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Multiple Heavy Metal and Antibiotic Resistance of *Acinetobacter baumannii* Strain HAF – 13 Isolated from Industrial Effluents

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Abstract The emergence of multiple metal/antibiotic resistance among bacterial populations poses a potential threat to human health. Heavy metal and antibiotic resistance have been shown to have a strong correlation in nature, and their inter-relation is an important subject of study. The present study had an objective to isolate and identify multiple metal/antibiotic resistant bacteria from industrial wastewater of a Plastic Factory at Hafar Al Baten governorate, Saudi Arabia. Initially a total of 14 bacterial cultures (coded HAF – 1 to HAF – 14) were isolated on nutrient agar plates supplemented with different concentrations; 10, 15, 25, 25 and 30 mg/l of the five heavy metals; Hg^{2+}, Pb^{2+}, Cd^{2+}, As^{5+} and Cr^{6+} respectively. Out of 14 isolates, 5 (35.71 %) isolates (HAF – 2, HAF – 6, HAF – 7, HAF – 9 & HAF – 13) were selected as a multiple heavy metal resistant (MHMR) organisms with maximum tolerable concentrations (MTCs); 75 – 100 mg/l for Hg^{2+}, 125 – 175 mg/l for Pb^{2+}, 200 mg/l for Cd^{2+} and 200 – 250 mg/l for Cr^{6+} and As^{5+}. Antibiotic resistance pattern of the selected MHMR isolates was determined by Kirby-Bauer disc diffusion method against 15 different antibiotics belonging to 10 classes. Out of 5 isolates, 4 (80 %) isolates were multiple antibiotic resistance (MAR) with varying degrees. Among them isolate, HAF – 13 showed a wide range of resistance to all tested antibiotics; Amikacin, Augmentin, Ceftazidime, Chloramphenicol, Ciprofloxacin, Clindamycin, Cotrimoxazole, Erythromycin, Gentamicin, Levofloxacin, Oxacillin, Tetracycline, Vancomycin and Penicillin Gexcept Imipenem. Strain HAF – 13 was selected for its multiple metal/antibiotic resistance and identified by morphological, physiological and biochemical characteristics in addition to the phylogenetic analysis of the nucleotide sequence of 16S rRNA gene, which indicated that this strain is belonged to the genus *Acinetobacter* with high similarity 98% to *Acinetobacter baumannii* (accession number KU310899.1) and designated *Acinetobacter baumannii* strain HAF – 13.

Keywords: *Acinetobacter baumannii strain HAF – 13, industrial wastewater, multiple metal/antibiotics resistance*

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1. Introduction

Heavy metals are natural constituents of the environment, but indiscriminate use for human purposes has altered their geochemical cycles and biochemical balance. This results in excess release numerous heavy metals into natural resources like the soil and aquatic environments. Prolonged exposure and higher accumulation of such heavy metals can have deleterious health effects on human life and aquatic biota [1].

Heavy metals are the major toxic constituent of various industrial wastewaters and pose greater risk for the environment if not treated properly prior to their disposal [2]. Amongst all heavy metals, lead, cadmium, mercury, and chromium are known to be extremely toxic even at low concentrations [3]. Untreated industrial wastewater discharged to the nearby water bodies may cause severe ground water and environmental pollution [4].

Microbial survival in polluted environments depends on intrinsic biochemical and structural properties, physiological and/or genetic adaptation including morphological changes of cells, as well as environmental modifications of metal speciation [5]. Microbes apply various types of resistance mechanisms in response to heavy metals [6]. Several metal resistance mechanisms have been identified which is responsible for alteration of normal cell physiology leading to development of drug resistance in microorganisms [7].

The matter of public concern that needs to be underlined is the potential for the development and amplification of antimicrobial resistance genes via horizontal transfer of genetic elements encoding antimicrobial resistance [8]. This type of transfer may occur from relatively innocuous commensal strains where antimicrobial resistance develops

and can then be transferred to pathogens resulting in multiple-drug- and metal resistant pathogens. This can take place in a variety of environments especially highly polluted area [9].

Antibiotic resistance studies in the bacterial community of the metal contaminated areas are very important because many researchers suggested that metal exposure indirectly selects for bacteria resistant to unrelated toxicants, particularly antibiotics [10]. Bacterial resistance to antibiotics and heavy metals is an increasing problem in today's society [11].

Among microbial communities involved in different ecosystems such as soil, freshwater, wastewater and solid wastes, several strains belonging to the genus of *Acinetobacter* have been attracting growing interest from medical, environmental and a biotechnological point of view [12]. Bacteriaof this genus are known to be involved in biodegradation, leaching and removal of several organic and inorganic manmadehazardous wastes [13].

Although a link between antibiotic and metal resistance has been established in many bacteria [14,15,16,17] but epidemiological surveys carried out on *Acinetobacter* do not comment on metal ion resistance [18]. This may be because metal ion resistances are of less clinical concern than antibiotic resistances. However, such association is significant since knowledge of metal resistance may provide useful information on mechanism(s) of antibiotic resistance [19].

Heavy metal and antibiotic resistance have been shown to have a strong correlation in nature, and their inter-relation is an important subject of study. Therefore, there is a need to isolate and characterize metal/antibiotic resistance in the microorganisms that exist and interact in a contaminated environment. The present work was aimed to isolate and characterize the multiple metal/antibiotic-resistant bacteria from industrial wastewater of a Plastic Factory at Hafar Al Baten governorate, Saudi Arabia. Identification of the selected isolate was carried out by morphological, physiological and biochemical tests in addition to phylogenetic analysis.

2. Materials and Methods

2.1. Chemicals and Media

The elemental salts employed in this study include: cadmium chloride $CdCl_2$ [Cd^{2+}], chromium trioxide CrO_3 [Cr^{6+}], mercuric chloride $HgCl_2$ [Hg^{2+}], lead nitrate $Pb(NO_3)_2$ [Pb^{2+}] and sodium arsenate $AsHNa_2O_4.H_2O$ [As^{5+}] (all from Sigma Aldrich, St. Louis, MO, USA). The media used were nutrient agar for isolation of bacteria, Mueller Hinton agar for antibiotic assay (all from HiMedia Laboratories Pvt. Ltd. India).

2.2. Collection of Samples

The industrial effluent samples were collected from outlet of a Plastic Factory at Hafar Al Baten governorate, Saudi Arabia in the month of December 2014. Sub-surface (0 - 20 cm) three wastewater samples (500 ml in volume) were collected in a screw cap sterilized plastic bottles[20]. The samples were kept in an icebox containing ice packs where the sample temperature was maintained at approximately 4°C to prevent from contamination and allow the sample to stay longer [21,22]. The samples were transported to the laboratory of Microbiology, Department of Biology, Faculty of Science and Arts, Northern Border University for bacteriological analysis where they were analysed within 24 h of collection. For heavy metal analysis samples were acidified with concentrated HNO_3 and stored at 4°C [23].

2.3. Physicochemical Analysis of the Collected Samples

Physicochemistry of the samples was determined by standard methods described by APHA [24], Nelson and Sommers [25] and AOAC [26]. The analyzed parameters included; turbidity, pH, dissolve oxygen (DO), total dissolved solids (TDS), total suspended solids (TSS), biological oxygen Demand (BOD) were assayed at Water Research Center (WRC), King Abdulaziz University, Saudi Arabia.

2.4. Heavy Metal Analysis

The samples were treated as recommended by Grimalt [27] by acid digestion using 0.6 ml of concentrated HNO_3, 0.25 ml of 75% H_2SO_4 and 100 ml of unfiltered water. Each sample was then evaporated, diluted to 25 ml and analyzed for metal content using atomic absorption spectrophotometer (Perkin Elmer Analyst 2380). The heavy metal analysis of the collected samples was carried out at Water Research Center (WRC), King Abdulaziz University, Saudi Arabia.

2.5. Preparation of Heavy Metal Stock Solutions

Stock solutions (1000 mg/l) of the five heavy metals; Hg^{2+}, Pb^{2+}, Cd^{2+}, Cr^{6+} and As^{5+}in the form of their salts $HgCl_2$, Pb $(NO_3)_2$, $CdCl_2$, CrO_3 and $AsHNa_2O_4.H_2O$ respectively were prepared. A weight of each of these heavy metal salts that gave a corresponding 1g of each of the respective heavy metal was weighed and dissolved in 1000 ml of deionised water. These were left to stand for 30 mins to obtain complete dissolution then sterilized by filtration through 0.22-µm membrane filters (Nucleopore Corp., Pleasanton, CA, USA), and stored in sterile flasks in the dark at 4°C for no longer than 1 month.

2.6. Screening for Multiple Heavy Metal Resistant Bacteria

Heavy metals resistant bacteria were recovered from the collected samples on nutrient agar medium supplemented with different concentrations; 10, 15, 25, 25 and 30 mg/l of the five heavy metals; Hg^{2+}, Pb^{2+}, Cd^{2+}, As^{5+} and Cr^{6+} respectively in the form of their salts. The nutrient agar medium was sterilized at 121°C for 15 min and allowed to cool 40 – 45 °C then the metals (with the above mentioned concentrations) were added to medium before plating. The collected wastewater samples were serially diluted from 10^{-2} to 10^{-6} using 0.9% sterile saline then 0.1 ml from each dilution was spread on the surface of the agar plates and incubated at 37 °C for 48 h. Individual bacterial colonies showing and having different morphological appearance on nutrient agar plates were picked up and purified by repeated streaking on nutrient

agar media supplemented with the same concentrations of the tested heavy metals. Pure bacterial cultures were kept on slants of the same medium and stored at 4°C for further studies [28,29].

2.7. Determination of Maximum Tolerable Concentrations (MTCs)

The maximum tolerable concentration of heavy metal was selected as the highest concentration of heavy metal that allows growth after 2 days [30]. The MTCs of the tested heavy metals were determined for the obtained bacterial isolates on Tris-minimal salts (TSM) agar medium [31]. The medium was consisted of (g/l): D-glucose (10), Tris-HCl (6.06), NaCl (4.68), KCl (1.49), NH_4Cl (1.07), Na_2SO_4 (0.43), $MgCl_2.2H_2O$, (0.2), $CaCl_2.2H_2O$ (0.03), pH was adjusted to 7 using HCl. The isolated bacterial cultures were primary screened for MTCs on TSM agar plates individually supplemented with different concentrations of the tested heavy metals. The tested concentrations were; 25 – 125 mg/l for Hg^{2+}, 25 – 225 mg/l for Pb^{2+}, 50 – 250 mg/l for Cd^{2+} and 100 – 300 mg/l for Cr^{6+} and As^{5+}. The plates were incubated at 37 °C for 48 h then results were recorded.

The obtained results on TSM agar were confirmed on TSM broth medium. 0.1 ml of overnight broth culture (OD_{620}= 0.8) of each isolate was inoculated in 10 ml sterile TSM broth supplemented with individual concentration of the metals under study. For measurement the growth of the tested organisms, negative control (culture media containing the same concentration of metals without inoculation) and blank (culture media neither inoculated with bacteria nor heavy metal addition). After 48 h, bacterial growth was measured as optical density values at a wave length of 620 nm using (UNICO 2100 U.V. visible spectrophotometer). All experiments were performed in triplicates and the average values were determined by Microsoft Office Excel 2013.

2.8. Antibiotics Resistance Pattern

The antibiotic resistance pattern of the multiple heavy metal resistance isolates was studied by Kirby-Bauer disk diffusion method [32] towards fifteen antibiotics (belonging to 10 classes). The antibiotics (µg/disc) were AK: Amikacin (30), AMC: Augmentin (Amoxicillin /Calvunic acid) (30), CAZ: Ceftazidime (30), C: Chloramphenicol (30), CIP: Ciprofloxacin (5), DA: Clindamycin (2), SXT: Cotrimoxazole (Sulfamethoxazol/Trimithoprim) (25), E: Erythromycin (15), CN: Gentamicin (10), IPM: Imipenem (10), LEV: Levofloxacin (5), OX: Oxacillin (1), TE: Tetracycline (30), VA: Vancomycin (30) and P: Penicillin G (benzylpenicillin) (10 IU) [33]. Antibiotic discs used in this study were fromH iMedia Company at A-516, Swastik Disha Business Park, ViaVadhani Ind. Est., LBS Marg, Mumbai-400086, India.

Antibiotic impregnated discs were placed over freshly prepared Mueller Hinton agar seeded with the bacterial strains under study. All 15 antibiotic disks were placed on each of the seeded plates at appropriate distances from one another (5 disc/plate) then plates were incubated at 37 °C for 24 h. The isolate was classified as resistant or sensitive by the presence/absence of inhibition zone of growth around antibiotic discs. Zones of inhibition were obtained by measuring the diameter across the center of each zone in millimeters. The resistance break points were those recommended by Clinical and Laboratory Standards Institute (CLSI) [34].

2.9. Identification of the Selected Multiple Heavy Metal/Antibiotic Resistant Isolate

2.9.1. Phenotypic Identification

2.9.1.1. Morphological characteristics

Morphological characteristics namely, colony morphology (color, shape, margin, elevation and surface) cell morphology (shape and gram reaction) of the selected isolate were studied.

2.9.1.2. Physiological and biochemical characterization

The biochemical characteristics of the selected isolate were identified using Vitek-2 automated machine (BioMerieux®, France) [35] at laboratory of Microbiology, Faculty of Pharmacy, Northern Border University, Saudi Arabia. The physiological parameters; catalase, oxidase, temperature and pH growth ranges were identified. The biochemical and physiological tests used to identify the target isolate were compared to Bergey's Manual of Determinative Bacteriology [36].

2.9.2. Molecular and Phylogenetic Identification

2.9.2.1. Isolation of genomic DNA

Total DNA was extracted by a modified method of Moore et al. [37]. In brief, bacterial cells were collected by centrifugation at 13,000 rpm for 2 min followed by suspension in 564µl Tris-HCl-EDTA buffer and incubation with 10µg lysozyme (50 mg/ml) at 37 °C for 30 mins. 6µl proteinase K (20 mg/ml) and 30µl of 10% SDS were added, mixed and incubated for 1 h at 37 °C. To the lysis solution, 100µl of 5M NaCl was added followed by incubation for 2 min at 65°C. This was followed by an addition of 80µl CTAB/NaCl and a further incubation for 10 min at 65 °C. The mixture was treated with phenol/chloroform/isoamyl alcohol (25:24:1). The supernatant was collected and precipitated with isopropanol by keeping at – 20 °C overnight. Genomic DNA was washed in 70% ethanol and dissolved in 100µl TE buffer. RNase treatment was carried out to remove traces of RNA from the sample.

2.9.2.2. Amplification of 16S rDNA Genes

The 16S rDNA genes were amplified with bacterial universal primers specific for eubacterial 16S rDNA gene (Forward, AGTTTGATCATGGCTCAG) and (Reverse, TTACCGCGGCTGGCA) according to the method described by Hookoom and Puchooa [38]. The PCR (50µl) contained 0.5µl of each forward and reverse primer, 1.5mM of 10X Taq buffer (stock 20mM), 0.125 mM (2.5µl) of each deoxynucleotide (ddATP, ddGTP, ddCTP and ddTTP), 1.25 units of Taq DNA polymerase (5units/µl) and 5µl DNA. PCR conditions were as follows: denaturation at 94 °C for 3 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72°C for 1 min, and a final extension step at 72 °C for 10 min. The PCR products obtained from DNA extracted from the samples were first analyzed by electrophoresis in

1.5% agarose gel and was stained with ethidium bromide and visualized under short-wavelength UV light.

2.9.2.3. Nucleotide sequencing and alignment

A DNA fragment was eluted by using QIAgen Gel Extraction Kit. PCR product was sequenced by 3730x1 DNA synthesizer (Applied Biosystems, California, USA). The part of DNA isolation and purification, 16S rRNA gene amplification and sequencing was carried out at Sigma Scientific Services Co, Lebanon Square, El Giza, Egypt. Sequences were matched with previously published bacterial 16S rDNA sequences in the National Center for Biotechnology Information (NCBI) database using the GenBank BLAST search available through the center's website (http://www.ncbi.nlm.nih.gov/BLAST). The 16S rDNA sequences were then submitted to the Gene Bank using the Sequin service. Further phylogenetic tree, similarity index was generated and compared with known sequences using MEGA 4 software [39].

3. Results and Discussion

3.1. Physicochemical Characteristic of Industrial Effluent Samples

The results of physicochemical analysis of the analyzed wastewater sample exhibited that, the effluent was reddish brown in color, pH of the effluent was 4.92 this indicated that the effluent sample were slightly acidic in nature. Turbidity of the effluent was found to be 10.22 ntu. Dissolve oxygen was found to be 6.7 mg/l. while the total dissolved solids (TDS) and total suspended solids (TSS) in the effluent sample were 29.4 and 34.6 mg/l respectively, the BOD was found to be 45.6 mg/l. The physicochemical parameters of the effluent sample were recorded in Table 1. From the standpoint of the samples' physicochemistry, it is apparent that they lack adequate treatment processes hence, are not fit for discharge into the environment. Physical characteristics of industrial wastewater vary depending on the type of industry. The measurement of the concentration of waste organic materials in a wastewater is important in the design of the treatment plant and in the control of its operation [40].

Table 1. Physico-chemical characters of effluent sample

No.	Parameter[a]	Effluent
1	pH	4.92
2	Turbidity	10.22 ntu
3	Dissolve oxygen (DO)	6.7 mg/l
4	Total Dissolved solids (TDS)	29.4 mg/l
5	Total suspended solids (TSS)	34.6 mg/l
6	Biological oxygen Demand (BOD)	45.6 mg/l

[a]Values represent averages of three replicate determinations.

Heavy metals are one of the environmental pollutants, where they pose health hazards to man and aquatic lives if their concentration exceeds allowable limits [41]. In the present study, the analyzed effluent sample exhibited higher concentrations of all metals assayed where concentration of the measured heavy metals were; 12.56, 23.90, 24.12 & 28.23 mg/l for lead, cadmium, arsenic and chromium respectively. Data of the heavy metal analysis are recorded in Table 2. Elevated concentrations of heavy metals are introduced into the environment through industries manufacturing textile, allied chemicals, electroplating, batteries, paints, plastics and petrochemicals. The discharge of effluents containing heavy metals mounts pressures on the ecosystem and consequently causing health hazards to plants, animals, aquatic life and humans [42].

Table 2. Heavy metal ions concentrations in effluent sample

No.	Metal ions[a]	Concentration (mg/l)
1	Pb^{2+}	12.56
2	Cd^{2+}	23.90
3	As^{5+}	24.12
4	Cr^{6+}	28.23

Introduction of certain concentrations of heavy metals into the environment kills the majority of the microflora, thereby selecting for a few cells that would have evolved resistance mechanisms to the metals.

3.2. Isolation of Heavy Metal Resistant Bacteria from the Collected Samples

A total of 14 different bacterial cultures coded HAF – 1 to HAF – 14 were isolated on nutrient agar medium–supplemented with 10, 15, 25, 25 and 30 mg/l of the five heavy metals; Hg^{2+}, Pb^{2+}, Cd^{2+}, As^{5+} and Cr^{6+} respectively. Description the growth of the obtained isolates on the isolation nutrient agar medium was varied where; 5 (35.71 %) isolates (HAF – 2, HAF – 6, HAF – 7, HAF – 9 & HAF – 13) showed good (+++) growth; 6 (42.85 %) isolates (HAF – 1, HAF – 3, HAF – 5, HAF – 8, HAF – 10 & HAF – 11) showed moderate (+) growth while, 3 (21.42 %) isolates (HAF – 4, HAF – 12 & HAF – 14) showed weak (W) growth. Wastewater contains a high content of enteric pathogens, including bacteria, viruses and helminthes, which are easily transmitted through water [43]. The emergence of resistant microbial strains from the indigenous community participates in various self-recovery processes which occur in such polluted habitat. Some microorganisms have been reported to have evolved mechanisms to detoxify heavy metals thereby, becoming resistant to such metals [44].

3.3. Maximum Tolerable Concentrations (MTCs) of the Tested Heavy Metals for the Obtained Bacterial Isolates

3.3.1. Determination of MTCs on Solid Media

The isolated 14 bacterial strains were screened for the maximum tolerable concentrations (MTCs) toward increased concentrations of the previously tested heavy metals using TSM agar medium. Out of 14 isolates, 5 (35.71 %) isolates (HAF – 2, HAF – 6, HAF – 7, HAF – 9 & HAF – 13) were selected as a multiple heavy metal resistant (MHMR) organisms where it showed a high degree of resistance to all tested heavy metals with maximum tolerable concentrations (MTCs); 75 – 100 mg/l for Hg^{2+}, 125 – 175 mg/l for Pb^{2+}, 200 mg/l for Cd^{2+} and 200 – 250 mg/l for Cr^{6+} and As^{5+}. The plates were investigated and results were recorded as (R=Resistant/tolerated) for the grown cultures and (-=Sensitive/inhibited) for non-growing ones (Table 3). The microbial level of resistance of each concentration of

heavy metal was obtained by the level of growth on the agar plates [45].

Table 3. MTCs of the different heavy metals for MHMR isolates on TSM agar medium

Isolates	Heavy metals concentrations (mg/l)																								
	Hg^{2+}					Pb^{2+}					Cd^{2+}					Cr^{6+}					As^{5+}				
	25	50	75	100	125	25	75	125	175	225	50	100	150	200	250	100	150	200	250	300	100	150	200	250	300
HAF – 1	R	-	-	-	-	R	R	-	-	-	-	-	-	-	-	R	-	-	-	-	R	R	-	-	-
HAF – 2	R	R	R	R	-	R	R	R	-	-	R	R	R	R	-	R	R	R	R	-	R	R	R	R	-
HAF – 3	R	R	-	-	-	R	-	-	-	-	R	R	R	-	-	R	R	-	-	-	R	R	R	-	-
HAF – 4	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	R	-	-	-	-	R	R	-	-	-
HAF – 5	R	R	-	-	-	R	R	-	-	-	R	R	R	-	-	R	R	R	-	-	R	R	R	-	-
HAF – 6	R	R	R	R	-	R	R	R	R	-	R	R	R	R	-	R	R	R	R	-	R	R	R	R	-
HAF – 7	R	R	R	R	-	R	R	R	-	-	R	R	R	R	-	R	R	R	R	-	R	R	R	-	-
HAF – 8	R	R	-	-	-	R	R	-	-	-	R	R	-	-	-	R	R	R	-	-	R	R	R	-	-
HAF – 9	R	R	R	-	-	R	R	R	R	-	R	R	R	R	-	R	R	R	-	-	R	R	R	R	-
HAF – 10	R	-	-	-	-	R	R	-	-	-	R	R	R	-	-	R	-	-	-	-	R	-	-	-	-
HAF – 11	R	R	R	-	-	R	R	-	-	-	R	-	-	-	-	R	R	-	-	-	R	R	-	-	-
HAF – 12	R	-	-	-	-	R	R	-	-	-	R	-	-	-	-	-	-	-	-	-	R	-	-	-	-
HAF – 13	R	R	R	R	-	R	R	R	R	-	R	R	R	R	-	R	R	R	R	-	R	R	R	R	-
HAF – 14	R	-	-	-	-	R	R	-	-	-	-	-	-	-	-	R	R	-	-	-	R	R	R	-	-

(R)=Resistant (tolerated), (-)=Sensitive (inhibited).

3.4. Determination of MTCs on Broth Media

For confirmation the results of MTCs on solid media, the selected multiple heavy metal resistant (MHMR) isolates (HAF – 2, HAF – 6, HAF – 7, HAF – 9 & HAF – 13) were inoculated on TSM broth medium supplemented with the same concentrations as in solid media. Hussein and Joo [46] reported that, in order to isolate the most resistant strains, the MIC experiment was conducted on plates of Tris-minimal medium and was confirmed on broth medium. In this study TSM broth medium was used in order to minimize the complexation of heavy metals and to give an accurate estimation for MTCs [47]. Generally all MHMR isolates exhibited the same resistance pattern to the tested concentrations of the different heavy metals as in solid media, but it was observed a slight decrease in MTCs for some isolates in broth medium than in solid medium and this may be due to one of the diffusion factors for the solid medium. Data of MTCs on broth medium are represented in Figure 1a-e.

The obtained results are in agreement with those by Hassan et al. [47], who stated that the difference in toxicity toward bacterial isolates could be explained by the conditions of bacterial isolation and the nature and physiological characteristics of each bacterial isolate. In this study, values of MTCs may agree or differ with other reports that discussed MTCs of the heavy metal resistant bacteria. Any way the obtained MTCs are in the range of MTCs obtained by Rohini and Jayalakshmi [48], where they discussed MTCs of *Bacillus cereus* isolated from industrial wastewater against nickel, lead, cobalt, chromium, cadmium and mercury. The strain showed a MTCs of 100 mg/l against cobalt and cadmium, 400 mg/l against chromium, 500 mg/l of nickel and lead respectively making it a very potential. The organism was highly susceptible to mercury as it is the most toxic heavy metal.

3.5. Antibiotic Resistance Pattern of MHMR Isolates

The antibiotic resistance pattern of the multiple heavy metal resistant (MHMR) isolates (HAF – 2, HAF – 6, HAF – 7, HAF – 9 & HAF – 13) was studied by Kirby-Bauer disk diffusion method against 15 different antibiotics. Several reports discussed antibiotic resistance pattern in the heavy metal resistant bacterial strains isolated from industrial effluents [45,49,50,51,52].

Out of 5 isolates, 4 (80 %) isolates were multiple antibiotics resistance (MAR) where it showed high resistance degrees to the tested antibiotics. These isolates were; HAF – 9 (resistance to 10 antibiotics), HAF – 6 (resistance to 12 antibiotics), HAF – 2 (resistance to 13 antibiotics) and HAF – 13 (resistance to 14 antibiotics). While one isolate, HAF – 7 showed a weak resistance degree (resistant to 4 antibiotics only), data of antibiotic resistance pattern of MHMR isolates are recorded in Table 4 and represented in Figure 2. The high levels of antibiotic resistance among MHMR isolates establish the link between antibiotic and metal resistance in the nature. Yamina et al. [51] reported that, 13 heavy metal resistant bacteria isolated from industrial wastewater were resistant to zinc, lead, chromium and cadmium with minimum inhibitory concentration (MIC) ranged from 0.1 to 1.5 mg/l, these isolates showed co-resistance to other heavy metals and antibiotics, of which 15% were resistant to one antibiotic and 85% were multi- and bi-antibiotics resistant. Many reports established the correlation exists between metal tolerance and antibiotic resistance in bacteria because of the likelihood that resistance genes to both antibiotics and heavy metals may be located closely together on the same plasmid in bacteria and are thus more likely to be transferred together in the environment [53,54].

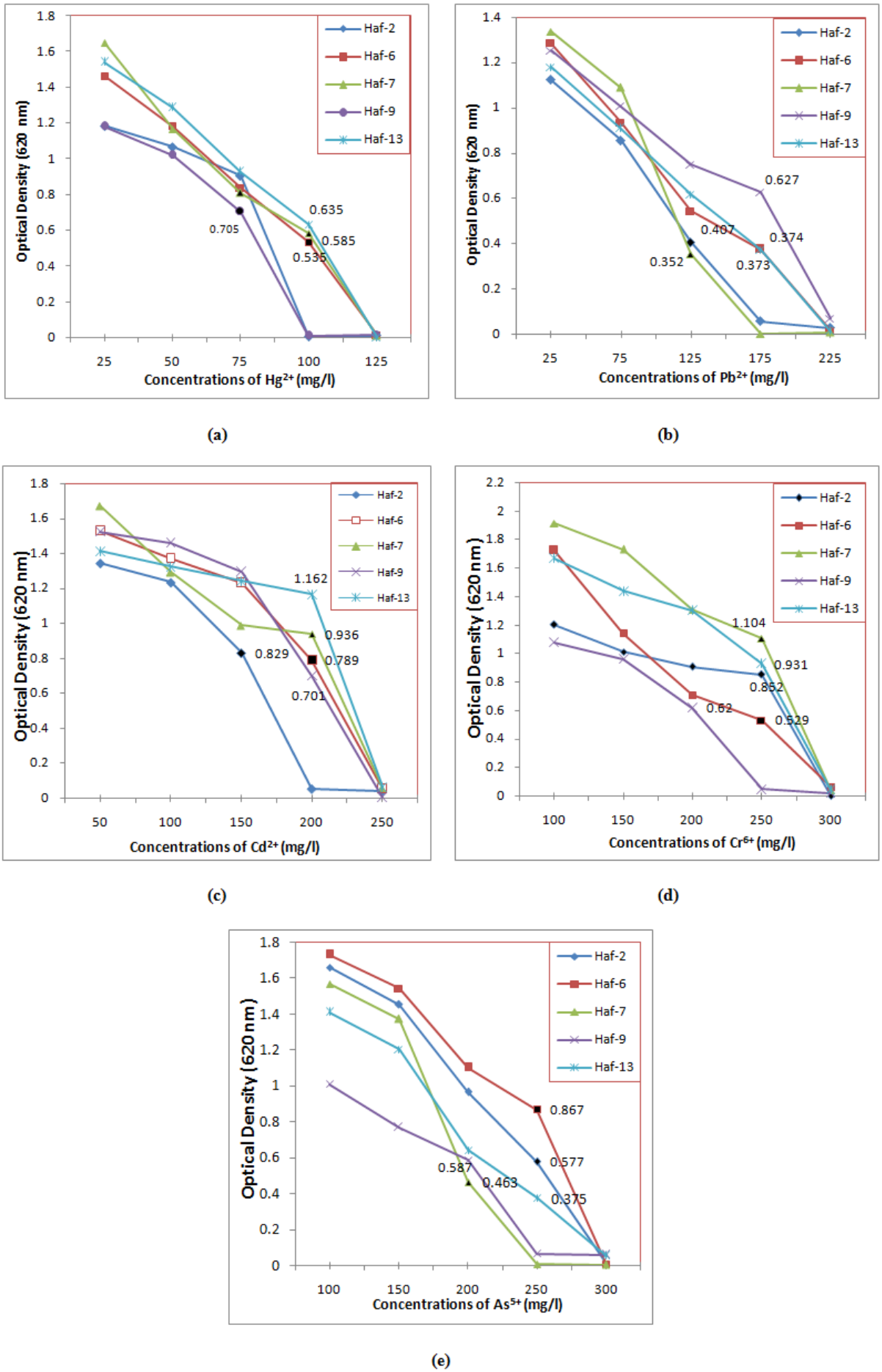

Figure 1a-e. MTCs of different heavy metals; (a) Hg^{2+}, (b) Pb^{2+}, (c) Cd^{2+}, (d) Cr^{6+} and (e) As^{5+} for MHMR isolates on TSM broth medium

Table 4. Antibiotic resistance pattern of the MHMR isolates

Isolates	Tested antibiotics														
	Amikacin	Augmentin	Ceftazidime	Chloramphenicol	Ciprofloxacin	Clindamycin	Cotrimoxazole	Erythromycin	Gentamicin	Imipenem	Levofloxacin	Oxacillin	Tetracycline	Vancomycin	Penicillin G
	AK	AMC	CAZ	C	CIP	DA	SXT	E	CN	IPM	LEV	OX	TE	VA	P
HAF – 2	R	R	R	R	R	R	R	R	R	-	R	-	R	R	R
HAF – 6	R	R	R	-	R	R	R	R	R	-	R	R	R	-	R
HAF – 7	R	-	R	-	R	R	-	-	-	-	-			-	-
HAF – 9	R	R	R	-	-	R	-	R	-	-	R	R	R	R	R
HAF – 13	R	R	R	R	R	R	R	R	R	-	R	R	R	R	R

R=Resistance, -= Sensitive.

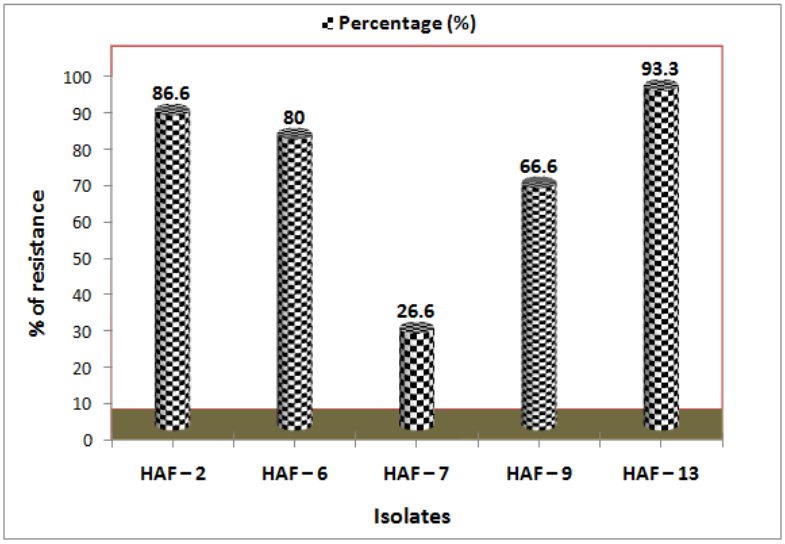

Figure 2. Antibiotic resistance pattern of MHMR isolates against 15 antibiotics

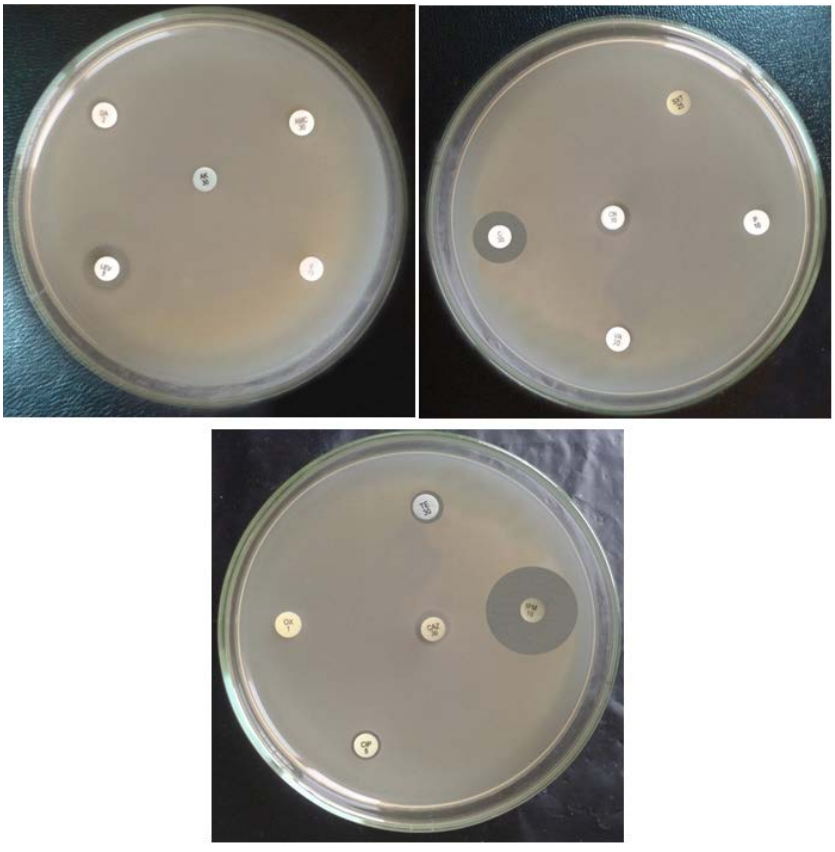

Figure 3. Antibiotic resistance assay of HAF–13 isolate

The isolates (HAF – 2, HAF – 6, HAF – 9 & HAF – 13) were selected as MAR isolates. Among them, HAF – 13 Isolate was the highest antibiotic resistant isolate where it showed a wide range of resistance to all tested antibiotics; Amikacin, Augmentin, Ceftazidime, Chloramphenicol, Ciprofloxacin, Clindamycin, Cotrimoxazole, Erythromycin, Gentamicin, Levofloxacin, Oxacillin, Tetracycline, Vancomycin and Penicillin G except Imipenem (Figure 3). Microorganisms resistant to antibiotics and metals appear as the result of exposure to metal-contaminated environments which cause coincidental coselection of resistance factors for antibiotics and heavy metals. Heavy metal tolerance in the environment may contribute to the maintenance of antibiotic resistance genes by increasing the selective pressure of the environment [52].

Based on the previous screening studies of multiple metal/antibiotic resistance among the bacterial strains isolated from the collected industrial wastewater samples, the isolate HAF – 13 was selected as a target organism. The wastewater coming from industrial sources is the appropriate environment where the microorganisms can develop resistance to heavy metals and antibiotics [55]. The isolate HAF – 13 was selected for further studies regarding to its identification.

3.6. Identification the Selected Isolate HAF – 13

The identification of the multiple heavy metal/antibiotic resistance HAF – 13 isolate was done on the basis of morphological, physiological and biochemical characteristics as well as phylogenetic analysis of 16S rRNA gene.

3.6.1. Morphological Characteristics

Morphological characteristics of HAF – 13 isolate was studied through macroscopic examination of grown colonies on nutrient agar plates that showed white, circular and smooth colonies with entire margin. While the microscopic examination of the stained cells showed Gram-negative, coccobacilli cells.

3.6.2. Biochemical and Physiological Characteristics

Biochemical characteristics of HAF – 13 isolate were identified using automated vitek 2 system. Generally the results of biochemical characterization exhibited that the isolate was positive for lipase, tyrosinase and urease enzymes, also the organism was able to utilize of carbon sources; D-glucose, D-mannose, citrate and malonate. The results of the biochemical testes otaibed by Vitek-2 system indicated that the isolate under study is closely related to *Acinetobacter baumannii*with excellent identification Level (99 % probability). Vitek-2 system is an efficient biochemical test to confirm identification of *A. baumannii* [56,57].

Physiological characteristics examined by microbiological methods were exhibited that, the organism was positive for catalase and negative for oxidase also, it was able to grow at pH values between 6.5 and 7.5 with optimum pH 7.2, and in the temperature range 33–45°C, and the optimal growth temperature was 38 °C. The physiological and biochemical characteristics of HAF – 13 isolate are recorded in Table 5. Morphological, physiological and biochemical characteristics of the isolate HAF – 13 were compared with data of the genus *Acinetobacter* in Bergey's Manual of Determinative Bacteriology [36] and indicated that the isolate HAF – 13 is closely related to *Acinetobacter baumannii*.

Table 5. Biochemical and physiological characteristics of the isolate HAF – 13 using automated identification VITEK-2 system and microbiological techniques

Well	Biochemical tests		HAF–13	Well	Biochemical tests		HAF–13
2	Ala-phe-pro-arylamidase	APPA	-	**35**	D-trehalose	dTRE	-
3	Adonitol	ADO	-	**36**	Citrate (sodium)	CIT	+
4	L-Pyrrolydonyl-arylamidase	PyrA	-	**37**	Malonate	MNT	+
5	L-Arabitol	lARL	-	**39**	5-Keto-d-gluconate	5KG	-
7	D-Cellobiose	dCEL	-	**40**	L-lactate alkalinisation	lLATk	+
9	Beta-galactosidase	BGAL	-	**41**	Alpha-glucosidase	AGLU	-
10	H_2S production	H_2S	-	**42**	Succinate alkalinisation	SUCT	+
11	Beta-n-acetyl glucosaminidase	BNAG	-	**43**	Beta-n-acetyl-galactosaminidase	NAGA	-
12	Glutamylarylamidasepna	AGLTp	+	**44**	Alpha-galactosidase	AGAL	-
13	D-glucose	dGLU	+	**45**	Phosphatase	PHOS	-
14	Gamma-glutamyl-transferase	GGT	-	**46**	Glycine arylamidase	GlyA	-
15	Fermentation/ glucose	OFF	-	**47**	Ornithine decarboxylase	ODC	-
17	Beta-glucosidase	BGLU	-	**48**	Lysine decarboxylase	LDC	-
18	D-Maltose	dMAL	-	**53**	L-histidine assimilation	lHlSa	-
19	D-mannitol	dMAN	-	**56**	Coumarate	CMT	+
20	D-mannose	dMNE	+	**57**	Beta-glucoronidase	BGUR	-
21	Beta-xylosidase	BXYL	-	**58**	O/1 29 resistance (comp.vibrio.)	O129R	-
22	Beta-alanine arylamidasepna	BAlap	-	**59**	Glu-gly-arg-arylamidase	GGAA	-
23	L-proline arylamidase	ProA	-	**61**	L-malate assimilation	lMLTa	-
26	Lipase	LIP	+	**62**	Ellman	ELLM	-
27	Palatinose	PLE	-	**64**	L-lactate assimilation	lLATa	-
29	Tyrosine arylamidase	TyrA	+	**Range of growth**			
31	Urease	URE	+		Temperature (33–45 °C)		+
32	D-sorbitol	dSOR	-		pH (6.5 – 7.5)		+
33	Saccharose/sucrose	SAC	-		Catalase		+
34	D-tagatose	dTAG	-		Oxidase		-

(+=positive, - =negative).

3.6.3. Molecular and Phylogenetic Identification

The morphological and biochemical identification of isolate HAF – 13 was confirmed by 16S rRNA gene sequencing. The genomic DNA was extracted and 16S rRNA gene was amplified by PCR then examined by agarose gelelectrophoresis (Figure 4). The nucleotide sequence of 16S rRNA gene was obtained as a partial sequence (937bp). The sequence was submitted to the GenBank database and deposited under GenBank accession number KU310899.1.

According to a sequence homology analysis of GenBank by BLASTN, the highest score was found with *Acinetobacter* species. Among them strain of *A. baumannii* TU04 have the highest identities 98%. In order to understand the phylogenetic position of the strain HAF – 13, we constructed a phylogenetic tree based on comparison of 16S rRNA sequences of the isolate and those of reference *Acinetobacter* strains (Figure 5). These results confirmed that the isolate HAF – 13 is a strain of *Acinetobacter baumannii* and designated as *A. baumannii* strain HAF – 13. These results are in accordance with results of several studies which established the dominance of *A. baumannii* in heavy metal polluted areas [18,56,57,58].

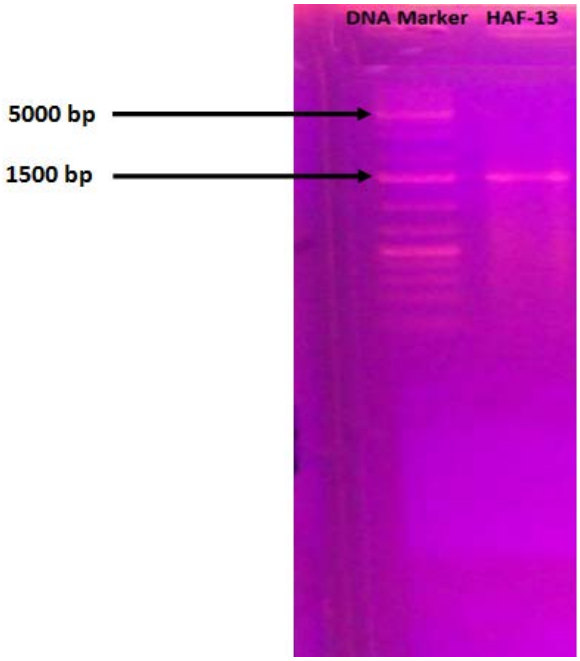

Figure 4. Gel Electrophoresis of PCR-product of 16S rRNA gene of the isolate HAF–13 and DNA ladder marker

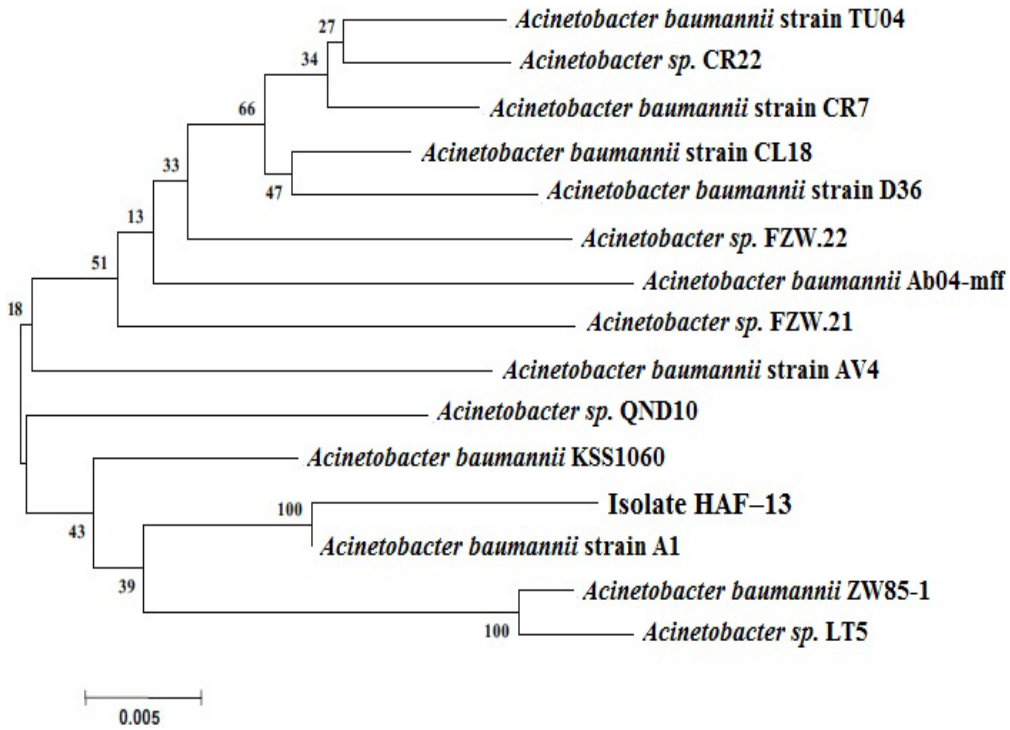

Figure 5. Phylogenetic tree showing genetic relationship of *Acinetobacter baumannii* strain HAF–13 KU310899.1 with taxonomically similar species based on 16s rRNA sequences using MEGA 4 software

4. Conclusion

The industrial use of mercury, cadmium, lead and other heavy metals have led to the pollution of the environment. The concentration of toxic metals that affects the growth and survival of different microorganisms varied greatly. It is clearly indicated that industrial wastewater is responsible for the development of bacterial resistance along with the risk of human health and environment. The long term effect of pollutants has led to emergence of multi-metal and multi-antibiotic resistant bacteria in the study area. Therefore, dual expressions of heavy-metal and antibiotic resistance from *A. baumannii* strain HAF – 13 make this isolate, a potential seeds for decommissioning of sites polluted with industrial effluents rich in heavy metals, since this isolate will be able to withstand in situ antibiosis that may prevail in such ecosystems.

References

[1] Dixit R., Wasiullah, Deepti M., Kuppusamy P., Udai B.S., Asha S., Renu S., Bhanu P.S., Jai P.R., Pawan K.S., Harshad L. and Diby P. Bioremediation of heavy metals from soil and aquatic

environment: an overview of principles and criteria of fundamental processes. *Sustainability* (2015), 7(2): 2189-2212.

[2] Bhattacharya A. and Gupta A. Evaluation of *Acinetobacter sp.* B9 for Cr (VI) resistance and detoxification with potential application in bioremediation of heavy-metals-rich industrial wastewater. *Environmental Science and Pollution Research* (2013), 20:6628-6637.

[3] Coen N., Mothersill C., Kadhim M. and Wright E.G. Heavy metals of relevance to human health induce genomic instability. *The Journal of Pathology* (2001), 195(3):293-299.

[4] Gleick P.H. *The World's Water Volume 8: The Biennial Report on Freshwater Resources*, Island Press (2014).

[5] Wuertz S. and Mergeay M. *The impact of heavy metals on soil microbial communities and their activities*. In J. D. van Elsas, E. M. H. Wellington, & J. T. Trevors (Eds.), Modern soil microbiology. New York: Marcel Decker (1997), (pp. 1-20).

[6] Nies D.H. Efflux-mediated heavy metal resistance in prokaryotes. *FEMES Microbiology Reviews* (2003), 27(2-3):313-339.

[7] Garhwal D., Vaghela G., Panwala T., Revdiwala S., Shah A. and Mulla S. Lead tolerance capacity of clinical bacterial isolates and change in their antibiotic susceptibility pattern after exposure to a heavy metal. *International Journal of Medicine and Public Health* (2014), 4(3):253-256.

[8] Boerlin P. and Reid-Smith R.J. Antimicrobial resistance: its emergence and transmission. *Animal Health Research Reviews* (2008), 9:115-126.

[9] Jakobsen L., Kurbasic A., Skjot-Rasmussen L., Ejrnaes K., Porsbo L.J. and Pedersen K., et al. *Escherichia coli* isolates from broiler chicken meat, broiler chickens, pork, and pigs share phylogroups and antimicrobial resistance with community-dwelling humans and patients with urinary tract infection. *Foodborne Pathogens and Disease* (2010), 7:537-547.

[10] Summers A.O. Generally overlooked fundamentals of bacterial genetics and ecology. *Clinical Infectious Diseases* (2002), 34(Suppl 3):S85-92.

[11] Kawane R.S. Studies on antibiotics and heavy metal resistance profiling of *Escherichia coli* from drinking water and clinical specimens. *Bioscience Discovery* (2012), 3(3):292-295.

[12] Abdel El-Haleem D. *Acinetobacter*: environmental and biotechnological applications. Review article. *African Journal of Biotechnology* (2003), 2 (4):71-74.

[13] Ying W., Bin H., Hua-bing Z. and Bin and BAO–li A. Biodegradation of phenol by free and immobilized *Acinetobacter* sp. strain PD12. *Journal of environmental sciences* (2007), 19(2): 222-225.

[14] Foster TJ. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiological Reviews* (1983), 47(3):361-409.

[15] Witte W., Green L., Misra T.K. and Silver S. Resistance to mercury and the cadmium in chromosomally resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* (1986), 29(4):663-669.

[16] Wang Y., Mahler I., Levinson H.S. and Halvorson H.O. Cloning and expression in *Escherichia coli* of chromosomal mercury resistance genes from a *Bacillus sp. Journal of Bacteriology* (1987), 169(10):4848-4851.

[17] Silver S. and Misra T.K. Plasmid mediated heavy metal resistances. *Annual Review of Microbiology* (1988), 142:717-743.

[18] Dhakephalkar P.K. and Chopade B.A. High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of *Acinetobacter*. *BioMetals* (1994), 7(1): 67-74.

[19] Trevors J.T., Oddie K.M. and Belliveau B.H. Metal resistance in bacteria. *FEMS Microbiology Reviews* (1985), 1(1):39-54.

[20] APHA. *Standard Methods for the Examination of Water and Wastewater*. 18th ed. American Public Health Association, Washington, DC (1992).

[21] Dick E.M. Water and Wastewater Sampling for Environmental Analysis. Environmental sampling for trace analysis, (1994), 255.

[22] Halimoon N. and Yin R.S. Removal of heavy metals from textile wastewater using zeolite. *Environment Asia* (2010), 3:124-130.

[23] Sabry S.A., Ghozlan H.A. and Abou-Zeid D.M. Metal tolerance and antibiotic resistance patterns of a bacterial population isolated from sea water. *Journal of Applied Microbiology* (1997), 82(2):245-252.

[24] APHA. *Standard Methods for the Examination of Water and Wastewater*. 21st ed. American Public Health Association. Washington, DC. Part, (2005), 8000:94-100.

[25] Nelson D.W. and Sommers L.E. Total Carbon, Organic Carbon and Organic Matter. In A. L. Page, R. H. Miller, D. R. Keeney (Eds.), Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties. 2nd ed. Agronomy Monogram (1982), 9: 539-580.

[26] AOAC. Official methods of analysis. Washington DC: Association of Official Analytical Chemists, (1990).

[27] Grimalt J.G. Sampling, sample handling, and operational methods for the analysis of trace pollutants in the marine environment. In Marine Pollution ed. Albaiges, J. New York, Washington, Philadelphia, London: Hemisphere Publishing Corp, (1989).

[28] Jamaluddin H., Zaki D.M., Ibrahim Z., Tan S., Zainudin N. and Yusoh O. Isolation of metal tolerant bacteria from polluted wastewater. *Pertanika Journal of Tropical Agricultural Science* (2012), 35(3):647-662.

[29] Pandit R., Patel B., Kunjadia P., and Nagee A. Isolation, characterization and molecular identification of heavy metal resistant bacteria from industrial effluents. *International Journal of Environmental Science* (2013), 3(5):1689-1699.

[30] Coral M.N., Korkmaz H., Arikan B. Plasmid heavy metal mediated resistances in *Enterobacter* spp. Isolated from *Sofulu landfill*. *Annals of Microbiology* (2005), 55(3):175-179.

[31] Fasim F., Ahmed N., Parsons R. and Gadd G.M. Solubilization of zinc salts by a bacterium isolated from the air environment of a tannery. *FEMS Microbiology Letters* (2002), 213(1): 1-6.

[32] Bauer A.W., Kirby W.M.M., Sherries J.C. and Truck M. Antibiotic susceptibility testing by a standardized single disc diffusion method. *American Journal of Clinical Pathology* (1966), 45(4):493-496.

[33] Loureiro M.M., De Moraes B.A., Quadra M.R.R., Pinheiro G.S. and Asensi M.D. Study of multi-drug resistant microorganisms isolated from blood cultures of hospitalized newborns in Rio De Janeiro City, Brazil. *Brazilian Journal of Microbiology* (2002), 33(1): 73-78.

[34] Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; 21st inform. suppl. CLSI document M100-S21. CLSI, Wayne, Pennsylvania (2011).

[35] Vitek® microbiology reference manual, Loiaison, appendix F: Method for determining duplicate isolates for Data Trac Reports. BioMerieux France 12, (2004).

[36] Holt J.G., Krieg N.R., Sneath H.A., Stanley J.T. and Williams S.T. *Bergeys Manual of Determinative Bacteriology*. (9thed), Baltimore; Wiliams and Wilkins, USA (1994).

[37] Moore E., Arnscheidt A. and Kruger, A. et al. Simplified protocol for the preparation of genomic DNA from bacterial cultures. Molecular Microbial Ecology Manual Second Edition (2004), 3–18.

[38] Hookoom M. and Puchooa D. Isolation and identification of heavy metals tolerant bacteria from industrial and agricultural areas in mauritius. *Current Research in Microbiology and Biotechnology* (2013), 1(3): 119-123.

[39] Tamura K., Dudley J., Nei M. and Kumar S. MEGA4: molecular evolutionary genetics analysis (mega) software version 4.0. *Molecular Biology and Evolution* (2007), 24(8):1596-1599.

[40] Ademiluyi F., Amadi S. and Nimisingha J. Adsorption and treatment of organic contaminants using activated carbon from waste Nigerian Bamboo. *Journal of Applied Sciences and Environmental Management* (2009), 13(3): 39-47.

[41] Igwe J.C. and Abia A.A. a Bioseparation process for removing heavy metals from wastewater using biosorbents. *African Journal of Biotechnology* (2006), 5(12):1167-1179.

[42] Pazirandeh M., Wells B. and Ryan R.L. Development of bacterium-based heavy metal biosorbents: Enhanced uptake of cadmium and mercury by *Escherichia coli* expressing a metal binding motif. *Applied and Environmental Microbiology* (1998), 64(10): 4068- 4072.

[43] Simon T. PCR and the detection of microbial pathogens in water and wastewater. J. Water Research (1999), 33(17): 3545-3556.

[44] Clausen C.A. Isolating metal-tolerant bacteria capable of removing copper, chromium, and arsenic from treated wood. *Waste Management and Research* (2000), 18(3): 264-268.

[45] Mishra A. and Prasad K.M. Bacterial response as determinant of oxidative stress by heavy metals and antibiotic. *Journal of Innovations in Pharmaceuticals and Biological Sciences* (2015), 2(3): 229-239.

[46] Hussein K.A. and Joo J.H. Heavy metal resistance of bacteria and its impact on the production of antioxidant enzymes. *African Journal of Microbiology Research* (2013), 7(20): 2288-2296.

[47] Hassan S.H.A., Abskharon R.N.N., Gad El-Rab S.M.F. and Shoreit A.A.M. Isolation, characterization of heavy metal resistant strain of *Pseudomonas aeruginosa* isolated from polluted sites in Assiut city, Egypt. *Journal of Basic Microbiology* (2008), 48(3): 168-176.

[48] Rohini B. and Jayalakshmi S. Bioremediation potential of *Bacillus cereus* against copper and other heavy metals. *International Journal of Advanced Research in Biological Sciences* (2015), 2(2): 200-209.

[49] Sevgi E., Coral G., Gizir M.A., Sangün M. Kemal. Investigation of heavy metal resistance in some bacterial strains isolated from industrial soils. *Turkish Journal of Biology* (2010), 34(4): 423-431.

[50] Ganiyu O.O., Matthew O.I., Sunday A.A., Oluwafemi S.O. and Olukayode O.A. Bacteria with dual resistance to elevated concentrations of heavy metals and antibiotics in Nigerian contaminated systems. *Environmental Monitoring and Assessment* (2010), 168(1):305-314.

[51] Yamina B., Tahar B. and Marie L.F. Isolation and screening of heavy metal resistant bacteria from wastewater: a study of heavy metal co-resistance and antibiotics resistance. *Water Science and Technology* (2012), 66(10): 2041-2048.

[52] Aktan Y., Tan S. and Icgen B. Characterizations of lead-resistant river isolate *Enterococcus faecalis* and assessment of its multiple metal and antibiotic resistance. *Environmental Monitoring and Assessment* (2013), 185(6): 5285-5293.

[53] Sobecky P.A. Plasmid ecology of marine sediment microbial communities. *Hydrobiologia* (1999), 401(0): 9-18.

[54] Endo G., Narita M., Huang C.C. and Silver S. Microbial heavy metal resistance transposons and plasmids: potential use for environmental biotechnology. *Journal of Environmental Biotechnology* (2002), 2(2): 71-82.

[55] Virender S., Chauhan P.K., Kanta R., Tejpal D. and Vinod K. Isolation and characterization of *Pseudomonas* resistant to heavy metals contaminants. *International Journal of Pharmaceutical Sciences Review and Research* (2010), 3(2): 164-167.

[56] Rodríguez C.E., Quesada A. and Rodríguez E. Nickel biosorption by *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolated from industrial wastewater. *Brazilian Journal of Microbiology* (2006), 37(4):465-467.

[57] AL-Saffar A.K., Alahmed S.G., Alwesawei Y.A. and Mahdi R.K. *Acinetobacter baumannii*: isolation, identification and antimicrobial resistance. *International Journal of Recent Scientific Research* (2015), 6(4):3766-3773.

[58] Shiri Z., Kermanshahi R.K., Soudi M.R. and Farajzadeh D. Isolation and characterization of an n-hexadecane degrading *Acinetobacter baumannii* KSS1060 from a petrochemical wastewater treatment plant. *International Journal of Environmental Science and Technology* (2015), 12(2):455-464.