Development of Plant Growth Promoting Rhizobacterial Consortium for Remediation of Cadmium (Cd) and Lead (Pb) contaminated soil by *Canna indica* and *Zea mays* L.

SUMMARY OF

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

LUCKNOW

BABASAHEB
BHIMRAO
AMBEDKAR
UNIVERSITY

LUCKNOW

प्रज्ञा शील करुणा

ESTABLISHED 1996

FOR THE DEGREE OF

Doctor of Philosophy

IN

ENVIRONMENTAL MICROBIOLOGY

Submitted By

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(Enrolment no. 188/10)

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UTTAR PRADESH, INDIA

2019

Soil's heavy metal contamination due to various natural and anthropogenic activities has been recognized as a serious environmental hazard and considered a major barrier to sustainable development, particularly in developing or underdeveloped nations. Amongst all the heavy metals, cadmium (Cd) and lead (Pb) have emerged as serious toxic environmental pollutants in the past few years because of their excessive use in manufacturing and agricultural industries. Their elevated concentrations in the soil, enormously pollute the natural ecosystem as well as alter or destruct the soil texture by reducing its fertility and nutrient availability. With the growing concern and more industrialization, there is an urgent need to address this severe problem because of their continuous accumulation in agricultural soils and water resources that pose a great threat to contaminate the food chain and cause potential risk to human health and ecosystem. Different conventional approaches such as physical, chemical, thermal and electrokinetic techniques have been developed by the researchers in recent years to mitigate the contamination of heavy metals from soil but unfortunately they all have failed with several demerits such as cost, non-eco-friendly nature, labour intensive, generate secondary pollutants that are more complex than previous contaminants. Taking into consideration all these problems, a plant based biological technique "Phytoremediation" has been identified around five decades as a low cost emerging, eco-friendly and sustainable solution for the remediation of heavy metal contaminated soils. Phytoremediation is a widely accepted technique in which plants are used as a remediator or accumulating agents for removal of toxic heavy metals from contaminated soils. Plants used in this technique have an increased rate of heavy metal uptake, rapid translocation from root-to-shoot and excellent ability to detoxify

and sequester heavy metals in their aerial parts. Because of sustainable features, phytoremediation has been considered as a best alternate for removal of heavy metals from contaminated soil without affecting the biological activity, structure and fertility.

Although phytoremediation is easily applicable and cost-effective technique, but it does have some inherent technical constraints like, it is restricted to the site with low pollutant concentration; the higher concentration of contaminant may check the plant growth. Moreover, hyperaccumulator plants are usually limited to their slow growth and low biomass. Limited bioavailability of tightly bound fraction of metal ions from the soil is another demerit of phytoremediation technology. Slow transfer rate of metal from soil to root and root to shoot makes this technique inefficient. To mitigate these drawbacks use of beneficial soil microbiota colonizing the rhizosphere, often known as Plant Growth Promoting Rhizobacteria (PGPR), endowed with the unique property of heavy metal resistant and plant growth promotion, have been considered as an important tool to promote phytoremediation technology by increasing plant biomass. PGPR's increase plants biomass by various mechanisms such as fixation of atmospheric nitrogen, mitigate stress by utilization of 1 aminocyclopropane-1-carboxylic acid (ACC) as a sole N source, production of siderophores and anti-pathogenic substances, production of plant growth regulators (phytohormones, such as auxins), and also through the transformation of nutrient elements like phosphorous and potassium.

The present study entitled "**Development of Plant Growth Promoting Rhizobacterial Consortium for Remediation of Cadmium (Cd) and Lead (Pb) contaminated soil by *Canna indica* and *Zea mays* L."** was carried out with the aim of isolation and characterization of Cd and Pb resistant plant growth promoting rhizobacterial strains and their exploitation in the phytoremediation of Cd and Pb contaminated soil using *Canna indica* and *Zea mays* L.

Total thirty morphologically distinct bacteria were isolated initially from industrially contaminated sites based on primary screening which involved the notable growth in Cd and Pb supplemented media at 100 ppm. Further, out of thirty isolates, 94% isolates were growing at 100 to 200 ppm of Cd concentration, and 4% were growing at 200 to 600ppm Cd concentration, while only 1% isolates were growing up to 1200 ppm of Cd concentration. However, only 1% isolates were growing at 100 to 1500 ppm Pb and 2% isolates were growing at 100 to 750 ppm of Pb concentration, rest isolates were growing at ≤500 ppm of Pb concentration. Moreover, at the last of this experiment, it was screened-out that out of the total thirty isolates, three isolates (SA, PC1 and PC3) were growing at higher concentration (1200 ppm Cd and 1500 ppm Pb concentration) of both cadmium and lead. On the basis of maximum tolerance concentration of Cd and Pb, three isolates *viz.*, SA, PC1 and PC3 were finally selected for further study.

Further, these three selected rhizobacterial isolates (SA, PC1 and PC3) were characterized for their plant growth promoting attributes *viz.* ACC deaminase activity, IAA production, phosphate solubilization, siderophore, extracellular polysaccharide production, ammonia and HCN production. Results revealed that the selected bacterial isolates (SA, PC1 and PC3) were showing multiple plant growth promoting activities in the absence and presence of heavy metal (Cd and Pb) ions till 500 ppm concentration. Beyond this concentration, all the strains lost their PGP properties. Hence, 500 ppm concentration of both (Cd and Pb) metal ions were selected for further experiments. Some of the plant growth promotory traits (ACC deaminase and siderophore) enhanced in the presence of heavy metals while some traits (IAA, EPS and phosphate solubilization) decreased, while some (Ammonia and HCN) remained unaffected.

All three test isolates depicted capability to cleave ACC as a sole source of nitrogen by producing ACC deaminase enzyme in DF salt media which is essential to balance the ethylene level in cells. Further, quantitative assessment of ACC-deaminase activity was determined and it was observed that isolate PC1 and PC3 enhanced the activity by 19.28% and 23.44%, respectively in the presence of Cd. While, in the presence of Pb ions, isolate PC1 increased the activity by 21.42%, strain PC3 by 27.70% and strain SA by 42.32%. On the other hand, it was observed the activity was unaffected by strain SA in the presence of Cd metal ions. Siderophore production by the selected isolates revealed that strains SA and PC3 were able to produce siderophore with and without Cd and Pb ions amended media. While strain PC1 was found negative for siderophore production in both conditions i.e. in absence and presence of heavy metal ions. The quantitative assessment with strains SA and PC3 revealed that 99.01 and 54.74 µM of siderophore were released in the culture supernatant in control conditions while, siderophore production increased by 6.056% in case of isolate SA in the presence of Pb metal ions. Moreover, it was observed that the selected isolates were able to produce IAA by utilizing L-tryptophan as a precursor compound in the presence of Cd and Pb metal ions. Quantitative estimation showed that IAA production increased as the concentration of tryptophan increased but at a certain concentration, it decreased gradually. Further, the highest amount of IAA was produced by strain PC1 (38.737 µg mL^{-1}) followed by strain PC3 (35.556 µg mL^{-1}) and SA (31.48 µg mL^{-1}) at 500µgml^{-1} tryptophan concentration. Phosphate solubilization test showed that all the selected heavy metal resistant rhizobacterial strains were able to solubilize inorganic phosphate which was confirmed by brown colour halo zone on NBRIP media agar plate. Further quantitative assessment at different time intervals showed that solubilization was increased as the incubation period increases but after a certain time period, it decreases gradually. Further, selected rhizobacterial isolates produced HCN and Ammonia in the presence and absence of Cd and Pb amended media. Observed results revealed that HCN production by isolates, SA, PC1 and PC3 were positive in the absence of Cd and Pb heavy metals. However, in the presence of Cd, strain PC1 was negative for HCN production. For the production of ammonia, selected isolates showed positive results in the absence and presence of Cd and Pb. Extracellular polysaccharide production revealed that all the three heavy metal resistant rhizobacterial isolates were able to produce extracellular polysaccharide (EPS) in the absence and presence of Cd and Pb metal on LB agar plates. The mucoid gummy colonies on LB agar plate and increased viscosity in LB broth indicated the ability of strains to produce EPS. Further, EPS produced by bacterial isolates, SA, PC1 and PC3 characterized by FTIR depicted different characteristic peaks between 3401-805cm^{-1}. The stretching vibration at 3402, 3072 and 3040 cm^{-1} indicating O-H (hydroxyl) group and hydrogen bonding, a characteristic of polysaccharide ring.

Further, morphological characterization of selected isolates showed that strain SA possessed large hairy colony while PC1 and PC3 test isolates possessed tiny round-shaped colony, with convex elevation and margins were entire for one while undulate for other test isolate i.e. they depicted different morphological features. All test isolates were Gram negative, rod shaped and motile. Scanning electron microscopic (SEM) study of the selected isolates confirmed that all isolates were rod shaped. Further, biochemical characterization tests showed that all the three test isolates were negative for indole and methyl-red test; PC1 and PC3 were positive for vogesproskauer test, while SA was negative for it. All the three isolates were positive for catalase, cellulase and citrate utilization while negative for urease production test. However, in case of oxidase test, isolate SA was positive and isolates PC1 and PC3 were negative. Isolate SA (*Pseudomonas aeruginosa*) was positive for lipase production while other two (*Pantoea agglomerans*, *Enterobacter cloacae*) failed to produce the same. By 16S rRNA partial gene sequencing and similarity index it was observed that strain PC1, PC3 and SA were closest to *Pantoea agglomerans*, *Enterobacter cloacae* and *Pseudomonas aeruginosa* respectively, showing 98-100% homology. The obtained sequences were submitted to Gene Bank (NCBI) under the accession number KX668493 (PC1), KX668494 (PC3) KX668495 (SA). For the evolutionary relationship, a phylogenetic tree was constructed using neighbor joining (NJ) method for the strains PC1, PC3 and SA. Antibiotic sensitivity pattern of selected isolates were showing resistant towards several antibiotics such as Piperacillin, Gentamycin and Chloramphenicol, while showing intermediate response for Cefoxitin antibiotic. Among the species, *Pantoea agglomerance* (PC1) and *Enterobacter cloacea* (PC3) were showing resistance pattern for most of the antibiotics.

To prepare successful microbial consortium compatibility test is an important test and was performed by agar diffusion method. Three rhizobacterial strains, PC1, PC3, and SA showed compatibility with each other.

Further, each individual strain and their consortium were tested for seed germination of *Canna indica* and *Zea mays* L. plant under Cd and Pb stress with suitable control. The results of seed germination tests revealed that each individual strain and their consortium increased germination percentage as well as seed vigour index of *Canna indica* and *Zea mays* L. under heavy metal (Cd and Pb) stress. Moreover, findings of seed germination tests depicted that consortium gave better results than individual strains.

The study was undertaken to investigate the interactive effects of Cd and Pb on the growth and development of *Zea mays* L. and *Canna indica* plant species treated with consortium as well as individual strains, SA, PC1, PC3 at different time interval using pot experiments. Findings depicted that as the concentration of heavy metal (Cd and Pb) increases, the physiological growth parameters of plants decrease linearly. However, inoculation of rhizobacterial strains as well as their consortium increases the plant growth in each concentration of Pb and Cd contaminated soil. The root-shoot length and biomasses were directly related to plant growth which was observed to decrease consequently under Cd and Pb stress, while in presence of three test isolates and consortium, there was an increasing trend in all the three parameters where consortium was better than individual strains. Enhancement in plant length and biomass under metal stress condition, inoculated with PGPR strains and consortium depicted reduction in toxicity through diverse mechanisms such as ACC deaminase, IAA synthesis, siderophore and phosphate solubilisation.

Chlorophyll ‘a’ and ‘b’ content in leaves of both the plants was found to be higher at low concentration of cadmium and lead. However, with increase in concentration of Cd and Pb in soil, chlorophyll content declined. Similar to the chlorophyll inhibition pattern, present study also investigated the inhibition of carotenoid content in both the plant leaves under the toxic effects of Cd and Pb. Marked distortion of chloroplast ultrastructure leads to disturbed shape and inflated thylakoids and this decreases carotenoid content severely affecting photosynthetic performance and ultimately plant growth reduction. However, due to the inoculation of PGPR strains (SA, PC1 and PC3) and consortium, carotenoid content enhanced that helped plant overcome the toxic effects of Cd and Pb. Proline is well known for osmolytic activity which protects the cells from the deleterious effects of ROS generated by cells under the influence of environmental stresses such as heavy metals, drought, and salinity. Observations of present study showed that the foliage proline content progressively increased with increasing concentration of Cd and Pb. However, after inoculation of rhizobacterial strains as well as their consortium, the proline content decreased successively in each treatment.

Uptake and accumulation of Cd and Pb in shoot and roots of *Canna indica* and *Zea mays* L. plants were directly correlated with the exposure time and concentration of heavy metals in the soil. It was observed that there was a linear increase of Cd and Pb concentration in response to increased exposure time as well as concentration (100-500 mg Kg^{-1}) of heavy metals in the soil by *Canna indica* and *Zea mays* L. The maximum Root Metal Content (RMC), shoot metal contents (SMC) and total metal accumulation (TMA) in *Canna indica* and *Zea mays* L. plants were observed at 500 ppm concentration of Cd and Pb at 90 DAS. Interestingly, inoculation of rhizobacterial strains as well as their consortium further increased the RMC, SMC and TMA as compared to their respective control. Bioconcentration factor (BCF) describes the ability of plant to uptake metal ions from the soil. It is an important factor required to estimate the accumulation potential of a particular plant species for the remediation of heavy metals. BCF in this study depicted that the value for *Canna indica* and *Zea mays* L. plants under Cd and Pb stress were >1 at the concentration range from 100 ppm to 500 ppm while at 0 ppm concentration, the values were below one (<1) at all exposure time (30, 60, and 90 DAS) under four different treatments (T1, T2, T3, and T4). The value of BCF >1 indicated that the plants have good potential for remediation of Cd and Pb contaminated soil. Further, the translocation factor (TF) of the plant describes the ratio of heavy metal (Cd and Pb) concentration in shoot tissues over heavy metal concentration in root tissues. Results from this study revealed that the TF value of *Canna indica* and *Zea mays* L. plants under Cd and Pb stress were <1 at all the concentration and all exposure times under four different treatments i.e. T1, T2, T3, and T4. Further, it was observed when rhizobacterial consortium as well as individual strains were applied, slight increase in TF values were obtained. The increase in TF value in the presence of rhizobacterial consortium indicated that rhizobacterial consortium help the plant in phytoextraction process of heavy metal contaminated soil. Moreover, a comparative phytoextraction study between *Canna indica* and *Zea mays* L. was carried out with the help of principle component analysis; results yielded twelve components, that explained 100% of the total variance, the loading factor with score plot indicate that the component-1 is associated with RMC, SMC, TMA, BCF and TF of both the plants under higher concentration of Cd concentration at 90 DAS. While, the second component showed positive association with TDB and SMC under controlled condition as well as lower concentration of Cd concentration (upto 200) at all the sampling periods. However, in the presence of Pb metal ion, the loading factor with score plot indicated that the component-1 is associated with RMC, SMC, TMA, BCF and TF of both the plants under higher concentration of Cd concentration at 60 and 90 DAS. While the second component (Principal Component 2; PC2) represents the positive association with TDB and BCF under control condition as well as lower concentrations of Pb (upto 200) at all the sampling periods.

With these findings, study concluded that isolated rhizobacterial strains and their consortium have the ability to detoxify heavy metals as well as promote growth of *Canna indica* and *Zea mays* L. by enhancing nutrient availability, efficiently diminishing the high level of ethylene, proline and other oxidative stress caused by heavy metals. Out of *Zea mays* L. and *Canna indica*, *Zea mays* L. is better for remediation with rhizobacterial strains but *Canna indica* holds promise owing to more accumulation of cadmium and lead in the tissues as compared to *Zea mays* L. Further, these rhizobacterial candidates and their consortium could be targeted for environmental management studies wherein they could efficiently cleanup toxic metal contaminated sites and concurrently improve the plant growth even in metal polluted soils. Moreover, there are lot of hidden mechanisms that naturally occur in heavy metal contaminated soils that are still to be understood, but the findings of this study will form a strong foundation and may serve as a baseline data for increasing the growth of plants in heavy metal contaminated soils, for bio-management of such soils using heavy metal resistant plant growth promoting rhizobacteria under sustainable environmental and agricultural development and reclamation of sites contaminated with heavy metals.