IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)
e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 9, Issue 7 Ver. I (July. 2015), PP 70-76
www.iosrjournals.org

Isolation and Characterization of Mercury Resistant Bacteria from Fly Ash Sample of Mejia Thermal Power Plant, W. B, India for Application in Bioremediation and Phytoremediation

Soumi Ghosh, Pritam Mukherjee, Pallabi Pati, Roopali Roychowdhury, Atanu Roy, Madhumita Roy*
Department of Biotechnology; Techno India University; Salt Lake; Sector V; Kolkata-91; W. B; India

Abstract: *Mercury (Hg) is the most toxic heavy metal among all the heavy metals and is the only heavy metal that is liquid at standard conditions of temperature and pressure. It can disrupt cellular function even at very low concentration. Fly ash dump site is a source of environmental mercury contamination as fly ash generated from coal burning retains this toxic metal of coal. The present work is aimed to isolate and characterize mercury resistant bacterial strains from settled fly ash sample of Mejia Thermal Power Plant of DVC, located at Bankura, West Bengal, India. Three types of isolates were recovered by enrichment culture technique and all of them showed resistance for Hg up to 150 ppm. The three bacterial isolates identified by morphological, biochemical and molecular assay were identified to belong to two genera: Staphylococcus and Micrococcus and were named as Staphyloccus haemolyticus strain MR1, Staphylococcus pasteuri strain MR2 and Micrococcus sp. strain MR3. All of the isolates were found to be resistant to multiple antibiotics and possessed acid producing and phosphate solubilisation ability. These mercury tolerant strains can serve as potential candidates for bioremediation and phytoremediation of ash ponds and other mercury contaminated aquatic or soil samples.*
Keywords: *Antibiotic resistance, Heavy metal, Mercury resistant bacteria (MRB), MIC (minimum inhibitory concentration), Phosphate solubilization.*

I. Introduction

Mercury (Hg), the most toxic metal among all heavy metals exists in several forms: elemental or metallic mercury, inorganic mercury compounds, and organic mercury compounds. Elemental or metallic mercury is a shiny, silver-white metal and is liquid at room temperature. Mercury, an element of the earth crust is found in many rocks including coal [1]. When coal is burned, mercury is released into the environment. Coal-burning thermal power plants are the largest anthropogenic source of mercury emissions to the air. Fly ash produced by coal combustion also retains mercury. As huge amount of fly ash generated during power generation is mostly stored in ash dykes or ash ponds, leaching of Hg from ash ponds and surface run offs can contaminate surrounding soil, surface water and groundwater exceeding maximum contaminant level (MCL) of 2 ppb (0.002 mg/L). Concern has been raised regarding the potential for release of this Hg to the environment from the fly ash dump sites [2]. Mercury binds to sulfhydryl groups of enzymes and proteins inactivating their functions. Mercury pollution becomes a serious threat when it settles into water bodies where it builds up in aquatic organisms through biomagnifications and will end up in our body when we will eat them. In human body, excessive mercury acts as a neurotoxin and mostly harms our brain and nervous system. Even in low doses, Hg can affect a child's development. In adults, Hg poisoning can adversely affect all organs and particularly hamper fertility, memory loss, tremors, blood pressure regulation and vision [3]. Microbes which are resistant to mercury convert inorganic mercury to methylmercury, which has higher toxicity level. Thus a small environmental concentration of mercury and subsequent presence of MRB (mercury resistant bacteria) at a site increases the chances of accumulation of mercury in higher levels of food chain through biomagnifications [4]. The aim of this study was to isolate and characterize Hg-resistant bacteria from fly ash sample of Mejia Thermal Power Plant of DVC located in Bankura of West Bengal and see whether these Hg resistant bacteria can be used as potential agents for bioremediation of mercury toxicity. Three mercury resistant bacteria (MRB) were isolated and identified by morphology, biochemical and molecular studies. Bacterial tolerance to Hg compounds was evaluated by determination of minimal inhibitory concentrations (MIC). All the strains appeared to be multiple antibiotics resistant and mega plasmids were found in two of the isolates suggesting the role of plasmids in their acquisition of Hg resistance and antibiotic resistance. Acid production ability in presence of metal was observed suggesting its role in metal mobility and uptake.

II. Materials And Method

2.1. Sample Collection

Sample was collected from ash pond of Mejia Thermal Power Station of DVC (MTPS-DVC) located in Durlovpur area of Bankura, West Bengal, India. The samples were collected in sterile glass containers and transported immediately to the laboratory and kept at 4°C for microbial analysis.

2.2. Isolation of Mercury Resistant Bacteria

Isolation of mercury resistant bacteria (MRB) was done by enrichment culture. 1 gram (g) of settled fly ash sample was inoculated in nutrient broth, (composition: peptone 5.0g, yeast extract 2.0g, beef extract 1.0g, NaCl 5.0g; final pH 7.0). To induce selective growth of only mercury resistant bacteria about 20ppm $HgCl_2$ salt was added to sterile medium prior to inoculation. Culture flasks were incubated at 30^0C in shaker. Growth was determined by turbidity. The 10^{-2}-10^{-6} dilutions were spread plated on NB agar plates amended with $HgCl_2$. Pure MRBs (mercury resistant bacteria) were isolated by repeated subculturing of the individual separated colonies from agar plates to culture flasks and again re-streaking them on agar plates with $HgCl_2$. Single colonies were obtained following this procedure and proceeded for taxonomical identification. The pure cultures of isolated strains were preserved in glycerol stocks (50%).

2.3. Morphological and Physiological Characterization of the Isolates

Gram staining was conducted for morphology study and identifying Gram nature of the isolates. After staining they were viewed under phase contrast microscopy. Various biochemical tests were also conducted for determination of motility, haemolysis activity on blood agar plate, presence of other enzyme activities like urease, phosphatase, oxidase, catalase, coagulase etc [5, 6].

2.4. Genomic DNA Isolation

Total DNA was isolated from the enrichment cultures by protocol of Sambrook et al., 1989 [7]. 150ul culture was centrifuged and pelleted cells were suspended in 500 µl TE buffer (Tris 25 mM, EDTA 10 mM). 50 µl 10% SDS was then mixed gently. After 15 min incubation at 65°C, 300 µl ice-cold 5M potassium acetate was added into the tubes. The genomic DNA was extracted and then precipitated with isopropanol (v/v), pelleted by centrifugation, ethanol washed, and then resuspended in 50 µl TE buffer.

2.5. Ribotyping and Phylogenetic Tree Construction

The final identification of the isolates were done by 16s rDNA analysis. As the strains were all gram positive we could not get any bands on colony PCR using cell lysate prepared by boiling cells in H_2O. So we used genomic DNA for doing 16s PCR. The final identification of the isolates were done by using 27f (5'-GAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGTTACCTTGTTACGACTT-3') universal primers, 1X PCR buffer, dNTPs and Taq Polymerase of Merck-Genei. Amplification was performed using 20 ng of genomic DNA in 50 ul of total reaction mixture. The reaction mixtures were incubated at 95^0C for 90 s and then cycled 35 times through the following temperature profile: 95^0C for 30 s, 50^0C (annealing temperature) for 30 s and 72^0C for 1.5 min. Finally the mixtures were incubated at 72^0C for 10 min. The Thermal Cylcer ABI2720 was used for this process. After successful amplification the 16s bands were cut from gel and gel extracted with Quiagen gel extraction kit and send for Sanger sequencing.

2.6. Analysis of Sequence Data

The sequences obtained were searched for homology by using the NCBI-Blast Program. Phylogenetic tree construction was performed using PhyML software package [8]. The tree represents the phylogenetic position of our strains with some related published strains of *Staphylococcus* and *Micrococcus*. The characterized sequences have been shown in bold.

2.7. Optimization of Bacterial Growth Condition and Drawing of Bacterial Growth Curve

To study the optimum temperature and pH for growth of the MRB isolates about 100µl of overnight grown cultures were dispensed into 10 ml NB containing test tubes and incubated at different temperature like 4^oC, 25^0C, 30^oC, 37^0C, 45^0C and at different pH from 4.0-9.0 for 24-48 hours. Optical density (OD) was measured at 600nm using uv-visible spectrophotometer (Systronics UV/VIS Spectrophotometer) and result were recorded. After optimization the strains were monitored for growth for 7 days in NB medium amended with Hg and without Hg to study the effect of Hg on microbial growth pattern.

2.8. Determination of Minimum Inhibitory Concentration (MIC)

The MIC values were determined by dispensing 10ml of NB broth into the test tubes and the tubes were amended with increasing concentrations of Hg from 50ppm to 150ppm. For each strain 7 test tubes with

different concentration of mercury (20ppm, 50ppm, 100ppm, 120ppm, 150ppm, 200ppm, 250ppm) were inoculated with 100µl of bacterial suspension. Optical density was recorded at 600nm to see growth pattern.

2.9. Antibiotic Resistance Assay

The antibiotic resistance ability of the isolated MRBs was studied by broth dilution method [9]. Stock solutions were prepared for Streptomycin, Ampicillin, Chloramphenicol, Penicillin, and Rifampicin. Earlier the potencies of all the antibiotics used in the study were confirmed using susceptible *E. coli* and *Bacillus* strains. Agar plates were prepared with the antibiotics and the isolates were streaked and monitored for growth.

3.0. Phosphate Solubilisation Assay

Pikovskaya's agar medium containing calcium phosphate as the inorganic phosphate was used in this assay. Streaking were done on the plates and kept for incubation at 28°C for 7 days.

3.1. Isolation of Plasmid

Plasmid DNA was isolated by miniprep metod of Sambrook et al [7]. The isolated plasmids were run by 0.7% agarose gel electrophoresis performed through horizontal slab gel of 0.8% agarose submerged in Tris-HCl, acetic acid, and EDTA (TAE) running buffer at 70 V for 2 h. DNA bands were then stained with ethidium bromide for 15 min and visualized on a UV transilluminator.

III. Result and Discussion

3.1. Isolation of the MRBs

Colonies appeared in $HgCl_2$ containing NB agar plates after 48 hours of incubation. The well isolated colonies were further checked for pure characters and based on the morphological and biochemical differences three types of isolates were finally selected for further characterization.

3.2 Morphological and Biochemical Features of the Isolates

Size, shape, colour and elevation of the bacterial colonies were observed for 24-48 hours incubated on agar plates containing 20ppm $HgCl_2$. Cells were observed under microscope after Gram staining and motility test were done to check presence of flagella. Figure 1 shows the phase contrast microscopic view of the isolates and morphology of the colonies. Table 1 shows the biochemical features of the isolates.

3.3. 16s Identification and Phylogenetic Tree

For 16s identification we extracted genomic DNA from the strains (Fig. 4A shows the genomic DNA bands). Figure 4C shows the PCR bands of 16s gene obtained with 27f and 1492r universal primers. The isolates were identified as *Staphylococcus haemolyticus*, *Staphylococcus pasteuri* and *Micrococcus* sp. by partial sequencing the 16S rRNA and comparing the sequences with sequences present in the 16s ribosomal database (RDB) of NCBI by blast searching. As the strains were identified by their mercury resistance we have named the three isolates as *Staphylococcus haemolyticus* strain MR1, *Staphylococcus pasteuri* MR2 *and Micrococcus* sp.MR3. Figure 2 shows the phylogenetic tree of cluster analysis prepared by Phylogeny.fr software. Multiple sequence alignment was done by MUSCLE program (3.8) and the Newick tree created by the software was uploaded in TreeviewX to create the final tree.

3.4. Determination of Optimal Growth Condition

Maximum cell density within a particular time was obtained with cultures incubated at 37^{o}C and pH 7-8. All the strains displayed wide growth range and we found growth at 25^{o}C and 45^{o}C and all the pH values. However OD was highest with 37^{o}C and pH 7-8. No growth was observed at 4^{o}C and initial pH of 4.0. After optimization of growth conditions effects of the metals on growth pattern were investigated. Figure 3 shows the growth curve of the three isolates at optimum pH and temperature in the presence and absence of mercury salts (100ppm).

3.5. Determination of Minimum Inhibitory Concentration (MIC) of Mercury

The viability of cells following exposure to high levels of mercury was studied. The three isolates were grown at different concentrations of $HgCl_2$ containing NB broth dispensed in test tubes at 37°C for 48 hr. All the three isolates showed growth upto 150ppm of Hg and with further increase in Hg concentration the growth were severely reduced. So 150ppm was selected as their MIC values.

3.6. Antibiotic Sensitivity

Staphylococcus haemolyticus strain MR1 was found to be a multiple antibiotic resistant strain. It grew in presence of almost all antibiotics we tested. The rest of the two isolates also showed resistance to multiple antibiotics as shown in table 2.

3.7 Phosphate Solubilisation Ability

Distinct hollow zones were observed around the bacterial colonies indicating the solubilisation of phosphate.

3.7. Plasmid Isolation

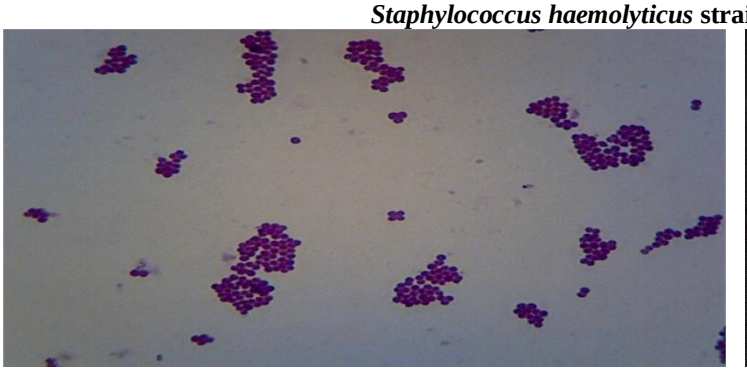
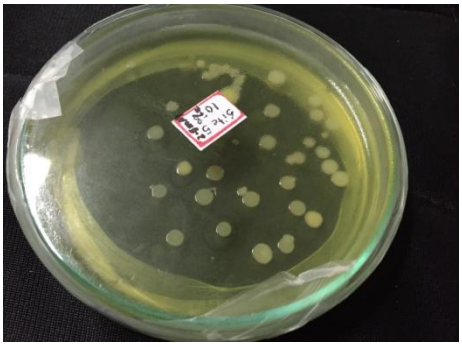
Plasmid DNA was isolated by miniprep method of Sambrook [7]. The two *Staphylococcal* isolates had megaplasmids but we could not recover megaplasmid from *Micococcus*. The isolated plasmids were run alongside the genomic DNA (Fig. 4B). As exact size determination was not possible without PFGE (Pulse Field Gel Electrophoresis) so we could not say the exact size. But as the genomic DNA and plasmid DNA were run side by side and plasmid band was present just below the genomic DNA, we understood that the plasmid was a megaplasmid.

In this study three isolates belonging to *Staphylococcus* and *Micrococcus* were identified based on 16s DNA and other morphological and biochemical analysis. All the isolated strains could resist upto 150ppm of $HgCl_2$ in NB agar media. The growth curve analysis (Fig. 3) showed that in presence of Hg lag phase state prevailed for a long time in comparison with the NB grown culture without metal stress. Growth started to begin after 24 hours and full turbidity was attained or maximum OD was reached after 48 hours. However *Micrococcus* attained maximum OD after 24 hours. After 72 hours OD started to fall (36 hours in case of *Micrococcus*) and after 96 hours (4 days) it showed minimum and after that no more readings were taken. The pH tolerance studies showed that optimum growth was found with medium pH value between 7 and 8. But we observed pH 4 in a 7 day old culture. With subsequent investigations we found the isolates were responsible for acid production and due to this the medium pH was decreasing. Figure 3D shows the rate of reduction of pH with increasing incubation time for all the three isolates. However when the same isolates were grown in NB without Hg no such acid production was observed. It is well known matter that metal solubilisation by acid production is a microbial strategy for detoxification [10]. Observing the acid production activities of the bacteria we tested them for phosphate solubilisation ability. All of the strains produced distinct hollow zones in phosphate containing agar medium. Temperature sensitivity test showed that although highest OD was obtained at 37^0C but all of them grew quite well in all experimental temperatures except 4^oC ranging from 25^oC to 42^oC. All the three isolates showed distinct morphology as shown in Fig. 1. Table 1 and table 2 show their biochemical features and antibiotic resistance profiles respectively. All strains possessed multiple antibiotic resistance ability. In many cases it has been found that strains that acquire heavy metal resistance also acquire antibiotic resistance and in most cases these are plasmid borne and spread among microbes through horizontal gene transfer (HGT). Continuous exposure to toxic mercury make few bacteria adapt various mechanisms for resistance and after possessing resistance they spread this property through HGT. MRBs possess a variety of resistance mechanisms to combat the toxic effects of mercury. One most important type among them is resistance based on clustered genes in an operon (mer operon) that allows bacteria to detoxify Hg^{2+} into volatile mercury by enzymatic reduction. Genes coding mercuric reductase enzyme is mostly located in mer operon and this mer operon in most cases have been found in plasmid [10] and sometimes in chromosomes [11] or sometimes as components of transposons [12]. Two of our *Staphylococcal* strains possess mega plasmids giving indication that mega plasmid may play role in mercury resistance and antibiotic resistance. In later investigation we would try to explore the molecular mechanisms behind mercury resistance.

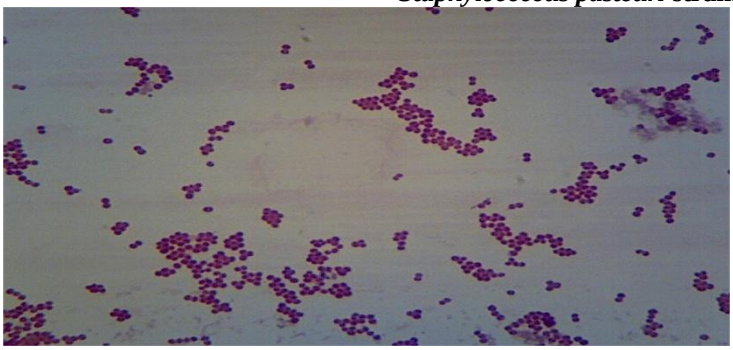
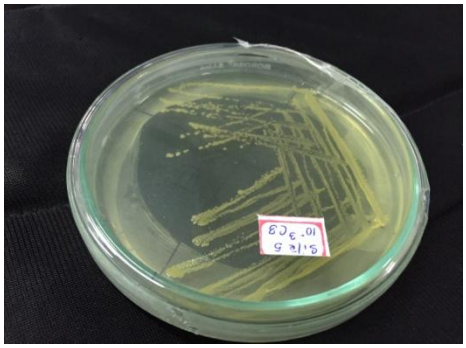
III. Tables and Figures

Phase contrast microscopic view after gram staining (A) **Colony morphology (B)**

***Staphylococcus haemolyticus* strain MR1**

***Staphylococcus pasteuri* strain MR2**

***Micrococcus* sp. strain MR3**

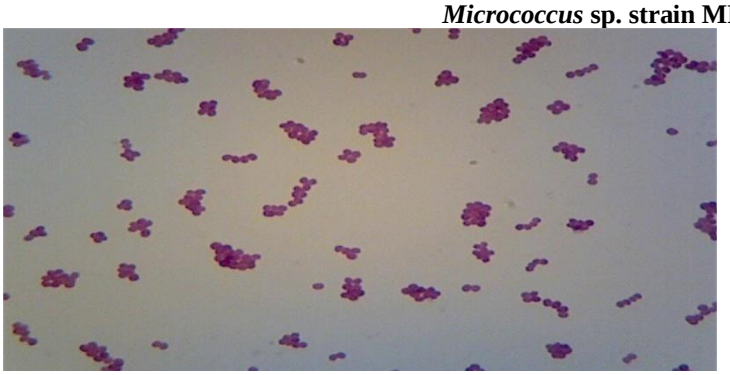
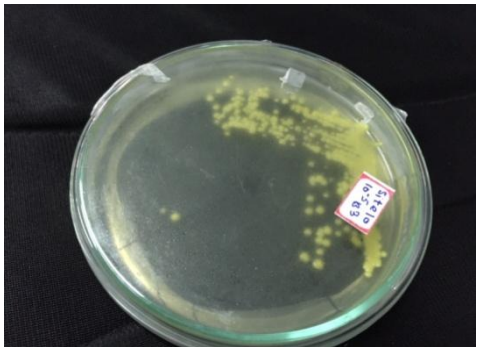



Fig. 1: Phase contrast microscopic view (A) and colony morphology (B) of the isolates.

Table 1: Biochemical features of the isolates.

Biochemical features	***S.haemolyticus* strain MR1 (Phylum Firmicutes)**	***S.pasteuri* strain MR2 (Phylum Firmicutes)**	***Micrococcus* sp. strain MR2 (Phylum Actinobacterium)**
Gram reaction	Gram positive	Gram positive	Gram positive
Motility	Non motile	Non motile	Non motile
Sporulation	Non sporulating	Non spore forming	Nd
Cell shape	Coccus (0.8-1.3uM)	Cocci (0.5-1.5uM)	Cocci (0.5-3.5uM) Normally in tetrads
Colony Morphology	Off white glossy circular colonies with flat elevation	Golden yellow circular with convex elevation	Bright yellow circular with convex elevation
Coagulase	Negative	Negative	Negative
Catalase	Positive	Positive	Positive
Oxidase	Negative	Negative	Positive
Urease	Positive	Positive	Positive
Phosphatase	Positive	Positive	Nd
Haemolysis in blood agar	Positive	Positive	Positive
Indole acetic acid production	Positive	Positive	Positive

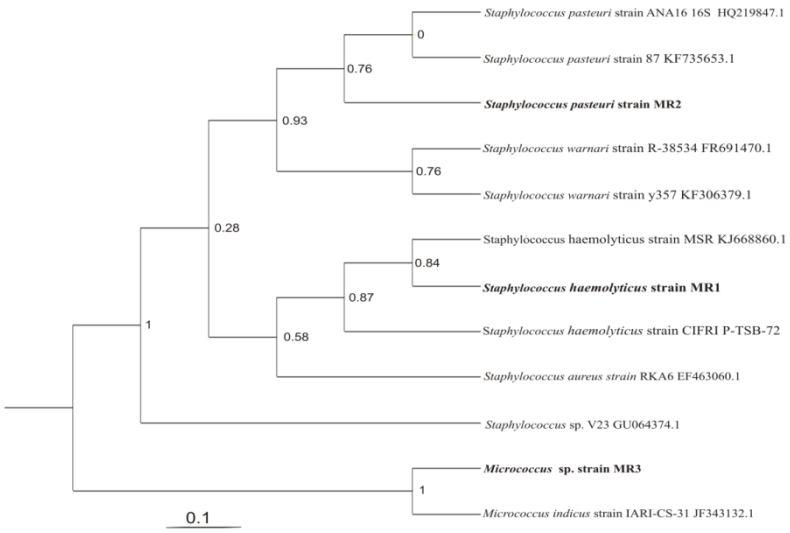


Fig. 2: Phylogenetic tree of the isolates with related representative strains

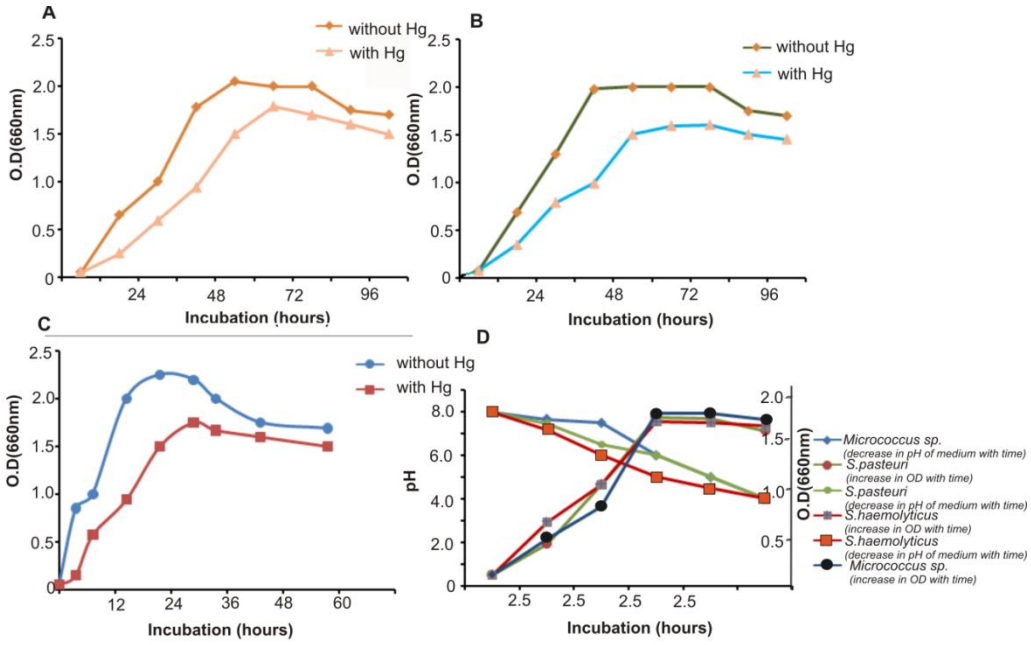


Fig. 3: Growth curve of the isolates in presence and absence of mercury. (A) Growth curve of *S.haemolyticus* with and without Hg (B) Growth curve of *S.pasteuri* with and without Hg (C) Growth curve of *Micrococcus* sp with and without Hg (D) Decrease of pH level with increase of optical density in presence of Hg for all the three isolates.

Antibiotic used	***S.haemolyticus* strain MR1 (Phylum Firmicutes)**	***S.pasteuri* strain MR2 (Phylum Firmicutes)**	***Micrococcus* sp. strain MR2 (Phylum Actinobacterium)**
Penicillin	R	R	S
Streptomycin	R	R	R
Tetracyclin	R	R	R
Kenamycin	R	R	R
Chloramphenicol	R	S	S
Rifampicin	R	S	R
Ampicillin	R	S	S
Vancomycin	R	S	S

Table 2: Antibiotic resistance profile of the isolates. R indicates resistivity and S indicates sensitivity.

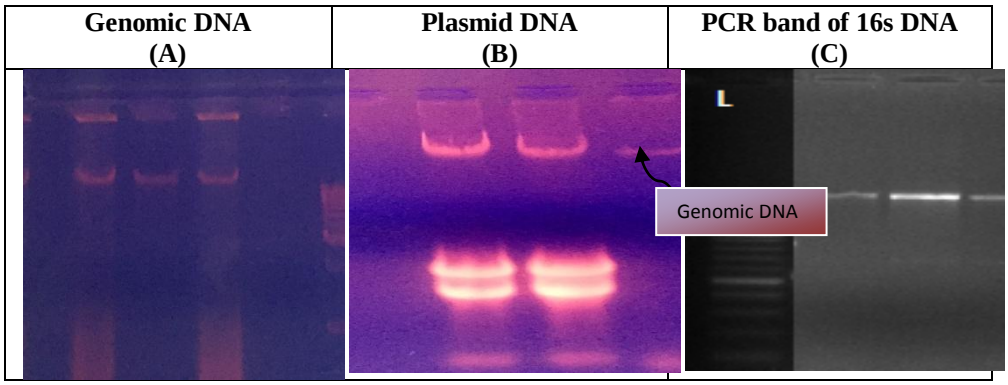


Fig. 4: Agarose gel electrophoresis image of the DNA bands of genomic DNA (A), plasmid DNA (B) and 16s PCR band (C)

IV. Conclusion

The study successfully isolated three mercury resistant bacterial strains (MRBs). Based on 16srDNA analysis they were identified to belong to two genera: *Staphylococcus* and *Micrococcus*. These isolates have high potential to remove Hg from Hg contaminated environment and can tolerate Hg contamination up to 150 ppm which can be further increased with adaptation by continuous subculturing in Hg containing media. As two of them possess megaplasmids and they are resistant to multiple antibiotics it can be suggested that like most other MRBs these two isolates also possess MRB operons in plasmids. Acid producing ability of the isolates may have a role in metal solubilisation. Future investigations can be done at genetic level to understand the Hg resistance mechanism and find out the correlation of their antibiotic resistance properties. Due to the possession of phosphate solubilising ability they can also be used as plant growth promoting bacteria. Thus this study offered us three potential candidate strains for bioremediation and phytoremediation of mercury contaminated environments.

Acknowledgements

We are thankful to DVC-MTPS authority as without their cooperation work on this would not be possible. We are particularly grateful to Kalyani Pyne, Junior engineer of DVC-MTPs for her constant help and support.

References

[1]. AB Mukherjee and R Zevenhoven R, Mercury in coal ash and its fate in the Indian subcontinent: A synoptic review, Sci Total Environ; 368(1): 2006, 384-92.
[2]. Z Wei, G Wu, RSu, C Li and P Liang, Mobility and contamination assessment of mercury in coal fly ash, atmospheric deposition, and soil collected from Tianjin, China, Environ Toxicol Chem.30(9): 2011,1997-2003.
[3]. TW Clarkson, L Magos, The toxicology of mercury and its chemical compounds, Crit Rev Toxicol **36** (8): 2006, 609– 62.
[4]. JJ Calomiris, JL Armstrong, RJ Seidler, Association of metal tolerance with multiple antibiotic resistances of bacteria isolated from drinking water. Appl Environ Microbiol. 47(6):1984, 1238-42.
[5]. LM Prescott and JP Harley, 2002. Laboratory Exercises in Microbiology. 1st Edn., McGraw Hill Publ., New York, USA.
[6]. Bergey, D. H. (David Hendricks), Breed, Robert S. (Robert Stanley), Bergey's manual of determinative bacteriology American Society for Microbiology; (1957)
[7]. J Sambrook , EF Fritsch, and T Maniatis (1989).Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
[8]. S. Guindon and O. Gascuel, A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood, Systematic Biology, 52: 2003, 696–704
[9]. AW Bauer, WM Kirby, JC Sherris and M Turck, Antibiotic susceptibility testing by a standardized single disk method, Am J Clin Pathol.; 45(4): 1966, 493–496
[10]. C White, JA Sayer and G.M Gadd, Microbial solubilization and immobilization of toxic metals: key biogeochemical processes for treatment of contamination, FEMS Microbiology Reviews, 20: 1997, 503–516.
[11]. P Radstrom,O Skold, G Swedberg, J Flensburg, PH Royand L Sundstrom, TransposonTn5090 of the plasmid R751,which carries integron, is related to Tn7, Mu, and the retroelements. J. Bacteriol. 176:1994, 3257-3268.
[12]. C Inoue, K Sugawara and T Kusano, The merR regulatory gene in Thiobacillus ferrooxidans is spaced apartfrom the mer structural genes. Mol. Microbiol. 5: 1991, 2707-2718.
[13]. GY Kholodii, OV Yurieva, OL Lomovskaya, ZM Gorlenko, SZ Mindlin and VG Nikiforov, Tn5053, a mercury resistance transposon with integron ends. J. Mol. Biol. 230: 1993, 1103-1107.