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ISOLATION AND CHARACTERIZATION OF HEAVY METALS AND ANTIBIOTIC RESISTANT BACTERIA FROM ENVIRONMENTAL SAMPLES

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ABSTRACT

In current years toxic heavy metal pollution has become one of the most critical environmental problems. In the present study, screening, isolation and characterization of heavy metal and antibiotic resistant bacteria from different environmental samples was carried out. Those bacterial isolates which shows >512 µg/ml. minimum inhibitory concentration were examined for antibiotic resistance activity. Antibiotics such as Ciprofloxacin, tetracycline, amoxicillin and erythromycin were taken for antibiotic resistance activity. Concentration of antibiotics were taken >16 to >64 µg/ml. Then characterization of selected bacterial isolate was carried out by 16S rDNA technique. The bacteria which has more resistivity towards both heavy metal and antibiotics that was identified by 16S rDNA sequencing as *Pseudomonas aeruginosa* and is the most heavy metal and antibiotic tolerant bacteria found in the environment.

KEYWORDS: Heavy metals, MIC, 16S rDNA.

INTRODUCTION

Heavy metal is a common collective term, which utilizes to the group of metalloids and the metals with their atomic density greater than 4000 Kg/m³. Environmental pollution by heavy metal comes from mining application of pesticides containing metal, fertilizer and the disposal of the environmental wastes by several industries.[1] Heavy metal can collect in biological systems and eventually be introduced into food web via different mechanisms. In

the environment several different forms of heavy metal can originate considerable modifications of their microbial activities and communities.[9, 14, 17] Industrial wastes cause toxic effects in aquatic organisms especially in fishes. Bioremediation is based on the plants, microorganisms or biological systems provide a cost- effective and their friendly environment for metal clean-up.[8, 16] Heavy metals are depends on their concentration and availability of metals on the actions of complex processes to regulate by multiple factors. Heavy metal such as chromium (Cr), lead (Pb), zinc (Zn), and copper (Cu) were toxic to aquatic organisms and danger in the environment.[18] The heavy metal tolerance bacteria has been declared as an indicator of potential toxicity of metals to other form of life. Heavy metals are naturally occurring but toxic levels are very rare. Most common problem are *cationic* metals (metallic elements whose forms in soil are positively charged cations e.g., Pb2+) are mercury, cadmium, lead, nickel, copper, zinc, chromium, and manganese. Some aerobic bacteria have been recognized for their degradative abilities such as *Pseudomonas, Alcaligenes, Sphingomonas, Rhodococcus,* and *Mycobacterium.* It has been reported that these microbes have been often used to degrade heavy metals, pesticides and hydrocarbons compounds. Generally, Working or living near an industrial site which utilizes these metals and their compounds increases ones risk of exposure, as does living near a site where these metals have been improperly disposed. Higher risks of exposure can be imposed also due to subsistence lifestyles and health impacts because of hunting and gathering activities. Therefore, the present study was initiated to analyse the heavy metal and antibiotic tolerance of these microorganisms achieved from environmental samples.

MATERIALS AND METHODS

Collection of Environmental samples

Collected soil samples were from various waste disposal sites of the urban environment of Bangalore city such as Contaminated soil (peenya industrial area, Sunkadakatte and Chikkabanavara), Vegetative leaves (coriander, Mint and Palak leaf), Industrial solid waste (Textile, Polymer, and Wood industry), Industrial liquid waste (Textile, paint, and chemical industry).

Heavy metals sources

Lead (Pb) – Lead acetate Pb $(C_2H_3O_2)_2$

Chromium (Cr) – Potassium dichromate $K_2Cr_2O_7$

Copper (Cu) – Copper sulphate ($CuSo_4$)

Zinc (Zn) – Zinc sulphate ($ZnSo_4$)

Isolation of total viable microflora from waste samples and heavy metal resistant bacteria by Pour plate technique

Microorganisms are isolated by serial dilution technique. Enumeration of total viable colonies bacteria were recorded. The environmental samples were collected in standard sized sterile plastic bags and carry for further bacteriological analysis. Based on total viable count the sample was diluted and inoculated into heavy metals – $ZnSO_4$, $CuSO_4$, $K_2Cr_2O_7$ and Pb $(C_2H_3O_2)_2$ (@ 100 PPM concentration) incorporating with nutrient agar by pour plate technique.

Identification of heavy metal and Antibiotic resistant bacteria

a. Determination of MIC (Minimal Inhibitory Concentration) for heavy metals in selected bacteria

MIC is described as the lowest concentration of antimicrobial agent required to inhibit growth of the organism. Heavy metal ion resistance studied was done by 64-512mg/l determining the Minimal Inhibitory Concentration (MIC) of the metals ions in tryptone broth media.

b. Micro broth dilution technique

Test Concentrations

Heavy metal stock solution was incorporated into tryptone broth. The heavy metal were serially diluted in sterile tryptone broth to obtain a final concentration of 64 ppm, 128 ppm, 256 ppm, 512 ppm by twofold dilution. The % inhibition of growth in treated was calculated using the formula given below. The concentration of heavy metal giving 50 % inhibition compared to control is considered as MIC.

% Inhibition = (Absorbance control – Absorbance sample / Absorbance control) X 100.

c. Determination of Micro broth dilution technique for Antibiotic resistant bacteria in heavy metal tolerant bacteria

The bacterial isolates whose minimum inhibitory concentration (MIC) were >512 those isolates were taken or selected for antibiotic resistance test.Heavy metal tolerant cultures were tested for antibiotic resistance by micro broth dilution technique.

Preparation of stock solution: Ciprofloxacin, Erythromycin, Tetracycline, Amoxicillin.

By using conventional technique pure bacterial cultures that were sub-cultured were further processed for identification. Gram staining [(Hi-media kit: Gram's Crystal Violet (SO12), Gram's Iodine (S013), Gram's Decolourizer (SO32), Safranin, 0.5% w/v (SO27)], and various biochemical tests such as Lactose fermentation, motility testing, Catalase, Citrate utilization test, Triple sugar iron, Urease, Oxidase, Carbohydrate fermentation tests were carried out for bacterial identification.

Molecular Characterization of heavy metal and Antibiotic resistant bacteria by 16S rDNA Sequencing

a. Genomic DNA extraction: Genomic DNA was isolated by using the InstaGeneTM Matrix Genomic DNA isolation kit (Catalog # 732-6030).

b. PCR amplification of 16S gene: Target gene fragment was amplified using MJ Research PTC-225 Peltier Thermal Cycler The following universal prmiers UNI_IL forward primer (5'-GGTGGAGCATGTGGTTTA-3'), UNI_IR reverse primer (5'-CCATTGTAGCACGTGTGT-3') (synthesized at Sigma Aldrich, Bangalore) targeting 16S gene were used.

c. Gene sequencing and phylogenetic tree construction: The PCR amplicons were purified and sent for sequencing at Vimta Labs (Hyderabad, India). The gene sequences obtained were analyzed by using the BLAST search programme and aligned with closing matching gene sequences from Genebank by multiple sequence alignment using the CLUSTAL W Program . The aligned sequences were analyzed by Mega 5 program and phylogenetic tree was constructed.

RESULTS AND DISCUSSIONS

Table 1: Total viable count by pour plate technique

SOURCE	SAMPLES	TOTAL VIABLE COUNT
A- vegetative leaf	1. Coriander leaf	$490x10^{-3}$/g
	2. Palak leaf	$421x10^{-3}$/g
B- Contaminated soil	3. Mint leaf	$594x10^{-3}$/g
	4. sunkadakatte	$5x10^{-3}$/g
	5. penya	$29x10^{-3}$/g
	6. chikabanawara	$70x10^{-3}$/g
C- Industrial waste(liquid)	7. peenya	$365x10^{-3}$/g
	8. Penya (paint industry)	$150x10^{-1}$/g
	9.Penya(chemicals)	$280x10^{-2}$/g

D- Industrial waste(solid)	10. penya	$9x10^{-3}$/g
	11. polymer	$80x10^{-2}$/g
	12. wood	$545x10^{-1}$/g

Table 2: Total viable count of heavy metal resistant bacteria by pour plate technique

SOURCE	SAMPLES	NO. OF HEAVY METAL RESISTANT BACTERIA			
		Pb	Cu	Kr	Zn
A- vegetative leaf	1. Coriander leaf	32	4	0	23
	2. Palak leaf	3	0	0	0
	3. Mint leaf	76	2	0	42
B- Contaminated soil	4. sunkadakatte	178	42	1	43
	5. peenya	405	-	165	198
	6. chikkabanawara	295	37	307	252
C- Industrial waste(liquid)	7. peenya	24	0	0	2
	8. Peenya(paint industry)	500	60	5	37
	9.Penya(chemicals)	245	50	80	277
D- Industrial waste(solid)	10. peenya	35	60	73	135
	11. polymer	522	26	447	258
	12. wood	820	354	656	395

Above table number 1 and 2 explains that the total viable count of bacterial colonies was done by serial dilution technique and pour plate technique(incl. heavy metal) respectively. That was collected from four different environmental samples e.g.vegetative leaf, contaminated soil, industrial waste (liquid), industrial waste (solid).

Minimum inhibitory concentration

Table 3: Minimum inhibitory concentration of bacteria with Lead acetate.

Isolate no.	Minimum inhibitory concentration(µg/mL)
	Lead acetate
1	512
2	>512
3	>512
4	>512
5	>512
6	>512
7	>512
8	512
9	512
10	256

Table 4: Minimum inhibitory concentration of bacteria with Potassium Di- chromate.

Isolate Number	Minimum Inhibitory Concentration (µg/mL)
	Potassium Di-chromate
11	128
12	512
13	>512
14	>512

Table 5: Minimum inhibitory concentration of bacteria with Copper sulphate.

Isolate Number	Minimum Inhibitory Concentration (µg/mL)
	Copper Sulphate
17	>512
18	>512
19	64
20	128
21	512
22	512
23	128
24	>512

Table 6: Minimum inhibitory concentration of bacteria with Zinc sulphate

Isolate Number	Minimum Inhibitory Concentration (µg/mL)
	Zinc Sulphate
25	512
26	>512
27	>512
28	>512
30	128
31	512
33	64
34	128
35	256

Table 3 explains that among the isolate tolerant to Lead, isolates 2, 3, 4, 5, 6 and 7 show maximum tolerance with MIC of >512 µg/ml. Similarly **Table 4** among the isolates tolerant to chromium, maximum tolerance with MIC of >512 µg/ml was observed in isolates13 and 14. Than **Table 5** among the isolates 13 and 14 tolerant to copper, maximum tolerance with MIC of >512 µg/ml. After that **Table 6** among the isolates 26, 27 and 28 tolerant to Zinc, maximum tolerance with MIC of >512 µg/ml. The concentration of heavy metal giving 50 % inhibition compared to control is considered as MIC.

Antibiotic resistance

Table 7: Minimum inhibitory concentration of bacteria with Antibiotic Resistance activity

Isolate No.	Minimum Inhibitory concentration (µg/mL)				
	Ciproflaxacin	Tetracyline	Erythromyclin	Amoxycilin	Heavy metal
2	16	>64	>64	>64	Pb
3	4	64	64	>64	Pb
4	>16	>64	16	>64	Pb
5	>16	>64	16	>64	Pb
6	>16	>64	16	4	Pb
7	>16	>64	16	4	Pb
13	4	64	64	4	Cr
14	4	64	>64	>64	Cr
17	>16	>64	>64	4	Cu
18	4	>64	>64	4	Cu
24	16	64	>64	64	Cu
26	4	64	4	4	Zn
27	>16	64	64	4	Zn
28	4	64	64	4	Zn

Table 7 explains that among all the selected bacterial isolates, isolate no. 4, 5, 6, 7, 17, and 27 that were carried out for MIC for antibiotic resistance shows maximum Ciproflaxacin tolerance. Similarly, for Tetracyline isolate no. 2, 4, 5, 6, 7, 17, and 18 shows maximum tolerance. For Erythromyclin, isolate no. 2, 14, 17, 18, and 24 shows maximum tolerance and for Amoxycilin isolate no. 2, 3, 4, 5, and 14 shows maximum tolerance.

Identification of bacteria through biochemical test

Table 8: Identification of bacteria through Biochemical test

isolate no.	Gram	Type	Motility	Lactose	Citrate	H_2S	Urease	Catalase	Oxidase	Sucrose	Mannitol	Arabinose	Inositol	Identification
3	-	Bacilli	-	+	+	-	-	-	-	+	+	+	+	*Klebsiella*
4	-	Bacilli	+	-	+	-	-	+	+	+	+	+	+	*Pseudomonas*
7	+	Bacilli	-	-	+	-	-	+	-	-	-	-	-	*Bacillus*
13	-	Bacilli	+	+	+	-	-	+	-	+	+	+	+	*Enterobacter*
14	+	Cocci	-	-	+	-	-	+	-	+	+	-	-	*Staphylococcus*
17	+	Cocci	-	-	+	-	-	-	-	+	-	-	-	*Streptococci*
27	-	Bacilli	+	+	+	-	-	-	-	+	+	+	+	*Enterobacter*
28	+	Bacilli	+	-	-	-	-	+	-	-	-	-	-	*Bacillus*

Thus, **Table 8** shows various genuses of bacteria that were identified through various biochemical tests. Bacteria such as *Pseudomonas, Bacillus, Enterobacter, Klebsiella, Staphylococcus, Streptococci*

Molecular Characterization of heavy metal and Antibiotic resistant bacteria by 16S rDNA Sequencing

Heavy metal resistant Isolate was identified by 16S rDNA sequence analysis using universal primers. Identification to the species level was done by Phylogenetic analysis. Genus and Species was identified based on16S rDNA sequence similarity with that of the published sequence in Gen Bank.

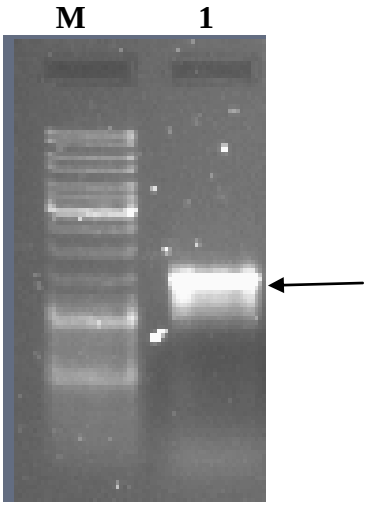

Figure 1: Agarose Gel Electrophoresis image of HMR04 genomic DNA 16S rDNA Amplicon

M: 3000bp ladder (Fermentas), 1-HMR04 amplicon

Figure 1 arrow indicates that 287bp.

Gene sequencing and phylogenetic tree construction

For determining the nucleotide sequences of 16S rDNA and antibiotic resistance determinants, the PCR amplified products were purified using PCR purification kit (Sigma Aldrich), DNA sequencing of the purified product was performed by dideoxy chain termination method using gene specific primers at the sequencing facility of Vimta Labs (Hyderabad, India). The gene sequences obtained were analyzed by using the BLAST search programme. The sequence of the PCR product was compared with known 16S rRNA gene sequences in the Gene Bank by multiple sequence alignment using the CLUSTAL W Program (Thompson *et al*., 1994) and the phylogenetic tree was constructed using Mega 5

program.

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene base sequences of the HMR04 were blasted and phylogenetic tree was constructed with closely matched aligned sequence. The phylogenetic tree reveals that isolate closely relates to *P.aeruginosa* strains – SRP2696 and 2604 sequences . The phylogenetic tree of 16S rRNA gene of isolate is presented in Figure 2. The phylogenetic tree confirmed the isolate as *P. aeruginosa*.

HMR04_16S Raw Sequence(273 bp)

>GCGCCTAACACTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTG
GTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCA
CCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGG
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GAA

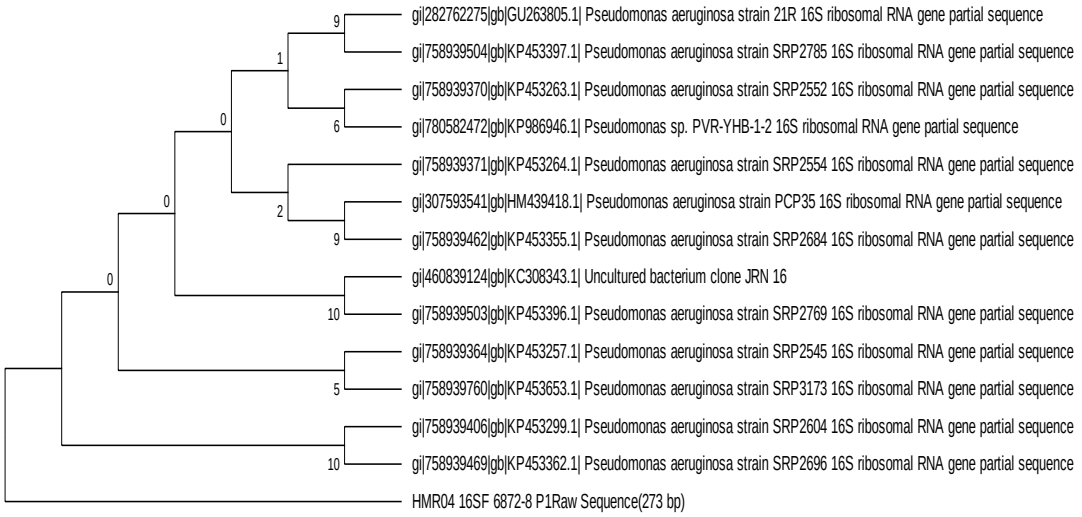

Figure 2. Phylogenetic Tree of HMR04 Isolate

CONCLUSION

Pollution of environment is a serious problem of the modern world. In this current review, an attempt was made to study the heavy metal tolerant bacteria isolated from the industrial effluents (solid, liquid), vegetative leaf, and contaminated soil of urban environment of Bangalore city. Than Enumeration, isolation, identification, characterisation of bacteria was done by which heavy metal tolerant bacteria were identified. Different types of bacteria were

identified which are specially present in environment. and common bacteria found were *Pseudomonas, Bacillus, Enterobacter, Klebsiella, Staphylococcus, Streptococci.* Minimum inhibitory concentration of heavy metals in selected bacteria was done and heavy metal tolerant cultures were tested for antibiotic resistance by micro broth dilution technique. Antibiotics resistance was determined on Mueller Hinton agar plates (Hi Media, India). The concentration of heavy metal giving 50 % inhibition compared to control is considered as MIC.

Isolates which shows heavy metal resistance will be selected and it's morphological, biochemical and molecular characterization 16S Rdna will be done. Heavy metal resistant Isolate was identified by 16S Rdna sequence analysis using universal primers. Identification to the species level was done by Phylogenetic analysis. Genus and Species was identified based on16S Rdna sequence similarity of 99% respectively with that of the published sequence in GenBank. The phylogenetic tree reveals that isolate closely relates to *P.aeruginosa* strains – SRP2696 and 2604 sequences .These isolates can further be used for bioremediation of heavy metals form industrial effluent/ wastes.

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