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INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Unravelling the characteristics of beneficial bacteria isolated from a polluted site and their application in soil quality enhancement

A thesis submitted

By

SUDHA SAHU

In partial fulfillment of the requirements for the award of the degree of

Doctor of Philosophy

School of Agro & Rural Technology
Indian Institute of Technology Guwahati, Assam
Guwahati-781039, Assam, India

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Dedicated to my Dear Husband (Tulsi)
and Lovely Son (Advait)

SCHOOL OF AGRO & RURAL TECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

STATEMENT

I, undersigned hereby declare that the research embodied in this thesis entitled "**Unravelling the characteristics of beneficial bacteria isolated from a polluted site and their application in soil quality enhancement**" is the result of experiments carried out at the School of Agro & Rural Technology (SART), Indian Institute of Technology Guwahati, India, under the supervision of Prof. Sudip Mitra.

In keeping with the general practice of reporting scientific observations, due acknowledgments have been made wherever the work described is based on the finding of other research.

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CERTIFICATE

It is certified that the work described in this thesis entitled **“Unravelling the characteristics of beneficial bacteria isolated from a polluted site and their application in soil quality enhancement”** by Sudha Sahu (Roll No. 176154004) for the award of degree of Doctor of Philosophy is an authentic record of the result obtained from the research work carried out under my supervision at the School of Agro & Rural Technology, IITG. The work carried out in this thesis has not been submitted elsewhere for a degree.

Date: 25/07/2022

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Table of Content

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Abbreviations

AA = Acetic acid
AAS= Atomic absorption spectrophotometer
ACC = Acetyl-CoA carboxylase
ACP = Acid phosphatase
AET= Agro ecotechnology
As= Aresnic
ATP = Adenosine triphosphate
Av K=Available potassium
Av N=Available nitrogen
Av Na=Available sodium
Av P=Available phosphorus
BC=Biochar
BNF = Biological nitrogen fixation
BLAST = Basic local alignment tools
C = Carbon
CA = Citric acid
Ci = Initial concentration
Ce = End concentration
Cd= Cadmium
CEC=Cation exchange capacity
cFDA = Carboxyfluorescein diacetate
CFU = Colony-forming unit
Chla=Chlorophyll a
Chlb=Chlorophyll b
CK= Control
Cr=Chromium
Cu=Copper
D1= Dumping site sampling location
DAI = Day after incubation
rDNA = Ribosomal deoxynucleic acid
DTPA= Diethyl triamine pentaacetic acid
EDX= Energy dispersive X-ray spectroscopy
EPS = exopolysaccharide
FAO = Food and Agriculture Organization
FESEM= Field emission scanning electron microscopy
FETEM = Field emission transmission electron microscopy
FTIR= Fourier transform infrared
GA = Gluconic acid
HM=Heavy metals
IR = Infrared
IARC = International Agency for Research on Cancer
MA = Malonic acid
MBC=Microbial biomass carbon
MBP = microbial biomass phosphorus

MIC = Minimum inhibitory concentration
Mn=Manganese
N = Nitrogen
NCBI = National center for biotechnology information
Ni=Nickel
NFB = Nitrogen fixing bacteria
OC=Organic carbon
OD = Optical density
OM=Organic matter
OTUs = Operational taxonomic units
P = Phosphate
PI = Propidium iodide
K = Potassium
HCN = Hydrogen cyanide
Pb=Lead
PCR= Polymerase chain reaction
PSB = Phosphate solubilizing bacteria
PVK = Pikovskaya
rRNA = Ribosomal ribose nucleic acid
rDNA = Ribosomal deoxynucleic acid
SEM-EDX = Scanning electron microscopy-energy dispersive X-ray
SI = Solubilizing Index
TCA = Tricarboxylic acid
^{232}Th = Thorium - 232
^{238}U = Uranium - 238
UV–Vis Spectrophotometer = UV-Visible Spectrophotometer
^{210}Po = Polonium - 210
XRD= X-Ray diffraction
YEMA = Yeast extract mannitol agar
Zn=Zinc

Units

° C= Degree centigrade
cm= Centimeter
cm^{-1}= Wavenumber
cmol kg^{-1}= Centimole of charge per 1 kilogram
d year^{-1}= Days per year
g = gram
g cm^{-1}= Gram per centimeter
g L^{-1} = Gram per litter
g kg^{-1}= Gram per kilogram
g= Gram
h= Hours
K min^{-1}=Kelvin per minute
Kg = Kiligram
kg ha^{-1}=Kilogram per hectare
m= Meter
mg g^{-1} = Microgram per gram
nm = Nanometer
µM = Micromolar
µ mol L^{-1} h^{-1} = Micromolar per litter per hour
µg g^{-1}=Microgram per gram
µg= Microgram
µg C g^{-1} = Microgram per centigram
mg L^{-1} = microgram per litter
M=Molar
mol L^{-1} = Molar per litter
mL=Mililiter
mg = Miligram
mg cm^{-2} h^{-1}= Miligram per centimeter per hour
mg d^{-1}=Miligram per day
mg gm^{-1}=Miligram per gram
mg kg^{-1}= Miligram per kilogram
mg L^{-1}=Milligram per liter
mg= Miligram
min=Minutes
mM= Milimolar
mS cm=Micro Siemens
mS cm^{-1} = Micro Siemens per centimeter
mol kg^{-1}. = Molar per kilogram
nm=Nanometer
1L Acre^{-1} = One litter per acre
ppm= Parts per million
2θ ° = Two theta
t ha^{-1}= Tonnes per hectare
v/v=Volume/ Volume
w/v= Weight/ Volume
wt= Weight

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Abstract

In today's world, emerging economies such as India face a greater challenge toward sustainable agriculture. Farmers have been encouraged to use chemical fertilizers and pesticides indiscriminately to grow more food in less area. As a result, the yield has increased, but these benefits have been negated by environmental damages. It also harmed soil fertility, which in turn had a negative impact on agricultural yield. On the other side, uncontrolled population growth, urbanization, and socioeconomic progress have boosted living standards. These generate massive amounts of municipal solid trash worldwide, particularly in India. It primarily pollutes soil and water with various pollutants such as heavy metals and other comparable things. Both of these scenarios devastate the earth's environment and increase the toxicity in the foodecosystems. Biofertilizers with pollution remediation properties are being considered as a possible solution to this persistent problem. Present thesis research aims to address this issue. Soil samples were collected from the Boragaon dumping site in Guwahati, Assam contaminated with municipal solid waste (MSW) and has a common border with the ecologically sensitive Deepor Beel area (Ramsar site No. 1207). According to the analysis, these soil samples had the least soil nutrition and the high heavy metals level. With selective media, approximately 400 Phosphate solubilizing bacteria (PSB) and 350 N fixing bacteria (NFB) were isolated from soil samples.

Following molecular characterization, the diversity of *Bacillus* sp., *Enterobacter* sp., and *Paraburkholderia fungorum* was discovered. Furthermore, the PSB were cultured in Pikovskaya (PVK) media, resulting in organic acid and enhanced acid phosphatase (ACP) activity. SM_ SS8 had the best Cd and Cu tolerance capacity among all the tested strains (up to 100 mg L^{-1}). The growth pattern of SM_SS8 was investigated using optical density (OD) and kinetic growth measurements. Infrared spectroscopy (IR) and X-Ray Diffraction (XRD) studies were used to examine the strain's Cd and Cu degradation capability. The optimum concentration of Cd and Cu bio-precipitation in the SM_SS8 cell has been studied using SEM-EDX. In Cd-treated bacterial cell cultures, flow cytometry analysis revealed that 70.92%, 46.93%, and 20.4% of viable SM_SS8 bacterial cells were identified in 10, 50, and 100 mg L^{-1}, respectively. The potential of biochar, biofertilizer, and their formulation to remediate HMs-contaminated soils and improve soil quality and plant production (like tomato) was also investigated through a pot experiment. The pot experiment results demonstrated that biofertilizer and biochar have a synergistic impact, resulting in improved soil nutrition and plant growth. Simultaneously, bacterial taxonomic analysis of harvested soil samples revealed that, the treated samples had higher diversity when compared to CK. The findings showed that a single biofertilizer and its formulation were best for various soil features such as physico-chemical, microbial diversity, and biochemical parameters.

Keywords: Bioremediation; Municipal solid waste (MSW); Phosphate solubilizing bacteria (PSB); Nitrogen Fixing Bacteria (NFB); Heavy metals (HMs).

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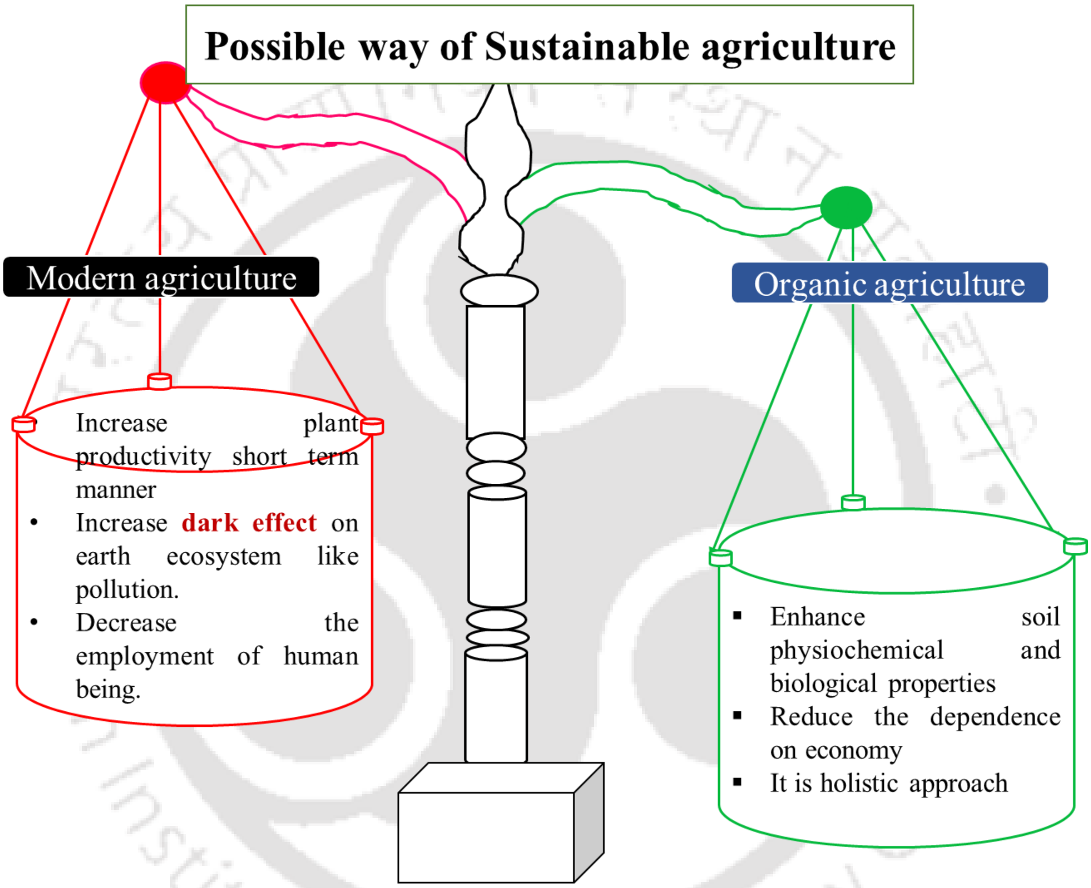
CHAPTER 1

Introduction



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This chapter describes how to improve soil quality, the effect of synthetic fertilizers and heavy metals on soil quality, and how these factors affect the ecosystem and human health. It also deliberates the specific objectives and structure of the thesis.

1. Introduction

1.1 Background

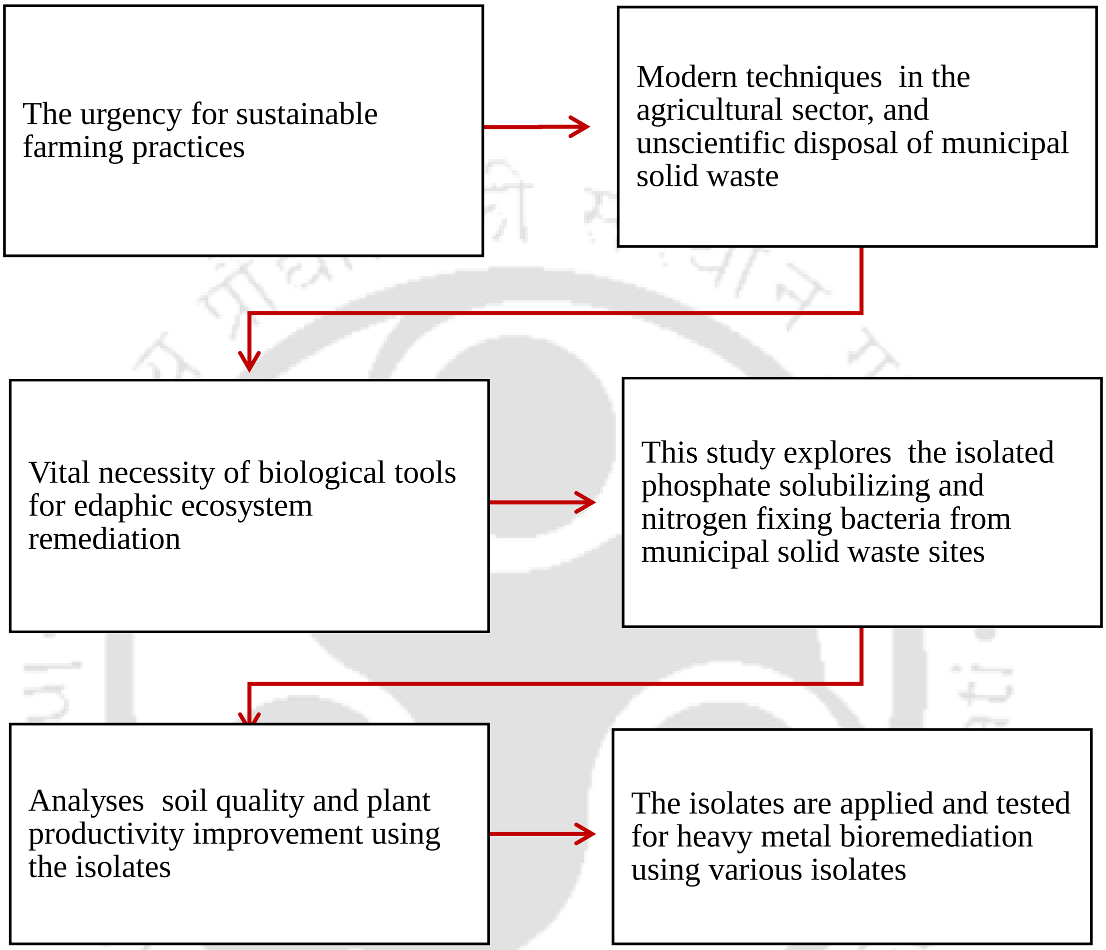

Fig. 1.1 A brief introduction to the thesis.

As per the UN Food and Agriculture Organization (FAO), food production will require an increase of 70% by 2050 to feed the world's population (Dorling, 2021; FAO and UNICEF, 2020). On the other hand, the dual pressures from population growth and economic development have escalated the degradation of the edaphic environment. Farmers remain vulnerable and inadequately equipped to deal with the rapid changes, particularly in nations that form part of the low income group. A quick fix solution has been the global acceptance and application of chemical fertilizers and pesticides according to the world food program (Fig. 1.1).

These chemical fertilizers are synthesized artificially with prescribed amounts of N, P, and K (Bhardwaj et al., 2014). The application of chemical fertilizers has frequently increased short-term productivity while simultaneously lowering both soil quality and long-term output. As a result, long-term exploitation would pollute natural resources such as soil, water, and air (Fig. 1.1) (Sahu et al., 2022). Pesticides are made up of synthetic or organic substances, while chemical fertilizers mostly contain phosphate, nitrate, ammonium, and potassium salts. The fertilizer industry is also a possible source of natural radionuclides and heavy metals (HMs). It contains many HMs such as Hg, Cd, As, Pb, Ni, and Cu and natural radionuclides such as ^{238}U, ^{232}Th, and ^{210}Po. In addition, arsenic (calcium arsenate and lead arsenate) and a fumigant hydrogen cyanide were used in the first generation of pesticides (Sahu et al., 2022).

On the other hand, India has multiple issues due to its rapid rampant urbanization and ever-increasing urban population. The increased generation of non-segregated municipal solid waste (MSW) and its unregulated dumping in open dumping sites is a major fallout of these issues (Joshi and Ahmed, 2016; Karak et al., 2012). As a repository for organics and HMs, an open dumping site poses a serious threat to human health and the environment, including the soil. These degraded soil will eventually endanger the long-term viability of agricultural production, besides seriously impacting and cause long-term harm to the environment and human health

(Khorshid and Thiele-Bruhn, 2016). HMs are cytotoxic, buried, persistent, and biological accumulators (Sinha et al., 2009). Consequently, soil quality which is determined by various biological and non-biological processes, is adversely influenced by HMs (Gujre et al., 2021a, 2021b). The current demands for food and sustainable agriculture, could be resolved by using soluble fertilizers and soil amendments, which can increase food production while simultaneously improving soil fertility and avoiding environmental damage (Bera et al., 2017).

The use of biological systems such as plants and plant growth promoting rhizobacteria (PGPR) to remediate (HMs) damaged environments is an increasingly important area of research (Yuan et al., 2017). Microorganism-based bioremediation of HMs is more efficient, cost-effective, and environmentally friendly. The ensuing reduction metal toxicity and improvement in soil quality, will lead to increased food production over time (Sowmya et al., 2020). In addition, the microbes possess an inherent ability to scavenge and fix macronutrients such as P, K, and N in the soil and make them available to plants (Nacoon et al., 2020; Parastesh et al., 2019). MSW dumping sites are among the most difficult, as evidenced by changing temperatures, acidic pH, precipitation, and the disposal of various types of wastes (Ben Hamed et al., 2020).

Agroecotechnological (AET) techniques are frequently seen as the most effective means of addressing challenges related to agriculture and the environment. Some of the strategies adopted for the AET are therefore listed.

- Development of cyclic, sustainable, and chemical-free agritechniques.
- Conservation of resources during farming cultivation.
- Protection and sustaining environmental biodiversity during the farming process.
- Implementation of a multi-crop, integrated management system.
- Application of biological mechanisms to improve soil quality.

- Maintenance of a balanced input-output system by the AET lab.

1.2 Objectives of the study

Following objectives of the present study:

1. To isolate and screen N fixing and P solubilizing bacteria from the municipal dumping site's soil.
2. To characterize N fixing and P solubilizing bacteria through morphological, biochemical and molecular analysis.
3. To evaluate the remediation potentials and carrying out the enzymatic assay of screened P solubilizing bacteria
4. To evaluate the qualitative and quantitative impact of N fixing and P solubilizing bacteria on soil and plant.

1.3 Overview of thesis organization

Chapter 1:

The first chapter of the thesis presents a brief discussion on the problems and challenges for sustainable agriculture and soil quality management. Special emphasis is brought to bear on the two major sources of soil pollutants generated by chemical fertilizers and MSW dumping sites. The scope of characterization of P solubilizing and N fixing bacteria isolated from MSW site soils and application in soil amendment is delineated with reference to the objectives of the present thesis.

Chapter 2:

A comprehensive and in-depth overview of the literature on the current state of soil quality in India and around the world is the mainstay of this chapter. The previous studies, background information, and existing research gaps are discussed in relation to the present thesis. In addition, the challenges encountered in soils contaminated by MSW waste, chemical fertilizers, chemical pesticides, along with biofertilizers and their role in improving soil quality are critically examined.

Chapter 3:

This chapter focuses on collecting soil samples from multipollutant areas like MSW sites. The physico-chemical parameters of the collected contaminated soil are examined in order to determine its quality status. In addition, N fixing and P solubilizing bacteria are isolated based on their ability to grow on selective media. The screening of isolated strains, which is also done on selective growth media to understand the potency for various soil quality upliftment, is a very important part.

Chapter 4:

Fourth chapter of the thesis focuses on the characterization of selected N fixing and P solubilizing bacteria. Basically, morphological, biochemical and molecular characterizations were carried out. The bare eyes method is employed for morphological characterization. Furthermore, biochemical examination for a particular bacterial strain is conducted using the gram staining method. Four PSB and four NFBs were chosen for molecular analysis based on morphological and biochemical selection criteria.

Chapter 5:

Furthermore, microbes have long been recognized as effective biodegradation instruments for improving soil quality. However, there are not enough reports on extracting beneficial bacteria from MSW-contaminated soils. Hence, the fifth chapter focuses on the analysis of the P solubilizing activity by isolated PSB. Therefore, acid phosphatase, organic acid assay, microbial biomass phosphate, and pH assays are used to assess P solubilizing activity. In addition, selected PSB are used in the Cd and Cu bioremediation. Multiple growth models are used to understand the HMs bioremediation potency of selected PSB on different concentrations of Cd and Cu stress. Cell viability study by flow cytometer is used to assess the selected strain Cd and Cu bioremediation capacity, then cross-verified by FT-IR, XRD and EDX investigation.

Chapter 6:

Sixth chapter illustrates the qualitative and quantitative impacts of N fixing and P solubilizing bacteria on soil and plants. Further, the soil quality upliftment and bacterial biodiversity enhancement are computed from applications based on pot experiments. Different formulations of isolated strains and BC with plant growth-promoting qualities are applied in the contaminated MSW site soil. Tomato (*Solanum lycopersicum*), a flowering plant of the nightshade family (*Solanaceae*), is also cultivated and used to remediate Cd-contaminated soil. The life cycle of tomato cultivation is completed around four months. To evaluate the impact of formulations, various analyses of post-harvest soil and plant were carried out.

Chapter 7:

This is the last chapter, which explains about the learning out of this thesis research and future research needs. It is worked out by adding up all the results of the research objectives as listed elsewhere in this thesis. This section goes into greater detail on the benefits of using biofertilizers. In addition, the importance of biofertilizer and its application in combination with biochar in different economic and environmental conditions is discussed.

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The organization of entire thesis is depicted in the below Fig. 1.2.

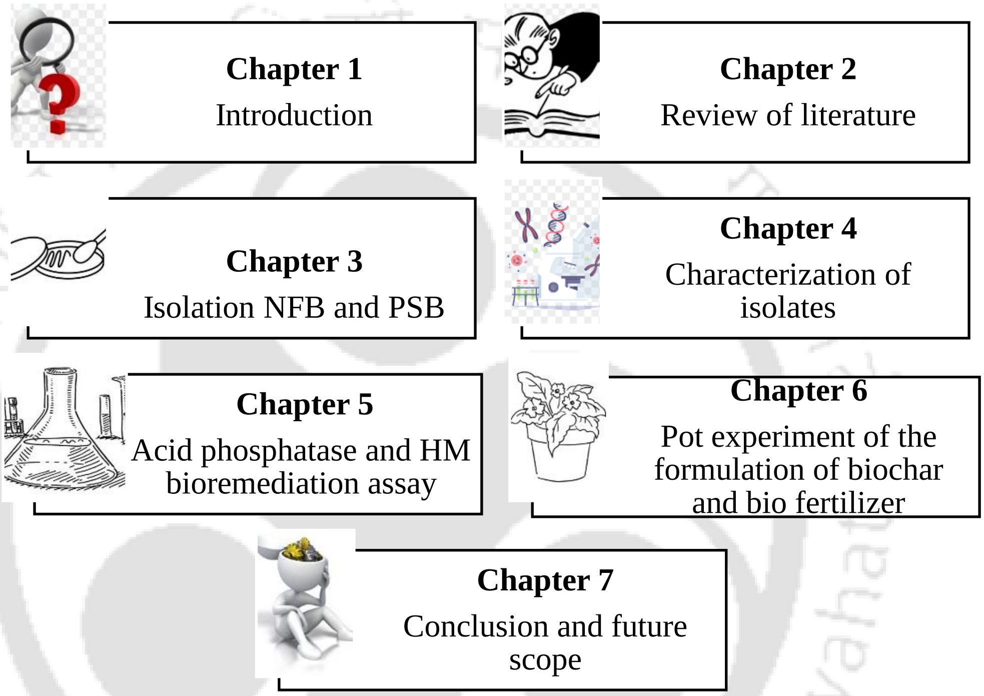

Fig. 1.2 Overview of thesis chapters.

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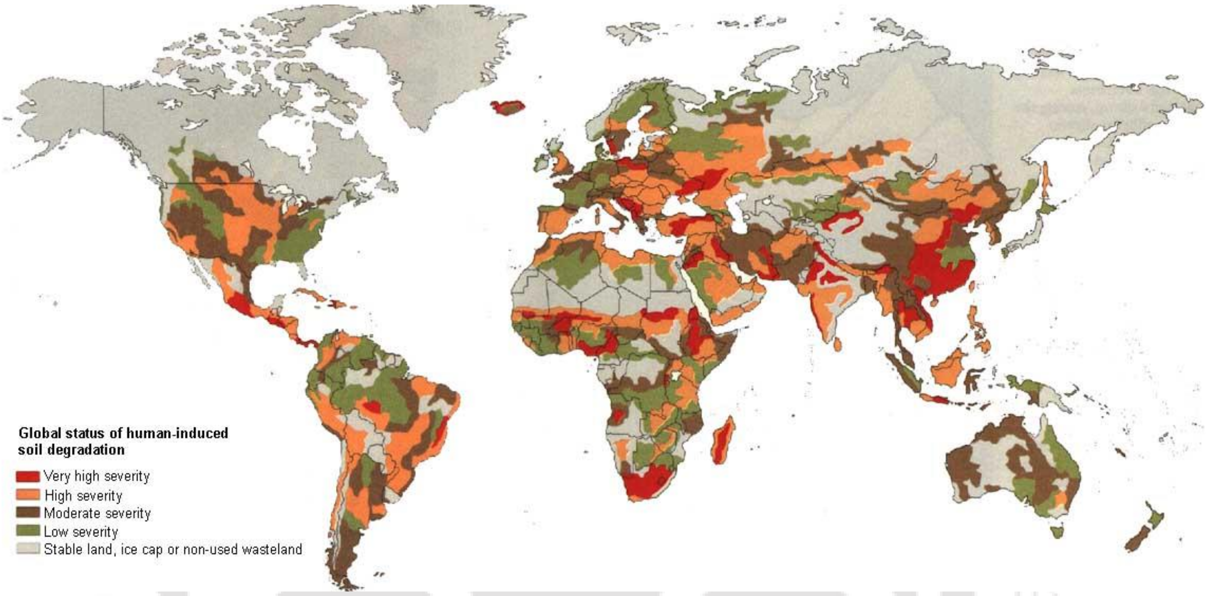
CHAPTER 2

Review of literature



Global status of human induced soil degradation **(**Sources: SET, 2021**)**

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This chapter includes a comprehensive and in-depth overview of the literature on the current state of soil quality in India and around the world. It describes the challenges of soil that have been contaminated by MSW waste, chemical fertilizers, and chemical pesticides. This chapter also discusses biofertilizers and their role in improving soil quality.

2. Review of literature

2.1 Need for soil quality management

The limited amount of land available for food support is severely constrained and damaged by the enormous demand for food, rapid population increase, and economic development (Belay et al., 2002). Since the Green Revolution of the 1960s, agriculture practices involving an exponential rise in application of chemical fertilizers and pesticides have been adopted to satiate the humungous human population. Therefore, the International Year of Soils in 2015, endorsed the dual concerns of soil sustainability and scientific land management to target deep concerns of soil health to support a food secure world. In the same year, Brito et al.,(2015) drew attention to land deterioration by labelling it as a "global pandemic", wherein soil erosion, salinity , acidity, and compaction are facets of soil degradation.

2.2 Chemical fertilizer and its effect on the environment

Chemical fertilizers and pesticides are both non-organic chemicals. Fertilizers generally contain phosphate, nitrate, ammonium, and potassium salts, while pesticides are made up of synthetic or organic compounds. According to an analysis from the Department of Agriculture, chemical fertilizers can assist increase food production while meeting economic criteria for boosting soil fertility and avoiding environmental damage (Mahanty et al., 2017). As a result, efficient solutions are needed to lessen the environmental impact of chemicals while also increasing plant growth and productivity in the long-term (Pretty and Bharucha, 2015).

Natural radionuclides like ^{238}U, ^{232}Th, and ^{210}Po and HMs like Hg, Cd, As, Pb, Cu, Ni, and Cu and are surmized to have arisen in abundant quantities from fertilizer industry. HMs at unsafe levels have been discovered in MSW dumpsites in developing nations. They are recognized as a global environmental threat by their uniq ue qualities of remaining concealed, long-term degradability, and irreversibility (Fig. 2.1) (Ma et al., 2020). HMs are easily can be biomaginifed in living things and constitute a health hazard to humans (Gujre et al., 2021a, 2021b; Huang et al., 2019; Ma et al., 2020). In vertebrate and invertebrates, HMs have been reported to induce oxidative stress, interfere in protein folding, and lead to failure of physiological functions (Abdu et al., 2017).

Among the different HMs, Cd is the most toxic to living organisms even at a very low concentration of <5 µM (Jeong et al., 2013). Moreover, it responds very slowly to the bioremediation processes; consequently, it remains in the soil for a prolonged time period. In addition, cadmium has been classified as carcinogenic to humans by the World Health Organization and the International Agency for Research on Cancer (IARC, 2018). Every kg of commercial phosphate fertilizer adds approximately 200 mg Cd in soil. Electronic wastes and deposited material are the two major sources of Cd in the agricultural soil, and it is a severe threat to food safety (Tansel, 2017). Wastes generated from different industries, such as pulp and paper, cement, automobile tyres, lubricants, mining and metallurgy are also the major Cd sources (Borah et al., 2018).

Thus , the development of new methods and strategies for the remediation of environmental pollution due to HMs critical (Sowmya et al., 2020). Phytoremediation is emerging to be the most preferred method to remediate soil contaminated with HMs, but its application is limited due the slow process and inefficient removal of HMs (Sahu et al., 2022; Teng et al., 2019). Compared with phytoremediation and conventional physico-chemical methods, microorganisms based bioremediation of HMs is considered more efficient and cost-effective as well as eco-friendly (Gadd, 1992; Teng et al., 2020).

Amongst the different microorganisms, bacteria and fungi have been part of bioremediation strategies to reduce metal toxicity and increase soil quality, thereby sustainably increasing food productivity (Sowmya et al., 2020). However, there are some concerns connected with HMs toxicity that could subject microorganisms (mostly bacteria) to grave danger during bioremediation, even at very low concentrations (Bertin and Averbeck, 2006; Choi, 2009). HMs are credited with altering the variety, population number, and general biological system of microbial communities in the pedosphere at very low concentrations (Meena et al., 2020; Nies, 1992).

2.3 Biofertilizer and its use

Soluble fertilizers and soil amendments can help improve food production while meeting economic criteria for improving soil fertility and avoiding environmental damage, according to a report by the Department of Agriculture (Fig. 2.1) (Mahanty et al., 2017). Biofertilizers are soil-borne living cells or latent cells of successful microorganism strains that help agricultural plants absorb nutrients more efficiently (Fig. 2.1) (Ashrafuzzaman et al., 2009; Mahanty et al., 2017). When administered through seed or soil, it uses biological mechanisms in the rhizosphere to convert unavailable nutrient forms to available nutrient forms (Malusá et al., 2012; Malusá and Vassilev, 2014). The use of biofertilizers to remediate HM polluted sites has garnered a lot of interest in the last decade (Fig. 2.1) (Yuan et al., 2017).

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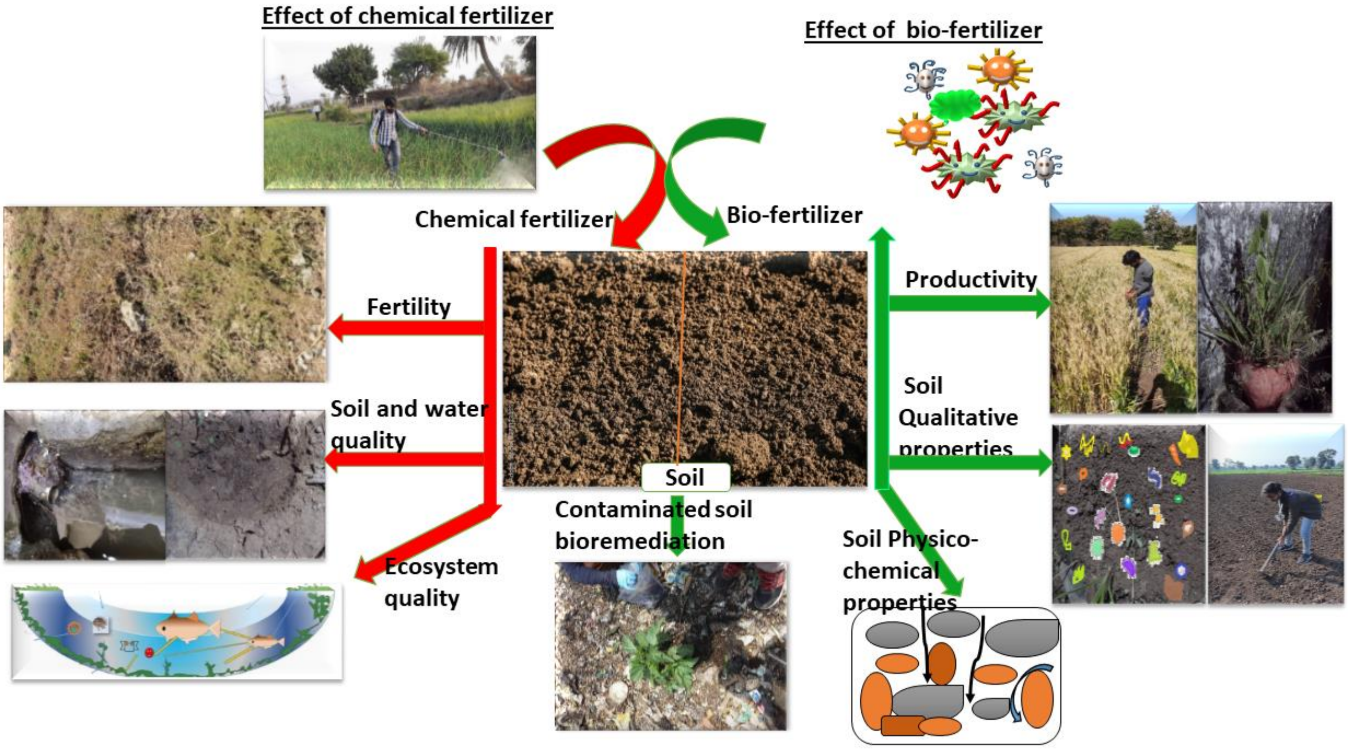

Fig. 2.1 Effect of biofertilizer and chemical fertilizer on plant, soil and ecosystem.

For example, Cyanobacteria and Proteobacteria fix the N from the atmosphere. The bioavailability of N, P, K, and Fe and soil aggregation were studied using inocula of Cyanobacteria and Proteobacteria with fungus (Rashid et al., 2016). *Bacillus*, *Azospirillum*, *Azocarus*, and *Azorhizobium* assisted in N fixation while also creating a variety of plant growth-promoting compounds (Ochoa-Velasco et al., 2016; Karimi et al., 2018; Sabry et al., 2018; Ochoa-Velasco et al., 2018). Many bacterial genus such as *Bradyrhizobium*, *Pseudomonas*, *Bacillus*, *Leclarcia*, and *Enterobacter* sp. SM _SS8 aid in the availability of P and the production of siderophores, IA, and HCN (Afzal & Bano, 2008; Yu et al., 2020; Mukhtar et al., 2017; Teng et al., 2019; Sahu et al., 2022). Additionally, *Pseudomonas* sp., *Paenibacillus* sp., *Bacillus* sp., and *Aspergillus* sp. aid in P and K-solubilization, increasing plant production and nutrient availability and absorption (Lynn et al., 2013; Sangeeth et al., 2012; Ali et al., 2021; Muthuraja & Muthukumar, 2022). Another group of bacteria, including *Enterobacter*, *Agrobacterium*, *Serratia*, *Pseudomonas*, *Bacillus*, and *Bradyrhizobium*, can bioremediation HMs such as Cd, Pb, U, Zn, Ni. HMs bioremediation has been completed by several mechanisms such as bioprecipitation, biotransformation, bioaccumulation, bioleaching, and biomineralization, according to various studies. These bacteria also produce compounds that aid plant development and production (Sahu et al., 2022; Jiang et al., 2020; Li et al., 2020; Li et al., 2020; Ma et al., 2020; Sowmya et al., 2020).

To speed up microbial processes that reduce the cost of nutrients that plants can easily assimilate (Fig. 2.1). To improve soil fertility by fixing atmospheric nitrogen, solubilizing insoluble phosphates, and producing plant growth-promoting substances in the soil (Mazid and Khan, 2015). They also harvest a naturally occurring biological nutrient mobilization system, which dramatically improves soil fertility and as a result crop productivity (Pandey and Singh, 2012). Several investigations on the role of biofertilizers in bioremediation of metal toxicity have indicated important roles essayed by a varied spectrum of microorganisms (Dixit et al., 2015). Consequently, the potential of biofertilizers in several areas, such as agriculture, ecology, and pollution remediation has positioned biofertilizers as promising agents for long-term agriculture development.

2.3.1 Nitrogen fixing bacteria (NFB)

One of the most important nutrients for living things is nitrogen. It is a component of living macromolecules such as nucleic acid, protein, vitamins, and lipids. Plants and their products provide a simple source of nitrogen that aids in growth and development. Although 78% of N_2 is present in the atmosphere, plants cannot use it. The nitrogen must first be transformed to a useful form that plants can easily ingest via the biological nitrogen fixation (BNF) process (Fig. 2.2) (Tairo and Ndakidemi, 2013). NFB convert atmospheric nitrogen to ammonia during biological nitrogen fixation (BNF) utilizing an enzyme complex known as *Nitrogenase*

(Mehes-Smith et al., 2013). Organisms that fix nitrogen might be symbiotic or non-symbiotic. Members of the *Rhizobiaceae* family form a symbiotic connection with leguminous plants (Table 2.1) (Ahemad and Khan, 2012). Similarly, microorganisms such as Cyanobacteria, *Azospirillum*, and *Azotobacter* are examples of non-symbiotic organisms (Bhattacharyya and Jha, 2012). However, the identified microorganisms like *Azocarus, Azorhizobium Beijerinckia* are having good amount of N fixing potency whereas the some bacterial species like *Burkholderia, Bacillus licheniformis* and *Cyanobacteria, Proteobacteria* are also having other properties like antibiotics production and plant growth promoting activity and many more (Table 2.1).

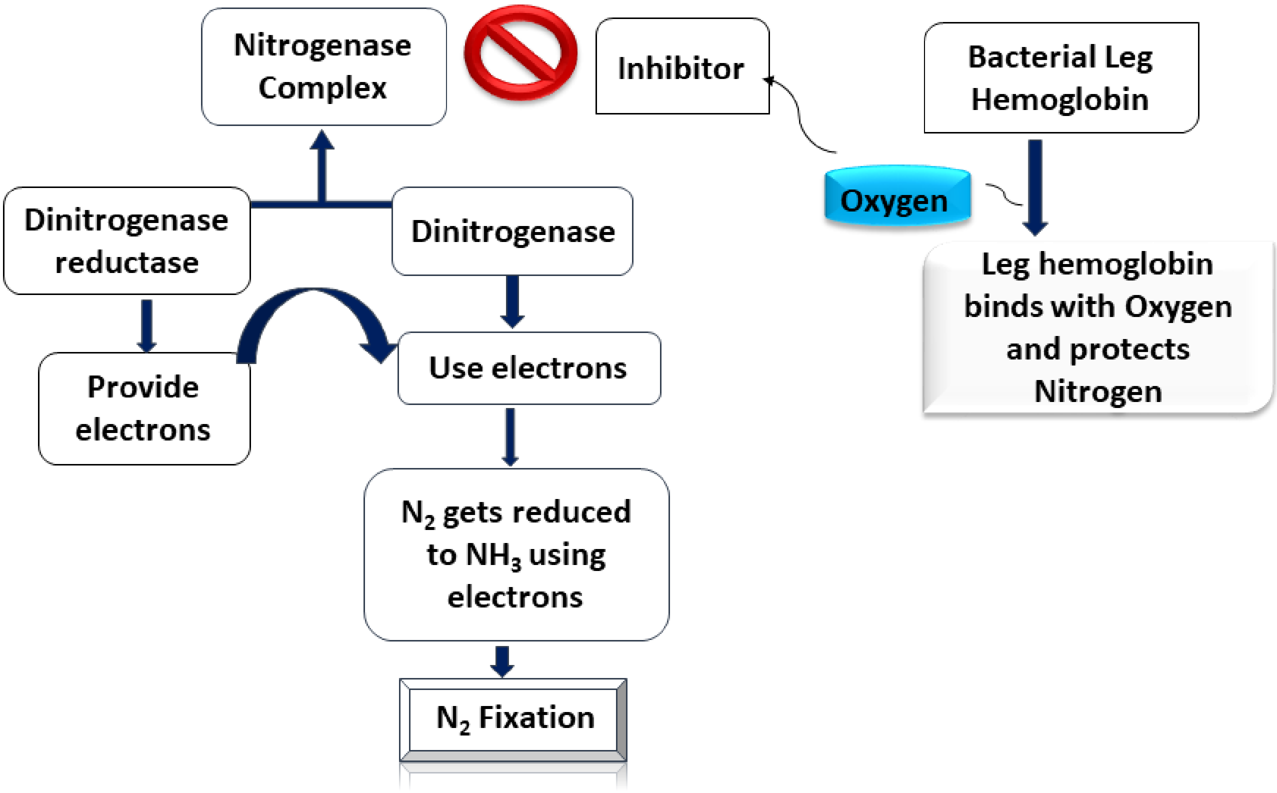

Fig. 2.2 Mechanisms of nitrogen fixation by NFB.

Table 2.1 Roles of the NFB for plant growth promotion.

S. No	Type of biofertilizers	Role in plant growth promotion	References
1	*Azocarus*	N fixation	(Reinhold-Hurek and Hurek, 1998)
2	*Azorhizobium*	N fixation	(Sabry et al., 1997)
3	*Beijerinckia*	N fixation	(De Felipe et al., 2006)
4	*Burkholderia*	N fixation, Producing antibiotics	(Govindarajan et al., 2008)
6	*Frankia*	N fixation	(Simonet et al., 1990)
7	*Gluconacetobacter*	N fixation	(Muñoz-Rojas and Caballero-Mellado, 2003)
8	*Herbaspirillum*	N fixation	(Elbeltagy et al., 2001)
9	*Streptomyces*	N fixation	(Dahal et al., 2017)
10	*Rhizospheric bacteria*	N fixation	(Navarro-Noya et al., 2012)
11	*Azolla*	N fixation	(Yao et al., 2018)
12	*Cyanobacteria, Proteobacteria*	N fixation (bacterial and fungal inocula prepared bioavailability {N, P, K and Fe} and aggregation of the soil.)	(Rashid et al., 2016)
13	*Bacillus licheniformis*	N fixation and antioxidant	(Ochoa-Velasco et al., 2016)
14	*Cyanolichens*	N fixation	(Darnajoux et al., 2017)
15	*Azospirillum*	N fixation	(Karimi et al., 2018)
16	*Azocarus*	N fixation	(Reinhold-Hurek and Hurek, 1998)
17	*Azorhizobium*	N fixation	(Sabry et al., 1997)

2.3.2 Phosphate solubilizing bacteria (PSB)

All living things require phosphorus. It is found in the form of phosphates (PO_4) and functions as a molecule that stores energy (e.g., ATP), besides being a constituent of biomolecules like nucleic acids, lipids, vitamins, and certain proteins. Plants can only take phosphorus in two soluble forms: monobasic (e.g., NaH_2PO_4) and dibasic (e.g., Na_2HPO_4). Inositol phosphate (soil phytate), phosphomonoesters, and phosphotriesters are examples of insoluble phosphorus in both inorganic and organic forms (Mahdi et al., 2012). The mechanism of P and K solubilization from insoluble form using bacteria has been outlined (Fig. 2.3). Some bacteria can solubilize the P and K by their biological mechanism and improve soil nutrition and plant productivity (Table 2.2 & 2.3). Some species of *Mesorhizobium*, *Bradyrhizobium*, *Arthrobacter*, *Acinetobacter*, *Leclarcia*, *Bacillus* were showing production and secretion of siderophores, IA, HCN, antibiotics, cell wall degrading enzymes, chitinases, exopolysaccharides, ACC deaminase, IAA, Antifungal activity and organic acid other than P solubilizing and K mobilization (Table 2.2 & 2.3)

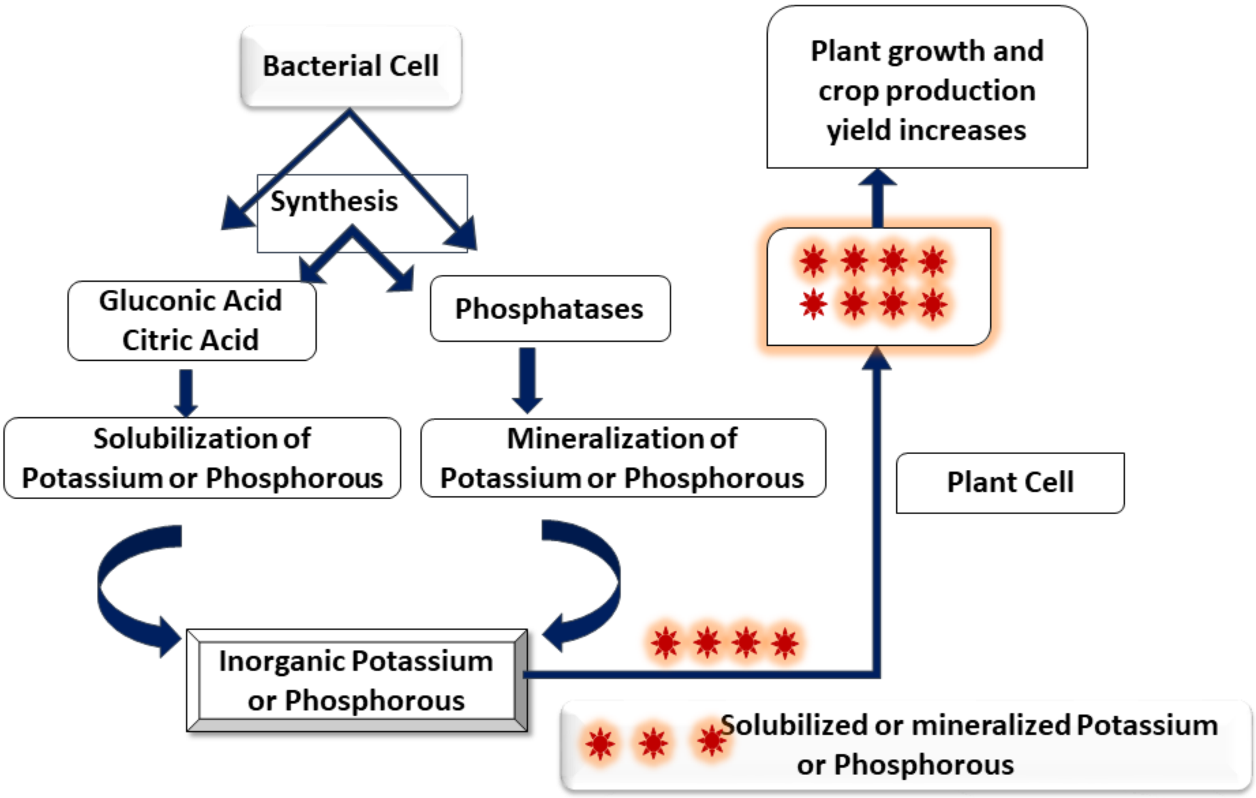

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Fig. 2. 3 Mechanisms of P and K solubilization by PSB and KSB.

Table 2.2 Roles of the PSB for plant growth promotion.

S. No	Type of biofertilizers	Role in plant growth promotion	References
1	*Rhizobium leguminosarum*	Solubilization of minerals such as phosphorous and cytokinin, Secreting antibiotics and cell wall degrading enzymes	(Afzal and Bano, 2008)
2	*Mesorhizobium mediterraneum*	Phosphate solubilization	(Afzal and Bano, 2008)
3	*Bradyrhizobium* sp.	P solubilization, siderophores and IA, Producing HCN	(Afzal and Bano, 2008)
4	*Bradyrhizobium japonicum*	P solubilization, siderophores and IA, Secreting antibiotics and cell wall degrading enzymes	(Afzal and Bano, 2008)
5	*Arthrobacter*	P solubilization, Chitinases	(Bhattacharyya and Jha, 2012)
6	*Burkholderia*	P solubilization	(Bhattacharyya and Jha, 2012)
7	*Enterobacter asburiae*	P solubilization, siderophores and IA, HCN, exopolysaccharides	(Ahemad and Khan, 2010)
8	*Acinetobacter sp.*	P solubilization, ACC deaminase and IAA, Antifungal activity	(Indiragandhi et al., 2008)
9	*Flavobacterium*	P solubilization	(Bhattacharyya and Jha, 2012)
10	*Microbacterium pseudomonas*	P solubilization	(Bhattacharyya and Jha, 2012)
11	*Rhodococcus*	P solubilization	(Bhattacharyya and Jha, 2012)
12	*Erwinia*	P solubilization, Synthesizing Ethylene	(Bhattacharyya and Jha, 2012)
13	*Pantoea ananatis Rahnella aquatilis and Enterobacter sp.*	P solubilization and IAA production	(Bakhshandeh et al., 2017)
14	*Bacillus megaterium, Staphylococcus haemolyticus and B. licheniformis*	P solubilization, IAA production, ammonium ion production	(Biswas et al., 2018)
15	*Pseudomonas prosekii* YLYP6	P solubilization	(Yu et al., 2020)
16	*Bacillus sps.*	P solubilization	(Mukhtar et al., 2017)
17	*Leclarcia adecarboxylata*	By phosphate solubilization, organic acid	(Teng et al., 2019)
18	*Enterobacter sp. SM_SS8*	P solubilization, different organic acid	(Sahu et al., 2022)

2.3.3 K Solubilizing bacteria (KSB)

After nitrogen and phosphorus, potassium (K) is the third most important nutrient for plant development and growth (Hu et al., 2006; Mazahar and Umar, 2022; Parmar and

Sindhu, 2013). When present in the soil solution, K plays a role in various physiological and biochemical functions in plants, including cell osmotic control and enzyme activation (Valmorbida and Boaro, 2007). The soil solution, or soluble K fraction, is, nevertheless, quite low in the soils. This is primarily due to insoluble rocks, minerals, and other deposits that contain a major portion of the K (Goldstein, 1995).

Table 2.3 Roles of the K solubilizing bacteria for plant growth promotion.

S.No.	Type of biofertilizers	Role in plant growth promotion	References
1	*Paenibacillus glucanolyticus*	Enhanced K uptake and dry weight in inoculated plants	(Sangeeth et al., 2012)
2	*Bacillus edaphicus*	Improved plant growth and yield	(Paau, 1989)
3	*Bacillus. mucilaginosus KCTC 3870*	Increased P and K uptake	Paau (Paau, 1989)and Lee, 2005
4	*Ectomycorrhizal fungi (UFSC-Pt22 and UFSC-Pt186)*	Enhanced N, P, K content and plant growth	(Alves et al., 2010)
5	*Pseudomonas putida and P. fluorescens*	Increased root and shoot growth and increase the nutrition x	(Glick and Bashan, 1997)
7	*B. mucilaginosus, Azotobacter chroococcum*	Higher K mobilization, root shoot growth and yield	(Singh et al., 2010)
8	*Enterobacter hormaechei, Aspergillus terreus*	Increased root, shoot growth and K-uptake	(Prajapati et al., 2013)
9	*Bacillus pasteurii*	Significant increase in K availability	(Youssef et al., 2010)
10	*Bacillus mucilaginosus*	Higher biomass yield, uptake and % K-recovery, increased root-shoot growth	(Basak and Biswas, 2010)
12	*Bacillus mucilaginosus*	Higher biomass accumulation and nutrient acquisition	(Basak and Biswas, 2010)
13	*Arbuscular mycorrhizae*	Increased plant height, root and shoot weight, root length and P, N contents	(Clark et al., 1999)
14	*Pseudomonas putida*	Tea quality parameters like theaflavin, thearubigin, highly polymerized substances, total liquor colour, were improved	(B. Bagyalakshmi, 2012)
14	*Enterobacter cloacae, Klebsiella variicola*	Inoculation improved plant dry weight and enhanced nutrient uptake	(Zhang and Kong, 2014)
15	*Pseudomonas sp.*	P and K solubilizers significantly improved tomato yield	(Lynn et al., 2013)
16	*Paenibacillus glucanolyticus*	Enhanced K uptake and dry weight in inoculated plants	(Sangeeth et al., 2012)
17	*Bacillus cereus*	Growth and yield of potato, increased the availability and uptake of nutrients	(Ali et al., 2021)
18	*Bacillus licheniformis* *Aspergillus violaceofuscus*	High salt tolerant and K solubilizing bacteria	(Muthuraja and Muthukumar, 2022)

2.3.4 In the impact of biofertilizers on heavy metal bioremediation

The recent decade, the use of biofertilizers such as P and K solubilizing and N fixing bacteria to remediate heavy metal polluted sites has received a lot of attention (Fig.2.4) (Table 2.4) (Muthuraja and Muthukumar, 2022; Roy Chowdhury, 2022). Metals are the natural part of soils, and many of these metals are required for plant growth as they act as micronutrients and macronutrients. Due to rapid industrialization, activities like the overuse of chemical fertilizers and pesticides, in the agricultural sector has led to the release of pollutants such as (HMs), toxic wastes, and organic contaminants. These are inorganic contaminants that are water-soluble and accumulate in the soil biosphere due to their inability to degrade (Ahmad et al., 2014). Zinc (Zn), arsenic (As), chromium (Cr), cadmium (Cd), mercury (Hg), copper (Cu), nickel (Ni), and lead (Pb) are examples of these elements. Plants require metals as micronutrients, but the excessive accumulation of HMs are harmful to most of them. When these metal ions are present in high concentrations in the environment, plant roots absorb them quickly and translocate them to the shoots and leaves, causing stress and possibly death (Mehes-Smith et al., 2013).

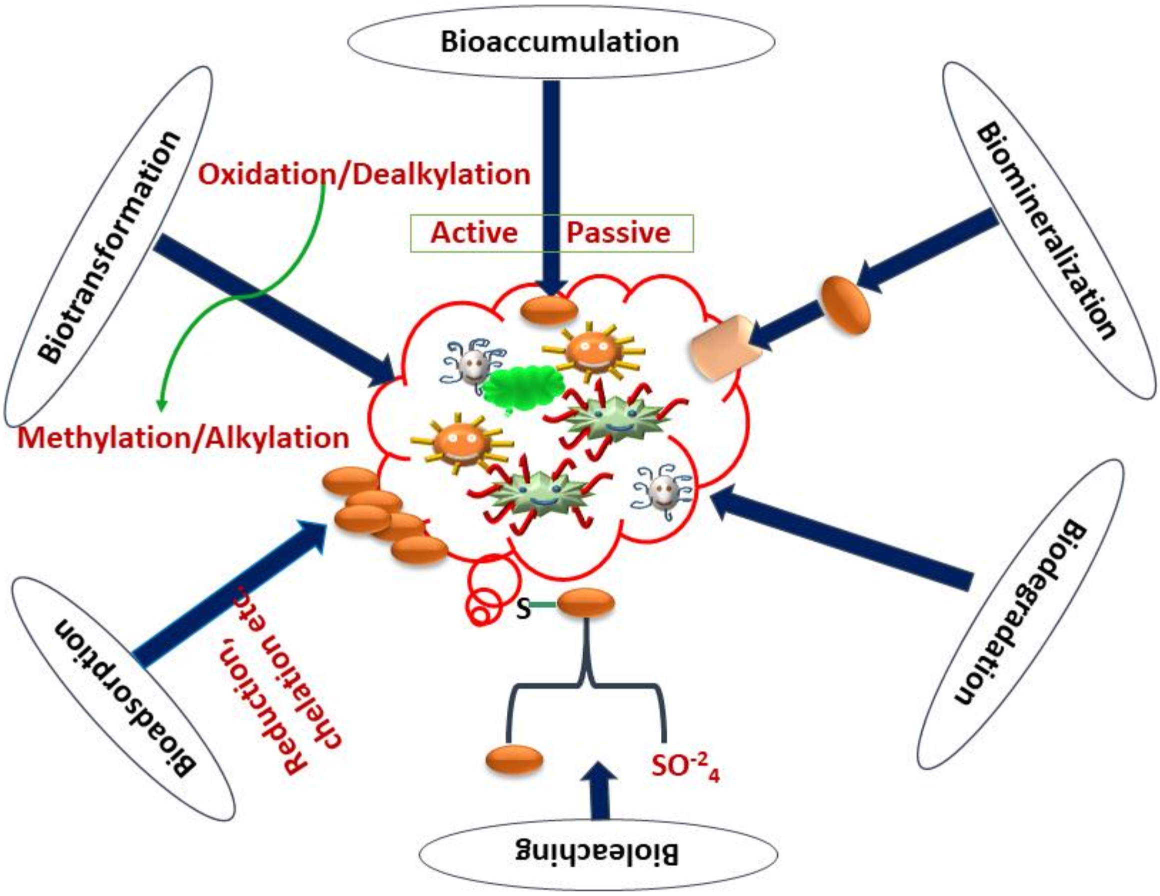

Fig. 2. 4 Mechanisms of HMs bioremediation by PGPR.

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Table 2.4 Roles of the biofertilizers for HMs bioremediation.

S.No	PGPR involved in HM remediation	HMs	N, P and K PGPR or bacteria	Reference
1	*Bacillus sp. PSB10*	Cr	NFB	(Wani and Khan, 2010)
2	*Bacillus subtilis SJ-101*	Ni	-	(Zaidi et al., 2006)
3	*Bradyrhizobium sp.750, Pseudomonas sp.,*	Cu, Cd, Pb	-	(Dary et al., 2010)
4	*Brevibacillus sp. (N fix)*	Zn	-	(Mahanty et al., 2017)
5	*Pseudomonas aeruginosa, Ralstonia Metallidurans (N fix)*	Pb, Cr	-	(Braud et al., 2009)
6	*Rhizobium sp. RP5 (N fix)*	Ni	-	(Wani et al., 2007)
7	*Psychrobacter sp. SRS8*	Ni	-	(Shinwari et al., 2015)
8	*Leclercia adecarboxylata and Pseudomonas putida*	Pb	PSB	(Teng et al., 2019)
9	*Enterobacter sp.*	Cd	-	(Sahu et al., 2022)
10	*Enterobacter sp.*	Pb and Cd	-	(Jiang et al., 2020)
11	*Agrobacterium sp.*	Cd	-	(Li et al., 2020)
12	*Bacillus sp. TZ5*	Cd	-	(Ma et al., 2020)
13	*Serratia sp.YU-SS-B-18, Enterobacter sp. YU-SS-B-51, Pseudomonas sp. YU-SS-B-65*	U	-	(Sowmya et al., 2020)
14	*Bacillus subtilis KUPSB16*	Pb, Cr, Cd, and Zn	-	(Paul and Sinha, 2015)
15	*Enterobacter sp., Bacillus sp., and Lactococcus sp.*	Pb and Cd	-	(Yuan et al., 2017)
16	*Leclercia adecarboxylata capsules containing tricalcium phosphate*	Pb	-	(Zhang et al., 2020)
17	*Pseudomonas sp.*	Pb	-	(Wang et al., 2020)
18	*Leclercia adecarboxylata*	Pb	-	(Teng et al., 2021)
19	*Leclercia adecarboxylata*	Pb	-	(Zhang et al., 2020)
20	*Bacillus sp. PS-6*	Cu, Zn, Cd, Mn, Ni, Pb, and As	PSB	(Sharma et al., 2021)

The harmful HMs must be removed from the polluted soil, changed, or immobilized. For microorganisms and their hosts to live and grow in soil full of metals, they must be tolerant of the particular polluted soil. Tolerance is the unique ability of microorganisms to deal with the toxicity of pollutants through detoxifying mechanisms triggered in direct response to the excess amounts of the corresponding pollutant (Fig. 2.4) (Ahemad and Khan, 2009) Consequently, in environments heavily stressed with HMs, bacteria have evolved various mechanisms to immobilize, mobilize, or transform metals, leaving them inactive (Nies, 1999). Since bacterial resistance mechanisms are often found on plasmids and transposons, they accrue resistance to HMs by acquiring a new gene or changing their extant genes. In gram-negative bacteria, for example, the czc system is responsible for cadmium, zinc, and cobalt resistance (e.g., Ralstonia eutropha). The czc genes code for a metal-exporting cation-proton antiporter (CzcABC) (Nies et al., 2006).

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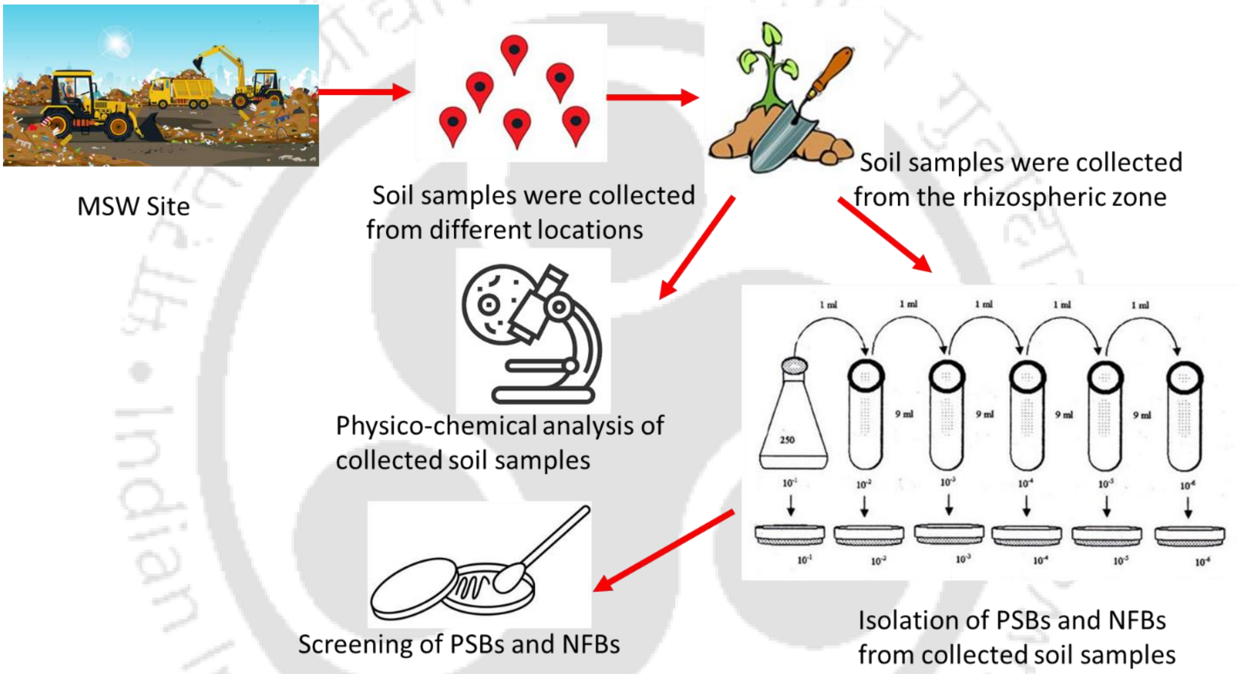
Isolation and screening of N fixing and P solubilizing bacteria in soils from the municipal dumping site



This chapter covers the physico-chemical evaluation of the soils collected from contaminated sites, namely MSW dumping site in Boragaon, Guwahati, Assam, as well as the isolation and screening of PSB and NFB from the collected soils using selective medium.

3. Isolation and screening of N fixing and P solubilizing bacteria in soils from the municipal dumping site

3.1 Introduction

Soil is a natural growth medium made up of unconsolidated mineral materials from the earth's outer crust. A variety of elements, generically classified as edaphic, genetic, and environmental; or more specifically the, parent material, climate, surface contamination, organisms, and topography, all contribute to soil formation and characteristics. Since soil characteristics directly impact the immediate environment and human health, soil profile analysis supports optimal usage and management (Basu, 2011). Consequently, the two main parts of the present thesis are profiling basic soil characteristics and testing the ability of rhizospheric bacteria to reduce pollutants harmful to human health. The physical and chemical characteristics of soil collected from the municipal dumping site were profiled prior to isolation and screening of N fixing and P solubilizing bacteria.

The physical parameters of the soil were evaluated by measurement of pH, electrical conductivity (EC), and cation exchange capacity (CEC). The bioavailability of nutrients and HMs bioremediation, are influenced by soil pH and the EC. The EC also determines ionic transport in a solution, and indicates the soluble mineral salt concentration of the soil. The CEC is the total number of exchangeable cations that a specific soil may retain and is a critical physical feature influenced by clay and organic matter content.

The chemical parameters investigated were moisture content (MC) organic carbon (OC), organic matter (OM), available nitrogen (AvN), sodium (AvNa), phosphorus (AvP), potassium (AvK), and microbial biomass carbon (MBC). OM is made up of dead organism tissue, organic material smaller than 2 mm, and microbes at various stages of decomposition. OM is important for soil stabilization, water retention, and nutrient recycling, making it essential for agricultural

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production and environmental resilience (Rindi et al., 2018). Although OC availability depends on the level of transformation or decomposition, it is the primary source of energy for soil microbes (Datta et al., 2021). The OC/OM content of the soil is thought to have a variety of roles in delivering ecosystem processes and services.

AvN in the soil is primarily in the organic form (97-99%) and is linked to the action of microorganisms that enable organic matter breakdown (NH_4-N and NO_3-N). On the other hand, denitrification accelerates the release of AvN from soil organic materials. AvP promotes legume development by increasing the activity of nitrogen-fixing bacteria. It also aids in the creation of seeds and fruits, as well as stimulating root growth and development. AvK helps in creating and synthesizing amino acids and proteins from ammonium ions taken from the soil, which is crucial for leaf photosynthetic activity. All living things require phosphorus. It is found in the form of phosphates (PO_4) and serves as an energy storage molecule (e.g., ATP). It is made up of biomolecules such as nucleic acids, lipids, vitamins, and some proteins. Plants can only accept two soluble forms of phosphorus: monobasic (NaH_2PO_4) and dibasic (e.g., Na_2HPO_4). Inorganic and organic forms of insoluble phosphorus include inositol phosphate (soil phytate), phosphomonoesters, and phosphotriesters. Similarly, N is one of the most critical nutrients for living organisms, nucleic acid, protein, vitamins, and lipids are examples of living macromolecules that contain it. Plants and their products provide an easy source of nitrogen for growth and development (Sahu et al., 2022). Similarly, even though there is 78% of N_2 in the atmosphere, plants cannot use it. The biological nitrogen fixation (BNF) mechanism must first convert the nitrogen to a form that plants can easily consume (Tairo and Ndakidemi, 2013). During BNF, N fixing bacteria use an enzyme complex called Nitrogenase to convert nitrogen to ammonia. The N fixing organisms can be symbiotic or non-symbiotic.

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3.2. Materials and methods

3.2.1 Study area, soil sampling and analysis

Soil samples contaminated with MSW were collected from, Boragaon, Guwahati, Assam (latitude: 26° 06. 915’ N and longitude: 91° 40. 669' E) following the stratified random sampling method. The sampling site is situated about 18 km southwest of Guwahati city, on National Highway 31, and located in the vicinity of Deepor Beel (a large aquatic body and Ramsar site famous for migratory birds). The MSW discarding site comprised of heaps of waste, composting plant, and metal and glass segregation units, from where approximately 0.5 kg of soil were collected from the rhizospheric zone of the plants available in that area). The soil adhering to the roots of plants growing in the MSW site was separated by gentle tapping using forceps and collected in sterilized labelled sampling bags. The soil samples were then processed and stored at 4 °C till further analysis.

The soil samples were air-dried for 4-5 days, ground and homogenized, and passed through 2 mm and 0.2 mm brass sieves before being subject to analysis of various soil parameters. In addition to pH and moisture content, the soil was assessed for cation exchange capacity (CEC) and electrical conductivity (EC). Using an HI 3221 pH meter (Hanna Instruments Inc., USA) and soil-to-water ratio of 1:2.5 (*w/v*), the pH, CEC, and EC of the soil were determined (Biswas et al., 2010). The gravimetric method was used to measure moisture content (MC). The sodium saturation method was used to calculate cation exchange capacity (Bower et al., 1952). The Walkley and Black, (1934) method was used for estimating organic carbon (OC). Microbial biomass carbon (MBC) was determined through the chloroform fumigation method (Station, 2002). Available nitrogen (AvN) was determined using Kjeldahl assembly (Subbiah and Asija, 1956) from Velp Scientifica UDK 129, USA. The flame photometer Systronics Model 126, India, was used to determine the available sodium (AvNa) and potassium (AvK) (Knudsen et al., 1982). Using a Genesys 10S UV-Vis Spectrophotometer, available phosphorus (AvP) was calculated using the Olsen method for alkaline soil Olsen, (1954); and Bray's method Bray and Kurtz, (1945) for acidic soil.

3.2.2 Isolation of PSB from the contaminated soil

The isolation of PSB was carried out following the standard dilution-plating procedure, using phosphate saline buffer for dilution (Liu et al., 2015). The soil suspension was serially diluted and spread over the Pikovskaya (PVK) agar media, which contained $Ca_3(PO_4)_2$ 5 g; glucose, 10 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4.7H_2O$, 0.1 g; NaCl, 0.2 g; KCl, 0.2 g; $MnSO_4.H_2O$, 0.002 g; yeast extract, 0.5 g; $FeSO_4.7H_2O$, 0.002 g with agar, 15 g L^{-1} (pH 7.2–7.4) (Pikovskaya, 1948). These media plates were then incubated for 4–7 days at 28 °C for growth and isolation of bacterial colonies. For screening, colonies were selected according to clear halo zones in the PVK plates, and further purified using the same media.

3.2.3 Isolation of NFB from the contaminated soil

The isolation of NFB was carried out following the standard dilution-plating procedure. According to the standard method, the dilution was carried out using saline buffer (Liu et al., 2015). The soil suspension was serially diluted and distributed over N free Winogradsky agar media. The composition of salt solution in per litre of distilled water was KH_2PO_4 50 g, $MgSO_4.7H_2O$ 25 g, NaCl 25.0 g, $FeSO_4.7H_2O$ 1.0 g, $MnSO_4.4H_2O$ 1.0 g, $Na_2MoO_4.4H_2O$ 1.0 g and $CaCO_3$, trace organic matter (sucrose or glucose) 10.0 g, Agar 20.0 g. The plates containing the media were then incubated for 4-7 days at 28 °C to locate bacterial colonies growth and isolation. For screening, colonies were selected according to the growth condition on plates, and further purified using the same media (Dahal et al., 2017).

3.2.4 Screening of PSB and NFB

The screening of PSB and NFB were followed according to Pikovskaya, (1948) and Dahal et al., (2017) PVK and YEMA media respectively.

3.3 Results and discussions

3.3.1 Physico-chemical properties of the contaminated soil

The majority of soil nutrients are known to be accessible in the pH range of 6.5–7.5 (Waseem et al., 2019), whereas the pH of the MSW site soil was in the moderately to the slightly acidic range (5.5 ± 0.30 – 6.9 ± 0.39) (Table 1.1). The suggested EC limit is 1.10–5.70 mS cm^{-1}, according to Suwal, (2018), however, EC at the MSW site displayed lower values that ranged from 0.06 ± 0.01 to 1.24 ± 0.10 mS cm^{-1}. Generally, clayey soil has higher CEC values which range from 10 to 150 C mol kg^{-1}. In the present study, CEC ranged from 3.34 ± 0.44 to 33.53 ± 2.52 C mol kg^{-1}. The level of soil nutrients in the site was adjudged on basis of available phosphate, nitrogen, potassium and sodium (AvP, AvN, AvK and AvNa respectively). According to Suwal, (2018), the optimal concentration of AvP for fertile soil ranges between 72 and 137 kg ha^{-1}. As the AvP concentration of the site ranged from 5.03 ± 0.45 to 14.08 ± 0.84 kg ha^{-1} (Table 1.1), the entire area displayed low AvP levels AvN, a macronutrient for plants, constitutes approximately 1–4% of the plant dry weight. However, the range of AvN content observed in the experimental soils was 0.03 ± 0.00–1.46 ± 0.16 kg ha^{-1}, well below the requisite range. AvK is essential for photosynthesis, protein synthesis, starch formation, and translocation of sugars. In the present study, AvK ranged from 7.28 ± 0.55 to 77.28 ± 6.18 kg ha^{-1} (Table 1.1), which is far below the recommended limit of 120 kg ha^{-1}. The AvNa is another nutrient that is bioavailable in exchangeable and water-soluble forms. The exchangeable part depends upon the surrounding environment, while water-soluble fraction relies on the salinity of the water. The AvNa affects soil permeability by causing swelling and dispersion of clay particles and clogging soil pores. In the study area of Boragaon the AvNa values ranged from 18.70 ± 2.06 to 187.77 ± 15.02 kg ha^{-1} which is below than recommended ranged of AvNa is from 14.00 to 35.50 kg ha^{-1}.

SMC in the soil of MSW site ranged from 6.58 ± 2.06 to 37.50 ± 3.64% whereas the recommended limit is 66.41% (Bisht and Neupane, 2015). OC an essential soil factor, displayed a wide divergence from 1.20 ± 0.10 to 54.71 ± 4.65 g kg^{-1} in the present assessment, whereas the recommended ranged of MBC content was from 0.87 ± 0.09 to 88.47 ± 8.85 µg C g^{-1}. Higher OC in the soils of MSW site may be due to the organic decomposition of organic wastes, plant and animal residues, root exudates, living and dead microorganisms, and soil biota (Bisht and Neupane, 2015). The concentrations of Cu, Cr, and Cd in soil were revealed 142, 1.55, and 466.55 mg kg^{-1} respectively. According to Canadian soil quality guidelines CCME, (2007), the content of HMs (such as Cu, Cd and Cr) were found to be higher than the permissible limits in the MSW soils under investigation.

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Table 3.1 Physico-chemical properties of collected soil

S. No.	**Parameters for collected soil**	**Results**	**Recommended limits**	**Reference**
1	pH	(5.5 ± 0.30 - 6.9 ± 0.39)	6.5 to 7.5	Khan et al., (2019)
2	EC (mS cm^{-1})	0.06± 0.01 to 1.24 ±0.10	1.10-5.70	Suwal, (2018)
3	CEC C mol kg-^{1}	3.34 ± 0.44 to 33.53 ± 2.52	10 to 150	Suwal, (2018)
4	AvK kg ha^{-1}	5.03 ± 0.45 to 14.08 ± 0.84	72 and 137	Suwal, (2018)
5	AvN kg ha^{-1}	0.03 ± 0.00 - 1.46 ± 0.16	0.28-2.80	Bisht and Neupane, (2015)
6	AvP kg ha^{-1}	7.28 ± 0.55 to 77.28 ± 6.18	120	Suwal, (2018)
7	AvNa kg ha^{-1}	18.70 ± 2.06 to 187.77 ± 15.02	14.00- 35.50	Suwal, (2018)
8	Moisture content (in %)	6.58 ± 2.06 to 37.50 ± 3.6	66.41	Bisht and Neupane, (2015)
9	OM g kg^{-1}	1.20 ± 0.10 to 54.71 ± 4.65	39	Bisht and Neupane, (2015)
10	MBC µg C g^{-1}	0.87 ± 0.09 to 88.47 ± 8.85	2.13-27.27	Bisht and Neupane, (2015)
11	Cu mg kg^{-1}	142		Canadian soil quality guidelines, CCME, (1999)
12	Cd mg kg^{-1}	466.55		
13	Cr mg kg^{-1}	1.55		

3.3.2 Isolation and screening of the PSB

All the collected rhizospheric samples exhibited robust and prolific bacterial growth in PVK media. These PSB were characterized based on their ability to solubilize tricalcium phosphate ($Ca_3(PO_4)_2$), on PVK agar media by forming a visible clear hollow zone (Fig 3.1). Approximately 400 PSB isolates have been identified from the contaminated soil (Table 3.2). Further studies were done on colonies identified by clear zone formation and colony morphology. Some isolates showed a clear hollow zone around the PVK agar colonies when screened for phosphate solubilizing activity, and four of these strains were selected for further analysis. The selected isolates were coded as P1, P2, P3, and P4. As mentioned earlier, the phosphate-solubilizing potential of the four PSB strains was measured using the phosphate solubilizing index (SI) based on (Premono et al., (1996). The SI of phosphate of all four

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isolates were 0.43 ± 0.03, 0.53 ± 0.03, 1.20 ± 0.26, and 2.30 ± 0.10 cm, respectively, in PVK agar media in comparison to CK.

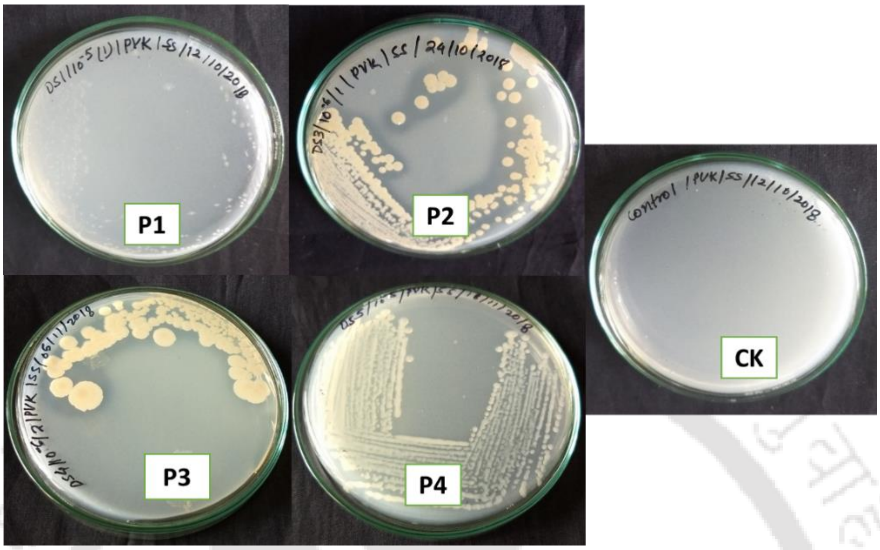

Fig 3.1 Screening PSB in PVK culture plate.

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Table 3.2 Number of P solubilizing isolates from different sampling sites

Sr. No.	**Sample identification code**	**Number of colonies in different dilutions**								
		10^{-4}			10^{-5}			10^{-6}		
		1	2	3	1	2	3	1	2	3
1	DS1	1	2	3	2	1	1	2	2	1
2	DS1.1	1	2	1	2	1	1	2	1	2
3	DSR1	4	2	2	2	1	2	1	1	1
4	DS2	3	2	3	2	3	1	2	2	1
5	DS3	3	2	2	3	1	1	2	3	1
6	DS3.1	2	1	2	2	2	1	2	2	3
7	DS4	1	4	2	2	1	2	2	2	2
8	DS5	4	2	2	3	3	2	3	2	2
9	DS6	2	2	3	2	3	1	3	2	2
10	DS7	2	2	3	2	3	2	2	2	2
11	DSR7	3	3	3	2	3	3	2	1	1
12	DS8	2	2	1	2	3	3	3	3	3
13	DS9	2	2	2	2	2	1	2	2	2
14	DS10	2	3	1	2	1	1	2	3	2
15	DS11	2	2	2	2	2	1	2	2	3
16	DS12	2	2	2	2	2	2	2	3	1
17	DS13	2	3	1	2	2	2	2	2	2
18	DS14	2	3	3	3	2	2	1	3	2
19	DS15	3	2	2	2	2	2	2	2	2
20	DS16	1	2	2	2	2	2	3	3	2
21	DS17	2	2	1	3	3	3	2	2	2
22	DS18	3	2	2	3	1	2	4	2	2
23	DS19	3	2	3	3	2	2	2	3	2
24	DS20	1	2	1	2	3	1	3	2	2

3.3.3 Isolation and screening of NFB

Approximate 350 N fixing bacterial strains have been isolated from multipollutant (mainly heavy metal) collected soil (Table 3.3). The media plates contain visible colonies in the Winogradsky agar media (Fig 3.2), which is an N-free media. This result shows that the isolates can fix the atmospheric nitrogen for the completion of the life cycle. Four NFB were selected for further study based on the growth parameters in Winogradsky media. The selected NFB isolates were coded as **N1**, **N2, N3,** and **N4.**

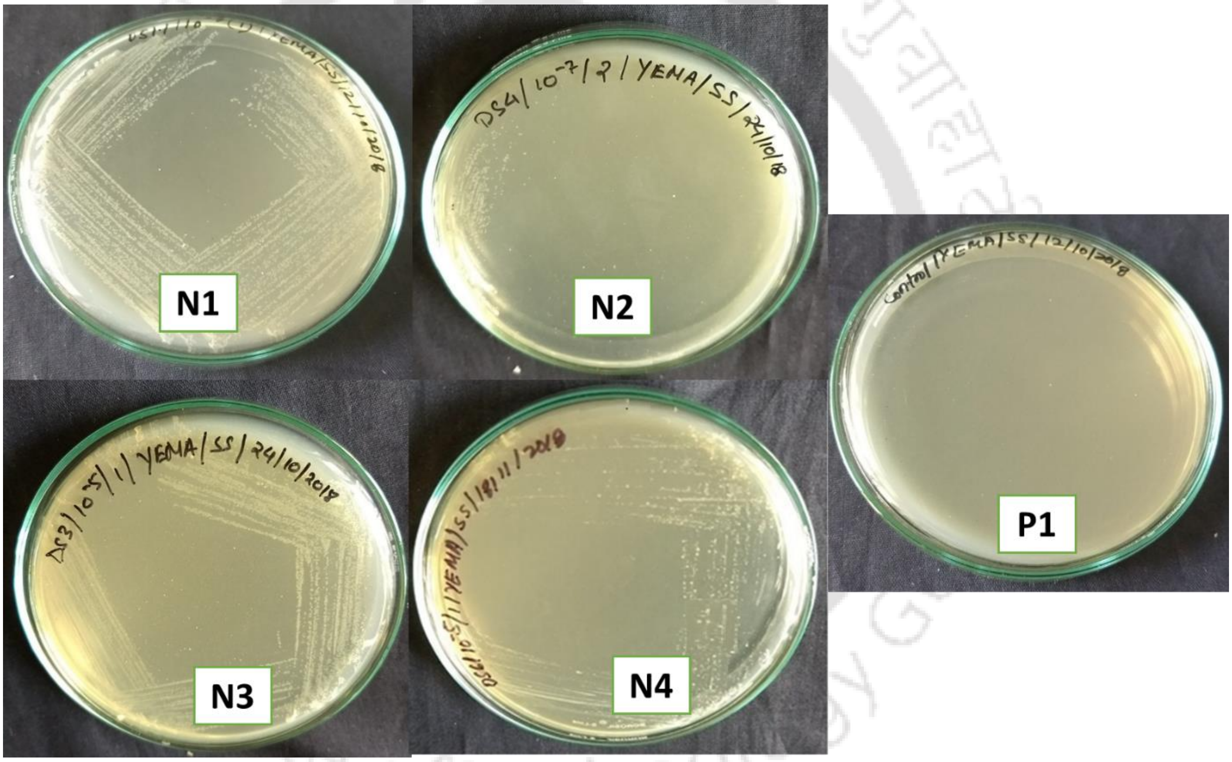

Fig 3.2 Screening of NFBs in YEMA culture plates.

Table 3.3 Number of NFB isolates from different sampling sites

Sr. No	Sample identification code	Number of colonies in different dilutions								
		10^{-4}			10^{-5}			10^{-6}		
		1	2	3	1	2	3	1	2	3
1	DS1	1	1	1	1	1	1	1	2	1
2	DS1.1	2	2	1	2	2	2	1	1	2
3	DSR1	2	2	2	1	3	1	1	1	1
4	DS2	2	2	2	1	2	1	2	2	1
5	DS3	2	2	2	2	1	1	2	2	1
6	DS3.1	2	2	2	2	1	1	3	3	3
7	DS4	2	2	2	2	5	2	2	2	2
8	DS5	2	2	3	2	2	2	2	2	2
9	DS6	2	3	3	3	2	1	2	1	2
10	DS7	2	2	1	2	3	2	3	2	2
11	DSR7	2	2	1	2	3	3	2	2	1
12	DS8	3	1	1	1	2	3	3	3	3
13	DS9	3	2	2	3	2	1	2	2	2
14	DS10	1	3	1	3	3	1	2	2	2
15	DS11	2	1	2	3	2	1	1	1	3
16	DS12	2	2	2	1	1	2	2	2	1
17	DS13	1	2	1	3	1	2	1	0	2
18	DS14	2	2	2	1	5	2	2	2	2
19	DS15	1	2	2	4	2	2	2	2	2
20	DS16	2	1	2	1	2	2	2	2	2
21	DS17	1	1	1	2	1	3	2	3	2
22	DS18	2	2	1	1	3	2	2	1	2
23	DS19	3	2	1	1	3	2	2	1	2
24	DS20	1	1	1	3	2	1	2	2	2

3.2.3.1 Screening of NFB isolates in the ammonium free atmosphere

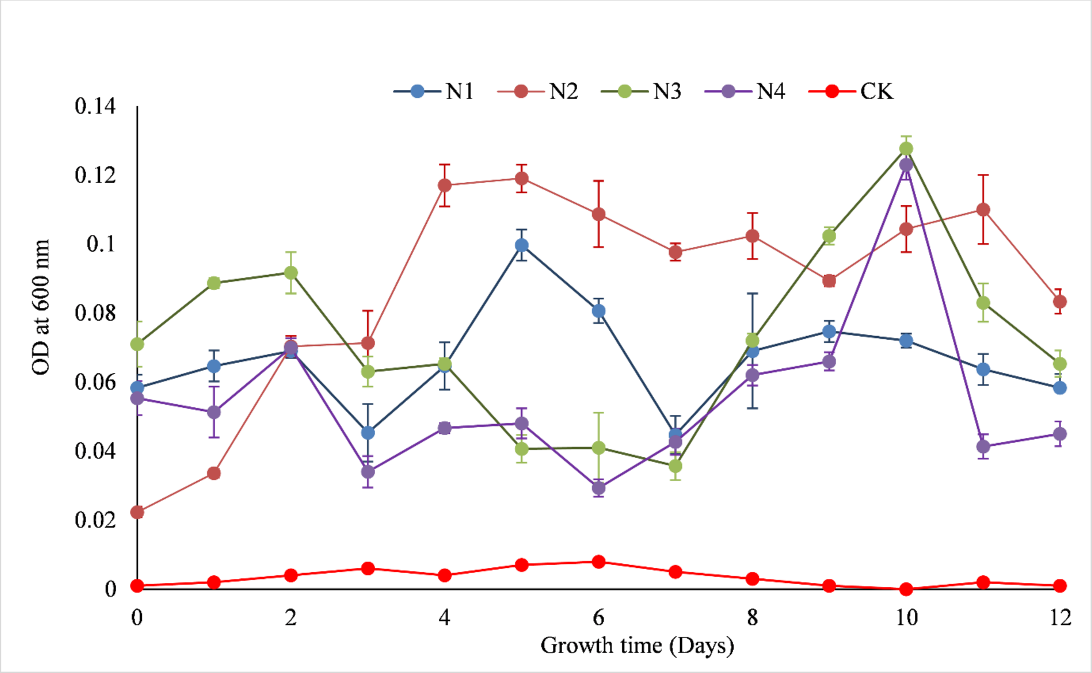

Fig. 3.3 Nitrogen fixation by isolated NFB in ammonium depleted atmosphere.

The isolates were incubated liquid cultures in a sealed atmosphere container with the zeolite clinoptilolite. It has previously been proven to bind residual ammonia to prevent the possibility of growth using ambient ammonia (Dahal et al., 2017). Fig. 3.3 represents the ability of all four NFB strains to fix atmospheric nitrogen during their life cycle. The strains were grown completely in liquid Winogradsky media (Dahal et al., 2017). On such a clear background, their growth appeared as discrete clumps, and when plated on NFM, the clumps expanded. The growth of the isolates was varied which could be related to adaptation or the fact that the time taken for nitrogen fixation of each isolate is not the same.

In a prior investigation, the fungus *Aureobasidium pullulan*s grew in NFM but not in an ammonia deficient atmosphere with clinoptilolite. In contrast, bacterial isolates grew in liquid NFM in an ammonia depleted atmosphere, showing the potential to fix nitrogen (Dahal et al., 2017). It is unknown how the other isolates were able to grow in a medium free of combined nitrogen and in an ammonia-depleted environment, according to (Dahal et al., 2017).

3.4 Conclusion

The soil samples from the MSW site displayed moderate to slightly acidic pH. Further, the soil nutrients were adjudged based on available phosphate, nitrogen, potassium, and sodium (AvP, AvN, AvK and AvNa, respectively) analysis. The soil moisture content was low against the recommended limits. The higher OC content in the soil samples may be due to the organic decomposition of organic wastes, plant and animal residues, root exudates, living and dead microorganisms, and soil biota. The concentrations of Cu, Cr, and Cd in the soil samples were higher than the permissible limits. On the other hand**,** all the collected rhizospheric samples exhibited robust and prolific bacterial growth in PVK and Winogradsky media for PSB and NFB, respectively. Approximately 400 and 350 viz. PSB and NFB isolates have been identified from the contaminated soil. The screening was performed in the PVK and YEMA media for PSB and NFB, respectively.

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CHAPTER 4

Characterization of N fixing and P solubilizing bacteria through morphological, biochemical and molecular analysis

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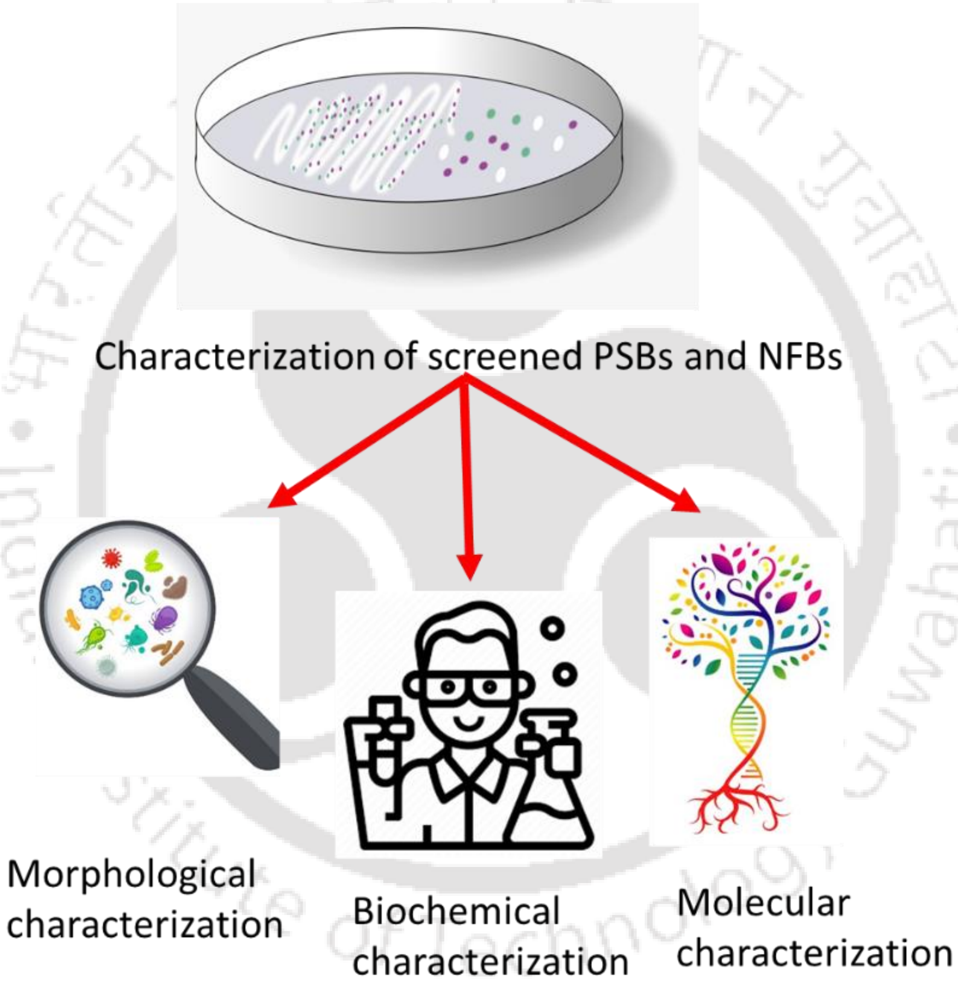

This chapter discusses the characterization of isolated and screened colonies using morphological, biochemical, and molecular techniques

4. Characterization of N fixing and P solubilizing bacteria through morphological, biochemical and molecular analysis

4.1 Introduction

Characterization of unknown bacteria is one of the crucial steps in the field of bacteriology. In the present study, morphological, biochemical, and molecular characterization of bacteria were conducted. The morphological characterization of the selected strains was done using naked eyes. Further, the gram staining method was performed for the biochemical analysis. After morphological and biochemical analysis, four PSB and four NFB were chosen for molecular analysis from those colonies. The molecular characterization was completed using 16s rRNA gene sequencing followed by the phylogenetic analysis to track down the taxonomic status of the four PSB, and four NFB selected bacteria.

Microbiologists can define and differentiate cells using their growth phenotype or morphology. The PhD dissertation of L.E. den Dooren de Jong, who worked on the project first under M.W. Beijerinck and then under A.J. Kluyver at the Technological University of Delft in the Netherlands, established the power of phenotypic description of bacteria in a systematic method (Den Dooren de Jong, 1926). Researcher, demonstrated that bacteria could be identified easily by using growth experiments on agar media with hundreds of C and N sources.

According to the first edition of Bergey's Manual of Determinative Bacteriology, systematic classification and definition of bacterial species have been decided based on the list of phenotypes (Bergey, 1994). However, the gram staining allows for the determination of the gram reaction, morphology, and arrangement of the organism. Although this information provides a few good clues, it does not allow the determination of the species or even genus of the organism with certainty. Thus, microbiologists use molecular characterization or analysis, among which 16S rRNA gene sequences benefit from being universally applicable, and taxonomically predictive in microbial taxonomy. Phenotypic testing is also taxonomically predictive and globally applicable and provides useful information about the biological features of cells (Bochner, 2009).

4.2 Materials and methods

Morphologically different, well developed, single colonies were selected and sub-cultured using the same media until pure isolates were obtained. The individual colonies appearing on the plates were then preserved in PVK agar slant with glycerol stock (30%) for further investigations (Yu et al., 2019).

4.2.1 Morphological and biochemical characterization

The morphological characteristics was studied according to the protocols of Bergey's Manual of Determinative Bacteriology, published in (1994). The biochemical characterization applying gram staining has been carried out following the methodology of Graham and Parker, (1964), and Vincent, (1970).

4.2.2 Taxonomic identification of isolates and phylogenetic analysis

The 16S rRNA gene sequencing was used to identify the isolated bacterial strains. In this experiment, all the bacterial isolates were cultured in Luria-Bertani (LB) media for 18 h at 28 °C temperature, and 120 rpm agitation. DNA Kit (e. g. AI10015 N and AHG0382A) protocol from Bioserve Biotechnologies (India) was used to extract crude DNA. The partial sequences of the 16S rRNA genes of the bacterial strains were obtained using the universal primer sets 27F/16SR (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R/16SR (5′-TACGGYTACCTTGTTACGACTT-3′). The 16S rDNA region of bacterial DNA was amplified using emerald amp GT PCR master mix (RR310). The PCR product was purified later by Gel elution/SAP. The purified product was sequenced by Sanger's method of DNA sequencing using a standard protocol (Bioserve-D/Seq-02 to 06). Furthermore, BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) (Awais et al., 2017) was used for comparing the sequencing results with the NCBI nr-database. Multiple alignments of the nucleotide sequences were carried out using Clustal X (Thompson et al., 1997); the badly aligned sequences were deleted using the Gblocks tool (https://github.com/atmaivancevic/Gblocks). The finally aligned sequence was employed to construct the phylogenetic tree using neighbor-joining method with 500 bootstrapping using MEGA X tool (https://www.megasoftware.net/).

4.3 Results and discussions

4.3.1 Morphological and biochemical characterization

In the morphological analysis, different characters of different strains such as the opacity, elevation, margin and texture were studied. In addition, differential staining techniques, 16 sRAN analysis backed by NCBI identification were also used. Thus, opacity was graded as transparent, translucent, or opaque; their texture when tested with a needle could be butyrous (buttery texture), viscous (gummy), or dry (brittle or powdery). In addition, the surface character could be smooth (shiny glistening surface), rough (dull, bumpy, granular, or matte surface), or mucoid (slimy or gummy appearance) (Bergey, 1994). Gram staining is a differential staining technique that differentiates bacteria into two groups: gram positive and gram negative. In the present study, both bacteria have been identified. The results were also supported by 16 sRAN analysis and its identification by NCBI.

4.3.2 Taxonomic identification of isolates and phylogenetic analysis

Taxonomic characteristics and phylogenetic analysis using 16S rRNA gene sequencing were used to track down the taxonomic status of the four PSB, and four NFB selected bacteria.

The results revealed the isolates belonged to two phyla (*Firmicutes* and g-*Proteobacteria*), and three genera (*Bacillus*, *Paraburkholderia,* and *Enterobacter*) (Fig 4.1 & 4.2). P1, N2 and P3 belonged to the *Bacillus* genus, and N1, N3 and N4 belonged to *Paraburkholderia fungorum*, whereas P2 and P4 belonged to the *Enterobacter* genus. Hence, P1 and N2 were identified as *Bacillus ceresus* (SM_SS1) and (SM_SS2), N1, N3, and N4 were identified as *Paraburkholderia fungorum* viz (SM_SS1), (SM_SS3) and (SM_SS4), P2 as *Enterobacter* sp. (SM_SS6), P3 as *Bacillus* sp. (SM_SS7), and P4 as *Enterobacter* sp (SM_SS8) (Fig. 4.1 & 4.2).

The *Bacillus* sp. was identified as gram positive bacteria. Some of these species are known to cause pathogenicity in humans as well as in animals (Okutani et al., 2019). The other bacteria from the contaminated soil have been identified as *Enterobacter* sp. which are gram negative bacteria and classified as a facultative anaerobe. However, pathogenic potential of the strain isolated in this study is based on Edberg, (1991), who reported that *Bacillus* species generally produce toxic compounds in negligible amounts but are not virulent against animals or plants. Similarly, some *Enterobacter* species are considered plant pathogens, whereas some bacteria belonging to the same species are beneficial to plant hosts (Robinson, 2014). The pathogenicity of the strain isolated in the present study was verified to establish its health risk to humans and other organisms. Additionally, these isolates are multipollutant resistant strains that have occasionally spilled over into the community, infecting other individuals as well (Davin-Regli et al., 2019). Though they are pathogenic, but they possess phosphate solubilizing, nitrogen fixing, and HM remediation abilities, which are very important for rejuvenating soil quality. Thus, in the present study, we attempted to explore and utilize the abilities of the bacteria for soil quality enhancement.

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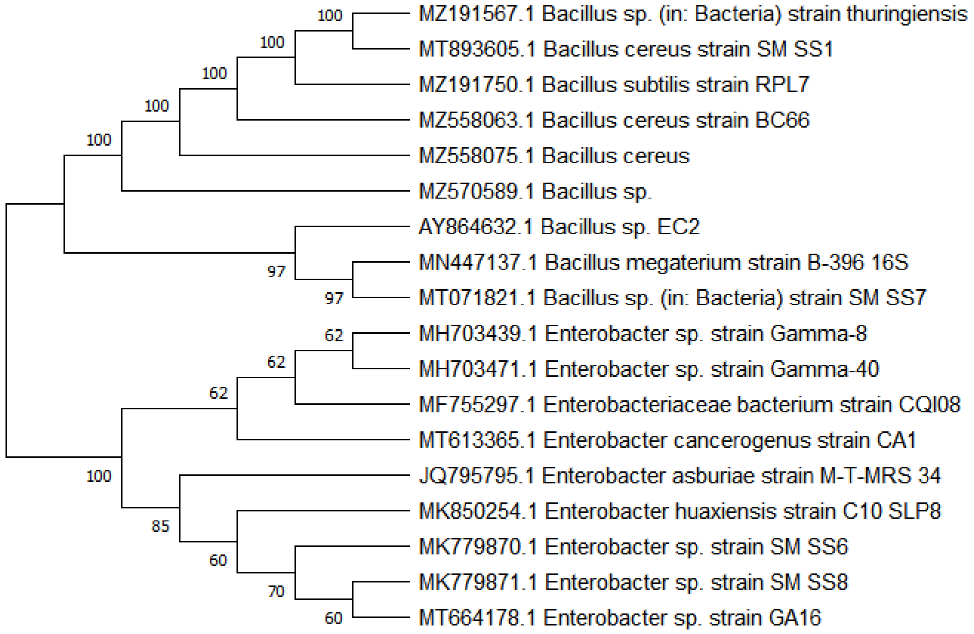

Fig. 4.1 The distance based phylogenetic tree of PSB "*Bacillus* sp. strain SM_SS1 (P1)" "*Enterobacter* sp. strain SM_SS6" (P2), "*Bacillus* sp. strain SM_SS7" (P3) and "*Enterobacter* sp. strain SM_SS8" (P4).

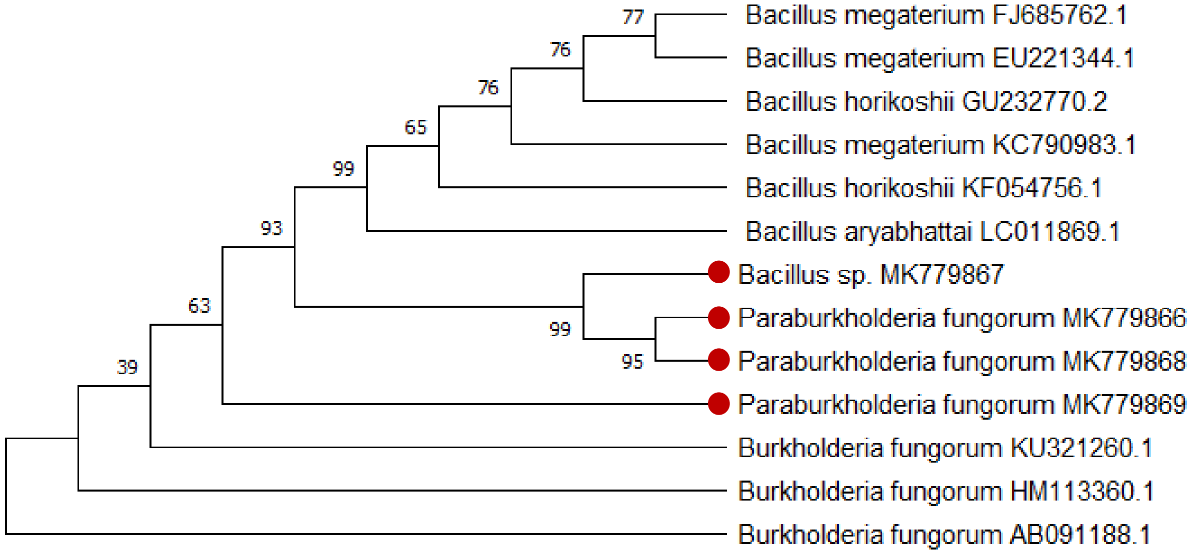

Fig. 4.2 The distance based phylogenetic tree of NFB "*Paraburkholderia fungorum* SM_ SS1 (**N1**)" "*Bacillus sp.* strain SM__SS2 (**N2**)" "*Paraburkholderia fungorum* SM_SS3 (**N3**)" "*Paraburkholderia fungorum* SM_SS4 (**N4**)".

4.4 Conclusion

In the morphological analysis, different strains show various characteristics such as the opacity of the colonies; transparent, translucent, or opaque, their texture when tested with a needle: butyrous (buttery texture), viscous (gummy), or dry (brittle or powdery, elevation, and margin. Also, both gram-positive and gram-negative bacteria have been identified after gram staining. Similarly, based on molecular characterization data, the selected strains indicated two phyla (Firmicutes, g-Proteobacteria, and Proteobacteria) and three genera (*Bacillus*, *Enterobacter*, and *Paraburkholderia*). Some strains among the characterized isolates have pathogenic characters, but they possess phosphate solubilizing, nitrogen fixing, and HM remediation abilities, which are very important for rejuvenating soil quality. Thus, in the present study, we attempted to explore and utilize the abilities of the bacteria for soil quality enhancement.

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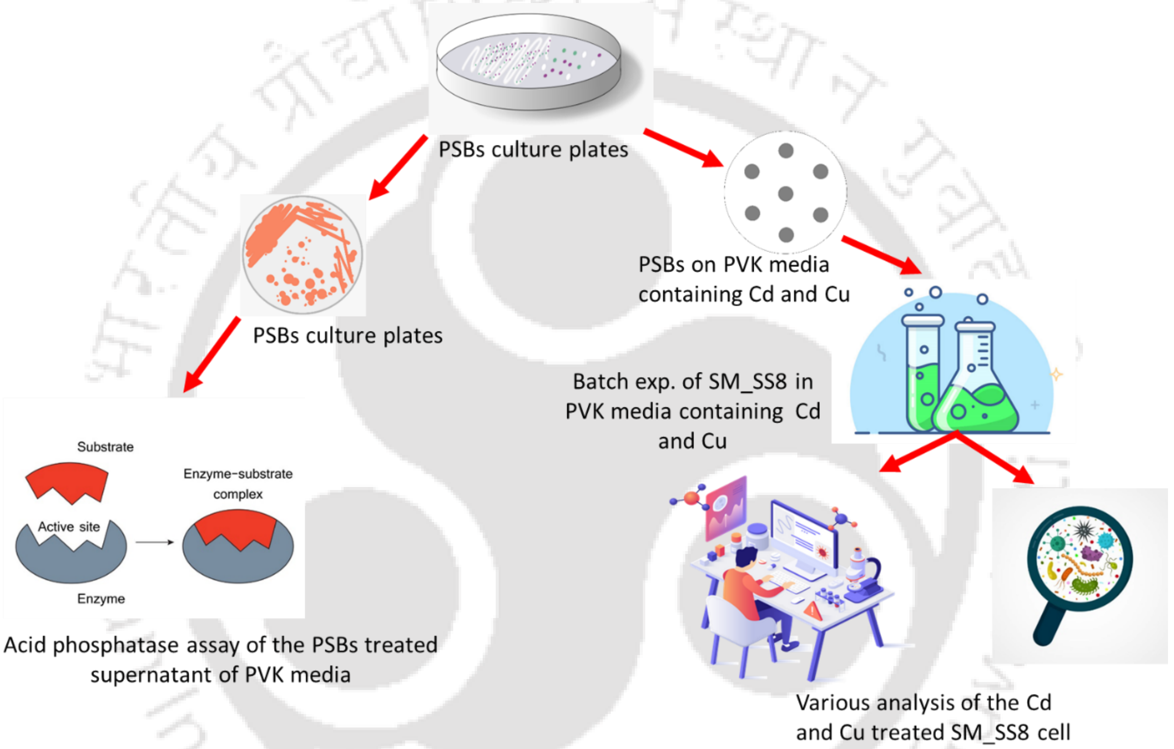
CHAPTER 5

Evaluation of the remediation potentials and carrying out the enzymatic assay of screened P solubilizing bacteria



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The purpose of this chapter is to determine the effectiveness of P solubilization by characterized PSB and to investigate the potency of the SM SS8 strain for bioremediation of various Cd and Cu dosages.

5. Evaluation of the remediation potentials and carrying out the enzymatic assay of screened P solubilizing bacteria

5.1 Introduction

The dangerous levels of HM contamination in agricultural land and MSW dump sites have necessitated urgent evaluation of bioremediation strategies. Presence of HMs are attributed to both geological and anthropogenic activity (Kumar et al., 2017; Zerizghi et al., 2022). Thus, the natural geological processes of bedrock weathering and volcanic eruptions that release HMs are exacerbated by anthropogenic industrial and agricultural activity. Substantial contributions to the HM load in the natural aquatic and terrestrial habitat originate from fertilizers, insecticides, metal smelting, mining, and other metallurgical operations (Kumar et al., 2017). High levels of HMs are particularly prevalent in the open MSW locations in developing countries (Biswas et al., 2010; Borah et al., 2020). Cu and Cd which are extremely hazardous to living things (Pavlaki et al., 2016), continue to display an unabated increase due to industrial pollutants and uncontrolled application of pesticides and fertilizers in agricultural soils (Aadil et al., 2014). Cd is much more dangerous than other divalent HMs like Pb, Zn, and Cu. At concentrations of more than 5 M, the toxicity of Cd to vital human organs (kidney, testis, brain), and the circulatory system shows a sharp rise (Jeong et al., 2013). On the other hand, the copper displays two facets in the human system. It is one of many metal ions required for important biological processes, but its excess or lack can harm human health. Cu deficiency leads to significant disorders such as anaemia and neutropenia (Oliver et al., 1997). However, excessive amounts of Cu induce liver and Alzheimer's disease and brain breakdown (Oliver et al., 1997).

According to the World Health Organization and the International Agency for Research on Cancer, HMs ions mimic the vital constituents of the human body and disrupt metabolic processes (Rindi et al., 2018). As HMs can damage cellular membranes, their presence exacerbates cellular stress (Brito et al., 2015). Additionally, they can influence enzyme activity, gene expression, and protein formation by changing the shape of active biomolecules or altering metabolic pathways (Saurav and Kannabiran, 2011).

Precipitation, oxidation processes, and reverse osmosis are effective HM treatment methods. However, these processes have a variety of drawbacks that make them unsuitable for managing larger volumes (Sahu et al., 2022). As a result, it is vital to design effective green methods for cleaning up HMs. The hunt for green techniques in environmental restoration is a crucial as the microbes at the MSW are becoming increasingly resistant to HMs stimulated by constant changes in the dumping sites. Microbes can also acclimatize to adverse environments more easily than other organisms, making them more stable and resistant to HMs. As a result, PSB isolated from MSW dumping sites, such as *Enterobacter* sp., are likely to perform better in HM bioremediation (Sahu et al., 2022). *Halomonas* BVR 1 was previously described as a highly resistant HM microbe capable of eliminating up to 200 mg L^{-1} of Pb, Cd, and Zn. Microorganisms can also be used to improve soil quality through biodegradation (Sahu et al., 2022).

The kinetic study is a tool that has been used to evaluate the impact of harsh environments on bacteria cell viability (Peleg, 1995; Vázquez et al., 2005). The classic Dosage-Response study technique is based on the idea that a sigmoid model predicts how some quantity that can be calculated from growth data (typically the maximal specific growth rate) fluctuates with dose (Riobó et al., 2008).

Pollution, toxicity, and ecotoxicity have been mostly related to "HMs" in the environment (Duffus, 2002). In reaction to HMs in the environment, bacteria have evolved resistance mechanisms (Bruins et al., 2000). Although the processes in all bacteria species are likely to be similar (Ji and Silver, 1995), it is envisaged that different bacterial species or groups will respond to the same harmful dose in different ways, and the bacteria might be environmentally friendly.

On the other hand, the PSB are important for mobilizing and converting available phosphorus (AvP), which is one of the most important nutrients for plant and animal growth. Despite the availability of 95-99 % phosphorus in the soil, the majority of it is insoluble and unavailable, due to the significant sorption and fixation of metal oxides in the soil matrix. Under these circumstances, plant growth-promoting bacteria scavenge the P in the soil and make it bioavailable to the plants (Nacoon et al., 2020). PSB has been discovered to be capable of converting insoluble P into soluble forms by various processes, including acidification, chelation, and exchange processes, and the production of polymeric compounds with the help of different phosphatase enzymes. These reactions are linked to the synthesis of organic acids, the release of their protons (H^+), and the presence of redox-active metals (Delvasto et al., 2006).

One of the most significant biological activities in the soil environment is soil phosphorus cycling. Soil phosphatase catalyze the hydrolysis of ester phosphate bonds, converting organic phosphorus into an inorganic form that plants and microbes can easily absorb from the soil solution (Nannipieri et al., 2011). As a result, phosphatase is essential for phosphorus cycling. Meanwhile, soil enzymes are a sensitive indicator of natural and anthropogenic changes in ecosystems and they utilized to assess the influence of numerous pollutants in the soil, including heavy metals (Ciarkowska, 2015; Rao et al., 2014). HMs limit enzyme activity in various ways, including masking catalytically active regions, denaturing protein structure, and competing with metal ions (Karaca et al., 2010). Additionally, microorganisms have been traditional and powerful tools of soil quality improvement through biodegradation. However, there is a lack of sufficient in-depth studies on isolating useful bacteria from soils contaminated with MSW. Hence, this work focuses on the acid phosphate assay of isolated PSB from an MSW discarding site for Cd and Cu bioremediation.

5.2 Materials and methods

5.2.1 *Qualitative and qualitative estimation of phosphate solubilization*

Phosphate solubilizing potential of isolated strains was measured according to Premono et al., (1996). After three days of post inoculation on a PVK agar media, the bacterial isolates were picked up. The point inoculation was done on new plates of PVK media and incubated at 32 ± 2 °C. After 3–5 days of incubation, the diameter of the bacterial colony and the halo zone were recorded. Solubilization Index (*SI*) was estimated using equation (1), where the diameter was measured in cm.

$$SI = \frac{Total\ diameter\ of\ \ the\ growth}{diameter\ of\ \ the\ colony} \qquad (1)$$

For quantitative estimation, 100 mL of PVK broth containing 5 g L^{-1} tricalcium phosphate was prepared in triplicates for all four bacterial strains. About 1.0 mL of seed inoculum of PSBs was inoculated in the broth, while the uninoculated media served as the control in this experiment. The bacterial cultures were incubated at 28 °C for 7 days at 120 rpm, and the homogenized suspensions were sampled every day, post incubation. The Molybdenum-blue method was used to analyze the released inorganic phosphate in the supernatant (Chen et al., 1956).

5.2.2 *Evaluation of physico-chemical properties of PSB strains*

The physico-chemical properties of PSB strains were analyzed using a standard protocol. The insoluble culture media was precipitated, and suspensions were left for 30–40 min. Using a spectrophotometer, the optical density (OD) of the culture at 600 nm was measured to determine the biomass growth of individual PSB in the supernatant (Genesys 10S UV–Vis Spectrophotometer, USA). 10 mL of the culture samples were taken and centrifuged at 5000 g (Remi R–4C, DX, India). The pH, P concentration, acid phosphatase activity, and organic acid concentration were all determined using the supernatant. The sedimented part of the bacterial culture was washed twice with 10 mL of 0.1 mol L^{-1} HCL to eliminate leftover tricalcium P on the bacterial cell surface (Xiang et al., 2011). The microbial cells were then killed by exposure to 121 °C for 30 min, and subsequently digested for 48 h 5 mL of 6% H_2O_2, held at 60 °C in a water bath. The microbial biomass phosphorus (MBP) content of the solution containing digested cells was measured by weighing the sedimented collected after centrifugation at 5000 g for 20 min (Sahu et al., 2022).

5.2.3 Assessment of organic acids and acid phosphatase activity

The organic acids were detected and quantified by High Performance Liquid Chromatography (HPLC) (Agilent Technologies, 1260 infinity HPLC USA). It was calibrated with a flow rate of 0.5 mL min^{-1} at 35 °C of operating temperature, using 0.1% ortho-phosphoric acid as a mobile phase. According to Tabatabai and Bremner, (1969) ACP assay method, 1 mL, 25 mM p-nitrophenyl phosphate with 4 mL modified universal buffer (MUB) (pH 6.5) was added to the bacterial supernatant, followed by incubation at 37 °C for one hour. After adding 1 mL 0.5 mol L^{-1} $CaCl_2$, and 4 mL 0.5 mol L^{-1} NaOH, the reaction was terminated. OD of reaction mixtures was taken at 420 nm using a spectrophotometer for measuring the enzyme activity.

5.2.4 Effect of Cd and Cu on SM_SS8 strain growth metabolism

The SM_SS8 PSB strain based on optimum phosphate solubilization properties and highest MIC value was chosen to assess the role of isolated PSB on Cd and Cu bioremediation. For bioremediation of the metals, 10,000 mg L^{-1} stock solutions of $CuSO_4.5H_2O$ and $Cd(NO_3)_2.4H_2O$ were prepared in Millipore water. The filter sterilization was done and stored for one month at 4 °C (Vignaroli et al., 2018). This study was carried out in PVK agar plate with concentrations ranging from 10, 25, 50, 75, 100 mg L^{-1} of $Cd(NO_3)_2.4H_2O$ and $CuSO_4.5H_2O$ in triplicate. About 1 mL of pre-inoculum of PSB in PVK media was spread in PVK and metal-containing agar plate, and the uninoculated media served as blank. The plates were incubated at 28 °C for 4 days and visually inspected for microbial growth. After incubation, the colony forming unit (CFU) was measured for viable bacterial cells using a hemocytometer (Labtronics 37 Colony counter Panchkula India). Furthermore, 1 mL of isolated SM_SS8 PSB strain was inoculated into 100 mL broth media; in 250 mL conical flasks containing 10, 25, 50, 75, 100 mg L^{-1} of both metals from seed culture. The flasks were incubated at 28 °C and agitated at 120 rpm. Samples were taken on alternate days after incubation for carrying out various analysis. The OD_{600}, pH, soluble phosphorus, and residual Cd concentrations were measured according to standard protocol (Teng et al., 2019).

5.2.5 Cell viability analysis of cadmium treated SM_SS8 bacterial cells

To analyse the viability of SM_SS8 bacterial cells grown in the presence of Cd, samples were collected after seven days of incubation. They were initially stained with 5-carboxyfluorescein diacetate (cFDA) followed by incubation and final addition of propidium iodide (PI). The samples were diluted so as to maintain 10^6-10^7 cells population, and 2 μL (0.5 mg mL^{-1} working concentration) of the respective dyes were added to 0.5 ml of the samples. The cFDA stained samples were incubated in a water bath at 50 °C for about an hour,

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and the PI stained samples were incubated in room temperature (37 °C) for 10 minutes. Both the stained samples were centrifuged and washed thoroughly prior to analysis using Cytoflex flow cytometer (Beckman coulter). The resultant flow cytometry data were analyzed using FlowJO_V10 software to obtain the live and dead cell percentage in the samples. Appropriate positive and negative controls, along with the dual staining, were taken for this cell viability analysis.

5.2.6 Growth models of SM_SS8 for different Cu and Cd concentrations

In this present study different Growth models have fitted with the data namely logistic model, modified logistic model, Gompertz's model, modified Gompertz's model, Richards's model and Stannard model (Annadurai et al., 2000; Zwietering et al., 1990). Although these models are being widely used for growth study but these models are limited by the fact they do not represent all the 4 phases of the microbial growth. Therefore, a new multiplicative model, proposed by Munoz-Lopez et al., (2015) has been applied for the given dataset, which represent all the 4 growth phases namely lag, growth, stationary and decay phase. The proposed model is given as

$$y = p.t^q - a.t^b$$

Where, $y = \ln\left(\frac{N}{N_0}\right) = \ln\left(\frac{OD}{OD_0}\right)$, q and b are empirical exponents

$p = \left(\frac{1}{t_{cg}}\right)^{1/q}$ and $a = \left(\frac{1}{t_{cd}}\right)^{1/b}$ where t_{cg} and t_{cd} are characteristic time parameters for growth and decay phase respectively.

Table 5.1. The expressions of the models are given in the following table

Name	**Mathematical expression**	**Parameters**
Logistic	$y = \frac{a}{1 + \exp(b - cx)}$	a, b, c
Modified Logistic model	$y = \frac{A}{1 + \exp\left[\frac{4\mu_m}{A}(\lambda - x) + 2\right]}$	A, μ_m, λ
Gompertz model	$y = a.\exp(-\exp(b - cx))$	a, b, c
Modified Gompertz model	$y = A\exp\left[-\exp\left\{\frac{\mu_m e}{A}(\lambda - t) + 1\right\}\right]$	A, μ_m, λ
Multiplicative model	$y = p.t^q - a.t^b$	p, q, a, b

5.2.7 AAS of Cu and Cd treated SM_SS8 samples

The supernatant solution of bacterial samples was taken for AAS analysis. The solution was centrifuged at 5000 rpm for 10 min. The supernatant was taken, and concentrated Cd and Cu heavy metal on the bacterial cell were removed using 5.0 mL of 1.0 M HCl. The sample was recentrifuged, and the supernatant was taken out for AAS.

$$S(\%) = \frac{Ci - Ce}{Ci} \times 100$$

The percentage of metal ions extracted from the media suspension (S%) was determined by comparing its concentrations before, Ci(mg L^{-1}) and after extraction, Ce(mg L^{-1}).

5.2.8 XRD and FTIR analyses of Cd and Cu treated bacterial cells

The PVK media with different doses of Cd along with the SM_SS8 PSB strain was incubated for seven days for XRD and FTIR analyses. At the end of the incubation period, the culture was centrifuged at 5000 rpm for 15 min, followed by 3 times washing of pellet using phosphate saline buffer (pH 7.2). The sample was then lyophilized for 48 h at -20 °C and slowly ground to obtain a fine powder (approx. 200 mesh). For XRD analysis (Micromax-007HF, Rigaku), the dried mass was used, whereas for FTIR analysis (PerkinElmer spectrum 2 FTIR spectrophotometer), it was mixed with potassium bromide (KBr) prior to the analysis (Rodríguez-Sánchez et al., 2017).

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5.2.9 SEM-EDX of Cd and Cu treated SM_SS8 cell

The 10 ml of bacterial broth culture was taken from suspension media**.** The bacterial cells were centrifuged at 5000 rpm for 20 min. The pellet was collected then washed, two to three times with phosphate buffer saline and centrifuged at 5000 rpm for 10 min. The pellet was left in centrifuge tube and maintained at -20 °C till completely dry. After it was completely dry, the sample was placed in the sample holder and with the help of carbon tap. To attain ideal measurement conditions, i.e., low background level and high sensitivity, for the analysis of the elements with lower atomic numbers and at, such as C, N, O, Ca, S, P, Cl, H. K, Fe, Cu and Cd, accelerating voltages of 1.5kV, 2 kV and 5kV were used (Khan et al., 2020). The working distance and lens aperture sizes were 8 mm and 30 µm, respectively. To acquire a statistically significant number of counts in the X-ray spectra, a distinctive quantifying time of 30s for each point was used. During area (0.05 µm × 0.05 µm) mode analysis, the measurement was carried out for a minimum of 10 min.

5.3 Results and discussions

5.3.1 Quantitative estimation of phosphate solubilization

The solubilization of phosphates by PSB is mostly due to organic acids and chelation of ligands, acidification, and redox processes (Wei et al., 2018). The formation of extracellular polymeric compounds, and the synthesis of various phosphatase enzymes are both dependent on the acidification of the media (Delvasto et al., 2006; Wei et al., 2018). The concentration of solubilized P in culture filtrate increased significantly, which decreased pH, and produced some organic acids. It was observed that the P solubilization by the isolates ranged from 46 to 277.66 mg L^{-1} (Fig. 5.1a).

The concentration of soluble phosphate of P1 strain was a maximum of 159.66 mg L^{-1}, with the least soluble potential detected compared to other strains. In previous studies, some researchers have documented 8.82 mg L^{-1} as the minimum solubilized phosphorus (Teng et al., 2019), whereas the maximum was 1226.6 mg L^{-1} (Sowmya et al., 2020) on 7th DAI (days after incubation). In the present study, the identified strains showed optimum P solubilizing ability compared to the previous research. Furthermore, *Enterobacter* sp. (SM_SS8) had the maximum phosphate solubilizing capacity among the isolated strains, with maximum P released into the media (reaching 277.66 mg L^{-1} after 4th DAI). In the surveyed literature, Yuan et al., (2017) reported the highest phosphate solubilizing capacity (up to 472.7 mg L^{-1}), but used a consortium of HMs tolerant *Enterobacter* sp., *Bacillus* sp., and *Lactococcus* sp. In this study, microbial biomass phosphorus was positively correlated with solubilized P. Additionally, present work evinced that the tricalcium phosphate present in the media was converted to a solubilized form; which appeared either in microbial biomass, or in the bacterial culture supernatant. Overall, it was observed that different PSB strains had unique, and different phosphate solubilization mechanisms, attributed to the significant differences in the production of organic acids, or other metabolites by the PSB strains (Zhao et al., 2022).

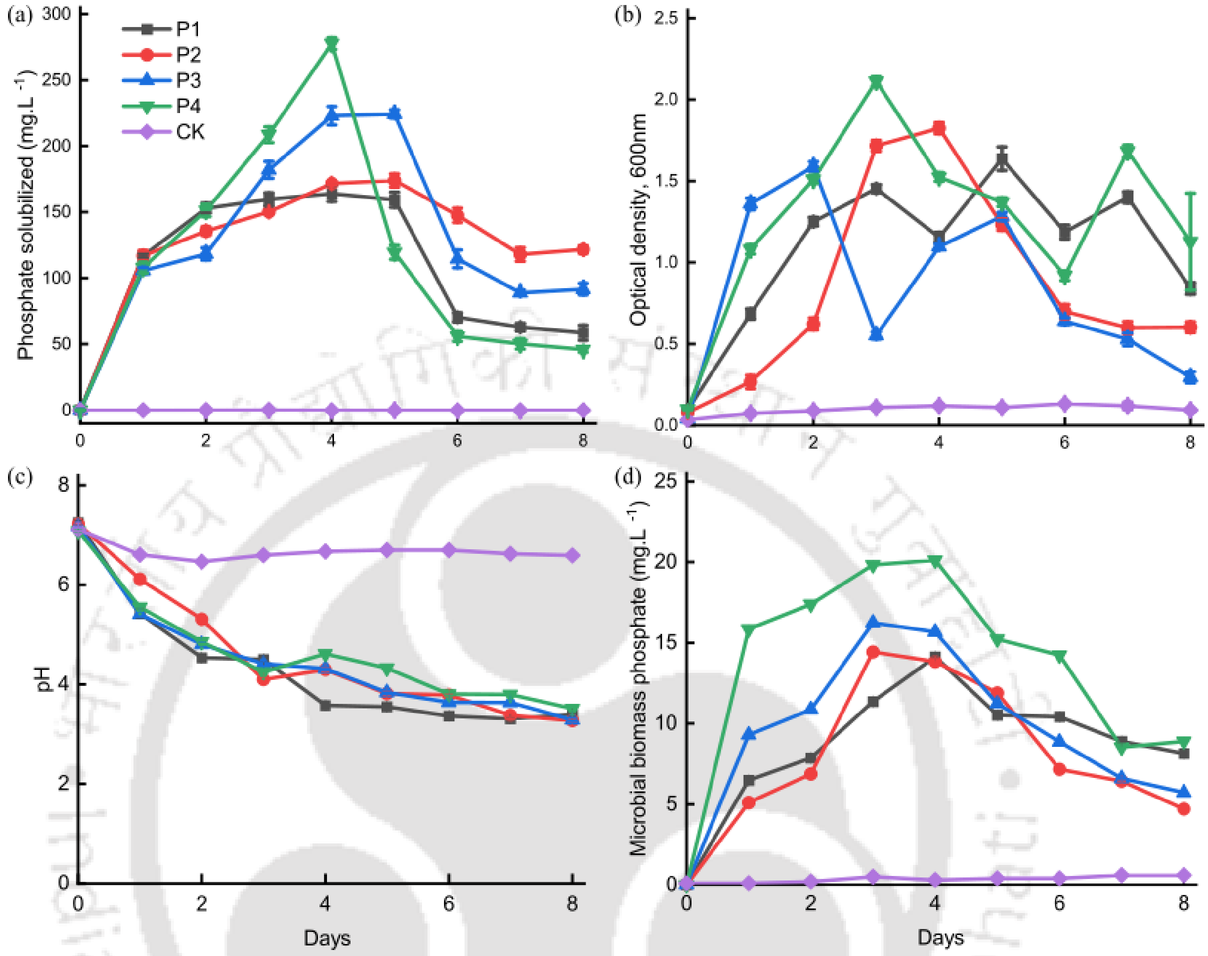

Fig. 5.1 The phosphate solubilization ability by four isolated PSB strains in the PVK broth media with $Ca_3(PO_4)_2$. The supernatant of culture media was aseptically collected every 24 h: (a) Concentration of solubilized phosphate (b) Optical density at 600 nm, (c) pH, and (d) phosphorus in microbial biomass (samples in triplicates).

5.3.2 Evaluation of physico-chemical properties of PSB strains

All the four PSB strains exhibited more than 1.5 OD at 600 nm, whereas SM_SS8 had a maximum of 2.15 OD after a day of incubation (Fig. 5.1 b). The OD of the strains fluctuated, which could have been set off by adaptation of bacteria, in a milieu of reduced pH, solubilized P, and freshly produced organic acids. Optimum bacterial growth is determined by its ability to solubilize the phosphate in tricalcium containing PVK media. Previous reports have revealed that the drastic alteration in the colour of the culture supernatant was closely

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associated with the solubilization of $Ca_3(PO_4)_2$ (Zhao et al., 2022) (Fig. 5.1 c). However, in the present study, the pH gradually dropped, and ranged from 7.22 to 3.32 in all the strains. The pH shapes the microbial life cycle; it affects the environmental conditions and plays a crucial role in enzymatic activity involving growth and biosynthesis (Sahu et al., 2022). P solubilization facilitates the release of organic acids and decreases the pH (Nacoon et al., 2020). As a result, the solubilization of $Ca_3(PO_4)_2$ in broth culture media was predicted using the low pH. As a result, pH, followed by organic acid concentration, is the most important factor in P solubilization (Sahu et al., 2022).

In the context of the current study, the PSB strains released a significant amount of phosphate, accompanied by a drop in the pH of the PVK media. An inverse relationship between phosphate solubilization and media pH has been previously reported, indicating the acidification of the culture media. Furthermore, Ca^{2+} (proton) substitution facilitates the release of orthophosphate from mineral phosphate (Nacoon et al., 2020). The microbial biomass phosphorus is also a critical parameter to analyze the soluble phosphorus in the media. The concentration of MBP increased significantly until the 4th DAI and then declined (Fig. 5.1 d). This reduction could be attributed to nutrient depletion in the media at the same time. As a result, after microbes complete their life cycle, biomass P is thought to be released directly into the ecosystem. It decomposes quickly and becomes readily available to plants (Kouno et al., 2002).

5.3.3 Assessment of ACP activities and organic acid

5.3.3.1 Acid phosphatase (ACP) activity in PSB strains

The ACP activities are reported for seven days of incubation time in the four PSB strains (Fig. 5.2). The metabolism of the bacteria gets strengthened during the growth phase, which leads to enhanced ACP activities in the media (Behera et al., 2017). The ACP activity in this study ranged from 29 to 70 μmol L^{-1} h^{-1}. The ACP activities, on the other hand, changed throughout time, and the time it took each strain to reach its maximum activity was not consistent or synchronous. The PSB's ability to hydrolyze the unavailable phosphate into the available form (such as tricalcium phosphate into phosphoric acid), corroborated previously reported results (Zhao and Zhang, 2015). From the 0th day to the 3rd day, the ACP activity increased gradually, but after 6th day, a slight decline was observed for all strains, except the SM_SS8 strain. In the present study, the P2 strain had the highest (68 µmol L^{-1} h^{-1}) ACP activity, whereas the activity of P4 was reported to be the lowest (50.6 µmol L^{-1} h^{-1}), compared to other strains on the 7th day of assessment. The P4 strain revealed an intermediate ACP activity in the entire study period. The study suggested that ACP activity is related to organic acid production by the bacteria and pH of the media (Fig. 5.2).

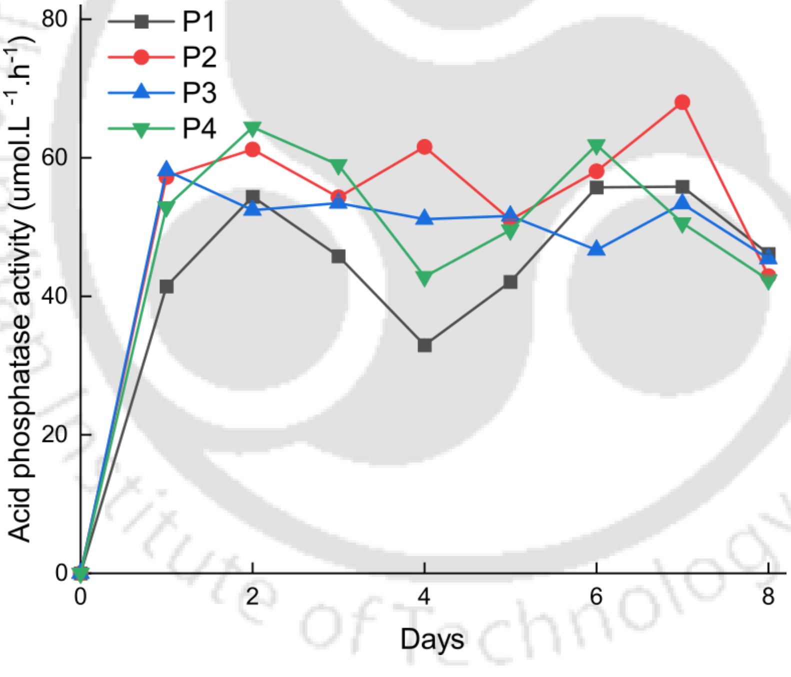

Fig. 5.2 ACP assay in the supernatant of PVK broth media every 24 h of four isolated PSB strains.

5.3.3.2 Production of organic acids by PSB

Isolated PSB bacteria secreted different organic acids in the PVK supernatant. The majority of these acids also have small molecular weight. The most often released organic acids are the Gluconic, lactic, malonic, tartaric, formic, and acetic acids (Sahu et al., 2022). Glycolysis and

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tricarboxylic acid (TCA) cycle pathway produce organic acids as intermediate products of metabolism. Some other routes also support the biological system to produce organic acids, such as tartaric acid and support the metabolism of fatty acids (Yin et al., 2015). Most of the intermediate acids of the TCA cycle (oxalic and tartaric acid), are expected to reinforce Cd mobilization via a suitable complexation capacity, and dissolution of acids (Wei et al., 2017). The glycolysis and TCA pathways contain the majority of the enzyme activities involved in organic acid metabolism. This enzymatic activity is altered by intracellular pH, substrate content, and environmental conditions. Organic acids produced by PSB acidify the media and in turn $Ca_3(PO_4)_2$ is converted in to accessible phosphate via protonation or chelation processes (Wei et al., 2018). Similarly, the amount of AA and MA secreted were higher as compared to GA and CA (Fig. 5.3 a & b). However, the concentration of different organic acids secreted by the isolated PSB strains varied with the increasing incubation period. P3 and P4 strains produced significantly higher levels of AA.

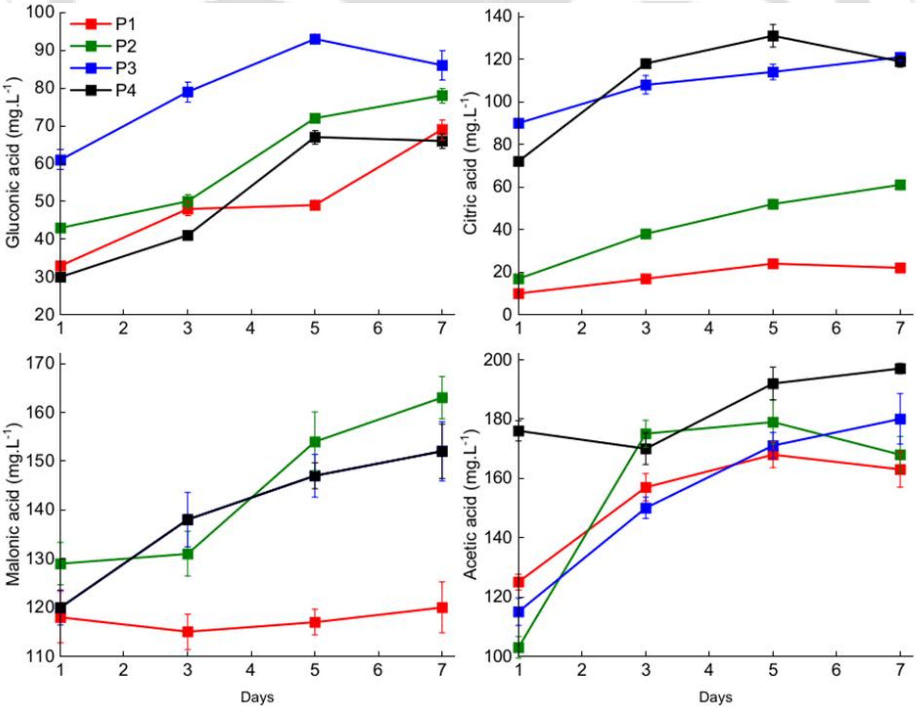

Fig. 5.3 Production of organic acids (a) Gluconic acid, (b) Citric acid, (c) Malonic acid and (d) The acetic acid in mg L $^{-1}$ in the supernatant of PVK broth media at the range of incubation period by four isolated PSB strains.

5.3.4 Effect of the Cd on SM_SS8 phosphate solubilization

After studying the ability of P solubilization and initial trials in different doses of Cd, the SM_SS8 strain (*Enterobacter* sp.) was selected for an in-depth assessment of Cd remediation. The MIC was found to be more than 100 mg $L^{-1,}$ in case of Cd, which is more dangerous than Pb to *Enterobacter* sp. (Jiang et al., 2020). The bacterium could not survive under Cd stress (up to 200 mg L^{-1}) because Cd particles were tightly adsorbed onto its cell wall (Jiang et al., 2020). In the current study, we assessed the impact of Cd on the growth pattern of the PSB isolate (SM_SS8), in $Ca_3(PO_4)_2$ in the liquid media (Fig. 5.4 a). The bacterial biomass was measured using absorbance at 600 nm wavelength, which declined linearly as Cd concentration increased, with CK as a reference. The OD of Cd-treated bacterial cells indicated that the P4 strain (SM_SS8) could withstand high Cd stress (Fig. 5.4 b). Even though Cd has a MIC of 50 mg L^{-1} in PSB $Ca_3(PO_4)_2$ agar, it did not prevent bacterial viability at a concentration of 100 mg L^{-1} in the broth media.

Furthermore, it was observed in the present study, that the pH of the media for all treatments tend to drop, which is reported to be favourable for Cd bioremediation (Sahu et al., 2022). Nonetheless, the rate of decline was lower than in Cd untreated PVK medium (Fig. 5.4 b). Therefore, the pH result of Cd treated samples might be attributed to the reaction of Cd ions with hydroxide and phosphate ions, which tend to increase the pH of the media. Besides, it is interesting to note that the pH decreased sharply in the presence of Cd ion, during post-inoculation in the PSB treated media. In general, Cd has various environmental and ecological negative effects on the species, by impacting their activity, biomass, and growth metabolism (Ma et al., 2020). The pH of Cd untreated PSB is lower than that of Cd treated PSB (Fig. 5.4 b). The higher pH of Cd treated PSB might be due to the sequestration of Cd ions with the organic acids, in particular AA, produced by the bacterium, a hypothesis that requires further affirmation. The study of phosphate solubilization in the presence of Cd in the broth is also a crucial parameter for PSB. The results revealed that the efficiency of phosphate solubilization by SM_SS8 strain, decreased with an increased concentration of Cd (Fig. 5.4 c). According to the Cd bioremediation assessment of SM_SS8 strain, phosphate solubilization property is directly proportional to OD, but inversely proportional to pH.

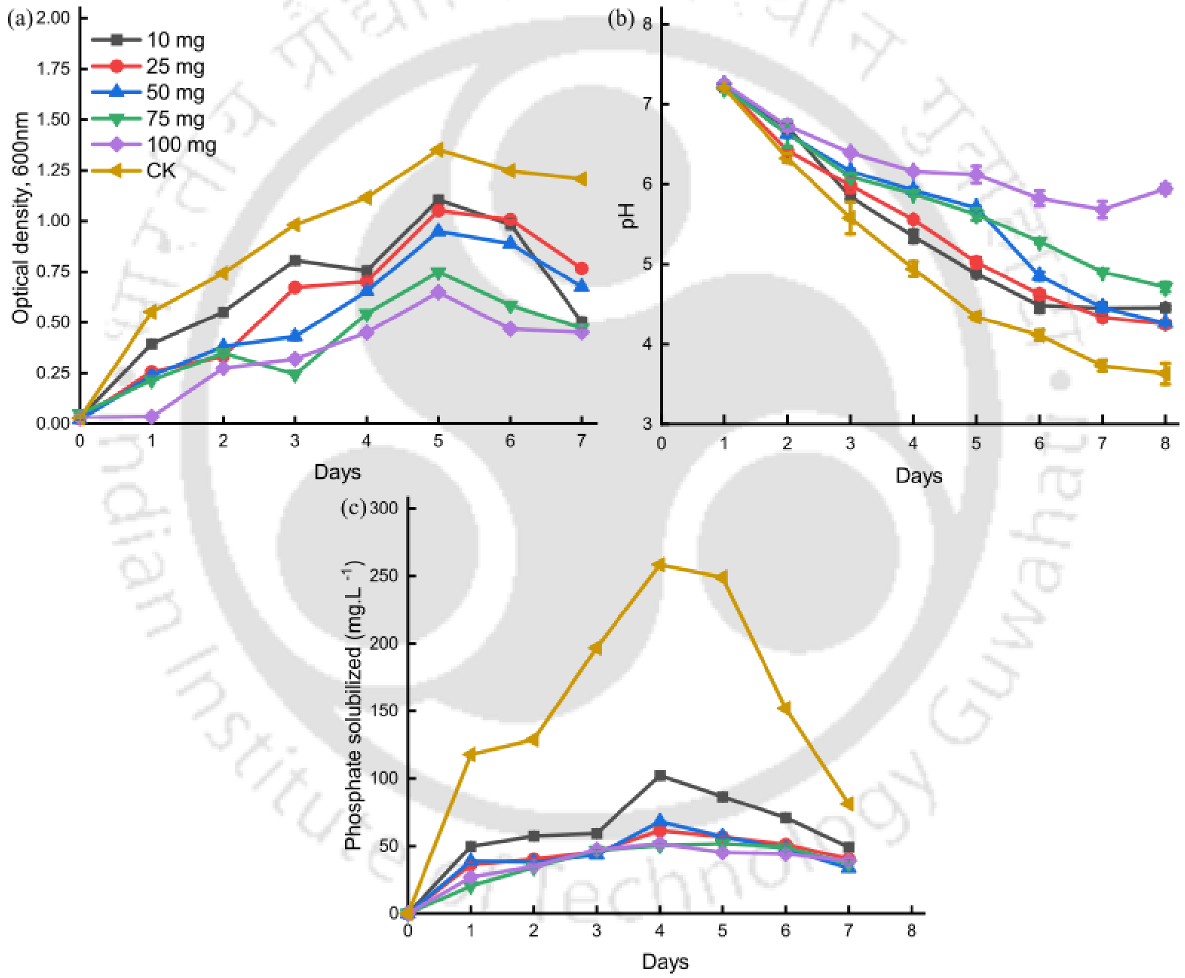

Fig. 5.4 Growth pattern of *Enterobacter* sp. strain SM_SS8 on PVK broth media with at the concentrations 10, 25, 50, 75 and 100 mg L^{-1} Cd. Optical density at 600 nm (a), pH (b), solubilized phosphate concentrations (c), CK represents the aseptic contrast experiment (sample containing the $Ca_3(PO_4)_2$ media without Cd).

5.3.5 Cell viability of Cd treated SM_SS8

Flow cytometry was employed for viability assessment of the SM_SS8 bacterial cells under HM stress. Quadrant gating for the control and treated samples are shown in Fig. 5.5. the resultant viability of SM_SS8 bacterial cells was ~46.93% at 50 mg L^{-1} concentration of Cd. In most cases, HMs might enter PSB and other organisms using multiple strategies. Several toxic metals which occur as divalent cations, such as Cd, can pass through the plasma membrane via cation transporters. On the other hand, sparingly soluble metal complexes may be taken up by phagocytosis, resulting in significant intracellular accumulation of these metals, following the slow breakdown in the lysosomes (Beyersmann and Hartwig, 2008). According to Beyersmann and Hartwig, (2008), these metals interrupt the normal metabolism, and work via three mechanisms to enhance toxicity: (1) Interference with cellular redox regulation and induction of oxidative stress, which cause oxidative DNA damage; or trigger signalling cascades leading to stimulation of cell growth. (2) Inhibition of major DNA repair processes causing genomic instability, and the accumulation of important mutations. (3) Induction of signalling pathways or inactivation of growth regulators, causing disruption of cell proliferation.

For viability assessment, three test samples of different Cd concentrations were considered (10, 50 and 100 mg L^{-1}). Metal-free and heat killed cells were considered as controls for live and dead cells, respectively. In the first treatment, the initial concentration of Cd was analyzed to observe the impact of Cd toxicity on SM_SS8 bacterial cells. Biosorption of Cd expending bacterial biomass is one of the most favoured alternatives, as this method is effective, inexpensive, reasonable, and eco-friendly (Khan et al., 2016; Ma et al., 2020). Earlier studies have reported that microorganisms possess a strong metal-binding capacity (Fein et al., 2019). In the present investigation, metal binding capacity of isolated SM_SS8 bacterial cells were analyzed in the context of their tolerance and endurance in a Cd vitiated milieu. Flow cytometry data suggested that the viability of the SM_SS8 bacterial cells was about 70.92%, 46.93%, and 20.4% in 10, 50 and 100 mg L^{-1} of Cd treated PSB cell culture. MIC value of Cd was found to be 50 mg L^{-1} for the SM_SS8 bacterial strain. Metal-free live-cell population of SM_SS8 bacterial examined as controls (untreated) were characterized by a dense green colour, endorsing cell viability (Fig. 5.5 C1, 6C2 & 6C3). The viability of SM_SS8 bacterial cells significantly decreased when the cells were treated with higher concentrations of Cd (Fig. 14 T1, 6T2 & 6T3). Consequently, cell viability was compared with Cd free live cells with ~34.8% nonviable cells and ~46.93% viable cells in 50 mg L^{-1} Cd concentration. These results are in close agreement with previous research, suggesting that the gram negative bacteria have a higher ability to tolerate HMs stress (Giovanella et al., 2017).

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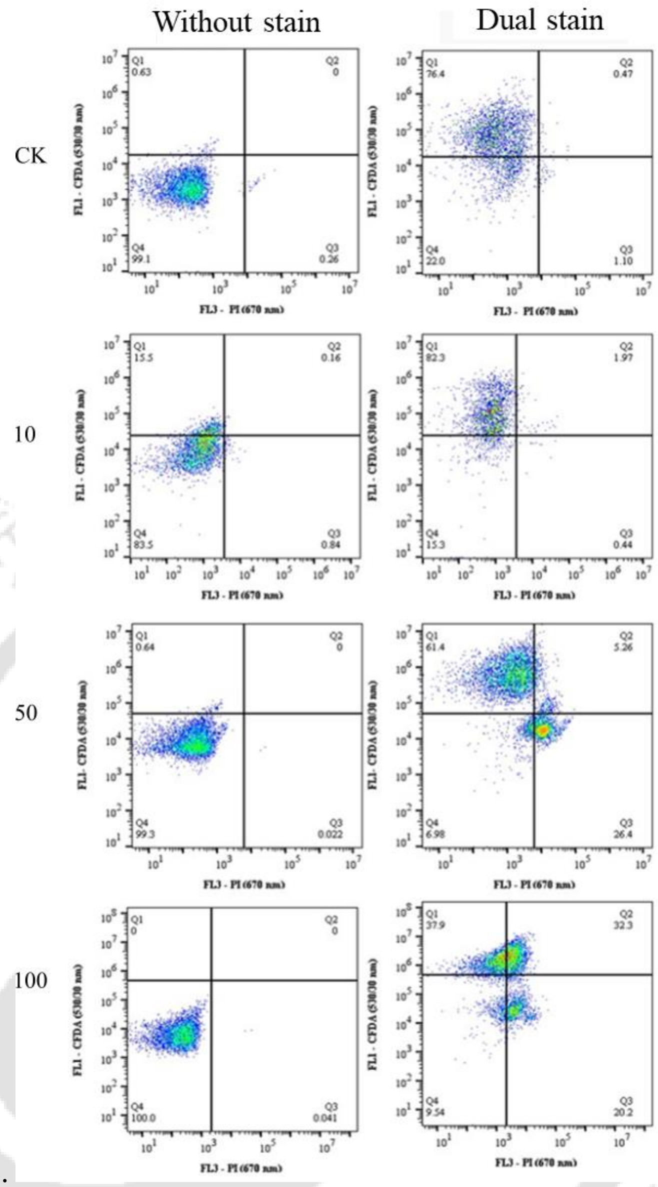

Fig. 5.5 Quadrant gating for control (untreated) and treated Enterobacter sp. strain SM_SS8 to staining with cFDA and PI. Bacterial cells treated with different concentration of 10, 50 and 100 mg L^{-1} Cd as shown in figure T1, T2, and T3 respectively. The viability of Enterobacter sp. strain SM_SS8 are illustrated in the dual stained Q1 quadrant. Dead cells displayed red colouration in the dual stained Q3 quadrant. Whereas, damaged cells, with partial permeability to PI, retained some green fluorescence due to cFDA, found in the dual stained Q2 quadrant.

5.3.6 Effect of Cd and Cu on SM_SS8 strain growth

The *Enterobacter* sp. (SM_SS8) has been chosen with the reference of P solubilization and Cd bioremediation in the PVK media (Sahu et al., 2022). The growth of SM_SS8 has been studied in the PVK media. Consequently, the growth curve of the SM_SS8 in the different growth model has been fitted with the data namely logistic model, modified logistic model, Gompertz's model, modified Gompertz's model, Richards's model and Stannard model (Annadurai et al., 2000; Zwietering et al., 1990). Although these models are widely used for growth study, they are limited by their inability to represent all 4 phases of microbial growth. Therefore, a new multiplicative model, proposed by Munoz-Lopez et al., (2015) has been applied for the given dataset, representing all 4 phases, namely lag, growth, stationary, and decay phase (Fig. 5.6). Initially, the significant growth of *Enterobacter* (SM_SS8) was detected in the different doses (up to 100 mg L^{-1}) of Cd and Cu in the growth curve (Fig. 5.7 & 5.8). In addition, the SM_SS8 also demonstrated the optimum amount of P solubilization (Sahu et al., 2022). A significant growth difference in the PVK media with various doses of Cd and Cu has been found in the initial trial. The ability of Cd and Cu bioremediation and the initial trials in different doses of Cd and Cu showed a significant difference in the growth curve of SM_SS8 strain (*Enterobacter* sp.). A previous study also revealed that the low pH of the medium also facilitates the bioremediation of HMs such as Cd and Cu by the PSB (Sahu et al., 2022).

Furthermore, it was observed in the present study that the pH of the media for all treatments tends to drop, which is reported to be favourable for Cd bioremediation (Li et al., 2020). The growth rate of *Enterobacter* sp. in both heavy metals displayed a decreasing rate, when compared with CK, as described in both growth models (Fig. 5.7 & 5.8). The MIC value was found to be more than 50 mg L^{-1} in the Cu metal, where as in case of Cd less than 50 mg L^{-1}. Previous researches have confirmed that Cd ion is more toxic to *Enterobacter sp*. than Pb^{2+} (Jiang et al., 2020). *Enterobacter* sp. could not survive under Cd stress (up to 200 mg L^{-1}), as Cd particles are tightly adsorbed by their cells (Jiang et al., 2020). In the case of Cu, *Pseudomonas stutzeri* LA3 removed about 50% of Cu (II) at 50 mg L^{-1} of concentration. In the current study, the result was in direct contrast to the study conducted by Teng et al., (2019), which stated that the viability of bacteria is more suitable for the toxicity of Pb in the liquid media (Rodríguez-Sánchez et al., 2017).

5.3.7 Effect of Cd and Cu on SM_SS8 strain growth model

Cd and Cu stress was studied using different models viz. logistic models, modified logistic models, Gompertz's model, and modified Gompertz's model. Based on the previously discussed limitations of these growth models. The multiplicative model was utilized for the kinetic study of Cd and Cu treated SM_SS8 strain. The comparative study between different models are shown in Table 5.2. It was found that no significant difference is observed in the performance criteria of logistic and Modified logistic models, and between the Gompertz and modified Gompertz models (Table 5.2). Significantly higher Nash Sutcliffe Efficiency (NSE) shows that the Multiplicative model performs better than the other four models in presenting the 4 phases of bacterial growth study: lag, growth, stationary, and decay phase. SM_SS8 strain also demonstrated its Cd and Cu remediation potentials (Fig. 5.7 & 5.8).

From the Mann Kendall's (MK) test, it is found that with the increase of Cu concentration μ follows a decreasing trend ($Q = 0.002$), whereas in the case of Cd, no significant trend has been found for μ with different Cd concentrations (Table 5.2). It is also observed from the MK test that with the increase of the Cu concentration, the specific growth rate of bacteria (μ) decreases significantly; however, no such significant pattern is observed in case of an increase in Cd concentration. These models therefore revealed that the survival of SM_SS8 increased with the increase in concentrations of both metals. The strain reduction capacity in both metals was found relatively in similar concentrations (e. g. 50 mg L^{-1}). In contrast, in the previous study, the data for Cd showed a growth reduction capacity of less than 5 mg L^{-1}, whereas for

Cu, it was nearly 50 mg L^{-1} against the *Bacillus* sp. (Zeng et al., 2020). The results of MIC showed that *Enterobacter* have the ability to survive under the stress created by Cd and Cu up to certain limits as indicated by the data. Extracellular polymeric substance (EPS) acts as the primary barrier in protecting the cell from direct contact with heavy metals, and reducing' the toxic effect of heavy metals on microorganisms (Aquino and Stuckey, 2004). Moreover, heavy metal ions can be precipitated onto the cell surfaces by functional EPS groups (Ueshima et al., 2008). Bacteria, therefore, have been thought to produce more EPS under metal stress (Zeng et al., 2020). The modified logistic and Gompertz model represents the negative value of growth time (λ) as shown in Table 5.2. The negative value of growth time was unusual and this value was not best fitted in all four phases of bacterial growth. But in the case of the Multiplicative model, value of growth time (λ) was positive as displayed in Table 5.2., and the four phases have been represented. In addition, the significantly higher Nash Sutcliffe Efficiency (NSE) shows that the Multiplicative model performs better than the other four models in presenting the 4 phases of bacterial growth study.

Table 5.2: Statistical output of different growth models

Model Name	Parameters	Value	95% confidence interval		Performance (in simulating actual vs predict OD) (Mean value)	
			Lower bound	Upper bound		
Logistic	*a*	5.351	5.092	5.611	MAE	0.093
	b	1.197	0.863	1.531	RMSE	0.113
	c	0.287	0.217	0.357	NSE	-0.812
Modified logistic	*A*	5.351	5.092	5.611	MAE	0.093
	μ_m	0.383	0.300	0.467	RMSE	0.113
	λ	-2.803	-4.548	-1.057	NSE	-0.809
Gompertz	*a*	5.427	5.179	5.675	MAE	0.074
	b	0.522	0.346	0.699	RMSE	0.089
	c	0.218	0.172	0.264	NSE	-0.127
Modified Gompertz	*A*	5.427	5.179	5.675	MAE	0.075
	μ_m	0.435	0.357	0.514	RMSE	0.09
	λ	-2.191	-3.375	-1.007	NSE	-0.141
Multiplicative Model	p	1.101	0.983	1.218	MAE	0.014
	q	0.359	0.297	0.422	RMSE	0.018
	a	$1.191\ 10^{-5}$	$-5.228\ 10^{-5}$	$7.610\ 10^{-5}$	NSE	0.955
	b	3.636	2.000	5.272		

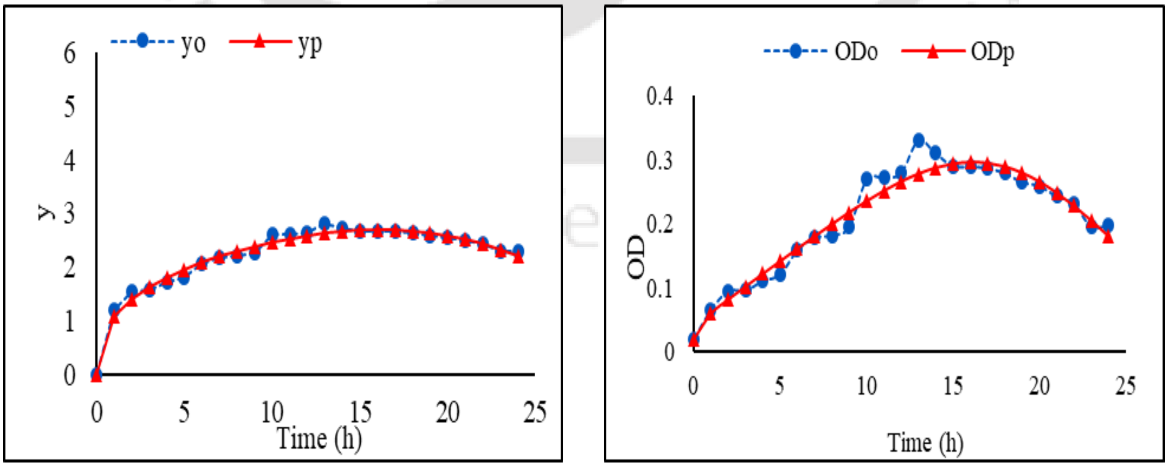

Fig. 5.6 (a) y vs. time and (b) OD vs time for 4 stage Multiplicative model for normal growth of SM_SS8.

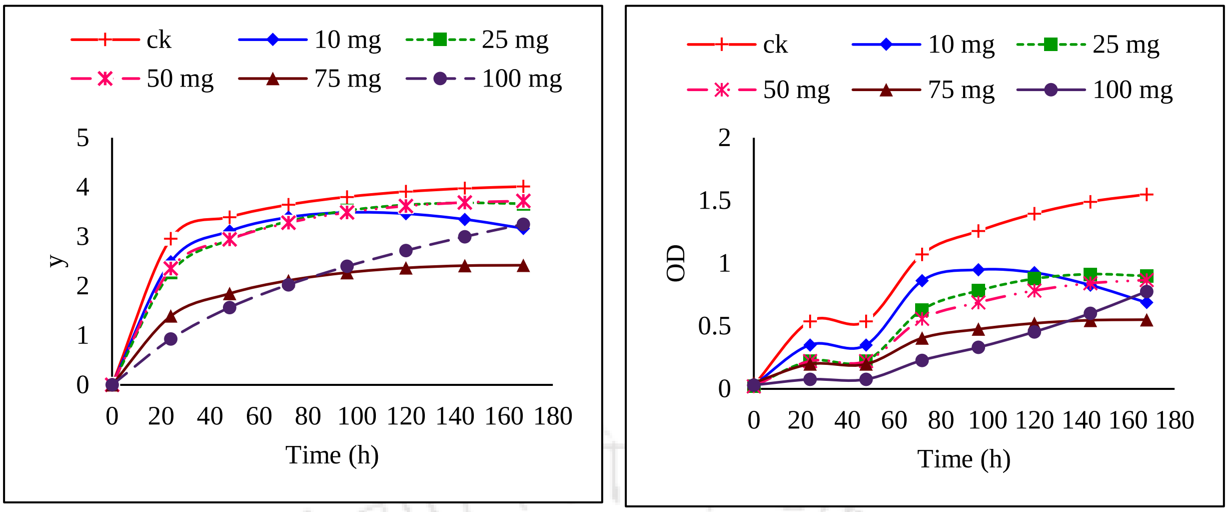

Fig. 5. 7 (a) y vs. time and (b) OD vs time for 4 stage Multiplicative model for Cu treated SM_SS8 cell biomass.

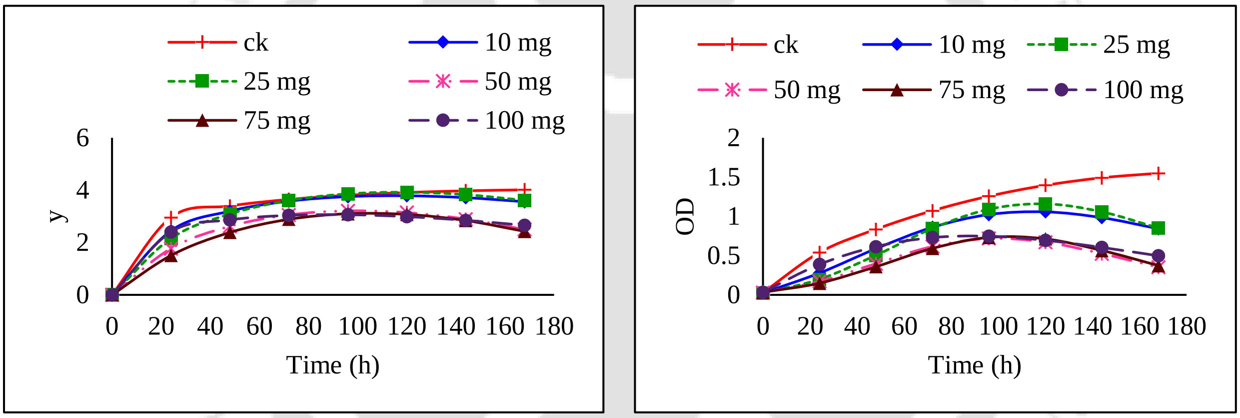

Fig. 5.8 (a) y vs. time and (b) OD vs time for 4 stage multiplicative model for Cd treated SM_SS8 cell biomass.

5.3.8 AAS of the supernatant of Cd and Cu treated SM_SS8 cell sample

After AAS analysis, the percentage of Cd and Cu remediation decreased with the increased concentration of both heavy metals Fig. 5.9. The value of 79.60% is the highest percentage of HM remediation by SM_SS8 for Cd at 10 mg L^{-1}, whereas 19.56% of Cd remediation occurred at 100 mg L^{-1}. Previous studies have confirmed that the HM reduces or inhibits the physiological and biological activities of microorganisms (Brito et al., 2015; Gujre et al., 2021b). The *Halomonas sp.* MG was found to be resistant against As and Cu, along with Pb.

It can resist up to 1000, 800, and 500 ppm of Pb, As, and Cu, respectively (Govarthanan et al., 2015), but in the case of the present study, the resistant properties of SM_SS8 against the Cd and Cu did not exceed 100 mg L^{-1}. The possibility of Ca in the PVK media being exchanged with Cd and Cu in the present study, could affect accurate analysis of Cd and Cu by AAS. The SEM-EDX of different doses of Cd and Cu treated SM_SS8 cell, indicating that some quantity of Cd and Cu were exchanged with Ca, lent support to this conjecture. In addition, Österås and Greger (2006), reported concentration of toxic heavy metals in the forest ecosystems might be reduced by the competition between higher calcium and heavy metals ions Similarly, the lead-resistant strain of *Enterococcus facealis* displayed resistance against various harmful heavy metals (Aktan et al., 2013). Thus, the obtained results suggested that the microorganisms isolated from heavy metal contaminated sites have developed adaptive mechanisms to survive in the metal-contaminated environment. Nanda et al., (2019) confirmed that microorganisms have mutual transporters for the export and import of heavy metals. Further, PbrA is a very well-known P-type ATPase transporter present in *C. metallidurans* CH_3 used for Cd, Zn, and Pb (Hynninen et al., 2009).

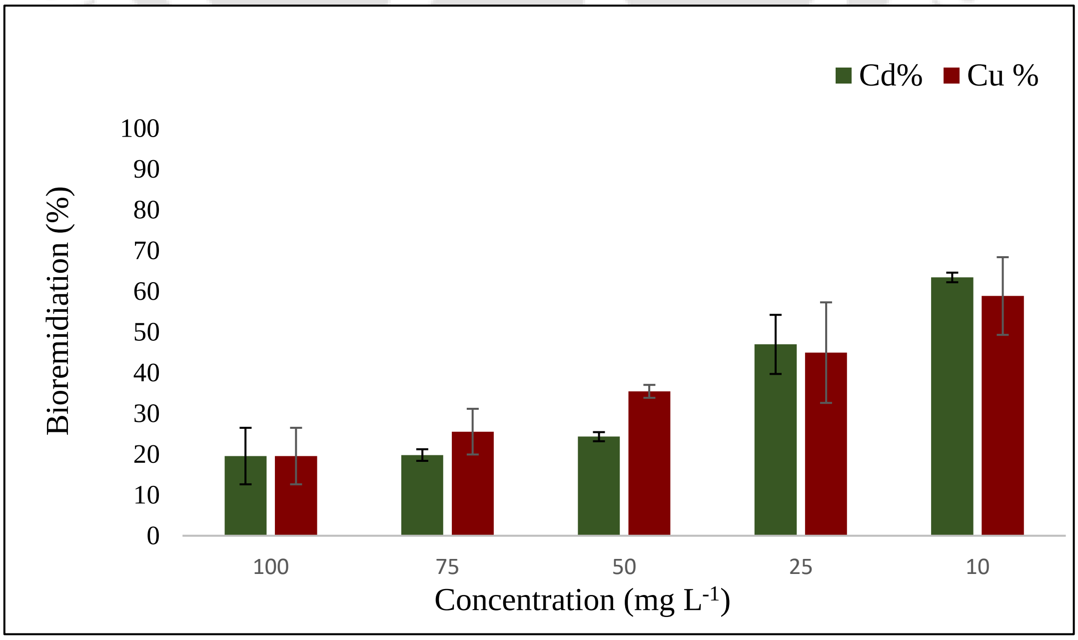

Fig. 5.9 Pecentage of Cd and Cu remediation by SM_SS8 cell.

5.3.9 FTIR analysis of the Cd and Cu treated SM_SS8 cell

The absorption capacity of metal ions in the microbial biomass mainly dependant on the functional groups are present on the active sites of the bacterial cell surface, and best accessed by FTIR and XRD analysis. In addition, bioprecipitation of Cd and Cu is dependent on the physiochemical properties of the media. The FTIR spectra of Cd (Sahu et al., 2022) and Cu treated and untreated PSB biomass in the range of 1000–4000 cm^{-1} were taken to ascertain the presence of functional groups that could be involved in the biosorption process. The FTIR spectrum revealed a sharp peak at 3262.28 cm$^{−1}$ representing OH, N-H, and ≡C-H stretching. The HM treated sample displayed more absorption intensity than the control sample. Among both metals, the peak of Cu treated samples has a wide range with the highest absorption intensity (Fig. 5.10 b). The C-H stretching of -CH_2, and -CH_3 occurred at 3000 cm^{-1}; with some Cd treated samples displaying a visible peak at this range (Sahu et al., 2022), while the Cu treated samples were not represented. The peak observed around 1870-1540 cm$^{−1}$ was reported due to C=O stretching (Doshi et al., (2007).

The common involvement of carboxyl functional group for Cd and Cu adsorption on the bacterial cell wall is derived from the bands stretching at 1642–1651 cm^{-1} (10 mg L^{-1}), 1652 cm^{-1} (25 mg L^{-1}), and 1650 cm^{-1} in Cd treated samples (Fig. 5.10 a). In addition, all doses of Cu treated samples also revealed a similar range of spectra. The IR spectra in this analysis evinced shifting in stretching bands indicating greater IR absorption intensity for the treated samples than CK (e.g., 1060 cm^{-1}). The shift might be attributed to the interaction and absorption of Cd along with phosphate groups.

It was also found that the peak transmittance in the different doses of Cd and Cu treated cells was considerably lower than that of the untreated PSB cell. These changes suggest that bond stretching is lowered by the presence of Cd, and consequently, peak transmittance is reduced. In agreement with our findings, similar results have been reported by Huang et.al., (2013). The formation of varying spectra following adsorption of the Cd and Cu ions on PSB biomass conclusively validated the contribution of functional groups in metal binding. However, the phosphate attached to Cd and Cu did not alter the functional groups on the PSB surface, except for cation exchange between either Cd or Cu with Ca, as compared to CK. Those functional groups were changed under the association of tricalcium phosphate and Cd or Cu, indicating that the functional groups played a crucial role in Cd and Cu ion bioprecipitation. The elemental and spectrum analysis of SM_SS8 cell wall by EDX also supported the HMs bioremediation by bioprecipitations.

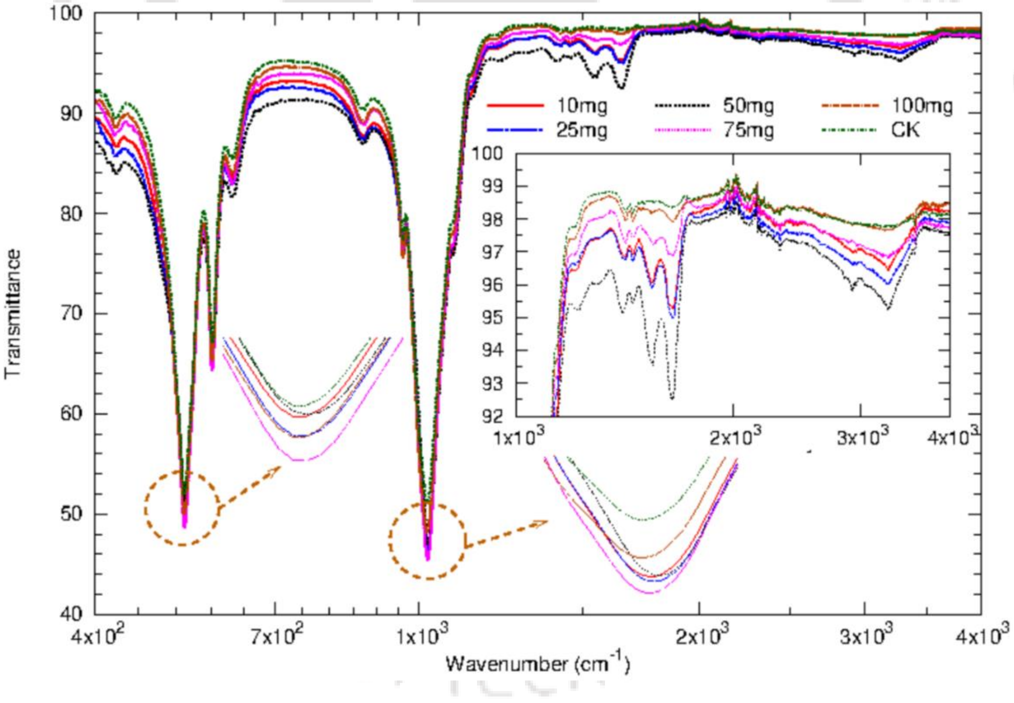

Fig. 5.10 a FTIR spectra of different concentrations of Cd treated *Enterobacter* sp. strain SM_SS8, and PSB cell with reference of CK for analysing Cd bound in the bacterial cell

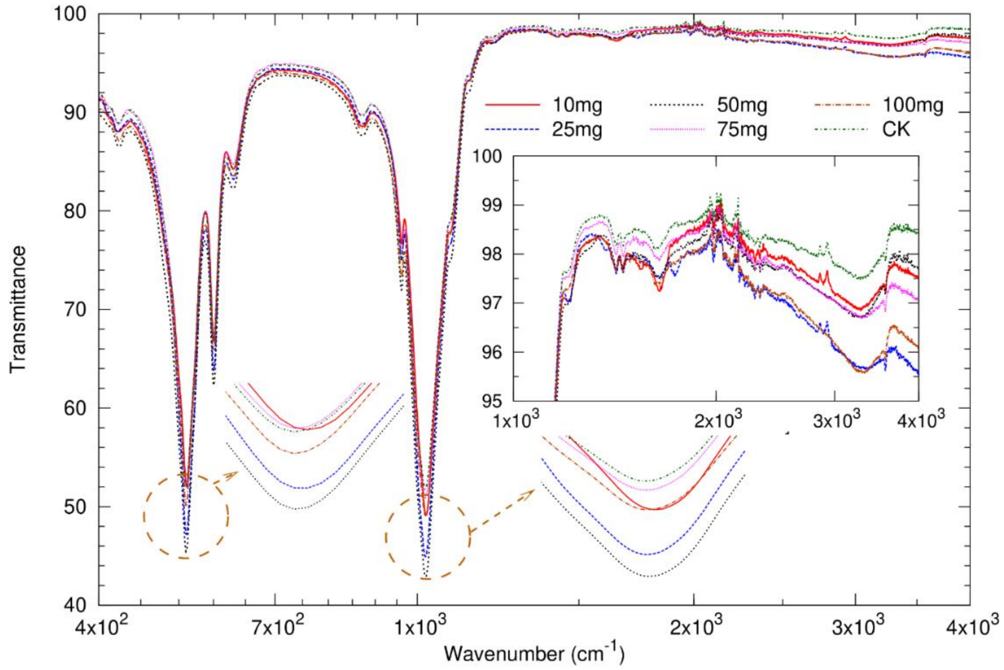

Fig. 5.10 b FTIR spectra of different concentrations of Cu treated *Enterobacter* sp. strain SM_SS8, and PSB cell with reference of CK, for analysing Cd bound in the bacterial cell

5.3.10 XRD analysis of the Cd and Cu treated SM_SS8 cell

The XRD results showed intensity of Cd treated PSB cells were lower (Fig. 5. 11 a) Sahu et al., (2022), with a greater shift to 2θº. Whereas in Cu treated PSB cells, the shift towards 2θ is less, and the intensity is also lower compared to CK (Fig 5.11 b). XRD patterns showed a range of prominent peaks in all treated PSB at 23.58, 30.26, and 49.67 2θº. In the case of Cu, the prominent peaks are around 31.3, 31.4, 32, 32.3 32.4, 32.5, 33, 33.1, 40, 48 and 50 2θº. The peaks of Cu treated samples appeared before the control. For example, the band of Cu treated samples have appeared at 32.3 and 32.4 2θº, whereas the CK peak was visible at 32.5 2θº. The differences of the peaks against control might have occurred due to the formation of Cd and Cu compounds in combination with phosphate, calcium, and carbonate. The diffraction of the Cd-treated sample peaks occurred due to $CdCO_3$ (Sahu et al., 2022; Shan et al., 2015). The greater shifting of 2θº in the Cu treated bacterial sample might attributed to the formation of calcium copper orthophosphate ($Ca_{10}Cu(PO_4)_7$), which is a higher molecular weight compound (Yanov et al., 1994).

The results indicated that the Cd and Cu immobilization have taken place via the formation of several types of inorganic cadmium and copper compounds (Mire et al., 2004; Yanov et al., 1994). Moreover, the Cu immobilization on the *Enterobacter* cell wall might be attributed to formation of copper (II) Phosphate cupric, phosphate copper (I) dihydrogen Phosphate and calcium copper orthophosphate. Similarly, in case of Cd, it is plausible that immobilization occurred due to the formation of $Cd_5(PO_4)_3OH$ on the bacterial surface (Chen et al., 2020). The Cd^{2+} accumulated on the surface of PSB could end as various cadmium and phosphate salts. Overall, the results revealed that several types of inorganic cadmium phosphates like cadmium orthophosphate ($Cd_3(PO_4)_2$), diphosphoric acid ($H_4P_2O_7$), and cadmium salt ($CdO_7P_2^{-2}$) are formed on the surface of PSB, which provided important evidence of Cd immobilization or Cd adsorption by PSB (Mire et al., 2004).

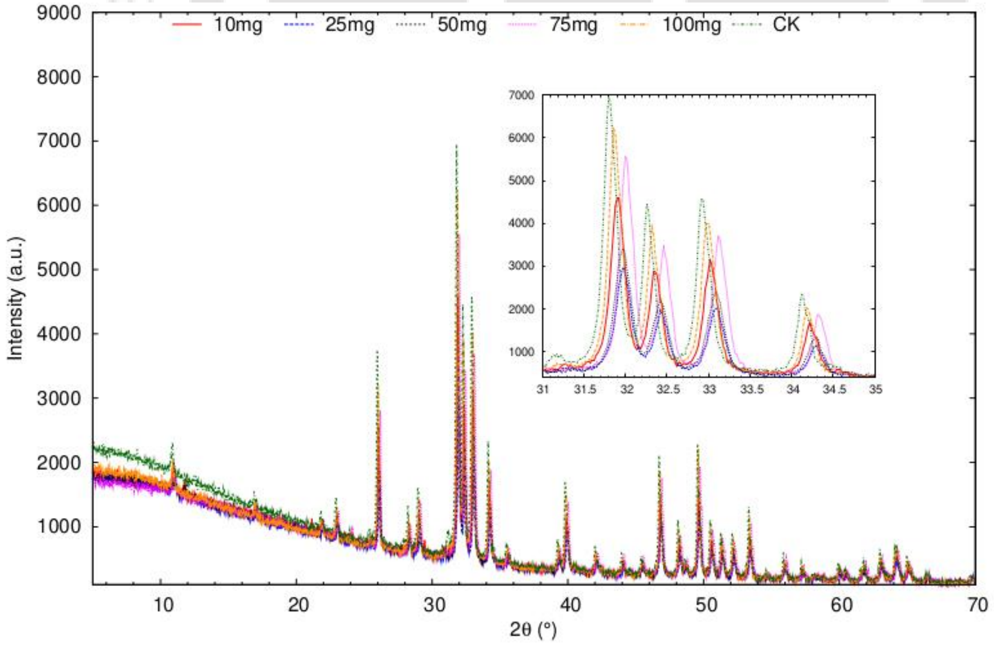

Fig. 5.11 a XRD spectra of different concentrations of Cd treated *Enterobacter* sp. strain SM_SS8, and PSB cell with reference of CK to analyze Cd bound in the bacterial cell.

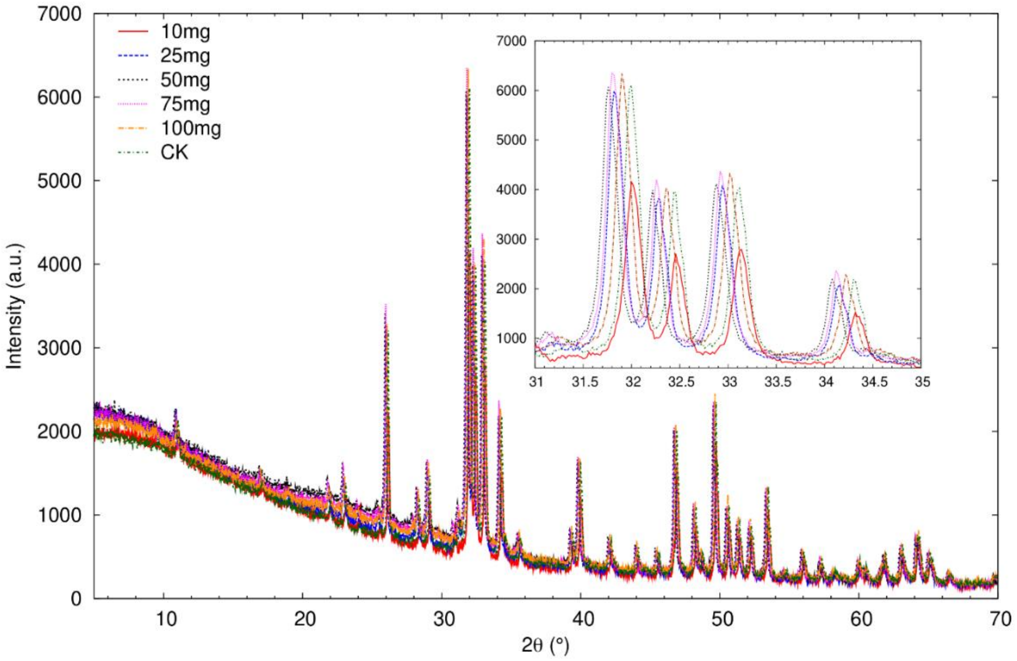

Fig. 5.11 b XRD spectra of different concentrations of Cu treated *Enterobacter* sp. strain SM_SS8, and PSB cell with reference of CK.

5.3.11 EDX analysis of Cd treated bacteria cell

The elemental composition and morphology of the metal bioadsorption or bioprecipitation were analysed using EDX. The results shown in Fig. 5.12 reveal the presence of cadmium and copper in the bacterial cell biomass, clearly signalling the bioremediation of cadmium and copper. Further, other peaks engendered by nutritional elements like C, N, O, Na, and Fe are displayed in the Fig. 5.12 a & b., supported by the reports of Kiran et al., (2017) and present as nutrients in the PVK media. The presence of different macro elements along with the HMs Cd, and Cu is confirmed by the EDS spectrum taken from the spots on the outer cell surface of the bacteria (Fig 5.12 a & b). Metal precipitates in the vicinity of the bacterial cell surface are clearly visible in the spectrum taken up by EDX. Spectrum of different doses of Cd and Cu treated bacterial cell revealed different concentrations. For example, the intensity of 25 mg L^{-1} Cd treated bacterial cell have the higher intensity than the lower intensity exhibited by 75 mg L^{-1} Cd treated as shown in Fig. 5.12 a (a) & (c). Whereas the peak intensity of 25 mg L^{-1}

Cu treated cells revealed an average peak intensity. It might be the concentration of Cu was lower quantity and it was utilized for the various physiological function by the bacterial cell. The flow cytometer analysis of SM_SS8 also supported the maximum viable cell at 10 and 50 mg L^{-1} as compared to the 100 mg L^{-1} treated (Sahu et al., 2022).

The analysis of FT-IR and XRD has shown that Cd was sequestered with phosphate and carbonate as the main functional groups present in the PSB cell surface for its immobilization (Sahu et al., 2022). In addition, the intensity of Cu 25 mg L^{-1} was higher in comparison to the other doses of Cu (Fig. 5.12 b). It was also revealed that, the maximal intensity of the Cu peak in Cd treated cells as well as the CK, could be attributed to the presence of Cu as a nutrient in the media. Similarly, the PVK media containing Cu as a micronutrient was probably responsible for the small peak of Cu visible in the Cd treated samples (Fig. 5.12 b). The study of Kiran et al., (2017) suggested that, the anaerobic sulphide reducing bacteria have the potency to bioprecipitated the HMs like Cd, Ni, Zn, Cu on to the bacterial cell biomass from HMs contaminated site.

The TEM-EDS and FESEM-EDX generated data of the metal removal and precipitation by the bacteria were connected with the bacterial cell surface. The capacity of the sulphate reducing bacteria to reduce HMs was linked to their elimination process, which resulted in the metals precipitating as sulphide salts (Kumar and Pakshirajan, 2020). According to Kumar and Pakshirajan, 2020 it can be clearly seen from the FETEM images that nanoparticles are accumulated outside as well as on the cell wall of the bacteria. The images also exhibited layer like structure surrounding the bacteria. It has been reported that such a naturally produced layers made up of exopolysaccharide (EPS) that bind around the bacterial cell physically or electrostatically. The bacteria secrete EPS as a defence mechanism against the HM toxicity.

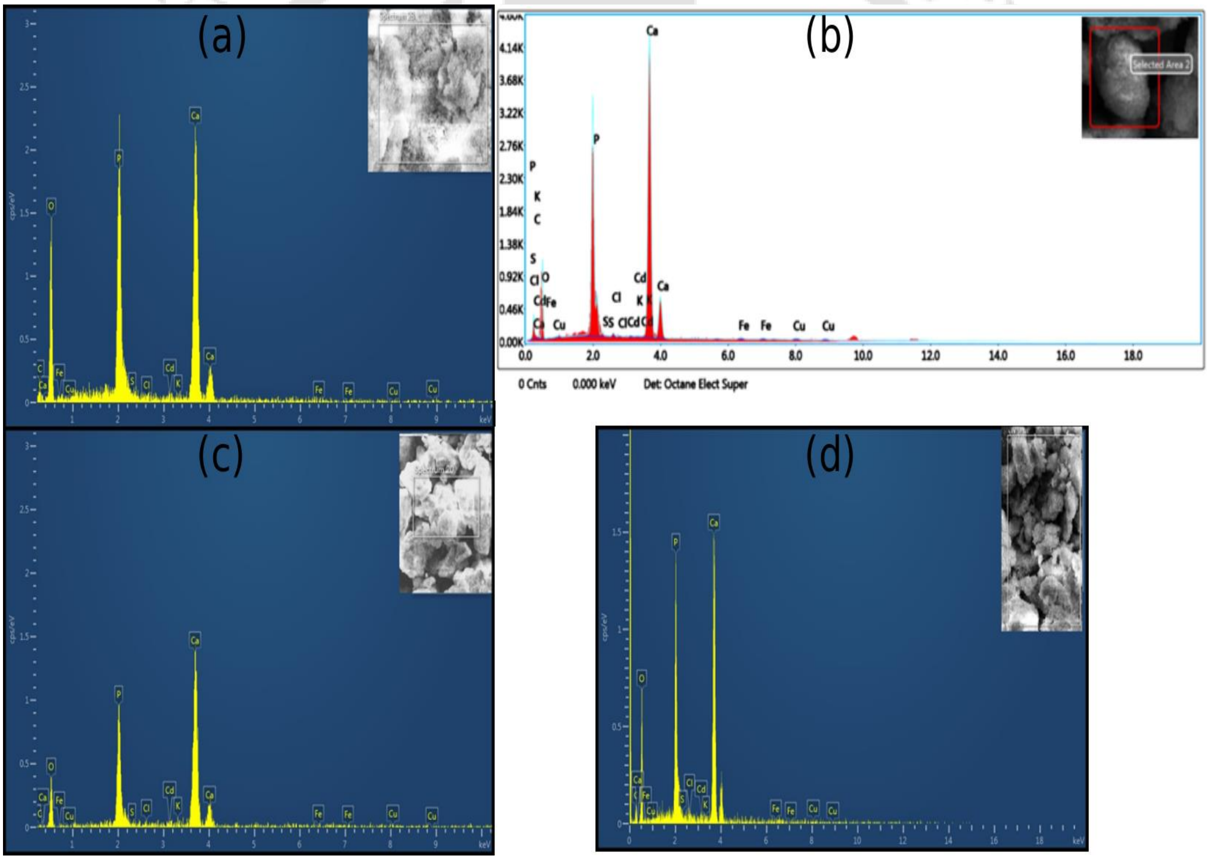

Fig. 5.12 a EDX spectra of Cd (a) 25 mg L^{-1} (b) 50 mg L^{-1} (c) 75 mg L^{-1} (d) 100 mg L^{-1} treated *Enterobacter sp.* strain SM_SS8, and PSB cell with reference to CK (Fig 5.12 b).

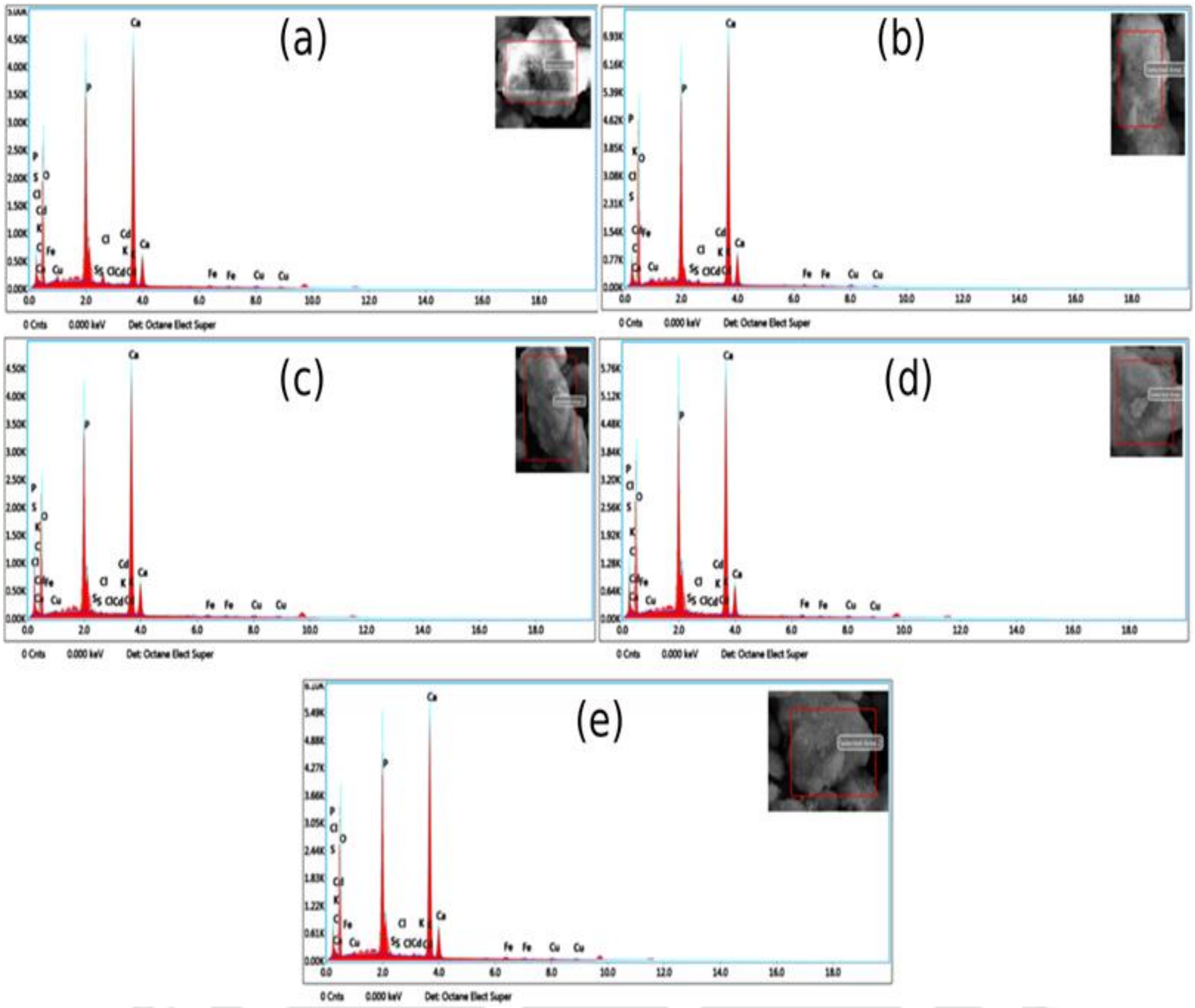

Fig. 5.12 b EDX spectra of Cu (a) 25 mg L^{-1} (b) 50 mg L^{-1} (c) 75 mg L^{-1} (d) 100 mg L^{-1} treated *Enterobacter sp.* strain SM_SS8, and PSB cell with reference to CK (e).

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5.4 Conclusion

Enterobacter sp. (SM-SS8) had the maximum phosphate solubilizing capacity among the isolated strains, with maximum P released into the media (reaching 277.66 mg L^{-1} after 4th DAI). The pH gradually dropped, and ranged from 7.22 to 3.32 in all the strains. The concentration of MBP increased significantly until the 4th DAI and then declined. From the 0th day to the 3rd day, the ACP activity increased gradually, but after 6th day a slight decline was observed for all strains, except the SM_SS8 strain. The P4 strain revealed an intermediate ACP activity in the entire study period. The amount of AA and MA secreted were higher as compared to GA and CA. However, the concentration of different organic acids secreted by the isolated PSB strains varied with the increasing incubation period. The results of P solubilization showed that it is correlated with pH, ACP activity, and organic acid. On the other hand, the multiplicative model has a better fit than the other four models, as it can capture more precisely all the four stages of growth under Cd and Cu stress. Cell viability study by flow cytometer was used to assess the Cd and Cu bioremediation capacity of the discovered P4 strain, and then cross-verified by FTIR and XRD investigation. In Cd treated PSB cells, 70.92 %, 46.93 %, and 20.4 % viable PSB cells were identified in 10, 50, and 100 mg L^{-1}, respectively. The P4 strain (SM_SS8) had the best phosphorus solubilization efficiency and excellent Cd and Cu remediation capabilities.

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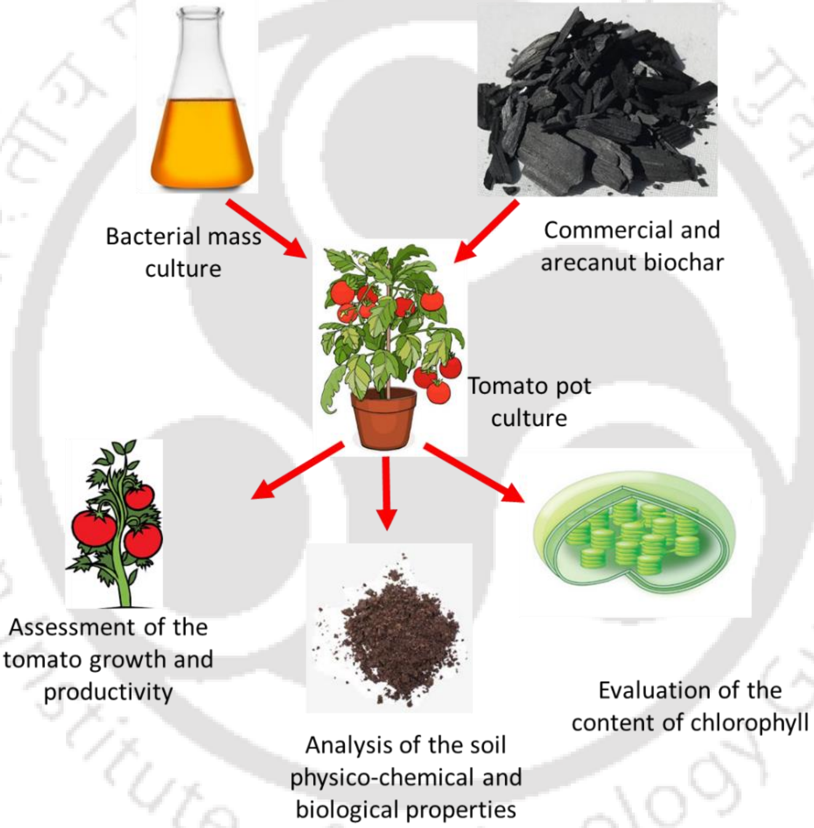
CHAPTER 6

Evaluation of the qualitative and quantitative impact of N fixing and P solubilizing bacteria on soil and plants



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The current chapter addresses the use of biochar and biofertilizer formulations in soils contaminated with Cd. Tomato plants are cultivated to validate the improvement soil quality, plant production, and HMs bioremediation. In addition, the microbiological diversity of harvested soil is studied.

6 Evaluation of the qualitative and quantitative impact of N fixing and P solubilizing bacteria on soil and plant

6.1 Introduction

The amendment of degraded soils with biofertilizers and biochar (BC) has received worldwide acceptance as a potential option for improving soil fertility and the functioning of the edaphic ecosystem (Tariq et al., 2022). Biofertilizers and biochar are able to bioremediate the HMs in a more efficient, cost-effective, and environmentally friendly manner. They reduce metal toxicity and improve soil quality, subsequently increasing food production over time (Sowmya et al., 2020). In addition, biofertilizers are soil-borne living cells or latent cells of successful microorganism strains that help agricultural plants absorb nutrients more efficiently (Ashrafuzzaman et al., 2009; Bera et al., 2017). Wood chips, agricultural wastes, nutshells, seed mill screens, and algal biomass are examples of traditional feedstock that can be pyrolyzed in an oxygen-depleted environment to produce biochar. The feedstock and pyrolysis settings determine the physical and chemical features of biochar (Gujre et al., 2021).

The use of biofertilizers to remediate heavy metal polluted sites have garnered much interest in the last decade (Yuan et al., 2017). Multifaceted benefits are derived from the application of biofertilizers, leading to the improvement of pH, CEC, soil buffering, porosity, water retention capacity, and bulk density of contaminated soil (Yuan et al., 2019). Further, the microbes scavenge and fix macronutrients such as P, K, and N in the soil and make them available to plants (Nacoon et al., 2020; Parastesh et al., 2019). Microorganisms may reduce heavy metal toxicity in plants by reducing metal uptake or by reducing the levels of harmful stress ethylene generated by heavy metals without affecting their uptake (Madhaiyan et al., 2007). Metal-resistant microorganisms with plant growth-promoting traits, might be increase the availability of heavy metals for plant absorption by solubilizing or mobilizing, and are therefore useful for phytoremediation (Madhaiyan et al., 2007). In addition, the application of biofertilizers and BC not only increase the diversity of beneficial soil microorganisms, but simultaneously remediate a variety of pollutants from the edaphic ecosystem. The use of biological systems like PGPR to remediate pollution of various HMs from the environment is a case in point (Yuan et al., 2017).

Similarly, the application of BC helps in regulating various biogeochemical processes in the soil, including the carbon (C), phosphorus (P), and nitrogen (N) cycle. However, biochar can be used as a promising and cost-effective soil amendment technique. It improves crop productivity with its greater surface area, highly porous structure, added oxygen functional groups, and higher cation exchange capacity (CEC) with the property of HMs reduction (Dai et al., 2020).

Cd is one of the heavy metals that is considered harmful to plants. Cd is a phytotoxic element with uncertain biological function in plants, causing various plant-related problems (Madhaiyan et al., 2007). We investigated the endurance of isolated strains and BC with plant growth-promoting properties to various Cd metals. In the present study, soil was taken from MSW site and spiked with the Cd. *Bacillus* sp. strain SM_SS7 (**P3**), *Enterobacter* sp. strain SM_SS8 (**P4**), *Paraburkholderia fungorum* SM_ SS1 (**N1**) *and Bacillus* sp. strain SM__SS2 (**N2**) with the formulation of biochar has been applied for soil treatment. Tomato, (*Solanum lycopersicum*), a flowering plant of the nightshade family (Solanaceae) was cultivated and used for remediation of the soil contaminated with Cd.

6.2 Materials and Methods

6.2.1 *Collection and treatment of the soil sample*

The soil used for this study was collected from the IIT Guwahati campus (10-20 cm). The soil was spiked with $CdCl_2$ (50 mg L^{-1}) to determine the effect of biochar, biofertilizer and the combination of biochar and biofertilizer on plant growth and production. The spiked soils were left for the incubation for a month at greenhouse. Before spiking physio-chemical properties of collected soil was analyzed.

6.2.2 Preparation of the seed and mass culture

After screening and characterization of NFB and PSB on Winogradsky and Pikovskaya media (PVK), N1 and N2 and P3 and P4, respectively, were selected for the pot experiment. The Winogradsky and PVK media were used to prepare seed and mass culture of NFB and PSB respectively; subsequently, the concentration and growth condition of bacterial culture were monitor by OD at 600 nm at specific intervals.

6.2.3 ***Heavy metal tolerance by the bacterial strains***

The ability of the strains to grow under increasing concentrations of $CdCl_2$ was tested by plate and broth assays. For the determination of minimal inhibitory concentrations, strains were streaked on agar plates supplemented with $CdCl_2$ from 1.0 to 100 mg L^{-1} and checked for their growth after appropriate incubations. In the broth assay, pre-cultures in media without heavy metal were used to inoculate the media containing different concentrations (1.0 to 100 mg L^{-1}) of $CdCl_2$. The growth after every 24 h incubation with shaking (200 rpm) at 37 °C was recorded by measuring the absorbance at 600 nm.

6.2.4 ***Preparation of Biochar as a carrier and incubation of the collected soil***

Two types of biochar were sourced to act as carriers for the biofertilizers. The characterised Areca nut biochar (A), was sourced from the SM AEL laboratory at IITG was used as a carrier for N1 = *Paraburkhendria* (SM_SS1), N2 = *Bacillus* (SM_SS2), P3 = *Bacillus* (SM_SS7), and P4 = *Enterobacter* (SM_SS8**).** Similarly, the second was a commercially available biochar (CB)**,** was also used as a carrier for among all four strains**..** The biochars were tightly packed in fresh biohazards polyethylene bag and autoclaved for 20 min at 121°C for three consecutive days to stop the contaminations.

Each pot was filled with five kg of Cd spiked soil. The 10^6-10^8 mL^{-1} concentration of biofertilizers were properly mixed with the spiked soil, at a proportion of one L $acre^{-1}$. The pots were done placed for incubation for 8 days. After that, each pot was sowed with two tomato saplings. The control samples (CK) were prepared without Cd-spiked soil, biochar, and biofertilizer. All the pots were allowed to incubate for a period of 8-days, post which each pot was planted with two tomato saplings, and watered regularly to maintain 70% moisture. After every 14 days, thinning was done and only healthy saplings were allowed to grow. The experiment was conducted in a completely randomized block design with four replicates for each treatment. At particular intervals, morphological plant characteristics were noted. Number of flowers, and later the fruit yield was also calculated. After completion of life cycle, the plant samples were thoroughly washed with ultrapure water and oven dried at 70 °C for 72 h. The dry biomass of root and shoot was measured using the weighing machine.

6.2.5 Physico-chemical properties of soil collected from pot culture

The physico-chemical properties of the pot soil after tomato harvesting, were analysed to determine the effects of bioformulation (biofertilizer and biochar) on the soil fertility status. Briefly, the pH was measured using a handheld HI 3221 pH-EC meter (Hanna Instruments Inc., USA) and the CEC was measured following the method of Biswas et al., (2010). The flame photometer Systronics Model 126, India, was used to determine the available sodium (AvNa) and potassium (AvK) (Knudsen et al., 1982). Available nitrogen (AvN) was determined using Kjeldahl assembly (Subbiah and Asija, 1956) sourced from Velp Scientifica UDK 129, USA. Using a Genesys 10S UV-Vis Spectrophotometer, the available phosphorus (AvP) was calculated by the Olsen (1954) method for alkaline soil and the Bray and Kurtz, (1945) method for acidic soil.

Plant heights were measured in cm. To assess the effect of biochar and biofertilizer on plant growth, the biomass of tomato plants was determined. After cultivation, the shoot and roots portions of the tomato plants were collected separately and washed thoroughly with ultrapure water, and then measure with the help of scale. The harvesting of tomato during pot experiment was also recorded.

6.2.6 Content of Chlorophyll a, Chlorophyll b, and total Chlorophyll of tomato leaves

The leaf samples from each treatment were taken to determine chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (total Chl) content. According to Arnon, (1949), and Krick and Allen, (1965) method, chlorophyll extracts were determined with spectrophotometer at 663 645, and 470 nm, respectively.

Following Formulae used for Chl estimation:

- Chl a (mg g^{-1} FW) = (12.7 ×D663) - (2.69×D645)
- Chl b (mg g^{-1} FW) = (22.9 ×D645)- (4.68×D663)
- Total chl (mg g-1 FW) = (1000D470-1.9×chla-63.14×chlb/ 214)

6.2.7 AAS of the soil collected from pot experiment

Three composite soil samples were collected from each pot culture. Soil samples were collected into transparent plastic bags using a steel Auger from 0–15 cm depth. Samples were air-dried in the laboratory by spreading out on transparent plastic on a bench for several days. Further, the different HMs analyses by AAS were performed according to (Gujre et al., 2021b).

6.2.8 Taxonomic analysis of bacterial population in the soils of pot experiment

Metagenomics analysis of harvested soil for bacterial diversity has been completed according to (Baird and Hajibabaei, 2012; Wang et al., 2007; Winand et al., 2019).

6.3 Results and discussion

6.3.1 Effect of biofertilizer and biochar application on soil parameters

Table 6. 1 Effect of biofertilizer and biochar application on physico-chemical properties of soil collected from pot culture

Sr. No.	**Code of samples**	**pH**	**AvK in the pot soil (mgKg^{-1})**	**AvP in the pot soil (mgKg^{-1})**	**AvN in the pot soil (mgKg^{-1})**	**AvNa in the pot soil (mgKg^{-1})**	**CEC mol kg^{-1} of soil**
1	AN1	7.0±0.1	7.99±0.90	50.97±4.10	1.52±0.07	19.34±0.99	86.08±1.70
2	AN2	7.3±0.1	7.37±0.71	53.57±6.68	1.59±0.04	20.14±0.86	91.49±1.43
3	AN1N2	7.2±0.2	8.46±1.23	55.57±4.56	1.78±0.07	26.12±1.17	65.45±2.14
4	CN1	7.4±0.2	7.51±0.97	52.07±5.65	1.41±0.05	20.84±1.12	60.56±1.38
5	CN2	7.2±0.1	7.76±1.06	53.50±3.25	1.57±0.04	24.90±1.30	68.38±2.91
6	CN1N2	7.5±0.1	7.40±0.85	59.33±2.35	1.60±0.05	24.86±0.77	70.83±2.25
7	N1	7.3±0.1	6.67±0.83	48.00±2.89	1.37±0.04	20.67±0.81	62.20±2.11
8	N2	7.1±0.1	6.83±0.79	49.40±3.97	1.33±0.04	20.49±0.85	88.66±3.45
9	N1N2	7.4±0.1	8.58±1.19	48.83±3.12	1.48±0.04	21.09±1.12	86.61±3.67
10	A	7.4±0.1	7.79±0.78	41.57±3.43	1.31±0.02	25.55±0.88	87.82±5.34
11	C	7.5±0.1	6.79±0.85	39.57±3.00	1.23±0.03	25.10±0.76	54.48±3.85
12	AC	7.4±0.1	6.92±0.71	42.50±4.21	1.27±0.06	30.68±0.77	84.08±2.67
13	AP3	7.3±0.1	7.77±0.92	71.75±5.14	1.43±0.06	26.15±0.64	50.73±5.16
14	AP4	7.3±0.1	7.91±0.78	79.00±3.16	1.35±0.06	27.33±1.71	45.17±2.78
15	AP3P4	7.5±0.1	8.39±0.88	76.70±5.31	1.54±0.04	35.00±0.67	59.31±2.64
16	CP3	7.4±0.1	7.72±0.93	68.17±2.54	1.33±0.03	25.48±1.05	40.90±2.08
17	CP4	7.2±0.1	7.29±0.69	79.00±4.84	1.40±0.08	20.70±0.61	51.86±1.38
18	CP3P4	7.6±0.1	7.87±0.95	77.67±1.29	1.47±0.04	34.49±1.20	34.43±1.00
19	P3	7.1±0.4	7.51±0.42	56.60±3.20	1.34±0.04	20.11±0.13	51.07±1.81
20	P4	6.9±0.4	7.80±0.65	62.27±6.27	1.36±0.05	19.64±0.77	67.95±0.72
21	P3P4	7.0±0.2	8.15±0.49	76.53±3.29	1.37±0.05	25.53±1.30	65.40±1.20
22	CK	6.0±0.2	6.25±0.89	26.23±5.40	0.85±0.06	15.41±0.79	29.81±0.78

*A = Arecanut biochar, N1= Nitrogen fixing bacteria strain 1 (SM_SS1), N2 = Nitrogen fixing bacteria strain 2 (SM_SS2), C = Commercial biochar, P3 = Phosphate solubilizing bacteria strain 3 (SM_SS7), P4 = Phosphate solubilizing bacteria strain 4 (SM_SS8), CK = control

The results of the pot trials from this study indicated that using biofertilizers and biochar enriched soil can improve tomato yield and soil quality. This result is comparable to the yield of cherry tomatoes grown on chromosol soil with wastewater sludge biochar (Hossain et al., 2010). The Cd spiked soil selected for the study had reduced nutrient availability, which resulted in lower agricultural qualities of the soil used in the pot experiment (Table 6.1). When compared to the untreated soil sample, the AvN and AvP increased dramatically in the treated samples (Table 6.1). The characterized *Paraburkhendria* (SM_SS1), and *Bacillus* (SM_SS2), exhibited the largest amount of accessible N. The optimum quantity of AvP was found in the presence of the P solubilizing bacteria *Bacillus* (SM_SS7), and *Enterobacter* (SM_SS8). N fixing capabilities were also reported to be abundant in *Bacillus* and *Enterobacter* sp. (Mowafy et al., 2021). The N fixation, P solubilization, and HMs bioremediation abilities of the aforementioned bacterial species matched the extant reports (Mowafy et al., 2021; Sahu et al., 2022).

In contrast to other treatments, the highest improvement in the amount of phosphate was generated by the *Enterobacter* containing its formulation, followed by the *Bacillus* in the harvested soil. The *Paraburkhondria* and *Bacillus* sp. were identified as NFB in the current investigation and demonstrated substantial P solubilization, as also reported by Agustiyaniet et. al., (2022).

The combination of the P4 strain *Enterobacter* (SM_SS8) as shown in Table 6.1, were critical in improving the physico-chemical properties of pot soil. Additionally, N2 and its biochar formulation had the most beneficial effect on the soil. In the soil of N2 treated strain *Bacillus* (SM_SS2), the AvK, AvNa, ECE, and pH were found to be 6.83±0.79 mg Kg^{-1}, 20.49±0.85 mg Kg^{-1}, 88.66±3.45 C mol kg^{-1}, and 7.4±0.01, respectively. The P4 formulation containing *Enterobacter* (SM_SS8), yielded 8.150.49 mg Kg^{-1}, 25.53±1.30 mg Kg^{-1}, 65.40±1.20 C mol kg^{-1} of soil, and 6.9±0.4 viz AvK, AvNa, ECE, and pH. The largest amounts of soil AvK, AvNa, ECE, and pH were found with the Arecanut biochar (A) at 7.79±0.78 mg Kg^{-1}, 25.55±0.88 mg Kg^{-1}, 87.82±5.34 C mol kg^{-1}, and 7.4±0.1 C mol kg^{-1}, respectively. CK was less effective than treatments in terms of physico-chemical qualities; Wasim Akram et al., (2019) have documented that the bulk of the nutrients are available, when the pH is between 6.5 and 7.5.

The soil pH was found to be slightly basic to neutral (7.6 – 7.0), and all treated samples, except for CK, fit into this range. On the other hand, clay soil has greater CEC values, ranged from 10 to 150 C mol kg^{-1}. However, the values in the current investigation ranged from 29.81±0.78 to 91.49±1.43 C mol kg^{-1}. According to Suwal, (2018), an adequate concentration of AvK was required for photosynthesis, protein synthesis, starch biosynthesis, and sugar translocation. In the current study, AvK ranged from 6.25±0.89 to 8.581.19 mg kg^{-1}, whereas the recommended limit is 120 mg kg^{-1}. AvNa, which comes in exchangeable and water-soluble forms is another easily available nutrient. Water-soluble nutrients are affected by water salinity, whereas exchangeable nutrients are affected by the surrounding environment. By causing clay particles to inflate and disperse, the AvNa lowers soil permeability and blocks soil pores.

Biochar has been shown to have a beneficial liming impact when applied to low pH soils. As a result, applying biochar to acidic soils raises the soil pH and enhances nutrient usage efficiency. On the other hand, biofertilizers increase the microbial diversity, nutritional availability, and physical qualities of the soil in a sustainable manner (Gujre et al., 2021b; Sahu et al., 2022).

6.3.2 Effect of biochar and biofertilizer application on tomato stem and root growth as well as the productivity

The growth of each plant with all the treatments was measured from the 3rd to the 16th week. The results revealed that the formulation containing A and the P4 strain *Enterobacter* (SM_SS8), had the largest impact on the stem and root height (Fig. 6.1). According to Fig. 6.1, A containing P3 and P4 formulations performed much better than A with N1 and N2 formulations. For example, 90.8±2.25 and 21.60±1.64 cm viz shoot, and root growth was found at the end of the week in AP3P4 treatment. In contrast, stem and root growth of AN1 was 65±2.36 and 18.33±1.70 cm, respectively. The pot shoot and root growth of CK was inhibited, which might be the effect of the Cd treatment. The highest stem growth rate was reported for P3 and A formulations during the 10th week of the tomato life cycle, while CP3P4 and P4 formulations were seen during the 12th week. According to Hossain et al.,(2010), the maximum growth of tomato plants was observed at the 8th and 9th weeks of the life cycle when treated with wastewater sludge biochar. Our data also support the higher plant productivity of tomatoes when grown in biochar and biofertilizer treated samples, than in the control (Fig. 6.2).

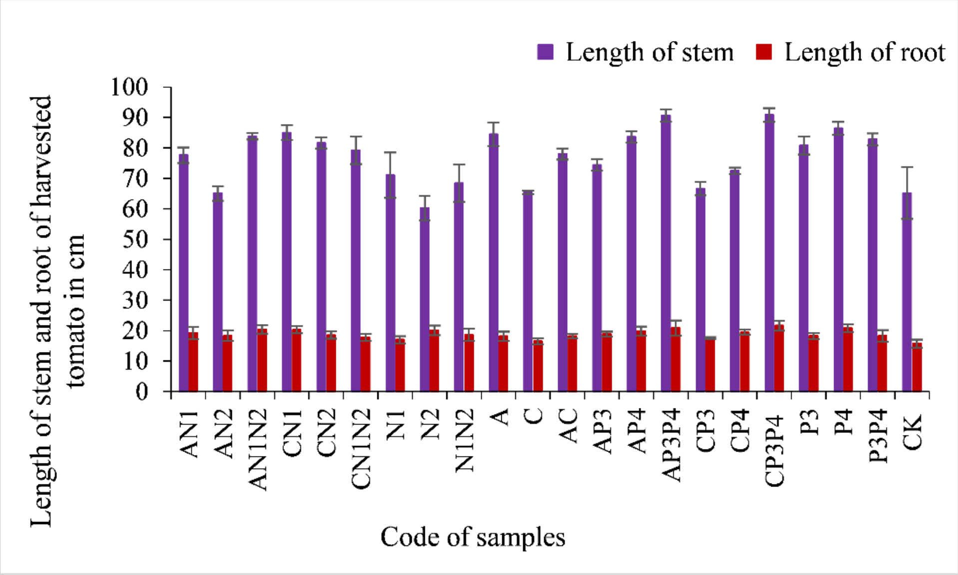

Fig. 6.1 Stem and root length of tomato plants after harvesting from soil treated with biochar and biofertilizer combination in a pot experiment

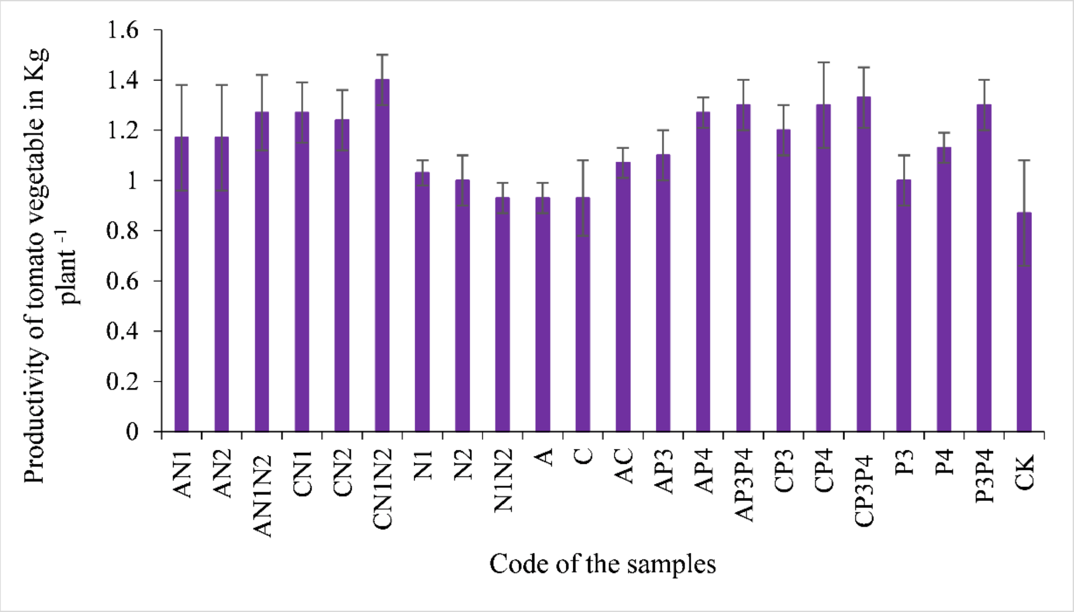

Fig. 6.2 Productivity of cultivated tomatoes in soil treated with various formulations of biochar and biofertilizer in comparison to CK.

6.3.3 Effect of biochar and biofertilizer on the content of Chl a, Chl b, total Chl of tomato leaves

The chlorophyll content of treated samples was significantly enhanced compared to the control. AP3 evinced 1.707±0.02, 0.73±0.008 and 2.437±0.003 (mg g^{-1} of the leaf) of Chl a, Chl b and total Chl content, respectively. These were followed by the P3P4 1.012±0.04, 0.57±0.004, and 1.578±0.005 (mg g^{-1} of the leaf) of Chl a, Chl b and total Chl content, respectively (Fig. 6.3). Additionally, The P4 showed the optimum effect on the content of Chl a (0.877±0.00), Chl b 1.49±0.021 and total Chl 2.364±0.004 mg g^{-1} of the leaf. However, AN1 has revealed the minimum content of the Chl e. g. 0.240±0.01, 0.17±0.004, and 0.415±0.004 (mg g^{-1} of the leaf) of Chl a, Chl b and total Chl content, respectively. The different treatments of the pot experiment showed significant variations in the content of Chl (Fig. 6.3). According to Li et al., (2018), the quantities of Chl a and Chl b in fertile soil were 0.87–15.92 mg g^{-1} leaf and 0.32–6.42 mg g^{-1} leaf, respectively. However, since the current study focused on Cd-spiked soil, so the content of Chl a and Chl b in the soil was lowered (Li et al., 2018).

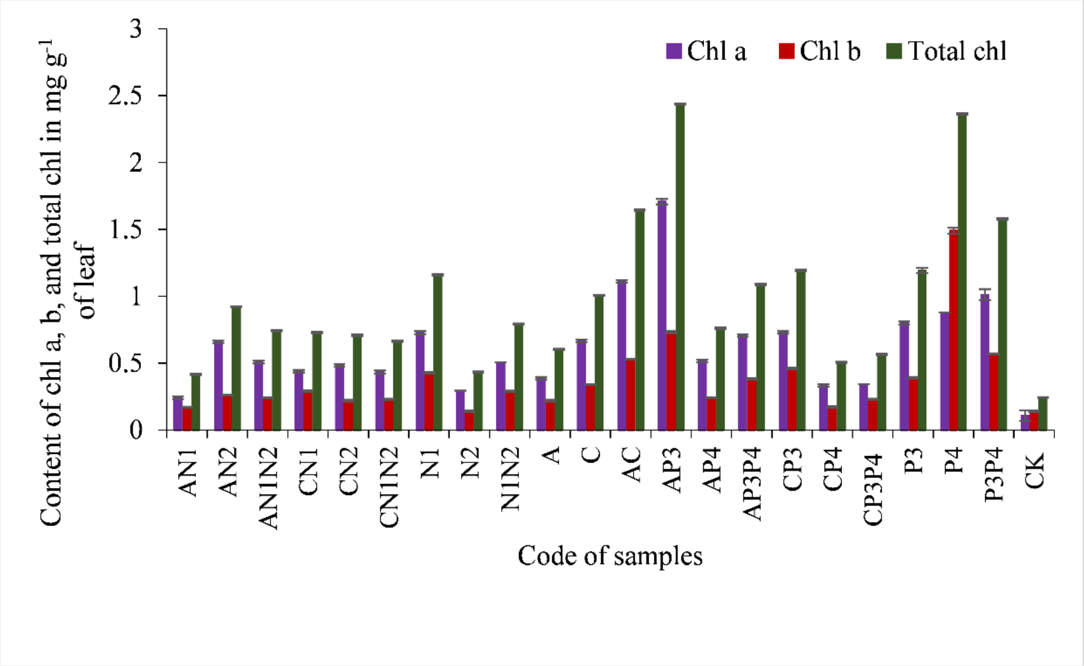

Fig. 6.3 Content of chl a, b, and total chl of the cultivated tomato leaves after the treatment of soil with different formulation of biofertilizer and biochar.

6.3.4 Concentration of HMs in soils after the harvest of tomato from pot culture

Highly hazardous HMs (Ni, Pb, and Cd) were found in very low concentrations in all biochar and biofertilizer treated soil samples (post-harvest) in the pot experiment. `1 On the other hand, microorganisms and plants have biological processes for bioremediating soil HMs through biosorption, bioavailability, and sequestration. Furthermore, soil microbes can be employed to remediate contaminated soils directly. Microbial activity affects the solubility and bioavailability of heavy metals by changing soil physical properties like soil structure and biochemical properties like pH, soil redox state, and soil enzymes. Because Ni, Cd and Pb were not detected in the harvested soil samples in the current study, it is likely that previously described mechanisms remediated them. However, all the treated and control samples had Mn and Cu concentrations within the acceptable range Fig. 6.4.

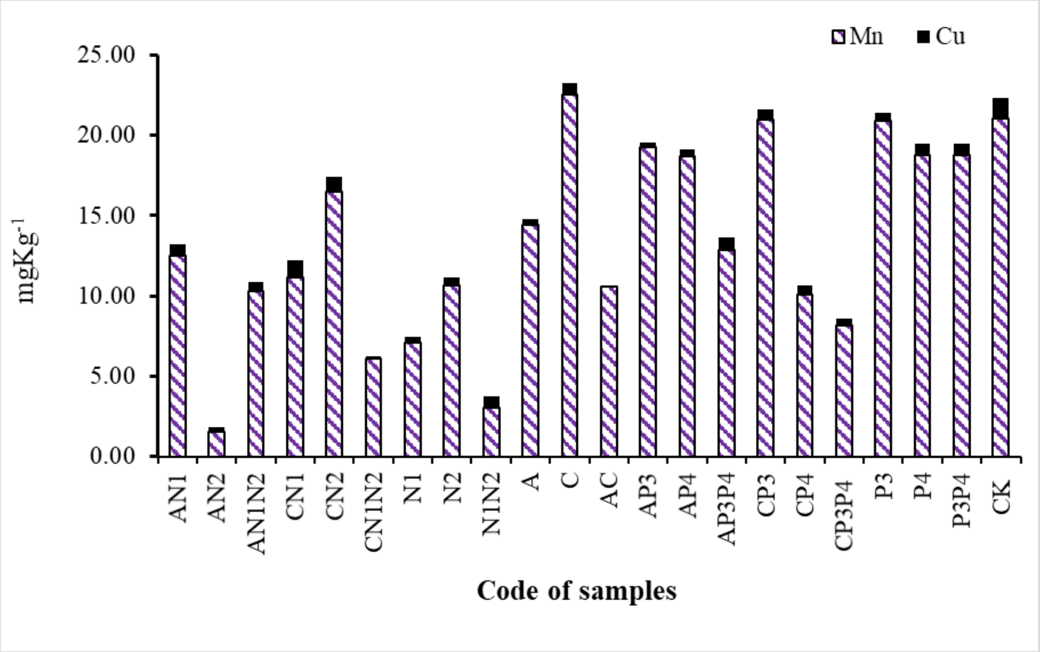

Fig. 6.4 Concentration of Mn and Cu in the soil harvested from the different formulation of biofertilizer and biochar.

6.3.5 *Taxonomic analysis of bacterial population in the soils of pot experiment*

The kingdom of bacteria and archaea were found in the P4, AP4, CP4, and CK treated soil samples (Fig. 6.5 a). In addition, the sequences of the four samples were divided into eleven phyla. Each sample exhibited a higher abundance of the Actinobacteria phylum, while Deinococcus-Thermus was rare. Simultaneously, the Nitrospirae and Deinococcus-Thermus phylum were discovered in the only CP4-treated soil sample, while Patescibacteria was discovered in P4-treated and Control soil (Fig. 6.5 b). Across all treatments, the Proteobacteria evinced the second highest abundance in the current investigation. On the other hand, the genus of *Nocardioides, Bacillus,* and *Streptomycin* were found in all the samples with maximum abundance, while *Solirubrobacter* and *Lysobacter* displayed low occurrence. (Fig. 6.5 c). Similarly, microorganisms were abundant in HM contaminated soil, and many of them had multiple physiological activities. On the other hand, HMs pollutants such as Cd can limit microbial diversity and alter the community structure in the soil. However, the examination of the functions of key microorganisms (e.g., *Proteobacteria, Sulfuricella,* and *Thiobacillus*) revealed that these bacteria and their physiological functions were critical for the soil community to cope with HM contamination (Feng et al., 2018).

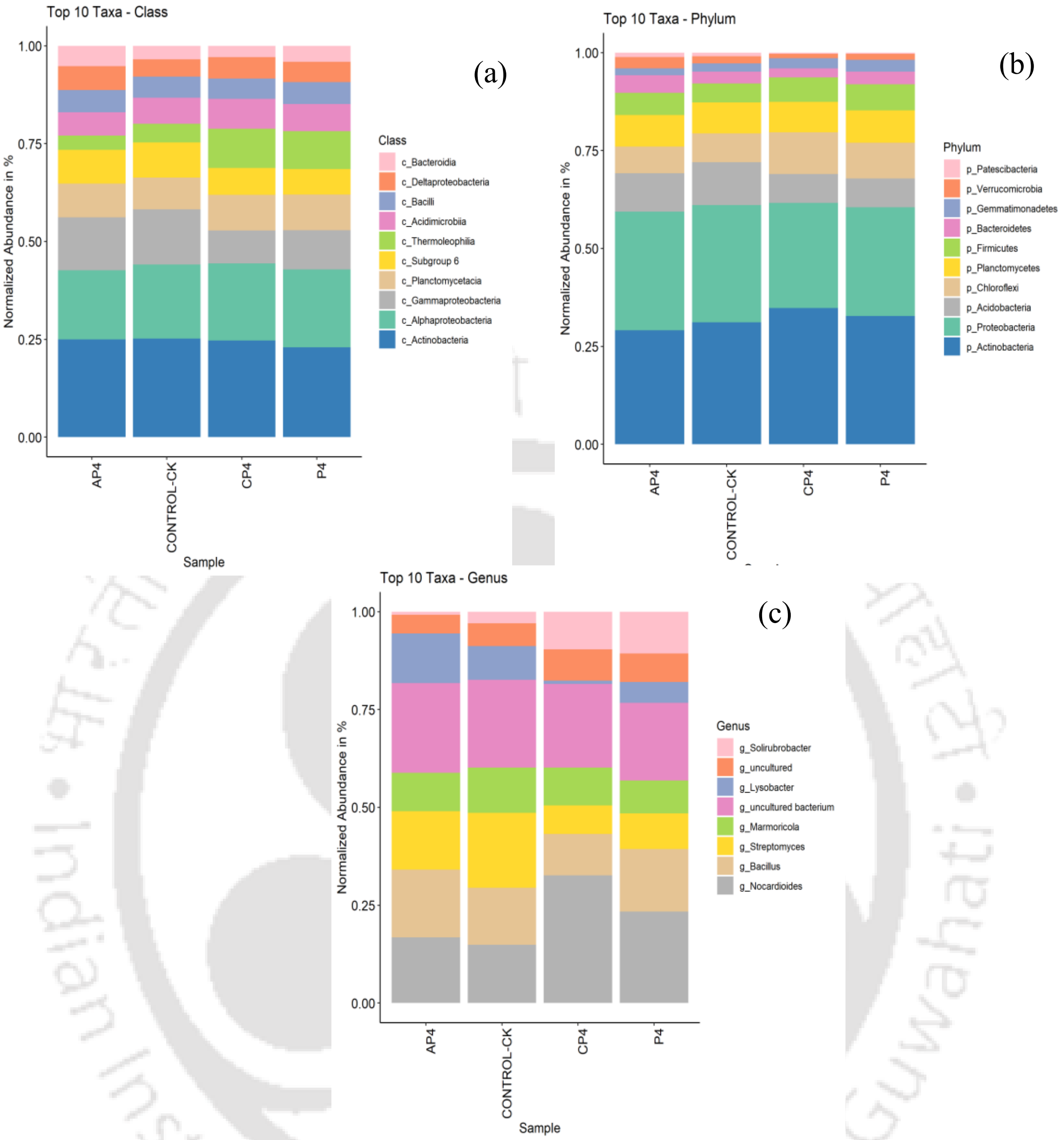

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Fig. 6.5 Bar plots showing the proportion of reads of the 4 samples in this study at the phylum (a) Class (b) and Genus (c) levels. The samples are sorted on the bases of OTUs abundance.

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6.3.6 *Bacterial diversity of soil samples collected from pot culture*

6.3.6.1 Alpha diversity analysis by Venn diagram *Chao1, Observed species, Shannon's index:* and *Simpson's index:*

The Venn diagram displayed the unique and shared bacterial OTUs of Cd-contaminated soil samples treated with P4, AP4, CP4, and CK (Fig. 6.6 a). Soil samples treated with P4, AP4, and CP4 contained 483, 423, and 391 unique OTUs, respectively, whereas CK contained 376. (Fig. 6.6 a). Simultaneously, the nineteen OTU were shared among the all samples. Whereas, the P4, AP4, and CP4 shared 60, 66, and 46 OTUs respectively with CK. However, investigations revealed that the abundance of OTUs is low in the untreated soil samples and the CP4 soil sample evinced the least shared microbial diversity.

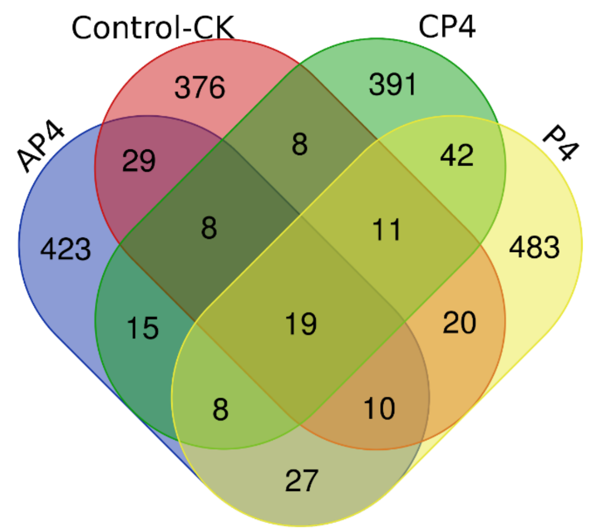

Fig. 6.6 a Venn diagram of OTU numbers in soil samples. Different colours represent different samples, the numbers of overlapping sections represent the number of species common in multiple samples, and the numbers of non-overlapping sections represent the number of species unique to the corresponding sample.

When the Shannon, InvSimpson, Fisher, and Simpson indexes (Table 2, coverage estimator 99%) were employed to quantify the variety of microbial communities, the stimulation performance was also demonstrated, when the index values of the control

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showed the least diversity. The Ace and Chao richness indexes of soil bacteria treated with P4 and its formulation, were greater than the CK of Cd-containing soil (Fig. 6.6 b). However, the bacterial, and biochar treatment can promote soil bacterial diversity. Additionally, Vitousek and Hooper (1993) suggested that microbial richness was linked to the functional qualities of the ecosystem, along with C and N mineralization, and a rise in total C, N, and P storage.

These relationships suggest that a shift in richness could disrupt the soil environment. Furthermore, microbial variety and richness are thought to be inherent properties of microbial communities that protect them from outside intervention. Yet, from such varied perspectives, only a small portion has been studied to determine the effects of various heavy metals, biochar, and biofertilizer on the soil microbial population (Fig. 6.6 b).

Table 6.2. Alpha-diversity of the soil harvested from the tomato pot experiment

Sample Id	**Observed**	**Chao1**	**se.chao1**	**ACE**	**se.ACE**	**Shannon**	**Simpson**	**Inv Simpson**	**Fisher**
AP4	539	539	0.00	539.00	7.19	6.06	0.99	357.73	100.76
CK	481	481	0.49	481.17	6.10	5.96	0.99	326.41	89.39
CP	502	502	0.25	502.17	7.30	5.99	0.99	342.17	94.07
P4	620	621	2.34	620.35	7.33	6.21	0.99	417.91	115.92

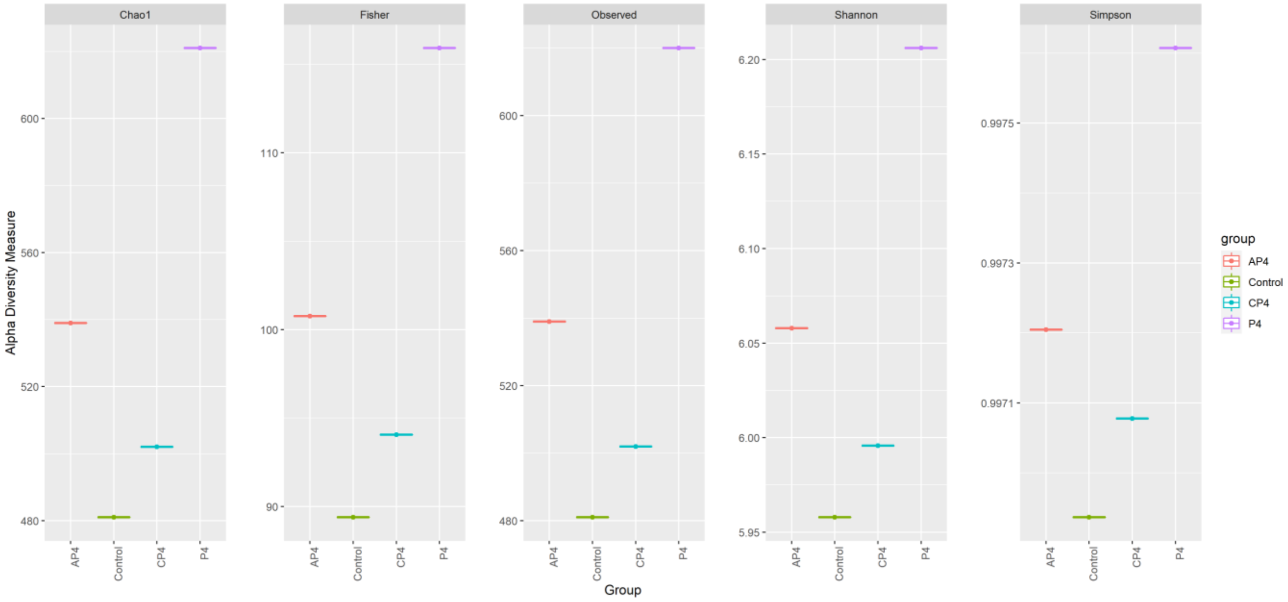

Fig. 6.6 b Representing the Alpha diversity: Chao1: estimates the species richness. Observed species: measures unique OTUs in the sample. Shannon’s index: measures both richness and evenness Simpson’s index: measures both richness and evenness, but less affected by the presence of rare species when compared to Shannon’s index of all four samples with the groups.

6.3.6.2 Beta diversity by PCoA (Principle Coordinate Analysis)

The 3-Dimensional PCoA plot shows the distinct clustering of samples from the four different groups. However, the P4 and its group samples were clustered close together; in fact, the AP4 and its group cluster were very close. In contrast, the CP4 and its group samples cluster were farther from each other (than any of the others), but evinced distinct clusters that were separate from the other samples (Fig. 6.7). However, the changes in the composition of ground plant species growing above the soil are more connected with similarities in many ecosystem services in grasslands around the world, than the changes in soil microbial composition, (Liu et al., 2021). Importantly, abiotic variables have mostly indirect impacts. These indirect effects show that changes in the climatic and edaphic factors effecting ecosystem functioning are mediated by their considerable implications on plant and soil microbial populations in the past and present (Liu et al., 2021). Similarly, in the present study the beta diversity of treated samples increased significantly as compared to the CK.

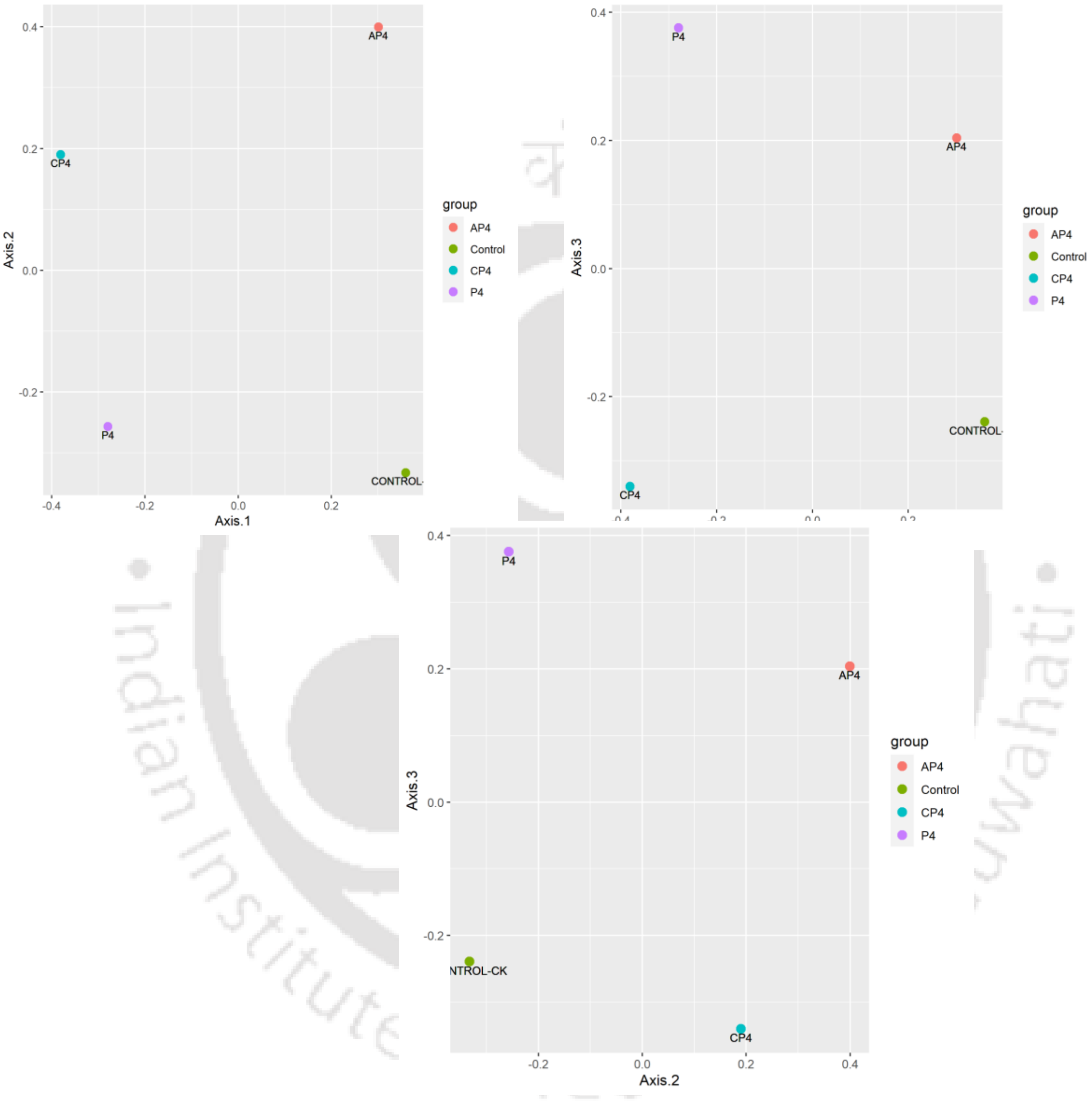

Fig. 6.7 Principal Coordinate Analysis plot. The four samples (P4, AP4, CP4, and CK) are presented in the PCoA analysis with four groups.

6.4 Conclusion

Biofertilizers and biochar application in soil can improve tomato yield, soil quality, and microbial diversity. Compared to the untreated soil samples, the AvN and AvP in the treated samples increased. Biochar-treated samples were greater than CK samples but lower than bacterial-treated samples. The most available N was found in the characterized N fixing bacteria. The optimum amount of AvN was also discovered in the characterized P solubilizing bacteria. Furthermore, the *Enterobacter* and its formulation treated samples improved the most in terms of phosphate, followed by the *Bacillus* in the harvested soil. Similarly, in biochar and biofertilizer treated samples, tomato plant productivity was shown to be higher than in CK samples. Furthermore, the *Enterobacte*r strain and its formulations were found to be highly important parameters in improving different physico-chemical characteristics such as AvK, AvNa, ECE, and soil pH, followed by *Bacillus* sp. and its biochar formulation. In terms of physico-chemical properties, CK was less effective than treatments. According to the findings, arecanut biochar, *Enterobacter* strain, and their formulation had the greatest impact on stem and root height. The shoot and root growth in the CK pot, on the other hand, was inhibited, which could be due to the Cd treatment. Furthermore, compared to the CK, the chlorophyll content of treated samples increased considerably. The findings showed that biofertilizers and biochar work together to improve soil nutrition and plant growth. However, the highly hazardous HMs (Ni, Pb, and Cd) were found in very low concentrations in all biochar and biofertilizer treated soil samples (post-harvest) in the pot experiment. Simultaneously, taxonomic analysis of harvested soil samples demonstrated that the diversity of cultivated and uncultured microorganisms increased when compared to CK. The results showed that a single biofertilizer and its formulation was effective for physico-chemical, microbial diversity, and biochemical parameters.

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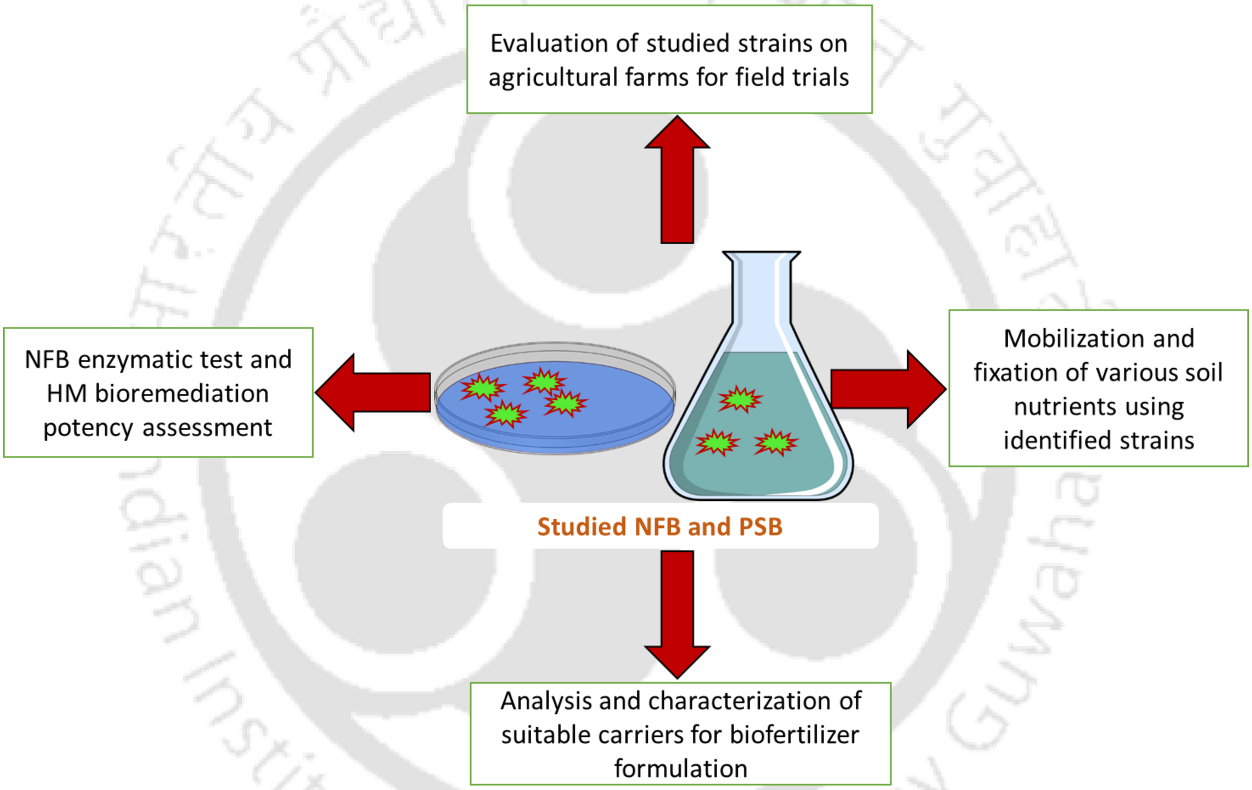
CHAPTER 7

Summary and future recommendations



This chapter discusses the challenges and future recommendations generated by the present study. It also summarizes the main findings of the current study.

Emerging economies, such as India, confront more significant challenges in achieving sustainable agriculture in the 21st century. Farmers have been urged to use chemical fertilizers and pesticides ad libitum to grow more food on less land. The negative environmental fallout chemicals outweigh the subsequent massive increase in yield. An immediate aftermath is an adverse effect on soil fertility, severely impacting agricultural productivity. Concurrently, uncontrolled population expansion, urbanization, higher standards of living, and socioeconomic progress have generated humungous quantities of MSW, especially in India and worldwide (Sahu et al., 2022). MSW predominantly pollutes soil and water with a variety of pollutants, including heavy metals and other similar substances, wreaking havoc on the biosphere and raising the toxicity of food ecosystems (Gujre et al., 2021). The use of biofertilizers in pollution remediation is considered a viable solution to this long-standing concern. The research embodied in the present thesis aims to address this critical issue.

The study area where MSW contaminates is located within the Boragaon dumping site in Guwahati, Assam. One side of this dumping site is contiguous with the ecologically sensitive Deepor Beel area (Ramsar site No. 1207), adding a critical implication to the outcome of this study. The soil collected from MSW dumping sites exhibited moderate to slightly acidic pH. Similarly, the moisture content of collected soil samples was found to be less than the standard limit. Organic carbon was in the higher range and HMs like Cu, Cr, and Cd were found to be higher than the permissible levels in the soil. However, the soil nutrients AvP, AvN, AvK, and AvNa, were found below the recommended range.

From the same site, I have made a comprehensive effort to explore and utilize the abilities of the bacteria for soil quality enhancement. Approximately 400 PSB and 350 NFB isolates have been identified from the contaminated soil. All the collected rhizospheric samples exhibited robust and prolific bacterial growth in PVK and Winogradsky media for PSB and

NFB, respectively. The molecular characterization data of the selected strains indicated two phyla, the Firmicutes and Proteobacteria (included g-Proteobacteria); and three genera (*Bacillus, Enterobacter,* and *Paraburkholderia*). Although some of the identified isolates exhibited pathogenic characteristics, they also possessed phosphate solubilizing, Nfixing, and HM remediation properties, all of which are critical for soil rejuvenation. *Enterobacter* sp. (SM_SS8) showed the highest phosphate solubilizing capacity among the isolated bacteria, and released the maximum amount of P into the media. Furthermore, ACP activity grew significantly from day zero to the 3rd day, but after the 6th day, all strains, save the SM SS8, showed a modest reduction. The results also revealed that AA and MA were secreted in higher quantities than GA and CA.

The bioremediation potential of *Enterobacter* sp. (SM_SS8) was assessed by evaluating Cd and Cu treated SM_SS8 cells in comparison to SM SS8 cells without HMs. The viable cells of SM_SS8 were found 70.92 %, 46.93 %, and 20.4 % of 10, 50, and 100 mg L^{-1} Cd treated samples respectively after flow cytometer analysis. Further, Cd and Cu bio-absorption during bioremediation was cross-verified by FTIR, EDX and XRD. Finally, the multiplicative model was utilized in the study of HMs bioremediation by the SM_SS8 strain, for precise appraisal of all four bacterial growth stages under Cd and Cu stress. Thus, the present assessment is critical in the development of biofertilizers and biochars to improve the soil nutrient and simultaneously reduce HM pollution.

Enterobacter sp. strain and its formulations were found to be extremely helpful in increasing physico-chemical features such as AvK, AvNa, ECE, and soil pH, followed by *Bacillus* sp., and its biochar formulation. Thus, the increase in AvN and AvP was the highest in biofertilizers treated samples, followed by samples treated with biochar, and were lowest in the untreated or control samples. This clearly indicated that the characterized N fixing and P solubilizing bacteria could increase more AvN and AvP than biochar.

Furthermore, experiments with pot culture of tomato plants exhibited improved soil quality, microbial diversity and tomato yield. Consequently, tomato plant production was shown to be higher in samples treated with biofertilizer and biochar than in the CK. In addition, the most significant positive changes in stem and root height were obtained by a combination of areca nut biochar and *Enterobacter sp* strain. Furthermore, the chlorophyll content and the diversity of microorganisms of treated samples increased significantly compared to the CK. The findings revealed that a single biofertilizer and its formulation act synergistically to promote soil parameters (physico-chemical, biochemical and nutrient profile) besides enhancing microbiological diversity and plant growth.

The practical strategy of isolating P solubilizing and N fixing bacteria from contaminated soil in the present investigation could help rural communities make good use of abandoned agro-waste for soil quality improvement. Yet, at this juncture, further critical research and evaluation of the multiple factors that impact the features and applications of biofertilizers are vital for the success of this alternative and effective agro-technology. The followings are some of those to be mentioned:

1. Evaluation of studied strains on agricultural farms for field trials
2. NFB enzymatic test and HM bioremediation potency assessment
3. Analysis and characterization of suitable carriers for biofertilizer formulation
4. Mobilization and fixation of various soil nutrients using identified strains
5. Increasing efficiency of biofertilizers by combining biochar and organic compost with beneficial microbes

Biofertilizer technologies have crucial social, economic, and cultural implications that are yet to be investigated via multidisciplinary research. The biofertilizer nurturing methodology given here is customized to the Indian context of soil quality improvement. It focuses on soil quality assessment and remediation, utilizing biofertilizers and biochar formulations, and it primarily analyses the dynamics between the soil and plant. The global benefits that may be accrued from viable, widespread, and sustainable biofertilizer technology are the dual benefits of reducing dependence on chemical fertilizers and facilitating bioremediation of HMs. On the ground, investigations must be followed by widespread dissemination of the enormous potential of biofertilizers to gain acceptance among the potential stakeholders.

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Publication in peer-reviewed journals:

1. **Sahu, S.,** Rajbonshi, M. P., Gujre, N., Gupta, M. K., Shelke, R. G., Ghose, A., Rangan, L, Pakshirajan, K. & Mitra, S. **(2022**). Bacterial strains found in the soils of a municipal solid waste dumping site facilitated phosphate solubilization along with cadmium remediation. **Chemosphere**, 287, 132320.
2. **Sahu, S**. et al. (2022) A dynamic study of different doses Cd and Cu treated phosphate solubilizing bacteria (under prep).

Publications in conferences proceedings:

1. **Sahu, S.,** Gupta, D., Rajbonshi, M.P., Rangan, L., and Mitra, S., (**2021**) Bacterial strain isolated from a municipal dumping site showed potentials for integrated solutions: phosphate solubilisation along with heavy metals remediation in soil, International Conference on Biotechnology for Sustainable Agriculture, Environment and Health (**BSAEH-2021**), (April 04-08, 2021).
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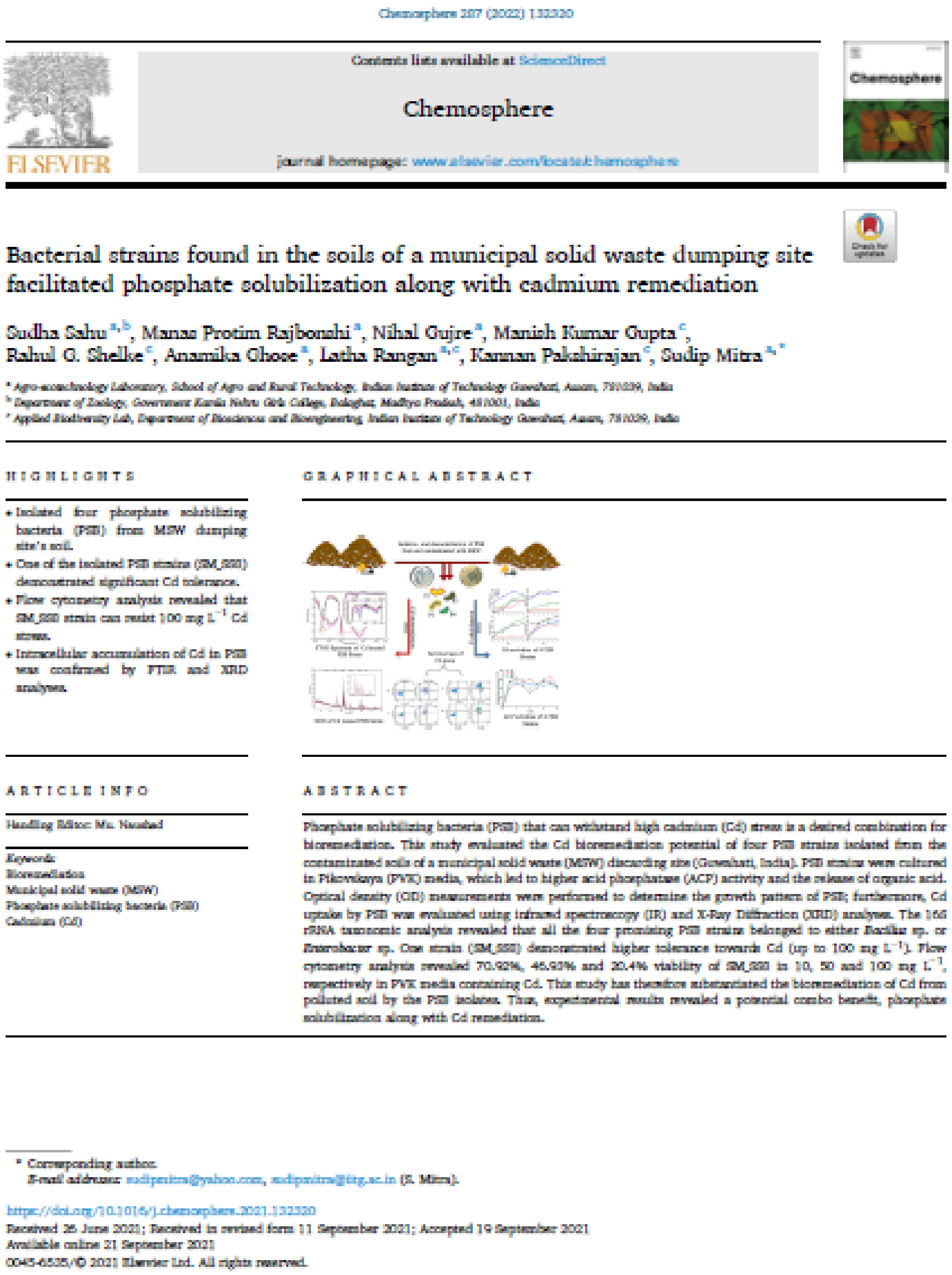

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Bacterial strains found in the soils of a municipal solid waste dumping site facilitated phosphate solubilization along with cadmium remediation

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HIGHLIGHTS

- Isolated four phosphate solubilizing bacteria (PSB) from MSW dumping site's soil.
- One of the isolated PSB strains (SM_SS5) demonstrated significant Cd tolerance.
- Flow cytometry analysis revealed that SM_SS5 strain can resist 100 mg L^{-1} Cd stress.
- Intracellular accumulation of Cd in PSB was confirmed by FTIR and XRD analyses.

GRAPHICAL ABSTRACT

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ABSTRACT

Phosphate solubilizing bacteria (PSB) that can withstand high cadmium (Cd) stress is a desired combination for bioremediation. This study evaluated the Cd bioremediation potential of four PSB strains isolated from the contaminated soils of a municipal solid waste (MSW) discarding site (Guwahati, India). PSB strains were cultured in Pikovskaya (PVK) media, which led to higher acid phosphatase (ACP) activity and the release of organic acid. Optical density (OD) measurements were performed to determine the growth pattern of PSB; furthermore, Cd uptake by PSB was evaluated using infrared spectroscopy (IR) and X-Ray Diffraction (XRD) analyses. The 16S rRNA taxonomic analysis revealed that all the four promising PSB strains belonged to either *Bacillus* sp. or *Enterobacter* sp. One strain (SM_SS5) demonstrated higher tolerance towards Cd (up to 100 mg L^{-1}). Flow cytometry analysis revealed 70.92%, 46.93% and 20.4% viability of SM_SS5 in 10, 50 and 100 mg L^{-1}, respectively in PVK media containing Cd. This study has therefore substantiated the bioremediation of Cd from polluted soil by the PSB isolates. Thus, experimental results revealed a potential combo benefit, phosphate solubilization along with Cd remediation.

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