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HEAVY METAL RESISTANCE OF MICRORGANISMS ISOLATED FROM COAL MINING ENVIRONMENTS OF NEYVELI

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ABSTRACT
"Heavy metal" is a general collective term, which applies to the group of metals and metalloids with atomic density greater than 5 times more than water. Heavy metals are widespread pollutants of great concern as they are non-degradable. Heavy metals are highly persistent pollutants in the environment. These metals are used in various industries from which effluents are consequently discharged into the environment. Introduction of metals into the environment can produce many of microbial communities and will affect their activities. Contamination of surface sediments and natural aquatic recaptors with heavy metals is a major environmental problem all over the world. Excessive metal concentration cause threat of carcinogenesis, neuralgia, encephalopathy, respiratory cancer, and mutagenesis, cardiovascular and gastrointestinal diseases. Some of the heavy metals are essential trace elements most can be at high concentrations, toxic to all forms of life, including microbes, humans and animals. Heavy metals cannot be degraded or destroyed because they are stable and persistent as environmental contaminants. By affecting the growth, morphology and biochemical activities, heavy metals influence the Microbial population and results in decreased biomass as well as diversity. Therefore microbes have developed mechanisms to tolerate the metals either by presence of heavy metals through efflux or reduction of metal ions or to use them as terminal electron acceptors in anaerobic respiration. Most mechanism reported involves the efflux of metal ions outside the cell, and genes for tolerance mechanisms have been found on both chromosomes and plasmids. Bacteria that are resistant to and grow on metals play an important role in the biogeochemical cycling of those metal ions. The present study investigates the resistant of Zinc to microorganisms isolated from coal mining environments of Neyveli.

KEYWORDS: Heavy Metals, Contamination, Microbial Population, Zinc and Bacteria.

INTRODUCTION
Metals in general are a class of chemical elements that form lustrous solids that are good conductors of heat and electricity. The heavy metals have been defined in different ways according to the concept, "***heavy metals can be defined as elements with metallic properties and atomic weight more than 20***" (**Ghane, M *et. al.*, 2013**). There are 90 naturally occurring elements, which are heavy metals, but not all of them are biologically significant (**Mohammed Umar Mustapha and Normala Halimoon, 2015).** Based on their solubility under physiological conditions, 17 heavy metals may be available for living cells and are of importance for organisms and ecosystems. Among these metals, Fe, Mn are important as micronutrients (**Neha *et. al.*, 2015).** Zn, Ni, Cu, Co, Cr, W are toxic elements with high or low importance as trace elements. The metalloids exert different toxic effects than the metals because they have different chemistries (**Hossein Zolgjarnein *et. al.*, 2007).** Metals are predominantly present as cationic species and metalloids are predominantly present as anionic species. Elements such as zinc and copper are essential for the normal plant growth and development since they are constituents of many enzymes and other proteins. However the concentrations of both essential and nonessential metals can result in growth inhibition and toxicity symptoms (**Karamat Mahmood *et. al.*, 2012).**

Based on the metal bioavailability, metals in the environment can be classified into two categories
1) Bioavailable (soluble, nonsorbed, and mobile) and
2) Non-bioavailable (precipitated, complexed, sorbed and Non - motile)
3) The bioavailable metal concentration is taken up and thus it is toxic to the biological system.

Most of the research on metal bioavailability has been done in soil because of understanding the rate of metal in soil and sediment and also used to determine the effect of metals on the biota, metal leaching to ground water and metal transfer up the food chain (**B. Volesky*** and **Z. R. Holant, 1995**). The environmental hazards posed by metals are directly linked to their concentrations in the soil solution. High concentrations in the soil solution results in uptake of many plants, where metals that are retained in the soil phase show a great reduced environmental hazard (**Haynes and William M., 2011).**

Metals usually accumulate at higher concentration in the soil than in water because it is diluted in soil than in water easily. Soils are composed of minerals which can naturally contain high concentrations of metals (**Joshi B.H. and Modi K.G, 2013)**. Capacity of soils will allow metals to attach to soil particles in response to ionic attractions and accumulate. Several biotic and abiotic factors can influence the specification and toxicity of metals in the soil. These factors interact to influence metal speciation, bioavailability and overall toxicity. Thus, it must be emphasized that determination of the total concentration of a metal in a soil is not enough to predict toxicity in biological systems **(Amalesh Samantha *et. al.*, 2012).**

Zinc is an abundant metal found in earth's crust with myriad industrial and biological uses. At room temperature zinc is brittle and blue-white in colour, but can be polished to a bright finish (**Shazia Iram *et. al.*, 2012).** It belongs to the group 12 and d-block. Atomic weight of zinc is 65.38.Zinc have been dated as far back as 500BC, and zinc was first intentionally added to copper to form brass around 200-300BC. Brass supplemented bronze during the Roman Empire in the manufacture of coins, weapons, and art. Alessandro Volta created the first battery in 1800 using zinc plates, StanislawSorel has named his new process of zinc plating. Galvanization, after luigiGalvani, who has discovered the animating effect of electricity while autopsying frog **(Bharagava *et. al.*, 2014).**

MATERIALS AND METHODS

Soil samples were collected from coal mining industry in Neyveli.The samples were collected in sterile plastic containers and stored.Soil samples were taken from the soil surfaces (0-5 cm) and from a depth (20 cm). Soil samples were transported in sterile bags to the microbiology laboratory and stored at 4°C for further study.

Sterilization of glassware and other materials

All glassware used were thoroughly washed with deionized water and detergent, rinsed and allowed to dry. The glassware were then enfolded with aluminium foil and sterilized. The distilled water used for serial dilutions, was autoclaved at 121 °C for 15 minutes. The workbench was cleansed with 75% alcohol prior and after every experiment and all experiments were conducted in three replicate and the results were statistically analysed using mean, standard deviation and student t test to examine the significance differences.

Isolation of microorganisms from sample

10 gm of the soil sample was added to 90 ml of sterilized water and was mixed with a magnetic blender for 30 minutes to separate the microorganisms from the soil completely. After being deposited for 20 minutes, 1ml of suspension was added to the broth and was incubated at 37°C for 24 hours.

Serial dilution

The incubated tubes were taken for serial dilution. 9 ml of saline was added to 10 sterilized test tubes. 1 ml from the incubated test tubes was added to the first test tube that gives 1:10 dilution. The tube was mixed well and 1 ml from the first test tube was transferred to the second tube. This was continued till the eighth tube. And 1 ml from the eighth tube was discarded. Dilutions such as 10^4, 10^5, 10^6 and 10^7 were chosen for plating.

Spread plate technique

Once the plates solidified, 0.1 ml from 10^4 dilution was taken and added to the petri plate, L-rod was flame sterilized using ethanol and it was used to spread the sample on agar surface. The same procedure was repeated for 10^5, 10^6 and 10^7 dilutions. 1 plate was used as control and the plates were incubated at 37°C for 24 hrs. After the incubation period (24 - 48hrs) the plates were observed for growth on the media.

Study of Colonial Morphology

The isolated colonies of the purified strain grown on solidified agar plates were observed and the data was recorded regarding the form (circular, filamentous, irregular); elevation (flat, convex); margin (entire, undulate, filamentous) and optical feature (opaque, transparent, translucent) of the colonies.

Biochemical Characterization

Biochemical screening was done by performing tests such as Indole, Methyl Red Test, Citrate Utilization Test, Urease Test, Oxidase Test and Catalase Test.

Physiological Characterization

Physiological characteristics were screened by supplying wide range of growth temperature such as 10°C - 50°C and 4-12 pH and was tested. To examine the ability of isolate to resist heavy metals, Spot inoculation was done on nutrient agar plate provided with different concentrations (0.5, 1.0, 3.0 and 5.0 mM) of heavy metal of Zinc in Zinc sulphate and was incubated at 37°C for 24 hours and the cell growth were observed. Antibiotic Susceptibility Test was carried out using Disc Diffusion Method and Plasmid Isolation by Electrophoresis.

RESULT AND DISCUSSION

Isolation of Microorganisms

Pale Blue colonies and white Bacterial colonies were observed for Zinc Heavy Metal.

Identification of Microorganisms

Lactophenol Cotton Blue Staining

Lacto phenol cotton blue stain was used to identified the Fungal strain *Penicillium spp.* It was observed under 40X magnification (**Figure 1**).

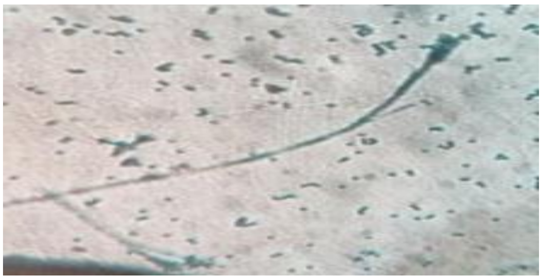
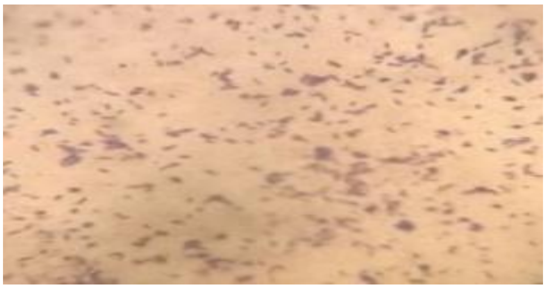


Figure. 1: Lactophenol Cotton Blue Staining.

GRAM STAINING



Gram positive rods.

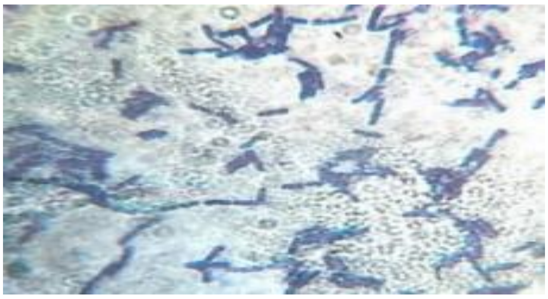


Figure. 2: Gram positive rods.

Gram staining result showed purple colour long rods. The bacterium was identified to be gram positive (Figures 1 – 2 & Table 1).

BIOCHEMICAL TESTS

Table. 1: Biochemical Tests for Zinc.

S. No.	Tests	*Bacillus spp., A*	*Bacillus spp., B*
1	Methyl red test	+	+
2	Indole production	+	+
3	Citrate Utilization	+	+
4	Urea hydrolysis	+	+
5	Oxidase	+	+
6	Catalase	+	+

pH PROFILE FOR ZINC

Growth response of isolates at different pH was estimated by complexometric titration. The results indicate that optimum growth of *Aspergillus flavus* was found to be at pH 1.5 and growth starts within a pH range of 1.0-8.0 (**Table 2 & Figure 3**).

Table. 2: pH Profile for Zinc.

ORGANISMS	pH- 1	pH-2	pH-4	pH-6	pH-8
Bacillus spp.A	0.9 mg	3.1 mg	3.5 mg	4.5 mg	4.9 mg
Bacillus spp.B	2.4 mg	3.6 mg	3.8 mg	4.0 mg	4.4 mg
Penicillium spp.	2.1 mg	2.3 mg	2.5 mg	2.9 mg	3.0 mg

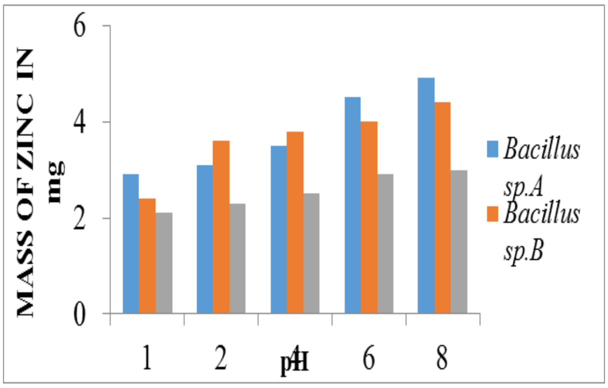


Figure. 3: pH profile of *Bacillus spp.A, Bacillus spp. B*, *Penicillium spp.*, X-axis depicts temperature range and Y-axis depicts Fungal growth.

TEMPERATURE PROFILE FOR ZINC

Growth response of isolates at different Temperature was estimated by complexometric titration. The results indicates optimum growth to be at pH 1.5 and growth starts within a pH range of 1.0-8.0 (**Figure 4**).

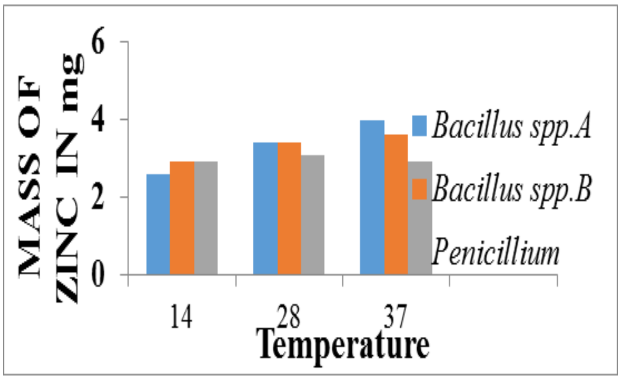


Figure. 4: Zinc Temperature profile of *Bacillus spp.A, Bacillus spp.B and Penicillium spp.*, X-axis depicts temperature range and Y-axis depicts Bacterial growth.

ANTIBIOTIC SUSCEPTIBILITY TEST

In order to determine the resistance to antibiotics, isolated strains were tested against antibiotics by disc diffusion method. After incubating for 36-48 hours, the plates with antibiotics disc were observed for zone of inhibition and resistance pattern of each isolate were recorded (**Table 3 and Figure 5**).

Sensitivity test for bacterial isolates

Table. 3: Antibiotic Resistance Pattern.

Antibiotic Discs	*Bacillus spp. A*	*Bacillus spp. B*
Tetracycline	Sensitive	Resistant
Penicillin	Resistant	Resistant
Ciproflaxin	Resistant	Resistant

Figure. 5: Zone of inhibition for Bacteria.

Strains of *Bacillus SP. A* and B showed highest resistance to Tetracycline(10µg/disc), Penicillin (25µg/disc) and Ciproflaxin (30µg/disc) and *Bacillus B* showed highest resistance against antibiotics. Strains of *Bacillus spp. A* showed sensitive to Tetracycline (10µg/disc). This result indicates that the strains exhibit a wide spectrum of antibiotics, i.e.; multiple drug resistance patterns.

Sensitivity test for fungal isolates

Table. 4: Antibiotic resistance pattern of Fungal metal resistant isolates.

ANTIBIOTIC DISCS	*Penicillium spp.,*
Penicillin	**Resistant**

Penicillium spp. showed highest resistance to Penicillin (25µg/disc). This result indicates that the strains exhibit wide spectrum of antibiotics, i.e.; multiple drug resistance patterns (**Table 4**).

PLASMID ISOLATION

The plasmid DNA was successfully isolated from *Bacillus spp. A* and *Bacillus spp.B* (**Figure 6**).

Figure. 6: Plasmid profile of isolated strains subjected to Electrophoresis on Agarose Gel.

A) DNA ladder
B) *Bacillus sp.*B
C) *Bacillus spp.*A

To determine the plasmid contents in bacteria (*Bacillus spp.*A and *Bacillus spp.*B) from the soil samples, plasmid DNA were isolated from the bacterial strains that showed the highest maximum resistant values for each heavy metal. According to the electrophoretic separation, 28KB plasmids were detected in *Bacillus spp.* strains with resistance to Zinc.

SUMMARY AND CONCLUSION

Microbial bioremediation is a process of removal of toxic substances and also heavy metals from the environment with the help of microbes. Elements with atomic weight more than twenty and having these properties like conductivity, specificity as cations are referred to as heavy metals. Out of 90 naturally occurring heavy metals, 17 could be found in biological system. However the heavy metals selected here is Zinc. The soil sample was collected from coal mining environments of Neyveli. Isolation was done by incorporating heavy metals. The microorganisms isolated from the sample were *Penicillium spp.*, *Bacillus spp. A* and *Bacillus spp. B.* The biochemical characterization was done for the bacterial species. The physiological characterization like pH and Temperature was done by adjusting the pH (1,2,4,6,8) and Temperature (14˚C, 28˚C, 37˚C) range for the metal Zinc. Antibiotic Susceptibility test were assessed. Plasmid DNA was isolated for the bacterial species which gave high tolerant resistance.

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