Mechanistic Elucidation of Plant Growth Promoting Bacteria in Biosorption and Phytoremediation of Heavy Metals

By

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A thesis submitted in partial fulfillment of the requirements for the degree

of Doctor of Philosophy

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2022

DEDICATED

TO

MY BELOVED PARENTS

AND

ALL WELL-WISHERS WHO HELPED ME IN THIS JOURNEY

APPROVAL CERTIFICATE

This is to certify that the research work presented in this thesis, entitled **"Mechanistic Elucidation of Plant Growth Promoting Bacteria in Biosorption and Phytoremediation of Heavy Metals"** was conducted by Mr. Javed Ali under the supervision of Dr. Hassan Javed Chaudhary.

No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Plant Sciences, Quaid-I-Azam University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Sciences, Department of Plant Sciences, Quaid-I-Azam University, Islamabad, Pakistan.

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List of abbreviations

Abbreviations	Full name
ACC	1-Aminocyclopropane-1-carboxylate
AIDS	Acquired immunodeficiency syndrome
q_e	Adsorption capacity at equilibrium
ATSDR	Agency for toxic substances and disease registry
ANOVA	Analysis of variance
AAS	Atomic absorption spectrophotometer
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
Cd	Cadmium
CAT	Catalase
Cm	Centimeter
Chl a	Chlorophyll a
Chl b	Chlorophyll b
CAS	Chrome azurol S
Cr	Chromium
CFU	Colony forming unit
CRD	Completely randomized design
C	Control
R^2	Correlation coefficient
DDW	Double distillation
DW	Dry weight
EC	Electrical conductivity
ELL	Electrolyte leakage
EF	Erlenmeyer flasks
EPS	Exopolysaccharide

FTIR	Fourier transform infrared spectrophotometric
FW	Fresh weight
GGDC	Genome-to-Genome Distance Calculator
G	Gram
HMs	Heavy metals
H	Hour
HCN	Hydrogen cyanide
H2O2	Hydrogen peroxide
IAA	Indole acetic acid
H	Initial adsorption rate
N	Intercept
IARC	International Agency for Research on Cancer
LSD	Least significant different
LB	Luria-Bertani
MDA	Malondialdehyde
MS	Mean square
Qt	Metal ions adsorbed at time
µm	Micrometer
Mg/kg	Milligram per kilo gram
mL	Milliliter
Mm	Millimeter
nm	Nano meter
Ni	Nickel
NF	Nitrogen fixation
OD	Optical density
OM	Organic matter
PPM	Part per million
%	Percentage
POD	Peroxidase

PSB	Phosphate solubilization bacteria
PVK	Pikovskaya
PG	Plant growth
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
PAH	Polycyclic aromatic hydrocarbon
ROS	Reactive oxygen species
RWC	Relative water content
rRNA	Ribosomal RNA
RL	Root length
SEM	Scanning electron microscopic
SWT	Shapiro Wilk test
SL	Shoot length
Y	Slope
SOD	Superoxidase dismutase
TBA	Thiobarbituric acid
TI	Tolerance index
Total chl	Total chlorophyll
TF	Translocation factor
TCA	Trichloroacetic acid
TSB	Tryptic Soy Broth
Rpm	Revolution per minute
αKB	A-ketobutyrate

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ABSTRACT

The sustainable agricultural, global food security and maintenance of ecological regimes, native bacterial species can be scrutinized for their capability to tolerate heavy metals stress along with plant growth promotion. Four experiments were performed to assess the step-by-step ability of isolated PGPB for heavy metals (HMs) tolerance.

The first investigation rhizospheric soil samples were collected from five different districts: Larkana, Qambar Shahdadkot, Shaheed Benazirabad, Naushahro Feroze, and Jacobabad, Sindh Pakistan. Twenty bacterial strains were isolated, screened and characterized for plant growth promoting activities and initial heavy metals tolerance potential. These isolates were confirmed to produce phosphate solubilization, hydrogen cyanide, indole acetic acid, zinc, ammonia, and siderophore Additionally, they also showed positive results to produce various extracellular enzymes i.e., ACC-deaminase, cellulase, catalase, amylase, protease, pectinase enzyme and exopolysaccharides. Among all, bacterial strain PM21 exhibited maximum ACCD (1.56-1.75 µM/mg protein/h), exopolysaccharides (2.73-2.98 mg/mL), and indole acetic acid (IAA) production (99-119 µM/mL) under normal and metal stressed conditions. The studied bacterial strains (PM21, PM22, PM23, PM24, and PM25) were identified via 16S rRNA gene sequencing technique. These bacterial strains were identified as *Bacillus anthracis* (PM21), *Bacillus safensis* (PM22), *Enterobacter cloacae* (PM23), *Bacillus sonorensis* (PM24) and *Bacillus thuringiensis* (PM25). The presence of *nifH* gene responsible for nitrogenase activity was confirmed in two strains (PM21 and PM23) through its polymerase chain reaction (PCR) amplification. Further, PCR amplification of *acds* gene in all strains verify their ability of heavy metal tolerance. These promising bacterial strains were proceeded further to check heavy metals biosorption potential.

Assessment of mechanisms and abilities of plant growth promoting bacterial stains (PM21, PM22, PM23, PM24, and PM25) were conducted in second study for biosorption of cadmium (Cd), chromium (Cr), and nickel (Ni). Growth curve analysis of five bacterial strains, based on best performance, were plotted at different levels of Cd, Cr and Ni stresses. Bacteria were challenged with high concentrations of Cd 100-800, Cr 100-300, and Ni 100-500 mg/L. In batch biosorption experiments, the maximum adsorption value was obtained for *Bacillus anthracis* PM21 for the applied heavy metals at the optimum pH i.e., 8 for Cd, 6 for Cr, and 4 for Ni respectively. The

maximum adsorption values for Cd, Cr, and Ni were recorded after 60 min for all the bacterial strains i.e., PM21, PM22, PM23, PM24, and PM25. The maximum adsorption capacities (qe) of PM21 were observed 5-35 mg/g for Cd, 4-24 mg/g for Cr and 3-24 mg/g for Ni under 200 mg/L heavy metals. All the applied models supported the results of Cd and Cr biosorption with highest correlation coefficient (R^2) values as compared to Ni. The pseudo-second order kinetic model accurately represented the biosorption processes of biosorbents, indicating that heavy metal biosorption was primarily chemisorption. The participation of functional groups in metal ion adsorption was anticipated by FTIR surface characterization of bacteria. The scanning electron microscope (SEM) results revealed that application of 200 mg/L of Ni showed damaging effects on cell surface morphology. While in case of Cd and Cr (200 mg/L) the cells maintained their shape and size. The existence of CzcD gene responsive for Cd and Cr resistant, in four strains (PM21, PM22, PM24, and PM25), was confirmed by its PCR amplification. Based on plant growth promoting and biosorption potential strain PM21 was further applied on legume plant species, *Sesbania sesban* L. under heavy metals (Cd and Cr) stress to study seed germination of *Sesbania sesban* L.

In third study, the seed germination test of *Sesbania sesban* L. was evaluated by the application of isolated strain PM21 in the absence and presence of cadmium and chromium. The PM21 inoculation to seedlings enhances seed germination (97.01%), length of roots (59.51%), length of shoot (5.03%), chlorophyll a (20%), b (16%) and total chlorophyll content by 18%, under Cd stress as compared to control. According to the findings, *B. anthracis* PM21 was able to withstand metal stress by maintaining antioxidant activity homeostasis, which had a beneficial impact on *S. sesban* L. growth and biomass. All the physiological, biochemical and growth parameters proposed that PM21 could better remediate Cd as compared to Cr. After germination experiment the strain PM21 was also applied to remediate Cd spiked soil with *Sesbania sesban* L. greenhouse study.

The fourth study was conducted to investigate the plant growth promoting rhizobacterial assisted phytoremediation of cadmium (Cd). Phytoremediation potential of *S. sesban* L. was explored in Cd contaminated soil inoculated with *Bacillus anthracis* PM21. Application of *B. anthracis* PM21 significantly enhanced the studied plant attributes under normal and Cd stress conditions. Application of *bacillus anthracis*

PM21 increased morphological and physiological parameters as compared to uninoculated ones. Application of *B. anthracis* PM21 significantly ($p \leq 0.05$) enhanced Cd uptake in root, shoot translocation factor, bioconcentration factor 118.6, 73.4 mg/kg, 0.61, and 0.36 respectively.

After assessment of data acquired in the four current studies, the analysis of comparison with the reported data was performed with the best of our knowledge. The data showed following parameters with significant increase which were bioconcentration factor, seed germination (%), root length, chlorophyll a, b.

For the first time, plant growth promoting *Bacillus anthracis* PM21 have been evaluated for heavy metal tolerance potential. The tolerance level of *Bacillus anthracis* PM21 was reported as 800 and 300 mg/L cadmium and chromium, respectively. To best of our knowledge, *Bacillus anthracis* PM21 showed maximum tolerance towards Cd and Cr. So, its efficiency to tolerate dual metal stress under *in-vitro* conditions has been reported in our study. Furthermore, *Bacillus anthracis* PM21 was also applied for the first time in biosorption and phytoremediation that enhanced plant growth of *Sesbania sesban* L. under heavy metals stress at different levels.

Chapter 1
Introduction and Literature Review

1. General Introduction and Review of Literature

Pollution is one of the big environmental issues in current era. It is the most serious problem and causes illness and death, worldwide. According to reports, pollution caused 9 million premature deaths, which is more than three times the number of deaths caused by tuberculosis, acquired immunodeficiency syndrome and malaria combined (Landrigan et al., 2017). Poverty and illiteracy contribute to environmental pollution being worse in developing countries as compared to developed countries (Muralikrishna and Manickam, 2017). Deforestation, bush burning, dispose of household waste in water bodies, agrochemicals in aquatic, and excessive disposal of electronic wastes are all examples of contamination of the air, soil, and water. Environmental pollution is caused by a variety of factors, including industrialization, urbanization, population growth, exploration, and mining. Microbial bioremediation has gotten a lot of attention recently, probably because it is a feasible and environmentally safe way to restore the ecosystem. There are different forms of pollution, but the three most common types are: organic, inorganic and air pollution. Air pollution is most deleterious and emerging environmental constraint in the 21st century and is badly affecting our ecosystem (World Bank, 2016; UNECE, 2020). About 6.5 million deaths have been reported annually worldwide due to air pollution (WHO 2016). Air pollution badly affected Asian continent especially South, Southeast and West Asia as revealed by the occurrence of highest particulate matter concentrations in the air (World Air Quality, 2019). Transportation and industrial emissions, agricultural and biomass burnings, and coal combustion are all major polluting agents (IQAir, 2019). South Asian countries such as Bangladesh, Nepal Pakistan, and India reported highest annual $PM_{2.5}$ exposures in 2017 (Institute of Health Metrics and Evaluation., 2019). There are several organic contaminants such as organochlorine pesticides, polycyclic aromatic hydrocarbon (PAH's), phthalate esters and polychlorinated biphenyls which affect environment due to their persistent nature (Sun et al., 2018). Ground water contamination results due to extensive use of chemicals (organic and inorganic). Organic contaminants are the most persistent and highly hazardous organic pollutants as compared to other pollutants (Li et al., 2018). The potential cause of organic pollution is the use of chlorinated organic pollutants (Odabasi et al., 2014). The organic pollutants in agricultural soil adversely affect the

soil quality, plant growth and plant yield (Sun et al., 2018). Organic pollutants are highly toxic and adversely affect human health (Chen et al., 2016).

1.1. Inorganic pollution

Heavy metals (HMs) are knowns as metallic, natural elements having relatively high atomic weight and density than water. Due to socio - economic and industrial activity, heavy metal contamination is one of the world's most important environmental challenges (Singh et al., 2020; Ahmed et al., 2020). Heavy metal pollution in sediments can be caused by industrial, deposit, agricultural effluents, weathering of limestone and atmospheric deposition (Dukes et al., 2020). Various environmental factors influence the release of heavy metal from sediments into the water bodies thus causing serious threats to living being (Superville et al., 2014; Rehman et al., 2018). Heavy metals concentration is also high in crops cultivated on polluted soils (Hembrom et al., 2020; Muhammad et al., 2019). Heavy metals, identified as chemical elements with a density greater than 5 g/cm^3, naturally found in soils, rocks, and water (Kocadal et al., 2021). After reading the published data and assessing environmental studies in Pakistan we have selected three metals cadmium, chromium, and nickel (Alamgir et al., 2016; Khuhawar et al., 2018; Imran et al., 2019; Lanjwani et al., 2020). The Cd, Cr, and Ni are the fourth, fifth, and seventh hazardous heavy metals, respectively, according to the Agency for Toxic Substances and Disease Registry (ATSDR) (Fuet al., 2019).

Table 1.1. Important list of selected HMs (2105) designed ATSDR, (2019)

S. #	HMs	Position
1	Cadmium (Cd)	4^{th}
2	Chromium (Cr)	$5th$
3	Nickel (Ni)	7^{th}

Impact and origin of three HMs on environment and more specifically to plant growth are briefly described below.

1.1.1. Cadmium

Cadmium is a phytotoxic heavy metal (HM), with a highwater solubility leading to its bioaccumulation in the food chain and is classified as a carcinogen by the International Agency for Research on Cancer (IARC) (Tiwari and Lata, 2018). It is a transition

element with a relative melting point three hundred twenty one °C, atomic number forty eight, boiling point 765 °C, atomic mass of 112.14 (Rahimzadeh et al., 2017). Many enzyme processes are adversely affected thus causing chlorosis, fluctuation in antioxidant enzymes, leaf epinasty, lipid peroxidation, inhibition in pollen generation and tube development, and reduced photosynthesis are some of the recorded problems associated with Cd in plants (Afzal et al., 2018; Gill and Tuteja, 2011; (Pei et al., 2017).

1.1.2. Chromium

Chromium belongs to group -VI on the periodic table with relative atomic mass 51.99u and atomic number of 24, and melting point of 1907°C (Terentyev et al., 2020). Plants require chromium (Cr) for normal growth and development, but higher levels of Cr have toxic effects not only on plants but also on other species (Lajayer et al., 2019; Lilli et al., 2019). Cr (VI) is more mobile, listed as a 'A' human carcinogen by the US Environmental Protection Agency, and extremely toxic to plants (Arshad et al., 2017). When the amount in intolerant plants exceeds 100 mg/kg dry weight, it induces negative effects including phytotoxicity (stunted growth, decreased germination, and changes in antioxidant enzymes) (Shanker et al., 2005; Arshad et al., 2017; Lilli et al., 2019; Jain et al., 2015).

1.1.3. Nickel

Nickle lies in group VIII on periodic table having relative atomic mass of 58.69 and atomic number 28, and melting point 1,455°C (Pi et al., 2020). Nickel (Ni) is an essential constituent that plants need as a micronutrient to thrive, but high levels have been linked to phytotoxicity (Rehman et al., 2016). Inactivation of photosystem I and II, inhibition of electron transport, blockage of chlorophyll synthesis, reduced protein content, higher proline content, production of reactive oxygen species (ROS) in the root and leaves, and hampered CO_2 fixation, are among the known phytotoxic effects (Sirhindi et al., 2016). These impacts were reported at concentration as low as 1.5 mg/kg dry weight of plant (Kamran et al., 2016).

1.2. Sources of heavy metals in soil

The toxicity and contamination by heavy metals is constantly elevating due to nonbiodegradable nature and high solubility (Desai et al., 2008). Among igneous rocks, such as basalt and shales ingenious rocks, contains higher concentration of HMs like

Cd, Chromium, Nickel (Imran et al., 2017). These heavy metals are found generally in soil in the form of carbonates, sulfides, oxides, or salts. Progress in agricultural and industrial sector has a lot of contribution in elevating the level of contamination in water resources and in soil (He et al., 2005; Sarwar et al., 2010; Czarnecki and Düring, 2015). Depending on the rock phosphate source, calcium phosphate and triple super-phosphate fertilizers contain different concentration of heavy metals (Cd).

1.2.1. Toxicity of heavy metals on human

Even at low concentration, heavy metals are harmful to human health (Kara, 2005). *Different metals* have different effects on human health, some of which are:

- Cadmium (Cd): Cadmium is carcinogenic, mutagenic, and teratogenic and also interferes with calcium therefore it (Awofolu, 2005).
- Chromium (Cr): Chromium causes nose infections, nasal congestion, issues with breathing and fall of hairs.
- Nickel (Ni): Nickel causes neurotoxicity, genotoxicity, allergic dermatitis, inhalation problem and ultimately lung and throat cancer (Mishra et al., 2010).

Table 1.2. Different type of heavy metals and effects of human beings (Abbas et al., 2014)

Heavy metals	Major sources	Toxic effects
Cadmium	Mining, pesticide welding, refining, Plastic, and fertilizer	Damage to the liver, bronchitis, gastrointestinal disease
Chromium	Textile paints and pigments, steel fabrication and dyeing,	Mutagenic, carcinogenic, teratogenic
Nickel	non-ferrous metal Porcelain enameling, electroplating and paint formulation	Chronic bronchitis, decreased activity of the lungs

1.3. Current scenario of heavy metals in Sindh

The rising contamination of Pakistan's drinking water supplies, as well as the implications on environmental and human health, is a major source of concern. Environmental pollution is leading towards of the land resources in Pakistan (Khan et al., 2012). Although most of the Pakistan's population (70 percent) gets their water from

underground aquifers, surface water is also a major source of water for drinking and other domestic purposes (Aziz, 2005). In major cities of Pakistan, such as Karachi, Hyderabad, Larkana, Kamber Shahdadkot groundwater is the primary source of drinking, farming, and industrial needs (Daud et al., 2017; Imran et al., 2019; Lanjwani et al., 2020a,b). One-third of the world population consumes about 65% of groundwater for drinking (Adimalla et al., 2019; Salehi et al., 2018). Demand of water resources is drastically increasing because of increasing population. In addition, the groundwater sources are contaminated and becoming incompatible for consumption due to some anthropogenic activities (Barilari et al., 2020). Contamination of bacteria, toxic metals like cadmium, chromium, , nickel are posing major threats to water quality in Pakistan. According to Lanjwani (2020a) Cd, Cr and Ni were found more than permissible limit with 57.14, 57.14 and 52.38 mg/L in Larkana, Kamber Shahdadkot and Jacobabad. In contrast, 0.17 mg/L of Cd was found in groundwater of Thatta and Hyderabad Sindh, Pakistan (Alamgir et al., 2016) Waseem et al., 2014). In Pakistan, <0.001–9.8 mg/L of Cr content was noticed in ground water (mean value 2.12 mg/L) and 0.16–0.29 mg/L in surface water. During soil sample analysis of Pazang site and Hyderabad 0.84 mg/kg of Cd was found (Waseem et al., 2014). Institute for health metrics and evaluation proposed that most of the deaths and disabilities observed in Pakistan during the year 2017 was caused by air pollution. Moreover, air pollution ranked 5th among factors responsible for deaths and disabilities in Pakistan.

Table 1.3. Permissible limit of heavy metals in Pakistan (Khan et al., 2010), India (Tiwari et al., 2015), China (Pan et al., 2016)

Permissible limits	Soil (mg/kg)			Water (mg/L)		
	Cd	Cr	Ni	Cd	Cr	Ni
Pakistan (WHO)	0.8	100	35	0.1	0.10	75
India (WHO)	3.00	2.0	40-60/3.0	0.003	2	-
China (WHO)	0.3–0.6	150–300	1.0	0.03	0.5	1.0

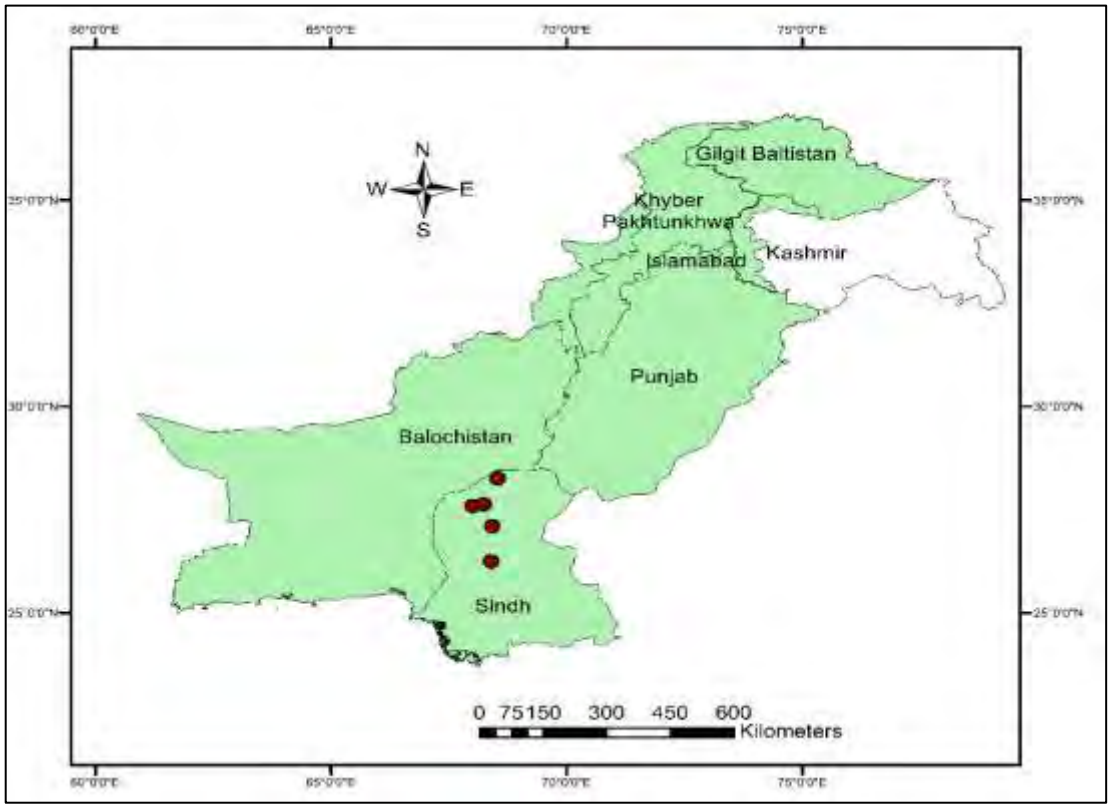


Figure 1.1. Map of Sindh, Pakistan showing areas of heavy metals contamination

1.4. Methods are divided in two categories

1.4.1. Conventional techniques

One method like surface capping, vitrification, soil flushing, landfilling, electro-kinetic extraction (Liu et al., 2018; Das et al 2017). Various methods among these are cost effective and technically complex; furthermore, these physiochemical methods affect soil richness and biodiversity (Khalid et al., 2017).

1.4.2. Biobased approach

Other is focused on biobased approach such as bioremediation and phytoremediation. Bioremediation has certain recognized benefits of high public acceptance, high efficacy, low cost, easy accessibility, environment friendly, which has also been widely established in the remedy of combined HMs (Zhang et al.,2020). Various approaches implemented by plants to cope with toxic HMs include metal sequestration, exclusion and inactivation by organic ligand exudation, compartmentalization in some cell organelles (Choppala et al., 2014).

1.5. Bioremediation

The removal of heavy metals to decrease their level of toxicity as well as mobility in soil is the primary goal of remediation strategies (Pathak et al., 2020). Bioremediation is the use of organisms to eliminate or decline the detrimental compounds in soil. Naturally occurring and genetically engineered organisms can possibly be used for bioremediation. The primary organisms used in this process are fungi, bacteria, algae, plankton, plants, and protozoa, which either eliminate organic substances or convert metals to a stable form. Bioremediation is classified based on various types of living organisms involved in the process, including microbial remediation (Jan et al., 2014), phytoremediation (Ojuederie and Babalola 2017), and combined remediation. Bioremediations involve bioaccumulation biocrystallization and phytoremediation. Heavy metals resistant rhizobacteria also play a significant role in soil fertility preservation (Kumar et al., 2015) since, they react quickly in adverse situations and are extremely sensitive to environmental changes. (Yu et al., 2014). Rhizobacteria are also known as important bioindicators of soil quality (Valverde et al., 2011). Biological, soil

remediation methods are useful because they are cost-effective, and environmentally friendly (Pirzadah et al., 2015; Pandey et al., 2016; Megharaj and Naidu,2017).

1.5.1. Biosorption

Biosorption involves remediation of heavy metals or metalloid species, particles from aqueous solutions by living organisms (Wang and Chen, 2009). Remediation of heavy metals can be carried out by dead biomass and living or through cellular products like exopolysaccharides (EPS). Heavy metals containing waste is discharged into environment by surface finishing, leather tanning, metal surface treating, mining of metalliferous, metallurgy, electroplating, electrolysis, electric appliance manufacturing etc. Therefore, heavy metals are causing serious environmental pollution and are dangerous for human health as well as ecosystem (Wang and Chen, 2006). There are many kinds of methods and technologies used for heavy metal removal from aqueous solution such as biological, physical, and chemical. Whereas chemical techniques include precipitation, membrane technologies, filtration, ion exchange, electrochemical treatment, evaporation, and activated adsorption of carbon are the most common approaches (Volesky, 1990).

1.5.2. Biosorption mechanism

It is difficult to understand the process by which microorganisms absorb heavy metals (HMs) due to the complexity of the biomaterials in cell wall (Yu et al., 2018a). Ligands found in the cell wall of microorganisms are involved in the biosorption process (Giovanella et al., 2017). Several other mechanisms i.e., Van der wall forces, covalent factor, electrostatic attractions, ion exchange, complexation, microstructure sequestration and surface adsorption also play their role in the process of biosorption (Esmaeili and Beni, 2018). The biosorbent based heavy metal ions adsorption process occur in two phases including solute mass transfer having the ion to the surface of adsorbent, which is relatively a quicker process (Esmaeili and Beni, 2015). The next step is dissolved component transfer from the surface of the adsorbent to the core active sites, that is comparatively slowest procedure (Mahindrakar and Rathod, 2018). Many chemical groups make cell wall polymers such as, sulfhydryl, hydroxyl, carbonyl, imidazole, sulfonate, imine, amine, amide, carboxyl, thioether, phosphodiester, and phosphonate (Hossain et al., 2015, Mahindrakar and Rathod, 2018).

1.5.3. Bacterial biosorbent

Among the microorganism's bacteria make a remarkable portion of the whole biomass of terrestrial ecosystem ~ 10^{18} g (Wang and Chen 2009). Some microorganisms are considered to assemble elements of metal with extreme potential(Vijayaraghavan and Yun, 2008). Bacteria are used as biosorbent due to their small size, ubiquity, and their resistance to wide range of environmental conditions (Wang and Chen 2009). Several species of bacteria like *Bacillus*, *Escherichia*, *Pseudomonas*, *Micrococcus*, *Streptomyces*, etc have been verified for uptake of heavy metals. Under laying mechanism include physical sequestration, exclusion, detoxification, and complexation. Ligand binds with heavy metal and inhibit their entry into the bacterial cell (Ahemad and Kibret, 2013). Other mechanisms include siderophores production thus removing the toxic effects of heavy metal. Uptake of heavy metals is predicted by experimental values and Langmuir model.

Table 1.4. Absorption capacity of different bacterial strains for Cd, Cr and Ni

Biosorbent	Heavy Metals	Adsorption Capacity in mg/g	Reference
Staphylococcus xylosus	Cd	250	(Ziagova et al., 2007)
Bacillus subtilis	Cd	208.08	(Wang and Sun, 2013)
Rhizobium leguminosarum bv. Vicia	Cd	167.5	(Abd-Alla et al., 2012)
Pseudomonas sp.	Cd	150	(Ziagova et al., 2007)
Alteromonas macleodii NR	Cd	150	(Moselhy et al., 2013)
Bacillus jeotgali U3	Cd	99.9	(Green-Ruiz et al., 2008)
Ochrobactrum sp. GDOS	Cd	83.33	(Khadivinia et al., 2014)
Staphylococcus cohnii GC	Cd	83.034	(Kalkan et al., 2013)
Baccilus laterosporus	Cd	72.6	(Zouboulis, 2004)
Arthrobacter nicotianae IAM12342	Cd	56.0405	(Tsuruta et al., 2014)
Bacillus megaterium	Cd	40.32	(Ziagova et al., 2014)
Rhodobacter sphaeroides	Cd	30–40	(Bai et al., 2008)
Bacillus subtilis IAM1026 292	Cd	42.0303	(Tsuruta et al., 2014)
Bacillus megaterium IAM1166	Cd	38.0437	(Tsuruta et al., 2014)

Micrococcus luteus IAM1056	Cadmium	39.5245	(Tsuruta et al., 2014)
Corynebacterium equi IAM1038	Cadmium	37.702	(Tsuruta et al., 2014)
Bacillus subtilis var niger IAM1633	Cadmium	36.9047	(Tsuruta et al., 2014)
Brevibacterium helovolum IAM1637	Cadmium	36.6769	(Tsuruta et al., 2014)
Escherichia coli IAM1264 166	Cadmium	34.0571	(Tsuruta et al., 2014)
Pseudomonas aureofaciens IAM12353	Cadmium	20.2748	(Tsuruta et al., 2014)
Deinococcus proteolyticus IAM12141	Cadmium	18.908	(Tsuruta et al., 2014)
Pseudomonas putida IAM1506	Cadmium	18.7941	(Tsuruta et al., 2014)
Pseudomonas maltophilia IAM1554	Cadmium	14.8074	(Tsuruta et al., 2014)
Nocardia erythropolis IAM1399	Cadmium	9.5679	(Tsuruta et al., 2014)
Tsukamurella paurometabola A155	Cadmium	16.89	(Limcharoensuk et al., 2015)
Halomonas BVR 1	Cadmium	12.023	(Rajesh et al., 2014)
Bacillus circulans	Cr (VI)	34.5	
Bacillus megaterium		32.0	(Srinath et al., 2002)
Bacillus coagulans		39.9	
Ochrobactrum anthropic	Cr (VI)	86.20	(Ozdemir et al., 2003)
Aeromonas caviae	Cr (VI)	284.4	(Loukidous et al., 2004)

Staphylococcus xylosus	Cr (VI)	143	(Ziagova et al., 2007)
Corynebacterium glutamicum	Cr (VI)	95	(Park et al., 2008)
Escherichia coli	Cr (VI)	64.36	(Gabr et al., 2009)
Pseudomonas aeruginosa *Bacillus subtilis*	Cr (VI)	1.44	(Tarangini et al., 2009)
Arthrobacter viscosus	Cr (VI)	17.0	(Silva et al., 2012)
Bacillus salmalaya	Cr (VI)	20.35	(Dadrasnia et al., 2015)
Ochrobactrum sp.	Cr (VI)	30.2 ± 0.8	(Chen et al., 2016)
Alteromonas sp.	Cr (VI)	215.2 ± 5.1	(Zhang et al., 2017)
Arthrobacter viscosus	Cr (VI)	20.37	(Hlihor et al., 2017)
Kocuria sp.	Cr (VI)	43.5	(Akbarpour Nesheli et al., 2018)
Sinorhizobium sp.	Cr (VI)	285.71	(Jobby et al., 2019)
Pseudomonas sp.	Cr (VI)	32.0	(Chang et al., 2019)
Halomonas sp.		150.7	(Kalola and Desai, 2020)
Parapedobacter sp.	Cr (VI)	33.783	(Tyagi et al., 2020)
Pseudomonas alcaliphila	Cr (VI)	10	(El-Naggar et al., 2020)
Lysinibacillus sp. BA2	Ni	238.04	(Prithviraj et al., 2014)

Acinetobacter sp. FM4	Ni	66.7	(Masood and Malik, 2015)
Stenotrophomonas maltophilia	Ni	54.3	Wierzba, (2015)
Bacillus subtilis		57.8	
Bacillus subtilis 1612WTNC	Ni	152.534	(Al-Gheethi et al., 2014)
Pseudomonas fluoresens 1353WTNC		147.82	
Curtobacterium sp. FM01	Ni	140.99	(Masoumi et al., 2016)
Streptomyces roseorubens	Ni	208.39	(Long et al., 2018)
Halo bacillus sp. KN57	Ni	111.11	(Kardel and Torabi, 2019)
Planococcus sp.	Ni	0.47	(Hoseini et al., 2020)
Saccharomyces caravesae	Ni	46.3	Shamim, 2018
Streptomyces rimosus	Ni	32.9	Sahmoune, 2018
Bacillus subtilus	Ni	328.7	Rizvi et al., 2020
Pseudomonas aeruginosa	Ni	167.8	Rizvi et al., 2020
Streptomyces roseorubens SY	Ni	208.3	Long et al., 2018
Pseudomonas fluorescens	Ni	12.4	Uzel Ozdemir 2009

1.5.4. Adsorption and absorption isotherm models

Sorption is characterized as an association of solid-phase chemicals. Comparing adsorption and absorption, adsorption is characterized by the binding of molecules to a 2-D matrix while, absorption is characterized by formation of 3-D matrix of molecules (Nouri et al., 2007; Prasad and Srivastava, 2009; Qi et al., 2017).

1.5.5. Langmuir isotherm model

Adsorption isotherm model is based on solid phase gas adsorption process (Foo and Hameed, 2010). The adsorption mechanism on a solid surface involves irresistible molecular bombardment on the surface with desorption or evaporation of counterpart molecules and zero-accumulation rate at the surface (Al-Ghouti and Da'ana).

The adsorption and desorption rates, in other words, should be equivalent. The Langmuir isotherm model has been conventionally used to measure and contrast the adsorption potential of various biosorbents (Kundu and Gupta, 2006; Foo and Hameed, 2010).

1.5.6. Freundlich isotherm model

Freundlich isothermal model represents the reversible and non-ideal adsorption mechanism. The Freundlich model is based on both monolayer and multilayer adsorption as compared to the Langmuir isothermal model (Al-Ghouti and Da'ana 2020). The heterogeneity of the surface and the exponential distribution of the energies on the active sites is described by the expression of the Freundlich isothermal model.

1.5.7. Adsorption kinetics

Adsorption kinetic study provide a key role in the demonstration of efficacy of the process. Factors effecting the kinetic processes in equilibrium attainment in a specific time and chemical processes are explored by kinetic studies. There are following kinetic models are used.

1.5.8. kinetic model of pseudo-first order

The Langmuir model is applicable in demonstration of absorption rate in system of liquid phase. Langmuir model (pseudo-first order) states that ions attached to single

sorption sites on the absorbent surface (Ghaedi et al. 2013; Gupta and Bhattacharyya 2011)

1.5.9. kinetic model of pseudo-second order

The equation of pseudo-second order is known to be a specific type of Langmuir kinetics (Gupta and Bhattacharyya 2011). The pseudo second order model depends on the filling of absorbent surface by adsorbate molecules attached to adsoption site at any time. The presence of adsorption sites in deep tiny pores make it difficult for the adsorbate molecules to reach adsorption sites. Hubbe et al., (2011) also reported the same results. Numerous studies stated that the utmost suitable model for the kinetics of metal sorption is pseudo-second (Benguella and Benaissa, 2002, Celekli and Bozkurt, 2011). The following is equation of pseudo-second-order model (Ho, 2006).

1.5.10. Intra-particle diffusion model

Weber-Morris described the intra-particle diffusion model. He found that in several adsorption processes the solute particles uptake depends on I instead of contact time (Alkan et al., 2007; Weber and Morris 1963).

1.6. Phytoremediation

Mechanisms of phytoremediation are phytoextraction, phytoaccumulation, Phyto-stabilization, and phytovolatilization and rhizo-filtration (Jadia and Fulekar, 2009; Liu et al., 2020). Phytoremediation is a form of bioremediation, in which plants are used to extract toxic HMs as a remediator or accumulating agent (Ali et al., 2013). Plant achieved this by binding, extracting and remediating pollutants from the environment (phytoremediation) (Ojuederie and Babalola, 2017). In the metabolic processes, plants remove the contaminants through root systems, translocate them to shoots and then (mostly) accumulate (heavy metals) or mineralized them. Phytoremediation is an ecofriendly and economical process that can be used without affecting soil fertility and biodiversity for the contaminated soil reclamation (Ahmad et al., 2016; Zloch et al., 2017; Sarwar et al., 2017; Liu et al., 2018; Xiao et al., 2019).

Table 1.5. Selected studies on phytoremediation of Cadmium, Chromium and Nickel

Plant species	Metals	References
Brassica rapa	Cadmium	Khan et al., 2017
Solanum nigrum L.	Cadmium	Tang et al., 2017
Pisum sativum L.	Cadmium	Cardoso et al., 2017
Zea mays L.	Cadmium	Hayat et al., 2020
Soybean *Glycine max*	Cadmium	Movahed et al., 2020
Pisum sativum L.	Cadmium	Cheraghi-Aliakbari et al., 2020
Trifolium repens L.	Cadmium	Lin et al.,2021
Alfalfa *(Medicago sativa)*	Cadmium	Li, et al., 2021
Pennisetum	Cadmium	Kamal et al., 2021
Sesbania sesban L.	Chromium	Din et al., 2020
Genipa americana	Chromium	Santana et al., 2019
Brachiaria mutica	Chromium	Akram et al.,2020
Tithonia diversifolia and *Helianthus annuus*	Chromium	Farid, et al.,2020
Amaranthus mangostanus L.	Nickel	Jia et al.2016
Raphanus sativus L.	Nickel	Akhtar et al., 2018
Salix viminalis	Nickel	Korzeniowska and stanislawska-Giubiak 2019

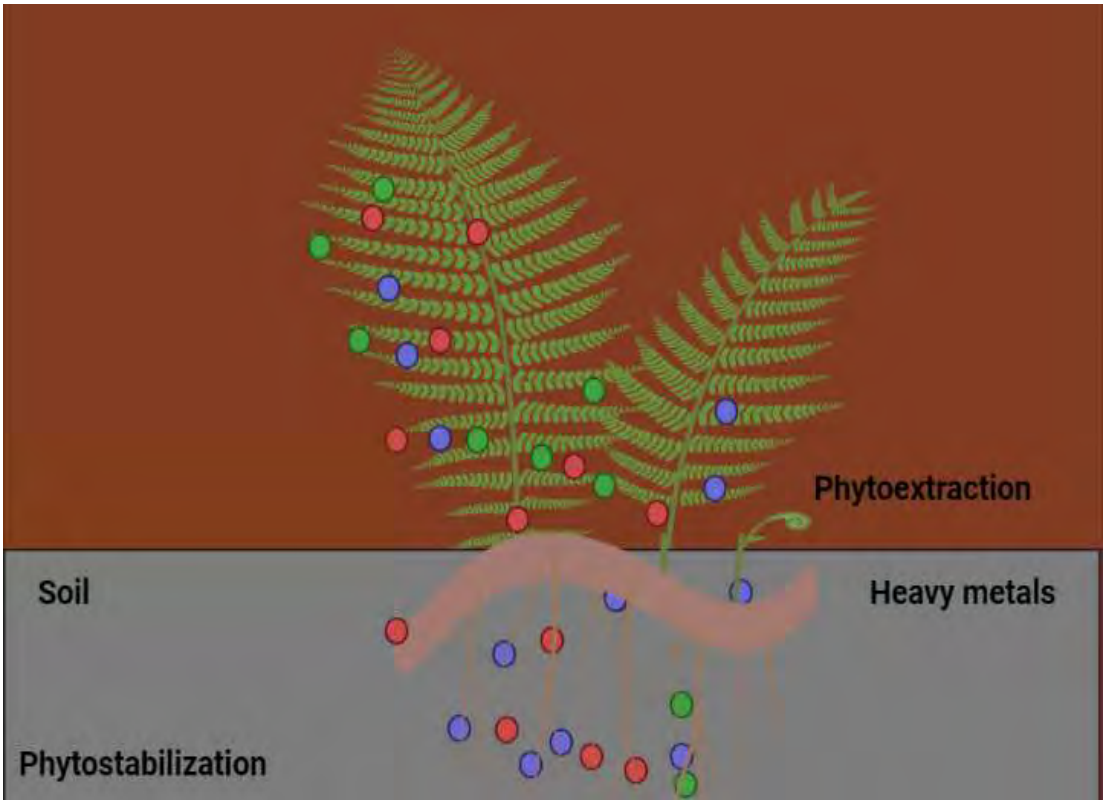


Figure 1.2. Phytoremediation mechanism adopted by plants to remediated heavy metals

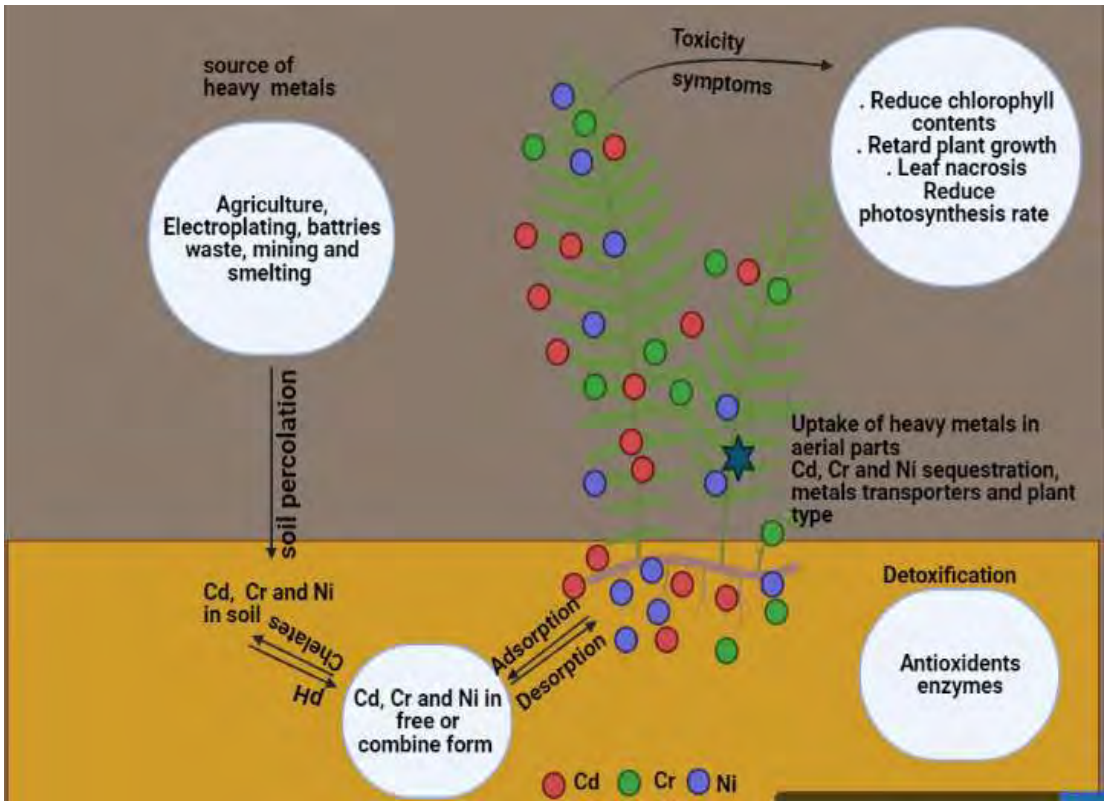


Figure 1.3. Phytoremediation mechanism of Cd, Cr and Ni adopted by plant

1.6.1. Microbial assisted phytoremediation

Microbial aided phytoremediation is a biologically based integrated technology (Girolkar et al., 2021). As a result, it has lately been utilized to clean up a variety of contaminated locations, including heavy metals (Rahman and Singh, 2020). Restoration of wasteland productivity, increased biomass, carbon sequestration, and a high plant survival rate are among the environmental and social benefits of this strategy (Juwarkar, 2012). For pollutant remediation, the method/technology employs both plants and bacteria, as the name implies. The effectiveness of bacteria-assisted phytoremediation to accumulate and degrade pollutants depends on the ability of plants and microbes to accumulate and degrade pollutants at high concentrations. Plants that accumulate heavy metals are known as hyperaccumulators, and several plants contain enzymes that breakdown organic molecules (Glick, 2010; Ma et al., 2011).

Table 1.6. Microbial assisted phytoremediation of HMs (Cd, Cr and Ni)

Bacteria	Plant	Heavy metals	References
Bacillus xiamenensis PM14	*Sesbania sesban* L.	Chromium	Din et al., 2020
Eichhornia sp. *Pistia ssp.*	aquatic macrophytes	Chromium	Mondal and Nayek 2020
Pseudomonas aeruginosa strain OSG41	Chickpea (*Cicerarietinum*)	Chromium	Oves et al., 2013
Bacillus cereus, Pseudomonas moraviensis	*Triticum aestivum*	Ni, Cd and Cr	Hassan et al., 2017
Klebsiella pneumoniae	*Oryza sativa*	Cadmium	Pramanik et al., 2017
Enterobacter, Leifsonia, Klebsiella, Bacillus	*Zea mays*	Cadmium	Ahmad et al., 2016
Bacillus sp.E1S2 and *Bacillus pumilus* E2S2	*Sedum plumbizincicola*	Cadmium	Ma et al., 2015
Micrococcus sp. MU1 and *Klebsiella sp.* BAM1	*Helianthus annus*	Cadmium	Prapagdee et al., 2013
Alcaligenes faecalis	*Brassica juncea*	Ni, Cd and Cr	Robinson Junior
Bacillus subtilis			Ndeddy Aka and
Pseudomonas aeruginosa			Olubukola Oluranti
			Babalola (2016)
Pseudomonas putida	*Eruca sativa*	Cd	Kamran et al., 2015
Bacillus species PSB10	Chickpea (*Cicerarietinum*)	Cr	Wani and Khan 2010
Psychrobacter sp. SRA1, *Bacilluscereus* SRA10	*Brassicajuncea*, B*rassicaoxyrrhina*	Ni	Ma et al., 2009b
Psychrobacter sp. SRA1, *Bacilluscereus* SRA10	*Brassicajuncea*, *Brassicaoxyrrhina*	Ni	Ma et al., 2009b
Pseudomonas sp. A3R3	*Alyssumserpyllifolium*, *Brassicajuncea*	Ni	Ma et al. 2011a

1.6.2. Phytoextraction

Phytoextraction is the most significant phytoremediation approach for removing HMs from sediments, water, polluted soil, biosolids (He et al., 2005; Seth, 2012; Ali et al., 2013). The transference of HMs from soil and water to the upper section of the plant is known as phytoextraction (Rafati et al., 2011). The plants chosen for phytoextraction should have the following characteristics: rapid growth rate, resistance to diseases and pests, tolerance to heavy metal toxicity, increased biomass production, and adaptability to changing climatic conditions (Tong et al., 2004; Shabani and Sayadi, 2012; Ali et al., 2013).

1.6.3. Phyto-filtration

In phyto-filtration the polluted soil surface and wastewater are cleaned through plants (Sangeeta and Maiti, 2010). Phyto filtration is characterized for the adsorption of contaminants and minimizing their movement to ground water.

1.6.4. Phyto-stabilization

Another technique used in phytoremediation is phyto stabilization by which soil pollutants can be stabilized. Phytostabilization refers primarily to the use of plants capable of reducing the bioavailability or mobility of a metal, thus preventing it from moving to groundwater or to entering the food chain (Erakhrumen, 2007). Plants can decrease the toxic effects of heavy metals or prevent complexation, precipitation, and sorption from entering the soil (Wuana and Okieimen, 2011).

1.6.5. Phyto-volatilization

Phytovolatilization is the release of metal into the atmosphere through stomata by converting metal into volatile form (Ghosh and Singh, 2005). Basically, plant roots take the toxins from the soil and convert them from the plant body into volatile compounds that can be quickly released into the atmosphere. It is one of the most appropriate methods for the removal of organic compounds found in the soil (Padmavathiamma and Li, 2007).

1.7. General introduction and phytoremediation potential of *Sesbania sesban* L.

Sesbania sesban L. belongs to a legume family also known as Egyptian river rod and is called *janthi* and *janther* in *urdu*. It is among the fast-growing perennial plants (legume tree). It stems can reach to 8 meter in height and 12 cm in diameter. It has the shallow root system. leaves are pinnate compound type comprises of 6 to 27 pairs of oblongs, linear approximately 5 mm long and 26 mm leaflet. Furthermore, *Sesbania sesban* L. possesses raceme type inflorescence with 2 to 20 of yellow flowers and is up to 30 cm long. It possesses pods at maturity stage consisting of 10 to 15 small light black or gray color seeds. It is mostly grown along stream banks, and swamp edges, about 2300 m from the sea level. It is cultivated throughout sub humid tropical regions and semi-arid area and is mostly dispersed. Normal growth conditions are 17ºC to 20ºC (average annual temperature) and 500-2000 mm of annual rainfall. One of the significant features of plant is that it has ability to grow in alkaline, saline, and acidic soils as well as at low pH levels. Besides this, S*esbania* can also tolerate waterlogging conditions except at first seedling stage. Interestingly, it can withstand with cool temperatures and can grow in the higher tropic elevations (Brussaard et al., 2007). Soil can be cheaply improved by fast growing nitrogen fixing leguminous plants such as *Sesbania sesban* (L.) (Sultan et al., 2012; Sohrawardy and Hossain, 2014). *Sesbania* can be utilized as organic fertilizer and green manure (Mahmood et al., 2008; Nigussie and Alemayehu, 2013). Moreover, it has the high efficiency for wastewater treatment (Dan et al., 2011).

1.8. Microbial role in metal extraction and growth of plant

The PGPB enhance growth of plant under abiotic stress. Much research showed that the microbes not only induce plant growth but also increases the biomass and plants nutrient status. In stressful conditions plant produce ethylene, which adversely affects the growth of plant. In order to overcome this situation, PGPR produces 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Glick, 2004). In the soil environment microbes interaction stabilize the agriculture (Nadeem et al., 2014). Generally, PGPR'S are applied as biofertilizers, a promising technology for future sustainable farming systems and they also facilitate nutrient availability especially nitrogen and phosphorus (Schütz et al., 2018; Ghosh et al., 2011). For example *Bacillus subtilis* produce Indole acetic acid (IAA), to improve growth of plant and also helps in

accumulation of nickel (Zaidi et al., 2006). The *Pseudomonas* strain that improved brown mustard growth and increased trace element extraction by producing IAA (Rajkumar et al., 2005).

The study will evaluate heavy metals which will be selected based on local environmental assessments. The research design will examine the tolerance of isolated strains against selected metals and later elaborate biosorption mechanism and microbial assisted phytoremediation in the control environment. The different biochemical attributes like ammonia, hydrogen cyanide, indole acetic acid, siderophore and solubilization of phosphate, exopolysaccharide and zinc isolated strains will be executed. Extracellular enzymes such as ACC-deaminase, cellulase, catalase, amylase, protease, pectinase by studied bacterial strain will also be carried out. The current study could present a potential solution in the form of results that could be interpreted for the possible remediation of environmental pollutants selected in our research design that is cadmium, chromium, and nickel.

1.9. Aims and Objectives

Keeping in mind above facts regarding negative impact of heavy metals on us in environment and then presenting a solution in the form of microbial assisted phytoremediation, current research work was design with following objectives:

- **a.** Sampling, isolation, and biochemical characterization of bacterial strains
- **b.** Screening and biosorption studies against three heavy metals cadmium, chromium, and nickel by isolated bacterial strains
- **c.** To study effects of inoculation of best isolated strain on germination of seeds of *sesbania sesban* L. under heavy metals stress
- **d.** To evaluate the microbial assisted phytoremediation of heavy metal with *Sesbania sesban* L.

Chapter 2

Sampling, isolation, and biochemical characterization of bacterial strains

2.1. Introduction

The PGPB growth promoting and suppress the adverse effects of environmental toxins and diseases. Additionally, PGPR has also been used for bioremediation of various environmental pollutants (Sheng et al., 2012; Etesami and Beattie, 2017). Plant roots influence the biological and chemical aspects of soils in the rhizospheric zone, which is rich in microorganisms. Bacteria can have a symbiotic or non-symbiotic interaction with rhizospheric plants, depending on their mechanism of activity (Kundan et al., 2015).

Under both natural and stress conditions, PGPR also plays a crucial role in increasing plant production (Yaish et al., 2015; Choudhary et al., 2016). Furthermore, PGPR enables the absorption of plant nutrients from surrounding ecosystem through different processes, including the production of siderophores to sequester iron, phosphate solubilization and nitrogen fixation (Etesami and Beattie, 2017). Furthermore, the enzyme 1-aminocyclopropane-1-caroxilate (ACC) deaminase can influence growth of plant by producing hormones like indole acetic acid (IAA) or decreasing the action of ethylene on plants (Glick, 2014). When plants counteract or prevent damage caused by pathogenic pathogens, indirect mean plant growth promotion by PGPR occurs (Compant et al., 2005).

Comprehensive studies were conducted regarding the application of PGPR by utilizing different bacterial strains like *Pseudomonas fluorescens* and *Pseudomonas putida* having the potential to scavenge cadmium ions from soil and reduced the drastic effect of heavy metals in barley (Baharlouei et al., 2011). It has been documented that PGPR enhanced the water status of leaf under abiotic stresses (Ahmad et al., 2014).

The isolation of rhizospheric bacteria and the selection of promising plant PGPR is a complex process because of the isolation methods, screening, and the organization of a huge data (Barnett, et al., 2017). There are a lot of research methods that study the isolated strains and characterization from rhizosphere. The main and widely used method is culture-dependent method in which large number of microorganisms are isolated, characterized and identified. At the end, the best promising bacterial strains are selected for biosorption and pot experiment. This method is a step-by-step technique with various phases including at least four levels of investigation. These levels include isolation in laboratory, bacterial biosorption of heavy metals, *in-vitro*

seed germination and greenhouse experiment (Yan et al., 2018). It is also based on meta-genomic studies in which population diversity and phylogenetic analysis of each family focused (Petruzzi, et al., 2014). In the current study the rhizospheric bacteria were isolated. Further characterization of bacterial isolates was done against PGP traits and heavy metals stress. The finally selected isolates were identified with the help of 16S rRNA sequencing technique.

2.2. Materials and Methods

2.2.1. Soil sampling

Soil samples were collected from five different districts of Pakistan; Sindh, Larkana (27.628396ºN, 68.240394ºE), Qambar Shahdadkot (27.585919ºN, 68.006018ºE), Shaheed Benazirabad (13.0359ºN, 8.3163ºE), Naushahro Feroze (27.096586ºN, 68.423359ºE) and Jacobabad (28.251700ºN, 68.539061ºE) in sterilized petri plate and were kept in a refrigerator at 4ºC (Naim 1965).

2.2.1.2. Microbial strains isolation

Bacterial strains were isolated from rhizospheric soil through serial dilution method. Luria-Bertani (LB) agar plates were used as growth medium (Majeed et al., 2015). Soil (1 g) was taken in autoclaved dispensation flasks containing autoclaved water and thoroughly mixed to recover microorganisms from soil. A 100 µl of aliquot was poured on LB media, then incubated at 32 ± 2 ºC for 3 d in incubator (SHP-160, Biobase, Shanghai, China). Individual colonies were selected and streaked on LB plates for purification. Isolated pure bacterial strains were further characterized both morphologically and biochemically as given below.

2.2.2. Biochemical characterization of isolated bacterial strains

2.2.2.1. Indole acetic acid (IAA)

Falcon tubes containing Luria Broth modified with tryptophan at the rate of 1mg/mL (precursor of IAA) were used for bacterial culture growth. After incubation for 3 days the cultures were centrifuged and in 2 mL of supernatant, Kovac's reagent was mixed in test tube for confirmation of IAA (Hussain et al., 2019).

2.2.2.2. Phosphate solubilization (PS)

The PS of bacteria was checked by spot inoculating a bacterial colony in the center of plate in Pikovskaya (PVK) medium containing $Ca_3(PO_4)_2$ as insoluble source of phosphate and looking for the development of zone of inhibition across colony after incubation (Amna et al., 2020).

2.2.2.3. Ammonia production

Using peptone water, bacterial isolates were tested for ammonia production (Dinesh et al., 2015). Twenty-four hours grown cultures were inoculated in separate tubes having 10 mL peptone water (NaCl 5g/L, peptone 10g and pH 7 ± 0.2) and incubated for 48–72 h at 35 ± 2 ºC. The tubes were then treated with 0.5 mL of Nessler's reagent. Ammonia development was verified by a change of color from brown to yellow. The composition of Nessler's reagent was as followed 50g of KI was dissolved in the least possible quantity of water. A saturated solution of $MgCl_2$ (22g in 350 mL of H_2O) was added into KI solution till the appearance of precipitate. Next 200mL of 5N sodium hydroxide was added and, after appearance of precipitation, the solution was filtered.

2.2.2.4. Hydrogen cyanide (HCN) determination

Isolated strains were screened out for HCN production by streaking bacteria on a plate containing LB agar added with glycine 4.4 g L^{-1}. The filter papers were cut in round shape. A solution comprised of picric acid (0.5%) and sodium carbonate (2%) was soaked and fixed in the upper lid of plates. Parafilm was used to seal the plates to avoid gas discharge and incubated for 5 days at 30 ºC (Kumar et al., 2012).

2.2.2.5. Siderophore production

The proposed protocol of Louden et al. (2011) was implemented for the siderophore production. A loop full of selected isolates was inoculated on selective media (without iron) amended with CAS-substrate and kept incubated at 30 ºC for 7 days. The formation of siderophores was shown by a yellow to orange-colored region around bacterial colonies.

2.2.2.6. Nitrogen fixation

Nitrogen fixing ability of bacterial isolates was performed on nitrogen free medium (10g L^{-1} sucrose, 5g L^{-1} malic acid, 0.1g L^{-1} NaCl, 0.1g L^{-1} Dipotassium phosphate, 0.4 g L^{-1} Monopotassium phosphate, 0.2 g L^{-1} Magnesium sulfate, 0.02g L^{-1} Calcium chloride, 0.01g L^{-1} Iron (III) chloride, 0.002g L^{-1} Sodium molybdate, 2mL bromothymol blue as an indicator, 15 g L^{-1} agar). For growth media, freshly developed bacterial isolates were streaked over nitrogen-free medium and incubated at 28 -30°C (Elbeltagy et al., 2001).

2.2.3. Extracellular enzyme activities

2.2.3.1. Protease production

Skim milk agar medium was used to evaluate the protease activity. Media was prepared by adding 1g glucose, 2g peptone, 5g yeast extract, 1g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 5g skimmed milk and 15g agar in 1000 mL distilled water. Media was autoclaved and plates were prepared. Bacterial strains were spot inoculated on skimmed milk media plates and placed in an incubator at 30 ºC for 2-3 days. Formation of halo zone is a positive indication of protease production (Chang et al., 2009).

2.2.3.2. Pectinase production

All isolates were screened for their pectinase producing capability by spot inoculation on agar plates containing 1g yeast extract, 2g NH_4SO_4. 6g Na_2HPO_4, 3g KH_2PO_4, 5g pectin from citrus peer and 15g of agar in 1 Liter of distilled water. After inoculation, dishes were wrapped with parafilm and incubated for two days at 30 ºC. After completion of incubation period, plates are flooded with iodine solution, and the formation of halo zone is a positive indication of pectinase production (Tiru et al., 2013).

2.2.3.3. Amylase production

Amylase production following the method of Ashwini al. (2011). A loop full of the bacterial colony was spot inoculated in agar plate prepared by adding 1g yeast extract, 0.1g $MgSO_4$, 7g K_2HPO_4, 2g KH_2PO_4, 1g $(NH_4)_2SO_4$, 5g NaCl, 5g starch and 15g agar in one liter of distilled water. Incubation of plates was done for 2 days at 28ºC. Plates were saturated with iodine solution after the incubation process was completed, and a halo zone formed around the bacterial colony. Formation of halo zone is positive result of amylase production (Ashwini and Gaurav, 2011)

2.2.3.4. Catalase and cellulose production

For testing catalase enzyme (CAT) activity, a single cell of bacteria from 24 h old bacterial culture was placed on glass slide and a drop of 30% (H_2O_2) hydrogen peroxide was added upon it. Formation of gas bubbles indicated the production of CAT enzyme (Naseem and Bano, 2014). Cellulose production was performed according to Kumar and Turner, 2015.

2.2.3.5. Quantitative analysis of ACCD

The ACCD activity was quantified under normal and heavy metal stress conditions following a previously published protocol (Nadeem et al., 2020); as outlined here. Bacterial culture was grown in 5 mL of Tryptic Soy Broth medium at 24 h for 32 ℃ in shaking incubator (DKS-1020, N-Biotek, Gyeonggi-Do, Korea) at 120 rpm. Bacterial cell pellets were extracted by centrifugation at 3000 rpm for 5 minutes, washed twice, and resuspended in 0.1 M Tris-HCl pH 7.5. Spot inoculations of these cultures were then made on Petri plates containing DF media supplemented with and without ACC. As a positive control, ammonium sulphate plates were used. Growth on ACC supplemented plates was similar to growth on positive and negative controls plates after three days.

2.2.3.5.1 Quantitative analysis

After qualitative the quantitative determination of induce ACCD activity was carried out using late log phase culture. After washing cell pellet with 0.1M Tris-HCl and pH 7.5, amended with DF minimal medium including 3mM ACC under normal and heavy metal stress and incubation was carried out for 72 h at 120 rpm shaking. To determine the concentration of α-KB, the extracted cell pellet was labialized with toluene (5%) and supplemented with 0.3M ACC. The toluinized cells (50 l) were used without ACC as a negative control. In each sample, a 0.56 N HCl solution (500 µl) was blended and vortexed. Bacterial suspension was centrifuge at 12000 rpm for 5 min. 500 ml of each sample's supernatant was amended with DNF and 0.56 N HCl solution. After 30 minutes of incubation, the absorbance was measured at 540 nm. After that, each sample received 1 mL of 2N NaOH before being tested for absorbance. α-ketobutyrate was used to develop standard curve through the known values of αKB concentration. Amount of protein was assessed in toluinized cells according to Bradford (1976).

2.2.4. Amplification of *acds* gene

Universal primers described by Duan et al., (2009) were used to amplify *acds* gene, responsible to produce ACC deaminase enzyme. Primer sequences were, Forward: 5'GGCAAGGTCGACATCTATGC-3', Reverse: 5'- GGCTTGCCATTCAGCTATG3'. The initial denaturation of DNA was performed at 94 ºC for 180 sec, subsequent

denaturation for 60 sec at 94ºC (30 cycles), annealing for 60 sec at 58 ºC, extension for 180 sec at 72ºC and a final primer extension at 72 ºC for 30 sec (Singh et al., 2015).

2.2.4.1. Agarose electrophoresis

The polymerase chain reaction products were observed in 2% agarose gel made in 1X TAE buffer. Samples were run at 85V for 35 min in a horizontal electrophoresis unit and were visualized in a gel doc system (Satyanarayana et al., 2017).

2.2.5. Qualitative and quantitative assay of exopolysaccharide (EPS) production

The ATCC medium No. 14 was used to determine exopolysaccharide production by the bacterial isolates (Subair, 2015). Formation of slimy layer around the bacterial colonies after three days of incubation confirmed the production of EPS.

2.2.6. Amplification of *nifH* gene

A set of universal primers (5-TATGATCCAAAAGCAGA-3' and 3'-ATAGCCATCATTTCACC-5') were employed to amplify the *nifH* gene. Initial denaturation at 97°C for 3 minutes; 97°C, 55°C for 50s, and 72°C for 35s, 40 cycles; and final extension at 72°C for 5 minutes were the PCR reaction conditions. Gel electrophoresis was used to examine the PCR products (Zehr et al., 1998).

2.2.7. Morphological characterization

Fresh colony of each isolate was streaked on LB agar plates and kept in incubator at35±2ºC for 24 h.

2.2.7.1. Morphology of bacteria

The agar medium was used to culture bacteria for 24 h and morphological traits of colonies including color, margins, surface texture, shape, and elevation of bacterial colony (Rohomania et al., 2015).

2.2.7.2. Gram staining and cell morphology

The purified isolates were further confirmed by Gram staining technique (Etesami etal., 2017). Gram staining of purified bacteria was performed for their purity check and morphological studies. Throughout the gram staining technique, the following steps were followed. Drop of distilled sterilized water was put on a clean glass slide. Single

bacterial colony was picked and spread with drop of water on the glass slide. The slide was gently heated, till the isolated colony was fixed well. The dried slide was flooded with crystal violet solution for 1 minute (min). After this step, the slide was washed with sterilized water. Then, slide was flooded for 1 min with Iodine solution and was washed with sterilized water. For the decolorization, the decolorizing mediator ethanol was used for washing the slide. After this step safranin was flooded on the slide for about 10 to 20 sec. Again, washed with sterilized water and allowed to dry for about 3 to 4 sec. The cover slip was fixed on the bacterial colonies. The slide was examined under the microscope, (Claus et al., 1992).

2.2.8. Molecular profiling

For molecular characterization, bacterial genomic DNA was extracted following the method of Ahmed et al. (2014). The extracted DNA was used as template for 16S rRNA gene amplification through polymerase chain reactions using universal primer 27F (5'-AACTGAAGAGTTTGATCCTGGCTC-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') primers. Thermal cycling consisted of an initial denaturation phase at 96 °C for 5 minutes, followed by 30 cycles of denaturation at 96 °C for 1 minute, primer annealing at 56 °C for 1 minute, and extension at 72 °C for 1 minute. The final extension step was carried out at 72 °C for 10 minutes before the reaction was cooled to 4 °C. The sequence obtained were analyzed by using online Genome-to-Genome Distance Calculator (GGDC) 2.1 (Meier-Kolthoff et al., 2013).

2.3. Results

2.3.1. Isolation of rhizobacteria

A total of 20 bacterial strains were isolated from the rhizosphere. Bacterial strains were finalized for morphological analysis. The purified isolates using 18% glycerol at -20°C until further studies at Plant Microbe Interactions Lab, Quaid-I-Azam University, Islamabad, Pakistan.

2.3.2. Biochemical characterization of isolated bacterial strains

Screening of bacterial strains were done against various plant growth promoting assays (Table 2.1).

2.3.2.1. Indole acetic acid (IAA) production

Among all 20 bacterial isolates 13 isolates showed positive results for IAA production with the formation of the cherry re d ring on the top of the test tube Table 2.1 and Figure 2.1.

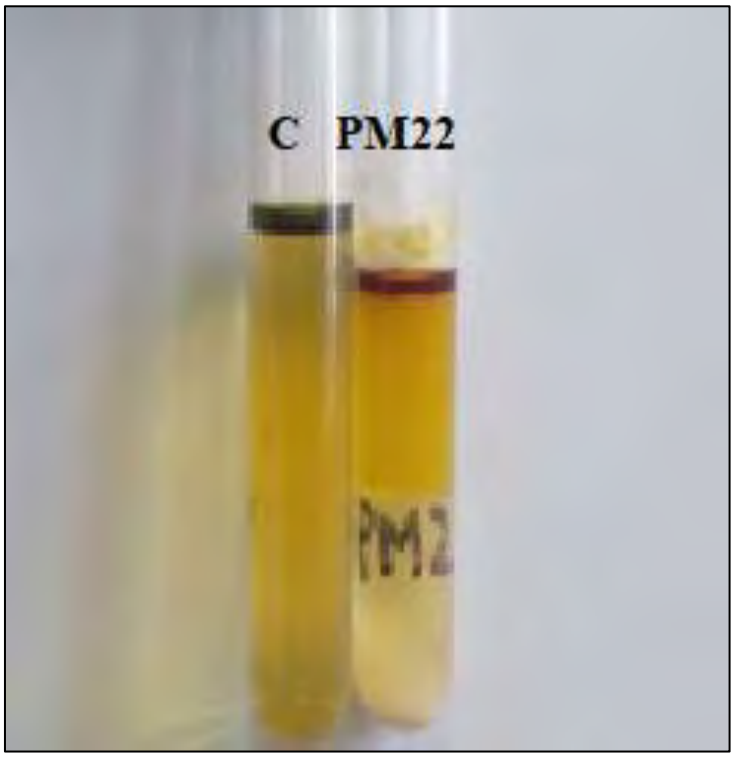


Figure 2.1. Indole acetic acid production by isolated bacteria: C: control

2.3.2.2. Phosphate solubilization

One bacterial isolate (PM21) out of twenty showed a positive result of P-solubilization Table 2.1. The appearance of clear halo-zone surrounding colonies was indication of phosphate solubilization Figure 2.2.

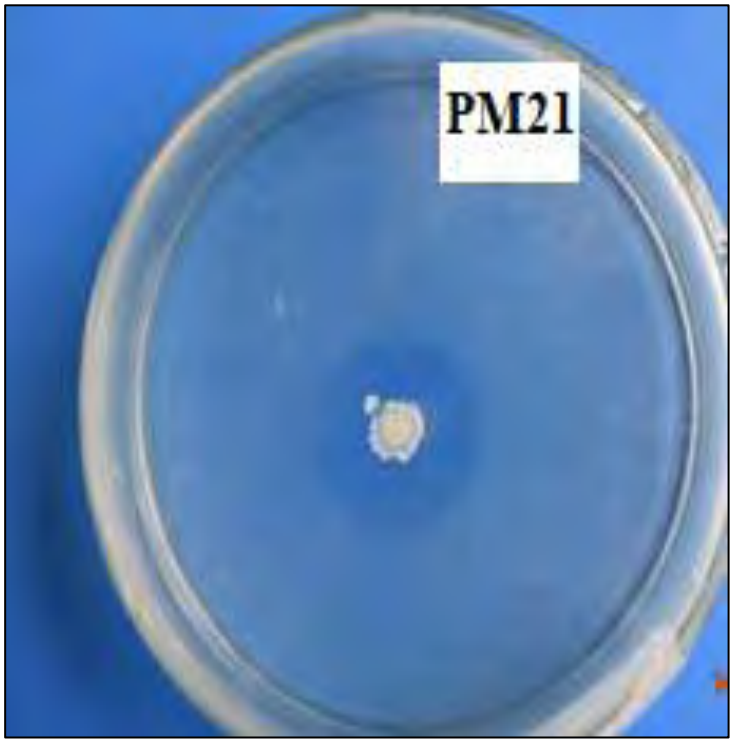


Figure 2.2. Phosphate solubilizing activity of isolated bacterial strain

2.3.2.3. Ammonia production

All 20 isolates showed positive result regarding ammonia production by the formation of brown to yellow color upon addition of Nessler reagents Table 2.1 and Figure 2.3.

2.3.2.4. Siderophore production

Twelve bacterial isolates out of twenty showed positive results for siderophores production Table 2.1 and Figure 2.4.

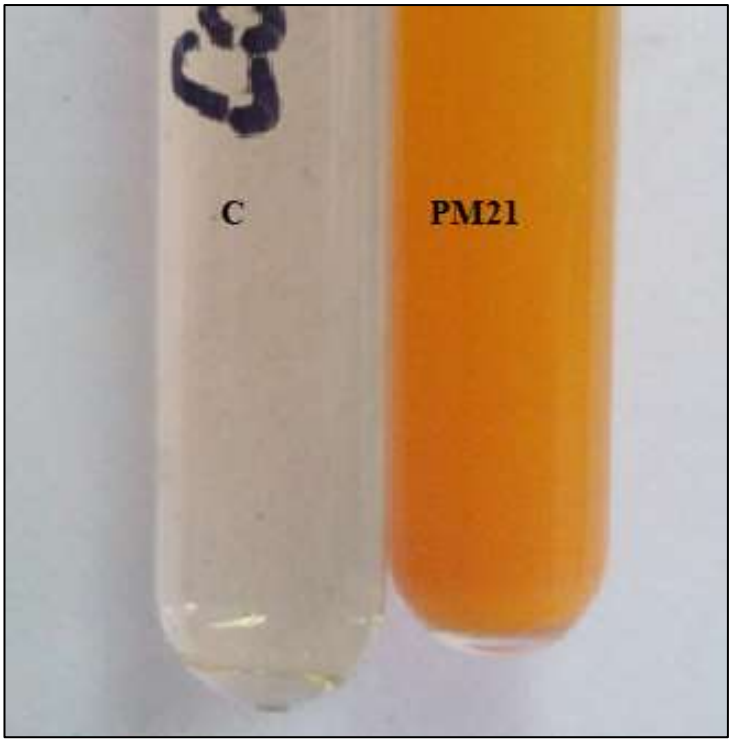


Figure 2.3. Ammonia production

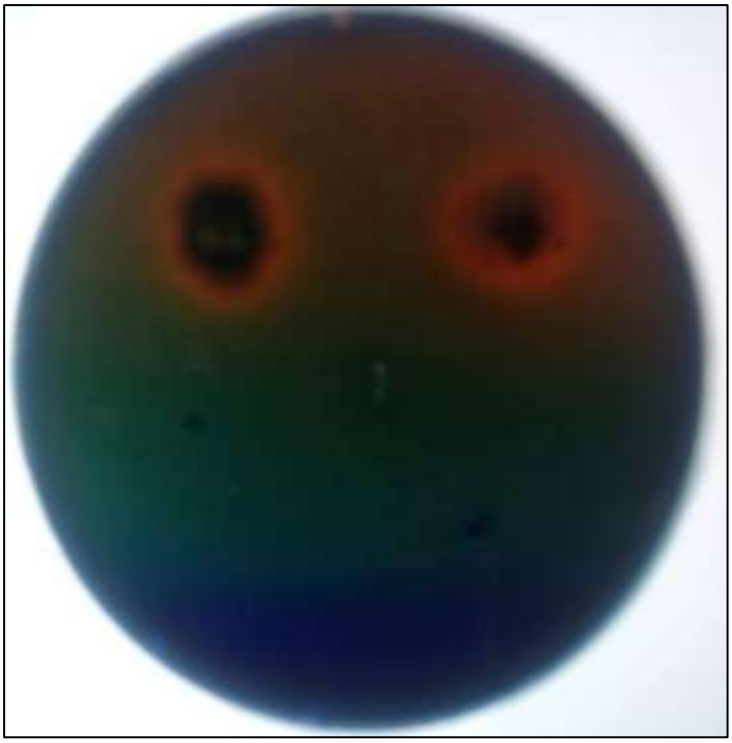


Figure 2.4. Siderophore production

2.3.2.5. Zinc Solubilization

Eleven bacterial isolates out of twenty showed positive results for zinc Solubilization by forming halo zone around bacterial colony on respective medium containing zinc oxide Table 2.1 and Figure 2.5.

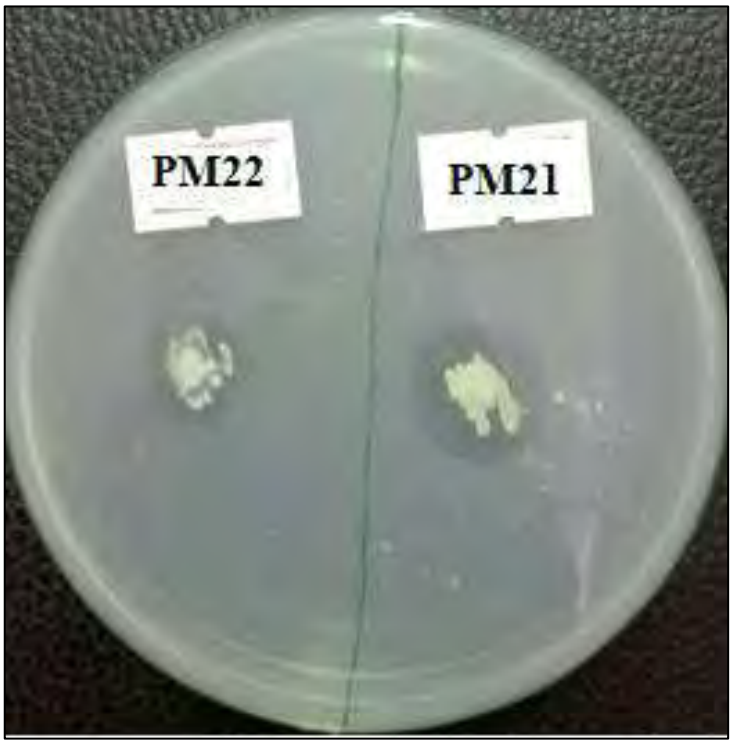


Figure 2.5. Zinc solubilization

2.3.2.6. HCN production

One bacterial isolate (PM21) out of twenty, showed the positive result for HCN production. Table 2.1 and Figure 2.6.

Figure 2.6. Hydrogen cyanide production

2.3.3. Extracellular enzyme tests

2.3.3.1. Protease production

Ten strains showed positive response for protease production by the formation of halo zone around the bacterial colony Table 2.1 and Figure 2.7.

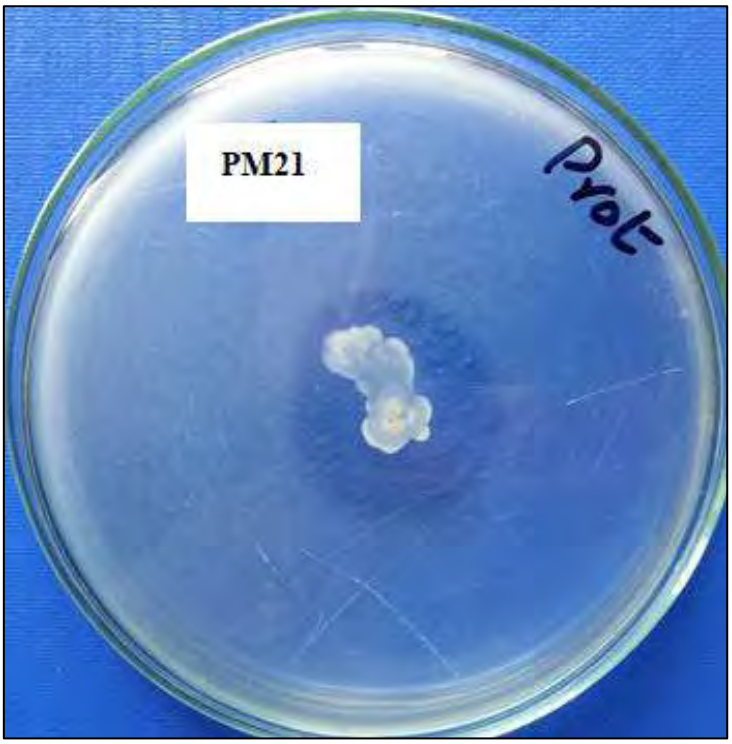


Figure 2.7. Protease production by bacterial isolates

2.3.3.2. Pectinase production

Sixteen bacterial isolates showed positive result for pectinase production by forming halo zone around bacterial colony after flooding with 50 mM iodine solution Table 2.1 and Figure 2.8.

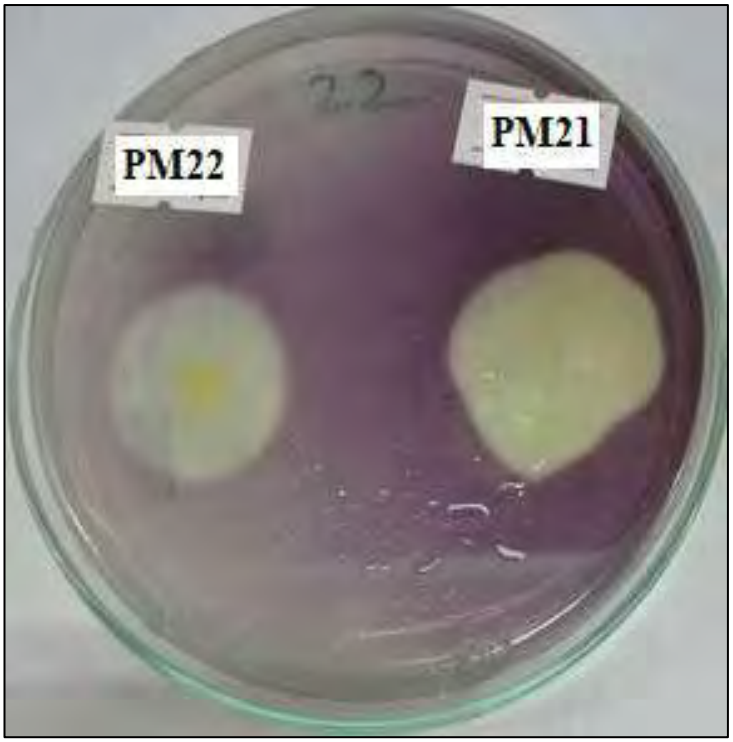


Figure 2.8. Pectinase test of the bacterial isolates

2.3.3.3. Amylase production

Fourteen isolates out of total 20 isolates showed positive results for amylase production Table 2.1 and Figure 2.9.

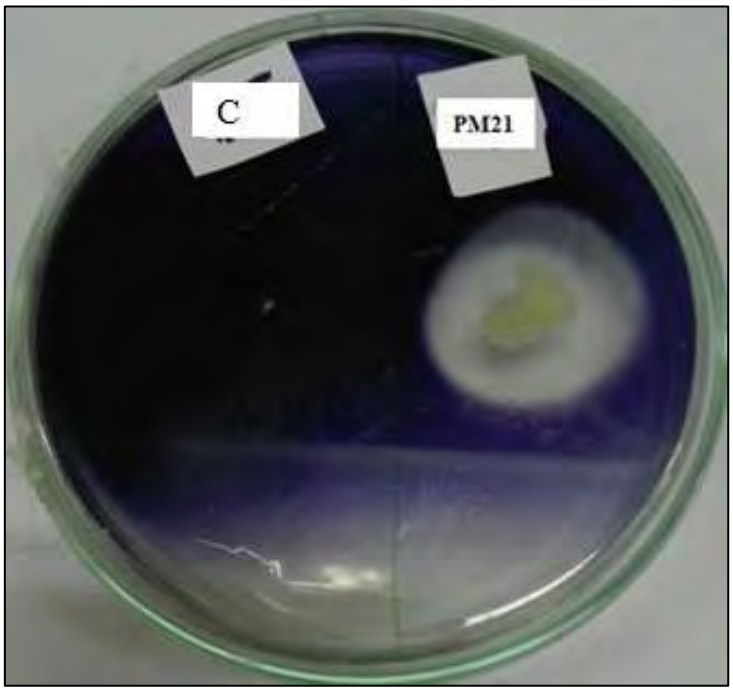


Figure 2.9. Amylase production of bacterial isolates C: control

2.3.3.4. Catalase production

Out of total 20 isolates 15 isolates showed bubble formation demonstrating that they have ability to produce catalase Table 2.1 and Figure 2.10.

2.3.3.5. Cellulose

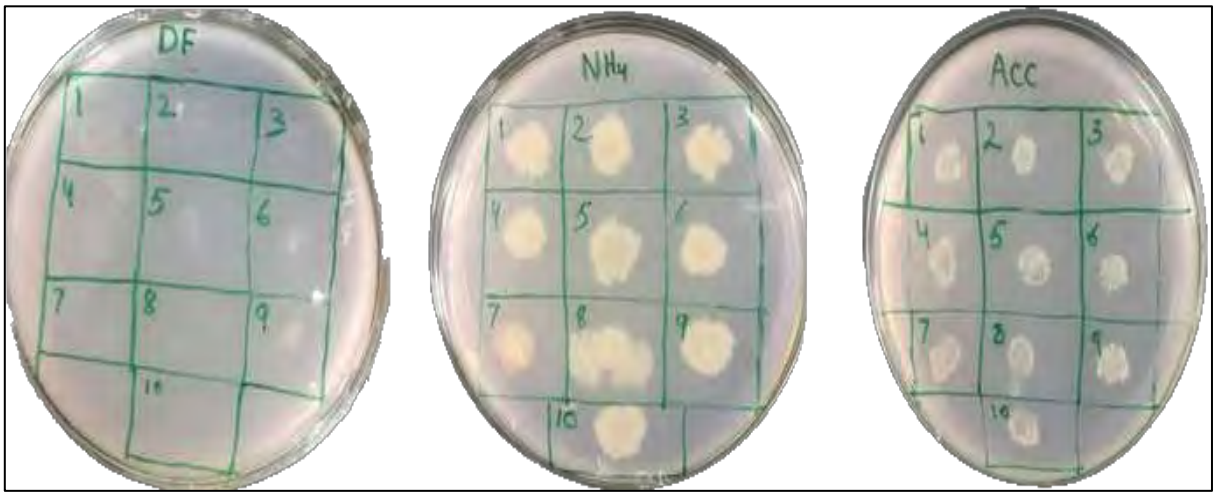
Out of total 20 isolates 16 isolates produced cellulose as indicated by the formation of halo zone on respective medium Table 2.1 and Figure 2.11.

2.3.4. ACC Deaminase Activity

Efficient growth was observed on plates with ammonium sulfate serving as a positive control compared to growth on plates with only DF medium Table 2.1. However, variation in growth pattern of all isolates at agar plates supplemented with ACC was observed. Eleven out of twenty isolates showed positive results for ACC deaminase activity Figure 2.12.

Figure 2.10. Catalase activity of bacterial isolates

Figure 2.11. Cellulose of bacterial isolates



Negative Control **Positive Control** **ACC deaminase activity**

Figure 2.12. ACC Deaminase Activity

2.3.5. Exopolysaccharides (EPS) production

In case of qualitative EPS production, selected bacterial isolates (PM21, PM22, PM23, PM24, PM25, J04 and J05) showed positive result (Table 2.1), by the formation of mucoid transparent colonies as shown in Figure 2.13.

Figure 2.13. Exopolysaccharide's production

Table 2.1 Qualitative assay of important PGP traits in the bacterial strains isolated from rhizospheric soil from Sindh, Pakistan.

PGP Traits	PM21*	J02	J03	J04	J05	PM22	J07	J08	J09	J10	J11	PM23	J13	J14	J15	PM24	J17	J18	J19	PM25
Indole Acetic Acid	+	-	-	+	+	+	+	-	+	+	+	+	-	-	+	+	-	+	-	+
Phosphate solubilization	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Siderophore	+	-	-	-	-	+	+	+	+	+	-	+	-	-	+	+	+	+	-	+
Exopolysaccharide	+	-	-	+	+	+	-	-	-	-	-	+	-	-	-	+	-	-	-	+
Zinc solubilization	+	-	-	+	+	+	-	-	+	+	-	+	-	-	+	+	-	-	+	+
ACC-deaminase	+	+	-	+	+	+	-	+	+	-	+	+	-	+	-	+	+	-	-	+
Ammonia production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrogen cyanide	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase test	+	-	+	+	-	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+
Amylase test	+	-	+	+	+	+	+	+	-	-	+	+	+	+	-	+	-	-	+	+
Protease	+	+	-	+	-	+	-	-	-	+	+	+	-	-	+	+	-	-	-	+
Pectinase	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-	+	+	+
Cellulase	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	+	+	+	+	+
Nitrogen fixation	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-

(+) Present (-) absent; **the strain number with red font have maximum number of traits present.*

2.3.6. Quantitative determination of ACC-deaminase, exopolysaccharides, and indole acetic acid production

Among the five best performing bacterial stains PM21 exhibited maximum ACC deaminase activity (1.56-1.75 µM/mg protein/h), exopolysaccharides (2.73-2.98 mg/mL), and Indole acetic acid (IAA) (99-119 µM/mL) in normal and metal stress conditions (Table 2.2).

Table 2.2. Quantitative assays PGP traits of bacteria strains under metal stress

Strain code	ACC-deaminase (µM/mg protein/h)		Exopolysaccharide (mg/mL)		Indole acetic acid (µM/mL)	
	Normal	Under stress	Normal	Under stress	Normal	Under stress
PM21	**1.56±0.07**†	**1.75±0.01**	**2.73±0.01**	**2.98±0.01**	**99±4.07**	**119±1.47**
PM22	1.11±0.03	1.32±0.03	2.53±0.02	2.73±0.01	82±1.63	101±1.37
PM23	1.01±0.01	1.11±0.04	2.51±0.02	2.69±0.03	76±1.72	96±0.97
PM24	0.91±0.08	1.11±0.02	2.31±0.04	2.42±0.04	74±1.62	89±0.84
PM25	0.92±0.02	0.99±0.01	2.12±0.02	2.33±0.01	85±1.65	99±0.95

†Values are presented as means ± *SE* (n=3).

2.3.7. *NifH* gene amplification

Nitrogen fixation capacity of bacterial strains was supported with amplification of *nifH* gene in two strains (PM21 and PM23) out of five bacterial strains (Figure 2.3).

2.3.8. *Acds* gene amplification

Using a universal set of primers, the PCR mediated amplification of the *acds* gene was carried out for five bacterial strains (Figure 2.4.). While ACC deaminase enzyme activity was quantitatively confirmed in all strains, its activity was further checked by gene amplification in the selected strains using the collection of primers.

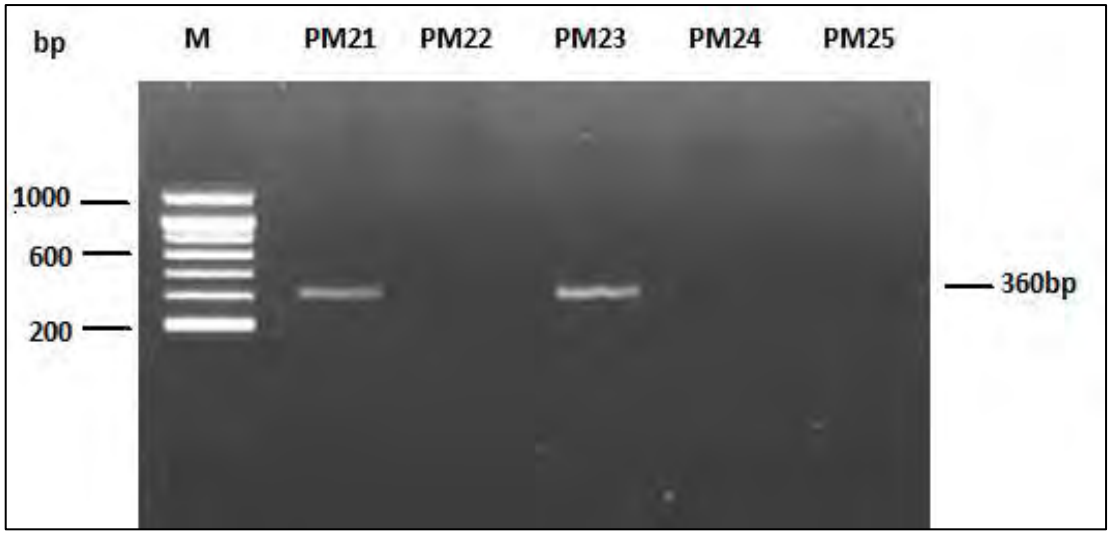


Figure 2.14. Image of an agarose gel showing amplification of the *nifH* gene in M: ladder PM21, PM22, PM23, PM24 and PM25

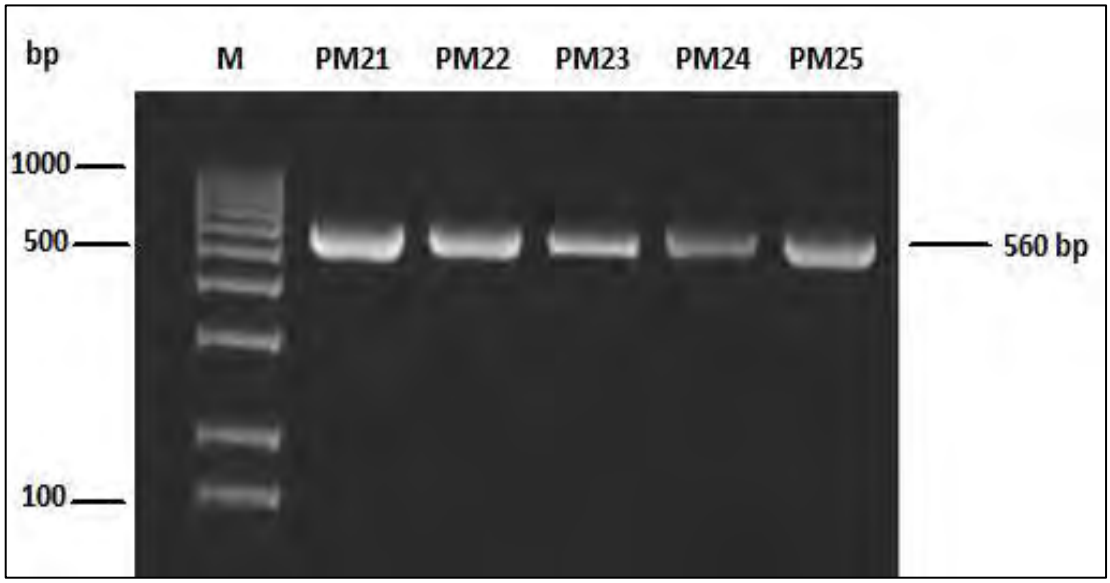


Figure 2.15. Gel image showing the amplification of *acds* gene in the selected bacterial strains

2.3.9. Morphological characterization

Bacterial isolates showed their characteristics through cell morphology, color, shape and Gram staining reaction, (Table 2.3).

Table 2.3. Morphological characterization PM21 strain

Bacterial traits	Results
Morphological features	Minor slimy, glossy colony, irregular edge, whitish,
PM21	Under the microscope, Gram-positive showed scattered arrangements of short rod-shaped cells

2.3.10. Molecular profiling

Molecular profiling of the selected five bacterial strains via16S rRNA were confirmed as *Bacillus anthracis* (PM21) *Bacillus safensis* (PM22), *Enterobacter cloacae* (PM23), *Bacillus sonorensis* (PM24), and *Bacillus thuringiensis* (PM25) respectively (Table 2.4).

Table 2.4. Molecular profiling of the five selected bacterial strains

Serial number	Isolates Code	Scientific name
1	PM21	*Bacillus anthracis*
2	PM22	*Bacillus Safensis*
3	PM23	*Enterobacter cloacae*
4	PM24	*Bacillus sonorensis*
5	PM25	*Bacillus thuringiensis*

2.4. Discussion

Plant growth is severely retarded in soil contaminated with heavy metals. It has been well documented that microorganisms residing in rhizosphere, support plant growth under abiotic stress condition (Patel etal., 2017; Ali et al., 2021). The screening, selection, and application of abiotic stress tolerant PGPR gained attention to enhance agricultural yield and to overcome detrimental impacts of HMs (Khani et al., 2010; Amna et al., 2020). The rhizospheric soil samples were collected from Larkana, Qambar Shahdadkot, Shaheed Benazirabad, Naushahro Feroze and Jacobabad Sindh-Pakistan (Ali et al., 2021). The use of heavy metal stress resistant PGPR will reduce the adverse effects of heavy metal on the food chain as well as human health. A total of 20 bacterial strains were isolated from the collected samples and characterized for essential plant growth-promoting traits in the current analysis.

Among the 20 isolated bacterial strains, five strains (PM21, PM22, PM23, PM24, and PM25) exhibited PGP traits i.e., phosphate and zinc solubilization, indole acetic acid, siderophore production, exopolysaccrides production ACC-deaminase enzyme ammonia, hydrogen cyanide, Cellulase, including several activities such as catalase, amylase, protease , and pectinase. Phosphate solubilization and IAA production are beneficial for growth (Akhtar et al., 2018; Gupta et al., 2018). Siderophore, and ACC-deamination production, and zinc solubilization also support plant growth (Kamran et al., 2017). Ethylene concentration is increased in plant tissues exposed to abiotic stresses (Ali et al., 2014). The ACC-deaminase enzyme produced by microbes reduce ethylene production by cleaving ACC to α-ketobutyrate and ammonia in plants under HMs (Glick et al., 2007). The strain *B. anthracis* PM21 showed high ACC-deaminase (1.75 µM/mg protein/h) activity, IAA (119 µM/mL) and EPS (2.98 mg/mL) production in metal stress, which confirms its ability to better adapt to metal stress conditions. Observation of ACC-deaminase activity in PM21 was also supported by previous reports (Zainab et al., 2020). Dixit et al. (2020) reported 16.19 nM/mg protein/h of IAA production in *B. safensis* under stress conditions; this value is far lesser than our current results (119 µM/mL). The current pattern of enhanced EPS production under stress agrees with the finding of Dixit et al. (2020) in *B. safensis*. The EPS enhances water retention and uptake around the roots by forming a biofilm; that helps to stabilize soil aggregates and regulates sources of nutrients and organic carbon. These traits directly

enhance growth of plants both normal and metal stress conditions (Costa et al., 2018). Isolation and characterization of metals tolerant bacterial strains have been well reported (Gupta et al., 2004; Ganesan, 2008; Wani and Ayoola, 2015; Karthik et al., 2016; Shi et al., 2020).

Genotypic characterization was carried out by 16S rRNA gene sequencing of promising bacterial strains, which is a standard tool used to classify bacterial organisms Clarridge, (2004). This is the first study that explore genus *Bacillus* and *Enterobacter* for heavy metals (Cd, Cr and Ni) tolerance and plant growth promotion in Pakistan. It is observed that the rhizobacteria affects growth by various direct mechanisms (Mushtaq et al., 2021). During current study, the five promising bacterial strains (PM21, PM22, PM23, PM24 and PM25) showed greater potential for tolerating heavy metals Cd, Cr and Ni. These strains could be suggested for further evaluation regarding biosorption of heavy metal, *in vitro* seed germination and greenhouse experiment.

2.5. Conclusion

Twenty bacterial isolates were characterized, maintained, and tested for heavy metal resistance and plant growth promotion. Several PGP traits were observed in the isolates, including the synthesis of IAA, ammonia, HCN, siderophores, protease, amylase, pectinase, cellulose, catalase, and phosphate and zinc solubilization. ACC-deaminase, IAA, and exopolysaccharide synthesis were analyzed qualitatively and quantitatively in these bacterial strains. Five out of twenty strains were selected, based on initial heavy metals tolerance. These isolated bacterial strains were distinguished through 16S rRNA gene sequencing likewise with their strongly related species i.e., *Bacillus anthracis* PM21, *Bacillus safensis* PM22, *Enterobacter cloacae* PM23, *Bacillus sonorensis* PM24 and *Bacillus thuringiensis* PM25. The study provides data of potential plant growth promoting bacterial isolates *Bacillus anthracis* PM21, *Bacillus safensis* PM22, *Enterobacter cloacae* PM23, *Bacillus sonorensis* PM24 and *Bacillus thuringiensis* PM25 for further evaluating the biosorption of heavy metals, *in-vitro* seed germination and greenhouse experiment.

Chapter 3

Screening and biosorption studies against three heavy metals cadmium, chromium, and nickel by isolated bacterial strains

3.1. Introduction

Industrialization imparts negative effects on the environment and human health by releasing toxic compounds into aquatic and terrestrial ecosystems. The released compounds might be dyes, oils, organic pollutants, heavy metals, pesticides etc. Most of these pollutants are persistent in nature. The industrial wastewater contains elevated levels of cadmium, chromium, nickel, and other metals which are considered to be the most dangerous chemicals (Egbosiuba et al., 2021). Plants and animals need some metals as a part of their nutrition, but the exceeded concentrations of metals are thought to have a deleterious effect on life of plant and animals (Ofomaja et al., 2010; Ekere et al., 2016). The phrase "heavy metal" refers to a group of metals and metalloids with an atomic density larger than 5 g/cm^3, or five times that of water (Islam et al., 2019).

Toxicity of HMs have been reported to human health and environment when they exceed their limit. Some of the heavy metals are regarded as carcinogenic and mutagenic (Diels, 2002; Farouk, 2011; Ali et al., 2019). Harmful nature of heavy metals is due to long lasting nature, transformation from less toxic to more toxic forms with passage of time, and disruption in cell physiology. Heavy metals are toxic even at very less level such as Cd 0.001-0.1 mg L^{-1} is toxic concentration (Alkorta et al., 2004; Wang et al., 2002). The Cr results in distortion of DNA and proteins, Cd distorts proteins which produces thiol and methyl groups derivatives (Jaishankar et al., 2014). The metal contaminated food and water causes the accumulation of HMs in the animal's manure (Chen et al., 2020). The Cr (III) is released into the environment by industrial discharge, and it is more toxic than Cr (VI). Increased amount of Cr in plants causes stunted growth and abnormality of roots while in animals it results in cancer and skin allergy (Volesky, 2001; Chaudhary et al., 2016). Cadmium (Cd) is hazardous because of its stable nature; thus, it accumulates in food chain and causes serious illnesses even in low concentrations (Alkorta et al., 2004).

Nickle is toxic and causes different diseases such as bronchitis, lungs cancer, and dermatitis in animals. While in plants, it alters their metabolic activities such as inhibition of enzymatic activities, electron transport and biosynthesis of chlorophyll (Sreekanth et al., 2013). Therefore, heavy metals must be remediated from soil to keep

human health safe from their negative effects and to sustain environment for the next generations (Glick, 2010).

In the past 10 years, different economical and efficient bio-sorbents (bacteria) for metal ions absorption have been applied (Shamsipur et al., 2013; Rajabi et al., 2015). Common water treatment approaches include precipitation, evaporation, electrochemical process, reverse osmosis, membrane filtration, ion exchange and solvent extraction (Kurniawan et al., 2006; Azimi et al., 2017). Due to the processing of secondary metabolites, high expense, solvent depletion, complex activity, higher energy requirements, and the inability to fully extract metals, these methods are somewhat disadvantageous (Lin et al., 2005; Joshi, 2018). Biosorption is regarded as a viable and alternative method of removing heavy metals from aqueous solutions (Yahaya et al., 2009). It is the method of biological materials separating metals/metalloids species, compounds, and other particulates from solution (Escudero et al., 2019). Instead of traditional processes, the biosorption approach is innovative, and it has the ability to be used in a variety of industries (Beni and Esmaeili, 2020).

Biosorbents can be of several types and are developed from different raw biomasses like bacteria (*Bacillus thuringiensis*), fungi *Botrytis cinerea* and algae *Anabaena sphaerica* (Vijayaraghavan and Yun, 2008; Akar and Tunali, 2005; Abdel-Aty et al., 2013). In the biosorption process, both dead and live biomass can be utilized, and it is independent, reversible process (Tabak et al., 2005; Yu et al., 2020).

Rhizospheric soil is inhabited by several bacterial species which promote plant growth, increase the availability of nutrients, and help in resistance and defense against soil borne pathogens. Such bacteria are classified as PGPR (Mhatre et al., 2019). In a field situation, the plant is not an individual, but a complex culture exists there (Lundberg et al., 2012). A community of microorganisms, is well-structured, regulated, and interrelated with the plants (Bulgarelli et al., 2015). The microbes utilize the root exudates as nutrients for their growth (Ismail et al., 2020). In bioremediation, various treatment methods can be carried out, such as biofiltration, bioventing, bioaugmentation and bio-stimulation. Ex-situ conservation, such as bioreactor compositing, land forming (da Conceição Gomes et al., 2016). Plants and beneficial microbes eliminate toxic HMs from polluted soil through phytoextraction,

phytostabilization and transformation (Lebeau et al., 2008; Glick, 2010). Beside this, plants also have ability of accumulating heavy metals, for example *Eleocharis acicularis* stock up pb, Cu, As and Zn in their parenchyma (Ha et al., 2011). Therefore, there is an urgent need to prepare some novel low-cost bacterial adsorbents with strong capability to remove toxic metals from aqueous solution.

3.2. Materials and Methods

The bacterial isolates from the rhizosphere were screened for heavy metal tolerance and later the selected isolates were evaluated in biosorption studies.

3.2.1. Screening of bacteria for heavy metals tolerance potential

At a pH of 7.4 ± 0.2, all isolates were cultured in nutritional broth medium containing 10 gL^{-1} peptone, 3 gL^{-1} beef extract, and 5 gL^{-1} sodium chloride. Heavy metal stock solutions up to 1000 mgL^{-1} were made by liquifying Cr ($K_2Cr_2O_7$), Cd ($CdCl_2$), and Ni ($NiCl_2$) salts in double distilled water separately. Bacterial growth curves were generated in nutrient broth media in the existence of different concentration of Cr (100, 200, 300 mgL^{-1}), Cd (100, 200, 300, 400, 500, 600, 700 and 800 mgL^{-1}) and Ni (100, 200, 300, 400 and 500 mg/L) salts. For this purpose, a starter culture of 2 mL of exponentially growing bacteria (OD_{600}=1.0) was suspended into 100 mL nutrient broth containing various levels of heavy metals stress (Cr, Cd and Ni) in 250 mL conical flasks and incubated for 40 h at 32 ± 2°C in an orbital shaker at 150 rpm). A spectrophotometer (752N UV-VIS, Beijing, China) was used to monitor bacterial growth by recording light absorbance at 600 nm wavelength (Huang et al., 2014).

3.2.2. Biosorption experiments

3.2.2.1. Preparation of the bacterial biomass

The bacterial strains were cultured on Luria–Bertani (LB) agar medium. These strains were inoculated in LB broth (100 mL) in a conical flask 250 mL on 150 rpm shaking for biomass production at 35°C ± 2°C. after that centrifugation of culture was performed at 4,000 rpm for 20 min at 4°C to harvest cell. The collected pellet was used directly for further experiments (Gillania et al., 2017).

3.2.2.2. Heavy metals stock solution

The 1000 mgL^{-1} stock solution of various heavy metals was obtained by dissolving cadmium, chromium, and nickel salts in double distillation (DDW). The dilution of stock solutions was used to prepare different concentrations of heavy metals for the experiment.

3.2.3. Adsorption studies

The batch experiment for adsorption studies of heavy metal ions was carried out to study the elements affecting the capacity of metal uptake capability of bacteria . The factors like influence of pH, contact time and metal concentration were optimized Erlenmeyer flask of 250 mL. The whole experiment was carried out in triplicate.

Following equation was used to study the heavy metals sorption efficiency of the bacterial strains.

$$q_e = \frac{\mathbf{Co - Ce}}{\mathbf{M}} \times \mathbf{V} \quad (\mathbf{3.1})$$

where *q* gives the mass in mg that is adsorbed at the applied biosorbent (g), Co is the concentration of metal at initial stage in mg L^{-1}, C_e represents the final concentration of metal in mg L^{-1}, V is the volume of heavy metal solution taken in liters, and M shows the biosorbent mass in grams (Vishan et al., 2019).

3.2.3.1. A study of the effect of pH on adsorption

Adsorption was investigated at pH levels ranging from 2 to 10. 0.1 N HNO_3 and 0.1N NaOH reagents were used to keep the pH of the solution constant. After 1 hour of shaking, the culture was centrifuged at 4000 rpm for 10 minutes. Using an Atomic Absorption Spectrophotometer, the metal content in the supernatant was determined (AAS-240FS Varian) (Gillania et al., 2017).

3.2.3.2. Study of the effect of varying contact times on adsorption

Contact time of HMs with bacteria was studied by taking bacterial culture at different time intervals (0, 20,40,60,80,100 and 120 min), at optimal pH and at fixed concentration (50 mg/L) of each metal ion using batch experiment in a conical flask 0f 250 mL volume.

3.2.3.3. Optimization of initial metal concentration on adsorption study

At constant pH and contact time, the effect of initial metal ion concentrations on bacteria's metal biosorption capacity was investigated. Five different initial metal

concentrations (25, 50, 100, 150, and 200 mg/L) were utilized for HMs. All trials were carried out at a constant temperature of 35 °C ± 2 °C (Gillania et al., 2017).

3.2.4. Adsorption Isotherm modeling

In batch experiments, adsorption study of metal ions was conducted to explore the effect of different factors influencing adsorption and metal uptake capacity. In Erlenmeyer flasks mounted in the shaking incubator at 150 rpm, batch experiments were performed at optimum conditions. All tests were carried out in triplicate.

3.2.5. Characterization

3.2.5.1. Fourier transform infrared spectrophotometric (FTIR) analysis

Using a Fourier transform infrared spectrophotometer, the functional groups involved in the adsorption of cadmium, chromium, and nickel ions on the bacterial cell surface were discovered (FTIR). Initially, bacterial cultures were inoculated in LB broth with and without the addition of 200 mg/L Cd, Cr, and Ni. Bacterial cultures were centrifuged for 10 minutes at 8000 rpm at 4 °C after 24 hours of incubation. The bacterial cells were palleted and purified after the supernatant was discarded. Bacterial cells were washed three times with sodium chloride solution (0.85%), then twice with purified water H_2O until being dried at 50 °C (Kamnev et al., 1997). Furthermore, using a manual hydraulic press at 100 kg cm^{-2} pressure, 1 mg of crushed, dry bacterial cells and 400 mg of potassium bromide were fully combined, ground into fine powder, and put to transparent sample discs (10 min). These discs were then placed on the FTIR Spectrophotometer (Nicolet TM, Thermo Science, USA). The FTIR spectrum was noted at 400-4000 cm^{-1} to observe unique functional groups (François et al., 2012).

3.2.5.2. Scanning electron microscopic (SEM) analysis

The effect of Cd, Cr, and Ni sorption on bacterial cell morphology was investigated using SEM. Bacterium was grown in LB broth at 120 rpm for 24 hours at 30 °C in the presence (200 mg/L) and absence (Cd, Cr, Ni).The LB broth which does not contain Cd, Cr, and Ni was considered as control treatment. Centrifugation 8000 rpm of bacterial cells was performed at 4°C for 10 min at 24 h of incubation. The pelleted bacterial cells were washed three times in phosphate buffer saline (PBS). The washed

bacterial cells were pre-fixed for 4-6 hours at 4°C with glutaraldehyde (2.5%) (Bharagava and Mishra 2018). The PBS (pH 7.2) was again used to wash two times the pre-fixed bacterial cells. For post-fixation, osmium tetraoxide (1%) was used for 1 hour, followed by PBS washing and dehydration with acetone (v/v); 20%, 40%, 60%, 80%, and 100%. Before being studied with a scanning electron microscope (SEM), bacterial cells were dried using a critical point drier (CPD) and a platinum-coated ion sputter coater (JEOL, Japan, JFC 1600 Auto Fine Coater) (JEOL JSM-6490LV).

3.2.6. Amplification of heavy metal resistance (CzcD) gene

For bacterial strains PM21, PM22, PM23, PM24, and PM25, heavy metal encoding genes were tested against cadmium and chromium (CzcD). The CzcD primer set has the following sequence: (F- CAGGTCACTGACACGACCAT), (R-CATGCTGATGAGATTGATGATC) having 398 base pair amplicons (Ayangbenro et al., 2019). Initial denaturation was performed at 95 °C for 5 minutes, followed by 34 cycles of 94 °C for 90 seconds, 52 °C for 90 seconds, 72 °C for 2 minutes, and a final extension step of 72 °C for 7 minutes (Nies et al., 1989).

3.2.7. Statistical analysis

The data was examined with SPSS tools and analysis of variance (ANOVA) at 0.05 significance level (IBM SPSS Statistics 21).

3.3. Results

3.3.1. Heavy metals tolerance assay

All bacterial isolates were screened against heavy metals Cadmium, Chromium and Nickel. Among twenty bacterial isolates five strains previously resulted best PGPR were tolerant towards 800 mgL^{-1} Cd, 300 mgL^{-1} Cr and 500 mgL^{-1} Ni Table 3.1.

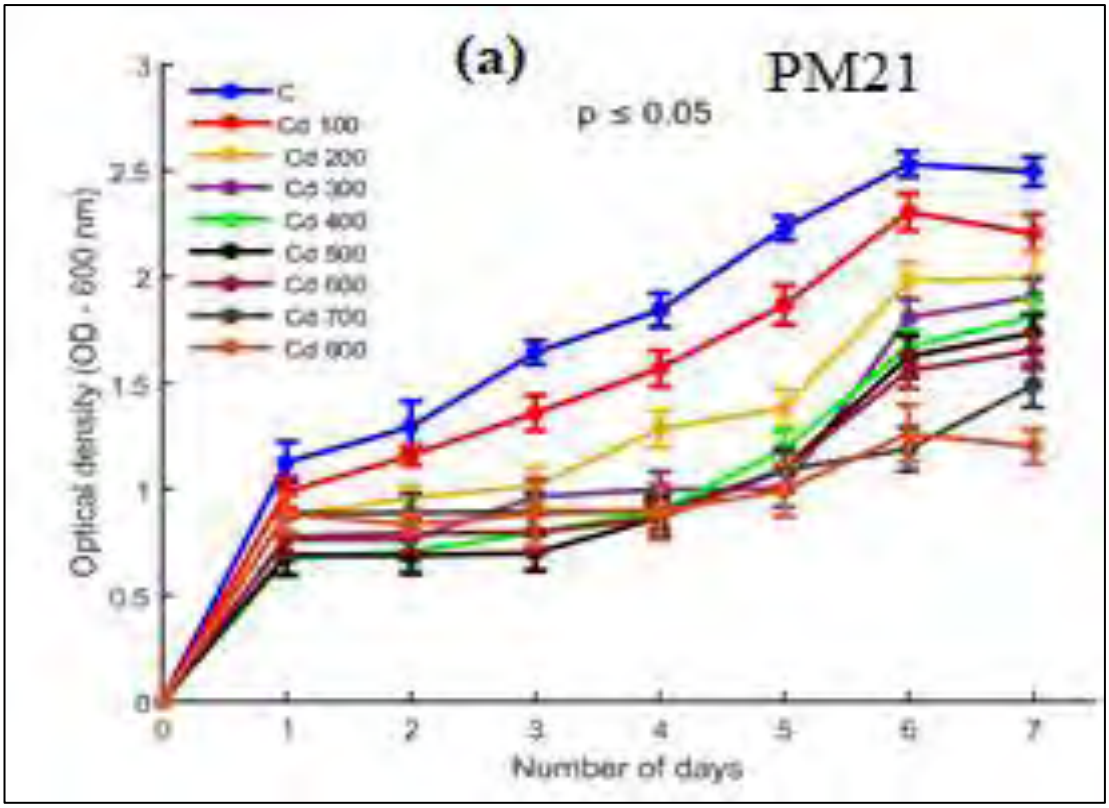
3.3.2. Growth curve analysis of selected bacterial strains in heavy metals stress

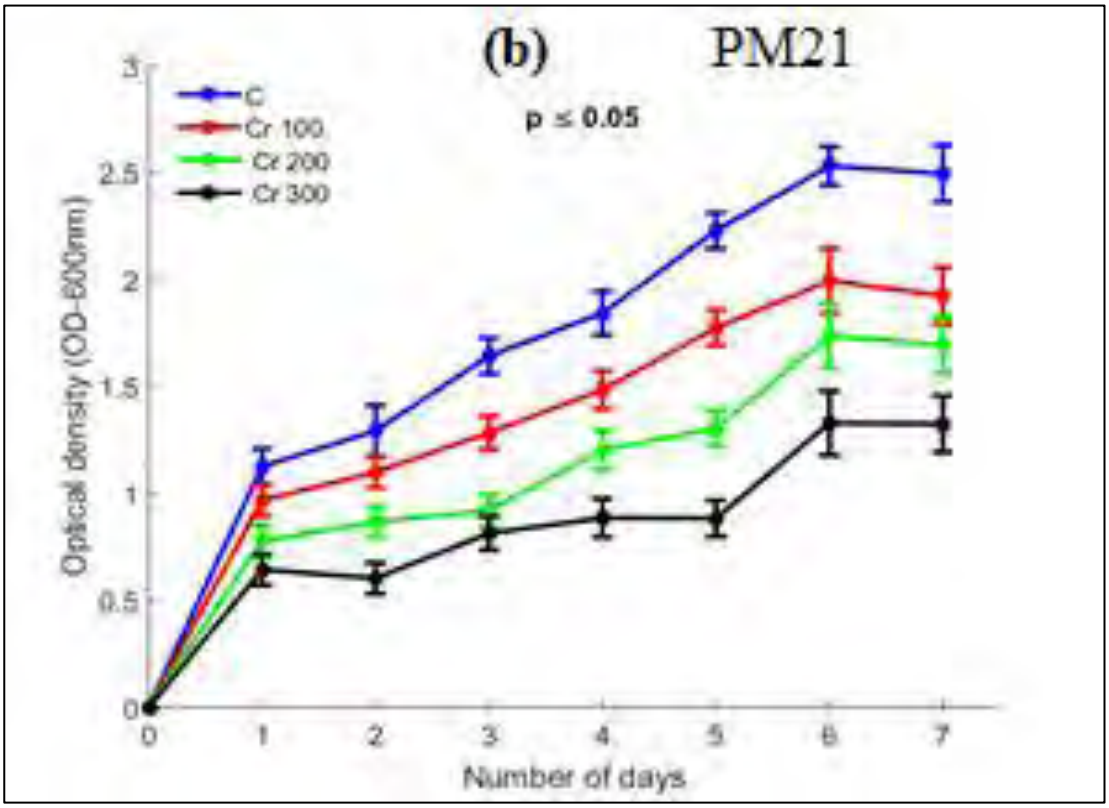
The isolated bacterial strains were tested for their growth under different levels of Cd, Cr and Ni over a period of 7 days (Table 3.1 and Figure 3.1). Bacterial strains (PM21, PM22, PM23, PM24 and PM25) proved to be tolerant for Cd, Cr and Ni although its growth remained negatively proportional to heavy metals concentration. Maximum growth, of the best performing strains, at sixth day of incubation was observed in the following order PM21> PM22 > PM23> PM25> PM24.

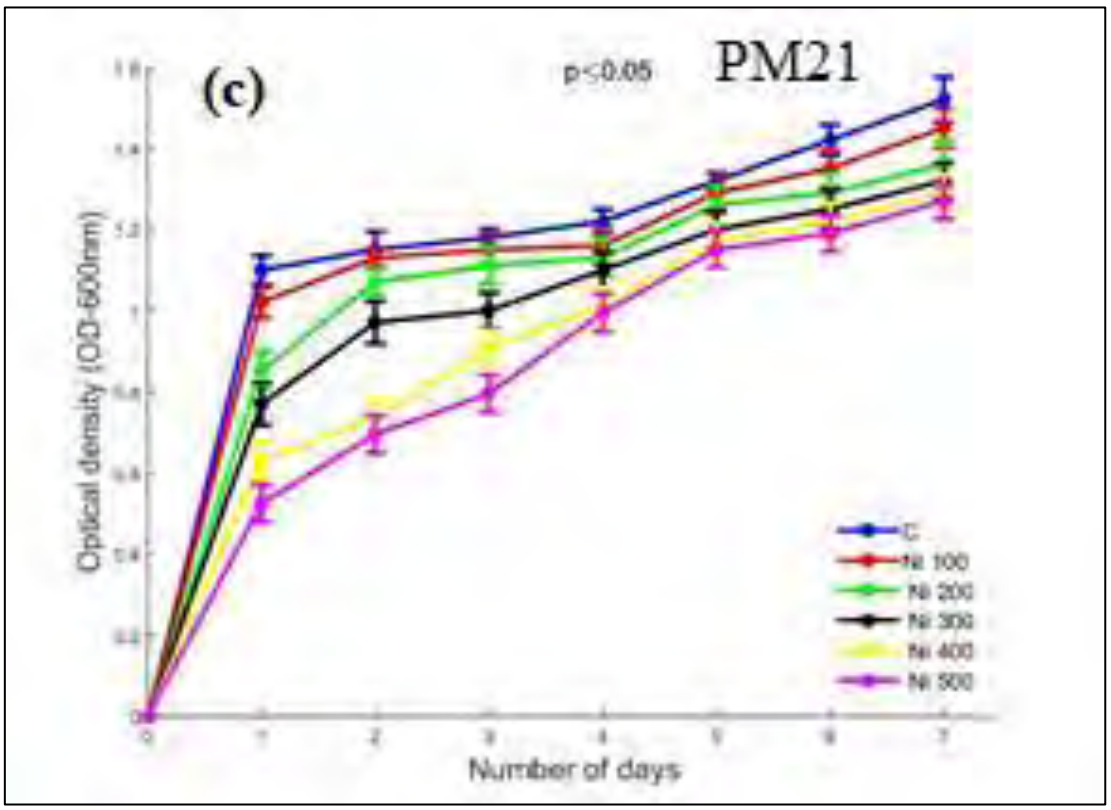
Table 3.1. Initial screening of heavy metals tolerant bacterial strains

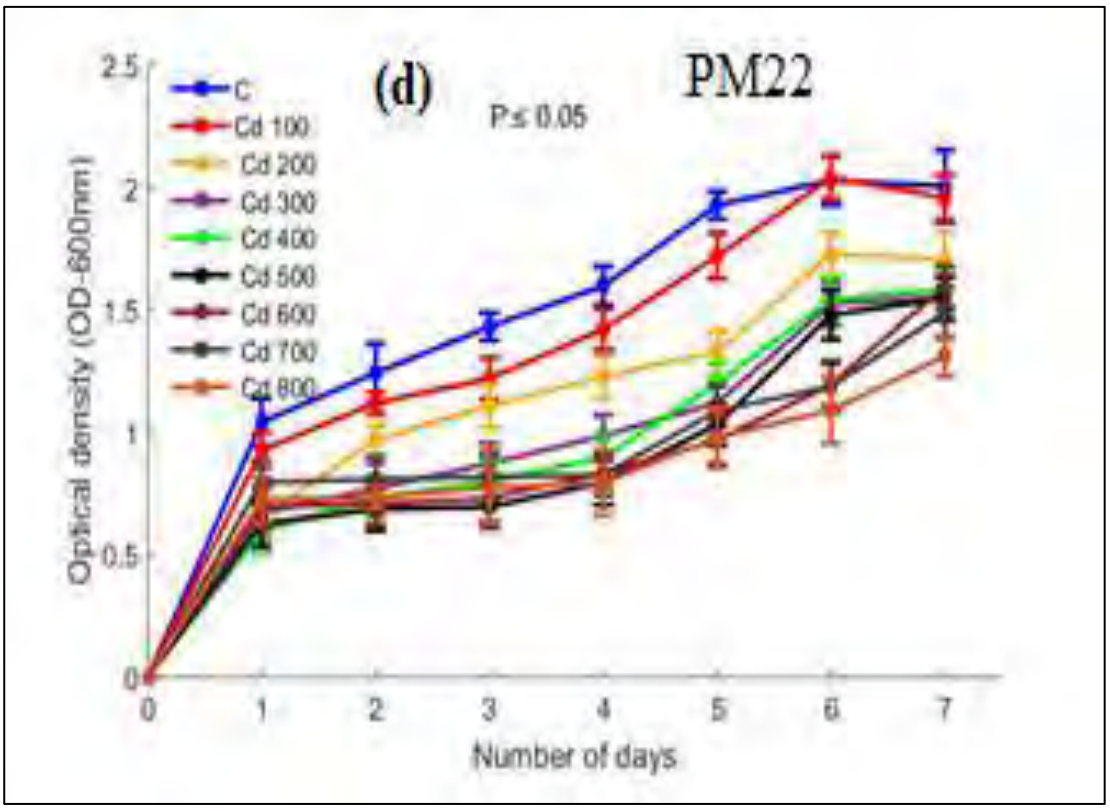
Strains	PM21	PM22	PM23	PM24	PM25	J02	J03	J04	J05	J07	J08	J09	J10	J11	J13	J14	J15	J17	J18	J19
Cd mg/L																				
Cd 100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cd 200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cd 300	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cd 400	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cd 500	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cd 600	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cd 700	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cd 800	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cr mg/L																				
Cr 100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cr 200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cr 300	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cr 400	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ni mg/L																				
Ni 100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ni 200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ni 300	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ni 400	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ni 500	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ni 600	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

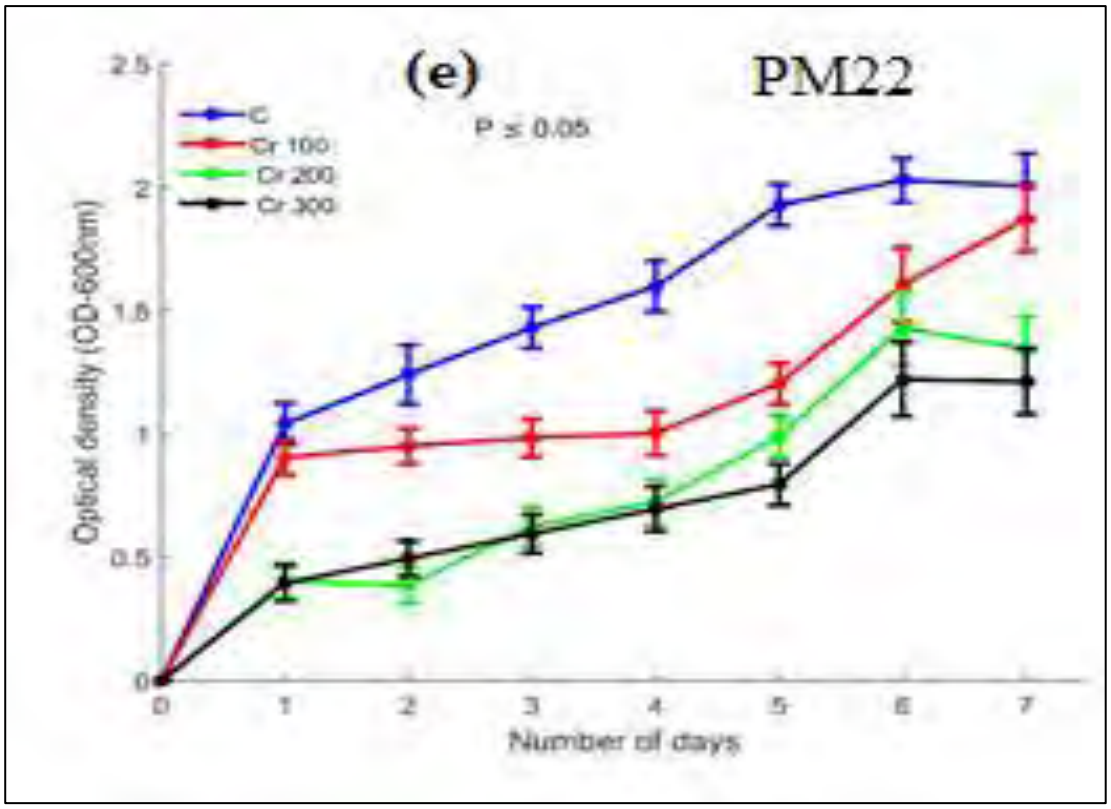
(+) present (-) absent

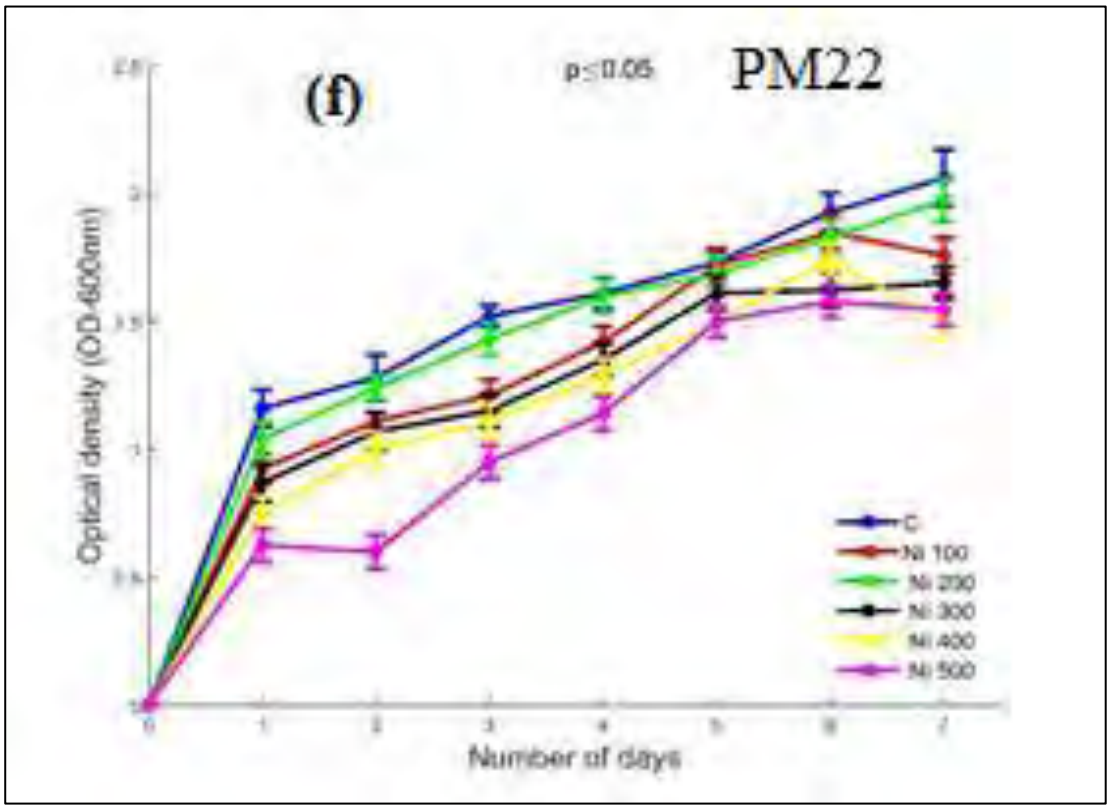

(a)
PM21
p ≤ 0.05
C
Cd 100
Cd 200
Cd 300
Cd 400
Cd 500
Cd 600
Cd 700
Cd 800
Optical density (OD - 600 nm)
Number of days

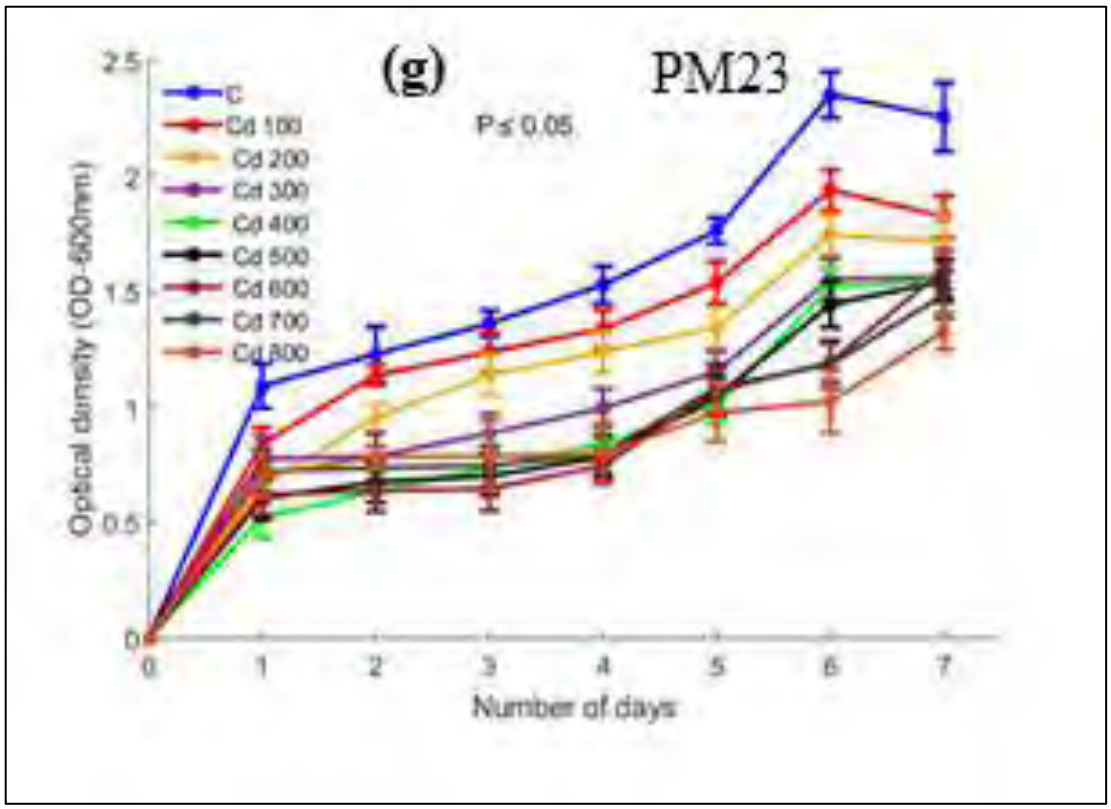

(b)
PM21
p ≤ 0.05
C
Cr 100
Cr 200
Cr 300
Optical density (OD-600nm)
Number of days

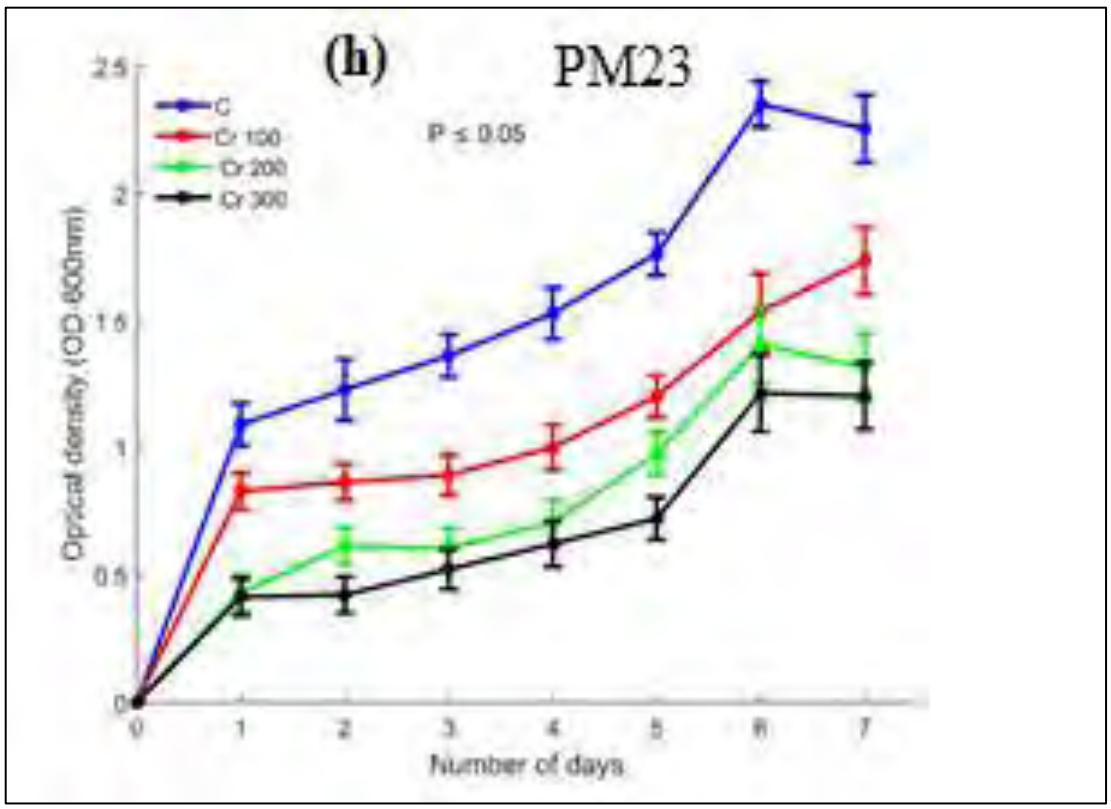

(c)
p<0.05
PM21
Optical density (OD-600nm)
Number of days
C
Ni 100
Ni 200
Ni 300
Ni 400
Ni 500

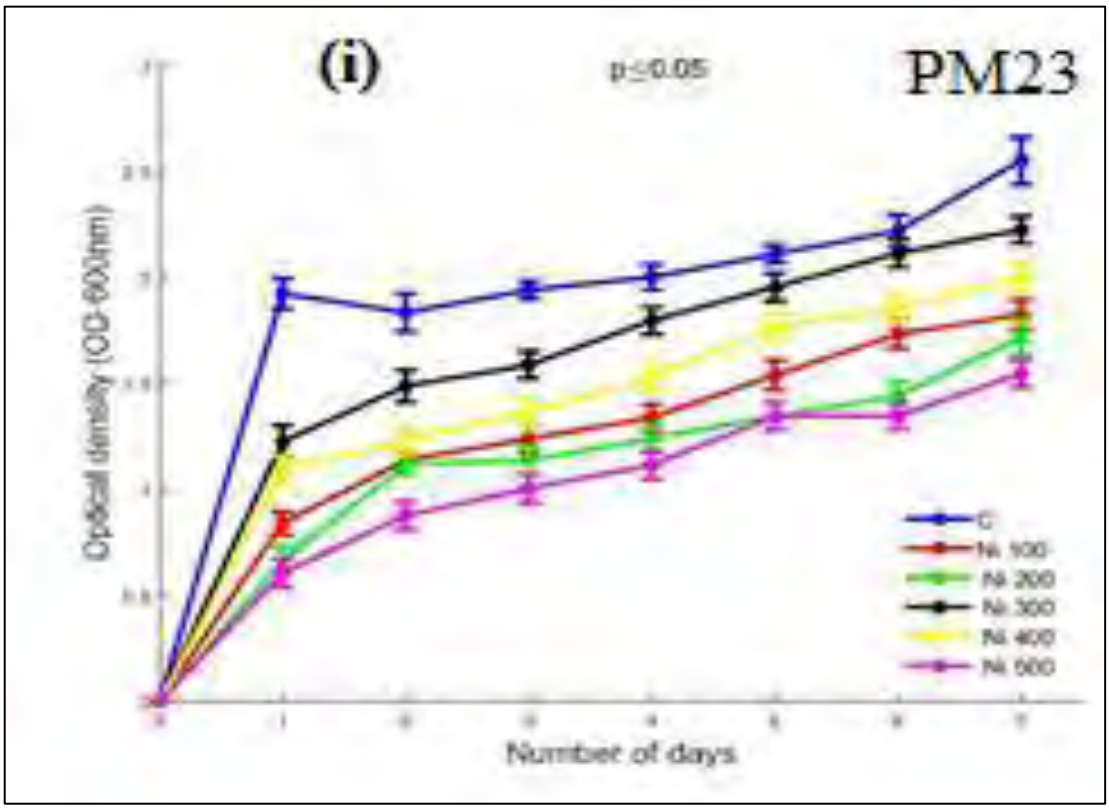

(d)
P≤ 0.05
PM22
Optical density (OD-600nm)
Number of days
C
Cd 100
Cd 200
Cd 300
Cd 400
Cd 500
Cd 600
Cd 700
Cd 800

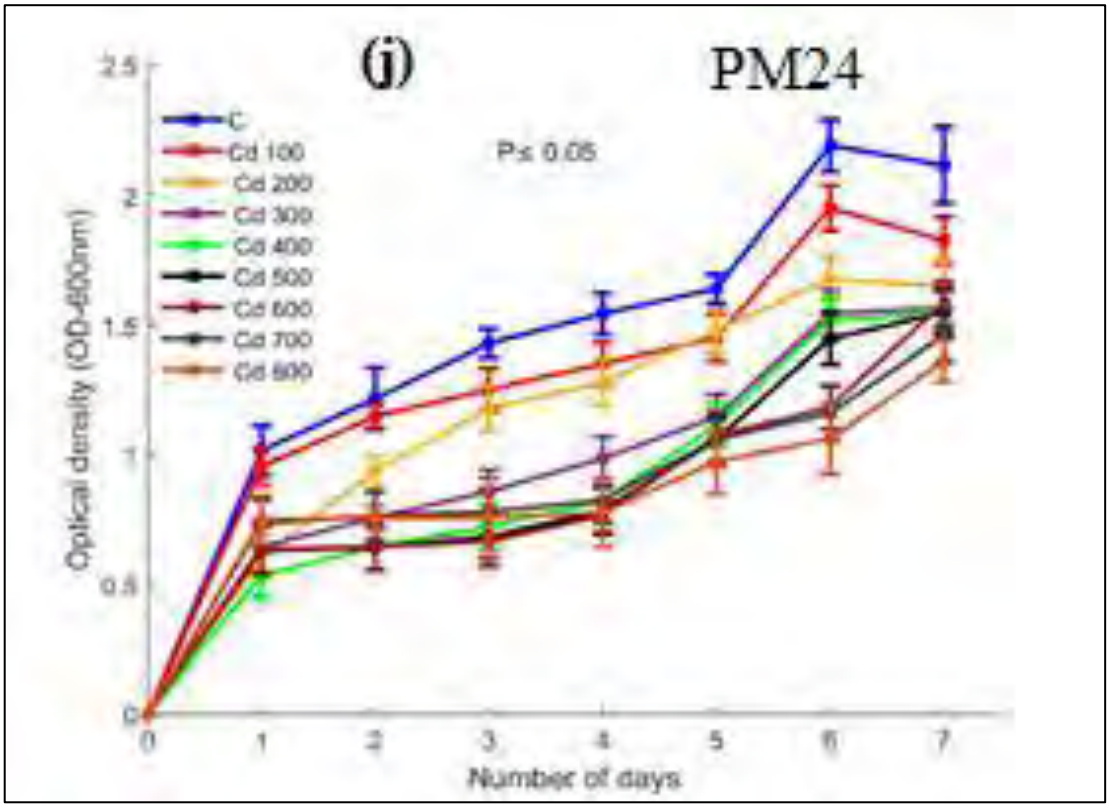

(e)
PM22
P ≤ 0.05
C
Cr 100
Cr 200
Cr 300
Optical density (OD-600nm)
Number of days

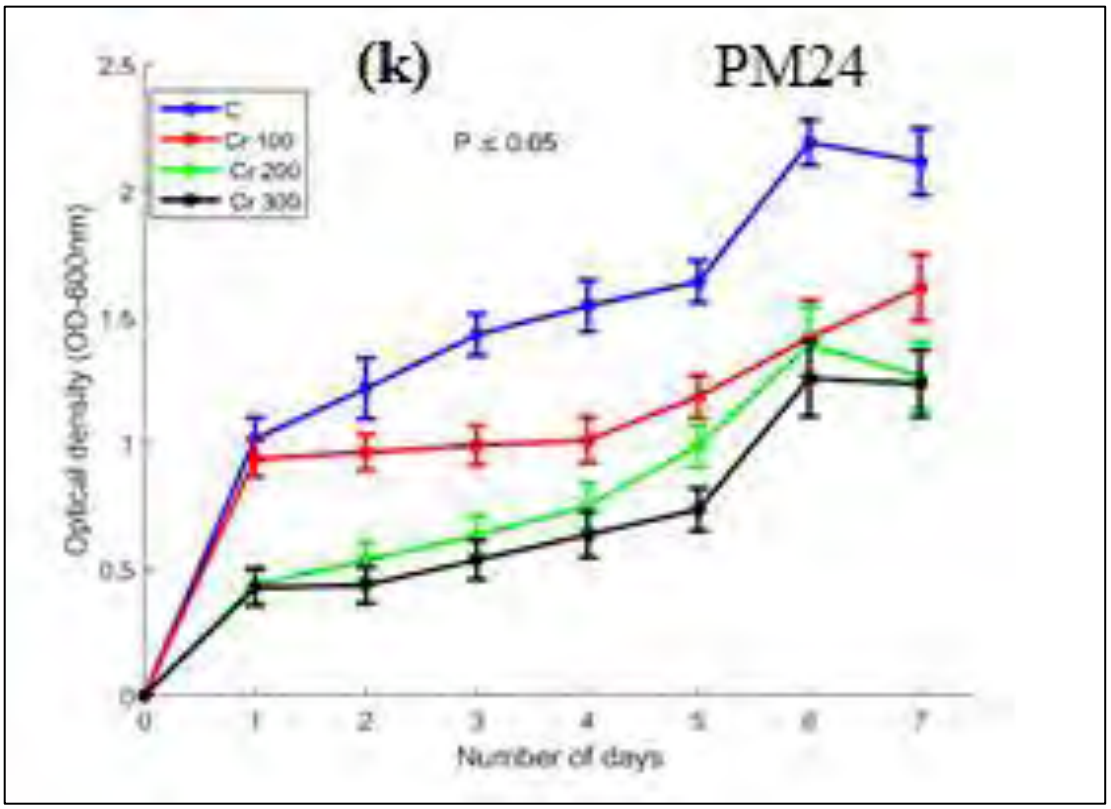

(f)
p≤0.05
PM22
C
Ni 100
Ni 200
Ni 300
Ni 400
Ni 500
Optical density (OD-600nm)
Number of days

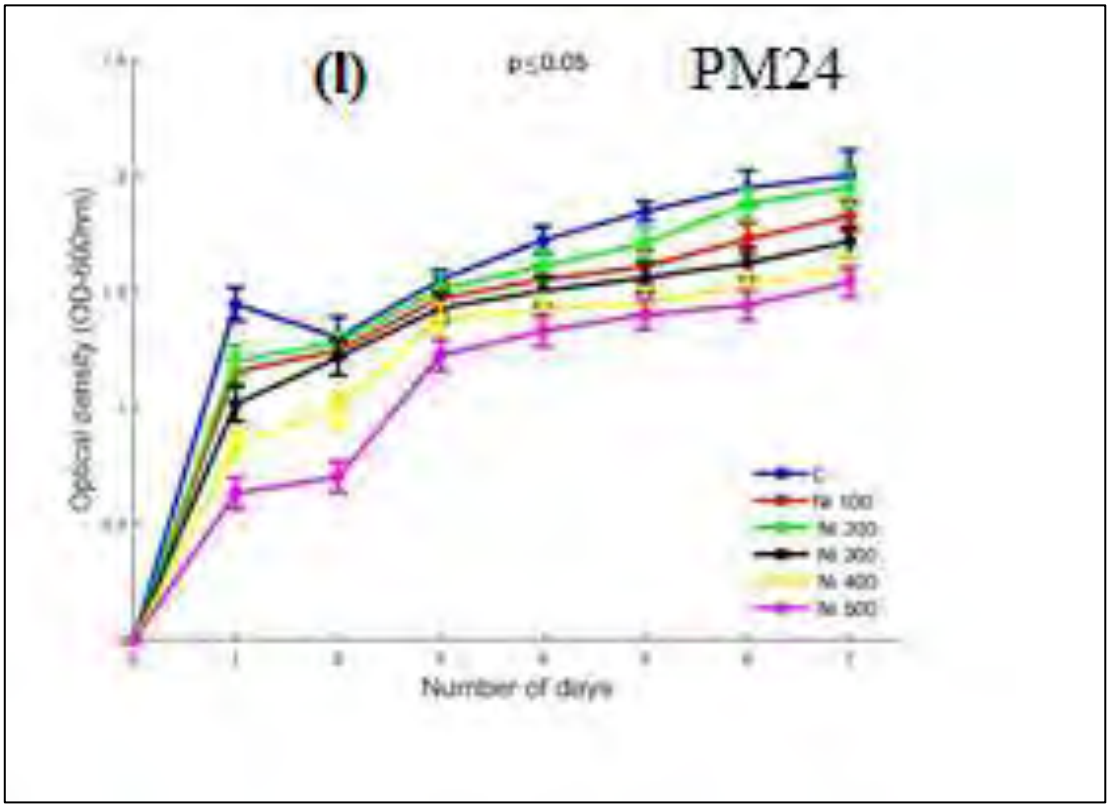

(g)
PM23
P≤ 0.05
C
Cd 100
Cd 200
Cd 300
Cd 400
Cd 500
Cd 600
Cd 700
Cd 800
Optical density (OD-600nm)
Number of days

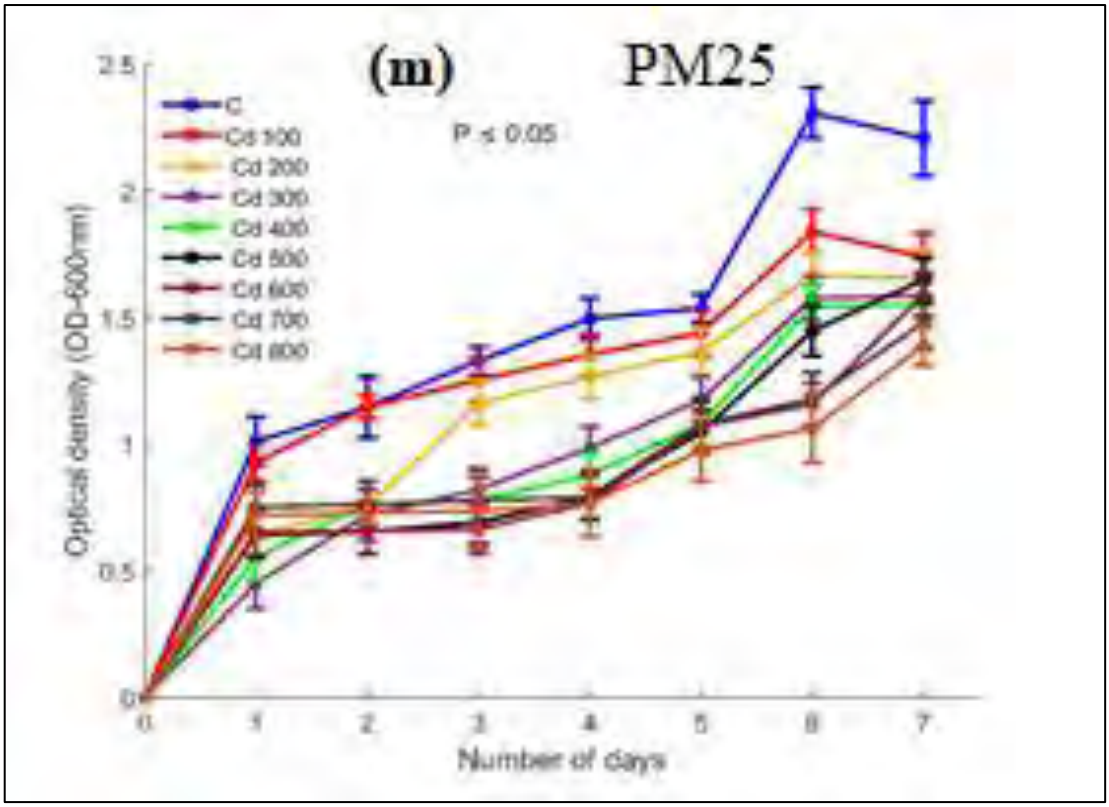

(h)
PM23
P ≤ 0.05
C
Cr 100
Cr 200
Cr 300
Optical density (OD-600nm)
Number of days

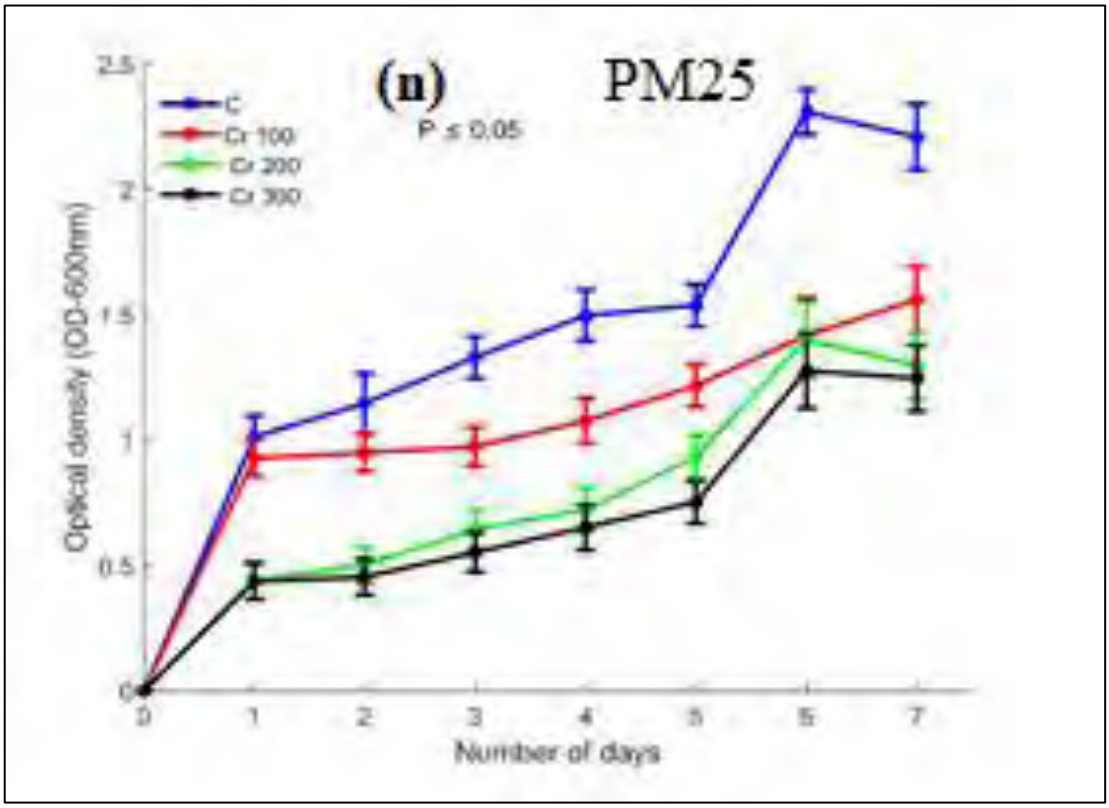

(i)
p≤0.05
PM23
Optical density (OD-600nm)
Number of days
C
Ni 100
Ni 200
Ni 300
Ni 400
Ni 500


(j)
PM24
P≤ 0.05
Optical density (OD-600nm)
Number of days
C
Cd 100
Cd 200
Cd 300
Cd 400
Cd 500
Cd 600
Cd 700
Cd 800


(k)
PM24
P ≤ 0.05
C
Cr 100
Cr 200
Cr 300
Optical density (OD-600nm)
Number of days


(l)
p≤0.05
PM24
Optical density (OD-600nm)
C
Ni 100
Ni 200
Ni 300
Ni 400
Ni 500
Number of days


(m)
PM25
P ≤ 0.05
C
Cd 100
Cd 200
Cd 300
Cd 400
Cd 500
Cd 600
Cd 700
Cd 800
Optical density (OD-600nm)
Number of days


(n)
PM25
P ≤ 0.05
C
Cr 100
Cr 200
Cr 300
Optical density (OD-600nm)
Number of days

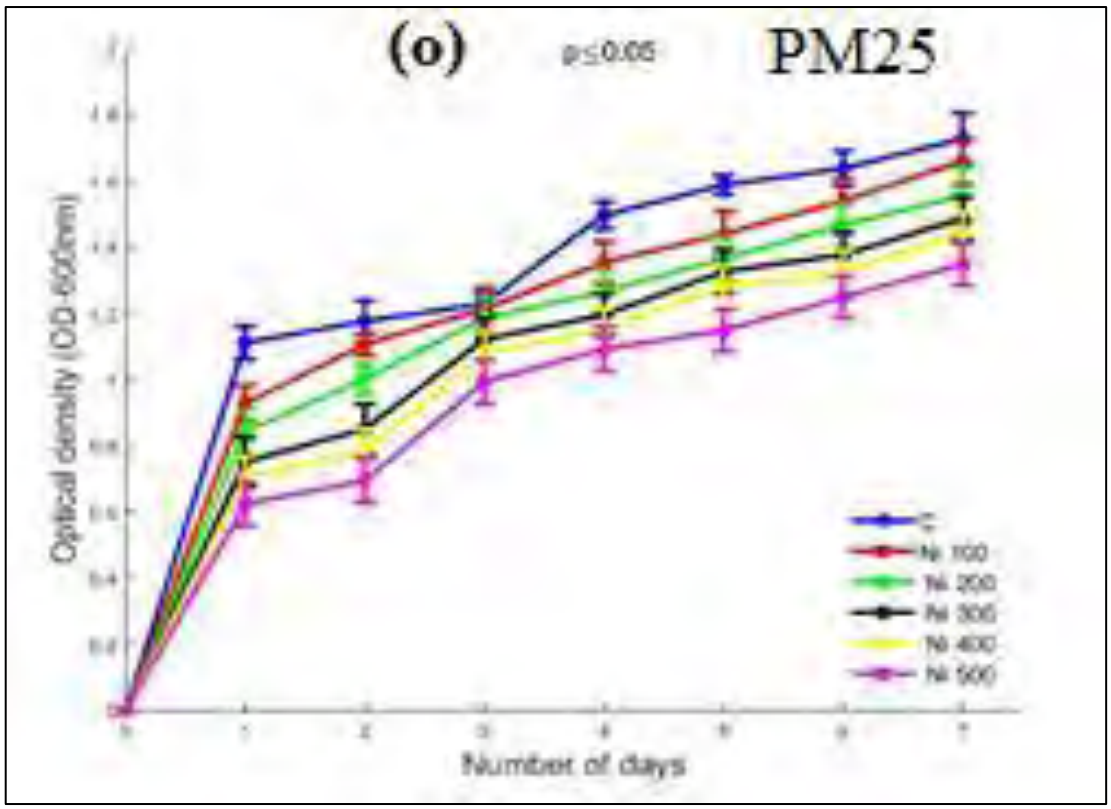
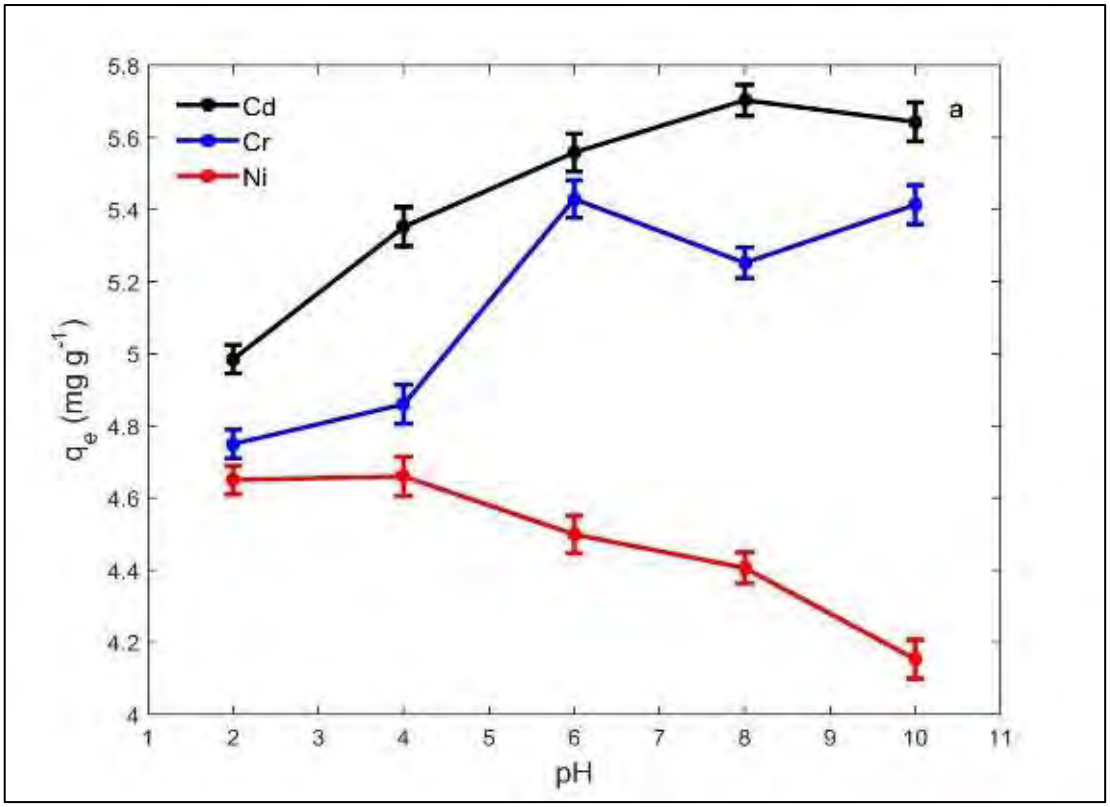
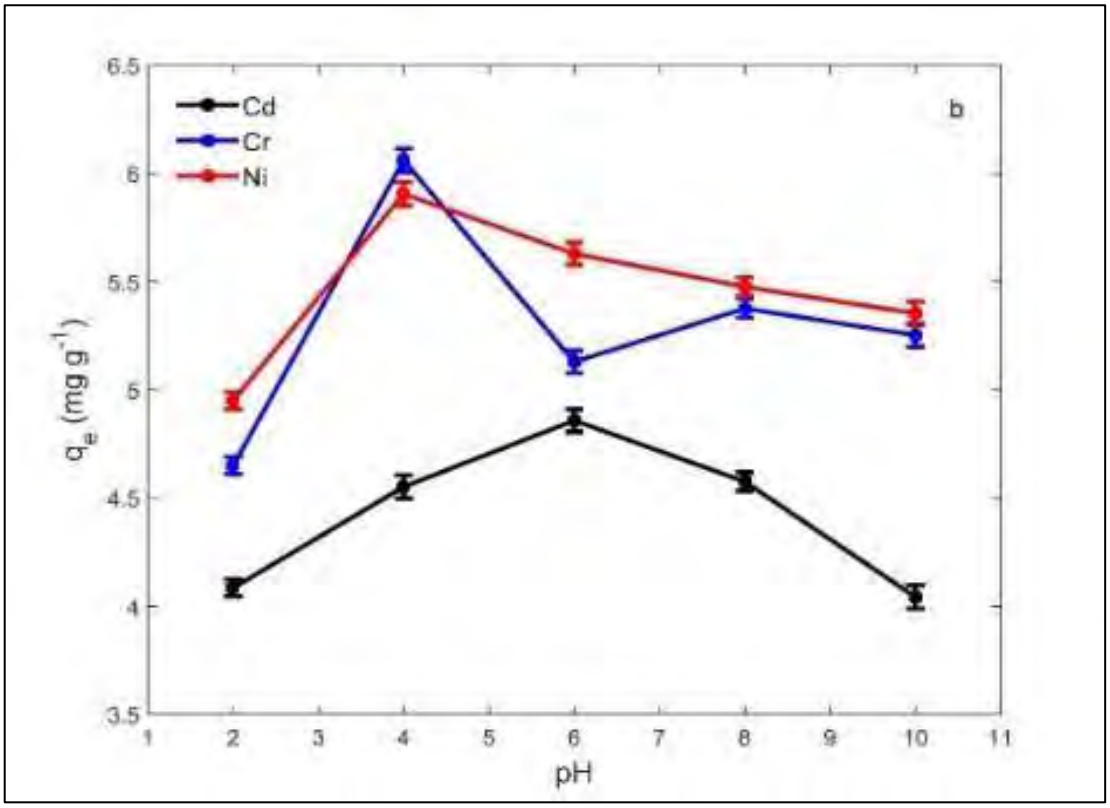


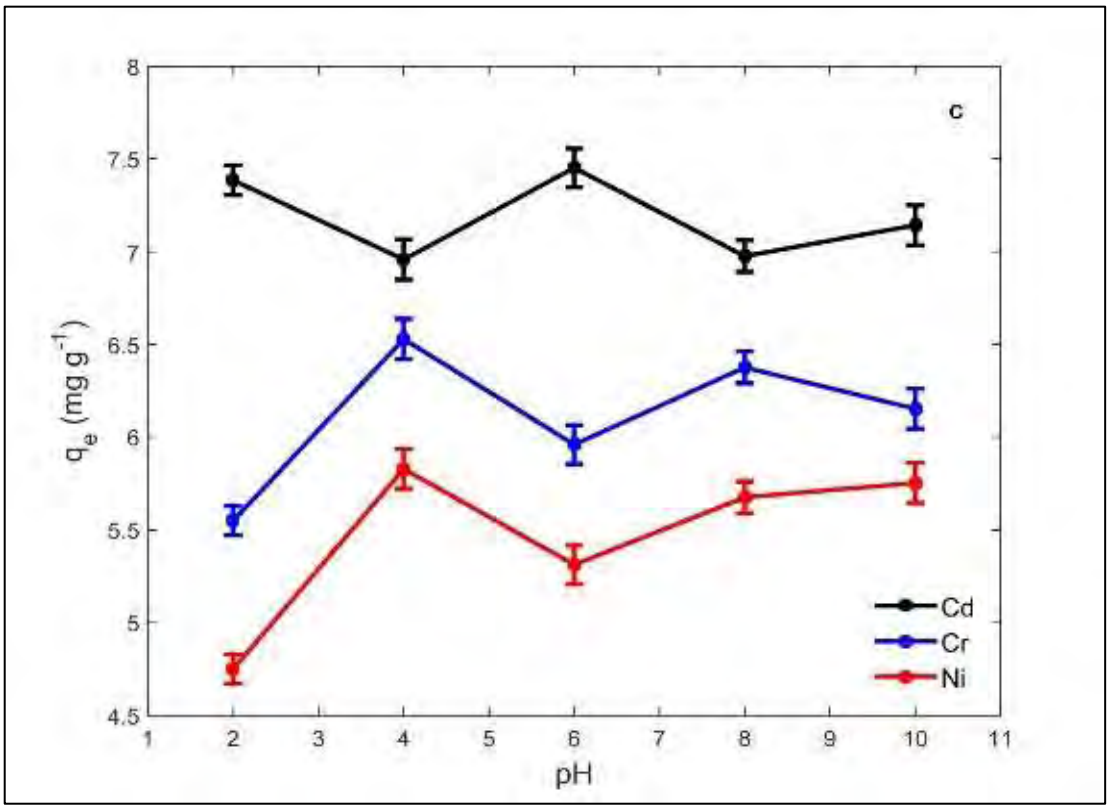
Figure 3.1. Growth curve analysis of bacterial strains PM21 (a, b, and c), PM22 (d, e, and f), PM23 (g, h and i), PM24 (j, k, and l) and PM25 (m, n and 0) in control (C: inoculation without stress) and different concentrations of Cd (100-800 mg L^{-1}), Cr (100-300 mg L^{-1}) and Ni (100-500 mg L^{-1}) stress. Error bars presented as SE (n=3)

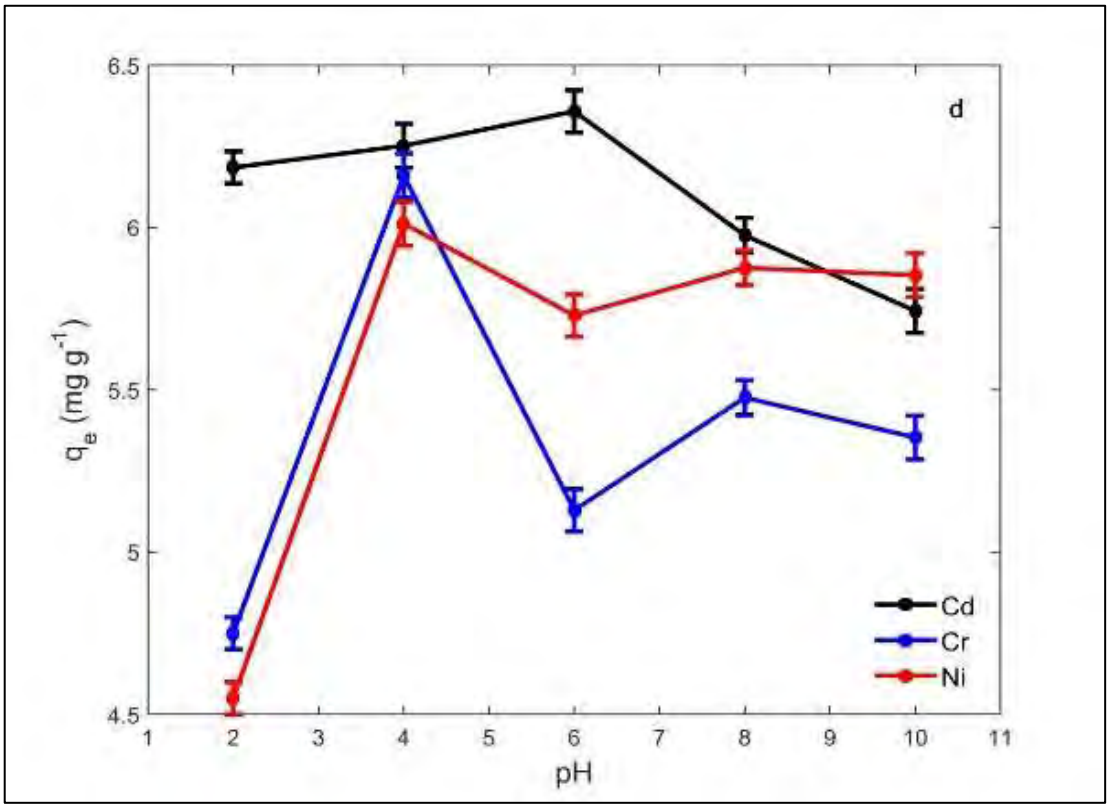
3.3.3. Influence of varying pH on adsorption study

The influence of pH on Cd, Cr and Ni ions adsorption by PM21, PM22, PM23, PM24 and PM25 was evaluated at pH level of 2 to 10 with interval of two with bacterial density of 0.5 g/L, and initial concentration (50 mg/L) of metals. Maximum adsorption potential for bacterial strain *Bacillus anthracis* PM21 was obtained at pH value 8 for Cd, pH-6 for Cr, and pH-4 for Ni. In similar way, pH value for biosorption capacity of Cd, Cr and Ni by PM22, PM23, PM24, and PM25 was determined as 6 for Cd, 4 for Cr, and 4 for Ni (Figure 3.2).


Cd
Cr
Ni
a
q_e (mg g^{-1})
pH
5.8
5.6
5.4
5.2
5
4.8
4.6
4.4
4.2
4
1
2
3
4
5
6
7
8
9
10
11


Cd
Cr
Ni
b
q_e (mg g^{-1})
pH
6.5
6
5.5
5
4.5
4
3.5
1
2
3
4
5
6
7
8
9
10
11


c
8
7.5
7
6.5
6
5.5
5
4.5
1
2
3
4
5
6
7
8
9
10
11
pH
q_e (mg g^{-1})
Cd
Cr
Ni


d
6.5
6
5.5
5
4.5
1
2
3
4
5
6
7
8
9
10
11
pH
q_e (mg g^{-1})
Cd
Cr
Ni

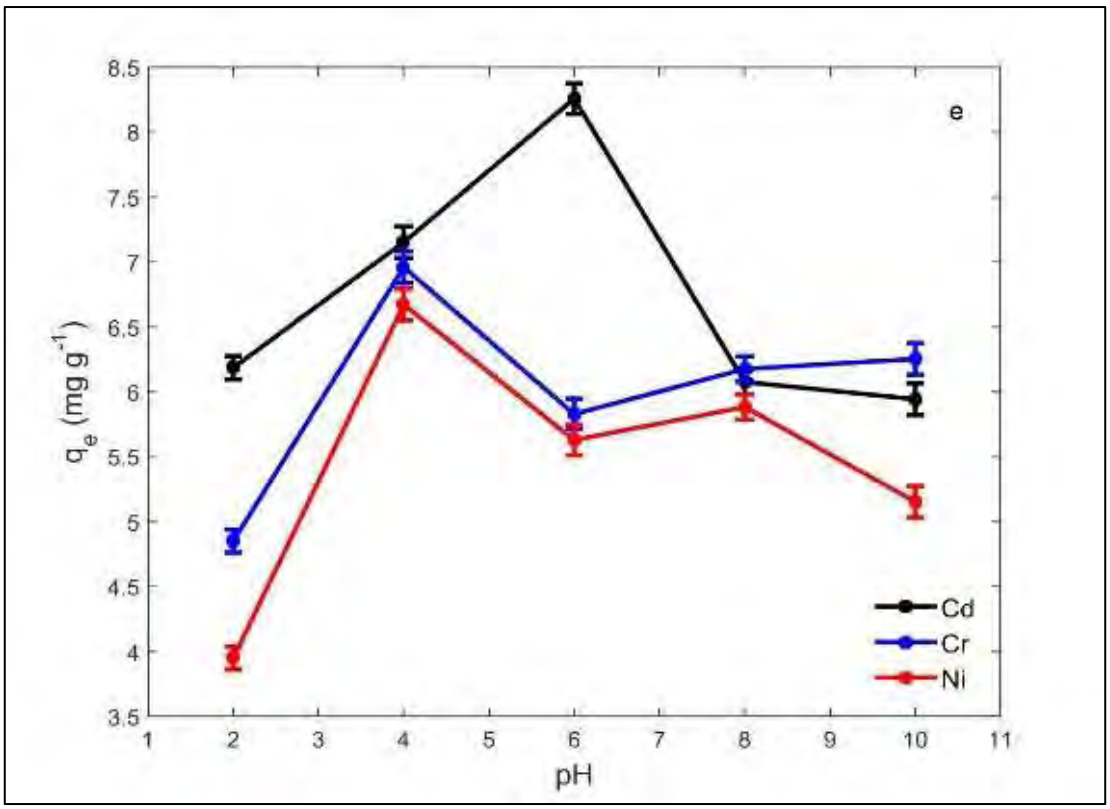
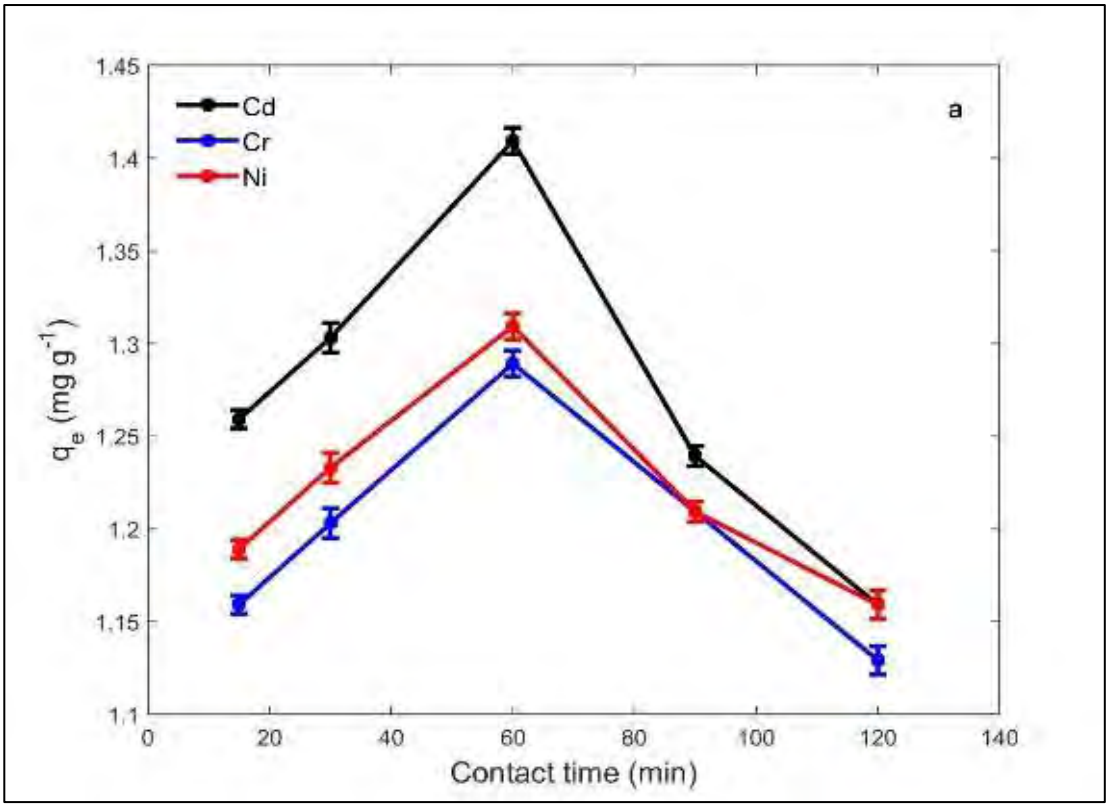
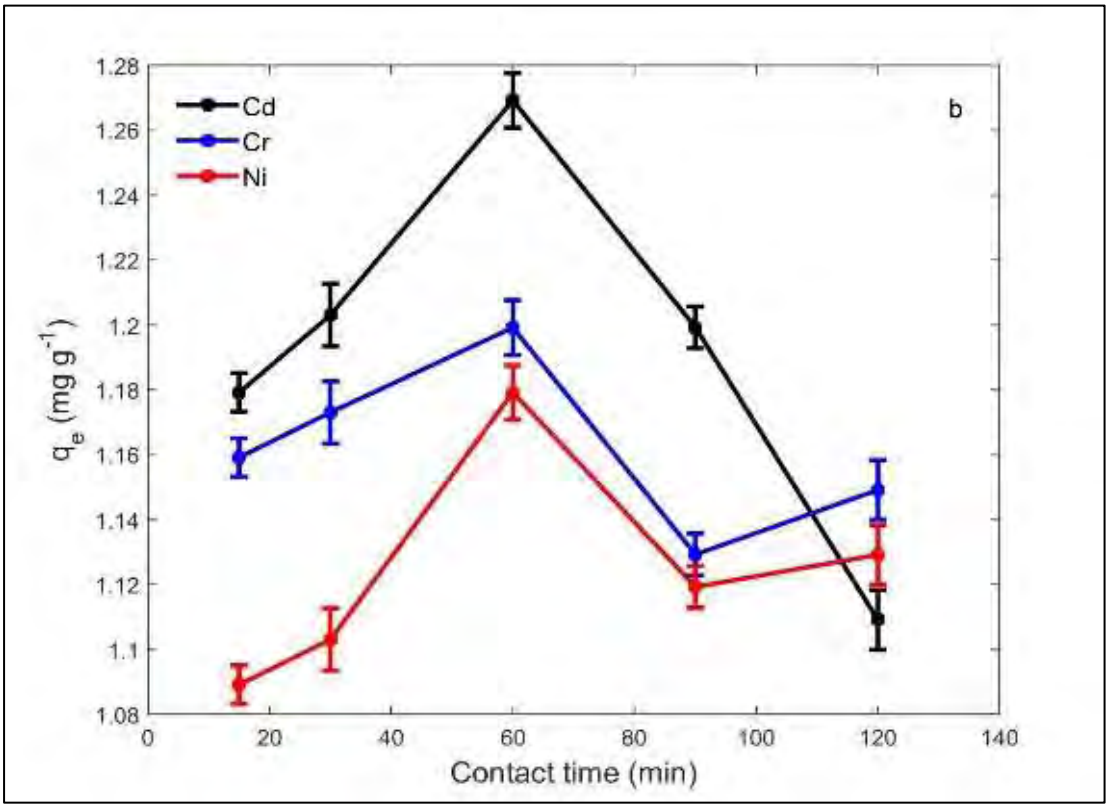


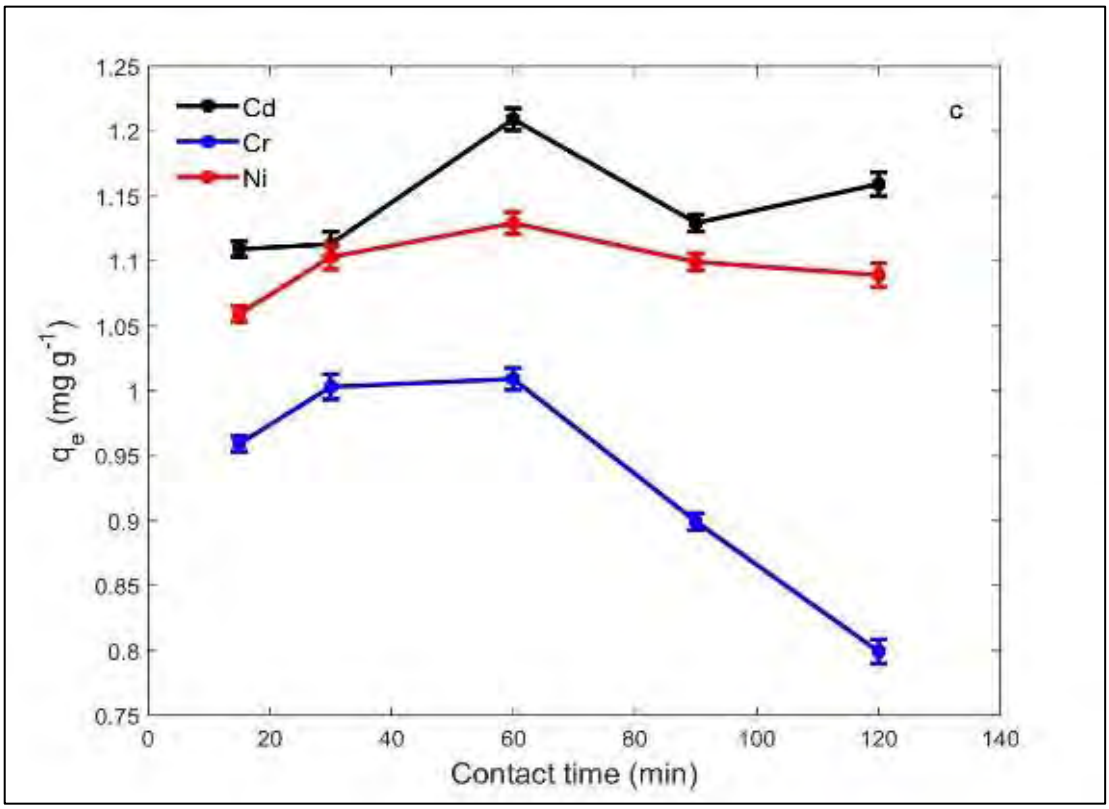
Figure 3.2. Effect of different pH on different heavy metals adsorption PM21(a), PM22 (b), PM23 (c), PM24 (d) and PM25 (e).

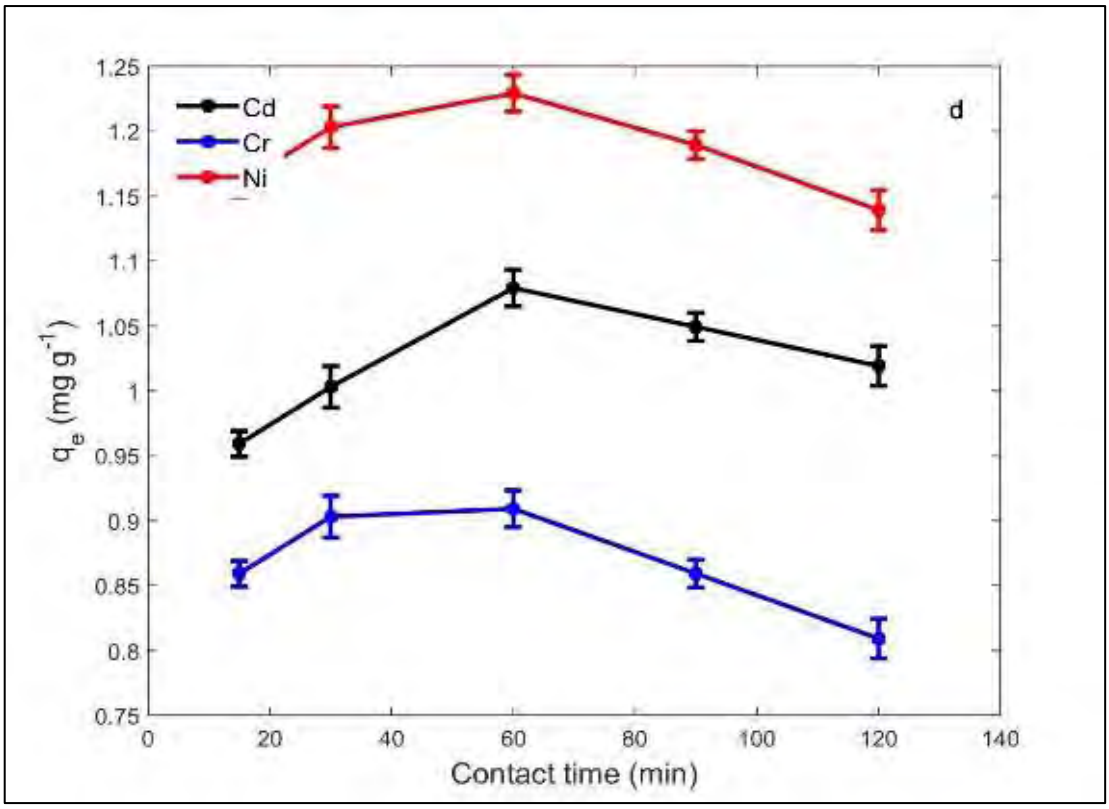
3.3.4. Influence of different contact time on adsorption

The adsorption capacity of heavy metals (Cd, Cr and Ni) was determined at different time intervals i.e., 0, 20,40,60,80,100 and 120 min. Results showed maximum adsorption of Cd, Cr and Ni was recorded after 60 min for all the bacterial strains i.e., PM21, PM22, PM23, PM24, and PM25 (Figure 3.3).


Cd
Cr
Ni
a
q_e (mg g^{-1})
Contact time (min)
1.45
1.4
1.35
1.3
1.25
1.2
1.15
1.1
0
20
40
60
80
100
120
140


Cd
Cr
Ni
b
q_e (mg g^{-1})
Contact time (min)
1.28
1.26
1.24
1.22
1.2
1.18
1.16
1.14
1.12
1.1
1.08
0
20
40
60
80
100
120
140


c
Cd
Cr
Ni
q_e (mg g^{-1})
Contact time (min)
1.25
1.2
1.15
1.1
1.05
1
0.95
0.9
0.85
0.8
0.75
0
20
40
60
80
100
120
140


d
Cd
Cr
Ni
q_e (mg g^{-1})
Contact time (min)
1.25
1.2
1.15
1.1
1.05
1
0.95
0.9
0.85
0.8
0.75
0
20
40
60
80
100
120
140

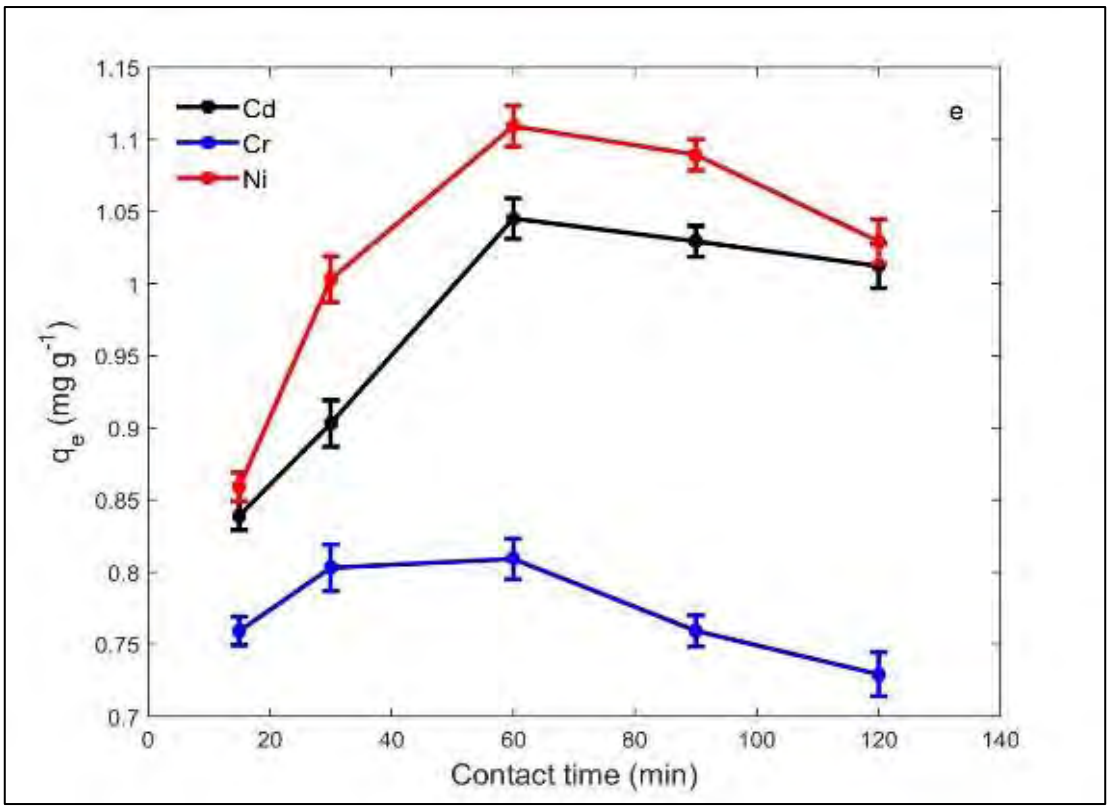
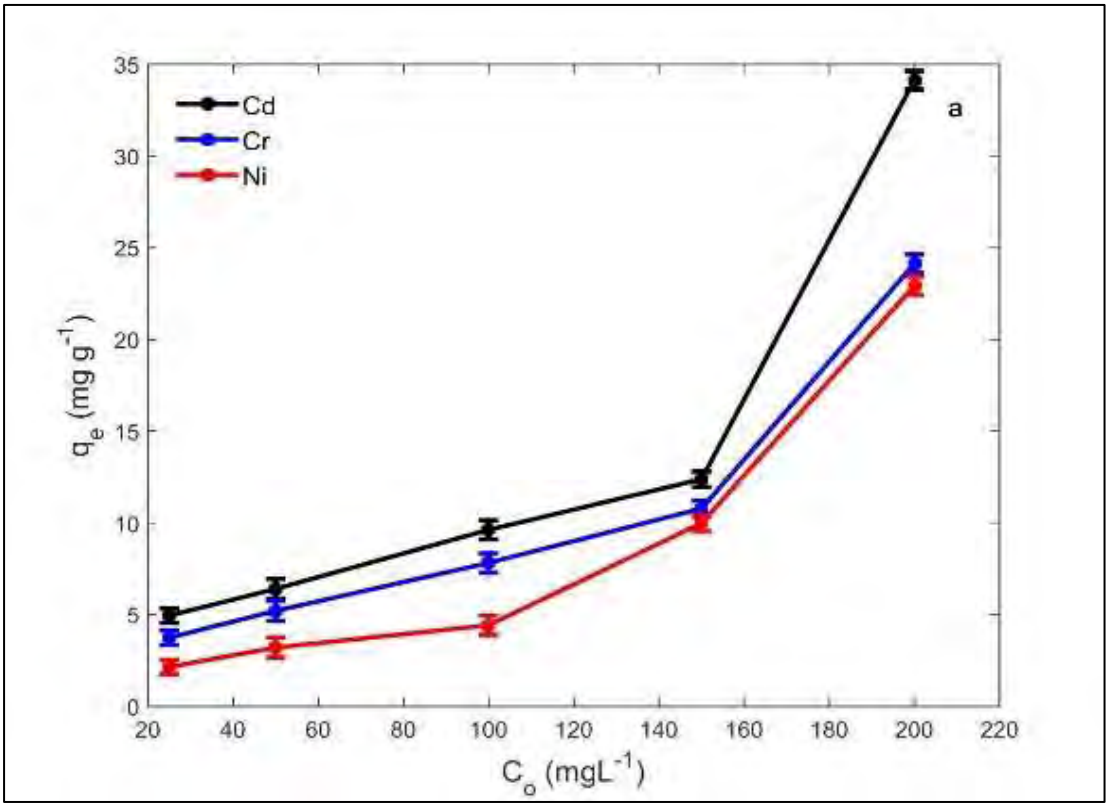
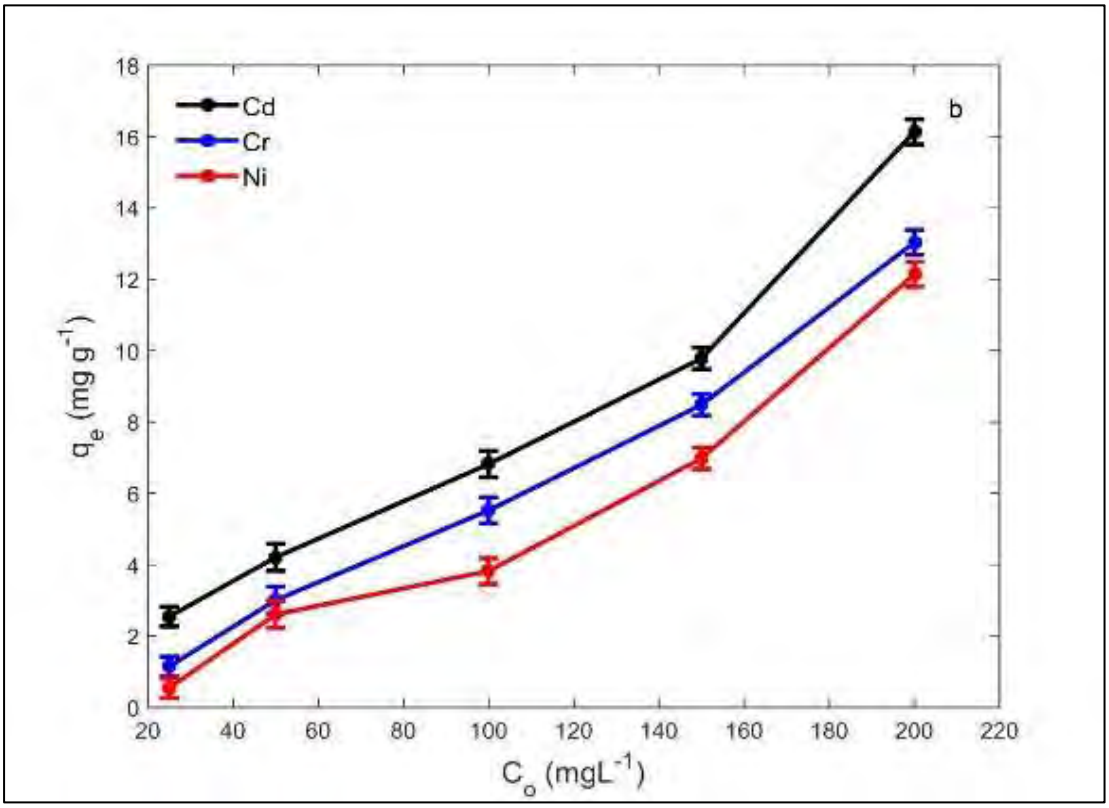


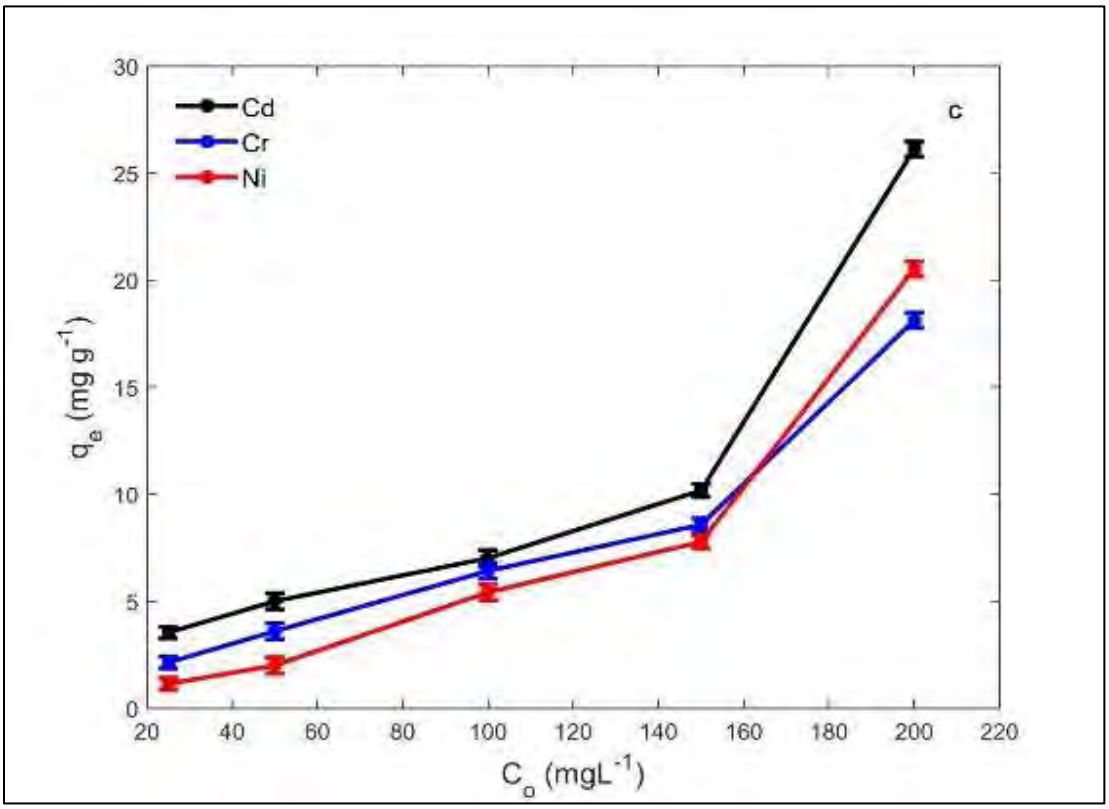
Figure 3.3 Effect of contact time on different heavy metals adsorption PM21(a), PM22 (b), PM23 (c), PM24 (d) and PM25 (e).

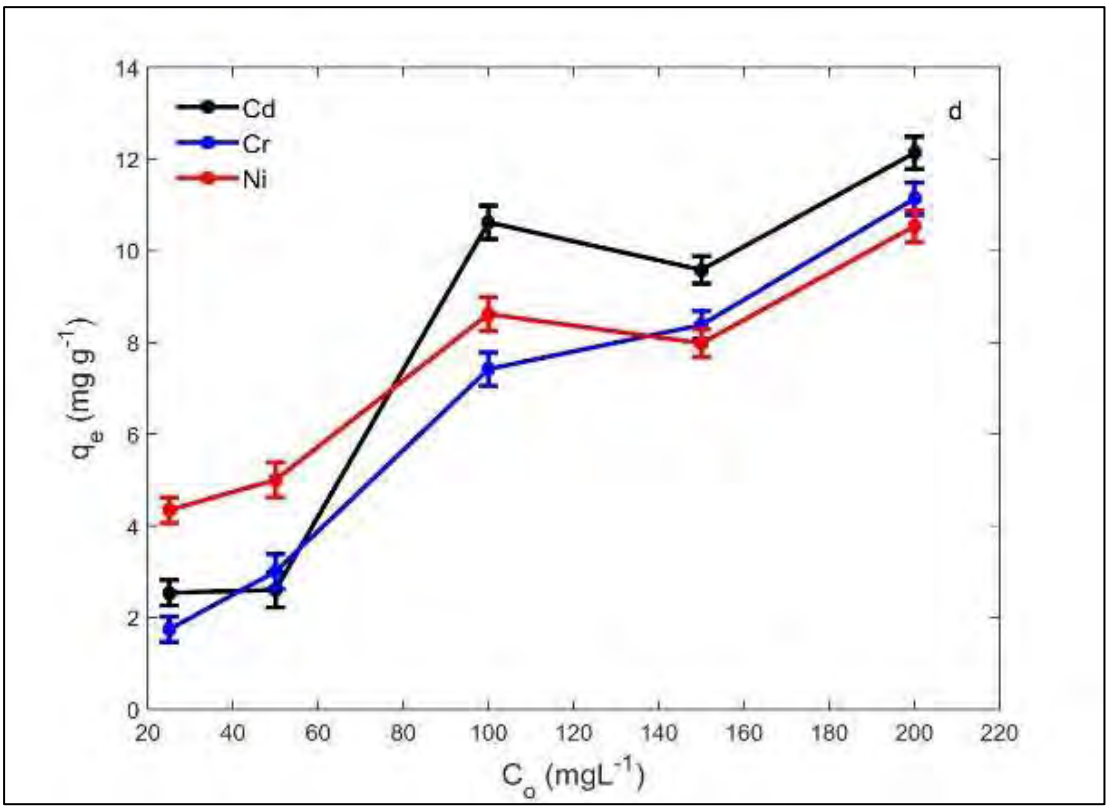
3.3.5. Initial metal concentrations

Effect of different HMs (25-200 mg L^{-1}) of Cd, Cr, and Ni was used to check their adsorption kinetic under optimum conditions of temperature (35°C ± 2°C), sorbent value 0.5 g/L, time duration 60 min and at optimum pH (8 for Cd, 6 for Cr, and 4 for Ni) for PM21. Initial concentrations of heavy metals (25, 50,100, 150, and 200 mg/L), temperature (35°C ± 2°C), dose (0.5 g/L), and time (60 min) were also kept same for bacterial strains PM22, PM23, PM24, and PM25, while pH was kept at 6 for Cd, 4 for Cr, and 4 for Ni, respectively. Bacterial strain PM21 exhibited highest adsorption capacity among the five bacterial strains. The adsorption capacities (qe) of PM21 increased (5-35 mg/g for Cd, 4-24 mg g for Cr, and 3-24 mg g for Ni) with increasing initial heavy metals concentration of 200 mg/L, respectively (Figure. 3.4)


Cd
Cr
Ni
a
q_e (mg g^{-1})
C_o (mgL^{-1})
0
5
10
15
20
25
30
35
20
40
60
80
100
120
140
160
180
200
220


Cd
Cr
Ni
b
q_e (mg g^{-1})
C_o (mgL^{-1})
0
2
4
6
8
10
12
14
16
18
20
40
60
80
100
120
140
160
180
200
220


Cd
Cr
Ni
c
q_e (mg g^{-1})
C_o (mgL^{-1})
0
5
10
15
20
25
30
20
40
60
80
100
120
140
160
180
200
220


Cd
Cr
Ni
d
q_e (mg g^{-1})
C_o (mgL^{-1})
0
2
4
6
8
10
12
14
20
40
60
80
100
120
140
160
180
200
220

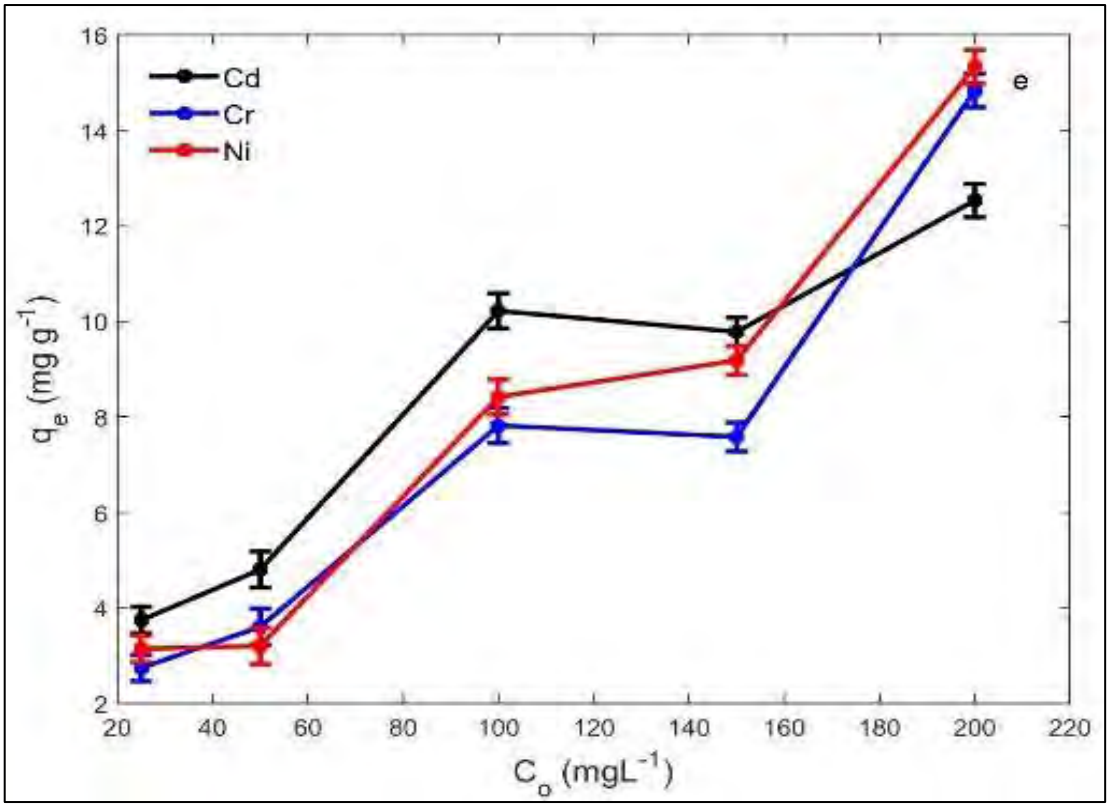


Figure 3.4. Influence of initial metal concentration on different heavy metals adsorption PM21(a), PM22 (b), PM23 (c), PM24 (d) and PM25 (e).

3.3.6. Equilibrium isotherms and models

Both very considerable adsorption equilibrium isotherms known as Langmuir and Freundlich models were used to determine the relationship among sorption and aqueous concentrations of metals ions i.e., Cd, Cr, and Ni. The study was carried out at pH of 8, 6, and 4 temperature 35±2ºC, biosorbent density (0.5 g/L) and various treatments of heavy metals given as 25, 50, 100, 150 and 200 mg/L.

3.3.6.1. Langmuir isotherm model

This isotherm model was applied to relate the equilibrium concentrations of heavy metals ions PM21, PM22, PM23, PM24 and PM25 with uptake capacity (q_e), and equilibrium concentrations of heavy metals in the solution (C_e). various values of C_e and q_e were examined for various initial concentration of metals i.e., (25, 50, 100, 150 and 200 mg/L). The numeric values obtained were then utilized to set Langmuir isotherm plot following the equation given below.

$$\frac{\mathbf{1}}{\boldsymbol{q_e}} = \frac{\mathbf{1}}{\boldsymbol{Q_{max}}} + \frac{\mathbf{1}}{\boldsymbol{Q_{max}\, bC_e}} \qquad (\mathbf{3.1})$$

Graph of *1*/*qe* compared with *1*/*Ce* generated straight lines for heavy metals as mentioned in (Figure 3.5), which reveal consistent adsorption. $Q_{max,}$ *b* and correlation coefficient (R^2) were estimated from graph as shown in (Table 3.2). The slope of the graph and intercept was used to determine values of *b* and Q_{max} respectively ($1/Q_{max}b$ = slope and $1/Q_{ma\ x}$ = intercept).

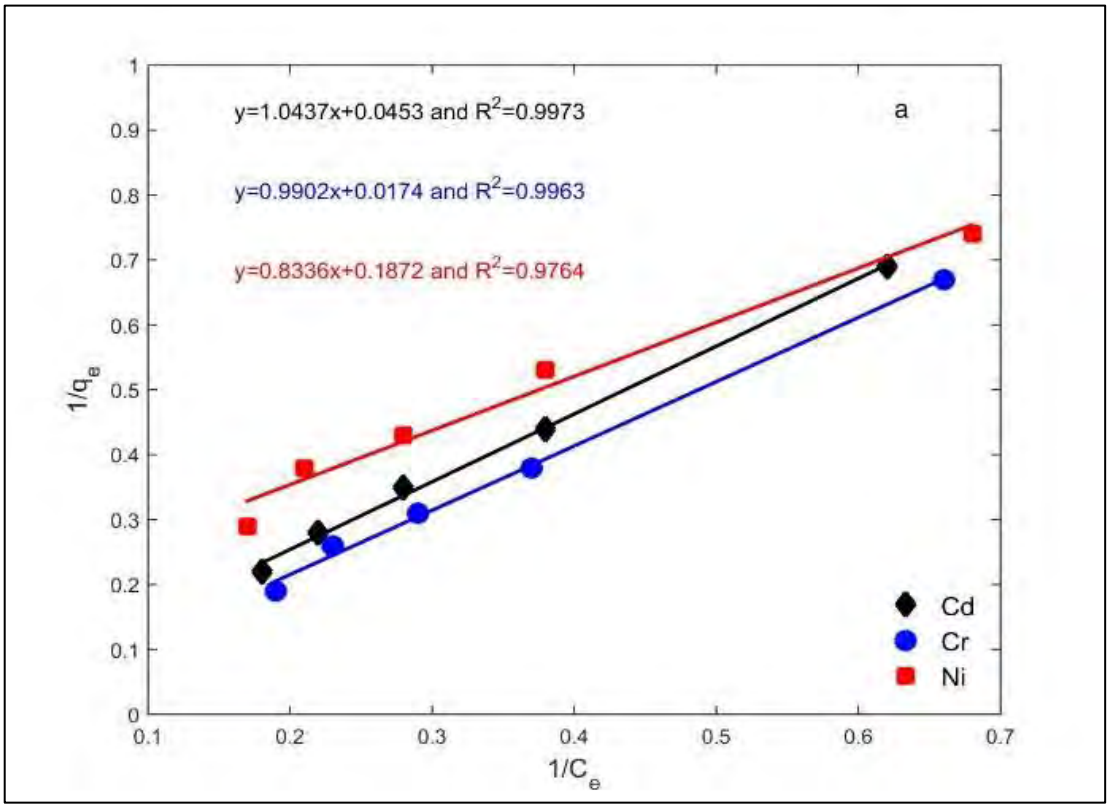
This model, Q_{max} (mg/g) represents highest adsorption of *Bacillus anthracis* for heavy metals ions. Adsorption capacity Q_{max} for bacterial strains were determined for different heavy metals (Table 3.2).

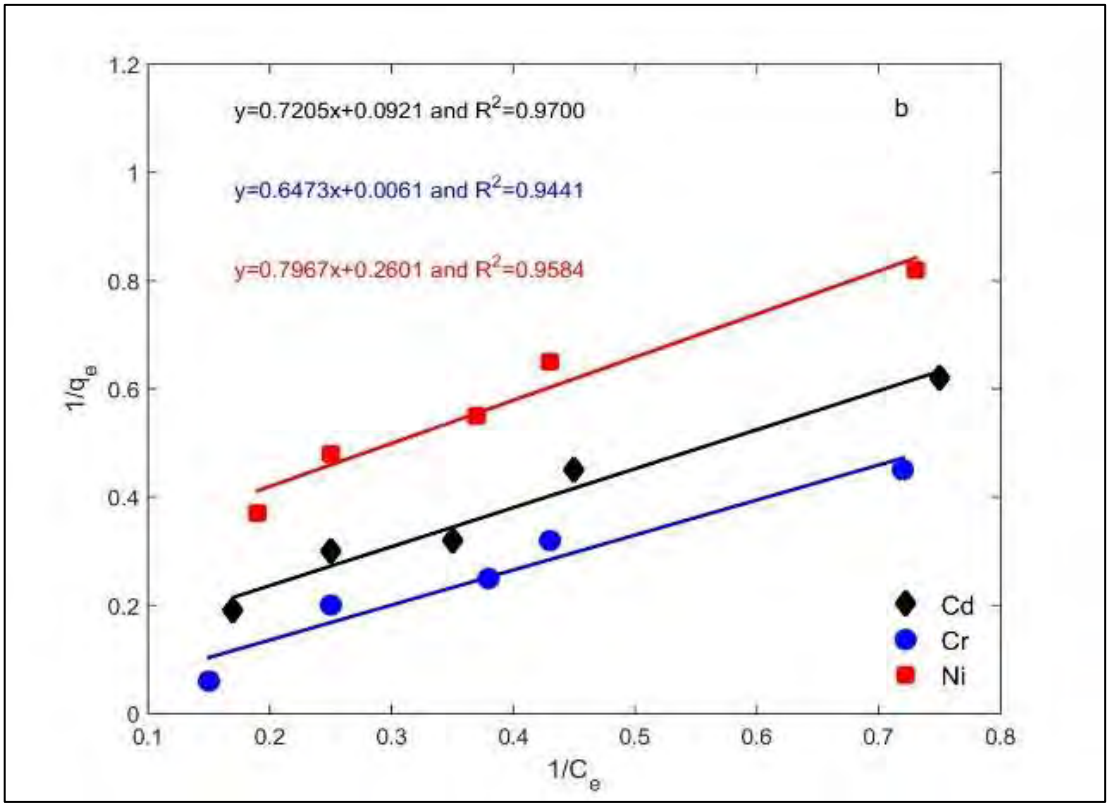
The Langmuir constant, b (L m/g), is used to calculate adsorption free energy. A highest b value suggests that HMs ions have a stronger affinity for bacterial strain biomass. Current experiment, the values of *b* were estimated as cadmium (0.043), chromium (0.017), and nickel 0.001for PM21, cadmium 0.04, chromium 0.009, and nickel 0.032 for PM22, cadmium 0.039, chromium 0.061, and nickel 0.263 for PM23, Cd 0.061, chromium 0.617, nickel 0.378 for PM24 and cadmium 0.066, chromium 0.061, and nickel 0.263 for PM25 respectively).

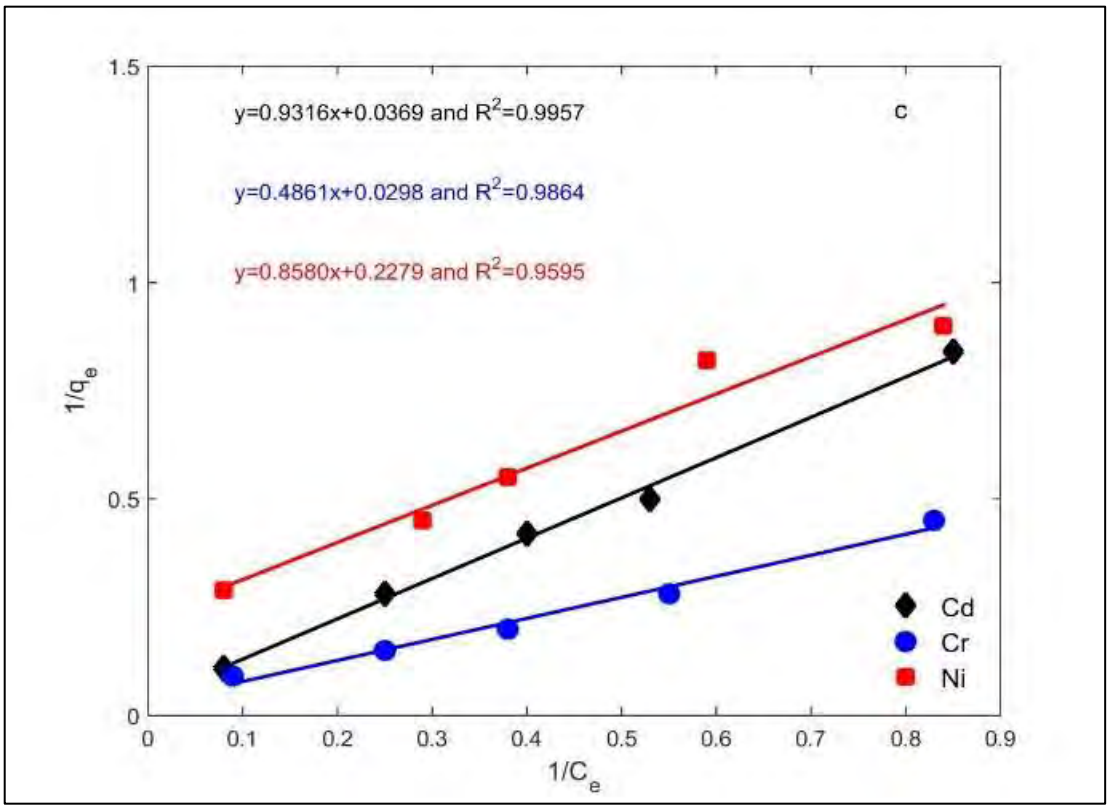
R_L is a dimensional element that estimates the essence of the mechanism of adsorption. R_L can be estimated from the following equation:

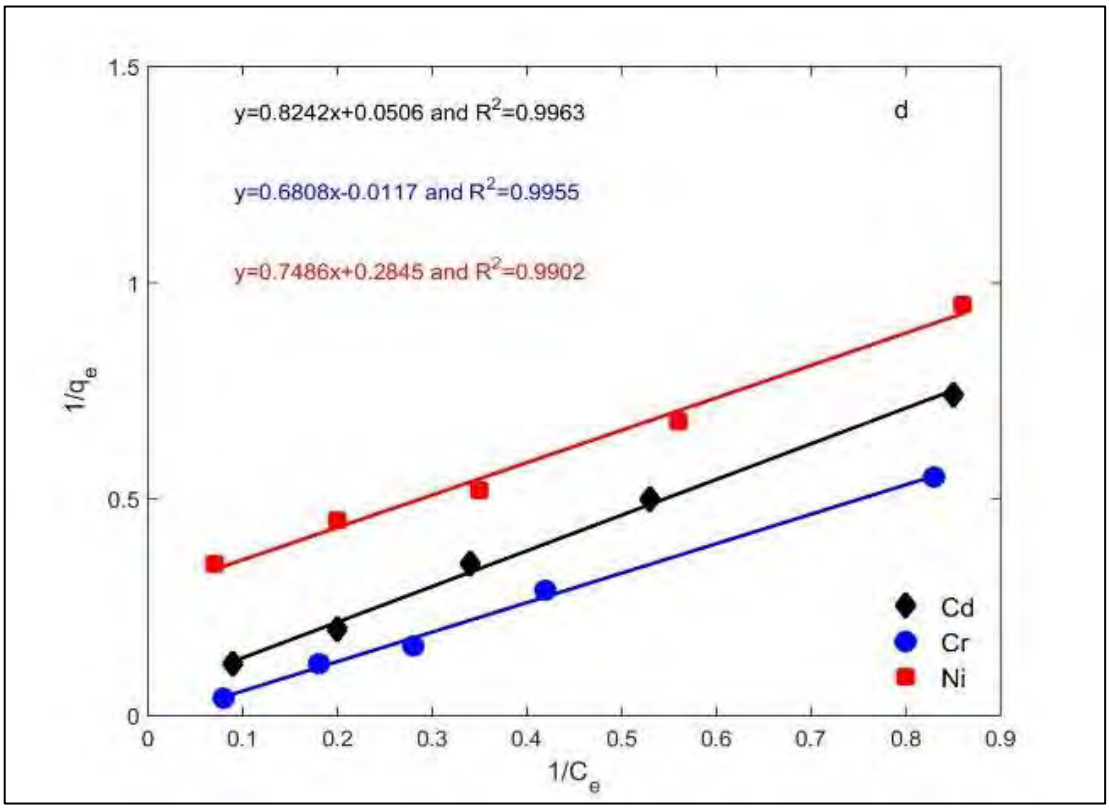
$$\boldsymbol{R_l} = \frac{\mathbf{1}}{\mathbf{1} + (\boldsymbol{b} \times \boldsymbol{C_o})} \qquad (\mathbf{3.2})$$

Where the initial concentrations of heavy metals was *Co* (mgL), *b* (mgL) was a langmuir isotherm constant. R_L values higher than 1 was considered to be unfavorable, favorable if the R_L value was within the range (0-1), if the R_L value was equal to 1 then linear and irreversible if the value R_L is 0 (Haq et al., 2016). From Table 3.2 it is shown that R_L values were all positive, greater than 0 and less than 1 for different heavy metal ions at different C_o suggesting the heavy metal adsorption.


y=1.0437x+0.0453 and R2=0.9973
y=0.9902x+0.0174 and R2=0.9963
y=0.8336x+0.1872 and R2=0.9764
a
Cd
Cr
Ni
1/qe
1/Ce


y=0.7205x+0.0921 and R2=0.9700
y=0.6473x+0.0061 and R2=0.9441
y=0.7967x+0.2601 and R2=0.9584
b
Cd
Cr
Ni
1/qe
1/Ce


y=0.9316x+0.0369 and R2=0.9957
y=0.4861x+0.0298 and R2=0.9864
y=0.8580x+0.2279 and R2=0.9595
c
Cd
Cr
Ni
1/qe
1/Ce
0
0.5
1
1.5
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9


y=0.8242x+0.0506 and R2=0.9963
y=0.6808x-0.0117 and R2=0.9955
y=0.7486x+0.2845 and R2=0.9902
d
Cd
Cr
Ni
1/qe
1/Ce
0
0.5
1
1.5
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

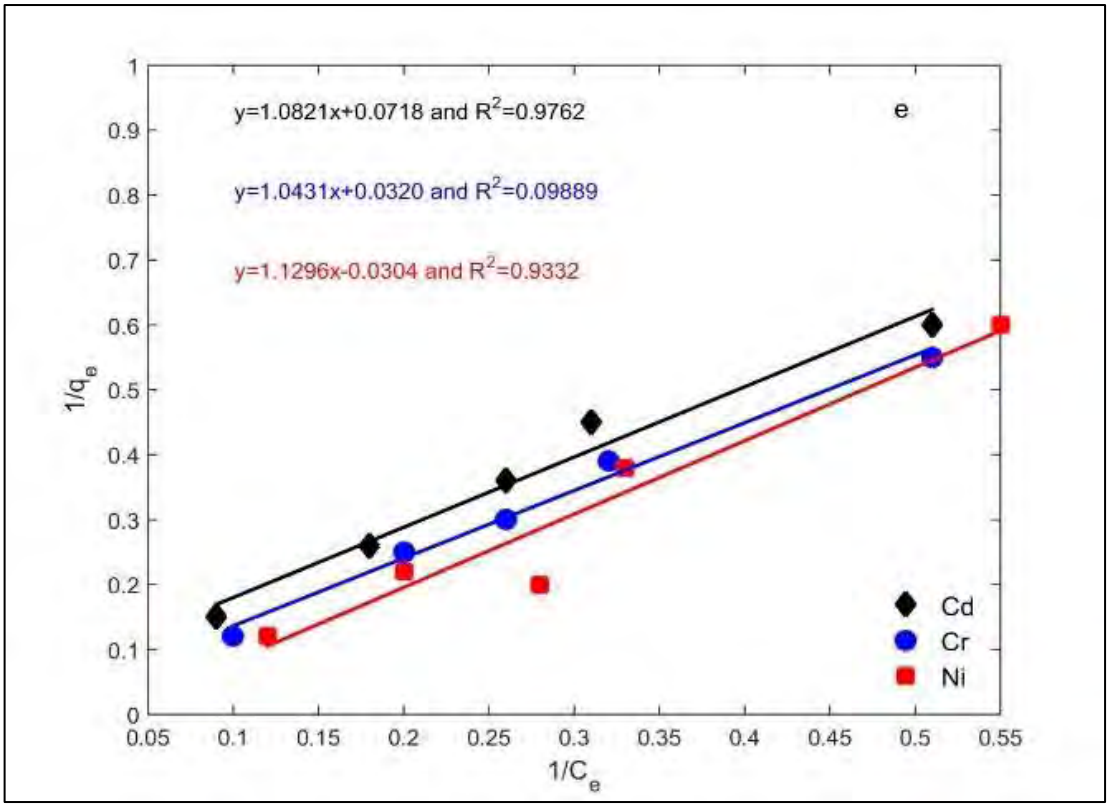


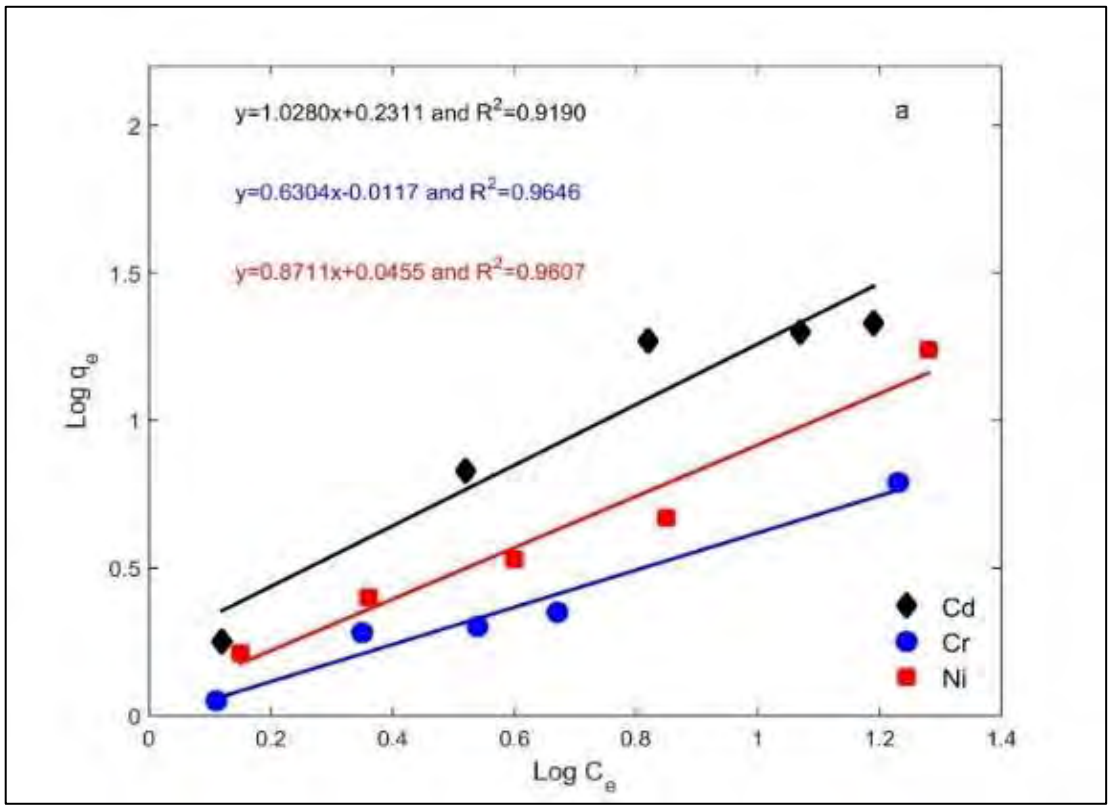
Figure 3.5 Linearized Langmuir model for heavy metals removal PM21(a), PM22 b), PM23 (c), PM24 (d) and PM25 (e) .

3.3.6.2. Freundlich isotherm

Freundlich model explains heterogeneous adsorption on the adsorbent surface. The linear equation of Freundlich adsorption isotherm is given as under:

$$\boldsymbol{logq_e = logk_f + \frac{1}{n}\, logC_e} \qquad (\mathbf{3.3})$$

Here, K_f represents the constant for Freundlich binding the adsorption ability of the absorbent, and n is an empirical parameter associated with the strength of adsorption (Figure 3.6). All the calculated values of *Kf* and *n* to be (1.7, 1, 1.1), (0.97, 1.58 and 1.14) for Cd, Cr and Ni by PM21. Regression coefficient (R^2) values by PM21 were 0.919, 0.964 and 0.96, for Cd, Cr and Ni. Similarly, Kf, n and R2 values for PM22, PM23,PM24 and PM25 are shown in Table 3.3. Furthermore, Q_{max} values for strain PM21 against Cd, Cr and Ni was noted as 22.07, 57.04 and 5.34, respectively.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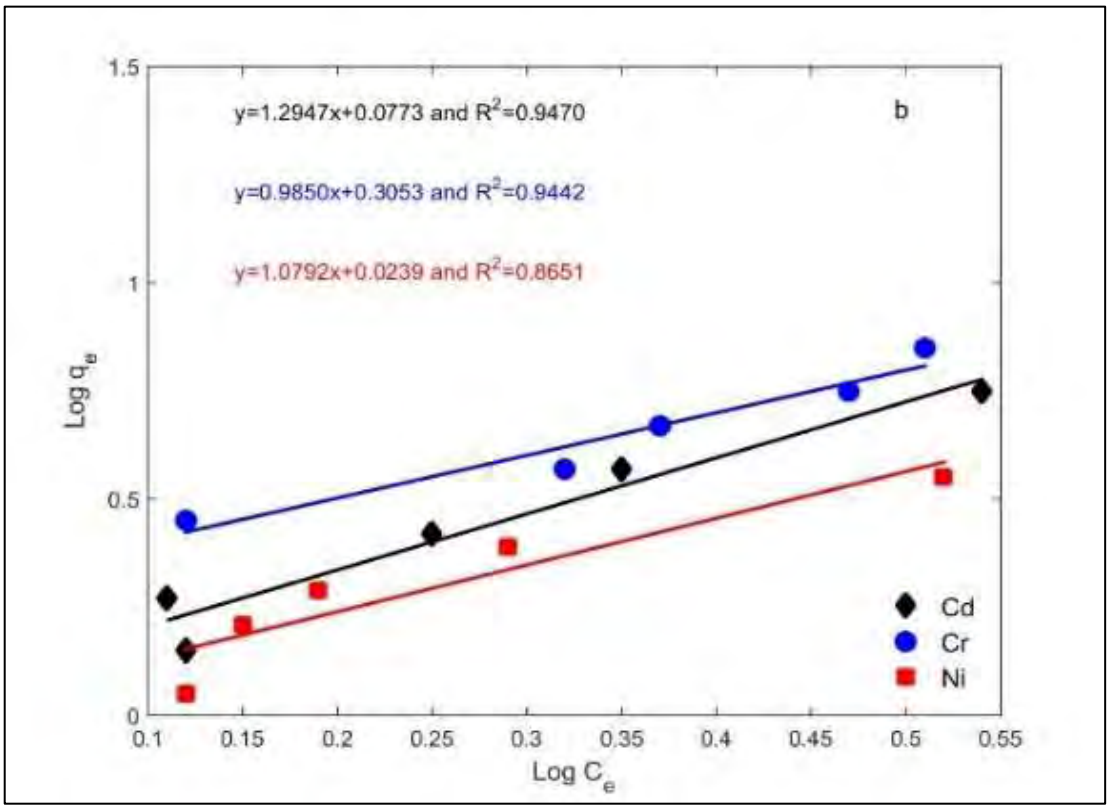
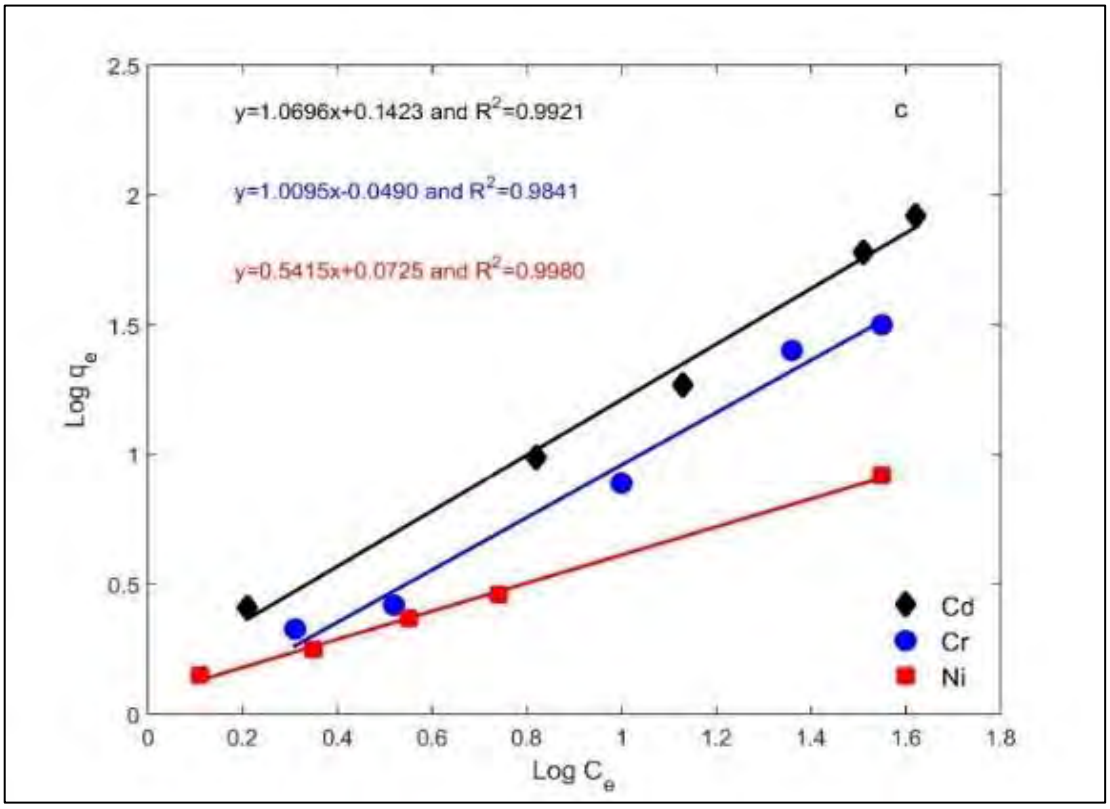
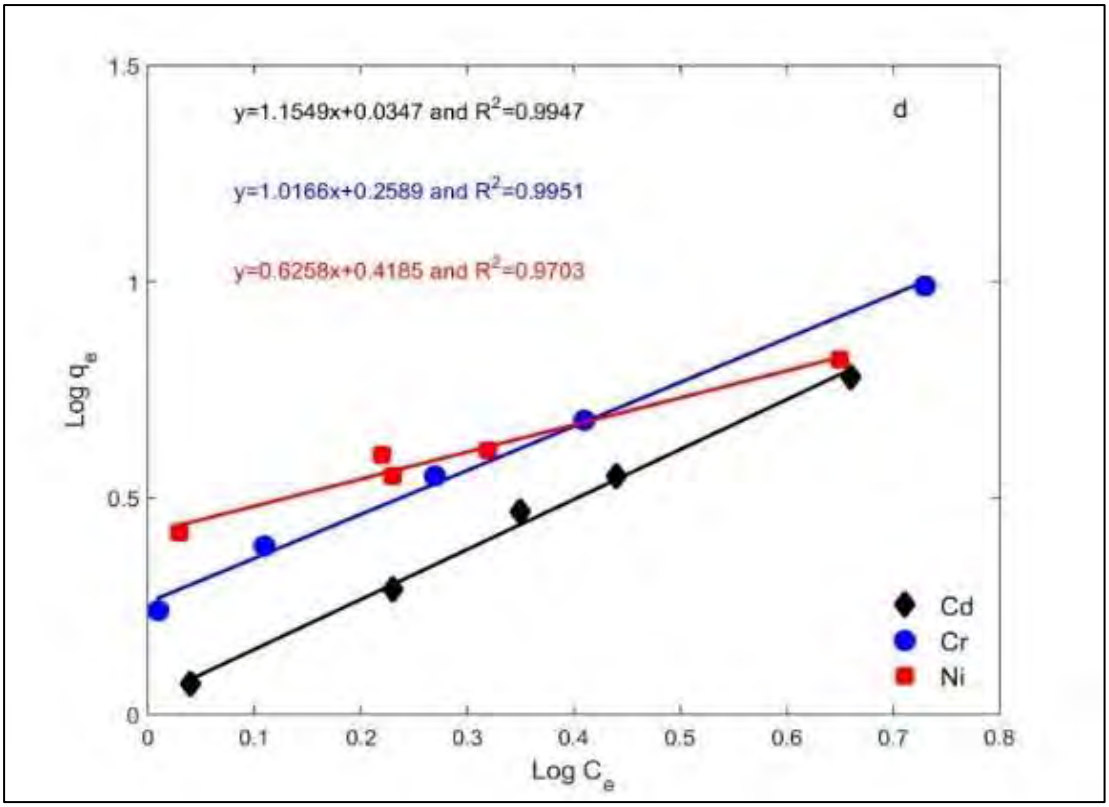
a
y=0.6304x-0.0117 and R2=0.9646
y=0.8711x+0.0455 and R2=0.9607
Log qe
Log Ce
Cd
Cr
Ni
0
0.5
1
1.5
2
0.2
0.4
0.6
0.8
1.2
1.4


y=1.2947x+0.0773 and R2=0.9470
y=0.9850x+0.3053 and R2=0.9442
y=1.0792x+0.0239 and R2=0.8651
b
Cd
Cr
Ni
Log qe
Log Ce
1.5
1
0.5
0
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
0.55


y=1.0696x+0.1423 and R2=0.9921
y=1.0095x-0.0490 and R2=0.9841
y=0.5415x+0.0725 and R2=0.9980
c
Cd
Cr
Ni
Log qe
Log Ce
2.5
2
1.5
1
0.5
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8



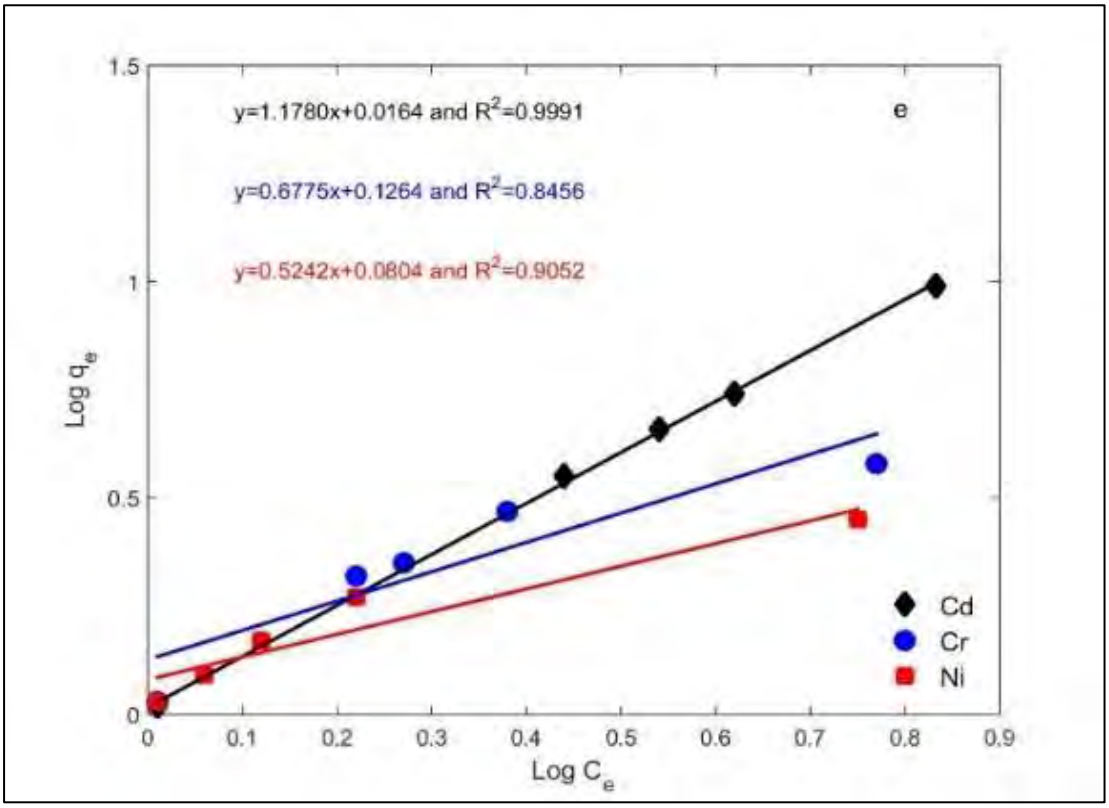


Figure 3.6 Linearized Freundlich model for heavy metals removal on PM21(a), PM22 (b), PM23 (c), PM24 (d) and PM25 (e).

Table 3.2 Coefficients for Langmuir and Freundlich Isotherms

	Langmuir model					Freundlich model		
Strain's name	**Adsorbate**	**Q_{max}**	**b (Lmg^{-1})**	**R_L**	***R^2***	**K_f mgg^{-1}**	**n**	***R^2***
PM21	Cd	22.07	0.043	0.317	0.997	1.7	1.14	0.969
	Cr	57.04	0.017	0.541	0.996	1.1	1.58	0.964
	Ni	5.34	0.001	0.952	0.976	1	0.97	0.91
PM22	Cd	10.8	0.04	0.333	0.97	1.19	0.77	0.947
	Cr	13.9	0.009	0.690	0.944	2	1.01	0.944
	Ni	3.8	0.032	0.385	0.958	1.05	0.92	0.865
PM23	Cd	27.1	0.039	0.339	0.995	1.38	0.93	0.992
	Cr	33.5	0.061	0.247	0.986	1.11	0.99	0.984
	Ni	4.3	0.263	0.071	0.959	1.18	1.84	0.998
PM24	Cd	19.7	0.061	0.247	0.996	1.08	0.87	0.994
	Cr	15.4	0.617	0.031	0.995	1.81	0.98	0.995
	Ni	3.5	0.378	0.050	0.99	2.61	0.61	0.97
PM25	Cd	13.9	0.066	0.233	0.976	1.03	0.84	0.999
	Cr	31.2	0.061	0.247	0.098	1.33	1.47	0.845
	Ni	29.4	0.263	0.071	0.933	1.2	1.9	0.9052

3.3.7. Kinetic study of adsorption

The study of the rate of sorption and the factors that influence it in a sufficient amount of time to reach equilibrium is known as biosorption kinetics. It also provides information on the adsorption function and an estimation of equilibrium adsorption loading. Data are used to predict biosorption kinetics using intra-particle diffusion and pseud-second order. The following linearized equation is the kinetic equation of pseudo-second-order (Aly et al., 2014).

$$\frac{t}{q_t} = \frac{1}{k_2 q_e 2} + \frac{t}{q_e} \times t \qquad (3.4)$$

In this equation qt and q indicate the mass in mg g^{-1} of metal ions adsorbed at equilibrium and time t (time of interaction in minutes), and k1 shows the pseudo-second-order rate constant in g mg^{-1} min^{-1} figure 3.7.

The following equation, the initial adsorption rate, *h* (mg/g/min), was determined.

$$h = k_2 q_e 2 \qquad (3.5)$$

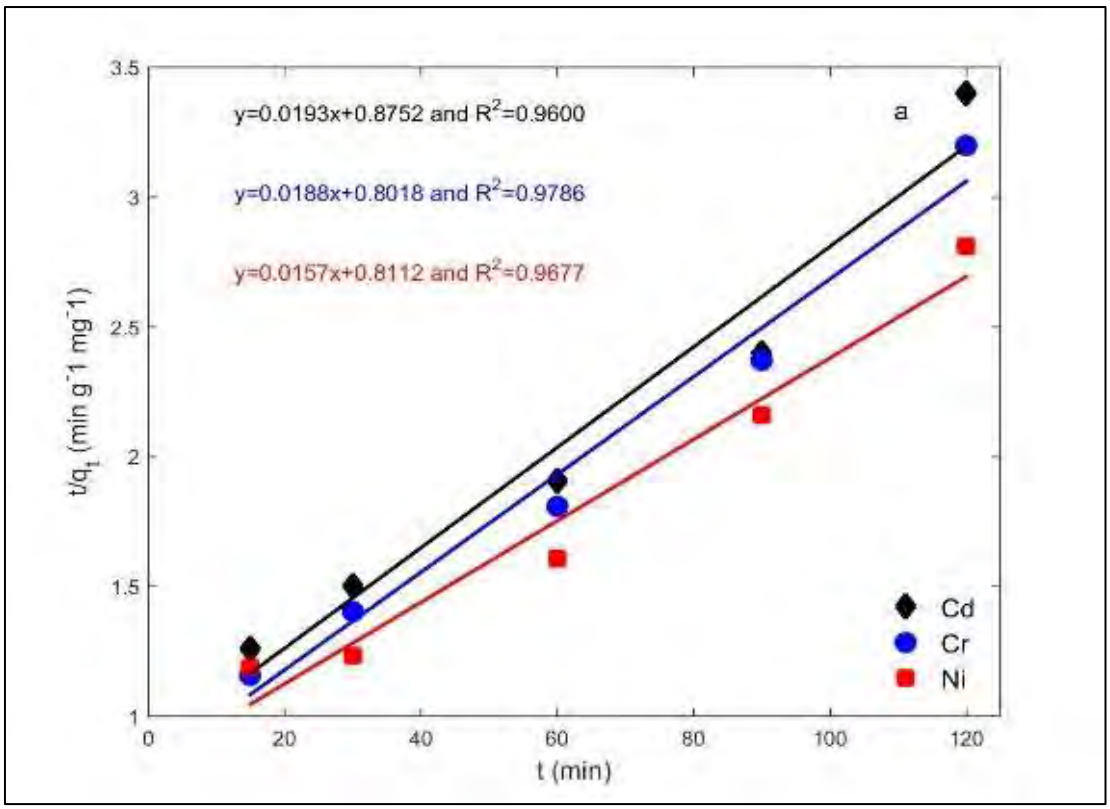
The correlation co-efficient R^2 are shown in (table 3.3). The intraparticle diffusion model was applied to the obtained data. Intra-particle diffusion model equation is given below.

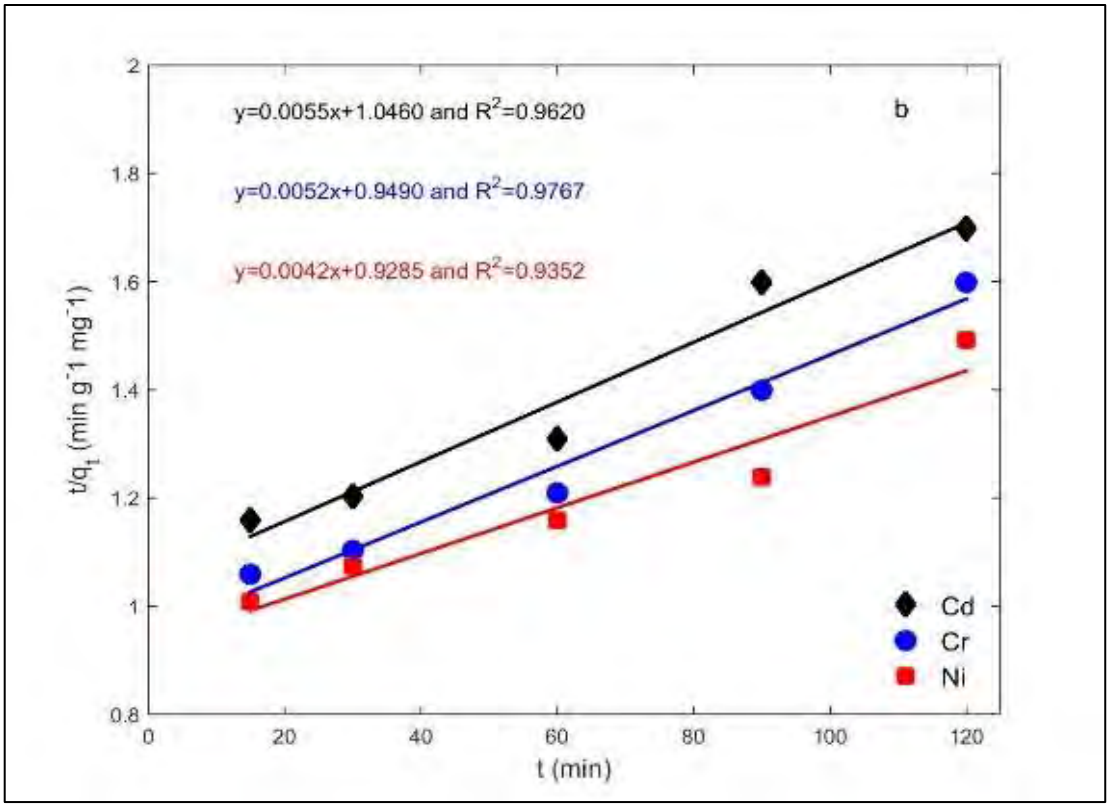
$$q_t = k_{int} \frac{t1}{2} + C_i \qquad (3.6)$$

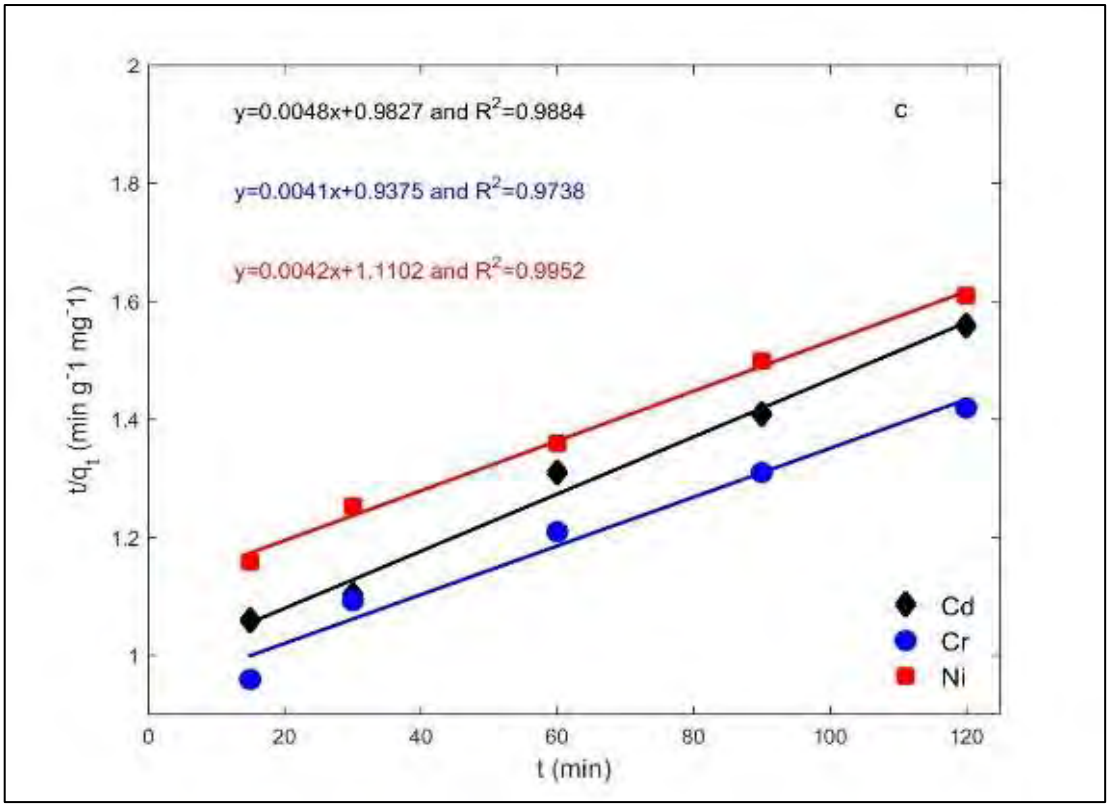
Where, k_{int} is the constant of the intra-partial diffusion rate and *Ci* was the intercept describing the border layer thickness.

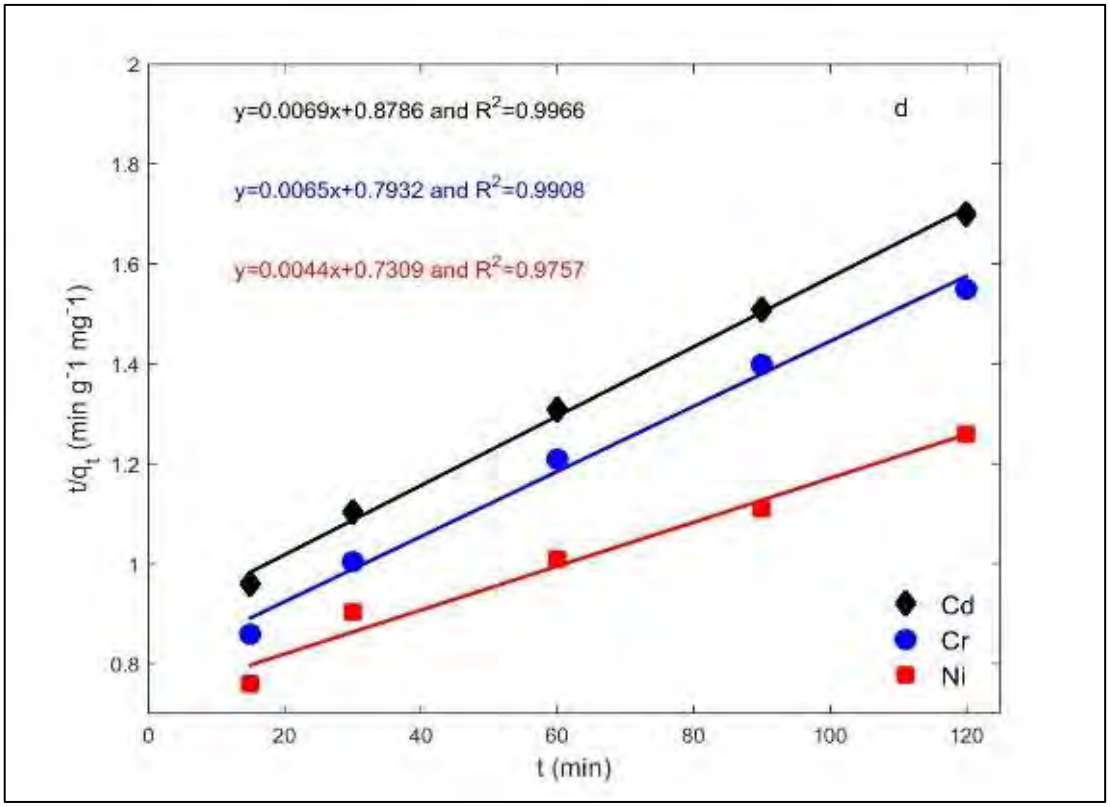
Table 3.3. Kinetic factors of Pseudo-second order and Intra-particle diffusion models for heavy metals

	Metals	Exp. Value	Pseudo-second order			Intra-particle diffusion			
Strain name	**Adsorbate**	q_e **mgg^{-1}**	**k_2**	**qe mgg^{-1}**	**H**	R^2	**k_{int}**	**C_i**	R^2
	cadmium	1.2	0.013	1.41	0.019	0.96	0.018	1.193	0.993
PM21	chromium	1.3	0.011	1.27	0.018	0.978	0.021	1.079	0.895
	Nickel	1.0	0.011	1.03	0.016	0.957	0.019	1.084	0.878
	cadmium	0.55	0.018	1.27	0.006	0.962	0.015	1.104	0.665
PM22	chromium	0.5	0.021	1.2	0.006	0.976	0.005	1.249	0.638
	Nickel	0.63	0.011	1.17	0.005	0.935	0.021	0.994	0.803
	cadmium	1.62	0.002	1.22	0.009	0.988	0.005	1.159	0.434
PM23	chromium	1.6	0.002	1.01	0.005	0.973	0.031	0.677	0.853
	Nickel	1.54	0.002	1.13	0.004	0.995	0.109	0.511	0.516
	cadmium	0.68	0.015	1.08	0.007	0.996	0.085	1.085	0.768
PM24	chromium	0.74	0.012	0.9	0.006	0.99	0.007	0.822	0.963
	Nickel	0.69	0.009	1.23	0.004	0.975	0.084	0.063	0.468
	cadmium	0.85	0.007	1.04	0.005	0.983	0.014	1.042	0.736
PM25	chromium	0.77	0.010	0.8	0.006	0.927	0.005	0.747	0.761
	Nickel	0.74	0.008	1.11	0.004	0.96	0.006	1.142	0.693


y=0.0193x+0.8752 and R2=0.9600
y=0.0188x+0.8018 and R2=0.9786
y=0.0157x+0.8112 and R2=0.9677
a
Cd
Cr
Ni
t/qt (min g-1 mg-1)
t (min)


y=0.0055x+1.0460 and R2=0.9620
y=0.0052x+0.9490 and R2=0.9767
y=0.0042x+0.9285 and R2=0.9352
b
Cd
Cr
Ni
t/qt (min g-1 mg-1)
t (min)


y=0.0048x+0.9827 and R2=0.9884
y=0.0041x+0.9375 and R2=0.9738
y=0.0042x+1.1102 and R2=0.9952
c
t/qt (min g-1 mg-1)
t (min)
Cd
Cr
Ni


y=0.0069x+0.8786 and R2=0.9966
y=0.0065x+0.7932 and R2=0.9908
y=0.0044x+0.7309 and R2=0.9757
d
t/qt (min g-1 mg-1)
t (min)
Cd
Cr
Ni

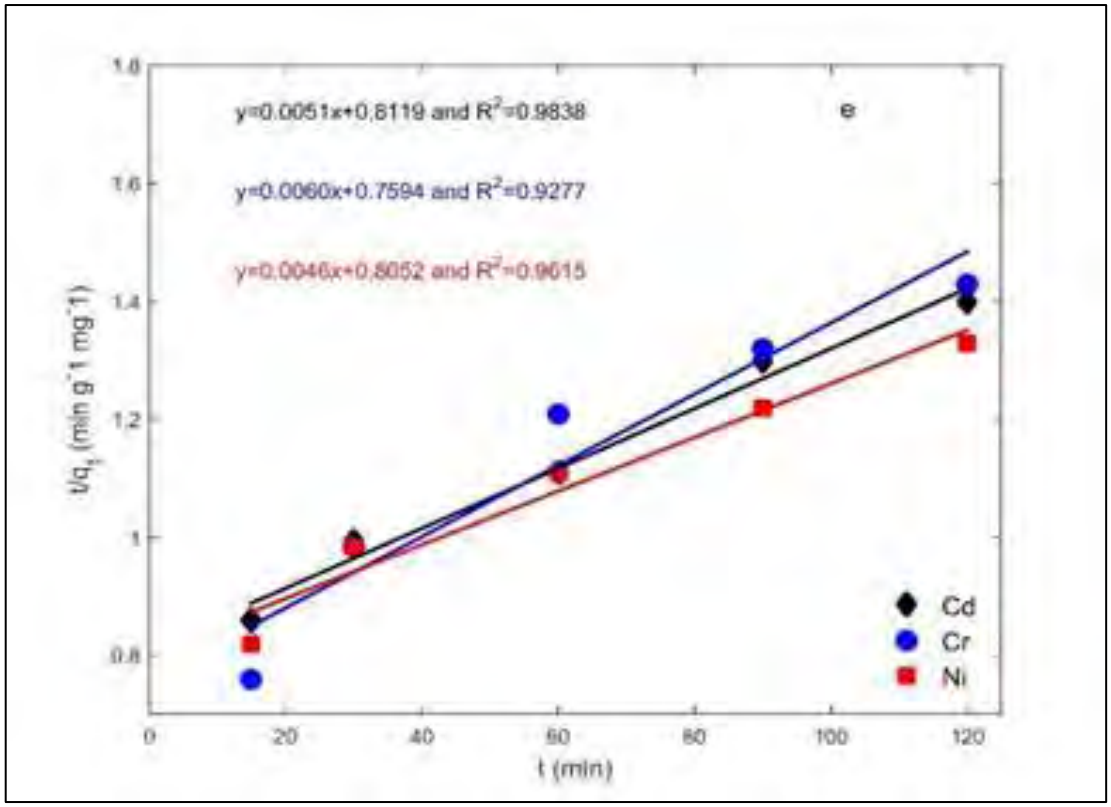


Figure 3.7. Pseudo second order model for heavy metals removal on PM21(a), PM22 (b), PM23 (c), PM24 (d) and PM25 (e) (*qt* = metal ions adsorbed at time; Y= Slope, R^2 = correlation coefficient)

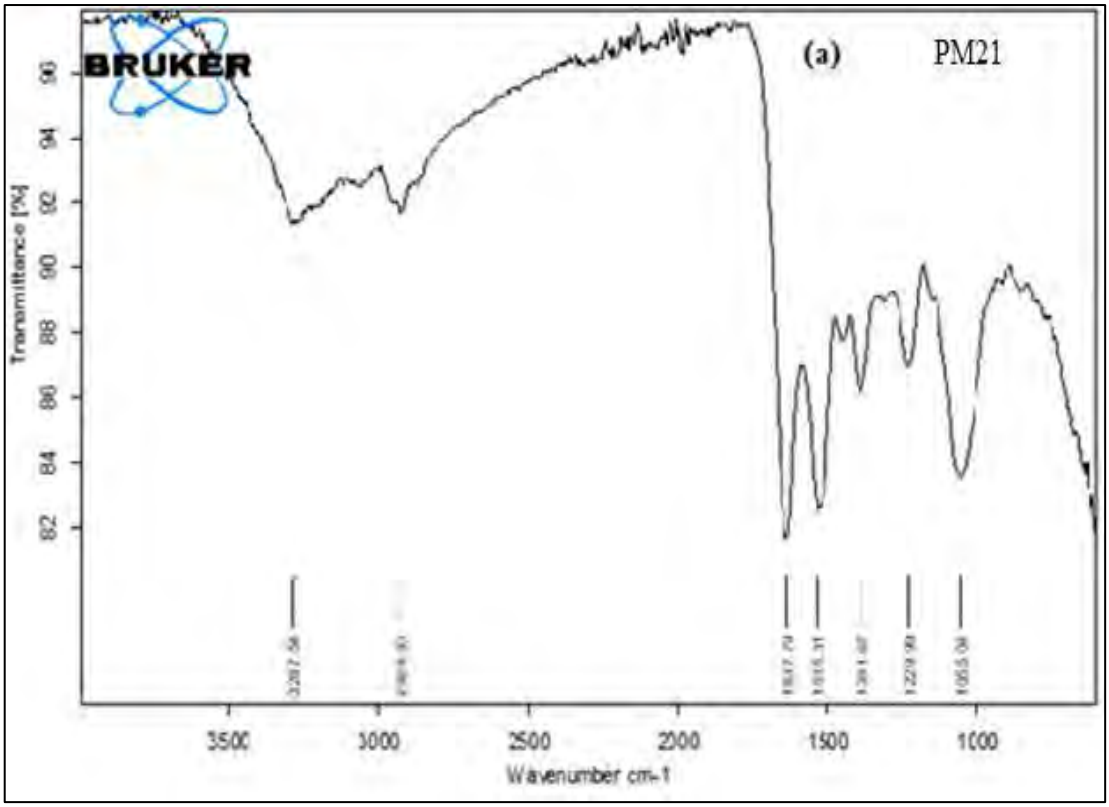
3.3.8. Characterization

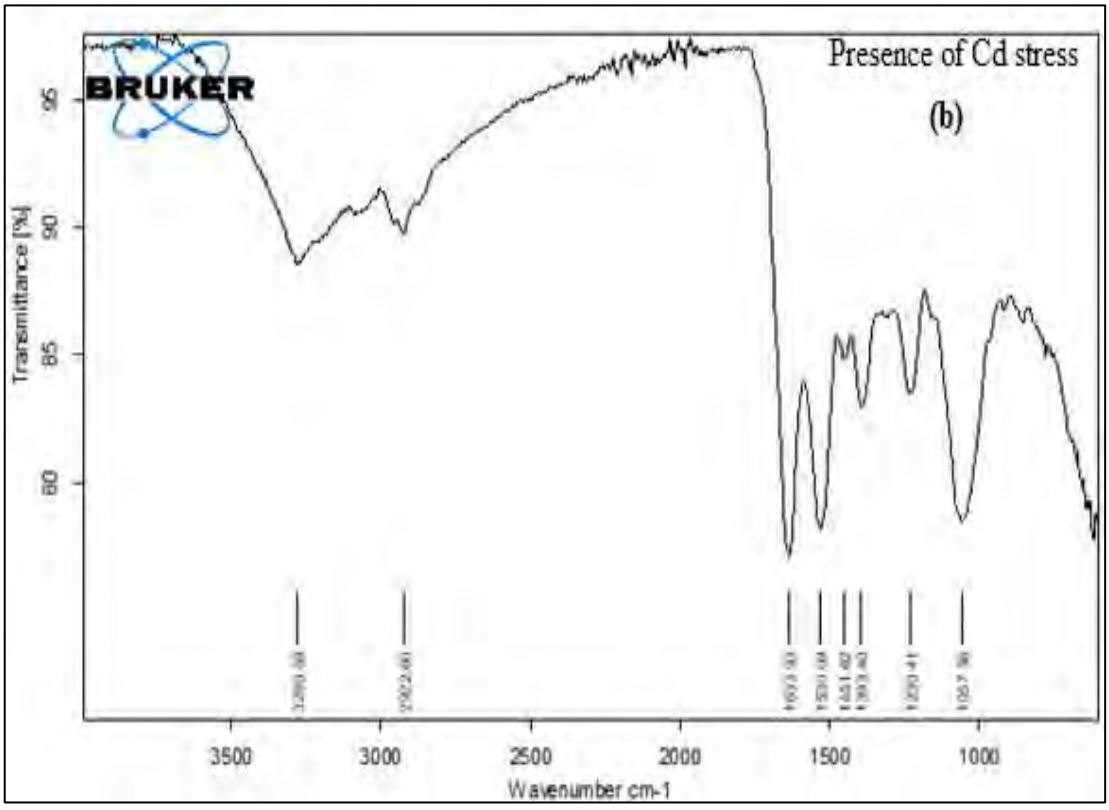
3.3.8.1. Fourier transform infrared spectrometer (FTIR)

The samples were assessed by Fourier transform infrared spectrometric (FTIR) analysis before and after the application of heavy metals. The O-H stretching (3287.54 cm^{-1}) indicated bonding with alcohols, C-H stretching (2929.53 cm^{-1}) with alkanes, C=C stretching (1637.79 cm^{-1}) with alkenes, N-O stretching (1535.31 cm^{-1}) with nitro compounds, O-H bonding (1319.67 cm^{-1}) with phenols, C-O stretching (1229.99 cm^{-1}) with alkyl aryl ethers and C-O stretching (1055.04 cm^{-1}) with primary alcohol of *Bacillus anthracis* PM21 at different wavelengths (Figure. 3.7a). However, O-H stretching (3280.89 cm^{-1}) showed bonding with alcohols, C-H stretching (2922.60 cm^{-1}) with alkanes, C=C stretching (1633.93cm^{-1}) with alkenes, N-O stretching(1530.09cm^{-1}) with nitro compounds, C-H bending (1451.62 cm^{-1}) with alkanes, S=O stretching (1393.40 cm^{-1}) with sulfonyl chlorides, C-O stretching (1230.41 cm^{-1}) with alkyl aryl ethers and C-O stretching (1057.56 cm^{-1}) with primary alcohols on bacterial cell surface grown under Cd 200 mg/L stress at different wavelengths (Fig. 3.7b). Similarly, O-H stretching (32.82.84, 32.80.46 cm^{-1}) exhibited bonding with alcohols, C-H stretching (2923.52, 2920.35 cm^{-1}) with alkanes , C=C stretching (1634.48, 1631.11 cm^{-1}) with alkene, N-O stretching(1519.14, 1529.65 cm^{-1}) with nitro compound, C-H (1449.95, 1439.28 cm^{-1}) with alkanes, S=O stretching (1389.28, 1379.26 cm^{-1}) with sulfonyl chlorides, C-O stretching (1227.25, 1231.36 cm^{-1}) with alkyl aryl ethers and C-O stretching (1061.22, 1041.50 cm^{-1}) with primary alcohols on bacterial cell surface grown under Cr and Ni 200 mg/L stress at different wavelengths figure 3.3 (Fig. 3.7 c and d).

Table 3.4 In terms of functional groups involved in the biosorption process, the range of FTIR spectra is extensive

Absorptions in standard table	Without stress	Stress with Cd	Stress with Cr	Stress with Ni	Functional group	References
3550-3200	3287.54	3280.89	3282.84	3280.46	O-H stretching Alcohol	Liu et al., 2015
3000-2800	2929.53	2922.60	2923.52	2920.35	N-H-CH stretching Amine, alkanes	Liu et al., 2018
1650-1600	1637.79	1633.93	1634.48	1631.11	C=C Stretching Alkene	Tejeda-Serrano et al., 2020
1550-1500	1535.31	1530.09	1519.14	1529.65	N-O stretching Nitro compound	Ramu et al., 2021
1465	1391.67	1451.62, 1393.41	1449.95, 1389.28	1439.28, 1379.26	C-H, S=O Bending Alkane, sulfonyl chlorides	Safari et al., 2013; Xia et al., 2016
1275-1200	1229.99	1230.41	1227.25	1231.36	C-O, stretching Alkyl aryl ether,	Fereidouni et al., 2009
1124-1087	1055.04	1057.56	1061.22	1041.50	C-O stretching Secondary Alcohol	Fereidouni et al., 2009


BRUKER
(a)
PM21
Transmittance [%]
Wavenumber cm-1


BRUKER
Presence of Cd stress
(b)
Transmittance [%]
Wavenumber cm-1

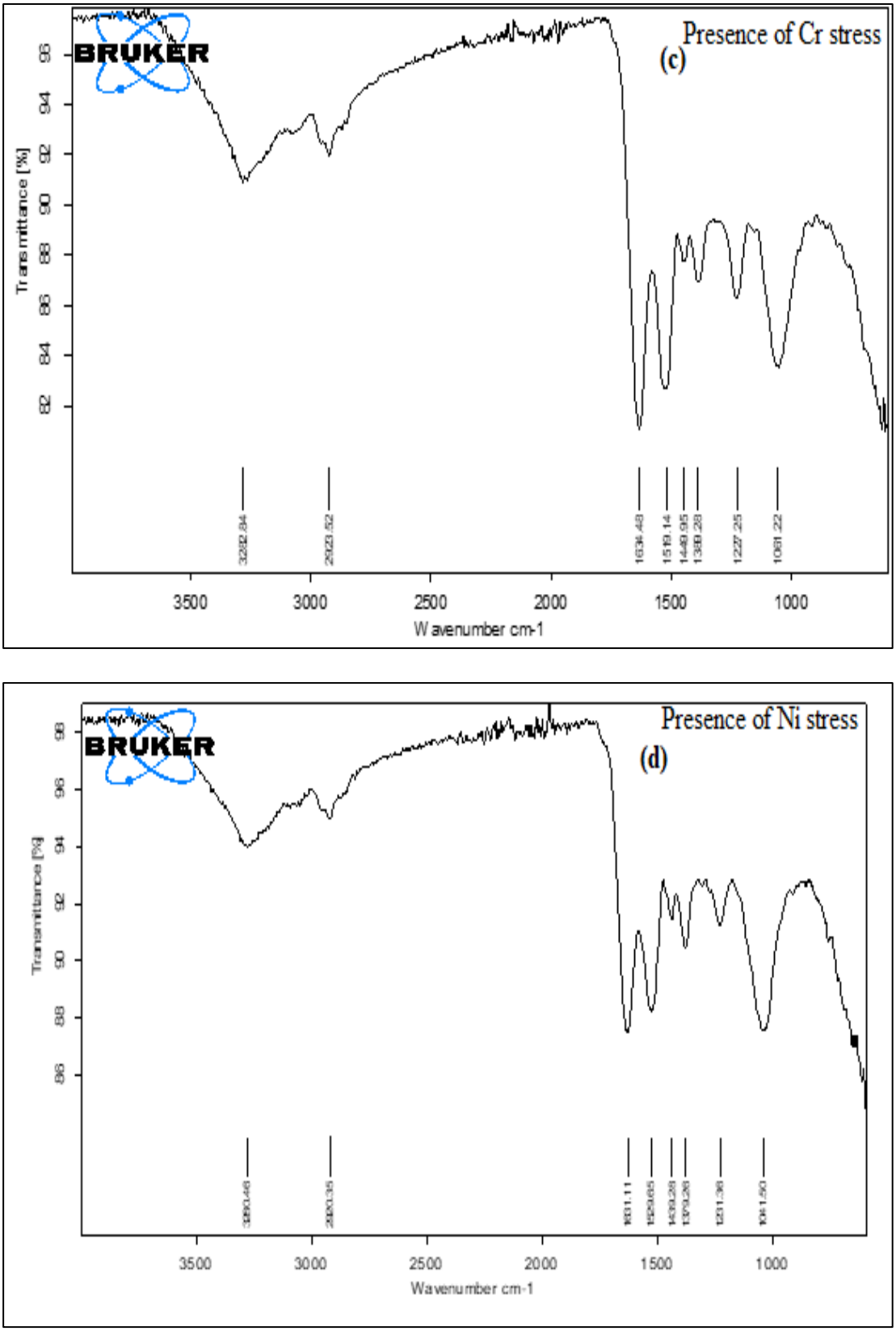
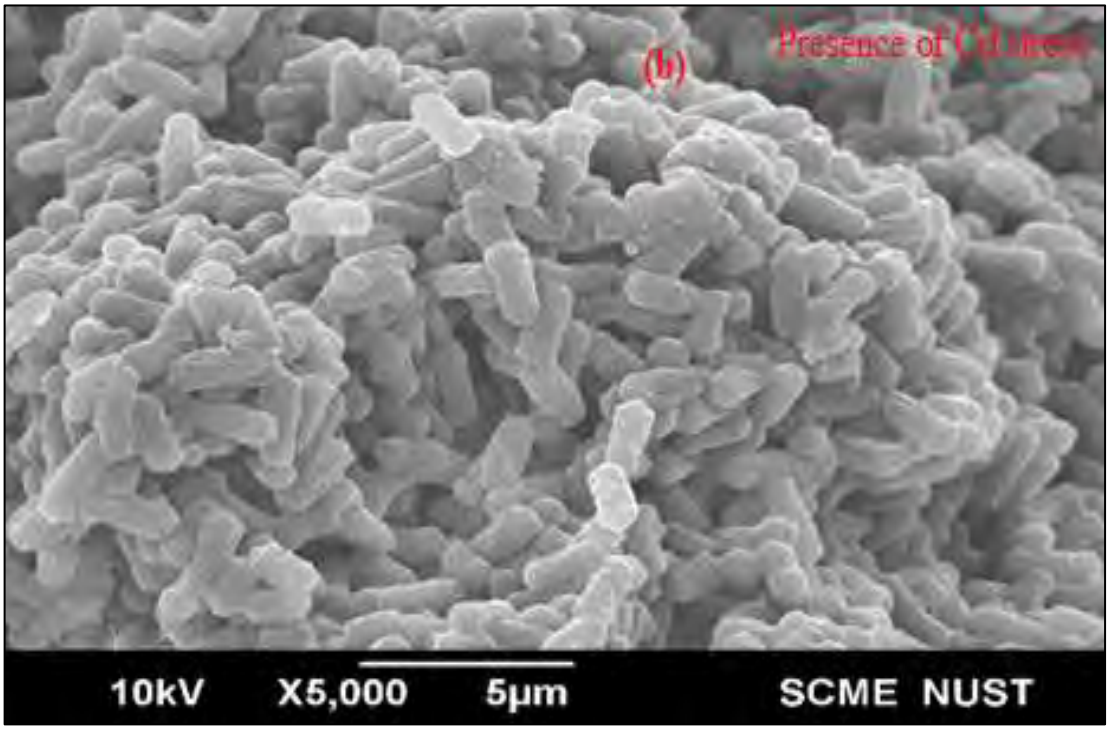


Figure 3.8. FTIR analysis of *Bacillus anthracis* PM21 (a), Cd (b), Cr (c), Ni (d) treated 200 mg L^{-1} cells of *Bacillus anthracis* PM21

3.3.7.2. Scanning electron microscope (SEM)

Figure 3.8 a presents unexposed cell of PM21 with smooth surface. The Scanning electron microscope analysis showed that with surface depressions, the bacterial cells exposed to Cd showed increase cell size and retained their shape relative to unexposed cells (Figure 3.8 b). Cr and Ni treated cells were rough with reduction in cell size (Figure 3.8 c and d).


(a)
(M2)
10kV X5,000 5µm SCME NUST


(b)
Presence of Cd (II)
10kV X5,000 5µm SCME NUST

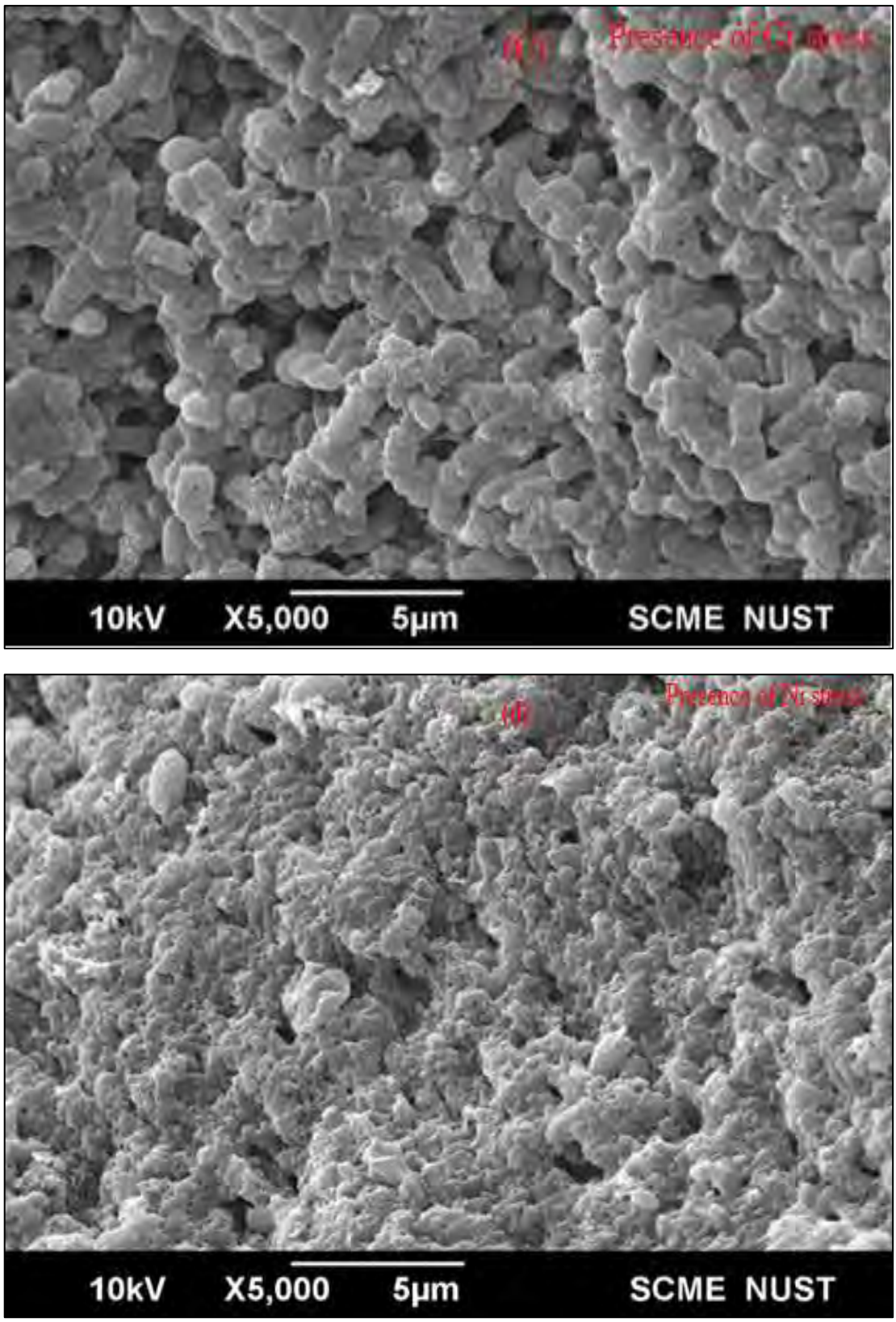


Figure 3.9. SEM analysis of *Bacillus anthracis* PM21 (a), Cd(b), Cr (c), Ni (d) treated 200 mg L^{-1} cells of *Bacillus anthracis* PM21

3.3.9. Amplification of (CzcD) gene

The gene responsible for Cd and Cr tolerance in bacteria, CzcD was amplified in the bacterial strains PM21, PM22, PM23, PM24 and PM25 previously showed tolerance against Cd and Cr in batch experiment (Figure 3.8).

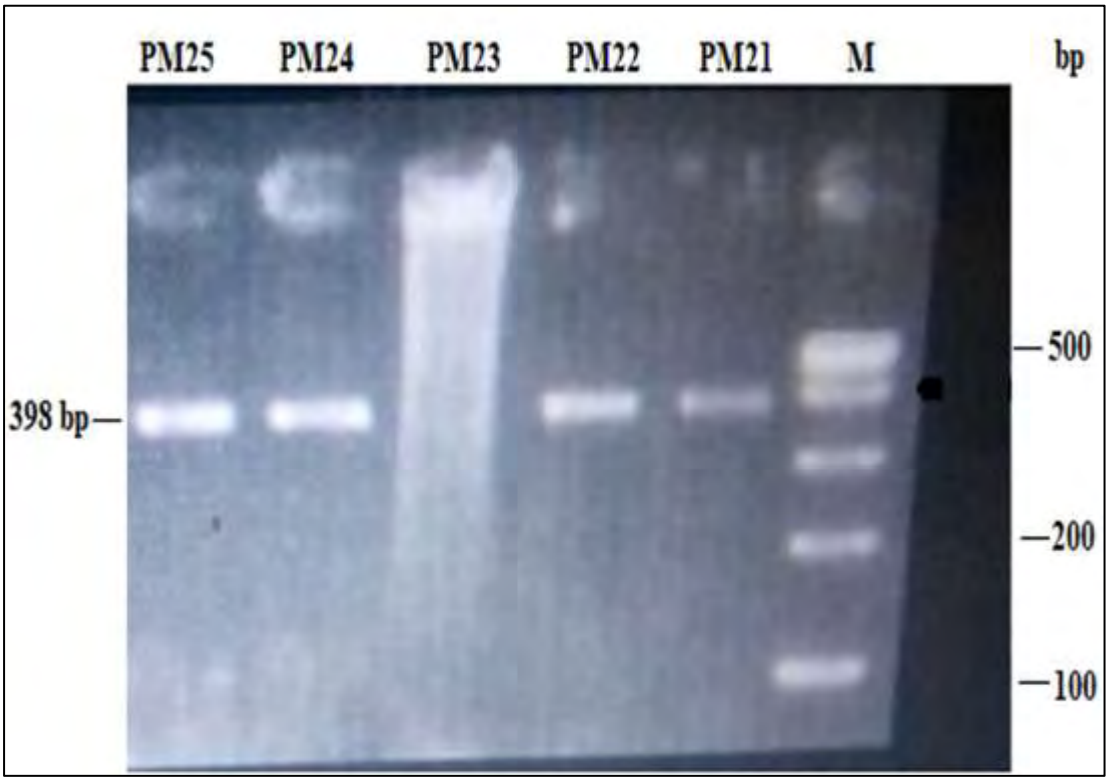


Figure 3.10. Image of an agarose gel showing amplification of the CzcD gene in M: ladder PM21, PM22, PM23, PM24 and PM25

3.4. Discussion

Plant growth promoting rhizospheric bacterial strains showed best results against different HMs Cadmium (Cd), chromium (Cr) and Nickel (Ni) (Table 3.1). The growth curve analysis of selected bacterial strains in the current study demonstrates their ability to tolerate high levels of Cr, Cd, and Ni (Figure 3.1). The Cd, Cr and Ni tolerance of *Bacillus cereus* RC-1 was previously reported (Arshad et al., 2017; Huang et al., 2014). However, we demonstrated its tolerance at metals concentrations higher than previous reports. High resistance potential of bacterial strains (PM21, PM22, PM23, PM24 and PM25) to several ions of metals and its growth status study counter to Cd, Cr and Ni mention it is capability to be utilized in biosorption of HMs.

Current experiment, some of the important parameters, for instance, contact time, and initial metal concentrations, affecting biosorption ability of different HMs. Among all major factors, pH is an important environmental factor affecting adsorption mechanism of heavy metals (Zhang et al., 2020). Structure of bacterial cell wall was studied to investigate various functional groups including carboxylic group, amino group, imidazole, organic acid, and phosphate (De-Farias et al., 2020). A minor fluctuation in pH range may alter the chemistry of solution and can influence the degree of ionization up to certain limit of the said functional groups (Gillani et al., 2017; Peng et al., 2018). The recent investigation revealed highest adsorption at pH 8 for Cd, 6 for Cr, and 4 for Ni by PM21 (Al-Dhabi et al 2019). While bacterial strains PM22, PM23, PM24, and PM25 showed highest adsorption capacity at pH 6 for Cd, 4 for Cr, and 4 for Ni, respectively. Our findings were in line with results of Gillania et al. (2017) in which *Agrobacterium tumefaciens* showed an adsorption of Cr and Pb at pH of 4 and 6, respectively. A minor fluctuation in pH affects the positive charges on the cell surface, which retard the attachment of metal ions (He et al., 2020).

Among all the factors affecting biosorption, the contact time of metal with biomass is another important factor that influences the biosorption potential (Haq et al., 2016). Biosorption experiment was carried out at various ranges of contact time (0–120 min) for all studied heavy metals at 35 ± 2 °C with fixed concentration of heavy metals (50 mg L^{-1}). Our results indicated that plant growth promoting rhizobacteria (PGPRs) exhibited maximum adsorption capacity at 60 min. Our results are strongly supported

by the research work of Gillania et al. (2017) and Ogata et al. (2020) as they also reported maximum adsorption capacity by bacterial strains at 60 min. In the initial, adsorption of target heavy metals increased quickly because of abundant accessibility of active binding sites of bacterial strains (Liu et al., 2018). However, with steady possession of active sites, the biosorption appeared less efficient (Pillai et al, 2013). Similar results were obtained previously using different sorbents (Kumar et al., 2006). Initial concentrations (25-200 mg L^{-1}) of HMs were used to check their adsorption potential under constant conditions of temperature, pH, and contact time. The adsorption capacity dramatically increased with increasing initial concentrations of metals. Increase in adsorption might be due to electrostatic connections between sorbent surface sites and metal ions (Al-gami, 2005; Bueno et al., 2008). The greater initial heavy metal concentration provides additional accessibility for the adsorption of metal ions. In addition, the rise in the different metal concentration allows all bacterial biomass transfer resistance of HMs to overcome increased push intensity. It also increases the possibilities the collision among metal ions and sorbent (Vimala and Das, 2009).

Adsorption is regarded as one of the very commonly used method for removal of pollutants from polluted water. Both isotherm constants of Freundlich and Langmuir indicated that heavy metals adsorption is favorable for PM21, PM22, PM23, PM24 and PM25 biomass. Langmuir, Freundlich linearized and Pseudo-second-order models were plotted between the Cd, Cr and Ni uptake (q_e) and the Cd, Cr, and Ni concentration in the solution C_e (Figure 3.3 a,b and table 3.2). Under the experimental circumstances used, the application of Langmuir isotherms to the biosorption of Cd, Cr, and Ni by PM21, PM22, PM23, PM24, and PM25 revealed monolayer biosorption and a homogeneous energy distribution of the active sites on the bacterial surface (Chi et al., 2020). In the case of PM21, the Freundlich model yielded correlation coefficients (R^2) of 0.9190 for cadmium, 0.9646 for chromium, and 0.9607 for nickel. PM22 (R^2 = cadmium 0.9470, chromium 0.9442, and nickel 0.8651), PM23 (R^2 = cadmium 0.9921, chromium 0.9841, and nickel 0.9980), PM24 (R^2 = cadmium 0.9947, chromium 0.9951, and nickel 0.9703), and PM25 (R^2 = cadmium 0.991, chromium 0.8456, and nickel 0.9052) are the correlation coefficients from the Freundlich model for various bacterial strains. The binding site affinities on the biomass surface are thought to fluctuate with

the interactions between the adsorbed molecules in the Freundlich model (David et al., 2003). The current investigation, the highest biosorption capacity (Q_{max}) in PM21 was found to be 22.08, 57.47, 5.34 mg/g for cadmium, chromium, and nickel, respectively. The Q_{max} for PM21 found in the study, on the other hand, was comparable to values found in previous literature using various biosorbents to remove a variety of HMs (Haq et al., 2016).

The uptake rate of sorbate is described by a biosorption kinetic analysis, and this rate obviously regulates the sorbate's residence time at the solid-liquid interface (Akinyeye et al., 2020). Rate limiting steps are determined by the useful kinetic parameters in the biosorbent study of HMs on the bacterial surfaces (Shehzad et al., 2020). External mass transfer of particle diffusion can be used to describe the solute transfer mechanism of solid-liquid biosorption (Aravindhan et al., 2009). Numerous independent methods, such as bulk film diffusion, chemisorption, and intra-particle diffusion, can be used to elucidate biosorption kinetics. The pseudo-second order model suited the biosorption data of the five bacterial strains well, with R^2 values of 0.960 for Cd, 0.978 for Cr, and 0.967 for Ni by PM21. Moreover, R^2 values for PM22, PM23, PM24 and PM25 are presented in (Table 3.2). The findings revealed that the rate of Cd, Cr, and Ni biosorption was governed by chemical interactions between functional groups on the surface of the biosorbent and HMs (Bulgariu and Bulgariu, 2012; Li et al., 2017). Metal ions will diffuse into the interior section of the cell via the cell membrane during the biosorption process (Sinha et al., 2018). As a result, biosorption data was fitted to the intra-particle diffusion model. The PM21 biosorption results fit the intra-particle diffusion model better than PM22, PM23, PM24, and PM25's, with R2 values of 0.993 for Cd, 0.895 for Cr, and 0.878 for Ni in this experiment. These findings demonstrated that bacterial strain PM21 displayed surface adsorption during the biosorption of Cd, Cr, and Ni. These effects could be attributed to metabolic and enzymatic activities that actively transport metal ions into the cell surface (Naik and Dubey, 2013; Mohapatra et al., 2019). The FT-IR spectral analysis was conducted for *Bacillus anthracis* PM21 and it demonstrated different functional groups such as alcohols, alkanes, alkenes, nitro compounds, sulfonyl chlorides, alkyl aryl ethers and primary alcohols of bacterial biomasses. During their interaction with HMs, these functional groups were intended to relate to polysaccharides and proteins on the cell surface (Du et al., 2012). The

measured peak values of 3280.89 cm^{-1} and 2922.60 cm^{-1} suggested that the alcohols were in the O-H stretching mode. On the surface of bacterial cells, the overlapping of alcohol and hydroxyl stretching was clearly visible (Mungasayalli et al., 2007; Albert et al., 2021). However, the Cd stress exposed cells, an unchanged absorption peak was observed at 2929.53 cm^{-1}, suggesting the O-H stretching of, polysaccharides, cell wall, proteins, nucleic acid, and lipids (Du et al., 2012). There was also a minor variation in the absorption peak from 1633.93 cm^{-1} to 1637.79 cm^{-1}, indicating the presence of C=C stretching predominantly connected with the mode of C=C deformation (Doshi et al., 2007; Yoon et al., 2017).

The SEM images of strain *B. anthracis* PM21 showed clear adsorption of Cd, Cr and Ni. Surface morphology was changed in case of Cr, and Ni (200 mg L^{-1}), indicating its dependence on the absorption of HMs. Surface complexation changes the surface structure, resulting in a progressive increase in cell surface roughness. Adsorption of heavy metal on bacterial surfaces is dependent on the parameters surface complexation changes the surface architecture, resulting in a progressive increase in cell surface roughness (Du et al, 2012). It has been well documented about the structural changes in reaction to stress as an adaptive response (Chakravarty et al., 2007; Nithy et al., 2011). Reduced surface area of bacteria was noted that is responsible for lowering the toxicity of environmental stresses (Nathya et al., 2011). It was attributed to decreased Cd precipitation or adsorption on the surface of bacterial cells (Dhal et al., 2010; Kumari et al., 2016). The bacterial strains PM21, PM23, PM24, and PM25 own the CzcD operon, which is responsible for Cd and Cr resistance. The CzcD operon is used to detoxify Cd and Cr by exporting it into the extracellular medium from the cytoplasm and/or periplasm (Legatzki et al., 2003). It is well documented that *Bacillus anthracis* PM21 *and Enterobacter cloacae* PM23 have *nifH* gene with ability to produce nitrogenase enzyme which perform significant role in nitrogen (N_2) fixation (Goswami et al., 2016; Yousuf et al., 2017; Jabir et al., 2020).

3.5. Conclusion

The *Bacillus anthracis* PM21 was selected from among the five bacterial strains based on its adsorption potential, according to the findings of the current investigation. The maximum adsorption capacity for the bacterial strain *Bacillus anthracis* PM21 was obtained in batch biosorption experiments at the optimum pH-8 for Cd, pH-6 for Cr, and pH-4 for Ni. After 60 min, maximum Cr, Cd, and Ni adsorption was observed for all the bacterial strains. The adsorption capacity (*qe*) of PM21 increased by 5-35 mg g for Cd, 4-24 mg g for Cr, 3-24 mg g for Ni with increasing initial heavy metals concentration. The biosorption of biosorbents have been well defined by pseudo-second order. It clearly suggested that the biosorption of heavy metals occurred through the chemisorption phenomenon. The presence of unique functional groups of bacterial cell walls in metal ion adsorption were predicted by Fourier-Transformed Infrared Spectroscopy. The Scanning Electron Microscopy results revealed the changes in cell surface morphology depending on the heavy metal concentration. This study suggested that the bacterial strain PM21 showed promising biosorption potential for the remediation of *Sesbania sesban* L. against Cadmium and Chromium.

Chapter. 4

To study effects of inoculation of best isolated strain on germination of seeds of *sesbania sesban* L. under heavy metals stress

4.1. Introduction

Metal exerts toxic effects not only on plant growth but also on proliferation of bacteria, however, metals tolerant plant growth promoting bacteria could survive, and promote plant growth. Wastewater generated due to industrialization and urbanization contain heavy metals and is discharged into the agricultural land (Malar et al., 2014; Mushtaq et al., 2020). Among all HMs cadmium (Cd) contamination has become a serious issue in various countries including Thailand, South Korea, China, Pakistan, Turkey, and India (Rafique et al., 2019; Latif et al., 2020). Highest level of Cd in the soil deteriorates plant growth and ultimately reduces the yield at seed germination as well as vegetative stage (Sharma et al., 2019; Ahmad et al., 2016). Cadmium induces ultra-structural changes and reduces the activities of antioxidant enzymes (Rizwan et al., 2016). Another toxic heavy metal is Cr (IV), that is broadly used in electroplating, tanneries, various textile coloring, and metal processing industries. Chromium concentration in the industrial effluents can spike as high as 270,000 ng/L (Gowd and Govil, 2008). Chromium (VI) is also regarded as a mutagen for humans (Edition, 2011). It is absorbed by the roots and transferred to the shoots and leaves of plants by sulphate transporter channels, where it can stop cell division (Plugaru et al., 2016). Chromium (VI) reaches to the hazardous levels at concentrations of 0.5-5 mg/kg in crops and 5–100 mg/kg in the soil (Plugaru et al., 2016). The Cr inhibits seed germination, hampers root and shoot development, causes leaf chlorosis, and leads to other negative physiological and biochemical changes (Plugaru et al., 2016). As both Cd and Cr are usually present together in same environment, phytoremediation of both metals by potential host plants can help in restoration of contaminated soils.

Various physical and chemical approaches are used to restore polluted soils, despite the fact that such approaches are expensive and detrimental for microbial diversity in soil (Gupta et al., 2016). Phytoremediation is an economical and ecofriendly approach to eradicate heavy metal contamination from the soils and water bodies (Ahmaruzzaman and Gupta, 2011; Rezania et al., 2016; Burakov et al., 2018; Rafique et al., 2019). Phytoremediation, coupled with ecofriendly bacteria, is a superior approach for recovering polluted environments (Gheju and Balcu, 2017; Sharma et al., 2018; Zainab et al., 2020, Din et al., 2020). Some rhizospheric microorganisms can support phytoremediation process due to their positive interactions with plants (Shameer and

Prasad, 2018; Kaur et al., 2019; Fatemi et al., 2020). Al-Baldawi et al. (2017) showed that bio-augmentation of *B. anthracis* as PGPR to a sedge species *Scirpus grossus* removed 84% of total petroleum hydrocarbons. The *B. anthracis* strain MHR2 promoted plant growth and conferred resistance to multiple metals and antibiotics in *Bracissa juncea* L. (Mukherjee et al., 2017). Heavy metal stress leads to conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, which is prime phytohormone involved in leaf senescence. However, some bacteria possess ACC-deaminase activity and divert the ethylene production pathway therefore increasing root and shoot dry biomass of plants (Utami et al., 2018). *Bacillus* sp. was reported to produce ACC-deaminase in addition to indole acetic acid (IAA), a growth promoting phytohormone, and enhance HM uptake in radish (Akhtar et al., 2018). To circumvent various abiotic stresses, bacteria produce exopolysaccharides (EPS) and biofilms, which enable bacteria to thrive by forming micro-colonies (Rafique et al., 2015; Amna et al., 2019; Bali et al., 2019).

Sesbania sesban L. (family Fabaceae) is a leguminous plant species, distributed in tropical countries (Gomase, 2012). Rhizospheric bacteria associated with the roots of *S. sesban* L. perform nitrogen fixation that can help plant to grow well in heavy metal contaminated soil (Gomase, 2012; Varun et al., 2017). However, mechanistic insight of *S. sesban* L. under dual metal stress (Cd and Cr combined) remains unclear. It was hypothesized that *S. sesban* L. can tolerate single as well as dual stress of Cd and Cr at seedling stage, which is important for its phytoremediation potential in natural conditions. Moreover, owing to its strong metal tolerance, harboring ACC-deaminase, IAA and EPS production activities, PM21 was introduced to *S. sesban* L. The objectives of this study were to evaluate (i) the effect of single as well as dual metal stress on seed germination, seedling growth and antioxidant activities of *S. sesban* L., (ii) to investigate metal stress tolerance of *S. sesban* L. with and without *B. anthracis* supplementation, and iii) to explore the phytoremediation potential of bacterial associated *S. sesban* L.

4.2. Materials and methods

4.2.1. Selection of heavy metals

Based on results of biosorption studies further remediation of cadmium and chromium was carried out using *Sesbania sesban* L. as a test plant with inoculation of *Bacillus anthracis* PM21.

4.2.2. Seed sterilization

The Sesbania sesban L. seeds were initially soaked for 5 min in 75% ethanol and then sterilized with 0.1% HgCl2 for 1 min (Ali et al., 2018). Bacterial cells were harvested from the broth culture by centrifugation at log phase, rinsed with (0.85g) sodium chloride and re-suspended in DDH_2O to maintain their population up to 10^9 CFU/mL (OD_{600}=1.0). In consecutive treatments, 2 mL of this bacterial suspension was applied to S. sesban L. seeds for 2-4 hours.

4.2.3. *In-vitro* seed germination of *S. sesban* L. seedlings

Seeds inoculated with *B. anthracis* PM21 (10 seeds/plate) were placed in autoclaved petri plates lined with double layer of Whatman No.1 and allowed to grow at different levels of Cr (25, 50, 75 mg/L at the rate of 3 mL/day), Cd (100, 150, 200 mg/L at the rate of 3 mL/day) and dual stress of Cr + Cd (25+100, 50+150, 75+200 mg/L at the rate of 1.5 mL/day each). For control treatments, the filter paper was moistened by adding double distilled water at the rate of 3 mL/day. Another set of Petri plate was kept with similar conditions except that the seeds were provided with 2 mL sterilized nutrient broth. Petri plates were placed in growth chamber at 60% relative humidity; 24±2ºC temperature; 14 h day and 10 h night photoperiod. Seed germination was assessed after every 24 h for 10 days consecutively.

4.2.4. Morphological parameters and germination percentage %

Seedlings were harvested after 10 days to determine their various biochemical and growth parameters (Muslu and Ergun, 2013). Percentage of seed germination was measured after 10 days using following formula (Ahmad et al., 2012):

$$\%\ Germination = (Germinated\ seeds)/(total\ seeds\ )\ \times 100$$

Root and shoot lengths of *S. sesban* L. seedlings were measured using a measuring scale. Fresh weight of seedlings was taken using an analytical balance at the time of harvest, while the dry weight (biomass) was taken after drying the seedlings in an oven at 70 °C for 3 days (Sapre et al., 2018).

4.2.5. Physiological attributes

The photosynthetic pigments of *S. sesban* L. seedlings were determined at the time of harvest, according to Pérez-Patricio et al. (2018).

$$\boldsymbol{Chlorophyll\ a\ =\ (12.7 * A663) - (2.49 * A645)}$$

$$\boldsymbol{Chlorophyll\ b\ =\ (12.9 * A645) - (4.7 * A663)}$$

$$\boldsymbol{Total\ Chlorophyll\ =\ (8.2 * A645) + (20.2 * A645)}$$

4.2.6. Electrolyte leakage (ELL)

Electrolyte leakage (ELL) was measured as reported by Ahmad et al. (2016):

$$\boldsymbol{Electrolyte\ leakage\ (ELL) = \frac{Electrical\ conductivity1}{Electrical\ conductivity2} \times 100}$$

4.2.7. Proline

Proline content was measured following standard protocols (Ahemad, 2012; Sing et al., 2020). In a nutshell, 0.1 g of leaf was grinded in 4 mL sulphosalicylic acid of 3% concentration and left overnight. After centrifugation of mixture at 3000 × g for 5 min, glacial acetic acid and ninhydrin were added (Pro Economy, Centurion Scientific, UK). The suspension was then heated in a water bath at 100°C for 1 hour before cooling in an ice bath. Proline from the suspension was obtained using toluene as solvent and quantified by recording absorbance at 520 nm. The proline concentration was measured as μg/g of fresh weight by standard curve analyses and expressed.

$$\boldsymbol{Proline\ =\ \frac{k\ value \times dilution\ factor \times absorbance}{sample\ weight}}$$

4.2.8. Malondialdehyde (MDA)

The malondialdehyde (MDA) activity was determined using Thiobarbituric acid reactive substances (TBARS), as reported previously (Prochazkova et al., 2001).

$$\boldsymbol{MDA = 6.45\,(A532 - A600) - 0.56\,A440}$$

4.2.9. Antioxidant's enzymes

The SOD activity was determined following previously published protocols (Afridi et al., 2019; Sing et al., 2020). The method of Khalilzadeh et al. (2020) was used for the determination of peroxidase and catalase activity.

4.2.10. Analysis of Cr and Cd contents in *S. sesban* L. seedlings

Atomic absorption spectrophotometry-FAAS (Varian FAAS-240, Triad Scientific, USA) based Cr and Cd concentrations in seedlings of *S. sesban* L. were quantified from sample prepared through wet acid digestion method (Wan et al., 2012). The FAAS instrument was calibrated with standards (R^2=0.9999) provided by the company (SRM 3108 for Cadmium; SRM 2701 for hexavalent Chromium) to ensure quality along with the blank. The accuracy of Cr and Cd measurements was ensured by periodic standardization of FAAS instrument at intervals of every 10 samples with standards of both metals. Detection limit of FAAS instrument is 0.002 µg/L for Cd and 0.004 µg/L for Cr.

4.2.11. Statistical analysis

The data obtained followed normal pattern of distribution (p>0.05) according to Shapiro–Wilk test (Table 4.2) (Wang and Riffel, 2011), was executed on SPSS software (IBM SPSS Statistics 21). Microsoft Excel sheet was used for data compilation and statistical analysis. Treatments (9 levels of heavy metal) and bacterial inoculation (2 levels) were arranged in completely randomized design (CRD)-factorial with three replications. Analysis of variance (ANOVA) (Table 4.1) was performed in Statistix version 8.1. Data were presented as means ± *SE*; statistical significance among the treatments was determined using LSD value at p≤0.05.

4.3. Results

4.3.1. Analysis of variance and Shapiro Wilk test

For all of the parameters tested, the analysis of variance revealed extremely significant differences (Table 4.1). Shapiro Wilk test exhibited normal distribution for the studied parameters among 10 treatments (Table 4.2). All the parameters exhibited significant value of either 0.05 or greater than 0.05 that suggested the normal distribution of the data.

Table 4.1. For the study variables, analysis of variance revealed a highly significant difference

Source	**MS**
Germination % un-inoculated	550.459**
Germination % inoculated	740.148**
Root length un-inoculated	3.14116**
Root length inoculated	6.33515**
Shoot length un-inoculated	4.15689**
shoot length inoculated	4.43719**
Fresh weight un-inoculated	0.13617**
Fresh weight inoculated	0.19152**
Dry weight un-inoculated	0.00536**
Dry weight inoculated	0.00877**
Chlorophyll a un-inoculated	0.01995**
Chlorophyll a inoculated	0.04332**
Chlorophyll b un-inoculated	0.46076**
Chlorophyll b inoculated	0.75337**
Total Chl un-inoculated	0.07652**
Total Chl inoculated	0.13529**
Proline un-inoculated	0.17457**
Proline inoculated	0.02052**
ELL % un-inoculated	293.637**
ELL % inoculated	208.938**
SOD un-inoculated	0.41475**
SOD inoculated	0.11698**
POD un-inoculated	0.03941**
POD inoculated	0.01257**
Catalase un-inoculated	0.02097**
Catalase inoculated	0.00203**
MDA un-inoculated	0.02331**
MDA inoculated	0.01327**

**, **= Significantly 5 and 1 % probability level, respectively. ns = significant.*

Table 4.2. Shapiro Wilk test showed normal distribution for all the studied parameters

Parameters	Treatments	Shapiro-Wilk Test		
		Statistic	Df	Sig.
Germination	un-inoculated	0.933	10	0.478
	Inoculated	0.914	10	0.309
R L	un-inoculated	0.855	10	0.067
	Inoculated	0.957	10	0.754
SL	un-inoculated	0.903	10	0.237
	Inoculated	0.877	10	0.12
FW	un-inoculated	0.738	10	0.06
	Inoculated	0.817	10	0.24
DW	un-inoculated	0.824	10	0.08
	Inoculated	0.982	10	0.975
Chl a	un-inoculated	0.891	10	0.176
	Inoculated	0.919	10	0.349
Chl b	un-inoculated	0.935	10	0.494
	Inoculated	0.959	10	0.779
Total Chl	un-inoculated	0.839	10	0.053
	Inoculated	0.874	10	0.821
Electrolyte leakage	Inoculated	0.899	10	0.212
	Inoculated	0.97	10	0.887
MDA	un-inoculated	0.977	10	0.946
	Inoculated	0.757	10	0.054
SOD	un-inoculated	0.983	10	0.98
	Inoculated	0.947	10	0.636
POD	un-inoculated	0.755	10	0.064
	Inoculated	0.873	10	0.109
CAT	un-inoculated	0.714	10	0.051
	Inoculated	0.957	10	0.752
Cr	un-inoculated	0.81	10	0.051
	Inoculated	0.783	10	0.059
Cd	un-inoculated	0.834	10	0.058
	Inoculated	0.835	10	0.059
BAF Cr	un-inoculated	0.88	10	0.132
	Inoculated	0.863	10	0.083
BAF Cd	un-inoculated	0.635	10	0.053
	Inoculated	0.873	10	0.11

RL= root length, SL= shoot length, Cd = cadmium, Cr = chromium, POD = peroxidase, SOD = superoxidase dismutase, CAT = catalase, MDA = malondialdehyde, chl a = chlorophyll a, chlorophyll b, total chlorophyll, BAF= Bioaccumulation factor

4.3.2. Analyses of *in-vitro* seed germination

High concentrations of Cr and Cd metals significantly reduced *in-vitro* seed germination of *S. sesban* L., whether these were applied individually or combined (Fig. 2; Table 3). Maximum seed germination was observed in bacterial-inoculated and un-inoculated treatments without metal stress (control treatments). Severe reduction in seed germination (89, 78 and 61%) was observed in plants under maximum stress (100, 150 and 200 mg/L) of Cd. Seeds inoculated with *B. anthracis* PM21 showed high seed germination (97, 87 and 71%) than their respective un-inoculated control seeds (Table 4.3).

4.3.3. Seedling length and biomass

Root and shoot lengths of *S. sesban* L. were negatively correlated with Cr and Cd concentrations (Figure 4.1 and Table 4.3). Seedlings without bacterial inoculation in treatment T9 (Cr 75 mg/L + Cd 200 mg/L) exhibited severe decrease in root and shoot lengths in comparison to their respective controls. Inoculation of bacteria led to increase in root and shoot lengths even under heavy metals stress.

Fresh weight of *S. sesban* L. was reduced to 54% in treatment T9 in un-inoculated treatment as compared to control. However, inoculated treatment produced significantly high (16.66%) fresh weight (Table 4.3). Application of Cr and Cd significantly reduced plant dry weight. Maximum reduction in dry weight of plant was observed in un-inoculated (89%) and inoculated (85%) seedlings in treatment T6. Maximum plant dry weight (0.23 g) was recorded for the inoculated control plants (treatment T1); dry weight decreased with increasing metal stress. Minimum dry weight (0.03 g) was recorded for seedlings in treatment T6 (Table 4.3).

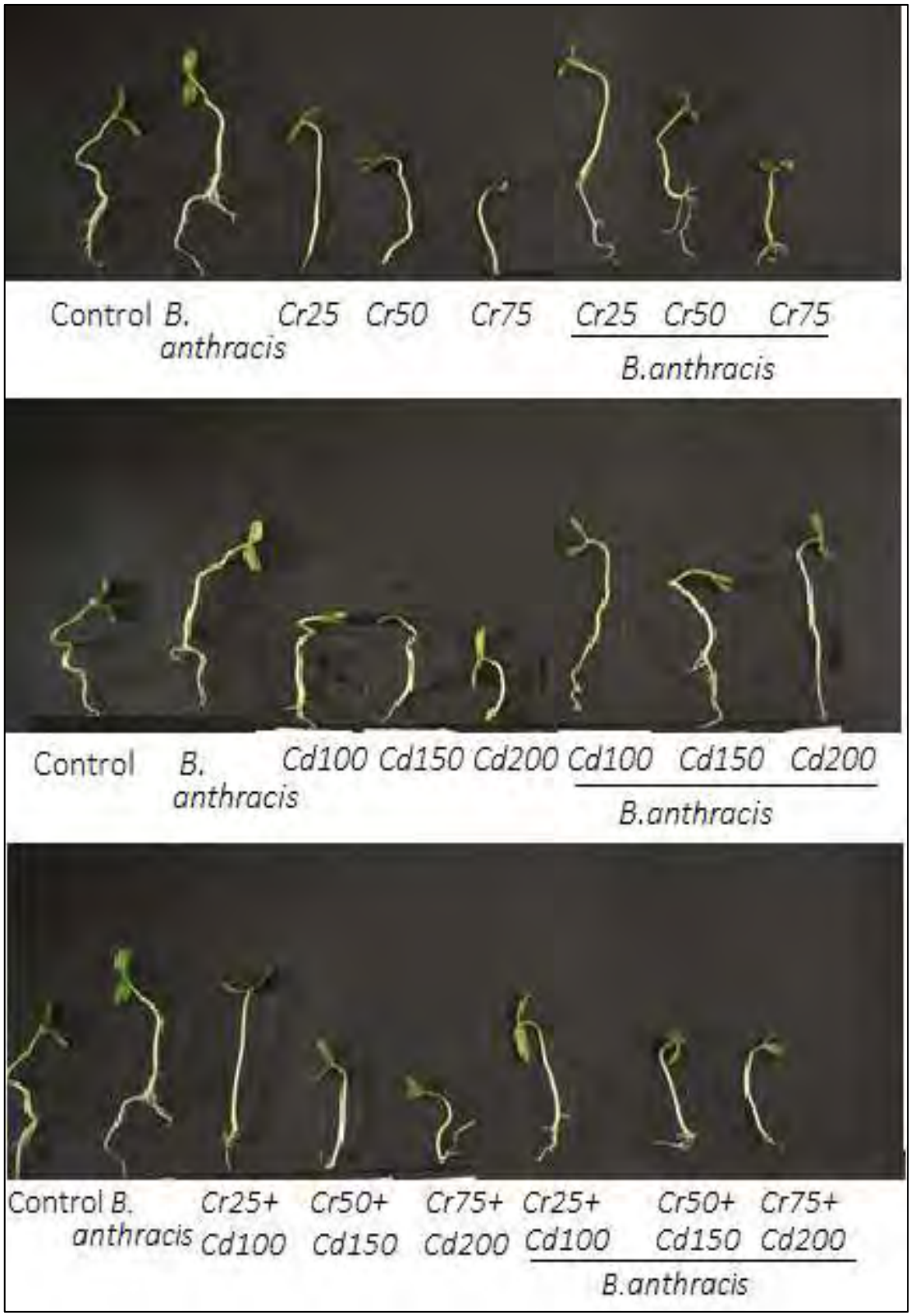


Figure 4.1. Qualitative PM21 effects of *Bacillus anthracis* inoculation on growth of *S. sesban* L. seedlings under various levels of Cr (25-75 mg/L) and Cd (100-200 mg/L).

Table 4.3. Comparison among percentage, growth parameters of *S. sesban* L. seedlings with and without inoculation of *Bacillus anthracis* PM21 grown under variable heavy metal stress conditions

Treatments	Seed germination (%)		Root length (cm)		Shoot length (cm)		Fresh weight (g)		Dry weight (g)	
	Un-inoculated	Inoculated	Un-inoculated	Inoculated	Un-inoculated	Inoculated	Un-inoculated	Inoculated	Un-inoculated	Inoculated
T0	94.00±0.86^{c}	99.00±1.92^{a}	4.10±0.03^{c}	5.13±0.04^{a}	6.60±0.03^{c}	8.33±0.03^{a}	0.26±0.01^{f}	0.35±0.06^{e}	0.16±0.03^{b}	0.23±0.02^{a}
T1	89.00±1.92^{d}	96.00±1.36^{b}	2.30±0.01^{f}	4.90±0.02^{b}	5.70±0.04f^{g}	6.10±0.05d^{e}	0.17±0.01^{hi}	0.24±0.03^{fg}	0.12±0.05^{d}	0.14±0.02^{c}
T2	78.67±2.31^{g}	84.67±3.81^{e}	1.97±0.02^{gh}	3.13±0.02^{d}	4.03±0.06^{n}	5.10±0.04^{ij}	0.12±0.02^{ijk}	0.20±0.05^{gh}	0.07±0.04^{gh}	0.11±0.22^{e}
T3	64.33±4.33^{j}	66.33±4.75^{i}	0.87±0.05^{lm}	1.70±0.03^{i}	3.47±0.04^{o}	4.37±0.09^{m}	0.10±0.04^{k}	0.16±0.04^{hij}	0.06±0.04^{ij}	0.08±0.00^{fg}
T4	**89.67±2.21^{d}**	**97.01±2.30^{b}**	**2.32±0.03^{f}**	**5.73±0.04^{d}**	**5.87±0.07e^{f}**	**6.23±0.04^{d}**	**0.77±0.01^{b}**	**0.90±0.01^{a}**	**0.17±0.04^{hi}**	**0.19±0.00^{f}**
T5	**78.33±3.27^{g}**	**87.00±3.48^{f}**	**1.99±0.04^{g}**	**3.17±0.00^{e}**	**4.80±0.08k^{l}**	**6.03±0.04^{de}**	**0.47±0.08^{d}**	**0.67±0.04^{c}**	**0.08±0.02l**	**0.16±0.05^{gh}**
T6	**61.33±2.50^{i}**	**71.00±3.48^{k}**	**1.37±0.01^{l}**	**2.40±0.03^{f}**	**3.50±0.15^{o}**	**5.30±0.08h^{i}**	**0.27±0.07^{f}**	**0.47±0.06^{d}**	**0.09±0.02^{m}**	**0.13±0.06^{l}**
T7	74.33±2.79^{h}	84.00±3.94^{f}	1.50±0.01^{j}	1.87±0.05^{h}	6.07±0.12^{de}	8.30±0.04^{b}	0.14±0.04^{ijk}	0.17±0.03^{hi}	0.08±0.01^{fg}	0.12±0.02^{dl}
T8	59.67±0.40^{m}	66.00±1.73^{k}	1.20±0.02^{k}	1.73±0.04^{i}	5.03±0.15^{jk}	5.53±0.04^{gh}	0.12±0.05^{ijk}	0.14±0.04^{ijk}	0.07±0.03^{hi}	0.08±0.01^{fg}
T9	56.00±7.41^{n}	59.00±0.14^{o}	0.57±0.02^{n}	0.83±0.03^{m}	3.53±0.15^{o}	4.56±0.02^{lm}	0.10±0.04^{jk}	0.12±0.01^{ijk}	0.04±0.11^{kl}	0.05±0.01^{jk}

T0: control (without any metal stress); T1–T3 represent treatments under Cr stress at 25, 50 and 75 mg/L concentrations, respectively; T4–T6 represent treatments under Cd stress at 100, 150 and 200 mg/L, respectively, while T7–T9 represent treatments with combined stress of Cr and Cd at 25+100, 50+150, and 75+200 mg/L for Cr+Cd, respectively. The superscripts indicate significance between inoculated and un-inoculated conditions at $p \leq 0.05$ level. Values are presented as means ± *SE* (n = 3).

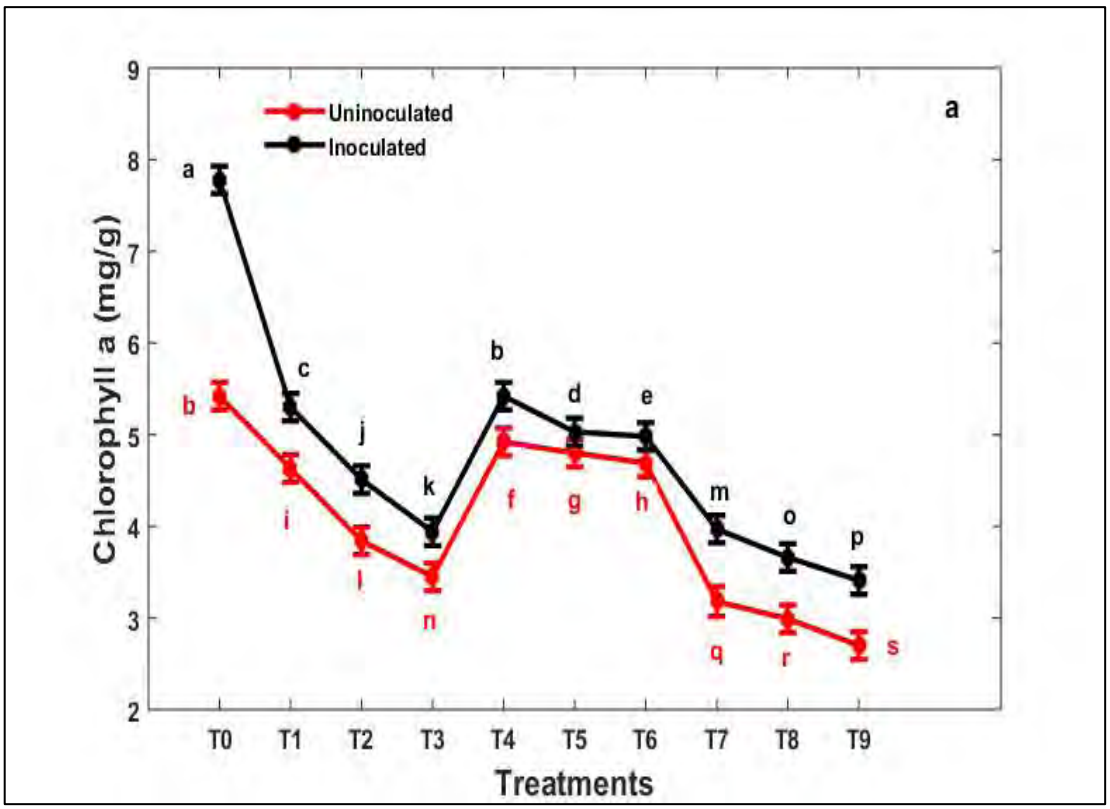
4.3.4. Quantification of photosynthetic pigments, proline content and electrolyte leakage

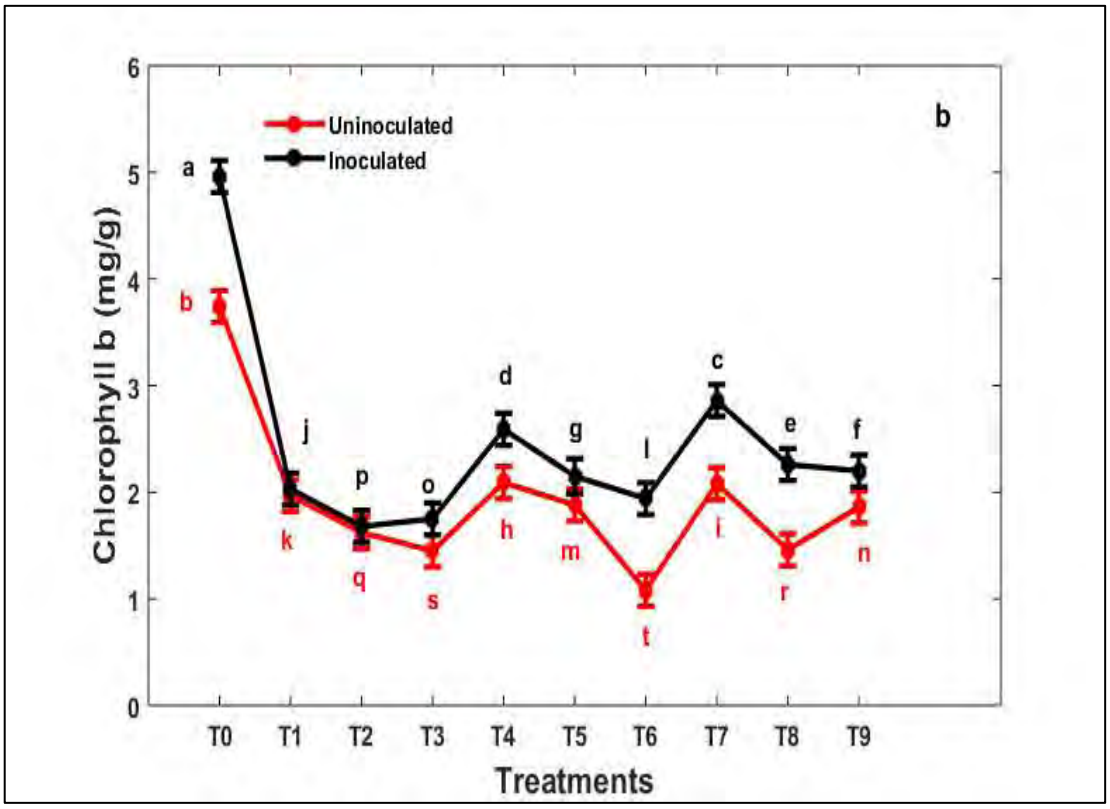
Chlorophyll *a* and chlorophyll *b* content of *S. sesban* L. seedlings were significantly lowered with either individual or combined application of Cr and Cd ($P > 0.05$) (Figure 4.2a and 4.2b, respectively). Exposure of Cr (75 mg/L) reduced chlorophyll *a* content up to 36%; Cd (200 mg/L) reduced it up to 14%, while both metals in combination (75+200 mg/L) reduced chlorophyll *a* up to 50%, as compared to their un-inoculated control ($P > 0.05$). However, inoculation of *B. anthracis* PM21 significantly increased chlorophyll content. Chlorophyll *a* and *b* increased by 21% and 16% respectively, contrasted to control ($P > 0.05$) (Figure 4.2a and 4.2b).

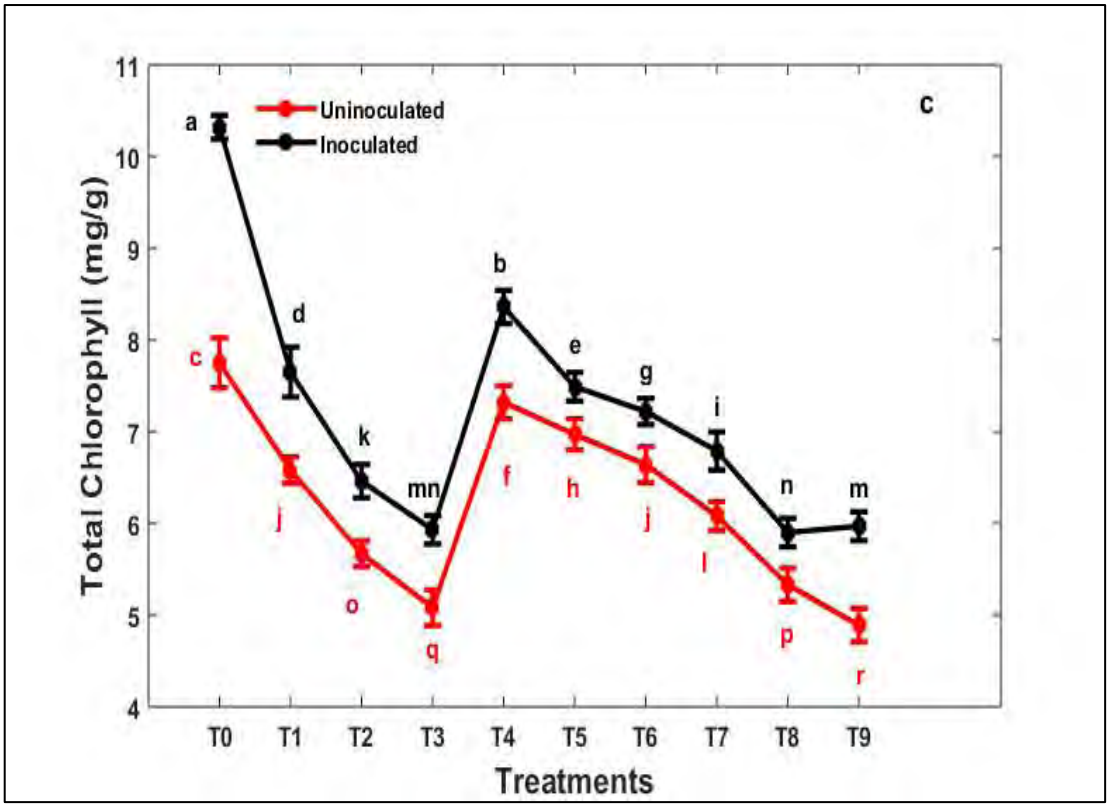
Total chlorophyll content also decreased significantly with individual and combined application of Cr and Cd in *S. sesban* L. seedlings ($P > 0.05$) (Figure 4.2c). Exposure of Cr (75 mg/L) caused 34% reduction in total chlorophyll, Cd (200 mg/L) reduced it by 14%, while dual metal stress of Cr+Cd (75+200 mg/L) reduced total chlorophyll up to 41%, in comparison to un-inoculated controls ($P > 0.05$). However, *B. anthracis* PM21 inoculation significantly increased total chlorophyll content by 18% as compared in un-inoculated control ($P > 0.05$).

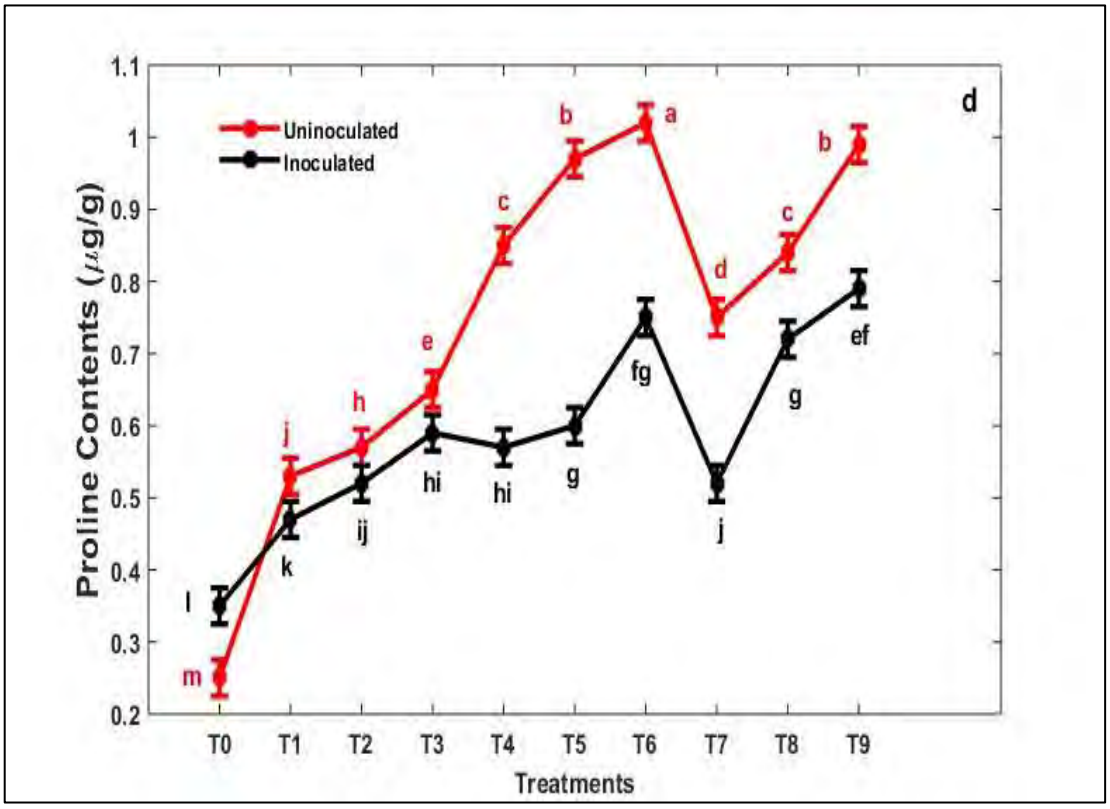
Proline as an amino acid is synthesized in high quantity under stress conditions. Proline concentration was noticed as directly proportional to individual and combined application of Cr and Cd (Figure 4.2d). Exposure of Cr (75 mg/L) increased proline content by 10%, Cd (200 mg/L) up to 36%, while both Cr+Cd (75+200 mg/L) increased proline content up to 25% in un-inoculated treatments, as compared to inoculated controls ($P > 0.05$). Observed data shows significantly beneficial effects of *B. anthracis* PM21 strain on photosynthetic pigments of *S. Sesban* L. seedlings in the study.

Dual metal stress of Cr+Cd (75+200 mg/mL) significantly enhanced electrolyte leakage in comparison to their control ($P > 0.05$) counterparts (Figure 4.2e). The application of bacterial strain (PM21) significantly reduced (50%) electrolytic leakage from the seedling membrane (Figure 4.2e).


Uninoculated
Inoculated
a
Chlorophyll a (mg/g)
Treatments
T0 T1 T2 T3 T4 T5 T6 T7 T8 T9


Uninoculated
Inoculated
b
Chlorophyll b (mg/g)
Treatments
T0 T1 T2 T3 T4 T5 T6 T7 T8 T9


c
Uninoculated
Inoculated
Total Chlorophyll (mg/g)
Treatments
T0 T1 T2 T3 T4 T5 T6 T7 T8 T9
a d k mn b e g i n m
c j o q f h j l p r


d
Uninoculated
Inoculated
Proline Contents (μg/g)
Treatments
T0 T1 T2 T3 T4 T5 T6 T7 T8 T9
l k ij hi hi g fg j g ef
m j h e c b a d c b

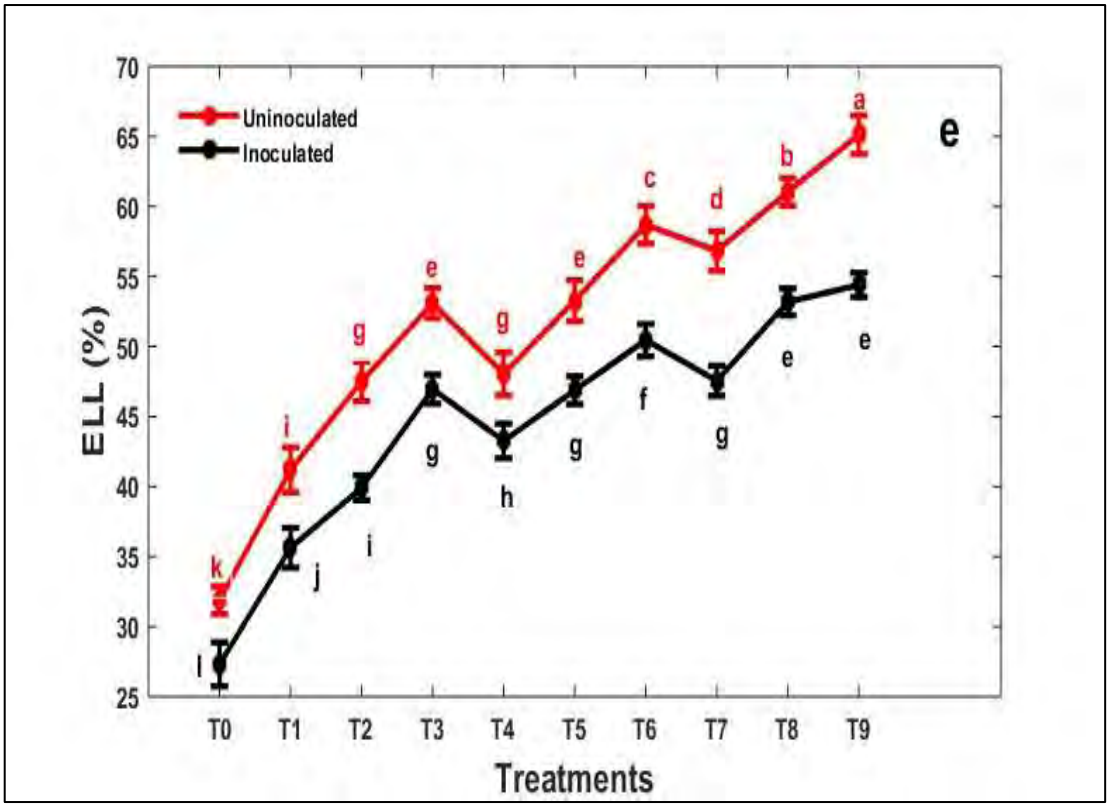
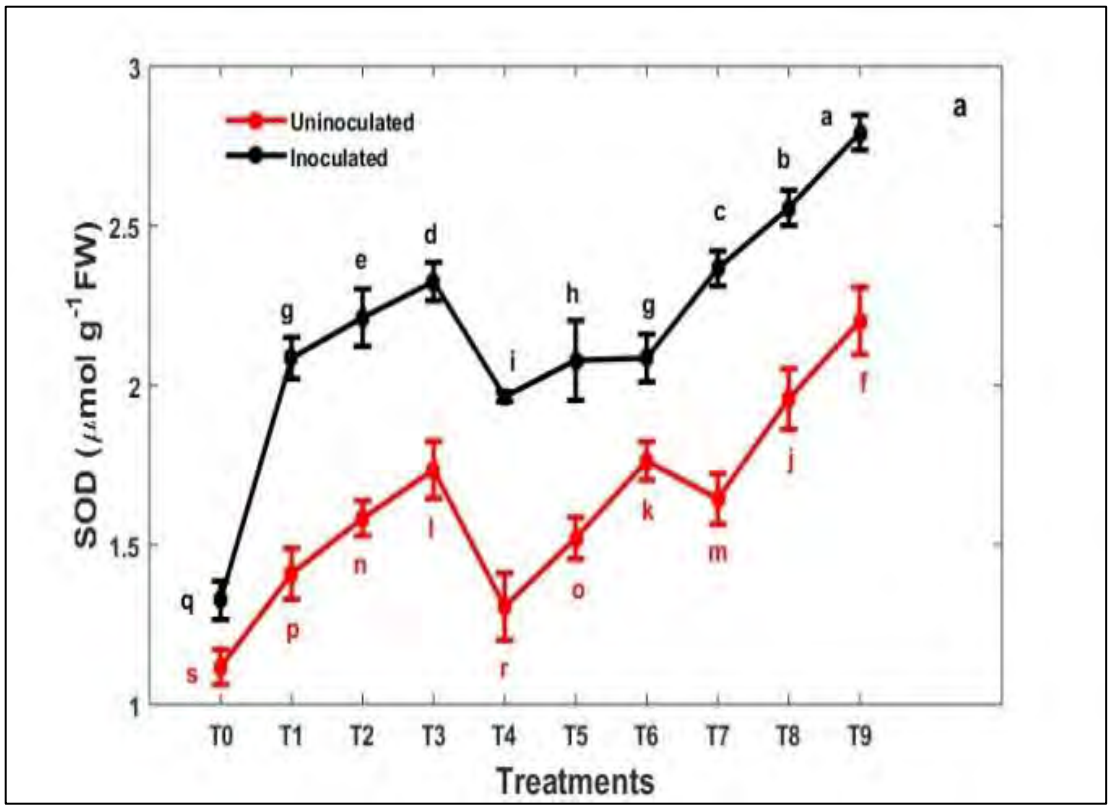


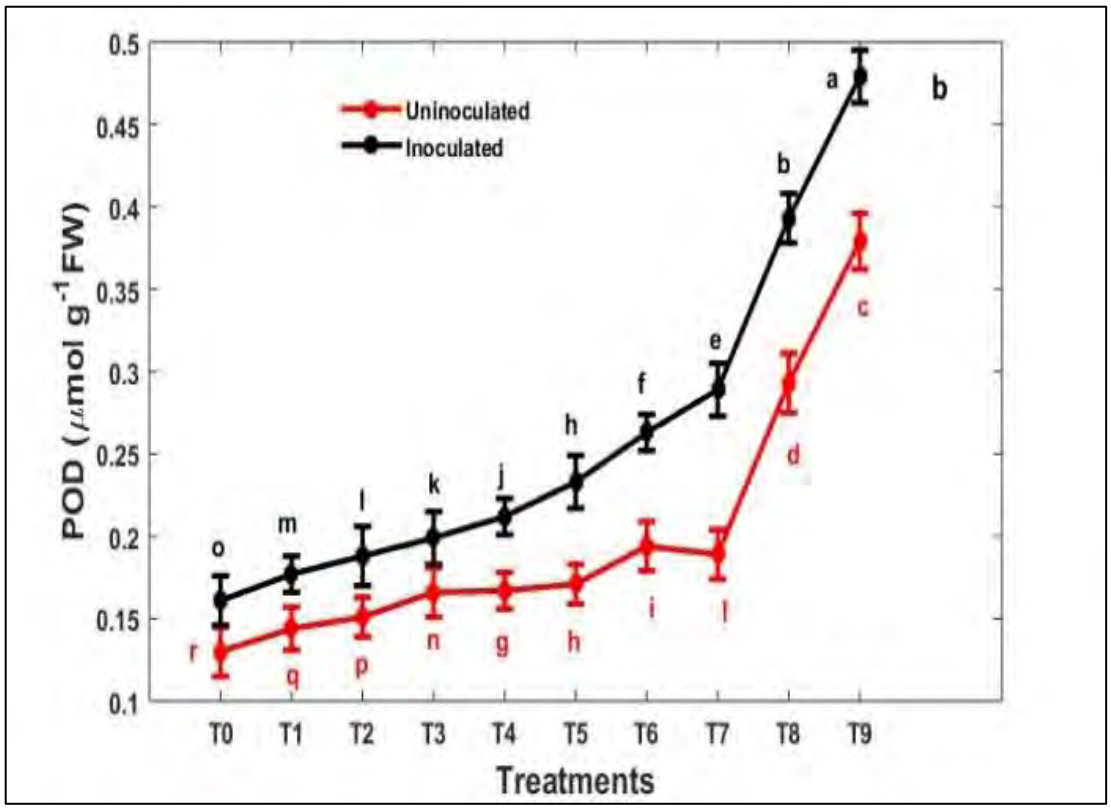
Figure 4.2. Effects of *Bacillus anthracis* PM21 inoculation coupled with heavy metal stress on chlorophyll *a* (a), chlorophyll *b* (b), total chlorophyll (c) proline content (d) and electrolyte leakage (ELL) (e) of *S. sesban* L. seedling. T0: control (without any metal stress); T1–T3 represent treatments under Cr stress at 25, 50 and 75 mg/L concentrations, respectively; T4–T6 represent treatments under Cd stress at 100, 150 and 200 mg/L, respectively, while T7–T9 represent treatments with combined stress of Cr and Cd at 25+100, 50+150, and 75+200 mg/L for Cr+Cd, respectively. Each treatment value is presented as means of three replicates (n = 3) with *SE*. Different letters at each treatment indicate significance between inoculated and un-inoculated conditions at $p \leq 0.05$ level.

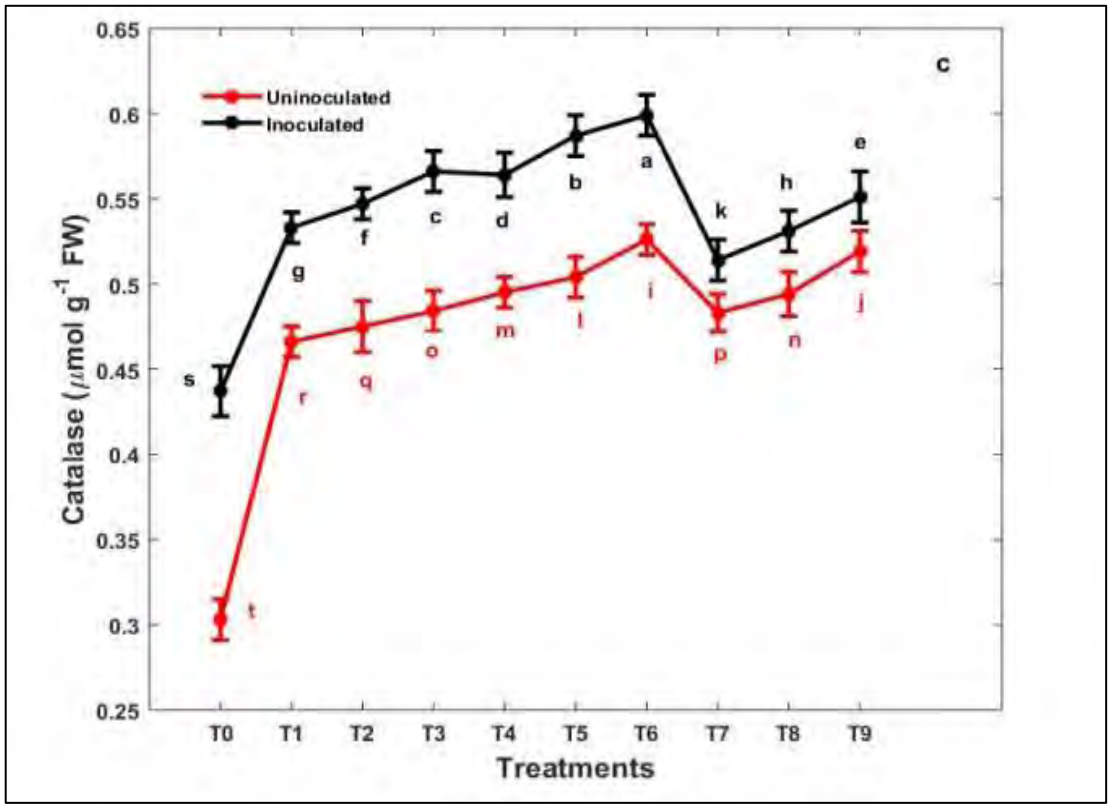
4.3.5. Quantification of antioxidant enzyme activities

Antioxidant activities of SOD, POD and CAT enzymes were determined in *S. sesban* L. seedlings exposed to single (Cr, Cd) and dual metal stress (Cr+Cd) conditions (Figure 4.3). The exposure of seedlings at different levels of Cr, Cd or Cr+Cd induced significant increase in all antioxidants as compared to control treatment (T0) ($P > 0.05$). Dual metal stress as Cr+Cd (75+200 mg/L) enhanced activities of SOD (36%), POD (66%) and CAT (27%) enzymes, as compared to un-inoculated control (T0). In addition, inoculation of *S. sesban* L. seedlings with *B. anthracis* PM21 strain further

enhanced SOD, POD and CAT activities in all treatments as compared to their un-inoculated counterparts ($P > 0.05$) (Figure 4.3a-c). Maximum increase in SOD (34%) and POD (35%) were observed for combined application of Cr+Cd (75+200 mg/L), while CAT activity (31%) was observed in inoculated seedlings treated with Cd (200 mg/L), as compared to un-inoculated counterparts. Contrary to these observations, malondialdehyde (MDA) content of *S. sesban* L. decreased in all treatments of bacterial inoculation. Maximum decrease in MDA was observed for inoculated seedlings with Cr+Cd (75+200 mg/L) as compared to their respective un-inoculated treatments (Figure 4.3d).




b
Uninoculated
Inoculated
POD (μmol g-1 FW)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
a
b
c
d
e
f
h
j
k
l
m
o
r
q
p
n
g
h
i
l
T0 T1 T2 T3 T4 T5 T6 T7 T8 T9
Treatments


c
Uninoculated
Inoculated
Catalase (μmol g-1 FW)
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
a
b
c
d
e
f
g
h
k
s
t
r
q
o
m
l
i
p
n
j
T0 T1 T2 T3 T4 T5 T6 T7 T8 T9
Treatments

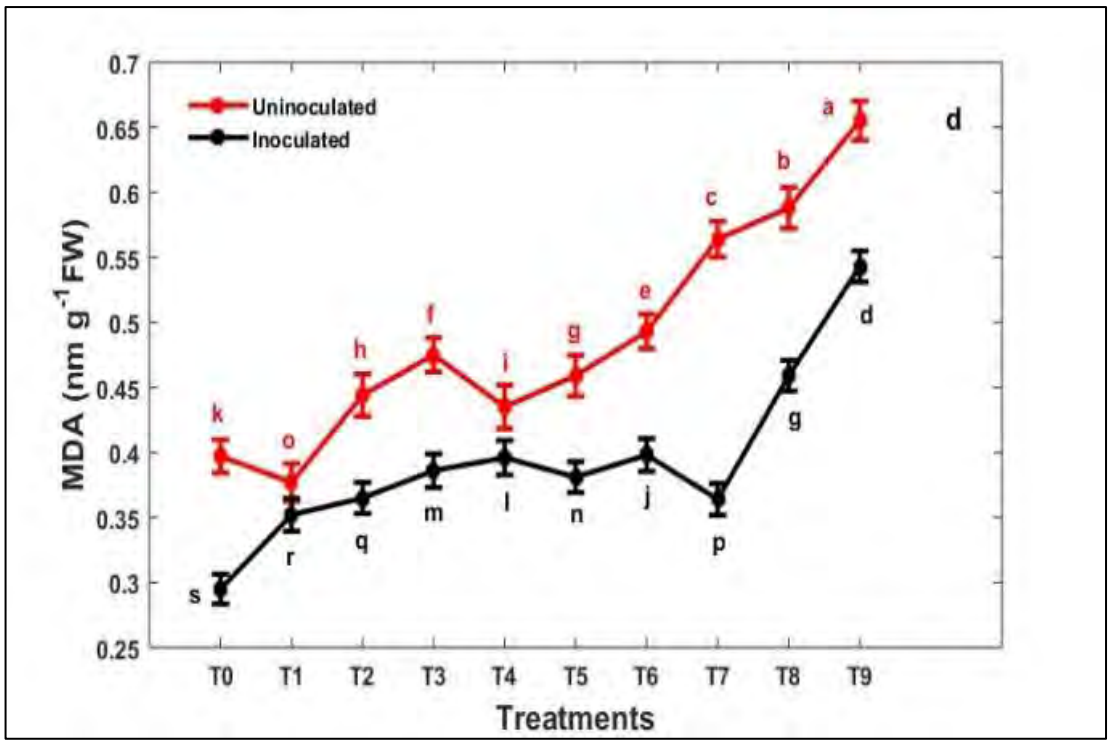


Figure. 4.3. Effects of *Bacillus anthracis* inoculation and heavy metals on SOD (a), POD (b), Catalase (c) and MDA (d) of *S. sesban* L. seedling. T0: control (without any metal stress); T1–T3 represent treatments under Cr stress at 25, 50 and 75 mg/L concentrations, respectively; T4–T6 represent treatments under Cd stress at 100, 150 and 200 mg/L, respectively, while T7–T9 represent treatments with combined stress of Cr and Cd at 25+100, 50+150, and 75+200 mg/L for Cr+Cd, respectively. Each treatment value is presented as means of three replications (n = 3) with *SE*. Different letters at each treatment indicate significance between inoculated and un-inoculated conditions at $p \leq 0.05$ level.

4.3.6. Increase in metal assimilation in germinating seedlings after bacterial inoculation

The concentrations of Cr and Cd in *S. sesban* L. seedlings with and without inoculation of PM21 strain was shown in Table 4.2. Plant tissues exhibited an increase in Cr assimilation after bacterial inoculation by 36% (25 mg/L Cr), 31% (50 mg/L Cr) and 29% (75 mg/L Cr) and increase in Cd assimilation by 25% (25 mg/L Cd), 28% (50 mg/L Cd) and 29% (150 mg/L Cd), as compared to their respective un-inoculated counterparts. Tissue assimilation of Cr+Cd in combination increased by 29+14% (25+100 mg/mL), 25+17% (50+150 mg/L) and 21+19% (75+200 mg/L), in seedlings after bacterial inoculation as compared to their respective un-inoculated treatment.

Table 4.4. Assimilation of heavy metals in whole plant under different treatments

Treatments	Cr content (μg/g)		Cd content (μg/g)		BAF	
	Un-inoculated	Inoculated	Un-inoculated	Inoculated	Un-inoculated	Inoculated
T0	0.00 ± 0.00^{i}	0.00 ± 0.00^{i}	0.00 ± 0.00^{h}	0.00 ± 0.00^{h}	0.00 ± 0.00^{l}	0.00 ± 0.00^{l}
T1	11 ± 0.03^{h}	15 ± 0.04^{f}	-	-	0.44±0.02b	0.60 ± 0.01^{a}
T2	13 ± 0.04^{g}	17 ± 0.03^{e}	-	-	0.26 ± 0.03^{d}	0.34 ± 0.03^{c}
T3	15 ± 0.03^{e}	19 ± 0.06^{c}	-	-	0.22 ± 0.01^{f}	0.29 ± 0.04^{d}
T4	-	-	17 ± 0.03^{g}	22 ± 0.04^{e}	0.16 ± 0.03^{g}	0.20 ± 0.02^{e}
T5	-	-	18 ± 0.04^{f}	23 ± 0.03^{d}	0.12 ± 0.04^{i}	0.15 ± 0.04^{g}
T6	-	-	21 ± 0.04^{e}	27 ± 0.04^{c}	0.10 ± 0.03^{k}	0.13 ± 0.02^{h}
T7	17 ± 0.03^{e}	22 ± 0.06^{c}	22 ± 0.06^{d}	25 ± 0.04^{c}	0.13 ± 0.02^{h}	0.17 ± 0.04^{f}
T8	20 ± 0.04^{d}	25 ± 0.05^{b}	23 ± 0.04^{d}	27 ± 0.03^{b}	0.10 ± 0.03^{j}	0.12 ± 0.04^{i}
T9	24 ± 0.05^{b}	29 ± 0.04^{a}	26 ± 0.04^{b}	31 ± 0.02^{a}	0.08 ± 0.04^{k}	0.10 ± 0.05^{k}

Effects of different treatments on heavy metal uptake of *S. sesban* L. seedling grown in Petri plates. The treatments T0 through T9 have been defined above in the Table 3 caption Each treatment value is presented as means of three replications (n = 3) with *SE*. Different letters at each treatment indicate significance between the inoculated and un-inoculated conditions at $P \leq 0.05$ level.

4.4. Discussion

The screening, selection and application of abiotic stress tolerant PGPR have gained attention to enhance agricultural yield and to overcome detrimental impacts of climate change (Khani et al., 2010; Amna et al., 2020). Use of stress tolerant PGPR can significantly decrease harmful effects of synthetic agrochemicals on the food chain and human health. In the current study, exploration of biochemical and growth-related effects of supplementing a leguminous species *S. sesban* L. at its germination stage with a heavy-metal stress tolerant *B. anthracis* PM21 bacterial strain was performed.

Inoculating the bacterial strain *B. anthracis* PM21 to the seedlings demonstrated prominent results for various plant growth promoting traits, including IAA, ammonia, HCN, siderophore and EPS production, phosphate, and zinc solubilization, and ACC-deaminase activity. Phosphate solubilization and IAA production are beneficial for plant growth (Rajkumar et al., 2008; Ahmad et al., 2014). Siderophore, and ACC-deamination production, and zinc solubilization also support plant growth (Kamran et al., 2017). The ACC-deaminase enzyme produced by microbes reduce ethylene production by cleaving ACC to α-ketobutyrate and ammonia in plants under heavy metals stress (Glick et al., 2007). The bacterial strain *B. anthracis* PM21 exhibited significant IAA production and ACC-deaminase activity which might be involved in Cd and Cr alleviation from environment and elongate shoots and roots of *S. sesban* L. seedlings (Jalali et al., 2020). The inoculated bacteria synthesize IAA in normal and stress conditions, which positively affect root elongation by increasing uptake of water and nutrients by roots (Ahmad et al., 2016; Akhtar et al., 2018). Similarly, EPS helps to stabilize heavy metals in roots, which is indicated by increased Cr and Cd concentration in tissue of *S. sesban* L.*,* in our study. We demonstrated that the plant physiological parameters such as chlorophyll and proline content were improved under normal and Cr + Cd stress conditions upon inoculation of *B. anthracis* (Bashir et al., 2021). It results due to the production of EPS compounds by *B. anthracis* under heavy metals stress. Being hydrated compounds containing 97% water in the form of polymer matrix, EPS can directly protect seedlings from desiccation (Amna et al., 2019). Plant growth was directly enhanced due to these traits both under normal and stress condition. Besides the enhanced nutrient availability and water storage, PGPR provide cell

adhesion, cell-cell signaling and protection from heavy metals stress (Bramhachari et al., 2018). Under abiotic stress, seeds or plants produce ACC, which is absorbed by microbes and hydrolyzed into α-KB and ammonia (Dubey et al., 2021). Reduction in intracellular ACC level decreases production of stress-induced ethylene in plants and enhances plant growth (Kumawat et al., 2020). Ammonium-N increased by integration of ACC improved chlorophyll content of *S. sesban* L. (Barreiro-Vescovo et al., 2020).

Toxicity of HMs such as Cd, and Cr causes profound deterioration of seed germination in wheat (Ahmad et al., 2012; Gang et al., 2013). In the present study, high concentrations of Cr and Cd showed significant decline of *S. sesban* L. seed germination with the lowest germination rate in dual metal stress treatment (T9). However, PM21 significantly enhanced seed germination both under normal and heavy metal stressed conditions. Our findings agree with those of Sobariu et al. (2017) who demonstrated that germination percentage enhanced with application of PGPR under heavy metals stress. The inoculated PGPR showed promised ability to safeguard the plant from hazardous effects of Cr and Cd. Excessive Cd stress reduces root and shoot growth in plants (Ahmad et al., 2014, 2016; Maqbool et al., 2019; Din et al., 2020; Rafique et al., 2020). The toxicity of Cr in *S. sesban* L. plant was manifested as reduced growth parameters under variable Cr levels, but negative effects were mitigated by supplementing seedlings with PM21, in the current study. Application of CPSB21 under Cr^{6+} stress also enhanced all plant growth attributes in sunflower (Gupta et al., 2018).

Heavy metals directly interfere with photosynthetic pigments by altering their normal metabolic processes (Souri et al., 2019). The reduction of photosynthetic pigments in *S. sesban* L. treated with single and dual stress of Cr and Cd was due to impairment of aminolaevulinic-acid dehydratase activity. The accessory pigments i.e., total chlorophyll content was also reduced under Cr stress in *Ocimum tenuiflorum* L. and in other plants (Rai et al., 2004). Similarly, Cd stress also led to a drastic decrease in photosynthetic pigments (Farooq et al., 2013). Our finding in *S. sesban* L. also agree with this trend of decline in photosynthetic pigments due to Cd stress. However, PGPR inoculation (*B. anthracis* in our case) under Cr and Cd stress markedly improved photosynthetic pigments, as already reported (Hassan et al., 2017; Khanna et al., 2019).

Proline biosynthesis in plants elevates under stress (Khan et al., 2020). In our study, the proline content was positively correlated with Cr and Cd concentration, but it declined in treatments where bacteria were inoculated in *S. sesban* L. seedlings as compared to their un-contaminated and un-inoculated controls possibly reflecting bacterial ability to alleviate the metal stress (Jan et al., 2019).

Proper cell functioning was achieved by optimum electrolyte levels and water content inside the cell (Jia et al., 2002). Elevated levels of ELL have been observed due to Cr (Gonzalez-Mendozaa et al., 2009) and Cd (Singh and Shah, 2014) contaminations. In the present study, significant increase (up to 51%) in ELL was noted at maximum Cr + Cd treatment (T9). The formation of reactive oxygen species from stable compounds like, DNA-DNA cross talks, Cr-proteins, Cr-DNA, and DNA double or DNA single stranded breaks in cells may be the cause of this elevated ELL (Din et al., 2020). The ELL was markedly improved (up to 49.8% in treatment T9) in seedlings with bacterial inoculation in this study. There was a striking improvement in ELL compared to only 18% after inoculation of PGPR under heavy metal stress conditions in radish plants (Ahmed et al., 2018). Our results demonstrate that the bacterial strain can cope significantly under heavy metal stress, possibly by strengthening membrane stability index.

Important antioxidant activities including CAT, SOD, and POD play significant roles in decreasing accumulation of ROS, maintain integrity of cell membrane, prevent cell peroxidation of lipids and protect internal cell structures (Zainab et al., 2020). These enzymes are also known to protect plants from devastating effects of heavy metal (Gill et al., 2015). Majority of plants are unable to produce adequate amount of antioxidant enzymes to deal with destructive effects of ROS under stress condition (Latef et al., 2020). High concentrations of CAT, SOD, and POD enzymes indicate amelioration of oxidative stress caused by the heavy metal toxicity (Din et al., 2020).

Under Cd stress, increase in the activity of POD and CAT is correlated with high production of ROS species (Guo et al., 2019; Rahbari et al., 2020). The inoculation of *S. sesban* L. seedlings with PGPR in all treatments significantly enhanced SOD, POD and CAT activities and reduced MDA content as compared to their un-inoculated counterparts. These findings agree with Ahmed et al. (2018) who demonstrated decline

in the MDA content after PGPR inoculation under heavy metal stress in radish plant. Production of MDA is directly linked with membrane lipid peroxidation due to oxidative stress in plants (Anjum et al., 2011). The MDA content reacts with amino groups, which produces proteins, biomolecules and causes peroxidation of lipids, leading to cell necrosis (Islam et al., 2016). Reduction in MDA and ELL content after PGPR inoculation in current study indicates ability of PM21 to evade the metal stress. Production of different enzymes in *Pseudomonas putida* and *B. anthracis* PM21 enhance the potential uptake of heavy metals (Yousaf et al., 2010; Din et al., 2020). Application of PM21 causes production of various organic acids and chelators that promote redox alteration, thereby enhancing uptake of heavy metals (Diarra et al., 2020). Current investigations demonstrate an increase in Cd and Cr assimilation in *in-vitro* grown *S. sesban* L. seedlings following inoculation with PM21. It will be premature to recommend this strain for phytoremediation in environment in the absence of data for controlled soil experiments (Gan et al., 2020). The Cd pollution is becoming a major threat to food security, careful application of the stress tolerant bacteria to eliminate heavy metals from contaminated soils could contribute towards global food security (Amna et al., 2020).

4.5. Conclusion

This study shows that growth of *in vitro* grown *S. sesban* L. seedlings were arrested due to Cd and Cr toxicity. Heavy metal toxicity also contributed to down-regulating important biochemical and physiological parameters of the seedlings. Our findings highlighted marked improvements after inoculation of seedlings with *B. anthracis* PM21 as a PGPR. The result revealed that *Bacillus anthracis* PM21 performed best under Cd stress as compared to Cr stress condition. In comparison to un-inoculated seedlings, *B. anthracis* PM21 inoculation greatly improved ($p \leq 0.05$) seed germination percentage (97.01%), shoot length (5.03%), root length (59.51%), photosynthetic pigments under Cd stress. Currently, large number of industries are releasing pollutants such as heavy metals in atmosphere and lithosphere. These heavy metals are threating environment and agroforestry. The PGPR such as *B. anthracis* PM21 having bioremediation potential can open a new endeavor for clean environment and food. This strain *Bacillus anthracis* PM21 presents striking potential for use as PGPR and biosorption of heavy metal. Carefully designed greenhouse experiment is required to evaluate its full potential in supporting microbial assisted phytoremediation.

Chapter. 5

To evaluate the microbial assisted phytoremediation of heavy metal with *Sesbania sesban* L.

5.1. Introduction

Environmental pollution is drastically increasing due to anthropogenic activities (Gavrilescu et al., 2015). As a result of anthropogenic activities, the contamination of heavy metals (HMs) causes serious threats to the environment (Gavrilescu et al., 2015). Being non-degradable and persistent in nature, HMs can easily bio-accumulate in the food chain and eventually induce ill effects on the health of humans. Contamination of HMs in the soil has become a critical problem in Pakistan, and various HMs have been detected in the soil of Malakand Agency and Vehari (Chen et., 2015). Additionally, heavy metals are merely the result of weathering process. According to the Environmental Protection Agency (EPA) (Cd) the most widespread in the environment (Yadav et al., 2017a; Chauhan and Mathur, 2020).

Level of Cd contamination in agricultural soil of Faisalabad and Lahore have been detected as 0.29 and 184 mg/kg respectively that is above permissible limits of WHO (Waseem et al., 2014). Heavy metals polluted soil can be remediated by utilizing various biological and physico-chemical methods. Phytoremediation is a cost-effective method to remediate HM contaminated soils (Sarwar et al., 2017). Soil contaminated with HMs is regarded as a different substrate than air and water. It might be due to persistent nature of heavy metals present in soil than other components of the biosphere (Mahar et al., 2016; Rizwan et al., 2017; Vardhan et al., 2019).

Phytoremediation is emerged as promising, ecofriendly, and cheap approach for the extraction, and immobilization of pollutants from ground water and soil sediments (Adrees et al., 2015). Methods such as phyto-stabilization, phytoextraction, rhizofiltration and phytovolatilization can be used for the purpose of phytoremediation . Hyperaccumulator plants are used for phytoextraction as they can better tolerate and accumulate various heavy metals (Liang et al., 2009). Hyperaccumulator plants are used for phytoextraction, as they can better tolerate and accumulate various HMs (Ali et al., 2021). The tolerance of plants to HM toxicity, the interaction between HMs and the native bacterial community, and the uptake of available soil HMs are key factors of phytoremediation (Gomase et al., 2012).

Sesban (*Sesbania sesban* (L.) Merr.) belongs to the Fabaceae or Leguminosae family that has huge medicinal importance and is widely distributed in tropical regions,

worldwide (Gomase, 2012). It is also used for fodder and fuel purposes and has high biomass (Dubis et al., 2020). *Sesbania sesban* L. is reported to survive in contaminated environments (Liang et al., 2021). The *Sesbania sesban* L. could be an effective option for reclamation due to potential of nitrogen fixation in root nodules (Chan et al., 2003). This research work was mainly focused to highlight the ability of *B. anthracis* PM21 for Cd tolerance as well as in bacterial assisted phytoremediation of Cd spiked soil. Additionally, to evaluate the differential effects of *B. anthracis* PM21 inoculation on important morphological, physiological, and biochemical attributes of *Sesbania sesban* L. under different Cd concentrations.

5.2. Materials and methods

Based on results of germination experiment of previous study the *Bacillus anthracis* PM21 was further evaluated for assisted phytoremediation of cadmium under greenhouse condition.

5.2.1. Antibiotic resistance of *Bacillus anthracis* PM21

Disk diffusion method was used for the detection of antibiotic resistance (Amna et al., 2019). Bacterial culture was allowed to grow in broth for 24 h. The antibiotic discs were fixed on the LB agar plates on that 24 h grown culture (100 µL) was spread. Incubation of inoculated Petri plates was done overnight at 35^{o}C. Bacterial resistant to antibiotic was quantified through measuring the zone diameter of inhibition around the antibiotic disks.

5.2.2. Inoculum preparation and seed treatment

The Bacillus anthracis PM21 was inoculated in 250 mL Erlenmeyer flask having 100 mL Luria-Bertani liquid medium at 35°C with 120 rpm for 24 h. The value of optical density was recorded at 600 nm and cells were maintained up to 10^9 colony forming unit (CFU/mL). The *Sesbania sesban* L. seeds were initially soaked for 5 min in 75% ethanol and then sterilized with 0.1% $HgCl_2$ for 1 min (Ali et al., 2018). Seeds were washed for 5 to 6 times with double distilled water (DDW). Seed priming was carried out via immersion in bacterial inoculum for 3-4 h. Control treatment contain seeds that were soaked only in double distilled water.

5.2.3. Green house experiment

Soil was collected from agricultural and non-contaminated field of National Agricultural Research Center, Islamabad-Pakistan (33.6701°N, 73.1261°E). The collected soil was autoclaved at 121°C for 1 h. Nature of the soil was found as loamy. Properties of experimental soil prior to experiment were checked and given in Table 5.1.

Experimental pots each having 23 cm diameter and 19 cm length, were filled with autoclaved soil (5 kg). Cadmium was applied in solutions with two levels (100 and 200 mg/kg) in pot treatments. Treatments containing Cd were thoroughly mixed for two

weeks for metal stabilization to maintain the even metal concentration in soil before sowing (Din et al., 2020). Completely randomized design having three replicates, was implemented to manage the experimental treatments. Experimental treatments include Control (T0), *Bacillus anthracis* (T1), Cd 100 mg/kg (T2), Cd 100 mg/kg + *B. anthracis* (T3), Cd 200 mg/kg (T4), and Cd 200 mg/kg + *B. anthracis* (T5) respectively.

Table 5.1. Soil properties of experimental soil prior to experiment

Soil properties	Agricultural soil
Soil texture Loamy	Loamy
Clay (%)	15
Silt (%)	42.5
Sand (%)	42.5
pH of soil	7.06
Electrical conductivity (dS/m)	2.28
Organic matter (%)	0.7
Phosphorous (mg/kg)	156
Potassium (mg/kg)	3.27
Nitrate-nitrogen (mg/kg)	0.02
Extractable Cd (mg/kg)	0.4

5.2.4. Growth variables

Growth variables were observed from plants harvested after 65 days of sowing from each experimental pot. The root and shoot length, fresh (FW) and dry weight (DW) were recorded as plant growth traits. Plant parts were kept in oven for three days at 70°C till dry weight estimation (Ali et al., 2016).

5.2.5. Photosynthetic pigments of *Sesbania sesban* L.

Leaf sample of 0.5 g was mixed with 6 mL of 80% acetone (C_3H_6O) for determination of photosynthetic pigments i.e., Chlorophyll *a*, chlorophyll *b*, and total chlorophyll content. Centrifugation of the extract was done, and supernatant was assorted in 6 mL

of C_3H_6O followed by centrifugation. Chlorophyll *a*, *b*, and total chlorophyll content was determined with help of optical density (Pérez-Patricio et al., 2018).

5.2.6. Assessment of electrolyte leakage and relative water content

Fresh leaves (0.5 g) from each treatment were maintained in H_2O at 4°C for 4 h to evaluate relative water content. Similarly, plant samples were dried in oven (80°C) to obtain dry weight (DW) (Ahmad et al., 2016). The protocol suggested by Ahmad et al. (2016) was implemented to measure electrolyte leakage (ELL) via formula given below.

$$\textbf{Electrolyte leakage (ELL)} = \frac{\textbf{Electrical conductivity1}}{\textbf{Electrical conductivity2}} \times \textbf{100} \quad (\textbf{5.1})$$

5.2.7. Estimation of proline content, malondialdehyde (MDA), and antioxidant activity

The proline content was determined using Ninhydrin (Amna et al., 2019). A 4 mL of sulphosalicylic acid (3%) was used to ground the leaf sample (0.5 g) and placed overnight. Centrifugation of the crushed samples was done for 5 min at 3000 rpm and then ninhydrin and glacial acetic acid were added to the previously prepared. The obtained mixture was heated at 100°C for 1 h in water bath. Mixture was transferred into ice bath to cool it. Toluene was used for extraction of mixture and its absorbance was noted at 520 nm. Standard curve was used to measure proline concentration and expressed in mmol/g. Fresh leaf sample of 0.5 g was grinded in 10 mL of 0.1% trichloroacetic acid (TCA) to measure MDA (Prochazkova et al., 2001). Centrifugation of the sample was carried out at 15000×g for 15 min and the upper layer was removed and combined with 4 mL of 0.5% thiobarbituric acid and 20% trichloroacetic acid. The samples were centrifuged at 10000 rpm for 10 min and absorbance was noted at 440, 532 and 600 nm, respectively. MDA equivalents were estimated using formula given below.

$$\textbf{MDA} = \textbf{6.45(A532} - \textbf{A600)} - \textbf{0.56} * \textbf{A44}$$

Antioxidant enzyme superoxide dismutase (SOD) was assessed by following Afridi et al. (2019). In similar way, peroxidase activity (POD) was observed by following the

protocol of Afridi et al. (2019). The protocol suggested by Khalilzadeh et al. (2020) was implemented to determined catalase activity by H_2O_2 decomposition. The optical density was measured at 240 nm by using spectrophotometer.

5.2.8. Metal analysis of plants by wet acid digestion method

Heavy metal uptake was achieved employing the method of wet acid digestion (Wan et al. 2012). Plant material (1 g) was fine macerated via pestle and mortar and transferred into 100 mL conical flask. About 10 mL Perchloric and Nitric acid ($HClO_4$ - HNO_3 at 1:3 ratio) was added into the flak and kept overnight in fume hood. Then flasks were kept in fume hood for 60 min maintaining its temperature at 70°C. During this process, brown fumes turned into white. The flasks containing mixture was allowed cool for a few seconds. Distilled water was added to dilute the mixture. The obtained extract was filtered through Whatman No. 42 and double distilled water was added to raise total volume up to 50 mL. These samples were utilized to quantify Cd concentration with the help of flame AAS (Varian FAAS-240, Triad Scientific and New Jersy, USA).

5.2.9. Calculation of tolerance index (TI), translocation factor (TF) and bioconcentration factor (BCF)

The TI was measured as reported by Khan et al. (2016). Translocation factor (TF) determined the ability of a given plant species as phytoremediator. Translocation factor is the ratio between concentration (conc.) of an element present in plant shoot to root. The bioconcentration factor (BCF) was estimated to know metal uptake capacity from soil to plant tissues. The BCF was calculated for individual plant parts i.e., roots and shoots. The formulas proposed by Fitz and Wenzel, (2002) and Mendez and Maier, (2008) were used to calculate BCF and TF, respectively.

$$\textbf{\textit{Cd tolerance index}}\ (\%) = \frac{\textbf{\textit{Biomass of treated plants}}}{\textbf{\textit{Biomass of control plants}}} \quad (\mathbf{5.2})$$

$$\textbf{\textit{Bioconcentration Factor}}\ (\textbf{\textit{BCF}}) = \frac{\textbf{\textit{Concentration of metal in shoot}}}{\textbf{\textit{Concentraion of metals in soil}}} \quad (\mathbf{5.3})$$

$$\textbf{\textit{Translocation Factor}}\ (\textbf{\textit{TF}}) = \frac{\textbf{\textit{Concentration of metal in shoots}}}{\textbf{\textit{Concentraion of metals in roots}}} \quad (\mathbf{5.4})$$

5.2.9.1. Re-isolation of inoculated strain

Re-isolation was measured as reported by Vijay et al. (2020). The re-isolation experiment was performed for the confirmation of inoculated strain PM21.

5.2.10. Statistical analysis

The whole experimental was conducted in triplicate. The shapiro-wilk test of normality (Wang and Riffel, 2011) and analysis of variance were performed for all the studied parameters through SPSS software (IBM SPSS Statistics 21). Data was entered into excel software to analyze mean values and standard error. Least significant difference was used to find variation among treatments ($p \leq 0.05$) by implementing multiple comparison test.

5.3. Results

5.3.1. Antibiotic resistance of PM21

The bacterial strain was tested by using disk method for antibiotic resistance. The strain was resistant against the maximum number of antibiotics applied. A total of 20 antibiotics were used in for the test. Appearance of halo zone around the disk was considered as a sign of susceptibility of bacterial strain. Antibiotic resistant ability of strain is shown in (Table 5.2). Following the Table 5.2 it is analyzed that PM21 is highly resistant to antibiotics. It showed resistant against 15 antibiotics out of 20.

5.3.2. Soil properties of experimental soil prior to experiment

Pre-analyzed soil properties are given in Table 5.1. Nature of the soil was found as loamy (clay 15%, silt 42.4% and sand 42.5%) with pH (7.06), electrical conductivity (2.28 µs/cm), organic matter (0.93%), available phosphorous (156 mg/kg), extractable potassium (3.27 mg/kg), and available Cd (0.4 mg/kg).

5.3.3. Agro-morphological parameters

5.3.3.1. Root and shoot length

Significant increase in root length of *S. sesban* L. was observed in Cd stressed and non-stressed treatments with the inoculation of *B. anthracis* PM21 (Table 5.3). Both Cd contamination levels, 100 and 200 mg/kg (T2) caused significant decrease in root length (47.68%) and shoot length (33.29%) respectively as compared control. Inoculation of PM21 increased shoot and root length in both non-stress and Cd stress treatments (Table 5.3). Comparing level of Cd, maximum root length (38.39%) and shoot length (32.67%) was noted with 200 mg/kg Cd (T5) respectively under inoculation of PM21 as compared to control.

5.3.3.2. Fresh and dry weight

Fresh weight was reduced under contaminated treatments as compared to uninoculated control treatment. Cd contamination at the level of 200 mg/kg reduced fresh weight (44.95%) and dry weight (62.48%) as compared to control treatment. However, *S. sesban* L. inoculated with PM21 showed fresh weight (43.48%) and dry weight (50.84%) at 200 mg/kg of Cd (Table 5.3).

Tale 5.2 Antibiotic resistance of *Bacillus anthracis* PM21

Antibiotic	Concentration	Inhibition zone (mm)	Zone diameter		
			Susceptible	Intermediate	Resistant
Rifampicin	05 µg	4	-	-	+
Tobramycin	10 µg	5	-	-	+
Tetracycline	30 µg	12	-	+	-
Ceftazidime	30 µg	3	-	-	+
Neomycin	10 µg	8	-	-	+
Spectinomycin	25 µg	25	-	-	-
Kanamycin	30 µg	6	-	-	+
Streptomycin	10 µg	25	-	-	+
Cloxacillin	5 µg	5	-	-	+
Penicillin	01 µg	6	-	-	+
Nitrofurantoin	300 µg	6	-	-	+
Fosfomycin	50 µg	5	-	-	+
Gentamycin	10 µg	4	-	-	+
Lincomycin	15 µg	3	-	-	+
Ciprofloxacin	30 µg	5	-	-	+
Chloramphenicol	30 µg	12	-	+	-
Colistin	10 µg	3	-	-	+
Erythromycin	15 µg	13	-	+	-
Clindamycin	2 µg	25	+	-	-
Piperacillin	100 µg	6	-	-	+

(+): positive; (-): negative

Table 5.3. Influence of bacterium inoculum PM21 on important agronomic traits of *Sesbania sesban* L. in Cd spiked soil

Treatments	Root length (cm)	Shoot length (cm)	Fresh weight (g)	Dry weight (g)
T0	25.67±0.33^{b}	63.01±0.10^{b}	20.02±0.01^{ab}	7.73±0.08^{b}
T1	30.33±0.33^{a}	66.50±0.73^{a}	25.06±0.02^{a}	9.55±0.01^{a}
T2	15.16±0.29^{d}	46.5±0.17^{d}	13.04±0.01^{c}	3.04±0.08^{e}
T3	24.2±0.12^{c}	64.67±0.36^{c}	20.03±3.33^{bc}	6.51±0.01^{c}
T4	13.43±0.05^{e}	42.03±0.29^{f}	11.02±0.08^{c}	2.90±0.08^{ef}
T5	22.01±0.11^{d}	62.43±0.06^{e}	19.50±0.08^{b}	5.9±0.26^{cd}

T0 = Control, T1= *Bacillus anthracis* PM21, T2= Cd 100 mg/kg, T3= Cd 100 mg/kg + *Bacillus anthracis* PM21, T4= Cd 200 mg/kg and T5= Cd 200 mg/kg + *Bacillus anthracis* PM21. ±: Error bars (n = 3). Cm: Centimeter; g: gram.

5.3.3. Photosynthetic pigments of *Sesbania sesban* L.

Significant reduction in photosynthetic pigments was noted in Cd contaminated treatments of *S. sesban* as compared to control treatment (Table 5.4). Chlorophyll a, b and total chlorophyll contents were reduced by 27.34, 62.79 and to 43.71% respectively at Cd stress level of 200 mg/kg as compared to control treatment. Chlorophyll a, b and total chlorophyll content were considerably improved due to the application of PM21 as compared to respective control. Enhanced chlorophyll a (60.08), b (48.38%) and total chlorophyll content (17.65%) were noted with the application of PM21 at the level of Cd stress of 200 mg/kg.

Table 5.4. Effect of *Bacillus anthracis* PM21 on the chlorophyll content of *Sesbania sesban* L. under Cd stress.

Treatments	Chlorophyll *a* (mg/g)	Chlorophyll *b* (mg/g)	Total Chlorophyll (mg/g)
T0	1.28±0.03^{e}	0.43±0.03^{c}	12.10±0.05^{b}
T1	2.75±0.08^{a}	0.65±0.03^{a}	15.16±0.03^{a}
T2	1.47±0.03^{d}	0.35±0.05^{d}	9.35±0.01^{d}
T3	2.55±0.01^{b}	0.52±0.01^{b}	10.50±0.05^{c}
T4	0.93±0.01^{f}	0.16±0.08^{f}	6.81±0.01^{f}
T5	2.33±0.01^{c}	0.31±0.01^{e}	8.27±0.01^{e}

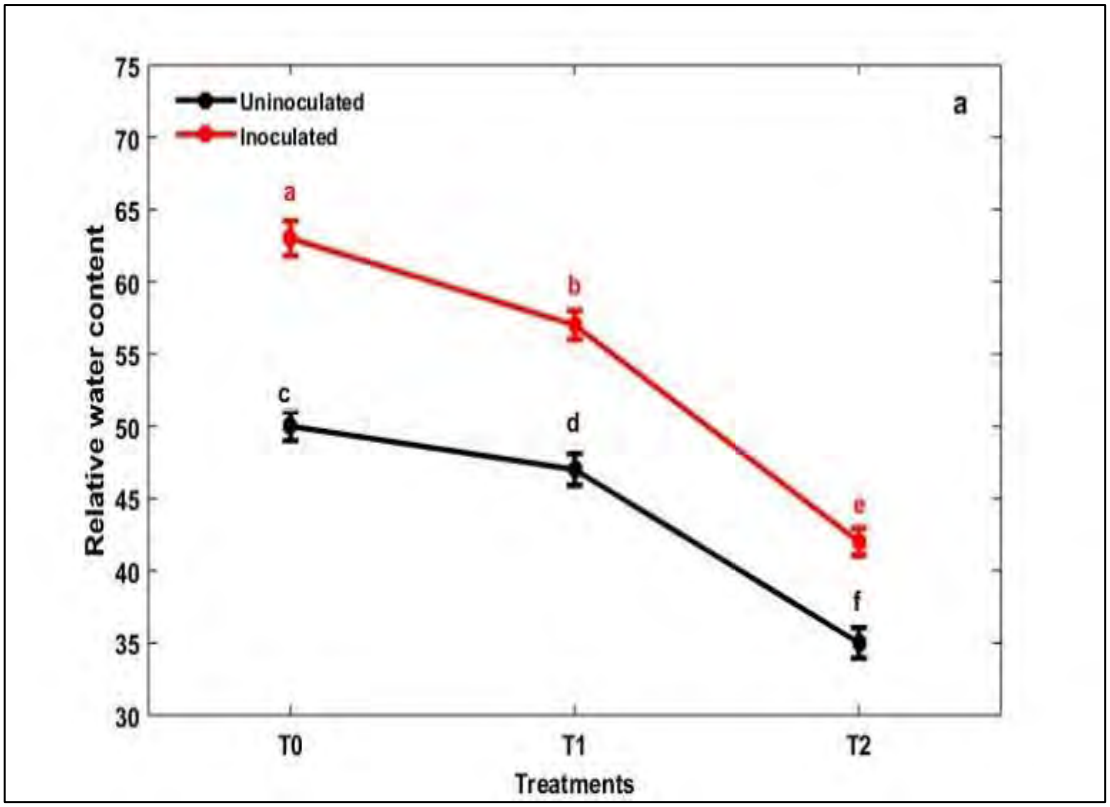
T0: Control; T1= *Bacillus anthracis* PM21, T2= Cd 100mg/kg, T3= Cd 100mg/kg + *Bacillus anthracis* PM21, T4= Cd 200 mg/kg and T5= Cd 200 mg/kg + *Bacillus anthracis* PM21.

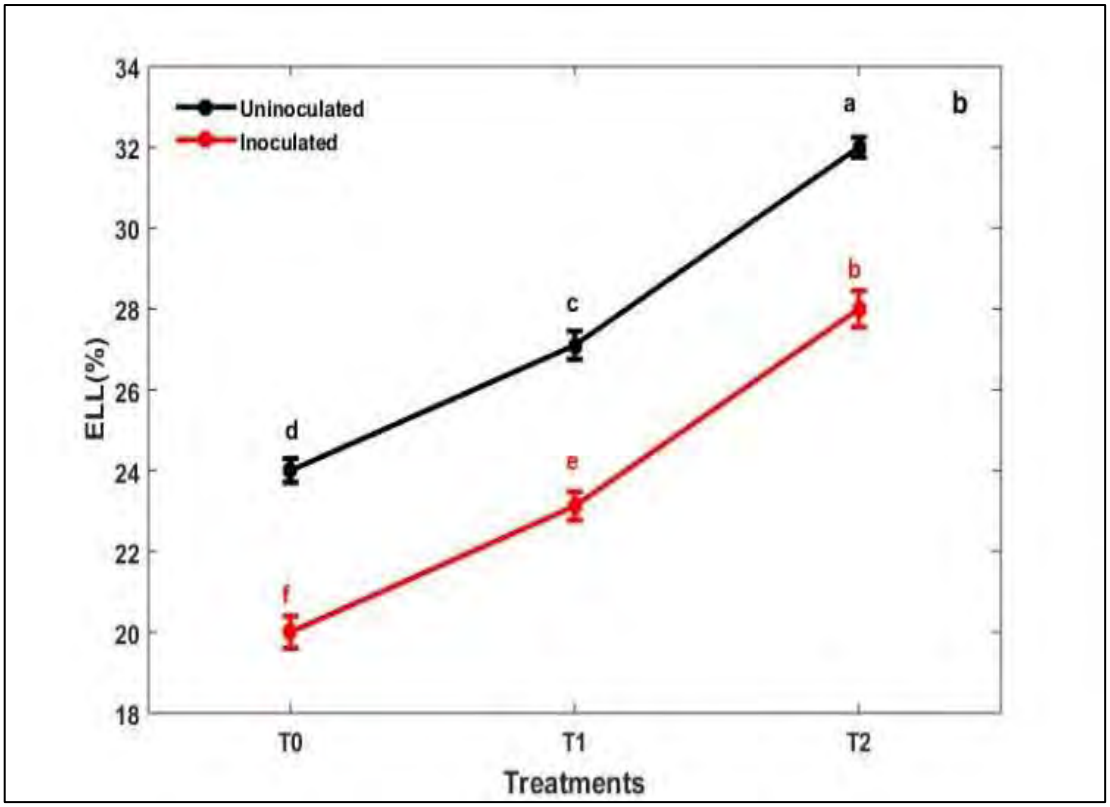
5.3.4. Relative water content, electrolyte leakage, MDA, and proline

Reduced RWC and enhanced electrolyte leakage was noted in Cd contaminated treatments as compared to control (Figure 5.1). Cd stress at the level of 200 mg/kg caused maximum reduction in RWC (16.66%) and enhanced membrane electrolyte leakage (14.28%) as compared to control treatment. Inoculation of PM21 under Cd stress at the level of 200 mg/kg resulted maximum increase in RWC (16.66%) and decease in ELL (12.5%). The Cd contamination enhanced proline and MDA contents of *S. sesban* L. (Figure. 3a, b). Cd stress at the level of 200 mg/kg recorded maximum proline content (37.75%), and MDA content (41.04%) as compared to control treatment. Bacterium inoculation under Cd stress at the level of 200 mg/kg observed maximum reduction in proline content (16.37%), and MDA content (12.65%).

5.3.5. Antioxidant's activity

Important antioxidant activities were significantly enhanced with the application of PM21 under control and Cd stress treatments (Figure 2). The PM21 inoculation significantly ($p < 0.001$) promoted antioxidant activities i.e., SOD (11.98%), POD (12.16%), and CAT (4.46%) at 200 mg/kg of Cd stress.


Uninoculated
Inoculated
a
Relative water content
75
70
65
60
55
50
45
40
35
30
a
b
c
d
e
f
T0
T1
T2
Treatments


Uninoculated
Inoculated
b
ELL(%)
34
32
30
28
26
24
22
20
18
a
b
c
d
e
f
T0
T1
T2
Treatments

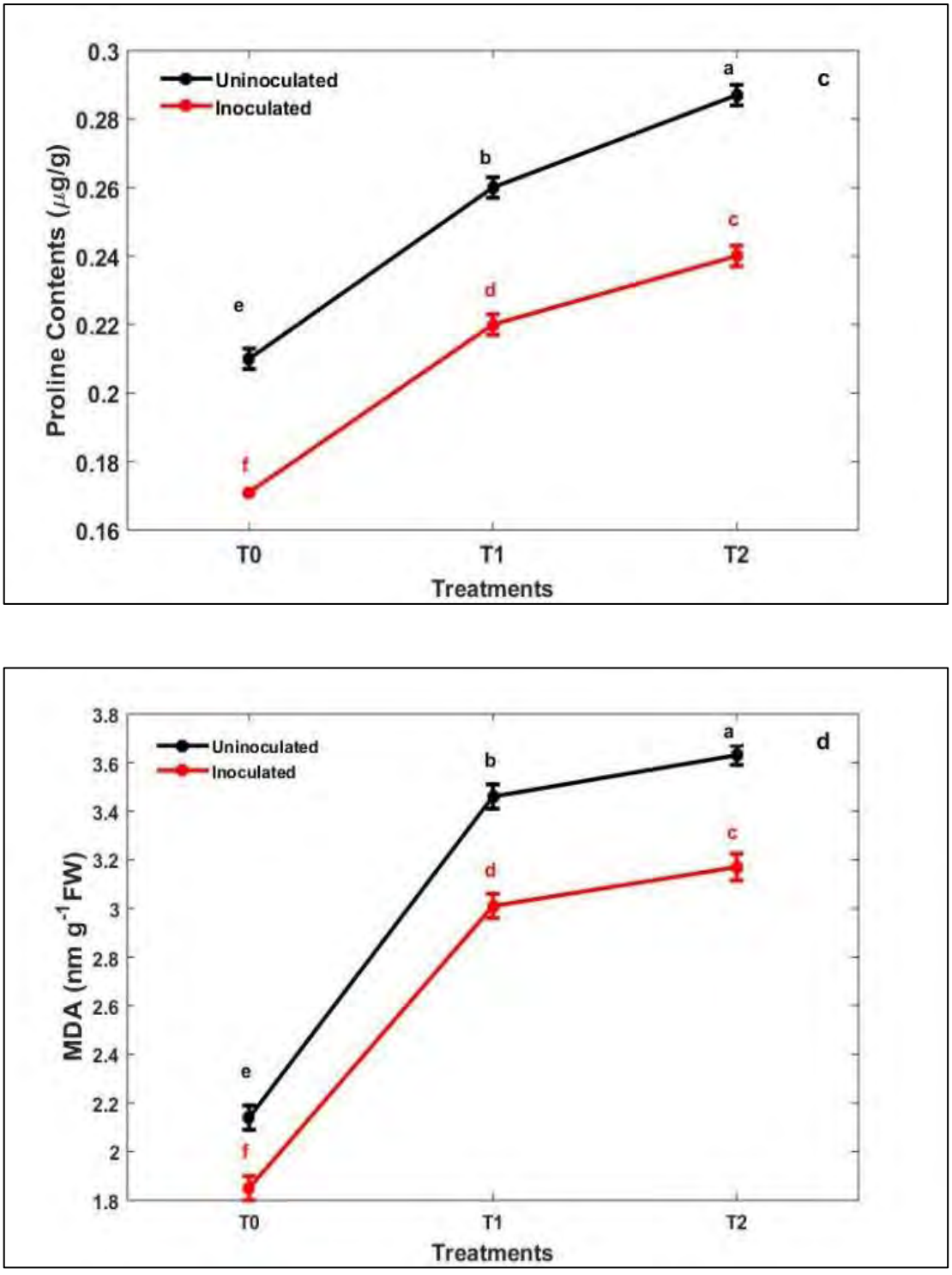
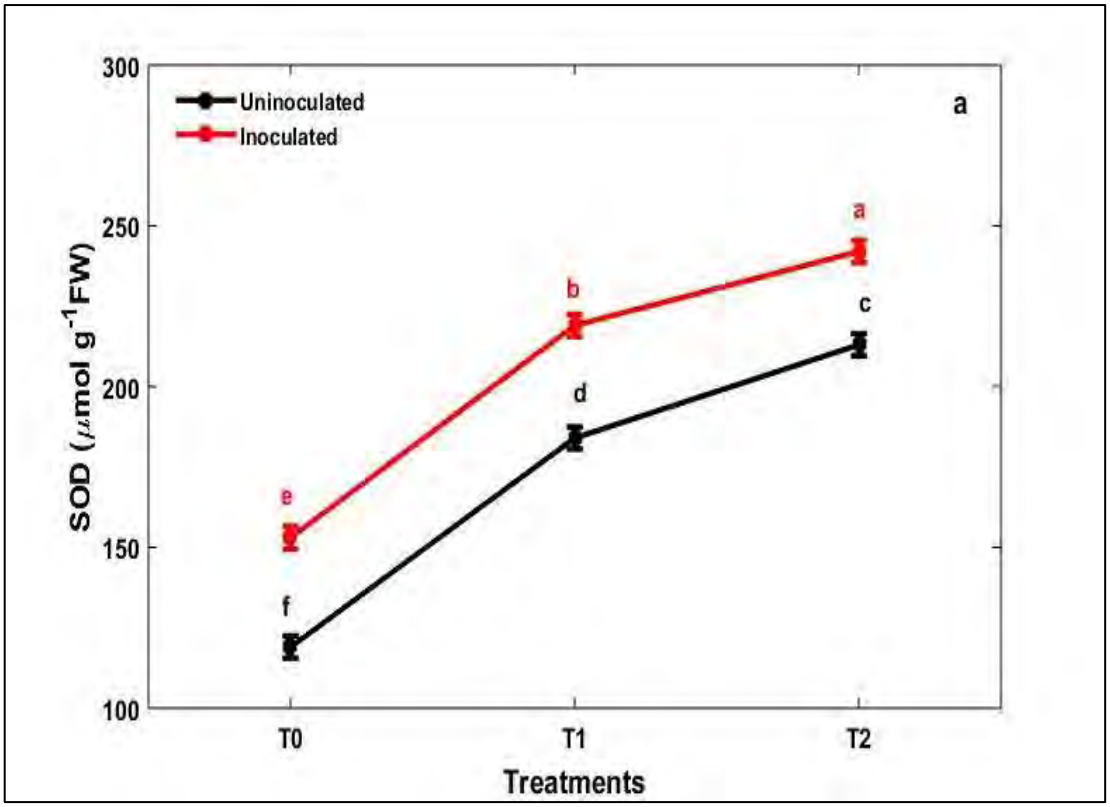
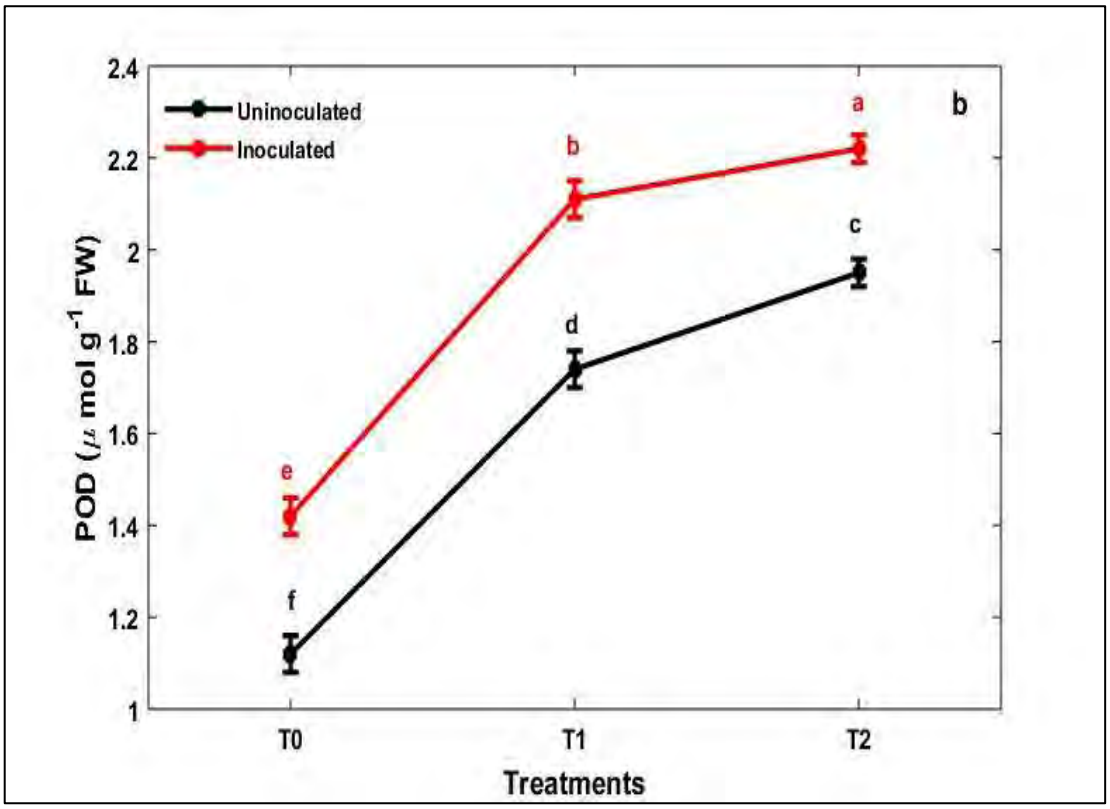


Figure 5.1. Influence of bacterium inoculum PM21 on (a) RWC, (b) membrane electrolyte leakage, (c) proline and (d) MDA of *Sesbania sesban* L. in Cd spiked soil. T0: Control, *B. anthracis* PM21; T1: Cd 100 mg/kg, *B. anthracis* PM21; T2: Cd 200 mg/kg, *B. anthracis* PM21. FW: Fresh weight. The superscripts indicate significance between inoculated and un-inoculated conditions at $p \leq 0.05$ level. Values are presented as means ± *SE* (n = 3).


Uninoculated
Inoculated
a
SOD (μmol g-1FW)
300
250
200
150
100
a
b
c
d
e
f
T0
T1
T2
Treatments


Uninoculated
Inoculated
b
POD (μ mol g-1 FW)
2.4
2.2
2
1.8
1.6
1.4
1.2
1
a
b
c
d
e
f
T0
T1
T2
Treatments

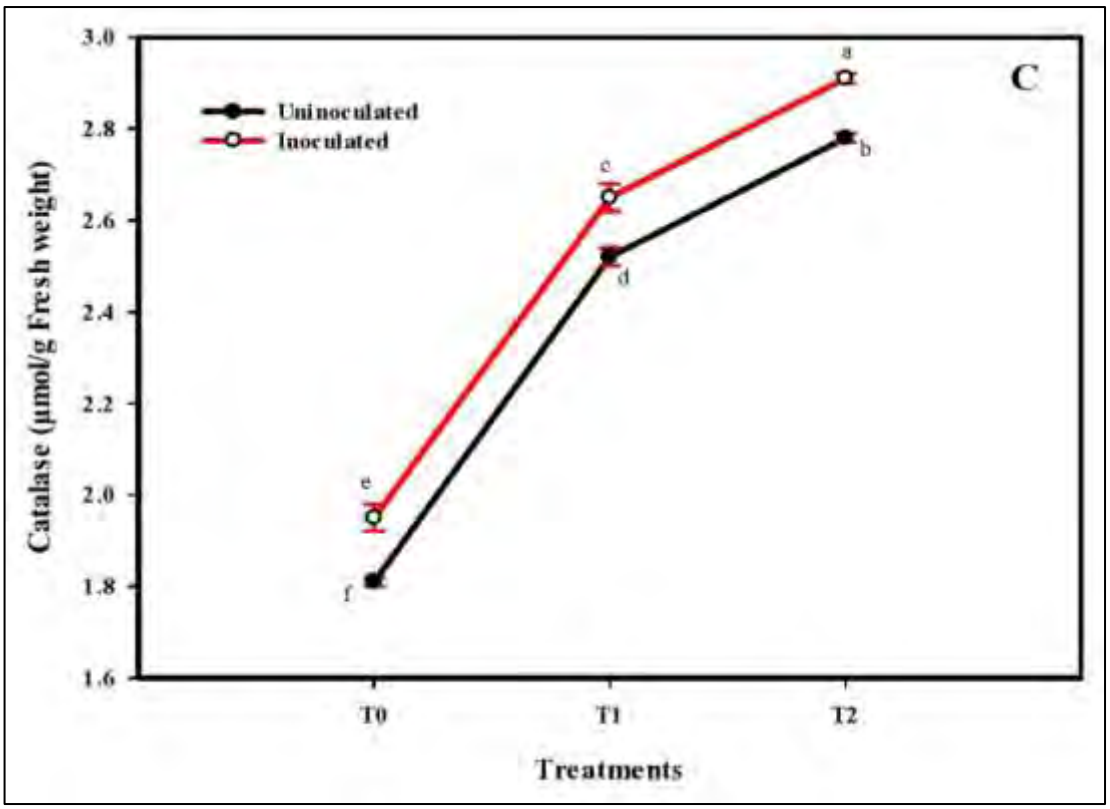


Figure 5.2. Influence of bacterium inoculum PM21 on antioxidant enzyme including SOD (a), POD (b) and Catalase (c) of *S. sesban* L. in Cd stress. T0: Control, *B. anthracis* PM21; T1: Cd 100 mg/kg, *B. anthracis* PM21; T2: Cd 200 mg/kg, *B. anthracis* PM21.

5.3.6. Tolerance index, translocation factor (TF) and bioconcentration factor (BCF)

The enhanced Cd tolerance and high biomass values were noted in plants inoculated with PM21 as compared to control (Table 5.4). *Sesbania sesban* L. plants shown to have a higher Cd absorption upon exposure to increasing levels of Cd (Table 5.4). The application of PM21 shown increased Cd absorption, resulting in best cadmium contents in inoculated Cd 200 plants. In all treatments, the roots of the plants were having higher Cd levels than the shoots. In comparison to roots of un-inoculated *Sesbania sesban* L. PM21 inoculated plants have 12.42, and 11.38% more Cd content in roots for Cd100, Cd 200 mg/kg, respectively. Inoculated plants have 24.58, and 18.39% more Cd in their shoots under Cd100, and Cd 200 mg/kg, respectively, as compared to respective control. The current findings showed that PM21 inoculated plants exposed to Cd100, and Cd 200 mg/kg have higher TF and BCF as compared to respective control. When compared to un-inoculated Cd 100 plants, the Cd100 mg/kg

with PM21 have 13.9% and 23.44% higher TF and BCF values, respectively. Cadmium 200 + PM21 plants showed 8.19 and 19.44% rise in TF and BCF, respectively, as compared to respective control (Table 5.4). The value of cumulative Cd accumulated in whole plants (mg/plant) was calculated by multiplying the Cd contents in plant tissues by the dry weight of the plants. The higher levels of Cd were exhibited by plants inoculated with PM21 in both treatments (Table 5.4).

5.3.6.1. Re-isolation of inoculated strain

The experiment result confirmed the inoculated strain PM21 by re-isolation and identification.

Table 5.5. Cadmium (Cd) uptake of *Sesbania sesban* L. plant material

Treatments	Cadmium uptake				
	Root	**Shoot**	**TI**	**TF**	**BCF**
T0	0.1±0.01e	0.01±0.01e	-	0.05 ± 0.01d	0.02 ± 0.02e
T1	0.2±0.05e	0.05±0.05e	-	0.1 ± 0.01d	0.12 ±0.01d
T2	46.5±0.11d	31.9±0.11d	72.36±0.02c	0.68 ± 0.01b	0.32 ±0.01c
T3	53.1±0.02c	42.3±0.12c	100.05±0.01a	0.79 ± 0.02a	0.42 ±0.02b
T4	105.1±0.03b	59.9±0.30b	61.15±0.01d	0.56 ± 0.01c	0.34 ±0.01c
T5	118.6±0.11a	73.4±0.05a	97.11±0.02b	0.61 ± 0.01c	0.36±0.01a

T0: Control, T1: *Bacillus anthracis* PM21; T2: Cd 100 mg/kg, T3: Cd 100 mg/kg+ *B. anthracis* PM21; T4: Cd 200 mg/kg; T5: Cd 200 mg/kg + *B. anthracis* PM21, TI: Tolerance index; TF: Translocation factor; BCF: Bioconcentration factor

5.4. Discussion

Agricultural soil contaminated with Cd poses major risks to crop productivity (Abhilash et al., 2016). Microbial assisted phytoremediation is considered as effective approach for the reclamation of polluted soils. Phytoremediation in combination with inoculation of PGPR can be used efficiently to manage the soil polluted under HMs (Rajkumar et al., 2012). The inoculation of stress tolerant PGPR can reduce detrimental impact of heavy metal in sustainable agriculture (Khani et al., 2010; Amna et al., 2019; Ali et al., 2021). Bacteria can tolerate heavy metals, which can enhance plant growth by producing antioxidants that can absorb metals and help in survival (Rajkumar e al., 2010). The current investigation explained the *Sesbania sesban* L. with its microbial (*Bacillus anthracis* PM21) assisted phytoremediation potential under Cd stress to analyze potential regulation of physiological, biochemical parameters and antioxidants enzymes (Ali et al., 2020). The applied of HM tolerant bacteria can be used as a promising technique to induce Cd stress tolerance mechanism in plants (Ahmad et al., 2016; Ali et al., 2021).

Antibiotic resistant PGPR can be utilized in the form of inoculum for reducing the competition. In current study, *Bacillus anthracis* PM21 showed resistance against 15 antibiotics. Beneduzi et al. (2012) reported that bacteria can be benefited by antibiotic resistance to enhance their colonization and niche with plants compared to other. Genes responsible for antibiotic resistance are considered to provide the key role in mechanism proposed for antibiotic resistance. The genes are either located in chromosome or in plasmid. The presence of multifunctional proteins which are involved in functions like efflux of metals or molecules are considered for antibiotic resistance (Ramakrishna et al., 2019).

Application of PM21 increased agro-morphological traits including fresh weight (FW), dry weight (DW), root length (RL), and shoot length (SL), of *Sesbania sesban* L. plant in presence and absence of Cd stress. The RL (47.68%), SL (33.29%), FW (44.95%) and DW (62.48%) of *S. sesban* L. were significantly decrease in Cd stress as compared to control treatment.

Previous results reported Cadmium treated plants at 150 µM Cd reduced root length (27.56%), shoot length (30.95%), fresh weight (28.46%) and dry weight (32.24%) as compared to the control (El-Esawi et al., 2020). Several studies have shown that Cd hamper various biological process in the plant including impregnation through ammonia and other compound like nitrogen. Cadmium stress also bring negative physiological, biochemical, and genetic changes in the plant which might be a reason for decreased growth parameters of *Sesbania sesban* L. under Cd stress (Ahmad et al., 2016). Furthermore, Cd level above permissible limit may cause inhibition of enzymatic functions leaf chlorosis reduced plant fresh and dry weight and sometime death (Faizan et al., 2012).

The increased growth parameters of *S. sesban* L. has been observed due to inoculation of PM21 at the concentration level of 200 mg/kg Cd. Inoculation of PM21 significantly increased RL, SL, FW, and DW up to 38.39, 32.67, 43.48, 50.84% respectively, under 200 mg/kg of Cd as compared control (Table. 5.2). This could be due to modulation of antioxidant mechanisms and photosynthetic pigments of the studied plant with application of strain PM21 (Khator e al., 2021). It has been well documented that inoculating Cd-stressed *Serratia marcescens* BM1 to soyabean enhanced root (14.58%) and shoot length (21.51%) and fresh (11.11%) and dry weight (5.11%) (El-Esawi et al., 2020). Usually, the applied bacteria benefit plant in phytoremediation under abiotic stress condition through increasing metal solubility with the synthesis of various organic acids, ACC-deaminase enzyme and exopolysaccharides produced by bacteria (Sharma, 2021). Moreover, reduction in plant growth and biomass of *Solanum nigrum* has been examined when plants were under stress with Cd contamination only, but application of *Serratia* sp. RSC-14 significantly reduced Cd stress and facilitate plant growth (Khan et al., 2015).

Chlorophyll a (27.34%), b (62.79%), and total chlorophyll content (43.71%) of *Sesbania sesban* L. were reduce in Cadmium spiked soil as compared to control treatment. In the same way and investigation conducted by Abas et al. (2020) chlorophyll a (29.25%), b (43.22%), and total chlorophyll content (61.73%) were decreased under Cd stress. Low synthesis of photosynthetic pigments could be due to change in structural compound and gas exchange factors, blocked under stress conditions (Wan et al., 2012; Rizwan et al., 2018). This reduction in chlorophyll

contents of *S. sesban* L. plant could be due to activation of chlorophylls and membrane damage (Ehsan et al., 2014; Ahmad et al., 2018). On the other hand, the chlorophyll a (60.08%), chlorophyll b (48.38%) and total chlorophyll content (17.65%) were positively improved with the application of PM21 (Table. 5.2). The chlorophyll contents of the maize plant were increased by bacterial inoculation under 30 µM of Cd (Abbas et al., 2020; El-Esawi et al., 2020). Plant growth promoting bacteria could enhance chlorophyll that upsurges nutrient uptake in plants through PSB and exudating essential substances that have a role in synthesis of photosynthetic pigments required for photo assimilation (Khanna et al., 2019). The comparison with the reported literature, our results showed significant improvement in the following growth parameters root, shoot length, fresh, dry weight and chlorophyll a, b, and total chlorophyll.

In the current study the electrolyte leakage (ELL) (14.28%) and relative water content (RWC) (16.66%) were significantly decreased upon exposure Cd as compared to control. Similar finding was observed with decreased ELL (20.14%) and RWC (29.44%) under Cr 200 mg/kg (Din et al., 2020). Positive correlation among enhanced ELL and decrease in RWC represent the membrane damage in *S. sesban* L. on exposure to Cd (Ahmed et al., 2018). Present investigation displayed that the inoculation of PM21 reduced ELL (12.5%) and enhanced RWC (16.66%). In comparison inoculation of (*B. xiamenensis* PM14) to *Sesbania sesban* minimized ELL (2.73%) and improved RWC (25.79%) (Din et al ., 2020). Bacterium inoculation enhanced water absorption due to improved root surface area which resulted in improved physiological parameters of host plant (Zainab et al., 2020). Under Cd exposure proline (37.75% and MDA content (41.01%) were significantly decreased *Sesbania sesban*. In the same way both physiological parameters proline (37.75%) and MDA contents (34.84%) were decreased in *Sesbania sesban* when exposed to Cd (Din et al ., 2020). Cadmium stress produced ROS that led to plant to oxidative stress with increased ELL and lipid peroxidation (Ekmekci et al., 2009). Malondialdehyde content is indication of cell membrane damage due to its reaction with amino groups of protein (Islam et al., 2016a, b). Improved physiological mechanisms like Malondialdehyde (MDA) and proline content have been documented in bacterial inoculated plants (Amna et al., 2019; Din et al., 2020). Increased proline content (16.37%) could be due exopolysaccharides

synthesis by PM21 under Cd stress condition (Amna et al., 2019). Bacterial inoculated *S. sesban* L. plants showed significant decrease in MDA content (12.65%) that could be due to the mitigation of Cd stress by application of ACC-deaminase producing microbial solution. Results presented by Din et al., 2019 were in line with current results in which proline (12.33%) was improved and MDA (29.53%) were decreased with *B. xiamenensis* PM14 in *Sesbania sesban*. Plant can tolerate Cd stress with the production of antioxidant enzymes which scavenge produced reactive oxygen species (ROS). Maximum oxidative stress in *S. sesban* L. is triggered with Cd stress applied at the level of 200 mg/kg which led to the enhanced production of important antioxidant activities (SOD, POD and CAT) (Figure 5.2) (Tanwir et al., 2021). However, inoculation with PM21 significantly improved antioxidants activities SOD (11.98%), POD (12.16%) and CAT (4.46%) at 200 mg/kg of Cd (Figure 5.2). It was previously reported that SOD (24.54%), POD (26.03%), CAT (30.54%) at (30 μM) Cd stress level increased by bacterial inoculation (Abbas et al., 2020). The *Acinetobacter* controls the activities of antioxidant enzymes as reported in previous research study, due to the initiation of antioxidant enzyme mRNA expression (Wu et al., 2018). As one of the mitigation strategies to deal with the drastic effects of metal stress, particularly in susceptible ones, the improved antioxidant enzyme under abiotic stress using PGPR could be predicted (Bhat et al., 2020). Enhanced activities of SOD, POD and CAT in inoculated chickpea improved plant growth through the protection of chloroplasts and other organelles in which the important metabolic mechanisms occur (Hashem et al., 2016).

Both bioconcentration factor (BCF) and translocation factor (TF) can be used as possible tools to identify the ability of plants to absorb metal ions. The TF and BCF of the PM21 inoculated *Sesbania sesban* L. plants have been shown in (Table 5.4). Compared to the control plants, inoculated *Sesbania sesban* L. showed increased root, shoot, TF, BCF 118.6, 73.4 mg/kg, 0.61 and 0.36 respectively. It was previously reported that accumulation of Cd increased in root (57.2 mg/kg) shoot (9.02 mg/kg), resulting in higher TF (0.18) and BAF (0.06) values of *Sesbania sesban* (Varun et al., 2017). Because of their growth-promoting biochemicals and boosting nutrient uptake capabilities, the PGPR can co-currently enhance phytoremediation and plant growth (Ma et al., 2011). It was observed that tolerance index (TI) of PM21 inoculated plants was 97.11% with 200 mg/kg of Cd. Tolerance index was 49.6% and 48.14% as reported

by Chauhan and Rai's (2009) and Khan et al., (2017) respectively. The growth and phytoextraction of *Sesbania sesban* L. were improved by inoculating *Bacillus anthracis* PM21. Moreover, plant growth promoting rhizobacteria boosted bioaccumulation, bioavailability, and plant biomass in inoculated plant (Chen et al., 2013; Khan et al., 2018). Bacteria are capable of producing chelating biochemicals, siderophores and phosphate solubilization which have role in improved Cd uptake and alleviation of stress inoculated plants (Tak et al., 2013). Current study suggests that PM21 is involved in bioavailability in Cd due to which accumulation and uptake of Cd is enhanced in *Sesbania sesban* L. (Abou-Shanab et al 2006; Khan et al 2018). In accordance with the reported literature, our results revealed considerable improvement in Cd uptake of root, shoot, TF, BCF and TI of *Sesbania sesban* L.

5.5. Conclusion

Our findings highlighted remarkable improvements after inoculation of plants with *B. anthracis* PM21 strain as a plant growth promoting rhizobacteria. Application of *bacillus anthracis* PM21 increased morphological and physiological parameters as compared to uninoculated ones. Application of *B. anthracis* PM21 significantly ($p \leq 0.05$) enhanced Cd uptake in root, shoot translocation factor, bioconcentration factor 118.6, 73.4 mg/kg, 0.61, and 0.36 respectively. The PGPR, like *B. anthracis* PM21 with bioremediation potential can be utilized to decontaminate heavy metals contaminated soil. The *Bacillus anthracis* PM21 was also applied for the first time in Microbial assisted phytoremediation that enhanced plant growth of *Sesbania sesban* L. under Cd stress at different levels. It is concluded that strain PM21 with plant growth promoting characteristics could ameliorate heavy metal stress in *Sesbania sesban* L. plant by altering the morphological, physiological, antioxidant activities, and can be successfully applied in phytoremediation strategies. To explore its full potential regarding microbial assisted phytoremediation, carefully planned and regulated field experiments are required.

Summary and Conclusion

Summary and Conclusion

Twenty bacterial isolates were described and tested for heavy metal resistance and plant growth promoting (PGP) activities. Several PGP activities like production of IAA, ammonia, HCN, siderophores, protease, amylase, pectinase, cellulose, catalase and solubilization of phosphate and zinc were observed in the isolates. These bacterial strains were further characterized for their qualitative and quantitative analysis of IAA, ACC-deaminase, and exopolysaccharide production. Five out of twenty strains were selected, based on initial heavy metals tolerance. Sequencing of 16S rRNA gene was used to distinguish these bacteria with their closely related species i.e., *Bacillus anthracis* PM21, *Bacillus safensis* PM22, *Enterobacter cloacae* PM23, *Bacillus sonorensis* PM24 and *Bacillus thuringiensis* PM25. The *nifH* gene responsible for nitrogenase activity was amplified in two strains (PM21 and PM23) and *acds* gene was polymerase chain reaction (PCR) amplified in all the five selected strains. This study provides a base line for potential plant growth promoting bacterial isolates *Bacillus anthracis* PM21, *Bacillus safensis* PM22, *Enterobacter cloacae* PM23, *Bacillus sonorensis* PM24 and *Bacillus thuringiensis* PM25, to be used further to evaluate the potential for biosorption of heavy metals, in-vitro seed germination and greenhouse experiment.

The second study was focused to explore the mechanisms and capabilities of plant growth-promoting microorganisms (PM21, PM22, PM23, PM24, and PM25) for biosorption of Cd, Cr, and Ni. In batch biosorption experiments, the maximum adsorption value was obtained for *Bacillus anthracis* PM21 for the applied heavy metals at the optimum pH i.e., 8 for Cd, 6 for Cr, and 4 for Ni. The maximum adsorption of Cr, Cd, and Ni was recorded after 60 min for all the bacterial strains i.e., PM21, PM22, PM23, PM24, and PM25. The maximum adsorption capacities (qe) of PM21 were observed 5-35 mg/g for Cd, 4-24 mg/g for Cr and 3-24 mg/g for Ni under 200 mg/L heavy metals. All the applied models supported the results of Cd and Cr biosorption with highest correlation coefficient (R^2) values as compared to Ni. The pseudo-second order kinetic model accurately captured the biosorption processes of biosorbents, indicating that HM biosorption was primarily chemisorption. The use of Fourier Transform Infrared Spectroscopy (FTIR) to characterization the surface of bacteria anticipated the involvement of particular functional groups in metal ion

adsorption. The Scanning Electron Microscope (SEM) results revealed that application of 200 mg/L of Ni has damaging effects on cell surface morphology. While in case of Cd and Cr (200 mg L^{-1}) the cells maintained their shape and size. The existence of CzcD gene responsible for cadmium and chromium resistance, in four strains (PM21, PM22, PM24, and PM25), was confirmed by PCR amplification. Plant growth is severely impeded by heavy metals like Cd, and Cr and it is well known that microorganisms inhibiting rhizosphere support plant growth under HMs. To elaborate the process of bacterial assisted phytoremediation, PM21 was applied on legume plant species, *Sesbania sesban* L. under heavy metals (Cd and Cr) stress.

In conclusion, this study shows that *in-vitro* growth of *Sesbania sesban* L. seedlings were arrested due to Cd and Cr toxicity. Heavy metals toxicity also negatively impacts important physio-chemical parameters of the growing seedlings.

Great potential of strain PM21 for biosorption of heavy metals further subjected to its *in-vitro* seed germination activity. After evidence revealed in 3rd study of *in-vitro* seed germination analysis, the strain was checked for its efficiency under control conditions. Our findings highlighted marked improvements after inoculation of seedlings with *B. anthracis* PM21 as a PGPR. The result revealed that *Bacillus anthracis* PM21 performed best under Cd stress as compared to Cr stress condition. The PM21 augmentation to seedlings significantly improved ($p \leq 0.05$) seed germination percentage (97.01%), root length (59.51%), shoot length (5.03%) and chlorophyll contents (total chlorophyll: 18%: Chlorophyll a: 20%; and Chlorophyll b: 16%), under Cd stress as compared to un-inoculated seedlings.

On the base of *in-vitro* seedling experiment, a pot experiment was conducted to explore the potential of PM21 in real time conditions. Our findings highlighted remarkable improvements after inoculation of plants with *Bacillus anthracis* PM21 strain as a plant growth promoting rhizobacteria. Application of *bacillus anthracis* PM21 increased morphological and physiological parameters as compared to uninoculated ones. Application of *B. anthracis* PM21 significantly ($p \leq 0.05$) enhanced Cd uptake in root, shoot translocation factor, bioconcentration factor 118.6, 73.4 mg/kg, 0.61, and 0.36 respectively. The PGPR, like *B. anthracis* PM21 with bioremediation potential can be utilized to decontaminate heavy metals contaminated soil.

The *Bacillus anthracis* PM21 was also applied for the first time in microbial assisted phytoremediation approach that enhanced plant growth of *Sesbania sesban* L. under Cd stress at different levels. It is concluded that strain PM21 with plant growth promoting characteristics could ameliorate heavy metal stress in *Sesbania sesban* L. plant by altering the morphological, physiological, antioxidant activities, and can be successfully applied in phytoremediation strategies. To explore its full potential regarding microbial assisted phytoremediation, carefully planned and regulated field experiments are required. Currently, large number of industries are releasing pollutants such as heavy metals in atmosphere and lithosphere. These heavy metals are threating environment and agroforestry. The PGPR such as *B. anthracis* PM21 having bioremediation potential can open a new endeavor for clean environment and food. This strain *Bacillus anthracis* PM21 presents striking potential for use as a plant growth promoting rhizobacteria and biosorption of heavy metal. Carefully designed field experiment is required to evaluate its full potential in supporting microbial assisted phytoremediation.

Future Recommendations

- The gene expression and quantification of CzcD in selected strains needed to be assessed by RT-PCR.
- The strains should be evaluated in future for multi-stress tolerance i.e., cold, drought, salt, and heat stress etc., in bench and field scale studies.
- The efficiency of phyto-accumulation of *Sesbania sesban* L. inoculated with PM21 should be evaluated in the field.

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Litrature Cited

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Appendices

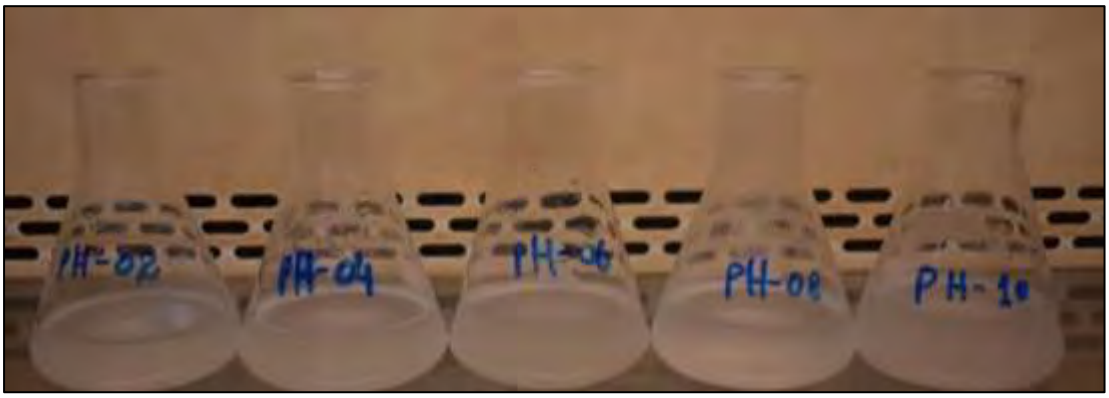

Plate 1: Effects of different pH on bacterial biosorption

Plate 2: Seed sterilization assay

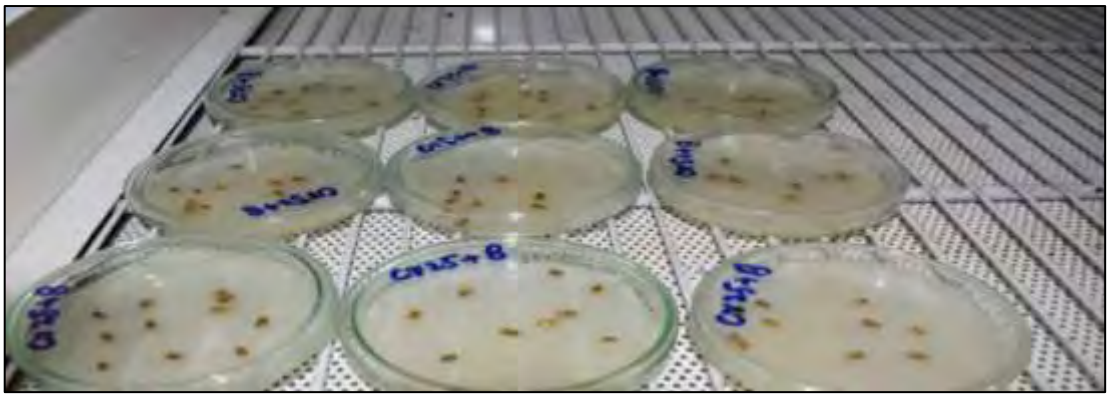

Plate 3: *In-vitro* seed germination activity

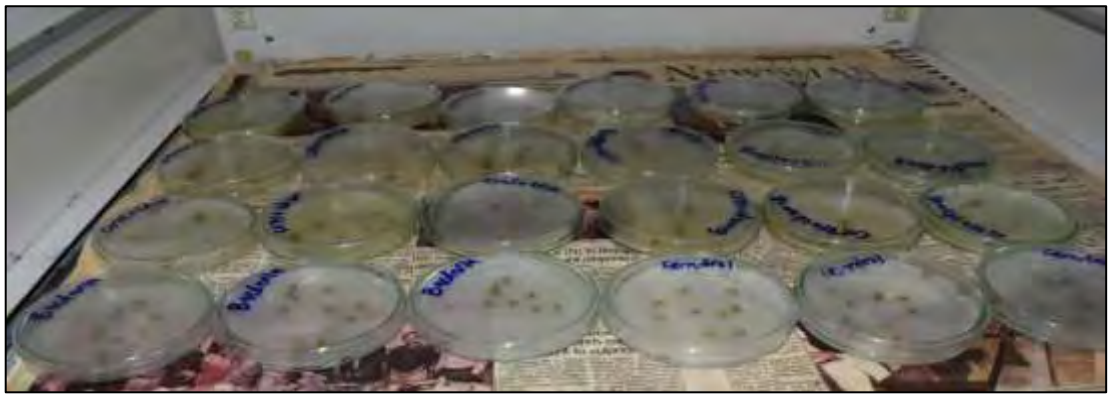

Plate 4: 1st day of seed germination on Petri plates

Plate 5: 2nd day of seed germination on Petri plates

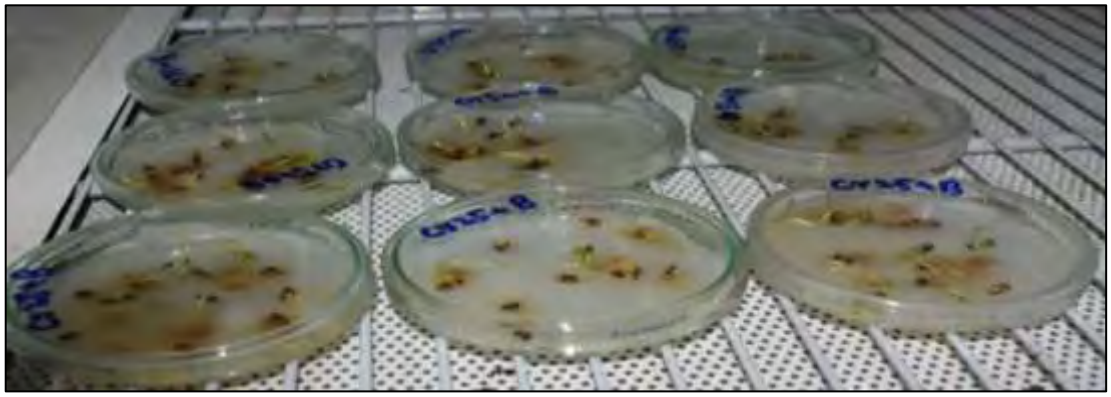

Plate 6: 3nd day of seed germination on Petri plates

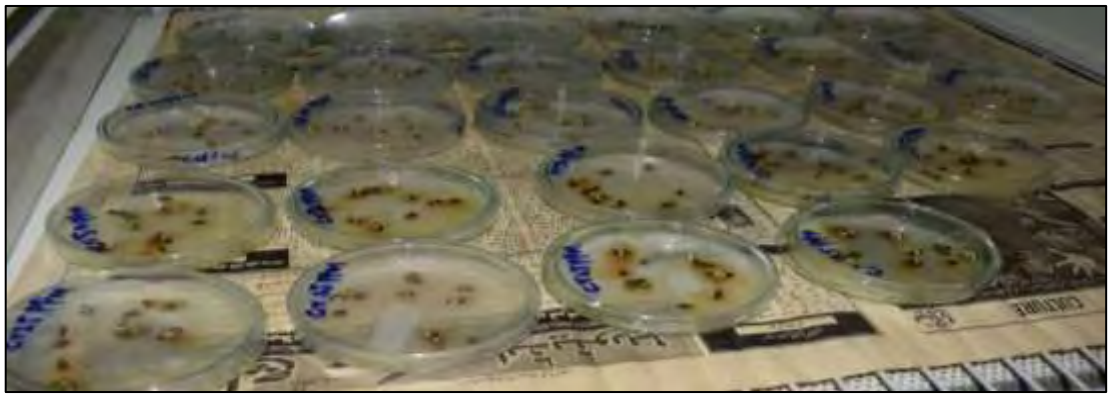

Plate 7: 4th day of seed germination on Petri plates

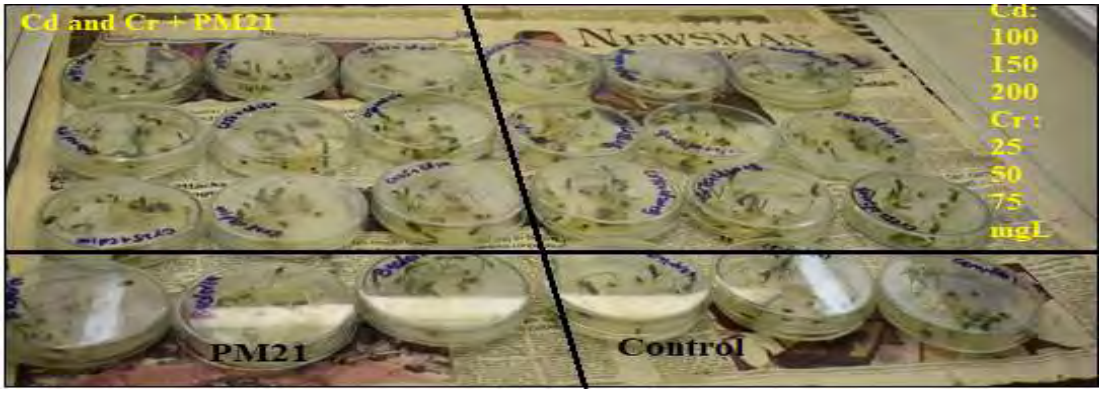

Plate 8: 3rd leaf stage of germinated seeds

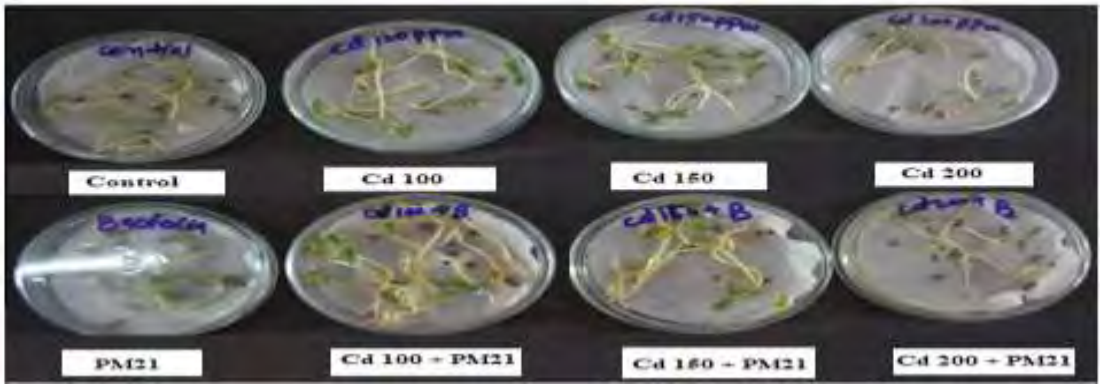

Plate 9: Cadmum effect on seed germiantion

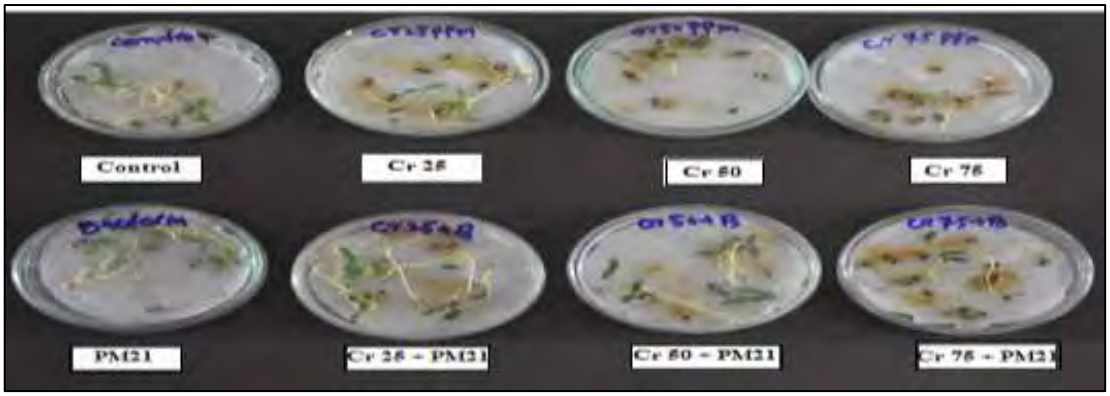

Plate 10: Effect of different chromium concentration on *Sesbania sesban*

Plate 11: Qualitative effects of *Bacillus anthracis* PM21 inoculation on growth of *Sesbania sesban* seedlings under various concentrations of Cd and Cr mg/L.

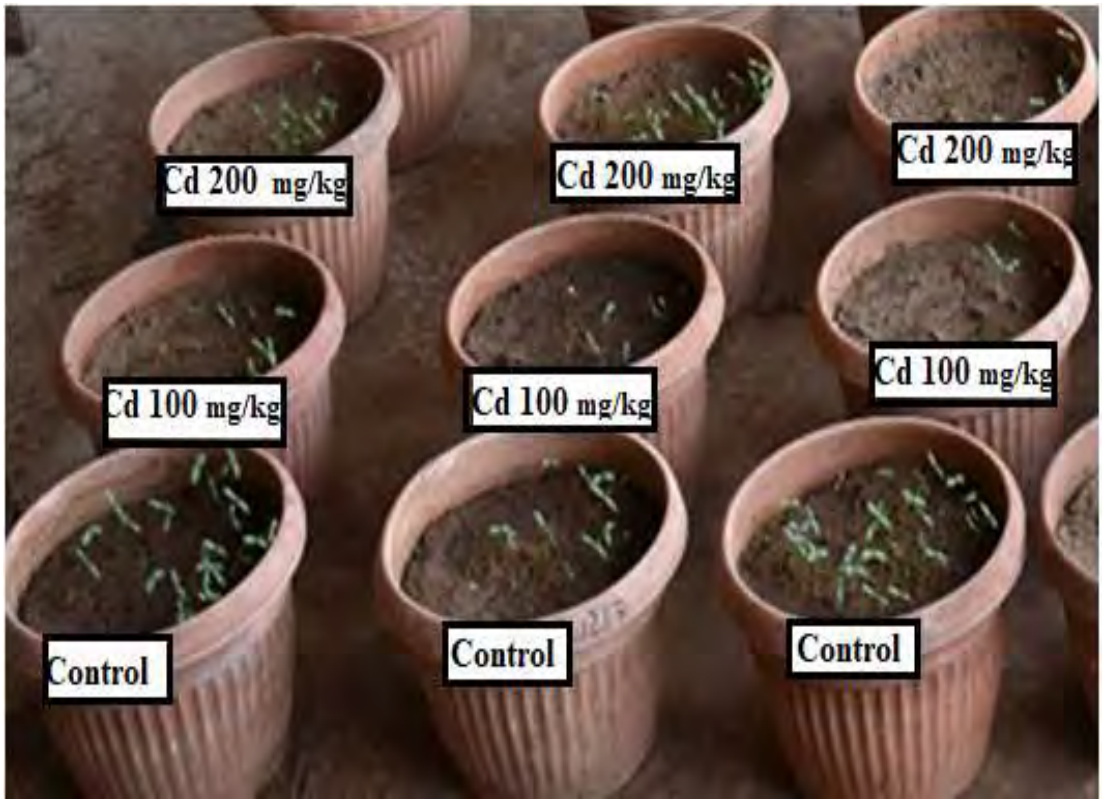

Plate 12: Growth condition of *Sesbania sesban* L. treated with cadmium

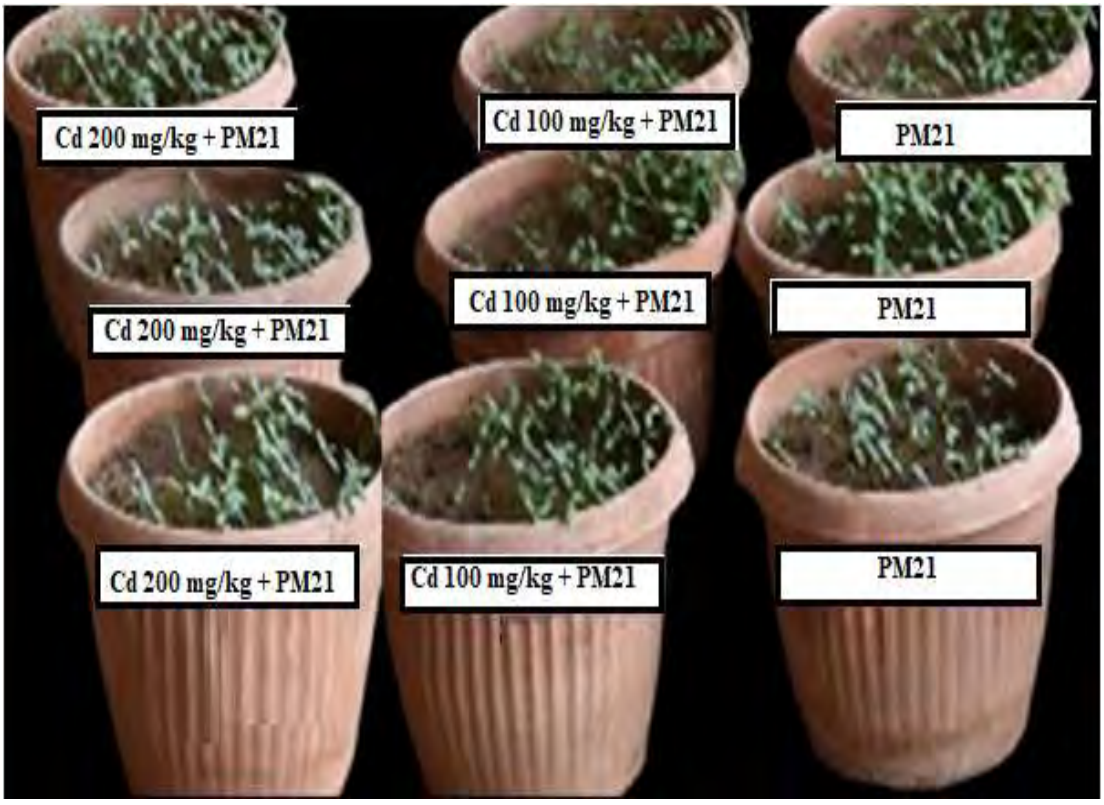

Plate 13: *Sesbania sesban* L. inoculated with *Bacillus anthracis* PM21 along with cadmium

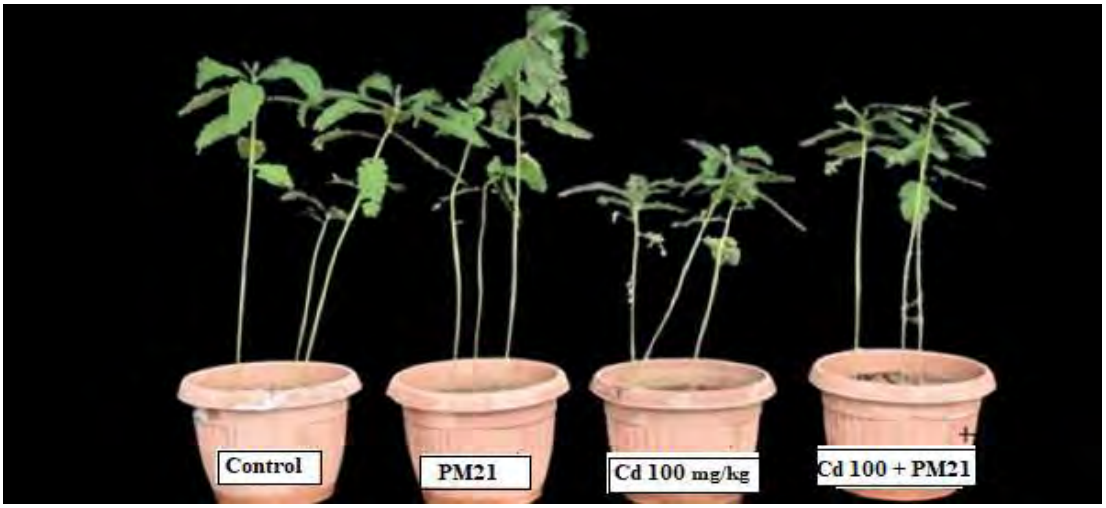

Plate 14: Comparison of plants of different treatments

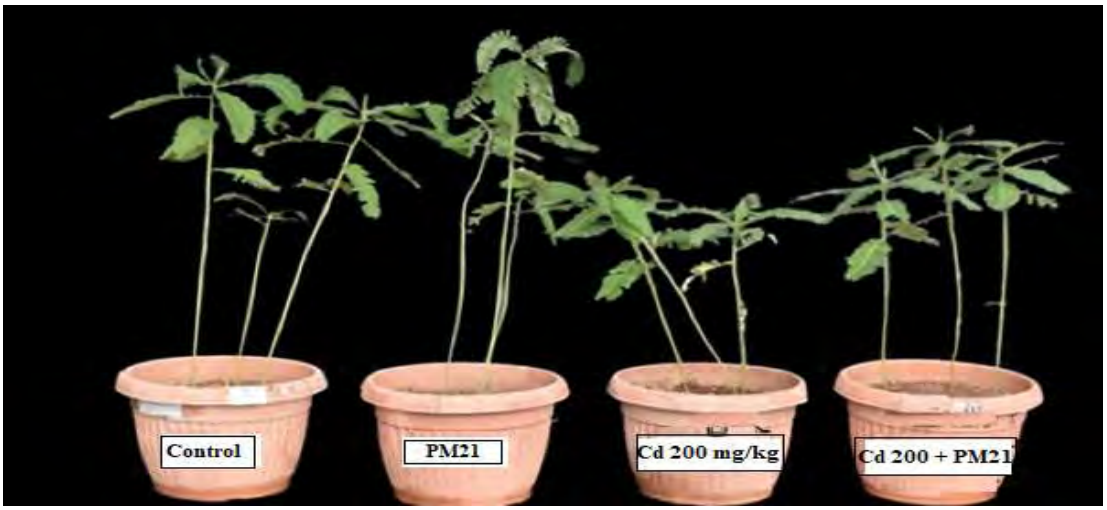

Plate 15: Comparison of plants of different treatments

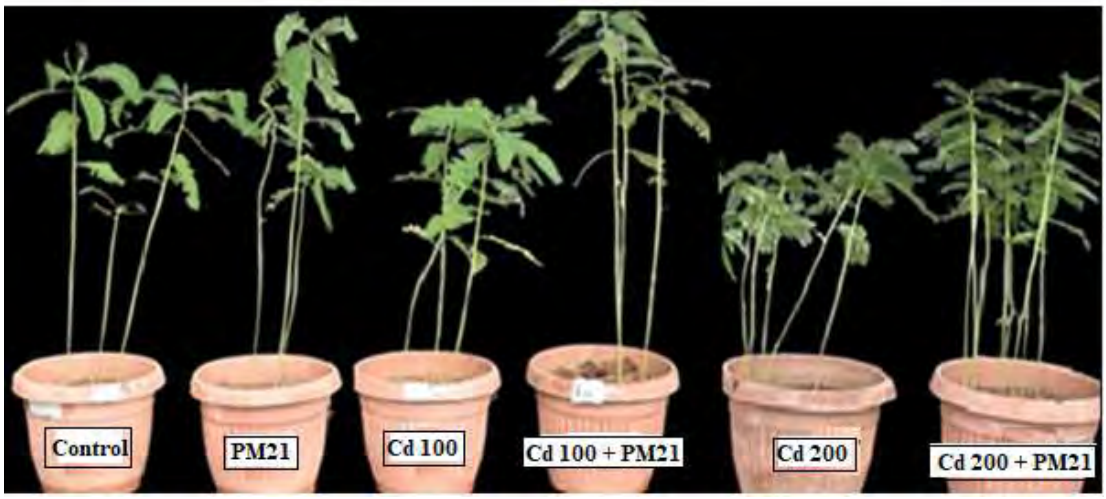

Plate 16: Showing effect of metal tolerant PM21 on growth of *Sesbania sesban* under normal and Cd stress condition

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Ecotoxicology and Environmental Safety

journal homepage:

Mechanistic elucidation of germination potential and growth of *Sesbania sesban* seedlings with *Bacillus anthracis* PM21 under heavy metals stress: An *in vitro* study

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ABSTRACT

Soils contaminated with heavy metals such as Chromium (Cr) and Cadmium (Cd) severely impede plant growth. Several chlorophenic microorganisms support plant growth under heavy metal stress. In this study, Cr and Cd stress was applied to in vitro germinating seedlings of a Leguminous plant species, *Sesbania sesban*, and investigated the plant growth potential in presence and absence of *Bacillus anthracis* PM21 bacterial strain under heavy metal stress. The seedlings were exposed to different concentrations of Cr (25–75 mg/L) and Cd (100–200 mg/L) in Petri plates. Growth curve analysis of *B. anthracis* PM21 revealed its potential to adapt Cr and Cd stress. The bacteria supported plant growth by exhibiting ACC-deaminase activity (1.57–1.75 μM of α-ketobutyrate/h/mg protein), producing indole 3 acetic acid (99–119 μM/mL) and exopolysaccharides (2.74–2.98 mg/mL), under heavy metal stress condition. Analysis of variance revealed significant differences in growth parameters between the seedlings with and without bacterial inoculation in metal stress conditions. The combined Cr + Cd stress (75 + 200 mg/L) significantly reduced root length (70%), shoot length (24%), dry weight (56%) and fresh weight (57%) as compared to control. Conversely, *B. anthracis* PM21 inoculation to seedlings significantly increased ($p < 0.05$) seed germination percentage (5%), root length (31%), shoot length (29%) and photosynthetic pigments (Chlorophyll *a* 20%; Chlorophyll *b* 16% and total chlorophyll 18%), as compared to control seedlings without *B. anthracis* PM21 inoculation. The *B. anthracis* PM21 inoculation also enhanced activities of antioxidant enzymes, including superoxide dismutase (52%), peroxidase (66%), and catalase (23%), and decreased proline content (56%), electrolyte leakage (50%), and malondialdehyde concentration (46%) in seedlings. The *B. anthracis* PM21 inoculated seedlings of *S. sesban* exhibited significantly high ($p < 0.05$) tissue deposition of Cr (17%) and Cd (36%) as compared to their control counterparts. Findings of the study suggested that *B. anthracis* PM21 relieved metal stress through assessment of antioxidant activities, and positively imparted *S. sesban* growth and biomass. Further experiments in controlled conditions are necessary for investigating phytoremediation potential of *S. sesban* in metal-contaminated soils in presence of *B. anthracis* PM21 bacterial strain.

1. Introduction

Many anthropogenic activities are continuously deteriorating soil health. Wastewater generated due to industrialization and urbanization is contaminating agricultural land (Maier et al., 2014; Rhodes et al., 2010). Cadmium (Cd) contamination in agricultural land has become a serious issue that is reported frequently in farming soils of various countries including Thailand, South Korea, China, Pakistan, Turkey and India (Rafique et al., 2019; Latif et al., 2020). High concentration of Cd in the soil deteriorates plant growth and ultimately reduces the yield of

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Phytoremediation of Cadmium Contaminated Soil Using *Sesbania sesban* L. in Association with *Bacillus anthracis* PM21: A Biochemical Analysis

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Abstract: Sustainable food production to feed nine to 10 billion people by 2050 is one of the greatest challenges we face in the 21st century. Due to anthropogenic activities, cadmium (Cd) contamination is ubiquitous with deleterious effects on plant and soil microbiota. In the current study, the phytoremediation potential of *Sesbania sesban* L. was investigated in Cd-spiked soil inoculated with *Bacillus anthracis* PM21. The Cd-spiked soil drastically reduced important plant attributes; however, inoculation of *B. anthracis* PM21 significantly ($p \leq 0.05$) enhanced root length (17.21%), shoot length (15.35%), fresh weight (37.02%), dry weight (28.37%), chlorophyll a (52.79%), chlorophyll b (48.38%), and total chlorophyll contents (17.65%) at the Cd stress level of 200 mg/kg as compared to the respective control. In addition, bacterial inoculation improved superoxide dismutase (11.98%), peroxidase (12.16%), catalase (25.26%), and relative water content (16.66%) whereas it reduced proline content (16.37%), malondialdehyde content (12.67%), and electrolyte leakage (12.5%). Inoculated plants showed significantly ($p \leq 0.05$) higher Cd concentration in the *S. sesban* root (118.6 mg/kg) and shoot (73.4 mg/kg) with a translocation (0.61) and bioconcentration factor (0.36), at 200 mg/kg Cd. Surface characterization of bacteria through Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) predicted the involvement of various functional groups and cell surface morphology in the adsorption of Cd ions. Amplification of the CzcD gene in strain PM21, improved antioxidant activities, and the membrane stability of inoculated *S. sesban* plants conferred Cd tolerance of strain PM21. In addition, the evaluated bacterial strain *B. anthracis* PM21 revealed significant plant growth-promoting potential in *S. sesban*; thus, it can be an effective candidate for phyto-remediation of Cd-polluted soil.

Keywords: antioxidant enzymes; Fourier transform infrared spectroscopy; scanning electron microscopy; CzcD gene; plant growth-promoting rhizobacteria

1. Introduction

Sustainability and food security are the main challenges of the current era. Environmental pollution is drastically increasing due to anthropogenic activities [1]. As a result of anthropogenic activities, the contamination of heavy metals (HMs) causes serious threats to the environment [1]. Being non-degradable and persistent in nature, HMs can easily bio-accumulate in the food chain and eventually induce ill effects on the health of humans