salts, vegetable and synthetic tannins, phenolic compounds, azo dyes, surface-active compounds, pesticides, and sulphonated oils, are being used to convert the raw hide/skins into the commercial leather or leather products (Saxena et al. 2016; Dixit et al. 2015). These chemicals are not fully taken up by hide/skins during leather processing and thus end up in tannery wastewater (TWW), which is a major source of environmental (soil/

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Green synthesis of TiO_2 nanoparticles using leaf extract of *Jatropha curcas* L. for photocatalytic degradation of tannery wastewater

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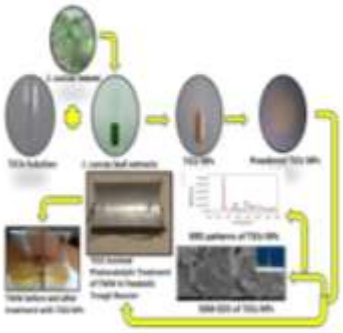
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Green synthesis
Titanium dioxide
Tannery wastewater
Photocatalysis
Chromium
Chemical oxygen demand

ABSTRACT

Green synthesis is a single, eco-friendly and emerging approach of synthesizing nanoparticles (NPs) and currently, attracting scientific community from around the world. The objective of the present study was to synthesize green titanium dioxide (TiO_2) NPs and evaluate its performance for the photocatalytic treatment of TWW after the secondary (biological) treatment process. TiO_2 NPs was synthesized using leaf extract of the biodiesel plant, *Jatropha curcas* L. in a one-step at room temperature to examine its treatability for tannery wastewater (TWW). Moreover, the green synthesized TiO_2 NPs was further characterized by UV-Visible spectrophotometer, Field Emission Scanning Electron Microscopy (FESEM), X-ray Energy Dispersive Spectroscopy (EDS), Fourier Transform Infrared (FT-IR) spectroscopy, X-ray Diffraction (XRD), Dynamic Light Scattering (DLS), Brunauer-Emmett-Teller (BET) and Barret-Joyner-Halenda (BJH) analysis. Results confirmed the synthesis and anatase phase of the spherical TiO_2 NPs and also unfold the presence of phytochemicals in leaf extract, which might involve in the capping/stabilization of NPs. Further, the green synthesized TiO_2 NPs was applied for the first time to testify its potential for the simultaneous removal of chemical oxygen demand (COD) and chromium (Cr) from secondary treated TWW. During the photocatalytic treatment of wastewater in a self-designed and fabricated Parabolic Trough Reactor (PTR), 82.26% removal of COD and 76.48% removal of Cr from TWW was achieved upon the treatment with green synthesize TiO_2 NPs, and thus, successfully employed for the

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Phytoremediation of Heavy Metal-Contaminated Sites: Eco-environmental Concerns, Field Studies, Sustainability Issues, and Future Prospects

Gaurav Saxena, Diane Purchase, Sikandar I. Mulla, Ganesh Dattatraya Saratale, and Ram Naresh Bharagava

Contents

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Microbial indicators, pathogens and methods for their monitoring in water environment

Gaurav Saxena, Ram Naresh Bharagava, Gaurav Kaithwas and Abhay Raj

ABSTRACT

Water is critical for life, but many people do not have access to clean and safe drinking water and die because of waterborne diseases. The analysis of drinking water for the presence of indicator microorganisms is key to determining microbiological quality and public health safety. However, drinking water-related illness outbreaks are still occurring worldwide. Moreover, different indicator microorganisms are being used in different countries as a tool for the microbiological examination of drinking water. Therefore, it becomes very important to understand the potentials and limitations of indicator microorganisms before implementing the guidelines and regulations designed by various regulatory agencies. This review provides updated information on traditional and alternative indicator microorganisms with merits and demerits in view of their role in managing the waterborne health risks as well as conventional and molecular methods proposed for monitoring of indicator and pathogenic microorganisms in the water environment. Further, the World Health Organization (WHO) water safety plan is emphasized in order to develop the better approaches designed to meet the requirements of safe drinking water supply for all mankind, which is one of the major challenges of the 21st century.

Key words | drinking water quality, fecal contamination, microbial indicator, molecular techniques, waterborne disease, water safety plan

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INTRODUCTION

Ensuring the safety of drinking water (DW) is an ongoing process. Water that looks suitable for drinking may be contaminated with pathogens that may cause serious health hazards. The microbiological examination of DW for the presence of indicator microorganisms (IMs) is key to determining microbiological quality and ensuring public health safety. The presence of IMs represents the fecal contamination of DW with pathogens and quality deterioration. The microbiological assessment of DW quality is based on the relationship between IMs and pathogens (Borrego *et al.* 2002a, 2002b; Koster *et al.* 2003; WHO 2008). However, DW illness outbreaks have occurred both in the presence and absence of IMs. This is because of either failure of treatment processes that do not completely eliminate the pathogens from DW or entry of contaminated water harboring pathogens into distribution systems through cracks/ leakage (Figueras & Borrego 2010). In spite of specific legislation, DW illness outbreaks are still occurring worldwide and the associated control measures are being carried out (Figueras & Borrego 2010).

The World Health Organization (WHO) has published several guidelines in collaboration with the International Water Association (IWA) and the Organisation for Economic Co-operation and Development (OECD) for improvement in DW quality (Dufour *et al.* 2003; EIWID 2003; WHO 2008). Water safety plans (WSPs) are the most recent document to create awareness among water quality professionals, so that they can develop the preventive strategies to protect public health (Bartram *et al.* 2009).

Many waterborne pathogens are still difficult to detect and/or quantify due to the lack of easy and reliable methods. The specific methods that are used to detect IMs have also

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BOOK REVIEW

Ram Chandra: advances in biodegradation and bioremediation of industrial waste

CRC Press, Taylor & Francis Group, Boca Raton/London/New York, 2015, 479 pp, Price: 108.00 £, ISBN: 13: 978-1-4987-0055-9

Gaurav Saxena[1] · Ram Naresh Bharagava[1]

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Environmental sustainability with rapid industrialization is one of the major challenges of the current scenario worldwide (Chandra 2015). Industries are the key drivers in the world economy, but these are also the major polluters due to discharge of hazardous wastes containing organic and inorganic pollutants, which cause environmental (soil and water) pollution and severe toxic effects in living beings (Maszenan et al. 2011; Chandra 2015). Being a low cost and eco-friendly clean technology, bioremediation can be a sustainable alternative to conventional technologies for the treatment and management of industrial wastes to protect the environment and human health (Megharaj et al. 2011; Maszenan et al. 2011). Bioremediation utilize microorganisms, plants or their enzymes to degrade/detoxify the pollutants in the environment (Kulshreshtha 2012). However, the mechanism of bioremediation technologies and their role in the environmental cleanup is in nascent stage. Therefore, in this perspective, the book, "Advances in Biodegradation and Bioremediation of Industrial Waste" provides a comprehensive knowledge on the fundamental, practical, and purposeful utilization of bioremediation technologies for the sustainable development. The book describes the microbiological, biochemical, and molecular aspects of biodegradation and bioremediation, including the use of microbial genomics and proteomics for the development of efficient bioremediation technologies for industrial wastes to combat the forthcoming challenges. The book contains 14 chapters exclusively focused on the different aspects of biodegradation and bioremediation of industrial wastes. Each chapter is concluded with an exhaustive list of references for readers interested to learn further details about the subject matter. All the chapters are accessible through internet to readers who would find this book most useful. For this book, many relevant topics have been contributed by the experts from different universities, research laboratories, and institutes. In general, the book is outstanding, except Chap. 11, which mainly describe the anaerobic biodegradation of tallow-slaughterhouse lipid (TSHL) waste.

The first chapter highlights the basic mechanisms used by plants in phytoremediation of heavy metals (HMs) from industrial waste polluted sites for environmental cleanup. In addition, the role of siderophore-producing plant growth promoting rhizobacteria in bioremediation and potential of genetically engineered plants that possess the required traits necessary under certain environmental conditions has been also discussed. Further, the phytoremediation has been suggested as a low cost alternative for developing countries like India, where funding is a big issue. The potential of microbial cells (dead or alive) as biosorbents for HMs removal from industrial wastewaters has been discussed in Chap. 2. The mechanisms used by microbial cell biosorbents and innovations to both live and dead cells through immobilization; growth manipulation and genetic engineering have been well described through figures in this chapter. Chapter 3 presents an overview on the toxicity, microbial degradation, and degradation pathways of polycyclic aromatic hydrocarbons (PAHs) and pesticides from industrial wastes for sustainable environment.

Enzymes are crucial for all life forms and play an important role in the biodegradation and bioremediation of

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Author's Proof

Chapter 18
Environmental Hazards and Toxicity Profile of Organic and Inorganic Pollutants of Tannery Wastewater and Bioremediation Approaches

Gaurav Saxena, Diane Purchase, and Ram Naresh Bharagava

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Persistent Organic Pollutants and Bacterial Communities Present during the Treatment of Tannery Wastewater

Gaurav Saxena and Ram Naresh Bharagava

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13 Pollutants in Tannery Wastewater, Pharmacological Effects and Bioremediation Approaches for Human Health Protection and Environmental Safety

Swetlana Gautam, Gaurav Kaithwas, Ram Naresh Bharagava, and Gaurav Saxena

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Chapter 4

Organic Pollutants in Tannery Wastewater and Bioremediation Approaches for Environmental Safety

GAURAV SAXENA AND RAM NARESH BHARAGAVA[1]

ABSTRACT

Leather industries (technically called as tannery industries) play an important role in the economy of developing countries, but these industries are also the major source

Chapter 21
Progresses in Bioremediation Technologies for Industrial Waste Treatment and Management: Challenges and Future Prospects

Ram Naresh Bharagava and Gaurav Saxena

Abstract Industrial wastewater treatment and management is a major challenge of the twenty-first century and essential to safeguard the environment and public health. Industrial wastewaters are considered as one of the major sources of environmental contamination because these carry a variety of environmental contaminants that may

Chapter 14
Organophosphate Pesticides: Impact on Environment, Toxicity, and Their Degradation

Sikandar I. Mulla, Fuad Ameen, Manjunatha P. Talwar, Syed Ali Musstjab Akber Shah Eqani, Ram Naresh Bharagava, Gaurav Saxena, Preeti N. Tallur, and Harichandra Z. Ninnekar

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Chapter 17
Green Synthesis of Nanoparticles and Their Applications in Water and Wastewater Treatment

Surya Pratap Goutam, Gaurav Saxena, Diptarka Roy, Anil Kumar Yadav, and Ram Naresh Bharagava

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Chapter 11

Role of Laccase Enzyme in Bioremediation of Industrial Wastes and its Biotechnological Application

PANKAJ CHOWDHARY, GAURAV SAXENA AND RAM NARESH BHARAGAVA[1]

Introduction

Laccase (EC 1.10.3.2 para-bezenediol:dioxygen oxidoreductases) is a multi-copper blue enzyme that couples the four electron reduction of oxygen with the oxidation of a broad range of organic substrates, including

Chapter 1
Genetically Modified Organisms (GMOs) and Their Potential in Environmental Management: Constraints, Prospects, and Challenges

Gaurav Saxena, Roop Kishor, Ganesh Dattatraya Saratale, and Ram Naresh Bharagava

Abstract Increasing environmental contamination with highly toxic chemicals is warning us to find sustainable technologies to protect the environment and human health, which is a key challenge of the current scenario. A variety of physicochem-

Chapter 3
Application of Microbial Enzymes in Degradation and Detoxification of Organic and Inorganic Pollutants

Gaurav Saxena, Roop Kishor, and Ram Naresh Bharagava

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Chapter 5
Emerging and Ecofriendly Technologies for the Removal of Organic and Inorganic Pollutants from Industrial Wastewaters

Gaurav Saxena, Surya Pratap Goutam, Akash Mishra, Sikandar I. Mulla, and Ram Naresh Bharagava

Abstract Environmental pollution is one of the major problems of the current world, and providing a sustainable solution to manage pollution is a key challenge. Industries are mainly responsible for the environmental pollution as they discharge highly toxic pollutants in the receiving environment and provide chance for exposure to mankind and, thus, may create toxicity in humans and animals. The

Chapter 16
An Overview of Nitro Group-Containing Compounds and Herbicides Degradation in Microorganisms

Sikandar I. Mulla, Ram Naresh Bharagava, Dalel Belhaj, Ganesh Dattatraya Saratale, Zabin K. Bagewadi, Gaurav Saxena, Ashok Kumar, Harshavardhan Mohan, Chang-Ping Yu, and Harichandra Z. Ninnekar

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Department of Biochemistry, Karnatak University, Dharwad, Karnataka, India

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Laboratory of Bioremediation and Metagenomics Research (LBMR), Department of

1 Industrial Wastewaters
The Major Sources of Dye Contamination in the Environment, Ecotoxicological Effects, and Bioremediation Approaches

Roop Kishor, Ram Naresh Bharagava, and Gaurav Saxena

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1 Bioremediation
An Eco-Sustainable Green Technology, Its Applications and Limitations

Ram Naresh Bharagava, Pankaj Chowdhary, and Gaurav Saxena

CONTENTS

Chapter 1
Introduction to Industrial Wastes Containing Organic and Inorganic Pollutants and Bioremediation Approaches for Environmental Management

Ram Naresh Bharagava, Gaurav Saxena, and Sikandar I. Mulla

Contents

Chapter 26

Applications of Metagenomics in Microbial Bioremediation of Pollutants: From Genomics to Environmental Cleanup

Ram N. Bharagava[1], Diane Purchase[2], Gaurav Saxena[1] and Sikandar I. Mulla[3]
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2 Organic and Inorganic Pollutants in Industrial Wastes, their Ecotoxicological Effects, Health Hazards and Bioremediation Approaches

Gaurav Saxena and Ram Naresh Bharagava

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Microbes an Ecofriendly Tools for the Treatment of Industrial Wastewaters

SURABHI ZAINITH[1*], SANDHYA, SUJATA, GAURAV SAXENA AND RAM NARESH BHARAGAVA[2**]

ABSTRACT

Industries play a major role in the economic growth of developing countries. However, these are also the major source of environmental pollution because all types of industries discharge a huge volume of wastewater into the environment, which causes serious soil and water pollution as well as serious

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विषविज्ञान संदेश

2013-14

विषविज्ञान संदेश

चमड़ा उद्योग के अपशिष्टों का पर्यावरणीय दुष्प्रभाव एवं उनका जैवीय अपघटन

गौरव सक्सेना[1], आकाश मिश्रा[1], राम चंद्र[2], अभय राज[2], राम नरेश भार्गव[1]

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चमड़ा उद्योग को हम तकनीकी रूप से "चमड़े का कारखाना" अथवा "टेनेरी" के नाम से भी जानते है। यह विश्व का एक सबसे पुराना व आम उद्योग है। भारत में

किया जाता है क्योंकि यह गंदा पानी पेड़-पौधों एवं जीवों के लिए काफी दुष्प्रभावी होता है और पर्यावरण में

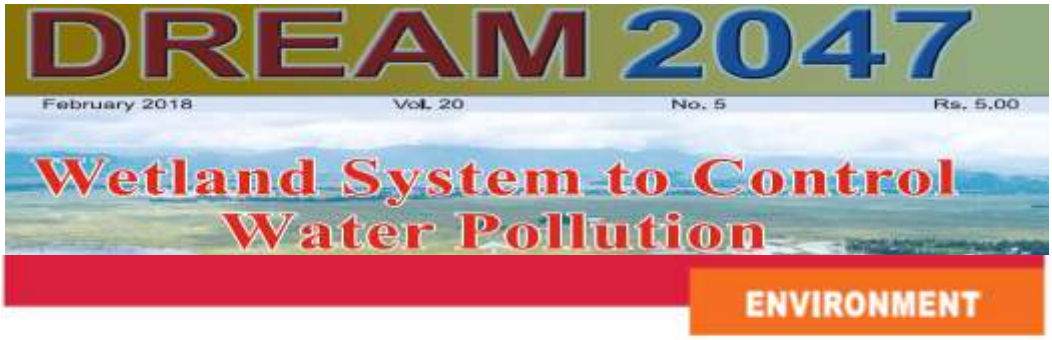
EVERYMAN'S SCIENCE

Vol. L No. 3 (Aug'15 - Sept'15)

PHYTOREMEDIATION: A GREEN TECHNOLOGY FOR RESTORATION OF HEAVY METAL CONTAMINATED SITES

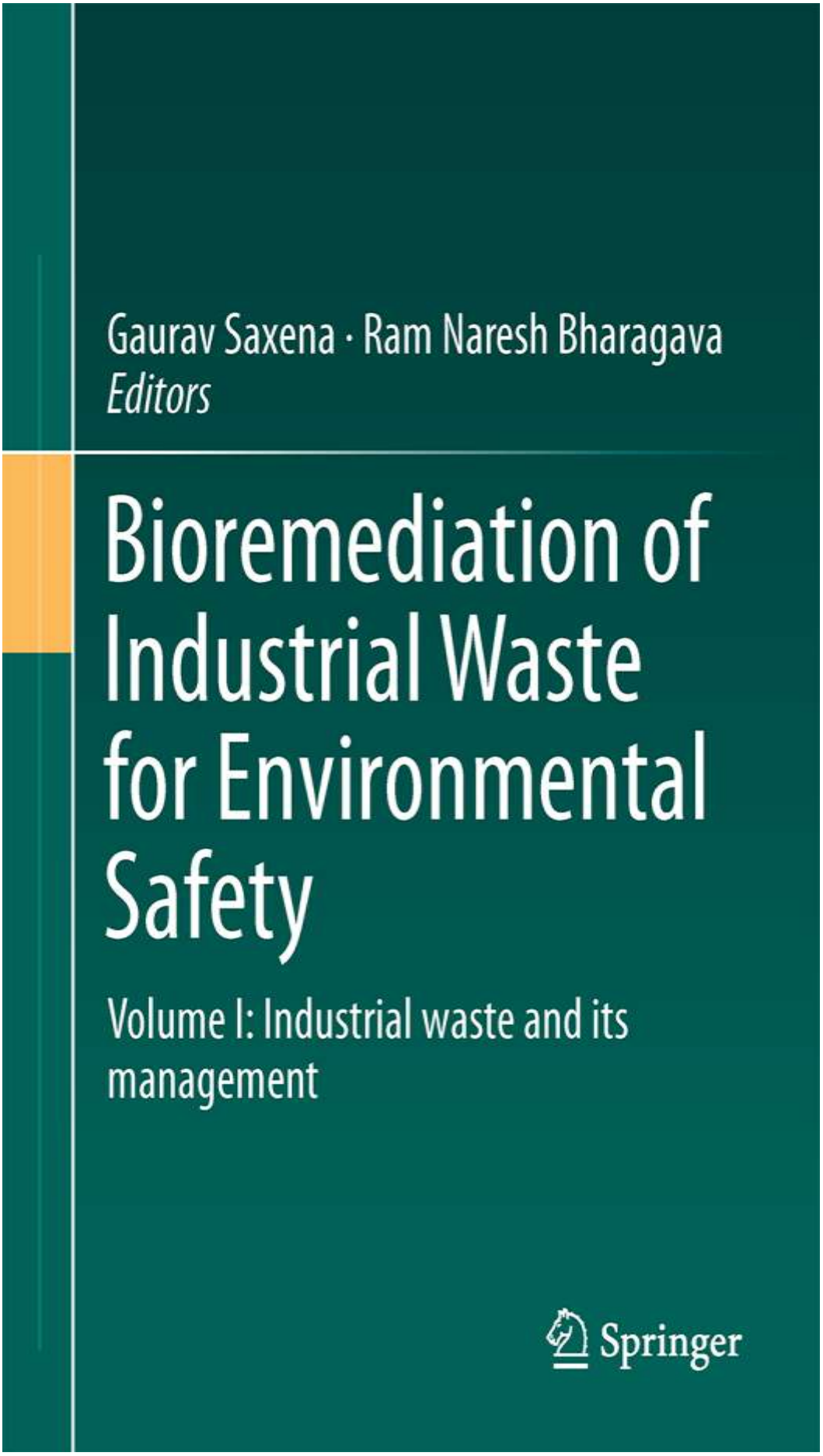
Gaurav Saxena and Ram Naresh Bharagava*

Heavy metal pollution is a major concern worldwide because metals are highly toxic, often non-biodegradable and may reduce the growth of microbial communities present in soil, thereby retard the bioremediation processes. Therefore, phytoremediation is a suitable approach for the restoration of heavy metal free environment.



Wetland System to Control Water Pollution

Gaurav Saxena and R. N. Bharagava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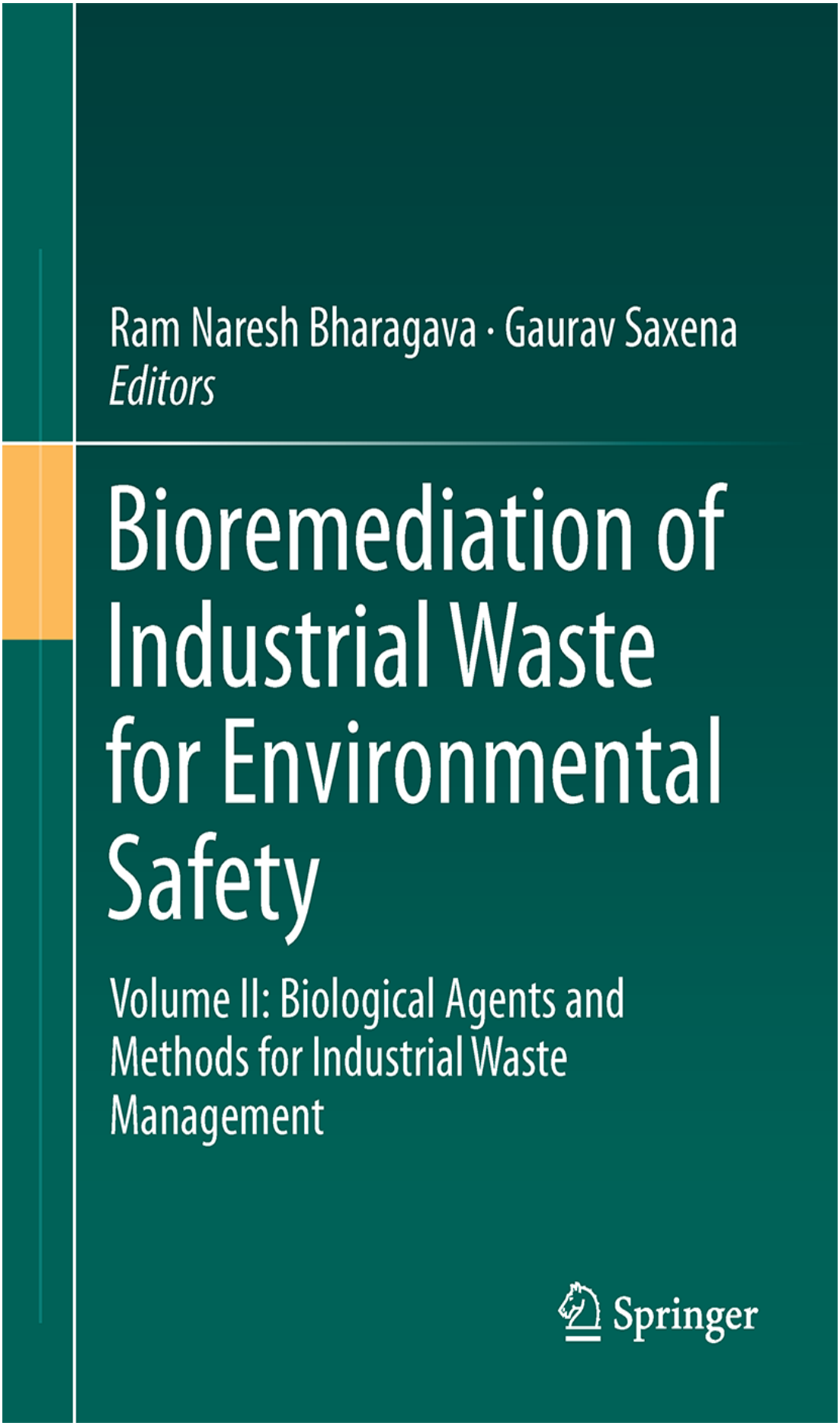
Volume I: Industrial waste and its management
Springer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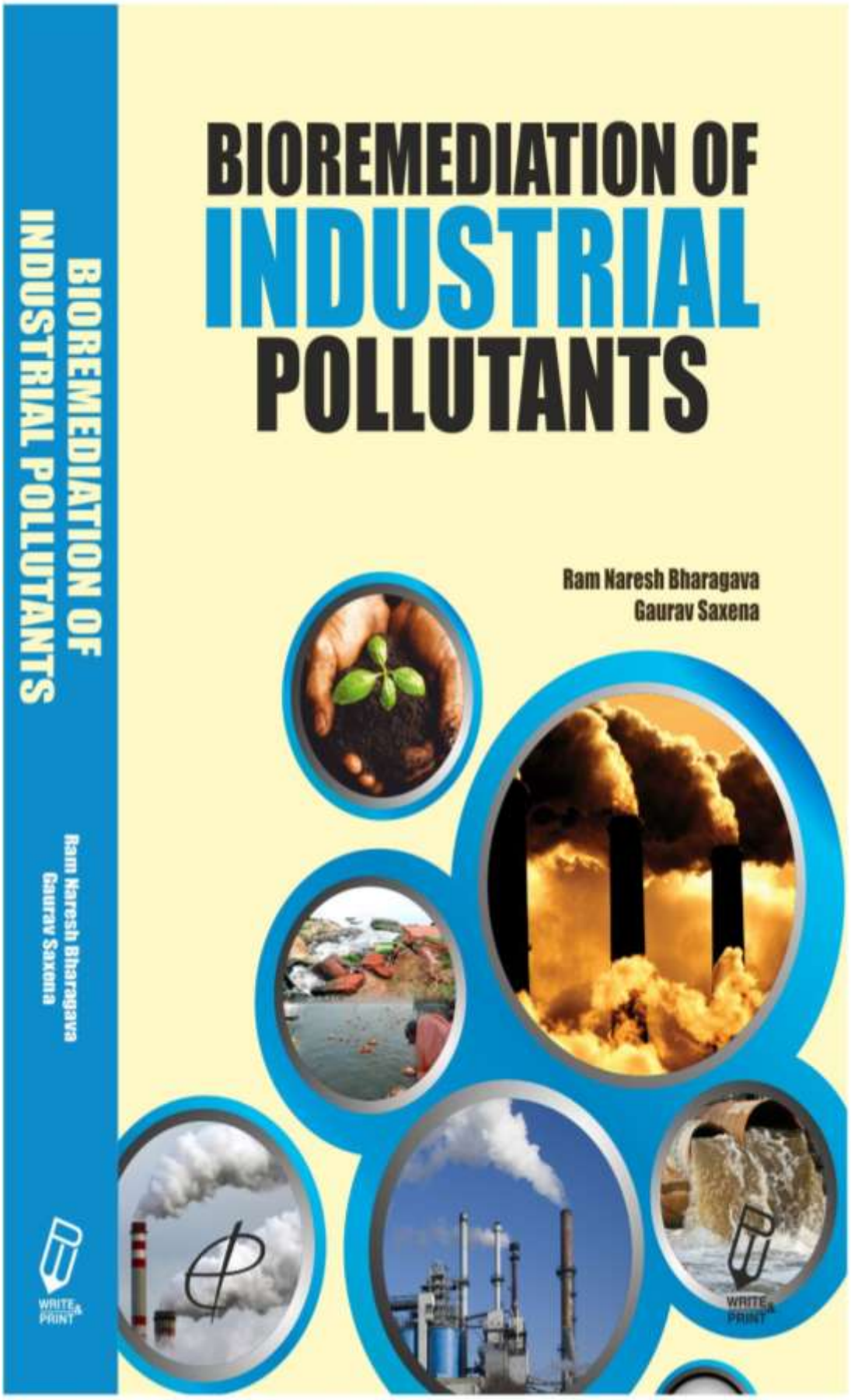
Editors
Bioremediation of Industrial Waste for Environmental Safety
Volume II: Biological Agents and Methods for Industrial Waste Management
Springer


BIOREMEDIATION OF
INDUSTRIAL
POLLUTANTS
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Gaurav Saxena
BIOREMEDIATION OF
INDUSTRIAL POLLUTANTS
Ram Naresh Bharagava
Gaurav Saxena
WRITE & PRINT

Appendices

Appendices I

Chemicals, Reagents, Solvents, and Microbiological Media's

All the required chemicals, reagents, and solvents were used of analytical grade (highest purity ≥ 99%) and purchased from **Sigma-Aldrich (St. Louis, MO, USA)** whereas microbiological media was purchased from **HiMedia Laboratories (Mumbai, MH, IN).**

Appendices II

Glasswares and Plasticware's

All the laboratory glassware's were purchased from **Borosil Glass Works Ltd.,** Mumbai, Maharashtra; **J-SIL Scientific Industries,** Agra, Uttar Pradesh; **Loba Chemie Pvt. Ltd.,** Mumbai, Maharashtra (India); and **ASGI (India) Industries,** Agra, Uttar Pradesh whereas the laboratory plastic wares were purchased from **Lab Line Traders** Traders, Lucknow, Uttar Pradesh, India.

Appendices III

Laboratory Tools and Equipments

Autoclave (SM-102, S M Scientific Instruments Pvt. Ltd., UP, IN)

Temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA)

Refrigerated centrifuge (REMI Instruments Pvt. Ltd., Mumbai, MH, IN)

Veriti™ 96-Well Thermal Cycler (Applied Biosystems™ Inc., CA, USA).

Spectrophotometer (Thermo Scientific™ Evolution 201, Australia)

Nicolet FT-IR Spectrometer (Model Nicolet 6700, Thermo Fisher Scientific, MA, USA).

515 HPLC system (Waters Corporation, Milford, MA, USA)

GC-MS (Thermo Fisher Scientific, FL, USA).

Biographical Sketch

Mr. Gaurav Saxena was born in 1989 and completed school education from Government Schools at Shahjahanpur, Uttar Pradesh (UP) India. He received B.Sc. degree (2010) in Industrial Microbiology, Zoology, Botany and Chemistry from Hemwati Nandan Bahuguna Garhwal (Central) University (HNBGU), Srinagar (Garhwal), Uttarakhand (UK), India and then moved to Babasaheb Bhimrao Ambedkar (Central) University, Lucknow (UP) India where completed M.Sc. degree (2013) in Environmental Microbiology. Because of his keen interest in environmental protection, he joined Babasaheb Bhimrao Ambedkar (Central) University, Lucknow (UP) India to further pursue doctoral research work in environmental microbiology at the Laboratory for Bioremediation and Metagenomics Research (LBMR), Department of Microbiology (DEM). Presently, Mr. Saxena is engaged in developing eco-friendly treatment solutions for hazardous tannery wastewater. His area of research includes Environmental Microbiology and Toxicology, Bio/Phytoremediation of Environmental Contaminants/Industrial Effluents, and Metagenomics and Wastewater Treatment. He has been qualified (2015) National Eligibility Test (NET) and also selected as a Junior Research Fellowship (JRF) of Department of Science and Technology (DST), Government of India (GOI), India. He has been awarded "*Young Environmentalist Award-2018*" by Agro-Environmental Development Society (AEDS), India in recognition of his scientific work. He has been also conferred *"InSc Young Achiever Award-2019"* for best paper selected by Institute of Scholars, Bengaluru (Karnataka) India. He has some good publications to his credit including books. He is also on the editorial board of *Frontiers in Microbiology* journal and serving as a *Review Editor*. He is also serving as a potential reviewer for various international and national scientific journals in his research areas. He also holds the life memberships of the Association of Microbiologists of India (AMI), and Indian Science Congress Association (ISCA) and Agro-Environmental Development Society (AEDS), India.