Remediation of Cadmium Contaminated Soil with PGPR Consortia and Hyperaccumulator Plants

Thesis

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Summary

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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