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Original Article

Bioremediation of Heavy metal contaminated soil by the Exigobacterium and Accumulation of Cd, Ni, Zn and Cu from Soil environment

Ajaz haja mohideen[a], R., Thirumalai Arasu[c], V., Narayanan[b], K. R and M.I.Zahir Hussain[d]
[a] Dept of Microbiology, Sadakathullah Appa College, Tirunelveli – 627 011, India
[b] Department of Adv. Zoo. and Biotechnology, Sri Paramakalyani College, Alwarkurichi – 627 412
[c] Department of Microbiology, Ayya Nadar Janaki Ammal College, Sivakasi -626123, India
[d]- Dept of Adv. Zoo. and Biotechnology, Sadakathullah Appa College, Tirunelveli-627011, India
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Abstract

The progressive accumulation of metals may inhibit the degradation of organic pollutants or of humic substances in the environment. In our study area, Sivakasi is well known for crackers, printing and Match factories. Fireworks and match work industries are predominant one and the explosion of fireworks has been discovered to be a source of intense heavy metal release that is being addressed in Sweden and other countries. Two bacterial isolates were examined for their ability to degrade the Cd, Ni and Zn in soils and for their effect on metals uptake by Millet and Green gram plant. Restriction fragment length polymorphic DNA (RFLP) analysis was used to show that the bacterial cultures were genetically diverse. Bacterial isolates AZ T4 and AZ T12 had 16S rRNA gene sequences that were most similar to Bacillus cereus and Bacillus thuringiensis, based on 100% similarity in their 16S rDNA sequence, respectively. Filtrate liquid media that had supported B. cereus and B. thuringiensis growth significantly increased Cd and Zn extraction from soil polluted with metal industry effluent and from Cd-rich soil, respectively. The highest concentrations of Cd (0.14 mg-kg^{-1}), Cu (13.6 mg-kg^{-1}) Ni (22.03 mgkg^{-1}), Pb (42.88 mg-kg^{-1}) and Zn (58.1 mgkg^{-1}) were accumulated in shoots of greengram grown on Cd-rich soil inoculated with *B. cereus* and *B. thuringiensis*. The highest concentration of Ni (22.03 mgkg^{-1}), was accumulated in Millet roots grown in metal industry effluent polluted soil inoculated with a mixed inoculums of bacterial strains. These results show that bacteria play an important role in increasing soil fertility by removal of heavy metal and accumulation of soil Environment.

Keywords: Bacteria, Heavy Metal, RFLP, 16S rDNA, Millet, Green gram

Introduction

Due to urbanization and land degradation, the area of agricultural land is continuously decreasing. Proficient use of available agricultural land resources is important to overcome the deficiency. Soil is a non-renewable dynamic resource and acts as an interface between agriculture and the environment. Maintaining soil quality is the vital factor to improve crop yield and productivity. Among the soil quality maintance heavy metals plays an imperative role to sustain its eminence properties (Mc Grath *et al.*,1995; Cheng, 2003). In India many industries are using heavy metals in their process and exiled out without proper treatment. Metals are released into the environment leads wide spectrum of anthropogenic activities such as smelting of metallic ores, industrial fabrication and commercial application of metals, which are polluting our aquatic bodies.

Though, several metals are essential for biological systems and must be present in a certain concentration range. Too low concentrations lead to a decrease in metabolic activity. At too high concentrations these metals lead to toxicity. Nonessential metals are tolerated at very low concentrations and inhibit metabolic activity at higher concentrations. The many uses of heavy metals in several applications lead to their wide distribution in soil, silt, waste, and wastewater. Such a pollution of the environment by toxic metals and radionuclide arises as a result of many human activities, largely industrial, although sources such as agriculture and sewage disposal also contribute. Historical emissions of old nonferrous factories in the neighbourhood of Vlaamse Installing voor Technologic Onderzoek (VITO) in the Kempen lead to large geographical areas (>100 km^2 with Cd concentrations higher than 3mg/kg soil) of

contaminated sites. Zinc (Zn) was the most abundant pollutant creating phytotoxicity while public health was mostly endangered by the presence of the toxic metal Cd. Besides Zinc (Zn), Nickel (Ni) and cadmium (Cd) the metals copper (Cu) and lead (Pb) were also present in the contaminated sandy soils.

A promising, relatively new technology for heavy metal contaminated sites is phytoremediation. Phytoremediation employs the use of plants to remove organic and/or inorganic contaminants from biota (phytoextraction), volatilization of contaminants by plants from the soil into the atmosphere (phytovolatilization), or stabilization of an inorganic into a less soluble form (phytostabilization). Phytoremediation is inexpensive, effective, can be implemented in situ, and is environmentally friendly (Chaney *et al.,* 1997; Trapp and Karlson,2001; Zavoda *et al.*,2001). A large proportion of metal contaminants are unavailable for root uptake by field grown plants. Methods of increasing heavy metal contaminant phytoavailability in soil and its transport to plant roots are vital to the success of phytoremediation in the field (Ernst 1996; Kukier *et al.*,2004; Abou-Shanab *et al.,* 2006). Microbial populations are known to affect trace metal mobility and availability to the plant, through release of chelators, acidification, and redox changes (Smith and Read,1997; Abou-Shanab *et al.,* 2003). The presence of rhizosphere bacteria has been reported to increase the concentrations of Zn, Cu, Pb or Cr in plants (Whiting *et al.*,2001; Chen *et al.,* 2005; Abou-Shanab *et al.*,2007).Improvement of the interactions between plants and beneficial rhizosphere microorganisms can enhance biomass production and tolerance of the plants to heavy metals, and is considered to be an important component of phytoremediation technologies (Wenzel and Jockwer,1999; Glick,2003). The present work has been focused on examined bacterial isolates for their ability to solubilize Cd, Zn, Ni, and Cu, as measured by extraction in soil and their effect on metal uptake by millet and green gram.

Materials and Methods

Preparation of Soil sample

Soil samples were taken from different site of industrial area present in Sivakasi. It contained high concentrations of Cu, Zn, Cd, Ni and Pb due to smelter emissions. Soil samples were mixed in large containers and air-dried at room temperature, then crushed and sieved to remove rocks and undecomposed organic materials. Soil pH was determined after mixing 1g of soil in 2.5mL water for about 5min, allowed ionic exchange to reach equilibrium prior to measuring pH (Forster 1995). Total metals in soil were determined by digesting 200 mg of soil in a mixture of concentrated HCl /HNO_3 (4:1, v/v) (McGrath and Cunliffe,1985). Water extractable metals were measured by shaking 10 g (dry wt) moist field soil for 2h in 20mL deionized water (Angle *et al.*,1993). Sample were filtered and acidified with HNO_3 before analysis. Metal concentrations in the acid digest and solutions were analysed by Atomic absorption spectrophotometry (AAS).

Metal extraction from soil using the products of bacterial growth

This experiment tested the ability of the products of bacterial growth to solubilize Cu, Zn, Cd, Ni and Pb in soil. Two bacterial isolates were tested for the effects on Zn, Ni, and Cd extraction from industrial-effluent-polluted soil and soils collected from site exposed to a Cd smelter. These bacteria were originally isolated from metal-contaminated soils (Abou-Shanab *et al.*,2006). Bacterial strains were grown overnight in 500-mL Conical flasks containing 250 mL of sterilized nutrient broth (Wollum,1982) on a shaker at 100 rev/min at 37°C until late log phase. Another flask containing sterile nutrient broth was uninoculated as a sterile (axenic) control. The medium in each flask was centrifuged at 8000 rpm for 15min; The supernatant was decanted and vacuum filtered through a sterile filter (0.22ml pore size). The ability of the filtrate to extract metal from the soil was determined by shaking four replicate 2-g samples of the soil with 10mL of each of the bacterial or axenic filtrates for 2h. The soil suspensions were centrifuged at 4000 rpm for 15 min and filtered. The concentration of metals in the HNO_3 acidified filtrate was determined by AAS, and the mass of soil was determined after drying at 80°C.

Electron microscopic studies

The scanning electron microscope (SEM) will scan the ultra image of the specimen. The size of the bacterial strain were estimated

with SEM. Sample photos were taken by a Quanta 200 scanning electron microscope made by FEI, at magnification of 5000 X and 10,000X.The samples are dried with platinum coating under coating holder. This dried specimen was tested under high vacuum in natural state, without sputtering a platinum layer on the sample. We estimate the size of the specimen. The ultra structure of the specimen was recorded with a computer analyzer (Fig.1).

16S ribosomal RNA (rRNA) gene sequencing

PCR on the extracted DNA was performed in a 100-µl volume. Oligonucleotide primers with specificity for eubacterial 16S rRNA genes, primers 16S rDF CGCTGGCGGCAGGCTTAACA); 16S rDR (CCAGCCGCAGGTTCCCCT) were used to amplify the 16S rRNA gene fragments with template DNA originating from bacteria. The following conditions were used for DNA amplification: 35 cycles consisting of denaturation at 94°C for 0.5 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min followed by a final extension at 72°C for 3 min. Amplified PCR products of the proper size (about 1500 base pair fragment) were confirmed by electrophoresis of 10µL sub samples through a 1% horizontal agarose gel containing 0.5µg/mL Ethidium bromide. Gels were examined under UV light and photographed. PCR products were purified using QIAquick Spin columns (Qiagen Inc., Chatsworth, CA). A Perkin Elmer 377 DNA sequencer, in combination with Dye Deoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, CA) was used for sequencing the purified PCR products by the help of MWG bio informatics centre. Nucleotide sequences were compared with sequences in the National Centre for Biotechnology information (NCBI).GenBank database using the BLASTn program and Ribosomal Database Project (RDP) database using the sequence matching program.

PCR-RFLP analysis

Restriction Fragment Length Polymorphism (RFLP) analysis was carried out in PCR amplified product of 16S rDNA sequence. Genomic DNA of bacterial isolates was extracted from 10mL bacterial cultures grown overnight according to the method described by McSpadden *et al.,* (2000).The required quantity of 16S rDNA was digested with respective restriction endonucleases in the presence of appropriate 1X reaction buffer. Reaction mixture was incubated at 37°C for 3 h and if necessary, the reaction was stopped by heat inactivation as recommended by the manufacturer. The PCR products were analysed on 1.5%agarose gel and visualized by ultraviolet illumination after staining with 0.5lg/mL Ethidium bromide. The fingerprint patterns resulting from RFLP analysis were scored for each template DNA by recording the presence or absence of bands.

Pot experiment

Bacterial inoculation of Millet and Green gram

Bacterial cells were grown overnight in 500-mL Conical flasks containing 250mL of sterilized nutrient broth on a shaker at 100 rev/min at 37° C until late log phase. Soil was sterilized by autoclaving at 15 lbs pressure at 121° C for 15 min. The seeds were surface sterilized and coated with test microorganisms. The sterilized soil was transferred to the pots ($3/4^{th}$). The seeds were sowed into the soil. Water was poured onto the pots regularly (Morning and Evening). Once the germination gets start, the culture was centrifuged and the pellets were suspended with saline, it was used as inoculums for pot. Without culture (only saline) was used as inoculum for control.

Chlorophyll determination

The chlorophyll content of plant leaves was measured by the method of Arnon, (1949). 100 mg of leaf samples were ground with 80% acetone followed by centrifugation at 3000 g for 5 min. Absorbance of the supernatant was detected at 645 and 663 nm.

Total Chlorophyll mg L^{-1}
= 20.2 x A645 + 8.02 x A663 x Vol/1000 x weight
Carotenoid content mg L^{-1}
= A480 + (0.114+A638) x A645

Root and Shoot growth

Grown plants were removed carefully from the soil by mixing the total in sterilized distilled water and remove without any damage to the root. The length of the root and shoots were estimated with commercially available scale. Further the fresh weight and dry weight was weighed in electronic balance. The later

one is weighed after drying in oven for overnight.

Results and Discussion

Soil physicochemical properties

Total metal content is important because it determines the size of the metal pool in the soil and thus the potential for metal uptake (Ibekwe *et al.,* 1995). Therefore, soil samples were analysed for total and water extractable Ni, Cu, Cd and Zn. Each soil exhibited a high concentration in one or more of the metals. The results (Table-2) also revealed a high variability of concentration of Cd, Ni, and Zn in different land uses. The concentration of the Nickel and cadmium was higher (3.9 and 4.1 ppm respectively) in sample 1 (S1) than remaining four samples (S2, S3, S4 and S5). Similarly the concentration of the zinc was higher in sample 3 (S3) than others. soil pH analysis 80% of the samples showed the pH ranged from 7.2 to 8.21 and remaining 20% of them in acid soils pH<6.7 (Table-1). This indicates that the pH for agricultural soils in Sivakasi has a tendency to be higher than natural and acidic soils pH. Organic matter and sand, silt, and clay contents vary significantly among soil samples.

Table-1: Soil characteristic feature of agriculture soil samples taken from Sivakasi

Sample	Soil pH	Organic C content	Soil conductivity
S1	8.5 ± 0.1	0.9 ± 0.25	35.8 ± 0.7
S2	8.4 ± 0.2	10.6 ± 0.18	36.8 ± 0.7
S3	7.9 ± 0.1	12.3 ± 0.34	29.0 ± 0.8
S4	6.3 ± 0.3	3.5 ± 0.68	15.8 ± 0.9
S5	7.8 ± 0.1	11.5 ± 0.45	37.1 ± 0.7

PCR RFLP analysis

After digest of PCR product of 16S rDNA which is amplified from soil DNA was restricted with the enzyme *HindIII.* The gel profile was visual compared and this show the bacterial communities shows distinctly different profiles (Fig.1).

Fig -1: Showing scanning Electron Microscope analysis of AZT4 strain structure at X750 magnifying condition

A thick intense prominent band was present in all samples irrespective of metal or organic concentrations, and few other slight bands were present in certain samples. But the size of the band is differed. The DNA band located in the control soil DNA template was visible in the sample DNA extracted from the soil of rhizosphere taken after 15 days.

PCR amplification of 16S rRNA coding gene

The 16S rRNA gene of the AZT4 strain was PCR amplified using the 16S rDNA universal primers and both the strands were sequenced. The sequences were compared with the 16S rDNA sequences available in the RDP database (http://rdp.cme.msu.edu/). Sequence analysis revealed that the strains were phylogenetically closely related to the genus *Exiquobacterium.* BLAST analysis of the 16S rDNA sequence of AZT4 isolates revealed that it is more similarity score bit with *Exiquobacterium* sp. Though the isolates had a close similarity, the dendrogram constructed based on their phylogenetic relationship revealed that all the isolates were distinctly placed under separate clusters. (Fig -2)

Fig -2: PCR amplification of 16 SrRNA gene from AZT4 Strain using Universal 16SrDNA specific primer.

Fig-3: RFLP analysis of PCR amplified 16 rDNA product of soil DNA isolated from two different plant (Greengram and Millet) seeding Root adhere soil of AZT4 and AZT12 inoculations.AZT4 -Lane 1-Control (0 day);Lane 2-After 5 days; Lane3-After 10 days;Lane4-After 15 days ; AZT12-Lane5-After 5 days; Lane6-After 10 days; Lane7-After 15 days.

Effect of bacterial filtrate on the solubilisation of metal in soils

Soil microorganisms are ubiquitous in soils to which hyper accumulators are native, even in those soils containing high concentrations of metals (Schlegel *et al.,* 1991; Ghaderian *et al.*,2000). Soil microorganisms can produce iron chelators and siderophores that ensure iron availability, reduce soil pH, and/or solubilize metal-phosphates (Abou-Shanab *et al.*, 2003). To support the hypothesis that bacteria facilitate an increase in the solubility of no labile Cd, Zn, Cu and Ni in the soil was obtained by measuring the concentration of Cd, Zn, Cu and Ni extracted from soil by bacteria in nutrient liquid media. The concentrations of Cd solubilized from the max soil by nutrient filtrated media after 24 h of bacterial growth (*B. cereus* and *B. thuringiensis*) were significantly higher than those extracted by the sterile growth media. Also, the products of *B. cereus* and *B. thuringiensis* growth increased Zn, Cu and Ni solubilization in the metal contaminated soils, indicating that the products of bacterial growth could mobilize these metals in the soils. These observations are in agreement with that obtained by (Abou-Shanab *et al.,*2003) in which they reported that the concentration of extractable Ni was increased from a high-Ni soil of 2.2-2.6 mg kg^{-1} when the soil was inoculated with *B. cereus* siderophore production and phosphate solubilization by the bacterial isolates facilitated Ni solubility in the nonsterile, Ni-rich soils. Siderophore production can also be stimulated by the presence of heavy metals (van der Lelie *et al.,* 1999) and they possibly affect bioavailability as well. For instance, it was reported that in Azobacter vinelandii, siderophore production is increased in the presence of Zn (II) (Huyer and Page 1988). Iron-chelating hydroxamic acid production by *Bacillus thuringiensis* is increased by exposure to Cu, Cr, Cd, Zn and Ni were found to increase siderophore production in Pseudomonas aeruginosa (Gilis 1993; Hassan 1996). The same effect was found for Zn and Al in *P. fluorescens* ATCC17400 (Gilis,1993). These results indicate that the activity of the bacteria in the soil would very likely have had a significant effect on increasing the mobility of metals in the rhizosphere of the plants in soils.

Effect of metal resistant bacterial culture in Green gram plant growth

Effect of MRB isolate AZT4 and AZT12 was analyzed with three different plants namely green gram, millet and rice seedling. Inoculation of these strains for the improvement on plant growth parameters such as root length; shoot length and chlorophyll content were examined. Treatment of this heavy metal resistant strain AZT4 with green gram seedlings has significantly improved the shoot height (45.5 mm) compared to the plant treated with the AZT12 strain (34.0mm) and corresponding control (29.0 mm). Similarly the root length of the plants treated with the AZT4 was significantly higher (46.0 mm) compared to the plants treated with AZT12 (35.0mm) and control (30.0 mm) (Table-3). Correspondingly the dry weight and fresh weight of the individual plant is observed and tabulated in table 3

Table-2: AAS analysis of Heavy metal in Agricultural soil samples

Soil sample	Heavy metals	Concentration (ppm)
S1	Nickel	3.9
	Zinc	2.5
	Cadmium	4.1
S2	Nickel	2.6
	Zinc	3.2
	Cadmium	3.9
S3	Nickel	2.7
	Zinc	4.1
	Cadmium	3.3
S4	Nickel	2.9
	Zinc	3.2
	Cadmium	3.7
S5	Nickel	3.4
	Zinc	2.8
	Cadmium	3.7

Values are represented as ppm concentration of heavy metal (Mixture of five random samples from single site).

Table -3: Effect of metal resistant bacterial culture in Green gram, Millet and rice seedling plant growth

Characters	**Plant growth**			
		Green Gram	Millet	Rice
	Control	29	40	34.5
Shoot height (mm)	AZT4	45.5	43.5	36.5
	AZT12	34	30.5	31.5
	Control	30.5	40.5	69.5
Root length (mm)	AZT4	46.0	50.5	112
	AZT12	35.0	43.5	104
	Control	0.54	0.35	1.03
Fresh weight (mg)	AZT4	1.63	0.75	0.59
	AZT12	0.75	0.62	1.10
	Control	0.16	0.12	0.22
Dry Weight (mg)	AZT4	0.76	0.32	0.45
	AZT12	0.24	0.15	0.52
	Control	0.432	0.493	0.412
Total Chlorophyll (mg gfw^{-1})	AZT4	0.634	0.937	0.775
	AZT12	0.613	0.856	0.654

Effect of metal resistant bacterial culture in Millet plant growth

Treatment of this heavy metal resistant strain AZT4 with green gram seedlings has significantly improved the shoot height (45.5 mm) compared to the plant treated with the AZT12 strain (33.5mm) and corresponding control (40.0 mm). Similarly the root length of the plants treated with the AZT4 was significantly higher (50.5 mm) compared to the plants treated with AZT12 (43.5 mm) and control (40.5 mm) (Table- 3). In this the strain inoculated with AZT12 showed degreased shoot length than control. Correspondingly the dry weight and fresh weight of the individual plant is observed and tabulated in table- 3.

Effect of metal resistant bacterial culture in rice plant growth

Treatment of this heavy metal resistant strain AZT4 with green gram seedlings has significantly improved the shoot height (36.5 mm) compared to the plant treated with the AZT12 strain (31.5 mm) and corresponding control (34.5 mm). Similarly the root length of the plants treated with the AZT4 was significantly higher (112.0 mm) compared to the plants treated with AZT12 (104.0 mm) and control (69.5 mm) (Table-3). Similar with millet in this also the strain AZT12 degreases the shoot length (31.5) than control. Correspondingly the dry weight and fresh weight of the individual plant is observed and tabulated in table 3.

Chlorophyll content

The plants treated with the ATZ4 showed significantly higher level of leaf chlorophyll content in the leaf of green gram, millet and rice is 0.634; 0.937 and 0.775mg gfw^{-1} respectively. In the plant treated with the AZT12 inoculated showed 0.613; 0.856 and 0.654 mg gfw^{-1}) and the corresponding control 0.432;0.493 and 0.412mg gfw^{-1}) (Table-3). Similar trend was observed with respect to the chlorophyll *a* and chlorophyll *b*.

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