ISOLATION AND IDENTIFICATION OF HEAVY METAL RESISTANCE BACTERIA FROM RHIZOSPHERE AND SCREENING FOR THEIR PLANT GROWTH PROMOTING POTENTIAL

A dissertation submitted in partial fulfillment of the requirements for the degree of Master of Philosophy

Farzana Ahmad

MZU Registration No:1505351

M.Phil. Registration No:MZU/M.Phil./532 of 17.05.2019

Department of Biotechnology

School of Life Science

Mizoram University

October 2021

ISOLATION AND IDENTIFICATION OF HEAVY METAL RESISTANCE BACTERIA FROM RHIZOSPHERE AND SCREENING FOR THEIR PLANT GROWTH PROMOTING POTENTIAL

BY

Farzana Ahmad

Department of Biotechnology

Bhim Pratap Singh

Submitted

In partial fulfillment of the requirement of the Degree of Master of Philosophy in Biotechnology of Mizoram University, Aizawl.

DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF LIFE SCIENCES
MIZORAM UNIVERSITY
(A CentralUniversity)
TANHRIL, AIZAWL – 796004: MIZORAM, INDIA

Dr. Bhim Pratap Singh,Ph.D, PDF (UK)*Assistant Professor*

Phone : 0389-2330859/2330861(O)
Fax : 0389-2330642 (O), 09436353807
E-mail : bhimpratap@mzu.edu.in
bhimpratap@gmail.com

CERTIFICATE

This is to certify that the dissertation entitled **"Isolation and identification of heavy metal resistance bacteria from rhizosphere and screening for their plant growth promoting potential"**submitted to the Mizoram University for the award of Master of Philosophy in Biotechnology by **Farzana Ahmad** Registration no. MZU/M.Phil./532 of 17.05.2019,Research scholar in the Department of Biotechnology,is a record of research work carried out by her during the period from 2018 to 2020 of study, under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma or other similar titles in this University or any other University or institution of higher learning.

It is further certified that the scholar fulfilled all the requirements as laid down by the University for the purpose of submission of M. Phil. dissertation.

Date:

Place: **Bhim Pratap Singh**

DECLARATION OF THE CANDIDATE

Mizoram University

January 2021

I Farzana Ahmad, hereby declare that the subject matter of this dissertation is the record of work done by me, that the contents of this dissertation did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/ Institute.

This is being submitted to the Mizoram University for the degree of Master of Philosophy in Biotechnology.

Farzana
Ahmad

(Candidate)

Prof. N Senthil Kumar

(Head, Department of Biotechnology)

Dr. Bhim Pratap Singh

(Supervisor)

ACKNOWLEDGEMENT

First and foremost, I am grateful to the Almighty God, giving me His blessings and good health and strength to undertake this research task and enabling me to its completion. I would like to pay my deepest gratitude to my supervisor **Dr. Bhim Pratap Singh** for his guidance, support, and help and for inspiring me throughout my research work, and also for providing all the required facilities to complete my research work.

I would like to thank Dr. Th Robert Singh, Head, Department of Biotechnology, Mizoram University for his kind cooperation and all the faculties and technical staff of the Department for their constant help and support.

I express my heartiest gratefulness to Dr. Gajanan Mehetre for assisting me and my lab members Purbajyoti Deka, Yogesh Malvi, Lalrokimi, Lallawmsangi,Ruth and William for their cooperation throughout my research.

At last, I would like to thank all my adorable parents and all my family members for their love and support.

BIODATA

NAME **:** Farzana Ahmad

DATE OF BIRTH **:** 7th October 1992

EDUCATIONAL QUALIFICATIONS

2015 Bachelor of Science (B.Sc)

Institution : Manipur University, Imphal

Specialisation : Biotechnology

2018 Masters of Science(M.Sc)

Institution : Mizoram University

Specialisation :Biotechnology

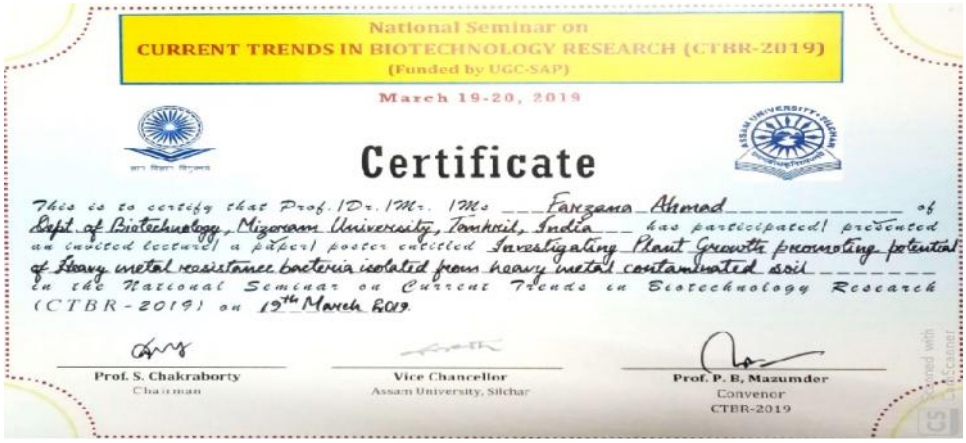
Master of Pholisophy (M.Phil)

Institution : Mizoram University

Registration no : MZU/M.Phil./532of 17.05.2019

Conference/Seminar/Workshops attended

- Presented a paper entitled "**Investigating Plant growth Promoting potential of heavy metal resistance bacteria isolated from heavy metal contaminated site**" in the national seminar on Current trends in biotechnology Research (CTBR-2019) at Assam University, Silchar


National Seminar on
CURRENT TRENDS IN BIOTECHNOLOGY RESEARCH (CTBR-2019)
(Funded by UGC-SAP)
March 19-20, 2019

Certificate

This is to certify that Prof./Dr./Mr./Ms Farzana Ahmed of Dept of Biotechnology, Mizoram University, Tanhril, India has participated/ presented an invited lecture/ a paper/ poster entitled Investigating Plant Growth promoting potential of Heavy metal resistance bacteria isolated from heavy metal contaminated soil in the National Seminar on Current Trends in Biotechnology Research (CTBR-2019) on 19th March 2019.

Prof. S. Chakraborty
Chairman

Vice Chancellor
Assam University, Silchar

Prof. P. B. Mazumder
Convener
CTBR-2019

- Workshop on "**Statistical tools in biomedical research**" (23-24 Nov 2018) organized by Bioinformatics Infrastructure Facility (BIF), Department of biotechnology, Mizoram University.

BIOINFORMATICS INFRASTRUCTURE FACILITY (BIF)
DEPARTMENT OF BIOTECHNOLOGY
Mizoram University
(Accredited with 'A' Grade by NAAC)
Aizawl - 796004

Certificate

Certified that Farzana Ahmad Participated / ~~acted as a Resource person~~ in the National Workshop on Statistical tools in Biomedical Research held during 22-24 Nov 2018 organized by Bioinformatics Infrastructure Facility (BIF), Department of Biotechnology, Mizoram University sponsored by Department of Biotechnology (DBT), New Delhi.

(Prof. K.R.S. Sambasiva Rao)
Vice-Chancellor
Mizoram University

(Prof. N. Senthil Kumar)
Coordinator
Bioinformatics Infrastructure Facility (BIF)
Mizoram University

CONTENTS

LIST OF TABLES

ABBREVIATIONS

Pb	Lead
Cd	Cadmium
Cr	Chromium
ROS	Reactive oxygen species
DNA	Deoxyribonucleic acid
VOC	Volatile organic compounds
PGPR	Plant growth promoting rhizobacteria
IAA	Indole acetic acid
ACC	1-aminocyclopropane-1-carboxylic acid
rRNA	ribosomal RNA
ACCD	1-aminocyclopropane-1-carboxylic acid deaminase
PLP	Pyridoxal 5’ phosphate
P	Phosphorus
PSB	Phosphate solubilizing bacteria
TE	Tris EDTA
HCl	Hydrochloric acid
SDS	Sodium dodecyl sulfate
TAE	Tris acetate EDTA
UV	Ultraviolet rays

PCR	Polymerase chain reaction
HCN	Hydrogen cyanide
rpm	Revolution per minute
DNPH	2,4-Dinitrophenylhydrazine
PSI	Phosphate solubilizing index
$Ca_3(PO_4)_2$	Tricalcium phosphate
Ppm	Parts per mellion
OD	Optical density
CAS	Chrome azurol S
MTCC	Microbial Type Culture Collection
TSA	Tryptic soya agar
EC	Electrical conductance
V/V	Volume by volume
NCBI	National Center for Biotechnology Information
LB	Luria–Bertani

Introduction:

Heavy metal contamination is a greatconcern forenvironmental pollution. Industrialization, urbanization, increasing population and technology advancement has accelerated the use of heavy metals that lead to environmental contaminations(Oves*et al*, 2016). Terrestrial environments that harbor diverse range of micro-flora and fauna often contaminated with heavy metals due to anthropogenic activities like release of waste water fromchemical fertilizer industries, sewage sludge, smelting and mining industries as well as with the useof pesticides in agricultural sectors, etc. Heavy metal contamination contributes a rigorous hazard to agriculture, environment and consequently via the food chain to human and animal health (Robinson *et al*., 2001).Among various heavy metals used in industries are lead, mercury, arsenic, cadmium, chromium, thallium, etc. are more toxic and released in the environment.

Heavy metals like lead (Pb)and cadmium (Cd)are the chief contaminants found in the environment and they are awfully toxic to humans, plants, microbes and animals. They can damage plasmalemma, modify specificity of enzymes and destruct the organization of DNA (Olaniran*et al*., 2013). This toxicity is formed by the dislocation of crucial metals from their native binding sites or ligand binding (Igiri*et al*., 2018).Cadmium can inhibit the growth of roots and shoots, affect nutrient absorption and homeostasis, and often accumulate in major crops (Sanita di Toppi and Gabrielli, 1999). Cadmiummay get into the human or animal body through the various food sources that are contaminated with heavy metal which may cause severe diseases and toxicity. Soil pollution by cadmium also has a negative impact on the biodiversity and activities of soil microbial populations (McGrath, *et al*., 1994). Chromium is another highly toxic heavy metal easily diffuses across the plasmalemma, reduced inside the cells producing reactive oxygen species (ROS) and free radicals which further intensify its toxicity. These ROS compounds damage the root meristem activity and chlorophyll destruction (Foreman *et al.,* 2003).

The heavy metal contamination is a great environmental concern and several research studies are involved for its remediation. In general, various methods such as physical, chemical and biological methods are employed. Physical methods like adsorption, membrane separation, ionexchange are employed. Chlorination, chemical extraction, etc., are employed and methods like phytoremediation, bioleaching, etc., were employed under biological methods.

Huge amount of heavy metals cannot be removed easily from soil and most of the toxic metals were recognised as a major inhibitor of biodegradation of organic pollutants (Olaniran*et al*., 2013). The toxicity of heavy metals mainly results from the interactions of metals with enzymes and blocks the biological reaction of metabolic pathways (Volland*et al*., 2012).

In addition to metal toxicity, there are several other factors thatlimit the growth of plant in contaminated soils, including dry environment, poor soil quality, pest,insufficient irrigation, and nutritional deficiencies. To prevent all these problems chemical fertilizers and pesticides are used which are not eco-friendly and cause major problems to the environment. Therefore alternatives to the use of chemical fertilizers and pesticides are required. For these microbial inoculants, environmentally friendly microbial formulations that act as phytostimulants, biofertilizers and microbial biocontrol agents can be used (Olubukola*et al.,* 2012).

Plant growth promoting bacteria (PGPB) are considered beneficial to plants and they colonizes plants root or it can grow in, on, or around plant tissues, stimulating plant growth by a range of mechanisms, either directly such as synthesis of plant hormones, nitrogen fixation, solubilisation of inorganic phosphate and mineralization of organic phosphate and other nutrients, and indirectly by antagonistic properties against phytopathogenic microorganisms (Esitken*et al.,* 2010). These bacteria trigger plants to produce different plant growth hormones like auxin, cytokinin and gibberellin as well as volatile organic compounds (VOCs). These bacteria can also produce growth regulators like siderophore, which fix nitrogen, solubilize organic and inorganic phosphate

(Numan*et al.,* 2018).Plant-bacteria-soil interaction is a determinant of soil fertility and plant health. Effective use of beneficial microorganisms requires a perfect understanding of the complex interactions between the various components of soil-microorganisms (Hani Antoun, 2012). The term rhizobacteria was first defined by Kloepper*et al*., in 1979. Rhizobacteria are bacteria that colonies in the plant rhizosphere and directly or indirectly involved in promoting plant growth and development through the production and secretion of various regulatory substances,hormones, enzymes, etc., (Kloepper*et al*., 2003). Khan *et al*., (2007) mentioned the following inherent PGPR capabilities: (i) they must be able to colonise the root surface (ii) they must survive, reproduce and compete with other microorganisms, at least for the time required to express their plant growth promoting activity inside; and (iii) can promote plant growth (Khan *et al*., 2007). Overall, PGPR functions in three different ways; by synthesizing plant-specific compounds, promoting the absorption of certain nutrients from the soil and boosting plant immunity (Rifat *et al*., 2010). These beneficial effects are directed upon interaction between PGPR and hosts plants. They have the ability to produce plant growth promoters such as indoleacetic acid, gibberellins, ethylene and cytokinin (Yahya and Azawi, 1998), through the production of serarubin (Parul and Dharmendra, 2014), antibiotics (Arshad and Frankenberger, 1993) to combat plant pathogenic microorganisms.PGPR are an alternative to chemical fungicides and chemical fertilizers (Rais*et al*., 2017).

There are certain soil bacteria which can grow in heavy metal contaminated environments using various mechanismslike bioaccumulation, precipitation, complex formation etc. Amongst these heavy metal tolerating bacteria, there are also a group of them which even shows plant growth promoting factors. These bacteria can reduce toxic form of heavy metal to less toxic forms.

These organisms reduces stress in plants by secreting compounds like IAA, ACC deaminase, siderophore, phosphates, acidifying the soil, increasing root surface area (Khanna *et al*., 2019). So these types of organisms become a target for researchers in the era of heavy metal contamination. These types of organisms can be exploited in

agricultural fields nearby industries which are contaminated by heavy metal or in barren fields where excess chemical fertilizers have used before.

In addition, PGPRs have different mechanisms of heavy metal tolerance like biosorption, precipitation or bioaccumulation in external and intracellular spaces. Due to toxicity of heavy metals , most of the agricultural land is not under cultivation. The rhizospheric bacteria with heavy metal detoxification abilities could be an alternative source to decontaminated such land and use for plant development. As most of such bacteria are possess plant growth promoting abilities and therefore could help to in agricultural cultivation in heavy metal contaminations sites. In this context, we aim a study for isolation and characterization of such bacteria from various environments. The isolated bacteria will be screed for both heavy metal tolerance and plant growth promoting abilities and potent strains will be sued for developing microbial consortia for agricultural cultivations.

Aim and Objectives:

1. Isolation and screening of bacteria for heavy metal tolerance against lead, cadmium and chromium.
2. Molecular identification of the potential isolates using 16SrRNA gene amplification.
3. Selection of the potent organisms and evaluation of their *in vitro* and *in vivo* plant growth promoting potentials.

Review of Literature:

Heavy metals are a constituent of soil and some of the heavy metals like copper, chromium etc., are incorporated in cofactor and enzymes which are essential for plant growth and metabolism. Trace amount of heavy metal are present in the soil.Soil becomes contaminated by heavy metal when the concentration of heavy metal is more than the normal range. Heavy metalcontaminated sites are vast, and they are toxic to humans, plants and animals.Human activities related to industrialization and intensive agriculture have caused environmental pollution and have become a widespread problem in the globe. The most common contaminants include non-biodegradable metals and metalloids, which tend to persist in the environment due to their biotoxicity and pose a serious threat.Toremediate such contaminated soil or wastewater systems, several methods including those that arephysical and chemical are being developed. Physical methods are not cost effective therefore are not considered for a long term remediation program. While chemical methods generates huge wastes and it alters soil structure, soil fertility, soil ecosystem and microbial biodiversity (Kumari and Thakur, 2018). Biological approaches of removing heavy metal or bioremediation uses a living organism like plants or microorganisms. These organisms develop reduces the amount of toxic heavy metal from the surrounding by various process. This process includes bioaccumulation, reduction of heavy metal to a less toxic state, bioadsorption, bioprecipitation, etc. Biological treatment based on living microorganisms or plants can reduce the content of toxic metals to an ecologically acceptable level in a cost-effective and environmentally friendly manner (Kadukova and Vircikova 2005). The microbial repair of metal ions is mainly carried out through three main processes: cell surface absorption (biological precipitation). Bioabsorption is a process unrelated to metabolism, fast and reversible. Bioaccumulation is an active intracellular accumulation process that depends on cell metabolism, and the third process, bioprecipitation, can occur as a passive or active process depending on microorganisms and metal ions (Machalova*et al*., 2015, Kadukova, 2016).

The soil bacteria that colonise the roots of plants and increase plant growth are Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroth, 1978).As plants grow older, the growing roots are surrounded by firmly bound soil particles, and the area where this soil interacts with the roots is called the rhizosphere (Hani Antoun, 2012). It is the main place to absorb nutrient and also the place for important physiological, chemical and biological activities. Bacteria are the most abundant microorganisms present in the rhizosphere. The roots of plant release organic matter in the rhizosphere and support the activity of microorganisms, which Hitler calls the rhizosphere (Flaishman*et al*., 1996). However, not all bacterial strains of the rhizosphere in a given genus or species have beneficial effects on plants with PGPR (Penrose and Glick, 2003; Glick, 2014).Therefore, to develop effective microbial inoculants that promote plant growth and yield increase, it is very important to conduct reliable screening and selection of PGPR.

Certain groups of plant growth promoting rhizobacteria (PGPR) enhance the accumulation of heavy metals in plants by mechanisms like bioaccumulation or biosorption, etc. (HassanEtesamiandDinesh K.Maheshwari, 2018).Bacillus species can produce spores that are resistant to stress and secreted metabolites. These spores stimulate plant growth and prevent pathogen infection (Radhakrishan*et al*., 2017). Therefore the use of microorganisms in the rhizosphere is a method to increase tolerance to abiotic stress, especially environmental stress caused by climate change. *Bacillus subtilis* also plays an important role in improving tolerance to biological stress. The induction of disease resistance involves the expression of certain genes and hormones, such as 1-aminocyclopropane-1-carboxylate deaminase (ACCD). Ethylene can reduce the growth of roots and stem and help maintain homeostasis. The degradation of ethylene precursor (ACC) by bacterial ACCD helps to reduce plant stress and maintain normal growth under stress conditions (Glick *et al*., 2007). While ethylene is needed by many plants during their development, high levels of ethylene after germination are inhibitory for root elongation in order to break seed dormancy. The enzyme 1-amino-cyclopropane-1-carboxylate (ACC) deaminase, a pyridoxal 5' phosphate (PLP)-

dependent enzyme that splits the plant ethylene precursor ACC into ammonia and alpha-ketobutyrate, can be produced by a variety of PGPR strains, thus reducing the level of ethylene and the associated stress in plants. (Penrose and Glick, 2003; Blaha *et al.,* 2006). ACC deaminase is a multimeric enzyme with a molecular mass of around 35–42 kDa in the monomeric subunit. It is a sulfhydryl enzyme that, as an essential co-factor, utilises pyridoxal 5-phosphate. Pyridoxal phosphate is closely bound to the enzyme at approximately one molecule per subunit; at 418 nm, it shows a characteristic visible absorbance of pyridoxaldimine. Although several D-amino acids can act as substrates for ACC deaminase, notably D-serine and D-cysteine,L-serine and L-alanine are effective competitive inhibitors of the enzyme (although less efficiently than ACC). ACC deaminase catalyses an ACC cleavage that involves fragmentation of the cyclopropane ring and deamination of ACC to form α-ketobutyrate and ammonia (Glick, 2005).In order to effectively protect plants against a broad range of abiotic stresses, such as drought, salinity, heat, flooding or water logging, and heavy metal stress, ACC deaminase which is produced by plant growth-promoting bacteria helps in reducing the abiotic stress. ACC deaminase activity has been reported to date by rhizobacteria belonging to the genera *Pseudomonas*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter* and *Kluyverahave* (Saleem *et al*., 2007).As a result of inhibition of seed germination , seedling growth, vigour, and flowering due to the accumulation of ethylene stress, heavy metal stress inhibits plant growth. ACC deaminase-positive PGPR decreases the ethylene stress level and confers tolerance for abiotic stress in these plants (Gontia-Mishra *et al*., 2014). This element is necessary for almost all major metabolic processes of plant growth and development.

Bacterial siderophores have been shown to have direct benefits for plant growth promotion by serving as a direct source of iron and making it available to plants, among the many characteristics responsible for considering a strain as PGPR (Vansuyt*et al*., 2007).Microorganisms develop siderophores (low molar mass biomolecules − 0.5–1.5 kDa) in an iron-limited state to scavenge ferric ions, which bind with high affinity to unique outer membrane receptors. Hydroxamates, thiohydroxamates and catecholates

are several donor groups for the chelation of Fe^{3+} (Richardson *et al*., 1999). Most bacteria can synthesise their own chelator for iron acquisition or use other microbial and plant siderophores (Grusak*et al*., 1999).A virulence factor for human and animal pathogenic bacteria is believed to be the iron acquisition mechanism (Neilands, 1995). Studies conducted by Neema*et al*., (1993) in the case of plants (e.g. *Saintpauliaionantha* leaves) confirm that siderophore chrysobactin developed by *Erwinia chrysanthemi* was involved in the process of the disease.In different crops in the past, siderophore-producing microbial inoculants have been shown to have a direct plant growth-promoting effect.

Phytohormone, indole-3-acetic acid (IAA), is another very significant microbial metabolite involved in direct plant growth promotion through free-living PGPR. Many free-living PGPRs, such as *Azospirillum* and fluorescent *Pseudomonas*, secrete IAA to support root development and growth (Figueiredo*et al*., 2010).By derepressing the IAA signalling in the plant, bacterial IAA helps to bypass the host defence; IAA may also have a direct effect on bacterial survival and its plant defence resistance (S. Spaepen, J. Vanderleyden, R. Remans, 2007). The effectiveness of host invasion and survival also needs that bacteria resolve responses to plant defence caused after microbial recognition, a method involving surface polysaccharides, antioxidant systems, inhibitors of ethylene biosynthesis and genes of virulence (M.J. Soto, J. Sanjua'n, J. Olivares, 2006).It can be hypothesised that the development function of IAA is part of the technique used to bypass the plant protection mechanism by IAA-producing bacteria (S. Spaepen, J. Vanderleyden, R. Remans, 2007). IAA is a plant hormone with no obvious bacterial cell function, and it can be hypothesised that the development of IAA can enhance the fitness of the relationship of plant-bacterium (C.L. Patten, B.R. Glick, 2002).Since the first phase of the invasion of plant root bacteria includes the attachment of isolates to the root surface epidermal cells, where the root hair zone shows one of the key sites of primary colonisation (mainly on the basal region of emerging hairs), it is likely that IAA can colonise plant roots better than other bacteria by raising the root system. Furthermore, IAA levels weaken mechanisms of plant defence that promote colonisation (E.K. James,

2002; F. Chi, 2005; C. Chaintreuil, 2000; M. Sevilla, 2001).Bacterial IAA can loosen the walls of plant cells and, as a consequence, encourage a growing amount of root exudation that provides additional nutrients to sustain rhizosphere bacteria growth (E.K. James, 2002; F. Chi, 2005). Since endophytic microbial communities derive from the soil and rhizosphere (M. Elvira-Recuenco, J.W.L. VanVuurde, 2000), by increasing the amount of root exudation, bacterial IAA can attract more rhizosphere bacteria.Since bacterial IAA promotes the growth of the host plant's root system (I.E.G. De Salamone, 2005), isolate-producing IAA may enhance the fitness of interactions between plant and microbe (I.E.G. De Salamone, 2005; M.T. Brandl, S.E. Lindow, 1998). Bacterial IAA is known to loosen the walls of plant cells and, as a consequence, increases a growing amount of root exudation that provides additional nutrients to promote the growth of rhizosphere bacteria (E.K. James, 2002; F. Chi, 2005).IAA promotes the overproduction in plants of root hair and lateral roots and the release of saccharides during elongation from plant cell walls (P.J. Davies, 2004). Saccharides are a source of microorganism nutrients and may increase the capacity of plant-associated bacteria to colonise (M.T. Brandl, S.E. Lindow, 1998). The bacterial IAA increases the area and length of the root surface and thus allows the plant greater access to soil nutrients and water absorption (J.K. Vessey, 2003).

Phosphorus (P) together with nitrogen is the second most important macronutrient required by plants. Phosphorus is completely oxidised in the form of phosphate, but it always forms a large amount of insoluble chemical complexes with calcium, iron and aluminium, and forms insoluble phosphates present in the soil. Compared to other essential macronutrients, phosphorus is the least fluid nutrient in plant and soil (Gyaneshwar, 2002). Phosphorus soil dynamics has the characteristics of physical chemistry, desorption and adsorption biology, immobilisation and mineralisation (Mc Vickar, 1963). The inorganic form of phosphorus is dissolved by a group of heterotrophic microorganisms that secrete organic acids that can dissolve phosphate minerals and/ or chelate cationic P ion partners that can release P directly in

solution (He *et al*., 2002). While there are significant quantities of inorganic and organic phosphates in most agricultural soils, most of these are immobilised and inaccessible to plants. Several PGPR strains have been reported to have the ability to solubilize certain insoluble inorganic phosphate compounds, such as *Pseudomonas, Bacillus*, *Burkholderia, Rhizobium* and *Flavobacterium*.The use of these phosphate-solubilizing bacteria as inoculants could increase the absorption of P by plants and thus provide the advantage of direct promotion of plant growth (Bashan and de Bahsan, 2010; Saharan and Nehra, 2011).

The overall contribution to phosphorus plant nutrition includes root development, stem and stem resistance and growth, flower and seed formation, resistance to plant diseases, crop quality, maturity and yield. Fixing N is the main task related to the nutrition of P in beans (Deepshikha*et al*., 2014). The solubilisation of insoluble phosphate in the rhizosphere is one of the most common ways of plant growth promoting bacteria (PGPB), which can improve the utilisation of plant nutrients (Richardson *et al*., 2001). PSB can release various organic acids including oxalic acid, citric acid, gluconic acid, fumaric acid, adipic acid and 2-ketoglucose Acid (Rose, 1957).

Gluconic acid and 2-ketogluconate seems to be the most common solubilising inorganic phosphates (Deubel*et al*., 2000; Song *et al*., 2008). Other organic acids have been found in phosphate solubilisers, such as acetic acid, citric acid, lactic acid, propionic acid, glycolic acid, oxalic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, etc., (Ahmed and Shahab, 2011). These organic acids are the products of microbial metabolism, mainly produced by oxidative respiration or fermentation of organic carbon sources (such as glucose) (Trolove*et al*., 2003).

An alternative to this method is to strictly regulate the utilisation of phosphate in the rhizosphere. The solubilisation of minerals and organophosphates can become an important part of the system. Modern methods of applying chemical fertilisers can be cultivated, but the potential is often sacrificed to improve efficiency and environmental safety, thereby attempting to change the chemical composition of the soil solution in the

root zone. Even more encouraging is that many new cultivation techniques and fertilizer formulations have been developed to increase the effectiveness of phosphorus.

Many PhoshateSolubilisingBacteria proved to be effective biological fertilizers or biological control agents. PSB can be considered as a broad-spectrum biological fertilizer (Gupta, 2004). PSB such as *Bacillus megaterium, Bacciluscirculans, Bacillus subtilis* and *Pseudomonas strase* are effective biological fertilizers.Phosphatesolubilising microorganism are a better choice to solve this problem.This is an ecological and profitable agricultural technology used to improve agricultural production. Phosphorus is the second most important macronutrient after nitrogen , and plays an important role in the metabolism of high energy transfer, cell division, photosynthesis, biological oxidation, plant vegetative growth, reproduction and absorption. It is oxidized in the form of phosphate, but it always forms a large amount of insoluble chemical complexes with calcium, iron and aluminium, and forms insoluble phosphates present in the soil, making this nutrient a paradox. The availability of phosphorus in many soil is about 1molbut plant need 30 mol to reach maximum yield.

Biological control using beneficial microorganisms is an excellent way to reduce the harmful effects of pathogens on plant health and productivity. Great efforts have been made to identify microbial biological control agents that can inhibit plant pathogens, especially those related to soil-borne diseases, and can increase agricultural productivity (Cazorla *et al*., 2007). The genus *Bacillus* is considered to be safe bacterium that produces substances beneficial to the cultivation and production of industrial compounds (Stein, 2005). In addition, *Bacillus* also produces endospore to help bacteria survive under adverse environmental conditions, can use various environmental signals for germination, can preserve biological control agents for a long time, and reduce the complexity of the formulation process (Collins and Jacobsen, 2003). It should be noted that *Bacillus* used in the rhizosphere can also be used as a plant endophyte (McSpaddenGardener, 2004) and can also protect plants from pathogens (Romero *et al.,* 2004). Bacillus bacteria produce antimicrobial metabolites, which can

replace synthetic chemicals or be used as additives for bio-pesticides and biofertiliers against plant disease (Ongena*et al*., 2005). The success of biological control methods depends on the correct selection of effective biological control agents and their ability to provide protection against specific pathogens in specific crops.

MATERIALS AND METHODS:

Collection of Soil and Isolation of Bacteria:

Rhizospheric soils were collected from fieldsRamrikawn, farms of Mizoram University, of Aizawl district, Mizoram, India and stored at refrigerator 4ºC until used for experiment. The soil sample was serially diluted. 1 gm of soil was added in 9 ml distilled water, diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}dilutions respectively. 100 µl of 10^{-4} dilution was spread on six different nutritional media, viz., Starch Casein Agar (SCA), Tryptic Soya Agar (TSA), Luria Bertani (LB), ISP2, ISP5 and Tap Water Yeast Extract Agar (TWYE). The spread plates were incubated at 28ºC for 4-5 days. Based on difference in morphology, colour, texture and growth pattern, bacteria were selected; streaked and pure cultures were obtained.

Screening for Heavy Metal Tolerance:

The bacterial isolates were screened for heavy metal tolerance. Three heavy metals namely, lead, cadmium and chromium were used. These heavy metals were used in their salt form as lead nitrate (formula) cadmium chloride (formula) and chromium chloride. A range of concentration of lead nitrate was used (1mM - 26mM). Most of the bacteria do not grow above 25mM, so bacteria that growing on medium containing 25 mM lead nitrate were further screened for cadmium and chromium heavy metals at 15 mM concentration of each cadmium nitrate and chromium chloride. The isolates showing tolerance to these heavy metals were used for further studies.

Heavy Metal Biodegradability Assay:

The protocol described by Marzan*et al.*, 2017 was followed. Bacterial isolates were cultured in a shake flask containing LB broth for one hour in a rotary shaker at 150 rpm. The pH and temperature were maintained at 7.0 and 37°C respectively. After an optical density of 0.6 (λ = 600 nm) was achieved, 100 ppm of sterilised heavy metal (lead nitrate, cadmium chloride or chromium chloride) was applied to each culture flask

separately and incubated again in the same condition for 24 h. The culture broth was then centrifuged for 15 min at 5000 rpm. The supernatants was collectedand combined with twice the concentrated HNO_3.Then the mixture was heated on a hotplate to 100 °C to achieve acid digestion before the final decrease in volume and down to the initial volume of supernatant. The extract was filtered through a filter paper (Whatman No. 1) to extract any insoluble substance and collected into a volumetric flask and then diluted. This extract was analyzed by the Atomic Absorption Spectrophotometer for complete heavy metal reduction.

Identification and Characterization of Potent Isolates:

Based on heavy metal screening of the three heavy metals, 7 potent isolates were selected for molecular identification. For molecular identification, DNA isolation is carried out followed by 16S rRNA sequencing. The selected isolates used for DNA isolation using protocol of Green and Sambrook, (2012) method.

1.5 ml of grown culture of bacterial sample was taken in eppendroff tube and it was centrifuged at 10,000 rpm at 4˚C for 15 mins and the pellet was collected by discarding the supernatant.

⇩

The pellet was resuspended in 500µl of TE buffer pH-8.0 with HCl) and 50 µl of 10%SDS and 10µl of 10mg/ml proteinase K was added. The sample was mixed properly and incubated in water bath at 37˚C for 1 hour.

⇩

The water saturated Phenol: Chloroform:Isoamylalcohol (25:24:1) was added (500µl) in the sample by inverting the tube until the phase are completely mixed. After that, the

⇩

mixture was gently vortexed and centrifuged at 10,000 rpm for 15mins at room temperature.

The upper aqueous phase was transferred to a new microcentrifuge and again phenol: chloroform: isoamylalcohol(500µl) was added and mixed well.After that, the mixture was gently vortexed and centrifuged at 10,000 rpm for 15mins at room temperature.

The upper aqueous phase was transferred to a new microcentrifuge tube and 1/10 volume of sodium acetate (pH- 5.2) was added. After that 600 µl of isopropanol in the sample until the DNA precipitates.

In freeze it was kept for 1 hour if no pellete was visible and the mixture was centrifuged at 10,000 rpm for 10mins at 4˚C.

The pellet was washed with 1 ml of 70% ethanol for 30 sec and again centrifuged at 10,000 rpm for 5mins at 4˚C.

The pellet (DNA) was air dried inside the laminar airflow and dissolved in 80-100µl TE buffer and stored at 4˚C.

Quantification of Genomic DNA by Gel Electrophoresis (Agarose gel):

0.8% agarose was dissolved in 100 ml of 1X TAE buffer from the stock of 50X TAE buffer. 4µl of 10 mg/ml of ethidium bromide was added. It was allowed to solidify and DNA sample was run usually at 50 volt.

Amplification of 16S rRNA gene:

All the isolates were subjected to the amplification of 16S rRNA gene by using universal primers (forward 16S rRNA primer 5'-AGAGTTTGATCCTGGCTCA-3' and reverse 16S rRNA primer 5'-ACGGCTACCTTGTTACGACT-3') (Cui *et al*.,2008). Reactions were performed in a Veriti thermal cycler (Applied Bio-systems, Singapore) in a total volume 25µl consisting of 1.0µl genomic DNA (50ng), 0.5µl of each primer (10 pmol), 2.0 µl of deoxynucleotide triphosphate (2.5mM each), 2.5µl of 1X PCR buffer, 1.0 µl of taq DNA polymerase (1U/µl) and 17µl milliQgrade water. PCR was performed under the following conditions: initial denaturation step at 94˚C for 5 min, followed by 34 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚Cfor 40 sec and extension at 72˚C for 1.3 min with afinal extension step at 72˚C for 10 min. A negative control reaction mixture without DNA template of bacterial isolate was also included with each set of PCR reactions. The amplified PCR products were analysed by electrophoresis through 1.2% agarose gels made in TAE buffer. The PCR bands were analysed under UV light and documented using Bio-rad Gel Doc XR^{+} systems (Hercules, CA, USA) and were purified using Purelink PCR Purification Kit (Invitrogen, USA), and sequencing was done commercially at Sci-Genome Pvt. Ltd., Cochin, India.

PGPR Activities of Potent Strains:

The 7 potent bacterial strains were further evaluated for their plant growth promoting abilities. It includes ACC deaminase activity, phosphate solubilisation, IAA, siderophore, antagonistic properties, extracellular enzymes, protease, ammonia, HCN and pot experiment was carried out.

Activity of ACC Deaminase:

Bacterial isolate were screened for 1-Aminocyclopropane-1-carboxylate(ACC) deaminase activity based on the ability to use ACC as a sole source of nitrogen. Isolates were grown on aminimum agar medium supplemented with 3mM ACC instead of (NH4)2SO4 as its source of nitrogen. An increasing growth on minimal salts of agar

mediumby isolates will indicate the ACC deaminase activity. (2013: Shrivastava and Kumar).

Quantification of ACC Deaminase:

The samples were inoculated in a 50 ml flask containing 20 ml fresh solution and kept in the shaker for 24 hours at 180 rpm at 28˚C. 30ml fresh medium was added and incubated for 12hours 180 rpm at 28˚C. It is then centrifuged for 10 min at 8000 rpm at 4˚C and pellets were washed twice with 5 ml DF salts minimal and transferred to 50 ml sterile flask. 45µl of 0.5M ACC solution was then added to the bacterial samplesand was shaken with 180 rpm at 30˚Cfor 24 hours. It was then centrifuged for 10 min at 8000 rpm at 4˚C and cells were washed twice with 5 ml 0.1M Tris HCl, pH 7.6. Finally it was re-suspended in 1 ml 0.1M Tris HCl, pH 7.6 and transferred into10 ml centrifuge tubesand centrifuge for 5 min at 16000 rpm at 4˚C and pellets were suspended in 600µl of 0.1M Tris HCl, pH 8.5. 30 µl toluene was added and vortex intensely for 30 s and transferred 200µl of the suspension in 3 tubes (1 blank, 2 replica). 20 ml of 0.5M ACC solution was added in 2 tubes and water was added in the blank tube as control. The tubes were vortex and kept at 30˚C for 15 min. 1 ml of 0.56M HCl was added. It was thencentrifuged at16000 rpm at room temperature for 5 min. 1 ml of supernatant was vortex in the presence of 800µl of 0.56M HCl. 300µl of DNPH (0.2% DNPH was added in 2M HCl) and incubated for 30 mins at 30˚C. 2ml NaOH (2M) was added and absorbance was observed at 540nm.The activity of ACC deaminase was calculated by estimating the amount of alpha- ketobutyrate released from ACC degradation (Shrivastava and Kumar, 2013).

Phosphate Solubilization:

For the screening of rhizobacterial isolates for P-solubilising ability, Pikovskaya's agar (PA;Pikovskaya, 1948) was used. For 7-14 days at 30 ° C, the inoculated plates were incubated. Isolates showing clearance zones on PA have been reported as positive.

The phosphate solubilisation index (PSI) primarily defined the ability of rhizobacterial isolates to solubilize insoluble phosphate: the ratio of total diameter (colony + halo zone) to colony diameter on PA incubated at 30°C (Edi-Premono*et al*., 1996). The calculation was performed on incubation day 7.

For Quantification of Soluble Phosphorus:

The isolates were grown individually overnight in LB broth medium and the OD 600 nm was adapted to 1.0.0. The cells were inoculated with insoluble tricalcium phosphate ($Ca_3(PO_4)_2$) in the Pikovskaya broth growth medium. Until autoclaving, the pH of the Pikovskaya broth was changed to 6.75 ± 0.25. In 20 ml vials containing Pikovskaya broth, the strains were inoculated and incubated for 5 days at 30°C in a shaker incubator (150 rpm). Owing to the presence of suspended particles of insoluble $Ca_3(PO_4)_2$ in the supernatant, to obtain a transparent supernatant, the broths were centrifuged for 10 min at 13,000 rpm. In clean, dry, acid washed test tubes, triplicate aliquots of the supernatant (100 µl) were transferred. Using Barton's reagent, soluble phosphate was determined.

Preparation ofBarton's Reagent:

The reagent (Sundara Rao and Sinha, 1963) was prepared as per the following procedure:

Solution A: 25 g of ammonium molybdate was dissolved in 400 ml of water.

Solution B: 1.25 g of ammonium metavanadate was dissolved in 300 ml of boiling water, cooled and then 250 ml of concentrated nitric acid (HNO3) was added. Afterwards, A and B solutions were mixed and the volume was made up to one litre. A standard curve was prepared by dissolving 0.2195 g of potassium dihydrogen orthophosphate (KH_2PO_4) in water. The solution was made up to one litre (1 ml = 59 ppm phosphorus). Further dilution of 10 ml into 250 ml was made so that 1 ml is equivalent to 2 ppm of P. Aliquots of 2, 3,4,5,6, 8,10,15 and 20 ml ofthe 2 ppm stock

solution were taken in 50 ml volumetric flasks to which 2.5 ml ofBarton's reagent was added and the volume was made up to 50 ml with distilled water. After 10 minutes, yellow colour developed was read in a colorimeter at 430 nm. A standard graph was then prepared from which phosphorus values for experimental samples were calculated (Koenig and Johnson, 1942).

Briefly, a 2.5 ml volume of Barton's reagent was added to each tube along with respective samples. The tubes were vortexed and then incubated for 30 min at room temperature. After that, the solution was read using a spectrophotometer at 498 nm and the phosphate level was measured by extrapolating against the typical phosphate curve prepared. To evaluate the final pH, a subsample of this supernatant was used.

IAA Production:

Indole-3-acetic acid production was estimated by growing the bacteria in ISP2 broth and Salkowski reagent was used. (Sheng *et al.,* 2008).TheGlickmann and Dessaux method (1995) was used to quantitatively estimate the IAA provided by the selected bacterial isolate. In a rotary incubator shaker, bacterial cultures were separately grown in DM broth supplemented with 0.5 percent L-tryptophan at 32^{o}C and agitated at 120 rpm. The culture suspensions were centrifuged for 10 minutes at 10000 rpm after 36 hours, and the supernatant (2 ml) was combined with 2 ml of freshly prepared Salkowski reagent (left in the dark for 20 minutes). IAA production was indicated by the formation of the pink colour and was measured in OD at 530 nm using a spectrophotometer. To approximate the IAA concentration per ml basis, the OD values were interpolated using a standard curve.

Siderophore Production:

For the screening of siderophore production, the cultures were streaked in Chrome Azurol S agar plates (Ames-Gottfred*et al.,* 1989), the appearance of an orange halo zone around the cultures was observed after 48 hours incubation at 28°C which indicates formation of siderophores.

The isolate-produced siderophore was quantified using the Payne, (1994) described chrome azurol S (CAS) shuttle assay. Test isolate, supplemented with 1 % (w /v) glucose, was grown in iron-free King's B medium. The CAS assay solution was combined in equal proportion with culture supernatant and allowed to stand for 20 minutes. Siderophore presence removes the iron from the dye complex and causes the blue colour strength, which is recorded at 630 nm, to decrease. Medium was used as blank for the measurements and percent siderophore units were determined by the following formula-[(Ar − As)/Ar] 100 = percent siderophore units, where Ar = reference absorption (media + CAS assay solution) and As = sample absorption.

Antagonistic Potential:

All fungal plant pathogens were obtained from the Microbial Type Culture Collection, MTCC (Institute of Microbial Technology, IMTEC Chandigarh) culture collection. Antagonism of bacterial isolates toward phytopathogenic fungi was performed *in vitro* using PDA plates. Six fungal pathogen were used for screening, namely *Fusarium lycopersicum, Fusarium graminearum, Fusarium udum, Fusarium proliferatum, Macrophominaphaseolina*and*Tricophytonmetagrouphytes.* An agar plug of the growing fungal mycelium was placed on the center of the PDA plate, and then two isolates were inoculated on the plate at two equidistance sites, 3 cm apart from the colony of each of the fungal pathogen in the center. In the absence of bacterial culture spots, negative monitoring consisting of fungal agar discs was also carried out. At 30°C for 7 days, the Petri plates were then incubated. The following formula suggested by Alenezi*et al*., 2016; has measured the percentage of fungal growth inhibition.

$$\% \text{ of fungal growth inhibition} = 1 - (a/b) \times 100\%$$

where *a* is the distance between fungal growth edge (from the bacterial side) and the bacterial isolate growth edge (from the fungus side) and *b* is the distance between the fungal upper growth edge and the upper edge of the control Petri dish.

Production of Extracellular Enzymes:

The screening for cellulase and xylanase was done using Congo red assay (Teatherand Wood, 1982). Both the medium containing carboxy methyl cellulose (CMC) and the water soluble oat split xylan extract were inoculated into the 7 bacterial isolates and incubated at 37 ° C for 2 days. The presence of extracellular cellulase and xylanase after cell growth was detected by halozoneformation by flooding the plates with 0.3 percent Congo red solution for 15 minutes and the plates with 0.1 percent NaCl for 15 minutes. For the halo zone indicating cellulase and xylanase development, the plates were visualized.

Protease Activity:

In nutrient agar medium, modified with 1 percent of skim milk, protease activity of the bacterial isolate was determined (Pereira and Castro, 2014). Isolates were incubated for 3 days at 28°C. Positive proteolytic activity is demonstrated by the strong halo around the cell.

Ammonia Production:

To estimate ammonia, the Nesslerization response defined by Cappucino and Sherman (1992) was used. The bacterial isolate was grown for five days at 29 ± 2 ° C in the peptone water broth. Culture supernatant (0.2 ml) was combined with Nessler's reagent (1 ml) and up to 8.5 ml of this mixture amount was obtained by adding purified water free of ammonia. A spectrophotometer was used to test the development of the brown to yellow colour at 450 nm.

HCN Production:

Cyanide production was detected as described by Alvarez *et al*, 1995. An agar medium containing 10% TSA supplemented with 4.4g/l glycine was prepared. The seven bacterial isolates were streaked, a filter paper impregnated with 0.5% picric acid

and 2% sodium carbonate was placed on the lid and sealed and incubated for 4-7 days. Change in colour from yellow to orange indicates cyanide production.

Preparation of Soil for Pot Experiment:

The soil for conducting potexperiment were collected from garden, transferred into polythene bags and brought into the laboratory. The soil samples were ground properly, air dried in a hot air oven at 105˚C for 24 hours and sieved at 2mm sieve.

Physico-chemical analysis of soil

Lists of Physico-chemical parameters:

Parameters	**Description/Methods**
pH	pH meter
ECelectrical conductometer	
Water holding capacityKeen's box	

Determination of pH in Soil:

10 gm of soil was added in 20 ml distilled water and stirred properly with a glass rod for around 30 minutes. Particles were allowed to settle down. Using standard buffer of known pH, the electrode was adjusted the pH of the soil was recorded.

Determination of Electrical Conductivity in Soil:

10 gm of soil was added in 20 ml distilled water and stirred properly with a glass rod for around 30 minutes. Particles were allowed to settle down. The instrument was calibrated and the EC was recorded.

Determination of Water Holding Capacity:

The water holding capacity of the soil was calculated using Keen Raczkowski box. A filter paper was placed at the bottom of the box and soil was filled and covered with a lid. Then the Keen Raczkowski box was placed on a petri dish containing water and left overnight. The box containing the saturated soil was removed from the petri dish and weight was measured. It is then dried in a hot air oven at 105˚C overnight and weight was recorded.

EFFECT OF POTENT STRAIN ON SEED GERMINATION:

Germination Assay:

Uniform size, viable seeds of chick pea were selected and surface sterilized with sodium hypochloride followed by ethanol 70% and washed twice with double distilled water. The sterilized seeds were soaked in sterile water inside laminar hood for 8 hours.After 8 hours of soaking, the seeds were treated in three different sets. Set I(control), Set II(5mM heavy metal (Pb or Cd or Cr)) and Set III(treated with each HM 5mM concentration along with bacterial inoculum(613 and 642 respectively))(Mitra *et al*. 2018).

Experimental set up for seed germination assay

Table 1: Experimental Set up for Seed Germination Assay

Sets	Set 1	Set 2			Set 3					
Conditions	Control	Pb	Cd	Cr	Pb+613	Pb+642	Cd+613	Cd+642	Cr+613	Cr+642
Conc.(mM)	0	2	2	2	2	2	2	2	2	2

500 gm of the collected soil sample were measured and kept in each polypots. Three of the polypots were labeled as control.Triplicatepolypots were labeled as Lead, Cadmium, Chromium, Lead+613, Lead+642, Cadmium+613, Cadmuin+642, Chromium+613,

Chromium+642 respectively. Lead, Cadmium and chromium were added to the labeled heavy metal respectively.

Determination of Plant Physical Parameters:

On the 31st day the grown plants were removed from the pots. Roots were separated slowly from the soil and washed in running tap water. The root and shoot length was measured and the number of branches were counted. The fresh weight of the plant was measured using weighing balance

Estimation of Chlorophyll Content:

The method given by Arnon (1949) was followed to estimate the photosynthetic pigments.Approximately 1 gm of the leaf sample was cut into small pieces and homogenised using 80 percent (V / V) acetone in a pre-cooled mortar and pestle. While grinding, a pinch of calcium carbonate was applied. The extract was centrifuged for 15 min at 3000 rpm and produced up to 25 ml of 80 percent (V / V) acetone. The transparent solution was transferred to a colorimeter tube and the optical density was calculated against an 80% acetone blank in a spectrophotometer at 645 nm and 663 nm, respectively. The chlorophyll 'a' and chlorophyll 'b' levels were calculated using the following equation:

Chlorophyll ‘a’ (μ/g/ml) = (12.7 x O.D. at 663 nm) – (2.69 x O.D. at 645 nm)

Chlorophyll ‘b’ (μ/g/ml) = (22.9 x O.D. at 645 nm) - (4.08 x O.D. at 663 nm)

Total chlorophyll (μ/g/ml) = (20.2 x O.D. at 645 nm) + (8.02 x O.D. at 663 nm)

The chlorophyll content was expressed as mg chlorophyll per gram fresh weight of the leaf.

RESULTS

Collection of Soil and Isolation of Bacteria:

Rhizosphericsoil was collected from fields of Ramrikawn and farms of Mizoram University.In total, 600 bacterial isolates were obtained using six nutritional media viz., Starch Casein Agar (SCA), Tryptic Soya Agar (TSA), Luria Bertani (LB), ISP2, ISP5 and Tap Water Yeast Extract Agar (TWYE). The isolates were selected based on their morphological characteristicsMaximum bacterial isolates were obtained using SCA media (n=235; 39.1%) followed by ISP7 (n=141; 24.2%), ISP5 media (n=67; 11.0%), LB (n=60; 10.2%), TSA (n=54; 8.9%) and TWYE (n=43; 7.2%).

Screening for Heavy Metal Resistance:

All isolates were screened againstthree different heavy metals (Lead, Chromium and Cadmium)using variousconcentrations ranging from 1mM to 26mM.

Out of 600 isolates, **60 showed tolerance against lead, 34 against chromium and 9 showed** tolerance against cadmium.

Among them seven bacterial isolate (**457, 498, 519, 521, 613, 642, 665**) showed tolerance against all tested three heavy metals and were taken for further studies

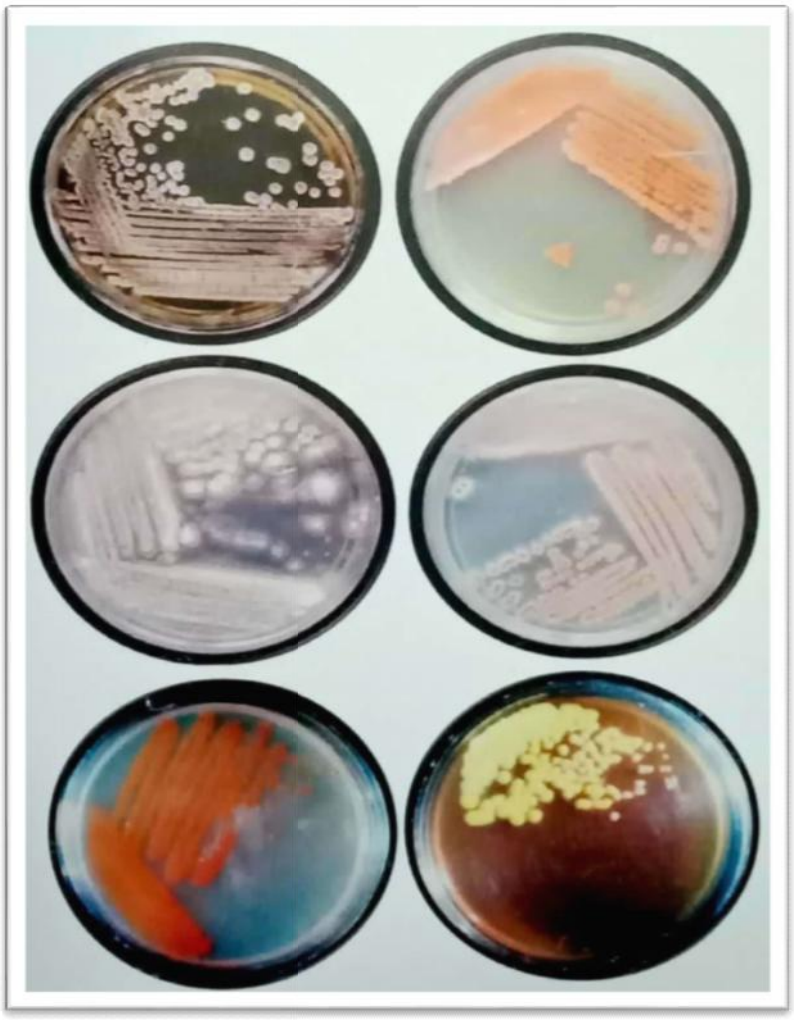


Figure 1: Morphological characteristics of the bacterial isolates

Molecular Characterization of the Isolates:

DNA isolation of the bacterial isolates were carried out manually following the protocol of Green and Sambrook, 2012 and 16s rRNA PCR was run and was sent for sequencing commercially. After sequencing the obtained sequences were deposited in NCBI database and checked for the closest similarities. The isolates were identified as shown in the table below.

Table 2: Identification of the Isolates

Isolate name	NCBI GenBank accession no.	Closest species	Similarity
457	MK968182	*Streptomyces coelicoflavus*	99.9%
519	MK968183	*Bacillus thuringensis*	100%
521	MK968184	*Bacillus cereus*	98.92%
613	MK968185	*Bacillus sp.*	99.95%
642	MK968186	*Bacillus cereus*	100%
665	MK968187	*Lysinibacillus fusiformis*	99.82%

Heavy Metal Biodegradability Assay:

Heavy metal biodegradability assay was conducted by growing the isolates in LB broth to log phase and then 100 ppm of lead nitrate was added to the broth and grown for 24 hours. Acid digestion was done and analysed by AAS. Isolate *Bacillus thuringensis* showed highest reduction of lead nitrate.

Table 3: Amount of heavy metal reduced in broth (analysed by AAS)

Isolates	% of HM(Pb) utilized
457	97.89±0.17
498	96.12±1.37
519	98.31±1.19
521	90.10±0.87
613	94.08±1.16
642	97.17±1.14

665	97.84±0.95

SCREENING FOR PGP ATTRIBUTES:

The bacteria isolated were screened for various plant growth promoting potentials.It includes ACC deaminase, phosphate solubilisation, IAA production, siderophore, ammonia production, HCN production, protease activity, extracellular enzyme activity,etc.

ACC Deaminase:

The 7 strains were tested for ACC deaminase activity and were found to be positive for all 7 strains. The ACC deaminase activity quantification showed large variations (Table 1). The highest activity was observed in cell-free extracts obtained from *Bacillus sp.*(613) (177 µmolα-ketobutyrate mg protein^{-1} h^{-1}) followed by 498 (104 µmolαa-ketobutyrate mg protein^{-1} h^{-1}) followed by*Bacillus cereus*(642) and *Lysinibacillus fusiformis*(665) (98 µmolα-ketobutyrate mg protein-1 h-1) followed by *Streptomyces coelicoflavus* (457) (30 µmolα-ketobutyrate mg protein-^{1} h^{-1}) contaminated cadmium (Table 1).

Figure 1: ACC deaminase activity shown by*Streptomyces coelicoflavus,Bacillus thuringensis,Bacillus cereus,Bacillus sp. and Lysinibacillis fusiformis*

Table 4: ACC deaminase Produced by the Bacteria(mg protein^{-1} h^{-1})

Amount of µmol α-ketobutyrate (mg protein^{-1} h^{-1})							
Treatment & Samples	**457**	**498**	**519**	**521**	**613**	**642**	**665**
No heavy metal	82±0.95	104±0.91	85±0.37	84±0.63	**177**±0.04	98±0.64	98±0.88
Lead	60±1.01	84±1.20	55±0.82	54±0.64	**63**±0.77	59±0.37	79±1.36
cadmium	30±0.05	52±0.68	26±0.36	38±0.64	**55**±0.84	51±0.63	42±0.82
chromium	76±0.99	75±1.10	58±0.51	6±0.80	**84**±0.73	78±0.84	84±0.91

Mean ± standard error

Phosphate Solubilization:

After incubation of the rhizospheric strain for 7 days at 30°C, the strains showed great variation in phosphate-solubilization capacity (Table 2). All seven isolates showed halo zone formation for phosphate solubilisation using PKV agar. *Bacillus cereus*(521) contaminated with lead solubilises phosphorus and *Lysinibacillus fusiformis(*665) contaminated with cadmium also solubilises phosphorus.

The highest phosphate solubilisation was shown by isolate 498 with 3.90 PSI solubilises 92.87µg/ml followed by isolate *Bacillus sp.*(613) as 3.69 PSI solubilises 87.21µg/ml. And the lowest PSI was shown by isolate *Bacillus cereus*(521) contaminated with lead which solubilises 22.55 µg/ml of phosphorus.

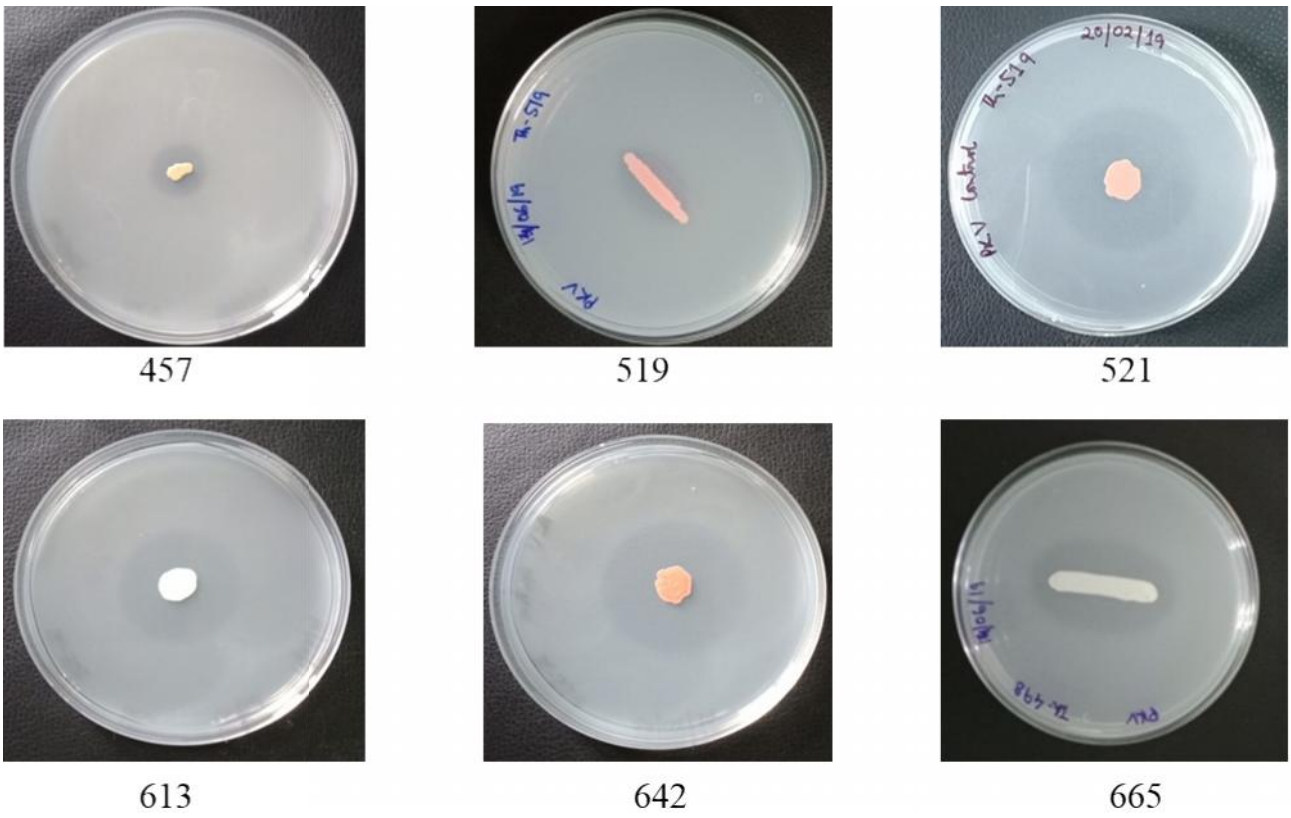


Figure 2: Phosphate Solubilisation shown by*Streptomyces coelicoflavus*, *Bacillusthuringensis*, *Bacillus cereus*, *Bacillus sp.*,*Bacillus cereus*,*Lysinibacillus fusiformis*

Table 5: Phosphate solubilisation index (PSI) and amount of inorganic phosphate solubilized by the isolates

Bacterial isolates	Treatment	Colony diameter	Halo diameter	PSI	Amount (µg/ml)
457	No Heavy metal	8	11	2.37	66.48±2.08

498	No Heavy metal	11	32	3.90	**92.87±2.02**
519	No Heavy metal	9	11	2.22	72.10±0.86
521	No Heavy metal	8	15	2.62	85.73±2.54
613	No Heavy metal	13	22	3.69	**87.21±3.52**
642	No Heavy metal	11	21	3.36	**87.11±1.67**
665	No Heavy metal	10	12	2.20	64.57±3.35
521	**Lead**	13	15	2.15	22.55±1.62
665	**Cadmium**	7	11	2.57	54.18±1.70

IAA Production:

The bacterial isolates tested have different abilities in secreting IAA levels. The ability of rhizospheric bacterial isolates in IAA production is presented in Table 6. Seven bacterial isolates were tested for IAA production. The three heavy metals (lead, cadmium and chromium) were also treated to confirm whether the production of IAA is taking place or not in presence of the heavy metals. The table showed that all isolates analyzed were able to produce IAA with different heavy metals. The highest IAA concentration is produced by *Bacillus thuringensis*(519(chromium)) is about 126 µg/ml, while the smallest IAA concentration is produced by*Lysinibacillus fusiformis*(665 (lead))which is 34.1 µg/ml. The second and third highest IAA concentrations were

produced by *Streptomyces coelicoflavus* (457 (chromium))of 125 µg/ml and *Bacillus cereus*(642(chromium)) isolates of 123µg/ml. Presence or absence of heavy metals affects the production of IAA. Presence of chromium increases the production of IAA, whereas absence of lead and cadmium produces more IAA. Presence of cadmium drastically decreases the production of IAA whereas lead doesn't affect much in the production of IAA.

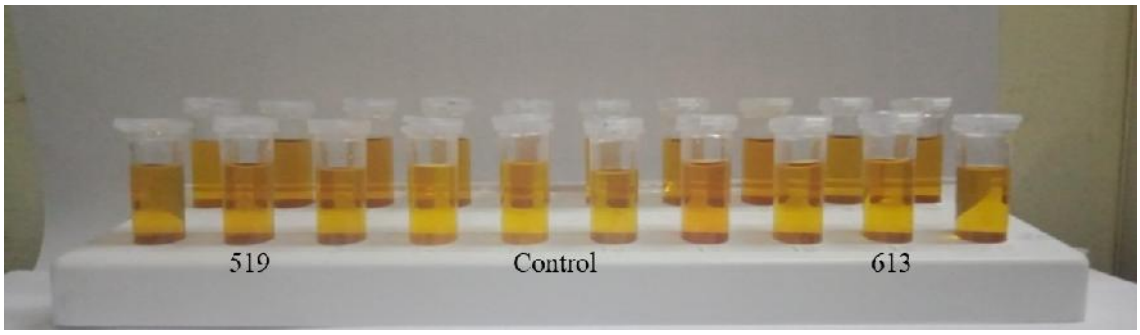


Figure 3: Change of colour from light yellow to pinkish red by the bacteria

Table 6: Amount of IAA produced by the bacteria in µg/ml concentration.

Samples	Lead		Cadmium	Chromium
457	101±2.08	102±0.99	40.3±0.99	125±0.64
498	100±0.99	106±1.62	53.9±2.08	117±0.99
519	103±0.64	110 0.77	56.8±0.64	126±0.05
521	98.3±0.99	120±0.99	36.5±0.99	115±1.62
613	103±1.62	39.5±0.64	34.5±1.62	119±0.05

642	102±0.05	90.9±2.08	34.6±0.05	123±0.99
665	41.80.77	34.1±0.05	57.7±2.08	103.07±1

Siderophore Production:

Out of 7 isolates, three isolates *Bacillus sp.*(613), *Bacillus cereus*(642) and *Lysinibacillus fusiformis*(665) shows positive to siderophore production by forming orange halozone on CAS agar media. The production of siderophore was quantified for the three isolates. *Bacillus sp.*(613) produces 7.87%, *Bacillus cereus*(642) produces 20.12% and *Lysinibacillus fusiformis*(665) produces 6.56% respectively.

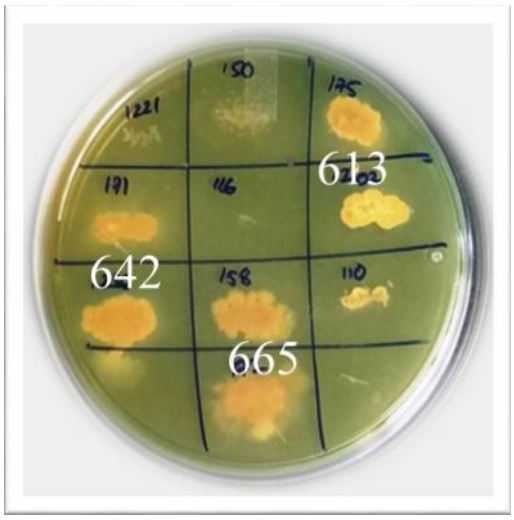
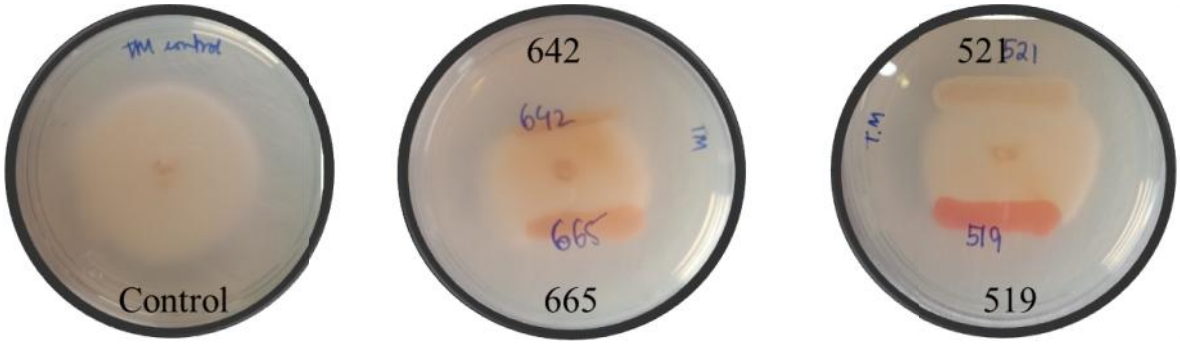


Figure 4: Formation of orange colourhalozone on blue colour CAS agar medium

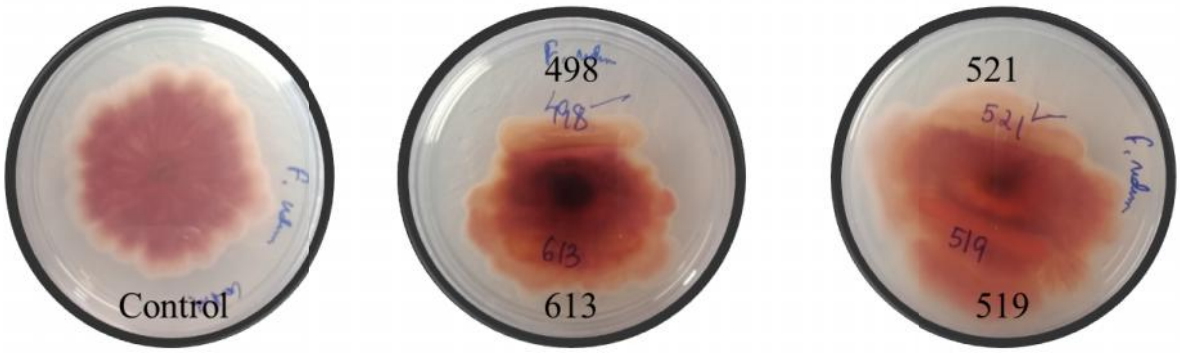
Antagonistic Potential of the Isolates:

Out of 7 isolates, isolatenumber 498 and showed antagonistic potential againstfour fungal pathogens. Isolate 498 showed antagonistic potential to *Fusarium lycopersicum*, *Fusarium graminearum, Fusarium udum*and*Macrophominaphaseolina. Bacillus cereus* showed antagonistic potential to *Fusarium lycopersicum, Fusariumlycopersicum, Fusarium graminearum, Fusarium udum, Fusarium*

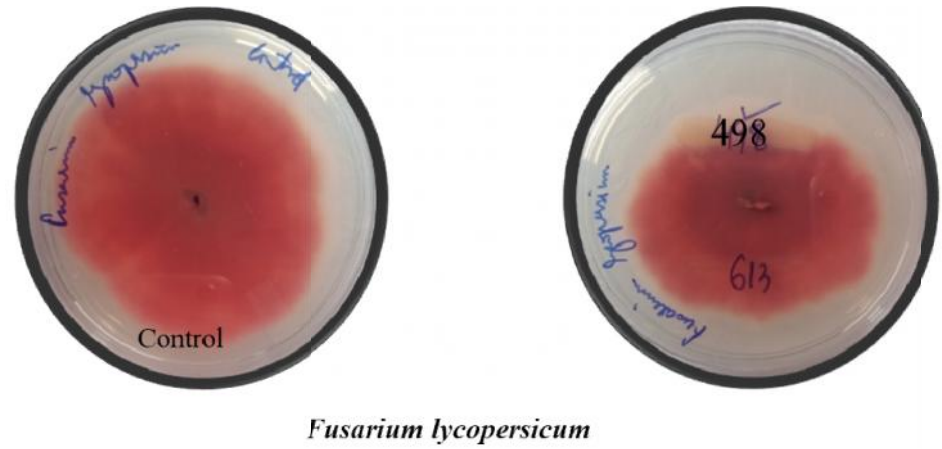
poliferatum, Tricophytonmetagrouphytes.Bacillus thuringensis, *Bacillus cereus* and *Lysinibacillus fusiformis* also showed antagonistic activity to *Tricophytonmetagrouphytes.*



Trichophyton mentagrophytes



Fusarium udum



Fusarium lycopersicum

Macrophomina phaseolina

Figure 5: Antifungal activity shown by *Tricophytonmetagrouphytes*, *Fusariumudum*, *Fusarium lycopersicum*, *Macrophominaphaseolina*

Table 7: Antagonistic Potential shown by Isolates to plant fungal pathogen

Fungal pathogen	457	498	519	521	613	642	665
Fusarium lycopersicum	-	+	-	+	-	-	-

Fusarium graminearum	-	+	-	+	-	-	-
Fusarium udum	-	+	-	+	-	-	-
Fusarium poliferatum	-	-	-	+	-	-	-
Macrophominaph aseolina	-	+	-		-	-	-
Tricophytonmetag rouphytes	-	-	+	+	-	+	+

EXTRACELLULAR ENZYMES:

Cellulase and Xylanase screening:

- Isolate 613(*Bacillus sp.*) showed cellulase and xylanase activity in presence of the heavy metal**.**

Table 8: Extracellular Activity

Cellulase							
Treatment & Samples	**457**	**498**	**519**	**521**	**613**	**642**	**665**
No heavy metal	+	-	-	-	+	+	-
lead	-	-	-	-	+	-	-
cadmium	-	-	-	-	+	-	-
chromium	-	-	-	-	+	-	-
Xylanase							

Treatment &Samples	**457**	**498**	**519**	**521**	**613**	**642**	**665**
No heavy metal	+	-	-	-	+	+	-
lead	-	-	-	-	+	-	-
cadmium	-	-	-	-	+	-	-
chromium	+	-	-	-	+	+	-

Table 9: Hydrolysis capacity shown by the bacteria

Treatments	Isolate	[A] Size of the colony (cm)	[B] Destained Zone (cm)	[B/A] Hydrolysis capacity
Cellulase				
No treatment	**613**	**0.5**	**1**	**2**
Lead	**613**	**0.5**	**1**	**2**
Cadmium	**613**	**0.5**	**0.9**	**1.8**
chromium	**613**	**0.4**	**0.8**	**2**
No treatment	457	0.4	0.3	0.75

No treatment	642	0.6	0.3	0.5
Xylanase				
No treatment	**613**	**0.5**	**1.2**	**2.4**
lead	**613**	**0.6**	**1.1**	**1.83**
cadmium	**613**	**0.6**	**1.0**	**1.6**
chromium	**613**	**0.6**	**1.0**	**1.6**
No treatment	457	0.5	0.5	1.0
chromium	642	0.4	0.3	0.75
No treatment	642	0.6	0.6	1.0
Chromium	642	0.6	0.5	0.83

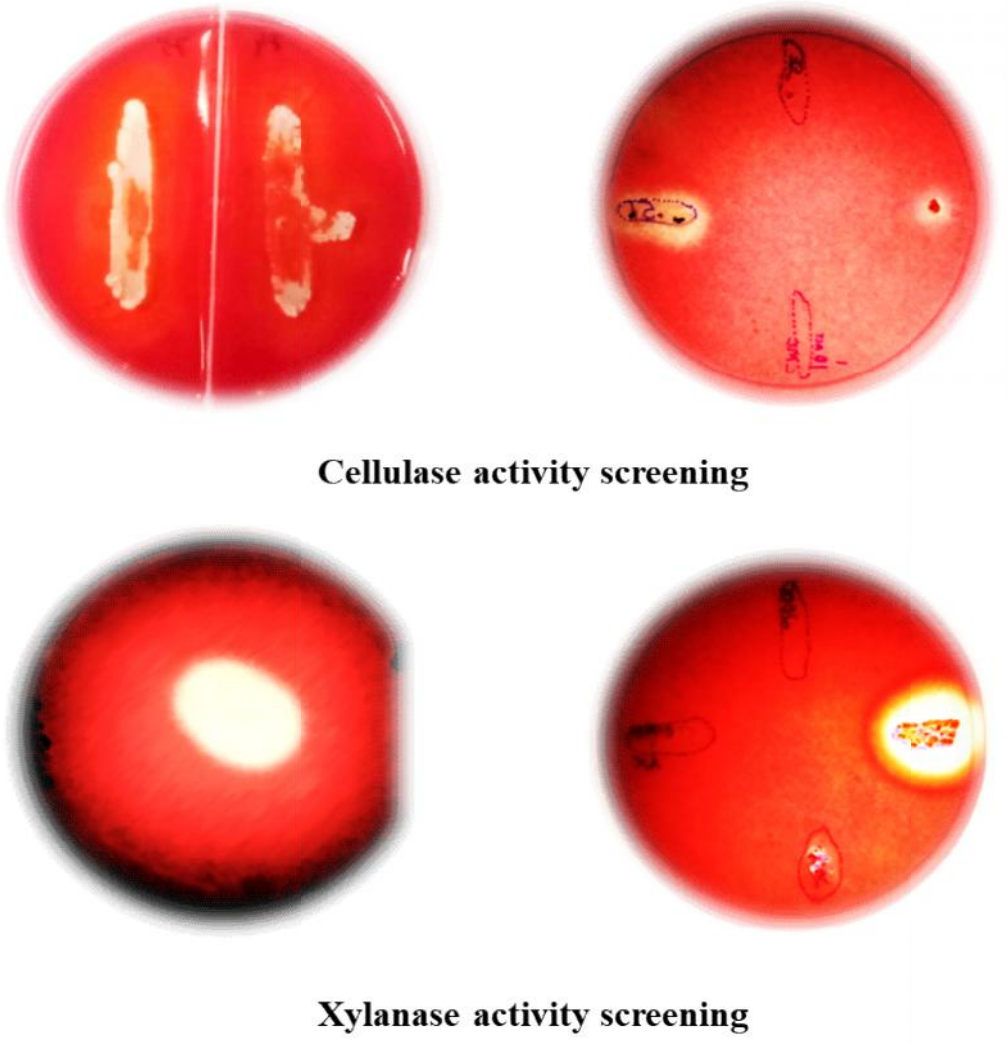


Figure 6: Extracellular Enzyme Activity

- *Bacillus sp.*(613)showed the cellulase and xylanase production ability in the presence of all three heavy metals, however isolate *Streptomyces coelicoflavus*(457) and *Bacillus cereus*(642) showed the enzymatic production in the presence chromium.

Screening for Protease Activity:

Streptomyces coelicoflavus(457), *Bacillus sp.*(613), *Bacillus cereus* (642) and *Lysinibacillus fusiformis* (665)has protease activity. When treated with 2mM of lead, cadmium and chromium each, *Streptomyces coelicoflavus* showed protease activity in presence of lead, and isolates *Bacillus sp.*, *Bacillus cereus* and *Lysinibacillus fusiformis* showed protease activity in presence of lead, cadmium and chromium.

Figure 7: Formation of clear zone on skim milk agar (protease activity)

Ammonia Production:

All 7 isolates were tested for ammonia production, out of which 6 isolates were found to produce ammonia. *Bacillus thuringensis*(519), *Bacillus cereus*(521), *Bacillus sp.*(613)and*Bacillus cereus*(642) produces brown colour when Nessler reagent was added indicating good amount of ammonia production, followed by*Streptomyces coelicoflavus*(457) and (498) respectively. The four isolates *Bacillus thuringensis*,*Bacillus cereus*, *Bacillus sp.* and *Bacillus cereus* produces ammonia when treated with heavy metals whereas isolates *Streptomyces coelicoflavus* and 498 produces little ammonia under lead and chromium treatment but no ammonia production under cadmium treatment.

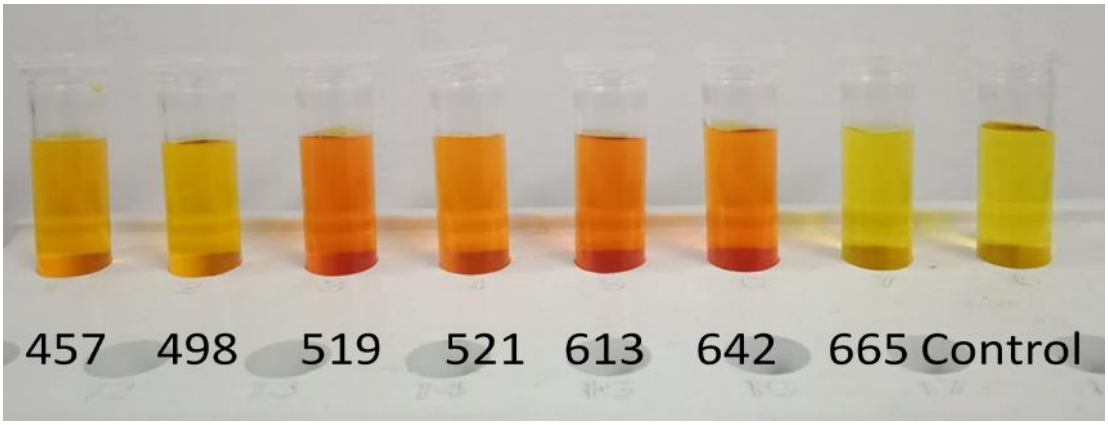


Figure 8: Ammonia production: Change of light yellow supernatent into dark brown colour

6 isolates showed positive in ammonia production. 4 isolate *Bacillus thuringensis*(519), *Bacilluscereus*(521), *Bacillus sp.*(613) and *Bacillus cereus*(642). *Streptomyces coelicoflavus*(457) and 498 produces ammonia but lesser. No ammonia production is shown by *Lysinibacillus fusiformis*(665).

Table 10: Production of ammonia by the bacteria

Isolates	No HM	Lead	Cadmium	Chromium
457	Light brown	Light brown	-	Light brown
498	Very light brown	Very light brown	-	Very light brown
519	Brown	Brown	Brown	Brown
521	Brown	Brown	Brown	Brown
613	Brown	Brown	Brown	Brown
642	Brown	brown	Brown	Brown
665	-	-	-	-

Ammonia Production:- Very Light brown- minimal; Light brown-less; Brown-high

Screening for HCN production:

Two isolates *Streptomyces colicoflavus*(457) and *Bacillus cereus*(642) showed HCN production. Both isolate *Streptomyces coelicoflavus*(457) and *Bacillus cereus*(642) changed the yellow colour picric acid impregnated filter paper into reddish brown indicating the production of HCN. When treated with 2mM each of lead, cadmium and chromium, *Streptomyces coelicoflavus*(457) and *Bacillus cereus*(642) showed HCN productionin lead and chromium treatment.

Figure 9: Change of yellow colour impregnated filter paper into reddish brown due to the production of HCN

Table 11: Selection of Two potent plant growth promoting rhizobacterial Strains

PGP activity	**613 (***Bacillus sp.***)**	**642 (***Bacillus cereus***)**
ACC deaminase	**1.77**±0.04µg/ml	0.98±0.64µg/ml
Phosphate solubilisation	87.21 µg/ml	87.11µg/ml
IAA production	119 µg/ml	123 µg/ml
Extracellular enzymes	Produced in all HM treatment	Produced(with Cr only)
Ammonia production	High ammonia	High ammonia
Protease	Showed activity in all HM treatment	Showed activity in all HM treatment
HCN	-	+

Siderophore	Positive	Positive
HM degradation	94%	97%
Seed germination	90%	95%

Physico-Chemical Analysis of Soil:

The physico chemical properties of the soil were analyzed. It includes pH, water holding capacity, electrical conductance. The pH of the soil was found to be 7.87. Electrical conductance was found to be 180.9 MHOS/cm and water holding capacity was found to be 42.27.

Seed Germination Assay:

Seed were surface sterilized and soaked in water for 8 hours. Three sets of treatment were given-Set I-only hoagland solution, SetII- Hoagland solution+ HM(Pb, Cd or Cr) and Set III-Hoagland solution+ HM(Pb, Cd or Cr)+ bacterial isolate(613(*Bacillus sp.*) or 642(*Bacillus cereus*)).

Table 12: Germination Assay

Samples	Germination %
Control	80
Pb	70
Pb+613	80
Pb+642	80
Cd	60
Cd+613	70
Cd+642	75
Cr	80

Cr+613	85
Cr+642	85
613	90
642	95

Control

Lead

Cadmium

Chromium

Figure 10: Seed Germinationassay carried out with heavy metals

Pot experiment:

Pot experiment was carried out for the best two PGP isolates. The germinated seeds were seeded on corresponding pots and grown for at least 30 days.

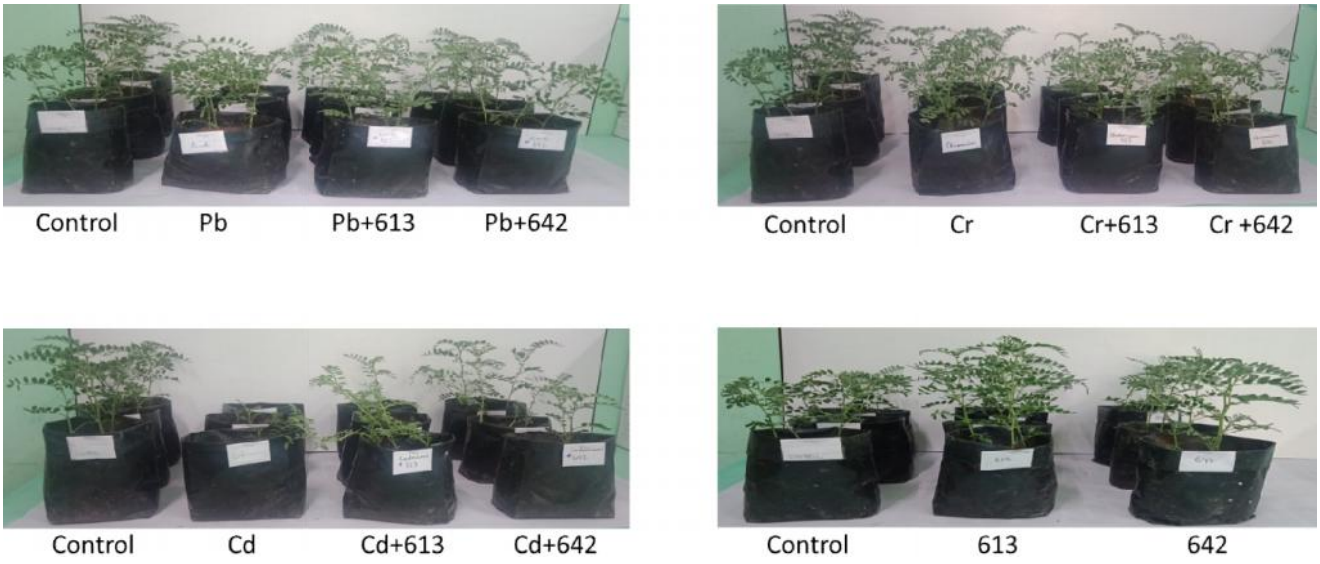


Figure 11: Pot experiment carried out on Chick pea plant against three heavy metal and two potent bacterial strain.

Plant physical parameters:

After around 30 days of growing, the growing plant was separated from the soil and the roots was washed in running water. Length of the root, length of shoot and no of branches was counted as shown in the table.

Figure 12:Plants physical parameters observed after pot experiment

Table 13: Plant physical parameters (shoot length, root length and branches)

Isolates	Shoot Length	Root Length	Branches
Control	15.93±1.25	16.86±2.50	8.33±0.58
Lead	15.03±1.27	14.16±1.26	8.66±1.15
Lead+613	19.06±1.29	16.80±2.54	9.33±1.53
Lead+642	20.83±1.26	16.93±2.75	10.00±1.00
Cadmium	14.00±0.50	10.33±4.16	8.33±0.58
Cadmium+613	15.16±0.29	12.00±1.00	8.66±0.58
Cadmium+642	16.33±1.04	14.00±1.00	8.73±0.58
Chromium	19.33±1.15	18.33±4.07	9.00±0.00
Chromium+613	18.00±1.00	17.33±4.93	10.00±1.00
Chromium+642	18.00±1.00	19.00±3.61	9.33±1.53
613	19.36±1.21	17.06±1.01	10.33±1.15
642	19.50±0.50	18.33±1.04	10.66±0.58

Chlorophyll Content:

Chlorophyll was extracted in 80% acetone and the absorbance was read at 663 and 645 nm in a spectrophotometer. Using the absorption coefficient given by Arnon, 1949, the amount of chlorophyll was calculated.

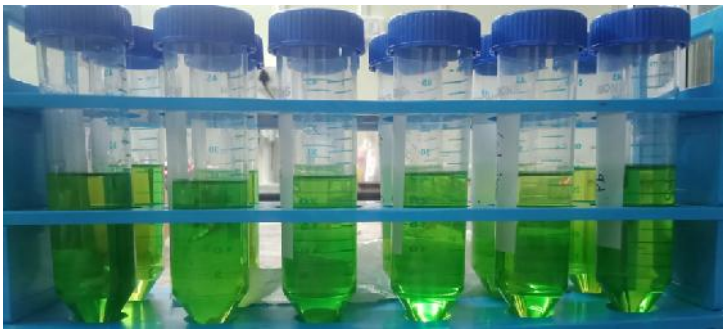


Fig:Chlorophyll extraction with 80% ethanol

Table 14: Estimation of Chlorophyll

Treatments	Chlorophyll a (mg/g of tissue)	Chlorophyll b (mg/g of tissue)	Total chlorophyll (mg/g of tissue)
Pb	1.23±0.56	0.46±0.85	1.70±0.97
Pb+613	1.17±0.34	0.42±0.63	1.59±0.96
Pb+642	1.17±1.02	0.57±0.74	2.24±0.53
Cd	0.99±0.76	0.39±0.01	1.39±0.54
Cd+613	0.96±0.56	0.40±0.95	1.36±0.64
Cd+642	1.21±0.86	0.44±0.37	1.64±0.85
Cr	1.30±0.54	0.51±0.54	1.87±0.67
Cr+613	1.10±0.01	0.40±0.43	1.52±0.83
Cr+642	1.18±0.96	0.40±0.76	1.61±0.38
613	1.19±0.76	0.43±0.43	1.61±0.01
642	1.36±0.34	0.47±0.56	1.78±0.75

Control	1.14±0.87	0.41±0.96	1.55±0.74

Discussion:

Heavy metals are present in traces in the soil naturally and they are harmful to plants microbes and human if it exceeds in the environment. Through plants growing in heavy metal contaminated sites, it comes to us from our food chain. They need to be removed from the agricultural field or have to convert it from its toxic form to a non-toxic form. Biological approaches becomes a better option as compared to physical and chemical methods of removing the heavy metals. Blaylock et al., (1997) concluded that when 1 acre of lead polluted agricultural field was bioremediated, 50-65 % saving as compared to conventional methods like landfill or excavation was done. Some plants and microorganisms are capable of bioremediating the toxic heavy metals from polluted soil. Amongst these, there are groups of microorganisms which can remove heavy metals as well as enhances plants growth promotion in various ways including phosphate solubilisation, ACC deaminase activity, IAA production, HCN production, siderophore production, protease, ammonia production, etc., (Glick 1995; El-Deeb*et al*., 2012). These organisms could become a target as biofertilisers as well as useful in bioremediation.

In the present study, we focused on microbes capable of tolerating heavy metal stresses with plant growth promoting properties. We have obtained more than 7 strains of rhizospheric bacteria that showed both heavy metal tolerance as well as plant growth promotion. The presence of the enzyme ACC deaminase becomes an important characteristics features of stress tolerating microorganisms. Abiotic stress such as drought, heavy metal toxicity stress, salt stress, etc., can be tolerated to some extend if they have ACC deaminase. It increases the level of ethylene under stress thereby tackling the stress by producing ACC deaminase. All seven isolates showed ACC

deaminase activity. *Bacillus sp.* (613) showed the highest ACC deaminase activity. It produces 177 μmol α -ketobutyrate mg protein-1 h-1 of ACC deaminase activity. Previously studies also suggested that presence of ACC deaminase activity showed increase in plant growth as well as biomass and in addition to this, it improves systemic resistance induction and increases the activity of antioxidant enzymes in wheat, Arabidopsis, tomatoes and other plants treated with Enterobacter ludwigii and Flavobacterium sp. (Gontia-Mishra et al., 2014). So these PGP bacteria used in this study can be exploited in heavy metal contaminated sites as well as it can be exploited to drought, salt contaminated sites, water logging areas, etc.

IAA is a result of the metabolism of microorganisms using L-tryptophan. IAA manufacturing is thus directly dependent on the availability of L-tryptophan for many PGPRs (Ahmad et al. 2008). Other than IAA, L-tryptophan metabolism can produce IBA, indole-3-pyruvate, indole-3-acetamide, tryptamine, and indole-3-acetonitrile (Godinho et al., 2010). In addition, there have been records of these L-tryptohan metabolites reacting and giving colour reaction to Salkovaski reagent (Glickmann&Dessaux 1995; Szkop&Bielawski 2013).

IAA is a phytohormone that affects plant growth in many plant parameters, such as elongation and cell division, apical dormancy, and vascular tissue differentiation (Kloepper&Schroth 1978). Godinho et al., 2010 reported that isolated from Goa sand dunes, Kocuria rosea developed IAA 26 μg ml-1. Vicene et al., 2012 reported unnamed Kocuria sp. 35μg ml−1 developed PWN-228A isolated from Pinewood pinaster. Ahmad et al., 2008 reported that IAA of up to 20 μg ml−1, 25 μg ml−1, and 10 μg ml−1 were developed by several species of Azotobacter, Pseudomonas, and Bacillus. The highest IAA concentration is produced by Bacillus thuringensis (519(chromium)) is about 126 μg/ml, while the smallest IAA concentration is produced by Lysinibacillus fusiformis (665 (lead)) which is 34.1 μg/ml. Presence or absence of heavy metals affects the production of IAA. Presence of chromium increases the production of IAA, whereas absence of lead and cadmium produces more IAA. Presence of cadmium drastically

decreases the production of IAA whereas lead doesn't affect much in the production of IAA.

Other than IAA production, phosphate solubilization, siderophore production and peptone ammonia production were also shown by the 7 isolates. The two primary nutrients needed by plants are phosphate and nitrogen. There is a significant factor responsible for phosphate solubilization in the secretion of organic acid by rhizobacteria (Buch et al. 2010). The root surface population of rhizobacteria is 10 times greater than that of bulk soil. Such rhizobacteria primarily use glucose, sucrose, fructose, xylose, and L-arabinose sugars that are present in root exudates to metabolise and generate organic acid that allows phosphate solubilization (Archana et al., 2012).

The highest phosphate solubilisation was shown by isolate 498 with 3.90 PSI solubilises 92.87µg/ml followed by isolate Bacillus sp. (613) as 3.69 PSI solubilises 87.21µg/ml. And the lowest PSI was shown by isolate Bacillus cereus 521 contaminated with lead which solubilises 22.55 µg/ml of phosphorus.

Another essential function of PGPR is the production of ammonia, in which an organism can break down complex nitrogen materials such as peptones and release ammonia into the soil. As a nutrient source, released ammonia is consumed by plants. There may be an accumulation of ammonia in soils rich in nitrogen, causing an alkaline state of the soil that suppresses the growth of some fungi (Jha *et al*., 2012). Ammonia formed by strain such as 519, 521, 613 and 642 showed resemblances with strains like *Bacillus, Pseudomonas, Rhizobium, Azotobacter, and Enterobacter* (Joseph *et al*., 2007).

Siderophores are compounds that can chelate iron and induce iron deficiency in the plant rhizosphere to increase pathogens, indirectly suppressing the growth of these pathogens. Jan et al., 2011 vividly explains the significance of siderophore development in Pseudomonas organisms, where it induced systemic resistance in watermelon against gummy stem rot while the siderophore-negative mutants failed to induce resistance. Out of 7 isolates, three isolates Bacillus sp. (613), *Bacillus cereus* (642) and *Lysinibacillus*

fusiformis (665) shows positive to siderophore production by forming orange halozone on CAS agar media. The production of siderophore was quantified for the three isolates. Bacillus sp. (613) produces 7.87%, *Bacillus cereus* (642) produces 20.12% and *Lysinibacillus fusiformis* (665) produces 6.56% respectively.

Biocontrol activity against the fungal pathogens *Fusarium lycopersicum, Fusarium graminearum, Fusarium udum, Macrophominaphaseolina Fusarium poliferatum, Tricophytonmetagrouphytes* was also shown. Under the current analysis, *Bacillus sp.* (613) and *Bacillus cereus* (642) with substantial PGPR characteristics showed growth promotion in heavy metal soil-containing chick pea growth. Therefore, *Bacillus sp.* and *Bacillus cereus* strains isolated from rhizospheric soil can be regarded as PGPR from the evidence collected. Out of 7 isolates, isolate number 498 and showed antagonistic potential against four fungal pathogens. Isolate 498 showed antagonistic potential to *Fusarium lycopersicum, Fusarium graminearum, Fusarium udum*and*Macrophominaphaseolina. Bacillus cereus* showed antagonistic potential to *Fusarium lycopersicum, Fusarium lycopersicum, Fusarium graminearum, Fusarium udum, Fusarium poliferatum, Tricophytonmetagrouphytes. Bacillus thuringensis, Bacillus cereus* and*Lysinibacillus fusiformis* also showed antagonistic activity to *Tricophytonmetagrouphytes.*

Therefore, the use of microorganisms in the rhizosphere is a technique for the tolerance to abiotic stress, in particular to climate-change-induced environmental stress. In order to enhance biological stress tolerance, *Bacillus sp.* also plays an important role. Induction of disease tolerance is necessary for the regulation of certain genes and hormones, such as 1-aminocyclopropane-1-carboxylate deaminase (ACCD). Ethylene can reduce the growth of the root and stem and help preserve homeostasis.

Conclusion

Heavy metal contamination is the great concern to both the environmental and human health. Contaminated lands are mostly abounded, which leads to reduced use of agricultural lands. Therefore, remediation of such land or environments is the main concern for improving the agricultural productivity in such lands. In the present study, we tried to isolate and identify of heavy metal tolerating bacteria from soil that may also have other plant growth promoting properties. Isolation was done using heavy metal enrichment techniques followed by isolation and characterization for heavy metal tolerance and plant growth properties. In total, 60 bacteria were isolated that showed tolerance to 25mM of lead (Pb). These isolates were further screened for cadmium (Cd) and chromium (Cr) and maong those, 34 isolates showed tolerance to 15mM of chromium and other 9 isolate found tolerance to cadmium. Among all heavy metak tolerating isolates, seven 7 isolate found to be tolerance to all the three heavy metals at a higher concentrations. These isolates were identified using molecular characterization and the sequences obtained were submitted at GenBank. These bacteria were identified as *Streptomyces coelicoflavus*, *Bacillus thuringensis*, two *Bacillus cereus*, *Bacillus sp*. and *Lysinibacillus fusiformis*. These bacteria were then evaluated for their plant growth promoting properties. We found that ACC deaminase production ranges from 177µmol α-ketobutyrate mg protein^{-1} h^{-1} to 30 α -ketobutyrate mg protein^{-1} h^{-1}). All 7 isolates solubilise inorganic phosphate, additionally *Bacillus cereus* and *Lysinibacillus fusiformis* solubilises inorganic phosphate in presence of lead and cadmium respectively. Out of 7 isolates, 498 and *Bacillus cereus* showed antagonistic potential against more than one fungal pathogen. *Bacillus thuringensis, Bacillus cereus* and *Lysinibacillus fusiformis* also showed antagonistic activity to *Tricophytonmetagrouphytes*. All isolate showed positive in IAA production. Quantitative analysis of IAA production ranges from 126 µg/ml to 34.1 µg/ml. *Bacillus thuringensis* produced the highest amount of IAA. 6 out of 7 isolates produces ammonia. 3 isolates produces extracellular enzymes (cellulose and xylanase). 3 isolates Bacillus sp., Bacillus cereusand *Lysinibacillus fusiformis* were positive for siderophore. *Bacillus sp.* and *Bacillus cereus* and 642 exhibits broad range of PGP activities and were further selected for pot experiment. Two best isolates

Bacillus sp. and *Bacillus cereus* based on PGP activity were selected and pot experiment was carried out with these two strains. The present study showed that the bacteria with both the heavy metal tolerance as plant growth properties could be found useful for remediating the heavy metal contaminated lands that can be used for agricultural purpose to increase the productivity.

ABSTRACT

ISOLATION AND IDENTIFICATION OF HEAVY METAL RESISTANCE BACTERIA FROM RHIZOSPHERE AND SCREENING FOR THEIR PLANT GROWTH PROMOTING POTENTIAL

FARZANA AHMAD

DEPARTMENT OF BIOTECHNOLOGY

MIZORAM UNIVERSITY

INTRODUCTION

Heavy metal contamination becomes a serious concern. It arises due to various anthropogenic activities like mining, sewage from industries, tanning of leather, etc.These contaminated heavy metals need to be removed from the environment as it is very toxic to humans, plants and animals. Various methods are employed to remove these heavy metals from the soil such as physical, chemical and biological methods. Amongst these methods, biological methods became the most desirable one as it is cost effective and environment friendly. Microorganisms is one of the most desiredorganism to study bioremediation. Some microorganisms which shows heavy metal bioremediation also shows plant growth promoting characteristics also. This study aims at Isolation and screening of bacterial isolates for their heavy metal resistance ability against lead, cadmium and chromium, Determination of *in vitro* and *in vivo* plant growth promoting potential of selected isolates and Molecular identification of the potential isolates using 16SrRNA gene amplification.

MATERIALS AND METHODS

Isolation of bacteria was done from rhizospheric soil by using serial dilution technique (Manivannan*et al*., 2012) and spread on different nutritional media.The obtained isolates were screened against three heavy metals (lead, cadmium and chromium) and isolates which showed tolerance to all three heavy metal was selected for PGPR studies. Genomic DNA of the selected isolates was extracted by the protocol of Green and Seabrook (2012) method and was identified on the basis of 16S ribosomal RNA sequence. The extracted DNA was amplified using 16S rRNA universal primers - PA: (5′-AGA GTT TGATCC TGG CTC AG-3′) and PH: (5 ′- AAG GAG GTG ATC CAG CCG CA-3′) according to Qin *et al*., (2009).Aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme assay was done by following the protocol of Shrivastava and Kumar (2013).IAA production was estimated according to Bric *et al*., (1991).Insoluble phosphate solubilisation was carried out on Pikovskaya agar plate and also quantified(Pikovskaya, 1948). Cellulase and xylanase degrading activity of the obtained bacterial isolates was screened by using induction medium supplemented with CMC and xylan respectively (Gupta *et al*., 2004). For siderophore production ability, 1 µL of the bacterial suspension which was grown overnight in Luria broth was spotted on Chrome Azurol S agar plates (Ames-Gottfred*et al*., 1989). Formation of orange halozone confirms siderophore

production. All the selected bacterial isolates was screened for their antifungal activity using four fungal pathogens (*Fusariumlycopersicum, Fusariumgraminearum, Fusariumoxysporium*and*Fusariumpoliferatum*) according to Passari*et al.,* (2016). To estimate ammonia, the Nesslerization response defined by Cappucino and Sherman (1992) was used. Cyanide production was detected as described by Alvarez *et al.,* 1995. For conducting the pot experiment, the soil were collected from garden, transferred into polythene bags and brought into the laboratory and pots were prepared and kept for atleast two months. Germination assay was carried out by soaking the chickpea seeds for 8 hours, the seeds were treated in three different sets. Set I(control), Set II(5mM heavy metal (Pb or Cd or Cr)) and Set III(treated with each HM 5mM concentration along with bacterial inoculum(613 and 642 respectively)) (Mitra*et al*., 2018). The germinated seeds were planted on pots and kept it for growing for one months. On the 31st day the grown plants were removed from the pots. Roots were separated slowly from the soil and washed in running tap water. The root and shoot length was measured and the number of branches were counted. Chlorophyll content of the plant were estimated. The method given by Arnon (1949) was followed to estimate the photosynthetic pigments.

RESULTS

Out of 600 isolates, 60 showed tolerance against lead, 34 against chromium and 9 showed tolerance against cadmium. The 7 strains were tested for ACC deaminase activity and were found to be positive for all 7 strains. The highest activity was observed in cell-free extracts obtained from*Bacillus sp.* (613) (177 µmol α -ketobutyrate mg protein^{-1} h^{-1}) followed by 498 (104 µmol α a-ketobutyrate mg protein^{-1} h^{-1}). All seven isolates showed halo zone formation for phosphate solubilisation using PKV agar. *Bacillus cereus* (521) contaminated with lead solubilises phosphorus and *Lysinibacillus fusiformis* (665) contaminated with cadmium also solubilises phosphorus. The highest IAA concentration is produced by *Bacillus thuringensis*(519 (chromium)) is about 126 µg/ml, while the smallest IAA concentration is produced by *Lysinibacillus fusiformis* (665 (lead)) which is 34.1 µg/ml. Out of 7 isolates, three isolates *Bacillus sp.* (613), *Bacillus cereus* (642) and *Lysinibacillus fusiformis* (665) shows positive to siderophore production by forming orange halozone on CAS agar media. Out of 7 isolates, isolate number 498 showed antagonistic potential against four fungal pathogens *Fusariumlycopersicum*, *Fusariumgraminearum, Fusariumudum*and *Macrophominaphaseolina.*

Bacillus cereus showed antagonistic potential to *Fusariumlycopersicum, Fusariumlycopersicum, Fusariumgraminearum, Fusariumudum, Fusariumpoliferatum, Tricophytonmetagrouphytes.Bacillus thuringensis*, *Bacillus cereus* and *Lysinibacillus fusiformis* also showed antagonistic activity to *Tricophytonmetagrouphytes.*

Bacillus sp.(613)showed the cellulase and xylanase production ability in the presence of all three heavy metals, however isolate *Streptomyces coelicoflavus* (457) and *Bacillus cereus* (642) showed the enzymatic production in the presence chromium. *Streptomyces coelicoflavus*(457), *Bacillus sp.* (613), *Bacillus cereus* (642) and *Lysinibacillus fusiformis* (665). *Bacillus thuringensis*(519), *Bacillus cereus*(521), *Bacillus sp.* (613) and *Bacillus cereus* (642) produces brown colour when Nessler reagent was added indicating good amount of ammonia production, followed by *Streptomyces coelicoflavus* (457) and (498) respectively. The four isolates *Bacillus thuringensis*, *Bacillus cereus*, *Bacillus sp.* and *Bacillus cereus* produces ammonia when treated with heavy metals whereas isolates *Streptomyces coelicoflavus* and 498 produces little ammonia under lead and chromium treatment but no ammonia production under cadmium treatment as protease activity. Two isolates *Streptomyces colicoflavus* (457) and *Bacillus cereus* (642) showed HCN production.

CONCLUSION

Heavy metal contamination is the great concern to both the environmental and human health. Contaminated lands are mostly abounded, which leads to reduced use of agricultural lands. Therefore, remediation of such land or environments is the main concern for improving the agricultural productivity in such lands. In the present study, we tried to isolate and identify of heavy metal tolerating bacteria from soil that may also have other plant growth promoting properties. Isolation was done using heavy metal enrichment techniques followed by isolation and characterization for heavy metal tolerance and plant growth properties. In total, 60 bacteria were isolated that showed tolerance to 25mM of lead (Pb). These isolates were further screened for cadmium (Cd) and chromium (Cr) and maong those, 34 isolates showed tolerance to 15mM of chromium and other 9 isolate found tolerance to cadmium. Among all heavy metals tolerating isolates, seven 7 isolate found to be tolerance to all the three heavy metals at a higher concentrations. These isolates were identified using molecular characterization and the sequences obtained were submitted at GenBank. These bacteria were identified as *Streptomyces*

coelicoflavus, *Bacillus thuringensis*, two *Bacillus cereus*, *Bacillus sp*. and *Lysinibacillus fusiformis*. These bacteria were then evaluated for their plant growth promoting properties. We found that ACC deaminase production ranges from 177µmol α-ketobutyrate mg protein^{-1} h^{-1} to 30 α -ketobutyrate mg protein^{-1} h^{-1}. All 7 isolates solubilise inorganic phosphate, additionally *Bacillus cereus* and *Lysinibacillus fusiformis* solubilises inorganic phosphate in presence of lead and cadmium respectively. Out of 7 isolates, 498 and *Bacillus cereus* showed antagonistic potential against more than one fungal pathogen. *Bacillus thuringensis, Bacillus cereus* and *Lysinibacillus fusiformis* also showed antagonistic activity to *Tricophytonmetagrouphytes*. All isolate showed positive in IAA production. Quantitative analysis of IAA production ranges from 126 µg/ml to 34.1 µg/ml. *Bacillus thuringensis* produced the highest amount of IAA. 6 out of 7 isolates produces ammonia. 3 isolates produces extracellular enzymes (cellulose and xylanase). 3 isolates Bacillus sp., Bacillus cereusand *Lysinibacillus fusiformis* were positive for siderophore. *Bacillus sp.* and *Bacillus cereus* exhibits broad range of PGP activities and were further selected for pot experiment. Two best isolates *Bacillus sp*. and *Bacillus cereus* based on PGP activity were selected and pot experiment was carried out with these two strains. The present study showed that the bacteria with both the heavy metal tolerance as plant growth properties could be found useful for remediating the heavy metal contaminated lands that can be used for agricultural purpose to increase the productivity.

REFERENCES

Alvarez-Clare, S; Mack, M C; Brooks, M (2013). A direct test of nitrogen and phosphorus limitation to net primary productivity in a lowland tropical wet forest. Ecology, 94(7):1540-1551.

Ames-Gottfred, N. P., Christie, B. R., and Jordan, D. C. (1989). Use of the Chrome Azurol S agar plate technique to differentiate strains and field isolates of *Rhizobium leguminosarum*(*biovartrifolii*). *Appl.Environ.Microbiol*. 55:707–710.

Arnon. D.I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol*. 24:1-5

Bric, J M., Bostock, R M., Silverstone, S E. (1991). Rapid *in situ* assay for indole acetic acid roduction by bacteria immobilised in nitrocellulose membrane. *Appl. Environ. Microbiol*. 57: 537-538

Gupta, A. K. (2004). The complete technology book on biofertilizers and organic farming. National Institute of Industrial Research Press India

Manivannan, M., Ganesh, P., Suresh, K. R., Tharmaraj, K., and Shiney, R. B. (2012). Isolation, screening, characterization and antagonism assay of PGPR isolates from rhizosphere of rice plants in Cuddalore district. *International Journal of Pharmaceutical and Biological Archive*, 3(1):179–185.

Passari, A. K., Mishra, V. K., Leo, V. V., Gupta, V. K., and Singh, B. P. (2016) Phytohormone production endowed with antagonistic potential and plant growth promoting abilities of culturableendophytic bacteria isolated from ClerodendrumcolebrookianumWalp. *Microbiol. Res.* 193, 57–73.

Pikovskaya, R. I. (1948). Mobilization of phosphorus in soil connection with the vital activity of some microbial species.*Microbiologia*17:362–370.

Qin, S., Li, J., Chen, H. H., Zhao, G. Z., and Zhu, W. Y. (2009).Isolation, diversity and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna, China.Applied and Environmental Microbiology, 75, 6176 –6186.

Shrivastava, U.P., Kumar, A., 2013. Characterisation and optimisation of 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity in different rhizospheric PGPR along with *Microbacterium sp*. Strain ECI-12A. *Int J. Appl. SciBiotecnol.* 1, 11-15.