Remediation of Cadmium Contaminated Soil with PGPR Consortia and Hyperaccumulator Plants

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

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Certificate

This is to certify that the thesis titled "**Remediation of Cadmium Contaminated Soil with PGPR Consortia and Hyperaccumulator Plants"** submitted by **Miss Chhaya Verma** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

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

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DECLARATION

I, Chhaya Verma, hereby declare that the dissertation work entitled "**Remediation of Cadmium Contaminated Soil with PGPR Consortia and Hyperaccumulator Plants"** is my own work carried out under the guidance of **Prof. Rajesh Kumar, Head, Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University (A Central University), Vidya Vihar, Rae Bareli Road, Lucknow-226 025.** The matter embodied in this thesis work is written by me and has not been submitted to any other university for the fulfillment of the requirement of any other Degree or Diploma.

Place: - **Lucknow** (**Chhaya Verma**)

Date: -

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Chhaya Verma

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylic acid
AMF	Arbuscular Mycorrhizal Fungi
bp	Base pair
CAS	Chrome Azural S
CAT	Catalase
Cd	Cadmium
CDA	Czapek - Dox Agar
CFU	Colony forming units
EDTA	Ethylene diamine tetraacetic acid
EPS	Extracellular polymeric substances
FP	Fluorescent Pseudomonad
FPs	Fluorescent Pseudomonad
FTIR	Fourier Transform Infra-Red spectroscopy
H_2O_2	Hydrogen Peroxidase
HCN	Hydrogen Cyanide
HM	Heavy metal
HPLC	High performance liquid Chromatography
hr	Hours
IAA	Indole Acetic acid
ICTV	International committee on the taxonomy of viruses
Kg	Kilogram
LB	Luria Bertani
LMWOAs	Low molecular weight organic acids
MR	Methyl red
MAR	Multiple antibiotic resistant
MHA	Muller Hinton Agar
MIC	Minimum inhibitory Concentration
MR-VP	Methyl Red- Voges Proskauer
MT	Metalothionein
MIC	Minimum inhibitory concentration
NA	Nutrient agar

NB	Nutrient broth
OD	Optical density
PBS	Phosphate buffer saline
PGPR	Plant growth promoting rhizobacteria
PGP	Plant Growth Promoting
POX	Peroxidase
PPM	Parts per million
PS	Phosphate solubilisation
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Scanning electron microscope
SIM	Sulfide Indole Motility
SOD	Superoxide dismutase
SU	Siderophore unit
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSS	Total Soluble sugar

Introduction

Chapter 1 Introduction

Industrialization has resulted in increased influx of toxic and non-toxic substances in vital segments of environment i.e. air, soil and water. Metals, amongst them existed from ages. However, heavy metals released from industries and its impact is matter of global concern. Various heavy metals such as arsenic (As), cadmium (Cd), copper (Cu), chromium (Cr), mercury (Hg), lead (Pb) and zinc (Zn) are reported in environment and these heavy metals at low concentration play an important role for many life processes such as in enzyme productivity (Cheng, 2003). But, above the threshold limits, these heavy metals can be dangerous for life. Heavy metals are a group of 65 metallic elements with density greater than 5 g/cm^3, exhibiting diverse properties with a potential to exert toxic effects on microorganisms and other forms of life. Organic pollutants can be degraded by microbes, but heavy metals accumulate in soil for several years with persistent quality (Xu et al., 2012).

Heavy metals are very dangerous for all living beings because it can form some potentially toxic compounds in cationic form, e.g. Hg^{2+}, Cd^{2+} and Ag^{+}. Among heavy metals, As (Arsenic), Al (aluminium), Zn (zinc), Mn (manganese), Cr (chromium), Cu (copper), Cd (cadmium), Pb (lead) and Hg (mercury) are the common toxic metals (Emamverdian et al., 2015). Amongst all the heavy metals, cadmium has deleterious effects on agricultural ecosystem, environment and human health (Wagner, 1993). Cadmium (Cd) enters in soil by various routes, but mainly through anthropogenic source such as release of phosphate fertilizers, emissions from power stations, metal and cement industries, vehicles exhaust (Bolan et al., 2013) zinc smelting and municipal wastes (Lima et al., 2006). Cadmium compounds are relatively more soluble than those of other metals, this property make the cadmium highly mobile in the water-soil-plant system (Nordic Council of Ministers, 2003).

From soil, cadmium enters into plants through the root cortical tissues and reaches the xylem via a symplastic and/or apoplastic pathway (Salt et al., 1995). Plant tissue concentration of cadmium is greater than 5–10 mg/kg (dry matter) and becomes toxic to most plants (White and Brown, 2010).

In case of plants, cadmium can cause growth inhibition by reduction of carbon fixation through inhibition of photosynthetic rate and chlorophyll concentration. Cadmium also induces water stress in plants, results in the inhibition of stomatal conductance, transpiration rate, and leaf relative water content (Chen and Huerta, 1997). Such water stress may result from physiological damages such as inhibition of size and number of xylem vessels, intracellular spaces, and the number of chloroplasts in leaves. In addition to this, cadmium also causes cell enlargement by the inhibition of phytochelatin synthase (Kasim, 2005). The heavy metals including cadmium are non-degradable and persist in the soil for approximately 15–1100 years (Kabata and Pendias, 1993). Stored cadmium accumulates in the harvestable (edible) part of plants (Baker et al., 1994) via translocation. High accumulation level generally causes growth inhibition and finally death of plant as well as cell.

Heavy metal as well as cadmium contamination of soils has received considerable attention in the contemporary science due to its toxicity. Therefore, it is important to develop methods to remediate the heavy metals. Various engineering methods such as excavation, landfill, thermal treatment, leaching and electro-reclamation are used (Tangahu et al., 2011). However, these methods are not fully satisfactory as they destroy the biotic and abiotic components of the soil, and are also technically difficult and expensive to use (Zubair et al., 2016). All these techniques such as thermal processes physical separation, electrochemical methods, washing, stabilization/solidification and burial for remediation and detoxification of metal

contaminated soils are generally too expensive and often affect the diversity of microbial community in soil (Ma et al., 1993; McGrath et al., 1995; Pulford and Watson, 2003). In addition to this, various biological processes such as phytoremediation, bioremediation and mixed methods are also used for remediation of cadmium and other heavy metal, but they also have challenging task because heavy metals including cadmium cannot be degraded and hence persist in the soil for several years (Kidd et al., 2009; Rajkumar et al., 2010; Ma et al., 2011).

Among all these methods, phytoremediation is best and has been a highly acceptable method from several years. Plant based clean-up process for cadmium is commonly referred to as "phytoremediation". It has been proposed as best alternative method for removal of pollutants from air, soil and water and does not affect soil biological activity, structure and fertility (Raskin et al., 1997; Salt et al., 1998). 'Phytoextraction', is one of the key processes of phytoremediation in which metal accumulating hyper-accumulator plants are involved for removal of metals from soil by concentrating them in harvestable parts of the plant. Second most successful process of phytoremediation is 'Phytostabilization' in which metal tolerant plants arrest the leaching of heavy metals through the thick mat of adventitious roots and rhizosphere microbes.

The success of phytoremediation is dependent on the potential of the plants to yield high biomass and tolerate metal stress. Efficiency of metal as well as cadmium translocation and phyto-stabilization process is dependent on the bioavailability of metal in rhizospheric soil (Ma et al., 2011). For improving the bioavailability of metal, various types of chemicals are used such as EDTA, Limestone etc. (Barrutia et al., 2010; Wu et al., 2011). However, uses of these chemicals such as EDTA have some limitations because they have toxic nature for the plants (Evangelou et al., 2007)

as well as plant growth promoting microorganisms of root region (Ultra et al., 2005; Mühlbachová, 2009).

Phytoremediation process is dependent on hyper-accumulator plants. For phytoremediation, selection of hyper-accumulator plants is an important factor because most of the hyper-accumulator plants are slow-growing and usually produce limited amounts of biomass. Plants that accumulate metals at high concentration are called hyper-accumulators (Visoottiviseth et al., 2002). If the shoots of plants contain >100 mg Cd kg^{-1}, >1000 mg Ni, Pb and Cu kg^{-1} or >10,000 mg Zn and Mn kg^{-1} (dry wt), then they are known as hyper-accumulators (Baker and Brooks, 1989). There are approximately 45 hyper-accumulator plant families and 500 plants belonging to these. Some of the important families to which these plants belong, includes Brassicaceae, Euphorbiaceae, Asteraceae, Fabaceae, Lamiaceae and Scrophulariaceae etc. (Ghosh and Singh, 2005).

Amongst all the plants, Indian mustard has good capacity to extract, sequester, or detoxify the heavy metals from contaminated soils. However, their sequestration capacity depends on metal mobility, plant factors and crop management factors. Indian mustard (*Brassica juncea*) is an important oilseed crop in India. It contributes maximum in domestic edible oils. According to Ministry of Agriculture (2015) it is cultivated in 6.28 Mha with 7.46 Mt production and 1188 kg ha^{-1} of its productivity. The phytoremediation process by using Indian mustard is done by two mechanisms viz. phytoextraction and phytostabilization. The extraction capacity of plants can be induced by agronomic interventions such as selection of cropping systems, use of chelators, fertilizers, and irrigation (Ma et al., 2001; Diwan et al., 2008).

Other hyper-accumulator plant 'Maize' has good capacity to remediate toxicant (Wuana and Okieimen, 2010) and maize can grow in diverse climates in high

mountain plains or arid desert plains. Maize plant is a major source of food for both humans and animals, and is grown in many countries. The majority of the maize crop is used as livestock feed. The remainder is processed into a range of food and industrial products such as ethanol as a fuel, starch, sweeteners (fructose maize syrup) and maize oil. Although use of hyper-accumulator plants has several advantages in phytoremediation, but due to some properties it has some limitations. These plants generally accumulate one specific element with limited root system and this limitation makes its use irrelevant (Begonia et al., 2005) for remediation purpose.

Remediation of contaminants such as heavy metal, dye, xenobiotics, and hydrocarbon etc. is also done by microorganisms is called as "Bioremediation". In this remediation process microorganisms use the chemical contaminants as nutrients for energy and transform the contaminants into less toxic or harmless products via metabolic processes in most of the cases. Hence, bioremediation works as an alternative technique for remediation of contaminants via biological mechanisms (Kamaludeen et al., 2003). Similarly, the uses of microorganisms that occur in root area are also an effective tool for the remediation of heavy metals from the contaminated site. Among all the known remediation processes: bioremediation phytoremediation and rhizoremediation could be more applicable or acceptable method to remediate the cadmium and other heavy metals from soil. Presently, use of biotechnology in this area can be helpful for making remediation process more relevant and applicable by developing genetically modified microorganisms (Wang and Chen, 2009). But then, they also have limitations due to biosafety issues and their stability (gene stability) in the environment in presence and absence of abiotic stresses.

Use of microbial isolates or microbial assisted phyto-remediation can be better tool wherein microbial metabolites are used (Sharma and Archana, 2016). These microbial metabolites offer a promising alternative of chemical amendments as they are less toxic and easily biodegradable. This is a microbe-mediated process in the rhizosphere, in which the microbial metabolites affect plant metal uptake by changing the mobility and bioavailabity of metal (Wenzel, 2009; Rajkumar et al., 2010; Verma et al., 2017; Glick, 2010; Ma et al., 2011; Aafi et al., 2012; Ullah et al., 2015). It may be possible to produce microbial metabolites *in-situ* at rhizosphere region by inoculation of PGP (Plant Growth Promoting) microbes. In aspect of this, plant growth promoting substances/metabolites such as siderophore, plant growth hormones (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enhance the growth and metal tolerance of the plant in metal contaminated soils (Wu et al., 2006; Glick et al., 2007; Lebeau et al., 2008; Glick, 2010; Rajkumar et al., 2010; Kuffner et al., 2010; Babu and Reddy, 2011; Ma et al., 2011; Miransari, 2011; Wang et al., 2011). In addition to this microbial activities in the rhizosphere soil also induces the effectiveness of phytoremediation processes in metal contaminated soil by two complementary ways: i) Direct promotion of phytoremedation by facilitating phytoextraction or reduction of phytostabilization and (ii) Indirect promotion of phytoremediation in which the microbes induces metal tolerance and/or enhance the biomass production in plant for removal/accumulation of the metal.

Bio-inoculants are living organisms including bacteria, fungi, or algae, that enhance the growth of plant, decompose organic contaminants and improve phytoremediation capacity of various plants including mustard and maize. Besides this, metal resistant plant growth promoting endophytes have also been reported that are resistant to different heavy metals (Li et al., 2012). Anwar et al. (2012) reported

that bio-inoculants induce the dry matter of shoots and roots of Indian mustard in metal contaminated soil. For support of this finding Luo et al. (2009) suggested that application of beneficial microorganisms especially arbuscular mycorrhizal fungi (AMF) and the plant growth promoting rhizobacteria (PGPR) are environment friendly techniques for improvement of phytoremediation. These microbial communities are effective in enhancing metal availability to plants through alterations in rhizospheric microbial complex, consisting the release of chelators, acidification and redox changes (Sheng and Xia, 2006; Zaidi et al., 2006; Sinha and Mukhrjee, 2008; Sheng et al., 2008; Ma et al., 2009).

Hence, this study focused on remediation of cadmium contaminated soil as well as growth promotion of mustard and maize plants by using cadmium resistant PGPRs (fluorescent pseudomonads). To see the role of cadmium resistant PGPRs on the mustard and maize plants and remediation of cadmium, pot experiment conducted in 100 ppm cadmium amended soil. For cadmium resistant analysis of bacteria the growth pattern and morphology of bacteria were analysed in presence and absence of bacteria. To check the efficiency of PGPRs for remediation of cadmium accumulation test of isolates was also done. Isolates used in this study were good PGPRs with multiple plant growth promoting properties and have high cadmium resistant properties. Before applying in the pot, all the isolates were tested and characterized for best result. In this study it was found that the used PGPRs were helpful for remediation of cadmium by rhizosphere accumulation of cadmium and growth promotion of mustard and maize plant in presence of cadmium as well as in absence of cadmium. The findings also established that use of PGPR strains resulted in stabilization of cadmium in the root zone with less concentration in the edible part of the plant.

Review of Literature

Mid of twentieth century led to an increase in industrialization to meet the demands of growing population but this resulted in release of various toxicants in the water, air and soil. The toxicants have posed various uncompromising and fatal effects on human health and the stability of the ecosystem. Of the many toxicants/pollutants which includes hazardous wastes like PAH, e-waste, paints and solvents, automobile waste, pesticides, hospital waste, fertilizer waste, plastic material, industrial effluents containing many carcinogenic dyes and other materials including heavy metals (Saluja et al., 2011; Li et al., 2015). All these hazardous waste including heavy metals finds their way into the food chain through various roots. These heavy metals contaminating the agricultural soil are of immense concern throughout the world, particularly in the suburban areas of developing cities. All the metals have fixed regulatory limit as listed in Table-2.1.

Table-2.1- Concentration range and regulatory limit of toxic heavy metal (Source: Salt et al., 1995):

Heavy Metal	**Concentration Range mg/kg**	**Regulatory Limit mg/kg**
Arsenic (As)	0.1-102	20
Cadmium (Cd)	0.1-345	100
Chromium (Cr)	0.005-3950	100
Copper (Cu)	0.03-1550	600
Mercury (Hg)	0.001-1800	270
Lead (Pb)	1-6900	600
Zinc (Zn)	0.15-5000	1500

2.1.Heavy Metal:

The term ‘heavy metal’ has different definitions, but it is mostly used in the context of environmental pollution. Among others, Shaw et al. (2004) explained four

criteria in distinguishing the groups of heavy metal: 1) Relatively abundant in the earth's crust; 2) reasonable extraction and usage; 3) having direct contact with people; and 4) toxic to humans. Another definition describes heavy metals as the metals which have a specific gravity of more than 4 or 5 (Nieboer and Richardson, 1980). Most heavy metals are categorized as toxic and accessible, based on the classification of Wood (1974), and their concentrations in soil vary between 1 to 100,000 mg/kg (Blaylock and Huang, 2000). From the bibliographical survey made by Duffus (2002), the term heavy metals appears to be commonly applied to elements of density higher than 3.5–7 g/cm and high atomic number (higher than 20), and includes transition metals, some metalloids, lanthanides and actinides.

Among all the heavy metals Cd (cadmium), Cr (Chromium), Cu (Copper), Pb (Lead), Hg (Mercury), As (Arsenic), Zn (Zinc), Aluminium (Al) and Mn (Manganese) are the very common hazardous metals (Emamverdian et al., 2015). Excess use of heavy metals is toxic while lower concentration of certain heavy metals like zinc, copper, manganese, iron etc. are required by plants as micronutrients. These heavy metals show toxic effects on terrestrial and aquatic ecosystem that enhance the risk related with physiology (Chen et al., 2015; Roy and McDonald, 2015). In comparison to all other heavy metals, there are metals which are more toxic than others and cadmium is one of them. Regular use of untreated waste water of industry for irrigation purposes enhances the level of cadmium in soil (Murtaza et al., 2008). Studies have shown that application of industrial effluents, sewage sludge, phosphate fertilizers and wastewater for irrigation has increased the levels of heavy metals in the agricultural soils and also in edible portions of vegetable crops.

2.2.Cadmium:

Cadmium was discovered by Friedrich Stromeyer and Karl Hermann in 1817 simultaneously with the samples of zinc oxide. Cadmium forms complex with other metal and not found in pure (free) form in nature. It exists in nature in the form of cadmium carbonate, hydroxide, sulphide, or chloride because cadmium easily reacts with the water vapour, carbon dioxide, sulphur di or tri oxide and hydrogen chloride. Cadmium has toxic effects on all the living organisms when used in excess (Jackson and Alloway, 1992). Cadmium affects the plant growth by accumulation in edible parts of the crop (Khan and Lee, 2013). Cadmium has been rated as number 7 pollutant amongst the 275 hazardous toxicants such as benzene (Organic) and arsenic, vinyl chloride, polychlorinated biphenyls, lead, mercury (Inorganic) in aspect of toxicity (ATSDR; Agency for Toxic Substances and Diseases Registry, 2007).

2.3.Source of Cadmium:

Cadmium enters into the environment by various sources categorised into natural and anthropogenic pathways. Cadmium is listed as a pollutant all over world and has existed in earth crust as heavy metal (IPCS, 1992). Cadmium is released from various sources such as electroplating industries, phosphate fertilizers, timber industries, and stainless steel industries etc. (Bolan et al., 2003; Saluja et al., 2011; Choppala et al., 2013).

In nature cadmium occurs generally with the zinc ores sphalerite and it is the source of cadmium for commercial production. According to Loganathan et al. (2012) cadmium is also found in soil forming rocks such as igneous rocks, sandstones, and limestone at low concentration. Cadmium is highly taken up by crops because it is found in soil in an exchangeable phase. Application of phosphatic fertilizers continuously enhances the concentration of cadmium in agricultural field and cause

pollution when used excessively (Taylor and Perciva, 2001). Fig-2.1 depicts various sources of cadmium release in the environment and their level.

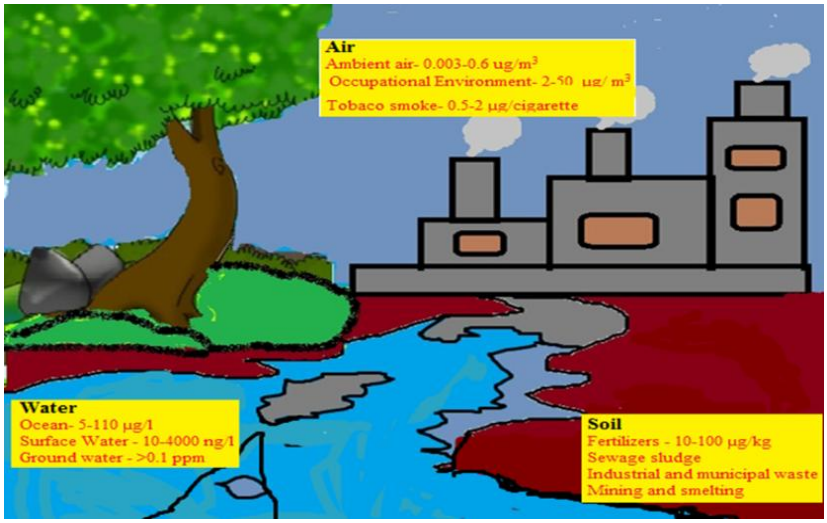


Fig-2.1-Source and level of cadmium in environment (Source- Verma et al., 2017)

2.4.Toxicity of Cadmium:

2.4.1. Cadmium Toxicity to Animals:

According to ATSDR (Agency for Toxic Substances and Diseases Registry, 2007), cadmium is a potent toxic metal amongst all the toxicants. It is a nephrotoxic heavy metal found in a number of occupational and environmental settings. After entering systemic circulation, Cd localizes primarily in the kidneys and the liver (Robinson et al., 1993; Zalups and Ahmad, 2003; Longe, 2005), with a biological half-life of about 20 years, and leads to pulmonary emphysema and renal tubular damage (Ryan et al., 1982). Extreme cases of chronic Cd toxicity can result in osteomalacia and bone fractures, as characterized by the disease called Itai-Itai in Japan in the 1950s and 1960s, where local populations were exposed to Cd-contaminated food crops, principally rice. It is a ubiquitous environmental toxin which may plausibly contribute to cardiovascular disease (CVD), it may exert its adverse cardiovascular effects by promoting atherosclerosis and by inducing disadvantageous cardiac functional and

metabolic changes (Kopp et al., 1983; Houtman, 1993). Due to cadmium exposure, damage of lungs, liver, and kidneys in cats and humans have been reported (Prodan, 1932). Acute poisoning of cadmium affects the lung and causes the severe bronchial and pulmonary irritation, lung emphysema, and, in the most severe situations, even death may occur.

Low levels of cadmium are generally excreted in faeces and urine. Commonly the detoxification of cadmium is done by MT (Metallothionein) through high binding affinity of the metal to MT. Nordberg (2009) reported that MT has many functions such as metal homeostasis, regulating gene expression, tissue generation and scavenging ROS (reactive oxygen species) and all participate to MT protection against cadmium. In intestine, cadmium generally affects the absorption of calcium in the villi of intestinal epithelium because cadmium competes with the Ca^{+2} ions. It also affects the metabolisms of vitamin D_3 indirectly. Cadmium absorption is also affected by intake of calcium with diet (Bredderman and Wasserman, 1974; Washko and Cousins, 1977). Table-2.2 lists the various toxicity symptoms in animals.

2.4.2. Cadmium Toxicity in Plants:

Growth and development of various plants is affected by cadmium because of its high solubility (Ragan and Mast, 1990). Many studies have proved the toxic nature of cadmium. Cadmium causes inhibition of lateral root formation while the main root becomes brown, rigid and twisted (Krantev et al., 2008; Yadav, 2010; Rascio & Navari-Izzo, 2011). It also affects the leaf due to alteration of chloroplast ultrastructure, low content of chlorophyll etc. and finally photosynthesis restriction occurs (He et al., 2008; Lee et al., 2010; Liu et al., 2010; Miyadate et al., 2011). In case of rice, presence of cadmium causes inhibition of root growth and alteration of morphogenesis (Rascio et al., 2008).

Many studies related to cadmium toxicity confirm that the primary sites for action of cadmium are photosynthetic pigments (Prasad, 1995). Various plants such as *Beta vulgaris* (Greger & Ögren, 1991), *Vigna radiata* (Keshan & Mukherji, 1992), *Phaseolus vulgaris* (Padmaja et al., 1990) are affected by cadmium ion where cadmium participates in alteration of chloroplast function. Cadmium also affects the photosynthetic carboxylation reactions PSII and especially oxygen evolving complex are affected due to its high sensitivity against cadmium (Clijsters & Assche, 1985). Main target of cadmium effects is two main enzymes of carbon dioxide fixation and the enzymes are phosphoenolpyruvate carboxylase (PEPCase), and ribulose-1, 5-bisphosphate carboxylase (RuBPCase). Functions of membrane are affected by cadmium through alteration of fatty acid and lipid composition (Ouariti et al., 1997; Popova et al., 2009). Table-2.2 lists the various toxicity symptoms to plants.

Table-2.2- Toxicity of cadmium in plants and animals:

Toxic effects of cadmium in animals	**Toxic effects of cadmium in plants**
Itai- Itai diseases, osteomalacia,, osteoporosis	Reduction of shoots and root elongation
Respiratory stress, injury in respiratory tract, emphysema, anosmia and chronic rhinitis.	Change the fatty acid and lipid composition of membrane
Affects cardiovascular system, atherosclerosis, peripheral and vascular diseases	Rolling of leaves
Acute inflammation of gastrointestinal tract	Chlorosis, Alteration of chloroplast structure, Reduction of chlorophyll
Damage testicular function, oxidative induction impaired the antioxidant defence mechanism, alters prostate function	Inhibit lateral root formation, main root become brown, rigid and twisted
	Root browning and decomposing
Cadmium toxicity affects immune response	Affects carboxylation reaction

Metals including cadmium produce ROS (Reactive oxygen species) such as hydrogen peroxide (H_2O_2), hydroxyl radical (HO), superoxide radical (O_2^-) and enhance the oxidative damage in cells. In absence of protective mechanisms, ROS can react with amino acid, proteins, lipid, and nucleic acids and can lead to irreparable metabolic dysfunction, damaged cell structure and function (Moller et al., 2007; Gill and Tuteja, 2010). Cadmium has many toxic effects when enter into cell as shown in Fig-2.2.

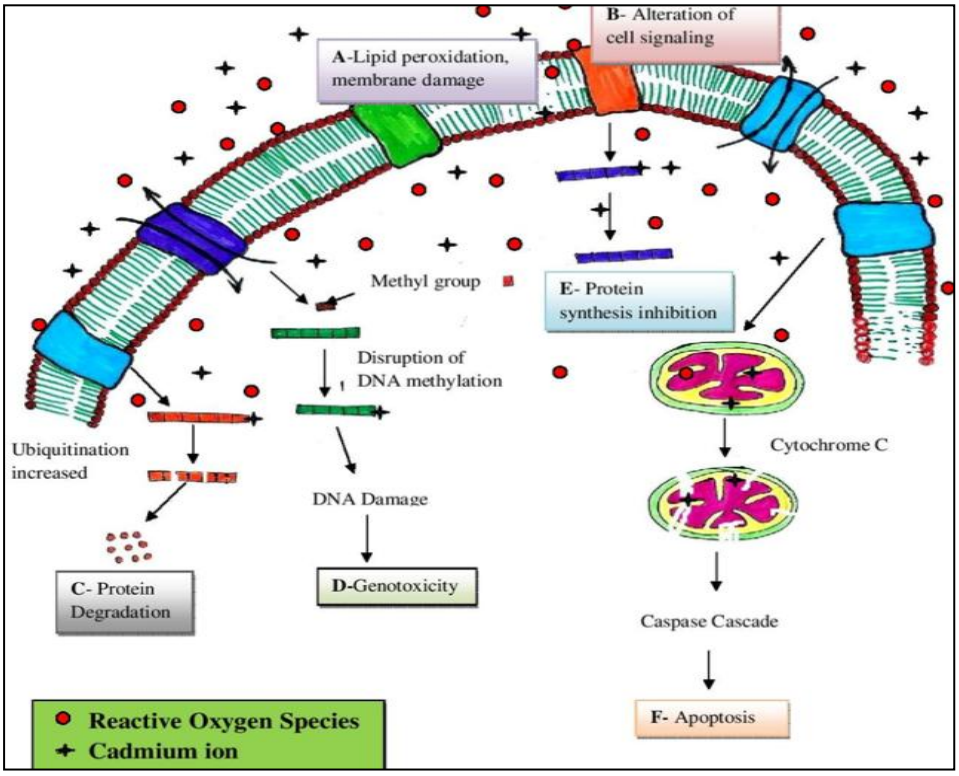


Fig-2.2- Cellular toxicity of cadmium A- Lipid peroxidation of cell membrane, B- Alteration of cell signalling, C- Protein degradation, D- Genotoxicity, E- Inhibition of protein synthesis, F- Apoptosis (Source- Verma et al., 2017)

2.4.3. Cadmium Toxicity in Microorganisms:

Toxicity in microbes is exhibited due to various metal-microbe interactions. Almost every index of microbial metabolism and activity can be adversely affected by elevated levels of heavy metals **(**Nies, 1999; Gadd, 2000). Cadmium is toxic to most microbes, probably by binding to essential respiratory proteins and through oxidative damage of reactive oxygen species (Lee et al., 2001). Even low concentration of free

cadmium is toxic to bacteria's such as *Desulfovibrio* sp. and *Desulfotomaculum* sp. (Poulson et al., 1997). Besides these, cadmium is also toxic to other microbes like Gram negative *Escherichia coli* as well (Feriance et al., 1998). In case of many Gram negative bacteria, cadmium affects the DsbA gene in periplasm (Rensing et al. 1997). The toxic effects of cadmium on microorganism are well documented and derived from several mechanisms. Disruption of protein function can occur through binding of cadmium to sulfhydryl groups (Cunninghan and Berti, 1993; Jungmann et al., 1993)**.** In addition, cadmium competes with several divalent ions such as Ca, Zn and Mn for metal binding sites in biological systems. Binding of cadmium to nucleotides leads to single strand break in cellular DNA **(**Mitra and Bernstein, 1978; Hughes and Poole, 1989). This potent toxic effect can result in prolonged lag phase, decreased growth rate, lower cell density or death of bacteria & algae at level as low as 1ppm of cadmium (Aiking et al., 1982; Les and Walker, 1984). Cadmium enters the bacterial cell in the form of divalent cation using Zn^{2+}, Ca^{2+}, Mn^{2+} transport systems and shows toxicity at very low concentration (Nies 1999). According to Nies, (1992) cadmium becomes toxic to microorganisms, when the level of cadmium rises above the threshold level and alters the growth and properties of the organism (Roane and Kellogg, 1996; Giller et al., 1998).

2.5.Cadmium Resistance and Tolerance:

Plants and microorganisms have evolved various mechanisms to tolerate cadmium which are discussed in subsequent sections.

2.5.1. Cadmium Tolerance in Plants:

In case of plants, cadmium uptake is by various divalent uptake systems of Ca^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} transporters (Clemens et al., 1998; Rascio & Navari-Izzo, 2011). Hyper-accumulator plants have no symptoms of toxicity because they have ability to

minimise the toxicity of cadmium by detoxification mechanisms. Hyper-accumulator plants are those plants that can tolerate large amount of cadmium and other metal exceedingly from soil. As per the data available, around 450 plant species of angiosperm family have been reported as Hyper-accumulators for various metals (As, Cd, Co, Cu, Mn, Ni, Pb, Sb, Se, Tl, and Zn) (Rascio and Navari-Izzo, 2011). Detoxification of metal in these types of plants is by various mechanisms that are commonly based on chelation and sub-cellular compartmentalisation (Yadav, 2010). Cadmium transportation from root to shoot is different in hyper-accumulator and non-hyper-accumulator plants. Cadmium is chelated by root in cytoplasm or stored in vacuoles and rapidly trans-located to the shoot through xylem.

Heavy metals including cadmium are bounded by the organic molecules present in root of most of the plants especially hyper-accumulator plants. Heavy metal detoxification and sequestration is done in the epidermis, cuticle and trichomes of plants, thereby reducing the damage to the photosynthetic machinery. Binding of ligands with metal and removal of metal from metabolically active cytoplasm through compartmentalization in vacuole are also the key factors for detoxification process in plants (Rascio & Navari-Izzo, 2011). Plants have high resistant power against oxidative damage with high antioxidant concentration (Gill and Tuteja, 2010). Boojar and Goodarzi (2007) reported that antioxidant enzyme activities of superoxide dismutase (SOD) and catalase (CAT) of *Chenopodium* plants are able to protect the proteins, chlorophylls and lipids of some part of such plants.

2.5.2. Cadmium Resistance in Microbes:

Microorganisms exhibit tolerance and resistance properties against cadmium and other metals by developing several mechanisms to control the negative impact of heavy metals. Gram negative soil bacteria *Pseudomonas* is widely used bacteria due

to its high metal resistant properties. For survival mechanisms bacteria in metal stressed soil makes the metal inactive by developing various methods and with the help of these methods bacteria can mobilize or transform metals (Nies, 1992). Such methods are extrusion (Ahemad et al., 2009), accommodation (Ahemad and Khan, 2011), bio-transformation (Vidali, 2001), exclusion (Tabak et al., 2005), methylation and de-methylation (Kamaludeen et al., 2003). These mechanisms work as defence mechanisms and allow the bacteria to function metabolically in contaminated environments (Fig-2.3).

In Gram positive bacteria, Cd-efflux ATPase mechanism is involved in cadmium resistant properties. However, cyanobacteria contain metallothioneins (Olafson et al., 1979) and amplification of smt MT locus enhances the cadmium resistance (Gupta et al., 1992). In cyanobacteria, RNA and P type transport systems may also be useful for cadmium resistant bacteria. RND-driven systems such as Czc (Zinc exporter) and Ncc (Nickel exporter) are responsible for detoxification of cadmium in Gram negative bacteria (Nies, 1995; Nies and Silver, 1989; Schmidt and Schlegel, 1994). In Gram positive bacteria *CadA* pump is responsible for cadmium exportation in *S. aureus* (Nucifora et al., 1989; Silver et al., 1989). According to Liu et al. (1996) cadmium resistant property of gram positive bacteria is driven by CadA proteins. In *S. Cerevisiae,* cadmium bis-glutathionato complex is formed by binding of glutathione and transported to the vacuole (Li et al., 1997). Cadmium is accumulated by magnesium system in *S. cerevisiae* (Liu et al., 1997) and in *Ralstonia* sp. CH34 (Nies and Silver, 1989). Cadmium resistant property of microorganisms is based on cadmium efflux systems. Manganese uptake system is also responsible for cadmium transportation in the microbes (Laddaga et al., 1985; Tynecka and Malm, 1995).

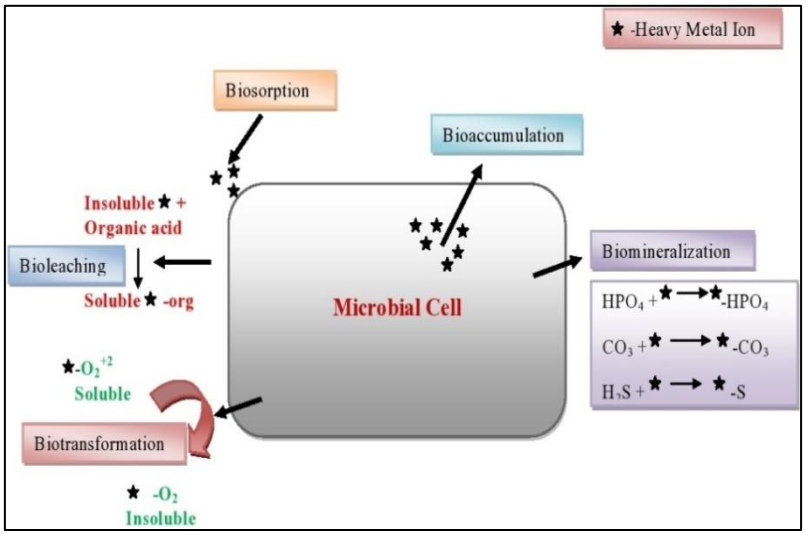


Fig-2.3- Resistant mechanisms of microbes for heavy metals in environment through Bio-sorption, Bioleaching, Bioaccumulation, Bio-mineralization, Biotransformation mechanisms (Source: Verma et al., 2016)

2.6.Remediation of Heavy Metals:

Action of remedying something, in particular of reversing or stopping environmental damage is termed as 'Remediation'. It deals with the removal of pollutants or contaminants from environmental media such as soil, groundwater, sediment, or surface water. Various physico-chemical and biological methods are used for remediation and named accordingly (Fig-2.4)

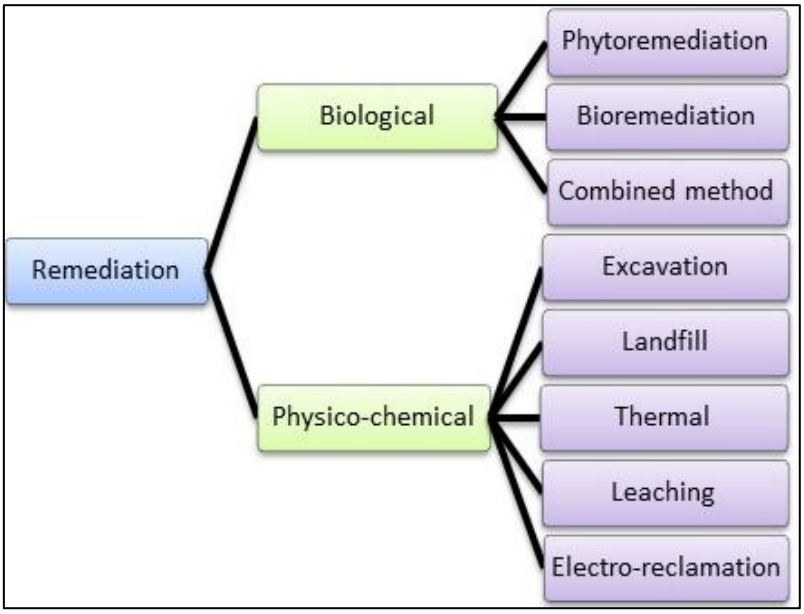


Fig-2.4- Remediation methods (Physico-chemical and Biological) for heavy metals

Remediation of cadmium as well as other metal is very important for controlling the toxic effects of heavy metals to preserve the environment (Taj and Rajkumar, 2016). Remediation of heavy metals from the environment is very challenging task due to technical complexity and cost (Mahar et al., 2016). Various types of methods are used from several years and some are under pipeline. These methods are categorised into chemical, biological and physical processes (Lim et al., 2014; Hasegawa et al., 2016).

2.6.1. Physico-chemical Methods for Remediation and Their Limitations:

Metal contaminated soils are conventionally treated via on-site management or excavation and disposal to landfill sites for removal of metal. Soil washing is another method for remediation. Electro-reclamation, leaching, landfill, thermal treatment, excavation are included in physico-chemical approaches. Chemical and physical methods have limitations including alterations of native soil flora, reduction of soil properties, are costly and labour intensive. Chemical methods of remediation generate secondary pollution, are expensive and produce large quantities of secondary products (Tangahu et al., 2011; Zubair et al., 2016). Physical and chemical methods are not helpful to remediate the cadmium and it shifts the problems from one place to another. In these treatments, resultant pollutant rich residues need further action for treatment. Physico-chemically treated soils become unsuitable for growth of plant due to inhibition of biological activities (Gaur and Adholeya, 2004; Tangahu et al., 2011). Physico-chemical approaches are not an absolute solution to this problem of metal remediation and detoxification. These approaches only change the form of the problem and fail to remediate the pollutants thoroughly (Gomes et al., 2016). These methods therefore do not qualify as a technology for remediation and only change the form of problem.

2.6.2. Biological Methods for Remediation:

In biological remediation of heavy metals, living organisms such as microbes and plants participate and is commonly known as bioremediation (Hasegawa et al., 2016). Bioremediation is defined as a collective range of clean-up methods by using natural microorganism (such as bacteria, plant, Fungi, etc.) to degrade hazardous organic contaminants or convert hazardous inorganic contaminants to environmentally less toxic or nontoxic compounds to safe levels in soils, subsurface materials, water, sludge, and residues.

Kang et al. (2016) reported that biological methods of remediation have several advantages and are natural and eco-friendly processes with low cost and high public acceptance. Biological methods include Bio-stimulation, bio-augmentation, composting, bioreactors, land farming, bioventing, phytoremediation, (Mani and Kumar, 2014). Biological methods are preferred methods over others because these methods conserve natural soil properties and utilize solar energy (Beskoski et al., 2011; Kang et al., 2016). Kumar et al. (2011) reported use of *Pseudomonas* and *Bacillus* strains used in remediation of Zn and Cu through bioremediation process. Of these, plant assisted remediation (phytoremediation) and microbe assisted remediation (bioremediation) are discussed below.

2.6.2.1.Phytoremediation:

Although the use of plants to remediate radionuclide-contaminated soils was explored in the 1950s, the term phytoremediation was not invoked until the 1980s, and rapid expansion in this field only began in the last decade (Willey, 2007). Phytoremediation has now emerged as a promising strategy for *in situ* removal of many contaminants including heavy metals but only to a certain extent of their level in the soil (Salt et al., 1998; Susarla et al., 2002; Pulford and Watson, 2003; Pilon-Smits, 2005). The term

phytoremediation, from the Greek phyto, meaning ‘‘plant’’, and the Latin suffix remedium, ‘‘able to cure’’ or ‘‘restore’’, was coined by Raskin in 1994, and is used to refer to plants which can remediate a contaminated medium.

Phytoremediation is a green biological technology as discussed above and worked as an emerging technology in which plants are used to accumulate different amounts of heavy metals form agricultural and non-agricultural soil (Chaturvedi et al., 2016). Hyper-accumulating plants play an important role for accumulation of toxic metals in biomass (Gomes et al., 2016). Soils polluted with heavy metals can be easily remediated by phytoremediation and biomass produced during phytoremediation can be used for biodiesel production. Thus, bioenergy crops like Brassica and maize species that hyper accumulate toxic heavy metals such as Cd, Cu, Cr and Pb could be suitable option for such remediation purpose. According to Ma et al. (2016), phytoremediation is associated with the ability of plants for accumulation of enhanced levels of metal in soils.

Phytoremediation takes advantage of the plant’s ability to remove pollutants from the environment or to make them harmless or less dangerous (Raskin et al., 1996). It can be applied to a wide range of organic (Anderson and Coats, 1995) and inorganic contaminants. It is defined as the process of utilizing plants to absorb, accumulate and detoxify contaminants in soil through physical, chemical and biological processes (Cunningham and Ow, 1996; Saxena et al., 1999; Wenzel et al., 1999). Uptake of metals is mainly influenced by their bioavailable fraction rather than by the total amount in soil. Metal availability depends on (1) the intensity of adsorption to soil particles; (2) the ability of plants to desorb and transfer metals to their tissues; and (3) interactions with soil microorganisms (Salt et al., 1998; Lasat, 2002). It helps to prevent landscape destruction and enhances activity and diversity of soil

microorganisms to maintain healthy ecosystems. Phytoremediation is a general term including several processes (Fig-2.5), among which phytoextraction and phytostabilisation are the most reliable method for heavy metals remediation.

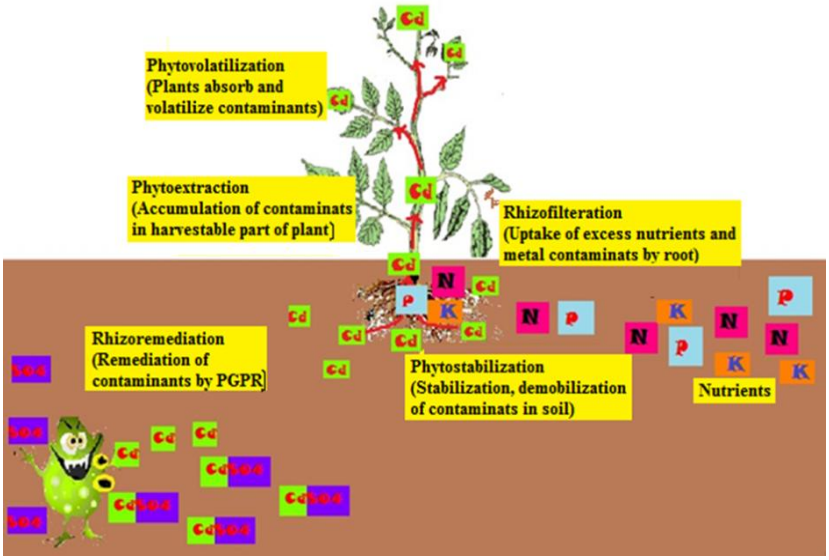


Fig-2.5- Methods of Phytoremediation including Phytovolatilization, Phytoextraction, Phytostabilization, Rhizoremediation and Rhizofilteration (Source-Verma et al., 2017)

2.6.2.2.Phytoextraction:

Phytoextraction is a green technology, born 34 years ago from the studies of Raskin et al. (1994) and later of Brooks et al. (1998), which exploits the ability of plants to translocate a great fraction of metals taken up to harvestable biomass. Phytoextraction is a phytoremediation process whereby plant roots absorb, translocate and store contaminants along with other nutrients and water. Fig-2.6 shows a schematic representation of metal uptake and accumulation in plants. Contaminated biomass may be used for energy production, whereas remaining ashes are dumped, included in construction materials, or subjected to metal extraction (phytomining; Brooks et al., 1998).

Plants show different morphological and physiological responses to soil metal contamination. Most are sensitive to very low concentrations; others have developed

tolerance, and a reduced number show hyper-accumulation (Baker and Brooks, 1989; Brooks et al., 1998). The latter capacity has practically opened up the way to phytoextraction (Garbisu and Alkorta, 2003). Metal accumulation is expressed by the metal biological absorption coefficient (BAC), i.e. the plant (harvestable)-to-soil metal concentration ratio (Blaylock et al., 1997). Besides convenient BAC, both the high bioconcentration factor (BCF, root-to-soil metal concentration ratio) and the translocation factor (TF, shoot-to-root metal concentration ratio) can positively affect phytoextraction. Tolerant plant species tend to restrict soil–root and root–shoot transfers, and therefore have much less accumulation in biomass, whereas hyper-accumulators actively take up and translocate metals into above-ground tissues. Plants with high BAC (greater than 1) are suitable for phytoextraction; those with high BCF (higher than 1) and low TF (lower than 1) have potential for phytostabilisation (Yoon et al., 2006).

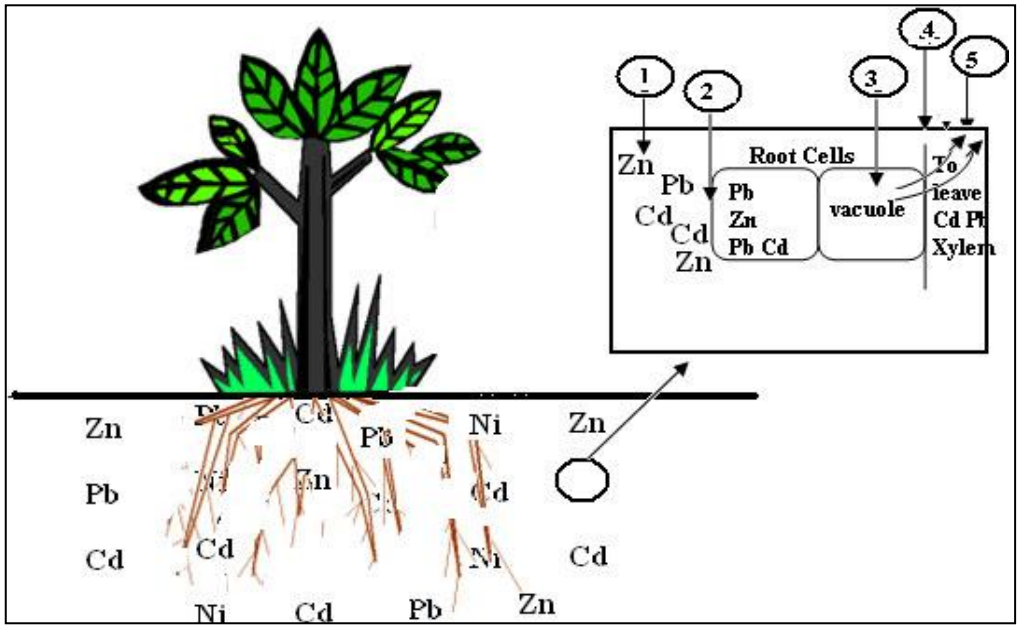


Fig-2.6-Metal uptake and accumulation in plants: (1) metal fraction is adsorbed at root surface, (2) bio-available metal moves across cellular membrane into root cells, (3) fraction of the metal absorbed into roots is immobilized in the vacuole, (4) intracellular mobile metal crosses cellular membranes into root vascular tissue (xylem), (5) metal is trans-located from the root to aerial tissues (Lasat, 2002).

2.7.Hyper-accumulator plants:

In environment various types of plants are present that accumulate heavy metal in high level and are known as 'Hyper-accumulator plants'. Hyper-accumulation limit of plants is different for different metals. Plants that accumulate more than 100 mg/kg dry weight of Cd and 1000 mg/kg dry weight of Pb, Cu, or Ni etc from metal polluted soil are termed as Hyper-accumulator plants. In environment, 45 families are reported as hyper-accumulator plants for heavy metals such families are Fabaceae, Asteraceae, Euphorbiaceae and Brassicaceae, Lamiaceae and Scrophulariaceae and used in phytoremediation process. Jaffre et al. (2009) reported that approximately 450-500 plant species come under the category of hyper-accumulator plants. *Thlaspi caerulescens*, is a plant that has been reported to accumulate high concentrations of metal such as Ni Cd and Zn highly (Lasat, 2002; Assunçao et al., 2003).

2.7.1. Mustard (*Brassica juncea*):

Mustard (*Brassica juncea*) plant is suitable plant for phytoextraction of heavy metal with high biomass production and metal concentration in above ground portions (Rathore et al., 2013). Indian mustard is a main oil seed crop of India and contributes maximum in edible oils domestically. Mustard is cultivated in 6.28 Mha with 7.46 Mt production and 1188 kg/ha productivity (Ministry of agriculture, 2015). Mustard is a good phytoextracting plant with higher biomass. Higher biomass and alteration in lipid composition of membrane makes the mustard a good choice as phytoextracting plant and is ideal for extraction of several heavy metals like Ni, Cd, Hg, Se, Cr and Se. Mustard plants gain maximum phytoextraction efficiency (PE) and phytoextraction rate (PR) when grown in metal contaminated soils (Hall, 2002; Ansari et al., 2015). Heavy metals are stored in vacuoles and tonoplast after extraction i.e. localised in limited area (Bhoominathan and Doran, 2003; Ma et al., 2005). This plant

tolerates the metal by chelation and compartmentalization into the vacuole. They may also volatilize and accumulate the metal in safe tissue and intracellular space like epidermis. Mustard plants develop enzymatic mechanisms for reduction or elimination of oxidative damage caused by heavy metal uptake using enzymes such as carnitine-acylcarnitine translocase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and superoxide dismutases (SOD) (Mani et al., 2013).

2.7.2. Maize (*Zea mays*):

Maize (*Zea mays* L.), an annual cereal crop is spread worldwide with high growing capacity. It produces extensive fibrous root system with high biomass and number of seed in adverse conditions. This is a heavy metal tolerant and metal accumulating plant with moderate bioaccumulation factor. Maize has good phyto-extraction potential in metal contaminated soils through translocation from roots to shoots (Nascimento and Xing, 2006; Wuana and Okieiman, 2010). Maize has good capacity to remediate toxicant and maize can grow in diverse climates in high mountain plains or arid desert plains. Maize plant is a major source of food for both humans and animals, and is grown in many countries. The majority of the maize crop is used as livestock feed. The remainder is processed into a range of food and industrial products such as ethanol as a fuel, starch, sweeteners (fructose maize syrup) and maize oil. Maize plant is used to accumulate certain heavy metals such as Cd (Kimenyu et al., 2009) and Pb (Pereira et al., 2007) at high level. Máthé-Gáspár and Anton (2005) grouped the maize plants as an accumulator and a metal tolerant plant especially for Cd and Zn on the basis of capability of heavy metal uptake and sensitivity to high metal pollution.

Both hyper-accumulator plants, mustard and maize belong to different families but have the potential to remediate cadmium contaminated soil. Table-2.3 gives the detailed classification of both the plants.

Table-2.3- Classification of mustard and maize:

Kingdom	Plantae	Plantae
Subkingdom	Tracheobionta	Tracheobionta
Super division	Spermatophyta	Spermatophyta
Division	Magnoliophyta	Magnoliophyta
Class	Magnoliopsida	Liliopsida
Subclass	Dilleniidae	Commelinidae
Order	Capparales	Cyperales
Family	Brassicaceae	Poaceae
Genus	*Brassica*	*Zea*
Species	*Juncea*	*Mays*

2.8.Limitations of Phytoremediation:

Practical use of hyper-accumulator plants has several advantages in phytoremediation, but some properties induce limitations. These plants generally accumulate one specific element with limited root system and this limitation makes its use irrelevant (Begonia et al., 2005; Verma et al., 2017). In phytoremediation, various accumulator plants have high level of contaminants in harvestable parts and incineration is used after harvesting (Oh et al., 2011). Phytoremediation is a plant-based technology that is applicable in low-concentration areas having longer treatment time (Wenzel et al., 1999). Therefore, high cost is involved in traditional phytoremediation (without the involvement of microorganisms) and the owner of the polluted area does not get any benefits; he rather incurs losses. In phytoremediation, hyper-accumulator plants play

an important role to enhance the removal of heavy metals from the soil through high growth rate and yield, but depletion of nutrients is responsible for reduction in plant growth under stress.

2.9.PGPR-assisted phytoremediation:

Phytoremediation is a biological method for remediation of heavy metals through plants, but it has some limitations. For enhancement of phytoremediation properties, microbes play an important role (Afzal et al., 2014; Chang et al., 2014). Simultaneous use of hyper-accumulator plants and metal tolerating plants gained considerable attention for remediation. In this process microbes accumulate metals by affecting metal mobilization with higher plant growth and metal uptake at lower part of plants (Xun et al., 2015). This process is termed as 'PGPR assisted phytoremediation'. PGPR are those heterogeneous groups of microorganisms which are found in rhizosphere of plants and can enhance growth of plants in stress as well as the normal condition through direct or indirect manner (Tica et al., 2011). This field of study is very new and termed as PGPR assisted phytoremediation.

According to Wang et al. (1989) the success of phytoremediation is depends on plants, PGPR and level of heavy metals in soil and PGPRs have special quality to survive in heavy metal contaminated soil (Belimov et al., 2005). These PGPRs exceptionally promote the plant growth by various mechanisms such as nitrogen fixation, mineral solubilisation, siderophore production, phyto-hormone and transformation of nutrients (Glick et al., 1999). Glick (2003) reported that enhancement of plant-microbe interaction in PGPR assisted phytoremediation is very helpful. It can enhance biomass and tolerance of plants to heavy metals. Fig-2.7 depicts the role of PGPR's in metal tolerance and growth promotion of plants.

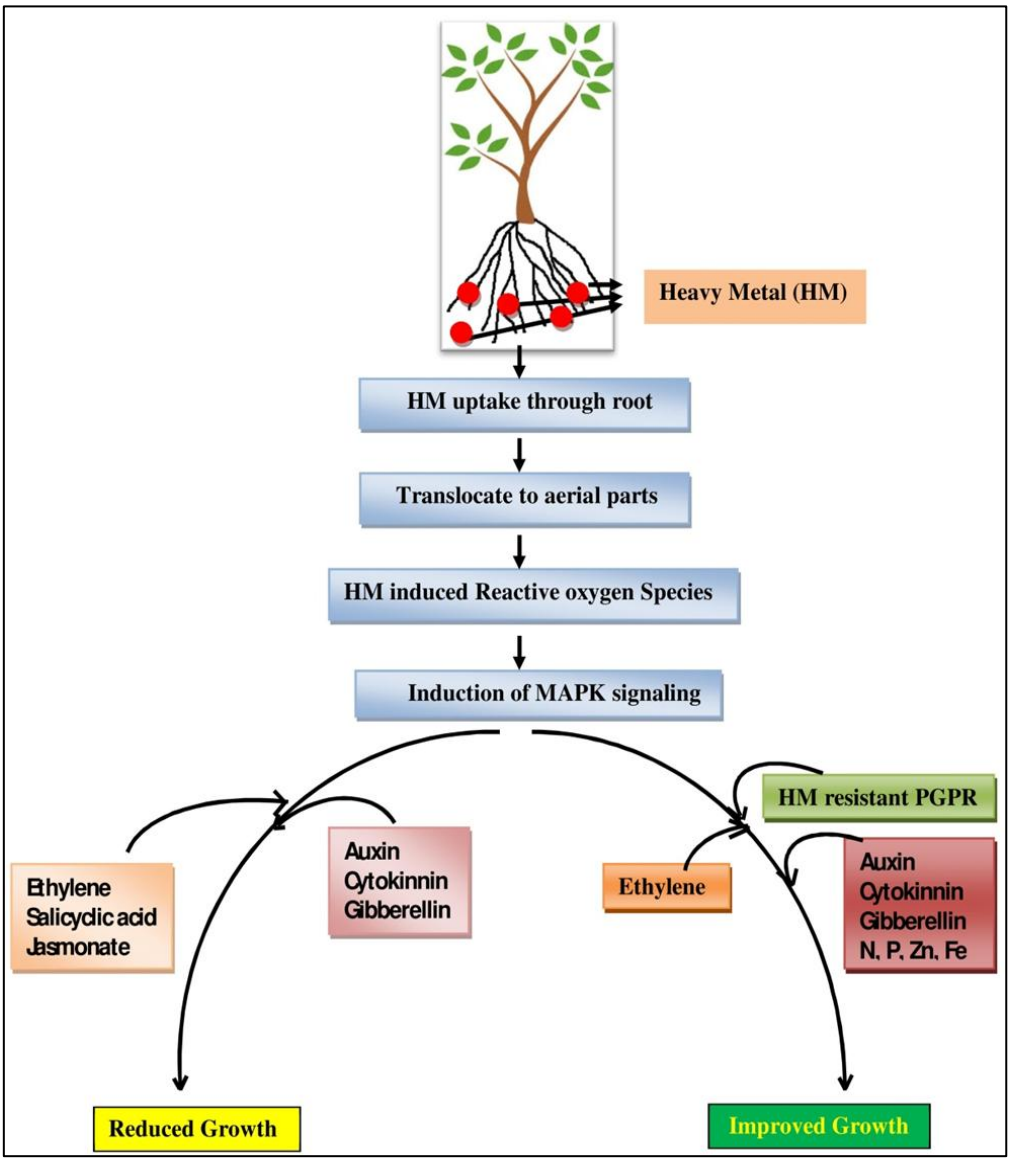


Fig-2.7- Role of PGPR in metal tolerance and growth promotion of plants

To remove the limitations of phytoremediation by using microorganisms (PGPRs) there is an important need to select suitable plants with high biomass production and metal tolerance property. Plants of brassicaceae family like mustard are commonly used as accumulator and hyper-accumulator plant for remediation purpose (Kumar et al., 1995). Saxena et al. (1999) reported that *Brassica juncea* is one of the best plants among all the plants for phytoremediation with higher growth rate. PGP bacteria directly enhance the bioavailability, solubility and heavy metal accumulation.

However, in indirect mechanisms they improve plant growth and protection against pathogens that further facilitate the accumulation of heavy metals. They are also helpful for development of root and nodule formation which significantly induce the plant growth and heavy metal tolerance (Ma et al., 2016; Taj and Rajkumar, 2016). Consequently, healthy plants have more efficiency than weak plants in

aspect of heavy metal tolerance as well as other organic and inorganic toxicants accumulation or extraction. PGPR assist the phytoremediation process by secreting various metabolites as shows in Table-2.4.

Table-2.4-Function of metabolites secreted by PGPRs in PGPR-assisted phytoremediation: (Ahemad, 2015)

Metabolites	**Function**
Indole acetic acid	By development of physiological changes induce the metal tolerance in plants Induce plant growth and development Improve absorption of nutrients and heavy metals in root by proliferation of root
Organic acid	Solubilize and mobilize heavy metals
ACC deaminase	Reduce the ethylene level produced after metal stress Enhance the PGPR assisted phytoremediation efficiency of plants by improving the length and density of root
Siderophore	Induce the suppression of chlorophyll biosynthesis by providing iron to stressed plants Enhance heavy metal accumulation in lower part of plants
Bio-surfactants	Induce the bioavailability of heavy metals Bind effectively to toxic metals with high affinity
Polymeric substances and glycoprotein	Bind the metal through complex formation

Heavy metal resistant PGPRs improve the plant growth and heavy metal tolerance via several mechanisms such as stimulation of nutrients acquisition, metal toxicity reduction, immobilization of mobilize heavy metals, recycling of nutrients, bio-control of pathogens and enhancement of plant growth and development (Sheng et al., 2008a; Lebeau et al., 2008; Glick, 2010; Hayat et al., 2010; Kuffner et al., 2010; Aafi et al., 2012; Rajkumar et al., 2010; Ma et al., 2011; Miransari, 2011; Orłowska et al., 2011; Wu et al., 2011). Among all the used bacteria for remediation of metal, rhizosphere bacteria deserve special attention because they can directly enhance the phytoremediation mechanisms of plants through alteration of the bioavailability of metal by changing the release of chelators such as organic acids, siderophores, pH of soil and oxidation/reduction reactions (Gadd, 2000; Khan et al., 2009; Kidd et al., 2009; Uroz et al., 2009; Wenzel, 2009; Rajkumar et al., 2010; Ma et al., 2011). PGPR assisted phytoremediation takes place through various mechanisms and several factors enhance the capacity of this remediation process. These factors are as:

2.9.1. Remediation of Metal through Plant Growth Promotion:

Growth of plants is suppressed by heavy metal contamination in soil, because metal contamination affects nutrient uptake of plants (Ouzounidou et al., 2006). PGP bacteria play an important role because they possess ability to increase the availability of required nutrients to plants in metal contaminated soils. Some bacteria are also helpful for nitrogen fixation even in presence of toxicants like metal. Nonnoi et al. (2012) reported that *Rhizobium bv trifolii* is helpful for nitrogen fixation in heavy metal polluted soil. After nitrogen, phosphorous is also very essential nutrient and deficiency of phosphorous can reduce growth of plants (Ullah et al., 2015) under metal contamination. They also produce phyto-hormones (gibberellines, cytokinins, IAA) in abiotic stress conditions (Verbon and Liberman, 2016). Various studies

supported these findings related to plant-associated microbes in protection of plants under heavy metal stress condition and proved that colonization of bacteria induce the nutrient uptake and plant biomass (Dimkpa et al., 2008; Mastretta et al., 2009; Ma et al., 2010; Luo et al., 2011; Maria et al., 2011; Luo et al., 2012). Some study reported that heavy metal contamination often interferes with nutrient (Fe, P, Mg, Ca, Zn) uptake potential of roots and functions; finally retardation of growth occurs (Parida et al., 2003; Ouzounidou et al., 2006). Under such nutrient limiting conditions, PGPRs enhance the acquisition of plant nutrient through mobilization of nutrients and make the essential nutrient available for plants. Study on coffee plant proved that AMF minimizes the stress of Cu and Zn in the rhizosphere and as a result nutrition is improved with higher growth of seedlings.

2.9.2. Remediation of Metal by Metal Reduction and Oxidization Process:

Metal oxidation by rhizospheric microbes is particularly interesting in view of phyto-extraction. Certain plant-associated microorganisms have ability to change the heavy metals mobility by oxidation or reduction reactions. In addition to these, Shi et al. (2011) reported that sulphur oxidizing rhizospheric bacteria enhance mobilization of copper and its uptake in plant tissue under stress soils. This study proved that the sulphur oxidizing bacteria reduce pH of soil via oxidation of sulphur to sulphates, and making Cu available for plant uptake. Similarly Chen and Lin (2001) have also reported that Fe/S oxidizing bacteria have the potential for enhancement of bioavailability of metal in the soils by acidification reaction.

2.9.3. Bio-Sorption Mechanisms involved in Metal Remediation:

The plant-associated microbes may also contribute in plant metal uptake through bio-sorption mechanism. Ma et al. 2011 reported that bio-sorption is microbial adsorption of metals by a metabolism independent or dependent, and passive or active process.

Various studies prove that bacterial bio-sorption mechanism is used for reduction of metal uptake in plants. In support of this finding, Madhaiyan et al. (2007) reported that inoculation of metal binding bacteria *Magnaporthe oryzae* and *Burkholderia* sp are helpful for reduction of Ni and Cd accumulation in roots and shoots of tomato plant. Treatment of *Brevibacillus* sp B-I in *Trifolium repens* causes the reduction of zinc concentration in shoot tissues in comparison to control (Vivas et al., 2006).

2.9.4. Remediation of Metal by Antioxidant Mechanisms:

Various studies favoured inoculation of beneficial microbes that help plants to minimize stress of heavy metal by induction of antioxidant enzymes (Kavita et al., 2008; Wani et al., 2008; Ma et al., 2010; Wang et al., 2011). Bacterial inoculations in *R. communis* and *H. annuus* grown in soils amended with nickel have shown higher activities of antioxidant enzymes like POX (peroxidase) and CAT (Catalase). Similar study was done by Wang et al. (2011) where they reported that inoculation of As-resistant and plant growth–promoting *Agrobacterium radiobacter* in *Populus deltoides* LH05-17 in arsenic contaminated soil, significantly increased the length of plant, dry weight of roots, chlorophyll contents and soluble sugar with the SOD and CAT activities. The mycorrhizal fungi can also alter plant tolerance against heavy metals by changing the antioxidant enzyme activities on the basis of physiological and biochemical activities. Recently, Azcón et al. (2010) reported that AMF and/or plant growth promoting bacteria inoculation in multi-contaminated soil enhance the growth of plant by antioxidant mechanisms. They found that AMF inoculation significantly enhanced CAT, ascorbate peroxidase, or GR activities and helped plants to limit oxidative damage to biomolecules in response to metal stress.

2.9.5. Role of ACC Deaminase in Metal Remediation:

Ethylene is known to play important role in regulating various physiological responses of plants, but higher production of ethylene is lethal for plants. According to Hassan et al. (2016) certain plant growth promoting (PGP) bacteria have ability to produce ACC deaminase, that breaks the ACC into α-ketobutyrate and ammonia (Glick et al., 2007). Thus, bacteria having ACC deaminase activity via ACC utilization generates ammonia that works as a nitrogen source for the plants. Glick (2012) reported that ACC utilizing bacteria protect plants from pathogen through bio-control activity. Moreover, PGP bacteria also act as bio-control agents because these bacteria protect plants from phyto-pathogens (Glick, 2012). In stressed conditions, bacteria maintain the equilibrium between the rhizosphere and root interior ACC levels; and plants release ACC that results in the reduction of ethylene level (Adams and Yang, 1979). Recent studies proved that plants inoculated with rhizosphere bacteria having ACC were better survivor in metal polluted soils (Rodriguez et al., 2008). Bio-inoculation of ACC utilizing *M. oryzae* strain CBMB20 in Ni and Cd amended soil increased the growth of seedlings of tomato plants by reducing the ethylene production (Madhaiyan et al., (2007). Zhang et al. (2011) also confirmed this finding and reported that Pb-resistant and ACC deaminase-producing endophytic bacteria favoured the growth and stress minimization of rapeseed plant by reduction of stress ethylene. Table-2.5 lists the cadmium hyper-accumulating plants and associated microorganisms.

Table-2.5- List of cadmium hyper-accumulating plants and associated microorganisms (Verma et al., 2017):

Microbes	Plants	Mechanisms	Reference
Variovorax sp, *Rhodoccus* sp., *Flavobacterium* sp.	Canola	Increased root length; IAA, siderophores, ACC deaminase	Belimov et al., 2005
P. putida	Mung bean	Increased biomass and decreased metal uptake; siderophores	Tripathi et al., 2005
Rhizobacteria	Graminaceae grasses	IAA, siderophore, ACC deaminase	Dell Amico et al., 2005
P. brassicacearum, *P. marginalis*	Pea	Increased biomass and nutrient uptake; ACC deaminase	Safranova et al., 2006
Pseudomonas sp., *Bacillus* sp.	Canola	Increased biomass and metal uptake; IAA	Sheng and Xia, 2006
Mesorhizobium huakuii	Chinese milk vetch	Increased metal accumulation; bacterium expresses phytochelatin and metallothionein	Ike et al., 2007
Burkholderia cepacia	*Sedum alfredii*	Increased biomass, metal uptake and translocation of metal to shoots	Li et al., 2007
P. aeruginosa	Black gram	Increased biomass and rooting, and decreased cadmium uptake; IAA, siderophore, ACC deaminase, phosphate solubilisation	Ganesan, 2008
Pseudomonas sp, *Bacillus* sp.	Tomato	Increased root length, above ground biomass and above ground metal; siderophore, IAA, ACC deaminase	He et al., 2009
Streptomyces tendae	Sunflower	Decrease metal uptake and siderophore	Dimpka et al., 2009

2.9.6. Role of Siderophore in Metal Remediation:

Microorganisms secrete chelating agents in the form of siderophore that bind iron as well as heavy metals and increase bioavailability of metal via complex formation (Gadd, 2010; Rajkumar et al., 2010). Siderophores are low-molecular mass (400–

1,000 Daltons) secondary metabolites of microorganisms and are also produced by plants phytosiderphores), but the affinity of chelating iron under stressed conditions of bacterial siderophores is better than phytosiderophores. Bacterial siderophores form can also form stable complexes with other metals, such as copper, cadmium lead etc. (Schalk et al., 2011). On the basis of functional group, siderophores are broadly classified into three main groups catecholates (enterobactin), hydroxamates (desferrioxamines), and (α-hydroxy-) carboxylates (aerobactin). Siderophore is produced by various groups of bacteria. However, PGP bacteria are better known for siderphore production in adverse environmental conditions such as nutrient limiting or elevated levels of toxic metals (Rajkumar et al., 2010; Ullah et al., 2015).

In addition to this it is believed that siderophore producing microbes may enhance the phyto-extraction of heavy metal because siderophore solubilize unavailable forms of heavy metal which also contain nutrients (Braud et al., 2009a; Dimkpa et al., 2009; Rajkumar et al., 2010). For example production of pyoverdine and pyochelin by *Pseudomonas aeruginosa*, increase the concentrations of bioavailable Cr and Pb in the rhizospheric region of maize plant (Braud et al., 2009b). Consequently, Sinha and Mukherjee (2008) reported that the inoculation of siderophore producing *P. aeruginosa* strain KUCd1 decreases the uptake of cadmium in roots and shoots of *Brassica juncea* and *Cucurbita pepo*. Various factors including availability of iron, pH, nutrient status of soils, type and concentration of heavy metals affects the production of siderophore by microorganisms and heavy metals increase the production of siderophore in case of *P. aeruginosa* in iron-limited succinate medium amended with Al, Cu, Ga, Mn and Ni (Braud et al., 2009b). Braud et al. (2010) also reported that in presence of heavy metals like Cu, Ni and Cr,

siderophore production was enhanced even in presence of sufficient level of iron in soil.

2.9.7. Organic Acids involved in Metal Remediation:

Production of low molecular weight organic acids (LMWOAs) such as citric, oxalic and gluconic acid are done by PGP bacteria (Ullah et al., 2015) and these acids are helpful for enhancement of solubility and mobility of heavy metals. According to Jones, (1998), organic acids are CHO containing compounds categorised by the presence of one or more carboxyl groups with a high molecular weight (300 Daltons) and have ability to bind heavy metal through complexation reaction. However, the stability of the complex (ligand: heavy metal) depends on various factors including number of carboxylic groups, binding form of the heavy metals and pH of soil solution (Jones, 1998; Ryan et al., 2001). Various studies describe biosynthesis and excretion mechanisms of organic acid in case of bacteria and fungi (Sauer et al., 2008). In this context, derivative of gluconic acid, 5-ketoglutonic acid, synthesized by *Gluconacetobacter diazotrophicus* improves the solubilisation of Zn compounds (Rajkumar et al., 2010; Ullah et al., 2015). For instance, Han et al. (2006) demonstrated the role of organic acids, acetic acids and malic acids in inducing Cd uptake by maize roots.

2.9.8. Role of Bio-surfactants in Metal Remediation:

Plant growth promoting bacteria have ability to involve in enhancement of mobility and subsequent phytoremediation of toxic heavy metals by production of bio-surfactants. These bio-surfactants form complexes with different heavy metals at surface of soil by stimulation of desorption of metals from soil matrix with high solubility and availability of heavy metals (Gadd, 2010; Rajkumar et al., 2012). Produced bio-surfactants are amphiphilic molecules including hydrophobic non-polar

tail and a hydrophilic polar/ionic head. Ullah et al. (2015) reported that bio-surfactants increase mobility and solubility of Cd and Pb which is produced by *Pseudomonas aeruginosa* BS2. For instance Venkatesh and Vedaraman (2012) assessed the ability of rhamnolipids produced by *P. aeruginosa* for Cu mobilization in polluted soils and found that 2% rhamnolipids removed 71% and 74% of Cu from soil.

Consequently, Sheng et al. (2008b) used the bio-surfactant producing bacterial strain *Bacillus* sp. J119 and reported bio-surfactant of this strain enhanced the uptake of cadmium by rape, maize, sudan grass and tomato in soil artificially amended with 0-50 ppm of the metal cadmium. Interestingly, various studies suggest that the microorganism producing surfactants enhance the heavy metal mobilization in heavy metal polluted soil (Juwarkar et al., 2007; Sheng et al., 2008b; Venkatesh and Vedaraman, 2012).

2.9.9. Role of Polymeric Substances and Glycoprotein in Metal Remediation:

Polymeric substances such as extracellular polymeric substances (EPS), muco-polysaccharides and proteins are secreted by plant-associated microbes. These substances can also play an important role in complexing toxic metals and in increasing their availability in rhizospheric region. For instance, Joshi and Juwarkar (2009) studied on the immobilization of Cd and Cr by inoculation of EPS producing *Azotobacter* sp. They found that this isolate have the ability to bind 15.2 mg g^{-1} of Cd and 21.9 mg g^{-1} of Cr. Similarly arbuscular mycorrhizal fungi (AMF) produce insoluble glycoprotein glomalin, which binds the heavy metal by complex formation (Gonzalez-Chavez et al., 2002).

As evident from the above review of the research done till date, researchers worldwide are still working on standardisation of the bio assisted phytoremediation technique as no concrete finding or technology has emerged till date due to some or

the other reasons but interestingly, plant growth promoting bacteria's have emerged as interesting candidate to be used in remediation of heavy metal (cadmium) affected soils along with plants, especially the hyper-accumulator plants. Mechanisms of cadmium resistance in these organisms have also been extensively studied. But, every microbe having potential to synthesise various metabolites that help in combating abiotic stress posed by cadmium will interact differently with different plant. So, the present study was undertaken using four different potential stress (cadmium) tolerant fluorescent pseudomonad strains, having multi growth promoting properties with two different hyper-accumulator plants: mustard and maize and their remediation potential with the following objectives:

1. Isolation of cadmium resistant plant growth promoting fluorescent pseudomonads from contaminated industrial sites.
2. Morphological, biochemical and molecular characterization of the isolates.
3. Development of microbial consortia and their cadmium remediation potential *in situ* with mustard and maize crop.
4. To compare the accumulation and tolerance power of mustard plant with maize plants for bio-assisted phytoremediation of contaminated site.

This study will help in better understanding and deciding about the selection of hyper-accumulator crop for remediation of cadmium contaminated soil with the four PGPR strains isolated in the present study.

Materials and Methods

This chapter provides the detail discussion about the methodology of the work to address the objectives of the current study. The objective of the present study is remediation of cadmium contaminated soil with PGPR consortia and hyper-accumulator plants which is accomplished in the following sections:

3.1.Experimental Site and Glasswares:

The recent study was conducted in the Rhizosphere Biology Laboratory of the Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University (A Central University), Vidya Vihar Raebareli Road, Lucknow (U.P.), India. Glasswares used in this study were of Borosil and Rankem and were properly autoclaved at a pressure of 15 lb/inch^2 for 20 min before use.

3.2.Chemical /Reagents and Media:

All the media used for research work were of high quality obtained from Hi Media, India, while the chemicals and reagents were of analytical grade obtained from Merck, India and Sigma, India. All the chemicals, reagents and media used for the study were of analytical grade and instruments were of limit of precise accuracy. The following types of media / broth were used:

A. Nutrient Agar Media (NA): Used for the preparation of plates and for isolation of bacteria

Composition-

Peptone-5.0 g

Beef extract-3.0 g

Sodium chloride NaCl-5.0 g

Agar-15.0 g

Distilled water-1000.0 ml

Final pH (at 25 °C) - 7.0±0.2

B. Mueller-Hinton Agar Media (MHA): Used to check the susceptibility of microorganisms against antimicrobial agent

Composition-

Beef infusion-300.0 g

Casein acid hydrolysate -17.50 g

Starch -1.50 g

Agar-17.0 g

Distilled water-1000.0 ml

Final pH (at 25 °C) - 7.4±0.2

C. Skim Milk Agar: Used for casein hydrolysis test

Composition-

Skim milk powder-100.0 g

Agar -15.0 g

Distilled water- 1000.0ml

Final pH (at 25 °C) - 7.00±0.2

D. Starch Agar Media: Used for the detection of amylase production

Composition-

Starch (soluble) - 20.0 g

Peptone- 5.0 g

Beef Extract- 3.0 g

Agar- 15.0 g

Distilled water- 1000.0 ml

Final pH (at 25 °C) - 7.0±0.2

E. Nutrient Broth: Used for general cultivation of bacteria

Composition-

Peptone-5.0 g

Beef extract-3.0 g

NaCl -5.0g

Distilled water-1000.0 ml

Final pH (at 25 °C) - 7.0±0.2

F. Simmons's Citrate Agar: Used for citrate utilization

Composition-

Ammonium dihydrogen phosphate- 1.0g

Dipotassium hydrogen phosphate -1.0g

Sodium chloride-5.0g

Sodium citrate-2.0 g

Magnesium sulphate - 0.2g

Bromo thymol blue- 0.08 g

Agar -15.0 g

Distilled water-1000.0 ml

Final pH (at 25 °C) – 6.6±0.2

G. Phenol Red Dextrose Agar: Used to check the dextrose metabolism/fermentation

Composition-

Trypticase – 10.0g

Dextrose- 5.0 g

NaCl- 5.0 g

Phenol Red- 0.018 g

Agar- 15.0 g

Distilled water- 1000.0 ml

Final pH (at 25 °C) - 7.3±0.2

H. MR-VP Broth (Methyl Red- Voges Proskauer): Used for the Methyl red and Voges Proskauer test to differentiate coli-aerogens group.

Composition-

Peptone- 7.0 g

Potassium phosphate- 5.0 g

Dextrose- 5.0 g

Distilled water- 1000.0 ml

Final pH (at 25 °C) – 6.9±0.2

I. SIM (Sulfide Indole Motility) Agar: Used to check the H_2S production and motility

Composition-

Peptone- 30.0 g

Beef Extract- 3.0 g

Ferrous ammonium sulphate- 0.2 g

Sodium thio-sulphate- 0.025 g

Agar- 3.0 g

Distilled water- 1000.0 ml

Final pH (at 25 °C) - 7.3±0.2

J. Urea Agar: Used to check urease production

Composition-

Peptone- 1.0 g

NaCl- 5.0 g

Potassium monohydrogen (or dihydrogen) phosphate- 2.0 g

Agar- 20.0 g

Distilled water- 1000.0 ml

Final pH (at 25 °C) – 6.8±0.2

K. King's Media B Base: Used for non-selective isolation and pigment production of *Pseudomonas* species.

Composition-

Proteose peptone – 20.00 g

Dipotassium hydrogen phosphate – 1.50 g

Magnesium sulphate, $7H_2O$ – 1.50 g

Agar -20.00 g

Distilled Water- 1000.0 ml

Final pH (at 25 °C) - 7.3±0.1

L. Tryptone Water: Used for detection of indole production of microorganisms

Composition-

Sodium chloride- 5.0 g

Casein enzymic hydrolysate- 10.0 g

Distilled water- 1000.0 ml

Final pH (at 25 °C) - 7.5±0.2

M. Pikovskaya Agar: Recommended for detection of phosphate solubilization of microorganisms

Composition-

Yeast extract- 0.50 g

Dextrose- 10.0 g

Calcium phosphate- 5.0 g

Ammonium sulphate- 0.50 g

Potassium chloride- 0.20 g

Magnesium sulphate- 0.20 g

Manganese sulphate- 0.0001 g

Ferrous sulphate- 0.0001 g

Distilled water- 1000.0 ml

Final pH (at 25 °C) - 7.4±0.2

N. Pseudomonas Agar: Used for detection of fluorescein production by *Pseudomonas* sp

Composition-

Casein enzymic hydrolysate- 10.00 g

Protease Peptone- 10.00 g

Dipotassium Phosphate- 1.50 g

Magnesium Sulphate- 1.50 g

Agar- 20 gm

Distilled Water- 1000 ml

Final pH (at 25 °C) - 7.0±0.2

O. Luria Bertani Agar: and used for cultivation of recombinant microorganisms

Composition-

Casein enzymic hydrolysate-10.00 g

Yeast extract-5.00 g

Sodium chloride-10.00 g

Agar-15.00g

Distilled water-1000 ml

Final pH (at 25 °C) - 7.5±0.2

P. Gelatin Agar: Used to detect the gelatinase production in bacteria

Composition-

Peptone-5.00 g

Beef extract -3.00 g

NaCl -5.00 g

Gelatin agar- 40.00 g

Distilled water- 1000 ml

Final pH (at 25 °C) - 7.2±0.2

Q. Carboxy Methyl Cellulose-Czapek Dox Agar: Used to check the cellulose production by microorganisms

Composition-

Part A-

Ammonium phosphate- 1.00 g

Magnesium sulphate- 1.00 g

Potassium Chloride- 0.2 g

Yeast extract- 1.00 g

Part B-

CMC- 26.00 g

Agar- 3.0 g

Distilled Water- 1000.0 ml

Final pH (at 25 °C) - 7.2±0.2

R. Citrate Agar: Used for detection of iron bacteria from soil samples that use citrate as a sole carbon source

Composition-

Magnesium sulphate- 0.2 g

Sodium chloride- 5.0 g

Ammonium dihydrogen phosphate- 1.0 g

Dipotassium hydrogen phosphate- 1.0 g

Sodium citrate (citric acid) - 2.0 g

Bromothymol blue- 0.08 g

Agar- 20.0 g

Distilled water- 1000 ml

Final pH (at 25 °C) – 6.7±0.1

S. Tween 80 Agar: Used for lipase production test

Composition-

Solution A:

Peptone- 10.0 g

NaCl- 5.0 g

$CaCl_2$ x 2 H_2O- 0.1 g

Agar- 20.0 g

Distilled water- 900.0 ml

Solution B:

Tween 80- 10.0 g

Distilled water- 100.0 ml

Final pH (at 25 °C) - 7.2±0.2

Both solution A and B were sterilized separately by autoclaving and were mixed after cooling to 50 °C.

T. CAS (Chrome Azurol S Agar: Used to detect the siderophore producing microorganisms.

Composition-

Chrome Azurol S- 60.5mg/50ml distilled water

Hexadecyltrimethyl ammonium bromide- 72.9 mg/40ml distilled water

King's Medium B base- 42.23

Distilled water- 900.0 ml

Final pH (at 25 °C) – 6.8±0.2

Chrome Azurol S solution and hexadecyltrimethyl ammonium bromide (HDTMA) solution were mixed and added in 10 ml of 1mM $FeCl_3.6H_2O$ solution prepared in 10 mM HCl. Add the final solution to 900.0 ml of King's Medium B base.

U. Peptone Water: Used as a growth medium and as a base for carbohydrate fermentation media.

Composition-

Peptic digest of animal tissue- 10.0 g

Sodium chloride- 5.0 g

Distilled Water- 1000 ml

Final pH (at 25 °C) - 7.2±0.2

3.3.Chemical Reagents:

Hydrogen peroxide, iodine, alcohol, safaranin, hydrochloric acid, crystal violet, Pikovskaya reagent, methyl red, ortho-phosphoric acid, Salkowski reagent, Kovac's reagent, VP reagent-i (napthol solution), VP reagent-ii (40% koh), mercuric chloride, hexadecyltrimethyl ammonium bromide, congo red, oxidase reagent, chrome azurol reagent, Nesseler's reagent, standard Indole acetic acid (IAA), pyocyanin pigment, perchloric acid, acetic acid, sulphuric acid, glucose, acetone, phenol, nitric acid, primers, ethanol, ethyl acetate, methanol, oxidase disc, antibiotic discs

3.4.Requirements:

Petri dishes, autoclavable bag, forceps, inoculating needle and loop, micropipette, scissor, sprit lamp, centrifuge tube, beaker, conical flask, test tubes, boiling tubes, measuring cylinder etc.

3.5.Instrument Used:

Autoclave, pH meter, laminar, shaking incubator, refrigerator, microscope, oven, chemical balance, spectrophotometer, centrifuge, hot plate etc.

Methods and protocols used in this study have been described in this chapter and most of them are standard and modified protocols. The present chapter also describes the source of bacteria used in the study and their isolation and characterization techniques. Methodology of the work is shown in Fig-3.1.

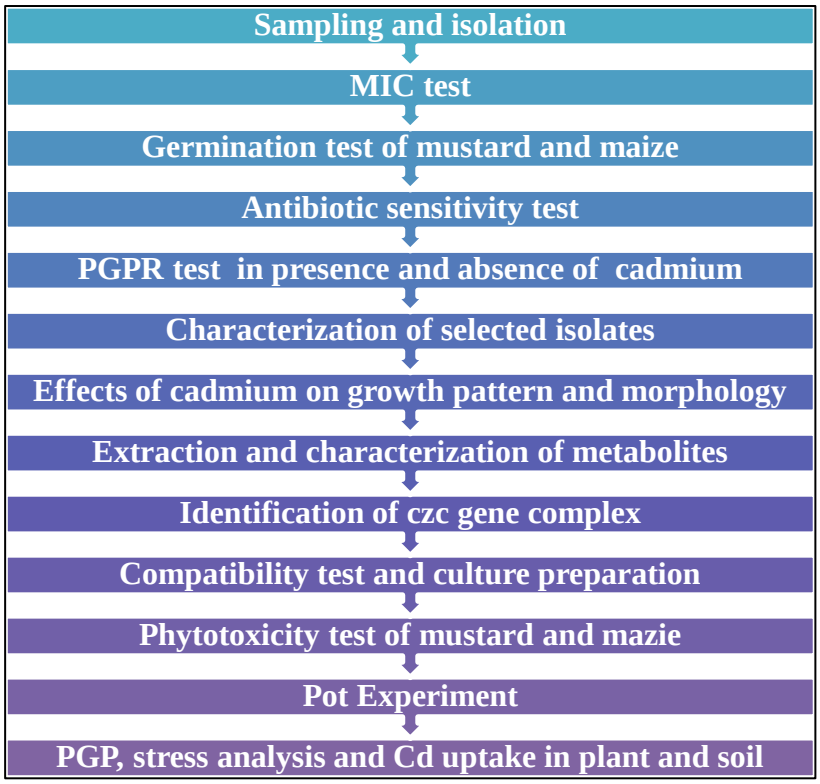


Fig.3.1- Methodology of work

We also studied about the application of isolated bacteria in the remediation of cadmium and growth promotion and stress tolerance of mustard and maize plants. The protocols used in this study are described here:

3.6.Sampling and Isolation of Cadmium Resistant Fluorescent Pseudomonads:

Sampling was done from rhizospheric region (mustard and maize plants) and non-rhizospheric region of industrial area (Fig-3.2). In all twenty six soil samples were

collected from different rhizospheric and non-rhizospheric region of India such as Jamshedpur, Lucknow, Kanpur, and Delhi shown in Table-3.1 and Fig-3.3. All the 26 sapling sites were listed in Table.3.2.

Fig.3.2- Rhizospheric and non-rhizospheric sampling sites

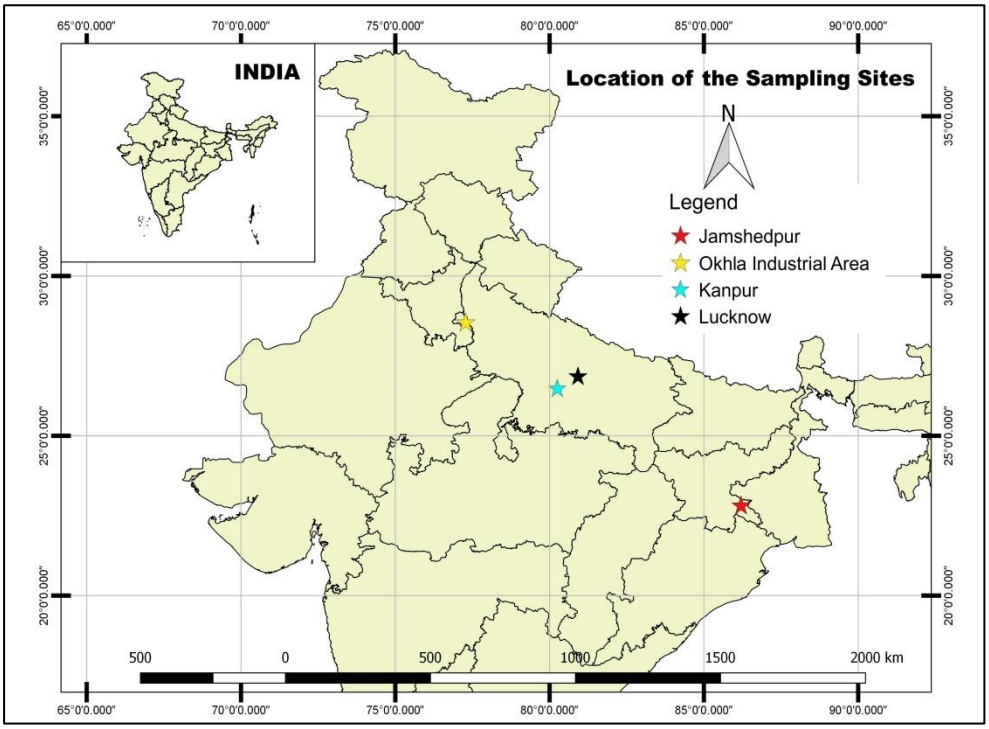


Fig3.3. Map showing sampling sites

Table.3.1- Details of sampling sites:

Sampling Sites	Location	No. of samples
Lucknow	Latitude:26° 51' 4.752"	17
	Longitude:80° 55' 0.4908"	
	Elevation:123 Meters above sea level	
Kanpur	Latitude:26°28'4.52"	2
	Longitude:80°14'50.47"	
	Elevation: 128.62 Meters above sea level	
Delhi	Latitude:28°31'54.14"	3
	Longitude: 77°16'35.61"	
	Elevation: 219.12 Meters above sea level	
Jamshedpur	Latitude:22° 48' 20.2248"	4
	Longitude: 86° 12' 11.1960"	
	Elevation: 151 Meters above sea level	

Table.3.2-List of sampling sites:

S No.	Sampling sites
1.	Avadh Vihar Yojna, Lucknow
2.	Behind Ultratech Cement Industry, Mpohanlalganj, Lucknow
3.	Near Lok Nirman Vibhag. Mohanlalganj Lucknow
4.	Near Radhaswami Satsang Bhawan, Lucknow
5.	Near Railway track , Mohanlalganj, Lucknow
6.	Bijnaur, Lucknow
7.	Okhla Industrial area-II near railway track, Delhi
	Okhla Industrial area-II near iron factory, Delhi
8.	Wajirganj, Delhi
9.	Okhla Industrial area-II near concore, Delhi
	Okhla Industrial area-II near metro station, Delhi
10.	Chandrawal, Lucknow
11.	Noornagar , Lucknow
12.	Sarojaninagar Industrial area Phase-I, Lucknow
13.	Banthara, Lucknow
14.	Sisandi Lucknow
15.	Makkakheda Lucknow
16.	Makdumpur Lucknow
17.	Sarojaninagar Industrial area Phase-II, Lucknow
18.	UPDESCO, Sarojaninagar Industrial area Lucknow
19.	Salex chemiocals Pvt Ltd, Sarojaninagar Industrial area
20.	Kankaha Mohanlalganj, Lucknow
21.	Thermal Power Plant, Panki Kanpur
22.	Oil Mil, Jamshedpur
23.	Manfit Industry, Jamshedpur
24.	Telco Industry, Jamshedpur
25.	Tube Division, Jamshedpur
26.	Concore Industry, Jamshedpur

3.7.Identification of Isolates as Fluorescent Pseudomonad (FPs):

For selection and identification of fluorescent pseudomonads, all the pure colonies were streaked on pseudomonas agar and King's B agar media. Both types of media were specific for fluorescent pseudomonas bacteria. On these media, the *Pseudomonas* bacteria were distinct by the fluorescent and transparent colonies. Among all the isolates, 55 were identified as fluorescent pseudomonads by the production of fluorescent transparent colonies on agar. King's B broth was also used to check the production of fluorescent pigment.

All the 55 isolates were maintained on Luria Bertani agar medium and broth medium (Hi Media, India) at 4 °C for further study. While for the long-term storage cultures were mixed with glycerol stock and kept at -20 °C.

3.8.Cadmium Resistance Test:

For cadmium resistance, MIC (Minimum Inhibitory Concentration) test was performed by plate dilution method (Summers & Silver, 1972; Malik & Ahmad, 1994). The concentration of metal that inhibited the bacterial growth is termed as 'Minimum Inhibitory Concentration' (MIC) for the specific tested bacteria. The heavy metal cadmium was used as cadmium nitrate $Cd(NO_3)_2.7H_2O$ in different concentrations ranging from 0-2100 ppm (mg/l). For preparation of media with various concentrations (0-2100 ppm), stock solution of cadmium with 10^6 ppm concentration was used, prepared in autoclave using double distilled water. All these concentrations were maintained in nutrient agar media in gradually increasing order (in multiples of 100). The isolated bacteria were inoculated by streaking on these cadmium amended plates and incubated at 30 °C for 3-4 days (Sarathambal et al., 2017**)**. The concentration of cadmium at which growth of bacteria was inhibited was recorded as its MIC for the strain under study. Similar study related to cadmium

resistance test of eubacteria was done by various researchers (Malik & Jaiswal, 2000; Aleem et al., 2003; Malik & Ahmad, 1994; Trevors et al., 1985).

3.9.Antibiotic Sensitivity Test:

This test was performed by using Kirby- Bauer disc diffusion method for testing the sensitivity of microorganism against antibiotics (Bauer, 1966). The effectiveness is based on size of inhibition zone, and zone may vary due to diffusibility of drug, size of inoculums, type of medium etc. In the present study, this test was performed to develop an identification profile of the isolates. Muller-Hinton agar medium was used for antibiotic sensitivity test. This medium was originally formulated for isolation of pathogenic bacteria. Now-a-days it is more commonly used in conjunction with high potency antibiotic discs for determination of antibiotic sensitivity patterns of bacteria. Antibiotics used for the study were ampicillin (A^{25}), amikacin (AK^{30}), aztreonam AT^{30}, ciprofloxacin (CIP^{5}), gentamicin (GEN^{30}), nystatin (NS^{10}), cefepime (CPM^{30}), Co-trimoxazole (COT^{25}), oxacillin (OF^{5}), norfloxacin (NX^{10}), linezolid (LE^{5}), Gatifloxacin (GAT^{5}), Nitrofurantoin (NIT^{300}) and streptomycin (S^{30}).

Procedure:

1. A lawn of bacterial isolate was prepared on MHA (Muller Hinton Agar) media with the help of sterilized cotton swab.
2. Antibiotic discs were placed on this lawn, pressed gently with sterile forceps to ensure firm contact with the agar surface and incubated at 37 °C for 24 hrs.
3. After incubation, zone of inhibition was observed around the antibiotic disc.

3.10.Seed Germination Test:

Based on cadmium resistance, six isolates having highest concentration to cadmium were selected from the 55 isolates and with these selected six isolates, seed germination test was performed. For germination test, healthy seeds of mustard

(breeder seeds) and maize were surface sterilized by using 2% mercuric chloride solution, followed by five washings with sterile distilled water. Petri plates with filter paper were autoclaved then used for germination analysis. Ten seeds of mustard and five seeds of maize were placed on filter paper of each treatment. Treatments for seed germination were as given in Table-3.3 for both mustard and maize plant. Further 3 ml distilled water and 3 ml bacterial suspension was poured in each treatment except control. In control, 6 ml distilled water was poured in plate. Plates were incubated in dark at 30 °C for 5 days. After incubation, number of germinated seed was recorded.

Table-3.3- Treatments for seed germination test of mustard and maize:

S. No.	Treatments for germination test of mustard and maize
1.	G_1
2.	K_1
3.	H_1S
4.	G_2
5.	A_1
6.	C_3
7.	Control

3.11.Morphological characterization of bacteria:

Four best bacterial isolates as per seed germination data were selected for further characterization and studies.

Morphological characterization of bacterial cell (G_1, G_2, K_1 and C_3) was done by Gram staining and SEM (Scanning electron microscopy) analysis. Colony characteristics of bacteria were analysed after growing them on growth media.

3.11.1. Colony Characterization:

For colony characterization, selected bacteria G_1, G_2, K_1 and C_3 were streaked on nutrient agar media and incubated at 30 °C for 24-48 hrs. Afterwards, colony morphology of bacteria was recorded by observing the growth of bacteria on agar surface.

3.11.2. Cell Characterization by Gram Staining (Gram, 1884):

This is an empirical method to differentiate the bacteria into two groups named as Gram positive and Gram negative. In Gram staining firstly crystal violet added to the heat fix smear and after 1 minute pouring of iodine on smear was done. Then, added decolourizer after washing with distilled water. Finally, safarnin was poured for counter staining. After 45 seconds washing of slide was done. After drying, viewing of slide was done under the microscope. In this test, the bacteria that retains the primary stain (crystal violet) are called as Gram +ve where as those that loose the crystal violet colour and counter stained by safranin (counter stain), referred to as Gram –ve**.**

3.11.3. Cell Morphology Characterization- Scanning Electron Microscopy (SEM) of Bacteria:

For scanning electron microscopy, fresh culture of isolates G_1, G_2, K_1, and C_3 were grown in nutrient agar media. Fresh bacterial cultures were inoculated in 1 ml of 5% sterilized glutaraldehyde in eppendorf tube and all the tubes were kept in refrigerator at 4 °C for fixation of cells. Once fixed, contents were centrifuged at 3000 rpm for 10 minutes at 4 $^{\circ}$C for removal of gluteraldehyde which comes as a supernatant while bacterial pellet settles down. After removal of gluteraldehyde, cells were washed with buffer saline. After this step, 1 ml of 30% ethanol was added and the contents were again centrifuged at 10,000 rpm for 10 minutes at 4 $^{\circ}$C. Supernatant was disposed off.

Same procedure was repeated with 50, 70, 80, 90 and 100% of ethanol. These steps of alcohol treatment were necessary for dehydration purpose. Further, dehydrated cells suspended in 100% ethanol, were mounted on stubs coated with carbon tape with help of micropipette. After drying, mounted samples were observed under the scanning electron microscope. Results were recorded.

3.12.Biochemical Characterization of Bacterial Isolates:

For biochemical characterization, various tests namely amylase test, catalase test, oxidase test, gelatinase test, protease test, urease test, SIM agar analysis, cellulose test, carbohydrate metabolism test, citrate agar test, citrate utilization test etc. were performed on respected media to confirm the identity of isolates as described by Aneja (2003) and Cappucino and Sherman (1992). All the biochemical tests are as:

3.12.1. Citrate Utilization Test:

For citrate utilization test, Simmons citrate agar was used. In this test, microorganism use citrate as a carbon source for their energy. Utilization of citrate involves the enzyme 'Citrase', which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and CO_2. The bacteria were aseptically inoculated into sterile Simmon's citrate agar containing sodium citrate and a pH indicator bromothymol blue. Inoculated plates were incubated at 30 °C for 24 hours. After incubation, colour change of media from green to blue confirmed the utilization of citrate by bacteria (Cappuccino and Sherman, 2002).

3.12.2. Catalase Test:

Catalase test detects the production of 'catalase' enzyme in cytochrome containing aerobic bacteria. They form hydrogen peroxide as an oxidative end product of the aerobic breakdown of sugars. Catalase decomposes hydrogen peroxide to water and oxygen. For catalase test, smear of fresh culture was made by loop on glass slides.

Few drops of 3% hydrogen peroxide were added to the culture. A positive reaction is indicated by the formation of bubbles (Cappuccino and Sherman, 2002).

3.12.3. Hydrogen Sulphide Production Test:

Sulfur-indole (SIM) agar media is used to check the production of 'Cysteine desulfurase' by the isolates. Cysteine desulfurase breaks down sulfur containing amino acids to pyruvate, ammonia and hydrogen sulfide. Iron in the medium reacts with hydrogen sulfide by producing black precipitatation, which is a positive indication for hydrogen sulfide production by the bacteria (Cappuccino and Sherman, 2002). For hydrogen sulfide production test, bacterial isolates were inoculated in Sulfur-Indole Motility agar by stubbing through the sterilized media. This set up was then incubated at 30 °C for 24 hours and hydrogen sulfide production was confirmed by blackening of the media.

3.12.4. Methyl Red-Voges-Proskauer test (MR-VP):

The MR-VP test was done to determine the ability of bacterial isolates to oxidize glucose with the production and stabilization of high concentration of acids as end product. The bacteria were aseptically inoculated into sterilized MR-VP broth and incubated at 30 °C for 24 hrs. After incubation, half culture media of each tube were transferred to other set of tubes equally. Further, MR reagent was added in one set and VP reagent (I & II) was added in another set of tubes. Development of yellow colour and red colour indicated the positive reaction for MR test and VP test respectively (Cappuccino and Sherman, 2002).

3.12.5. Amylase Test:

Amylase production test was done on starch agar media. Amylase is an exo-enzyme that hydrolyses starch into disaccharide and some monosaccharide such as glucose. Amylase production was tested to determine the absence or presence of starch in the

medium by using iodine solution as an indicator. For this test, bacterial isolates were spot inoculated on sterilized media. Then, incubation was done for 24-48 hours at 37º C. After incubation, iodine solution was poured on plate for 30 Sec. Starch in the presence of iodine produced a dark-blue colouration of the medium, and yellow zone formation around the growth indicated amylolytic activity.

3.12.6. Casein Hydrolysis Test:

Casein is the major protein found in milk and some microbes have ability to degrade this protein by producing exo-enzyme 'Caseinase' that breaks the peptide bond CO-NH by introducing water into the molecule. Casein hydrolysis by bacteria was analysed on sterilized skim milk agar media. The medium was opaque due to the presence of casein in colloidal suspension. For casein hydrolysis, bacterial isolates were spot inoculated on media and allowed to incubate at 37^{o}C for 24-48 hrs. Formation of clear zone around the growth confirmed the production of enzyme caseinase resulting in casein hydrolysis.

3.12.7. Gelatin Hydrolysis Test:

Gelatin is a protein produced by hydrolysis of collagen. It is a major component of connective tissues and tendons in humans and other animals. It dissolves in warm water (50 °C), exists as a liquid above 25 °C and solidifies when cooled below 25 °C. For geletinase test, gelatin agar media was used. Firstly, the bacterial isolates were inoculated on sterilized media and incubated at 37 ^{o}C for 24-48 hrs. After incubation, formation of a clear zone around growth is an indication of gelatinase activity on addition of $HgCl_2$.

3.12.8. Urease Test:

Urea is a major organic waste product of protein digestion in most vertebrates. Urea is excreted in the urine. Some micro-organisms have the ability to produce the enzyme

'Urease'. The urease is a hydrolytic enzyme that attacks the carbon and nitrogen bond of amide compounds with the liberation of ammonia. Urease test is performed by growing the test organism on urease agar media. For this test, bacterial isolates were inoculated in slants of urea agar media and incubated at 37 °C for 24-48 hrs. Production of pink colour in slants is a confirmation of urease production.

3.12.9. Carbohydrate Utilization Test:

Carbohydrates are organic molecule that contain carbon, hydrogen and oxygen in the ratio of **$(CH_2O)n$**. There are some bacteria, fungi and yeasts which have the ability to break down these carbohydrate molecules. Organisms use carbohydrates differently depending upon their enzyme compliment. Carbohydrate (dextrose) utilization of all the bacterial isolates was checked by using phenol red dextrose agar media. Bacterial isolates were spot inoculated on this media. After inoculation, incubation was done at 37^o C for 24-48 hrs. Formation of yellow colour around the growth confirmed the dextrose utilization by tested bacteria.

3.12.10. Cellulase Test:

Degradation of cellulose is brought about by fungi, bacteria and actinomycetes by the secretion of extracellular enzyme 'Cellulase'. It is a complex enzyme composed of at least three components *viz.* endoglucanase, exoglucanase and β-glucosidases. In this test, CMC-CDA agar media was used. For this test, bacterial isolates were spot inoculated on CMC-CDA media and incubated at 30 °C for 24-48 hrs. After incubation, congo red dye was poured on plates. Formation of clear zone around the growth confirmed the cellulose degradation capacity of tested bacterial isolates.

3.12.11. Citrate Agar Test:

The iron bacteria oxidize ferrous iron to ferric state, which precipitate as ferric hydroxide around cells. The ferric hydroxide deposits give a brown or rust red colour

to the organisms. Citrate agar is recommended by Subba Rao, 1977 for the isolation and detection of iron bacteria. Di-potassium phosphate provides buffering to the medium. Magnesium sulphate, ammonium sulphate and calcium chloride are sources of ions that stimulate metabolism. Ferric ammonium citrate is used as a source of carbon and sodium nitrate acts as a source of nitrogen. For this test, citrate agar media was used for inoculation of bacterial isolates. After inoculation, incubation was done at 30° C for 3-4 days. Development of brown or rust red colour is an indication of positive test for citrate.

3.12.12. Indole Production Test:

Tryptophan is an essential amino acid, oxidized by some bacteria using the enzyme 'tryptophanase' into indole, pyruvic acid and ammonia. Production of indole by bacteria is performed by inoculation of bacterial isolates in tryptone water. After inoculation, tubes are allowed to incubate at 35^{o}C for 48 hrs. Indole production can be detected by addition of Kovac's reagent (dimethyl aminobenzaldehyde) and development of cherry red reagent layer, indicative of indole production.

3.12.13. Oxidase Test:

The oxidase test is used for the identification of bacteria that produce cytochrome C oxidase, an enzyme of the bacterial transport chain. When present, the cytochrome C oxidase oxidises the reagent tetramethyl-p-phenylenediamine to indophenols (purple coloured end product), while in the absence of the enzyme production, the reagent remains reduced and is colourless.

For this test, oxidase discs, impregnated with the dye, tetramethyl-p-phenylenediamine dihydrochloride (TMPD) were oversprayed with the fresh bacterial culture and observed after 5-10 sec. Discs that were colourless were taken as negative while the ones appearing purple were recorded as positive.

Based on morphological and biochemical characteristics, all the four isolates (G_1, G_2, C_3, and K_1), were identified according to the key of Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). For further confirmation, they were subjected to molecular analysis as given in subsequent section.

3.13.Molecular Characterization of Bacterial Isolates:

For further identification, molecular analysis was performed. For this, PCR product was subjected to 16s rRNA was used for sequencing of the DNA using universal primers as given below:

In this method, 5 µl of genomic DNA was extracted. Extracted DNA was amplified in PCR using universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (CGGTTACCTTGTTACGACTT) as template. For sequencing, 785F (GGATTAGATACCCTGGTA) and 1492R (5′ TACGGYTACCTTGTTACGACTT 3′) primers were used. The total PCR mixture was 25 µl containing- 1 µl template, 2.5 µl of taq DNA polymerase buffer, 5mM $MgCl_2$, 1 µl of dNTP at 2.5 mM, 0.5 µl of 2.5 unit Taq DNA polymerase 3.75 pmol primers (each).

The PCR was performed at 95 ^oC for 0.5 minutes, 52 ^oC for 0.5 min and 72 ^oC for 2 min of 35 cycles followed by initial denaturation at 95^oC for 2 min and final extension at 72 ^oC for 15 minutes. All the obtained sequences were aligned with gene bank database (http://www.ncbi.nlm.nih.gov/Blast) as well as Ez taxon BLAST. The neighbour-joining method was used to infer the evolutionary history (Saitou and Nei, 1987) through phylogenetic tree construction. Evolutionary analysis was conducted in MEGA6 (Tamura et al., 2013).

3.14.Gene Bank Submission and G+C content analysis:

The partial homologous and aligned sequence of all the selected isolates G_1, G_2, K_1 and C_3 were submitted to NCBI genbank and the accession number were assigned. G+C content of obtained partial sequence was also calculated by online software.

3.15.Evaluation of PGP Traits:

Qualitative and quantitative analysis of plant growth promoting (PGP) characteristics of cadmium resistant fluorescent pseudomonads C_3, G_1, K_1 and G_2 were analysed by following the standard methods in presence and absence of cadmium. Plant growth promotory characteristics play an essential role in plant growth promotion and heavy metal remediation. Plant growth promotory traits tested for the selected isolates are given below in subsequent sections:

3.15.1. Production of Indole Acetic Acid:

Production of Indole Acetic Acid (IAA) was checked by following the method of Brick et al. (1991). In this test, nutrient broth amended with 1 mg/ml tryptophan was used. After sterilization of the media, selected isolates were inoculated and incubated at 30 $^{\circ}$C for 72-96 hrs. After incubation, few drops of ortho-phosphoric acid were added, followed by addition of Salkowski reagent in culture filtrate. Tubes were again incubated in dark for 30 min. Development of pink colour indicates the presence of IAA in the tubes. For quantitative analysis, the absorbance of the tubes was recorded at 530 nm wavelength. Concentration of produced IAA was calculated by calibrating standard graph of IAA (Loper and Scroth, 1986).

3.15.2. Ammonia Production:

Bacterial isolates were tested for the production of ammonia in sterilized peptone water broth. For this test, inoculated tubes were incubated for 72-96 hrs. After incubation, few drops of Nessler's reagent were added in the culture filtrates.

Development of brown colour (+++), faint yellow colour (++) and light yellow colour (+) in tested broth tubes confirmed the production of ammonia (Cappucino and Sherman, 1992).

3.15.3. Production of HCN (Hydrogen cyanide):

All isolates were screened for the production of hydrogen cyanide (HCN) by using the standard method of Lorck (1948). In this test, King's B media was amended with 4.4g glycine/l. Sterilized Whatman filter paper soaked in 2% sodium carbonate and 0.5% picric acid solution was placed in lid of the plate after inoculation. Then, plates were properly sealed with parafilm and incubated at 28±2 ^{o}C for 72-96 hrs. Turning of yellow colour to orange colour, dark brown colour and light brown colour of filter paper confirmed the strong production, moderate and low production of HCN respectively.

3.15.4. Determination of Phosphate Solubilisation:

For qualitative analysis of phosphate solubilisation, Pikovskaya agar and modified Pikovskaya agar was used (Pikovskaya, 1948; Mehta and Nautiyal, 2001). In modified pikovskaya agar media, bromophenol blue dye was added. Both types of agar plates were spot inoculated and incubated at 28±2^{o}C for 72-96 hrs. After incubation, plates were observed for inhibition zone and red colour zone in Pikovskaya and modified Pikovskaya agar plates respectively around the growth. The inhibition zone and red colour zone was measured and used for calculation of phosphate solubilisation index- PSI (Edi-Premono et al., 1996).

Quantitative analysis of phosphate solubilisation of tri-calcium phosphate in liquid medium was done as described by King (1932). For quantitative analysis, tri-calcium phosphate amended media was used. Selected isolates, G_1, G_2, K_1 and C_3 were inoculated in 25 ml Pikovskaya's broth and incubated for 72-96 hrs at 28±2 °C. After

incubation, growth media were centrifuged at 15,000 rpm for 30 min. One ml supernatant was mixed with 10 ml of chloro-molibidic acid and the volume was made up to 45 ml with sterilized distilled water. Then, 0.25 ml of chloro-stannous acid was added to maintain the volume up-to 50 ml with distilled water. The absorbance of the developed blue colour was read at 600 nm and the amount of soluble phosphorus was detected from the standard curve of KH_2PO_4.

3.15.5. Analysis of Siderophore:

Siderophore production test was done on CAS (Chrome Azural S) agar media by following standard protocol (Schwyn and Neilands, 1987). Fresh cultures of bacteria were spot inoculated on CAS agar media and incubated for 72-96 hrs at 28±2^{o}C. After incubation, formation of orange zone around the growth indicated the siderophore production.

The quantitative estimation of siderophore produced by *Pseudomonas* was done by CAS-shuttle assay (Schwyn and Nielands, 1987). In this method, the strain was grown on nutrient broth medium and incubated for 24-48 hrs at 28^{o}C with constant shaking at 120 rpm on rotator shaking incubator. After incubation, the fermented broth was centrifuged at 10,000 rpm in referigerated centrifuge at 4^{o}C for 10 minute. From this, 0.5 ml of cell free supernatant was collected and mixed with 0.5 ml of CAS solution. After 20 minutes of incubation, obtained colours absorbance was recorded at 630 nm using spectrophotometer. Tube containing 0.5ml un-inoculated nutrient broth medium and 0.5 ml CAS solution were used as reference/ control tubes.

The percentage of siderophore units was estimated as the proportion of shifting of dye CAS colour from blue to orange using the folrmula:

$$PSU\ OR\ \%\ SU = [(Ar - As)/Ar] \times 100$$

Where, Ar is the A_{630}nm of reference (CAS assay solution+ un-inoculated media) and As is the A_{630} nm of the sample (CAS assay solution+ supernatant).

3.15.6. ACC Utilization Test:

ACC (Aminocyclopropane-1-carboxylate) deaminase production was checked by following the modified method of Li et al. (2011). In this method, ACC utilizing capacity of bacterial strains is determined. For this test, 24 hrs pure bacterial cultures were grown in Luria Bertani medium and centrifuged at 8000 rpm for 5 min. Obtained cell pellet was washed with DF medium. Then, inoculated in 2 ml DF-ACC medium and incubated for 24 hrs at 28 °C. One tube was remaining un-inoculated as control. One ml of this culture medium was centrifuged at 8000 rpm for 5 min and 100 µl of supernatant was diluted to 1 ml with liquid DF medium. Sixty microliter of diluted supernatant was mixed with 120 µl of ninhydrin reagent in a tube. All tubes were put on water bath for 30 min and heated till boiling temperature. Tubes were observed for Ruhemann's purple colour formation. Two ml content of the each tube was used for measuring the absorbance at 570 nm using spectrophotometer. Intense purple colour is indication of high ACC deaminase activity and recorded with a rise in absorbance value while less intense purple colour is indicative of less ACC deaminase activity.

3.15.7. Zn Solubilisation Test:

For zinc solubilisation test, the nutrient agar media was mixed with D-glucose and different insoluble zinc compounds ZnO, $Zn_3(PO_4)_3$ and $ZnCO_3$ at a concentration of 0.1% Zn by following the standard method (Fasim et al., 2002). Zinc containing agar plates were spot inoculated by using sterile loop and kept in an incubator at 30 °C for 7 days. After incubation, halo zone around the growth confirmed the positive test for Zn solubilisation.

3.15.8. Production of EPS (Exo-polysachharide):

Four selected bacterial strains G_1, G_2, K_1 and C_3 were used for EPS production, extraction and characterization by following standard protocol. For extraction of EPS, the bacterial strains were inoculated in 20 ml nutrient broth and incubated for 4 days at 30 °C in shaking incubator. After incubation, the broth was centrifuged at 10,000 rpm for 15 min. Obtained supernatant was mixed with three volumes of ethanol slowly along the side wall of the flask and allowed to stand at 4 °C overnight to precipitate the EPS. The precipitated EPS was filtered and measured after drying at 80 °C (Hong et al., 2002).

3.16.Pyoverdine Production, Extraction and Characterization:

For pyoverdine production, fresh culture was used to inoculate 10 ml broth. After 30 hrs of incubation, bacterial culture was centrifuged at 10,000 rpm for 15 min. Supernatant was collected and filtered through membrane filter and pH was adjusted in the range 5 – 5.5. Production of pyoverdine was checked by spectrophotometer at 403 nm wavelength and HPLC (High Performance Liquid Chromatography) technique was used to confirm the production of pyoverdine by comparing the peak of samples against standard pyoverdine.

3.17.Effects of Cadmium on Morphology and Growth Pattern of Bacteria:

For determination of cadmium resistance pattern analysis of bacteria, nutrient broth amended with 0, 100 ppm of cadmium was used for inoculation. Then, incubation of bacteria was done at 28 °C upto 106 hrs with 100 rpm shaking. Growth analysis of isolates was done by measuring OD at 600nm at different time interval. After different time interval, 5 ml media was removed from each treatment and bacterial growth was analysed by turbidometric analysis in reference to control of each

concentration without inoculation. To analyse the effect of cadmium on morphology of bacteria, SEM analysis of bacteria was done in presence and absence of cadmium.

3.18.Accumulation analysis of Cadmium by bacterial cells:

In cadmium accumulation analysis the bacterial strains C_3 and G_1 were first grown in 500 ppm cadmium amended nutrient broth for one week, then centrifuged at 10, 000 rpm for 15 min. After centrifugation, the obtained pellet was washed with buffer; obtained pellet and supernatant was digested with acid. Further both digested supernatant and pellets were used to analyse cadmium level by AAS (Atomic Absorption Spectroscopy) analyser (Chakraborty and Das, 2014).

3.19. Characterization of Cadmium Resistance Gene: Genomic DNA Isolation and PCR Amplification of czcABC Gene:

Isolation of genomic DNA was carried out by following standard protocol (Sambrook et al., 1989). For amplification of conserved region of czc genes, primers F_1-czc 5′TCCTCAAATCCGAACTGGGC3′ and F_2-czc5′GCTCGATGGCGAATTGGATG3′ were used. The primers used in this study were designed with NCBI primer blast and synthesized by Sigma Aldrich, India. For amplification, 25 µl of total volume was used in thermal cycler. PCR mixture contained 1 Unit/µl Taq polymerase, 0.5µM of each primer, 1× enzyme buffer, 200 µM of each dNTP and 1.25 mM $MgCl_2$. In this reaction, initial denaturation was at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 72 °C for 2 min and final extension of 72 °C for 10 min. Further, the PCR product was analysed by using gel electrophoresis and visualized in gel documentation system.

3.20.Extraction and Characterization of Metabolites:

3.20.1. Extraction and Characterization of Siderophore:

For study of chemical structure, siderophore was extracted in crystal form. For crystallization, 30 hrs old cultures were centrifuged at 10,000 rpm for 15 min and cell

free supernatant was added with saturated $FeSO_4$ solution. Further, H_2SO_4 and 50% ammonium sulphate solution was added for deproteinization. The aqueous phase of the solution was kept at 4° C in refrigerator, followed by concentration using rotary vacuum evaporator. The filtrate was neutralized, reduced and extracted in methanol in crystal form. The crystals were then separated out on Whatman filter paper No.44. Crystals obtained were characterized by FTIR (Fourier-transform infrared spectroscopy) analysis (Tank et al., 2012). In this analysis, 200 mg of crystallized siderophore was mixed with dry potassium bromide (KBr) and pressed into mold for IR spectroscopy by using the IR spectrophotometer. This technique is helpful for analysis of C=O and –OH bonds. Obtained data was analysed by comparing with the FTIR of standard hydroxamic acid crystals (Fuchs and Budzikiewz, 2001).

3.20.2. IAA Extraction, Purification and Characterization:

For IAA extraction, four flasks containing 100 ml of IAA producing media with 15 mg/ml of L-tryptophan were inoculated with the organism namely G_1,G_2, K_1 and C_3 (Bharucha et al., 2013). Inoculated flasks were incubated in shaking incubator for 4 days at 30 °C. After incubation, media was centrifuged at 10,000 rpm for 15 min, and the supernatant was acidified with 1 N HCl to pH 2.5. Then, acidified filtrate was mixed with equal volume of ethyl acetate and shaken vigorously by vortex. This process was repeated again and produced IAA was extracted. Further sample were allowed to dry at 40 °C in rota-vapour. Extracted and dried IAA was mixed with 3 ml of methanol and kept at 4 °C for characterization. HPLC (High Performance Liquid Chromatography) analysis was also done for confirmation of IAA produced by isolates G_1, G_2, K_1 and C_3.

3.20.3. EPS Characterization:

For characterization of EPS, FTIR technique was used. In this analysis, 200 mg of EPS was mixed with dry potassium bromide (KBr) and pressed into mold for IR spectroscopy by using the IR spectrophotometer. This technique is helpful for analysis of C=O and –OH bonds.

3.21.Compatibility Test:

The compatibility test of all the selected PGPR strains G_1, G_2, K_1 and C_3 was done before making the consortia. The nutrient agar medium was used for this test. Firstly, one isolate was streaked on one side of the plate and other isolate streaked perpendicularly up to the test isolate as cross pattern. Streaked plates were incubated for 48 hrs at 30 °C and observed for the growth of isolates in respect of each other. Growth inhibition of one isolate by other isolate was considered as negative i.e. no compatibility while simultaneous growth of the isolates indicates the compatibility of tested isolates (Sarathambal et al., 2017). Based on the results of compatibility study, three consortium were developed as:

Consortium1 ($Cons_1$) - G_1, K_1, G_2

Consortium2 ($Cons_2$) - G_2, K_1, C_3

Consortium3 ($Cons_3$) - G_1, K_1, C_3

3.22.Culture Preparation and Seed Inoculation:

Luria Bertani medium was used for culture preparation of *Pseudomonas* sp. G_1 (KU947109), *Pseudomonas putida* G_2 (KX681787), *Pseudomonas guariconensis* K_1 (KX681789) and *Pseudomonas aeruginosa* C_3 (KU947108). This broth was incubated on an orbital shaker at 28° C for 48 hrs with a speed of 100 rpm. The incubated broth was centrifuged for 15 min at 3000 rpm and pellets were washed with distilled water. Pellets were suspended in distilled water having 1.0 OD (10^8 cells/ ml) at 610 nm

wavelength. For pot analysis and root elongation assay, suspension (10^8cells/ml) of isolates G_1, G_2, K_1 and C_3 and their consortia (G_1, K_1, G_2-$Cons_1$; G_2, K_1, C_3-$Cons_2$; G_1, K_1, C_3-$Cons_3$) was used.

3.23.Root Elongation Assay:

Nuclear seeds of *Brassica juncea* and *Zea mays* obtained from CRC, GBPUAT, Pantnagar were used. Root elongation assay was done by placing surface sterilized seeds on sterilized filter paper containing petri plate and allowed to grow after treatment (Belimov et al., 2005). Complete method of the test is described below:

Surface sterilization of seeds was done by using mixture of absolute ethanol and 30% H_2O_2 (1:1) for 15 min. To the surface sterilized seeds, 3 ml of bacterial suspension and 3 ml of sterilized distilled water was applied on each plate. In plates without cadmium additional 2 ml of distilled water was also poured, but in plates with cadmium 2 ml of 100 ppm cadmium was applied on each treatment for cadmium amendment. For control without cadmium and bacteria, only 8 ml of distilled water was applied in plates and control with cadmium contained 6 ml of distilled water and 2 ml 100 ppm cadmium. All treatments applied in this test are given in Table-3.4 for mustard and maize. All plates were incubated for 5 days at 30 ^oC and length of the root of both plants were recorded after incubation for root elongation assay.

3.24.Pot Experiment:

3.24.1. Preparation of Cadmium Contaminated Soil:

Soil was collected from field at 15 cm depths and it was air dried, homogenized and sieved with 0.2 mm sieve. For preparation of cadmium contaminated soil 100 ppm cadmium solution of cadmium nitrate was added in air dried soil and makes the soil cadmium contaminated with 100 ppm concentration. This soil was kept for 3-4

months for cadmium solubilisation. The physicochemical properties of soil were also analysed.

3.24.2. Preparation of Seedlings and Inoculation of Bacteria in Pot:

Seeds of *Brassica juncea* and *Zea mays* were firstly surface sterilized and germinated in uncontaminated agricultural soil in pot of 15 cm diameter. Seedlings with 10 cm height of mustard plant and 15 cm height of maize plants were transplanted in control and cadmium contaminated soil in pots filled with 5 kg of soil. At cultivation period chemical fertilizers were also applied in pot to avoid limiting of nutritional value and 20 ml of fresh bacterial culture of mustard and maize was poured in respected pot after one week of sowing. To avoid the leaching of cadmium, bottom of pots were sealed off. The pot experiment was CRD (Completely randomized design) in arrangement consists of sixteen treatments eight with cadmium and eight without cadmium in three replicates as shown in Table-3.4. This experiment was done in greenhouse chamber and watered daily.

3.24.3. Cadmium Uptake Analysis:

All plants were harvested and analysed to check the cadmium concentration in root and shoot region of both plants. Soil near the root region was also used to check the cadmium concentration. For this analysis, the plant and soil samples were oven dried at 80 $^{\circ}$C and then digested in nitric acid and per-chloric acid (Burd et al., 1991). Digested samples were used for cadmium analysis by atomic absorption spectroscopy (AAS).

3.25.Statistical Analysis of Data:

All the statistical analyses were performed using SPSS software package. All the triplicate data were analysed through a one way analysis of variance (ANOVA) and treatment means were compared and separated by Duncan's test. Data were also

represented as mean±SD (Standard deviation) of triplicates. All analyses were performed at the P≤0.5 level.

Table-3.4-Treatments used in pot experiment of mustard and maize:

Treatments				**Code of treatments (In Graph)**
Mustard		**Maize**		**Mustard and Maize**
Control Mz-C_0-Cd_0	Control Mz-C_0-Cd_{100}	Control Mu-C_0-Cd_0	Control Mu-C_0-Cd_{100}	C
Mz-G_1-Cd_0	Mz-G_1-Cd_{100}	Mu-G_1-Cd_0	Mu-G_1-Cd_{100}	G_1
Mz-G_2-Cd_0	Mz-G_2-Cd_{100}	Mu-G_2-Cd_0	Mu-G_2-Cd_{100}	G_2
Mz-K_1-Cd_0	Mz-K_1-Cd_{100}	Mu-K_1-Cd_0	Mu-K_1-Cd_{100}	K_1
Mz-C_3-Cd_0	Mz-C_3-Cd_{100}	Mu-C_3-Cd_0	Mu-C_3-Cd_{100}	C_3
Mz-$Cons_1$-Cd_0	Mz-$Cons_1$-Cd_{100}	Mu-$Cons_1$-Cd_0	Mu-$Cons_1$-Cd_{100}	$Cons_1$
Mz-$Cons_2$-Cd_0	Mz-$Cons_2$-Cd_{100}	Mu-$Cons_2$-Cd_0	Mu-$Cons_2$-Cd_{100}	$Cons_2$
Mz-$Cons_3$-Cd_0	Mz-$Cons_3$-Cd_{100}	Mu-$Cons_3$-Cd_0	Mu-$Cons_3$-Cd_{100}	$Cons_3$

Where Mz-Maize; Mu-Mustard, Cd_0-without cadmium; Cd_{100}-with 100ppm cadmium; C_0-without bacterial culture; G_1, G_2, K_1 and C_3 are names of bacterial cultures used; $Cons_1$-Consortium G_1, G_2 and K_1; $Cons_2$- Consortium G_2, K_1 and C_3; $Cons_3$-Consortium G_1, K_1 and C_3

Results

Chapter 4 Results

This chapter embodies the results obtained in the present study entitled "**Remediation of Cadmium Contaminated Soil with PGPR Consortia and Hyperaccumulator Plants".**

4.1.Isolation of Cadmium Resistant Bacteria:

For isolation of cadmium resistant fluorescent pseudomonads (FPs), the rhizospheric and non-rhizospheric soil samples were collected from various region of India (Lucknow, Kanpur, Delhi and Jamshedpur). From these soil samples, various types of bacteria were isolated, but only FPs were selected for further study as shown in Fig-4.1. Primary identification of bacteria as FPs was done by observing their fluorescent green colour colonies on pseudomonas agar and King's B agar. Out of 89 isolates, only 55 (61.75%) bacteria belong to FPs group at 50 ppm of cadmium.

4.2.Identification of Isolates as Fluorescent Pseudomonad:

On pseudomonas agar and King's B agar, selected bacterial isolates G_1, G_2, K_1, C_3 H_1S and A_1 formed fluorescent green colonies. On the basis of cultural, morphological and biochemical characteristics, total of 4 isolates G_1, G_2, K_1 and C_3, were grouped into *Pseudomonas* family as described in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

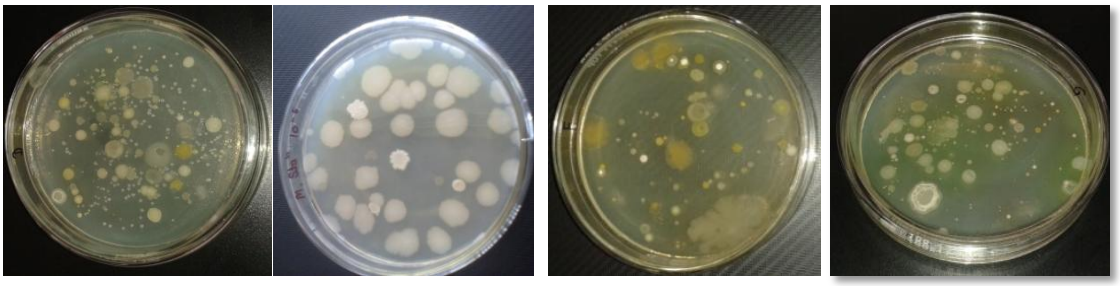


Fig-4.1- Isolation of bacteria

4.3 Cadmium Resistance Test:

All the 55 bacterial isolates were tested for cadmium resistance from 100-2100 ppm of cadmium (Fig-4.2 and 4.3). Of the total, 21% of the isolates were resistant upto 1500 ppm and only 3.6% were resistant till 2000 ppm of cadmium. Only one isolate, H_1S could grow at a concentration of 2100 ppm of cadmium. Amongst the 55 isolates, six showing maximum cadmium tolerance (G_1, G_2, K_1, C3, A_1 and H_1S) were selected for seed germination test as shown in Fig-4.4.

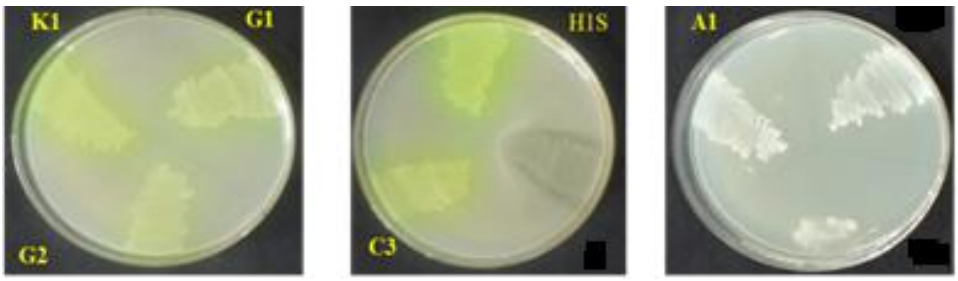


Fig-4.2-Cadmium resistant analysis

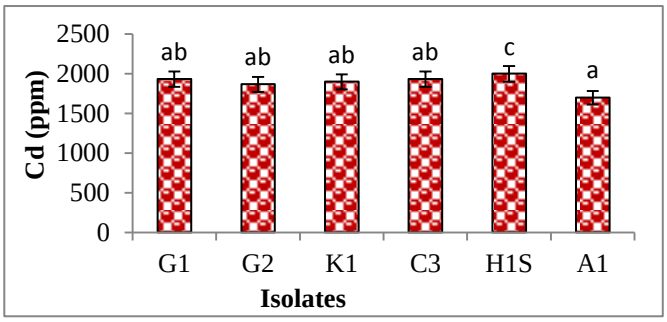


Fig.4.3- MIC of isolates against cadmium

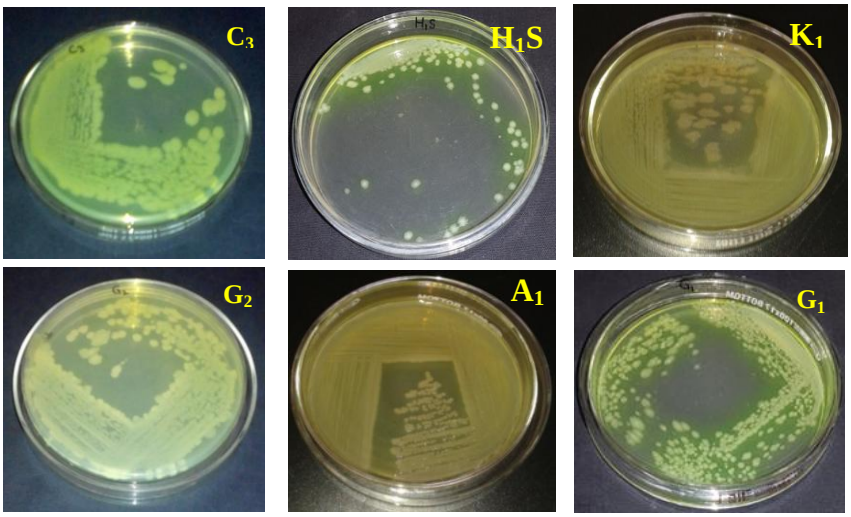


Fig-4.4- Pure culture of cadmium resistant isolates

4.4.Antibiotic Resistance Test:

Result of antibiotic susceptibility test was interpreted by measuring the inhibition zone (Fig-4.5) according to the National Committee for Clinical Laboratory Standards **(NCCLS)** for obtaining the bacterial category (Sensitive, Resistant and Intermediate). On the basis of this test, we find that the isolates A_1 had similarity with K_1 and H_1S was similar with C_3. But the isolates G_1 had very different types of antibiotic resistant pattern in comparison of other isolates and G_2 isolates showed slightly similar pattern with G_1 isolate. All the isolates were sensitive for Streptomycin (S), Gatifloxacin (GAT), Ofloxacin (OF) and Amikacin (AK) antibiotic and resistant for Ampicillin (A), Cefepime (CPM), Nystatin (NS), Aztreonam (AT) and Nitrofurantoin (NIT) as shown in Table-4.1. However, all the isolates were intermediate for Gentamicin (GEN) while with Ciprofloxacin (CIP), Cotrimoxazole (COT), Norfloxacin (NX), Linezolid (LE) antibiotics, the isolates depicted different sensitivity.

Table-4.1- Response of bacteria against tested antibiotics:

Isolates	**G_1**	**G_2**	**K_1**	**A_1**	**H_1S**	**C_3**
A25	R	R	R	R	R	R
AK30	S	S	S	S	S	S
CPM30	R	R	R	R	R	R
CIP5	S	I	S	I	S	S
S25	S	S	S	S	S	S
GEN10	I	I	I	I	I	I
NS100	R	R	R	R	R	R
COT25	I	R	R	R	R	R
OF5	S	S	S	S	S	S
NX10	S	S	I	S	S	S
LE5	S	I	S	S	S	S
AT30	R	R	R	R	R	R
GAT5	S	S	S	S	S	S
NIT300	R	R	R	R	R	R

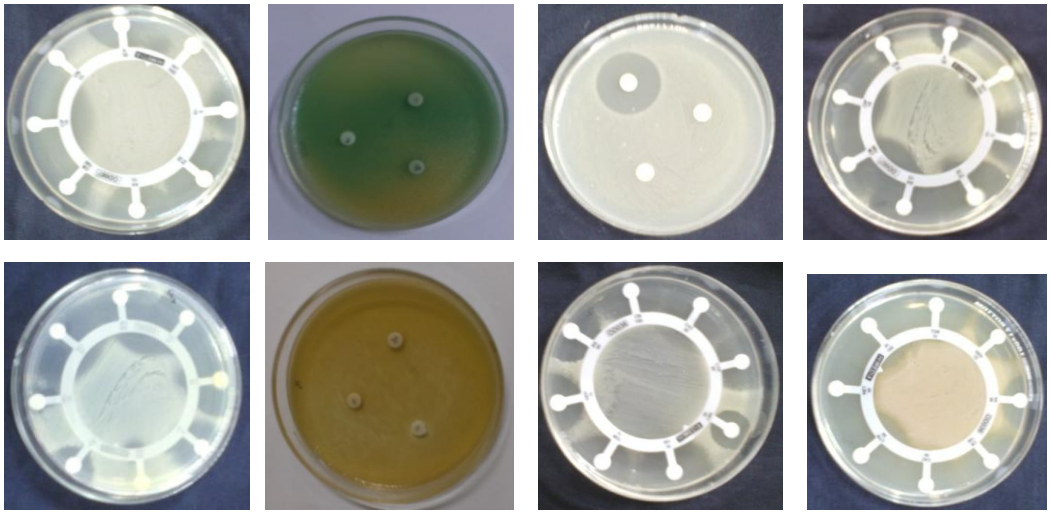
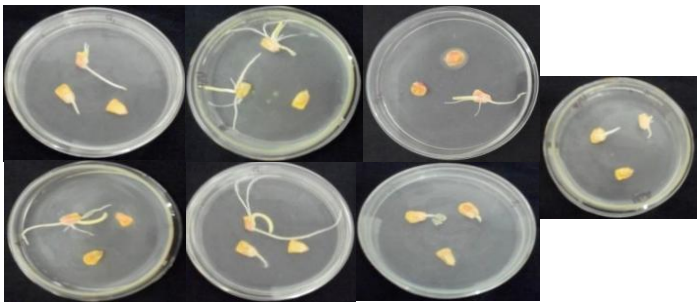


Fig.4-5- Response of bacteria against tested antibiotics

4.5.Seed Germination Test:

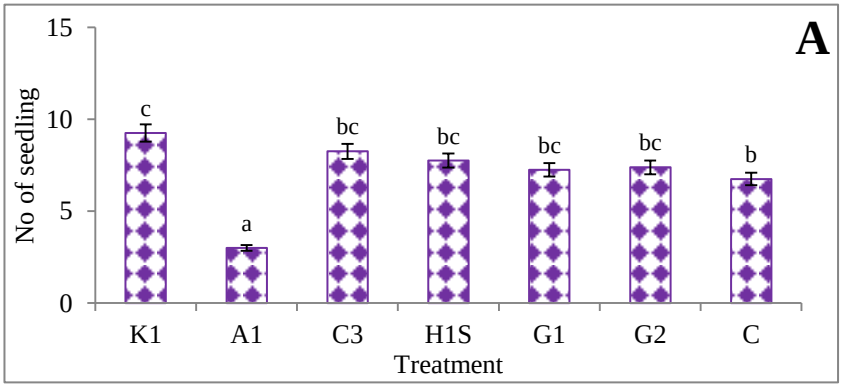
All the six isolates were tested for their seed germination efficiency with mustard and maize. The results for the same are depicted graphically in Fig. 4.7 A and B. Minimum germination percentage was reported in case of A_1 in both mustard and maize plants. In presence of this isolate, germination percentage was reduced by 32.5% and 35.62% in mustard and maize, respectively, over the control. Maximum germination percentage of 94.9% was observed in case of mustard seeds with the isolate, K_1 and C_3 showed 83.2% while in case of maize, 84% was found after treatment in case of K_1 and C_3 isolates Fig-4.6 (A, B) and 4.7 (A, B). Findings of this test showed that isolates G_1, G_2, K_1 and C_3 performed better than H_1S and A_1 in germination analysis. Thus, selective elimination was done on the basis of germination test. Among six isolates G_1, G_2, K_1, C_3, H_1S and A_1 four isolates G_1, G_2, K_1 and C_3 were selected because these bacteria were applicable for enhancement of germination percentage of both mustard and maize seeds with high MIC value.

(A)



(B)

Fig-4.6 A & B- Plates showing germination of Mustard (A) and Maize (B)



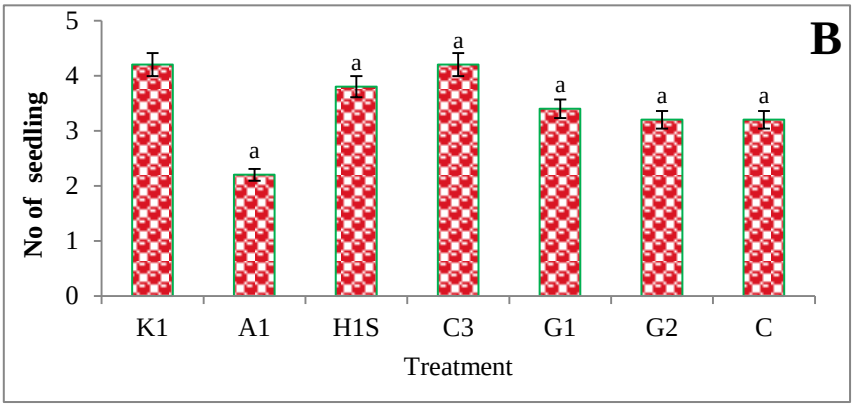


Fig-4.7 A & B-Germination test of Mustard (A) and Maize (B)

4.6.Characterization of Bacteria:

4.6.1. Morphological Characterization:

Colony of all the isolates, G_1, G_2, K_1 and C_3 were green, fluorescent and transparent. These isolates were gram negative, rod shaped bacteria as confirmed by Gram staining and SEM (Scanning Electron Microscopy) analysis (Fig-4.8).

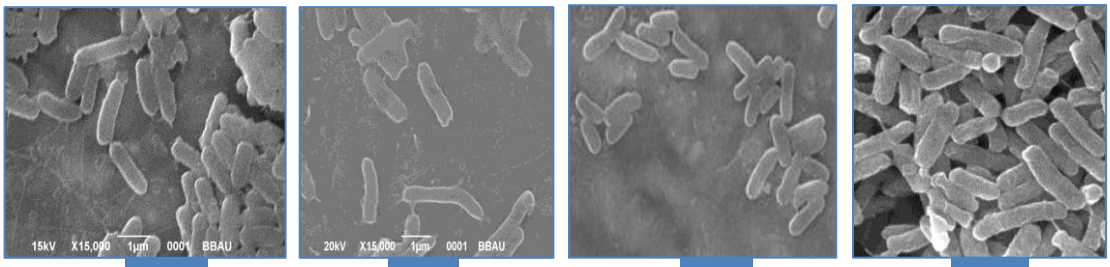


Fig-4.8- Scanning Electron microscopic image of selected isolates

4.6.2. Biochemical Characterization:

In biochemical characterization, all the isolates G_1, G_2, K_1 and C_3 were positive for citrate utilization, cellulose test, and protease test and negative for VP and indole test. For remaining test, isolates showed different results. Isolates G_1 and C_3 were positive for gelatinase test and negative for amylase test. For citrate agar test and lipase test, only G_2 showed negative results, while for H_2S production, only K_1 showed negative results. In phenol red dextrose agar test, all the isolates G_2, K_1 and C_3 showed positive results except G_1. Detailed results of biochemical tests are shown in Fig-4.9 and Table-4.2.

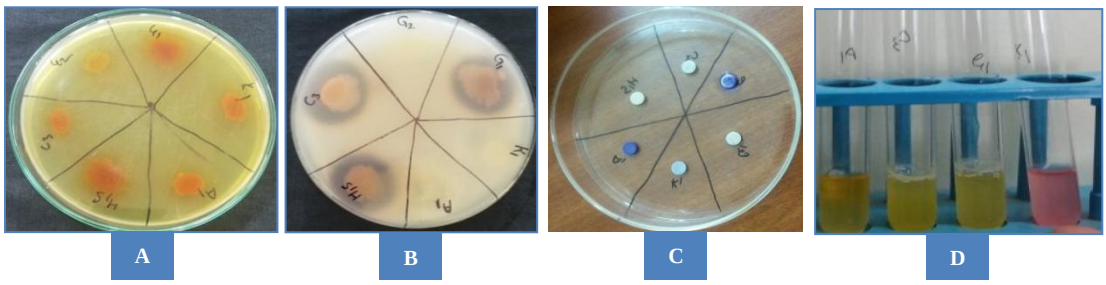


Fig-4.9- Biochemical Tests A- Citrate Agar, B- Gelatinase test, C- Oxidase Test, D- MR test

Table-4.2- Results of biochemical tests:

Tests	G_2	G_1	K_1	C_3
Amylase	+	-	+	-
Simmons citrate	+	+	+	+
MR	-	-	+	-
VP	-	-	-	-
Indole	-	-	-	-
Citrate agar	-	+	+	+
Phenol red	+	-	+	+
Cellulase	+	+	+	+
Lipase	-	+	+	+
H2S	+	+	-	+
Urease	+	-	+	-
Skim milk	+	+	+	+
Gelatinase	-	+	-	+

4.6.3. Molecular Characterization:

Further, in molecular characterization all the isolates were characterized as *Pseudomonas* species (Fig.4.10). Because, they showed close similarity to the genus *Pseudomonas* after comparative analysis of obtained sequence and already submitted sequence. In identification process, by using EZ-taxon and BLAST analysis, it was confirmed that isolate G_1 showed 96.60% similarity to *Pseudomonas putida*, isolate G_2 had 99.35% similarity to *P. putida*, isolate C_3 showed 99.18% similarity to *P. aeruginosa* and 99.59% similarity to *P. guariconensis* was reported in case of K_1 isolate Table.4.3. Phylogenetic tree of all the isolates is depicted in Fig.4.11 (A, B, C and D). Results of BLAST through EZ taxon (Yoon et al., 2017) and NCBI confirmed that there is a possibility of novel strains G_1 because it showed only 96.16% close similarity to *Pseudomonas putida*. There is a need of more study for complete

characterization of this bacterium as novel. The isolate K_1 is also a new strains and was recently isolated by Toro et al. (2014).

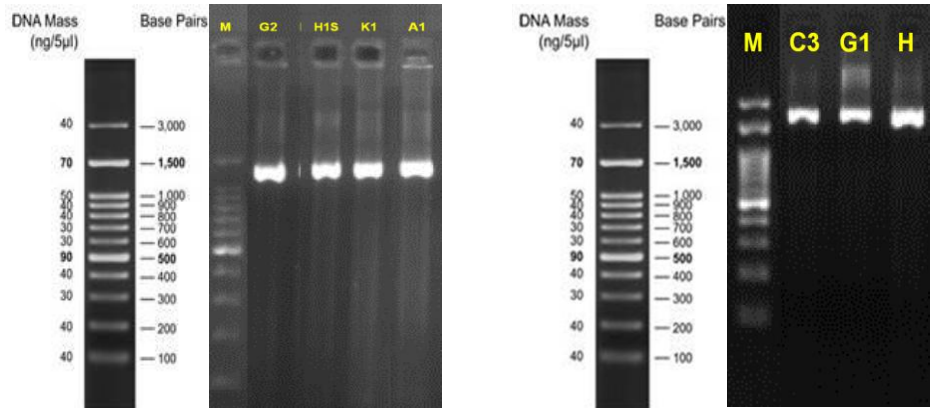
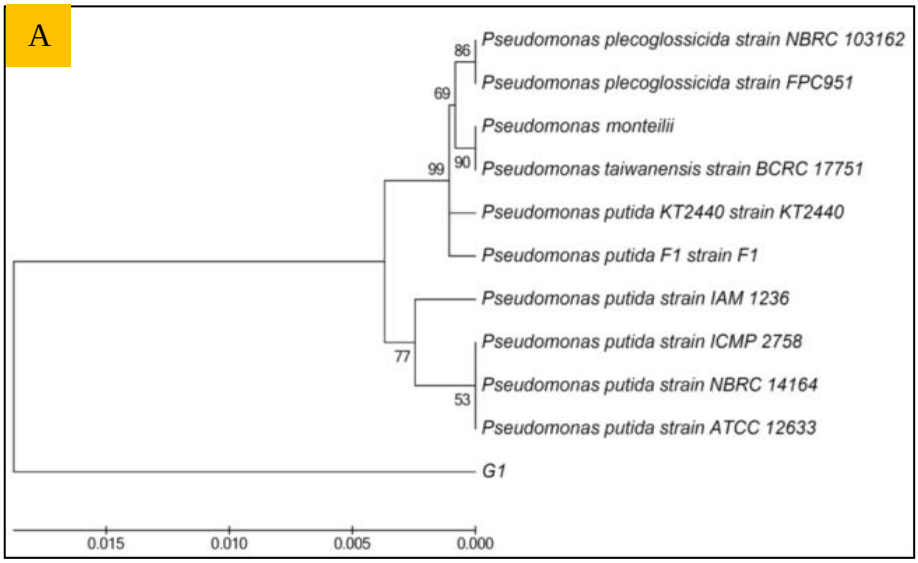
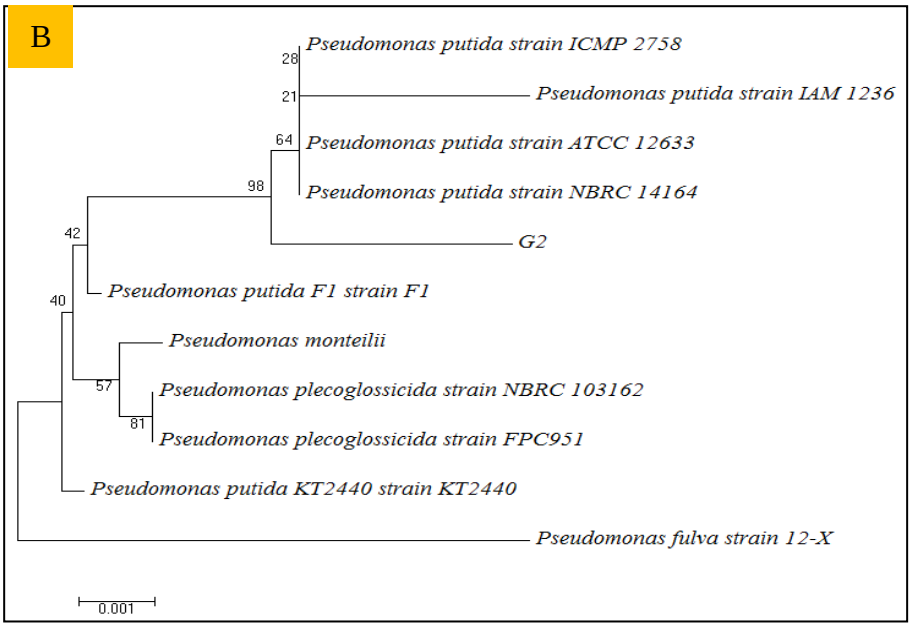


Fig.4.10-Eleterophoresis band showing isolated DNA





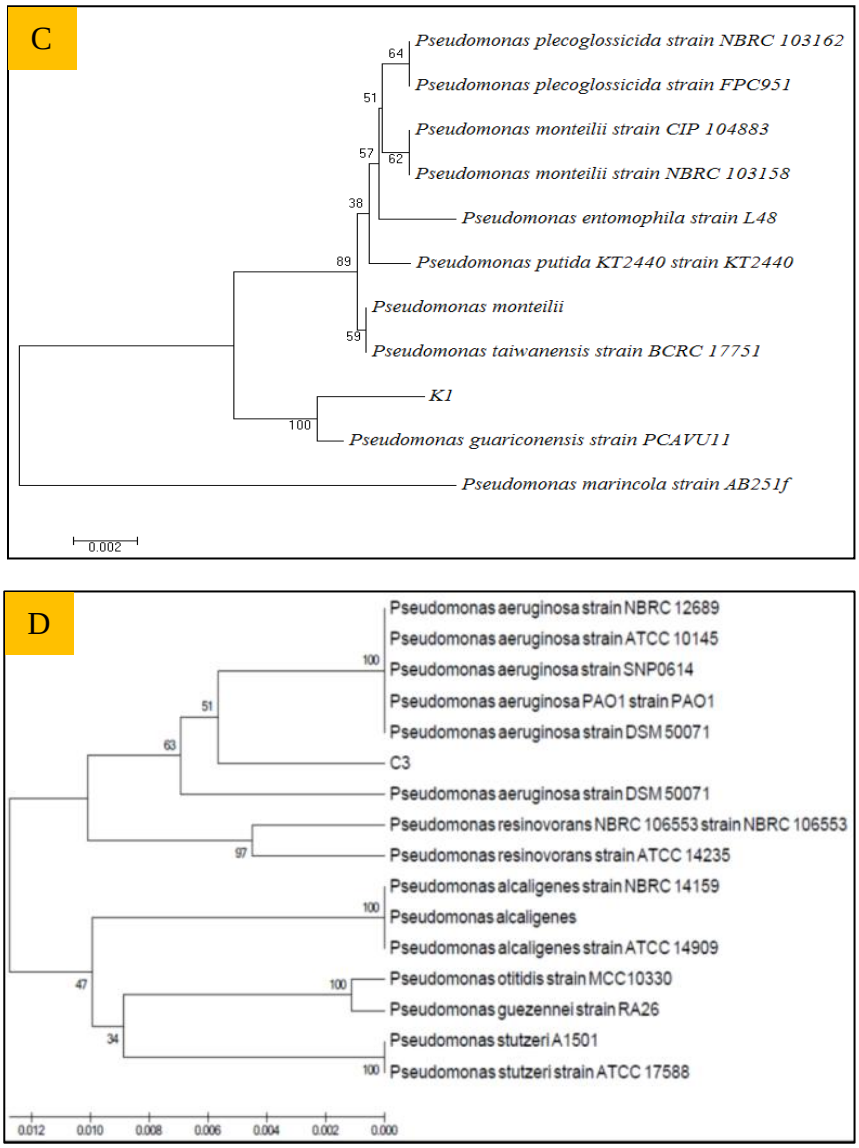


Fig-4.11 A, B, C & D- Phylogenetic tree of isolates A-G_1 (*Pseudomonas* sp), B-G_2 (*Pseudomonas putida*), C-K_1 (*Pseudomonas guariconensis),* D-C_3 (*Pseudomonas aeruginosa)*

Partial sequence of all the bacteria G_1 (*Pseudomonas* sp), G_2 (*Pseudomonas putida*), K_1 (*Pseudomonas guariconensis*) and C_3 (*Pseudomonas aeruginosa*) were submitted to NCBI and accession number KU947109, KX681787, KX681789 and KU947108 was assigned respectively (Table-4.3). In addition to this G+C content value was higher in C_3 isolate (53.6) and minimum in G_1 (52.2) isolate as shown in Table-4.3.

Table-4.3- Accession number and similarity index of isolates G_1, G_2, K_1 and C_3:

Isolates	**Accession no.**	**Base pair (G+C)**	**Similarity**
Pseudomonas sp. (G_1)	KU947109	1245 (52.53)	**96.60**% similar to *Pseudomonas putida* NBRC 14164
Pseudomonas putida (G_2)	KX681787	1227 (52.81)	**99.35**% to *Pseudomonas putida* NBRC 14164
Pseudomonas guariconensis (K_1)	KX681789	1219 (52.99)	**99.59**% similar with *Pseudomonas guariconensis* LMG 27394
Pseudomonas aeruginosa (C_3)	KU947108	1222 (53.51)	**99.18**% similar to *Pseudomonas aeruginosa* JCM 5962

4.7.Evaluation of PGP Traits in Presence and Absence of Cadmium:

PGP (Plant Growth Promoting) characterization of isolates is an important step. In this study, it was observed that all the fluorescent pseudomonads, G_1, G_2, K_1 and C_3 showed multiple plant growth promoting activities such as production of siderophore, HCN, ammonia, ACC deaminase, IAA and solubilisation of zinc and phosphate in presence and absence of cadmium. Some PGP properties were significantly elevated in presence of cadmium than in the absence of cadmium such as production of siderophore and ACC deaminase. In addition to these, NH_3 production was less affected by presence of cadmium and other PGP properties such as production of

IAA, HCN and phosphate solubilisation were reduced in the presence of cadmium except few isolates.

4.7.1. IAA (Indole Acetic Acid) Production Test:

In qualitative analysis, indole acetic acid production was maximum in K_1 isolate than other isolates in absence of cadmium. But in presence of cadmium, maximum production was achieved by isolate G_1 and C_3, while minimum production was found in K_1 isolate (Fig-4-12 A and Table-4.4, 4.5).

On the other hand, in quantitative analysis maximum IAA production of 1.71 mg/ml was observed in case of K_1 isolate and minimum production 0.44 mg/ml was found in G_1 isolate in absence of cadmium. However, at 100 ppm of cadmium G_1 and C_3 isolates produced maximum IAA 0.29 mg/ml and 0.23 mg/ml respectively as shown in Table-4.6 and 4.7**Ammonia (NH_3) Production Test:**

Ammonia production was almost similar in all the isolates, both in presence and absence of cadmium (Fig-4.12B and Table-4.4, 4.5). Higher production was found in case of isolates K_1, C_3 and G_1 in presence and absence of cadmium than G_2.

4.7.2. Hydrogen Cyanide (HCN) Test:

In HCN production test, maximum production was obtained in C_3 isolate in absence of cadmium (Fig-4.12C and Table-4.4, 4.5) while others depicted HCN production at a lower level. But, in presence of cadmium, no production was reported in case of K_1 and G_2 isolates and very less production was found in C_3 isolate, while maximum production was achieved by G_1 isolate.

4.7.3. Siderophore Test:

Siderophore production was affected by cadmium presence and production was confirmed by orange colour zone formation around growth as shown in Fig-4.12 D. In qualitative analysis, three isolates G_1, K_1, C_3 were positive except G_2, but at 100 ppm

of cadmium G_2 showed positive reaction (Table-4.4 and 4.5). However, enhancement of siderophore production in presence of cadmium was non-detectable in qualitative analysis and therefore was confirmed by quantitative analysis.

In quantitative analysis at 0 ppm cadmium, C_3 isolate produced highest amount 36.71 SU of siderophore, but in presence of cadmium highest production 37.39 SU was found in G_1 isolate. On the other hand minimum production 32.50 SU and 15.04 SU was found in case of K_1 isolate both in absence and presence of cadmium respectively, while G_2 showed negative results in both conditions. In case of two isolate C_3 and G_1 siderophore production was increased with cadmium concentration (Table-4.6 and 4.7).

4.7.4. Phosphate Solubilisation (PS) Test:

Phosphate solubilisation was affected by cadmium concentration in all the isolates, wherein solubilisation of phosphate was affected by the presence of cadmium (Table 4.4, 4.5, 4.6 and 4.7). Figure Fig-4.12E depicts phosphate solubilisation by isolates on Pikovskaya and modified Pikovskaya plates. In absence of cadmium, isolate G_1 and C_3 gave better result than other isolates G_2 and K_1. However, at 100 ppm of cadmium phosphate solubilization was reduced in case of all isolates G_1, G_2, K_1 and C_3 (Table-4.4 and 4.5).

In quantitative analysis maximum value 86 µg/ml and 69.66 µg/ml was found in C_3 isolate and minimum value 34.0 µg/ml and 23.0 µg/ml was found in G_1 isolate at 0 ppm and 100 ppm cadmium respectively (Table-4.6 and 4.7).

4.7.5. ACC Utilization Test:

Development of purple colour in tubes indicated ACC deaminase production as shown in Fig-4.12F. As per qualitative analysis, same amount was produced by all the isolates except G_2 in absence of cadmium, it showed maximum production than other

isolates. Cadmium affects the ACC deaminase production and in presence of cadmium ACC utilization was enhanced in all isolates K_1, G_2 and C_3 except G_1. In presence of cadmium, K1 showed maximum production, while no production was observed by G_1 isolate (Table-4.4 and 4.5).

In quantitative analysis of ACC utilization test maximum OD was found in case of G2 isolate 0.131 and K isolate 0.568 at absence and presence of cadmium respectively. But lowest value 0.075 was reported in case of C_3 and G1 isolate in absence of cadmium and in presence of cadmium G1 showed negative result (Table-4.6 and 4.7).

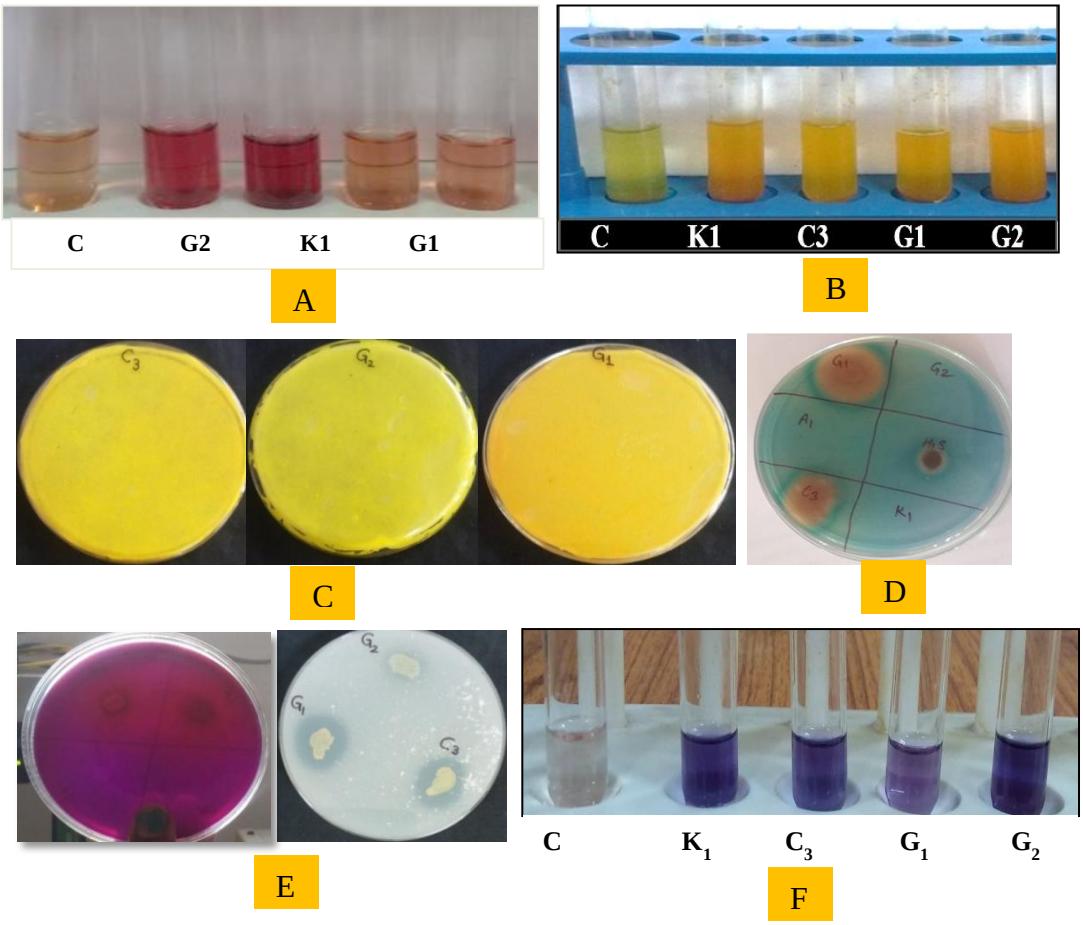


Fig-4.12 (A, B, C, D, E, F)- PGP tests A- Indole Acetic acid, B- Ammonia production, C- HCN production, D- Siderophore test, E- Phosphate solubilisation, F- ACC deaminse test

4.7.6. EPS (Exo-polysaccharide) Production Analysis:

EPS production was reduced in the presence of cadmium in case of all the four isolates G_1, K_1, G_2 and C_3. EPS production was maximum in case of G_1 isolate (195.33 ug/ml) and minimum value 152.66 ug/ml was produced by K_1 isolate in absence of cadmium. In presence of cadmium C_3 isolate produced maximum EPS 170.33 ug/ml and minimum value 150.33 ug/ml was produced by G_2 isolate (Table-4.6 and 4.7.).

Table-4.4- Results of PGP (plant growth promoting) test at 0 ppm:

Isolates	PS	IAA	NH_3	S	HCN	ZS	ACC D
G_1	++	++	+++	+++	+	++	+
G_2	+	++	++	-	+	-	++
K_1	+	+++	+++	++	+	-	+
C_3	++	++	+++	+++	++	++	+

Table-4.5- Results of PGP (plant growth promoting) test at 100 ppm:

Isolates	PS	IAA	NH_3	S	HCN	ZS	ACC D
G_1	+	++	+++	++	+++	+	-
G_2	+	+	++	+	-	-	++
K_1	+	+	+++	++	-	-	++
C_3	+	++	+++	++	+	+	++

Table-4.6- Results of quantitative PGP test at 0 ppm:

Isolates	IAA mg/ml	(SU)	PS µg/ml	EPS µg/ml	ACC OD
G_1	0.46 ± 0.04^{a}	33.47±0.41c	34.00 ± 4.00^{a}	195.33 ± 5.50^{c}	0.075 ± 0.004^{a}
G_2	0.44 ± 0.03^{a}	0.00 ± 0.00^{a}	67.66 ± 2.51^{c}	171 ± 3.60^{b}	0.131 ± 0.008^{b}
K_1	1.71 ± 0.26^{b}	32.50 ± 0.44^{b}	47 ± 3.60^{b}	152.66 ± 2.51^{a}	0.078 ± 0.006^{a}
C_3	0.55 ± 0.04^{a}	36.71 ± 0.31^{d}	86 ± 3.60^{d}	190.33 ± 5.50^{c}	0.075 ± 0.003^{a}

Table-4.7- Results of quantitative PGP test at 100 ppm:

Isolates	IAA mg/ml	(SU)	PS µg/ml	EPS µg/ml	ACC OD
G_1	0.29±0.03^{b}	37.39±3.24^{c}	23.00±2.00^{a}	180±3^{d}	0.165±0.037^{a}
G_2	0.015±0.004^{a}	0.00±0.00^{a}	37.00±2.00^{c}	150.33±5.50^{b}	0.376±0.037^{b}
K_1	0.015±0.002^{a}	15.04±0.66^{b}	30.33±1.52^{b}	130.66±4.04^{a}	0.568±0.037^{d}
C_3	0.23±0.026^{b}	41.46±0.84^{d}	69.66±6.11^{d}	170.33±3.51^{c}	0.408±0.011^{bc}

Note- In above table values are mean of three independent experiment and mean±SD (standard deviation) followed by same letter within a row are not significantly different (P≤0.5) according of DMRT (Duncan's multiple range test).

4.8.Production and Characterization of Pigment:

Colour and fluorescent properties of bacteria changed from media to media and time to time. Bacteria G_1 and C_3 formed green, fluorescent pigment and isolate K_1 and G_2 produced yellowish fluorescent pigment in broth. Pigment production of isolates G_1, K_1, G_2, and C_3 was enhanced by presence of cadmium. Development of reddish brown pigment was also reported in case of C_3 and G_1 isolates (Fig-4.13). Pigment of C_3 and G_1 was extracted and characterized by HPLC (Fig-4.14). After this analysis, it was revealed that the extracted product was pyoverdine. Pyoverdine production was confirmed because similar dominant peak was observed at 14.52 min and 24.75 min in case of C_3 and in case of G_1 at 14.47, 21.63, 24.60 and 26.27 min. They showed similar peak with standard pyoverdine peak (Table-4.8).

Table-4.8- Similar peak of extracted pigment of C_3 and G_1 with standard

Standard	G_1	C_3
14.49	14.47	14.52
21.16	21.63	
24.72	24.60	24.75
27.70	26.27	

Fig-4.13- Fluorescent green pigment by G_1 and C_3 isolate

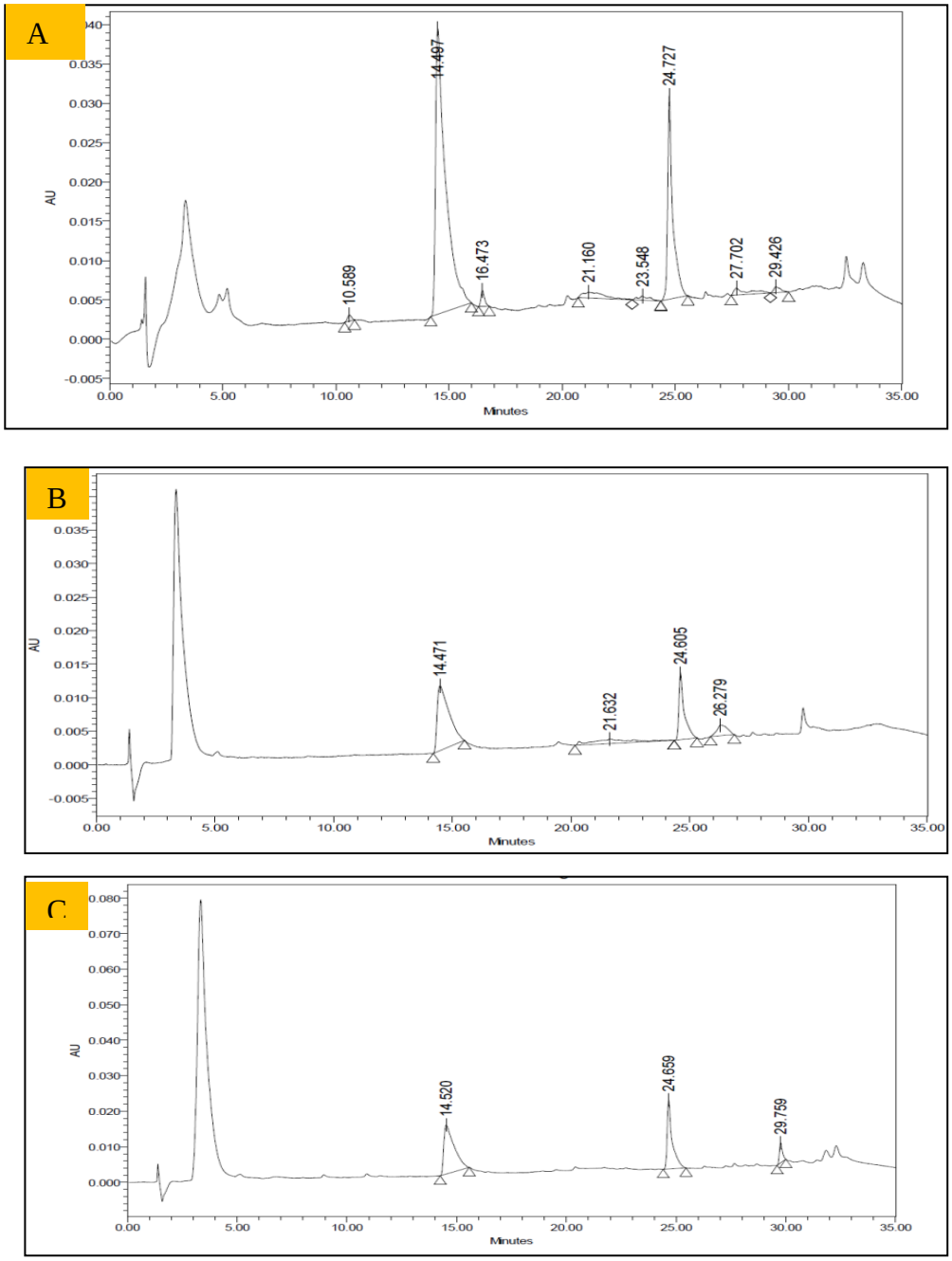


Fig-4.14 (A, B, C)- HPLC peak of standard pyoverdine, and extracted pigment of C_3 and G_1 (where Fig. A-Standard; Fig B-C_3 and Fig. C-G_1)

4.9.Effects of Cadmium on Morphology and Growth Pattern of Bacteria:

Presence of cadmium alters the morphology of bacteria. Surface of bacteria was affected by cadmium as shown in Fig-4.15. In presence of cadmium, surface of bacteria became rough due to absorption of cadmium.

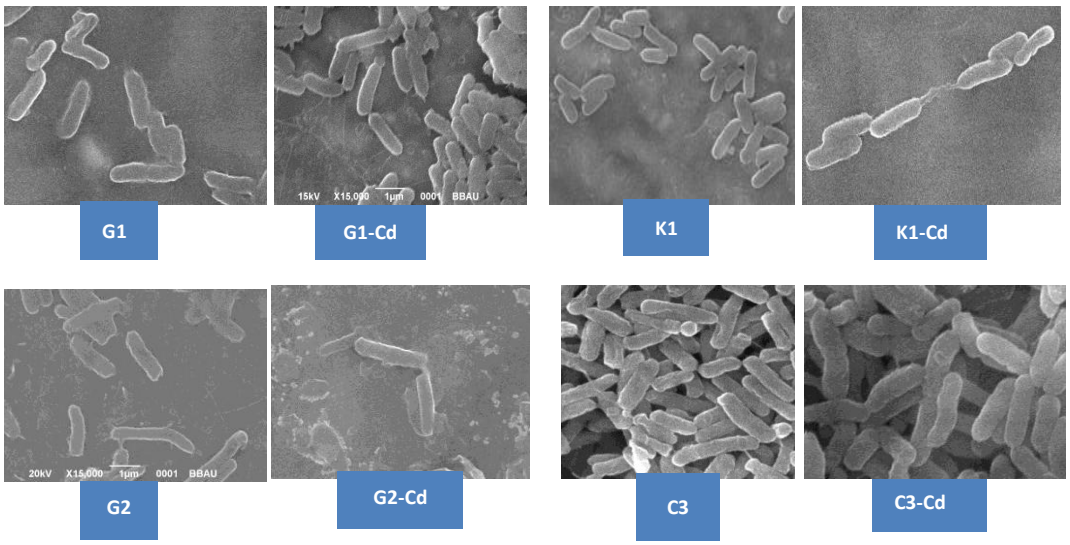


Fig-4.15- SEM pictures of isolate G_1, G_2, K_1 and C_3 in presence and absence of cadmium

Growth of bacteria was also affected by cadmium and different incubation days. In growth pattern analysis we found that all the strains showed different growth patterns in both presence and absence of cadmium (Fig-4.16). In G_1 and G_2 isolates, pattern of growth was affected by cadmium slightly and was slightly reduced. In case of other isolate named as K_1, cadmium affects the growth and growth pattern as well. Cadmium reduced the growth of isolate K_1 at 30 hrs incubation, but after 30 hrs it increased and was less affected by cadmium indicating towards the development of resistance during this period.

On the other hand, growth pattern of C_3 was different from all the strains. In this case, growth was maximum at 24 hrs and then reduced upto 106 hrs incubation in absence of cadmium. But in presence of cadmium it becomes changed and growth of bacteria suddenly enhance at 96 hrs incubation. Growth pattern of this isolate in presence and absence of cadmium showed that it was very less affected by cadmium.

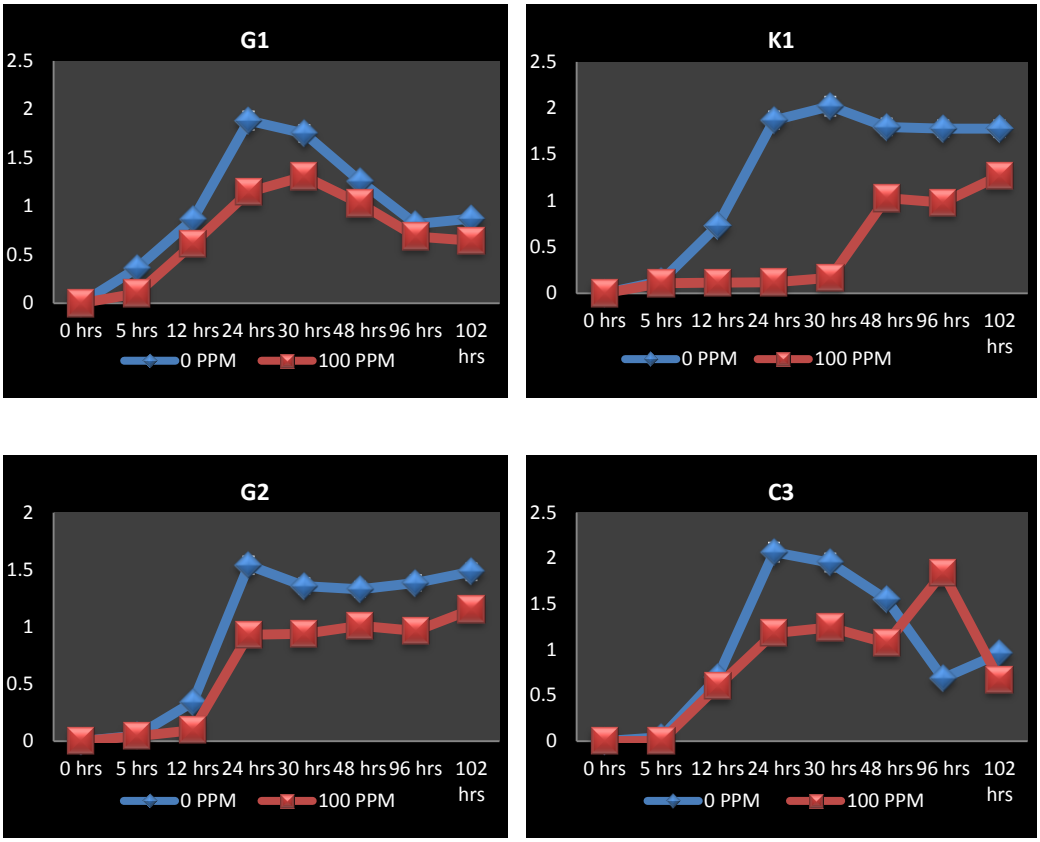


Fig-4.16- Growth pattern of isolate G_1, K_1, G_2 and C_3 in presence and absence of cadmium

4.10. Accumulation Analysis of Cadmium in Isolates:

In cadmium accumulation analysis it was found that the strain G_1 has good capacity to accumulate cadmium than C_3 strain. Isolate C_3 accumulated 40.825 mg/l cadmium, while other isolate G_1 accumulated 190.9 mg/l of cadmium out of 500 mg/l. The supernatant of C_3 contained 84.5 mg/l and supernatant of G_1 contained 106.2 mg/l cadmium (Table-4.9).

Table-4.9- Cadmium accumulation by isolate G_1 and C_3:

Isolate	Cd **in pellet** (mg/l)	Cd **in broth** (mg/l)
G_1	190.9 mg/ml	106.2
C_3	40.82 mg/ml	84.5

4.11. Characterization of Cadmium Resistant Gene:

For czc analysis, genomic DNA of all the isolates G_1, G_2, K_1 and C_3 was amplified with cadmium resistant gene primer and found that only C_3 isolate had czc gene (Fig-4.17). Cadmium resistant gene was not found in other three isolates. This gene is responsible for the cadmium resistant properties of bacteria. This finding showed that there is a chance that they may have new cadmium resistant gene. For this analysis primer designing is required because no primers are available in market and can be a subject of further research.

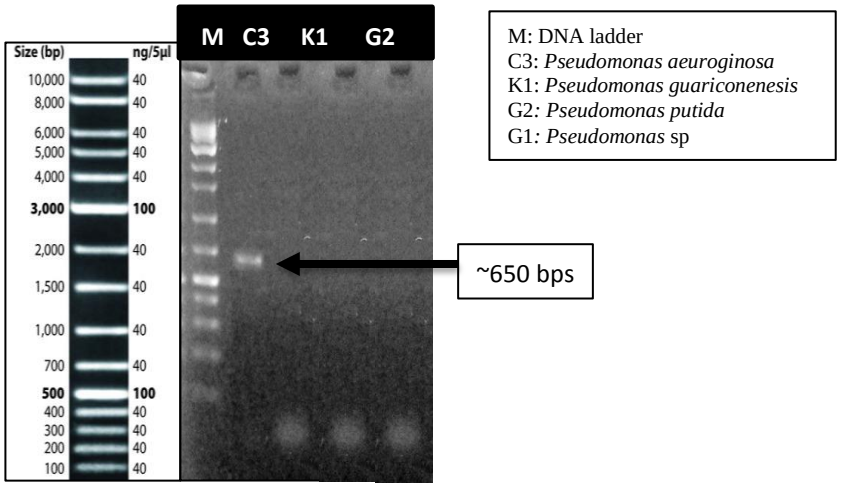


Fig.4.17- Electrophoresis band showing the czc gene

4.12. Characterization of Metabolites:

Since metabolites of bacteria such as IAA, EPS, siderophore etc. have metal binding affinity and plant growth promoting ability. Thus analysis of these responses related to metabolites is very helpful for PGPR assisted phytoremediation. Characterization of metabolites is given in subsequent sections:

4.12.1. IAA Characterization:

Production of IAA was confirmed by HPLC analysis (Fig-4.18 A, 4.18B, 4.18C and 4.18D) because HPLC peaks of produced IAA of all the isolates were similar with standard peaks. Similarity of peaks is shown in Table-4.10.

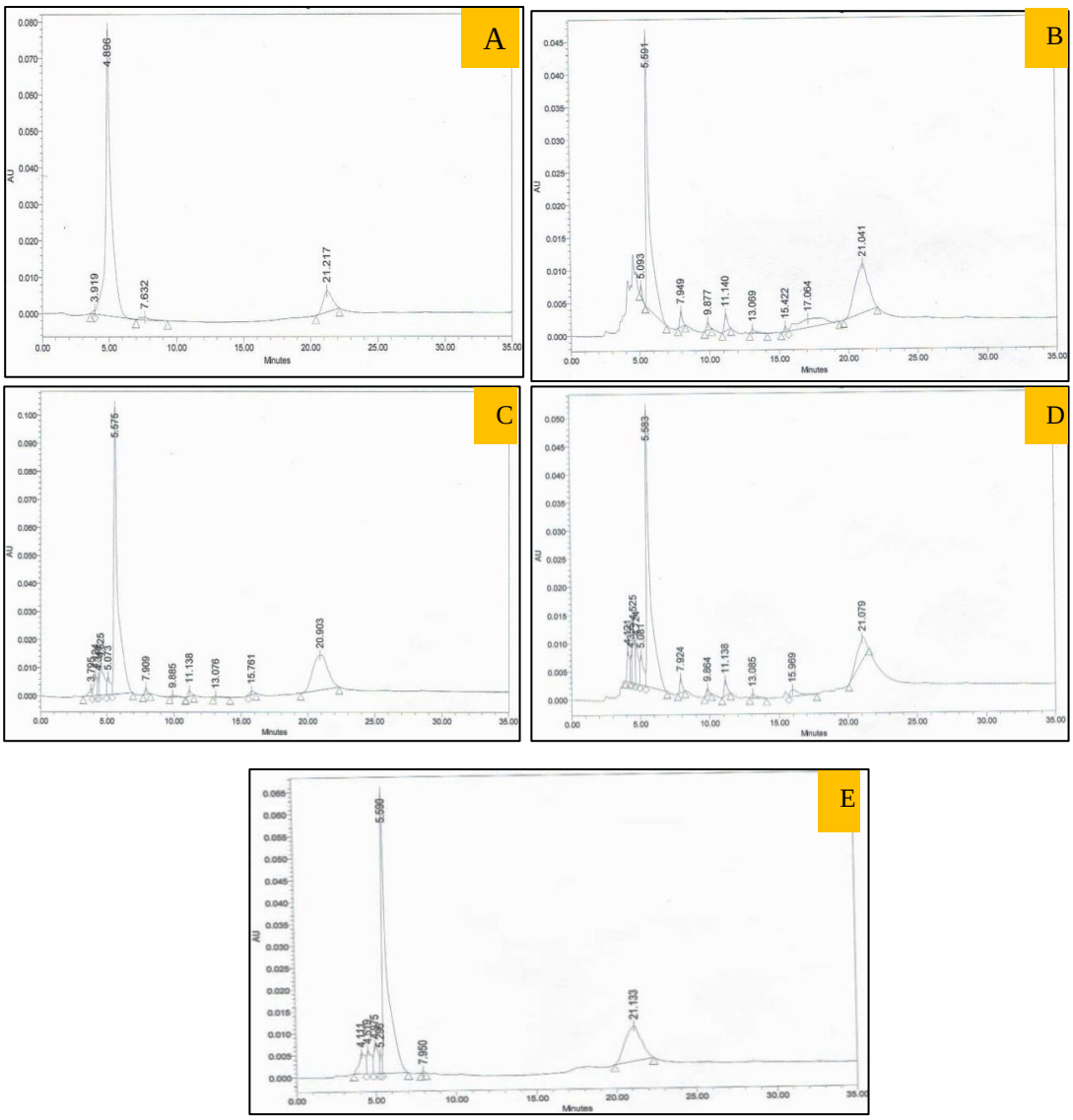


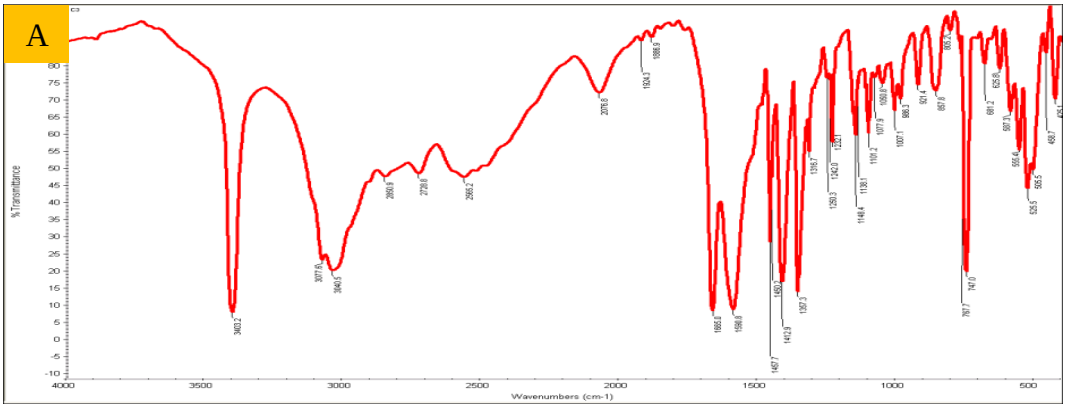
Fig-4.18 (A, B, C, D)-HPLC peak of A-standard IAA and IAA produced by B-C_3, C-G_1, D-K_1, E-G_2

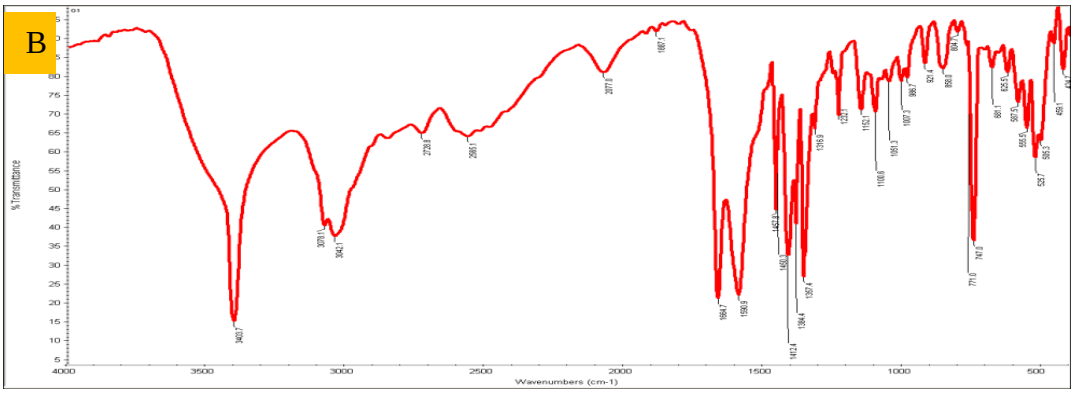
Table-4.10- Similar peak obtained in IAA characterization by HPLC:

STD	**G_1**	**G_2**	**K_1**	**C_3**
4.95	4.12, 4.34, 4.25	4.97, 4.11, 4.51	4.13, 4.52	
5.56	5.07, 5.57	5.29, 5.59	5.05, 5.58	5.90, 5.59
8.11	7.90	7.95	7.906	7.94
10.59	11.13		11.14	11.14
21.11	20.90	21.13	21.15	21.04

4.12.2. EPS Characterization by FTIR:

FTIR analysis was used to identify the functional groups found in the exoploysaccharide produced by *Pseudomonas sp* (G_1), *Pseudomonas aeruginosa* (C_3), *Pseudomonas putida* (G_2), and *Pseudomonas guariconenesis* (K_1). The FTIR analysis of EPS of all the isolates showed similar absorption peaks at 3403 (O-H stretching and hydrogen bonding), 3041-3078 (Aliphatic CH stretching), 1664-1665 (C=O asymmetric stretching of –NH-CO-R and/or N-H bonding of H_2N-CO-R), 1590-1592 (N-H bonding of –NH-(Amide II) and/or C =C stretching of aromatic ring), 1353-1357 (C=O symmetric stretching of carboxylate and/or C-OH stretching of phenolic OH), 1232 (P=O stretching of phosphate PO_4^{3-} and/or C-O stretching of –O-COR), 1050 (C-O-C group vibrations in the cyclic structures of carbohydrates), 804-805 (C-O-S stretching of –O-SO_4). Presence of these peaks showed the presence of hydrogen bond compound, acid, amides, alkynes or amine salt (Fig.4.19, (A, B, C, D) and Table-4.11).





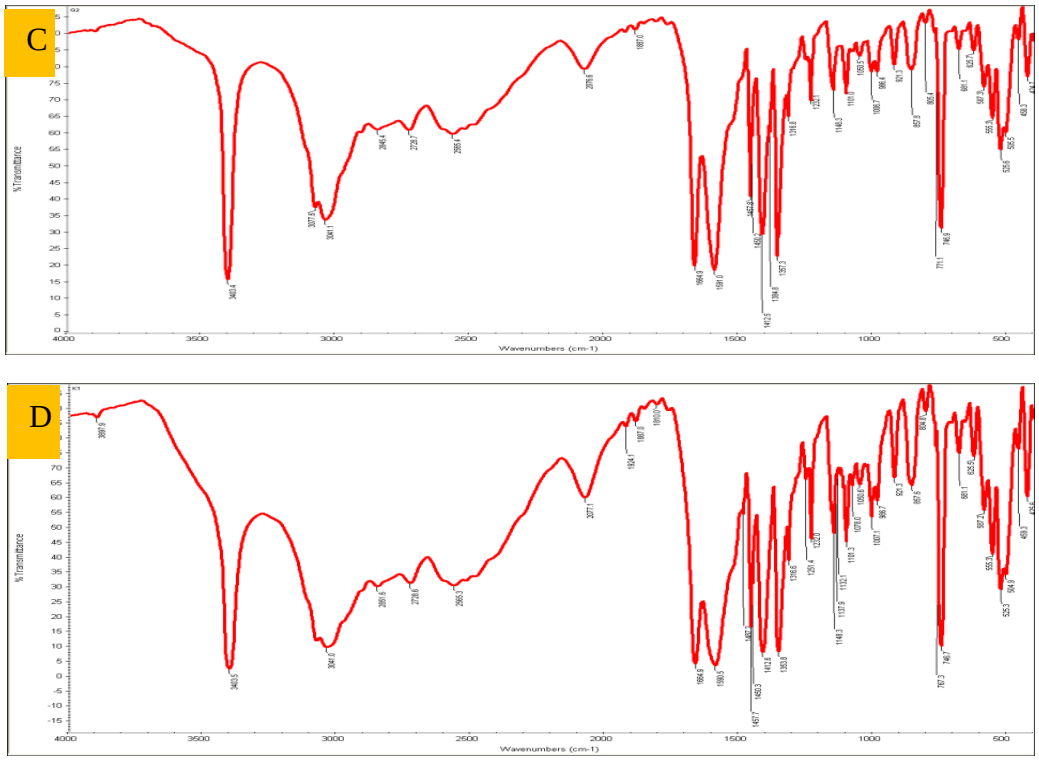


Fig-4.19 (A, B, C, D)-FTIR peak of extracted EPS of A-C_3, B-G_1, C-G_2 and D-K_1

Table-4.11-FTIR peak of extracted EPS produced by strains G_1, G_2, K_1 and C_3:

Wave numbers	**Functional Group**
3403	O-H stretching and hydrogen bonding), 3041-3078 (Aliphatic CH stretching
1664-1665	C = O asymmetric stretching of –NH-CO-R and/or N-H bonding of H_2N-CO-R
1590-1592	N-H bonding of –NH-(Amide II) and/or C =C stretching of aromatic ring
1353-1357	C = O symmetric stretching of carboxylate and/or C-OH stretching of phenolic OH
1232	P = O stretching of phosphate PO_4 3- and/or C-O stretching of –O-COR
1050	C-O-C group vibrations in the cyclic structures of carbohydrates
804-805	C-O-S stretching of –O-SO_4

4.12.3. Characterization of Siderophore:

Extracted siderophore crystal (Fig.4.20) were analysed through FTIR analysis with KBr pellets between the range of 2.5 to 14 (4000-400cm) as shown in Fig.4.21. Results of FTIR showed that obtained crystals had hydroxamate functional group

which correlated with the peaks obtained from FTIR analysis of PBHA crystals. Peaks were observed at 3211.2, 1677.8, and 723.7 which are same as FTIR analysis of PBHA crystal. But, along with these peaks, many other peaks were also observed at 168.6, 1433.5, 1087.4 and 979.7 wave number and revealed the presence of many functional groups as given in Table-4.12.

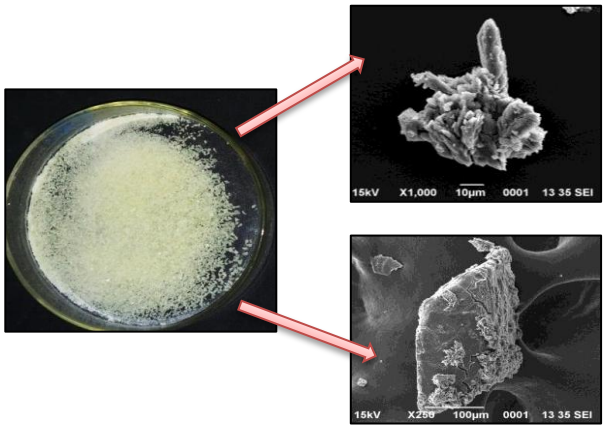


Fig.4.20- Siderophore crystal and their SEM pictures

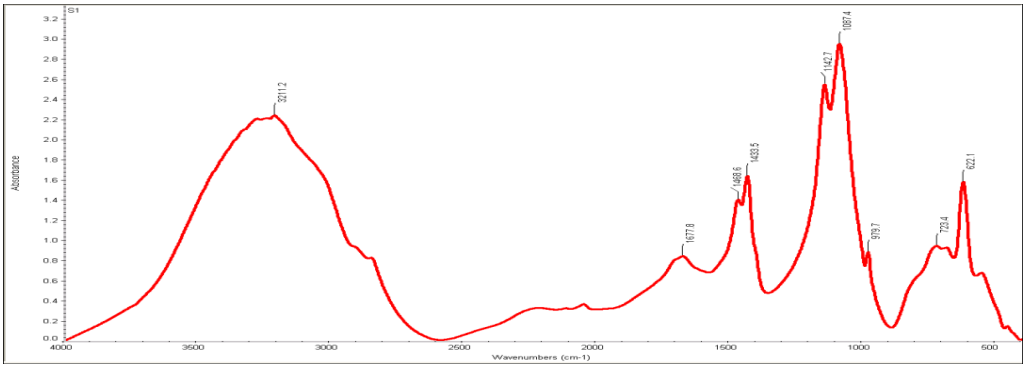


Fig.4.21- FTIR peak of extracted siderophore

Table-4.12- FTIR peak of siderophore:

Peak	**Functional Group**
3211.2	Ar-OH
1677.8	O-H
1468.6	>C=O Stretching
1433.5	C-H, C=O Bending
1142.7	Ar-O-stretching of phenolic OH atom
10874	C=N stretching
979.7	M substitute partial
723.7	C-H bonding in aromatic ring

4.13. Compatibility Test:

This test was done to make the consortium of compatible strains. In compatibility test, it was found that all the isolates, G_1, K_1, G_2, and C_3 were compatible with each other because they grew simultaneously (Fig-4.22).

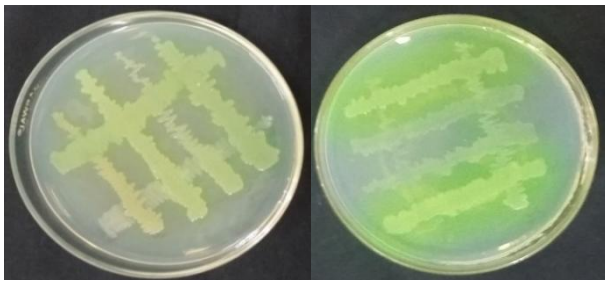


Fig-4.22- Compatibility of isolates G_1, K_1, G_2 and C_3

4.14. Root Elongation Assay:

It was conducted in plates with individual strains as well as consortium, prior to pot experiment; results of the study are summarized:

Identified strain G_1, G_2, K_1 and C_3 and their consortia affected root elongation of *B. juncea* and *Zea mays* in the presence and absence of Cd. In addition to this, 100 ppm Cd to the filter paper affected root elongation of un-inoculated seedlings. As compared to control (without culture) in presence of cadmium, treatments having bacterial cultures stimulated root elongation and it was more pronounced. In the root elongation analysis, it was found that all the strains G_1, G_2, K_1 and C_3 and their consortia (G_1, K_1, G_2-$Cons_1$; G_2, K_1, C_3-$Cons_2$; G_1, K_1, C_3-$Cons_3$) in presence and absence of cadmium worked as very good bio inoculant and enhance the root length of both the plants mustard and maize.

In case of mustard, this test confirmed that maximum root length 14.33 cm and 14.26 cm was obtained in treatment with Consortium (C_3) and culture C3 respectively at 0 ppm cadmium. However, slightly similar result was found in presence of 100 ppm of cadmium and the maximum value is 13.66 cm was found in mustard plant after treatment of C_3 isolate. On the other hand minimum value 12.26 cm was observed in

C_3 and 6.55 cm value was observed in treatment G_2 at 0 ppm cadmium and 100 ppm cadmium respectively (Fig-4.23).

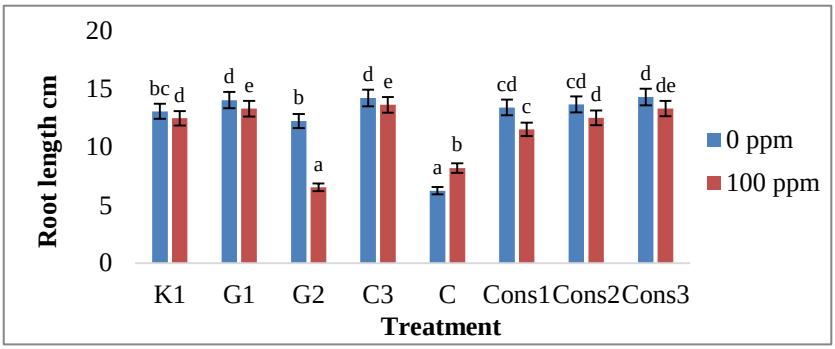


Fig-4.23- Root length of mustard in presence and absence of cadmium

In the case of second plant maize, the minimum root length 26.04 cm and 5.32 cm was found in treatment of G_2 at 0 ppm and 100 ppm cadmium respectively. While, maximum value 21.33 cm was found after treatment of $Cons_3$ isolate in absence of cadmium and in presence of cadmium consortia also gave best result and enhanced the root length upto 21.62 cm after Consortium ($Cons_3$) treatment (Fig-4.24).

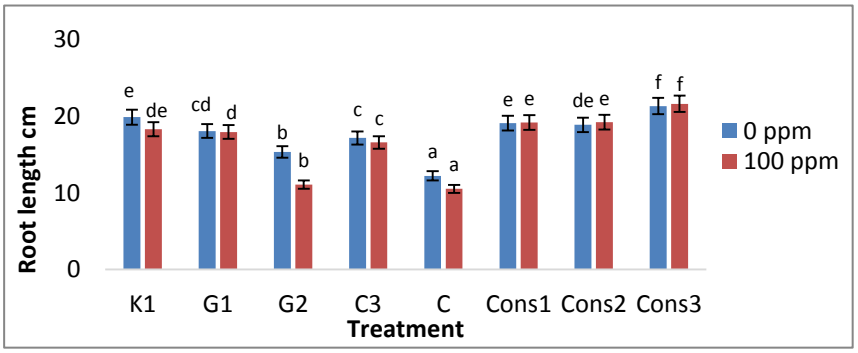


Fig-4.24- Root length of maize in presence and absence of cadmium

Findings of root elongation assay showed that consortia gave better results than single isolates. Elongation of root of both plants was very slightly affected by cadmium and no significant differences were observed.

4.15. Pot Experiment:

In pot study, plant growth potential of inoculants and their consortia G_1, G_2, K_1, C_3, $Cons_1$, $Cons_2$ and $Cons_3$ was examined and found that they enhanced the growth in

presence and absence of cadmium and cadmium tolerance of mustard and maize plant. After harvesting, cadmium uptake in root, shoot and rhizospheric soil was also analysed and found that cadmium accumulation is centred in and around root of both mustard and maize plants. In edible part of plants, very less concentration of cadmium was present. For food security purpose this technique is very useful than other biological remediation technique because they enhanced the quality of phytoremediation.

4.15.1. PGP (Plant Growth Promotion) analysis:

In present study, effects of *Pseudomonas* of four different species K_1, G_1, G_2 and C_3 and their consortia on growth of *Brassica juncea* (Fig-4.25 and Fig-4.26) and *Zea mays* (Fig.4.27) was analysed under stressed and normal conditions.

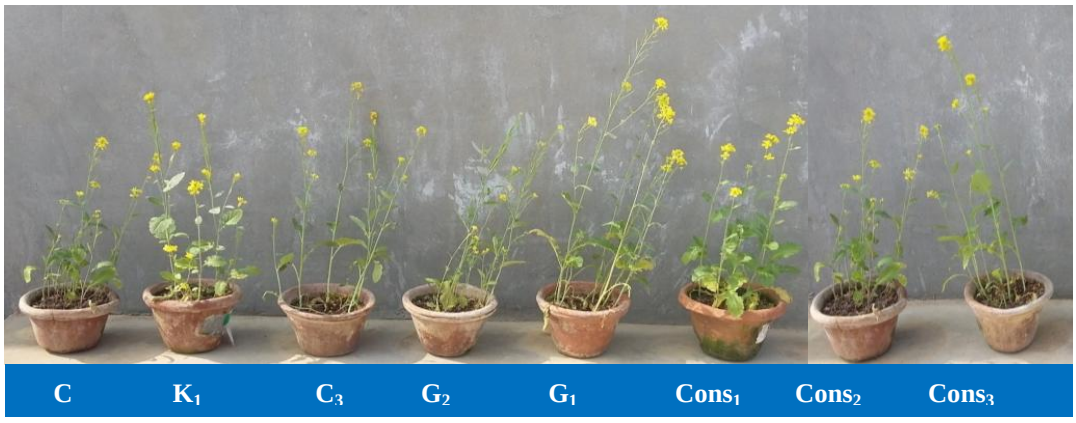


Fig-4.25- Pots of mustard plant treated with single strain K_1, C_3, G_2, $G_1$and their consortia Cons1, $Cons_2$ and $Cons_3$ in absence of cadmium

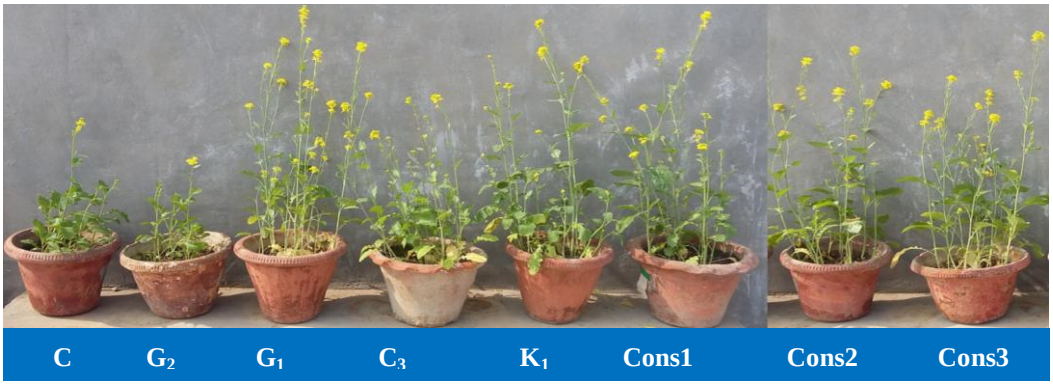


Fig-4.26- Pots of mustard plant treated with single strain G_2, G_1, C_3, K_1 their consortia $Cons_1$, $Cons_2$ and $Cons_3$ in presence of cadmium

Fig-4.27- Harvested maize plant treated with single strain G_1, K_1, G_2 and C_3 and their consortia $Cons_1$, $Cons_2$ and $Cons_3$ in presence of cadmium

4.15.1.1.Shoot Length Analysis in presence and absence of cadmium in mustard and maize plant

In case of mustard, the shoot length was highest in presence of $Cons_1$ in both conditions like presence and absence of cadmium. But, in case of maize, finding was different (Fig-4.28 A and 4.28 B). This treatment (with $Cons_1$), enhanced the shoot length of mustard plant by 50.57% and 48.78% in absence and presence of cadmium respectively over the control treatment (Fig. 4.33A). In case of maize, maximum shoot length was found in case of $Cons_1$ and G_1 in absence and presence of cadmium, respectively. In this finding, bacterial isolate G_1 enhanced shoot length by 27.85% and cons1 by 21.52% over control (Fig. 4.28 B).

In mustard, minimum shoot length 56.33 cm in absence of cadmium and 56.00 cm in presence of cadmium was observed in treatment G_2. However, in case of maize, minimum value 133.66 cm and 136.00 cm were reported in presence and absence of cadmium respectively in treatment C_3. Findings of this parameter showed that treatment $Cons_1$ and G_1 was more effective than other inoculant and other treatments did not show significant results.

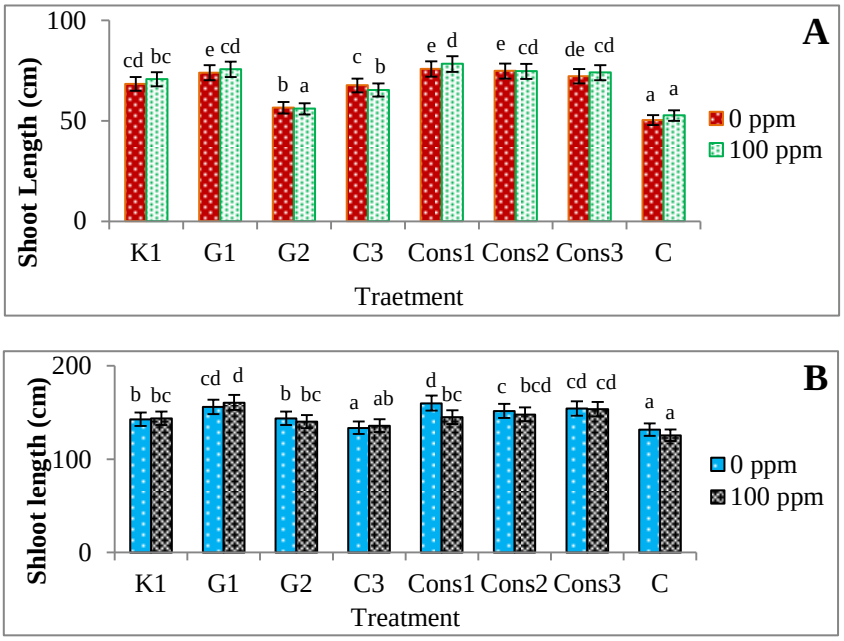


Fig-4.28 (A & B) - Shoot length of A- mustard and B-maize at 0 ppm and 100 ppm

4.15.1.2.Root Length Analysis in presence and absence of cadmium in mustard and maize plant:

Root length was also affected by different treatments in presence and absence of cadmium. In mustard plant, root length was maximum in treatment with Consortium ($Cons_3$) and ($Cons_2$) in absence and presence of cadmium respectively. In addition to this, minimum value for root length was found at same treatment C_3 for presence and absence of cadmium (Fig-4.29 A and 4.29 B). In the maize plants, the case was slightly different and maximum length was at treatment with Consortium ($Cons_1$) and G_1 in absence of cadmium and in presence of cadmium, respectively. Minimum root length was found in treatment C_3 for both conditions i.e. absence and presence of cadmium at 100 ppm level.

In mustard plant, root length was enhanced upto 106.44% by $Cons_1$ treatment and 83.97% by $Cons_3$ treatment in presence and absence of cadmium respectively. Minimum enhancement 17.04% at 0 ppm and 16.07% at 100 ppm level of cadmium was observed in treatment C_3 in case of mustard plant.

In case of maize plants, bacterial culture at 0 ppm level of cadmium enhanced the root length over the control treatment except the treatment C_3 (Fig.4.29B).

However, at 100 ppm of cadmium, root elongation was observed in all the cases of treatment over the control with G_1 giving maximum enhancement in root length (Fig. 4.29B). The percentage increase in case of treatments at 100 ppm cadmium level ranged from 4.91% for C_3 to 50.02% for G_1 over the control.

On the basis of these findings it is clear that for root elongation purpose, G_1 inoculant is best in both normal and metal stressed condition in maize. However, in case of mustard, consortium ($Cons_1$) gave significant and applicable results for root length enhancement.

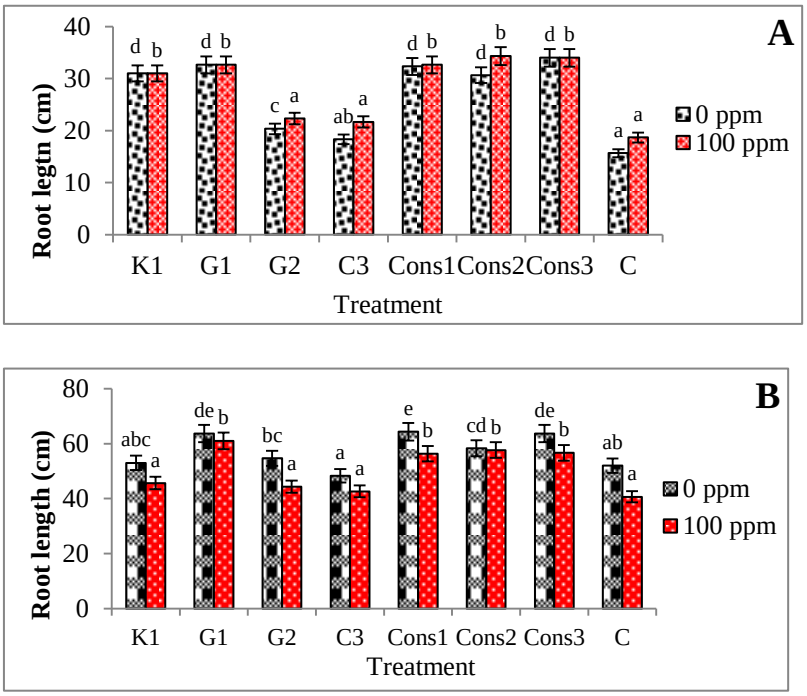


Fig-4.29 (A&B) - Root length of A- mustard and B-maize at 0 ppm and 100 ppm

4.15.1.3.Dry weight analysis of shoot in presence and absence of cadmium in mustard and maize plant:

Dry weight of shoot observations were not uniform in all the treatments. In case of mustard plant, in absence and presence of cadmium, dry weight of shoot was less in case of culture G_2 and C_3 as compared to control plants while in other cases there was

a significant enhancement in weight in absence and presence of cadmium (Fig-4.30 A).

In case of maize plant also, both the treatments with culture C_3 and G_2 were inferior to control in dry wright of shoot parameter. At 0ppm level of cadmium, culture C_3 was less as compared to control while in presence of 100 ppm cadmium, culture G_2 was less than control. Rest of the cultures and consortium were significantly better in dry weight biomass. The average value ranged from 2.75% for G_2 to 29.16% for Consortium ($Cons_1$) in absence of cadmium while in presence of cadmium, percentage increase in dry shoot weight ranged from 2.87% for C_3 to 35.70% for consortium ($Cons_3$; Fig-4.30B). On the basis of these findings it is clear that treatment G_2 and C_3 are not applicable for dry weight of shoot in case of both plants. However, Consortia and G_1 treatments are very applicable.

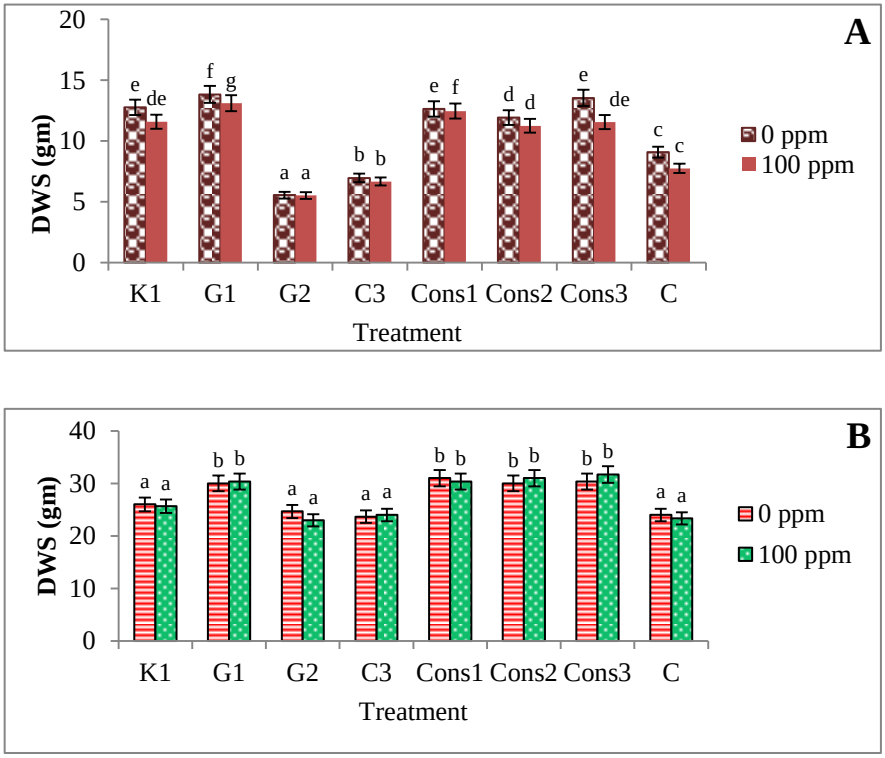


Fig-4.30 (A & B)- Dry weight of shoot of A- mustard and B-maize at 0 ppm and 100 ppm

4.15.1.4.Dry weight analysis of root in presence and absence of cadmium in mustard and maize plant:

In maize, the maximum dry weight of root was found in Consortium ($Cons_3$) treatment and minimum was found in G_2 treatment in both absence and presence of cadmium. While in mustard plant maximum dry weight of root was found in case of $Cons_2$ at 0 ppm of cadmium and minimum value was found at same treatment C_3. In maize, dry weight of root was enhanced upto 10% by $Cons_3$ and reduction of value upto 20 % was in G_2 treatment in absence of cadmium. Similar effects of treatments were observed in presence and absence of cadmium (Fig-4.31A and 4.31B). In presence of cadmium, dry weight of root was enhanced upto 27.63% by Cons3 and reduced upto 13.26%.

In mustard plant, treatment Cons3 enhanced the parameter upto 90.12% in absence of cadmium, but in presence of cadmium other treatment K_1 improves the dry weight of root upto 74.29%. In comparison to maize reduction of dry weight of root was not observed in mustard plant.

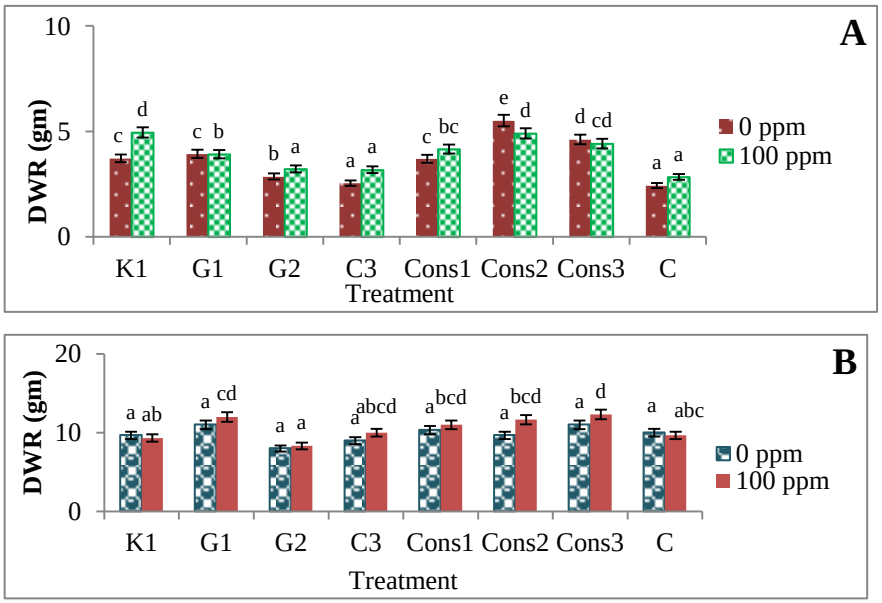
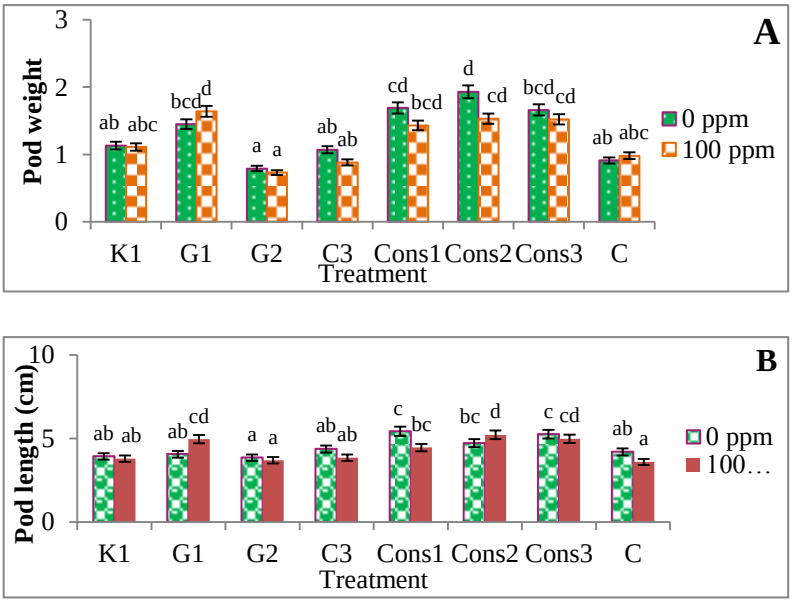


Fig-4.31 (A&B) - Dry weight of root of A- mustard and B-maize at 0 ppm and 100 ppm

This finding cleared that the consortia of the selected strain give the better result than single strain, but the isolate G_1 also give best result when used as single for both plants and in presence and absence of cadmium as well.

4.15.2. Effects of Cadmium on Productivity of Mustard Plants:

Productivity of mustard plant was analysed in terms of pod weight, pod length and no. of seeds in pod. Results of this analysis are shown in Fig-4.32 A, 4.32B and 4.32C. Weight of pod was maximum in treatment $Cons_2$ 112.80% in absence of cadmium. In presence of cadmium, G_1 showed maximum value enhancement of 67.34%. Reduction of value upto 13.18% was observed in treatment G_2 in absence of cadmium and in presence of cadmium 10.20% reduction in pod weight was found. In case of pod length, maximum value 5.43 cm was found in treatment $Cons_1$ and 5.22 cm in $Cons_2$ in absence and presence of cadmium respectively. Number of seeds in pod was also affected by cadmium level and different bacterial treatments. Maximum seed number was found in treatment $Cons_1$ (11.39 cm) and $Cons_3$ (16.66 cm) in absence of cadmium and in presence of cadmium respectively. Results of productivity indicated that the consortia treatment is better than individual treatment, because number of seed and weight of pod increased in presence of consortia.



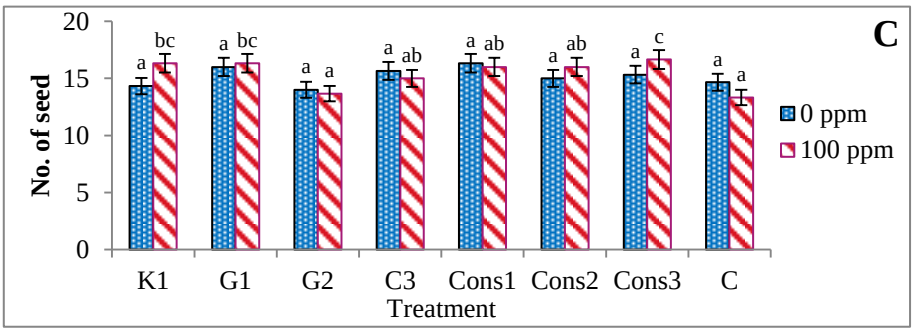


Fig-4.32 (A, B & C)- Effects of cadmium on productivity of mustard A- Pod weight, B- Pod length and C- No. of seed in pod

4.15.3. Stress Tolerance:

Inoculants used in this study protected the plant significantly from the toxic effects of cadmium and results are summarized below:

4.15.3.1.Proline (µ moles/g of fresh weight) production in mustard and maize :

In mustard plant, at 30th day, by analysis of proline it is clear that in presence of $Cons_3$ 195.97% and 202% of proline production was enhanced in absence and in presence of cadmium, respectively than control. However, in few treatments, production of proline was reduced and maximum reduction was reported in treatment C_3 (23.07%) in absence of cadmium and G_1 (20.26%) in presence of cadmium. In case of maize plant these values were different and found that in presence of consortia, production of proline was reduced over control, while in few treatments, this parameter was enhanced (Fig-4.33 & 4.33 B).

In addition to this, proline production was maximum in mustard plant than maize as compared to control in presence and absence of cadmium. In maize plant, proline production differed slightly between all treatments. In maize, maximum reduction of proline was found in treatment C_3 (29.18%) and $Cons_3$ (33.39%) in absence and presence of cadmium, respectively. Proline reduction was not found in treatment G_2 in both absence and presence of cadmium and enhanced 20.01% in absence of cadmium

and 5.25% in presence of cadmium. In maize, proline production was reduced in all the treatments and increased only in G_2 treatment.

On the basis of these findings it is clear that in mustard plant, proline production was maximum than maize under different treatments. In maize plant, proline production was reduced and maximum reduction was found under consortia treatments.

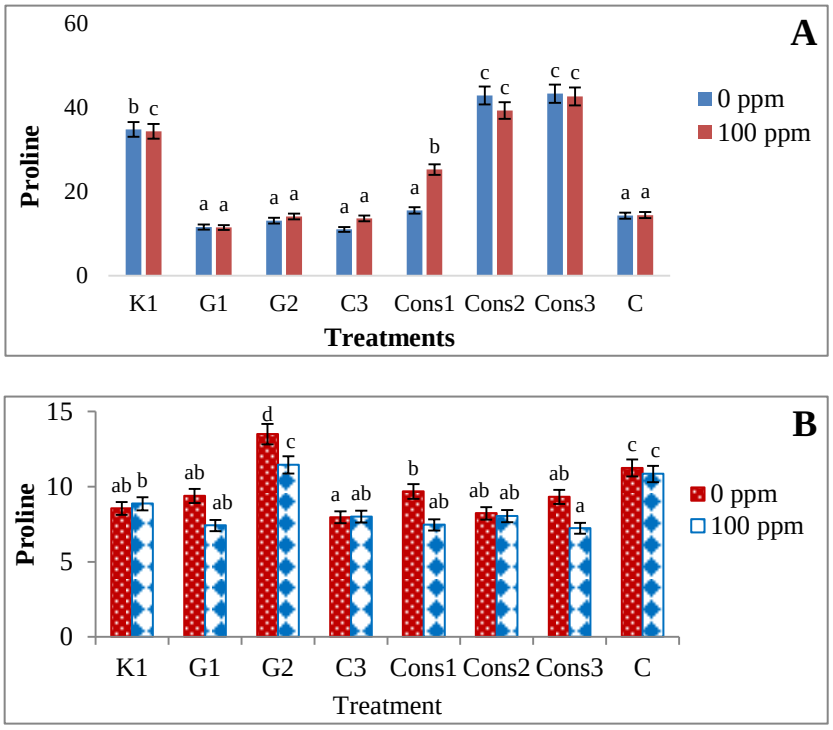


Fig-4.33 (A&B) - Proline production in A-Mustard and B- Maize

4.15.3.2. Chlorophyll production analysis in mustard and maize:

Chlorophyll content was also affected by presence of cadmium. In case of mustard, maximum chlorophyll was in treatment G_1 in absence and presence of cadmium. While minimum production was in treatment G_2. This finding show that, G_1 isolate gives good result for enhancement of chlorophyll content than other treatments. However, in maize plant, chlorophyll content was very slightly affected by treatment of PGPR both in presence and absence of cadmium. In maize, maximum value of chlorophyll was found at treatment $Cons_3$ and K_1 in absence of cadmium and presence

of cadmium respectively while minimum value was found at treatment C_3 in both presence and absence of cadmium (Fig-4.34 A& 4.34 B).

Findings of chlorophyll analysis showed that chlorophyll product4on was enhanced in mustard as compared to maize in both presence and absence of cadmium. Average value of chlorophyll content was higher in mustard than maize plant. Chlorophyll of mustard plant enhanced upto 85.89% and 141.78% in absence and presence of cadmium respectively. Minimum value 18.58% and 4.79% was reported in G_2 treatment at 0 ppm and 100 ppm respectively.

In maize plant, with different treatments, mixed findings were there. In some cases, chlorophyll content enhanced while in some, it reduced. In both presence and absence of cadmium the chlorophyll content was different with different treatments (Fig-4.34 B) On the basis of these findings it is clear that chlorophyll content is not significantly affected by treatments in presence as well as absence of cadmium.

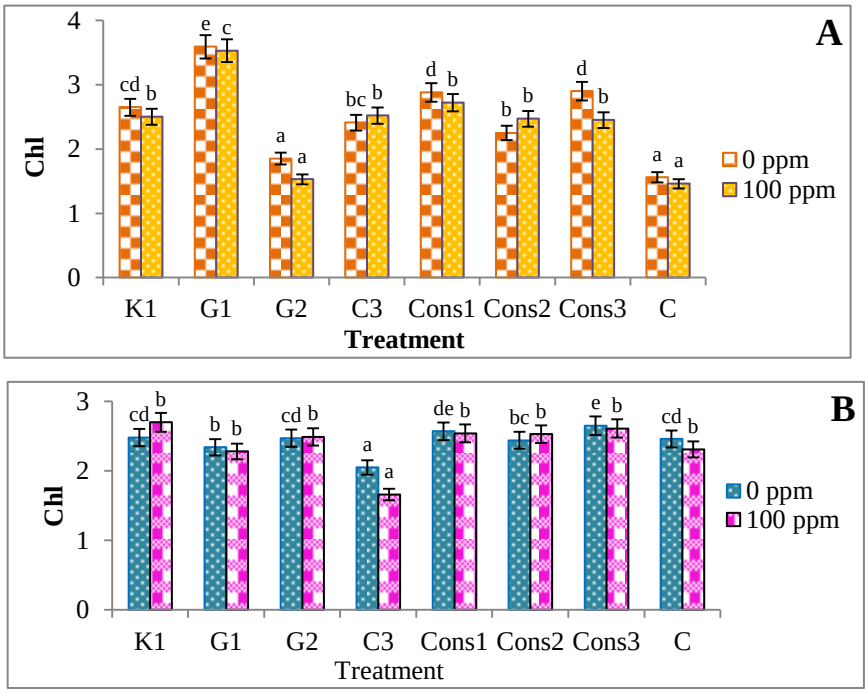


Fig-4.34 (A & B) - Chlorophyll production in A-Mustard and B- Maize

4.15.3.3. Production of Total Soluble Sugar:

In mustard plant, total soluble sugar increased approximately by 32.21% and 15.40% in absence and presence of cadmium, respectively in K_1 treatment and minimum value was found in treatment with consortium ($Cons_1$ and $Cons_2$) in absence and presence of cadmium, respectively (Fig-4.35).

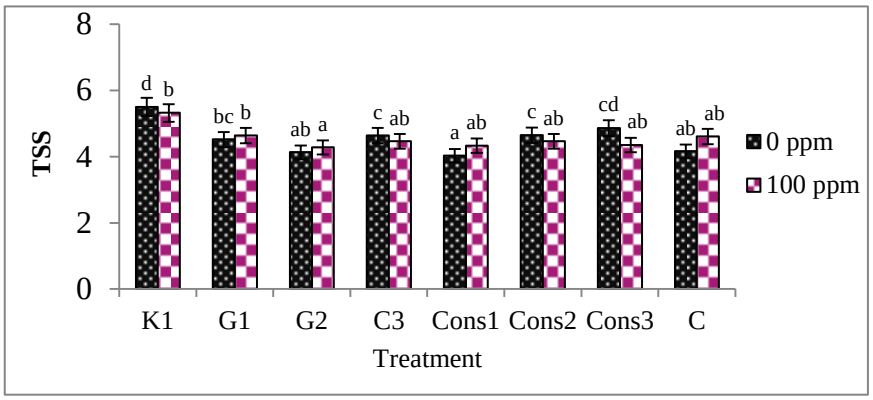


Fig-4.35- Total soluble sugar production by mustard at 0 ppm and 100 ppm

4.15.4. Cadmium Accumulation Analysis:

Results of pot experiment showed that isolates G_1, K_1 G_2 and C_3 and their consortia were helpful in combating cadmium stress. They are applicable in stress minimization and growth promotion of plants.

4.15.4.1. Cadmium Accumulation in Mustard:

In cadmium uptake analysis of mustard plant, it was found that in shoot, maximum uptake 11.86 ppm was reported in treatment K_1 and minimum value 2.47 ppm was found in G_2 treatment. Cadmium uptake in shoot was reduced in all treatments with bacterial cultures over control (without bacterial cultures). On the other hand, in root region, the cadmium uptake was higher than shoot in all the treatments and maximum uptake 81.93 ppm was achieved in treatment with consortium ($Cons_2$) and minimum uptake 11.64 ppm was found at treatment $Cons_3$. In case of soil, maximum accumulation of 98.12 ppm of cadmium was found at treatment $cons_3$ and minimum

accumulation 12.97 ppm was observed in treatment $Cons_2$. Results of this uptake study showed that maximum cadmium was accumulated in rhizospheric region i.e. process of cadmium stabilization is working (Fig.4.36 A)

4.15.4.2. Cadmium Accumulation in Maize:

Cadmium accumulation analysis of maize plants revealed, that in shoot maximum uptake of 28.29 ppm of cadmium was observed in treatment C_3, while minimum uptake was in treatment $Cons_2$. However, in root region, maximum uptake of 77.82 ppm was found in treatment $Cons_2$ and minimum uptake 37.97 ppm was in presence of G_2 and value was reduced than control. In soil, maximum accumulation 64.69 ppm was found at treatment G_2 and minimum accumulation 12.11 ppm in treatment $Cons_2$ (Fig-4.36B).

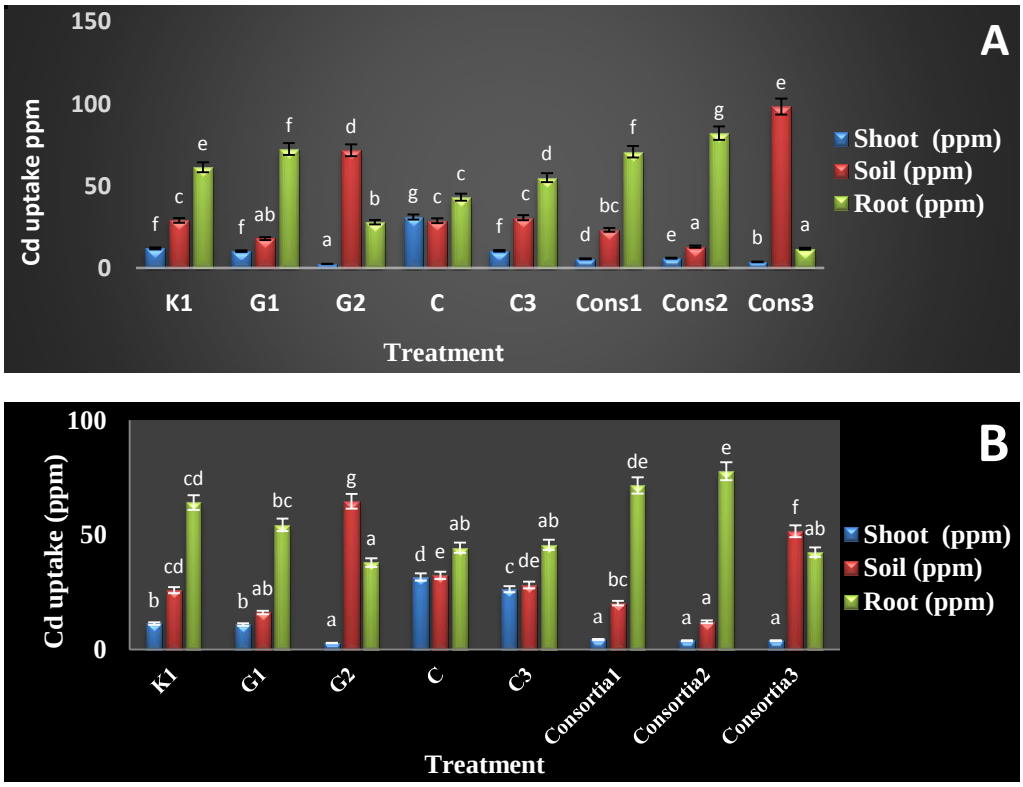


Fig-4.36 (A&B) - Cadmium uptake in shoot, root and rhizospheric soil of A- mustard, B- maize

Overall, it can be concluded from the results that all the four individual cultures and consortium behaved differently with both the hyper-accumulator plants, mustard and

maize but accumulation of cadmium in rhizospheric region was more as compared to plants i.e. the process of stabilization or rhizo-accumulation or rhizo-remediation is active in the present study. These results are discussed in detail with the findings reported till date by other researchers in the next chapter entitled "Discussion".

Discussion

Enhanced contamination of agricultural soil, irrigation water is a resultant of rapid development in last few decades. This development on one hand has made human life easier while on the other hand they has resulted in enormous amount of polluted effluent, contamination of groundwater, and of the soil by various hazardous wastes including heavy metals. Cadmium is one such hazardous metal, whose widespread occurrence is due to excessive use of phosphatic fertilizers, pesticides, disposal of industrial wastes etc. ultimately threatening the ecosystem health (Azevedo et al., 2012; Gallego et al., 2012). Cadmium and other heavy metals are released from various industrial sources like agrochemicals and sewage sludge, mining, discharge of battery and paint forming industry, phosphatic fertilizer etc. All the heavy metals including cadmium are non-degradable and stay in environment for long time. In this study, fluorescent pseudomonads (FPs) were isolated from heavy metal contaminated industrial sites of various regions of India and supported that heavy metal contaminated sites contain highly resistant microorganisms (Desouza et al., 2006; Xie et al., 2010).

Cadmium affects the beneficial soil microbial population by several ways and it reduces the growth of most of the bacteria. However, some bacteria develop tolerance mechanisms due to selection pressure and resistant mechanisms. In this study, only 3.63% bacteria (FPs) tolerated the 2000 ppm of cadmium and finding is supported by earlier work done by various researchers (Bhagat and Shrivastva, 1991; Campbell et al., 1995; Dell Amico et al., 2005). In this study, fluorescent pseudomonads were used because they are the key model of high cadmium resistant properties and are found predominantly in soil as bio-indicators for heavy metals (Hassen et a., 1998; Dell Amico et al., 2008; Chakraborty and Das, 2014; Pereira et al., 2015; Vacheron et al.,

2016). Four fluorescent bacterial isolates, G_1, G_2, K_1 and C_3, used in the present study were selected on the basis of germination test and cadmium resistant properties, out of 55 fluorescent isolates from different regions of the country (Delhi, Kanpur, Lucknow and Jamshedpur). Used isolates, enhanced the germination percentage of mustard and maize. Similar findings were observed by Almaghrabi et al. (2014) and they reported that in presence of PGPR (*Pseudomonas*) germination percentage is enhanced over control.

Selected isolates, G_1, G_2, K_1 and C_3 , isolated form rhizospheric region of mustard and maize plants showed high MIC (Minimum Inhibitory concentration) value for cadmium (in the form of cadmium nitrate) upto 2000 ppm. Similar findings were given by various researchers (Malik and Jaiswal, 2002; Belimov et al., 2005). Horitsu et al. (1986) isolated *Pseudomonas putida* which showed 1280 ppm MIC value. All the four isolates, also showed multiple antibiotic resistant properties against A (Ampicillin), CPM (Cefepime), NS (Nystatin), AT (Aztreonam) and NIT (Nitrofirantoin) etc. On the basis of cultural, morphological and biochemical characteristics, total four isolates G_1, G_2, K_1 and C_3, were grouped into *Pseudomonas* family as described in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Results of 16s rRNA sequencing showed that the selected isolates G_1, G_2 K_1 and C_3 were *Pseudomonas* sp, *Pseudomonas putida, Pseudomonas guariconensis* and *Pseudomonas aeruginosa*, respectively. Sequences were deposited to NCBI gene bank and accession number for isolates G_1, G_2, K_1 and C_3 have been obtained as KU947109, KX681787, KX681789 and KU947108, respectively (Table-4.3). Based on the biochemical, morphological and molecular characterization, it may be possible that isolate G_1 may be a newer strain, belonging to genera *Pseudomonas*, because it

depicted only 96.60% similarity to *P. putida.* This bacterium also showed different types of biochemical tests than *P. putida* strain and was more efficient than other three isolates G_2, K_1 and C_3 in aspect of MIC and PGP (Plant growth promoting) traits. The isolate K_1, is also a very new bacterium and has been reported in 2013 by Toro et al. (2013) as *Pseudomonas guariconensis* (K_1). This strain has been used earlier by Patel et al. (2015) as a biocontrol agent for control of collar root disease of *Arachis hypogea.* As a bioremediator of cadmium along with hyper-accumulator plants, it has been used for the first time in the present study.

All the selected isolates have multiple plant growth promoting properties in presence and absence of cadmium. PGPRs are helpful for controlling the toxic effects of metal in plants (Belimov et al., 2005; Dell Amico et al., 2005; Dell Amico et al., 2008; Ganesan, 2008; Ullah et al., 2015; Sharma and Archana, 2016; Verma et al., 2017; Lal et al., 2018) through various PGP properties such as production of IAA (Indole acetic acid), HCN (Hydrogen cyanide), NH_3 (Ammonia), siderophore, ACC (1-aminocyclopropane-1-carboxylate) deaminase and solubiliztion of zinc and phosphate. Presence of cadmium enhances the ACC deaminase activity and production of siderophore in some bacteria. Other properties such as phosphate solubilisation, production of IAA etc., were reduced in presence of cadmium. Research, worldwide is being carried out for the isolation of more and more potent plant growth promontory bacterial strain for detoxification of heavy metal in the soil and bio assisted phytoremediation (Ganesan, 2008; Wani and Khan, 2010; Ma et al., 2011; Verma et al., 2017).

These PGPRs exceptionally promote the plant growth by various mechanisms such as nitrogen fixation, mineral solubilisation, and production of siderophore, phyto-hormone and transformation of nutrients (Glick et al., 1999; Ullah et al., 2015;

Sharma and Archana, 2016) nitrogenase activity, phosphate solubilisation (Ahemad and Khan, 2012) and siderophore production (Tian et al., 2009). Like fluorescent pseudomonads, diverse group of symbiotic (*Rhizobium, Mesorhizobium* and *Bradyrhizobium*) and non-symbiotic (*Pseudomonas, Bacillus, Klebsiella, Azotobacter, Azomonas*) rhizobacteria are now being used around the globe as inoculants to promote plant growth and plant productivity under various stresses like heavy metals (Wani and Khan, 2010; Ma et al., 2011). PGPRs are those heterogeneous groups of bacteria which are found in rhizospheric region of plants and can enhance growth of plants in stressed as well as normal conditions, directly or indirectly (Tica et al., 2011; Ullah et al., 2015).

All the cadmium resistant bacteria used in this study possessed plant growth promotory and metal tolerance properties. Similar findings of fluorescent pseudomonads having plant growth as well as metal tolerance ability have been reported by Burd et al. (2000) and Belimov et al. (2005) and the fluorescent strains have been used for bioremediation also.

In case of two isolates, C_3 and G_1, siderophore production was increased with increasing cadmium concentration and ACC deamianse production was improved in case of all the isolates, except G_1 (Table-4.5 to 4.8). This finding was unanimous with earlier findings of Dell Amico et al. (2008), wherein they reported that siderophore production was enhanced by cadmium in *Pseudomonas* bacteria. On the other hand, other properties such as production of IAA, EPS, solubilisation of phosphate and zinc, etc. were reduced in presence of cadmium (Dell Amico et al., 2008).

For cadmium resistant gene (czc) analysis, genomic DNA of all the isolates G_1, G_2, K_1 and C_3 was amplified with cadmium resistant gene primer and it was found that, only C_3 isolate had czc gene. This finding is similar to earlier finding by Zeng et al.

(2012), Chakraborty and Das (2014), where czc complex was reported by them in *Pseudomonas* strain. CZC complex responsible for the cadmium resistant properties by pumping of Cd metal from cytoplasm to EPS (Nies, 1992). On the other hand, cadmium resistant gene czc was not found in other three isolates G_1, K_1 and G_2. This finding showed that there is a chance that they may have new cadmium resistant gene. For this analysis primer designing is required because no primers are available in market and can be a subject of further research.

Furthermore, cadmium accumulation test was confirmed by Chakraborty and Das (2014), they also studied about cadmium accumulation in biomass of *Pseudomonas.* In cadmium sorption analysis of two isolates, G_1 and C_3 it was found that the strain G_1 had good capacity to accumulate cadmium than C_3 strains. The C_3 isolate, accumulated 40.82 mg/l of cadmium, while other isolate G_1 accumulated 190.9 mg/l of cadmium out of 500 mg/l. Similar to this findings *Pseudomonas* used by Chakraborty and Das (2014) accumulated cadmium 58.76 % of 1000 ppm. Findings of present study showed that G_1 strain accumulates approximately 38.18 % of 500 ppm cadmium (Doyle et al., 1975). They also concluded that cadmium was immobilized by bacteria in broth.

Pigment production by the isolates, G_1, G_2, K_1 and C_3 was analysed and found that production was enhanced by the presence of cadmium. Pigment of C_3 and G_1 was extracted and characterized by HPLC. After this analysis, it was revealed that the extracted product was pyoverdine (Tank et al, 2012). Pyoverdine production is a specific feature of fluorescent pseudomonads and this finding supported that the bacterial strains used in present study are fluorescent pseudomonad and are involved in iron transport as pyoverdine is also a type of siderophore (Meyer & Hornsperger, 1978). In SEM analysis of morphology, it was confirmed that surface of bacteria

became perturbed due to release of membrane fragments (Higham et al., 1986). Presence of cadmium alters the morphology of bacteria and makes the surface rough due to absorption of cadmium. Growth of bacteria was also affected by the presence of cadmium and different incubation period (0 h-106 h). As can be seen in Fig. 4.16, growth of all the isolates increased but all four isolates had different patterns of growth and overall it was less as compared to control (absence of cadmium). In growth pattern analysis, we found that all the strains showed different growth patterns in both presence and absence of cadmium (Doyle et al., 1975). They said that cadmium was immobilized by bacteria in broth.

In the root elongation analysis, it was found that all the strains G_1, G_2, K_1 and C_3 and their consortia ($Cons_1$-G_1, K_1, G_2; $Cons_2$-G_2, K_1, C_3; $Cons_3$-G_1, K_1, C_3) in presence and absence of cadmium worked as good bio-inoculant and enhanced the root length of both the plants mustard and maize. These results are in confirmation as that reported by Belimov et al. (2005). Before making consortium, compatibility test of isolates was done and found that all the isolates G_1, G_2, K_1 and C_3 were compatible with each other. Results of root elongation assay showed that consortium were better than individual strains in promoting root length in absence as well as presence of cadmium. Study of Sarathambal et al. (2017) also stressed upon use of consortium over individual strains in performance from plant growth promotion under cadmium stressed conditions. They reported that consortia of *Bacillus* sp. along with mycorrhizae inoculation effectively enhanced the growth, antioxidants enzymes, and cadmium uptake in *A. donax* plant than *Bacillus* alone.

The bacterial interaction on soil root interface play a pivotal role in transformation, solubilisation and mobilization of essential nutrients and cadmium by production of various metabolites. In this study, various metabolites like EPS, siderophore and IAA

were extracted and characterized by FTIR (Fourier-transform infrared spectroscopy) and HPLC (High-performance liquid chromatography) analysis. Many studies supported that the metabolites of *Pseudomonas* like siderophore, rhamnolipids, organic acids and EPS were able to bind or chelate the metal ion and reduced the phyto-extraction of metal in edible parts of plants. These metabolites were secreted in rhizospheric region, due to this the maximum amount of cadmium was concentrated in lower parts of the plant (Mulligan 2001; Rajkumar et al., 2010; Upadhyay et al., 2011; Pacwa-Płociniczak et al., 2011). Idris et al. (2004) reported that metabolites of PGPR participate in metal mobilization.

Metabolites of PGPR directly participate in remediation by production of metal chelating substances such as siderophore, organic acids, bio-surfactant, bio-methylation etc. (Ullah et al., 2015). These metabolites bind the metal and enhance the rhizo-accumulation of cadmium in rhizosphere (Juwarkar et al., 2007; Rajkmuar et al., 2010; Rajkumar et al., 2012).

Plants grown in cadmium contaminated soil can easily accumulate cadmium in their tissues and these cadmium accumulated plants become hazardous for human when consumed directly or indirectly. To overcome problem of cadmium contamination, there is an emergent need to remediate the agricultural soil. Amongst all the used remediation technologies, phyto-remediation, a green technology to remediate the cadmium and other heavy metals is a highly acceptable technology for years (Chaney et al., 1997; Ma et al., 2011; Kamran et al., 2014). This method of remediation has various limitations and one most important limitation is that accumulation of metals in plants is responsible for irreversible damage of plant tissues and finally results in retardation of plant growth (Barosci et al., 2003). Remediation of cadmium as well as

other metal is very important for controlling the toxic effects of heavy metals to preserve the environment (Glick, 2010; Taj and Rajkumar, 2016;).

Remediation of heavy metals from the environment is a very challenging task due to technical complexity and cost (Sheoran et al., 2011**;** Mahar et al., 2016). Various types of methods have been used since years and some are under pipeline. These methods are categorised into chemical, biological and physical processes (Lim et al., 2014; Hasegawa et al., 2016). In this area, PGPRs become more useful for improvement of phytoremediation without any risk. This technology is termed as 'PGPR assisted phytoremediation' for the remediation of contaminated sites (Verma et al., 2017; Ullah et al., 2015). This field of study is very new and needs exploration to obtain various potent PGPR's that improve phytoremediation. Various researchers reported that enhancement of plants microbe interaction in PGPR assisted phytoremediation is very helpful; it can enhance biomass production and tolerance of plants to heavy metals (Ullah et al., 2015; Sharma and Archana, 2016; Verma et al., 2017; **Lal et al., 2018**).

Improvement of phytoremediation can be done by using efficient microorganisms and selection of suitable plants having high metal tolerance ability and high biomass production. In the present study, hyper-accumulator plants like mustard and maize were selected for remediation of cadmium contaminated soil. Kumar et al. (1995) reported that plants belonging to Brassicaceae family are commonly used accumulators with hyper-accumulating ability. Amongst all the plants, *Brassica juncea* is one of the best plants which is used in phytoremediation with higher growth rate (Kumar et al., 1995; Saxena et al., 1999) and maize plant is also a good hyper-accumulator plant (Wuana and Okieimen, 2010).

Plant growth promontory traits present in all the four test isolates are helpful in plant growth promotion and abiotic stress management by assisting phytoremediation. They have ability to confer metal tolerance to plants, plant biomass enhancement and confer protection against phytopathogens as also reported by Luo et al. (2012). Production of ACC deaminase is helpful for mitigating stress because they convert stress enzyme ACC to ammonia and α-ketobutyrate (Malekzadeh et al., 2010) as shown in Fig-5.1. This ammonia is used by plant as a nitrogen source and increasing the root length in cadmium polluted soil (Safronova et al. 2006). Siderophore production removes the iron deficiency of plants and indirectly alleviates heavy metal toxicity (Burd et al., 2000; Malekzadeh et al. 2010). In addition to this, IAA production enhances the length of plants and bioavailability of nutrients and metals (Patten and Glick, 2002) and participates in minimization of heavy metal stress. Collectively all the PGPRs with above properties are good bio-inoculant for plant growth promotion and stress minimization. All these properties are inter-related as shown in Fig-5.1.

Pot experiment of mustard and maize was performed with 100 ppm cadmium amended soil in green house chamber to study the effect of four different species of *Pseudomonas viz.* G_1, G_2, K_1 and C_3 and their consortia on growth of *Brassica juncea* and *Zea mays* under stressed and normal conditions.

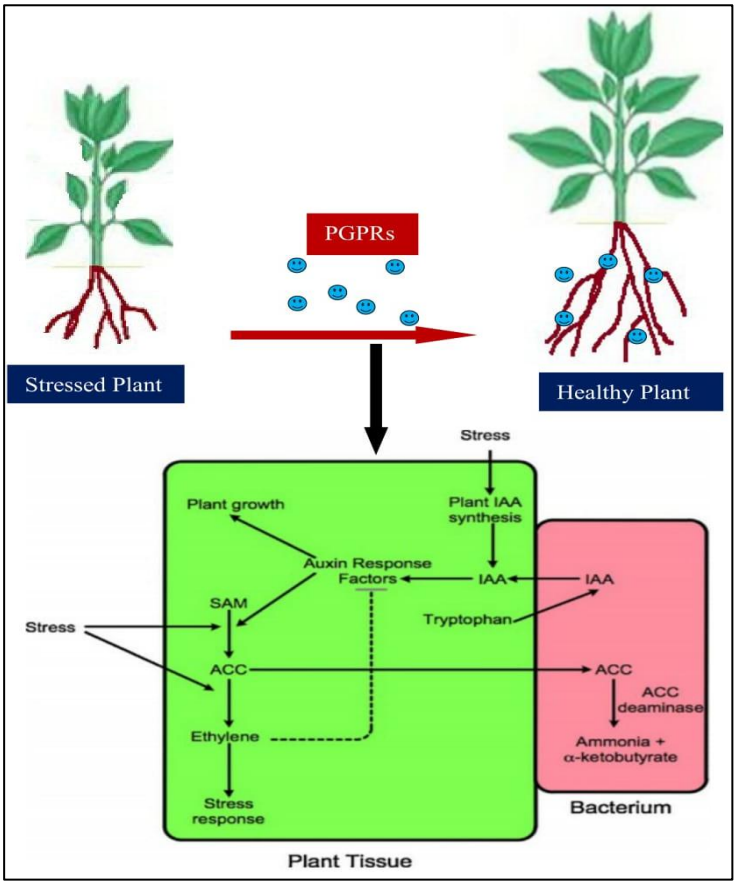


Fig-5.1- Facilitation of plant growth by IAA and ACC deaminase producing PGPR (Plant Growth Promoting Rhizobacteria; Source- Glick, 2014)

Presence of consortium and individual strains improved plant growth under normal as well as stressed conditions. Plants, in general were better in most of the studied agronomic parameters like length of root and shoot, dry weight of root and shoot, productivity of plants, chlorophyll, proline and TSS (total soluble sugar) etc., over the control (Fig-4.28 to 4.35).

In present study, cadmium did not had any negative effect upon chlorophyll content of plant, rather it improved in the presence of cadmium, while prior studies demonstrated that the increased concentration of cadmium reduced the chlorophyll content (Simonova et al., 2007).

Proline production, another stress tolerant parameter, was better in case of mustard as compared to maize. In maize plant, proline production was reduced and maximum reduction was found under consortia treatments. Osmotic turgor in plant is maintained by accumulation of compatible osmolytes such as proline and soluble sugars for minimization of abiotic stress (Grover et al., 2011; Tiwari et al., 2016). Lata et al. (2010) reported that accumulation of proline and TSS has increases manifold under stress conditions in chickpea cultivars. Recent studies revealed that the strains isolated from heavy metal rich habitats have higher tolerance and application in agriculture and industrial soils (Idris et al., 2004; Barzanti et al., 2007; Dell Amico et al., 2008; Rajkumar and freitas, 2008). Moreover, rhizospheric microorganisms can promote biomass production and tolerance of plants to heavy metal in stressed environment (Sheng and Xia, 2006; Dell Amico et al., 2008). Similar results were found in this experiment and prove that it can be an efficient method for protecting seeds from growth inhibition caused by toxic Cd^{+2} concentration.

After harvesting, cadmium uptake in root, shoot and rhizospheric soil was analysed by AAS Atomic absorption spectroscopy) to check remediation efficiency of the four strains with mustard and maize. It was found that the level of cadmium was restricted in shoot region plants treated with test isolates (PGPR) over control. Maximum amount of cadmium accumulated in rhizospheric region (Fig-4.36 A & 4.36 B). In this case also, bacterial consortia were better than individual strains in localization of cadmium in the soil or roots only. In mustard and maize plant consortium2 ($Cons_2$) and consortium3 ($Cons_3$) were the best consortia for rhizoremediation purpose. In mustard plant, in case of $Cons_2$ 81.93 ppm of cadmium was concentrated in root, while only 5.93 ppm was present in shoot region. On the other hand in case of $Cons_3$

98.12 ppm cadmium accumulated in soil and only 3.76 ppm was entering in shoot of mustard.

In case of maize, 77.82 ppm of cadmium was accumulated in root, while only 3.86 ppm was accumulated in shoot region in $Cons_2$ treatment. On the other hand in case of $Cons_3$ 51.65 ppm and 42.27 ppm of cadmium localised in soil and root respectively. The reduced uptake of cadmium might be due to accumulation of cadmium by bacterial cells or immobilization by metabolites produced by bacterial cells. Findings are supported by study of Ullah et al. (2015), Sharma and Archana (2016), Verma et al. (2017). These cadmium resistant PGPRs play role in rhizoremediation and same results were obtained by Malekzadeh et al. (2012) and many other studies. With increasing population, shrinking land and enhanced contamination of agricultural land, this technique can be helpful to meet the nutritional challenges and the food security of the increasing population. Findings of pot experiment such as growth and productivity of plants, cadmium tolerance and cadmium uptake in rhizospheric region depict that consortia treatments are better than all the individual treatments (Malekzadeh et al., 2012, Sarathambal et al., 2017).

The capacity of *B. juncea* to accumulate cadmium and mechanism of translocation in the harvestable parts has been described by Salt et al. (2000). In the present study, however this mechanism of translocation of cadmium was there but it was restricted due to the accumulation of cadmium by bacterial cells and their metabolites in the rhizosphere. These results strengthen the suggestion that *B. juncea* may be effective in the remediation of cadmium polluted soils using microbes (PGPR's). Use of maize plants in PGPR assisted phytoremediation technology was supported by Malekzadeh et al. (2012). They reported that the consortium of *B. mycoides* and *M. roseus* was more effective in phyto-stabilization of cadmium without entering in food chain.

According to Glick (2003), enhancement of plants microbe interaction in PGPR assisted phytoremediation is very helpful and it can enhance biomass production and tolerance of plants to heavy metals. Use of PGPR's in phytoremediation process offer an economically attractive and ecologically sound means of reducing the cadmium uptake in edible parts of plants and improvement of plant may occur.

With regard to uptake analysis, bacterial metabolites like Indole Acetic Acid (IAA) and ACC deaminase both were playing an effective role because they promote root and shoot length and enhance plant biomass (Belimov et al., 2005). In addition to these properties, they enhance root surface area so that more and more cadmium can be accumulated with the result that even low amount of cadmium accumulated is almost negligible as compared to large surface and high biomass. This type of remediation has been reported by Wu et al. (2006) and Sheng and Xia (2006). They reported that cadmium resistant PGPR's like *Pseudomonas* and rhizobia are able to accumulate cadmium intra-cellularly and favour phytoremediation process.

In comparative study of accumulation and tolerance power of mustard plant with maize plant for bio –assisted phytoremediation, it is concluded that performance of maize plant was better than mustard plant in aspect of studied objectives. The reason behind this is better biomass production in case of maize than mustard. Accumulation of cadmium in rhizospheric region was maximum in case of maize because of high quantity of biomass.

On the basis of proline, chlorophyll and total soluble sugars (TSS) content, tolerance of the plants mustard and maize was compared. Out of these, proline is a stress parameter and mainly synthesised under stressed conditions to combat abiotic stress. Based on these, it was found that in case of mustard, all the three parameters were better than maize and therefore it can be concluded that mustard is having better

tolerance power than maize plant. In conclusion both the plants are good phyto-remediators with maize being better in biomass production (hence more metal accumulation) and mustard being more tolerant to cadmium and therefore helpful for remediation of cadmium by rhizospheric accumulation of cadmium in presence of PGPRs.

The increased growth of *Brassica juncea* and *Zea mays* plants even in the presence of cadmium might have been due to several factors like-

1. Production of IAA, ACC deaminase, siderophore, HCN, ammonia, EPS and solubilisation of zinc and phosphate by selected *Pseudomonas* species (G_1, G_2, K_1 and C_3),
2. Cadmium accumulation ability of tested strains,
3. Ability of bacterial strains to overcome the abiotic stress due to cadmium contamination in soil and shoot region of plants.

Overall this study concludes that the used bacterium FP_S protected the plants from the toxicity of cadmium, enhancing plant biomass, root and shoot length, nutrient assimilation, chlorophyll, proline content and seed yield. In other aspect, they participated in remediation of cadmium as well as minimization of cadmium in edible parts of mustard and maize.

Based on above properties, all the bacterial strains could be developed as bio-inoculant for enhancing growth and yield of plants as well as the phytoremediation of cadmium in soil through minimization of cadmium toxicity. Metabolites of PGPR's and PGPR itself may be responsible for the rhizospheric accumulation of cadmium and it has been proved by many researchers.

Effective and safe phytoremediation could be accomplished by PGPRs having potential of solubilizing cadmium, promoting growth of plant and minimizing stress in plant through rhizospheric accumulation of cadmium. The present study indicated that cadmium tolerant and plant growth promoting PGPRs isolated from metal

contaminated rhizospheric soils could increase the cadmium availability in cadmium amended soils. Pot experiment demonstrated that isolated PGPR strains, G_1, G_2, K_1, C_3 and their consortia ($Cons_1$, $Cons_2$ and $Cons_3$) could significantly promote growth, stress tolerance and productivity of mustard and maize. They also promote rhizo-accumulation of cadmium by minimization of cadmium uptake in edible parts of plants by enhancing bioavailability of cadmium.

Results of interaction with the strains vary from plant to plant in remediation potential as found in this study and also reported in the past with other strains. These fluorescent bacterial strains are better as compared to other strains as they not only help plants combat abiotic stress but also promote plant growth and restrict the movement of toxic metal like cadmium, keeping it localized to rhizospheric region and can be used for remediation purposes. Further, studies are required for exploration of more PGPRs, because in environment, various potent microorganisms are present and there is a need to explore them. In addition to this, productive efficiency of specific plant growth promoting rhizobacteria to remediate cadmium further increased with the optimization and acclimatization according to environmental condition and physicochemical properties of soil because environment and soil may affect the properties of PGPRs.

More research is required in this field to know the potentials of already known PGPRs and their mechanisms to support the PGPR assisted phytoremediation. Further research and understanding of mechanisms of PGPR-assisted phytoremediation would pave to find out more competent rhizobacterial strains which may work under diverse climatic conditions and to improve the efficiency and acceptance of this technology numerous field trials are required, before it could be transferred.

Conclusion

Industrialization has resulted in increased influx of toxic and non-toxic substances in vital segments of environment i.e. air, soil and water. Metals, amongst them existed from ages but anthropogenic activities in last few decades have resulted in release of more and more of them in the environment. Release of these heavy metals from industries and their impact on human beings is a matter of global concern. Amongst all the heavy metals, cadmium has deleterious effects on agricultural ecosystem, environment and human health. Cadmium is one of the heavy metal which is considered as most hazardous and is included in top 10 hazardous metals as per the US EPA. From contaminated soil, cadmium enters into plant via roots and is accumulated in the harvestable part of plants, resulting in growth inhibition and finally cell/plant death. Heavy metal as well as cadmium contamination of soils has received considerable attention in the contemporary science due to its toxicity. Therefore, it is important to develop methods to remediate the heavy metals. Application of PGPR (Plant growth promoting rhizobacteria) is helpful for stress minimization and remediation of cadmium. Therefore, the current topic PGPR assisted phytoremediation was selected for research work.

In the present study four fluorescent plant growth promotory, cadmium resistant strains G_1, G_2 K_1 and C_3 were selected for final study. Selected bacteria were morphologically, biochemically and molecularly characterized by following standard protocol. After characterisation, it was confirmed that all bacteria G_1, G_2, K_1 and C_3 were gram negative rod shaped and belonging to *Pseudomonas* genera named as *Pseudomonas* sp. (KU947109), *Pseudomonas putida* (KX681787), *Pseudomonas guariconenesis* (KX681789) and *Pseudomonas aeruginosa* (KU947108), respectively. For plant growth promotion ability the PGP properties such as production of IAA

(Indole acetic acid), HCN (Hydrogen cyanide), NH_3 (Ammonia), siderophore, EPS (Exo-polysaccharide), ACC (1-aminocyclopropane-1-carboxylate) deaminase and solubilisation of zinc and phosphate solubilization were checked in both presence and absence of cadmium and found that all strains (G_1, G_2, K_1 and C_3) have multiple plant growth promoting properties.

For efficiency analysis of bacteria, growth pattern and morphology of bacteria in presence and absence of cadmium was analysed. Accumulation of cadmium by bacteria were also checked and found that they have good tolerance and accumulation capacity. Cadmium resistant gene complex czc was reported in C_3 isolate, while, in other isolates G_1, K_1, and G_2 no gene complex was found, hinting towards the presence of other gene complex responsible for resistance. Pyoverdine was produced by C_3 and G_1 isolate confirmed by HPLC (High-performance liquid chromatography) analysis. All the selected isolates produced various metabolites such as EPS, siderophore, pigment and IAA and this is confirmed by the FTIR (Fourier-transform infrared spectroscopy) and HPLC characterisation.

Pot experiment was done to check the role of bacteria in growth promotion of plants and their remediation efficiency over control and also the efficiency of each plant type in phytoremediation. Besides this, comparative studies for remediation potential between individual strains and consortium was also recorded. In pot experiment, growth of plants (mustard and maize) increased in presence of bacteria over control in presence as well as absence of cadmium. Productivity was also enhanced by inoculants. Stress tolerance factors like chlorophyll, proline and TSS (Total Soluble Sugar), responsible for imparting tolerance to the plants were more in case of mustard as compared to maize. In cadmium uptake analysis, it was found that more of cadmium was localized in rhizospheric region or roots as compared to shoots.

This effect of localization in the soil was more pronounced in case of bacterial treated plants over the control. Amongst the individual cultures, bacterial culture G_2 and C_3 were not very effective. Comparison of individual cultures with consortium in cadmium uptake, consortia were better than individual strains in restricting cadmium movement to the harvestable part of both the plants. Overall, consortium3 ($Cons_3$) and consortium2 ($Cons_2$) in case of mustard and maize was the best because they improved the localisation of cadmium in rhizosphere (root and soil).

Above properties are helpful for the remediation of cadmium by accumulation in rhizospheric region. In all, the treatment consortium gave good results than individual strains in case of both mustard and maize plants. In conclusion, both plants are good phyto-remeditors and helpful for remediation of cadmium by rhizospheric accumulation of cadmium in presence of PGPRs (G_1, G_2, K_1 and C_3). The results of present study may serve as baseline data for selecting PGPRs for remediation of cadmium as well as other heavy metals by improvement of phytoremediation technology. Also the consortium can be used as bio-innoculant for remediation of cadmium affected soil with mustard and maize as the agricultural land is shrinking and the wasteland is increasing with more and more industrialization.

Summary

Metals with density greater than 5g cm^{-3} are termed as heavy metals. A few of the heavy metals, in general are useful for the environment or the plants only in trace amounts while others are either of no use or least use. As the concentration of these heavy metals increases in the environment, their toxicity also increases. Cadmium is one such heavy metal whose presence in general is toxic to the environment and for human beings. Rapid industrialization in the last few decades and enhanced use of phosphatic fertilizers for increasing agricultural productivity has resulted in the contamination of the biosphere at a rapid speed. This contamination of the environment, particularly soil and water with cadmium is highly toxic for the animals and humans as it causes brittle bones and cancer.

Increasing contamination of agricultural land and use of irrigation water contaminated with these heavy metals especially needs attention. Various attempts have been made by various researchers in the past including conventional techniques and a newer technology such as phytoremediation, which has been used for years (Chaney et al., 1997; Ma et al., 2011), but owing to certain limitations, bacterial assisted remediation known as bioremediation is gaining importance where plant growth promotory rhizobacterial strains are becoming more useful for improvement of phytoremediation without any risk (Verma et al., 2017).

PGPRs are those heterogeneous groups of bacteria which found in rhizosphere region of plants and can enhance growth of plants in stress as well as normal condition through direct or indirect manner (Tica et al., 2011; Ullah et al., 2015). This technology is termed as 'PGPR assisted Phytoremediation' for the remediation of contaminated sites. This field of study is very new and needs exploration to obtain various potent PGPR that improve phytoremediation. Improvement of

phytoremediation was done by using efficient microorganisms and selection of suitable plants should have high metal tolerance property and high biomass production is an important need. Among all the hyper accumulator plant *Brassica juncea* and *Zea mays* plants are best hyper-accumulator plants (Wuana and Okieimen, 2010; Kumar et al., 1995; Saxena et al., 1999).

With this, present study entitled "**Remediation of Cadmium Contaminated Soil with PGPR Consortia and Hyperaccumulator Plants**" was carried out during the year 2012-17. The study was aimed to isolate and characterize cadmium resistant plant growth promoting fluorescent pseudomonads native to rhizospheric soil and had ability to remediate the cadmium through minimization of its toxicity to edible parts of mustard and maize. Growth and productivity of plants were also checked in this study. The salient features of the investigations are as follows:

There are various types of bacteria and only cadmium resistant fluorescent pseudomonads were selected for experiment because they are the key model for the present study. Out of 89 isolates from soil samples of industrial sites of Lucknow, Delhi, Kanpur and Jamshedpur, India, only 55 isolates belonged to this group of fluorescent pseudomonads. All the fifty five isolates showed varying degree of cadmium tolerance when tested between 0-2100 ppm of cadmium and only 3.63% bacteria (FPs) tolerated 2000 ppm of cadmium level while only isolate H_1S could tolerate 2100 ppm of cadmium. Out of these, six isolates showing high tolerance to cadmium were selected *viz.* G_1, G_2, K_1, C_3, H_1S and A_1 for seed germination and antibiotic resistance test. For 14 different antibiotics tested, isolate G_2 depicted resistant property against most of the antibiotics. This test gives idea about multiple antibiotic resistant properties.

In germination test of mustard and maize seeds, isolate K_1 and C_3 enhanced the germination rate in case of both mustard and maize than other isolates. On the other hand isolates A_1 and H_1S did not show any effective results in germination analysis and therefore were dropped from the further studies.

Morphological characterization, confirmed that all the isolates were Gram negative, rod shaped bacteria with fluorescent and transparent colonies. Biochemical tests were also done to characterize bacteria. Morphological and biochemical characterization of isolates confirmed that all the isolates, G_1, G_2, K_1 and C_3 belonged to *Pseudomonas* group of bacteria. This finding was confirmed by molecular characterization by 16S rRNA sequencing. The obtained sequences were aligned with already submitted sequence using BLAST program of NCBI and EZ taxon and confirmed the above finding based on morphological and biochemical features that all the isolates belonged to the *Pseudomonas* family. Results of sequencing showed that the selected isolates G_1, G_2 K_1 and C_3 were *Pseudomonas* sp, *Pseudomonas putida*, *Pseudomonas guariconensis* and *Pseudomonas aeruginosa* respectively. Obtained sequences deposited to NCBI gene bank and accession number for isolates G_1, G_2, K_1 and C_3 have been assigned as KU947109, KX681787, KX681789 and KU947108, respectively. Results of BLAST through EZ taxon and NCBI confirmed that there is a possibility that G_1 is a newer and a novel strain because it showed only 96.16% similarity to *Pseudomonas putida*. Further study (FAME analysis, DNA-DNA hybridisation and Maldi TOF analysis) is required for complete characterization and its reporting as a novel bacterium. The isolate K_1 is also a new strain and was recently isolated by Toro et al., (2013). G+C contents of used partial sequence was also calculated by online software and found that the maximum G+C content was in C_3 isolate, than other three isolates.

Analysis of Plant Growth Promoting (PGP) attributes of strains G_1, G_2, K_1 and C_3 is an important step. In this study, it was observed that all the fluorescent pseudomonads showed multiple plant growth promoting activities such as production of siderophore, HCN, ammonia, ACC deaminase, IAA and solubilisation of zinc and phosphate in presence and absence of cadmium. Some PGP properties were significantly elevated in presence of cadmium than in the absence of cadmium such as production of siderophore and ACC deaminase. In addition to these, NH_3 production was less affected by presence of cadmium and other PGP properties such as production of IAA, HCN and phosphate solubilisation were reduced in the presence of cadmium in most of the isolates.

Quantitative analysis of PGPR attributes showed that K_1 produced maximum IAA (1.41 mg/ml) while other strains depicted maximum production of other metabolites like siderophore (36 SU) by C_3, phosphate solubilisation in C_3 (85 ug/ml) and maximum EPS (195 µg/ml) was produced by G_1, maximum utilization of ACC (0.131) was by G_2 isolate in absence of cadmium. In presence of cadmium, all these properties were changed for all the isolates. In case of two isolates, C_3 and G_1 siderophore production increased with cadmium concentration while other properties such as production of IAA, EPS, solubilisation of phosphate and zinc, etc., were reduced. On the other hand, in presence of cadmium ACC utilization was enhanced in all isolates while G_1 showed negative results and in HCN production C_3 and G_1 showed higher production than others in absence and presence of cadmium respectively.

Pigment production of isolates G_1, K_1, G_2, and C_3 was analysed and it was found that production was enhanced by presence of cadmium. Pigment of G_1 and C_3 were extracted and characterized by HPLC and were found to be pyoverdine. All the

selected isolates produced various metabolites such as EPS, siderophore and IAA. All the produced metabolites such as EPS, siderophore and indole acetic acid (IAA) were extracted and characterized by HPLC and FTIR. Characterization confirmed the presence of EPS, siderophore and IAA by all the four strains G_1, G_2, K_1 and C_3.

Presence of cadmium alters the morphology of bacteria and makes the surface rough due to absorption of cadmium and release of membrane fragments. Growth of bacteria was also affected by the presence of cadmium and different incubation period (0 h-106 h). Growth of all the isolates increased but all four isolates had different patterns of growth and overall it was less as compared to control (absence of cadmium). In growth pattern analysis, we found that all the strains showed different growth patterns in both presence and absence of cadmium. In cadmium sorption analysis of two isolates G_1 and C_3 we found that the strain G_1 has better capacity to accumulate cadmium than C_3 strains. The C_3 isolate accumulate cadmium 40.825 mg/l, while other isolate G_1 accumulate 190.9 mg/l of cadmium out of 500 mg/l. In other aspect the growing liquid media of C_3 contained 84.5 mg/l cadmium and in case of other isolate G_1 this value was 106.2 mg/l.

For czc analysis, genomic DNA of all the isolates G_1, G_2, K_1 and C_3 was amplified with cadmium resistant gene primer and found that only C_3 isolate had czc gene. Cadmium resistant gene was not found in other three isolates. This finding showed that there is a chance that they may have new cadmium resistant gene. Before making consortium, compatibility test was done and it was found that all the isolates, G_1, G_2, K_1 and C_3 were compatible with each other because they grew simultaneously.

Identified strain G_1, G_2, K_1 and C_3 and their consortia affected root elongation of *B. juncea* and *Zea mays* in the presence and absence of Cd. In the root elongation analysis, it was found that all the strains G_1, G_2, K_1 and C_3 and their consortia (G_1, K_1,

G_2-$Cons_1$; G_2, K_1, C_3-$Cons_2$; G_1, K_1, C_3-$Cons_3$) in presence and absence of cadmium worked as good bio inoculant and enhance the root length of both the plants mustard and maize. Findings of root elongation assay depicted that consortia gave better results than single isolates. Elongation of root of both mustard and maize plants was slightly affected by cadmium and no significant differences were observed.

Under pot experiment, effects of *Pseudomonas* of four different species G_1, G_2, K_1 and C_3 and their consortia on growth of *Brassica juncea* and *Zea mays* was analyzed under stressed (100 ppm cadmium amended) and normal conditions. All individual strains as well as consortium (G_1, G_2, K_1, C_3, $Cons_1$, $Cons_2$ and $Cons_3$) enhanced the growth of maize and mustard in presence and absence of cadmium. In all the parameters studied (except chlorophyll content), mustard growth improved in presence of cadmium over the control indicating towards the tolerance of mustard. This tolerance could be attributed to the presence of proline and TSS. While in case of maize all parameters depicted reduction in presence of cadmium over the control. Overall consortia were better in both mustard and maize in absence and presence of cadmium.

After harvesting, cadmium uptake in root, shoot and rhizospheric soil was also analyzed by AAS (Atomic absorption spectroscopy) to check remediation efficiency of mustard and maize. In this study, cadmium accumulation in roots, shoot and rhizospheric soil was compared and it was observed that more cadmium was localized in root or the rhizospheric region. In shoots also, the level of cadmium was reduced in bacterial culture treated plants over control and the amount deducted was almost negligible. In this case also, bacterial consortia were better than individual strains in localization of cadmium in the soil or roots only. In mustard and maize plant consortium2 ($Cons_2$) and consortium3 ($Cons_3$) were the best consortia for

rhizoremediation purpose. In mustard plant, in presence $Cons_2$ 81.93 ppm of cadmium was concentrated in root while only 5.93 ppm was present in shoot region. On the other hand in case of $Cons_3$ 98.12 ppm cadmium accumulated in soil and only 3.76 ppm was entering in shoot of mustard. In case of maize, 77.82 ppm of cadmium was accumulated in root while only 3.86 ppm was accumulated in shoot region in $Cons_2$ treatment. On the other hand, in case of $Cons_3$ 51.65 ppm and 42.27 ppm of cadmium localised in soil and root respectively (maize). For food security purpose, this technique is very useful than other biological remediation techniques because they enhanced the quality of phytoremediation.

Accumulation of cadmium in rhizospheric region was maximum in case of maize because of high quantity of biomass. The ratio of cadmium in plant to its biomass was very low and therefore it can be concluded that the amount of cadmium was almost negligible. While in case of mustard the ratio was slightly more (because of less biomass) but the cadmium tolerance level of mustard is better and therefore both mustard and maize can be effectively used for remediation of cadmium affected soil in presence of PGPR.

Overall this study concludes that the used bacterium FP_S protected the plants from the toxicity of cadmium leading thereby to a considerable increase in the biomass, root and shoot length, nutrient assimilation, chlorophyll, proline content and seed yield. In other aspect, they participate in remediation of cadmium as well as minimization of cadmium in edible parts of mustard and maize. The increased growth of *Brassica juncea* and *Zea mays* plants even in the presence of cadmium is due to several factors like production and release of plant growth promoting substances such as phyto-hormone, siderophore, HCN, NH_3 and phosphate solubilisation EPS production, Zn solubilisation by *Pseudomonas,* cadmium tolerance and accumulation ability.

Based on above properties, all the bacterial strains could be developed as bio-inoculant for enhancing growth and yield of plants as well as the phytoremediation of cadmium in soil through minimization of cadmium toxicity. Metabolites of plant growth promotory rhizobacterial strains or the strain itself is responsible for rhizospheric accumulation of cadmium and it has been proved by other researchers working with other strains of pseudomonads. Further, studies are required for exploration of more PGPRs, because in environment various potent microorganisms are presents and there is a need to explore them. More research is required in this field to know the potential of already known PGPRs and their mechanisms to support the plant growth promotory rhizobacterial (PGPR) assisted phytoremediation and further field evaluation for commercialization of the technology.

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Publication

PGPR-assisted phytoremediation of cadmium: an advancement towards clean environment

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One of the major problems, that the world is facing today due to rapid industrialization is environmental pollution caused by several factors, including heavy metals. Among the heavy metals, cadmium is a hazardous carcinogenic element. From contaminated soil, cadmium enters the plants through the roots and is accumulated in the harvestable (edible) parts, and thus gains entry into the food cycle. Phytoremediation plays a beneficial role in the remediation of cadmium contamination from soil, but becomes less effective with increasing toxicity. Even hyperaccumulator plants fail to perform under these conditions. Plant growth promoting rhizobacteria (PGPR), inhabitants of the plant rhizosphere, play a supporting role and promote bioremediation of soil by accumulation or transformation of contaminants, thereby enhancing plant growth and development. This article focuses on cadmium contamination and PGPR-assisted phytoremediation of cadmium-contaminated soils.

Keywords: Cadmium, phytoremediation, plant growth promoting rhizobacteria, toxicity.

INDUSTRIAL revolution is the main factor for metal pollution in the biosphere[1]. Heavy metal contamination is a serious environmental hazard for agricultural soils, plants, animals and human beings. The most toxic heavy metals are Pb, Hg, As, Cd, Sn, Cr, Zn and Cu[2]. These are a group of 65 metallic elements with density greater than 5 g/cm^3, exhibiting diverse properties with a potential to exert toxic effects on microorganisms and other forms of life. Among the heavy metals, cadmium has deleterious effects on agricultural ecosystem, environment and human health[3]. There are many sources that can cause cadmium contamination. They include use of Cd-containing sewage sludge, industrial emission, application of phosphatic fertilizers and municipal waste[4]. The heavy metals including cadmium are not degradable and persist in the soil for approximately 15–1100 years (ref. 5) and accumulate in the harvestable (edible) part of plants[6]. High accumulation rate generally causes growth inhibition and finally death of plant as well as cell[7].

Therefore, it is important to develop methods to remediate the heavy metal entry of toxic elements into the food chain. Various engineering methods (excavation, landfill, thermal treatment, leaching and electro-reclamation) presently being used are not fully satisfactory as they destroy the biotic and abiotic components of the soil, and are also technically difficult and expensive to use. According to Prasad[8], phytoremediation is defined as the use of plants to destroy, sequester and remove toxic pollutants from the environment. However, this method also has many drawbacks[8]. Therefore, phytoremediation associated with rhizospheric microorganisms has emerged as an acceptable agronomic remediation technology[9].

The relationships that exist between plants and microbes in the rhizosphere play a key role in enhancing the efficacy of phytoremediation[10] through a process known as 'bio-assisted phytoremediation'. In the soil, microorganisms present in and around the roots are called plant growth promoting rhizobacteria (PGPR); they use many types of mechanisms to promote plant growth and minimize stress. PGPR are helpful for plant growth enhancement and bioremediation of contaminated soil through sequestering or degrading heavy metals and other toxicants[11,12]. Bioremediation is, therefore, an option that offers the possibility to destroy or render harmless, various contaminants using natural biological activity. PGPR assist phytoremediation directly or indirectly through

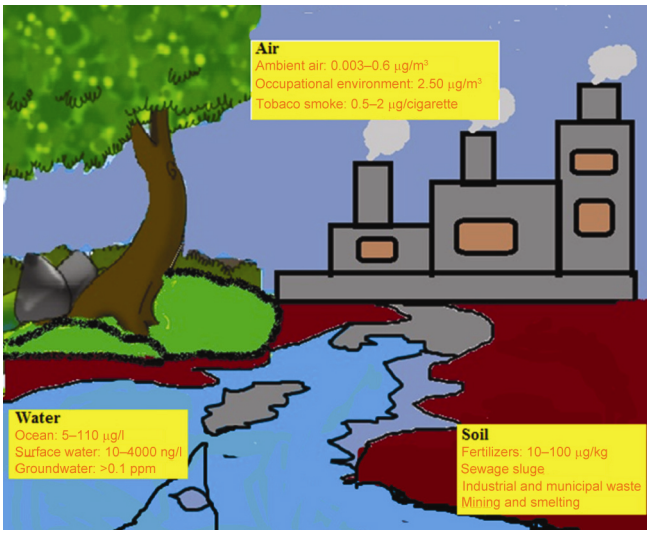

Figure 1. Level of cadmium in the environment.

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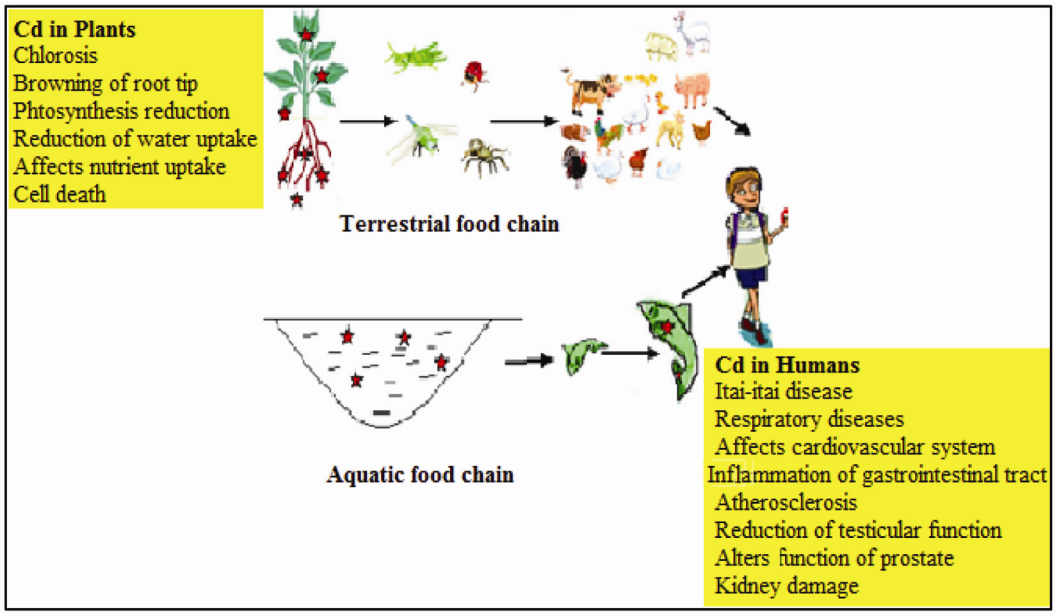

Figure 2. Movement of cadmium in the food chain and effects of the heavy metal on plants and humans.

several mechanisms, such as increased nutrient uptake, suppressing pathogens by producing antibiotics and siderophores or bacterial and fungal antagonistic substances (hydrogen cyanide, HCN), phytohormone production (indoleacetic acid, IAA) and nitrogen fixation[11–14]. The present article focuses on the role of PGPR in remediation of cadmium from cadmium-contaminated soils and enhancement of phytoremediation in hyperaccumulator plants.

Cadmium toxicity in plants and animals

Cadmium has received special attention due to high persistent properties in the environment with an extremely long biological half-life (6–38 years in the human kidneys and 4–19 years in the liver). Cadmium level is different in soil, water and air as shown in Figure 1. It causes damage by moving up the food chain and finally accumulating in human beings and causes several damaging effects (Figure 2). The World Health Organization (WHO)[15] has set-up biotoxic limits of cadmium for human beings at 100–200 μg g^{-1} wet wt. The International Agency for Research on Cancer[16] has characterized cadmium as one of the 126 priority contaminants and the US-EPA considers it as human carcinogen. Cadmium is implicated as carcinogenic, mutagenic and teratogenic for a large number of animal species above threshold limit[17]. Cadmium poisoning could affect the kidney, cardiovascular system, liver and reproductive system, and cause renal damage, osteomalacia and lung cancer. Long-term exposure to high doses of cadmium causes itai-itai disease mainly in women and is characterized by severely impaired tubular and glomerular function and generalized osteomalacia and osteoporosis[18]. About 1–2 μg of cadmium is present in cigarette smoke; 10–20% of this is introduced into the lungs of a smoker in a complex form. It affects passive smokers as well. In passive smokers it causes the risk of sudden infant death syndrome, ear disease, asthma, respiratory illnesses, lung cancer and coronary heart diseases (Figure 3).

Storage of cadmium occurs in the liver, kidney, testis, spleen, heart, lungs, thymus, salivary glands epididymis and prostate. However, 50% of the cadmium is stored in the liver and kidneys in the form of CdMT (metallothionein) complex[19]. In kidney storage of cadmium, especially in the cortical part increases with long-time exposure to low doses (below 5 μg/g of creatinine)[15,20]. After exposure to cadmium, Cd^{2+} ions are present in the form of inorganic salts, e.g. $CdCl_2$ than as CdMT complex in the liver, kidney or bones. In urine, cadmium concentration approximately 5 μg/g of creatinine is considered as a safe limit[15]. The acidic environment (pH 4.5–5.5) of the gastro-intestinal tract is favourable for cadmium transportation with the help of proton metal co-transporter DMT1 (ref. 21). Low content of nutrients in the diet increases cadmium absorption in gastrointestinal tract.

Cadmium toxicity is also responsible for the production of reactive oxygen species (ROS) and reduction of antioxidant properties at the cellular level (Figure 4). Cadmium in the environment negatively affects biodiversity and the activity of soil microbial communities[22], and results in change in the qualitative and quantitative structure of the soil[23]. Regulatory limit of cadmium in agricultural soil is 100 ppm (ref. 24). Cadmium forms complex

ions, but in a soil solution it occurs as Cd^{2+}. In plant–soil system, cadmium is the more mobile heavy metal; it easily enters into the plants and has no essential function[25]. Cadmium accumulation in plants affects root and shoot growth, inhibits nutrient uptake and homeostasis[26]. These negative effects cause physiological and morphological alteration in the cells, such as stunted growth, chlorosis and decreased reproducibility, by interacting with chlorophyll biosynthesis and biomolecules[27]. Symptoms of cadmium toxicity in plants are indicated by reduced growth, browning of root tips, chlorosis and finally death[28]. Alcantara *et al.*[29] reported that photosynthesis is affected by cadmium via inhibition of root Fe(III) reductase.

Cadmium disturbs the transport, uptake and use of various elements (K, P, Ca and Mg) and water. Reduction in absorption and transportation of nitrate from root to shoot is observed in cadmium-contaminated plants[30]. In *Silene cucubals*, reduction in the activity of nitrate reductase occurs under cadmium stress[31]. In the nodules of soyabean root, nitrogen fixation and assimilation of NH_3 are altered by cadmium toxicity[32]. It can also alter the permeability of plasma membrane and reduce water content in a cell[33]. Fodor *et al.*[34] reported that ATPase activity of plasma membrane is affected by cadmium in wheat and sunflower roots. Cadmium toxicity causes lipid peroxidation in cell membrane through reduction of functions of the membrane[34]. Cadmium toxicity also affects chloroplast metabolism by reducing the enzymes which are involved in CO_2 fixation[35].

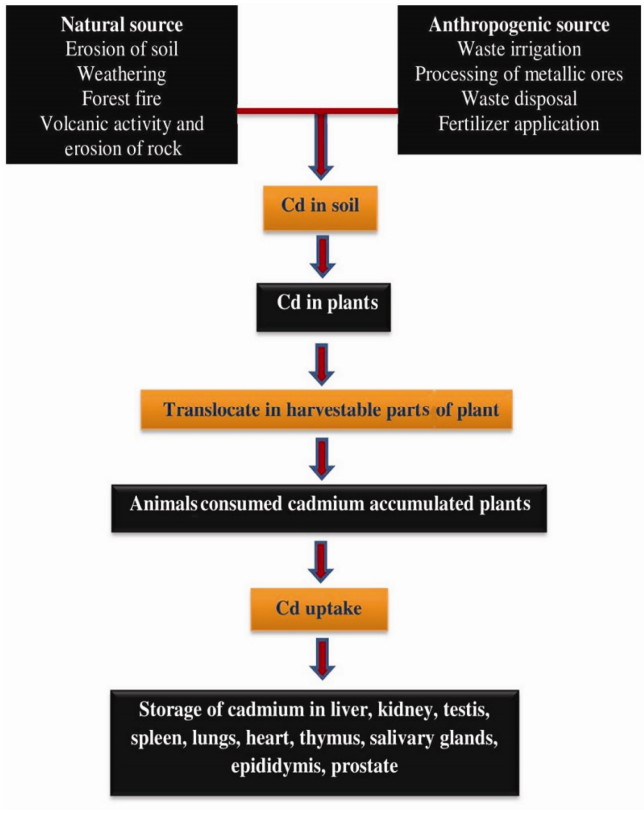

Figure 3. Transport and storage of cadmium.

Phytoremediation of cadmium and its limitations

Contamination of cadmium makes the soil unsuitable for agricultural and other uses. Therefore remediation of such soil types is important. High cost and failure or incomplete removal of heavy metals through various physicochemical and biological techniques have prompted the researchers to develop alternative low-cost methods. Phytoremediation is a novel, low cost, efficient and ecofriendly remediation strategy that has good public acceptance[36]. Many factors affect the phytoremediation efficiency such as area, contaminants, plants, etc. (Figure 5). In this process, plants accumulate high levels of contaminant heavy metals in their rhizosphere and root tissues[37]. Phytoremediation technique applied in the field using biofuel plants like maize, sunflower, soybean, barley and wheat, etc. enhances the quality of agricultural soil and make it highly relevant for agricultural use. For phytoremediation many alternate strategies can be used. The cultivation of ornamental plants, floriculture crops, tree plantations and growing of aromatic grasses was used to remediate soil[38]. However, this method is not widely accepted because of the issue of pollution transfer from soil to plant and heavy metal content in biomass[39].

Hyperaccumulator plants should be used which have high biomass production, and enhanced metal tolerance and metal uptake potential. However, most of hyperaccumulator plants are slow-growing and usually produce limited amounts of biomass. Selection of plants either as accumulators or hyperaccumulators is important in phytoremediation[40]. Plants that accumulate metals at high concentration are called hyperaccumulators[41]. If the shoots of plants contain >100 mg Cd kg^{-1}, >1000 mg Ni, Pb and Cu kg^{-1} or >10,000 mg Zn and Mn kg^{-1} (dry wt), then they are known as hyperaccumulators[42]. Hyperaccumulation is generally expressed on a dry weight basis; about 0.2% for more toxic elements like Cd, Pb, As, Hg, Cr and above 2% for the less toxic elements like Zn, Ni and Cu. There are approximately 45 hyperaccumulator plant families and 500 plants are reported in the literature – some important families are *Brassicaceae*, *Euphorbiaceae*, *Asteraceae*, *Fabaceae*, *Lamiaceae* and *Scrophulariaceae*[43].

Practical use of hyperaccumulator plants has several advantages in phytoremediation, but some properties induce limitations. These plants generally accumulate one specific element with limited root system and this limitations makes its use irrelevant[44]. In phytoremediation, various accumulator plants have high level of contaminants in harvestable parts and incineration is used after harvesting[45]. Phytoremediation is a plant-based technology that is applicable in low-concentration areas having longer

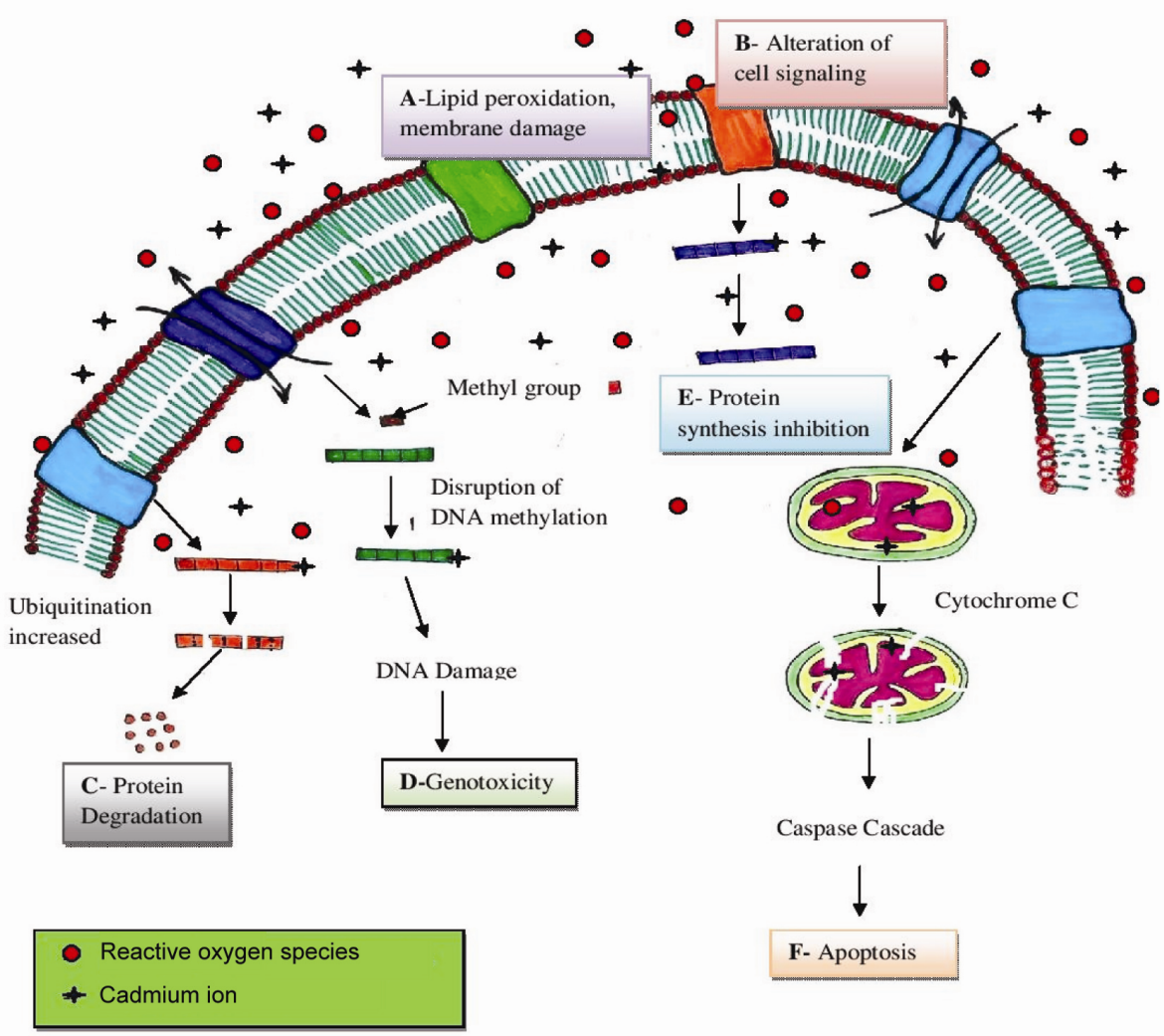

Figure 4. Mechanisms of cadmium toxicity at the cellular level.

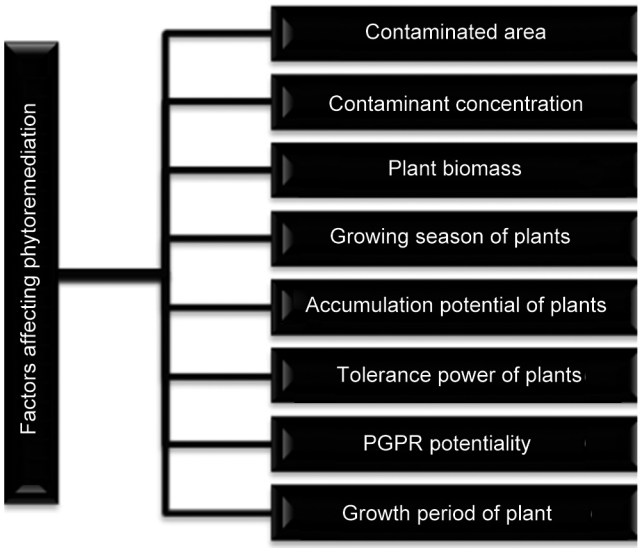

Figure 5. Factors affecting phytoremediation clean-up time.

treatment time[46]. Various mechanisms involved in this process are shown in Figure 6. Therefore, high cost is involved in traditional phytoremediation (without the involvement of microorganisms) and the owner of the polluted area does not get any benefits; he rather incurs loss. In phytoremediation, hyperaccumulator plants play an important role to enhance the removal of heavy metals from the soil through high growth rate and yield, but depletion of nutrients is responsible for reduction in plant growth under stress.

Role of microorganisms in the enhancement of phytoremediation

In recent years, bio-assisted phytoremediation or rhizoremediation plays an important role in decontamination of the soil. Rhizoremediation is the most emerging, eco-friendly and potentially effective process of biodegradation

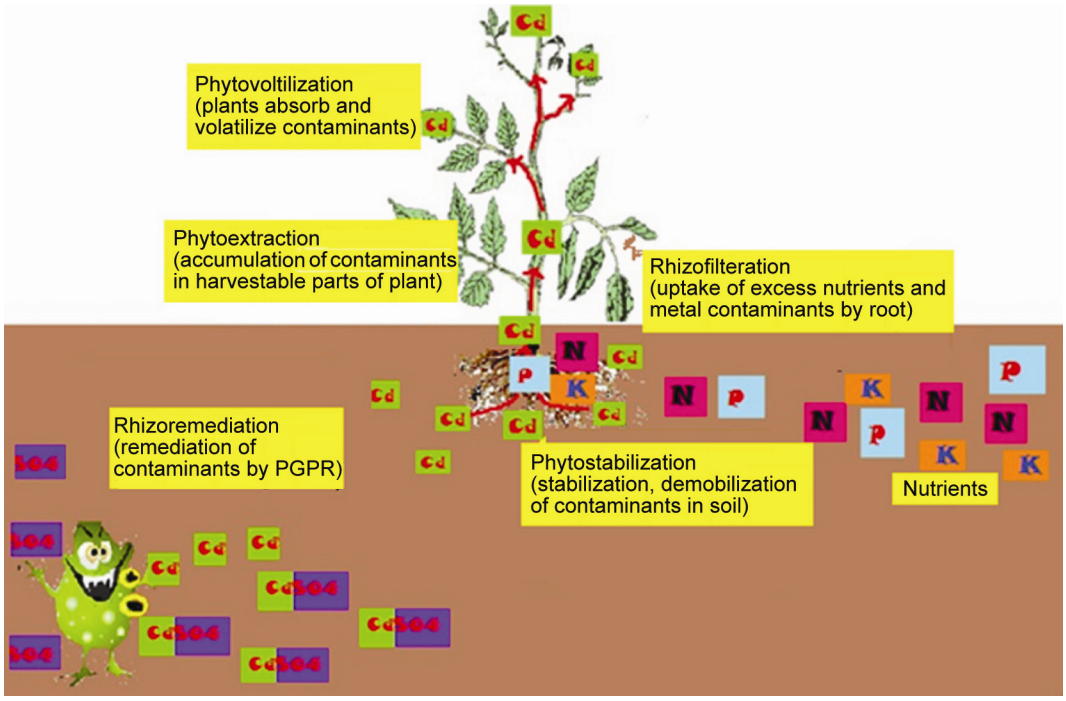

Figure 6. Mechanisms involved in the phytoremediation process.

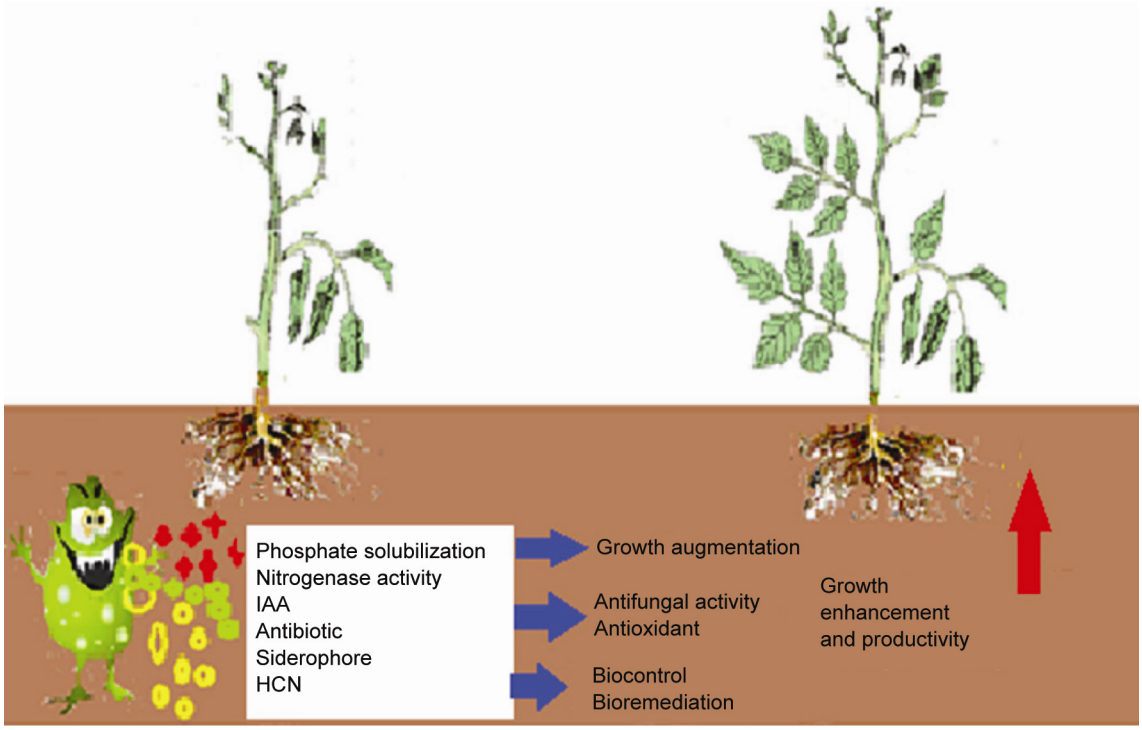

Figure 7. Mechanisms of growth promotion of plant by plant growth promoting rhizobacteria (PGPR).

of cadmium in the soil. It involves removal of specific contaminants from contaminated sites by mutual interaction of plant roots and suitable microbial species[47] (Figure 7). Rhizosphere is a micro-environment where microorganisms (PGPR) form special types of communities with plant growth promoting capabilities[48], and remove the toxic contaminants[49]. Glick[50] studied the interactions between plants and PGPR, and reported that the remediation technologies are developed by improving accumulation of metals and biomass production through

Table 1. List of phytoremediating plants and associated microorganisms with their mechanisms

Microorganisms	Plants	Metals	Mechanisms	Reference
Kluyvera ascorbata	Canola	Ni	Increased biomass; ACC deaminase	70
Psuedomonas	Canola	Cu	Increased biomass; IAA	69
Brevibacillus sp.	*Trifolium pratense*	Pb	Decreased lead uptake; IAA	71
Enterobacter aerogenes, *Rahnella aquatilis*	Indian mustard	Ni, Cr	Increased biomass and metal uptake; IAA, siderophores, ACC deaminase, phosphate solubilization	72
Achromobacterxyl osoxidans	Indian mustard	Cu	Increased root and shoot length and biomass; ACC deaminase, phosphate solubilization, IAA	73
Flavobacterium sp.	*Orychophragmus violaceus*	Zn	Increased root length, biomass, metal uptake	74
Bacillus edaphicus	Indian mustard	Pb	Increased biomass; IAA, siderophores, ACC deaminase	75
Pseudomonas putida	Canola	Ni	Increased seed germination and biomass; siderophores, IAA, ACC deaminase	76
Enterobacter sp.	Indian mustard	Ni, Zn, Cr	Increased biomass and metal uptake; IAA, siderophores, ACC deaminase, phosphate solubilization	77
Bacillus subtilis	Indian mustard	Ni	Increased nickel uptake; IAA, phosphate solubilization	78
Bacillus licheniformis, *Bacillus biosubtyl*, *Bacillus thurnigiensis*	Indian mustard	Se, Cd, Cr	Increased metal uptake depending upon specific metal–bacteria combination; mechanism unknown	79
Pseudomonas putida	Canola	Ni	Increased biomass in the field; IAA, ACC deaminase	80

the activities of rhizospheric microorganisms. According to Gadd[51], many types of bacteria improve the mobilization and immobilization of metals and tolerance power of the plants, but only few types of interactions between rhizospheric microbes and hyperaccumulating crops are important for decontamination purpose (Table 1). According to Amico *et al.*[52] soil bacteria transform metals into simple form by different types of mechanisms. Various types of soil microorganisms (PGPR) involved in rhizospheric biodegradation, and natural substances that are released by the plant roots increase the activity of these types of microorganisms[53].

PGPR were first used to promote the growth of plants, now they play a relevant role in remediation of cadmium-contaminated soils. PGPR assist in phytoremediation by the production of soluble minerals such as phosphorus and potassium[54], siderophore for iron and heavy metal chelation[14], phytohormones such as IAA and cytokinin[55], ACC deaminase for lowering stress ethylene[56], EPS and osmoprotectants[57], rhamnolipid[58] and immobilization of heavy metals[59]. Rhizobacteria such as *Pseudomonas cepacia*, *P. fluorescens* and *Streptomyces aurantiacus* were reported to increase crop yield up to 25% more than control[60]. Indian mustard and canola (*Brassica campestris*) seeds were grown in the presence of a PGPR strain in Ni, Pb and Zn-contaminated regions[61]. According to Belimov *et al.*[48], growth of canola (*Brassica napus*) plant improved on inoculating recalcitrant PGPR. Rhamnolipid is a commercially available amphiphilic biosurfactant produced by *Pseudomonas aeroginosa* (Table 2). It is extensively used in remediation of the soil through extraction of cadmium or other metals[58].

AMF are important endophytic fungi living in the roots of most terrestrial plants. They reduce metal toxicity to plants through decreasing translocation of heavy metals and their concentration[62]. In stress condition increasing interaction of plants and microbes enhances the availability of metal as well as growth of plant. According to Idris *et al.*[63] metal mobility and availability to the plants are enhanced by rhizospheric microorganisms releasing chelating agents, acidification, phosphate solubilization and redox changes. PGPR affect the bioavailability of cadmium and other metals by secretion of various metabolites such as siderophore, rhamnolipid, EPS and organic acids like oxalic acid, malic acid and citric acid, which chelate the cadmium ions and reduce their toxicity (Figure 8). Vulcanizing bacteria produce H_2S and alter the bioavailability of cadmium by precipitation of metal[64].

Cadmium bioavailability in rhizosphere region can be reduced by application of clay and modified clay minerals to cadmium and other heavy metal contaminated sites[65]. This method reduce cadmium toxicity in the plants as well as the rhizosphere region by accumulation of the heavy metal. Mycorrhizal species improve the bioavailability of toxic metals by affecting the root–rhizosphere system[66]. Siderophore, an iron-chelating complex synthesized by the PGPR helps in chelating iron,

Table 2. List of cadmium hyperaccumulating plants and associated microorganisms

Microorganisms	Plants	Mechanisms	Reference
Variovoraxparadoxus, *Rhodoccus* sp., *Flavobacterium* sp.	Canola	Increased root length; IAA, siderophores, ACC deaminase	61
Pseudomonas putida KNP9	Mung bean	Increased biomass and decreased metal uptake; siderophores	81
Rhizosphere bacteria	Graminaceae grasses	IAA, siderophore, ACC deaminase	52
Pseudomonas putida	Sunflower	Increased cadmium uptake and decreased toxicity; bacterium expresses a metal-binding peptide	64
Pseudomonas brassicacearum, Pseudomonas marginalis	Pea	Increased biomass and nutrient uptake; ACC deaminase	82
Pseudomonas sp., *Bacillus* sp.	Canola	Increased biomass and metal uptake; IAA	83
Mesorhizobium huakuii	Chinese milk vetch	Increased metal accumulation; bacterium expresses phytochelatin and metallothionein	84
Burkholderia cepacia	*Sedum alfredii*	Increased biomass, metal uptake and translocation of metal to shoots	85
Bacillus sp.	Canola, Corn, Sudan grass, tomato	Some increased biomass and cadmium uptake, IAA, siderophore, biosurfactant production	86
Pseudomonas aeruginosa	Black gram	Increased biomass and rooting, and decreased cadmium uptake; IAA, siderophore, ACC deaminase, phosphate solubilization	87
Pseudomonas sp, *Bacillus* sp.	Tomato	Increased root length, aboveground biomass and aboveground metal; siderophore, IAA, ACC deaminase	88
Streptomyces tendae	Sunflower	Decreased metal uptake and increased iron content; siderophores	89

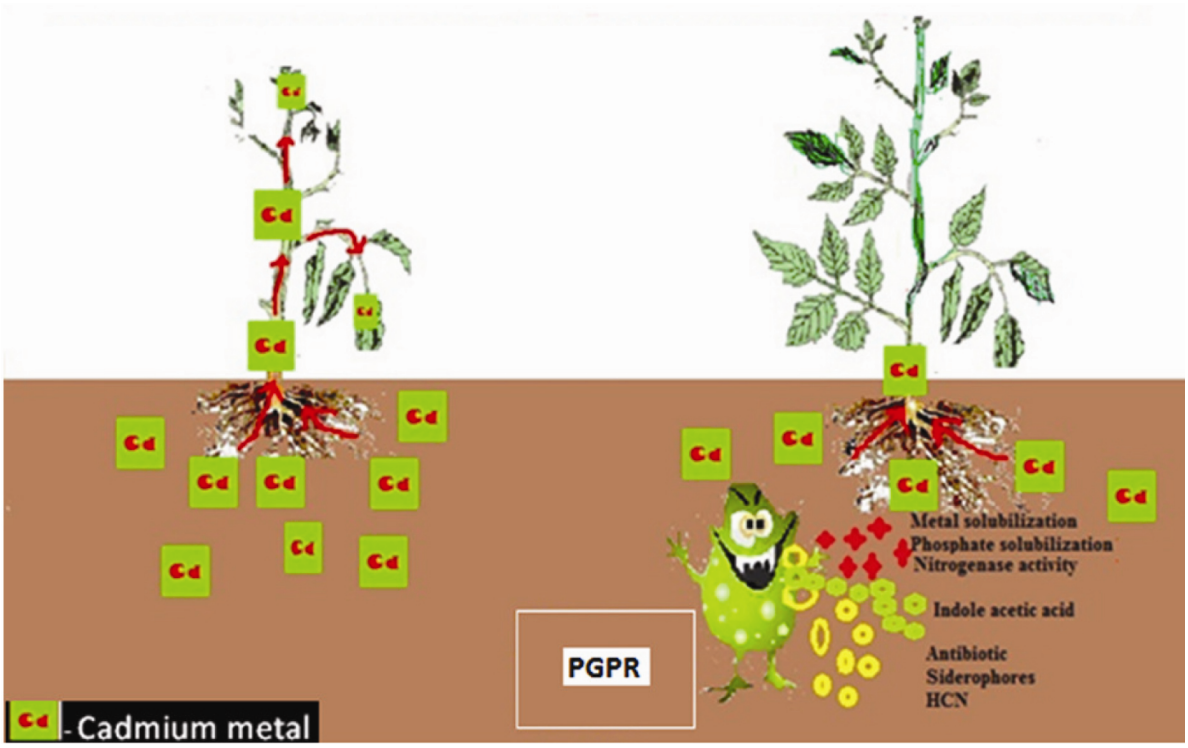

Figure 8. Mechanisms of PGPR action in the improvement of phytoremediation.

especially under cadmium and other heavy metal stress conditions. Microbial siderophore is more potent than phyto-siderophore for chelation of iron and heavy metals. So, under stress conditions, where phyto-siderophores fail to sequester iron for the plants, microbial siderophores help the plants avoid chlorotic conditions of leaves by improving chlorophyll synthesis through chelation of iron.

Siderophores also form complexes with heavy metals like Cd, Al, Cu, Ga, Pb, Zn, radionuclides, including U and Np (refs 67, 68). Binding of the siderophore to a metal increases the soluble metal concentration and hence bacterial siderophores help alleviate the stress imposed on plants by high levels of these heavy metals in the soil[14]. Ethylene is important for normal plant development, as well as for their response to stress. However, high levels of ethylene lead to inhibition of root elongation. PGPR strains possessing ACC deaminase activity get bound to seeds or roots of seedlings and can reduce the amount of plant ethylene by breaking it into ammonia and alpha ketogutarate, thereby reducing the extent of its inhibition on root elongation[56]. Growth of crop plant is improved by PGPR that help in decreasing the plant stress related to phytoremediation methods[69]. Selection of highly potential microbial combination is a big challenge for developing phytoremediation strategies. Once PGPR are established in the rhizospheric zone, native plants do not require fertilizers, pesticides or excess water; they restore wetlands and other habitats, and are helpful for creating natural parks, sanctuaries and other green areas.

Conclusion

In the natural environment, cadmium has several deleterious effects on the diversity of flora, fauna and microbial communities. Contamination of agricultural soils by cadmium results in its easy entry into the food chain, thereby affecting animal and human health. Various conventional strategies discussed in this article have some disadvantages and even green technology using plants alone, sometimes fails. Under these conditions, PGPR assist plants in remediation of cadmium from the contaminated environment (abiotic stress management) by production of various metabolites. These metabolites relieve plants from stress by various mechanisms such as supplying nutrients like iron and phosphate, lowering of stress ethylene, promotion of apical growth, etc. in normal as well as under stressed conditions. PGPR also help in biotic stress management in the rhizospheric zone of plants (indirect plant growth promotion–hydrogen cyanide production, rhamnolipids and other biosurfactants for biocontrol of pathogens) and enhance plant growth and biomass (direct plant growth promotion by modulating plant growth hormones and solubilizing phosphate, sequestering nutrients like iron, nitrogen, phosphorus and other essential minerals from the environment) which are key factors for further extraction of cadmium from contaminated soils by plants. Siderophores synthesized by these PGPR also improve bioavailability under normal as well as cadmium or other heavy metal stress conditions. Microorganisms isolated from cadmium metal stress sites are more adapted to peculiar soil environment and can be commercially and effectively used to assist in phytoremediation. On the basis of the above discussions, we can conclude that PGPR-assisted phytoremediation technique (Rhizoremediation) for treating cadmium-contaminated sites/soils is useful with high acceptance compared to other methods.

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Chapter 7

Genetically Modified Organisms (GMOs): Utility, Prospects and Challenges in Bioremediation of Environmental Pollutants

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SUMMARY

Rapid industrialization and agricultural practices are responsible for the release of huge amount of pollutants in the environment. All these are mutagenic and carcinogenic in nature with persistent property or degrade very slowly as well as cause a variety of serious toxic effects in living beings. Currently, bioremediation is a highly promising eco-friendly cost effective technology to remediate these pollutants. In this process, microorganisms use their enzymes to degrade the pollutants or enhance degradation rate either *in situ* or *ex situ*. The rate of biodegradation not only depends on structure of contaminant and soil, but also on catabolic potential of microorganisms. In this regard, the use of genetically modified organisms (GMOs) is increasing in the recent years. GMOs are applied in various fields such

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as human health, agriculture, medicine, bioremediation and in many other industries. Better understanding about degradative mechanisms, respective enzymes, biochemical reaction of microorganisms may provide a good opportunity for the construction of the potent engineered microorganisms. However, engineered microorganisms are not so applicable in degradation under the field trial because of many types of risk associated with this technology. Therefore, this chapter reviews the construction and applications of GMOs in commercial scale as well as in the bioremediation of organic and inorganic pollutants for environmental sustainability.

Introduction

Modernization is a consequence of progress in science and engineering. Every day, new inventions are occurring in science and engineering. We could hardly control over the technologies because they have turned up our hope and expectations. Today one of the hot issues is the use of genetically modified organisms (GMOs) in our daily life. GMOs can also be referred as genetically engineered microorganisms (GEMs) or genetically altered organisms (GAOs), all of these terms are interchangeable. In the natural environment, genes are changing, exchanging and manipulating (mutation) every day. Human beings are exploiting these processes for their own purposes for centuries. For example, everything we eat is derived from livestock, crops and microorganism. DNA has never been static neither in natural nor in the artificial environment (Jones, 1999). Genetic modification also allows the individual genes to be specifically switched off, through the antisense approach.

GMOs are living organism (bacteria, virus, plants and animals) whose genetic material is manipulated in laboratory by the genetic engineering for commercial purpose. Genes with specific traits synthetically move from one organism to other organisms are well known and called as transgene. Transgene are novel, synthetic genes that have never existed in nature; minimally, they are composed of a target gene sequence flanked by a promoter and other elements that may come from different organisms (Snow, 2003). Transgenic organisms are often called as "Genetically Modified Organisms" (GMOs). It is the engineering aspect of transgenic organisms that distinguish them from previous varieties (Snow, 2003). Boyer and Cohen were the first to manipulate gene in the year 1973 using biotechnological techniques (Cohen *et al*., 1973). From 1976, the technology became commercialized, with companies producing and selling the genetically modified foods and medicines. The first genetically engineered crops were tobacco plants, which

was manipulated in 1986 to resistant against the herbicides. In 1995, FDA approved GE corn, soy, cotton, canola, potato, squash and tomato for commercial purposes.

However, the GMOs are highly promising in the bioremediation of environmental pollutants. Environmental pollutants can be divided into organic and inorganic pollutants. Generally, arrays of microbes are employed for the bioremediation of environmental pollutants. The degradation and detoxification of pollutants mainly depends on the metabolic potential of microbes, mainly the enzymes and genes, which are responsible for the catabolism of pollutants. Microbes mainly utilize the pollutants as a carbon and energy source. Organic pollutants can be easily and completely degraded and detoxified by microbes. However, the bioremediation of inorganic pollutants (heavy metals) is not an easy task for microbes because of the existence of heavy metals in different oxidation states (Garbisu and Alkorta, 2001). Bioremediation is refers to a process that uses microorganisms and their enzymes to promote degradation and removal of contaminants from the environment. Microorganisms decompose or transform hazardous substances into less toxic metabolites or degrade to non-toxic end products. Further, the metabolic engineering/modification of microbes (modification in genes responsible for the catabolism of pollutants), mainly bacteria may a suitable approach for the enhanced bioremediation of environmental pollutants. Therefore, this chapter provides details on the construction and use of GMOs in bioremediation of environmental pollutants. Further, the risk and challenges associated with the use of GMOs for bioremediation are also discussed.

Approaches for the Construction of Genetically Modified Organisms (GMOs)

Genetic modification has been made possible because of similar chemical content of DNA present in all organisms. Due to similarity in DNA material, gene of two organisms can be cut (restriction enzymes) and rejoined with the help ligases. Such type of gene manipulation is possible in extra-chromosomal genetic material called as plasmid DNA. With the help of restriction and ligase enzymes, foreign genetic material can be inserted into the plasmid DNA and after modification recombinant plasmid is mixed with host bacteria for the intake under appropriate conditions (Jones, 1999). This modified plasmid multiplies within the bacteria in appropriate conditions and the product of modified gene can be used for commercial purposes.

Nowadays, the genetic engineering is a modern technology, which is used to design the microorganisms, having capacity to degrade specific contaminants. This technology gives an opportunity to make artificial set of genes that do not exist together in the natural environment. Genetic

engineering techniques include engineering with single genes or operons, pathway construction and alterations in the sequences of existing genes (Dale and Park, 2007). In environment, many types of organisms are found that are used in genetic engineering technology. But bacteria, belonging to the genus *Pseudomonas* are especially the major object of genetic manipulations for environmental purposes. Chromosomal and plasmid DNA of *Pseudomonas* may carry genes for metabolism of contaminants. Therefore, such microorganisms are the important source of catabolic genes for genetic engineering (Davison, 2005). Williams and Murray reported the first catabolic plasmid TOL (117 bp) from *Pseudomonas putida* mt-2. Later, various plasmid-born catabolic genes for the degradation of contaminants were reported that are often located in transposons for example, in Tn4653 from *P. putida* mt-2, Tn4655 from *P. putida* G7 and Tn4656 from *P. putida* MT53 (Top *et al*., 2002). In genetic engineering, plasmids are commonly used as cloning vectors to multiply or express the particular genes**.** The scheme for the construction of a recombinant cell or a GMO and its application in different sectors has been well shown in Fig. 1.

Identification of Suitable Organisms

This is the first approach for the construction of GMOs and is done by modification with applicable genes. For example, those microorganisms that are fully adapted for terrestrial environment may not be able to survive sin aquatic environment and cannot be used relevantly in different environments. Therefore, aquatic microorganisms can be used for construction of GEMs to remediate aquatic sites. The application of such organisms would minimize the supplementation of nutrients to the inoculated environment and reducing the costs incurred and maintenance that is required in this process.

Pathway Construction, Extension and Regulation

In development of GEMs, catabolic pathway is enhanced or extended for the degradation of some other pollutants, which are not easily degraded by wild strain. Due to gene sequence alteration, the efficiency and efficacy of catabolic pathways can be improved and constructed GEMs would have high degradation abilities than others.

Enzyme Specificity and Affinity Modification

Enzymes are synthesized by transcription and translation of specific genes and control the metabolic pathways. GMOs alter the enzymatic activity and enzyme substrate specificities. GMOs are synthesized by hybrid gene clusters, which encode the enzyme transforming capability.

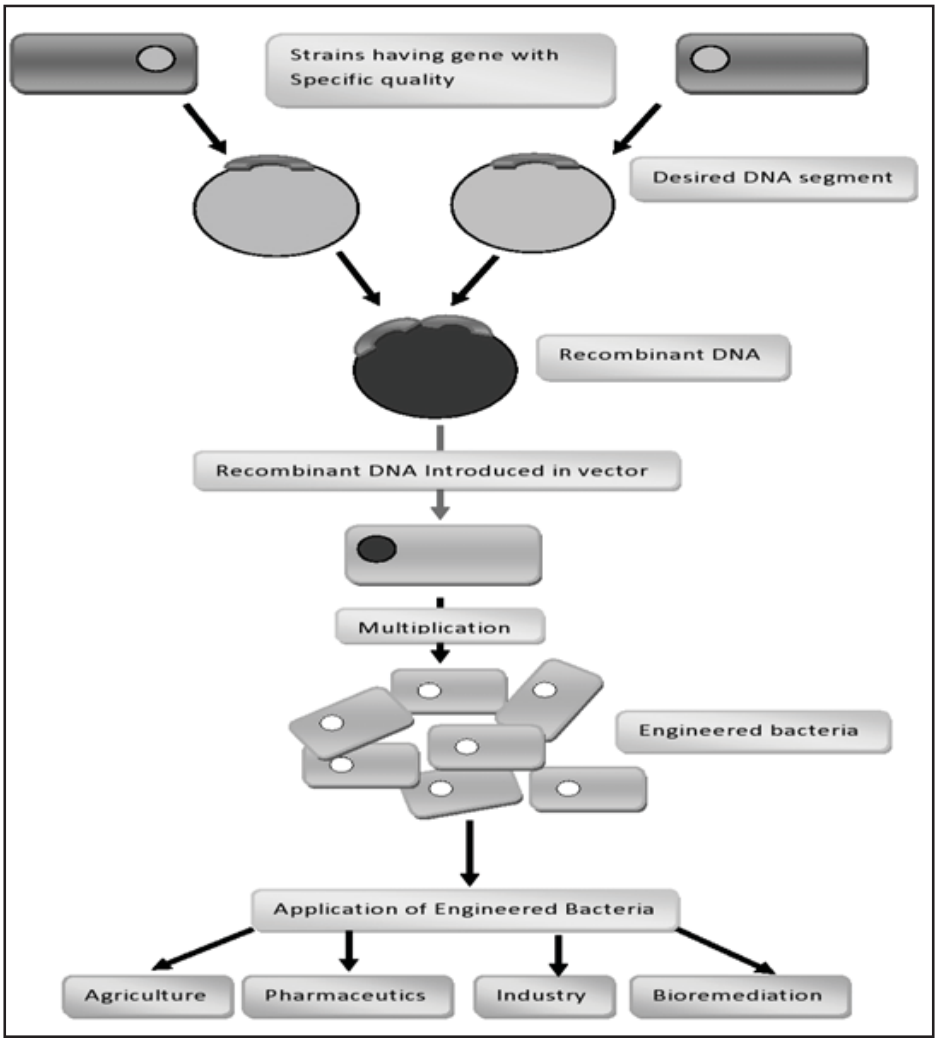

Fig. 1: Construction of genetically engineered bacteria and their application

Selection and Identification of GMOs

Presently, various molecular techniques are used for the selection and identification of GMOs such as FISH (fluorescent in situ hybridization), *in situ* PCR (in situ polymerase chain reaction), DGGE (denaturing gradient gel electrophoresis), TGGE (temperature gradient gel electrophoresis), T-RFLP (terminal restriction fragment length polymorphism) and ARDRA (amplified rDNA restriction analysis). All these methods are based on the detection of specific DNA or RNA sequences, especially conservative fragments in bacterial 16S rRNA (Urung-Demirats *et al*., 2006; Mrozik and Pitrovaskya, 2010).

Application of Genetically Modified Organisms in various Fields

Genetically altered microorganisms, transgenic plants and transgenic animals have finded many applications in different fields as presented in Table 1 & 2. However, a list of transgenic crops approved for commercial use in USA is presented in Table 3.

GMOs in Bioremediation of Environmental Pollutants

A variety of contaminants such as pesticides, dyes, hydrocarbons, heavy metals and radionuclides are discharged from industries and other sources into the environment. Nature has its own quality to remediate environmental contaminants with the help of natural sources such as microorganisms, plants etc., but rapid industrialization has resulted in enhanced pollution above the tolerance limit. Therefore, there is an urgent need of safe and economic methods to clean the environment. Various types of physical, chemical and biological methods, which are reported by various researchers to remediate the contaminants (organic and inorganic) are presented in Fig. 2, but they have many limitations and drawback with high cost value.

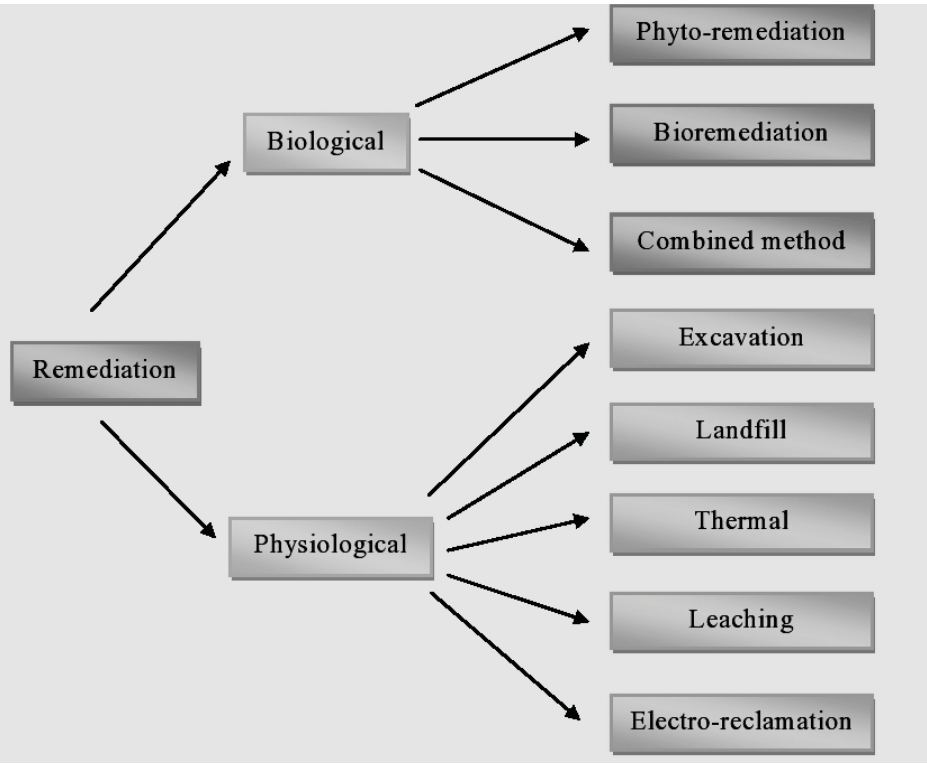

Fig. 2: Different types of remediation method applied for environmental contaminants

Table 1: Approved transgenic products being used in various sectors globally

Product	Therapeutic indication	Produced in host cells	Brand name	Year of approval
Interferon IFN- α2a	Hairy cell leukemia	E. coli	Roferon A	1986
IFN- β1b	Multiple sclerosis	E. coli	Betaferon	1995
IFN- ϒ1b	Chronic granulomatous disease	E. coli	Actimmune	1990
Interleukin IL-11	Prevention of chemotherapy-induced thrombocytopenia	E. coli	Neumega	1997
OspA(lipoprotein)	Lyme disease vaccine	E. coli	Lymerix	1998
Combination vaccine containing HbsAg	Vaccination against hepatitis B, diphtheria, tetanus and pertusis	*S. cerevisiae* as one component	Tritanrix-HB	1996
Vaccine Hepatitis B surface antigen	Hepatitis B prevention	S. cerevisiae	Merck	1986
Platelet derived growth factor (PDGF)	Lower extremity diabetic neuropathic ulcers	S. cerevisiae	Regranex	1997
Glucagon	Hypoglycemia	S. cerevisiae	Glucagen	1998
Human growth hormone	hGH deficiency in children	E. coli	Nutropin Genotropin Humatrope	1994 1995 1987
Insulin Insulin Aspart. Insulin lispro	Diabetes mellitus	E. coli	Insuman Novorapid Humalog	1997 1999 1996
Streptokinase Tissue plasminogen activator	Actual myocardial infarction	E. coli	Haverkinase Rapilysin	1990 1996
Recombinant hirudin	Prevention of venous thrombosis	S. cerevisiae	Revacs	1997

Table 2: Approved therapeutic products being used in India

Product Name	Company	Trade name
Human insulin	Eli Lily Knoll (Boots) (Novo Nordisk) Hoechst Marion Roussel Sarabhai MJ Pharmaceuticals Ltd.	Humalog, Human Insulin Lispro Actrapid Insuman Prodica, Repodice, Repromax, Z insulin -
Hepatitis B vaccine (All recombinant surface antigen protein based)	Neon Lab Ltd., Transgene Vaccine M/S V H Bhagat Smithkline Beecham Bharat Biotech International Cadila Healthcare Panacea Biotec Ranbaxy Wockhardt Shantha Biotechnics	- - - Engerix B - HB Vac Enivac-HB - Biovac-B Shanvac
Human growth hormone	Eli Lilly Ranbaxy Ltd. Elvina Lab Ltd. Serum Institute Novo Nordisk	Humatrope Genotropin Saizen Norditropin
Human interleukin	Wyeth Lederle Ltd. Ambala Sarabhai Enterprises Ltd.	- -
Granulocyte colony stimulating factor (GCSF)	Rhone Poulenc Fullford	Granocyte -
Tissue Plasminogen Activator	German Remedies Ltd. Boehringer Ingelheim	ACTILYSE™ -
Follicle stimulating hormone	Serum Institute of India Infar India Limited	Gonal-F Recogon
α-Interferon	CIMMCO Lupin Labs Shantha Biotechnics Fulford (India) Ltd.	- - Virferon -
Streptokinase	CIMMCO Kee Pharma	Streptase™ (Herberkinase) -

Bioremediation is a biological method and has many advantages over all physico-chemical methods of remediation. In bioremediation process, microorganisms or their enzymes are used for the degradation and or removal of contaminants from environment. Bioremediation is an eco-

Table 3: List of transgenic crops approved for commercial use in USA

Product Name	Genetically altered traits with introduced genes along with origin of genes	Company	Product Name	Year of Approval
Canola	Altered oil composition 12.0 acyl carrier protein thioesterase gene from *Umbellularia californica*	Calgene Inc	Laurical™	1995
Cotton	Bt gene incorporated plants CRY1 A (c) gene from *Bacillus thuringiensis kurstaki*	Monsanto Co.	Bollgard™	1995
	Resistant to bromoxynil A nitrilase gene from *Klebsiella ozaenae*	Calgene Inc	BXN Cotton™	1995
	Resistant to glyphosphate Enolypyruvylshikimate-3-phosphate synthase gene from *Agrobacterium sp.* CP4	Monsanto Co.	Round up ready™	1996
Maize	Bt gene incorporated CRY 1A (b) gene from *Bacillus thuringiensis kurstaki*	Ciba-Geigy Crop	Maximizer™	1995
	Glufosinate resistant phosphinothricin acetyl transferase gene from *Streptomyces viridochromo*	AgoEvo Inc.	Liberty Link™	1996
	Bt gene incorporated (resistant to cornborer) CRY 1A (b) gene from *Bacillus thuringiensis kurstaki*	Monsanto Co.	Yield Gard™	1996
Papaya	Resistant to virus coat protein gene of p type of PRSV HA 5-1-from Hawaii	Cornell Unvi. USA	-	1997
Potato	Bt gene incorporated CRY III (A) gene from *Bacillus thuringiensis tenebrionis*	Monsanto Co.	New leaf ™	1995
Soyabean	Resistant to glyphosphate Enolpyruvylshikimate-3-phosphate synthase gene from *Agrobacterium sp. CP4*	Monsanto Co.	Round up ready™	1995
Squash	Resistant to viruses coat protein genes of watermelon mosaic virus 2 and Zucchini yellow mosaic virus	Asgrow Seed Co.	Freedom II™	1995
Tomato	Delayed ripening, gene sequence for polygalactouranase production in tomato rearranged and reversed to minimise its expression by antisense technology	Calgene Inc.	Flavr savr™	1995

friendly and low cost technology for the remediation of pollutants as compared to other physical, chemical and biological processes (Perpetuo and Sauza, 2011). Biodegradation is one of the most recommended cost effective and environment friendly technology, which minimize the pollution problem by using microorganisms and their enzymes to improve the degradation and remediation of pollutants from environment. Biodegradation is an enzyme catalyzed reduction in complexity of chemical compounds (Alexander, 1994) and at "mineralization" process, biodegradation is complete. In this mechanism, various microorganisms consisting of bacteria, fungi and yeasts can degrade, transform or accumulate the organic and inorganic substrates e.g., hydrocarbons, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), radionuclides and heavy metals by metabolic or enzymatic mechanisms and these mechanisms are based on growth and co-metabolism.

Table 4: Some major environmental pollutants and their toxicity

Pollutant	Toxicity
Heavy Metals	ROS production, membrane damage, protein modification, DNA inactivation, alteration of cellular antioxidant system, destabilization of bio-molecules, mutagenesis, genetic disorder and cancer etc.
Hydrocarbons	damages system of body like nervous, circulatory, reproductive, endocrine etc., liver and kidney damage, cause tumor and cancer, accumulate in body etc.
Pesticides	Accumulate in fatty tissues, cancer, thyroid disruption, neurodegenerative disease and reproductive disorder etc.
Dyes	Cancer, hypersensitivity, behavioral defects and nervous disorder etc.

Biodegradation is mostly applied in respect of ecology, waste management and often related with remediation of environment (Marinescu *et al*., 2009). Biodegradation process is completed by three methods such as: (a) Natural attenuation (no human involvement, microorganisms reduce the contaminants), (b) Biostimulation (systems effectiveness is improved by improving the nutrients and oxygen supply) and (c) Bioaugmentation (effective microorganisms are introduced into the environment). For remediation of environmental contaminants, a remedial technology first requires microorganisms, which have high capability for quick adaptation and utilization of pollutants as a sole source of carbon and nitrogen in a reasonable time (Seo *et al*., 2009). Genetic engineering is a highly relevant technology to achieve the useful microbes for the removal of contaminants from the environment for sustainable development. The efficiency of bioremediation can be improved by developing GMOs with unique characteristics.

Table 5: GMOs reported for the bioremediation of organic pollutants

GMOs	Introduced gene(s)	Organic pollutants	Reference
Pseudomonas florescens HK44	*luxCDABE*	Naphthalene	Strong *et al*. (2000)
Burkholderia cepacia L.S.2.4	pTOD plasmid	Toluene	Barac *et al*. (2004)
Escherichia coli AtzA	Atrazine chlorohydrolase	Atrazine	Strong *et al*. (2000)
Pseudomonas putida KT2442(pNF142::Tn*Mod* OTc)	pNF142 plasmid, *gfp*	Naphthalene	Filonov *et al*. (2005)
Pseudomonas florescens F113rifpcb*rrnB*P1::*gfp*mut3	operon *bph*, *gfp*	Chlorinated biphenyls	Boldt *et al*. (2004)
Rhodococcus sp. RHA1(pRHD34::*fcb*)	*fcbABC* operon	2(4)-chlorobenzoate2(4), 2(4)-chlorobiphenyl	Rodrigues *et al*. (2006)
Burkholderia cepacia VM1468	pTOM-Bu61 plasmid	Toluene	Taghavi *et al*. (2005)
Comamonas testosteroni SB3	pNB2::*dsRed* plasmid	3-chloroaniline	Bathe *et al*. (2009)
Pseudomonas putida PaW85	pWW0 plasmid	Petroleum	Jussila et a. (2007)
Pseudomonas putida PaW340(pDH5)	pDH5 plasmid	4-chlorobenzoic acid	Massa *et al*. (2009)
Escherichia coli JM109 (pGEX-AZR)	Azoreductase gene	Decolorize azo dyes,C.I. Direct Blue 71	Jin *et al*. (2009)

Adapted from Wasilkowski *et al*. (2012).

Environmetal pollutants are highly toxic in nature and causes severe toxic effects in the environment. The toxicity of major environmental pollutants is summarized in Table 4. Major biodegradable contaminants in environment comes either from natural or artificial origin or among all artificial substances, the most toxic substances are BTEX (benzene, ethylbenzene, toluene and xylene), chlorophenols, nitrophenols, PAHs (polychlorinated biphenyls polycyclic aromatic hydrocarbons), organic solvents and heavy metals. All of these contaminants have persistent properties and remain in environment for several years with carcinogenic and mutagenic effects. Organic compounds such as fuels, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides and dyes are highly toxic contaminants. Further, the GMOs, which are reported

for the bioremediation of organic pollutants, are summarized in Table 5. However, radionuclides and heavy metals like Cd, Hg, As, Pb, Zn, etc. are some other chemicals that are extremely resistant to biodegradation through existing flora as compared to naturally occurring organic compounds that are readily degraded, when enter into the environment. The GMOs, which are reported for the bioremediation of inorganic pollutants, are summarized in Table 6.

Table 6: GMOs reported for the bioremediation of inorganic pollutants

Bacteria	Modified gene expression	Heavy metals	Reference
Deinococcus radiodurans strains	Hg (II) resistance gene (merA)	Hg (Radioactive waste sites from nuclear weapons)	Brim *et al*. (2000)
P. fluorescens 4F39	Ni transport system	Ni (In laboratory)	Lopez *et al*. (2002)
Mesorhizobium huakuii B3	Phytochelatin synthase (PCS) gene expression	Cd^{2+} (From rice fields)	Sriprang *et al*. (2003)
P. putida strain	Chromate reductase (ChrR)	Cr (Bacterial cultures as well as cell suspensions)	Ackerley *et al*. (2004)
Acidithiobacillus ferrooxidans strain	Hg ion transporter gene expression	Hg (In laboratory)	Sasaki *et al*. (2005)
Achromobacter sp AO22	Hg reductase expressing mer gene	Hg (In situ bioremediation of contaminated sites)	Ng *et al*. (2009)
Methylococcus capsulatus (Bath)	CrR genes for Cr (VI) reductase activity	Cr (VI) (Cell-associated Cr removal in laboratory conditions)	Hasin *et al*. (2010)
Caulobacter crescentus JS4022/p723-6H	RsaA-6His fusion protein	Cd (II) (From the bacterial growth medium)	Patel *et al*. (2010)
E. coli strain	Metalloregulatory protein ArsR (overexpressing ELP153AR)	As (Contaminated drinking and ground water)	Kostal *et al*. (2004)
B. subtilis BR151 (pTOO24)	Luminescent Cd sensors	Cd (Naturally polluted soils)	Ivask *et al*. (2011)
Sphingomonas desiccabilis and *Bacillus Idriensis* strains	Overexpression of ArsM gene	As (Laboratory conditions)	Liu *et al*. (2011)
Ralstonia eutropha CH34	Metallothionein (MT)	Cd^{2+} (In laboratory)	Valls *et al*. (2000)

Adapted from Singh *et al*. (2011)Several mechanisms such as uptake, adsorption, methylation, oxidation and reduction are involved in the protection of microorganisms from heavy metal toxicity. The different mechanism for the bioremediation of heavy metals is presented in Fig. 3. Dissimilatory reduction process is involved in reduction of metals (Fernandez *et al*., 2012), in which bacteria utilize metals as terminal electron acceptors for anaerobic respiration. In addition, there are other mechanisms are also reported in bacteria that are not coupled to respiration e.g., reduction of Se(VI) to elemental Se, reduction of Cr(VI) to Cr(III) under aerobic or anaerobic conditions, reduction of Hg(II) to Hg(0) and reduction of U(VI) to U(IV).

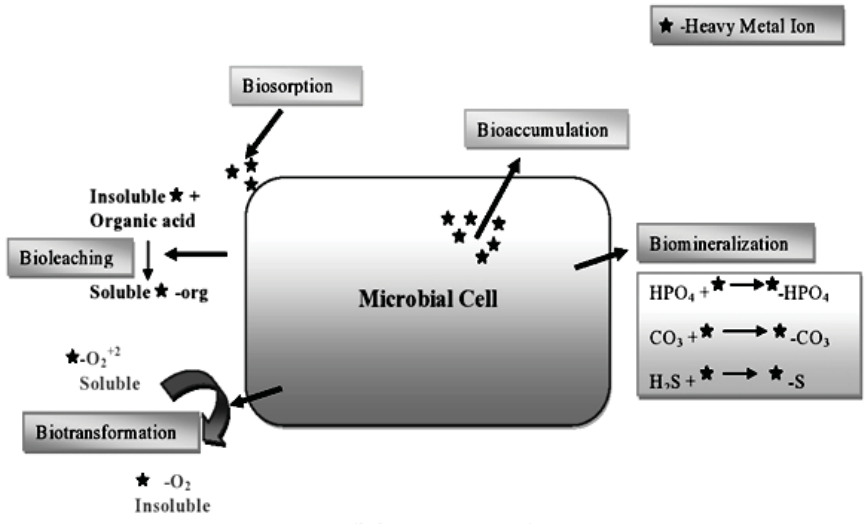

Fig. 3: Bioremediation mechanisms used by microbial cell to remediate heavy metals from contaminated site

In degradation of pollutants, bioaugmentation and biostimulation methods are very significant for enhancement of recovery of polluted sites. In 1970s and 1980s, scientist worked on cloning of genes that encoded catabolic enzymes for persistent compounds and applied in various fields. The fusion of traditional biochemistry, ecology, microbiology and genetic engineering is a very auspicious solution for bioremediation. In present time, many microbiologists and molecular biologists realize about the potential of genetic engineering to improve *in-situ* biodegradation (Cases and Lorenzo, 2005). GMOs have capability for bioremediation of soil, activated sludge and groundwater, and showed the increased degrading capabilities for a wide range of chemical contaminants (Sayler and Ripp, 2000). GMOs have various types of applicability in several fields such as human health,

agriculture, bioremediation and in various industries (Cases and Lorenzo, 2005). Biosafety and risk assessment is required for releasing genetically modified microorganisms for bioremediation. At this duration, intensive study of metabolic potential of microorganisms and development of genetic engineering technique gives an idea to design the genetically modified microorganisms (GMMs).

The construction of pollutant degrading GMMs is possible because of several degradative pathways, enzymes and their respective genes and biochemical reactions are well understood. This study gives an idea for development of GMMs with new metabolic pathways and this is an innovative approach to remediate the toxic organic compounds from environments (Cases and Lorenzo, 2005). In 1981, *Pseudomonas aeruginosa* (NRRL B-5472) and *Pseudomonas putida* (NRRL B-5473) were first patented as genetically modified strains in USA. They were developed by Chakrabarty in early 70s and have genes for naphthalene, salicylate and camphor degradation. In addition, the naphthalene-degrading *Pseudomonas fluorescens* HK44 was the first genetically engineered microorganism containing pUTK21 plasmid (Sayler and Ripp, 2000). Jussila *et al*., (2007) studied the possibility of plasmid pWW0 transfer from *Pseudomonas putida* PaW85, which plays an important role in the degradation of petroleum hydrocarbons by rhizosphere bacteria.

Generally, in presence of toxic compounds in soil, plant growth-promoting bacteria (PGPB) are not able to promote plant growth (Cases and Lorenzo, 2005; Pimentel *et al*., 2011). For controlling these, Yang *et al*., (2011) tried to develop genetically modified bacteria that could degrade phenol and enhance the maize growth simultaneously. The recombinant P13 was constructed through horizontal gene transfer between *Pseudomonas aeruginosa* SZH16 that was not able to promote plant growth and *Pseudomonas fluorescens* having no ability to degrade phenol. In other study, Barac *et al*., (2004) transferred the toluene-degrading plasmid pTOD from donor *Burkholderia cepacia* G4 into natural endophytic strain of yellow lupine *Burkholderia cepacia* L.S.2.4 through genetic engineering to improve the efficiency of toluene detoxification. The results explained that recombinant bacteria had capability for toluene degradation as well as reduced transpiration rate. There are several strategies reported that optimize the bioremediation mechanism of contaminants. One approach is bio-augmentation, which enhances the growth and population of pollutant degrading microorganisms. This mechanism can be completed either by introducing microorganisms that naturally contain catabolic genes or those that have been genetically modified organisms (GMOs).

Improvement of Remediation in Plant Growth Promoting Rhizobacteria

Use of recombinant endophytic and rhizospheric bacteria in plant-associated remediation of hazardous compounds in agricultural and non-agricultural soil is recommended as best alternative for the remediation of contaminated soils (Diyva and Deepak, 2011). For selecting the favorable strain to construct recombinant bacteria, many criteria are used, out of which some are as given:

- Target gene shows high expression and after cloning, the strain should be stable
- The strain ,which is used that should be tolerant or insensitive for the contaminant;
- Some strains can grow only in several specific plant rhizosphere (Huang *et al*., 2004).

In rhizoremediation, rhizospheric microorganisms are targeted for the degradation of pollutants. There are many bacteria present in the rhizospheric region of plants having less ability to degrade the organic pollutants. To solve this problem, genetic engineering is used by many workers to engineer the rhizobacteria with the pollutant degrading genes to start the rhizoremediation (Glick, 2010). Sriprang *et al*., (2003) used molecular mechanisms to degrade contaminants such as trichloroethylene (TCE) and PCBs.

Challenges Associated with the use of GMOs in Bioremediation Processes

The specific qualities of biotechnological applications have clearly necessitated the construction of recombinant strains to tackle the new challenges in environment. Field release of GMMs for bioremediation with a satisfactory degree of environmental assurance is major concern. There are many substances that are degraded or transformed by microorganisms such as synthetic compounds and other chemicals (hydrocarbons and heavy metals) with eco-toxicological effects. Many of the genetically altered microorganisms are helpful in bioremediation process in laboratory condition, but when these strains are applied in natural environment, they fail to perform such functions (Sayler and Ripp, 2000). There are many physical, chemical and biological constraints responsible for the failure of genetically modified microorganisms in environment. Adaptation and competition with other organisms is one such major factor, which can be managed by using indigenous microbial population with genetically altered traits for enhanced biodegradation. Fast growing and biomass producing organisms can be better performers. According to Wackett, (2004), the bacterial strains *Pseudomonas, Rhodococcus* are fast growing and support the biodegradation in natural environment.

Use of Stable Isotope Probing (SIP) and Equivalent Methods in Microbial Ecology to Determine the Performance of Engineered Bacteria in Environment

There is a need to examine the performance of engineered bacteria in terms of their potential of horizontal gene transfer, which may affect the indigenous microflora. However, there is no evidence that the release of recombinant bacteria in environment for bioremediation has caused adverse impact on the natural microbial community, but survival and performance of the genetically engineered bacteria (GEB) in complex natural environment (Singh *et al*., 2011). In some cases, when natural or recombinant bacteria are introduced into the environment, they show little differences due to unfamiliar territory. Iwasaki *et al*., (1993) reported that introduction of bacterial biomass in an existing niche may develop a pleasant niche for protozoa, which inhibit the bacterial growth above certain level. An innovative approach is required to avoid these issues and regarding this problem encapsulation of the inoculums in a polymeric matrix or protection in plastic tubing is used (Foster *et al*., 2002).

Development of Containment System (S-GEMs) to Reduce the Risks Associated with GMOs

There are two avenues regarding the release of GEMs in the environment: First is to complete the task prescribed by the designer and second is the elimination from the environment (GAO, 1988). In order to overcome above limitations, researchers have developed various constructs termed ''Bacterial Containment Systems'' that would resolve the unlimited proliferation and survivability of GEMs and also restrict horizontal gene transfer to the existing microbial community (Kolata, 1985; Diamand, 1999; Molin *et al*., 1993; Atlas, 1992). Bacterial containment system was designed by exploring the knowledge of several plasmid addiction systems and a wide range of catabolic regulatory gene(s).

Matin, (1994), reported that stationary phase promoters or starvation promoter have an important role in respect to above problem and the expression of biodegradation genes can be artificially uncoupled from growth. In addition to these problems, Pandey *et al*., (2005) gave a new concept to construct the "suicidal-genetically engineered microorganisms" (S-GEMS) for minimization of contaminants to achieve the applicable and safer remediation of contaminated sites. In this model, the cognate antidote would be expressed under the tight control of a promoter that would be inducible with the pollutant, while the killer gene would be continuously expressed. In this killer system, as soon as the pollutant is sensed by the S-GEM (the inducible promoter), antidote synthesis would continue and neutralize the lethal action of toxins.

However, after depletion of pollutant or lowering down to a level below the detection limits of the S-GEM, the antidote synthesis would be checked, causing immediate cell death. In alteration to these several promoter-antidotes fusion systems would be placed on the chromosome, which will result in cell death on degradation of pollutants. This development is applicable for those sites polluted with heterogeneous mixture of contaminants and would not be useful in case of random mutations in the antitoxin or its regulatory circuit because constitutive expression of the killer genes would lead to immediate cell death in such a case. Use of two killer-anti killer systems would reduce the threat of 'escapers' since simultaneous inactivation of both constitutively expressed killer genes by random mutations would have an extremely low probability.

Horizontal gene transfer (HGT), is another major issue related with the release of GEMs that could also be reduced by applying the killer and antidote gene(s) in a plasmid and chromosome of S-GEM in question respectively. Plasmid containing killer gene is a rare event of horizontal gene transfer (HGT), which would cause immediate death of recipient due to the constitutive expression of the killer gene. A field application of *Pseudomonas fluorescens* HK44 for bioremediation is very successful, when conducted on moderately large-scale and under controlled conditions (Ripp *et al*., 2000). However, the future implementation of genetically engineered bacteria for decontamination will not be free from the challenges related with their release in the environmental conditions. The major issues in complete bioremediation technology exist for aggressive field conditions in case of engineered microbes. Besides, the molecular techniques are generally limited for only few well characterized bacteria such as *E. coli*, *Pseudomonas putida*, *Bacillus subtilis*, etc. and there is further scope for the construction of genetically modified microorganisms from other bacterial strains.

Conclusion

At present, genetically engineered organisms are being applied in various fields such as in agriculture, human health, bioremediation, industries like pharmaceuticals, food, textile etc. Now-a-day's bioremediation of organic and inorganic pollutants is a big issue for environmentalist. Bioremediation is a complex process and is completed by many stages. Due to rapid industrialization, concentration of contaminants is increasing day by day and it is not possible to remediate all the contaminant from environment through biodegradation/bioremediation. To enhance the degradation efficiency of microorganisms, genetic engineering is a relevant method. This technique is successful in laboratory scale under favorable conditions, but unfortunately, there are many drawbacks and certain limitations when GEMs are applied in field due to various factors such as competition with

the native microflora (Biological constrain), insufficient supply of essential nutrients, unfavorable external environment (Physical constrain) and less bioavailability of contaminants. So there is a need to solve this problem and environmental biotechnology is helpful for this. In spite of these, there are many risk associated with the use of GEMs in field. To cope up with the risks associated with release of genetically modified organisms in the environment, researchers have come out with a new concept "suicidal genetically engineered microorganisms" (S-GEMS), which will be self destroyed after the complete degradation of pollutant. However, more research is required with other potent microorganisms including plant growth promoting rhizobacteria, which can be genetically altered for the effective remediation of environmental pollutants.

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REVIEW

Bacterial biosurfactants can be an ecofriendly and advanced technology for remediation of heavy metals and co-contaminated soil

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Abstract Environmental pollution due to heavy metals has become a significant drawback as a result of their ecotoxicity. Hence, their remediation is of pressing concern. Many technologies are planned for their remediation; however, most of them are highly expensive and result in incomplete removal of contaminants. So, massive attention has paid to the event and application of the latest biologically techniques, that is effective in remedy and cost, not harming the prevailing surroundings. Hence, application of biosurfactant in heavy metal remediation is one among the recent ecofriendly technique. The present review critically highlights bacterial biosurfactants as a best alternative technique for heavy metals remediation. The review also emphasizes that bacterial biosurfactants can open up a new vista in remediation of metal-contaminated soil.

Keywords Heavy metal · Biosurfactant · Remediation · Soil washing · Soil flushing · Co-contaminated soil

Introduction

The fate of heavy metals is of immense environmental concern due to their persistent occurrence in nature and toxic properties. Heavy metals are electronegative elements with a density greater than 5 g/cm^3 (Duffus 2002). They are non-biodegradable in nature which is the main reason responsible for their prolonged persistent in the environment, and as a result, they pass from one level to another in the food chain causing many diseases and blocking the biological pathways (Tangahu et al. 2011). Comparably, accumulations of toxic heavy metals in soil and water bodies also have a detrimental effect on the ecosystem (Baeicii and Stotzky 1983; Sobolev and Begonia 2008). Hence, the presence of trace amount of heavy metals in the soils has been found to have serious hazardous effect. There are various techniques for remediation of heavy metal such as physical, chemical, biological and phytoremediation, but most of them are quite expensive and risky. So, large amount of attention has been paid on the development and implementation of new biologically techniques, which should be effective in remediation, easily available, not harm the existing environment, ecofriendliness and cost-effectiveness an alternative of conventional techniques, which are efficient at lower levels of contamination. Hence, application of biosurfactant in remediation of heavy metals is one of the recent ecofriendly techniques. Biosurfactants are diverse group of surface-active compound produced by microorganisms, which possess both hydrophilic and hydrophobic moieties. Structurally, they possess a hydrophobic moiety comprising of saturated or unsaturated fatty acids or hydrocarbon chains and a hydrophilic moiety of peptide cations or anions, mono-, di- or polysaccharides acid (Kiran et al. 2010; Muthusamy et al. 2008). Biosurfactants are potential compounds use in environment management, food industry, petroleum industry, pharmaceutical industry and other industries as these are environment friendly, easily degradable, economical and stable at elevated pH, temperatures and salt concentrations as compared to their

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Review Paper

Arsenic in the Environment effectuates Human Health: An Imperative Need to Focus

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Abstract

Arsenic is omnipresent in our environment and humans are always and inevitably revealed to this toxic metalloid. Smelting and mining and processes contribute to arsenic contamination because arsenic is a natural component of copper, zinc, lead, and gold ores. Arsenic Exposure is increasing in our environment day by day which directly or indirectly leads to health defects contributing highest carcinogenic and non-carcinogenic effects on human. The present review focuses on the Arsenic in environment, its health defects and prevention to control the exposure.

Keywords: Arsenic, environment, carcinogenic, non-carcinogenic.

Introduction

Arsenic is an element belongs to heavy metal group having properties of metal and non- metal and widely spread earth's crust[1]. It is an omnipresent element found in the soil, natural waters, atmosphere, organisms and rocks and marshaled through natural processes like biological activity weathering reactions, volcanic emissions and through anthropogenic activities[2]. Insecticides, wood preservatives, pesticides livestock dips and herbicides are anthropogenic sources applied to soil and plant system. Arsenical pesticides undiscriminating use during the early to mid 1900s results extensive deterioration of soils quality worldwide[3]. Smelting and mining processes impart arsenic contamination since arsenic is a natural constituent of zinc, lead, gold and copper ores. Arsenic subsists in −3, 0, +3 and +5 oxidation states. Arsenious acids, dimethylarsinic acid, arsenites, arsenates, methyl arsenic acid, arsenic acids and arsine etc are different form of arsenic found in environment. Arsenic (III) is a hard acid and form complexes with nitrogen and oxides, whereas arsenic (V) behaves is a soft acid forms complexes with sulfide. Arsenic has two valency states, trivalent and pentavalent, which can from a vast range of compounds either in organic and inorganic forms. Dimethyl arsenic acid and Monomethylarsenic acid are the organic forms of arsenic whereas arsenite and arsenate are inorganic forms arsenic. Arsenate and arsenite are more toxic than dimethyl arsenic acid and monomethyl arsenic acid. World Health Organization (WHO) has set a standard for arsenic in drinking water i.e. 10 µ g/l (0.01 mg/l), but increased Arsenic (As) level in groundwater is a major health concern in Asia and world now days. Arsenic is ubiquitous in our environment and humans are always and unavoidably exposed to this toxic metalloid. It may enter into human body by accidental ingestion from a variety of environmental sources like soil, water, air and food.

Arsenic in the Environment

Arsenic in Water: Increased concentration of arsenic in groundwater is an environmental concern due to its risk poses to animals, plants and human health. The EPA is in the process of setting the new arsenic standard for drinking water at 10 ppb (µg/L) to protect humans against the effects of long-term, chronic exposure to arsenic in drinking water. Approximately 5% of community water systems serving 11 million people will have to take corrective action to lower the current levels of arsenic in their drinking water. Higher concentration of arsenic are reported in groundwater sources as compared to surface-water sources and arsenate comprises about 50 percent of the total Arsenic in groundwater[4-6]. Many countries such as Argentina, Bangladesh, Chile, Hungary, India, Taiwan and Mexico have accounted wide arsenic contamination in their groundwater supplies. Arsenic contaminated groundwater has also been accounted in the Mid-west, New England, California and Oklahoma and in Bangladesh it is reported to contaminate up to 2mg/L. Utilization of contaminated water for irrigation purpose has resulted elevated concentrations of arsenic in agricultural soils [7-11]. In northern La Pampa Province of central Argentina arsenic contamination in groundwater surmount the World Health Organization (WHO) suggested value of 10 µg/L. Groundwater arsenic correlated positively with alkalinity, pH, V and F, whereas weaker correlations were observed with Mo, B, Be and U.

Arsenic in soil: Soil with arsenic levels below 40 mg/L is considered to be normal soil and distribution of arsenic in the soil is anthropogenic or natural.Arsenic toxicity soils acquaint

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Comparative Study of Co-Resistance Pattern of Bacteria Isolated from Waste Water of Hospital Discharge and Soil of Industrial Area

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ABSTRACT

The rise of heavy metal pollution and antibiotic resistant bacteria in soil and water is a key challenge to worldwide public health. The aim of current study is assessment of microbial load and co-resistance (cadmium and antibiotics) of bacteria isolated from the water of hospital waste discharge and soil of industrial of Lucknow and Allahabad (U.P. India). Soil samples were collected from different location of Uttar Pradesh. The samples were analyzed for presence of microbial load by heterotrophic plate count (HPC) ranged from 160cfu/ml–400x10^5cfu/ml. Isolated bacteria assessed for multiple antibiotics and heavy metal resistance. The study was carried out on the basis of maximum threshold limit for the cadmium by the isolates. MIC test of isolates was performed for detection of tolerance against cadmium metal. From 34 potent isolates only ten isolates (AIA_2, AIA_4, $BBAU_2$, $BBAU_4$, SIA_2, BSS_1, BSS_2, BSS_4, HWS_2, and HWS_1) were able to grow at high cadmium level. While, out of ten only two isolates BSS_2 and BSS_4 found to grow at maximum threshold limit at 1100ppm. In antibiotic resistance test all the isolates had resistant property against cpm and most of the heavy metal resistant isolates had high multiple antibiotic resistant (MAR) index value. High MAR values were seen in BSS_2 and AIA_4 isolates.

Keywords: *Heavy metal, Co-resistance, Pollution, Antibiotics, MAR (Multiple Antibiotic Resistance), Cadmium*

INTRODUCTION

Today industrialization and release of uncontrolled hospital waste are very big challenges to environmentalist. They are responsible for heavy metal pollution and evolving of multiple drug resistant (MDR) bacteria. Heavy metals arise unsurprisingly in rocks and soil, but concentration is often eminent as a result of pollution. They are also called as trace elements, which are toxic towards living organisms on extreme concentration. However, some metals including Zn, Mn etc., are worked as micronutrients at significant concentration. At elevated concentrations, these micronutrients damage DNA and membrane as well as thrashing of functions of enzyme. Heavy metals like As, Cd, Ni, Hg and Pb cause oxidative stress, lipid peroxidation, carcinogenesis, and neurotoxicity on flora and fauna at low concentrations[1]. Out of these heavy metals cadmium is one of the most toxic metal present in earth crust. Many types of anthropogenic activities are responsible for cadmium contamination such sources are the non-ferrous metal industry, mining, production, use and disposal of batteries, metal-contained wastes and sludge disposal and use of pesticides and phosphate fertilizers lead to dispersion

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Review Paper

Utilization of Distillery Waste Water in Fertigation: A Beneficial Use

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Abstract: *Distilleries are largest polluter discharging a huge amount of waste water called spent wash, which are difficult and costly to treat and dispose. Quality and the characteristics of molasses spent wash were depending on the method of processing. Disposal of waste water from industries environment regulatory authorities have strict rule. When distillery waste water discharged in water bodies causes environmental pollution because it contains high organic and inorganic content which results in depletion of oxygen because of microbial growth in water and causing widespread mortality of aquatic organisms. In soil distillery waste reduces the soil alkalinity and inhibits the seed germination, growth and development. Fresh water is consumed at every step of alcohol manufacturing and processing. Due to high organic and inorganic content if distillery waste water used after treatment and dilution, then it gives very relevant results for use of distillery in irrigation and this method of irrigation is called as fertigation. Fertigation is an emerging field for agricultural purpose because it applies water and fertilizer simultaneously. Use of distilleries waste water in fertigation could lead to proper management of waste water and conservation of ground water. This focused on distillery effluent, its composition and application of distillery effluent as irrigational water and fertilizer without any adverse effects.*

Keywords: Distillery, Waste water, Fertigation, Fertilizer, Water quality

Introduction

One of the most important environmental problems faced by the world is the pollution that is mostly generated by industries. India is the most sugar producing country in world in recent time and integrated with distilleries and distillery waste have hazardous effects. Hence, safe disposal of distillery effluents is an important need to reduce the hazardous effects of distillery waste, mostly in India. Bagasse, press mud and molasses are waste material produced in sugar manufacturing process mostly in sugar cane producing rural areas. Bagasse used in paper industry and in boilers as fuel, press mud has indirect use in industry and molasses is most significant and economically important due to its cheapness and availability used as a raw material in distillation of alcohol production [1]. Alcohol production mainly concentrated in states of U.P., Karnataka and Maharashtra.

Distilleries is the most polluting agro based industry in the world and produced a huge quantity of brown colored effluent called as spent wash, which require safe disposal and have a large amount of organic and inorganic load. In distillation processes raw molasses diluted with water up to sugar level 15%, pH 4-4.5, then nutrient and yeast suspension added for alcohol production by microbial activity and the fermented liquid containing alcohol and waste material. Molasses spent wash is the most difficult waste products to dispose [2] because of low pH, high temperature, dark brown color, high ash content and high percentage of dissolved organic and inorganic matter. Fresh water is consumed at various stages of alcohol manufacturing process such as yeast preparation, dilution of molasses, bottle washing, adjusting the alcohol to the required strength for potable purpose and dilution of treated effluent [3].

For disposal of waste water from industries environment regulatory authorities have strict rule. In India CPCB constituted task force on CREP (Corporate Responsibility for Environmental protection) this stated that distillery industry had been told to achieve zero discharge of spent wash by Dec. 2005 (According to charter of CPCB 2003). In India there are around 400 distillery units with total production capacity of about 3800 million liters of alcohol [4]. On one liter of alcohol production around 8-12 liters waste water are produced depending on process and quality of molasses, etc.[5]. Further, a maximum of $15m^3$ of effluent can be generated per kilolitre of alcohol produced according to the Water (Prevention and Control of Pollution) Cess Rule, 1978 specified by the Government of India. The pollution

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RESEARCH ARTICLE

Comparative Study of PGPR Isolated from Crop Plants (Mustard and Maize) and Wild Medicinal Plant (Lantana) and their Potency for Enhancement of Wheat Plant

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are those beneficial bacteria which colonize the rhizosphere region of the root and increase the plant growth activity by the various mechanisms. PGPR induced the production of plant hormones (IAA), ammonia, siderophore, HCN and phosphate solubilisation to enhance the plant growth and development. The aim of this study was to isolate the microorganisms from rhizosphere soil of crop plant (Mustard and maize) and wild medicinal plants (Lantana) of different areas of Lucknow and Kanpur (UP, India). Out of thirty strains, three were giving best PGPR result in which two isolates from wild plant (VY_1 and RC_2) and one from crop plant (PM_1) were selected for the pot experiment. Subsequently, an experiment was conducted in plastic cups containing soil in which seeds of wheat were sown in each cup and treated with selected PGPR to analyze the effect of PGPR on the growth of wheat (*Triticum* sp.) plant. Present study results that PGPR of wild plant give the significant result with increasing the shoot length, root length and dry weight than crop's PGPR. Hence, it is expected that in future PGPRs of wild plant is also very effective as other PGPR and are used as bio-fertilizer to enhance the growth and yield of plants.

Key words: PGPR, IAA, Soil microorganisms, Phosphate Solubilisation, Lantana, Wheat.

INTRODUCTION

Lanatana camara (Lantana) is a type of an ornamental plant which is used in traditional medicine for the treatment of various diseases (Banik, 2007). All the parts of lantana (root, stem and leaves) have various medicinal value and they contain several compounds like allelopathic, antimicrobial, nematicidal and insecticidal activities (Achhireddy & Singh, 1984; Begum *et al*., 2000; Abdel-Hady *et al*., 2005; Marongiu *et al*., 2007; Sharma *et al*., 2007). In agricultural field various studies has been done and some are in working condition on the side effects of chemical fertilizer. Hence, on the basis of present literature and data we can say that intensive introduction of chemical fertilizers in agricultural field causes the reduction of crop productivity and yield. This destructive effect is exhibit through the changing of physicochemical properties of soil and other biological changing (Adediran *et al*., 2004). For controlling these destruction microorganisms work as relevant agent by promoting agricultural yield and productivity and minimize the use of chemical fertilizer and pesticides.

Microbes affect the plant growth in various ways some microbes cause diseases and inhibit plant growth; whereas others can directly or indirectly promote the plant growth through a various mechanisms such as Nitrogen fixation, Phosphate solubilisation, Production of siderophore, phytohormone and ACC deaminase (Glick 2003; Bais *et al*., 2006). A large array of bacterium including species of Pseudomonas, Azospirillum, Azotobacter, Burkholderia, Bacillus, Klebsiella, Enterobacter, Alcaligens, Arthrobacter, and Serratia have reported as plant growth promoting rhizobacteria to enhance plant growth (Kloepper *et al*., 1989). Theses microbes inhabiting in and around the root and enhance the soil qualities also

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Isolation and characterization of heavy metal resistant PGPR and their role in enhancement of growth of wheat plant under metal (cadmium) stress condition

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ABSTRACT

In present time excessive use of chemical fertilizers in agricultural field causes the environmental hazards and affects the human and animal health. There is an urgent need to reduce the application of chemical fertilizers. Plant growth promoting rhizobacteria (PGPR) is the best alternative of chemical fertilizers, present in root zone of plant and enhanced the plant growth in stress as well as the normal condition. This study was done on heavy metal resistant PGPR, isolated from heavy metal contaminated soil of industrial and agricultural area of Lucknow, Kanpur and Ambedkar Nagar. Out of 27 isolates only 6 were grown on high concentration of cadmium metal. Isolate PBB_1 showed 1000 ppm MIC of cadmium and remaining isolates namely PP_3, PP_2, SNA_3, and SNA_5 were grown on 800 ppm MIC of cadmium. PGPR screening was done for selection of best PGPR to enhance the growth of wheat plant by production of IAA, ammonia and phosphate solubilization. The pot study was done with 100 ppm cadmium amended soil. When cadmium resistant PGPR were applied on seeds of wheat the growth and germination of plants were enhanced. On comparison of finding result with literature, it may be possible that all the isolates may be fluorescent pseudomonads. The result of pot study showed that the Pseudomonas sp. SNA_5 gave the best result in comparison of other isolates.

Keywords: Cadmium, PGPR, *Pseudomonas*, Heavy metal resistant microbes, Pollution

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INTRODUCTION

Heavy metal pollution is increases day by day due to industrialization in all over world. Pollution is generated due to metallic ferrous ores mining and smelting, fossil fuels, sewage, municipal wastes, pesticides and fertilizers [1]. Among all the heavy metal cadmium (Cd) is a very poisonous metal and has 7th rank in all 20 toxin categories because it is highly toxic for cellular enzymes [2]. According to Naidu et al., [3] cadmium shows toxicity against humans, animals, and plants, with long biological life and it alters the cell differentiation, proliferation, apoptosis, and improves activation of oncogene in carcinogenesis mechanisms. In environment sources of cadmium contamination in soil are usage of industrial effluents, phosphatic fertilizers, and municipal sewage sludge and city compost in agricultural field [4, 5]. Alloway [6] said that in human approximately 70% of cadmium intake is occur through vegetable foods and cadmium stay in environment for several years.

In cadmium contaminated soil, microorganisms evolved several mechanisms by which they can survive in stress environment such mechanisms are metal exclusion by barrier of permeability, cellular sequestration that is