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Isolation, Characterization and Identification of Bacteria from Hosur Industrial area and their Tolerance to Antibiotics and Heavy Metals

Mahilarasi A[1], Veeramanikandan Veeramani[1*], Jaianand Kannaiyan[2], Balaji Paulraj[2]
[1]PG & Research Centre in Microbiology, MGR College, Dr. MGR Nagar, Hosur, Tamil Nadu, India
[2]PG & Research Centre in Biotechnology, MGR College, Dr. MGR Nagar, Hosur, Tamil Nadu, India

ABSTRACT

In the current situation heavy metal contamination is becoming a serious threat for plants, animals, humans and whole nature, the main sources of soil and water pollution are solid waste and waste water sometimes with extremely toxic level of hazardous substances. To manage these wastes, the decomposition of it by the potent microbial action is the only safer, natural and cheap way. Because all the microbes are isolated from the nature and being studied by the scientist weather they don't have any type of negative impact on soil, plant, animal, humans or nature in any way. The present study deals with isolation, identification and characterization of heavy metal resistant bacteria was isolated from different metal dumping sites collected in and around Hosur. Initially, a total of 27 isolates were screened from the effluent after screening experiments. The five isolates were selected based on high level of heavy metal and antibiotic resistances. On the basis of morphological, biochemical analysis revealed that, the isolates were authentically identified as Acinetobacter pittii, Escherichia coli, Fictibacillus nanhaiensis, Lysinibacillus xylanilyticus and Planococcus maritimus. The identified isolates were resistant to Aluminium nitrate ($Al(No_3)_3$), Calcium chloride ($CaCl_2$), Nickel sulphate ($NiSo_4$), Cobalt chloride ($CoCl_2$), Mercury chloride ($HgCl_2$), Potassium dichromate ($K_2Cr_2O_7$), Zinc sulfate ($ZnSo_4$) and Copper(II) sulfate ($CuSO_4$). The minimum inhibitory Concentration (MIC) of the effluent isolates was determined in solid media. The multiple metal resistances of these isolates were also associated with Antibiotics Ampicillin, Penicillin, Bacitracin, Novobiacin, Erythromycin, Methicillin, Rifampicin, Clindamycin, Linezolid, Imipenem, Azithromycin and Trimetholinin. The identified heavy metal resistant bacteria could be useful for the bioremediation of heavy metal contaminated sites.

Keywords : Antibiotics, Bio-Remediation, Bacteria, Heavy Metals

I. INTRODUCTION

The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. In hazardous substances the main target are the chemical pesticide residues and heavy metals. From last two to three decades, chemical pesticides are used abundantly in all over the India. In a number of crops side effect of pesticide and heavy metals are being observed. There are some sources of heavy metals like e-waste, are also present in solid waste which releases heavy metals e.g. arsenic, chromium, cadmium and lead above the toxic level in the nature which goes into the soil, ground waste or rivers of India and finally gives a negative impact.

Furthermore, recent studies suggest that antibiotic resistant bacterial strains may arise in the

environment through cross- or co-resistance to metals or resistance pathway co-regulation. Antibiotic resistance is the ability of a microorganism to resist the effects of an antibiotic to which it is generally liable. Bacteria resistance to antibiotics and other antimicrobial agents is a growing delinquent in today's civilization. The existing antibiotics are becoming less useful due to in distinguish use of these antibiotics against pathogenic bacteria, infectious diseases are becoming more challenging to treat. The antibiotic resistant bacterial strains may arise in the environment through cross- or co-resistance to metals or resistance pathway co-regulation[1].

Global attention has been drawn on ways to sustain the environment using microorganism to remediate environmental pollutants because physical and chemical treatment are costly and can lead to production of toxic substance[2]. Bioremediation involves the use of microorganism to reduce or remove the pollutants from contaminated area which may lead to restoration of the original natural substance without further disruption to the local environment[3]. Mostly, the oxidized products of organic materials provide energy and carbon as a nutrient source for cell growth[4]. Bioremediation is an economical, eco-friendly and requires less expensive techniques for water pollution[5]. Several researchers focused on selected consortium was significantly improved the degradation for the different industrial wastes and degradation degree achieved by consortium and using the stains individually[6]. Microorganisms and Microbial products can be highly efficient bioaccumulations of soluble and particulate forms of metals especially dilute external solutions. Microbes related technologies may provide an alternative or addition to conventional method of metal removal or metal recovery. These microbial communities appeared to play important roles in removing nutrients, odour and reducing hazarders heavy metals in the contaminated sites without chemical pre-treatment[7]. This present study revealed that the bacterial isolates were able to tolerate different concentration of heavy metals recovered from the different contaminated sites and the isolates were resistant to the different antibiotics. Thus the present study defend that heavy metal resistance leads to antibiotic resistance, also these bacterial isolates can be used for the bioremediation of heavy metals.

II. METHODS AND MATERIAL

A. Sample collection

A total of 10 samples were collected from various industries at metal dumping site located in District Krishnagiri, Hosur. Effluent samples were collected in dry, sterile polypropylene containers and transported immediately to Microbiology Research Laboratory at the Department of Microbiology, MGR college Hosur. These containers were maintained at 4^{o}C to ensure the minimal biological activity. Processing of samples for isolation of bacteria was carried out within 3h of sample collection.

B. Isolation of Bacteria

The bacterial species were isolated from the collected water samples with the help of conventional serial dilution technique[8]. For the pure culture of bacteria single colonies were picked and streaked on the nutrient agar plates containing different concentrations (200 to 2000 ppm) of different heavy metals (Aluminium nitrate ($Al(No_3)_3$), Calcium chloride ($CaCl_2$), Nickel sulphate ($NiSo_4$), Cobalt chloride ($CoCl_2$), Mercury chloride ($HgCl_2$), Potassium dichromate ($K_2Cr_2O_7$), Zinc sulfate ($ZnSo_4$) and Copper(II) sulfate ($CuSO_4$) (all from Sigma Aldrich, St. Louis, MO)) under sterile conditions. These concentrations were selected on the basis of previous studies reported in the literature. Pure cultures of strains which showed growth on plates containing 2000 ppm heavy metal concentration were grown on slants by stab and streak method for storage and subsequently for identification and biochemical characterization of bacterial isolates. These tests were

used to identify the isolates referring to the Bergey's manual of systematic bacteriology[8], determinative bacteriology and probabilistic identification matrix.

C. Bio-Chemical Analysis

Biochemical tests were performed in order to establish the identities of different isolates such as indole test, citrate utilization test, nitrite reduction test, catalase test, MR–VP, urease test, sugar fermentation test (such mannose, sucrose, galactose glucose, lactose and fructose) of all collected samples were analyzed and also heavy metal degradation analysis was carried out in the laboratory[9].

D. Identification and characterization of the bacterial isolate

Heavy metal resistant bacterium obtained was initially characterized in terms of colony morphology (color, shape, size, elevation, margin, consistency, opacity) and basic microscopic observations (gram stain, spore stain, size). Further to this confirmative identification these isolated species were inoculated in nutrient broth containing heavy metal concentration (2000 ppm) incubated for three different selection was done on the basis of (1) Final counts of CFU taken after 24 hrs from LB supplemented with heavy metal concentration of different respective metals (2) Minimum Inhibitory Concentration (MIC) of the metals which they can resist, (3) Antibiotic susceptibility test on 9 different antibiotic discs (ampicillin (10 mg), penicillin (30 mg), bacitracin (30 mg), novobiacin (30 mg), erythromycin (15 mg), methicillin (30 mg), rifampicin (30 mg), clindamycin (2 mg), linezolid (30 mg), imipenem (10/750 mg), azithromycin (15 mg), trimetholinin (30 mg) (Himedia, India). The isolates which have shown growth on heavy metal concentration (2000 ppm) were considered as potential degraders. Among the isolated bacteria, 5 isolates were found potent heavy metal degraders. Hence, for cumulative identification these isolates were sent to Yaazh Xenomics, Coimbatore for the 16S rDNA sequence analysis.

E. 16S rDNA sequence determination

A colony PCR method was used for amplification of 16S rDNA. A single colony of bacterial isolate was suspended into 100µl sterile distilled water. Boiled for 25 minutes at 95˚C and centrifuged at maximum speed in microcentrifuge for 15 minutes to pellet cell debris. 5 µl of the supernatant was used as DNA templates for PCR. Bacterial 16S rDNA was amplified from the extracted genomic DNA by using the universal bacterial 16S rDNA primers 8F forward primer (5'AGAGTTTGATCCTGGCTCAG3') and 1541R reverse primer (5'AAGGAGGTGATCCAGCCGCA3'). PCR was performed with a 25µl reaction mixture containing 5µl of DNA extract as the template, 1.5µL of Forward Primer and Reverse Primer, 5µL of deionized water, and 12µL of Taq Master Mix. Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).

F. PCR amplification

The PCR product was sequenced using the primers. Sequencing reactions were performed using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems) and consists of an initial 95°C denaturation for 2 min followed by 25 cycles of 95°C for 30sec, 55°C for 30sec, 72°C for 2 min, followed by a final extension at 72°C for 10 min. Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to ectrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

G. Nucleotide sequencing, alignment, and phylogeny

Sequences were matched with previously published bacterial 16S rDNA sequences in the National Centre for Biotechnology Information (NCBI) databases using

Basic local alignment search tool using nucleotide query (BLASTN)[10]. Based on the scoring index, the program MUSCLE 3.7 was used for multiple alignments of sequences[11]. The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions[12]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as substitution model. PhyML was shown to be atleast as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering[13].

III. RESULTS AND DISCUSSION

H. Isolation, Identification and characterization of the bacterial isolate

A total of 27 isolates were recovered from metal dumping sites from Hosur region (Figure 1) and 9 isolates were screened after heavy metal resistance experiment. The isolates were further identified on the basis of morphological characteristics (size, colour and texture). Also the microscopic examination of these isolates revealed that out of 9 isolates, 3 were gram positive cocci, 6 were gram negative bacilli (Table: 1 and Table 2). Finally, five best isolates are picked from samples (C1, D1, D2, E1, and E2) which were tolerant to high concentrations of the heavy metals under study. All the five isolates were able to grow at the highest concentrations at 2000ppm of $Al(No_3)_3$ 18mm, $CaCl_2$ 19mm, $CoCl_2$ 25mm, $K_2Cr_2O_7$ 26mm, $HgCl_2$ 15mm, $Niso_4$ 20mm, $ZnSo_4$ 25mm and $CuSO_4$ 26mm.

Figure 1: Heavy metal sample collection site located in District Krishnagiri, Hosur.

Table 1. Colony characterization and microscopic examination of isolated bacteria

Isolates	Colony colour	Pigmentation	Colony size and texture	Gram staining	Motility test
A1	Light white	-	Small size and smooth	(+) short rod	Motile
A2	Light cream	-	Medium size and smooth	(-) short rod	Non-motile
B1	Light White	Orange	Small size and smooth	(-) short rod	Non-motile
B2	Light yellow	-	Medium size and smooth	(+) long rod	Non Motile
C1	White	-	Small size and smooth	(+) long rod	Non motile
C2	Light cream	Orange	Small size and smooth	(-) cocci	Motile
D1	Light White	-	Small size and smooth	(+) long rod	Non-motile
D2	Cream	Orange	Large size and smooth	(-) short rod	Motile
E1	Light White	-	Small size and smooth	(-) short rod	Motile
E2	Light cream	-	Small size and smooth	(-) short rod	Non motile

Note: Gram's positive bacteria (+), Gram's negative bacteria (-).

I. 16S rDNA sequence determination

Based on the morphological, physiological, biochemical characteristics (Table 1 to Table 3) showed that the strain is close to the members of genus *Pseudomonadales, Enterobacterales* and *Bacillales.* The partially amplified for five different samples correspondingly 1245bp, 1266bp, 980bp, 840bp and 908bp fragment of 16S rDNA sequence was submitted to NCBI database search using Blastn to confirm the species of the bacterium. The highest sequence similarity revealed that it is closely related to *Acinetobacter pittii* accession number MH496640, *Escherichia coli* accession number MH496639, *Fictibacillus nanhaiensis* accession number MH496638, *Lysinibacillus xylanilyticus* accession number MH496636 and *Planococcus maritimus* accession number MH496637.

Table 2. Biochemical characterization of the isolated bacteria

Isolates	A1	A2	B1	B2	C1	D1	D2	E1	E2
Indole	(+)	(-)	(+)	(-)	(+)	(-)	(-)	(+)	(-)
Methyl red	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(-)	(+)
VP	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(-)	(+)
Citrate	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)
Catalase	(+)	(-)	(+)	(-)	(+)	(+)	(-)	(+)	(+)
Oxidase	(-)	(+)	(-)	(+)	(-)	(-)	(+)	(-)	(-)
Urease	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)
TSI	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
Gas	(+)	(-)	(+)	(+)	(+)	(+)	(-)	(+)	(+)
H2s	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)
Glucose	A/G	A	A/G	A/G	A/G	A/G	A/G	A/G	A/G
Fructose	A/G	A	A/G	A/G	A/G	A/G	A/G	A/G	A/G
Sucrose	A/G	A	A/G	A/G	A/G	(-)	A/G	(-)	A/G
Lactose	A/G	(-)	(-)	(-)	(-)	A/G	(-)	A/G	A/G
Mannose	A/G	(-)	A/G	A/G	A/G	A/G	A/G	A/G	(-)
Galactose	A/G	A	A/G	A/G	A/G	A/G	A	A/G	A/G

Note: Positive result (+), Negative result (-), Acid (A), Acid/ Acid (A/A), Acid /Gas (A/G) production respectively.

The bacterial isolates were tested for their sensitivity to 19 different commonly used antibiotics. Almost all the bacterial isolates were resistant to most of the antibiotics. In the antibiotic cultural sensitivity assay, five isolates were found resistant to four or more different groups of antibiotics. Such isolates were regarded as multidrug resistant. This number further increased when the intermediate resistance was also accounted for among resistant strains. (Table 3).

Table 3. Antibiotic susceptibility of heavy metal resistant bacteria isolates

Antibiotic disc name	A1	A2	B1	B2	C1	D1	D2	E1	E2
Cefepime [CPM] 30 mg		21 (R)		18 (I)		14 (R)	12 (R)		
Tobramycin [TOB] 10 mg		10 (R)				10 (R)	24 (R)		
Chloramphenicol [C] 30 mg	10 (R)	14 (I)		17 (R)		17 (R)	11 (R)	24 (R)	

Piperacillin [PC) 100mg		14 (R)		11 (R)		< 10 (R)	10 (R)		
Ciftazidine [CA] 30 mg		11 (R)		11 (R)		< 10 (R)	11 (R)		
Ampicillin [A] 10 mg	< 10 (R)	10 (R)		< 10 (R)		< 10 (R)	< 10 (R)	< 10 (R)	
Cefixime/ clavulanic acid [CMC] 5/100 mg		26 (R)		14 (R)		10 (R)	16 (R)		
Ampicillin/ sulbactum [AS] 10 mg		14 (I)		10 (R)		< 10 (R)	10 (R)		
Penicillin-G [P] 30 mg			< 10 (R)		< 10 (R)				< 10 (R)
Amoxicillin [AC] 10 mg		12 (R)				< 10 (R)	< 10 (R)		
Bacitracin [B] 30 mg			< 10 (R)		< 10 (R)				< 10 (R)
Novobiocin [NV] 30 mg			< 10 (R)		< 10 (R)				< 10 (R)
Erythromycin [E] 15 mg	< 10 (R)		< 10 (R)		< 10 (R)			< 10 (R)	< 10 (R)
Methicillin [M] 30 mg			< 10 (R)		< 10 (R)				< 10 (R)

Note: All numerical values of diameter in mm and represents Resistant (R), Sensitive (S), Intermediate (I)

J. Nucleotide sequencing, alignment, and phylogeny

The multiple sequence alignment and the phylogenetic relationship confirmed the highest sequence similarity with *Acinetobacter pittii* (MH071327.1) with distribution of the top 155 blast hits, *Escherichia coli* (CP028589.1) with distribution of the top 200 blast hits, *Fictibacillus nanhaiensis* (KY082742.1) with distribution of the top 106 blast hits, *Lysinibacillus xylanilyticus* (MH496636.1) with distribution of the top 200 blast hits and *Planococcus maritimus* (KR063196.1) with distribution of the top 108 blast hits on 100 subject sequences.

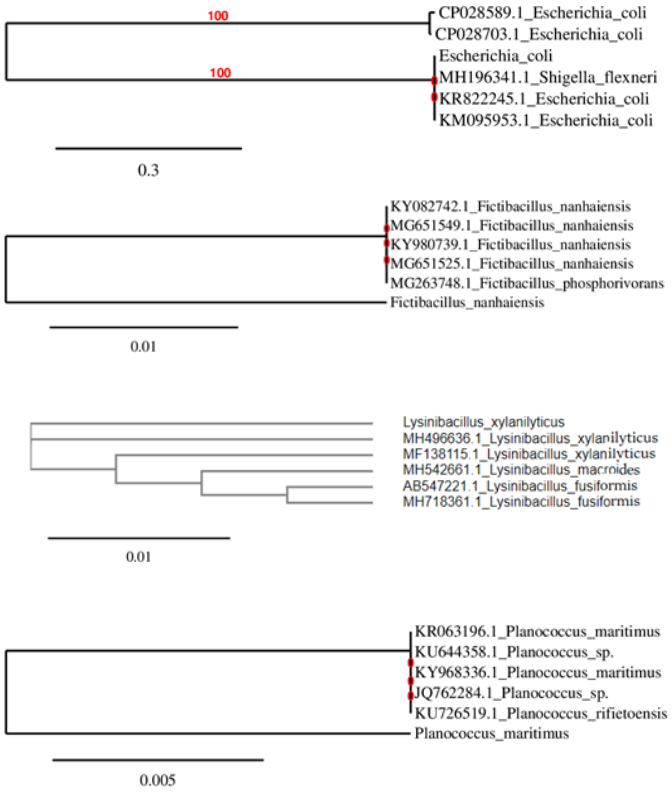

Figure 2. Phylogenetic relationship based on 16S rDNA sequence comparison showing the position of Acinetobacter pittii (MH496640), Escherichia coli (MH496639), Fictibacillus nanhaiensis (MH496638), Lysinibacillus xylanilyticus (MH496636) and Planococcus maritimus (MH496637). The GeneBank accession numbers for the 16S rDNA sequences are given after the strain in parenthesis. while scale bar corresponds to nucleotide sequence difference.

The calculation and classification of phylogenetic relationship was determined and PhyML was shown to be atleast as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering (Figure 2).

To conclude that the present study revealed that the bacterial isolates were able to tolerate of the discussed different concentration of heavy metals recovered from the contaminated sites. Furthermore, the isolates were resistant to the different antibiotics which were used against the isolates. Thus, the present study justify that heavy metal resistance leads to antibiotic resistance, also these bacterial isolates can be used for the bioremediation of heavy metals.

IV. CONCLUSION

The present study highlights the identities of the bacteria occurring in the metal dumping sites and reports the isolates as Multiple Drug Resistant (MDR). There appears to be a direct correlation between the heavy metal tolerance and antibiotic resistance. However, further studies are required to establish this fact with certainty. Also, studies relating to the presence of different communities of bacteria in such sites with relation to their phylogeny, metabolic activities need to be further explored. Also links between phylogenetic and metabolic diversities must be established in order to attribute the measured microbial processes to a specific phylogenetic group. Further investigations are also needed to completely evaluate the potential of the contaminated soils to constitute a reservoir of heavy-metal resistant microorganisms or heavy-metal resistance/tolerance genes. The search must be extended to genetic determinants conferring resistance to several heavy metals. Culture dependent and culture independent methodologies must be combined to accomplish this objective. Novel culturing procedures must be applied to assess a higher diversity of culturable bacteria and to determine not only resistance phenotypes but also their genetic content.

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