Chapter 1

Introduction

1. Introduction:

The modern-day industrialization and urbanization have led to the deposition of heavy metals in the soil, water, and the atmosphere. Several heavy metals like Chromium, Cadmium, Cobalt, are predominant environmental pollutants, specially, in regions with elevated human-induced activities. These heavy metals are very harmful even if they applied in lesser amount because of their high density [Singh et al. 2011]. These pollutants seriously affect the lungs, kidney, cardiovascular and central nervous systems and even lead to cancer. Accumulation of high concentration of heavy metals also affects plant growth and metabolism adversely [Goyal et al. 2020]. Bioremediation is a suitable method to combat the problem which helps degrade, remove, or utilize organic contaminants like heavy metals, oils, and other substances from waste by the activity of selected plants or microorganisms. The microbes growing in urbanized sewage sites can resist heavy metal toxicity and detoxify soil by developing different specialized mechanisms including efflux, accumulation, heavy metal reduction, utilization of metal ions in physiological processes, for instance as terminal electron acceptors during respiration or acid production, [Nageswaran et al. 2012; Valverde et al. 2011] to remove heavy metals from the environment. Many prior reports have demonstrated the role of waste land resident soil microbes in metal abolition [Kelly et al. 1998; Vargas-Garcia et al. 2010; Kamika and Momba, 2013; Gupta et al. 2014].

1.1 Review of previous literature:

Heavy metal uptake by bacterial cells is an important technique used for the removal of heavy metals from polluted environments. Previous literature reported that, microorganisms degrade different metal pollutants by applying different approaches to fulfil their physiological requirements [Tiedje, 1993] but still it's not a common practice. Previous finding also demonstrated that soil microbes can accumulate metals within their cells at a concentration 50 times higher than that of their habitat [Pumpel et al. 2001]. Microorganisms exhibit a number of metabolism-dependent and -independent processes for the uptake and accumulation of heavy metals. If bacteria are unable to permanently accumulate or assimilate the absorbed heavy metals within their cells, it returns to the environment again, thereby polluting it. It is therefore important to study the fate of the absorbed metal in bacterial bioremediation studies.

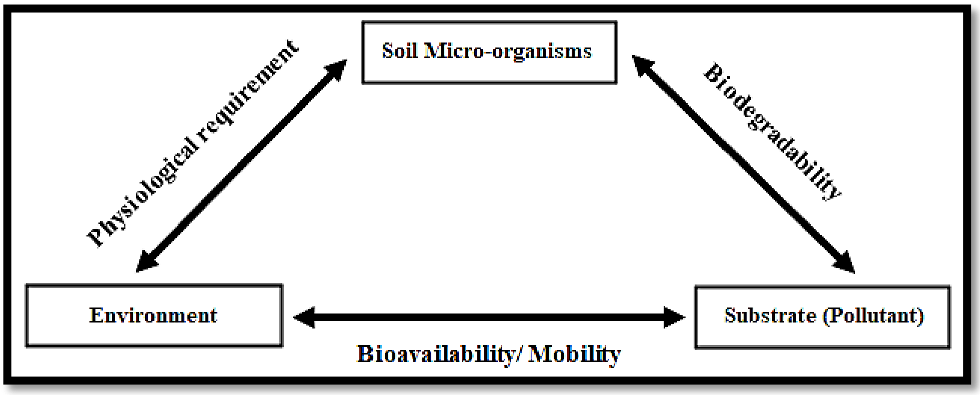

Figure 1.1.1: Microorganisms used to degrade metal contaminants through different strategies for their physical requirements and it's very inexpensive in situ approach; [Figure constructed by grasping the basic idea from: Tiedje, J. M. (1993)].

Although the soil is the natural habitat of millions of soil-borne microbes, contamination with different hazardous pollutants reduce the concentration of soil microbes. However, the microbes in the plant rhizospheres can withstand polluted environments, since a considerable amount of soil microbes can survive in the heavy metal contaminated soil of the plant rhizospheres [Whipps, 1990; Bais et al. 2006; Badri and Vivanco, 2009a; Badri et al. 2009b]. Plant growth promoting rhizospheric microbes act as abiotic stress reliever in heavy metal contaminated, nutrient deficient or saline soil by improving water and nutrient uptake, source-sink relationship, metabolite synthesis, protein function, proton transport, osmolyte assembling and antioxidant activity [Ilangumaran and Smith, 2017]. Different signaling pathways have also invented between plant-microbe interactions which contribute in soil salinity tolerance [Hasegawa et al. 2000]. Plant growth promoting capacity of the plant rhizospheric soil microbes should investigated to confirm their plant growth promoting rhizobacteria (PGPR) like traits and measured the plant bioconcentration factor (BCF) in presence of hazardous heavy metals to check their role in decreasing plant metal accumulation. So, if heavy metal degrading bacteria can aid plant growth and reduce the levels of accumulated heavy metals in plants, they can be implemented in poorly cultivated heavy metal contaminated environments [Atieno et al. 2013].

The Plant Growth Promoting Rhizobacteria (PGPR) also could be a good candidate for promoting plant growth because they instigate plant growth by fabricating certain extracellular plant growth stimulating substances such as Indole Acetic Acid (IAA), Indole Butyric Acid (IBA), siderophore, and phosphate degrading enzymes in the plant rhizospheric soil. In this way, they decreased the dependency on hazardous agricultural chemicals and PGPR also increased the mobility of soil nutrient and make them available to plants. Previously many siderophore producing *Bacillus*, *Azotobacter* [Xianmei et al. 2011] and Indole compound producing *Enterobacter, Klebsiella, Pseudomonas* have already been reported [Idris et al. 2007; Chen et al. 2009; El-Sayed et al. 2014]. Because of rhizospheric microbial activity in the vicinity of plant root regions, the hazardous metal accumulation by the plant remains lower. If plant accumulate huge amount of hazardous heavy metals from soil, large amount of ROS (reactive oxygen species) has produced which ultimately leads to programmed cell death (PCD). But the plant root rhizospheric bacteria developed different strategies like metal chelation and compartmentalization to overcome the heavy metal stress.

The plant growth promoting rhizobacteria can induce plant growth directly or indirectly. In direct way, they produce different extracellular substances such as siderophore, phytohormone, phosphate solubilizing enzyme which acquire different mechanisms to cause plant growth. Consequently, the microbes produced different substances like EPS (Exopolysaccharide), different lytic enzymes like gelatinase, amylase and different antibiotics [Gopalakrishnan et al. 2015] which induce plant growth by acquiring indirect mechanisms. The Indole compound containing phytohormone auxin is one of the best examples of microbe secreting phyto-simulator which is synthesized by plants and a few microbes and plays a key role in both root and shoots development by its centrifugal movement and also helps in plant cell division and elongation. Some plant rhizospheric bacteria produce different indole-containing plant hormones including Indole Acetic Acid (IAA) and Indole Butyric Acid (IBA), which stimulate and facilitate plant growth. Kloepper (1980) first introduced the role of rhizospheric bacteria secreted chelating compound siderophore in plant growth promotion by making complex with environmental iron and make it less available to the aboriginal microflora. Siderophores are low molecular weight iron-chelating compounds secreted by bacteria, fungi, and plants under the conditions of iron starvation, and make iron available for microbial and plant cells. Siderophores also have the ability to bind a variety of metals in addition to iron, and they have a wide range of chemical structures and specific properties. Siderophores act as bioremediating and chelation agents due to their heavy metal mobilization property, in addition to their important role in weathering soil minerals and enhancing plant growth due to metal uptake in rhizospheric regions [Ahmed and Holmström, 2014].

To observing the role of rhizospheric soil microbes in lowering plant metal absorbtion, soil to plant bioconcentration ratio (BCR) should be investigated. Bioconcentration ratio (BCR) or bioconcentration factor (BCF) can be investigated following different models among which Trapp and Matthies (1995) one compartment model is quite convenient which give a long term BCF from soil to leaf through transient assessment technique.

The BCF of plant rhizospheric soils used to describe the ratio of the concentration measured in the vegetation to the concentration in the soil supporting that vegetation. The bioconcentration factor (BCF) or bioconcentration ratio (BCR) of dry plant mass respective to dry soil should be calculated by applying the following formula:

$$BCR_{d,ss} = \frac{\text{Concentration in dry plant tissue (mg/kg)}}{\text{Concentration in dry soil (mg/kg)}}$$

Actually, the BCF values stated how much contaminants are consumed from the depository [Wang, 2016]. A lesser than one plant BCF value indicates that they probably only absorb but do not accumulate heavy metals within the cell. The BCF of the plant have expressed their potentiality in metal accumulation from growth substrate [Qihang et al. 2011].

The BCF of some edible plants grown at near vicinity of the heavy metal containing sewage reservoirs and consumed by poor people should be investigated to found whether bioaccumulation and biomagnification of heavy metal through the food chain have taken place or not. The hazardous metals can accumulate within the plants and enter the food chain through human and animal consumption. The heavy metals enter and accumulate within human body through two main routes: inhalation and food consumption. Heavy metal intake by human population through food chain has been reported many times and it's a potential threat for human society [Gall et al. 2015]. Since there is no good mechanism for their elimination, chronic low-level intake of heavy metal has severe damaging effects on human being and other animals. But the metal resistant and polluted soil inhabiting bacteria perform a significant role in cleaning up or remediating metal contaminated environments [Spain and Alm, 2003] as well as help in plant growth at heavy metal contaminated environment through lowering plant heavy metal accumulation from soil [Ahemad, 2019].

Invention of new bacterial strains which implied different environmental heavy metal degrading strategies to survive and withstand in their tricky heavy metal polluted natural habitat is notable by means of environmental pollution cleaning. The present study reported that municipal solid waste (MSW), agricultural compost, household sewage and most importantly industrial biproducts have polluted different sewage canals of Kolkata with heavy metals. The metals like chromium, cobalt, cadmium is generally emitted from battery manufacturing, skin processing (tannery), electroplating, organic chemical and dye forming industries and polluted sewage reservoirs. Metal contaminated sewage soil dwelling bacteria can tolerate and bypass the toxic effects of hazardous heavy metals. Thus, employing these microbes in environmental heavy metal removal opens up the possibility of decontaminate as well as recycle metals. Microbe remove environmental heavy metals through different strategies like bio-accumulation, retention and metabolism-dependent or -independent reduction. The present study significantly investigated the plant-soil-microbe interrelationship as well metal resistance pattern and biochemical mode of action strategies implemented by eco-friendly heavy metal resistant bacteria isolated from different sewage sites of Kolkata.

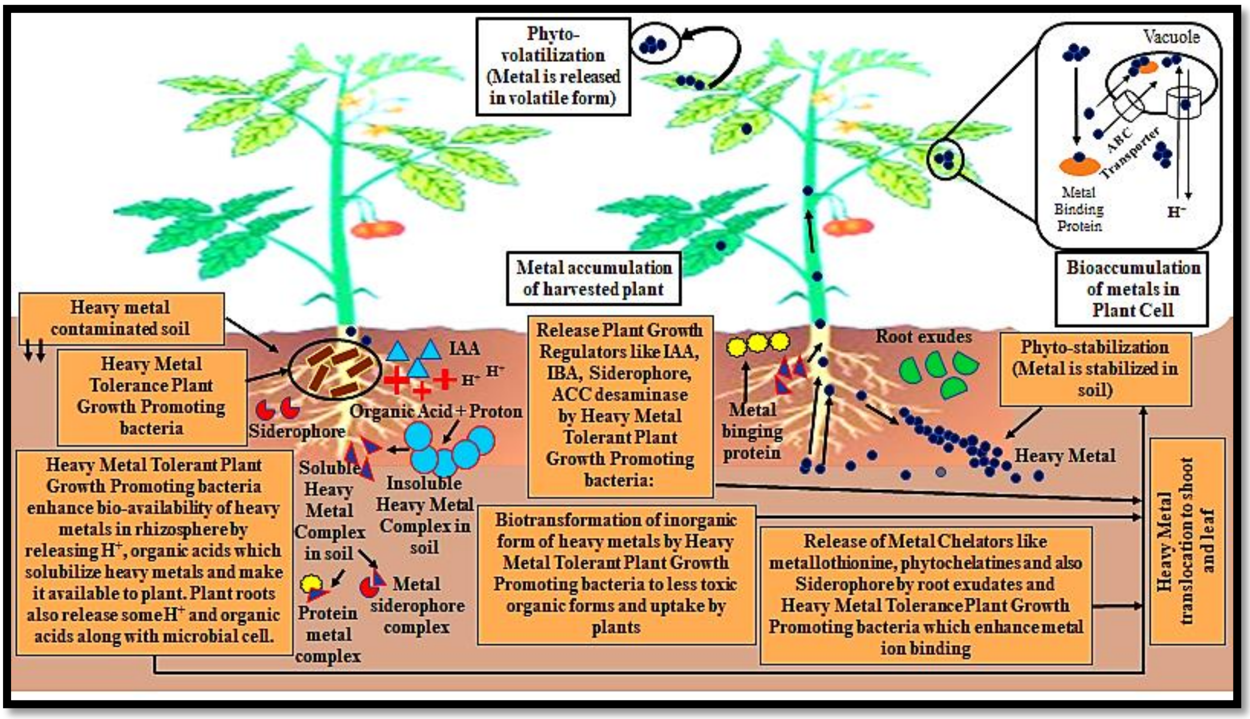

Figure 1.1.2: **The composite roles of heavy metal tolerant plant growth promoting rhizobacteria in soil heavy metal detoxification and plant growth promotion represented schematically [Figure constructed based on the findings of Mishra et al. (2017)].**

Previously Ray Chaudhuri et al. (2012) reported that the hot and humid tropical climate of Kolkata aids the sewage microbial communities to thrive in the heavy metal contaminated environment of East Kolkata Wetland and Gupta, Chatterjee**,** Gupta (2012) predicted the role of soil microbe in lowering plant heavy metal accumulation. But there is no detailed report on the study of heavy metal degrading mechanisms implied by microbes present in the different sewage canals of Kolkata. It is also unclear from previous studies whether the plant rhizospheric microbes can diminish plant heavy metal accumulation and promote plant growth or not.

1.2: Aims and Objectives:

Take the present scenario and previous background studies in mind; we wanted to investigate heavy metal implied bioremediation by microbial inhabitant of different sewage sites of Kolkata. We were also interested about the role of sewage sludge inhabiting rhizospheric microbes in lowering plant heavy metal accumulation.

The present study was aimed to investigate heavy metal implied bioremediation by microbial inhabitant of different sewage sites of Kolkata. The main objectives of the study enclosed the following:

1. Characterization and identification of soil microbial isolates of heavy metal contaminated sewage sites.

2. Monitoring heavy metal resistance capability of isolated soil microbes and predict their possible mode of action to remove sewage soil heavy metal contaminants.

3. Role of the isolated soil microbe in plant growth promotion and in decreasing plant heavy metal accumulation.

To fulfil the objectives, soil microbes were isolated from sewage sludge of three different sewage canals of Kolkata. As a reason of the hot and humid environment throughout the year, the waterbodies of Kolkata are the natural reservoir of different pollutant tolerant soil and water dwelling microbes and because of different anthropogenic chemical byproducts like fossil fuel, medical waste, municipal solid waste (MSW) and most importantly industrial waste the environment become contaminated with different hazardous heavy metals. We had chosen three most polluted sewage sites of south, north and east Kolkata viz. Circular canal, East Kolkata Wetland and Kestopur Khal based on the previous reports (Gupta et al. 2012; Ray Chaudhuri et al. 2012; Saha et al. 2014). To check the diversity in community composition among bioremediating microbes present in different wetlands of Kolkata, soil microbes were isolated from the air-dried sewage sludge. The isolated microbes were maintained in pure and replica plates as well as glycerol stalk, identified by morphological, physiological, biochemical and molecular means, there heavy metal resistance capability and probable biochemical mode of action behind metal resistance capability was investigated accordingly. Their role in decreasing plant heavy metal accumulation was also monitored.

Different soil physic-chemical parameters like soil pH, Chemical Oxygen Demand (COD), Dissolved oxygen (DO), dehydrogenase (DHA) activity of soil, should be investigated before isolate soil bacteria to determine the interaction type between different soil components with the soil inhabiting microbes. Because different factors like soil category, soil biotic ingredient percentage, the pH of soil, percentage of soil clay matter and many more biochemical and chemical characteristics affect the threshold of heavy metal dietary toxicity in soil-crop system [Islam et al. 2007]. Soil heavy metal toxicity threshold is an important factor which affects heavy metal tolerating limit of soil environment and determine heavy metal cumulative loading limit. For soil-plant system, the heavy metal toxicity is the highest permissible content in the soil (total or bioavailable concern) that does not produce any phytotoxicity or the heavy metal in the edible part of crops does not exceed the critical dietary heavy metal threshold for human health. The pH of soil is one of the most important parameters for microbial growth. Generally, pH values ranging between 4.5 and 8.3 are optimum for microbial growth owing to the suitability of mineral constituents and availability of carbon source [Andersson and Nilsson, 2001]. The Chemical Oxygen Demand (COD) of sewage is the amount of oxygen that is consumed by the sewage bacteria or is utilized in other chemical reactions. It is used to quantify the number of oxidizable pollutants found in the surface of wastewater [Hu and Grasso, 2005]. This test is based on the fact that a strong oxidizing agent, under acidic conditions, can fully oxidize almost any organic compound to carbon dioxide. Dissolved oxygen (DO) is an important parameter in wastewater treatment. The oxygen content of sewage water decreases when there is an increase in the amount of nutrients and organic materials from industrial wastewater, sewage discharges, and runoffs from the lands [Patel and Vashi, 2015]. The determination of DHA activity of soil provides a large amount of information about the biological characteristics of the soil as, soil dehydrogenase is a bacterial extra-cellular enzyme that strongly increases under anaerobic conditions [Wolińska and Stępniewska, 2012].

Microbial isolates were chosen and differentiated from mixed sewage population according to their morphological, biochemical, physiological and molecular parameters to fulfil the criteria of polyphasic classification of bacterial identification [Wang et al. 2019]. The cellular morphology of the isolated microbes was determined by the conventional Gram staining procedure [Bartholomew and Mittwer, 1952]. Each microbial species possesses a well-defined set of unique enzyme-controlled biochemical metabolic activities. Intracellular enzymes are the basis of cellular metabolism, the products of which are formed and excreted outside cells [Passalacqua et al. 2016]. Extracellular enzymes are excreted by the bacteria into the surrounding environment to hydrolyze the complex biomolecules for their own benefits. Culture conditions should be optimized by standardizing different parameters such as temperature, initial value of pH, and the inoculum percentage, to obtain a suitable environment for bacterial growth and survival [Andersson and Nilsson, 2001]. Antibiotic screening is also a routine procedure for bacterial characterization. The aims of antibiotic screening are to detect possible antibiotic resistance in isolated soil microbes for identification purpose, and also to assure the susceptibility to the antibiotic of choice for the particular microbes because the multi- or bi-antibiotic resistance genes show co-occurrence with heavy metals [Yamina et al. 2012; Chen et al. 2019]. Heavy metal tolerant microbes might also have a significance contribution in maintaining antibiotic resistant genes as they can tolerate huge selective force of environment [Spain and Alm, 2003].

After initial characterization, analyze bacterial heavy metal resistance efficiencies and find out their possible mode of action in removing heavy metal from environment. The disk-diffusion or agar cup method [Rose and Miller, 1939] involves the diffusion of the antimicrobial agent from where the agent has been applied to an agar plate that previously inoculated with the test microorganism, tests the effectiveness of heavy metals or antibiotics on a specific microorganism. In order to measure the bacterial Minimum Inhibitory Concentration (MIC) [Andrews, 2001], they were treated with elevated concentrations of heavy metal solutions till their growth was completely inhibited [Yilmaz, 2003].

Incidentally, most of the isolated microbes showed resistance against chromium. That's why we have chosen chromium for further investigation. According to previous findings, the second stable environmental state of chromium, the hexavalent chromium (Cr^{6+}), revealed different hazardous effects on human, animal [Shrivastava et al. 2002] and plants [Oliveira, 2012], should be removed from the environment. The hazardous hexavalent chromium absorption and reduction capability of the microbial isolates was monitored. Also described the probable biochemical mode of action behind chromium reduction through which respiratory chain linked electron transport. Many prior investigations have reported different species of *Bacillus*, *Staphylococcus*, *Pseudomonus*, *Microbacterium*, *Micrococcus*, *Mangrovibacter yixingensis*, *Klebsiella*, *Desulfovibrio* and *Escherichia coli*, *Agrobacterium radiobactor*, *Aeromonus dichromatica* participate in metal bioremediation and most of them exhibit reduction of hexavalent chromium (Cr^{6+}) to less toxic trivalent chromium (Cr^{3+}) [Sanjay et al, 2020; Wang and Shen,1995; Upadhyay et al. 2017; Kaushik et al. 2012].

As, microbial metal uptake by metabolism-dependent and -independent processes has a considerable impact on toxic metal removal from polluted environment, chromium accumulation and retention capability of isolated microbial stains was determined and measured implying Transmission Electron Microscopy (TEM) [Tripathi and Singh, 2016], Energy Dispersive X-Ray Fluorescence (EDXRF) spectrometer [Adams, 2019] and Atomic Absorption spectroscopy (AAS) [Menzies, 1960]. Viability and survival of metal resistance strains up to certain concentration of toxic hexavalent chromium was detected by Scanning Electron Microscope (SEM) images [Hubbs et al. 2013] besides inspecting the zone of inhibition and minimal inhibitory concentrations. Later, the acceleration deceleration rates of chromium reduction were also investigated in presence and absence of growth stimulator Dinitrophenol (DNP) and growth inhibitor sodium azide to predict the significance of chromium reduction in aerobic respiration and respiratory chain linked electron transport [Goto and Anraku, 1974; Cabrol et al. 2017].

Role of the isolated microbe in plant heavy metal accumulation reduction was also inspected as plant growth promoting heavy metal tolerant microbes can absorb the bioavailable heavy metal in acidic soil pH [Merdy et al. 2009] and most of the root habiting soil microbe produce extracellular organic acid as chelating substance which help them to accumulate soil heavy metals [Turnau and Kottke, 2005; Seneviratne et al. 2017]. Soil dwelling heavy metal tolerant rhizospheric microbes can rescue plant from heavy metal stress by developing several strategies like accumulation, detoxification or transformation of soil heavy metals [Mishra et al. 2017]. Plant rhizospheric microbe can bind heavy metals at their outer surface or change soil metal mobility [Ehrlich, 1997].

Figure 1.2.1: Some edible plants at their heavy metal contaminated natural habitat. [A] *Cleome rutidospernmum* (Capparidaceae) at the vicinity of Circular canal; [B] *Colocasia esculenta* (Araceae) at East Kolkata Wetland region; [C] *Eichornia crassipes* (Pontederiaceae) at Kestopur Khal; [D] *Amaranthus viridis* (Amaranthaceae) in mixed vegetation of East Kolkata Wetland [E] *Coccinia grandis* (Cucurbitaceae) within mixed vegetation at Kestopur Khal area

So, the significance of the present study lies in implying eco-friendly heavy metal remediating bacteria from the different sewage sites of Kolkata.