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# Isolation and Identification of Mercury Tolerant Bacteria from Mercury Contaminated Areas

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***Abstract:*** *Bacteria posses distinctive characteristics, hence they were exploited by different scientists for various applications. In this piece of work, bacteria were isolated from mercury contaminated soils, and were further screened for superior mercury resistant bacteria for using them in bioremediation. Initially, mercury tolerant bacterial strains were isolated from mercury contaminated soils, and labelled them as HG 1, HG 2, HG 3 and HG 4. In order to derive maximum mercury tolerance, all the four isolates were inoculated on nutrient broth, supplemented with a vast range of concentrations of mercuric chloride (100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm and 600 ppm). All the isolates were found adaptable to the concentration of mercury up to 300 ppm. Among them, only one isolate (HG 2), due to its potential mercury resistance and high Minimum Inhibitory Concentration (MIC) values, was found to be the relatively most tolerant and was selected for molecular characterization. Further phylogenetic analysis using the MEGA 6.0 software revealed that the selected isolate HG 2 belongs to the genus Brevundimonas. These mercury tolerant bacterial strains would be of immense use in the bioremediation of metal contaminated soils, in future research and biotechnological development.*

***Key Words:*** *Bioremediation, mercury, phylogenetic analysis, contaminated soils.*

## 1. INTRODUCTION:

As it is a known fact that, industrial growth is directly proportional to the rise in heavy metal contamination in agricultural soils. Among all the heavy metals, mercury is identified as the most toxic metal. It is ubiquitous and can be transported through the atmosphere as it is very volatile in nature (1). Mercury can easily enter the water bodies through the process of precipitation. Subsequently it enters the food chain of aquatic animals, and ultimately reaches the human beings, which leads to bioaccumulation. Biomagnification is evident from the fact that the high concentration of mercury is found in carnivorous fish (2, 3 & 4). Mercury is the most toxic to all organisms including plants and animals due to the fact that they exhibit a strong affinity for thiol groups of proteins which are present in organisms (5 & 6). Mercury contamination adversely effects the growth and development of plants. It causes inhibition in germination (7), results in decrease of biomass production (8), and hinders protein function (9 & 10*)* shows negative impact on DNA (11) and inhibits photosynthesis in many plants (12). Considering the above ill effects caused by the mercury, we should prioritize the need of the sustainable techniques to mitigate its hazardous effects. Many methods are being used but the bioremediation is quite efficient, environment friendly and cost effective in decontaminating the environment (13, 14 &15). Several studies have shown that extensive variability of micro organisms (bacteria, fungi, yeast and algae) has distinct and natural capacities to absorb toxic heavy metals (16, 17 & 18). Bacterial bioremediation provides us certain advantages such as it is easy to culture; they multiply faster and can accumulate metals efficiently in various conditions (19 & 20).

Bacteria evolved to exist in metal contaminated areas (21). Various studies affirm that bacteria acquire resistance towards heavy metals (22 & 23), to survive in extreme environmental stress condition (24). In this connection, bacterial application in bioremediation of heavy metals needs to be studied and high mercury resistant bacteria needs to be isolated.

The aim of this study is to identify the efficient mercury resistant bacteria, isolated from high mercury contaminated soils by 16S rRNA gene sequencing analysis. Construction of phylogenetic tree for taxonomic classification of bacteria using 16S rRNA gene was initially performed by various researches (25).

## 2. Methods and materials:

**Sample Collection:** Mercury resistant bacteria isolated from soil collected near pharmaceutical industries in Visakhapatnam.

**Bacterial isolation:** Mineral salts medium (MSM) supplemented with 100 ppm mercuric chloride, was used for isolation and characterization of mercury tolerant bacteria. The MSM has the following composition in (g/L): $KH_2PO_4$, 4.8; $K_2HPO_4$, 1.2; $NH_4NO_3$, 1.0; $MgSO_4.7H_2O$, 0.2; Ca $(NO_3)_2.4H_2O$, 0.04; and Fe $(SO_4)_3$, 0.001 with pH 7.0. Nutrient Agar medium was used for pure culture maintenance. The composition of the media are as follows (g/L); sodium chloride 5.0; peptone 1.5; yeast extract 1.5; agar 15.0; (pH-7.4).

**Culture enrichment and isolation of mercury tolerant bacteria from soil samples:**

Five samples were serially diluted and third dilution was inoculated into MS media which contains 100 ppm of $HgCl_2$ and 0.25% of glucose as carbon source and incubated in orbital shaker for three days at $37^0$C and 240 rpm. Then 100 µl of samples from the above treatments were transferred into fresh tube containing MS media with 250 ppm $HgCl_2$ and 0.5% of glucose and incubated in orbital shaker for three days at $37^0$C and 240 rpm. Then from the above treatments 100 µl of sample was transferred into test tube containing MS media with 500ppm of $HgCl_2$ and 0.75% of glucose and incubated in orbital shaker for three days at $37^0$C and 240 rpm. Finally 100 µl of sample was inoculated into test tube containing MS media with 1000 ppm of $HgCl_2$ and incubated in orbital shaker for three days at $37^0$C and 240 rpm. After incubation, 100 µl from each treatment were spread separately onto mineral agar plates supplemented with $HgCl_2$ were incubated at $37^0$C for 24 h.

**Identification of the colonies:** Based on variation in physical appearance and morphological characters, few of the bacterial colonies were selected and were inoculated onto nutrient agar plates. The bacteria were allowed for biochemical and molecular characterization

**Biochemical characterization of isolated bacteria:** The individual bacterial colonies that grew on the medium were sub cultured onto mineral agar containing chlorpyrifos of the same concentration until pure cultures were obtained. Bacterial isolates were subjected to morphological and biochemical studies.

**Selection of metal tolerant bacteria:** In order to isolate potential mercury tolerant bacteria, the selected bacterial isolates (HG 1, HG 2, HG 3 and HG 4) were grown in nutrient broth supplemented with different concentrations of Hg such as 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm and 600 ppm. Control bacterial cultures maintained without metal ion. 5% inoculums were added in every tube. The inoculated tubes were incubated at $37^0$C for 48 hours in orbital shaker incubator at 180 rpm. Culture medium was taken as a blank for every reading. The results were presented in Figure 3. Growth kinetics was observed after 48 hours of incubation using a spectrophotometer at 600 nm OD for the conformation of the bacterial density.

**Molecular characterization of specific isolate:** The strains used in this study were grown in shaking flasks containing Nutrient broth at 37°C.

**Isolation of chromosomal DNA:** Chromosomal DNAs were isolated by a versatile quick-prep method for genomic DNA of bacteria (26) with some modifications. Mycelia (1–2 ml) grown in a GYM broth cultures were centrifuged, rinsed with TE and resuspended in 0.4 ml SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). Lysozyme was added to a concentration of 1 mg/ml and incubated at 37°C for 30 min–1 h. Then 0.1 vols of 10% SDS and 0.5 mg Proteinase K $ml^{-1}$ were added and incubated at 55°C with occasional inversion for 2 h. One-third volume 5 M NaCl and 1 vol. chloroform were added and incubated at room temperature for 30 min with frequent inversion. The mixture was centrifuged at 4500 rpm for 15 min and the aqueous phase was transferred to a new tube using a blunt-ended pipette tip. Chromosomal DNA was precipitated by the addition of 1 vol. 2-propanol with gentle inversion. The DNA was transferred to a new tube, rinsed with 70% ethanol, dried under vacuum and dissolved in a suitable volume (about 100 ml) of distilled water. The dissolved DNA was treated with 20 mg RNase-A $ml^{-1}$ at 37°C for 1 h. Samples were extracted in the same volume of phenol/chloroform/isoamyl alcohol (25: 24 : 1) and precipitated with 2.5 vols cold ethanol and 0.1 vols 3 M sodium acetate. The pellets were washed with 70% ethanol, dried and dissolved in TE or distilled water.

**PCR Amplification and sequence analysis of 16S rRNA gene:** Potential mercury tolerant bacterial isolate (HG 2) selected for molecular study, to identify the species. Bacterial genomic DNA was extracted from the selected isolate (HG 2). With the above isolated genomic DNA, the PCR amplification of the 16S rRNA gene was performed using 16S rRNA specific two universal primers, 27F (5'AGAGTTTGATCMTGGCTCAG3') and 492R (5'TACGGYTACCTTGTTACGACTT3'). PCR reaction mixture of 25 µl total volume, containing 1/10 volume 10× *Taq* buffer, 2 mm $MgCl_2$, 1 unit *Taq* DNA polymerase, 0.2 mM dNTP, 20 pmol forward primer, 20 pmol reverse primer and 100 ng DNA. DNA amplification was carried out in a Biorad Mini thermocycler with the following PCR conditions: an initial denaturing step at 94°C for 5 min; 40 cycles for 1 min at 94°C (denature), 1 min at 48°C (annealing), 2 min at 72°C (extension) and a final elongation step at 72°C for 5 min. PCR products were separated by electrophoresis on 1.5% agarose gel containing 0.5 µg/ml ethidium bromide, and photographed. The standard DNA samples (100 bp DNA ladder marker) were used as molecular size marker. The purified PCR products were subjected to Sanger's dideoxy sequencing, in both forward and reverse directions, using Big Dye terminator v 3.1 cycle sequencing kit on ABI Prism 3700 DNA Analyzer (Applied Biosystems Inc., USA) as per manufacturer's instructions. Sequencing of the cloned 16S r RNA gene of strain HG 2 resulted in a virtually complete 1417 bp long sequence. The

resulting 16S r DNA gene sequence (1417 bp) was used to search in the Gen Bank/ EMBL/DDBJ database with the BLAST program to determine the relative phylogenetic positions. Phylogenetic analysis was conducted using MEGA 6.0 (27) by first generating a complete alignment of 16S r DNA gene sequences of the isolates and type strains of all valid species. A phylogenetic tree was inferred using neighbour-joining tree algorithms (28). 16S r DNA datasets were cooperatively analyzed using MEGA 6.0 (27) which was used to calculate evolutionary distances and similarity values. Topography of the constructed tree was evaluated by bootstrap analysis with 1000 replicates (29).Only nodes with bootstrap values over 50% were considered to be significant.

**Phylogenetic analysis:** Multiple sequences were aligned using CLUSTAL W version 1.8 software packages (30). The resultant sequence alignment can then be used for the neighbour-joining (29), maximum-parsimony (31) and maximum-likelihood (32) methods using the MEGA 6.0 (27) package; all were implemented within the server. The alignment can be exported for use by external programs including MEGA 6.0 (27) and BioEdit (version 7.2.0) (33). An evolutionary distance matrix was generated for the neighbour-joining as described by (28).The resultant tree topology was evaluated by a bootstrap analysis (34), with 1000 re samplings from the neighbour-joining dataset using Seqboot and Consense from the Phylip package (35)

## 3. RESULT DISCUSSION:

**Isolation and identification of mercury tolerant bacteria:**

The bacterial isolates were characterized on the basis of morphological and biochemical assays.

**Morphological characterization of the bacterial isolates:** Colony morphology of all the isolates was examined in this study. Characteristics including colour, shape, elevation and colony surface were studied (Table 1). The colonies (HG 1 and HG 2), which were selected visually based on differences with naked eye, were identified as white in colour, while the remaining two were observed to be yellow coloured. It was observed that the shapes of the three colonies of bacterial isolates (HG 1, HG 2 and HG 3) were mostly circular, whereas HG 4 is in irregular form. The surface characteristics of bacterial isolates HG 1 and HG 2 were found to be smooth, while surface was found to be rough in the case of HG 3 and HG 4. In the case of the HG 3 and HG 4 flat margins were observed, however in HG 1 and HG 2, there was a visible elevated growth of the colony.

**Biochemical characterization of bacterial isolates:**

Biochemical characteristics of the bacteria isolated from the soil samples were shown in Table 2.

**Grams staining:** Following the protocols of standard Gram staining, it was found that, all the four selected isolates belong to Gram negative bacteria. Based on the previous reports it was known that Gram negative bacteria are more resistant to heavy meals than Gram positive bacteria (36).

**Starch hydrolysis:** Among the four isolates, HG 3 showed positive result for starch hydrolysis (capacity to utilize starch) and remaining (HG 1, HG 2 and HG 4) showed negative test results.

**Citrate test:** HG 1 and HG 3 exhibited positive test results for citrate utilization, where as other isolates HG 2 and HG 4 found to be negative.

**Nitrate test:** All the three bacterial isolates (HG 1, HG 2 and HG 4) except HG 3 have the ability to reduce nitrate to nitrite, and provided positive results for nitrate reductase test but HG 3 has no ability to reduce nitrate and hence it was negative.

**Methyl red test:** No isolate showed positive to methyl red test.

**Voges proskauer test:** Volkes Prosker test is positive for bacteria which form acetoin. This
Test was positive for HG 1 and HG 4. The other HG 2 and HG 3 are not able to synthesize acetoin hence they are negative for the test.

**Catalase test:** Isolates HG 1 and HG 2 were found to be catalase positive as they showed bubble formation on addition of $H_2O_2$. The bacterial isolates HG 3 and HG 4 were found to be catalase negative as no bubbles were formed.

**Salt tolerance:** Bacterial isolates HG 1, HG 3 and HG 4 were identified as salt sensitive isolates, where as HG 2 noticed as salt tolerant strain.

**Gelatin hydrolysis:** Only HG 2 was observed as negative to gelatin hydrolysis test, where as remaining three isolates (HG 1, HG 3 and HG 4) were positive for the test.

**Growth kinetics of bacterial isolates:**

The present study was designed to evaluate the adaptation capabilities of soil bacteria under high mercury concentrations. HG 1, HG 2, HG 3, and HG 4, were the bacterial isolates used for the experiment. The growth of these bacterial isolates, were screened on nutrient broth supplemented with various concentrations ranging from 100 ppm to 600 ppm of $HgCl_2$. All the bacterial strains were capable of withstanding up to 300 ppm of $HgCl_2$ concentration. Mercury has no significant effect on growth of bacteria at low concentrations, but higher concentrations of mercury negatively influence the growth of bacterial colony (37). The growth of bacterial strains steadily decreased with increase

in mercury concentration. The Minimum Inhibitory Concentration all the isolates in nutrient broth was 600 pp of $HgCl_2$. From the figure 1, it was inferred that bacterial growth was inversely proportional to concentration of mercury. The first notable difference observed at the lowest concentration of mercury (100 ppm), at which HG 4 and HG 2 exhibited the highest growth, but at the increasing concentration (400 ppm), growth of HG 4 decelerated and HG 2 showed the highest tolerance even at 600 ppm of $HgCl_2$. When HG 1 was moderately tolerant, HG 3 was more susceptible and ceased to grow at 400 ppm of mercury chloride. The analysis of growth kinetics of selected bacterial isolates cultured in different mercury concentrations were presented in figure 1, demonstrate that one bacterial isolate (HG 2) possess more efficient mechanisms of resistance in comparison to the other isolates. It showed growth up to 600 ppm mercury chloride concentration in growth medium. From these results we can deduce that isolate HG 2 was the most tolerant bacterial isolate. It was observed that strain HG 2 could resist up to 600 ppm of $HgCl_2$ concentration, which revealed the strong mercury resistance ability. Therefore molecular study was performed on this strain.

**Identification of Mercury tolerant bacterial strain by 16S rRNA analysis:**

The strain HG 2 was selected for further phylogenetic analysis of 16 S r RNA gene sequence. The DNA of mercury tolerant bacterial strain HG 2 was amplified with primers 1492R and 27F. The 16S rDNA gene sequence of bacterial isolate HG 2 was PCR amplified and products were detected by 0.8% agarose gel electrophoresis with ultraviolet (UV) light. The length of object fragment is about ~1417 bp (Figure 2). Sequence analysis of the 16S r RNA gene has been considered a fast and accurate method to identify the phylogenic position of bacteria. The 16S r RNA gene sequence (Figure 3) was deposited in NCBI Genbank with an accession number MT072131.Comparative analysis of the sequences with already available database showed that the strains were closed to the members of genus *Brevundimonas* and it was classified in the branch of *Brevundimonas sp*. A phylogenetic tree was constructed using MEGA 6.0 software which showed 97.47% homology with the genus *Brevundimonas.* Hence the bacterium which shows highest tolerance to mercury was identified as *Brevundimonas sp.* The occurrence of *Brevundimonas sp* in mercury contaminated areas was confirmed by the earlier reports (38) and reports of several authors (39 & 40) suggested that Brevundimonas accumulates high amounts of mercury by removing it from the surrounding medium. The mercury resistant bacteria have various remediation mechanisms of heavy metals which help to convert them to less toxic forms may provide the way to check the entry into the food chain. In this way it might lessen the hazardous metals to affect the health of human beings and other organisms. It is obvious from the earlier reports that bacteria that tolerate high concentrations of mercury could serve to effectively remediate mercury pollution (41 & 42).

## 4. RESULTS:

Table 1: Morphological characters of the HgCl2 tolerant bacterial isolates.

| Morphological characters | Bacterial isolates | | | |
|---|---|---|---|---|
| | Hg1 | Hg2 | Hg3 | Hg4 |
| Colony Colour | White | White | Yellow | Yellow |
| Colony shape | Circular | Circular | Circular | Irregular |
| Elevation | Raised | Raised | Flat | Flat |
| Colony Surface | Smooth | Smooth | Rough | Rough |

Total 4 bacteria were isolated from the above samples studied. The biochemical characters were mentioned below

| Isolate | Hg 1 | Hg 2 | Hg 3 | Hg 4 |
|---|---|---|---|---|
| Grams staining | Negative rods | Negative rods | Negative cocci | Negative rods |
| Starch hydrolysis | - | - | + | - |
| Citrate test | + | - | + | - |
| Nitrate test | + | + | - | + |
| Methyl red test | - | - | - | - |
| Voges proskauer test | + | - | - | + |
| Catalase test | + | + | - | - |
| Salt tolerance | - | + | - | - |
| Gelatin | + | - | + | + |

Table 2: Biochemical characters of the HgCl2 tolerant bacterial isolates.

+: positive test; - : Negative test

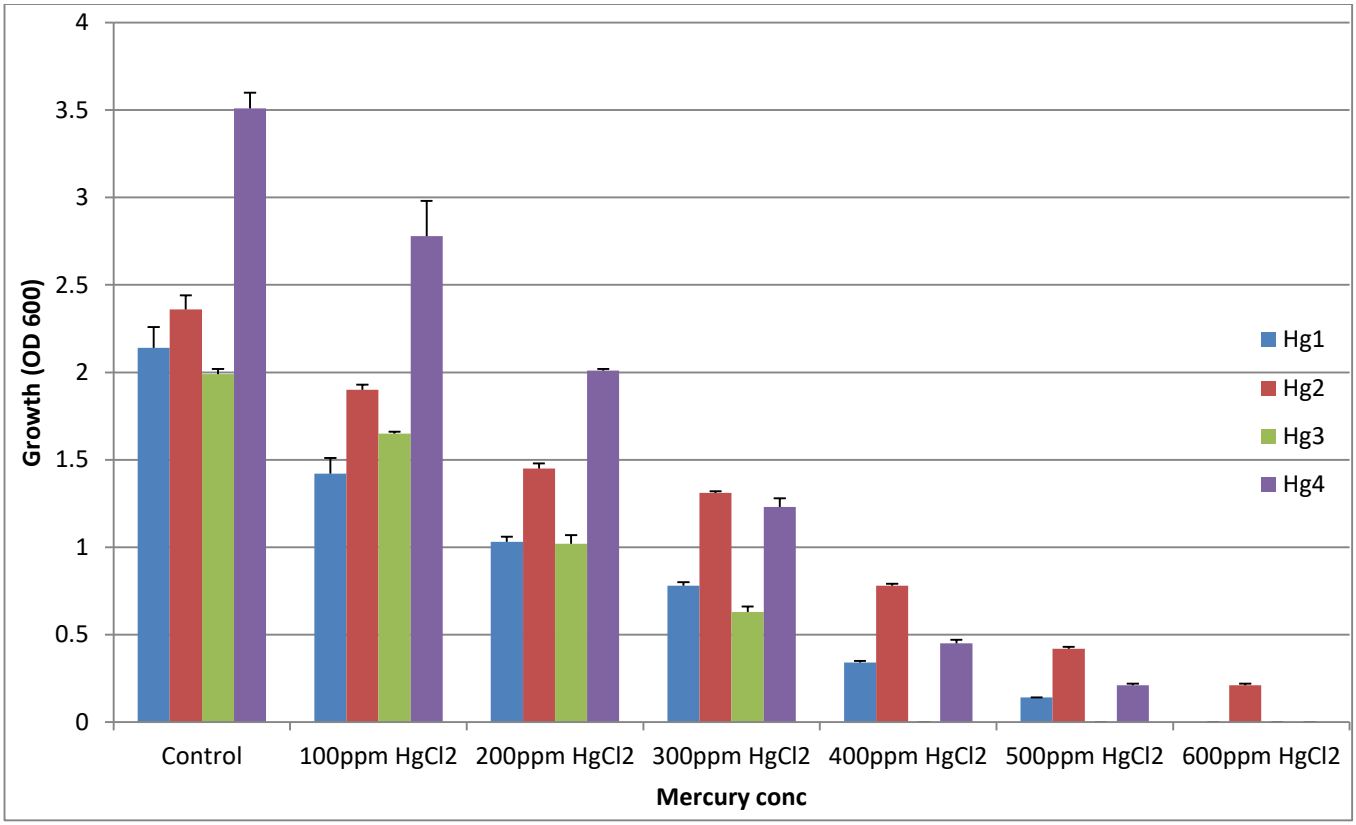
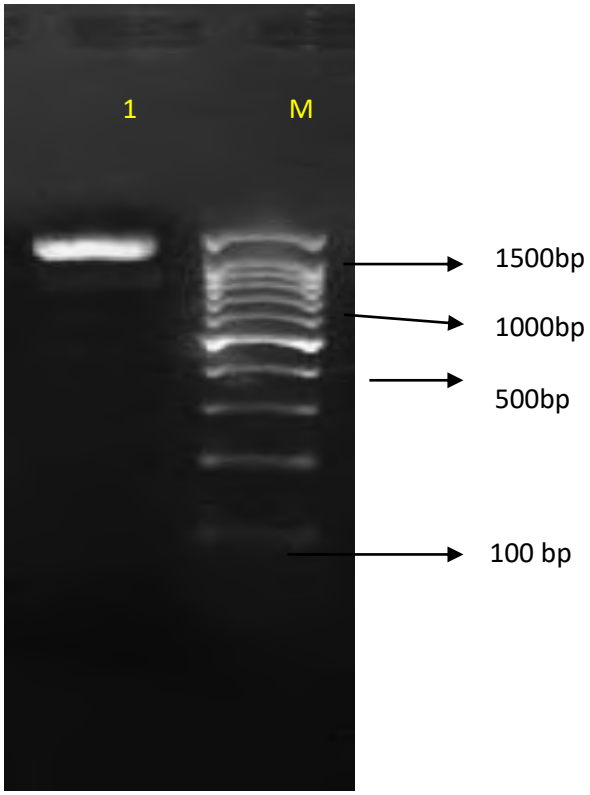



Figure 1: Growth characterization of four bacterial isolates at different mercury concentrations.

Molecular Characterization:




1: lane 1 M: Lane M

Figure 2: Agarose gel electrophoresis for 16s rRNA gene amplified product.
Lane M: 100 bp Ladder marker
Lane 1: DNA of bacterial isolate HG 2

Based on the molecular characterization the highest $HgCl_2$ tolerant isolate (HG 2) belongs to *Brevundimonas spp.*
16 S ribosomal RNA sequence of Hg 2 isolate:

AGCGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGACCCTTCGGGGTTAGTGGCGGACGGG
TGAGTAACACGTGGGAACGTGCCCTTTGGTTCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGAATGT
GCCCTTCGGGGGAAAGATTTATCGCCATTGGAGCGGCCCGCGTCTGATTAGCTAGTTGGTAGTGTAACG
GACTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGC
CCAAACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTGACGCAGCCATGCC
GCGTGAATGATGAAGGTCTTAGGATTGTAAAATTCTTTCACCGGGGACGATAATGACGGTACCCGGAGA
AGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATTA
CTGGGCGTAAAGGGAGCGTAGGCGGACATTTAAGTCAGGGGTGAAATCCCGGGGCTCAACCTCGGAAT
TGCCTTTGATACTGGGTGTCTTGAGTATGAGAGAGGTATGTGGAACTCCGAGTGTAGAGGTGAAATTCG
TAGATATTCGGAAGAACACCAGTGGCGAAGGCGACATACTGGCTCATTACTGACGCTGAGGCTCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGATTGCTAGTTGTTGGG
AAGTTTACTTCTCGGTGACGCAGCTAACGCATTAAGCAATCCGCCTGGGGAGTACGGTCGCAAGATTAA
AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCA
GAACCTTACCACCTTTTGACATGCCCGGACCGCCACAGAGATGTGGCTTTCCCTTCGGGGACTGGGACA
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTCGCCATTAGTTGCCATCATTTAGTTGGGAACTCTAATGGGACTGCCGGTGCTAAGCCGGAGGAAGGT
GGGGATGACGTCAAGTCCTCATGGCCCTTACAGGGTGGGCTACACACGTGCTACAATGGCGACTACAG
AGGGTTAATCCTTAAAAGTCGTCTCAGTTCGGATTGTCCTCTGCAACTCGAGGGCATGAAGTTGGAATC
GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACAC
CATGGGAGTTGGTTCTACCCGAAGGCGGTGCGCTAACCAGCAATGGAGGCAGCCGACCACGGTAGGGT
CAGCGACTGGGGTGAAGTCGTAACAAGGTGCCGTAGGGGAACCTGC

Figure 3 Complete sequence of HG 2 isolate

**Phylogenetic tree:**

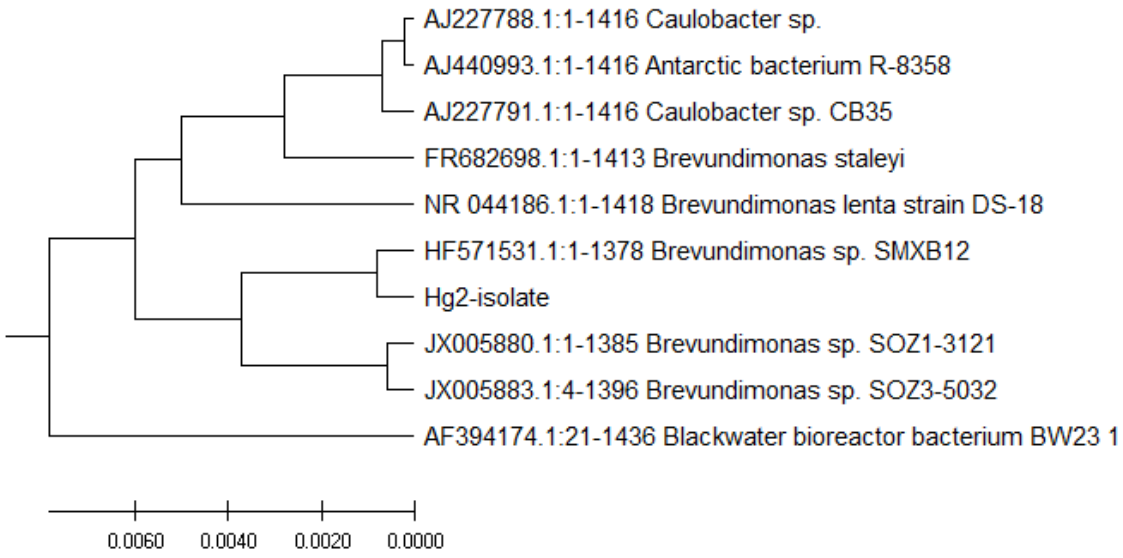



Figure 4: Phylogenetic tree based on 16S r DNA gene sequence showing the relationship with the most closely related bacterial strains.

## 5. CONCLUSION:

Despite the alarming present scenario of degrading ecological systems, there is a ray of hope from microbes as an emerging efficient technique in remediation. The selected bacterial strain (*Brevundimonas*) was capable of withstanding high concentration of mercury. Hence it is necessary to study its possible *in situ* application in reclaiming environments contaminated with heavy metals. It is crucial to study the mechanisms and the operating pathways

enabling bacteria to survive in metal rich areas and genes that are controlling them for better utilization in cleaning the hazardous metals present in the environments.

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