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ISOLATION AND CHARACTERIZATION OF INDIGENOUS MICROBES FROM HEAVY METAL-IMPACTED SITES

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ABSTRACT

The surge in industrial activities has significantly increased the release of industrial waste, particularly heavy metals, into the environment, primarily impacting soil and water. Heavy metals, prevalent in various industrial processes, contribute to soil pollution via methods like atmospheric deposition and industrial runoff. Contaminated soil often exhibits reduced microbial mass and soil fertility. Heavy metals pose dire health risks, being toxic even in small amounts, with elements like arsenic and cadmium causing cell damage and cancer. Biological methods, including biosorption and bioaccumulation, show promise for heavy metal removal, exploiting microbial capabilities for cleanup. Ambient conditions significantly affect microbial response to metal concentrations. Specific bacteria, such as *Bacillus subtilis*, demonstrate notable resistance and ability to bioaccumulate heavy metals in various settings. The isolates obtained from multiple sampling sites, were assessed for antimicrobial activity resistance through various methods like Agar cup method and Agar ditch method using a range of selected heavy metals like Copper (Cu), Cadmium (Cd), Chromium (Cr) and Lead (Pb). In this study, a genera of various gram positive and negative microorganisms like *Bacillus* spp., *Escherichia* coli, *Micrococcus* spp., *Klebsiella* spp., *Staphylococcus* and *Arthrobacter*., were isolated and identified. These microbes displayed varied levels of resistance to the heavy metals. The investigation has widened the scope for research and development of metal tolerance and antibiotic resistance from bacterial origin. The future prospect lies in the application of these microorganisms for purposes like heavy metal remediation and potential use in extracting rare metals from dilute solution or removing toxic metals from industrial effluents.

KEYWORDS: Heavy metals, bioaccumulate, resistant, microorganisms.

INTRODUCTION

The increase in industrial activity has led to more industrial waste being released into the environment. This primarily affects soil and water, causing pollution and heavy metal buildup (Endo et al. 1997). Heavy metals are common in many industrial processes (Valdam et al. 2001). They gradually deplete through leaching and erosion. Heavy metal pollution in soil happens through several pathways. These include atmospheric deposition, irrigation, animal waste, industrial runoff, and the use of fungicides. Analyses of contaminated soil show a decrease in microbial mass and soil fertility (Kuperman et al. 1997).

The uncontrolled release of heavy metals into soil and water poses serious global health risks. These metals are persistent and toxic, even in low amounts. Metals like arsenic and cadmium can damage cells, cause cancer, and induce genetic mutations. Cadmium, a major pollutant from human activity, enters the food chain (Kalcher et al. 1993). It is highly mutagenic, while nickel can hinder plant growth and development and cause birth defects (Chen and Lin et al., 1998). Several techniques exist for removing heavy metals. These include chemical precipitation and membrane technology, but many methods struggle with concentrations below 100 mg/L. The solubility of heavy metal salts in water makes it hard to separate them using physical methods. Additionally, physico-chemical techniques can be expensive and not be effective at low concentrations.

Biological methods, such as biosorption and bioaccumulation, could be potential alternatives to traditional methods for removing heavy metals. Using microorganisms for cleanup represents a sustainable way to improve soil conditions. The response of microbial communities depends on metal concentration and various environmental factors (Ruchita Dixit et al. 2015).

Due to the growth of industry, there has been a increase in the discharge of industrial waste to the environment, chiefly soil and water, which results in the pollution and also large amount of the accumulation of heavy metals (Endo et al. 1997). Heavy metal pollution of soil happens through many routes including settling from atmosphere, through irrigation, waters, in animal manures, run off from industries, application of heavy metal containing fungicides. Analysis of soils with metal contamination show decrease in microbial mass and decreased in microbial mass and soil fertility (Kuperman et al, 1997).

The indiscriminate release of heavy metals into the soil and waters is a major health concern worldwide, as they cannot be broken down to non-toxic forms and therefore have long-lasting effects on the ecosystem. Many of them are toxic even at very low concentrations; arsenic, cadmium, chromium, copper, lead, mercury, nickel, zinc *etc.* are not only cytotoxic but also carcinogenic and mutagenic in nature. Cadmium is major anthropogenic heavy metal pollutant and enters into human through food chain (Kalcher et al. 1993). Cadmium is highly mutagenic. Nickel is another toxic metal; retards plant growth and development apart from being teratogenic. (Chen and Lin et al., 1998).

Microorganisms' uptake heavy metals actively (bioaccumulation) and/or passively (adsorption). Among various microbe-mediated methods, the biosorption process is more feasible for large scale application compared to the bioaccumulation process (Ruchita dixit et al., 2015). The heavy metal ion resistance is based on three mechanisms, which may include active extrusion of heavy metal toxicant from cell, formation of complex thiol-containing compounds as well as oxidation into less toxic oxidation (Nies et al., 1999).

Metals can be sequestered either intracellularly or extracellularly, where intracellular exclusion is mediated by metallothioneins and cystein rich proteins that bind metal ions. Extracellular sequestration is carried by excretion of some compounds such as glutathione which has a very high affinity for heavy metals and binds and keep metal outside the cell (Silver et al., 1989).

In many foreign countries it is proposed activated sludge system augmented with the acclimatized strains is the best choice to ensure high treatment efficiency and performance under metal stresses especially when industrial effluents are involved (Ebtesam E. et al, 2012). Probiotic bacteria have the capacity to bind many toxic compounds like aflatoxins to bind lead and cadmium from water (T. Halttunen et al. 2007). A thymidine incorporation technique was used to determine the tolerance of a soil bacterial community to Cu, Cd, Zn, Ni, and Pb.(Montserrat Diaz-ravina et al. 1994).

In one of study some selected isolated microbes from Orita-aperin and Awotan dumpsites leachate to bioaccumulate heavy metal were investigated. the combination of the two bacteria (*Bacillus subtilis and Micrococcus luteus*) in the experimental set-up when left un-agitated showed relatively high accumulation ability for copper and nickel with 86.71% and 83.3% respectively (Adebisi Musbaudeen Sulaimon, Adebowale Toba Odeyemi, Adeniyi Adewale Ogunjobi, Ismail Olasunkanmi Ibrahim et al, 2014).

In Spain a bacterial strain able to use cyanide as the sole nitrogen source under alkaline conditions has been isolated. The bacterium was classified as *Pseudomonas pseudoalcaligenes*. bacterium stoichiometrically converted cyanide into ammonium in the presence of L-methionine-D, L-sulfoximine, a glutamine synthetase inhibitor. Cyanase activity was induced during growth with cyanide or cyanate, but not with ammonium or nitrate as the nitrogen source. This result suggests that cyanate could be an intermediate in the cyanide degradation pathway, but alternative routes cannot be excluded. (Victor M. Luque-Almagro, Marı´a-J. Huertas, Manuel Martı´nez-Luque, et al.2005). In another study bioremediation of heavy metal-contaminated effluent using optimized activated sludge bacteria. Results revealed that using the proposed activated sludge with the resistant bacterial mixture was more efficient for heavy metal removal compared to the activated sludge alone.

Certain members of the gut microbiota, such as lactobacilli used in food applications, as they have resistance mechanisms which are effective in preventing damage to the cells by sequestering heavy metals to their cell surfaces, thus removing them through defecation. Active expulsion of toxic metals from the cytosol has been shown in the presence of *mer* and *ars* operons in *Lactobacillus* and other gut-associated species which encode efflux transporters. Gut microbiotas provided the first line of defense to the body by converting toxic Cr (VI) to a less-toxic Cr (III) (Shrivastava et al).

This study was aimed to isolate and characterize various different heavy metal tolerating bacteria from varied environmental conditions. Few microbes like Bacillus subtilis were found to be resistant to all the four heavy metals exposed in 0.5mg/l.

METHODOLOGY

1. Sample Collection

Table 1 - Samples used alongside their source and locations.

Sr. no.	Sample	Source and Location
a.	Water	Ulhas River, Ulhasnagar
b.	Sludge and effluent	CETP, Koparkhairane
c.	Soil	Agricultural Land, Ulhasnagar C. H. M. College Garden, Ulhasnagar

Fig 1: Sampling sites: A- Ulhas River; B - CETP, Koparkhairane; C – CHM College Garden; D – CHM College.

1. Strains isolation and Purification of selected isolates

The samples were selectively enriched for heavy metal like Cadmium (Cd), Copper (Co), Chromium (Cr) and Lead (Pb) respectively. Firstly, 10g of sample was inoculated in 100ml of Nutrient Broth (NB) with individual addition of 20µg/ml chosen heavy metals in their respective flasks. Flasks were incubated at 37°C for 24hrs on Shaker (Higham and Sadler *et. al*.1984). Supernatants were diluted, 10^{-1} and 10^{-2} were spread onto Nutrient Agar plates containing 20µg/ml of above heavy metals and incubated at 37°C for 72hrs. The selected colonies were screened at higher concentrations (20-800µg/ml) of each heavy metal. Finally, three strains of Cadmium, Copper, Lead, Chromium were selected as resistant isolates and purified on Nutrient Agar for further studies.

2. Morphological characterization

The isolates were streaked on Nutrient Agar plates and the size, margin, elevation, colour, opacity and gram's nature of the colonies were studied. Biochemical tests like IMViC, gelatin, nitrate reduction, catalase, oxidase and acid production tests like glucose, maltose, xylose, melibiose and arabinose.

3. Antimicrobial Activity assessment

Antimicrobial activity is the ability of a substance to inhibit growth of microorganisms. In this study, the antimicrobial activity of the heavy metals was studied in the isolates using various methods namely, Agar cup method (Rose and Miller 1939), Agar ditch method (Bowers and Jeffries, 1995), Micro dilution assay (Lalitha, 2005), MIC/MBC determination using Microtiter Plate Technique (Amsterdam, 1996). The heavy metal resistant isolates obtained from various different samples was tested for the resistance in variable concentrations of heavy metals. the Minimum Inhibitory Concentration (MIC) of the heavy metals aided to decipher the use of such resistant microbes for bioremediation in locations with heavy metal pollution within the MIC range.

a. Agar Cup Method (Rose and Miller 1939)

Sterile molten nutrient agar (15 ml) was seeded with 1 ml of suspension (10^8 cells per ml) and poured in a sterile plate. Once solidified, using a sterile Cork Borer of 8 mm diameter, agar cups were bored aseptically and different dilutions of heavy metals under study were added into the wells. The plates were kept in refrigerator for 10 minutes to facilitate diffusion and then incubated for 24hrs at 37°C. After the incubation, zone of inhibition was observed and measured. The inoculum can be poured or spread onto the plates. Wells are made with a cork borer and a drop of the heavy metal solution is added.

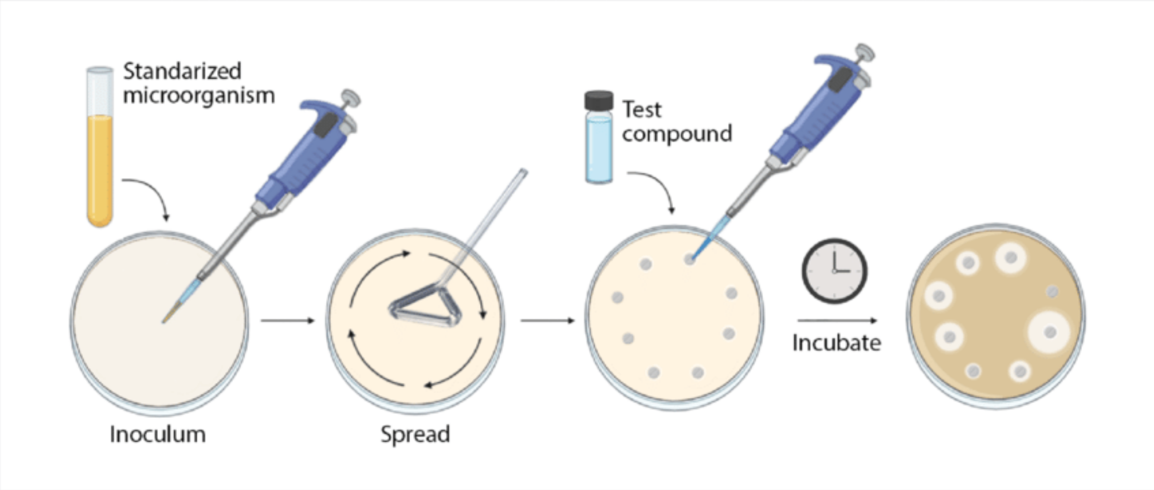

Fig 2: Agar Cup Method.

b. Agar ditch method (Bowers and Jeffries, 1995)

Agar ditch method is generally used to screen the effect of some water insoluble or partially soluble compounds or drugs (Baur et al, 1965). A sterile agar plates was prepared using 20 ml of sterile, molten medium. A ditch of 8.0 cm x 1.5 cm was cut out aseptically in medium. The weighed quantity of the compound was dissolved in 0.5 ml of alcohol or water and was added to 10 ml of sterile melted agar medium to obtain the required concentration. It was mixed thoroughly and aseptically poured into the ditch such that the surfaces of the agar in the plate and that in the ditch were even. The plates were kept in refrigerators for 10 minutes for proper setting of agar. The test strains were streaked perpendicular to the ditch and parallel to each other. The plates were incubated at 37°C for 24 hrs. Next day, growth was observed on ditch and near to it, along the line of streak of cultures. Appropriate solvent controls were maintained and tested to eliminate the errors that may arise due to these solvents.

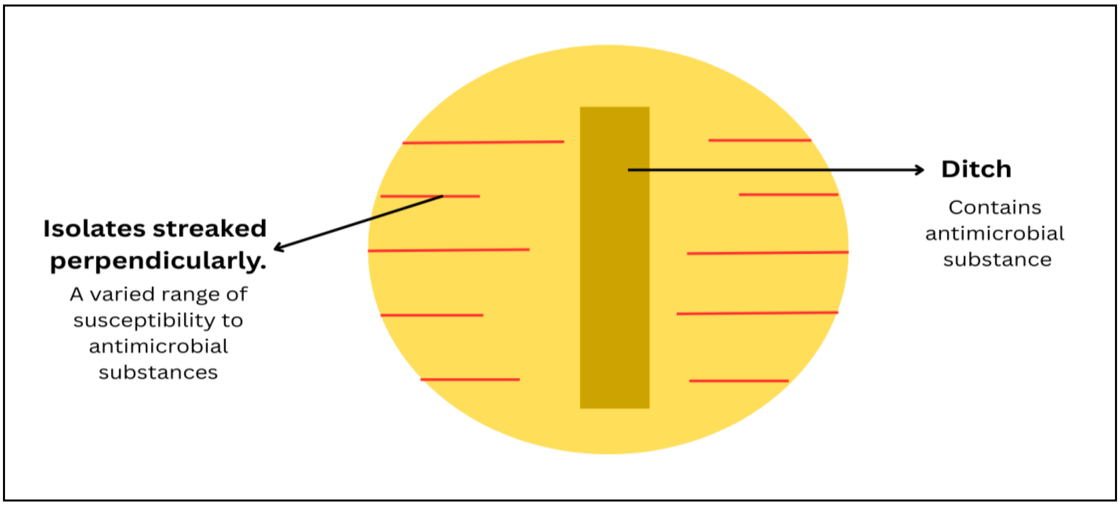

Fig 3: Agar ditch method.

RESULTS AND DISCUSSIONS

1. Isolation of bacteria

A total of 62 colonies were obtained from primary enrichment whereas only 10 isolates through secondary selective enrichment, in the presence of heavy metals with the concentration of 20µg/ml. These isolates were purified on nutrient agar and used for further characterization.

Table 2: Isolates and their respective designate obtained from different sampling sites.

Sample sites	Total no. of colonies	No. of isolates	Designate
Sludge sample collected from CETP plant, Belapur	22	3	S-1, S-2 S-3
Water sample collected from Ulhasnagar River	17	3	S-4, S-5 S-6
Effluent sample collected from CETP plant, Belapur	23	2	S-7 S-8

2. Identification and characterization

The isolates were coded with a uniform coding of 'S'. Isolates were classified into 2 major groups on basis of various parameters for characterization. Parameters like Gram staining, colony morphology (size, shape, opacity, texture, elevation, form and margin surface), pigment production, biochemical properties, which included simple substrate utilization step which confirmed the presence or absence of some specialized tests. The strains were characterized according to the Bergey's Manual of Determinative Bacteriology.

Among gram positive isolates *Bacillus subtilis*, *Bacillus cereus* and *Bacillus* sp. were observed with varying sizes of rods. *Bacillus subtilis* was found to be highly resistance to all the four heavy metals used. Other gram-positive cocci were *Micrococcus sp.*, *Micrococcus luteus*. *Staphylococcus aureus* and *Arthrobacter*. Gram negative rods observed was identified as *Klebsiella*, *Escherichia coli.* They were identified on basis of oxidase test, nitrate test, citrate test and mode of utilization of other sugars.

3. Agar cup method

The compound to be tested is filled in the agar cup and diffusion of that substance from the cup into the media inhibits the test organism which inoculated in that media. Thus, by measuring the zone of inhibitions results can be interpreted. This test also used for study of MIC. The lowest concentration at which no growth observed is taken as MIC.

4. **Agar ditch method**

The various concentration of heavy metals used are 0.5mg/ml, 0.75mg/ml, 1mg/ml, 1.25 mg/ml and 1.50 mg/ml. According to results obtained all isolates showed growth for concentrations of Chromium except for S-1 showed no growth in 1.5mg/ml concentration. All organisms were able to tolerate as well as degrade the Chromium. The isolates degraded and tolerated all the concentrations of Lead Nitrate. The isolates S-2, S-5, S-6 and S-8 showed tolerance to all concentration of Copper and Cadmium. Rest all organisms showed sensitivity pattern to Copper and Cadmium. Isolate S-5 and S-6 were observed to be slightly sensitive at 1.25 mg/ml and 1.5 mg/ml for Cadmium. As a result, S-2 and S-8 exhibited great tolerance to all four of the chosen heavy metals.

Future Prospects

The effort to provide a successful solution to pollution was found to be successful under laboratory condition. The future prospect lies in the application of this microorganism for purposes like heavy metal remediation and potential use in extracting rare metals from dilute solution or removing toxic metals from industrial effluents (QA. Affan, E. Shoeb, U. Badar, J. Akhtar, 2009). These studies inform us that the isolated ***Bacillus spp., Escherichia coli, Micrococcus spp., Klebsiella spp., Staphylococcus and Arthrobacter.,*** have the properties to resist a wide range of heavy metals and antibiotics; it may be harmful to human being as well as to the animals. The investigation has widened the scope for research and development of metal tolerance and antibiotic resistance from bacterial origin.

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A- (https://www.hindustantimes.com/rf/image_size_640x362/HT/p2/2016/04/04/Pictures/rishikesh-edition-kalyan-january-choudhary-river-wednesday_65acc096-fa8c-11e5-89a7-e0427befb59e.jpg)

B- (https://5.imimg.com/data5/SELLER/Default/2022/11/YY/BO/QH/148747805/demineralizers-1000x1000.png)

C- (https://ruaecospaces.com/wp-content/uploads/2023/07/CHM-5.jpg)

D- (https://www.chmcollege.in/wp-content/uploads/2022/05/1.jpeg)