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Screening of Zinc Resistant Bacteria Isolated from Coal Mine Overburden Soil

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Abstract

Heavy metal pollution of coal mine spoils is a significant environmental problem affecting both flora and fauna. These degraded soils can be remediated by using heavy metal resistant microorganisms. Zinc has been usually reported in high concentration from coal mine spoils. The present study, therefore, deals with screening of Zinc, resistant bacteria isolated from coal mine spoils. The bacterial isolates were isolated from coal mine spoils of Tinsukia district of Assam. For the isolation of bacteria, King's B Medium and Pikovskaya's Agar Media were used. Heavy metal resistance of the bacterial isolates against zinc was tested on nutrient agar and sucrose-minimal salt low phosphate (SLP) medium. The results showed that out of the 20 test isolates 11 isolates were resistant. The minimum inhibitory concentration (MIC) ranged from 0.001 M to 0.02 M. Antibiotic resistance was studied for two most resistant isolates. The optimal growth conditions with reference to pH and temperature of the two most resistant bacterial isolates were evaluated. Growth pattern of the most resistant isolates was determined in different concentrations of zinc amended broth media. On the basis of cultural and biochemical characters, the resistant isolates were identified as *Pseudomonas sp*, *Bacillus sp* and *Rhizobium sp*. Some of these isolates also exhibited plant growth promoting (PGP) traits and phosphate solubilizing ability.

Key words: Heavy metal; Resistance; Zinc; Minimum inhibitory concentration; PGP traits.

Open cast coal mining is one of the major factors leading to acidification and heavy metal contamination, which has poised a great threat to both biotic and abiotic factors (Kabata-Pendias, 1992; Jarup, 2003). Heavy metal contamination of soil is receiving increasing attention, particularly in developing countries (Yanez et al., 2002). The remediation of such soils is important because these usually cover large areas that are rendered unsuitable for agricultural and other human use. Moreover, elevated concentration of heavy metals can form complex toxic compounds and cause oxidative stresses, alter protein structure which severely influence biological functions (Rajbanshi, 2008). Many microorganisms can survive in harsh acidic and heavy metal contaminated soil as they can mitigate the toxic effects of heavy metals through secretion of acids, proteins, phytoantibiotics & other chemicals (Han et al., 2005). These indigenous heavy metal resistant microorganisms can play an important role in reclamation process (Ledin et al., 1996) as they are potent in biosorption, bioprecepitation, extracellular sequestration, transport mechanisms and chelation (Mergeay, 1991; Hughes and Poole, 1991). This study, therefore, aimed to isolate bacteria from coal mine overburden soil and to screen them with respect to zinc resistance and some growth promoting traits.

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Materials and Methods

Study area and sampling

The study area for the present work was Tirap colliery of Makum coal fields (latitudes 27°13'-27°23'N and longitudes 95°35'-96°00'E) of Tinsukia, district of Assam, India. The overburden soil was 15-20 yrs old, disorganized, poor in supportive and nutritive capacity. The rhizospheric soil samples of 12 different plants were collected during winter season 2010. Soil samples were aseptically collected using an auger from a depth of 10-15 cm according to V-shaped method (Bashan and Wolowelsky, 1987). Samples were taken to the laboratory in sterile plastic bags. The samples were then mixed in equal proportion to make a composite sample. The composite sample was sieved through a 4mm mesh sieve and was kept at field moisture content at 4°C for until further analysis.

Soil physico- chemical analysis

Soil pH was determined using a digital pH meter (Cyberscan 510). Temperature of the overburden spoils were recorded by inserting a soil thermometer at a depth of 15 cm for 10 minutes. Moisture content (%) was calculated after drying 10 g of soil at 105°C for 24 hrs. Soil N was estimated by the alkaline permanganate method (Black et al., 1965). Available soil P was determined by the procedure of Jaiswal (2006) and available K was determined using flame photometer technique (Toth, S. J and A. L. Prince, 1949). The available Ca and Mg were determined by EDTA titration method (Tucker, B.B & L.T. Kurtz, 1961). Soil organic carbon was estimated by dichromate oxidation method (Walkley and Black, 1934).

Estimation of Zinc

Concentration of Zinc in the spoil was determined with the help of Atomic Absorption Spectrophotometer (Perkin Elmer AA200) by the triple acid mixture method (Jackson, 1967). Analytical conditions of atomic absorption spectrophotometer (AAS) used were as: wavelength - 213.86 nm, silt width - 2.7/1.80 mm, operating working range - 0.01 – 2 ppm, HC lamp current -5 mA, type of flame - Air– C_2H_2, fuel gas flow rate -1 L/min, air flow rate - 3.5 L/min.

Isolation of bacteria

Kings B media (HIMEDIA, Mumbai) and Pikovskaya's agar (HIMEDIA, Mumbai) media were used for the isolation of bacteria from the samples. Bacterial isolates were isolated by spread plate and streak plate method. Plates are incubated at 28°C for three days. All the pure culture isolates were maintained in the laboratory by subculturing on nutrient agar slant at an interval of 15 days.

Screening and Determination of minimum inhibitory concentration

(MIC) of zinc resistant bacteria

The isolated strains were screened for zinc ($ZnCl_2$) resistance by agar dilution method (Cervantes et al., 1986) using Nutrient agar and Sucrose-minimal salts low phosphate agar (SLP) media. Zinc chloride amended agar plates of various concentrations were inoculated with 0.5 ml of 1.0 O.D. actively growing cell suspension. Heavy metal tolerance was determined by the appearance of bacterial growth after incubating the plates at 30°C for 24-48 h. The lowest concentration that prevented bacterial growth was considered as the minimum inhibitory concentration (MIC) (Summer and Silver, 1972; Aleem et al., 2003). Characterization of the resistant isolates was done using Bergey's Manual of Systematic Bacteriology (1994) and Microbiology A Laboratory Manual (Cappuccino & Sherman, 2006).

Antibiotic sensitivity of heavy metal resistant isolates

Antibiotic sensitivity and resistance of the isolated heavy metal resistant isolates were assayed on Mueller Hinton agar (HIMEDIA, Mumbai) plates according to the Kirby-Bauer disc diffusion method (Bauer et al., 1996). Plates were seeded with 0.5 ml of 1.0 O.D. actively growing cell suspension and allowed to dry. Antibiotic discs in triplicates were placed over the plates. After incubation at 30°C for 24-48 h, isolates were classified as sensitive or resistant to an antibiotic according to the diameter of inhibition zone as described in standard antibiotic disc chart.

Growth studies

Growth pattern of the two most resistant isolates was studied in 0.1 M zinc chloride amended nutrient broth and un-amended control. Flasks were inoculated with 0.5ml of standard active inoculum and incubated in a rotary shaker (150 rev/ min) at 30°C. Growth was monitored as a function of biomass by measuring the absorbance at 600 nm using spectrophotometer (Systronics, Double beam, 2202).

Determination of optimal growth conditions

The optimal growth conditions of the two most resistant strains with reference to pH and temperature were determined. The isolates were grown in nutrient broth with range of pH values i.e. 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 and different incubation temperatures i.e. 25°C, 30°C and 37°C. The bacterial growth was determined by measuring the optical density of the log phase growing cultures at 600 nm.

Characterization of resistant rhizobacteria for PGP traits

Resistant bacterial isolates were also studied for their plant growth promoting traits. Production of indole acetic acid was (IAA) was detected as by the method of Brick et al., (1991). Ammonia and catalase were detected by the method as described by Cappuccino and Sherman (1992); phosphate solubilizing activity of the strains was determined by plate assay method (Pikovskaya, 1948).

Results

Physico-chemical characteristics of overburden spoils

Physico-chemical properties of coal mine spoils are presented in (Table-1). The soil was highly acidic and pH was found to be as low as 4.37. Soil temperature was 19.27°C having very low moisture in to it (4.56%). The spoil had high percentage of organic carbon (1.87%) but was poor in available P (43.68 Kg/ha. Total Nitrogen and available K were respectively recorded as 634.50 Kg/ha and 36.77 Kg/ha. Available Ca was 240.48 ppm while the available Mg 16.16 ppm. The concentration of zinc in the spoil was estimated to be 210.5 ppm.

Table 1 Physico-chemical characteristics of the rhizospheric overburden soil.

Physico-chemical Characteristics									
pH	Tem.	M (%)	C org. (%)	N total (Kg/ha)	Avail. P (Kg/ha)	Avail. K (Kg/ha)	Avail. Ca (ppm)	Avail. Mg (ppm)	Zn (ppm)
4.37	19.27	4.56	1.87	634.5	43.68	36.77	240.48	16.16	210.5

Table 2 Zinc sensitivity profile of the bacterial isolates.

Sl. No.	Isolates	0.5mM ZnCl2	Remarks
1	TP1	+	Resistant
2	TP2	+	Resistant
3	TP3	+	Resistant
4	TP4	+	Resistant
5	TP5	+	Resistant
6	TP6	+	Resistant
7	TP7	+	Resistant
8	TP8	-	Sensitive
9	TP9	+	Resistant
10	TP10	-	Sensitive
11	TP11	-	Sensitive
12	TP12	+	Resistant
13	TP13	+	Resistant
14	TP14	+	Resistant
15	TP15	-	Sensitive
16	TP16	-	Sensitive
17	TP17	-	Sensitive
18	TP18	-	Sensitive
19	TP19	-	Sensitive
20	TP20	-	Sensitive

Note: +, growth; -, no growth

Isolation, screening and characterization of resistant bacteria

Twenty bacterial isolates were isolated and screened for zinc resistance initially at 0.5 mM concentration. Out of the test isolates, eleven were found resistant, capable to grow on 0.5 mM zinc amended agar plates (Table-3). On the basis of cultural and biochemical traits, zinc resistant isolates belong to the genus *Bacillus*, *Pseudomonas* and *Rhizobium* (Table-3). Species level identification and molecular characterization are in progress. The minimum inhibitory concentration (MIC) of the resistant isolates was determined (Fig.-1). Two resistant isolates, *Pseudomonas* and *Bacillus* had highest MIC (20 mM $ZnCl_2$ conc.)

Antibiotic resistance, optimal growth and growth pattern

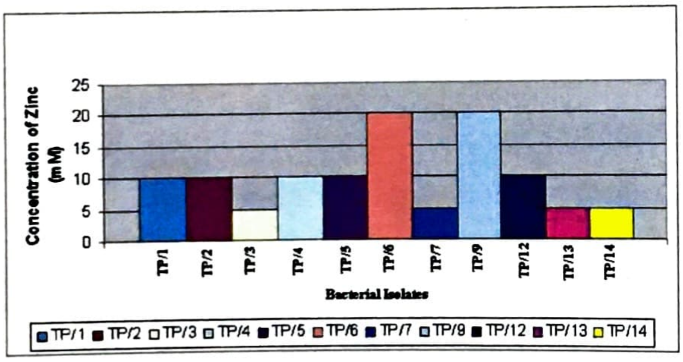
Antibiotic resistance, optimal growth and growth pattern of the two most zinc resistant isolates were evaluated. Strain TP6, characterized as *Pseudomonas* sp., and was found resistant to

Table 3 Morphological and biochemical characteristics of resistant bacterial strains.

Morphological and biochemical characteristics	*Bacillus* (5)*	*Pseudomonas* (5)*	*Rhizobium* (1)*
Gram reaction	G +ve	G -ve	G -ve
Shape	rods	rods	rods
Pigments	-	+	+
Dextrose	+	+	-
Sucrose	+	+	-
Mannitol	+	-	+
Oxidase	-	+	+
H2S production	-	+	+
Indole	-	-	+
Methyl red	-	-	+
Vogues Proskauer	+	-	+
Citrate utilization	+	+	-
Nitrate reduction	+	+	+
Starch hydrolysis	+	+	+
Gelatin hydrolysis	+	-	-

*Number within parenthesis shows no. of strains.

Fig.1- Effect of different concentrations of Zinc on the growth of Zinc resistant bacterial isolates.



tetracycline and ampicillin while, strain TP9 was resistant to ampicillin and streptomycin (Table-4). Optimal pH for growth of TP6 and TP9 strains was 6.5 and 7.0 respectively. The strains TP6 and TP9 required 28°C and 31°C respectively for optimum growth. Growth pattern of the strains TP6 and TP9 were studied both in control and 0.1 mM $ZnCl_2$ amended broth media (Fig. 3 and 4). The strains TP6 and TP9 respectively required 18 to 22 hrs and 22 to 24 hrs to reach the stationary phase. However, the growth of the two strains was slightly less in metal amended media than in control.

Plant growth promoting traits

All the resistant strains except strain TP3 (*Bacillus* sp) showed PGP traits (Table-5). Among the resistant isolates phosphate solubilizing activity was exhibited by strain TP5 (*Pseudomonas sp*), TP13 & 14 (*Bacillus sp*) and TP12 (*Rhizobium sp*).

Table 4 Antibiotic sensitivity profile of the two highest zinc resistant isolates.

Sl. No.	Isolate Code	Organism	Sensitive to	Resistant to
1	TP6	*Pseudomonas* sp.	Ciprofloxacin, Chloramphenicol, Streptomycin	Tetracycline, Ampicillin.
2	TP9	*Bacillus* sp.	Chloramphenicol, Ciprofloxacin, Tetracycline	Ampicillin, Streptomycin.

Fig. 3 Growth pattern of 20mM zinc resistant *Pseudomonas* sp.

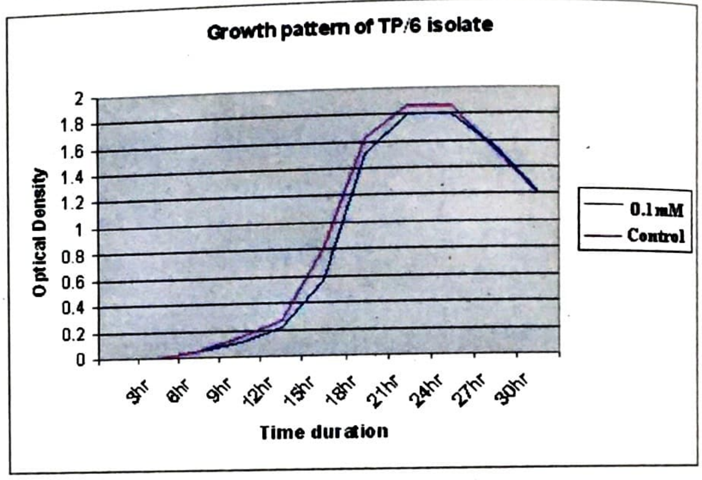


Fig. 4 Growth pattern of 20mM zinc resistant *Bacillus* sp.

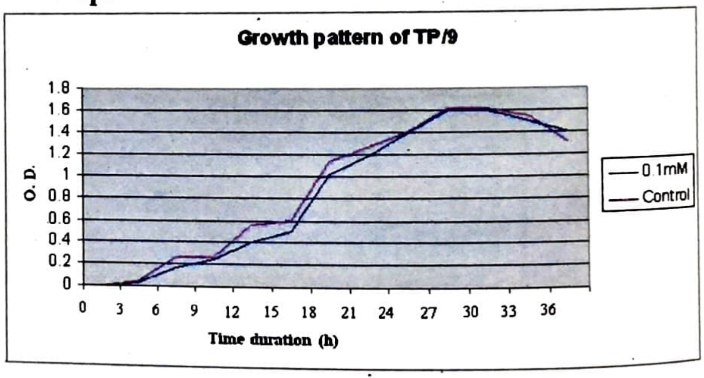


Table 5 Plant growth promoting traits (%) of the zinc resistant isolates.

Bacteria	No. of Isolates	*PGP characteristics (%)*		
		IAA production	Ammonia production	Catalase production
Pseudomonas spp.	5	100	100	100
Bacillus spp.	5	100	80	100
Rhizobium spp.	1	100	100	100

Discussion

This study reveals that the coal mine overburden soil was acidic. All the soil physico-chemical parameters studied were more than optimum except the available phosphorus level that was low. This may be one of the critical factors limiting plant growth. Zinc concentration of the spoil soil was high. In the present investigation >50% of the isolates showed resistance to zinc. The isolates might have acquired varied mechanisms of zinc resistance ranging from reduced uptake to uptake and efflux, external and internal sequestration and in some cases transformation of metals to less toxic form due to zinc resistance genes and metalloregulatory proteins over a period of time. Presence of isolates having zinc resistance as well as phosphate solubilizing abilities might play an important role to increase the phosphate level of the phosphate deficient spoil soil. Acidity, zinc toxicity, nutrient availability and distorted soil texture may be the critical barriers on the way of revegetation of mine spoils. The indigenous resistant bacteria having plant growth promoting traits might have applications in the reclamation of degraded mine spoils. This needs to be explored properly so that appropriate ecofriendly techniques can be developed for the revegetation of degraded mine spoils.

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