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ASSESSING THE POTENTIAL OF RHIZOBACTERIA IN MINE SPOIL REMEDIATION

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ABSTRACT
Phytoremediation of mine land offers a great challenge, to restore its productivity and fertility. It is one of the widely used emerging techniques for soil remediation, to remove pollutants from the environment or to stabilize them**.** Conventional phytoremediation techniques mostly involve growing hardy and tolerant plants, to remove pollutants or to stabilize the contaminated site. But long-term sustainability on mine spoil dumps requires a scientific approach. Inoculation of selective, site specific microorganisms at such sites increases the better survivability, growth and biomass of the plants. In this study, lignite mine spoil was collected from lignite mines of Neyveli Lignite Corporation, Neyveli. Heavy metal resistant rhizobacteria were isolated from plants obtained from revegetated mine area. The production of growth promoting factors (Indole Acetic Acid and siderophores) of the isolated strains was determined. Pot trials, with selected strains and suitable plant species were conducted to determine the plant growth in polluted mine spoil sample.

KEYWORDS: Mine spoil, Phytoremediation, Heavy metal resistance.

INTRODUCTION

Lignite is brownish-black in color and has a carbon content around 60–70 percent. It has high inherent moisture content as high as 75 percent, and an ash content ranging from 6–19 percent compared with 6–12 percent for bituminous coal. Lignite mines are present all over the world. In India, lignite is extracted at Neyveli lignite mines in Cuddalore district, Tamil Nadu, Giral (Barmer) Mine in Western Rajasthan, Matasukh and Kasnau mines in Central Rajasthan including five lignite mines present in Gujarat. Mine spoil dump generated due to mining activities poses drastic physicochemical and biological constraints for sustainable vegetation. A wide variety of heavy metals such as Zinc, Lead, Copper and Cadmium have been detected in soil at mining sites, that are presenting a major threat to the environment and population. Heavy metals cannot be biologically degraded and indefinitely persist in the environment. Heavy metals transferred through the food chain are a serious hazard to human health (Mazej *et al.*, 2010). Due to contamination by heavy metals, mining sites are surrounded by large barren areas. The awareness of the detrimental heavy metal contamination at mining sites has been increased in recent years. Phytoremediation has been effectively used to remediate heavy metal-polluted sites as a sustainable remediation approach. Augmenting the nutritive potential of the mine soil using organic wastes from domestic and industrial resources, namely paper mill sludge, biosludge, sewage sludge, compost and introduction of biofertilizer inoculants could provide a cost effective solution for waste dump reclamation. These waste materials contain substantial amount of organic matter, which favors establishment of vegetation and microbial proliferation. Plant-microbe partnerships may be utilized to improve biomass production and remediation. To have a functional role in remediation, bacteria in heavy metal-contaminated soil must first overcome the heavy metal stress. Microorganisms tolerate heavy metals by immobilizing metals on cell surfaces or transforming metals into less toxic forms, for example by precipitation, acidification and oxidation-reduction .

Phytoremediation utilizes heavy metal-tolerant plant species with metal accumulation ability. Since the addition of Indole Acetic Acid (IAA) to soil can enhance the growth of plant, bacteria-producing IAA have been used to assist the phytoremediation of soil contaminated with heavy metals. Restoration of microbial activity on these dumps enhances biogeochemical cycles. Further, use of biofertilizers and mycorrhizae has proved beneficial for long-term fertility and ecosystem development (Hooda, 2007). The application of biofertilizers containing the suitable heavy metal resistant rhizobacteria to improve the nutrient potential of mine soil dump is indispensable in today's need to conserve the depleting natural resources.

MATERIALS AND METHODS

Lignite Mine spoils and Fenugreek seeds are two important materials of the study. The mine soil was assessed for physico chemical properties like pH, bulk density, electrical conductivity, Cation Exchange Capacity Total Nitrogen, Total Phosphorus, Organic Carbon and Heavy metals like Cadmium, Chromium, Cobalt, Copper, Lead, Mercury, Nickel according to standard protocol. Rhizosphere soil samples were collected from roots of *Capsicum annuum*, one of the plant species found in the revegetated area of Mine 1 and Mine 1A of Neyveli Lignite Corporation, Neyveli, Tamil Nadu, India. The plants were uprooted carefully and brought to the laboratory in polythene bags in cold insulated container.

The soil samples were processed immediately or stored at 4^0C for the isolation of bacterial isolates. The Chilli plants *(Capsicum annuum Family:* Solanaceae) were collected from 35 year old plantations from Mine 1A of Neyveli Lignite Corporation, Neyveli.

Isolation and identification media includes Nutrient agar and *Pseudomonas* isolation agar. The above said media were prepared, sterilized and poured into plates. Assay media used were Nutrient broth amended with 2% of tryphtophan for Indole Acetic Acid (IAA), Chrome Azurol Sulphonate Medium (CAS Agar) for Siderophore production and Nutrient agar with the heavy metals Chromium and Cobalt for determining heavy metal resistance. Determination of PGPR activity of the isolates was done by estimation of Indole acetic acid (IAA) and siderophore production. Production of IAA by the bacterial isolates was determined by procedure as described by Gordon and Weber, (1951). Estimation of Siderophore was done by Chrome Azurol Sulphonate Assay (Schwyn and Neilands, 1987). Productions of ammonia by the isolates were checked by method described by Cappucino and Sherman, 1992. This is followed by analysis of heavy metal resistance and determination of minimum inhibitory concentration (MIC) of heavy metals, Chromium and Cobalt. Chromium was added in the form of Potassium dichromate (K_2 Cr_2 O_7) and Cobalt in the form of Cobaltous chloride ($CoCl_2$). Both the metals were added separately in the concentrations of 50ppm, 100ppm, 150ppm and 200ppm. Pot trials were conducted with heavy metal resistant, Siderophore and IAA producing isolates. Individual broth culture of these isolates was prepared using nutrient broth in Mac Farland Standard. The Fenugreek seeds were immersed in each of the broth culture for 30 minutes at room temperature and sown in the pots consisting of mine spoil. The root and shoot length were compared with the control for assessing growth promotion by the test strains.

RESULTS & DISCUSSION

Soil was subjected to physicochemical analysis that include pH, ,bulk density, cation exchange capacity, electrical conductivity, Total nitrogen , Total phosphorus, Organic carbon, the concentrations of heavy metals like Chromium, Cobalt ,Cadmium, Copper, Lead, Mercury and Nickel. The results are tabulated (Table 1.1).

TABLE 1. Analysis of mine spoil

S.No	Physico-Chemical Parameters	Methods	Values
1.	pH	IS 2720(part 26): 1987	7.59
2.	Bulk density	Cylinderic method	1.27 g/cc
3.	Cation Exchange Capacity	IS 2720 (part 24): 1976	163 mg/kg
4.	Electrical Conductivity	IS 14767: 2000	62.5 µmhos/cm
5.	Total nitrogen as N	IS 10158: 1982	73.8 mg/kg
6.	Total phosphorus as P	IS 10158:1982	21 mg/kg
7.	Organic carbon	Walkley and Black method	1.5%
8.	Chromium	EPA 200:8	1.27 mg/kg
9.	Cobalt	EPA 200:8	1.81mg/kg
10.	Cadmium	EPA 200:8	< 1 mg/Kg
11.	Copper	EPA 200:8	4 mg/Kg
12.	Lead	EPA 200:8	< 1 mg/Kg
13.	Mercury	EPA 200:8	< 1 mg/Kg
14.	Nickel	EPA 200:8	1.8 mg/Kg

Ten different isolates selected for the study were identified based on colony morphology, preliminary and biochemical tests (Table 1 & Fig. 1). *Bacillus* and *Pseudomonas* strains were predominant among the isolated strains. The ten strains were further subjected for the production of Indole acetic acid (IAA), Siderophore and Ammonia. Table 2 shows that all the ten strains were positive for siderophore production which was confirmed by the orange zone around the spot inoculums on Chrome Azurol Sulphonate (CAS) medium. All the strains were positive for ammonia production (Table 3).

TABLE 2: Identification of the strains

S. No.	Strain No.	Name of the strain
1.	LV1	*Enterobacter sp*
2.	LV2	*Klebsiella*
3.	LV3	*Bacillus subtilis I*
4.	LV4	*Serratia*
5.	LV5	*Bacillus subtilis II*
6.	LV6	*Bacillus sp.*
7.	LV7	*Pseudomonas sp*
8.	LV8	*Pseudomonas sp*
9.	LV9	*Pseudomonas fluorescens*
10.	LV10	*Bacillus subtilis III*

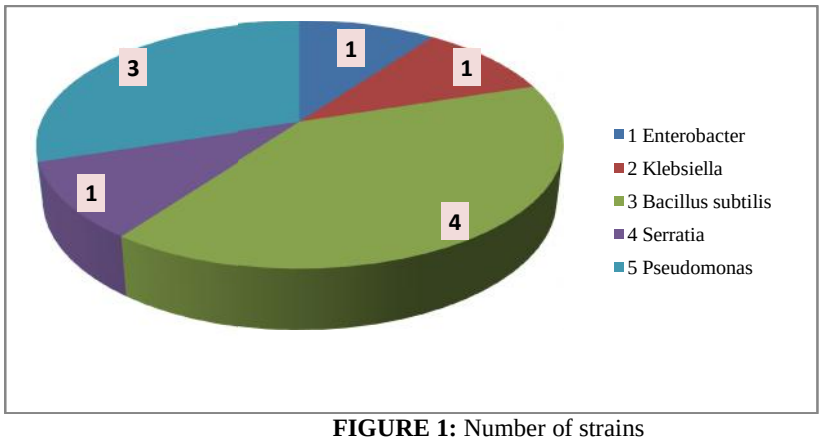

FIGURE 1: Number of strains

The Indole acetic acid production was determined by inoculating the ten strains in nutrient broth tubes in the presence and absence of tryptophan. The IAA production and its concentration can be interpreted from the Fig. 1.2 and 1.3. Maximum concentration of IAA in medium with tryptophan was produced by strains *Pseudomonas fluorescens* followed by *Bacillus subtilis III* and minimum production was by *Klebsiella*. But maximum concentration of IAA in medium without tryptophan was produced by *Bacillus subtilis I*, followed by *Serratia* and minimum production was by *Bacillus sp*. The ten strains were subjected to heavy metal resistance assay and their resistance was determined by their growth in the presence of heavy metals in medium with different concentrations. The Minimum Inhibitory Concentration of the heavy metals Chromium (Cr) and Cobalt (Co) at concentrations of 50ppm, 100ppm, 150ppm and 200ppm was determined.

TABLE 3. Identification of siderophore and ammonia production among the isolated strains

S. No.	Strain No	Name of the strain	Indole Acetic Acid (µg/ml)		Siderophore production	Ammonia Production
			With Tryptophan	Without Tryptophan		
1.	LV1	*Enterobacter sp*	24±100	16	Positive	Positive
2.	LV2	*Klebsiella*	21±1.52	14	Positive	Positive
3.	LV3	*Bacillus subtilis I*	31±0.50	30	Positive	Positive
4.	LV4	*Serratia sp*	40±0.98	24	Positive	Positive
5.	LV5	*Bacillus subtilis II*	32±0.52	4	Positive	Positive
6.	LV6	*Bacillus sp*	31±0.76	2	Positive	Positive
7.	LV7	*Pseudomonas putida*	32±100	4	Positive	Positive
8.	LV8	*Pseudomonas sp*	30±0.49	2	Positive	Positive
9.	LV9	*Pseudomonas fluorescens*	62±2.50	14	Positive	Positive
10.	LV10	*Bacillus subtilis III*	57±1.05	12	Positive	Positive

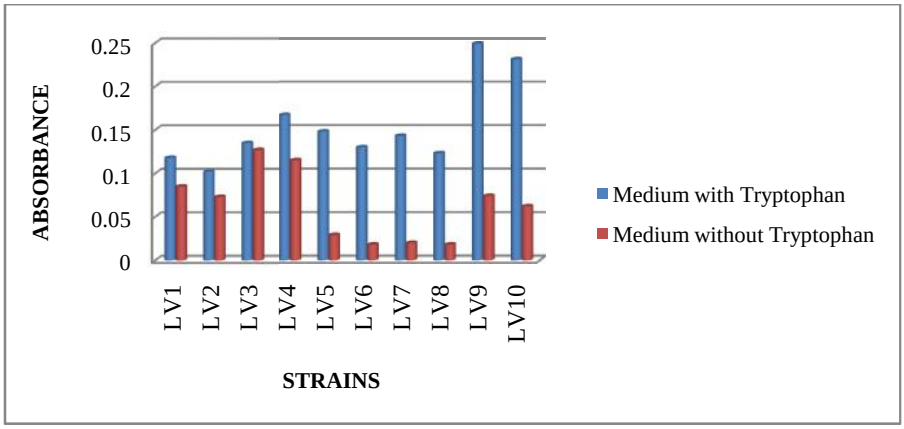

FIGURE 2: Estimation of Indole Acetic Acid Production

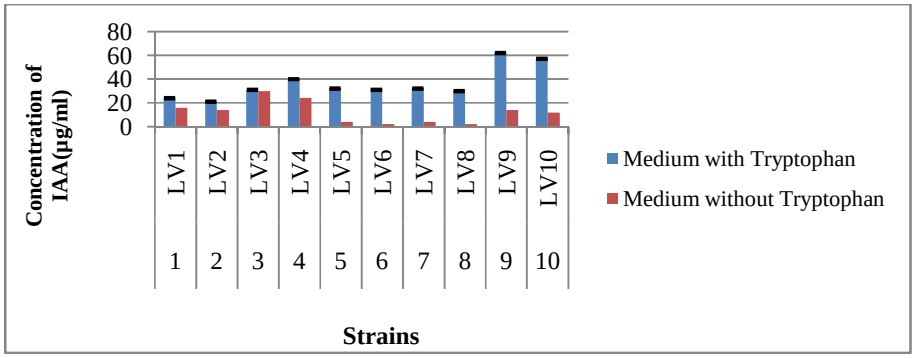

FIGURE 3: Concentration of Indole Acetic Acid

All the ten strains readily exhibited growth in media containing Chromium at all concentrations (Table 4). This indicated that the heavy metal chromium is exerting least toxicity in the test strains. While determining the resistance against Cobalt, it was found that all the ten strains grew in medium containing 50ppm Cobalt (Table 4). In medium containing 100ppm of Cobalt, *Enterobacter sp* did not grow and *Bacillus subtilis II* showed minimum growth while the rest of the other strains showed growth on the medium. In medium containing150 ppm and 200 ppm of Cobalt, all the strains showed no growth the reason may be due to the absence of plasmids conferring the heavy metal resistance(for cobalt) and explains that cobalt is more toxic than chromium.

TABLE 4. Heavy metals resistant pattern exhibited by the isolated strains

S. No.	Strain No	Strain name	Chromium (Cr)				Cobalt (Co)			
			50 ppm	100 ppm	150 ppm	200 ppm	50 ppm	100 ppm	150 ppm	200 ppm
1.	LV1	*Enterobacter sp*	++	++	++	++	+	-	-	-
2.	LV2	*Klebsiella*	++	++	++	++	+	+	-	-
3.	LV3	*Bacillus subtilis I*	++	++	++	++	+	+	-	-
4.	LV4	*Serratia sp*	++	++	++	++	+	+	-	-
5.	LV5	*Bacillus subtilis II*	++	++	++	+	+	-	-	-
6.	LV6	*Bacillus sp*	++	++	++	++	+	+	-	-
7.	LV7	*Pseudomonas putida*	++	++	++	++	+	+	-	-
8.	LV8	*Pseudomonas sp*	++	++	++	++	+	+	-	-
9.	LV9	*Pseudomonas fluorescens*	++	++	++	++	+	+	-	-
10.	LV 10	*Bacillus subtilis III*	++	++	++	++	+	+	-	-

+ indicates minimum growth, ++ indicates good growth

In pot trial experiment, Fenugreek plants treated with the broth culture LV1 *(Enterobacter sp)*, LV3 (*Bacillus subtilis I)*, LV8 (*Pseudomonas sp)* LV9 (*Pseudomonas fluorescens)* and LV10 (*Bacillus subtilis III)* showed quick and effective growth than the control plants. No effective results was found in the growth pattern of Fenugreek plants treated with the broth culture LV2 *(Klebsiella sp)*, LV4 (*Serratia sp)*, LV5 (*Bacillus subtilis II)*, LV6 (*Bacillus sp)* and LV7 (*Pseudomonas putida)*.The root length was observed to be maximum with LV10 (*Bacillus subtilis III)* (4 cm) and minimum root length was found with strains LV2 *Klebsiella*, LV4 *Serratia sp*, LV6 *Bacillus sp* and LV7 *Pseudomonas putida* (3 cm) which was similar to that of the control plant. The shoot length was observed to be maximum with LV10 (*Bacillus subtilis III* (Table 5.) and LV8 *Pseudomonas sp*. (5cm). Minimum shoot length was found with strains LV6 *Bacillus sp* (3.8 cm) which was similar to that of the control plant.

TABLE 5. Root and shoot length of the fenugreek plant

S. No.	Strain No.	Name of the strain	Root Length (cm)	Shoot Length (cm)
1.	LV1	*Enterobacter sp*	3.5	4.8
2.	LV2	*Klebsiella*	3	4.1
3.	LV3	*Bacillus subtilis I*	3.4	4.6
4.	LV4	*Serratia sp*	3	4
5.	LV5	*Bacillus subtilis II*	3.9	4.8
6.	LV6	*Bacillus sp*	3	3.8
7.	LV7	*Pseudomonas putida*	3	3.9
8.	LV8	*Pseudomonas sp*	3.5	5
9.	LV9	*Pseudomonas fluorescens*	3.5	4.4
10.	LV10	*Bacillus subtilis III*	4	5
11.	Control	-	3	4

In this study, it was determined that all the ten strains were effective in Siderophore, Ammonia and Indole acetic acid production with better resistance towards Chromium and Cobalt. Among the ten isolated strains, LV10 (*Bacillus subtilis III)* had the best siderophore and Indole acetic acid production with better resistance towards Chromium and Cobalt. It was also found that Fenugreek plant with the broth cultureLV10 (*Bacillus subtilis III)* showed maximum root and shoot length (Table 5). These bacteria exhibited PGPR traits and are responsible for the plant growth in lignite mine spoil and can also be used for soil fertility restoration purpose.

CONCLUSION

From this study it was concluded that, plant growth promoting (PGPR) bacteria are present in lignite mine spoil. They play an indispensable role in the growth of plants in such mine spoils, irrespective of the presence of heavy metals, less nutrition and other unfavourable conditions.

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