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Isolation, Characterization, and Evaluation of the MIC and Antibiotic Resistance of Heavy Metal-Resistant Bacteria (Copper, Lead & Zinc) From a Municipal Dumping Site in Guwahati

Satyakam Agarwala[1,3], Arijit Bora[2*], Ankita Kalita[3], Srijana[3]

[1]Dept. of Bioengineering & Technology, Gauhati University, Gopinath Bordoloi Nagar, Jalukbari, Guwahati, Pin 781014
[2]Deputy Secretary, University Classes, Gauhati Univeristy, Gopinath Bordoloi Nagar, Jalukbari, Guwahati, Pin 781014
[3]Dept. of Applied Biology, School of Biological Sciences, University of Science and Technology Meghalaya, Ribhoi, Bariduwa, Pin – 793101, India

Corresponding Email: arijitbora@gauhati.ac.in

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ABSTRACT:

Urbanization and industrial development have led to the dumping of solid Municipal wastes releasing many toxic, mutagenic and non-biodegradable heavy metals into the environment either directly or indirectly, contributing to environmental pollution. Their spread and accumulation have proved to be hazardous and dangerous to living organisms. Microbes have evolved ways to absorb and accumulate these metals, thereby contributing to its remediation. Municipal dumping site at Paschim Boragaon, Guwahati, was selected as the study site. The current investigation aimed at isolation and characterization of the indigenous microbes and their antimicrobial susceptibility and minimum inhibitory concentration was also assessed. Four isolates were identified to their genus level based on their morphological and biochemical characters as Bacillus sp., and Lysinibacillus sp against Copper, Lead, and Zinc. Molecular characterization based on 16S rDNA analysis identified the isolates as Bacillus cereus, Bacillus paramycoides, and Bacillus velezensis, all showed varying level of MIC against the metals. Bacillus paramycoides showed MIC for Pb at 900 ppm, Bacillus cereus for Zn at 500 ppm, whereas Bacillus toyonensis showed moderate tolerance for Cu at 300 ppm respectively. Antibiotic susceptibility of the isolates showed resistance towards Ampicillin and Penicillin G and a varying susceptibility towards chloramphenicol, Tetracycline, Azithromycin, Norfloxacin, Ciprofloxacin and Streptomycin. All the isolates showed optimum growth for heavy metals at 35°C and a pH between 6.0– 7.5. Heavy metals tolerance might be plasmid mediated, which can be utilised as a tool for plasmid transformation transferring heavy metal accumulation potential, thereby, offering a viable method for bioremediation of contaminated soil.

Keywords: Municipal Waste, Characterization, Heavy Metal Resistant, MIC, Antibiotic Sensitivity, Bacillus

1. Introduction

In the human society, especially in the developing countries, environmental pollution is becoming the biggest concern. Over the time, this has led to the detrimental effect on the natural resources and human settings (Abbasi et al., 2023). The immediate impact on the environment due to pollution has caused an extensive harm as well as it has led to the disruption of organic food chain that are identified over extended periods (Naveed et al., 2023a, Naveed et al., 2023b,

Zameer et al., 2023). The major outcome is the expansion of municipal solid waste due to rapid industrialization and urbanization as well as growth of human populations and economic development (Abdel-Shafy et al., 2018, Adamović et al., 2018, Chen et al., 2018). The hazardous contents of municipal solid wastes include domestic, medical, agricultural waste or any form of garbage which are dumped directly at landfills and open dumping ground without segregation and treatment (UNEP, 2017). Leachates coming out of the decomposition of landfill wastes not only destabilises the environmental balance but also affects human health (Afolagboye et al., 2020). Additionally, other terrestrial and aquatic creatures are also affected including microbes, which tend to maintain their number and diversity for ecosystem sustainability through their biogeochemical function (McMichael and A. J., 2020). Heavy metals in the leachates of the dump site are one of the major pollutants which can bioaccumulate and bio-magnify in cells and tissues of plants and animals (Agamuthu et al., 2010). Numerous reports have suggested that leachates and soils from dumpsites are the major reservoirs of different types of heavy metals (Odukoya et al., 2010). Extensive use of pesticides and fertilisers have also contributed to the increasing concentrations of heavy metals which remain constant due to their stable structures (Ince et al., 2017, Ertan, B and Efe, D., 2019). Trace amounts of heavy metals remain less toxic to living organisms, however, higher levels have been known to be toxic particularly to humans, plants and microbes (Lima et al., 2012). Because of the non-biodegradability nature of heavy metals, land contamination is posing several health issues and is a continuous source of environmental anxiety (Tatah et al., 2020, Otitoju et al., 2022). World health organization's top 10 list of toxic heavy metals, which are of public concern includes cadmium (Cd), mercury (Hg), lead (Pb) and arsenic (As). Other examples are also included namely manganese, chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), selenium (Se), silver (Ag), antimony (Sb) and thallium (Ti) (Alam et al., 2012). Lead is one the dangerous and most pervasive in nature owing to its tendency to cause serious health issues and environmental problems, thereby raising a concern not only to humans but also to plants and microbes (Wani et al., 2015, Raj et al., 2023). The extent of lead toxicity is determined by the length, intensity of exposure, and mode of ingestion. Consumption may be through contaminated water, or food or just by breathing it in straight from the surrounding (Kushwaha et al., 2018). Arsenic is an omnipresent metalloid distributed both in land and water bodies as well they are released by humans to the environment through activities leading to pollution, thereby, posing a wide range of health issues in humans and animals too (Nriagu et la., 2007, Akopyan et al., 2018). As(III) and As(V) are taken up by a cell's aquaglyceroporins and phosphate transporters from the surroundings (Garbinski et al., 2019). Once inside, As induces oxidative stress causing disruption of protein structure as well as damage to DNA (Lemire et al., 2013). Microbial growth is controlled by heavy metals, either directly or indirectly. Interaction of most of the heavy metals with microbial cells and their accumulation due to physic-chemical mechanisms is directly or indirectly dependent on metabolism (Fourest and Roux., 1992). In most cases, higher concentrations of heavy metals forced the microbes to adjust themselves to the presence of heavy metals pollution (Habi and Daba., 2009). Heavy metals typically cause the death of microorganisms by damaging the functional groups of metal ions as well as displacing the metal ions. They also alter the active conformation of biological components. However, as co-factors for enzymes and metalloproteins, microbes depend on low concentration of specific metals (Olawale et al., 2023).

Microbial plasmids are most often the determining properties of metal tolerance and their detoxification by bacteria (Adekanmbi et al., 2019). Co-regulation of resistance genes not only triggers co selection of heavy metals due to environmental pollution but also increases the tolerance towards antibiotics. This co-regulation of genes is also responsible for reducing antibiotic susceptibility (Baker-Austin et al., 2006). As confirmed by many reports about the correlation between metal and antibiotic resistance, metal resistance being far more prevalent,

the genes for both metal and antibiotic resistance are present in the same plasmid (Ansari and Malik., 2007). Moreover, genes encoding metal resistance are found in plasmids and transposons and the trait is transferred not only between different genera or between different types of bacteria but also between indigenous microflora (Zhema et al., 2022).
Many reports have suggested that microbial tolerance towards heavy metal concentration can play an important role for the restoration of contaminated soil (Carrasco et al., 2005, Wei et al., 2009). In response to high metal stress, microorganisms including bacteria have evolved various adaptation strategies, both intra and extracellular mechanisms resulting in environmentally beneficial means of bioremediation. Such adaptations include production of various extracellular molecules leading to metal sorption, outflow of toxic metals from the cell, extracellular precipitation on the surface, enzymatic reduction of heavy metals to a less toxic state and mineralization (Ramesh et al., 2023, Pham et al., 2022, Teng et al., 2022).

The current study's objective was to isolate and identify heavy metal resistant bacteria (Pb, Zn, As) from metal contaminated site and to investigate and evaluate minimum inhibitory concentration of the isolates in order to ascertain their resistance against a high concentration of metals. Furthermore, isolates were analysed for antibiotic susceptibility against a broad range of antibiotics. Taxonomic characterizations further identified the species of bacteria present in the region. For the present investigation, the study was conducted in the Municipal dumping site at Boragoan situated at the outskirts of Guwahati city.

2. Materials and Methods

Sample Collection Site and Study Area

Solid waste of anthropogenic origin is disposed of in large amounts every day in the Municipal waste dumping site making it an ideal study area for isolation and investigation of metal resistant bacteria. Keeping the idea in mind, Paschim Boragaon situated at the outskirts of Guwahati city, Kamrup (M) district, Assam (*26.06.872° N latitude, 91.40.896° E longitude)* was chosen as the collection site for the current study. Sludge or leachate was collected in sterile falcon tubes (50.0mL) and transferred to the laboratory immediately. The tubes were then stored under cold temperature (-20 ºC) for further analysis.

Isolation of Metal Resistant Bacteria

Isolation of resistant bacteria (Pb, Cu, and Zn) from sludge was performed using the method of serial dilution and spread plate. In this method, 1.0mL of the sludge was added to a tube containing 9.0mL of sterilized distilled water blanks and was serially diluted from 10^{-1} to 10^{-9}. Nutrient media (HiMedia, India) incorporated with 30 ppm each of Lead Nitrate ($PbNO_3$), Zinc Chloride ($ZnCl_2$) and Copper Sulphate ($CuSO_4$) was prepared and 0.1mL of the sample was spread over the sterilized nutrient agar medium. The plates were incubated at 35ºC for 24 to 48 hours and were observed for growth. Individual colonies were identified and studied and sub-cultured (Pandit et al., 2013) for further studies and analysis.

Biochemical Characterization of Isolated Bacteria

Screening and identification of bacterial isolates were performed based on its phenotypical and biochemical characteristics. The isolates were tested for Methyl red, Vogues Proskauer (MRVP), citrate utilization, indole production, oxidase, catalase, and starch hydrolysis (Cappucino and Sherman., 2005). The selected bacterial isolate was characterized using Bergey's Manual of Determinative Bacteriology (Murray et al., 2005).

Antibiotic Sensitivity Assay

There are numerous reports supporting the association of metal resistance and antibiotic resistance (Rani et al., 2010). Bacterial isolates were tested for Antibiotic susceptibility against broad range antibiotics and were carried out on Muller Hinton agar medium. Susceptibility of the isolates were studied using the Kirby Bauer Disc diffusion method (Bauer et al., 1966). A total of eight antibiotic discs (HiMedia, India) of standard concentration (ranging from 5 mcg to 30 mcg) were taken and includes: tetracycline (30mcg), streptomycin (10mcg), chloramphenicol (30mcg), ampicillin (10mcg), penicillin G (10mcg), azithromycin (15mcg), norfloxacin (10mcg), and ciprofloxacin (5mcg) respectively. For the study, overnight grown isolates were spread over autoclaved Muller Hinton agar medium and incubated at 35ºC for 24 hours. Zone of inhibition was observed and noted by measuring the diameters of the zone and the strains were classified as sensitive (*S*), intermediate (*I*), or resistant (*R*) following the standard antibiotic disc given by (Wayne and P.A., 2003).

Minimum Inhibitory Concentration Assay

Assessment of Minimum inhibitory concentration was carried out using overnight grown cultures of metal resistant bacterial cells, which were harvested (OD_{600} = 0.7) and adjusted to approx 1.1 x 10^8 cells mL^{-1}. Bacterial culture (0.1mL) was introduced to autoclaved nutrient broth (HiMedia, India) comprising of increasing concentration of heavy metals, Lead Nitrate ($PbNO_3$), Copper Sulphate ($CuSO_4$), and Zinc Chloride ($ZnCl_2$) until the isolates failed to grow or showed impaired growth. The concentration of heavy metal was increased from 100 ppm till 1900ppm. The culture was cultivated for 24 hours at 35°C in a shaker incubator at 200 rpm. Absorbance of bacterial culture was taken at 600nm using multiplate reader (GeNei Laboratories, Elisa Reader). MIC was defined as the concentration at which specific strain of bacteria ceased to grow (Filali et al., 2000, Rajbanshi., 2008).

Taxonomic Characterization and Phylogenetic Analysis

Isolation of genomic DNA was employed for molecular characterization of the bacteria using a DNA extraction kit (HiMedia, India). Quality of the DNA band was evaluated by running the DNA on a 1.2% agarose gel and observing a high molecular weight single DNA band followed by determination of the concentration of the band using Nanodrop (Biotech Instruments, USA). Isolated DNA was stored at -80ºC for further analysis. Veriti® 96-well Thermal Cycler was employed to amplify isolated DNA using a specific universal primer for 16S rRNA. The experiment was carried out using a master mix (total volume 25µl) comprising of 10.0pmol of forward and reverse primers (27F–GAGTTTGATCATGGCTCAG and 149R–TACGGTTACCTTGTTACGACTT), 2.5 mM of $MgCl_2$, 200µM of deoxyribonucleotide triphosphates (dNTPs), Taq DNA polymerase of about 0.5 U, PCR buffer of a concentration of 1X (Invitrogen Life Technologies, Brazil) along with 50-100 ng of isolated bacterial genomic DNA. The process begins with the denaturation of the DNA template at a high temperature of 95 °C for a period of 5 min. A total of 39 denaturation cycles of the DNA strands was carried out at the temperature of 95 °C lasting for a period of 30 secs. It is followed by annealing for a period of 45 secs and then elongation for 60 secs at a temperature of 72°C and a final extension lasting for 7 mins. Agarose gel electrophoresis using 1.2% agarose gel was carried out to check the resulting amplicons. Genetic analyser (BDT v3.1) along with Cycle sequencing kit on ABI 3730xl was used for DNA sequencing, both forward and reverse, of the PCR products by employing both forward and reverse primers. A consensus sequence of the rDNA was created with the help of an aligner software and the sequence data information. Using the rDNA sequence data and known database of NCBI Genbank, the rDNA sequence was employed to carry out BLAST in order to determine the genus and species of the bacterial isolates. On the basis of maximum identity score, first 10 sequences were chosen and were

aligned using ClaustalW multiple alignment software. Phylogenetic analysis was done MEGAXI software and construction of phylogenetic tree was carried out using the neighbour-joining tree method (Saitou and Nei., 1987). A distance matrix was developed using the model as given by (Tamura and Nei., 1993).

Statistical analysis

The means and standard error of the data are shown using Duncan's test to assess significant differences, with an alpha of 0.05.

3. Results and Discussions

Environmental pollution in major cities is becoming a huge concern to the people, especially people living near to waste dumping sites. Such municipal solid waste dumping site comprises of wastes generated from various sources including domestic, commercial and industrial materials of anthropogenic origin, some of which are the major sources of heavy metals being dumped (Imron et al., 2021, Afolagboye et al., 2020). Being a major reservoir of heavy metals, municipal waste dumping site (Ojiego et al., 2022) is usually taken as the study area for the isolation of metal resistant bacteria and to assess the tolerance capability of the isolates for a better understanding of the isolates towards using them as a potential bioremediation agent. For the current investigation, Municipal dumping site at Paschim Boragaon, situated at the outskirts of Guwahati was chosen for the study, where, on a daily basis large amounts of solid wastes are dumped (Fig-1). Nutrient medium supplemented with heavy metals ($PbNO_3$, $CuSO_4$ and $ZnCl_2$) was formulated and were observed for the growth. Four colonies, based on their considerable tolerance and resistance, were selected for further studies. The isolates were grown and sub-cultured on separate nutrient medium containing 30 ppm of heavy metals, Pb(II), Cu(II), Zn(II) and were identified as isolates resistant to the respective metals (Fig-2). The isolates were subjected to identification using Bergey's Manual of Determinative Bacteriology (Murray et al., 2005) based on their biochemical interpretations. All the four isolates were found to be gram positive rods, endospore forming with similar morphology and characteristics. In regard to these attributes, they were identified as *Bacillus* sp, and *Lysinibacillus* sp. The isolates were designated as CuS1, PbS2, and ZnS3. Table-1 displays the results of morphological and biochemical findings. The results of the gram staining closely match previous research showing that the majority of bacteria resistant to heavy metals were gram positive, particularly *Bacillus* (Ndeddy et al., 2017). The most suitable temperature of all the four isolates was found to be 35ºC. For PbS2, CuS1and ZnS3, the ideal pH range was found to be between 6.0 and 7.0.

Further molecular characterization based on 16S rDNA sequence of the isolates confirmed the organisms. After the alignment of 16S rDNA sequences of isolates using BLAST, the result was found to have a 100% query coverage and 99–100% high level identity with known data of 16S rDNA sequences in GenBank. Phylogenetic tree further concluded the degree of similarities between 16S rDNA sequences of the isolates with bacteria collected from database (Fig-3). The isolates were identified to be closely related to CuS1 *Bacillus toyonensis* (99.69%), PbS2 *Bacillus paramycoides* (99.87%), and ZnS3 *Bacillus cereus* (99.92%). Accession number of the isolate as listed in GenBank are shown in Table-2. Predominance of the genera *Bacillus* in the soil of various ecosystems can be concluded as reported by several studies (Saxena et al., 2019). Furthermore, the genetic identities of the isolates coincided with the outcomes of their biochemical reactions.

Ther are several studies reporting that heavy metal resistant genes are responsible for coregulation of resistance towards antibiotic resistance and a decrease in antibiotic susceptibility (Zhai et al., 2016). Many reports also suggested a corelation between metal resistance to antibiotic resistance of a bacteria (Summers and Silver., 1972). Once microbe

develops metal resistance, this helps in acquiring antibiotic resistance as well. This has been attributed to efflux pumps on their membrane [Abskharon et al., 2010]. Zone of inhibition against various antibiotics were studied and measured for the isolates as given in Table - 3. Diameter of zone on inhibition was used as the basis to separate the strains as resistant, and sensitive. From eight antibiotics tested, all the strains showed multiple resistance towards ampicillin and even penicillin G. The study also showed that *Bacillus toyonensis*, *Bacillus cereus* expressed a high degree of susceptibility against both norfloxacin and ciprofloxacin, as well towards streptomycin. Whereas, *Bacillus paramycoides*, *Bacillus cereus* was found to be sensitive towards streptomycin. Azithromycin and tetracycline showed almost less susceptibility for the isolates. Furthermore, *Bacillus cereus* showed far less susceptibility towards tetracycline, norfloxacin and azithromycin. Previous reports have inferred the resistance of *Bacillus cereus* towards b-lactam antibiotics (Yim et al., 2015) such as penicillin, ampicillin, carbenicillin, amoxicillin, oxacillin, cefazolin, and ceftriaxone. Production of β-lactamase may be the contributing factor the antibiotic resistance (Park et al., 2009). As compared to other strains, *Bacillus paramycoides* showed sensitivity towards the antibiotics to a lesser extent. In most cases, environmental pollution remains the contributing factor that triggers heavy metal co selection and enhanced level of tolerance to antibiotics (Nath et al., 2019). Some previous work has put more emphasis on the possibility of not less than a random occurrence of co expression of meatal resistance and antibiotic resistance in *Bacillus cereus* but rather a result of environmental metal selection (Nath et al.,2018, Shammi and Ahmed.,2016).

Metal stress has made bacteria to evolve resistance mechanism as its surviving factor towards heavy metals in the environment. Microbes produce a wide range of metal-binding compounds, some specific and others more generic, in response to high concentrations of harmful metals. As a result, heavy metal tolerant bacteria have evolved a variety of mechanisms of resistance and detoxification, the majority of which are plasmid mediated. The concentration of heavy metals, bacterial diversity, and the geographic location of the area, all have a major impact on the toxicity level in the soil (Rajkumar et al., 2010). The study revealed different metal tolerance by CuS1 *Bacillus toyonensis*, PbS2 *Bacillus paramycoides,* and ZnS3 *Bacillus cereus* (Fig-4). Table – 4 shows MIC of the isolates, *Bacillus paramycoids,* which showed tolerance rate at 900 ppm (0.305±0.02) towards Lead. Furthermore, tolerance to a lesser extent was shown by *Bacillus toyonensis* at 300 ppm for Cu (0.217±0.01), whereas *Bacillus cereus* was tolerant towards Zn at 500 ppm (0.227±0.02). Previous studies on *Lysinibacillus sphaericus* have shown the presence of metal resistant genes for metals including arsenic, nickel, cobalt, iron, manganese, chromium, cadmium, lead and zinc which codes to almost a total of 123 proteins that are responsible in the transportation of metal ions into the cells by binding to them (Rahman et al., 2015). Some studies have even reported biosorption of arsenic, cobalt, iron and chromium by *Lysinibacillus sphaericus*, either living or dead (Velásquez and Dussan., 2019). Based on its capability of metal tolerance, *Bacillus paramycoides* can be used as an effective tool for degrading complex pollutants coming from paper and pulp industry waste, thereby, can be used as an agent for bioremediation (Verma et al., 2022). Many studies have put forwarded species that are responsible in removing Copper from the environment when there is an exceeding level of the metal, which includes *B. thuringiensis*, *B. cereus*, *B. licheniformis*, and *B. sphaericus* and even *Bacillus subtilis* (Raj et al., 2018, Rohini and Jayalakshmi., 2015, Oves et al., 2013). Many research has been conducted to explain the rate of accumulation of heavy metals in microbial cells, which usually surpasses the rate at which they are lost extracellularly (Prabhakaran et al., 2016). While the synthesis of metalloproteins and cell wall architectures of the cells governs the varying concentrations of metal accumulation by different isolates (Chatterjee et al., 2020).

4. Conclusion

Over the years there has been a huge accumulation of waste, both solid and liquid in the environment, which can be categorised of anthropogenic origin. Both domestic and industrial sources are the major players contributing to elevated levels of toxic metals in the soil n water bodies. This accumulation of metals has caused a devastation to the ecosystem, not only affecting the human health but also of the animals and microbes living in that area. Based on the effect of heavy metals on microbes, the present study was aimed at investigating the endurance level of bacteria isolated from sludge against heavy metals namely copper, lead, and zinc. Biochemical and molecular characterization of four isolates revealed the presence of *Bacillus toyonensis*, *Bacillus paramycoides*, and *Bacillus cereus* with varying level of tolerance against Cu, Pb, Zn and As respectively. MIC was carried out for the endurance level of the isolates and *Bacillus paramycoides* showed MIC for Pb at 900 ppm, *Bacillus cereus* for Zn at 500 ppm and *Bacillus toyonensis* for Cu at 300 ppm. Antibiotic susceptibility of the bacteria against different antibiotics revealed co regulation of genes of heavy metal tolerance with antibiotic resistance and sensitivity. All the isolates showed optimum growth for heavy metals at 35°C and a pH between 6.0 – 7.5. Further studies on the metal degrading capabilities of the microbes, especially on Municipal dumping grounds of major cities, can open doors for bioremediation of toxic metals and to minimise the level of metal contamination from the environment.

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Conflict of interest
Conflict of interest has not been declared by the authors.

Tables and figures

Growth conditions and Biochemical Tests	**Isolates**		
	CuS1	PbS2	ZnS3
pH	7.5	7.0	6.0-7.0
Temperature	37ºC	35ºC	35ºC
Gram	+	+	+
Shape	Rods	Rods	Rods
MR	+	+	-
VP	-	-	+
Indole	-	_	-
Citrate utilization	-	+	+
Catalase	+	+	-
Starch hydrolysis	+	+	+
Carbohydrate fermentation			
Glucose	+	+	-
Sucrose	+	+	+
Fructose	+	+	+

Table – 1 Morphological and Biochemical characteristics of the isolates. Symbols: (+) positive; (-) negative

Sl. No.	Isolates	Identified strains	Accession number
1.	CuS1	Bacillus toyonensis	MT279535.1
2.	PbS2	Bacillus paramycoides	MT373523.1
3.	ZnS3	Bacillus cereus	PP064862.1

Table – 2: Accession numbers of the isolates

Antibiotics	CuS1 Bacillus toyonensis	PbS2 Bacillus paramycoides	ZnS3 Bacillus cereus
Tetracycline	(26) S	(14) I	(22) S
Ampicillin	R	R	R
Chloramphenicol	(17) S	(16) S	(17) S
Penicillin G	R	R	R
Ciprofloxacin	(30) S	(20) S	(30) S
Streptomycin	R	(20) S	(23) S
Azithromycin	(14) I	(13) I	(15) S
Norfloxacin	(25) S	(14) I	(26) I

Table–3: Antibiotic susceptibility and resistance patterns of isolates. (*S* sensitive, *I* intermediate, *R* resistance)

(a) (b)

Fig – 1: Images showing (a) Dumping site at Paschim Boragaon (b) Satellite view of the site

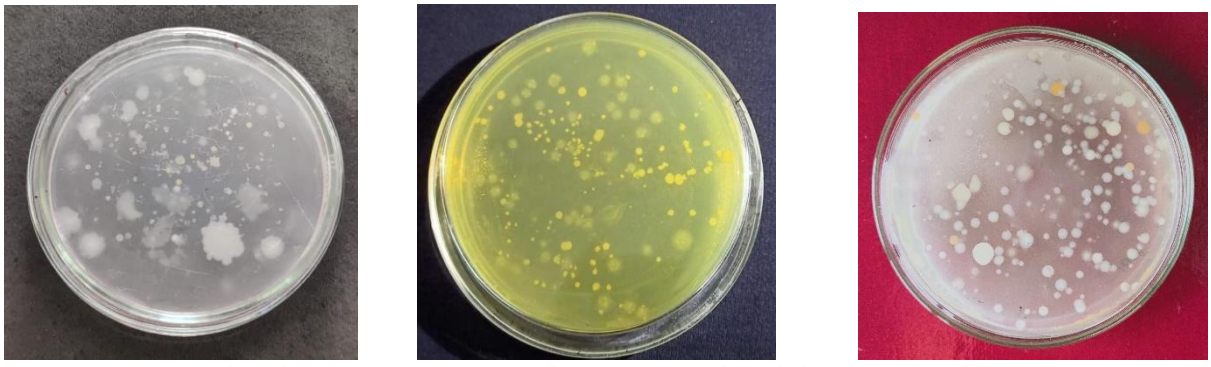

Fig – 2: Bacterial isolates on Nutrient media augmented with heavy meatals (30ppm) (a) Cu, (b) Zn, (c) Pb

Heavy metals and isolates	MIC (ppm)
Cu (CuS1)	300 (0.072±0.010)

Pb (PbS2)	900 (0.101±0.020)
Zn (ZnS3)	500 (0.075±0.020)

Table – 4: Minimum Inhibitory Concentration pattern of the isolates against metals. (Each value is the mean of three readings (n = 3))

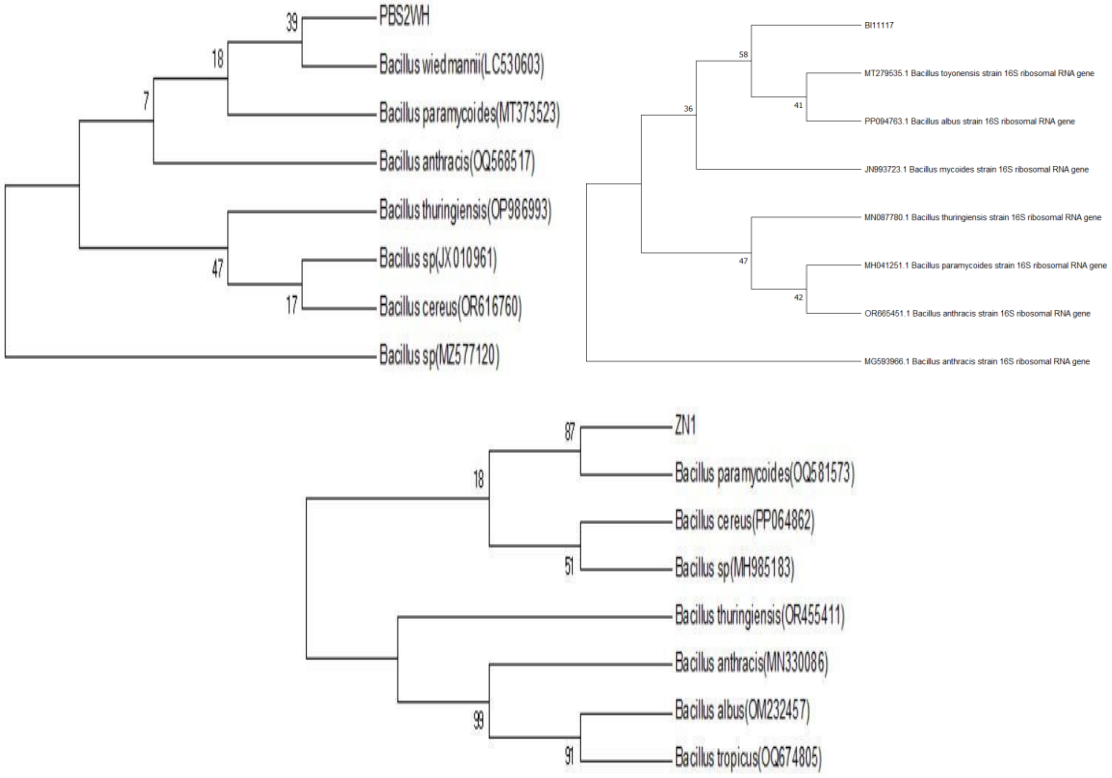

Fig – 3: Neighbour joining tree analysis showing evolutionary link between the bacterial isolates and the reference data from GenBank databases based on 16S rDNA sequences. At branching points, the numerical values correspond to bootstrap values obtained from 1000 repeats (Bi11117 – *Bacillus toyonensis*, PbS2WH – *Bacillus paramycoids*, Zn1 – *Bacillus cereus*)

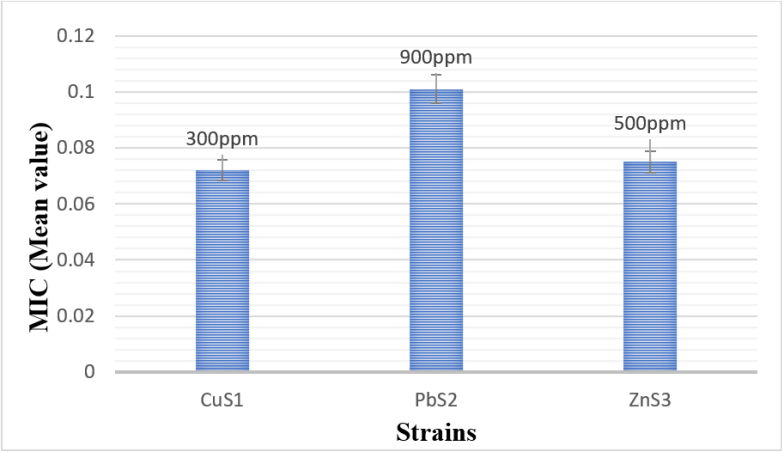

Fig – 4: Minimum inhibitory concentration measurements at absorbance 600nm of Cu, Pb and Zn resistant bacteria at different concentration of the heavy metal. Measurements were done in triplicates. Error bars indicate ±SD.

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