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Identification and Isolation of Heavy Metal (Copper) Resistant Bacteria

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ABSTRACT

Over the years, with the active spread and development of the industries, Heavy Metal, which are either used, or produced as byproducts by numerous manufacturing, industrial, refining and mining processes have become ubiquitous, persistent environmental pollutants. India too not exempted from such devasting environmental degradation caused by these pollutants. This study develops a method to accelerate the process of removal by encouraging the microbial and associated biota to degrade and/ or remove pollutants from the identified sites and also helps in isolation and cellular characterization of bacterial isolates. The area under study is the main sewage of Aligarh, U.P., lock industry in Talanagri and Surendra Nagar, Aligarh, from where soil in sterilized plastic bags and effluent samples in polypropylene bottles were collected. Processing of the samples for the isolation of tolerant strains was carried out in Mangalayatan University, Aligarh, which is followed by their cellular characterization.

Keywords: Heavy Metal, Environmental Degradation, Environmental Pollutants, Lock Industry, Soil Pollution.

INTRODUCTION

Pollution due to chemicals including heavy metals is a problem that may have negative consequences on the biosphere. The most abundant pollutants in the wastewater and in sewage are heavy metals [9]. Human activities such as mining operations and the discharge of industrial wastes have resulted in the accumulation of metals in the environment and eventually are accumulated through the food chain, leading to serious ecological and health problems.

Consequences of Heavy Metal Contamination

In recent years, ground soil and other materials polluted with heavy metals have become a serious environmental problem throughout the world due to their use in many manufacturing

processes, and up as waste in industrial effluent, through which heavy metals can enter water cycle, and then in the food chain where they are concentrated ultimately reaching the toxic levels [17]. Use of industrial wastewater for irrigation is a common practice in most of the third world countries which could alter the fertility of soil [3]. Moreover, accumulation of heavy metals in vegetation due to irrigation with waste water could affect human health [11].

Toxicity of Heavy Metals

Metals play a vital role in biological systems as a living cell cannot exist without metal ions. Trace amounts of heavy metals are also required by living organism including copper, cobalt, iron. Excessive levels of essential metals however can be toxic to the organism [5][7]. Essential heavy metal ions present a dual challenge to both eukaryotic and prokaryotic cells in that they are useful but can be lethal also. Therefore, a cell must meet its physiological requirement for essential metal ions while preventing their deleterious effects [1]. Availability of heavy metals in the cell must be carefully controlled due to their potential to form radicals and their tendency to bind to biological macromolecules [4]. Microorganisms use a number of mechanisms to maintain the correct equilibrium, including the uptake, chelation and extrusion of metals [14][16].

While some of the heavy metals are purely toxic with no known cellular role [15], other metals are essential for life at low concentration but become toxic at high concentrations [2], high concentration of all the heavy metals inhibits the activity of sensitive enzymes [10]. Wide range of essential cell components is potential targets for metal induced damage such as DNA for replication as a result of which cell death can occur [12]. Heavy metal toxicity can result in damaged or reduced central nervous function, lower energy levels and damage to blood composition, lungs, kidneys, liver and other vital organs. Long term exposure may result in slowly progressing physical and neurological degenerative process that mimic Alzheimer's disease, Parkinson's disease, Muscular dystrophy and Multiple sclerosis. Allergies are not uncommon and repeated long term contact with some metals or their compounds may even cause cancer [8]. If unrecognized or remain untreated, toxicity can result in significant illness and reduced quality of life.

Why bacteria equipped resistant systems to deal with these heavy metals?

In order to survive in the wild, bacteria need to develop different mechanisms to confer resistances to these heavy metals. There is no general mechanism for resistances to all heavy metal ions. Three generalizations may be made:

- The specificities of plasmid-determined metal resistances.
- Heavy metal resistance systems have been found on plasmids in almost every bacterial group (the absence of known resistance determinants in any group probably reflects insufficient effort).
- Two general resistant mechanisms were found, efflux pumping and enzymatic detoxification (generally red - ox chemistry to convert more toxic to less toxic metal-ion species).

How bacterial heavy-metal resistances evolved?

The general believes are these resistances arisen as a result of human pollution in recent centuries. However, it seems more likely that these resistances arose soon after life began, in a world already polluted by volcanic activities and other geological sources. Similar to antibiotic

resistances are preexisted in the pre-antibiotic era. Alternatively, certain heavy metal resistance might be evolved from pre-existing genes (through accumulating mutations).

Why bacterial heavy-metal resistances evolved?

Bacteria develop heavy-metal resistance mostly for their survivals, especially a significant portion of the resistant phenomena was found in the environmental strains (with or without the presence of heavy metals). One theory for bacterial heavy-metal resistance evolved is due to the use of antibiotics. For example, bacterial antibiotic-plasmids (sometime these plasmids are very big and called mega plasmid) existed in bacteria before the antibiotic era but their presence was brought into prominence by the use of antibiotics, which selected for antibiotic resistant strains. Subsequently, the range of genes carried on these plasmids (frequently associated with these heavy metal resistant determinants) was shown to extend far beyond those coding for antibiotic resistance. Similarly, heavy metals are also widespread in the environment; exert a selective pressure for the population of these plasmid-harboring bacteria. Although most of these resistances were linked to the plasmids, some are chromosomal origins.

Significance of Microbial Tolerance to Heavy Metals

Although microbial tolerance to heavy metals has been studied over the past 30 years, the last 15 years have been outstanding with respect to discoveries at molecular level [8].

3 possible uses of comprehensive metal resistance studies in biotechnology:

- Metal resistance can be added to a microorganism in order to facilitate a biotechnological process.
- Metal resistant bacteria can be used in bio – mining of expensive metals (bioleaching).
- Metal resistant bacteria can be utilized in bioremediation of metal – contaminated environments.

We need a better understanding of the microbial tolerance mechanisms in order to reduce the overall effect of toxic heavy metals in the environment.

MATERIALS AND METHODS

Sample collection, preparation and analysis:

Sampling of soil samples

All lab ware and sampling apparatus were pre-soaked by distilled water for a day prior to sampling to remove trace concentrations of metals. Contaminated soil samples were collected from Industrial Area, Talanagari, Lock Factory, Surendra Nagar, Aligarh, U.P, and around the main sewage of Aligarh near Aligarh College of Engineering and Technology (ACET). Soil samples were collected in sterilized plastic bags and were then transported to Institute of Biomedical Education and Research (IBMER), Mangalayatan University, Beswan, Aligarh. These containers were maintained at 4^{o}C or less to ensure minimal biological activity. To provide homogenized soil samples the soil was thoroughly mixed. The soil samples were dried for 24 hours, then finely ground and sieved through a 200 mesh sieve. The area under study for this research work was identified based on need, diversity and extent of pollutants produced.

Sampling of effluent samples

The effluent samples were collected in dry sterilized polyethylene bottles from Industrial Area, Talanagari, Lock Factory, Surendra Nagar, Aligarh, U.P. and from the main sewage of Aligarh near Aligarh College of Engineering and Technology (ACET), Mathura road, Aligarh. Effluents samples were collected by immersion by hand of a polyethylene sampling bottles.

Preparation of Medium:

Table No. I. Ingredients of M9 Minimal Medium

Composition	Gram/l
Na_2HPO_4	12.8 g
KH_2PO_4	3.10 g
NaCl	00.5 g
NH_4Cl	1.00 g
$MgSO_4$	00.5 g
Glucose	00.4 g
Agar	15.0 g
Distill Water	1.00 L

Each prepared petri plate has different concentration of HM, i.e. $CuSO_4$ according to 100µg/ml initially. In this study the maximum tolerable concentration (MTC) of each heavy metal was determined to all tolerant isolates using various concentration of each heavy metal by increment of **100µg/ml** starting from initial consternation of **200µg/ml** on media.

Processing of Samples for Isolation of Bacterial Strains

Bacterial strains were isolated from soil and effluent of Metal Industry and Sewage. Isolation was achieved by serial dilution [6] method.

- For stock solution 1gm of soil was dissolved in 10ml distill water.
- Five test tubes each with 9ml of distill water was prepared and marked as 1 to 5.
- One ml from stock solution was aseptically transferred to first test tube making the dilution 10^{-1}.
- Solution in the test tube was mixed and 1ml from this tube (10^{-1}) was transferred aseptically to next making it as 10^{-2}.
- Similar transfers were made till 10-5 dilution was achieved.
- From 10^{-2}, 10^{-3}, 10^{-4}, a loop full sample was sticked to sterile M9 Minimal Medium agar plates and spread properly in three different petri plates or in one by making segments, as available.
- The plates were then kept for incubation at 32 $\pm 1^{o}$C for 24 – 48 hrs.

Identification and Characterization of Bacterial Isolates

Morphological Characterization

Morphological studies of the isolates were done on the basis of colonies size, color, shape, diameter, elevations, and whether opaque, transparent or translucent.

Cellular Morphology

To observe cellular morphology cells were observed under Grams Staining under the microscope (oil immersion, 100X) by making smear of purified colony grown on M9 Minimal medium. Staining was carried out by the standard procedure of Gram staining for shape of cells (cocci, bacilli and cocco – bacilli), arrangement of cells (scattered, bunches and chains) and Gram (+) or gram (-) bacteria.

- Thin smear were prepared on clean glass slides.
- Smears were stained with crystal violet for 30 seconds and rinsed with sterile distill water.
- Films were flooded with gram Iodine for 30 seconds and again rinse with sterile distill water.
- Decolorization was done with 95 % alcohol and rinsed again with sterile distil water.
- Counter staining was done with Safranin for 20 – 30 seconds, finally rinsed with sterile distill water and blot dried.
- Slides were observed under microscope.

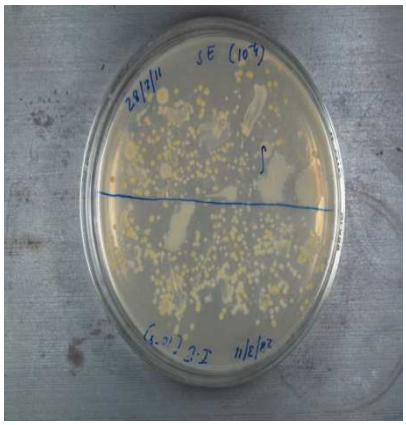
RESULTS AND DISCUSSION

Isolation and Identification of Microorganisms

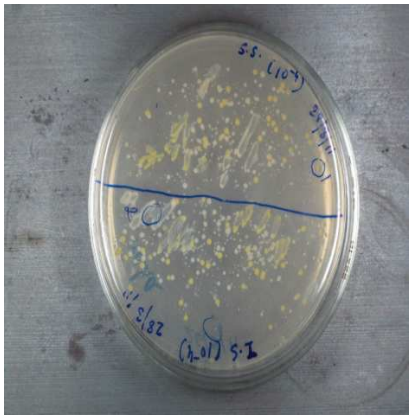
Two types of isolates were observed (table 2) [fig (a), (b)], when samples in different dilutions were sticked onto petri plate at HM concentration of 100µg/ml. All samples have same type of colonies ranging from 0.5mm – 1mm in diameter, circular with entire margins, slightly elevated and appearing yellow and white in color.

Table 2.Describing Sample Collection and Isolates

SAMPLES COLLECTION				
S.NO.	**Sites**	**Types**	**Dilution**	**Number of Isolates**
1.	Sewage near Aligarh College of Engg. &Tech., Mathura Road, Aligarh, U.P, India.	a. Soil	10^{-4}	2
		b. Effluent	10^{-4}	2
2.	Metal Industry, Talanagari, Aligarh, U.P, India	a. Soil	10^{-4}	2
		b. Effluent	10^{-4}	2
3.	Lock Factory, Surendra Nagar, Aligarh	a. Soil	10^{-4}	2
		b. Effluent	10^{-4}	2



(a)



(b)

Table 3. Showing Difference in Growth of Bacteria under different HM concentration

HM Concentration (µg/ml)	% Resultant Growth	**% Reduction in growth** = [Control (C)-Treated (T)/Control] *100
100 (C)	100	0
200 (T)	85	15
400 (T)	70	30
600 (T)	65	35
800 (T)	55	45
1000 (T)	40	60
1200 (T)	35	65
1400 (T)	25	75
1600 (T)	20	80
1800(T)	15	85
2000 (T)	0	100

Identification of Bacterial Isolates

Cellular Characterization

After performing Grams Staining, it was observed that the isolates may be Gram Negative Bacteria, as they appear pink in color and was rod shaped.

The first theme of this study was to isolate metal (copper) resistant organisms. For which samples were taken from sewage (soil and water) and industrial effluent along with soil. This sample size was chosen as all the domestic and small scale industrial waste is dumped in that sewage, which can be habitat of different types of micro organisms, so there is a possibility to find any novel micro organism during the study. Metal Industry samples were collected for more specification in isolation. For the isolation the growth media used was M9 minimal media as its components were easily available in the campus.

The isolated colonies were white and yellow in color, i.e. two types of isolates were identified. According to color, it can be predicted that white ones may be species of *Pseudomonas* and white of *Bacillus* species. Later while performing MTC only white colonies were identified.

CONCLUSION

The tests carried out in the laboratory have demonstrated that the levels of extractable metals in soil and water can be reduced by microbes. The leaching of metals and hydrocarbons to the environment can be reduced by these isolates. This study has revealed the MTC of isolates which will help in bioremediation process. The strains isolated from the soil and effluent samples (a total of 4) collected from the Sewage, near Aligarh College of Engineering and Technology, Mathura road, Aligarh, U.P and Metal Industry, Talanagari, and Lock factory, Aligarh, U.P. The Maximum tolerable bacterial isolate (at 2000µg/ml) was obtained mainly from sewage samples which were able to grow in the media containing high concentrations of heavy metal (Cu) under laboratory conditions within 24 - 48 hrs of incubation. On comparison of our result with literature, the isolate was suspected to belong with *Pseudomonas.*

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