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Comparison of Heavy Metal Resistance of Microrganisms Isolated from Coal Mining Environments of Neyveli

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Abstract:

Heavy metals are classified into three classes : metals, heavy metals and metalloids. Metals in general are a class of chemical elements that form lustrous solids that are good conductors of heat and electricity. There are 90 naturally occurring elements, which are heavy metals, but not all of them are biologically significant. 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. 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. 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 non - essential metals can result in growth inhibition and toxicity symptoms. The present study investigates and compares the resistance of Heavy metals isolated from coal mining environments of Neyveli.

Keywords — **Heavy metals, Zinc, Copper, Cobalt, Coal Mining Environment of Neyveli.**

INTRODUCTION

Common sources of heavy metal pollution include discharge from industries. These pollutants are released from various industries by some of the processes such as electroplating, metallurgy, tannery, battery manufacturing etc. The effluents contain considerable amount of metals, i.e. Zinc (Zn), Copper (Cu), Cobalt (Co), Cadmium (Cd), Chromium (Cr). This adversely affects soil health, fresh water resources and ground water quality. Contamination of surface sediments and natural aquatic recaptors with heavy metals is a major environmental problem all over the world (Joshi B.H. and Modi K.G, 2013). Excessive metal concentration cause threat of carcinogenesis, neuralgia, encephalopathy, respiratory cancer, 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 persistentas environmental contaminants (Bharagava et. al., 2014). By affecting the growth, morphology and biochemical activities, heavy metals influence theMicrobial 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, complexation, or reduction of metal ions or to use them as terminal electron acceptors in anaerobic respiration (B. Volesky*, and Z. R. Holant, 1995). 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 (Hossein Zolgjarnein et. al., 2007).

There are two main natural sources of heavy metals; the underlying parent material and atmosphere. In recent times, due to anthropogenic activities, like mining, ore refining, combustion of fossil fuels, metal-working industries, battery manufacturing, paints, preservatives, insecticides and phosphate fertilizers, etc., it has led to the emission of heavy metals and the accumulation of these compounds in ecosystems causing serious threat to the environment Amalesh Samantha et. al., 2012).
Mining and milling of metal ores coupled with industries have bequeathed many countries, the legacy of wide distribution of metal contaminants in soil. During mining, tailing are directly discharged into the environment in elevated concentrations. Zinc ore mining and smelting are resulted in contamination of soil that shows risk to humans and ecological health. Soil heavy metal environmental risks to humans is related to bioavailability.

In naturally polluted environments, the microbe's response to heavy metals toxicity depends on the concentration and the availability of metals and on the action of factors such as the type of metal, the nature of medium and microbial species (Ghane et. al., 2013). Fungi and yeast can tolerate heavy metals. They are versatile group, they can grow under various extreme conditions of pH, temperature and nutrient availability, as well as high metal concentrations. The surface soil is a rich habitat of all major groups of microorganisms, i.e., bacteria, fungi, and algae (Mohammed Umar Mustapha and Normala Halimoon, 2015).

These microorganisms convert toxic organic and inorganic compounds to harmless products often carbon dioxide and water. In order to survive in heavy-metal polluted environments, many microorganisms have developed means of resistance to toxic metal ions. These mechanisms include: metal exclusion by permeability barriers, active transport of the metal away from the cell organism, intracellular sequestration of the metal by protein binding, extracellular sequestration, enzymatic detoxification of the metal to a less toxic form and reduction in metal sensitivity of cellular targets (Karamat Mahmood et. al., 2012). The detoxification mechanisms may be directed against one metal or a group of chemically related metals (Shazia Iram1 et. al., 2012). The tolerance of some microorganisms to a variety of heavy metals is well documented. In some cases it produces intracellular / extracellular enzymes to resist the metal concentration or they possess the processes of valence transformation, active uptake, complexation, crystallization and bio sorption to cell walls. Species like Penicillium, Aspergillus, Pseudomonas, Sporophyticus, Bacillus, Phanerochaete, etc. are found to be very useful forthe removal of heavy metals such as chromium and nickel (Neha et. al., 2015).

The present study investigates and compares the resistance of Heavy metals isolated from coal mining environments of Neyveli.

MATERIALS AND METHODS

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, 3.0and 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).

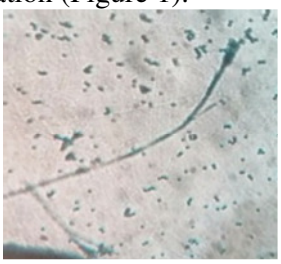
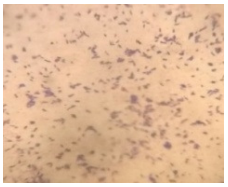


Figure 1 : Lactophenol Cotton Blue Staining

GRAM STAINING



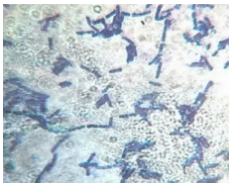


Figure 2 : Gram positive rods Gram positive rods

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

BIOCHEMICAL TESTS

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	+	+

Table 1 : Biochemical Tests for Zinc

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).

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

Table 2 : pH Profile for Zinc

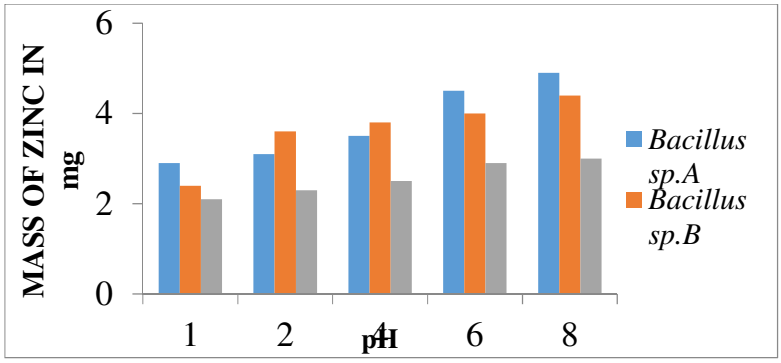


Figure 3 : pH profile of Bacillus spp.A,Bacillus spp.B, Penicillium spp., X-axis depicts temperature range and Y-axis depicts Fungal growth.

pH PROFILE of COPPER

Growth response of isolates at different pH was analyzed colorimetrically at 590nm. The result indicates optimum growth of Aspergillus niger and Yeast at pH 5 and growth starts within a pH range of 3.8 - 8.0 (Figure 4).

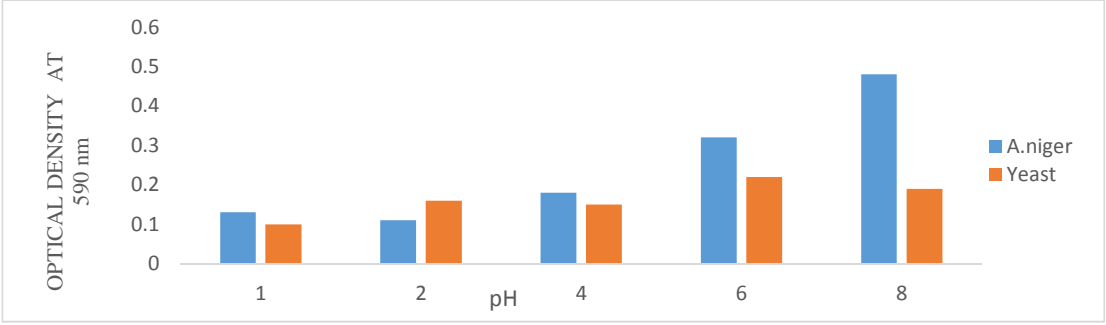


Figure 4 : Copper pH profile of Aspergillus niger& Yeast. X-axis depicts pH range and Y-axis depicts Fungal & Yeast growth in µg/ml

pH PROFILE FOR COBALT

Growth response of isolates at different pH was analyzed spectrophotometrically at 440nm. The result indicates optimum growth of Aspergillus flavus at pH 6 and growth starts within a pH range of 2.0 - 6.0 (Table 3 & Figure 5).

ORGANISM	pH-1	pH-2	pH-4	pH-6	pH-8
A.flavus	0.12µg/ml	0.23µg/ml	0.37µg/ml	0.47µg/ml	0.44µg/ml

Table 3 : pH Profile for Cobalt

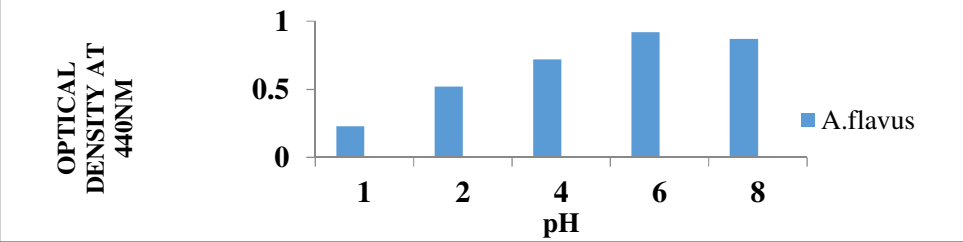


Figure 5 : Cobalt pH profile of *Aspergillus flavus*. X-axis depicts pH range and Y-axis depicts fungal growth

TEMPERATURE PROFILE FOR ZINC

Growth response of isolates at different Temperature was estimated by complexometric titration. The results indicates optimum growth to be at pH1.5 and growth starts within a pH range of 1.0-8.0 (Figure 6).

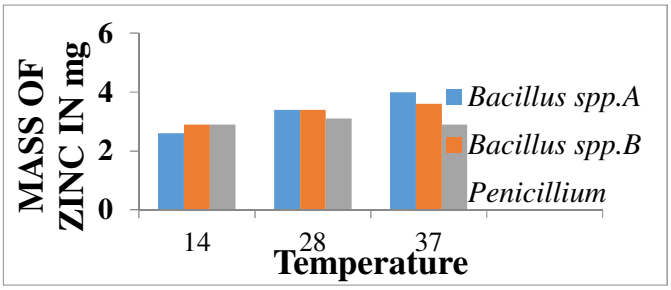


Figure 6 : Zinc Temperature profile of *Bacillus spp.A, Bacillus spp.B and Penicillium spp.*, X-axis depicts temperature range and Y-axis depicts Bacterial growth.

TEMPERATURE PROFILE OF COPPER

Growth response of isolates at different temperature was analyzed colorimetrically at 590nm. The result indicates optimum growth of *Aspergillus niger*and Yeast at 28°C and growth starts between the range of 15ºC - 28ºC (Table 4 & Figure 7).

ORGANISMS	14ºC	28ºC	37ºC
A.niger	**0.17µg/ml**	**0.37µg/ml**	**0.29µg/ml**

Yeast	**0.09µg/ml**	**0.23µg/ml**	**0.14µg/ml**

Table 4 : Temperature profile of *Aspergillus niger* & Yeast

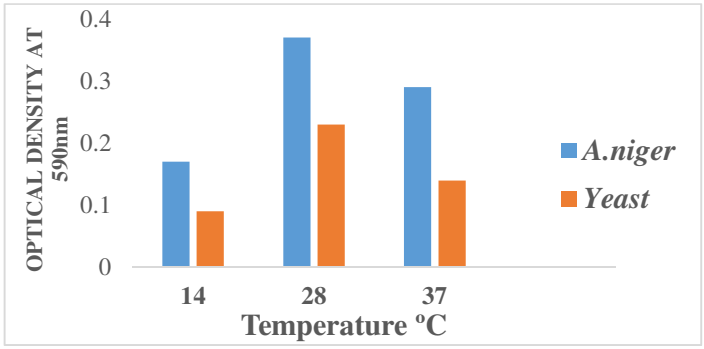


Figure 7 : Copper Temperature profile of *Bacillus spp.A, Bacillus spp.B and Penicillium spp.*, X-axis depicts temperature range and Y-axis depicts Bacterial growth.

TEMPERATURE PROFILE FOR COBALT

Growth response of isolates at different temperature was analyzed Spectrophotometrically at 360-440nm. The result indicates optimum growth of *Aspergillus flavus* at 37°C and growth starts between the range of 15ºC - 37ºC (**Table 5 & Figure 8**).

ORGANISM	14 ºC	28 ºC	37ºC
A.flavus	**0.39**µg/ml	**0.74**µg/ml	**0.87**µg/ml

Table 5 : Temperature Profile for Cobalt

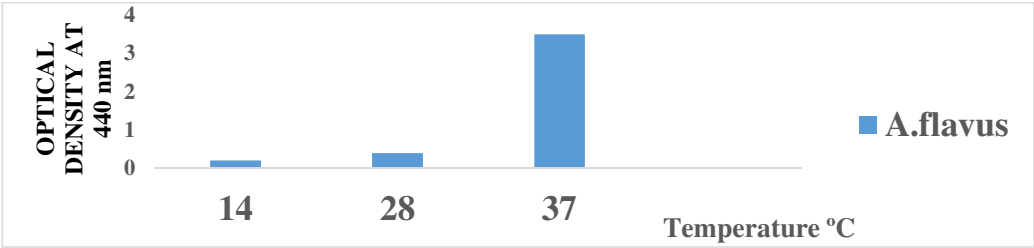


Figure 8 : Copper temperature profile of *Aspergillus flavus*. X-axis depicts temperature range and Y-axis depicts Fungal growth

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 6 and Figure 9**).

SENSITIVITY TEST FOR BACTERIAL ISOLATES

ANTIBIOTIC DISCS	*Bacillus spp. A*	*Bacillus spp. B*
Tetracycline	Sensitive	Resistant
Penicillin	Resistant	Resistant
Ciproflaxin	Resistant	Resistant

Table 6 : Antibiotic Resistance Pattern

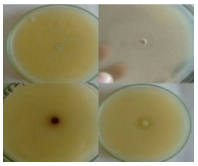


Figure 9 : Zone of inhibition for Bacteria

SENSITIVITY TEST FOR YEAST ISOLATE

ANTIBIOTIC DISCS	YEAST
Amphotericin B	Resistant

Table 7 : Antibiotic Resistant Pattern of Yeast

ANTIBIOTIC RESISTANCE PATTERN OF YEAST METAL RESISTANT

Figure 10 : Zone of inhibition For Yeast

Strain of yeast showed resistance to Amphotericin B antibiotics (**Table 7 & Figure 11**).

SENSITIVITY TEST FOR FUNGAL ISOLATES

ANTIBIOTIC DISCS	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium spp.,*
Amphotericin B	Resistant	Resistant	
Penicillin			Resistant

Table 7 : Antibiotic resistance pattern of Fungi

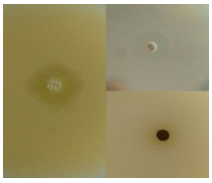


Figure 11 : Zone of inhibition for Fungi

Strains of Aspergillus niger and Aspergillus flavus showed highest resistance to Amphotericin B (10µg/disc) and Penicillium spp. showed highest resistance to Penicillin (25µg/disc). The results indicate that these strains exhibit wide spectrum of antibiotics, i.e.; multiple drug resistance patterns (Table 7 & Figure 11).

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

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

Table 8 : Antibiotic resistance pattern of Fungal metal resistant isolates.

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 8**).

PLASMID ISOLATION

The plasmid DNA was successfully isolated from *Bacillus spp. A* and *Bacillus spp.B* (**Figure 12**).

A B C

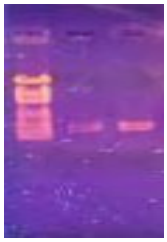


Figure 12: Plasmid profile of isolated strains subjected to Electrophoresis on Agarose Gel.

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.

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 for studies are zinc, copper and cobalt. The soil sample was collected from coal mining environments of Neyveli.
- Isolation was done by incorporating heavy metals. The microorganisms were isolated from the sample such as. *Aspergillus niger, Aspergillus flavus, Penicillium spp.,* Yeast*, 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 all the three metals.
- Antibiotic Susceptibility test were assessed.
- Plasmid DNA was isolated for the bacterial species which gave high tolerant resistance.

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