Study the role of bacterial laccase enzyme for detoxification of residual organic pollutants in pulp and paper mill wastewater after secondary treatment

Thesis

SUBMITTED TO
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
LUCKNOW

BABASAHEB
BHIMRAO
AMBEDKAR
UNIVERSITY
LUCKNOW
प्रज्ञा शील करुणा
ESTABLISHED 1996

FOR THE DEGREE OF

Doctor of Philosophy
IN
ENVIRONMENTAL MICROBIOLOGY

Submitted By
Adarsh Kumar
Enrolment No. 055/13

Under the Supervision of
Prof. Ram Chandra

DEPARTMENT OF ENVIRONMENTAL MICROBIOLOGY
SCHOOL FOR ENVIRONMENTAL SCIENCES
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
(A CENTRAL UNIVERSITY)
VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW-226 025
UTTAR PRADESH, INDIA
2021

Dedicated

to

My Parents

CERTIFICATE

This is to certify that the thesis entitled ***"Study the role of bacterial laccase enzyme for detoxification of residual organic pollutants in pulp and paper mill wastewater after secondary treatment"*** submitted by **Mr. Adarsh Kumar** 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.) regulation-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: 14/10/2021.

Supervisor

PROF. RAM CHANDRA
Head & Supervisor
Deptt. of Environmental Microbiology
B.B. Ambedkar University
(A Central University)
Lucknow-226025 (U.P.)

(Head of the Department)

PROF. RAM CHANDRA/प्रोफेसर रामचंद्रा
Head / विभागाध्यक्ष
Department of Environmental Microbiology
पर्यावरणीय सूक्ष्मजैविकीय विज्ञान विभाग
Babasaheb Bhimrao Ambedkar University
बाबासाहेब भीमराव अम्बेडकर विश्वविद्यालय
Lucknow, U.P./लखनऊ, यूपी-226025

DECLARATION

I, Adarsh Kumar hereby declare that the work which is being presented in the thesis entitled ***"Study the role of bacterial laccase enzyme for detoxification of residual organic pollutants in pulp and paper mill wastewater after secondary treatment"*** in partial fulfilment of the requirement for the award of the Degree of Doctor of Philosophy and submitted in the Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow, Uttar Pradesh is an authentic record of my work carried out during the period from November 2016 to October 2021 under the supervision of **Prof. Ram Chandra**, Professor and Head, Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow.

The matter presented in this thesis has not been submitted by me for the award of any other degree in any other University/Deemed University without proper citation.

I also declare that the thesis is essentially free from all kinds of plagiarisms.

aKumar
(ADARSH KUMAR)
Department of Environmental Microbiology
School for Environmental Sciences,
Babasaheb Bhimrao Ambedkar
(A Central) University
Vidya Vihar Raebareli Road,
Lucknow-226025, Uttar Pradesh, India

ACKNOWLEDGEMENT

This thesis would not have been possible without the inspiration and support of a number of wonderful individuals — my thanks and appreciation to all of them for being part of this journey and making this thesis possible.

First and foremost I would like to thanks to my guide ***PROF. RAM CHANDRA****, Professor, Head and Ex. Dean, Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow, for his guidance, constructive criticism, endless patience, affection and cheerful encouragement throughout this study. His altruistic, benevolent and magnanimous nature has left an indelible mark in my heart and mind. Sir, you would always remain a source of inspiration in my whole life.*

I owe gratitude to ***Prof. Sanjay Singh,*** *Vice-Chancellor, Babasaheb Bhimrao Ambedkar University, Lucknow, for providing basic facility and research environment at the university.*

I extend my deep gratitude to ***University Grants Commission, New Delhi*** *for financial support during this research work without which this study could not have been undertaken.*

I wish to express my sincere gratitude to ***Prof. Rajesh Kumar, Assist. Prof. R.N. Bhargawa, Assist. Prof. J. Shankar, Assist. Prof. V.S. Baghel*** *and other faculty members, Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow.*

I am extremely thankful to my colleagues ***Mr. Ajay Kumar Singh, Ms. Prerna, Ms. Sonam Tirpathi and Mr. Kshitij Singh*** *for their valuable help and cooperation.*

I express my thanks to my lab staff ***Mr. Sarju Singh*** *who was always helped whenever I required during experiments. They also supported to me for the completion of the thesis time to time as required.*

Further, I would like to acknowledge ***Mr. Subhash Chandra,*** *Department of Chemistry, Indian Institute of Technology (IIT) Banaras Hindu University for providing me with the analysis of Copper and Iron nanoparticles.*

Finally, and most of all, I would like to give my special thanks to my parents, my sisters, my brothers, which always inspired me. It was their constant support, love, care, blessings, advice and prayers that have been a driving force behind my all efforts that brought me to this stage. I am forever indebted to my parents for giving me the opportunities and experiences that have made me who I am. They selflessly encouraged me to explore new directions in life and seek my own destiny. This journey would not have been possible without them, and I dedicate this milestone to them.

(ADARSH KUMAR)

CONTENTS

LIST OF TABLES

Chapter 6 Part A

Chapter 6 Part B

LIST OF FIGURES

Chapter 6 Part A

Chapter 6 Part B

ABBREVIATIONS AND SYMBOLS

AAS	**:**	Atomic absorption spectrophotometer
ABTS	**:**	2, 20-azinobis-3-ethylbenzothiazoline-6-sulphonic acid
Al	**:**	Aluminium
AlCl3	**:**	Aluminium chloride
ANOVA	**:**	Analysis of variance
AOX	**:**	Adsorbable organic halogens
APS	**:**	Ammonium Persulfate
ARW	**:**	Agro-residues wastes
BLAST	**:**	Basic local alignment search tool
BOD	**:**	Biochemical oxygen demand
BSA	**:**	Bovine serum albumin
BSTFA	**:**	N,O-bis(trimethylsilyl)trifluoroacetamide
BUPMW	**:**	Bleached and unbleached paper mill wastewater
C	**:**	Carbon
$CaCl_2$	**:**	Calcium chloride
CaO	**:**	Calcium oxide
CCD	**:**	Central composite design
Cd	**:**	Cadmium
COD	**:**	Chemical oxygen demand
Cr	**:**	Chromium
Cu	**:**	Copper
$CuSO_4$	**:**	Copper sulfate
Cys	**:**	Cysteine
DCM	**:**	Dichloromethane
DNA	**:**	Deoxyribo nucleic acid
DO	**:**	Dissolved oxygen
ECF	**:**	Elemental chlorine-free
EDC	**:**	Endocrine distrusting chemicals
EDTA	**:**	Ethylenediaminetetraacetic acid
EDX	**:**	Energy dispersive X-ray spectrometer
EPR	**:**	Electro-paramagnetic resonance
EtBr	**:**	Ethidium bromide

F : Forward

Fe : Iron

$FeCl_3$: Ferric chloride

$FeSO_4$: Ferrous sulfate

Fig. : Figure

FT-IR : Fourier-transform infrared spectroscopy

g : Grams

$g\ L^{-1}$: Gram per liter

GC-MS : Gas chromatography–mass spectrometry

h : Hours

H_2O_2 : Hydrogen peroxide

H_2S : Hydrogen sulfide

HBT : 1-hydroxybenztriazole

HCl : Hydrochloric acid

$HgCl_2$: Mercuric chloride

His : Histidines

HNO_3 : Nitric acid

HPLC : High performance liquid chromatography

K_2HPO_4 : Dipotassium hydrogen orthophosphate

kDa : Kilo Dalton

LASs : Linear alkylbenzene sulfonates

LiP : Lignin peroxidase

Ltd. : Limited

M : Mole

MBSS : Minimal basal salt solution

MBT : Methylene bisthiocyanate

MCOs : Multicopper oxidases

MEGA : Molecular evolutionary genetics analysis

mg : Milligrams

$MgSO_4$: Magnesium sulfate

mL : Milliliter

mM : Milimolar

Mn : Manganese

MnP : Manganese peroxidase

MSM	**:**	Mineral Salts Medium
Na_2HPO_4	**:**	Disodium phosphate
NaCl	**:**	Sodium chloride
NaOH	**:**	Sodium hydroxide
NCBI	**:**	National Center for Biotechnology Information
NH_4NO_3	**:**	Ammonium nitrate
Ni	**:**	Nickel
NIST	**:**	National Institute of Standard and Technology
nm	**:**	Nanometer
NPs	**:**	Nanoparticles
O	**:**	Oxygen
OD	**:**	Optical density
OFTA	**:**	One-factor-at-the-time
PAGE	**:**	Polyacrylamide gel electrophoresis
PAH	**:**	Polycyclic aromatic hydrocarbons
Pb	**:**	Lead
PCB	**:**	Polychlorinated biphenyl
PCDD	**:**	Polychlorinated dibenzo-para-dioxin
PCDF	**:**	Polychlorinated dibenzofuran
PCP	**:**	Pentachlorophenol
PCR	**:**	Polymerase chain reaction
PEO	**:**	Polyethylene oxide
PEPs	**:**	Persistent environmental pollutants
pH	**:**	Potential of hydrogen
ppm	**:**	Parts per million
PPMW	**:**	Pulp and paper mill wastewater
ROPs	**:**	Residual organic pollutants
rpm	**:**	Revoultion per minute
rRNA	**:**	Ribosomal ribonucleic acid
RSM	**:**	Response surface methodology
RT	**:**	Retention time
SD	**:**	Standard deviation
SDS	**:**	Sodium dodecyl sulfate
SDS-PAGE	**:**	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM	**:**	Scanning electron microscope
SmF	**:**	Submerged fermentation state
Sp.	**:**	Species
SSF	**:**	Solid-state fermentation
TCDD	**:**	Tetrachloro-dibenzo-para-dioxin
TCF	**:**	Total chlorine-free
TDS	**:**	Total dissolved solids
TEM	**:**	Transmission electron microscopy
TEMED	**:**	N, N,N’N’-tetramethylethylenediamine
TEMPO	**:**	2,2,6,6-tetramethyl- 1-piperidinyloxy
TOC	**:**	Total organic carbon
TS	**:**	Total solids
U	**:**	Unit
UASB	**:**	Upflow sludge blanket
UV	**:**	Ultraviolet
v/v	**:**	Volume/ volume
VA	**:**	Veratryl alcohol
VOCs	**:**	Volatile organic compounds
VP	**:**	Versatile peroxidase
w/v	**:**	Weight/volume
XPS	**:**	X-ray photoelectron spectroscopy
XRD	**:**	X-ray powder diffraction
µg	**:**	Microgram
µL	**:**	Microlitre
µm	**:**	Micrometer
%	**:**	Percentage
<	**:**	Less than
>	**:**	Greater than
^{0}**C**	**:**	Degree centigrade
3D	**:**	Three-dimensional
3′	**:**	Three prime
5′	**:**	Five prime

Chapter One

General Introduction

1.1 Introduction

The rapid population growth due to increased demand for an industrial establishment to meet human needs has created problems such as overexploitation of available resources, resulting in land, air, and water pollution. The paper industry has been considered a major user of natural, resources, that is, water, wood, and power (fossil fuels, electricity) and a major contributor to pollutants directly released into the environment (Singh and Chandra, 2019). The paper industry ranks sixth among the world's most polluting industries, producing very large scale toxic wastewater (Ugurlu et al., 2007). According to the Central Pulp Paper Research Institute's annual report from 2016, India's overall pulp paper industry consists of about 850 units. Some major industries have been listed in table 1.1. These industries are generating a huge amount of contaminated wastewater that includes organic and inorganic residual organic pollutants. Approximately 190-200 m^3 of freshwater is utilized per ton of paper production (Chandra and Singh, 2012b). From the original mass of raw material, about 40–45 % reports to pulp, while the remainder is released in wastewater that contains cellulose, hemicellulose, tannins, resin acids, and chlorophenols, as well as chloro-lignins produced by bleaching and washing of the pulp. The persistent dark brown hue of lignin and its derivatives, such as chlorolignin, in the wastewater generated during the pulping process, is one of the most serious issues (Prasongsuk et al., 2009). Because it contains a high amount of biochemical oxygen demand (BOD), chemical oxygen demand (COD) and several other parameters beyond the permissible limits for CPCB guideline, 2012.

Primary treatment of the wastewater involves screening of pulp fibers and pH adjustment. The resulting mixed wastewater is then subjected to a secondary effluent treatment process using activated sludge. A recent study revealed that complex organic pollutants are retained as residual organic pollutants in discharged pulp and paper mill wastewater, even after secondary treatment (Kumar et al., 2020b). The common residual organic pollutants detected were 2-Methyl-4-keto-2-pentan-2-ol 1TMS, Pentadecanoic acid, ethyl ester, β-Sitosterol trimethylsilyl ether, Lactic acid, trimethylsilyl ether, trimethylsilyl ester, Phenol,4-ethyl-2-methoxy, Tetradecanoic acid methyl ester, Phenol,4-ethyl-2-methoxy, 1-Tetradecene, Hexadecanoic acid, trimethylsilyl ester or Palmitic acid TMS, Hexadecanoic acid, and β-Sitosterol trimethylsilyl ether (Kumar et al., 2020b). The indicated these compounds exhibited an endocrine-disrupting effect on aquatic organisms due to the presence of endocrine-disrupting chemicals (EDCs) (Chandra and Singh, 2019); however, detailed knowledge regarding the estrogenic and androgenic potential of these compounds toward aquatic organisms is lacking. Many of these pollutants are known to cause acute or chronic toxicity. Chlorinated organic chemicals like dioxins and furans can cause genetic alterations in exposed species (Chandra and Singh, 2019). As a result, there is growing worried about genotoxicants' potential to harm aquatic biota and public health by contaminating drinking water, recreational water, or edible organic species (Chandra et al., 2009, 2012). The public's increased awareness of these toxins' fates, together with the strict laws imposed by various authorities and agencies, are forcing the industry to handle wastewater at the level of enforcement required before it is released into the environment (D’Souza et al., 2006).

The pulping procedure is the first step in the paper-making process. Debarking, chopping wood, washing chips, digestion of chips, screening pulp, thickening, and drying are the main steps in these segments. Pulping is done using both mechanical and chemical processes all over the world. Mechanical methods include mechanical disc refiners, heating and light chemical treatments to boost pulping yield, wood chips, cooking in high-temperature pulping liquors, and cooking under pressure (Sumathi and Hung, 2006). In addition, mechanical and chemical processes can be mixed in specific applications. Mechanical procedures have a greater yield (90-95 %) than chemical processes (40-50 %). Despite this, the pulp produced by mechanical operations is of poor quality, being brightly coloured and containing short fibers (Pokhrel and Viraraghavan, 2004). Chemical pulping is therefore often preferred in alkaline or acidic settings. In an alkaline medium known as the kraft phase, the wood chips are cooked in liquor containing sodium hydroxide (NaOH) and bisulfide ions (NaS_2). The sulphide process uses a combination of sulfuric acid (H_2SO_4) and bisulfide ions (HSO_3) in acidic environments.

I. During fiber processing, roughly 5-10 % of lignin comes from raw materials that cannot be extracted and these are responsible for the dark colour.

Table 1.1 National pulp and paper industries

S.N.	Industry Name	Location	Capacity	Types of production
1.	Seshasayee Paper and Boards	Madurai Tamil Nadu	1,15,000 TPA	Writing and printing paper
2.	M/S Century Pulp & Paper	Lalkua-Nainital Uttrakhand	99,000 TPA	Writing & printing paper
3.	M/S Banwari Paper Mills Ltd.	Kashipur Uttrakhand	12,600 TPA	Writing & printing paper
4.	Shree Shyam Pulp & Board Mills Ltd	Kashipur, U.S. Nagar Uttrakhand	48,600 TPA	Writing & printing paper and copier
5.	M/s Multiwal Pulp & Board Mill (P) Ltd	US Nagar Uttrakhand	23,400 TPA	duplex boards, craft paper, Writing g printing paper
6.	K. R. Pulp & Papers	Shahjahanpur UttarPradesh	1,20,000 TPA	High quality Writing & printing paper,
7.	Kuantum Papers Ltd	Hoshiarpur Punjab	148500 TPA	Writing and printing paper
8.	Hindustan Paper Corporation Ltd 2Units	Township Area Panchgram - Cachar Paper Mill, Panchgram, Assam	1,00,000 TPA 1,00,000 TPA	Writing and printing paper and newsprint
9.	Nagaon Paper Mills	Kagaj Nagar, Jagiroad, Assam	1,00,000 TPA	Writing and printing paper and newsprint
10.	Amaravathi Sri Venkatesa Paper Mills	Palani, Tamil Nadu	2,88000 TPA	duplex boards, craft paper, Writing printing paper
11.	International Paper APPM Ltd.	East Godavari Dist Andhra Pradesh	240,000 TPA	Writing and printing paper
12.	Ballarpur Industries Limited (2 Units)	Gurugram, Haryana Dehradun, Uttarakhand	3,60,000 TPA	Writing and printing paper paperboard
13.	Bindals Papers Mills Ltd.	Muzaffarnagar UttarPradesh	1,00,000 TPA	Writing and printing paper
14.	Delta Paper Mills Limited	Hyderabad, Telangana	51,100 TPA	Writing & Printing papers
15.	Multiwalgroup of industries	Kashipur, Uttarakhand	2,16000 TPA	Writing & Printing paper
*****	Other 15 major industries for paper production	Different places in India	2,854,705 TPA	Writing, Printing

II. Debarking is the process of breaking down the plant's fiber into smaller pieces and removing the bark. The presence of raw materials employed in this stage, such as hardwood, softwood, and agricultural leftovers, aids in the conversion of tannins, resin acids, and other compounds found in the bark to process water. Softwoods, for example, have far more resin acids than hardwoods, but agro wastes may be devoid of resin acids.

III. The pulping process converts the chips to the pulp. This procedure removes the majority of the lignin and hemicellulose content from the raw material, resulting in a cellulose-rich pulp. Pulping can be carried out in a variety of ways, including hydraulic, semi-chemical, kraft, and sulfite pulping, and the raw material can be reused (Ali and Sreekrishnan, 2001).

IV. During the washing stage, remove the bleaching agents from the pulp. To remove colour and bleaching chemicals from the pulp, an alkali sodium hydroxide and caustic soda were typically utilised, and this stage is also known as the alkali extraction stage (Ali and Sreekrishnan, 2001).

V. Bleaching is done to the brown pulp obtained after pulping in order to achieve the desired colour specified by the product standards. Hydrogen peroxide, chlorine dioxide, chlorine, ozone, oxygen, and other bleaching chemicals can be employed separately or in combination. Lignin, phenols, resin acids, and other xenobiotics are chlorinated and converted into very hazardous xenobiotics during this process.

VI. After that, the washed pulp is mixed with suitable fillers (calcium carbonate, titanium dioxide, and clay) and sizing agents such as rosins and starch to make paper and paper products.

The wastewater produced by the pulping process consists of various wooden compounds such as cellulose, hemicellulose, lignin, pectin, carbohydrate, extractives and it is difficult to biologically treat (Kumar and Chandra, 2020). In addition, to these, certain toxic compounds such as resin acids, unsaturated fatty acids, terpene alcohols and chlorinated resin acids may occur in the process wastewater (Pokhrel and Viraraghavan, 2004). The most critical reaction in the bleaching stage is chlorine oxidation, and chlorinated organic compounds or AOX are the main problems with the wastewater materials (Singh and Chandra, 2019). The toxic effects of these by-products on the environment were analyzed in the wastewater. Various studies have documented the toxic/lethal effects on daphnia, shrimp, planktons in the water bodies receiving wastewaters of the pulp and paper industry (Chandra et al., 2009, 2012a; Singh and Chandra, 2019; Erisction and Larsson, 2000; Schnell et al., 2000b; Kovacs et al., 2002).

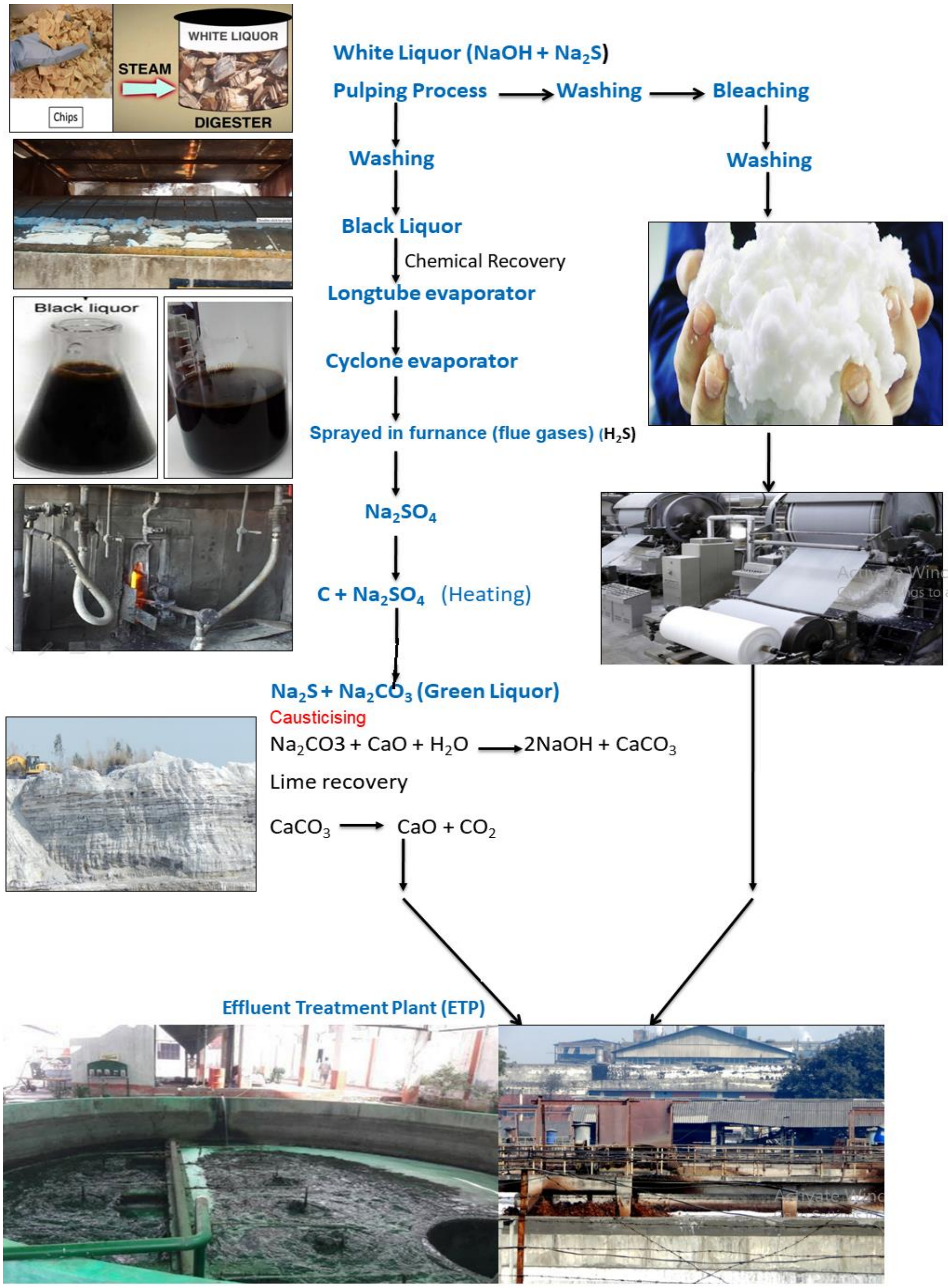


Fig. 1.1 Process flow diagram for pulp and paper production

The digestion and pulping process of the wood in pulp and paper mill wastewater (PPMW) discharge containing a lot of residual organic pollutants such as adsorbable organic halides (AOX), ammonium nitrogen (NH_4^{1-} N), chlorophenol, ditolyethane, di-iso-propyl naphthalene, bis- (methylphenoxy)-ethane, bis(methylphenoxy)ethane, terphenyl, benzylnaphthylether, chloromethyl-phenoxy-methyl-phenyl-ethane, benzyl-biphenyl, and grease, etc. and the black liquor produced after the digestion of wood majorly consists of cellulose, hemicellulose, lignin, resins, and fatty acids along with various other phenolic compounds (Lacorte et al., 2003; Sponza, 2003). In addition to this various studies have also reported the presence of a high amount of lignin, sulfate, phenolic, nitrate compounds, and another monomer of lignins and fatty acids along with plant steroids during the chlorine bleaching process (Chandra and Abhishek, 2011), whereas Romeo et al. (2000) reported about the presence of several fatty acids such as tridecanoic acid, tetradecanoic acid, pentadecanoic acid, 9-hexadecenoic acid, hexadecanoic acid, heptadecanoic acid, 9-octadecenoic acid, octadecanoic acid, nonadecanoic acid, heneicosanoic acid, and docosanoic acid), sterols (campesterol, sitosterol, stigmastanol, fucosterol, and cycloartenol); hydrocarbons (squalene and stigmasta-3-5-diene), steroid ketones (stigmasta-3,5-dien-7- one, stigmast-4-en-3-one, stigmastan-3-one and stigmasta-3,6-dione), and waxes in the effluent during the total chlorine-free bleaching process of pulp from the Eucalyptus globulus wood (Romeo et al., 2000). Several other studies have also revealed the presence of several residual organic pollutants in the discharged pulp paper industry wastewater and their respective discharge sites (Yadav and Chandra, 2018).

Wood extractives include lipophilic compounds such as fatty and resin acids, sterols, sterol esters, triglycerides, and hydrophilic compounds such as lignans, low-molecular-mass lignins, lignin-like substances, and hemicelluloses which are water-soluble and dissolve in water during paper production. Among these extractives, resin and fatty acids are mainly responsible for lowering the machine functioning and decreasing the physical properties of the paper (tensile strength, opacity, brightness, etc.) as they form pitch deposits (Gutierrez et al., 2006). Different types of resin and fatty acids are known, such as linoleic acid, stearic acid, palmitic acid, margaric acid, isopimaric acid, dehydroabietic acid, and dichlorodehydroabietic acid. Although these are not removed by primary flocculation, a decrease of 50 % or more is observed after the biological treatment of resins and fatty acids which are toxic for aquatic life, for example, causing jaundice in rainbow trout (Rigol et al., 2003a).

The wastewater from the pulp and paper industry consists of surfactants, such as linear alkylbenzene sulfonates (LASs) and alkylphenol ethoxylates (APEOs) that are used as cleaning agents or additives in antifoams, drinkers, dispersants, etc (Singh and Chandra, 2019). The several chlorinated compounds, such as chlorolignin compounds, chlorophenols such as pentachlorophenol (PCP), chlorobenzenes, chlorinated acetic acids, chlorinated thiophenes, chloroguaiacols, chlorosyringol, chlorovanillin, chlorocatechol, polychlorinated dibenzo-para-dioxin (PCDD), polychlorinated dibenzofuran (PCDF), polychlorinated biphenyl (PCB), etc. have been detected in the effluents and sludge of the pulp and paper industry (Singh and Chandra, 2019). Chlorophenols are highly lipophilic and are less degradable (Virkki et al., 1994). Chloroveratoles and anisoles are extremely toxic constituents of effluents of pulp and paper wastewater with the tendency to bioaccumulate. In addition to this chlorophenols and trichloroacetic cause growth inhibition to a variety of plants and are considered to be toxic (Kumar et al., 2020b). Chlorolignins convert into chlorocatechols and chloroguaiacols, which are thought to be precursors of tetrachloro-dibenzo-para-dioxin (TCDD), and chloroveratoles after degradation in effluents, along with polychlorinated dibenzothiophenes, which are extremely toxic to the environment (Munawar et al., 2000; Sinkkonen et al., 1992).

Various biocides, which are classified, based on their modes of action, such as membrane-active biocides, cell wall inhibitors, cytotoxic agents, and genotoxic agents, are widely used in the pulp paper industry to reduce microbial, fungal and algal growth (Latorre et al., 2003; Petra et al., 2015). Different types of biocides are commonly used, such as 5-chloro-2-methyl-4- isothiazolinone-3-one, halogenated hydantoin, 2-bromo-2-nitropropane-1,3-diol, n-octylisothiazolin-3-one, methylene bisthiocyanate (MBT), glutaraldehyde, and 2,2-dibromo-2-cyanoacetamide in the papermaking process (Appling et al., 1955; Kasra and David, 2002). The biocides, dioxins, and furans cause several adverse effects on reproduction and cell development, especially in the early stages of life, and are neurotoxins and carcinogenic to the receiving water environment (Oller et al., 2011; Blair et al., 1993; Hileman, 1994; Petra et al., 2015). In addition, chlorine is majorly used as a bleaching agent during the pulp cleaning process in paper-making industries. A well-known toxic compound, dioxins, and furans have been reported to be present in the pulp paper effluent sediment and various discharge sites from the paper industry (Munawar et al., 2000).

The pulp paper mills are considered to discharge various organometallic and nonmetallic compounds into the receiving water bodies. Calcium (Ca), chromium (Cr), copper (Cu), ferrous (Fe), nickel (Ni), and manganese (Mn) are some of the major metals which are discharged by these industries, and nonmetallic compounds include potassium, sodium (Na), chlorine (Cl), sulfate phosphate, calcium, and magnesium, etc. (Chandra and Abhishek., 2011; Chandra et al., 2017; Yadav and Chandra, 2018). This can result in the toxicity risk in water bodies due to the presence of other inorganic nonmetallic compounds and the hardness of PPMW along with other metals (Commonwealth of Australia, 2015). Different types of toxicity have been reported by the waste generated from these PPMW discharge, such as copper toxicity in various aquatic organisms, that is, Rainbow trout (*Salmo gairdneri*) and channel catfish (*Ictalurus punctatus*), has been reported in the presence of calcium and high pH (William and Peter, 1994; Miller and Mackay, 1980). In addition, pre-exposure with beta-naphthoflavone increased the toxicity of Cu in the form of oxidative stress and genotoxicity to European eels (*Anguilla anguilla* L.) that occurred in the aquatic ecosystem (Gravato et al., 2006). The toxic effects were reported of bioaccumulation of heavy metals in different organs of *Anabas testudineus* on exposure to pulp and paper industry effluent (Prasanta, 2014). In the exchangeable component, the sequential leaching properties of various heavy metals from the green liquor dregs of the pulp paper industry after the causticizing process showed a strong extracting tendency for manganese (Mn) and a moderate tendency for the Zn, Ni, and Ba (i.e., leachable with CH_3COOH). This also reflects the source of contamination for the groundwater in the vicinity of the pulp paper industry (Nurmesniemi et al., 2005). Arsenic is a universal factor that occurs in various industrial activities, including the pulp paper industry. Arsenic is highly toxic to aquatic life and bioaccumulates in some animals. Arsenic’s inorganic forms, such as arsenite and arsenate, are more harmful to human health, are extremely carcinogenic, and can cause lung, kidney, prostate, and skin cancer (Chowdhury et al., 2000). In addition, several metals can accumulate in aquatic organisms, including fish that take up metals through the gills digestive tract, and body surface (Tao et al., 2001; Kamunde et al., 2002) and metals persist in water and sediments (Luoma and Rainbow, 2008). Metals also cause oxidative stress in fishes. The generation of reactive oxygen species in aquatic organisms due to oxidative stress from metals in fish occurs primarily through two mechanisms: redox-active metals produce reactive oxygen species through redox cycling, while metals without redox activity inhibit antioxidant defences, especially those of antioxidants and enzymes containing thiol (Stohs and Bagchi., 1995). The metal-mediated production of free radicals induces various changes to DNA bases, increased lipid peroxidation, and altered calcium as well as homeostasis of sulfhydryl (Sevcikova et al., 2011). Therefore, other than the heavy metals and other toxic compounds present in the effluent of the pulp and paper industry, various air pollutants, and gas emissions are the other concerns that are studied in-depth. Apart from water vapour, particulates, nitrogen oxides, volatile organic compounds (VOCs), sulfur oxides, and total reduced sulfur compounds are also produced by these industries which are a cause for concern for the environment.

The PPMW is considered as one of the most polluting industry contributing 100 million kg of toxic pollutants that are being released every year in the environment (Dey et al., 2013). More than 260 chemicals have been identified present in paper and pulp mill effluents which are produced at different stages of papermaking (Hawkins et al., 2002). The presence of organic pollutants in the final effluent discharge indicated that the microbial community present in the wastewater was unable to degrade them due to lack of adequate nutrients; hence, optimization of bacterial growth conditions by addition of nutrient or environmental conditions may be an effective approach for detoxification of wastewater (Kumar and Chandra, 2021). The major challenges of PPMW are the removal of colour contributing compounds like lignin, phenols, fatty acids, and their metabolic products as well as inorganic compounds before their safe disposal into the environment. Different feedstocks, fermentation processes and biological treatments may bring about inconsistent structures of individual colourants (Kumar et al., 2020b). The toxic nature is derived from the presence of several naturally occurring and xenobiotic compounds which are formed and released during various stages of processing (Sharma et al., 2007; Singh and Thakur, 2006). The wastewater coming from paper mill containing a large amount of tannins, these tend to absorb more light and heat and retain less oxygen than unprocessed water, thereby negatively affecting the aquatic flora and fauna. It was established that condensed tannins from spruce bark are toxic, not only to methanogens at concentrations present in the paper mill wastewater but also to aquatic organisms, like fish and change their behavioural response, development and growth, impacts on the immune system, impact on enzymes and reproductive (Singh et al., 2021).

Typically, the pulp and paper industry produces a huge amount of wastewater that must be properly treated before being discharged into the environment; otherwise, it poses a significant environmental and economic burden. The pulp and paper industry's environmental challenges are not limited by its excessive water use. The various sources of wastewater, solid waste generation, wastewater treatment plants, and other treatment methods are all important. Various industries collect large solid waste such as lime mud, lime slaker grits, furnace ash and boiler, residual wood processing, scrubber sludge, and wastewater treatment sludge. Because of the high presence of chlorinated organic compounds, microorganisms, and several heavy metals, these solid wastes are disposed of and cause environmental hazards (Chandra et al., 2017). Environmental effects have been due to chemicals added during the production process, to natural compounds released from plant material, interactions of these compounds with each other, and interactions with biota in industry effluent during wastewater generation and the treatment process (Hewitt et al., 2008).

Therefore, in recent years, microbial approaches for the wastewater degradation process to optimize different parameters under laboratory conditions have drawn the significant attention of many researchers around the world to explore viable, cost-effective and sustainable wastewater treatment technology (Kaushik et al., 2010). Certain potential bacterial strains have been reported as more effective than fungal strains for bioremediation of complex environmental pollutants due to their good environmental adaptability and biochemical versatility for their growth. Because of the development of microbial enzymes such as laccase, lignin peroxidase, and manganese peroxidase, most of the fungi were found to have high ligninolytic activity (Kumar and Chandra, 2020). But these techniques large scale implementations have their restriction due to the low pH range (3.0–5.0), slow growth period, huge spore formation, and adverse submerged conditions for fungal growth (Arimi et al., 2014). Pulp paper mill wastewater (PPMW), pH values are generally alkaline (pH 7.0–9.0) and the requirement to reduce the pH before fungal inoculation maintains the conditions there is an additional cost. Unlike fungi, bacteria that survive in acidic to alkaline pH can play a key role in the bioremediation of PPMW without needing to adjust pH. Bacteria isolated from compost soil, viz., *Azotobacter* and *Serratia marcescens*, were reported capable of degradation and decolorization of lignin compounds (Morii et al., 1995). A comparative study of lignin biodegradation under aerobic conditions by *Bacillus subtilis* and Bacillus sp. isolated from soil was reported (Abd-Elsalam and El-Hanafy, 2009). Three potential aerobic bacterial strains were isolated from pulp and paper mill effluent sludge for kraft lignin degradation, identified as *Paenibacillus* sp. (AY952466), *Aneurinibacillus aneurinilyticus* (AY856831), and *Bacillus* sp. (AY952465) (Chandra et al., 2007). These bacterial strains showed decolorization of kraft lignin at 500 mg/L and their metabolic products were characterized by gas chromatography with mass-spectrometric detection (GC–MS) (Raj et al., 2007). *Bacillus* sp. and *Serratia marcescens* have also been reported as effective for pentachlorophenol and pulp and paper mill effluent degradation up to 94 % in the presence of sources of supplemented carbon (1 % glucose) and nitrogen (0.5 % peptone m/v) under optimized laboratory conditions (Singh et al., 2008). These studies provide strong evidence for the degradation and detoxification of chlorolignin-containing pulp and paper mill wastewater by bacterial consortia (Chandra et al., 2007; Chandra and Singh, 2012); however, the detoxification and in-situ bioremediation of pulp and paper mill effluent after secondary treatment has not yet been reported. Several previous workers were reported bacterial species for capable of lignin, PPMW degradation, and detoxification (Chandra et al., 2007). Bacterial laccases are a broad range of substrate utilization and are active at high pH and temperatures (Wang et al., 2010). But, there was a still gap and require knowledge about ligninolytic enzymes capable of degradation and detoxification of PPMW at optimized environmental and nutritional conditions.

The thesis of study has been compiled into the following chapters;

The chapter first has introduced the basic information on the topic of the thesis. The information related to the number of pulp and paper mills installed in India and their wastewater generated in the environment. Further, this chapter also provides comprehensive information on the pulp and paper mill wastewater colourants, as well as their toxicological effects on the environment, human and animal health.

Chapter two of this thesis has described the objectives of this study, keeping in view exploring the novel methodology and scientific findings for scale-up to develop a new method for industrial-scale application has also been described.

Chapter three of this thesis has described the comprehensive existing information on various physico-chemicals, biological (i.e. aerobic and anaerobic), enzymatic treatment as well as emerging techniques for the treatment of pulp and paper mill wastewater.

Chapter four has described the investigation of unknown residual organic pollutants from bleached and unbleached paper mill wastewater (BUPMW) and their environmental effects after secondary treatment. In addition, this section has also described the characterization and residual organic pollutants of pulp and paper mill wastewater after liquid-liquid and solid-liquid extraction and analyzed by GC-MS to reveal the nature of pollutants present in this waste. The conventional toxicity test was also done to assess the toxicity of BUPMW on *Phaseolus aureus* and *Tubifex tubifex* worms. Hence, the study will be more effective and expand the knowledge of residual organic pollutants present in BUPMW for environmental safety.

Chapter five of this study the isolation, screening and identification of potential laccase producing bacteria from pulp and paper mill waste. The 26 isolated bacterial strains were purified on the MSM agar plate by the streak plate technique. All bacterial strains were screened to produce laccases on modified B and K agar medium containing 5 mM guaiacol. Among these, six bacterial strains (i.e. BL1 (*Bacillus* sp. strain AKRC01), BL6 (*Bacillus aquimaris* strain AKRC02) and BL9 (*Bacillus cereus* strain AKRC03) demonstrated the laccase activity.

Chapter six of this study comprised the study of the effects of various nutrients for the production of bacterial laccase in different pH and temperatures. The present work was evaluated the utilization of various kinds of agro wastes such as rice bran, wheat bran, sawdust, banana peel, orange peel, potato peel, pea peel and sugarcane bagasse to increase laccase production from *Bacillus* sp. AKRC01 and *Bacillus aquimaris* strain AKRC02. This process makes use of another material that requires less energy, reduces the pollutants in the environment, as cost-effective and ecofriendly. The best-suited agro-waste was optimized by using the statistical tool response surface methodology (RSM). RSM is a set of valuable statistical and mathematical methods for designing laccase production processes, enhancing and optimizing variables. Central composite design (CCD) is used for the optimization of laccase production in submerged fermentation state (SmF).

Chapter seven of this study deals with the Purification and characterization of bacterial laccase enzymes of different molecular weights in different nutritional and environmental conditions. Partial purification of the laccase enzyme was carried out using the ammonium sulfate precipitation method, Sephadex column (G-100) chromatography and SDS-PAGE molecular weight determination.

Chapter eight has described study the effect of the cupper (Cu) and iron (Fe) nanoparticle for bacterial laccase production. The nanoparticles were synthesised and characterized through XRD, XPS, SEM, TEM and FTIR. After-effects of laccase production were observed in different concentration NPs and immobilized laccase with magnetic nanoparticles. The effects of free and immobilized laccase were observed activity and their stability.

Chapter nine of this study deals with the optimization of nutrient and environmental conditions for the production of bacterial laccase for detoxification and degradation of residual lignocellulosic waste. In this study, we have tried to address this gap by laccase producing bacteria isolated from pulp paper mill sludge and optimized environmental and nutritional conditions for degradation and detoxification of PPMW. The bacterial treated and untreated PPMW was analyzed by UV–Vis analysis has been correlated for their structural changes and GC-MS analysis of identified residual organic pollutants (ROPs) for degraded/transformation. The toxicity reduction of bacterial treated PPMW was also assessed by using the seed germination of *Phaseolus mungo* L and *Tubifex tubifex* worm.

Chapter ten has summarized the findings of the thesis. This section has mentioned the brief findings of each chapter.

Chapter eleven 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

Chapter twelve has been annexed with the title page of published papers and other scientific output of work. The cover page of each published original research paper in National and International journal has also been attached.

Chapter Two

Objectives

Objectives

The objectives of my study were as below;

1. **Investigation of residual organic pollutants from different pulp and paper mill wastewater and their environmental effects after secondary treatment**
2. **Screening and identification of potential laccase producing bacteria from pulp and paper mill waste**
3. **Study the effects of various nutrients for production of bacterial laccase in different pH and temperature**
4. **Purification and characterization of bacterial laccase enzymes of different molecular weight in different nutritional and environmental conditions**
5. **Study the effect of cupper (Cu) and iron (Fe) nanoparticle for production of bacterial laccase**
6. **Optimization of nutrient and environmental conditions for production of bacterial laccase for detoxification and degradation of residual lignocellulosic waste**

Chapter Three

Review of Literature

Review of literature

A wide range of lignocellulosic waste is produced all over the world, including whole plants, plant parts (e.g. seeds, roots, and stems) and processing by-products of pulp and paper mill wastes. Paper mill effluent can induce chronic toxic effects and endocrine disruption caused by largely unknown residual organic pollutants that are released into the environment. Due to the high chemical diversity of the residual organic pollutants and solvents are present in pulp and paper mill wastewater caused toxic effects on crops as well as aquatic communities have been observed in recipient watercourses. Therefore, the effluent discharged from various industries after secondary treatment does not meet the requirement for the environmental safety guidelines. There is a need to search the more potential organisms and reveals the mechanisms of their degradation process along with their inhibitory residual organic pollutants.

3.1 Physico-chemical methods for the treatment of pulp and paper mill wastewater

Physico-chemical treatment methods are the combination of physical and chemical technologies. Removal of suspended solids from the pulp paper mill wastewater is physical operation while reduction of the dissolved solid is a chemical process. Both operations are done on pulp paper mill wastewater by adding chemicals. Several Physico-chemical methods have been shown to decrease the pollutants load of paper mill wastewater. These include adsorption, ozonation, advanced oxidation processes, thermolysis, coagulation and precipitation to material such as chitosan and activated carbon.

Activated carbon is the most widely studied adsorbent, and it has been found to adsorb a variety of materials such as metals, phenols, dyes, and other organic compounds and bio-organisms, and is therefore used for the removal of pollutants from wastewaters by adsorption. The activated carbons were prepared from agro-residues such as sugarcane bagasse, fly ash, rice husk ash, wood ash, and wood saw dust. In pulp paper mill wastewater treatment, the interface is between the liquid and solid surface that is artificially provided. The material removed from the liquid phase is called the adsorbate and the material providing the solid surface is called the adsorbent. This process has been also found successful in removing harmful parameters like COD and colour from pulp and paper mill wastewater (Swamy et al., 1997). Decolourisation of pulp and paper mill wastewater includes adsorption on commercial as well as indigenously prepared activated carbons have been reported by various researchers (Satyawali and Balakrishnan, 2009). The adsorption method is very effective for colour and organic pollutants removal from the wastewater of different industries. Different-2 absorbents are also used for the colour and lignin removal from wastewater of pulp paper mill and these adsorbents are activated carbon, silica and coal ash, etc. (Kamali and Khodaparast, 2015; Murthy et al., 1991). The same results were also reported by Shawwa et al., (2001) for used activated coke for the colour and COD removal of 90 % of the bleached paper mill wastewater through adsorption methods. Lignin removal was also recorded through the adsorption process by the use of blast furnace dust (80.4 %) and slag (61 %) (Das and Patnaik, 2000). The 41.38 % colour and 60.87 % COD were also removed by the use of polyaluminum silicate chloride (400 mg/L), cheap adsorbent bentonite (450 mg/L) through tertiary adsorption coagulation treatment (Xilei et al., 2010).

In recently membrane techniques is most effective and extensively used for the treatment of various industrial wastewater. But it is very difficult to use this system on a wide scale because of technological limitations and high costs (Greenlee et al., 2010). Therefore, by using pretreatment methods, the efficiency of this method can be increased. The removal of colour, BOD, TDS and COD through the membrane electrochemical reactor from paper mill wastewater has been also reported by Chanworrawoot and Hunsom (2012). As the study of that COD (89 %), sulfate (97 %), total hardness (83 %), coefficient of spectral absorption (95 %), and conductivity (50 %) were removed from the paper mill wastewater water by the technique Ultrafiltration (UF) membranes at pH 10.

The ozonation techniques are used for disinfection of the wastewater with a broad range of applications in wastewater treatment. But, According to Yamamoto (2001) ozonation process was used for the decolorization and detoxification of colour, COD and various contaminants from the industrial wastewater. Moreover, photocatalysis and ozonation methods were effective for the treatment of COD, TOC, colour and toxicity of the pulp paper industry effluents (Torrades et al., 2001). If the high dose of ozone for 15 m were utilized for the removal of colour, it decreased up to 95-97 % (Sevimli and Sarikaya, 2002). Furthermore, the ozonation technique for the pulp paper mill wastewater treatment improved BOD, COD and lignin by 40 %, 11 %, and 46 % respectively but it was insufficient for the colour removal (Ruas et al., 2007). The degradation and removal of lignin depend on the ozone dose during the ozonization phase of the solution of alkali lignin (Michniewicz et al., 2012). It is estimated that at an ozone dose of 0.1 and 3.6 mg O_3/mg COD, respectively, the concentration of lignin is reduced by around 40 to 96.6 %, followed by a reduction in COD in the range of 8.8-69.6 %. As an efficiently applied advanced oxidation process large scales for the treatments of the pulp paper industry wastewater, while broadly reviewed the ozonation process by Kamali et al. (2019).

The advanced oxidation process is successful for lignin removal from the pulp paper industry wastewater. This process is also known for the detoxification of the organic substances from the wastewater and is also effective for the non-biodegradable compounds which are not degraded by microorganisms properly. Balcioglu and Ferhan (1999) reported on photocatalytic oxidation of kraft pulp bleaching wastewater showing that the removal largely depended on the concentration of COD and chloride below a certain level. Zamora et al. (1998) reported on the use of horseradish peroxide to decolorize kraft effluent by 50% within three hours of reaction time. The degradation of phenolic and polyphenolic compounds present in the bleaching effluent was studied using advanced oxidation systems such as photocatalysis with O_2/ZnO/UV, O_2/TiO_2/UV, O_3 and O_3/UV. The authors concluded that O_2/ZnO/UV and O_2/TiO_2/UV were the best systems to oxidize the effluent in a short period. Perez et al. (2002c) reported that the combination of Fenton and photo-fenton reactions proved to be highly effective for the treatment of bleaching kraft mill effluent. Verenich et al. (2000) reported on the improvement in biodegradability of effluent from 30 % to 70 % by the wet oxidation method. Hassan and Hawkyard (2002) studied the removal of colour by combined oxidation with ozone and Fenton's reagent and stated that 100 % colour removal was achieved at a pH of 4 – 5 in the case of ferral (derived from natural clay sources, which contains 2 % ferric sulfate and 6 % aluminum sulfate) and ferric sulfate. Dufresne et al. (2000) reported on the oxidation of total reduced sulfur (TRS) giving odour free products by catalytically enhanced oxidation.

Coagulation and flocculation are normally employed in the tertiary treatment in the case of pulp and paper mill wastewater treatment and are not commonly adopted in the primary treatment. Tong et al. (1999) carried out a comparative study of horseradish peroxide (chitosan) and other coagulants such as ($Al_2(SO_4)_3$), hexamethylene diamine epichlorohydrin polycondensate (HE), polyethyleneimine (PEI), to remove adsorbable organic halides (AOX), total organic carbon (TOC), and colour. The authors indicated that modified chitosan was far more effective in removing these pollutants than other coagulants. Wagner and Nicell (2001) investigated the treatment of foul condensate, defined by phenolic compounds, and toxicity using microtox assay from kraft pulping by horseradish peroxide and H_2O_2 and found a total phenol reduction below 1 mg/L and toxicity (microtox assay) reduction by 46 %. Dilek and Gokcay (1994) reported 96 % removal of COD from the paper machine, 50 % from the pulping, and 20% for bleaching effluents by using alum as a coagulant. Rohella et al. (2001) stated polyelectrolytes were better than the conventional coagulant alum to remove turbidity, COD, and colour. Sheela and Distidar (1989) reported on black liquor treatment by precipitation with $CaSO_4.2H_2O$ in the presence of CO_2. The removal of dissolved solids was reported to be 63 %. However, Wang and Pan (1999) reported that the use of coagulants such as polyethylene oxide (PEO), worsened the settlability and increased COD levels, turbidity, and suspended solids of the treated effluent when the dose was between 25 and 250 ppm. Chernoberezhskii et al. (1994) reported that coagulation with aluminum sulfate or modified adsorbents was the best option for colour removal from the sulfate and sulfite wood pulp and paper industry.

3.2 Biological methods for the treatment of pulp and paper mill wastewater

Biological treatment of pulp and paper mill wastewater by several microorganisms such as actinomycetes, bacteria, fungi, and cyanobacteria have been reported which are capable to degrade lignocellulosic waste and other wood containing fibers. There is a growing concern among researchers to isolate bacteria and fungi directly from lignocellulosic waste contaminated sites due to acclimatized microbial genome pool with secreted ligninolytic enzymes i.e. laccase, lignin peroxidase and manganese peroxidase are involved in the detoxification and biodegradation of lignin and other residual organic.

Several actinomycetes, isolated from different soil samples were tested for their ability to utilize sulphite in PPMW. The adsorbable organic halides values of the higher molecular weight fractions were also reduced. Extracellular peroxidase and cell wall-bound catalase activities were produced during the growth of the microorganisms on bleach effluents (Chandra et al., 2012). Some potential bacterial species were used for the delignification of paper mill wastewater such as lignin degradation of 70–80 % (*P. putida and A. calcoaceticus*), COD removal (*A. formicans* and *Bacillus* sp.), etc., (Gupta et al., 2001; Raj et al., 2007; Brown and Chang, 2014). *Pseudomonas putida*, *Citrobaterer* sp. and *Enterobacter* sp. can decolorized effluent up to 97 % and also can reduce BOD, COD, phenolics and sulphide up to 96.63 %, 96.80 %, 96.92 % and 96.67 % respectively within 24 h of growth and the heavy metals were removed up to 82-99.80 % (Keharia and Madamwar, 2003).

Pseudomonas aeruginosa is capable of reducing PPMW colour by 26-54 % or more under aerobic conditions (Ramsay and Nguyen, 2002). Tiku et al. (2010) and Raj et al. (2007) were tested *Bacillus cereus* and two strains of *Pseudomonas aeruginosa* for the decolorization of PPMW. *Streptomycetes badius* and *S. viridosporous* were able to use a commercial kraft lignin as sole carbon source which was characterized by Fourier transformed infrared spectroscopy, amino acid analysis, and elemental analysis for C, H, N, and high performance size enclusion chromatography (Chandra et al., 2011). Apart from the investigation, mixed consortia of aerobic and anaerobic microbes for resin acid degradation, researchers have employed pure cultures of several bacteria which include *Bacillus* sp., *E. coli, Flavobacterium* sp., *Pseudomonas, Acaligenes eutrophus, Anthrobacter, Sphinomonas, Zooglea, Commamonas, Mortierella isabella, Chaetomium cochliolidae, Corticum sasaki* and *Fomesannosus* (Tiku et al., 2010; Raj et al., 2014). Wilson et al. (1996) isolated two species of *Pseudomonas*, IpA-1 and IpA-2 which were capable of growing on isopimaric acid as the sole carbon source and an electron donor. These isolates were also found to grow on pimaric acid and dehydroabietic acid. *Bacillus* SAM3 producing high levels of cellulose free xylanase active and stable at alkaline pH (Chandra and Singh, 2012). Moreover, several other researchers have also reported that the *Paenibacillus* sp., *Bacillus* sp., and *Streptomyces* sp. were reported to be involved in lignin degradation process (Niladevi and Prema, 2005).

Table 3.1 Various ligninolytic bacterial strains isolated from lignocellulosic waste containing sites

Bacterial Strain	**Source of isolation**	**Lignocellulose substrates supporting Growth**	**Relevant characteristics**	**Growth conditions**	**References**
Psudomonas sp.	Soil	Effluents	Enhanced degradation of benzene and p-xylene in the presence of toluene	aerobic	Alvarez and Vogel (1991)
Aneurinibacillusaneurinilyticus (AY856831)	Pulp paper mill effluent	Kraft lignin	Decolorize kraft lignin, produce low-molecular-weight compounds	Facultative anaerobe/ microaerophilic	Chandra et al. (2007); Raj et al. (2007)
Azotobacter	Soil		Decolorize and solubilize lignin	Aerobic	Morii et al. (1995)
Bacillus cereus	Pulp paper mill effluent	Phenol (with glucose)	Degrade phenol and pentacholorophenol pollutants	Aerobic	Singh et al. (2009)
Bacillus megaterium	Soil		Decolorize and solubilize lignin		Morii et al. (1995)
Bacillus sp. (AY952465)	Pulp and paper sludge	Kraft lignin	Decolorize kraft lignin, produce low-molecular-weight compounds	Facultative anaerobe, 10 % NaCl	Chandra et al. (2007); Raj et al. (2007)
Citrobacterfreundii	Pulp paper mill effluent	10 % Black liquor	Decolorize lignin	Microaerophilic	Chandra and Abhishek (2011)
Citrobacter sp.	Rayon grade pulp black liquor	10 % Black liquor		Requires oxygen	Chandra et al. (2011)
Enterobacter	Soil	Lignin model compounds	Oxidative ligninolytic enzymes	Requires ABTS	Yadav et al. (2014)
Escherichia coli	Soil	Lignin model compounds	Oxidative ligninolytic enzymes	Requires ABTS	Yadav et al. (2014)
Klebsiella pneumonia	Rayon grade pulp black liquor	10 % Black liquor		Requires oxygen	Chandra et al. (2011)
Paenibacillus sp. (AY952466)	Pulp and paper sludge	Kraft lignin, phenol (with glucose)	Decolorize kraft lignin produce low-molecular-weight compounds	Facultative anaerobe, 3 % NaCl	Chandra et al. (2007); Raj et al. (2007)
Pantoea sp.	Pulp paper mill effluent		Decolorize, reduce COD and BOD,degrade lignin and chlorophenol, ligninolytic enzymes	Aerobic, pH 7	Chandra and Singh (2012)
Pseudochrobactrum glaciale	Pulp paper mill effluent		Decolorize, reduce COD and BOD, degrade lignin and chlorophenol, ligninolytic enzymes	Aerobic, pH 9	Chandra and Singh (2012)
Pseudomonas putida	Pulp paper mill effluent	Effluent	Remove color, phenolics, and sulfide	Aerobic	Chandra (2011)
Serratia marcescens	Soil; rayon grade pulp black Liquor	10 % Black liquor	Decolorize and solubilize lignin	Requires oxygen	Morri et al. (1995); Chandra et al. (2011)

Interestingly, microbes are well recorded in their capacity to degrade aromatic compounds such as lignin building blocks (Fuchs et al., 2011). Though the prevalence of ligninolytic enzyme has been reported in fungus, due to their growth limitations restricted large scale application. In contrast the bacterial open an ample opportunity for industrial application due to the versatile nature of their nutrients and environmental adaptions.

The most efficient lignin-degradation by a white-rot fungus has been reported due to their capability for secreting ligninolytic enzymes are also received our attention (Leonowicz et al., 1999). This fungus was used of lignin as a carbon source and degrades lignin. Moreover, several *Basidiomycetes* fungal species are also reported for lignin degradation by their extracellular ligninolytic enzymes which require low-molecular-weight mediators (Kumar and Chandra, 2020; Kumar et al., 2020a, b). This is the advanced biological technique for the removal of lignin by the use of *A. foetidus*, *P. chrysosporium* and *T. versicolor* (Fu and Viraraghavan, 2001). Furthermore, the fungus *P. chrysosporium* is well-known for the potential of degrading and detoxified xenobiotic contaminants present in wastewater (Kumar and Chandra, 2020). Basidiomycete *Bjerkandera adusta* is mostly found in Europe. This fungus can degrade xenobiotic compounds and is also capable of lignin degradation of paper mill wastewater (Sodaneath et al., 2017). It has been already reported that *B. adusta* has the ability to lignin biomineralization of soil and decolorization of industrial dye due to its lacquer and manganese peroxidase activity, but not to date at the industrial stage (Anastasi et al., 2010). The nutrients can improve the decolourizing efficiency of the fungus and also reduce BOD and COD of the effluent. Sucrose was found to be the best co-substrate for the degradation of lignin (Tiku et al., 2010; Kamali and Khodaparast, 2015). The use of *Tinctoria borbonica* has removed 90-99 % colour in 4 days of incubation (Abd El-Rahim and Zaki, 2005). Schizophyllum commune can be removed the 90 % colour of effluent and also reduced BOD and COD by 70 % and 72 % respectively under optimum conditions in 2 days incubation (Saritha et al., 2010). Fungus *Coriolus versicolor* im-mobilized in calcium alginate beads have been used in airlift bioreactor for the treatment of PPMW (Verma and Madamwar, 2002). The *Cyrus stercoreus* can also degrade lignin as efficiently as like as other white rot fungi did (Saritha et al., 2010). The *Trametes versicolor* strain B7 oxidized the chromophores present in PPMW in the presence of the carbohydrates, and highest decolourization was reported in the presence of glucose at optimum condition, pH 4.5-5.5 and temperature 30 ^{0}C (Diez et al., 1999). Decolourization of PPMW was reported maximum de-colourization of 34 % by *Trametes versicolor* on third-day incubation in effluent supplemented with 1.0 % (w/v) glucose as co-substrate and 0.2 % (w/v) urea as nitrogen source (Singhal and Thakur, 2009).

The optimum conditions for fungal growth are quite different from degradation. The pH range for optimum growth was 4.3 to 4.8 and degradation is retarded below pH 4.0 and above pH 5.0 due to lowering growth. Therefore, suggesting that the pH does not play a critical role in the treatment of PPMW (Saritha et al., 2010). The optimum temperature for the growth of fungus was 40 °C whereas the treatment is not much affected to the same narrow range of the temperature but slightly decrease in the rate at a temperature as low as 25 ^{0}C (Tiku et al., 2010). The fungal degradation needs oxygen and a co-substrate but not requires the addition of nitrogen source (Verma and Madamwar, 2002).

Microalgae treatment is of superior importance in today's era. It attracts scientists not only by treating waste but also by its products/by-products which are in high demand. Cyanobacteria from pulp-and-paper waste-treatment systems can contribute to the organic load of wastewater through photosynthesis, but can also remove simple organic compounds (i.e., sugars) through mixotrophy (Kirkwood et al., 2003). Unlike the heterotrophic bacteria, cyanobacteria do not appear to play an important role in organic waste removal during biological treatment (Kirkwood et al., 2003). However, the presence of large cyanobacterial communities in pulp-and-paper waste-treatment systems does suggest that their interactions with the heterotrophic community influences degradation of contaminants, and thus final wastewater toxicity. It has been reported that some algae like *Microcystis* sp. can decolourize diluted bleach kraft mill effluents (Iyovo et al., 2010; Sharma et al., 2014). According to Chandra and Singh (2012), pure and mixed algal cultures removed up to 70 % of colour within two months of incubation. Most cultures showed a similar colour reduction pattern consisting of a phase with declining rate. The decolorization was maximum remedial during the first 15-20 days of incubation and then gradually declined.

3.3 Molecular structure and mechanisms of ligninolytic enzymes

The most common lignocellulosic waste degrading enzymes are Laccase (EC 1.10.3.2), Lignin peroxidase (EC 1.11.1.14), Manganese peroxidase (EC 1.11.1.13) and Versatile peroxidase (EC 1.11.1.16). Recently, researches have been revealed the indirect role of several other enzymes such as feruloyl esterase (EC 3.1.1.73), aryl-alcohol oxidase (EC 1.1.3.7), quinone reductases (EC 1.6.5.5), lipases (EC 3.1.1.3), xylanase (EC 3.2.1.8) and catechol 2, 3-dioxygenase (EC 1.13.11.2) which facilitate the ligninolytic enzyme process for degradation of environmental pollutants (Guillen et al., 1997; Pothiraj et al., 2006; Ozer et al., 2019). Hydroxycinnamic acid, which is replaced by feruloyl esterase during lignin degradation, acts as a mediator for the laccase enzyme (Ozer et al., 2019). Aryl-alcohol oxidase and quinone reductases are involved in lignin degradation by various bacteria and fungi (Pothiraj et al., 2006). It was proposed to act on the reduction of quinones, which can use by ligninolytic enzymes or in support of a peroxidase reaction (Guillen et al., 1997). Similarly, lipase enzymes can be used as first-rate biocatalysts for in-situ per-acid formation in nonaqueous media, preliminary from carboxylic acids with diluted hydrogen peroxidase. Moreover, immobilized lipase was effectively used in a per acid-mediated lignocellulosic delignification method in a non-aqueous solvent: dimethyl carbonate was used for lipase-mediated oxidation both as a solvent and as an acyl-donor reagent (Wiermans et al., 2013; Pothiraj et al., 2006). Xylanase and catechol 2, 3-dioxygenase enzyme did not release significant amounts of lignin; however, the enzyme facilitated the extraction of the lignin fraction because breaking down monocyclic aromatic linking between xylan and lignin (Varnai et al., 2011; Eltis and Bolin, 1996). The ligninolytic enzymes are found in different molecular weights based on amino acid sequence and co-factor composition.

3.3.1 Laccase: structure and extremophilic properties

Laccase (EC 1.10.3.2) is copper-containing extracellular enzyme that consists of monomeric, dimeric and tetrameric glycoproteins. This is predominantly present in microorganisms i.e. bacteria, fungi, and actinomycetes (Janusz et al., 2017). The extremophilic adaptive features such as different compositions of amino acids, hydrophobic interaction, surface charges, tighter packing (compactness), a deleted loop, saturated/unsaturated fatty acid, salt bridge, disulfide bridge, hydrogen bond, several ions, and α-helical, as well as, cysteinyl-tRNA synthetase enzyme to maintain their active stability for catalytic functionalities in extreme conditions (Kumar and Chandra, 2020). Several workers have been reported different isoenzymes having an average molecular weight of 50–300 kD (Chandra and Chowdhary, 2015). Molecular characterization of laccase enzyme contains approximately 500 amino acid residues, consecutive three domains, with a greek key β barrel topology that is circulated in a single molecule (Matera et al., 2008). The first domain contains initial 150 amino acids, the second domain possesses between 150 and 300 residues and the third domain contains 300 to 500 amino acids (Chandra and Chowdhary, 2015). The structure is stabilized generally into two disulfide bridges bond localized between domains I to II and domains I to III are also present (Matera et al., 2008; Ferraroni et al., 2007; Bertrand et al., 2002). While some laccase is also present in three disulfide bridges bond. *Melanocarpus albomyces* has disulfide bridges inside domain I, another between domain I and domain III, and the last one between domain II and III (Hakulinen et al., 2002). Laccase has three types of copper atoms present in variations linked with coordinate with each other's and maintain the active site of the amino acids. The first domain (T1 Cu) is also called substrate reducing site shows a diverse triangular planar coordination similarly to other multicopper oxidases (Dwivedi et al., 2011). They displayed active site contains a sequence of the amino acid such as one cysteine (Cys) and two histidines (His) as equatorial ligands. In contrast, other multicopper oxidases enzyme contains an additional axial ligand with methionine (Matera et al., 2008; Ferraroni et al., 2007; Bertrand et al., 2002; Hakulinen et al., 2002). In another way, T2 Cu exhibits coordination with two His amino acids and a water molecule. While, in T3 Cu, the two Cu molecules with six His are present in two groups of three active sites to share oxygen molecule that reduced into a water molecule and split out (Matera et al., 2008; Ferraroni et al., 2007; Bertrand et al., 2002) (Figure 3.1). Laccase is considered to be an ideal “green catalyst” because of its oxidizing property in a vast variety of compounds using O_2 and liberating H_2O as the only by-product (Alcalde et al., 2007; Giardina et al., 2010). In Table 3.2, the detail of bacterial species strain/laccase-like protein and their functions are mentioned.

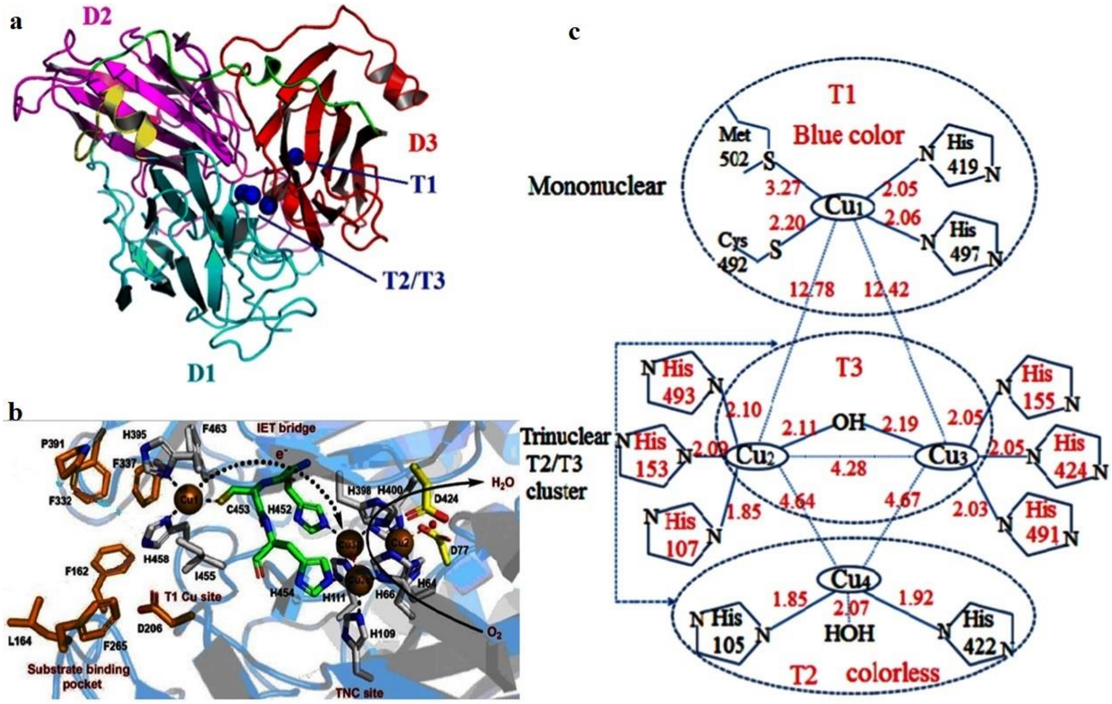


Fig. 3.1 Overall structure and copper centers of *B. subtilis* CotA-laccase (PDB code 1GSK). **(a)** Domains, T1, and T2/T3 copper (domains1, 2, and 3 represented in cyan, magenta, and red, respectively). D2 acts as a bridge between D1 and D3. A short α-helical fragment shown in yellow connects D1 and D2. A large loop segment shows D2 and D3 in green links) (Guan et al., 2017). **(b)** Representation of the laccase mechanism of action in the active site of *Trametes versicolor* laccase (PDB 1KYA) (Arregui et al., 2019). **(c)** Schematic of T1 and T2/T3 centers, including interatomic distances among all relevant atoms.

Table 3.2 Bacterial strain/laccase- like protein and their function

Strains name	**Protein**	**Functions**	**References**
Aquifexaeolicus	SufI-2983586	Work as cell division protein	Deckert et al. (1998)
Bacillus SF		Particularly stable at high temp. and pH if still bound to spore, Total decolorization of several phenolic dyes	Held et al. (2005)
Streptomyces griseus	EpoA	Morphogenesis	Endo et al. (2002), Endo et al. (2003)
Streptomyces lavendulae		Thermostable broad substrate specificity	Suzuki et al. (2003b)
Bordetella pertussis	Contig-449e		
Campylobacter jejuni	Contig-1e		
Caulobactercrescentus	Contig-122e		
Escherichia coli	PcoA-1073341		
Escherichia coli	YacK-	Cu^{2+} oxidation of	Kim et al. (2001);

	2506227	phenolate-siderophores ferrooxidase activity	Roberts et al. (2002)
Escherichia coli	CueO		
Mycobacterium avium	Contig-982e		
Mycobacterium tuberculosum	Rv0846c-2916905		
Pseudomonas putida	CumA-4580028	Mn^{2+} oxidation, Decolorization of violacein and azodyes	Brouwers et al. (1999); Senan and Abraham, (2004)
Pseudomonas syringae	CopA - 116921	Cu^{2+} resistance activity	Cha et al. (1991)
Pseudomonas aeruginosa	Contig-52e		
Rhodobactercapsulatus	3128288		
Xanthomonascampestris	CopA-1073083	Cu^{2+} resistance	Lee et al. (1994)
Yersinia pestis	Contig-768e		
Bacillus subtilis,	Cot A	Pigmentation of spores, UV and H_2O_2 resistance, Oxidation of substituted phenols	Hullo et al. (2001)
S.cerevisiae	RAD9		
Marinomonasmediterranea	PpoA	Pigmentation	Sanchez-Amat et al. (2001)

3.3.1.1 Natural mediators and synthetic mediators

The low redox potential of laccase hinders them from directly taking part in the catalysis of the complex compounds. Due to the uses of several natural and synthetic mediators should be possessing properties to cyclic bring efficient, low-cost, non-toxic, stable, oxidizing and reducing forms that enzyme reaction should not be inhibited (Morozova et al., 2007; Call and Mucke, 1997; Chandra, 2015). However, the redox mediators should be able to constantly control and conserve the cyclic redox process of conversion. A mediator is a small chemical compound that is continuously oxidized by the enzyme and then reduced by the substrate. As the compound especially lignocellulose complex, enzyme active site cannot penetrate, due to low affinity of the enzyme. Due to this enzyme cannot directly oxidize the substrate, for uses several mediators of the oxidizing substrate (Christopher et al., 2014). However, mediators for laccase induction are a component of lignin and it can be removed by several enzymes i.e. feruloyl esterase. This enzyme acts as a mediator and also helps in dissolving lignin polysaccharide complexes (Ozer et al., 2019). The laccase enzyme activities are decreased with increasing the substrate size, this substrate accessibility is overcome through the use of suitable laccase mediators. In the first step of the reaction, mediators are oxidized for unstable intermediates within high redox potential through laccase and followed by controlled reaction kinetics. The oxidized mediator diffuses away from the enzyme active site, and its small size can penetrate the pores of the plant cell walls to reach the target substrate. On the other hand, the vast range of substrate oxidized through laccase and can be further accelerated by a mediator. In some cases, the mediator is very reactive unstable cationic radicals which can oxidize more complex substrate before returning into their original state. Some ideal mediators like TEMPO (2,2,6,6-tetramethyl- 1-piperidinyloxy) and its analogs favor the ionic way and mediators such as HBT (1-hydroxybenztriazole) and ABTS [2, 20-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] react via radical paths (Wells and Teria, 2006; Fabbrini et al., 2002) shown in Figure 3.2.

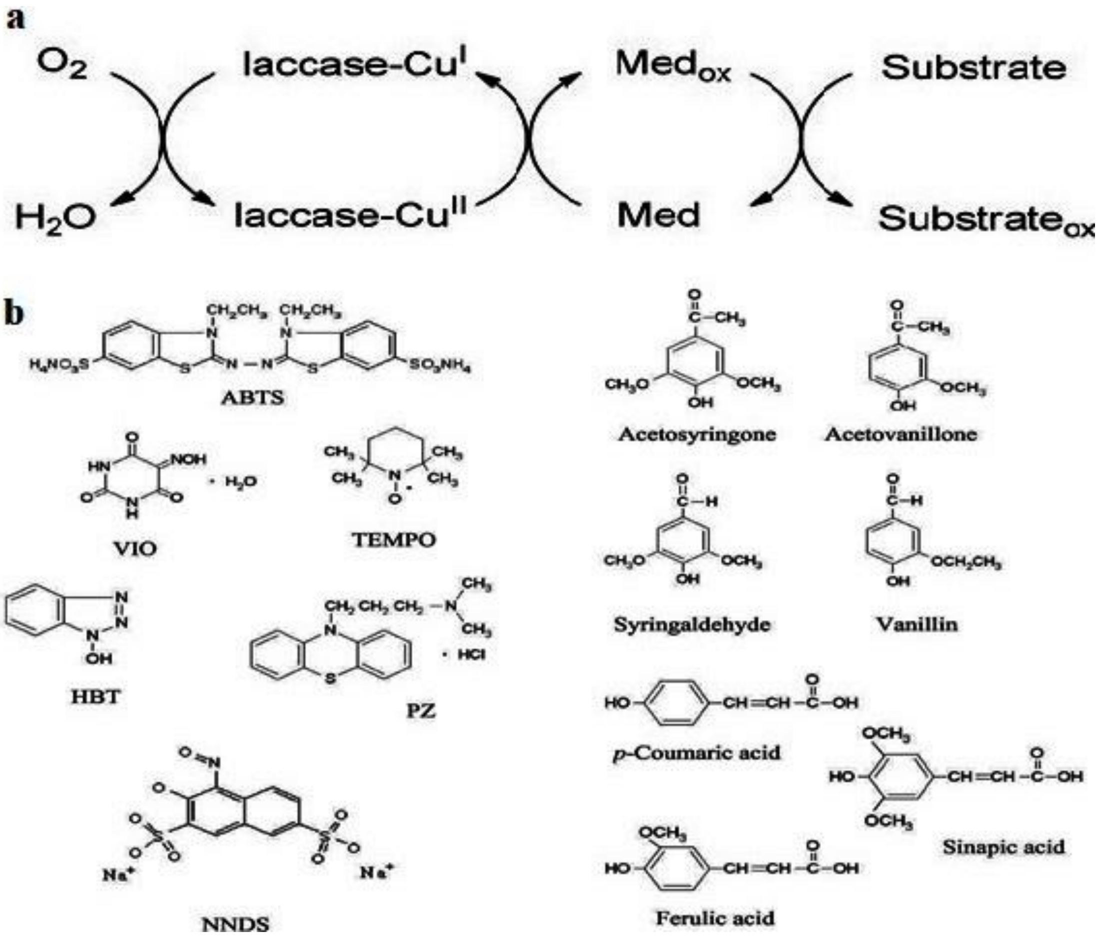


Fig. 3.2 (a) Laccase- mediator system, **(b)** Structures of some representative artificial (ABTS, HBT, violuric acid -VIO-, TEMPO, promazine -PZ- and 1-nitroso-naphthol-3,6-disulfonic acid -NNDS-) and natural mediators (AS, SA, vanillin, acetovanillone, p-coumaric acid, ferulic acid, and sinapic acid) (Kunamneni et al., 2008).

Enzyme inhibitors are small, low molecular weight molecule that binds to an enzyme and inhibiting activity by blocking the active site. However, decreasing the enzyme's activity can kill a pathogen or correct a difference in metabolic functions (Fabbrini et al., 2002). Several drugs may also consider as enzyme inhibitors. Some most common inhibitors are vanillin, catechol, 4-methyl catechol, vanillic acid, 4-Hydroxybenzaldehyde, 4-Hydroxybenzoic acid, syringic acid, ferulic acid, 2-furaldehyde, formic and acetic acid.

3.3.1.2 Oxidation mechanism of laccase: direct and in-direct

Laccase catalyzed reactions by two types: one is direct and the second is indirect substrate oxidation. The direct oxidation contains the oxidation of substrate to the similar radical as a result of direct contact that occurs with copper cluster (Matera et al., 2008) shown in Figure 4. In some reactions, direct oxidation is not possible because T1 copper ion contains low redox potential (Morozova et al., 2007).

However, in some cases limitation of the enzyme does not directly catalyze the substrate and proceeded by using several mediators which, two-step process: the first enzyme catalyzes the mediator and then the mediator oxidized the substrate known as the indirect substrate oxidation process shown in Figure 3.3.

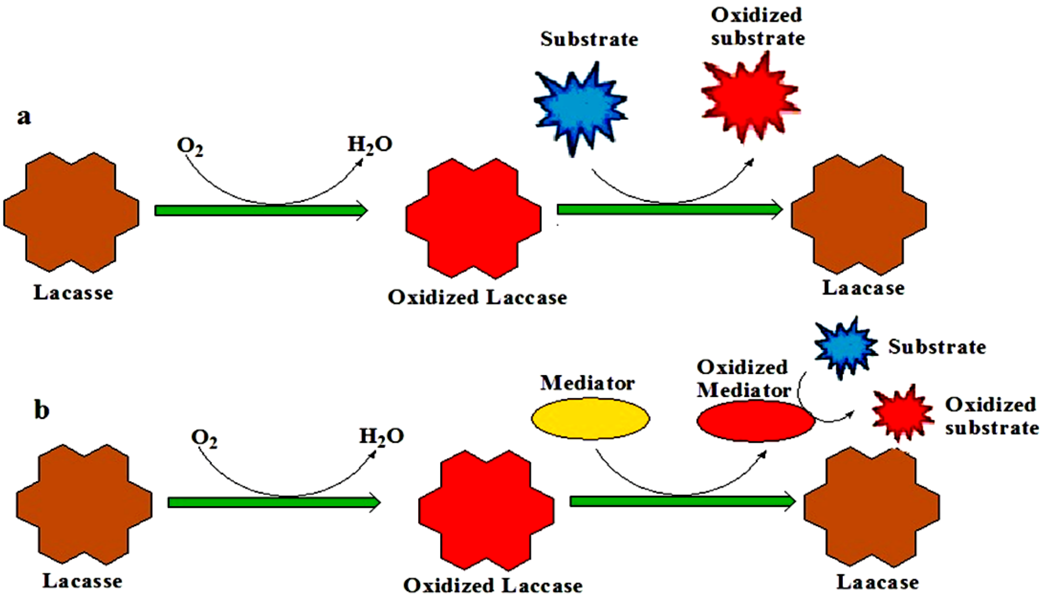


Fig. 3.3 Catalyzed cycle by laccase; **(a)** direct oxidation: the substrate is oxidized and **(b)** in-direct oxidation: the substrate is oxidized in the presence of a mediator (Komal et al., 2018).

To occurrence of reaction without any barrier, certain characteristics should be shown by mediators. These include a good substrate for laccase both in its oxidizing and reducing forms do not inhibit the enzymatic reaction, and conversion must be cyclic in nature (Komal et al., 2018).

3.3.1.3 Catalytic mechanisms of laccase

Laccase enzyme is broadly divided into three categories based on its functions: (1) ring cleavage of organic compounds, (2) degradation of biopolymers and (3) cross-linking structure of monomers (Kawai et al., 1988; Dwivedi et al., 2011), depends upon the Cu atoms dispersion among the three altered binding sites. These Cu atoms play a crucial role in the enzyme catalytic mechanisms. Laccase active site is well conserved it contains three copper sites namely type 1 (T1, one Cu atom), type 2 (T2, one Cu atom) and type 3 (T3, two Cu atoms) per molecule of laccase (Chandra and Chowdhary, 2015; Kumar and Chandra, 2020). T1Cu has been showing electro-paramagnetic resonance (EPR) and blue colour protein absorbance at about 600 nm. T2Cu does not show any colour but it can be detectable through electro-paramagnetic resonance (EPR) spectroscopy. And T3Cu are binuclear contains a pair of Cu atom that gives a weak absorbance (330 nm) and cannot be detected by EPR spectroscopy (Chandra and Chowdhary, 2015). T1Cu is the substrate-binding site while T2 and T3 Cu bind the inducer/inhibitor and oxygen binding site. The O_2 molecule binds to both Type 2 and Type 3 Cu active site that consists of a trinuclear cluster for the asymmetric activation. Molecular oxygen acts as an electron acceptor that represents one catalytic cycle of substrate oxidation. The electrons are transferred internally from the T1Cu site to a trinuclear cluster made up of Type 2 and Type 3 Cu sites, in which O_2 is involved in the enzyme catalytic mechanism (Chandra and Chowdhary, 2015; Kumar and Chandra, 2020). Hydrogen peroxidase is not detected outside of laccase during steady-state laccase catalysis which represented that a four-electron reduction of oxygen performed splitting of the water molecule (Gianfreda et al., 1999). Laccase enzyme has the ability to operate the array of reducing molecule oxygen-storing electrons from separate substrate oxidation to reduce the reaction of molecular oxygen shown in Figure 3.4. There unique broad range of substrate specificity acidic to alkaline conditions (Dwivedi et al., 2011; Kumar and Chandra, 2020).

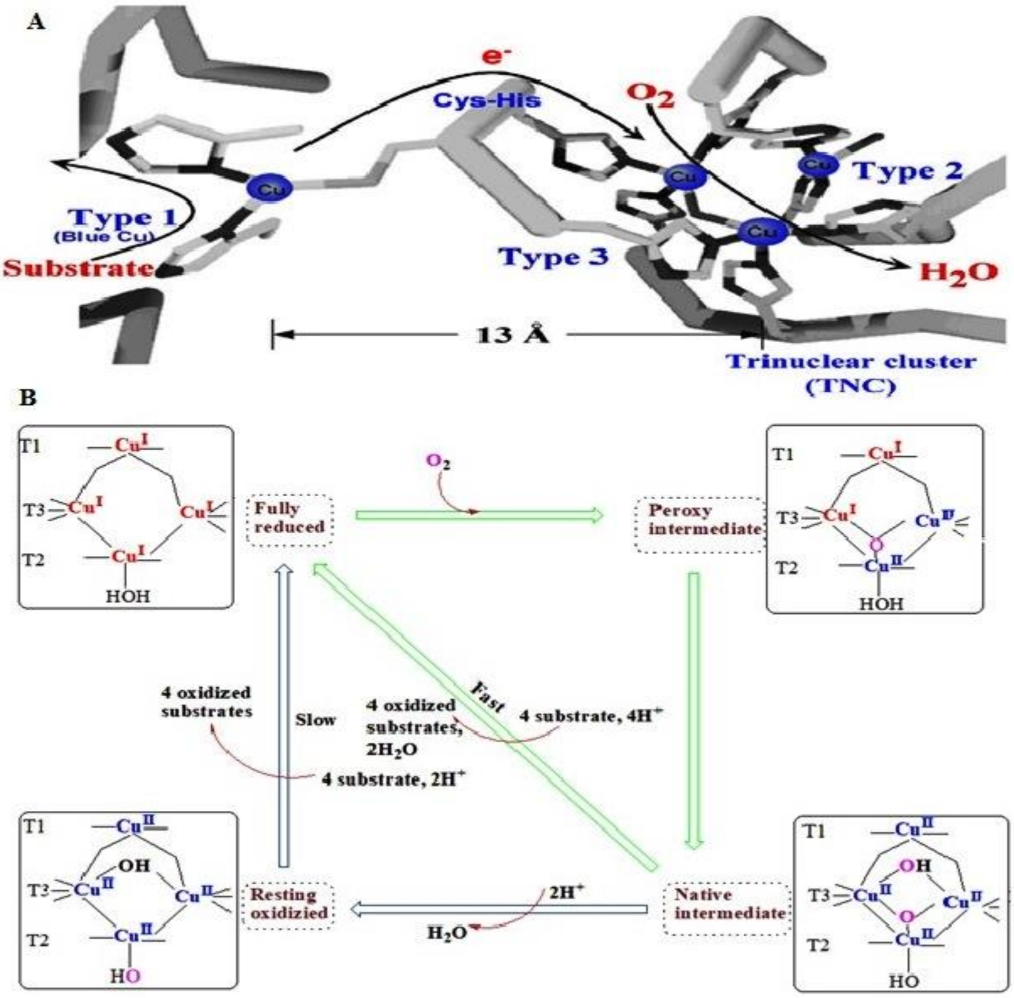


Fig. 3.4 (A) Laccase active site with arrows marking the flow of substrates, electrons (e-), and O_2, (B) mechanism of bacterial laccase (Solomon et al., 2008).

3.3.1.4 Role of nanoparticles in laccase production

The role of nanoparticles in the augmentation of laccase production and catalytic efficiency is an interesting topic from the research point of view. Because nanoparticles have a large surface area, they have a strong potential to have a wide range of applications. Enzymatic protein adsorption on these nanoparticles can boost enzyme activity. This also enhances the stabilities of enzymes in extremophilic conditions (Galhaup and Haltrich, 2001; Shah et al., 2010). The highest enzyme activity and stability can be achieved with adequate modifications and scaling of nanoparticles (Shah et al., 2010). The increased production and subsequent usage of engineered nanoparticles increase the probability of these substances being exposed to the natural environment (Weisner et al., 2006). Several metal ions such as Ag^+, Mn^{2+}, Cd^{2+}, Hg^{2+}, Cu^{2+} and Zn^{2+} and their influence on laccase as inducers have already been studied (Galhaup and Haltrich, 2001). Some of these have been shown to cause oxidative stress in the mycelia (Galhaup and Haltrich, 2001). On the fourth day of cultivation, all of the possible inducers were used directly in actively developing fungal cultures at a concentration of one millimolar (Galhaup and Haltrich, 2001). Some metal ions, such as Ag^+, Cd^{2+}, and Hg^{2+}, were extremely hazardous to organisms at specific optimal quantities, causing full growth suppression following their addition. According to Galhaup and Hltrich (2001), considerable enhancement of laccase activity was observed after a consistent increase in copper concentration. The optimized concentration of copper was between 1.5-2.0 mM for the laccase production by *Trametes pubescence*. Laccase growth was not inhibited at low $CuSO_4$ concentrations, while laccase activity was maximum at higher copper concentrations. $CuSO_4$ was added to the culture after days with a suitable concentration of glucose (13 g/l) in the medium, which resulted in higher laccase activity (Galhaup and Haltrich, 2001).

The copper-containing enzyme laccase immobilised on carbon was used to catalyse bioelectrocatalytic reduction of O_2 to H_2O under conditions of direct electron transfer communication between the electrode and the enzyme (Tsujimura et al., 2007; Blanford et al., 2008; Ivnitski et al., 2010). This appears to be particularly advantageous in the development of direct electron transfer biofuel cells (Kamitaka et al., 2007; Cracknell et al., 2008; Miyake et al., 2011; Zebda et al., 2011; Nazaruk et al., 2012). The bioelectrocatalytic reduction of O_2 at the intrinsic redox potentials of the enzyme active site, ideally near to the E^0 for O_2/H_2O reduction, is preferred by direct electron transfer electron coupling of laccase with the electrode. This is highly useful in the production of high potential biocathodes that work in ambient conditions and reduces the system's complexity when compared to mediated enzyme electrodes because it avoids issues like toxicity, leakage, and stability associated with the mediator in use (Soukharev et al., 2004; Balkenhohl et al., 2008; Cracknell et al., 2008; Nazaruk et al., 2009).

Laccase production can be easily stimulated by using small amounts of Cu and iron nanoparticles in millimolar amounts. What effect will copper and iron nanoparticles have on laccase activity and production by bacteria? This is something that should be investigated further. The effects of iron and copper-based nanoparticles on ligninolytic bacterial strains were demonstrated for laccase production.

3.3.2 Properties of lignin peroxidase

Lignin peroxidases (EC 1.11.1.14) belong to family oxidoreductase, which degrades lignin and its derivatives in the presence of H_2O_2 (Kumar and Chandra, 2020). These are heme-containing enzymes secreted mainly by higher fungi and some bacteria, which degrade the polymer via an oxidative process (Pothiraj et al., 2006). In Addition, some insects have used this enzyme to digest higher woody debris such as *Reticulitermes flavipes* and eastern subterranean termite. Chemical structure of lignin peroxidases (LiPs) are monomeric glycosylated containing enzyme that has molecular weight (40–68 kDa) with four carbohydrates, 370 water molecules, 343 amino acids residues, two calcium ions, and heme group (Choinowski et al., 1999). On the other hand, LiP is helicoidal in nature contains major and minor eight helixes, two anti-parallel beta-sheets and two domains at both sides of the heminic group. The heme; includes 40 residues, that connects to protein by hydrogen bridges. This group supplements the protein but has two small channels for solvent accessibility (Choinowski et al., 1999). Furthermore, heme iron is associated with His amino acid and the high redox potential of the enzyme. Distance between each heme group and His amino acid increases enzymatic redox potential and there creates an electronic deficiency in the porphyrin ring of the iron (Choinowski et al., 1999; Hammel and Cullen, 2008).

LiPs enzymes are produced by several ligninocellulytic microorganisms that are utilized in the efficient degradation of lignocellulosic waste. The overall LiP catalyzed mechanism is a two-step reaction involving the native enzyme of the ferric resting state, (1) the radical cation oxoferryl unstable intermediate compound I and (2) the impartial oxoferryl intermediate compound II (Castro et al., 2016). These enzymes also can oxidize substrates through electron transfer in the multi-step process (Wong, 2009). LiP is capable of oxidizing various aromatic organic compounds with high redox potentials than 1.4V by one electron abstraction, but this redox mechanism is not fully understood yet (Piontek et al., 2001). LiPs are also degrading various phenolic compounds as well as lignin in the presence of co-substrate of the H_2O_2 and mediators like veratryl alcohol (VA). The reducing veratryl alcohol is formed [Fe(IV) ¼ O, LiP-II] compound II and a VA radical cation ($VA^{\cdot+}$) and enzyme then return native state through 1^{e^-}reduction followed by finalizing the catalytic cycle (Pollegioni et al., 2015). The reaction of LiP-I with a reducing substrate to form LiP-II is pH-dependent and the rate decreased with increasing pH. Certainly, the pH dependence of reducing LiP–I to LiP-II rather than forming LiP–I determine the unusual low pH and optimized for the enzyme. The porphyrin π-cation radical first accepts an electron from the substrate in the first step of decrease and concomitant with a proton transfer to the distal His, LiP-II thus formed together with porphyrin field by the donor substrate is one-electron oxidation equal above the native LiP (Wong, 2009). In some cases, LiP–I can also return to the native state of the enzyme through a direct two-electron reduction (Castro et al., 2016) shown in Figure 3.5.

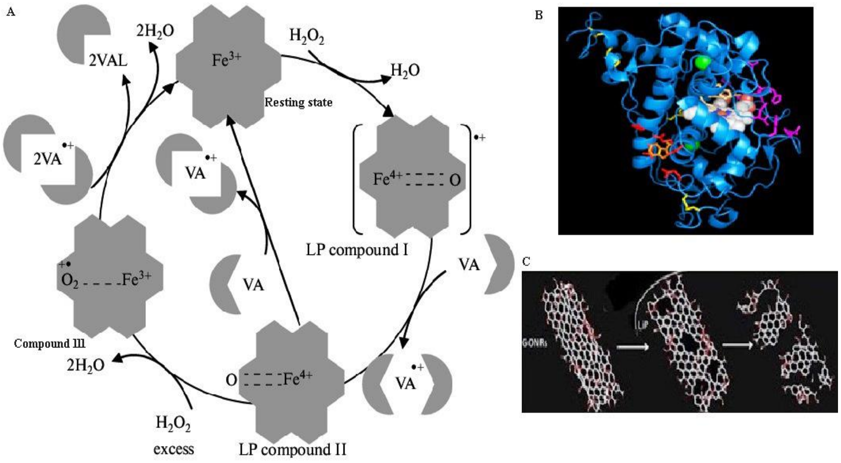


Fig. 3.5 a) Catalytic mechanisms of lignin peroxidase (Kulikova et al., 2011) b) LiP molecular structure c) LiP degradation of the compound structure.

3.3.3 Manganese peroxidase: structure and properties

Manganese peroxidase (MnP) is a heme-containing enzyme that belongs to the oxidoreductases family (Kuwahara et al., 1984). Ligninolytic microorganisms secrete MnP enzymes in solid and liquid state into their microenvironments (Hatakka et al., 2003). The production of MnP isozymes averages molecular mass of 40–50kDa has been reported in several bacteria, basidiomycetous fungi, and algae that utilize various genes after coding and regulating (Wong, 2009). MnP has been commonly observed as a crucial enzyme for lignin degradation. The molecular structure contains the MnP enzyme has two Ca^{2+} ions and five disulfide-bridging elements, that are responsible for maintaining the active site structure of the enzyme (Sutherland et al., 1997; Carmona-Ribeiro et al., 2015) as shown in Figure 3.6. The MnP enzyme active site comprises various amino acids such as proximal histidine ligand (His), H-bonded to an aspartic acid residue (Asp) and a distal side peroxidase-binding pocket containing catalytic His and arginine (Arg) residues. MnP enzyme is the crystal structure of the substrate-binding site and their mutants reveal that there is only one Mn^{2+} binding site, and consists of two water molecules, three acidic ligands and a heme propionate (Sundaramoorthy et al., 1997; Hofrichter, 2002). Ligninolytic microorganisms such as basidiomycetes, white-rot fungi, and bacteria oxidized Mn^{2+} to Mn^{3+} in a multistep process. The Mn^{2+} accelerates and triggers the functions and production of the substrate by MnP enzymes. Subsequently, the Mn^{3+} has been generated through the MnP enzyme, which acts as a mediator in the process of oxidation for several phenolic and non-phenolic compounds. The Mn^{3+} chelates oxalate is very small in size to diffuse into the active site of the enzyme. Certain case analogous structures of lignin, such as recalcitrant compounds buried deep within the soil, which is not inevitable for the enzymes (Ten Have and Teunissen, 2001). MnP enzymes possess specific, that act like an oxidase and peroxidase (Singh et al., 2011). Moreover, MnP not only catalyzes lignin and its derivative compounds but also catalyze the various non-phenolic compounds such as polycyclic aromatic hydrocarbons (PAH) through oxidation in the presence of H_2O_2 as an oxidant into the Mn^{2+} to Mn^{3+} (Steffen et al., 2003; Shin et al., 2005).

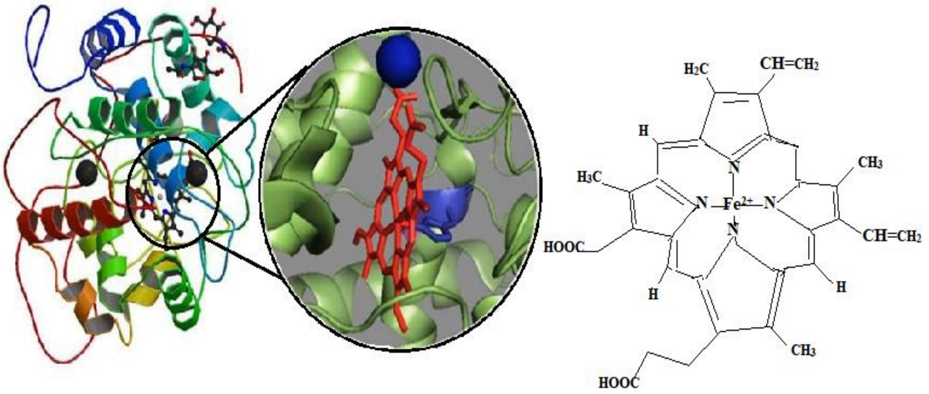


Fig. 3.6 Molecular structure and active site of MnP (Carmona-Ribeiro et al., 2015).

The MnP catalytic cycle is started by H_2O_2 through binding to the resting ferric enzyme from an iron-peroxide complex (Hofrichter, 2002). Cleavage of peroxide enzyme O–O bond needs a two-electron transfer from the heme-porphyrin that leads to the formation of unstable intermediate MnP Compound I, (Fe^{4+} oxo-porphyrin radical cation). Consequently, dioxygen bond is cleaved heterolytically and one water molecule is spelled out (Hofrichter, 2002). A mono chelated Mn^{2+} ion is oxidized to Mn^{3+} and acts as one electron donor for this porphyrin to form unstable intermediate Compound II. Reduction of this intermediate Compound II proceeds in a similar way that another Mn^{3+} is formed from Mn^{2+} of leading to return of its resting state enzyme and second water molecule has been spelled out (Hofrichter, 2002) shown in figure 3.7.

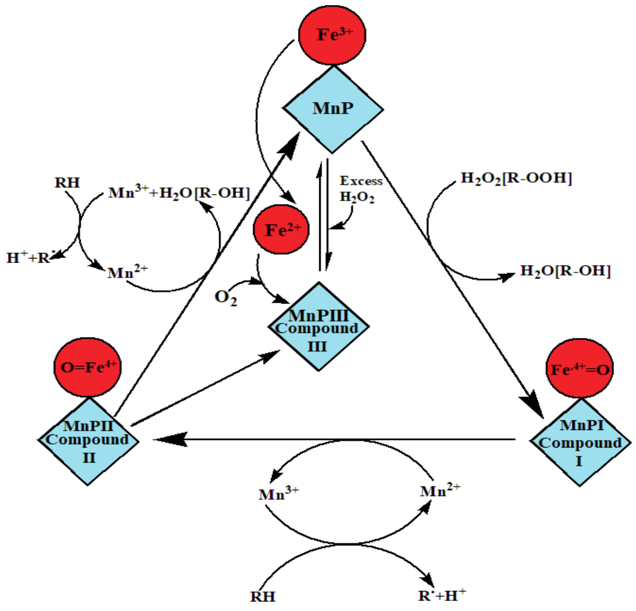


Fig. 3.7 Catalytic cycle of MnP (Hofrichter, 2002).

3.4 Role of ligninolytic enzymes in lignin degradation

Several microorganisms are producing ligninolytic enzymes that degrade lignocellulosic waste. These are major enzymes such as laccase and heme-containing peroxidases, for example, lignin, manganese, and multifunctional versatile peroxidase. In the last decade, there are several new approaches for delignification through enzyme activities have been reported (Figure 3.8). These enzymes have potential applications in biotechnology for biodegradation of lignin and other organic compounds. Recently, various residual lignocellulosic compounds are identified which are degraded by several bacteria and fungi through ligninolytic enzyme. The extracellular ligninolytic enzymes attack firstly on various types of bonds such as β-O-4 ether bond, biphenyl bond, O-demethylation enzyme systems, and aromatic organic pollutants performs one-electron oxidation to generate free cation radicals (Hammel, 1992). These cation radicals might suffer chemical reactions spontaneously such as hydroxylation or C–C bond cleavage resulting in hydrophilic products

(Hammel and Moen, 1991). In lignin structure, several linkages are present such as β-O-4 aryl-ether linkage and biphenyl linkage is a fundamental part of lignin (Bugg et al., 2011). According to Masai et al. (1999), bacterial species such as *S. paucimobilis* SYK-6 has the potential to grow under the stress condition of lignin derivatives such as vanillate and syringate as the carbon source. In this regard, vanillate and syringate have been altered into protocatechuate and 3-O-methylgallate through the O-demethylases LigM and DesA respectively (Kasai et al., 2005; Sato et al., 2009). Various fungal species are also metabolized those products that are taken up as a source of carbon to carbon dioxide (Hammel and Moen, 1991). The degradation mechanisms of ligninolytic enzymes are complex and connecting some cofactors low molecular weight compounds that can assist the redox mediators (Tunde and Ming, 2000). These are the multifaceted route i.e. hydroxylation, oxidation, reduction, and methylation for the degradation. On the contrary, the mechanisms of the ligninolytic enzymes for the degradation of lignin-containing compounds are not fully studied.

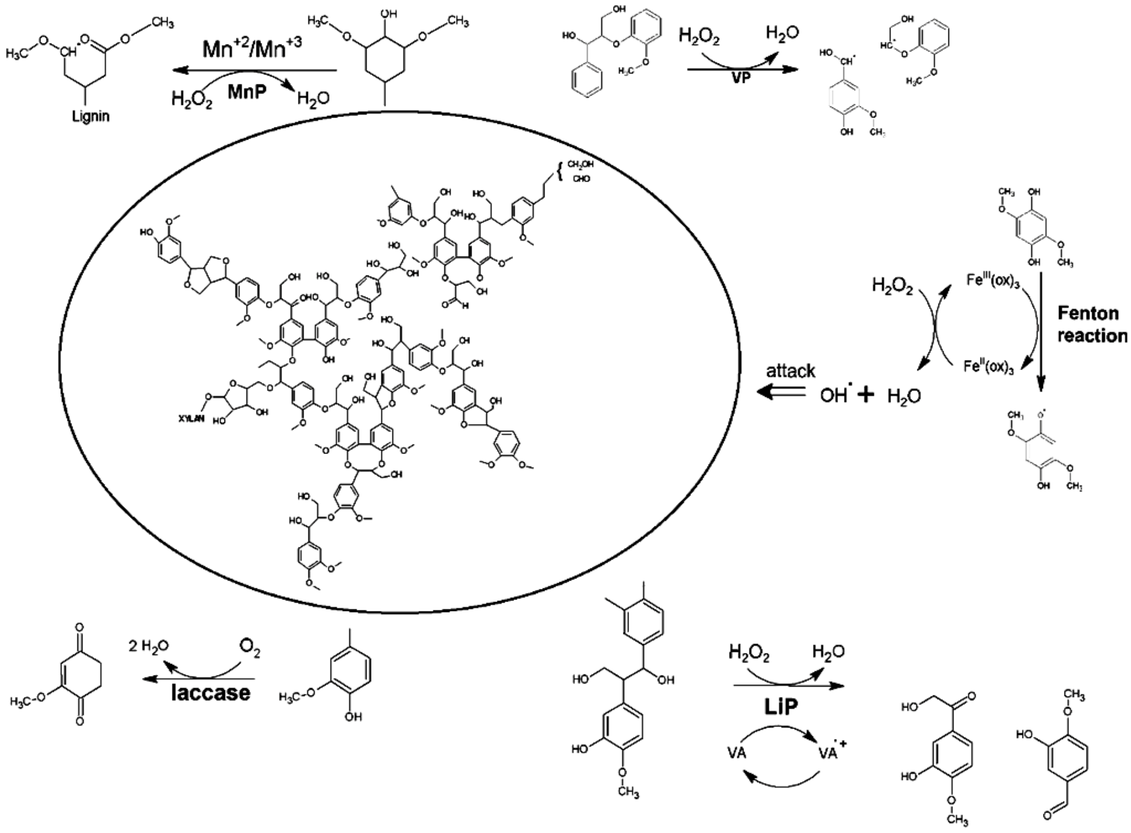


Fig. 3.8 Comparison of lignin degradation by laccase and peroxidases manganese peroxidase (MnP), versatile peroxidase (VP), lignin peroxidase (LiP) (Janusz et al., 2017).

3.5 Improvement strategies for laccase enzyme production

The successful and attractive ligninolytic enzymes of high yields and excellent performance have significant importance in the industrial sector. There are several traditionally as well as modern technologies that have been used for enhancing the degradation capacity of ligninolytic enzymes. The traditional technologies using ligninolytic enzymes have application in biotechnology. These are principally based on the screening of producing organisms and their optimization, purification of the enzyme and their biochemical characterization. However, modern technologies have been used for improved ligninolytic enzymes shown in Table 3.3: (1) firstly ligninolytic organisms are used in genetic engineering, somewhere a heterologously produced molecule can be altered at the level of DNA. The secondary steps were developments in protein engineering to improve strategies for the specificity, stability, and economy for chemically modifying the wild type enzyme immobilization PEGylation, glycosylation and hydrophobization.

Table 3.3 Improvement strategy for ligninolytic enzymes

Improvement strategy	Improved Characteristics	Reference
Novel inducer with agitated submerged cultures	increase in enzyme production	Liebeskind et al. (1990)
Immobilization on electrodes	High electronic transfer	Gutierrez Sanchez et al. (2012)
PEGylation	Improved thermal stability	Lopez-Cruz et al. (2010)
Immobilization and orientation on MWCNT electrodes	Efficient electron transfer	Lalaoui et al. (2016); Chen et al. (2016)
Substrate binding-pocket engineering	Improved catalytic efficiency	Gupta and Farinas (2009)
Genetic engineering and purification	Enzymes orientated immobilization	Pinar et al. (2017)
Codon optimization, heterologous expression, and computational analysis	Enhanced laccase production and substrate affinity	Rivera-Hoyos et al. (2015)
Heterologous expression, recombinant expression, and affinity chromatography	Improvement in production, time, and cost	Lambertz et al. (2016)
Immobilization and orientation on MWCNT electrodes	Efficient electron transfer	Lalaoui et al. (2016)
Solid-state and submerged fermentation	Production cost and optimization	Vantamuri and Kaliwal (2015)

3.6 Industrial applications of laccase enzyme

Extracellular ligninolytic enzymes have enormous potential for industrial applications in various areas including food processing, cosmetic, degradation and detoxification of pulp paper, textile, distillery effluent, and biosynthesis of various fine chemicals, biofuel production (Malherbe and Cloete, 2002; Maciel et al., 2010). Laccase-mediator system has been reported for depolymerization of lignin; delignify wood pulps, bleaching of kraft pulps as potential applications in the pulp and paper industry (Widsten and Kandelbauer, 2008). In the food industry, laccase can be used to certain process that enhances the colour appearance of food and beverages and eliminate the undesirable phenolic compounds. The major role of laccase in biodegradation of various polycyclic aromatic hydrocarbons (PAHs) and xenobiotics compounds (Anastasi et al., 2009; Wen et al., 2009; Robles-Hernandez et al., 2008). Its role has also been seen in the manufacturing of medical, and pharmaceuticals utensils as well as polymers production, coupling of phenols and steroids, complex natural products synthesis, personal hygienic products, biosensors and bioreporters areas (Maciel et al., 2010; Kunamneni et al., 2008; Mikolasch and Schauer, 2009; Barbosa et al., 2008; Lee et al., 2006; Ponzoni et al., 2007). The most important has been reported in the conversion of lignocellulose waste material into value-added products such as animal feeds, composite, pulp and paper, biofuels, fine chemicals and several enzymes (Malherbe and Cloete, 2002).

Conclusion

Lignocellulosic waste being a major pollutant of the environment, containing the complex composition of cellulose, hemicellulose, and lignin along with plant resins and fatty acids. Due to its complex structure; lignin, resin and plant fatty acid they are not easily degradable by microbial communities. Therefore, these constituents of lignocellulosic waste have been detected as residual organic pollutants discharged from agro-waste industries. Hence, these pollutants require special attention for their complete degradation. Moreover, the lignocellulosic waste has a strong binding tendency with cationic compounds i.e. heavy metals and salts, which aggravate their toxicity properties with the formation of complex structures. The natural capabilities of microorganisms to degrade lignocellulosic waste efficiently due to highly effective enzymatic systems are attractive as new strategies for the development of industrial processes. Therefore, the group of ligninolytic enzymes i.e. laccase, lignin peroxidase, manganese peroxidase, and versatile peroxidase involve in their sequential degradation and transformation. Due to their structural (i.e. copper, iron, manganese and calcium ions) specificity (hydrophobic interaction, salt bridge, disulfide bridge, and hydrogen bond) it retains extremophilic property and stability. But the detailed mechanism is still unknown? In addition, Ligninolytic enzymes are potential applications for the degradation of various environmental pollutants including polycyclic aromatic hydrocarbons, synthetic dyes, pesticides, herbicides, polychlorinated biphenyls, xenobiotics, and several other lignocellulosic compounds. Ligninolytic enzymes are able to penetrate inside the structural unit of lignocellulosic waste for degradation. This process makes use of another material that requires less energy, reduces the pollutants in the environment, as cost-effective and ecofriendly. Currently, several types of research across the planet worked on the biodegradation of industrial lignocellulosic waste using various microorganisms. The present study evaluated important information about the key role of ligninolytic enzymes in the biodegradation of lignocellulosic waste and their application in the field of biotechnology.

Chapter Four

Investigation of residual organic pollutants from different pulp and paper mill wastewater and their environmental effects after secondary treatment

Investigation of residual organic pollutants from different pulp and paper mill wastewater and their environmental effects after secondary treatment

4.1 Introduction

Environmental pollution is mainly caused due to the disposal of untreated or partially treated industrial waste to the aquatic ecosystem (Singh and Chandra, 2019). The Paper mill industry can be categorized mainly into two forms as pulp and paper mill (bleached paper) and Kraft paper mill (unbleached paper) depending upon their raw material and finished product. The pulp and paper mill produces the writing paper after using high amount of bleaching chemicals at the multistage bleaching process due to variation in their process and raw materials. During the pulping process, the cellulose fibers are separated due to solubilization of lignin (Hubbe et al., 2016). However, lignin remains up to 6 % as a major constituent of pulp even after chemical digestion (Julkapli and Bagheri, 2016). Therefore, multistage bleaching is required to increase the brightness of cellulose fibers. The cellulose fibers after bleaching become high tensile strength, this absorbed the additives used to modify pulp into chemically stable while paper and board products (Julkapli and Bagheri, 2016). But during washing and bleaching of pulp the poor quality of cellulosic fibers are washed away alone with wastewater containing the bleached chemical. Thus wastewater discharged from these industries is known as bleached paper mill wastewater (Hubbe et al., 2016). While the Kraft paper mills either do semi bleaching or do not use any bleaching process. The Kraft paper mills may also use recycled paper as raw material with pulp of wood for the production of different types of packaging paper or brown paper (Hubbe et al., 2016). Therefore, the pollutants present in discharged wastewater from both categories of the industry remain different due to variation process and raw materials. In the pulp and paper manufacturing process there is consumption of a large amount of water and energy, therefore, releases approximately 47,000-80,000 gallon wastewater per ton of paper production (Chandra et al., 2011). In India, there are approximately 859 paper industries as per the annual report of Central Pulp & Paper Research Institute (CPPRI, 2017-18). This reflects the magnitude of the entire environmental problems of the country. Though there is the globally known process of elemental chlorine-free (ECF) and total chlorine-free (TCF) bleaching but in India, the majority of bleached paper industry uses multistage bleaching process by using in sequence of chlorine (Cl_2), sodium hypochlorite (NaOCl), sodium hydroxide (NaOH), chlorine dioxide (ClO_2), oxygen (O_2), hydrogen peroxide (H_2O_2), ozone (O_3), acid washing (SO_2) and sodium dithionite (NaS_2O_4) along with biobleaching process by using xylanases respectively. Initially released wastewater from paper machine known as black liquor and contains the high amount of lignin, cellulose, hemicellulose, tannic acid, resin acids, plastids, chlorinated phenol, chlorinated hydrocarbon, surfactant, and biocides, etc. during pulping and papermaking process from industries in the form of black liquor (Chandra et al., 2011). The black liquor undergoes the primary and secondary biotreatment process in the industry for its degradation. But, during the secondary treatment process some biopolymers i.e. cellulose, hemicellulose, lignin, and chlorolignin are degraded up to certain extent but some compounds are only biotransformed as the metabolic product (Maurya et al., 2015). While the various organic pollutants which do not change much during secondary treatment and it is discharged either in their original form or with minor change in the paper mill wastewater even after secondary treatment is known as residual pollutants in wastewater of paper industry. This directly influences aquatic life due to their carcinogenic and mutagenic properties (Ericson and Larsson, 2000, Yadav and Chandra, 2018). Recent studies have highlighted their endocrine-disrupting chemicals (EDCs) properties that have been reported the world over for their adverse effect on the reproductive system of freshwater fish (Ericson and Larsson, 2000; Yadav and Chandra, 2018). Besides, due to the high chemical diversity of the organic pollutants present in bleached paper mill wastewater, the various toxic effect on the crop plant and other aquatic life have been also evaluated in recipient water sources (Rios et al., 2012; Yadav and Chandra, 2018). Furthermore, some residual organic pollutants present in this wastewater tend to persist in nature and showed toxicity to aquatic life with genotoxic and clastogenic properties. It has also high potential to migrate widely throughout the ecosystem due to their high solubility in water, which subsequently accumulated in the fatty tissue of animals including humans through food chain (Singh and Chandra, 2019). A significant amount of various heavy metals have been detected in the discharged wastewater of the paper industry after secondary treatment which makes more residual due to their strong binding tendency with various organic polymers present in the paper industry wastewater (Cailian et al., 2019). The organic pollutants with heavy metals have aggravated the toxicity in the environment and also influence the food chain (Cailian et al., 2019). Thus the paper mill wastewater is not only hazardous to the aquatic ecosystem but also health hazards to the downstream flora and fauna including the human (Rai et al., 2019). The accumulations of heavy metals and distribution among crop plants in a developing country are also reported (Rai et al., 2019). But, the detailed knowledge of various pollutants present in the paper industries and their toxicity in the environment are still not much known. Molecular dibenzophenol (dioxins and furans) and polychlorinated dibenzodioxins, recalcitrant compounds in paper mill wastewater are highly toxic and tend to persist in nature (Hubbe et al., 2016). However, the toxicity and lipophilicity of these compounds in higher animals can be enhanced by anaerobic bacteria that methylate chlorinated organic compounds (Yadav and Chandra, 2018). Hence, the US Environmental Protection Agency has levelled these residual organic pollutants (POPs) as priority pollutants. The discharged several pollutants from the paper industry not only affecting the surface water but it is also influencing the underground water quality (Kim, 1996; Singh and Chandra, 2019). Hence, the study has focused to identify the unknown residual organic pollutants from bleached and unbleached paper mill wastewater (BUPMW) after secondary treatment. The conventional toxicity test was also done to assess the toxicity of BUPMW on *Phaseolus aureus* and *Tubifex tubifex* worms. Hence, the study will be more effective and expand the knowledge of residual organic pollutants present in BUPMW for environmental safety.

4.2 Material and Methods

4.2.1 Sample collection

Bleached and unbleached paper mill wastewater sample collected after secondary treatment from the M/s Star Paper Mill in Saharanpur, UP India, and M/s Naini paper mill Ltd. Uttrakhand, India, they are located at Latitude: 29° 56’ 10.89” N to Longitude: 77° 34’ 13.51” E and Latitude 29°11'47.23" to 29°12'06.90"N. Longitude respectively (Fig. 4.1). This industry uses wheat straw, sugarcane bagasse, and eucalyptus, bamboo, popular wood as raw materials for paper making. Wastewater samples were collected in pre-sterilized jerry cane of 20 liters capacity and temperature was maintained at 4 ^{0}C and transported to the laboratory for analysis within 24 hours.

4.2.2 Physico-chemical analysis of BUPMW

The PPMW was processed within 24 h for analysis of different physicochemical parameters including colour, odor, pH, BOD, COD, TS, TDS, phenol, sulfate, phosphate, and heavy metals based on the standard methods for the examination of water and wastewater (APHA, 2012).

Fig. 4.1 Sample collecting site of **(a)** Star paper mill (bleached) and **(b)** Naini paper mill (unbleached).

4.2.2.1 Color (Visual Comparison Method)

Colour in water may be resulted due to the presence of natural metallic ions (Fe and Mn), humus and peat materials, plankton, weeds and industrial wastes. Colour is removed to make water suitable for general and industrial applications. Coloured industrial wastewater may require colour removal before discharge into water courses. In some highly coloured industrial wastewaters like pulp and paper mill wastewater, colour is principally contributed by lignins.

(a) Principle

Colour was determined by the visual comparison method of the sample with known concentration of colored samples. The comparison may also be made with special, properly calibrated, colored glass disks. The platinum-cobalt method of colour measurement is the standard method, the unit of colour being that produced by 1.0 mg platinum/l in the form of chloroplatinate ion. The ratio of cobalt to platinum may be varied to match the hue in special cases.

(b) Apparatus

a. Nessler tubes: Matched, 50 mL, tall form.

b. pH meter: for determining sample pH

(c) Reagents

a. Potassium chloroplatinate (K_2PtCl_6)

b. Cobaltous chloride ($CoCl_6.6H_2O$)

c. Hydrochloric acid (HCl)

(d) Procedure

a. Preparation of standards: Dissolved 1.246 g potassium chloroplatinate, K_2PtCl_6 (equivalent to 500 mg metallic Pt) and 1.0 g crystallized cobaltous chloride, $CoCl_2.6H_2O$ (equivalent to about 250 mg metallic Co) in distilled water containing 100 mL of concentrated HCl and diluted to 1000 mL with distilled water. This stock standard has a colour of 500 units. Then, prepared standards having colour of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, and 70 by diluting 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, and 7.0 mL stock standard with distilled water to 50 ml in Nessler tubes and protected these standards against evaporation and contamination when not in use.

(e) Calculation

Calculated the color units by the following equation

$$\text{Colour units} = \frac{A \times 50}{B}$$

Where,

A = Estimation colour of a diluted sample.

B = mL sample taken for dilution.

4.2.2.2 Biological oxygen demand (5-Day BOD Test)

(a) Principle

The method consisted of filling with samples to overflowing an airtight bottle of the specified size and incubating it at the specified size and incubating it at the specified temperature for 5 days. Dissolved oxygen (DO) is measured initially and after incubation, and BOD is computed from the difference between initial and final D.O. Because the initial D.O. is determined immediately after the dilution is made, all oxygen uptake, including that occurred during the first 15 min is included in BOD measurement.

(b) Apparatus

a. Incubation bottles: 300 mL capacity.

b. Air incubator or water bath: Thermostatically controlled at 20±1 ^{0}C exclude all light to prevent the possibility of photosynthetic production of DO.

(c) Reagents

a. Phosphate buffer solution: Dissolved 8.5 g KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $Na_2HPO_4.7H_2O$ and 1.7 g NH_4Cl in about 500 mL distilled water and diluted to 1000 mL. The pH should be 7.2 without further adjustment.

b. Magnesium sulfate solution: Dissolved 22.5 g $MgSO_4.7H_2O$ in distilled water and diluted to 1000 mL.

c. Calcium chloride solution: Dissolved 27.5 g $CaCl_2$ in distilled water and diluted to 1000 mL.

d. Ferric chloride solution: Dissolved 0.25 g $FeCl_3.6H_2O$ in distilled water and diluted to 1000 mL.

e. Sodium sulfite solution: Dissolved 1.575 g Na_2SO_3 in 1000 mL distilled water.

f. Manganous sulphate solution: Dissolved 480 g $MnSO_4.4H_2O$ in 1000 mL distilled water.

g. Alkai-iodide azide reagent: Dissolved 500 g NaOH and 135 g NaI in 1000 mL distilled water.

h. Starch: Dissolved 2 g starch in 1000 mL distilled water.

i. Standard sodium thiosulphate: Dissolved 6.025 g $Na_2S_2O_3.5H_2O$ and 0.4 g NaOH in 1000 mL distilled water.

(d) Procedure

a. Preparation of dilution water: Placed 300 mL sample water in a suitable bottle and added 1mL each of phosphate buffer, $MgSO_4$, $CaCl_2$ and $FeCl_3$ solutions/l of water, seeded dilution water 1mL L^{-1}. The temperature was maintained at 20±1 ºC.

b. Determination of initial D.O.: To the sample collected in a 300 mL bottle, added 1.0 mL $MnSO_4$ solution followed by addition of 1.0 mL alkaline-iodide-azide reagent, mixed by inverting the bottle a few times. Let the precipitate settle sufficiently and added 1.0 mL concentrated H_2SO_4 and mixed again by inverting bottle several times until dissolution was completed, titrated with 0.025 M $Na_2S_2O_3$ solution to a pale straw colour. Added few drops of starch solution and continued titration till disappearance of blue colour.

c. Incubation: Incubated at 20±1 ^{0}C BOD bottles containing 1000 × dilution, seed controls and dilution water blanks.

d. Determination of final D.O.: After 5 day incubation period determined dissolved oxygen in sample dilution as described above.

(e) Calculation

$$\mathrm{BOD_5\,(mg\,L^{-1})} = \frac{(D_1 - D_2) - (B_1 - B_2)}{P}$$

Where,

D_1 = D.O. of diluted sample immediately after preparation in mg L^{-1}

D_2 = D.O. of diluted sample after 5-day incubation at 20 ^{0}C in mg L^{-1}

P = Decimal volumetric fraction of sample used

B_1 = D.O. 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 sample to seed in seed control

4.2.2.3 Chemical oxygen demand (Open Reflux Method) (COD)

(a) Principle

The majority of organic matter is oxidized by a boiling mixture of chromic and sulfuric acids. A sample is refluxed in a strongly acid solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). 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.

(b) Apparatus

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.

b. Hot plate: Having sufficient power to produce at least 1.4 w/cm^2 of heating surface.

(c) Reagents

a. Standard potassium dichromate solution, 0.0417 M: Dissolved 12.259 g $K_2Cr_2O_7$, previously dried at 103 ^{0}C for 2 h, in distilled water and diluted to1000 mL.

b. Sulphuric acid reagent: Added 5.5 g Ag_2SO_4 in 1000 g sulphuric acid (H_2SO_4). Let stood for 1 to 2 days to dissolve Ag_2SO_4.

c. Ferroin indicator solution: Dissolved 1.484 g phenonthroline monohydrate and 695 mg $FeSO_4.7H_2O$ in distilled water and diluted to 100 mL.

d. Standard ferrous ammonium sulphate (FAS), titrant, 0.25N: Dissolved 98 g ferrous ammonium sulphate [$Fe(NH_4)_2(SO_4)_2.6H_2O$] in distilled water. Added 20 mL of concentrated sulphuric acid (H_2SO_4), and diluted to 100 mL. Standardized the solution against standard $K_2Cr_2O_7$ solution as follows: Diluted 10 mL standard $K_2Cr_2O_7$ up to 100 mL. Added 30 mL concentrated hydrogen sulfides and cooled and titrated with FAS titrant using 0.10 to 0.15 mL (2 to 3 drops) ferroin indicator.

$$\text{Molarity of FAS solution} = \frac{\text{Volume of 0.0417M } K_2Cr_2O_7 \text{ solution titrated (mL)} \times 0.25}{\text{Volume of FAS used in titration (mL)}}$$

e. Mercuric sulphate: Powdered mercuric sulphate ($HgSO_4$).

(d) Procedure

Taken 1.0 mL of sample 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, (added very slowly), with mixing to dissolve $HgSO_4$. Cooled while mixing to avoid the loss of volatile materials and added 25 mL of 0.0417M $K_2Cr_2O_7$ solution and mixed. Attached the flask to a condenser and turned on cooling water. The remaining sulphuric acid reagent (70 mL) was added through open end of condenser, continued swirling and mixing while adding sulphuric acid reagent. The open end of 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 distilled water. Disconnected reflux condenser and diluted mixture to about twice its volume with distilled water. Cooled to room temperature and titrated excess $K_2Cr_2O_7$ with FAS using 2-3 drops (0.10 to 0.15 mL) ferroin indicator. The first sharp change from blue-green to reddish-brown was taken as the end point of the titration. In same manner, refluxed and titrated a blank containing the reagents and a volume of distilled water equal to that of sample.

(e) Calculation

$$\mathrm{COD\ (mg\ O_2\ /L)} \quad = \frac{(\mathrm{A} - \mathrm{B})\ \times\ \mathrm{M}\ \times\ 8000}{\mathrm{mL\ of\ sample}}$$

Where,

A = mL FAS used for blank

B = mL FAS used for sample

M = Molarity of FAS

4.2.2.4 Total dissolved solids (dried at 180 ^{0}C)

(a) Principle

A well-mixed sample is filtered through a standard glass fiber filter, and the filtrate is evaporated to dryness in a weighed dish and dried to constant weight at 180 ^{0}C. The increase in dish weight represents the total dissolved solids. This procedure may be used for drying at other temperatures.

(b) Apparatus

a. Evaporating dishes: Dishes of 100 mL capacity made of one of the following materials:

1) Porcelain, 90 mm diam.

2) Platinum-Generally satisfactory for all purposes.

3) High-silica glass.

b. Muffle furnance for operation at 550 ^{0}C.

c. Steam bath

d. Desiccator provided with a desiccant containing a colour indicator of moisture concentration or an instrument indicator.

e. Drying oven, for operating at 103 to 105 ^{0}C.

f. Analytical balance, capable of weighing to 0.1 mg.

g. Magnetic stirrer with TFE stirring bar.

h. Wide-bore pipets

i. Graduated cylinder

j. Low form beaker

k. Glass-fiber filter disks without organic binder

l. Filtration apparatus with reservoir and coarse (40 to 60 µm) fritted disk as filter support. Suction flask of sufficient capacity for sample size selected.

4) Drying oven, for operation at 180±2 ^{0}C.

(c) Procedure

Heated clean dish to 103 to 105 ^{0}C for 1 h. Cooled in desiccator and weighed immediately. Now 50 ml of well-mixed sample was taken in a pre-weighed dish and evaporated to dryness in a drying oven at approximately 2 ^{0}C below boiling to prevent splattering. Dried evaporated sample for at least 1 h in an oven at 103 to 105 ^{0}C, cooled dish in desiccators to balance temperature and weighed. Repeated cycles of drying, cooling, desiccating and weighing till a constant weight was obtained, or until weight change was less than 4 % of previous weight or 0.5 mg.

(d) Calculation

$$\text{mg total dissolved solids/L} \quad = \frac{(\mathrm{A} - \mathrm{B}) \times 1000}{\text{Sample volume, mL}}$$

Where,

A= weight of dried residue + dish, mg, and
B= weight of dish, mg

4.2.2.5 Total solids (Dried at 103-105 ^{0}C)

(a) Principle

A well-mixed sample is evaporated in a weighed dish and dried to constant weight in an oven at 103 to 105 ^{0}C. The increase in weight over that of the empty dish represents the total solids.

(b) Apparatus

a. Evaporating dishes: Dishes of 100 mL capacity made of one of the following materials:

1) Porcelain, 90 mm diam

2) Platinum-Generally satisfactory for all purposes.

3) High-silica glass.

b. Muffle furnace for operation at 550 ^{0}C.

c. Steam bath.

d. Desiccator, provided with a desiccant containing a colour indicator of moisture concentration or an instrumental indicator.

e. Drying oven, for operating at 103 to 105 ^{0}C.

f. Analytical balance, capable of weighing to 0.1 mg.

g. Magnetic stirrer with TFE stirring bar.

h. Wide-bore pipets.

i. Graduated cylinder

j. Low-form beaker

(c) Procedure

Heated clean dish to 103 to 105 ^{0}C for 1 h. Cooled in desiccator and weighed immediately. Now 50 ml of well-mixed sample was taken in a pre-weighed dish and evaporated to dryness in a drying oven at approximately 2 ^{0}C below boiling to prevent splattering. Dried evaporated sample for at least 1 h in an oven at 103 to 105 ^{0}C, cooled dish in desiccator to balance temperature and weighed. Repeated cycles are drying, cooling, desiccating and weighing till a constant weight was obtained, or until weight change was less than 4 % of previous weight or 0.5 mg.

(e) Calculation

$$\text{mg total solids/L} \quad = \frac{(\text{A} - \text{B}) \times 1000}{\text{Sample volume, mL}}$$

Where,

A= weight of dried residue + dish, mg, and
B= weight of dish, mg

4.2.2.6 Estimation of phenolics

(a) Principle

Phenols, defined as hydroxy derivatives of benzene and its condensed nuclei may occur in domestic and industrial waste waters. Steam-distillable phenols react with 4-amino antipyrine at pH 7.9±0.1 in presence of potassium ferricyanide to form a coloured 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}.

(b) Apparatus

a. Photometric equipment: A spectrophotometer.

b. Filter funnels: Buchner type with fritted disk.

c. Filter paper: alternatively, an appropriate 11 cm filter paper for filtering chloroform ($CHCl_3$) extracts instead of the buchner-type funnels and anhydrous Na_2SO_4 can be used.

d. pH meter.

e. Separatory funnels 1000 mL Squibb form, without ground-glass stoppers and TFE stopcocks.

(c) Reagents

Prepared all the reagents in distilled water, it should be free of phenols and chlorines.

a. Stock phenol solution: Dissolved 100 mg 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 distilled water to 100 ml; [1 mL = 10 μg phenol, Prepared daily].

c. Standard phenol solution: Diluted 50 ml intermediate phenol solution to 500 ml with freshly boiled and cooled distilled water. [1 mL = 1 μg phenol, Prepared with in 2 h of use].

d. Bromate-bromide solution: Dissolved 2.784 g anhydrous potassium bromo-oxide ($KBrO_3$) in distilled water. Then, added 10 g 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 fresh concentrated NH_4OH to 1000 ml with distilled water.

g. Standard sodium thiosulphate titrant, 0.025 M: Dissolved 6.025 g $Na_2S_2O_3.5H_2O$ and 0.4 g NaOH in 1000 mL distilled water.

h. Starch solution: Dissolved 2.0 g laboratory-grade soluble starch and 0.2 g salicylic acid, as a preservative, in 1000 ml hot distilled water.

i. Phosphate buffer solution: Dissolved 104.5 g K_2HPO_4 and 72.3 g KH_2PO_4 in water and dilute to 1000 mL distilled water (pH=6.8).

j. 4-aminoantipyrine solution: Dissolved 2.0.g 4-aminoantipyrine in water and diluted to 100 mL. Prepared daily.

k. Potassium ferricyanide solution: Dissolved 8.0 g $K_3Fe(CN)_6$ 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).

n. Potassium iodide: Laboratory grade potassium iodide (KI) crystals.

(d) Procedure

A measured volume (25 mL) of the 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 500 mL of blank and a series of 500 mL phenol standards 5, 10, 20, 30, 40, and 50µg. The treated samples, blank and standards 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 amino antipyrine solution, mixed well followed by addition of 3 ml $K_3Fe(CN)_6$ solution. Mixed the solution well and let colour 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 $CHCl_3$ 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.

(e) Calculation

$$\text{Phenol } (\mu\text{g L}^{-1}) \quad = \frac{\text{A x 100}}{\text{B}}$$

Where,

A = µg phenol in sample, from calibration curve

B = mL original sample

4.2.2.7 Chloride (Cl^-) (Argentometric Method)

(a) Principle

In a neutral or slightly alkaline solution, potassium chromate can indicate the end point of the silver nitrate titration of chloride. Silver chloride is precipitated quantitatively before red silver chromate is formed.

(b) Apparatus

a. Erlenmeyer flask, 250 mL.

b. Buret, 50 mL.

(c) Reagents

a. Potassium chromate indicator solution: Dissolve 50 g K_2CrO_4 in a little distilled water. Add $AgNO_3$ solution until a definite red precipitate is formed. Let stand 12 h, filter, and dilute to 1 L with distilled water.

b. Standard silver nitrate titrant, 0.0141M (0.0141N): Dissolve 2.395 g $AgNO_3$ in distilled water and dilute to 1000 ml. Standardize against NaCl by the procedure described in 4b below; 1.00 mL = 500 µg Cl^-. Store in a brown bottle.

c. Standard sodium chloride, 0.0141M (0.0141N): Dissolve 824.0 mg NaCl (dried at 140^0C) in distilled water and dilute to 1000 mL; 1.00 mL = 500 µg Cl^-.

d. Special reagents for removal of interference:

1) Aluminum hydroxide suspension: Dissolve 125 g aluminum potassium sulfate or aluminum ammonium sulfate, $AlK(SO_4)_2{\cdot}12H_2O$ or $AlNH_4(SO_4)_2.12H_2O$, in 1 L distilled water. Warm to 60 ^{0}C and add 55 mL conc. ammonium hydroxide (NH_4OH) slowly with stirring. Let stand about 1 h, transfer to a large bottle, and wash precipitate by successive additions, with thorough mixing and decanting with distilled water, until free from chloride. When freshly prepared, the suspension occupies a volume of approximately 1 L.

2) Phenolphthalein indicator solution.

3) Sodium hydroxide, NaOH, 1N.

4) Sulfuric acid, H_2SO_4, 1N.

5) Hydrogen peroxide, H_2O_2, 30 %.

(d) Procedure

a. Sample preparation: Use a 100-mL sample or a suitable portion diluted to 100 mL. If the sample is highly coloured, add 3 mL $Al(OH)_3$ suspension, mix, let settle and filter. If sulfide, sulfite, or thiosulfate is present, add 1 mL H_2O_2 and stir for 1 min.

b. Titration: Directly titrate samples in the pH range 7 to 10. Adjust sample pH to 7 to 10 with H_2SO_4 or NaOH if it is not in this range. For adjustment, preferably use a pH meter with a non-chloride-type reference electrode. (If only a chloride-type electrode is available, determine amount of acid or alkali needed for adjustment and discard this sample portion. Treat a separate portion with the required acid or alkali and continue analysis.) Add 1.0 mL K_2CrO_4 indicator solution. Titrate with standard $AgNO_3$ titrant to a pinkish-yellow end point. Be consistent in end-point recognition. Standardize $AgNO_3$ titrant and establish reagent blank value by the titration method outlined above. A blank of 0.2 to 0.3 mL is usual.

(e) Calculation

$$\text{mg Cl}^-/\text{L} = \frac{(\text{A} - \text{B}) \times \text{N} \times 35450}{\text{Sample volume, mL}}$$

Where,

A= mL titration for sample

B= mL titration for blank, and

N= normality of $AgNO_3$

mg NaCl/L = (mg Cl^-/L) × 1.65

4.2.2.8 Phosphate

(a) Principle

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 orthophosphates in biological treatment processes or by receiving water biota. Molybdophosphoric acid is formed and reduced by stannous chloride to intensely coloured 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.

(b) Apparatus

a. Colorimetric equipment: Spectrophotometer (400-490 nm).

b. Acid washed glassware: Cleaned all glassware with hot diluted HCl and rinsed well with distilled water,

c. Filtration apparatus and filter paper: Whatman No. 42 or equivalent.

(c) Reagents

a. Phenolphthalein indicator aqueous solution.

b. Strong-acid solution: Slowly added 300 ml of concentrated H_2SO_4 to about 600 mL distilled water. When cold, added 4.0 mL concentrated HNO_3 and diluted to 1000 mL.

c. Ammonium molybdate reagent I: Dissolved 25 g $(NH_4)6MO_7O_{24}.4H_2O$ in 175 ml distilled water. Cautiously added 280 ml concentrated H_2SO_4 to 400 ml distilled water. Cooled, added molybdate solution and diluted to 1000 mL.

d. Stannous chloride reagent I: Dissolved 2.5 g fresh $SnCl_2.2H_2O$ in 100 mL 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 anhydrous KH_2PO_4 in distilled water and diluted to 1000 mL; 1.00 mL= 50.0 μg PO_4^{3-} - P.

f. Reagents for extraction:

1. Benzene-isobutanol solvent: Mixed equal volumes of benzene and isobutyl alcohol.

2. Ammonium molybdate reagent II: Dissolved 40.1g $(NH_4)_6MO_7O_{24}.4H_2O$ in approximately 500 ml distilled water. Slowly added 396 mL ammonium molybdate reagent I. Cooled and diluted to 1000 mL

3. Alcoholic sulphuric acid solution: Cautiously added 20 mL conc. H_2SO_4 to 980 mL methyl alcohol with continuous mixing.

4. Diluted stannous chloride reagent II: Mixed 8 mL stannous chloride reagent I with 50 mL glycerol. The reagent was stable for at least 6 months.

(d) Procedure

A measured volume (20 mL) of 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.

(e) Calculation

$$\text{P (mg L}^{-1}) \;=\; \frac{\text{mg P (in approximately 104.5 ml final volume)} \times 1000}{\text{mL of sample}}$$

4.2.2.9 Sulfate (SO_4^{2-}) (Gravimetric Method with Drying of Residue)

(a) Principle

Sulfate is precipitated in a 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 $BaSO_4$.

(b) Apparatus

a. Steam bath.

b. Drying oven, equipped with thermostatic control.

c. Muffle furnace, with temperature indicator.

d. Desiccator.

e. Analytical balance, capable of weighing to 0.1 mg.

f. Filter: Use one of the following:

1) Fritted-glass filter, fine (‘‘F’’) porosity, with a maximum pore size of 5 µm.

2) Membrane filters, with a pore size of about 0.45 µm.

b. Vacuum oven.

(c) Reagents

a. Methyl red indicator solution: Dissolve 100 mg methyl red sodium salt in distilled water and dilute to 100 mL.

b. Hydrochloric acid, HCl, 1 + 1.

c. Barium chloride solution: Dissolve 100 g $BaCl_2 \cdot 2H_2O$ in 1 L distilled water. Filter through a membrane filter or hard- finish filter paper before use; 1 mL is capable of precipitating approximately 40 mg SO_4^{2-}.

d. Silver nitrate-nitric acid reagent: Dissolve 8.5 g $AgNO_3$ and 0.5 mL conc. HNO_3 in 500 mL distilled water.

(d) Procedure

a. Precipitation of barium sulfate: A measured volume of sample (50 mL) was taken in a flask and pH was adjusted to 4.5 to 5.0 with HCl using a pH meter or the orange colour 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 ^{0}C.

b. Filtration and Weighing: Filtered $BaSO_4$ through a pre-weighed membrane filter at room temperature. Washed precipitate with several small portions of 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 ^{0}C. Cooled in desiccator and weighed.

(e) Calculation

$$\text{mg } SO_4^{2-}/\text{L} = \frac{\text{mg } BaSO_4 \times 411.5}{\text{mL sample}}$$

4.2.2.10 Nitrate (4500-NO_3^-) (Cadmium Reduction Method)

(a) Principle

NO_3^- is reduced almost quantitatively to nitrite (NO_2^-) in the presence of cadmium (Cd). This method uses commercially available Cd granules treated with copper sulfate ($CuSO_4$) and packed in a glass column. The NO_2^- produced thus is determined by diazotizing with sulfanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly coloured azo dye that is measured colorimetrically. A correction may be made for any NO_2^- present in the sample by analyzing without the reduction step. The applicable range of this method is 0.01 to 1.0 mg NO_3^--N/L.

The method is recommended especially for NO_3^- levels below 0.1 mg N/L where other methods lack adequate sensitivity.

(b) Apparatus

a. Reduction column: Purchase or construct the column*#(2) (Figure 4500-NO3 –:1) from a 100-mL volumetric pipet by removing the top portion. The column also can be constructed from two pieces of tubing joined end to end: join a 10-cm length of 3-cm-ID tubing to a 25-cm length of 3.5-mm-ID tubing. Add a TFE stopcock with metering valve1 to control flow rate.

b. Colorimetric equipment: One of the following is required:

1. Spectrophotometer, for use at 543 nm, providing a light path of 1 cm or longer.

2. Filter photometer, with light path of 1 cm or longer and equipped with a filter having maximum transmittance near 540 nm.

(c) Reagents

a. Nitrate-free water: The absorbance of a reagent blank prepared with this water should not exceed 0.01. Use for all solutions and dilutions.

b. Copper-cadmium granules: Wash 25 g new or used 20- to 100-mesh Cd granules with 6N HCl and rinse with water. Swirl Cd with 100 mL 2 % $CuSO_4$ solution for 5 min or until blue colour partially fades. Decant and repeat with fresh $CuSO_4$ until a brown colloidal precipitate begins to develop. Gently flush with water to remove all precipitated Cu.

c. Color reagent: Prepare as directed in Section 4500-NO_2^-.

d. Ammonium chloride-EDTA solution: Dissolve 13g NH_4Cl and 1.7g disodium ethylenediamine tetraacetate in 900 mL water. Adjust to pH 8.5 with conc NH_4OH and dilute to 1 L.

e. Dilute ammonium chloride-EDTA solution: Dilute 300 mL NH_4Cl-EDTA solution to 500 mL with water.

f. Hydrochloric acid, HCl, 6N.

g. Copper sulfate solution, 2 %: Dissolve 20 g $CuSO_4 .5H_2O$ in 500 mL water and dilute to 1 L.

h. Stock nitrate solution

i. Intermediate nitrate solution

j. Stock nitrite solution

k. Intermediate nitrite solution

l. Working nitrite solution: Dilute 50.0 mL intermediate nitrite solution to 500 mL with nitrite-free water; 1.00 mL = 5 mg NO_2^--N.

(d) Procedure

a. Preparation of reduction column: Insert a glass wool plug into bottom of reduction column and fill it with water. Add sufficient Cu-Cd granules to produce a column 18.5 cm long. Maintain water level above Cu-Cd granules to prevent entrapment of air. Wash column with 200 mL dilutes NH_4Cl-EDTA solution. Activate column by passing through it, at 7 to 10 mL/min, at least 100 mL of a solution composed of 25% 1.0 mg NO_3^--N/L standard and 75% NH_4Cl-EDTA solution.

b. Treatment of sample:

1. Turbidity removal—for turbid samples

2. pH adjustment—Adjust pH to between 7 and 9, as necessary, using a pH meter and dilute HCl or NaOH. This insures a pH of 8.5 after adding NH_4Cl- EDTA solution.

3. Sample reduction - To 25.0 mL sample or a portion diluted to 25.0 mL, add 75 mL NH_4Cl-EDTA solution and mix. Pour mixed sample into column and collect at a rate of 7 to 10 mL/min. Discard first 25 mL. Collect the rest in the original sample flask. There is no need to wash columns between samples, but if columns are not to be reused for several hours or longer, pour 50 mL dilute NH_4Cl-EDTA solution onto the top and let it pass through the system. Store the Cu-Cd column in this solution and never let it dry.

4. Color development and measurement - As soon as possible, and not more than 15 min after reduction, add 2.0 mL colour reagent to 50 mL sample and mix. Between 10 min and 2 h afterwards, measure absorbance at 543 nm against a distilled water-reagent blank.

NOTE: If NO_3^- concentration exceeds the standard curve range (about 1 mg N/L), use remainder of reduced sample to make an appropriate dilution and analyze again.

c. Standards: Using the intermediate NO_3^-- N solution, prepare standards in the range 0.05 to 1.0 mg NO_3^--N/L by diluting the following volumes to 100 mL in volumetric flasks: 0.5, 1.0, 2.0, 5.0, and 10.0 mL. Carry out reduction of standards exactly as described for samples. Compare at least one NO_2^- standard to a reduced NO_3^- standard at the same concentration to verify reduction column efficiency.

(e) Calculation

Obtain a standard curve by plotting absorbance of standards against NO_3^--N concentration. Compute sample concentrations directly from the standard curve. Report as milligrams oxidized N per liter (the sum of NO_3^--N plus NO_2^--N) unless the concentration of NO_2^--N is separately determined and subtracted.

4.2.2.11 Lignin estimation

(a) Klason and Acid-Soluble Lignin Determination

Samples were ground frozen with a blade-mill (Polymix PX-A10). The dry solids content of the milled wood samples was determined at 103 °C. The samples of air-dried wood powders (3 g) were extracted with acetone using a Soxhlet apparatus for 6 h (Dence, 1992). After evaporation of the solvents, the residues were dried at 103 °C, allowed to cool in a desiccator and then weighed. The amount of acid-insoluble lignin was determined by the Klason method (Dence, 1992). The samples of the extracted wood powders (300 mg) were treated with 3 cm3 of 72 % sulfuric acid under vacuum for 1 h. The mixtures were diluted with about 82-cm^3 portions of water and autoclaved at 125 °C for 1 h. The precipitates were collected with glass fiber filter SS GF 52 ×47 mm by suction filtration and washed with water. The filters with the acid-insoluble lignin (Klason lignin) were dried at 103 ^{0}C, cooled in the desiccator and weighed. In order to determine the amount of acid-soluble lignin, the filtrates were diluted with water to 250 cm^3. Absorption of the acid solutions with the dissolved lignin was measured at 203 nm using sulfuric acid of the same concentration as a blank. The absorbance readings were obtained with a Shimadzu UV-2401 PC UV-VIS Recording spectrophotometer. The total lignin (Klason lignin + acid-soluble lignin) content was calculated from the unextracted wood as follows: Klason lignin% $= p(100 - u)/m$, in which p = precipitate (g), u = extractives (%) and m = calculated dry weight of extracted sample (g). The acid-soluble lignin content was calculated using a lignin absorptivity of 128 $L \cdot g^{-1} \cdot cm^{-1}$ and corrected because of the absorption of carbohydrates according to (Hatfield et al., 1999). The total lignin content of each sample was determined as the mean of the duplicate measurements; this is referred to as the measured lignin content of the sample.

(b) AcBr-Based Determination

The powder was either lyophilized (birch tissues) or dried at 60 °C for 48 h (birch clones), and 5- or 10-mg samples were extracted with acetone to remove soluble extractives. Lignin contents were determined using the acetyl bromide method (Rolando et al., 1992; Hatfield et al., 1999). A sample of 5 or 10 mg of phloem and inner bark tissue was weighed into a 10-mL screw-capped test tube and sonicated with 5 mL of acetone for 30 min. The extract was pipetted off, and the extractive free sample was dried. It was redissolved in 5 mL 20 % (v/v) AcBr-acetic acid solution (containing 100 µL 70 % perchloric acid, a hazardous chemical) and the sample was kept in a block heater at 50 °C for 3 h with regular shaking (Rolando et al., 1992). After treatment, the sample was frozen at −20 °C for 15 min to stop the reaction. The melt solution was transferred to a 50-mL volumetric flask containing 5 mL 2 M NaOH and 12 mL 100 % acetic acid. The solution was diluted into 50 mL with acetic acid. The UV spectrum was measured with a Shimadzu UV-2401 spectrometer at 280 nm. Lignin content was calculated using the following expression:

$$\text{Lignin \%} = 100\,(A_s - A_b)V/aW$$

Where,

A_s = absorbance of sample

A_b = absorbance of blank

V = volume of solution

W = weight of sample

a = the absorptivity of a lignin standard calculated for each analysis series

AcBr determination was calibrated with the total lignin content (Klason + acid-soluble) of a silver birch wood sample (lignin standard).

4.2.2.12 Metals [Inductively Coupled Plasma (ICP) Emission Spectroscopy Method]

(a) Principle

To reduce interference by organic matter and to convert metals associated with particulates to form (usually the free metal ions) that can be determined by electrothermal atomic absorption spectrometry or inductively coupled plasma spectroscopy. This method is designed to determine trace metals and metalloids in surface, ground and drinking waters also.

(b) Apparatus

a. Hot plate

b. Conical (Erlenmeyer) flask, 125 mL, or griffin beakers, 150 mL, acid washed and rinsed with water

c. Volumetric flask, 100 mL

d. Watch glass, ribbed and unribbed

e. Safety shield

f. Safety goggles

(c) Reagents

a. Concentrated Nitric acid (HNO_3) and Perchloric acid ($HClO_4$)

b. Acid water solution containing HCl and water in a 1:1 ratio.

(d) Procedure

1. 1g of soil/water sample was placed in a 250 mL beaker

2. HNO_3 and $HClO_4$ in 3:1 ratio were added to the sample.

3. The sample was digested on a hot plat at 100 ^{0}C for 4-5 hrs until a whitish brown dry mass was obtained.

4. The sample after digestion was treated with acid water mixture and filtered through Whatman No. 42 filter paper.

5. The filtrate was analyzed for total Fe, Mn, Ni, Zn, Cu, Pb and Cd by using atomic absorption spectrophotometer.

(e) Calculations

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

Results were calculated up to three significant figures.

4.2.3 SEM EDXs and FT-IR Spectroscopy

The scanning electron microscope was used for the EDXs analysis in which 10 mg BUPMW dried sample was spotted on aluminum stubs (Yadav and Chandra, 2018). In elemental analysis of wastewater, an area was selected, for the elements in the sediment sample were analyzed by a high-resolution scanning electron microscope which was equipped with EDXs system (SEM, QUANTA FEG 450, Netherland).

The FT-IR analysis was done for analysis of functional groups present in organic chemicals of bleached and unbleached paper mill wastewater, and then the sample was dried at 104 to 106 ^{0}C in the oven. To perform this analysis, the absorbance spectra were recorded by FT-IR Spectrometer (Thermo Fisher Scientific, Model Nicolet 6700, USA). The whole analysis was performed in the mid-infrared region from 4000 to 400 cm^{-1} (Yadav and Chandra 2018). The data processing was performed using the software OMNIC™ (v7.4).

4.2.4 Extraction and identification of various residual organic pollutants from BUPMW

4.2.4.1 Liquid-liquid extraction

The organic pollutants present in BUPMW were extracted by dichloromethane under acidic conditions (pH <2.0) as previously described (Bharagava and Chandra, 2009). To extract the organic pollutants, a fixed volume (10 mL) of BUPMW was acidified with 35% hydrochloric acid and then placed in a separating funnel (100 mL), after which an equal volume of dichloromethane was added and the mixture was shaken continuously for 5 h with intermittent rests for liquid-liquid extraction (Fig. 4.2). The extraction was repeated successively three times to complete extraction of organic pollutants. The organic layers obtained from BUPMW were kept together and concentrated on a rotatory evaporator until complete solvent evaporation (Rotavapor RE 120, Buchi, Flawil, Sweden) at ≤40 ^{0}C. A similar process was followed for the extraction of various organic compounds products from PPMW. An aliquot of the concentrate was dissolved in 3.0 mL of dichloromethane, filtered through 0.22 μm syringe filters (Millipore Ltd., Bedford, MA, USA), and used for further GC-MS analysis.

4.2.4.2 Liquid-solid extraction

The organic pollutants present in sludge samples were extracted by the suitable solvent such as dichloromethane under acidic conditions (pH <2.0) as previously described (Kumar et al., 2020b). The procedure of liquid-solid extraction was the same as described in section 4.2.4.1.

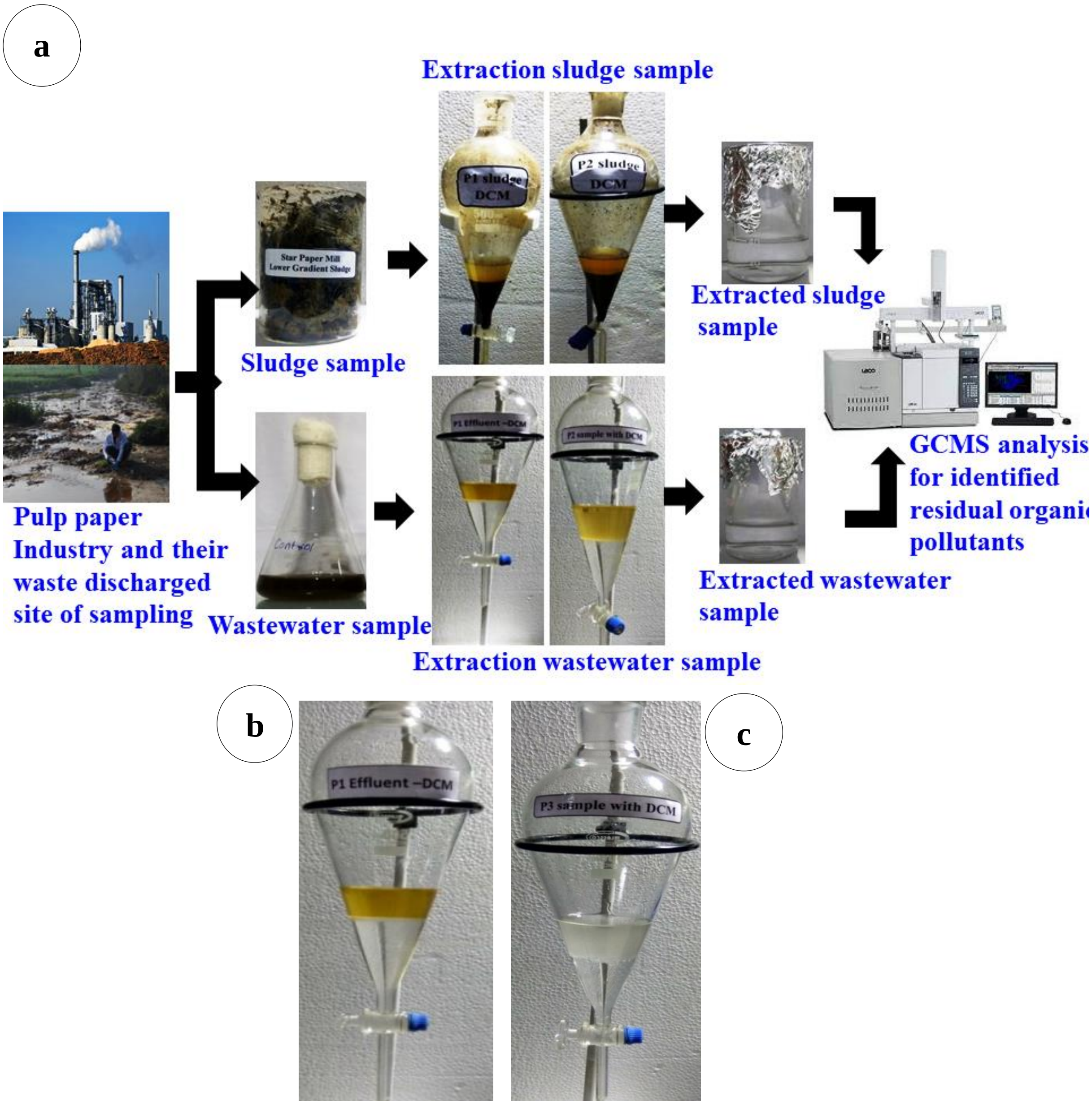


Fig. 4.2 **(a)** Systematic overview for extraction of organic pollutants by using liquid-liquid and liquid-solid extraction procedure for sample preparation GCMS analysis. **(b)** Star pulp and paper mill (Bleached) and **(c)** Naini paper pulp and paper mill wastewater (Unbleached) after intermittent rests for liquid-liquid extraction organic layer was formed organic layer obtained from paper mill wastewater + solvent mixture

4.2.4.2 GC-MS analysis

In GC-MS analysis, the extracted samples were derivatised with trimethylsilyl (TMS) as described by Kumar et al. (2020b). In this method, the extracted samples (300 μL) were transferred into GC vials and evaporated to dryness with nitrogen gas. Derivatization was performed by adding 50 μL of pyridine to the sample and then subjecting it to silylation with 80 μl trimethylsilyl BSTFA and TMCS. Next, the mixture was heated at 70 ^{0}C for 30 min with periodic shaking to dissolve the residues, after which it was subjected to GC-MS analysis. The system consisted of 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 Scientific, FL, USA). An aliquot (2.0 μl) of silylated sample was automatically injected into DB-5MS capillary column [30 m length × 0.25 μm I.D. × 0.25 mm film thickness of 5 % phenyl and 95 % methylpolysiloxane (v/v)] with helium as the carrier gas at a flow rate of 1.1 mL min-1. The initial oven temperature was 65 ^{0}C (hold for 2 min), after which it was increased to 230 ^{0}C at 6 ^{0}C min and then to 290 ^{0}C (hold for 20 min) at a rate of 10 ^{0}C min. The transfer line temperature and ion source temperature were kept at 290 and 220 ^{0}C, respectively. The mass spectrometer was operated in positive electron ionization (+EI) mode at an electron energy of 70 eV with a solvent delay of 7 min. Initially, derivatization of organic compounds was conducted in full scan mode using a mass range of 45–800 amu. The NIST mass spectral database library (version 1.0.0.12) available with the instrument was used to identify selected peaks.

4.2.5 Phytotoxicity assay of BUPMW

The phytotoxicity of any environmental sample using higher plants is a basic and authentic tool for toxicity evaluation of any environmental sample. Therefore, the toxicity of BUPMW against *P. mungo* L. seed germination was studied as previously described (OECD 2003). Before preparation of various concentrations of BUPMW, the pH was adjusted to 7.0 with 1N NaOH solution. For the seed germination experiment, BUPMW was applied at 10, 25, 50, 75 and 100 % (*v/v*). The surfaces of the seeds were sterilized with 0.1 % HgCl for 2 min to avoid any fungal contamination, after which they were subjected to repeated washings with sterilized distilled water (Santal et al., 2011). Subsequently, 10 seeds of *P. mungo* L. were placed separately in sterilized glass petri dishes of uniform size lined with two Whatman no. 1 filter paper disks. These filter discs were then moistened with 10 mL of tap water for control and with the same volume of BUPMW, after which they were incubated at room temperature for three consecutive days. A similar process was followed for the phototoxicity assessment of BUPMW. Germination, which was considered the visible protrusion of the radical from the seed coat, was expressed as a percentage as previously described (Bharagava and Chandra, 2010). Different seed germination parameters like germination percentage, relative toxicity percentage, phytotoxicity percentage, and stress tolerance index of seedling were also studied sing the following formula as described by David Noel and Rajan, (2015).

$$\text{Germination (\%)} = \frac{\text{No. of seed planted}}{\text{No. of seed germinated}}$$

Relative toxicity (%) = {(x-y)/x} × 100

Where,

x= Germination percentage in control at particular hours of incubation

y= Germination percentage in presence of effluent at the same hours of incubation

$$\text{Phtotoxicity (\%)} = \frac{\text{Radical length of control} - \text{radical length of test of tested} \times 100}{\text{Radical length of control}}$$

$$\text{Stress tolerance index} = \frac{\text{Mean length of longest root in treatment}}{\text{Mean length of longest root in control}}$$

4.2.5.1 Assay for α-amylase activity and determination of molecular weight by SDS-PAGE

According to Bharagava and Chandra, (2010), for the preparation of enzyme extract, seeds were taken from each concentration and homogenized with sodium acetate buffer solution (0.1 M, pH 4.8), filtered by two layers of cheesecloth 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 ^{0}C. The enzyme assay was performed with reaction mixture containing 0.5 ml of enzyme extract, 1.0 ml of 0.1 M acetate buffer, pH 4.8 and 1.0 ml of 0.1 % soluble starch. The reaction mixture was incubated for 10 min at room temperature followed by adding 1.0 mL of 0.1 % iodine reagent and 3.0 mL of 0.05 N HCl. The optical density (OD) was read at 620 nm and the decrease in absorbance was expressed in terms of amylase activity (Beri et al., 2007).

The alpha-amylase was purified from germinated seeds and determined molecular weight by denatured sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on 10 % polyacrylamide gel. To determine the molecular weight of the purified enzyme was compared with the band of standard α-amylase enzyme (Sigma–Aldrich, USA) and protein ladder (Bangalore Genei, India). After 10 hours of staining the gel was destined and imagined and stored in a gel documentation system (GeNei^{TM} UVITEC Cambridge).

4.2.6 Aquatic toxicity: *Tubifex tubifex*

Tubifex worms were collected from Gomati River Lucknow, from natural sources, and acclimatized to laboratory conditions for 7 days before experiments. The wastewater will be using *tubifex* worm because this worm is an important member of lentic fauna in the aquatic environment (Hellawell, 1986). For the toxicity evaluation to worms of *tubifex* will be inoculated into different concentration (Control) 0 %, 25 %, 50 %, 75 % and 100 % (v/v) of BUPMW sample used. Tests were conducted in 200 mL beakers containing 100ml of test water. Ten tubifex worms were exposed to each concentration and each concentration was tested in replicates of three. The test worms were considered dead when there were complete immobilization and no response to pressing with a blunt glass rod (Singh et al., 2007; Auston, 1973). Death was further confirmed by transferring worms back to fresh control tap water (Khangarot, 1991). Exposure to higher concentration results in a considerable disintegration of the hind part of the body in some worms (Nikon Corporation Tokyo Japan model ECLIPSE Ci-S 402397). Test water was renewed every 24 hours.

4.3 Results and Discussion

4.3.1 Physico-chemical analysis of BUPMW

The physico-chemical characteristics of the wastewater sample collected from the discharged site of bleached and unbleached paper mill wastewater (BUPMW) are shown in Table 4.1. The discharged wastewater showed high pollution parameters along with various heavy metals. Due to various pollutants in wastewater, it contributed high BOD and COD which were beyond the permissible limit (USEPA, 2012). The high values of BOD (bleached 225.0 ± 2.24 and unbleached 112.0 ± 1.14) and COD (bleached 543.04 ± 1.22 and unbleached 413.5 ± 0.81) in both wastewater samples might be due to the presence of high amount of complex organic compound in discharge wastewater. Because of bleached paper mill wastewater contents mixture of various salt and chlorinated compounds along with plant extract. Moreover, the presence of metallic contents of wastewater is also responsible for creation of organometallic compounds. Therefore, these complex compounds do not break during the wastewater treatment process and are discharged on paper mill wastewater. Hence, the toxic effect of discharged paper mill wastewater has been reported globally towards fish, microorganisms, flora, and fauna (Bajpai et al., 2015; Ugurlu et al., 2007). Unbleached paper mill wastewater is showed two-fold of suspended solid (270.0 ± 0.28) and dark colour than the bleached (164.0 ± 0.42) paper mill wastewater this might be due to the utilization of recycled and sugarcane bagasse's as raw material for the production of paper (Hubbe et al., 2016). Moreover, high content of soluble lignin (bleached 578 ± 0.13 and unbleached 285 ± 0.20) and its derivatives at alkaline pH (bleached 7.68 ± 0.21 and unbleached 7.95 ± 0.16) contributed dark brown colour (bleached 1275 ± 3.11 and unbleached 4180 ± 2.10), which blocks the sunlight penetration in receiving water bodies, and reduce photosynthetic activity, dissolved oxygen content rejuvenation, and thus negatively affect the aquatic life (Savant et al., 2006). The higher sulfate present in bleached paper mill wastewater than unbleached paper mill wastewater might be due to the application of high amount of sodium sulfate during the wood digestion and pulping process. Similarly, the high contents of phenol in wastewater might be due to release from the plant cell wall and other depository material in the wood digestion and pulping process (Chandra et al., 2011). The total phenol (bleached 13.195 ± 0.52 and unbleached 11.691 ± 0.82) and its derivatives induce toxicity as a carcinogen, immunotoxic, genotoxic, and a physiological effect on fish has been reported by Michael et al., (2010). The other pollution parameters like total solids, dissolved solids, suspended solids, fixed solids, volatile solids, chloride, phosphate, nitrate, and various heavy metals were also detected above the permissible limits (Table 4.1). Moreover, the high contents of iron, copper, chromium, lead and other heavy metals are also detected beyond the permissible limit that is also environmental hazards that aggravate the toxicity of organic pollutants (Phukan and Bhattacharyaa, 2003, Cailian et al., 2019). The contents of Fe and Cu might be in the wastewater from iron pipes and with other equipment due to corrosion during the beaching and washing of pulp. The copper toxicity to aquatic organisms i.e. Rainbow trout (*Salmo Gairdneri*), channel catfish (*Ictalurus punctatus*) has been also reported in presence of calcium and high pH in paper industry wastewater previously by other researchers (William and Peter, 1994). The bioaccumulation pattern of different heavy metals and their toxic effect in different organs of *Anabas testudineus* has been also reported on exposure to paper mill wastewater (Prasanta, 2014).

Table 4.1 Physicochemical analysis of bleached and unbleached paper mill wastewater

S. no	Parameters	Bleached paper mill wastewater	Unbleached paper mill wastewater	Permissible limits USEPA,2012
1.	pH	7.68 ± 0.21	7.95 ± 0.16	5–9
2.	BOD	225.0 ± 2.24	112.0 ± 1.14	40
3.	COD	543.04 ± 1.22	413.5 ± 0.81	120
4.	Total solids	3280.0 ± 1.32	698.0 ± 2.34	300 mg/l
5.	Dissolved solid	3110.0 ± 2.42	584.0 ± 1.82	500 mg/l
6.	Suspended solids	164.0 ± 0.42	270.0 ± 0.28	100
7.	Fixed Solids	2900.0 ± 2.35	566.0 ± 1.12	-
8.	Volatile solids	380.0 ± 0.31	132.0 ± 0.22	-
9.	Chloride	2350.0 ± 1.14	1740.0 ± 1.10	1500
10.	Phosphate as P	2.56 ± 0.12	1.1 ± 0.20	-
11.	Sulphate as SO_4^{-2}	713.1 ± 1.11	316.71 ± 0.81	250
12.	Nitrate	210.08 ± 2.32	47.17 ± 1.10	50
13.	Color	1275 ± 3.11	4180 ± 2.10	Colorless
14.	Total phenol	13.195 ± 0.52	11.691 ± 0.82	0.50

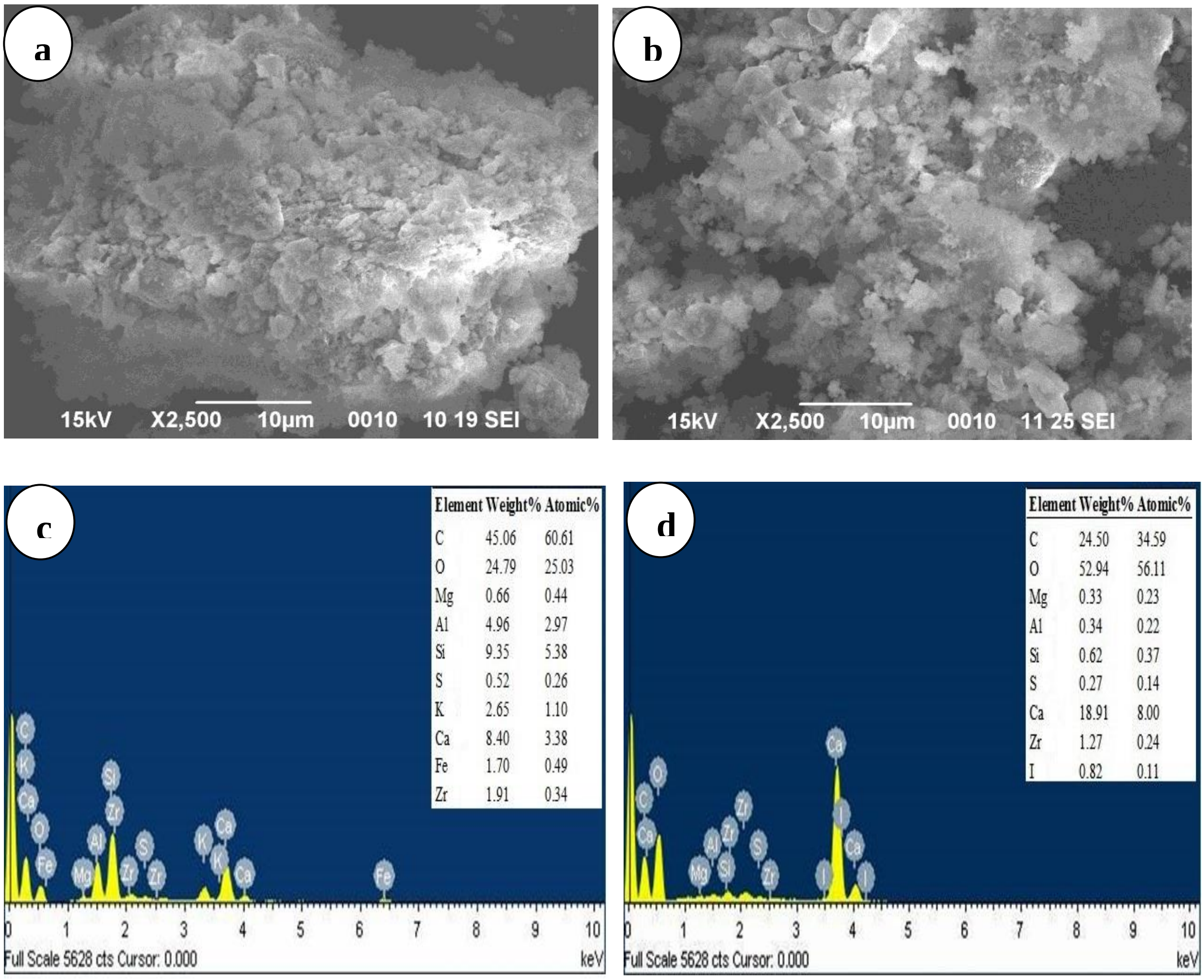
15.	Lignin	578 ± 0.13	285 ± 0.20	0.05
Heavy metals				
16.	Lead (Pb)	0.2550 ± 0.12	0.1360 ± 0.42	0.05
17.	Iron (Fe)	1.8598 ± 0.90	0.4232 ± 1.02	2
18.	Chromium (Cr)	0.3058 ± 0.01	0.0834 ± 0.11	0.05
19.	Copper (Cu)	1.3814 ± 0.16	0.8971 ± 0.20	0.5
20.	Cadmium (Cd)	0.08632 ± 0.13	0.219 ± 0.10	0.01
21.	Zinc (Zn)	0.0945 ± 1.21	0.0408 ± 0.82	2.00

Note: - All the parameters are in mg/l except pH and colour (Pt-co).

4.3.2 Characterization of residual organic pollutants

4.3.2.1 Scanning electron microscope (SEM-EDX)

The scanning electron microscope (SEM) analysis revealed the morphology of organic pollutants present in BUPMW as shown in figure 4.3a, b. The organic pollutants present in bleached paper mill wastewater showed compact particle and crystalline structure. While the unbleached paper mill wastewater showed abundantly presence of rough and large particulate matter scattered on the surface sample as shown in figure 4.3a, b. The compact irregular crystalline structure in bleached paper mill wastewater might be due to the fine particle of various organic pollutants generated either in pulping and bleaching or in the secondary treatment process of wastewater (Cherian and Siddiqua, 2019). While in unbleached paper mill wastewater the presence of large particles and scattered rough structure of various suspended particulate matter of raw material used in paper manufacturing. The finding has also correlated with the previous studies observed in paper mill wastewater reported by Yadav and Chandra, (2018). Further, the EDX analysis showed the elements present in weight % and atomic % in BUPMW as shown in figure 4.3c, d. The bleached paper mill wastewater major elements have C, O, Si, Al, Ca found, while unbleached paper mill wastewater major elements show the presence of O, C, and Zr. The presence of these elements has been also reported in previous studies but some toxic elements are beyond the permissible limit (Yadav and Chandra, 2018; Chandra et al., 2018, USEPA, 2012). Besides, heterogeneous particles allowed the adsorption of elements are released from the BUPMW which directly and indirectly, affects the surface water as well as groundwater and causes health hazards.



Element	Weight%	Atomic%
C	45.06	60.61
O	24.79	25.03
Mg	0.66	0.44
Al	4.96	2.97
Si	9.35	5.38
S	0.52	0.26
K	2.65	1.10
Ca	8.40	3.38
Fe	1.70	0.49
Zr	1.91	0.34

Element	Weight%	Atomic%
C	24.50	34.59
O	52.94	56.11
Mg	0.33	0.23
Al	0.34	0.22
Si	0.62	0.37
S	0.27	0.14
Ca	18.91	8.00
Zr	1.27	0.24
I	0.82	0.11

Fig. 4.3 SEM and EDX analysis of organic pollutants present in bleached paper mill wastewater (**a** and **c**) and unbleached paper mill wastewater **(b** and **d)** was done and observed in the surface sludge sample of wastewater.

4.3.2.2 Analysis of UV–Vis spectroscopy

The UV–Vis spectroscopy absorption scanning between 200-700 nm of BUPMW sample is showed absorption maxima at 280 nm and 230 nm respectively shown in figure 4.4. The absorption pattern of bleached paper mill wastewater showed maximum absorption spectra within the range 200-400 nm while the unbleached paper mill wastewater showed maximum absorption range between 200-300 nm. This revealed that bleached paper mill wastewater content majority of soluble lignocellulosic waste along with various chemicals. But, in unbleached paper mill waste contents prevailing with particulate matter which settled and soluble organic pollutants are restricted within the range of 200-300nm. This also indicated the presence of conjugated double bond of organic pollutants. The organic pollutants might be chlorophenols, resin acids, anthracene, fatty acids and aromatic ring containing pollutants (Chandra et al., 2018). The absorption of chlorolignin and its derivative compound, several functional chemical groups such as hydroxyl, methoxyl, carboxyl compounds also have been reported within the range of 200-400 nm (Jablonsky et al., 2015; Skulcova et al., 2017).

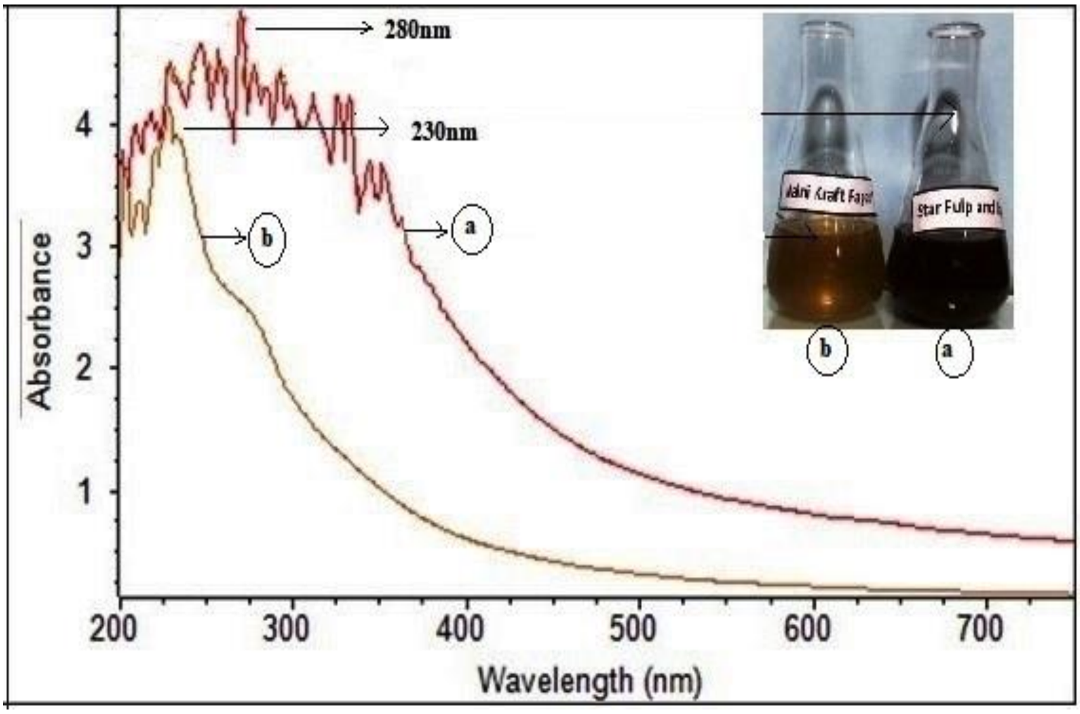


Fig. 4.4 Scanning Uv-Vis absorption spectrum analysis for pollutants present in paper mill wastewater **(a)** bleached paper mill wastewater and **(b)** unbleached paper mill wastewater collected after secondary treatment.

4.3.2.3 FT-IR spectroscopy

The FT-IR analysis of organic pollutants of BUPMW has shown in figure 4.5a, b. further their detail of the functional group presented in Table 4.2. The bleached paper mill wastewater analysis bandwidth range from 654.7-3402.2 cm^{-1} was done that showed similar to some extent with unbleached paper mill wastewater analysis bandwidth range 460.2-3410.0 cm^{-1} based on their functional group. BUPMW band range at 3402.2 and 3410.0 cm^{-1} represent the (O-H) functional group of alcohol and phenol which are generated from the plant extracts. Phenols are major component of plant cell walls and are also reported in previous studies (Chandra et al., 2011b). However, both sample band range 2933.2 and 2924.0 cm^{-1} represent the functional group (C-H) bond stretching showed the long chain of aliphatic compounds, which are generated from the plant's alkaloid that is used as a raw material in paper industries. The bleached paper mill wastewater showed broadband at 1653.4 cm^{-1} represented the (C=N stretch variable) these functional groups of ketone and amide, while the unbleached paper mill wastewater showed several functional groups (O-H) at broadband 2516.4 cm^{-1} corresponds the several carboxyl group's. It might be after biological treatment, in effluent treatment plant similar pattern for the generations of compounds are reported (Oller et al., 2011). The absorption band of BUPMW around 1539.5-1430.3 cm^{-1} (C=C aromatic skeletal lignin) is generated depending upon the raw material used for paper industries, while lignin is recalcitrant in nature and major component of wood (Chandra et al., 2018). However, bleached paper mill wastewater absorption band around the 1390.0 cm^{-1} (C-O-C stretch bending) in the spectrum biologically treatment of wastewater are very intensive indicating the abundant presence of ether and amine groups (Oller et al., 2011), while, unbleached paper mill wastewater band around 873.6 cm^{-1} (=C-H stretch bending) generally correspond to alkyl halide this might be generated during pulp bleaching (Yadav and Chandra, 2018). Besides, bleached paper mill wastewater less intense band at 1236.0 cm^{-1} (SO_3H symmetrical) sulphonic acids, while unbleached paper mill wastewater band 710.8 cm^{-1} (C-S stretch) sulfide group are present, which, these compound were released from the wood digestion during kraft recovery and sulfide recovery process (Philip et al., 2011). BUPMW absorption band 1063.1-460.2 cm^{-1} (=C-H bending) indicating the functional group of alkyl halides and alkanes, this is a major group of aliphatic compounds generated from plants source (fatty acids, resin acids) during the papermaking process. Bleached paper mill wastewater sample, it can be found that absorbance band around 654.7 cm^{-1} (C-Cl stretch) generally represent alkyl halides, which may be generated during pulp beaching and chlorination process of papermaking steps (Yadav and Chandra, 2018).

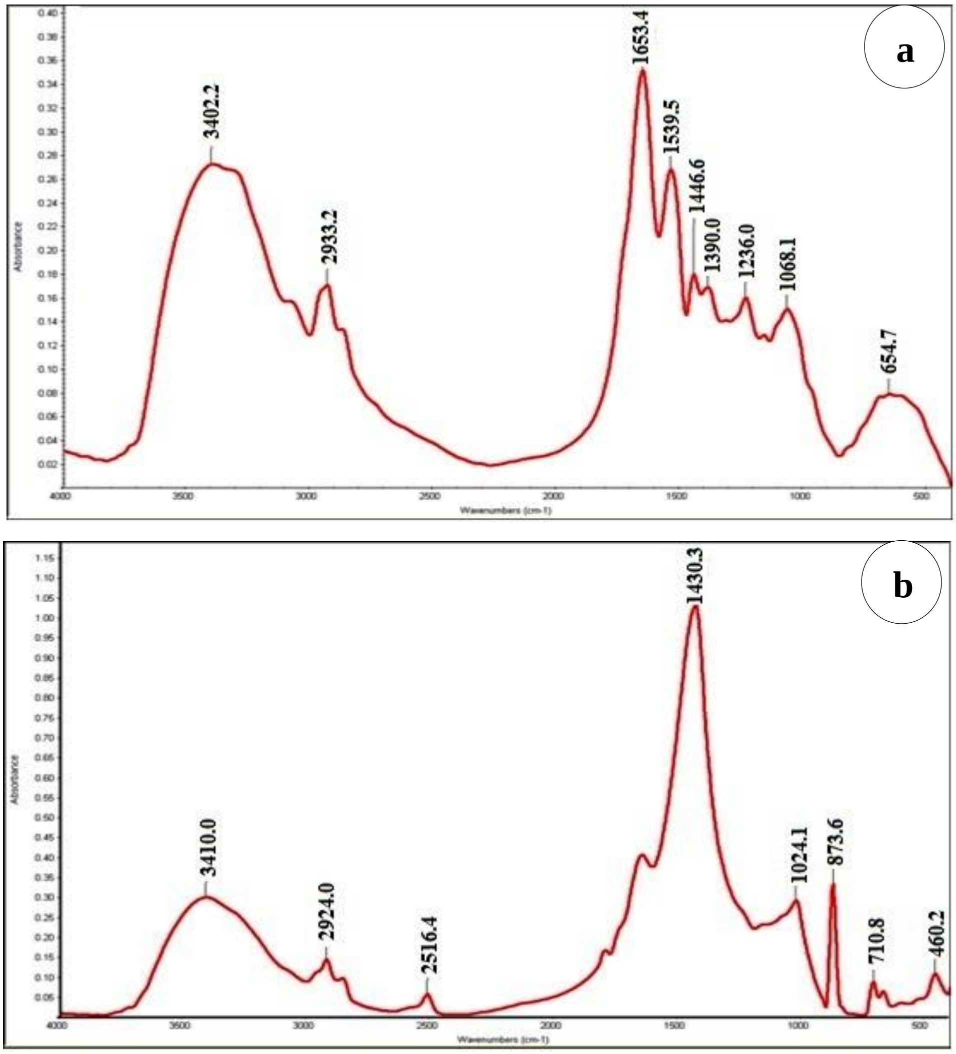


Fig. 4.5 FT-IR **a**nalysis of organic pollutants present in **(a)** bleached paper mill wastewater and **(b)** unbleached paper mill wastewater.

Table 4.2 FTIR bandwidth range were observed in bleached and unbleached paper mill wastewater and their functional group present in wastewater

Bleached paper mill wastewater			
Peak no	**Range (cm^{-1})**	**Bond**	**Functional groups**
1.	3402.2	O-H Stretch, Strong,broad	OH in alcohol, phenols
2.	2933.2	C-H Stretch Strong	Aliphatic chain CH_3, CH_2
3.	1653.4	C=N Stretch Variable	Ketones,amide
4.	1539.5	C=C Stretch medium	Aromatic skeletal in lignin
5.	1446.6	C-N Stretch	Amide group
6.	1390.0	C-O-C Stretch Bending Strong	Ether, amine group
7.	1236.0	SO_3H symmetrical	Sulphonic acid

8.	1063.1	=C-H Bending	Alkene
9.	654.7	C-Cl Stretch Strong	Alkyl halide
Unbleached paper mill wastewater			
1.	3410.0	O-H Stretch,H bond, Strong,broad	Alcohol, Phenols, carboxylic acids
2.	2924.0	C-H Stretch Strong	Long chain of aliphatic CH_2
3.	2516.4	O-H Stretch	Carbonyl acids,
4.	1430.3	C=C Stretch	Aromatic skeletal in lignin
5.	1024.1	Carbon ring in cyclic compounds	Aromatic rings
6.	873.6	=C-H Stretch Bending Strong	Alkyl halide
7.	710.8	C-S stretch	Sulfide group
8.	460.2	=C-H Bending	Alkene

4.3.2.4 Dichloromethane extract of bleached and unbleached paper mill wastewater

GC-MS analysis of bleached paper mill wastewater extracted with dichloromethane revealed various peaks at a different retention time (RT) (Fig. 4.6a and Table 4.3), the major peaks noted at retention time (RT) 9.50, 27.82, and 30.70, which corresponded to Silane,[1-cyclohexene-1,2-diylbis(oxy)]bis[trimethyl-(CAS); Hexadecanoic acid; trimethylsilyl ester; and Diethyl 3,4-dihydro-2-naphthyl-phosphonate, respectively. Some minor peaks were also noted at different RT 40.03, 43.03, 43.79, 45.02, 47.36 which corresponded to Methyl ester of [1'R-[1'à,4'á,8'à(E),8a'á]]-3-(8'-ethyl-1,2,2',3',6',7',8',8a'-octahydro-1-methyl-2-oxospiro[3H-indole-3,1'(5'H)-inndolizin-8'-yl)-2-propenoic acid N-oxide; 2-(1-Methyl-1H-2-pyrrolyl)quinoline; Cyclotrisiloxane, hexamethyl- (CAS); 5,11,17,23-Tetra-t-butyl-25,26,27,28-tetrahydroxycalix-4-arene; a-Fluoro-(p-methyl)chalcone respectively. These compounds have been reported previously also as residual organic pollutants generated from the papermaking process and chemicals used for storage (Yadav and Chandra, 2018). Moreover, some residual organic pollutants are also were used as a preservative and these compounds cause acute toxicity, respiratory tract irritation in human beings (Yadav and Chandra, 2018). These pollutants were discharged as wastewater after recovery and secondary treatment, as shown in Table 4.3. However, the detected residual organic compounds i.e. Dodecane, 1-iodo-; Decane, 1-iodo-; Tetracosane; Heneicosane; Hexadecanoic acid, trimethylsilyl ester; and Eicosane have been listed as potential endocrine-disrupting chemicals (EDCs) reported by the various workers (USEPA, 2012; Kim et al., 2016; Yadav Chandra, 2018). Moreover, Octadecane, 1-iodo- has been reported as acute lung injury toxicity to human beings (Yadav and Chandra, 2018). Hence, the other residual organic pollutants which were detected from GC-MS technique their fate in the environment along with the detected pollutants is still unknown because these pollutants might be intermediate compounds that were formed from the residual mixture of chlorine, resins, fatty acids and biocides used in paper industry during papermaking.

GC-MS analysis of unbleached paper mill wastewater extracted with dichloromethane showed various peaks at different retention times (RT) (Fig. 4.6b). The major peaks at RT 9.50, 27.82 and 30.69 were characterized as 7,8-Dimethyl-4-trifluoromethyl-(1H,5) benzodiazepine; Hexadecanoic acid, trimethylsilyl ester (CAS); Octadecanoic acid, trimethylsilyl ester, respectively (Table 4.4). In addition, some minor peaks were also noted at different RT showed in figure 4.6 and table 4.4. Previously reported of octadecadienoic acid at RT 30.69 with ethanol extractives of *Eucalyptus camaldulensis*, it causes acute lung injury and skin irritation reported by various worker (Peng and Wu, 2008; Kim et al., 2016; Yadav and Chandra, 2018). Mostly the nonadecane (CAS); dodecane, 1-iodo-; tricosane (CAS); and tetradecane were noted as EDC, effect nervous system, and odor nuisance reported in (USEPA, 2012). Sulfurous acid, 2-ethylhexyl hexyl ester and (3R)-3-Phenyl-2,3-dihydro-1H-isoindol-1-one) were released during the wood digestion in unbleached paper mill wastewater; and the sulphuric acid is also reported as EDC compounds according to (USEPA, 2012). Moreover, the various residual organic pollutants detected in the extracted wastewater and their toxicity was still unknown.

Major of the residual organic pollutants detected in BUPMW were of residual in nature because these compounds were not degraded during the secondary treatment process and directly discharged into the environment. Initially, these pollutants have originated during the process of wood digestion, pulping stages and recycling of paper in industries of bleached and unbleached paper mill wastewater. In addition, few residual organic pollutants might be generated by reactions with each other and metabolite is formed, moreover the bio-degradation or bio-transformation of highly residual organic pollutants via microbial activity might be generated in the movement of wastewater discharge drain. The detail of residual organic pollutants which are identified through GC-MS analysis with different RT (retention time) are listed in

Tables 4.3 and 4.4, their generating source with their toxicity has been also reported by different researchers in the world. However, the toxicity profile of several residual organic pollutants detected in BUPMW has not been explored until now and requires further investigation.

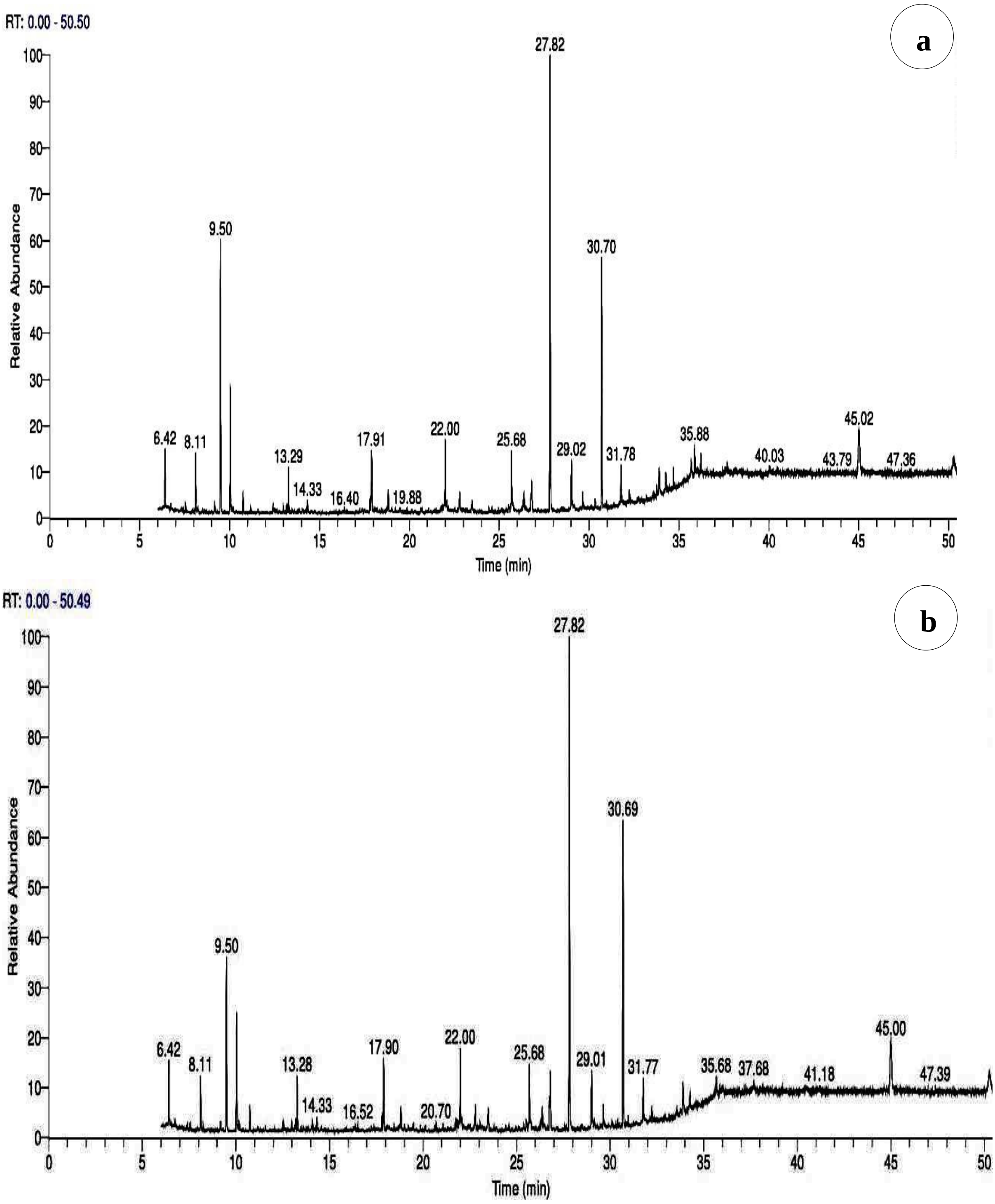


Fig. 4.6 GC-MS chromatogram of extracted organic pollutants from **(a)** bleached paper mill wastewater and **(b)** unbleached paper mill wastewater.

Table 4.3 Identified residual organic pollutant by GCMS analysis in bleached paper mill wastewater extract with dichloromethane (DCM)

S. No.	RT (min)	Identified compounds	Sources	Toxicity
1.	6.42	Silanol, trimethyl-, triester with boric acid (H3BO3) (CAS)	-	Causes skin, eye irritation
2.	8.11	D-LACTIC ACID-DITMS	Bacterial production	Diabetic ketoacidosis
3.	9.50	Silane,[1-cyclohexene-1,2-diylbis(oxy)]bis[trimethyl-(CAS)	Paper preservative agents	Irritate the skin and eyes, Breathing, nose and throat
4.	13.29	Dodecane, 1-iodo-	Plant chemical	EDC
5.	14.33	Decane, 1-iodo-	Used as a solvent and to make other chemicals.	EDC, Cell membrane integrity
6.	16.40	Tetracosane (CAS)	Plant chemical	EDC, effects nervous system, odor nuisance
7.	17.91	Heptacosane	Plant chemical	Effects of heart, kidneys, muscle and adipose bovine tissues.
8.	19.88	Pentan-1,3 dioldiisobutyrate, 2,2,4-trimethyl	Primary blowing agents used in the production of polystyrene foam and other foams for bleaching stage	May be fatal if swallowed and enters airways, Acute lung injury
9.	22.00	Octadecane, 1-iodo-	plant sources	Acute lung injury
10.	25.68	Heneicosane	plant sources	EDC
11.	27.82	Hexadecanoic acid, trimethylsilyl ester	Fatty acids in plant sources react other compounds make a complex structure	EDC, Skin irritation Respiratory tract irritation
12.	29.02	Eicosane (CAS)	Plant materials of paraffin waxes	EDC, Carcinogenic effects
13.	30.70	Diethyl 3,4-dihydro-2-naphthyl-phosphonate	Chemical reactions in bleaching stages	May cause long-term adverse effects in the aquatic environment
14.	31.78	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	Fatty acids in plant sources	EDC, may be fatal if swallowed and enters airways
15.	35.88	1,2,3,4,5-Pentaisopropylbis(cyclopentadienyl) cobalticimium (Cobaltocene)	Cobaltocene is prepared by the reaction of sodium cyclopentadienide (NaC_5H_5) with anhydrous cobalt(II) clorinations chloride in THF solution.	Suspected of causing genetic defects, Germ cell mutagenicity, Suspected causing cancer

Table 4.4 Identified residual organic pollutant by GCMS analysis in unbleached paper mill wastewater extract with dichloromethane (DCM)

S. No.	RT (min)	Identified compounds	Sources	Toxicity
1.	6.42	2-Phenyl-N-propyl-4-quinazolinamine	Alkaloid compound mixture bacteria and other chemicals	Nervous system, liver abnormalities
2.	8.11	Sulfurous acid, 2-ethylhexyl hexyl ester	Recovery process	EDC, Teratogenic
3.	9.50	7,8-Dimethyl-4 trifluoromethyl-(1H,5)benzodiazepine	Mixture of compounds formed various stage of pulping process	central nervous system, ataxia, and altered mental status
4.	13.28	Nonadecane (CAS)	Plant sources	EDC, pulmonary edema
5.	14.33	Dodecane, 1-iodo-	Plant chemical	EDC
6.	16.52	3,6-Dioxa-2,7-disilaoctane,	Mixture of compounds in bleaching stages	narcosis, depressed respiration, bradycardia
7.	17.90	Tricosane (CAS)	Plant sources	EDC, eye and skin irritation
8.	20.70	7,7-diphenyl-3,5-dioxo-7-hydroxyheptanenitrile	-	Acute toxicity, oral, dermal, inhalation
9.	22.00	Tetradecane	Plant chemical	EDC, effects nervous system, odor nuisance
10.	25.68	Eicosane (CAS)	Plant materials of paraffin waxes	EDC
11.	27.82	Hexadecanoic acid, trimethylsilyl ester (CAS)	Fatty acids in plant sources react other compound make a complex structure	EDC, Skin irritation Respiratory tract irritation
13.	30.69	Octadecanoic acid, trimethylsilyl ester	plant sources	Acute lung injury Skin irritation
14.	31.77	Eicosane (CAS)	Plant materials of paraffin waxes	EDC
15.	37.68	4-(Chloromethyl)-3-methyl-5-phenylisoxazole	Mixture of compounds in chlorination stage	Target organ toxicity, single exposure; Respiratory tract irritation
16.	41.18	N(2)-[2,5-di(t-butyl)phenyl]-N(1)-ethyl-3,4:9,10-perylenetetracarboxydiimide	Digestion stage are formed mixture of compounds	Respiratory tract irritation
17.	45.00	3,5,7-Tris(trimethylsiloxy)-2-[3,4-di(trimethylsiloxy)phenyl]-4H-1-benzopyran-4one	Mixture of compounds in Biocide	hepatic and renal cell damage
18.	47.39	(3R)-3-Phenyl-2,3-dihydro-1H-isoindol-1-one	Bleaching stage mixture of compounds	Acute exposure to hexadecane causes irritation, CNS depression

4.3.3 Phytotoxicity evaluation

The collected samples after discharged from industry were assessed for its phytotoxicity using *Phaseolus aureus* seed germination test. The result of germination as well as seedling growth of *Phaseolus aureus* seeds in different concentrations (25, 50, 75 and 100 %) BUPMW and sludge samples as presented in figure 4.7 and table 4.5. The seedling growth compared to controls, root lengths and shoots of seedling were highest at 10 % (v/v) wastewater but the further increase of wastewater concentration showed decrease of percent seed germination and shoot, root length also. The phytotoxicity percentage (PP) was maximum at higher wastewater concentration and minimum at lower wastewater concentration. This might be associated with the presence of highly toxic residual organic pollutants, toxic metals in bleached and unbleached paper mill wastewater. The toxicity wastewater might be responsible for inhibiting the seedling growth parameters. It has been reported that the various highly toxic residual organic pollutants along with metals act as an inhibitor for several phytohormones, such as gibberellins, auxins, and cytokinins, which act a crucial role in seed germination (Chandra et al., 2011a). Additionally, significant changes were observed in several physiological parameters and reveal more accurate information about the harmful effects of BUPMW in the tested seeds. The speed of germination (SG) was more reduced from lower to a maximum concentration of wastewater, which additionally provided evidence of having more toxic compounds in bleached and unbleached paper mill wastewater. In another way, the toxic nature of BUPMW was confirmed through seedling mortality rate (SLM), which was a higher concentration of wastewater. Moreover, mean daily germination (MDG), root shoot ratio (RSR), and seed vigour index (SVI) varied from maximum to minimum for lower to higher wastewater concentrations. The germination index (GI) was 100 % at the control and diverse from lower to higher concentrations of BUPMW sample. The treated seeds with control revealed both shoot and root growth, while seeds treated with 100 % BUPMW in different concentrations with the extremely short root (0.06 and 0.16 cm) and short shoot (0.06 and 0.36 cm). Also, the α-amylase activity recorded in germinating seeds evidently justified the toxicity of treated with BUPMW on seed germination and seedling growth parameters.

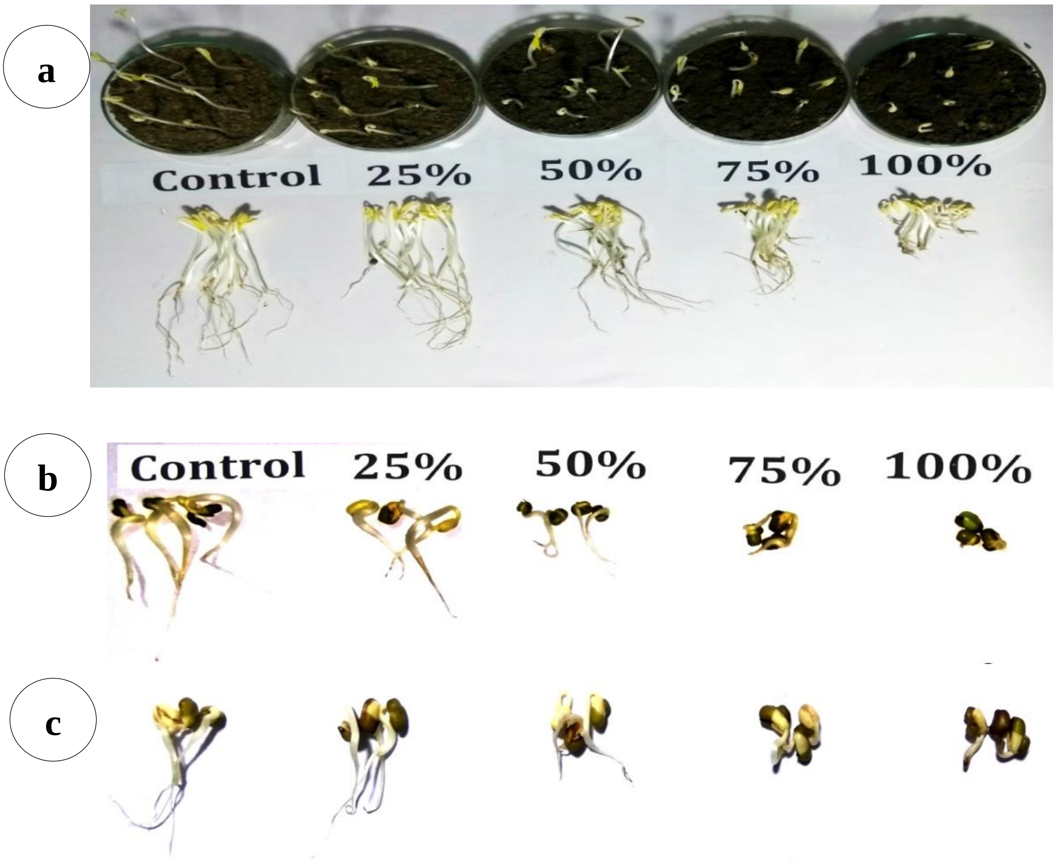


Fig. 4.7 Toxicity effect of sludge and wastewater on seedling growth of *Phaseolus mungo* L. seeds cultivated with **(a)** sludge **(b)** Bleached and **(c)** unbleached paper mill wastewater.

Some important physiological parameters of seedling growth and seed germinations in BUPMW were compared by Pearson's correlation coefficient at the $P<0.05$ significance level shown in Table 4.6. The results indicated that there appeared to be bleached paper mill wastewater a positive and unbleached paper mill wastewater some parameters negative correlation between the lower concentration of wastewater. Mostly several parameters are significant in different concentrations of wastewater. However, a positive significant correlation in all parameters of seed germination (germination percentage, speed of germination, shoot length, and root length) significant correlation was observed between 0 %, 25 %, 50 %, 75 % and 100 % different concentrations of bleached mill wastewater ($P<0.05$) and some negative significant correlation was observed between germination percentage, speed of germination, shoot length, and root length at 0 % to 25 % and positive significant correlation observed between germination percentage, speed of germination, shoot length, and, as well as root length at 50 %, 75 % and 100 % unbleached paper mill wastewater concentration ($P<0.05$).

Table 4.5 Effect of different concentrations of bleached and unbleached paper mill wastewater on physiological parameter of seed germination and α-amylase activity in *Phaseolus aureus*

Parameters	**GP (%)**	**GI (%)**	**SG (s/d)**	**MDG (s/d)**	**SL(cm)**	**RL(cm)**	**SLM (%)**	**RSR**	**SVI**	**PP (%)**	**α Amylase activity (unit grains $^{-1}$)**
Bleached paper mill wastewater (%) used for seed irrigation											
0% (TP)	98.35±0.57	100	16.5±0.50	3.93	2.2±0.10	2.1±0.10	0.34±0.00	0.95±0.07	845.38	0	0.203
25 %	88.30±0.57	59.85	14.55±3.53	3.53	1.8±0.10	1.47±0.11	2.33±0.00	0.81±0.23	577.80	30	0.152
50 %	63.35±1.52	22.37	9.77±0.38	2.53	0.7±0.52	0.73±0.11	7.33±0.00	1.04±0.02	181.03	65.23	0.068
75 %	36.65±0.57	3.54	4.96±0.53	1.46	0.5±0.10	0.2±0.10	12.67±0.00	0.04±0.21	51.31	90.47	0.027
100 %	13.30±0.57	0.38	1.8±0.57	0.53	0.06±0.05	0.06±0.05	17.33±0.00	0.00±0.00	0.159	97.14	0.018
Unbleached paper mill wastewater (%) used for seed irrigation											
0% (TP)	98.3±0.57	100	16.16±1.70	3.93	2.3±0.10	2.06±0.15	0.67±0.00	0.89±0.03	857.17	0	0.203
25 %	88.30±0.57	82.99	12.61±0.53	3.53	2.06±0.15	1.80±0.05	1.33±0.00	0.87±0.21	720.66	12.62	0.198
50 %	76.65±1.15	36.34	10.22±0.25	3.06	1.16±0.05	0.96±0.11	4.67±0.00	0.82±0.07	324.99	53.39	0.137
75 %	55±10	11.68	6.85±0.69	2.20	0.73±0.05	0.43±0.05	9.0±0.00	0.58±0.08	127.60	79.12	0.072
100 %	30±10	2.37	4.08±0.66	1.20	0.36±0.05	0.16±0.05	14.0±0.00	0.44±0.05	31.20	92.23	0.053

All values are mean of triplicate (n=3) ± SD

Control (TP) tap water, *GP* germination percentage, *SG* speed of germination, *MDG* mean daily germination, *GI* germination index, *SL* shoot length, *RL* root length, *SLM* seedling mortality rate, *RSR* root shoot ratio, *SVI* seed vigour index, *PP* plant phytotoxicity

Table 4.6 Pearson correlation coefficient among various physiological parameters of seed germination of *Phaseolus aureus* in different concentrations of bleached and unbleached paper mill wastewater

Bleached paper mill wastewater (%)				
Control (TP)	**GP**	**SG**	**SL**	**RL**
Germination percentage	1	0.971**	0.949**	0.947**
Speed of germination	0.971**	1	0.958**	0.962**
Shoot length	0.949**	0.958**	1	0.991**
Root length	0.947**	0.962**	0.991**	1
25 %				
Germination percentage	1	0.961**	0.960**	0.937**
Speed of germination	0.961**	1	0.982**	0.976**
Shoot length	0.960**	0.982**	1	0.984**
Root length	0.937**	0.976**	0.984**	1
50 %				
Germination percentage	1	0.976**	0.968**	0.847**
Speed of germination	0.976**	1	0.979**	0.972**
Shoot length	0.968**	0.979**	1	0.981**
Root length	0.947**	0.972**	0.981**	1
75 %				
Germination percentage	1	0.986**	0.981**	0.961**
Speed of germination	0.986**	1	0.983**	0.974**
Shoot length	0.981**	0.983**	1	0.981**
Root length	0.961**	0.974**	0.981**	1
100 %				
Germination percentage	1	0.988**	0.982**	0.952**
Speed of germination	0.988**	1	0.985**	0.969**
Shoot length	0.982**	0.985**	1	0.979**
Root length	0.952**	0.969**	0.979**	1
Unbleached paper mill wastewater (%)				
Control (TP)				
Germination percentage	1	-0.082^{NS}	0.124^{NS}	0.603**
Speed of germination	-0.082^{NS}	1	-0.427^{NS}	-0.715^{NS}
Shoot length	0.124^{NS}	-0.427^{NS}	1	0.292^{NS}
Root length	0.603**	-0.715^{NS}	0.092^{NS}	1
25 %				
Germination percentage	1	0.689*	0.578*	0.675**
Speed of germination	0.689*	1	0.537^{NS}	0.284^{NS}
Shoot length	0.578*	0.537^{NS}	1	0.329^{NS}
Root length	0.675**	0.284^{NS}	0.329^{NS}	1
50 %				
Germination percentage	1	0.859**	0.902**	0.914**
Speed of germination	0.859**	1	0.836**	0.789**
Shoot length	0.902**	0.836**	1	0.955**
Root length	0.914**	0.789**	0.955*	1
75 %				
Germination percentage	1	0.942**	0.944**	0.961**

Speed of germination	0.942**	1	0.928**	0.917**
Shoot length	0.944**	0.928**	1	0.982**
Root length	0.961**	0.917**	0.982**	1
100 %				
Germination percentage	1	0.962**	0.946**	0.945**
Speed of germination	0.962**	1	0.958**	0.947**
Shoot length	0.946**	0.958**	1	0.989**
Root length	0.945**	0.947**	0.989**	1

NS not significant, *TP* tap water
*Correlation is significant at the $P < 0.05$ level (2-tailed)
[a] Correlation cannot be computed because at least one of the variables is constant

4.3.3.1 Alpha-amylase activity and SDS-PAGE

Alpha-amylase activity, as a function of seed germination, is a residual biochemical process in plants that can be affected by several environmental factors. In germinating seeds, starch is the main component of the world's crop yield hydrolyze is initiated through α-amylase (Juliano et al., 1969) release maltose and finally, β-glucosidase breaks down maltose into glucose providing energy to germinating seeds (Bharagava et al., 2010). In the present study, α -amylase activity in tap water (0 %) was recorded (0.203 U) and optimum α amylase activity (0.209 U) was recorded in 10 % bleached paper mill wastewater and optimum α amylase activity (0.223U) was recorded in 10 % unbleached paper mill wastewater. In addition, this study is also correlated with Yadav and Chandra, (2015) amylase activity (0.3) during seed germinating conditions. Afterwards, a continued decline in alpha-amylase activity was observed at higher concentrations (100 %) of bleached and unbleached paper mill wastewater. The results obtained at 10 % (v/v) wastewater concentration were invariably better as compared to (0 %) control. It seems that this concentration of wastewater acts as a liquid fertilizer. Additionally, the denaturing SDS-PAGE of α amylase enzyme extracted from germinating seeds for different concentrations of BUPMW has yielded bands of different molecular weights and intensities. Band intensity has indicated the α-amylase enzyme produced in germinating seeds in different concentrations of bleached and unbleached paper mill wastewater. Further, the molecular weight of α-amylase isoenzymes of (*Phaseolus aureus* L) determined through comparing with α-amylase standard and protein marker has indicated that the molecular weight separated by SDS-PAGE was approximately 50.8 and 51.5 kDa, respectively shown in Figure 4.8. According to Bharagava et al. (2010), the α-amylase activity and molecular weight of the α-amylase enzyme were determined by SDS-PAGE approximately 47.5kDa.

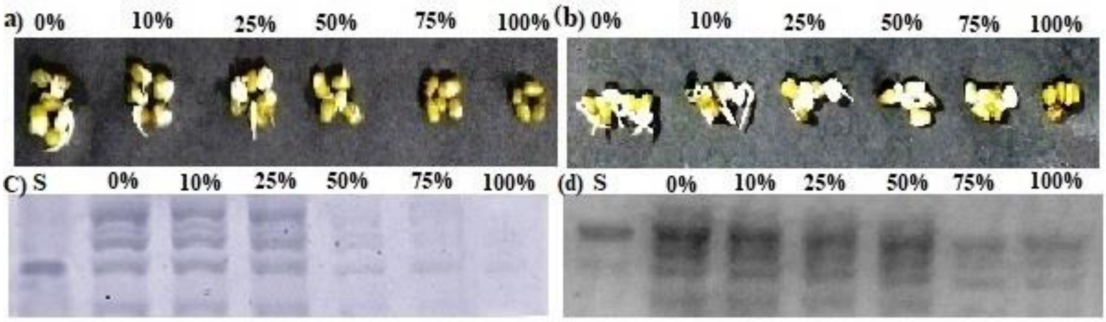


Fig. 4.8 showing the effect of organic pollutants of paper mill wastewater on *Phaseolus aureus*. **(a)** effect on seeds of *Phaseolus aureus* due to bleached paper mill wastewater; **(b)** effect on seeds of *Phaseolus aureus* due to unbleached paper mill wastewater; **(c)** effects of bleached paper mill wastewater on alpha-amylase activity; **(d)** effects of unbleached paper mill wastewater on alpha-amylase activity; **Lane S** amylase standard; **0 %** seed treated with tap water.

4.3.4 Aquatic toxicity on *Tubifex tubifex*

Tubifex worms are useful indicators of varying degrees of aquatic pollution (Auston, 1973). The freshwater Tubifex worm (*Tubifex tubifex*) is an important link in the aquatic food chain (Khangarot, 1991). The BUPMW are used for the toxicity analysis in different concentrations (25, 50, 75 and 100 %) with a different interval of the period as presented in the table (Table 4.7). The whole experiment was carried out in triplicate. The toxicity was minimum at <25 % of concentration up to 48-hour exposure and maximum after >50 % concentration of wastewater at 48 hours of exposure. The wastewater contains different mutagenic and carcinogenic residual organic pollutants which are described in Tables 4.3 and 4.4. Due to the presence of these pollutants in wastewater, there was an enhanced mortality rate of *Tubifex*, physical damage, bursting of cells and whole-cell damage of *Tubifex* worms. Hence, these organo-chlorinated pollutants are highly toxic to the benthic ecosystem. While in control, Tubifex worms remained active during the test period. They were clustered at the bottom of the test container and showed typical *Tubifex* movement. This might be associated with the presence of highly toxic residual organic pollutants and toxic metals in BUPMW that cause the toxicity of the worm (Fig. 4.9). This study revealed the bleached paper mill wastewater is highly toxic than unbleached paper mill wastewater shown in table 4.7**.**

Table 4.7 Toxicity on Tubifex worm against bleached and unbleached paper mill wastewater

Bleached paper mill wastewater % toxicity

Concentration/ Time (hrs)	0 h	24 h	48 h	72 h	96 h	Experiment type	Measurement	Effects	LC50
0 %	19.66±0.57	19.33±1.15	18.66±1.52	19±1	17.66±2.08	Static	Mortality	No change	Non-Toxic
25 %	19.33±1.15	17.33±1.15	10.66±2.08	8.33±2.08	1.33±1.5	Static	Mortality	Physical damage	Slightly Toxic
50 %	18±1	15.66±1.52	9±2	6.66±1.52	0.66±0.57	Static	Mortality	Break down of cell membrane	Moderately Toxic
75 %	17.66±1.52	12.66±1.52	7±2	4±2	0±00	Static	Mortality	Cell damage	Highly Toxic
100 %	16.33±2.08	10.66±2.08	4±4.35	1.33±1.52	0±00	Static	Mortality	Burst and cell damage	Extremely Toxic

Unbleached paper mill wastewater % toxicity

Concentration/ Time (hrs)	0 h	24 h	48 h	72 h	96 h	Experiment type	Measurement	Effects	LC50
0 %	19.33±0.57	18.33±1.52	17.66±2.08	17.33±2.30	15.66±3.78	Static	Mortality	No change	Non-Toxic
25 %	19±1	18±1.73	14.33±1.52	10.66±2.08	3.33±2.51	Static	Mortality	Physical damage	Slightly Toxic
50 %	18.66±1.52	16.33±2.08	13.66±1.52	8.66±2.08	2.33±2.08	Static	Mortality	Break down of cell membrane	Moderately Toxic
75 %	18±1	12±3.60	11.33±1.52	6.66±2.08	1±1	Static	Mortality	Cell damage	Moderately Toxic
100 %	17.66±1.52	11.33±4.04	8.66±2.08	4.66±2.08	1±1	Static	Mortality	Cell damage	Highly Toxic

At the lethal concentrations of wastewater after 24h of exposure, the hemoglobin content was less and the rear part of the body became white, with the disintegration of the body observed. In general, disintegration starts from the rear part of the body and advances towards the front part.

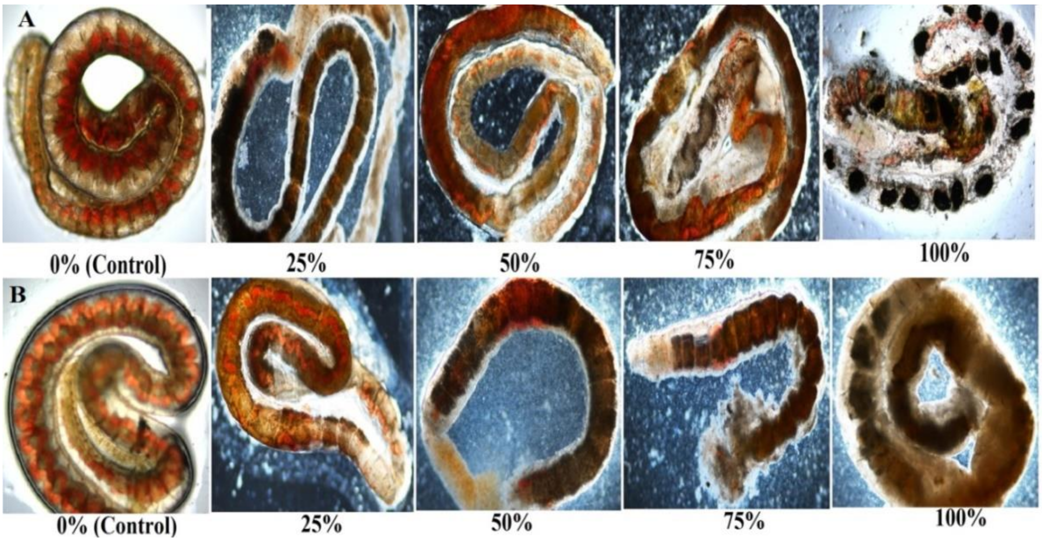


Fig. 4.9 showing **e**ffect of paper mill wastewater (A) bleached paper mill wastewater and **(B)** unbleached paper mill wastewater on *Tubifex tubifex* body sections in acute toxicity**,** Control (0 %) worm in healthy conditions**,** treated with 25 %, 50 %, 75 % and 100 % concentration of wastewater

Conclusion

Discharged wastewater from BUPMW after secondary treatment retains various organic pollutants which are hazardous to the aquatic ecosystem. Some of the detected organic pollutants were found with mutagenic, carcinogenic, and endocrine-disrupting chemicals (EDCs) properties. The wastewater discharged from bleached paper mill was found more toxic than unbleached paper mill wastewater due to chemical pulping, bleaching process. The various chemical used in paper manufacturing process make the residual and do not completely degrade. The toxicity test with *Phaseolus aureus* and *Tubifex tubifex* given strong evidence that discharged wastewater is inhibited to enzyme responsible for seed germination and adversely affect aquatic life also. Therefore, wastewater prior to discharge in the environment should be detoxified at the tertiary stage of treatment.

Chapter Five

Screening and identification of potential laccase producing bacteria from pulp and paper mill waste

Screening and identification of potential laccase producing bacteria from pulp and paper mill waste

5.1 Introduction

The laccase (EC 1.10.3.2) is a copper-containing enzyme that consists of monomeric, dimeric and tetrameric glycoproteins. In recent years, laccase has gained significant applications in the field of textile, pulp paper mill, and food industry (Kumar and Chandra, 2020). However, laccases can also degrade both phenolic and non-phenolic compounds (Kumar and Chandra, 2020). They also can detoxify a broad range of environmental pollutants i.e. xenobiotics compounds, dyes degradation (Nyanhongo et al., 2002a), biobleaching (Arias et al., 2003) pulp delignification, biosensors (Kuznetsov et al., 2001) and biofuel cells development (Palmore and Kim, 1999). These enzymes are belonging to the oxidoreductase family and widely distributed among bacteria, archaebacteria, fungi, and plants (Shraddha et al., 2011). The capability of laccase production was reported in several bacteria and fungi such as *Azospirillum lipoferum*, *P. putida, P. desmolyticum* NCIM 2112, *B. halodurans, Bacillus* sp. HR03, *B. pumilus, B. subtilis* WP1 and *B. subtilis* MTCC 2414 (Kumar and Chandra, 2020) and *Termetes versicolor, Pleurotous ostreatous, Phanerochaete chrysosporium* respectively (Chandra and Chowdhary, 2015). The bacterial laccases are active in a wide range of temperatures (30–85 ^{0}C) and pH values (3.0–9.0), but the most extremophilic laccases found so far are of bacterial origin, which is a fact of special importance for industrial applications (Chandra and Chowdhary, 2015).

The Discovery of novel laccases with different substrate specificities and improved stabilities is important for industrial applications. Microbes that produce laccases have been screened for either on solid media containing coloured indicator compounds that enable the visual detection of laccase production or with liquid cultivations monitored with enzyme activity measurements (Arora et al., 2002; Kumar et al., 2020b). The use of coloured indicators is generally simpler as no sample handling and measurement is required. As laccases oxidize various types of substrates, several different compounds have been used as indicators for laccase production. With guaiacol, a positive reaction is indicated by the formation of a reddish-brown halo (Kumar et al., 2020b), while with tannic and gallic acid the positive reaction is a dark-brown coloured zone (Harkin and Obst, 1973). The traditional screening reagents tannic and gallic acid (Harkin and Obst, 1973) have nowadays mostly been replaced with synthetic phenolic reagents, such as guaiacol and syringaldazine (Nishida et al. 1988; De Jong et al. 1992) or with the polymeric dyes Remazol Brilliant Blue R (RBBR) and Poly R-478 (D'Souza et al. 1999; Raghukumar et al. 1999).

In this study, laccase-producing bacteria were isolated from pulp and paper mill sludge samples using four different indicator compounds in agar plates. The screening results with different indicators were also compared to reduce the number of indicator compounds needed in future screening procedures. In addition, the production of laccase by the positive strains was monitored in submerged fermentation state (SmF). The laccases that were produced in significant amounts were preliminarily characterized.

5.2 Material and Methods

5.2.1 Collection of pulp and paper mill sludge sample

The paper mill sludge sample was collected from the star paper mill Saharanpur, UP India, KR paper mill Shahjahanpur, UP India, Century pulp and paper mill Lalkuan, UK India and Naini paper mill Kashipur UK, India in a sterile bag transported to the department of environmental microbiology (DEM) laboratory Babasaheb Bhimrao Ambedkar University (BBAU) in the icebox and stored at 4 ^{0}C for microbiological study.

5.2.2 Isolation and Screening of laccase producing bacteria in different substrate

The bacterial strains were isolated from 10 grams of sludge sample and transferred to an Erlenmeyer flask (250 mL) containing 100 mL minimal salt media (MSM) as described by Kumar and Chandra (2020a). The flask was incubated in incubator shaker (Orbitek, Scigenic Biotech, India) at 37±1 ^{0}C at 120 rpm for 7 days. Then, the serial dilution was performed in which 1.0 mL sample was taken and diluted (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) than 0.1 mL diluted sample was spread out in the MSM agar plate medium and the streak plate technique was used for isolation of pure culture. The laccase activity of bacterial strain was appeared due to the existence of guaiacol (Sigma, USA) in B and K agar media (Kumar et al., 2020a). Another substrate is used for laccase screening was 0.1 % gallic acid, 0.5 % tannic acid and 0.1 % catechol as indicator compounds and incubated at 37 ^{0}C for 3 days.

5.2.3 Identification of bacterial strains

5.2.3.1 Morphological and biochemical characterization

The isolated bacterial strains were identified based on morphological and biochemical characteristics by using the standard procedure described by Barrow and Feltham (2003).

5.2.3.1.1 Gram Staining

(a) Principle

Christian Gram discovered gram staining, a differential staining method that differentiates bacteria either into Gram-positive or Gram-negative. Staining is based on the principle of component of the cell wall of the bacterial cell. The Gram-negative bacterial cell wall is thin, complex, multilayered structure and contains relatively high lipid contents in addition to protein and mucopeptide. 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 decolourization 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 mucopeptide. This cell wall, when treated with alcohol causes dehydration and closure of cell wall pores thereby not allowing the loss of crystal- iodine complex and cells remains 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

Safranine

Safranine : 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 the 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 decolourized with a few drops of acetone and washed thoroughly with water. Counter stain the slides with 0.5 % safranin for half min, washed again and stand slide on end to drain or blot dry and then observed microscopically.

(e) Interpretation

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

5.2.3.1.2 Motility

(a) Principle

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

(b) Composition of Motility Media (g L^{-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 ^{0}C 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.

5.2.3.1.3 Catalase

(a) Principle

Catalase acts as a catalyst to break down 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 the presence of the 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.

$$H_2O_2 \xrightarrow{\text{Catalase}} H_2O+O_2$$

(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.

5.2.3.1.4 Oxidative-fermentative (O-F) test

(a) Principle

O.F. medium contains low concentration of peptone, high concentration of carbohydrate and low concentration of agar as comparison to carbohydrate fermentative medium. The lower protein to carbohydrate ratio reduces the formation of alkaline amines that can neutralize the small quantities of weak acid that may form oxidative metabolism. The relatively large amount of carbohydrates serves to increase the amount of acid that can potentially be formed. The semi-solid consistency of the agar permits acids that form on the surface of agar to permeate through the medium, making interpretation of pH shift of the indicator easier to visualize.

(b) Composition of O.F. medium (g L^{-1})

Peptone : 2 g
NaCl : 5 g
K_2HPO_4 : 0.3 g
Agar : 3 g
Glucose : 1 %
BCP : 0.2 % aqueous solution (15 mL)
Distilled water : 1000 mL

(c) Procedure

O.F. medium constituents were dissolved in 1000 mL double distilled water, heated to dissolved the solids and pH was adjusted to 7.1, filtered and added the indicator, sterilized at 115 ^{0}C for 20 min. Added a sterile solution of the appropriate carbohydrate aseptically to give a final concentration of 1.0 %. Mixed and distributed aseptically into sterile tubes of not more than 16 mm diameter. Glucose was autoclaved separately to low temperature after that these were mixed aseptically to medium, inoculated the duplicate tubes by stabbing with a sterilized straight wire. To one of the tubes added a layer of melted soft liquid paraffin to a depth of about 1 cm. Incubated and examined daily for up to 14 days.

(d) Interpretation

Open Tube		**Covered tube**
Oxidation	Acid (yellow)	Alkaline (green)
Fermentation	Acid (yellow)	Acid (yellow)
Non-Sacchrolytic	blue or green	Alkaline (green)

5.2.3.1.5 Indole Production

(a) Principle

The test organisms were grown in a media containing tryptophan. Indole is a volatile substance (easily vaporized) and its production was detected by Kovac’s reagent, which contained (p) dimethylamino benzaldehyde. This reacted with indole producing red coloured compound.

(b) Kovac's reagent for indole (g L^{-1})

p-dimethylamino benzaldehyde	:	5 g
Amyl alcohol	:	75 mL
Conc. HCl	:	25 mL

(c) Procedure

Dissolved p-dimethylamino benzaldehyde in alcohol by gently warming in a water bath at 50-55^{0}C, cooled and added the acid with care. Protected from light and stored at 4 ^{0}C. Inoculated peptone water or nutrient broth and incubated for 48 h. Added 0.5 mL Kovac's reagent for indole production, shaken well and examined after about 1 min.

(d) Interpretation

The red colour in the reagent layer indicates indole production.

5.2.3.1.6 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 gram-negative rod. KB002 is the comprehensive test system used for the 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 ^{0}C for 24 h. The indices obtained after reading and results were interpreted using the Hi-Media result interpretation chart supplied with the

Biochemical Identification kit. The organisms were identified to species level. The bromothymol blue pH indicator is 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 the positive result.

(c) Interpretation

Blue color and streak of growth: Citrate utilized
Original green color of medium: Citrate not utilized

5.2.3.1.7 Lysine utilisation

(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. The 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 same as described in section 5.2.3.1.6.

(c) Procedure

The strips were inoculated with isolated bacterium suspension and incubated at 37 ^{0}C for 24 h. The indices obtained after reading the results were interpreted using the Hi-Media result interpretation chart supplied with the 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.

5.2.3.1.8 Ornithine utilization

(a) Principle

Ornithine Decarboxylase is used for detection of the ability of microorganisms to decarboxylate ornithine. Decarboxylation is the process in which bacteria that possess specific decarboxylase enzymes 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 same as described in section 5.2.3.1.6.

(c) Procedure

The procedure of this test was same as described in section 5.2.3.1.7.

(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.

5.2.3.1.9 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 colour of the indicator to red-pink.

$$(NH_2)_2CO + H_2O \rightarrow CO_2 + 2NH_3$$

(b) Reagents and media

The reagent and media used in this test were same as described in section 5.2.3.1.6.

(c) Procedure

The procedure of this test was same as described in section 5.2.3.1.7.

(c) Interpretation

Pink color and streak of growth: Urea Utilized.

Organish yellow color of medium: Urea not utilized.

5.2.3.1.10 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 same as described in section 5.2.3.1.6.

(c) Procedure

The procedure of this test was same as described in section 5.2.3.1.7.

(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 colour. The green colour indicates the positive result.

5.2.3.1.11 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 is further 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 residua 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 same as described in section 5.2.3.1.6.

(c) Procedure

The procedure of this test was same as described in section 5.2.3.1.7.

(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 colour within 5 min, added powdered zinc and allowed to stand.

Pinkish Red color: Nitrate present in medium (i.e. not reduced by the organism).

Absence of red color (colourless): Nitrate absent in medium (i.e. reduced by the organism to nitrite, which in turn was itself reduced).

5.2.3.1.12 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 was same as described in section 5.2.3.1.6.

(c) Procedure

The procedure of this test was same as described in section 5.2.3.1.7.

(d) Interpretation

Black colour: H_2S produced by organism

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

5.2.3.1.13 Sugar fermentation test

(a) Principle

The sugar fermentation tests were performed for the detection of acid and gas production by isolated bacterium. The test was performed in a fermentation tube as well as kit that contained Durham tube (a small tube placed in 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 5.2.3.1.6.

(b) Procedure

To perform sugar fermentation test (i.e. Glucose, adonitol, lactose, arabinose, sorbitol) the biochemical strips were inoculated with isolated bacterium suspension and incubated at 37 ^{0}C for 24-48 h. The indices obtained after reading the results were interpreted using the Hi-Media result interpretation chart supplied with the Biochemical Identification kit. In addition, to perform sugar fermentation test total of seven fermentation mediums were prepared that contain different sugar (Glucose, Sucrose, Maltose, Lactose, Cellobiose, Mannitol, Raffinose). Inoculation of the isolated bacteria into each fermentation tube and keep one an inoculated tube of each fermentation broth as a comparative control. After inoculation, the fermentation tubes were incubated at 35 ^{0}C for 24-48 hours.

(d) Interpretation

Yellow colour: Glucose, adonitol, lactose, arabinose, and sorbitol was fermented by bacteria

Pinkish Red/Red: Glucose, adonitol, lactose, arabinose, and sorbitol was not fermented by bacteria

5.2.3.2 Molecular characterization

The isolated potential laccase producing bacterial strains were characterized by 16S rRNA gene sequence analysis. The total genomic DNA from the overnight growth culture of bacterial strains was extracted using the method described by Kapley et al. (2001). Briefly, about 5 μL DNA sample was used to amplify the 16S rDNA gene using universal eubacterial 27F and 1492R primers in a reaction mixture which contained 1×PCR buffer, 3.0 mM $MgCI_2$, 200 μM of each of dNTPs, and 2.5U of Taq polymerase in a final volume of 50 μL. The thermocycling program was as follows: initial denaturation at 94 ^{0}C for 1 min, primer annealing at 55 ^{0}C for 1 min, and a final extension at 72 ^{0}C for 3 min for a total of 35 cycles. The PCR amplicon was electrophoresed through 1.2 % (w/v) agarose gel in 1× TAE buffer using 1 Kb DNA ladder (M/s Merck Biosciences, India), then visualized by staining with ethidium bromide. Finally, the amplified 16S rDNA gene amplicon was purified from gel using a PCR cleanup kit (Merck Biosciences, India) and sequenced using 1492R and 27F eubacterial primer in an automated DNA sequencer. A phylogenetic tree was generated using the MEGA software (version 6.0) by the Neighbor-Joining method (Tamura et al. 2013).

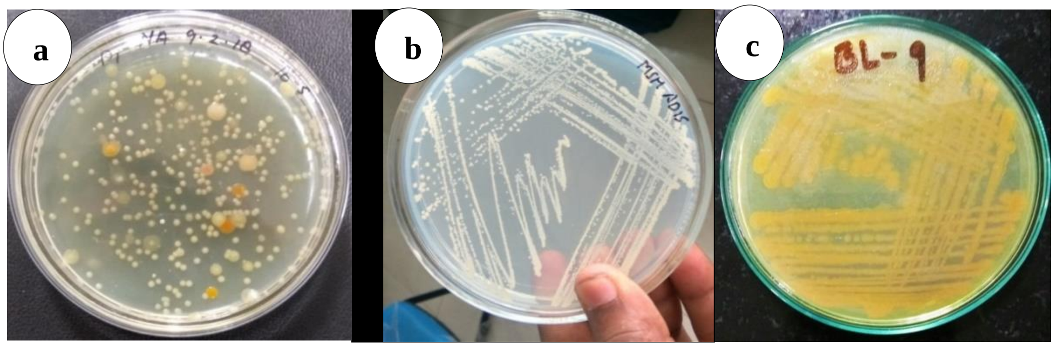
5.3 Results and discussion

5.3.1 Isolation and screening of bacterial strains

The 26 bacterial strains isolated were purified on the MSM agar plate by the streak plate technique (fig. 5.1a, b and c). All bacterial strains were screened to produce laccases on modified B and K agar medium containing 5 mM guaiacol. Among these, six bacterial strains (i.e. BL1, BL6, BL9, BL12, BL15, and B16) demonstrated the laccase activity as shown in Table 1 and (fig. 5.1). Furthermore, the isolated three bacterial strains showed the highest laccase production, resulting in the reddish-brown colour on the medium around the culture growth due to guaiacol oxidation (Fig. 5.1e). This apparently indicated the production of extracellular laccase enzyme, which resulted in due to oxidation of 2 methoxyphenol (Guaiacol). The oxidations of guaiacol by laccase have also been reported from fungal sources but the laccase production from bacterial sources is limited (Senthivelan et al., 2019). In addition, the isolated strain was found to be capable of depolymerizing the other polyphenolic compounds i.e., tannic acid ($C_{76}H_{52}O_{46}$), gallic acid ($C_6H_2(OH)_3COOH$), and catechol ($C_6H_4(OH)_2$) (Table 1 and fig. 5.1d). The appearance of brown and yellow colour was observed in the growth medium due to the degradation of these substrates that indicated the broad range substrate utilization of isolated strain for degradation of phenolic compounds present in the lignocellulosic waste as environmental pollutants. The degradation of tannic and gallic acid by laccase has been reported from fungal sources (Kiiskinen et al., 2004; Shrestha et al., 2016). Similarly, the isolated strain also converted the catechol in the growth medium into yellow colour (Tavakoli and Hamzah, 2016) that may be due to Ortho or Meta cleavage.

Table 5.1 Laccase activity in different substrate

Bacterial strains	**Substrates**			
	Guaiacol	**Catechol**	**Gallic acid**	**Tannic acid**
BL-1	+++	+	-	-
BL-6	+++	+	++	-
BL-9	+++	++	+++	-
BL-12	++	+	+	-
BL-15	+	-	+	-
BL-16	++	++	+	-



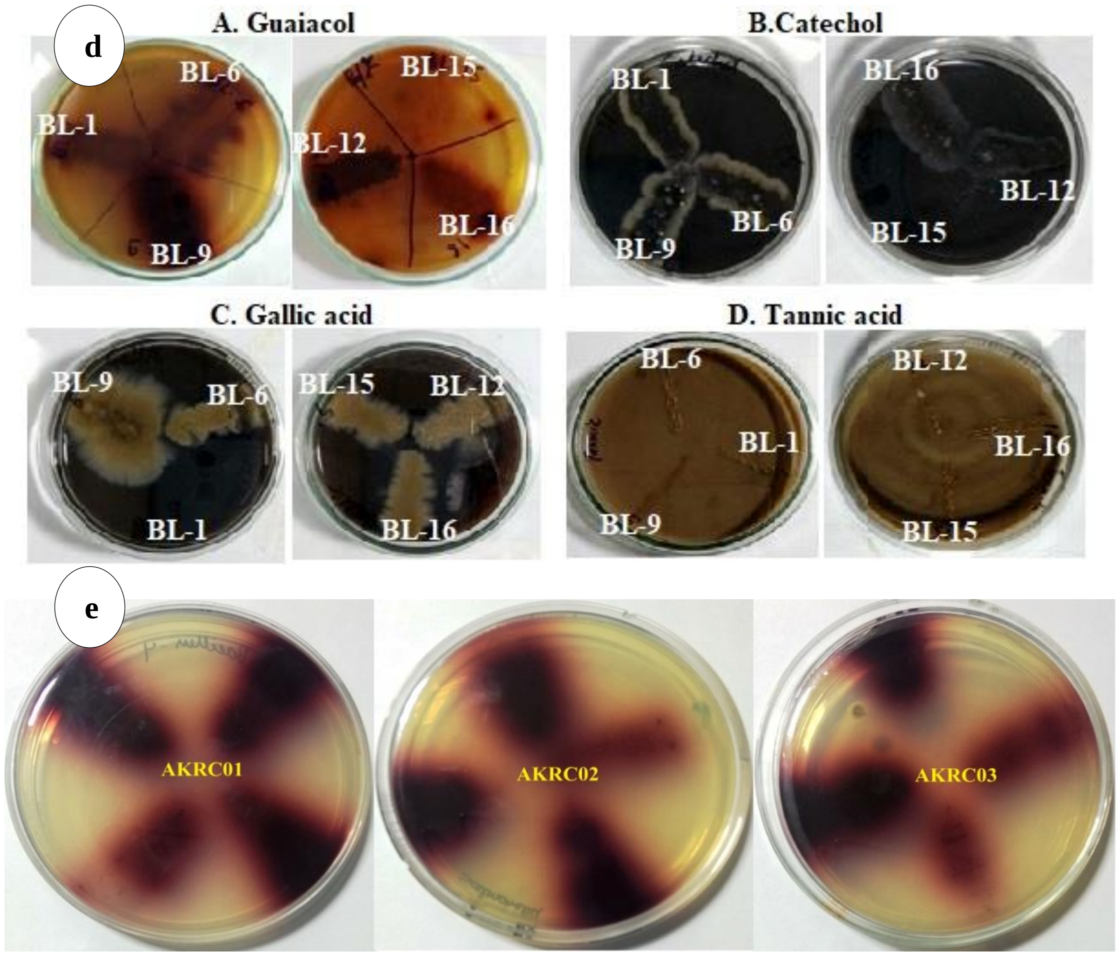
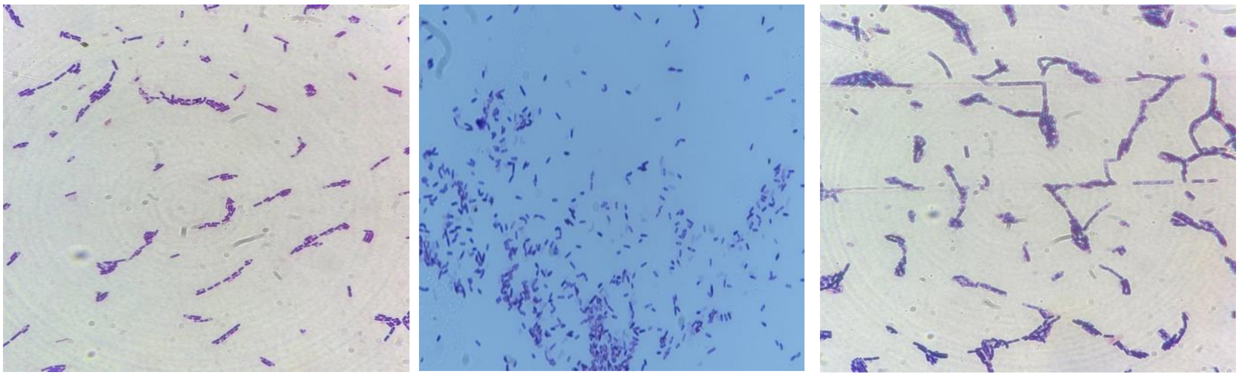


Fig. 5.1 (a) Spreading, **(b and c)** Streaking, **(d)** Screening of laccase activity in the different substrates and **(e)** Potential bacterial strains AKRC01, AKRC02, and AKRC03 showing laccase activity on guaiacol amended B&K agar plates.

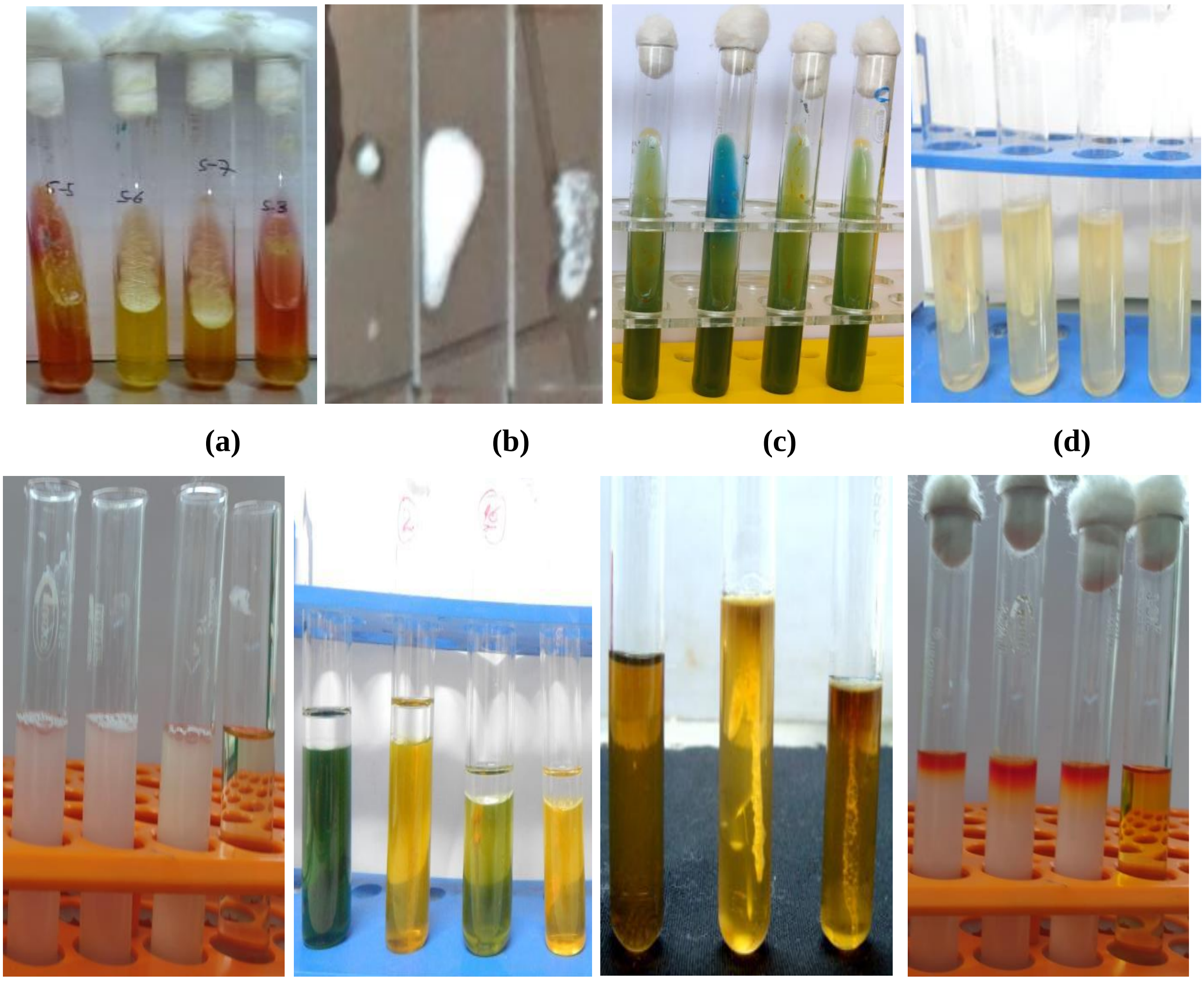


(a) **(b)** **(c)**

Fig. 5.2 Morphological characteristics of isolated laccase producing potential bacterial strains **(a)** AKRC01, **(b)** AKRC02 and, **(c)** AKRC03.

5.3.2 Biochemical characterisation of bacterial strains

The screening of laccase producing potential bacterial strains is characterized by morphological and biochemical tests, such as Gram-positive, rod-shaped, motile bacterium, and salt tolerant. The isolated strain was capable of utilizing a broad range of carbon compounds, including sucrose, mannitol, glucose, arabinose, trehalose, malonate, O-F test, indole production, and catalase. While, these strains show negative tests i.e. lysine, ortho-nitrophenyl-β-galactoside (ONPG), phenylalanine deaminase, nitrate reduction, citrate utilization and arginine. But AKRC02 showed the positive result of H_2S and citrate utilization. In a previous report, aesculin, hypoxanthine, tyrosine, and xanthine were not hydrolyzed (Jung-Hoon et al., 2003). The morphological, physiological, and biochemical characteristics of bacterial strains AKRC01, AKRC02 and AKRC03 are in detail as shown in Figures 5.2, 5.3, and 5.4 and Table 5.2.



(a) **(b)** **(c)** **(d)**

(e) **(f)** **(g)** **(h)**

Fig. 5.3 Various morphological and biochemical reactions showed by isolated bacterial strains AKRC01, AKRC01 and AKRC01 **(a)** Carbohydrate test, **(b)** catalase test, **(c)** Citrate utilization, **(d)** motility test, **(e)** Lysine utilization, **(f)** Oxidative-fermentative test, **(g)** H_2S production test, **(h)** Indole production.

(a) (b)

(c) (d)

Fig. 5.4 Biochemical characterization of isolated bacterial strains using HiMedia Rapid Biochemical Identification kit **(a)** control **(b)** AKRC01 **(c)** AKRC02 **(d)** AKRC03

Table 5.2 Morphological and biochemical characteristics of isolated bacterial strains

Characteristics	Bacterial Isolates		
	AKRC01	**AKRC02**	**AKRC03**
Morphological test			
Gram stain	+ ve	+ ve	+ ve
Shape	Rod	Rod	Rod
Arrangement	In chains	In chains	In chains
Motility	Motile	Motile	Motile
Biochemical test			
Catalase	+	+	+
Oxidative Fermentation (O-F)	+	+	+
Indole production	+	+	+
Citrate utilisation	+	-	+
Lysine utilisation	-	-	-
Ornithine utilisation	-	-	-
Urease	-	+	-
Phenylalanine deaminase	-	-	-
Nitrate reduction	+	+	+
H_2S production	-	+	-
Sugar fermentation test			
Glucose	+	+	+
Sucrose	+	+	+
Mannitol	+	+	+
Arabinose	+	+	+
Trehalose	+	+	+

+ve: Gram positive; **-ve:** Gram negative **+:** Positive; **-:** Negative

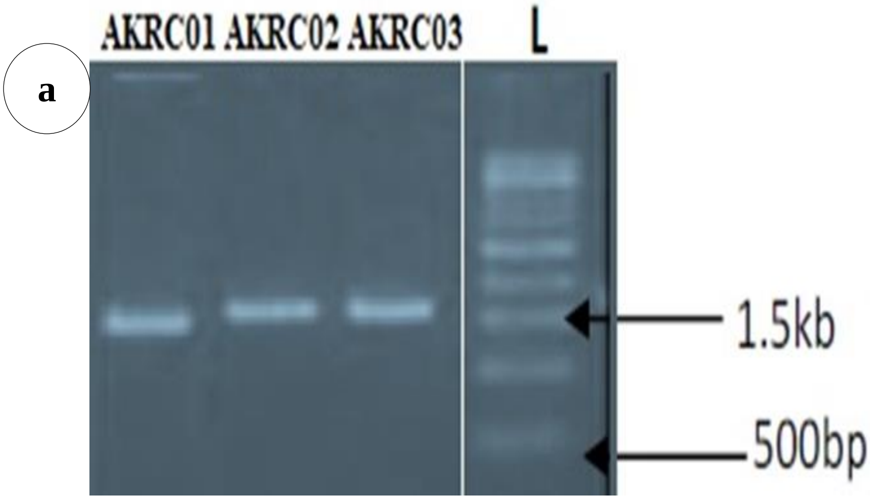
5.3.3 16s RNA identification of bacterial strains

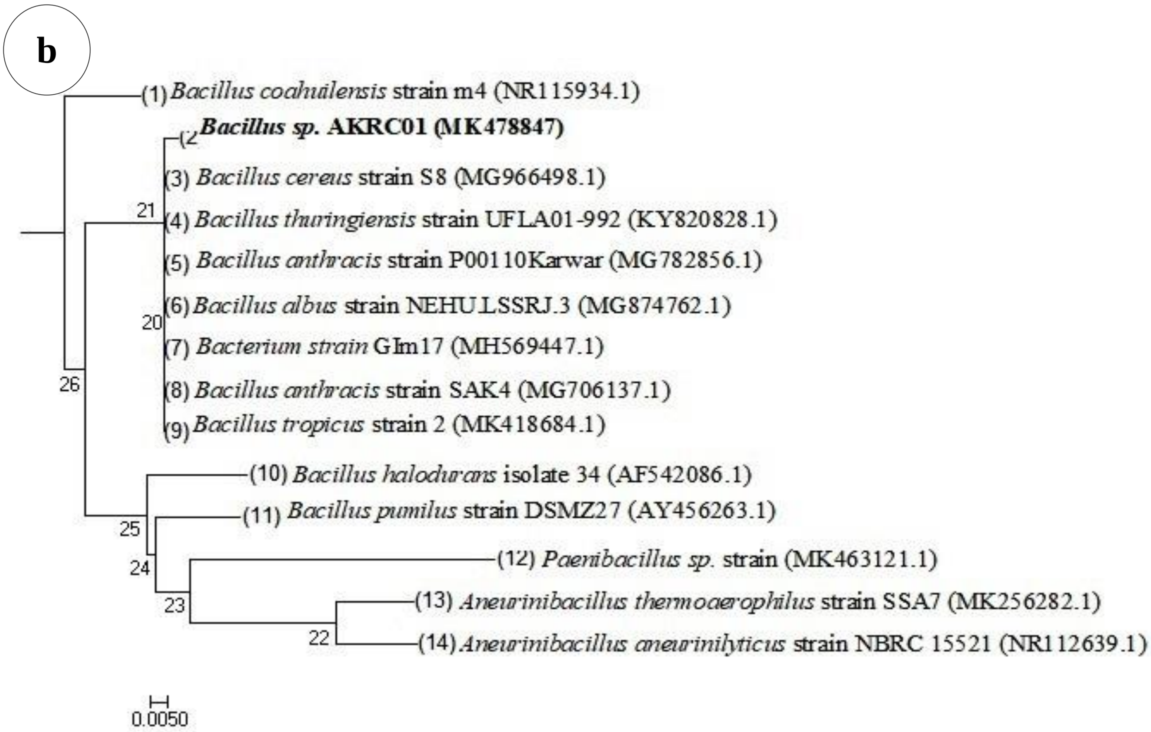
Further, the molecular identification of screened bacterial strains AKRC01, AKRC02, and AKRC03 showed specific amplification of a single band of around 1500 bp of

16S rRNA gene as shown in Fig. 5.5a. The bacterial strain was selected based on laccase activity and analyzed by sequencing the amplified 16S rRNA gene for identification. The identified bacterial strain was confirmed by Chromous Biotech Pvt Ltd, Bangalore, India. The 16S rRNA sequence of bacterial strain *Bacillus* sp. AKRC01 was deposited in Gene Bank under the accession number MK478847.1 (fig. 5.5b). Moreover, 16S rDNA analysis of isolate AKRC01 indicated 99 % homology with *Bacillus anthracis* strain SS1 and 97 % *Bacillus* sp. MH01. Although 16S rDNA sequencing does not provide authentic differentiation and identification *Bacillus* sp. AKRC01 which is closely associated such as *Bacillus cereus* strain 67, *Bacillus cereus* strain QCG4. It can be used for initial associations.

The bacterial strain was selected based on enzyme production in the comparative screening with the different substrates of isolated strains. The 16S rRNA sequence of isolates showed 99.6 % similarity with *Bacillus aquimaris* strain TF-12 (NR025241.1). Based on NCBI blast data of nucleotide sequences, the isolate was identified as *Bacillus aquimaris* AKRC02 (MK478945.1) (fig. 5.5c). *Bacillus aquimaris* has been primarily isolated from seacoast marine habitats as a halotolerant (Jung-Hoon et al., 2003). But it has shown a broad range of adaptions towards various pollutants in different habitats (Shivanand and Jayaraman, 2009). This bacterium has also been isolated from the shrimp gut responsible for carotenoid pigment production (Ngo et al., 2016), indicating the broad range of adaptions for protein and cellulose digestion. Therefore, our isolate from pulp and paper waste showed a related characterization due to the abundant presence of various salts i.e., sodium hydroxide (NaOH), CaO, $Ca(OH)_2$, sodium sulfide (Na_2S), and $CaCO_3$, etc. (Kumar et al., 2020b). The presence of cellulose waste in pulp paper waste also corroborated with the production of endoglucanase for the cellulose degradation property of bacteria as adaptive features in the polluted environment (Khalid et al., 2017). Thus, this indicated the direct involvement of isolated strain for bioremediation of pulp and paper mill waste containing lignin, cellulose with various salt and metallic compounds.

Therefore, another bacterial strain was identified the maximum laccase production. Further, 16S rRNA sequence of isolated was done which showed 93 % similarity with *Bacillus tropicus* strain SA65 (MK467571.1) (fig. 5.5d). The isolate was identified as *Bacillus cereus* strain AKRC03 (MN72058.1) based on NCBI blast data of nucleotide sequence. Based on partial 16S rDNA sequence contrasts, the phylogenetic tree of the *Bacillus cereus* strain AKRC03 and their related genera have been linked. Sequence assessments among different species indicated the degree to which they are linked. This was performed using a neighbor-joining technique to construct the phylogenetic tree.





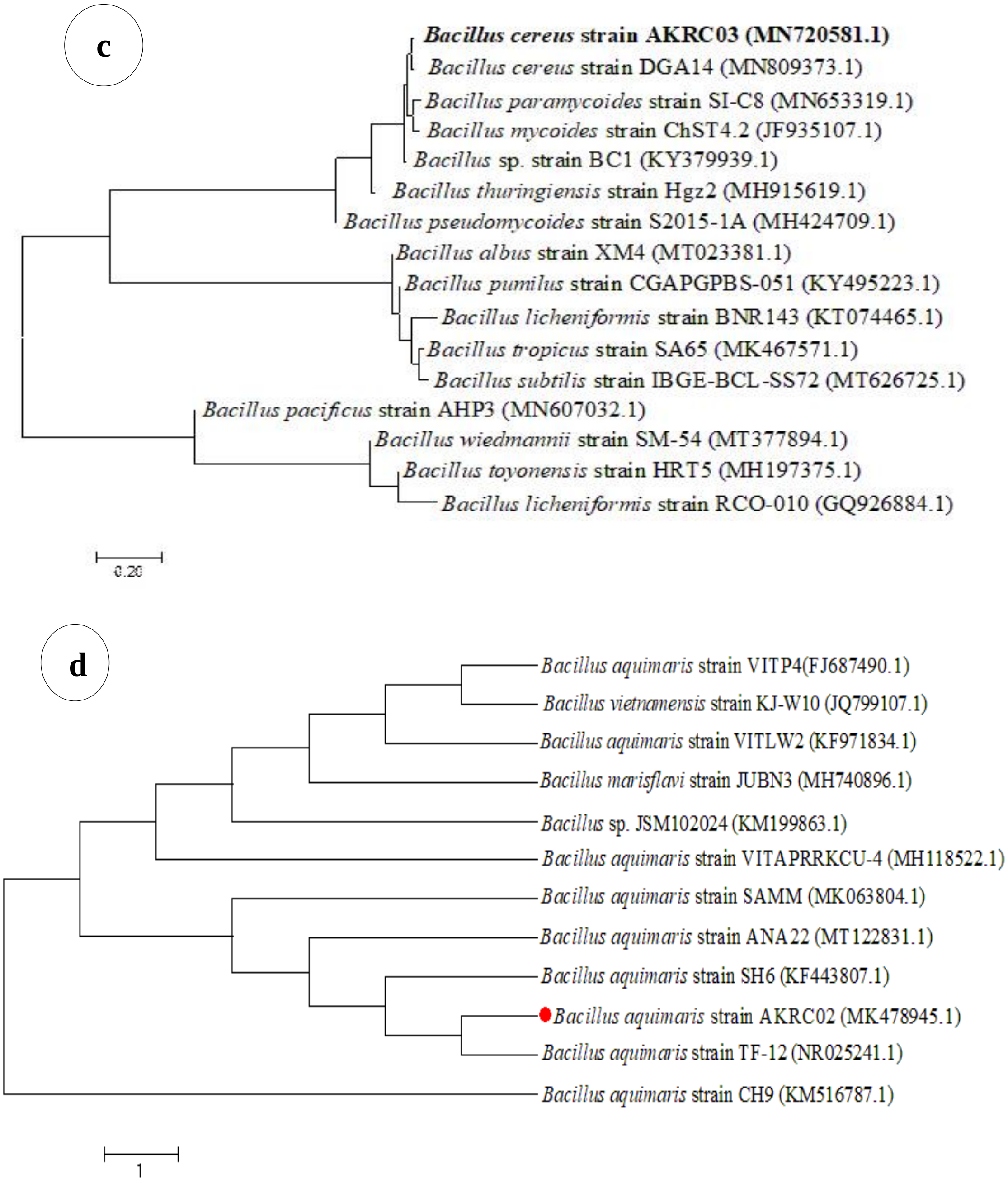


Fig. 5.5 (a) PCR amplification of 16S rRNA gene of isolated bacterial strains; L 500bp DNA Ladder, Phylogenetic tree of the **(b)** *Bacillus* sp. strain AKRC01, **(c)** *Bacillus aquimaris* strain AKRC02 and **(d)** *Bacillus cereus* strain AKRC03 and their related genera have been linked based on partial 16S rDNA sequence comparisons. Their names and respective accession numbers are given in the tree.

Conclusion

Isolation and screening of laccase producing potential bacterial strains *Bacillus* sp. strain AKRC01, *Bacillus aquimaris* strain AKRC02 and *Bacillus cereus* strain AKRC03 are characterized by morphological and biochemical tests, such as Gram-positive, rod-shaped, motile bacterium, and salt tolerant. The Isolated of bacterial strains from pulp paper mill sludge indicated the broad range adaptation of bacteria for its remediation potential in the presence of various salts, i.e., sodium hydroxide (NaOH), CaO, $Ca(OH)_2$, sodium sulfide (Na_2S), $CaCO_{3,}$ and organometallic compounds.

Chapter Six

Study the effects of various nutrients for production of bacterial laccase in different pH and temperature

Study the effects of various nutrients for production of bacterial laccase in different pH and temperature

6.1 Introduction

Laccase is a copper-containing enzyme, which consists of monomeric, dimeric, and tetrameric glycoproteins. In recent years, laccase has gained significant attention due to its broad-spectrum applications in the food industry, distillery, textile, and pulp paper industries (Kumar and Chandra, 2020). This enzyme belongs to the oxidoreductase family and is widely distributed in archaebacteria, bacteria, fungi, and plants (Shraddha et al., 2011). Laccase can catalyze the degradation of both phenolic and non-phenolic compounds along with recalcitrant lignocellulosic waste materials (Kumar and Chandra, 2020). It also plays a very crucial role in the detoxification of other environmental pollutants i.e., xenobiotics and dyes degradation, biobleaching of pulp, biosensors, and biofuel production (Kuznetsov et al., 2001; Nyanhongo et al., 2002; Arias et al., 2003; Mathur et al., 2021). The majority of extremophilic laccases for industrial applications are found from bacterial origin (Chandra and Chowdhary, 2015). For large-scale laccase production, two approaches have been adapted (i) enzymes immobilization on solid supports with reuse properties (Syahlan et al., 2021), and (ii) enzyme productions using cheap substrates (Libra et al., 2003; Narayanan and Murugan, 2014; Chandra and Chowdhary, 2015; Kumar and Chandra, 2020). Besides, the bacterial laccases are determined to be active in a diverse range of temperatures (30–85 ^{0}C) and pH values (3.0–9.0). Though white-rot fungi are better candidates for high extracellular laccase production (Nyanhongo et al., 2002), they require a solid-state fermentation (SSF) process than the submerged process due to growth limitations. Also, bacterial laccases are often stable to tolerate a varied range of environmental habitats with faster growth than fungus (Harms et al., 2011). Some potential bacteria have shown the maximum laccase production in submerged conditions due to their growth suitability and proper nutrient availability. Therefore, the selection of bacterial strains for the production of laccase in the submerged fermentation (SmF) process using a cheap substrate is of supreme interest for large-scale applications. Current research is mainly focused on the development of sustainable technology for laccase production and SmF technique is easily available and economically valuable than other methods. This mode of fermentation has many advantages in controlling various process parameters and scaling up processes regarding product purity and easiness. This is usually preferred for large-scale industrial fermentation for the biosynthesis of enzymes, amino acids, antibiotics, organic acids, baker’s, and distiller’s yeast (Davis and Blevins, 1979).

Optimization of different process parameters has been reported using the conventional laccase production method (Songulashvili et al., 2007). Further, the environmental and nutritional composition of the medium, incubation period, pH, temperature, carbon (C), and nitrogen (N) source also plays a significant role in laccase production (Lorenzo et al., 2002; Ghosh and Ghosh, 2017). It is regarded as the most suitable and effective approach for optimizing the multiple combinations of variables for maximum laccase production. Production of bacterial laccase in a commercial manner is a prime necessity; therefore, the agricultural residue waste may be a promising option as a substrate for laccase production due to rich sources of carbon, nitrogen, and phosphorus (Awasthi et al., 2015). India produces approximately 600 metric tons of waste from various agricultural sources (Asokan et al., 2010). A huge amount of agricultural waste is generated from different sources i.e. wheat straw, the husk of coconut waste, sugarcane industry, vegetable waste, oil industry waste, and food products (Hafid et al., 2021). These wastes are causing serious environmental pollution due to a lack of proper management strategies. The screening of potential bacteria for laccase production by using agricultural waste would be a cheap alternative source for nutrients (Wang et al., 2019). Recently, the production of laccase enzyme using agro-residue substrate has received great significance because of their applications in the degradation and reuse of waste that can also minimize the disposal problems (Poonam and Pandey, 2009).

The present work evaluated the screening and identification of laccase-producing bacteria from paper mill sludge. Suitable agro residue wastes were screened for laccase production under SmF. Different environmental and nutritional parameters were standardized by CCD-RSM for maximum laccase production.

6.2 Material and Methods

6.2.1 Selection and chemical analysis of agro-residues waste

Agro-residues wastes (ARW) i.e. potato peel, banana peel, sawdust, pea peel, wheat bran, orange peel, and rice bran were collected from the local area around Lucknow.

All these agro-residues were washed three times with distilled water, boiled up to 15 min for removal of dust particles, and dried in an oven at 60 °C. After drying, these ARW were milled using a domestic mixer grinder (Maharaja Mx-116A), and the screened (40 µm mesh) ARW powder was stored at 4 °C. ARW chemical composition i.e. lignin acid detergent fiber (ADF), hemicellulose, neutral detergent fiber (NDF), cellulose, ash content, and carbohydrates were used for other natural substrates as per the O'Dwyer, (1923); and Norman and Jenkins, (1933). The effects of substrate concentrations (1–4 % w/v) on the production of laccases were also evaluated.

6.2.2 Laccase assay

Laccase activity was determined as described earlier by Arora et al. (2002). In reaction mixtures containing 3.8 mL acetate buffer (50 mM, pH 4.6) and 1 mL guaiacol (2 mM), 0.2 mL of the crude enzyme was added and incubated at room temperature for 2 h. The reaction was observed by measuring the absorbance at 465 nm using a UV-Vis spectrophotometer (Thermo science evolution-201). The total enzyme activity was calculated as the quantity of oxidized one micromole guaiacol (substrate) per minute at room temperature. Every experiment was conducted twice with guaiacol (extinction coefficient = 12,000 mM^{-1} cm^{-1}) (Teigiserova et al., 2021) and the activity of laccase was calculated in U/mL.

$$(\mathrm{U/mL}) = \frac{\Delta \mathrm{A}_{465/\mathrm{min}} \times 4 \times \mathrm{V_t} \times \text{dilution factor}}{\varepsilon \times \mathrm{V_s}}$$

6.2.3 Screening of the ARW for laccase production under SmF condition

The BL-9 (*Bacillus aquimaris* AKRC02) bacterial strain was selected to enhance the laccase production using different ARW. Two grams of each ARW were taken in separate flasks and mixed with 100 mL of mineral basal salt solution (MBSS). The composition of MBSS was in gL^{-1} dextrose 10.0, peptone 3.0, K_2HPO_4 0.4, $ZnSO_4$ 0.01, $MnSO_4$ 0.5, KH_2PO_4 0.6, $FeSO_4$ 0.0005, and $MnSO_4$ 0.5. Precisely, 2.76×10^8 CFU of *Bacillus aquimaris* AKRC02 was inoculated in the sterilized flask and incubated at 37 °C for 144 h. After incubation, 50 mM glycine-NaOH buffer (pH 8.0) was mixed thoroughly, and centrifuged at 10,000 g for 10 min at 4 °C. The resulting cell-free supernatant obtained was used for enzyme assay by taking OD at 465 nm to calculate enzyme activity (Muthukumarasamy et al., 2015).

6.2.4 Delignifiying activity of rice bran

Exactly, two-gram of rice bran fermented for 120 h with *Bacillus aquimaris* AKRC02 was air-dried, and the fermented rice bran particles were observed under SEM to evaluate modifications of their surface structure of rice bran. SEM analysis of unfermented rice bran was also observed and used as a control sample (Niladevi et al., 2007). The remaining fermented and unfermented rice bran was sterilized and re-inoculated with *Bacillus aquimaris* AKRC02 and monitored for laccase production.

6.2.5 Scanning electron microscope (SEM) of bacterial growth during enzyme optimization conditions

During the enzyme optimization, the optical density for bacterial growth was measured continuously for 144 h at 24 h intervals with a UV–Vis spectrophotometer at 620 nm. The bacterial biomass was collected after centrifugation at 6000 g for 10 min and fixed with 2 % glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.5) overnight (Chandra et al., 2018). After fixation, the bacterial cells were again fixed with osmium tetra-oxide (Sigma Aldrich, USA) to dissolved in 50 mM sodium phosphate buffer (pH 6.5) for 30 min and dehydrated with ethanol from 30 % to 100 % (10 min at every concentration) (Chandra et al., 2018). The dehydrated bacterial cells were coated with a thin layer of gold using ion sputter (CoaterIB-2, Gike Engineering, Japan) and analyzed for morphological characterization under SEM analysis (model: JSM-6490LV, Make: JEOL, Japan).

6.2.6 Statistical optimization of laccase production

SmF process is generally suitable for the bacterial system due to proper oxygen circulation and nutrients availability of the culture medium. The selected ARW i.e., rice bran (two grams) was used to attain a high yield of laccase production at optimized environmental conditions (incubation period, pH, and temperature) and different nutrients i.e., carbon (glucose, sucrose, starch, maltose, lactose) and nitrogen sources (peptone, urea, yeast extract, sodium nitrate, and ammonium sulfate) were used to enhance the enzyme activity. The impact of each variable on the production of the enzyme has been studied in five different concentrations: -α, -1, 0, + 1, + α. The separated 20 sets of studies on rice bran were also conducted in triplicates (Fatma et al., 2011). All variables were considered to be zero at a core coded value. An analysis of variance (ANOVA) was performed using Design-Expert software-11 (student version) (Stat-Ease, Minneapolis, MN, USA) to analyze the data obtained from RSM on the laccase production. RSM findings were used to match a polynomial second-order Eq.1.

$Y= \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1 \beta_1 A^2 + \beta_2 \beta_2 B^2 + \beta_3 \beta_3 C_2 + \beta_1 \beta_2 AB + \beta_1 \beta_3 AC + \beta_2 \beta_3 BC$ (Eq. 1)

Where Y is the variable, β_0 intercept coefficients, β_1, β_2, β_3 linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$, $\beta_{3,3}$ square coefficients, $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$ interactive coefficients, and A, B, C is the linear effect, A_2, B_2, C_2 square effect and AB, AC, BC autonomous interactive coefficients.

6.2.7 Effects of supplementary carbon and nitrogen sources for enzyme production

In the culture medium, the nature and amount of carbon and nitrogen sources are essential for bacterial growth which increased the production of extracellular laccase enzymes. The production medium was enriched with different concentrations (1.0 % 1.5 % and 2.0 %) of carbon sources (glucose, sucrose, maltose, starch, and lactose) depend upon the carbon chain and structural difference. Nitrogen source used based on percentage and structural liberty (peptone, urea, and yeast extract, ammonium sulfate and sodium nitrate) in 250mL Erlenmeyer flasks containing 50 mL of MBSS medium with 2.0 g (v/w %) of rice bran. The flasks were sterilized, cooled at room temperature, and inoculated with 3.1×10^6 CFU of *Bacillus* sp. AKRC01 and $2.76 \text{ x } 10^8$ of *Bacillus aquimaris* AKRC02 and incubated at optimized environmental conditions with the agro-waste substrate. The contents of the flasks were centrifuged at 10000 rpm for 10min at 4 ^{0}C and the supernatant was used to assay for production of the enzyme at 450 nm.

Part A

6.3 Results and discussion

6.3.1 Analysis of agro-residues waste

Selected agro-residues waste (i.e. rice bran, wheat bran, sawdust, orange peel, banana peel, potato peel, and pea peel) were suitable for the production of laccase enzyme in submerged fermentation (SmF) state. The chemical compositions of the different agro-residues substrates are shown in Table 6.1.

Table 6.1 Selection of agro residue substrate and their chemical compositions

Natural substrates	**Composition analysis (%)**						
	Cellulose	**Hemicellulose**	**Lignin**	**Carbohydrate**	**NDF**	**ADF**	**Ash**
Rice bran	33.43	20.99	18.25	45.9	61.12	45.8	5.64
Wheat bran	27.21	13.29	10.64	48.1	49.39	36.1	4.28
Saw dust	22.31	10.2	11.76	22.3	35.8	25.6	3.46
Banana peel	25.8	12.17	8.21	51.84	40.37	28.2	5.31
Orange peel	13.6	20.46	8.0	12.0	38.28	18.32	5.18
Pea peel	62.3	8.2	3.5	2.6	74.0	65.8	4.80
Potato peel	2.5	34.7	3.8	64.47	41.0	6.3	6.34

NDF: neutral detergent fiber; ADF: acid detergent fiber

6.3.2 Screening of different agro-residues waste for laccase production

Screening of better agro-residue substrates has been done based on laccase production. These substrates are cheap required lower cost, promote to produce large quantities of enzyme and have significant biotechnological applications. In the current study, several agro-residues substrates (2.0 %) (i.e. rice bran, wheat bran, sawdust, orange peel, banana peel, pea peel, and potato peel) were used with MBSS media for laccase production. Each flask was maintained at (pH 8.0) and incubated for 144 hours and activity recoded after 24 hour hours intervals. Figure 6.1 showed that the maximum activity of laccase (4.58 U/mL) was recorded in rice bran followed by wheat bran (3.74 U/ mL), sawdust (2.07 U/ mL) orange peel (0.96 U/ mL), banana peel (0.94 U/ mL), pea peel (0.85U/ mL) and potato peel (1.24U/ mL) respectively.

Among the others, rice bran is the most effective agro-waste substrate for laccase production. The production of extracellular laccases by bacteria with the utilization of agro-industrial waste is a rare task. The agro-residues substrates have not only reduced the problem of disposal but also reduce the probability of pollution from the discharge in the environment. Earlier, a study on *Pseudomonas putida* indicated the laccase activity (4.25 U/ mL) after an incubation period of 108 hours (Kuddus et al., 2013).

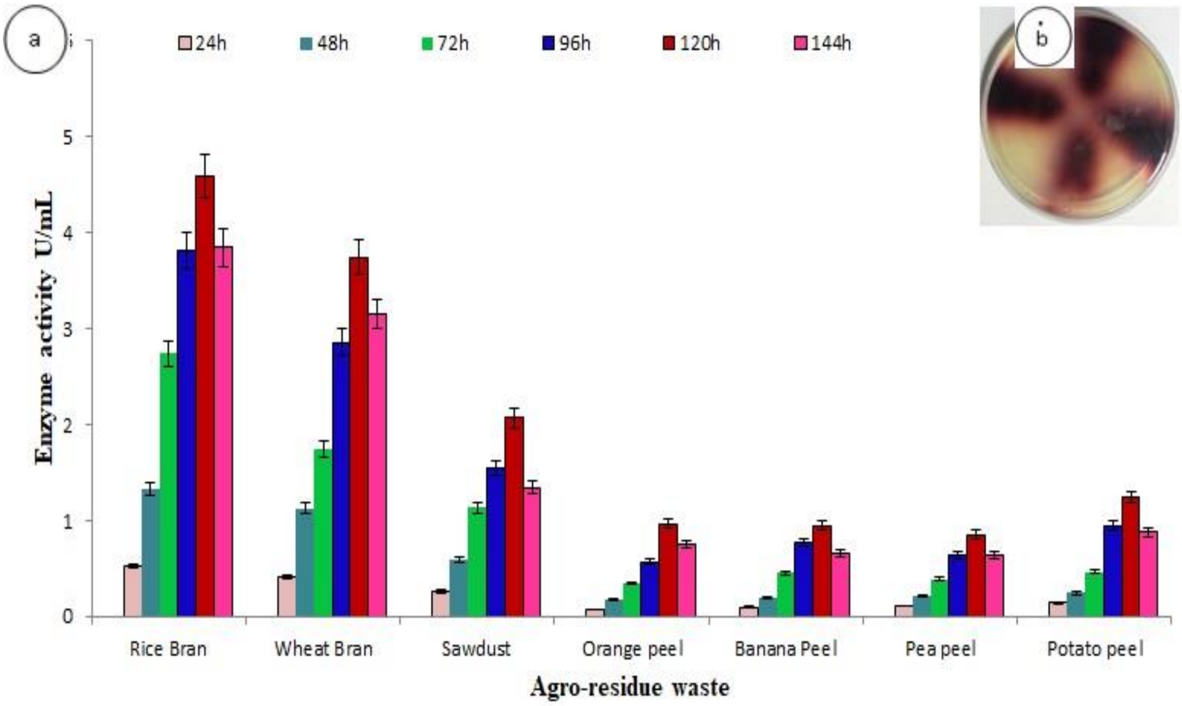


Fig. 6.1 (a) Effect of incubation period in different agro-residue for laccase production (b) Plate assay of laccase

6.3.3 Delignifiying activity of rice bran

The production of laccase by *Bacillus aquimaris* AKRC02 in the presence of rice bran resulted in delignification/distortion of the cell wall layers. Several structural alterations were observed in rice bran during the fermentation with *Bacillus aquimaris* AKRC02. The peeling and channeling mechanism detected in unfermented (control) rice bran (Fig. 6.2a) as compared to the fermented rice bran was noted at 48 and 120 h incubation (Fig.6.2b and c). Experimental results suggested strong evidence of delignification activity in the fibers that change the surface structure and became destroyed and modified (Fig. 6.2b, c). The utilization of lignin as a carbon source by the bacteria has also been reported for laccase production in previous studies (Niladevi and Prema, 2008). This showed the surface structural change as supporting evidence for lignin utilization by *Streptomyces psammoticus.* Our observation of destruction and modification of the fibrous structure corroborated the lignin utilization. Enhanced porosity further exposes the cellulosic portion for efficient hydrolysis. SEM images of biologically treated samples of *A. mangium* wood chips with *P. coccineus, Phellinus sp. Daedalea sp.* and *T. versicolor* showed the degradation mechanism of lignin due to the activities of fungal-derived ligninolytic enzymes (Liew et al., 2011). Lignin is recalcitrant in the ARW substrate, which not only acts as a barrier to hydrolytic enzymes (cellulases and hemicellulases), but also hampers the fiber digestibility of ruminant (Niladevi and Prema, 2008; Kumar et al., 2020a). Several researchers are working on the up-gradation of rice bran for ruminant feed (Huttermann et al., 2000). The present work provides better prospects that the rice bran can be potentially used to produce laccases and fodder with greater nutritional and digestibility potentials.

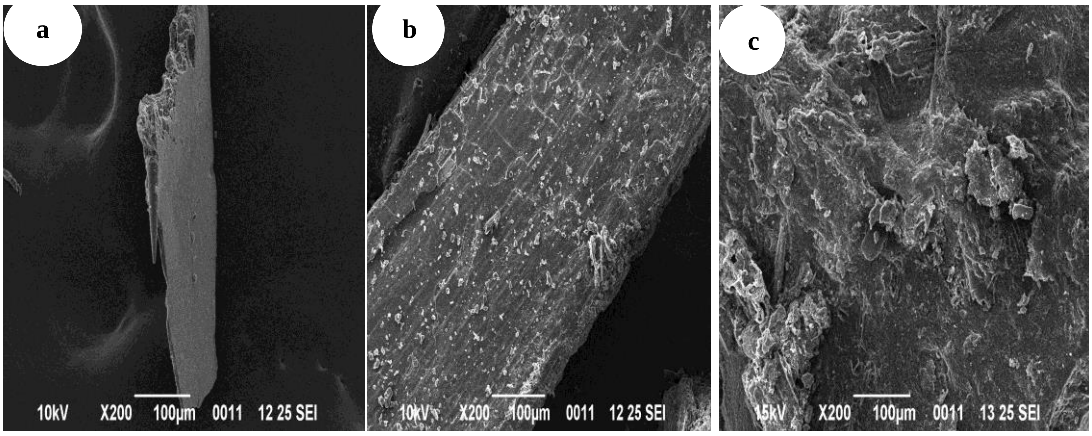


Fig. 6.2 showing scanning electron micrograph of rice bran (**a**) control surface structure of unfermented rice bran, (b) appearance rice bran after 48 h, (**c**) with *Bacillus aquimaris* AKRC 02.

6.3.4 Bacterial growth

Bacterial growth was evaluated periodically to confirm the utilization of agro-residue as nutrients by growing bacterial cells. The maximum optical density of the growing culture was noted to be 3.52 (CFU/mL 2.76 x 10^8) at 35±1 °C after 120 h incubation at 620 nm (Fig. 6.3a and b). These conditions were selected for further optimization of laccase in various agro-residues as nutrients and environmental conditions.

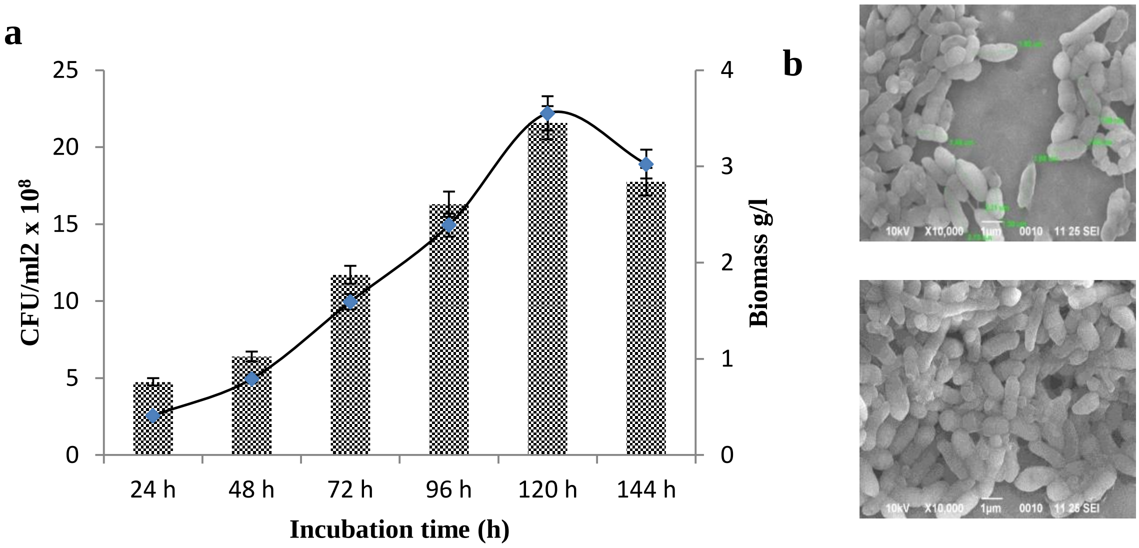


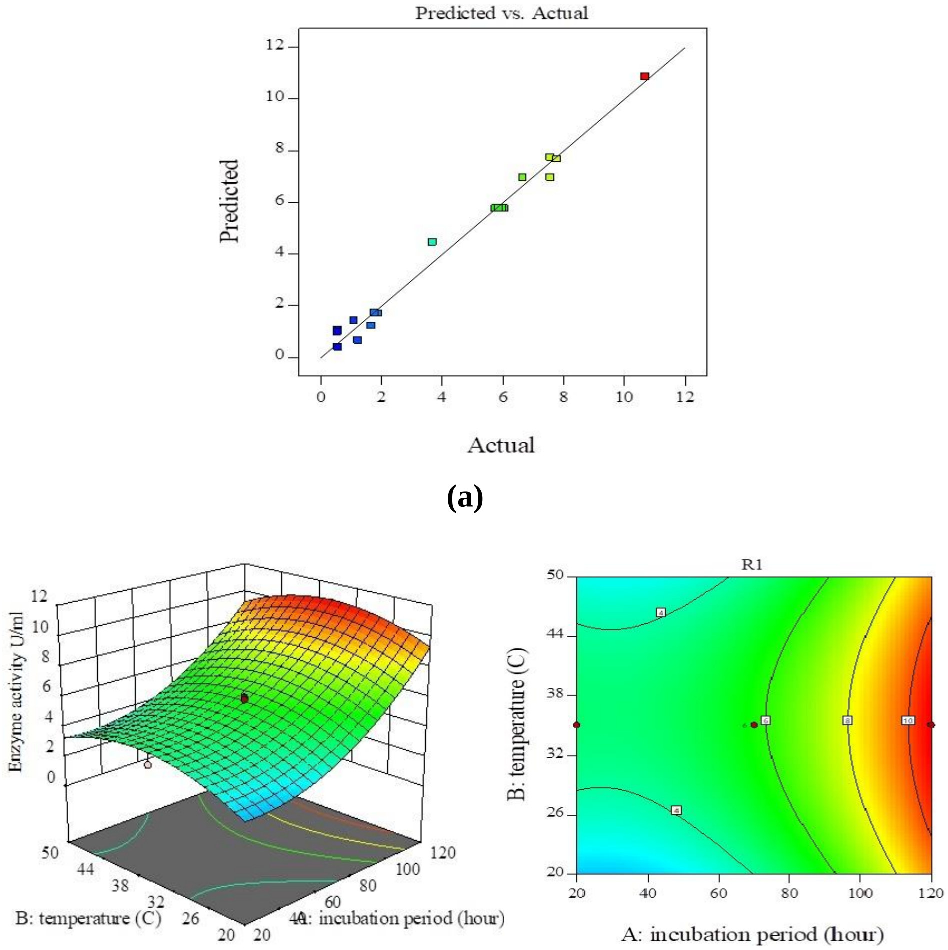
Fig. 6.3 (a) Bacterial growth (CFU/ mL 2.76 x 10^8) pattern during enzyme optimization and biomass estimation **(b)** scanning electron micrograph of bacterial cells

6.3.5 Optimization of laccase production using RSM-CCD

In conventional optimization processes, each factor has been varied, while other factors remain constant that represents the one-factor-at-the-time (OFTA) process. The OFTA model is not capable of explaining the interactive behaviour of variables (Ahmad et al., 2018). Thus, nonconventional methodologies, statistical and mathematical response surface methodologies have become promising tools to determine the collective effects of two or more factors in the experimental design (Antunes Barros et al., 2019). In this study, the different physicochemical activities for laccase production in rice bran were investigated with the SmF process using the central composite design (CCD) (Fatma et al., 2011; Kumar et al., 2020a). However, the incubation period, temperature, and pH were determined based on experimental optimization parameters. CCDs are designs of the response surface methodology (RSM) that can fit a complete quadratic model (Niladevi et al., 2007). The RSM-CCD was used to identify the suitable values of the variables on laccase production by *Bacillus aquimaris* AKRC02 using rice bran in SmF process. The three most significant model term parameters, including incubation period, temperature (℃), and pH were chosen and subjected. The optimum combination of process parameters was conducted by the CCD experimental design (Table 6.2) for the maximum laccase production (Fatma et al., 2011). The experimental data were statistically significant at

$P<0.05$ level and represent a good fit with the polynomial equations of the second order. For laccase production, ANOVA results indicated ‘F-value’ 76.61 for rice bran implying that the model was significant (Table 6.3). The model terms having 'Prob>F' values less than 0.05 were considered important. Through the determination of the coefficient (R^2), the model goodness of fit was checked (Table 6.3). The R^2 value for rice bran was 0.98, suitable for the adjusted R^2 value of 0.97 (Fig. 6.4a). The coefficient of variation (CV) was also lower as 11.33 in rice bran that showed fewer deviations between predicted and experimental values. The adequate precision value (29.46) for design space indicated an adequate sign that recommends the usefulness of the model (Table 6.3). In the present study, A, A^2, B^2, C^2 were model term values ($p>0.05$) that showed the significant conditions of the model. Lack of Fit F-value (45.81) in rice bran expressed that the lack of fit was significant. There was only a 0.04 % chance that a Lack of Fit F-value becomes insignificant due to noise.

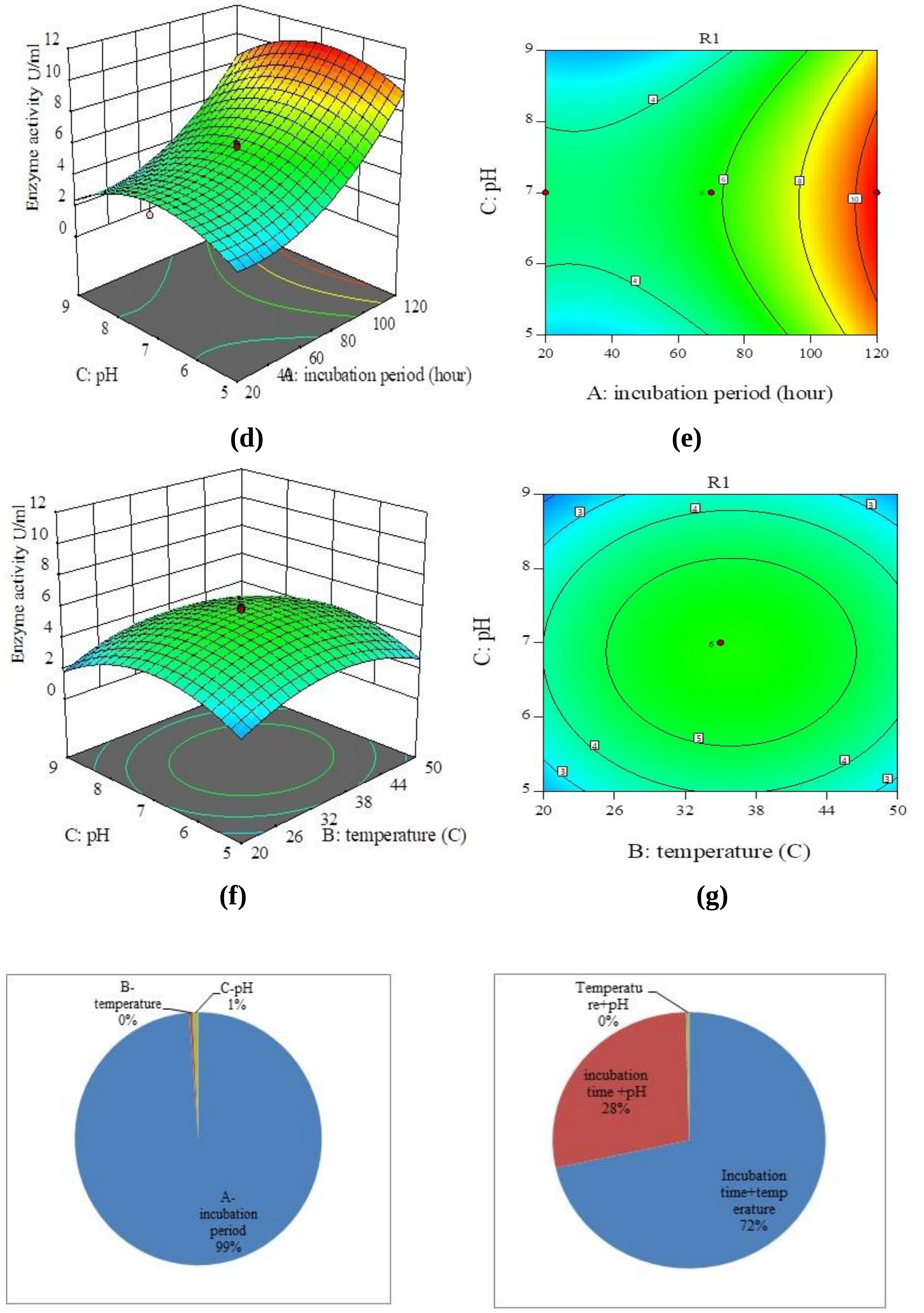
Environmental factors i.e., temperature and pH play a dynamic role in the metabolic activity of bacterial cells for enzyme production. The 3D response surface plots for the interaction between different environmental variables were used and the optimum conditions of each factor for the maximum laccase production by *Bacillus aquimaris* AKRC02 are shown in figure 6.4 b-g. During the temperature optimization for *Bacillus aquimaris* AKRC02 to produce laccase (6.624 U/mL) using rice bran, the maximum activity of laccase was recorded at 35 °C for 120 h (Fig. 6.4 b, c and d, e). Increasing temperature resulted in decreased activity of the laccase. Similar results were also reported in *Pseudomonas putida* where the maximum yield of laccase (8.845 U/mL) was achieved at 40 °C (Kuddus et al., 2013). The pH is another crucial parameter that affects enzymatic reactions during bacterial growth. The pH of the culture medium highly affects enzyme productions due to the existence of various ionizable groups. It influences the hydrogen ions (H^+) concentration throughout the permeability of the cell membrane. The effect of pH on the growth of *Bacillus aquimaris* AKRC02 and production of laccase is shown in figure 6.4 d, e. 3D response surface plots revealed the best optimal values at incubation period 120 h, temperature 35 °C with pH 7.0 in rice bran to induce the maximum laccases activity (10.88 U/mL) (Fig. 6.4 f, g). The 3D response surface plot for the interaction between different environmental variables was used and the optimum conditions of each factor for the maximum laccase production by *Bacillus aquimaris* AKRC02 was displayed in figure 6.4 b-g. Moreover, the effects of percent contribution of individual factors and percent contribution of interactive factor for laccase production showed in Figures 6.4h and I respectively. The enzyme activity showed increasing trends in pH 6.0 to 9.0 and after that, the enzyme activity decreases significantly. A similar result was also noted by Ghosh and Ghosh, (2017), where different fermentation variables i.e. temperature 30 °C and pH 5.0 were found to be significant for laccase yield (5.93 U/mL) by *A. flavus* PUF5. Generally, fungal laccase is reported at low pH values (pH 3-5). In a previous study, laccase production (3.832 U/mL) by *Bacillus sp.* AKRC01 was recorded in the presence of rice bran at optimized conditions of 96 h at pH 8.0 and 35 °C (Kumar et al., 2020a). In comparison to previous observations, the bacterial strain is found better than fungal for laccase production in a wide range of environmental conditions.



(a)

(b)

(c)



(d) (e)

(f) (g)

(h) (i)

Fig. 6.4 (a) Relation between predicted and actual responses **(b-g)** 2D and 3D interaction plot of incubation time temperature and pH for laccase activity **(h)** percent contribution of factor individually **(i)** percent contribution of interactive factor

Table 6.2 Central composite experiments design matrix with experimental and predicted values for laccase production in rice bran from *Bacillus aquimaris* AKRC02

Factor			Rice bran	
A:incubation period	**B:temperature**	**C:pH**	**Observed**	**Predicted**
20	20	5	1.23	0.672
120	20	5	7.56	7.747
20	50	5	1.09	1.432
120	50	5	7.78	7.677
20	20	9	0.56	0.414
120	20	9	7.56	6.969
20	50	9	1.67	1.234
120	50	9	6.65	6.959
20	35	7	3.67	4.468
120	35	7	10.67	10.868
70	10	7	0.54	1.086
70	60	7	1.9	1.712
70	35	4	1.76	1.737
70	35	10	0.54	1.005
70	35	7	5.934	5.772
70	35	7	5.876	5.772
70	35	7	5.743	5.772
70	35	7	6.04	5.772
70	35	7	5.98	5.772
70	35	7	5.86	5.772

Table 6.3 Analysis of variance (ANOVA) for laccase production in the second-order polynomial model

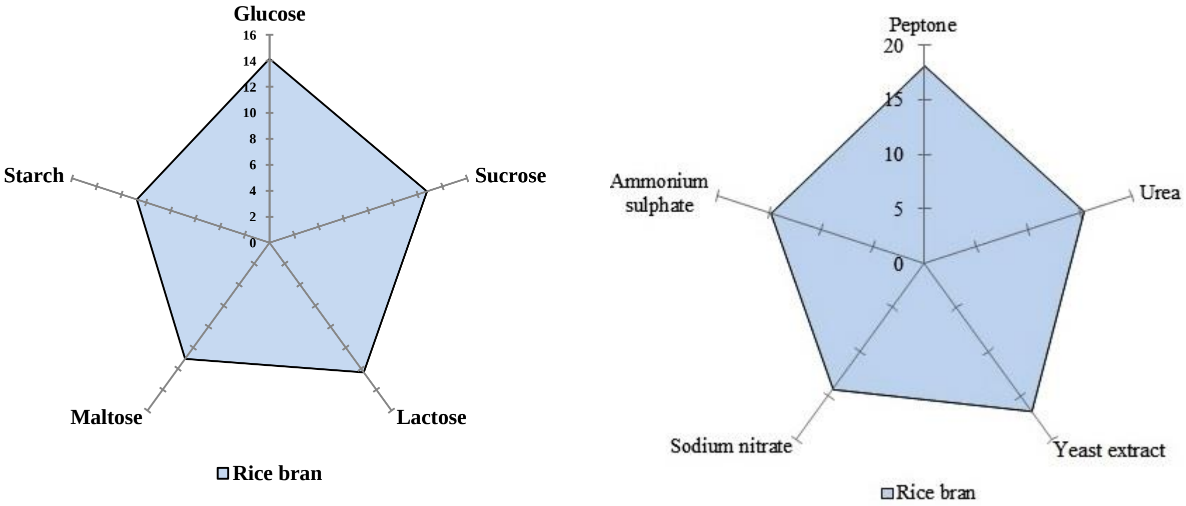
		Rice bran			
Source	**df**	**Sum of Squares**	**Mean Square**	**F-value**	**p-value**
Model	9	173.64	19.29	76.61	< 0.0001*
A-incubation period	1	102.40	102.40	406.62	< 0.0001*
B-temperature	1	0.4784	0.4784	1.90	0.1982
C-pH	1	0.7442	0.7442	2.96	0.1163
AB	1	0.3444	0.3444	1.37	0.2693
AC	1	0.1352	0.1352	0.5369	0.4806
BC	1	0.0018	0.0018	0.0071	0.9343
A^2	1	16.48	16.48	65.43	< 0.0001*
B^2	1	34.35	34.35	136.40	< 0.0001*

C^2	1	36.88	36.88	146.46	< 0.0001*
Residual	10	2.52	0.2518		
Lack of Fit	5	2.46	0.4929	45.81	0.0004
Pure Error	5	0.0538	0.0108		
Cor Total	19	176.16			
Mean	4.43				
Std. Dev.	0.52				
C.V. %	11.33				
$\mathbf{R^2}$	0.98				
$\mathbf{R^2}_{\mathbf{Adjusted}}$	0.97				
$\mathbf{R^2}_{\mathbf{Predicted}}$	0.87				

* Significant

6.3.6 Optimization of carbon and nitrogen sources for laccase production

Optimized environmental conditions (incubation time 120 h, temperature 35 ℃ and pH 7.0) were used for further optimization of carbon source (1.0 %, w/v) (fructose, glucose, maltose, sucrose, and starch) in the culture medium to enhance bacterial growth for the maximum enzyme production. Among the carbon sources tested, supplementation of glucose (1.0 % w/v) induced the maximum activity of the enzyme (14.164 U/mL) in rice bran (Fig. 6.5 a). In previous studies, the addition of 2.0 % glucose results in the maximum production of bacterial laccase (4.967 U/mL) (Kumar et al., 2020a). Ghosh and Ghosh, (2017) recorded the highest activity of the enzyme (9.21 U/mL) in the presence of soluble starch (1.0 %) followed by carboxymethyl cellulose. The effect of additional carbon sources acts as an inducer for bacterial growth for laccase production. Chemically different types of suitable carbon sources are the most important factors for the fermentation process due to enhanced enzyme productions. Different organic and inorganic nitrogen sources (0.5 %, w/v), such as peptone, urea, yeast extract sodium nitrate, ammonium sulfate, were supplemented to rice bran containing medium for enhanced production of laccase. The maximum laccase production was noted (18.124 U/mL) in the presence of peptone 0.5 % w/v along with glucose supplementation (1.0 %) at optimized environmental conditions (Fig. 6.5b). However, inorganic sources of nitrogen, such as sodium nitrate and ammonium sulfate have been considered less significant to produce laccase than organic nitrogen (peptone, yeast extract, and urea). Nitrogen has an active involvement in the synthesis of amino acids that consists of proteins and other substances. In some studies, agro-waste was employed as a nutritional supplement by using other bacterium *Bacillus sp.* AKRC01 for enhanced laccase production (Kumar et al., 2020a). Kumar et al. (2020a) found an optimized carbon source with 2.0 % glucose (4.967 U/mL) and nitrogen source 1.0 % peptone (6.236 U/mL) for the enhanced bacterial laccase production. According to Niladevi et al. (2007), selected nitrogen sources and yeast extract showed higher activity of the enzyme (34.8 U/mL) in rice bran. Muthukumarasamy et al. (2015) noted sucrose and peptone as significant sources for laccase production by *Bacillus subtilis* MTCC2414 during the optimization process through SSF. The results of this study and previous reports revealed that the nutritional supplement that efficiently increases the production of laccases depends on the individual microbes for their specific growth conditions.



(a) **(b)**

Fig. 6.5 Qualitative optimization of different nutritional factor **(a)** carbon, and **(b)** nitrogen sources

Conclusion

The study revealed that the identified bacterium *Bacillus aquimaris* AKRC02 is primarily a halotolerant with potential laccase-producing capabilities. Isolation of AKRC02 from pulp paper mill sludge indicated the broad range adaptation of bacteria for its remediation potential in the presence of various salts i.e., sodium hydroxide (NaOH), CaO, Ca(OH)$_2$, sodium sulfide (Na$_2$S), CaCO$_3$, and organometallic compounds. The SmF process makes it suitable for the bacterial system due to oxygen circulation and nutrients availability. Rice bran appeared as an effective substrate for the maximum production of bacterial laccase in SmF process The maximum laccase production was recorded at optimized environmental (incubation time 120 h: 4.58 U/mL, temperature 35 °C: 6.624 U/mL, pH 7.0: 10.142

U/mL) and nutritional conditions (glucose 1.0 %: 14.164 U/mL, peptone 0.5 %: 18.12 U/mL). Production of industrial enzymes in an economical manner is important. Traditionally, laccase production has been carried out using synthetic mediums, which increases cost and difficult its industrial applications. Thus, this study highlighted the use of SmF process as the tangible and cost-effective process for laccase production, employing agro-residues as natural, renewable, and rich carbon sources that avoid their deposition in landfills and negative environmental impact.

Part B

6.3 Result and discussion

6.3.1 Agro waste substrate screening for laccase production in SmF

Exactly 2 g of each agro-waste substrate added in the different flask containing MBSS medium and inoculated 3.1× 10^6 CFU of *Bacillus* sp. AKRC01. Each flask having pH 7.0 was incubated for 120 hours and laccase production was measured at 24 hours intervals. Results of fig. 6.1 showed that the maximum production of laccase (1.954 U/mL) was noted in the rice bran followed by wheat bran (1.625 U/ mL), sawdust (0.954 U/mL), potato peel (1.326 U/mL), orange peel (0.864 U/mL), pea peel (1.448 U/mL), banana peel (1.322 U/mL) and sugarcane bagasse (1.365 U/mL). Furthermore, studies were carried out using rice bran as an effective substrate for enzyme production in SmF. From this, it is evaluated that the agro wastes reduce the disposal problem and prevent probable environmental pollution.

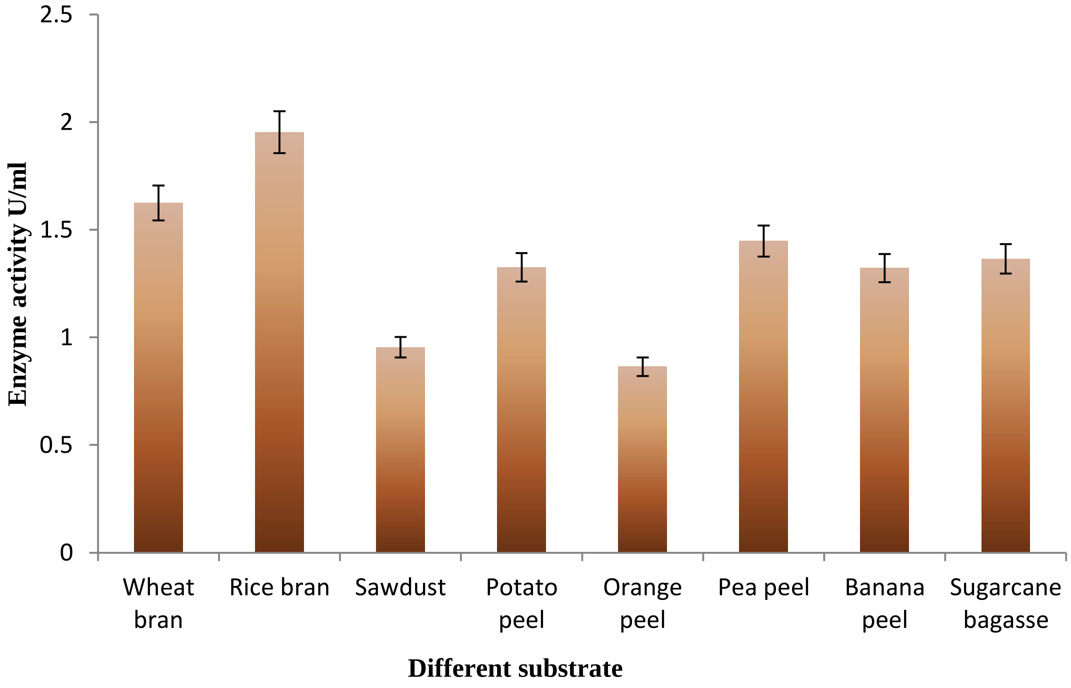


Fig. 6.1 Screening of agro wastes for laccase production

6.3.2 Optimization by RSM

The three significant environmental parameters (incubation time, temperature and pH) that influenced the maximum production of laccase were used as central values to design experiments for central composite response surface design (table 6.1). A predictive quadratic polynomial equation (2) using multiple regression analysis on the experimental data as shown in fig.4a was designed to describe the actual and predictive correlation between laccase production and three significant parameters as follows:

Laccase production (U/mL) = -23.89210+0.098215 Time+0.453983 Tempereature+ 4.11600 pH+4.68750E-06 Time* Temperature -0.000078 Time* pH+0.004250 Temperearture* pH-0.000471Time^2-0.006991Tempereature^2-0.301163pH^2

Table 6.1 Central composite experiments design matrix with observed and predicted values of laccase production in rice bran by *Bacillus* sp. AKRC01

Factors					Rice bran	
Std	**Run**	**A:Incubation time (hours)**	**B:Temperature (^{0}C)**	**C:pH**	**Observed value**	**Predicted value**
8	1	48	25	5	0.354	0.432
20	2	144	25	5	1.202	1.342
9	3	48	45	5	0.452	0.398
7	4	144	45	5	0.645	0.698
19	5	48	25	9	0.524	0.643
13	6	144	25	9	0.678	0.78
15	7	48	45	9	0.298	0.342
10	8	144	45	9	1.125	1.24
6	9	24	35	7	0.214	0.236
4	10	168	35	7	1.823	1.976
3	11	96	20	7	2.023	2.02
18	12	96	50	7	1.752	1.65
5	13	96	35	4	0.456	0.454
17	14	96	35	10	0.987	0.923
1	15	96	35	8	3.832	3.986
14	16	96	35	7	3.452	3.654
12	17	96	35	7	3.457	3.53
11	18	96	35	7	3.565	3.64
2	19	96	35	7	3.645	3.78
16	20	96	35	7	3.512	3.43

where, coded values are the A (incubation time), B (temperature), and C (pH). The variance analysis for the quadratic response surface model is presented in table 6.2. The Model F-value of 37.74 implies the model is significant. There is only a possibility (0.01 %) that this large F-value might happen due to noise. P-values below 0.05 indicate significant terms for the model. In this case, A, A^2, B^2, C^2 were significant model terms. p values greater than ($p>0.05$) indicate the not significant model terms. In this case, A, A^2, B^2, C^2 were significant model terms. The determination coefficient R^2 (0.8120) showed good compatibility between the values of experimental and predicted. The adjusted R^2 value (0.9457) indicated that the enzyme activity variation (94.57 %) was attributed to the independent variables and the model could not describe the total variation only (0.87 %).

Table 6.2 Analysis of variance (ANOVA) for laccase production in second-order polynomial model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	34.08	9	3.79	37.74	< 0.0001*	significant
A-Time	1.57	1	1.57	15.68	0.0027	
B-Temperature	0.0332	1	0.0332	0.3312	0.5777	
C-pH	0.0854	1	0.0854	0.8506	0.3781	
AB	0.0000	1	0.0000	0.0004	0.9844	
AC	0.0005	1	0.0005	0.0045	0.9479	
BC	0.0578	1	0.0578	0.5760	0.4654	
A^2	12.13	1	12.13	120.92	< 0.0001*	
B^2	5.03	1	5.03	50.17	< 0.0001*	
C^2	14.59	1	14.59	145.43	< 0.0001*	
Residual	1.00	10	0.1003			
Lack of Fit	0.9774	6	0.1629	24.95	0.0039	significant
Pure Error	0.0261	4	0.0065			
Cor Total	35.09	19				
Std. Dev.	0.3168					
Mean	1.70					
C.V. %	18.64					
R^2	0.9714					
Adjusted R^2	0.9457					
Predicted R^2	0.8120					
Adeq Precision	15.9472					

***** significant

6.3.3 Perturbation plot

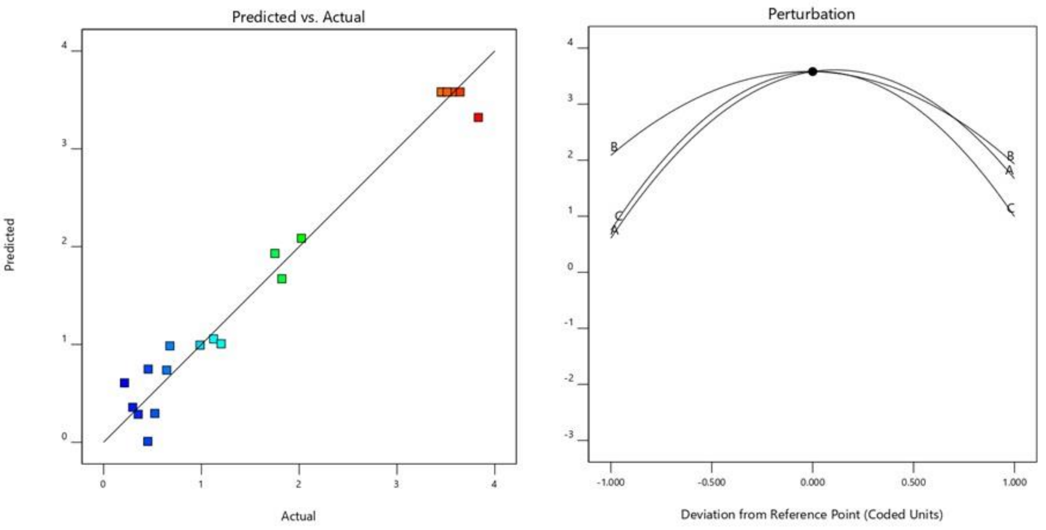
The perturbation plot demonstrates how the response change as each factor passes from the selected reference point, with other variables at the reference value being kept constant (fig. 6.2a). Laccase production was affected by the incubation period followed by temperature and pH (Fig. 4). Increasing the incubation period up to 96 hours resulted in elevated laccase production while increasing the temperature (35 ^{0}C) and pH (8.0) up to the coded reference point (0.21) also increases the laccase productions. However, the increase in these parameters beyond the coded point may decrease the laccase production.

6.3.3.1 Diagnosis of the statistical model

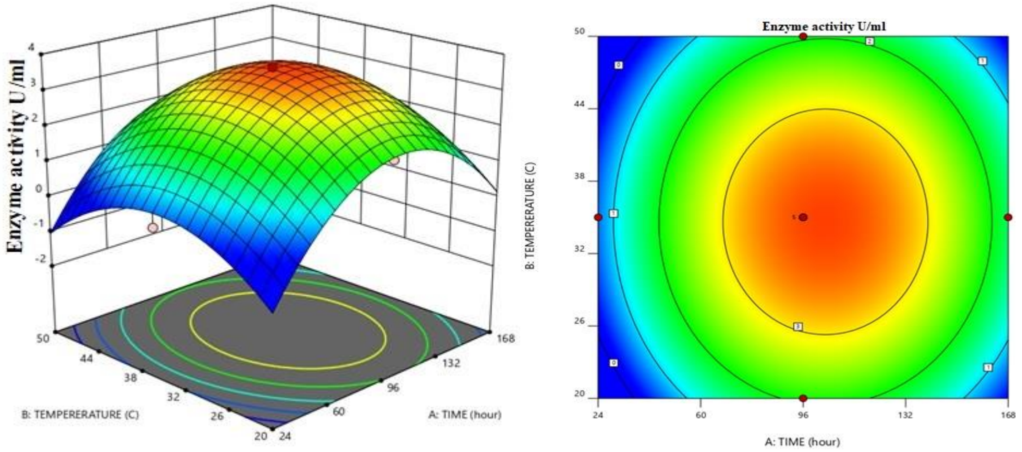
The normal residual plot can analyze the statistical properties of the model and data points should be nearly linear. A non-linear pattern in the error (such as an S-shaped curve) denotes non-normality that can be adjusted by a conversion. The residual from the production of laccase indicates the normal distribution (Fig. 6.2b). No data abnormalities are indicated by their alignment on the drawn line.

6.3.3.2 Interaction between variables

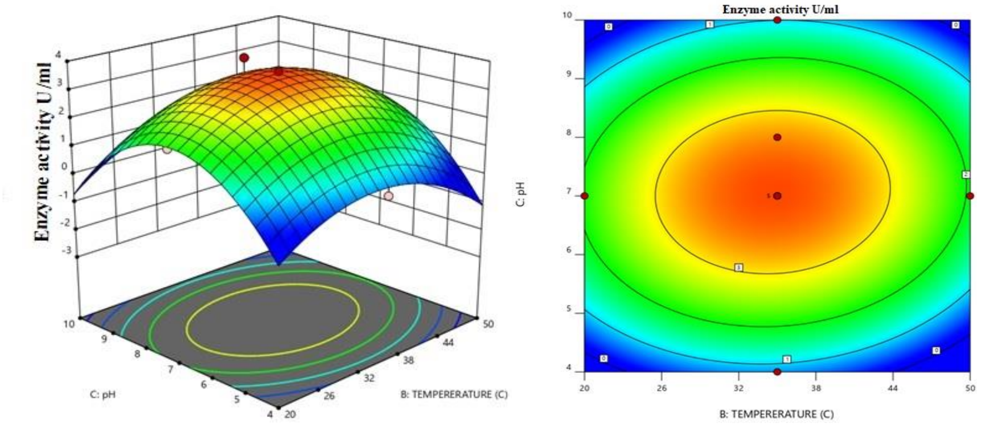
When the third is kept at its optimum value, the interaction between two factors was shown in 3D and 2D graphs (Fig. 6.2c-h). The 3D and 2D graphs showed how the production of laccase influenced by the variability of two variables at a time, maintaining the other in optimum conditions. Laccase enzyme production increased with increase optimum incubation time up to (96 hours). Incubation time, temperature and pH were played a major role in enzyme production in SmF conditions (Fig. 6.2c-d). Moreover, initial water amounts adversely impacted the microbial activity. This may be because the porosity of the medium reduces as the liquid content increases leading to easy oxygen transfer (Aslam et al., 2012). According to Niladevi et al. (2007), different variables fermentation such as incubation time, temperature and pH were found to have a major role for laccase production by *Streptomyces psammoticus.*


Predicted vs. Actual
Predicted
Actual
0
1
2
3
4
Perturbation
Deviation from Reference Point (Coded Units)
-1.000
-0.500
0.000
0.500
1.000
A
B
C
-3
-2
-1
0
1
2
3
4

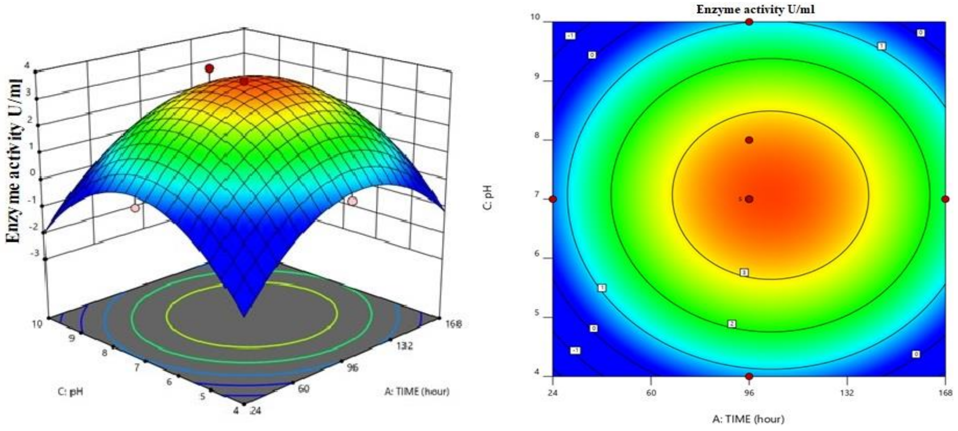
(a) **(b)**


Enzyme activity U/ml
B: TEMPERERATURE (C)
A: TIME (hour)
20
26
32
38
44
50
24
60
96
132
168
-2
-1
0
1
2
3
4

(c) **(d)**


Enzyme activity U/ml
C: pH
B: TEMPERERATURE (C)
4
5
6
7
8
9
10
20
26
32
38
44
50
-3
-2
-1
0
1
2
3
4

(e) **(f)**



(g) (h)

Fig. 6.2 (a) Three factors affecting laccase production of perturbation graph, **(b)** Relation between actual and predictive data, **(c-h)** 3D and 2D interaction plot of incubation time temperature and pH for laccase activity.

6.3.4 Qualitative optimization of carbon and nitrogen sources using RSM Optimized Value

In present study physical factors, optimized value (incubation period 96-hour, temperature 35 ^{0}C and pH 8.0) were used for further qualitative optimization of carbon sources (glucose, fructose, maltose, starch, and sucrose) and nitrogen source (peptone, urea, yeast extract, and ammonium sulfate, sodium nitrate). The use of different concentrations of carbon and nitrogen source for laccase production in SmF has also been well-documented (Niladevi et al., 2007; Nandal et al., 2013). In this optimization process, the most influencing additional nutrient source affected laccase production and combined with glucose 2.0 % and peptone 1.0 %. Optimized nutritional conditions with 2.0 % glucose (4.967 U/mL) and 1.0 % peptone (6.236 U/mL) supplements gains maximum production of extracellular bacterial laccase (Fig. 6.3a and 6.3b). According to Muthukumarasamy et al. (2015), they reported that 3 % of sucrose showed the maximum activity with rice bran. Similarly, nitrogen source as peptone showed higher activity with rice bran. Annova was used to examine the experiment results and to evaluate how many differences each variable contributed.

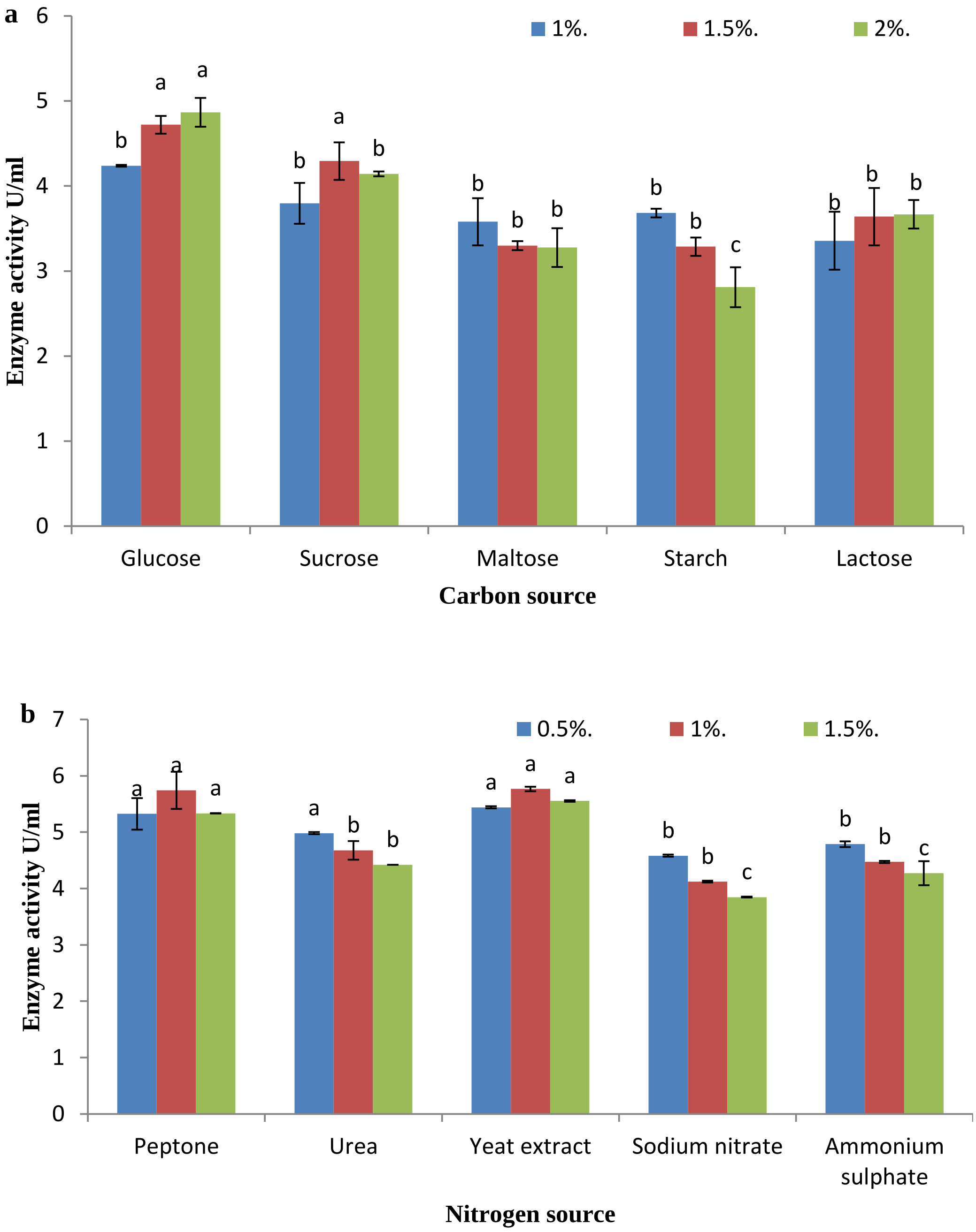


Fig. 6.3 Effect of **(a)** carbon and, **(b)** nitrogen sources on laccase activity using rice bran as a substrate

6.3.5 Validation of the model

The statistical tool of response surface model for all experimental design has been validated. To study laccase optimization, a random set of the experimental combination was used. The validation experiment clearly demonstrates that the experimental production value of 6.236 U/mL and predicted value 5.935 U/mL was in close agreement with the statistically. The results validated that the preceding model with different parameters such as incubation time (96 hours), temperature (35 ^{0}C), pH (8.0) and additional carbon (2.0 %) and nitrogen sources (1.0 %) was the optimum conditions for maximum laccase production, confirming the model's precision.

Conclusion

The purpose of this study for maximum production of bacterial laccase enzyme in submerged fermentation state is a viable approach to the cost-related problems encountered in the production of enzymes. The optimized environmental factors (incubation period 96 h, temperature 35 ^{0}C and pH 8.0) and nutritional conditions (glucose 2.0 % and peptone 1.0 %) are the crucial parameters enhancing the maximum laccase production (6.236 U/mL) by *Bacillus* sp. AKRC01 in the presence of rice bran as an effective agro-waste substrate. The production of laccase by bacteria has been broadly studied due to their secretion of enzyme and their growth using agro-waste as a cheap substrate.

Chapter Seven

Purification and characterization of bacterial laccase enzymes of different molecular weight in different nutritional and environmental conditions

Purification and characterization of bacterial laccase enzymes of different molecular weight in different nutritional and environmental conditions

7.1 Introduction

Laccases are multicopper oxidases (MCOs) which catalyze the oxidation of a wide variety of organic and inorganic compounds with concomitant four-electron reduction of molecular oxygen to water. In general, laccases oxidize phenols and aromatic amines such as methoxyphenols, phenols, polyphenols, anilines, aryl diamines, hydroxyindols, benzenethiols and some cyanide complexes of metals. Laccases are very useful enzymes concerning their applications in industry. They have found use in industrial and biotechnological applications such as in biobleaching, xenobiotics bioremediation, textile dyes decolorization, biosensors, food industry etc (Shraddha et al., 2011).

They have been found in almost all spheres of life but have been most extensively studied in fungi including *Ascomycetes, Basidiomycetes* and *Deuteromycetes* (Brijwani et al., 2010). Fungal laccases are not stable in extreme conditions like temperature, pH, salt etc. which exists in the industry. Moreover, the production of fungal laccases in large quantities is problematic due to the accumulation of large amounts of fungal biomass. Bacterial laccases have several significant properties which are not characteristics of fungal laccases like stability at high temperature and pH as well as salt tolerance (Zhang et al., 2013) etc. Only a few bacterial laccases have been characterized till date but they could not be exploited on an industrial scale as most of them are intracellular or spore bound (Sharma et al., 2007). There are some reports of extracellular laccase from *Sterptomyces* (Niladevi and Prema, 2007; Molina-Guijarro et al., 2009; Gunne and Urlacher, 2012), which are difficult to produce in large quantity because of the problems associated with their filamentous growth (Van Wezel et al., 2006), slow growth rate and expensive downstream processing (Gomes and Menawat, 1998), thus limiting their applications. Therefore, there is a need to study laccases that are thermo-alkali-stable and produced extracellularly by bacteria. In this regard, previously we have isolated *Bacillus tequilensis* SN4 from the activated sludge of paper mill effluent treatment plant which produces a laccase extracellularly in the culture supernatant. Moreover, on preliminary analysis, this laccase was found to be highly thermostable (optima at 80–90 ^{0}C) and alkali-stable which makes this laccase a potential candidate for application in the industry (Sondhi et al., 2014). In this study, we have purified and characterized the enzyme with respect to properties which are important for its industrial applications.

7.2 Materials and methods

7.2.1 Purification of laccase

The culture supernatant was collected by centrifugation of 24 h grown culture of *Bacillus* sp. AKRC01 and *Bacillus aquimaris* AKRC02 at 5000 rpm for 20 min. The chilled acetone was added slowly with constant stirring at 30 % saturation into the culture supernatant and kept overnight at the cold condition to precipitate the proteins. The precipitate formed after the treatment of acetone was dissolved in 50 mM potassium phosphate buffer (pH 8.0) and dialyzed against the same buffer overnight. The dialyzed enzyme was applied on the DEAE-cellulose an anion exchange column (cylindrical glass column with 15cm height and 1 cm diameter) equilibrated with the same buffer at a flow rate of 1mL min^{-1}. The retained proteins were eluted with a linear NaCl gradient from 0 to 0.4M over 100 min. The purified enzyme fractions were loaded and analyzed by SDS-PAGE (10 %) and run at 120 volts (Laemmli, 1970). After running, the samples were stained with coomassie brilliant blue R 230, and protein bands were visualized under the gel documentation system (GeNeiTM UVITEC Cambridge).

7.2.2 Gel Filtration Chromatography using Sephadex (G-100)

7.2.2.1 Requirement:

- Double Distilled water
- Micropipette (1mL and 100 µL)
- Microtips (1mL and 100 µL)
- Beaker (08)
- Sodium phosphate buffer (4.5)
- Sodium chloride
- Sephadex G-100 (**Swelling:**1 g swells to 15-20 mL gel; **Bead Size:**40-120 µm)

7.2.2.2 Gel Matrix Preparation

1. Prepare at least 20-bed volumes (10Vt) of buffer and filter it through 0.22 µm filter in a filtration unit.

2. Calculate the amount of dry matrix required for packing the column:

a. The swelling factor of the gel matrix is provided by the manufacturer.

b. The amount of dry gel required in grams $= \frac{Bed\ volume\ (ml)}{Swelling\ factor\left(\frac{ml}{g}\right)}$

3. Weigh slightly more than the calculated amount of the gel matrix.

4. Take 2-bed volumes (2Vt) of buffer in an Erlenmeyer flask with a thick side-arm for applying vacuum.

5. Transfer the dry gel matrix into the buffer present in the Erlenmeyer flask.

6. Stir the suspension gently using a glass rod (Important: Do not use a magnetic stirrer as it can disrupt the soft gel particles).

7. Cover the flask with a rubber stopper that fits well in the flask mouth; seal the side arm with parafilm.

8. Allow the gel to swell overnight at room temperature.

9. During swelling, the gel settles down in the beaker. The upper buffer region may contain broken beads and looks hazy. Remove the hazy buffer portion by decanting.

10. Suspend the settled gel in 2 – 4 fold excess of buffer and allow ~95 % gel to settle down. Decant the buffer that contains non-settled gel particles. Repeat this process until the gel matrix settles as a sharp zone (usually 4 – 5 times is sufficient).

7.2.2.3 Packing of the column

1. Dilute the gel slurry two-fold by adding approximately the same volume of the buffer as that of the settled gel.

2. If more than 50 % of the column tube needs to be packed, attach an extension on top of the column tube that can, together with the column, hold the entire volume of the gel slurry.

3. Mount the column tube on a stable laboratory stand. The column along with its attachments.

4. Remove air from the bottom adapter tubing of the column by attaching a buffer-filled syringe and forcing the sufficient buffer volume up through the bed support net.

5. Insert the bottom adapter to the desired level in the column tube, remove the syringe, tighten the adapter, and attach the stop plug.

6. Ensure that the column is vertical using a carpenter's level or a plumb line.

7. Swirl the gel slurry and pour the entire slurry into the column along a glass rod that is in contact with the inner wall of the column or column extension.

8. Fill the remaining space with the buffer by pouring it carefully along the glass rod so that the gel layer is not disturbed**.**

9. Open the top cap first and then the bottom cap of the column to prevent air from entering the resin. Allow the buffer to drain out of the column, under gravity, to a waste container. Ensure that the resin settles evenly in the column.

7.2.2.4 Equilibrate the column

1. Apply 100 μL Gel Filtration Buffer to the top of the column and allow it to drain out freely into a waste container. Repeat this step 9 more times, so that a total of 1 mL Gel Filtration Buffer has been added.

2. Carefully load 3 mL Gel Filtration Sample to the column without disturbing the column surface. Allow the sample to enter the column.

7.2.2.5 Elute the sample

1. Apply 100 μL Gel Filtration Buffer to the top of the column and allow it to drain freely into a waste container.

2. Repeat step 5 until sample begins to elute from the column. When the sample starts to elute from the column, position a centrifuge tube under the column and collect 0.2-0.3 mL elution into the tube.

3. Collect consecutive fractions eluting from the column in new tubes.

7.2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE)

7.2.3.1 Preparation of gel

1. Clean the surface thoroughly on which work has to be done.

2. Clean the glass plates with soap and water, then with ethanol. Assemble the glass plates and spacers.

3. Assemble the glass plates by putting the shorter glass plate in front of the longer one.

4. Fix the glass plates and fill them with distilled water to check for any leakage from the bottom. If leakage occurs, seal the end of the plate with molten agar.

5. First the resolving gel is prepared which is usually more basic and has higher polyacrylamide content than the loading gel.

6. Take a small beaker; add distilled water, Tris-Cl (pH-8.8), acrylamide. SDS with the help of pipette.

7.2.3.2 Resolving Gel (10 %)

Ammonium persulphate and TEMED are loaded when the gel is ready to be polymerized.

1. Mix it properly by moving the beaker in circular motion on the surface.

2. Pour the gel between the plates before it polymerizes, then add water to the surface to remove the unpolymerized gel.

3. Similarly prepare stacking gel using the following composition.

H_2O	:	4 mL
Tris 1.5M	:	2.5 mL
SDS 10 %	:	100 µL
Acrylamide 40 %	:	3.33 mL
APS 10 %	:	50 µL
TEMED	:	15µL

7.2.3.3 Stacking Gel (5 %)

1. When resolving gel polymerizes, remove the water by using tissue paper, pour the stacking gel and place the comb to create the wells.

2. After the stacking gel ploymerizes, remove the comb and the gel is ready for electrophoresis.

H_2O : 3.075 mL
Tris 1.5M : 1.25 mL
SDS 10 % : 0.025 mL
Acrylamide 40 % : 3.33 mL
APS 10 % : 0.67 mL
TEMED : 0.005 mL

7.2.3.4 Preparation of samples

Mix the sample with an equal volume of 2X sample buffer, Boil the sample in boiling water for 5 minutes, cool to room temperature before loading, if particulate is present, centrifuge samples for 2 minutes.

7.2.3.5 Loading the sample

1. Clamp the gel plates properly and fill the buffer chamber with running buffer.

2. Load 50µL prepared sample into the wells. The tip is wiped with distilled water every time the next sample is to be loaded

3. First marker is loaded in the first lane to determine the size of the desired protein.

4. Then the samples are loaded in the adjacent lanes. Bubbles are avoided in the tip. Lane 9: Protein marker; Lane 3: fractional precipitation by $(NH_4)_2SO_4$ 60 %; Lane 5: Crude Enzyme and lane 7, partial purified L-asparaginase on Sephadex G-200.

Sample loading Dye for 1mL

DdH_2O : 125 µL
0.5 % M Tris, pH (6.8) : 125 µL
50 % Glycerol : 300 µL
10 % SDS : 400 µL
2-βmercaptoethanol : 50 µL
Pinch of Bromophenol Blue

7.2.3.6 Running the gel

1. Place the lid on top of the buffer chamber.

2. Connect the electrical leads to the power pack with the proper polarity (black to black and red to red) and run the gel at 60V.

3. Run the gel till the dye reaches the end of the gel.

4. As soon as the dye reaches at the end, switch off the power.

Running Buffer for (1 L)

Tris **:** 3.002 g
Glycine **:** 14.40 g
SDS **:** 1 g

7.2.3.7 Staining the gel

1. Take out the glass plates from the electrophoretic tank and tear apart the two plates using spatula.

2. Carefully place the gel into staining solution overnight.

3. Incubate the gel into this staining solution overnight.

Staining Solution

Coomassie blue **:** 0.2 % w/v

Methanol: Acetic acid: water **:** 5**:**1**:**5

Dissolve Coomassie Blue in Methanol: Acetic acid: Water.

Make up the final volume to 500mL.

7.2.3.8 Destaining the gel

1. Once the gel is stained, the gel is then transferred from a staining solution to a destaining solution.

2. Destaining is done until all the extra dye is removed from the gel except the dye bound to protein in the form of bands.

3. After destaining clear bands of protein are seen and compared to corresponding bands of the ladder to determine the molecular weight of the protein.

Destaining Solution

Methanol **:** Acetic acid **:** water 5**:**1**:**5

Make up the final volume to 500 mL.

7.2.4 Stability of laccase enzyme in different temperature

Laccase was incubated in various temperature ranges (25, 35, 45, 55, 65, 75, and 85 ^{0}C) for different time intervals (0, 2, 4, 6, 8, 10, 12, 14, and 16 h) to determine the thermal stability. The residual laccase activity was calculated by evaluating the oxidation of guaiacol at 100 mM acetate buffer pH 5.0 at 465 nm (Navada and Kulal, 2020).

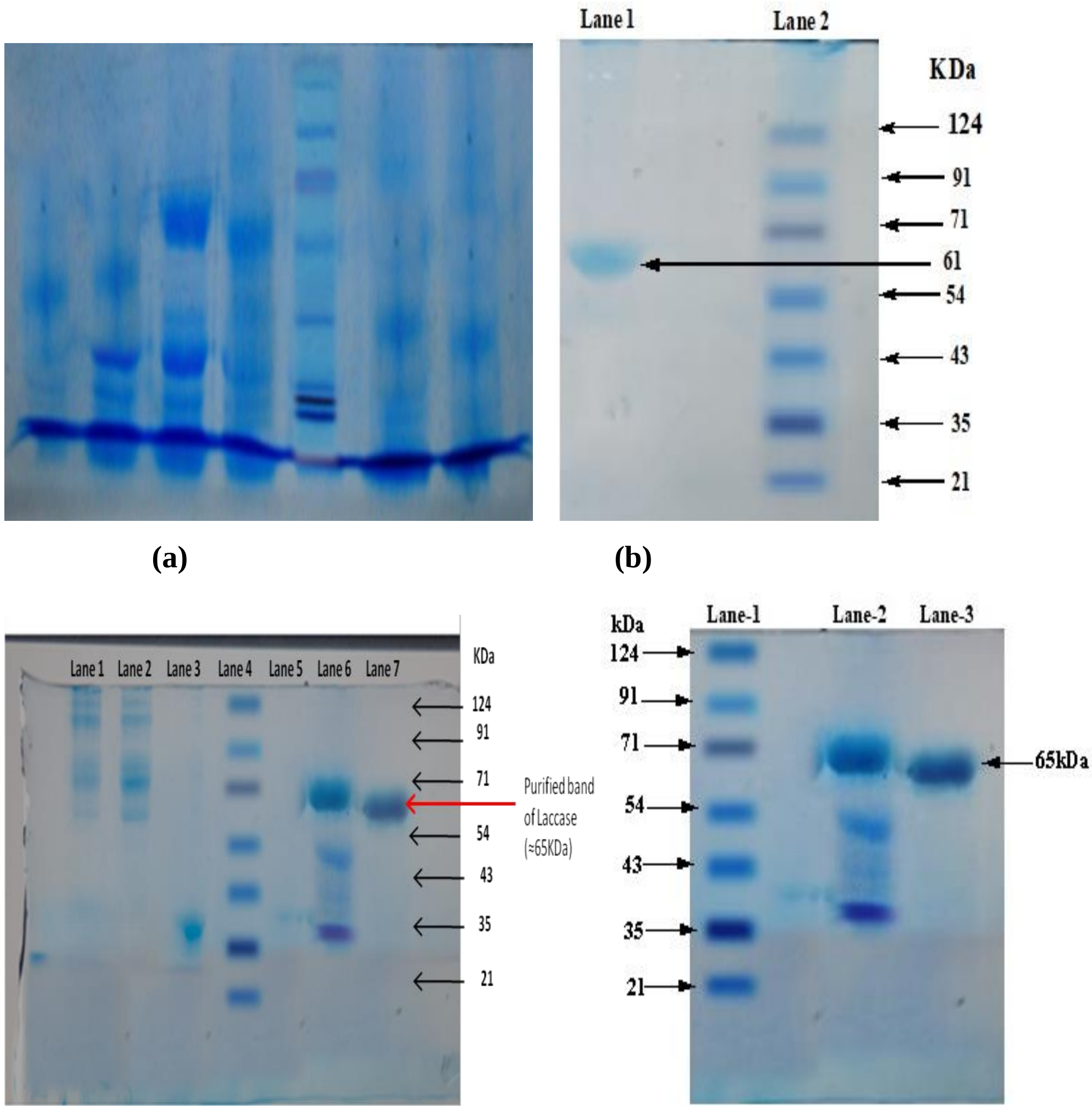
7.3 Result and discussion

7.3.1 Purification of bacterial laccase and determining molecular weight

Extracellular laccase was purified from a culture of *Bacillus* sp. AKRC01 and *Bacillus aquimaris* AKRC02 in optimized conditions for enzyme production. The laccase enzyme was purified from bacterial culture and determined molecular weight by denaturing 10 % SDS-PAGE performed. The molecular weight of laccase produced by *Bacillus* sp. AKRC01 in the presence of rice bran was found to be 61 kDa (Fig. 7a and b), while *Bacillus aquimaris* AKRC02 was determined to be 65 kDa (Fig. 7b and c), which is inconsistent with the previous research. The elution profile of purified laccase showed a well-resolved single peak of enzyme activity. Approximately 38.08-fold purification was achieved with the specific activity of 228.34 U/mg (Table 7.1). Niladevi and Prema, (2007) discovered that *Streptomyces psammoticus*, unusual halotolerant-alkaline laccase having a molecular weight of about 43kDa and *Bacillus subtilis* WPI was also reported for a laccase production and determined the molecular weight of 55 kDa (Sheikhi et al., 2012). Similar results were also documented from *Bacillus subtilis* MTCC 2414 was found to be 52 to 55 kDa using rice bran and wheat bran (Muthukumarasamy et al., 2015). Another melanogenic bacterium *Bacillus* HR03 having laccases of 50 kDa molecular weight (Dalfard et al., 2006).

Table 7.1 Purification of laccase from *Bacillus aquimaris* AKRC02

Purification steps	**Total protein concentration (U/mg)**	**Total activity (Units)**	**Specific activity (U/mg)**	**Yield (%)**	**Purified (fold)**
Concentrated crude enzyme	48.26	816.12	16.86	100	1
$(NH_4)_2SO_4$ precipitation	15.60	310.61	19.91	29.51	1.26
Ion exchange chromatography Q-Sepharose FF 100	0.6	114.18	228.34	18.32	38.08



(a) (b)

(c) (d)

Figure 7.1 SDS-PAGE of partially purified laccase from **(a)** Crude laccase enzyme, **(b)** *Bacillus* sp. AKRC01. **Lane 1:** Purified laccase, **Lane 2:** Protein markers**, (c)** Crude laccase enzyme**, (d) Lane 1:** Protein ladder, **Lane 2:** Crude laccase, **Lane 3**: Purified laccase from *Bacillus aquimaris* AKRC02.

7.3.2 Laccase stability at different temperature

Purified laccases of *Bacillus aquimaris* AKRC02 were stable at 45 ^{0}C for 8 h with an enzyme activity loss of just 50 % in comparison to the enzyme activity at the optimum temperature. About 28 % of its activity was maintained by the enzyme even after 16 h at 45 ^{0}C. The half-life of laccases at higher temperatures of 55 and 65 ^{0}C was approximately 6 h. The activity of the enzyme dramatically decreased and displayed about 90 % loss of activity at a higher temperature of 85 ^{0}C after 16 h. At

25 and 35 ^{0}C, the laccase was very stable with a minimum loss of 5 % in its activity, even after 16 h. After 16 h, there was approximately 76, 84, 88 % loss in enzyme activity at 55, 84, 88 ^{0}C, respectively (Fig. 7.2) entailing the enzyme stability at the optimal temperature (35 ^{0}C). These findings were consistent with the earlier *T. hirsuta* laccase enzyme study, which showed that the enzyme's half-life was 4 h at 60 to 70 ^{0}C and the enzyme was thermo-stable at 20 and 30 ^{0}C even after 16 h, with minimal loss of activity (Navada and Kulal, 2020). Earlier reports showed that the heterologically expressed recombinant laccase of *T. trogii* BAFC 463 in *P. pastoris* was thermo-stable with a half-life of 45 min at 70 ^{0}C (Campos et al., 2016). However, it was evident in the present study that laccases produced from *Bacillus aquimaris* AKRC02 showed the highest thermal stability even at 75 ^{0}C with a half-life of 4 h.

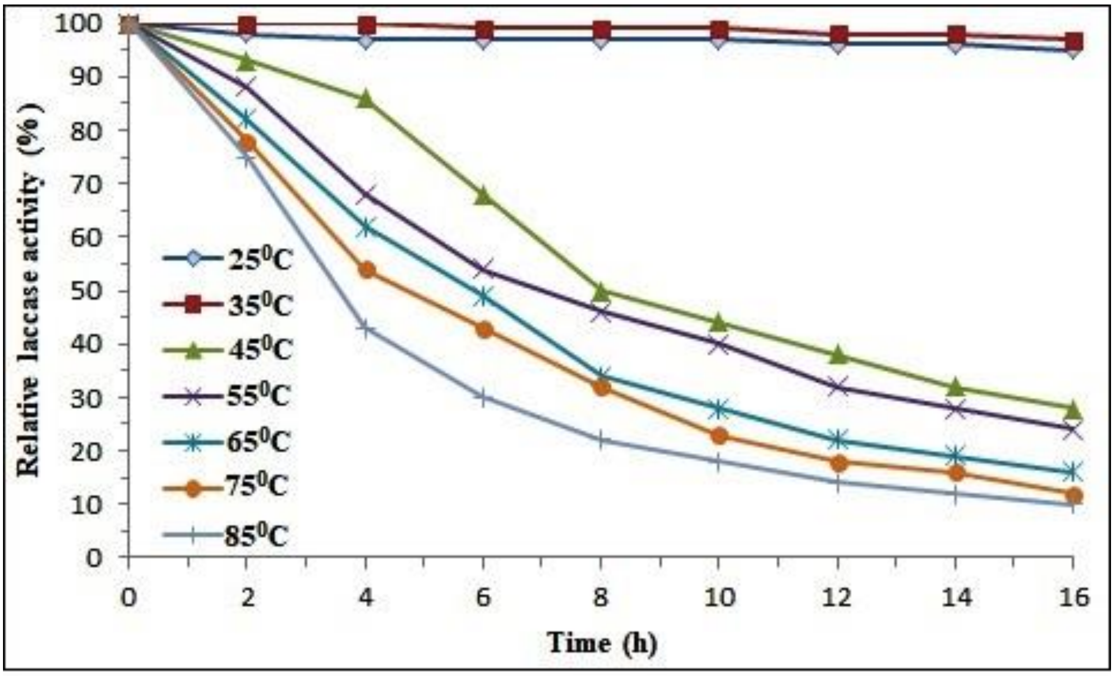


Fig. 7.2 Stability of laccase at different temperatures and time intervals.

Conclusion

Laccase enzyme was partially purified from culture of *Bacillus* sp. AKRC01 and *Bacillus aquimaris* AKRC02 in optimized conditions for enzyme production via ammonium sulfate precipitation method and chromatography using the Sephadex column and determination of the molecular weight of 61 kDa and 65 kDa compared with the protein ladder was done by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purified laccase showed activity for broad range temperature stability. Production of industrial enzymes in an economical manner is important.

Chapter Eight

Study the effect of copper (Cu) and iron (Fe) nanoparticles for production of bacterial laccase

Study the effect of copper (Cu) and iron (Fe) nanoparticles for production of bacterial laccase

8.1 Introduction

Laccases are member of copper-containing oxidases that are involved in the oxidation of a range of aromatic compounds including amines and phenolic units present in lignin (Leonowicz et al. 2001; Majeau et al. 2010). These are multicopper proteins that belong to the blue-copper protein family and present in several plants, fungi, and bacteria (Shraddha et al., 2011). Laccases have got a lot of interest from academicians in the recent decade because of their high substrate specificity and capability to oxidize a range of phenolic compounds. They also exhibited a crucial role in the degradation and detoxification of industrial wastewaters (Couto and Herrera, 2006). Recently, the application of bacterial laccases has been growing because of their several distinctive aspects from an industrial perspective, such as capability to work in wide-ranging pH and temperature and remains highly stable against several inhibitory agents (Guan et al., 2015). Furthermore, bacterial laccases offer several added benefits due to their lower industrial use cost such as high substrate specificity, rapid synthesis and ease of cloning and expression in the host etc.(Fernandes et al., 2014; Prins et al., 2015). They are used in bio-bleaching of pulp, textile dye decolorization, degradation, and development of biosensor (Mathews et al., 2016). Laccases have also been utilized in biotransformation processes, wastewater treatment, and the development of biofuel cells due to its strong catalytic activity (Wang et al., 2008; Nguyen et al., 2016; Patel et al., 2018). Nevertheless, the commercial use of laccases is still limited due to their high manufacturing cost, and low reusability. The immobilization of laccase molecules on various supporting substances for example activated carbon, chitosan, magnetite, mesoporous silica, halloysite clay nanotubes, and polymers offers a generic method to overcome these shortcomings (Wang et al., 2008; Menale et al., 2012; Wang et al., 2013; Nguyen et al., 2016; Tully et al., 2016; Patel et al., 2018; Pylypchuk et al., 2018).

Earlier research has revealed that covalent bonds might be bind laccase to enhance the stability and prevent their desorption (Zhu et al., 2007). However, due to the conformational changes within them, the immobilization procedure could reduce catalytic activity. The active sites of enzymes are not easily degraded by laccase tethering by adsorption process. Most carriers, on the other hand, are nonselective, and the low physical adsorption forces could result in loss of laccase and lower working stability (Xia et al., 2016). Consequently, the interaction among metal ions and complexing agents through metal chelated adsorption can resolve these problems. It is reported that laccase contains high concentration of histidine residues that can develop coordination interactions with Cu^{2+} ions (Patel et al., 2019). More critically, this high binding capacity can reduce desorption and distortion of laccase. As a result, adsorption through metal chelation emerges as a pleasing approach for immobilizing the laccase (Sun et al., 2019; Wang et al., 2019; Zhang et al., 2020).

For the recycling of biocatalysts and promising carriers, the magnetic nanoparticles are considered efficient (Xia et al., 2016; Kadam et al., 2017). In this study, we have developed Cu/FeO nanoparticles for laccase immobilization. The Cu/FeO nanoparticles were discovered to have a simple immobilization technique, high loading capacity, and high enzyme activity, stabilities, and reusability of the immobilized laccase. Immobilized laccase was also shown to be a viable biocatalyst for the degradation of a variety of organic pollutants.

8.2 Materials and Methods

8.2.1 Chemicals and reagents

All the media, chemicals and reagents used in the following experiments were purchased from Hi-media (India) and Sigma-Aldrich (USA) until specified.

8.2.2 Synthesis of Cu/FeO nanoparticles

Copper nitrate [$Cu(NO_3)_2$], and iron nitrate [$Fe(NO_3)_2$], Sodium hydroxide (25 wt % in H_2O) were obtained from Merck scientific Ltd India. The chemicals used for this experimental work are of analytical grade. For the synthesis of catalyst, the required amount of $Cu(NO_3)_2$. $3H_2O$ and $Fe(NO_3)_2.9H_2O$ were dissolved separately using deionized water. The prepared solutions were mixed in a single beaker and stirred continuously for 7 hours at room temperature. The temperature of the stirred solution was then elevated to 50 ^{0}C with dropwise additions of liquid Sodium hydroxide solution to keep the pH of the solution between 8 and 12. To obtain a better precipitate, the reaction mixture was further stirred. The synthesised catalyst was maintained in an oven at 110 ^{0}C for 10-12 h and calcined in a muffle furnace at 900

^{0}C after suitable precipitation that was filtered and washed with deionizedwater multiple times to obtain the desired catalyst. The schematic representation of catalyst systhesis process, characterisation and application is shown in Scheme 1.

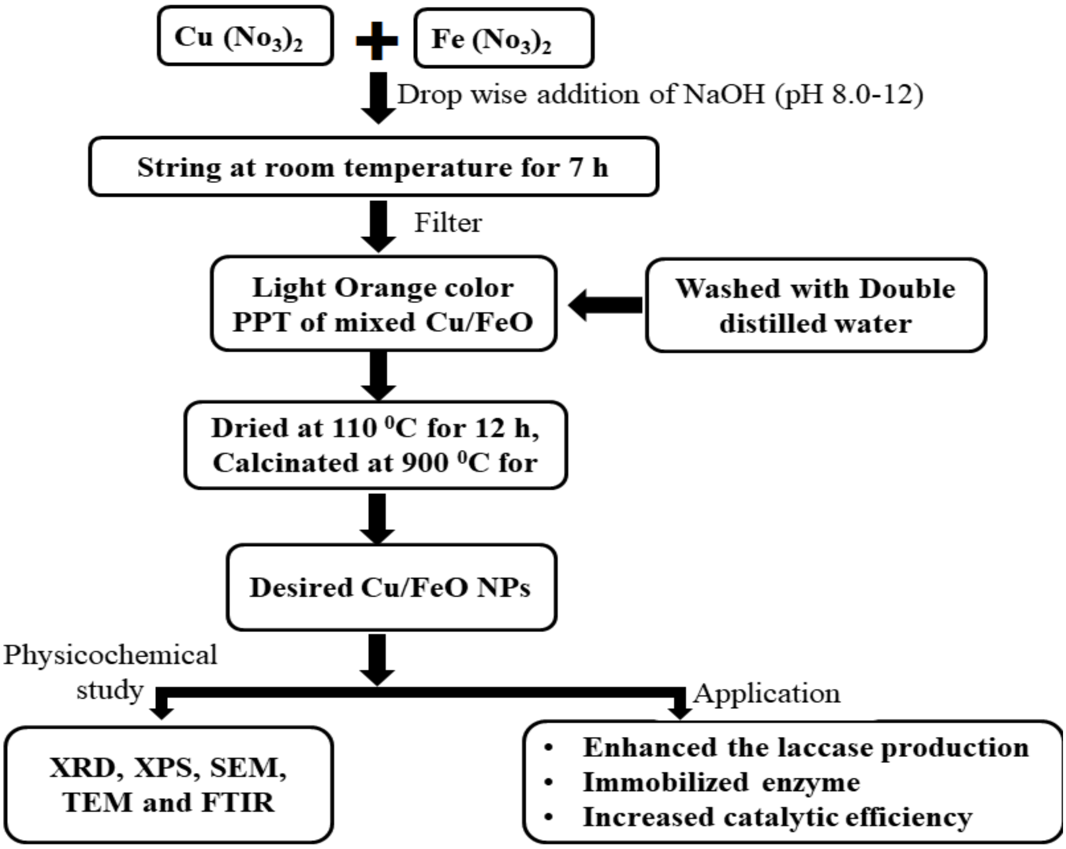


Fig. 8.1 Systematic synthesis of Cu/FeO NPs.

8.2.3 Characterizations of the Copper and Iron Nanoparticles

8.2.3.1 X-ray diffraction (XRD) analysis of Cu/FeO NPs

X-ray diffraction (XRD) patterns were examined through Rigaku smart lab diffractometer using high-power Cu Ka radioactive source (k = 0.154 nm) at 40 kV/40 mA having 2θ range of 10^0-90^0 with a step function of 0.02. The joint committee on powder diffraction standards (JCPDS) file and Scherrer equation were used to determine the amorphous phase and amorphous size of the designed catalysts, respectively.

8.2.3.2 XPS analysis of Cu/FeO NPs

To evaluate the surface elemental valence of spent Cu/FeO inside a range of about 10 nm, X-ray photoelectron spectroscopy (XPS) was used with physical electronics quantum (VG ESCALAB 250,). However, aliphatic hydrocarbons C 1s peak was used at 284.6 eV to calibrate the spectral binding energy.

8.2.3.3 SEM, TEM and FT-IR analysis of Cu/FeO NPs

The surface morphology of the specimen was investigated through a field emission scanning electron microscope (FE-SEM- JSM-7610F) provided by the JEOL, while the shape and size of the manufactured nanoparticles were investigated using a high-resolution transmission electron microscope (HR-TEM - TECHNAI G2 20 S-TWIN). The images and spectra were attained at various magnifications with working voltage of 30 kV.

The absorbance spectra were recorded using an FT-IR spectrometer (Thermo Fisher Scientific, Model Nicolet 6700, USA) for NPs analysis. Using KBr pellets, the entire study was carried out in the mid-infrared range between 4000 and 400 cm^{-1}. The pellets were made by crushing KBr and mixing it with a small amount of solid samples, then recording the spectra at a resolution of 4 cm^{-1}. The data processing was done with the software OMNIC^{TM} (v7.4).

8.2.4 Effect of Cu/FeO nanoparticles on laccase production

Submerged fermentation state (SmF) was conducted in 250-mL Erlenmeyer flasks with rice bran as substrate previously reported by Kumar et al., (2021a). The medium was added with varying concentrations (0.5-3.0 mM) of Cu/FeO NPs to investigate the effect of NPs on laccase synthesis. However, a medium lacking NPs was used as a control and the pH of the medium was maintained at 7.0 in the beginning. Flasks were then autoclaved to sterilize, cooled, and inoculated with 4 % bacterial culture comprising 2×10^7 CFU/mL and incubated for 120 h at 35 ^{0}C. After cultivation of the bacterial isolate in the fermentation medium, 50 mL of sodium citrate buffer (pH 4.8) was added and the mixture was shaken in a vortex shaker for 30 min and then centrifuged for 10 min at 5,000 rpm with temperature 4 ^{0}C. After centrifugation, the supernatant was utilized directly for enzyme assays.

8.2.5 Laccase assays and protein determination

The activity of immobilized and free laccase in reaction mixture comprising 2 mL of ABTS (1 mmol/L) as substrate and 2 mL sodium citrate buffer (0.1 mol/L, pH 4.0) was measured spectrophotometrically at 420 nm and temperature 25^0C (Hu et al., 2015). The spectrophotometer (TU-1900, Beijing Puxi, China) was used to detect the oxidation of the substrate to $ABTS^+$ over 5 minutes. The quantity of laccase necessary to oxidize 1 mol of ABTS per minute was explained as one unit (U) of laccase activity. The ultimate activity of immobilized laccase was measured in units per milligram of laccase.

8.2.6 Immobilization of laccase

Following the procedure outlined below, the enzyme was immobilized onto Cu/FeO nanomaterials. Initially, ultrasonication was used to disperse 0.5 g of amine-functionalized particles in 45 mL of 0.1 M Na_2HPO_4-NaH_2PO_4 buffer solution having pH 8.0 (Tavares et al., 2013). After that, 50 % glutaraldehyde (GA) solution was mixed in buffer having dispersed particles to obtain final volume of 5 % (v/v). At room temperature, the reaction mixture was constantly agitated at 150 rpm for 2 hours. The GA-modified particles were collected after magnetic separation, washed with phosphate buffer, and used to react with the enzyme's amino groups (Wlizło et al., 2020). One milligram of laccase (10 U) was dissolved in 1 mL of a 100 mM citrate buffer solution to make the final enzyme solution (pH 4.5). Finally, ultrasonication was used to re-disperse 5 mg of GA-modified Cu/FeO NPs in the enzyme solution. The enzyme-bound magnetic nanoparticles were then collected using a magnet after stirring the reaction mixture at 25 ^{0}C for 120 minutes. Followed by the rinse with a citrate buffer solution (100 mM) eliminated the unbound enzymes. The enzyme loading and enzyme activity were determined using enzyme-bound particles. In this work, independent factors such as pH (4.0–9.0) and temperature (30–55 ^{0}C) were chosen to optimize the conditions for maximal immobilization of the enzyme.

The quantity of laccase immobilized on the nanoparticles was determined using the Bradford, (1976) test at 595 nm after the parameters were optimized. However, Immobilization efficacy was evaluated using equation 1.

$$\text{Immobilization efficiency (\%)} = (A_0 - A_1)/A_0 \times 100 \qquad (1)$$

Where, A0 and A1 signify the amounts of total protein presented for immobilization and the quantity of protein existing in the filtrate after immobilization, respectively.

The yield of immobilization was also considered as prescribed in equation 2.

$$\text{Immobilization yield (\%)} = (B_0 - B_1)/B_0 \times 100 \quad (2)$$

Where, B0 and B1 represent the activity of specific enzymes within the solution before and after immobilization.

8.2.7 Thermal and pH stability of immobilization and free laccase

The thermal and pH stability of immobilized and free laccase solutions (pH 4.0–9.0) was studied by incubating them for 6 hours at temperatures extended from 30 ^{0}C to 55 ^{0}C. However, for the modification of pH, 0.1 M solution of different buffers such as citrate (pH 2.0–6.0), phosphate (pH 6.5–8.0), and Tris HCl (pH 8.5–10.0) were used. The remaining activity was then tested using the usual ABTS oxidizing reaction as defined in Section 8.2.6.

8.2.8 Solvent stability of immobilization and free laccase

The activity of immobilized and free laccase was evaluated at 25 ^{0}C by ABTS as substrate. These were incubated in several organic solvents such as acetone, ethanol, methanol, acetonitrile, and dimethyl sulfoxide (DMSO), at 25 ^{0}C for 2 hours to measure the tolerances activity. For this, 3 mL of these free and immobilized laccases were supplemented in the substrate solution, statically stirred for 5 min, and the rise in clarity of solution was measured at 420 nm. The shift in optical density per mol of protein per minute supplied in substrate solution (3 mL) via 1 cm cell path length was defined as one unit of relative activity.

8.2.9 Reusability and storage stability of immobilization and free laccase

Repetitive oxidation reactions of ABTS in the citrate buffer across multiple cycles were used to test the operational steadiness of the immobilized laccase. The immobilized laccase was isolated by magnet at the end of each oxidation cycle, washed thrice with the same buffer, and the technique was restarted with a new aliquot of the substrate under identical reaction conditions. In the first cycle, the activity of immobilized enzymes was assumed to be 100 % and the residual activity of the enzyme in the reaction mixture was used to define activity in each cycle. All of the tests were carried out in replicates. However, both the immobilized and free laccases were stored in 0.1 M sodium citrate buffer having pH 4.5 at 25 ^{0}C for many days to assess their storage stability. The enzyme's residual activity was then assessed under controlled conditions.

8.2.10 Catalyzing the oxidation of syringic acid and guaiacol through immobilized laccase

It is known that lignin poses a three-dimensional polymer structure that combines with structural units of phenylpropane via an ether and carbon-carbon double bond. Because of the complexity of lignin's structure, model compounds of lignin have been utilized in the previous investigation (Kumar and Chandra, 2020). Because the phenolic lignin was considered as the particular substrate for laccase, therefore, the usual model compound of lignin such as guaiacol and syringic acid was chosen in the current study to determine the catalytic effectiveness of the immobilized laccase. Experiments were carried out at 40 ^{0}C using immobilized laccase in sodium citrate buffer having pH 3.5 with mechanical stirring to catalyze the oxidation of guaiacol and syringic acid. For this, 10.0 mg of laccase was mixed with 20 ml of reaction media containing 2 mM guaiacol and syringic acid, and incubated for 1 hour. The proportion of syringic acid and guaiacol catalytic oxidation efficacy was measured at various intermissions by isolating the laccase with a magnet. Finally, the absorbance of the upper solution was measured at 262 and 470 nm via UV–visible spectrophotometer (Bergbauer, 1991; Volkova et al., 2012; Hu et al., 2015).

8.2.11 Statistical analysis

All the experimental data existing here were presented as mean and standard deviation (SD). However, the MS office Excel program (version, 2010) was used to calculate the data.

8.3 Results and discussion

8.3.1 XRD analysis of Cu/FeO NPs

Figure 8.2 shows an XRD analysis of Cu/FeO nanoparticles. A Rigaku D/Max 2500 VBZ$^+$/PC diffractometer was used to perform X-ray diffraction (XRD) measurements. The angle range was 200–600 degrees. The study revealed that there was no discernible peak in the spectra. The nanoparticles were amorphous rather than crystalline, according to the findings. Cu/FeO nanoparticles had previously been found to display similar diffraction patterns (Irshad et al., 2017). In comparison to crystalline iron nanoparticles, the amorphous form of iron nanoparticles enhances catalytic activity in different reactions and pollutants degradation rates.

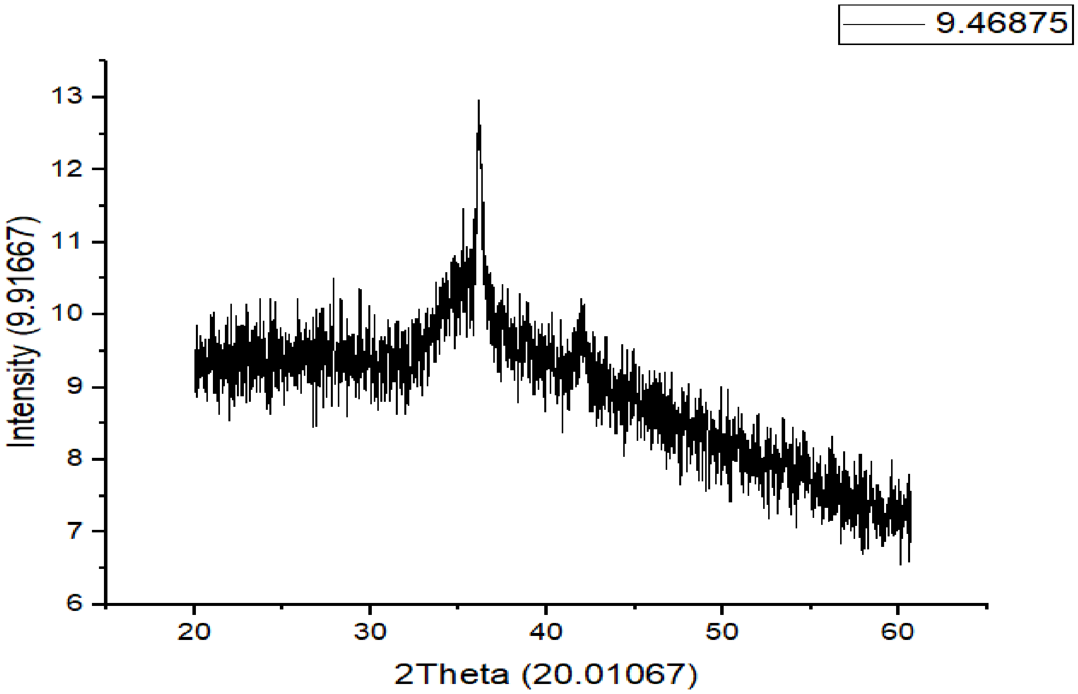
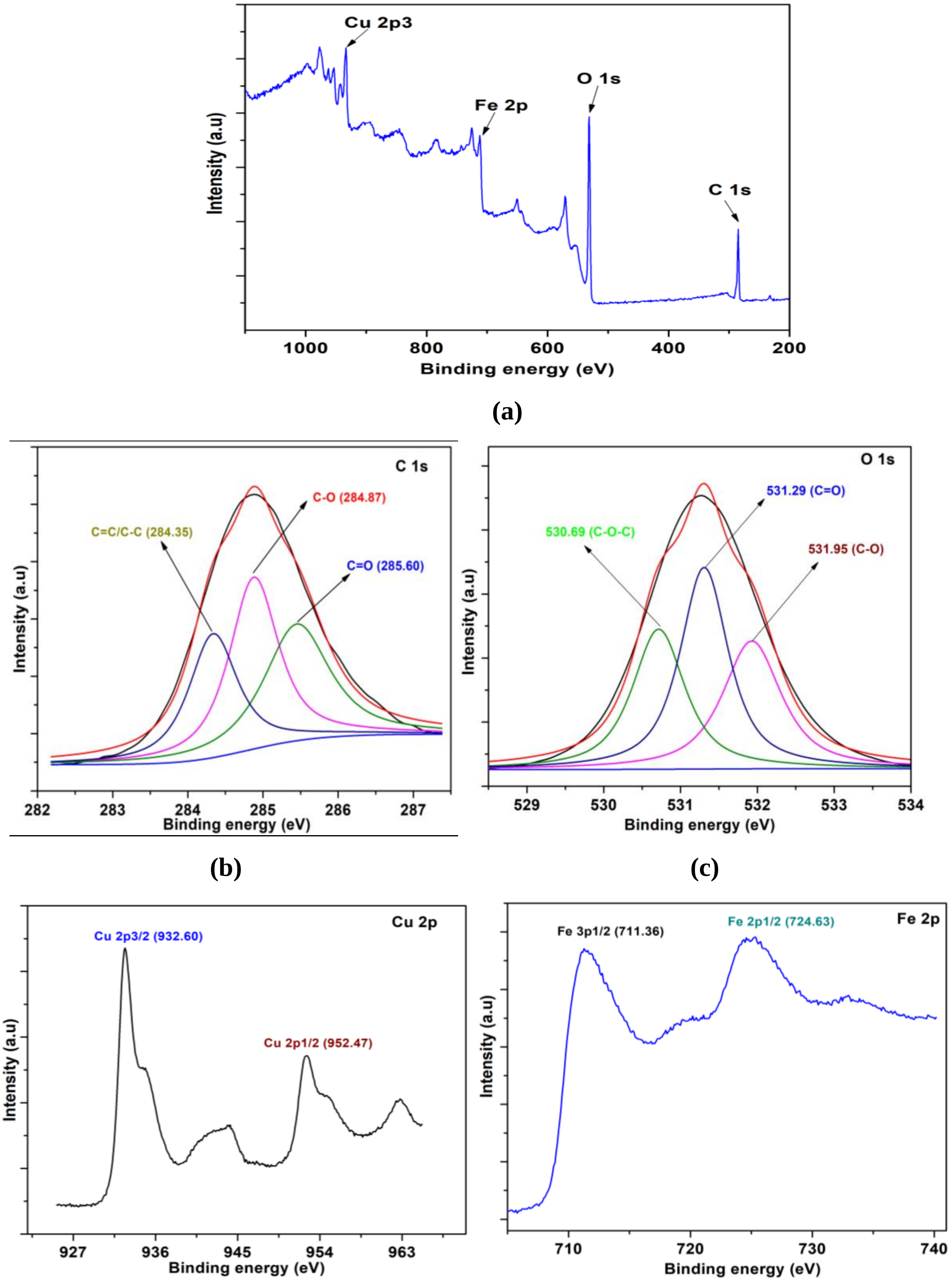


Fig. 8.2 XRD pattern of the synthesized Cu/FeO NPs.

8.3.2 XPS analysis of Cu/FeO NPs

The Cu/FeO nanoparticles' typical C 1s, O 1s, Cu 2p, and Fe 2p X-ray photoelectron spectroscopy (XPS) spectra are shown in fig. 8.3. Three peaks at 284.35, 284.87, and 285.60 eV were fitted to the C 1s peak, which correspond to carbon in the C=C/C-C, C-O, and C=O groups, respectively (Chen et al., 2019). The O 1s peak may be divided into two peaks at 530.69, 531.29, and 531.95 eV, respectively, attributable to the O atoms in the C-O-C, C=O, and C-O groups (Li et al., 2017). Fe 2p peaks at 711.36 and 724.63 eV have been attributed to Fe 3p1/2 and Fe 2p1/2 of Fe_3O_4 respectively (Xue et al., 2011). Cu 2p3/2 and Cu 2p1/2 of Cu^{2+} are assigned to the peaks at 932.60 and 952.47 eV in the high-resolution Cu 2p spectra, suggesting that Cu^{2+} ions were chelated onto the surface of Fe_3O_4@C nanoparticles (Parmigiani et al., 1992; Wang et al., 2015).



(a)

(b) (c)

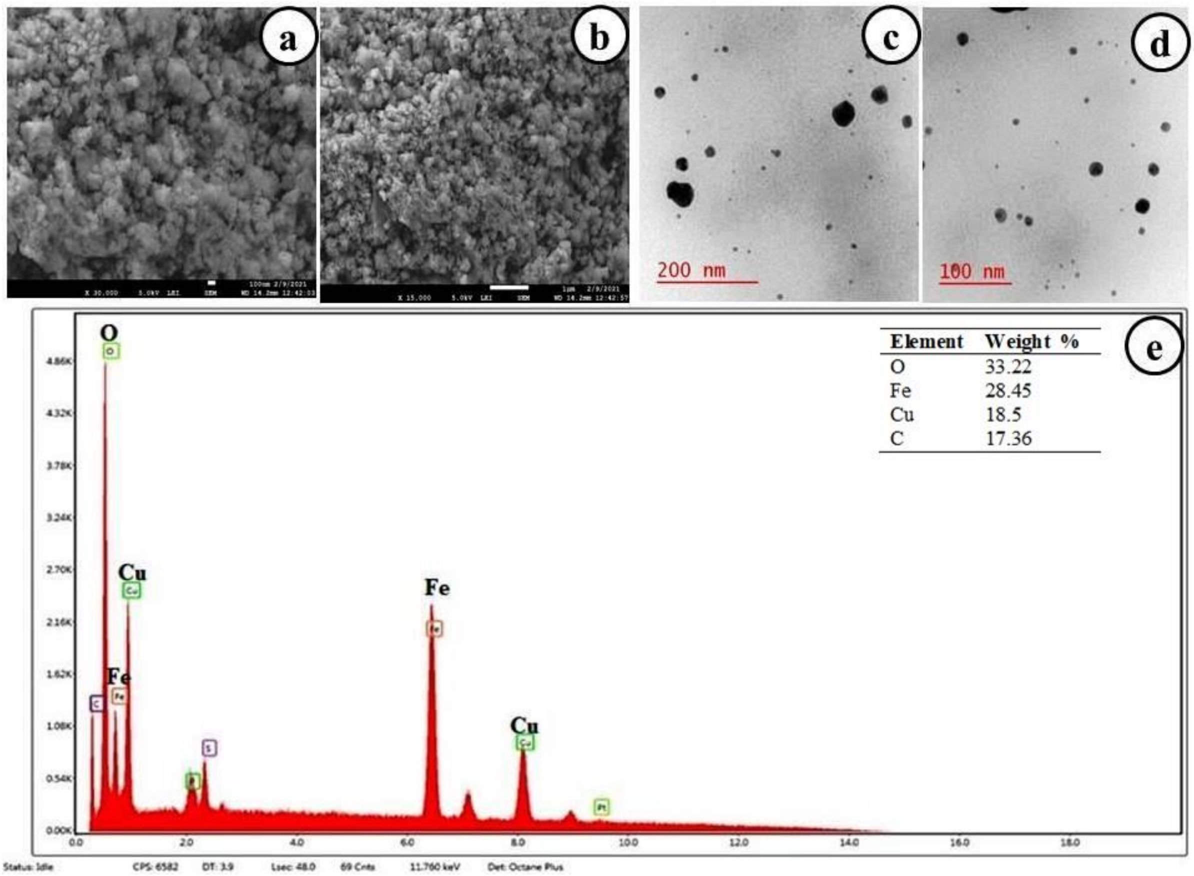
(d) (e)

Fig. 8.3 (a) XPS survey spectrum, **(b)** C 1s, **(c)** O 1s, **(d)** Cu 2p and **(e)** Fe 2p XPS spectra of Cu/FeO NPs.

8.3.3 SEM and TEM analysis of Cu/FeO NPs

Cu/FeO nanoparticles are seen in SEM images in Figures 8.4a and b. It can be observed that the particles are evenly dispersed over the whole micrograph and have grain sizes ranging from 14 to 100 nm. Cu/FeO NPs size was examined further using a transmission electron microscope (TEM). The TEM images of the Cu/FeO nanoparticles in figure 8.4c and d reveal that the nanoparticles are spherically formed. EDS was used to analyze the elemental composition of Cu/FeO nanoparticles (8.4e). A scanning electron microscope was used to acquire the EDS spectra. The existence of Cu/FeO nanoparticles was indicated by iron, copper, and oxygen peaks in the study (Fig. 8.4). The presence of a carbon peak further supports the hypothesis that the organic component acted as a capping agent. Similar evidence has previously been provided in several investigations (Irshad et al., 2017).

Characteristic bands were identified in the FTIR spectrum of Cu/FeO (Fig. 8.5). The hydroxyl groups covering the surface of the particles are responsible for the peak at 3328.80 and 3389.64 cm^{-1}, which correspond to the O-H stretching vibration (Fig. 8.5a) (Chu et al., 2013). The anti-symmetric and symmetric stretching vibrations of the -COO- group were attributed to the peaks at 1637.27 and 1625.36 cm^{-1}, respectively (Xuan et al., 2011). The stretching vibration characteristic for Cu/FeO has a peak at 672.14, 619.33, 698.78, and 687.66 cm^{-1} C–C stretching (strong) (Fig. 8.5) (Abboud et al., 2015). In the region of 760–1,114.76 cm^{-1}, C–C stretching (strong) and C–N stretching (medium) exist. This region encloses the complex skeleton modes involving few vibration local modes due to modification of the length of the bonds and angles between the bonds. All the spectra were normalized to the intensity of the Fe–O peak at 488.78 cm^{-1} to compare the intensity of peaks at different spectra regions for various adsorbed molecules. The peak at 1625.36 cm^{-1} is attributed to the C = O mode of stronger interaction with MNPs (Zheng et al., 2012). All of these findings show that the Cu/FeO nanoparticles' surfaces were functionalized with a considerable number of -OH and –COO- groups, allowing laccase immobilization. The FT-IR data showed that glutaraldehyde was successfully connected to the carrier via amine groups as a cross-linker for protein binding.



Element	Weight %
O	33.22
Fe	28.45
Cu	18.5
C	17.36

Fig. 8.4 (a) and (**b)** SEM micrograph, **(c)** and **(d)** TEM micrograph of Cu/FeO NPs (e) EDX analysis of Cu/FeO.

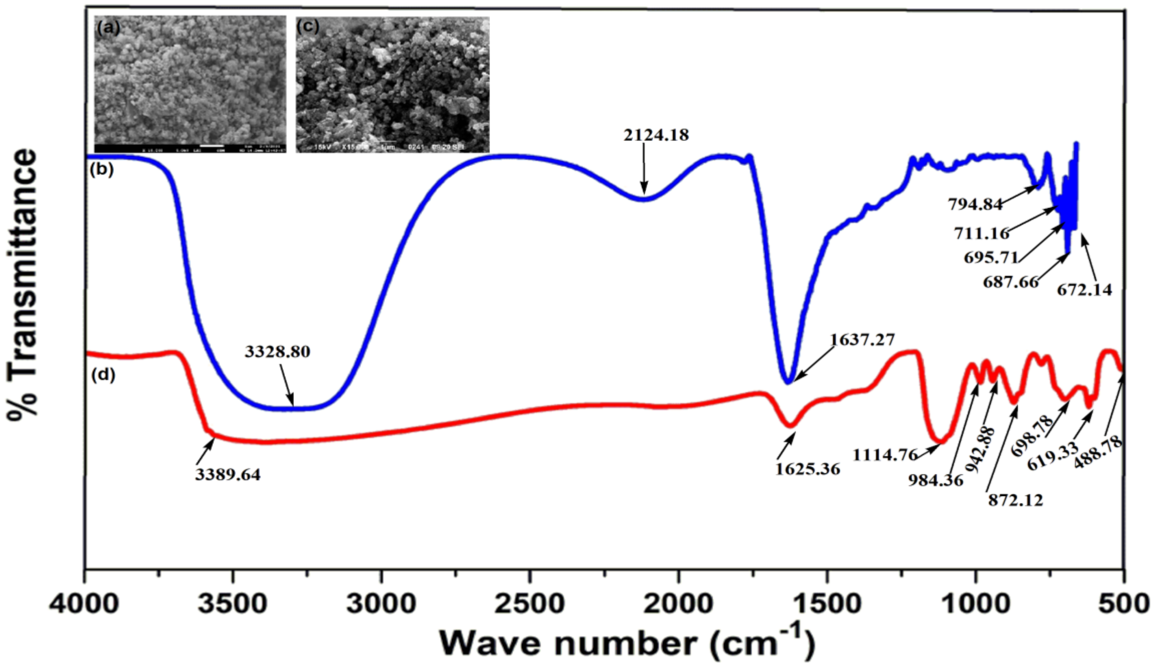


Fig. 8.5 (a) SEM analysis, **(b)** FT-IR spectra of FeO, and **(c)** SEM analysis, **(d)** FT-IR spectra of Cu/FeO.

8.3.4 Effect of Cu/FeO NPs for the laccase production

In previously reported *Bacillus aquimaris* AKRC02 for laccase production in different environmental and nutritional conditions in the presence of rice bran (Kumar et al., 2021a). Different concentrations of Cu/FeO NPs were utilized in the production medium to understand the impact of Cu/FeO NPs on laccase production. The Cu/FeO NPs utilized in this work were not stabilized with surfactants or other protective coatings and were thus introduced to the manufacturing medium as such. Laccase activity was enhanced in the presence of NPs in the medium compared to the control (Fig. 8.6), with the highest laccase production recorded at 1.5 mM NP concentration. To assess laccase activity, several concentrations of Cu/FeO (0.5mM, 1.0mM, 1.5mM, 2.0mM, 2.5mM, and 3.0mM) were added to the liquid medium. Figure 8.6 indicates the findings, although a greater concentration of copper inhibited bacterial growth, laccase activity increased as the Cu/FeO concentration in the medium enhanced. After adding 1.5 mM Cu/FeO to the medium for 96 hours, the highest laccase activity (20.321 2.14 U/L) was found, which was nearly 2 times greater than that of the control group.

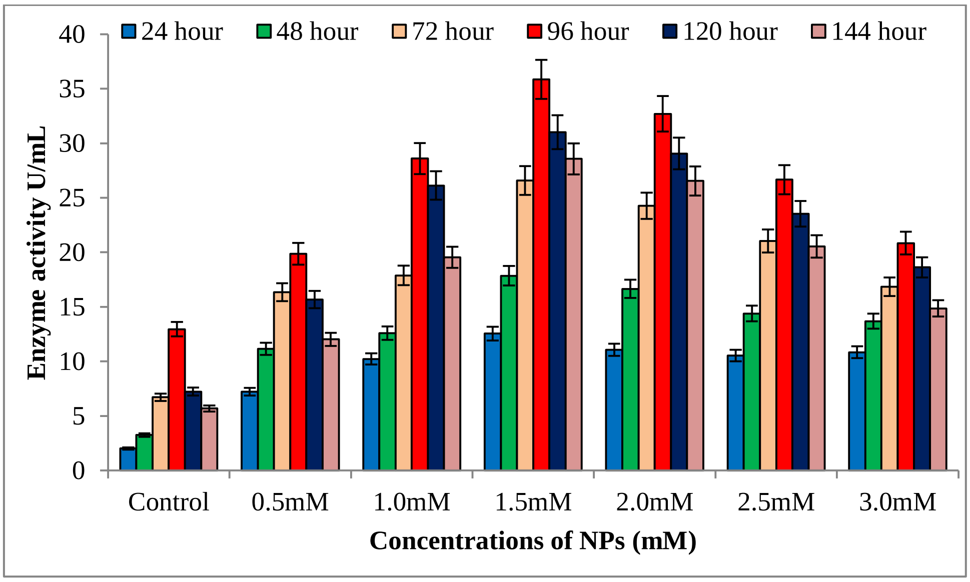


Fig. 8.6 Effects of different concentrations Cu/FeO NPs for maximum laccase production.

8.3.5 Laccase immobilization

Multiwalled carbon nanotubes are effective for immobilizing enzymes, increasing their catalytic activity, reusability, and ease of separation from aqueous solutions using a magnetic field. The nanocomposite was used as a support for laccase immobilization, and the ideal conditions for laccase immobilization onto Cu/FeO were established indirectly by evaluating the enzyme's relative activity. The best immobilization conditions were found to be 40-minute incubation duration, a pH of 7.5, and temperature of 45 ^{0}C (Fig. 8.7). The percentages of immobilization yield and immobilization efficiency were reported as 74.22 % and 86.45 % respectively, after the attachment period and pH temperature was optimized (Fig. 8.7a and b). To activate the support before immobilizing the enzyme, glutaraldehyde was used as a cross-linker. Because of the strong connection between the substrate and enzyme molecule, glutaraldehyde increases the reusability and stability of the enzyme and inhibits enzyme leaching throughout the reaction process; moreover, glutaraldehyde is a flexible and affordable reagent. This cross-linker has two aldehyde functional groups, one of which can covalently react with the support and the other with the amino groups of enzymes, resulting in enzyme immobilization (Ranjan et al., 2019; Zhou et al., 2019; Rouhani et al., 2020).

When glutaraldehyde concentrations are too high, more aldehyde groups form magnetic multi-walled carbon nanotubes (Cu/FeO), and too many aldehyde groups can conjugate with laccase's NH_2 groups, resulting in the laccase enzyme becoming more and more densely immobilized in Cu/FeO. The activity of laccase that had been immobilized in Cu/FeO was then augmented. There were more reaction sites between the enzyme molecules and activated Cu/FeO when glutaraldehyde concentrations were increased further. The synthesis of Schiff's bases smoothed the binding of more laccase enzymes in Cu/FeO, but this flexibility was lost for reaction performance (Costa et al., 2019; Zhou et al., 2019). Furthermore, high glutaraldehyde content may cause the enzyme's structure to be destroyed or result in uncontrolled polymerization (Zhou et al., 2019).

As a result, the optimal glutaraldehyde concentration was 1.5 wt. %, which was utilized in this research. Rouhani et al., (2020) observed that increasing the glutaraldehyde content from 2 to 5 % increased laccase activity. The optimum glutaraldehyde concentration for optimal activity performance was discovered to be 5 %, resulting in an 86 % recovery activity. The recovery activity dropped dramatically beyond the optimum concentration (i.e. 5 to 8 %). Similar findings were reported by Ren et al. (2020) using glutaraldehyde concentrations range from 1 to 6 %, with a 5 % glutaraldehyde concentration being optimum. Using an optimum glutaraldehyde concentration of 2.5 %, Kadam et al. (2020) obtained comparable results, with a recovery activity of 95.13 % and laccase loading of 100.12 mg g1. Kashefi et al. (2019) found similar results with a 156.5 mg/1 laccase loading and 64.6 % immobilization yield utilizing a 3 % (v/v) glutaraldehyde concentration and 0.9 mg/mL starting laccase concentration. Similar findings were made by Zhou et al. (2019) when the optimal glutaraldehyde concentrations were 0.8, 1.2, and 1.6 %, respectively, with 66, 5431, and 63,436 U/g support activities for immobilized nitrile hydratase (NHase), *Candida antarctica* lipase B (CALB), and penicillin G acylase (PGA). Several papers have been published in the literature that describes new supports for laccase immobilization under various circumstances and with various laccase sources. As a result, comparing these immobilization findings is very difficult.

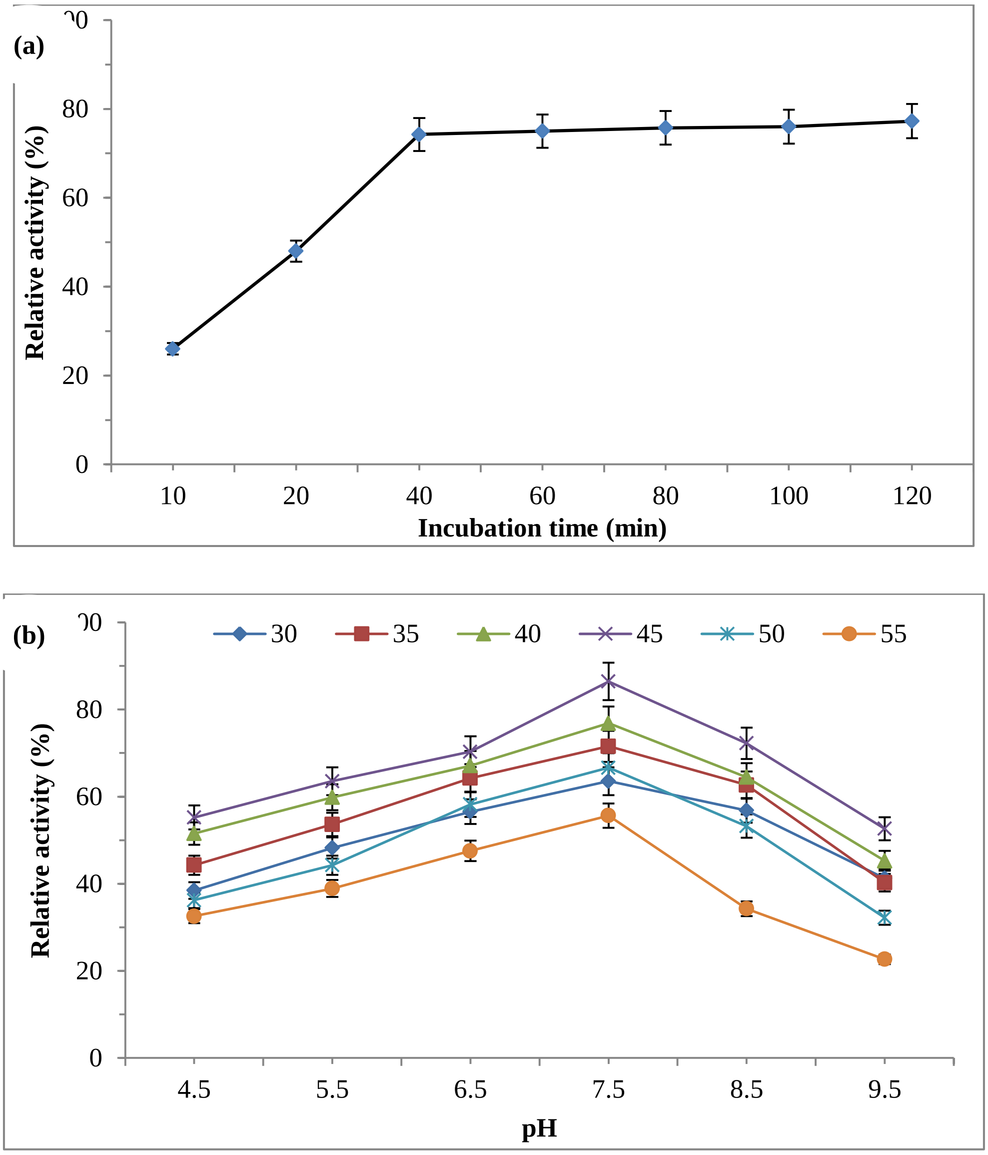


Fig. 8.7 Effect of **(a)** incubation time and **(b)** pH temperature on the relative activity profile of the immobilized laccase.

8.3.6 Thermal stability on laccase activity

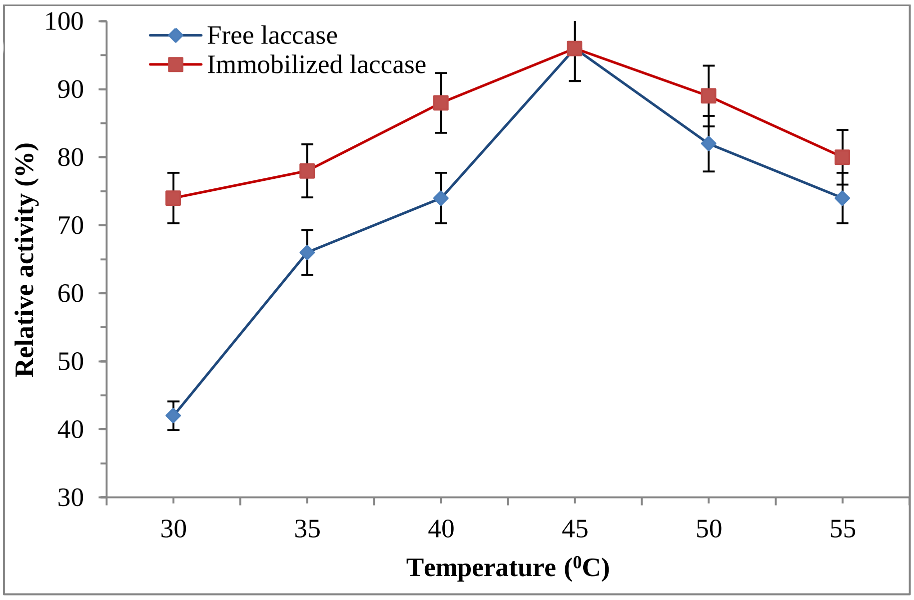
The temperature is a critical component to monitor during the enzyme immobilization process since it has a major impact on the enzyme's activity (Darwesh et al., 2019; Zofair et al., 2020). The effect of temperature on the activity of immobilized and free laccase was studied in this study. As shown in fig 8.8a, increasing the temperature increases the activity of free and immobilized laccase within the temperature range of 30 to 45 ^{0}C, with the immobilized laccase having slightly higher activity than the free laccase. But, further increasing the temperature from 45 to 55 ^{0}C decreased the activity of both enzymes, though only slightly for the immobilized laccase. 45 ^{0}C was the optimum temperature for both immobilized and free enzymes. Moreover, the laccase immobilized in Cu/FeO NPs is more stable at a high temperature contrasted to the free laccase. The free laccase maintained 74 % of its relative activity at 55 ^{0}C, while the immobilized enzyme maintained 80 % of its relative activity at the same temperature (10 min for each reaction). This is consistent with previously published research (Zofair et al., 2020; Tuncay et al., 2020), which can be interpreted as follows: Although an increase in temperature is advantageous to the laccase's strong conjugation to the support, it may also encourage the enzyme's molecular shifting to high activity (Zhou et al., 2019; Yuan et al., 2020). However, further increases in the temperature might be to the loss of enzyme activity due to the denaturation of the enzyme structure (Muthuvelu et al., 2020; Zofair et al., 2020).

8.3.7 pH stability of free and immobilized laccase

The pH is an important factor because it might influence enzyme activity due to ionization changes in enzyme functional groups (Darwesh et al., 2019). The ideal pH for laccases is also said to be highly dependent on the type of substrate employed as well as its redox potential (Zhou et al., 2020). Furthermore, the pH stability of bacterial laccase is dependent on the alkaline medium, and the pH stability varies significantly depending on the enzyme's origin (Zdarta et al., 2018; Zhou et al., 2020; Daronch et al., 2020). The influence of pH on laccase activity was investigated in this study throughout a wide pH range (4.0–9.0). Both the free and immobilized enzymes had their highest relative activity at pH 7.5, as shown in figure 8.8b. In all cases, the free enzyme had lower activity levels than the immobilized form, which implying that the immobilized enzyme is extremely stable at all pH levels.

At pH 8.0 and 9.0, immobilized laccase maintained 96 and 87 % relative activity, respectively, while the free laccase maintained 92 and 72 % relative activity (Fig. 8.8b). It was also discovered that at pH 7.5, both free and immobilized laccase have the highest activity. On the other hand, immobilized laccase was slightly higher activity on both acidic and alkaline media than the free laccase, which could be attributable to its stability and strong interaction with the support materials (Darwesh et al., 2019; Daronch et al., 2020). However, in alkaline media, the relative activity of both immobilized and free laccase was reduced, though only slightly for the immobilized laccase. This is due to hydroxyl anion inhibition of type 2/type 3 copper on laccase centers at higher pH, which limits oxygen binding and results in reduced activity (Zofai et al., 2020; Zhou et al., 2020; Daronch et al., 2020). Therefore, the free laccase activity decreases with the increasing pH. The relative activity is retained at an alkaline medium with pH 8.0 for free laccase 26 %, whereas the immobilized enzyme retains 52 %. It has also been found that the immobilised enzyme has a higher activity profile for a wider range of pH values (Zdarta et al., 2018; Zofair et al., 2020; Muthuvelu et al., 2020). According to these findings, immobilised laccase is more adaptable to acidic and alkaline conditions, which is advantageous in a wide range of applications.

(a)



(b)

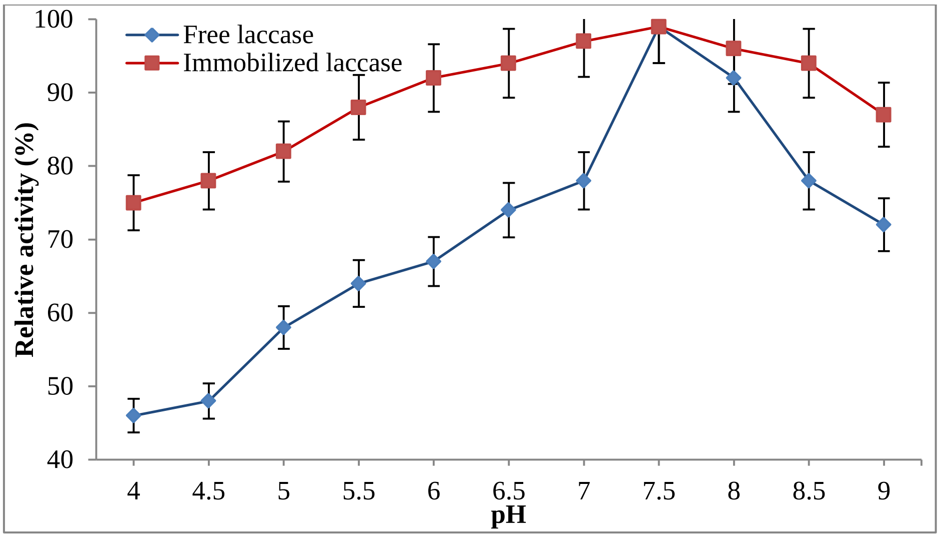


Fig. 8.8 (a) Thermal and **(b)** pH stabilities of free and immobilized laccases.

8.3.8 Effect of solvent for laccase activity

Organic solvents decrease enzyme activity, which is a significant aspect of practical applications. The free and immobilized laccases were incubated in various organic solvents at 25 ^{0}C for 2 hours to determine their tolerances to organic solvents. In acetone, ethanol, methanol, acetonitrile, and dimethyl sulfoxide (DMSO), free laccase lost 30–48 % of its activity (Fig. 8.9). On the other hand, immobilized laccase, retained 65–88 % of its initial activity in the same organic solvents. Organic solvents, in general, have a direct effect on laccase activity by removing the tightly bound water layer from the enzyme, causing structural and functional changes (Qiao and Liu, 2019). The improvement in immobilized laccase resistances suggested that magnetic carriers could increase enzyme rigidity and prevent laccase conformation transition in organic solvents (Zhang et al., 2020).

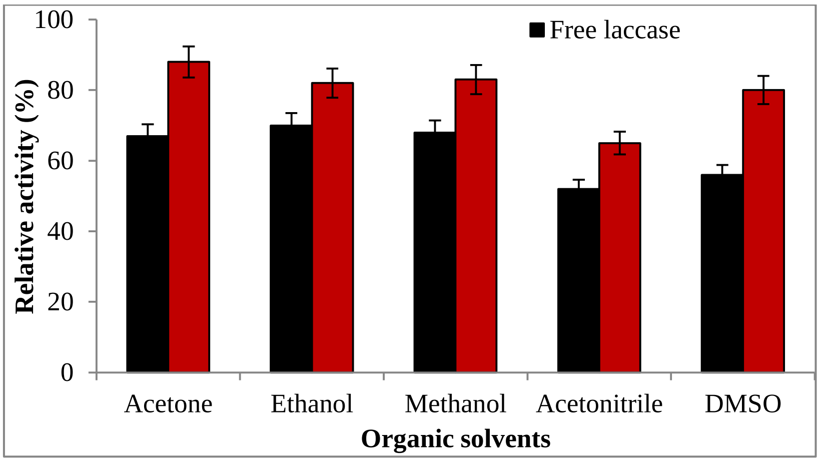


Fig. 8.9 Organic solvents stability on free and immobilized laccases.

8.3.9 Reusability and storage stability of immobilization and free laccase

The reuse stability of the immobilized laccase was investigated using the ABTS oxidation technique for ten consecutive cycles. The immobilized laccase lost around 16 % of its initial activity after three reuses, as showed in figure 8.10a. As the number of cycles grew, the enzyme activity decreased. The immobilized laccase's residual activity was at 54 % after ten reuses. During use, the laccase leaks, resulting in a decrease in activity. In terms of biotechnological application economics, the reusability of immobilized laccase is important. Previously published similar studies have been immobilized laccase using 10 consecutive cycles by Moshtaghioun et al. (2011).

Enzyme storage is a potential key factor to consider because enzymes can be influenced by the external microenvironment and may be deactivated when stored under certain conditions (Zofair et al., 2020; Yusof et al., 2020). Enzymes can be protected from the environment by adopting immobilization technology, and their activity can be maintained to some degree. As a result, the storage stability of the enzyme was examined by storing both enzymes at room temperature for 16 days in a buffer (pH 8.0) (Fig. 8.10b). The findings revealed that immobilized laccase can maintain 59 % relative activity for up to 16 days, whereas free laccase can only maintain 16 % relative activity under the same conditions. Based on these findings, the synthesised Cu/FeO might shield enzymes from the external microenvironment by providing an activity from the buffering solution (Zofair et al., 2020; Muthuvelu et al., 2020). Moreover, enzyme leakage from the support material, as well as microbial degradation and changes in the surrounding microenvironment, could all contribute to a decrease in immobilized laccase activity (Zdarta et al., 2018; Zhou et al., 2020; Daronch et al., 2020). As a result, the enzyme immobilized in Cu/FeO can be used in a wide range of environmental and industrial applications.

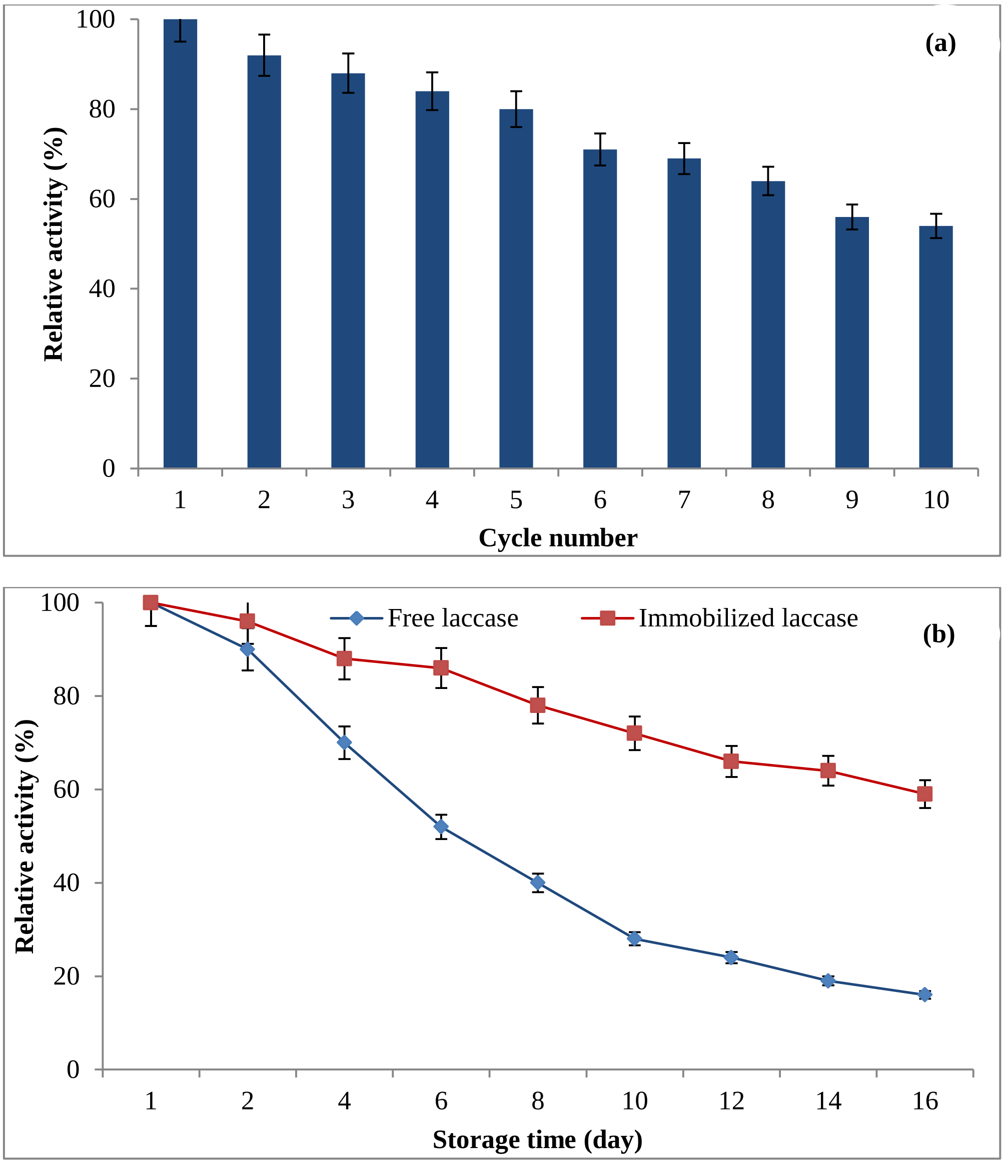


Fig. 8.10 (a) Reusability of immobilized laccase in the batch oxidation of ABTS and (b) comparison of storage stability of free and immobilized laccase under pH 8.0 at 25 ^{0}C for 16 days.

8.3.10 Catalysing the oxidation of syringic acid and guaiacol by immobilized laccase

The immobilized laccase was used to assess the catalytic efficiency of syringic acid and guaiacol, a typical phenolic lignin model compound. Immobilized laccase quickly oxidised syringic acid and guaiacol, as shown in figure 8.11a and the optimum catalytic efficiency was reached after 30 and 40 minutes, respectively. The process of immobilized laccase catalysing syringic acid and guaiacol catalysis is depicted in figures 8.11b and 8.11c.

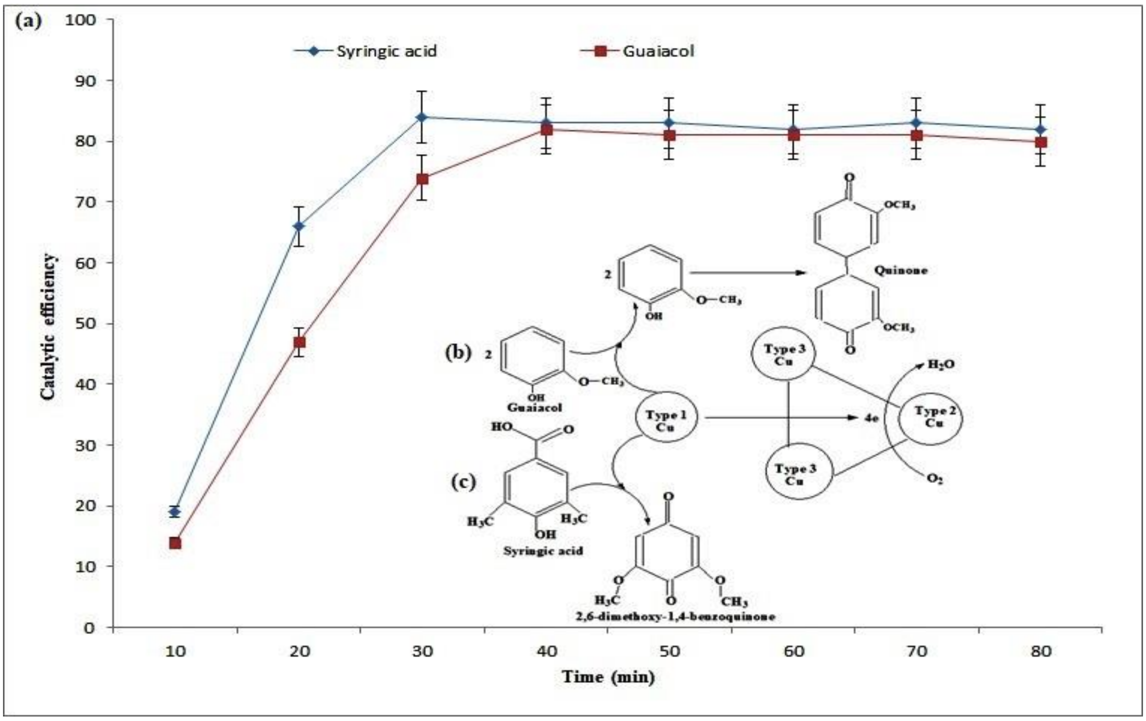


Fig. 8.11 Catalytic efficiency of syringic acid and guaiacol by immobilized laccase. **(a)** The curves of the catalytic efficiency as a function of time; **(b)** The mechanism of catalytic the guaiacol into quinone by immobilized laccase; **(c)** Catalytic mechanism syringic acid to 2,6-dimethoxy-1,4-benzoquinone by laccase.

Syringic acid and guaiacol are converted to 2,6-dimethoxy-1,4-benzoquinone and quinone, respectively, by the laccase. Because syringic acid and guaiacol have high catalytic effectiveness, utilising immobilized laccase to catalyze lignin is a potential and unique technique for usage in the agro-waste industry. Laccases have a low oxidation potential compared to lignin peroxidase, which can only oxidise phenolic end groups in lignin. A phenolic end-group lignin model is syringic acid (Lopretti et al., 1998; Rahouti et al., 1999) that is oxidized by laccase to create stable quinonic oxidation products (Liu et al., 1981) that can be further linked to a variety of amino-acid compounds (Tatsumi et al., 1994). The wood structure units, such as wood fibers and particles, can be joined together under appropriate hot-pressing conditions and pressed into eco-friendly wood-based composites without the emission of formaldehyde, due to radical polymerization between the phenoxy radicals. Following that, research is being done on how to make eco-friendly wood-based composites employing immobilization laccase technology.

Conclusion

In this paper, we describe a simple and effective chemical crosslinking approach for immobilizing *Bacillus aquimaris* AKRC02 laccase on magnetic nanoparticles. The core-shell structures of the magnetic Cu/FeO NPs were characterizations using XRD, SEM, TEM, XPS and FTIR that proved to be a suitable magnetic carrier for laccase immobilization. The factors that determine the catalytic activity of immobilized laccase on magnetic carriers have been thoroughly examined. According to enzyme activity measurements, the activity recovery of immobilized laccase was around 86.45 %. Temperature and pH stabilities, as well as tolerances to organic solvents, were all much higher in the immobilized laccase than free laccase. Furthermore, the immobilized laccase retained 54 % of its original activity after ten reuses. Immobilized laccase demonstrated 59 % storage stability after 16 days. When compared to free laccase, immobilized laccase had substantially superior thermal stability, storage stability, and operational stability of the biocatalysts, as well as show up to a wider range of pH and temperature values. Therefore, the immobilized laccase showed high catalytic efficiency for the oxidation of syringic acid and guaiacol, a lignin model compound, indicating that using the immobilized laccase to catalyze lignin and fabricate wood-based composite materials through radical polymerization is a very promising and novel technology that deserves to be explored further in the field.

Chapter Nine

Optimization of nutrient and environmental conditions for production of bacterial laccase for detoxification and degradation of residual lignocellulosic waste

Optimization of nutrient and environmental conditions for production of bacterial laccase for detoxification and degradation of residual lignocellulosic waste

9.1 Introduction

The pulp and paper mill is one of the largest freshwater consumers and generates huge amounts of wastewater worldwide (CPCB, 2015; Kumar et al., 2015). The pulp and paper mills use various types of chemicals i.e. sodium hydroxide, sodium carbonate, and chlorine compounds during washing, pulping, bleaching steps (Kumar et al., 2020b). After this process, the industry directly releases dark brown-coloured wastewater containing high BOD, COD, and as well as toxic chlorinated compounds, fatty acids, resin acids, tannins, suspended solids, dissolved solids, and inorganic salts along with lignin derivatives (Pokhrel and Viraraghavan, 2004; Raj et al., 2007). Moreover, chlorinated phenols, lignin, and their derivatives are main contaminants that are produced from the paper industries. Lignin is major recalcitrant compound responsible for offensive colour, as well as for inhibiting phototrophic species growth by reducing the transmission of sunlight in wastewater (Karrasch et al., 2006). Besides, the chlorinated compounds and organic halides can be bioaccumulated in fish tissue, resulting in causes of carcinogenic, clastogenic, endocrine, and mutagenic effects that, after consumption of contaminated fish, may also pose problems for humans (Savant et al., 2006). Moreover, exposure to furans and dioxins can cause reproductive effects and skin disorders i.e. skin cancer. Considering their bioaccumulation, bio-magnification, and recalcitrance properties of ROPs, the presence of toxic pollutants in wastewater is significant from a toxicological perspective.

Keeping in mind the above, experts have not advocated for environmental effect schemes in zero discharge of water-intensive mills. Therefore, water management experts from the protection aspects of the river and other aquatic resources are stressing the disposal of ecologically sustainable industrial wastewater. The conventional methods of treatment, such as activated sludge and aerated lagoons plants are unsuccessful in removing phenolics and colour. In most cases, this untreated wastewater containing high COD, BOD, and toxic pollutants are directly discharged into the environment and also causing problems to the aquatic and terrestrial environment (Raj et al., 2014; Kumar et al., 2020b). Moreover, the farmers are directly irrigating their crops in many developing countries as water sources that could be harmful effects on crop productivity as well as soil fertility. This leads to threats of toxicant bioaccumulation and bio-magnification through the food chain. Thus, before its final discharge, the treatment of PPMW is required. While there are many physical and chemical methods were available for treating wastewater or a combination of different methods in sequence, they are more energy-intensive and less desirable than the cost-effective biological process (Yang et al., 2008). So, affordable, energy-efficient, and environmentally friendly technologies are still required.

Consequently, in recent years, microbial approaches for wastewater degradation process to optimize different parameters under laboratory conditions have drawn the attention of many researchers around the world to explore viable technology as it can lead to cost-effectiveness and eco-sustainability (Kaushik et al., 2010). Because of the development of microbial enzymes such as laccase, lignin peroxidase, and manganese peroxidase, most of the fungi were found to have high ligninolytic activity (Kumar and Chandra, 2020). But, these techniques large scale implementations have their restriction due to low pH range (3.0-5.0), slow growth period, huge spore formation, and adverse submerged conditions for fungal growth (Arimi et al., 2014). Pulp paper mill wastewater (PPMW), pH values are generally alkaline (pH 7.0-9.0) and the requirement to reduce the pH before fungal inoculation maintains the conditions there is an additional cost. Unlike fungi, bacteria that survive in acidic to alkaline pH can play a key role in the bioremediation of PPMW without needing to adjust pH. Bacterial laccases are broad range of substrate utilization and are active at high pH and temperatures (Raj et al., 2014; Kumar and Chandra, 2020). Several previous workers were reported bacterial species capable of lignin, PPMW degradation, and detoxification (Raj et al., 2007; Chandra et al., 2007; Singh et al., 2011). However, there was a still gap and require knowledge about ligninolytic enzymes capable of degradation and detoxification of PPMW at optimized environmental and nutritional conditions (Hullo et al., 2001; Wang et al., 2010).

In this study, we have tried to address this gap by laccase-producing bacteria isolated from pulp paper mill sludge and optimized environmental and nutritional conditions for degradation and detoxification of PPMW. The bacterial treated and untreated PPMW was analyzed by UV–Vis analysis has been correlated for their structural changes and GC-MS analysis of identified residual organic pollutants (ROPs) for degraded/transformation. The toxicity reduction of bacterial-treated PPMW was also assessed by using the seed germination of *Phaseolus mungo* L and *Tubifex tubifex* worm.

9.2 Materials and methods

9.2.1 Paper mill sludge and wastewater sample collection

Wastewater and sludge sample was collected (through the grab sampling type) separately in pre-sterilized plastic jerry-can (20L capacity, Tarsons Production Pvt., USA) from M/s. Century Pulp and Paper mill, which is a large contaminated site located in Lalkuan (29.08°N 79.52°E) Uttrakhand, India. The sample was transported to the laboratory for microbiological study. This paper mill uses raw material i.e. bagasse, bamboo, eucalyptus, poplar, veneer waste, and waste paper recycled to manufacture its products. It discharges 80,000 gallons/day of toxic wastewater into the environment, which ultimately reaches to mix aquatic bodies.

Fig. 9.1 (a) Century Pulp and Paper mill wastewater discharge site, **(b)** Sample collecting site.

9.2.2 Physico-chemical analysis of the pulp paper mill wastewater before and after bacterial treatment

Bacterial treated and untreated PPMW samples were analyzed of several physicochemical parameters as per standard methods for the examination of water and wastewater (APHA, 2012). Physico-chemical parameters i.e. total solid, dissolve solid, COD by open reflux method, BOD 5 days methods, total phenol estimation by chloroform extraction method, sulfate by $BaCl_2$ precipitation method, and phosphate by the colorimetric method (APHA, 2012). Moreover, colour was measured by UV–Vis spectrophotometer (Thermo science evolution-201) following the standard method at 465 nm (APHA, 2012), pH was measured by using pH meter. Lignin was estimated according to Pearl and Benson, (1940), and ions were also analyzed by ion meter (Orion Model 960) using the selective ion electrodes. Simultaneously, heavy metals (Cr, Cd, Cu, Ni, Zn, Fe and Pb) in treated and untreated PPMW were analyzed by atomic absorption spectrophotometry (AAS) (ZEEnit 700, Analytic Jena, Germany).

9.2.3 Wastewater decolorization assay

The experiment was conduct in Erlenmeyer flasks (250 mL) containing 99 mL of wastewater addition with mineral salts (g/L) (glucose 5.0, peptone 3.0, Na_2HPO_4 2.4, K_2HPO_4 2.0, $CaCl_2$ 0.01, NH_4NO_3 0.1 and $MgSO_4$ 0.01 were pH adjusted 7.0 and autoclaved at 121 ^{0}C for 20 min (Kumar and Chandra, 2018). The bacterial culture grown overnight (18 hours) having an inoculums size of 3.2×10^6 CFU/mL was 1 mL and 99 mL PPMW added in flasks for inoculation. The culture inoculated and without inoculated flasks was incubated for 144 hours at 37±1 ^{0}C with agitation 120 rpm. The decolorization of PPMW was monitored using UV–Vis spectrophotometer (Evolution-201, Thermo Scientific, USA) to measure the change in absorbance maxima of the lignin at 200-280nm (Raj et al., 2014). The experiments were conducted in triplicate and data presented are mean ± SD.

$$\text{Decolourisation (\%)} = \frac{\text{initial absorbance } (\mathrm{A}_0) - \text{final absorbance } (\mathrm{A}_1)}{\text{Initial absorbance } (\mathrm{A}_0)} \times 100$$

9.2.4 Optimization for culture conditions for degradation and decolorization of PPMW

9.2.4.1 Optimization of nutritional condition

The influence of varied carbon sources 1.0 % (w/v) i.e. glucose, starch, sucrose, dextrose, and lactose was estimated for the PPMW degradation and decolorization. Moreover, different nitrogen sources 0.5 % (w/v) i.e. yeast extract, urea, peptone, sodium sulfate, and ammonium chloride have been added to the MSM medium for evaluating the maximum decolorization. The result of carbon and nitrogen sources at different concentrations of viz (0.1-1.0 w/v) was optimized for PPMW decolorization and degradation.

9.2.4.2 Optimization of environmental conditions

To evaluate the effect of different environmental conditions the same experiment was carried out at different temperatures (17–57 ^{0}C), pH (4–9), and shaking speed (100–200 rpm).

9.2.5 UV–Vis spectroscopic analysis

The UV–Vis spectral scans were performed for untreated and bacterial treated PPMW using spectrophotometer (Model Evolution 201, Thermo Fisher Scientific, USA) in the wavelength range 200–700 nm. Before, the spectral scan the untreated and treated samples were maintained at pH 7.0. A quartz cuvette with a length of 1.0 cm of the path was used to scan the wavelength. At a scanning rate of 0.1 nm and 1000 nm min^{-1} scan speed, all spectra were observed.

9.2.6 Metabolites characterization by GC-MS analysis

Untreated and bacterial treated PPMW samples were centrifuged at 12000×g for 10 min to segregate bacterial biomass and particulate matter to assess degradability. PPMW was extracted with a suitable solvent i.e. ethyl acetate in acidic conditions (pH 2.0) supernatant obtained (Chandra and Abhishek, 2011; Kumar et al., 2020b). The organic layer collected was dewatered and filtered by Whatman no. 54 filter paper over anhydrous sodium sulfate (Na_2SO_4). Moreover, samples were dried at room temperature and dissolved in ethyl acetate of GCMS analysis for identified pollutants present in untreated and bacterial treated PPMW. Besides, dioxane (100 μL) and pyridine (10 μL) were used for GC-MS analysis of the extracted sample, followed by 50 μL of N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) having trimethylchlorosilane (TMCS) (Raj et al., 2014). To dissolve the residue solution was subsequently heated up to 60 ^{0}C with periodic shaking for 15 minutes. Finally, the silylation sample (1 μL) was injected in GC-MS (PerkinElmer, UK) with a PE-5MS capillary column and helium with a flow rate of 1.0 mL/min as the carrier gas. The column temperatures were set at 50 ^{0}C for 5 min, then ramped at 50 to 300 ^{0}C and kept for 5 min. At 200 and 250 ^{0}C, respectively, the transfer line and the ion-source temperatures were maintained.

9.2.7 Effects of treated and untreated PPMW with *Phaseolus aureus* L.

The toxicity assessments of treated and untreated PPMW were using seed germination bioassay (Wang 2003; Kumar et al., 2020b). Black gram (*Phaseolus aureus* L.) seeds were surface-sterilized done mercuric chloride ($HgCl_2$) 0.1 % for 2 min, to prevent other contamination. The *P. aureus* L. seeds homogenized were placed in different concentrations (25- 100% v/v) of untreated and treated PPMW. The seeds were incubated at 28±1 ^{0}C in dark conditions for 48 hours. PPMW toxicity assessment was detected in terms of seeds germination percent and α-amylase activity of *P. aureus* L. The tested seeds were kept on two layers of filter paper in each petri dish soaked with different concentrations of PPMW. Each concentration of tested seeds was crushed with sodium acetate buffer (0.1 M, pH 4.8), and filtered by cheesecloth to large particles remove then centrifuged at 15,000×g for 20 min after supernatant was obtained. All experiment preparation was carried out at 4 ^{0}C. The α-amylase assay was conducted with reaction mixture containing enzyme extract (0.5 mL), 0.1 M acetate buffer (1.0 mL, pH 4.8), and 0.1 % soluble starch (1.0 mL). The reaction mixture was incubated at room temperature for 10 min then adding 0.1% iodine reagent (1.0 mL) and 0.05 N HCl (3.0 mL) to stop the reaction (Kumar et al., 2020b). The using UV-Vis spectrophotometer was read the optical density (OD) at 620 nm and amylase activity expressed the decrease in absorbance (Beri and Gupta, 2007).

9.2.8 Tubifex toxicity test

Tubifex worm is a significant member of lentic fauna in the aquatic ecosystem, the treated and untreated PPMW can use for toxicity analysis (Kumar et al., 2020b). Tests were performed in beakers (200 mL) containing 100 mL of treated and untreated PPMW. The toxicity assessment of tubifex worms will be inoculated into different concentrations (25-100 %) of treated and untreated PPMW and control as tap water. Each concentration of the tested worm picture was visualized under the phase-contrast microscope. Ten tubifex worms were exposed to three replicates of each concentration tested. When there was no response and complete immobilization to pressing with a blunt glass rod, the tested tubifex worms were considered dead

(Lagauzere et al., 2009). Besides, moving worms back to tap water (control), death was further confirmed (Khangarot, 1991).

9.3 Result and discussion

9.3.1 Wastewater characterization

The physicochemical parameters were characterizations of PPMW as showed in Table 9.1. The wastewaters were dark brown and generally alkaline which turned into light brown colour after bacterial treatment. The analysis of the wastewater sample showed several physicochemical parameters beyond the permissible limits along with heavy metals. Therefore, several parameters i.e. (mg/L^{-1}) i.e. pH (8.1 ± 0.12), Total solid (1946.0 ± 1.02), Dissolved Solid (1784.0 ± 1.24), COD (752.0 ± 0.78), BOD (380.0 ± 0.45), Lignin (416.0 ± 5.12), phosphate (34.0 ± 0.46), nitrate (695.5 ± 1.05), total phenol (29 ± 0.95) and heavy metals i.e. Fe (5.6982 ± 1.02), Ni (0.956 ± 0.58) and Zn (3.2346 ± 0.89). However, a sharp reduction pH (7.64 ± 0.11), Total solid (314.0 ± 1.12), Dissolved Solid (652.0 ± 2.15), COD (186.0 ± 0.16), BOD (52.0 ± 0.58), Lignin (30.0 ± 1.02), phosphate (14.5 ± 0.21), nitrate (112.75 ± 0.12), total phenol (6.27 ± 0.00) and heavy metals i.e. Fe (3. 1462 ± 1.00), Ni (0.6217 ± 0.91) and Zn (1.1462 ± 0.98) was noted as shown in Table 1. Phenols and lignin are recalcitrant in nature and major plant constituents of PPMW. Due to lignin causes the dark brown colour, high value of COD and phenols are toxic for the aquatic and terrestrial environment, even at relatively low levels in discharged PPMW (Gupta et al., 2017). Phenol at a higher concentration blocked the photosynthesis of blue algae, diatom, and concentration range of 100-400 g/mL also induced full inhibition of photosynthesis (Duan et al., 2017). Moreover, sodium sulfite, which is used during the pulping process, may be the source of sulfate ions in wastewater, and the nitrate found in the wastewater may be generated from lignin (Singhal and Thakur, 2009). The metal content in wastewater samples can be attributed to the bioaccumulated metals by plants, and these plants are used as raw materials, as well as to the different chemicals using the pulping and bleaching process of papermaking.

Table 9.1 Physicochemical characteristics of pulp and paper mill wastewater. Values are mean ± SD of triplicate samples.

Parameters	**Untreated**	**Treated**	**Permissible limits USEPA,2012**
pH	8.1 ± 0.12	7.64 ± 0.11	5–9
Total soild	1946.0 ± 1.02	314.0 ± 1.12	300
Dissolved Solid	1784.0 ± 1.24	652.0 ± 2.15	500
SS	462.0 ± 0.84	122.0 ± 0.26	100
VS	434.0 ± 0.96	78.0 ± 0.21	-
FS	1512.0 ± 1.46	466.0 ± 0.98	-
Total nitrogen	14.0 ± 0.12	1.84 ± 0.12	-
COD	752.0 ± 0.78	186.0 ± 0.16	120
BOD	380.0 ± 0.45	52.0 ± 0.58	40
Lignin	416.0 ± 5.12	30.0 ± 1.02	-
Chloride	2613.0 ± 2.34	1825.0 ± 1.87	1500
Sulphate	970.0 ± 1.02	342.0 ± 1.06	250
Phosphate	34.0 ± 0.46	14.5 ± 0.21	-
Nitrate	695.5 ± 1.05	112.75 ± 0.12	50
Fluoride	6.75 ± 0.23	3.5 ± 0.06	-
Bromide	14.0 ± 0.24	3.73 ± 0.09	-
Sodium	422.0 ± 0.98	81.5 ± 0.81	-
Potassium	37.0 ± 0.25	14.5 ± 0.14	-
Manganissium	28.25 ± 0.29	9.5 ± 0.82	-
Calcium	3.5 ± 0.16	1.0 ± 0.11	-
Total phenol	29 ± 0.95	6.27 ± 0.00	-
Sulphide	120.0 ± 0.63	23.0 ± 0.91	-
NH_4	422.0 ± 1.48	70.0 ± 0.58	-
Heavy metals			-
Cr	0. 1202 ± 0.16	0.0842 ± 0.63	0.05
Cd	0.0435 ± 0.21	0.0320 ± 0.11	0.01
Cu	2.5851 ± 0.96	0.8245 ± 0.58	0.50
Ni	0.956 ± 0.58	0.6217 ± 0.91	0.50
Zn	3.2346 ± 0.89	1.1462 ± 0.98	2.00
Fe	5.6982 ± 1.02	3. 1462 ± 1.00	2.0
Pb	0.3015 ± 0.58	0.1156 ± 0.96	0.05

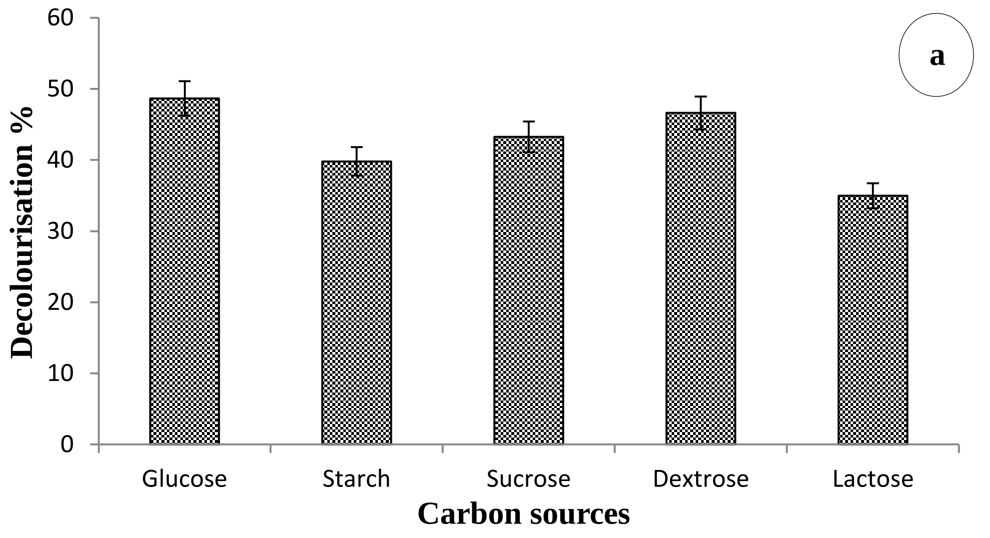
Note: - All the parameters are in mg/L except pH

9.3.2 Effect of different nutritional and environmental parameters

9.3.2.1 Effect of different carbon and nitrogen sources

PPMW decolorization and degradation were observed in different carbon sources for 120 h of incubation period results as shown in figure 9.2a. The most suitable carbon source of glucose 1.0 % (w/v) was found for optimum decolorization (48.66 %). Moreover, starch, sucrose, dextrose, and lactose other carbon sources were found less effective than glucose exhibited decolorization up to 46.61-39.81 %. Whereas the observed bacterial growth was slow in the absence of glucose in culture medium. This showed that bacteria do not readily use the carbon source present in PPMW. But the added glucose in the media was easily available for bacterial growth. This showed strong evidence that incubated bacteria are incapable of using the PPMW for growth and metabolism. In the case of processed PPMW related finding has been published by the previous worker (Sonkar et al., 2019). This indicated our bacteria were more effective in decolorizing and degrading PPMW. This may be the maximum production of laccase enzymes which facilitated the decolorization process (Raj et al., 2014).

The effect of the various nitrogen sources along with glucose (1.0 % w/v) showed that peptone (0.5 % w/v) was the most suitable organic nitrogen source which increased PPMW degradation and decolorization up to 52.33 % (Fig. 9.2b). Further, the peptone concentrations were increases in the medium resulted in the continuous reduction of the degradation and decolorization capability of the bacterium due to inhibited the decolorization process. Since, peptone is the limited amount needed by bacteria because of its abundance of free short peptides and amino acids, which promote bacterial growth with low carbon source consumption rate and reduce by-product accumulation (Lau et al., 2004). In contrast, the other organic nitrogen sources i.e. yeast extract could show degradation and decolorization of PPMW up to 41.72 % only, whereas urea could show decolorization up to 30.79 %, while inorganic nitrogen sources such as ammonium chloride and sodium sulfate could show decolorization up to 27.29 and 21.72 % respectively. Similarly, the effect of nutritional parameters has been reported by previous researchers for the degradation and decolorization of PPMW (Chandra and Singh, 2012; Sonkar et al., 2019). Therefore, previous study reported the presence of glucose (1.0 %) and peptone (0.5 %) for COD reduction up to 85 and 72 % and colour after 144 h of treatment by *Serratia liquefaciens* (Haq et al., 2016). Thus, the BOD, COD reduction, and colour decolorization previously reported by several researchers, the bacteria developed using tiny amounts of suitable carbon and nitrogen sources for easily available for their growth.



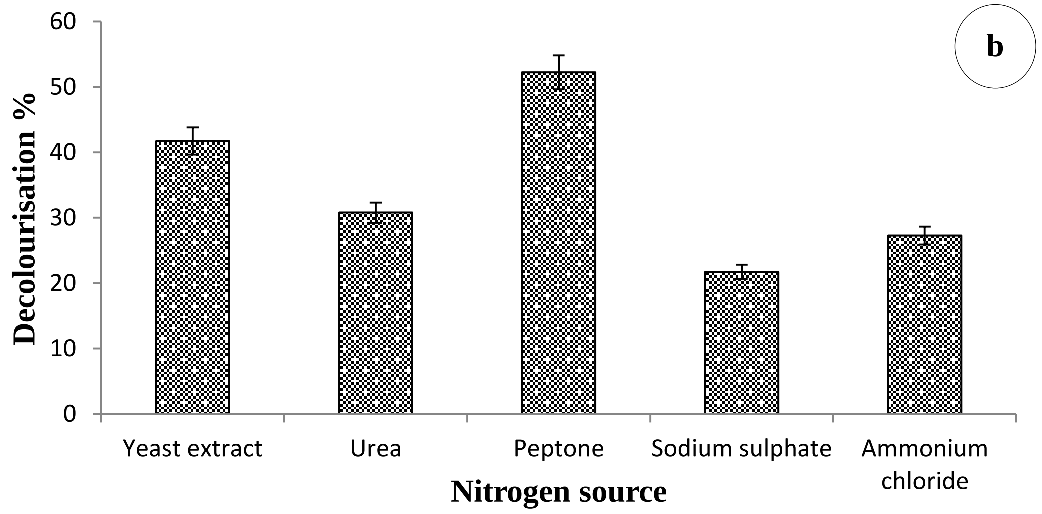


Fig. 9.2 Effect of different nutritional parameters on degradation and decolourisation of pulp paper mill wastewater **(a)** effect of carbon sources **(b)** effect of nitrogen sources

9.3.2.2 Effect of different pH, shaking speed and temperature

The degradation and decolorization process of PPMW was specifically affected by the pH, shaking speed, and temperature of culture conditions. The effect of different pH and shaking speed of PPMW optimum decolorization showed (72.67 %) in pH (7.0) with agitation 180rpm after 120 h incubation (Fig. 9.3a). Further, the increase and decrease in pH inhibited the degradation and decolorization of PPMW. This might be due to the inhibition of bacterial enzyme production. In a previous report, the optimum pH for PPMW degradation and decolorization by *Paenibacillus* sp. was noted at pH 7.2 (Raj et al., 2014). However, the PPMW were significantly affected by degradation and decolorization by various shaking speeds also. The result of shaking speeds of 100 to 200 rpm demonstrated maximum degradation and decolorization were reported at 180 agitations as shown in figure 9.3a. Besides, an increase in shaking speeds resulted in the degradation and decolorization rate decreasing. This may be due to the mechanical injury of bacterium cells at higher shaking speeds (Chandra and Singh, 2012; Tiwari et al., 2014). It was also found that temperature increase (17-37 ^{0}C) increased PPMW degradation and decolorization up to 34.16 to 78.64 % respectively (Figure 9.3b). Although the increased temperature up to 37 to 57 ^{0}C were adversely affected the bacterial growth due to inhibiting the degradation and decolorization of PPMW. Our findings correlated with some previously reported reports (Hooda et al., 2018).

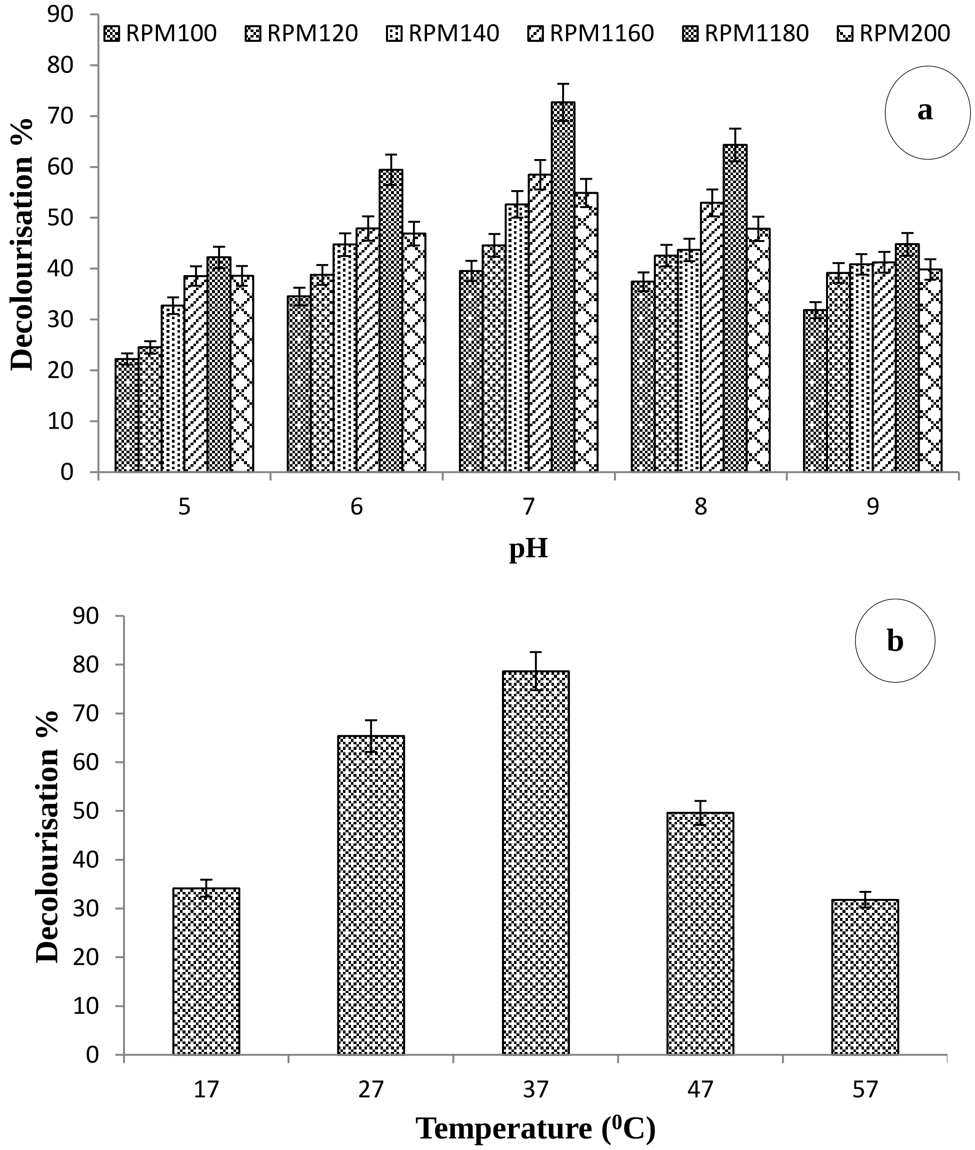


Fig. 9.3 Effect of different environmental parameters on degradation and decolorization of pulp paper mill wastewater **(a)** effect of pH and shaking speed **(b)** effect of temperature

9.3.3 UV–Vis spectroscopy analysis of treated PPMW

The UV–Vis spectral scan of bacterial treated PPMW samples at different time intervals (24-120 h) was done to identify the change in absorbance during degradation and decolorization by *Bacillus cereus* strain AKRC03. Figure 9.4 shows the UV-Vis scan of untreated and bacterial treated PPMW samples. Around 215 and 230 nm can be seen in the two prominent absorption peaks. The identified absorbance peaks decreased during the course of treatment and peaks almost disappeared after 120 hours. The UV-Vis spectral analysis assisted the degradation of ROPs and lignin derivatives compounds by *Bacillus cereus* strain AKRC03. The absorbance for lignin derivatives and its chlorine dioxide derivative was observed around this region (Nie et al., 2014; Kumar et al., 2015). This study correlated with previously published reports related to the UV-Vis spectrum was observed during the degradation of the PPMW by *Bacillus* sp. IITRDVM-5 compared to the present study and also observed decolorization and degradation of chlorophenols (Sonkar et al., 2019; Patel and Kumar, 2016).

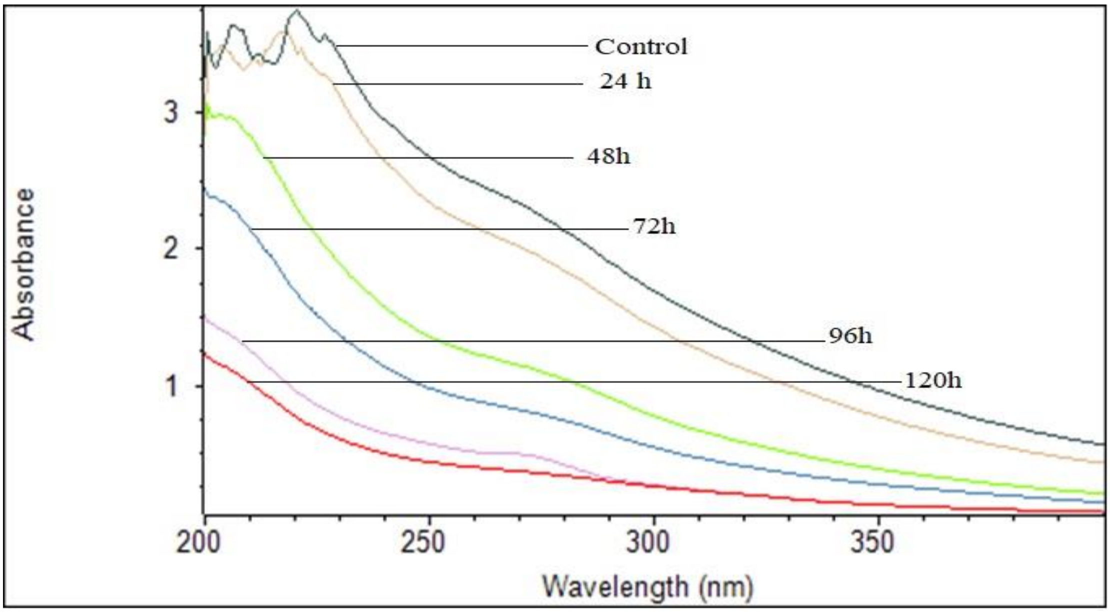


Fig. 9.4 UV–Vis spectroscopy analysis of pulp and paper mill wastewater contamination degradation during the course of treatment under optimized condition by *Bacillus cereus* strain AKRC03.

9.3.4 GCMS analysis of metabolites present in PPMW

To identify the ROPs present in PPMW for degraded or transformed by *Bacillus cereus* strain AKRC03. The total ion chromatogram (TIC) of ROPs present in the bacterial treated and untreated sample as shown in Figures 9.5a and 9.5b and their identified pollutants are represented in Table 9.2. After treatment, the peak intensity reduction compared to the untreated sample, and the appearance of new peaks can be observed in the treated sample. The major ROPs identified in untreated sample at different RT were (cis)-1-Butyl-2-undecylcyclopropane (RT: 12.86), 1-Hexadecanol (RT: 16.87), 1-Octadecanol (RT: 20.52), 1-Dotriacontanol (RT: 24.03), Heptacosane (RT: 26.58), 1-Heptadecanol (RT: 28.45), 9-Octadecenoic acid (RT: 29.28), Hexatriacontane (RT: 29.94), Heptasiloxane, hexadecamethyl (RT: 30.77), Heptadecane, 2-methyl (RT: 31.10), Tricosane (RT: 32.09), Dodecane, 1,1'-oxybis (RT: 32.98), 1-(2-Hydroxyethoxy)tridecane (RT: 33.80), Docosanoic acid, 1,2,3-propanetriyl ester (RT: 34.37), Pentatriacontane (RT: 38.39), Diethyl a-trimethyloxy-2,4,6-trimethylbenzylphosphonate (RT: 41.99), Pentatriacontane (RT: 42.32), N-[(methyl phenyl)methylene]-2-methyl-2-propanamine N-oxide (RT: 44.07), Heptasiloxane, hexadecamethyl (RT: 45.71), 3-(Dimethylphenysilanyl)-6,11-dihydroxy-3-methyl-1, 2,3,4-tetrahydrobenz[a]anthracene-7,12-dione (RT: 50.78). However, the investigation of bacteria treated PPMW sample has showed the existence of various ROPs such as 3,6,9-Trioxa-2,10-disilaundecane,n2,2,10,10-tetramethyl (RT: 9.74), Silane, (dodecyloxy)trimethyl (RT: 16.46), 1,1,1,3,5,5,7,7,7-Nonamethyl-3-(trimethylsiloxy)tetrasiloxane (RT: 21.56), (1á,3á,4à)-3,4-Bis[dimethyl(4-methyl phenyl)silyl]cyclo pentan-1-yl acetate (RT: 23.34), Hexadecanoic acid, trimethylsilyl ester (RT: 24.68), (2S,3R)-(3-Tetradecyloxiranyl)methanol (RT: 26.93), Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester (RT:31.39), Diethyl(1E,3Z)-3-cyano-4-naphthylbuta-1,3-dienylphosphonate (RT: 32.72), Heptacosane (33.19), (S,S)-1,1-Bis(ethoxycarbonyl)-2,2-bis-p-tolylsulfinyl-1-ethanol (RT: 36.24), 2,4,6-Tri[4,5-(methylenedioxy)phenyl]-s-triazine (RT: 40.08) and (5S,6S)-4,5-Dimethyl-6-phenyl-3,4,5,6-tetrahydro-2H- 1,3,4-oxadiazin-2-one (RT: 44.08) at different RT. In the untreated (control) sample, these pollutants were completely absent, PPMW was degraded, and a few were new metabolites are formed. Besides, the bacterial treated samples one organic compound i.e. Silane, butoxytrimethyl (RT: 7.52) could not be metabolized. The presence of various ROPs, phenolics, chlorinated, and aliphatic compounds in wastewater were previously reported by several researchers for the degradation through bacterium (Sonkar et al., 2019). However, the after-bacterial treated PPMW containing ROPs are shown in Table 2. Similarly, various ROPs of transformed or degraded phenolic, non-phenol halide derivatives as well as lignin derivatives from wastewater have been identified in PPMW (Raj et al., 2014; Chandra and Singh, 2012). Therefore, after bacterial treatment, some ROPs identified in the untreated sample were diminished. This indicated that ROPs and other pollutants were degraded with the help of bacterial strain for produced laccase enzyme. These ROPs were used by bacteria as the primary energy source and play a key role in the degradation and decolorization of PPMW. The result indicates that *Bacillus cereus* strain AKRC03 transformed or degraded chlorinated, phenolic, non-phenolic as well as lignin derivatives from the wastewater.

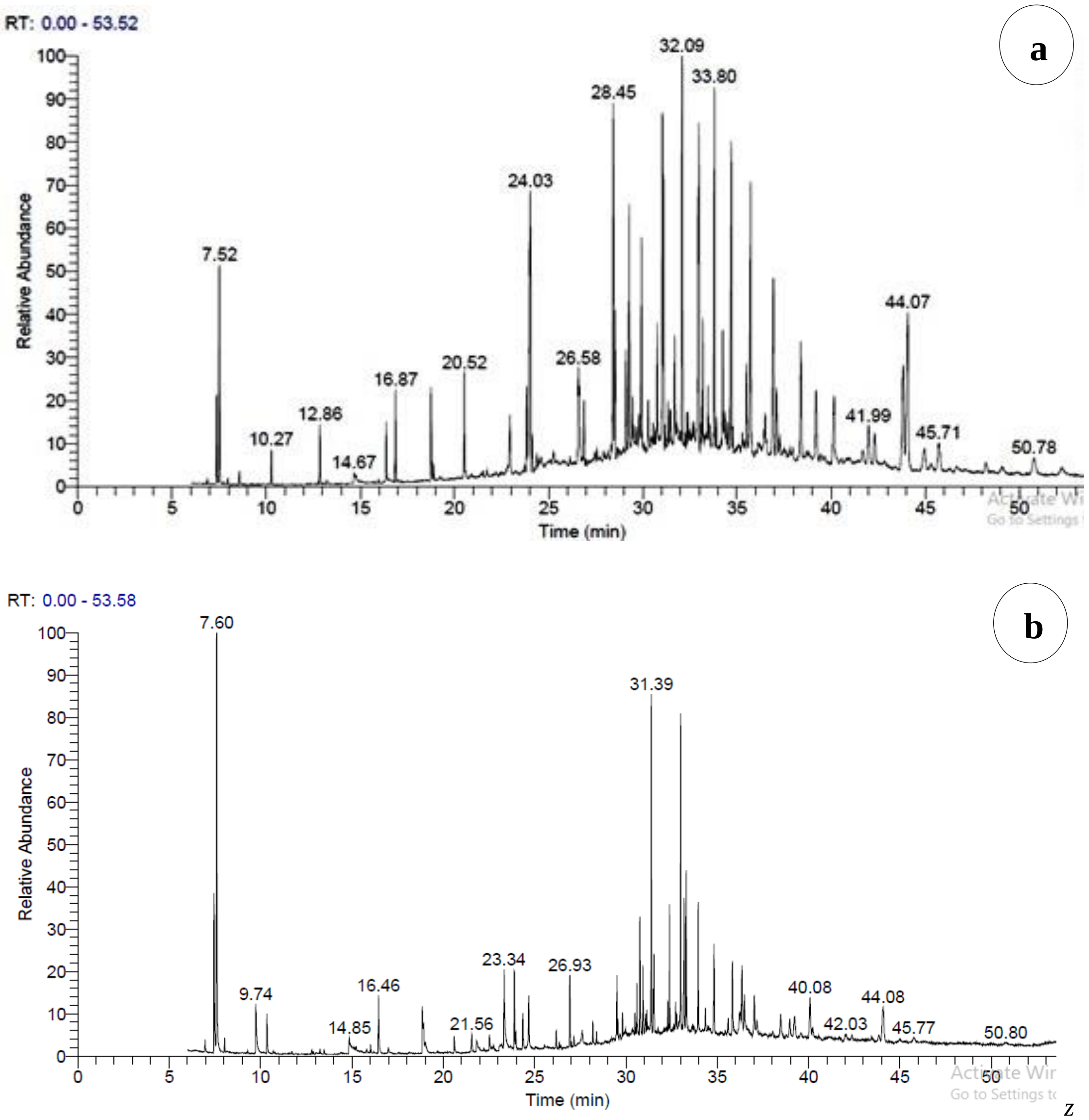


Fig. 9.5 GC–MS chromatograms of compounds extracted with ethyl acetate from **(a)** Untreated and **(b)** treated wastewater by *Bacillus cereus* strain AKRC03.

Table 9.2 Compound identified as trimethylsilyl (TMS) derivatives in ethyl acetate extract from (a) untreated and (b) treated wastewater by *Bacillus cereus* strain AKRC03

S. No.	Retention time (RT)	Compound Name	Untreated (0 h)	Treated (120 h)
1.	7.52	Silane, butoxytrimethyl	+	+
2.	7.60	Silane, butoxytrimethyl	+	+
3.	9.74	3,6,9-Trioxa-2,10-disilaundecane,n2,2,10,10-tetramethyl	-	+
4.	12.86	(cis)-1-Butyl-2-undecylcyclopropane	+	-
5.	14.85	1-Hexadecanol	-	+
6.	16.46	Silane, (dodecyloxy)trimethyl	-	+
7.	16.87	1-Hexadecanol	+	-
8.	20.52	1-Octadecanol	+	-
9.	21.56	1,1,1,3,5,5,7,7,7-Nonamethyl-3-(trimethylsiloxy)tetrasiloxane	-	+
10.	23.34	(1á,3á,4à)-3,4-Bis[dimethyl(4-methylphenyl)silyl]cyclo pentan-1-yl acetate	-	+
11.	23.84	1-Hexadecene	+	-
12.	24.03	1-Dotriacontanol	+	-
13.	24.68	Hexadecanoic acid, trimethylsilyl ester	-	+
14.	26.58	Heptacosane	+	-
15.	26.93	(2S,3R)-(3-Tetradecyloxiranyl)methanol	-	+
16.	28.45	1-Heptadecanol	+	-
17.	29.28	9-Octadecenoic acid	+	-
18.	29.94	Hexatriacontane	+	-
19.	30.77	Heptasiloxane, hexadecamethyl	+	-
20.	31.10	Heptadecane, 2-methyl	+	-
21.	31.39	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	-	+
22.	32.09	Tricosane	+	-
23.	32.72	Diethyl(1E,3Z)-3-cyano-4-naphthylbuta-1,3-dienylphosphonate	-	+
24.	32.98	Dodecane, 1,1'-oxybis	+	-
25.	33.19	Heptacosane	-	+
26.	33.80	1-(2-Hydroxyethoxy)tridecane	+	-
27.	33.96	Docosane	-	+
28.	34.37	Docosanoic acid, 1,2,3-propanetriyl ester	+	-
29.	36.24	(S,S)-1,1-Bis(ethoxycarbonyl)-2,2-bis-p-tolylsulfinyl-1-ethanol	-	+
30.	36.50	Docosanoic acid, 1,2,3-propanetriyl ester	+	-
31.	38.39	Pentatriacontane	+	-
32.	40.08	2,4,6-Tri[4,5-(methylenedioxy)phenyl]-s-triazine	-	+
33.	41.99	Diethyl a-trimethyloxy-2,4,6-trimethylbenzylphosphonate	+	-
34.	42.32	Pentatriacontane	+	-
35.	44.07	N-[(methylphenyl)methylene]-2-methyl-2-propanamine N-oxide	+	-
36.	44.08	(5S,6S)-4,5-Dimethyl-6-phenyl-3,4,5,6-tetrahydro-2H-1,3,4-oxadiazin-2-one	-	+
37.	45.71	Heptasiloxane, hexadecamethyl	+	-
38.	50.78	3-(Dimethylphenysilanyl)-6,11-dihydroxy-3-methyl-1,2,3,4-tetrahydrobenz[a]anthracene-7,12-dione	+	-

(+) Present; (−) Absent.

9.3.5 Toxicity assessment for environmental safety

Toxicity assay of untreated and bacterial treated PPMW showed an effect in seeds germination of *P. mungo* L. in 3 days observations as showed in figure 9.6a. The test indicated the removal of toxicity based on germination or suppression of seed and early growth of seeds. The various parameters of seed germination were observed with untreated and bacterial treated wastewater has been summarized in Table 9.3. The toxicity test of *P. mungo* L. seeds with different concentrations of untreated PPMW was more toxic showed only 86, 60, 40, and 20 % germination respectively. While the bacterial treated PPMW toxicity was significantly 70 % reduction in seed germination test of *P. mungo* L. The α-amalyse is a key role in seed germination and after exposure; the different concentrations of wastewater are detailed in figure 6b. The α-amylase activities were decreased when increases higher concentrations of untreated PPMW. Also, when seeds were exposed to untreated wastewater, the results were more pronounced compared to bacterial treated wastewater (Fig. 9.6b). The presence of more toxic pollutants and dissolved solids that are absorbed by the seeds before germination and effect of the different physicochemical and biochemical parameters of seeds due to germination suppression at high concentrations of PPMW (Sonkar et al., 2019). Increased germination percent in treated PPMW may be due to decreased and detoxified ROPs that have formed suitable environmental conditions for seed germinations and utilization of nutrients present in wastewater. Recently, reported toxicity analysis of untreated PPMW through seed germination tests and results were correlated with several previous studies (Kumar et al., 2020b).

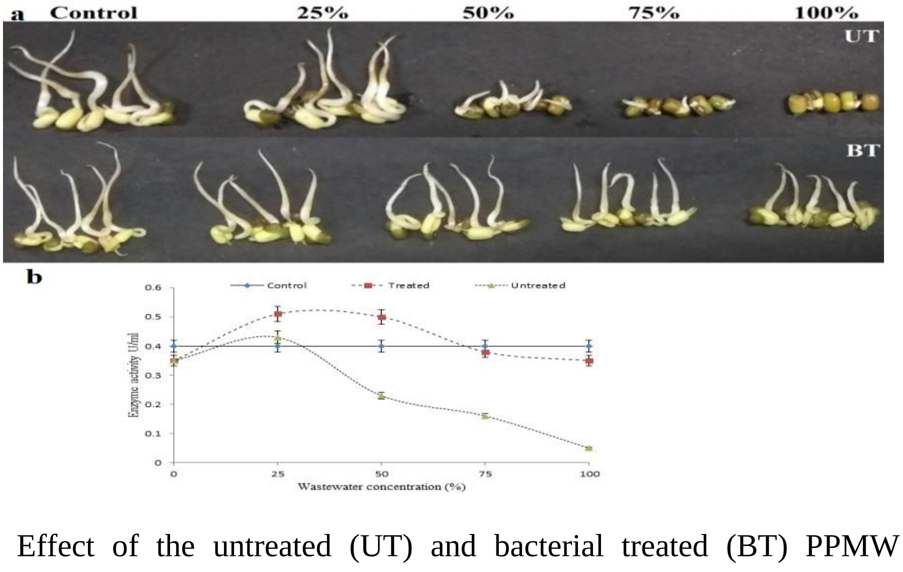


Fig. 9.6 Effect of the untreated (UT) and bacterial treated (BT) PPMW at the concentration of 0, 25, 50, 75 and 100 % on **(a)** seed germination and **(b)** alpha amylase activity

Table 9.3 Toxicity analysis of PPMW by *Phaseolus mungo* L. seed germination

Samples	Treatment (%)	Germination (%)	Radical length (cm)	Phytotoxicity (%)	Stress tolerance index (%)	Relative Toxicity (%)	Seedling vigor index
Untreated	25	86.60±0.57	1.90±0.1	10.79±0.02	89.20±0.04	13.40±0.01	164.54±0.02
	50	60.0±1.0	0.9±0.1	57.74±0.02	42.25±0.03	40.0±0.01	54.0±0.04
	75	40.0±1.0	0.3±0.1	85.91±0.04	14.08±0.02	60.0±0.03	12.0±0.01
	100	20.33±0.57	0.13±0.05	93.89±0.01	6.1±0.03	79.67±0.03	2.64±0.02
Treated	25	100±0.00	2.1±0.1	1.4±0.51	98.59±0.1	00	210±0.01
	50	83.33±0.57	1.93±0.05	9.38±0.01	89.2±0.04	16.67±0.01	160.82±0.03
	75	80.0±1.00	1.73±0.05	18.77±0.05	81.22±0.01	20±0.01	138.4±0.02
	100	70.0±1.00	1.46±0.05	31.45±0.01	68.54±0.01	30±0.051	102.2±0.05
Control	0	100±0.00	2.13±0.05	00	00	00	213±0.01

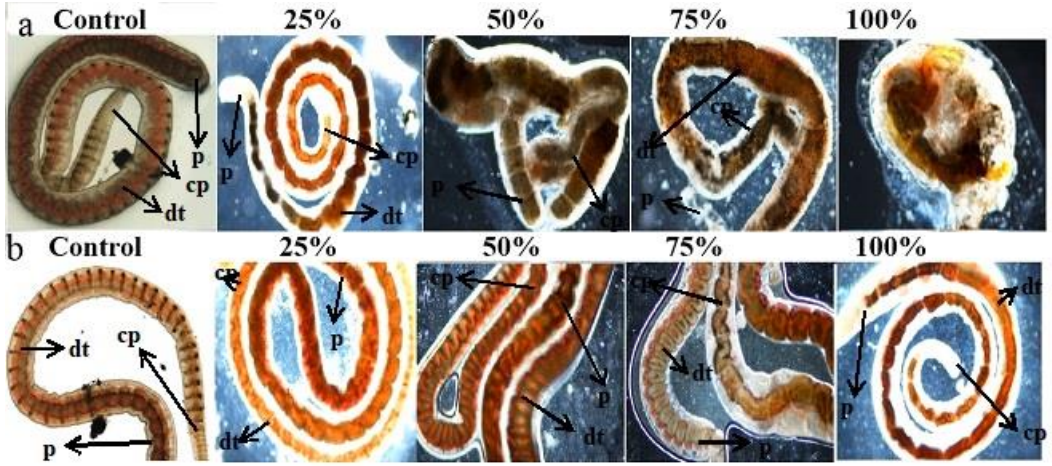


Fig. 9.7 *Tubifex tubifex* malformations after exposer to PPMW **(a)** untreated and **(b)** treated in different concentration 25 %, 50 %, 75 %, 100 % and control as 0 % as a tap water. Morphology of worm with intact prostomium (p), caudal part (cp) and digestive tract (dt).

9.3.6 Effects of PPMW of *Tubifex tubifex*

Tubifex worms are a significant link in the aquatic food chain and are valuable indicators of variable degrees of aquatic contamination due to freshwater worms (Khangarot, 1991). Toxicity assay of untreated and bacterial treated PPMW effect on *Tubifex tubifex* in 96-hour observations as shown in figure 9.7. In control, the Tubifex worm showed the typical behaviour of healthy worm i.e. no mortality and straight movement was observed. The toxicity test of untreated PPMW exposures at <25 % remarkably reduced the movement of the tested Tubifex worm. Exposure to higher untreated PPMW concentration resulted in lower hemoglobin content, the blackness of the rear part of the body, substantial disintegration of the hind portion body damage of Tubifex worm (Fig. 9.7a). While the bacterial treated PPMW sample was significantly reduced the toxicity exposures at <75 and 100 % reduced remarkably the movement of test organisms as shown in figure 7b. According to a report of USEPA (2012) an annual report of CPCB, (2015), the untreated PPMW showed several physicochemical parameters beyond the permissible limits along with heavy metals (showed in table 1) and different mutagenic and carcinogenic ROPs (showed in table 2) are responsible for toxicity. Because of the presence of these pollutants in PPMW, the rate of mortality from physical injury, cell bursting, and whole-cell damage has been increased (prostomium, digestive tract, and caudal part) of Tubifex worms (Kumar et al., 2020b; Lagauzere et al., 2009). This may be correlated with the existence of more toxic ROPs and toxic metals that cause worm toxicity in untreated PPMW. This study revealed the untreated PPMW is more toxic than bacterial treated PPMW as shown in figure 9.7b. Some researchers previously reported various heavy metals (Cu, Ag, Pd, Zn, and Uranium) caused intoxication was reduced tactile movement, degeneration, and segmentation of the Tubifex worm and death appeared without noticeable signs of the worms (Khangarot, 1991; Lagauzere et al., 2009).

Conclusion

This study has been revealed that the identified toxic ROPs present in the PPMW due to the cause of aquatic and terrestrial toxicity. PPMW sample was collected from Centaury Paper Mill Uttrakhand, India. Further, the isolated potential laccase-producing bacterium *Bacillus cereus* strain AKRC03 was capable of the degradation and detoxification of ROPs present in PPMW. The bacterium was used to degrade and decolorize PPMW up to 78.67 % in the suitable supplementary nutritional source (glucose: 1.0 %, peptone: 0.5 %) and environmental parameters (pH: 7.0, temperature: 37 ^{0}C, agitation: 180 rpm) at optimized incubation period 120 h. UV-Vis spectral scan and GC–MS analysis confirmed the decolorization and degradation as well as toxicity reduction of pollutants present in PPMW for safe aquatic and terrestrial environments. Further, the toxicity assessment using *Phaseolus mungo* L. seeds and *Tubifex tubifex* worm confirmed the toxicity reduction of wastewater after bacterial treatment. Therefore, *Bacillus cereus* strain AKRC03 could be used as promising in decolorization and detoxification of ROPs present in PPMW for its safe disposal.

Chapter Ten

Summary

Summary

A recent study revealed that complex organic pollutants are retained as residual organic pollutants in discharged pulp and paper mill wastewater, even after secondary treatment. Pulp and paper mill wastewater is a threat to environment for its safe disposal due to presence of residual organic pollutants, cellulose, hemicellulose, lignin, pectin, carbohydrate, extractives resins, fatty acids, terpene alcohols, phenolic compounds. The most critical reaction in the bleaching stage is chlorine oxidation, and chlorinated organic compounds or adsorbable organic halides are the main problems with the wastewater materials. The toxic effects of these by-products on the environment were analyzed in the wastewater. Various studies have documented the toxic/lethal effects on daphnia, shrimp, and planktons in the water bodies receiving wastewaters of the pulp and paper industry. Lignin is a major colourant of pulp and paper mill wastewater. It has also been reported that lignin has net negative charges; therefore, various heavy metals strongly binds with lignin to form large organo-metallic complex molecules. Among the different physicochemical and biological processes available for the treatment of pulp and paper mill wastewater. The wastewater after secondary treatment becomes dark brown colour due to complexation of lignin along with organic and inorganic residual organic pollutants present in pulp paper mill wastewater. The discharged wastewater after secondary treatment causes environmental problems in the aquatic and terrestrial ecosystem which ultimately affect aquatic flora and fauna. Therefore, adequate treatment of the pulp and paper mill wastewater is warranted prior to its safe discharge into environment. Hence, the detailed chemical properties of various pollutants present in pulp paper mill wastewater before and after secondary treatment have not yet to be elucidated in detail.

The above information has been systematically elaborated in the first chapter of the thesis as introduction. Subsequently, the second chapter has mentioned objectives of the thesis. Further, chapter three is the review of literature on the topic that has been elaborated pulp and paper mill wastewater treatment through the ligninolytic enzyme such as laccase. Ligninolytic enzymes play a key role in the degradation and detoxification of lignocellulosic waste in environment. The major ligninolytic enzymes are laccase, lignin peroxidase, manganese peroxidase, and versatile peroxidase. The structurally laccase is isoenzymes with monomeric or dimeric and glycosylation levels (10–45 %). This contains four copper ions of three different types. The enzyme catalyzes the overall reaction: 4 benzenediol + O_2 to 4 benzosemiquinone + $2H_2O$. While, lignin peroxidase is a glycoprotein molecular mass of 38–46 kDa containing one mole of iron protoporphyrin IX per one mol of protein, catalyzes the H_2O_2 dependent oxidative depolymerization of lignin. The manganese peroxidase is a glycosylated heme protein with molecular mass of 40–50kDa. It depolymerizes the lignin molecule in the presence of manganese ions. The versatile peroxidase has broad range substrate sharing typical features of the manganese and lignin peroxidase families. Although laccase enzyme has broad range of industrial applications especially the degradation and detoxification of lignocellulosic waste discharged from various industrial activities, its large scale application is still limited due to lack of limited production. Further, the extremophilic properties of laccase enzyme indicated their broad prospects in varied environmental conditions.

Chapter four has mentioned the physico-chemical analysis and identification of various residual organic pollutants from the bleached and unbleached paper mill wastewater and both sources showed endocrine-disrupting compounds. The result has revealed that bleached paper mill wastewater showed high BOD (225.0 ± 2.24), COD (543.04 ± 1.22), TS (3280.0 ± 1.32), DS (3110.0 ± 2.42), fixed solid (2900.0 ± 2.35), chloride (2350.0 ± 1.14), total phenol (13.195 ± 0.52), sulfate (713.1 ± 1.11), nitrate (210.08 ± 2.32), phosphate (2.56 ± 0.12) and lignin (578 ± 0.13 mg L^{-1}) levels with alkaline pH (7.68 ± 0.21), and dark brown colour. In addition, several major heavy metals were detected, including Fe (1.8598 ± 0.90), Pb (0.2550 ± 0.12), Cr (0.3058 ± 0.01), Cu (1.3814 ± 0.16), Cd (0.08632 ± 0.13) and Zn (0.0945 ± 1.21 mg L−1). While the result has revealed that unbleached paper mill wastewater showed high BOD (112.0 ± 1.14), COD (413.5 ± 0.81), TS (698.0 ± 2.34), DS (584.0 ± 1.82), fixed solid (566.0 ± 1.12), chloride (1740.0 ± 1.10), total phenol (11.691 ± 0.82), sulfate (316.71 ± 0.81), nitrate (47.17 ± 1.10), phosphate (1.1 ± 0.20) and lignin (285 ± 0.20 mg L^{-1}) levels with alkaline pH (7.95 ± 0.16), and dark brown colour. In addition, several major heavy metals were detected, including Fe (0.4232 ± 1.02), Pb (0.1360 ± 0.42), Cr (0.0834 ± 0.11), Cu (0.8971 ± 0.20), Cd (0.219 ± 0.10) and Zn (0.0408 ± 0.82 mg L^{-1}). The GC-MS analysis of bleached paper mill wastewater revealed the presence of toxic residual organic pollutants i.e. Silanol, trimethyl-, triester with boric acid (H_3BO_3) (CAS); D-LACTIC ACID-DITMS; Dodecane, 1-iodo; Decane, 1-iodo;

Tetracosane; Heptacosane; Pentan-1,3 dioldiisobutyrate, 2,2,4- trimethyl; Octadecane, 1-iodo; Heneicosane; Hexadecanoic acid, trimethylsilyl ester; Eicosane; Diethyl 3,4-dihydro-2-naphthyl-phosphonate; Hexadecane, 2,6,10,14-tetramethyl; 1,2,3,4,5-Pentaisopropylbis(cyclopentadienyl) cobalticimium (Cobaltocene); Methyl ester of [1'R-[1'à,4'á,8'à(E),8a'á]]-3-(8'-ethyl-1,2,2',3',6',7',8,8a'- octahydro-1 -methyl-2-oxospiro[3H-indole-3,1'(5'H)-inndolizin-8'-yl)-2-propenoic acid N-oxide; 2-(1-Methyl-1H-2-pyrrolyl)quinolone; Cyclotrisiloxane, hexamethyl; 5,11,17,23-Tetra-t-butyl-25,26,27,28-tetrahydroxycalix-4-arene and a-Fluoro-(p-methyl)chalcone. While, unbleached paper mill wastewater i.e. 2-Phenyl-N-propyl-4-quinazolinamine, Sulfurous acid, 2-ethylhexyl hexyl ester, 7,8-Dimethyl-4 trifluoromethyl-(1H,5)benzodiazepine, Nonadecane, Dodecane, 1-iodo, 3,6-Dioxa-2,7-disilaoctane, Tricosane, 7,7-diphenyl-3,5-dioxo-7-hydroxyheptanenitrile, Tetradecane, Eicosane, Hexadecanoic acid, trimethylsilyl ester, Octadecanoic acid, trimethylsilyl ester, 4-(Chloromethyl)-3-methyl-5-phenylisoxazole, N(2)-[2,5-di(t-butyl)phenyl]-N(1)-ethyl-3,4:9,10- perylenetetracarboxydiimide, and (3R)-3-Phenyl-2,3-dihydro-1H-isoindol-1-one. The toxicity test with *Phaseolus aureus* seed germination showed inhibition of seed germination and alpha-amylase activity >25 % in bleached and unbleached paper mill wastewater. The LC50 of *Tubifex tubifex* was noted of >50 % after 48 hours incubation test. This revealed that the wastewater discharged from bleached paper mills is more toxic than unbleached paper mill waste this might be due to the use of more chemicals during bleaching and pulping process.

Chapter five of the thesis has mentioned the isolation, screening and identification of laccase producing bacteria from pulp paper mill sludge samples. The 26 isolated bacterial strains were purified on the MSM agar plate by the streak plate technique. All bacterial strains were screened for laccase production on modified B and K agar medium containing 5 mM guaiacol. The three bacterial strains (i.e. BL1, BL6 and BL9) demonstrated laccase activity. The study revealed that the identified potential laccase-producing bacterial strains *Bacillus* sp. AKRC01, *Bacillus aquimaris* AKRC02 and *Bacillus cereus* AKRC03.

Based on the potentiality of isolated laccase producing bacterial strains were optimized environmental and nutritional conditions which constitute chapter six of my thesis. The result revealed that screening of suitable agro residues waste (potato peel, banana peel, sawdust, pea peel, wheat bran, orange peel, and rice bran) for maximum laccase production. Among these, rice bran supported the maximum laccase production. The *Bacillus* sp. AKRC01 were under submerged fermentation state using rice bran as the substrate. The optimized environmental conditions such as incubation period 96 hours, temperature 35 ^{0}C and pH 8.0 were obtained maximum enzyme production 3.832 U/mL. In addition, optimized the carbon and nitrogen sources such as 2.0 % glucose (4.967 U/mL) and 1.0 % peptone (6.236 U/mL) resulted in maximum enzyme production with rice bran as an effective agro-waste substrate. While, *Bacillus aquimaris* AKRC02 maximum laccase production optimized environmental conditions (incubation time 120 h; 4.58 U/mL), 35 ^{0}C; 6.624 U/mL) and pH 7.0; 10.142 U/mL) and nutritional sources (glucose 1.0 %; 14.164 U/mL and peptone 0.5 %; 18.124 U/mL) significantly enhanced the laccase production.

As per the objective, the purified laccase using culture supernatant was collected by centrifugation of 24 h grown culture of *Bacillus* sp. AKRC01 and *Bacillus aquimaris* AKRC02 at 5000rpm for 20 min. The elution profile of purified laccase showed a well-resolved single peak of enzyme activity. Approximately 38.08-fold purification was achieved with the specific activity of 228.34 U/mg Extracellular laccase was purified from a culture of *Bacillus aquimaris* AKRC02 in optimized conditions for enzyme production. The laccase enzyme was purified from bacterial culture and determined molecular weight by denaturing 10 % SDS-PAGE performed. The molecular weight of laccase produced by *Bacillus* sp. AKRC01 in the presence of rice bran was found to be 61 kDa and *Bacillus aquimaris* AKRC02 was determined to be 65 kDa. Thermo stability of purified laccase was incubated in various temperature ranges (25, 35, 45, 55, 65, 75, and 85 ^{0}C) for different time intervals (0, 2, 4, 6, 8, 10, 12, 14, and 16 h) to determine the thermal stability. The residual laccase activity was calculated by evaluating the oxidation of guaiacol at 100 mM acetate buffer pH 5.0 at 465 nm. Purified laccases of *Bacillus aquimaris* AKRC02 were stable at 45 ^{0}C for 8 h with an enzyme activity loss of just 50 % in comparison to the enzyme activity at the optimum temperature. About 28 % of its activity was maintained by the enzyme even after 16 h at 45 ^{0}C. The half-life of laccases at higher temperatures of 55 and 65 ^{0}C was approximately 6 h. The activity of the enzyme dramatically decreased and displayed about 90 % loss of activity at a higher temperature of 85 ^{0}C after 16 h. At 25 and 35 ^{0}C, the laccase was very stable with a minimum loss of 5 % in its activity, even after 16 h. After 16 h, there was approximately 76, 84, 88 % loss in enzyme activity at 55, 84, 88 ^{0}C, respectively entailing the enzyme stability at the optimal temperature (35 ^{0}C).

Chapter eight has described Study the effect of the copper (Cu) and iron (Fe) nanoparticles on the production of bacterial laccase. The nanoparticles were synthesised and characterised through XRD, XPS, SEM, TEM and FTIR analysis. After-effects of laccase production were observed in different concentration NPs and immobilized laccase with magnetic nanoparticles. Under mild conditions, an effective technique for improving catalytic activity and stability of immobilised laccase via condensation reaction with magnetically separable and reusable magnetite-supported copper (nanocat- Cu/FeO) nanoparticles. Immobilizing a *Bacillus aquimaris* AKRC02 laccase in magnetic multiwalled carbon nanotubes (Cu/FeO), an excellent substrate for immobilising enzymes, resulted in a new magnetically separable laccase immobilised system. According to our findings, Cu/FeO nanoparticles had a high loading amount of bovine serum albumin (BSA, 436 mg/g support) and laccase activity recovery of 86.45 % after immobilisation. It should be noted that the immobilised laccase's temperature and pH stabilities, as well as tolerances to organic solvents, were significantly higher than those of the free laccase. Furthermore, after 10 reuses, the immobilised laccase maintained 54 % of its original activity. After 16 days, immobilised laccase had 59 % storage stability. The use of an immobilised system to catalyse the phenolic lignin model compounds syringic acid and guaiacol was studied. Because of the high catalytic effectiveness for guaiacol, using immobilised laccase to catalyse lignin is a very promising method for further application in the wood industries.

Further, as per the objective of the study, the laccase producing bacterial degradation of PPMW. The physic-chemical analysis and identification of various residual organic pollutants were present in PMMW before and after secondary treatment. The result has revealed that i.e. (mg/L^{-1}) i.e. pH (8.1 ± 0.12), Total solid (1946.0 ± 1.02), Dissolved Solid (1784.0 ± 1.24), COD (752.0 ± 0.78), BOD (380.0 ± 0.45), Lignin (416.0 ± 5.12), phosphate (34.0 ± 0.46), nitrate (695.5 ± 1.05), total phenol (29 ± 0.95) and heavy metals i.e. Fe (5.6982 ± 1.02), Ni (0.956 ± 0.58) and Zn (3.2346 ± 0.89). However, a sharp reduction pH (7.64 ± 0.11), Total solid (314.0 ± 1.12), Dissolved Solid (652.0 ± 2.15), COD (186.0 ± 0.16), BOD (52.0 ± 0.58), Lignin (30.0 ± 1.02), phosphate (14.5 ± 0.21), nitrate (112.75 ± 0.12), total phenol

(6.27 ± 0.00) and heavy metals i.e. Fe (3. 1462 ± 1.00), Ni (0.6217 ± 0.91) and Zn (1.1462 ± 0.98) was noted. The main focus of this work is to study the potential laccase producing bacterium *Bacillus cereus* AKRC03 (accession no. MN720581.1) for biodegradation and toxicity reduction from pulp paper mill wastewater. The laccase producing *Bacillus cereus* AKRC03 exhibited up to 78.67 % of decolorization and degradation capability for hazardous residual organic pollutants at different nutritional (glucose: 1.0 %, peptone: 0.5 %) and environmental conditions (pH: 7.0, temperature: 37 ^{0}C, agitation: 180 rpm, incubation period: 120 h). The absorption peak of the UV-Vis spectral scan identified the decolorization and degradation pattern for pollutants present in wastewater during treatment. Furthermore, the transformation of major residual organic pollutants was exhibited through GC-MS analysis. These were 1-dotriacontanol, 1-heptadecanol, tricosane, 1-(2-hydroxyethoxy) tridecane, and n-[(methylphenyl) methylene]-2-methyl-2-propanamine n-oxide. While silane, (dodecyloxy)trimethyl, (1á,3á,4à)-3,4-bis[dimethyl(4-methylphenyl)silyl]cyclo pentan-1-yl acetate, (2S,3R)-(3-tetradecyloxiranyl)methanol, hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, and 2,4,6-tri[4,5-(methylenedioxy)phenyl]-s-triazine appeared as metabolic product after degradation of wastewater. The reduction of toxicity was measured up to 70 % for treated wastewater with *Phaseolus mungo* L seeds and *Tubifex tubifex* worms. From these findings, it is concluded that the isolated bacterium may be used in the bioaugmentation process for the further detoxification and degradation of discharged pulp paper mill wastewater for environmental safety.

Conclusion

The study concluded that pulp and paper mill wastewater after secondary treatment contains various residual organic pollutants which showed aquatic and terrestrial toxicity such as *Phaseolus aureus, Phaseolus mungo* L seeds and *Tubifex tubifex.* The isolate laccase producing *Bacillus* sp. AKRC01, *Bacillus aquimaris* AKRC02 and *Bacillus cereus* AKRC03 from pulp and paper mill sludge samples. Extracellular laccase was purified from a culture of *Bacillus* sp. AKRC01 and *Bacillus aquimaris* AKRC02 in optimized environmental and nutritional conditions for enzyme production. The molecular weight of extracellular purified laccase produced by *Bacillus* sp. AKRC01 in the presence of rice bran was found to be 61 kDa and *Bacillus aquimaris* AKRC02 was found to be 65 kDa through SDS-PAGE. In addition, the effects of laccase production were observed in different concentrations of copper (Cu) and iron (Fe) NPs and immobilized laccase with magnetic nanoparticles. Under mild conditions, an effective technique for improving catalytic activity and stability of immobilised laccase via condensation reaction with magnetically separable and reusable magnetite-supported copper Cu/FeO nanoparticles. It should be noted that the immobilised laccase's temperature and pH stabilities, as well as tolerances to organic solvents, were significantly higher than those of the free laccase. Furthermore, after 10 reuses, the immobilised laccase maintained 54 % of its original activity. Therefore, the laccase producing *Bacillus cereus* AKRC03 exhibited up to 78.67 % of decolorization and degradation capability for hazardous residual organic pollutants at different nutritional and environmental conditions. The transformation of major residual organic pollutants was exhibited through GC-MS analysis such as 1-dotriacontanol, 1-heptadecanol, tricosane, 1-(2-hydroxyethoxy) tridecane, and n-[(methylphenyl) methylene]-2-methyl-2-propanamine n-oxide. While silane, (dodecyloxy)trimethyl, (1á,3á,4à)-3,4-bis[dimethyl(4-methylphenyl)silyl]cyclo pentan-1-yl acetate, appeared as metabolic product after degradation of wastewater. The reduction of toxicity was measured up to 70 % for treated wastewater with *Phaseolus mungo* L seeds and *Tubifex tubifex* worms.

Based on work, nine original research papers have been published in high impact journals and three research papers are under consideration. While five conference papers were presented at national and international conferences. Besides, three book chapters are published in firm of reputed publishers such as Elsevier and Springer.

Chapter Eleven

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Chapter Twelve

Scientific Publications and Achievements

Scientific Publications and Achievements:

(a) Research Papers published high impact Journals of International Repute:

(1) **Kumar, A.,** Singh, A.K., Bilal, M., Chandra, R. **(2021b).** Extremophilic ligninolytic enzymes- a versatile tool with huge biotechnological promise. Catalysis letters, doi :10.1007/s10562-021-03800-8.

(2) **Kumar, A.,** Singh, A.K., Bilal, M., and Chandra, R. **(2021a).** Sustainable production of thermostable laccase from agro-residues waste by *Bacillus aquimaris* AKRC02. Catalysis Letters, doi.org/10.1007/s10562-021-03753-y.

(3) **Kumar, A.,** Chandra, R. **(2021a).** Biodegradation and toxicity reduction of pulp paper mill wastewater by isolated laccase producing *Bacillus cereus* AKRC03. Cleaner Engineering and Technology 4 100193. https://doi.org/10.1016/j.clet.2021.100193.

(4) Singh, A.K., **Kumar, A.,** Bilal, M., Chandra, R. **(2021).** Organometallic pollutants of paper mill wastewater and their toxicity assessment on Stinging catfish and Sludge Worm. Environmental Technology and Innovation 24, 101831. https://doi.org/10.1016/j.eti.2021.101831.

(5) **Kumar, A.,** Chandra, R. **(2020).** Ligninolytic enzymes and its mechanisms for degradation of lignocellulosic waste in environment. Heliyon 6, e03170.

(6) **Kumar, A.,** Singh, A.K., and Chandra, R. **(2020b).** Comparative analysis of residual organic pollutants from bleached and unbleached paper mill wastewater and their toxicity on *Phaseolus aureus* and *Tubifex tubifex*. Urban Water Journal vol. 17, 10, 860–870.

(7) **Kumar, A.,** Singh A.K., Ahmad, S., Chandra, R. **(2020a).** Optimization of Laccase Production by *Bacillus* sp. Strain AKRC01 in Presence of Agro-waste as Effective Substrate using Response Surface Methodology. J. Pure Appl. Microbiol., 14(1), 351-362.

(8) Singh, A.K., **Kumar, A.** and Chandra, R. **(2020a).** Detection of refractory organic pollutants from pulp paper mill effluent and their toxicity on *Triticum aestivum*; *Brassica campestris* and *Tubifex-tubifex*. Journal of Experimental Biology and Agricultural Sciences, 8(5) 663 – 675.

(9) Singh, A.K., **Kumar, A.,** Chandra, R. **(2020b).** Residual organic pollutants detected from pulp and paper industry wastewater and their toxicity on *Triticum aestivum* and *Tubifex-tubifex* worms, Materials Today: Proceedings, https://doi.org/10.1016/j.matpr.2020.10.862.

(b) Communicated Research Papers(s)

(1) **Kumar, A.,** Singh, A.K., Bilal, M., Chandra, R. **(2021).** Improved performance of immobilized laccase on Cu/FeO nanoparticles and its role for biodegradation lignin derivatives compounds. International journal of biological micromolecules (Under Review).

(2) Singh, A.K., **Kumar, A.,** Bilal, M., Chandra, R. **(2021).** Bacterial population dynamics and their relationship with toxic organometallic pollutants of pulp and paper mill contaminated sludge. Environmental science and pollution research (Under review).

(3) Singh, A.K., **Kumar, A.,** Bilal, M., Chandra, R. **(2021).** Environmental pollutants of paper industry wastewater and their toxic effect on human health and ecosystem. Groundwater for Sustainable Development, (Under review).

(c) Book Chapter(s)

(1) **Kumar, A.,** Chandra, R. **(2021b).** Extremophilic nature of microbial ligninolytic enzymes and their role in biodegradation. Ed. Das S, Dash R. Microbial Biodegradation and Bioremediation, and Elsevier.

(2) **Kumar, A.,** Singh, A.K., and Chandra, R. **(2021c).** Recent advances in physicochemical and biological approaches for degradation and detoxification of industrial wastewater. Ed. Haq I., Kalamdhad A., Emerging Treatment Technologies for Waste Management, Springer.

(3) **Kumar, A.,** Saxcen, G., Kumar, V., Chandra, R. **(2020c).** Environmental contamination, toxicity profile and bioremediation approaches for treatment and detoxification of pulp paper industry effluent. Ed. Saxcena G, Kumar V, Shah M. Bioremediation for Environmental Sustainability Toxicity, Mechanisms of Contaminants Degradation, Detoxification, and Challenges, Elsevier.

(d) Research Paper Presented in National /International symposium and conferences

(1) **Kumar, A.,** Chandra, R. **(2020).** Optimization of laccase production by Bacillus sp. strain AKRC01 in presence of agro-residue waste as effective substrate using response surface methodology. National online conference on “Environment, human health and sustainable development goals” and world environmental day 2020 celebration 5-6 June 2020.

(2) **Kumar, A.,** Singh, A.K., and Chandra, R. **(2019).** Complex organic pollutants discharged from pulp paper and kraft paper mill wastewater: toxicity to *Phaseolus aureus*. National Seminar on the occasion of National Unity Day “Horticulture-A Boon for Indian Economy”.

(3) **Kumar, A.,** Chandra, R. **(2018).** Bacterial laccase involved in lignin degradation and potential biotechnological applications. 1st North Indian Science Congress

(4) **Kumar, A.,** Chandra, R. **(2017).** Production, Purification and Industrial uses of Bacterial Laccase. Conference: 58th annual conference of Association of Microbiologists of India & international symposium.

(5) Singh, A.K., **Kumar, A.,** and Chandra, R. **(2017).** Persistent organic pollutants release after secondary treatment from pulp and paper mill effluent and their aquatic toxicity. Conference: 58th annual conference of Association of Microbiologists of India & international symposium.

(e) Memberships of Scientific Societies

(1) Life Member of **Association of Microbiologists of India (AMI)**

(2) Life Member of **The Biotech Research Society, India (BRSI)**

(3) Life Member of **International Society of Environmental Relationship and Sustainability (ISERS)**

Reprints

URBAN WATER JOURNAL
https://doi.org/10.1080/1573062X.2020.1836238

Taylor & Francis
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RESEARCH ARTICLE

Comparative analysis of residual organic pollutants from bleached and unbleached paper mill wastewater and their toxicity on *Phaseolus aureus* and *Tubifex tubifex*

Adarsh Kumar, Ajay Kumar Singh and Ram Chandra

Department of Environmental Microbiology, School for Environmental Sciences Babasaheb Bhimrao Ambedkar (A Central) University, Lucknow, India

ABSTRACT

The paper industry wastewater even after bio-treatment at industrial scale is a major source of aquatic and soil pollution due to various unknown compounds. Hence, the study has focused on the detection of residual organic pollutants from the bleached and unbleached paper mill wastewater and both sources showed endocrine-disrupting compounds. The toxicity test with *Phaseolus aureus* seed germination showed inhibition of seed germination and alpha-amylase activity >25% in bleached and unbleached paper mill wastewater. The LC_{50} of *Tubifex tubifex* was noted of >50% after 48 hours incubation test. This revealed that the wastewater discharged from bleached paper mill is more toxic than unbleached paper mill waste this might be due to the use of more chemicals during bleaching and pulping process. This study has revealed that there is need to treat both paper mill wastewaters adequately prior to discharge.

ARTICLE HISTORY
Received 6 June 2019
Accepted 5 October 2020

KEYWORDS
Organic pollutants; paper mill wastewater; endocrine disrupting chemical; *Phaseolus aureus*; *Tubifix tubifix*

1. Introduction

Environmental pollution mainly caused due to the disposal of untreated or partially treated industrial waste to the aquatic ecosystem (Singh and Chandra 2019). The Paper mill industry can be categorized mainly into two forms as pulp and paper mill (bleached paper) and Kraft paper mill (unbleached paper) depending upon their raw material and finished product. The pulp and paper mill produces the writing paper after using high amount of bleaching chemicals at the multistage bleaching process due to variation in their process and raw materials. During the pulping process, the cellulose fibers are separated due to solubilization of lignin (Hubbe et al. 2016). However, lignin remains up to 6% as a major constituent of pulp even after chemical digestion (Julkapli and Bagheri 2016). Therefore, the multistage bleaching is required to increase the brightness of cellulose fibers. The cellulose fibers after bleaching become high tensile strength, this absorbed the additives used to modify pulp into chemically stable while paper and board products (Julkapli and Bagheri 2016). But during washing and bleaching of pulp the poor quality of cellulosic fibers are washed away alone with wastewater containing the bleached chemical. Thus wastewater discharged from these industries is known as bleached paper mill wastewater (Hubbe et al. 2016). While the Kraft paper mills either do semi bleaching or do not use any bleaching process. The Kraft paper mills may also use recycled paper as raw material with pulp of wood for the production of different types of packaging paper or brown paper (Hubbe et al. 2016). Therefore, the pollutants present in discharged wastewater from both categories of the industry remain different due to variation process and raw materials. In the pulp and paper manufacturing process there is consumption of a large amount of water and energy, therefore, releases approximately 47,000–80,000 gallon wastewater per ton of paper production (Chandra et al. 2011a). In India, there are approximately 859 paper industries as per the annual report of Central Pulp & Paper Research Institute (CPPRI, 2017-18). This reflects the magnitude of entire environmental problems of country. Though, there is globally known process of elemental chlorine-free (ECF) and total chlorine-free (TCF) bleaching but in India, majority of bleached paper industry uses multistage bleaching process by using in sequence of chlorine (Cl_2), sodium hypochlorite (NaOCl), sodium hydroxide (NaOH), chlorine dioxide (ClO_2), oxygen (O_2), hydrogen peroxide (H_2O_2), ozone (O_3), acid washing (SO_2) and sodium dithionite (NaS_2O_4) along with bio-bleaching process by using xylanases respectively. Initially released wastewater from paper machine known as black liquor and contains the high amount of lignin, cellulose, hemicellulose, tannic acid, resin acids, plastids, chlorinated phenol, chlorinated hydrocarbon, surfactant and biocides, etc. during pulping and papermaking process from industries in the form of black liquor (Chandra et al. 2011a). The black liquor undergoes the primary and secondary biotreatment process in industry for its degradation. But, during the secondary treatment process some biopolymers i.e. cellulose, hemicellulose, lignin and chlorolignin are degraded up to certain extent but some compounds are only biotransformed as the metabolic product (Maurya, Singla, and Negi 2015). While the various organic pollutants which do not change much during secondary treatment and it is discharged either in their original form or with minor change in the paper mill wastewater even after secondary treatment is known as residual pollutants in wastewater of paper industry. This directly influences the aquatic life due to their carcinogenic and mutagenic properties (Ericson and Larsson 2000; Yadav and Chandra 2018). Recent studies have highlighted regarding their endocrine-disrupting chemicals (EDCs) properties that have been reported world over for

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CONTACT Ram Chandra rc_microitrc@yahoo.co.in

Supplemental data for this article can be accessed here.

their adverse effect on the reproductive system of freshwater fish (Ericson and Larsson 2000; Yadav and Chandra 2018). Besides, due to the high chemical diversity of the organic pollutants present in bleached paper mill wastewater, the various toxic effect on the crop plant and other aquatic life have been also evaluated in recipient water sources (Rios, Perez, and Sandoval 2012; Yadav and Chandra 2018). Furthermore, some residual organic pollutants present in this wastewater tend to persist in nature and showed toxicity to aquatic life with genotoxic and clastogenic properties. It has also high potential to migrate widely throughout the ecosystem due to their high solubility in water, which subsequently accumulated in the fatty tissue of animals including the humans through food chain (Singh and Chandra 2019). A significant amount of various heavy metals have been detected in the discharged wastewater of the paper industry after secondary treatment which makes more residual due to their strong binding tendency with various organic polymers present in the paper industry wastewater (Cailian et al. 2019). The organic pollutants with heavy metals have aggravated the toxicity in the environment and also influence the food chain (Cailian et al. 2019). Thus the paper mill wastewater is not only hazardous to the aquatic ecosystem but also health hazards to the downstream flora and fauna including the human (Rai et al. 2019). The accumulations of heavy metal and distribution among crop plants in a developing country are also reported (Rai et al. 2019). But, the detailed knowledge of various pollutants present in the paper industries and their toxicity in the environment are still not much known. Molecular dibenzophenol (dioxins and furans) and polychlorinated dibenzodioxins, recalcitrant compounds in paper mill wastewater are highly toxic and tend to persist in nature (Hubbe et al. 2016). However, the toxicity and lipophilicity of these compounds in higher animals can be enhanced by anaerobic bacteria that methylate chlorinated organic compounds (Yadav and Chandra 2018). Hence, the US Environmental Protection Agency has leveled these residual organic pollutants (POPs) as priority pollutants. The discharged several pollutants from the paper industry not only affecting the surface water but it is also influencing the underground water quality (Kim 1996; Singh and Chandra 2019). Hence, the study has focused to identify the unknown residual organic pollutants from bleached and unbleached paper mill wastewater (BUPMW) after secondary treatment. The conventional toxicity test was also done to assess the toxicity of BUPMW on *Phaseolus aureus* and *Tubifex tubifex* worms. Hence, the study will more effective and expend the knowledge of residual organic pollutants present in BUPMW for environmental safety.

2. Material and methods

2.1. Sample collection and physico-chemical analysis

Bleached and unbleached paper mill wastewater sample collected after secondary treatment from the M/s Star Paper Mill in Saharanpur, UP India, and M/s Naini paper mill Ltd. Uttrakhand, India, they are located at Latitude: 29° 56′ 10.89″ N to Longitude: 77° 34′ 13.51″ E and Latitude. 29°11′47.23″ to 29° 12′06.90″N. Longitude respectively. This industry uses wheat straw, sugarcane bagasse and eucalyptus, bamboo, popular wood with as raw material for paper making. Wastewater samples were collected in pre-sterilized jerry cane of 20 liters capacity and temperature was maintained at 4°C and transported to the laboratory for analysis within 24 hours.

All the physicochemical parameters BOD, COD, total solids, dissolved solids, suspended solids, chloride, phosphate, nitrate and total phenol analysis of the BUPMW sample were done as per the standard methods for the examination of water and wastewater (APHA 2015). The lignin content was estimated according to the method of Pearl and Benson (1990). Simultaneously, heavy metals in the wastewater samples were analyzed with standard methods (American Public Health Association APHA 2015) by atomic absorption spectrophotometry (AAS) (ZEEnit 700, Analytic Jena, Germany) after nitric acid-perchloric acid digestion method no. 3030 H.

UV-Vis spectrophotometer scanning of wastewater sample was done between ranges of the electromagnetic spectrum at 200 to 700 nm (Model Evolution 201, serial no 5A4R304008, Thermo Fisher Scientific, USA).

2.2. SEM EDXs and FT-IR spectroscopy

The scanning electron microscope was used for the EDXs analysis in which 10 mg bleached and unbleached paper mill wastewater dried sample was spotted on aluminum stubs (Yadav and Chandra 2018). In elemental analysis of wastewater, an area was selected, for the elements in the sediment sample were analyzed by a high-resolution scanning electron microscope which was equipped with EDXs system (SEM, QUANTA FEG 450, Netherland).

The FT-IR analysis was done for analysis of functional groups present in organic chemicals of bleached and unbleached paper mill wastewater, then the sample was dried at 104 to 106°C in the oven. To perform this analysis, the absorbance spectra were recorded by FT-IR Spectrometer (Thermo Fisher Scientific, Model Nicolet 6700, USA). The whole analysis was performed in the mid-infrared region from 4000 to 400 cm^{-1} (Yadav and Chandra 2018). The data processing was performed using the software OMNIC™ (v7.4).

2.3. Characterization of residual organic pollutants by GC-MS analysis

The residual organic pollutants present in bleached and unbleached paper mill wastewater were extracted by dichloromethane in acidic conditions (pH <2.0) as previously described by Bharagava and Chandra (2009). Further, the GC–MS sample analysis of the extracted sample was processed by using dioxane (100 µL) and pyridine (10 µL) to the samples, followed by 50 µL BSTFA [N,O-bis(trimethylsilyl) trifluoroacetamide) containing TMCS (trimethylchlorosilane) (Raj, Reddy, and Chandra 2007). Subsequently, the solution was heated up to 60°C with periodic shaking for 15 min to dissolve the residue. Finally, the silylated samples (1 µL) were injected into a GC–MS (PerkinElmer, UK) equipped with a PE-5 MS capillary column (20 m × 0.18 mm internal diameter, 0.18-µm film thickness) and using helium at a flow rate of 1 mL/min as the carrier gas. The column temperature was set at 50°C for 5 min, then ramped from 50 to 300°C at 10°C min and held for 5 min. The transfer line and ion-source temperatures were maintained at 200 and 250°C, respectively.

2.4. Phytotoxicity assessment of *Phaseolus aureus*

For phytotoxicity, the experiment was carried out at different concentrations (Control 0%, 10%, 25%, 50%, 75% and 100% v/v) of bleached and unbleached paper mill wastewater. The seeds surface was sterilized with 2.0% $HgCl_2$ solution for 2 min to avoid any fungal contamination and then washed three times with distilled water. Thereafter seeds were placed in sterilized glass Petri dishes with double Whatman No. 1 filter paper discs. The percent germination and germination index of seeds were calculated with formula as proposed by Czabator (1962), Naeem et al. (2015) and Wang et al. (2004) respectively. However, phytotoxicity percentage (PP) and seed vigor index (SVI) were calculated according to Abdul-Baki and Anderson (1973).

2.5. Assay for α-amylase activity and determination of molecular weight by SDS-PAGE

According to Bharagava and Chandra (2010), for the preparation of enzyme extract, seeds were taken from each concentration and homogenized with sodium acetate buffer solution (0.1 M, pH 4.8), filtered by two layers of cheesecloth 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 enzyme assay was performed with reaction mixture containing 0.5 mL of enzyme extract, 1.0 ml of 0.1 M acetate buffer, pH 4.8 and 1.0 mL of 0.1% soluble starch. The reaction mixture was incubated for 10 min at room temperature followed by adding 1.0 mL of 0.1% iodine reagent and 3.0 mL of 0.05 N HCl. The optical density (OD) was read at 620 nm and the decrease in absorbance was expressed in terms of amylase activity (Beri and Gupta 2007).

The alpha-amylase was purified from germinated seeds and determined molecular weight by denatured sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on 10% polyacrylamide gel. To determine the molecular weight of the purified enzyme was compared with the band of standard α-amylase enzyme (Sigma–Aldrich, USA) and protein ladder (Bangalore Genei, India). After 10 hours staining the gel was destined and imagined and stored in a gel documentation system (GeNei^{TM} UVITEC Cambridge).

2.6. Aquatic toxicity: *Tubifex tubifex*

Tubifex worms were collected from Gomti river Lucknow, from natural sources, and acclimatized to laboratory conditions for 7 days before experiments. The wastewater will be using *tubifex* worm because this worm is an important member of lentic fauna in the aquatic environment (Hellawell 1986). For the toxicity evaluation to worms of *tubifex* will be inoculated into different concentration (Control) 0%, 25%, 50%, 75% and 100% (v/v) of BUPMW sample used. Tests were conducted in 200 mL beakers containing 100 mL of test water. Ten tubifex worm were exposed to each concentration and each concentration was tested in replicates of three. The test worms were considered dead when there were complete immobilization and no response to pressing with a blunt glass rod (Singh, Chandra, and Sharma 2007; Auston 1973). Death was further confirmed by transferring worms back to fresh control tap water (Khangarot 1991). Exposure to higher concentration results in a considerable disintegration of the hind part of the body in some worm (Nikon Corporation Tokyo Japan model ECLIPSE Ci-S 402397). Test water was renewed every 24 hours.

2.7. Statistical analysis

All the laboratory experiment data analysis was carried out in triplicate and the results were presented as the mean of three independent observations. The criterion of Pearson's correlation coefficient was used to determine the relationship between different physiological parameters of seed germination. The $^{*}P < 0.05$ was considered as the statistically significant data, calculated using IBM SPSS Statistics Software (v20.0.0).

3. Results and discussion

3.1. Physico-chemical characteristics of wastewater

The physico-chemical characteristics of the wastewater sample collected from the discharged site of bleached and unbleached paper mill are shown in Table 1. The discharged wastewater showed high pollution parameters along with various heavy metals. Due to various pollutants in wastewater, it contributed high BOD and COD which were beyond the permissible limit (USEPA 2012). The high values of BOD (bleached 225.0 ± 2.24 and unbleached 112.0 ± 1.14) and COD (bleached 543.04 ± 1.22 and unbleached 413.5 ± 0.81) in both wastewater samples might be due to the presence of high amount complex organic compound in discharge wastewater. Because of bleached paper mill wastewater contents mixture of various salt and chlorinated compounds along with plant extract. Moreover, the presence of metallic contents of wastewater is also responsible for creation of organometallic compounds. Therefore, these complex compounds do not break during the wastewater treatment process and discharged on paper mill wastewater. Hence, the toxic effect of discharged paper mill wastewater has been reported globally towards fish, microorganisms, flora and fauna (Bajpai 2015; Ugurlu et al. 2007). Unbleached paper mill wastewater is showed two-fold of suspended solid (270.0 ± 0.28) and dark color than the bleached (164.0 ± 0.42) paper mill wastewater this might be due to the utilization of recycled and sugarcane bagasse's as raw material for the production of paper (Hubbe et al. 2016). Moreover, high content of soluble lignin (bleached 578 ± 0.13 and unbleached 285 ± 0.20) and its derivatives at alkaline pH (bleached 7.68 ± 0.21 and unbleached 7.95 ± 0.16) contributed dark brown color (bleached 1275 ± 3.11 and unbleached 4180 ± 2.10), which blocks the sunlight penetration in receiving water bodies, and reduce photosynthetic activity, dissolved oxygen content rejuvenation and thus negatively affect the aquatic life (Savant, Abdul-Rahman, and Ranade 2006). The higher sulfate present in bleached paper mill wastewater than unbleached paper mill wastewater might be due to the application of high amount of sodium sulfate during the wood digestion and pulping process.

Table 1. Physicochemical analysis of bleached and unbleached paper mill wastewater.

S. no.	Parameters	Bleached paper mill wastewater	Unbleached paper mill wastewater	Permissible limits USEPA,2002
1.	pH	7.68 ± 0.21	7.95 ± 0.16	5–9
2.	BOD	225.0 ± 2.24	112.0 ± 1.14	40
3.	COD	543.04 ± 1.22	413.5 ± 0.81	120
4.	Total solids	3280.0 ± 1.32	698.0 ± 2.34	300 mg/l
5.	Dissolved solid	3110.0 ± 2.42	584.0 ± 1.82	500 mg/l
6.	Suspended solids	164.0 ± 0.42	270.0 ± 0.28	100
7.	Fixed Solids	2900.0 ± 2.35	566.0 ± 1.12	-
8.	Volatile solids	380.0 ± 0.31	132.0 ± 0.22	-
9.	Chloride	2350.0 ± 1.14	1740.0 ± 1.10	1500
10.	Phosphate as P	2.56 ± 0.12	1.1 ± 0.20	-
11.	Sulfate as SO_4^{-2}	713.1 ± 1.11	316.71 ± 0.81	250
12.	Nitrate	210.08 ± 2.32	47.17 ± 1.10	50
13.	Color	1275 ± 3.11	4180 ± 2.10	Colorless
14.	Total phenol	13.195 ± 0.52	11.691 ± 0.82	0.50
15.	Lignin	578 ± 0.13	285 ± 0.20	0.05
Heavy metals				
16.	Lead (Pb)	0.2550 ± 0.12	0.1360 ± 0.42	0.05
17.	Iron (Fe)	1.8598 ± 0.90	0.4232 ± 1.02	2
18.	Chromium (Cr)	0.3058 ± 0.01	0.0834 ± 0.11	0.05
19.	Copper (Cu)	1.3814 ± 0.16	0.8971 ± 0.20	0.5
20.	Cadmium (Cd)	0.08632 ± 0.13	0.219 ± 0.10	0.01
21.	Zinc (Zn)	0.0945 ± 1.21	0.0408 ± 0.82	2.00

All the parameters are in mg/l except pH and color (Pt-co).

Similarly, the high contents of phenol in wastewater might be due to release from the plant cell wall and other depository material in the wood digestion and pulping process (Chandra, Abhishek, and Sankhwar 2011). The total phenol (bleached 13.195 ± 0.52 and unbleached 11.691 ± 0.82) and its derivatives induce toxicity as a carcinogen, immunotoxic, genotoxic and a physiological effect on fish has been reported by van den Heuvel et al. (2010). The other pollution parameters like total solids, dissolved solids, suspended solids, fixed solids, volatile solids, chloride, phosphate, nitrate, and various heavy metals were also detected above the permissible limits (Table 1). Moreover, the high contents of iron, copper, chromium, lead and other heavy metals are also detected beyond the permissible limit that is also environmental hazards which aggravate the toxicity of organic pollutants (Table 1) (Phukan and Bhattacharyya 2003; Cailian et al. 2019). The contents of Fe and Cu might be in the wastewater from iron pipes and with other equipment due to corrosion during the beaching and washing of pulp. The copper toxicity to aquatic organisms i.e. Rainbow trout (*Salmo Gairdneri*), channel catfish (*Ictalurus punctatus*) has been also reported in presence of calcium and high pH in paper industry wastewater previously by other researchers (Wurts and Perschbacher 1994). The bioaccumulation pattern of different heavy metals and their toxic effect in different organs of *Anabas testudineus* has been also reported on exposure to paper mill wastewater (Prasanta 2014).

3.2. Characteristics of organic pollutants

3.2.1. Scanning electron microscope (SEM-EDX)

The scanning electron microscope (SEM) analysis revealed the morphology of organic pollutants present in BUPMW as shown in supplementary figure S1(a,b). The organic pollutants present in bleached paper mill wastewater showed compact particle and crystalline structure. While the unbleached paper mill wastewater showed abundantly presence of rough and large particulate matter scattered on the surface sample as shown in Supplementary figure S1(a,b). The compact irregular crystalline structure in bleached paper mill wastewater might be due to the fine particle of various organic pollutants generated either in pulping and bleaching or in secondary treatment process of wastewater (Cherian and Siddiqua 2019). While in unbleached paper mill wastewater the presence of large particle and scattered rough structure of various suspended particulate matter of raw material used in paper manufacturing. The finding has also correlated with the previous studies observed in paper mill wastewater reported by Yadav and Chandra (2018). Further, the EDX analysis showed the elements present in weight% and atomic% in BUPMW as shown in Supplementary figure S1(c, d). The bleached paper mill wastewater major elements have C, O, Si, Al, Ca found, while unbleached paper mill wastewater major elements show the presence of O, C and Zr. The presence of these elements has been also reported in previous studies but some toxic elements are beyond the permissible limit (Yadav and Chandra 2018; Chandra et al. 2018; USEPA 2012). Besides, heterogeneous particles allowed the adsorption of elements are released from the BUPMW which is, directly and indirectly, affects the surface water as well as groundwater and causes health hazards.

3.2.2. Analysis of UV–Vis and FT-IR spectroscopy

The UV–Vis spectroscopy absorption scanning between 200 nm and 700 nm of BUPMW sample is showed absorption maxima at 280 nm and 230 nm respectively shown in Supplementary Figure 2. The absorption pattern of bleached paper mill wastewater showed maximum absorption spectra within range 200–400 nm while the unbleached paper mill wastewater showed maximum absorption range between 200 nm and 300 nm. This revealed that bleached paper mill wastewater content majority of soluble lignocellulosic waste along with various chemicals. But, in unbleached paper mill waste contents prevailing with particulate matter which settled and soluble organic pollutants are restricted within the range of 200–300 nm. This also indicated the presence of conjugated double bond of organic pollutants. The organic pollutants might be chlorophenols, resin acids, anthracene, fatty acids and aromatic ring containing pollutants (Chandra et al. 2018). The absorption of chlorolignin and its derivative compound, several functional chemical groups such as hydroxyl, methoxyl, carboxyl compounds also have been reported within the range of 200–400 nm (Jablonsky, Botkova, and Adamovska 2015; Skulcova et al. 2017).

The FT-IR analysis of organic pollutants of BUPMW has shown Supplementary figure S3(a,b). further their detail of the functional group presented in Supplementary Table 1. The bleached paper mill wastewater analysis bandwidth range from 654.7 cm^{-1} to 3402.2 cm^{-1} was done that showed similar to some extent with unbleached paper mill wastewater analysis bandwidth range 460.2–3410.0 cm^{-1} based on their functional group. BUPMW band range at 3402.2 and 3410.0 cm^{-1} represent the (O-H) functional group of alcohol and phenol which are generated from the plant extracts. Phenols are major component of plant cell walls and are also reported in previous studies). However, both sample band range 2933.2 and 2924.0 cm^{-1} represent the functional group (C-H) bond stretching showed the long chain of aliphatic compounds, which are generated from the plant's alkaloid that is used as a raw material in paper industries. The bleached paper mill wastewater showed broadband at 1653.4 cm^{-1} represented the (C = N stretch variable) these functional groups of ketone and amide, while the unbleached paper mill wastewater showed several functional groups (O-H) at broadband 2516.4 cm^{-1} corresponds the several carboxyl group's supplementary figure S3(a,b). It might be after biological treatment, in effluent treatment plant similar pattern for the generations of compounds are reported (Oller, Malato, and Sanchez-Perez 2011). The absorption band of BUPMW around 1539.5–1430.3 cm^{-1} (C = C aromatic skeletal lignin) is generated depending upon the raw material used for paper industries, while lignin is recalcitrant in nature and major component of wood (Chandra et al. 2018). However, bleached paper mill wastewater absorption band around the 1390.0 cm^{-1} (C-O-C stretch bending) in the spectrum biologically treatment of wastewater are very intensive indicating the abundant presence of ether and amine groups (Oller, Malato, and Sanchez-Perez 2011), while, unbleached paper mill wastewater band around 873.6 cm^{-1} (=C-H stretch bending) generally correspond to alkyl halide this might be generated during pulp bleaching (Yadav and Chandra 2018). Besides, bleached paper mill wastewater less intense band at 1236.0 cm^{-1} (SO_3 H symmetrical) sulfonic acids, while unbleached paper mill wastewater band 710.8 cm^{-1} (C-S stretch) sulfide group are present, which, these compound were released from the wood digestion during kraft recovery and sulfide recovery process (Scott et al. 2011). BUPMW absorption band 1063.1–460.2 cm^{-1} (=C-H bending) indicating the functional group of alkyl halides and alkanes, this is a major group of aliphatic compounds generated from plants source (fatty acids, resin acids) during the papermaking process. Bleached paper mill wastewater sample, it can be found that absorbance band around 654.7 cm^{-1} (C-Cl stretch) generally represent to alkyl halides, which may be generated during pulp beaching and chlorination process of papermaking steps (Yadav and Chandra 2018).

3.2.3. Gas chromatography-mass spectrometry characterization of residual organic pollutants

GC-MS analysis of bleached paper mill wastewater extracted with dichloromethane revealed various peaks at a different retention time (RT) (Figure 1(a) and Table 2), the major peaks noted at retention time (RT) 9.50, 27.82 and 30.70, which corresponded to Silane,[1-cyclohexene-1,2-diylbis(oxy)]bis[trimethyl-(CAS); Hexadecanoic acid, trimethylsilyl ester; and Diethyl 3,4-dihydro-2-naphthyl-phosphonate, respectively. Some minor peaks were also noted at different RT which is listed in Figure 1(a) and Table 2. These compounds have been reported previously also as residual organic pollutants generated from the papermaking process and chemicals used for storage (Yadav and Chandra 2018). Moreover, some residual organic pollutants are also were used as a preservative and these compounds cause acute toxicity, respiratory tract irritation in human beings (Yadav and Chandra 2018). These pollutants were discharged as wastewater after recovery and secondary treatment, as shown in Table 2. However, the detected residual organic compounds i.e. Dodecane, 1-iodo-; Decane, 1-iodo-; Tetracosane; Heneicosane; Hexadecanoic acid, trimethylsilyl ester; and Eicosane have been listed as potential endocrine-disrupting chemicals (EDCs) reported by the various workers (USEPA 2012; Kim et al. 2016; Yadav and Chandra 2018). Moreover, Octadecane, 1-iodo- has been reported as acute lung injury toxicity to human beings (Yadav and Chandra 2018). Hence, the other residual organic pollutants which were detected from GC-MS technique their fate in the environment along with the detected pollutants is still unknown because of these pollutants might be intermediate compounds which were form from the residual mixture of chlorine, resins, fatty acids and biocides used in paper industry during papermaking.

GC-MS analysis of unbleached paper mill wastewater extracted with dichloromethane showed various peaks at different retention times (RT) (Figure 1(b)). The major peaks at RT 9.50, 27.82 and 30.69 were characterized as 7,8-Dimethyl-4-trifluoromethyl-(1 H,5) benzodiazepine; Hexadecanoic acid, trimethylsilyl ester (CAS); Octadecanoic acid, trimethylsilyl ester, respectively (Table 3). Besides, some minor peaks are shown in Figure 1(b) and Table 3. Previously reported of octadecadienoic acid at RT 30.69 with ethanol extractives of *Eucalyptus camaldulensis*, it causes acute lung injury and Figure 3Figure 2skin irritation reported by various workers (Peng and Wu 2008; Kim et al. 2016; Yadav and Chandra 2018). Mostly the nonadecane (CAS); dodecane, 1-iodo-; tricosane (CAS); and tetradecane were noted as EDC, affect the nervous system and odor nuisance reported in (USEPA 2012). Sulfurous acid, 2-ethylhexyl hexyl ester and (3 R)-3-Phenyl-2,3-dihydro-1 H-isoindol-1-one) were released during the wood digestion in unbleached paper mill wastewater; and the sulfuric acid is also reported as EDC compounds according to (USEPA 2012). Moreover, the various residual organic pollutants detected in the extracted wastewater and their toxicity was still unknown.

Table 2. Identified of residual organic pollutant by GCMS analysis in bleached paper mill wastewater extract with dichloromethane (DCM).

S. No.	RT (min)	Identified compounds	Sources of generations
1.	6.42	Silanol, trimethyl-, triester with boric acid (H3BO3) (CAS)	-
2.	8.11	D-LACTIC ACID-DITMS	Bacterial production
3.	9.50	Silane,[1-cyclohexene-1,2-diylbis(oxy)]bis[trimethyl-(CAS)	Paper preservative agents
4.	13.29	Dodecane, 1-iodo-	Plant chemical
5.	14.33	Decane, 1-iodo-	Used as a solvent and to make other chemicals.
6.	16.40	Tetracosane (CAS)	Plant chemical
7.	17.91	Heptacosane	Plant chemical
8.	19.88	Pentan-1,3 dioldiisobutyrate, 2,2,4- trimethyl	Primary blowing agents used in the production of polystyrene foam and other foams for bleaching stage
9.	22.00	Octadecane, 1-iodo-	Plant sources
10.	25.68	Heneicosane	Plant sources
11.	27.82	Hexadecanoic acid, trimethylsilyl ester	Fatty acids in plant sources react other compounds make a complex structure
12.	29.02	Eicosane (CAS)	Plant materials of paraffin waxes
13.	30.70	Diethyl 3,4-dihydro-2-naphthyl-phosphonate	Chemical reactions in bleaching stages
14.	31.78	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	Fatty acids in plant sources
15.	35.88	1,2,3,4,5-Pentaisopropylbis(cyclopentadienyl) cobalticimium (Cobaltocene)	Cobaltocene is prepared by the reaction of sodium cyclopentadienide (NaC_5H_5) with anhydrous cobalt(II) clorinations chloride in THF solution.
16.	40.03	Methyl ester of [1 R-[1 à,4 á,8 à(E),8a'á]]-3-(8 -ethyl -1,2,2 ,3 ,6 ,7 ,8 ',8a'-octahydro-1-methyl-2-oxospiro[3 H-indole-3,1 (5 H)-inndolizin-8 - yl)-2-propenoic acid N-oxide	The catalytic performance of the as-synthesized catalyst series was evaluated during the transesterification of cooking palm oil with methanol to produce fatty acid methyl esters
17.	43.03	2-(1-Methyl-1 H-2-pyrrolyl)quinoline	Used for preservation as a biocides.
18.	43.79	Cyclotrisiloxane, hexamethyl- (CAS)	Plants sources
19.	45.02	5,11,17,23-Tetra-t-butyl-25,26,27,28-tetrahydroxycalix-4-arene	Wood Digestion stage complex mixture formed
20.	47.36	a-Fluoro-(p-methyl)chalcone	Precursors of flavonoids

Major of the residual organic pollutants detected in BUPMW were of residual in nature because these compounds were not degraded during the secondary treatment process and directly discharged into the environment. Initially, these pollutants have originated during the process of wood digestion, pulping stages and recycling of paper in industries of bleached and unbleached paper mill wastewater. Also, few residual organic pollutants might be generated by reactions with each other, and metabolite is formed, moreover the bio-degradation or bio-transformation of highly residual organic pollutants via microbial activity might be generated in the movement of wastewater discharge drain. The detail of residual organic pollutants which are identified through GC-MS analysis with different RT (retention time) are listed in Tables 2 and 3, their generating source with their toxicity has been also reported by different researchers in the world. However, the toxicity profile of several residual organic pollutants detected in BUPMW has not been explored until now and requires further investigation.

3.3. Phytotoxicity evaluation

The collected samples after discharged from industry were assessed for its phytotoxicity using *Phaseolus aureus* seed germination test. The result of germination as well as seedling growth of *Phaseolus aureus* seeds in different concentrations (10, 25, 50, 75 and 100%) as presented in Table 4. The seedling growth compared to controls, root lengths and shoots of seedling were highest at 10% (v/v) wastewater but the further increase of wastewater concentration showed decrease of percent seed germination and shoot, root length also. The phytotoxicity percentage (PP) was maximum at higher wastewater concentration and minimum at lower wastewater concentration. This might be associated with the presence of highly toxic residual organic pollutants, toxic metals in bleached and unbleached paper mill wastewater. The toxicity wastewater might be responsible for inhibiting the seedling growth parameters. It has been reported that the various highly toxic residual organic pollutants along with metals act as an inhibitor for several phytohormones, such as gibberellins, auxins and cytokinins, which act a crucial role in seed germination (Chandra et al. 2011b). Additionally, significant changes were observed in several physiological parameters and reveal more accurate information about the harmful effects of BUPMW in the tested seeds. The speed of germination (SG) was more reduced from lower to a maximum concentration of wastewater, which additionally provided the evidence of having more toxic compounds in bleached and unbleached paper mill wastewater. In another way, the toxic nature of BUPMW was confirmed through the seedling mortality rate (SLM), which was a higher concentration of wastewater. Moreover, mean daily germination (MDG), root shoot ratio (RSR) and seed vigor index (SVI) varied from maximum to minimum for lower to higher wastewater concentrations. The germination index (GI) was 100% at the control and diverse from lower to higher concentrations of the BUPMW sample. The treated seeds with control revealed both shoot and root growth, while seeds were treated with 100% BUPMW in different concentrations with the extremely short root (0.06 and 0.16 cm) and short shoot (0.06 and 0.36 cm). Also, the α-amylase activity recorded in germinating seeds evidently justified the toxicity of treated with BUPMW on seed germination and seedling growth parameters.

Some important physiological parameters of seedling growth and seed germinations in BUPMW were compared by Pearson's correlation coefficient at the *P < 0.05* significance level shown in Supplementary Table 2. The results indicated that there appeared to be bleached paper mill wastewater a positive and unbleached paper mill wastewater some parameters negative correlation between the lower concentration of wastewater. Mostly several parameters are significant in different concentrations of wastewater. However, a positive significant correlation in all parameters of seed germination (GP, SG, SL and RL) significant correlation was observed between at 0%, 10%, 25%, 50%, 75% and 100% different concentration of bleached mill wastewater (*P < 0.05*) and some

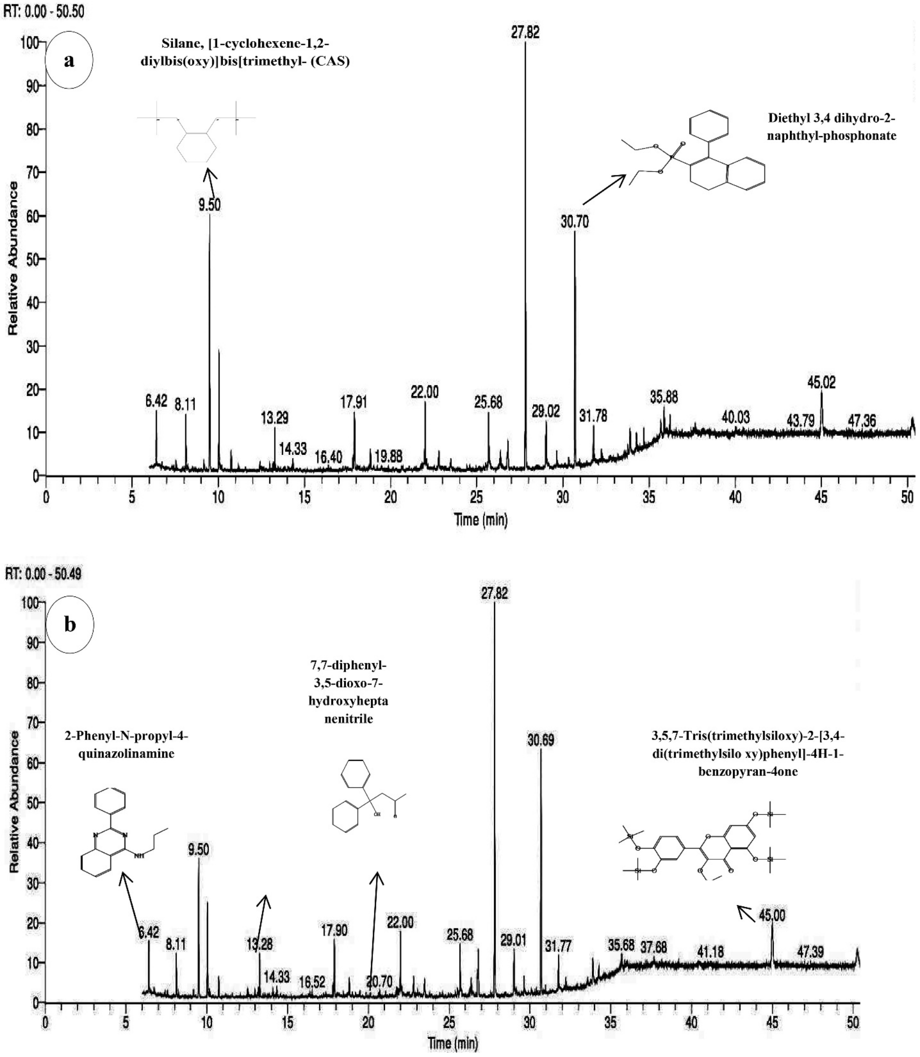

Figure 1. GC-MS chromatogram of extracted organic pollutants from (a) pulp paper mill wastewater and (b) kraft paper mill wastewater.

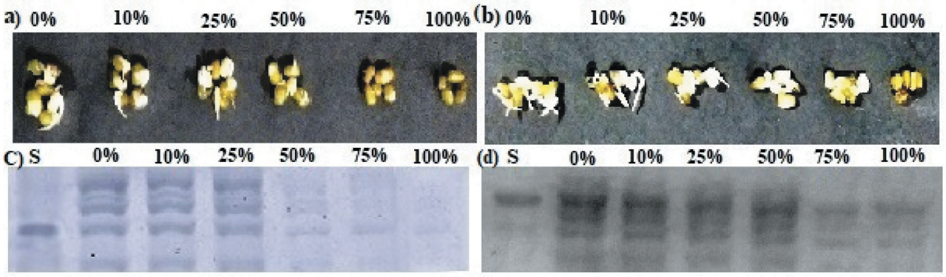

Figure 2. Showing effect of organic pollutants of paper mill wastewater on *Phaseolus aureus*. (a) effect on seeds of *Phaseolus aureus* due to pulp paper; (b) effect on seeds of *Phaseolus aureus* due to kraft paper; (c) effects of kraft paper mill wastewater on alpha amylase activity; (d) effects of kraft paper mill wastewater on alpha amylase activity; Lane S amylase standard; 0% seed treated with tap water.

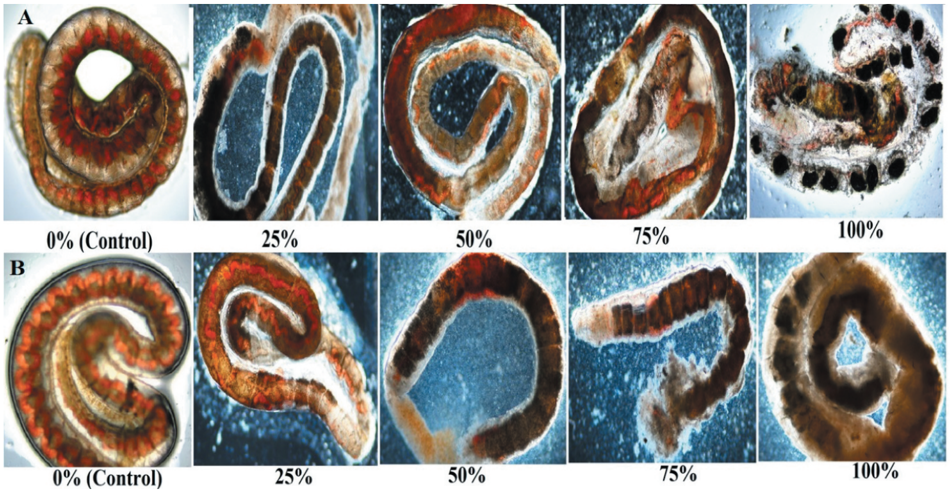

Figure 3. Showing effect of paper mill wastewater (A) (a) pulp paper mill wastewater and (b) kraft paper mill wastewater on *Tubifex tubifex* body sections in acute toxicity, Control (0%) worm in healthy conditions, treated with 25%, 50%, 75% and 100% concentration of wastewater.

negative significant correlation was observed between GP, SG, SL and RL at 0% to 10% and positive significant correlation observed between GP, SG and SL, as well as RL at 25%, 50%, 75% and 100% unbleached paper mill wastewater concentration ($P < 0.05$).

3.4. Alpha-amylase activity and SDS-PAGE

Alpha-amylase activity, as a function of seed germination, is a residual biochemical process in plants that can be affected by several environmental factors. In germinating seeds, starch is the main component of the world's crop yield hydrolyze is initiated through α-amylase (Juliano and Varner 1969) release maltose and finally, β-glucosidase breaks down maltose into glucose providing energy to germinating seeds (Bharagava and Chandra 2010).

In this study, α -amylase activity in tap water (0%) was recorded (0.203 U) and optimum α amylase activity (0.209 U) was recorded in 10% bleached paper mill wastewater and optimum α amylase activity (0.223 U) was recorded in 10% unbleached paper mill wastewater. In addition, this study is also correlated with Yadav and Chandra (2015) amylase activity (0.3) during seed germinating conditions. Afterward, a continued decline in alpha-amylase activity was observed at higher concentrations (100%) of bleached and unbleached paper mill wastewater. The results obtained at 10% (v/v) wastewater concentration were invariably better as compared to (0%) control. It seems that this concentration of wastewater acts as a liquid fertilizer. Additionally, the denaturing SDS-PAGE of α amylase enzyme extracted from germinating seeds for different concentrations of BUPMW has yielded bands of different molecular weight and intensity. Band intensity has indicated the α-amylase enzyme produced in germinating seeds in different concentrations of bleached and unbleached paper mill wastewater. Further, the molecular weight of α-amylase isoenzymes of (*Phaseolus aureus* L) determined through comparing with α-amylase standard and protein marker has indicated that the molecular weight separated by SDS-PAGE was

Table 3. Identified of residual organic pollutant by GCMS analysis in unbleached paper mill wastewater extract with dichloromethane (DCM).

S. No.	RT (min)	Identified compounds	Sources of generations
1.	6.42	2-Phenyl-N-propyl-4-quinazolinamine	Alkaloid compound mixture bacteria and other chemicals
2.	8.11	Sulfurous acid, 2-ethylhexyl hexyl ester	Recovery process
3.	9.50	7,8-Dimethyl-4 trifluoromethyl-(1 H,5)benzodiazepine	Mixture of compounds formed various stage of pulping process
4.	13.28	Nonadecane (CAS)	Plant sources
5.	14.33	Dodecane, 1-iodo-	Plant chemical
6.	16.52	3,6-Dioxa-2,7-disilaoctane,	Mixture of compounds in bleaching stages
7.	17.90	Tricosane (CAS)	Plant sources
8.	20.70	7,7-diphenyl-3,5-dioxo-7-hydroxyheptanenitrile	-
9.	22.00	Tetradecane	Plant chemical
10.	25.68	Eicosane (CAS)	Plant materials of paraffin waxes
11.	27.82	Hexadecanoic acid, trimethylsilyl ester (CAS)	Fatty acids in plant sources react other compound make a complex structure
13.	30.69	Octadecanoic acid, trimethylsilyl ester	Plant sources
14.	31.77	Eicosane (CAS)	Plant materials of paraffin waxes
15.	37.68	4-(Chloromethyl)-3-methyl-5-phenylisoxazole	Mixture of compounds in chlorination stage
16.	41.18	N(2)-[2,5-di(t-butyl)phenyl]-N(1)-ethyl-3,4:9,10-perylenetetracarboxydiimide	Digestion stage are formed mixture of compounds
17.	45.00	3,5,7-Tris(trimethylsiloxy)-2-[3,4-di(trimethylsilo xy)phenyl]-4 H-1-benzopyran-4one	Mixture of compounds in Biocide
18.	47.39	(3 R)-3-Phenyl-2,3-dihydro-1 H-isoindol-1-one	Bleaching stage mixture of compounds

Table 4. Effect of different concentrations of bleached and unbleached paper mill wastewater on physiological parameter of seed germination and α-amylase activity in *Phaseolus aureus.*

Parameters	GP (%)	GI (%)	SG (s/d)	MDG (s/d)	SL(cm)	RL(cm)	SLM (%)	RSR	SVI	PP (%)	α Amylase activity (unit grains $^{-1}$)
Bleached paper mill wastewater (%) used for seed irrigation											
0% (Tp)	98.35 ± 0.57	100	16.5 ± 0.5	3.93	2.2 ± 0.1	2.1 ± 0.1	0.34 ± 0.00	0.95 ± 0.07	845.38	0	0.203
10%	96.65 ± 0.57	97	17.55 ± 0.95	3.86	2.1 ± 0.05	2.2 ± 0.1	0.67	0.95	842.78	4.76	0.209
25%	88.3 ± 0.57	59.85	14.55 ± 3.53	3.53	1.8 ± 0.1	1.47 ± 0.11	2.33 ± 0.00	0.81 ± 0.23	577.8	30	0.152
50%	63.35 ± 1.52	22.37	9.77 ± 0.38	2.53	0.7 ± 0.52	0.73 ± 0.11	7.33 ± 0.00	1.04 ± 0.02	181.03	65.23	0.068
75%	36.65 ± 0.57	3.54	4.96 ± 0.53	1.46	0.5 ± 0.1	0.2 ± 0.1	12.67 ± 0.00	0.04 ± 0.21	51.31	90.47	0.027
100%	13.3 ± 0.57	0.38	1.8 ± 0.57	0.53	0.06 ± 0.057	0.06 ± 0.05	17.33 ± 0.00	0.00 ± 0.00	0.159	97.14	0.018
Unbleached paper mill wastewater (%) used for seed irrigation											
0% (Tp)	98.3 ± 0.57	100	16.16 ± 1.7	3.93	2.3 ± 0.1	2.06 ± 0.15	0.67 ± 0.00	0.89 ± 0.039	857.17	0	0.203
10%	96.65 ± 0.57	96.88	14.60 ± 0.58	3.86	2.26 ± 0.05	2.2 ± 0.1	0.67	0.89	829.25	1.45	0.223
25%	88.30 ± 0.57	82.99	12.61 ± 0.53	3.53	2.06 ± 0.15	1.8 ± 0.05	1.33 ± 0.00	0.87 ± 0.21	720.66	12.62	0.198
50%	76.65 ± 1.15	36.34	10.22 ± 0.25	3.06	1.16 ± 0.05	0.96 ± 0.11	4.67 ± 0.00	0.82 ± 0.07	324.99	53.39	0.137
75%	55 ± 1	11.68	6.85 ± 0.69	2.2	0.73 ± 0.05	0.43 ± 0.05	9.0 ± 0.00	0.58 ± 0.08	127.6	79.12	0.072
100%	30 ± 1	2.37	4.08 ± 0.66	1.2	0.36 ± 0.05	0.16 ± 0.05	14.0 ± 0.00	0.44 ± 0.05	31.2	92.23	0.053

All values are mean of triplicate (n = 3) ± SD.

Control (TP) tap water, *GP* germination percentage, *SG* speed of germination, *MDG* mean daily germination, *GI* germination index, *SL* shoot length, *RL* root length, *SLM* seedling mortality rate, *RSR* root shoot ratio, *SVI* seed vigor index, *PP* phytotoxicity.

approximately 50.8 and 51.5 kDa, respectively as shown in Figure 2. According to Bharagava and Chandra (2010), the α-amylase activity and molecular weight of the α-amylase enzyme were determined by SDS-PAGE approximately 47.5kDa.

3.5. *Aquatic toxicity on Tubifex Tubifex*

Tubifex worms are useful indicators of varying degrees of aquatic pollution (Auston 1973). The freshwater Tubifex worm (*Tubifex Tubifex*) is an important link in the aquatic food chain (Khangarot 1991). The BUPMW are used for the toxicity analysis in different concentration (25, 50, 75 and 100%) with a different interval of the period as presented in the table (Table 5). The whole experiment was carried out in triplicate. The toxicity was minimum at <25% of concentration up to 48-hour exposure and maximum after >50% concentration of wastewater at 48 hours of exposure. The wastewater contains different mutagenic and carcinogenic residual organic pollutants which are described in Tables 2 and 3. Due to the presence of these pollutants in wastewater, there was enhanced the mortality rate of *Tubifex*, physical damage, bursting of cell and whole-cell damage of *Tubifex* worms. Hence, these organo-chlorinated pollutants are highly toxic to the benthic ecosystem. While in control, Tubifex worms remained active during the test period. They were clustered at the bottom of the test container and showed typical *Tubifex* movement. This might be associated with the presence of highly toxic residual organic pollutants and toxic metals in BUPMW causes the toxicity of worm (Figure 3). This study revealed the bleached paper mill wastewater is highly toxic than unbleached paper mill wastewater shown in Table 5. At the lethal concentrations of wastewater after 24 h of exposure, the hemoglobin content less and the rear part of the body became white, with the disintegration of the body observed. In general, disintegration starts from the rear part of the body and advances towards the front part.

4. Conclusion

Discharged wastewater from BUPMW after secondary treatment retains various organic pollutants that are hazardous to the aquatic ecosystem. Some of the detected organic pollutants were found with mutagenic, carcinogenic and endocrine-

Table 5. Toxicity on Tubifex worm against bleached and unbleached paper mill wastewater.

Concentration/ Time (hrs)	0 h	24 h	48 h	72 h	96 h	Experiment type	Measurement	Effects	LC50
Bleached paper mill wastewater % toxicity									
0%	19.66 ± 0.57	19.33 ± 1.15	18.66 ± 1.52	19 ± 1	17.66 ± 2.08	Static	Mortality	No change	Non-Toxic
25%	19.33 ± 1.15	17.33 ± 1.15	10.66 ± 2.08	8.33 ± 2.08	1.33 ± 1.5	Static	Mortality	Physical damage	Slightly Toxic
50%	18 ± 1	15.66 ± 1.52	9 ± 2	6.66 ± 1.52	0.66 ± 0.57	Static	Mortality	Break down of cell membrane	Moderately Toxic
75%	17.66 ± 1.52	12.66 ± 1.52	7 ± 2	4 ± 2	0 ± 00	Static	Mortality	Cell damage	Highly Toxic
100%	16.33 ± 2.08	10.66 ± 2.08	4 ± 4.35	1.33 ± 1.52	0 ± 00	Static	Mortality	Burst and cell damage	Extremely Toxic
Unbleached paper mill wastewater % toxicity									
0%	19.33 ± 0.57	18.33 ± 1.52	17.66 ± 2.08	17.33 ± 2.30	15.66 ± 3.78	Static	Mortality	No change	Non-Toxic
25%	19 ± 1	18 ± 1.73	14.33 ± 1.52	10.66 ± 2.08	3.33 ± 2.51	Static	Mortality	Physical damage	Slightly Toxic
50%	18.66 ± 1.52	16.33 ± 2.08	13.66 ± 1.52	8.66 ± 2.08	2.33 ± 2.08	Static	Mortality	Break down of cell membrane	Moderately Toxic
75%	18 ± 1	12 ± 3.60	11.33 ± 1.52	6.66 ± 2.08	1 ± 1	Static	Mortality	Cell damage	Moderately Toxic
100%	17.66 ± 1.52	11.33 ± 4.04	8.66 ± 2.08	4.66 ± 2.08	1 ± 1	Static	Mortality	Cell damage	Highly Toxic

disrupting chemical (EDCs) properties. The wastewater discharged from the bleached paper mill was found more toxic than unbleached paper mill wastewater due to the chemical pulping and bleaching process. The various chemical used in paper manufacturing process make the residual and do not completely degrade. The toxicity test with *Phaseolus aureus* and *Tubifex tubifex* given strong evidence that discharged wastewater is inhibited to enzyme responsible for seed germination and adversely affect aquatic life also. Therefore, wastewater prior to discharge in the environment should be detoxified at tertiary stage treatment.

Acknowledgements

This work was supported from the Department of Biotechnology New Delhi, Letter No. BT/PR18896/BCE/8/1372/2016 dated 28-03-2018, University Grant Commission, New Delhi, to Mr. Adarsh Kumar is also highly acknowledged and NFPwD (F No.01-01/2019-Sch.) fellowship to Mr. Ajay Kumar.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Department of Biotechnology New Delhi [BT/PR18896/BCE/8/1372/2016]; Ajay Kumar Singh [NFPwD (F No.01-01/2019-Sch.)]; Adarsh Kumar [NON NET Fellowship, University Grant Commission Ne].

ORCID

Adarsh Kumar http://orcid.org/0000-0002-3350-151X
Ajay Kumar Singh http://orcid.org/0000-0002-6716-331X
Ram Chandra http://orcid.org/0000-0001-7070-6074

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Catalysis Letters
https://doi.org/10.1007/s10562-021-03753-y

Sustainable Production of Thermostable Laccase from Agro-Residues Waste by *Bacillus aquimaris* AKRC02

Adarsh Kumar[1] · **Ajay Kumar Singh**[1] · **Muhammad Bilal**[2] · **Ram Chandra**[1]

Received: 24 May 2021 / Accepted: 20 July 2021

Abstract

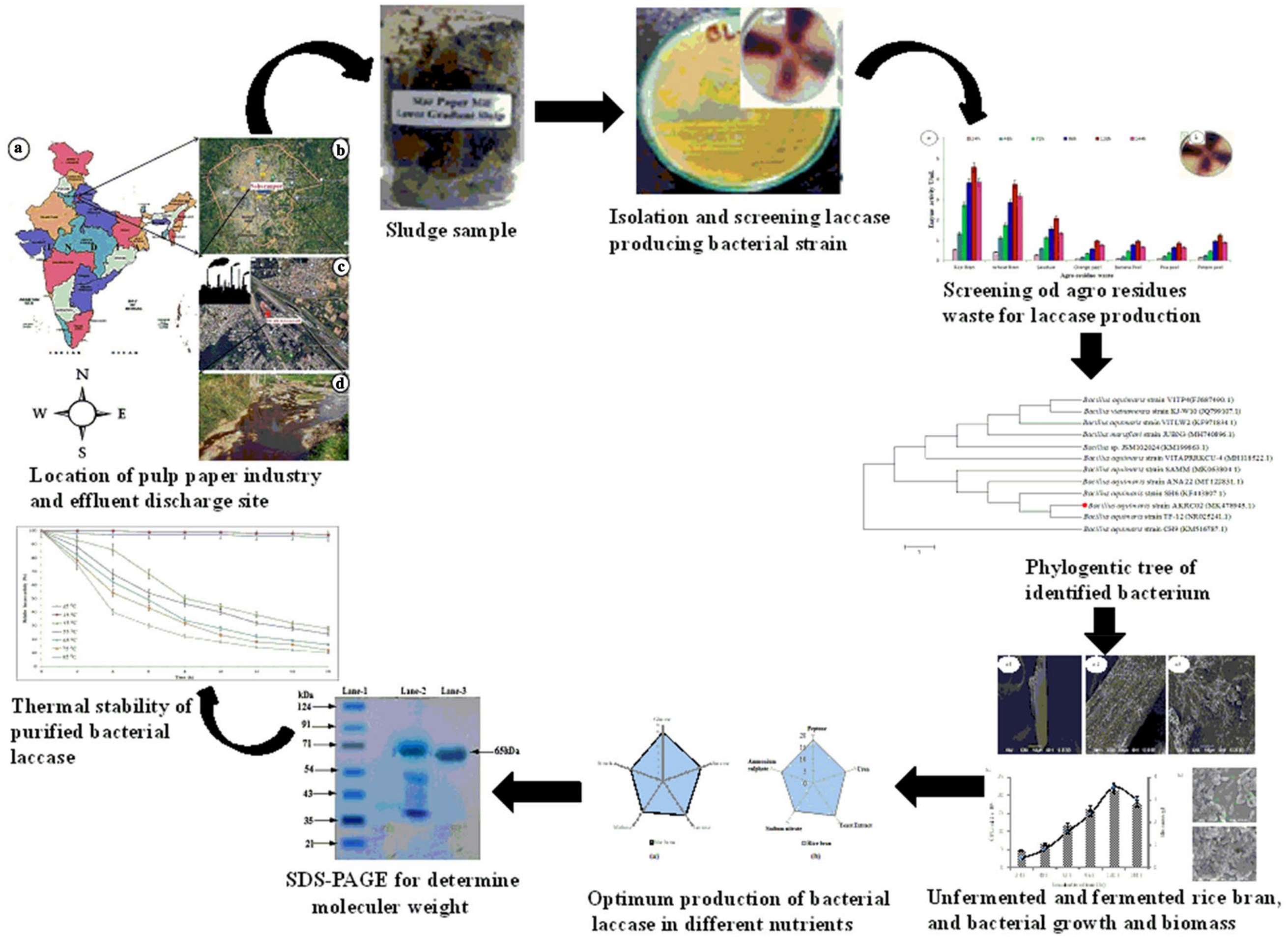
Laccase is a versatile enzyme that plays a major role in the remediation of various environmental pollutants. In this work, a thermo-tolerant halophilic *Bacillus aquimaris* AKRC02 was isolated from pulp and paper mill waste sludge for efficient laccase production. Various agro-industrial waste residues, including potato peel, banana peel, sawdust, pea peel, wheat bran, orange peel, and rice bran, were screened to produce laccase using a submerged fermentation process. Among these, rice bran supported the maximum laccase production (4.58 U/mL). The optimized environmental conditions (incubation time 120 h; 4.58 U/mL), 35 ^{0}C; 6.624 U/mL) and pH 7.0; 10.142 U/mL) and nutritional sources (glucose 1.0%; 14.164 U/mL and peptone 0.5%; 18.124 U/mL) significantly enhanced the laccase production. Purified laccase showed a specific activity and purification fold of 228.34 U/mg and 38.08, respectively. The purified enzyme showed a molecular weight of 65 kDa and high thermal stability at 45 ^{0}C for 8 h. In conclusion, the remarkable properties of the newly isolated bacterium may provide a significant opportunity for degrading environmental contaminants, making it an attractive biocatalyst for industrial applications.

✉ Ram Chandra
prof.chandrabbau@gmail.com

[1] Department of Environmental Microbiology, School of Earth & Environmental Sciences, Babasaheb Bhimrao Ambedkar (A Central) University, Vidya Vihar, Raebareli Road, Lucknow, Uttar Pradesh 226025, India

[2] School of Life Science and Food Engineering, Huaiyin Institute of Technology, Huai'an 223003, China

Graphic Abstract



Keywords Laccase · *Bacillus aquimaris* AKRC02 · Agro-residues waste · Process parameters · Optimization · Stability

Abbreviations

RSM	Response surface methodology
CCD	Central composite design
SmF	Submerged fermentation
3D	Three dimensional
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
MSM	Minimal salt media
ARW	Agro-residues wastes
SEM	Scanning electron microscope
MBSS	Mineral Basal Salt Solution

1 Introduction

Laccase is a copper-containing enzyme, which consists of monomeric, dimeric, and tetrameric glycoproteins. In recent years, laccase has gained significant attention due to its broad-spectrum applications in the food industry, distillery, textile, and pulp paper industry [1]. This enzyme belongs to the oxidoreductase family and is widely distributed in archaebacteria, bacteria, fungi, and plants [2]. Laccase can catalyze the degradation of both phenolic and non-phenolic compounds along with recalcitrant lignocellulosic waste materials [1]. It also plays a crucial role in detoxifying other environmental pollutants, i.e., xenobiotics compounds, dyes degradation, biobleaching of pulp, biosensors, and biofuel production [3]. The majority of extremophilic laccases for industrial applications are found from bacterial origin [4]. For large-scale laccase production, two approaches have been adapted (i) enzymes immobilization on solid supports with reuse properties [5], and (ii) enzyme productions using cheap substrates [6–8]. Laccase production has been reported in several bacteria, such as *A. lipoferum*, *B. pumilus*, *P. putida, P. desmolyticum* NCIM 2112, *B. halodurans*, *Bacillus* sp. HR03, *B. subtilis* WP1, *B. subtilis*

MTCC 2414, and fungi, *Termetes versicolor*, *Pleurotous ostreatous*, *P. chrysosporium* [9–12]. Very little literature is available on the potential effects of bacterial laccases in applying in the bioremediation field. White-rot fungus is more attractive because of its high production rate of laccase enzymes, but they require a solid-state fermentation (SSF) process [11]. However, bacteria are more stable at wider pH values (3.0–9.0), tolerate a broader range of habitats (temperatures 30–85 ^{0}C), and grow faster than the fungal system [13]. Some potential bacteria have shown the maximum laccase production in submerged conditions due to their growth suitability and proper nutrient availability. Therefore, the selection of bacterial strains for laccase production in the submerged fermentation (SmF) process using a cheap substrate is of supreme interest for large-scale applications. Current research mainly focuses on developing sustainable, economically valuable technology for laccase production in SmF technique due to efficient oxygen and nutrients availability of the culture medium. This fermentation mode has many advantages in controlling various process parameters and scaling up product purity and easiness processes. This is usually preferred for large-scale industrial fermentation for the biosynthesis of enzymes, amino acids, antibiotics, organic acids, baker's, and distiller's yeast [14].

Optimization of different process parameters has been reported using the conventional laccase production method [15]. Further, the environmental and nutritional composition of the medium, incubation period, pH, temperature, carbon (C), and nitrogen (N) source also plays a significant role in laccase production [16, 17]. It is regarded as the most suitable and effective approach for optimizing the multiple combinations of variables for maximum laccase production by response surface methodology with central composite design (RSM-CCD). Production of bacterial laccase in a commercial manner is a prime necessity; therefore, the agricultural residue waste may be a promising option as a substrate for laccase production due to rich sources of carbon, nitrogen, and phosphorus [18, 19]. In India produces approximately 600 metric tons of waste from various agricultural sources [20]. A considerable amount of agricultural waste is generated from different sources, i.e. wheat straw, the husk of coconut waste, sugarcane industry, vegetable waste, oil industry waste, and food products [21, 22]. These wastes are causing serious environmental pollution due to a lack of proper management strategies. The screening of potential bacteria for laccase production using agricultural waste would be a cheap alternative nutrient source [8, 23, 24]. Recently, the production of laccase enzymes using agro-residue waste has received great significance because of their applications in the degradation and reuse of waste, minimizing the disposal problems [22, 24–27].

The present work evaluated the screening and identification of laccase-producing bacteria from paper mill sludge. Suitable agro residue wastes were screened for laccase production under SmF. Different environmental and nutritional parameters were standardized by CCD-RSM for the maximum laccase production. The laccase enzyme was subjected to partial purification by chromatography using Sephadex column (G-100), and molecular weight was determined through SDS-PAGE as well as investigated the enzyme's thermostability.

2 Materials and Methods

2.1 Sampling Site

The sample was collected from the M/S Star paper mill, a large contaminated site located in Saharanpur (29.97°N, 77.55°E), Uttar Pradesh, India (Fig. 1). This paper mill uses agro byproducts such as "bagasse" as feedstock. Bagasse is a fibrous material, such as wheat straws and rice straws, and supplement wood for paper production purposes. This industry has a manufacturing capacity of 75,000 tons per annum of different writing types, printing paper, and paperboard products. It discharges 406.0 m3/day (metric ton per day) of sludge and 1700 m^3/day of effluent into the environment as soil pollutants, which ultimately reaches to mix Hindon river, a tributary of Yamuna River. To isolate potential strains from the acclimatized environment, the sludge sample was collected from the disposal site adjacent to the industry in 10 kg sterile plastic bags (Tarsons Production Pvt., USA). The collected samples were preserved in an icebox and transported to the lab for microbiological study.

2.2 Isolation and Screening of Laccase Producing Bacteria in Different Substrate

The bacterial strains were isolated from 10 g of sludge sample and transferred to an Erlenmeyer flask (250 mL) containing 100 mL minimal salt media (MSM) [28]. The flask was incubated in a temperature controller incubator shaker (Orbitek, Scigenic Biotech, India) at 37 ± 1 ^{0}C with agitation 120 rpm for 7 days. Subsequently, the serial dilution was performed in which 1.0 mL of sample was taken, diluted (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}), and then 0.1 mL diluted sample was spread out in the MSM agar plate medium. The streak plate technique was used for the isolation of pure bacterial culture. The laccase activity of bacterial strain was revealed due to the existence of guaiacol (4 mM) (Sigma, USA) in B and K agar medium containing dextrose 1.0%, peptone 0.5%, NaCl 0.5%, beef extract 0.3%, agar 1.5% and $CuSO_4$ (1 mM) [29, 30]. Another substrate was used for laccase screening, i.e., gallic acid 0.1%, tannic acid 0.5%, and catechol 0.1% as an indicator compound, and incubated at 37 ^{0}C for 3 days.

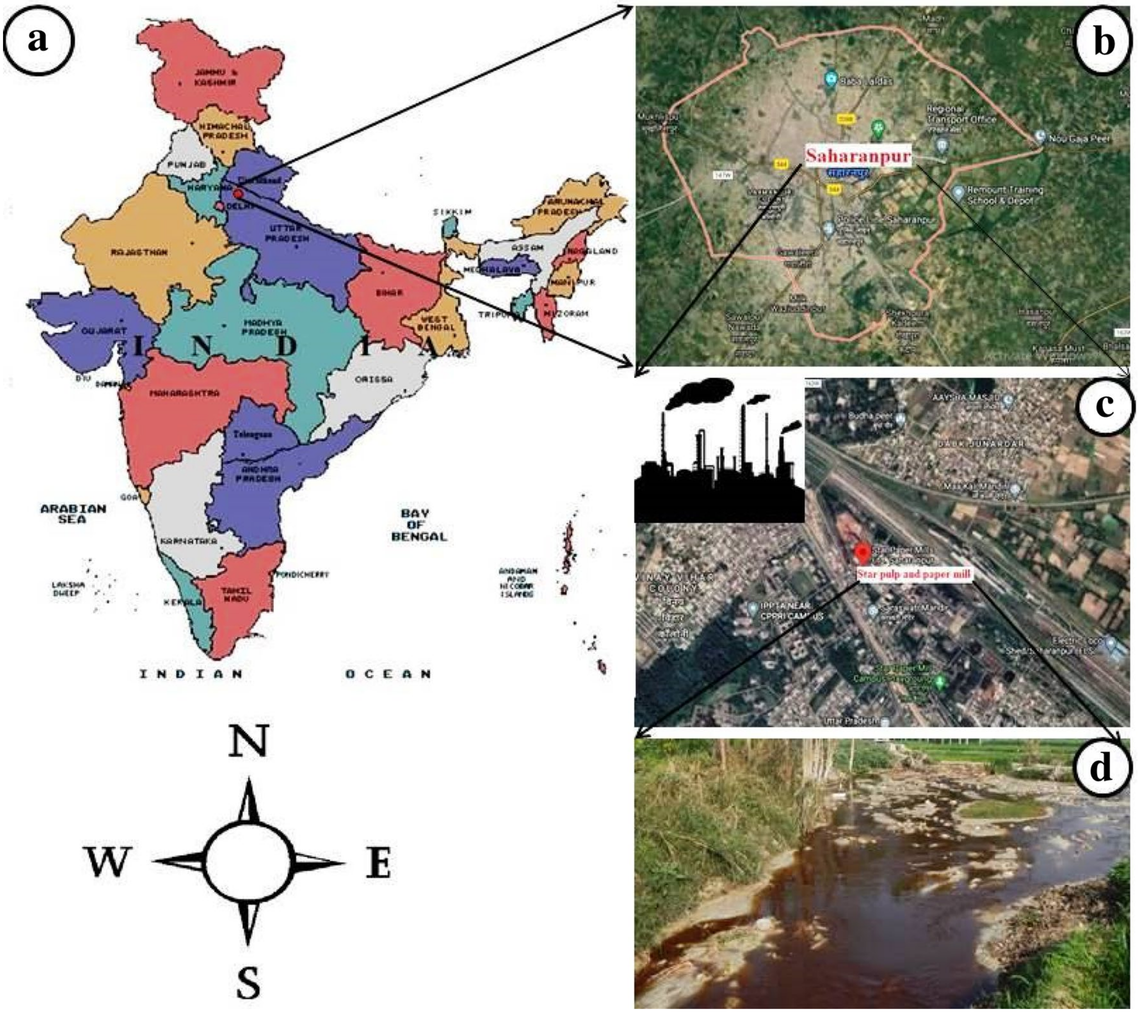


Fig. 1 View of location map of Star paper mill Ltd. Saharanpur, Uttar Pradesh, India **(a, b** and **c),** and **(d)** sampling site

2.3 Identification of Bacterial Strain

2.3.1 Morphological and Biochemical Characterization

The characterization of screened laccase-producing bacterial strain was performed by Bergey's Manual of Systematic Bacteriology [31]. Biochemical parameters, *i.e.* gram staining, shape, motile bacterium, catalase, Voges-Proskauer (VP), citrate, ortho-nitrophenyl-β-galactoside (ONPG), malonate, nitrate reduction, and carbohydrate utilization were performed as described in Crowan and Steels, manual for identifying medical bacteria [32].

2.3.2 16S rRNA Sequence for Identification

The isolated potential laccase-producing bacterium was identified through 16Sr RNA gene sequence analysis. Genomic DNA was extracted from the overnight grown culture of isolated strain, according to Kapley et al. [33]. The bacterial DNA was purified and extracted by an extraction kit (Bioneer, Korea). The quality of bacterial DNA was evaluated through an agarose gel (1.0%) with a single band and high molecular weight. The amplification process was performed for 16S rDNA fragment through polymerase chain reaction (PCR) with primer 27F & 1492 R. After all observed, PCR amplicon on agarose gel was 1 kb with ethidium bromide ($C_{21}H_{20}BrN_3$) visualization. In addition, PCR cleanup kits were used with gel to purify 16S rDNA gene amplified sequence and automated DNA sequencer of eubacteria with primers 5'-TACGGYTACCTTGTACGACTTT–3' and 5'-AGAGTTTGATCMTGCTCAG-3'. Finally, the phylogenetic tree was created by MEGA software (version 6.0) with the neighbour-joining technique [34].

2.4 Chemical Analysis of Agro-Residues Waste

Agro-residues wastes (ARW), i.e. potato peel, banana peel, sawdust, pea peel, wheat bran, orange peel, and rice bran, were collected from the local area around Lucknow. All these ARW were washed three times with distilled water, boiled up to 15 min to remove dust particles, and dried in an oven at 60 ^{0}C. After drying, this ARW were milled using a domestic mixer grinder (Maharaja Mx-116A), and the screened (40 µm mesh) ARW powder was stored at 4 ^{0}C. ARW chemical composition, i.e. lignin acid detergent fiber (ADF), hemicellulose, neutral detergent fiber (NDF), cellulose, ash content, and carbohydrates were used for other natural substrates as per the O'Dwyer, [35]; and Norman and Jenkins, [36]. The effects of substrate concentrations (1–4% w/v) on the production of laccases were also evaluated.

2.5 Laccase Assay

Laccase activity was determined as described earlier by Arora et al. [37]. In reaction mixtures containing 3.8 mL acetate buffer (50 mM, pH 4.6) and 1 mL guaiacol (5 mM), 0.2 mL of the crude enzyme was added and incubated at room temperature for 2 h. The reaction was observed by measuring the absorbance at 465 nm using a UV–Vis spectrophotometer (Thermo science evolution-201). The total enzyme activity was calculated as the quantity of oxidized one micromole guaiacol (substrate) per minute at room temperature. Every experiment was conducted twice with guaiacol (extinction coefficient = 12,000 $\text{mM}^{-1}\ \text{cm}^{-1}$) [38], and the activity of laccase was calculated in following equation:

$$\text{U/mL} = \frac{\Delta \text{A}_{465/\text{min}} \times 4 \times \text{V}_\text{t} \times \text{dilution factor}}{\varepsilon \times \text{V}_\text{s}}$$

where,

$\Delta A.\text{min}^{-1}$ = Absorbance change per minute at 465 nm.

4 = Derived from unit definition and principle.

Vt = Final volume of reaction mixture (mL).

ε = Extinction coefficient of guaiacol $\text{mM}^{-1}\ \text{cm}^{-1}$.

Vs = Enzyme volume (mL).

2.6 Screening of the ARW for Laccase Production under SmF Condition

The BL-9 (*Bacillus aquimaris* AKRC02) bacterial strain was selected to enhance the laccase production using different ARW. Two grams of each ARW were taken in separate flasks and mixed with 100 mL of mineral basal salt solution (MBSS). The composition of MBSS was in gL^{-1} dextrose 10.0, peptone 3.0, K_2HPO_4 0.4, $ZnSO_4$ 0.01, $MnSO_4$ 0.5, KH_2PO_4 0.6, $FeSO_4$ 0.0005, and $MnSO_4$ 0.5. Precisely, 2.76×10^8 CFU of *Bacillus aquimaris* AKRC02 was inoculated in the sterilized flask and incubated at 37 ^{0}C for 144 h. The bacterial culture was taken continuously for 144 h at 24 h intervals and centrifuged at 10,000 rpm for 10 min at 4 ^{0}C, and the resulting cell-free supernatant obtained was used for enzyme assay by taking optical density (OD) at 465 nm to calculate enzyme activity [39].

2.7 Delignifiying Activity of Rice Bran

Exactly, two-gram of rice bran fermented for 120 h with *Bacillus aquimaris* AKRC02 was air-dried, and the fermented rice bran particles were observed under a scanning electron microscope (SEM) to evaluate modifications of the surface structure of rice bran. SEM analysis of unfermented rice bran was also observed and used as a control sample [40]. The remaining fermented and unfermented rice bran was sterilized and re-inoculated with *Bacillus aquimaris* AKRC02 and monitored for laccase production.

2.8 SEM Analysis of the Bacterial Growth during Enzyme Optimization Conditions

During the enzyme optimization, the optical density for bacterial growth was measured continuously for 144 h at 24 h intervals with a UV–Vis spectrophotometer at 620 nm. The bacterial biomass was collected after centrifugation at 6000 g for 10 min and fixed with 2.0% glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.5) overnight [41]. After fixation, the bacterial cells were again fixed with osmium tetra-oxide (Sigma Aldrich, USA) to dissolved in 50 mM sodium phosphate buffer (pH 6.5) for 30 min and dehydrated with ethanol from 30 to 100% (10 min at every concentration) [41]. The dehydrated bacterial cells were coated with a thin layer of gold using ion sputter (CoaterIB-2, Gike Engineering, Japan) and analyzed for morphological characterization under SEM parameters such as voltage 10kv, magnification X10,000 and beam 1125 SEI (model: JSM-6490LV, Make: JEOL, Japan).

2.9 Statistical Optimization of Laccase Production

Statistical experiment designs are important tools for the rapid analysis of key factors from a multivariable system. For the interactive approach of variables on response using a response surface methodology (RSM), the statistical design is an ideal model for designing experiments [42]. The conventional one-factor time approach (OFTA) fails to explain the interactions between multiple factors. Optimization has been done by response surface methodology with central composite design (RSM-CCD) were carried out by the non-conventional OFTA approaches [17]. It is a suitable and effective approach for optimizing the multiple combinations of variables for maximum laccase activity. SmF process is generally suitable for the bacterial system due to proper oxygen circulation and nutrients availability of the culture medium. The selected ARW, i.e., rice bran (two grams) was used to attain a high yield of laccase production at optimized environmental conditions (incubation period, pH, and temperature) and different nutrients, i.e., carbon (glucose, sucrose, starch, maltose, lactose) and nitrogen sources (peptone, urea, yeast extract, sodium nitrate, and ammonium sulfate) were used to enhance the enzyme activity. The impact of each variable on the production of the enzyme has been studied in five different concentrations: -α, -1, 0, + 1, + α. The separated 20 sets of studies on rice bran were also conducted in triplicates [43]. All variables were considered to be zero at a core coded value. An analysis of variance (ANOVA) was performed using Design-Expert software-11 (student version) (Stat-Ease, Minneapolis, MN, USA) to analyze the data obtained from RSM on the laccase production. RSM findings were used to match a polynomial second-order Eq. 1.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1\beta_1 A^2 + \beta_2\beta_2 B^2 + \beta_3\beta_3 C_2 + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_2\beta_3 BC \tag{1}$$

where Y is the variable, β_0 intercept coefficients, β_1, β_2, β_3 linear coefficients, $\beta_{1},_{1}$, $\beta\ _{2,2}$, $\beta\ _{3,3}$ square coefficients, $\beta\ _{1,2}$, $\beta\ _{1,3,}$ $\beta\ _{2,3}$ interactive coefficients, and A, B, C is the linear effect, A_2, B_2, C_2 square effect and AB, AC, BC autonomous interactive coefficients.

2.10 Purification and Determination of Molecular Weight

The isolated bacterial strain was grown in a liquid medium under optimized conditions and centrifuged at 10,000 g for 20 min at 4 ^{0}C for cell elimination. The cell-free supernatant was concentrated by 80% ammonium sulfate precipitation, resuspended in 50 mM sodium phosphate buffer (pH 8.0), and dialyzed again in the same buffer for 24 h [43–46]. The partially purified enzyme was applied to ion-exchange chromatography on a column of Q-Sepharose FF (100 u16 mm; Amersham Biosciences) that was pre-equilibrated with the same buffer [47]. A gradient of 0.4 M NaCl in 0.15 mM tris buffer (pH 8.0) was run for 7 min to the elution; the gradient was then increased for 5 min to 100% at a 1.0 mL/min flow rate. The purified enzyme fractions were loaded and analyzed by SDS-PAGE (10%) and run at 120 V [45]. After running, the samples were stained with coomassie brilliant blue R 230, and protein bands were visualized under the gel documentation system (GeNei™ UVITEC Cambridge).

2.11 Stability of Laccase Enzyme in Different Temperature

Laccase was incubated in various temperature ranges (25, 35, 45, 55, 65, 75, and 85 ^{0}C) for different time intervals (0, 2, 4, 6, 8, 10, 12, 14, and 16 h) to determine the thermal stability. The residual laccase activity was calculated by evaluating the oxidation of guaiacol at 100 mM acetate buffer pH 5.0 at 465 nm [48].

2.12 Statistical Data Analysis

All the experimental results were presented as the average of three independent observations. ANOVA was carried out using Design-Expert software-11 (student version) (Stat-Ease, Minneapolis, MN, USA).

3 Results and Discussion

3.1 Isolation and Screening of Laccase Producing Bacterial Strains

The 18 isolated bacterial strains were purified on the MSM agar plate by the streak plate technique. All bacterial strains were screened for laccase production on modified B and K agar medium containing 5 mM guaiacol. The six bacterial strains (i.e. BL1, BL6, BL9, BL12, BL15, and B16) demonstrated the laccase activity as shown in Table 1. Among the isolated bacterial strain, BL9 showed the highest laccase production, resulting in the reddish-brown colour on the medium around the culture growth (Fig. 3b). This indicated the production of extracellular laccase enzyme, resulting in the oxidation of 2 methoxyphenol (Guaiacol). The oxidations of guaiacol by laccase have also been reported from fungal sources, but the laccase production from bacterial sources is limited [49]. In addition, the isolated strain was found to capable of oxidation of the other polyphenolic compounds, i.e., tannic acid ($C_{76}H_{52}O_{46}$), gallic acid ($C_6H_2(OH)_3COOH$), and catechol ($C_6H_4(OH)_2$) (Table 1). The appearance of brown and yellow colour was observed in the growth medium due to the oxidation of these substrates

Table 1 Qualitative screening of the laccase activity in different substrate

Bacterial strains	Substrates			
	Guaiacol	Catechol	Gallic acid	Tannic acid
BL-1	+ + +	+	—	—
BL-6	+ + +	+	+ +	—
BL-9	+ + +	+ +	+ + +	+ +
BL-12	+ +	+	+	—
BL-15	+	—	+	—
BL-16	+ +	+ +	+	—

Note: + + + highly production, + + medium production, + less production,—no production that indicated the broad range substrate utilization of isolated strain for degradation of phenolic compounds present in the lignocellulosic waste as environmental pollutants. The degradation of tannic and gallic acid by laccase has been reported from fungal sources [50, 51]. Similarly, the isolated strain also converted the catechol in the growth medium into a yellow colour that may be due to ortho or meta cleavage [52]. Two different metabolic routes for the aerobic breakdown of catechol are now well established in bacteria. The meta cleavage route for the degradation of catechol has been studied in *Pseudomonas* species, and two different pathways have been reported [53]. In some bacterial strains, oxidized catechol undergoes intra-diol fission by a catechol 1,2 oxygenase leading to the formation of cis & muconic acid (ortho cleavage), whereas other bacteria possess an extra-diol oxygenase which produces 2-hydroxy-muconic semialdehyde (meta cleavage). Ornston and Stanier [53] have fully characterized the enzymatic steps that convert catechol through cis, cis muconate and 2-oxoadipate into succinate as well as acetyl-CoA (ortho pathway or 2-oxoadipate pathway).

3.2 Biochemical and Identification Characterization of Bacteria

Based on laccase producing potential bacterial strain *Bacillus aquimaris* AKRC02 is characterized by morphological and biochemical tests, such as Gram-positive, rod-shaped, motile bacterium, and salt tolerant. The isolated strain was capable of utilizing a broad range of carbon compounds, including sucrose, mannitol, glucose, arabinose, trehalose, malonate, Voges-Proskauer (VP), nitrate reduction, and catalase. Citrate, ortho-nitrophenyl-β-galactoside (ONPG), and arginine were not hydrolyzed as shown in Table 2. In a previous report, aesculin, hypoxanthine, tyrosine, and xanthine were not hydrolyzed [54].

The bacterial strain was selected based on enzyme production in the comparative screening with the different substrates of isolated strains (Table 1). The 16S rRNA sequence of isolates showed 99.6% similarity with *Bacillus aquimaris* strain TF-12 (NR025241.1). Based on NCBI blast data of nucleotide sequences, the isolate was identified as *Bacillus aquimaris* AKRC02 (MK478945.1), as shown in Fig. 2. *Bacillus aquimaris* AKRC02 has been primarily isolated from seacoast marine habitats as a halotolerant [54]. It has demonstrated a broad range of adaptions towards various pollutants in different habitats [55]. This bacterium has also been isolated from the shrimp gut responsible for carotenoid pigment production [56], which might be indicated the broad range of adaptions for protein and cellulose digestion. Therefore, our isolate from pulp and paper waste showed a related characterization due to the abundant presence of various salts, i.e., sodium hydroxide (NaOH), CaO, $Ca(OH)_2$, sodium sulfide (Na_2S), and $CaCO_3$, etc. [29, 57]. Thus, this indicated the direct involvement of isolated strain for bioremediation of pulp and paper mill waste containing lignin with various salt and metallic compounds [58, 59].

3.3 Chemical Composition and Screening of Different ARW for Laccase Production

Selected ARW were screened for the optimum laccase production in the SmF process. The chemical compositions of different ARW are shown in Table 3. In the current study, (2.0%) selected ARW (i.e. rice bran, wheat bran, sawdust,

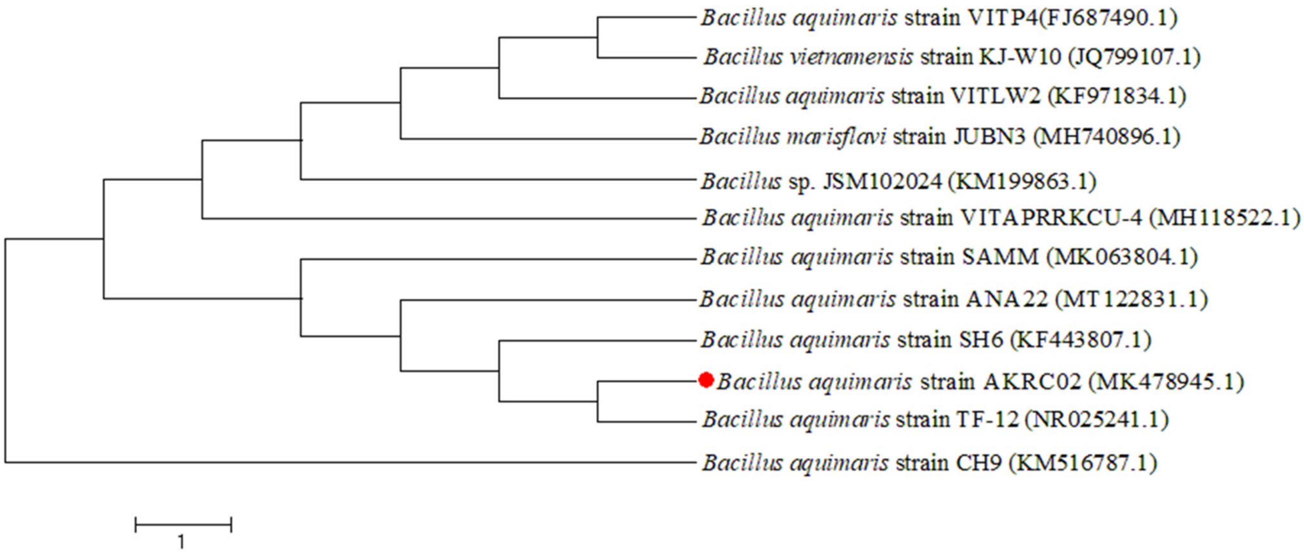


Fig. 2 Phylogenetic tree of the potential laccase producing *Bacillus aquimaris* strain AKRC02 and their related genera linked based on 16S rDNA sequences. The tree was generated using Neighbour-Joining method and scale bar shows 1% sequence divergence

Table 2 Biochemical characterization of isolated bacterial strains using Hi-Media Rapid Biochemical Identification kit

Test parameters	Results
Shape	Rods
Gram reaction	+ ve
Malonate	+
Voges-Proskauer	+
Citrate	−
ONPG	−
Nitrate reduction	+
Catalase	+
Arginine	−
Sucrose	+
Mannitol	+
Glucose	+
Arabinose	+
Trehalose	+
NaCl (3.0%)	+

Note: + Positive; − Negative

orange peel, banana peel, pea peel, and potato peel) was used with MBSS media for laccase production in laboratory conditions. Each flask culture media was maintained at pH 7.0 and incubated for 144 h to record enzyme activity. Results revealed that rice bran furnished the maximum enzyme activity (4.58 U/mL) followed by wheat bran (3.74 U/mL), sawdust (2.07 U/mL) orange peel (0.96 U/mL), banana peel (0.94 U/mL), pea peel (0.85 U/mL) and potato peel (1.24 U/mL). Among, the all the selected ARW such as rice bran was found the most effective for laccase production (Fig. 3). The production of extracellular laccase by *Bacillus aquimaris* AKRC02 using agro-industrial waste is a robust biotechnological approach. These substrates are cheap and easily available as agro-residue for enzyme production. Various researchers have harnessed the potential of ARW for laccase and other ligninolytic enzyme production through fungal sources [17, 60]. However, the use of ARW to produce the ligninolytic enzyme through bacteria is limited. Moreover, *Bacillus aquimaris* AKRC02 growth on lignocellulosic waste indicated its notable contribution to pulp paper mill waste bioremediation. Utilizing agro-residues as a substrate of bacterial laccase production would be a new way for waste minimization in the environment contaminations. The maximum production of laccase by *Pseudomonas putida* (4.25 U/mL) has been reported in the presence of guaiacol broth after incubation up to 108–120 h in pH 7.0 and at 27 ^{0}C [44]. In comparison to the previous report, the isolated strain showed more potential for laccase production in the presence of an inexpensive source of nutrients.

3.4 Delignifiying Activity of Rice Bran

The production of laccase by *Bacillus aquimaris* AKRC02 in the presence of rice bran resulted in delignification/distortion of the cell wall layers. Several structural alterations were observed in rice bran during the fermentation with *Bacillus aquimaris* AKRC02. The peeling appearance observed on the fermented rice bran was noted at 48 and 120 h incubation (Figs. 4a2 and a3) compared to the unfermented rice bran (Fig. 4a1). Experimental results suggested strong evidence of delignification activity in the fibers that changed the surface structure and became destroyed as well as modified (Figs. 4a2, a3). The utilization of lignin as a carbon source by bacterium has been reported for laccase production in previous studies [61]. This showed the surface structural change as supporting evidence for lignin utilization by *Streptomyces psammoticus.* Our observation of destruction and modification of the fibrous structure corroborated the lignin utilization. Enhanced porosity further exposes the cellulosic portion for efficient hydrolysis. Several bacterial species have been reported, such as *Enterobacter* sp. for the hydrolysis of different agricultural lignocellulosic substrates, which can produce multiple cellulolytic enzymes [62]. The SEM images of biologically treated samples of *A. mangium* wood chips with *P. coccineus*, *Phellinus sp*. *Daedalea sp*. and *T. versicolor* showed the degradation mechanism of lignin due to the activities of fungal-derived ligninolytic enzymes [63]. Previously, various researchers have reported that lignin present in the lignocellulosic materials acts as a barrier to hydrolytic enzymes like cellulases

Table 3 Selection of natural substrate and their chemical compositions

Natural substrates	Composition analysis (%)						
	Cellulose	Hemicellulose	Lignin	Carbohydrate	NDF	ADF	Ash
Rice bran	33.43	20.99	18.25	45.90	61.12	45.80	5.64
Wheat bran	27.21	13.29	10.64	48.10	49.39	36.10	4.28
Saw dust	22.31	10.20	11.76	22.30	35.80	25.60	3.46
Banana peel	25.80	12.17	8.21	51.84	40.37	28.20	5.31
Orange peel	13.60	20.46	8.00	12.00	38.28	18.32	5.18
Pea peel	62.30	8.20	3.50	2.60	74.00	65.80	4.80
Potato peel	2.50	34.70	3.80	64.47	41.00	6.30	6.34

NDF: neutral detergent fiber; ADF: acid detergent fiber

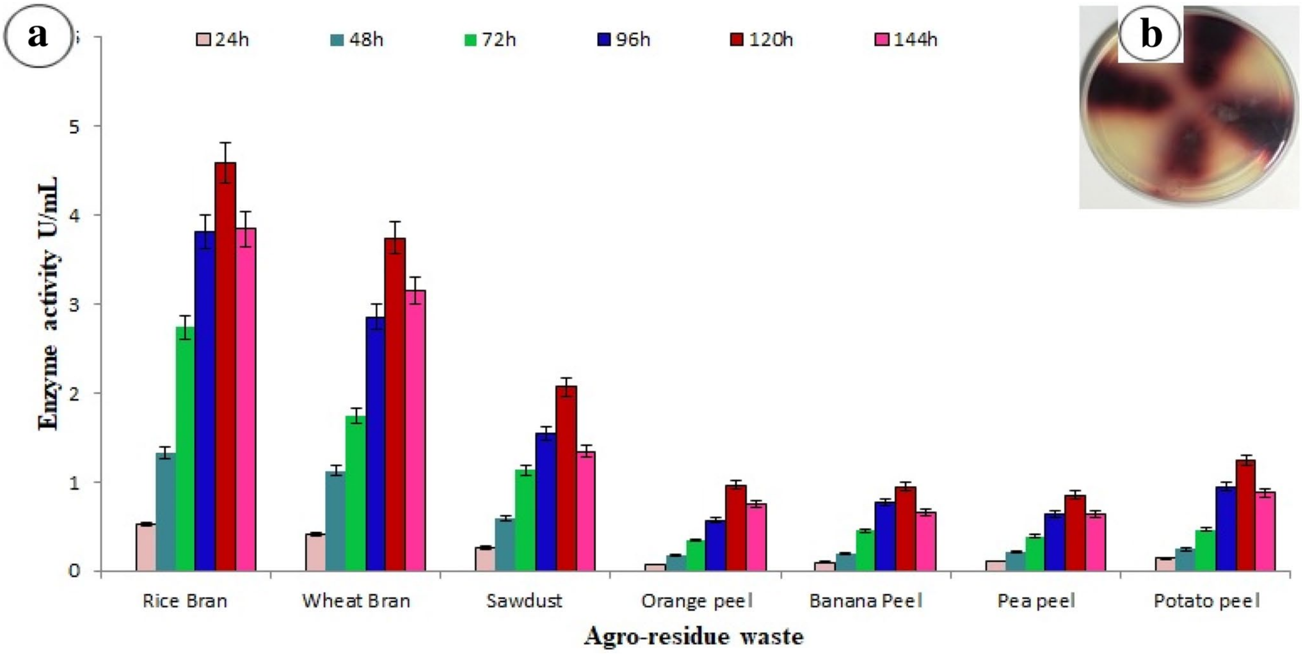


Fig. 3 **(a)** Screening of the agro-residues waste for laccase production in different incubation periods **(b)** Plate assay of laccase

and hemicellulases in attacking their respective substrates also reduces the digestibility of the fiber by ruminants [30, 61]. Several researchers are working on the up-gradation of rice bran for ruminant feed [59, 64, 65]. The present work provides better prospects that the rice bran can be potentially used to produce laccases and fodder with greater nutritional and digestibility potentials.

3.5 Bacterial Growth

Bacterial growth was evaluated periodically to confirm the utilization of rice bran as nutrients by growing bacterial cells. The maximum optical density of the growing culture was noted to be 3.52 (CFU/mL 2.76×10^{8}) at 35 ± 1 ^{0}C after 120 h incubation at 620 nm (Figs. 4b1 and b2) as well as bacterial biomass 0.02 g/mL. These conditions were selected for further optimization of laccase production in different types of nutrients sources and environmental conditions.

3.6 Optimization of Laccase Production using RSM-CCD

Environmental factors, i.e., temperature and pH, play a dynamic role in the metabolic activity of bacterial cells for laccase production. Figure 5a showed the relation between predicted and actual responses of laccase production. During the temperature optimization for *Bacillus aquimaris* AKRC02 to produce laccase (6.624 U/mL) using rice bran, the maximum laccase activity was recorded at 35 ^{0}C for 120 h (Fig. 5b and c). Increasing temperature resulted in decreased activity of the laccase. Similar results were also reported in *Pseudomonas putida* where the maximum yield of laccase (8.845 U/mL) was achieved at 40 ^{0}C [44]. The pH is another crucial parameter that affects enzymatic reactions during bacterial growth. The pH of the culture medium highly affects enzyme productions due to various ionizable groups. The effect of pH on the growth of *Bacillus aquimaris* AKRC02 and laccase production is shown in Fig. 5d. The enzyme activity showed increasing trends in pH 6.0 to 9.0, and after that, the enzyme activity decreases significantly. It influences the hydrogen ions (H^{+}) concentration throughout the permeability of the cell membrane. Three dimensional (3D) response surface plots for the interaction between different environmental variables were the best optimal values at incubation period 120 h, temperature 35 ^{0}C with pH 7.0 in rice bran to induce the maximum laccases production (10.88 U/mL) by *Bacillus aquimaris* AKRC02 (Fig. 5b, d). Ghosh and Ghosh, [17] also noted a similar result where different fermentation variables, i.e. temperature 30 ℃ and pH 5.0, were significant for laccase yield (5.93 U/mL) by *A. flavus* PUF5. Generally, fungal laccase is reported at low pH values (pH 3–5). In a previous study, laccase production (3.832 U/mL) by *Bacillus sp.* AKRC01 was recorded in the presence of rice bran at optimized conditions of 96 h at pH 8.0 and 35 ^{0}C [30]. Compared to previous observations, the bacterial strain is found better than fungal for laccase production in a wide range of environmental conditions.

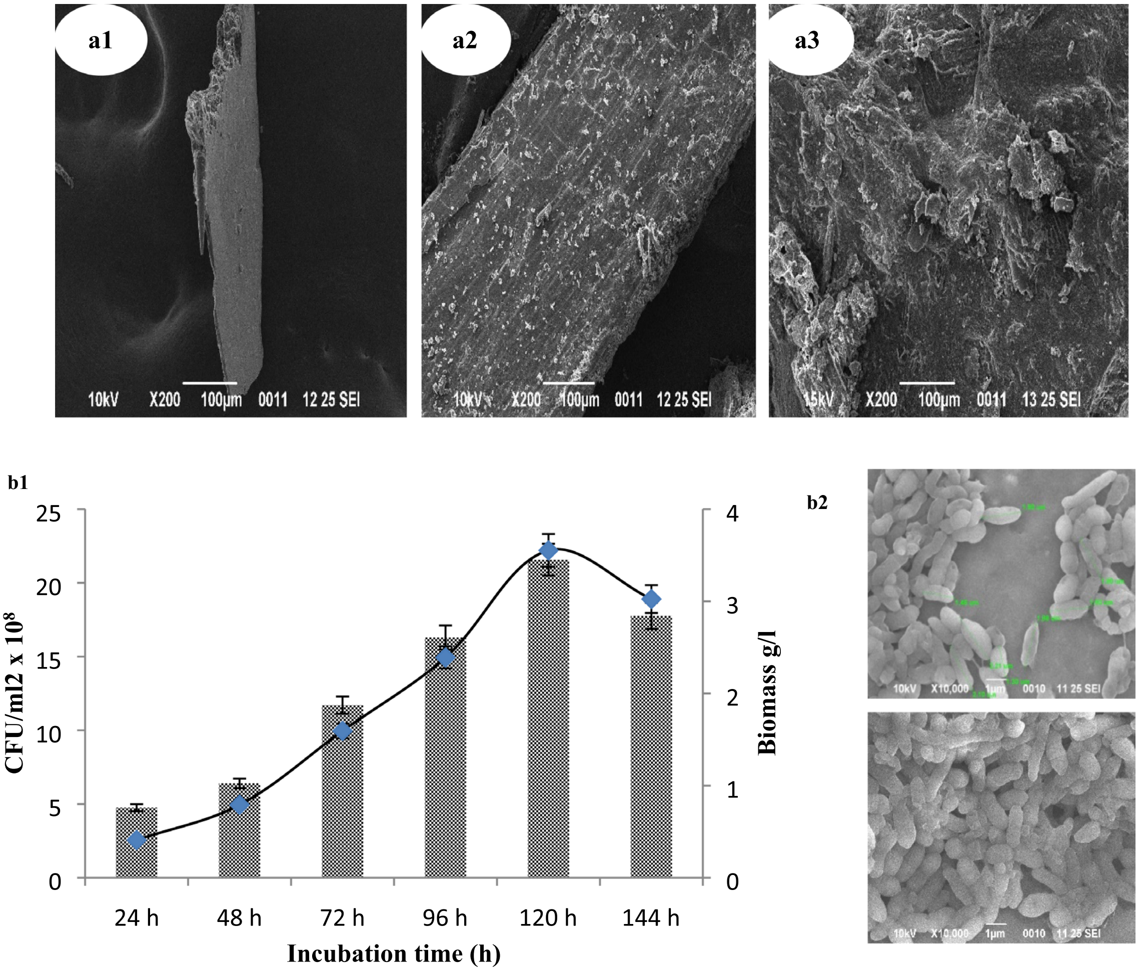


Fig. 4 Scanning electron micrograph of rice bran **(a1)** control surface structure of unfermented rice bran, **(a2)** appearance of the rice bran after 48 h, (**a3**) 120 h with *Bacillus aquimaris* AKRC02, **(b1)** Bacterial growth (CFU/mL 2.76×10^8) pattern during enzyme optimization and biomass estimation **(b2)** scanning electron micrograph of bacterial cells

In conventional optimization processes, each factor has been varied, while other factors remain constant representing the one-factor-at-the-time (OFTA) process. The OFTA model is not capable of explaining the interactive behaviour of variables [66]. Thus, nonconventional methodologies, statistical and mathematical response surface methodologies have become promising tools to determine the collective effects of two or more factors in the experimental design [42]. In this study, the different physicochemical activities for laccase production in rice bran were investigated with the SmF process using the central composite design (CCD) [30, 41, 43]. However, the incubation period, temperature, and pH were determined based on experimental optimization parameters. CCDs are designs of the response surface methodology (RSM) that can fit a complete quadratic model [40]. The RSM-CCD was used to identify the suitable values of the variables on laccase production by *Bacillus aquimaris* AKRC02 using rice bran in SmF process. The three most significant model term parameters, including incubation period, temperature (^{0}C), and pH were chosen and subjected. The optimum combination of process parameters was conducted by the CCD experimental design (Table 4) for the maximum laccase production [43]. The experimental data were statistically significant at the $P < 0.05$ level and represented a good fit with the polynomial equations of the second order. ANOVA results indicated an ‘F-value’ of 76.61 for rice bran for laccase production, implying that the model was significant (Table 5). The model terms having 'Prob > F' values less than 0.05 were considered important. By determining the coefficient (R^2), the model goodness of fit was checked (Table 5). The R^2 value for rice bran was 0.98, suitable for the adjusted R^2 value of 0.97 (Fig. 5a). The coefficient of variation (CV) was also lower as 11.33 in rice bran that showed fewer deviations between predicted and experimental values. The adequate precision value (29.46) for design space indicated a good sign that recommends the

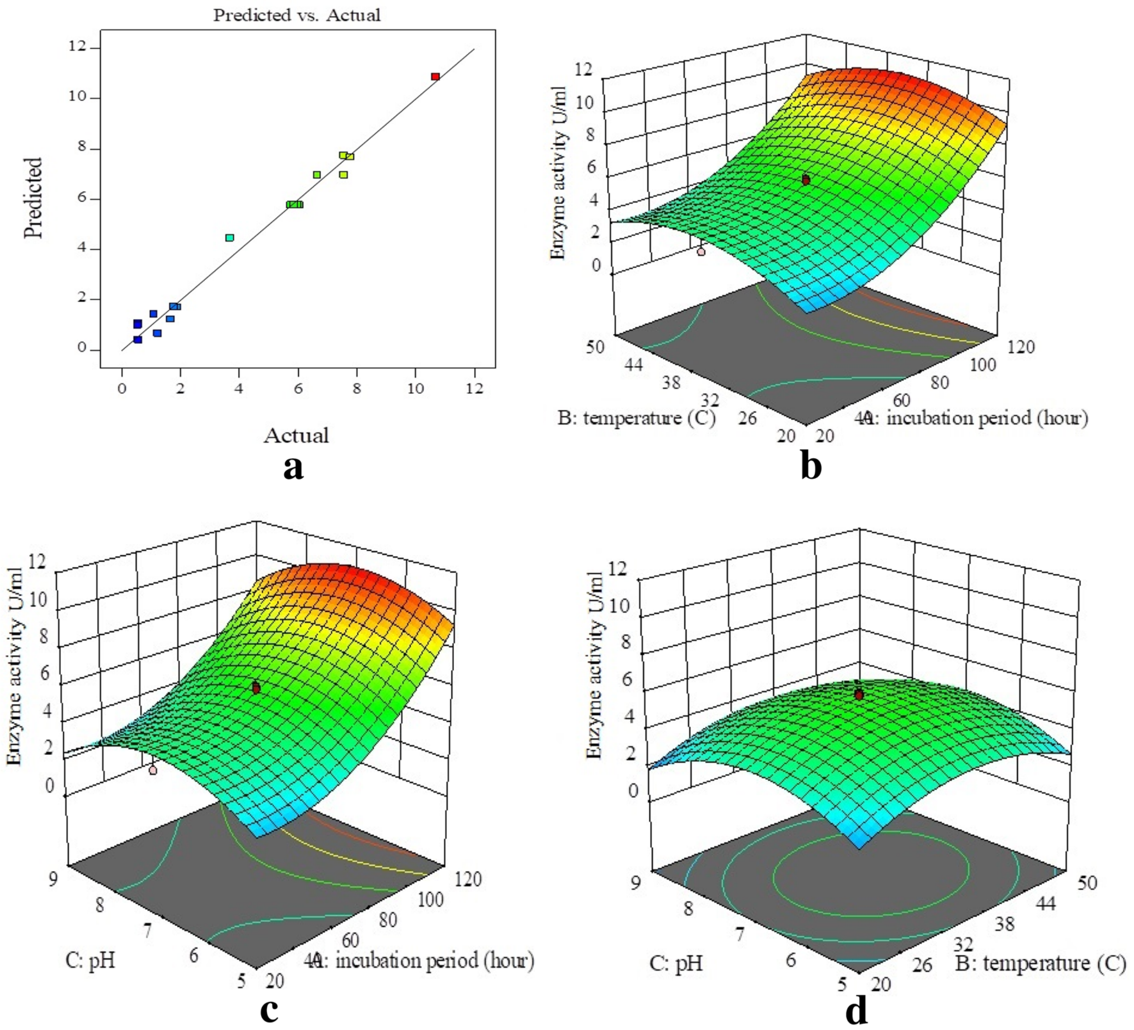


Fig. 5 **(a)** Relations between predicted and actual responses **(b-d)** 3D interaction plot of the incubation period, temperature and pH for the laccase production

model's usefulness (Table 5). In the present study, A, A^2, B^2, C^2 were model term values ($p > 0.05$) that showed the model's significant conditions. Lack of Fit F-value (45.81) in rice bran expressed that the lack of fit was significant. There was only a 0.04% chance that a Lack of Fit F-value becomes insignificant due to noise.

3.7 Optimization of Carbon and Nitrogen Sources for Laccase Production

Optimized environmental conditions (incubation time 120 h, temperature 35 ^{0}C and pH 7.0) were used for further optimization of supplementation carbon source (1.0%, w/v) (fructose, glucose, maltose, sucrose, and starch) in the culture medium to enhance bacterial growth for the maximum enzyme production. Among the carbon sources tested, glucose (1.0% w/v) induced the maximum activity of the enzyme (14.164 U/mL) in rice bran (Fig. 6a). In previous studies, the addition of 2.0% glucose results in the maximum production of bacterial laccase (4.967 U/mL) [30]. Ghosh and Ghosh [17] recorded the highest activity of the enzyme (9.21 U/mL) in the presence of soluble starch (1.0%) followed by carboxymethyl cellulose. The effect of additional carbon sources acts as an inducer for bacterial growth for laccase production. Chemically different types of suitable carbon sources are the most important factors for the fermentation process due to enhanced enzyme production. Moreover, various organic and inorganic nitrogen sources (0.5%, w/v), such as peptone, urea, yeast extract sodium nitrate, ammonium sulfate, were supplemented to rice bran containing medium for enhanced laccase production. The maximum laccase production was noted (18.124 U/mL) in the presence of peptone 0.5% w/v

Table 4 Central composite experiments design matrix with experimental and predicted values for laccase production in rice bran from *Bacillus aquimaris* AKRC02

Factor			Rice bran	
A:incubation period	B:temperature	C:pH	Observed	Predicted
20	20	5	1.23	0.672
120	20	5	7.56	7.747
20	50	5	1.09	1.432
120	50	5	7.78	7.677
20	20	9	0.56	0.414
120	20	9	7.56	6.969
20	50	9	1.67	1.234
120	50	9	6.65	6.959
20	35	7	3.67	4.468
120	35	7	10.67	10.868
70	10	7	0.54	1.086
70	60	7	1.9	1.712
70	35	4	1.76	1.737
70	35	10	0.54	1.005
70	35	7	5.934	5.772
70	35	7	5.876	5.772
70	35	7	5.743	5.772
70	35	7	6.04	5.772
70	35	7	5.98	5.772
70	35	7	5.86	5.772

Table 5 Analysis of variance (ANOVA) for laccase production in the second-order polynomial model

	Rice bran				
Source	df	Sum of Squares	Mean Square	F-value	p-value
Model	9	173.64	19.29	76.61	< 0.0001*
A-incubation period	1	102.40	102.40	406.62	< 0.0001*
B-temperature	1	0.4784	0.4784	1.90	0.1982
C-pH	1	0.7442	0.7442	2.96	0.1163
AB	1	0.3444	0.3444	1.37	0.2693
AC	1	0.1352	0.1352	0.5369	0.4806
BC	1	0.0018	0.0018	0.0071	0.9343
A^2	1	16.48	16.48	65.43	< 0.0001*
B^2	1	34.35	34.35	136.40	< 0.0001*
C^2	1	36.88	36.88	146.46	< 0.0001*
Residual	10	2.52	0.2518		
Lack of Fit	5	2.46	0.4929	45.81	0.0004
Pure Error	5	0.0538	0.0108		
Cor Total	19	176.16			
Mean		4.43			
Std. Dev		0.52			
C.V. %		11.33			
R^2		0.98			
R^2_{Adjusted}		0.97			
$R^2_{\text{Predicted}}$		0.87			

*Significant

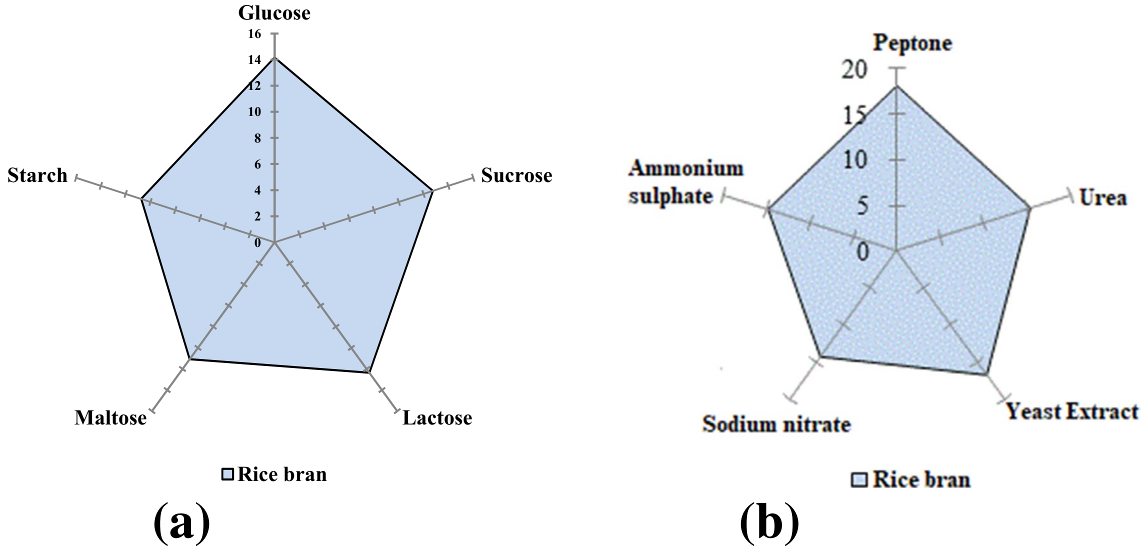


Fig. 6 Optimization of the different nutritional factors **(a)** carbon, and **(b)** nitrogen sources for laccase production

along with glucose supplementation (1.0%) at optimized environmental conditions (Fig. 6b). However, inorganic nitrogen sources, such as sodium nitrate and ammonium sulfate have been considered less significant to produce laccase than organic nitrogen (peptone, yeast extract, and urea). Nitrogen has an active involvement in the synthesis of amino acids that consists of proteins and other substances. In some studies, agro-waste was employed as a nutritional supplement by using other bacterium *Bacillus sp.* AKRC01 for enhanced laccase production [30]. Kumar et al. [30] found an optimized carbon source with 2.0% glucose (4.967 U/mL) and a nitrogen source of 1.0% peptone (6.236 U/mL) for the enhanced bacterial laccase production. According to Niladevi et al. [40], selected nitrogen sources and yeast extract showed higher enzyme activity (34.8 U/mL) in rice bran. Muthukumarasamy et al. [39] noted sucrose and peptone as significant sources for laccase production by *Bacillus subtilis* MTCC2414 during the optimization process through SSF. The results of this study and previous reports revealed that the nutritional supplement that efficiently increases the production of laccases depends on the individual microbes for their specific growth conditions.

3.8 Purification of Bacterial Laccase and Determining Molecular Weight

The elution profile of purified laccase showed a well-resolved single peak of enzyme activity. Approximately 38.08-fold purification was achieved with the specific activity of 228.34 U/mg (Table 6). The molecular weight of the laccase enzyme produced by *Bacillus aquimaris* AKRC02 was determined to be 65 kDa (Fig. 7) that showed inconsistency with the previously published reports. The purified laccase from *Bacillus* sp. AKRC01 was reported molecular weight 61 kDa in the presence of rice bran [30], and laccase from *Bacillus subtilis* MTCC 2414 was found to be 52 to 55 kDa using rice bran and wheat bran [39]. Similar results were also documented from the melanogenic bacterium *Bacillus* HR03 having laccases of 50 kDa molecular weight [67].

3.9 Laccase Stability at Different Temperature

Purified laccase of *Bacillus aquimaris* AKRC02 were stable at 45 ^{0}C for 8 h with an enzyme activity loss of just 50% compared to the enzyme activity at the optimum temperature. The enzyme maintained about 28% of its activity

Table 6 Purification of laccase from *Bacillus aquimaris* AKRC02

Purification steps	Total protein concentration (U/mg)	Total activity (Units)	Specific activity (U/mg)	Yield (%)	Purified (fold)
Concentrated crude enzyme	48.26	816.12	16.86	100	1
$(NH_4)_2SO_4$ precipitation	15.60	310.61	19.91	29.51	1.26
Ion exchange chromatography Q-Sepharose FF 100	0.6	114.18	228.34	18.32	38.08

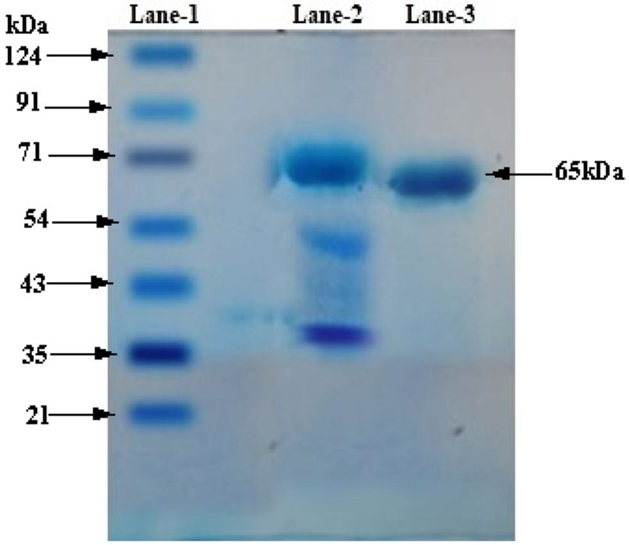


Fig. 7 SDS-PAGE of purified laccase **Lane 1:** Protein ladder, **Lane 2:** Crude laccase, **Lane 3**: Purified laccase from *Bacillus aquimaris* AKRC02

even after 16 h at 45 ^{0}C. The half-life of laccases at higher temperatures of 55 and 65 ^{0}C was approximately 6 h. The enzyme activity dramatically decreased and displayed about 90% loss of activity at a higher temperature of 85 ^{0}C after 16 h. At 25 and 35 ^{0}C, the laccase was very stable with a minimum loss of 5% in its activity, even after 16 h. After 16 h, there was approximately 76, 84, 88% loss in enzyme activity at 55, 84, 88 ^{0}C, respectively (Fig. 8), entailing the enzyme stability at the optimal temperature (35 ^{0}C). However, it was evident in the present study that laccases produced from *Bacillus aquimaris* AKRC02 showed the highest thermal stability even at 75 ^{0}C with a half-life of 4 h. These findings were consistent with the earlier reported *Alcaligenes faecalis* purified laccase revealed its optimum activity at 80 ^{0}C and displayed remarkable stability in the range of 70–90 ^{0}C [68]. A novel extracellular active thermo-alkali-stable laccase is active at 90 ^{0}C using 2,6-dimethoxyphenol as the substrate from *Bacillus tequilensis* SN4. The *Bacillus tequilensis* SN4 laccase retained 27% activity for 5 min at 100 ^{0}C and more than 80% activity for 24 h at 70 ^{0}C [69]. Furthermore, the previously reported fungus *T. hirsuta* laccase enzyme study showed that the enzyme's half-life was 4 h at 60 to 70 ^{0}C and enzyme, which was thermo-stable 20 and 30 ^{0}C even after 16 h, with minimal loss of activity [70]. Earlier reports showed that the heterologically expressed recombinant laccase of *T. trogii* BAFC 463 in *P. pastoris* was thermo-stable with a half-life of 45 min at 70 ^{0}C [71]. The purified laccases stability at various temperatures shows

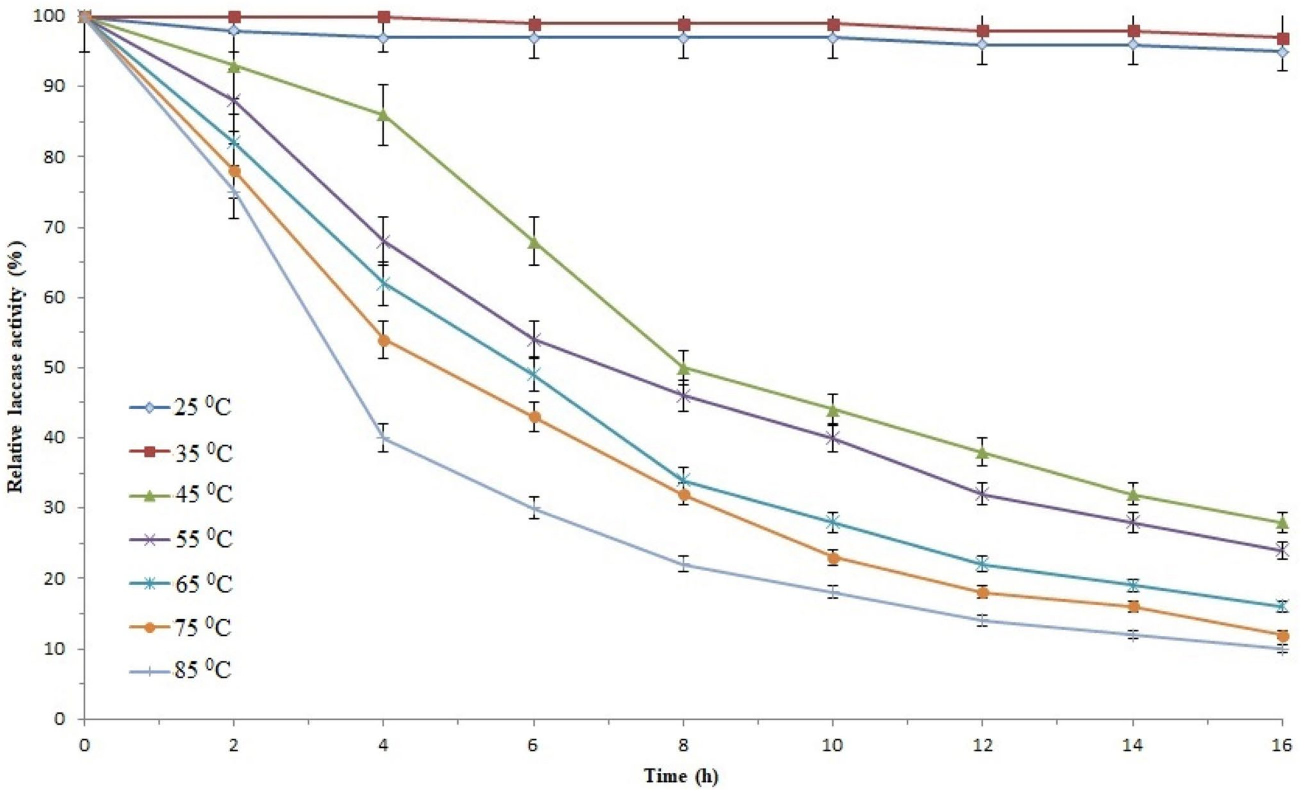


Fig. 8 Stability of purified bacterial laccase at different temperatures and time intervals

the potential for industrial application, i.e. biopolymers (polyhydroxyalkanoates as an alternative to synthetic plastics), fine-chemicals (methanol, ethanol, etc. as fuel or industrially important chemicals), biofuels (hydrogen as a clean fuel, and methane as biofuel) through the single or multi-stages process from biowastes [1].

4 Conclusion

This study revealed that the identified bacterium *Bacillus aquimaris* AKRC02 is primarily a halotolerant with potential laccase-producing capabilities. Isolation of AKRC02 from pulp paper mill sludge indicated the broad range adaptation of bacteria for its remediation potential in the presence of various salts, i.e., sodium hydroxide (NaOH), CaO, $Ca(OH)_2$, sodium sulfide (Na_2S), $CaCO_{3,}$ and organometallic compounds. The SmF process makes it suitable for the bacterial system due to oxygen circulation and nutrients availability. Rice bran appeared as an effective substrate for the maximum production of bacterial laccase in the SmF process. The maximum laccase production was recorded at optimized environmental (incubation time 120 h, temperature 35 ^{0}C, pH 7.0) and nutritional conditions (glucose 1.0%, peptone 0.5%). The purified laccase 65 kDa and showed activity for broad range temperature stability. Production of industrial enzymes in an economical manner is important. Traditionally, laccase production has been carried out using synthetic mediums, increasing cost and difficult industrial applications. Thus, this study highlighted the use of SmF process as the tangible and cost-effective process for laccase production, employing ARW as natural, renewable, and rich carbon sources that avoid deposition in landfills negative environmental impact.

Acknowledgements This work was supported by University Grant Commission, New Delhi, to Mr. Adarsh Kumar is also highly acknowledged, NFPwD (F No.01-01/2019-Sch.) fellowship to Mr. Ajay Kumar and the Department of Biotechnology New Delhi, Letter No. BT/PR18896/BCE/8/1372/2016 dated 28-03-2018 to Prof. Ram Chandra.

Author contributions Adarsh Kumar performed the experiments, drafting, editing the manuscript, Ajay Kumar Singh helped during the research work, and Muhammad Bilal editing and reviewing the manuscript. Ram Chandra supervised the work.

Data Availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no any conflict of interest.

Ethics Approval Not applicable.

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Cleaner Engineering and Technology 4 (2021) 100193

Contents lists available at ScienceDirect

Cleaner Engineering and Technology

journal homepage: www.sciencedirect.com/journal/cleaner-engineering-and-technology

Biodegradation and toxicity reduction of pulp paper mill wastewater by isolated laccase producing *Bacillus cereus* AKRC03

Adarsh Kumar, Ram Chandra *

Department of Environmental Microbiology, School for Environmental Science, Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Raebareli Road, Lucknow, Uttar Pradesh, 226025, India

A R T I C L E   I N F O

Keywords:
Laccase
Bacillus cereus strain AKRC03
Wastewater treatment
GC-MS
Bioremediation
Toxicity assessments

A B S T R A C T

Wastewater discharged from pulp and paper mills play a major role in environmental pollution and have significant effects on aquatic and terrestrial components. The main focus of this work is to study the potential laccase producing bacterium *Bacillus cereus* AKRC03 (accession no. MN720581.1) for the biodegradation and toxicity reduction from pulp paper mill wastewater. The isolated strain of *Bacillus cereus* AKRC03 exhibited up to 78.67% of decolorization and degradation capability for hazardous residual organic pollutants at different nutritional (glucose: 1.0%, peptone: 0.5%) and environmental conditions (pH: 7.0, temperature: 37 °C, agitation: 180 rpm, incubation period: 120 h). The absorption peak of the UV–Vis spectral scan identified the decolorization and degradation pattern for pollutants present in wastewater during treatment. Furthermore, the transformation of major pollutants was exhibited through GC-MS analysis. These were 1-dotriacontanol, 1-heptadecanol, tricosane, 1-(2-hydroxyethoxy) tridecane, and n-[(methylphenyl) methylene]-2-methyl-2-propanamine n-oxide. While silane, (dodecyloxy)trimethyl, (1á,3á,4à)-3,4-bis[dimethyl(4-methylphenyl)silyl] cyclo pentan-1-yl acetate, (2S,3R)-(3-tetradecyloxiranyl)methanol, hexadecanoic acid, 2,3-bis[(trimethylsilyl) oxy]propyl ester, and 2,4,6-tri[4,5-(methylenedioxy)phenyl]-s-triazine appeared as metabolic product after degradation of wastewater. The reduction of toxicity was measured up to 70% for treated wastewater with *Phaseolus mungo* L seeds and *Tubifex tubifex* worms. From these findings, it is concluded that the isolated bacterium may be used in the bioaugmentation process for the further detoxification and degradation of discharged pulp paper mill wastewater for environmental safety.

1. Introduction

The pulp and paper mill is one of the largest freshwater consumers and generates huge amounts of wastewater worldwide (CPCB, 2015). According to the Central Pulp & Paper Research Institute (CPPRI, 2017–18), there are approximately 859 pulp and paper mills in India that discharge continuously contaminated water containing different categories of pollutants. The pulp and paper mills use various types of chemicals i.e., sodium hydroxide, sodium carbonate, and chlorine compounds during washing, pulping, and bleaching steps (Kumar et al., 2020b). After these processes, the industry directly releases dark brown-coloured wastewater containing high BOD, and COD as along with toxic chlorinated compounds, fatty acids, resin acids, tannins, suspended solids, dissolved solids, inorganic salts and lignin derivatives (Pokhrel and Viraraghavan, 2004). In which, chlorinated phenols, lignin, and their derivatives are the main contaminants produced from the paper industries (Raj et al., 2007). Lignin is a major recalcitrant compound responsible for offensive colour, as well as for inhibiting phototrophic species growth by reducing the transmission of sunlight in wastewater (Karrasch et al., 2006). Besides, the chlorinated compounds and organic halides can be bioaccumulated in fish tissues, resulting in carcinogenic, clastogenic, endocrine, and mutagenic effects (Kumar et al., 2020b). Consumption of these contaminated fishes may also pose several serious problems for humans through the food chain (Savant et al., 2006). Moreover, exposure to furans and dioxins can also cause reproductive effects and skin disorders i.e., skin cancer. Considering the bioaccumulation, bio-magnification, and recalcitrance properties of ROPs, the presence of toxic pollutants in wastewater is significant for a toxicological perspective.

Keeping in mind the above, experts have not advocated for environmental effect schemes in zero discharge of water-intensive mills. Therefore, there is a significant concern among the water management

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* Corresponding author.
E-mail address: prof.chandrabbau@gmail.com (R. Chandra).

https://doi.org/10.1016/j.clet.2021.100193
Received 3 December 2020; Received in revised form 14 June 2021; Accepted 1 July 2021

experts regarding the protection aspects of the river and other aquatic resources towards the disposal of industrial wastewater. The conventional methods of treatment such as activated sludge and aerated lagoons plants are unsuccessful in the removal of phenolics and colour. In most cases, untreated wastewater containing high COD, BOD, and toxic pollutants are directly discharged into the environment that causes severe problems to the aquatic environment (Kumar et al., 2020b). Besides, the farmers in several developing countries are directly irrigating their crops from untreated wastewater that can cause harmful effects on crop productivity as well as soil fertility (Raj et al., 2014). This leads to the bioaccumulation and bio-magnification of toxicant through the food chain. Thus, before its final discharge, the optimum treatment of PPMW is required. Several physical and chemical methods or a combination of different methods in the sequence are available for the treatment of wastewater. However, these methods are not feasible because they are energy-intensive and less desirable as compared to the cost-effective biological processes (Yang et al., 2008). Consequently, affordable, energy-efficient, and environmentally friendly technologies are still required to be established.

In recent years, microbial approaches for the wastewater degradation process to optimize different parameters under laboratory conditions have drawn the significant attention of many researchers around the world to explore viable, cost-effective and sustainable wastewater treatment technology (Kaushik et al., 2010). Because of the development of microbial enzymes such as laccase, lignin peroxidase, and manganese peroxidase, most of the fungi were found to have high ligninolytic activity (Kumar and Chandra, 2020). But these techniques large scale implementations have their restriction due to the low pH range (3.0–5.0), slow growth period, huge spore formation, and adverse submerged conditions for fungal growth (Arimi et al., 2014). Pulp paper mill wastewater (PPMW), pH values are generally alkaline (pH 7.0–9.0) and the requirement to reduce the pH before fungal inoculation maintains the conditions there is an additional cost. Unlike fungi, bacteria that survive in acidic to alkaline pH can play a key role in the bioremediation of PPMW without needing to adjust pH. Bacterial laccases are a broad range substrate utilization and active at high pH and temperatures (Kumar and Chandra, 2020). Several previous workers were reported bacterial species for capable of lignin, PPMW degradation, and detoxification (Chandra et al., 2007). However, there was a still gap and require knowledge about ligninolytic enzymes capable of degradation and detoxification of PPMW at optimized environmental and nutritional conditions (Wang et al., 2010).

In this study, we have tried to address this gap by laccase-producing bacteria isolated from pulp paper mill sludge sample as well as optimized environmental and nutritional conditions for degradation and detoxification of PPMW. The untreated and bacterial treated PPMW was analyzed by UV–Vis spectrophotometer and measured structural changes, and GC-MS analysis has been performed to identify the degraded/transformed residual organic pollutants (ROPs). The toxicity reduction of bacterial-treated PPMW was also assessed using the seed germination of *Phaseolus mungo* L and *Tubifex tubifex* worm.

2. Materials and methods

2.1. Sample collection

Wastewater and sludge samples were collected (through the grab sampling type) separately in pre-sterilized plastic jerry-can (20L capacity, Tarsons Production Pvt., USA) from M/s. Century Pulp and Paper mill, located in Lalkuan (29.08°N 79.52°E) Uttarakhand, India. The samples were transported to the laboratory for microbiological study. This paper mill uses raw material i.e., bagasse, bamboo, eucalyptus, poplar, veneer waste, and waste paper recycled to manufacture its products. It discharges 80,000 gallons/day of toxic wastewater into the environment, which ultimately reaches to mix aquatic bodies.

2.2. Physico-chemical analysis of PPMW

Bacterial treated and untreated PPMW samples were analyzed to several physicochemical parameters as per standard methods for the examination of water and wastewater (APHA, 2012). Physico-chemical parameters i.e., total solid, dissolve solid, COD by open reflux method, BOD 5 day methods, total phenol estimation by chloroform extraction method, sulfate by $BaCl_2$ precipitation method, and phosphate by the colorimetric method (APHA, 2012). Moreover, the colour was measured by UV–Vis spectrophotometer (Thermo science evolution-201) following the standard method at 465 nm (APHA, 2012), and pH was measured by using a pH meter. Lignin was estimated according to Pearl and Benson (1940), and ions were also analyzed by ion meter (Orion Model 960) using the selective ion electrodes. Simultaneously, heavy metals (Cr, Cd, Cu, Ni, Zn, Fe and Pb) in treated and untreated PPMW were analyzed by atomic absorption spectrophotometry (AAS) (ZEEnit 700, Analytic Jena, Germany).

2.3. Isolation and screening laccase producing bacterial strains

Isolation of bacterial strains from 10g sludge sample was transferred to a Erlenmeyer flask (250 mL) containing MSM broth i.e. glucose 1.0%, peptone 0.1%, NH_4Cl 0.4%, K_2HPO_4 0.1%, $FeSO_4.7H_2O$ 0.001%, NaCl 0.01%, $CaCl_2$ 0.01%, $MgSO_4.7H_2O$ 0.05%, in 100 mL (Raj et al., 2014). The flasks were incubated at 37 ± 1 °C in a refrigerated incubator shaker (Orbitek, Scigenic Biotech, India) at 120 rpm for 7 days. The flask containing the sample showing the decolorization was selected. Subsequently, an aliquot (1.0 mL) was taken serially diluted in order of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}, then after diluted sample (0.1 mL) was spread on MSM agar medium plates. Further, the bacterial colonies showing clear zone on agar plates were purified on MSM agar plate by streak plate method. The screening of laccase-producing bacteria on guaiacol containing B and K agar plates containing glucose 1.0%, peptone 0.3%, yeast extract 0.1%, NaCl 0.5%, Cu^{2+} 0.0025%, agar 1.6% and pH = 7.6 was autoclaved, afterwards guaiacol 0.02% (Sigma) was added (Chandra and Singh, 2012). Each laccase-producing bacterial strain was inoculated spot on B and K agar plates were incubated at 34 ± 1 °C for 4 days. Laccase productions on the B and K agar plate were visually indicated the reddish-brown coloured zone around the colonies (Kumar et al., 2020a).

2.4. Quantitative assay

Based on laccase production bacterial isolate was grown in a minimal basal salt solution (MBSS) medium for quantification (Kumar et al., 2020a). The selected bacterial isolates were inoculated in Erlenmeyer flasks (250 mL) containing MBSS medium (pH 7.6) and incubated for 132 h at 34 ± 1 °C with agitation 120 rpm. The bacterial culture broth was taken continuously for 132 h at 12 h intervals and centrifuged at 10,000×*g* for 15 min after supernatant are obtained for laccase assay (Arora and Sandhu, 1985). The total reaction mixture was made up of 3.8 mL acetate buffer (50 mM, pH 4.6) and 1 mL guaiacol (2 mM). In which 0.2 mL of the crude laccase was added and incubated at room temperature for 2 h. The reaction was monitored by a UV–Vis spectrophotometer measuring the absorbance at 465 nm. The enzyme activity was calculated that the oxidized 1 μmol substrate per minute at room temperature.

2.5. Identification of bacteria

The isolated laccase-producing bacterial strain was identified by 16S rRNA gene sequence analysis. The complete bacterial genomic DNA extracted from the overnight grown bacterial culture using the method earlier described by Kapley et al. (2001). Using DNA extraction kit (Bioneer, Korea) for bacterial DNA extracted and purified. The 16S rDNA fragment was amplified using 27F & 1492 R primers by

Polymerase Chain Reaction (PCR). When resolved on agarose gel (1.0%) (Mark Biosciences, India), alone distinct PCR band of amplicon 1 kb DNA was observed, then visualized with ethidium bromide. Moreover, PCR amplicon staining with forward and reverse DNA sequencing reaction was performed with an automated DNA sequencer using the eubacterial primer of 5′-TACGGYTACCTTGTACGACTTT-3 ′and 5′-AGAGTTTGATCMTGCTCAG-3'. To construct a phylogenetic tree for the neighbor-joining technique, MEGA software (version 6.0) was used (Tamura et al., 2013).

2.6. *Wastewater decolorization assay*

The experiment was conduct in Erlenmeyer flasks (250 mL) containing 99 mL of wastewater addition with mineral salts (g/L) (glucose 5.0, peptone 3.0, Na_2HPO_4 2.4, K_2HPO_4 2.0, $CaCl_2$ 0.01, NH_4NO_3 0.1 and $MgSO_4$ 0.01 were pH adjusted 7.0 and autoclaved at 121 °C for 20 min (Kumar and Chandra, 2018). The bacterial culture grown overnight (18 h) having an inoculums size of 3.2×10^6 CFU/mL was 1 mL and 99 mL PPMW added in flasks for inoculation. The culture inoculated and without inoculated flasks was incubated for 144 h at 37 ± 1 °C with agitation 120 rpm. The decolorization of PPMW was monitored using a UV–Vis spectrophotometer (Evolution-201, Thermo Scientific, USA) to measure the change in absorbance maxima of the lignin at 200–280 nm (Raj et al., 2014).

Decolorization (%) = initial absorbance (A_0) − final absorbance (A_1) × 100.

Initial absorbance (A_0).

2.7. *Optimization for culture conditions for degradation and decolorization of PPMW*

2.7.1. *Optimization of nutritional condition*

The influence of different carbon sources 1.0% (w/v) i.e. glucose, starch, sucrose, dextrose, and lactose was analysis for the degradation and decolorization of PPMW. Moreover, different nitrogen sources 0.5% (w/v) i.e. yeast extract, urea, peptone, sodium sulfate, and ammonium chloride have been added to the MSM medium for evaluating the maximum decolorization. The result of carbon and nitrogen sources at different concentrations (0.1–1.0 w/v) was optimized for the decolorization and degradation of PPMW.

2.7.2. *Optimization of environmental parameters*

The same experiment was performed at different temperatures (17–57 °C), pH (5.0–9.0) with agitation (100–200 rpm) to assess the effect of environmental conditions.

2.8. *UV–Vis spectroscopic analysis*

The UV–Vis spectral scans were performed for untreated and bacterial treated PPMW using a spectrophotometer (Model Evolution 201, Thermo Fisher Scientific, USA) in the wavelength range 200–700 nm. Before, the spectral scan the untreated and bacterial treated samples were maintained pH 7.0. A quartz cuvette with a length of 1.0 cm of the path was used to scan the wavelength. At a scanning rate of 0.1 nm and 1000 nm min^{-1} scan speed, all spectra were observed.

2.9. *GC-MS analysis for characterization of metabolites*

Untreated and bacterial treated PPMW samples were centrifuged at 12,000×*g* for 10 min to segregate bacterial biomass and particulate matter to assess degradability. PPMW was extracted with a suitable solvent i.e. ethyl acetate in acidic conditions (pH 2.0) supernatant obtained (Chandra and Abhishek, 2011). The organic layer collected was dewatered and filtered by Whatman no. 54 filter paper over anhydrous sodium sulfate (Na_2SO_4). Moreover, samples were dried at room temperature and dissolved in ethyl acetate for GCMS analysis through identified pollutants present in untreated and bacterial treated PPMW. Besides, dioxane (100 μL) and pyridine (10 μL) were used for GC-MS analysis of the extracted sample, followed by 50 μL of N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) having trimethylchlorosilane (TMCS) (Raj et al., 2014). To dissolve the residue solution was subsequently heated up to 60 °C with periodic shaking for 15 min. Finally, the silylation sample (1 μL) was injected in GC-MS (PerkinElmer, UK) with a PE-5MS capillary column and helium with a flow rate of 1.0 mL/min as the carrier gas. The column temperatures were set at 50 °C for 5 min, then ramped at 50 to 300 °C and kept for 5 min. At 200 and 250 °C, respectively, the transfer line and the ion-source temperatures were maintained.

2.10. *Effects of treated and untreated PPMW with Phaseolus aureus L*

The toxicity assessments of treated and untreated PPMW were using seed germination bioassay (Wang 2003). Black gram (*Phaseolus aureus* L.) seeds were surface-sterilized done mercuric chloride ($HgCl_2$) 0.1% for 2 min, to prevent other contamination (Kumar et al., 2020b). The *P. aureus* L. seeds homogenized were placed in different concentrations (25–100% v/v) of untreated and bacterial treated PPMW. The seeds were incubated at 28 ± 1 °C in dark conditions for 48 h. PPMW toxicity assessment was detected in terms of seeds germination percent and α-amylase activity of *P. aureus* L. The tested seeds were kept on two layers of filter paper in each Petri dish soaked with different concentrations of PPMW. Each concentration of tested seeds was crushed with sodium acetate buffer (0.1 M, pH 4.8), and filtered by cheesecloth to large particles remove then centrifuged at 15,000×*g* for 20 min after supernatant was obtained. All experiment preparation was carried out at 4 °C. The α-amylase assay was conducted with a reaction mixture containing enzyme extract (0.5 mL), 0.1 M acetate buffer (1.0 mL, pH 4.8), and 0.1% soluble starch (1.0 mL). The reaction mixture was incubated at room temperature for 10 min then adding 0.1% iodine reagent (1.0 mL) and 0.05 N HCl (3.0 mL) to stop the reaction (Kumar et al., 2020b). The using UV–Vis spectrophotometer was read the optical density (OD) at 620 nm and amylase activity expressed the decrease in absorbance (Beri and Gupta, 2007).

2.11. *Tubifex toxicity test*

Tubifex worm is a significant member of lentic fauna in the aquatic ecosystem (Kumar et al., 2020b). Tests were performed in beakers (200 mL) containing 100 mL of untreated and bacterial treated PPMW can use for toxicity analysis. The toxicity assessment of tubifex worms will be inoculated into different concentrations (25–100%) of untreated and bacterial treated PPMW and control as tap water. Each concentration of the tested worm picture was visualized under the phase-contrast microscope. Ten tubifex worms were exposed to three replicates of each concentration tested. When there was no response and complete immobilization to pressing with a blunt glass rod, the tested tubifex worms were considered dead (Kumar et al., 2020b). Besides, moving worms back to tap water (control), death was further confirmed (Singh et al., 2020a,b).

2.12. *Statistical data analysis*

All the experiments were carried out in triplicate and the findings were presented as an average of three independent observations. Pearson's correlation coefficient criterion was used to evaluate the relationship between different physiological parameters of seed germination. The statistically significant $^*P < 0.05$ was considered to be the data, calculated software by IBM SPSS (v20.0.0).

3. Results and discussion

3.1. Wastewater characterization

The physicochemical characterization of PPMW was shown in Table 1. The wastewater was dark brown and alkaline in nature, which turned into light brown colour after bacterial treatment. The analysis of the wastewater sample showed several physicochemical parameters beyond the permissible limits along with heavy metals. Therefore, several parameters in (mg/L^{-1}) i.e., pH (8.1 ± 0.12), Total solid (1946.0 ± 1.02), Dissolved Solid (1784.0 ± 1.24), COD (752.0 ± 0.78), BOD (380.0 ± 0.45), Lignin (416.0 ± 5.12), phosphate (34.0 ± 0.46), nitrate (695.5 ± 1.05), total phenol (29 ± 0.95) and heavy metals such as Fe (5.6982 ± 1.02), Ni (0.956 ± 0.58) and Zn (3.2346 ± 0.89). However, a sharp reduction in pH (7.64 ± 0.11), Total solid (314.0 ± 1.12), Dissolved Solid (652.0 ± 2.15), COD (186.0 ± 0.16), BOD (52.0 ± 0.58), Lignin (30.0 ± 1.02), phosphate (14.5 ± 0.21), nitrate (112.75 ± 0.12), total phenol (6.27 ± 0.00) and heavy metals such as Fe (3. 1462 ± 1.00), Ni (0.6217 ± 0.91) and Zn (1.1462 ± 0.98) was noted as shown in Table 1. Phenols and lignin are recalcitrant in nature and major plant constituents of PPMW. Due to lignin causes the dark brown colour, high value of COD and phenols are toxic for the aquatic and terrestrial environment, even at relatively low levels in discharged PPMW (Gupta et al., 2017). Phenol at a higher concentration blocked the photosynthesis of blue algae, diatom, and concentration range of 100–400 g/mL also induced full inhibition of photosynthesis (Duan et al., 2017). Moreover, sodium sulfite, which is used during the pulping process, may be the source of sulfate ions in wastewater, and the nitrate found in the wastewater may be generated from lignin (Singhal and Thakur, 2009). The metal content in wastewater samples can be attributed to the bioaccumulated metals by plants, and these plants are used as raw materials, as well as to the different chemicals using the pulping and bleaching process of papermaking.

Table 1
Physicochemical characteristics of pulp and paper mill wastewater.

Parameters	Untreated	Treated	Permissible limits CPCB annual report, (2015)
pH	8.1 ± 0.12	7.64 ± 0.11	5–9
Total solid	1946.0 ± 1.02	314.0 ± 1.12	300
Dissolved Solid	1784.0 ± 1.24	652.0 ± 2.15	500
COD	752.0 ± 0.78	186.0 ± 0.16	120
BOD	380.0 ± 0.45	52.0 ± 0.58	40
Lignin	416.0 ± 5.12	30.0 ± 1.02	NS
Chloride	2613.0 ± 2.34	1825.0 ± 1.87	1500
Sulphate	970.0 ± 1.02	342.0 ± 1.06	250
Phosphate	34.0 ± 0.46	14.5 ± 0.21	NS
Nitrate	695.5 ± 1.05	112.75 ± 0.12	50
Calcium	3.5 ± 0.16	1.0 ± 0.11	NS
Total phenol	29 ± 0.95	6.27 ± 0.00	NS
Sulphide	120.0 ± 0.63	23.0 ± 0.91	NS
NH_4	422.0 ± 1.48	70.0 ± 0.58	NS
Heavy metals			
Cr	0. 1202 ± 0.16	0.0842 ± 0.63	0.05
Cd	0.0435 ± 0.21	0.0320 ± 0.11	0.01
Cu	2.5851 ± 0.96	0.8245 ± 0.58	0.50
Ni	0.956 ± 0.58	0.6217 ± 0.91	0.50
Zn	3.2346 ± 0.89	1.1462 ± 0.98	2.00
Fe	5.6982 ± 1.02	3. 1462 ± 1.00	2.00
Pb	0.3015 ± 0.58	0.1156 ± 0.96	0.05

All values are mean (n = 3) ± SD in mg L^{-1} except pH.
COD Chemical oxygen demand, *BOD* Biological oxygen demand.
NS not specified.

3.2. Isolation and identification laccase producing bacteria

Isolated laccase producing bacteria from pulp and paper mill sludge sample. Seven bacterial strains induced coloured reddish-brown zones on the guaiacol agar plate indicate the production of laccase (Fig. 1a). Based on laccase production seven potential isolates named BL-1, BL-2, BL-3, BL-4, BL5, BL-6, and BL-7, were selected for quantification of the enzyme. Out of seven isolates, BL-1 was found to be the most potential isolate producing 6.09 U/mL laccase production followed by BL-3 (5.36 U/mL), BL-5 (4.55 U/mL), and BL-2, BL-4, BL-6, BL-7 produced only 2.94, 3.12, 1.84, and 2.53 U/mL, respectively. Therefore, the oxidation of guaiacol is one of the most suitable substrates for enzyme assays used by bacteria to produce laccase enzyme (Kaushik and Thakur, 2014). Hence, the formation of the reddish-brown colour is induced by laccase due to the oxidative polymerization of guaiacol (Kumar et al., 2020a).

Studies of the laccase production were performed in a liquid medium and selected the highest producing strains after 120 has shown in Fig. 1b. The isolate BL1 was found the maximum laccase production among the seven isolates for further study. The isolate BL1 was identified as *Bacillus cereus* strain AKRC03 (MN720581.1) based on NCBI blast data of nucleotide sequence. Furthermore, a 16S rRNA sequence of isolated strain was also showed 93% similarity with *Bacillus cereus* strain DGA14 (MN809373.1). Based on partial 16S rDNA sequence contrasts, the phylogenetic tree of the *Bacillus cereus* strain AKRC03 and related genera have been linked, as shown in Fig. 2.

3.3. 3Effect of different nutritional and environmental parameters

3.3.1. Effect of various carbon and nitrogen sources

PPMW decolorization and degradation were observed in different carbon sources for 120 h of incubation period results as shown in Fig. 3a. The most suitable carbon source of glucose 1.0% (w/v) was found for optimum decolorization (48.66%). Moreover, starch, sucrose, dextrose, lactose and other carbon sources were found less effective than glucose exhibited decolorization up to 46.61–39.81%. Whereas the observed bacterial growth was slow in the absence of glucose in the culture medium. This showed that bacteria do not readily use the carbon source present in PPMW. But the added glucose in the media was easily available for bacterial growth. This showed strong evidence that incubated bacteria are incapable of using the PPMW for growth and metabolism. In the case of processed PPMW related finding has been published by the previous worker (Sonkar et al., 2019). This indicated our bacteria were more effective in decolorizing and degrading PPMW. This may be the maximum production of laccase enzymes which facilitated the decolorization process (Raj et al., 2014).

The effect of the various nitrogen sources along with glucose (1.0 w/v) showed that peptone (0.5% w/v) was the most suitable organic nitrogen source which increased PPMW degradation and decolorization up to 52.33% (Fig. 3b). Further, the peptone concentrations were increases in the medium resulted in the continuous reduction of the degradation and decolorization capability of the bacterium due to inhibited the decolorization process. Because, peptone are rich in free amino acids and short peptides, which support cell growth with a low consumption rate of the carbon source and minimize the accumulation of by-products (Lau et al., 2004). Moreover, the other organic nitrogen sources i.e. yeast extract could show degradation and decolorization of PPMW up to 41.72% only, whereas urea could show decolorization up to 30.79%, while inorganic nitrogen sources such as ammonium chloride and sodium sulfate could show decolorization up to 27.29 and 21.72%

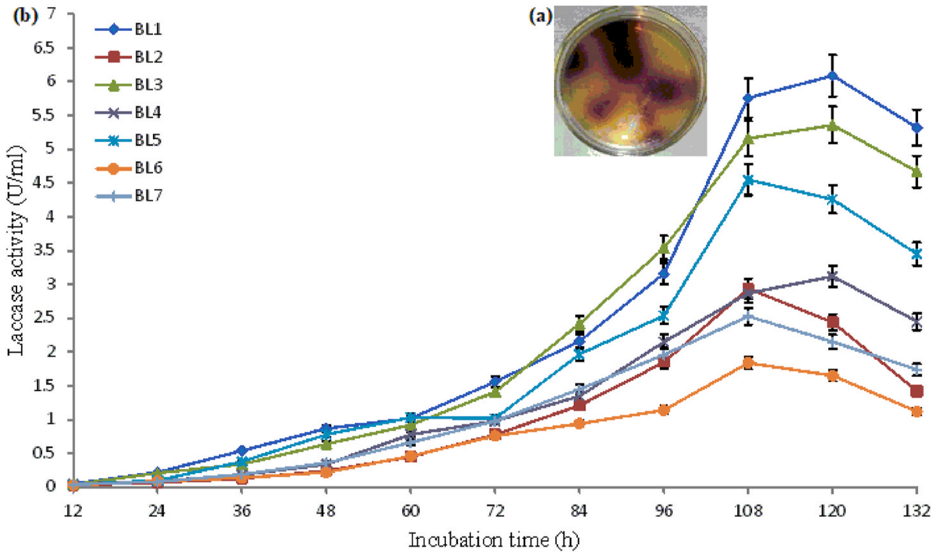


Fig. 1. Screening for laccase producing bacteria. **(a)** Laccase activity on the guaiacol-agar plate and **(b)** Production of laccase enzyme at different time duration (pH 7.0 and temperature 35 °C).

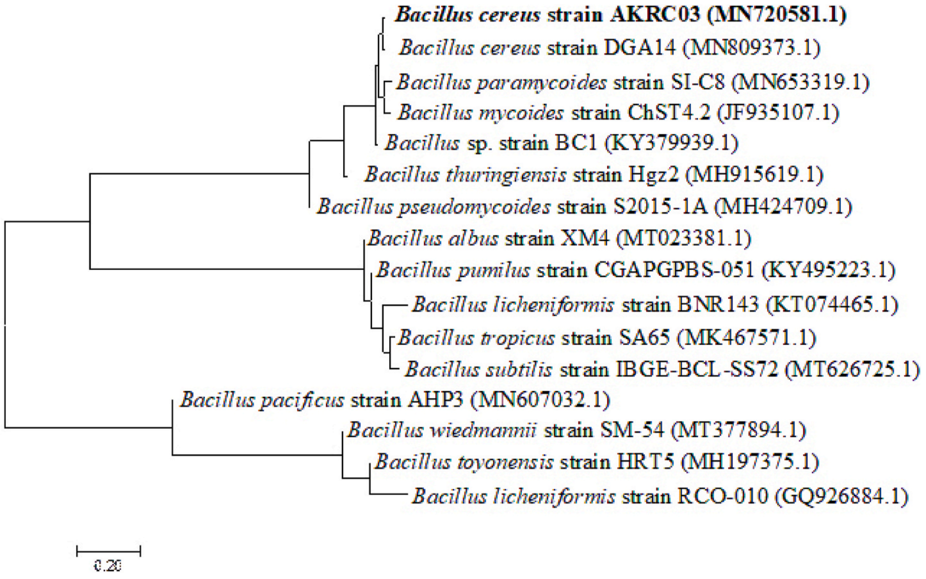
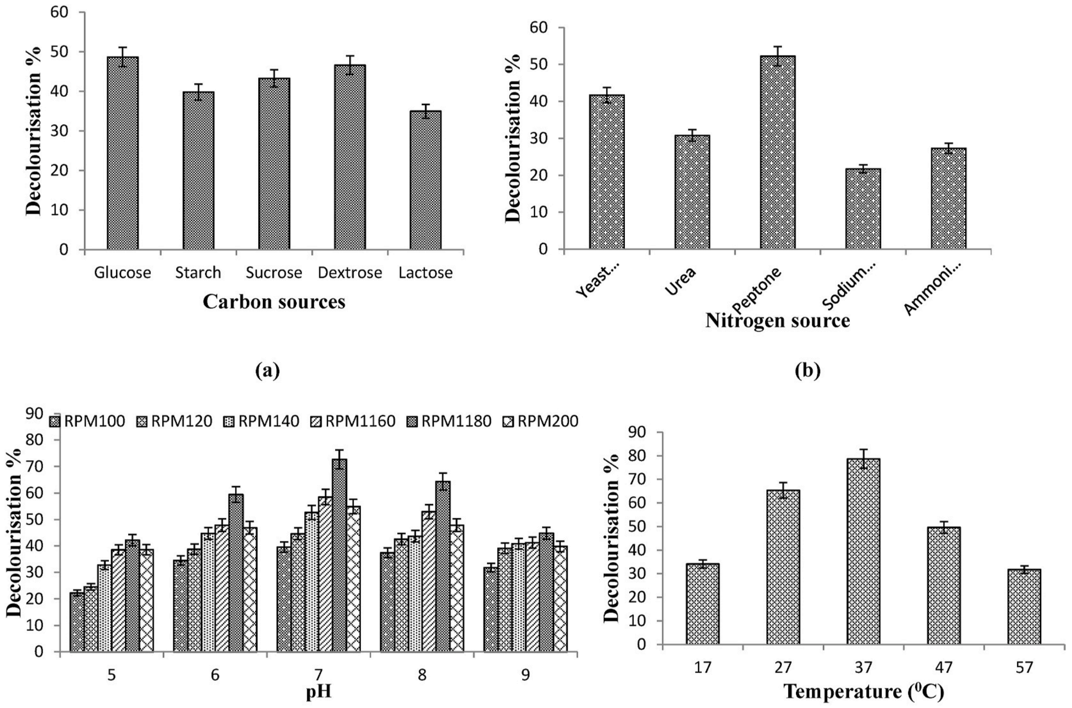


Fig. 2. Phylogenetic tree of the *Bacillus cereus* strain AKRC03 *(*strain BL1) has been linked based on partial 16S rDNA sequence comparisons. Their names and respective accession numbers are given in the tree.

respectively. Similarly, the effect of nutritional parameters has been reported by previous researchers for the degradation and decolorization of PPMW (Chandra and Singh, 2012). Therefore, a previous study reported the presence of glucose (1.0%) and peptone (0.5%) for COD reduction up to 85 and 72% and colour after 144 h of treatment by *Serratia liquefaciens* (Haq et al., 2016). Thus, the reduction in BOD, COD and colour decolorization reported previously by several researchers through the bacteria using suitable carbon and nitrogen sources for their growth (Sonkar et al., 2019).

3.3.2. Effect of different pH, shaking speed and temperature

The degradation and decolorization process of PPMW was specifically affected by the pH, shaking speed, and temperature of culture conditions. The effect of different pH and shaking speeds of PPMW showed optimum decolorization (72.67%) at pH (7.0) with agitation 180 rpm after 120 h incubation (Fig. 3c). Further, the increases and decreases pH due to inhibited the degradation and decolorization of PPMW. This might be due to the inhibition of the bacterial laccases production. In a previous report, the optimum pH for the PPMW degradation and decolorization by *Paenibacillus* sp. was noted at pH 7.2 (Raj et al., 2014). Moreover, the PPMW were significantly affected by degradation and decolorization by various shaking speed also. The result of shaking speeds of 100–200 rpm demonstrated that the maximum degradation and decolorization were reported at 180 agitations as shown in Fig. 3c. Besides, an increase in shaking speed resulted in a decrease in degradation and decolorization rate. This may be due to the mechanical injury of bacterium cells at higher shaking speeds (Chandra and Singh, 2012). Besides, it was also found that the increases temperature (17–37 °C) can also enhance the degradation and decolorization of PPMW from 34.16 to 78.64% (Fig. 3d). However, the increase in temperature from 37 to 57 °C can adversely affect bacterial growth by inhibiting the degradation and decolorization of PPMW. Therefore, our findings correlated with some previously reported reports (Hooda et al., 2018).



(a) (b)

(c) (d)

Fig. 3. Effect of different nutrients and environmental parameters on pulp paper mill wastewater decolorization. **(a)** Carbon source, **(b)** nitrogen source, **(c)** pH, and shaking speed, (d) temperature for 24–120 h incubation.

3.4. UV–Vis spectroscopy analysis of treated PPMW

The UV–Vis spectral scan of bacterial treated PPMW samples at different time intervals (24–120 h) was done to identify the change the absorbance during degradation and decolorization by *Bacillus cereus* strain AKRC03. Fig. 4 shows the UV–Vis scan of untreated and bacterial treated PPMW samples. Around 215 and 230 nm can be seen in the two prominent absorption peaks. The identified absorbance peaks decreased during the course of treatment and peaks almost disappeared after 120 h. The UV–Vis spectral analysis assisted the degradation of ROPs and lignin derivatives compounds by *Bacillus cereus* strain AKRC03. The absorbance of lignin and its derivatives as well as chlorine dioxide derivative was observed around this region (Nie et al., 2014). This study correlated with several previous studies related to the UV–Vis spectrum observed during the degradation of PPMW by *Bacillus* sp. IITRDVM-5 (Sonkar et al., 2019). They also observed decolorization and degradation of chlorophenols (Patel and Kumar, 2016).

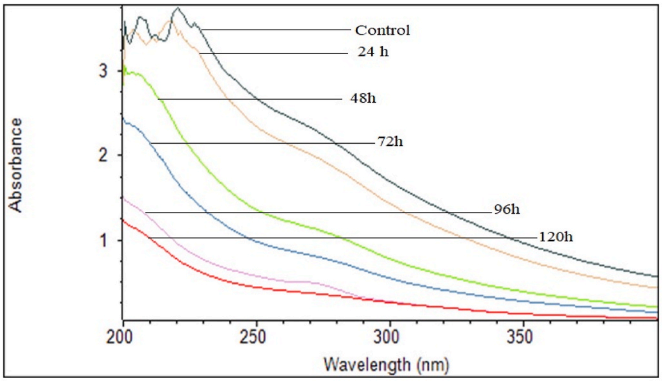


Fig. 4. UV–Vis spectroscopy analysis of pulp and paper mill wastewater contamination degradation during treatment under optimized condition by *Bacillus cereus* strain AKRC03.

3.5. GCMS analysis of metabolites present in PPMW

To identify the degraded or transformed ROPs present in PPMW by *Bacillus cereus* strain AKRC03. The total ion chromatogram (TIC) of ROPs present in the bacterial treated and untreated samples as shown in Fig. 5a and b and their identified pollutants are represented in Table 2. After treatment, the peak intensity reduction compared to the untreated sample, and the appearance of new peaks can be observed in the treated sample. The major ROPs identified in untreated sample at different RT were (cis)-1-Butyl-2-undecylcyclopropane (RT: 12.86), 1-Hexadecanol (RT: 16.87), 1-Octadecanol (RT: 20.52), 1-Dotriacontanol (RT: 24.03), Heptacosane (RT: 26.58), 1-Heptadecanol (RT: 28.45), 9-Octadecenoic acid (RT: 29.28), Hexatriacontane (RT: 29.94), Heptasiloxane, hexadecamethyl (RT: 30.77), Heptadecane, 2-methyl (RT: 31.10), Tricosane (RT: 32.09), Dodecane, 1,1′-oxybis (RT: 32.98), 1-(2-Hydroxyethoxy) tridecane (RT: 33.80), Docosanoic acid, 1,2,3-propanetriyl ester (RT: 34.37), Pentatriacontane (RT: 38.39), Diethyl a-trimethyloxy-2,4,6-trimethylbenzylphosphonate (RT: 41.99), Pentatriacontane (RT: 42.32), N-[(methyl phenyl)methylene]-2-methyl-2-propanamine N-oxide (RT: 44.07), Heptasiloxane, hexadecamethyl (RT: 45.71), 3-(Dimethylphenysilanyl)-6,11-dihydroxy-3-methyl-1, 2,3,4-tetrahydrobenz[a]anthracene-7,12-dione (RT: 50.78). However, the investigation of bacteria treated PPMW sample has showed the existence of various ROPs such as 3,6,9-Trioxa-2,10-disilaundecane,n2,2,10,10-tetramethyl (RT: 9.74), Silane, (dodecyloxy)trimethyl (RT: 16.46), 1,1,1,3,5,5,7,7,7-Nonamethyl-3-(trimethylsiloxy)tetrasiloxane (RT: 21.56), (1á,3á,4à)-3,4-Bis [dimethyl(4-methyl phenyl)silyl]cyclo pentan-1-yl acetate (RT: 23.34), Hexadecanoic acid, trimethylsilyl ester (RT: 24.68), (2S,3R)-(3-Tetradecyloxiranyl)methanol (RT: 26.93), Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester (RT:31.39), Diethyl(1E,3Z)-3-cyano-4-naphthylbuta-1,3-dienylphosphonate (RT: 32.72), Heptacosane (33.19), (S,S)-1,1-Bis(ethoxycarbonyl)-2,2-bis-p-tolylsulfinyl-1-ethanol (RT: 36.24), 2,4,6-Tri[4,5-(methylenedioxy)phenyl]-s-triazine (RT:

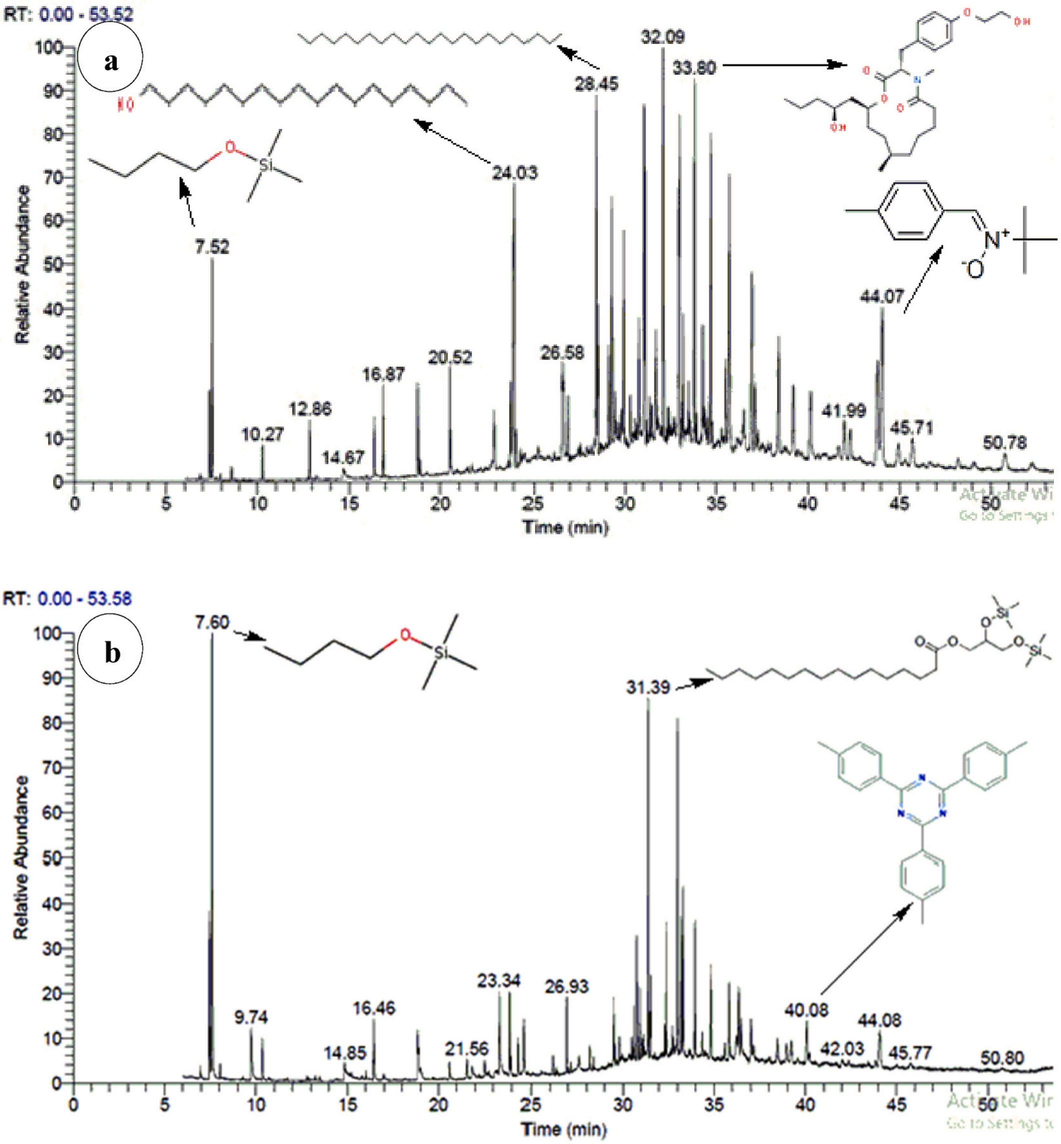


Fig. 5. GC–MS chromatograms of compounds extracted with ethyl acetate from **(a)** Untreated and **(b)** Treated wastewater by *Bacillus cereus* strain AKRC03.

40.08) and (5S,6S)-4,5-Dimethyl-6-phenyl-3,4,5,6-tetrahydro-2H-1,3,4-oxadiazin-2-one (RT: 44.08) at different RT. In the untreated (control) sample, these pollutants were completely absent due to the complete degradation of PPMW and some new metabolites were formed. Besides, in the bacterial treated samples, one organic compound i.e., Silane, butoxytrimethyl (RT: 7.52) could not be metabolized. The presence of various ROPs, phenolics, chlorinated, and aliphatic compounds in wastewater were previously reported by several researchers for the degradation through bacterium (Sonkar et al., 2019). However, the after-bacterial treated PPMW containing ROPs are shown in Table 2. Similarly, various ROPs of transformed or degraded phenolic, non-phenol halide derivatives as well as lignin derivatives from wastewater have been identified in PPMW (Raj et al., 2014). Therefore, after bacterial treatment, some ROPs identified in the untreated sample were diminished. This indicated that ROPs and other pollutants were degraded with the help of bacterial strain for produced laccase enzyme. These ROPs were used by bacteria as the primary energy source and play a key role in the degradation and decolorization of PPMW. The result indicates that *Bacillus cereus* strain AKRC03 transformed or degraded chlorinated, phenolic, non-phenolic as well as lignin derivatives compounds from the wastewater.

3.6. Toxicity assessment for environmental safety

Toxicity assay of untreated and bacterial treated PPMW showed in effect of seeds germination of *P. mungo* L. in 3 days observations (Fig. 6a). The various parameters of seed germination were observed with untreated and bacterial treated wastewater (Table 3). The test indicated the removal of toxicity based on germination or suppression of seed and early growth of seeds. The toxicity test of *P. mungo* L. seeds with different concentrations of untreated PPMW was more toxic showed only 86, 60, 40, and 20% germination respectively. While the bacterial treated PPMW toxicity was significantly 70% reduction in seed germination test of *P. mungo* L. The α-amylase is a key role in seed germination and after exposure; the different concentrations of wastewater are detailed in Fig. 6b. The α-amylase activities were decreased when increases higher concentration of untreated PPMW. Also, when seeds were exposed to untreated wastewater, the results were more pronounced compared to bacterial treated wastewater (Fig. 6b). The presence of more toxic pollutants and dissolved solids that are absorbed by the seeds before germination and the effect of the different physicochemical and biochemical parameters of seeds due to germination suppression at high concentrations of PPMW (Sonkar et al., 2019). Increased germination

Table 2
Compound identified as trimethylsilyl (TMS) derivatives in ethyl acetate extract from (a) untreated and (b) treated wastewater by *Bacillus cereus* strain AKRC03.

S. No.	Retention time (RT)	Compound Name	Untreated (0 h)	Treated (120 h)
1.	7.52	Silane, butoxytrimethyl	+	+
2.	7.60	Silane, butoxytrimethyl	+	+
3.	9.74	3,6,9-Trioxa-2,10-disilaundecane,n2,2,10,10-tetramethyl	-	+
4.	12.86	(cis)-1-Butyl-2-undecylcyclopropane	+	-
5.	14.85	1-Hexadecanol	-	+
6.	16.46	Silane, (dodecyloxy)trimethyl	-	+
7.	16.87	1-Hexadecanol	+	-
8.	20.52	1-Octadecanol	+	-
9.	21.56	1,1,1,3,5,5,7,7,7-Nonamethyl-3-(trimethylsiloxy)tetrasiloxane	-	+
10.	23.34	(1á,3á,4à)-3,4-Bis[dimethyl(4-methylphenyl)silyl]cyclo pentan-1-yl acetate	-	+
11.	23.84	1-Hexadecene	+	-
12.	24.03	1-Dotriacontanol	+	-
13.	24.68	Hexadecanoic acid, trimethylsilyl ester	-	+
14.	26.58	Heptacosane	+	-
15.	26.93	(2S,3R)-(3-Tetradecyloxiranyl) methanol	-	+
16.	28.45	1-Heptadecanol	+	-
17.	29.28	9-Octadecenoic acid	+	-
18.	29.94	Hexatriacontane	+	-
19.	30.77	Heptasiloxane, hexadecamethyl	+	-
20.	31.10	Heptadecane, 2-methyl	+	-
21.	31.39	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	-	+
22.	32.09	Tricosane	+	-
23.	32.72	Diethyl(1E,3Z)-3-cyano-4-naphthylbuta-1,3-dienylphosphonate	-	+
24.	32.98	Dodecane, 1,1′-oxybis	+	-
25.	33.19	Heptacosane	-	+
26.	33.80	1-(2-Hydroxyethoxy)tridecane	+	-
27.	33.96	Docosane	-	+
28.	34.37	Docosanoic acid, 1,2,3-propanetriyl ester	+	-
29.	36.24	(S,S)-1,1-Bis(ethoxycarbonyl)-2,2-bis-p-tolylsulfinyl-1-ethanol	-	+
30.	36.50	Docosanoic acid, 1,2,3-propanetriyl ester	+	-
31.	38.39	Pentatriacontane	+	-
32.	40.08	2,4,6-Tri[4,5-(methylenedioxy)phenyl]-s-triazine	-	+
33.	41.99	Diethyl a-trimethyloxy-2,4,6-trimethylbenzylphosphonate	+	-
34.	42.32	Pentatriacontane	+	-
35.	44.07	N-[(methylphenyl)methylene]-2-methyl-2-propanamine N-oxide	+	-
36.	44.08	(5S,6S)-4,5-Dimethyl-6-phenyl-3,4,5,6-tetrahydro-2H- 1,3,4-oxadiazin-2-one	-	+
37.	45.71	Heptasiloxane, hexadecamethyl	+	-
38.	50.78	3-(Dimethylphenysilanyl)-6,11-dihydroxy-3-methyl-1, 2,3,4-tetrahydrobenz[a]anthracene-7,12-dione	+	-

(+) Present; (−) Absent.

percent in treated PPMW may be due to decreased and detoxified ROPs that have formed suitable environmental conditions for seed germinations and utilization as a nutrients present in wastewater. Recently, reported toxicity analysis of untreated PPMW through seed germination tests and results were correlated with several previous studies (Kumar et al., 2020b).

3.7. *Effects of PPMW of Tubifex tubifex*

Tubifex worms are a significant link in the aquatic food chain as well as valuable indicators of variable degrees of aquatic pollution due to freshwater worms (Singh et al., 2020a). Toxicity assay of untreated and bacterial treated PPMW effect on *Tubifex tubifex* in 96-h observations as showed in Fig. 7. In control, the Tubifex worm showed the typical behaviour of a healthy worm i.e., no mortality and straight movement was observed. The toxicity test of untreated PPMW exposures at <25% remarkably reduced the movement of the tested Tubifex worm. Exposure to untreated PPMW with higher concentration resulted in low hemoglobin content, blackness of the rear part of the body and substantial disintegration of the hind portion body damage of Tubifex worm (Fig. 7a). While the bacterial treated PPMW sample was significantly reduced the toxicity exposures at <75 and 100% remarkably the movement of test organisms as shown in Fig. 7b. According to the reports of USEPA (2012) and an annual report of CPCB (2015), the untreated PPMW have several wastewater contaminants beyond the permissible limits along with heavy metals (Table 1). Together with these, different mutagenic and carcinogenic ROPs are also present (Table 2) have been listed in the USEPA report (2012). Because of the presence of these pollutants in PPMW, the rate of mortality from physical injury, cell bursting, and whole-cell damage has been increased (prostomium, digestive tract, and caudal part) of Tubifex worms (Kumar et al., 2020b). This may be correlated with the existence of more toxic ROPs and toxic metals that cause worm toxicity in untreated PPMW. This study revealed the untreated PPMW is more toxic than bacterial treated PPMW as shown in Fig. 7. Some researchers previously reported various heavy metals (Cu, Ag, Pd, Zn, and Uranium) caused intoxication was reduced tactile movement, degeneration, and segmentation of the Tubifex worm and death appeared without noticeable signs of the worms (Singh et al., 2020b).

4. Conclusion

This study revealed that PPMW contains several EDCs, as well as toxic ROPs that are generated during the paper manufacturing process, which directly discharged into the aquatic resources. PPMW sample was collected from Century Paper Mill Uttarakhand, India. Further, the isolated of potential laccase-producing bacterium *Bacillus cereus* strain AKRC03 was studied in terms of degradation and detoxification of ROPs present in PPMW. The bacterium was capable to degrade and decolorize PPMW up to 78.67% with suitable supplementary nutritional source (glucose: 1.0%, peptone: 0.5%) and environmental parameters (pH: 7.0, temperature: 37 °C, agitation: 180 rpm) at optimized incubation period 120 h. The degradation and decolorization of pollutants present in PPMW confirmed by UV–Vis spectral scan and GC–MS analysis. The toxicity assessment using *Phaseolus mungo* L. seeds and *Tubifex tubifex* worm was also confirmed the toxicity reduction of wastewater after bacterial treatment. Therefore, it is concluded that the *Bacillus cereus* strain AKRC03 could be used as a promising agent for the decolorization and detoxification of ROPs present in PPMW for its safe disposal.

Authors' contributions

Adarsh Kumar: Conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools, or data; drafting the manuscript and final correction of the manuscript.

Ram Chandra: Supervised the work.

Disclosure statement

The authors declare that there is no conflict of interest.

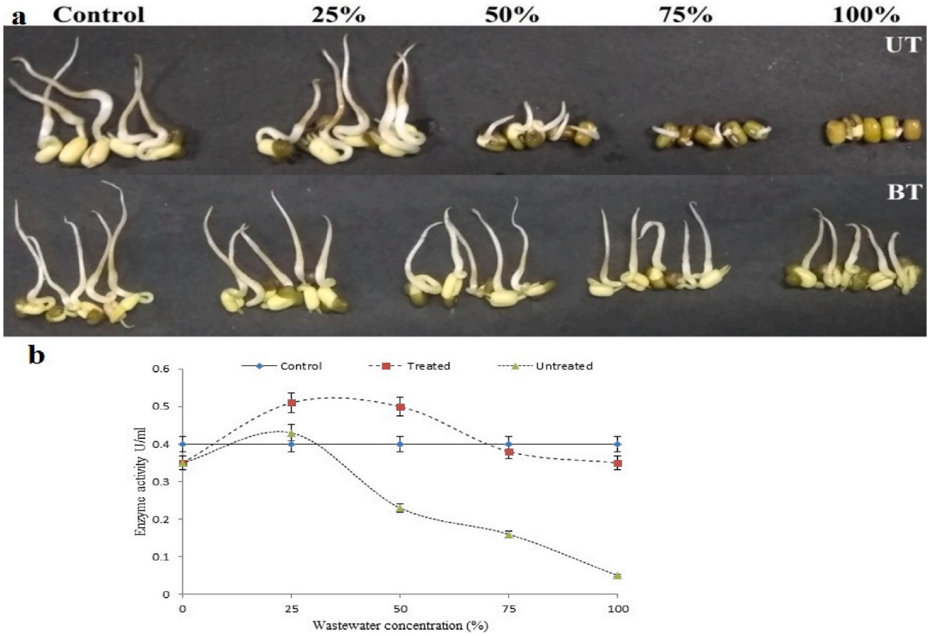


Fig. 6. Effect of the untreated (UT) and bacterial treated (BT) PPMW at the concentration of 0, 25, 50, 75, and 100% on **(a)** seed germination and **(b)** alpha-amylase activity.

Table 3
Toxicity analysis of PPMW by *Phaseolus mungo* L. seed germination.

Samples	Treatment (%)	Germination (%)	Radical length (cm)	Phytotoxicity (%)	Stress tolerance index (%)	Relative Toxicity (%)	Seedling vigor index
Untreated	25	86.60 ± 0.57	1.90 ± 0.10	10.79 ± 0.02	89.20 ± 0.04	13.40 ± 0.01	164.54 ± 0.02
	50	60.0 ± 1.00	0.90 ± 0.10	57.74 ± 0.02	42.25 ± 0.03	40.00 ± 0.01	54.00 ± 0.04
	75	40.0 ± 1.00	0.30 ± 0.10	85.91 ± 0.04	14.08 ± 0.02	60.00 ± 0.03	12.00 ± 0.01
	100	20.33 ± 0.57	0.13 ± 0.05	93.89 ± 0.01	6.10 ± 0.03	79.67 ± 0.03	2.64 ± 0.02
Treated	25	100 ± 0.00	2.10 ± 0.10	1.40 ± 0.51	98.59 ± 0.10	00	210 ± 0.01
	50	83.33 ± 0.57	1.93 ± 0.05	9.38 ± 0.01	89.20 ± 0.040	16.67 ± 0.01	160.82 ± 0.03
	75	80.0 ± 1.00	1.73 ± 0.05	18.77 ± 0.05	81.22 ± 0.01	20 ± 0.01	138.40 ± 0.02
	100	70.0 ± 1.00	1.46 ± 0.05	31.45 ± 0.01	68.54 ± 0.01	30 ± 0.051	102.20 ± 0.05
Control	0	100 ± 0.00	2.13 ± 0.05	00	00	00	213 ± 0.01

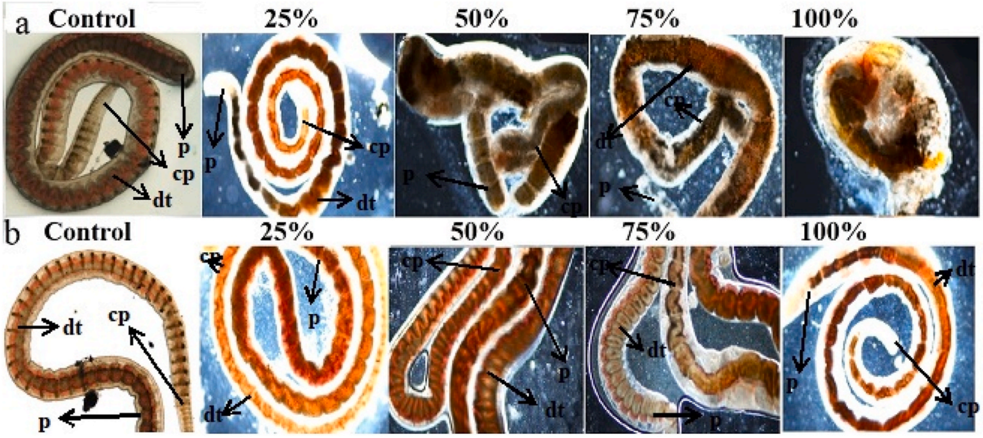


Fig. 7. *Tubifex tubifex* malformations after exposer to PPMW (a) untreated and (b) treated in different concentration 25%, 50%, 75%, 100% and control as 0% as a tap water. Morphology of worm with intact prostomium (p), caudal part (cp) and digestive tract (dt).

Declaration of competing interest

Manuscripttitle: Biodegradation and toxicity reduction of pulp paper mill wastewater by isolated laccase producing *Bacillus cereus* AKRC03.

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Acknowledgments

The financial assistance from University Grant Commission, New Delhi, to Mr. Adarsh Kumar, Ph.D. scholar is highly acknowledged to the financial support of Department of Biotechnology (DBT) vide project Letter No. BT/PR18896/BCE/8/1372/2016 dated 28-03-2018 is also acknowledged.

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Heliyon 6 (2020) e03170

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Review article

Ligninolytic enzymes and its mechanisms for degradation of lignocellulosic waste in environment

Adarsh Kumar, Ram Chandra *

Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar (A Central) University, Vidya Vihar, Raebareli Road, Lucknow, Uttar Pradesh, 226025, India

A R T I C L E I N F O

Keywords:
Laccase
Lignin peroxidase
Manganese peroxidase
Versatile peroxidase
Lignocellulosic waste
Degradation and detoxification
Environmental science
Microbiology

A B S T R A C T

Ligninolytic enzymes play a key role in degradation and detoxification of lignocellulosic waste in environment. The major ligninolytic enzymes are laccase, lignin peroxidase, manganese peroxidase, and versatile peroxidase. The activities of these enzymes are enhanced by various mediators as well as some other enzymes (feruloyl esterase, aryl-alcohol oxidase, quinone reductases, lipases, catechol 2, 3-dioxygenase) to facilitate the process for degradation and detoxification of lignocellulosic waste in environment. The structurally laccase is isoenzymes with monomeric or dimeric and glycosylation levels (10–45%). This contains four copper ions of three different types. The enzyme catalyzes the overall reaction: 4 benzenediol + O_2 to 4 benzosemiquinone + $2H_2O$. While, lignin peroxidase is a glycoprotein molecular mass of 38–46 kDa containing one mole of iron protoporphyrin IX per one mol of protein, catalyzes the H_2O_2 dependent oxidative depolymerization of lignin. The manganese peroxidase is a glycosylated heme protein with molecular mass of 40–50kDa. It depolymerizes the lignin molecule in the presence of manganese ion. The versatile peroxidase has broad range substrate sharing typical features of the manganese and lignin peroxidase families. Although ligninolytic enzymes have broad range of industrial application specially the degradation and detoxification of lignocellulosic waste discharged from various industrial activities, its large scale application is still limited due to lack of limited production. Further, the extremophilic properties of ligninolytic enzymes indicated their broad prospects in varied environmental conditions. Therefore it needs more extensive research for understanding its structure and mechanisms for broad range commercial applications.

1. Introduction

Lignocellulosic waste is considered as a chief component of renewable biomass on the Earth (Saini et al., 2015). This consists of three major components such as cellulose (40%–50%), hemicelluloses (25%–30%) and lignin (15%–20%) (Chaurasia, 2019). Cellulose is the primary polysaccharide as constituent of lignocellulosic materials that consist of hundred to over ten thousands β-1, 4 linked D-glucose units in unbranched linear chains. This is known as microfibrils with 3–5nm wide and many micrometers in length (Horn et al., 2012). Each cellulose chain-linked side by side through hydrogen bonding and van der waals interactions into microfibrils arrangement which are reported to consist 24 to 36 chains based on x-ray scattering data and the information available regarding the cellulose synthesis (Anwesha et al., 2011; Horn et al., 2012). Each cellulose microfibrils is synthesized from the spontaneous bundling and crystallization with dozens of the linked β-D glucan chains, each of which is made by a cellulose synthase (CESA) protein (Cosgrove, 2005). While hemicellulose is arranged in forms of strips tightly linked with cellulose and lignin in plant cell walls, it contains branched polysaccharide mainly xylans and mannans. It contains branched heterogenous polysaccharides of pentose (xylose and arabinose), hexoses (glucose, mannose, and galactose) and sugar acids (e.g. acetic, galacturonic and glucuronic) (Dimarogona et al., 2012). There is the presence of five monomeric sugars namely D-glucose, D-mannose, D-galactose, D-xylose and L-arabinose linked by β-1, 4-glycosidic bonds, that consist of both liners and branched heteropolymers. The major hemicelluloses in hardwoods are xylans (15%–30% dry weight), a polysaccharide having β-1,4 linked D-xylose units, which can be substituted with other monosaccharide units. However, softwood contains hemicelluloses mainly in the form of glactoglucomannan (15%–20% dry weight), a polysaccharide having β,1–4 linked D-glucose, and D-galactose units (Bugg et al., 2011).

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* Corresponding author.
E-mail address: prof.chandrabbau@gmail.com (R. Chandra).

https://doi.org/10.1016/j.heliyon.2020.e03170
Received 6 July 2019; Received in revised form 4 October 2019; Accepted 31 December 2019

Catalysis Letters
https://doi.org/10.1007/s10562-021-03800-8

Extremophilic Ligninolytic Enzymes: Versatile Biocatalytic Tools with Impressive Biotechnological Potential

Adarsh Kumar[1] · **Ajay Kumar Singh**[1] · **Muhammad Bilal**[2] · **Ram Chandra**[1]

Received: 21 July 2021 / Accepted: 12 September 2021

Abstract

Extremophilic microbial communities have developed several physiological and molecular strategies to survive and grow in extreme environments. This review explores the adaptive mechanisms of extremophilic ligninolytic enzymes such as laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP). The extremophilic ligninolytic enzyme adaptive features such as different compositions of amino acids, hydrophobic interaction, surface charges, tighter packing (compactness), a deleted loop, saturated/unsaturated fatty acid, salt bridge, disulfide bridge, hydrogen bond, several ions, and α-helical, as well as, cysteinyl-tRNA synthetase enzyme to maintain their active stability for catalytic functionalities in extreme conditions. The biochemical properties of extremophilic laccase are monomeric, dimeric, and trimeric glycoprotein with a molecular weight range of 50 to 97 kDa, while glycosylated haem proteins, LiP, and MnP, display a molecular weight ranging from 38 to 62.5 kDa (LiP: 38–46 kDa; MnP: 38–62.5 kDa). The optimum activity of laccase in bacteria and fungus was noted at pH 4.0–10.0. Moreover, the exquisite LiP and MnP activities in fungus occurred at pH 3.0–5.0, while bacteria adopted the diverse range of pH 4.0–9.0. Finally, concluding remarks, the current research needs to highlight the emerging applications of ligninolytic enzymes in broad range of industrial and biotechnological potentials.

✉ Ram Chandra
prof.chandrabbau@gmail.com

[1] Department of Environmental Microbiology, School of Earth & Environmental Sciences, Babasaheb Bhimrao Ambedkar (A Central) University, Vidya Vihar, Raebareli Road, Lucknow, Uttar Pradesh 226025, India

[2] School of Life Science and Food Engineering, Huaiyin Institute of Technology, Huai'an 223003, China

Environmental Technology & Innovation 24 (2021) 101831

Contents lists available at ScienceDirect

Environmental Technology & Innovation

journal homepage: www.elsevier.com/locate/eti

Organometallic pollutants of paper mill wastewater and their toxicity assessment on Stinging catfish and sludge worm

Ajay Kumar Singh [a], Adarsh Kumar [a], Muhammad Bilal [b], Ram Chandra [a,*]

[a] *Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar (A Central) University, Vidya Vihar, Raebareli Road, Lucknow, Uttar Pradesh 226025, India*
[b] *School of Life Science and Food Engineering, Huaiyin Institute of Technology, Huai'an 223003, China*

ARTICLE INFO

Article history:
Received 21 April 2021
Received in revised form 28 June 2021
Accepted 29 July 2021
Available online 31 July 2021

Keywords:
Water pollution
Organometallic pollutants
Aquatic toxicity
Pulp paper mill
Tubifix-tubifix
Health hazards

ABSTRACT

Paper mill wastewater is a complex mixture of different organometallic pollutants with alkaline pH and toxicity to aquatic organisms. This study investigates major physico-chemical parameters such as BOD (814.0 ± 2.84), COD (2289.0 ± 3.54), TDS (4128.0 ± 5.21), dissolved solid (3520.0 ± 4.85), suspended solids (608.0 ± 1.53), lignin (628 ± 2.24) and heavy metals along with organometallic pollutants from paper mill wastewater detected by gas chromatography and mass spectrophotometer technique. The organometallic pollutants such as decane, 2,9-dimethyl, eicosane, 2-bromotetradecane, 1,2-dihydro-1,4-diphenylphthalazine, benzene acetic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester, and docosane were detected via. gas chromatography and mass spectrophotometry (GC–MS). These pollutants are highly toxic and cause significant adverse effects on aquatic organisms. The toxic effects of organometallic pollutants were tested on *Heteropneustes fossilis* (Stinging catfish) and *Tubifex-tubifex* (sludge worm) using different concentrations of wastewater (25%, 50%, 75%, and 100%). Due to wastewater exposure *Heteropneustes fossilis*, toxic effects were observed, showing notable changes in the secondary lamellae (SL); primary lamellae (PL); mucous cell (MC); epithelial cell (EC); chloride cell (CHC). In *Tubifex tubifex,* minimum toxic effects were noted at the concentration of less than 25% up to 24 h of exposure and maximum at 48 h of exposure at 50% of the wastewater concentration. After 24 h wastewater exposure, the hemoglobin content was decreased, the body's rear part became white, and body disintegration was observed in worms. In conclusion, the present study investigates that paper mill wastewater is toxic to the aquatic ecosystem and must be treated and assessed before discharge.

1. Introduction

The paper mill is one of the most pollution-causing mills all over the world, including India. The pulp paper mill releases cellulose fibers, lignin, synthetic chemicals, and other plant materials into the wastewater during the papermaking process (Singh, 2015; Aniyikaiye et al., 2019; Yadav and Chandra, 2018). Many organic pollutants produced during the wood digestion and pulping process are directly discharged into the aquatic system without proper treatment (Cunha et al., 2020). Due to the high polluting contents in the wastewater, biological oxygen demand (BOD) is increased in the aquatic systems (Singh and Chandra, 2019; Aniyikaiye et al., 2019). Most of the organic compounds in the paper mill

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* Corresponding author.
E-mail address: prof.chandrabbau@gmail.com (R. Chandra).

https://doi.org/10.1016/j.eti.2021.101831

Materials Today: Proceedings xxx (xxxx) xxx

Contents lists available at ScienceDirect

Materials Today: Proceedings

journal homepage: www.elsevier.com/locate/matpr

Residual organic pollutants detected from pulp and paper industry wastewater and their toxicity on *Triticum aestivum* and *Tubifex-tubifex* worms

Ajay Kumar Singh, Adarsh Kumar, Ram Chandra

Department Environmental Microbiology, School for Environmental Science, Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Raibarely Road, Lucknow, 226025, India

ARTICLE INFO

Article history:
Received 18 October 2020
Accepted 28 October 2020
Available online xxxx

Keywords:
Paper industry wastewater
Residual organic pollutant
Toxicity assessment
T. aestivum
Tubifex-tubifex

ABSTRACT

Paper industry wastewater is the significant source of water and soil contamination due to various unknown residual organic pollutants directly discharged into the environment without proper treatment. Therefore, the study has focused on the detection of residual organic pollutants (N-(1,2,2,2-tetrachloroethyl) trimethylacetamide, octadecane, 1-iodo, Oleic acid, trimethylsilyl ester, eicosane; hexadecane, 2-methyl-; octadecane, docosane; tricosane) from paper industry wastewater sources showed endocrine-disrupting compounds and their toxicity. This experiment were carried out the effect of paper mill sludge amendments with garden soil (25%, 50%, 75%, and 100%) and garden soil was utilized as a control in a pot experiment. This study revealed the growth parameters of *Triticum aestivum* in seeds germination percentage and germination index values decreased with rising in sludge concentration. Further, soil amended with 25% paper industry sludge slightly reduced the growth in root length, shoot length, biomass, photosynthetic and 50% sludge amended soil had variable effects on the root, shoot, and photosynthetic pigment of *T. aestivum.* Moreover, the toxicity of paper industry wastewater on *tubifex-tubifex* worms after 48 h of exposure (>50% concentration), worms showed various changes such as movement; haemoglobin content disappeared, cell bursting, and the back of the body became white with body disintegration at the lethal concentrations of paper industry wastewater. This article has revealed that the toxicity of the organic pollutants in the terrestrial and aquatic life due to paper industry wastewater there is needed to treat adequately prior to discharge.

Selection and peer-review under responsibility of the scientific committee of the Emerging Trends in Materials Science, Technology and Engineering.

1. Introduction

During manufacturing processes, paper industries released lignocellulosic waste material as wastewater into the environment. Mostly 150–200 m3 wastewaters released per ton of paper manufacturing, it indicates the pollution load which is directly discharged without proper treatment [1]. The presence of high amount of lignin color and organic pollution content is the main issue of the paper industry. Wastewater contain chlorophenolic compounds which are formed during bleaching process and low molecular weight compounds are also generated and ligninolitic compounds are mixed together and forms organic compounds [2].

The ring structure of chlorine and pentachlorophenol is persistent to aerobic biodegradation but, aromatic structures of compounds with chlorine are biodegradation in nature [3]. The US Environmental Protection Agency has put pentachlorophenol on the list of priority contaminants hence, the pesticides prepared by using pentachlorophenol are banned to sale in the market globly. They have also banned the sale and use of pesticide products containing. The secure permissible limit of pentachlorophenol in water is 0.30 µg / l-1. Pulp paper mill effluent, however, even after industrial-scale treatment, retains pentachlorophenol well above the appropriate level [4]. Furthermore, pentachlorophenol has been identified as mutagenic even at low levels of concentration. However, the pentachlorophenol is accumulating in the human body via food chain but, we know these pollutants are considered as mutagenic in nature [5]. In different countries of the world, the toxic impact of waste water from the paper industry on the fish reproductive system has been documented, but due to insufficient awareness of the contaminants of wastewater from the paper

https://doi.org/10.1016/j.matpr.2020.10.862
2214-7853/
Selection and peer-review under responsibility of the scientific committee of the Emerging Trends in Materials Science, Technology and Engineering.

Please cite this article as: Ajay Kumar Singh, A. Kumar and R. Chandra, Residual organic pollutants detected from pulp and paper industry wastewater and their toxicity on *Triticum aestivum* and *Tubifex-tubifex* worms, Materials Today: Proceedings, https://doi.org/10.1016/j.matpr.2020.10.862

Journal of Experimental Biology and Agricultural Sciences, October - 2020; Volume – 8(5) page 663 – 675

Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

DETECTION OF REFRACTORY ORGANIC POLLUTANTS FROM PULP PAPER MILL EFFLUENT AND THEIR TOXICITY ON *Triticum aestivum*; *Brassica campestris* AND *Tubifex-tubifex*

Ajay Kumar Singh, Adarsh Kumar, Ram Chandra

Department Environmental Microbiology, School for Environmental Science, Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Raibarely Road, Lucknow, 226025

Received – August 11, 2020; Revision – September 28, 2020; Accepted – October 16, 2020
Available Online – October 25, 2020

DOI: http://dx.doi.org/10.18006/2020.8(5).663.675

KEYWORDS

Pulp paper mill

Organic pollutant

Toxicity assessment

Tubifex-tubifex

T. aestivum and *B. campestris*

ABSTRACT

The pulp and paper industry released different chemical pollutants that are directly discharged into the environment without proper treatment. This experiment was carried out to study the effect of pulp paper mill sludge amendments with garden soil (25%, 50%, 75%, and 100%), and garden soil was utilized as a control in a pot experiment. This study revealed the growth parameters of *Triticum aestivum* and *Brassica campestris* in germination percentage and germination index values decreased with rising in sludge concentration. Further, Soil amended with 25% paper mill sludge slightly reduced the growth in root length, shoot length, biomass, photosynthetic, and 50% sludge amended soil had variable effects on the root, shoot, and photosynthetic pigment of *T. aestivum*and *B. campestris*. However, the toxicity of effluent on *tubifex-tubifex* worms was also carried out at selected concentrations (25%, 50%, 75%, and 100%). Results of the study suggested that after 24 hours of exposure (>25% concentration), tubifex worms showed various changes such as morphological changes; hemoglobin content disappeared at the lethal concentrations of pulp paper mill effluent; cell bursting, and the back of the body became white with body disintegration at the lethal concentrations of pulp paper mill effluent. This article mainly focuses on the toxicity of the organic pollutants in terrestrial and aquatic life due to pulp paper mill effluent.

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* Corresponding author

E-mail: prof.chandrabbau@gmail.com (Prof. Ram Chandra)

Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI]
(http://www.horizonpublisherindia.in/).

Kumar et al., *J. Pure Appl. Microbiol.,* **14**(**1**), 351-362 | March 2020
Article 5992 | https://doi.org/10.22207/JPAM.14.1.36

Print ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE **OPEN ACCESS**

Optimization of Laccase Production by *Bacillus* sp. Strain AKRC01 in Presence of Agro-waste as Effective Substrate using Response Surface Methodology

Adarsh Kumar[1], Ajay Kumar Singh[1], Shamshad Ahmad[2] and Ram Chandra[1]*

[1]Department of Environmental Microbiology, [2]Department of Environmental Science, School for Environmental Science, Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Raebareli Road, Lucknow - 226 025, Uttar Pradesh, India.

Abstract

Laccases are the widely distributed oxidative enzymes belong to the group of phenoloxidases. These are considered as model enzymes and widely distributed in nature. Bacterial laccases have extremophilic nature and several other unique properties that are not features of fungal laccase such as high-temperature stability and pH. The present study focused on the optimization of laccase production from *Bacillus* sp. AKRC01 under submerged fermentation state using agro-waste as the substrate. The optimized environmental conditions such as incubation period 96 hours, temperature 35°C and pH 8.0 were obtained maximum enzyme production 3.832 U/ml. In addition, optimized the carbon and nitrogen sources such as 2.0% glucose (4.967 U/mL) and 1.0% peptone (6.236 U/mL) resulted in maximum enzyme production with rice bran as an effective agro-waste substrate. Laccase enzyme was partially purified via ammonium sulfate precipitation method and chromatography using the sephadex column and determination of the molecular weight of 61 kDa compared with the protein ladder was done by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The findings of this study demonstrated the ability of *Bacillus* sp. AKRC01 using low-cost agro-waste for laccase production. Laccases are important enzymes that have several industrial applications such as bioremediation, bio pulping and food industry.

Keywords: Agro-waste, Bioremediation, Laccases, Optimization, Partial purification, SDS-PAGE

***Correspondence:** prof.chandrabbau@gmail.com

(Received: December 12, 2019; accepted: January 30, 2020)

Citation: Adarsh Kumar, Ajay Kumar Singh, Shamshad Ahmad and Ram Chandra, Optimization of Laccase Production by *Bacillus* sp. Strain AKRC01 in Presence of Agro-waste as Effective Substrate using Response Surface Methodology, *J. Pure Appl. Microbiol.,* 2020; 14(1):351-362. https://doi.org/10.22207/JPAM.14.1.36

Recent Advances in Physicochemical and Biological Approaches for Degradation and Detoxification of Industrial Wastewater

Adarsh Kumar, Ajay Kumar Singh, and Ram Chandra

1.1 Introduction

During the past few decades, water resources are getting scarcer due to exponential growth in population, urbanization, agriculture, and industrialization (Kumar et al. 2020b). The dwindling supplies of natural resources of water have made this a serious constraint for industrial growth and a reasonable standard of urban living. This has made water treatment more costly and is becoming a massive burden for industries to comply with the discharge quality requirement itself. Owing to the release of poor quality wastewater, the degradation of water resources poses a significant hazard to human beings and aquatic life as they depend on water for sustenance (Singh and Chandra 2019; Singh et al. 2020a, b). Most water-intensive industries are the major contributors to cause environmental pollutions. For example, the traditional paper industry needs 300 m^3 of water per ton of product and often generates toxic huge amount of wastewater (Singh and Chandra 2019). Similarly, the mining industry requires 40 m^3 of water per ton of ore, and the oil industry needs 10–300 m^3 of water per ton of output (Chaphekar 2013). The synthetic fertilizer industry also requires a considerable contribution, needing up to 270 m^3 of water per ton of product, and the iron and steel industry needs 20–60 m^3 of water per ton of product (Chaphekar 2013). However, traditional methods for one-liter alcohol

Chapter 29

Extremophilic nature of microbial ligninolytic enzymes and their role in biodegradation

Adarsh Kumar and Ram Chandra
Department of Environmental Microbiology, School for Environmental Science, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, India

29.1 Introduction

Microorganisms are ubiquitous in nature, grown and survive under "extreme" conditions of all the habitats known on the earth. The extreme conditions of the environment affected by various unusual factors for a normal life are hot springs, thermal vents, glaciers, deep oceans, lakes, etc. (Cantrell, Dianese, Fell, Gunde-Cimerman, & Zalar, 2011). The extremophilic microorganisms were classified into psychrophiles, thermophiles, acidophiles, alkaliphiles, barophiles, halophiles, and radiophiles, as shown in Fig. 29.1 (Rampelotto, 2013; Satyanarayana, Raghukumar, & Shivaji, 2005). Extremophiles have built a range of tactics to deal with the effects of the usual adverse parameters through different pathways, that is, cell structure modifications and biochemical activities. In general, to prevent temperature stress, psychrophiles and thermophiles prefer to change their lipid content in the cell membrane, halophiles accumulate osmolytes in the cytoplasm to mitigate the effect of high salt concentrations, acidophiles and alkaliphiles use transport pumps for cell homeostasis, and barophiles withstand high pressures by increasing unsaturated fatty acid content (Padan, Bibi, Ito, & Krulwich, 2005; Reed, Lewis, Trejo, Winston, & Evilia, 2013).

CHAPTER

15

Environmental contamination, toxicity profile and bioremediation approaches for treatment and detoxification of pulp paper industry effluent

Adarsh Kumar[1], **Gaurav Saxena**[2,3], **Vineet Kumar**[2] **and Ram Chandra**[1]
[1]*Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar (A Central) University, Lucknow, India*
[2]*Environmental Microbiology and Biotechnology Laboratory, School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India*
[3]*Laboratory of Microbiology, Department of Microbiology, School of Life and Allied Sciences, Baba Farid Institute of Technology, Dehradun, India*

15.1 Introduction

The rapid population increase due to increased demand for an industrial establishment to meet human needs has created problems such as overexploitation of available resources, resulting in land, air, and water pollution. The paper industry has been considered a major user of natural, resources, that is, water, wood, and power (fossil fuels, electricity) and a major contributor to pollutants directly released into the environment (Kumar and Chandra, 2020). The paper industry ranks sixth among the world's most polluting industries, producing very large-scale toxic effluent (Ugurlu et al., 2007). As per the 2016 annual report of the Central Pulp Paper Research Institute, India's total pulp paper industry comprises about 850 units. Such industries produce effluent that includes organic and inorganic residual compounds. Lignocellulosic waste is discharged from raw material by 55%–60%, while only 40%–45% of pulp is obtained during the pulping process. Approximately 190–200 m^3 of freshwater is utilized per ton of paper production (Chandra and Singh, 2012). The environmental problems of the pulp and paper industry are not limited by the high water consumption. The several sources of wastewater,

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Production, purification and industrial uses of bacterial laccase

Adarsh Kumar, Ajay Kumar Singh and Ram Chandra
*Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb BhimraoAmbedkar University
(A Central University), VidyaVihar, Raebareli Road, Lucknow,
Uttar Pradesh-226025, India
E-mail: prof.chandrabbau@gmail.com

Laccases (EC 1.10.3.2) are copper containing and chemically extracellular monomeric, dimeric and tetrameric glycoproteinsbelongs to the family of blue multicopper oxidases that has three domain structures and usually contains four copper atoms. Laccase have mostly been isolated and characterized from many fungi, bacteria, some higher plants and insects. Laccase producingmicroorganismsculture condition such as period of incubation, pH, temperature and various nutrition parameters such as carbon source, nitrogen source, copper sulphate concentration and solvents were optimized. Guaiacol and acetate buffer were used to assay laccase production. Laccase activity was highest when operated at the following conditions, 72 h incubation, 40°C temperature, and pH-7, 2% Glucose as carbon source and 2% peptone as nitrogen source in the production medium. The enzyme was partially purified by ammonium sulfate precipitation and dialysis method. Laccases are considered to be ideal "*green catalysts*" since they are capable of oxidizing a wide variety of phenolic and nonphenolic compounds, as well as diamines and aromatic amines with concomitant reduction in a straight forward manner, using O_2 from the air and releasing H_2O as the only by

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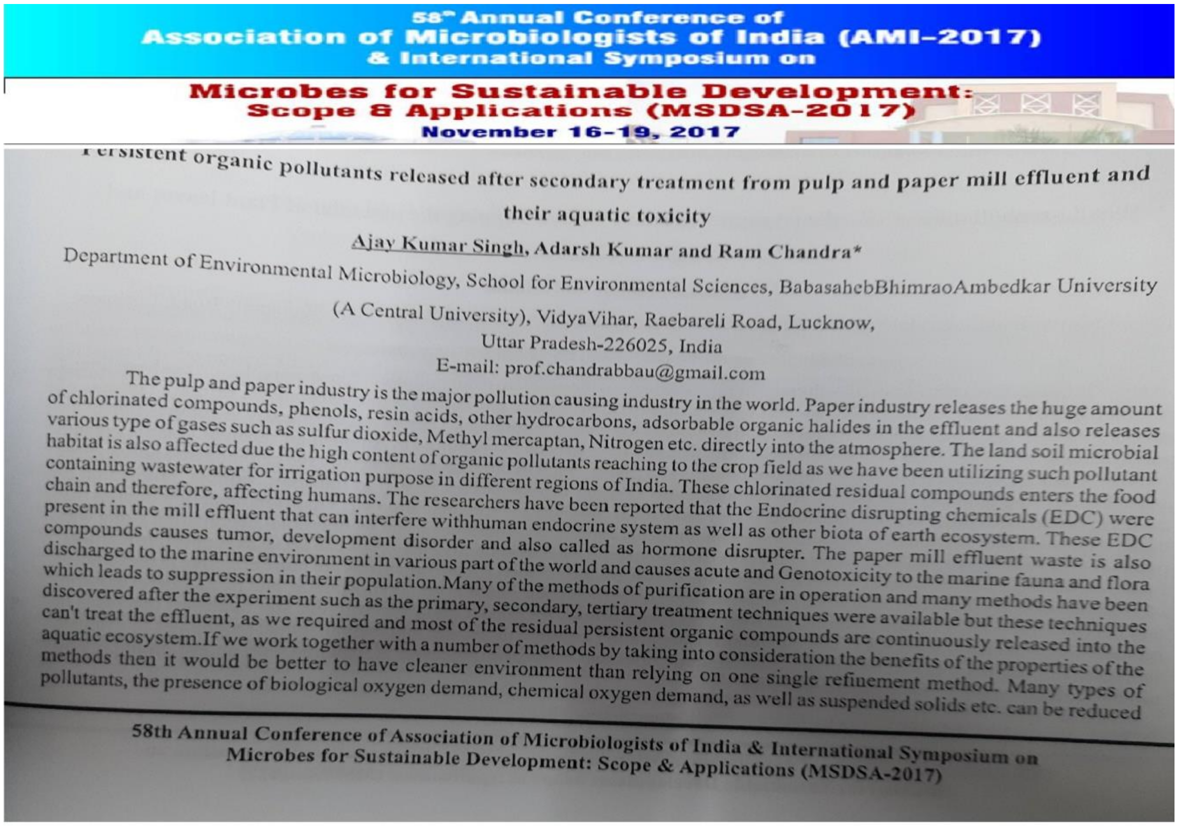

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Persistent organic pollutants released after secondary treatment from pulp and paper mill effluent and their aquatic toxicity

Ajay Kumar Singh, Adarsh Kumar and Ram Chandra*
Department of Environmental Microbiology, School for Environmental Sciences, BabasahebBhimraoAmbedkar University
(A Central University), VidyaVihar, Raebareli Road, Lucknow,
Uttar Pradesh-226025, India
E-mail: prof.chandrabbau@gmail.com

The pulp and paper industry is the major pollution causing industry in the world. Paper industry releases the huge amount of chlorinated compounds, phenols, resin acids, other hydrocarbons, adsorbable organic halides in the effluent and also releases various type of gases such as sulfur dioxide, Methyl mercaptan, Nitrogen etc. directly into the atmosphere. The land soil microbial habitat is also affected due the high content of organic pollutants reaching to the crop field as we have been utilizing such pollutant containing wastewater for irrigation purpose in different regions of India. These chlorinated residual compounds enters the food chain and therefore, affecting humans. The researchers have been reported that the Endocrine disrupting chemicals (EDC) were present in the mill effluent that can interfere withhuman endocrine system as well as other biota of earth ecosystem. These EDC compounds causes tumor, development disorder and also called as hormone disrupter. The paper mill effluent waste is also discharged to the marine environment in various part of the world and causes acute and Genotoxicity to the marine fauna and flora which leads to suppression in their population.Many of the methods of purification are in operation and many methods have been discovered after the experiment such as the primary, secondary, tertiary treatment techniques were available but these techniques can't treat the effluent, as we required and most of the residual persistent organic compounds are continuously released into the aquatic ecosystem.If we work together with a number of methods by taking into consideration the benefits of the properties of the methods then it would be better to have cleaner environment than relying on one single refinement method. Many types of pollutants, the presence of biological oxygen demand, chemical oxygen demand, as well as suspended solids etc. can be reduced

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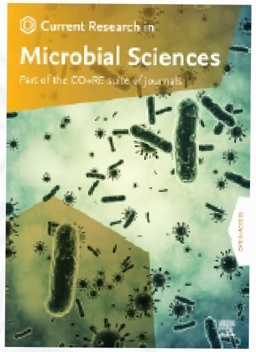

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